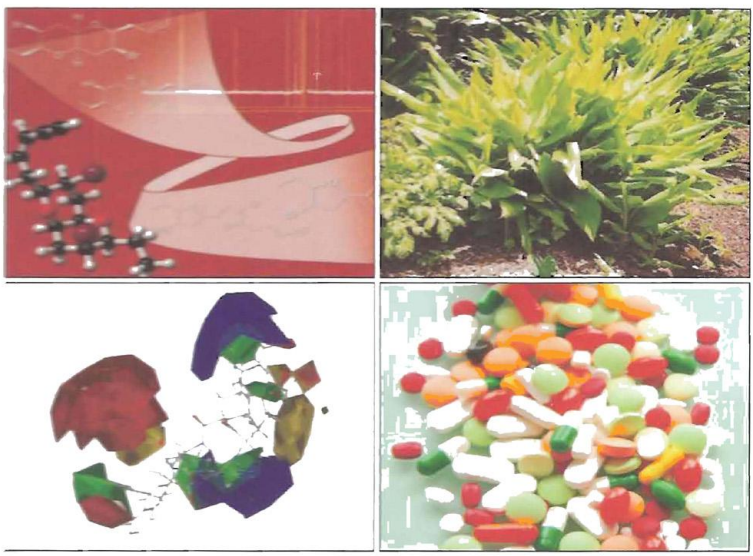
Comprehensive

Bioactive Natural Products

Vol 7 Structural Modifications & Drug Development



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Studium Press

Comprehensive
Bioactive Natural Products

Volume 7
Structural Modifications & Drug Development

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About the Series

Nature abounds with a rich potential heritage of bioactive natural products as that have been exploited for effective and beneficial use either as a therapeutic resource against a human disease or as a chemopreventive, antioxidant, nutraceutical or diet supplement. A natural product is a substance or a chemical compound produced by a living organism found in nature that usually has a pharmacological or biological activity. Some of these agents are derived from terrestrial plants, whereas, others are obtained from microorganisms, marine or freshwater organisms and even the higher animals.

Past few decades have witnessed an unprecedented worldwide growth in the interest of bioactive natural products based medicines, both in developed and developing countries, and offer a resource-pool for lead identification in drug discovery owing to the reason that isolation of a novel molecule is easier than *de novo* synthesis, especially when the molecular structure of the compound is very complex. On account of such advantages, pharmaceutical companies are opting for bioactive natural products based drugs. More than 20 new drugs, launched world over between 2000 and 2005, originate from natural products. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases (infectious and non-infectious). The development of new bioassay techniques, biotechnology methods, screening and high performance analytical methods have introduced new concepts and possibilities of rational drug design and drug discovery.

Interest in bioactive natural products research is growing more strongly and can be attributed to several factors, including remarkable diversity of natural products, unmet therapeutic needs, the development of novel and sensitive techniques to detect, isolate, purify and structurally characterize these active constituents. This has opened opportunities for multidisciplinary research that joins the forces of natural product chemistry, molecular and cellular biology, synthetic and analytical chemistry, bio-chemistry, bio-technology, general biology, and pharmacology to exploit the vast diversity of bioactive natural products.

Taking cognizance of these facts, we have initiated efforts in editing the present series "**Comprehensive Bioactive Natural Products**" and brought eight volumes (1 to 8) providing edited information from over 139 original research and review articles by eminent scientists and researchers

from India and abroad, representing 40 countries of the world on wide range of topics in the area of natural products categorized under the themes *viz.,*

1. *Potential & Challenges*
2. *Efficacy, Safety & Clinical Evaluation I*
3. *Efficacy, Safety & Clinical Evaluation II*
4. *Antioxidants & Nutraceuticals*
5. *Immune-modulation & Vaccine Adjuvants*
6. *Extraction, Isolation & Characterization*
7. *Structural Modifications & Drug Development*
8. *Quality Control & Standardization*

These volumes critically evaluate the present state-of-art, current status and future prospects in a well-illustrated manner. It is hoped that this series, which reflects the contributor's research results as well as world wide reviews will be widely read and used by all interested in these fields and shall open new vistas of research and academic pursuits for those engaged in the fields of bioactive natural products.

I would like to take this opportunity to thank each of the authors who have contributed in the preparation of this book series.

Jammu, India

V.K. Gupta
Series Editor

AMITY INSTITUTE FOR HERBAL
AND BIOTECH PRODUCTS DEVELOPMENT

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Prof. (Dr.) P. PUSHPANGADAN,
M.Sc. M.Phil. Ph.D., FBRS FES. FNRS, FNSE, FNESA, FNAASc, FNASc.,
(UN Equator Initiative Laureate)
Director General & Senior Vice President, RBEF
(Former Director, NBRI, Lucknow)

08-06-2009

Foreword to the Series

Medicine among the ancient civilizations was a blend of Religion and Science. Natural products played an important role throughout the world in the prevention and treatment of diseases. These natural products originated from varied sources such as plants, animals, micro-organisms, marine organisms, terrestrial vertebrates and invertebrates. In man's quest for discovering new and potent remedies for diseases, he keenly observed his immediate neighbourhood and experimented not only with organic substances but also with in-organic materials such as metals, minerals, ores etc. Over the years, the accumulated medical wisdom was compiled into excellent treatises and these have later developed into the various classical systems of medicine associated with the ancient civilizations and cultures of the world. Apart from the classical systems of medicines, which by its nature and practice had universal applications, there existed another stream of medicine in the countryside, among the less privileged class of people, among the forest dwellers and among the nomadic tribes which were location specific, confined to small communities, tribes and families. These medical systems were mostly perpetuated by oral traditions. Nevertheless, the value of the oral traditions cannot be overlooked as these localized knowledge systems are veritable treasures of invaluable information on the properties and uses of hundreds of plant, animal and mineral products.

Ethno medical information is now increasingly being utilized in the search for novel bioactive molecules from natural products. Several well known plants such as Licorice (***Glycyrrhiza glabra***) myrrh (***Commiphora sps***) and poppy capsule latex (***Papaver somniferum***) were mentioned in the clay tablets dating back to 2600 B.C, obtained from Mesopotamia. These plants and their derivatives are still used in various forms as herbal drugs as well as pure isolates in different systems of medicine. For instance,

codeine, papaverine, morphine etc isolated from ***P. somniferum*** are still used in modern medicine. Similarly hemisuccinate carbenoxolone sodium, a semi synthetic derivative of glycyrrhetic acid isolated from licorice is used for the treatment of gastric and duodenal ulcers. Plants, especially those with ethno medical history have been the major source of bio active molecules. According to a study by Fabricant and Famsworth (2001), out of 122 plant derived drugs, 80% had their origin from plants with ethno medical use. Since secondary metabolites from plants are products of biosynthetic reactions occurring in living systems, bioactive molecules from natural sources are excellent drug candidates as they are perceived to show more biological friendliness than synthetic drugs.

The ever increasing popularity and acceptance of natural products have evinced an extra ordinary interest and scope in the subject in recent years. Compendium of Bioactive Natural Products- a series of eight books edited by Dr. V.K. Gupta (Volumes 1,2,3 and 5) and Dr. V.K. Gupta, Dr. S.C. Taneja, Dr. B.D. Gupta and Dr. A.K.Verma (Volumes 4,6,7 and 8), published by M/S. Studium press LLC, U.S.A are an excellent compilation, giving comprehensive and reliable data on the current development in the field of bioactive natural products. This series contain 139 original research and review articles contributed by a galaxy of eminent scientists and academicians from 40 countries around the world. The editors have taken care to invite eminent scholars in the relevant areas to contribute each chapter in the series. The series deals with wide ranging topics related to natural products such as extraction, isolation and characterization, structural modifications and drug development, quality control and standardization and, immune modulation and vaccine adjuvants, antioxidants and nutraceuticals, efficacy, safety and clinical evaluation (Parts-1 and 2) and potentials and challenges. The editors of this series Dr. V.K. Gupta, Dr. S.C. Taneja, Dr. B.D. Gupta and Dr. Anil K. Verma are known to me for more than three decades and they are all stalwarts in natural products and related areas.

It is gratifying to know that this series is dedicated to the memory of Col. Sir Ram Nath Chopra, the Founder Director of Regional Research Laboratory, now known as Indian Institute of Integrative Medicine (IIIM), Jammu.

Col. Sir R.N. Chopra (1882-1973) was a pioneer of systematic studies of indigenous drugs, promoter of Indian Systems of Medicine and Patron of Pharmacy in India. Most of the medicinal plants, he studied were in use, in Ayurveda for thousands of years. The major fields of Cot. Chopra's research were general pharmacology, chemotherapy, indigenous drugs, drug addiction and drug assays. At the Calcutta school of Tropical Medicine, he developed a pattern of research in studying the action of medicinal plants which influenced an entire generation of pharmacologists. Pharmacists and physiologists in India. In the words of Dr. G.V. Satyawati, Former Director General of Indian Council of Medical Research, the credit of kindling the interest of Indian Chemists and Pharmacologists in Medicinal Plants should rightfully go to

Sir Ram Nath Chopra who, has been claimed as the Father of Indian Pharmacology'.

I had the privilege and good fortune of meeting Col, Sir. Ram Nath Chopra along with Prof. Dr. E.K. Janaky Ammal at his residence in 1969 immediately after my joining Regional Research Laboratory as a Scientific Assistant. My meeting with Col. Sir R.N. Chopra had left a lasting impression of a great scientist in my memory.

It is a fitting tribute to Col. Sir. R.N. Chopra that the editors have decided to dedicate these eight volumes to his memory. I congratulate the editors for bringing out this series which is timely, relevant and important to all those who are engaged in the study of natural products and development of therapeutically useful bioactive molecules. I am sanguine that these volumes will receive wider acceptance among the scientific community, especially among those engaged in Natural Product research.

(P. Pushpangadan)

About the Editors

Dr. Vijay Kumar Gupta

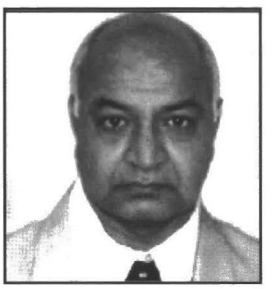
Dr. Vijay Kumar Gupta, Ph.D., FLS, London (born 1953-) Deputy Director & Head, Animal House, Indian Institute of Integrative Medicine (CSIR), Jammu, India. He did his M.Sc. (1975) and Ph.D. (1979) in Zoology both from University of Jammu, Jammu-India. His research capabilities are substantiated by his excellent work on histopathology, ecology and reproductive biology of fishes, turtles, birds and mammals, which has already got recognition in India and abroad. Dr. Gupta has to his credit more than 75 scientific publications and review articles which have appeared in internationally recognized Indian and foreign journals. Founder fellow, life member and office bearer of many national societies, academies and associations. He has successfully completed a number of research/consultancy projects funded by various governments, private and multinational agencies. His current areas of interest are histopathology, toxicology, pre-clinical safety pharmacology and reproductive efficacy studies of laboratory animals. He is also Editor-in-chief of the books 1) Utilisation and Management of Medicinal Plants 2) Medicinal Plants: Phytochemistry, Pharmacology and Therapeutics 3) Perspectives in Animal Ecology and Reproduction (Vols.1-6). The Editor-in-chief of the American Biographical Institute, USA, has appointed him as *Consulting Editor* of *The Contemporary Who's Who*. Dr. Gupta also appointed as Nominee for the *Committee for the Purpose of Control and Supervision of Experiments on Animals* (CPCSEA, Govt. of India). Recently the *Linnaean Society of London, U.K.* has awarded fellowship to him in November 2009 in recognition of his contribution towards the cultivation of knowledge in Science of Natural History.

Dr. Subhash Chandra Taneja

Dr. Subhash Chandra Taneja (born 1950-) obtained his masters degree in Organic Chemistry in 1971 and thereafter completed Ph.D. in Birla Institute of Technology & Science, Pilani, India in 1975 under the guidance of Prof.

H.P. Tiwari. He is scientist of eminence and presently working as Scientist 'G', Indian Institute of Integrative Medicine (CSIR), Jammu. Dr. Taneja has over 100 publications in international journals, two book chapters and two review articles, 38 patents of which 14 are USP. He has also visited Institute of Organic Chemistry, Warsaw, Poland (1989-1990) under CSIR Scientist Exchange Programme. His areas of interest are chemistry and bioactivities of medicinal plants and generation of semi-synthetic libraries based on natural product scaffolds; design and synthesis of bioactive lead molecules in the area of inflammation, oncology, infectious diseases, immunomodulation; exploitation of microbial biodiversity for the identification newer sources of biocatalysts *e.g.* lipases, esterases, glucosidases, nitrilases, oxido-reductases etc. and development of new throughput screening techniques; synthesis and kinetic resolution of important dugs/intermediates using chemo-enzymatic methods; development of green methodologies for the synthesis of mono terpenes and perfumery molecules; development of new glycosylation methodologies; *C*- and *O*-glycosylation techniques; new synthetic protection-deprotection methodologies for carbohydrates, and their conversion to bioactive molecules and organic synthesis of natural products and bioactive molecules.

Dr. Bishan Datt Gupta



Dr. Bishan Datt Gupta (born 1951-) obtained his M.Sc. in chemistry in 1973 from Jammu University and then did his Ph.D. at Regional Research Laboratory, Jammu (now IIIM, Jammu) under the guidance of Dr. C. K. Atal. He is a well known scientist in the area of natural product chemistry and is at present working as Scientist 'G' at Indian Institute of Integrative Medicine (CSIR), Jammu. Dr. Gupta has more than sixty publications in international journals, one book chapter and eighteen patents. He has visited France under CSIR-CNRS Scientist Exchange Programme and has also participated in two international conferences at Kuala Lumpur and Hanoi. His areas of interest are natural product chemistry, especially drug development based on natural products which includes activity guided fractionation for isolation of pure biomolecules,

their structure elucidation, semi-synthesis as well as structure modifications. His work areas also include standardization of herbal drugs/formulations using modern analytical techniques (GC-MS, HPTLC, HPLC, LC-MS) on the basis of marker compounds.

Preface

Since the dawn of human civilization, man had been using plants and other sources of food for survival and other necessities. The plants find their use in human civilization since Middle Paleolithic age some 60,000 years ago. It is estimated by the World Health Organization that approximately 80% of the World's population uses plant based medicines either in part or entirely. The natural medicines are safer, gentler and better for human health than synthetic drugs. This is probably so because human beings have co-evolved with plants for millions of years. India and China are the two largest users of herbal medicines since ancient times. Indian Ayurvedic system uses some 1400 plant species and traditional Chinese medicine uses over 5000. In present day also drugs derived from natural source have been used as anti-infective/antibiotic, anti-inflammatory, anti-cancer, analgesic, anti-malarial etc. The first break through in the antibiotic drugs was discovery of penicillin G, from a fungal source *Penicillium notatum,* that made possible the treatment of diseases which were otherwise thought to be fatal. The discovery of heparin which is derived from mucosal tissues of slaughtered meat animals as a potent anticoagulant highlighted the importance of new sources in drug discovery. Camptothecins, podophyllotoxins, taxol, vinca alkaloids etc. all belong to natural class of compounds used for the treatment of cancer. Despite the fact that there is a huge cache of natural products which are yet to be associated with biological activities or having prospect of being developed into drugs, the focus of chemist had always been towards the simpler semi-synthetic modifications of these natural products to prepare more potent analogues with lesser side effects. The semi-synthetic compounds may also be active against the resistant cell lines. In fact most of the currently used cancer drugs have been synthesized by simple modification of natural products. There are innumerable examples of the semi-synthetically modified natural products which are far more potent than their parent compounds.

The present volume ***"Structural Modifications & Drug Development"*** in the series "**Comprehensive Bioactive Natural Products**" is devoted to specific aspects of structural modifications of some natural products. The topics have been contributed by the experts in the field with relevant and up-to-date information. Some of the interesting studies included in the volume are: Synthetic and clinical status of marine derived anticancer peptides; Microwave- assisted non-convential esterification of polysaccharides; Structural modifications of parthenin: a sesquiterpene lactone of biological potential; Prospects for the development of polyphenolic acetate as the potential drug candidate: a review; Mining bioactive conformations: a novel methodology for computing predictive 3D-QSAR models; Synthesis and structure-activity relationships of some taxoids as multidrug resistance modulator in MDR cancer cells; Antioxidant and

neuroprotective effects of synthetic curcumin analogues and natural phenolics; Action of plant proteinase inhibitors using biological models; Plant hormone conjugates; Biological prospective of pintol and its structurally modified products; Drug sensitivity of curcumin analogues and bioconjugates; Structural based drug design of estrogen receptor beta selective ligands.

We believe that the present volume will be a useful compendium of knowledge for natural product chemists, pharmacologists, pharmaceutical chemists as well as other researchers in medicinal chemistry.

Jammu, India

V.K. Gupta
S.C. Taneja
B.D. Gupta

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Synthetic and Clinical Status of Marine Derived Anticancer Peptides†

DIWAN S. RAWAT*[1], RAM SINGH[1], NITIN KUMAR[1], MUKUL SHARMA[1] AND M.S.M. RAWAT[2]

ABSTRACT

Ocean has been a rich source of structurally diverse class of compounds ranging from simple linear peptides to complex macrocyclic polyethers and has demonstrated wide range of biological activities. Several marine derived compounds are currently in clinical trials and some of the compounds are in the advance stage of clinical trials and some have been launched for the treatment of cancer, neuropathic pain, schizophrenia, Alzheimer's disease. Numbers of peptides have been isolated from marine sources, and many of the compounds showed promising anticancer activity. Didemnin was the first marine peptide that has entered in human clinical trials in US for the treatment of cancer, and other anticancer peptides such as kahalalide F, hemiasterlin, dolastatins, cemadotin, soblidotin, didemnins, and aplidine have entered in the clinical trials. This article describes the synthetic and clinical status of marine derived anticancer peptides.

Key words : Clinical status, marine peptides, synthesis

INTRODUCTION

The role of medicinal plants in the treatment of human diseases is well documented since ancient times (Bhakuni & Rawat, 2005; Newman, 2008; Butler, 2005; Singh *et al.*, 2008; Proksch *et al.*, 2003). Approximately one

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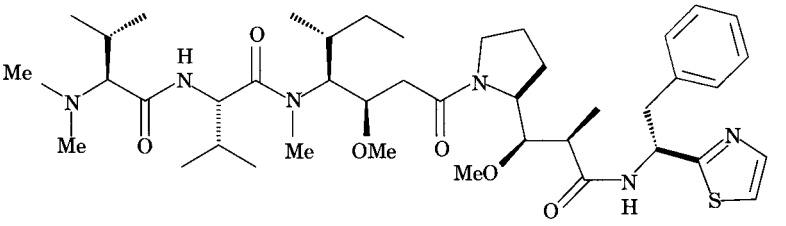
† Dedicated to Dr. D.S. Bhakuni on the occasion of his 78th Birthday.

third of best selling drugs are either natural products or inspired from natural products and over 60% of the anticancer drugs in the market are of natural origin (Jimeno *et al.*, 2004). Development of AZT as an anti AIDS drug, which is the synthetic analogue of sponge-derived nucleosides spongothymidine (Proksch *et al.*, 2003; Bergmann & Feeney, 1951), sparked the interest of medicinal chemist on marine natural products, and that resulted in the discovery of over 16,000 organic compounds from marine sources (Bhakuni & Rawat, 2005). Extensive research in the field of marine natural products in last five decades resulted in approximately 7,000 publications, and in addition, there are approximately another 9,000 publications on the subject dealing with syntheses, reviews, biological activity studies, ecological studies, besides over 300 patents have been issued on bioactive marine natural products (Bhakuni & Rawat, 2005; Amador *et al.*, 2003; Blunt *et al.*, 2005). Sponges, mollusks, bryozoans, and tunicates have been the main source of marine natural products, but various other marine microorganisms such as fungi, bacteria, and cyanobacteria have also produced number of biologically relevant molecules (Luesch *et al.*, 2002; Blunt *et al.*, 2008). Marine organisms have produced structurally diverse class of compounds (Faulkner, 2002; Wakeham, 1996; Blunt *et al.*, 2007; Newman & Cragg, 2004; Simmons *et al.*, 2005) from simple linear peptides to complex macrocyclic polyethers exhibiting a wide range of biological activities (Rawat *et al.*, 2006; Zhang *et al.*, 2006; Mayer & Hamann, 2004; Mayer & Gustafson, 2006; Singh & Pelaez, 2008; Herbst *et al.*, 2003; Behenna *et al.*, 2008; Romero *et al.*, 2008). Over a dozen of marine derived antitumor compounds are currently in various phases of human clinical trials for treatment of different types of cancer (Fenical, 2006; Jimeno *et al.*, 2006). Some of the prominent marine derived compounds in clinical trials includes squalamine lactate (amino steroid from shark) (Jimeno *et al.*, 2006; Bhargava *et al.*, 2001), yondelis (isoquinolone from sea squirt) (Lau *et al.*, 2005; Krasner *et al.*, 2008; Rinehart *et al.*, 1990), aplidin (cyclic depsipeptide from sea squirt) (Yao, 2003), bryostatin-1 (polyketide from bryozoan) (El-Rayes *et al.*, 2006; Pettit, 1996), discodermolide (polyketide from sponge) (Gunasekera *et al.*, 1990; Mickel, 2004; Florence *et al.*, 2008), dolastatin 10 (peptide from sea slug) (Mast *et al.*, 2005), ILX-651 (peptide from sea slug) (Ebbinghaus *et al.*, 2004), LU 103793 (Kerbrat *et al.*, 2003), cemadotin (peptide from sea slug) (Pettit, 1996), cryptophycin-52 (synthetic analogue of cryptophycin-1) (Liang *et al.*, 2005), epothilone B (macrolactone from myxobacterium) (Lee & Swain, 2008; Okuno *et al.*, 2005; Altmann, 2005), HTI-286 (tripeptide from sponge) (Loganzo *et al.*, 2003), KRN-7000 (α-galactosylceramide from sponge) (Yao, 2003; Yang *et al.*, 2004; Ishikawa *et al.*, 2005), and kahalalide F (cyclic depsipeptide from sea slug and alga) (Hamann, 2004). The present article summarizes the synthetic and clinical status of anticancer peptides isolated from the marine source, and their synthetic analogs.

DOLASTATIN 10

Dolastatins have been isolated from a mollusk *Dolabella auricularia* (Pettit *et al.*, 1993; Pettit, 1997) and dolastatins 10 (**1**) is the most active of the series. Structure determination of dolastatin 10 took fifteen years due to poor yield of the natural product (~ 1.0 mg/100 kg). Dolastatin 10 was most potent antiproliferative agent ($ED_{50} = 4.6 \times 10^{-5}$ μg/mL) known at the time of its discovery (Pettit *et al.*, 1987).



1

Out of the many cytotoxic peptides of the series, linear pentapeptide dolastatin 10 and the depsipeptide dolastatin 15 were selected for drug development programme (Pettit *et al.*, 1995; Pettit *et al.*, 1998). Most of the peptides of the series inhibit cell proliferation, and induce apoptosis in numerous malignant cell and cancer cell lines (Haldar *et al.*, 1998). It has been reported that dolastatin 10 (**1**) exerts potent cytotoxic effects in animals having intraperitoneal tumors. In addition to this, dolastatins also exhibited synergistic antitumor activity with tubulin interactive agent's vinca alkaloids and bryostatin-1 (Beck *et al.*, 2004; von Mehren *et al.*, 2004). Dolastatin 10 (**1**) entered into phase I clinical trials in early 1990s under the auspices of the NCI (Kindler *et al.*, 2005; Bai *et al.*, 1990; Garteiz *et al.*, 1998) and has been evaluated in various phase I clinical trials (Garteiz *et al.*, 1998). Preliminary data indicated that 40% of patients developed moderate peripheral neuropathy and patients with underlying neuropathy are at increased risk for this side effect.

Due to novel mode of action, limited activity of other anti-microtubular agents, the multi-institutional phase II study on dolastatin 10 (**1**), was conducted (von Mehren *et al.*, 2004). In phase II clinical study, dolastatin 10 was given *iv* at a dose level of 400 mg/m^2 and the dose was repeated every 21 days. During this study it was observed that dolastatin 10 caused hematological toxicity, but gastrointestinal, hepatic or renal toxicities were not observed. The respiratory failure death of one person was reported during this study. Based on data in phase II trials, further studies of dolastatin 10 were not recommended in advanced or metastatic soft tissue sarcomas. Although phase I and phase II clinical results were discouraging, but this offered a basis for structure activity relationship study on dolastatin 10. The synthetic programme around dolastatin resulted in the discovery

of TZT-1027, a potent anticancer agent. The synthetic programme was initiated with the aim to retain the anticancer activity of the lead compound but to reduce the toxicity profile. TZT-1027 fulfilled these criteria, and it later entered into clinical trials (Horti *et al.*, 2008). Phase I clinical trials on TZT-1027 (soblidotin) were conducted with the aim to study the dose-limiting toxicity (DLT), maximum tolerated dose (MTD), and pharmacokinetics, in Japanese patients with advanced solid tumors when administered on days 1 and 8 in 3-week courses. MTD was observed to be 1.5 mg/m^2 that was lower than the value of 2.4 mg/m^2 in European patients. Interestingly, antitumor activity was observed at low doses. This study further confirmed that TZT-1027 (soblidotin) is a promising new tubulin polymerization inhibitor and was recommended for further investigations in phase II studies (Tamura *et al.*, 2007).

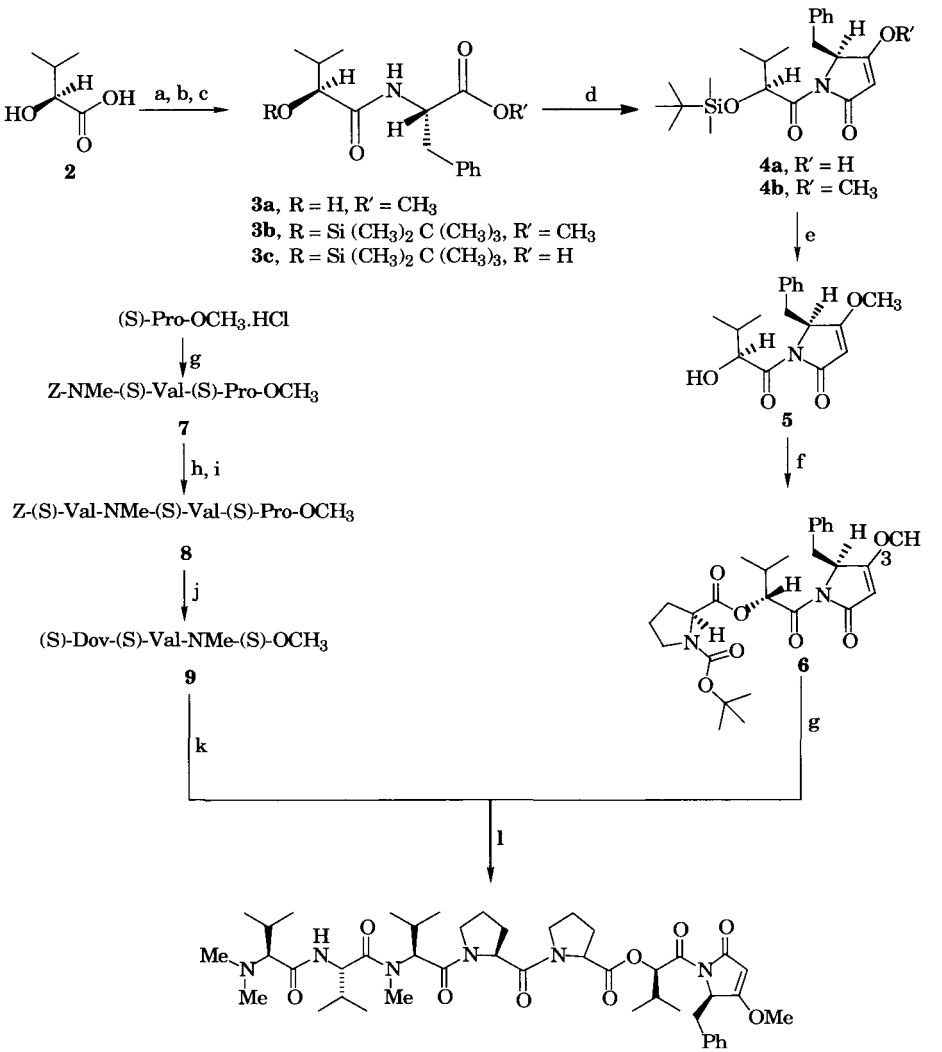
Total Synthesis of Dolastatin 15 (10)

Synthesis of the key Dpy intermediate **5** was achieved as summarized in the Scheme 1 (Pettit *et al.*, 1991). The hydroxyisovaleric acid (Hiva) component was obtained from (S)-Val *via* diazotization sequence. The condensation of unprotected (S)-Hiva with (S)-Phe-OMe, using diethyl phosphorocyanidate leads to the formation of (S)-Hiva-(S)-Phe-OMe **(3a)** in 60% yield. Protection using *tert*-butyldimethylsilyl chloride, saponification and further reaction of the resulting carboxylic acid with pentafluorophenyl trifluoroacetate followed by Meldrum's ester (Jouin *et al.*, 1987) leads to the formation of pyrrolidone **(4a)** as a viscous oil. Reaction with dimethyl sulfate provided the corresponding methyl ether **(4b)**. Cleavage of the silyl group with pyridinium polyhydrogen fluoride gave (S)-Hiva-(S)-Dpy **(5)** in 90% yield. Esterification of **6** with Boc-(S)-Pro gave depsipeptide **6** (74% yield). The stereochemical designations for the three chiral centers were determined to be C-4 (S), C-7 (S), and C-10 (S).

Tetrapeptide **9** was prepared by starting with dipeptide **7** using the procedure outlined in Scheme 1. Hydrogenolysis followed by pivaloyl mixed anhydride peptide bond formation results in the formation of tripeptide **8**, further hydrogenolysis and *in situ* reaction with (S)-Dov-OPfp led to tetrapeptide **9**. Finally, the carboxylic acid obtained by saponification of methyl ester **9** was condensed with the amine derivative of proline **6** to give, natural (–)-dolastatin 15 **(10)** in 68% yield.

Total Synthesis of Dolastatin 10

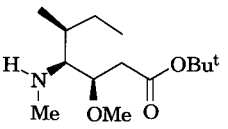
The synthesis of Dil component (3R, 4S, 5S)-Dil-OBut.HC1 (**11**) was achieved by methylating Z-(S, S)-isoleucine, followed by reduction with diborane-THF to N-Z-N-Me-(S, S)-isoleucinol, oxidation with DMSO-SO_3-Py to the aldehyde and aldol condensation with the lithium enolate (lithium diisopropylamide) from *tert*-butyl acetate yielded (3S, 4S, 5S)- and (3R, 4S,



(a) Phe-OMe, diethyl phosporocyanidate (DEPC), NMM, CH_2Cl_2; (b) TBDMS chloride, imidazole, DMF; (c) 1N NaOH, CH_3CH_2OH-H_2O; (d) Meldrum's ester, 4-DMAP, $C_6F_5O_2CCF_3$, CH_2Cl_2; CH_3OH, H_2; K_2CO_3, $(CH_3O)_2SO_2$, THF; (e) HF-pyridine; (f) Boc-(S)-Pro, DCCI, 4-pyrrolidino-pyridine, CH_2Cl_2; (g) Z-NMe.HCl-ether; (h) Z-(S)-Val, $(CH_3)_3$ CCOCl, NMM, $CHCl_3$; (i) (S)-Dov-OPfp, H_2, 10% Pd/C, dioxane; (j) NaOH, dioxane, H_2O, HCl; (k) TFA, CH_2Cl_2; (l) DEPC, TEA, DME, 0°C-RT.

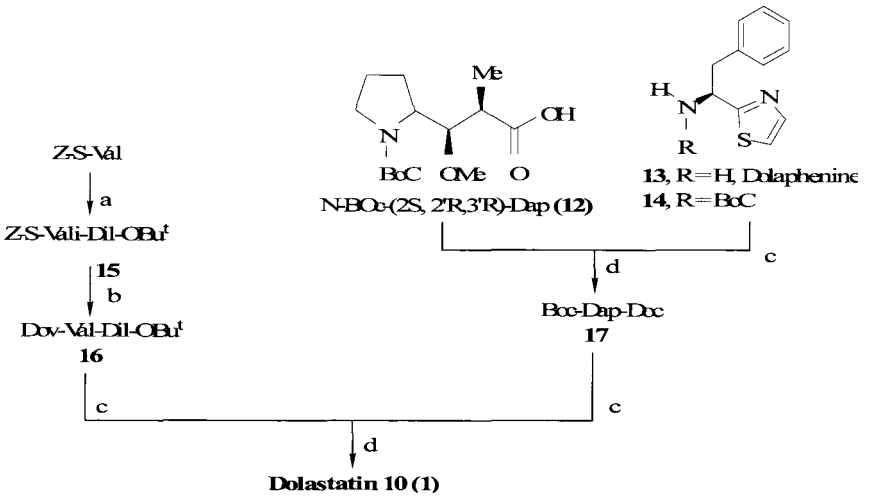
Scheme 1.

5S)-β-alcohols where the 3R-isomer was methylated with diazomethane-boron trifluoride etherate. The methylated product was subjected to hydrogenolysis to give **11** in 63% yield.



11

The more complex compound N-Boc-DAP **(12)** was synthesized in the following manner. The reduction of Boc-S-Pro with diborane followed by oxidation leads to Boc-S-prolinal. Aldol condensation of this aldehyde with the chiral enolate afforded nearly correct (2S, 2'S, 3'R)-isomer as the major product. Treatment of the major aldol product with boron trifluoride gave the (3'R) methyl ether derivative. Epimerization of the (2'S)-methyl group necessitated rather specific conditions, with potassium *tert*-butoxide in THF at –20°C to provide the (2'R) methyl epimer (57%). Hydrogenolysis with Pd/C of the benzyl ester led to N-Boc-(2S, 2'R, 3'R)-dolaproine (**12**). Mild treatment with trifluoroacetic acid led to natural (2S, 2'R, 3'R)-dolaproine as the trifluoroacetate salt. Preparation of dolaphenine **(13)** followed the probable biosynthetic route (Pettit *et al.*, 1982). Conversion of Boc-S-Phe to the corresponding N-Boc-5'-phenylalaninal was performed as above (Dil sequence). Condensation with 2-aminoethanethiol and dehydrogenation afforded N-Boc-S-Doe (**14**) (77%). The use of pivaloyl anhydride coupling (Wenger, 1985) for the N-methyl amino acid (Dil), and then proceeding as given in scheme via peptides **15**, **16** and **17**, natural (–)-dolastatin 10 (**1**) was obtained in 74% yield (Pettit *et al.*, 1989).

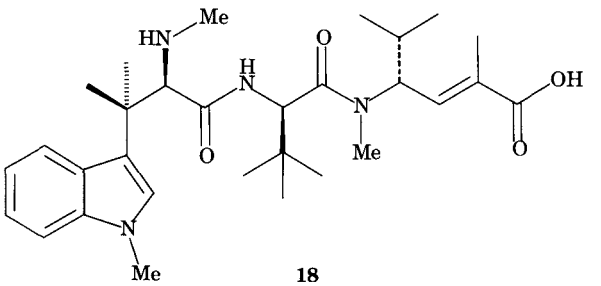


(a) $(CH_3)_3COCl$, NMM, (3R, 4S, 5S)-Dil-OBut.HCl, $CHCl_3$; (b) Dov-OPfp, 10% Pd/C, H_2, dioxane; (c) CF_3CO_2H, CH_2Cl_2; (d) diethylphosphorocyanidate (DEPC), TEA, DME, 1 h at 0°C, 2h, RT.

Scheme 2.

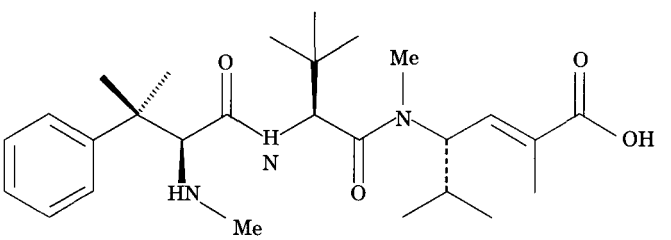
HEMIASTERLIN AND HTI-286

South African sponge *Hemiasterella minor* has been a source of many cytotoxic peptides such as geodiamolides A to F, hemiasterlin A and B and criamides (Jimeno, 2002). Hemiasterlin (**18**), a tripeptide was originally isolated from the South African sponge *Hemiasterella minor* (Talpir *et al.*, 1994). The same sponge has also been a source of geodiamolide TA and jaspamide.



18

Hemiasterlin and its derivatives A and B exhibit anti cancer activity due to their ability to depolymerize microtubule. The technology of hemiasterlins and its synthetic analogues was licensed for further development to Wyeth by Anderson *et al.* (2003). One of the analogues HTI-286 (**19**), emerged as a clinical candidate. HTI-286 has also been isolated from the sponge. Hemiasterlin derivatives have the property to overcome the P-glycoprotein mediated chemo-resistance. HTI-286 exhibits remarkable activity against paclitaxel and vincristine resistant solid tumors *in vitro* and *in vivo* and it is orally active peptide.



19

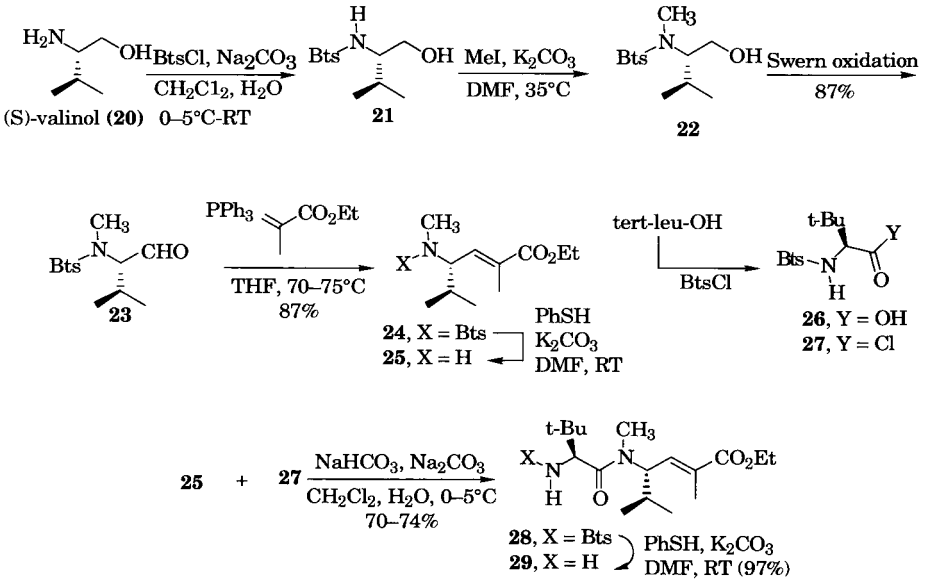
HTI-286 (**19**) inhibits the proliferation of tumor cells during mitosis, and exhibits antimitotic activity mainly due to its ability to bind at the Vinca peptide site in tubulin (Krishnamurthy *et al.*, 2003). Phase I clinical trial sponsored by Wyeth using HTI-286 as a single agent was started in the United States in March 2002, and phase I clinical trial in combination with carboplatin has also been started in the US for patients with solid tumors (Ratain *et al.*, 2003). Another phase I clinical trial in Japan is underway. HTI-286 has entered into a phase II clinical trial in October 2003 as a single agent for the treatment of patients with refractory non-small cell lung

carcinoma. HTI-286 and dolastatin 10 hybrids (Zask *et al.*, 2005) have been prepared and one of the hybrids was found to be much more active than dolastatin 10 in cells expressing the P-glycoprotein efflux pump. HTI-286 binds tubulin monomer and as a result oligomerizes the tubulin to 18.5 S species corresponding to a discrete ring structure consisting of about 13 tubulin units (Krishnamurthy *et al.*, 2003). It has been confirmed that both hemiasterlin (**18**) and HTI-286 (**19**) bind tubulin with nearly equal potency, but the stability of the tubulin oligomer is not identical under size-exclusion column chromatography conditions. In contrast to the microtubule-stabilizing effects of paclitaxel, both HTI-286 and hemiasterlin depolymerize preassembled microtubules at micromolar concentrations. HTI-286 also exhibits strong antitumor activity both in androgen-dependent and androgen-independent tumors (Hadaschik *et al.*, 2008). This study indicated that HTI-286 may act as a promising agent in second-line treatment for patients suffering from docetaxel-refractory prostate cancer (Hadaschik *et al.*, 2008).

Not much has been explored towards the total synthesis of hemiasterlin. Andersen *et al.* (1997) described the preparation of a protected tetramethyltryptophan (15 steps from indole-3-acetic acid) and an enoate (four steps from *N*-Boc-*N*-methylvaline), followed by a difficult coupling sequence. Later Vedejs *et al.* (2001) developed a shorter synthesis of an enantiomerically pure tryptophan derivative that could be used in place of protected tetramethyltryptophan. They started the work with the synthesis of unsaturated ester **25** from commercially available (*S*)-valinol (**20**) as summarized in Scheme 3.

The Bts-protected (*S*)-valinol (**21**) was prepared in 82% yield from BtsCl and (*S*)-valinol using biphasic conditions (CH_2Cl_2/water; 1.02 mol % of Na_2CO_3 as base). The methylation was carried out with iodomethane in the presence of K_2CO_3 in DMF (4 h, 35°C) to give **22** in 91% yield. The oxidation of **22** using either Dess-Martin periodinane (Dess & Martin, 1983) or Swern conditions (Dess & Martin, 1991) (oxalyl chloride/DMSO/Et_3N) provided the corresponding aldehyde **23** in good yield (> 90%). The unsaturated ester **24** was obtained from **23** as a 26:1 mixture of *E*:*Z* isomers using an excess of the ester-stabilized Wittig reagent Ph_3PdCH (Me) CO_2Et. The optimized conditions required treatment of the aldehyde **23** with 2 equiv of the Wittig reagent in refluxing THF (3 h). This procedure afforded the pure ester **24** in 87% yield, which was further converted to the free amino ester **25** in 93% yield. Compound **26** was prepared by the reaction of BtsCl and (*S*)-*tert*-leucine (heterogeneous conditions; suspension of BtsCl in aqueous NaOH). It was important to maintain the reaction pH in the range 10 to 10.5 and slow addition of aqueous NaOH as needed to neutralize the HCl byproduct. The use of excess BtsCl (1.6 equiv) was necessary due to competing hydrolysis of BtsCl in the aqueous medium. Under these optimized conditions, the Bts protected (*S*)-*tert*-leucine **26** was obtained in good yield (81%). Further, conversion to acid chloride **27** was carried out by routine

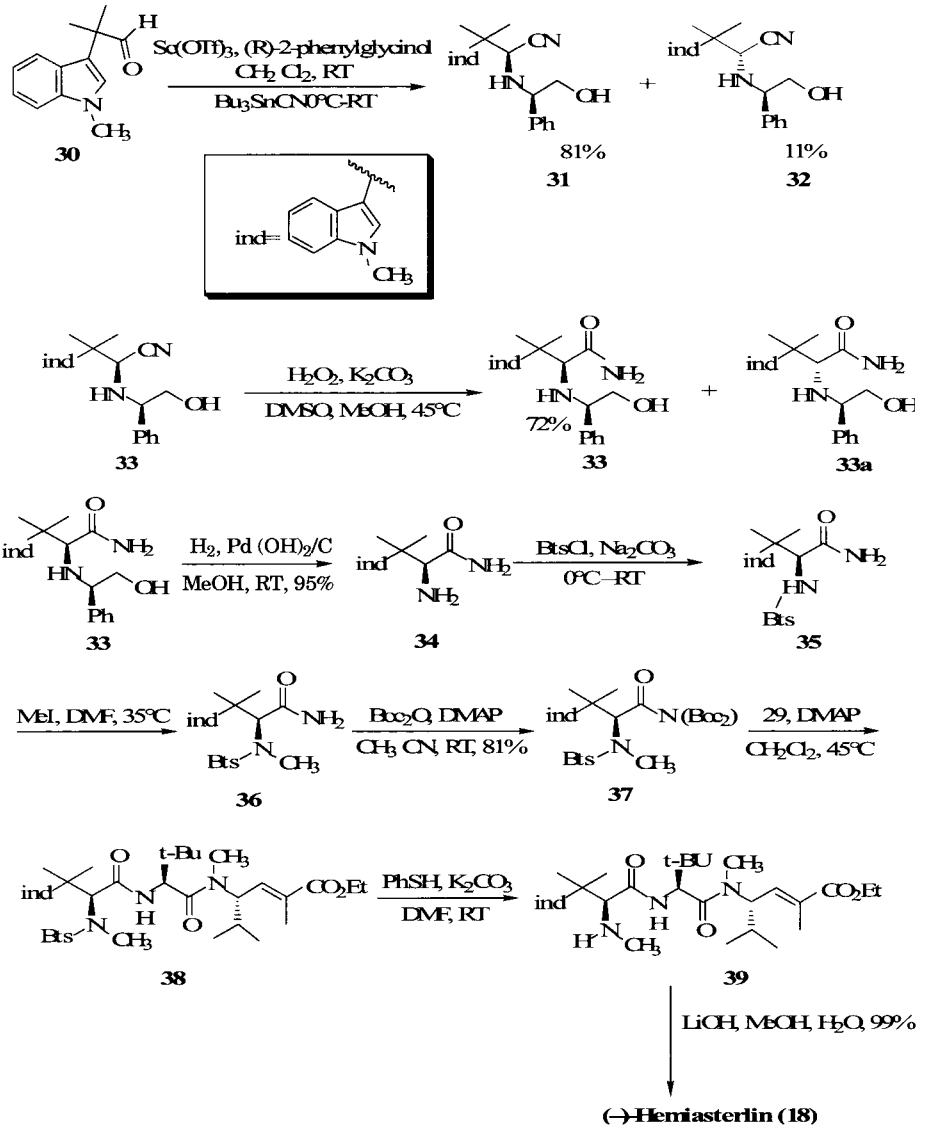
thionyl chloride treatment, and the crude **27** after removal of volatiles was used in the next step. The coupling of the amino ester **25** with **27** (1.5 equiv) was performed under biphasic conditions ($NaHCO_3$–Na_2CO_3, CH_2Cl_2–H_2O) and furnished the corresponding dipeptide **28** in 86% yield. The resulting material was deprotected using PhSH/K_2CO_3 in DMF to give **29** (97%).



Scheme 3.

The last step of hemistrein synthesis requires acylation of **29**, analogous to Andersen's intermediate. The Bts protected analogue to Andersen's intermediate was prepared from the aldehyde **30** (Scheme 4) (Mancuso *et al.*, 1978). Chiral amino nitriles **31** and **32** were prepared by the literature method.

The removal of chiral auxiliary from the major Strecker product **31** under oxidative conditions and conversion of nitrile to carboxylate derivative failed under variety of reaction conditions. So compound **32** was converted to an amide **33**. The amide diastereomers **33** and **33a** were separated by flash column chromatography. The chiral auxiliary was removed from **33** by hydrogenolysis over $Pd(OH)_2$ to yield amino amide **33a**. Treatment of **33a** with BtsCl gave **35** and methylation with excess MeI/K_2CO_3 in DMF afforded the *N*-Bts-*N*-methyl amide **36**. Finally, **32** was converted to bis-Boc derivative **33** by reaction with Boc_2O/DMAP in CH_3CN. The activated amide **33** was then treated with 1.2 equiv of the amino ester **29** in refluxing CH_2Cl_2 in the presence of DMAP for 18 h, resulting in the formation of tripeptide **38** in excellent yield (97%). The Bts cleavage under standard reaction conditions, yield the free amino ester **39**. Hydrolysis of **39** with LiOH in aqueous methanol afforded (–)-hemiasterlin (**18**).

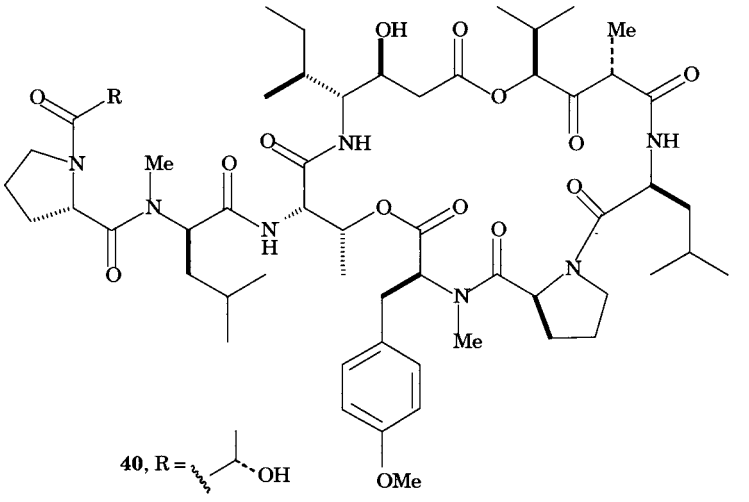


Scheme 4.

DIDEMNINS

Didemnins is one of the important classes of cyclic depsipeptides isolated from the Caribbean tunicate *Tridemnun solidum* (Rinehart Jr. *et al.*, 1981) and didemnin B (**40**) was found to be most potent anticancer agent amongst didemnins and has been selected for clinical development. Mechanistic

studies conducted on didemnin B revealed that it inhibits the synthesis of RNA, DNA and binds non-competitively to palmitoyl protein thioesterase (Rinehart Jr. *et al.*, 1981). Didemnin B (**40**) showed antitumor activity against variety of tumor cells such as breast, cervical, myeloma, glioblastoma/astrocytoma, lung and ovarian cell lines. Furthermore, preclinical study revealed that this compound exhibit potent dose dependent antitumor activity, and tolerable toxicity that provided the impetus for phase I clinical trials. The impressive *in vitro* and *in vivo* biological activities of the didemnins resulted in the first human clinical trials in the U.S. of a marine natural product against cancer (Chun *et al.*, 1986). The initial phase I clinical trials of didemnins evaluated different schedules of administration (Vera & Joullie, 2002; Dorr *et al.*, 1988). The toxicity profile of didemnins was quite similar across the trials. Nausea and vomiting were the most commonly reported dose-dependent side effects. Unfortunately, the phase II trials of didemnins B at the recommended doses were associated with poor efficacy, but trials using more aggressive regimens resulted in higher levels of toxicity, including cardiotoxicity (Shin *et al.*, 1991; Baker *et al.*, 2002). Despite potent activity against different type of cancers, the compound was too toxic, and clinical trials were terminated (Kucuk *et al.*, 2000). The experience gained from the study conducted on didemnins B helped in designing new molecules, that resulted in the discovery of more potent related molecule aplidine (**41**).



40, R = OH

41, R = O

Total Synthesis of Didemnin B (40)

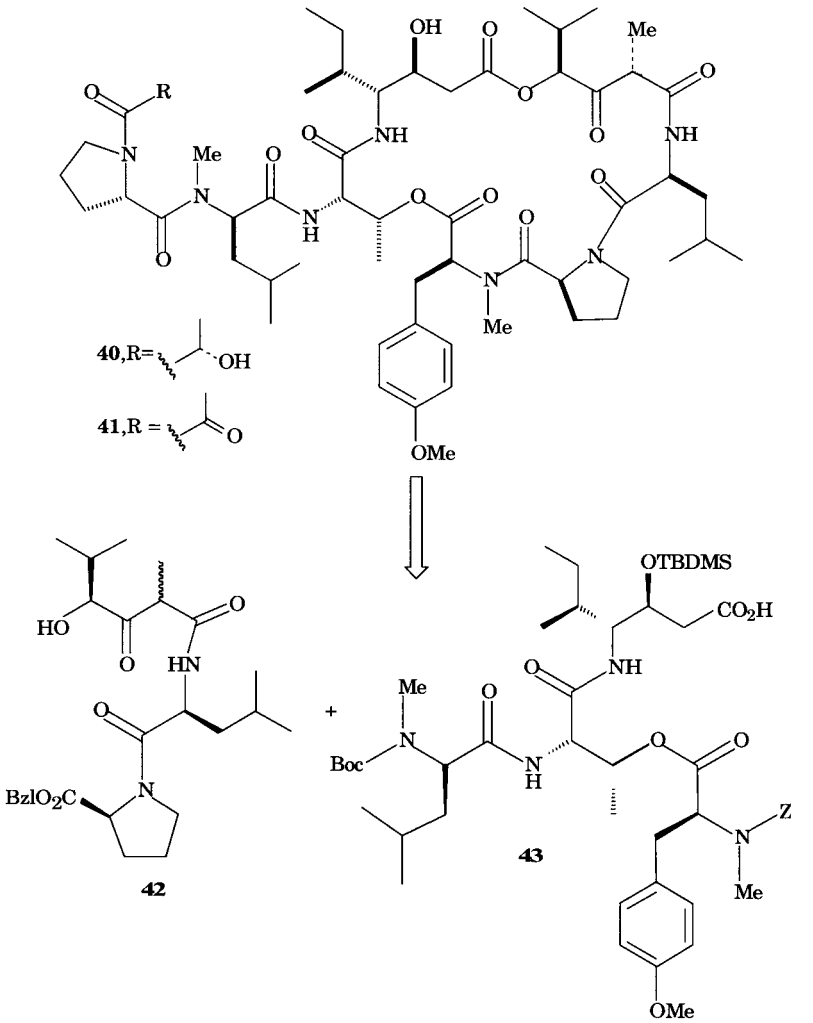
The synthetic approach leading to **40** was based upon the combination of two fragments **42** and **43** which contained the (hydroxyisovaleryl) propionyl (Hip) and isostatine (Ist) units (Scheme 5) (Nuijen *et al.*, 2000). The treatment of **44** with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) followed by alkaline treatment afforded **45,** which was condensed with Meldrum's acid by use of diethyl phosphorocyanidate (DEPC) to give **46** (Hamada *et al.*, 1989), which was further refluxed with benzyl alcohol in benzene to give the β-keto ester **47** and methylated to produce **48** (Scheme 6). The required compound **49** was synthesized according to Scheme 7.

The other required moieties were synthesized utilizing existing literature methodology. The N-methylamino acids, Boc-(R)-MeLeu-OH and Z-(S)-Me-Tyr (Me)-OH were easily prepared in 80–90% yields by treatment of Boc-(R)-Leu-OH and Z-(S)-Tyr-OH, respectively, with 3–5 equiv of NaH and 8–10 equiv of MeI in THF (Shioiri & Hamada, 1978).

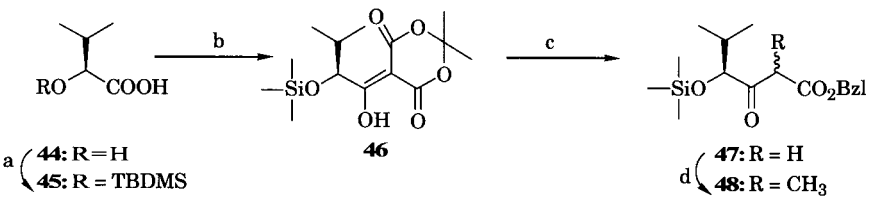
The fragment **42** was synthesized by condensing of Boc-(S)-Leu-OH with (S)-Pro-OBzl.HCl using the DEPC method, giving Boc-(S)-Leu-(S)-Pro-OBzl in 93% yield that was deprotected at the N-terminal with an acid to give HC1.H-(S)-Leu-(S)-Pro-OB (Scheme 8) (Nuijen *et al.*, 2000). The coupling of the deprotected dipeptide was done with TBDMS-(2RS,4S)-Hip-OH, obtained by the catalytic hydrogenolysis of **48**. TBDMS-(2RS,4S)-Hip-(S)-Leu-(S)-Pro-Obzl thus obtained afforded the fragment **42** after treatment with fluoride anion.

The fragment **43** was obtained from the lst derivative **49**, which, after deprotection with acid, was coupled with Boc-(S)-Thr(Bzl)-OH by the DEPC method to produce the required dipeptide. After removal of its Boc function, Boc-(R)-MeLeu-OH was introduced by the DEPC method to give the tripeptide. The hydroxyl function of the lst part was protected with TBDMS-C1. Catalytic removal of the benzyl function at the Thr part afforded Boc-(R)-Me-Leu-(S)-Thr-(3S, 4R, SS)-lst(TBDMS)-Otce that was condensed with Z-(S)-MeTyr (Me)-OH using DCC. The depsipeptide obtained quantitatively was treated with zinc powder to give the fragment **43**.

The coupling of the fragments **42** and **43** was achieved by DCC in 78% yield. Finally, didemnin B **(40)** was synthesized from didemnin A (Scheme 8). The reaction of ethyl (S)-lactate ((S)-Lac-OEt) with benzyl bromide in presence of silver oxide in DMF followed by saponification gave Bzl-(S)-Lac-OH in 76% yield. Condensation with (S)-Pro-OMe.HCl by the DEPC method yielded Bzl-(S)-Lac-(S)-Pro-OMe that was quantitatively hydrolyzed with 1 N aqueous NaOH in methanol. The coupling of the product with didemnin **A** by use of Bop-C1 followed by the transfer hydrogenation afforded didemnin B.

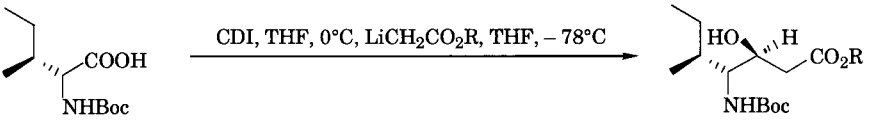


Scheme 5.



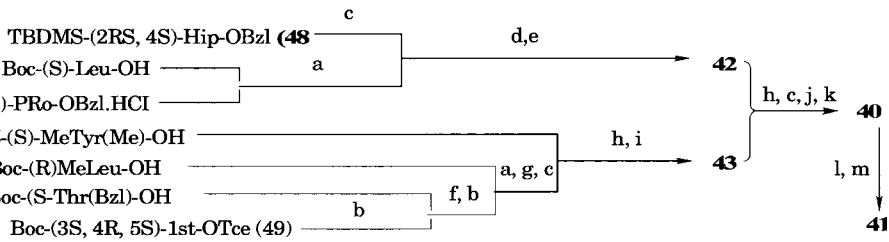
(a) TBDMS-Cl, imidazole, DMF, RT, 22 h, aq. K_2CO_3, RT, 4 h, 82%; (b) Meldrum's acid, DEPC, Et_3N, THF, 0°C, 2 h, RT, 20 h; (c) $PhCH_2OH$, benzene, reflux, 3 h, 69%; (d) MeI, NaH, RT, 20 h, 77%.

Scheme 6.



49: R = CI_3 CCH_2

Scheme 7.



(a) DEPC, Et_3N, DMF, 0°C, 4 h, RT, 20 h; (b) 4N HCl/dioxane, RT, 1 h; (c) H_3, 5% Pd-C, THF, RT, 2–3 h; (d) DCC, 1-hydroxybenzotriazole, N-methylmorpholine, THF/DMF (2:1), 0°C, 3 h, RT, 26 h, 79%; (e) $Bu_4N^+F^-$, THF, RT, 1 h, 90%; (f) DEPC, i-Pr_2EtN, DMF, 0°C, 4 h, RT, 20 h, 31%; (g) TBDMS-Cl, imidazole, DMF, RT, 37 h, 72%; (h) DCC, DMAP, CH_2Cl_2, 0°C, 3 h, RT, 16-22 h; (i) Zn, 1M, NH_4OAc (aqueous), THF, RT, 30 h, 89%; (j) Bop-Cl, Et_3N, CH_2Cl_2, 2°C, 3 days, 68%; (k) TMSOTf, CH_2Cl_2, 0°C, 3 h, 98%; (l) Bzl-(S)-Lac-(S)-Pro-OH, Bop-Cl, Et_3N, 2°C, 23 h; (m) Pd, HCO_2H, MeOH, 49%.

Scheme 8.

APLIDIN

Aplidin (dehydrodidemnin B, **41**) was isolated from the Mediterranean tunicate, *Aplidium albicans* (Cheung & Benoiton, 1977). PharmaMar reported the antitumor property of aplidin in 1996 (Rinehart & Lithgow Bertelloni, 1990). Aplidin was found to be active against solid tumors, and non-Hodgkins lymphoma. Detailed mechanistic studies conducted on aplidin revealed that it interferes with DNA synthesis, proteins and induces G1–G2 cell cycle arrest (Garcia-Fernandez *et al.*, 2002). Aplidin exhibits cyctotoxicity due to its ability to inhibit the ornithine decarboxylase, an enzyme that is critical in the process of tumor formation and angiogenesis. Recently, Taraboletti *et al.* (2002) showed that aplidin also inhibits the expression of the vascular endothelial growth factor gene, having antiangiogenic effects. In preclinical studies, aplidin was found to be more active than didemnins. Aplidin also exhibited substantial activity against a variety of solid tumor models, including tumors noted to be resistant to didemnins (Taraboletti *et al.*, 2004). On the basis of promising preclinical data aplidin entered into phase I and phase II clinical trials in Spain, Canada, UK, and France for the treatment of solid tumors, and non-Hodgkins lymphoma utilizing different schedules of administration (Faivre *et al.*, 2005; Rinehart, 2000; Caers *et al.*, 2008; Cuadrado *et al.*, 2003). Preclinical studies revealed that treatment with aplidin has generally been

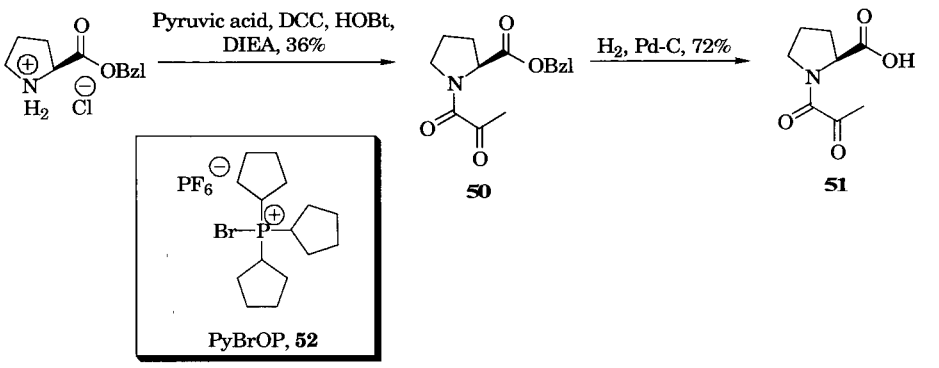
well tolerated. The most common side effects were asthenia, nausea, vomiting, transient transaminitis and hypersensitivity reactions. Interestingly aplidine does not induce hematological toxicity, mucositis or alopecia. The main dose-limiting factor was the occurrence of neuromuscular toxicity with the elevation of creatine kinase levels (Caers *et al.*, 2008; Cuadrado *et al.*, 2003). Aplidin selectively target and preferentially kill human leukemic cells in blood samples taken from children and adults. In these studies, it was observed that aplidin was more selective towards leukemia and lymphoma cells than towards normal cells. Recently this compound entered into Phase II clinical trials. Phase II trials are going on in Europe and Canada covering renal, head and neck, and medullary thyroid. The mode of action of this novel molecule is not yet known, but it appears to block VEGF secretion and blocks the corresponding VEGF-VEGF-Receptor-1 autocrine loop in leukemic cells (Erba *et al.*, 2002). Antimyeloma effect of aplidin was studied in the syngeneic 5T33MM model. *In vitro* results revealed that aplidin inhibits 5T33MMvv DNA synthesis with an IC_{50} of 3.87 nM. *In vivo* experiment was carried out by treating mice injected with 5T33MM, C57Bl/KaLwRij with vehicle or aplidin (90 μg kg^{-1} daily). These *in vivo* data indicated that aplidin is well tolerated and its antitumour and antiangiogenic effects support the use of the drug in multiple myeloma (Le Tourneau *et al.*, 2007).

Synthesis of Dehydrodidemnin B (Aplidin, 41)

The total synthesis of dehydrodidemnin B (Aplidin, **41**) has been achieved (Sakai *et al.*, 1996; Cuevas *et al.*, 2002). One such method requires the coupling of the (*S*)-Pro-Pyr side chain **51** to the amino group of the (*R*)-N(Me)-Leu residue of didemnin A (Cuevas *et al.*, 2002). The total synthesis of didemnin A is already discussed (Scheme 8). The side chain **51** was synthesized as shown in Scheme 9. The coupling that led the amide bond formation between H-Pro-Obzl and pyruvic acid was achieved using DCC in the presence of 1-hydroxybenzotriazole (HOBt), giving ester **50**, which was subsequently submitted to catalytic hydrogenolysis, producing **51**. The reaction of didemnin A with **51** using PyBrOP (**52**) afforded dehydro-didemnnin B (**41**) in a yield of 62% (Jou *et al.*, 1997).

KAHALALIDE F (53)

Kahalalide F (**53**), a depsipeptides has been isolated from the sacoglossan mollusk *Elysia rufescens* Pease 1871 (Hamann *et al.*, 1993; Faircloth & Cuevas, 2006), and an alga, a *Bryopsis* sp. (Bryopsidaceae) on which the mollusk feeds. Other depsipeptides such as kahalalide A-E, O (Horgen *et al.*, 2000), and liner peptide H, J (Hamann *et al.*, 1993) were isolated from *E. rufescens*, while kahalalide G was found in the diet of the animal. Kahalalide F (**53**) was isolated in poor yield from the animal, and the alga collected at the same site, and was found to be most active peptide in the



Scheme 9.

series (Horgen *et al.*, 2000; Rademaker-Lakhai *et al.*, 2005; Hamann *et al.*, 1996). The structure of kahalalide F (**53**) was initially determined by Hamann *et al.* (1993), and subsequent reinvestigation of stereochemical assignment of the originally proposed structure, confirmed that valine 3 should be D-valine and valine 4 should be L-valine, rather than the reverse as reported earlier (Bonnard *et al.*, 2003; Goetz *et al.*, 1999). Kahalalide F exhibits potent activity against human colon and lung cancers, it also shows activity against some pathogenic microorganisms that causes opportunistic infections of HIV/AIDS. Actual mode of action of this compound had not yet been fully determined, but it is known that kahalalide F target lysosomes (Jimeno *et al.*, 2004) and has selectivity for tumor cells such as prostate tumor. Kahalalide F (**53**) exhibits potent *in vitro* cytotoxic activity against various cell lines such as prostate, breast, colon carcinomas, neuroblastoma, chondrosarcoma, and osteosarcoma (Nuijen *et al.*, 2001; Garcia-Rocha *et al.*, 1996) with IC_{50} ranging from 0.07 mM (PC3) to 0.28 mM. Importantly, nontumor human cells such as MCF10A, HUVEC, HMEC-1, and IMR90 were found to be 5–40 times less sensitive to the drug (IC_{50} = 1.6–3.1 mM). Kahalalide F also exhibits potent cytotoxicity against human tumor specimens such as breast, colon, non-small cell lung, and ovarian carcinomas and has also shown *in vivo* activity against human prostate cancer xenografts (Sewell *et al.*, 2005). Flow cytometry analysis confirmed that kahalalide F induced neither cell-cycle arrest nor apoptotic hypodiploid peak and confocal laser and electron microscopic study revealed that kahalalide F treated cells underwent a series of profound alterations.

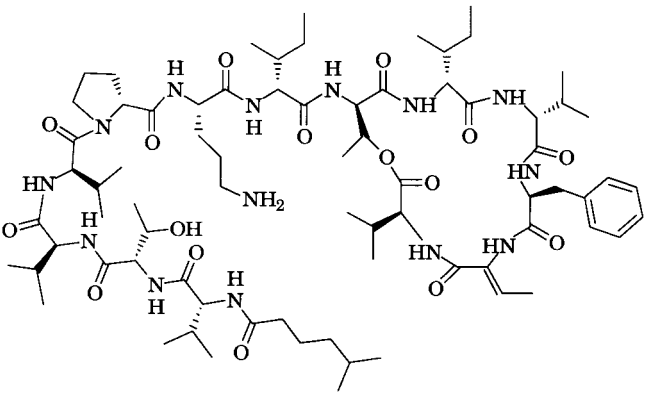
The compound was licensed to PharmaMar by University of Hawaii in the 1990's, and preclinical studies were conducted. On successful completion of preclinical studies, in December 2000 this compound entered in phase I clinical trials in Europe for the treatment of androgen independent prostate cancer (Medina *et al.*, 2001). Phase I clinical and pharmacokinetic study of Kahalalide F, administered weekly as a 1 h infusion to patients with advanced solid tumors have also been performed (Mealy & Bayes, 2005). The

maximum tolerated dose was found to be 800 $\mu g/m^2$. The dose-limiting toxicities with weekly kahalalide F, 1 h *iv* infusions were transient grade 3/4 increases in blood transaminase levels, and 650 $\mu g/m^2$ was declared as the recommended dose for phase II studies. The experimental evidence also suggested that kahalalide F may be active against more tumor types than was originally thought based on preclinical studies. The combination of both low toxicity and early evidence for clinical benefit in pretreated patients supported that kahalalide F may have a favorable therapeutic index and, therefore, deserves further clinical testing either as single agent or in combination.

In the preclinical developments, the compound was administered to male and female rats (*iv*) in multiple doses (daily for 5 days) and dose dependent toxicities were determined. Physiological changes such as clinical signs and body weight of the rats during this procedure were kept under observation. After the completion of the experiment, animals were necropsied, clinical signs and organ weights were recorded, and tissues were examined microscopically. It was observed that kahalalide F produced lethality at 375 and 450 mg/kg in males and females respectively, and the maximum tolerated dose (MTD) was determined to be 300 mg/kg (1800 mg/m^2). The nervous system was found to be a potential site of action for the lethality of this compound. In the organ toxicity, kidney was found to be the main target at dose level of 150 and 300 mg/kg. High dose of kahalalide F (300 mg/kg) caused necrotizing inflammation of bone marrow and peritrabecular osteocyte hyperplasia of bone on day four, which recovers thereafter. Due to local cytotoxicity, injury to blood vessels and surrounding tissue at the injection site was observed. When kahalalide F was administered once daily for five days at a dose of 80 mg/kg per day (400 mg/kg total dose), gain of slightly decreased body weight was the main drug related effect. Therefore, this drug is considered to be safe at or near 80 mg/kg per day. These results demonstrated that daily administration of kahalalide F for 5 days reduced drug-induced toxicity, so this compound was taken up for further clinical evaluation for the treatment of cancer. Currently this compound is in the phase II clinical trials for the treatment of prostate cancer (Brown *et al.*, 2002). Recently, conducted studies demonstrated that kahalalide F induces cell death *via* "oncosis" possibly triggered by lysosomal membrane depolarization in both prostate and breast cancer cell lines (Brown *et al.*, 2002).

The results of the phase II clinical study of kahalalide F in patients with advanced NSCLC, hepatocarcinoma (HC), and advanced malignant melanoma (AMM) have been reported at the 31st European Society for Medical Oncology Congress (ESMO), revealing an excellent tolerability profile with no serious adverse events (Pardo, 2008; Auld, 2008). A positive response and stable disease were observed in a number of patients. A Phase

II trial for the treatment of patients with severe psoriasis is also ongoing (Suarez *et al.*, 2003).



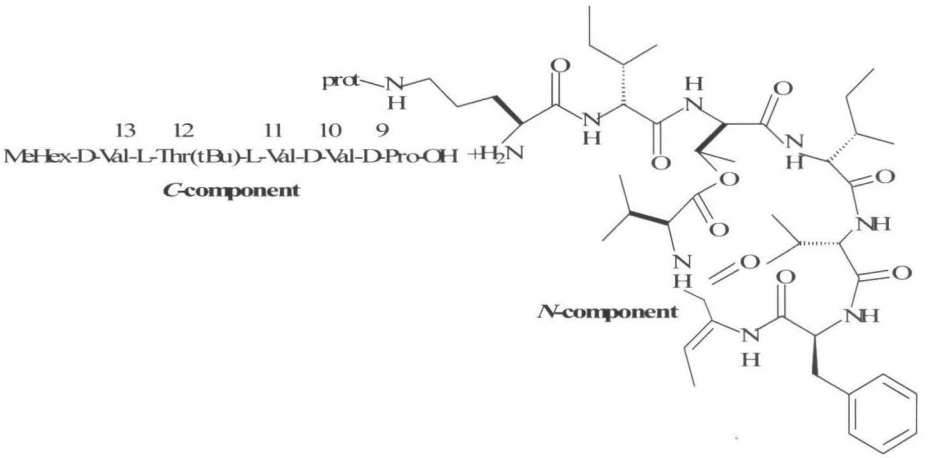
53

Synthesis of Kahalalide F

The compound was synthesized by linear solid phase peptide synthesis techniques involving cyclization and final deprotection performed in solution phase (Izquierdo Delso, 2004; Lopez *et al.*, 2005; Gracia *et al.*, 2006). Later several new convergent strategies for the synthesis of kahalalide F derivatives were acheived (Lopez-Macia *et al.*, 2001). Convergent strategies are defined as those in which peptide fragments are coupled to give the desired target molecules (Hamada & Shioiri, 2005). For the total synthesis of kahalalide F, different strategies were adopted. The most convenient synthetic strategy involves dividing the peptide in two fragments, the *C*-component and *N*-component (Scheme 10).

C-Component Synthesis

The main advantage of this strategy is that the hardly racemizable Pro is the *C*-terminal amino acid for the *C*-component. The fragment was synthesized on the CTC resin, starting with a limited incorporation of the first 9-fluorenylmethoxycarbonyl (Fmoc)-amino acid in the presence of *N, N*-diisopropylethylamine (DIEA) (Barlos & Gatos, 2000). The remaining resin chloride functions were capped with methanol (MeOH) to prevent the formation of tertiary amines after treatment with piperidine to remove the Fmoc group. The Fmoc-protecting group was removed with piperidine-*N, N*-dimethylformamide (DMF) (1:4) and the peptide chain was elongated with *N, N*-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. Finally, the peptides were cleaved from the resin with TFA-CH_2Cl_2 (1:99), characterized by HPLC and ESMS, and purified by semi preparative HPLC.



Scheme 10.

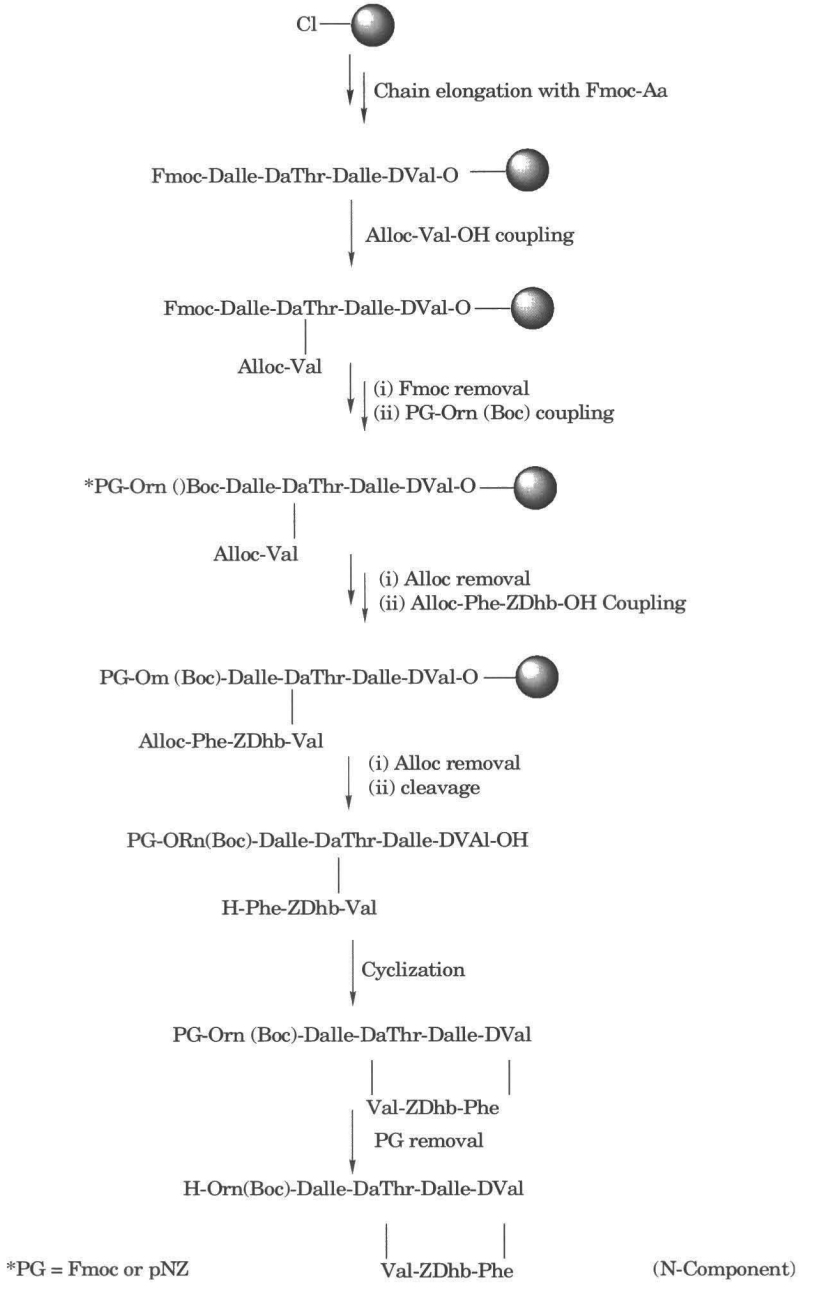
N-component Synthesis

The synthesis of *N*-component was based on the solid-phase synthesis of a branched peptide using an orthogonal protecting scheme and subsequent cyclization and deprotection of the *N*-terminal function in solution. The given strategy (Scheme 11) started with the incorporation of Fmoc-D-Val OH on the CTC resin, followed by elongation until the D-*allo*-Ile, esterification of the β-hydroxyl of the D-*allo*-Thr with Alloc-Val-OH. The incorporation of protected Orn-(Boc) residue on the main chain is then followed by the incorporation of the Alloc-Phe-*Z*Dhb-OH on the branched one.

Condensation of *C*-component and *N*-component

The coupling methods were standardized, which included the DIPCDI/7-aza-1-hydroxybenzotriazole (HOAt) (1.5 equiv each); 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC)/HOBt (3 equiv each); EDC/HOBt/*N*, *N*-(dimethylamino) pyridine (DMAP) (4 equiv, 4 equiv, 0.4 equiv); (7-azabenzotriazol-1-yloxy)-tris (pyrrolidino) phosphonium hexafluoro-phosphate (PyAOP)/DIEA (1 equiv, 3 equiv) + PyAOP (1 equiv after 1 h), and 1-[bis (dimethylamino)-methylene]-1*H*-1, 2, 3-triazolo-[4, 5-*b*] pyridinium hexafluorophosphate 3-oxide (HATU)/DIEA (1 equiv, 3 equiv) (Izquierdo Delso, 2004). On the basis of this screening, it was concluded that the method based on PyAOP gave the best results in terms of yields and the absence of side products in the HPLC chromatograms. Phosphonium derivatives such as PyAOP are more convenient for slow couplings compared with aminium/uronium reagents such as HATU, because the latter can terminate the peptide chain through a guanidination reaction (Albericio *et al.*, 1998). Furthermore, PyAOP contains HOAt, which is the most reactive benzotriazole. Each linear *C*-component was then condensed with its corresponding *N*-component in solution phase using PyAOP and DIEA. The

three protected peptides obtained were treated with TFA-H_2O (9:1) to remove the Boc and *t*-Bu group yielding kahalalide F.



Scheme 11.

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2

Microwave-Assisted Non-Conventional Esterification of Polysaccharides

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ABSTRACT

Amphiphilic polysaccharides attracted increased attention due to their multifunctionality, including surfactant properties. A brief review of previous and current studies on polymeric surfactants prepared from carboxymethyl starch, carboxymethyl cellulose, hydroxyethyl cellulose and beechwood glucuronoxylan by non-conventional esterification methods is presented. Transesterification of methyl and vinyl esters of lauric acid and fatty acid methyl esters of rape seed oil as well reaction with rape seed oil were applied, and the reactions performed in various media with and without catalysts and under 'solvent free' conditions. Microwave irradiation was used as heating source and for comparison experiments were performed by conventional heating. The obtained water-soluble amphiphilic polysaccharides showed a very low degree of esterification indicated by FT-IR spectroscopy. In spite of moderate surface tension-lowering effects, the derivatives exhibited excellent emulsifying efficiency for 'oil in water' type emulsions as well as good detergent performance properties, depending on the polysaccharide type. By microwave heating, the reaction time was significantly shortened from hours of conventional heating to several minutes, and in some cases the esterification was efficient at lower reaction temperatures. The polysaccharide derivatives represent biodegradable polymeric surfactants with potential applications in manufacture of consumer products and auxiliary materials for various technical and non-technical areas.

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Key words : Fatty acid methyl ester, microwave irradiation, surface activity, polymeric surfactants, transesterification

INTRODUCTION

During the last decades, a fast growing interest in natural, biodegradable and renewable materials has been noticed. As the current use of non-biodegradable surfactants is a drawback, there is a need to develop new families of 'green' surfactant molecules derived from plant resources. Carbohydrate-derived surfactants are of importance because they are potential substitutes of petroleum-derived synthetic products (Paik & Swift, 1995; Burczyk, 2002; Piasecki, 2002). Polysaccharides are attractive as natural and sustainable polymeric resources, which can be modified for special performance in various commercial applications. The covalent anchoring of hydrophobic side-chains in water-soluble polysaccharides affords water-soluble amphiphilic derivatives, that usually exhibit associative as well as surface active properties, giving liquids to control foaming or emulsion stability (Landoll, 1982; Sarkar, 1984; Schick, 1987). A series of water-soluble amphiphilic polysaccharide derivatives have been prepared from several commercial and non-commercial polysaccharides such as from hydroxyethyl cellulose (Landoll, 1982; Talába *et al.,* 1996; Sroková *et al.,* 2003), carboxymethyl cellulose (Sroková *et al.,* 1998, 2004; Cao *et al.,* 2002), carboxymethyl starch (Sroková *et al.,* 2001; Žoldáková *et al.,* 2005) and xylans (Skalková *et al.,* 2006). The derivatives exhibited surface-activity as well as surfactant performance properties.

Hydrophobization of various polysaccharides with long acyl chains to various degree of substitution has been usually realized by classical esterification with fatty acid chlorides (C_4–C_{18}) in different solvent systems (Sun *et al.,* 2000; Fang *et al.,* 2001). Recently, various alternative esterification methods have been developed aimed to avoid the use of the toxic acid halides, such as the esterification with mixed anhydrides prepared *in situ* and transesterification using fatty acid methyl or vinylesters in homogeneous and heterogenous conditions as well as under 'solvent free' conditions (Rooney, 1976; Vaca-Garcia *et al.,* 1999; Heinze *et al.,* 2000; Aburto *et al.,* 2005). Since fatty acid esters are found in nature as triacyl glycerides, attempts have been made to use them as non-expensive acylation reagents (Aburto *et al.,* 2005).

Nowadays attention has been paid to the new heating system—microwave radiation in organic and polymer chemistry (Galema, 1997; Koroskenyi & McCarthy, 2002; Bogdal *et al.,* 2003; Corsaro *et al.,* 2004) and has been reported to be very effective in polysaccharide chemistry as well (Galema, 1997; Lewandowicz *et al.,* 2000; Antova *et al.,* 2004). The most important advantage is that microwave-enhanced chemical reactions

are very rapid, lasting usually a few minutes, compared to h with classical heating methods. There are only few reports concerning the application of microwave heating in the esterification of cellulose and starch (Gourson *et al.,* 1999; Lewandowicz *et al.,* 2000; Satgé *et al.,* 2002; Shogren & Biswas, 2006).

The aim of this paper was to report on our previous and current studies concerning the preparation of polymeric surfactants from CMC, HEC, CMS and beechwood glucuronoxylan (GX) using non-conventional esterification methods and microwave radiation with controlled power as heating source. The results will be compared to corresponding polysaccharide derivatives prepared under classical heating.

MATERIALS AND METHODS

CMS (DS 0.3) was a gift from Prof. Th. Heinze (Friedrich-Schiller University of Jena, Germany). HEC, Tylose H 4000/P2 (DS 1.0, MS 2.5) was purchased from Hoechst, Frankfurt am Main (Germany) and CMC (DS 1.0) from Sigma–Aldrich, Steinheim (Germany). Beechwood glucuronoxylan (GX) was produced at the pilot plant of the Institute of Chemistry, SAS (Bratislava, Slovakia). It contains of xylose (87% related to neutral sugars) and 11.4% methylglucuronic acid, corresponding to a DS_{COOH} of ~ 0.1. Vinyl laurate (VL), sodium dodecyl sulfate (SDS), 4-toluenesulfonic acid (TSA) and Tween 20 (oxyethylated sorbitol monolaurate) were commercial products from Sigma-Aldrich Chemie (Steinheim, Germany). The methyl ester of lauric acid (ML) was kindly provided by Prof. Cvengroš (Slovak Technical University, Bratislava, Slovakia). The mixture of fatty acid methyl esters (MERO) prepared from a by-product of rape seed oil production was from VÚTCH-Chemitex, s.r.o. (Žilina, Slovakia). It contains oleic acid (58%), linoleyl acid (17%), linolenyl acid (17%), and saturated C_{14-20} fatty acids (8%). Rape seed oil (RO) was purchased from PALMA-TUMYS, a.s., Bratislava (Slovakia). All other chemicals used were of analytical grade.

FT-IR spectra were obtained by the NICOLET Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software with resolution of 4 cm^{-1} in KBr pellets.

The esterification reactions were carried out in the domestic microwave oven (SENCOR DAEWOO Electronics, maximum power 1000 W) without a pre-heating step. Some reactions were carried out in the microwave reactor Milestone (maximum power 1000 W) equipped with a magnetic stirrer (Sorisole, Italy) by a two-step procedure. It consists of a preheating stage (to reach the reaction temperature for 5 min) and following heating stage (starting the count of reaction time at the selected temperature). The application of microwave radiation is described in more detail in a current paper (Tomanová *et al.,* 2008).

Testing Methods

The surface tension of aqueous solutions of the derivatives (concentration range 0.015–5.0 kg/m^3) was determined at 25°C using the Du Nouy ring method and from the obtained concentration dependence the minimum surface tension (γ_{min}) and critical micelle concentration (c.m.c.) were determined graphically as described in a previous paper (Žoldáková *et al.*, 2005).

The emulsifying efficiency was tested on emulsions of the 'oil in water' (O/W) type. They were prepared by mixing 9 mL of water containing 0.05 g of the tested derivative and 1 mL of paraffinic oil dyed with SUDAN IV in a laboratory device (Heidolph DIAX 900) at 22 400 rpm for 1 min The stability of the emulsion was estimated at three different time intervals after the emulsions had been prepared, *i.e.* 5 min (h_1), 1 h (h_2), and 24 h (h_3) and expressed in terms of the height (mm) of oil and cream layers formed on the surface of the emulsion (Žoldáková *et al.*, 2005).

The performance properties—washing power (WP) and antiredeposition efficiency (ARE) of selected derivatives were tested at concentration 0.5 kg/m^3 (WP) and 5 kg/m^3 (ARE) by methods described in more detail by Stüpel (1957) and Žoldáková *et al.* (2005) using a model soil and cotton fabric (SK-standard 800 101). WP was assessed by determining the reflectance of an artificially soiled cotton fabric after washing in an alkaline surfactant bath (2 g/L Na_2CO_3). The washing time for WP was 10 min and for ARE 60 min; the washing temperature was 80°C in WP and 60°C in ARE tests.

Esterification Methods

The esterification of the polysaccharides using microwave radiation as heating source and conventional heating was performed using various reaction media and procedures:

(i) DMF/TSA, DMF/TSA—The polysaccharide was pre-activated by dissolving in the aprotic solvent containing TSA at 50–60°C for 30 min yielding the 'gel suspension' consistency (Vogt *et al.*, 1996). In some experiments the dissolution was performed without addition of TSA for 2 h. Thereafter the suspension was subjected to the esterification reaction at the selected conditions. The reaction product was poured into 4–6 volumes of ethanol. The precipitate was separated by filtration, thoroughly washed with ethanol, and extracted with ethanol in a Soxhlet apparatus for 6–8 h. In case of DMF the polysaccharide was dissolved at 50–60°C for 2–3 h, and further treated as described (Žoldáková *et al.*, 2005; Sroková *et al.*, 2004).

(ii) H_2O/K_2CO_3—The polysaccharide was dissolved in distilled water containing K_2CO_3 as catalyst according to Mormann and Al-Higari

(2004). The acylation agent was added and the mixture homogenized at 30,500 rpm for 30 s. Then, it was exposed to MWH in the domestic microwave oven SENCOR or the microwave reactor Milestone. The reaction product was poured into 8–10 volumes of acetone and the formed precipitate separated by filtration, thoroughly washed with acetone and extracted in Soxhlet apparatus with ethanol for 8 h. In the 'solvent-free' experiments the polysaccharide (CMS) was suspended in distilled water, containing sodium acetate as catalyst and the acylation agent was added. The reaction mixture was homogenized for 1 min at 22 400 rpm, followed by heating at 130°C for 30 min in order to evaporate the water. Then, the temperature was adjusted to 177°C reaction under conventional heating.

(iii) H_2O/DMF—The polysaccharide was dissolved in distilled water and stirred for 1 h at room temperature. Then, to the solution various amounts of the acylation agents dispersed in DMF were added and the reaction mixture homogenized at 30 500 rpm for 30 s. Afterwards it was exposed to MWH for 1 to 2 min, whereby the temperature increased to the reaction temperature (80–100°C). At this temperature, the reaction proceeded for 1–2 min In case of 'solvent-free' conditions, the reaction mixture after homogenization was heated at 130°C for 30 min to evaporate the water. In case of the esterification using rape seed oil (RO), sodium laurate and RO in DMF were added to the aqueous polysaccharide solution and after homogenization the water was evaporated as mentioned above. In one experiment, no DMF was added to the aqueous solution containing the acylation agent. The recovery of the derivatives from the reaction mixture was performed as described in (ii).

The derivatives of HEC were water-soluble and those of GX, CMS and CMC only in the carboxylate form of the glucuronic acid or the carboxymethyl substituents.

RESULTS AND DISCUSSION

The partial hydrophobization of HEC, CMS, CMC and GX was carried out in heterogeneous or homogeneous systems by varying the mass ratio, reaction time and power of microwave radiation, and the results compared with experiments performed under to CH as well as by the 'solvent- free' methods. The mass ratios of polysaccharide to acylation agents varied from 2:1 to 1:4. The reaction time varied from 1 to 5 min under microwave heating (MWH) and from 0.5 to 24 h using classical heating (CH), and the reaction temperature ranges were 80–135°C and 20–177°C, respectively. The reaction conditions, yield and surface active properties of the prepared polysaccharides derivatives are summarized in Tables 2.1–2.4.

Due to different structural features of the studied polysaccharides, which were ionic (CMS, CMC and GX) and non-ionic (HEC), differing mainly in

water solubility and, therefore, the most appropriate reaction media of the particular polysaccharide were selected. In case of CMS, which was partially soluble in water, the H_2O/K_2CO_3 system was applied for the transesterification using the VL, ML and MERO agents (Table 2.1). In case of VL, the positive effect of microwave heating (MWH) in comparison to the conventional heating (CH) was reflected by the reaction time, which decreased from 300 min of CH to 3 min of MWH at about 100°C, whereby the yields and achieved surface tension of the derivatives were similar. The effect was much higher, when for comparison the reaction was performed in the DMF/TSA medium using CH. Because high reaction temperatures were found to cause polysaccharide degradation in this medium (Žoldáková *et al.*, 2005), the reaction was performed at room temperature for 20 h.

Table 2.1. Reaction conditions of transesterification of carboxymethyl starch (CMS) using various acylation agents (A, B, C) and yield and surface active properties of the obtained fatty acid derivatives

Sample	CMS/AcA g/g	Reaction medium	MW power (W)	Temp. (°C)	Time (min)	Yield (g/g)	γ_{min} mN/m	c.m.c. g/L
CMS-A1	1 : 2	H_2O/K_2CO_3	600[a]	99	3	0.74	54.4	1.23
CMS-A2	1 : 4	H_2O/K_2CO_3	600[a]	97	3	0.80	53.4	1.13
CMS-A3	1 : 2	H_2O/K_2CO_3	CH	90	300	0.87	59.4	1.32
CMS-A4	1 : 4	H_2O/K_2CO_3	CH	100	300	0.96	55.1	0.55
CMS-A5[d]	1 : 1	DMF/TSA	CH	20	20 h	0.93	52.2	0.63
CMS-B1	1 : 2	H_2O/K_2CO_3	600[a]	92	2	0.61	54.2	0.45
CMS-B2	1 : 4	H_2O/K_2CO_3	600[a]	135	3	0.94	nd	nd
CMS-B3	1 : 4	Solvent-free[c]	CH	177	60	0.99	47.8	2.51
CMS-C1	1 : 2	H_2O/K_2CO_3	500[b]	99	3	0.40	60.4	No
CMS-C2	1 : 2	H_2O/K_2CO_3	CH	97	300	0.82	51.7	0.49
CMS-C3	1 : 4	Solvent-free[c]	CH	177	60	0.88	58.6	1.23

AcA, Acylation agents: vinyl laurate (A), methyl laurate (B) and MERO (C);

CH, Conventional heating;

[a] Microwave oven-SENCOR; [b] Microwave reactor-Milestone; [c] H_2O/CH_3COONa, evaporation of water at 130°C; [d] Žoldáková *et al.*, 2005.

The experiments with ML in the H_2O/K_2CO_3 system under MWH were compared with the reaction under 'solvent-free' conditions performed at 177°C by CH. Both the reaction time and temperature were lowered and similar yields were obtained. The surface tension depressing effect was somewhat lower in the MWH-assisted experiments. In case of the MERO-CMS derivatives prepared in the H_2O/K_2CO_3 medium, the reaction time was lowered using MWH. However, the yield of the derivative (CMS-C1) was very low indicating significant polysaccharide degradation and loss of material. A further advantage to use the H_2O/K_2CO_3 medium was that the transesterification reaction proceeded without the aprotic solvent, which has been generally used to enhance the reactivity of the polysaccharide by forming a 'gel suspension' (Vogt *et al.*, 1996).

Table 2.2. Reaction conditions of transesterification of carboxymethyl cellulose (CMC, DS = 1.0) using various acylation agents (A, C) and yield and surface active properties of the obtained fatty acid derivatives

Sample	CMS/AcA g/g	Reaction medium	MW power (W)	Temp. (°C)	Time (min)	Yield (g/g)	γ_{min} mN/m	c.m.c. g/L
CMC-A1	1 : 2	DMSO/TSA	300[b]	100	5	0.98	nd	nd
CMC-A2	1 : 4	DMSO/TSA	300[b]	90	5	1.03	64.6	0.63
CMC-A3[e]	2 . 1	DMSO/TSA	CH	40	180	0.75	59.9	No
CMC-C1[f]	1 : 2	DMF/TSA	500[b]	90	5	0.90	57.6	2.88
CMC-C2[f]	1 : 2	H_2O/DMF	300[b]	90	1	1.03	46.6	2.45
CMC-C3[f]	1 : 2	H_2O/DMF[g]	300[b]	90	3	1.02	49.9	0.74

TSA, 4-toluenesulfonic acid; [e] Sroková *et al.* 2004 (CMC, DS = 0.5); [f] Tomanová *et al.*, 2008; [g] K_2CO_3 as catalyst. For other footnotes see Table 2.1.

The esterification of CMC using VL was realized in the DMSO/TSA system (Table 2.2). In comparison to the experiment by CH, performed due to the above mentioned problems at a temperature of 40°C, *viz*-a-*viz* esterification under MWH performed in the microwave reactor, the reaction time was reduced from 180 min to 2–5 min, however, the reaction temperature was higher (~ 100°C) in MWH conditions. The yields of the derivatives were higher in the MWH-experiments, but the surface tension values were similar. The effect of different reaction media containing aprotic solvents on the reaction of CMC using MERO and MWH with different power was evaluated at constant CMC/MERO mass ratios, reaction temperatures and time between 1–5 min As seen, there were no differences in the yield of the derivatives, but lower surface tension values were achieved at 300 W. The addition of the catalyst had no effect on the yield and surface tension values of the derivatives (CMC-C2 and CMC-C3).

HEC was subjected to transesterification reaction of ML and MERO in the H_2O/DMF medium performed in the microwave reactor at controlled power of 660 W in the temperature range 80–96°C for 1–2 min (Table 2.3). The HEC/ML or MERO mass ratios varied from 2:1 to 1:2. Whereas the yield of the ML-HEC derivative was the lowest at the highest mass ratio, in case of the MERO-HEC the lowest yield was obtained at the lowest mass ratio. Somewhat lower surface tension values were observed with the ML-HEC derivatives.

Because the rape seed oil (RO) represents a mixture of triacyl glycerides, it was used as acylation agent as well (Table 2.3). The reaction was performed under MWH in the H_2O/DMF medium containing kalium laurate as catalyst at ~ 90°C for 2 min The same yield and surface tension values were observed at the HEC/RO mass ratios 2:1 and 1:1. When the raction was performed at the higher mass ratio without DMF or under 'solvent-free' conditions at 90°C and 177°C for 2 and 5 min, respectively, the derivatives showed about the same yields as obtained in the H_2O/DMF medium, but their surface tension lowering effect was much lower.

Table 2.3. Reaction conditions of transesterification of hydroxyethyl cellulose (HEC) using various acylation agents (B, C, D), and yield and surface active properties of the obtained fatty acid derivatives

Sample	HEC/AcA g/g	Reaction medium	MW power (W)	Temp. (°C)	Time (min)	Yield (g/g)	γ_{min} mN/m	c.m.c. g/L
HEC-B1	2 : 1	H_2O/DMF	660	84	1.5	1.00	47.3	1.25
HEC-B2	1 : 1	H_2O/DMF	660	80	2	1.01	46.2	No
HEC-B3	1 : 2	H_2O/DMF	660	90	2	0.77	50.7	No
HEC-C1	2 : 1	H_2O/DMF	660	93	1	0.69	60.2	0.13
HEC-C2	1 : 1	H_2O/DMF	660	96	2	1.06	49.8	0.03
HEC-C3	1 : 2	H_2O/DMF	250[b]	100	1	0.95	50.9	1.25
HEC-D1	2 : 1	H_2O/DMF[h]	660	85	2	0.81	50.0	0.62
HEC-D2	1 : 1	H_2O/DMF[h]	660	90	2	0.86	51.0	No
HEC-D3	1 : 1	No DMF	660	90	2	0.73	66.7	0.04
HEC-D4	1 : 1	Solvent-free[l]	660	177	5	0.86	66.5	No

D, Rape seed oil; [h] LaCOOK as catalyst; [l] H_2O/DMF/LaCOOK, evaporation of water at 130°C. For other footnotes see Table 2.1.

As GX is partially soluble in water, various reaction media were used (Table 2.4). The reaction with VL under MWH at the same reaction conditions in DMF with and without addition of sodium acetate as catalyst yielded derivatives with the same yield and surface active properties. Experiments performed in DMF under CH yielded derivatives with lower surface tension values, and the yield was particularly low with the derivative (GX-A4) obtained after 24 h at room temperature. Anyway, the application of MWH reduced the reaction time, but the applied reaction temperature was higher.

Table 2.4. Reaction conditions of transesterification of beechwood xylan (GX) using various acylation agents (A, B, C) and yield and surface active properties of the obtained fatty acid derivatives

Sample	HEC/AcA g/g	Reaction medium	MW power (W)	Temp. (°C)	Time (min)	Yield (g/g)	γ_{min} mN/m	c.m.c. g/L
HEC-B1	2 : 1	H_2O/DMF	660	84	1.5	1.00	47.3	1.25
GX-A1	1 : 3	H_2O/DMF[k]	200[a]	130	5	0.90	53.2	1.24
GX-A2	1 : 3	DMF	300[a]	120	5	0.90	52.9	1.21
GX-A3	1 : 3	DMF	CH	70	30	0.81	48.6	2.15
GX-A4	1 : 3	DMF	CH	23	24 h	0.66	42.6	1.39
GX-B1	1 : 1	Solvent-free[j]	600[b]	90	2	0.75	54.2	1.25
GX-B2	1 : 3	Solvent-free[j]	300[b]	110	2	0.68	53.7	1.20
GX-B3[l]	1 : 4	Solvent-free[j]	CH	177	30	0.45	49.3	0.55
GX-B4[l]	1 : 4	Solvent-free[j]	CH	177	120	0.70	45.9	0.52
GX-B5	1 : 2	H_2O/DMF[k]	600[a]	160	3	0.85	52.1	0.38
GX-B6	1 : 2	Solvent-free[j]	CH	177	120	0.51	47.4	0.46
GX-C1	1 : 1	H_2O/DMF[k]	450[b]	120	1	0.74	47.3	0.16
GX-C2	1 : 3	H_2O/DMF[k]	300[b]	110	2	0.76	53.2	1.25

[j] H_2O/DMF/CH_3COONa, evaporation of water at 130°C for 30 min; [k] CH_3COONa as catalyst; [l] Skalková *et al.* 2006. For other footnotes see Table 2.1.

The esterification of GX using ML under MWH and CH was compared using 'solvent-free' conditions, which were achieved by pre-activation of GX in the H_2O/DMF/CH_3COONa medium, followed by water evaporation. The product was subjected to esterification either under MWH or after adjusting the temperature to 177°C by CH. In comparison to the reaction under CH lasting 30 or 120 min, the reaction time was reduced by MWH to 2 min and the derivatives showed similar surface tension values. When ML was preformed at the same GX/ML mass ratio at high temperatures in the H_2O/DMF medium under MWH or under 'solvent-free' by CH, the reaction time-reducing effect of MWH was very high. The yield of the derivative (GX-B5) was higher. MERO-GX derivatives were prepared by MWH in the H_2O/DMF medium in presence of CH_3COONa in similar yields after 1–2 min The results indicate that the MERO-GX derivative of similar yield and surface activity can be prepared at lower dosage of MERO using a higher microwave power and *vice versa*.

The presence of ester groups in the polysaccharide derivatives was confirmed by FT-IR spectroscopy. In comparison to the non-modified polysaccharides, the ester carbonyl vibration appeared at ~ 1741 cm^{-1} separated as band or a shoulder. For this test, all derivatives prepared from the carboxyl groups containing polysaccharides were measured in their salt form in order to distinguish between the protonated carboxyl group (at ~ 1735 cm^{-1}) and its ester form. The spectra of some derivatives are illustrated in Fig 2.1a. For qualitative evaluation of the extent of esterification, the relative increase of the asymmetric and symmetric stretching vibrations of the fatty acid methylene groups at ~ 2932 cm^{-1} and 2868 cm^{-1}, respectively, were measured by differential FTIR spectra of the sample vs. parent polysaccharide (shown for some samples in Fig 2.1b). For comparison, the differential spectrum of the MERO-esterified CMC with DS ~ 0.06, estimated from the NMR spectral data (Tomanová *et al.*, 2008), is inserted. As none of the tested polysaccharide esters showed higher intensity differences, the esterification degree of the studied derivatives can be considered to be very low (DS < 0.1).

Summarizing the surface activity data in Tables 2.1–2.4, it can be shown that the surface tension values (γ_{min}) of the prepared derivatives, independently of the use of CH or MWH, ranged from 42.6 to 66.7 mn/m. Although they are much higher then the γ_{min} of the low molar mass surfactant SDS (32 mn/m), they indicate moderate ability of the derivatives to lower the surface tension of water (72.8 mn/m). The c.m.c. values of the ester derivatives, assigned as critical polymer concentration in case of polymeric surfactants, showed a very broad range, which were the lowest ones with the HEC derivatives. Similar surface tension data were reported also for other biopolymeric surfactants, such as CMC grafted with butyl or octylamines (Rosilio *et al.*, 2000), C-alkylated alginates (Kang *et al.*, 2002),

CMC (Sroková *et al.*, 1998) and CMS (Sroková *et al.*, 2001). Although the derivatives do not substantially lower the surface tension of water, they are able to form micelles. Vaca-Garcia *et al.* (1999) reported after measuring the contact angle that grafting of fatty acid chains to cellulose increases the hydrophobicity of the ester derivatives even at low degrees of esterification. In general, the abilities of polysaccharide surfactants to lower the surface and interfacial tension are much lower than those of classical surfactants (Garti & Leser, 2001). They depend on a number of factors, such as the type and content of the hydrophobic substituents, the chemical structure of the polysaccharide, its molecular mass, charge resulting from hydrophilic/hydrophobic balance (Jönnson *et al.*, 1998).

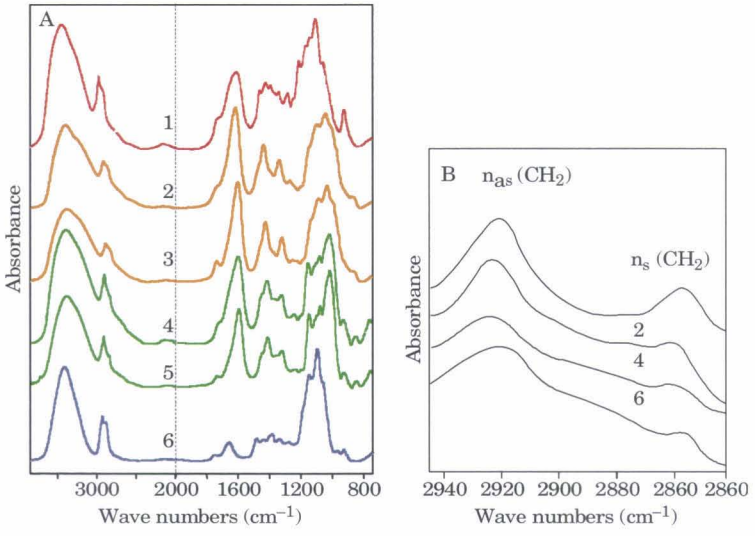

Fig 2.1. FTIR spectra of polysaccharide ester derivatives (a) FTIR spectra of derivatives (1) GX-C1, (2) CMC-C2 (DS 0.1, Tomanová *et al.*, 2008), (3) CMC-(C4), (4) CMS-B3, (5) CMS-C2 and (6) HEC-C2). (b) Differential FTIR spectra of (1) GX-C1, (2) CMC-C2, (4) CMS-B3 and (6) HEC-C2

Inspite their moderate surface tension-lowering effect, most of the derivatives showed remarkable emulsifying properties, comparable to control—the commercial emulsifier Tween 20 (Table 2.5). CMC (DS 1.0) and HEC exhibited a remarkable stabilizing effect of O/W-type emulsions even before the hydrophobic modification, what can be explained by strong associative properties (Zhang, 2001; Akiyama *et al.*, 2005), and in case of HEC also by its nonionic surfactancy imparted by the hydroxyethylene substitutents and high molar substitution (Landoll, 1982). Of importance is the fact that comparable emulsifying efficiencies were observed with corres-ponding derivatives of the particular polysaccharides prepared with MWH and CH.

Table 2.5. Emulsifying efficiency and foam formation of CMS, CMC, HEC and GX fatty acid derivatives

Sample	Oil/Cream (mm/mm)[a]		
	h_1	h_2	h_3
CMS-A1	0/0	0/0	0/6
CMS-A4	0/0	0/10	0/9
CMS-B1	0/0	0/0	0/7
CMS-B2	0/0	0/2	0/7
CMS-C1	0/0	0/10	0/12
CMS-C2	0/0	0/10	0/16
CMC-A1	0/0	0/6	0/12
CMC-A3	0/0	0/0	0/13
CMC-C1	0/0	0/10	0/14
CMC-C2	0/0	0/0	0/6
HEC-B1[b]	0/0	0/0	0/7
HEC-B2	0/0	0/0	0/2
HEC-C2	0/0	0/0	0/8
HEC-C3	0/0	0/0	0/12
HEC-D1	0/0	0/0	0/3
HEC-D3	0/0	0/0	0/10
HEC-D4	0/0	0/0	0/10
GX-A2	0/6	0/13	0/12
GX-A4	0/4*	0/8*	0/10
GX-B1	0/5	0/8	0/8
GX-B4	0/0*	0/7*	0/11
GX-C1	0/4	0/11	0/11
GX-C2	0/2	0/10	0/10
Tween	0/0	0/0	0/4
$CMS_{0.3}$[c]	0/0	0.5/0	0.5/15
$CMC_{1.0}$[f]	0/0	0/0	0/3
$CMC_{0.5}$[e]	0/0	1/18	3/8
HEC[h]	0/0	0/10	10/0
GX[i]	0/0	0.5/12	1/9

[a] Height of oil and cream layers formed on the surface of the O/W emulsion after (h_1) 5 min, (h_2) 1 h, and (h_3) 24 h; * Foam formation; [b] All HEC derivatives showed foam formation up to 1 h of rest; [f] Tomanová *et al.*, 2008; [h] Sroková *et al.*, 2003; [i] Skalková *et al.*, 2006.

The detergents performance of some of the esterified polysaccharide derivatives were tested by the washing power (WP) using as a standard sodium dodecyl sulfate (SDS) and by antiredeposition efficiency (ARE) compared to $CMC_{0.5}$ and $CMC_{1.0}$. CMC with a lower DS is often used as co-builder in detergents (Paik & Swift, 1995). The results illustrated in Fig 2.2 showed that WP of the esterified polysaccharides, particularly that of HEC

and CMS was very high and comparable to the standard, and not dependent on the type of heating source used for the esterification. The GX derivatives and VL-derivative of CMS (A1) showed lower WP. Excellent ARE values, higher than that of both CMC standards, were exhibited by the HEC derivatives. The ARE of the CMS derivatives was comparable to that of the $CMC_{0.5}$, whereas, that of the GX derivatives was very low.

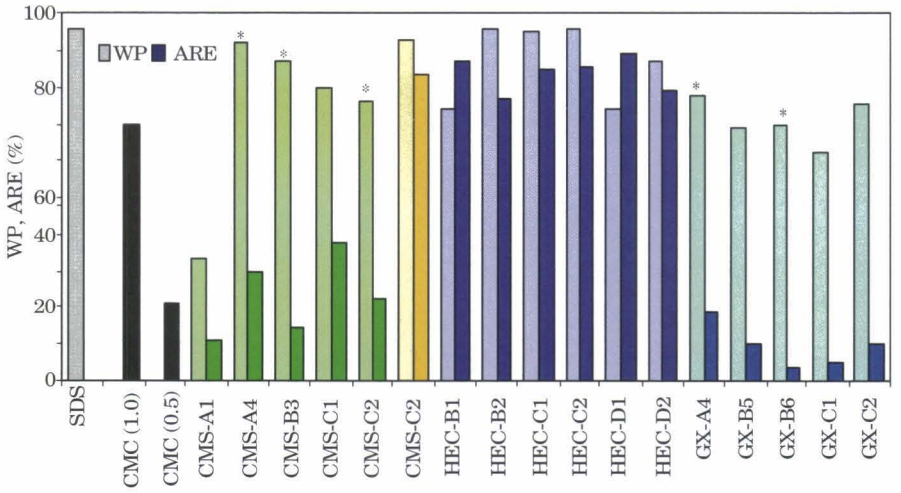

Fig 2.2. Washing power (WP) and antiredeposition efficiency (ARE) of some of the prepared esters and controls. WP (light color); ARE (dark color)

It can be concluded that the microwave-assisted transesterification methods possess several advantages in comparison to reactions performed under conventional heating. They reduced the reaction time down to several min In comparison to the very higher reaction temperatures applied during the transesterification under classical heating, namely using the 'solvent-free' method, microwave heating enabled to decrease also the reaction temperature. In combination with the very short reaction time this might be of interest for developing esterification technologies based on microwave heating. Promising results were obtained by esterification of CMC with MERO in H_2O/DMF medium in the presence of K_2CO_3 as a catalyst and HEC with vegetable oil (RO) in H_2O/DMF/LaCOOK. Despite the low degree of esterification, the prepared esters exhibited very good surface-active properties such as excellent emulsification efficiency. Of importance is the fact that derivatives prepared from the various polysaccharides showed comparable surface-activity and emulsifying properties as well as detergent performance properties, independently, if conventional or microwave heating sources had been applied. Such procedures might substitute the toxic, hazardous and time-consuming classical esterification processes in

preparing polysaccharide-based surfactants. Some reactions can be performed under 'solvent/free' conditions, thus omitting the usage of aprotic solvents, which is important in view of environmental protection. In addition, the proved applicability of MERO and vegetable oil as acylation agents contributes to a further valorization of the rape seed biomass.

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3

Structural Modifications of Parthenin: A Sesquiterpene Lactone of Biological Potential

BHAHWAL ALI SHAH[1] AND SUBHASH CHANDRA TANEJA[1,*]

ABSTRACT

Parthenin is the major sesquiterpene lactone of an exotic weed Parthenium hysterophorus belonging to the family Asteraceae (Compositae). Parthenium hysterophorus is mainly known for contact dermatitis in human and animals, and parthenin is believed to be the major compound responsible for it. Parthenium hysterophorus is also known for its biological activities like anti-cancer, anti-inflammatory, anti-malarial etc. and wide range of sesquiterpene lactones it possesses. The present review encompasses natural products isolated from Parthenium hysterophorus; biological activities of its constituents with a special focus on parthenin and its structurally modified products.

Key words : *Parthenium hysterophorus*, parthenim, allergy, sesquiterpene lactones, bio-active, anti-cancer, anti-inflammatory, *in vivo*

INTRODUCTION

Parthenium hysterophorus Linn. [syn. *Tanacetum parthenium* (L.) Sch. Bip.; *Chrysanthemum parthenium* (L.) Bernh.; *Matricaria parthenium* L., *M. parthenoides* Hort., *M. eximia* Hort., *M. odorata* Lam; *Pyrethrum parthenium* Smith] commonly known as congress weed, carrot weed, star

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weed, feverfew, white top, chatak chandani, bitter weed, ramphool, garghas etc. belongs to Asteraceae (Compositae) family. It is an annual weed with wide amplitude of ecological adaptability (Hedge & Patil, 1982) and different and challenging environments may lead to the expression of potentially beneficial genetic traits (Agrawal, 2001), some of which may promote invasiveness. Within the last 100 years, it has found its way to Africa, Australia and Asia. It is believed to have entered India accidentally in the mid 1950s from America through PL-480 wheat imports and is now considered as one of the most feared noxious weed species (Rao, 1956). The other species of parthenin are also known which include *P. glomeratum* Rollins, *P. incanum* HBK, *P. argentatum* Gray, *P. integrifolium* Linn., *P. alpinum* var. *tetraneuris* (Barneby) Rollins, *P. confertum* var. *lyratum* (Gray) Rollins, *P. confertum* cf. var. *microcephalum* Rollins, *P. cineraceum* Rollins, *P. fruticosum* var. *trilobatum* Rollins, *P. lozanianum* Blake, *P. bipinnatifidum* (Ortega) Rollins, *P. tomentosum* Linn., *P. tomentsum* var. *stramonium* (Greene) Rollins, *P. ligulatum* (Jones) Barneby, *P. fruticosum* Linn., *P. confertum* Gray, *P. schottii* Greenman, *P. hispidum* Raf. and *P. rollinsianum* Rzedowski. A large volume of research has accumulated on the isolation and identification of various constituents of all these species. In this review we are confined only to *P. hysterophorus,* its properties, constituents and modifications.

P. hysterophorus normally grows to a height of about 1 to 1.5 m. It is found in many parts of India and is prevalent in fertile lands, marshy lands and shady places with a remarkable power of regeneration. It is common in vertisols than alfisols. The plants start flowering in about a month after germination and keeps growing throughout the year under optimum conditions (Dhawan & Dhawan, 1996). The seeds are easily carried away by wind and are present in waste and crop lands. By this way it is well established in India and at present about 2.5 to 3 million hectares of land has been invaded by this weed. Prolific seeding habit of parthenium, non-dormancy and extreme light weight of its seeds armed with pappus is some of the characteristics which help its extensive spread and establishment. Parthenium produces enormous quantity of pollen which is carried away easily and settles on vegetative parts. The pollen grains inhibit fruit set in tomato, brinjal, beans etc. Presence of parthenium in cropped lands can cause yield reduction up to 40 percent.

ALLERGIES CAUSED BY PARTHENIUM

Parthenium causes severe human and animal health problems. It acts as allergen in allergic airway disease (Khurana *et al.,* 1999; Sohi *et al.,* 1979; Bapat *et al.,* 1978; Hausen, 1978; Bhutani & Rao, 1978; Lonkar *et al.,* 1974). *P. hysterophorus* rapid spread has caused an epidemic of contact dermatitis across globe (Burry & Kloot, 1982). Sesquiterpene lactones derived from plants of the family Asteraceae are well known for contact dermatitis

(Hosmani, 1995). Since plant hairs and the pollen contain sesquiterpene lactones, they can probably cause airborne contact dermatitis (Towers & Mitchell, 1983). Parthenium dermatitis, in its classical form is known as airborne contact dermatitis (Mahajan *et al.,* 2004). Allergy caused by parthenin is also known as compositae dermatitis, an allergic contact dermatitis caused by plant species of the Compositae family (Jovanovic & Poljacki, 2003). It has been found to sensitize as many as 56% of occupationally exposed persons without causing apparent dermatitis (Rao *et al.,* 1977). The exact mechanism of sensitization is unknown; the trichomes, pollen and plant dust containing sesquiterpene lactones, the major antigens, become airborne and cause airborne contact dermatitis in the majority. Direct or indirect contact sensitization is also possible in view of the rampant growth of the weed (Paulsen, 1992; Rodriguez *et al.,* 1976). Although pollen does not carry significant sesquiterpene lactones and detritus is plentiful only after plants die, sensitization occurs more frequently during the weed's growing season when there is little dust (Guin, 1989). The disease occurs in sensitized individuals and usually manifests as itchy, erythematous papules and plaques on exposed areas of the body, such as the face (including the upper eyelids), neck, "V" area of the upper chest, flexures of the forearms, and the antecubital and popliteal fossae (Sharma & Kaur, 1990; Verma *et al.,* 2001). The type of lesions and pattern of dermatitis may vary in some individuals (Verma *et al.,* 2002). Severe cases can lead to septicaemia infection. Parthenin is the major sesquiterpene lactone causing dermatitis (Lonkar *et al.,* 1976). *P. hysterophorus*, when fed to buffalo and bull calves resulted in acute toxicity leading to death. The former animals developed severe dermatitis. Autopsy revealed ulceration of alimentary tract. Extensive pathological changes were noticed in liver, kidney and skin (Narasimhan *et al.,* 1977). The correlation of titer of contact hypersensitivity (TCH) with disease severity and therapeutic response in patients of contact dermatitis caused by the plant *P. hysterophorus* revealed that it does not correlate with the clinical severity of contact dermatitis or response to treatment (Verma *et al.,* 2004). The positive patch test, prick test and elevated serum IgE suggest that both Type-I and Type-IV hypersensitivity may play a role in the induction and perpetuation of parthenium dermatitis in most patients. To date, delayed hypersensitivity was thought to be solely responsible for parthenium dermatitis. This study suggests that a combined type-I and type-IV hypersensitivity mechanisms may be operational (Lakshmi & Srinivas, 2007; Wedner *et al.,* 1987a). However the role of type-I hypersensitivity has been questioned later as the majority patients studied previously were atopics (Verma, 2007). Acetone extract has been found to be more appropriate for patch test (Sharma *et al.,* 2004). TCH in parthenin has been found to be reproducible (Raman *et al.,* 2000). The cross sensitivity between the four species of Compositae have also been studied with values differing for each plant in different patients (Nandakishore & Pasricha,

1994; Sriramarao & Rao, 1993). Parthenin had also been found to be the most frequent plant causing dermatitis when compared with other species of Compositae family (Sharma & Kaur, 1989). Cases of eczematous dermatitis caused by parthenium which generally escape clinical detection have been treated with a high-dose course of desensitization to underscore the probability of the entity in obscure cases of contact dermatitis and eczema (Kumar & Greval, 1993; Jeanmougin *et al.,* 1988).

In a study, persons suffering from parthenium dermatitis showed delayed immunologic reaction to the sesquiterpene lactone parthenin but not to its diastereomer, hymenin (Rao *et al.,* 1977; Rao *et al.,* 1978). Anti-idiotypic antibodies (Ab-2), which are the mirror images of idiotypic antibodies (Ab-1) have been used to diagnose parthenium dermatitis. The results have demonstrated that Parthenium-specific murine mouse (mAb-2) is of value as surrogate allergens in allergen standardization and for *in vitro* diagnosis (Sriramarao *et al.,* 1993a). Parthenium dermatitis has been sometimes found to be present with lesions very similar to or closely mimicking photosensitive lichenoid eruption in morphology and distribution (Verma *et al.,* 2002). The parthenin pollens extract has also been evaluated and found to cause allergy (Rodriguez *et al.,* 1991). A more measurable degree of hyposensitization can be induced by probably increasing the duration of oral ingestion of parthenium extract (Handa *et al.,* 2001). The airborne pollen of parthenium and its allergic potential suggest that a prolonged pollination season and perhaps increased allergenicity of unique parthenium pollen proteins allow parthenium to be a major allergen despite significantly less ambient pollen (Lewis *et al.,* 1991). The pollen of *P. hysterophorus* has been found to be a potential source of allergic rhinitis (Rao *et al.,* 1985; Sriramarao *et al.,* 1990). In a random clinical survey conducted on 2035 residents of Bangalore city with the aid of questionnaires and skin tests revealed that 7.1% of the study population was suffering from allergic rhinitis due to exposure to parthenium pollen. Nowhere in the world has such a high incidence of allergic rhinitis due to single pollen ever been reported (Sriramarao *et al.,* 1991). In a survey significant amount of parthenium pollens were found in atmosphere during the months of June and August (Seetharamiah *et al.,* 1981). In another study, 582 sera, contributed by 22 physicians from 18 Gulf Coast cities, were examined by ELISA for IgE directed against determinants in an aqueous extract of *P. hysterophorus* pollen, and these were compared to an extract of western ragweed (*Ambrosia psilostachya*). ELISA-inhibition analysis demonstrated that *P. hysterophorus* and *Ambrosia psilostachya* extracts contained unique allergenic epitopes. Clinical correlation of *in vitro* reactivity with skin testing and patients symptoms confirmed that *P. hysterophorus* sensitivity by ELISA was related in many cases to fall seasonal symptoms. Their data revealed that allergic reactivity to *P. hysterophorus* may be a significant contributor to fall allergic disease in

the U.S. Gulf Coast (Wedner *et al.,* 1989; Wedner *et al.,* 1987b). Allergens from the pollen of *P. hysterophorus*, responsible for high incidence of allergic rhinitis, were found by immunoprint analysis to be localized on the surface of the pollen grains. The allergens were released very rapidly when extracted *in vitro*. SDS-PAGE and Western blot analysis revealed that all the major pollen allergens with molecular weight 14, 28, 31, 37 and 45 kDa were eluted within 10 s of extraction. Periodate-Schiff staining showed that the 28, 31 and 45 kDa components of the pollen extract are glycoproteins. The pollen allergens released after different periods of extraction lost 75% of IgE binding activity when subjected to in situ sodium *m*-periodate oxidation under controlled conditions, while 80% of the allergenic activity was still retained after extensive proteolysis. The results supported the clinical observation of a rapid onset of symptoms of allergic rhinitis in patients sensitive to parthenium pollen, mediated predominantly by glycoproteins (Gupta *et al.,* 1995). Later a hydroxyproline-rich glycoprotein has been identified as the major allergen in parthenium pollen and it appeared that a group of soluble plant glycoproteins, which are related to the ubiquitous extensins, have certain carbohydrate-containing IgE-binding epitopes that may contribute to allergenic cross-reactivity among specific pollens and foods (Gupta *et al.,* 1996). A reference extract from the pollen of *P. hysterophorus*, responsible for allergic rhinitis has been generated and examined by skin test, radio-allergosorbent test inhibition and isoelectric focusing (Sriramarao *et al.,* 1993b). These reference reagents could not only be used for the quantization of parthenium-specific IgE in the sera of rhinitis patients but also for the evaluation of allergenic activity (relative potency and lot-to-lot variation) of different batches of parthenium pollen. Literature also finds reports of compositae dermatitis which are seasonal, worsening in summer, especially July and it clears on avoidance of contact, representing contact dermatitis due to oleoresins of Compositae plants (Guin & Skidmore, 1987). *P. hysterophorus*, inhibits 'state 3' respiration and stimulates 'state 4' respiration in rat liver and kidney mitochondria as well as ATPase activity in the presence of Mg^{2+} ions. These properties indicate that the toxic action of parthenin may be related to its interference with oxidative phosphorylation (Narasimhan *et al.,* 1985a). Indian childhood cirrhosis (ICC) is caused by hepatic copper accumulation but in experimental animals causes only modest liver damage. *P. hysterophorus* can be synergistic with copper in causing ICC as plant and fungal biocidal agents may be hepatotoxic, may increase hepatic copper concentration and may be secreted in milk of lactating animals (Tanner & Mattocks, 1987).

Amino acid, cysteine has been tried to treat parthenium dermatitis allergy in guinea pigs (Picman & Picman, 1985). Azothioprine has also been found to be effective against parthenium dermatitis (Verma *et al.,* 2006). It can be used for prolonged times with minimal side

effects (Verma *et al.,* 2000). Topical steroids, antihistamines, and avoidance of parthenium are the mainstay of treatment for localized dermatitis. Systemic corticosteroids and azathioprine are frequently needed for severe or persistent dermatitis (Sharma & Sethuraman, 2007).

Anopheles gambiae has been found to discriminately feeding on *P. hysterophorus* in a study conducted on thirteen plant species located within the vicinity of humans and larval habitat of *A. gambiae* in Kenya. The mosquitoes had a special preference for five species, parthenium being one of them and more often they fed from leaves and stems than from flowers (Manda *et al.,* 2007b). Later on attempt was made to establish a relationship between *A. gambiae's* preferences and its survival and fecundity. The corollary of results suggested a strong preference for *P. hysterophorus* for their feeding and higher fitness-related benefits (Manda *et al.,* 2007a).

ALLELOPATHIC EFFECTS OF PARTHENIUM

Parthenium competes strongly for soil moisture and nutrients and has been shown to interfere with crop growth (Tomado *et al.,* 2002). A yield decline of 40% for agricultural crops in India has been reported (Khosla & Sobti, 1981). The weed can reduce forage production in grasslands up to 90% (Nath, 1988). Parthenium has also been found to cause substantial loss in yields of *Helianthus annuus* (Sunflower) and *Sorghum bicolour* (sorghum) in Australia (Parsons & Cuthbertson, 1992), *Eragrostis tef* in Ethopia (Tefera, 2002) and one of the most important weeds in *Coffea arabica* (coffee) in Kenya (Njoroge, 1986). In South Africa, *P. hysterophorus* is a major nuisance in *Saccharum* spp. (Sugarcane) and *Musa* spp. (banana) orchards (Bromilov, 2006). *P. hysterophorus* is a prolific seed producer right up to senescence and one plant is reported to potentially produce between 15,000 and 25,000 seeds (Haseler, 1976; Joshi, 1991). *P. hysterophorus* seeds are capable of germination as soon as they have been released from the parent plant, although seeds may be induced into a state of conditional physiological dormancy by the ambient environmental conditions (Navie *et al.,* 1996). In a study on three different varieties (PD-10, PD-12 and PB) of paddy (*Oryza sativa* L.) for interference of parthenium on seed germination, seedling growth, and nutrient uptake, it was observed that it significantly interfered on the all the properties studied (Saxena *et al.,* 2004). In India, Pandey and Dubey (1989) observed *P. hysterophorus* seedlings in three consecutive cohorts in a single season, with seedling density and survival to maturity declining with successive cohorts. Parthenium also has inhibitory effects on the seed germination of different varieties of soyabean (Verma & Rao, 2006). Parthenin has proven its weed suppressing potential against weedy species *Amaranthus viridis*, *Cassia occidentalis*, *Echinochloa crus-galli*,

and *Phalaris minor* through a series of experiments conducted under laboratory or greenhouse conditions (Batish *et al.,* 2007). Parthenin has exhibited deleterious effects on Epigeic earthworms (*Eisenia fetida*) when fed on a diet of parthenin mixed with organic wastes amended with cattle manure (Biradar & Amoji, 2003).

It is widely believed that allelopathy also has an important role in the invasiveness of *P. hysterophorus*. Allelochemicals have been identified in all *P. hysterophorus* plant parts and several sesquiterpene lactones and phenolics have been identified and implicated as the principal allelochemicals in *P. hysterophorus* (Picman & Picman, 1984; Swaminathan *et al.,* 1990; Reinhardt *et al.,* 2004). Release of these allelochemicals from the plant into the environment can be achieved through the decomposition of plant residues. *P. hysterophorus* potentially uses all these mechanisms to release allelochemicals into the environment. Root mediated allelopathy depend on factors such as plant densities, root distributions, root densities and microbial activity (Ridenour *et al.,* 2001). The mobility of compounds in the soil may be less due to buffering or immobilization. Phenolics can interfere with plant growth directly by interfering with metabolic processes, affecting root symbionts and by affecting site through interference with decomposition, mineralization and humification (Van Andel, 2005). In grasses, *P. hysterophorus* extract have been demonstrated to be phytotoxic to *Eragrostis tef* (Belz *et al.,* 2006) and pure Parthenin was phytotoxic to *E. curvula* and *Echinochloa crus-galli* (Belz *et al.,* 2006). Parthenium has been also found to severely effect the germination, growth and some associated physiological changes in *Ageratum conyzoides* (Singh *et al.,* 2002). Allelopathy is an expression of a general phenomenon of chemical interaction and is known to inhibit seed germination by inhibiting hydrolysation of reserve food, cell division and several other factors (Rice, 1974). An experiment conducted on allelopathic effect of parthenium leaf extract on sunflower and sorghum revealed that the germination percentage, shoot and root length, dry weight and vigor index decreased with an increase in the concentration of parthenium leaf extract from one to 10 per cent (Murthy *et al.,* 1995).

ERADICATION OF PARTHENIUM

Pulling out the plant with root before flowering and burning is one of the easiest ways of control of parthenium. Mealy bug (*Ferris virgata*) can be used to control parthenium. It feeds on the root and the affected plant starts drying due to wilting. Another insect lantana bug (*Orthezia insignis*) is also effective in controlling it. *Zygogramma bicolorata* is another important beetle to control parthenium. It causes defoliation in the plant and finally destroys it. A leguminous weed, *Cassia sericea* inhibits the growth of parthenium by suppression. The use of monosodium methylene

arsenate (MSMA) at 4.4 kg per ha controls the weed. Due to growing awareness about environmental pollution and herbicide residue in soil and water, the sulphonyl urea herbicides like chlorimuron ethyl (20 kg/ha) and metasulfuron methyl (3.5 g/ha) which are needed in very small quantities can be used to control parthenium in non-cropped areas (Mishra & Bhan, 1994).

The emergence of the weed can be controlled by restoring the vegetative cover. The weed has shown to flourish in the areas bereft of vegetation (Kumar & Soodan, 2006). Cropping and competition exploits the differential growth habits, adaptabilities and competitive abilities of crops and crop varieties to prevent, suppress or reduce weed establishment. Growing of maize, sorghum and sunflower significantly suppressed the population of parthenium (Sankaran *et al.,* 1996). The 5 percent leaf extract of *Abutilon indicum* and *Tephrosia purpurea* reportedly causes maximum germination inhibition (90%) of parthenium and significantly reduces root and shoot elongation, dry matter production and vigour index. The aqueous foliar extracts of *Azadirachta indica*, *Aegle marmelos* and *Eucalyptus tereticomis*, which totally inhibit the seed germination of parthenium, can be exploited as an easy to obtain, cheap, effective bioherbicide for containing the spread of congress grass (Dhawan & Dhawan, 1995b). Citronellal, a monoterpene has been found to cause severe phytotoxicity to *P. hysterophorus* (Singh *et al.,* 2006). Seeds of *P. hysterophorus* failed to emerge even at 50 µg/g content. The post-emergent application of citronellal also caused visible injury in the form of chlorosis and necrosis, leading to wilting and even death of *P. hysterophorus*. It is also reported that leachates in various combinations of *Cassia fistula* L. and *Xanthium strumarium* L., *Azadirachta indica* A. Juss and *Lantana camara* L., *Salvadora oleoides Decne* and *Albizzia lebbeck* Benth, *Prosopis juliflora* (Sw) DC and *Cassia siamea* Lamk. *Datura alba* Nees and *Acacia nilotica* L., have allelopathic potential towards parthenium (Dhawan & Gupta, 1996b).

A bacterial leaf blight disease of *P. hysterophorus* has also been caused by the pathogen identified as *Xanthomonas campestris pv. parthenii pathovar nov.* The pathogenicity of bacterium is apparently limited only to parthenium (Chand *et al.,* 1995). In a study in Ethiopia several fungal isolates were obtained from seed and other parts of parthenium plants. However, pathogenecity test of the isolates obtained showed no or non-specific symptoms. It was concluded that these pathogens could be opportunistic with insignificant potential for biological control of parthenium. Two most important diseases associated with parthenium were, a rust disease, caused by *Puccinia abrupta* var. *partheniicola*, and a phyllody disease, caused by a phytoplasma of fababean phyllody (PBP) phytoplasma group. Phyllody and rust diseases of parthenium showed significant potential for classical biological control of parthenium (Taye *et al.,* 2002). In Tamil Nadu parthenium plants are generally affected by

two diseases, phyllody and powdery mildew. Phyllody affected plants show reduced growth, curved leaves and modified floral structures. No seed setting takes place, in infected plants. Tender parts are affected within 3–6 days. The entire plant is covered by powdery growth and later dries completely. Seed setting is reduced by 50 per cent. The pathogen is identified as *Oidium parthenii* and can not be controlled by chemical or cultural practices. Now biological control methods are gaining recognition as they are specific and not harmful to the eco-system (Doraiswamy & Manickarn, 1996).

In a study in Australia for the biocontrol of *P. hysterophorus*, biocontrol insects were evaluated. The leaf-feeding beetle *Zygogramma bicolorata* Pallister and the stem-galling moth *Epiblema strenuana* Walker were used as biocontrol insects and were tested at Mt. Panorama (central Queensland) and at Plain Creek (north Queensland). Biocontrol insects were more effective at Mt. Panorama than at Plain Creek (Dhileepan *et al.,* 2001). Variation of effectiveness of these strains was studied for a period of 4 year. It was revealed that defoliation and galling resulted in 70% reduction in the soil seed bank at Mount Panorama, but the reduction in the soil seed bank at Plain Creek due to galling was not significant. Effectiveness of *Z. bicolorata* and *E. strenuana* was dependent on weather conditions and as a result had only limited impact on the weed in three out of four years (Dhileepan *et al.,* 2003). The ecological adaptability and application as biological agent was also analyzed later. It was observed that 15–35°C was the suitable temperature regimes for *E. strenuana* to develop, and lower constraint temperature could reach –8 to –12°C. It was also observed that *E. strenuana* was an effective and safe agent for cereal and economic crops, ornamental plants, and indigenous natural enemy in the introduced region (Ma *et al.,* 2003).

POTENTIAL USES

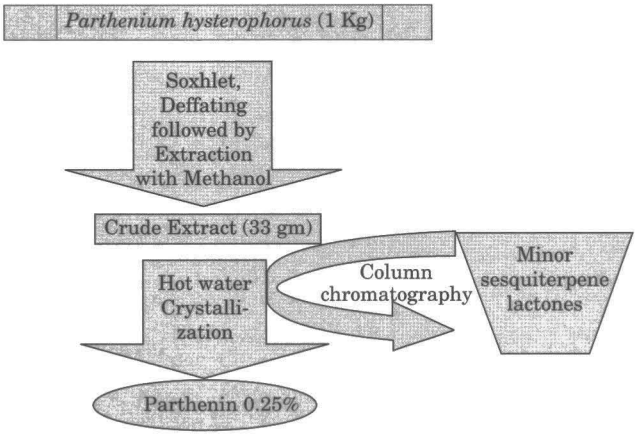
Instead of eradication of parthenium, constituents of parthenium can be utilized as green leaf manure, as a biopesticide and also as compost for crop plants. For the main crop of rice, the effect of parthenium green leaf manure on plant height has been shown to be comparatively less as compared to other green manures like leucaena and sun hemp, whereas, in the rice crop parthenium green leaf manure was superior in influencing the plant height (Sudhakar, 1984). Integrated use of *P. hysterophorus* as green manure with cattle manure and organic fertilizer has also been reported (Singh *et al.,* 2007). To assess the manurial value of parthenium and its composting value, a composting experiment has been conducted and compared with other organic wastes (Son, 1995). Incidence of leaf roller in rice crop was the highest with urea application, whereas it was the lowest with parthenium as green leaf manure application (Sudhakar, 1984). This bio-control behavior of parthenium can be exploited.

Thirteen fungal species such as *Aspergillus candidas*, *A. flavus*, *A. fumigatus*, *A. glaucus*, *A. niger*, *Altemaria alternata*, *Curularia pallescens*, *C. lunata*, *Fusarium equiseti*, *F. oxysporum*, *Penicillum notatum*, *Rlzizapus arrhims* and *Trichoderma ignorum* were isolated from the phyllosphere of young, mature and senescent but healthy leaves of parthenium. The total population of microrganisms showed considerable increase from young to mature to senescent but healthy leaves (Dhawan & Dhawan, 1995a). However, if some strain of any of these fungi is developed which meet the requirements of using them as bio-control agents *i.e.* host specificity and strong virulence, only then they can be considered as potential bio-herbicides for bio-control of parthenium (Dhawan & Gupta, 1996). Parthenin has been shown to act as a feeding deterrent to the adults of *Dysdercus koenigii* F, *Tribolium castaneum* Hbst, *Phthorimaea operculella* (Zell), *Callosobruchus chinensis* L2 and sixth-instar larvae of *Spodoptera litura* (F) (Datta & Saxena, 1997). Parthenin has also shown activity against termites and cockroaches (Tilak, 1977) as well as migratory grasshopper (Picman *et al.*, 1981). Whole plant extracts of *P. hysterophorus* showed insect-growth regulatory activity against the cotton stainer (*Dysdercus angulatus* F) (Kareem, 1984), fifth-instar larvae of *S. litura* (Rajendran & Gopalan, 1979; Balasubramanian, 1982) and toxic effects on cabbage leaf webber (*Crocidolomia binotalis* Zell), migratory grass hopper (*Melanoplus sanguinipes* F) (Fagoonee, 1983) and pulse beetle (*Callosobruchus maculatus* F) infesting cowpea seeds (Bhaduri *et al.*, 1985). Pure parthenin, as well as extracts from different parts of *P. hysterophorus* have shown phytotoxic effects on many aquatic (Pandey, 1996; Pandey, 1995; Pandey, 1984) as well as terrestrial weeds (Batish *et al.*, 1997; Khosla & Sobti, 1981; Singh *et al.*, 1992; Acharya & Rahman, 1997; Khosla *et al.*, 1980). Extracts of *P. hysterophorus* were also toxic to root knot nematodes *Meloidogyne incognita*; (Kofoid and White), Chitwood and *Helicotylenchus dihystera* (Cobb) Sher (Hasan & Jain, 1984). Crushed leaves admixed into the soil reduced root galling in papaya caused by *M. incognita* (Reddy & Khan, 1990). Pesticidal properties of parthenin derivatives have also been established (Datta & Saxena, 2001). Petroleum ether extracts of leaves, stem and inflorescence of *P. hysterophorus* at different concentrations were tested in the laboratory for their toxic effects on the mean life span and progeny production of adults of the mustard aphid, *Lipaphis erysimi* (Kalt). All the three plant parts tested decreased life span and progeny, but, the leaf extract exhibited the most significant effect (Sohal *et al.*, 2002).

ISOLATION OF PARTHENIN AND OTHER MINOR CONSTITUENTS FROM *P. HYSTEROPHORUS*

To isolate parthenin, the shade dried plant material including the flowers of *P. hysterophorus* is extracted with MeOH in a soxhlet extractor. The crude MeOH extract after concentration is defatted with *n*-hexane and the remaining extract is further extracted with chloroform and re-extracted

with MeOH. The crude chloroform extract after concentration is subjected to hot water extraction for the isolation of parthenin. The left over extract combined with re-extracted MeOH extract is subjected to column chromatography over a silica gel column to isolate other minor sesquiterpene lactones. The flow sheet diagram depicting the method for the isolation of parthenin and other minor constituents is given in Scheme 1.



Scheme 1.

P. hysterophorus mainly contains sesquiterpene lactones along with various other constituents. Infrared spectra of vegetative parts (leaf, stem and flower) of *P. hysterophorus* have been recorded and probable assignments of the bands were made with respect to the components present in the samples (Ramamurthy *et al.,* 2005). Parthenin (**1**), the major constituent of *P. hysterophorus* was isolated as early as 1959 by Herz *et al.* and its structure was provisionally assessed as a guainolide. Subsequently the structure was revised to that of a pseudoguainolide in 1962 (Herz *et al.*) by the same group. Detailed chemo profiling of *P. hysterophorus* revealed parthenin as its major constituent from various places like India, US, Mexico, Jamaica, Cuba, and Trinidad. However, Hymenin (**2**) was found to be the major constituent of plant population from southern Texas. In a follow up study of collection of *P. bipinnatifidum* from the high lands of central Mexico, Romo de vivar *et al.*, identified hysterin (**6**) as the major constituent. Later on hysterin was also found to be a major component of *P. glomeratums* from Argentina in 1975 by Rodriguez E. Coronopilin (**3**) has also been isolated from *P. hysterophorus* (Picman *et al.,* 1980). 11-Hydroxy-guaiene (**4**) and 2, 3 (H)-2, 3-epoxyambrosin (**5**) have been isolated from the roots of *P. hysterophorus* (Bohlmann *et al.,* 1977). Hysterin

(**6**) and dihydroisoparthenin (**7**) were also present in *P. hysterophorus* (Picman *et al.,* 1982). Sethi *et al.* (1987) reported three novel ambrosanolides namely 8β-hydroxycoronopilin (**8**), 2β-hydroxycoronopilin (**9**) and 11-H, 13-hydroxyparthenin (**10**) from flowers of *P. hysterophorus*. Chhabra *et al.* (1999) also reported three ambrosanolides 8β-epoxymethylacrylyloxy-parthenin (**11**), its 11β,13-dihydroderivative (**12**) and 8β-epoxymethyl-acrylyloxyambrosin (**13**). A *seco*-pseudoguaianolide namely charminarone (**14**) has also been isolated (Venkataiah *et al.,* 2003) (Fig 3.1).

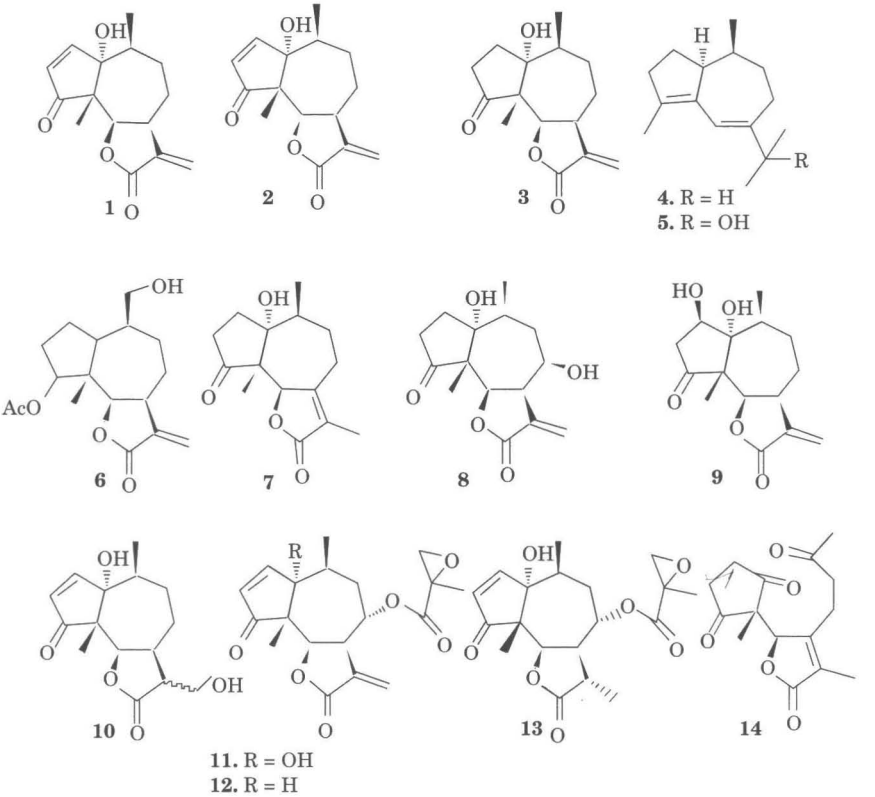


Fig 3.1.

Ramesh *et al.* (2003) identified four pseudoguaianolides, namely 12, 13-dihydro-10-α-hydroxyparthenin (**15**), 2, 3, 12, 13-tetrahydro-10-α-hydroxyparthenin (**16**), 12, 13-dihydro-4-deoxy 4β, 10-α-hydroxyparthenin (**17**) and 2, 3, 12, 13-tetrahydro-4-deoxy4β, 10-α-hydroxyparthenin (**18**) from *P. hysterophorus*. Besides, 8-β-acetoxyhysterone (**19**) (Das *et al.,* 2005a), deacetyltetraneurin (**20**) and 11,13-dihydro-4-deoxo-3,4-dihydroxyneo-ambrosin (**21**) (Das *et al.,* 2006) have also been reportedly identified. Recently four acetylated pseudoguaianolides (**22–25**) have been isolated by Das *et al.* (2007) from the flowers of the plant (Fig 3.2).

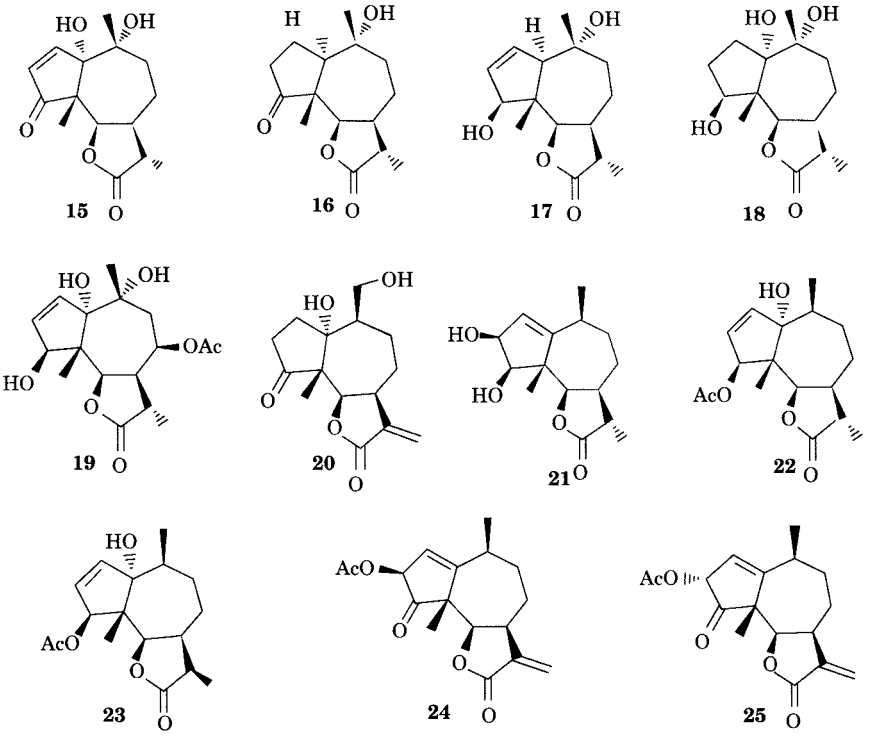
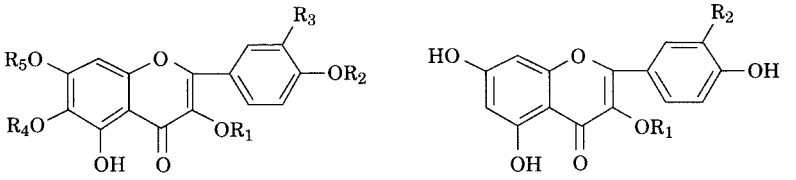


Fig 3.2.

Other than sesquiterpenes from parthenium, isolation of various flavanoids has also been reported (**26–30**) (Shen *et al.*, 1976) (Fig 3.3).



26. R_1 = Me, R_2 = H, R_3 = OH, R_4 = H, R_5 = Me
27. R_1 = Me, R_2 = H, R_3 = H, R_4 = H, R_5 = Me
28. R_1 = Glc, R_2 = OH
29. R_1 = Glc, R_2 = H
30. R_1 = Glc-O-Ara, R_2 = OH

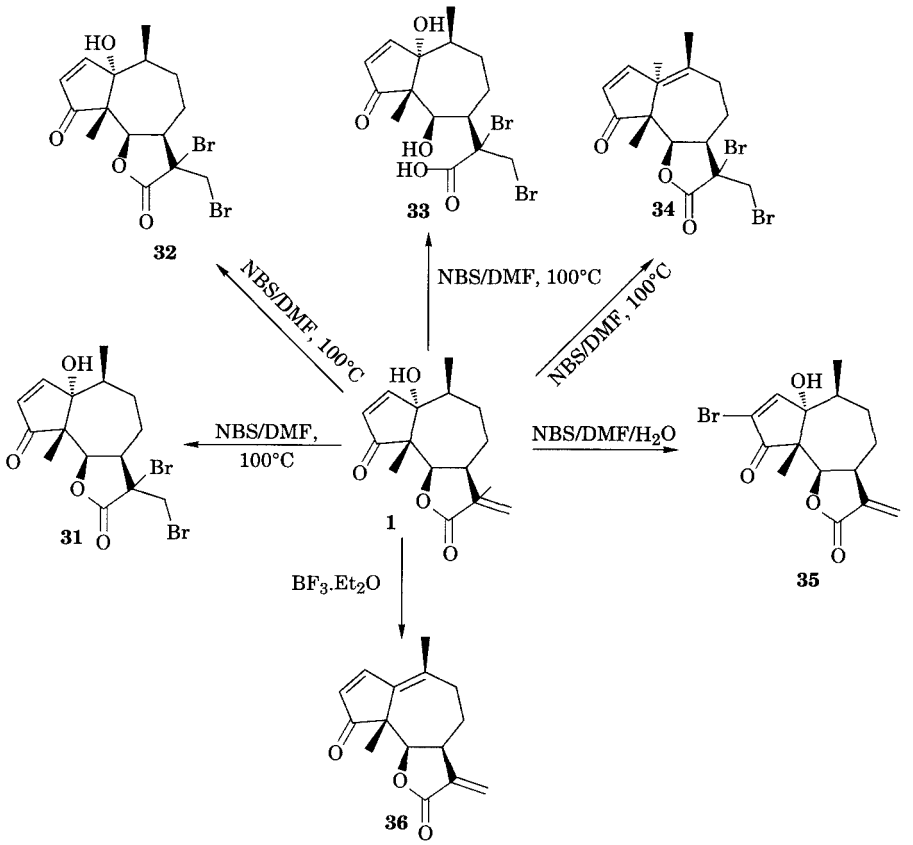
Fig 3.3.

STRUCTURE MODIFICATIONS OF PARTHENIN

Due to diverse biological activities of its major sesquiterpene lactone parthenin, *P. hysterophorus* has always been a centre of interest. The first ever attempt towards the total synthesis of parthenin was accomplished

by Asaoka *et al.* (1995). The reaction involved 17 steps staring from a cyclohexenone derivative with an overall yield of 1.6%. Later another total synthesis of parthenin was also accomplished by Shimoma *et al.* (1998). The synthetic scheme starting from 2-hydroxy-4-methylcycloheptatrienone involved in all 27 steps.

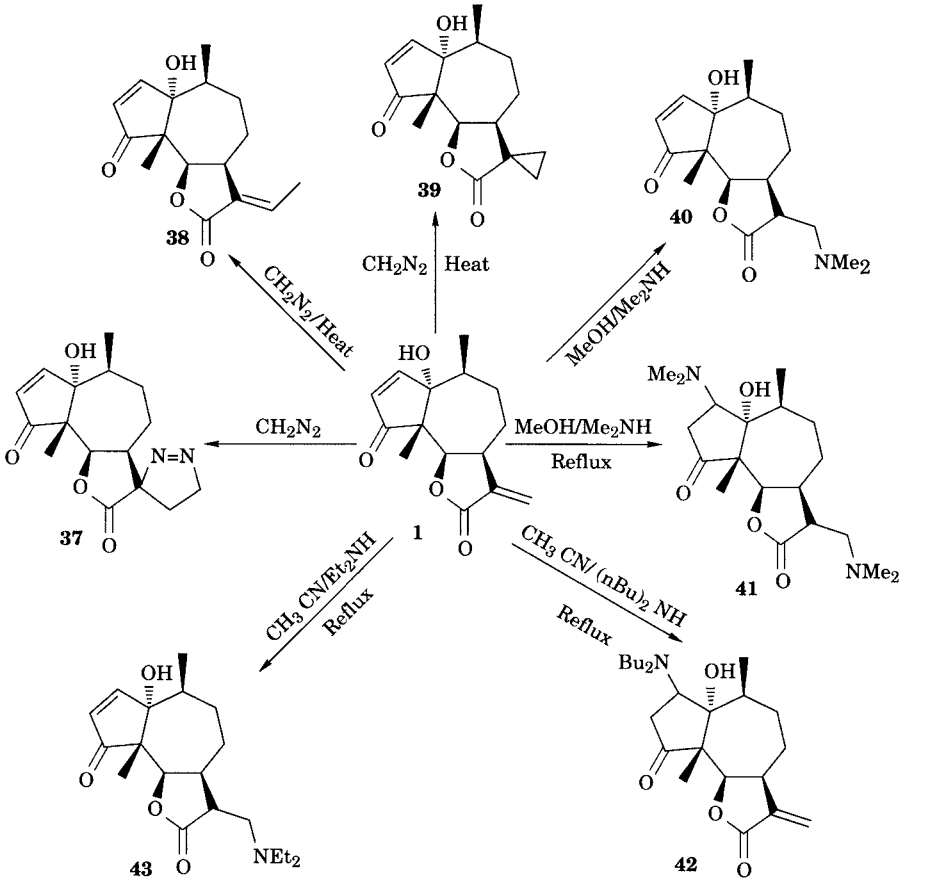
Though not much work has been done on structure modification of parthenin related to its biological activity however, a few publications have appeared in the past. Sethi *et al.* (1983) subjected parthenin to reactions with NBS and $BF_3.Et_2O$ to yield various analogues **31–36**. The structures of synthesized analogues are given below (Scheme 2).



Scheme 2.

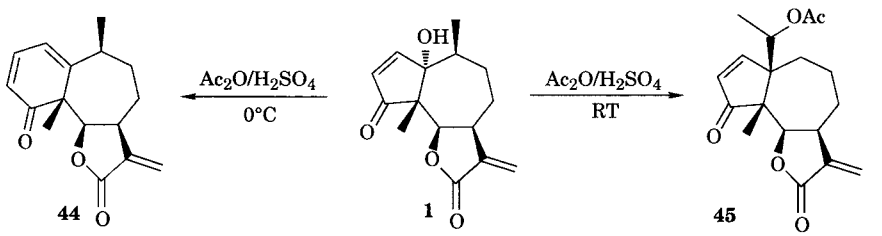
Parthenin has been treated with diazomethane to produce a pyrazoline derivative at exocyclic double bond **37** which on heating yielded compounds **38** and **39** (Saxena *et al.*, 1991). Secondary amine adducts of parthenin have also been prepared for their potential as antimalarial agent (Hooper *et al.*, 1990). The parthenin was subjected to different reaction conditions

with secondary amines like dimethyl amine, diethyl amine and dibutyl amine to produce amino adducts **40–43** (Bhonsle *et al.*, 1994) (Scheme 3).



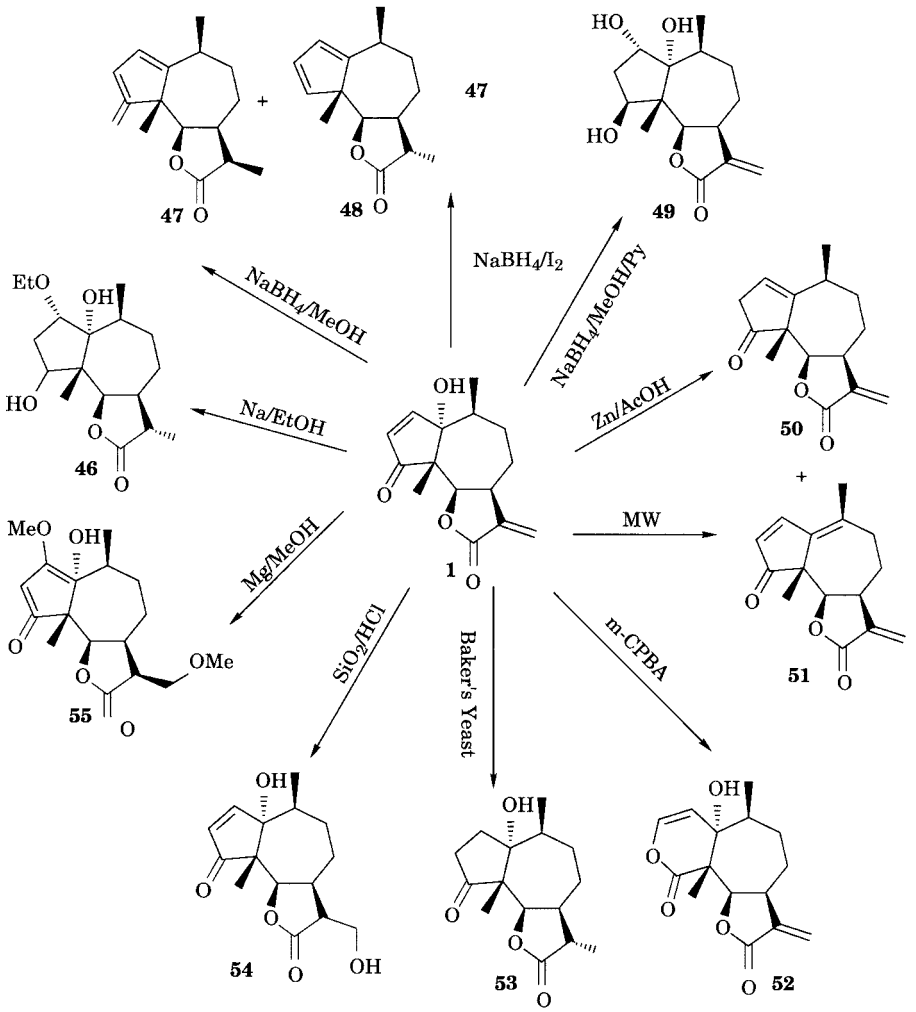
Scheme 3.

Chemical transformations on parthenin and related α-methylene-γ-lactones have also been carried out to establish a structure activity relationship (Dhillon *et al.*, 1994). Later parthenin was subjected to some acid catalyzed rearrangements to produce interesting molecules (Dhillon & Battu, 1995) (Scheme 4).



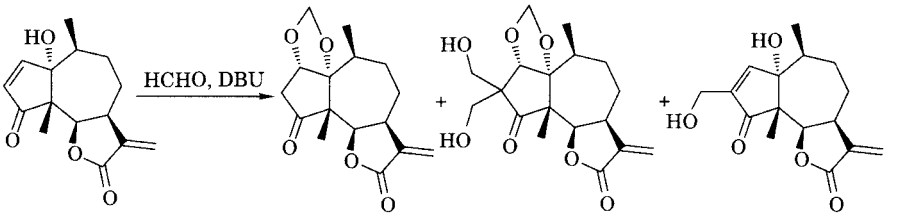
Scheme 4.

Das *et al.* (1999) have also brought out chemical and biochemical transformations on parthenin. Various transformation products **46–55** obtained through different reagents are summarized in Scheme 5.



Scheme 5.

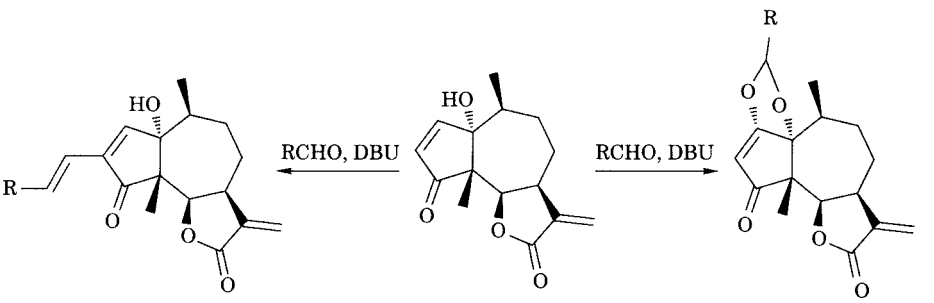
Analogues of parthenin have also been synthesized by utilizing Baylis-Hillman reaction (Shah *et al.*, 2007; Qazi *et al.*, 2008a, b). Baylis Hillman reactions were conducted using a series of aliphatic and aromatic aldehydes generating a library of novel compounds leading to the formation of 1,2 [2′-alkyl substituted 1′,3′-dioxolane] analogues, α-functionalized dehydrated Baylis Hillman adducts, 1,2 [2′-aryl substituted 1′,3′-dioxolane]. The reaction of parthenin with formaldehyde afforded three products (**56–58**) (Scheme 6).



1 **56** **57** **58**

Scheme 6.

The reaction was then extended to other higher aliphatic aldehydes. For example with lower aliphatic aldehydes *i.e.* paraldehyde and propionaldehyde, it lead to the formation of dioxolane derivative (**59** and **60),** whereas the reaction with higher homologues *i.e.* butyraldehyde, valeraldehyde and heptaldehyde produced normal dehydrated Baylis-Hillman product (**61–63**) (Scheme 7).



1

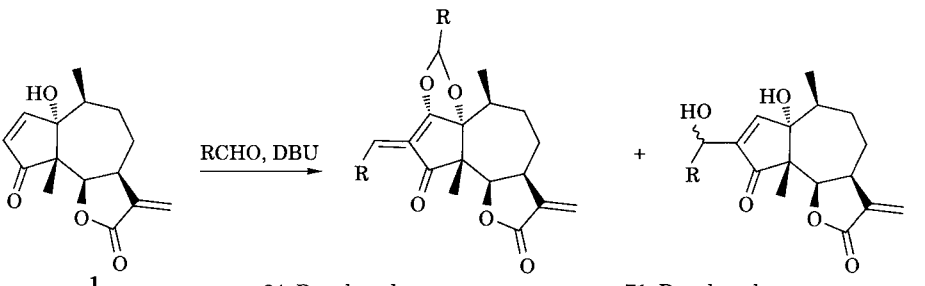
61. R = C_2H_5
62. R = C_3H_9
63. R = C_5H_{11}

59. R = CH_3
60. R = C_2H_5

Scheme 7.

The reactions with aromatic aldehydes lead to the formation of analogues **64–74** (Scheme 8).

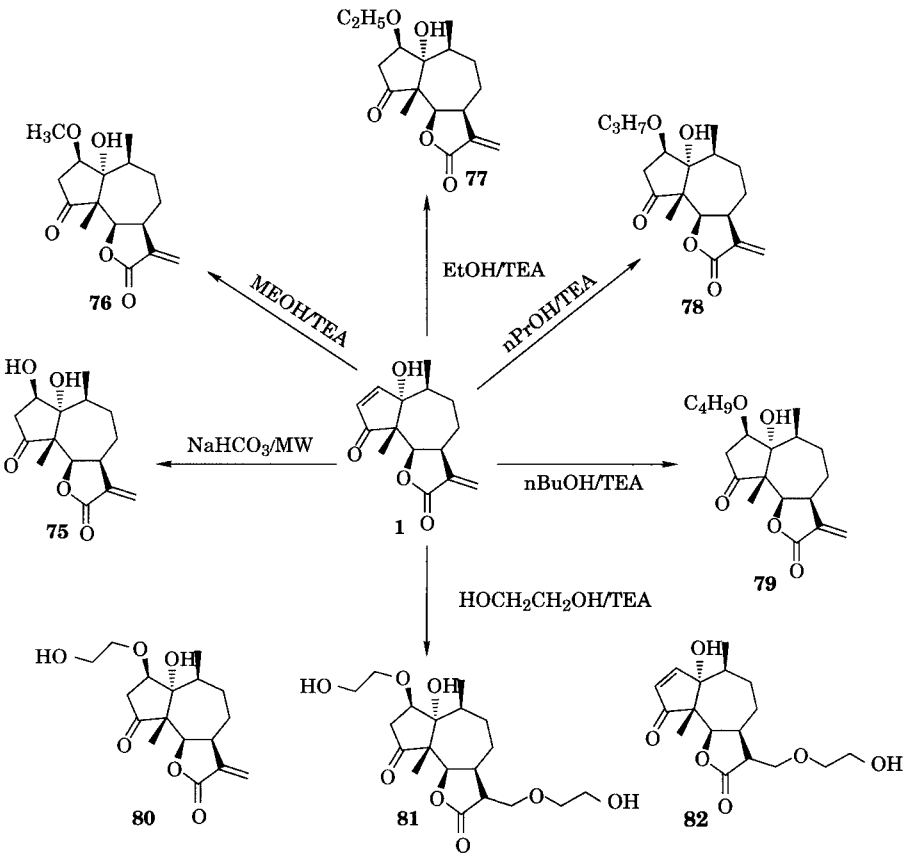
In addition, parthenin was also subjected to its reaction with various mono and dihydric alcohols thereby giving 2-β-alkoxy coronopilin analogues; while varying the ratios of NBS afforded different products (Qazi *et al.,* 2008a; Qazi *et al.,* 2008b). Parthenin on treatment with $NaHCO_3$ yielded 2β-hydroxy coronopilin, **75**. On extending the reaction with alcohols, seven new alkoxy analogues **75–82** were synthesized. The reaction of parthenin with 1 eq. of NBS in dioxane yielded **83**. On increasing the ratio of NBS to 1.5 and 2 eq. **84** and **85** respectively were isolated as the major products and on treatment with NBS and DMF, afforded **86** (Scheme 9).



64. R = phenyl
65. R = *p*-chloro phenyl
66. R = 3,4-methylenedioxy phenyl
67. R = *p*-methoxy phenyl
68. R = 3,4-dimethoxy phenyl
69. R = Styryl
70. R = Furfuryl

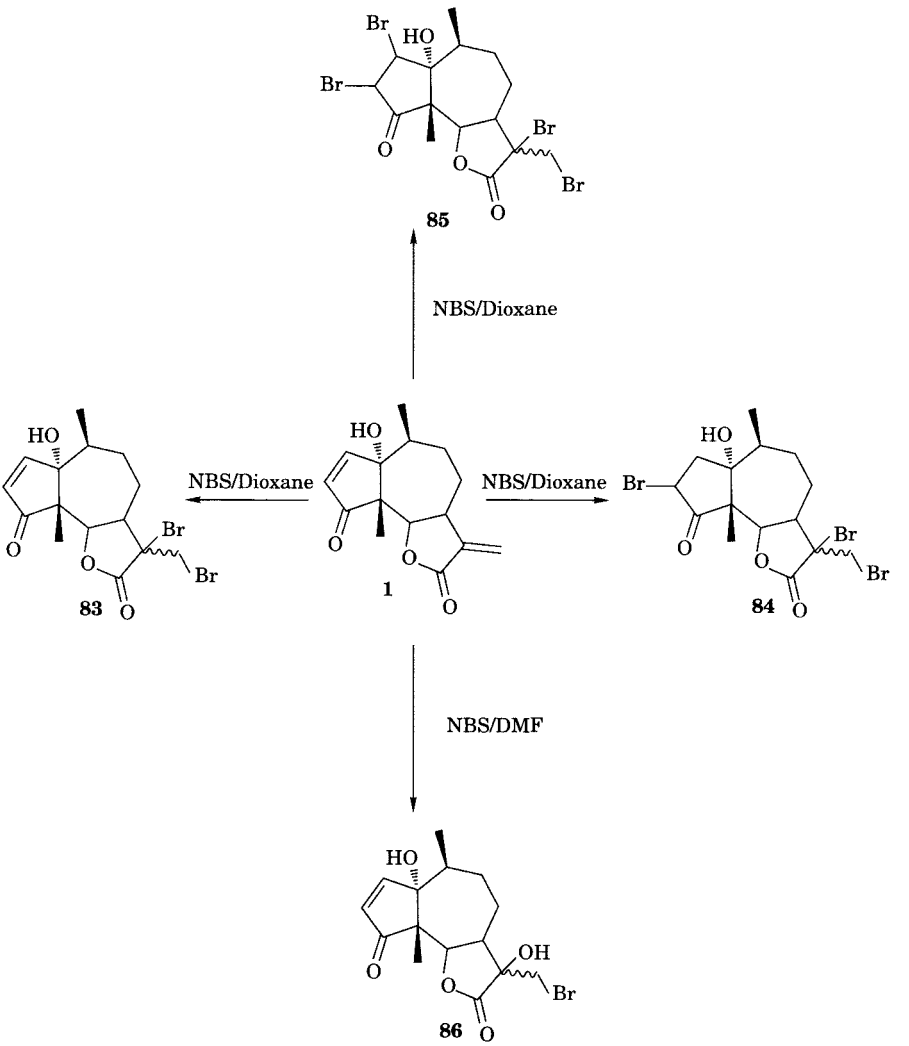
71. R = phenyl
72. R = *p*-choloro phenyl
73. R = 3,4-methylenedioxy phenyl
74. R = Styryl

Scheme 8.



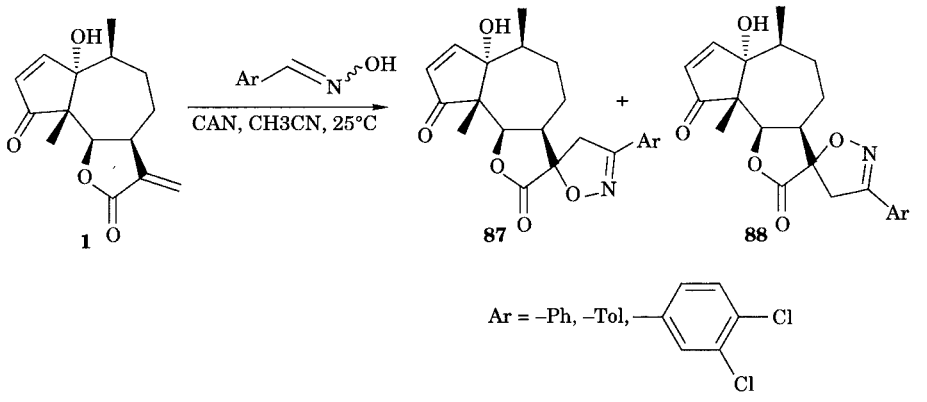
Scheme 9.

Parthenin was also treated with varying concentration of NBS to afford different products. Initially, parthenin was treated with 1 equivalent of NBS in dioxane yielding **83**. On increasing the ratio of NBS to 1.5 and 2 eq. **84** and **85** respectively were isolated as the major products. Parthenin was also treated with NBS in DMF, affording **86** (Scheme 10).



Scheme 10.

The reactions of parthenin with various aldoximes in the presence of ceric ammonium nitrate lead to the formation of novel spiro-2-isoxazolines (87) and (88) respectively (Das *et al.*, 2005b) (Scheme 11).

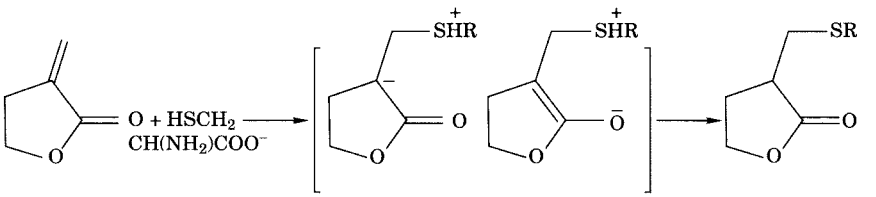


Scheme 11.

PARTHENIN AS ANTICANCER AGENT

Anticancer activities of sesquiterpenes are well known and had been a subject of reviews in recent past (Fraga, 2006; Modzelewska *et al.,* 2005). The biological activity of the complex natural products appears to be associated with their ability to act as alkylating agents by virtue of Michael addition of biological nucleophiles to α-methylene part of lactone moiety (Hanson *et al.,* 1970; Kupchan, 1970a; Kupchan, 1970b). It has been demonstrated that in contrast to α,β-unsaturated lactones which react rapidly with cysteine to form stable adducts, endocyclic α,β-unsaturated lactones react slowly to form unstable adducts (Kupchan *et al.,* 1970d). It was also postulated that "the reactions between α-methylene-γ-lactone and other conjugated systems with biologically important sulfhydryl groups may play a significant role in the mechanism by which these compounds exert their biological activities" (Kupchan *et al.,* 1970c). It has also been observed that an increase in cytotoxicity accompanies increase in lipophilicity. Furthermore, the presence of functionality in addition to α-methylene-γ-lactone, either in α-methylene-δ-lactone or in conjugated side chain ester as the second functionality increases cytotoxicity (Kupchan *et al.,* 1971). The importance of bifunctionality has also been revealed in synthesized α-methylene-γ-butyrolactones (Howie, 1976). The *cis* fused α-methylene-γ-lactones are highly strained molecules but the strain energy has little effect on the cytotoxicity. Moreover, if the transition state of cysteine addition has substantially more sp^3 character at the β carbon as shown in Scheme-12 in resonance structures, then one would expect an energy relief in these reactions (Cassady, 1978). Lee and co-workers have established that compounds of this type can be viewed as alkylating agents which exert their biological effects by inhibiting cellular enzyme activity and not by alkylating or impairing DNA function (Hall *et al.,* 1977; Lee

et al., 1977). These observations are further supported by the fact that elephantopin-bis (cysteine) adduct, which contains an endocyclic α, β-unsaturated lactone is inactive (Kupchan *et al.,* 1970).



Scheme 12.

The *in vitro* tests on parthenin have revealed that it was preferentially toxic to tumor cells when compared to normal murine cells that were either dividing or undergoing minimal cell division (Mew *et al.,* 1982). Moreover *in vivo* results exhibited antitumor activity at doses which was not lethal to mice. Parthenin has also been found to exhibit inhibitory effect on nitric oxide production (Morikowa *et al.,* 2006). The derivatives of parthenin synthesized by Qazi *et al.* (2008a, b) exhibited appreciable cytotoxicity against different human cancer cell lines. Some of these derivatives were also demonstrated to be potential inducer of apoptosis. The acetylated pseudoguaianolides isolated by Das *et al.* (2007) have also been demonstrated to possess promising anti-cancer activity. Though a lot of work has been done to establish the mechanism of action of sesquiterpene lactones (Hall *et al.,* 1977), but still parthenin to a larger extent remains an unexplored field.

OTHER BIOLOGICAL ACTIVITIES

Sesquiterpene lactones display a range of other biological activities besides anticancer activity, the most prominent being anti-inflammatory activity (Hall *et al.,* 1979). Parthenin has shown anti-inflammatory against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema. The ethyl phenyl propiolate (EPP)-induced mouse ear edema was also inhibited by parthenin (Recio *et al.,* 2000). Parthenin has been found to form a mono-adduct with L-cysteine through the alpha-methylene group of the gamma-lactone and a bi-adduct with the endocyclic double bond on the cyclopentenone ring. Thus, it supports the view that the types of adducts formed are correlated with the biological activity of the sesquiterpene lactones (Picman *et al.,* 1979). Parthenin, has also been reported to possess ameobicidal activity (Sharma & Bhutani, 1988). Parthenium extract has also exhibited trypanocidal activity. It exhibited *in vitro* trypanocidal activity at 5, 50, 500 and 1000 μg/mL. Its toxicity was significant at 1000 mg/kg dose when tested *in vivo* (Talakal *et al.,* 1995). The toxic effects of *P. hysterophorus* leaf extract (PLE) on the contractile properties of a frog

sciatic nerve–gastrocnemius muscle preparation have been reported (Hariharan *et al.,* 1982). But the studies were confined only to kymographic analysis of tension and fatigue studies. Later investigations on *in vitro* biochemical systems revealed anti-cholinesterase and insecticidal properties of PLE (Hariharan *et al.,* 1982; Rajakumar & Nanda, 1984; Nanda *et al.,* 1980a; Nanda Kumar *et al.,* 1980b). As anticholisternase causes a depolarizing type of neuromuscular blockade, electromyographic (EMG) and electrodiagnostic studies of PLE were made on sciatic nerve-anterior tibialis muscle *in vivo* in rats administered with a sublethal dose 100 mg/ 100 g body weight of rat (Vijayalakshmi *et al.,* 1999). The EMG studies revealed depolarizing neuromuscular junctional (NMJ) blocking effects of the leaf extract. Thus, confirming that the leaf extract contains promising new depolarizing NMJ blocker. *P. hysterophorus* has also been found to change neurotransmitter levels in brain (Verma *et al.,* 2007). In a study on adult mice it was found that it decreased all neurotransmitter levels. It was found to interfere in the functioning of the hypothalamo-hypophyseal axis by impairing the biogenic amine levels which may in turn affect the physiology of the peripheral endocrine glands. Crude extract of parthenium has also been assessed for mutagenicity in Salmonella/microsome (Ames) assay and the mouse bone marrow micronucleus test. Though mutagenicity was negative for all the strains tested but cytotoxicity was evident in all cases at 5000 μg/plate. When mutagenicity was monitored after column chromatography fractionation of the crude, one of the fractions was mutagenic. The crude extract exhibited *in vitro* pro-oxidant activity. It also inhibited lipid peroxidation ($IC_{50} = 4.1$ μg/mL) but failed to act as –OH scavenger (Ramos *et al.,* 2001). Parthenin also exhibits clastogenic effects. As screening of parthenin for oxidative mutagenesis with *E. coli* strains IC 188 and IC 203 gave negative results, but it induced chromosomal aberrations, mainly chromatid breaks, in blood lymphocytes exposed to 10–60 μm during 20 h (Ramos *et al.,* 2002). *P. hysterophorus* is also used as a tonic, analgesic, antipyretic, antiperiodic, febrifuge and emmenagogue in Mauritius, Rodrigues, Mexico, Belize and India (Hirschhorn, 1981; Hirschhorn, 1982). The plant has also been used as an ethnomedicine in the treatment of female reproductive problems (Lans, 2007). Parthenin has demonstrated cytotoxic effect on cultured bovine kidney cells, evident by its ability to markedly inhibit the activities of key cellular enzymes after *in vitro* treatment of the cells with the toxin for 24 h (Narasimhan *et al.,* 1985b).

CONCLUSIONS

Though *P. hysterophorus* is known to cause allergies and many more problems, but still can be used for numerous biological purposes. The cytotoxic and anticancer related activities of the major sesquiterpene lactone are also recorded in the literature. These properties together with the wide

spread availability of the weed in every nook and corner of the country makes it an ideal source of bioactive molecules which has not been fully exploited. Moreover, recognition of its uses for health and economic benefits may also indirectly facilitate its eradication in the long run.

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4

Prospects for the Development of Polyphenolic Acetate as the Potential Drug Candidate: A Review

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ABSTRACT

Polyphenols are produced by plants as secondary metabolites. These compounds are mainly synthesized by the highly branched phenylpropanoid pathway. They are ubiquitously present in foods of plant origin. The natural as well as synthesized polyphenols have a wide range of biological activities. Extensive literature search reveals little information on the biological activity of acetoxy polyphenols commonly called Polyphenolic Acetates (PA), which may be obtained by simple modification of parent polyphenols. Acetylation is a very important post translational modification of proteins. The acetylation of protein by acetyltransferases is increasingly considered a biologically relevant regulatory modification like phosphorylation. Acetylation of proteins

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is catalyzed by a wide range of acetyltransferases that transfer acetyl groups from acetyl-coenzyme A (acetyl CoA) to either the α-amino group of amino terminal residues or to the ε-amino group of lysine residue of target proteins. The extensive work carried out in our laboratory on the identification of a microsomal enzyme, a transacetylase, which catalyzed the transfer of acetyl group from PA, apart from acetyl CoA, to certain functional proteins such as microsomal cytochrome P-450, NADPH cytochrome c reductase and Nitric Oxide Synthase (NOS) leading to modulation of their catalytic activities and associated physiological effects. Accordingly, PA was found effective in the inhibition of cytochrome P-450 mediated activation of mutagens, vasorelaxation, inhibition of ADP-induced platelet aggregation, modulation of radiation-induced cytotoxicity and inhibition of Protein Kinase C (PKC) of lymphocytes of asthematic patients. Since the acetoxy derivatives of several classes of polyphenols were found to be the substrates for transacetylase, the enzyme was termed earlier Acetoxy Drug: Protein Transacetylase (TAase). Recently, TAase was purified to homogeneity from rat liver and human placenta. On the basis of N-terminal amino acid sequence analysis, TAase was found to be identical to mature Calreticulin (CR), a prominent Ca^{2+} binding protein of endoplasmic reticulum. Hence, TAase was termed Calreticulin Transacetylase (CRTAase). In this review, we have substantiated the role of CRTAase in mediating the aforementioned biological effects of PA which may be exploited for the development of candidate drugs.

Key words : Polyphenolic acetates, protein acetyl transferase, calreticulin transacetylase, nitric oxide, candidate drug, cytotoxicity

INTRODUCTION

Plant produces a large number of polyphenols as secondary metabolites. The majority of them are synthesized by the highly branched phenylpropanoid pathway (Chesson *et al.*, 1997), the initial compound being 4-hydroxy-cinnamic acid (*p*-coumaric acid) which is derived from phenylalanine (Chesson *et al.*, 1997). Substitution of the cinnamic acid with hydroxyl or methoxyl groups at the 3- and 5-positions yields caffeic, ferulic and sinapic acids. In addition, benzoic acid derivatives such as styrenes, acetophenones and gingerols are formed from hydroxycinnamic acid by chain shortening or elongation. The addition of cyclic esters at the side chain produces hydroxycoumarins and chromanones and various condensation reactions with malonyl residues produce xanthones, stilbenes and flavonoids. They are essential for the plants, being involved in diverse functions such as pigmentation, growth and development (Dewick, 1997). The main sources of polyphenols are fruits, vegetables, cereal, wine, seeds, grasses, flowers etc. Natural as well as synthesized polyphenols have wide range of biological activities such as antioxidant (Shen *et al.*, 2007; Molina *et al.*, 2003; Alia *et al.*, 2006; Fylaktakidou *et al.*, 2004; Padersen *et al.*, 2007), anti-inflammatory (Fylaktakidou *et al.*, 2004; Shakibaei *et al.*, 2007;

Tsai *et al.,* 1999), anticarcinogenic (Kunnumakkara *et al.,* 2007; Collett & Campbell, 2004; Anto *et al.,* 2002; Aoki *et al.,* 2007), anti-atherosclerosis, cardio protection (Petroni *et al.,* 1995; Gouedard *et al.,* 2004; Myhrstad *et al.,* 2002; Dedoussis *et al.,* 2004; Sato *et al.,* 2001), antiviral, antitoxic, hepatoprotective, cytoprotective coronary heart diseases and on several mammalian enzyme system (Elliot *et al.,* 2000). In this context, we found that nothing was known regarding the biological activity of PA which may be obtained by acetylation of polyphenols with acetic anhydride using a specific catalyst.

Post translational modifications (PTMs) are covalent processing events that change the properties of a protein by proteolytic cleavage or by addition of a modifying group to one or more amino acids. PTMs of a protein can determine its activity state, localization, turnover and interaction with other proteins (Cohen, 2000). Some common and important PTMs are phosphorylation, acetylation, methylation, acylation, glycosylation, sulfation, ubiquitation etc. (Matthias & Ole, 2003). Acetylation is a very important post translational modification of the proteins. The acetylation of protein by acetyltransferases is increasingly considered a biologically relevant regulatory modification like phosphorylation (Dormeyer *et al.,* 2005). Acetylation of proteins is catalyzed by a wide range of acetyltransferases that transfer acetyl groups from acetyl CoA to either the α-amino group of amino terminal residues or to the ε-amino group of lysine residues of various proteins (Allis *et al.,* 1985). The knowledge on protein acetylation independent of acetyl CoA was restricted to the non enzymatic protein acetylation by aspirin (Vane, 1971). The role of a transacetylase catalyzing the transfer of acetyl group from a small molecule to another small receptor molecule such as platelet-activating factor dependent transacetylase and arylamine N-acetyl transferases is well documented (Lee *et al.,* 1992; Weber & Henin, 1985).

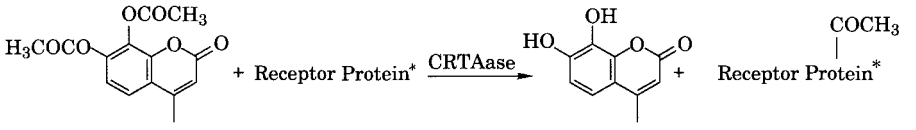
The persistent work carried out in our laboratory on the identification of a membrane bound enzyme, a transacetylase, which catalyzed the possible transfer of acetyl group from PA (Fig 4.1) to certain functional proteins leading to modulation of their catalytic activities (Raj *et al.,* 1998, 1999a, 2000; Khurana *et al.,* 2005; Kohli *et al.,* 2002a, 2004; Kumar *et al.,* 2005, 2007) and associated physiological effects. In this review, the emphasis was placed on biological functions of PA with special reference to the protein acetyltransferase function of CR and PA as an acetyl group donor.

Genesis of Our Investigation on PA: Discovery of the Biological Actions of PA

Antimutagenic Action

Our laboratory has been actively concerned on the mode of action of substituted 4-methylcoumarins for over a decade. These studies bear the origin to the chemoprevention of the activation of chemical carcinogens/

mutagens such as aflatoxin B_1 (AFB_1) and benzene as well as their subsequent mutagenic action. Of particular interest was the action of methoxy, hydroxy and acetoxy derivatives of 4-methylcoumarins on several liver microsomal cytochrome P-450-linked mixed function oxidases (MFO) mediating AFB_1 epoxidation, dealkylation of alkylated resorufin, oxidation of benzene and related clastogenic effects (Raj *et al.,* 1996, 1998a, 2001a, 2001b). Although all the aforementioned classes of coumarins were effective in causing the inhibition of AFB_1-binding to DNA, the acetoxycoumarins were alone effective in causing time dependent inhibition of AFB_1-DNA binding. We described the 7,8-Diacetoxy-4-methylcoumarin DAMC induced irreversible inhibition of MFO as pseudomechanism based inhibition (Raj *et al.,* 1998a) since, the inhibition differed from action of the classical inhibitors of cytochrome P-450 such as 21-chlorinated steroids (Halpert *et al.,* 1989), secobarbital (Lunetta *et al.,* 1989), dichloromethyl compounds (Halpert *et al.,* 1986) and *N*-alkyl-aminobenzotriazole (Mathews *et al.,* 1986). No oxidative metabolism of 7, 8-Diacetoxy-4-methylcoumarin (DAMC), a model PA was found necessary in effecting the mechanism based inhibition of rat liver microsomes catalyzed AFB_1-DNA-binding. The inhibition of AFB_1-DNA-binding by DAMC was totally abolished by thiol blocking agent p-hydroxy mercuribenzoate and iodoacetamide (Raj *et al.,* 1998a). These observations strongly indicated the existence of a new enzyme a "**transacetylase**" catalyzing the transfer of acetyl groups to possibly the lysines at the active center of apoprotein P-450 leading to the inhibition of MFO.



* Cytochrome P-450; NADPH Cytochrome P-450 Reductase; Nitric Oxide Synthase (NOS)

Fig 4.1. Protein acetyltransferase function of CRTAase

7, 8-Dihydroxy-4-methylcoumarin (DHMC), a deacetylated product of DAMC failed to exhibit the time dependent inhibition of microsomes catalyzed MFO (Raj *et al.,* 2000) indicating of the role of acetyl groups of PA in the inactivation of MFO. The genotoxicity observed in rats due to mutagenic chemicals such as benzene and AFB_1 was significantly reduced by the administration of DAMC (Fig 4.2a) where as DHMC had no such effect (Raj *et al.,* 2001a, b). The extent of inhibition of AFB_1/benzene-induced micronuclei by PA was found correlated with the specificity of microsomal TAase to PA (Fig 4.2b) (Raj *et al.,* 2001a, b; Kumar *et al.,* 2007). In a similar study, quercetin penta acetate was found superior to quercetin in the inhibition of AFB_1-induced clastogenic effect (Kohli *et al.,* 2002b).

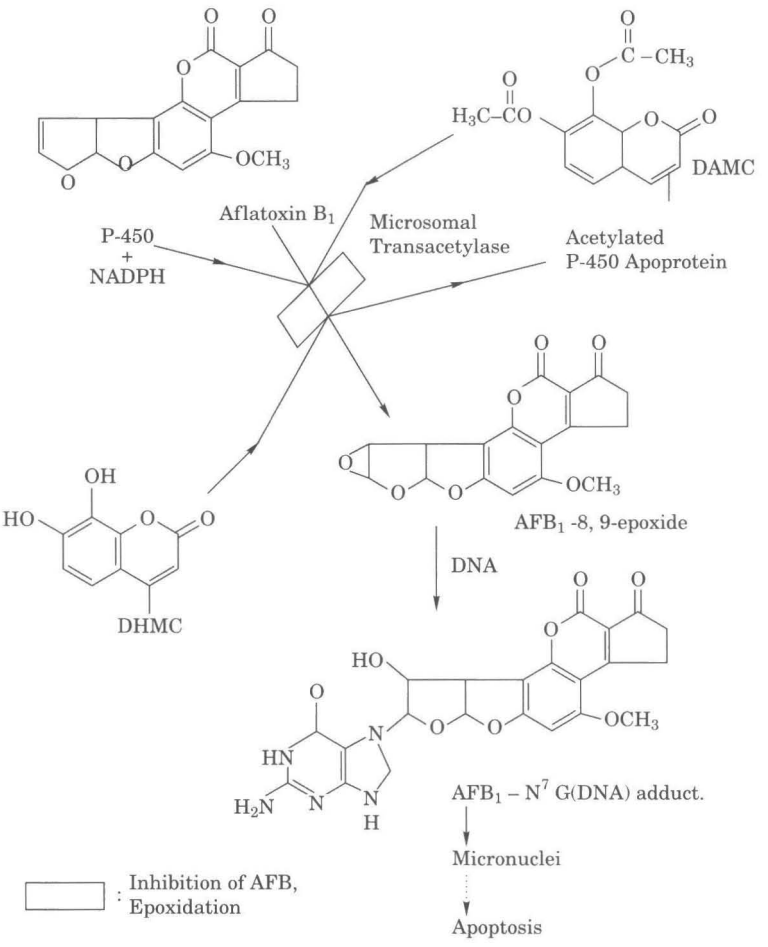
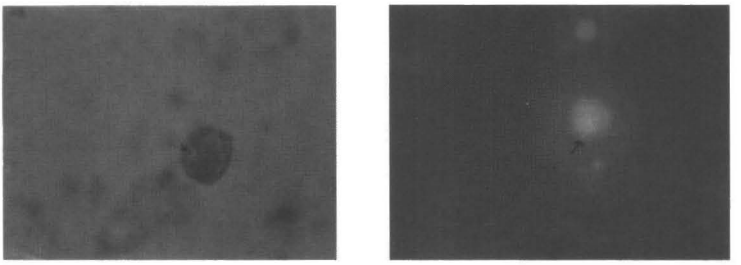


Fig 4.2a. Liver microsomal transacetylase-mediated chemoprevention of DNA damage due to AFB_1 by DAMC. Raj *et al.* (2001a). *Mutation Research*, **494**: 31–40



(i) (ii)

Fig 4.2b. AFB_1 induced formation of micronuclei in rat (i) rat bone marrow cells and (ii) lung cells obtained from bronchoalveolar lavage (BAL). Raj *et al.* (2001a). *Mutation Research*, **494**: 31–40

Antioxidant Properties

Our laboratory has been engaged in studying the chemistry and biological effects of 4-methylcoumarin derivatives (Raj *et al.,* 1998b, c, 1999b). We have systematically studied the effect of acetoxy, hydroxy and methoxy substituents in a wide variety of 4-methylcoumarin derivatives on initiation, propagation and chain termination of lipid peroxidation in rat liver microsomes (Raj *et al.,* 1998b) and free radical scavenging activity by electron paramagnetic resonance spectroscopy (EPR) (Padersen *et al.,* 2007). The inhibitory effects of monohydroxy- or mono acetoxycoumarins were very marginal, while there was a quantum jump in the potency of dihydroxy and diacetoxy derivatives to retard the initiation of lipid peroxidation. 4-Methylcoumarins, having two hydroxy or two acetoxy groups in the benzenoid ring at positions *ortho* to each other, have shown very strong antioxidant and radical scavenging properties (Raj *et al.,* 1998b). These compounds could abolish nearly 90% of lipid peroxidation. In addition, these dihydroxy- and diacetoxycoumarins were found to possess the ability to terminate the radical chain reaction and propagation of lipid peroxidation along with excellent radical scavenging potency. We have proposed a mechanism of action for their antioxidant effect (Raj *et al.,* 1998b). It is quite clear that such type of resonance-stabilized radicals in differently substituted flavonoids is known to confer radical scavenging properties to flavonoids. We believe that there exists the possibility of the conversion of 7,8-diacetoxy-4-methylcoumarin (DAMC) to 7,8-dihydroxy-4-methyl coumarin (DHMC) in the presence of the initiating free radical such as peroxy radical or superoxide radical through the formation of reactive ketene (Fig 4.3).

These properties of the coumarin derivatives highlight them as superior antioxidants. Investigations on several types of flavonoids allowed to conclude that the catechol moiety in their structure contributes to the antioxidant activity (Jha *et al.,* 1985; Husain *et al.,* 1987). The dihydroxy- and diacetoxy-4-methylcoumarins were found to possess maximum inhibitory effect. We have also used the pH metric techniques to study the mechanistic action of DAMC and DHMC to inhibit lipid peroxidation. These studies confirm the formation of a stable ADP-Fe-inhibitor (*e.g.* ADP-Fe-DHMC) mixed ligand complex, thereby inhibiting the reactive oxygen species (ROS), which is responsible for lipid peroxidation (Fig 4.4). Although polyphenolic compounds containing "free" hydroxyl groups are known to be antioxidants, the antioxidant action of the acetoxy compound DAMC was intriguing (Raj *et al.,* 1998c). Hence, pulse radiolysis studies were undertaken to explain the antioxidant action of DAMC. Accordingly, DAMC and DHMC were separately reacted with the system generating azide radicals and the resulting transient UV spectra were recorded (Raj *et al.,* 1999b). We proposed that the mechanism of scavenging of ROS by DAMC and DHMC is quite similar. These results suggest that the free radical mediated oxidation of DAMC initially produce a radical cation that may lose an acetyl carbocation to produce phenoxyl radical (Raj *et al.,* 1999b).

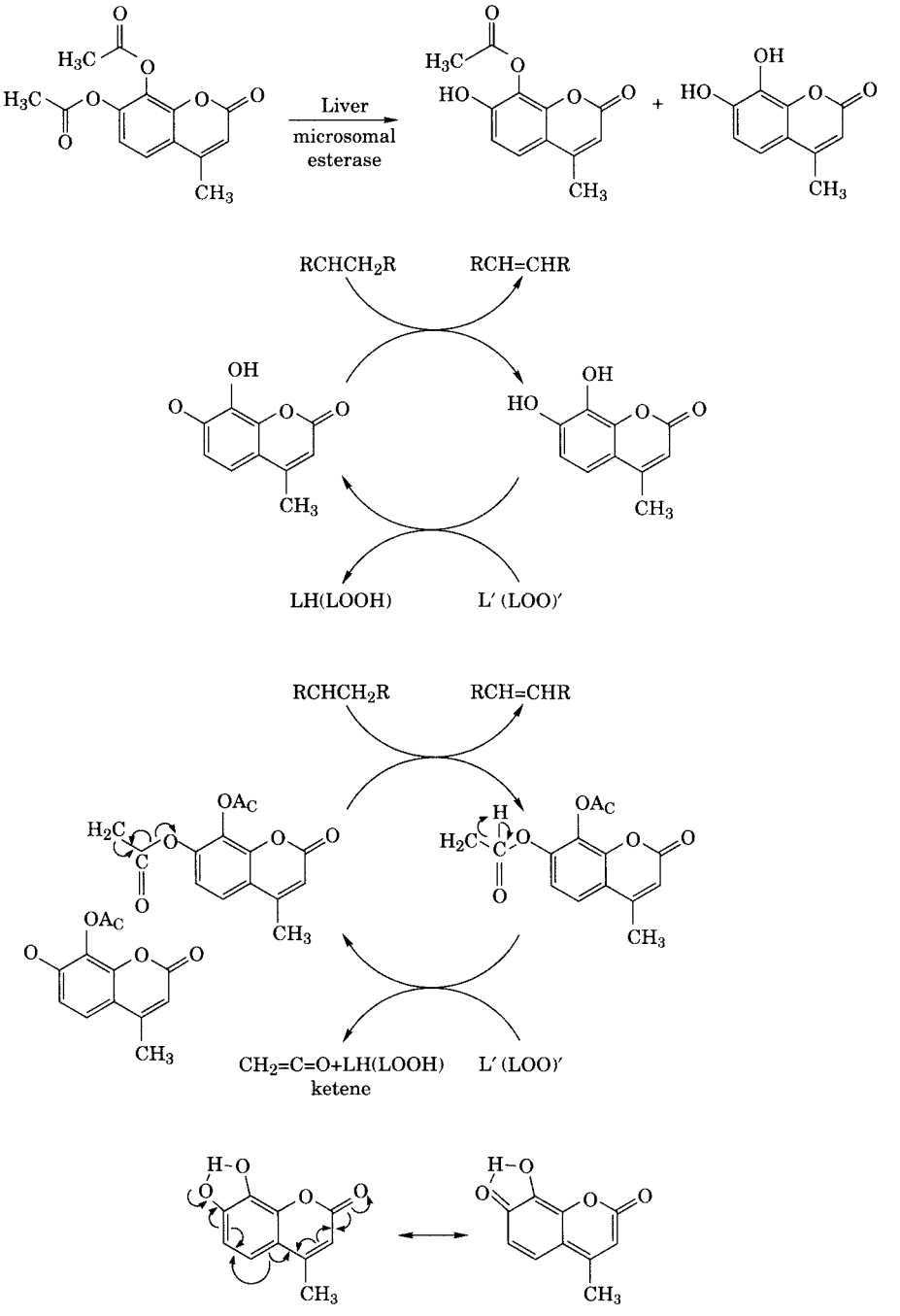


Fig 4.3. Suggested reaction of DAMC and DHMC showing radical structures. Raj *et al.* (1998b). *Bioorganic Medicinal Chemistry*, **6**: 833–9

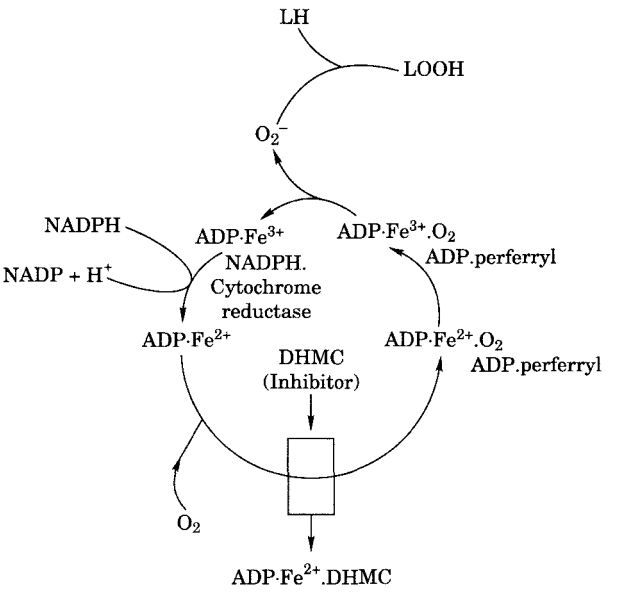


Fig 4.4. Inhibition of NADPH-dependent microsomal lipid peroxidation by DHMC. Raj *et al.* (1998c). *Bioorganic Medicinal Chemistry,* **6**: 2205–12. ADP-Perferryl ion formation is prevented by DHMC resulting in the production of a stable ternary mixed ligand (ADP-Fe-DHMC) which is the green chromophore complex LH: Lipid LOOH: Lipid hydroperoxide

Identification of Calreticulin as an Acetyltransferase

TAase was purified from rat liver microsomes to homogeneity and exhibited molecular weight of 55 kDa. TAase-catalyzed protein acetylation by PA was evidenced by the demonstration of immunoreactivity of the acetylated target protein such as NOS with anti-acetyl lysine antibody. The possible acetylation of human platelet NOS by PA as described above resulted in the N-terminal sequence of TAase was found to show 100% identity with N-terminal sequence of mature calreticulin (CR). The identity of TAase with CR, an endoplasmic reticulum (ER) protein, was evidenced by the demonstration of the properties of CR such as immunoreactivity with anti-calreticulin antibody, binding to Ca^{2+} ions and being substrate for phosphorylation by protein kinase c (PKC), which are the hallmark characteristics of CR (Raj *et al.,* 2006). Calreticulin is known to exhibit several biological functions such as chaperoning, Ca^{2+} homeostasis, modulation of cell adhesion, cardiogenesis and immunological functions. It is worth pointing out that the aforementioned work carried out in our laboratory ascribed the protein acetyl transferase function to CR for the first time (Fig 4.5). These observations convincingly attributed the transacetylase function to CR, which possibly plays an important role in protein modification by way of carrying out acetylation of various enzymes

through a biochemical mechanism independent of acetyl CoA. We have also purified and characterized CRTAase from other mammalian tissues such as human placenta (Seema *et al.*, 2007).

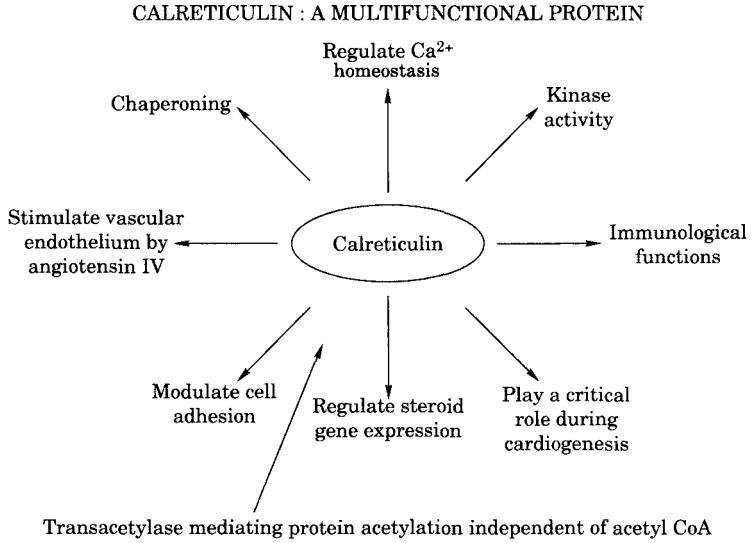


Fig 4.5. Novel TAase function of Calreticulin, Seema *et al.* (2007). *Cell Biochemistry & Biophysics,* **47**: 53–64

CRTAase Catalyzed Acetylation of Biologically Important Proteins

We have earlier discussed the possible acetylation of cytochrome P-450 by DAMC catalyzed by CRTAase. It was thought interesting to examine the outcome of the action of DAMC on another enzyme in the cytochrome P-450 cycle present in tissue microsomes, namely NADPH cytochrome c reductase. DAMC was found to cause kinetically discernible hyperbolic activation of liver microsomal NADPH cytochrome c reductase (Raj *et al.*, 1999a). The role of transacetylase in transferring the acetyl group from DAMC to amino acid at the active site of NADPH cytochrome c reductase causing irreversible enzymatic activation was postulated. Since NADPH cytochrome c reductase forms a domain of the NOS, it was thought interesting to examine whether DAMC could cause the activation of NOS activity. DAMC was indeed found to enhance the level of NO in human platelet when preincubated with DAMC (Khurana *et al.*, 2005). Further, the activity of CRTAase in platelet was established. An effort was made to confirm the acetylation of NOS by DAMC catalyzed by purified CRTAase. For this purpose, purified nNOS was incubated with DAMC and CRTAase, the modified nNOS was analyzed by nanoscale LC-MS/MS which recorded eleven distinct peptides with a significant score as acetylated on lysine residues. The distribution was in order: lysines-24, -33, -38, -131 and -229

of the PDZ domain, Lys-245 of the oxygenase domain, Lys-754 and -856 of FMN binding domain, Lys-989 of connecting domain and Lys-1300, -1321, -1371 of the NADPH binding domain were acetylated (Bansal *et al.,* 2008). We have reported for the first time activation of nNOS by way of acetylation. In addition, the acetylation of nNOS can be expected to potentiate the interaction with CR eventually leading to the augmented catalytic activity of NOS and expression of the related biological effects.

Increasing scientific evidence indicate that NO is suitable as a chemical messenger inside and between cells and with many important biochemical and physiological properties (Moncada *et al.,* 1991; Kuwabara *et al.,* 1995). This small reactive molecule convey biochemical signals that result in a wide spectrum of effects on different biological systems, including the central nervous, cardiovascular and immune systems, but also act as an important regulator of general cellular processes (Ignarro *et al.,* 1987; Moncada *et al.,* 1997). The formation of NO in blood platelets and the cell lining of the inner walls of blood vessels (endothelium) in response to certain chemical stimuli have been found to play a crucial role in maintaining the dilation of blood vessels-processes essential for the regulation of blood pressure. NO generated from L-arginine by NOS in the endothelium and in other cells plays a central role in several aspects of vascular biology (Brunori *et al.,* 1999).

NO Related Biological Effects Due to PA

Anti-platelet Action of PA

The role of NO in the inhibition of ADP-induced platelet aggregation stands out prominently. In this context, several PA were found to be profound enhancers of intracellular NO and were consequently considered to provide ample opportunities for the development of candidate drug endowed with the antiplatelet action. Accordingly, DAMC when preincubated with platelets was found to cause significant inhibition of platelet aggregation. Also, DAMC was found effective in causing antiplatelet action *in vivo* in rats. The potency of various PA to effect antiplatelet action was in tune with their affinity to platelet CRTAase (Khurana *et al.,* 2005; Raj *et al.,* 2006).

Vasoactivity of PA

There are several reports on the existence of Nitrite Reductase (NR) in mammalian cells. Mammalian NR activity is perceived due to NADPH cytochrome P450 reductase (CPR) and cytochromes P450 (Li *et al.,* 2006). Our earlier work has demonstrated the activation of cytochrome P450 reductase and NOS by PA mediated by CRTAase. Later, we turned our attention to NOS-independent formation of NO. We found nitrite dependent NO formation in platelets. Incubation of PA with the platelets caused

elevation of NO levels by nitrite. L-NAME failed to reverse the effect of PA. These results indicate CRTAase catalyzed activation of NR by PA, possibly by way of acetylation of CPR (Arora *et al.*, 2009). The deacetylated product of PA could not affect the NO levels in platelets caused by nitrite. We also found that NO generation by the activation of NR mediated the vasodilation of the de-endothelialized rings (Fig 4.6). Since the deendothelialized rings are devoid of NOS but retain the mitochondrial and ER enzymes, the vasorelaxation effect exhibited by PA can possibly be attributed to the enhancement of intracellular levels of NO due to activation of NR. The vasoactivity is known to be impaired in conditions such as diabetes and cardiovascular diseases and nitrite rich diet is being recommended to such patients. In this context, the effectiveness of PA in the activation of cellular NR may assume significance.

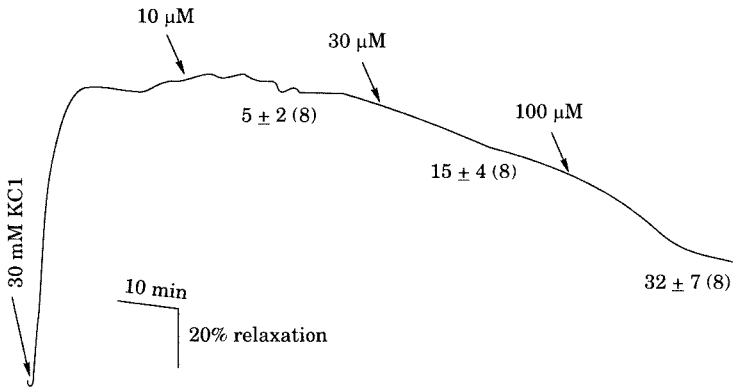


Fig 4.6. Relaxation of de-endothelialized aortic rings by DAMC de-endothelialized rings were hung in the organ bath in Kreb's solution. A tension of 3 g was applied to de-endothelialized rings and allowed to rest for 30 min and the tension corrected again to 3 g. The tissues were contracted 2x for 20 min. Once the contraction had reached plateau, DAMC was added to relax the tissue. DAMC in the range of 10–100 μM gradually relaxed the tissue in concentration dependent manner

The Effect of PA on IL-6 Production by Peripheral Blood Mono Nuclear Cells

Our investigations highlighted the modulation of TNF-α-induced activity of NOS and subsequent inhibition of the release of IL-6 by PA. Peripheral blood mono nuclear cells (PBMC) when treated with TNF-α resulted in the enhanced activity of NOS as compared to untreated PBMC. DAMC in conjunction with TNF-α was found to cause significantly higher levels of NO in PBMC as compared to that treated with TNF-α alone. Since TNF-α is known to cause the induction of cellular iNOS, further elevation of NO levels by DAMC is indicative of the activation of iNOS by way of acetylation. Incubation of iNOS specific inhibitor N-[3-(Aminomethyl) benzyl] acetamidine [1400W, Product of Sigma Chemical Company] with PBMC

one hour prior to adding TNF-α and DAMC resulted in the reduction of NO levels to that observed in case of control, confirming the activation of PBMC iNOS by PA. Further, DAMC was found effective in the inhibition of TNF-α-induced release of IL-6 in PBMC. Thus, PA might inhibit TNF-α-induced IL-6 production by PBMC through the prevention of TNF-α-stimulated activation of specific kinases (MAPK), which may eventually contribute to the anti-inflammatory effect of PA (Unpublished data).

Inhibitory Action of PA on Protein Kinase C

Our investigations have indicated protein kinase C (PKC) as another target for CRTAase catalyzed acetylation by PA. Human blood lymphocyte PKC was found to be irreversibly inhibited by DAMC (Gulati *et al.,* 2007). These studies were extended to the action of PA on the lymphocytes of the asthmatic patients. The activity of PKC in lymphocytes of asthmatic patients was found to proportionally increase with the severity of the disease. When PA was incubated with lymphocytes of normal patients, PKC was inhibited marginally. On the other hand, lymphocyte PKC of severe asthmatic patients was inhibited drastically. Several PAs inhibited PKC of asthmatic patients in tune with their specificity to CRTAase. DAMC was found to exert maximum inhibitory action on PKC, while 7, 8-dihydroxy-4-methylcoumarin (DHMC), the deacetylated product of DAMC, failed to inhibit PKC. Several inhibitors of PKC, such as calphostin C, tamoxifen, cyclosporine, etc. are known (Kanashiro *et al.,* 1998; McMillen *et al.,* 1989). Most of these are competitive inhibitors of PKC that cause inhibition by binding to the regulatory or the catalytic domain of PKC (McMillen *et al.,* 1989). Our investigations as described earlier highlighted for the first time that DAMC was an irreversible inhibitor of PKC possibly by acetylation of the protein. Also, there have been no reports on the modification of PKC by way of acetylation. The inhibition of PKC is known to result in diminished release of mediators of inflammation, such as IL-2 (Szamel *et al.,* 1993). PA can thus be expected to play an important role in controlling the inflammation in asthmatic conditions and may eventually serve as potential drug candidates.

Cytotoxic Effects of Polyphenolic Acetates

Acetylation of proteins, one of the important post translational modifications of proteins orchestrates the cellular responses both in normal and stressed conditions and also influences the cytotoxic effects of drugs and other agents including the anticancer therapeutics (Timmermann *et al.,* 2001; Dwaraknath *et al.,* 2008). PA like quercetin pentaacetate and acetylated coumarins have been shown to acetylate proteins such as nitric oxide synthase, GST etc. *in vitro* in a calreticulin dependent manner thereby altering their activities (Khurana *et al.,* 2005; Kohli *et al.,* 2002a). Therefore, these compounds have been investigated for their cytotoxic effects.

Moreover, since polyphenols are well known antioxidants, which under certain conditions show prooxidant activity in cells, the cytotoxic effects of these compounds have also been examined with reference to the oxidative stress generated by them. DAMC and quercetin penta acetates (QPA) have indeed shown a higher toxicity compared to their parent polyphenols in a breast carcinoma cell line (MDA-MB-468), which could only be partly attributed to the oxidative stress generated, suggesting thereby that the acetyl groups contribute to the cytotoxic effects (Koshy *et al.,* 2003). Furthermore, good correlation between the extent of inhibition of cell proliferation and the number of acetyl groups on the coumarin molecule. DAMC and 7-acetoxy- 4-methylcoumarin (7-AMC) have been compared for their cytotoxic effects on human cell lines. DAMC was found more toxic than 7-AMC (Table 4.1).

Table 4.1. Cytotoxic effects of polyphenolic acetates on BMG-1 cell line

Polyphenolic acetates	Concentration (μM)	Clonogenic survival	Relative growth	Apoptosis (% control)
None	—	1.00	100	4
DAMC	100	0.31	25	20
7-AMC	100	0.64	65	6

More recent studies have suggested that the cytotoxicity of polyphenolic acetates may be related to the higher mitochondrial activity induced by these compounds, as cells treated with DAMC showed a higher mitochondrial membrane potential as compared to 7-AMC (Dwarakanath *et al.,* 2007), which was accompanied by enhanced levels of ROS under these conditions (Koshy *et al.,* 2003). Interestingly, 7-AMC showed a higher prooxidant activity than DAMC, which also induced a higher level of NFκB (a prosurvival regulator) and acetylated histone H4 levels, thereby suggesting that acetylation of proteins (calreticulin dependent and independent) induced by PA indeed plays a role in cell signaling and changes in redox signaling. Initial studies with a calreticulin over-expressing cell line show a higher level of endogenous protein acetylation as compared to the parental cell line (Unpublished data). Although circumstantial evidences strongly suggest that alterations in protein acetylation leading to disturbances in cell signaling may be involved in the cytotoxic effects of these PA, further studies are required to unequivocally implicate the role of calreticulin in the action of these compounds.

The work carried out in our laboratory showed that polyphenolic acetates (PA) is a versatile donor of acetyl group for the modification of functional proteins by way of acetylation catalyzed by the CRTAase. The activation of NOS and inhibition of ADP induced platelets aggregation by several classes of PA may prove useful in producing hypotensive and vasorelaxing effects.

PA as Drug Candidate: Future Perspective

There is a plethora of information on the various aspects of biological action of polyphenols while, hardly anything was known about PA. The extensive investigations carried out by us characterized PA as biologically active compound. PA were found to possess properties distinct from the parent polyphenols or the former were superior in action. The intracellular enhancement of NO by PA stands out prominent and PA was found to promptly elicit NO related physiological response. The biological action of PA enumerated in this review is largely confined to DAMC, a model PA. In order to design PA as a drug, it is important to study its specific binding to the active site of CRTAase. There is need to systematically study the specificity of CRTAase to the structure of various acetoxy derivatives of polyphenols with a view to generate effective molecule which would merit as candidate drug endowed with antiplatelet, vasorelaxation and anti-inflammatory properties.

This Review is Dedicated to the Memory of Late Dr. Nizamuddin Ahmad, Biochemistry Division, Indian Veterinary Research Institute, Izatnagar (U.P.), India.

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5

Mining Bioactive Conformations: A Novel Methodology for Computing Predictive 3D-QSAR Models

JAYENDRA B. BHONSLE[1,*] AND DONALD P. HUDDLER[2]

ABSTRACT

The traditional approach of employing global minimum conformations or quantum mechanical geometry optimized conformations for development of QSAR models fails to deliver good predictive QSAR models, especially for flexible molecules. To address this issue we have developed a statistically driven methodology to mine through several conformations with energies within 20 Kcals / mol of the global minimum energy, where the conformations are such that they have good encompassment of the 3D space around some putative pharmacophoric moiety in the compound. Herein, we demonstrate the use of this novel methodology in the development of predictive 3D-QSAR models for antineoplastic cyclic pentapeptide CXCR4 inhibitors, DEET based insect repellents and antimicrobial peptides for MDR Staphylococcus aureus and Mycobacterium ranae. We also define and show the use of the novel concept of Descriptor Significance Percentage to elucidate the physical and biological significance towards the mechanism of action from the 3D-QSAR models.

Key words : 3D-QSAR, bioactive conformations, CXCR4, insect-repellent, antimicrobial peptides, Descriptor-Significance-Percentage

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INTRODUCTION

Antineoplastic Cyclic Pentapeptide CXCR4 Inhibitor 3D-QSAR Study

The human chemokine receptor (CXCR4) is the stromal cell-derived factor (SDF-1α) chemokine receptor. CXCR4 has been linked to several diseases such as AIDS (Deng *et al.*, 1996; Feng *et al.*, 1996), cancer metastasis & progression (Muller *et al.*, 2001) and rheumatoid arthritis (Matthys *et al.*, 2001). Fujii *et al.* (2003) reported several cyclic pentapeptides as potent CXCR4 inhibitors. The utilization of the bioactive conformation mining methodology for the CoMFA based 3D-QSAR study (Bhonsle *et al.*, 2005) of these cyclic pentapeptides and the related physical significance is discussed here.

DEET Based Insect Repellents 3D-QSAR Study

A variety of parasitic and pathogenic diseases including malaria, yellow fever and viral encephalitis are transmitted by mosquitoes and many other insects. (Brewste, 2001) Thus, keeping mosquitoes and insects away by using insect repellents is an important and significant strategy in the fight against these deadly diseases. There are three reported participating entities in the mechanism of action (Justice *et al.*, 2003) of DEET based insect repellents *viz.* the odorant-binding protein (OBP), the neuronal G-protein coupled receptors (GPCRs) and the odorant degrading enzymes (ODEs). In the first step the OBP binds to the odorants which are typically hydrophobic thereby facilitating their movement through the hydrophilic hemolymph towards the olfactory neuronal GPCRs. In second step, the OBP-odorant complex binds with the neuronal GPCR causing the repellency effect. Lastly, the ODE is reported to degrade the odorants thus preventing the continued stimulation of the olfactory receptors. The physicochemical properties based 3D-QSAR study (Bhonsle *et al.*, 2007) employing the bioactive conformation mining methodology, introduction to the novel descriptor significance percentage concept and the relationship of various physicochemical descriptors to the observed insect repellency effect is discussed here.

AMP Based Antibacterials 3D-QSAR Study

Antimicrobial peptides (AMPs) have evolved as a host defence mechanism against invading micro-organisms in many classes of living organisms. (Dennison *et al.*, 2005) Based on their mechanism of action, AMPs can be divided into two families as membrane-disruptors and non-membrane disruptors (Brogden, 2005). All membrane-disruptors are reported to follow specific steps in the process of binding to the target cells (Blondelle *et al.*, 1999). In the first step the AMPs are attracted to the surface of the membrane by the electrostatic interactions between the positively charged amino acids of the AMP and the negatively charged phospholipids of the cell

membrane (Dennison *et al.*, 2005). The following step involves the binding of the AMPs to the surface of the cell membrane further leading to cell lysis and death (Brogden, 2005). Based on the above assertions, our guiding hypothesis was that the target cell membranes (bacterial or mammalian) interacts with the approaching AMP in a specific way (via the bioactive conformation) through the mutually complementary 3D-physicochemical surface properties and thus define the resulting organism selectivity and potency. Here in we describe the effective use of the bioactive conformation mining methodology for the computation of 3D-QSAR models (Bhonsle *et al.*, 2007) for the *Staphylococcus aureus* ME/GM/TC resistant (ATCC 33592) (***SA***) and *Mycobacterium ranae* (ATCC 110) (***MR***) activity of AMPs. The use of Descriptor Significance Percentages for valuable insights in the mechanism of action is also presented.

MATERIALS AND METHODS

Antineoplastic Cyclic Pentapeptide CXCR4 Inhibitor 3D-QSAR Study

The data set, shown in Table 5.1, comprised of twenty five cyclic pentapeptides. The training set was selected to comprise of sixteen cyclic pentapeptides which had all of the D and L combinations of the four amino acids ($4^2 = 16$) *viz.* Tyr-Arg-Arg-Nal, where Nal is napthylalanine. The NMR based structural data, in the absence of the X-ray crystal structure of the ligand-receptor complex, is often employed for discovering the bioactive conformation (Guccione *et al.*, 2000). Therefore, the FC131 peptide NMR structure was used for the 3D coordinates of the cyclic pentapeptide backbone to serve as template for structure building of all the peptides. The structures of all the cyclic pentapeptides were built in Macromodel and minimized using the AMBER* force field (Ferguson & Kollman, 1991). The Polak–Ribiere conjugate gradient method (Polak & Ribiere, 1969) was employed with a gradient convergence criteria of 0.01 Kcal/Å-mol. The conformation of the central core cyclic pentapeptide ring was preserved, by employing positional constraint of 239.23 Kcal/mol-Å on the fifteen backbone ring atoms. The conformational search was performed in Macromodel using the Monte Carlo Multiple Minimum method (Chang *et al.*, 1989) (10,000 steps, 11.96 Kcal/mol energy window, with subsequent of minimization of 10,000 steps to ensure convergence). Water solvation was simulated using the Generalized Born (GB/SA) implicit solvation method (Still *et al.*, 1990). All the backbone bonds of the of the central pentapeptide ring (N-Cα and Cα-C) were fixed with a torsion constraint of 2,392.34 Kcal/mol-Å. Sybyl software was used to perform CoMFA analysis, employing the Tripos force field to calculate the steric (van der waals) and electrostatic (coulombic) interactions. The actual electrostatic value was replaced by the mean of the non-excluded electrostatic values, at all of the grid points, where the steric values exceeded 30 Kcal/mol. This was done for all the compounds, so that the sterically excluded points would not contribute to the PLS

calculations. The steric and electrostatic field cutoff values were set to 30 Kcal/mol. Thus, at any grid point if the steric and electrostatic field values exceeded 30 Kcal/mol then those values would be replaced by 30 Kcal/mol. This ensured that the steric and electrostatic field values would reach, at most, a plateau closer to the center of any atom. The molecules of the aligned Molecular Data Bases (MDBs) were placed in a cubic grid of 2 Å spacing, which extended 4 Å beyond every molecule. Further, a sp^3 carbon atom was used as the steric probe to calculate the steric field energies and a +1.0 net charge was used as the electrostatic probe to calculate the electrostatic field energies at all the grid points. All cross-validated PLS analyses were performed using the Leave-One-Out (CV-LOO) approach. The scaling option was set to CoMFA default. The column filtering value was set to 2.0 Kcal/mol. Thus, all of the CoMFA descriptor columns whose energy variance was less than 2.0 Kcal/mol were omitted in the PLS calculations. The number of components to explore was set to 6.

Table 5.1. Data set for CXCR4 inhibitors 3D-QSAR study

Peptide #	Peptide sequence	p (IC_{50})[(a)]
	Training set peptides	
FC08	L-Tyr—D-Arg—L-Arg—L-Nal—Gly	6.260
FC28	L-Tyr—L-Arg—D-Arg—L-Nal—Gly	4.959
FC48	D-Tyr—L-Arg—D-Arg—D-Nal—Gly	4.854
FC68	D-Tyr—D-Arg—L-Arg—D-Nal—Gly	8.810
FC130	L-Tyr—L-Arg—L-Arg—L-Nal—Gly	7.252
FC131	D-Tyr—L-Arg—L-Arg—L-Nal—Gly	9.495
FC132	L-Tyr—L-Arg—L-Arg—D-Nal—Gly	6.268
FC133	D-Tyr—L-Arg—D-Arg—L-Nal—Gly	6.260
FC134	D-Tyr—L-Arg—L-Arg—D-Nal—Gly	6.252
FC135	L-Tyr—D-Arg—D-Arg—L-Nal—Gly	6.268
FC136	L-Tyr—L-Arg—D-Arg—D-Nal—Gly	4.921
FC137	D-Tyr—D-Arg—D-Arg—L-Nal—Gly	6.268
FC138	L-Tyr—D-Arg—D-Arg—D-Nal—Gly	5.268
FC139	D-Tyr—D-Arg—D-Arg—D-Nal—Gly	5.260
FC91	L-Tyr—D-Arg—L-Arg—D-Nal—Gly	6.252
FC92	D-Tyr—D-Arg—L-Arg—L-Nal—Gly	8.979
	Test set peptides	
FC07	L-Arg—D-Tyr—L-Arg—L-Nal—Gly	6.301
FC10	L-Nal—D-Arg—L-Arg—L-Tyr—Gly	5.301
FC21	L-Tyr—L-Nal—D-Arg—L-Arg—Gly	5.268
FC27	L-Arg—L-Tyr—D-Arg—L-Nal—Gly	4.854

Table 5.1. *Contd.*

Peptide #	Peptide sequence	p (IC_{50})[(a)]
	Test set peptides	
FC30	L-Nal—L-Arg—D-Arg—L-Tyr—Gly	4.886
FC47	D-Arg—L-Tyr—D-Arg—D-Nal—Gly	4.824
FC50	D-Nal—L-Arg—D-Arg—D-Tyr—Gly	4.921
FC67	D-Arg—D-Tyr—L-Arg—D-Nal—Gly	5.26
FC70	D-Nal—D-Arg—L-Arg—D-Tyr—Gly	6.26

(a) IC_{50} values based on [^{125}I] SDF-1 binding to CXCR4.

DEET Based Insect Repellents 3D-QSAR Study

The collection of forty benzamides, benzyl amides and cyclohexyl amide DEET analogs and derivatives reported by Suryanarayana *et al.* (1991) was selected for this QSAR study. The chemical structures, vapor pressures @ 30°C and the protection time (PT) of all the compounds is summarized in Table 5.2. The molecular structures were build and minimized using the Cerius2 (C2) 3D-sketcher and minimizer employing Gasteiger-Marsili (Marsali & Gasteiger, 1980) charges and Drieding force field (Mayo *et al.*, 1990). The conformational models were computed by performing an exhaustive conformational search using the Grid Scan method (Accelrys, 2005) followed by cluster analysis based on the root mean squares (RMS) differences of the torsion angles. The common core amide group was used as a template to align the cluster nuclei. The cluster sizes were determined based on visual examination for good 3D sampling of the space (with little or no vacant volume and with much less crowding or over representation) around the amide group, which is the putative pharmacophoric moiety.

AMP Based Antibacterials 3D-QSAR Study

We selected 28 AMPs with diverse activity against *Staphylococcus aureus* ME/GM/TC resistant (ATCC 33592) (***SA***) and *Mycobacterium ranae* (ATCC 110) (***MR***) bacteria for this 3D-QSAR study (Hicks *et al.*, 2007). The amino acid sequences and their ***SA*** and ***MR*** activities are shown in Table 5.3. The peptides were constructed using the Biopolymer module of Insight II, energy minimized using the Steepest Descent Algorithm (Levitt & Lifson, 1969) and subjected to a brief (1000 cycles) MD simulation followed by exhaustive minimization to give the local minimum conformations. An exhaustive conformational search was performed using Monte Carlo Algorithm (Chang *et al.*, 1989). The conformations were clustered using Root Mean Squares (RMS) difference of torsion angles of the peptides (MSI, 1997). We selected sets of cluster nuclei that gave the best 3D spatial representations around the backbones, which were 20–30 conformers for some and 30–40 conformers

for the rest of the peptides. The conformers of all the peptides were aligned and added to a study table for descriptor computation with default settings. The correlation matrix was computed for all the descriptor values of all the conformers of all the peptides to obtain the cross correlation coefficients and correlation with bioactivity.

Table 5.2. Data set for DEET insect repellent 3D-QSAR study

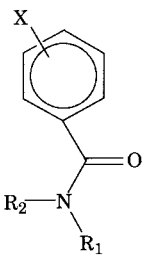
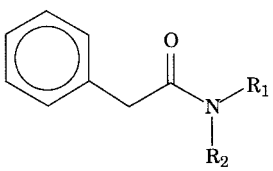
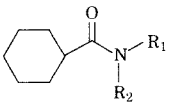
Compound structure	Compd #	X	R1	R2	PT Hrs	Training or Test set	VP
	1a	4-OCH_3	Et	H	0.08	Test	0.0062
	1b		CH_3	CH_3	1.00	Training	0.0039
	1c		Et	Et	1.00	Training	0.0037
	1d		iPr	iPr	1.17	Training	0.0155
	1e		C_5H_{10}		0.75	Training	0.1486
	2a	4-CH_3	Et	H	0.08	Training	0.0063
	2b		CH_3	CH_3	4.00	Training	0.0110
	2c		Et	Et	2.83	Training	0.0244
	2d		iPr	iPr	0.50	Test	0.0159
	2e		C_5H_{10}		1.00	Training	0.0313
	3a	H	Et	H	0.58	Training	0.0015
	3b		CH_3	CH_3	1.67	Test	0.0015
	3c		Et	Et	4.00	Training	0.1015
	3d		iPr	iPr	3.00	Training	0.0116
	3e		C_5H_{10}		3.00	Test	0.0559
	4a	3-CH_3	Et	H	0.67	Training	0.0013
	4b		CH_3	CH_3	3.00	Training	0.0055
	4c		Et	Et	5.00	Test	0.0260
	4d		iPr	iPr	2.67	Test	0.0151
	4e		C_5H_{10}		1.42	Training	0.0001
	5a	2-Cl	Et	H	0.58	Training	0.0006
	5b		CH_3	CH_3	5.00	Training	0.0076
	5c		Et	Et	3.00	Training	0.0602
	5d		iPr	iPr	1.00	Test	0.7728
	5e		C_5H_{10}		1.00	Training	0.0281
	6a	2-OEt	Et	H	0.08	Training	0.0003
	6b		CH_3	CH_3	2.83	Training	0.0264
	6c		Et	Et	3.50	Training	0.0012
	6d		iPr	iPr	1.08	Test	0.0144
	6e		C_5H_{10}		1.33	Training	0.0030
	7a	Et	H		1.00	Training	0.0058
	7b		CH_3	CH_3	2.17	Test	0.0020
	7c		Et	Et	6.00	Training	0.1043
	7d		iPr	iPr	1.00	Test	0.0014
	7e		C_5H_{10}		2.58	Training	0.1814

Table 5.2. *Contd.*

Compound structure	Compd #	X	R1	R2	PT Hrs	Training or Test set	VP
	8a		Et	H	0.50	Training	0.0168
	8b		CH_3	CH_3	3.00	Training	0.0136
	8c		Et	Et	4.00	Training	0.1638
	8d		iPr	iPr	2.00	Training	0.2843
	8e		C_5H_{10}		2.00	Training	0.0315

PT = Protection Time; **VP** = Vapor Pressure @ 30°C.

Table 5.3. Peptide amino acid sequence and their anti-bacterial activity

Pep#	Amino acid sequence	*SA* μM†	*MR* μM†
1	NH_2KLTcOcKTcOcFTcOcKTcOcFTcOcKTcOcKRNH_2	10	30
2	AcGFTcOcGKTcOcGFTcOcGKTcKKKK-NH_2	3	10
3	NH_2GFTcOcGKTcOcGFTcOcGKTcKKKK-NH_2	10	10
4	NH_2KLTcOcGKTcOcGFTcOcGKTcKKKK-NH_2	30	3
5	AcFTcOcKTcOcFTcOcKTcKKKKNH_2	3	30
6	AcFTcOcKTcOcFTcOcKTcKKKKKKNH2	3	3
7	AcGabaFTcOcGabaKTcOcGabaFTcOcGabaKTcKKKKNH_2	100	10
8	AcbAlaFTcOcbAlaKTcOcbAlaFTcOcbAlaKTcKKKKNH2	10	1
9	AcAhxFTcOcAhxKTcOcAhxFTcOcAhxKTcKKKKNH2	10	3
10	AcGabaFTcOcGabaKTcOcGabaFTcOcGabaKTcKKKKKNH_2	30	3
11	AcGTcOcKTcOcGTcOcKTcKKKKNH_2	10	3
12	AcGFOcGKOcGFOcGKKKKKNH_2	10^6	100
13	AcGFGOcGKGOcGFGOcGKGKKKKNH2	10^6	100
14	AcGFTcGKTcGFTcGKTcKKKKNH_2	10^6	30
15	AcGFTcGGKTcGGFTcGGKTcKKKKNH2	10^6	30
16	AcGFFOcGKFOcGFFOcGKFKKKKNH_2	10	10
17	AcGFTcOcGKTcOcGFTcOcGKTcKKKKKNH_2	3	3
18	AcGFTcOcGKTcOcGFTcOcGKTcOOOONH_2	10	10
19	AcGFpaTcOcGKTcOcGFpaTcOcGKTcKKKKNH_2	10	3
20	AcGFTcOcGOTcOcGFTcOcGOTcOOOONH_2	3	10
21	AcGFTcOcGKTcOcGFTcOcGKTcKKKKCONHCH_2CH_2NH_2	3	10
22	AcGFTcOcGKTcOcGFTcOcGKTcKKKKCONHCH_2CH_2CH_2NH_2	10	10
23	NH_2ELMNSTcOcGLTcOcGKTcOcGLTcOcGKTcOcELMNSNH_2	10^6	10^6
24	NH_2GKGLTcOcGKTcOcGFTcOcGKTcOcGFTcOcGKTcOcGKRNH_2	10	NT
25	NH_2GKGLTcOcGRTcOcGFTcOcGRTcOcGFTcOcGRTcOcGKRNH_2	10	10^6
26	NH_2GKGLTcOcGLTcOcGKTcOcGLTcOcGKTcOcGLTcOcGLRNH_2	100	NT
27	NH_2GKGLTcOcGKTcOcGLTcOcGKTcOcGLTcOcGKTcOcGKRNH_2	10	NT
28	NH_2GKGLTcOcFKTcOcKFTcOcFKTcOcKFTcOcFKTcOcFKRNH_2	30	10^6

Pep# = Compound #; Tc = Tetrahydroisoquinolinecarboxylic acid; Oc = Octahydroindolecarboxylic acid; Fpa = 4Fluoro Phenylalanine; Gaba = γAminobutyric acid; Ahx = εAminohexanoic acid; Ac = Acetyl; NT = Not Tested;† Since all analogs were screened in the concentration range of 0.1 μM to 100 μM, compounds with MIC of ≤ 100 μM, were deemed to be active compounds. For QSAR purposes all inactive compounds were assigned an MIC of 1.0 M

RESULTS AND DISCUSSION

Antineoplastic Cyclic Pentapeptide CXCR4 Inhibitor 3D-QSAR Study

The highly flexible cyclic pentapeptides have hundreds of possible conformers within 5 Kcals/mol energy range of their respective global minimum conformation energy. A set of the first or lowest energy 25 conformers (which were within 3 Kcals/mol of the global minimas) was selected for each of the training set peptide (Table 5.1) to mine the respective biologically active conformation. All of the 25 conformers of each of the top three most active peptides *viz*. FC131, FC68 and FC92 were chosen for discovering the biologically and statistically most significant alignment rule. The background of the multi-way Partial Least Squares (multi-way-PLS) method is as follows. The multi-way-PLS method was developed by Rasmus (1996), wherein each dimension (way) corresponded to the different compounds in the data set, their CoMFA field variables, the conformations of each compound and the different alignment rules. The set of conformers and the alignment rule that provided the best correlation of the observed and predicted values were computed as the solution of the mathematical equations. We have mimicked the multi-way-PLS approach by performing several two-way-PLS analyses. The set of 25 conformers for each peptide was one of the variable parameters (one way), whereas the 75 different alignments (25 conformers each of the top 3 most active compounds) was the other variable parameter (second way). Each progressive step is termed as 'generation' in our methodology. Thus, the first generation of the 75 Molecular Spread Sheets (MSSs) contained 25 conformers of each of the 16 peptides, totaling to 400 data points. After performing the first set of PLS analyses, we selected two conformers of each peptide with the minimum and next-to-minimum residual values (Residual = Predicted – Observed bioactivity). This resulted in the second generation of the 75 MSSs with 2 conformers of each the 16 peptides, totaling to 32 data points. PLS analyses of the second generation MSSs gave 75 QSAR models, all having $q^2>0.6$ with the best q^2 of 0.863. We selected one conformer for each of the peptide with the least residual value from the second generation MSSs to give the 75 third generation MSSs with 16 conformers (one per peptide) in each of the MSS. The third set of PLS analyses gave 28 QSAR models with $q^2>0.6$ with the best q^2 of 0.760. The optimal number of components was 6 for 19 models, 5 for 8 models and 3 for 1 model. The standard error of prediction was less than 1.0 for 5 models with a maximum value of 1.13 for the rest of the models. The fourth generation models were selected from the third generation models based on the cutoff q^2 value of 0.667 resulting in eight 3D-QSAR models. The leave-one-out cross-validated q^2 of the eight models varied from 0.644 to 0.734, whereas the non-validated r^2 varied from 0.998 to 1.0. The cross-validated standard error in prediction values range from 0.911 to 1.068, while the non-validated standard error in prediction values range from 0.036 to 0.082. The optimal number of components was 6 for

5 models and 5 for 3 models. The statistical measure F-value, which is the ratio of r^2 to $(1 - r^2)$ ranged from 954 to 4219. The overlay of all of the training set compounds biologically active conformers along with the template molecule 68_2 (third lowest energy conformer from the global minima of peptide FC68) is shown in Fig 5.1. The predictive r^2 based on the 9 test set peptides ranged from 0.829 to 0.990. The predicted *vs.* observed bioactivities plot of the most predictive QSAR model (model A), which was based on the FC68 peptide alignment rule for the training set and test set peptides for, is shown in Figs 5.2 & 5.3 respectively. The CoMFA contours of the QSAR model A along with the most active peptides (FC131, FC92 and FC68) bioactive conformations are depicted in Fig 5.4.

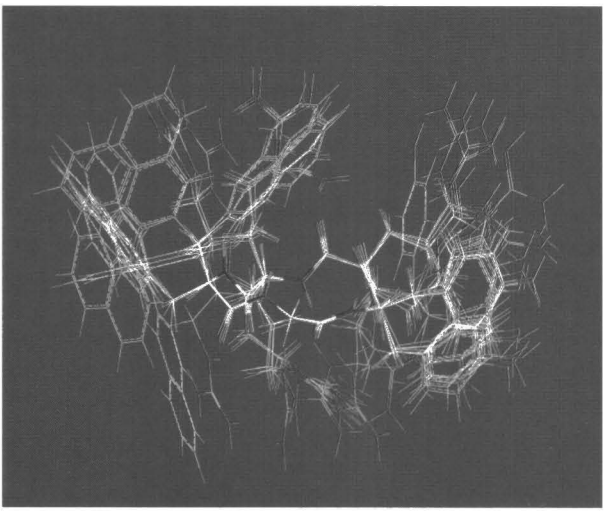


Fig 5.1. Alignment of the training set compounds along with the template molecule 68_2

The CoMFA interpretation is as follows. The positively charged arginines (Blue positive-charge favored regions) of all the peptides correlate with enhanced bioactivity. The bulky napthyl rings (green region) of FC131 and FC92 Nal residues contribute to enhanced activity, while the Nal napthyl ring of FC68, being placed outside the sterically favored (green) region, correlate with reduced bioactivity. In the same vein, the Tyr aromatic ring of the more potent FC131 and FC92 fall in the sterically favored (green) region, whereas for the relatively less potent FC68 it falls outside of the sterically favored region. The hydroxyl group of Tyr residue of the most potent FC131 falls in the negative-charge favored (red) region, while for the little less potent FC92 it falls a little away. The hydroxyl group of Tyr residue of FC68 falls far outside the negative charge favored (red) region correlating with reduced bioactivity. The other negative charge favored (red), steric or bulky groups favored (green) and steric or bulky groups

disfavored (yellow) regions where no portions of FC131, FC68 and FC92 occupy correlate the bioactivities of the other moderately active peptides.

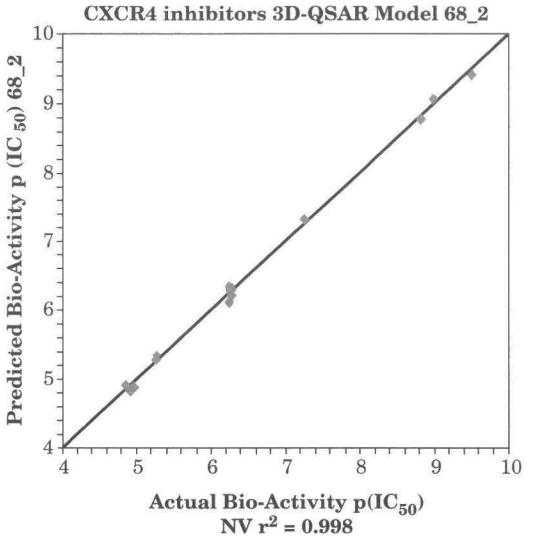


Fig 5.2. The predicted *vs.* actual bioactivities plot of the training set peptides for the best QSAR model 68_2

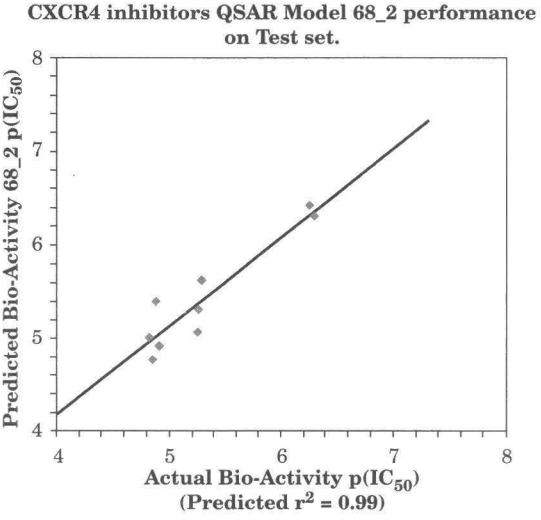


Fig 5.3. The predicted *vs.* actual bioactivities plot for the prediction of the test set compounds by the best QSAR model 68_2

Thus, the important conclusions from this 3D-QSAR study are that:

(i) the positively charged arginines provide the most significant interactions correlating to increased bioactivity,

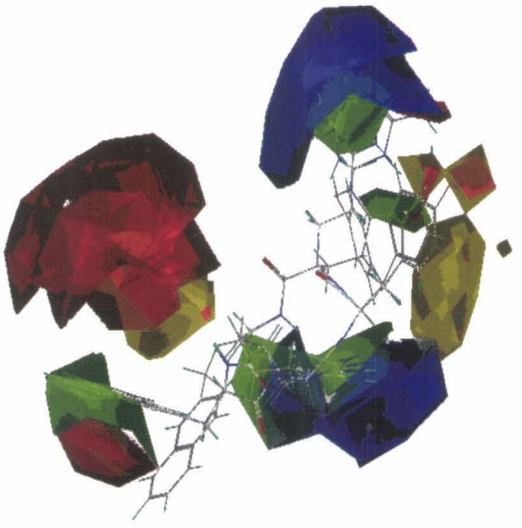


Fig 5.4. The CoMFA contours of the QSAR model A along with the peptides FC131, FC92 and FC68

(ii) the Tyr hydroxyl group provide the second most significant interactions for increased bioactivity, and

(iii) the Nal napthyl ring and Tyr aromatic ring provide the moderate interactions correlating to moderate increase in bioactivity.

In summary, this 3D-QSAR study demonstrates the use of brute force methods for discovering the bioactive conformations and biologically relevant alignment rules using CoMFA descriptors. The study provides valuable insight in the significant ligand and CXCR4 receptor interactions leading to enhance bioactivity. These results would help future researchers to design better and potent CXCR4 inhibitors.

DEET Based Insect Repellents 3D-QSAR Study

The data set of forty compounds was divided in two sets as training set with thirty compounds and test set of ten compounds. We computed a total of 127 descriptors comprising of ADME, electrotopological state (Kier & Hall, 1992), thermodynamic (Ghose & Crippen, 1986), Ghosh and Crippen atom types, Kiers shape indices (Kier, 1985), Jurs (Stanton & Jurs, 1990) partially charged surface areas, shadow indices (Rohrbaugh & Jurs, 1987) and quantum chemical descriptors. The stepwise method for the descriptor selection was adopted (Yao *et al.*, 2003) by first discarding all descriptors with poor correlation with bioactivity ($|r|<0.1$) followed by discarding the highly collinear descriptors with cross correlation coefficients greater than 0.9. We have juxtaposed the traditional 3D-QSAR methodology of the global minimum conformation

with our novel methodology by computing the 3D-QSAR models using the global minimas of the training set compounds with 127 descriptors and also with 30 selected descriptors using the genetic function algorithm (GFA), partial least square (PLS) and genetic partial least square (G/PLS) methods. These efforts gave models with non-validated R2 (nvR2) ranging from 0.792 to 0.935 and internal cross validation tests, leave-one-out (q^2_{LOO}), leave-10%-out and leave-20%-out greater than 0.7. However, all of these models performed poorly when subjected to the rigorous external validation with the test set compounds, as they yielded a predictive r^2 of 0.349 or less. The contemporary approach of using the global minimum conformation mostly does not furnish good QSAR models, probably because the bioactive conformations are quite different as compared to the global minimum conformations. Thus, the novel methodology we have devised to discover the bioactive conformation is by mining through a set of conformations that are within the energy range of 20 Kcals/mol of the global minimum such that the set of conformations have a good representation in the 3D space around some putative pharmacophoric moiety. The set of all 20–25 conformers of 30 training set compounds totaled to 706 conformations. The overlay of all the training and test set compounds aligned conformers is shown in Fig 5.5.

Fig 5.5. Overlay of all 940 conformers showing the alignment

The first generation 3D-QSAR model based on the selected 30 descriptors using PLS method for the 706 conformations gave a model with nvR^2 of 0.883, q^2_{LOO} of 0.877 and prediction error sum of squares (PRESS) of 200.06. The predicted residual (Predicted–Actual PT) values of several conformers showed identical values. On closer examination of the descriptor values of such conformers, we found that the descriptor values were also almost identical. On removal of such 'duplicate' conformers we got a set of 501

conformers, which on PLS analysis furnished the second generation 3D-QSAR model with nvR^2 of 0.879, q^2_{LOO} of 0.869 and PRESS of 135.01. The conformers selected for all subsequent generation models were the ones with least residual values. The next generations QSAR models were built by selecting aforementioned number of conformers from their respective previous generation QSAR models. Thus, 10 conformers for the third generation (300 conformers), 5 conformers for the fourth generation (150 conformers) and 2 conformers for the fifth generation (60 conformers) QSAR models were selected to give nvR^2 of 0.921, 0965 & 0.988, q^2_{LOO} of 0.911, 0.956 & 0.977 and PRESS values of 60.43, 15.12 & 3.10 respectively. For the sixth generation QSAR model the data was divided into two sets with most active PT cut off value of 3.0 h and not active PT values of less than 3.0. Thus, for the 9 compounds *viz.* **Compd#**(PT): **2b** (4.0), **3c** (4.0), **3d** (3.0), **5b** (5.0), **5c** (3.0), **6c** (3.5), **7c** (6.0), **8b** (3.0) and **8c** (4.0) two conformers were retained and for the remaining 21 training set compounds, the least residual value conformer were selected for the sixth generation 3D-QSAR model. The seventh generation 3D-QSAR model showed nvR^2 of 0.991, q^2_{LOO} of 0.974 and PRESS of 2.565. The final seventh generation QSAR model can be computed by choosing either one conformer for the nine most active compounds in 2^9 or 512 different ways. The computation of 512 3D-QSAR models using a Tcl-based Cerius2 script yielded six seventh generation models with q^2_{LOO} of 0.67 or larger. The best seventh generation 3D-QSAR model showed nvR^2 of 0.989, q^2_{LOO} of 0.701 and PRESS value of 20.37. Fig 5.6 shows the observed and predicted protection time plot for the best seventh generation QSAR model. The final 3D-QSAR model showed and excellent predictive r^2 of 0.845.

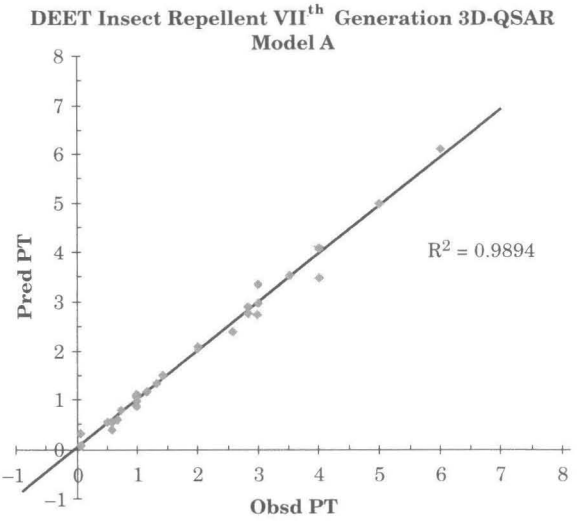


Fig 5.6. The observed *vs.* predicted protection time (PT) plot for the VIIth generation QSAR Model A

The gradual refinement of the set of conformers in the successively generated 3D-QSAR models computed by selecting the least residual value conformers, results in the conformers that best correlate with the observed bioactivity. Thus, we claim that these are indeed the bioactive conformations of the respective compounds. The shapes of these selected 'bioactive conformers' would then allude to the roles of the various moieties around the putative amide pharmacophore in the mechanism of action as also in the structure activity relationship. Based on the current study there are three important conclusions about the role of DEET analogs and derivatives in the insect repellency mechanism of action, *viz.*

1. The 3D-spatial location of the groups (phenyl, benzyl and cyclohexyl) attached to the carbonyl carbon does not have a significant effect on the bioactivity and thus this moiety is the one that probably dock with the OBP to form the complex.
2. There is a preferential positioning of the methyl, ethyl, isopropyl etc moieties on the amidic N within a narrow range of 60° to 70°, so we propose that these moieties probably interacts with the neuronal GPCR in the rate limiting step.
3. The compounds with poor hydrophobic group (*e.g.*, para or ortho methoxy/phenyl/benzyl) cannot dock effectively with the OBP and thus irrespective of the groups on the amidic N exhibit poor repellency activity, which probably also alludes to the competing nature of the OBP and ODE.

The Equation 1 describes the best 3D-QSAR model:

Equation 1:

Pred Bioactivity = 0.538 * ADME_Absorption_T2_2D – 0.682 * ADME_BBB_2D – 0.042 * Energy – 0.689 * ADME_BBB_Level_2D – 0.531 * S_dssC – 1.209 * ADME_Solubility_Level – 0.192 * S_aasC – 0.367 * S_ssNH + 0.054 * S_ssO + 0.531* Jurs-FNSA-2 + 0.001 * LUMO_MOPAC + 0.433 * DIPOLE_MOPAC + 0.004 * HF_MOPAC – 0.0001 * Jurs-DPSA-2 – 0.014 * Jurs-DPSA-3 + 1.288 * Jurs-FPSA-1 + 66.492 * Jurs-FPSA-3 + 0.536 * Jurs-RPCS + 12.508 * Jurs-RASA – 0.008 * Shadow-XY – 0.531 * Shadow-nu – 0.285 * Shadow-Xlength – 0.057 * Shadow-Zlength + 0.312 * Density – 0.001 * PMI-mag – 0.074 * Atype_C_5 + 0.196 * Atype_H_47 + 0.097 * Fh2o + 1.515 * JX + 1.299 * Kappa-3-AM – 12.4913.

The value and sign of the 3D-QSAR equation coefficients provide a qualitative insight into the correlation of the respective physicochemical (PC) property to the observed protection time. However, the quantitative contribution of any PC property to the protection time can only be judged from both the QSAR equation coefficient and the descriptor value quantifying it. So, we computed the mean descriptor values, for this purpose, as the arithmetic average of the descriptor values of all the training set compounds (*i.e.* MVD = [Σ descriptor value of all training set compounds]/ 30). The product of the QSAR equation coefficient (QEC) and the mean

descriptor value (MVD) would now provide the contribution of that PC property (CtoBA) to the protection time (*i.e.* CtoBA = QSAR coefficient * MVD). Further, the significance of any PC property *vis-à-vis* all other PC properties appearing in the QSAR equation can be computed as the ratio of CtoBA to the sum total of all CtoBA. The percentage value of this quotient, is what we have termed as the 'Descriptor Significance Percentage' DSP (*i.e.* DSP = CtoBA * 100/Σ abs (CtoBA)). Thus, the DSP values would now provide a more accurate insight into the quantitative contribution of each of the descriptors to the protection times. The list of descriptors and their QEC, MVD, CtoBA and DSP is presented in Table 5.4.

Table 5.4. Computation of descriptor significance percentage (DSP) for the DEET based insect repellent 3D-QSAR model

Descriptor	QEC	MVD	CtoBA	DSP
Jurs-RASA	12.508	0.882	11.028	25.038
Jurs-FPSA-3	66.492	0.074	4.916	11.161
JX	1.515	2.527	3.828	8.690
ADME_Solubility_Level	–1.210	3.100	–3.750	–8.514
Shadow-Xlength	–0.285	11.888	–3.393	–7.703
Kappa-3-AM	1.299	2.568	3.337	7.577
Energy	–0.042	51.357	–2.164	–4.912
Atype_H_47	0.196	7.933	1.555	3.530
DIPOLE_MOPAC	0.433	3.553	1.537	3.490
ADME_Absorption_T2_2D	0.538	2.736	1.473	3.344
Shadow-nu	–0.531	1.909	–1.014	–2.301
Jurs-FPSA-1	1.288	0.770	0.992	2.252
ADME_BBB_Level_2D	–0.689	1.300	–0.896	–2.034
Jurs-DPSA-3	–0.014	50.052	–0.717	–1.628
Shadow-XY	–0.008	59.513	–0.488	–1.109
Fh2o	0.097	–4.866	–0.473	–1.073
PMI-mag	–0.001	324.800	–0.438	–0.993
Shadow-Zlength	–0.057	6.306	–0.359	–0.815
Density	0.312	1.004	0.313	0.711
Jurs-RPCS	0.536	0.488	0.262	0.595
S_ssNH	–0.367	0.642	–0.236	–0.536
Jurs-FNSA-2	0.531	–0.438	–0.232	–0.528
S_aasC	–0.192	1.148	–0.221	–0.501
Jurs-DPSA-2	0.000	808.217	–0.131	–0.298
S_ssO	0.054	1.394	0.076	0.172
ADME_BBB_2D	–0.682	0.103	–0.071	–0.160
S_dssC	–0.531	0.114	–0.061	–0.138
HF_MOPAC	0.004	–11.390	–0.047	–0.107
Atype_C_5	–0.074	0.533	–0.040	–0.090
LUMO_MOPAC	0.001	0.094	0.000	0.000

QEC—QSAR Model A Equation coefficient values
MVD—Mean value of descriptors of all training compounds = (Σ descriptor_value/30)
CtoBA—Contribution of the descriptor to bioactivity = (QEC * MVD)
DSP—Descriptor significance percentage = [CtoBA * 100/Σ abs (C to BA)]

The top five descriptors Jurs-RASA, Jurs-FPSA-3, JX, ADME-Solubility level and Shadow-X length contribute to 62% of the protection time. The largest contribution to the protection time is from Jurs-RASA with a positive 25% contribution. Jurs-RASA is defined as the ratio between the total hydrophobic surface area (Jurs-TASA) and the total solvent accessible surface area (Jurs-SASA). This observation is consistent with the first step of the MOA where the odorant molecule binds to the OBP and hydrophobicity or lipophilicity plays a key role. ADME-Solubility level with negative 8.5%, Atype_H_47 with positive 3.5% and Fh2o with negative 1.1% also support the role of hydrophobicity in the MOA. This observation is also in agreement with the earlier reports (McIver, 1981; Suryanarayana *et al.*, 1991) that compound lipophillicity is directly related to repellency effect. The next largest contribution to protection time is from Jurs-FPSA-3 with a positive 11% value. Jurs-FPSA-3 is the quotient of Jurs-PPSA-3 and Jurs-SASA, where Jurs-PPSA-3 is the summation of the products of solvent accessible surface area and partial charge of all positively charged atoms. Thus, the 3D-QSAR model suggests that larger partial positive surface areas and larger partial positive charge along with smaller total solvent accessible surface area would correlate with higher protection time. This probably alludes to the second step of the mechanism of action where the odorant-OBP complex binds the neuronal GPCR peptide residues. The diffused or soft positively charged moiety's correlation with increased protection time is also corroborated by Jurs-FPSA-1 (Jurs-Fractional Positive Surface Area-1) defined as the sum of the solvent accessible surface area of all partial positively charged atoms with a positive 2.3% contribution and the positive 8.7% contribution from the Balaban index JX, which is inversely proportional to the electronegativities and covalent radii of the atoms in the repellent molecules. The fifth largest DSP contribution of negative 7.7% comes from the descriptor Shadow-X length, which is the measure of the projection of the molecule on the x-axis. The contribution of other shadow indices are shadow-Z length (projection measure on the z-axis) of negative 0.8%, shadow-XY (the area of the shadow of the molecule in the XY plane) of negative 1.1% and shadow-nu (ratio of the largest to the smallest shadow measures) of negative 2.3%. This combination of shadow indices indicate that elongated rectangular box (parallelepiped) like molecular structure correlate with protection times. This alludes to the shape of the binding pocket of the OBP involved in the first step of the mechanism of action.

AMP Based Antibacterials 3D-QSAR Study

The selection of descriptors was done in two steps with the removal of the descriptors that showed very poor correlation with bioactivity ($|r| < 0.1$) as the first step. The cross correlation matrix showed that 33 descriptors exhibited very high cross correlation coefficient values ($|r| > \sim 0.9$). The

second step of descriptor selection was the removal of the highly cross correlated descriptors to leave the final 22 and 21 descriptors for ***SA*** and ***MR*** QSAR models respectively. The list of the final descriptors for the two 3D-QSAR models is presented in Table 5.5. Our novel, gradual and stepwise bioactive conformer mining methodology mines the clustered conformations and identifies the bioactive conformers that most closely correlate with the observed bioactivity. Thus, the bioactive conformer mining method, over seven iterative generations (Bhonsle *et al.*, 2007) resulted in two conformers each for the 12 peptides (Pep# **1, 2, 5, 6, 17, 19, 20, 21, 22, 24, 25 & 27** for ***SA*** and Pep# **4, 6, 8, 9, 10, 11, 17, 18, 19, 20, 21 & 22** for ***MR***) and one conformer for each the remaining 16 peptides. There are 4096 (2^{12}) combinations or ways to select the best set of 12 conformers, from the 24 conformers. The 4096 eighth generation models were computed employing a Tcl-based Cerius2 script. The final ***SA*** and ***MR*** 3D-QSAR models, selected based on the best cross-validated r^2, showed non-validated r^2 of 0.988 and 0.997, leave-one-out cross-validated r^2 of 0.839 and 0.997 with PRESS values of 22.92 and 29.19 respectively. The 3D-QSAR equations for predicting the activity against ***SA*** is given in equation 2 and that against ***MR*** is given in equation 3. The correlation plots of the predicted *vs.* the observed antibacterial activities of the ***SA*** and ***MR*** 3D-QSAR models are shown in Figs 5.7 and 5.8 respectively. Internal validation (cross-validation) tests of the final 3D-QSAR models were performed at two levels. Both of the models showed $q^2_{LOO} > 0.83$ for the leave-one-out (LOO) cross-validation tests. For the leave-10%-out or leave-three-out (L10O) cross-validation tests, ***SA*** model showed q^2_{L10O} of 0.875, whereas ***MR*** model showed q^2_{L10O} value of 0.537. With more number of independent variables compared to the data points, it is well known that models obtained are prone to chance correlation. To ensure that the models we computed were not a result of chance correlation, we performed randomization tests of ninety-nine trials each at 99% confidence level for ***SA*** and ***MR*** 3D-QSAR models. None of the random r values were found to be larger than the non-random r values for either the ***SA*** or the ***MR*** models. The mean random r value for the ***SA*** model was 0.572 (r^2 = 0.327), and for the ***MR*** model was 0.617 (r^2 = 0.380). This proved that the ***SA*** and ***MR*** QSAR models are not obtained by chance.

Table 5.5. A rank ordering of the physicochemical properties defining ***SA*** and ***MR*** antibacterial activity

Physico-chemical property	*Staphylococcus aureus* QSAR_DSP	Physico-chemical property	*Mycobacterium ranae* QSAR_DSP
Jurs-FPSA-1	29.347	Density	–30.784
Density	–16.01	Jurs-RASA	16.827
Jurs-TASA	–14.762	Jurs-PPSA-1	–15.494
Jurs-PNSA-1	10.54	Jurs-TPSA	10.218
Jurs-RASA	7.886	Jurs-RPSA	–5.444

Table 5.5. *Contd.*

Physico-chemical property	*Staphylococcus aureus* QSAR_DSP	Physico-chemical property	*Mycobacterium ranae* QSAR_DSP
Jurs-SASA	4.12	Hbond donor	–3.905
Jurs-DPSA-2	3.093	Hbond acceptor	3.729
Jurs-PNSA-2	–2.911	Jurs-FPSA-1	–3.409
Jurs-RPSA	–2.492	Fcharge	2.892
Rotlbonds	–2.164	Jurs-PNSA-1	–1.244
Hbond acceptor	1.91	Rad of gyration	1.164
Jurs-FPSA-3	1.709	Rotlbonds	–1.156
Fcharge	–0.742	Apol	1.148
Jurs-RPCG	–0.726	Jurs-PPSA-2	1.016
Jurs-PPSA-1	0.555	Jurs-PNSA-2	–0.632
Jurs-FNSA-3	–0.426	Jurs-RNCG	0.4
Dipole-mag	0.162	Dipole-mag	0.298
RadOfGyration	–0.127	Jurs-FNSA-3	–0.127
Jurs-RPCS	–0.126	AlogP	0.051
Hbond donor	0.113	Conformer energy	0.037
Jurs-DPSA-3	0.053	Jurs-RPCG	–0.024
AlogP	–0.026	Jurs-DPSA-2	0

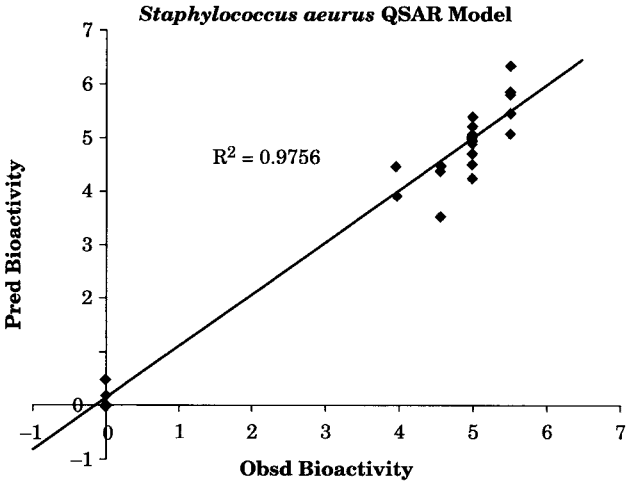


Fig 5.7. The correlation plot of predicted *vs.* observed anti-bacterial activities for the *Staphylococcus aureus* 3D-QSAR models

The ***SA*** 3D-QSAR model is described by **Equation: 2**

SA Predicted Activity = [(–1.49592 * Fcharge) + (0.0098147 * Dipole-mag) + (0.013993 * Jurs-SASA) + (0.00233 * Jurs-PPSA-1) + (0.187647 *

Jurs-PNSA-1) + (0.0021686 * Jurs-PNSA-2) + (0.00036919 * Jurs-DPSA-2) + (0.0015025 * Jurs-DPSA-3) + (438.251 * Jurs-FPSA-1) + (267.258 * Jurs-FPSA-3) + (120.432 * Jurs-FNSA-3) – (715.316 * Jurs-RPCG) – (12.8649 * Jurs-RPCS) – (0.065752 * Jurs-TASA) – (125.513 * Jurs-RPSA) + (125.513 * Jurs-RASA) – (183.99 * Density) + (1.03397 * Hbond acceptor) + (0.039473 * Hbond donor) – (0.306856 * Rotlbonds) + (0.114808 * AlogP) – (0.10004 * RadOfGyration) – 225.589].

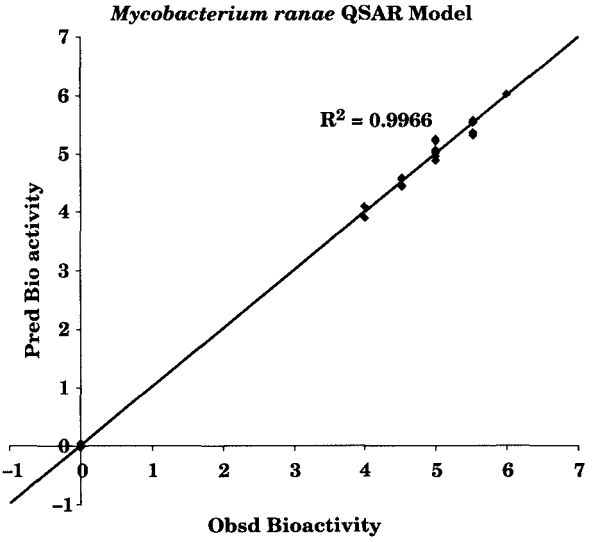


Fig 5.8. The correlation plot of predicted *vs.* observed anti-bacterial activities for the *Mycobacterium ranae* 3D-QSAR models

The ***MR*** QSAR model is described by **Equation: 3**

MR Predicted Activity = [(–0.0083585 * Conformer Energy) + (2.05758 * Fcharge) + (5.3259e-05 * Apol) + (0.0061422 * Dipole-mag) – (0.023941 * Jurs-PPSA-1) – (0.008252 * Jurs-PNSA-1) + (5.5381e-05 * Jurs-PPSA-2) + (0.00018566 * Jurs-PNSA-2) – (18.282 * Jurs-FPSA-1) + (13.321 * Jurs-FNSA-3) – (8.46841 * Jurs-RPCG) + (66.6262 * Jurs-RNCG) + (0.052889 * Jurs-TPSA) – (96.9761 * Jurs-RPSA) + (96.9761 * Jurs-RASA) – (127.577 * Density) + (0.768698 * Hbond acceptor) – (0.498282 * Hbond donor) – (0.060764 * Rotlbonds) – (0.075759 * AlogP) + (0.337835 * RadOfGyration) + 110.841].

The seventeen physiochemical properties appearing in the QSAR equations that are common to the ***SA*** and ***MR*** 3D-QSAR models are shown in Table 5.5. The five physicochemical properties specific to the ***SA*** QSAR model are Jurs–Fractional-Positive-Surface-Area–3 (Jurs-FPSA-3), Jurs-Relative-Positive-Charge-Surface-area (Jurs-RPCS), Jurs-Differential-Positively-charged-Surface-Area-3 (Jurs-DPSA-3), Jurs-total-Solvent-Accessible-Surface-Area (Jurs-SASA) and Jurs-TotAl-hydrophobic-Surface-

Area (Jurs-TASA). Whereas, the five physicochemical properties specific to the ***MR*** QSAR model are sum-of-all-atomic-polarizabilities (Apol), Conformer Energy, Jurs-Partial-Positively-charged-Surface-Area-2 (Jurs-PPSA-2), Jurs-Relative-Negative-CharGe (Jurs-RNCG), and Jurs-Total-Polar-Surface-Area (Jurs-TPSA). The commonality of physicochemical properties shows the minimal requirement for activity against ***SA*** and ***MR***. The importance of electrostatic potential for the AMP bioactivity is evident from the physicochemical properties such as Dipole-magnitude (Dipole-mag), Formal charge (Fcharge), Jurs-Fractional-Negatively-charged-Surface-Area (Jurs-FNSA-3), Jurs-Relative-Polar-Surface-Area (Jurs-RPSA), Jurs–Fractional-Positive-Surface-Area–1 (Jurs-FPSA-1), Jurs–Fractional-Negative-Surface-Area–1 (Jurs-PNSA-1), Jurs–Fractional-Negative-Surface-Area–2 (Jurs-PNSA-2), Jurs–Partially-Positive-Surface-Area–1 (Jurs-PPSA-1), and Jurs-Relative-Positive-CharGe (Jurs-RPCG). While the significance of the AMP molecular shape for bioactivity is seen from the physicochemical properties such as molecular Density (Density), number-of-H-bond-acceptors (H-bond acceptor), Jurs-RelAtive-hydrophobic-Surface-Area (Jurs-RASA), number-of-H-bond-donor (H-bond donor), molecular-Radius-Of-Gyration (RadOfGyration), and number-of-Rotatable-bonds (Rotlbonds). The importance of amphipathicity is alluded to by the physicochemical properties such as Jurs-RASA, Jurs-RPSA, and AlogP. The top six descriptors based on their DSP values are Jurs-FPSA-1 (29.35%), Density (–16.01%), Jurs-TASA (–14.76%), Jurs-PNSA-1 (10.54%), Jurs- RASA (7.89%), and Jurs-SASA (4.12%) which together account for 82% of the ***SA*** predicted activity. The correlation of non-polar surface area to bioactivity is evident from the descriptors such as Jurs-TASA with –14.76% DSP contribution and Jurs-RASA with 7.89% DSP contribution. The significant descriptors based on their DSP values accounting for 82% of ***MR*** predicted activity are Density (–30.78%), Jurs-RASA (16.83%), Jurs-PPSA 1 (–15.49%), Jurs-TPSA (10.22%), Jurs-RPSA (–5.44%), and H-bond donor (–3.91%). The correlation of the polar surface area to the ***MR*** bioactivity is evident from the descriptors Jurs-PPSA-1 with –15.5% DSP, Jurs-TPSA with 10.5% DSP contribution and Jurs-RPSA with –5.44% DSP contribution. The hydrophobicity and hydrophilicity correlation with the ***MR*** bioactivity is shown by the descriptors Jurs-RASA with 16.8% DSP contribution, and H bond donor with –3.9% DSP. The contribution of shape to ***MR*** predicted bioactivity comes from the descriptor density with –30.78% DSP contribution.

CONCLUSIONS

Computation of predictive 3D-QSAR models is a significant and challenging problem, which is further compounded for highly flexible compounds. We have successfully demonstrated the use of "bioactive conformation mining" methodology for discovering the biologically active conformation of the flexible compounds that best correlate with the observed bioactivity.We have demonstrated the application of this method for antineoplastic cyclic

pentapeptides CXCR4 inhibitors, DEET based insect repellents, AMPs for ***SA*** and ***MR*** antibacterial activities with cross-validate r^2 and predictive r^2 ranging from 0.6 to 0.99.

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Synthesis and Structure-Activity Relationships of Some Taxoids as Multidrug Resistance Modulator in MDR Cancer Cells

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ABSTRACT

*1, 7-Dideoxy-4-deacetylbacatin III (**12**) and its eight analogs **5–11, 13**, and their oxetane ring opened derivatives **14–17** were synthesized from taxinine (**1**). In them, compounds **6–9, 12–14, 16**, and **17** showed significant activity as MDR reversal agent by the assay of the calcein accumulation toward*

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MDR human ovarian cancer 2780AD cells. The most effective compound ***12*** *in this assay is efficient for the recovery of cytotoxic activity of paclitaxel, adriamycin (ADM), and vincristine (VCR) toward MDR 2780AD cells at the same level toward parental 2780 cells. Compound* ***12*** *is the most desirable compound for anti-MDR cancer reversal agent, because it has the highest accumulation ability of anticancer agent in MDR cancer cells and weak cytotoxic activity. Chemical modifications and biotransformations of taxuyunnanine C (****41****) and its analogs,* ***44****,* ***45****, and* ***50*** *gave a series of new generation taxoids. Compounds* ***43****,* ***45****,* ***46****,* ***48****, and* ***49a*** *showed significant activity toward calcein accumulation in MDR 2780AD cells. The most effective compound, 14β-cynnamoyloxy-2α, 5α-diacetoxy-10β-hydroxy-4 (20), 11-taxadiene (****49a****), was effcient for cellular accumulation of VCR in MDR 2780AD. The effective compounds* ***46*** *and* ***49a*** *in calcein assay are efficient for the recovery of cytotoxic activity of paclitaxel, ADM, and VCR toward MDR 2780AD. Since compounds* ***46*** *and* ***48*** *had no cytotoxicity, they were expected to be lead compound of MDR cancer reversal agents. On the contrary, compounds* ***43****,* ***45****, and* ***49a*** *showed cell growth inhibitory activity toward VA-13 and/or HepG2 as well as accumulation of calcein and/or vincristine in MDR 2780AD and they were expected to be lead compounds of new-type anticancer agents. Taxinine NN-1 (****39****) was synthesized from taxinine (****1****) in 45% overall yield in 11 steps. It showed the strongest activity toward VCR accumulation in MDR tumor cells in taxinine derivatives. The best condition of the production of taxinine NN-11 (****40****), which showed very strong activity toward VCR accumulation in MDR tumor cells, was established.*

Key words : Anti-MDR cancer reversal agent, 14β-cynnamoyloxy-2α, 5α-diacetoxy-4(20),11-taxadien-10β-ol, -1,7-dideoxy-4-deacetylbacatin III, MDR reversal agent, taxinine, taxinine NN-1, taxinine NN-11, taxuyunnanine C

INTRODUCTION

In cancer chemotherapy, occurrence of multidrug resistance (MDR) in cancer cells caused by repeated administration of anticancer agents is a serious problem. One mechanism of MDR is overexpression of the P-glycoprotein (P-gp), which is the efflux pump of anticancer drugs (Ueda *et al.*, 1987; Ueda & Komano, 1988). P-gp is a transporter for a wide range of reagents utilizing energy by hydrolysis of ATP. P-gp is expressed on the small intestine, capillary of brain, kidney, and liver. Its physiological role is considered as a defense mechanism against the toxic materials in the cell. When P-gp is expressed on the cell membrane of a cancer cell, it transports various kinds of anti-cancer agents from inside of the cell to the outside. Many taxane derivatives, which showed MDR reversal activity, have been reported (Shigemori & Kobayashi, 2004; Kobayashi *et al.*, 2000;

Kobayashi *et al.*, 1998; Kobayashi *et al.*, 1995; Ojima *et al.*, 2005; Brooks *et al.*, 2004; Minderman *et al.*, 2004; Brooks *et al.*, 2003; Geney, 2002). We have previously reported the isolation of MDR reversal agents from *T. caspidata* (Ando, 2001; Kosugi *et al.*, 2000; Sakai *et al.*, 2001), and their production by callus culture (Ando, 2001; Sakai *et al.*, 2002; Bai *et al.*, 2004; Bai *et al.*, 2005; Dai *et al.*, 2006). Effect of the substitution of the functional groups attached to baccatin III and taxinine on the MDR reversal activity was widely investigated. However, since baccatin III and taxinine analogs have no functional group at C-14, only a few reports were appeared about the effect of the substituent at C-14 on the MDR reversal activity. Ojima *et al.* (2005) reported structure-activity relationships of 14β-hydroxybaccatin III derivatives bearing a 1, 4-carbonate group. In this case, it is clearly indicated that modification of the C-7 position with hydrophobic group effected the MDR reversal activity (Ojima *et al.*, 1988). Under aforementioned background, we investigated the SAR of 1,7-dideoxy-baccatin III derivatives and taxuyunnanine C derivatives.

MATERIALS AND METHODS

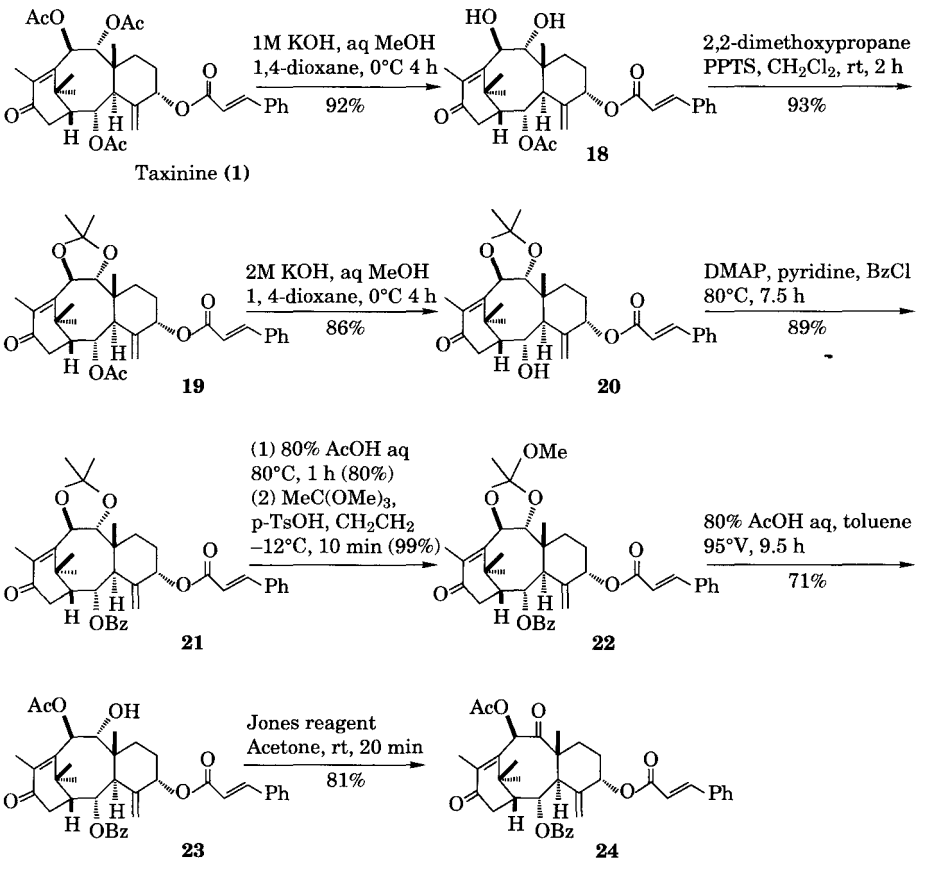
Preparation of Taxinine (1), the Starting Material of Substrate 5-17

Fresh needles and twigs of *T. cuspidata* (4.77 kg) collected in Sendai, Japan were extracted successively with hexane and EtOAc. The EtOAc extract was subject to acid and base extraction scheme. The crude neutral EtOAc extract (63.5 g) was separated repeatedly by column chromatography (silica gel) to give pure taxinine (**1**) (9.5 g, 0.2% yield).

Preparation of Substrates 5-17 (see Fig 6.1)

The starting material taxinine (**1**) is the major component of Japanese yew tree, *Taxus cuspidata*. Hydrolysis of **1** with 1M aqueous solution of KOH (1M KOH) in a mixture of methanol and dioxane at 0°C gave a diol **18** in 92% yield. Treatment of **18** with 2, 2-dimethoxypropane in the presence of *p*-toluenesulfonic acid (PPTS) gave an acetonide **19** in 93% yield. Further hydrolysis of **19** with 2M KOH in a mixture of methanol and 1,4-dioxane gave 2-hydroxy derivative **20** in 86% yield. Benzoylation of **20** with benzoyl chloride (BzCl) in the presence of 4-dimethylaminopyridine (DMAP) in pyridine afforded the corresponding benzoate **21** in 89% yield. Hydrolysis of **21** with 80% aqueous acetic acid (80% AcOH aq) at 0°C for 4 h, and successive treatment of resulting 9,10-diol with trimethy orthoacetate [$MeC(OMe)_3$] in the presence of PPTS in dichloro methane (CH_2Cl_2) at –12°C gave a 2-methoxy-1,3-dioxolane derivative **22** in 79% overall yield. Treatment of **22** with 80% AcOH aq in toluene at 95°C for 9.5 h gave 10β-acetoxy-9α-hydroxytaxane derivative **23** in 71% yield. Jones oxidation

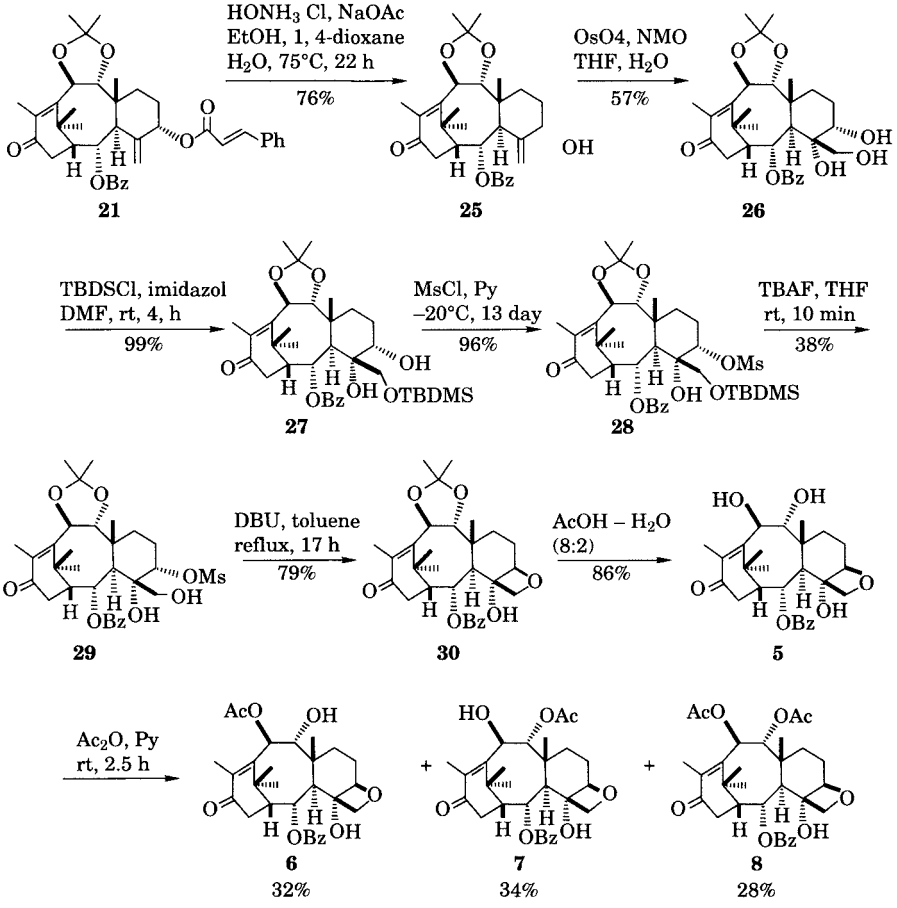
of **23** with Jones reagent (CrO_3, H_2SO_4, acetone) gave 9-oxo derivative **24** in 81% yield (Scheme 1).



Scheme 1.

Treatment of **21** with $HONH_3Cl$ and NaOAc in a mixture of EtOH-1,4-dioxane-water at 75°C for 22 h gave an alcohol **25** in 76% yield. Osmilation of **25** in the presence of *N*-methylmorpholine *N*-oxide (NMO) in a mixture of THF and water gave a triol **26** in 57% yield. Treatment of **26** with TBDMSCl in the presence of imidazole in DMF gave **27** in 99% yield. Mesylation of **27** with MsCl in pyridine at –20°C for 13 days gave mesylate **28** in 96% yield. Deprotection of TBDMS protecting group of **28** by the treatment with tetrabutylammonium fluoride (TBAF) in THF at room temperature for 10 min gave desired product **29** in 38% yield. Treatment of **29** with DBU in toluene at refluxing temperature for 17 h gave **30** in 79% yield. Treatment of **30** with an 8:2 mixture of AcOH and water at room temperature gave the substrate **5** in 86% yield. Acetylation of **5** with 1.1 equivalent of acetic anhydride (Ac_2O) in pyridine at room temperature

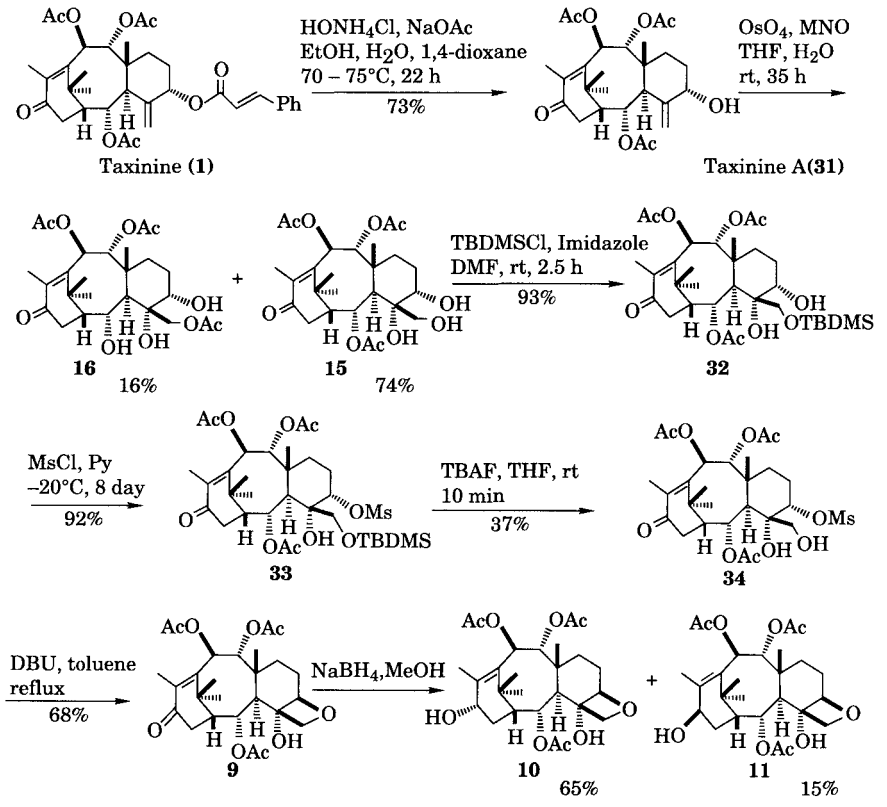
for 2.5 h gave substrate **6**, **7**, and **8** in 32, 34, and 28% yields, respectively (Scheme 2).



Scheme 2.

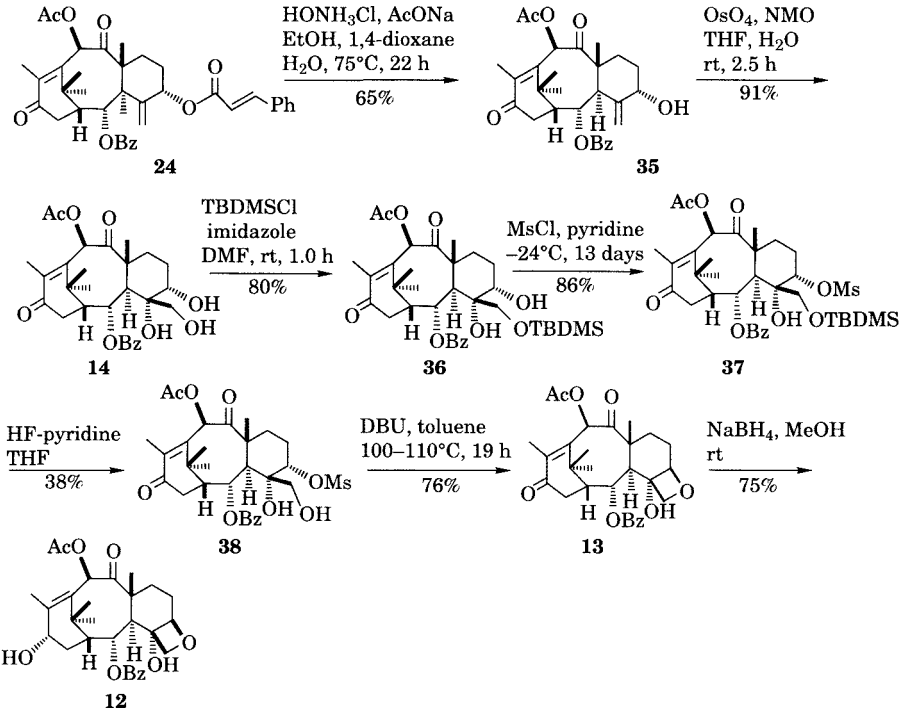
Treatment of taxinine (**1**) with $HONH_3Cl$ and NaOAc in a mixture of EtOH-1,4-dioxane-water at 70–75°C for 22 h gave taxinine A (**31**) in 73% yield. Dihydroxylation of **31** with 0.2 equivalent of OsO_4 in the presence of 2 equivalent of NMO in a 9:1 mixture of THF and water gave the substrates **15** and **16** in 74 and 16% yields, respectively. Treatment of **15** with TBDMSCl in the presence of imidazole in DMF at room temperature for 35 h gave **32** in 93% yield. Mesylation of **32** with MsCl in pyridine at –20°C for 8 days gave a mesylate **33** in 92% yield. Deprotection of TBDMS protecting group of **33** by the treatment with TBAF in THF at room temperature for 10 min gave desired product **34** in 37% yield. Treatment of **34** with DBU in toluene at refluxing temperature for 17 h gave the substrate **9** in 68% yield. Treatment of **9** with $NaBH_4$ in MeOH at room temperature

gave the substrates **10** and **11** in 65% and 15% yields, respectively (Scheme 3).



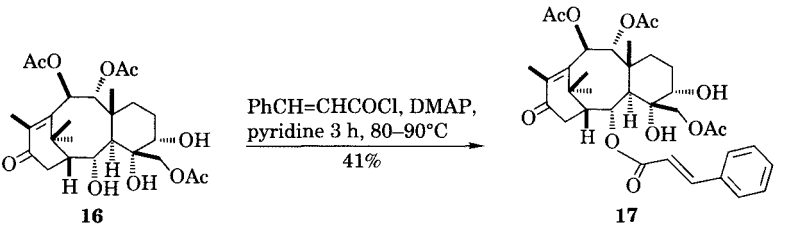
Scheme 3.

The selective hydrolysis of cinnamoyloxy group at C-5 of **24** was achieved by the treatment with hydroxylammonium chloride ($HONH_3Cl$) in the presence of sodium acetate (NaOAc) in a mixture of ethanol –1,4-dioxane-water at 75°C for 22 h to give **35** in 65% yield. Osmlation of **35** in the presence of *N*-methylmorpholine *N*-oxide (NMO) in a mixture of THF and water at room temperature for 2.5 h gave the substrate **14** in 91% yield. Treatment of **14** with *tertiary*-butyldimethylsilylchloride (TBDMSCl) in the presence of imidazole in dimethylformamide (DMF) gave **36** in 80% yield. Mesylation of **36** with methanesulfonyl chloride (MsCl) in pyridine at –24°C for 13 days gave mesylate **37** in 86% yield. Deprotection of TBDMS protecting group with HF-pyridine in tetrahydrofuran (THF) gave desired product **38** in 38% yield. Treatment of **38** with 1, 8-diazobicyclo [5.4.0] undec-7-ene (DBU) in toluene at 100–110°C for 19 h gave the substrate **13** in 76% yield. Reduction of 13-carbonyl group of **13** with sodium borohydride ($NaBH_4$) in methanol at room temperature gave the substrate **12** in 75% yield (Scheme 4).



Scheme 4.

The cinnamoylation of **16** with 8 equivalent of cinnamoyl chloride and 2 equivalent of DMAP as 0.1 M pyridine solution at 80–90°C for 3 h gave the substrate **17** in 41% yield (Scheme 5).



Scheme 5.

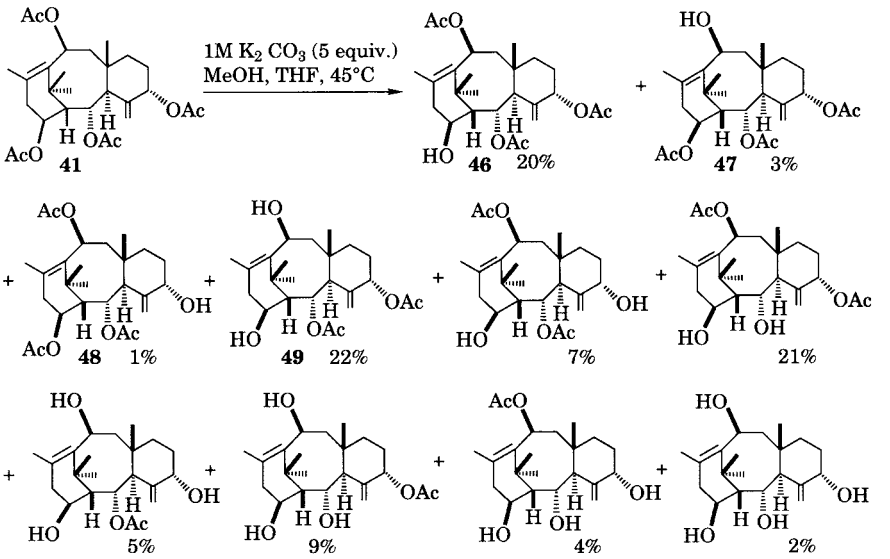
Preparation of Substrates 41-45 (see Fig 6.2)

The callus culture was induced from young stems of *T. cuspidata* collected in Sendai, Japan on modified Gamborg's B5 solid medium in the presence of 0.5 mg/L NAA. This callus culture (code-named CR-5) has the ability to grow continuously on the medium containing 0.5 mg/L NAA and showed fast growth compared with the callus cultures established on the medium with higher concentrations of NAA or other plant growth regulators such as 2,4-D and 4-Cl IAA (Sakai, 2002). The CR-5 callus line was subcultured

every 60 days. The calluses harvested for analysis were freeze-dried and extracted with hexane, EtOAc and methanol, successively. The methanol extracts were diluted with water and further extracted with $CHCl_3$. The hexane, EtOAc, and $CHCl_3$ extracts were separated by combinations of flash chromatography and normal- and reversed-phase HPLC to give the desired products **41–45**. The yields of substrates **41**, **42**, **43**, **44**, and **45** were 0.135%, 0.014%, 0.009%, 0.275%, 0.053% respectively based on the weight of dry callus. The yields of substrates **41**, **42**, **43**, **44**, and **45** were improved to be 0.805%, 0.055%, 0.050%, 0.0487%, and 0.04% respectively by the addition of acetic acid into the medium (1 mL/L).

Preparation of Substrates 46-49

Taxuyunnanine C (**41**) is the major metabolite of the callus cultures of *Taxus cuspidata*. The hydrolysis of **41** with 5 equivalent of 1M K_2CO_3 in a mixture of MeOH and THF at 45°C for 7 days gave the substrate **46**, **47**, **48**, and **49** in 20%, 3%, 1%, and 22% respectively (Scheme 6).



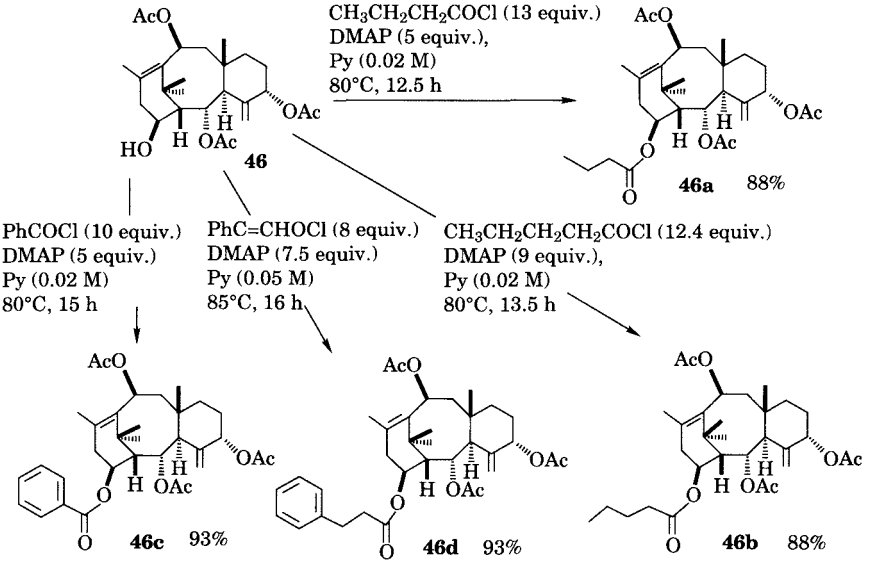
Scheme 6.

Preparation of Substrates 46a, 46b, 46c, and 46d

The butanoylation of **46** with 13 molar equivalent of butanoyl chloride and 5 molar equivalent of DMAP in pyridine at 80°C for 12.5 h gave the substrate **46a** in 88% yield.

The pentanoylation of **46** with 12.4 molar equivalent of pentanoyl chloride and 9 molar equivalent of DMAP in pyridine at 80°C gave the substrate **46b** in 88% yield.

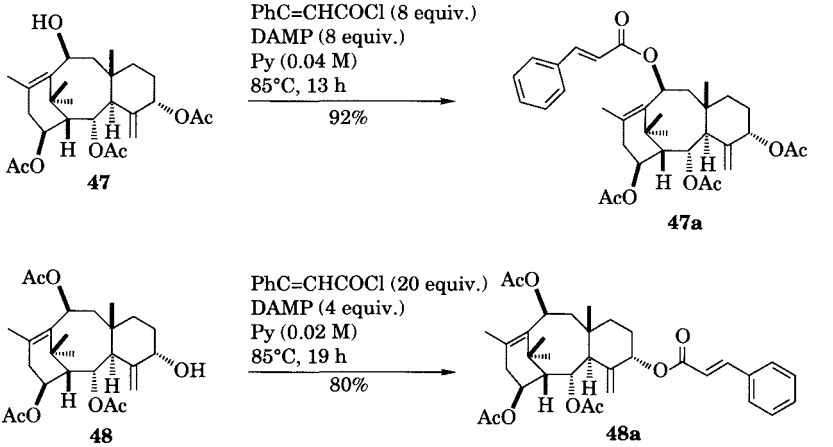
The benzoylation of **46** with 10 molar equivalent of benzoyl chloride and 5 molar equivalent of DMAP in pyridine at 80°C for 12.5 h gave the substrate **46c** in 93% yield (Scheme 7).



Scheme 7.

Preparation of Substrates 47a and 48a

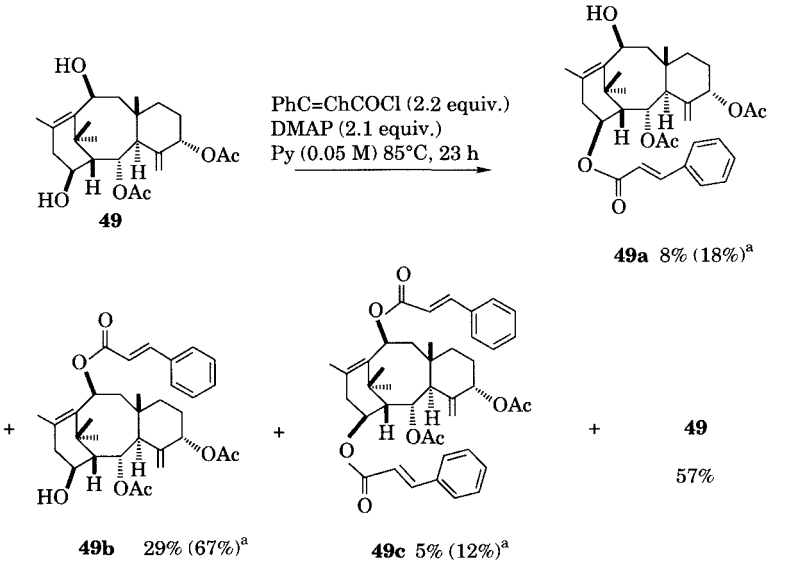
The cinnamoylation of **47** with 8 equivalent of cinnamoyl chloride and 8 equivalent of DMAP as 0.04 M pyridine solution at 85°C for 13 h gave the substrate **47a** in 92% yield.



Scheme 8.

The cinnamoylation of **48** with 20 equivalent of cinnamoyl chloride and 4 equivalent of DMAP as 0.02 M pyridine solution at 85°C for 19 h gave the substrate **48a** in 80% yield (Scheme 8).

Preparation of Substrates 49a, 49b and 49c



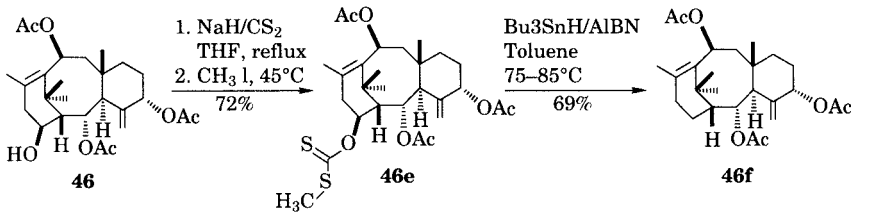
[a]The yields in parentheses are based on recovered starting material.

Scheme 9

The cinnamoylation of **49** with 2.2 equivalent of cinnamoyl chloride and 2.1 equivalent of DMAP as 0.05 M pyridine solution at 85°C for 23 h gave the substrate **49a**, **49b**, and **49c** in 18%, 67%, and 12% yields, respectively based on recovered **49** (Scheme 9).

Preparation of Substrate 46f

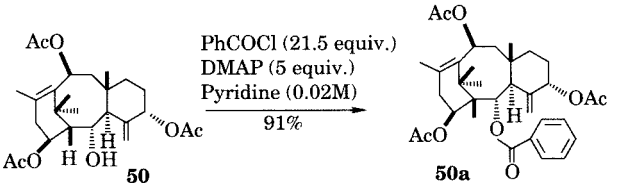
Treatment of **46** with 2.8 equivalent of NaH in the presence of 0.14 equivalent of imidazole in THF at refluxing temperature for 2.5 h gave, sodium alkoxide of **46**. Successive addition of 5.2 equivalent of CS_2 in the reaction mixture and heating at refluxing temperature for 30 min gave sodium salt of xanthate. Resulting salt was alkylated with 5.2 equivalent of methyl iodide (MeI) in THF at 45°C for 30 min to give methyl xanthate **46e** in 72% yield. Radical reduction of **46e** with 3 equivalent of *tri-n*-butyltin hydride in the presence of catalytic amount of 2,2′-azobisisobutyronitrile (AIBN) as radical initiator in toluene at 75–85°C afforded the substrate **46f** in 69% yield (Scheme 10).



Scheme 10.

Preparation of Substrate 50a

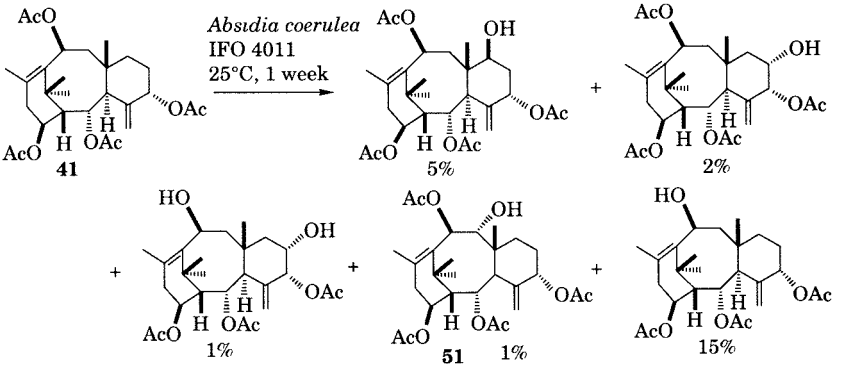
The benzoylation of **50** with 21.5 equivalent of benzoyl chloride and 5 equivalent of DMAP as 0.02 M pyridine solution at 85°C for 24 h gave the substrate **50a** in 91% yield (Scheme 11).



Scheme 11.

Preparation of Substrate 51

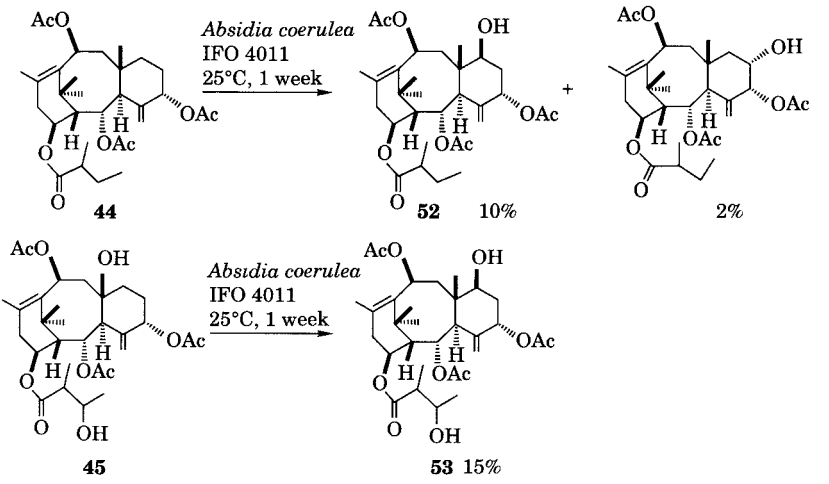
Incubation of **41** with *Absidia coeruelea* IFO 4011 at 25°C for one week gave the substrate **51** in 1% yield (Scheme 12).



Scheme 12.

Preparation of Substrates 52 and 53

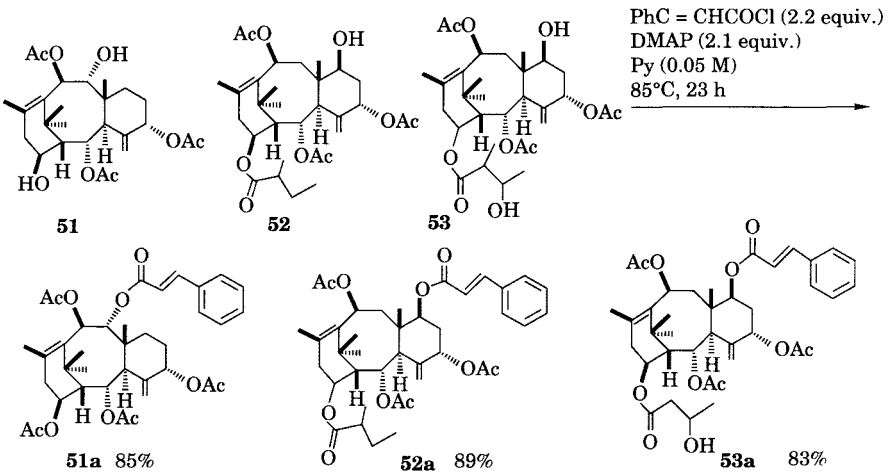
Incubation of **44** with *Absidia coeruelea* IFO 4011 at 25°C for one week gave the substrate **52** in 10% yield. Incubation of **45** with *Absidia coeruelea* IFO 4011 at 25°C for one week gave the substrate **53** in 15% yield (Scheme 13).



Scheme 13.

Preparation of Substrates 51a, 52a, and 53a

The cinnamoylation of **51**, **52**, and **53** with 2.2 equivalent of cinnamoyl chloride and 2.1 equivalent of DMAP as 0.05 M pyridine solution at 85°C for 23 h gave the substrate **51a**, **52a**, and **53a** in 85%, 89%, and 83% yields, respectively (Scheme 14).

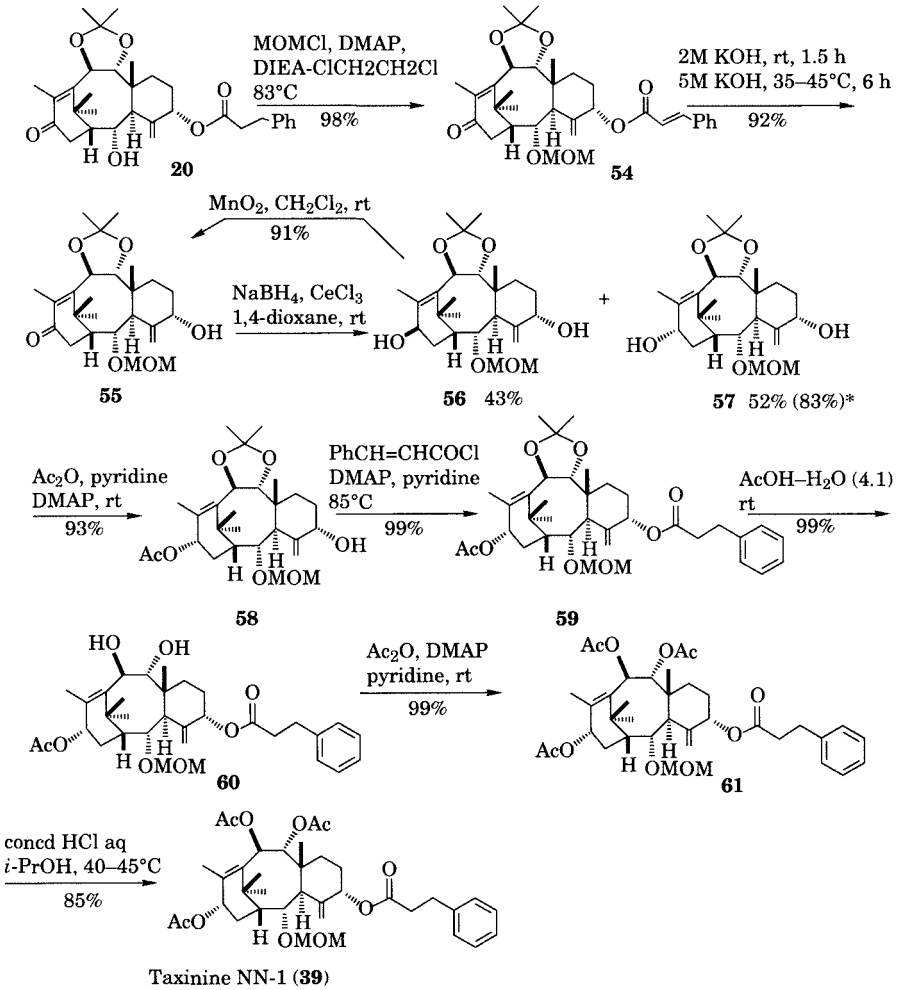


Scheme 14.

Preparation of Taxinine NN-1 (39)

Protection of **20** with MOMCl (methoxymethyl chloride) in the presence of DMAP in a mixture of DIEA (diisopropylethylamine) and dichloroethane

gave compound **54** in almost quantitative yield. Hydrolysis of **54** with 2M KOH in a mixture of 1,4-dioxane and MeOH gave 5-hydroxy derivative **55** in 92% yield. Reduction of **55** with 20 equivalent of $NaBH_4$ and 5 equivalent of $CeCl_3$ in 1,4-dioxane gave a mixture of desired 13α-hydroxyderivative **57** and its 13-epimer **56** in 52% and 43% yields, respectively. Since the regioselective oxidation of 13β-OH in 5α, 13β-diol **56** with active MnO_2 gave **55** in 91% yield, undesired epimer **56** was recycled by oxidation with MnO_2, and successive reduction with $NaBH_4$. The yield of **57** was improved from 52% to 83% by recycling the unwanted product **56** three times.



Scheme 15.

The regioselective acetylation of 13α-OH of **57** was achieved with 1.1 molar equivalent of Ac_2O and 2 molar equivalent of DMAP as 0.15M

pyridine solution at room temperature in 93% yield. The cinnamoylation of 5α-OH of resulting **58** with 8 molar equivalent of cinnamoyl chloride and 10 molar equivalent DMAP as 0.1M pyridine solution at 80–85°C gave desired 13α-acetoy-5α-cinnamoyloxy derivative **59** in almost quantitative yield. Deprotection of acetonide **59** with 80% aqueous acetic acid gave 9α,10β-diol **60** and successive acetylation of resulting **60** with 20 molar equivalent of Ac_2O and 1 molar equivalent DMAP gave triacetate **61** in 99% yield. Deprotection of 2-MOMO-group of **61** with concentrated HCl in *i*-PrOH at 40–45°C gave taxinine NN-1 (**39**) in 85% yield (Scheme 15).

Induction and Culture Conditions of Callus Culture of *Taxus cuspidata*

Gamborg's B5 medium supplemented with 2 × B5 vitamin and 20 g/L sucrose was used with either 0.5 mg/L NAA for CR-5 or combination with 1.0 mg/L NAA and 0.3 mg/L KTOS for CR-6 (All medium components except KTOS were purchased from Wako Pure Chemicals, Co., Ltd., Osaka, Japan). After the pH of the media was adjusted to 5.8 with 0.1M sodium hydroxide, 10 g/L agar was added. The media were autoclaved at 120°C for 15 min. The explants were placed on the solidified nutrient media and incubated in the dark at 25°C for 30–40 days. Then the initiated calluses were subcultured continuously every 60 days under the same conditions.

After the callus of CR-5 was subcultured eight times, one part of this callus line was harvested for analysis. The other part was subcultured subsequently on the medium supplemented with 100 μM methyl jasmonate (Wako Pure Chemicals, Co., Ltd.), and incubated for another 60 days.

After CR-6 was subcultured thirteen times, a part of this callus line was harvested for analysis. The remaining callus was divided into three parts, which were subcultured subsequently on the media supplemented with methyl jasmonate at concentrations of 100, 200, and 300 μM, respectively, and incubated for another 60 days.

KTOS

KTOS was provided by KNC Laboratories Co., Ltd., Kobe, Japan. It is a mixture of oligosaccarides that consist of 5 kinds of sugar, rhamnose, galactose, glucose, galacturonic acid, and glucuronic acid.

Cell Growth Inhibitory Activity of Compounds to WI-38 Fibroblast Cell, VA-13 Malignant Tumor Cell, and Hepg2 Human Liver Tumor Cell *In vitro*

Cells

WI-38 is the normal human fibroblast derived from female human lung. VA-13 is malignant tumor cells induced from WI-38 by infection of SV-40

virus. HepG2 is Human Liver Tumor Cells. These cell lines are available from the Institute of Physical and Chemical Research (RIKEN), Tukuba, Ibaraki, Japan. WI-38 and VA-13 cells were maintained in Eagle's MEM medium (Nissui Pharmaceutical Co., Tokyo, Japan) and RITC 80–7 medium (Asahi Technoglass Co., Chiba, Japan), respectively, both supplemented with 10% (v/v) fetal bovine serum (FBS) (Filtron PTY LTD., Australia) with 80 μg/mL of kanamycin. HepG2 cells were maintained in D-MEM medium (Invitrogen) supplemented with 10% (v/v) FBS (Filtron PTY LTD., Australia) with 80 μg/mL of kanamycin.

Procedures

100 μL medium containing ca. 5, 000 cells (WI-38, VA-13, HepG2) were incubated at 37°C in humidified atmosphere of 5% CO_2 for 24 h in 96 well micro plate. Then test samples dissolved in dimethyl sulfoxide (DMSO) were added to the medium and incubation was continued further for 48 h in the same conditions. Coloration substrate, WST-8 [2-(2-methyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodiun salt] was added to the medium. The resulting formazan concentration was determined by the absorption at 450 nm. Cell viability (%) was calculated as [(experimental absorbance–background absorbance)/(control absorbance–background absorbance)]×100. Cell viability at different concentration of compounds was plotted and 50% inhibition of growth was calculated as IC_{50}.

Cellular Accumulation of [^{3}H]-vincristine (VCR)

The multidrug-resistant 2780AD cells were maintained in PPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin. 2780AD cells (1×10^6 cells/well) were seeded in a 24-well plate and cultured for 18 h before the assay. The cells were treaded with 1×10^5 dpm of [^{3}H]-VCR (222 Gbq/mmol; Amersham Pharmacia Biotech, Tokyo, Japan) in the presence or absence of verapamil or taxiods. Immediately after incubation for 2 h at 37°C, the cells were washed five times with ice-cold phosphate-buffered saline containing 0.1 μg/mL of non-radioactive vincristine (VCR) and lysed with 500 μL of 0.2 M NaOH. After incubation for 45 min at 56°C, lysates were neutralized with 2 M acetic acid, and the radioactivity was counted in ACS II (Amersham Pharmacia Biotech).

Data Analyses of the Anticancer Activity of Compounds Based on the 39 Human Cancer Cell Lines Panel (HCC Panel)

Briefly, the cells were plated at proper density in 96-well plates in RPMI-1640 medium with 5% fetal bovine serum and allowed to attach overnight. The cells were exposed to drugs for 48 h. Then, the cell growth was

determined according to the sulforhodamine B assay. Three dose response parameters were calculated for each experimental agent. Those were growth inhibition of 50% (GI_{50}), which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation, the drug concentration resulting in total growth inhibition (TGI) and the drug concentration resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning (LC_{50}), which indicated a net loss of cells following treatment. The mean graph, which shows the differential growth inhibition of the drug in the cell line panel, was drawn based on a calculation using a set of GI_{50}. To analyze the correlation between the mean graphs of drug A and B, the COMPARE computer algorithm was developed. Peason correlation coefficients were calculated using the following formula: $r = (\Sigma (x_i - x_m)(y_i - y_m))/(\Sigma (x_i - x_m)^2 \Sigma (y_i - y_m)^2)^{1/2}$, where x_i and y_i are log GI_{50} of drug A and drug B, respectively, against each cell line, and x_m and y_m are the mean values of x_i and y_i, respectively.

Cellular Accumulation of Calcein

Adriamycin-resistant human ovarian cancer A2780 cells (AD10) were maintained in PRMI-1640 medium (Invitrogen) supplemented with 10 (%) fetal bovine serum (FBS) (Filtron PTY LTD., Australia) with 80 μg/mL of kanamycin. 100 μL medium containing ca. 1×10^6 cells were incubated at 37°C in humidified atmosphere of 5% CO_2 for 48 h in 96 well microplate. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS (–)). 50 μL of test samples were added to the medium and incubated for 15 min. Then, 50 μL of fluorogenic dye calcein acetoxymethyl ester [1 μM in PBS (–)] was added to the medium and incubation was continued further for 60 min. After removing the supernatant, each microplate was washed with 200 μL of cold PBS (–). The washing step was repeated two times and 200 μL of cold PBS (–) was added. Retention of resulting calcein was measured as calcein-specific fluorescence. The maximum absorption for calcein is 494 nm, the maximum emission is 517 nm.

RESULTS AND DISCUSSION

1,7-Dideoxy-4-deacetylbacatin III and Its Analogs as Multidrug Resistance Modulator in MDR Cancer Cells

We synthesized some taxane derivatives (**5–13**) with a 5β,20-epoxy ring (4,5-oxetane ring) and with 4α-hydroxyl group at the C-4 position and the corresponding oxetane ring opened 4α-, 5β-, 20-*tri*-oxygenated derivatives (**14–17**) as the substrates for the study of MDR reversal agents (Fig 6.1). The starting material is taxinine (**1**), which is the most abundant component of Japanese yew, *Taxus cuspidata*. We estimated the effects of substrates (**5–17**) as MDR reversal agents by increase of cellular accumulation of the

fluorogenic dye calcein, which was derived from calcein AM in the course of assay by enzymatic hydrolysis inside the cells and was used as an easily operated functional fluorescent probe for drug efflux protein. Verapamil is a well-known MDR reversal agent and it was used as a positive control.

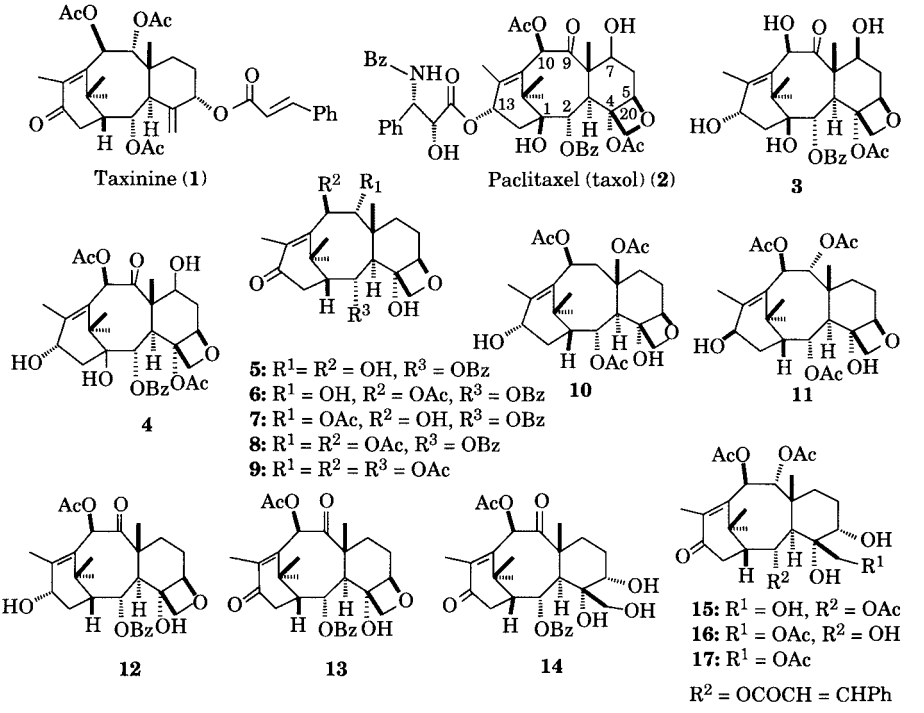


Fig 6.1. The structures of taxinine (**1**), paclitaxel (**2**), and substrates **3**-**17**

The effects of thirteen kinds of taxane substrates, (**5**–**17**), on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined comparing with 10-deacetyl baccatin III (**3**) and baccatin III (**4**) with a 5β,20-epoxy ring (4,5-oxetane ring) and with 4α-acetoxyl group at the C-4 position. The compounds **3** and **4** with acetoxy group at C-4 showed no activity toward calcein accumulation in MDR 2780AD cells. Among the compounds possessing an oxetane ring, compounds (**6**–**8**) with a benzoyloxy group at C-2, hydroxy group at C-4, an acetoxy group at C-9 and/or C-10, and α,β-unsaturated carbonyl group at C-13 showed significant effect on the calcein accumulation and the strength of their activities is almost the same. However, the corresponding compound **5** with hydroxyl groups at both C-9 and C-10 showed weaker activity than those of **6**-**8**. These results suggested that compound **5** possessing hydroxyl groups at both C-9 and C-10 positions decreased the activity of the cellular accumulation of calcein in MDR 2780AD cells (Table 6.1).

Compound **9** with a 13-carbonyl, 5,20-epoxy ring (4,5-oxetane ring), and three acetoxyl groups at C-2, C-9, and C-10 showed significant activity

toward calcein accumulation in MDR 2780AD cells. On the other hand, the corresponding 13α- and 13β-hydroxyl derivatives, **10** and **11** lost the activity.

Table 6.1. Effects of compounds on the accumulation of calcein in 2780AD cells, and cytotoxicity of compounds against WI-38, VA-13, and HepG2 cells

Compound	Calcein accumulation[a, b] (%control)			Cytotoxicity IC_{50}[c] (μg/mL)		
	0.25 (μg/mL)	2.5 (μg/mL)	25 (μg/mL)	WI-38	VA-13	HepG2
Verapamil	99	103	140	—	—	—
2	—	—	—	0.034	0.0043	6.9
3	95	90	93	—	—	—
4	93	84	81	16.1	0.84	9.91
5	105	110	106	> 100	> 100	67.7
6	99	102	140	42.3	> 100	77.7
7	115	118	134	0.67	67.0	43.2
8	97	119	135	0.82	51.9	7.32
9	115	120	123	77.0	> 100	> 100
10	94	95	95	8.39	81.5	> 100
11	82	95	96	76.2	> 100	> 100
12	96	129	163	54.2	77.0	69.9
13	101	120	137	7.38	7.93	30.1
14	88	114	112	57.4	59.6	33.4
15	79	94	88	85.3	9.73	> 100
16	88	97	126	>100	8.47	> 100
17	88	116	145	68.1	82.3	2.14

[a] The amount of calcein accumulated in multidrug-resistant human ovearian cancer 2780AD cells was determined compared with the control in the presence of 0.25, 2.5, and 25 μg/mL of test compounds and verapamil (positive control).

[b] The values are the relative amount of calcein accumulated in the cell compared with the control xperiment. The values represent the mean of triplicate determination.

[c] IC_{50} represents the mean of duplicate determination.

The structure change of **6** to **13** is the hydroxyl group at C-9 of **6** to a carbonyl group of **13**. Since the activity of **6** and **13** is almost the same, the effect of carbonyl and hydroxyl groups at C-9 showed the same efficiency. Compound **12** with structure change of the 13-carbonyl group of **13** to a 13α-hydroxyl group showed further stronger activity. These results showed that the 13α-hydroxyl group is more efficient than the 13-carbonyl group in this case. The structure-activity relationship of compounds **9-13** probably indicated that the existence of one carbonyl group at C-13 or C-9 is desirable for the expression of the activity.

Compound **12** showed the strongest activity among the compounds tested but **4** had no activity for calcein accumulation in MDR 2780AD cells. The

structure changes from **4** to **12** occur at the C-1, C-4, and C-7 positions. Two hydroxyl groups at C-1 and C-7 of **4** were lost in **12** and the acetoxyl group at C-4 of **4** was displaced by a hydroxyl group in **12**. These changes of functional groups of **4** to **12** enhanced remarkably the accumulation of calcein in MDR 2780AD cells. Compound **14** possessing 5α-, 20-dihydroxyl groups instead of a 5β, 20-epoxy ring (4, 5-oxetane ring) in **13** showed weaker activity than that of **13**. Analogously, compound **15** possessing 5α-, 20-dihydroxy groups in stead of 5*β*, 20-epoxy ring (4,5-oxetane ring) in **9** showed no activity of accumulation of calcein in 2780 AD cells, although **9** had significant activity. These results indicated that the 5*β*, 20-epoxy ring (4,5-oxetane ring) is an important functional group and 5α-, 20-dihydroxyl group has no effect for the expression of activity of the taxane derivatives above-mentioned. On the contrary, compound **16** possessing 5α-hydroxy-20-acetoxy groups instead of the 5α, 20-dihydroxyl groups of **15** showed significant activity. Compound **17**, α2-cinnamoyloxy derivative of **16,** showed more efficient activity than **16**. This enhancement of the activity was induced by the change of the functional group at C-2 from hydroxyl group to a cinnamoyloxyl group.

The above-mentioned compounds, (**5–17**), are all oxygenated at C-2, C-4, C-5, C-9, C-10, C-13, and C-20 positions. In fifteen taxane derivatives tested in this research, ten compounds showed moderate to strong activity on calcein accumulation in MDR 2780AD cells. Out of them, the most effective compound was 1,7-dideoxybaccatine III (**12**). These results seemed to indicate that the taxane skeleton has a special meaning toward the MDR reversal activity although the strength of activity depends on the combination of the functional groups on the taxane skeleton.

We tested the effect of the compound **12** on the cytotoxicity of taxol, adriamycin (ADM) and vincristine (VCR) toward MDR 2780AD cells and its parental cells (A2780) comparing with verapamil, which is a well-known MDR reversal agent. The IC_{50} values of taxol for A2780 and MDR 2780AD cells were 0.7 and 535 nM, respectively (Table 6.2). When compound **12** was added at a final concentration of 0.2, 2.0 and 10 μM, the IC_{50} values of taxol for MDR 2780AD cells were shifted to 375, 10 and 1 nM respectively. The enhancing effect of **12** for taxol was comparable to that of verapamil. Compound **12** is also effective for ADM and VCR. The IC_{50} values of ADM for A2780 and MDR 2780AD cells were 13 nM and 909 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 556, 107 and 17 nM in the presence of **12** at a final concentration of 0.2, 2.0 and 10 μM, respectively. The IC_{50} values of VCR for A2780 and 2780AD cells were 0.9 nM and 895 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 336, 30, and 7 nM in the presence of **12** at a final concentration of 0.2, 2.0, and 10 μM, respectively. The enhancing effects of **12** for taxol, ADM, and VCR were the same levels as that of verapamil

toward MDR 2780AD cells. On the other hand, compound **12** showed no enhancing effect for taxol, ADM, and VCR toward the parental 2780 cells. Thus, compound **12** can modulate the multidrug resistance of cancer cells as well as verapamil *in vitro*.

Table 6.2. Effect of compound 12 on the cytotoxicity of anticancer agents toward A2780 and MDR 2780AD cells[a]

Cell lines	MDR modulator (μM)		IC_{50} (nM) of anticancer agents		
			Taxol	ADM	VCR
2780	No modulator		0.7	13	0.9
	Verapamil	0.2	0.7	11	1
		2	0.8	9	0.8
		10	0.9	14	1.1
	12	0.2	0.7	8	1
		2	0.9	10	0.8
		10	0.8	7	0.8
2780AD	No modulator		535	909	895
	Verapamil	0.2	197	615	563
		2	11	109	29
		10	0.9	59	7
	12	0.2	375	556	336
		2	10	107	30
		10	1	17	7

The values represent the mean of triplicate determination.

[a] Enhancing effects of verapamil and compound **12** on the cytotoxicity of taxl, adriamycin (ADM), and vincristin (VCR) toward A2780 cells and MDR A2780 (2780AD) cells were determined in the presence of 0.2, 2.0, and 10 μM of each compound.

Cell growth inhibitory activity (IC_{50}) of compounds (**4–17**) to three different cell lines was examined (Table 6.1). The three cell lines employed in this experiment are human lung fibroblast cells (WI-38), malignant lung tumor cells (VA-13) induced from WI-38, and human liver cancer Hepatoma G2 cells (HepG2). Compound **8** showed the smallest IC_{50} values toward VA-13 and HepG2 in compounds (**5–8**). The results suggested that displacement of the acetoxyl group at C-9 and/or C-10 of **8** with a hydroxyl group induced the decrease of activity in **5**, **6**, and **7**. Cytotoxic activity of **9** against WI-38, VA-13 and HepG2 decreased remarkably on displacement of the 2α-benzoyloyl group of **8** with an acetoxyl group. Compound **13** showed moderate and weak activities to VA-13 and HepG2, respectively. Compound **14** with 5α, 20-dihydroxyl groups instead of 5β, 20-epoxy ring in **13** showed weaker activity to VA-13 than that of **13**. Compounds **15** and **16** with a 5α, 20-dihydroxyl and 5α-hydroxy-20-acetoxy groups respectively showed

significant activity toward VA-13. On the contrary, compound **17**,2-cinnamoyloxy derivative of **16,** showed significant activity not to VA-13 but HepG2. These results suggested that some modifications of the functional groups on the taxane skeleton induced a new cytotoxicity to a different cell line.

Compounds **6**, **9**, and **12** showed MDR reversal activities but have no cytotoxicity. These compounds are expected to be lead compounds of anti-MDR cancer reversal agents. On the other hand, cytotoxic activity of compounds **8** and **17** against HepG2 are the same level or more than that of taxol (**2**) or baccatin III (**4**). Since compounds **8** and **17** also showed significant MDR reversal activity, they are expected as lead compounds of new type anticancer agents or anti-MDR cancer agents. Compounds **13**, **15**, and **16** showed significant cytotoxic activity toward VA-13. Among them, **13** and **16** showed significant MDR reversal activity and are expected to be a new type of anticancer reagents or anti-MDR cancer agent.

Compound **13** with a carbonyl group at C-13 showed both MDR reversing and cytotoxic activities *in vitro*. On the other hand, the corresponding 13α-hydroxy derivative **12** showed efficient MDR reversing activity but its cytotoxic activity decreased drastically. Thus, compound **12** is a desirable compound as anti-MDR reversal agent. Introduction of an isoserine moiety to the 13α-hydroxyl group of taxane derivatives increases cytotoxic activity remarkably as shown in the change from baccatin III (**4**) to taxol (**2**) in Table 1. Compound **12** is also expected to be a lead compound of anti-MDR cancer reagents or anticancer reagents after introduction of the isoserine moiety on the13α-hydroxyl group of **12**.

Taxuyunnanine C Derivatives as Multidrug Resistance Modulator in MDR Cancer Cells

13-Oxygenated taxanes are the major component of *Taxus spesies*. In them, some compounds such as taxinine NN-1 (**39**) and taxinine NN-11 (**40**) are effective MDR modulators of MDR cancer cells. On the contrary, taxuyunnanine C (**41**) and its 14-acyloxy analogs (**42–45**) are the major metabolites from callus cultures of *Taxus* species in high yields (Fig 6.2). We reported their activity toward the accumulation of vincristine in MDR 2780AD cells and deduced that a hydrophobic less-hindered alkyl side chain of the C-14 acyloxy group of these compounds played some role to increase the activity (Bai *et al.*). We further investigated effect of the substitution of the functional groups at C-14 position on MDR reversal activity. For this purpose, we synthesized a series of new 14- oxygenated taxoids derived from taxuyunnanine C (**41**) and its analogs which are produced by callus cultures of *T. cuspidata* (Bai *et al.*, 2004; Menhard *et al.*, 1998; Ma *et al.*, 1994).

We obtained 14-deacetyltaxuyunnanine C (**46**), 10-deacetyltaxuyunnanine C (**47**), 5-deacetyltaxuyunnanine C (**48**) and 10,14-dideacetyltaxuyunnanine C (**49**) by hydrolysis of **41** (Scheme 6). 14-Butanoyloxy-(**46a**), 14-pentanoyloxy-(**46b**), 14-benzoyloxy-(**46c**), and 14-cinnamoyloxy-(**46d**) derivatives of **46** were obtained by the acylation of **46** by the corresponding acylchloride (Scheme 7). Cinnamoylation of 10-deacetyl-taxuyunnanine C (**47**) and 5-deacetyltaxuyunnanine C (**48**) gave **47a** and **48a** bearing a cinnamoyloxy group at C-10 and C-5, respectively (Scheme 8). Cinnamoylation of 10,14-dideacetyltaxuunanine C (**49**) gave 14- and 10-monocinnamoyloxy analogs, **49a** and **49b**, and a 10,14-dicinnamoyloxy analog **49c** (Scheme 9). 14-Deacetoxytaxuyunnanine C (**46f**) was obtained by Chugaev reaction of **46** (Scheme 10). 2-Benzoyloxy analog, **50a** was prepared from 2-deacetyltaxuyunnanine C (**50**), which was isolated from the callus culture of *T. cuspidata* (Scheme 11) as a minor product (Cheng, 1996).

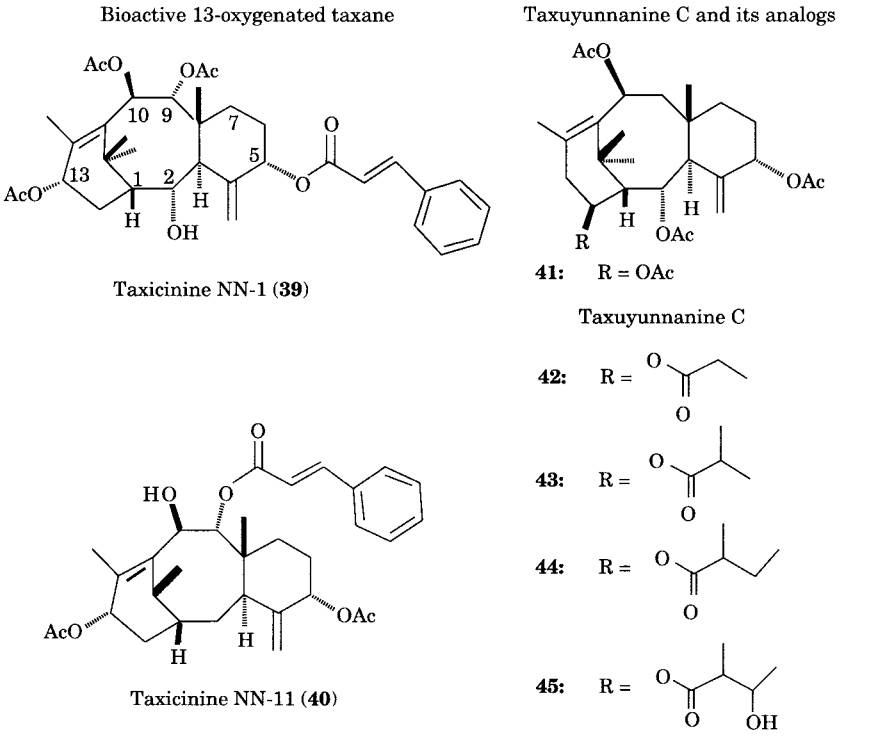


Fig 6.2. The structure of taxinines NN-1 (**A**) and NN-11 (**40**), and taxuyunnanine C (**41**) and its analog sis **42-45**

For the preparation of 7-or 9-oxygenated derivatives of **41** and its analogs **44** and **45**, we used a biotransformation. Thus, 14-acyloxytaxoids, (**51-53**), bearing a hydroxyl group at the C-7 or C-9 position was obtained by treatment of **41, 44,** and **45** with *Absidia coerulea* IFO 4011 (Schemes 12 & 13).

Compounds (**51-53**) were further converted to the corresponding cinnamoyl esters, **51a**, **52a**, and **53a** (Scheme 14).

Table 6.3. Effects of compounds on the accumulation of calcein in multidrug resistant cells 2780AD and cell growth inhibitory activities of compounds against WI-38, VA-13, and HepG2 cells

Compound	Calcein accumulation (% of control)[a, b]			Cell growth inhibitory activity (IC_{50} μM)[c]		
	0.25 (μg/mL)	2.5 (μg/mL)	25 (μg/mL)	WI-38	VA-13	HepG2
Paclitaxel				0.04	0.005	8.1
Adriamycin				0.66	0.38	1.2
41	130	141	127	130	53.1	18.5
42	104	98	119	>193	93.7	86.4
43	133	139	148	>188	90.9	28.0
44	100	86	119	>183	61.1	41.7
45	81	94	168	79.4	9.29	18.5
46	104	86	148	133	80	216
46a	97	98	114	73.4	17.2	18.5
46b	94	88	111	>183	56.7	16.6
46c	90	107	133	>176	>176	144
46d	123	126	125	154	>169	>169
46f	89	105	91	8.93	12.2	10.3
47	103	98	111	105	12.6	108
47a	92	123	126	>169	>169	106
48	118	118	141	149	105	183
48a	102	95	121	99.0	125	9.95
49a	107	132	190	13.5	37.4	15.7
49b	109	113	113	71.7	11.5	15.3
49c	83	80	80	>147	>147	>147
50	99	99	111	130.4	104	122
50a	98	96	125	125	175	51.9
51	102	101	110	129	>192	128
51a	102	103	125	151	>154	>154
52	94	98	110	81.6	79.1	75.2
52a	96	98	102	>144	>144	>144
53	87	104	107	140	>173	153
53a	97	102	130	62.4	49.9	80.4

[a] The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5, and 25 μg/mL of test compounds. [b] The values are the relative amount of calcein accumulated in the cell compared with the control experiment. The values represent the mean of triplicate determination. [c] IC_{50} represents the mean of duplicate determination.

The effects of 14-oxygenated taxoids mentioned above on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined (Table 6.3). Taxuyunnanine C (**41**), and its monodeacetyl analogs,

(**46–48**) and **50** and its 9-hydroxylated analog (**51**) showed weak to significant activity toward calcein accumulation in MDR 2780AD cells. In them, compounds **46** and **48**, in which the acetoxyl groups at C-14 or C-5 of **41** were displaced by a hydroxyl group respectively, showed stronger activity than that of **41**. On the other hand, compounds **47**, **50**, and **51**, in which the acetoxy groups at C-10 or C-2, or one hydrogen at C-9 of **41** were displaced by a hydroxyl group respectively, showed weaker activity than that of **41**. Taxoid **46f** in which the acetoxy group at C-14 of **41** was displaced by hydrogen, showed no activity. Compounds **52** and **53** with a hydroxyl group at the C-7 position showed lower activity than those of their parental compounds, **44** and **45**. These results of bioassay suggested that the substitution of the acetoxyl group at C-14 or C-5 of **41** with a more polar substituent such as a hydroxyl group is desirable to increase the activity toward calcein accumulation in MDR 2780AD cells. On the other hand, the substitution of the acetoxyl group at C-2, C-10 or one hydrogen at C-9 of **41** or at C-7 of **44** and **45** with a more polar substituent such as a hydroxyl group is an undesirable change to increase the activity.

Then, the activity of **41**, its seven kinds of C-14 acyloxy analogs (**42**, **43**, **44**, **45**, **46a**, **46b**, **46c**, and **46d**), 14-deacetyltaxuyunnanine C (**46**), and 14-deacetoxytaxuyunnanine C (**46f**) were compared. The activity order of the compounds was **45** > **46** = **43** > **46c** ≥ **41** = **46d** ≥ **42** = **44** ≥ **46a** ≥ **46b** >> **46f**. These results suggested that the compounds with hydrophilic substituents at C-14 such as **45** and **46** and the compounds with bulky acyloxy substituents at C-14 such as **43** and **46c** are desirable to increase the activity. On the contrary, the compounds possessing acyloxy groups with less hindered hydrophobic propyl and butyl side chains such as **46a** and **46b** showed lower activity than that of the parental compound **41**. Compound **46f**, in which the acetoxy group at C-14 of **41** was displaced by hydrogen, lost the activity completely.

We examined whether functional group at C-14 influenced the effect of structural change at different position of taxane skeleton on the calcein accumulating activity. When the functional group at C-14 is acetoxyl group such as **47** and **47a**, the activity of **47** with hydroxyl group at C-10 was less than that of **47a** with cinnamoyloxy group at C-10. On the other hand, when functional group at C-14 is cinnamoyloxy group such as **49a** and **49c**, the activity of **49a** with hydroxyl group at C-10 was higher than that of **49c** with cinnamoyloxy group at C-10. When the functional group at C-14 is 2-methylbutanoyloxy group such as **52** and **52a**, the activity of **52** with hydroxyl group at C-7 was slightly higher than that of **52a** with cinnamoyloxy group at C-7. On the other hand, in the case of **53** and **53a,** the activity of **53a** with cinnamoyloxy group at C-7 was higher than that of **53** with hydroxyl group at C-7, because 2-methyl-3-hydroxybutanoyloxy group at C-14 of **53a** is more hydrophylic group than cinnamoyloxy group at C-10. Thus, the influences of the functional groups at C-14 on the calcein accumulating activity seemed to be complicated.

Since taxinine NN-1(**39**) and taxinine NN-11 (**40**) bearing a cinnamoyloxy group on taxane skeleton exhibited strong MDR reversal activity, we expected the special effect of an aromatic acyloxy group such as a cinnamoyloxy group or a benzoyloxy group on taxusyunnanin C derivatives in calcein accumulation in MDR 2780AD cells. We examined the activity of ten kinds of cinnamoyloxy analogs of **41**. The order of the activity was **49a** > **53a** > **47a** = **46d** = **50a** = **51a** ≥ **48a** > **49b** > **52a** >> **49c**. In them, compound **49a** with a cinnamoyloxy group at C-14 and a hydroxyl group at C-10 showed the strongest activity. On the contrary, **49b**, regioisomer of **49a**, with a cinnamoyloxy group at C-10 and a hydroxyl group at C-14 showed weak activity. Compound **53a** with a cinnamoyloxy group at C-7 and a polar acyloxy group, 2-metyl-3-hydroxybutanoyloxy group at C-14 C-10, C-5, and C-9 showed significant activity. Interestingly, **52a** with a cinnamoyloxy group at C-7 and less polar 2-metylbutanoyloxy group at C-14 lost activity. Other analogs of **41**, **46d**, **47a**, **48a**, **51a** which possess one cinnamoyloxy group at C-14, C-10, C-5 and C-9-showed almost the same activity as **41**. Compound **49c** with two cinnamoyloxy groups at C-10 and C-14 showed no activity. In conclusion, compound **49a** with a cinnamoyloxy group at C-14 and a hydroxyl group at C-10 has the strongest activity in twenty-six test samples of taxuyunnanine C analogs toward calcein accumulation in MDR 2780AD cells.

Then, we tested the effects of several taxoids, **46**, **47**, **50**, **51**, **46d**, **47a**, **49a**, **49b** and **49c** on the accumulation of a widely used anticancer agent, vincristine (VCR) in MDR 2780AD cells using verapamil as a positive control (Table 6.4). Compound **49a** possessing a hydroxyl group at the C-10 position and a cinnamoyloxy group at the C-14 position showed the highest activity of cellular accumulation of vincristine in MDR 2780AD cells among the taxoids subjected to the test. Compound **49b**, regioisomer of **49a**, with a cinnamoyloxy group at C-10 and a hydroxyl group at C-14 showed weaker activity than that of **49a**. Taxoid **49c** bearing cinnamoyloxy groups at both C-10 and C-14 positions showed no activity. The activity of taxoid **47a** with a cinnamoyloxy group at the C-10 position was higher than that of taxioid **47** with a hydroxyl group at the corresponding positions. The results were similar to the calcein assay though there were some little differences.

Table 6.4. Effects of compounds on the accumulation of vincristine (VCR) in multidrug-resistant 2780AD cells[a]

Compound	Concd	Average[b] (μg/mL)	% (dpm/well)	Activity[d] of control[c]	Verapamil (%)[e]
46	0.1	360	102	±	94
	1	517	147	+	79
	10	858	242	+	53
46d	0.1	336	95	+	87
	1	494	140	+	75

Table 6.4. *Contd.*

Compound	Concd	Average[b] (mg/mL)	% (dpm/well)	Activity[d] of control[c]	Verapamil (%)[e]
	10	985	280	+	62
47	0.1	324	92	±	84
	1	420	119	+	64
	10	805	229	+	51
47a	0.1	404	132	+	130
	1	641	209	+	118
	10	1385	451	++	81
49a	0.1	425	121	+	111
	1	959	272	+	146
	10	1855	527	+++	116
49b	0.1	402	114	+	105
	1	669	190	+	102
	10	1749	497	++	110
49c	0.1	320	91	±	83
	1	325	92	±	49
	10	429	122	+	27
50	0.1	306	101	±	89
	1	436	144	+	84
	10	786	259	+	74
51	0.1	303	86	–	79
	1	379	108	±	58
	10	592	168	+	37

[a] The amount of VCR accumulated in MDR 2780AD cells was determined with the control in the presence of 0.1, 1, and 10 μg/mL of taxoids. [b] The values represents triplicated determinations. [c] The values are the relative amount of VCR accumulated in the cell compared with the control experiment. [d] The indices are expressed on a scale of five by the range of the relative amount of VCR accumulation as compared with the control experiment (%): +++, 501–1000%; ++ 301–500%; +, 111–300%; ±, 91–100%; –, < 90%. [e] The values are expressed as the relative amount of vincristine (VCR) accumulation in the cell as compared with that of verapamil.

The effects of compounds **46** and **49a** on the cytotoxicity of taxol, adriamycin (ADM) and vincristine (VCR) toward MDR 2780AD cells and its parental cells (A2780) were tested comparing with verapamil, which is a well-known MDR reversal agent (Table 6.5).

The IC_{50} values of taxol for A2780 and MDR 2780AD cells were 2.8 and 183 nM, respectively. When compound **46** was added at a final concentration of 0.2, 2.0 and 10 μM, the IC_{50} values of taxol for MDR 2780AD cells were shifted to 21, 26 and 5.3 nM respectively. At this time, cytotoxicity of compound **46** against 2780AD cells was equal to that of verapamil, showing more than 70% of cell viability at the concentration of 10 μM, while compoud **46** showed significant cytotoxicity against A2780 cells showing 20% of cell viability at the same concentration. From these results, compound **46**

enhanced the sensitivity to taxol in MDR 2780AD cells. The IC_{50} of taxol for 2780AD cells in the presence of 10 μM of compound **46** was equal to that for parental A2780 cells.

Compound **46** is also effective for ADM and VCR. The IC_{50} values of ADM for A2780 and MDR 2780AD cells were 5.7 nM and 298 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 215, 256 and 181 nM in the presence of **46** at a final concentration of 0.2, 2.0 and 10 μM, respectively. The IC_{50} values of VCR for A2780 and 2780AD cells were 3.1 nM and 311 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 55, 26, and 11 nM in the presence of **46** at a final concentration of 0.2, 2.0, and 10 μM, respectively. The enhancing effects of **46** for taxol, ADM, and VCR were the same levels as that of verapamil toward MDR 2780AD cells. Thus, compound **46** can modulate the multidrug resistance of cancer cells as well as verapamil *in vitro*.

Table 6.5. Effect of the compounds on the cytotoxicity of anticancer agents toward A2780 and MDR 2780AD cells

Cell lines	MDR modulator	(μM)	Viability of the cell (%)[a]	IC_{50} (nM) of anticancer agents[b]		
				Taxol	ADM	VCR
A2780	No modulator	0	100	2.8	5.7	3.1
		0.2	93	2.7	5.8	3.4
	Verapamil	2	82	2.9	6.0	2.0
		10	53	3.5	7.7	0.5
		0.2	97	2.6	7.0	2.6
	46	2	95	2.3	6.0	1.6
		10	20	6.6	7.8	4.8
		0.2	89	2.6	7.0	2.6
	49a	2	74	2.3	6.0	1.6
		10	27	6.6	7.8	4.8
2780AD	No modulator	0	100	183	298	311
		0.2	84	36	269	41
	Verapamil	2	90	5.5	201	25
		10	71	4.9	164	3.9
		0.2	101	21	215	55
	46	2	96	26	256	26
		10	77	5.3	181	11
		0.2	105	22	284	51
	49a	2	86	4.2	138	39
		10	38	3.9	73	23

[a] Cytotoxicity of the compounds were evaluated in the absence of the anticancer agents.
[b] Enhancing effects of the compounds on the cytotoxicity of taxol, adriamycin (ADM) and and vincristin (VCR) toward A2780 cells and MDR A2780 (2780AD) cells were determined in the presence of 0.2, 2.0, and 10 μM of each compounds.

Cytotoxicity of compound **49a** against 2780AD cells in the absence of anticancer agents was more significant than that of compound **46**, showing 38% of cell viability at the concentration of 10 μM, while the viability of the cell was 27% in the case of parental A2780 cells at the same concentration of compound **49a**. IC_{50} values of compound **49a** for MDR 2780AD and parental A2780 were calculated as 8.0 and 6.1 μM, respectively. Thus compound **49a** showed MDR reversal activity against 2780AD cells.

When compound **49a** was added at a final concentration of 0.2, 2.0 and 10 μM, the IC_{50} values of taxol for MDR 2780AD cells were shifted to 22, 4.2 and 3.9 nM respectively. These results showed that compound **49a** enhanced the sensitivity to taxol in MDR 2780AD cells.

Compound **49a** is also effective for ADM and VCR. The IC_{50} values of ADM for A2780 and MDR 2780AD cells were 5.7 nm and 298 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 284,138 and 73 nM in the presence of **49a** at a final concentration of 0.2, 2.0 and 10 μM, respectively. The IC_{50} values of VCR for A2780 and 2780AD cells were 3.1 nm and 311 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 51, 39, and 23 nM in the presence of **49a** at a final concentration of 0.2, 2.0, and 10 μM, respectively. Thus, compound **49a** can modulate the multidrug resistance of cancer cells as well as verapamil *in vitro*.

Cell growth inhibitory activity (IC_{50}) of taxoids to three different cell lines was examined (Table 6.3). The three cell lines employed in this experiment are human lung fibroblast cells (WI-38), malignant lung tumor cells (VA-13) induced from WI-38, and human liver cancer, Hepatoma G2 (HepG2) cells.

Taxuyunnanine C (**41**) and its derivatives **46-48, 50, 51** showed weak activity in many cases. Only taxuyunnanine C (**41**) and its C-10 deacetyl taxuyunnanine C (**47**) showed significant activity against HepG2 and VA-13, respectively. On the contrary, 14-deacetoxytaxuyunnanine C (**46f**) showed significant activities in all three cell lines. This result contrasted with the result of calcein assay and suggested that the modification of the C-14 position of the taxane skeleton significantly affected the biological activities.

The cytotoxic activity on HepG2 of taxuyunnanine C analogs, **42-44, 46a**, and **46b** bearing different kinds of acyloxy groups at C-14 increased according to the increase of hydrophobicity of their acyloxy group at C-14. In them, **46a** also showed significant cytotoxic activity on VA-13 in addition to the activity on HepG2. However, these compounds showed very weak or no cytotoxic activity against normal cell lines, WI-38. This is a desirable result in the screening of anticancer agents.

Compounds **46**, **48**, and **46c** showed the activity toward calcein accumulation in MDR 2780AD cells but have no cytotoxicity. These

compounds are expected to be lead compounds of MDR cancer reversal agents. On the other hand, cytotoxic activity of compounds **46f** and **48a** against HepG2 is at the same level of paclitaxel. Since compounds **48a** showed moderate activity toward calcein accumulation in MDR 2780AD cells, it is expected as lead compounds of new-type anticancer agents or anti-MDR cancer agent. Compounds **45**, **46a**, **47**, and **49b** showed significant cytotoxic activity toward VA-13.

Compounds **45** showed significant cytotoxic activity toward VA-13 and HepG2 along with the strong activity toward calcein accumulation in MDR 2780AD cells. Compounds **43** showed significant cytotoxic activity toward HepG2 along with the strong activity toward calcein accumulation in MDR 2780AD cells. Compounds **49a** showed significant cytotoxic activity toward WI-13 and HepG2 along with the strongest activity in tested samples toward calcein accumulation in MDR 2780AD cells. They are expected to be lead compounds of anti-MDR cancer agents or anticancer agents.

The Synthesis of Taxinine NN-1 and its Biological Activities as Anticancer Agent and Multidrug Resistance Modulator in MDR Cancer Cells

The staring material is **20**, which was synthesized from taxinine (**1**) as shown in Scheme 1. Protection of **20** with MOMCl ($MeOCH_2Cl$) in the presence of DMAP (4-dimethylaminopyridine) in a mixture of DIEA (diisopropylethylamine) and dichloroethane gave compound **54** in almost quantitative yield. Hydrolysis of **54** with 2M KOH in a mixture of 1,4-dioxane and MeOH gave 5-hydroxy derivative **55** in 92% yield. After various attempts to reduce **55,** we found that reduction with $NaBH_4$ gave the best results. Since the solubility of **55** in MeOH is low, we tried several solvents in reduction of **55** with $NaBH_4$. Reduction of C-13 carbonyl group of **55** with $NaBH_4$–$CeCl_3$ in 1,4-dioxane gave a mixture of desired 13α-hydroxyderivative **57** and its 13-epimer **56** in 52% and 43% yields, respectively. Although stereo-selectivity in reduction of **55** is low under this condition, the total yield of **56** and **57** is the best under the conditions tested by us. The inversion of the stereochemistry of 13β-OH of **56** by Mitsunobu reaction was unsuccessful probably because of the steric hindrance at C-13. Since the regioselective oxidation of 13β-OH in 5α, 13β-diol **56** with active MnO_2 gave **55** in 91% yield, undesired epimer **56** was recycled by oxidation with MnO_2, and successive reduction with $NaBH_4$. The yield of **57** was improved from 52% to 83% by recycling the unwanted product **56** three times (see Scheme 15).

The regioselective acetylation of 13α-OH of **57** was achieved with Ac_2O-DMAP in pyridine at room temperature, which proceed in 93% yield. The cinnamoylation of 5α-OH in **58** with cinnamoyl chloride-DMAP in pyridine at 80–85°C gave desired 13α-acetoy-5α-cinnamoyloxy derivative **59** in

almost quantitative yield. Deprotection of acetonide **59** with 80% aqueous acetic acid gave 9α,10β-diol **60** and successive acetylation of resulting **60** gave triacetate **61**. Deprotection of 2-MOMO-group of **61** with concentrated HCl in *i*-PrOH at 40–45°C gave taxinine NN-1 (**39**) in 85% yield. The overall yield of **39** from **1** was 45% in 11 steps.

The cytotoxic activity of taxinine NN-1 was examined toward WI-38, VA-13, and HepG2 cells (Table 6.6). It showed significant cytotoxic activity toward HepG2 (IC_{50} = 6.10 μg/mL). Since there is no effective anticancer agent to liver cancer, the assay result is interesting. Taxinine NN-1 on the accumulation of VCR in MDR 2780AD are examined (Table 6.7). It showed very strong activity (maximum verapamil % = 323% at 1 μg/mL). The cytotoxic activities of taxinine NN-1 (**39**) was examined on 39 human cancer cell lines panel (HCC manel). The effective concentration of 39 is low enough (MG-MID of $GI_{50} \leq 5$). Although the value was slightly weak, differential growth inhibition is recognized (Delta = 0.43, Range = 1.3). Since the result of COMPARE of **39** is negative ($\gamma < 0.5$), it possibly belongs to a new machanistic class and a new member of anticancer agent (Table 6.8).

Table 6.6. Cell growth inhibitory activities of taxinine NN-1 (**39**) against WI-38, VA-13 and HepG2 cells[a]

Compound	WI-38	VA-13	HepG2
	IC_{50} (μg/mL)	IC_{50} (μg/mL)	IC_{50} (μg/mL)
39	31.9	63.7	6.1
Paclitaxel	0.034	0.0043	6.9
ADM[b]	0.38	0.22	0.69

[a] IC_{50} values represent the means of duplicate determinations. Paclitaxel and ADM are positive controls [b] Adriamycin

Table 6.7. Effects of compound **39** on the accumulation of VCR in multidrug-resistant cells 2780AD

Compound	VCR accumulation[a]					Evaluation
	Concentration (μg/mL)	Average[b] (dpm/well)	% of control[c]	Activities[d]	Verapamil %[e]	Maximum verapamil % concentration
39	0.1	339	149	+	127	P[f]
	1	1527	670	+++	323	323%
	10	2143	940	+++	177	1 μg/mL
Control	0	228	100			
Verapamil	0.1	266	117	+	100	
	1	473	207	+	100	
	10	1211	531	+++	100	

[a] The amount of VCR accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.1, 1, and 10 μg/mL of taxoid.
[b] The values represent means of triplicate determination.
[c] The values are the relative amount of VCR accumulated in the cell compared with the control experiment.

[d] The indices are expressed on a scale of seven by the range of the relative amount of VCR accumulation as compared with the control experiment (%): +++++, > 2001%; ++++, 1000–2000%; +++, 501–1000%; ++, 301–500%; +, 111–300%; ±, 91–110%; –, < 90%.
[e] The values are expressed as the relative amount of vincristine (VCR) accumulation in the cell as compared with that of verapamil.
[f] positive.

Table 6.8. Summary of evaluation of compound **39** based on the 39 human cancer cell lines. Parameters of effective concentrations

	GI_{50}	TGI	LC_{50}
MG-MID	–5.33	–4.23	–4.03

Results of the COMPARE analysis

Rank	Compounds	γ	Molecular targets/ drug type
1	Interferon-α	0.488	Protein kinase C
2	UCN-01	0.411	Anti metabolight
3	Navelbine	0.404	Tubulin

GI_{50}, The 50% growth inhibition parameter; TGI, The total growth inhibition parameter; LC_{50}, The lethal concentration parameter; γ, Person correction coefficient; MG-MID, mean graph midpoint (MG-MID) is the average value of log GI_{50}, log TGI, and LC_{50} of compounds toward 39 human cancer cells expresd by M concentration. The mean graph of the tested compound was compared with those of 200 standard compounds using COMPARE analysis. Drugs were ordered according to the correlation coefficient.

The Production of Taxinine NN-11 by the Callus Cultures of *Taxus cuspidata* and Their Biological Activities as Anticancer Agent and Multidrug Resistance Modulator in MDR Cancer Cells

Taxinine NN-11 (**40**) was isolated from callus caltures of *T. cuspidata*. The callus culture was induced from young stems of *T. cuspidata* collected in Sendai, Japan on modified Gamborg's B5 solid medium in the presence of 0.5 mg/L NAA. This callus culture (code-named CR-5) has the ability to grow continuously on the medium containing 0.5 mg/L NAA and showed fast growth compared with the callus cultures established on the medium with higher concentrations of NAA or other plant growth regulators such as 2,4-D and 4-Cl IAA (Sakai, 2002). The CR-5 callus line was subcultured every 60 days. Compound **40** was not produced in culture CR-5. Although **40** was obtained from CR-5 in 0.0259% by addition of 100 μM methyl jasmonate into the medium, the growth rate of the culls became slower and the callus color turned dark brown. So it was not easy to improve the yield of **40** by further addition of methyl jasmonate. Since it was found that the callus culture (code-named CR-6) cultivated on the medium containing 0.3 μg/L KTOS and 1.0 μg/L NAA showed faster growth than CR-5, we attempted to use callus culture CR-6 for the production of **40** instead of CR-5. When methyl jasmonate was added at the concentration of either 100 or

200 μM to the medium of CR-6, the growth of the callus was markedly improved in comparison with that of the callus of CR-5 stimulated with 100 μM methyl jasmonate. The maximum content of **40** was obtained when the concentration of methyl jasmonate in the medium was 200 μM. However, when the concentration was increased to 300 μM, the callus turned completely dark brown and no trace of **40** was found (Table 6.9).

Table 6.9. Production of **40** using callus culture of CR-6 by addition of methyl jasmonate

Parameter	CR-5	CR-5 + methyl jasmonate 100 μM	CR-6	CR-6 + methyl jasmonate 100 μM	CR-6 + methyl jasmonate 200 μM	CR-6 + methyl jasmonate 300 μM
Growth rate[a]	1	0.68	1.36	1.06	0.98	0.67
Color degree[b]	1.9	3.5	1.6	2	2.2	5
Content of **40** (%)	no	0.0259	no	0.0256	0.0631	no

[a] The relative values were compared to those of CR-5. [b] The color of the callus cultures was evaluated in five degrees: 1 (colorless) to 5 (dark brown).

Table 6.10. Effects of compound **40** on the accumulation of VCR in multidrug-resistant cells 2780AD

Compound	VCR accumulation[a]					Evaluation
	Concentration (μg/mL)	Average[b] (dpm/)	% of control[c] %	Activities[d]	Verapamil %[e]	Maximum verapamil % concentration
	0.1	370	162	+	139	P[f]
40	1	909	399	++	191	191%
	10	1478	648	+++	122	1 μg/mL
Control	0	228	100			
Verapamil	0.1	266	117	+	100	
	1	473	207	+	100	
	10	1211	531	+++	100	

[a] The amount of VCR accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.1, 1, and 10 μg/mL of taxoid.

[b] The values represent means of triplicate determination.

[c] The values are the relative amount of VCR accumulated in the cell compared with the control experiment.

[d] The indices are expressed on a scale of seven by the range of the relative amount of VCR accumulation as compared with the control experiment (%): +++++, > 2001%; ++++, 1000–2000%; +++, 501–1000%; ++, 301–500%; +, 111–300%; ±, 91-110%; –, <90%

[e] The values are expressed as the relative amount of vincristine (VCR) accumulation in the cell as compared with that of verapamil.

[f] positive.

The effect of taxinine NN-11 (**40**) on the cellular accumulation of VCR in human ovarian cancer 2780 AD cells was examined and the results are summarized in Table 6.10. Compound **40** showed stronger activities than that of verapamil and exhibited about two times activity compared with that of verapamil. Since compounds **40** showed significant MDR cancer reversal activities, the cytotoxicic activities of **40** were examined in primary screening based on the 39 human cancer cell lines *in vitro* (Yamori, 1999; Boyd, 1995). The results are shown in Tables 6.11. Although the effective concentration of **40** is rather high, differential growth inhibition is recognized. Since the result of COMPARE of **40** is negative ($r < 0.5$) and it showed significant MDR reversal activity, therefore possibly it belongs to a new mechanistic class and a new member of anticancer agents.

Table 6.11. Summary of evaluation of compound **40** based on the 39 human cancer cell lines

Parameters of effective concentrations

	GI_{50}	TGI	LC_{50}
MG-MID	–4.65	–4.01	–4.00

Results of the COMPARE analysis

Rank	Compounds	γ	Molecular targets/ drug type
1	Interferon-α	0.335	
2	ICRF-193	0.318	Topoisomerase
3	W80	0.313	Tubulin

GI_{50}, The 50% growth inhibition parameter; TGI, The total growth inhibition parameter; LC_{50}, The lethal concentration parameter; γ, Person correction coefficient; MG-MID, mean graph midpoint (MG-MID) is the average value of log GI_{50}, log TGI, and LC_{50} of compounds toward 39 human cancer cells expressed by M concentration. The mean graph of the tested compound was compared with those of 200 standard compounds using COMPARE analysis. Drugs were ordered according to the correlation coefficient.

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Antioxidant and Neuroprotective Effects of Synthetic Curcumin Analogues and Natural Phenolics

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ABSTRACT

*In order to find more effective antioxidants and neuroprotective agents as well as to enrich the information of structure-activity relationship (SAR) of curcuminoids, fourteen curcumin analogues (compounds **1–14**) were synthesized and ten natural phenolics possessing similar structures of curcumin were isolated from Zingiber officinale (compounds **15–24**). The antioxidative activities of these compounds were investigated with four models: (1) the inhibitory effects on xanthine oxidase, (2) scavenging activities against superoxide anion and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, (3) anti-lipid peroxidative properties in rat brain homogenates, (4) chelating abilities of ferrous ion. Additionally, the neuroprotective effects evaluated by the potency of protecting rat pheochromocytoma cells (PC12 cells) from injury by hydroperoxide (H_2O_2) as well as by inhibiting acetylcholine esterase (AChE) were also carried out. Results demonstrated that the compounds **4**, **13**, **15**, **17** and **18** had remarkable antioxidant and neuroprotective properties through versatile experiment models. Meanwhile, from the study of preliminary SAR of the investigated compounds, it was observed that both ortho-diphenolic O–H and CH_2 groups played equally important roles on antioxidant activity. Further investigations of these active*

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compounds are required for the development of new chemical entities for the treatment of neurode-generative disorders, e.g. AD and Parkinson's disease (PD).

Key words: Acetylcholine esterase (AChE), antioxidants, enzyme inhibitors, lipid peroxidation, neuroprotective activity, rat pheochromocytoma, superoxide anion, xanthine oxidase

INTRODUCTION

Alzheimer's disease (AD), a progressive neurodegenerative disorder that is the most common form of dementia, is characterized by two pathological lesions termed senile plaques and neurofibrillary tangles and by the degeneration of selected neuronal populations (Perry & Smith, 1993). At the present time, there is no effective treatment for this disease, and the pathogenetic mechanism is not clearly defined. However, there are increasing evidences to support that free radical-induced oxidative damage may play an important role in the pathogenesis of AD. Free radicals are reactive oxygen compounds that may attack and damage lipids, proteins and DNA. The brain is especially sensitive to oxidative damage for its high contents of readily oxidized lipids, high use of oxygen and low levels of antioxidants (Practico & Delanty, 2000). Evidence for oxidative damage has been obtained from postmortem brain tissues as well as from living patients with AD. Such damages found in AD include lipid peroxidation adduction (Sayre *et al.*, 1997), advanced glycation end products (Smith *et al.*, 1994), nitration (Smith *et al.*, 1997) and carbonyl-modified neurofilament proteins and free carbonyls (Smith *et al.*, 1995). Although it is not known whether oxidative damage occurs first in the disease process or is a result of the disease itself, it does appear that it is a part of the pathophysiologic process of AD. Therefore, discovering effective antioxidants might serve as a way to prevent and to treat AD.

Curcumin (1, 7-bis(4-hydroxy-3-methoxyphenyl)-(1, 6-hepadiene-3, 5-dione) (Fig 7.1), isolated from a well-known Zingiberaceae plant, *Curcuma longa* L. (turmeric), has been wildly studied for its anti-inflammatory, anti-cancer, anti-oxidant, wound healing and anti-microbial effects (Maheshwari *et al.*, 2006). Curcumin exhibited strong antioxidant activity which is comparable to vitamin C and E. Recent studies demonstrated that curcumin could inhibit the formation of amyloid β oligomers and fibrils, and bind plaques and reduce amyloid β *in vivo* (Yang *et al.*, 2005). This makes curcumin become a promising candidate for the prevention and treatment of AD. Curcumin has a unique conjugated structure including two methoxylated phenols and an enol form of β-diketone (Fig 7.1). The antioxidant mechanism of curcumin has recently attracted much attention. The exact mechanism is, however, still not yet well understood. It has been reported that curcumin was a classical phenolic chain-breaking antioxidant,

donating H atoms from the phenolic groups instead of the methylene, and the phenolic OH was therefore essential for both antioxidant activity and free radical kinetics (Barcley & Vinqvist, 2000; Priyadarsini *et al.*, 2003). Furthermore, the analysis of oxidative coupling products of curcumin and ethyl linoleate indicated that curcumin could trap a radical by the phenolic group to form a curcumin radical which reacted with a peroxy radical of the ethyl linoleate at the 3′-position, affording a coupling product through a peroxyl linkage (Masuda *et al.*, 2001). This suggested that curcumin possesses a chain-breaking property against free radicals.

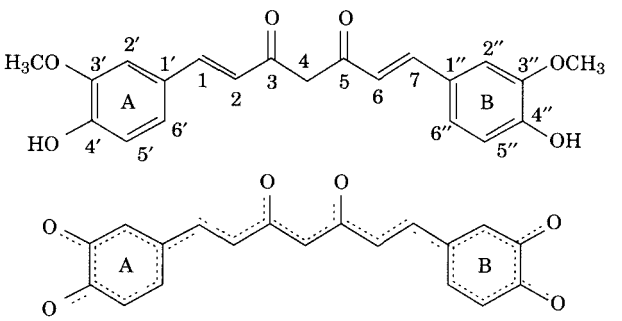


Fig 7.1. Structure of curcumin and its highly-conjugated system

In order to understand the structure-activity relationship (SAR) of curcumin derivatives, and to seek for more effective antioxidants including neuroprotective agents, three series (A, B and C) of curcumin analogues (**1–14**) were designed and synthesized. In series A (compounds **1–4**), the successively conjugated structure of curcumin was blocked by a CH_2 to examine the influence on anti-oxidative ability. In series B (compounds **5–8**), the alkene groups in compounds of series A were removed to observe the performance of the compounds owing only half of conjugated moieties on their antioxidant bioactivities (Fig 7.2). In series C (compounds **9–14**), the conjugated structure between two phenyls of curcumin was substituted by a totally saturated ester to study the effect of decreased conjugated system on anti-oxidative and neuroprotective activities. Moreover, substituents on the aromatic A and/or B rings, such as OCH_2OCH_3 to replace the OCH_3 groups and demethylation to expose the phenolic OH groups, were systematically introduced in the synthetic molecules. In addition, natural curcumin-based diarylheptanoids and gingerol related phenolics were also isolated from *Zingiber officinale*. *Z. officinale* belongs to Zingiberaceae family and contains high potent anti-oxidative ingredients as turmeric. This was aimed to obtain both diarylheptanoids and gingerols, since comparisons of natural phenolics with synthetic curcumin derivatives would be meaningful to construct the preliminary SAR concept of curcumin analogues. Thus ten natural compounds (**15–24**) were obtained from the rhizomes of *Z. officinale*.

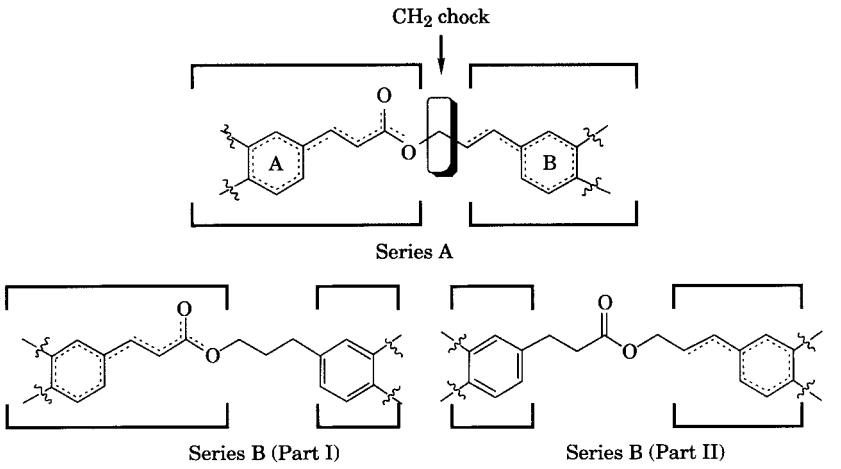


Fig 7.2. The design of synthetic curcumin analogues

The antioxidative activities and neuroprotective effects of all these compounds (**1–24**) were investigated with the following bioassay models: the inhibitory effects on xanthine oxidase; scavenging activities against superoxide anion and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals; anti-peroxidative properties in rat brain homogenates; chelating abilities of ferrous ion; the potency of protecting rat pheochromocytoma cells (PC12) from injury by hydroperoxide (H_2O_2).

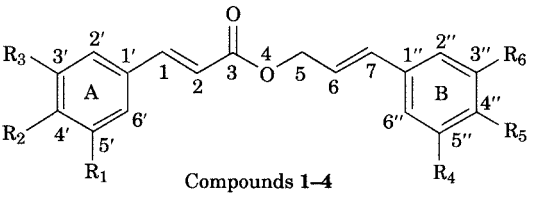
Moreover, it has been reported that most of the cortical acetylcholine esterase (AChE) activity was associated with senile plaques in AD brains (Kása *et al.*, 1997). AChE could promote the aggregation of Aβ peptide in amyloid fibrils and form a complex with the Aβ, which increases its cytotoxicity (Alvarez *et al.*, 1997). Bonnefont and colleagues (Bonnefont *et al.*, 1998) reported that it was possible that the association of AChE with the Aβ peptide during fibril formation in senile plaques accelerated a higher free radical generation than that observed in the presence of the Aβ peptide only, which induced enhancement of oxidative stress. Therefore, AChE inhibitory activities of both the natural (**15–24**) and the synthetic curcumin analogues (**1–14**) were assayed and the results are reported herein. In general, the present study would be beneficial for a deeper understanding of anti-oxidative mechanisms of curcumin analogues including phenolics from *Z. officinale*, for their potential application in treating AD.

MATERIALS AND METHODS

Materials

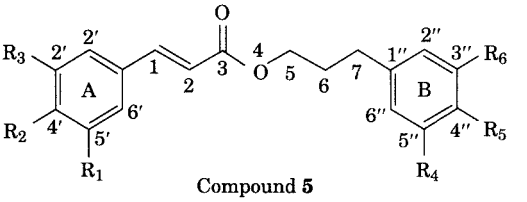
Ten known compounds (**1–4** and **9–14**) were synthesized according to the previously reported method (Hu *et al.*, 2005), which was reflected in Fig 7.3. The structures of the synthetic compounds were identified by MS and NMR

measurement, some of which were also directly compared with the authentic samples. Compounds (**15–24**) were isolated from the rhizomes of *Z. officinale*.

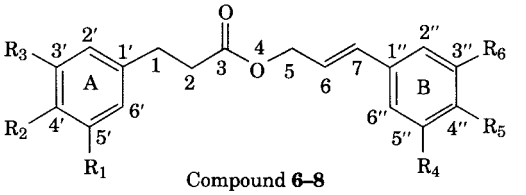


Compounds **1–4**

Series A	R_1	R_2	R_3	R_4	R_5	R_6
Compound **1**	H	NO_2	H	OCH_3	OCH3	H
Compound **2**	OCH_3	OCH_3	OCH_3	H	OCH_3	H
Compound **3**	H	OCH_2OCH_3	H	OCH_2OCH_3	OCH_2OCH_3	H
Compound **4**	OH	OH	H	H	H	H

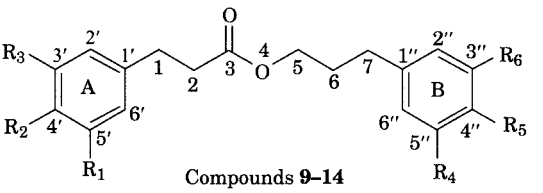


Compound **5**



Compound **6–8**

Series B	R_1	R_2	R_3	R_4	R_5	R_6
Compound **5**	H	CH_3	H	H	CH_3	H
Compound **6**	H	OCH_2OCH_3	H	H	H	H
Compound **7**	H	OH	H	H	H	H
Compound **8**	H	H	H	H	H	H



Compounds **9–14**

Series C	R_1	R_2	R_3	R_4	R_5	R_6
Compound **9**	OCH_3	OCH_3	H	OCH_3	OCH_3	H
Compound **10**	H	H	H	H	H	H
Compound **11**	OCH_2OCH_3	OCH_2OCH_3	H	H	OCH_2OCH_3	H
Compound **12**	H	OH	H	H	OCH_3	H
Compound **13**	OH	OH	H	H	H	H
Compound **14**	H	OH	H	H	H	H

Fig 7.3. Structures of three series of synthetic curcumin analogues

Silica gel (100–200 mesh and 200–300 mesh) used for column chromatography was supplied by the Qingdao Marine Chemical Factory, Qingdao, P.R. China. Melting points were measured on a Kofler hot-stage instrument and were uncorrected. ESIMS data were recorded on a Bruker Esquire 3000+ spectrometer. HRFABMS spectra were registered by a VG ZAB-HS spectrometer. The NMR spectra were obtained using a Bruker AM-400 FT-NMR spectrometer with TMS as internal standard. Preparative TLC was performed using silica gel GF_{254} and RP-18 plates (Merck, Darmstadt, Germany).

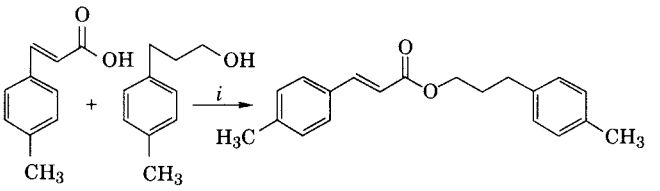
The whole plant of *Z. officinale* was collected in May 2000 at Luo Ping county, Yunnan province, China, and identified by Prof. Hua Peng at Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen is deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), allopurinol, xanthine, hydroperoxide (H_2O_2), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ferrozine were purchased from Sigma Chemical Co. (St. Louis, USA). Tris base, DMEM medium was obtained from Gibco (Invitrogen Beijing Office, Beijing, China). NADH (disodium salt) was supplied from Amresco Inc. (Solon, Ohio, USA). Acetylthiocholine iodine and dithio-bisnitro-benzoic acid were afforded from Acros. Quercetin was prepared in our laboratory (HPLC purity of 99%), and PC12 cells were provided by Cell Bank of Chinese Academy Sciences, Shanghai. All other reagents including curcumin were of the highest purity commercially available. Sprague- Dawley rats were obtained from the Zhejiang Center of Laboratory Animals, China. The use of animals was in accordance with Guideline for the Care and Use of Laboratory Animals of Zhejiang University.

Preparation of Compounds 5–8

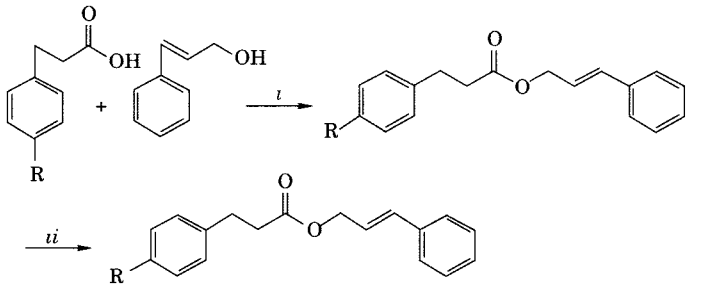
The synthetic route for compounds **5–8** is outlined in Schemes 1 and 2 (Yang *et al.*, 2009). Scheme 1 describes the procedure of preparing compound **5**. 4-Methyl-cinnamic acid was treated with 4-methylphenylpropyl alcohol under catalysis of carbonyldiimidazole (CDI) to afford **5** with a yield of 72%. The structure of **5** was elucidated mainly on the basis of its ^1H and ^{13}C NMR spectra as well as MS spectral data. Scheme 2 outlines the synthetic path of compounds **6–8**. 4-Methoxylmethoxylphenylpropionic acid, which was reacted with phenylallyl alcohol catalyzed by N,N-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to afford **6** with a yield of 70%. Compound **7** was obtained with a yield of 68% by removing the OCH_2OCH_3 group of **6** by treatment of 10% hydrochloric

acid. The preparation of **8** was similar to that of **6**, but with a different starting material, and its yield was 69%.



Reagents and conditions: (*i*) CDI, THF, reflux, 0.5 h; DBU, THF, reflux, 12 h.

Scheme 1. Synthetic route of compound **5**



Reagents and conditions: (*i*) DCC, CH_2C1_2, rt, 5 min; DMPA, rt, 12 h. (*ii*) 10% HC1, CH_3OH, reflux.

Scheme 2. Synthetic route of compounds **6–8**

(E)-4-Methylcinnamic acid-42-methyl phenylpropyl Ester (5): A mixture of 4-methylcinnamic acid (100 mg, 0.617 mmol), CDI (313 mg, 1.23 mmol) and dry tetrahydrofuran (THF, 10 mL) was refluxed for 30 min. The solution of 4-methylphenylpropanol (100 mg, 0.68 mmol) and 1, 8-diazabicyclo[5, 4, 0] undec-7-ene (DBU, 103 mg, 0.68 mmol) in dry THF (10 mL) was then added for another 12 h refluxing. The solvent was removed by evaporation and the resulted mixture was purified by column chromatography affording **5** (131 mg, 72%) as a colorless oil; *Rf* (petroleum ether/EtOAc = 3:1) 0.27; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (1 H, d, J = 16.0 Hz, H-1), 7.05 ~ 7.12 (8 H, m, ArH), 6.63 (1 H, d, J = 16.0 Hz, H-2), 4.09 (2 H, t, J = 6.4 Hz, H-5), 2.70 (2 H, t, J = 8.0 Hz, H-7), 2.34 (3 H, s, CH_3-4′), 2.33 (3 H, s, CH_3-4″), 1.92 (2 H, m, H-6); ESIMS m/z 374 ([M+NH_4]$^+$).

4-Methoxylmethoxylphenylpropionic Acid-(E)-phenylallyl Ester (6): The mixture of 4-methoxylmethoxylphenylpropionic acid (50 mg, 0.24 mmol) and DCC (54 mg, 0.26 mmol) in dry dichloromethane (8 mL) was stirred at room temperature for 5 min, and then phenylallyl alcohol (35 mg, 0.26 mmol) and 4-DMAP (5.8 mg, 0.048 mmol) were added. The whole mixture was stirred for another 12 h at room temperature. The white

insoluble substance was removed by filtering and the resulted mixture was purified by column chromatography to afford **6** (55 mg, 70%) as a colorless oil; *Rf* (petroleum ether/EtOAc = 3:1) 0.38; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 ~ 7.40 (9 H, m, ArH), 6.72 (1 H, d, J = 16.0 Hz, H-7), 6.63 (1 H, dt, J = 6.4, 15.6 Hz, H-6), 5.14 (2 H, s, CH_3OCH_2O-4′), 4.74 (2 H, d, J = 6.4 Hz, H-5), 3.47 (3 H, s, CH_3OCH_2O-4′), 2.93 (2 H, t, J = 8.0 Hz, H-3), 2.66 (2 H, t, J = 8.0 Hz, H-2); ESIMS m/z 344 ([M+NH_4]$^+$).

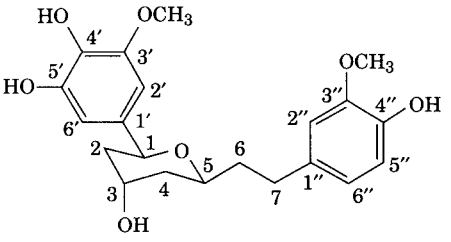
***4-Hydroxylphenylpropionic Acid-(E)-phenylallyl Ester (7)*:** Compound **6** (25 mg, 0.078 mmol) was dissolved in 6 mL methanol, and then 10% HCl (4 mL) was added to the solution. The mixture was refluxed for 40 min and was cooled to room temperature. Saturated sodium bicarbonate was then added to adjust the pH value to 7. After removing methanol by evaporation, the mixture was extracted by ethyl acetate for 3 times. The combined ethyl acetate layer was washed by saturated brine and dried over $MgSO_4$. Compound **7** (15 mg, 68%) was obtained as a colorless oil by column chromatography. *Rf* (petroleum ether/EtOAc = 3:1) 0.15; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.74 ~ 7.40 (9 H, m, ArH), 6.62 (1 H, d, J = 16.0 Hz, H-7), 6.38 (1 H, dt, J = 6.4, 15.6 Hz, H-6), 4.34 (2 H, d, J = 6.4 Hz, H-5), 2.88 (2 H, t, J = 8.0 Hz, H-3), 2.61 (2 H, t, J = 8.0 Hz, H-2); ESIMS m/z 300 ([M+NH_4]$^+$).

***Phenylpropionic Acid-(E)-phenylallyl Ester (8)*:** The preparation of **8** was similar to that of **6**: colorless oil; *Rf* (petroleum ether/ EtOAc = 3:1) 0.47; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21~7.40 (10 H, m, ArH), 6.63 (1 H, d, J = 16.0 Hz, H-7), 6.26 (1 H, dt, J = 6.4, 15.6 Hz, H-6), 4.74 (2 H, d, J = 6.4 Hz, H-5), 2.99 (2 H, t, J = 8.0 Hz, H-3), 2.70 (2 H, t, J = 8.0 Hz, H-2); ESIMS m/z 284 ([M+NH_4]$^+$).

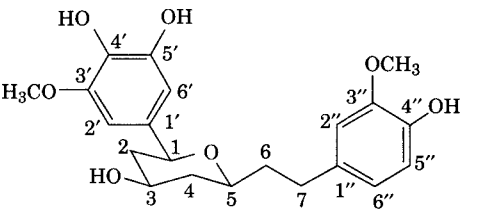
Extraction and Isolation of Phenols from *Zingiber officinale*

The air-dried rhizomes of *Z. officinale* (10 kg, dry weight) were powdered and extracted three times with 95% EtOH, and the alcoholic residue was suspended in H_2O and then partitioned successively with petroleum ether (60–90°C), EtOAc and BuOH. The EtOH portion (102 g) was mixed with silica gel (100 g, 100–200 mesh) and then subjected to column chromatography over 2.0 kg of silica gel (200–300 mesh) eluted with a petroleum ether-acetone gradient (0:1–1:0, v/v). Based on TLC analysis, 25 fractions were obtained. The fractions were chromatographed repeatedly. A new diarylheptanoid (compound **17**, 10.5 mg) and nine known compounds were obtained (Fig 7.4).

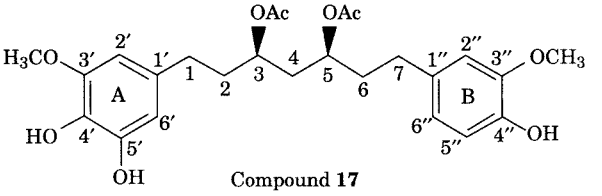
The ESI-MS spectrum of compound **17** exhibited its molecular ion peak at m/z 476 corresponding to the molecular formula of $C_{25}H_{32}O_9$. Its ^{1}H, ^{13}C-NMR and IR spectra were similar to those of the known compound (3*R*,5*S*)-3,5-Diacetoxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-



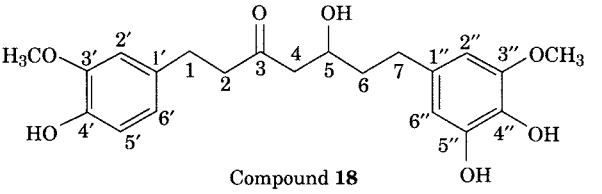
Compound **15**



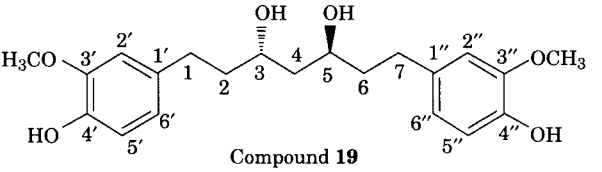
Compound **16**



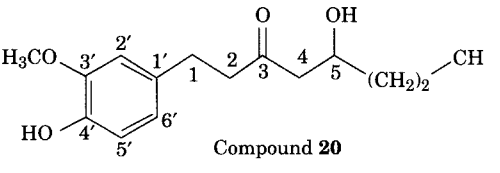
Compound **17**



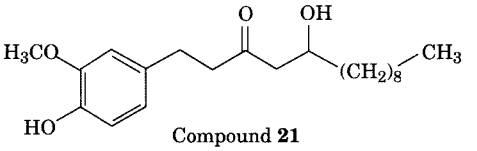
Compound **18**



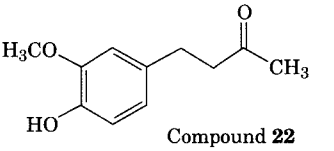
Compound **19**



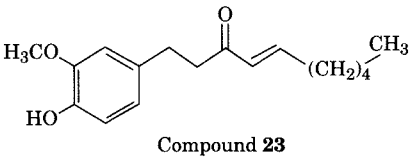
Compound **20**



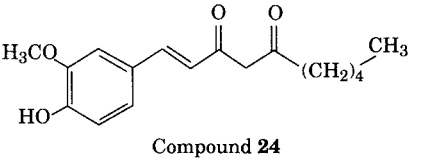
Compound **21**



Compound **22**



Compound **23**



Compound **24**

Fig 7.4. Structures of natural phenolics from *Zingiber officinale*

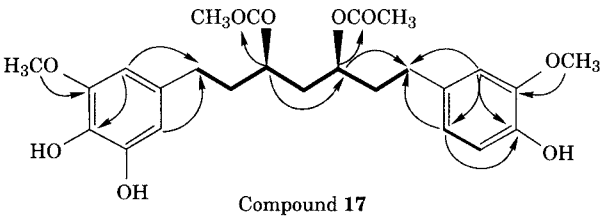
3-methoxyphenyl)-heptane (Kikuzaki *et al.*, 1991b) which was also obtained from the ethanol extract of the rhizomes of *Z. officinale*. However, the 5′-methoxy group of this known diarylheptanoid was absent in both ^{1}H and ^{13}C-NMR (Table 7.1) spectra of **17**. Instead, a hydroxy group was present, as suggested by the characteristic base peak at *m/z* 153 ($[CH_2C_6H_2(OH)_2(OCH_3)]^+$) in its EI-MS spectrum and the molecular ion peak (14 mass units lower than that of the known compound). The nearly identical NMR data of these two diarylheptanoids implied that they had the same stereochemistry. Therefore, **17** was assigned as (3*R*,5*S*)-3, 5-diacetoxy-1-(3-methoxy-4, 5-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-heptane. The structure was confirmed by HMBC and ^{1}H-^{1}H COSY correlations as shown in Fig 7.5.

Table 7.1. NMR spectral data of compound 17 [400 MHz (^{1}H) and 100 MHz (^{13}C) in CD_3OD with TMS as internal standard]

Positions	17	
	δ_C	δ_H (multi, J in Hz)
1	31.4	2.52 (dt, *J* = 15.6, 7.8), 2.54 (dt, *J* = 15.9, 7.8)
2	36.6	1.84 (q, *J* = 7.2)
3	69.8	4.97 (m)
4	38.5	1.76 (dt, *J* = 11.7, 5.7), 1.92 (dt, *J* = 14.0, 6.9)
5	69.8	4.97 (m)
6	36.5	1.84 (q, *J* = 7.2)
7	31.2	2.52 (dt, *J* = 15.6, 7.8), 2.54 (dt, *J* = 15.9, 7.8)
1'	133.1	
2'	108.4	6.36 (br s)
3'	146.8	
4'	130.5	
5'	143.8	
6'	103.2	6.26 (br s)
1"	133.2	
2"	110.9	6.66 (d, *J* = 1.6)
3"	146.3	
4"	143.7	
5"	114.2	6.80 (d, *J* = 8.0)
6"	120.7	6.62 (dd, *J* = 8.0, 1.6)
3-OAc	170.8, 21.1	2.00 (s)
5-OAc	170.8, 21.1	2.00 (s)
3'-OMe	56.1	3.84 (s)
3"-OMe	55.9	3.82 (s)

The structures of the known compounds were identified by comparing their ^{1}H and ^{13}C NMR and MS data with those reported in literatures as follows: 1, 5-epoxy-3-hydroxy-1-(3-methoxy-4,5-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-heptane (**15**) (Kikuzaki & Nakatani, 1996),

(1,5-epoxy-3-hydroxy-1-(3-methoxy-4,5- dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-heptane (**16**) (Kikuzaki & Nakatani, 1996), 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(3-methoxy-4,5- dihydroxyphenyl)-heptan-3-one (**18**) (Ma *et al.*, 2004), (*3S,5S*)-3,5-dihydroxy-1,7-bis-(4-hydroxy-3-methoxyphenyl)-heptane (**19**) (Kikuzaki *et al.*, 1991a), [4]-gingerol (**20**) (Denniff *et al.*, 1980; Shoji *et al.*, 1982), [10]-gingerol (**21**) (Denniff *et al.*, 1980; Shoji *et al.*, 1982), zingiberone (**22**) (Connel & Sutherland, 1969), [6]-shogaol (**23**) (Connel & Sutherland, 1969) and [1]-dehydrogingerdione (**24**) (Charles *et al.*, 2000).



Compound **17**

Fig 7.5. Structure and key ^{1}H-^{1}H COSY (in bold lines) and HMBC correlations (in arrows) of compound **17**

Bioactivity Assays

Inhibition of Xanthine Oxidase (XO): Inhibition of xanthine oxidase (XO) [EC 1. 1. 3. 22, from liver homogenate of Sprague-Dawley rats] was determined by the spectrophotometric measurement of the formation of formazan (Majklc-Singh *et al.*, 1987; Wang *et al.*, 1993). The reaction mixtures prepared in 96-well plates consisted of 540 μm of xanthine, 30 μl of XO, 100 μm of nitroblue tetrazolium (NBT), 100 μm of phenazine methosulfate (PMS), 0.4% (v/v) of Triton X-100 and a gradient of concentrations of test compounds (from 12.5–50 μg/mL). After 2 h of incubation at 37°C in water bath, the optical density (OD) values were measured at 550 nm in a multi-well plates reader (Synergy-HT, BioTek, Winooski, VT, USA). The blank samples contained phosphate buffer (0.1 M, pH 8.75) instead of xanthine. Allopurinol was used as the positive control for the assay.

Scavenging Superoxide Anion: The superoxide anion scavenging activity of the test compounds was assayed spectrophotometrically as reported with a slight modification (Robak & Gryglewski, 1988). Superoxide anion radicals were generated in a non-enzymic phenazine methosulfate-NADH system by following the reduction of nitroblue tetrazolium. In this assay, the superoxide anion radicals were measured in plates, which contained 78 μm of NADH, 50 μm of nitroblue tetrazolium, 5 μm of phenazine methosulfate and the test samples with gradient concentrations in 16 mm Tris-HCl buffer at pH 8.0 with a total volume of 250 μl. OD values were monitored at 560 nm after 5 min of the incubation at room temperature.

The wells without phenazine methosulfate were taken as the blank control. Quercetin was employed as the reference compound.

***Extinction of DPPH*:** Quenching of DPPH free radicals by test compounds was assayed spectrophotometrically at 517 nm against the absorbance of these stable radicals (Tapia *et al.*, 2004). The free radical scavenging efficiency of the investigated compounds was reflected by the decoloration of DPPH radicals. In brief, reaction mixtures contained various concentrations of test compounds which were dissolved in DMSO and DPPH (0.4 mg/mL) dissolved in methanol. The methanolic solution of DPPH served as a control and quercetin was applied as a reference. The absorbance was measured at 517 nm after incubating the mixture at 37°C for 30 min.

***Inhibition of Lipid Peroxidation*:** The formation of malondialdehyde (MDA) was used as an indicator of inhibiting lipid peroxidation and was determined by the thiobarbituric acid (TBA) assay on freshly prepared Sprague-Dawley rat brain homogenates using colorimetric analysis (Lee *et al.*, 2002). The reaction mixtures which composed of 200 µl of solution containing an aqueous $FeSO_4$ (4 µm), vitamin C (50 µm), 50 µl of rat brain homogenate and 5 µl of the test compounds (concentrations from 25 to 100 µg/mL), were incubated at 37°C in capped tubes for 1 h before 100 µl of trichloroacetic acid (20%, v/v) was added. The mixture reacted at room temperature for 30 min. Finally, 200 µl of HCl (0.1 M) and 100 µl of TBA (1%, w/v) were added into each tube and the mixture was incubated at 100°C for another 1 h. Centrifugation was then carried out at 5000 rpm for 5 min, and the absorbance of the supernatant was measured at 532 nm, quercetin was chosen as a positive standard.

***Protection of Rat Pheochromocytoma Cells (PC12 Cells) Against Hydrogen Peroxide-Induced Injury*:** The protective effects of the compounds on pheochromocytoma cells (PC12) cells against hydrogen peroxide-induced injury were assessed with MTT assay as reported previously (Xiao *et al.*, 1999). PC12 cells were maintained at 37°C in a humidified atmosphere containing 5% of CO_2. Cells were seeded into 96 well cell culture plates (Shengyou Biotechnology, Hangzhou, China) at a density of 8000 cells per well in DMEM medium which was supplemented with 10% heat-inactivated calf serum, 100 units/mL of penicillin and 100 µg/mL of streptomycin. Following protocols were carried out 48 h after cells were seeded. H_2O_2 was prepared in phosphate buffered saline (PBS) on the day of application to cultures at a final concentration of 600 µm. Samples were dissolved in DMSO and diluted with the medium. The PC12 cells were preincubated with samples 2 h before the H_2O_2 was added. A fresh solution of MTT (5 mg/mL) prepared in NaCl solution (0.9%) was added to the 96-well cell culture plates 3 h after H_2O_2 was added. The plates were incubated in the CO_2 incubator for 3 h, and were lysed with DMSO. The plates were analyzed in a multi-well-plate reader at 570 nm. Meanwhile,

the cytotoxicity of the test compounds to PC12 cells was also examined by MTT method.

***Chelation of Ferrous Ions*:** The ferrous ions chelating by the test compounds were estimated based on the method of Gülçin (Gülçin *et al.*, 2005). Briefly, the samples with concentrations ranging from 20 to 110 μg/mL were added to a solution of 2 mm $FeSO_4$ (5 μl) and 80% DMSO (200 μl). The reaction was initiated by adding of 5 mm ferrozine (10 μl), and the mixture was shaken vigorously before left standing at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm.

***Inhibition of AChE*:** The AChE inhibitory activities were determined by the colorimetric method described previously (Ellman *et al.*, 1961). The reaction system contained, in 0.1 M phosphate buffer at pH 8.0, 15 mm of acetylthiocholine, 40 μl of acetylcholine esterase which was obtained from the Sprague-Dawley rat brain homogenates, and various concentrations of test compounds dissolved in DMSO. After incubation at 37°C for 1 h, 10 mm of dithiobisnitrobenzoic acid (DTNB) was added to the mixture. The absorbance was recorded at 405 nm. Tacrine was chosen as a positive control.

Statistical Analysis

All experimental data derived from bioassays are expressed as means ± SD. Statistical analyses were carried out by the employment of ANOVA with Dunnet test for the difference between control and the studied compounds. Significant level was set as $p \leq .05$. The IC_{50} value was obtained by regression analysis.

RESULTS

Inhibition of Xanthine Oxidase

Co-incubation of xanthine oxidase and xanthine is one of the most important ways to generate free radicals *in vivo*. Xanthine oxidase (XO) catalyses the hydroxylation of purines (especially xanthine and hypoxanthine) to yield uric acid, superoxide anions and hydrogen peroxide. Imbalance between free radicals generated by XO and the ability of organism to defend them is referred to as oxidative stress (OS), which links various diseases, including AD (Pratico & Delanty, 2000). Thus, the inhibitory activities of synthesized and natural phenolics isolated from *Z. officinale* on XO were determined. As shown in Table 7.2, compounds **4**, **13**, **15** and **18** exhibited certain inhibitory activities at the concentration of 50 μg/mL. The activities of inhibiting the generation of superoxide anions of curcumin analogues were similar to those of phenolics from ginger. In general, the XO inhibitory activity declined when the highly-conjugated system between two aromatic

rings of curcumin was replaced by a saturated ester and was blocked by a CH_2 group. This suggested that the existence of a long-chain conjugated moiety in the test compounds might be essential for XO inhibitory activity. However, compounds **4** and **13** (Table 7.2), both without long-chain conjugated moieties but with *ortho*-diphenolic OH substituents on aromatic A ring also showed inhibitions. This indicated that the existence of *ortho*-diphenolic OH group might afford positive contribution to the antioxidant properties. Among all of the test compounds, compound **18**, a natural phenolic showed the most promising antioxidant property with an inhibition of 58.3 ± 4.6% at 50 μg/mL, while the inhibition of curcumin against XO was 56.1 ± 6.8% at this concentration. This might be due to the co-existence of its β-hydroxyl ketone moiety and rich phenolic hydroxy groups.

Table 7.2. Inhibitory effects of the test compounds (1–24) and curcumin on xanthine oxidase

Compounds	Inhibition (%) at 50 μg/mL
1	31.9%
2	–[b]
3	8.9%
4	49.9 ± 3.0%[a]
5	3.5%
6	17.3%
7	13.9%
8	13.5%
9	31.9%
10	5.3%
11	16.8%
12	12.2%
13	49.0 ± 4.2%[a]
14	3.1%
15	42.5 ± 3.5%[a]
16	27.9%
17	25.7%
18	58.3 ± 4.6%[a]
19	23.5%
20	27.2%
21	–[b]
22	15.5%
23	–[b]
24	–[b]
Curcumin	56.1 ± 6.8%[a]

[a] Data are expressed as means ± SD, $n = 3$; [b] no detectable activity. The IC_{50} value of allopurinol is 3.9 μg/mL in this experiment.

Free Radicals Scavenging Activities of the Test Compounds

***DPPH Assay*:** The DPPH is a stable free radical existed *in vitro*, and bleaching of DPPH absorption is representative of the capacities of the test compounds to scavenge free radicals with aryl and stable characters and independent from any enzyme activity. The obtained results of test compounds on DPPH assays are shown in Table 7.3. It could be observed that both of the curcumin analogues **4**, **13** and the natural phenolics from *Z. officinale* **15–24** showed noticeable DPPH scavenging activities (Table 7.3). It is worthy to point out that the inhibitory activities of compounds **13**, **15**, **18** and **19** are superior to that of curcumin (IC_{50} = 11.6 ± 1.8 μg/mL), and are even comparable with that of quercetin, a highly potent antioxidant with an IC_{50} value of 3.6 ± 0.1 μg/mL. Among the assayed compounds, compound **13** exhibited the most prominent DPPH scavenging activity.

Table 7.3. Free radicals scavenging activities of the test compounds and positive controls

Compounds	DPPH scavenging activity		Superoxide anion inhibition rate (50 μg/mL)
	Inhibition rate (50 μg/mL)	IC_{50} (μg/mL)	
1	11.2%		10.5%
2	18.6%		22.5%
3	16.8%		17.5%
4		10.6 ± 1.6[a]	44.0%
5	13.4%		41.9%
6	3.1%		26.2%
7	14.2%		15.3%
8	–[b]		27.9%
9	14.0%		19.7%
10	–[b]		45.9%
11	0.7%		11.0%
12	1.1%		2.2%
13		6.0 ± 1.2[a]	49.6%
14	–[b]		14.8%
15		11.4 ± 0.7[a]	50.4%
16		14.4 ± 0.3[a]	50.3%
17		24.4 ± 1.2[a]	48.6%
18		10.2 ± 1.1[a]	66.3 ± 1.7%[a]
19		8.5 ± 0.9[a]	–[b]
20		16.6 ± 3.1[a]	4.6%
21		12.7 ± 1.1[a]	–[b]
22		14.5 ± 0.7[a]	1.9%
23		17.2 ± 2.2[a]	6.6%
24		20.7 ± 0.3[a]	32.5%
Curcumin		11.6 ± 1.8[a]	61.3 ± 4.7%[a]
Quercetin		3.6 ± 0.13[a]	91.4 ± 2.8%[a]

[a] Data are expressed means ± SD, n = 3; [b] no detectable activity. IC_{50} values were generated only for those inhibitory rates greater than 50%.

***Superoxide Anion Assay*:** Superoxide anion radical is one kind of reactive oxygen species which could be generated endogenously, *e.g.* hypoxanthine subjected to xanthine oxidase. The anion radical has an extremely active property due to its unpaired electron. It can quickly capture an electron from other molecules and leads to generation of hydroxide radical, the most harmful free radical reported up to date. The superoxide anion radical scavenging activity was measured by the inhibition of NBT reduction. The experimental results are summarized in Table 7.3. It could be observed that compound **4** and **13** showed some inhibitory effects on scavenging superoxide anion, while some phenolics from *Z. officinale* demonstrated higher inhibition rates. By the performances of the investigated compounds on inhibiting XO and scavenging free radicals, it could be found that they exhibited the similar antioxidant tendency toward both the biological models. In general, radical-trapping ability would be markedly descended when the conjugated system of curcumin was interfered (Table 7.3). Nevertheless, compounds **4** and **13** still exhibited strong DPPH radicals scavenging activities and mild superoxide anion trapping abilities.

Antioxidant Properties of the Investigated Compounds in Rat Brain Homogenates

Hydroxyl and hydroperoxyl radicals are able to attack unsaturated fatty acids of phospholipids and other membrane lipids resulting in peroxidation. Such lipid peroxidation causes severe damage to the membrane structure and consequently, alters its fluidity and ability to function properly. Unbalanced oxidative homeostasis and lipid peroxidation were implicated in neurodegenerative diseases, *e.g.* AD and PD (Gilgun-Serki *et al.*, 2001). Therefore, inhibition of lipid peroxidation is a practical evaluation index of antioxidants. Brain homogenate is a widely adopted biomaterial for investigation of lipid peroxidation (Ko *et al.*, 1998). In this article, antioxidant properties of the studied compounds (**1–24**) in rat brain homogenates were carried out. As shown in Fig 7.6, compounds **4**, **13** and **21** exhibited potent antioxidant activities in rat brain homogenates, with their IC_{50} values of 25.7 ± 2.4, 29.7 ± 4.1 and 30.3 ± 2.8 μg/mL, respectively. The antioxidant properties of these compounds (**4**, **13** and **21**) exceeded that of the positive control, quercetin (IC_{50} = 47.6 ± 5.9 μg/mL) and are comparable to that of curcumin (IC_{50} = 25.5 ± 3.9 μg/mL). This suggested that these compounds might be considered as powerful lipid peroxidation inhibitors. Furthermore, compounds **15**, **18** and **24** also exhibited antioxidant activities with their IC_{50} values on lipid peroxidation model of 36.7 ± 3.8, 57.5 ± 4.7 and 36.2 ± 4.5 μg/mL, respectively. Compound **17** possessed about 54.1% inhibition at 100 μg/mL.

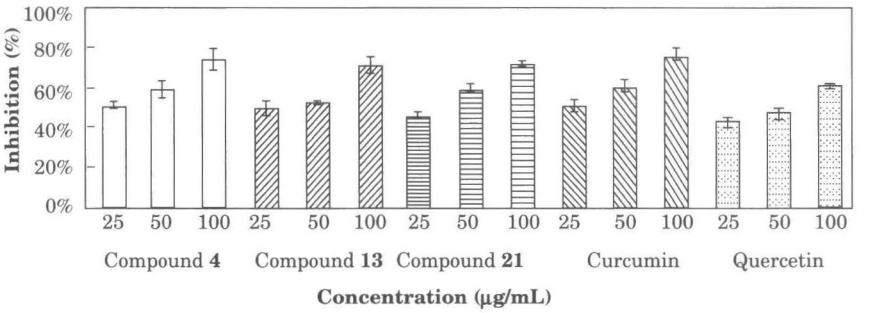


Fig 7.6. Inhibition of iron-induced lipid peroxidation in rat brain homogenates by selected compounds, including curcumin and quercetin as positive controls. TBARS formation was induced by 4 μM $FeSO_4$/50 μM ascorbic acid. The results are shown as the means ± SD, $n = 3$

Protective Effects on H_2O_2 Induced Insult in PC12 Cells

H_2O_2, a major form of reactive oxygen species (ROS), has been identified to be implicated in the pathogenesis of brain injuries and neurodegenerative diseases by damage of cells through direct oxidation of lipids, proteins and DNA or acting as a signaling molecule to trigger cellular apoptotic pathways (Butterfield & Lauderback, 2002; Lin *et al.*, 2004; Zhu *et al.*, 2005). Clonal cell lines, PC12 cells as an example, provide a useful model system for the investigation of neural injury. The protective effects of both the synthesized diarylheptanoid analogues and the natural phenolics on PC12 cells injured by direct application of hydrogen peroxide were performed. The results of morphological images of the PC12 cells cultivated together with compounds **4** and **13** are illustrated in Fig 7.7 (Yang *et al.*, 2009). According to images A and B in Fig 7.7, it could be found that there was an apparent decrease in the number of PC12 cells and most of the cells became round shapes, while the membranes of cells were impaired and became debris after 3 h exposure to 600 μm of H_2O_2. Though the round shapes of PC12 cells could also be observed in the presence of compounds **4** and **13**, the cell viabilities and integralities were remarkably improved in these cases (images C and D in Fig 7.7). This indicated that compounds **4** and **13** possess obvious protective effects against H_2O_2 insult. In addition, the cell viabilities of PC12 cells against H_2O_2-induced injury were also assayed (Fig 7.8). As shown in Fig 7.8, compounds **4**, **13**, **15**, **17** and **24** demonstrated significant neuroprotective activities in a dose-dependent manner. In this experiment, the protective efficacies of synthesized curcumin analogues compounds **4** and **13** not only surpassed those of the natural phenolics from *Z. officinale*, but also more effective than that of quercetin, the positive control. Meanwhile, other investigated compounds demonstrated only weak protective effects. The experiments of removing the compounds from the pre-incubated medium of PC12 cells prior to the injury initiated by H_2O_2 were also carried out. The neuroprotective effects of compounds **15**, **17** and

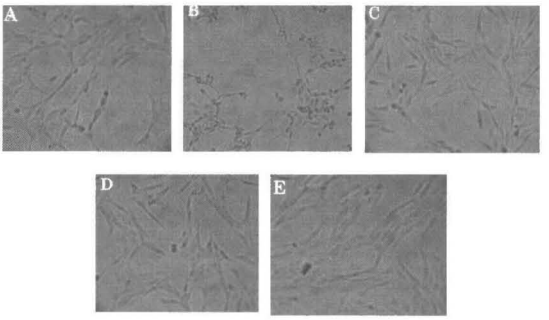


Fig 7.7. Effects of compounds **4** and **13** on PC12 cell injury induced by H_2O_2. (A) PC12 control cells. (B) PC12 cells exposed to 600 μM of H_2O_2 for 3 h. There is a significant decrease in cell number and most of the cells become round shapes. The membrane lesion of cells was also observed. (C, D and E) PC12 cells were pre-incubated with 20 μg/mL of compounds **4**, **13** and quercetin, respectively before exposed to 600 μM H_2O_2 for 3 h

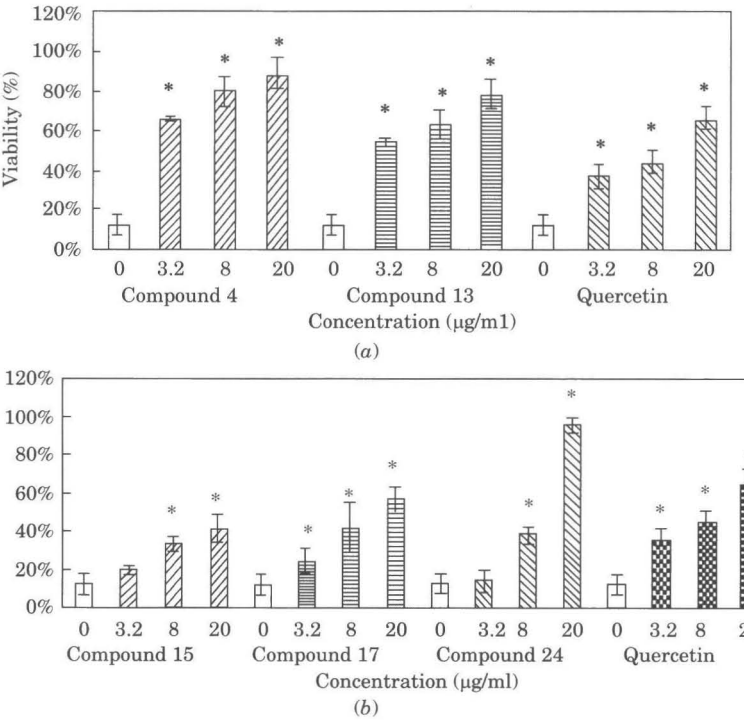


Fig. 7.8. Protective effects of test compounds and quercetin against H_2O_2 induced insult in PC12 cells (8×10^3 cells/well) which were incubated in medium for 48 h and then pretreated with the test compounds 2 h before addition of H_2O_2 (600 μM). Cell viabilities were assayed with MTT after 3 h of incubation. (**A**): the protection potencies of compounds **4** and **13**; (**B**): the protection potencies of compounds **15**, **17**, and **24**. Data are shown as means ± SD, n=3. *$p< .01$, compared with the negative control).

24 as well as quercetin declined dramatically in this case, however, the neuroprotective activity of compounds **4** and **13** still remained visible after the evacuation of pre-incubated medium containing these compounds. This suggested that compounds **4** and **13** might accumulate inside the PC12 cells to exert their protective effects intracellularly, while compounds **15**, **17** and **24** might interact directly with H_2O_2 in cultured medium extracellularly to protect PC12 cells. Additionally, the cytotoxicities of the active compounds were examined in parallel at the same concentrations. No noticeable cytotoxicities on PC12 cells were found among these hits. The protective mechanisms of the aforementioned active compounds on PC12 cells remained uncertain up to now. The most likely mechanisms might be supposed as that the active compounds protected PC12 cells through chelating of metal ions intracellular, or they defended PC12 cells by scavenging hydroxyl free radicals generated by H_2O_2. More evidences derived from further examinations are needed to clarify this mechanistic hypothesis.

Iron Chelate Property

It has been reported that iron is markedly elevated in the brain of AD patients and may have certain impact on the progress of AD through affecting the plaque formation and Aβ aggregation (Lovell *et al.*, 1998). The iron chelation properties of test compounds were therefore analyzed and the results were listed in Table 7.4. The results indicate that compound **4** had a strong iron affinity with the inhibition rate of 75.1 ± 4.9% at investigated concentration. However, none of the natural phenolics exhibited significant iron chelating properties.

Table 7.4. Iron chelation efficacies of the test compounds and positive controls

Compounds	Inhibition rate at 110 μg/mL
1	10.0%
2	2.4%
3	21.2%
4	75.1 ± 4.9%[a]
5	6.4%
6	10.4%
7	7.2%
8	4.8%
9	12.4%
10	11.2%
11	10.4%
12	14.4%
13	40.4%
14	14.8%
15	_[b]
16	_[b]

Table 7.4. *Contd.*

Compounds	Inhibition rate at 110 μg/mL
17	–[(b)]
18	15.6%
19	17.2%
20	12.0%
21	13.6%
22	13.2%
23	15.2%
24	19.6%
Quercetin	46.8%
Curcumin	31.2%

[(a)] Data are shown as means ± SD, $n = 3$; [(b)] no detectable activity.

Inhibition of AChE

AChE is another important pharmacological target for the treatment of AD. Therefore, the inhibitory effects of the test compounds on AChE were also investigated. Except that compound **18** had mild inhibitory effect on AChE with the inhibition rate of 59% at 50 μg/mL, no distinct inhibitory effects on AChE were found among other test compounds.

DISCUSSION

According to the highly-conjugated structure of curcumin, a reactive free radical can undergo electron transfer or abstract H-atoms from either phenolic OH or CH_2 group of curcumin. There are different hypothetical viewpoints presented in published literature about this process. Based on the findings of pulse radiolysis and related biochemical methods, the analysis of inhibiting styrene oxidation of numerous curcumin derivatives and other experiments (Barclay & Vinqvist, 2000; Gorman *et al.*, 1994; Kapoor & Priyadarsini, 2001; Khopde *et al.*, 1999; Priyadarsini, 1997; Priyadarsini *et al.*, 2003), it was suggested that the H atom from the phenolic OH was responsible for the antioxidant activity. However, Jovanovic and coworkers supported the standpoint that the hydrogen abstraction from the methylene CH_2 group of curcumin is responsible for the free radical scavenging activity (Jovanovic *et al.*, 1999; Jovanovic *et al.*, 2001).

According to the results reported in this article, it was observed that compounds with broken β-diketone moieties but intact phenolic OH groups did not show apparent antioxidant activities (*e.g.* compounds **7**, **12** and **14**). This suggested that the CH_2 group of a β-diketone moiety might play an important role in the process of antioxidation among these experimental models. This was further supported by the property of compound **19**, which lacks the β-diketone CH_2 group but possesses two phenolic OH groups

showing noticeable scavenging activity only to DPPH radicals in the six experimental models. Therefore, that compounds without intact β-diketone moiety and phenolic OH functionalities demonstrated inactivity against some models is not surprising. Among all the tested compounds, those with *ortho*-diphenolic hydroxyl groups possessed remarkable antioxidant activity (*e.g.* compounds **4**, **13** and **15–18**) and neuroprotective properties (*e.g.* compounds **4**, **13**, **15** and **17**). Moreover, compound **13** which possesses a saturated ester moiety between two phenyls and *ortho*-dihydroxy functionalities on its aromatic A ring, exhibited to be the most predominant DPPH scavenger. Among the natural phenolics from ginger, it could be observed that diarylheptanoids exhibited better bioactivity than those of gingerol analogues in this assay.

The mechanisms of ferrous ion-induced lipid peroxidation of brain homogenates were hypothesized as decomposition of lipid peroxides, the generation of hydroxyl radicals, or forming perferryl or ferryl species (Ko *et al.*, 1998). The antiperoxidative mechanism of compounds **15**, **18**, **21** and **24** might be due to their ability to directly scavenge free radicals. This presumption was further evidenced by the fact that compounds **15**, **17**, **18**, **21** and **24** did not directly chelate Fe^{2+} (Table 7.4). Meanwhile, the antiperoxidative activities of compounds **4** and **13** might be attributable to their Fe^{2+}-chelating properties in this study. Phenolic hydrogens might be responsible for the antioxidant activity of the test compounds. Furthermore, the reaction mechanisms of compounds **15–18** might be related to their direct free radicals scavenging abilities.

In addition, scrutiny on the experimental results (Tables 7.2 & 7.3) performed by compounds **15** and **16** which have similar structures with only a slight difference in the configurations of the 3-OH groups on the hexahydropyran rings led to a hypothesis that the isomer with an 3-OH located in axial orientation exhibited higher activity than that adopted an equatorial orientation. Furthermore, comparison of the structural similarity of compounds **17** and **18** confirmed an initial finding that compounds with a β-hydroxyl ketone moiety (**18**) performed stronger inhibitory effects on XO, DPPH, superoxide anion radicals and Fe^{2+} chelation models than those data displayed by the 1, 3-diol derivative (**17**) (Tables 7.2–7.4). Moreover, although the antioxidant capacity of compound **24** has been studied previously (Patro *et al.*, 2002; Patro *et al.*, 2005), few pieces of information were reported concerning its neuroprotective property. From the chemical point of view, compound **24** is similar to a half of curcumin, *i.e.* with only one phenolic hydroxyl group. This, however, did not influence its performance on neuroprotective effect. Conversely, the lack of another part of ferricyl part brought, interestingly, weaker cytotoxicity to PC12 cells than that of curcumin. Furthermore, though gingerol related compounds (**20–23**) demonstrated similar antioxidant efficacies in agreement with previous reports (Aeschbach *et al.*, 1994; Masuda

et al., 2004; Shin *et al.*, 2005), their activities are not competitive to diarylheptanoids.

As a summary, the synthetic curcumin analogues such as compounds **4** and **13** as well as the natural phenolics derived from *Zingiber officinale*, for instance, compounds **15, 17** and **18** have satisfactory antioxidant and neuroprotective properties through versatile experimental models. It is widely accepted that the antioxidant potency of curcumin is derived from its highly conjugated system, while our investigation indicated that the easily synthesized esters also possess significant antioxidant tendencies. The chocked curcumin analogues bearing specific functionalities presented even more notable activity than curcumin in the experiments of xanthine oxidase inhibition, superoxide anion and DPPH free radicals scavenging, and iron chelation investigation. It might be the first report which mentioned curcumin-like derivatives possessing a broken-conjugated-system still maintain the potency of antioxidant activities by versatile experiments. This implied the existence of other possibilities for discovering new candidates to enhance the anti-oxidant activity of curcumin analogues while to avoid the underlying imperfection arising from the cytotoxicity of curcumin itself. The study results suggested that in future it would be meaningful to further investigate the antioxidative mechanisms of these active compounds, and this might make more contribution to the prevention and treatment of injuries, disorders, and disease caused by free radicals. The detailed SAR investigations based on other synthetic series of curcumin analogues and pertinent findings are in progress.

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ABBREVIATIONS

AChE, acetylcholine esterase; AD, Alzheimer's disease; CDI, carbonyl-diimidazole; DBU, 1, 8-diazabicyclo-[5,4,0]-undec-7-ene; DCC, N, N-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMSO, dimethyl sulphoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DTNB, dithiobisnitrobenzoic acid; H_2O_2, hydroperoxide; MDA, malondialdehyde;

MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NBT, nitroblue tetrazolium; OD, optical density; OS, oxidative stress; PC12, rat pheochromocytoma cells; PD, Parkinson's disease; PMS, phenazine methosulfate; ROS, reactive oxygen species; SAR, structure-activity relationship; TBA, thiobarbituric acid; THF, tetrahydrofuran; XO, xanthine oxidase.

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8

Action of Plant Proteinase Inhibitors Using Biological Models

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ABSTRACT

Brazilian tropical forests, Pantanal region and Cerrado are considered to be great sources for natural products, which could potentially be beneficial to humanity. One component of this biodiversity is proteins obtained from leguminous seeds, which are capable of inhibiting diverse animal-originated proteinases, including human, and could therefore become models for developing compounds of physiopathological importance. Vegetable proteinase inhibitors can be purified by different procedures. Inhibitors from the Bowman-Birk family, isolated from Torresea cearensis (Tanaka et al., 1989, 1997), Torresea acreana (Tanaka et al., 1996) or Dioclea glabra (Bueno et al., 1999), have been characterized by their primary structure, specificity, kinetic properties and various biological properties. Kunitz-type trypsin inhibitors, obtained from Enterolobium contortisili-quum (Oliva et al., 1987; Batista et al., 1996, 2001), Leucaena leucocephala (Souza-Pinto et al., 1996; Oliva et al., 2000) and Swartzia pickelii (do Socorro et al., 2002) seeds have been characterized physicochemically and by proteinase specificity, primary structure and reactive site. Our group mostly studied the genus Bauhinia, or more specifically, the species B. ungulata (Oliva et al., 1999a, b; 2003), B. variegata (Andrade et al., 2003), B. bauhinioides (Oliva et al., 1999a, b; de Oliveira et al., 2001) and B. rufa (Nakahata et al., 2006), and in some species, we have characterized more than one inhibitor, exhibiting different properties (Oliva et al., 2001a, b; de Oliveira et al., 2001, Sumikawa et al., 2006; Nakahata et al., 2006). Although proteins from this group share high structural similarity, they present differences in proteinase inhibition, which

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we attempt to explore in studies using diverse biological models. This review presents the results systematically, independently from the order in which they were published.

Key words: *Bauhinia*, catepsin L, elastase, enzyme, inflammation, peptidase, proteinase inhibitor, trypsin

INTRODUCTION

Proteolytic enzymes are abundant in living cells and play important roles in intracellular proteolysis. Many studies have shown that proteinases are targets for the investigation of several diseases.

By cleaving proteins, proteinases are involved in the control of a large number of key physiological processes such as cell-cycle progression, cell proliferation, cell death, DNA replication, tissue remodeling, haemostasis (coagulation), wound healing and the immune response (Turk, 2006). For instance, in the case of cysteine proteinases, since the imbalance of their enzymatic activities causes serious diseases such as osteoporosis (Delaisse *et al.*, 1984) and tumor invasion (Denhardt *et al.*, 1987), the search for inhibitors that can moderately control their activity is desired for developing useful drugs. Also, these enzymes have been correlated with the invasion process of many parasites, which demonstrates important interactions with the host immune system (Renslo & McKerrow, 2006).

Over the past decade, there has been an increase in the understanding of the structure and function of proteinase inhibitors. The molecular basis of interactions between a variety of biological substrates or inhibitors and proteinases from different families provided insights into inhibitor protein-proteinase reactive site interactions. These inhibitors interact reversibly with proteinases forming stoichiometric complexes and competitively influencing the catalytic activity of these enzymes (Radisky *et al.*, 2004).

The protein inhibitors of serine proteinases inhibit enzymatic activity by binding extremely tightly to the enzyme's active site and resisting proteolysis (Laskowski & Kato, 1980; Bode & Huber, 1992).

Multiple molecular forms of these proteins have been characterized from microorganisms, animals and plants (Ryan, 2000; Birk, 2003).

Interest in enzyme inhibitors obtained from plants began in the 1940's, when Kunitz (Kunitz, 1946; Kunitz, 1947a, b) isolated and purified a protein from soybean, which inhibited trypsin. Since then, inhibitors have been the research subjects of many groups. Inhibitor proteins have been studied as model systems for elucidating proteinase inhibition mechanisms as well as for studying protein-protein associations.

These proteins have long been considered anti-nutritional factors and are believed to participate in various physiological functions such as the regulation of proteolytic cascades and safe storage of proteins, as well as act as defense molecules against plant pest and pathogens (Birk, 2003; Sumikawa *et al.*, 2008).

The best known groups of inhibitors obtained from seeds include serine proteinase inhibitors (EC. 3.4.21) such as trypsin (EC. 3.4.21.4), chymotrypsin (EC. 3.4.21.3) and subtilisin. Numerous examples of inhibitors are also known for cysteine proteinases (EC. 3.4.22), aspartyl proteinases (EC. 3.4.23) and metalloproteinases (EC. 3.4.12).

Kunitz-type proteinase inhibitors are found in large quantities in seeds from *Leguminosae* subfamilies, *i.e. Mimosoideae*, *Caesalpinoideae* and *Papilionoideae*. This type of inhibitor normally occurs as single polypeptide chains; however, inhibitors have also been shown to be dimeric proteins (Richardson, 1991; Krauchenco *et al.*, 2001, 2004).

In this paper, we review our recent data on the structure and function of plant Kunitz-type inhibitor interactions during the biochemical processes that are involved in some diseases.

PLANT KUNITZ-TYPE INHIBITORS

Plant Kunitz-type inhibitors are easily found in leguminous seeds. As mentioned previously, the first inhibitor from this family (SBTI) was obtained from *Glycine max* seeds and over the past three decades, a great number of other inhibitors have been purified and their primary structures determined. This lead to the conclusion that these inhibitors are not only restricted to the leguminous group but are also found in other plants (Richardson, 1991; Birk, 2003).

The structural study of biomolecules aims to obtain knowledge to develop products, which are potentially useful for solving biochemical problems, and eventually produce new therapeutic agents. Information on the structure of plant Kunitz-type inhibitors would allow us to analyze the mechanisms underlying their specificity for various coagulation factors, inflammation and tumors, and allow us to investigate which region of the protein is responsible for its biological activity and their use as pharmaceuticals in the future.

In the years 1996, Souza-Pinto *et al.* purified a Kunitz trypsin inhibitor from the leguminous plant *Leucaena leucocephala* (LlTI). Biochemical studies showed that LlTI blocks enzymes involved in blood clotting and fibrinolysis (Table 8.1) has anti-inflammatory effects and decreases bradykinin release.

Table 8.1. Inhibition effect (K_{iapp}, nM) of plant inhibitors on proteinases

Proteinase		Inhibitors	BuXI[a] 2Cys-Cys 1chain	BvTI[a] 2Cys-Cys 1chains	BbKI[b] 1Cys	BbCI[c] No Cys	BrTI[d] No Cys	gBrEI[e] 1Cys-Cys 1chain	EcTI[f] 2Cys-Cys 1chains	LITI[g] 2Cys-Cys 1chains
Cysteine-	Plant	Papain	ϕ	ϕ	ϕ	ϕ	—	ϕ	ϕ	ϕ
		Bromelain	ϕ	ϕ	ϕ	ϕ	—	ϕ	ϕ	ϕ
		Ficin	ϕ	ϕ	ϕ	ϕ	—	ϕ	ϕ	ϕ
	Mammalian	Cathepsin L	—	—	ϕ	0.22	—	—	—	—
		Cathepsin B	—	—	ϕ	ϕ	—	—	—	—
		Cathepsin V	—	—	ϕ	ϕ	—	—	—	—
		Cathepsin X	—	—	ϕ	ϕ	—	—	—	—
		Cathepsin K	—	—	ϕ	ϕ	—	—	—	—
	Parasite	Cruzipain[a]	—	—	ϕ	1.3	—	—	—	—
		Cruzain[a]	—	—	ϕ	0.3	—	—	—	—
Serine-	Mammalian	Bovine trypsin	28	2.1	2.0	ϕ	2.9	ϕ	0.88	2.5
		Bovine chymotrypsin	2.7	12	2600	ϕ	ϕ	ϕ	1.11	14
		Bovine pancreatic elastase	—	—	ϕ	40	ϕ	60	—	—
		Human neutrophil elastase	—	—	ϕ	5.3	ϕ	ϕ	55	—
		Human plasma kallikrein	6.9	23	2.4	ϕ	14	ϕ	6.1	6.3
		Human factor XIIa	74	110	ϕ	ϕ	—	—	—	—
		Human factor Xa	14	ϕ	ϕ	ϕ	ϕ	—	ϕ	ϕ
		Human plasmin	76	2.9	33	ϕ	ϕ	ϕ	9.36	0.32
		Porcine pancreatic kallikrein	ϕ	ϕ	200	ϕ	ϕ	ϕ	ϕ	ϕ
		Murine plasma kallirein	—	2.2	5.2	ϕ	13	—	—	—
		Thrombin	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	

"ϕ" No detectable inhibition. [a]Oliva *et al.* 2003; [b]Oliva *et al.* 2001a, b; [c]Oliveira *et al.* 2001; [d]Nakahata *et al.* 2006; [e]Sumikawa *et al.* 2006; [f]Batista *et al.*, 1996; [g]Souza Pinto *et al.* 1996. "nd" Dissociation Constants (K_{iapp}) not determined up to the moment. Inhibitor and proteinase were incubated at 37°C with one of the following proteinases and respective substrates: trypsin (7.0 nM in 0.05 M Tris/HCl, pH 8.0, 0.02% $CaCl_2$; 1.0 mM BAPA), chymotrypsin (76 nM in 0.1 M Tris/HCl, pH 8.0, 0.02% $CaCl_2$; 2.0 mM Suc-Phe-pNan), HuPK, human plasma kallikrein (67 nM in 0.05 M Tris/HCl, pH 8.0; 0.5 mM H-D-Pro-Phe-Arg-pNan); rPK, murine plasma kallikrein (5.0 nM in 0.05 M Tris/HCl, pH 8.0; 0.5 mM H-D-Pro-Phe-Arg-pNan); PoPK, porcine pancreatic kallikrein (2.6 nM in 0.1 M Tris/HCl, pH 8.0; 0.8 mM Ac-Phe-Arg-pNan), PPE, porcine pancreatic elastase (71 nM in 0.05 M Tris/HCl, pH 8.0, 0.5 M NaCl; 0.5 mM MeO-Suc-Ala-Ala-Pro-Val-pNan), HNE, human neutrophil elastase (25 nM

in 0.05 M Tris/HCl, pH 7.0, 0.5 M NaCl; 0.5 mM MeO-Suc-Ala-Ala-Pro-Val-pNan), factor Xa (56 nM in Tris/HCl 0.05 M, pH 8.0; 1.5 mM Boc-Ile-Glu-Gly-Arg-AMC), Factor XIIa (30 nM in 0.05 M Tris/HCl, pH 8.0; 40 μm H-D-Pro-Phe-Arg-AMC), and plasmin (3.5 nM in 0.1 M Tris/HCl, 0.2 M NaCl, pH 7.4; 1.0 mM H-D-Val-Leu-Lys-pNan). K_{iapp} values were determined by adjusting the experimental points to the equation for tight binding, using a nonlinear regression with the Grafit program (Morrison, 1982).

The known primary sequences of members of individual plant protein inhibitor families are highly similar. Several structural features are conserved in most Kunitz-type inhibitors: molecular mass of 20 kDa, four cysteine residues and the sequence neighboring the single reactive site, which in general is an Arg-Ser or Arg-Lys bond situated in a loop closed off by one disulfide bridge and involved in trypsin inhibition (Fig 8.1) whereas alanine or valine in the P1 position is essential for elastase inhibition. We have shown that serine proteinase inhibitors isolated from different species of *Bauhinia* seeds inhibit blood clotting enzymes, as well as other serine and cysteine proteinases (Table 8.1). We previously reported that the inhibitors, BbKI and BbCI, obtained from seeds of *Bauhinia bauhinioides*, a plant known in Brazil by the popular name of "paw cow" due the shape of its leaves, are 18 kDa proteins that present a high primary structure similarity with plant Kunitz-type inhibitors but differ by the absence of disulfide bridges and in their inhibition specificity (Table 8.1 & Fig 8.2) (Oliva *et al.*, 2001a,b, 2003; de Oliveira *et al.*, 2001; Neuhof *et al.*, 2003). The occurrence of a single S-S bridge inhibitor in *Swartzia pickellii* (do Socorro *et al.*, 2002), and inhibitors with none or only a single cysteine residue in *Bauhinia*, may suggest an evolutionary diversion from a Kunitz-type ancestral precursor.

Recently we showed that after the heterologous expression and production of BbCI and BbKI recombinants by *E. coli*, both proteins showed potent inhibitory activities towards their respective proteinases, similar to the wild-type proteins (Araújo *et al.*, 2005). BbCI inhibits the serine proteinases: human neutrophil elastase and pancreatic porcine elastase; and the cysteine proteinases: cathepsin L and cruzipain from *Trypanosoma cruzi*. Although BbKI presents a high primary structure identity to BbCI (84%), it differs by inhibiting plasma kallikrein, bovine trypsin and human plasmin (Table 8.1).

Subsequently, Hansen and co-workers reported the three-dimensional structure of recombinant BbCI at 1.7 Å resolution, and in comparison to the structures of BbKI and other plant Kunitz-type inhibitors, this showed that they share a common β-trefoil fold. Furthermore, the crystallographic structure of BbCI showed that maintenance of the canonical conformation of the reactive site loop is important for proper inhibitory function and that the protein scaffold plays an important role at this site. The absence of disulfide bridges in the structure of BbCI is compensated for by essential interactions that maintain its structural stability and preserve its biological function (Hansen *et al.*, 2007).

```
        1                                                          50
SBTI DFVLDNEGNP LENG.TYYIL  SDITAFGG.I  RAAPTGNERC  PLTVVQSRNE
LITI QVLVDLDGDP LYNGMSYYIL PVARGKGGGL  ELARTGSES C  PRTVVQTRSE
EcTI KELLDSDGDI LRNGGTYYI. PALRGKGGGL  ELAKTGDETC  PLNVVQARGE

       51                                                         100
SBTI LDKGIGTIIS  SPYRIRFIAE  GHPLSLKFDS  FAVIMLCVGI  PTEWSVVEDL
LITI TSRGLPARLA  SPYRILILGS  NIPLTIEFQP  QKPYSCHGHS  SRSLQWKVEK
EcTI TKRGRPAIIW  TPPRIAILTP  AFYLNIEFQT  DLPACL.....  REYSRLPRE

       101                                                        150
SBTI PEGPAVKIGE  NKDAMDGWFR  LERVSDDEFN  NYKLVFCPQQ  AED..DKCGD
LITI TQMVKIASSD  EEQRLFGPFQ  IQPYR....N  HYKLVYCESE  SRNHHDDCRD
EcTI EEHSEVKSDD  DS C KDLG...  .........  ...ISIAPKE  EAA...AFGX

       151                                                        186
SBTI IGISIDHDDG  TRRLVVSKNK  PLVVQFQKLD  KESL..
LITI LGISID.DQQ  NRLLVVKNGD  PLVVQFAKAN  RGGDDD
EcTI EKLKID.DEN  NRRLVVKDGD  PIAVRFVKAH  RRG...
```

Fig 8.1. Comparative sequences of related Kunitz inhibitors LITI-*Leucaena leucocephala* trypsin inhibitor (Oliva *et al.*, 2000); EcTI-*Enterolobium contortisiliquum* trypsin inhibitor (Batista *et al.*, 1996) and SBTI—soybean trypsin inhibitor. The cysteine residues are indicated by boxes and the P_1 residues of the reactive sites are underlined.

BrTI is a Kunitz proteinase inhibitor purified from *Bauhinia rufa* seeds, which contains the RGD sequence and inhibits trypsin and human plasma kallikrein but not other related enzymes. A variety of studies have demonstrated that proteinase inhibitors can suppress several stages of carcinogenesis, including tumor initiation, promotion and progression. Although their mechanism of action is not yet clear, in 2006, Nakahata and co-workers reported the inhibitory action of YLEPVARGDGGLA-NH_2, a synthetic peptide containing the RGD sequence derived from the structure of BrTI (Fig 8.2). This peptide inhibited the adhesion of B16F10 (a high-metastatic B16 murine melanoma cell line) and Tm5 (a murine melanoma cell line derived from a non-tumorigenic lineage of pigmented murine melanocytes, melan-a) to fibronectin. When Asp9 was changed to Glu (YLEPVARGEGGLA-NH2), cell attachment was not affected. Moreover, this peptide was only functional when the sequence present in the native protein was preserved, since changing Glu3 to Ile (YLIPVARGDGGLA-NH2) did not interfere with B16F10 cell adhesion and was less effective on the adhesion of Tm5 cells. YLEPVARGDGGLA-NH2, YLIPVARGDGGLA-NH2 nor YLEPVARGEGGLA-NH2 affected the interaction of RAEC (an endothelial cell line from rabbit aorta) with fibronectin. Interestingly, BrTI, different to other *Bauhinia* inhibitors, is the only one that exhibits insecticidal activity on *Callosobruchus maculatus* larvae.

```
          1                                                                  50
SbTI      DFVLDNEGN  PLEN..GTYY  ILSDITAFGG  .IRAAPTGNE  RCPLTVVQSR
BvTI      .DTLLDTDGE VVRNNGGPYY IIPAFRGNGG  GLTLTRVGSE TCPRTVVQAS
BuXI      .DIVLDTDGK PV.NNGGQYY IIPAFRGNGG  GLELTRVGRE TCPHTVVQAS
BbKI      .SVVVDTNGQ PVSNGADAYY LVPVSHGHAG  .LALAKIGNE  AEPRAVVLDP
BbCI      .SVILDTKGE PVSNAADAYY LVPVSKGEGG  .LALAKVGNE  AEPKAVVLDP
BrTI      .SVVLDTKGQ PVRNAADAVY LEPVARGDGG  .LALAKVGNE  AEPKAVVLDP
gBrEI     ASPVLDANGD PLVP.GGQYY VLPHIWPGPG  GLSFEKTGNQ  TCPVSVFQLP

          51                                                                 100
SbTI      N...ELDKGI GT.ISSPYRI RFIAEGHPLS  LKFDSFAVIM  LCVGIPTEWS
BvTI      S...EJSDGL PVVISALPRS LFISTSW.VT  IQFVVE...AP TCIPKPSFWH
BuXI      S. .EISNGL PVMIAALPRT MFISTAWRVS  IQFLK...VP TCTPKPSYWH
BbKI      H...HRP.GL PVRFESPLRI NIIKESYFLN  IKF.... .G  PSSSDSGVWD
BbCI      H  HRP.GL TV RFETPLAI AI ITESFFLN  IK F.....V  PSSSDSEVWD
BrTI      N...HRP.GL TV RFETPLRI NI IKESFFLN  IK F..... V PS SSESEVWE
gBrEI     RLPLNNGK   PL VFTPVSET DD INEDTAVE  IA F....AEP PSCAESGKWL

          101                                                                150
SbTI      EDLPEGP.   AV KIGENKDA MGW.....F     RLERVSDDE  NNYKLVFCPQ
BvTI      IPQDSELEG. AV KVGASDER FPL E.... ..... F RIERVSED... .TYKLMHCSS
BuXI      IPQDSDMEG. SV EVRV.DER FPL E..... F  RIEKVSED. .  .AYKLMHCPS
BbKI      VIQQDPIGL. AV KVTDTKSL LG.P.....F    KVEKEGE ...  .GYKIVYYPE
BbCI      VSKQYPIGL. AV KVTDTKSF VG.P.....F    RVEKEGE ...  .GYKIVYYPD
BrTI      VRQQPPEGL. AV KVTDTKSL VG.P.....F    RVEKEGE ...  .GYKIVYYPD
gBrEI     NDFKEEYW   SV GIGGPQDH EGYQTLTGYF    KIHKVGSF..   .AYMFSFLPF

          151                                                                194
SbTI      QAEDDKCGDI GISIDHDDGT RRLVVSKNKP  L.VQFQKLDK       ESL...
BvTI      TS.....DSCRDL GISID.E EGN RRLVVRDENP  LLVRFKKAQ     DSEK
BuXI      SS.. ...DSCRDL GIAID.E ENN RRLVVRDGKP  LLVRFKEANQ   DSE...
BbKI      RGO..TG L  DIGLVHRNDK YYLAVKDGEP  CVFKIRKATD       E.....
BbCI      RGO..TG..L DIGLVHRNDK YYLAATEGEP  FVFKIRKATY       E.. .....
BrTI      RGE..TG..L DIGLVHRNEK YYLAVKDGE.  .... .....       .... .....
gBrEI     VR... .... ..... ..... ..... ..... ... .....
```

Fig 8.2. Amino acid sequences of related Kunitz inhibitors. SBTI (soybean trypsin inhibitor); BvTI (*Bauhinia variegata* trypsin inhibitor); BuXI (*Bauhinia ungulata* factor Xa inhibitor); BbKI (*Bauhinia bauhinioides* kallikrein inhibitor); BbCI (*Bauhinia bauhinioides* cruzipain inhibitor); BrTI (*Bauhinia rufa* trypsin inhibitor); gBrEI (*Bauhinia rufa* elastase inhibitor, glycosylated form). Cysteine residues are in black boxes and the P_1 residues of the reactive sites are underlined

Several proteinase inhibitors have been isolated in our laboratory and one is the plant Kunitz-type inhibitor EcTI, which was purified from *Enterolobium contortisiliquum* seeds. EcTI appears to be an interesting inhibitor since it shows a strong capacity for inhibiting trypsin (K_{iapp} 0.88 nM) and because it also inhibits chymotrypsin (K_{iapp} 1.11 nM), plasma kallikrein (K_{iapp} 6.15 nM), plasmin (K_{iapp} 9.36 nM) and human neutrophil elastase (K_{iapp} 55.00 nM) (Oliva *et al.*, 1987; Batista *et al.*, 1996, 2001) (Table 8.1).

We are further evaluating the inhibitory capacity of these proteinase inhibitors in an approach to investigate their actions on the cell viability of different tumor cell lines.

Finally, we are also studying the influence of these substances on the cell viability of primary human fibroblasts and on the proliferation capacity of human mesenchymal stem cells, as well as continuing to investigate their mechanism of action on platelet aggregation, blood coagulation, fibrinolysis, and inflammation.

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9

Plant Hormone Conjugates

ANDRZEJ BAJGUZ[1,*] AND ALICJA PIOTROWSKA[1]

ABSTRACT

Plant growth and developmental processes as well as environmental responses require the action and cross talk of phytohormones including auxins, abscisic acid, brassinosteroids, cytokinins, ethylene, gibberellins, jasmonates, salicylates and polyamines. Active phytohormones are changed into multiple forms by acylation, esterification or glycosylation, for example. It seems that conjugated compounds could serve as pool of inactive phytohormones that can be converted to active forms by de-conjugation reactions. The concept of reversible conjugation of phytohormone suggests that under changeable environmental, developmental or physiological conditions these compounds can be a source of free hormones. Phytohormones metabolism may result in a loss of activity and decrease the size of the bioactive pool. All metabolic steps are in principle irreversible, except for some processes such as the formation of ester, glucoside and amide conjugates, where the free compound can be liberated by enzymatic hydrolysis. The nature of the plant hormone conjugates is discussed.

Key words : Conjugates, auxins, abscisic acid, brassinosteroids, cytokinins, ethylene, gibberellins, jasmonates, polyamines, salicylates

INTRODUCTION

Starling (1905) introduced the term hormone from the Greek *'horman'* (to stimulate) for the polypeptide secretin isolated from duodenum and stimulating secretion of the pancreatic juice *via* blood. Although 19th century plant physiologists such as Sachs had written of 'organ forming

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substances', it was Fitting (1909, 1910) who was the first to use the term 'hormone' in a botanical context, adopting it to describe postflowering phenomena in orchids. During the 20th century, a series of naturally occurring compounds with physiological activity were discovered, and the most important were classed as plant hormones and grouped according to chemical structure and/or physiological effect. Listed in order of the date of their first unequivocal identification in a plant tissue, the most notable include: ethene/ethylene (Gane, 1934); the auxins, *i.e.* IAA (Haagan-Smit *et al.,* 1942); the gibberellins, *i.e.* GA_1 (MacMillan & Suter, 1958); the cytokinins, *i.e.* zeatin (Letham, 1963); abscisic acid (Cornforth *et al.,* 1965); the brassinosteroids, *i.e.* brassinolide (Grove *et al.,* 1979); the jasmonates, *i.e.* jasmonic acid (Ueda & Kato, 1980).

Plant hormones are low-molecular-weight natural products that act at micromolar (or even lower) concentrations to regulate essentially all physiological and developmental processes during a plant's life cycle. These structurally diverse compounds include auxins, cytokinins, abscisic acid, gibberellins, ethylene, polyamines, jasmonates, salicylic acid, and brassinosteroids. This is evident that only auxins and cytokinins meet all criteria assumed at present, *i.e.* hormones are low-molecular compounds displaying their action at a distance and at very low concentrations. Several compounds in the biosynthetic and degradative pathways of plant hormones can exhibit biological activity, giving rise to a very complex network of signalling molecules at the cellular level. The concept of reversible conjugation of phytohormone suggests that under changeable physiological conditions these compounds can be a source of free hormones (Weyers & Paterson, 2001). The hormonal homeostasis has been defined as "the maintenance of a steady state concentration of the hormones in the receptive tissue appropriate to any fixed environmental condition" (Cohen & Bandurski, 1982). The reversible conjugation could be a mechanism to regulate the pool of the physiologically active hormones. Phytohormones metabolism may result in a loss of activity and decrease the size of the bioactive pool. All metabolic steps are in principle irreversible, except for some processes such as the formation of ester, glucoside and amide conjugates, where the free compound can be liberated by enzymatic hydrolysis (Fig 9.1). All-round studying the structure, functions, and action mechanism of phytohormones attracted the attention of many researchers both earlier and now (Weyers & Paterson, 2001).

ABSCISIC ACID CONJUGATES

Abscisic acid (ABA) is a 15-carbon sesquiterpenoid plant hormone. The naturally occurring form is *S*-(+)-ABA; the side chain of ABA is by definition 2-*cis*,-4-*trans*. *Trans*-ABA is biologically inactive, but *R*-(-)-ABA (a possible product of racemization *via* the catabolite ABA-*trans*-diol) has a biological activity (Fig 9.2). ABA plays important roles in many cellular processes

including seed development, dormancy, germination, vegetative growth, and environmental stress responses. These diverse functions of ABA involve complex regulatory mechanisms that control its production, degradation, signal perception, and transduction. Various factors determine the ABA concentration at the site of action, particularly the rate of synthesis, long-distance transport, tissue susceptibility, conjugation, and oxidative degradation of ABA (Giraudat *et al.*, 1994; Crozier *et al.*, 2000).

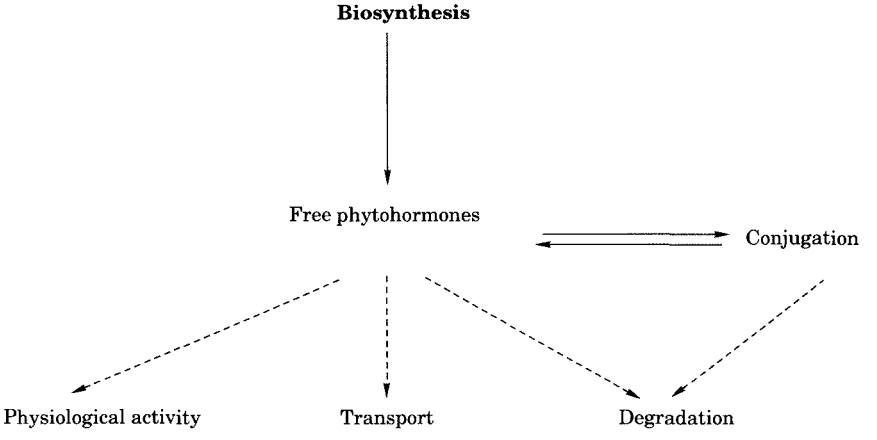


Fig 9.1. Diagrammatic representation of the plant hormone homeostatic model

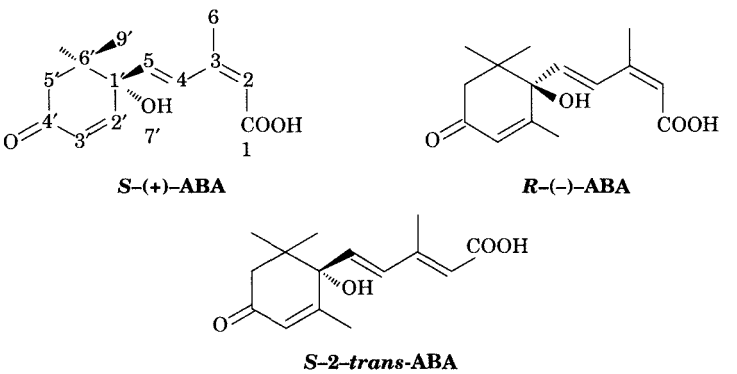


Fig 9.2. Naturally occurring *S*-enantiomer of ABA and the unnatural *R*-enantiomer

The ABA catabolism pathways, *via* phaseic acid and glucose-ester conjugation (Fig 9.3), were established before much was known about ABA biosynthesis, but subsequently our understanding of catabolism has proceeded surprisingly slowly. It seems to be the main inactivation pathway. It leads over 6-hydroxymethyl-ABA to phaseic acid, dihydrophaseic acid and some polar conjugates, with a side-branch from 6-hydroxymethyl-ABA to β-

hydroxy-β-methyl-glutarylhydroxy-ABA. Phaseic acid is usually present in plant tissues in small amounts, whereas accumulation of dihydrophaseic acid and also its conjugates has been observed in many plants, especially in association with stress and at some stages of germination. To a lesser extent, phaseic acid and dihydrophaseic acid can be conjugated to esters of the β-D-glucopyranoside and glucose type. An epimer of dihydrophaseic acid has also been shown to be a naturally occurring ABA metabolite although it usually occurs in lower concentration than dihydrophaseic acid. In addition, minor amounts of several other metabolites, (+)-7′-hydroxy-ABA, *trans*-ABA and the *cis*- and *trans*-1′, 4′-diols of ABA, have been detected in some plant species. In pea seedlings, ABA can be converted to ABA-1′, 4′-*trans*-diol, but in tomato, the diol is an ABA precursor rather than a catabolite (Harris & Dugger, 1986; Cutler & Krochko, 1999; Crozier *et al.,* 2000; Dietz *et al.,* 2000; Xiong & Zhu, 2003; Verslues & Zhu, 2005).

General, the two major pathways of ABA catabolism exist: (i) hydroxylation of ABA at the 8′ position by a P-450 type monooxygenase to give an unstable intermediate (8′-OH-ABA) that rearranges spontaneously to phaseic acid, and (ii) esterification of ABA to ABA-glucose ester. Conjugation of ABA to ABA-glucose ester is irreversible. Other known minor catabolites, such as the ABA-diols, 7′-hydroxy-ABA, ABA-1′-glucoside, 3-hydroxy-3-methylglutaryl conjugate of 8′-hydroxy-ABA, phaseic and dihydrophaseic glucose esters and glucosides occur in plant tissue (Fig 9.3). ABA β-glucosyl ester and ABA-1′-*O*-β-glucoside could act as storage products that can be hydrolyzed to release free ABA. However, it suggests that these conjugates are primarily deactivation products and do not supplement the endogenous ABA pool. While ABA-glucoside is derived from a small portion of the ABA pool. The ABA conjugate may accumulate in the aqueous phase of the cell wall. About 20% of total ABA is localized in plant vacuoles, and the vacuolar ABA is represented exclusively by its glucose ester (Harris & Dugger, 1986; Xiong & Zhu, 2003; Verslues & Zhu, 2005).

AUXIN CONJUGATES

Auxins were the first plant hormones discovered and have been extensively examined for many decades. In many bioassays, it has been shown that auxins play a critical role in plant growth and development. Auxins are thought to regulate or influence diverse responses on a whole-plant level, such as tropisms, apical dominance and root initiation, and responses on cellular level, such as cell enlargement, division, and differentiation (Hagen & Guilfoyle, 2002). Our knowledge about the physiological and molecular aspects of auxin role is rapidly expanding. Great advances have been also reported on the characterization of auxin metabolism, including conjugation and deconjugation.

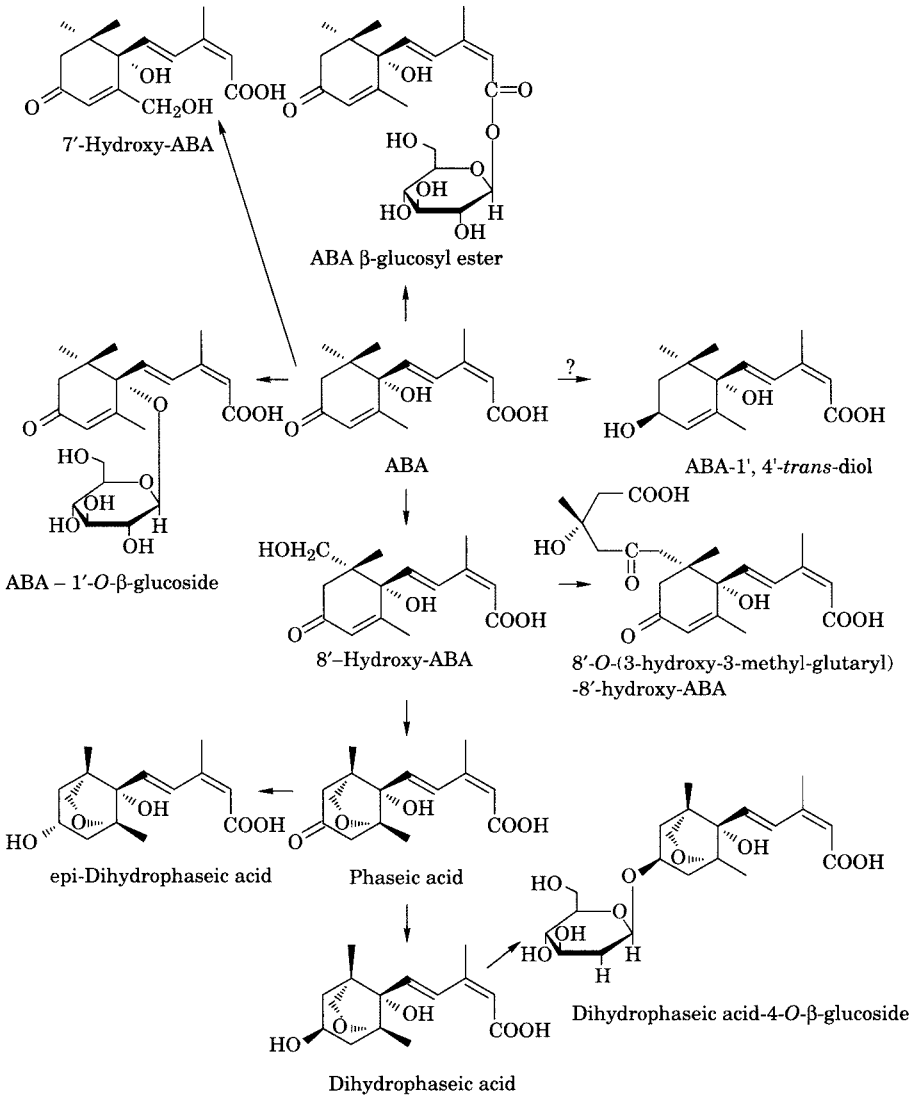


Fig 9.3. ABA metabolism pathway and glucose conjugates

Auxins belong to a chemically diverse of compounds, most of which have an aromatic system such as indole, phenyl or naphthalene ring with a side chain containing a carboxyl group attached (Fig 9.4). Indole-3-acetic acid (IAA) is the natural auxin commonly occurring in all vascular and lower plants (Cooke *et al.*, 2002). A chlorinated form of IAA with high auxin activity, 4-Cl-IAA, is found in several plant species (Slovin *et al.*, 1999). In addition to the indolic auxins, phenylacetic acid has been identified in plants and is an active auxin in the most bioassays. Certain IAA precursors, such as indole-3-acetonitrile and indole-3-pyruvic acid, exhibit also stimulating

properties of plant growth and development, presumably because of conversion in the tissue to the biologically active IAA (Cohen *et al.,* 2003). Similarly, indole-3-butyric acid, identical to IAA except for two additional methylene groups in the side chain, is effective in bioassays. Indole-3-butyric acid, originally classified as synthetic auxin, is in fact an endogenous plant compound (Bartel *et al.,* 2001). Two, main types of synthetic plant growth regulators with high auxin activity have been described: naphthalene-1-acetic acid and 2, 4-related compounds, *e.g.* 2, 4-dichlorophenoxyacetic acid. The isomer of naphthalene-1-acetic acid, naphthalene-2-acetic acid, shows lower biological activity. 2, 4-dichlorophenoxyacetic acid is commonly used herbicide (Kelley & Riechers, 2007).

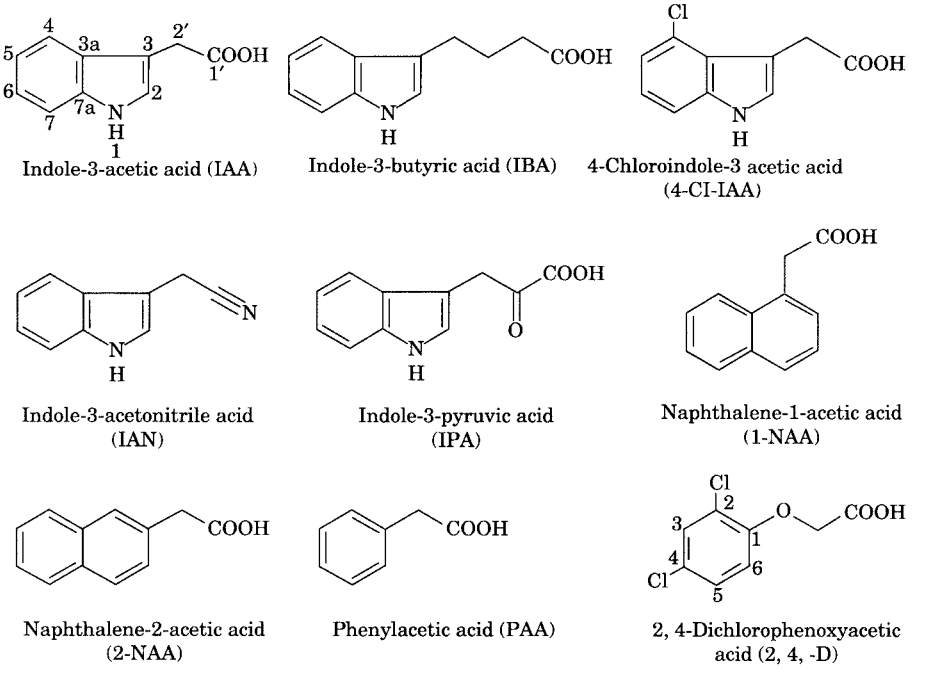


Fig 9.4. Selected chemical structures of natural and synthetic auxins

Plants use several mechanisms to control the levels of endogenous auxins, especially IAA. Despite, the regulation of synthesis and degradation of these phytohormones, plants may store auxins in the form of conjugates (Fig 9.5). Most of plant's endogenous IAA is found not as free and biologically active form, but conjugated at the carboxyl group. IAA is conjugated at the carboxyl group of monosaccharides, high molecular weight polysaccharides, *myo*-inositol, choline and the carbohydrate components of glycoproteins *via* ester bonds. IAA can also be conjugated to single amino acids, peptides or proteins *via* amide bonds. These mentioned conjugates are thought to be involved in IAA storage and transport, inactivation of the hormone pathways

to auxin catabolism, and as components of a homeostasis for the control of IAA levels. They can also protect IAA against peroxidative degradation, and detoxification of excess auxin. Hydrolysis of endogenous conjugates of IAA are likely to be important free IAA sources (Cohen & Bandurski, 1982; Fluck *et al.*, 2000).

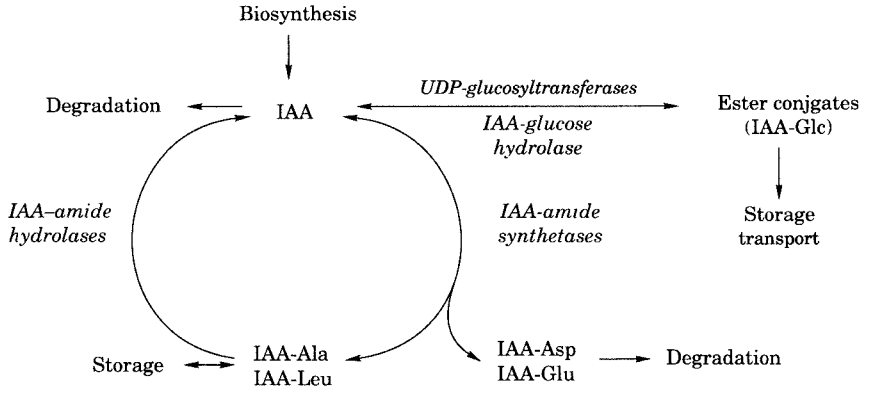


Fig 9.5. The role of indole-3-acetic acid (IAA) conjugates in auxin homeostatis

IAA conjugation is a ubiquitous process in both higher and lower plants and IAA-conjugated forms are synthesized rapidly in plant tissues when auxin homeostasis is perturbed. For example, the exogenous application of IAA and others natural and synthetic auxins generally increase the level of auxin conjugates. Moreover, conjugate formation and hydrolysis is developmentally regulated and varies significantly among plant tissues. Although some auxin conjugates exert stimulating properties of plant growth when applied exogenously, this biological activity generally correlates with the ability of plant tissue to hydrolyze these compounds to free active aglycones (Rampey *et al.*, 2004). Besides IAA, indole-3-butyric acid occurs as the free acid as well as in a variety of conjugated forms, although both are usually less abundant than IAA. Indole-3-butyric acid (IBA) is conjugated to other moieties through amide- and ester-linkages. On the other hand, ester conjugates of IBA dominates over amide forms of IAA. Moreover, IBA conjugates are more easily hydrolyzed and more slowly transported in different plant systems, perhaps leaving more phytohormones at the plant base in comparison with conjugates of IAA. In addition, certain IBA conjugates are very active in bioassays (Bartel *et al.*, 2001; Ljung *et al.*, 2002).

Different plant species have distinct IAA conjugate profiles. IAA conjugates have been identified in a number of plant species, ranging from algae to angiosperms. In general, monocots appear to accumulate ester conjugates, whereas dicots accumulate mostly amide conjugates (Slovin *et al.*, 1999; Cooke *et al.*, 2002; Jakubowska & Kowalczyk, 2004).

The maize seedlings contain primarily ester-linked conjugates including IAA-*myo*-inositol, IAA-*myo*-inositol glycosides, IAA-glucose and a large cellulosic glucan conjugates (Fig 9.6) (Cohen & Bandurski, 1982). The synthesis of IAA-glucose, followed by transacylation to *myo*-inositol

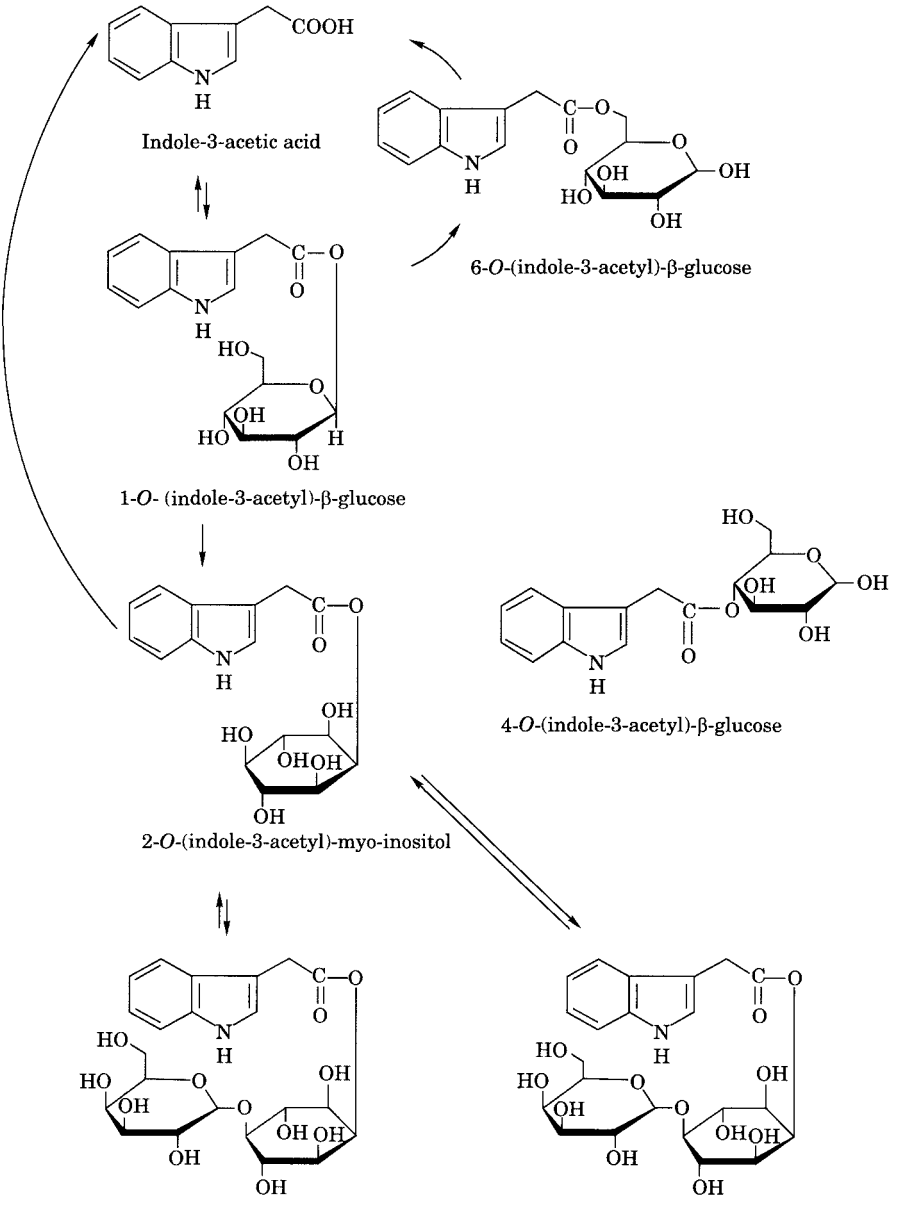


Fig 9.6. Biosynthesis and hydrolysis of indole-3-acetic acid ester conjugates in *Zea mays*

represents two potential regulatory steps for the control of IAA concentration by converting hormonally active free IAA into growth-inactive IAA ester. Since all maize tissues hydrolyze IAA-glucose isomers and, more slowly, IAA-*myo*-inositol to free IAA, the mechanism is interpreted as a shuttle to adjust the free pool of IAA *via* the temporal storage of IAA esters (Jakubowska & Kowalczyk, 2004). Glucosyltransferase from maize was the first plant protein with IAA-conjugating activity (Szerszen *et al.*, 1994). The formation of IAA-glucose from IAA and UDP-glucose by indole- 3-acetylglucose synthase is the first step in the series of reactions leading to the IAA-ester conjugates found in maize. Recently, an *Arabidopsis* and maize gene encoding UDP-glucosyltransferase that conjugate IAA to glucose was identified (Jackson *et al.*, 2001). Intracellular location of this enzyme is not clear, but the pH optimum and requirement of a redox status are consistent with being in the cytoplasm. Overexpressing gene encoding this specific UDP-glucosyltransferase renders plants resistant to exogenous IAA, and disturbs gravitropism, consistent with the role in IAA inactivation. This enzyme recognizes also indole-3-butyric acid (IBA) as a substrate and therefore is responsible for formation of IBA-glucose from radiolabeled IBA fed to *Arabidopsis* seedlings. Moreover, the stimulation of gene expression encoding IAA-glucose synthesis by auxin in maize coleoptiles has been observed (Kowalczyk *et al.*, 2002). In immature maize kernels, the energetically unfavourable synthesis of IAA-glucose is followed by an energetically favorable transacylation of the IAA moiety from IAA-glucose to *myo*-inositol. The synthesis of IAA-*myo*-inositol was observed for the first time *in vitro* in the seeds of maize, and the transferase catalyzing this reaction was partially purified and characterized (Kesy & Bandurski, 1990).

Conjugate profile of endogenous auxins in dicotyledonous plants differs during their growth and development. For example, the level of ester conjugates and free IAA in bean declines rapidly during seed maturation, so that in fully mature seeds, the ester-linked IAA represents about 13% of the total IAA pool, and only 6% is free IAA. It is noteworthy, that in seeds harvested at full maturity, IAA is conjugated to several polypeptides and proteins that approximate 80% of the total IAA pool (Walz *et al.*, 2002). IAA undergoes conjugation to yield both IAA-glucose and amide-linked IAA, and the preferential formation of either IAA-glucose or amide-linked IAA conjugates depends on the ripening stage of the fruit (Iyer *et al.*, 1997). Tobacco explants and leaf protoplasts produce mainly auxin-aspartate and auxin-glucose conjugates (IAA- or NAA-conjugates) (Smulders *et al.*, 1990). *Arabidopsis*, the model dicotyledonous plant, is also able to form the ester-linked IAA conjugates that constitute approximately 8–10% of the total IAA pool. However, amide-linked IAA conjugates constitute approximately 90% of the total IAA pool in *Arabidopsis*, and in other dicots as well (Tam *et al.*, 2000; Ljung *et al.*, 2002).

Many experiments have shown that conjugates of IAA with aspartate are the predominant IAA constituents present in most dicotyledonous plants,

e.g. Vicia faba seedlings and tomato pericarp discs (Fig 9.7). The principal IAA deactivation pathway in dicots converts IAA to *N*-(indole-3-acetyl)-L-aspartic acid. The indole ring of IAA-aspartate is oxidized to form *N*-(oxindole-3-acetyl)-L-aspartic acid, which is subjected to successive glycosylations (Iyer *et al.*, 1997; Crozier *et al.*, 2000).

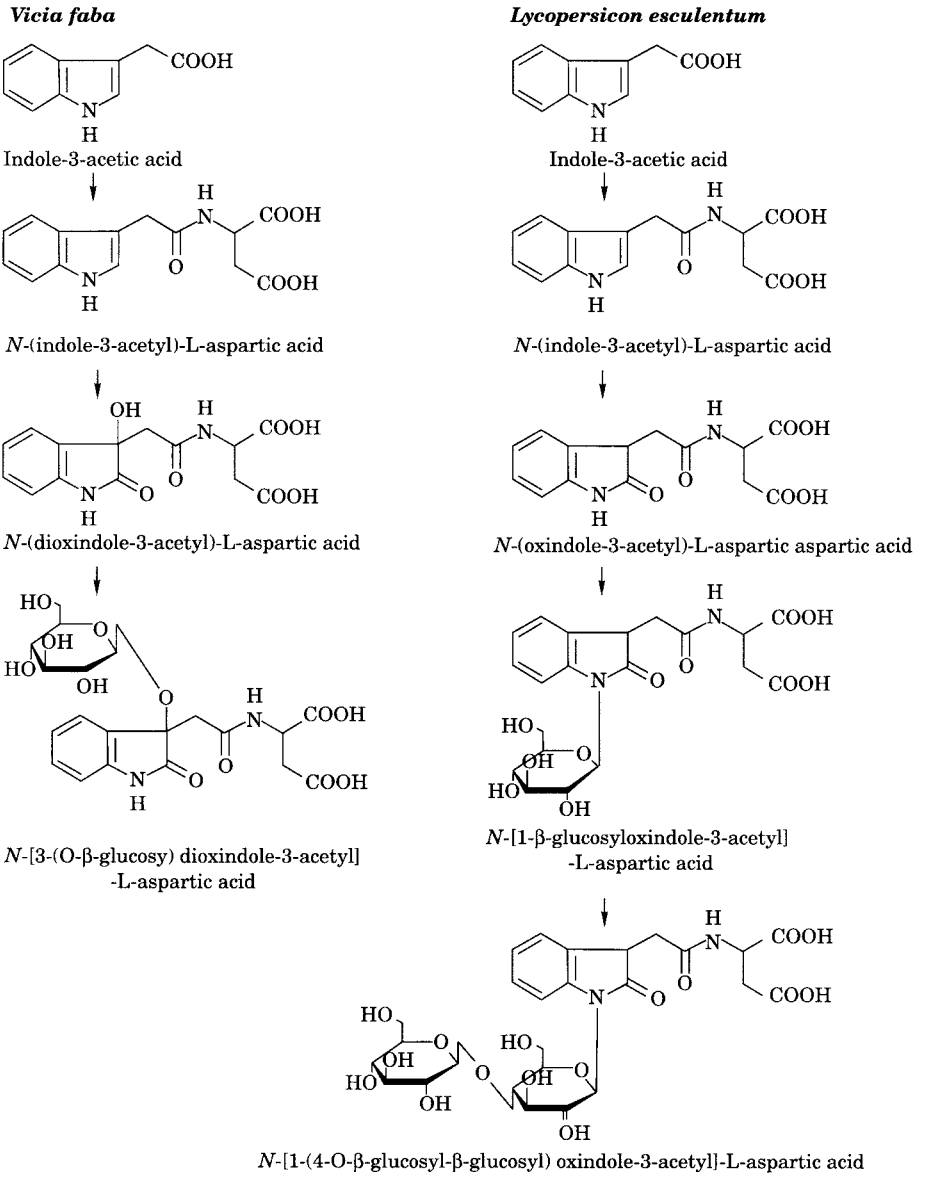


Fig 9.7. Non-decarboxylative catabolism and conjugation of indole-3-acetic acid in the seedlings of *Vicia faba* and tomato (*Lycopersicon esculentum*) pericarp discs

Amide conjugates with amino acids (Asp, Glu, Ala, Gly, Val and Leu) are present in a variety of plants, and conjugates with other amino acids may also occur (Fig 9.8). IAA-amino acid conjugates found in plant tissues may be classified into two groups: (i) based on bioassay activity and (ii) susceptibility to hydrolysis by specific enzymes in plant cell (Ljung *et al.*, 2002).

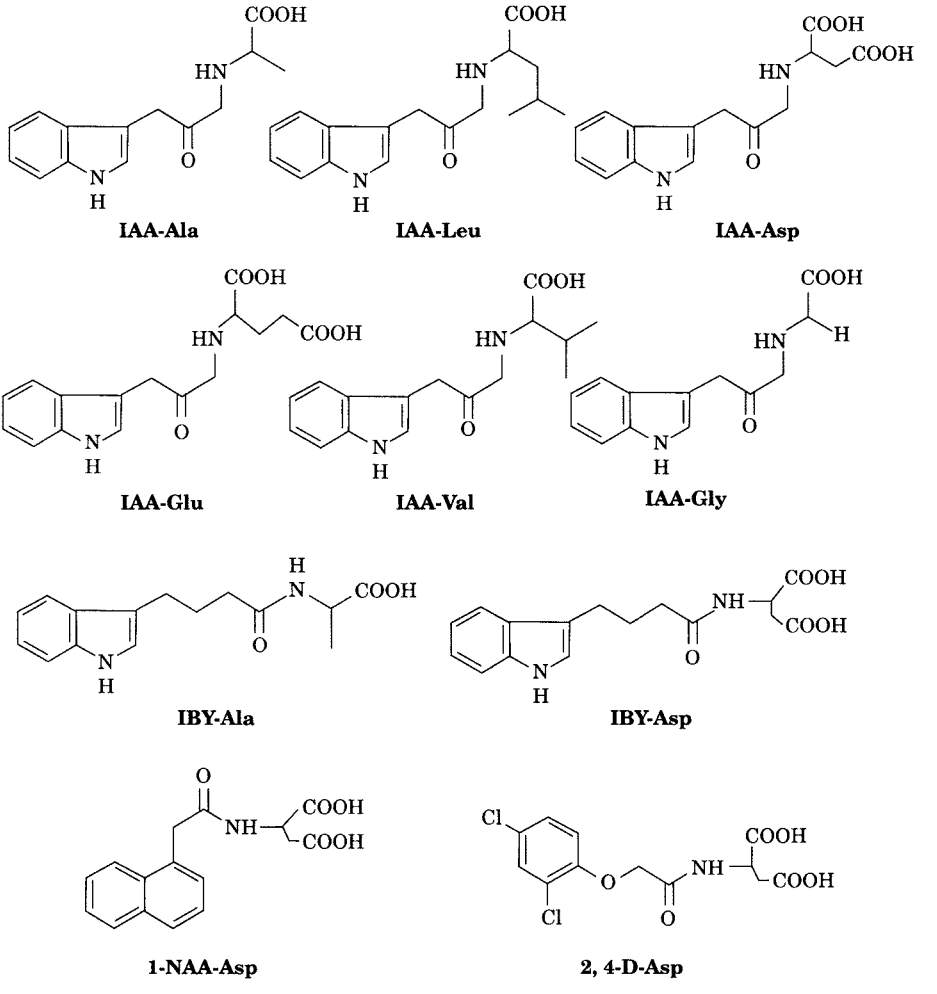


Fig 9.8. The structures of amide auxin conjugates

The first group of amide conjugates is represented by IAA-Ala and IAA-Leu. It is characterized by biological activity in bioassays. In *Arabidopsis*, IAA-Ala is present at highest levels in shoots, whereas IAA-Leu accumulates in roots (Ljung *et al.*, 2002). Both, IAA-Ala and IAA-Leu exhibit activity because they can be readily converted *via* amidohydrolases to active IAA and are likely storage forms of auxins. The conjugate hydrolysis is the

principal source of auxins in germinating seed and seedling development. Therefore, these conjugated forms of auxins contribute to the pool of active free auxins (Rampey *et al.,* 2004).

The second group of auxin conjugates is represented by IAA-Asp and IAA-Glu. These amide conjugates play role in IAA turnover and they are not appreciably hydrolyzed *via* amidohydrolases. Formation of these conjugates leads irreversibly to oxidation, followed by catabolism of IAA (Ljung *et al.,* 2002). Moreover, IAA-Glu and IAA-Asp are not characterized by biological activity in plant species tested (Slovin *et al.,* 1999). On the other hand, the conjugate of IAA with aspartate and its downstream metabolites must have an important function, because a mutant cell line (XIIB2) of Egyptian henbane (*Hyoscyamus muticus* L.) impaired in IAA-Asp biosynthesis rapidly dies at 33°C, a temperature to which, otherwise isogenic, wild-type cell cultures are resistant (Oetiker & Aeschbacher, 1997).

Amide conjugate synthesis may be uncharted, but some enzymes associated with hydrolysis of bioactive amide conjugates of IAA have been described. These enzymes cleave IAA-amino acid conjugates to release the active hormone. The conjugate hydrolases have different amino acid specificities both *in vivo* and *in vitro* when tested with battery of IAA-amino acid conjugates. They are also differentially expressed, implying that a variety of IAA conjugates exist and serve different roles at varied location throughout the plant (Cohen & Bandurski, 1982). For example, IAA-Ala is hydrolyzed by bean stem sections, *Arabidopsis* extracts, Chinese cabbage extracts and by the IAR3-amidohydrolase isolated from *Arabidopsis* tissue. IAA-Leu is hydrolyzed by the *Arabidopsis* ILR1-amidohydrolase which requires Mn or Co for its activity and is predicted to be localized to the endoplasmic reticulum lumen. Additionally, another protein, ILR3, is an apparent transcription factor important for IAA-conjugate responsiveness. These data suggested that ILR3 might regulate ILR1 activity because ILR3 may be directly or indirectly involved in metal homeostasis, especially metal cofactors availability (Mn, Co, Fe) which are necessary for ILR1 activity (Rampey *et al.,* 2006). Examined IAA-amidohydrolases in these plant species are targeted to the endoplasmic reticulum lumen, although the significance of such compartmentation is unclear because there is no clear evidence on the storage location of any conjugate compounds (Rampey *et al.,* 2004; Woodward & Bartel, 2005). Recently, the enzymes that conjugate IAA to amino acids were found in *Arabidopsis*. Those enzymes are in the luciferase superfamily and are related to the JAR1 enzyme that conjugates other plant hormone–jasmonic acid (JA) to amino acids, and are encoded by members of the *GH3* family of auxin-induced genes. The characterized GH3-like enzymes apparently prefer to synthesize biologically inactive conjugates of IAA (IAA-Asp, IAA-Glu) over hydrolysable and active in bioassay forms of amide conjugates (IAA-Ala, IAA-Leu) *in vitro* (Staswick *et al.,* 2005). Auxin substrate for

GH3 enzyme include also indole-3-butyric acid, indole-3-pyruvic acid, phenylacetic acid, 1-naphthalacetic acid. Moreover, amino acid conjugates of these phytohormones can also be glucosylated (Ljung *et al.,* 2002; Zazimalova & Napier, 2003).

Among amide conjugates of IAA, a 35-kDa IAA-peptide was identified in *Arabidopsis* seeds. The presence of peptide-bound IAA has been also demonstrated in bean seeds and strawberry fruit. The large size of this conjugate might contribute to the solvent insolubility of amino acid conjugates. Moreover, bean seeds apparently lack amino acid conjugates, and IAA is instead conjugated to several polypeptides ranging size of molecular weight from 3 to 60 kDa. Although, genes encoding peptides that conjugate to auxins have not been identified yet, one of these IAA-modified bean protein is similar to a soybean late seed maturation protein, suggesting that certain seed storage proteins may function in both amino acid and phytohormones storage (Walz *et al.*, 2002).

Auxin conjugates that accumulate during auxin feeding often differ from those apparently used for endogenous auxin storage forms. Exposure of plants to high exogenous level of natural IAA or synthetic 2, 4-dichlorophenoxyacetic acid with herbicide properties leads to detoxification of the excess auxins. *Arabidopsis* can inactivate high level of exogenous auxin by oxidation and conjugates to aspartic acid and glucose. This inactivation pathway is evolutionary conserved because different plant species apparently accumulate conjugates with aspartic acid and glucose in response to high concentration of exogenous auxins. These modified forms of auxins may be further oxidized for example to ox-IAA-conjugates, which permanently inactivate auxins (Kelley & Riechers, 2007).

Another conjugation process of auxin leads to methylation of IAA. An enzyme that methylates the carboxyl side chain of IAA is a member of a family of carboxyl methyltransferases which methylate various plant hormones, such as jasmonates and salicylates. Methylation may increase the volatility of IAA, but it is not clear whether this modification activates or inactivates of auxins (Woodward & Bartel, 2005).

A variety of indolic compounds and their conjugates are present in plant pathogenic and symbiotic microorganisms but their function in these organisms is largely unknown (Cohen & Bandurski, 1982). The enhanced synthesis of IAA in *Agrobacterium tumefaciens*-induced galls and tumors (Fig 9.9) results from expression of two bacterial genes that are transferred to the plant when the T-DNA integrates into the host genome. These genes are associated with a two-step tryptophan-dependent pathway to IAA. The *iaaM* gene encodes tryptophan monooxygenase, which converts L-tryptophan to indole-3-acetamide. On the other hand, the product of the *iaaH* gene, indole-acetamide hydrolase, catalyzes the conversion of indole-3-acetamide to IAA. Cognate genes with similar functions occur in the plant

pathogen *Pseudomonas savastanoi*, in which *iaaL* gene, encoding IAA-lysine synthase is also presents. When expressed in the cells of the plant host, this enzyme conjugates IAA and L-lysine to form ε-*N*-(indole-ε-acetyl)-L-lysine, which is metabolized further to ε-*N*-acetyl-ε-*N*-(indole-3-acetyl)-L-lysine. Although IAA-lysine formation reduces the pool of free IAA produced by the bacteria by about 30%, the role of these conjugates in gall formation has not been established (Glass & Kosuge, 1988; Roberto *et al.*, 1990; Stibon *et al.*, 1991).

Presented facts indicate the important role of amide and ester conjugates of auxins in the homeostatic control of hormone level. Nevertheless, the number of auxins, especially IAA, conjugates have been identified, a considerable proportion of total bound IAA and other endogenous auxins is still chemically unknown in many plants.

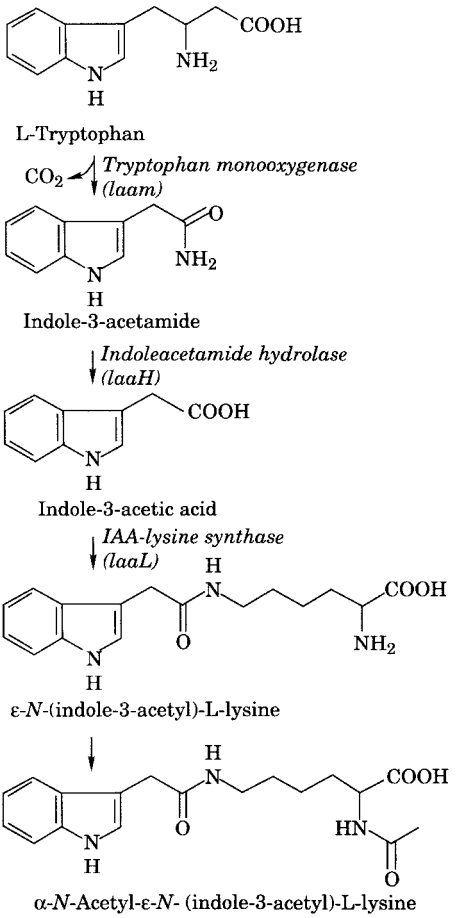


Fig 9.9. Indole-3-acetic acid biosynthesis and conjugation pathways in *Agrobacterium tumefaciens* and *Pseudomonas savastanoi*

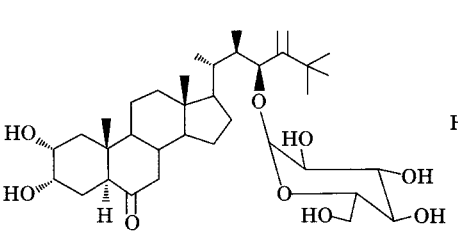
BRASSINOSTEROID CONJUGATES

Brassinosteroids are hydroxylated derivatives of cholestane and their structural variations comprise substitution patterns on rings A and B as well as the C-17 side-chain. These compounds can be classified as C_{27}, C_{28}, or C_{29} BRs, depending on the length of the side chain. Till now, 65 free brassinosteroids and five conjugates have been characterized from the plant kingdom (Bajguz & Tretyn, 2003).

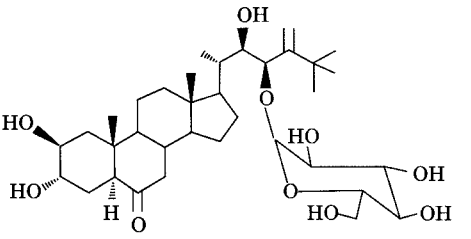
Brassinosteroids are important plant growth regulators in multiple developmental processes at nanomolar to micromolar concentration, including cell division, cell elongation, vascular differentiation, reproductive development and modulation of gene expression. They also influence various other developmental processes like germination of seeds, rhizogenesis, flowering, senescence, abscission and maturation. Brassinosteroid application include ethylene biosynthesis, membrane hyperpolarisation, enhanced DNA, RNA and protein synthesis, increased invertase activity, stimulation of photosynthetic activity, and changes in the balance of other endogenous phytohormones. They confer resistance to plants against various abiotic and biotic stresses (Sasse, 2003).

There are only a few papers presented on conjugation of the brassinosteroids. This field is in its very infancy and the low concentration of brassinosteroid conjugates may well represent a major difficulty in their detection and analysis. Among 65 free brassinosteroids, only three sugar and two fatty acid conjugates have been identified so far in plants (Bajguz & Tretyn, 2003). Although brassinosteroid molecules contain a series of functional groups, only hydroxyl groups at C-3, C-23 and C-25 have been found to be linked to acyl or glucosyl moieties. 25-Methyldolichosterone-23-*O*-β-D-glucoside (25-MeDS-Glu) and its 2β isomer from *Phaseolus vulgaris* seeds (Yokota *et al.,* 1987) and teasterone-3β-D-glucoside (TE-3-Glu), teasterone-3-laurate (TE-3-La) and teasterone-3-myristate (TE-3-My) from *Lilium longiflorum* pollen (Asakawa *et al.,* 1994, 1996; Soeno *et al.,* 2000b) were isolated as endogenous brassinosteroids (Fig 9.10).

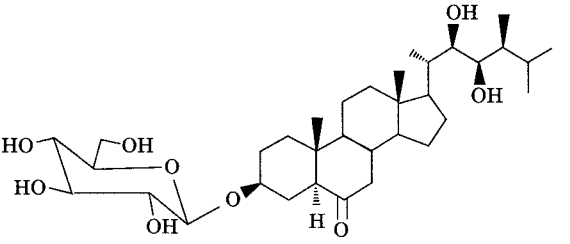
Conjugates of brassinosteroids have also been detected in metabolic studies in plants. Currently 42 brassinosteroid metabolites and their conjugates are known. Among them, 19 sugar, fatty acid and sulphuric acid conjugates (Fig 9.11) have been identified so far in plants. Metabolism of free brassinosteroids to conjugates can be divided into two categories: (i) structural changes to the steroidal skeleton at C-2 or C-3 and (ii) structural changes to the side-chain at C-22, C-23, C-25 or C-26. The most common modifications to the steroidal skeleton are esterification at C-3 (8 compounds) and glycosylation at C-3 (5 compounds); less common is glycosylation at C-2 (1 compound). The common modifications to the side-chain include glycosylation at C-23, C-25 or C-26 and sulfonation at C-22 (2 compounds) (Bajguz, 2007).



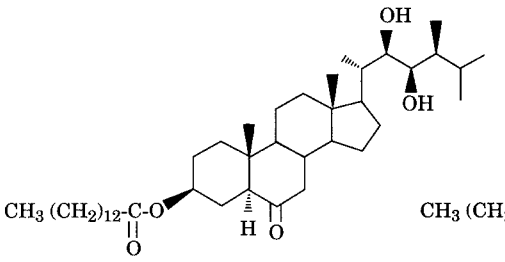
23-*O*-β-D-glucopyranosyl-25-methyldolichosterone (25-Me DS-Glu)



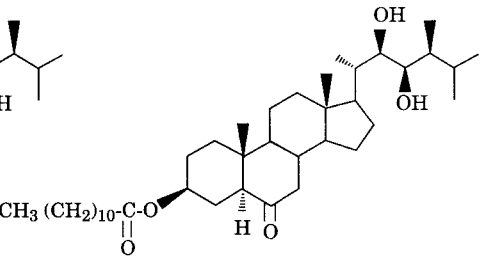
23-*O*-β-D-glucopyranosyl-2-epi-25-methyldolichosterone (2-epi-25-MeDS-Glu)



Teasterone-3-*O*-β-D-glucoside (TE-3-Glu)



teasterone-3-myristate (TE-3-My)



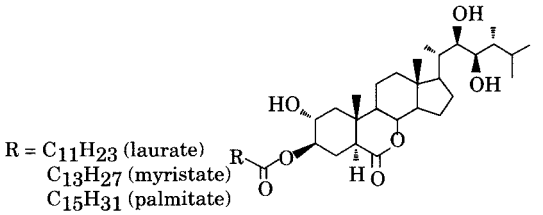
teasterone-3-laurate (TE-3-La)

Fig 9.10. Brassinosteroid conjugates naturally occurring in plants

After feeding brassinolide (BL) to mung beans (*Vigna radiata* or *Phaseolus vulgaris*), the corresponding 23-*O*-β-D-glycopyranosyl conjugate was identified (Suzuki *et al.*, 1993; Soeno *et al.*, 2006), while cell cultures of tomato (*Lycopersicon esculentum*) transform 24-epiBL to the 25-β-D-glucosyloxy derivative (Hai *et al.*, 1995). Although 23-*O*-β-D-glycopyranosyloxyBL is as active as the free BL in the rice lamina inclination test, there has been discussion suggesting that 23-O-glucosylation of brassinosteroids represents a regulatory deactivation step.

Interesting results have been obtained in the course of metabolic studies of 24-epicastasterone (24-epiCS) and 24-epiBL in cultured cells of tomato (*Lycopersicon esculentum*) (Schneider *et al.*, 1994; Hai *et al.*, 1995, 1996) and serradella (*Ornithopus sativus*) (Kolbe *et al.*, 1994, 1995, 1996, 1998).

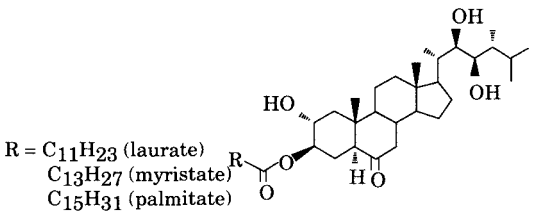
A. Brassinosteroid conjugates formed by esterification at C-3:



3, 24-diepibrassinolide-3β-laurate (3, 24-diepiBL-3β-La)

3, 24-diepibrassinolide-3β-laurate (3, 24-diepiBL-3β-La)

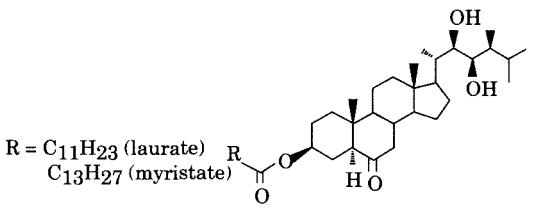
3, 24-diepibrassinolide-3β-palmitate (3, 24-diepiBL-3β-Pa)



3, 24-diepicastasterone-3β-laurate (3, 24-diepiCS-3β-La)

3, 24-diepicastasterone-3β-myristate (3, 24-diepiCS-3β-My)

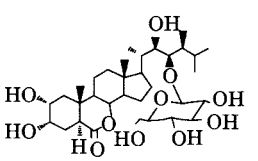
3, 24-diepicastasterone-3β-palmitate (3, 24-diepiCS-3β-Pa)



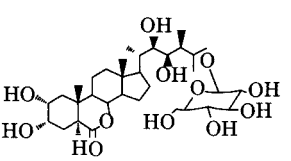
Teasterone-3β-laurate (TE-3β-La)

Teasterone-3β-myristate (TE-3β-My)

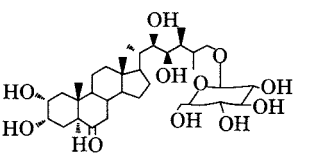
B. Brassinosteroid conjugates formed by glycosylation at C-2, C-3, C-23, C-25 or C-26:



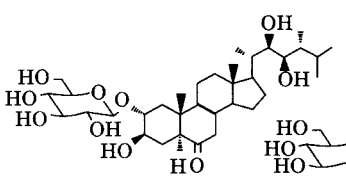
23-*O*-β-D-glucopyranosyloxy-brassinolide



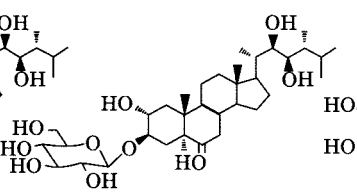
25-β-D-glucopyranosyloxy-24-epibrassinolide



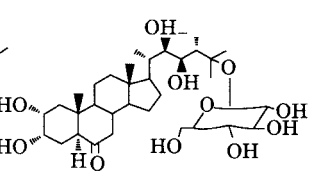
26-β-D-glucopyranosyloxy-24-epibrassinolide



2-*O*-β-D-glucopyranosyl-3, 24-diepicastasterone



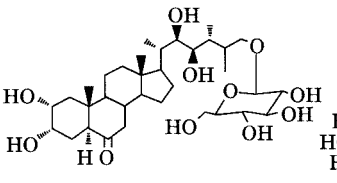
3-*O*-β-D-glucopyranosyl-3, 24-diepicastasterone



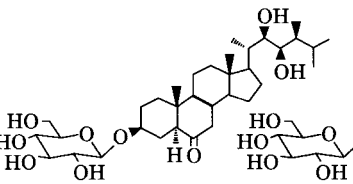
25-β-D-glucopyranosyloxy-24-epicastasterone

Fig 9.11. Brassinosteroid conjugates as metabolites of exogenously applied brassinosteroids to plants

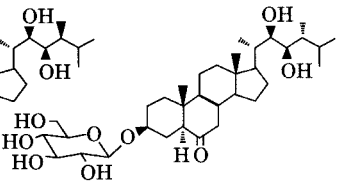
B. Continued



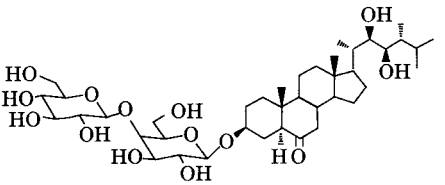
26-β-D-glucopyransyloxy-24-epicastasterone



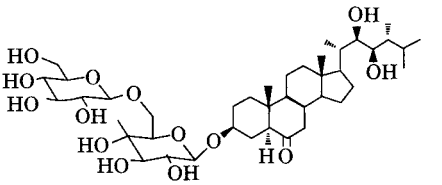
3-*O*-β-D-glucopyranosyl-teasterone



3-*O*-β-D-glucopyranosyl-24-epiteasterone

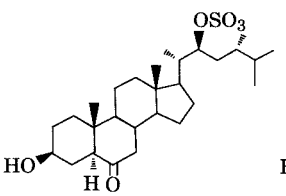


3-*O*-β-D-glucopyranosyl-(1- > 4)-β-D-galactopyranosyl-24-epicastasterone

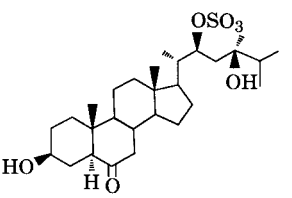


3-*O*-(β-D-glucopyranosyl-(1- > 6)-β-D-glucopyranosyl-24-epiteasterone

C. Brassinotoroid conjugates formed by sulfonation at C-22:



24-epicathasterone-22-sulfate (24-epiCT-22-sulfate)



24-epicathasterone-22-sulfate (24-epiTE-22-sulfate)

Fig 9.11. Brassinosteroid conjugates as metabolites of exogenously applied brassinosteroids to plants–*continued*

Structural changes to the steroidal skeleton of 24-epiCS and 24-epiBL occurred at the C-2 and C-3 positions, including dehydrogenation, hydroxylation, esterification and glycosylation. Furthermore, there are three metabolic reactions occurred to their side-chains, including hydroxylation at the C-20, C-25 and C-26 positions, and cleavage of the C-20/C-22 bond after hydroxylation at the C-20 position, and glycosylation at the C-25 and C-26 positions. In tomato cells, the major metabolites of 24-epiCS were 25-β-D-glucosyloxy- and 26-β-D-glucosyloxy-24-epiCS. On the other hand, 3, 24-diepiCS was converted to a mixture of 3-laurate, 3-myristate and 3-palmitate in serradella cells. This study represented the first report on fatty acid conjugates as metabolites of exogenously applied brassinosteroids. However, the esterification at the 3β-position has not been observed in tomato cells.

Teasterone (TE) and its derivative supplied to plant tissue are converted into several acyl and glucosides metabolites (Abe *et al.,* 1994; Asakawa *et al.,* 1994, 1996; Kolbe *et al.,* 1997; Soeno *et al.,* 2000a, b). TE was converted to a mixture of 3-laurate, 3-myristate and 3-glucoside, which were easily converted to free TE in lily (*Lilium longiflorum*) cell cultures. It indicated that the ester and glycosyl conjugations were a reversible reaction. They may act as a storage or transport component and are hydrolyzed for BL biosynthesis. In the time-course fluctuations of TE and TE-ester during pollen growth, a maximum concentration of the endogenous TE-ester appeared in the immature pollen but that of TE only in the mature stage. It indicates that the accumulated TE-ester must be hydrolyzed to release free TE and then converted into an active brassinosteroid, either CS or BL. That conversion is a part of the brassinosteroid biosynthetic pathway. Furthermore, conversion of 24-epiTE led to formation of a mixture of three glucosides. On the other hand, sulfonation of 24-epiTE and its precursor 24-epicathasterone (24-epiCT) has been shown in *Brassica napus* by steroid sulfotransferase which was expressed by *Escherichia coli* (Rouleau *et al.,* 1999). This enzyme catalyses the sulfonation of brassinosteroids specifically at position C-22. It exhibited the highest affinity for 24-epiCT, followed by 24-epiTE.

Cytokinin Conjugates

Cytokinins are a class of phytohormones that play an important role at all phases of plant development from seed germination to senescence. They act at the cellular level by inducing expression of some genes, promotion mitosis and chloroplast development but also on the organ level by releasing buds from apical dominance or by inhibiting root growth (Riefler *et al.,* 2006).

The naturally occurring cytokinins are N^6-substituted adenine derivatives that contain an isoprenoid or an aromatic derivative side chain (Fig 9.12). Among isoprenoid cytokinins, *trans*-zeatin is considered central due to its general occurrence and high activity in the most bioassays. Its stereoisomer, *cis*-zeatin, is characterized by weak activity in bioassays. However, *cis*-isomers can be dominant cytokinins at particular stages of development in plants such as *Cicer arietinum*, and *Lupinus albus*. Dihydrozeatin and N^6-(Δ^2-isopentenyl)-adenine are also commonly present in lower and vascular plants (Emery *et al.,* 1998, 2000; Sakakibara, 2006).

N^6-Benzyladenine and its derivatives, representing aromatic cytokinins, have been detected in a number of plant species as minor components of the total cytokinins. Hydroxylated derivatives of N^6-benzyladenine in *meta* or *ortho* position of benzyl group are commonly named as *meta*- and *ortho*-topolin, respectively (Strnad *et al.,* 1997). Kinetin, the most known cytokinin, has furfuryl ring at the N^6-position of adenine and was identified in both animal cellular DNA and plant tissue extracts (Barciszewski *et al.,* 2000).

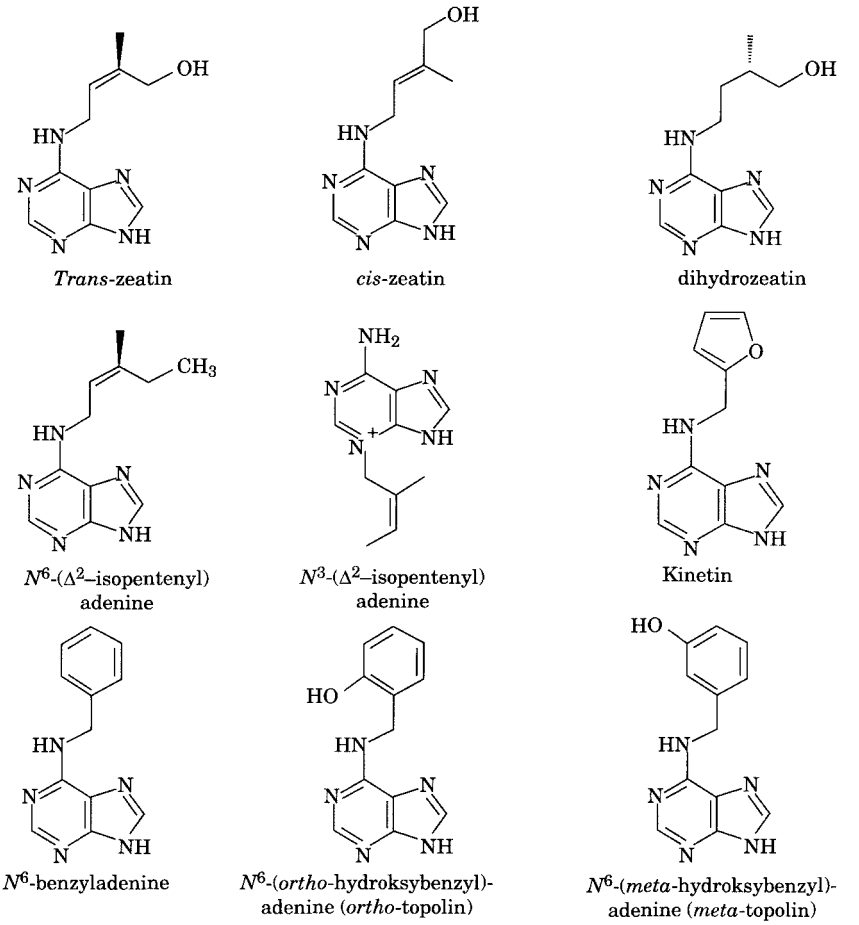


Fig 9.12. Selected chemical structures of naturally occurring cytokinins

Natural cytokinins behave as normal adenylate compounds in that they exist in the plant cell as mixtures of free bases, nucleosides (Fig 9.13), as well as mono-, di-, and trinucleotides in apparent equilibrium. All forms of cytokinins may be reversible or irreversible conjugated with sugars, and amino acids. In most bioassays, cytokinin bases are the most active, and therefore cytokinin conjugation contributes to regulation their activity. Cytokinin conjugates seem to serve as storage, transport, and deactivated forms because they are resistant to degradation by cytokinin oxidase/ dehydrogenase (Auer, 2002; Blagoeva *et al.*, 2004).

Cytokinin conjugate formation can be divided into two major categories: conjugation with the adenine moiety or the side chain. The most common modifications of the adenine molecule are *N*-glucosylation, and N^9-alanine conjugation. The adenine ring system can be glucosylated at the N^3-, N^7- and N^9-position (Fig 9.14). Glucosyl conjugates at N^7- and N^9-position

but not at the N^3-position of cytokinins such as *trans*-zeatin, dihydrozeatin as well as N^6-(Δ^2-isopentenyl)-adenine are usually inactive in the most of bioassays because their active free forms can't be released by hydrolysis. The relatively high activity of 3-β-glucosyldihydrozeatin in bioassay most probably reflects the presence of specific enzymes in the plant system studied which can convert its back to free active base. Additionally, *N*-glucosylated cytokinins may accumulate to even higher levels than the free bases under normal circumstances as well as stress conditions. However, the precise *in vivo* function of these cytokinin metabolites remains still unknown (Mok & Mok, 2001; Veach *et al.*, 2003).

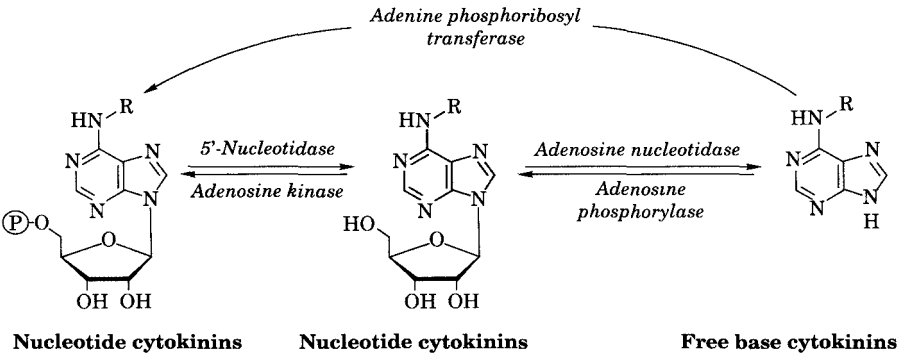


Fig 9.13. Possible interconversion of cytokinin bases, nucleosides, and nucleotides

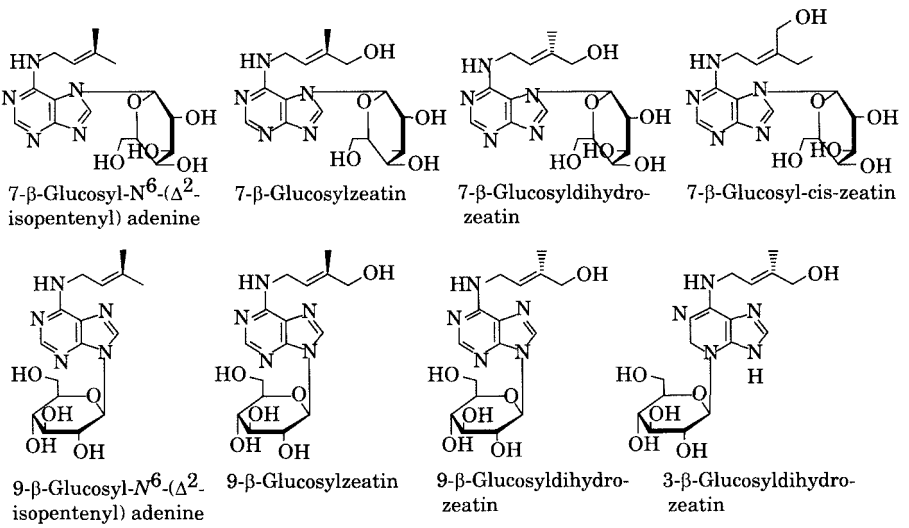
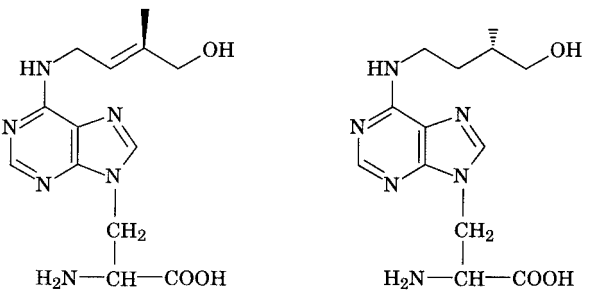


Fig 9.14. *N*-glucosylated conjugates of cytokinins

The formation of cytokinin glucosides at the N^7- and N^9-position is catalyzed in *Arabidopsis* by two specific glucosyltransferases (UGT76C1 and UGT76C2). However, both mentioned enzymes prefer glucosylation at

N^7- to that N^9-, which corresponds well with higher concentrations of various N^7-glucosylated forms of cytokinins in *Arabidopsis* plastids (Sakakibara, 2006). Although the enzymes recognize a large number of adenine derivatives as substrates, the rate of *N*-glucosylation is higher for compounds with N^6-side chains of at least three alkyl carbons and roughly correlates with cytokinin activity. Both, UDP-Glc and TDP-Glc can serve as glucosyl donors for formation of *N*-glucosylated cytokinins (Brzobochaty *et al.,* 1993).

Less common is alanine conjugation at N^9-position of adenine moiety (Fig 9.15). 9-Alanylzeatin and 9-alanyldihydrozeatin, commonly known as lupininc acid and dihydrolupininc acid, respectively, were identified in lupin (*Lupinus angustifolius*) seeds. A specific transferase which catalyses the conversion of *trans*-zeatin, and dihydrozeatin to their N^9-alanyl derivatives was discovered and purified from lupin seeds. The donor substrate is *O*-acetyl-*L*-serine. Alanine conjugates of isoprenoid cytokinins are characterized by low activity in bioassay because the lack of enzyme systems responsible for hydrolysis to active forms (Entsch *et al.,* 1983; Mok & Mok, 2001).

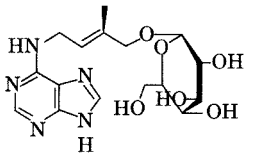


9-Alanylzeatin 9-Alanyldihydrozeatin

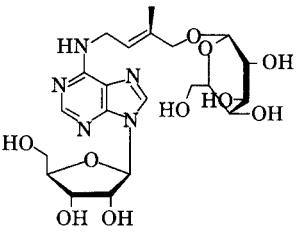
Fig 9.15. Amino acid (alanine) conjugates of cytokinins

Conjugation of cytokinins involves also *O*-glycosylation, and *O*-acetylation at the hydroxyl group of the side chains of cytokinins (Fig 9.16) (Martin *et al.,* 1999). The *O*-glycosylation of these phytohormones was extensively studied in different plant systems. *O*-glucosyl conjugates of isoprenoid and aromatic cytokinins are commonly present metabolites in various plants, although, the presence of *O*-xylosyl conjugates has been only observed in *Phaseolus* species (Martin *et al.,* 2000). Both, *O*-glucosylated and *O*-xylosylated cytokinins are considered important for storage, transport, and protection against degrading enzymes. Additionally, these metabolites can be easily converted into active cytokinin by specific β-glucosidases (Brzobochaty *et al.,* 1993). Thus, it is believed that *O*-glucosides and *O*-xylosides of these phytohormones play an important role in cytokinin homeostasis. Moreover, *O*-glycosylation of cytokinins may

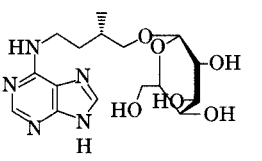
O-Glucosylated cytokinins



O-β-Glucosylzeatin



O-β-Glucosyl-9-ribosylzeatin



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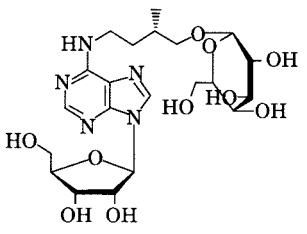
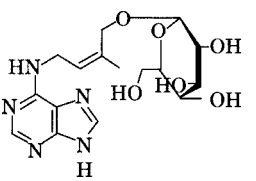
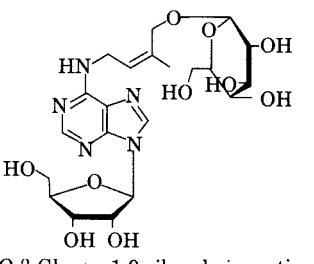


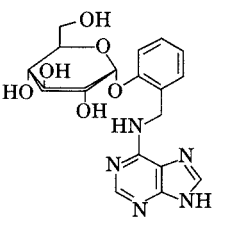
O-β-Glucosyl-9-ribosyldihydrozeatin



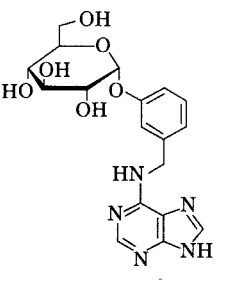
O-β-Glucosyl-*cis*-zeatin



O-β-Glucosyl-9-ribosyl-*cis*-zeatin

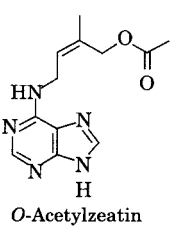


O-β-Glucosyl-N^6-(ortho-hydroksybenzyl)-adenine
(*O*-β-Glucosyl-*ortho*-toplin)



O-β-Glucosyl-N^6-(meta-hydroksybenzyl)-adenine
(*O*-β-Glucosyl-*meta*-toplin)

O-Acetylated cytokinin



O-Acetylzeatin

Fig 9.16. *O*-Glucosyl and *O*-acetyl conjugates of cytokinins

modify the activity of cytokinins. For instance, *O*-β-glucosylzeatin, and *O*-β-xylosylzeatin effectively stimulated callus growth in bean (*Phaseolus vulgaris*) and Lima bean (*Phaseolus lunatus*). These *O*-glycosides are relatively stable in plant tissues but they can be easily converted back to

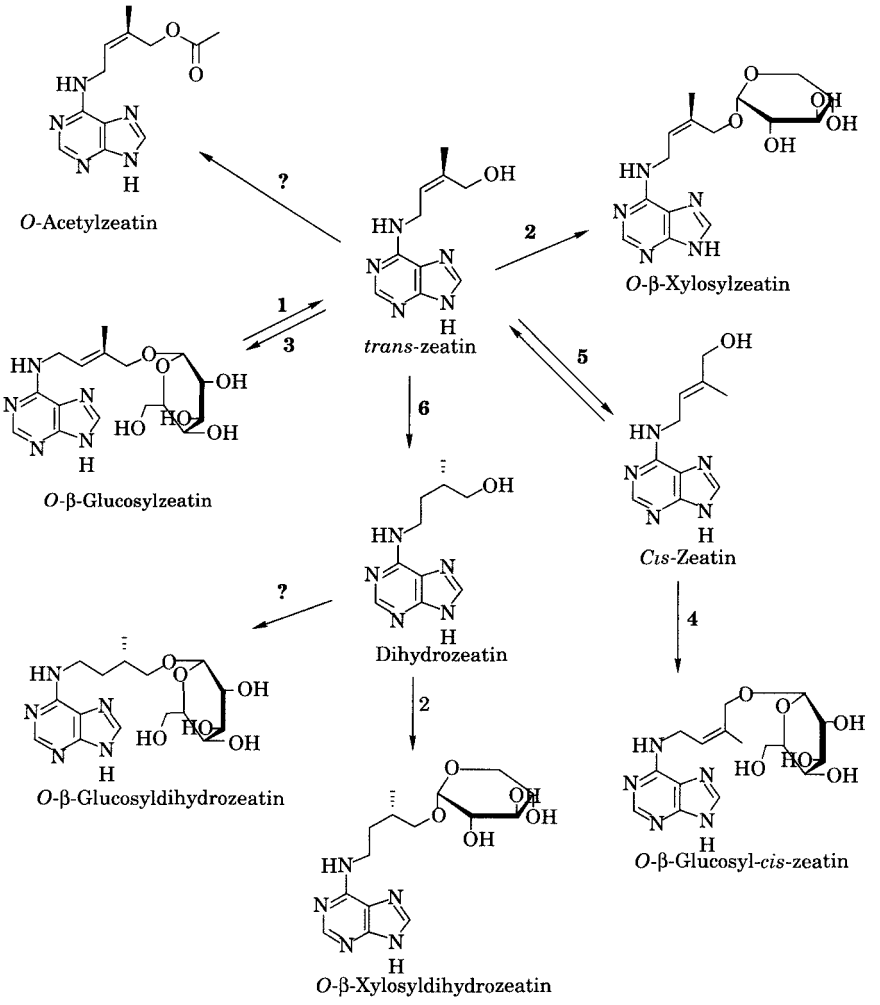
active *trans*-zeatin. The level of sugar derivatives shows also large fluctuations during plant development. For example, *O*-β-glucosylzeatin was the predominant cytokinin in fully developed leaves of *Urtica dioica*, whereas the dominance of free *trans*-zeatin was observed in young leaves (Wagner & Beck, 1992). The fact that *O*-glycosides may be need for stored cytokinins would mean that *trans*-zeatin biosynthesis does not necessarily coincide with the cytokinin requirement of the plant tissue. These conjugates are mainly localized in plant vacuoles, however the existence of mechanism controlling their transport across membranes when the need for cytokinin increases, is still unknown (Mok *et al.*, 1992). Moreover, it has been reported that the ratio of cytokinin conjugation process with glucose and xylose increases under the influence of stress conditions, for example in the presence of high concentrations of heavy metals such as Pb, Cu, Zn and Al as well as heat stress (Atanasova *et al.*, 2004; Wang *et al.*, 2004). In previous reports for tobacco callus tissue, levels of *O*-glucosides were also found to be markedly increased upon expression or de-repression of *ipt* gene encoding a key enzyme in cytokinin biosynthetic pathway (Redig *et al.*, 1996).

The transfer of glycosyl moiety from an activated glycosyl donor to hydroxyl group in the side chain of cytokinin can be mediated by specific glycosyltransferase enzymes (Fig 9.17) (Auer, 2002). The cytokinin glycosyltransferases are UDP-Glc- or UDP-Xyl-requiring enzymes and belong to family 1 of the 68 families of various glycosyltransferases. These enzymes are localized manly in plant vacuoles. Zeatin *O*-glucosyltransferase has been isolated from Lima bean (*Phaseolus lunatus*), soybean (*Gycine max*), rice (*Oryza sativa*), and tomato (*Lycopericon esculentum*), whereas zeatin *O*-xylosyltransferase was identified and purified from bean (*Phaseolus vulgaris*). The zeatin *O*-glucosyltransferase uses UDP-Glc and UDP-Xyl as donor substrates but has much higher affinity to UDP-Glc, whereas the zeatin *O*-xylosyltransferase exclusively utilizes UDP-Xyl (Mok & Mok, 2001; Meek *et al.*, 2008).

The cytokinin substrate recognition is also highly specific because only *trans*-zeatin, dihydrozeatin, and their respective nucleosides are *O*-glucosylated and *O*-xylosylated (Fig 9.17). The stringent substrate specificity for cytokinins and sugar donor suggests that conjugation process with glucose and xylose is precisely regulated during plant development. Moreover, the ribose moiety of cytokinin nucleosides can also be *O*-glucosylated, forming *O*-β-glucosyl-9-ribosylzeatin, and *O*-β-glucosyl-9-ribosyldihydrozeatin (Martin *et al.*, 1999).

The *O*-glucosylation of cytokinins is stereo-specific. The *O*-glucosyltransferase encoded by the *Phaseolus lunatus ZOG1* gene has high affinity for *trans*-zeatin as the substrate, whereas the enzyme encoded by the maize (*Zea mays*) gene prefers *cis*-zeatin (Veach *et al.*, 2003). The *cis*-zeatin-*O*-glucosyltransferase isolated from maize root specifically glucosylates *cis*-zeatin, and 9-ribosyl-*cis*-zeatin but do not recognize as a

substrate *trans*-zeatin and dihydrozeatin. Therefore, it could be assumed that *cis*-zeatin may have a greater importance in plant growth and development than previously believed (Martin *et al.*, 2001).



Enzymes:
1 Zeatin *O*-glucosyltransferase
2 Zeatin *O*-xylosyltransferase
3 β-glucosidase
4 *cis*-zeatin-*O*-glucosyltransferase
5 Zeatin *cis-trans* isomerase
6 Zeatin reductase
? Conversion occurs, enzyme unknown

Fig 9.17. The conjugation and deconjugation of the cytokinin N^6-isoprenoid side chain

Additionally, it was found that hydroxylated derivatives of N^6-benzyladenine (topolins) are also *O*-glucosylated (Fig 9.16). Structurally different isomers of topolins are recognized by distinct plant *O*-glucosyltransferases. Therefore, *meta*-topolin is the preferred substrate of zeatin *O*-glucosyltransferase, whereas *cis*-zeatin-*O*-glucosyltransferase recognizes *ortho*-topolin as the substrate. The *O*-glucosides of *meta*- and *ortho*-topolins are characterized by high activity in the *Phaseolus lunatus* callus bioassay. Probably, they can be easily converted back to active topolins. However, *para*-topolin cannot be metabolized by *O*-glucosyltransferases, and doesn't possess ability to promote growth of *Phaseolus lunatus* (Strnad *et al.*, 1997; Mok *et al.*, 2005).

Deconjugation process of *O*-glucosylated cytokinins involves the conversion of glucosides and xylosides to the correspondent active aglycones. This reaction is catalyzed by various substrate-specific β-glucosidase enzymes. Experiments have been shown that β-glucosidases utilize a broad spectrum of substrates, not only cytokinin conjugate. At this time, the only reported β-glucosidase from maize seedling leaves and root cells can hydrolyze endogenous cytokinin conjugates with sugars as well as number of other artificial and natural compounds (Vévodová *et al.*, 2001). For example, the β-glucosidase isolated from maize and encoded by *Zm-p60.1* gene cleaved the biologically inactive hormone conjugates such as *O*-β-glucosylzeatin and 3-β-glucosylkinetin, releasing active cytokinins. Tobacco protoplasts that transiently expressed *Zm-p60.1* gene could use the inactive cytokinin glucosides to initiate cell division. Product of *Zm-p60.1* gene was localized to the meristematic maize cells and may function *in vivo* to supply the developing embryo with active forms of cytokinins (Brzobochaty *et al.*, 1993). The low specificity of β-glucosidases to substrates suggests that the hydrolysis of cytokinin conjugates is not highly regulated, in contrast to the precise control of *O*-glycoside synthesis (Mok & Mok, 2001).

In addition to *O*-glucosylated cytokinins, the *O*-acetylation of side chain of cytokinins is less common in plants (Fig 9.16). For example, the *O*-acetylation of *trans*-zeatin have been observed in a few lupin species. This process does not reduce drastically stimulating properties of cytokinins. Probably, the ability of *O*-acetylzeatin to promote the growth and development of *Phaseolus lunatus* may be derived from the conversion this conjugate to free *trans*-zeatin. Although, *O*-acetylzeatin was identified as naturally occurring metabolite, its significance and synthesis is still unknown (Letham & Zhang, 1989).

In various lower and vascular plant species most cytokinins are thought to be present in both, free and as conjugated forms. Considerable efforts has been directed at clarifying the conjugation and deconjugation as well as factors contributing to cytokinin homeostasis, but the entire picture remains to be elucidated.

ETHYLENE PRECURSOR CONJUGATE

Ethylene is a small, readily diffusible hormone that has an important role integrating developmental events with external stimuli. It plays an active role in seed germination, tissue differentiation, the formation of root and shoot primordia, root elongation, lateral bud development, flowering initiation, anthocyanin synthesis, flower opening and senescence, pollination, fruit ripening and degreening, the production of volatile organic compounds responsible for aroma formation in fruits, storage product hydrolysis, leaf and fruit abscission. It is also an important stress hormone. Adverse biotic or abiotic stimuli usually lead to ethylene synthesis. Ethylene, in turn, slows down plant growth until the stress is removed (Arshad & Frakenberger Jr., 2002).

Ethylene is not known to form conjugates. In higher plants, ethylene biosynthesis follows the path: methionine → *S*-adenosylmethionine → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene. The final step is catalyzed by the enzyme ACC oxidase. ACC, the direct precursor of ethylene in higher plants (Adams & Yang, 1979; Yang & Hoffman, 1984; Yang, 1987) can be regulated by the respective rate of synthesis and conversion to ethylene as well as by conjugation to *N*-malonyl-ACC (Fig 9.18) (Yang & Hoffman, 1984), which was identified as a major metabolite of ACC in higher plants (Amrhein *et al.* 1981). ACC is conjugated to form *N*-malonyl-ACC. They are synthesized in the cytosol and then *N*-malonyl-ACC is transported into the vacuole by an ATP-dependent transtonoplastic carrier (Amrhein *et al.*, 1987; Pedreño *et al.*, 1991). In some tissues, such as pre-climacteric apples, more than 40% of the ACC synthesized in the skin and 5% in the flesh are diverted to *N*-malonyl-ACC (Lilièvre *et al.*, 1997). Feeding excised wheat leaves with labeled ACC and *N*-malonyl-ACC indicated that the

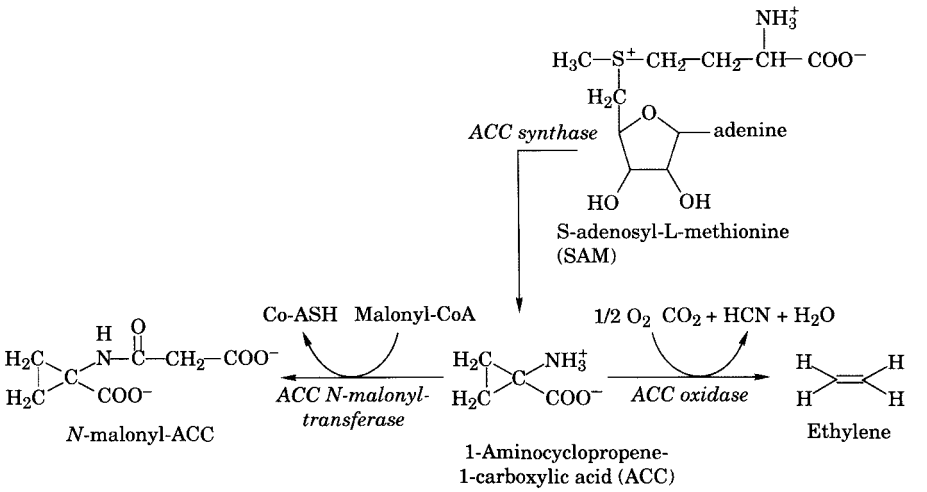


Fig 9.18. *N*-malonyl-ACC as a conjugate of ethylene precursor 1-aminocyclo-propane-1-carboxylic acid (ACC)

conjugation was essentially irreversible (Hoffman *et al.,* 1983). Under normal conditions *N*-malonyl-ACC is not converted back to ACC. However, under high concentrations in the plant tissue *N*-malonyl-ACC can be converted to ACC by inducible *N*-malonyl-ACC-hydrolase activity (Jiao *et al.,* 1986; Bergner *et al.,* 1987). Therefore, the rate of ethylene production could be affected by at least three reactions: the conversion of *S*-adenosylmethionine to ACC by ACC synthase, ACC to ethylene by ACC oxidase and ACC to *N*-malonyl-ACC by *N*-ACC-malonyltransferase. Ethylene, on the other hand, can regulate the activity of ACC oxidase, ACC synthase and ACC-malonyltransferase (Adams & Yang, 1979; Yang & Hoffman, 1984; Yang, 1987).

GIBBERELLIN CONJUGATES

Gibberellins are phytohormones extensively involved in flowering, stem elongation, leaf expansion, seed development, and germination. This large group of phytohormones, with 126 different compounds from GA_1 to GA_{126}, commonly occurs in plants, fungi as well as bacteria. Chemically, all known gibberellins are diterpenoid acids that are synthesized by the terpenoid pathway in plastids, and then modified in the endoplasmic reticulum and cytosol until they reach biologically active forms. All gibberellins are derived from the *ent*-gibberellane skeleton, but are synthesized *via ent*-kaurene (Sakamoto *et al.,* 2004).

Roughly 30% of all known gibberellins are biologically active. All higher plants contain presumably at least one, but usually several active, and inactive gibberellins that exist in different concentrations depending on the respective tissue. GA_1, GA_3, GA_4, and GA_7 are characterized by the most stimulating properties in higher plants (Hedden & Phillips, 2000). Many of other gibberellins are biosynthetic intermediates or catabolites of bioactive gibberellins. The amount of biologically active gibberellins in plant tissues can be affected by the rate of their synthesis, and oxidative degradation as well as the reversible or irreversible conjugation (Olszewski *et al.,* 2002).

The biosynthesis of gibberellin conjugates has long been recognized in various gymnospermous and angiospermous plant species (Nadeau & Rapaport, 1974; Moritz *et al.,* 1990). Their natural occurrence indicates on the important function in plant growth and development. It has repeatedly been suggested that conjugation followed by release *via* chemical or enzymatic hydrolysis could provide means of storing and regulating levels of the biologically active forms of these phytohormones. However, the conjugation of gibberellins to inactive metabolites may be a key step in the process of further catabolism. Because of their polar properties, they have been also considered to serve for the transport and compartmentation within the cell, and for the long distance transport throughout the whole plant. On the other hand, low activity of gibberellin conjugates in bioassays

suggests that these metabolites could be the waste products which are deposited in vacuoles. It has also been proposed that the presence of polar glucosyl moiety in conjugates causes a distorted orientation of gibberellin molecule within the membrane, prohibiting binding to specific receptor. First of all, the conjugation, and deconjugation of these phytohormones regulates the pool of endogenous gibberellins during plant growth and development (Schneider & Schliemann, 1994; Senns *et al.*, 1998).

The most commonly occurred conjugates of gibberellins in plants are glucosyl derivatives. These conjugates can be divided into two groups. In the first class, a hydroxyl group of the gibberellin skeleton is linked to glucose and they are known as glucosyl ethers, or glucosides (Fig 9.19).

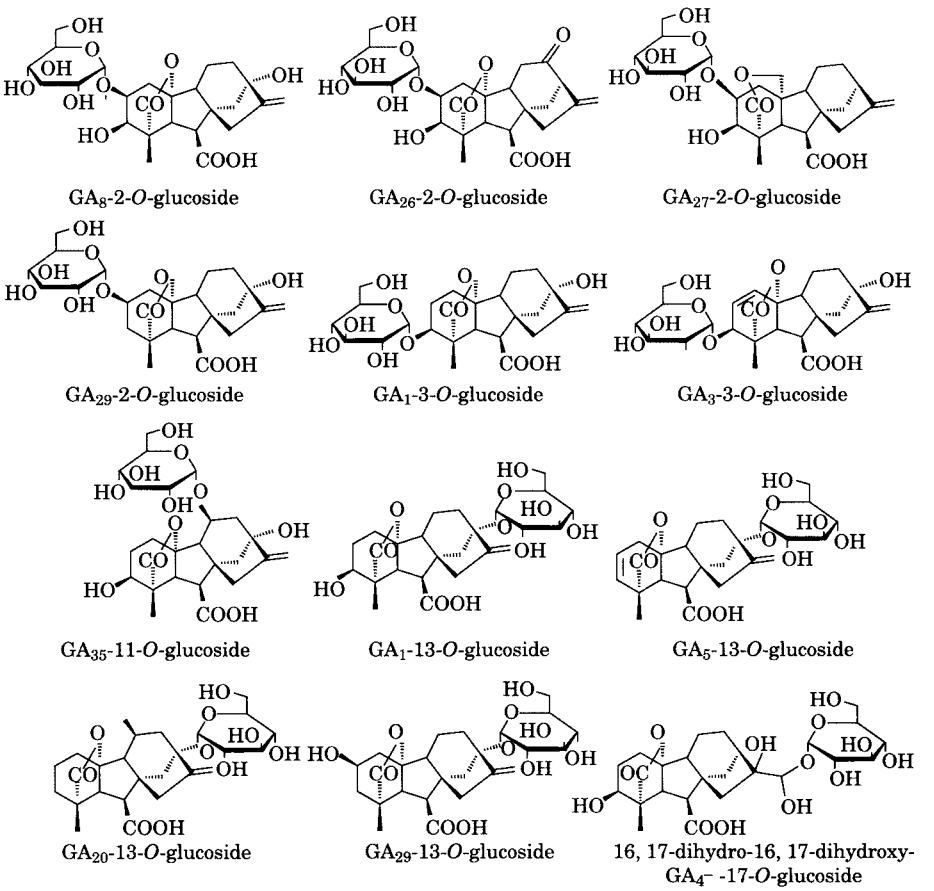


Fig 9.19. The structures of gibberellin glucosides (glucosyl ether type)

The glucosyl moiety can be linked either to 2-*O*-, 3-*O*-, 11-*O*-, 13-*O*- or the 17-*O*-position of the parent gibberellin molecule, generating a range of isometric forms. Glucosides such as GA_1 (-3-*O*-), GA_3 (-3-*O*-), GA_8 (-2-*O*-),

GA_{20} (-13-*O*-), GA_{26} (-2-*O*-), GA_{27} (-2-*O*-), GA_{29} (-2-*O*-), GA_{29} (-13-*O*-), and GA_{35} (-11-*O*-) (Fig 9.19) were identified physicochemically from several plant families (Koshioka *et al.*, 1983; Schneider *et al.*, 1992), for example, GA_1-3-*O*-glucoside is present in seedlings of maize (*Zea mays*), mature seeds and immature fruits of runner bean (*Phaseolus coccineus*), and mature caryopses of barley (*Hordeum vulgaris*) (Schliemann *et al.*, 1993). GA_8-2-*O*-glucoside was identified in milk-ripe ears, and mature caryopses of barley (*Hordeum vulgare*), immature seed of Japanese morning glory (*Pharbitis nil*), seedlings of pea (*Pisum sativum*), and maize, as well as immature pods, immature fruits, and mature seeds of runner bean (Hiraga *et al.*, 1974; Rivier *et al.*, 1981; Senns *et al.*, 1998). Barley, pea, wheat (*Triticum aestivum*), and maize were found to contain GA_{20}-13-*O*-glucoside and GA_{29}-2-*O*-glucoside in high concentrations (Schneider & Schliemann, 1994). These conjugates do not appear to be active in bioassays *per se*; rather their role is proposed to be as reserve, transport or entry to catabolite forms (Schneider & Schliemann, 1994; Olszewski *et al.*, 2002).

The second group of glucosyl conjugates is represented by glucosyl esters, where glucosyl moiety is linked to the 7-carboxyl group of different gibberellin compounds (Fig 9.20). Ester types of gibberellin conjugates are ubiquitously distributed in Leguminaceae family. For instance, GA_1, GA_4, GA_{37}, and GA_{38} glucosyl esters are found in high amounts in immature, and mature seeds as well as seedlings of bean (Hiraga *et al.*, 1974). Glucosyl esters were also identified and purified from gymnospermous plants such as needles, and shoots of Sitka spruce (*Picea sitchensis*), and Lodgepole pine (*Pinus contorta*) (Moritz *et al.*, 1990).

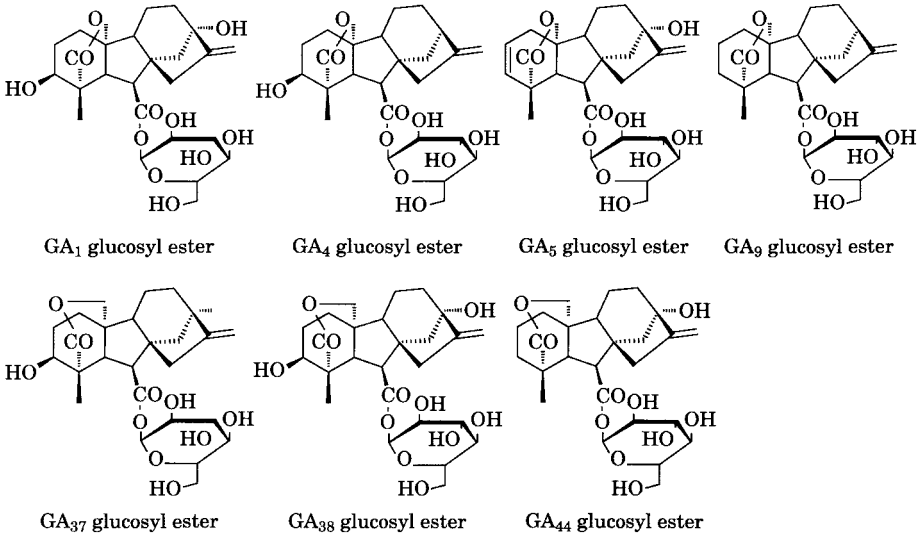


Fig 9.20. The structures of glucosyl esters of gibberellins (glucosyl ester type)

The biosynthesis of glucosyl conjugates with gibberellins was extensively studied in Leguminaceae family. High glucosylating activity was observed in maturing fruits of runner bean. It was found that the specific glucosyltransferase, identified from this plant tissue, utilizes preferably UDP-glucose as glucose donor. Additionally, the enzyme accepts GA_3, and has much lower affinity to glucosylation of GA_7, and GA_{30}, forming exclusively the GA_3-3-*O*-glucoside (Fig 9.19). However, the physiological significance of GA_3 conjugation with glucose is unknown, because this metabolite doesn't occur naturally in this plant tissue (Knöfel *et al.*, 1984). Additionally, a cytosolic enzyme fraction from cells of suspension culture of Peruvian tomato (*Lycopersicon peruvianum*) was tested for the process of glucosylation of various gibberellins in the presence of UDP-glucose. It was observed, that the specific glucosyltransferase conjugates preferably GA_7 and GA_9 to the corresponding glucosyl esters (Sembdner *et al.*, 1985).

Glucosyl conjugates are preferentially formed during seed maturation and often are present in relatively high levels in maturing seeds (Hiraga *et al.*, 1974). For example, in immature bean seeds, glucosylation of exogenously applied radiolabeled [^{3}H] GA_1 doesn't take place early; rather it occurs as seeds approach maturation. Further, upon germination of seeds, a slight decrease in radioactivity associated with [^{3}H]GA_1 glucosyl ester was observed, indicating that the reversible conjugation may have been occurring. During the germination, part of gibberellin conjugate pool was apparently released, and significant increase in the free GA_1 level, was noted (Yamane *et al.*, 1977). Therefore, gibberellin conjugates could be potential candidates to provide free gibberellins during the early stage of bean germination by their hydrolysis. Additionally, changes of the pool size of GA_{20}-13-*O*-glucoside and GA_8-2-*O*-glucoside was also observed in developing barley caryopses (Senns *et al.*, 1998). The level of GA_{20} and GA_8 glucosides was found to reach maximum at the beginning of ripeness and then declined until full ripeness. In mature barley caryopses the content of these glucosyl conjugates was three to four times higher than free forms of gibberellins. The pool of GA_{20}-13-*O*-glucoside and GA_8-2-*O*-glucoside increased dramatically during the final stages of ripening, indicating on the intensive metabolism of GA_{20}, and GA_8 during seed development (Senns *et al.*, 1998). Some indications are also reported for the conversion of gibberellin conjugates during maize germination. For example, after feeding radioactive GA_{20} to maturing maize cobs, metabolically formed glucosyl conjugates, was detected. The second step involved hydrolysis of the glucoside to release free GA_{20} moiety in germinating maize caryopses (Rood *et al.*, 1983; Schliemann *et al.*, 1993).

At least 18 glucosyl conjugates of gibberellins have been tested for bioactivity using a variety of bioassay systems. In general, gibberellin glucosides do not possess biological activity in plants, whereas glucosyl

esters show a range of bioactivity, depending on the gibberellin aglycone, and the assay system. For instance, the [^{3}H]glucosyl esters of GA_1, GA_3, and GA_4 are characterized by physiological activity in bean bioassay (Hiraga *et al.*, 1974). Probably, the bioactivity of gibberellin conjugates may be due to the free aglycone coming from the enzymatic hydrolysis of the conjugate molecule (Schneider & Schliemann, 1994). It was also found, that GA_8-2-*O*-glucoside represents similar activity compared with free GA_8 in several bioassay systems (Japanese morning glory, rice, maize, pea, cucumber), whereas GA_3-3-*O*-glucoside doesn't appear to stimulate plant growth (Fig 9.19) (Yokota *et al.*, 1971). This result can be supported by observation, where GA_8-2-*O*-glucoside was a 200 times faster hydrolyzed by a β-glucosidase isolated from dwarf rice (*Oryza sativa*) than GA_3-3-*O*-glucoside. Therefore deconjugation *via* hydrolysis may provide active forms of gibberellins (Schliemann, 1984). The specific β-glucosidase exhibiting a high hydrolyzing activity toward the endogenous GA_8-2-*O*-glucoside was also identified in maturing pods of runner bean. Moreover, the significant decrease in activity of this enzyme was observed in later stages of pod development which correlated well with the high increase in the gibberellin glucosylation in the same tissue. Probably, this enzyme may catalyze an important step of GA_8 glucose conjugate catabolism (Schliemann, 1988).

The interconversion between two types glucosyl conjugates, and free forms of gibberellins was demonstrated during feeding experiments in maize seedlings. The double labeled [6-^{2}H]glucosyl ester of [17-^{13}C,^{3}H]GA_{20} was applied to shoots of maize seedlings (Fig 9.21). The plant material was extracted 24 h later to identify the radioactive metabolites. The seedlings were shown to contain the ^{13}C labeled gibberellin metabolites such as GA_{20}, GA_{29}, GA_1, and GA_8 as well as three glucosides: GA_{20}-13-*O*-glucoside, GA_{29}-2-*O*-glucoside, and GA_8-2-*O*-glucoside (Fig 9.21). The first detected step of the metabolism of the exogenously applied [6-^{2}H]glucosyl ester of [17-^{13}C,^{3}H]GA_{20} is the endogenous hydrolysis at the ester linkage of this conjugate to GA_{20}. Then, GA_{20} may by hydroxylated to GA_{29} or chemically converted to GA_1, and GA_8. The second step involves formation of the gibberellin glucosides in reaction of reconjugation. The ^{2}H label present in the glucosyl moiety of the exogenous substrate was not detected in the synthesized conjugates, indicating that the detected GA-*O*-glucosides did not arise through direct transfer of the glucosyl moiety from the introduced radiolabeled molecule. This observation indicates on the rapid exchange between pools of free, and glucosyl conjugates (Schneider *et al.*, 1992; Schneider & Schliemann, 1994).

In contrast to our knowledge of the occurrence of gibberellin conjugates in various plant species, and conversion during feeding experiments, little is known about their function, biosynthesis and degradation *in vivo*, as well as factors involving in the gibberellin homeostasis.

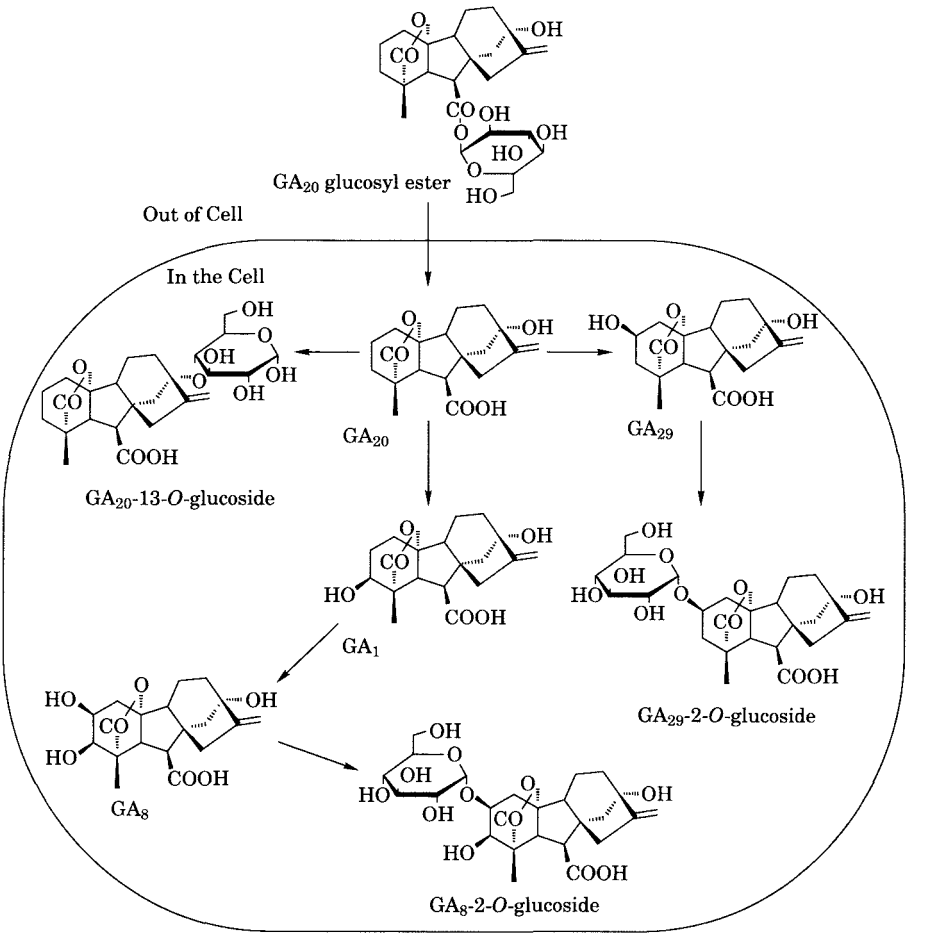


Fig 9.21. Conjugation and reconjugation of exogenously applied GA_{20} glucosyl ester to maize seedlings

JASMONATE CONJUGATES

The group of jasmonates, which are cyclopentanone derivatives, are widespread in the plant kingdom. (–)-Jasmonic acid (JA), its stereoisomer (+)-7-iso-jasmonic acid, (–)-9,10-dihydrojasmonic acid, 12-oxo-phytodienoic acid (the biogenetic precursor of JA), hydroxylated derivatives of JA (*i.e.* tuberonic acid, cucurbic acid) as well as methyl ester of jasmonic acid (MeJA) are the lead molecules of the whole jasmonates family (Fig 9.22). The structural positions essential for the biological activity of jasmonates are a planar pentanone ring, the pentenyl chain inserted at C-7, an acetyl and longer side chain at C-3, and the keto group at C-6 (Creelman & Mullet, 1997).

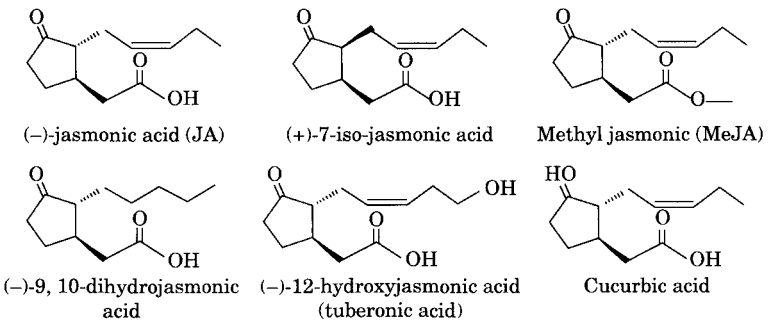


Fig 9.22. Structures of jasmonates

Jasmonates are inducers of variety of physiological processes such as seed germination, pollen development, ethylene synthesis, senescence, and tuber formation (Sembdner & Parthier, 1993). JA and MeJA are regarded as signaling substances responsible for the activation of signal transduction pathway in response to different kinds of biotic and abiotic stress. Moreover, both JA and MeJA accumulation as consequence of wounding, elicitation and mechanical stimulation has been observed in many plant tissues (Preston *et al.,* 2001; Cheong & Choi, 2003).

Jasmonates are detected throughout the plant, with highest concentrations in growing tissues such as shoot apex, root tips, immature fruits, and young leaves. Furthermore, these phytohormones were shown to occur conjugated with a variety of amino acids, as well as methyl, glucosyl, and gentobiosyl esters (Wasternack & Parthier, 1997). Although, the biological activity has been attributed to the free forms of jasmonates, the glucosylation and amino acid conjugation process is important for hormone homeostasis. Moreover, conjugates of JA with amino acids such as Ile, Leu, Val, Ala, Tyr, and Phe (Fig 9.23), may play essential role in the signal transduction pathway of a jasmonates-responsive event (Schaller *et al.,* 2005).

JA conjugates with amino acids like Tyr, Trp, and Phe occurred in flowers of the broad bean plant. JA conjugates with Ile, Leu, and Val were isolated as endogenous compounds from fruits and young leaves of *Vicia faba* and pollen of *Pinus mugo* (Brückner *et al.,* 1986, 1988; Knöfel & Sembdner, 1995). Interestingly, the level of JA amino acids conjugates was also found to increase in response to environmental stress. In wounded potato leaves, they represent up to 15% of all jasmonate compounds, and in osmotically stressed barley JA conjugated with Val, Leu or Ile accumulated simultaneously (Krammel *et al.,* 1997). Less common is an inactive conjugate of JA with tyramine, a biogenic amine formed by decarboxylation of tyrosine, which has been isolated from pollen of *Petunia* sp. (Krammel *et al.,* 1999; Miersch, 1999).

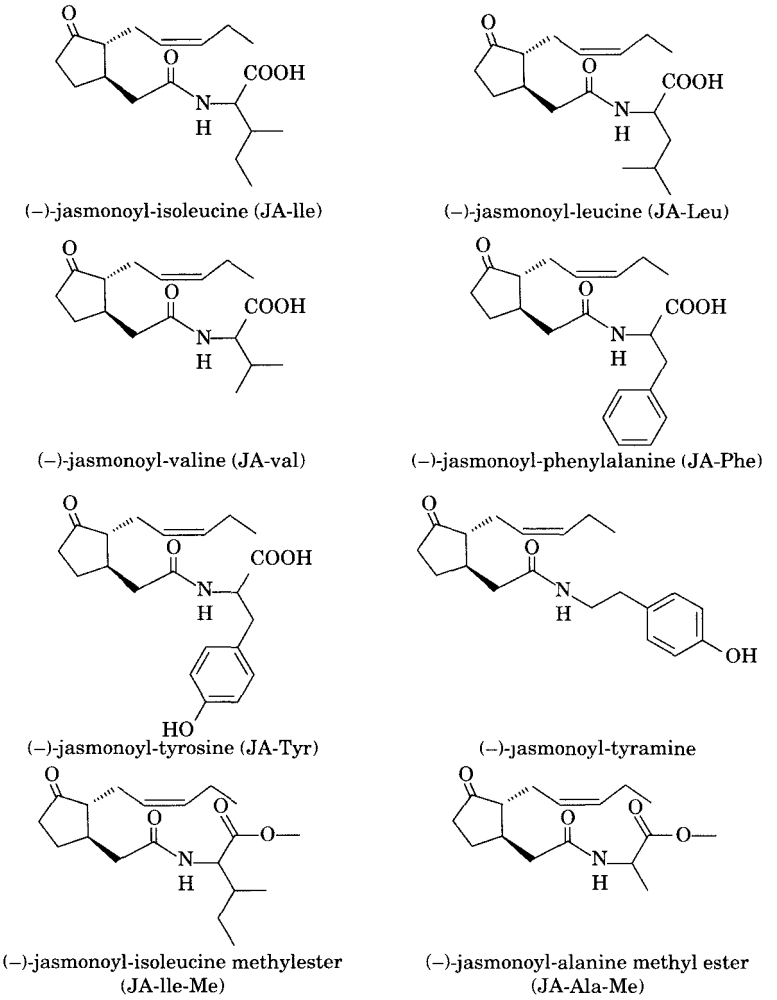


Fig 9.23. The conjugates of JA with amino acids and their derivatives

Amino acid conjugation is observed at C-1 of jasmonates and occurs in a two step reaction that utilizes ATP and Mg^{2+}. These enzymatic reactions in *Arabidopsis* are catalyzed by jasmonate:amino acid synthetase (JAR1). The hormone conjugating Mg-ATP-dependent enzyme catalyzes the activation of jasmonates and then transfers the acyl moiety onto the α-amino group of amino acid, *e.g.* isoleucine (Ile) (Fig 9.24) (Staswick & Tiryaki, 2004).

JAR1 + jasmonate + ATP ⇌ JAR1:jasmonoyl~pA + PPi

JAR1:jasmonoyl~pA + Ile → jasmonoyl-Ile + AMP + JAR1

Fig 9.24. Synthesis of jasmonate conjugate with amino acid by jasmonate:amino acid synthetase (JAR1)

JAR1 belongs to the acyl~adenylate-forming firefly luciferase superfamily and shows a strong preference for conjugating JA but it is also capable for synthesizing Ile conjugate of certain other jasmonates such as (+)-7-iso-jasmonic acid, (–)-9,10-dihydrojasmonic acid, and (–)-12-hydroxyjasmonic acid (tuberonic acid) (Guranowski *et al.,* 2007). On the other hand, methyl jasmonate (MeJA) is not a substrate for JAR1 because this enzyme requires the presence of free carboxyl group in the molecule. The importance of this enzyme for jasmonates metabolism and signaling confirms observation where mutation at *Arabidopsis JAR1* gene reduces plant sensitivity to JA, leads to male fertility, and limits systemic resistance pathways that protects plant against various pathogens. The mutant is insensitive to jasmonates and does not show root growth inhibition or vegetative storage protein induction in response to these phytohormones (Staswick *et al.,* 2002).

JA-Ile is the predominant amino acid conjugate in plants. Moreover, a systematic study with conjugates of JA with L- and D-amino acids demonstrated that conjugates of this phytohormone with L-amino acids are characterized by high biological activity in plants, whereas the conjugates with D-isomers are completely inactive. For example, conjugates of JA with L-amino acids are able to induce wound-responsive genes in tomato leaves and phytoalexin synthesis (Krammel *et al.,* 1997). Systematic permutation of the amino acid moiety in these conjugates showed that JA-Ile is the most effective in bioassays. In general, only aliphatic amino acids, resembling the size and polarity of L-isoleucine result in active conjugates, whereas the presence of heterocyclic, and aromatic group in the amino acid moiety is not tolerated (Miersch *et al.,* 1999; Mithöfer *et al.,* 2005).

Aliphatic amino acids conjugates of JA such as Ile-JA, Leu-JA, and Val-JA display activities in bioassays comparable to those observed for free JA and its chemical derivatives such as MeJA. For example, they have been shown to induce gene expression in tomato (*Lycopersicon esculentum*), and Lima bean (*Phaseolus lunatus*) (Krumm *et al.,* 1995; Reymond *et al.,* 2000). They can also stimulate the synthesis of jasmonate-inducible proteins (JIPs) in barley (*Hordeum vulgare*) leaf tissue (Krammel *et al.*, 1997). Moreover, methyl esters of JA conjugates with Ile and Ala exhibit also high activity and can alter the gene expression in barley leaves (Miersch *et al.,* 1999). JA conjugates seem to act without cleavage to JA, and amino acids because they are not hydrolyzed. Apparently, JA amino acid conjugates are found in plant cells lacking a cleaving enzymes, or the conjugates are inaccessible for cleavage by specific amidohydrolases. Therefore, these conjugates are active *per se*, and it is highly improbable that they are storage forms of jasmonates as describes for other phytohormones (Reymond *et al.,* 2000; Seo *et al.,* 2001; Staswick *et al.,* 2002).

In the light of presented facts, the question arises on the presence of inactive metabolites of JA in plants. Experiments indicated that esterification of jasmonates with glucose or gentobiose (JA-1-β-glucose, JA-1-β-gentobiose) (Fig 9.25) reduces their activity, and contributes to regulation of the jasmonate level (Sembdner & Parthier, 1993). For example, the presence of the glucose ester of JA, as the most abundant metabolite in jasmonate family, was found in a cell culture of tomato (Meyer *et al.*, 1989). Moreover, the increase in the concentration of JA ester conjugates was found in the feeding experiments in tobacco (*Nicotiana tabacum*) BY-2 cells (Świątek *et al.*, 2004).

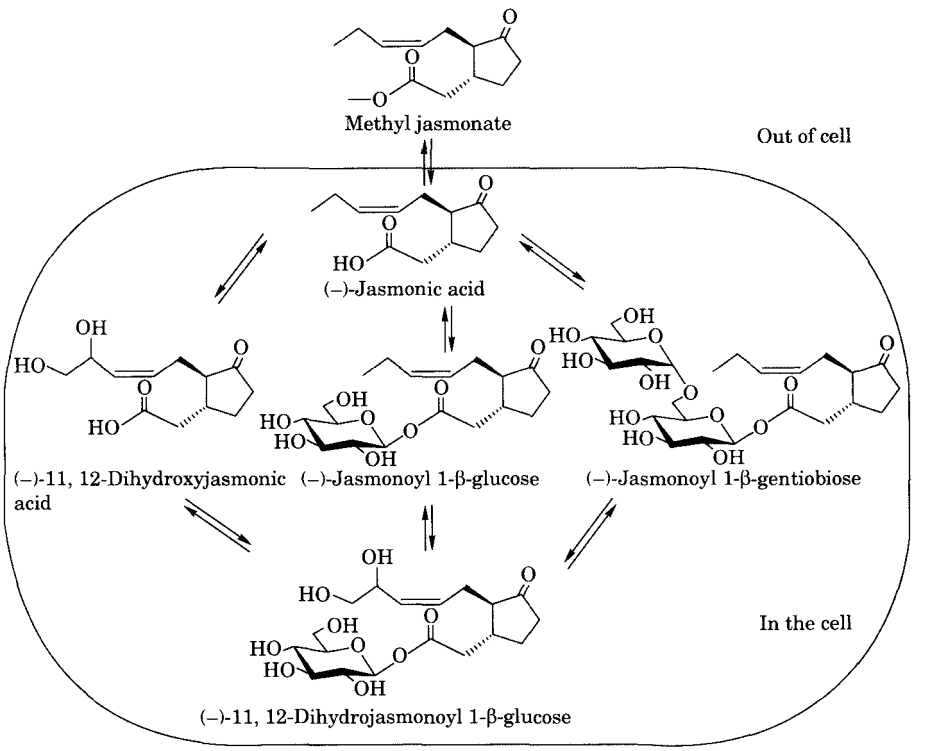
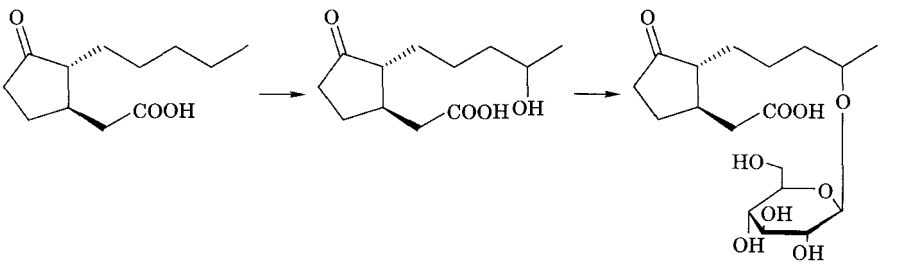


Fig 9.25. Metabolism of exogenous applied jasmonates

The first step in the exogenously applied JA and MeJA metabolism is most likely to be the conjugation to glucose and formation of a jasmonyl-1-β-glucose ester (JA-1-β-glucose) (Fig 9.25). This metabolite was synthesized within 24 h after treatment cells with JA as well as MeJA. The second step involves formation of the 1-jasmonyl-β-gentobiose (JA-1-β-gentobiose), possibly by the extension of the carbohydrate chain by a further glucose unit. Both compounds were shown to release free JA and might therefore serve as a JA-storage or -transport form because of their excellent solubility in water. Another conversion step involves the introduction of hydroxyl

group at C-11, and C-12 (Świątek *et al.,* 2004). Earlier feeding experiments, showed that treatment of ether-insoluble fractions of root and shoot extracts from *Nicotiana silvestris* with β-glucosidase released a pool of free JA from unidentified compounds. Additionally, both JA-1-β-glucose, and JA-1-β-gentobiose esters release free JA when treated with β-glucosidase, and they are also insoluble in ether (Zhang & Baldwin, 1997). Conjugation of jasmonates with sugar probably plays role in storage as well as in the transport of these phytohormones, and might be components of a pathway that inactivates excess jasmonic acid in plants. Interestingly, no significant amounts of MeJA or amino acid conjugates were observed in the JA-feeding experiments, and when tobacco cells were treated with MeJA, which was later rapidly hydrolyzed to JA and further processed like JA (Świątek *et al.,* 2004). On the other hand, barley shoots immersed in a (±)-[2-^{14}C]JA solution accumulated a mixture of several compounds, among which tuberonic acid, its β-glucoside, 11-hydroxyjasmonic acid, and amino acid conjugates of JA, and 11- and 12-hydroxylated jasmonates were identified (Meyer *et al.,* 1989). Probably, the nature of jasmonate conjugates during feeding experiments depends on the availability of the carbon source in the medium (Świątek *et al.,* 2004).

Feeding experiments have been carried out with radiolabeled (–)-9, 10-dihydroxyjasmonic acid (Meyer *et al.,* 1989; Miersch *et al.,* 1999). Excised barley shoots converted (–)-9, 10-dihydroxyjasmonic acid to (–)-11-hydroxy-9, 10-dihydroxyjasmonic acid and its 11-*O*-β-glucoside and smaller amounts of (–)-12-hydroxy-9, 10-dihydroxyjasmonic acid (Fig 9.26). Minor amounts of various amino acid conjugates of 9, 10-dihydroxyjasmonic acid and 11-hydroxy-9, 10-dihydroxyjasmonic acid, however undermined stereochemistry, also were formed. It has been shown that only (–)-metabolites were detected. They are assumed to have originated from the (–)-rather than the (+)-stereoisomer.



(–)-9, 10-dihydrojasmonic acid

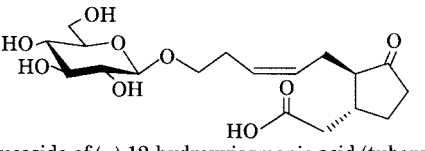
(–)-11-hydroxy-9, 10-dihydrojasmonic acid

(–)-11-hydroxy-9, 10-dihydrojasmonic acid 11-*O*-β-glucoside

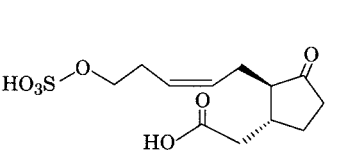
Fig 9.26. Metabolism of (–)-9, 10-dihydrojasmonic acid to glucose conjugate in barley shoots

Conjugation of jasmonates with glucose may be used for transport and storage of these phytohormones or alternatively might generate molecules

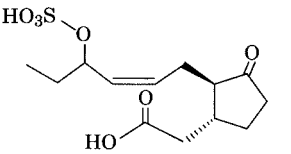
with new properties. For instance, the 12-*O*-glucoside of (–)-12-hydroxyjasmonic acid, commonly known as tuberonic acid, is characterized by high activity because it was shown to induce tuber formation *in vitro* (Fig 9.27). The presence of 12-*O*-β-glucoside of tuberonic acid has been detected in *Solanum tuberosum, Helianthus tuberosus,* and *Astragalus complanatus* (Yoshihara *et al.,* 1989).



12-*O*-β-glucoside of (–)-12-hydroxyjasmonic acid (tuberonic acid)



(–)-12-hydroxyjasmonic sulfate
(tuberonic acid sulfate)



(–)-11-hydroxyjasmonic sulfate

Fig 9.27. Glucose and sulfate conjugates of hydroxylated jasmonates

In addition to glucoside esters of jasmonates, a sulfate conjugate of tuberonic acid was found to accumulate in plants producing tubers, as well as in *Tribulus cistoides*, a member of Zygophylaceae family, and *Arabidopsis thaliana* which does not possess these properties (Achenbach *et al.,* 1994). The sulfonation of jasmonates has been suggested as a pathway for inactivation of these phytohormones. Recently, the gene *AtST2a* from *Arabidopsis* was reported to encode specific hydroxyjasmonate sulfotransferase which catalyzes the synthesis of sulfate conjugate of jasmonates. The enzyme AtST2a was found to exhibit strict specificity for (–)-11- and (–)-12-hydroxyjasmonic acid and does not accept their methyl esters (Fig 9.27). Therefore, the presence of a hydroxyl group at position C-11 or C-12 on the side chain as well as free C-1 in the molecule is required for binding at the active site of the enzyme. Moreover, the activity, and the level of the enzyme that sulfonate hydroxylate jasmonates was found to increase in *Arabidopsis* plants treated with JA, MeJA, and JA-Ile, indicating that AtST2a controls the concentration and hormonal activity of JA and its derivatives. The sulfonation of tuberonic acid might be irreversible considering the lack of evidence for the presence of genes encoding sulfatases in the genome of *Arabidopsis* (Gidda *et al.,* 2003).

Although, the biosynthesis and the biological function of the members of jasmonate family is extensively studied, the role of their particular metabolic modifications remains still unclear. There are also many contradictory data on the biological activity of jasmonate conjugates.

Unfortunately, the nature of compounds related to storage, inactivation, or transport of jasmonates seems to be still enigmatic.

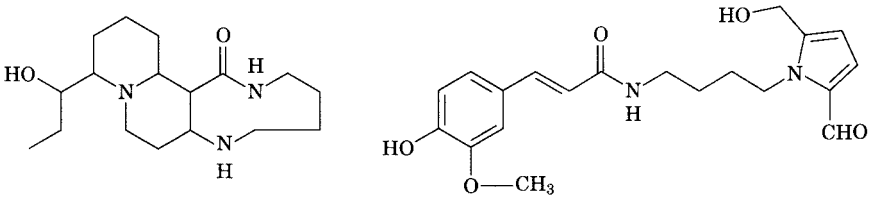
POLYAMINES CONJUGATES

Polyamines, mainly diamine putrescine, triamine spermidine, and tetraamine spermine, are polycationic compounds of low molecular weight that are present in plant and animal organisms. They have been proposed as a new category of plant growth regulators that are purported to be involved in a large spectrum of physiological processes, such as embryogenesis, cell division, morphogenesis, and development (Smith, 1985; Bais & Ravishankar, 2002; Liu *et al.,* 2006). In addition, they have been shown to be an integral part of plant stress response (Bouchereau *et al.,* 1999; Walters, 2003; Alcázar *et al.,* 2006). Though the physiological significance of polyamines in stress is not thoroughly understood, much progress has been made.

Polyamines occur as free molecular bases, but are often conjugated to small molecules like phenolic acids (conjugated forms) and also to various macromolecules like proteins (bound forms). The classification "free" and "bound" corresponds to the differential solubility of polyamine in trichloracetic acid (TCA) or perchloric acid (PCA), namely free polyamines (TCA- or PCA-soluble) and bound polyamines (TCA or PCA-insoluble). The first fraction covers free polyamines bases and their derivatives not precipitated in TCA or PCA; the second involves polyamines bound to high molecular compounds or subcellular structures and hence precipitated in the above media. Thus, according to their solubility, conjugated polyamines may fall in both fractions (Martin-Tanguy, 1997, 2001; Tiburcio *et al.,* 1997).

In animal and bacterial systems, polyamines can form part of a peptide (*e.g.* glutathionylspermidine), an amino acid (*e.g.* putreamine), and an antibiotic (*e.g.* bleomycin). Furthermore, polyamines can form an alkaloid with spermidine (*e.g.* palustrine) and putrescine (*e.g.* magnolamide) in plants (Fig 9.28). Polyamines are also conjugated by the formation of an amide linkage, utilizing esters of CoA for the provision of the activated carboxyl groups. This linkage is catalyzed by a class of enzymes known as transferases. These compounds occur as basic or as neutral forms. In the basic forms, the single amine group of an aliphatic amine is linked with a phenolic cinnamic acid, while in the neutral forms, each terminal amine group of an aliphatic amine is bound to a cinnamic acid (Fig 9.29). Post-translational covalent linkage of polyamines to proteins is catalyzed by transglutaminases, which have been localized both intra- and extra-cellularly (Martin-Tanguy, 1997, 2001). In plants, the hydroxycinnamic acid amides (HCAAs) are the common polyamine conjugates. Cinnamic acids, *e.g. p*-coumaric, ferulic and caffeic acids, are conjugated to polyamine by an amidic bond. Similar classification represents basic HCAAs, which typically contain the aliphatic di- and polyamines like putrescine, spermidine and

spermine, are water soluble, while neutral HCAAs, which contain aromatic amines like tyramine, octopamine, agmatine and tryptamine, are not soluble in water (Fig 9.30) (Facchini *et al.*, 2002). Conjugated polyamines, such as HCAAs (coumaroyl, feruloyl and caffeoyl conjugates), have also been shown to occur at high levels in plants and to correlate with developmental events. These phenolic amides accumulate in large amounts in meristems, flowers and seeds (Martin-Tanguy, 1997, 2001).



Palustrine Magnolamide

Fig 9.28. Examples of alkaloid polyamines

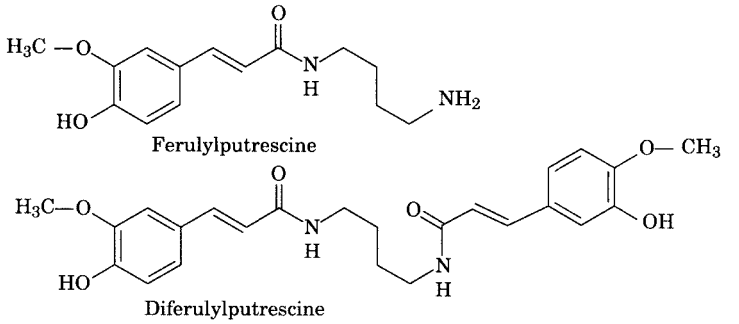


Fig 9.29. Examples of basic and neutral forms of polyamine conjugates. Ferulylputrescine is a basic conjugate of one molecule ferulic acid with one amino group of putrescine; diferulylputrescine is a neutral conjugate of two ferulic acids molecules with both amino groups of putrescine

The function of polyamines conjugates during cell division and cellular differentiation is still under discussion. Polyamines conjugates may act as a means for polyamine translocation (Martin-Tanguy, 1985). In many plants, during floral induction and floral development, polyamines conjugates are not metabolized and do not act as storage forms for polyamines. Probably, the major pathway of polyamine metabolism is *via* conjugation pathway (Martin-Tanguy, 2001). Conjugation of polyamines may regulate their interactions with inorganic cations such as Ca^{2+}, which might have an implication for the proposed role of polyamine on membrane stabilization. Neutral conjugates cannot be ionically bound to phosphate or other anionic groups on proteins, nucleic acids, or phospholipids. Conjugation reactions could regulate polyamine functions by affecting their binding and interaction with nucleic acids. The conjugates have been implicated in molecular signalling events in plant pathogen interactions (Galston *et al.*,

1997; Alcázar *et al.*, 2006).

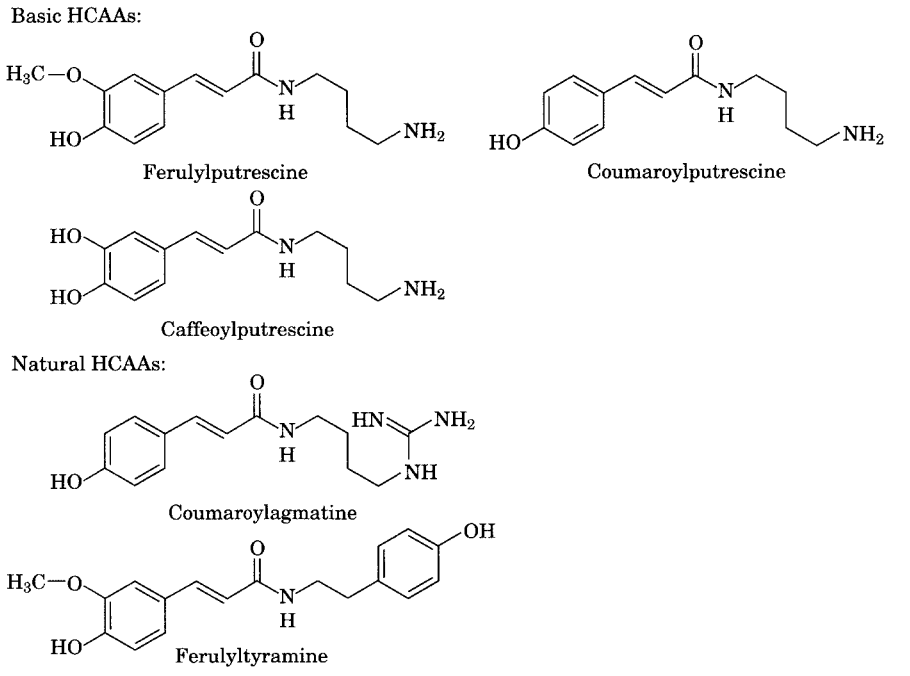


Fig 9.30. Examples of basic and neutral forms of the hydroxycinnamic acid amides (HCAAs)

SALICYLIC ACID CONJUGATES

Salicylic acid (SA) is one of numerous phenolic compounds, defined as compounds containing an aromatic ring with a hydroxyl group or its derivative, found in plants. There has been considerable speculation that phenolics in general function as plant hormones. Exogenously supplied SA was shown to affect a large variety of processes in plants, including stomatal closure, seed germination, fruit yield and glycolysis (Raskin, 1992; Klessig & Malamy, 1994; Hayat & Ahmad, 2007).

In plants, SA is synthesized from phenylalanine, which is converted to *trans*-cinnamic acid by phenylalanine ammonia lyase (PAL). PAL is a key enzyme in the phenylpropanoid pathway that yields phytoalexins, lignins and hydroxybenzoic acids. There are two proposed pathways for the conversion of *trans*-cinnamic acid to SA; they differ in the order of β-oxidation and *ortho*-hydroxylation reaction. β-Oxidation of *trans*-cinnamic acid produces benzoic acid, which can be hydroxylated to form SA. Alternatively, *ortho*-hydroxylation of *trans-cin*namic acid forms *ortho*-coumaric acid, which can be converted to SA *via* β-oxidation (Raskin, 1992; Yalpani *et al.*, 1993; Silverman *et al.*, 1995; Hayat & Ahmad 2007).

Most phenolic acids in plants exist in the form of sugar conjugates. There are glucose esters (glucose attachment through the carboxyl group) and glucosides (glucose attachment through the hydroxyl group). SA produced after tobacco mosaic virus (TMV) infection of tobacco is rapidly conjugated to glucose to form SA-2-*O*-β-glucoside (SAG) (Fig 9.31). Both free SA and SAG were found near the infected area, but only free SA was observed in distal portions of the leaf. It supports that free SA is the active signal in the induction of acquired resistance. Processes of formation of SAG and its hydrolysis may be physically separated in the cell, with the conjugate probably serving as a storage form of bioactive SA. The exogenously applied and endogenously produced SA induced UDP-glucose: SA glucosyltransferase in several plants (Enyedi *et al.*, 1992; Enyedi & Raskin, 1993; Hennig *et al.*, 1993; Yalpani *et al.*, 1993).

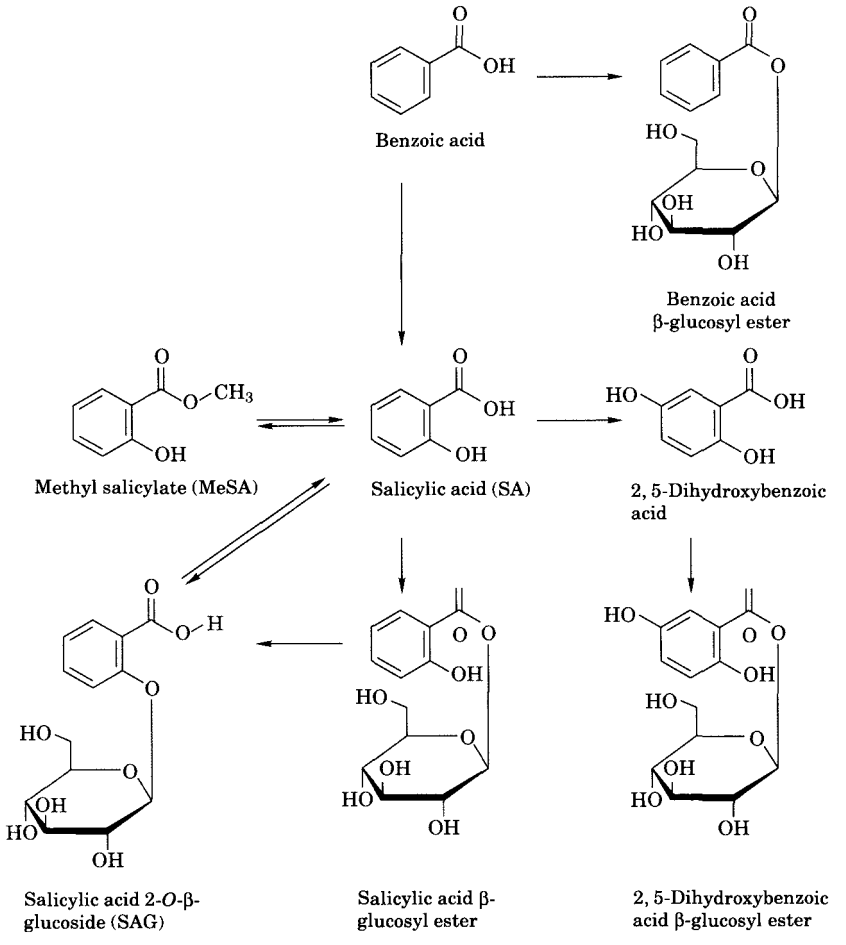


Fig 9.31. Metabolism of benzoic acid and salicylic acid in TMV-infected resistant tobacco

Recent studies, in which [^{14}C] SA was applied to mock- and TMV-inoculated Xanthi-nc tobacco leaves, have shown that SA is metabolized to SAG and 2, 5-dihydroxybenzoic acid β-glucosyl ester (Fig 9.31). Labeled 2, 5-dihydroxybenzoic acid (gentisic acid) and 2, 3-dihydroxybenzoic acid (*O*-pyrocatechuic acid) are found in leaves of various plants fed radioactive SA, cinnamic acid, or benzoic acid. In roots of buckwheat (*Fagopyrum esculentum*), SA is 5-hydroxylated to 2, 5-dihydroxybenzoic acid, followed by glucosylation at the hydroxyl group of the C5 position to form gentisic acid 5-*O*-β-D-glucoside. Exogenously supplied [^{14}C] benzoic acid in *Helianthus annuus* hypocotyls is converted to trace amounts of SA and large amounts of SAG. It suggests that glucosylation is a major route of SA metabolism in plants (Malamy & Klessig, 1992; Yalpani *et al.,* 1993).

In addition, significant amounts of SA derived volatile methyl salicylate (MeSA) (Fig 9.31) are released from TMV-inoculated Xanthi-nc tobacco. In contrast to methyl salicylate, glucosylated form(s) of SA accumulate only in and around hypersensitive response lesions formed during the incompatible interaction between plants and viruses, bacteria, or fungi. Gaseous MeSA is a major volatile in TMV-inoculated tobacco plants, and is produced in parallel with SA. MeSA may represent an airborne defense signal. MeSA is synthesized from SA and apparently acts by being converted back to SA (Shulaev *et al.,* 1997; Seskar *et al.,* 1998). MeSA is a constituent of the floral scent of *Clarkia breweri* flowers (Dudareva *et al.,* 1998), and is synthesized by an *S*-adenosylmethionine-dependent SA carboxyl methyltransferase that adds a methyl group to the carboxyl group of SA, not the hydroxyl group.

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10

Biological Prospective of Pinitol and its Structurally Modified Products

BHAHWAL ALI SHAH[1] AND SUBHASH CHANDRA TANEJA[1,*]

ABSTRACT

Pinitol is 3-O-methyl ether of D-chiro-inositol, belonging to the class of compounds known as inositols. The present review gives a comprehensive insight into the biological attributes of pinitol including its effect as an anti-diabetic, in sports medicine, in the treatment of polycystic ovary syndrome (PCOS) etc. Also its potential as an excellent starting material for the synthesis of many biologically active compounds is discussed.

Key words : Inositol, pinitol, D-*chiro*-inositol, TNF-α, anti-diabetic, anti-inflammatory, lipopolysaccharide, polycystic ovary syndrome (PCOS)

INTRODUCTION

Pinitol (**1**) is 3-O-methyl ether of D-*chiro*-inositol, also known as Mateziol or Sennitol or Cathrtomannitol. It belongs to the class of compounds known as inositols, which can be further sub-categorized under the class polyols. Inositols, unofficially referred as "vitamin B_8" are present in all animal tissues, with the highest levels in the heart and brain. The major naturally occurring inositol is *myo*-inositol. Other naturally occurring isomers (though in minimal quantities) are *scyllo*-, *chiro*-, *muco*-, and *neo*-inositol and other possible isomers are *allo*-, *epi*- and *cis*-inositol. Inositols and their derivatives have attracted a considerable attention due to their innumerable roles in living organisms (Posternak, 1962; Anderson, 1972), which include diverse biological activities (Michell & Drummond, 1989; Powis &

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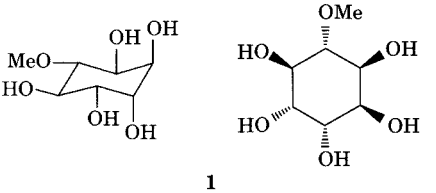
Kozikowski, 1994; Schedler & Baker, 2004). They have also served as a starting material in the synthesis of natural products (Balci *et al.*, 1990; Hudlicky, 1996) and may also be useful for depression (Levine *et al.*, 1995; Levine, 1997; Benjamin *et al.*, 1995a; Nemets *et al.*, 1999). Inositol has also been studied for bipolar disorder (Chengappa *et al.*, 2000) panic disorder (Benjamin *et al.*, 1995b; Palatnik *et al.*, 2001), bulimia (Gelber *et al.*, 2001), obsessive-compulsive disorder (Fux *et al.*, 1996; Fux *et al.*, 1999), Alzheimer's disease (Levine, 1997) and attention deficit disorder (Levine, 1997). It may also be helpful in improving various symptoms of polycystic ovary syndrome (PCOS), including infertility and weight gain (Gerli *et al.*, 2003; Gerli *et al.*, 2007). Inositol has also been found to reduce the symptoms of psoriasis triggered or made worse by use of the drug lithium (Allan *et al.*, 2004). Inositol is sometimes proposed as a treatment for diabetic neuropathy, but there have been no double-blind, placebo-controlled studies on this subject, and two uncontrolled studies had mixed results (Salway, *et al.*, 1978; Gregersen *et al.*, 1983). Inositol has also been investigated for potential cancer-preventive properties (Wattenberg, 1999; Dong *et al.*, 1999; Yang & Shamsuddin, 1995; Ishikawa *et al.*, 1999; Shamsuddin, 1999; Shamsuddin & Vucenik, 1999; Vucenik *et al.*, 1998; Shamsuddin & Vucenik, 1997). Inositol as the basis for a number of signaling and secondary messenger molecules is involved in a number of biological processes, including insulin signal transduction (Larner, 2002), cytoskeleton assembly, nerve guidance (Epsin), intracellular calcium (Ca^{2+}) concentration control, cell membrane potential maintenance, cerotonin activity modulation, breakdown of fats and reducing blood cholesterol and Gene expression (Shen, 2003; Steger, 2003). The myriad properties of inositol and its structural analogs make it truly an interesting subject to study.



1

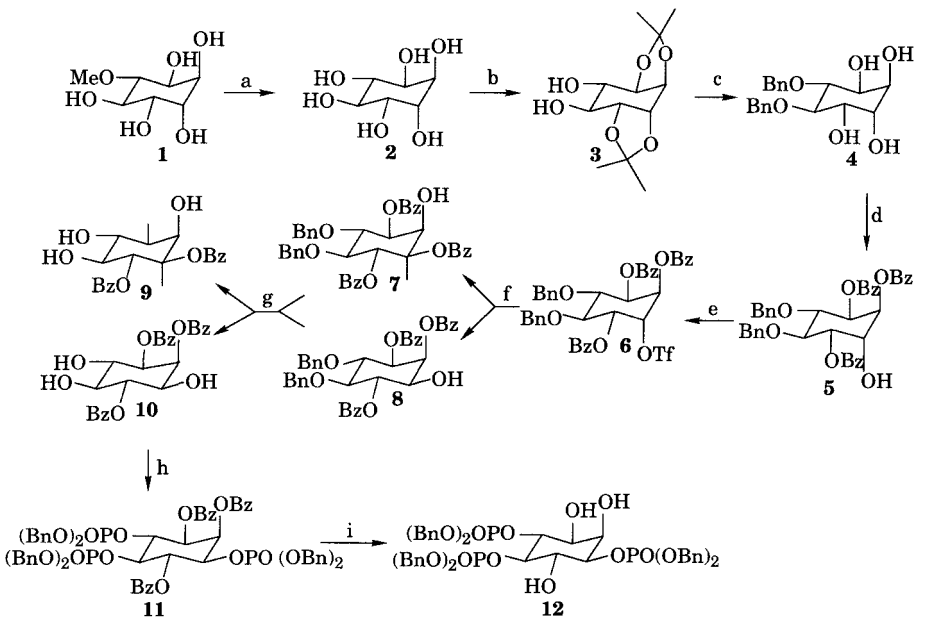
Pinitol has a widespread presence in plants. It occurs mainly in *Bougainvillea spectabilis* (Naryanan *et al.*, 1987) and *Pinus lambertiana* (Anderson, 1953). The pinitol has also been isolated from seeds from buck wheat (Szczecinski *et al.*, 1998; Steadman *et al.*, 2001) and jojoba beans (Ogawa *et al.*, 1997). It is a component common to many other species of Leguminosae, *Adesmia* spp. (Appel & Lobos, 1962), *A. intertexta* (Clark-Lewis *et al.*, 1961), *A. longissima*, *A. stolonifera* and *A. lasiopetala* (Rimington, 1935; Plouvier, 1949; Plouvier, 1950). Pinitol has also been reported from aerial parts of *Dianthus barbatus* (Cordell *et al.*, 1977). In a

study on secreted polysaccharides from soybean (*Glycine max*) roots grown for 15 days, pinitol has been found to be one of the main constituents (Timotiwu & Sakurai, 2002). Pinitol isolated from the methanol extracts of soybean leaves has been found to cause a 50% reduction in weight gain of *Heliothis zea* larvae at about 0.7% concentration when added to a synthetic diet (Dreyer *et al.*, 1979). It has been found to be the predominant sugar in leaves, petioles, roots and nodules of white clover (*Trifolium repens*) as identified by use of high-performance liquid chromatography and gas chromatography (Davis & Nordin, 1983). Pinitol has also been isolated from the leaves of *Gliricidia sepium* (Calle *et al.*, 1987) and aerial parts of *Tribulus cistoides* (Achenbach *et al.*, 1994; Achenbach *et al.*, 1996). The occurrence of pinitol has also been reported in *Zanha africana* (Cuellar *et al.*, 1997), *Genista ephedroides* (Pistelli *et al.*, 1998), *Medicago sativa* (Rendig & McComb, 1962), *Artemisia giraldii* (Tan *et al.*, 1999), *Genista corsica* (Pistelli *et al.*, 2000), Vetch (*Vicia villosa* roth.) seeds (Szczecinski *et al.*, 2001), *Onobrychis viciifolia* (Sainfoin) (Marais *et al.*, 2000), Jojoba seed meal (*Simmondsia chinensis*) (Van Boven *et al.*, 2001), *Detarium microcarpum* (Abreu & Relva, 2002), *Senna racemosa* (Mena-Rejon *et al.*, 2002), *Aristolochia arcuata* (Francisco *et al.*, 2003), *Cyclopia subternata* (Kamara *et al.*, 2004), *Glycyrrhiza glabra* (Biondi *et al.*, 2005), *Aristolochia contorta* (Yu *et al.*, 2005), *Anthyllis barbajovis* (Pistelli *et al.*, 2007) and *Tribulus macropterus* (Abdel-Hameed *et al.*, 2007). The pinitol has also been identified in *Acacia nilotica* and also reported is its chronic toxicity against *Aedes aegypti* and *Culex quinquefasciatus* IVth instar mosquito larvae (Chaubal *et al.*, 2005).

It is thought to protect the organelles and seeds during desiccation and storage (Horbowicz *et al.*, 1998). Pinitol and sucrose, accompanied by lower concentrations of glucose and fructose form the major components of black-wattle bark (Saayman *et al.*, 1965). Its simultaneous presence in the leaves, barks, sapwood and heartwood of the black wattle suggests a possible origin in the leaves, vertical translocation in the vascular tissues of the bark followed by radial translocation to the sapwood and heartwood. Other carbohydrates, sucrose (predominantly), glucose and fructose have the same concentration in the leaves and bark, but inversion of the sucrose predominates in the sapwood. The absence of these carbohydrates from the heartwood, leaving only pinitol suggests that the products of inversion, glucose and sucrose are either consumed or undergo transformation during tannin formation at the sapwood-heartwood interface.

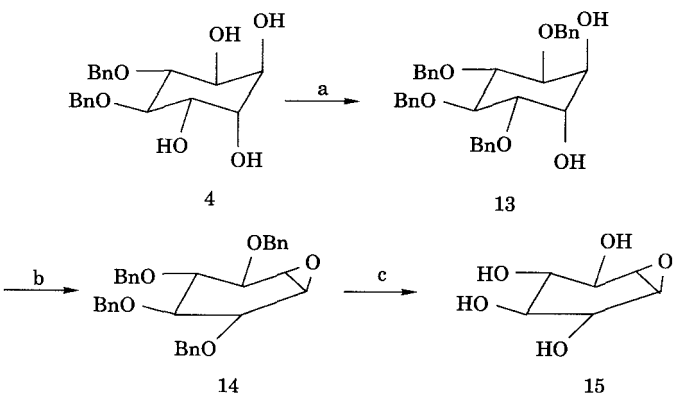
STRUCTURE MODIFICATIONS OF PINITOL

Pinitol has served as an excellent starting material for the synthesis of many important biologically important analogues. The synthesis of D and L-*myo*-inositol phosphate (**12**) from pinitol has been important step towards achieving that goal (Tegge & Ballou, 1989) (Scheme 10.1).



Scheme 10.1. Reagents and conditions: (a) 50% HI; (b) DME, pTSA; (c) (i) $C_6H_5CH_2Cl$, KOH (ii) CH_3COOH:H_2O (80:20); (d) C_6H_5COCl, pyr.; (e) triflic anhydride, pyr., 30°C; (f) 95% CH_3COOH/NaOAc, 40°C; (g) H_2-Pd, ethanol; (h) di-O-benzyl-di-N-isopropyl phosphoramidite in tetrazole-DCM, 23°C, m-CPBA; (i) H_2-Pd, ethanol, NaOH

D-pinitol has also been employed in the synthesis of 1-D-1,2-anhydro-*myo*-inositol (conduritol β-epoxide, **15**) (Falshaw *et al.*, 2004), which is known to have ability to act as an irreversible inhibitor of β-glucosidases (Legler, 1999) (Scheme 10.2).



Scheme 10.2. Reagents and conditions: (a) $(Bu_3Sn)_2O$, BnBr, Bu_4NBr, Toluene; (b) Ph_3P, DIAD, Toluene; (c) 10% Pd-C, H_2, 1:5 EtOAc : MeOH

Many of the actions of insulin have been suggested to be mediated by inositolphosphoglycans (IPGs), the number and structures of which have

not been completely evaluated (Jones & Varela-Nieto, 1999). The pseudodisaccharide $GlcNH_2$ α-1 → 6-D-*chiro*-inositols structural motif behaves as P-type naturally occurring IPGs in inducing differentiation in cultures of chicken embryo (Rademacher *et al.*, GB98/03847). The syntheses of 1-D-6-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-3-*O*-methyl-*chiro*-inositol (**16**), 1-D-6-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-3-*O*-methyl-*chiro*-inositol (**17**), 1-D-6-*O*-(2-amino-2-deoxy-α-D-galactopyranosyl)-3-*O*-methyl-*chiro*-inositol (**18**), and 1-D-6-*O*-(2-amino-2-deoxy-β-D-galactopyranosyl)-3-*O*-methyl-*chiro*-inositol (**19**), were also accomplished from the D-pinitol (Bonilla *et al.*, 2002) (Fig 10.1).

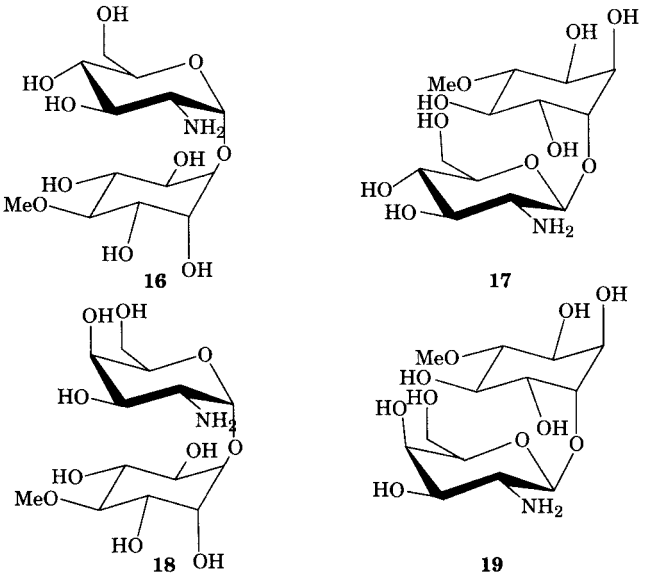


Fig 10.1. Structures of the synthesized pseudo-disaccharides from pinitol

Enzymatic β-D-galactopyranosylation of 1-D-*chiro*-inositol, D-pinitol, 1-D-3-*O*-allyl-4-*O*-methyl-*chiro*-inositol, 1-D-3, 4-di-*O*-methyl-*chiro*-inositol, 1-L-*chiro*-inositol and *myo*-inositol in combined yields ranging from 46% to 64% has been accomplished using β-galactosidase isolated from an anaerobic extreme thermophile, *Thermoanaerobacter* sp. strain TP6-B1 and p-nitrophenyl β-D-galactopyranoside as the donor (Hart *et al.*, 2004) (Fig 10.2).

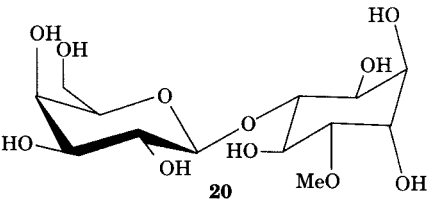
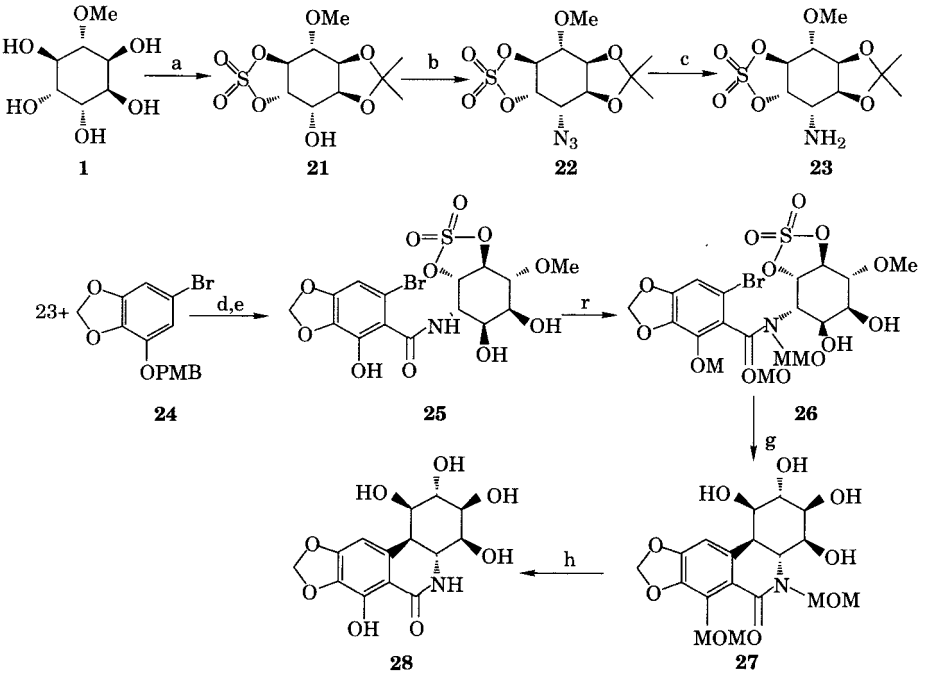


Fig 10.2. Structure of galactosylated derivative of pinitol

Pancratistatin (**28**) is a potent anti-cancer compound (Petit *et al.*, 1986) isolated from *Pancratium littorale* (Petit *et al.*, 1984). Its clinical evaluation has been hampered by the limited natural abundance. Pinitol, which occurs ubiquitously, can be employed as a building block in the synthesis of pancratistatin (Li *et al.*, 2006) (Scheme 10.3).



Scheme 10.3. Reagents and conditions: (a) (1) PPh_3, DEAD, CH_3SO_3H, CH_2Cl_2, 0°C to rt; (2) NaN_3, DMF, 60°C; (b) (1) TBAF, THF, 0°C to rt; (2) $SOCl_2$, Et_3N, CH_2Cl_2, 0°C; (3) $NaIO_4$, $RuCl_3$, aq CH_3CN; (c) PPh_3, aq THF, 0°C to rt; (d) CH_3CO_2H, 100°C; (e) $MgBr_2.OEt_2$, $COCl_2$, ether, 0°C; (f) K_2CO_3, MOMCl, DMF, rt; (g) *t*-BuLi, $CeCl_3$, ultrasound, THF, –78°C to rt; (h) (1) BBr_3, CH_2Cl_2, –78 to 0°C, 1 h; (2) MeOH, –78 to 0°C, 2 h

In past years, carbocyclic nucleosides, in which the O-atom of the furan ring is replaced by a CH_2 group, display a wide range of biological activities and have attracted particular attention for their antitumor (Zhan *et al.*, 2006) and antiviral (Boehme *et al.*, 1994) effects. Due to the absence of the glycosidic linkage between heterocycle and sugar, these compounds are more stable with respect to degradation by enzymes such as phosphorylases (Saunders & Cameron, 1995). Various 4/5-deoxy-4/5-nucleobase derivatives of pinitol have also been synthesized (Zhan *et al.*, 2006) (Fig 10.3).

Azole nucleosides of pinitol have also been synthesized and reported to have cytotoxic effects (Zhan & Lou, 2007) (Fig 10.4).

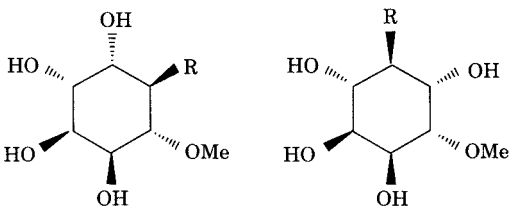


Fig 10.3. Structures of various deoxy derivatives of pinitol

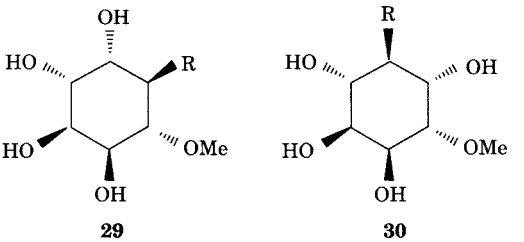
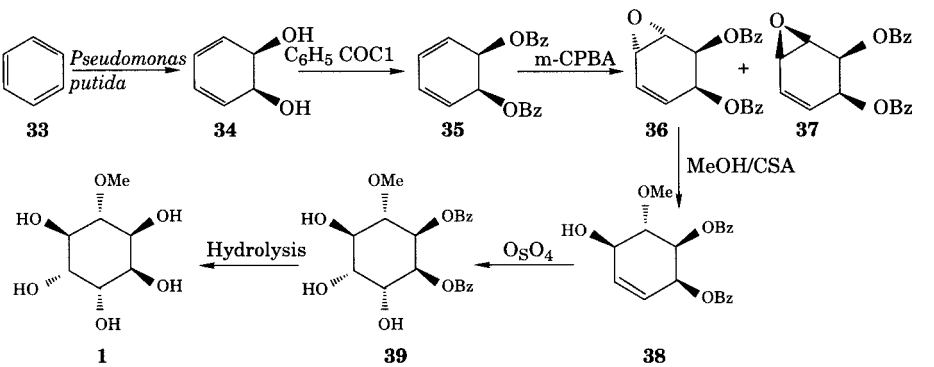


Fig 10.4. Structures of azole nucleosides of pinitol

TOTAL SYNTHESIS OF PINITOL

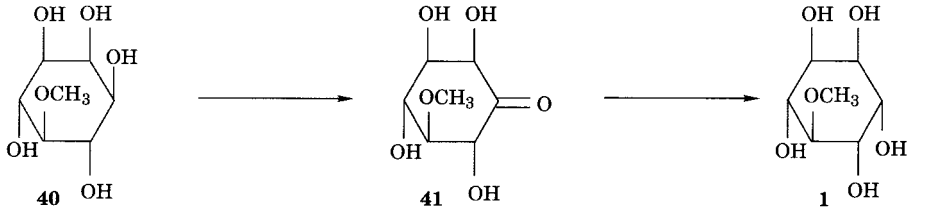
The total synthesis of pinitol has been carried out from benzene. One of the important steps in the total synthesis includes the transformation of benzene to *cis*-1, 2-dihydroxy-3, 5-hexadiene via biocatalytic oxidation (Scheme 10.4).



Scheme 10.4. Total synthesis of pinitol

The epimerization of sequoyitol (**40**, 5-O-methyl-myo-inositol) to D-pinitol is known to be the final step in the biosynthesis of the latter compound in *Trifolium incarnatum* (Scholda *et al.*, 1964). The reaction of sequoyitol (**40**) to pinitol has been demonstrated to proceed *via* a keto intermediate, D-5-O-methyl-2, 3, 5/4,6-pentahydroxycyclohexanone (**41**),

and is catalyzed by two dehydrogenases or one protein with two dehydrogenase actions, of which the first one is NAD-specific and oxidizes sequoyitol to the keto intermediate, whereas the other has the function to reduce the keto compound to D-pinitol using NADPH as hydrogen donor (Ruis & Ostenhoff, 1969). Both dehydrogenase actions as well as the overall epimerization reaction have been shown to be reversible (Scheme 10.5).



Scheme 10.5. Synthesis of pinitol

PINITOL AS AN ANTI-DIABETIC AGENT

IPGs are potentially important post-receptor mediators of insulin action (Kelly *et al.*, 1987; Larner *et al.*, 1988). *Bougainvillea spectabilis* has been traditionally used in the treatment of diabetes in Asia and the West Indies and is reported to reduce glucose concentrations in alloxan induced diabetic rats (Naryanan *et al.*, 1987). Pinitol appears to mimic the effects of insulin by acting downstream in the insulin signaling pathway (Fonteles *et al.*, 1996). D-Pinitol has now become one of the better studied insulin mimickers in the supplement industry and is proving to be an excellent aid in improving glucose metabolism. Pinitol has been shown to lower the blood glucose concentration in diabetic rats and in normal rats given glucose (Ortmeyer *et al.*, 1993). This isomer also increased the rate of glucose disappearance in insulin-resistant and hyperinsulinemic monkeys (Ortmeyer *et al.*, 1995), which suggests that it might improve glucose metabolism in addition to recover insulin resistance. In a four week study on 22 patients pinitol and D-*chiro*-inositol were administered to test the hypothesis that oral administration of soybean-derived pinitol would improve insulin sensitivity in obese subjects with diet-treated type II diabetes or glucose intolerance (Davis *et al.*, 2000). No toxicity due to the pinitol was observed during the study. Moreover it did not alter baseline glucose production, insulin-mediated glucose disposal, or rates of appearance of free fatty acids and glycerol in plasma. They further demonstrated that plasma levels of both pinitol and D-*chiro*-inositol are very responsive to pinitol ingestion, but insulin sensitivity does not increase after pinitol treatment in individuals with obesity and mild type II diabetes. D-pinitol also exerts an acute and chronic insulin-like antihyperglycemic effect in STZ-diabetic mice. Recent study indicated that pinitol does not augment the effect of insulin but might involve in an interaction with part of a cellular signaling pathway that links insulin with glucose transport (Bates *et al.*, 2000). In a clinical study

it has been proved that insulin sensitivity does not increase after pinitol treatment in individuals with obesity and mild type II diabetes (Davis *et al.*, 2000). The levels of inositols are elevated in the patients with diabetes and in a study with intent to measure the urinary excretion of inositols and to assess possible relationship with the metabolic control of glucose, in older non-diabetic men and women it has been found that all cases display differences in the urinary excretion of inositols with urinary excretion higher in men in all cases (Campbell *et al.*, 2001). A putative insulin mediator termed INS-2 (**42**), isolated from beef liver has been identified as inositol glycan pseudo-disaccharide Mn^{2+} chelate containing pinitol and galactosamine (Larner *et al.*, 2003) (Fig 10.5). Its role as an insulin mimetic was demonstrated by its action *in vivo* to decrease elevated blood glucose injected to low-dose streptozotocin diabetic rats in a stereospecific and dose-dependent manner. The pseudo-disaccharide also stimulated [^{14}C] glucose incorporation into [^{14}C]glycogen in a dose-dependent manner in H4IIE hepatoma cells in the presence of insulin, thus enhancing insulin action. Only when chelated to Mn^{2+} it activated pyruvate dehydrogenase phosphatase *in vitro* in a dose-dependent manner. The structure of the isolated pseudo-disaccharide was later confirmed by synthesizing the molecule. The chemically synthesized INS-2 has been shown to possess biological activity that significantly enhances insulin reduction of hyperglycemia in streptozotocin diabetic rats (Brautigan *et al.*, 2005). It was later proposed that INS-2 allosterically activates protein phosphatase 2C (PP2C), fulfilling the role of a putative mediator mimetic of insulin signaling to promote protein dephosphorylation and metabolic responses.

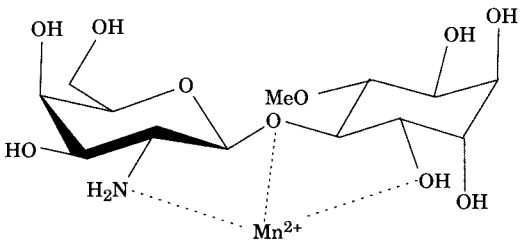


Fig 10.5. Structure of INS-2

Pinitol supplementation does not influence whole-body insulin-mediated glucose metabolism, muscle insulin receptor content and phosphorylation, in non-diabetic older people as demonstrated in a clinical study (Campbell *et al.*, 2004). The effects of soybean-derived pinitol on glycemic control and cardiovascular risk factors in Korean patients with type II diabetes mellitus suggest that it may be beneficial in reducing cardiovascular risk (Kim *et al.*, 2005a). Hyperglycemia and hyperlipidemia are causative factors of endothelial dysfunction (ED), which is an early feature of cardiovascular risk and diabetes. Excessive endothelial mitochondrial superoxide (ROS) production with hyperglycemia and hyperlipidemia is a key mechanism.

Inositols including pinitol prevented and reversed ED in rat and rabbit vessels, reduced elevated ROS in endothelial cells, potentiated nitrergic or vasculo-myogenic relaxations and preserved nitric oxide (NO) signaling. These effects are related to their metabolic actions, direct superoxide scavenging and enhancing and protecting NO signaling (Nascimento *et al.*, 2006). In a study on postprandial blood glucose response in fifteen patients with type II diabetes mellitus, it was observed that pinitol had no apparent effect on postprandial insulin levels (Kang *et al.*, 2006). In a study to evaluate the effect of pinitol therapy in type II diabetic patients who were poorly controlled with hypoglycemic drugs, such as sulfonylurea, metformin and/or insulin, twelve weeks of pinitol treatment in twenty patients altered glucose metabolism, but not lipid profiles or adipocytokine levels (Kim, *et al.*, 2007). Ethanolic extract of the roots of *Rhizophora apiculata* having pinitol as one of the main constituents, has also exhibited anti-hyperglycemic activity in rats (Lakshmi *et al.*, 2006).

PINITOL AS SPORTS SUPPLEMENT

As insulin is thought of as one of the most anabolic (muscle-building) hormones in our bodies and one of its main jobs is to regulate the uptake of glucose, a source of energy, into our cells. Cells that need glucose have specific insulin receptors on their surfaces and when the insulin binds to these receptors, it ensures correct glucose utilization. If insulin isn't working efficiently, our cells won't get energy, and so wouldn't we. Due to our eating habits, moods, or physical output, glucose levels can be up and down on a rollercoaster ride all day. When glucose levels are high, our body releases insulin to help regulate glucose to proper levels. When glucose levels are low, our body converts stored glycogen and fat into glucose, providing us the energy we need. For athletes, weight trainers and active people in general, proper glucose metabolism is essential, not only for ensuring prolonged periods of energy but also to increase the volume of muscles. This is where pinitol's role as a sport supplement comes in. Since pinitol has been shown to enhance glucose metabolism, efficient glucose metabolism can improve the storage of glycogen, which ultimately improves endurance, athletic performance, muscle volume, and post-workout recovery. Pinitol helps to improve glucose metabolism and increase cellular energy, thereby provides higher energy output (Nutritional supplement reviews, http://www.nutros.com/nsr-0204s.html).

Creatine, a natural nutrient found in animal foods, is alleged to be an effective nutritional ergogenic aid to enhance sport or exercise performance (Chanutin, 1926), and it is well known from the literature that creatine hyper-accumulation in muscle is insulin dependent. Pinitol has been shown to help enhance the uptake of creatine without the use of high-calorie simple carbohydrates. In a study, 20 men with no history of supplementation gave urine samples for four days. After one control day, subjects ingested in a

single-blind manner either a placebo containing 5 g dextrose four times/day, 5 g creatine monohydrate four times/day, creatine with low-dose pinitol (5 g creatine four times/day, 0.5 g pinitol twice/day), or creatine with high-dose pinitol (5 g creatine, 0.5 g pinitol four times/day) for three days. Additionally, another group took 0.5 g pinitol twice/day for five days followed by 5 g creatine four times/day and 0.5 g pinitol twice/day for three days. Creatine retention was estimated by subtracting total urinary creatine excretion from total creatine intake during the three-day period. Results revealed that cumulative creatine retention was significantly greater in the low-dose pinitol and prepinitol-loading groups. Researchers extrapolated a significantly greater percentage of creatine retention in the low-dose and preloading pinitol groups (creatine monohydrate equaled 61%; low-dose pinitol equaled 83%; high-dose pinitol equaled 61%; pre-loading pinitol equaled 78%). These preliminary findings suggest that ingesting D-pinitol during creatine loading or prior to and during creatine loading may augment whole-body creatine retention (Greenwood *et al.*, 2001). Due to its ability to considerably increase the ability of muscle cells to absorb glucose, with consequent benefits for sports where there are requirements for strength and endurance, with the added benefit of shortened recovery times. It also has a positive effect on the ability of muscles to store carbohydrates, allowing for muscle growth whilst permitting the burning of fat. Besides, pinitol has been shown not to add any calories when consumed and, additionally, it may exert hypoglycemic effect, which is critical to individuals with diabetes (http://www.sutherlandia.com/pinitol_frame.htm).

The ability of pinitol to increase muscle mass has strong applications to the treatment of wasting syndrome in cancer, HIV/AIDS and tuberculosis. Sutherlandia has been used in Africa for generations to treat sufferers of tuberculosis, with impressive results. However, it is suspected that it is not only the Pinitol in Sutherlandia that results in its remarkable properties. Whilst research is continuing, there is strong evidence that the interaction of Pinitol with the other bio-actively compounds in the Sutherlandia plant that are responsible for its remarkable properties (http://www.sutherlandia.com/pinitol_frame.htm).

DIFFERENT BIOLOGICAL ACTIVITIES OF PINITOL

Besides, aforementioned activities, Pinitol has other medicinal attributes as well. The pinitol has shown potential as an anti-inflammatory agent (Singh *et al.*, 2001). The pinitol isolated from the *Abies pindrow*, has been used as Ayurvedic remedy for fever, respiratory and inflammatory ailments (Chatterjee *et al.*, 1991; Singh & Pandey, 1997a; Singh & Pandey, 1997b; Singh *et al.*, 1998). The synergistic anti-inflammatory effects of pinitol and glucosamine have also been demonstrated in a study conducted either alone or in combination against carrageenan and cotton pellet-induced acute and sub-acute inflammation in rats (Kim *et al.*, 2005b). When 20 mg/kg of pinitol

was administered to rats paw, the edema induced by the carrageenan injection was significantly suppressed and the level of granuloma formation induced by the cotton pellet implantation was slightly reduced. When 25 mg/kg of glucosamine was administered, paw edema caused by the acute inflammation was slightly reduced and the level of granuloma formation caused by the sub-acute inflammation was strongly suppressed. Although the combined application of pinitol and glucosamine did not have an additional anti-inflammatory effect on the paw edema caused by acute inflammation, it did have an increased anti-inflammatory effect on the formation of granuloma induced by sub-acute inflammation. Therefore, pinitol and glucosamine have an anti-inflammatory effect on acute and sub-acute conditions. Pinitol has been shown to reduce allergic airway inflammation and hyper responsiveness due to the alteration of Th1/Th2 polarization via the suppression of GATA-3 and increase of T-bet expression (Lee *et al.*, 2007a). Therefore, pinitol might offer a new therapeutic approach to allergic airway diseases. Pinitol was demonstrated to inhibit phenotypic maturation and modulate cytokine production in dendritic cells (DC), resulting in a significant inhibition of Th1 development. Moreover, pinitol suppressed LPS induced co-stimulatory molecules/MHC class types and IFN-γ, under *in vivo* experimental conditions, thus illustrating its immune-pharmacological functions (Lee *et al.*, 2007b). Ethanolic extract of *Adesmia aegiceras* has been reported for antibacterial activity against *Micrococcus luteus* and eight pathogenic bacterial strains as well as antifungal activity against *Candida albicans*, which has been further reported to have pinitol along with quercetin, isorhamnetin-3-rutinoside, isovitexin and chlorogenic acid as main constituents (Agnese *et al.*, 2001).

Pinitol can also be employed in the treatment of polycystic ovary syndrome (PCOS). There is evidence that the insulin resistance seen in women with PCOS is due in part to a deficiency of D-*chiro*-inositol or to a defect in its utilization in the tissues (Baillargeon *et al.*, 2006). Pinitol has not been studied as a treatment for PCOS. However, because an estimated 33% of orally administered pinitol is converted in the body to D-*chiro*-inositol, and because it appears to have biochemical effects similar to those of D-*chiro*-inositol, pinitol might be beneficial for women with PCOS (Davis *et al.*, 2000). To test that possibility, 44 obese women with PCOS were randomly assigned to receive, in double-blind fashion, D-*chiro*-inositol (1,200 mg once a day) or placebo for eight weeks. Supplementation with D-*chiro*-inositol resulted in an improvement in insulin resistance and a 55% reduction in testosterone levels compared to the placebo group. Significantly more women ovulated in the D-*chiro*-inositol group than in the placebo group (86% vs. 27%). D-*chiro*-inositol supplementation decreased testosterone levels and improved ovulatory function, presumably by enhancing the action of insulin (Nestler *et al.*, 1999). Pinitol is probably converted to D-*chiro*-inositol in the body, as demonstrated by a 14-fold increase in the levels of D-*chiro*-inositol after administration of pinitol to

diabetic patients at a dose of 20 mg per kg of body weight per day for four weeks (Davis *et al.*, 2000).

Many reports have demonstrated varied results for insulin action in atherosclerosis development. Insulin can rescue many types of cells from apoptotic cell death (Barres *et al.*, 1992; Wu *et al.*, 1995) and inhibited apoptosis in THP-1 human and RAW 264.7 murine macrophage cell lines (Iida *et al.*, 2002). Satomi *et al.* (1985) showed that the exogenous administration of insulin inhibited TNF-α production in a dose-related manner in animals that had been challenged with lipopolysaccharide (LPS). In contrast, much evidence from the past decade has shown that high-insulin conditions cause atherosclerosis in patients with high insulin resistance or hyperinsulinemia (Reaven, 1988; Zavaroni *et al.*, 1989). TNF-α is a potent inflammatory mediator secreted from macrophages, and it is important in many macrophage associated reactions such as immune and inflammation processes (Belardelli, 1995). Insulin is able to up-regulate the expression of the TNF-α gene in macrophages derived from the THP-1 cell (Iida *et al.*, 2001) and TNF-α is involved in insulin resistance through the inhibitory effect on insulin sensitivity (Peraldi & Spiegelman, 1978). In a recent study it has been demonstrated that pinitol dose-dependently decreased the lipid laden foam cell formation in THP-1 derived human macrophages (Choi *et al.*, 2007). It slightly inhibited the lipid-laden foam cell formation by oxidized low-density lipoprotein (oxLDL). TNF-α and monocyte chemo-attractant protein-1 releases were significantly reduced by pinitol treatment (0.05–0.5 mm), whereas interleukin-1β (IL-1β) a pro-inflammatory cytokine that is produced primarily by microglia and macrophages (Bauer *et al.*, 1993), which in turn induces the production of IL-6 and TNF-α (Lee *et al.*, 1995). IL-8, which is secreted by activated macrophages and vascular endothelial cells, is also up-regulated in human atherosclerotic lesions secretions. Some doses of pinitol decreased both expression and production of IL-1β and IL-8 compared to the no-pinitol treatment. Thus, pinitol has some inhibitory effects on foam cell formation by reducing lipid accumulation, secretion, and expression of some cytokines and macrophage scavenger receptor expression via its insulin-like action.

CONCLUSIONS

The preceding discussion makes it apparent that pinitol is a fascinating and safe molecule with diverse biological activities. Moreover, there are currently no known examples of any adverse side effects with the use of pinitol at recommended dosage levels. The molecule has an immense potential ranging from supplementing with creatine as performance enhancer to a prospective drug for the treatment of diseases like PCOS, cancer, HIV, inflammatory disorders etc. It may also provide a ray of hope to the millions of people across the globe suffering from insufficient functioning of insulin in the body and the ailments that come with it.

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11

Drug Sensitivity of Curcumin Analogues and Bioconjugates

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ABSTRACT

Curcumin is a multitargeted wonder molecule having plethora of therapeutic applications. None other herbal molecule has received as much attention as curcumin for prevention and cure of multiple diseases. However, it has not been possible so far to assign a definite drug profile to this mystical molecule, due to it's limited bioavailability. The limitations in its use, mainly it's efflux from gut, fast metabolism, poor adsorption, low solubility and lack of targeted delivery have been overcome through it's formulations with other naturally occurring molecules like piperine, flavanoids and polyphenols. The bioconjugates of curcumin with different ligands have been prepared either to modify the structure to get more efficacious analogs according to QSAR, or the ligands may assist in cellular uptake and thus yield mutual prodrugs. Some of these bioconjugates are more efficacious than curcumin. Some naturally occurring analogs of curcumin have also been studied for their structural and functional similarity with curcumin. As a consequence, it appears that the antioxidant, pro-oxidant, inflammatory and radical scavenging activities are associated with the methoxylated phenolic function while the antitumor/anticancer, antiviral, antiparasitic activities are associated with the enone or dienone functions in the side chain or linker between the two aryl functions.

Key words : Curcumin, bioconjugates, curcumin-analogs, bioavailability, QSAR, targeted delivery

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INTRODUCTION

A drug is any chemical or substance, synthetic or non-synthetic, that is taken primarily for non-dietary needs and changes the way our bodies work. Thanks to medical and drug research, there are thousands of drugs that help people. Antibiotics and vaccines have revolutionized the treatment of infections. There are medicines to lower blood pressure, treat diabetes, and reduce the body's rejection of new organs. Medicines can cure, slow, or prevent disease, helping us to lead healthier and happier lives. Drugs can be designed so that they act selectively at the desired molecular targets, an area of medicinal chemistry known as Pharmacodynamics.

When drugs are introduced into the body (often by swallowing, applying externally, inhaling, or injecting), these find their way into bloodstream and are transported to different parts of the body. Drugs are designed so that they carry out this journey to reach the target, this area is known as Pharmacokinetics. Drugs are systemic, *i.e.* they act on all parts of the body that they are meant for. Some cannot cross the blood brain barrier (BBB) due to physiological reasons. Drugs which cross this barrier reach the brain and may either intensify or dull senses, alter sense of alertness, and sometimes decrease physical pain. A drug may be helpful or harmful. The effects of drugs can vary depending upon the kind of drug taken, how much is taken (dose), how often it is used, how quickly it gets to the brain, and what other drugs, food, or substances are taken at the same time (synergistic effects). Effects can also vary based on the differences in body size, shape, and chemistry.

Herbal products have a special place in the world of pharmaceuticals. Side effects of conventional medicine, efficiency of plant-derived drugs and growing interest in natural products has increased scientific interest in medicinal plants. A look at the research and investments that are going into medicinal plants looks like, they will continue to play an important role in human health. Most drug designing projects are based on lead molecules derived from plants.

Today, there are at least 120 distinct chemical substances derived from plants that are considered important herbal drugs. Statistical data available in USA shows that 41 percent of pharmaceutical development has originated from herbal drugs. As per WHO estimates, 80 percent of about 4000 million inhabitants on this planet rely on plant products. In addition, herbal medicines have a good potential in the emerging nutraceutical industry as these materials are often considered food additives, as well as, medicines. They are used in preventive and curative treatments throughout the world. Though there is anecdotal evidence supporting the use of herbal medicines, scientific studies in this field is in its infancy.

All the research on herbal products has triggered companies and research organisations lend an ear to the vast untapped potential source of

drugs. Over the decades, there have been several instances of drugs sourced from herbal medicines like reserpine, vincristine and vinblatine. Neem and turmeric have been patented abroad recently. In India, companies like Ajanta Pharmaceuticals, Dabur and The Himalaya Drug Company have launched several herbal remedies in the market. Herbal formulations are popular among the rural and urban community in India. The reason for the popularity and acceptability being the belief that all natural products are safe. In the literature, therapeutic efficacy or toxicity is some times attributed to the herb without describing the brand or its content of bio-active or quantitatively significant components. Pharmaceutical products used as medicines are usually single chemical entities with specific actions at receptors, enzymes and other cellular sites. These drugs or preparations are marketed after vigorous clinical trials to support rational pharmaco-therapy. Curcumin, the yellow pigment of turmeric is now being marketed as a food additive, specially as an antioxidant. In this review the drug like properties of some conjugates of this wonder molecule as well as some of it's naturally occurring analogs have been highlighted.

CURCUMIN AS A WONDER DRUG

Curcumin, 1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione, (diferuloylmethane) (Jayaprakasha *et al.,* 2002) is a natural polyphenolic component of turmeric, the powdered rhizome of the perennial herb *Curcuma longa* Linn. (Family, Zingiberaceae). Turmeric is widely used as a dietary spice and as a colourant in cooking as well as a herbal medicine in Ayurveda, the traditional Indian system of medicine. Curcumin is the principal curcuminoid found in turmeric, along with minor amounts of demethoxy [Ia] and bis demethoxy [Ib] analogues (1). It exists in two tautomeric structures (Keto-Enol forms).

Turmeric has been used in India for medicinal purposes for centuries. Extensive investigation over the last five decades has indicated that curcumin possesses antioxidant (Sreejayan *et al.,* 1994; Weber *et al.,* 2005), antiapoptotic (Singh *et al.,* 1995), antiangiogenic (Arbiser *et al.,* 1998; Thaloor *et al.,* 1998; Mohan *et al.,* 2000; Kim *et al.,* 2002; Shim *et al.,* 2003), anticancer (Pipier *et al.,* 1998; Lin *et al.,* 2001; Aggarwal *et al.,* 2003, 2005), antiinflamatory (Khopde *et al.,* 1999; Aggarwal *et al.,* 2003; Chainani-Wu *et al.,* 2003; Adams *et al.,* 2005), anticarcinogenic, anti-HIV (Sui *et al.,* 1993; Li *et al.,* 1993; Jordan *et al.,* 1996; Mazumder *et al.,* 1997; Barthelemy *et al.,* 1998), antileshmaniases (Saleheen, 2002), antidiabetogenic (Srinivasan *et al.,* 1972; Babu *et al.,* 1995, 1997; Suresh Babu *et al.,* 1998; Arun *et al.,* 2002) and antiatheroselerotic (Huang *et al.,* 1992; Chen *et al.,* 1998). Curcumin also reduces blood cholesterol (Rao *et al.,* 1970; Patil *et al.,* 1971; Keshavarz *et al.,* 1976; Soudamini *et al.,* 1992; Soni *et al.,* 1992; Hussain *et al.,* 1992; Ramirez-Tortosa, 1999; Asai *et al.,* 2001), suppresses thrombosis (Srivastava *et al.,* 1985), rheumatoid arthritis (Deodhar, *et al.,*

1980) and Alzheimer's disease (Frautschy *et al.,* 1980; Lim *et al.,* 2001). It enhances wound healing (Sidhu *et al.,* 1998; Phan *et al.,* 2001; Shahed *et al.,* 2001) protects from liver injury (Morikawa *et al.,* 2002) and increases bile secretion (Rao *et al.,* 1970). It protects from cataract formation, pulmonary toxicity and fibrosis (Venkatesan *et al.,* 1995, 1997, 2000; Punithavathi *et al.,* 2000), prevents platelet aggregation (Srivastava *et al.,* 1986; Srivastava *et al.,* 1995), myocardial infarction (Dikshit *et al.,* 1995; Nirmala *et al.,* 1996; Venkatesan *et al.,* 1998). Curcumin has also been reported to act as antiprotozoa (Bhavani Shankar *et al.,* 1979) and antiplasmodia (antimalarial) (Reddy *et al.,* 2005). It has recently been reported to reverse the multi drug resistance in human gastric carcinoma cell lines (Xiao-qing Tang *et al.,* 2005). Despite this plethora of therapeutic applications, no definite drug profile has so far been validated for this molecule.

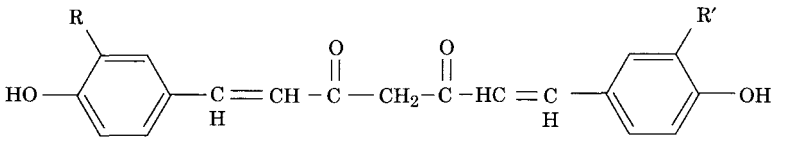


Fig 11.1. [I] R = R′ = -OCH_3; [Ia] R = -OCH_3 , R′ = –H; [Ib] R = R′ = -H

MOLECULAR TARGETS OF CURCUMIN

Various studies have shown that curcumin modulates numerous targets (Fig 11.2). These include the growth factors, growth factor receptors, transcription factors, cytokines, enzymes, and genes regulating apoptosis.

Cytokines and Growth Factors

Numerous growth factors have been implicated in the growth and promotion of tumors. Curcumin has been shown to down-regulate expression of several cytokines, including TNF, IL-6, IL-8, IL-12, and fibroblast growth factor 2 (Aggarwal *et al.,* 2005).

Receptors

HER2/neu and EGFR activity represent one possible mechanism by which curcumin suppresses the growth of breast cancer cells. Almost 30% of breast cancers overexpress the *HER2/neu* proto-oncogene (Slamon *et al.,* 1989) and both HER2 and EGF receptors stimulate proliferation of breast cancer cells. Overexpression of these two proteins correlates with progression of human breast cancer and poor patient prognosis (Slamon *et al.,* 1989). Curcumin has been shown to down-regulate the activity of EGFR and HER2/neu (Korutla *et al.,* 1995) and to deplete the cells of HER2/neu protein (Hong *et al.,* 1999).

Prostate cancer cell lines LNCaP and PC-3 when treated with curcumin, the results showed that curcumin down-regulated transactivation and expression of AR and cAMP response element-binding protein–binding protein. These findings suggested that curcumin has a potential therapeutic effect on prostate cancer cells through down-regulation of AR and AR-related cofactors (Nakamura *et al.,* 2002).

Transcription Factors

Curcumin may operate through suppression of various transcription factors, including NF-κB, STAT3, early growth response protein 1, AP-1, peroxisome proliferators–associated receptor gamma (PPAR-γ), and beta-catenin (Aggarwal *et al.,* 2005). These transcription factors play essential roles in various diseases. The constitutively active form of NF-κB has been reported in a wide variety of cancers. NF-κB is required for the expression of genes involved in cell proliferation, cell invasion, metastasis, angiogenesis, and resistance to chemotherapy. Bharti *et al.*, demonstrated that curcumin inhibited IL-6 induced STAT3 phosphorylation and consequent STAT3 nuclear translocation (Bharti *et al.,* 2003). Activation of PPAR-γ inhibits the proliferation of nonadipocytes. Xu *et al.* (2003) demonstrated that curcumin dramatically induced expression of the *PPAR*-γ gene and activated PPAR-γ. AP-1, another transcription factor closely linked with proliferation and transformation of tumor cells, has been shown to be suppressed by curcumin. Studies also suggest that curcumin has a potential therapeutic effect on prostate cancer cells through down-regulation of AR and AR-related cofactors (Aggarwal *et al.,* 2005).

Proinflammatory Enzymes

COX-2 and lipoxygenase (LOX) are important enzymes that mediate inflammation through production of prostaglandins and leukotrienes, respectively. Curcumin has been shown to suppress the expression of both COX-2 and LOX proteins as well as their enzymatic activities, most likely through the down-regulation of NF-κB, which is needed for COX-2 expression. Several groups have shown that curcumin down-regulates the expression of COX-2 protein in various tumor cells (Plummer *et al.,* 1999; Chen *et al.,* 1999). Plummer *et al.* measured COX-2 protein induction and prostaglandin E2 production in human blood after incubation with lipopolysaccharide. When 1 μm curcumin was added *in vitro* to blood from healthy volunteers, lipopolysaccharide-induced COX-2 protein levels and concomitant prostaglandin E2 production were reduced by 24% and 41%, respectively (Plummer *et al.,* 2001).

Protein Kinases

Curcumin suppresses a number of protein kinases, including MAPK, Jun N-terminal kinase, protein kinase A (PKA), protein kinase C (PKC), src

tyrosine kinase, phosphorylase kinase, IκBα kinase, JAK kinase, and the growth factor receptor protein tyrosine kinases. Treatment with curcumin inhibited highly purified PKA, PKC, protamine kinase, phosphorylase kinase, autophosphorylation-activated protein kinase, and pp60c-src tyrosine kinase (Reddy *et al.,* 1994). Phorbol myristate acetate–induced activation of cellular PKC is suppressed by curcumin (Liu *et al.,* 1993). Treatment of cells with curcumin inhibited tetradecanoylphorbol acetate–induced PKC activity without affecting the level of PKC. Curcumin inhibited the PKC activity *in vitro*, as well as in the cells (Hasmeda *et al.,* 1996).

Cell Cycle

Curcumin modulates cell-cycle–related gene expression. Specifically, curcumin induced G_0/G_1 and/or G_2/M phase cell cycle arrest, up-regulated cyclin-dependent kinase inhibitors p21WAF1/CIP1, p27KIP1, and p53, and slightly down-regulated cyclin B1 and cdc2. We found that curcumin can down-regulate cyclin D1 expression at the transcriptional and posttranscrip-tional levels (Reddy *et al.,* 1994; Liu *et al.,* 1993; Hasmeda *et al.,* 1996).

Adhesion Molecules

Curcumin inhibits inflammation by blocking the adhesion of monocytes to endothelial cells by inhibiting activation of these cell adhesion molecules. The expression of these molecules is in part regulated by NF-κB (Iademarco *et al.,* 1995). Gupta and Ghosh reported that curcumin inhibits TNF-induced expression of adhesion molecules on human umbilical vein endothelial cells (Gupta *et al.,* 1999). Jaiswal *et al.* (2002) showed that curcumin treatment causes p53- and p21-independent G_2 M phase arrest and apoptosis in colon cancer cell lines. Their results suggest that curcumin treatment impairs both Wnt signaling and cell-cell adhesion pathways, resulting in G_2/M phase arrest and apoptosis in HCT-116 cells.

Antiapoptotic Proteins

Curcumin is known to down-regulate expression of apoptosis suppressor proteins such as Bcl-2 and Bcl-X_L in several cancer cell lines. Cur cumin has been found to induce apoptosis through a mitochondrial pathway involving several caspases, Bid cleavage, and cytochrome C release. Aggarwal *et al.* have shown that Bcl-2 and Bcl-X_L are critical negative regulators of curcumin-induced apoptosis (Anto *et al.,* 2002). Curcumin suppresses the constitutive expression of Bcl-2 and Bcl-X_L in mantle cell lymphoma and multiple myeloma (Bharti *et al.,* 2003) cell lines. It also activates caspase-7 and caspase-9 and induces polyadenosine-5′-diphosphate-ribose polymerase cleavage in both cell lines. Thus, curcumin induces apoptosis by targeting several apoptotic pathways, inducing

cytochrome C release, Bid cleavage, and caspase-9 and -3 activation, and by down-regulating the antiapoptotic proteins Bcl-2 and $BclX_L$.

Multidrug Resistance

Multidrug resistance is a phenomenon that often is associated with enhanced drug efflux and thus decreased overall bioavailability of drug in tumor cells. It is often related to overexpression of P-glycoprotein on the surface of tumor cells, which reduces drug cytotoxicity. Curcumin has been shown to suppress the overexpression of P-glycoprotein in the multidrug-resistant human cervical carcinoma cell lines (Anuchapreeda *et al.,* 2002). Curcumin also down-regulates drug resistance by inhibiting expression of the *mdr* gene, which is responsible for this phenomenon (Limtrakul *et al.,* 2004).

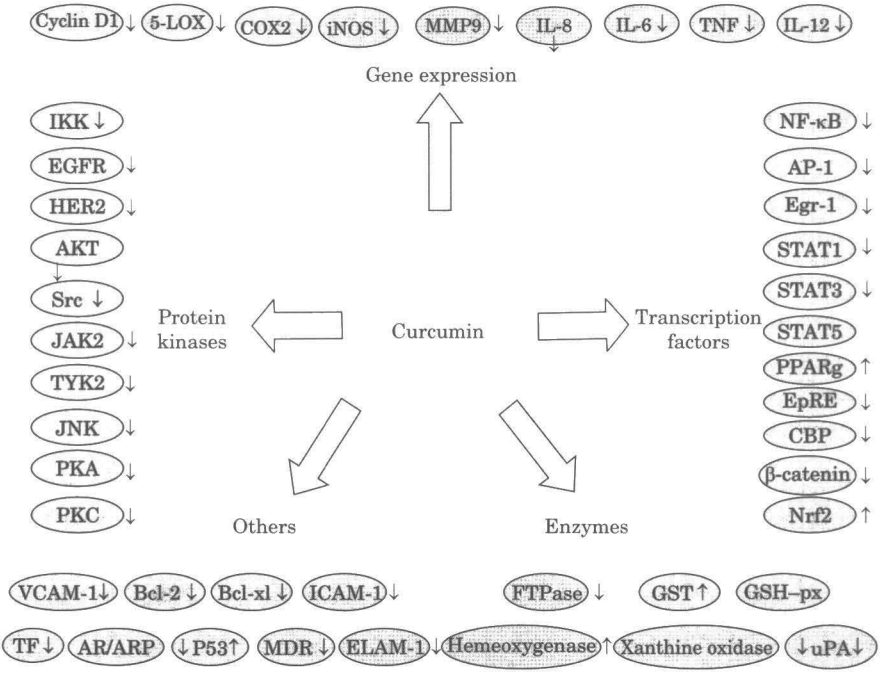


Fig 11.2. Molecular Targets of Curcumin

LIMITATIONS IN APPLICATION OF CURCUMIN AS A DRUG

The main limitations in the application of curcumin as a drug are:

- Low solubility
- Poor adsorbtion

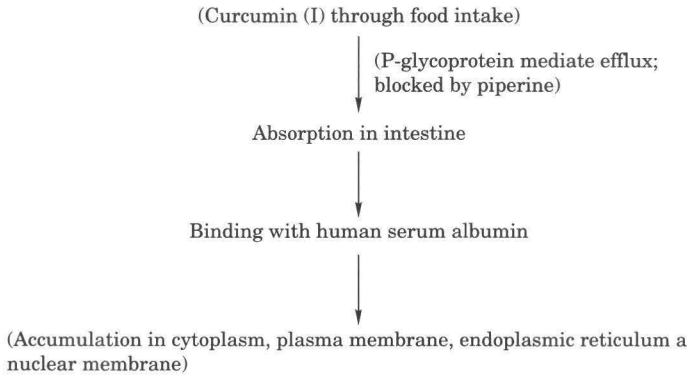
- Less bioavailability
- Fast metabolism
- Lack of targeted delivery.

SOLUBILITY

An ideal drug has to act both as lipophilic (fat soluble) to pass through the cell lining of the gut wall and also as water soluble in order to dissolve in the gut and blood serum. The two free phenolic hydroxyls in curcumin make it too polar, restricting its uptake into the cell. A solution to this problem was to mask the phenolics with an appropriate ligand which would make it less polar (hydrophobic) to facilitate its uptake from the gut and later the unmasking inside the cell to release the polar drug which can solubilize (hydrophilic) and bind with blood serum. This objective has been achieved by synthesizing its conjugates (esters) with a number or acids which are essential components of the human system and internalize well after release.

ADSORPTION

Although curcumin has a comparatively low molecular weight (368.37), but it is difficult for the molecule to squeeze through small gaps between the cells of gut wall. It has to pass from the gastrointestinal tract into the blood supply by traveling through the cell lining of the gut wall thus has to pass cell membrane on two occasions. The phospholipid bilayer of the gut and blood cells must be preventing the polar molecule from entering or leaving the cell. The absorption of curcumin is coupled with efflux of P-glycoprotein. Curcumin binds to P-glycoprotein and induces chemosensitivity (Romiti *et al.,* 1998), piperine enhances the bioavailability of curcumin by blocking this efflux via P-glycoprotein (Bhardwaj *et al.,* 2002).



Absorption of curcumin enhanced by piperine

BIOAVAILABILITY

Several studies have been carried out to determine the bioavailability of curcumin *in vitro* as well as *in vivo*. When administered orally, 75% of curcumin is excreted in the faeces while only traces appear in urine (Wahlstrom *et al.,* 1978; Ravindranath *et al.,* 1980). The *in vitro* studies were carried out to assess degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices was investigated (Ying-Jan Wang *et al.,* 1997). When curcumin was incubated in 0.1 M phosphate buffer and serum free medium, pH 7.2 at 37°C, about 90% decomposed within the 13 min. A series of pH conditions ranging from 3 to 10 were tested and the result showed that the composition was pH dependent and occurred faster at neutral-basic conditions. It is more stable in cell culture medium containing 10% fetal calf serum and in human blood; less than 20% of curcumin decomposed within 1 h, and after incubation for 8 h, about 50% of curcumin is still remained (Ying-Jan Wang *et al.,* 1997). However, several *in vivo* studies have also been reported, in one such study, patients with preinvasive malignant or high-risk premalignant conditions of the bladder, skin, cervix, stomach, or oral mucosa received 0.5–8 g curcumin by mouth daily for 3 months and their plasma curcumin concentrations were found to peak 1 to 2 h after intake and gradually declined within 12 h. The 8 g per day dose resulted in a peak serum concentration of one 1.75 ± 0.80 μM. When curcumin is micronized form was administered orally with orange juice at doses of 50–200 mg to 18 healthy volunteers, the average level of curcumin in the plasma was approximately 0.63 ng/mL. (Cheng *et al.,* 2001; Chainani-Wu, 2003). When curcumin in the form of "Curcuminoids C3" was consumed orally for upto 4 months at daily doses of curcumin between 0.45 and 3.6 g by 15 patients with advanced colorectal cancer, the average level of drug and glucuronide/sulfate conjugates detected in plasma was only 5 pmol/mL. (Mall *et al.,* 2005). When curcumin at 0.45, 1.8, or 3.6 g *per diem* was given to patients with colorectal cancer for 7 days prior to surgery, the mean concentrations of curcumin in normal and malignant colorectal tissue of patients who had ingested 3.6 g curcumin daily were 12.7 and 7.7 nmol/g tissue, respectively. These preliminary results in humans suggest that a daily dose of 3.6 g curcumin achieves measurable levels in colorectal tissue with negligible distribution outside the gut (Cheng *et al.,* 2001).

The data from these studies suggests that curcumin has low systemic bioavailability and rapid elimination from the body following oral consumption. Efficient intestinal metabolism of curcumin, particularly glucuronidation and sulfation, may explain its poor systemic availability when administered *via* the oral route. Thus, the challenge now is to present curcumin in dosage forms that will allow greater availability.

The bioavailability of curcumin is reported to be dramatically enhanced by co-ingestion of piperine (1-Piperoyl piperidine, an alkaloid present in

Piper nigrum Linn., black pepper) in both rats and humans (Khajuria *et al.,* 1998). Piperine enhances absorption by increasing intestinal brush border membrane (BBM) fluidity and microvilli length (Khajuria *et al.,* 2002). It inhibits CYP3A4 and blocks P-glycoprotein in rodents and humans, thereby, decreasing intestinal and hepatic glucuronidation and sulfation, and drug efflux. Piperine can downregulate intestinal and hepatic glucuronidation and sulfation and enhance bioavailability of curcumin by blocking it's efflux via P glycoprotein (Bhardwaj *et al.,* 2002). Interactions of curcumin with other food additives and polyphenols, specially flavonoids have also been studied. (Jesús Olivero-Verbel *et al.,* 2002; Shoskes, 1998; Shoskes *et al.,* 2006; Fujisawa *et al.,* 2006). Thus synergic effects of curcumin are possible in combination with other dietary constituents as well as drugs. Some more examples are enhancement of efficacy of anticancer drug cisplatin against fibrosarcoma and the enhancement of apoptotic effects of the pancreatic cancer drug, gemcitabine in cultured pancreatic cancer cells (Kunnumakkara *et al.,* 2007).

METABOLISM

The systemic low bioavailability of curcumin is attributable, at least in part, to it's fast metabolism. After intraperitoneal administration of curcumin (0.1 g/kg) in mice, approximately 2.25 μg/mL of curcumin appeared in the plasma within the first 15 min. After 1 h the levels of curcumin in intestine, spleen, liver, kidney and brain were 177.04, 26.06, 26.90, 7.51 and 0.41 μg/g respectively (Pan *et al.,* 1999). Thus it was observed that curcumin (I) was first biotransformed to dihydrocurcumin (III) and tetrahydrocurcumin (V) which subsequently get converted into monoglucuronide conjugates (Fig 11.3). Thus curcumin–glucoronide (II), dihydrocurcumin–glucoronide (IV), tetrahydrocurcumin–glucornide (VI) and tetrahydrocurcumin (V) are major metabolites of curcumin in mice (Lin *et al.,* 2000; Holder *et al.,* 1978). However, Ireson *et al.* (2001) reported that the major products of curcumin biotransformation in rat plasma were curcumin glucoronide and curcumin sulphate, whereas hexahydrocurcumin (VII), hexahydrocurcuminol (IX) and hexahydrocurcuminol-glucoronide (X) were present in small amounts.

Since the bioavailability of curcumin is very poor, it is required in large repetitive doses from the very low level of curcumin in blood and urine, it is currently concluded that curcumin possesses poor systemic bioavailability after oral dosing.

SYNTHETIC CONJUGATES OF CURCUMIN

In order to overcome the limitations of application of curcumin as a drug the alternatives were:

(A) either to modify the structure to get its analogs which could be more efficacious according to QSAR, or

(B) to attach it with such ligands which can assist in cellular uptake and thus yield mutual prodrugs.

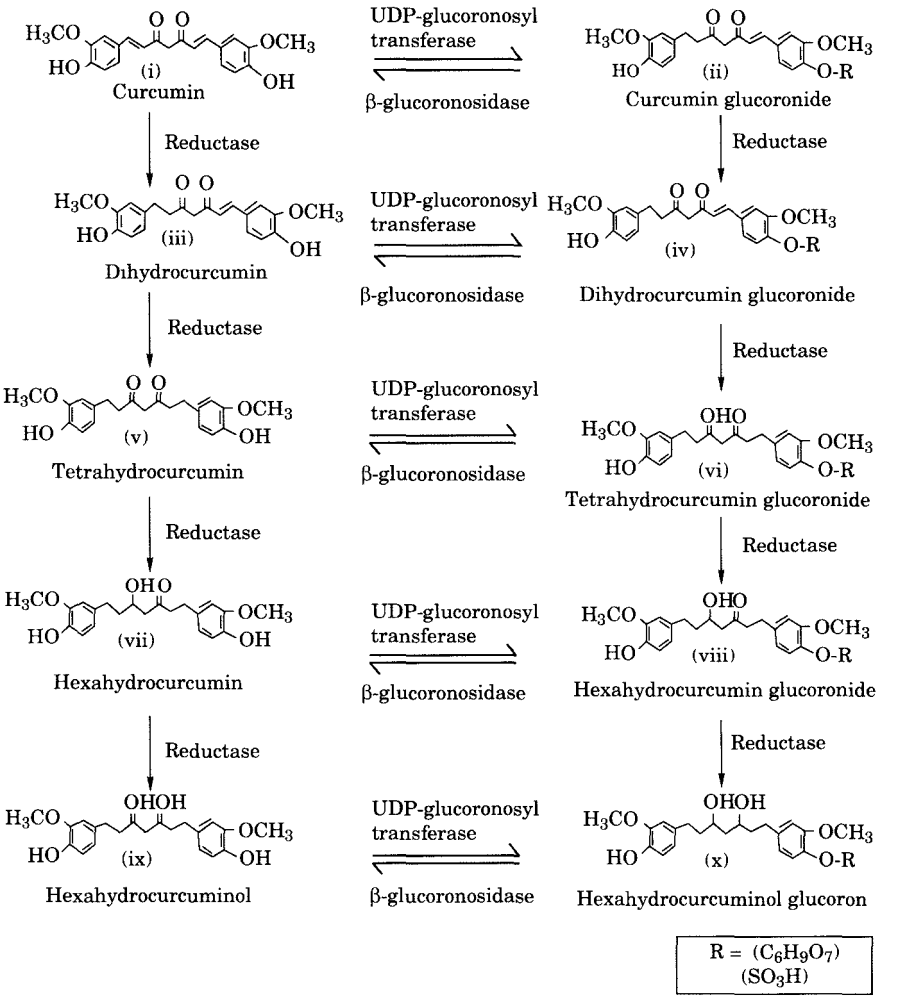


Fig 11.3. Metabolism of Curcumin

There is not much scope for structural variation in curcumin molecule. For enhancement of its activity, QSAR studies show that the presence of two phenyl rings having 3, 4-substitution is essential for activity (Shishodia *et al.*, 2006).

(A) The substitution of phenyl rings at 3 and 4 with hydroxy and methoxy functions also appear to be a must, since substitution with other groups diminishes activity. The recent reports of dimethyl curcumin having better antitumor/anticancer activity reflects doubt on the earlier presumption that phenolic groups are the sites involved in enzymes

activity at receptor site. However, the presence of free pheonolics appear to be essential for anti-inflammatory and antioxidant activities. It appears that size of the substituents in phenyl rings is more important rather than their hydrogen donor capacity. Bulkier groups are not favourable. The 2, 2′ positions should be unsubstituted.

The double bonds are essential for proper conformational flexibility of the molecule. The alternation in size of the 7-carbon linker diminishes activity.

Modifications involving this linker or substitution of active methylene group in general is unfavorable for any activity.

To summarize, it can be concluded that curcumin analogs as such have not been found to possess any significant enhancement in therapeutic activity than curcumin itself.

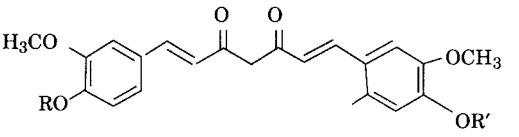
(B) The second alternative *i.e.* to attach curcumin with such ligands which can enhance its solubility and bioavailability, slow down metabolism, increase rate of cellular uptake and release the drug at target site, appears to be the method of choice. The site of attachment of ligand to curcumin molecule can be either at phenolic hydroxyls, keto groups (enol function) or active methylene group. The two phenolic hydroxyls could be utilized for generating biodegradable bonds *i.e.* esters and thus could prove as sites of choice. The main benefits for preparing the bioconjugates or mutual prodrugs of the lead compound curcumin are as follows:

- If the ligand is a natural guest, like nucleoside or amino acid, it will be recognized by its carrier protein which can then smuggle curcumin inside the cell.
- The masking of phenolic hydroxyls will delay the metabolism since glucoronide/sulfate formation can not occur unless the bond gets hydrolysed.
- Attachment of ligands may help in enhanced binding with blood serum albumin helping in their better transport and improved pharmacokinetics.
- The solubility and hydrophobicity of the mutual prodrugs might get improved.
- The biodegradable bonds may help in releasing the drug at the desired target.

Keeping in line with the above mentioned objectives, a large number of conjugates have been prepared in our laboratory and tested for their therapeutic activities. A classified account is being given in Table 11.1.

Table 11.1.

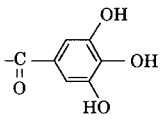
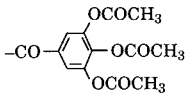
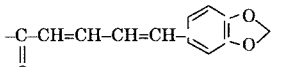
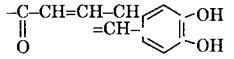
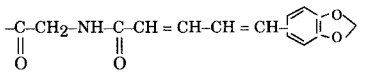
(i) Substitution at Phenolic Groups



(a) Esters

No.	R = R′	R	R′	Name of compound	References	Activity*
1.	–C(=O)–CH$_2$–NH$_2$			Di-O-glycinoyl curcumin	(Kumar *et al.*, 2001; Kumar *et al.*, 2001)	Ab, Af, Ao, Ap, Aap, Ac
2.		–C(=O)–CH$_2$–NH$_2$	H	Mono-O-glycinoyl curcumin	(Dubey *et al.*, 2007)	Ab, Af
3.	–C(=O)–CH$_2$–NH–CO–CH$_2$–NH$_2$			Tetra-O-glycylglycinoyl curcumin	(Kapoor *et al.*, 2006; Kapoor *et al.*, 2007)	Ab, Af, Ao, Ap, Ac
4.	–C(=O)–CH(CH$_3$)–NH$_2$ (D and L)			Di-O-(D & L)-alaninoyl curcumin	(Kumar *et al.*, 2001; Mishra *et al.*, 2005)	Ab
5.	–C(=O)–CH(CH(CH$_3$)$_2$)–NH$_2$			Di-O-valinoyl curcumin	(Dubey *et al.*, 2007)	Ab, Af
6.		–C(=O)–CH(CH(CH$_3$)$_2$)–NH$_2$	H	Mon-O-valinoyl curcumin	(Dubey *et al.*, 2007)	Ab, Af
7.	–CO–CH(COOH)–(CH$_2$)$_2$–NH$_2$			Di-O-glutamoyl curcumin	(Dubey *et al.*, 2007)	Ab, Af
8.	–O–C(=O)–CH(NH$_2$)–CH$_2$–S–S–CH$_2$–CH(NH$_2$)–C(=O)–O–			Cystinyl curcumin (cyclic structure)	(Mishra *et al.*, 2005; Kapoor *et al.*, 2004, 2006)	Ab, Af
9.	–C(=O)–CH(CH$_2$–SH)–NH$_2$			Di-O-cysteinyl curcumin	(Mishra *et al.*, 2005; Kappor *et al.*, 2004; 2006)	Ab, Af
10.	–C(=O)–CH$_2$–NH–C(=O)–CH(CH$_2$–SH)–NH$_2$			Di-O-cysteinyl-glycinoyl curcumin	(Mishra *et al.*, 2005; Kappor *et al.*, 2004; 2006)	Ab, Af
11.	–C(=O)–CH$_3$			Curcumin diacetate	(Mishra *et al.*, 2005; Kappor *et al.*, 2004; 2006)	Ab, Af Ap, Ao, Ai

Table 11.1. *Contd.*

No.	R = R′	R	R′	Name of compound	References	Activity*
12.				Di-O-galloyl curcumin	Kapoor *et al.*, 2004, 2006	Ab
13.				Di-O-(3, 4, 5-O-acetyl) galloyl curcumin	Kapoor *et al.*, 2004, 2006	Ab
14.				Di-O-piperoyl curcumin	Mishra *et al.*, 2005; Kappor *et al.*, 2004, 2006	Ab, Af
15.				Di-O-demethyl-enated piperoyl curcumin	Dubey *et al.*, 2007	Ab, Af, Ac
16.				Di-O-glycyl-piperoyl curcumin	Mishra *et al.*, 2005; Kappor *et al.*, 2004, 2006	Ab, Af
(b) Ethers						
17.	$-CH_3$			Dimethyl curcumin	Ohtsu *et al.*, 2002	Ac (Colon/ p rostate) Antiprot ozoan
18.	$-C_2H_5$			Diethyl curcumin	(Mukho-padhyay *et al.*, 1982)	Ai
(c) Salts						
19.	Na			Disodium curcumin	(Chattopad-hyay, *et al.*, 2004; Ghatak *et al.*, 1972)	Ab, Ai
(d) Glycosides						
20.	D-glucose			Curcumin-di-glucoside	(Mishra, S. *et al.*, 2005)	Ab

(ii) Substitution in Linker and Phenolic Functions

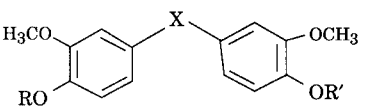
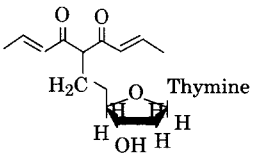
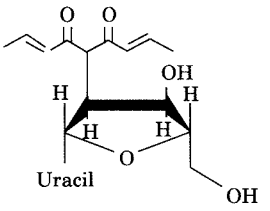


Table. 11.1. *Contd.*

No.	R = R′	R	R′	References	Activity*
21.		H	H	(Kumar *et al.*, 2001; Kumar *et al.*, 2000)	Ab
22.		H	H	(Kumar *et al.*, 2001; Kumar *et al.*, 2000)	Ab

*Ab = Antibacterial, Af = Antifungal, Ao = Antioxidant, Ac = Anticancer, Ap = Apoptotic, Aag = Antiangiogenic, Ai = Anti-inflammatory, Aap = Antiapoptotic

(iii) Analogs Conjugated with Oligonucleotides (DNA/RNA)

- The 4, 4′-O-diglycinoyl curcumin attached through a linker to a telomerase repeat sequence of DNA and tested on DU 145 prostate cancer cell lines(Mishra *et al.*, 2005).
- The 4,4′-O-tetraglycinoyl curcumin attached through a two-carbon linker to a telomerase repeat sequence of DNA, tested on KB and HeLa cell lines (Kapoor *et al.*, 2006). The 4, 4′-O-demethylenated piperoyl curcumin attached to a telomerase RNA template through phosphate group and tested on KB and HeLa cell lines (J. Drug Targeting, communicated).

The complexes in these three cases were effective in picomolar amounts, while the corresponding curcumin analogs under identical conditions were effective only in micromolar amounts.

NATURAL ANALOGUES OF CURCUMIN

Extensive SAR studies have been carried out on curcumin molecule and a large number of its synthetic analogs are known. However, curcumin molecule is unique in its physiological effects, having maximum number of molecular targets than any other molecule so far reported. In order to have a definite drug profile of this wonder molecule, it is necessary that along with its synthetic analogs, an exhaustive analysis of its naturally occurring analogs should also be carried out. Table 11.2 shows a number of naturally occurring bioactive compounds having some structural similarity to curcumin molecule, or at least having a pharmacophore containing one aryl function with 3, 4–substitution *i.e.* either methoxylated phenol or

catechol. Curcumin and its two main naturally occurring analogs *i.e.* demethoxy and bis-demethoxy curcumin together known as curcumin-3-complex or curcuminoids have 1,7 – diarylheptane as it's basic skeleton.

Table 11.2.

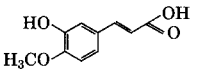
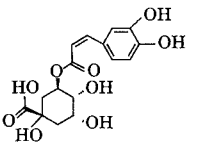
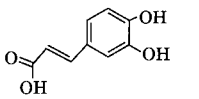
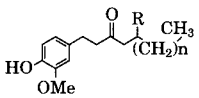
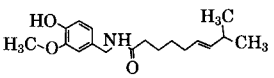
S. No.	Name of analog	Chemical structure	Biological activity	References
1.	Ferulic acid		Anticarcinogenic, reduces oxidative stress	Kampa *et al.*, 2004; Lee *et al.*, 2005; Lesca *et al.*, 1983; Mori *et al.*, 1999; Lin *et al.*, 2005; Mathew *et al.*, 2004
2.	Chlorogenic acid		Antioxidant, antiviral, antifungal, antibacterial, prevents Type-2 diabetes mellitus and cardio-vascular diseases and reduces hepatic glycogenolysis	Paynter *et al.*, 2006; Morton *et al.*, 2000; Jassim *et al.*, 2003; Rodriguez De Sotillo *et al.*, 1998; Bowels *et al.*, 1994; Johnston *et al.*, 2003; Clifford *et al.*, 1999
3.	Caffeic acid		Antioxidant, modifies gastrointestinal hormone secretion, glycemic effects & glucose tolerance	Paynter *et al.*, 2006; Rodrigue De Sotillo *et al.*, 1998; Bowels *et al.*, 1994; Johnston *et al.*, 2003; Clifford *et al.*, 1999
4.	Gingerols & Paradols	 6–Gingerol, n=4, R=OH; 8–Gingerol, n=6; R=OH; 6–Paradol; R=H; n=4	Antioxidant, antiinflammatory, antidiabetic, antihyperlipidemic, chemopreventive and cytostatic	Ernst *et al.*, 2000; Mustafa *et al.*, 1990; Ueki *et al.*, 2008; Ramesh, 2007; Lee *et al.*, 1998; Ishiguro *et al.*, 2007; Shishodia *et al.*, 2005; Nakamura *et al.*, 1983; Park *et al.*, 1998
5.	Capsaicin		Antioxidant, anticancer, pain reliever antilithogenic, mutagenic, modulates glycoprotein, inhibitor of lysozyme secretion and eicosonoids in rats	Kempaiah *et al.*, 2004; Mohamed *et al.*, 1994; Nagabhusan, 1986; Ahuja *et al.*, 2006; Joe *et al.*, 1994, 1997, 1997, 2000)

Table. 11.2. *Contd.*

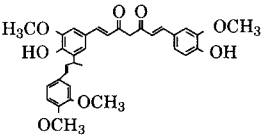
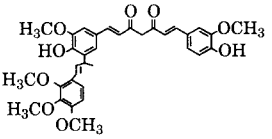
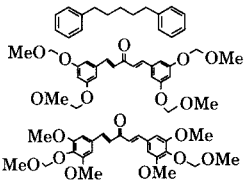
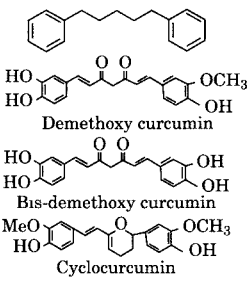
S. No.	Name of analog	Chemical structure	Biological activity	References
6.	Cassumunins A		Strong antioxidants and anti-inflammatory agents	Shishodia *et al.*, 2005; Takayuki *et al.*, 1997; Masuda *et al.*, 1980, 1998
	Cassumunins B			
7.	Diarylpentanoids Compounds with two aromatic rings joined through a 5-carbon linker		Antioxidants, Antileishmaniasis. Anticancer activities similar to diarylheptanoids	Hisatsugu *et al.*, 2006; Vignolio *et al.*, 2003
8.	Diarylheptanoids A group of compounds consisting of two aromatic rings separated by seven carbons (heptanes) and having various substituents. The best known members are curcuminoids	 Demethoxy curcumin Bis-demethoxy curcumin Cyclocurcumin	Antioxidants, anti-inflammatory, anticancer, cardioprotective, hepatoprotective, skindiseases, diabetes, rheumatoid arthritis, Nematocidal, multiplesclerosis, Alzheimer's disease, inflammatory bowel's disease, cystic fibrosis, anti-HIV, antimalarial, anti-leishmaniasis, nephrotoxicity preventive, etc.	Shishodia, *et al.*, 2005; Hyunsung *et al.*, 2006; Srivastava *et al.*, 1995; Padma, 2005; Kutluay *et al.*, 2008; Bourne *et al.*, 1999; Aggarwal *et al.*, 2004; Yang *et al.*, 2005; Ng *et al.*, 2006; Xu *et al.*, 2007; Bala *et al.*, 2006; Shoba *et al.*, 1998; US Patent, 2007; Bisht *et al.*, 2007; European patent, 2006; Kawanishi *et al.*, 2005; Moos *et al.*, 2004; Aggarwal *et al.*, 2003, 2005; Ahmed *et al.*, 2007; Campbell *et al.*, 2005; Chattopadhyay *et al.*, 2004; Goel *et al.*, 2007; Lee *et al.*, 1998; Lee *et al.*, 2005; Jurgens *et al.*, 1994; Alves *et al.*, 2003, 2003; Araujo *et al.*, 1999; Prasain *et al.*, 1998; Eunkyoung Song *et al.*, 2001

Table. 11.2. *Contd.*

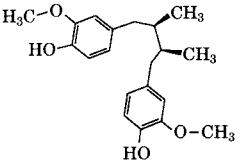
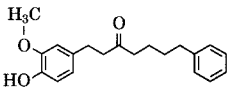
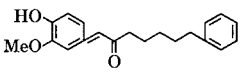
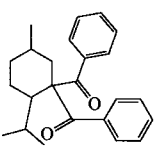
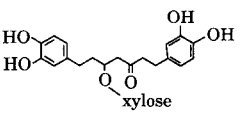
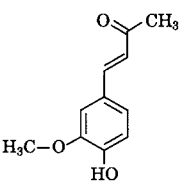
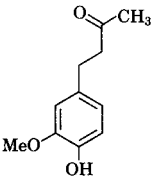
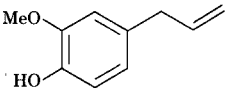
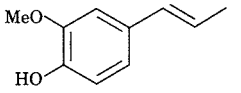
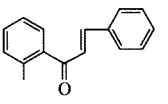
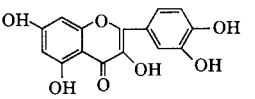
S. No.	Name of analog	Chemical structure	Biological activity	References
9.	Dihydro-guaiaretic acid		Prevents carcinogenesis, Antioxidant, inhibits primarily lipoxy-. genase, a weak inhibitor of cyclo-. oxygenase	Takaba *et al.*, 1997; Joseph, 1994
10.	Yakuchinone A		Suppress expre-ssion of COX-2 and iNOS, reduce production of tumor necrosis and hypocholeste-rolemic suppre-sses inducible Nitric oxide formation	Chun Kyung-Soo *et al.*, 2002; Chun *et al.*, 1999, 2002; Kyung-Soo Chun *et al.*, 1999; Young-Joon Surh *et al.*, 1999; Nakano *et al.*, 2004; Kenji *et al.*, 2001
	Yakuchinone B		-Do-	Chun Kyung-Soo *et al.*, 2002; Chun *et al.*, 1999, 2002; Kyung-Soo Chun *et al.*, 1999; Young-Joon Surh *et al.*, 1999; Nakano *et al.*, 2004; Kenji *et al.*, 2001
11.	Dibenzoyl-menthane		Induce apoptosis, suppress mammary tumorogenesis, nematocidal, antioxidant	Lin *et al.*, 2001; Singletary *et al.*, 1998; USA patent, 1999
12.	Oregonin 1, 7-Bis (3, 4-dihydroxy-phenyl)-3-heptanone-5-O xyloside		Antioxidant, anti-inflammatory, inhibits lipopolysaccha-ride-induced iNOS gene transcription and upregulates HO-1 expression in macrophages and microglia	Cheng-Jui Lee *et al.*, 2005; Rourke Ciara O' *et al.*, 2005; Guz *et al.*, 2002
13.	Dehydro zingerone,		Antioxidant, anti-inflammatory, prevents skin cancers, reduces lipid peroxidation, effective against nausea and motion sickness, anti-diarrheal, prevents heart attacks, self desesnsitization	Liu *et al.*, 1996; Affeltranger *et al.*, 2007; Prescott *et al.*, 1996; Chen Jaw-Chyun *et al.*, 2007; Sang-Guk Shin *et al.*, 2005; Prescott *et al.*, 1996

Table. 11.2. *Contd.*

S. No.	Name of analog	Chemical structure	Biological activity	References
	Zingerone			
14.	Eugenol		Antibacterials, antiseptics, anaesthetics, antioxidants, radical scavengers, inhibit NF-κB activation in macrophages, root canal sealer, used in dentistry & perfumery	Seiichiro *et al.*, 2004; Yoshinori *et al.*, 2007
	Isoeugenol			
15.	Flavanoids (1, 3-Diaryl-propenones)		Antioxidants, immunosuppressive, anticancer, anti-inflammatory, cholesterol lowering. Renoprotective, anti-HIV, used as mixture with curcumin	Jesús Olivero-Verbel *et al.*, 2002; Shoskes *et al.*, 1998, 2006; Fujisawa *et al.*, 2006
	Quercetin			

Besides this curcumin has two phenolic groups (at 4, 4′-positions on aromatic rings); two methoxy groups (at 3, 3′-positions on aromatic rings); two double bonds in the 7-C chain (linker); β–diketone and an active methylene group at C-4. The double bonds and ketonic functions are conjugated, accounting for the yellow color of curcumin. The structure explains the keto-enol tautomerism present in curcumin, although the amount of the enol form is much less than the stable keto form (Fig 11.1). It is evident that free phenolic groups in curcumin are hydrogen bond acceptors while those in bisdemethoxy curcumin are hydrogen bond donors, explaining the differential polarity of these curcuminoids, effecting their binding to receptors. However, free phenolic is essential for radical formation. Tetrahydrocurcumin has been found to be much stronger antioxidant than curcumin but weaker than tetrahydro bis-methoxy curcumin, indicating the importance of methoxy groups, which probably

may be due to their hydrophobic character. The chromophore in the linker, specially double bonds appear to be least important for ROS scavenging activity. Although no comparative studies on the antioxidant potential of different naturally occurring analogs of curcumin is available, a look at the Table 11.2 indicates that an ortho- methoxylated phenolic chromophore is desirable (Shoskes *et al.,* 2006; Fujisawa *et al.,* 2006; Venkatesan *et al.,* 2000), which may be present in a single aromatic ring *e.g.* ferulic acid, caffeic acid, chlorogenic acid, capsaicin, gingerols, zingerone, eugenols or may be in two aromatic rings *i.e.* oregonin, the potent nitric oxide synthase (iNOS) inhibitor, diaryl penta or heptanoids, flavonoids, dehydroguairetic acid, yakuchinones or cassumunins. The same chromophore is responsible for both *i.e.* anti and pro-oxidant properties of curcumin and analogs, which may be due to its radical generating or hydrogen bond donor/acceptor properties.

The anti-inflammatory activity is associated with para hydroxyl group and 3, 5-di alkyl groups, which should not be bulky, since derivatives with ethyl or tert-butyl groups have been found to be inactive. 5-n-Alkyl derivatives or 5-aryl substituted curcumin *i.e.* cassumunins A & B have been found to be good anti-inflammatory agents.

The linker, may be 7-membered (diarylheptanoids), 5-membered (diaryl pentanoids or 3-membered (flavonoids) appear to be responsible for antitumor, anticancer, antiparasitic activities in the same order. The inhibitory effect of tetrahydrocurcumin against tumor promoter induced ROS and Epstein-Bar virus activation was found to be much weaker than curcumin both *in vitro* and *in vivo.* The dimethylcurcumin has been reported to be more potent anticancer/antitumor agent than curcumin itself, indicating that free phenolics have least role to play, although the 3, 4-substitution of aryl ring is important. The combination of curcumin with flavonoids (quercetin) has been reported to enhance the antitumor or anti-viral activity, which needs more experimental work to be done before a proper explanation can be given.

Simon *et al.* (Sreejayan *et al.,* 1996) attributed the ability of curcumin to inhibit proliferation of breast tumor cells to diketone moiety. The naturally occurring enones (flavonoids, dehydrozingerone, oregonin, yokuchinone, chlorogenic acid etc.) or dienone (curcuminoids, cassumunins etc.) analogs of curcumin have been found to be having antiangiogenic properties (Ahsan *et al.,* 1999). As a consequence, it appears that the antioxidant, pro-oxidant, inflammatory and radical scavenging activities are associated with the methoxylated phenolic function of curcumin and its analogous naturally occurring biomolecules while the antitumor/ anticancer, antiviral, antiparasitic activities are associated with the enone or dienone functions in the side chain or linker between the two aryl functions.

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12

Structural Based Drug Design of Estrogen Receptor Beta Selective Ligands

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ABSTRACT

In preclinical research huge combinatorial libraries have generated a number of efficient drugs; increasing interest has been devoted to structure based drug design (SBDD). The advantage of this approach is that drug molecules can be 'tailor-made' to interact with the drug target. To apply SBDD, it is necessary to have access to structural information of the drug target. More than 30,000 high-resolution structures were deposited in public databases. Structural knowledge of proteins and their ligands has aided in improving drug potency and selectivity. Classically, SBDD has been exploited in lead optimization, a process that uses structure to guide the chemical modification of a lead molecule to give an optimized fit in terms of shape, hydrogen bonds and other non-covalent interactions with the target. More than 40 drugs originating from SBDD approaches have now entered clinical trials and seven of these had achieved regulatory approval and been marketed as drugs. In such examples, estrogen receptors (ER) are used as targets for the ligand development. ER are having wider role in many pathological conditions including cancer. Invention of ER β receptors opened new avenue for improvised cancer treatment. Many researches are focused on the development of selective ER β ligand, both synthetic and natural, to control cell proliferation and tumor growth. The current review mainly focus the structural difference between ER β and ER α, the binding pattern of estrogen agonists and ways to achieve ER β selectivity by using the ER ligand binding domain differences. The review will also discuss on designing drug molecule,

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protein active sites and related physicochemical properties taking estradiol and genistein as protype molecule.

Key words : Cancer, estradiol, estrogen receptors, genistein, phyto-estrogen, structure based drug design

INTRODUCTION

17β-Estradiol (E2) is a steroid hormone, which is primarily synthesized in the ovary of pre-menopausal women. E2, function as an endocrine signal, by exerting selective effects on different target tissues. E2 exerts its effects mainly in female reproductive system and in non-reproductive tissues such as the bone, cardiovascular, immune and central nervous systems. E2 elicits multiple tissue-specific responses throughout the body, resulting in beneficial but also detrimental responses (Dutertre & Smith, 2000). E2 mediate their effects through Estrogen Receptors (ERs) (Helguero *et al.*, 2005). In postmenopausal women, systemic E2 production is ceased and E2 is no longer able to function as an endocrine factor affecting distal tissues. However, both in postmenopausal women and men, E2 plays an important physiological role in a number of extra gonadal tissues. In clinical conditions E2 agonist and antagonist are employed in the treatment of hormone replacement therapy and breast cancer.

Estrogen Receptors

ERs are members of the steroid/thyroid hormone nuclear receptor super family that function as ligand-activated transcription factors (Katzenellenbogen, 1996). The biological effects of estrogens were mediated by binding to one of two nuclear ERs, namely ER α and ER β. Both ER α and ER β are ligand-dependent transcription factors that belong to the nuclear receptor family. ER α was the first ER sub-type identified in the 1960's by Jensen and his colleagues (Jensen *et al.*, 1967) and shown to be a ligand-activated transcription factor (O'Malley & McGuire, 1968; Means *et al.*, 1972). ER α was cloned in 1986 (Green *et al.*, 1986a; Greene *et al.*, 1986b) and it was generally accepted that this was the only receptor mediating the effects of estrogens. In 1996, a second genetically distinct receptor (ER β) was identified and cloned from rat prostate and ovary (Kuiper *et al.*, 1996), closely followed by cloning of human and mouse homologues (Mosselman *et al.*, 1996; Enmark *et al.*, 1997; Tremblay *et al.*, 1997) (Figs 12.1 and 12.2).

These receptor proteins share a common structural design of six distinct domains (A-F domains). These domains are responsible for ligand and DNA binding followed by transcriptional activation (Katzenellenbogen & Katzenellenbogen, 1996). In detail, the amino terminus (A and B domains) contains a transcriptional activation function (AF-1) that does not require ligand for activity (ligand independent). In stead, it is constitutively active

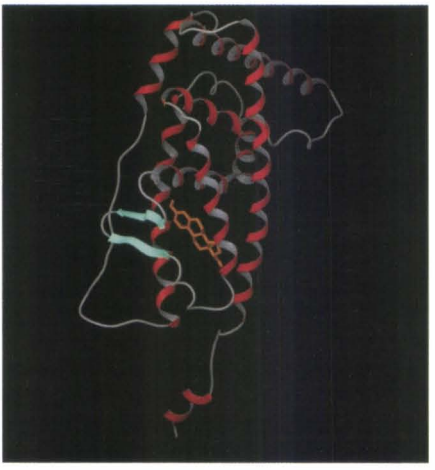


Fig 12.1.

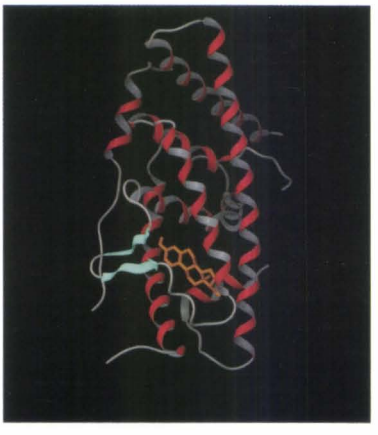


Fig 12.2.

when linked to a suitable DNA-binding domain (DBD) (Webb *et al.,* 1998). This linked DBD (C domain) consists of two zinc fingers that recognize specific DNA sequences, referred as estrogen response elements (EREs) (Schwabe *et al.,* 1990). Next to the DBD, there is a flexible hinge region (D domain) and a ligand-binding domain (LBD) (E domain). The ligand-binding cavity in association with the carboxy terminal region, which contains a ligand-dependent transcriptional activation domain (AF-2) (F domain) contributes to transcription activity (McInerney *et al.,* 1996). Upon ligand binding, conformational changes are induced leading to an interaction surface for cofactors (co-activators and co-repressors). Maximal activation of ER requires an interaction between the two activation domains AF-1 and AF-2, occurring when ligand and co-activator proteins are present. These different ER domains coordinately regulate ER mediated

transcription (Sathya *et al.,* 2002). In the initially described models, ERs reside in the cytoplasm in complex with heat shock protein 90 (HSP 90). Upon ligand binding, ERs dissociate from HSP 90, form dimers and interact with EREs within the promoter of their target genes to initiate transcription (McDonnell, 2004). ERs not solely function as transcription factors, but also serve as co-activators for other transcription factors. In addition, it seems likely that they have a function outside the nucleus to mediate very rapid cellular responses to E2. Therefore, E2 effects not only depend on the presence of its receptor, but also on the presence and abundance of several interactive proteins that are involved in these different ER pathways.

ERs are involved in several independent pathways.

1. ER initiates transcription via binding to ERE sites.
2. ER initiates or represses genes via interfering with other transcription factors.
3. ER rapidly induces effects by activating cytoplasmic proteins (phosphorylation) (Nilsson *et al.,* 2001).

Crystallographic Studies

Crystallographic studies with the LBDs of ER α and ER β revealed that both ER α and ER β share a similar overall architecture. The AF-2 interaction surface is composed of amino acids in helix 3, 4, 5, and 12 and the position of helix 12 is altered upon ligand binding. Amino acids within helices 3, 5 and 11 are important for ligand binding since mutation of these residues significantly decrease the binding affinity for E2 (Wurtz *et al.,* 1998). Deletion and mutation analysis have revealed that ER dimerization is mediated through helices 7-10 (Fawell *et al.,* 1990; Lees *et al.,* 1990).

The overall structure of the LBD is similar for several nuclear receptors and is composed of 12 helices, H1-H12, arranged together in an antiparallel, three-layered sandwich, which may include two to four β-strands (Wurtz *et al.,* 1996). Helices H1-H11 form the hydrophobic ligand-binding pocket whose entrance is guarded by H12 (Bourguet *et al.,* 1995; Uppenberg *et al.,* 1998). Agonist ligand binding induces a conformational change in many nuclear receptors resulting in alternate positioning of H12. This promotes recruitment of co-activators that interact with their short LXXLL-like motifs (where L is leucine and X is any amino acid) called NR-boxes. LXXLL-like motifs are present in many co-activators and are common motifs required for interacting with the LBD of nuclear receptors. The residues of the ligand-dependent activation function 2 (AF-2) are located in H12 (Danielian *et al.,* 1992). The structural data, together with transcriptional activation data, imply that the positioning of helix 12 is crucial for receptor activation (Warnmark *et al.,* 2003).

Crystal structures of the ER LBD complexed with E2, Diethyl stilbesterol (DES), the Selective Estrogen Receptor Modulators (SERMs) raloxifene and tamoxifen, and the pure antiestrogen ICI 164, 384, an analogue of fulvestrant (ICI 182,780), have been determined (Brzozowski *et al.,* 1997; Shiau *et al.,* 1998; Pike *et al.,* 1999, 2001). These structures reveal critical information regarding the agonist and antagonist activity of various ligands. They showed that the structure of helix 12 within AF-2 is sensitive to ligand binding. For example, when the ER α LBD is complexed with agonists such as E2, helix 12 is re-positioned over the ligand-binding pocket and it generates a functional AF-2 that interacts with LXXLL motifs of co-activators. In contrast, binding of antagonists such as raloxifene or tamoxifen with ER α-LBD results in displacement of helix 12 from its agonist position and this helix is re-positioned into the hydrophobic groove formed by helices 3, 4, and 5. This disrupts formation of the co-activator interaction surface (Shiau *et al.,* 1998; Pike *et al.,* 1999) and the ligand-dependent effects on helix 12 positioning is dependent on the agonistic or antagonistic activity of various ER ligands (Brzozowski *et al.,* 1997; Pike *et al.,* 2001). This forms the molecular basis for the action of selective estrogen receptor modulators.

Difference Between ER α and ER β Receptor

ER α and ER β receptor cavities are relatively flexible and depending on the nature of the bound ligand, the shape of the cavity may change significantly. Depending on the cellular and promoter context, certain ER ligands can function either as agonists or antagonists. For example, the high affinity ER ligand, tamoxifen, functions as an antagonist in breast and agonist in the uterus (Yamamoto *et al.,* 2003). In contrast, raloxifene functions as an antagonist in both tissues. One mechanism contributing to the tissue-selective effects of tamoxifen is the ratio of coactivators to corepressors expressed in the tissue. Binding of different agonists may cause small shifts in the position of H12 and thereby modulate the affinity of coactivators for nuclear receptors. Thus, ligands span a spectrum ranging from partial to full to super agonism (Pike *et al.,* 1999).

Other than helix 12 movements, several of the amino acid residue side chains, especially those in the pocket that harbors the C, D ring of steroids, can adopt alternative conformations. As the receptor-binding cavity is flexible, various ligands induce different conformations that may be transmitted to the outer surface of the protein. These changes can result in differential binding of various cofactors resulting in altered pharmacology. The plasticity of the receptor-binding cavity complicates the structure-based design of ligands (Pike *et al.,* 1999). As modifications of ligands are made to improve affinity or selectivity, unexpected changes in receptor conformation or ligand binding may occur.

The two ERs share great homology in their DNA binding domains (95%),

but their ligand binding domains share only 58% amino acid sequence identity. Although both subtypes bind to the natural ligand E2 with high and nearly equal affinity, the differences in their LBD's make the development of ER subtype-selective ligands possible. Such subtype-selective ligands are proving to be useful tools in probing the physiological function of each ER subtype and in studying structure-function relationships of the ligand-receptor complexes (Malamas *et al.,* 2004).

Number of ER subtype-selective ligands has been developed. Triaryl propyl pyrazole triol was found to be an ER α specific agonist, activating gene transcription only through ER α. By adding a basic side chain to the pyrazole core, a highly ER α selective antagonist, called methyl piperidino pyrazole was developed. Some of ER α selective ligands are effective agonists on ER α but are full antagonists on ER β (*e.g.* R, R-diethyl-tetrahydro-chrysene). Ligands that are ER β selective are also known. Some phytoestrogens such as genistein, coumesterol show some selectivity toward ER β, although they activate both ER α and ER β. They show modest selectivity towards ER β (Sun *et al.,* 2003).

Progress towards the development of subtype-selective ligands was significantly advanced with the reports of crystal structures of ER α and ER β. The two receptors differ in size, with ER α having 595 amino acids and ER β having 485 amino acids. The ligand binding pockets of the subtypes are similar but not identical. The ER β ligand-binding cavity is smaller (390 $Å^3$ versus 490 $Å^3$ for ER α). An analysis of the two receptors ligand binding cavity [each composed of 23 amino acids in direct vicinity (4Å) to E2] revealed two amino acid differences: Leu-384 and Met-421 in ER α are replaced by Met-336 and Ile-373, respectively, in ER β. The reduction in ligand binding cavity was due to the replacement of the leucine at position 336 {384} in ER α by a bulkier methionine in ER β. Although the volume of the sulphur-containing methionine side chain (volume = 85.9$Å^3$) is slightly larger than the branched amino acid side chains of leucine and isoleucine (volume = 82.6 $Å^3$ and 82.3 $Å^3$ respectively).

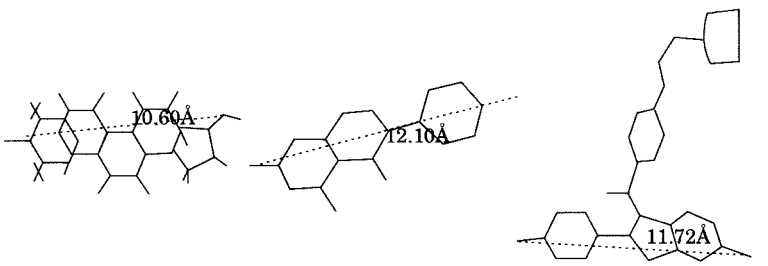
The increased flexibility of the linear methionine side chain would allow larger substituents to be accommodated. Notably, these two substitutions give rise to the selectivity of ligands for ER α and ER β (Hillisch *et al.,* 2004). Although ER β selective ligands have been designed but, only a small number of crystal structures are complexed with ER β and ER α.

Structural Requirements for Estrogen Receptor Binding

Estrogen receptors bind with steroidal as well as numerous nonsteroidal compounds. An aromatic ring and a hydroxyl group are important for binding effective and the remainder of the ER will accommodate hydrophobic groups (Anstead *et al.,* 1997). Structure-activity relationships may provide clues to the molecular basis for this agonism and antagonism (Brzozowski *et al.,* 1997). Important features that enable chemicals to bind

to an ER are the steric and hydrophobic properties of a compound, as well as the hydrogen bonding between the phenolic hydroxyl group and the ER binding site. Phytoestrogens are composed of a planar ring system that includes a *p*-hydroxy-substituted aromatic ring that is approximately 12 Å away from a second in-plane hydroxyl group (Hu & Aizawa, 2003). Two ring structures separated with two carbon atoms as well as spacing between hydrophobic and hydrogen bond interactions are also important in binding affinity to ERs (Brzozowski *et al.*, 1997). When the structures of the isoflavones metabolite equol and estradiol are overlaid, they can be virtually superimposed; the distance between the hydroxyl groups at each end of both molecules is virtually identical. Other characteristics for ER-binding affinity of a chemical are the degree and size of branching of the alkyl group and its location on the phenolic ring and the distribution range of electron density on the A ring (Hu & Aizawa, 2003).

Structure Activity Relationship of various ER ligands shows the presence of a hydrophobic ring system with polar groups (usually –OH) attached terminally. This configuration is required for the ligand to enter and bind to the ligand binding domain, a hydrophobic pocket, of the estrogen receptor. In addition ER-α antagonists (*e.g.* raloxifene) have a large side chain which hinders the movement of the Helix 12 of the receptor.

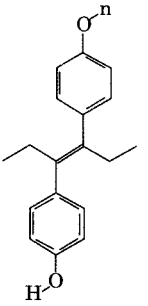


E2 (ER-α, β Agonist) **Genistein (ER-β Agonist)** **Raloxifen (ER-α Antagonist)**

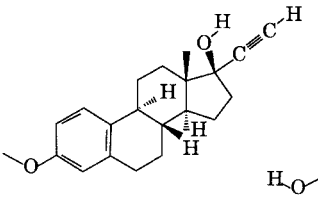
Interatomic distances between terminal hydroxyl atoms of Estradiol (ER-α, β agonist), Genistein (ER-β agonist) and Raloxifen (ER-α antagonist) were calculated using Deep View software program.

Recent efforts have been focused on the fine discrimination in ER–ligand interrelationships that allow different ligands to strongly discriminate between ER α and ER β, and the marked modulation of ER activity by co-regulators. In the development of optimal selective estrogen receptor ligands, one is aiming for tissue selective ligands that will be estrogen antagonists in the breast and uterus, thereby not stimulating cell proliferation in these tissues, while being good estrogen agonists in bone, the cardiovascular system, in terms of lipid profiles, and in the brain where cognitive functions and other activities may be regulated by estrogens. Small changes in ligand structure can result in major changes in the biological

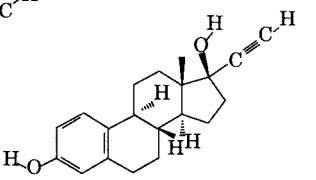
character of the receptor (Katzenellenbogen *et al.,* 2000).



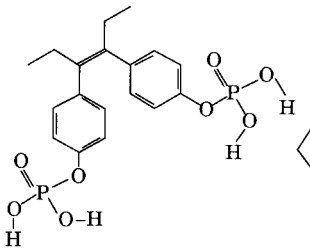
Synthetic ligands
Diethyl stilbestrol



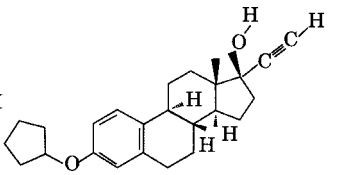
Mestranol



Ethinyl estradiol



Fosfestrol

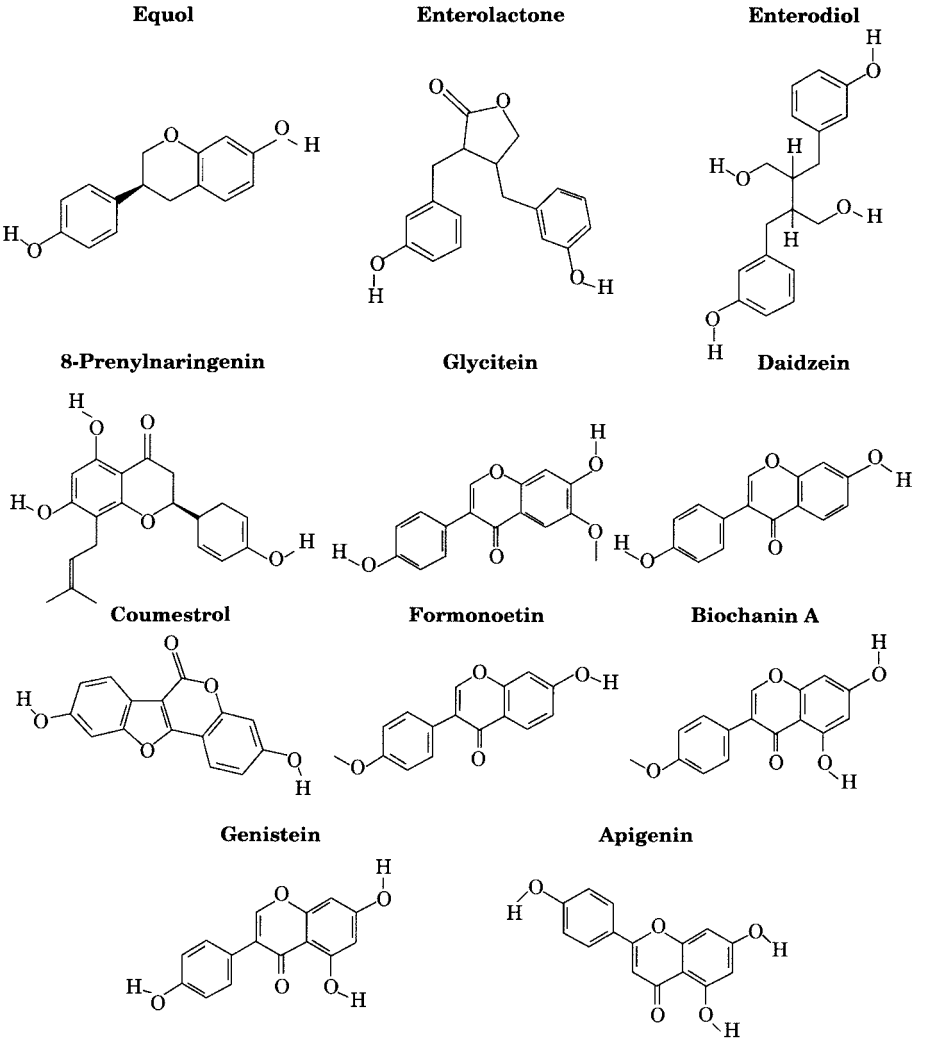


Quinestrol

Natural Estrogens

Phytoestrogens are able to interact with enzymes and receptors, and because of their stable structure and low molecular weight they can pass through cell membranes (Adlercreutz, 1998). These interactions allow them to bind to ERs, induce specific estrogen-responsive gene products, stimulate ER-positive breast cancer cell growth (Kurzer & Xu, 1997), interfere with steroid hormone metabolism or action (Adlercreutz, 1998) and alter ER structure and affect transcription (Santti *et al.,* 1998). Some genomic mechanisms of action include estrogenic and antiestrogenic effects on ERs, while other effects may not involve direct interaction with ERs (Messina & Loprinzi, 2001). Nongenomic effects that do not involve ERs include: induction of cancer cell differentiation, inhibition of tyrosine kinase and DNA topoisomerase activities, suppression of angiogenesis and antioxidant effects of phytoestrogens (Kurzer & Xu, 1997). Other effects can take place at the cellular and molecular level and potentially influence the biosynthesis and metabolism of steroids and fatty acids, the serum steroid carrier proteins (sex steroid binding proteins and α-fetoprotein), and the intracellular and transmembrane transfer of hormones to a membrane and to nuclear receptors (Benassayag *et al.,* 2002).

Below shown structures are some of the phytoestrogens which shows ER-β selectivity.



Tissue expression pattern ER α and ER β

Estrogens that lack marked selectivity for either ER, such as 17β-estradiol or 17α-ethinyl estradiol, have been well characterized for their effects on several organ systems. These include stimulation of the uterus, facilitation of proliferation/endbud development in the mammary gland, control of ovulation, maintenance of bone mineral density, and negative feedback to the hypothalamus and pituitary. As reviewed extensively, mice not expressing ER α lack many of these responses (Harris, 2007).

Since ER α and ER β have distinct transcriptional abilities, which could even be opposite to each other, their tissue specific expression pattern is a determinant of the E2 mediated effects. Both ERs are widely distributed

throughout the body. ER α is expressed primarily in the uterus, liver, kidney, and heart (Nilsson & Gustafsson, 2000), whereas ER β is expressed at high levels in other estrogen-target tissues such as the prostate, salivary glands, testis, ovary, vascular endothelium and smooth muscle, certain neurons in the central and peripheral nervous systems, and the immune system (Koehler *et al.,* 2005). Tissues, which express both ER α and ER β, are the mammary gland, the adrenals, bone, adipose tissue, vascular endothelium and smooth muscle cells and certain regions of the brain. In these tissues, there is a potential interplay between the two ERs, and thus their balance is important. For certain genes it has been found that, ER β exhibits an inhibitory activity on ER α-mediated gene expression (Kuiper & Gustafsson, 1997).

Uterus

ER β is expressed during development whereas ER α is expressed when the tissue matures (Nishihara *et al.,* 2000; Brandenberger *et al.,* 1997). In the immature uterus, ER α and ER β are expressed at comparable levels in the epithelium and stroma; E2 treatment decreases ER β in the stroma and increases ER α in both compartments (Weihua *et al.,* 2000). In the mature uterus, ER β plays a role in cervical ripening, which is essential for parturition and in decidualization, which is essential for implantation of the fetus. Endometriosis, an E2 dependent growth of uterine tissue in the abdominal cavity that is common in women during the reproductive years. Studies show that the ratio of ER α to ER β mRNA is increased in endometriotic tissue (Matsuzaki *et al.,* 2000). Because of its antiproliferative effects in the uterus, ER β may have a role to play in controlling growth of endometriotic lesions.

Breast

ER α is essential for ductal growth and in ER α-/-mice there is very little growth of mammary ducts (Lubahn *et al.,* 1993). ER β is more abundant in the normal breast and examination of the ductal epithelium of ER β-/-mice suggests that it is a prodifferentiative factor. Studies on the ER β-/-mouse mammary gland showed that ER β regulates levels of several proteins characteristic of differentiated cells (Forster *et al.,* 2002). Some but not all, proliferating cells express ER β, indicating that ER β is not essential for proliferation. *In vitro* experiments with MCF7 cells suggest that ER β is antiproliferative. ER α-positive MCF7 breast cancer cells respond to E2 with increased proliferation. When ER β is introduced in to these cells, E2 induced proliferation is inhibited (Strom *et al.,* 2004). Ductal cells in the mammary gland appear to be one example of cells where ER α and ER β oppose each other on proliferation and the proliferative response to E2 is determined by the ratio of ER α/ER β. The functions of ER β in the breast are probably related to its antiproliferative as well as its prodifferentiative

functions. Tamoxifen, ER antagonist is useful only in breast cancers that express ER α. The presence of ER β shows the promise that ER β selective ligands might be of some use in treatment of these cancers (Koehler *et al.*, 2005).

Prostate

In the prostate, both ER α and ER β exist with ER α in the stroma and ER β mainly in the epithelium. In humans, ER β has been found in normal, benign prostatic hyperplasia and prostatic cancer. During normal development, ER β is never expressed in proliferating epithelial cells of prostate. Further βERKO mice model favours an antiproliferative role of ER β. These observations suggest that ligands specific for ER β may be useful in the prevention and/or clinical management of prostatic hyperplasia and neoplasia (Weihua *et al.*, 2002). In the prostate, other than 17β estradiol there is another ER ligand, 5α-androstane-3 β, 17 β-diol (3 βAdiol), whose level is 100 times higher than that of estradiol in the prostate (Kuiper *et al.*, 1998). Many studies have shown that 3 βAdiol is a good ER ligand. Though the affinity of 3 βAdiol for ER is 10-fold lower than that of estradiol, its concentration in the prostate makes it the more likely endogenous ligand for ERs in the prostate (Weihua *et al.*, 2002).

Central Nervous System

Estrogen has influence on development, plasticity and survival of neurons. Estrogen is an important regulator of serotonergic, dopaminergic and cholinergic neurons. ER α is the predominant receptor subtype in the basal forebrain cholinergic neurons of the adult rat brain (Miettinen *et al.*, 2002). In the anterior dorsal raphe nucleus, estradiol increases the amount of the serotonin receptor mRNA and the serotonin transporter mRNA. ER β is expressed in serotonergic neurons in the dorsal raphe nucleus of the mouse. A species difference in ER β expression is seen in the dorsal raphe nucleus. The rat appears to be different from the mouse in that there is no ER β in the rat dorsal raphe, but in, primates, ER β is expressed in serotonergic neurons. In cynomolgus monkeys, phytoestrogens from soy, which are relatively selective for ER β, improve mood and enhance serotonergic transmission in the dorsal raphe (Shively *et al.*, 2003). In future, ER β may be an attractive target for novel antidepressant drugs. The dopaminergic system is also estrogen responsive. Within the nigrostriatal dopaminergic system, estrogen modulates tyrosine hydroxylase activity, dopamine metabolism and dopamine receptors. Parkinson's disease is more prevalent in males, and long-term estrogen therapy enhances dopaminergic responsiveness in postmenopausal women (Craig *et al.*, 2004).

Cardiovascular System

E2 is implicated in many vascular disorders. The risk of cardiovascular disease increases after the decline in E2 at menopause, and E2 replacement relieves perimenopausal hot flushes. Neonatal cardiomyocytes in culture express both ER α and ER β in their nuclei, but these receptors are not detectable until E2 was added to the culture medium (Grohe *et al.,* 1997). Some studies have reported that ER β cannot be detected in the adult heart (Jankowski *et al.,* 2001), whereas in others, ER β is abundant in the nucleus (Savolainen *et al.,* 2001) and in one study ER β was exclusively mitochondrial (Xu *et al.,* 2003).

Immune System and Lungs

ER β functions early in the bone marrow to regulate proliferation of the progenitor cells and loss of ER β in ER β-/-mice leads to myeloproliferative disease (Shim *et al.,* 2003). ER α has its important functions in the spleen and thymus, and loss of ER α leads to autoimmune disease with attendant glomerulonephritis (Shim *et al.,* 2004). ER β is very abundant in the alveoli of the lung, and ER α is abundant in the large bronchi (Koehler *et al.,* 2005).

S. No.	Tissue	ER α function	ER β function
1.	Uterus	Proliferative	In cervical ripening; antiproliferative.
2.	Breast	Ductal growth	Antiproliferative and prodifferentiative
3.	Prostate	Proliferative	Antiproliferative
4.	CNS	Predominant receptor subtype in the basal fore-brain cholinergic neurons	Predominantly expressed in serotonergic neurons in the dorsal raphe nucleus.
5.	Immune system and lungs	Have important functions in spleen and thymus.	In bone marrow to regulate proliferation of the progenitor cells.

CONCLUSIONS

Invention of software program in cheminformatics and bioinformatics made easier for the scientist to develop new chemical entity. As reports indicate that, the predictability on ligand receptor interaction is around 80%. Hence, it can be assumed that the success rate in new drug development through *in silico* method is fair enough. The invention of ER β receptor increased the interest among the researchers to develop newer selective ligands aiming anticancer activity. ER ligands having hydrophobic ring system with polar groups (usually –OH) attached terminally with low molecular ellipsoidal volume will have better selectivity to ER β.

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