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PREFACE

This volume of the series presents timely discussions on some of the important groups of alkaloids derived biosynthetically from phenylalanine and tyrosine. These authoritative chapters provide contemporary commentary on various aspects of alkaloid occurrence, synthesis, biosynthesis, and in one instance, efforts to enhance production to meet commercial needs.

Ravelo and coworkers offer a comprehensive overview of the Amaryllidaceae alkaloids present in the genus *Pancratium*, with an emphasis on using their NMR spectroscopic properties to distinguish the individual alkaloid types. Also covered in detail are important aspects of their synthesis, and the broad spectrum of biological properties which the alkaloids represent.

Another small and interesting group of alkaloids with a quite limited chemotaxonomic distribution is the *Erythrina* alkaloids. These alkaloids possess some very interesting structural diversity, which has inspired the creativity of the synthetic organic chemistry community. Parsons and Palframan provide an overview of this area for the first time since Volume 48.

The genus *Duguetia* produces a range of isoquinoline alkaloids, including those of the benzylisoquinoline type, the aporphinoid type, and the berberine type. Although only a small number of the species in the genus have been examined thus far, Pérez and Cassals have, for the first time, summarized these results, and the associated synthetic and biological efforts, and made chemotaxonomic comparisons with the botanically close genus *Guaterria*. Of particular interest is the diverse pharmacology, some of it potentially useful, which has recently been disclosed for these alkaloids.

The most recent alkaloid addition to pharmacotherapy is the introduction of galanthamine, an alkaloid from several genera in the Amaryllidaceae. A summarizing chapter from Heinrich on the historical aspects and background of galanthamine as a drug is followed by an extensive review of galanthamine production from the group of Stanilova based in Bulgaria. The chapter brings focus to the research efforts over many years to enhance the availability of galanthamine from natural sources. This is a classic issue for all natural product development, and this intimate and personal account of the broad diversity of approaches tried, and the sometimes unexpected results obtained, makes fascinating reading.

Geoffrey A. Cordell
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CHAPTER 1

Chemistry and Biology of *Pancratium* Alkaloids

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I. INTRODUCTION

The Amaryllidaceae is a widely distributed, monocotyledonous family, represented by 59 genera and over 850 species all over the world (Figure 1) (1,2). South America (28 genera) and South Africa (19 genera) are the regions with major diversity. The Mediterranean region, being the source of numerous horticultural introductions, has only eight genera; whereas Australia has only three genera. Plants in the Amaryllidaceae occupy many different habitats: seasonally dry places, ephemeral pools, rainforests understory, and rivers. Currently, molecular evidence places the most ancient lineages and the origin of the family in Africa (3).

Plants belonging to the Amaryllidaceae family are known for producing an exclusive group of alkaloids, named ''Amaryllidaceae alkaloids,'' isolated from plants of all genera of this family. Since the isolation of lycorine (**1**) from *Narcissus pseudonarcissus* in 1877, over 300 alkaloids have been isolated from plants of this family (4), including those alkaloids recently isolated from *Pancratium canariensis* (5). Although their structures appear to be very different, these alkaloids are known to be formed biogenetically from norbelladines.

A. Classification of the Amaryllidaceae Alkaloids

According to their structures, the Amaryllidaceae alkaloids are classified into eight skeleton types (6), for which the representative

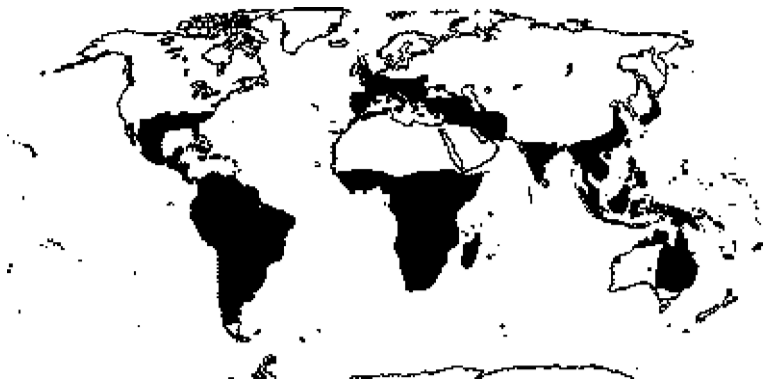


Figure 1 Distribution of plants of the Amaryllidaceae family.

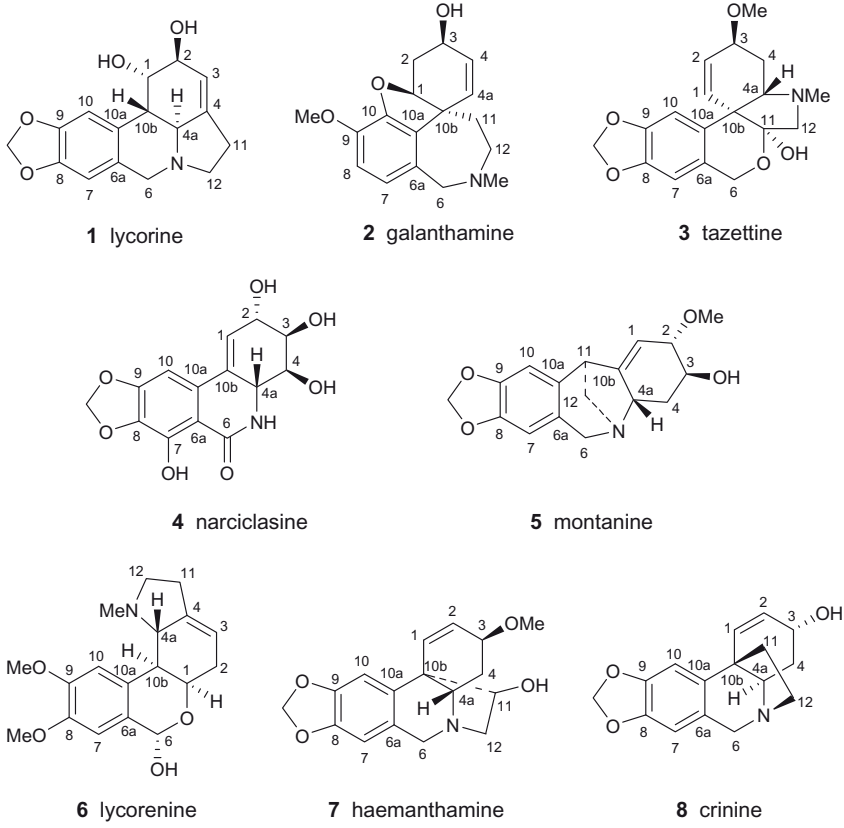


Figure 2 Amaryllidaceae alkaloid types.

alkaloids are: lycorine (**1**), galanthamine (**2**), tazettine (**3**), narciclasine (**4**), montanine (**5**), lycorenine (**6**), haemanthamine (**7**), and crinine (**8**) (Figure 2). Ghosal's model has been used for numbering each skeleton (7). Recently, Unver and Jin have proposed subgroups for some skeleton types, according to the structures of new alkaloids isolated from *Galanthus* species (8,9).

B. Biosynthesis

Except for galanthamine, most of the biosynthetic research on the Amaryllidaceae alkaloids was developed in the 1960s and 1970s. Biosynthesis of this class of alkaloids is the result of several intramolecular oxidative couplings of precursors related to norbelladine (**12**) (10). Norbelladine (**12**) is formed through the combination of 3,4-dihydroxybenzaldehyde (**10**) with tyramine (**9**); these two precursors arising from phenylalanine and tyrosine, respectively (Figure 3). After reduction of

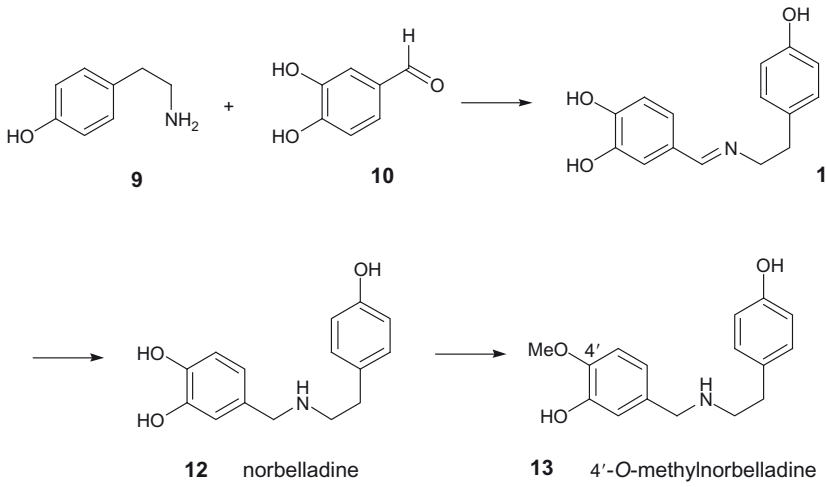


Figure 3 Biosynthesis of norbelladine (**12**) and 4′-*O*-methylnorbelladine (**13**) from tyramine (**9**) and 3,4-dihydroxybenzaldehyde (**10**).

the resulting Schiff's base **11** and methylation of norbelladine, 4′-*O*-methylnorbelladine (**13**) is obtained. Alkaloid **13** is considered the key intermediate in the elaboration of most of the alkaloids (11). 4′-*O*-Methylnorbelladine (**13**) can undergo three types of oxidative couplings: *ortho*−*para*′, *para*−*para*′, and *para*−*ortho*′ (Figure 4).

Lycorine (**1**) is formed through an *ortho*−*para*′ coupling, while the crinine (**8**) and haemanthamine (**7**) skeletons are formed from a *para*−*para*′ coupling. The galanthamine skeleton (**2**) is biosynthesized from a *para*−*ortho*′ coupling (Figure 4).

A precursor of lycorine (**5**), norpluviine (**14**) is also an intermediate in the formation of the lycorenine skeleton (**6**). In this case, norpluviine (**14**) is oxidized at the benzylic position to yield a cyclic hemiaminal group, which is in equilibrium with the corresponding open form. The rotation of the C-10a−C-10b bond of the amino aldehyde intermediate, followed by hemiacetal formation and methylation, provide the lycorenine core (**6**) (Figure 5) (12).

The alkaloid haemanthamine (**7**) is the precursor of the tazettine (**3**), montanine (**5**), and narciclasine (**4**) skeletons. Haemanthamine (**7**) is formed through a *para*−*para*′ coupling as the enantiomeric skeleton of crinine (**8**). The biosynthesis of tazettine (**3**) has been studied and described (Figure 6) (13). It involves the oxidation of haemanthamine (**7**) to haemanthidine (**15**) followed by ring opening to form the amino aldehyde equilibrium system, which, after hemiacetal formation and methylation, yields pretazettine (**16**) (14). Although pretazettine (**16**) has been identified in plants, it is unstable either in basic or acid solutions, and it converts slowly into tazettine (**3**) (15). Biosynthesis of the

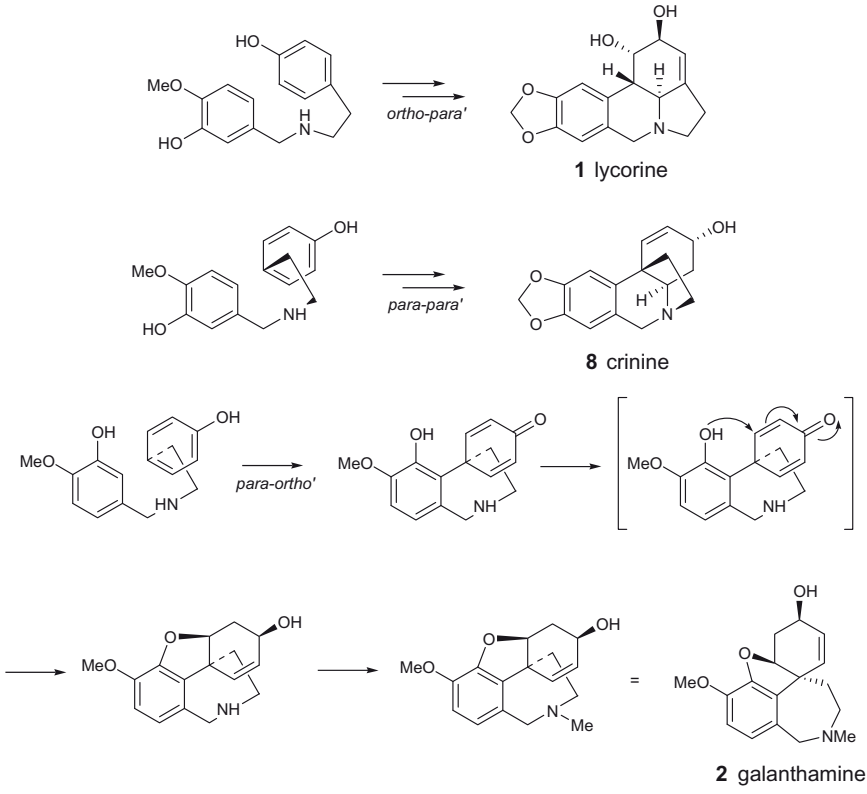


Figure 4 Phenol oxidative couplings of 4′-*O*-methylnorbelladine (**13**) to yield the lycorine, crinine, and galanthamine skeletons.

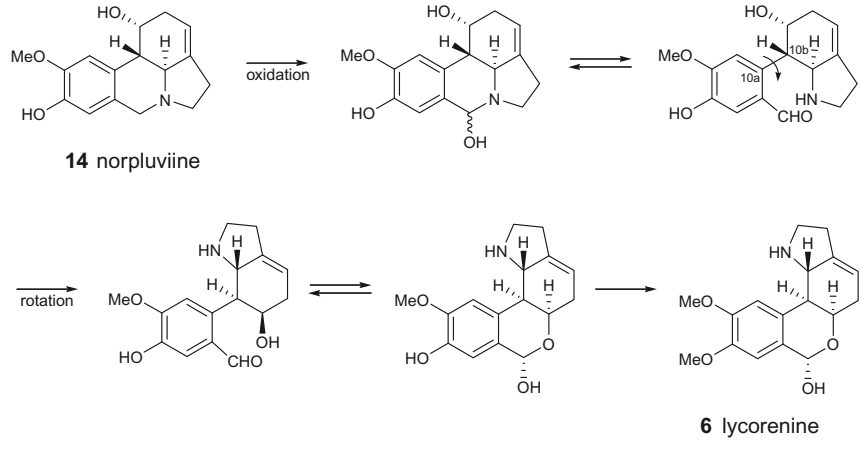


Figure 5 Biosynthesis of lycorenine (**6**) from norpluviine (**14**).

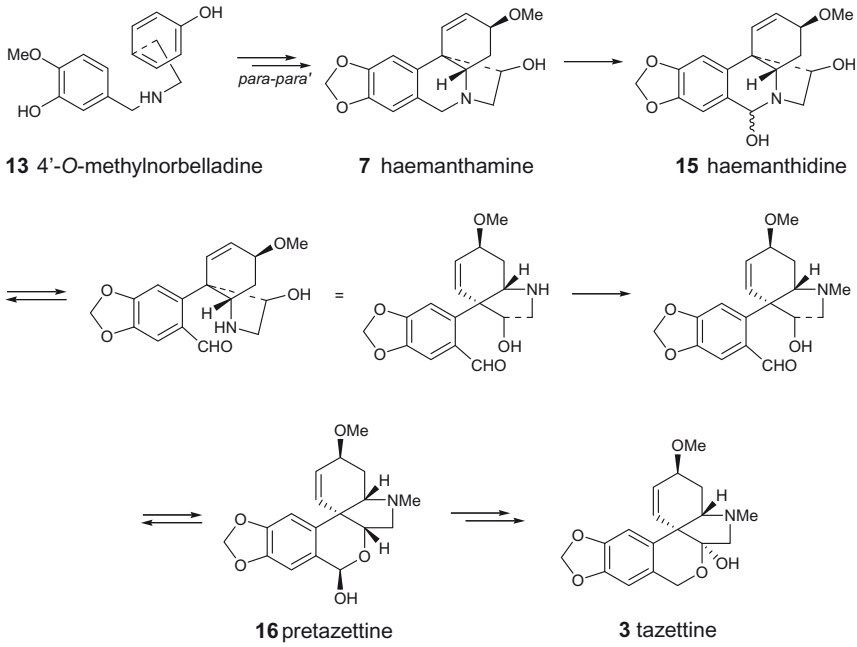


Figure 6 Biosynthesis of the tazettine skeleton from haemanthamine (**7**).

montanine (**5**) and narciclasine (**4**) skeletons still remains unknown, although some experiments involving isotopic species were performed (16).

II. *PANCRATIUM* GENUS

A. General Aspects of the *Pancratium* Genus

The name *Pancratium* derives from the Greek word ''pagkration,'' meaning almighty, perhaps because of the medicinal properties that species from this genus possess. *Pancratium* is the most widespread of all the genera in the Eurasian clade of Amaryllidaceae (3). Over 21 species have been identified in this genus, widely distributed exclusively around the Canary Islands, Northern Africa, the Mediterranean, and Indic areas (17). Table I shows the identified *Pancratium* species and the main areas where they can be located (17,18).

Some species, previously considered as members of the genus *Pancratium*, have been moved to other genera, such as *Proiphys* (19), *Vagaria* (20), and *Hymenocallis* (21) in the Amaryllidaceae. For example, *Pancratium littorale* was reclassified in 1993 as *Hymenocallis littoralis* (22).

Table I *Pancratium* species and their distribution

Species	Location
P. arabicum Sickenberger	Egypt to Sinai
P. biflorum Roxb.	India, Hong Kong, Sri Lanka
P. canariense [Ker-Gawl.]	Canary Islands
P. centrale (A. Chev.) Traub.	Central Africa, N.E. Cameroon to W. Ethiopia
P. donaldi Blatt.	Western India
P. foetidum Pomel	Northern Africa
P. illyricum L.	Italy, Island of Capri
P. landesii Traub.	Oman
P. longiflorum Buch.-Ham. ex Roxb.	Bangladesh, India
P. maritimum L.	Mediterranean, Canary Islands
P. maximum Forssk.	S. Arabian Penn., N.E. Sudan
P. parvicoronatum Geerinck	Zaire, Malawi
P. parvum Dalzell	Western India
P. sickenbergeri Asch. & Schweinf. ex Boiss.	Syria to the Arabian Penn.
P. st.-mariae Blatt. & Hallb.	Western India
P. tenuifolium Hochst.	Tropical and Southern Africa
P. tortuosum Herb.	Egypt to Eritrea, West Arabian Penn.
P. trianthum Herb.	West and Tropical Africa
P. triflorum Roxb.	Eastern India
P. verecundum [Soland.]	Pakistan to the Himalayas
P. zeylanicum L.	India to Malaysia

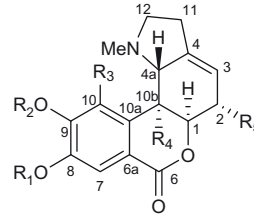
B. Botanical Description

General characteristics of the genus: the leaves are annual, sometimes hysteranthous, rarely finely pubescent (Figure 7). Spathe bracts 2, free or rarely fused into a tube. Flowers 1–10 (3), generally fragrant; perigone crateriform, white; tube slender, cylindrical below, funnel-form above; tepals lanceolate, spreading. Staminal filaments united into a staminal cup, usually toothed between each free filament, free filament incurved or spreading outwards. Stigma capitate to obscurely 3-lobed. Ovules numerous per locule. Seeds globose, subglobose, or wedge-shaped by compression, sometimes with a white elaiosome ($2n$=22) (2).

C. Alkaloids and Other Secondary Metabolites Isolated

The species *Pancratium maritimum* has been exhaustively studied and, consequently, over half of all of the alkaloids isolated from *Pancratium*


Figure 7 *Pancratium maritimum* (left) and *Pancratium canariense* (right).



17	R_1+R_2=-CH_2-, R_3=R_4=H, R_5=OH	hippeastrine
18	R_1=H, R_2=Me, R_3=R_4=R_5=H	(+)-9-*O*-demethylhomolycorine
19	R_1+R_2=-CH_2-, R_3=OH, R_4=H, R_5=OH	10-norneronine
20	R_1+R_2=-CH_2-, R_3=H, R_4=R_5=OH	pancratinine A

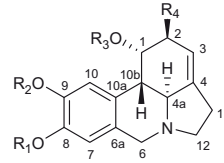
Figure 8 *Pancratium* alkaloid structures of the lycorenine type.

plants have been identified in this species. The alkaloids listed in the present review have been classified according to their skeleton types.

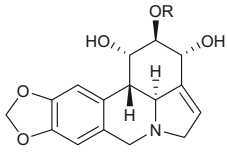
1. Lycorenine and Lycorine Types

Alkaloids of the lycorenine group are not very common in the Amaryllidaceae family. In fact, they are found only in genera such as *Brunsvigia*, *Crinum*, or *Boophane* (23). From the *Pancratium* genus four alkaloids with this skeleton have been isolated (Figure 8): hippeastrine (**17**) (7), (+)-9-*O*-demethylhomolycorine (**18**) (24), 10-norneronine (**19**) (25), and pancratinine A (**20**) (5). All of the alkaloids contain a lactone ring due to the oxidation of the hydroxyl group at C-6. Pancratinine A (**20**), which has an unusual oxygenated function at position 10b, has only been detected in a few species (5, 26).

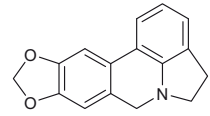
Seventeen lycorine-type alkaloids have been identified in *Pancratium* species (**1**, **21**–**36**) (5, 25) (Figure 9). Several of them have been isolated as



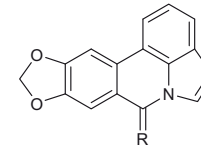
1	R_1+R_2=-CH_2-, R_3=H, R_4=OH	lycorine
21	R_1=Me, R_2=H, R_3=H, R_4=OH	pseudolycorine
22	R_1=H, R_2=Me, R_3=Ac, R_4=H	1-*O*-Acetyl-8-norpluviine
23	R_1=Me, R_2=H, R_3=R_4=H	9-norpluviine
24	R_1=Me, R_2=H, R_3=β-D-glc, R_4=OH	1-*O*-β-D-glycosillycorine
25	R_1+R_2=-CH_2-, R_3=β-D-glc, R_4=OH	1-*O*-β-D-glycosilpseudolycorine



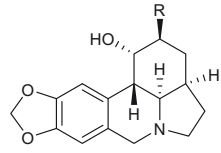
26 R=H pancrassidine
27 R=Me



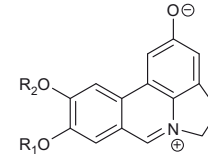
28 galanthane



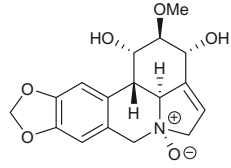
29 R=O hippadine
30 R=H_2 3,4-dihydroanhydrolycorine



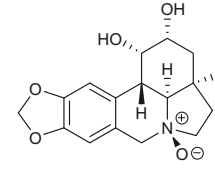
31 R=H dihydrocaranine
32 R=OH dihydrolycorine



33 R_1+R_2=-CH_2- ungeremine
34 R_1=R, R_2=Me zefbetaine



35 ungiminorine *N*-oxide



36 pancratinine D

Figure 9 *Pancratium* alkaloid structures of the lycorine type.

N-oxides (**35, 36**) (5,27), as zwitterionic compounds (**33, 34**) (5), in achiral form (**28**–**30**) (5,28,29), or with glycosidic substituents (**24, 25**) (30).

2. Montanine, Narciclasine, and Tazettine Types

Alkaloids belonging to the montanine (**5**), narciclasine (**4**), and tazettine (**3**) groups are unusual in the *Pancratium* genus. Only three alkaloids with

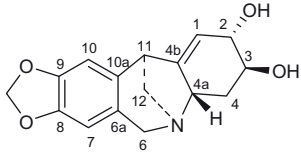
a montanine-type skeleton have been identified: pancracine (**37**) from *P. maritimum* (31) and *Pancratium sickenbergeri* (32), and pancratinine B (**38**) and pancratinine C (**39**) from *Pancratium canariense* (5) (Figure 10). These last two alkaloids represent the first examples of montanine-type alkaloids with a disubstituted double bond (C-1−C-2) instead of the typical trisubstituted double bond (C-1−C-4b).

From *P. maritimum* three narciclasine-type alkaloids have been isolated (Figure 11): pancratistatin (**40**) (33), well known due to its potent cytotoxic activity, narciclasine (**4**) (34), and its glycosidic derivative (**41**) (35).

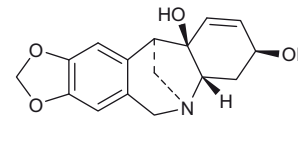
The tazettine-type alkaloids also constitute a limited group in the *Pancratium* genus. Tazettine (**3**) and deoxytazettine (**42**) isolated from *P. maritimum* (36), and pretazettine (**16**) from *Pancratium biflorum* (28) are the three alkaloids isolated from this genus (Figure 12).

3. Galanthamine Type

The galanthamine-type alkaloids are characterized by the presence of two *ortho* aromatic hydrogens. They are less common in this genus in comparison with other genera such as *Galanthus* or *Narcissus*. Seven different alkaloids (**2**, **43**−**48**) have been found in *P. maritimum*,

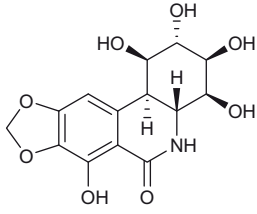


5 R=Me montanine
37 R=H pancracine

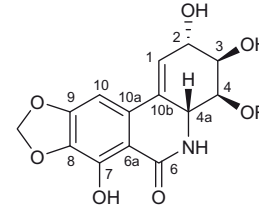


38 R=Me pancratinine B
39 R=H pancratinine C

Figure 10 *Pancratium* alkaloid structures of the montanine type.

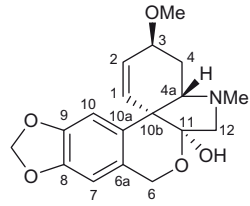


40 pancratistatin

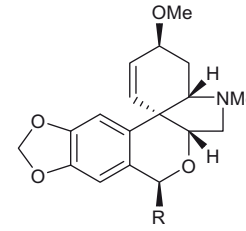


4 R=H narciclasine
41 R=β-D-glc 4-*O*-β-D-glycosylnarciclasine

Figure 11 *Pancratium* alkaloid structures of the narciclasine type.

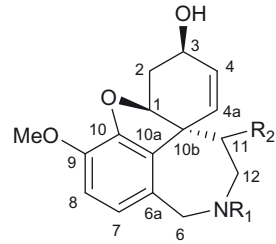


3 tazettine

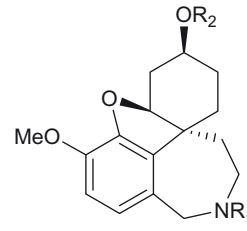


42 R=H 6-deoxytazettine
16 R=OH pretazettine

Figure 12 *Pancratium* alkaloid structures of the tazettine type.



2	R_1=Me, R_2=H	galanthamine
43	R_1=R_2=H	*N*-norgalanthamine
44	R_1=CHO, R_2=H	*N*-formylgalanthamine
45	R_1=Me, R_2=OH	habranthine



46	R_1=Me, R_2=H	lycoramine
47	R_1=R_2=H	*N*-norlycoramine
48	R_1=Me, R_2=Ac	3-*O*-acetyllycoramine

Figure 13 *Pancratium* alkaloid structures of the galanthamine type.

P. biflorum, and *Pancratium foetidum* (Figure 13) (28,35–37). The more abundant alkaloids are galanthamine (**2**) (37) and lycoramine (**46**) (38,39), which only differ in the presence of the C-4–C-4a double bond in **2**.

4. Crinine and Haemanthamine Type

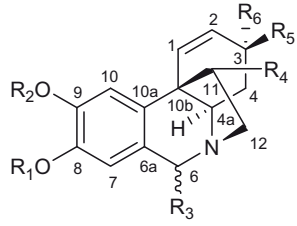
The crinine- and haemanthamine-type alkaloids, together with the lycorine-type alkaloids, are the most abundant alkaloids in *Pancratium* genus. Crinine-type alkaloids are unusual in other genera of Amaryllidaceae, such as *Narcissus* (40). Six different alkaloids have been found in *P. maritimum* (41,42) (Figure 14).

The most common type of alkaloid is the haemanthamine-type (Figure 15). Ten of these alkaloids have been isolated from *P. maritimum* (**7**, **54**–**55**, **61**) (25), *P. sickenbergeri* (**57**–**59**) (43), *P. foetidum* (**15**, **57**–**60**) (44), and *Pancratium tortuosum* (**56**) (45). The alkaloid haemanthidine (**15**) is found as a mixture of epimers in solution due to the presence of the aminal hydroxy group at C-6.

To date, 53 different alkaloids have been isolated from the *Pancratium* genus. Table II shows the number of isolated Amaryllidaceae alkaloids of each type and their corresponding percentage of contribution. It is important to note that several *Pancratium* species biosynthesize diverse alkaloids in large amounts (5).

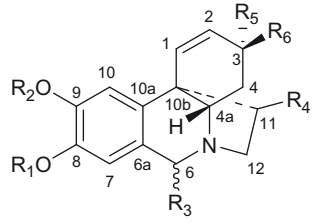
5. Other Secondary Metabolites

In addition to alkaloids, other secondary metabolites have also been isolated from the bulbs and the aerial parts of *Pancratium* species (Figure 16). For example, phenolic acids (46), chromones (47), chalcones (48), and flavonoids (49) have been identified in *P. maritimum*, and from *P. biflorum*, acetophenones (50) and glycosidic chromones (51) have been isolated.



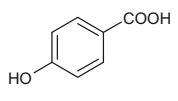
8	R_1+R_2=-CH_2-, R_3=R_4=R_5=H, R_6=OH	crinine
49	R_1+R_2=-CH_2-, R_3=R_4=H, R_5+R_6=O	crinan-3-one
50	R_1+R_2=-CH_2-, R_3=R_4=R_5=H, R_6=OMe	buphanisine
51	R_1=H, R_2=Me, R_3=R_4=R_5=H, R_6=OH	macowine
52	R_1+R_2=-CH_2-, R_3=R_4=OH, R_5=OMe, R_6=H	(-)-3β-methoxy-6,11-dihydroxycrinane
53	R_1+R_2=-CH_2-, R_3=H, R_4=R_5=OH, R_6=H	(-)-3β,11-dihydroxycrinane

Figure 14 *Pancratium* alkaloid structures of the crinine type.

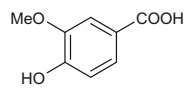


7	R_1+R_2=-CH_2-, R_3=H, R_4=OH, R_5=H, R_6=OMe	haemanthamine
54	R_1+R_2=-CH_2-, R_3=R_4=R_5=H, R_6=OH	vittatine
55	R_1+R_2=-CH_2-, R_3=H, R_4=OH, R_5=H, R_6=OH	11-hydroxyvittatine
56	R_1=R_2=Me, R_3=R_4=R_5=H, R_4=OH	maritidine
15	R_1+R_2=-CH_2-, R_3=R_4=OH, R_5=H, R_6=OMe	haemanthidine
57	R_1+R_2=-CH_2-, R_3=OH, R_4=R_5=H, R_6=OMe	*ent*-6-hydroxybufanisine
58	R_1=H, R_2=Me, R_3=R_4=R_5=H, R_6=OH	8-demethylmaritidine
59	R_1=Me, R_2=H, R_3=R_4=R_5=H, R_6=OH	9-demethylmaritidine
60	R_1+R_2=-CH_2-, R_3=H, R_4=OH, R_5=OMe, R_6=H	crinamine
61	R_1+R_2=-CH_2-, R_3=β-OMe, R_4=OH, R_5=H, R_6=OMe	6-*O*-methylhaemanthidine

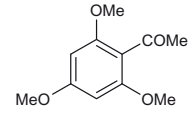
Figure 15 *Pancratium* alkaloid structures of the haemanthamine type.



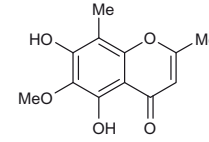
62 *p*-hydroxybenzoic acid



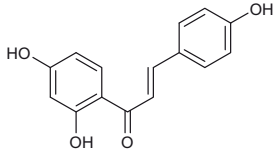
63 vanillic acid



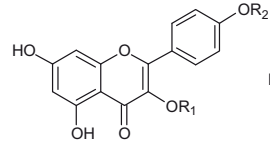
64 2,4,6-trimethoxy acetophenone



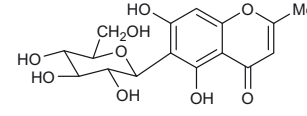
65 5,7-dihydroxy-6-methoxy-2,8-dimethylchromone



66 isoliquiritigenin



67 R_1= R_2= H; kaempferol
68 R_1= xyloside; R_2= H
69 R_1= R_2=xyloside



70 biflorine

Figure 16 Other secondary metabolites isolated from *Pancratium* species.

III. NMR STUDIES

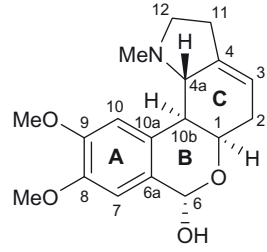
During the past decades, ^{1}H NMR and ^{13}C NMR spectroscopy, together with 2D NMR techniques, have contributed to elucidating and establishing the unequivocal assignments of the Amaryllidaceae alkaloids. In the

Table II Distribution of Amaryllidaceae alkaloids in the *Pancratium* genus

Alkaloid type	Number of isolated alkaloids	Contribution (%)
Lycorine	4	7.5
Lycorenine	17	32.1
Montanine	3	5.7
Narciclasine	3	5.7
Tazettine	3	5.7
Galanthamine	7	13.2
Crinine	6	11.3
Haemanthamine	10	18.8

next section, the most relevant ^{1}H NMR characteristics for each of the *Pancratium* alkaloid-types are discussed, indicating the key details used to identify and to discriminate between them (6).

A. Lycorenine-Type Alkaloids

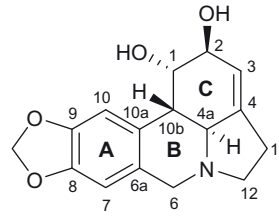


6 lycorenine

The absolute configuration of lycorenine (**6**) was established by X-ray studies (52). Most of the lycorenine-type alkaloids contain a lactone ring as the B ring due to the oxidation of the aminal hydroxy group at C-6. The following ^{1}H NMR characteristics are usual in these alkaloids:

- Two singlets for the aromatic hydrogens. Due to the presence of an oxygenated function at C-6, the signal of the hydrogen H-7 is located more downfield than the one corresponding to H-10.
- The orientation of the hydrogens implied in the B–C ring joint is always *cis*, consequently the coupling constant between H-1 and H-10b is very small or zero.
- The coupling constant J_{4a-10b} is $\sim$10 Hz because of the *trans* orientation of these protons.
- The methyl group attached to the nitrogen atom appears as a singlet at $\sim$2 ppm.

B. Lycorine-Type Alkaloids

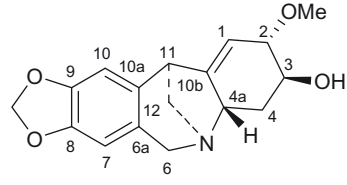


1 lycorine

The absolute configuration studies of the lycorine-type alkaloids have been carried out using X-ray, circular dichroism (CD), and optical rotatory dispersion (ORD) methods (53,54). The main ^{1}H NMR characteristics of the lycorine-type alkaloids are:

- The presence of three singlets, corresponding to two aromatic protons and one vinylic hydrogen, in the downfield region.
- The presence of an AB system for the benzylic hydrogens at C-6, with a large coupling constant (~16 Hz). In all cases, the hydrogen H-6α appears more downfield than H-6β.
- The stereochemistry of the B–C ring junction is *trans*, consequently a large coupling constant J_{4a-10b} (~10 Hz) is observed.
- When this type of alkaloid is isolated as the *N*-oxide, this moiety produces downfield shielding of the neighboring protons H-4a, H-6, and H-12.

C. Montanine-Type Alkaloids

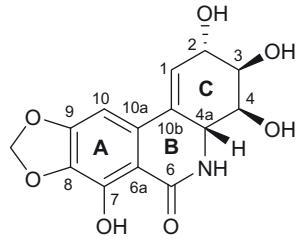


37 pancracine

The absolute configuration of pancracine (**37**) has been determined by CD (55). Montanine-type alkaloids present ^{1}H NMR data very similar to those possessing a lycorine skeleton. The alkaloids can be distinguished by the analysis of a COSY spectrum. The signals attributable to the H-4 hydrogens (the most upfield signals) in the montanine-type alkaloids

show correlation with the signals corresponding to H-3 (~4 ppm) and H-4a (~3.7 ppm). However, in a lycorine skeleton, the most upfield signals correspond to the H-11 hydrogens, and they only show correlation with the ABX system corresponding to the methylene hydrogens of C-12, which appear with a chemical shift lower than 3 ppm.

D. Narciclasine-Type Alkaloids

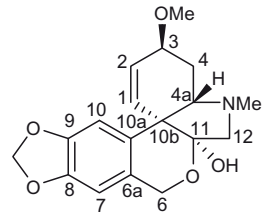


4 narciclasine

The narciclasine-type alkaloids present the highest degree of oxidation. The absolute configuration of the most studied alkaloid of this group, pancratistatin (**40**), was determined by X-ray diffraction (56). The main ^{1}H NMR characteristics of the narciclasine-type alkaloids are:

- These alkaloids possess only one aromatic hydrogen, which appears as a singlet with a chemical shift higher than 7 ppm.
- Those alkaloids with the double bond at C-1–C-10b hydrogenated possess a *trans* stereochemistry for the B–C ring junction, and consequently a large coupling constant value for J_{4a-10b}.
- The hydrogen attached to the nitrogen atom appears as a broad singlet with a chemical shift around 5 ppm, which disappears on the addition of D_2O.

E. Tazettine-Type Alkaloids



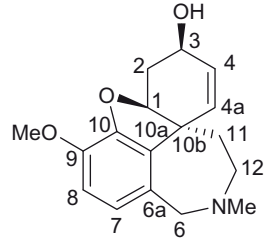
3 tazettine

The absolute configuration of these alkaloids was established by CD studies (57). This type of alkaloid possesses an aliphatic quaternary carbon (C-10b) and a disubstituted double bond similar to those present in the crinine and haemanthamine skeletons. However, the tazettine-type alkaloids display an NMe group as a singlet at ~2 ppm that allows for a discrimination from the previously mentioned skeletons.

Other relevant ^{1}H NMR data are:

- The methylene hydrogens at C-12 appear as an AB system with a large coupling constant (~10 Hz).
- In the case of tazettine (**3**), the methylene hydrogens at C-6 appear as another AB system. However, they are shifted more downfield and with a larger coupling constant (~15 Hz) that those of H_2-12. This observation allows for an easy distinction to be made between tazettine (**3**) and pretazettine (**16**).
- The orientation of the functional group at C-3 can be determined by the value of the coupling constant of H-3. In the case of a *J* value of ~5 Hz, the orientation of the substituent is β, while a smaller value of 0–1.5 Hz indicates an α orientation.

F. Galanthamine-Type Alkaloids



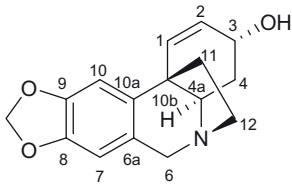
2 galanthamine

The absolute configuration of galanthamine (**2**) was determined by X-ray diffraction (58). The galanthamine-type alkaloids are the only *Pancratium* alkaloids that show two *ortho*-oriented aromatic protons. The general characteristic of their ^{1}H NMR spectra are:

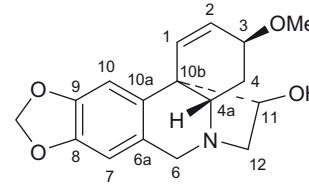
- Two doublets for the two aromatic protons with a coupling constant of ~8 Hz.
- The benzylic hydrogens at C-6 appear as an AB system with a large coupling constant (~15 Hz).

- The orientation of the substituent at C-3 can be established according to the value of the coupling constant of H-3. A β-disposition produces a *J* value of ∼5 Hz, while a *J* value between 0 and 1.5 Hz indicates an α-orientation.

G. Crinine and Haemanthamine-Type Alkaloids



8 crinine



9 haemanthamine

The absolute configurations of crinine (**8**) and haemanthamine (**7**) were established by X-ray diffraction studies (53,59). In the case of haemanthamine (**7**) CD studies (60) have been also carried out. The main NMR traits for this type of alkaloid are:

- Two singlets due to the aromatic hydrogens, in the downfield region.
- The benzylic protons at C-6 appear as an AB system with a large coupling constant (∼16 Hz).
- The orientation of the substituent at C-3, in the case of a haemanthamine skeleton, can be established according to the J_{2-3} value. A β-disposition produces a *J* value of ∼5 Hz, while an α-disposition produces a smaller coupling constant of 0−1.5 Hz. The same criteria can be applied to the enantiomeric skeleton crinine.

IV. PRODUCTION OF *PANCRATIUM* ALKALOIDS

A. Recent Synthetic Studies

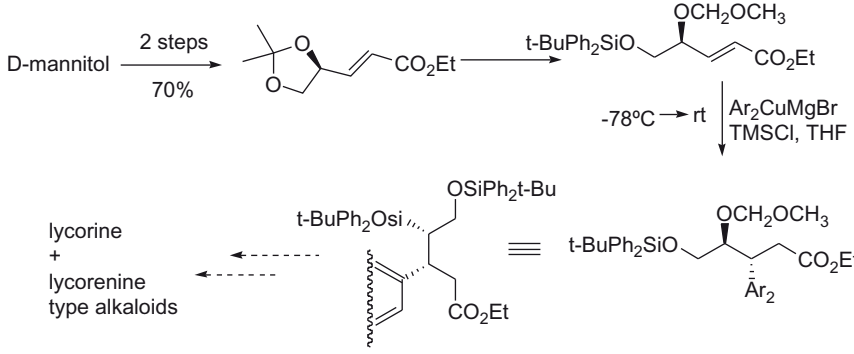
Because of the important properties of the Amaryllidaceae alkaloids, several efforts have been developed in the last years for an efficient production of these metabolites. Important reviews about synthesis of Amaryllidaceae alkaloids have been published (9,61,62−76,77). This section provides coverage of the chemical synthesis of the Amaryllidaceae alkaloids present in the genus *Pancratium* from 2005 to April 2009.

1. Synthesis of Lycorine-Type Alkaloids

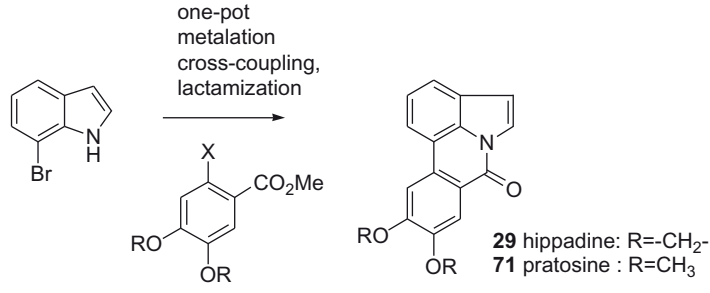
Kornienko *et al.* have synthesized several precursors of the lycorine and lycorenine-type alkaloids from the stereo-controlled addition of various substituted arylcuprates to a D-mannitol-derived conjugate ester (Scheme 1) (78).

A comparative study of the Kumada, Negishi, Stille, and Suzuki–Miyaura reactions in the synthesis of hippadine (**29**) and pratosine (**71**) has been carried out by Tonder *et al.* (Scheme 2) (79). The Suzuki–Miyaura coupling of the metalated indole substrates was found to deliver readily the target alkaloids in modest yields.

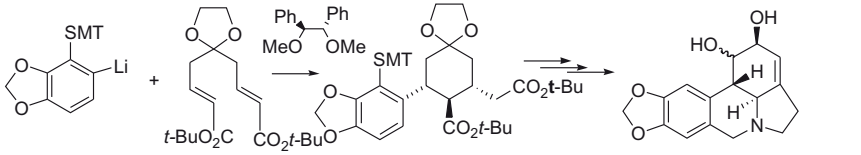
Total syntheses of (−)-lycorine (**1**) and (−)-2-*epi*-lycorine (**72**) were achieved using a chiral ligand-controlled, asymmetric cascade, conjugate addition methodology (Scheme 3) (80). This methodology enables the formation of two C–C bonds and three stereogenic centers in one pot to give synthetically useful, chiral cyclohexane derivatives. The synthetic strategy is flexible, and it can be applied to other natural and unnatural lycorine derivatives.



Scheme 1



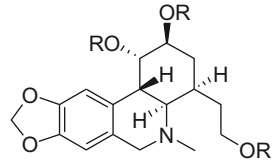
Scheme 2



1 (-)-lycorine, OH-α
72 (-)-2-*epi*-lycorine, OH-β

Scheme 3

Using lycorine (**1**) as the starting material, several 5,6-secolycorine derivatives (**73**) have been prepared. Some of them showed more potent inhibitory activity against acetylcholinesterase than galanthamine (**2**) (81).



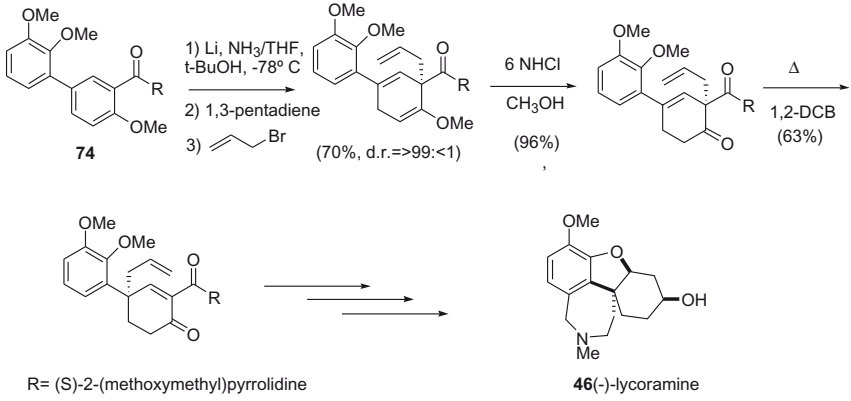
73 synthetic secolycorine derivatives

2. Synthesis of Galanthamine-Type Alkaloids

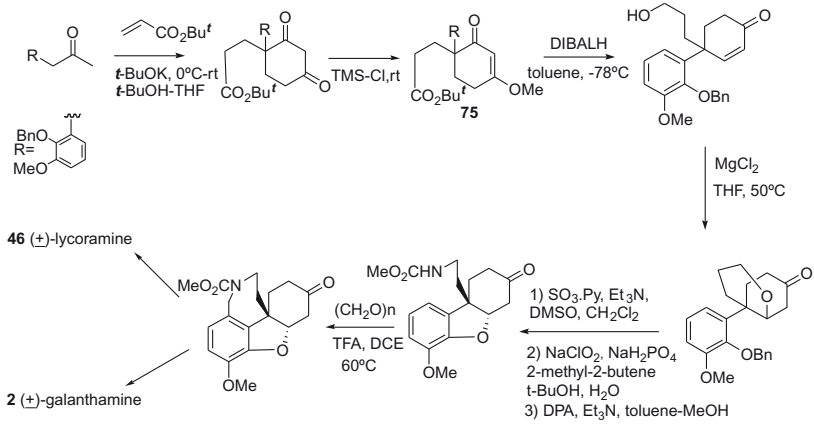
Galanthamine (**2**) as a selective, reversible, and competitive AChE inhibitor for the treatment of Alzheimer's disease (AD), is the first commercial natural product from the Amaryllidaceae family, and was launched onto the market in the European Union and the United States in 2001. Due to its biological activity and limited availability from natural sources, many synthetic strategies have been developed for this natural medicine (82,83).

Recently, several methods for the preparation of galanthamine and the related precursors have been patented (84–87). The first enantioselective synthesis of (−)-lycoramine (**46**) has been achieved in 14 steps and 5% overall yield from the biaryl compound **74** shown in Scheme 4. The synthesis features the application of the Birch–Cope sequence to efficiently generate the arylic quaternary chiral center with exceptionally high enantioselectivity (88).

The total synthesis of (±)-galanthamine (**2**) and (±)-lycoramine (**46**) using 4,4-disubstituted-1-alkoxy-1-cyclohexen-3-one (**75**) as the key intermediate has been reported (89). These intermediates are easily formed by a double Michael–Claisen cyclization from substituted acetone derivatives with acrylic acid esters (>200 mol%) in the presence of *t*-BuOK (200 mol%) in *t*-BuOH–THF (1:1) (Scheme 5).



Scheme 4 Birch–Cope sequence in (−)-lycoramine synthesis.



Scheme 5 Synthesis of (±)-galanthamine (**2**) and (±)-lycoramine (**46**).

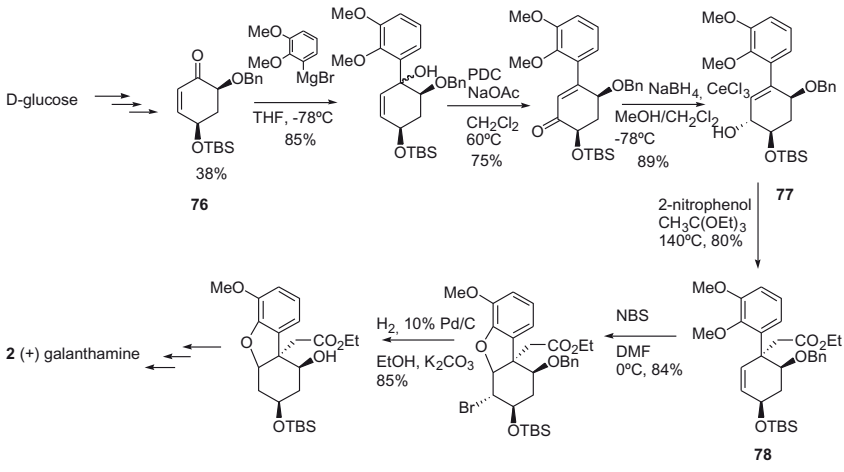
Node *et al*. synthesized (±)-galanthamine (**2**) in excellent yield by applying phenyliodine *bis*(trifluoroacetate) (PIFA)-mediated oxidative phenol coupling of *N*-(4-hydroxy)phenethyl-*N*-(3,4,5-trialkoxy)benzyl formamide as a key step (90). Because of the symmetrical characteristics of the pyrogallol moiety in this substrate, phenol oxidative coupling resulted in a single coupling product. On the basis of the successful results of the above strategy, (−)-galanthamine (**2**) was synthesized by employing a novel remote asymmetric induction, where the conformation of the seven-membered ring in the product of the phenol coupling was restricted by forming a fused-chiral imidazolidinone ring with D-phenylalanine on the benzylic C–N bond of the tri-*O*-alkylated gallyl amino moiety. An alternative total synthesis of (−)-galanthamine

hydrobromide, employing ecofriendly amidation, oxidative coupling, and classical resolution strategies has been also reported (91).

Trost *et al.* have developed an efficient divergent synthetic strategy for the synthesis of (−)-galanthamine (**2**) by employing a Pd-catalyzed asymmetric allylic alkylation (AAA) to set the stereochemistry (92). Three generations of syntheses of galanthamine (**2**) are discussed in detail with particular focus on the scope of the palladium-catalyzed AAA reactions and intramolecular Heck reactions. The pivotal tricyclic intermediate is available in six steps from 2-bromovanillin and the monoester of methyl 6-hydroxycyclohexene-1-carboxylate. This intermediate requires only two steps to convert to (−)-galanthamine (**2**).

The stereoselective total synthesis of (+)-galanthamine (**2**) starting from D-glucose has been described (93). The cyclohexene ring in (+)-galanthamine (**2**) was prepared in an optically active form from D-glucose using Ferrier's carbocyclization reaction to yield the cyclohexenone (**76**), and the critical quaternary carbon was stereoselectively generated via chirality transfer based on the Johnson−Claisen rearrangement of the cyclohexenol intermediate **77** (Scheme 6). The dibenzofuran skeleton was effectively constructed by the bromonium ion-mediated intramolecular cyclization of the cyclohexene possessing a phenolic ether function **78**. After the introduction of a carbon−carbon double bond, Pictet−Spengler type cyclization, followed by the reduction of the amide function, completed the chiral synthesis of (+)-galanthamine (**2**).

An enantioselective synthesis of (−)-galanthamine has been also reported from commercially available materials in 11 linear steps from isovanillin (94). Control of the absolute stereochemistry was achieved through the asymmetric reduction of a propargylic ketone. An efficient



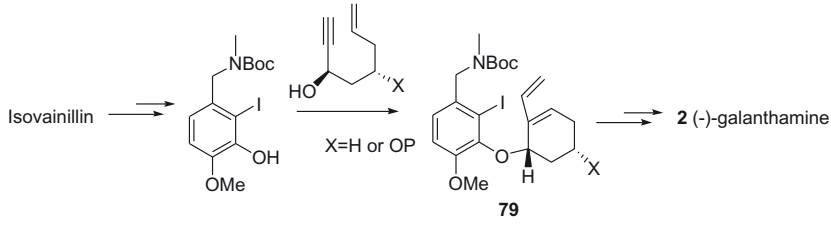
Scheme 6 Stereo-selective synthesis of (+)-galanthamine (**2**).

enyne metathesis reaction was used to close the B ring yielding the precursor (**79**) having the requisite functionality used in the formation of the D and C rings (Scheme 7).

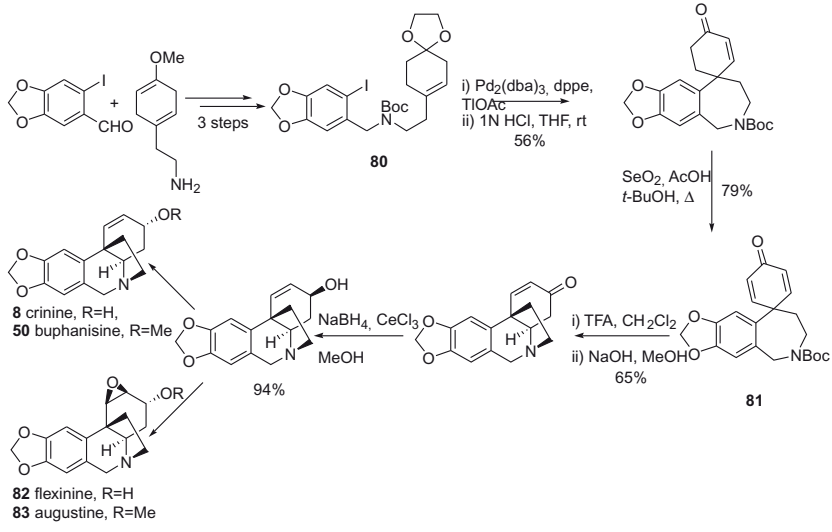
3. Synthesis of Crinine, Haemanthamine and Tazettine-Type Alkaloids

The total synthesis of (±)-crinine (**8**) and related alkaloids, such (±)-buphanisine (**50**), (±)-flexine (**82**), and (±)-augustine (**83**), has been described (95). The key intermediate is a tetracyclic spirocyclohexanone (**81**) generated by the cyclization of a bicyclic amine **80** via intramolecular Heck reaction, followed by an oxidation reaction (Scheme 8).

Chida *et al*. reported the first chiral synthesis of (+)-vittatine (**54**) and (+)-haemanthamine (**7**) from D-glucose. This approach demonstrated that the methodology involving Claisen rearrangement on the chiral



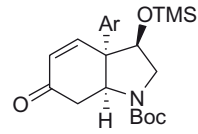
Scheme 7



Scheme 8 Synthesis of (±)-flexinine (**82**) and (±)-augustine (**83**).

cyclohexanol derived from D-glucose by way of the catalytic Ferrier's carbocyclization is effective for the stereoselective generation of quaternary carbons, and aminomercuration–demercuration, followed by Chugaev reaction, is a useful sequence for the construction of hexahydroindole skeletons (96).

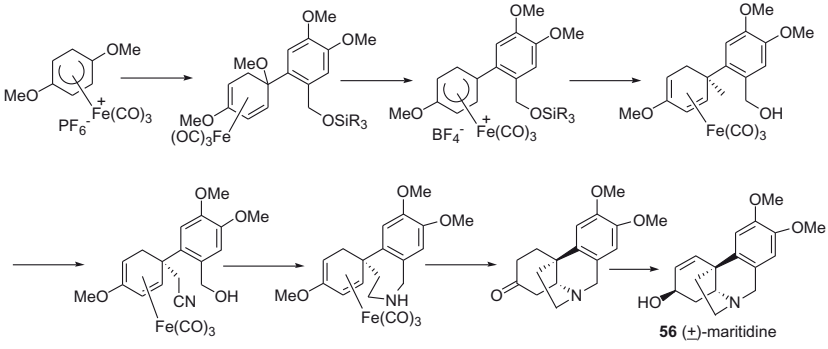
The total syntheses of (+)-haemanthidine (**15**), (+)-pretazettine (**16**), (+)-tazettine (**3**), and (+)-crinamine (**60**), were accomplished via a common intermediate **84** (97). This crucial precursor was achieved on the basis of the NBS-promoted semipinacol rearrangement and an intramolecular Michael addition, which efficiently constructed the sterically congested quaternary carbon center and the hydroindole skeleton of the crinine-type alkaloids, respectively.



84

Organoiron-mediated formal total synthesis of (±)-maritidine (**56**) has been carried out by Stephenson *et al.* (98). The reaction sequence is the first example of a successful application in the synthesis of an *ortho*-carbon substituent in the position required for Amaryllidaceae alkaloids of this type (Scheme 9).

Cho *et al.* have devised a new synthetic route to (±)-crinine (**8**) via the regioselective synthesis and Diels–Alder cycloaddition of 5-bromo-3-(3,4-methylenedioxyphenyl)-2-pyrone (**85**) with TBS vinyl ether. The following steps in the synthesis are illustrated in Scheme 10 (99).



Scheme 9 Synthesis of (±)-maritidine (**56**).

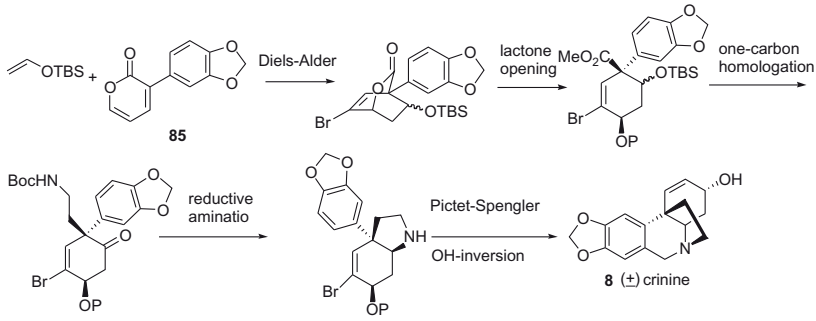
4. Synthesis of Narciclasine and Pancratistatin-Type Alkaloids

Over the past several decades there has been tremendous interest in the synthesis of narciclasine, lycoricidine, and pancratistatin due to their potent antitumor and antiviral activities (59–76). Recently, a two-step strategy involving Suzuki cross-coupling of boronic acid derivatives **86** with a diverse set of α-iodoenones **87**, followed by hydrogenation, was developed for the construction of highly oxygenated, ring [*c*]annulated, pancrastatin-like isoquinolines (Scheme 11) (100).

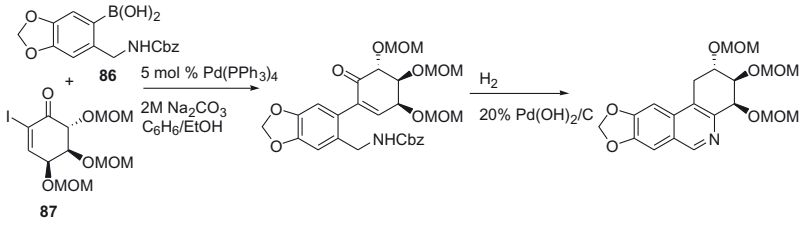
Hudlicky *et al.* have reported on the total synthesis of 7-deoxypancratistatin-1-carboxaldehyde (**88**) and carboxylic acid **89** via solvent-free, intramolecular aziridine opening and phenanthrene-to-phenanthridinone cyclization (Scheme 12) (101).

A chemoenzymatic total synthesis of *ent*-narciclasine has been described. This approach highlights the considerable utility of microbially derived *cis*-1,2-dihydrocatechols in the construction of a range of unnatural enantiomers of pancratistatin-type alkaloids and their analogues (102,103).

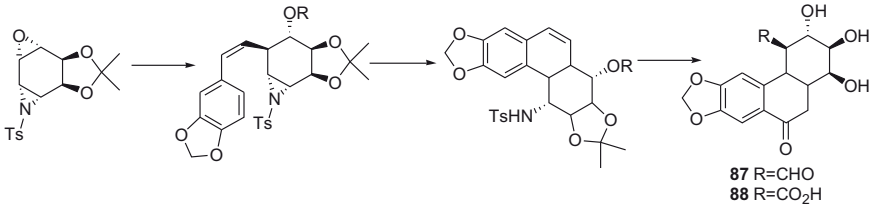
The total syntheses of (+)-lycoricidine (**90**) and (+)-7-deoxypancratistatin (**91**) were carried out through a one-pot Stille/intramolecular Diels–Alder cycloaddition cascade to construct the core skeleton. The



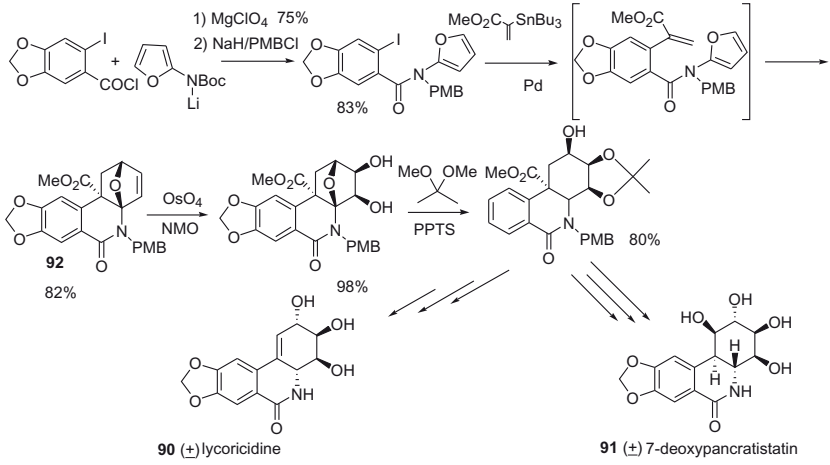
Scheme 10 Synthesis of (±)-crinine (**8**).



Scheme 11



Scheme 12

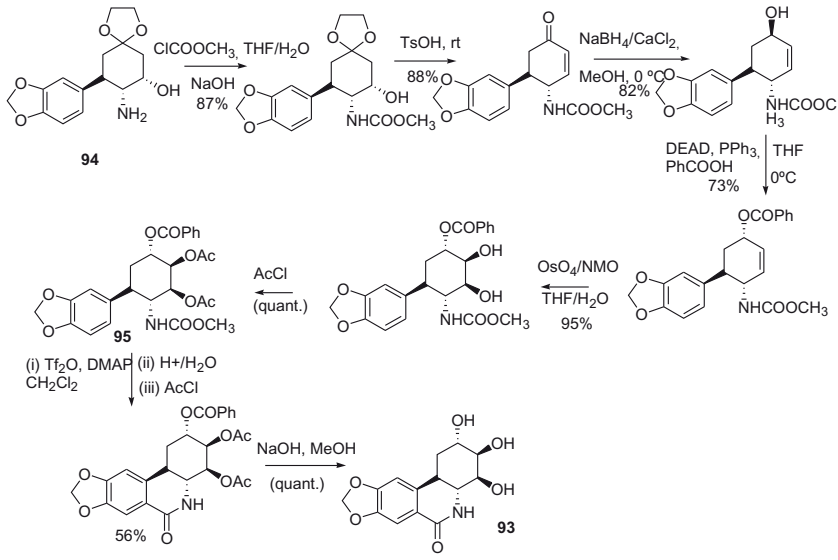


Scheme 13 Synthesis of (±)-lycoricidine (**90**) and (±)-pancratistatin (**91**).

resulting cycloadduct **92** was then used for the stereo-controlled installation of the other functionality present in the C ring of the target molecules (Scheme 13) (104).

Kadas *et al.* reported the stereoselective total synthesis of the antineoplastic (±)-7-deoxy-*trans*-dihydronarciclasine (**93**) (105). Starting from an arylcyclohexylamine-type precursor **94**, the C ring with the required stereochemistry was constructed using a chemo- and stereoselective enone reduction ($NaBH_4/CaCl_2$ system) and a Mitsunobu reaction. For the B ring closure, the Banwell modification of the Bischler−Napieralski reaction on the compound **95** was applied (Scheme 14).

The total synthesis of fully functionalized, polyhydroxyamide B,C-*seco*-analogues of pancratistatin has been described. Key steps include an Evans' $MgCl_2$-promoted anti-aldol reaction between a functionalized threose derivative and (*R*)-(+)-oxazolidinone to stereoselectively form the C-1−C-10 bond and a regiospecific radical-mediated oxidative fragmentation of a 1,3-benzylidene (106).



Scheme 14

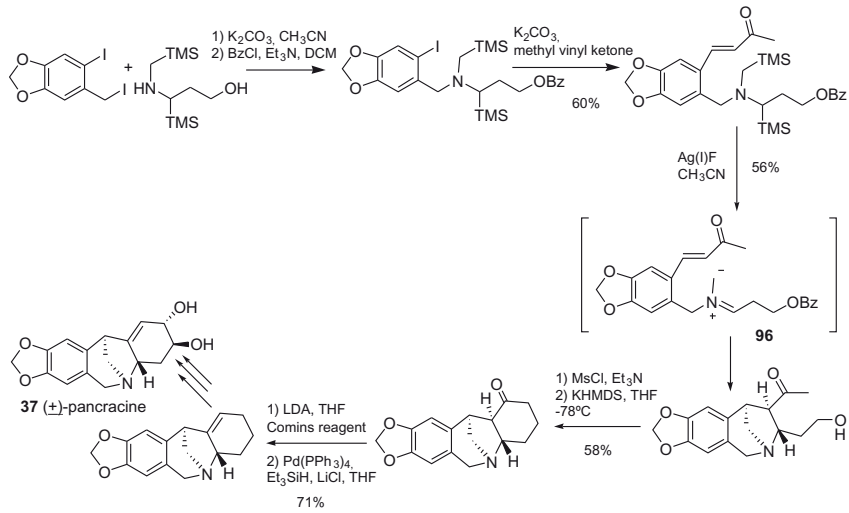
Cho *et al*. have described the total synthesis of (+)-*trans*-dihydronarciclasine through a highly *endo*-selective Diels–Alder cycloaddition of 3,5-dibromo-2-pyrone (107).

5. Synthesis of Montanine-Type Alkaloids

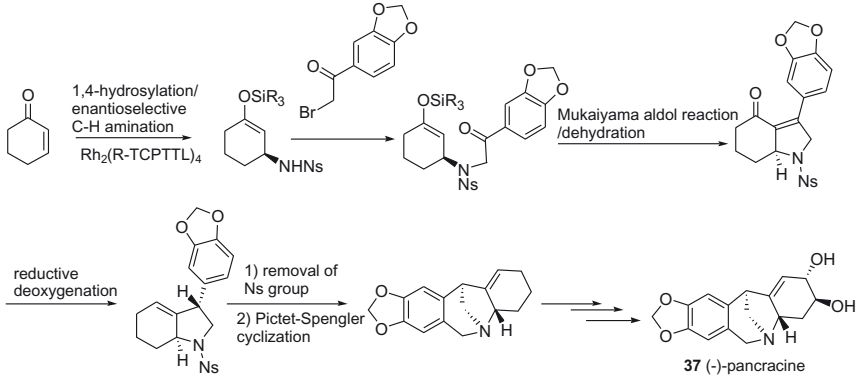
Pandey *et al*. have developed a short and conceptually new route for the stereo-specific construction of the core structure of 5,11-methanomorphanthridine alkaloids in one step. The key step is an intramolecular [3+2]-cycloaddition of the nonstabilized azomethine ylide **96**, and following this strategy (±)-pancracine (**37**) was synthesized (Scheme 15) (108).

An asymmetric formal synthesis of (−)-pancracine (**37**) via a catalytic, enantioselective C–H amination process has been described (109). The key steps involve: (a) a one-pot Rh_2(*R*-TCPTTL)$_4$-catalyzed sequential 1,4-hydrosylation/enantioselective C–H amination of 2-cyclohexen-1-one; (b) *N*-alkylation and subsequent intramolecular Mukaiyama aldol reaction/dehydration; and (c) a regio- and stereo-controlled reductive deoxygenation of bicyclic enone intermediate with migration of the double bond to create the C-1–C-11a double bond and the stereogenic center at C-11 of a 3-arylhexahydroindole derivative (Scheme 16) (109).

Sha *et al*. have developed a concise and expedient route toward the total synthesis of montanine-type alkaloids such as (−)-brunsvigine (**97**) and (−)-manthine (**98**). The synthetic approach features an efficient and stereo-controlled construction of a bicyclic enone **99** employing anionic cyclization. A sequence of synthetic transformations on bicyclic enone **99**



Scheme 15 Synthesis of (±)-pancracine (**37**).



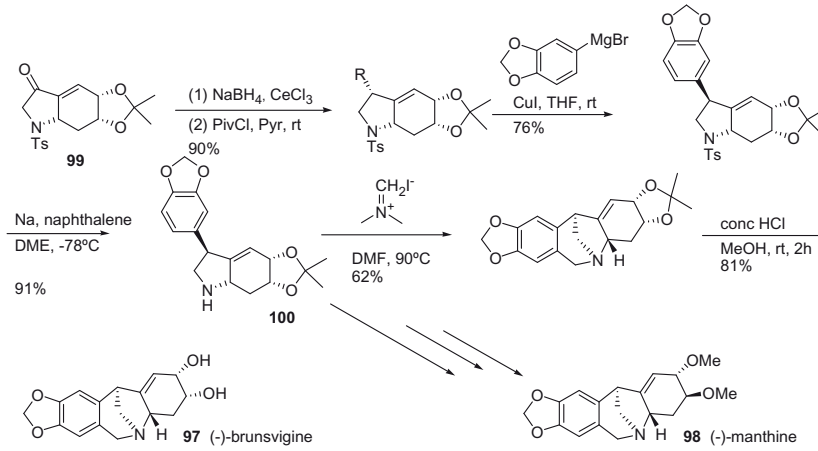
Rh_2(R-TCPTTL)$_4$ = dirhodium (II) tetrakis[N-tetrachlorophthaloyl-(S)-*tert*-leucinate

Scheme 16 Synthesis of (−)-pancracine (**37**).

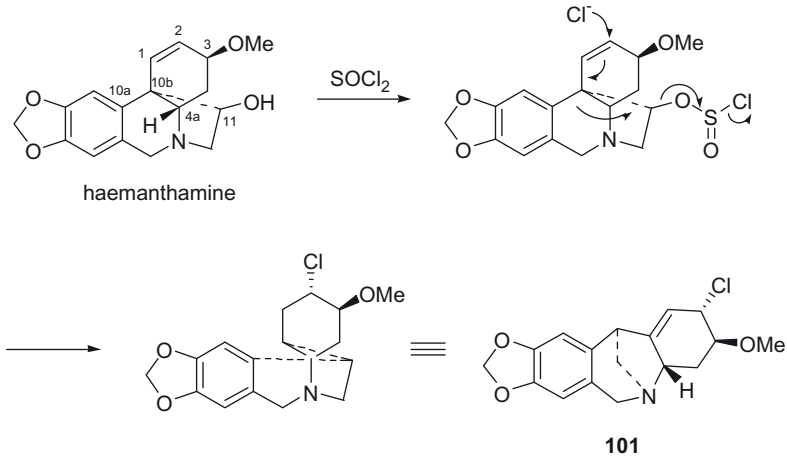
established the pivotal framework **100**. Pictet–Spengler cyclization was strategically applied to construct the 5,11-methanomorphanthridine ring system (Scheme 17) (110).

B. Interconversions between Skeletons

The less abundant skeletons, such as those of montanine (**69**) or tazettine (**3**), can be obtained from a rearrangement of haemanthamine (**7**)-type alkaloids, which are usually isolated in higher yields. Reaction of

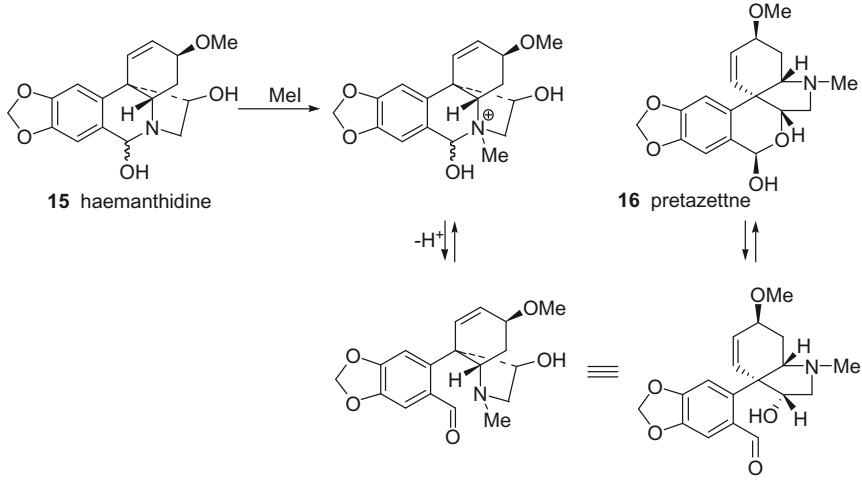


Scheme 17 Synthesis of (−)-brunsvigine (**97**) and (−)-montanine (**98**).



Scheme 18 Montanine skeleton obtained by rearrangement of haemanthamine (**7**).

haemanthamine (**7**) or haemanthidine (**15**) with halogenating agents produces a rearrangement of the molecule into a montanine-type compound **101**. For example, treatment of haemanthamine (**7**) with thionyl chloride produces the rearranged halogenated compound with the montanine skeleton (Scheme 18). The absolute configuration of the rearranged product **101** was determined by vibrational circular dichroism (VCD) as (*2S,3S,4aS,11S*)-**101**. The reaction requires an antiperiplanar disposition between the C-10a–C-10b bond and the leaving group at C-11 (111). This requirement was also observed by Wildman *et al.*, and consequently, when 11-*epi*-haemanthamine is used, the rearrangement



Scheme 19 Tazettine skeleton from haemanthidine (**15**).

fails (112). The substituent at C-2 can vary according to the nucleophile employed (111).

The haemanthamine-type alkaloids are also a source of tazettine-type compounds (113). For instance, tazettine (**3**) can be obtained by the treatment of haemanthidine (**15**) with methyl iodide (Scheme 19) (10).

C. *In Vitro* Culture Systems

The *in vitro* culture systems are an alternative source of alkaloids, especially in the case of galanthamine (**2**). However, the production *in vitro* of Amaryllidaceae alkaloids has only been performed for the species *Leucojum aestivum* and *Narcissus confusus*. There is only one study on the establishment of the *in vitro* culture of *P. maritimum* aiming at the production of bulbs (114). In this experiment, it was established that a culture medium supplemented with 80 g/L sucrose and 0.1 mg/L NAA gave the best result, as far as the percentage of bulb formation (93%) and their size (ave. wt. 263 mg) is concerned.

In vitro cultures from *L. aestivum* have produced 14 of the 24 alkaloids isolated from this plant. The major alkaloids were identified by HPLC as lycorine (**1**) and galanthamine (**2**) (115). However, the hairy root production of galanthamine (**2**) in *L. aestivum* was not possible (116). Galanthamine (**2**) has been obtained also from *in vitro* cultures of *N. confusus*, resulting in a production of 8.2 mg of the alkaloid per dry gram, almost the double the amount isolated from the bulbs (117). Due to the biological interest of some of the alkaloids found in *Pancratium* species, in the future, a biotechnological approach for the development of these species could be interesting.

V. BIOLOGICAL ACTIVITIES

Fifty-three different alkaloids have been isolated from *Pancratium* species. Most of them have also been found in other genera of the Amaryllidaceae, such as *Narcissus*. Because of the recent review on *Narcissus* alkaloids (4) in this series, the biological activities described for *Pancratium* alkaloids during the period 2005 until May 2009 will be described. These biological activities are mainly focused on anticancer and antiplasmodial activities.

A. Anticancer Activity

Evidente *et al*. have recently published on the cytotoxic activity of several Amaryllidaceae alkaloids and their derivatives (118). Table III shows the antiproliferative activity against HeLa and Vero cell lines, as well as the percentage of apoptotic cells, of the following *Pancratium* alkaloids: lycorine (**1**), pseudolycorine (**21**), 9-norpluviine (**23**), ungeremine (**33**), hippeastrine (**17**), tazettine (**3**), haemanthamine (**7**), 11-hydroxyvittatine (**55**), 9-demethylmaritidine (**59**), buphanisine (**50**), and narciclasine (**4**).

Most of the alkaloids that showed promising antiproliferative activities were also good apoptosis inducers. These were narciclasine (**4**), lycorine (**1**), pseudolycorine (**21**), and haemanthamine (**7**). Important exceptions are ungeremine (**33**), hippeastrine (**17**), and buphanisine (**50**), which may be strictly growth inhibitory. Hippeastrine (**17**), buphanisine

Table III Antiproliferative and apoptotic activity of *Pancratium* alkaloids

Skeleton type	Alkaloid	Cell viability (%)				Apoptosis (%)	
		HeLa		Vero		Jurkat	
		5 μM	25 μM	5 μM	25 μM	1 μM	25 μM
Lycorine	1	33±4	17±2	31±3	23±1	13±1	60±2
Lycorenine	21	52±6	18±3	42±6	28±2	6±4	54±3
9-Nor-pluviine	23	89±8	84±6	71±5	63±3	5±1	6±1
Ungeremine	33	83±5	46±15	78±3	78±9	5±1	7±1
Tazettine	3	95±3	99±5	82±2	83±5	3±1	3±1
Hippeastrine	17	78±5	57±2	78±7	48±2	3±1	5±1
Crinine	7	30±2	21±2	44±3	32±2	4±1	22±1
11-Hydroxy-vittatine	55	86±3	72±5	64±2	64±6	3±1	4±1
9-Demethyl-maritidine	59	90±7	82±7	87±4	82±5	5±1	4±1
Narciclasine	4	10±1	6±1	10±2	6±1	60±1	76±1
Buphanisine	50	100±8	99±6	74±7	79±6	3±3	5±1

(**50**) completely suppress cell invasion *in vitro* at nontoxic concentrations. The anti-invasive activity of buphanimine is particularly promising because this alkaloid is nontoxic to cells even at much higher concentrations.

Zupkó *et al.* reported on the MDR-reversing activity of pretazettine (**16**) on human MDR1-gene-transfected L5178 mouse lymphoma cells (119). This alkaloid significantly increased the intracellular concentration of Rh-123 and enhanced the antiproliferative activity of doxorubicin on the L5178 MDR cell line. Crinamine (**60**) and haemanthamine (**7**) were potent inducers of apoptosis in tumor cells at micromolar concentrations (120). Structure−activity relationships of a mini-library of natural and synthetic crinine-type alkaloids demonstrated the structural requirements to achieve high activity. The pharmacophoric elements are the alpha-C-2 bridge and a free hydroxyl group at C-11.

McLachlan *et al.* reported a detailed study on the effect of pancratistatin (**40**) treatment on cancerous and normal cells (121). The results indicated that pancratistatin-induced apoptosis selectively in cancer cells, and that the mitochondria may be the site of action of pancratistatin (**40**). To further explore the structure−activity relationship of pancratistatin-related compounds, the anticancer efficacy and specificity of two related natural alkaloids was investigated. Both of these compounds lack the polyhydroxylated lycorane element of pancratistatin, instead having a methoxy-substituted crinane skeleton. The results indicated that the phenanthridone skeleton in natural Amaryllidaceae alkaloids may be a significant common element for selectivity against cancer cells; furthermore, the configuration of the methoxy-side groups is responsible for higher binding affinity to the target protein/s thus making for a more efficient anticancer agent. The synergy of pancratistatin (**40**) and tamoxifen on breast cancer cells in inducing apoptosis by targeting mitochondria has been also reported (122).

The effects of lycorine (**1**) on the human multiple myeloma cell line, KM3, and the possible mechanisms of these effects have been studied (123). An MTT assay showed that lycorine (**1**) had significant inhibitory activity on KM3 cells. The growth rates of the KM3 cells exposed to lycorine evidently slowed down. Cell fluorescent apoptotic morphological changes, DNA degradation fragments, and a sub-G_1 peak were detected, indicating the occurrence of cell apoptosis after lycorine treatment. Furthermore, the release of mitochondrial cytochrome c, the augmentation of Bax with the attenuation of Bcl-2, and the activation of caspase-9, -8, and -3 were also detected, suggesting that the mitochondrial pathway and the death acceptor pathway were also involved. The results also showed that lycorine was able to block the cell cycle at the G_0/G_1 phase through the downregulation of both cyclin D1 and CDK4. In short, lycorine can suppress the proliferation of KM3 cells and reduce

cell survival by arresting cell cycle progression as well as inducing cell apoptosis (124). Lycorine and 1-acetyllycorine were tested against a mechanism-based bioassay utilizing genetically engineered mutants of the yeast *Saccharomyces cerevisiae* strains and only lycorine displayed moderate topoisomerase I inhibitory activity (125). Crinamine (**60**) showed potent, dose-dependent inhibition ($IC_{50} = 2.7\,\mu M$) of hypoxia inducible factor-1 (HIF-1) function (126).

The anticancer activity and preclinical studies of narciclasine (**4**) and its congeners has been gathered by Kornienko and Evidente in a recent review (127). Novel derivatives of narciclasine (**4**) have been prepared. Chemical modifications to the narciclasine backbone led to weakly active molecules or even to complete loss of antitumor activity *in vitro*. Only one narciclasine derivative (with a glycosidic substituent at C-7) demonstrated higher *in vivo* antitumor activity than narciclasine itself (128). The 3,4-*O*-cyclic phosphate salt of pancratistatin (**40**) is a novel, water-soluble synthetic derivative of pancratistatin which *in vivo* caused statistically significant tumor growth delays at its maximum-tolerated dose, and significant vascular shutdown and tumor necrosis were observed (129). This derivative offers a way of progressing this promising molecule into the clinic by greatly improving solubility without loss of antitumor activity.

B. Antiplasmodial Activity

The antiplasmodial activity of the *Pancratium* alkaloids: lycorine (**1**), crinamine (**60**), haemanthidine (**15**), haemanthamine (**7**), pancracine (**37**), and ungeremine (**33**) have been summarized in a recent review on alkaloids with antiprotozoal activity (130).

Several lycorine derivatives were examined for their inhibitory activity against *Trypanosoma brucei* and *Plasmodium falciparum*. Among them, 2-*O*-acetyllycorine showed the most potent activity against parasitic *T. brucei*, and 1-*O*-(3*R*)-hydroxybutanoyllycorine, 1,2-di-*O*-butanoyllycorine, and 1-*O*-propanoyllycorine showed significant activity against *P. falciparum* in an *in vitro* experiment (131).

C. Other Activities

The *in vitro* evaluation of the anti-inflammatory, antioxidant, and antimicrobial activities of the montanine alkaloids has been reported by Castilhos *et al.* (132).

V. CONCLUSIONS

The *Pancratium* genus is an important source of Amaryllidaceae alkaloids. The most abundant alkaloids belong to the lycorenine,

haemanthamine, galanthamine, and the crinine skeletons, and they can be obtained in high amounts from species such as *P. canariense*. Some representative *Pancratium* alkaloids exhibit significant antitumoral and antimalarial activities, which have stimulated the development of synthetic approaches to produce them. Future phytochemical analyses of unexplored *Pancratium* species will provide new bioactive alkaloids, which may shed light on elucidating the biosynthetic pathways, so far unknown.

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CHAPTER 2

Erythrina and Related Alkaloids

Andrew F. Parsons[1,*] and **Matthew J. Palframan**[1]

Contents

I. INTRODUCTION

Following the last review in this series in 1996 (1), and a subsequent review in 2004 (2), this review summarizes the literature relating to the alkaloids isolated from the genus *Erythrina* since 1996, including work not covered in the 2004 review, with a particular emphasis on the synthetic approaches to *Erythrina* and related alkaloids.

The *Erythrina* alkaloids have attracted interest because of their interesting structures and their range of useful biological activities. Some members of this family have curare-like activity, in addition to hypotensive, sedative, and CNS depressant properties. *Erythrina* alkaloids have characteristic polycyclic structures, and they have proven to be popular target molecules for synthetic chemists to exploit new methods for ring-forming reactions. The *Erythrina* alkaloids contain four linked rings, labeled A, B, C, and D, whose structures can be divided into

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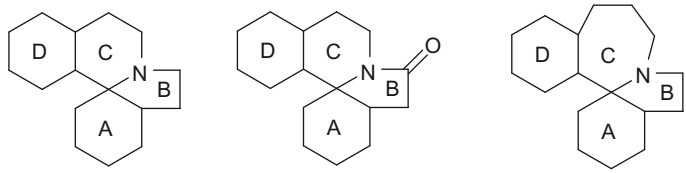


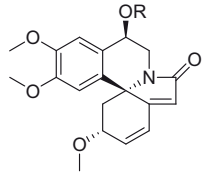
Figure 1 Some ring systems of the *Erythrina* alkaloids.

two groups – those whose structures contain an aromatic D ring, which is by far the largest group, and those whose structures contain an unsaturated lactone, or heteroaromatic ring, such as a furan or pyridine ring system. The 8-oxo *Erythrina* alkaloids are also widespread, while the homo-*Erythrina* alkaloids similarly contain four linked rings, in which the C ring is seven-membered (Figure 1).

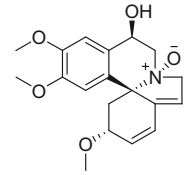
II. OCCURRENCE, DETECTION, AND ISOLATION

The *Erythrina* genus in the family Fabaceae is comprised of over 115 species of trees, shrubs, and herbaceous plants that possess orange or bright-red flowers. They are found throughout the tropical and subtropical regions of the world. In 1996, at the time of the last review in *The Alkaloids* series (1), there were 95 known *Erythrina* alkaloids; at the present the total number now stands at well over 110 alkaloids. Brief details of the isolation of the new alkaloids are presented.

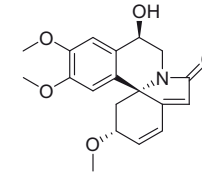
Majinda and coworkers isolated four novel *Erythrina* alkaloids from the flowers of the deciduous tree, *Erythrina lysistemon* Hutch., from South Africa, along with ten known *Erythrina* alkaloids (3). The alkaloids were isolated from the flowers of *E. lysistemon* by initial extraction with a mixture of chloroform and methanol, and subsequently purified by a combination of chromatographic procedures and recrystallization to yield the novel erythrinaline alkaloids: (+)-11β-hydroxyerysotramidine (**1**), (+)-11β-methoxy-erysotramidine (**2**), (+)-11β-hydroxyerysotrine *N*-oxide (**3**) (also known as erythrartine *N*-oxide), and (+)-11β-hydroxy-erysotrine (**4**). Their structures were deduced by examination of their EI-MS, IR, ^{1}H, ^{13}C DEPT, COSY, HMQC, and HMBC NMR spectra, and by comparison with known *Erythrina* alkaloids. The relative stereochemistries of the alkaloids were assigned based on *J* values.



R = H, **1**
(+)-11β-hydroxyerysotramidine
R = Me, **2**
(+)-11β-methoxyerysotramidine

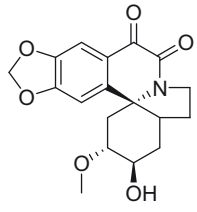


(+)-11β-hydroxyerysotrine *N*-oxide
3

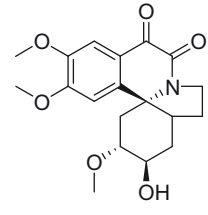


(+)-11β-hydroxyerysotrine
4

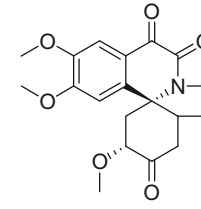
Rukachaisirikul and coworkers reported on the isolation of a further three new *Erythrina* alkaloids from the bark of *Erythrina subumbrans* Merr. (Fabaceae), a tree found in Thailand (4). The three new *Erythrina* alkaloids, (+)-10,11-dioxoerythratine (**5**), (+)-10,11-dioxoepierythratidine (**6**), and (+)-10,11-dioxoerythratidinone (**7**), are the first known examples of *Erythrina* alkaloids with carbonyl groups at both the C-10 and C-11 positions. Alkaloids **5**, **6**, and **7**, along with 14 other known *Erythrina* alkaloids, were extracted from the dried, powdered bark of *E. subumbrans* by successive Soxhlet extractions with hexane, dichloromethane, and methanol, subsequent filtration, and concentration *in vacuo*. The various alkaloids were separated by successive gradient column chromatography of the individual extracts. Alkaloids **5**, **6**, and **7** were inactive on biological evaluation for antiplasmodial, antimycobacterial, and cytotoxic activities.



(+)-10,11-dioxoerythratine
5



(+)-10,11-dioxoepierythratidine
6

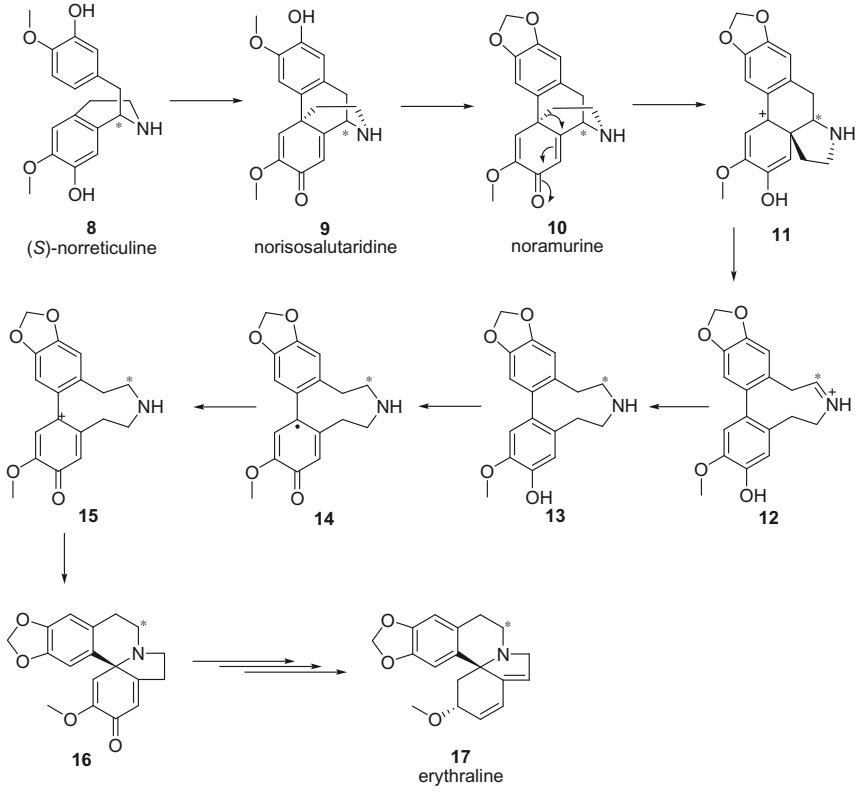


(+)-10,11-dioxoerythratidinone
7

III. BIOSYNTHESIS

Since the last reviews by Tsuda (1) and Reimann (2), little new work has been carried out into the biosynthesis of *Erythrina* and related alkaloids. Many years ago, Barton and coworkers proposed a biosynthetic pathway, however the low degree of incorporation of proposed precursors into the isolated alkaloids has prompted further investigation.

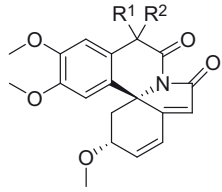
Zenk carried out ^{3}H and ^{13}C labeling studies to investigate the biosynthesis of *Erythrina* alkaloids isolated from *Erythrina crista-galli* L. (5). It was found that the fruit wall tissue was the major site of alkaloid biosynthesis, and it was subsequently shown that [1-^{13}C]-labeled (*S*)-norreticuline (**8**) was metabolized to give erythraline (**17**) with exclusive incorporation of ^{13}C at the C-10 position. It was proposed that the mechanism for the *Erythrina* alkaloid biosynthesis involves a *para*–*para* phenolic coupling of (*S*)-norreticuline (**8**), following a related proposal by Teetz (6), to afford morphinandienone and norisosalutaridine (**9**). Subsequent formation of the methylenedioxy group affords noramurine (**10**), which then undergoes rearrangement to form an asymmetric imine **12**. A subsequent two-electron oxidation process then gives the diallylic cation **15** that could be trapped by the nitrogen atom affording the *Erythrina* tetracyclic ring system **16**. Transformation to erythraline (**17**) is proposed to occur as previously described by Barton (7) (Scheme 1).



Scheme 1 Biogenesis of the *Erythrina* alkaloid erythraline (**17**).

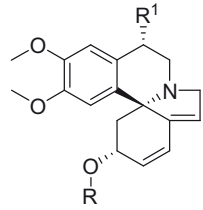
IV. PHARMACOLOGY

Following the observation that maize plants growing under the *E. latissima* tree were sparsely attacked by the stem borer insect, two new *Erythrina* alkaloids were isolated from *Erythrina latissima* E. Mey. (Fabaceae), a tropical and subtropical flowering tree, and their antifeedant activity explored (8). The roots and the stem were known to contain antimicrobial compounds (9), and the seeds, seedpods, and flowers contain erythraline alkaloids that are also known to have curare-like activity (10). The two new *Erythrina* alkaloids, (+)-11β-methoxy-10-oxoerysotramidine (**18**) and (+)-10,11-dioxo-erysotramidine (**19**) were extracted from the flowers, seeds, and seedpods of *E. latissima*. Subsequent studies found that leaves treated with **18** and **19** significantly reduced the amount of plant material eaten by the caterpillar of the *Spodoptera littoralis* moth.



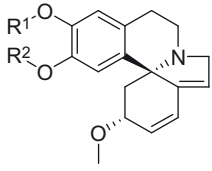
R^1 = H, R^2 = OCH_3, **18**
(+)-11β-methoxy-10-oxoerysotramidine
X-2 R^1 = R^2 = O, **19**
(+)-10,11-dioxoerysotramidine

Studies by Bolzani and Nuumes-de-Souza have demonstrated that the crude erythrinian alkaloids extracted from *E. mulungu* Mart. ex. Benth., a medium-branched tree native to Southern Brazil, and also known as the coral tree, due to its reddish flowers, possessed anxiolytic-like effects (antianxiety effects) in an elevated T-maze test (11). Two known erythrinan alkaloids, (+)-erythravine (**21**) and (+)-*R*-hydroxyerysotrine **22**, and the new erythrinan alkaloid (+)-11*R*-hydroxyerythravine (**20**) impaired the inhibitory avoidance task, with effects similar to those produced by diazepam, a well-know anxiolytic drug. It was suspected that the anxiolytic-like effects of the crude extract from *E. mulungu*, were due in some part to the effects of **20**, **21**, and **22**.

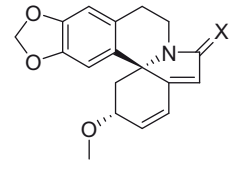


	R	R^1
20	H	OH
21	H	H
22	CH_3	OH

In work directed toward the discovery of biologically active compounds derived from tropical medicinal plants, the group of Ohsaki have explored the cytotoxicity of erythrinan alkaloids isolated from *Erythrina velutina* Willd. (12), a plant found in the north of Brazil. Traditionally, the bark is used for sedation, hypnogenesis, and the control of convulsions (13). They explored the synergistic activity of eight erythrinan alkaloids with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL selectively induces the apoptosis of a wide variety of cancer cells without damaging normal cells. Unfortunately, the clinical application of TRAIL is limited, due to resistance to the cytotoxicity. It was found that the six alkaloids, **23**–**28**, showed enhanced activity when combined with TRAIL. Of particular note were 8-oxo-erythraline (**28**), erysotrine (**23**), and glycoerysodine (**26**), which exhibited no cytotoxicity by themselves, but displayed significant cytotoxicity when combined with TRAIL (12).



	R^1	R^2
23	Me	Me
24	H	Me
25	Me	H
26	Glu	Me

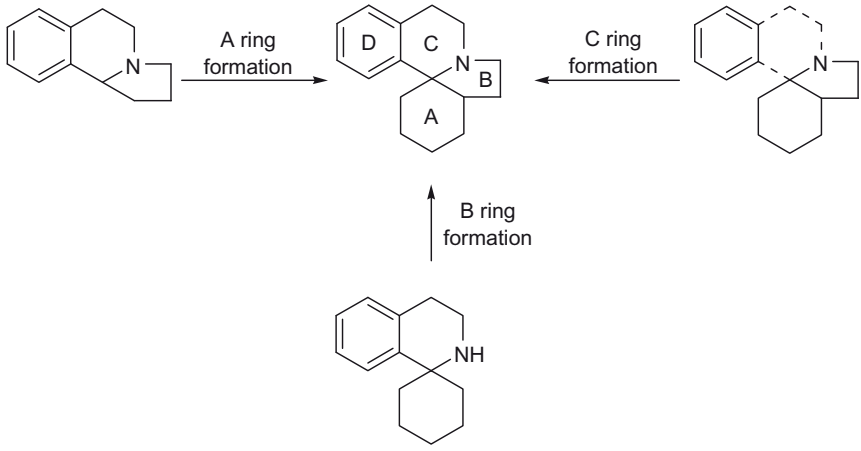


	X
27	H_2
28	O

V. SYNTHESIS

A. Aromatic *Erythrina* Alkaloids

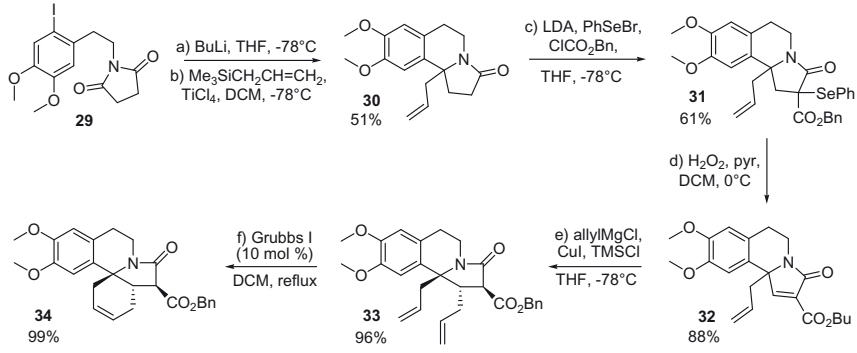
The discussion of the synthesis of the aromatic D-ring *Erythrina* alkaloids is organized by considering whether ring A, B, or C is formed in the last synthetic step (Scheme 2). Formation of the C ring in the last step is the most common strategy, and the discussion is separated into two subsections, based on whether the formation of the C ring is performed under acid conditions or not. An alternative approach is to form more than one ring in a single reaction process. For the formation of the A and B rings this is typically achieved by a metathesis approach. Alternatively, a multicomponent, domino reaction is typically used to construct the B and C rings. The discussion of the synthesis of nonaromatic *Erythrina* alkaloids will be considered on an individual basis (Section V, part B).



Scheme 2 Synthetic strategies for the formation of the erythrinan ring system.

1. Completion by Formation of the A Ring

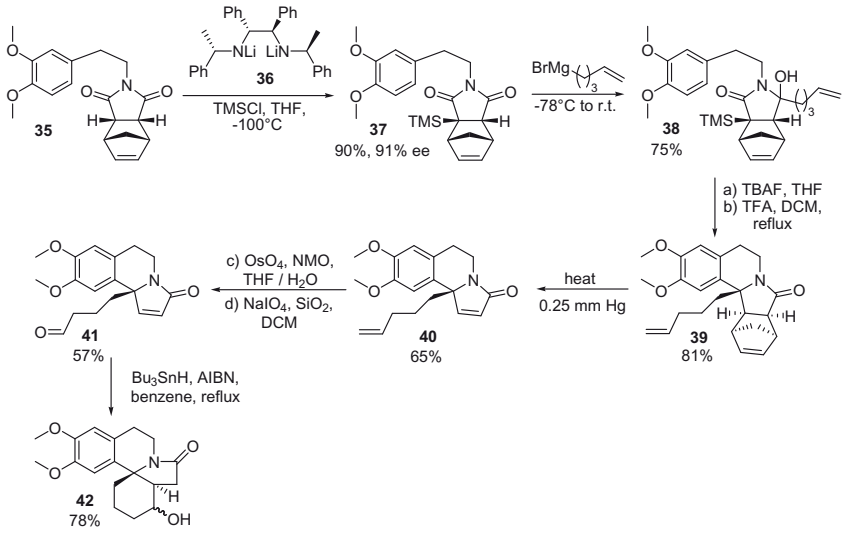
Lete and coworkers have reported a concise synthesis of the erythrnan ring system using a Grubb's ring-closing metathesis of a diene to form the A ring (Scheme 3) (14). The synthesis started with the cyclization of succinimide **29** under Parham conditions (15), to afford an α-hydroxylactam, which, on treatment with allyltrimethylsilane and titanium(IV) chloride, afforded the substituted tricycle **30**. Conversion to the α,β-unsaturated lactam **32**, was achieved in two steps by deprotonation alpha to nitrogen, quenching with benzyl chloroformate, and phenylselenyl bromide, and subsequent oxidative removal of the phenylselenide group. Addition of an organocuprate reagent occurred diastereoselectively



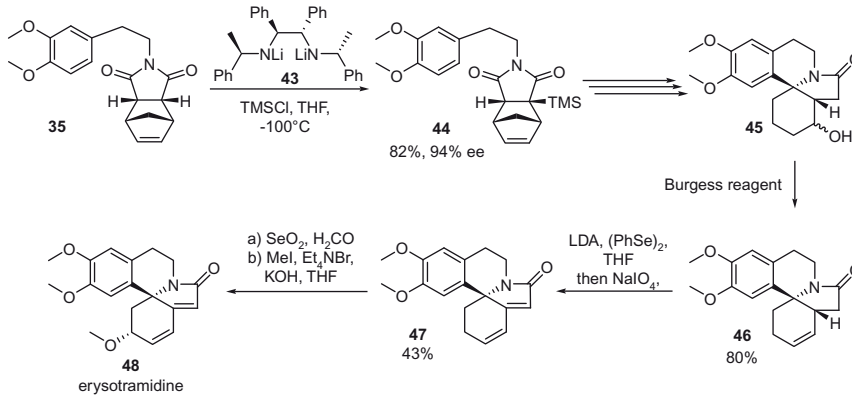
Scheme 3

to afford the *trans*-diene **33**. A final ring-closing metathesis reaction of diene **33** using Grubbs' first-generation catalyst cleanly afforded the erythrinan tetracyclic skeleton of **34**, as a single diastereomer in near quantitative yield.

Based on previous work of Lete (16), the group of Simpkins (17) has developed an asymmetric approach to the erythrinan alkaloid core, using the chiral base desymmetrization of an imide to form the BCD ring system, and a subsequent 6-*exo*-*trig* radical cyclization to form the A ring (Scheme 4). The key asymmetric step involves the deprotonation of imide **35** using the chiral bis-lithium amide **36**, and subsequent quenching of the resulting anion to give the desired silylated product **37** in 90% yield and 91% ee. Regioselective and stereoselective addition of a Grignard reagent to imide **37** is controlled by the trimethylsilyl group – the nucleophile adds distal to the trimethylsilyl group. Subsequent desilylation and heating in trifluoroacetic acid affords the pentacycle **39** with complete diastereocontrol. With the BCD ring synthesis complete, and all of the necessary stereochemistry present, attention turned to the installation of the A ring. This was accomplished by an initial retro-Diels–Alder reaction of **39** to afford diene **40**. Cleavage of the terminal alkene to the aldehyde was accomplished by oxidation to the diol using osmium tetroxide, followed by oxidative cleavage using sodium periodate to afford aldehyde **41**. A subsequent reductive cyclization mediated by tributyltin hydride afforded hydroxylactam **42**, to complete the synthesis of the ABCD core of the unnatural enantiomer of the *Erythrina* alkaloids.



Scheme 4



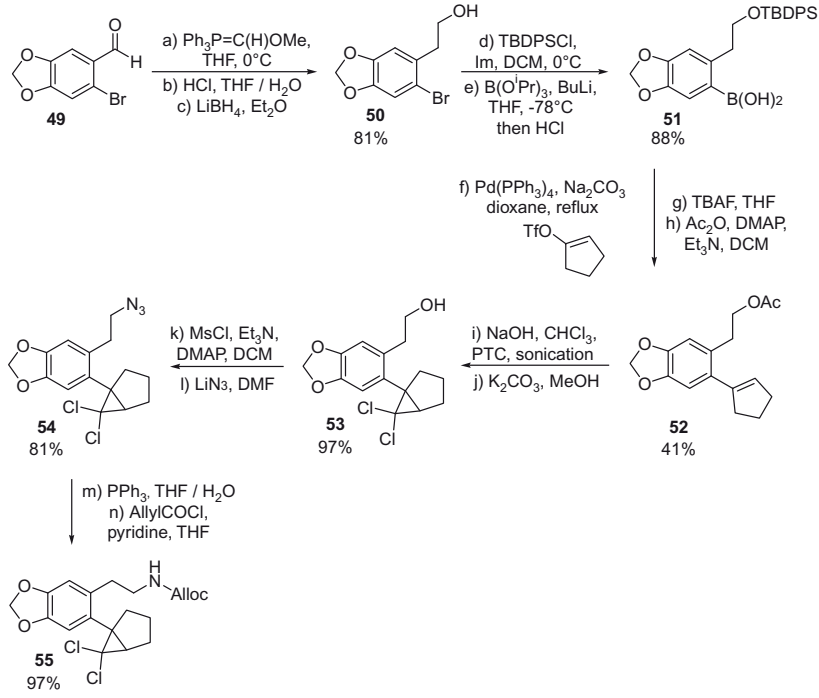
Scheme 5

Subsequent work within the Simpkins (18) group developed a route to the natural enantiomer of the *Erythrina* alkaloid erysotramidine (**48**), using the chiral base **43**, the opposite enantiomer of the chiral base **36**, in the asymmetric desymmetrization of imide **35** (Scheme 5). Following a similar sequence to that shown in Scheme 4, hydroxylactam **45** was prepared. Completion of the total synthesis of erysotramidine (**48**) was accomplished in four steps, involving initial dehydration of the hydroxylactam **45** with Burgess reagent to afford lactam **46**. This was followed by the introduction of a phenylselenide group, oxidation, and elimination of the resulting selenoxide, which then afforded the conjugated amide **47**. Finally, incorporation of the methoxy group was accomplished using a procedure developed by Padwa (19), involving allylic oxidation using selenium dioxide to give an allylic alcohol, although in low yield with predominant recovery of the starting material. Methylation afforded the *Erythrina* alkaloid erysotramidine (**48**) in 93% ee (18).

2. Completion by Formation of the B Ring

Banwell has accomplished the synthesis of the ABCD framework of *Erythrina* alkaloids by treatment of a *gem*-dichlorocyclopropane fragment with silver tetrafluoroborate to afford the ACD rings, and a subsequent tributyltin hydride-mediated cyclization that afforded the B ring (20). The synthesis of the *gem*-dihalocyclopropane **55**, required for the key electrocyclic ring-opening/spirocyclization sequence, was derived from the bromoaldehyde **49** (Scheme 6).

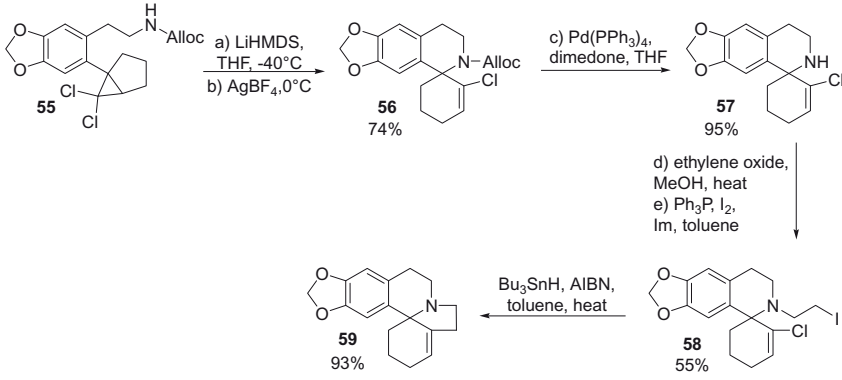
Treatment of bromoaldehyde **49** with (methoxymethylene)triphenylphosphorane gave the corresponding vinyl ether, which was hydrolyzed in aqueous acid to give an aldehyde that was subsequently reduced with lithium borohydride to afford **50**. *tert*-Butyldiphenylsilyl (TBDPS) protection of the primary alcohol was carried out before formation of the



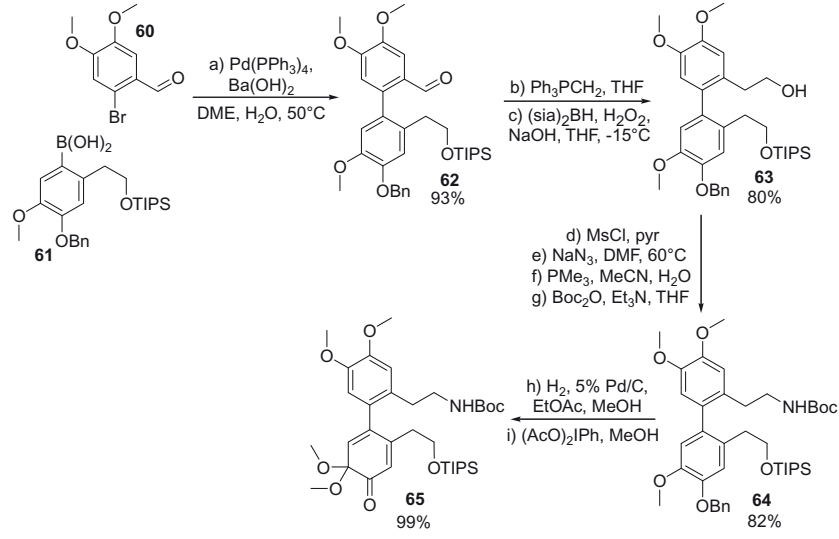
Scheme 6

boronic acid **51**. Subsequent Suzuki–Miyaura coupling (21) of boronic acid **51** with the enol triflate of cyclopentanone afforded the desired arylated cyclopentene, after which the silyl ether was converted into the corresponding acetate **52**. Treatment with dichlorocarbene, generated according to the procedure of Makosza (22), using phase-transfer catalysis (TEBAC) afforded the cyclopropane acetate, which was then converted into the free alcohol **53**. Conversion to the amine **55** was accomplished via mesylation, displacement with lithium azide, and a Staudinger reduction using triphenylphosphine. Alloc protection of the resulting primary amine afforded the key precursor **55** for spirocyclization.

Treatment of the Alloc-protected amine **55** with lithium hexamethyldisilazide, followed by silver tetrafluoroborate, afforded the desired spirocyclic compound **56**, via a mechanism involving *N*-deprotonation and subsequent nucleophilic attack on the cation derived from ring-opening of the cyclopropane ring (Scheme 7). Installation of the iodoalkyl chain was accomplished via removal of the Alloc-protecting group and subsequent addition of the resulting secondary amine to ethylene oxide, followed by conversion into the iodide **58**. Treatment of iodide **58** under standard tributyltin hydride reducing conditions afforded the ABCD framework of the *Erythrina* alkaloids in excellent yield (20).

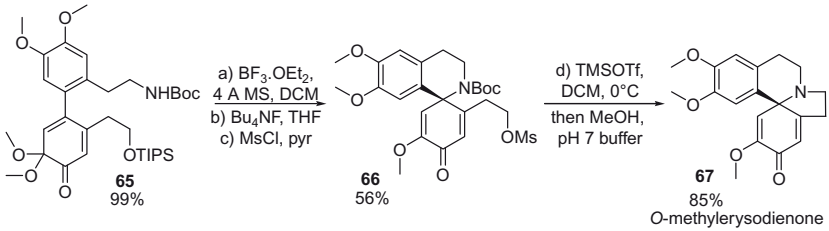


Scheme 7



Scheme 8

The group of Matsumoto has reported a novel approach to the synthesis of the erythrinan alkaloids using a biphenyl framework as the basis of the AD ring system, and a subsequent Lewis acid-promoted spirocyclization (an intermolecular attack of a nitrogen nucleophile in an S_N2' fashion) to install the C ring (Scheme 8) (23). The synthesis began by employing a Suzuki–Miyaura coupling of aryl bromide **60** with arylboronic acid **61** (24), to afford the biphenyl aldehyde **62**. Subsequent Wittig olefination, and oxidative hydroboration gave alcohol **63**, which was mesylated, displaced with sodium azide, reduced to the corresponding amine, and Boc protected to afford the biphenyl **64**. The A ring was



Scheme 9

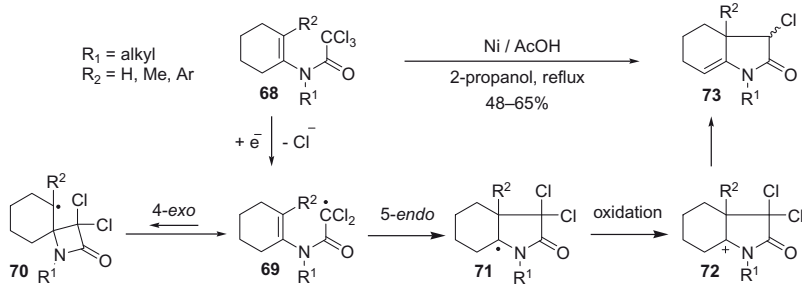
set up for the intermolecular spirocyclization through conversion to the quinone monoacetal **65** by removing the benzyl-protecting group and oxidation of the resulting substituted phenol.

With the quinone monoacetal **65** in hand, attention turned to the intermolecular spirocyclization to afford the C ring (Scheme 9). This was achieved cleanly by treatment of the quinone monoacetal **65** with boron trifluoride to give the spirocycle in an excellent 84% yield. Subsequent removal of the triisopropylsilyl group, and conversion of the primary alcohol into the mesylate afforded the protected tricycle **66**. Treatment of **66** with trimethylsilyl trifluoromethanesulfonate and methanol triggered the *N*-Boc deprotection and finally, cyclization yielded the erythrinan alkaloid *O*-methylerysodienone (**67**) (23).

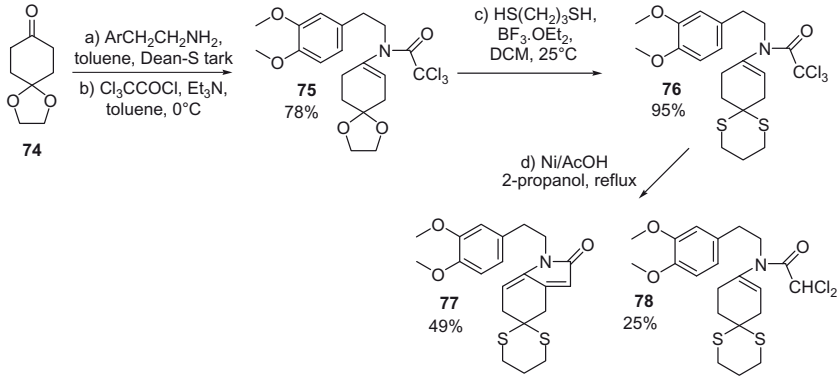
3. Completion by Formation of the C Ring

By Acid-Catalyzed Cyclization. Zard and coworkers have shown that nickel powder with acetic acid in refluxing 2-propanol can be used to generate radicals derived from trichloroacetamides, which are then able to undergo either 4-*exo* or 5-*endo* cyclizations (25). It was found that treatment of trichloroacetamides **68** with nickel and acetic acid resulted in reduction to form intermediate radical **69**, which cyclizes to give exclusively the *5-endo* product **71**, even though the *4-exo* cyclization would give the resonance-stabilized radical **70**. A subsequent oxidation of the 5-*endo* product **71** leads to the generation of a cation equivalent **72**, which undergoes elimination, and after further reduction, yields chlorolactam **73** (Scheme 10) (26).

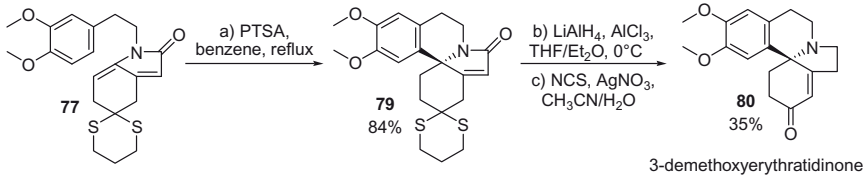
The use of a nickel/acetic acid-promoted cyclization was subsequently applied to the synthesis of the *Erythrina* alkaloid 3-demethoxyerythratidinone (**80**) (Schemes 11 and 12) (27). The trichloroacetamide **75** was obtained in three steps from cyclohexanone **74** by condensation with homoveratrylamine, followed by acylation with trichloroacetyl chloride. Trichloroacetamide **75** was treated with nickel and acetic acid; however, the cyclization was unsuccessful. Replacement of the ketal group with a dithioketal resulted in the isolation of the trichloroacetamide **76**. Subsequent treatment with nickel powder and acetic acid in



Scheme 10



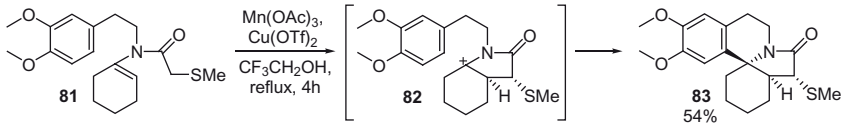
Scheme 11



Scheme 12

refluxing 2-propanol afforded the expected lactam **77** in 49% yield, together with a 25% yield of the direct reduction product **78**.

Treatment of the lactam **77** with *p*-toluenesulfonic acid (PTSA) afforded the desired tetracyclic *Erythrina* core **79** (Scheme 12). Finally, reduction of the amide moiety using alane (generated *in situ* from the



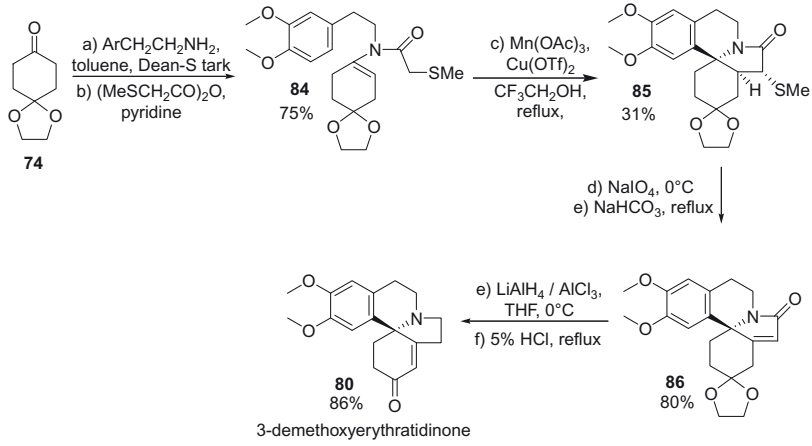
Scheme 13

reaction of lithium aluminum hydride and aluminum trichloride), and subsequent deprotection of the ketone with migration of the C═C bond, gave 3-demethoxyerythratidinone (**80**), in seven steps starting from the cyclohexadione monoethylene ketal **74** in an unoptimized 10% overall yield (27).

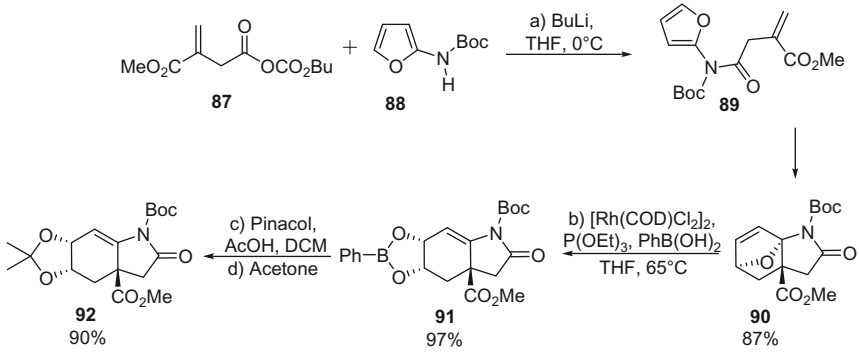
Ishibashi and coworkers have reported a similar approach to the tetracyclic core of *Erythrina* alkaloids. It was observed that the cyclization of methylthio amides could be achieved using an oxidative radical cyclization mediated by manganese(III) acetate in refluxing 2,2,2-trifluoroethanol in the presence of a copper(II) additive (28). Initial tests on a model system involved treatment of methylthio amide **81** with manganese(III) acetate and copper(II) triflate to afford the desired tetracyclic core **83** in 54% yield (Scheme 13) (29). It was rationalized that after radical cyclization the aromatic ring is able to attack a cationic intermediate **82**, formed by oxidation of the cyclized radical.

With the cyclization working on a model system, attention turned to the synthesis of 3-demethoxyerythratidinone (**80**) (Scheme 14). The key methylthio amide **84** was obtained from cyclohexanone **74** by condensation with homoveratrylamine followed by acylation with (methylthio) acetic anhydride. On treatment with manganese(III) acetate and copper (II) triflate in refluxing 2,2,2-trifluoroethanol, methylthio amide **84** afforded the expected *Erythrina* core **85** in 31% yield (30). Conversion of the *Erythrina* ring system of **85** into 3-demethoxyerythratidinone (**80**) was achieved using a previously developed four-step procedure, via oxidation of the sulfide **85** to the corresponding sulfoxide, thermal elimination to afford the amide **86**, and subsequent reduction with alane and deprotection to afford 3-demethoxyerythratidinone (**80**) (31).

Padwa has reported the synthesis of 3-demethoxyerythratidinone (**80**) using the Diels–Alder cyclization of an imidofuran (32) to prepare the A–B ring system, and subsequent *N*-alkylation and a Pictet–Spengler reaction to install the remaining C–D ring system (Schemes 15 and 16) (33). The synthesis of the A–B ring fragment (Scheme 15) was accomplished by coupling anhydride **87** with a lithiated furan derived from **88**, to afford the imidofuran **89**. Imidofuran **89** undergoes a rapid intramolecular [4+2]-cycloaddition/Diels–Alder cyclization to afford the oxabicyclo adduct **90**. A subsequent Rh(I)-catalyzed ring-opening



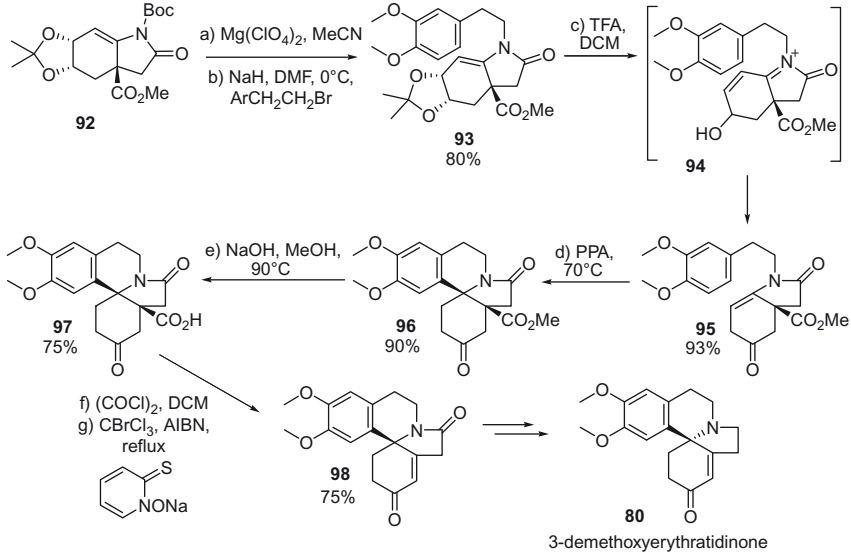
Scheme 14



Scheme 15

reaction, afforded the ring-opened boronate **91**, via nucleophilic addition distal to the bridgehead substituent (34). Conversion to the acetonide **92** was accomplished via treatment of **91** with pinacol and acetic acid, followed by reaction with acetone.

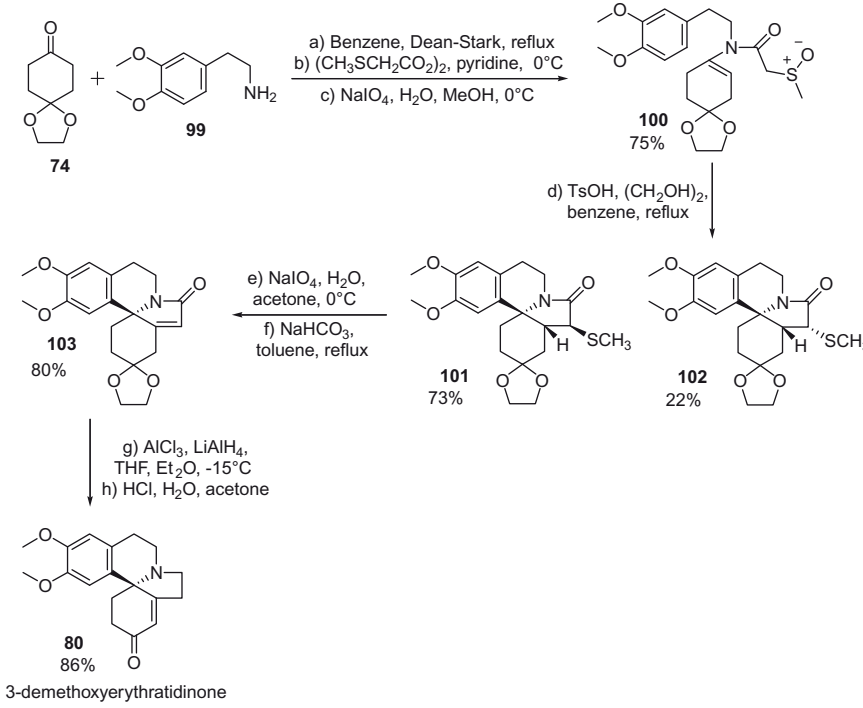
Treatment of acetonide **92** with magnesium perchlorate resulted in *N*-Boc deprotection and *N*-alkylation to give lactam **93** (Scheme 16). Subsequent treatment with trifluoroacetic acid-induced formation of the enamide **95**, presumably via acid-induced loss of acetone to afford *N*-acyliminium ion **94** as an intermediate. Treatment of **95** with polyphosphoric acid (PPA) triggered the desired Pictet–Spengler reaction to afford the tetracyclic erythrinan core **96**, and base-catalyzed hydrolysis



Scheme 16

gave carboxylic acid **97**. Decarboxylation and elimination of hydrogen bromide was accomplished using conditions developed by Barton (35) to give isoquinolinedione **98**, which was converted into 3-demethoxyerythratidinone (**80**) by a route previously developed by Tsuda (36).

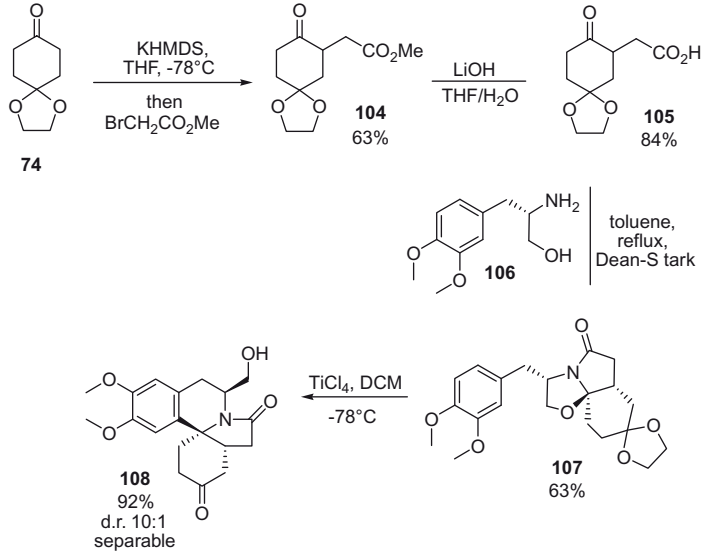
Ishibashi has reported the total synthesis of 3-demethoxyerythratidinone (**80**) in eight steps, and in an excellent overall yield of 39%, using the Pummerer rearrangement of a sulfoxide in the key step (Scheme 17) (31,37). The synthesis of the key sulfoxide **100** was achieved in three steps, via condensation of homoveratrylamine **99** with cyclohexanone **74**, subsequent acylation with (methylthio)acetic anhydride, and oxidation with sodium metaperiodate, which gave the sulfoxide **100** in 75% yield. Treatment with PTSA triggered the Pummerer reaction to produce an intermediate lactam, which then cyclized to afford the tetracyclic erythrinan ring system as a mixture of diastereoisomers **101** and **102**. Elimination of the methylthio group was accomplished by oxidation with sodium metaperiodate, to afford the sulfoxide, which then undergoes a thermal elimination to afford unsaturated lactam **103**. It should be noted that only the *cis* diastereomer **101** undergoes elimination due to the *syn*-mechanism for elimination (38). Finally, the synthesis was completed by reduction of the amide with alane, generated from lithium aluminum hydride and aluminum chloride, and deprotection of the ketal using aqueous acid to afford 3-demethoxyerythratidinone (**80**) (31).



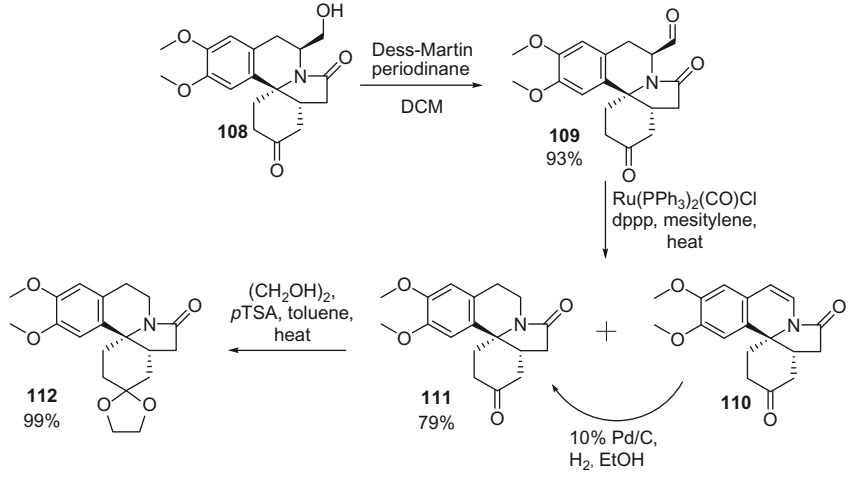
Scheme 17

Other Methods of Cyclization. Allin and coworkers have reported the formal asymmetric synthesis of 3-demethoxyerythratidinone (**80**) by the cyclization of a chiral, highly functionalized lactam as the key step (39). This approach follows from their previous work on the development of efficient and stereoselective routes to heterocyclic targets through the cyclization of chiral *N*-acyliminium intermediates (40). Synthesis of the key lactam **107** was achieved in three steps, starting from cyclohexanone **74**, using deprotonation followed by alkylation to afford the ester **104**. Subsequent hydrolysis gave carboxylic acid **105** (Scheme 18). Condensation, under Dean–Stark conditions, of the carboxylic acid **105** and the amino-alcohol **106**, obtained from reduction of the amino acid 3-(3,4-dimethoxyphenyl)-L-alanine, yielded the desired lactam **107** as a single diastereoisomer. Treatment of lactam **107** with the Lewis acid titanium (IV) chloride triggered the desired asymmetric cyclization, with the tetracyclic core **108** being obtained in an excellent 92% yield as a 10:1 mixture of separable diastereoisomers, with removal of the ketal-protecting group.

Completion of the formal synthesis was accomplished via removal of the hydroxymethyl substituent through Dess–Martin oxidation of the

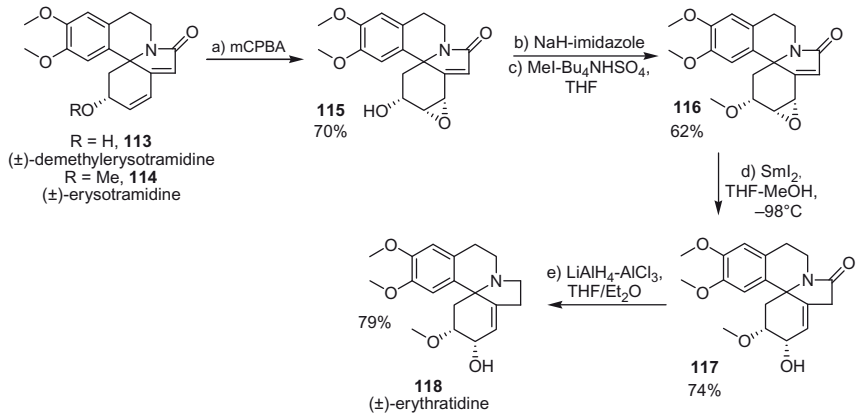


Scheme 18



Scheme 19

primary alcohol **108** to the aldehyde **109** followed by a rhodium-catalyzed decarbonylation. This afforded a mixture of the enamide **110** and the desired amide **111**, which, on hydrogenation of the crude reaction mixture, gave the desired amide **111** in 79% yield (Scheme 19). Finally, the formal asymmetric synthesis required reprotection of the

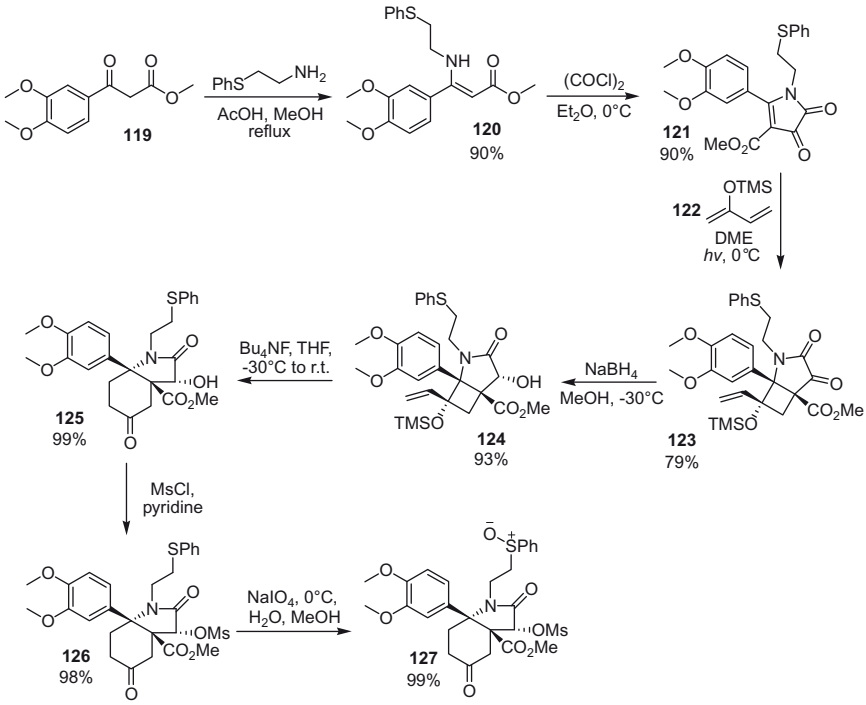


Scheme 20

ketone to form acetal **112**, from which Tsuda has described a four-step sequence to afford (+)-demethoxyerythratidinone (**80**) (41).

Tsuda and coworkers have directed their attention toward the synthesis of a range of erythranan alkaloids, which possess an aromatic D ring. One such example is the synthesis of (±)-erythratidine (**118**) (Scheme 20) (42). The synthesis commences from (±)-demethylerysotramidine (**113**) (43,44) for which the group had previously developed an efficient approach. A face-selective oxidation of (±)-demethylerysotramidine (**113**) with *m*-chloroperbenzoic acid yielded the epoxide **115**. Interestingly, similar attempts to epoxidize (±)-erysotramidine (**113**) (*R* = OMe) resulted in recovery of the starting material. Subsequent methylation of the epoxide **115** under phase-transfer conditions allowed the isolation of the *O*-methyl derivative **116**. A samarium diiodide-mediated radical opening of the epoxide ring, and subsequent migration of the C = C bond out of conjugation with the lactam, afforded the allylic alcohol **117** in 74% yield. The lactam was reduced through *in situ* generation of alane using lithium aluminum hydride and aluminum trichloride to afford (±)-erythratidine (**118**) in 79% yield (42).

Tsuda and coworkers have also described the synthesis of a cyclo-erythrinan utilizing a Pummerer (45) type reaction in a key step (46). Cyclo-erythrnans are known to be key synthetic intermediates for a range of *Erythrina* alkaloids (46). The synthesis of the Pummerer precursor **127** was accomplished by the initial amination of methyl 3,4-dimethoxybenzoyl acetate (**119**) with 2-phenylthioethylamine to furnish the enamine **120**. Subsequent condensation with oxalyl chloride gave *N*-(2-phenylthioethyl)dioxopyrroline (**121**) in 81% yield over the two steps (Scheme 21). A subsequent [2+2] photo-cycloaddition with 2-trimethylsilyloxybutadiene (**122**) afforded the desired vinylcyclobutane **123** in a

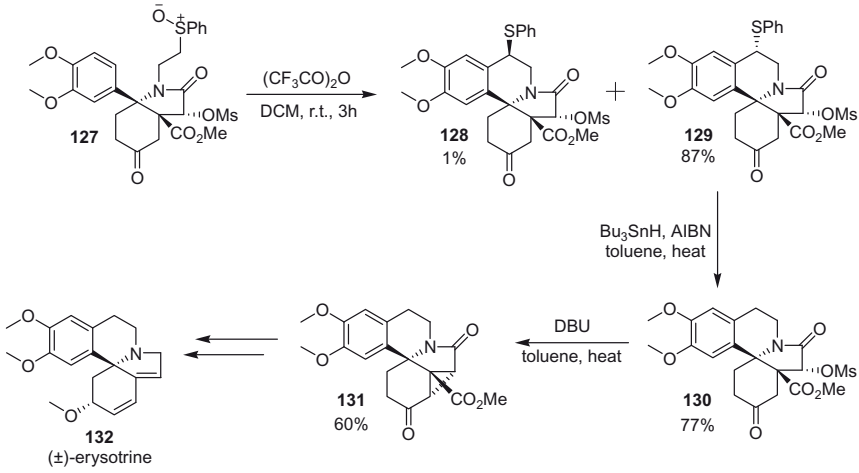


Scheme 21

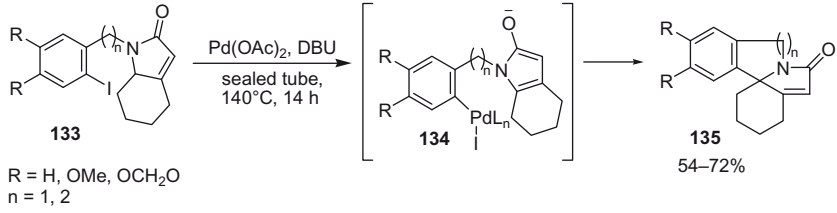
regioselective and stereoselective process. Sodium borohydride reduction of vinylcyclobutane **123** resulted in the stereoselective reduction of the C-4 ketone to afford alcohol **124** in 93% yield. Treatment of the silyl ether **124** with tetrabutylammonium fluoride resulted in an efficient 1,3-anionic rearrangement to yield the hydroindole **125** in 99% yield. To complete the synthesis of the Pummerer precursor **127** the alcohol **126** was mesylated, and a sodium periodate oxidation afforded the desired sulfoxide **127**.

The sulfoxide **127** was then subjected to the Pummerer cyclization by treatment with trifluoroacetic anhydride, which afforded the desired phenylthioerythinan **129** as one predominate isomer (Scheme 22). Cleavage of the phenylthio group was accomplished by a radical reduction using tributyltin hydride and AIBN, to yield amide **130**. Subsequent treatment with DBU afforded the cyclo-erythrinan **131**, in an overall yield of 35% over eight steps from methyl 3,4-dimethoxybenzoyl acetate (**119**). Previous reports have shown that cyclo-erythrinan **131** can be converted into erysotrine (**132**) (44).

Kim has developed a novel route for the synthesis of the tetracyclic core of *Erythrina* alkaloids by utilizing a palladium-catalyzed arylation of



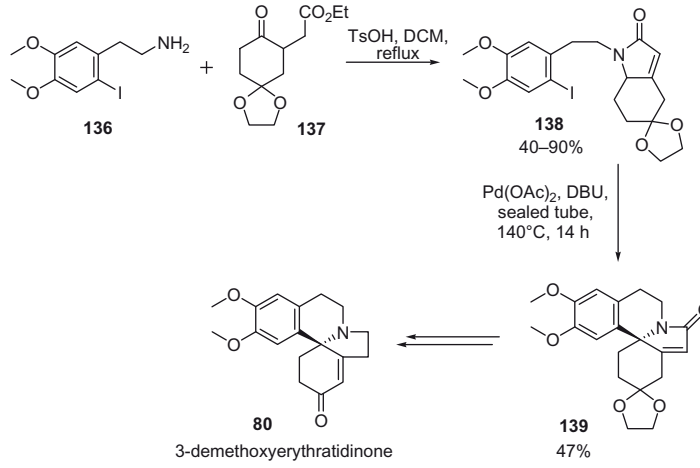
Scheme 22



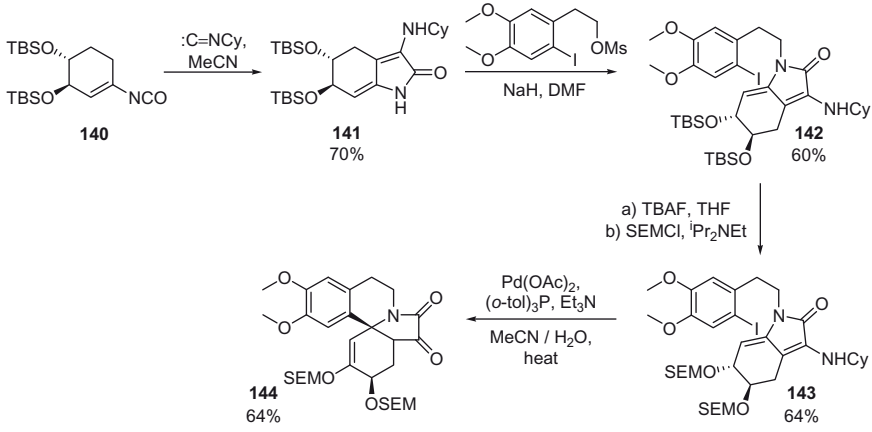
Scheme 23

an α,β-unsaturated γ-lactam (Scheme 23) (47). α,β-Unsaturated γ-lactams **133** can be prepared by condensation of the corresponding 2-iodo-arylamines with ethyl 2-oxocyclohexane acetate under reflux in toluene with PTSA in 40−90% yield (19). Treatment of these α,β-unsaturated γ-lactams **133**, under optimized conditions of 5 mol% of palladium(II) acetate and DBU in a sealed tube at 140°C affords the desired tetracycles **135**. A proposed mechanism for the cyclization involves the formation of enolate ion **134**, which reacts by insertion of the palladium into the C═C bond, followed by reductive elimination (48).

Subsequently, this methodology was applied to the synthesis of 3-demethoxyerythratidinone (**80**) (Scheme 24). The desired α,β-unsaturated γ-lactam **138** was prepared as described above from 2-iodo-arylamine **136** and ethyl 2-oxocyclohexane acetate (**137**). Treatment of the iodide **138** under the optimized conditions afforded the tetracyclic core **139**, which could be converted to 3-demethoxyerythratidinone (**80**) by reduction of the amide group with alane, followed by an aqueous acid-based deprotection of the ketal (31).

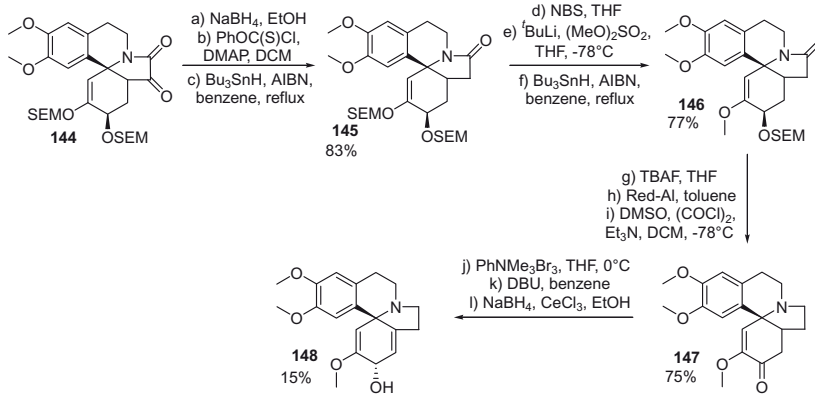


Scheme 24



Scheme 25

The *Erythrina* alkaloid, 2-epi-erythrinitol (**148**), has been synthesized in a sequence involving a [1+4]-cycloaddition followed by an intramolecular Heck reaction for assembly of the erythrinan ring system (Schemes 25 and 26) (49). The synthesis began with the [1+4]-cycloaddition between the substituted vinyl isocyanate **140** and cyclohexyl isocyanide (which is a 1,1-dipole equivalent) to afford the hydroindolone **141** (Scheme 25). Subsequent displacement of the mesylate by the sodium salt of lactam **141** afforded aryl iodide **142**, the planned precursor for the Heck reaction. However, initial attempts at the Heck reaction were unfruitful, being attributed to the steric bulk of the TBS groups. Exchanging the TBS

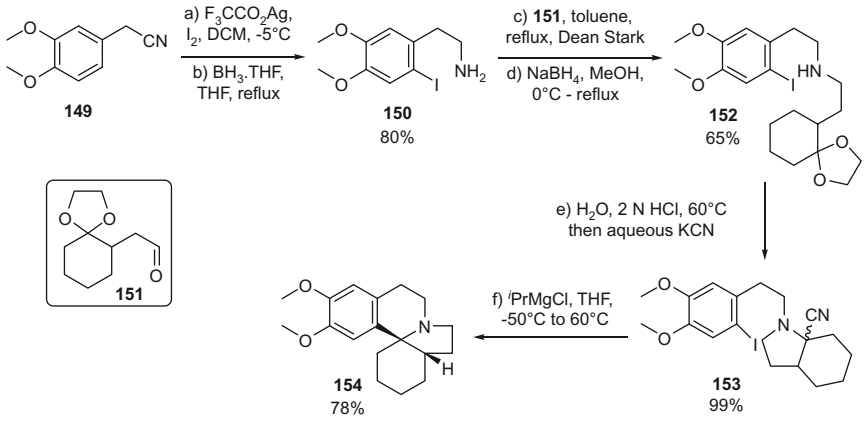


Scheme 26

ethers, for smaller SEM ethers, proceeded smoothly to afford the Heck precursor **143**. The palladium-mediated Heck reaction of the bis-SEM ether **143** proceeded as expected, and, after hydrolysis of the enamine, yielded the erythrinan core **144** as a single diastereomer.

With the carbon skeleton of erythrinitol in place, attention turned to correcting the oxidation levels of the ring systems (Scheme 26). Reduction of the ketone group was accomplished using sodium borohydride, and subsequent Barton–McCombie deoxygenation (50) afforded amide **145**. Conversion to the methyl enol ether **146** was achieved in three steps, via treatment with *N*-bromosuccinimide to give the α-bromoketone, *O*-methylation of the corresponding enol, and radical debromination using tributyltin hydride. Installation of the final C═C bond proved problematic, but was accomplished in six steps to afford 2-epi-erythrinitol (**148**). Removal of the SEM group, Red-Al reduction of the lactam, and a Swern oxidation gave the ketone **147**. Subsequent treatment with phenyltrimethylammonium bromide afforded the corresponding α-bromoketone, and elimination with DBU gave an α,β-unsaturated ketone, which, on reduction under Luche conditions, afforded 2-epi-erythrinitol (**148**) as a single diastereomer (49).

Reimann (51) has reported the synthesis of the Erythrinan core ring system using an intramolecular Strecker reaction, followed by an intermolecular Bruylants reaction (Scheme 27) (52). Their approach starts by iodination of the phenylacetonitrile **149**, followed by reduction of the nitrile group using the borane–tetrahydrofuran complex to afford the iodo-phenethylamine **150**. Subsequent condensation with aldehyde **151** gave the corresponding imine, which was then reduced with sodium borohydride, to afford the secondary amine **152**. With the secondary amine **152** in hand, the intramolecular Strecker reaction provided the



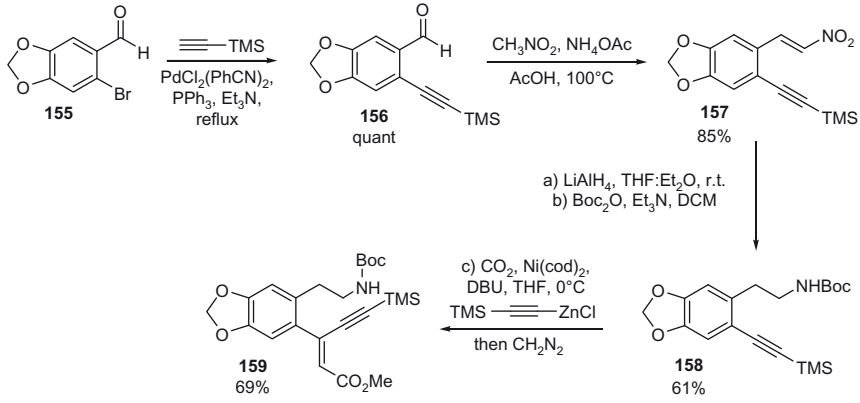
Scheme 27

bicyclic nitrile **153** as a mixture of diastereomers (7:3) in excellent yield. A subsequent halogen–magnesium exchange process triggered the Bruylants reaction leading to displacement of the nitrile group and formation of the desired erythrinan alkaloid **154** (51).

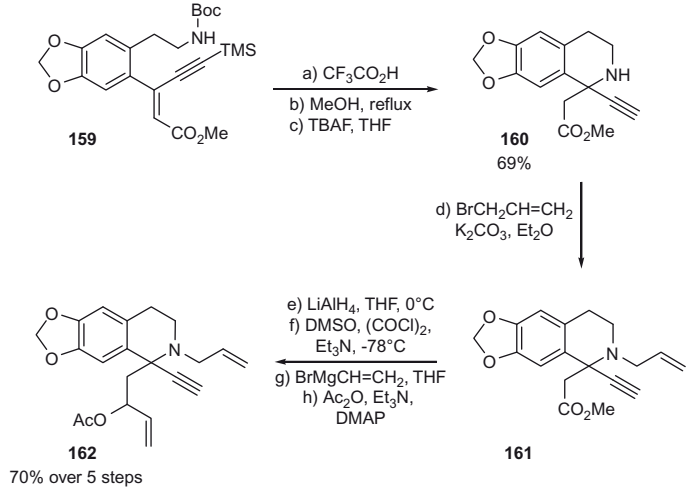
4. Multiple Ring Formation

By Metathesis. By extending previous work on nickel-mediated alkylative carboxylation reactions (53), Mori and coworkers have reported the total synthesis of erythrocarine (**164**). The synthesis uses a novel approach for preparing the desired isoquinoline C–D ring fragment, followed by a Grubb's first-generation ruthenium-catalyzed dienyne metathesis reaction in a key step (Schemes 28–30) (54). The synthesis of the first key alkyne **159** was achieved in five steps using an initial palladium-mediated (Sonogashira) coupling reaction of the commercially available bromoaldehyde **155**, with trimethylsilylacetylene, to afford alkyne **156** (Scheme 28). Subsequent condensation with nitromethane gave the nitroalkene **157** that was reduced with lithium aluminum hydride, and *N*-Boc protection afforded the precursor **158** for the nickel-mediated alkylative carboxylation. The carboxylation reaction using bis(cyclooctadiene)nickel(0) ($Ni(cod)_2$) proceeded smoothly, and this was followed by the addition of an alkynyl-zinc reagent, followed by hydrolysis and esterification with diazomethane to give methyl ester **159**.

The synthesis of the ring-closing metathesis precursor **162** was achieved in seven steps (Scheme 29). Initially, deprotection of the *N*-Boc group was followed by an intramolecular Michael addition to afford the isoquinoline core, then deprotection of the silyl group, and allylation of the secondary amine to afford **161**. Subsequently, reduction of the ester



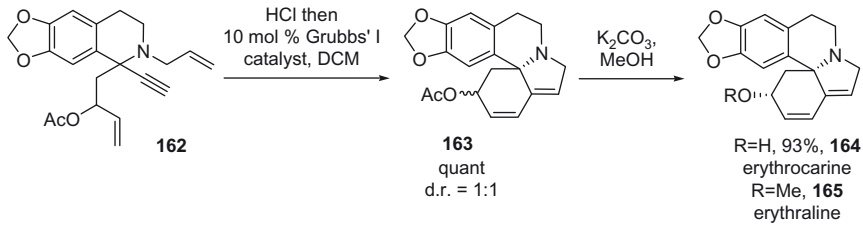
Scheme 28



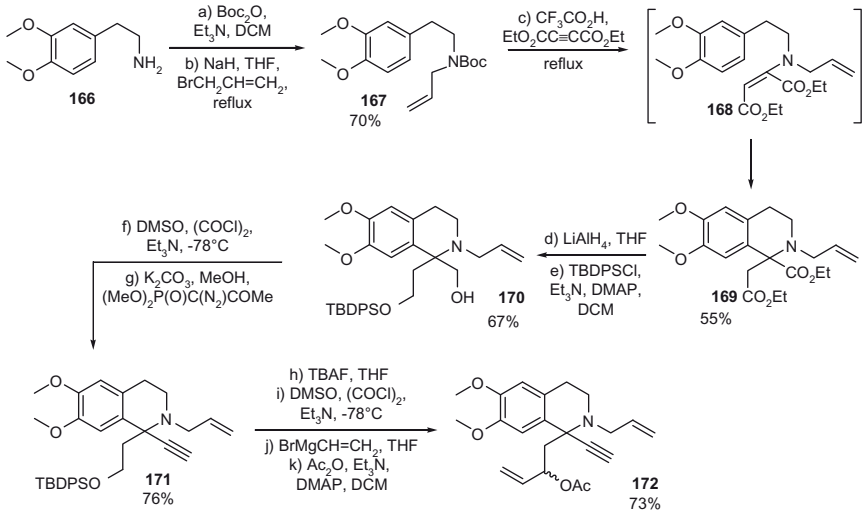
Scheme 29

(using lithium aluminum hydride) was followed by Swern oxidation to the aldehyde, nucleophilic addition of vinylmagnesium bromide, and finally, protection of the secondary alcohol gave acetate **162** as a 1:1 mixture of inseparable diastereomers.

To prevent coordination of the tertiary amine nitrogen to the ruthenium metal during metathesis, compound **162** was initially converted into the hydrochloride salt (Scheme 30). Reaction of the salt with Grubbs' first-generation ruthenium catalyst at room temperature cleanly afforded the expected tetracyclic compound **163** in near



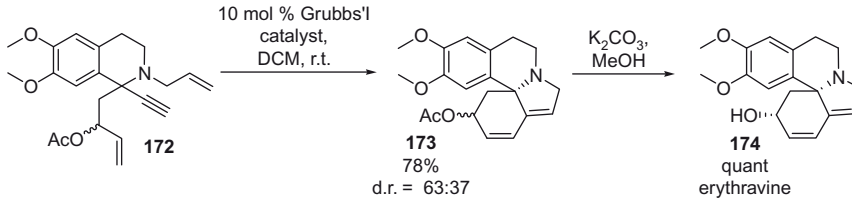
Scheme 30



Scheme 31

quantitative yield as a 1:1 mixture of separable diastereomers. Subsequent treatment of the desired diastereomer with potassium carbonate in methanol afforded the alcohol, erythrocarine (**164**) (54), whose methyl ether is erythraline (**165**).

Hatakeyama and coworkers have applied a similar ring-closing metathesis strategy to the first total synthesis of a related erythrinan alkaloid, (±)-erythravine (**174**) (Schemes 31 and 32) (55). The synthesis starts from 3,4-dimethoxyphenethylamine (**166**), which is first *N*-Boc protected and *N*-allylated. Subsequent addition to diethyl propiolate, followed by a Pictet–Spengler reaction in refluxing trifluoroacetic acid, gave the isoquinoline diester **169** (56). Reduction of both esters with lithium aluminum hydride was followed by selective TBDPS protection of the less hindered primary alcohol to give **170**. A Swern oxidation to the aldehyde, followed by a modified Horner–Wadsworth–Emmons-type (57) reaction afforded enyne **171**. Subsequent desilylation, Swern



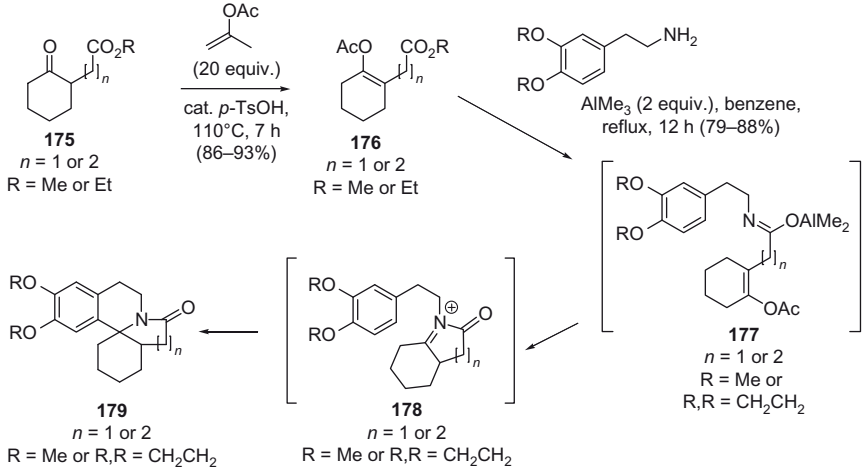
Scheme 32

oxidation, nucleophilic addition of vinylmagnesium bromide to the resulting aldehyde, and acetate formation yielded the dienyne **172** as a 1:1 mixture of diastereoisomers.

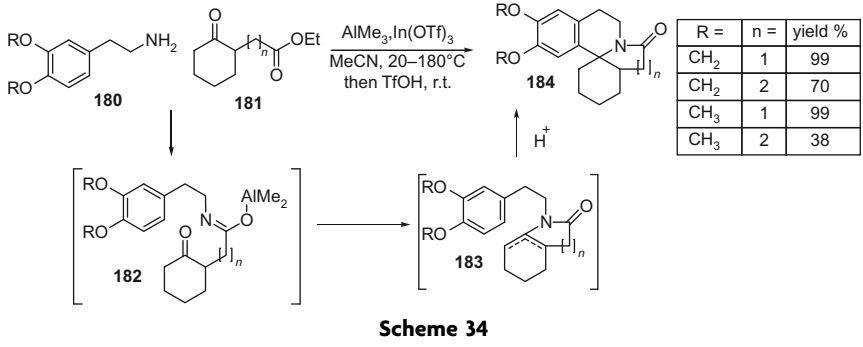
Treatment of acetate **172** with 10 mol% of Grubbs' first-generation ruthenium catalyst in refluxing DCM afforded the tetracycle **173** as a 63:37 mixture of diastereoisomers in 78% yield (Scheme 32). It was found that treatment of the free alcohol with Grubbs' catalyst in a range of solvents at room temperature or elevated temperature did not afford any cyclized material. It was proposed that the sluggish reaction of acetate **172** could be due to coordination of the free tertiary amine to the ruthenium catalyst. Finally, treatment of the tetracycle **173** with potassium carbonate in methanol afforded (±)-erythravine (**174**) (55).

Multicomponent Domino Reactions. A highly efficient synthesis of the *Erythrina* and β-homoerythrina skeleton has been developed using a trimethylaluminum-mediated domino reaction (58). First, enol acetates **176** were formed by reacting ketones **175** with isoprenyl acetate in the presence of a catalytic amount of an acid catalyst (Scheme 33). Treating the enol acetates **176** with a substituted aminoethylbenzene and trimethylaluminum was expected to form an intermediate aluminum amide enolate **177** that reacts with the enol ether to form an *N*-acyliminium ion **178**. Finally, cyclization of the *N*-acyliminium ion **176**, in an electrophilic substitution reaction, forms the *Erythrina* and β-homoerythrina alkaloids **179**. Overall, this domino reaction, which uses readily available starting materials, allows three sequential bonds to be formed in a one-pot reaction.

The group of Tietze (59) have reported a similar efficient, atom economical, domino reaction for the one-pot synthesis of the *Erythrina* and homoerythrina skeleton. The tetracyclic azaspiro core was formed by a Lewis acid-catalyzed reaction of an arylethylamine **180** with an oxocarboxylate **181**, in the presence of trimethylaluminum, to form the intermediate aluminum amide **182** (Scheme 34). The aluminum amide **182** then undergoes an intramolecular attack on to the C=O bond of the ketone to form a mixture of enamides **183**. Addition of triflic acid results



Scheme 33



R =	n =	yield %
CH_2	1	99
CH_2	2	70
CH_3	1	99
CH_3	2	38

Scheme 34

in protonation of the enamides to form an iminium ion. This ion undergoes a Pictet–Spengler reaction to afford the corresponding *Erythrina* or homoerythrina skeleton **184** (59).

B. Nonaromatic *Erythrina* Alkaloids

The syntheses of *Erythrina* alkaloids possessing an aromatic D ring, and especially those bearing oxygen atoms at C-15 and C-16, have been well documented. In contrast, the nonaromatic alkaloids consist of an oxa D ring, as in the erythroidines, or an aza D ring such as in erymelanthine (**191**) (Figure 2). The laboratory synthesis of the *Erythrina* alkaloids possessing a nonaromatic D ring has not been well studied. However, the

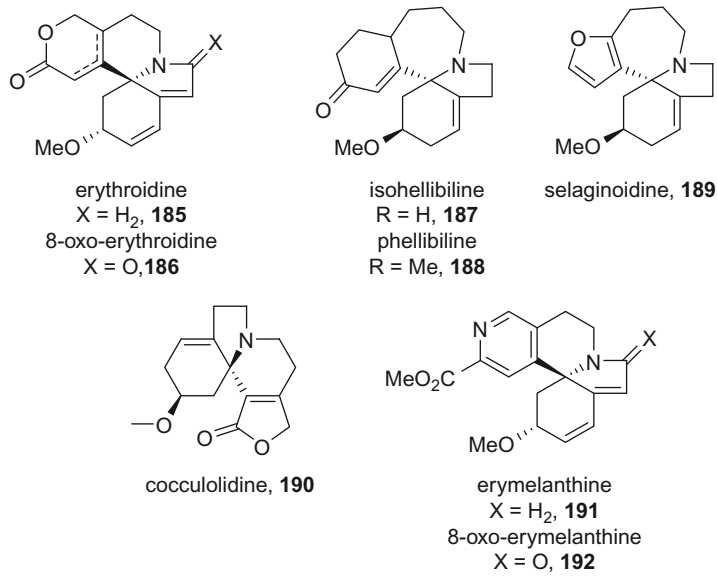
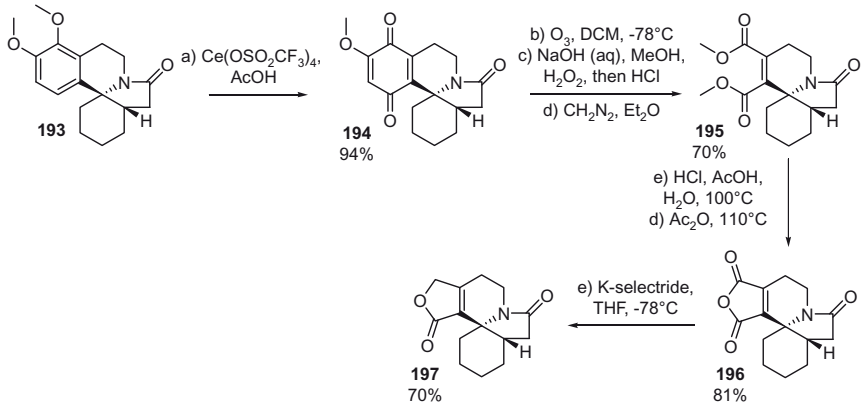


Figure 2 Examples of nonaromatic *Erythrina* alkaloids.

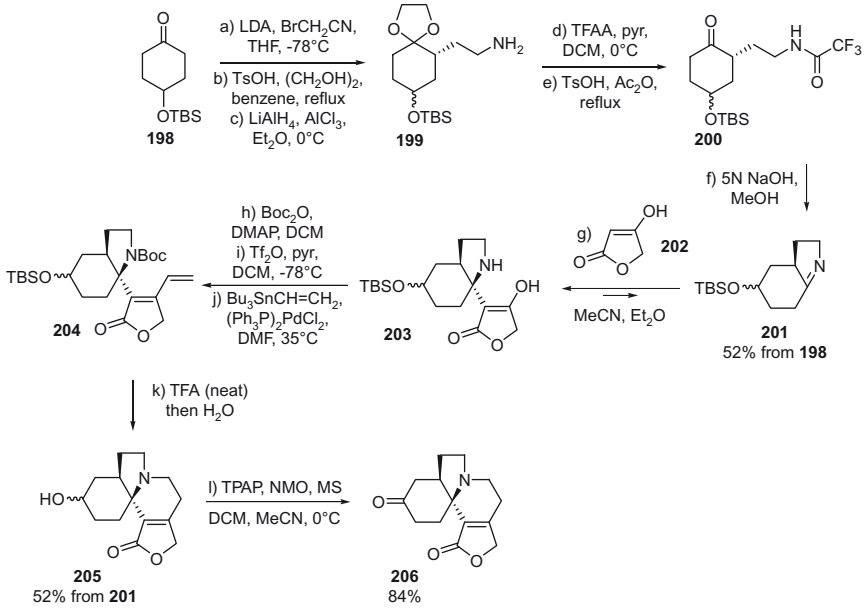
biosynthesis of the nonaromatic alkaloids is thought to involve oxidative cleavage of an aromatic D ring, followed by recyclization to afford the nonaromatic ring (60). This strategy is commonly employed in their total synthesis (61). The following are examples of recent synthetic approaches to nonaromatic *Erythrina* alkaloids.

1. Synthesis of Cocculolidine (**190**)

Tsuda and coworkers (62) have reported an efficient route to the synthesis of the carbon skeleton of cocculolidine (**190**), by the oxidative cleavage of the D ring of an aromatic *Erythrina* alkaloid (63). The starting point for the oxidation chemistry was dimethoxyerythrinan **193**, which is readily available from 2,3-dimethoxyphenethylamine (Scheme 35) (64). Treatment of dimethoxyerythrinan **193** with cerium(IV) trifluoromethanesulfonate (65), afforded the *p*-quinone **194**, which on ozonolysis and oxidative workup using hydrogen peroxide, followed by esterification with diazomethane afforded diester **195**. Subsequent hydrolysis afforded the diacid, which on treatment with acetic anhydride afforded the anhydride **196**. Treatment of anhydride **196** with potassium tri-*sec*-butylborohydride (K-selectride) regioselectively reduces the least hindered carbonyl to afford lactone **197** as the sole product. It should be noted that treatment of anhydride **196** with zinc borohydride affords a mix of regioisomers, due to competitive attack of the smaller hydride source at the more sterically hindered carbonyl.



Scheme 35

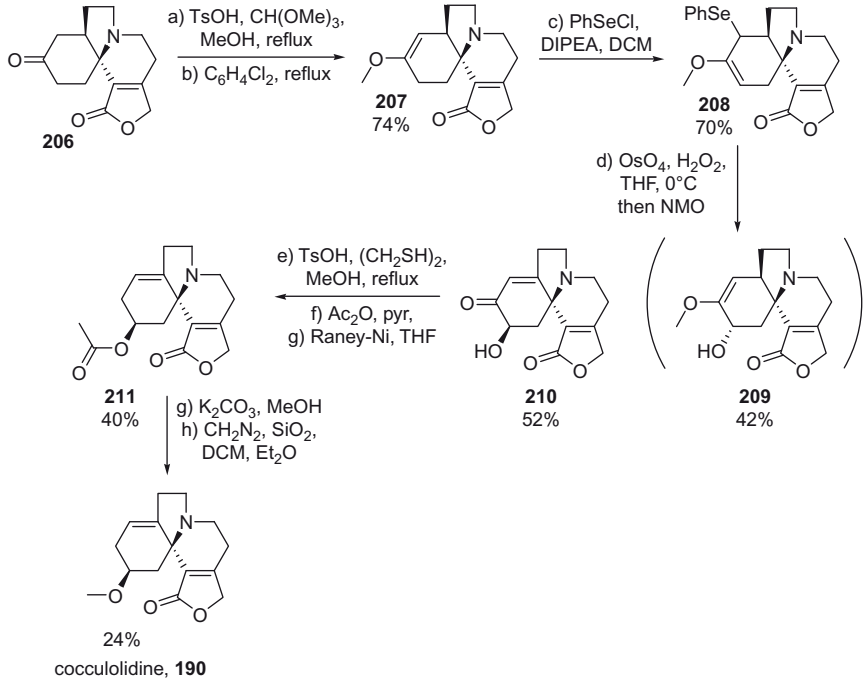


Scheme 36

Kitahara and coworkers have reported the synthesis of cocculolidine (**190**) (66), a nonaromatic *Erythrina* alkaloid with insecticidal activity, which involves the coupling reaction of the imine **201** with tetronic acid **202**, and a subsequent intramolecular 1,6-addition of unsaturated amine **204** (Scheme 36). The synthesis of the key imine **201** was achieved in six steps from 4-*tert*-butyldimethylsilyloxycyclohexanone (**198**), via α-alkylation with bromoacetonitrile, protection of the ketone as an acetal, and

reduction of the nitrile using lithium aluminum hydride and aluminum trichloride to afford the amine **199**. Protection of the amine as a trifluoroacetamide group and finally, deprotection of the acetal gave ketone **200**. Removal of the trifluoroacetyl group under basic conditions resulted in an intramolecular condensation to afford imine **201**. Coupling of the imine **201** with tetronic acid **202** proceeded in 91% yield with the precipitation of **203** driving the equilibrium to the product. Protection of the secondary amine of **203** with a Boc group was followed by conversion of the hydroxyl group into a trifluoromethanesulfonate, which, on reaction with vinyltributyltin under Stille (67) conditions, gave the unsaturated system **204**. Subsequent deprotection of the Boc group under acidic conditions triggered an intramolecular 1,6-addition to afford the desired tetracyclic ring system **205**. Oxidation of the secondary alcohol using tetrapropylammonium peruthenate (TPAP) and *N*-methylmorpholine-*N*-oxide (NMO) afforded ketone **206** (66).

Functionalization of the A ring was accomplished by initial transformation of ketone **206** into a dimethylacetal, followed by elimination of methanol by refluxing in dichlorobenzene to yield methyl enol ether **207**, along with 14% of its separable regioisomer (Scheme 37). Conversion of **207** into the seleno-enol **208** was accomplished with

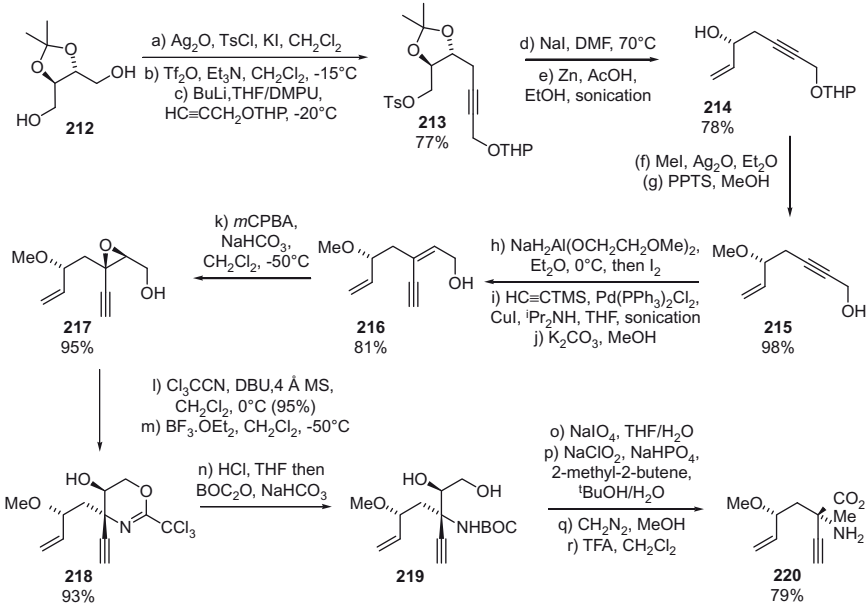


Scheme 37

phenylselenyl chloride and *N,N′*-diisopropylethylamine. Subsequent oxidation of the selenide with hydrogen peroxide and a catalytic amount of osmium tetroxide afforded the selenoxide, which on treatment with *N*-methylmorpholine-*N*-oxide gave enone **210**, through dihydroxylation and elimination, along with the enol ether **209**, which is formed by way of a sigmatropic rearrangement of the selenoxide. Direct attempts to convert enone **210** into cocculolidine (**190**) failed, so an alternative five-step procedure was developed. Enone **210** was first converted into a dithioacetal, then protection of the hydroxyl group as an acetate and reduction of the dithioacetal using Raney nickel afforded **211**. Removal of the acetyl group gave demethylcocculolidine, and methylation of the secondary alcohol was achieved under acidic conditions using diazomethane (68), to give cocculolidine (**190**), in 21 steps in an overall yield of 0.42% from 4-*tert*-butyldimethylsilyloxycyclohexanone (**198**) (66).

2. Synthesis of β-Erythroidine (185) and 8-oxo-β-Erythroidine (186)

The first total synthesis of (+)-β-erythroidine (**185**), a nonaromatic, dienoid-type *Erythrina* alkaloid, was developed using a tandem ring-closing metathesis reaction in the key step (69). First, the enantiomerically enriched, quaternary amino ester **220** was prepared from the readily available 2,3-*O*-isopropylidene-D-threitol **212** (Scheme 38). Following

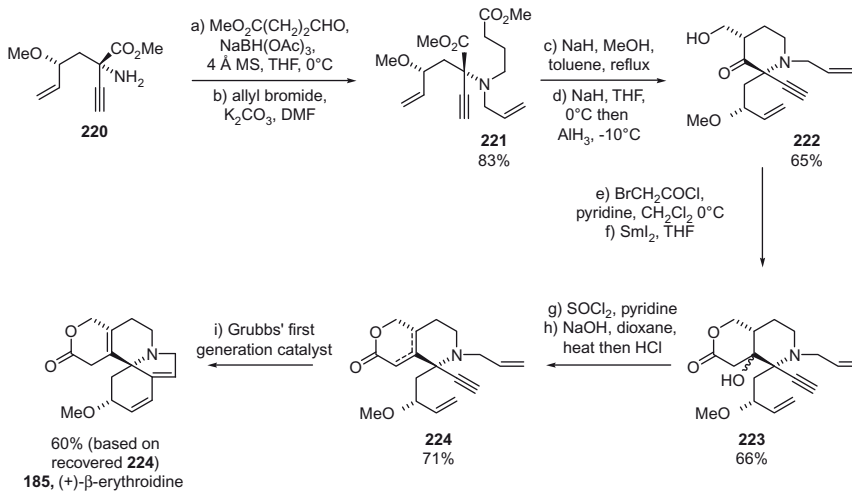


Scheme 38

sequential tosylation and triflation of **212**, reaction with lithiated propargyl tetrahydropyranyl ether gave tosylate **213**. Iodination of **213**, followed by reductive cleavage of the resulting iodide, gave the alcohol **214**, which, on methylation and deprotection of the tetrahydropyranyl group, gave propargyl alcohol **215**.

Treatment of alcohol **215** with $NaH_2Al(OCH_2CH_2OMe)_2$ followed by iodine resulted in the regioselective and stereoselective formation of a *Z*-iodoalkene. Reaction of the *Z*-iodoalkene with trimethylsilylacetylene in a Sonogashira coupling, allowed the introduction of the acetylene group, which, after desilylation, gave the *Z*-allylic alcohol **216**. Epoxidation of **216** using *m*-CPBA occurred with excellent diastereoselectivity to give epoxide **217**. The high diastereoselectivity is explained by a transition state in which the *m*-CPBA forms hydrogen bonds to the HO and MeO groups of **216**. Epoxy-alcohol **217** is converted into imidate **218** by reaction with trichloroacetonitrile and DBU, and the stereoselective introduction of a nitrogen atom (at a quaternary center) is achieved by cyclization of the epoxy-trichloroacetimidate promoted by $BF_3 \cdot OEt_2$. Acidic hydrolysis of **218**, followed by *N*-protection, gave the diol **219**, which was converted into amino ester **220** by oxidative cleavage of the diol, followed by aldehyde oxidation, esterification of the acid, and *N*-deprotection (69).

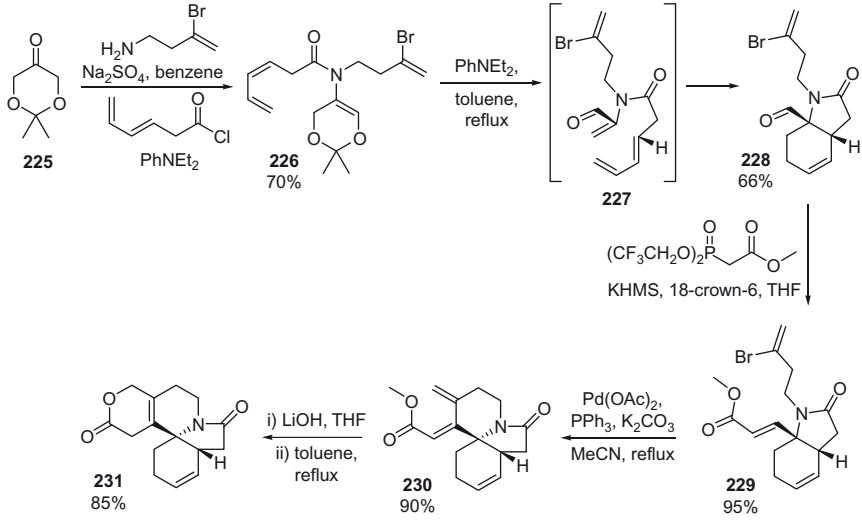
Reductive amination of **220** using methyl 3-formylpropanoate, sodium triacetoxyborohydride, and molecular sieves gave the corresponding secondary amine, which on *N*-allylation produced the tertiary amine **221** (Scheme 39). Heating **221** with sodium methoxide led to a



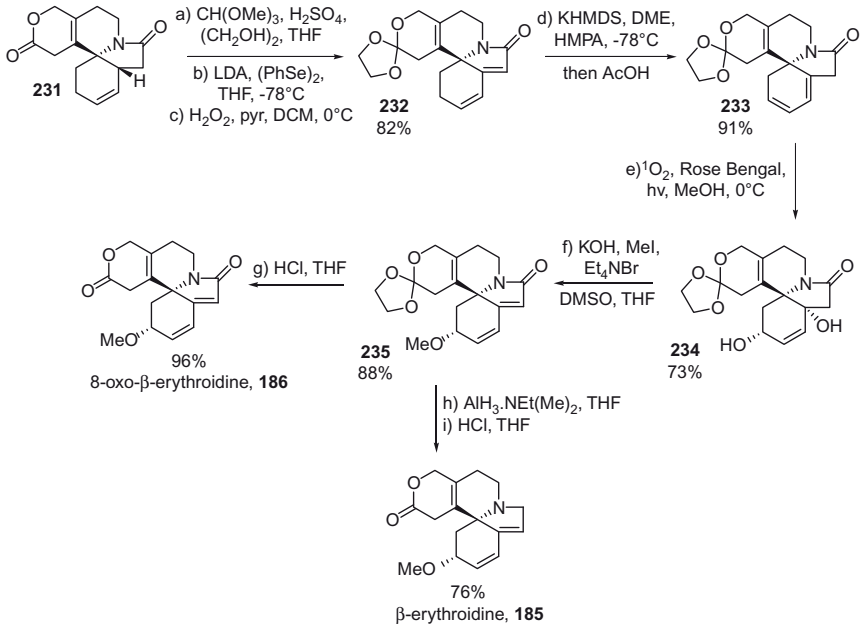
Scheme 39

Dieckmann condensation, and enolate ion formation followed by *in situ* reduction using AlH_3 gave β-hydroxy ketone **222**. Acylation with bromoacetyl chloride then gave the bromoacetate, which, on cyclization with samarium(II) iodide, led to the formation of lactone **223** (as a 60:40 mixture of epimers). Dehydration of **223**, using thionyl chloride in pyridine, gave a mixture of α,β- and α,γ-unsaturated lactones, which, on saponification followed by acidification, produced the β,γ-unsaturated lactone **224** in high selectivity (94:6). Finally, in the key step, treatment of β,γ-unsaturated lactone **224** with Grubbs' first-generation catalyst gave (+)-β-erythroidine (**185**) (69).

Based on previous work within their group (70), Funk and coworkers have reported the synthesis of β-erythroidine (**185**) and 8-oxo-β-erythroidine (**186**) using an intramolecular Diels–Alder cycloaddition of a 2-amidoacrolein **227** in the key step (71). First, the preparation of amidodioxin **226** was accomplished by condensing dioxanone **225** with 3-bromobut-3-en-1-amine to form the corresponding imine, and this was followed by *N*-acylation using hexa-3,5-dienoyl chloride (Scheme 40). Heating amidodioxin **226** affected a retro-cycloaddition to afford the intermediate 2-amidoacrolein **227** (together with acetone), which underwent a subsequent cycloaddition to afford, as the major product, the substituted indolone **228**. Completion of the erythroidine ring system was achieved in four steps. An initial Horner–Wadsworth–Emmons reaction of the aldehyde produced the α,β-unsaturated ester **229**, which,



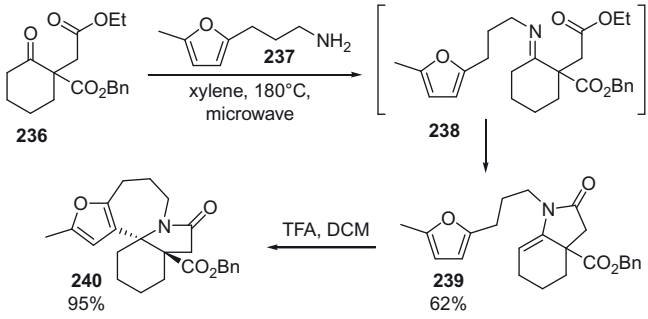
Scheme 40



Scheme 41

on Heck cyclization of the vinyl bromide, afforded tricycle **230**. Subsequent saponification and heating promoted an electrocyclic ring closure to give lactone **231**, which has the carbon skeleton of erythroidine.

Synthesis of lactam **235**, which was the divergent point for the preparation of both β-erythroidine **185** and 8-oxo-β-erythroidine (**186**), was achieved in five steps from lactone **231** (Scheme 41). Protection of lactone **231** as an *ortho* ester was followed by regioselective introduction of a C═C bond via selenylation alpha to the amide, followed by oxidative deselenylation, and subsequent deconjugation of the C═C bond (with the amide carbonyl) to afford the diene lactam **233**. A cycloaddition reaction of the diene **233** with singlet oxygen through stereoselective addition to the less hindered face and a reductive workup yielded diol **234** stereoselectively. Subsequent treatment with potassium hydroxide and methyl iodide allowed for simultaneous methylation of the C-3 hydroxyl and for elimination of the C-6 hydroxyl, to afford lactam **235**. Hydrolysis of the *ortho* ester of **235** afforded 8-oxo-β-erythroidine (**186**). Alternatively, reduction of the amide of **235** with alane-ethyldimethylamine complex, followed by hydrolysis afforded β-erythroidine (**185**), in 13% overall yield, from dioxanone **225** (71).



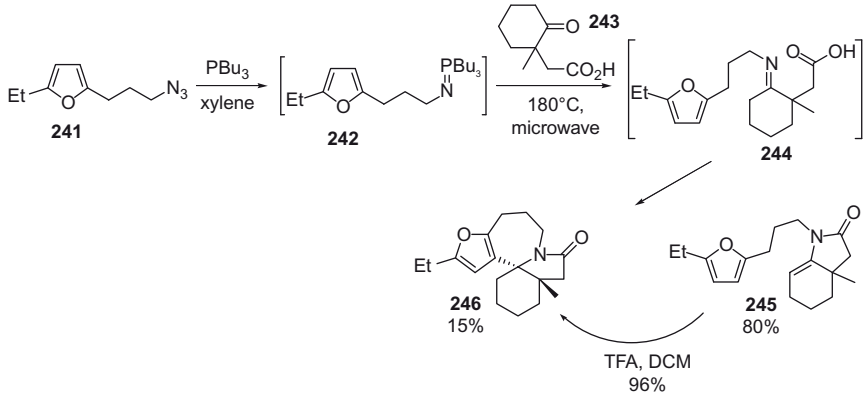
Scheme 42

3. Approaches to Selaginoidine (**189**)

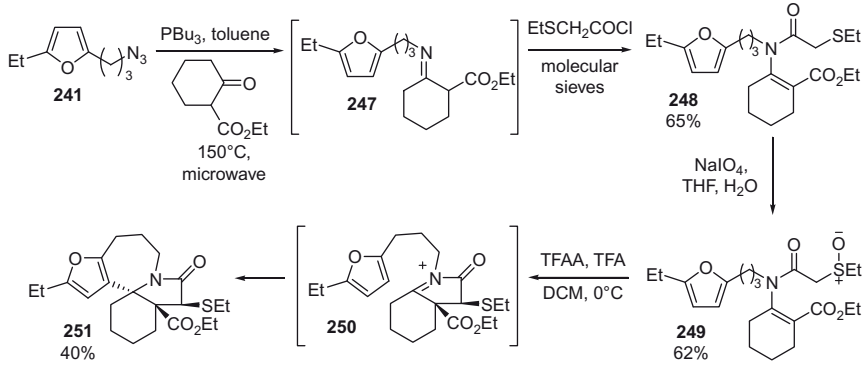
Selaginoidine (**189**) is a homoerythrina alkaloid possessing a furan-based D ring (Figure 2). To date the total synthesis of selaginoidine (**189**) has not been reported, however, Padwa and coworkers have developed several concise routes to the tetracyclic core of selaginoidine (**189**). It was envisaged that the furan ring could be incorporated using a Pictet–-Spengler reaction. To test this strategy, tetrahydroindolinone **239** was first prepared by the condensation of furan-amine **237** with the substituted cyclo-hexanecarboxylate **236** (Scheme 42). Subsequent treatment of tetrahydroindolinone **239** with trifluoroacetic acid initiated the desired Pictet–Spengler reaction, to give the tetracyclic ring system **240** in 95% yield (72).

Subsequent routes utilized an aza-Wittig reaction (73), to afford an advanced precursor in a one-pot procedure. It was found that reaction of furanyl azide **241** with tributylphosphine afforded iminophosphorane **242**, which, on treatment with ketoacid **243** in a microwave reactor, initiated the desired aza-Wittig reaction to generate the intermediate imine **244** (Scheme 43). A subsequent cyclization furnishes hexahydroindolinone **245** as the major compound, along with the desired tetracyclic furan **246** (74). Finally, treatment of hexahydroindolinone **245** with trifluoroacetic acid triggered the Pictet–Spengler reaction to give lactam **246**, which contains the core ring system of selaginoidine (**189**) (74).

An alternative route to selaginoidine (**189**) was based on earlier work within the Padwa group and utilized a combined Pummerer reaction and subsequent π-cyclization to form the alkaloid skeleton (Scheme 44) (75). Enamide sulfide **248** was first prepared by an aza-Wittig reaction of furanyl azide **241** with tributylphosphine, followed by *N*-acylation using ethylthioacetyl chloride. Sodium periodate-mediated oxidation of sulfide **248** afforded sulfoxide **249**, which, on treatment with trifluoroacetic



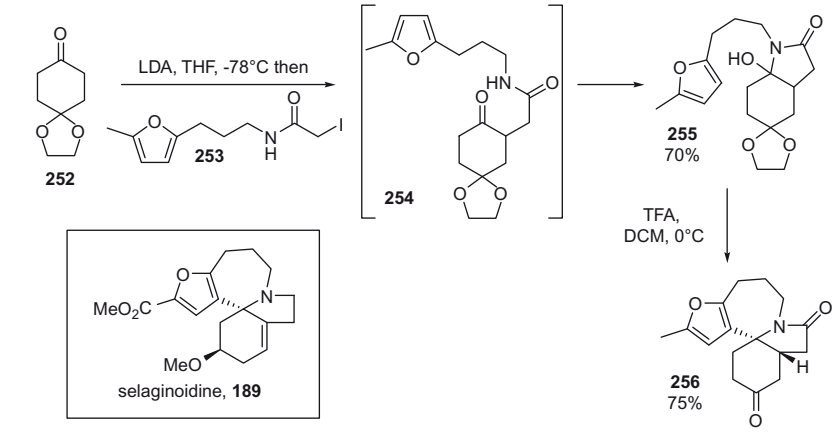
Scheme 43



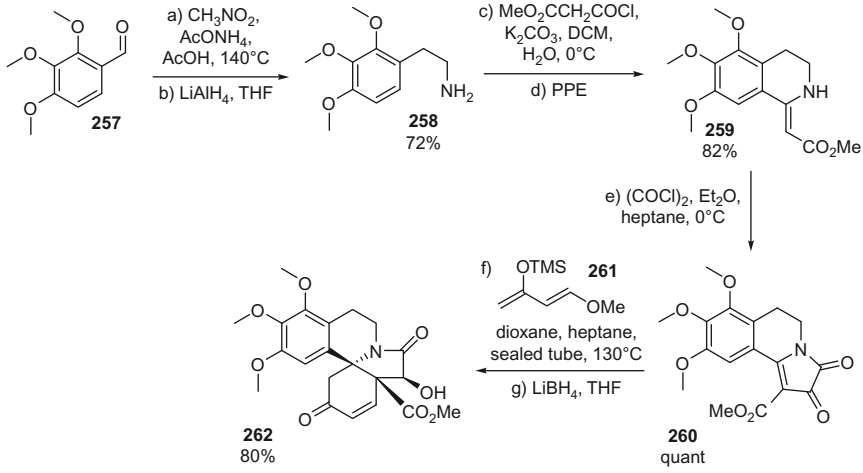
Scheme 44

anhydride and trifluoroacetic acid, triggered a Pummerer–Mannich cyclization. A subsequent Pictet–Spengler reaction of **250** afforded, as the major compound, tetracycle **251**, which contains the selaginoidine core ring system.

Both the groups of Padwa (72) and Tu (76) have reported the synthesis of the core ring system of selaginoidine (**189**), using a hydroxy amide **255**, as the precursor to a Pictet–Spengler reaction (Scheme 45). The preparation of hydroxy amide **255**, was accomplished by addition of the enolate ion formed from treatment of cyclohexanone **252** with lithium diisopropylamide, to iodo-furan **253** and subsequent cyclization of the amide onto the ketone. Treatment of hydroxy amide **255** with trifluoroacetic acid triggered the Pictet–Spengler reaction to afford the desired selaginoidine ring system **256** in good yield.



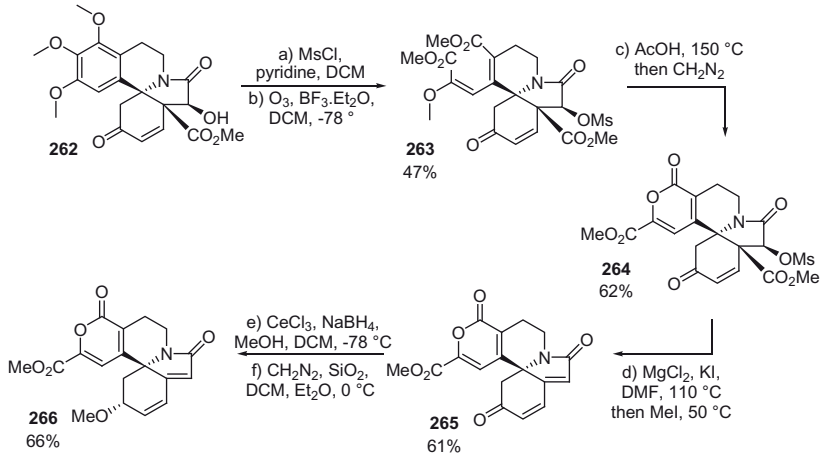
Scheme 45



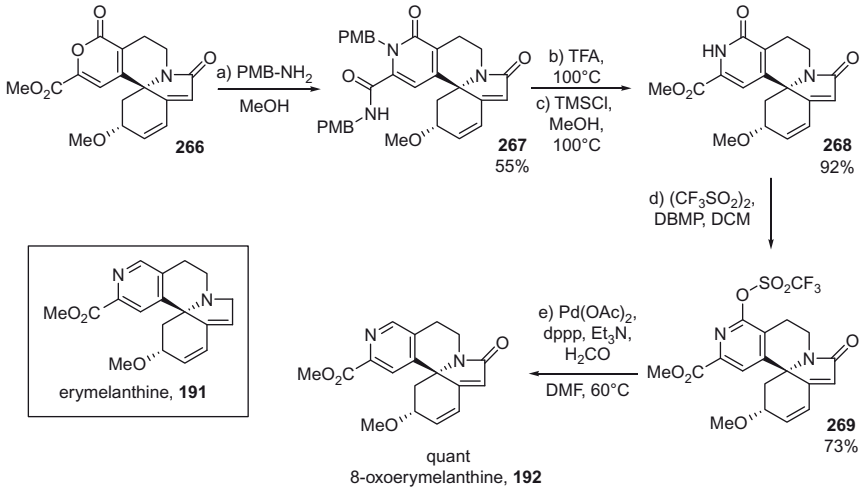
Scheme 46

4. Synthesis of Erymelanthine (**191**) and 8-Oxoerymelanthine (**192**)

Erymelanthine (**191**) and 8-oxoerymelanthine (**192**) are structurally unique *Erythrina* alkaloids as they possess a pyridine D ring. The biosynthesis of these alkaloids is believed to involve cleavage of an aromatic ring to give an intermediate dialdehyde that cyclizes with incorporation of ammonia. Yamamoto and coworkers have reported the only total synthesis of 8-oxoerymelanthine (**192**) using a similar biomimetic approach (77). Their strategy involved using a Diels–Alder reaction to construct the 6,5,6,6-tetracyclic ring system, followed by oxidative cleavage of the aromatic D ring and aminolysis (Schemes 46–48).



Scheme 47



Scheme 48

The synthesis of the 6,5,6,6-ring system **262** was accomplished in six steps starting from 2,3,4-trimethoxybenzaldehyde **257** (Scheme 46), utilizing a similar method to that described for the synthesis of aromatic erythrinan alkaloids (78,79). Treatment of 2,3,4-trimethoxybenzaldehyde **257** with nitromethane, followed by reduction of the unsaturated nitro group afforded amine **258**, which, on acylation with methyl malonyl chloride, yielded the corresponding amide. A Bischler–Napieralski reaction was then employed to form the isoquinolone core **259**. Subsequent reaction of **259** with oxalyl chloride gave dioxopyrroline **260**, which was

the precursor for the Diels–Alder reaction. Treatment of the dioxopyrroline **260** with the activated diene **261**, afforded the expected Diels–Alder adduct as a single diastereomer, which was reduced with lithium borohydride to afford enone **262** as the major product.

Oxidative cleavage of the aromatic D ring was accomplished via initial conversion of the alcohol **262** into the corresponding mesylate, and then an ozonolytic cleavage of the tri-methoxybenzene ring, in the presence of boron trifluoride etherate (80,81), to afford the triester **263** (Scheme 47). The fact that the enone functionality in the A ring is untouched, suggests that the boron trifluoride coordinates to the carbonyl oxygen of the enone and decreases the electron density of the C=C bond. Cyclization of triester **263** was achieved by heating in acetic acid, and subsequent treatment with diazomethane yielded diester **264**. Selective decarboxylation of the C-6 ester, together with elimination of the mesylate group, was achieved by heating **264** in the presence of magnesium chloride and potassium iodide to afford dienone **265**. A Luche reduction yielded a secondary alcohol, stereoselectively, which was then methylated using diazomethane to afford methyl ether **266**.

Attempts to directly convert pyrone **266** into pyridine **268** using ammonia failed, resulting in the development of an alternative three-step approach (Scheme 48). Treatment of pyrone **266** with *p*-methoxybenzylamine afforded pyridone **267**, which was deprotected using trifluoroacetic acid and converted into methyl ester **268** by treatment with trimethylsilyl chloride and methanol. The total synthesis of 8-oxoerymelanthine (**192**), was completed by conversion of pyridone **268** into triflate **269**, followed by a highly efficient palladium-catalyzed reduction of the triflate group. 8-Oxoerymelanthine (**192**) was obtained in 17 steps in 2.0% overall yield (77).

VI. SUMMARY

The chemistry of *Erythrina* and related alkaloids from 1996 through to mid-2009 has been reviewed, with a particular focus on the preparation of *Erythrina* alkaloids possessing an aromatic D ring. A number of synthetic approaches to alkaloids bearing an aromatic D ring have been reported, including some asymmetric routes. Recent studies have explored strategies toward *Erythrina* alkaloids possessing a nonaromatic D ring, although, as yet, few total syntheses have been published.

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CHAPTER 3

Alkaloids from the Genus *Duguetia*

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I. INTRODUCTION

Duguetia A. St.-Hil. (Annonaceae) is a genus of usually small, understory trees growing almost exclusively in the tropics of South America, with a small extension across the Panama Isthmus. It is now regarded as comprising close to 100 species, considering the recent inclusion of four African taxa, of which three were previously known as *Pachypodanthium* Engler & Diels. It is therefore one of the largest Annonaceous genera after *Guatteria* and *Annona*. Many studies have been conducted on the secondary metabolites present in different parts of *Duguetia* plants, from which essential oils, aromatic compounds, monoterpenes, diterpenes, triterpenes, flavonoids, and most typically alkaloids have been isolated and characterized. In common with the other ''primitive angiosperms,'' *Duguetia* species accumulate isoquinoline alkaloids, and more specifically 1-benzyl-1,2,3,4-tetrahydroisoquinolines, usually referred to simply as ''benzylisoquinolines,'' and their biosynthetic or biogenetically presumed derivatives. The literature reports studies on the alkaloids of about 16 *Duguetia* species (one of which was not clearly identified), resulting in the isolation and identification or characterization of 105 different alkaloids. Although many of these alkaloids are widely distributed, a few unusual groups of alkaloids appear to be specific to this genus.

II. BOTANICAL CONSIDERATIONS

The plants of the Annonaceae have traditionally been classed as part of the order Magnoliales. In the most recent consensus, the Magnoliales and Laurales constitute one of the two sister clades in the Magnoliidae, which are commonly regarded as the most ''primitive'' angiosperms in older classifications (1,2).

Regarding the occurrence of benzylisoquinoline alkaloids in the Annonaceae, other magnoliids, and more distantly related families, it is of interest to note that there is now good biochemical and molecular phylogenetic evidence for the evolution of benzylisoquinoline alkaloid biosynthesis in angiosperms from a common ancestor. Activity ascribable to the first enzyme in this biosynthetic tree, (*S*)-norcoclaurine synthase, occurs in 90 different plant species, and compares well with a molecular phylogeny. Phylogenetic analyses of norcoclaurine synthase, the berberine bridge enzyme, and several *O*-methyltransferases ''suggest a latent molecular fingerprint for benzylisoquinoline alkaloid biosynthesis in angiosperms not known to accumulate such alkaloids'' (3).

Duguetia was thought, on the basis of inflorescence and floral characters, to form an alliance with the very small neotropical genera *Duckeanthus*, *Fusaea*, and *Malmea*, and the African *Letestudoxa* (4). The monotypic *Pseudartabotrys* was later included and *Malmea* excluded (5), but incorporation of leaf, flower, fruit, and seed characters that had not been considered previously has led to a different grouping in which *Duguetia* (including *Pachypodanthium*) constitutes a clade of its own, close to a separate sister group including *Fusaea*, *Duckeanthus*, *Letestudoxa*, and *Pseudartabotrys* (6). Despite the inclusion of *Pachypodanthium* as ''African species of *Duguetia*,'' these plants still form a small, distinct cluster, perhaps not surprisingly together with *Duguetia riberensis* of Venezuela, in this cladistic analysis.

The genus has been further subdivided into 14 sections by Fries based on their morphological characters, but leaving some species in uncertain positions (7,8). These subdivisions have largely been upheld by a more recent study (9), and it is the system used in this review (Table I).

One third of all *Duguetia* species were analyzed in a study based on their genomic DNA sequences (41). That work supported the notion that *Duguetia*, like *Guatteria*, is monophyletic, with its most recent common ancestor dating back to 29.04 ± 4.52 million years ago (in the case of *Guatteria* this figure is 36.65 ± 2.50 mybp), although the authors concede that ''the accuracy of the absolute dates remains unassessed.'' A fossilized leaf from the middle Eocene period (about 38–48 mybp) from Western Tennessee, when the local climate was subtropical to tropical, has been classified as belonging to a *Duguetia* species (42), a conclusion that seems to conflict with the estimated DNA age of the genus. On the basis of its present geographic, trans-Atlantic distribution it was suggested that the *Duguetia* clade might predate the break-up of Gondwana (6). As the separation of Africa and South

America is believed to have been completed in the early Cretaceous (about 110 million years ago), and the age of the Annonaceae as a family is estimated to be as little as 82 million years (43), it seems necessary to assume long-distance dispersal over the widening early Atlantic Ocean, possibly across stepping-stones along the 80 million-year-old volcanic Sierra Leone Rise (to which the Ceará Rise should be added) (44) or, less likely, the more southerly Walvis Ridge (and Rio Grande Rise) (45). This hypothesis seems reasonable given the presence of Annonaceae in the Lesser Antilles, which would represent much more recent (Pliocene or even Pleistocene) events of a similar character (46).

III. ALKALOIDS FROM CHEMICALLY INVESTIGATED *DUGUETIA* SPECIES

The *Duguetia* species studied to date for their alkaloidal content are listed in Table I, ordered by sections, and in alphabetical order when appropriate. All of the alkaloids isolated from this genus have at least a formal isoquinoline-derived structure; including the 1-azaanthraquinone cleistopholine and the rare copyrine alkaloids, the 1-aza-7-oxoaporphines and 1-aza-4,5-dioxoaporphines. These alkaloids are classified as benzyltetrahydroisoquinolines, a single bisbenzyltetrahydroisoquinoline, berbines (tetrahydroprotoberberines), protoberberines, a morphinandienone, a proaporphine, and many aporphinoids and aporphinoid-related compounds. A large proportion of the aporphines are oxygenated at C7, a fairly common feature in the Annonaceae. 7-Methoxy derivatives are almost completely restricted to the African *Duguetia* species. Four *N*-formylnoraporphines have been identified. Three nitroso- or nitroaporphinoid derivatives isolated from *Duguetia furfuracea* might be artifacts, as discussed below. Several of the aporphinoids have the unusual 9,11-dioxygenation pattern in ring D which, aside from *Duguetia*, has only been found in one *Guatteria* species. As in *Guatteria*, some of the *Duguetia* aporphinoids bear a biogenetically intriguing carbon atom bonded to C7. Finally, a protoberberine–styrene adduct is a unique alkaloid from the African *Duguetia staudtii*. Table II lists the 105 alkaloids, including some possible artifacts, ordered according to their main structural features, as depicted in Figure 1 (Table III).

In many cases, the structures were known prior to their isolation from *Duguetia* species, or were very closely related to known alkaloids,

Table I Chemically investigated *Duguetia* species and their contained alkaloids

Section	Species	Alkaloid	Structure	Ref.(s)
Duguetia R. E. Fries	*D. furfuracea* (A. St.-Hil.) Benth. & Hook.	Reticuline	**1**	10
		Isochondodendrine	**3**	10
		Discretamine	**4**	10
		Isocorydine	**41**	10
		Norisocorydine	**40**	10
		Xylopine	**28**	10
		Obovanine	**30**	10
		Anonaine	**23**	10
		Asimilobine	**20**	10
		Atherospermidine	**86**	10
		Liriodenine	**83**	10
		Lanuginosine	**87**	10
		Duguetine	**76**	11
		N-Oxyduguetine	**77**	11
		Dicentrinone	**91**	11
		N-Methylglaucine	**36**	11
		N-Methyl-tetrahydropalmatine	**8**	11
		N-Nitrosoanonaine	**51**	12
		N-Nitrosoxylopine	**52**	12
		8-Nitroisocorydine	**42**	13
	D. odorata (Diels) J. F. Macbr.	Dehydrodiscretine	**16**	14
		Pseudopalmatine	**17**	14
		Oliveroline	**60**	14
		N-Methylguatterine	**66**	14

Table 1 *(Continued)*

Section	Species	Alkaloid	Structure	Ref.(s)
	D. stelechantha (Diels) R. E. Fries	Oxopukateine	**88**	15
		O-Methylmoschatoline	**85**	15
		Corypalmine	**5**	15
Hadrantha R. E. Fries	*D. hadrantha* (Diels) R. E. Fries	Hadranthine A	**99**	16
		Hadranthine B	**100**	16
		Imbiline-1	**101**	16
		Sampangine	**97**	16
		3-Methoxysampangine	**98**	16
Sphaerantha R. E. Fries	*D. calycina* Benoist	Discretamine	**4**	17
		10-Demethylxylopinine	**11**	17
		Xylopine	**28**	17
		Puterine	**31**	17
		O-Methylpukateine	**32**	17
		Obovanine	**30**	17
		Oxoputerine	**89**	17
		Atherosperminine	**94**	17
		Calycinine	**43**	17
		Noratherosperminine	**93**	18
		Duguecalyne	**54**	19
		N-Formylputerine	**53**	19
		Duguenaine	**47**	20
	D. obovata R. E. Fries	Xylopine	**28**	20
		Isolaureline	**29**	20

	N-Formylxylopine	**48**	20
	Buxifoline	**33**	20
	N-Methylbuxifoline	**34**	20
	N-Formylbuxifoline	**49**	20
	Anolobine	**27**	20
	Calycinine	**43**	20
	N-Methylcalycinine	**44**	20
	Duguevanine	**45**	20
	N-Formylduguevanine	**50**	20
	N-Methylduguevanine	**46**	20
	Oxobuxifoline	**90**	20
	Xylopinine	**12**	20
	Discretine	**10**	20
	(9*S*)-Sebiferine	**18**	20
D. spixiana Mart. (Colombia)	*N*-Oxycodamine	**2**	21,22
	N-Methylasimilobine	**21**	21
	Noroliveridine	**67**	21
	Oliveridine	**68**	21
	N-Oxyoliveridine	**70**	21
	Norpachyconfine	**56**	21
	Pachyconfine	**58**	21
	N-Oxypachyconfine	**59**	21
	Spixianine	**73**	21
	N-Oxyspixianine	**74**	21
	Duguexine	**71**	21
	N-Oxyduguexine	**72**	21

Table I *(Continued)*

Section	Species	Alkaloid	Structure	Ref.(s)
		Lanuginosine	**87**	21
		Atherosperminine	**94**	21
		N-Oxyatherosperminine	**95**	21
		Methoxyatherosperminine	**96**	21
		Spiduxine	**13**	21
		Duguespixine	**55**	21,23
	D. spixiana Mart. (Bolivia)	Anonaine	**23**	24
		Nornuciferine	**22**	24
		3-Hydroxynornuciferine	**25**	24
		O-Methylisopiline	**26**	24
		Noroliveridine	**67**	24
		Oliveridine	**68**	24
		N-Oxyoliveridine	**70**	24
		Duguexine	**71**	24
		Roemerolidine	**69**	24
		Nornuciferidine	**57**	24
		Rurrebanine	**63**	24
		Rurrebanidine	**62**	24
		Lysicamine	**84**	24
		Lanuginosine	**87**	24
		O-Methylmoschatoline	**85**	24
		Spiguetidine	**103**	24

		Spiguetine	**102**	24
		Xylopinine	**12**	24
		Tetrahydropalmatine	**7**	24
Calothrix R. E. Fries	*D. vallicola* J. F. Macbr.	*N*-Methyllaurotetanine	**37**	25
		Isocorydine	**41**	26
		Isoboldine	**38**	26
		Oliveridine	**68**	27
		Oliveroline	**60**	27
		Duguevalline	**92**	27
		O-Methylmoschatoline	**85**	27
		Xylopinine	**12**	26
		Discretine	**10**	26
		Pseudopalmatine	**17**	26
		Cleistopholine	**104**	27
		Glaziovine	**19**	26
Polyantha R. E. Fries	*D. eximia* Diels	*O*-Methylmoschatoline	**85**	28
		Oxopukateine	**88**	28
		Oxoputerine	**89**	28
Geanthemum R. E. Fries	*D. flagellaris* Huber	Nornuciferine	**22**	29,30
		Isopiline	**24**	29,30
		O-Methylisopiline	**26**	29,30
		Calycinine	**43**	29,30
		Duguevanine	**45**	29,30
		Pachypodanthine	**78**	29,30
		Oliveroline	**60**	29,30

Table 1 *(Continued)*

Section	Species	Alkaloid	Structure	Ref.(s)
		N-Oxyoliveroline	**61**	29,30
		Oliveridine	**68**	29,30
		Duguetine	**76**	29,30
Uncertain	*D. colombiana* Maas	*O*-Methylmoschatoline	**85**	31
	D. gardneriana Mart.	Discretamine	**4**	32
		Corypalmine	**5**	32
		Tetrahydropalmatine	**7**	32
	D. glabriuscula R. E. Fries	Polyalthine	**75**	33
		Oliveridine	**68**	33
		Oxobuxifoline	**90**	33
		Lanuginosine	**87**	33
	D. magnolioidea Maas	Discretamine	**4**	34
	D. trunciflora Maas	Reticuline	**1**	35
		Tetrahydropalmatine	**7**	35
		Corypalmine	**5**	35
		Discretamine	**4**	35
		Thaicanine	**14**	35
		Jatrorrhizine	**15**	35
Undetermined	*Duguetia* sp.	Norglaucine	**35**	36
		Dicentrine	**39**	36
		Duguetine	**76**	36
African species	*D. confinis*	Corypalmine	**5**	37
	(Engl. & Diels) Chatrou	Isocorypalmine	**6**	37

	Tetrahydropalmatine	**7**	37
	Govanine	**9**	38
	Discretine	**10**	38
	Oliveroline	**60**	37
	Guatterine	**64**	37
	N-Oxyguatterine	**65**	37
	Pachyconfine	**58**	37
	Pachypodanthine	**78**	38
	N-Acetylpachypodanthine	**80**	38
D. staudtii	Corypalmine	**5**	39
(Engl. & Diels) Chatrou	Isocorypalmine	**6**	40
	Discretine	**10**	39
	N-Methylpachypodanthine	**79**	39
	Pachystaudine	**82**	39
	Norpachystaudine	**81**	39
	Liriodenine	**83**	39,40
	Staudine	**105**	39,40
	Pachypodanthine	**78**	39,40

Table II Alkaloids isolated from *Duguetia* species

Alkaloid type and name	Structure	Molecular formula	MW	Species	Ref.(s)
Benzylisoquinolines					
(+)-Reticuline	**1**	$C_{19}H_{23}NO_4$	329	*D. furfuracea*	10
				D. trunciflora	35
cis-N-Oxycodamine	**2**	$C_{20}H_{25}NO_5$	359	*D. spixiana*[a]	21,22
Bisbenzylisoquinoline					
Isochondodendrine	**3**	$C_{36}H_{28}N_2O_6$	594	*D. furfuracea*	10
Berbines (Tetrahydroprotoberberines)					
(−)-Discretamine	**4**	$C_{19}H_{21}NO_4$	327	*D. calycina*	17
				D. gardneriana	32
				D. furfuracea	10
				D. trunciflora	35
				D. magnolioidea	34
(−)-Corypalmine (Tetrahydrojatrorrhizine)	**5**	$C_{20}H_{23}NO_4$	341	*D. gardneriana*	32
				D. stelechantha	15
				D. trunciflora	35
				D. staudtii	39
				D. confinis	37
(−)-Isocorypalmine	**6**	$C_{20}H_{23}NO_4$	341	*D. staudtii*	40
				D. confinis	37
(−)-Tetrahydropalmatine (Rotundine)	**7**	$C_{21}H_{25}NO_4$	355	*D. confinis*	37
				D. spixiana[b]	24
				D. gardneriana	32
				D. trunciflora	35

N-Methyltetrahydropalmatine	**8**	$C_{22}H_{28}NO_4$	370	*D. furfuracea*	11
(−)-Govanine	**9**	$C_{20}H_{23}NO_4$	341	*D. confinis*	38
(−)-Discretine	**10**	$C_{20}H_{23}NO_4$	341	*D. obovata*	20
				D. vallicola	26
				D. confinis	38
				D. staudtii	39
(−)-10-Demethylxylopinine	**11**	$C_{20}H_{23}NO_4$	341	*D. calycina*	17
(−)-Xylopinine	**12**	$C_{21}H_{25}NO_4$	355	*D. obovata*	20
				D. spixiana[b]	24
				D. vallicola	26
(−)-Spiduxine	**13**	$C_{21}H_{23}NO_5$	369	*D. spixiana*[a]	21
(−)-Thaicanine	**14**	$C_{21}H_{25}NO_5$	371	*D. trunciflora*	35
Protoberberines					
Jatrorrhizine	**15**	$C_{20}H_{20}NO_4$	338	*D. trunciflora*	35
Dehydrodiscretine	**16**	$C_{20}H_{20}NO_4$	338	*D. odorata*	14
Pseudopalmatine	**17**	$C_{21}H_{22}NO_4$	352	*D. odorata*	14
				D. vallicola	26
Morphinandienone					
(9*S*)-Sebiferine	**18**	$C_{20}H_{23}NO_4$	341	*D. obovata*	20
Proaporphine					
(−)-Glaziovine	**19**	$C_{18}H_{19}NO_3$	297	*D. vallicola*	26
Aporphines sensu stricto					
Asimilobine	**20**	$C_{17}H_{17}NO_2$	267	*D. furfuracea*	10
N-Methylasimilobine	**21**	$C_{18}H_{19}NO_2$	281	*D. spixiana*[a]	21

Table II *(Continued)*

Alkaloid type and name	Structure	Molecular formula	MW	Species	Ref.(s)
Nornuciferine	**22**	$C_{18}H_{19}NO_2$	281	*D. spixiana*[b]	24
				D. flagellaris	29,30
Anonaine	**23**	$C_{17}H_{15}NO_2$	265	*D. spixiana*[b]	24
				D. furfuracea	10
Isopiline	**24**	$C_{18}H_{19}NO_3$	297	*D. flagellaris*	29,30
3-Hydroxynornuciferine	**25**	$C_{18}H_{19}NO_3$	297	*D. spixiana*[b]	24
O-Methylisopiline	**26**	$C_{19}H_{21}NO_3$	311	*D. spixiana*[b]	24
				D. flagellaris	29,30
Anolobine	**27**	$C_{17}H_{15}NO_3$	281	*D. obovata*	20
Xylopine	**28**	$C_{18}H_{17}NO_3$	295	*D. calycina*	17
				D. obovata	20
				D. furfuracea	10
Isolaureline	**29**	$C_{19}H_{10}NO_3$	309	*D. obovata*	20
Obovanine	**30**	$C_{17}H_{15}NO_3$	281	*D. calycina*	17
				D. furfuracea	10
Puterine	**31**	$C_{18}H_{17}NO_3$	295	*D. calycina*	17
O-Methylpukateine	**32**	$C_{19}H_{19}NO_3$	309	*D. calycina*	17
Buxifoline	**33**	$C_{19}H_{19}NO_4$	325	*D. obovata*	20
N-Methylbuxifoline	**34**	$C_{20}H_{21}NO_4$	339	*D. obovata*	20
Norglaucine	**35**	$C_{20}H_{23}NO_4$	341	*Duguetia* sp.	36
N-Methylglaucine	**36**	$C_{22}H_{28}NO_4$	370	*D. furfuracea*	11
N-Methyllaurotetanine	**37**	$C_{20}H_{23}NO_4$	341	*D. vallicola*	25
Isoboldine	**38**	$C_{19}H_{21}NO_4$	327	*D. vallicola*	26

Dicentrine	**39**	$C_{20}H_{21}NO_4$	339	*Duguetia* sp.	36
Norisocorydine	**40**	$C_{19}H_{21}NO_4$	327	*D. furfuracea*	10
Isocorydine	**41**	$C_{20}H_{23}NO_4$	341	*D. furfuracea*	10
				D. vallicola	26
8-Nitroisocorydine	**42**	$C_{20}H_{22}N_2O_6$	386	*D. furfuracea*	13
Calycinine	**43**	$C_{18}H_{17}NO_4$	311	*D. calycina*	17
				D. flagellaris	29,30
				D. obovata	20
N-Methylcalycinine	**44**	$C_{19}H_{19}NO_4$	325	*D. obovata*	20
Duguevanine	**45**	$C_{19}H_{19}NO_5$	341	*D. obovata*	20
				D. flagellaris	29,30
N-Methylduguevanine	**46**	$C_{20}H_{21}NO_5$	355	*D. obovata*	20
N-Formylnoraporphines					
N-Formylputerine	**47**	$C_{19}H_{17}NO_4$	323	*D. calycina*	19
N-Formylxylopine	**48**	$C_{19}H_{17}NO_4$	323	*D. obovata*	20
N-Formylbuxifoline	**49**	$C_{20}H_{19}NO_5$	353	*D. obovata*	20
N-Formylduguevanine	**50**	$C_{20}H_{21}NO_6$	369	*D. obovata*	20
N-Nitrosonoraporphines					
N-Nitrosoanonaine	**51**	$C_{17}H_{14}N_2O_3$	294	*D. furfuracea*	12
N-Nitrosoxylopine	**52**	$C_{18}H_{16}N_2O_4$	324	*D. furfuracea*	12
7-Alkyl-substituted-6a,7-dehydroaporphines					
Duguenaine	**53**	$C_{19}H_{15}NO_3$	305	*D. calycina*	20
Duguecalyne	**54**	$C_{20}H_{17}NO_4$	335	*D. calycina*	19
Duguespixine	**55**	$C_{19}H_{17}NO_3$	307	*D. spixiana*[a]	21,23

Table II *(Continued)*

Alkaloid type and name	Structure	Molecular formula	MW	Species	Ref.(s)
7-Hydroxyaporphines					
Norpachyconfine	**56**	$C_{17}H_{17}NO_3$	283	*D. spixiana*[a]	21
Nornuciferidine	**57**	$C_{18}H_{19}NO_3$	297	*D. spixiana*[b]	24
Pachyconfine	**58**	$C_{18}H_{19}NO_3$	297	*D. confinis*	37
				D. spixiana[a]	21
N-oxypachyconfine	**59**	$C_{18}H_{19}NO_4$	313	*D. spixiana*[a]	21
Oliveroline	**60**	$C_{18}H_{17}NO_3$	295	*D. confinis*	37
				D. flagellaris	29,30
				D. vallicola	27
				D. odorata	14
N-Oxyoliveroline	**61**	$C_{18}H_{17}NO_4$	311	*D. flagellaris*	29,30
Rurrebanidine	**62**	$C_{18}H_{19}NO_4$	313	*D. spixiana*[b]	24
Rurrebanine	**63**	$C_{19}H_{21}NO_4$	327	*D. spixaina*[b]	24
Guatterine	**64**	$C_{19}H_{19}NO_4$	325	*D. confinis*	37
N-Oxyguatterine	**65**	$C_{18}H_{17}NO_5$	341	*D.confinis*	37
N-Methylguatterine	**66**	$C_{20}H_{22}NO_4$	340	*D. odorata*	14
Noroliveridine	**67**	$C_{17}H_{15}NO_3$	281	*D. spixiana*[a,b]	21,24
Oliveridine	**68**	$C_{19}H_{19}NO_4$	325	*D. spixiana*[a,b]	21,24
				D. glabriuscula	33
				D. flagellaris	29,30
				D. vallicola	27
Roemerolidine	**69**	$C_{18}H_{17}NO_4$	311	*D. spixiana*[b]	24
N-Oxyoliveridine	**70**	$C_{19}H_{19}NO_5$	341	*D. spixiana*[a,b]	21,24

Duguexine	**71**	$C_{18}H_{17}NO_4$	311	*D. spixiana*[a,b]	21,24
N-Oxyduguexine	**72**	$C_{18}H_{17}NO_5$	327	*D. spixiana*[a]	21
Spixianine	**73**	$C_{19}H_{19}NO_5$	341	*D. spixiana*[a]	21
N-Oxyspixianine	**74**	$C_{19}H_{19}NO_6$	357	*D. spixiana*[a]	21
Polyalthine	**75**	$C_{20}H_{21}NO_5$	355	*D. glabriuscula*	33
Duguetine	**76**	$C_{20}H_{21}NO_5$	355	*Duguetia* sp.	36
				D. flagellaris	29,30
				D. furfuracea	11
N-Oxyduguetine	**77**	$C_{20}H_{21}NO_6$	371	*D. furfuracea*	11
7-Methoxyaporphines					
Pachypodanthine	**78**	$C_{18}H_{17}NO_3$	295	*D. staudtii*	39,47
				D. confinis	38
				D. flagellaris	29,30
N-Methylpachypodanthine	**79**	$C_{19}H_{19}NO_3$	309	*D. staudtii*	39
N-Acetylpachypodanthine	**80**	$C_{20}H_{19}NO_4$	337	*D. confinis*	38
7-Methoxy-4-hydroxyaporphines					
Norpachystaudine	**81**	$C_{18}H_{17}NO_4$	311	*D. staudtii*	39
Pachystaudine	**82**	$C_{19}H_{19}NO_4$	235	*D. staudtii*	39
Oxoaporphines					
Liriodenine	**83**	$C_{17}H_9NO_3$	275	*D. furfuracea*	10
				D. staudtii	39,40
Lysicamine	**84**	$C_{18}H_{13}NO_3$	291	*D. spixiana*[b]	24
O-Methylmoschatoline	**85**	$C_{19}H_{15}NO_4$	321	*D. spixiana*[b]	24
				D. stelechantha	15

Table II *(Continued)*

Alkaloid type and name	Structure	Molecular formula	MW	Species	Ref.(s)
				D. eximia	28
				D. vallicola	27
				D. colombiana	31
Atherospermidine	**86**	$C_{18}H_{11}NO_4$	305	*D. furfuracea*	10
Lanuginosine	**87**	$C_{18}H_{11}NO_4$	305	*D. glabriuscula*	33
				D. furfuracea	10
				D. spixiana[a,b]	21,24
Oxopukateine	**88**	$C_{17}H_9NO_4$	291	*D. eximia*	28
				D. stelechantha	15
Oxoputerine	**89**	$C_{18}H_{11}NO_4$	305	*D. eximia*	28
				D. calycina	17
Oxobuxifoline	**90**	$C_{19}H_{13}NO_5$	335	*D. obovata*	20
				D. glabriuscula	33
Dicentrinone	**91**	$C_{19}H_{13}NO_5$	335	*D. furfuracea*	11
Duguevalline	**92**	$C_{20}H_{15}NO_6$	365	*D. vallicola*	27
Aminoethylphenanthrenes (6,6a-Secoaporphines)					
Noratherosperminine	**93**	$C_{19}H_{21}NO_2$	295	*D. calycina*	18
Atherosperminine	**94**	$C_{20}H_{23}NO_2$	309	*D. spixiana*[a]	21
				D. calycina	17
N-Oxyatherosperminine	**95**	$C_{22}H_{23}NO_3$	325	*D. spixiana*[a]	21
Methoxyatherosperminine	**96**	$C_{21}H_{25}NO_3$	339	*D. spixiana*[a]	21

Copyrine alkaloids					
1-Aza-7-oxoaporphines					
Sampangine	**97**	$C_{15}H_8N_2O$	232	*D. hadrantha*	16
3-Methoxysampangine	**98**	$C_{16}H_{10}N_2O_2$	246	*D. hadrantha*	16
1-Aza-4,5-dioxoaporphines					
Hadranthine A	**99**	$C_{18}H_{14}N_2O_4$	322	*D. hadrantha*	16
Hadranthine B	**100**	$C_{16}H_{10}N_2O_3$	278	*D. hadrantha*	16
Imbiline-1	**101**	$C_{17}H_{12}N_2O_3$	292	*D. hadrantha*	16
Azahomoaporphines					
Spiguetine	**102**	$C_{18}H_{16}N_2O_3$	308	*D. spixiana*[b]	24
Spiguetidine	**103**	$C_{19}H_{18}N_2O_3$	322	*D. spixiana*[b]	24
Azaanthraquinone					
Cleistopholine	**104**	$C_{14}H_9NO_2$	223	*D. vallicola*	27
Protoberberine–styrene adduct					
Staudine	**105**	$C_{31}H_{33}NO_7$	531	*D. staudtii*	39,48

[a] *D. spixiana* from Colombia.
[b] *D. spixiana* from Bolivia.

Table III Alphabetical list of alkaloids isolated from the genus *Duguetia* with their synonyms and structure numbers

Name	Structure	Name	Structure
N-Acetylpachypodanthine	**80**	Imbiline-1	**101**
Anolobine	**27**	Isoboldine (*N*-Methyllaurelliptine)	**38**
Anonaine	**23**	Isochondodendrine	**3**
Asimilobine	**20**	Isocorydine (Artabotrine, Luteanine)	**41**
Atherospermidine (Psilopine)	**86**	(−)-Isocorypalmine	**6**
Atherosperminine	**94**	Isolaureline (*N*-Methylxylopine)	**29**
Buxifoline	**33**	Isopiline	**24**
Calycinine	**43**	Jatrorrhizine	**15**
Cleistopholine	**104**	Lanuginosine (Oxoxylopine)	**87**
(−)-Corypalmine (Tetrahydrojatrorrhizine)	**5**	Liriodenine (Oxoushinsunine, Micheline B, Spermatheridine)	**83**
Dehydrodiscretine	**16**	Lysicamine (Oxonuciferine)	**84**
(−)-10-Demethylxylopinine	**11**	Methoxyatherosperminine	**96**
Dicentrine (*N*,*O*-Dimethylactinodaphnine, Eximine)	**39**	3-Methoxysampangine	**98**
Dicentrinone	**91**	*N*-Methylasimilobine	**21**
(−)-Discretamine	**4**	*N*-Methylbuxifoline	**34**
(−)-Discretine	**10**	*N*-Methylcalycinine	**44**
Duguecalyne	**54**	*N*-Methylduguevanine	**46**
Duguenaine	**53**	*N*-Methylglaucine	**36**
Duguespixine	**55**	*N*-Methylguatterine	**66**
Duguetine	**76**	*O*-Methylisopiline (*O*-Methylnorlirinine)	**26**
Duguevalline	**92**	*N*-Methyllaurotetanine (Lauroscholtzine, Rogersine)	**37**

Table III *(Continued)*

Name	Structure	Name	Structure
N-Oxyoliveridine	**70**	Staudine	**105**
N-Oxyoliveroline	**61**	(−)-Tetrahydropalmatine (Rotundine)	**7**
N-Oxypachyconfine	**59**	(−)-Thaicanine	**14**
N-Oxyspixianine	**74**	Xylopine (*O*-Methylanolobine)	**28**
Pachyconfine	**58**	(−)-Xylopinine	**12**
Pachypodanthine	**78**		

and in other instances the structure elucidations were straightforward, relying largely on the NMR spectra of the alkaloids. For this reason, in this section only the more problematic structure assignments will be discussed.

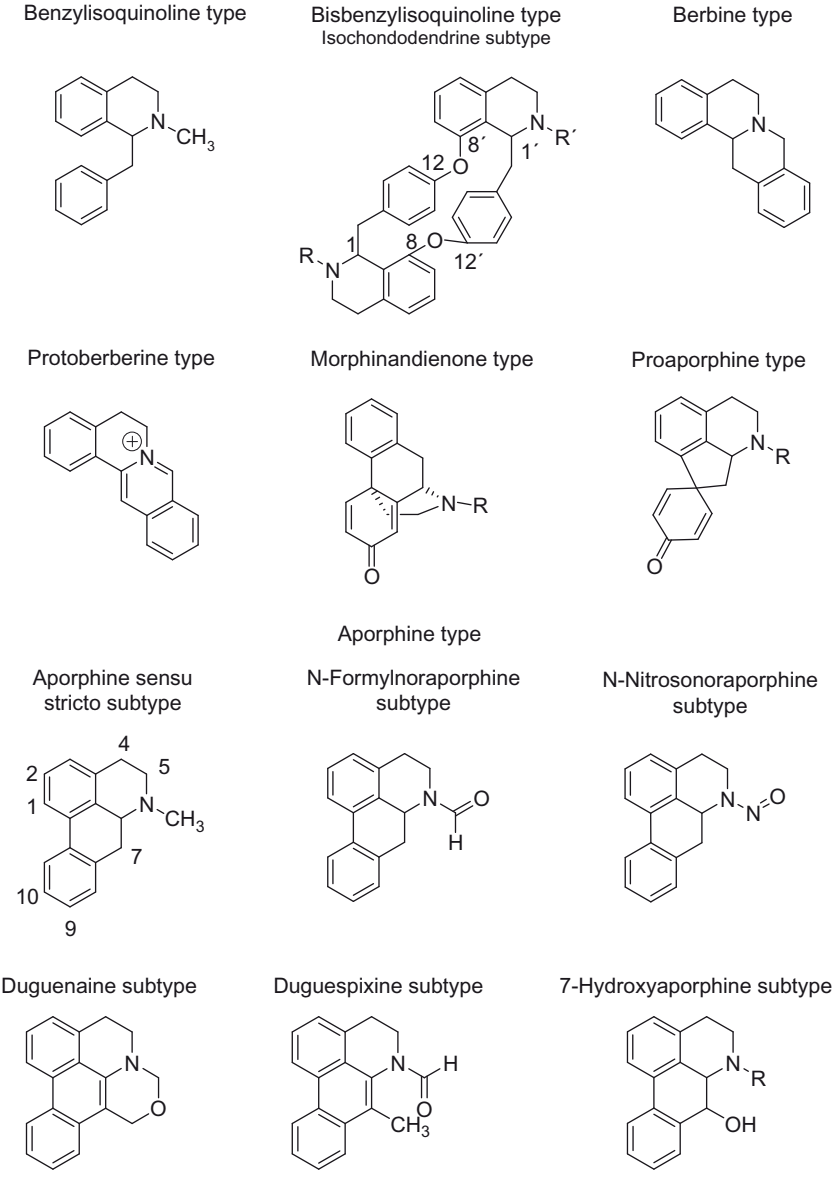


Figure 1 Structural types of alkaloids isolated from *Duguetia* species.

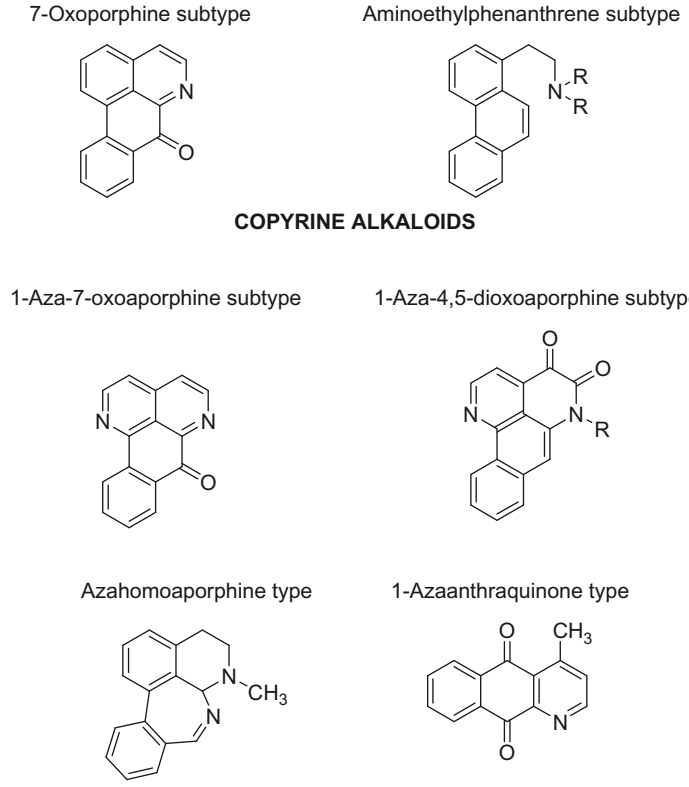
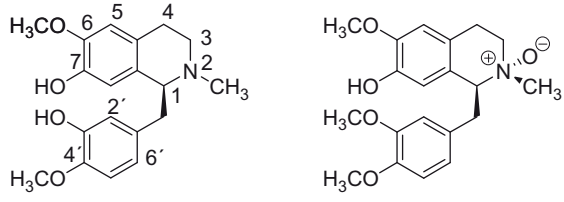


Figure 1 (*Continued*)

A. Benzyltetrahydroisoquinolines

Only two, unelaborated, benzyltetrahydroisoquinolines have been reported from the genus *Duguetia*, namely, reticuline (**1**), isolated from *Duguetia trunciflora* and *D. furfuracea* (10,35), and *cis-N*-oxycodamine (**2**), isolated from *Duguetia spixiana* (21,22).



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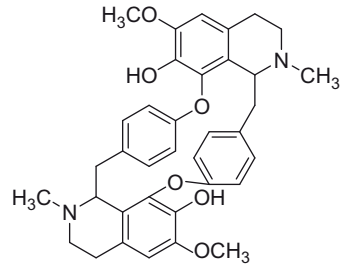
B. Bisbenzyltetrahydroisoquinolines

The head-to-tail/head-to-tail dimer isochondodendrine (**3**), isolated from *D. furfuracea* (10), is the only bisbenzyltetrahydroisoquinoline recorded to date from this genus.



3

C. Berbines and Protoberberines

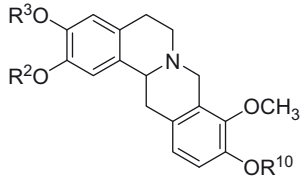
The berbines or tetrahydroprotoberberines appear to be widely distributed in the genus *Duguetia* (10 out of 15–16 species studied). Although quantitative analyses are lacking, it is noteworthy that these alkaloids comprise more than 50% of the mass of alkaloids isolated from the bark of the African *D. confinis*, and about 20% of *D. staudtii*, while they are apparently less abundant in the New World species. It is also noteworthy that, aside from their common precursor reticuline (**1**), the other five alkaloids isolated from *D. trunciflora* are members of this structural type, as do all three *Duguetia gardneriana* alkaloids. With the exception of spiduxine (**13**, only known so far from *D. spixiana*) and thaicanine (**14**, from *D. trunciflora*, but isolated previously from other, non-Annonaceous species), their structures are quite commonplace.

A single paper on the constituents of *D. trunciflora* reported the presence of reticuline (**1**), the berbines tetrahydropalmatine (THP) (rotundine) (**7**), tetrahydrojatrorrhizine (corypalmine) (**5**), discretamine (**4**), and thaicanine (**14**), and the protoberberine jatrorrhizine (**15**) (29). Although the optical rotations of the chiral members of this series were not published, all four berbines can be expected to have the usual *S*-configuration, and the same is true for the reticuline (**1**) isolated from this plant, if it is the biosynthetic precursor of the other isolates, and not, in this case, a dead-end metabolite with the *R* stereochemistry.

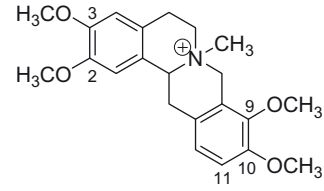
A report on the hypotensive and vasorelaxant effects of discretamine (**4**) from *Duguetia magnolioidea* Maas (34) refers to experimental details of the isolation ''according to the method described by Fechine *et al*. (2002)'' (35). Unfortunately, the report provides no information as to the location

where the plant was collected, its identification, or the existence of a voucher specimen.

In this genus, the quaternary *N*-methyltetrahydropalmatine (**8**) has only been isolated from *D. furfuracea*. Although its putative precursor, THP (also named rotundine, **7**) has not been reported from this species, its 3,10-dihydroxy analog discretamine (**4**) is present in *D. furfuracea*, *D. calycina*, *D. gardneriana*, *D. magnolioidea*, and *D. trunciflora* (10,17,32,34,35).



4 : $R^2 = CH_3$, $R^3 = R^{10} = H$
5 : $R^2 = R^{10} = CH_3$, $R^3 = H$
6 : $R^2 = H$, $R^3 = R^{10} = CH_3$
7 : $R^2 = R^3 = R^{10} = CH_3$



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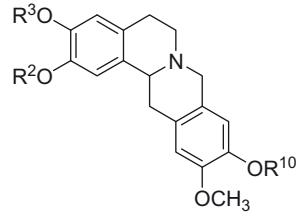
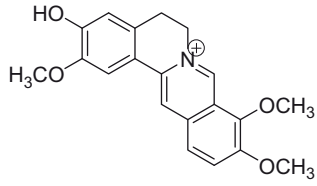


9 : $R^2 = H$, $R^3 = R^{10} = CH_3$
10 : $R^2 = R^{10} = CH_3$, $R^3 = H$
11 : $R^2 = R^3 = CH_3$, $R^{10} = H$
12 : $R^2 = R^3 = R^{10} = CH_3$

Protoberberines, easily formed nonenzymatically on prolonged exposure of berbines to air, have been isolated less often from *Duguetia*, but the co-occurrence of jatrorrhizine (**15**) and its tetrahydro analog corypalmine (**5**=tetrahydrojatrorrhizine) in *D. trunciflora*, and of pseudopalmatine (**17**) and the corresponding xylopinine (**12**) in *Duguetia vallicola* suggest that at least in these species they might be artifacts of storage or isolation. Thaicanine (**14**) is presumably a hydroxylation metabolite of THP (**7**). The C12-formylated spiduxine (**13**) from

Colombian *D. spixiana* is viewed as a (tetrahydro)retroprotoberberine (see Section V).



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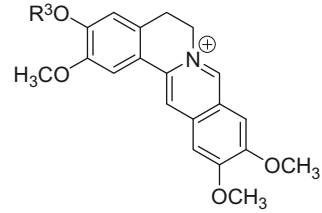
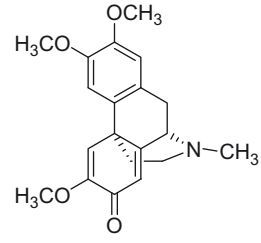


16 : $R^3 = H$
17 : $R^3 = CH_3$

D. Morphinandienone

(9*S*)-Sebiferine (**18**) is the only morphinandienone reported from this genus, as a constituent of *Duguetia obovata* (20).

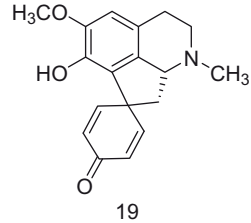


18

E. Aporphinoids

1. Proaporphines

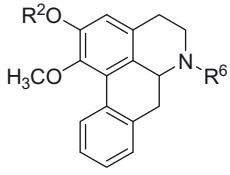
Proaporphines, like the morphinandienones, seem to be uncommon in *Duguetia*. Only glaziovine (**19**) has been reported from the leaves of *D. vallicola* in which it is quite abundant (26).



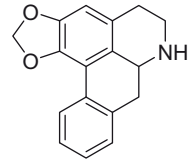
19

2. Aporphines *sensu stricto*

Aporphinoids in general are richly represented in the genus *Duguetia*. Aporphines *sensu stricto* **43**–**46**, *N*-formylduguevanine (**50**), the 7-hydroxyaporphines (**73**–**74**), and the oxoaporphine duguevalline (**92**), present the unusual 9,11-dioxygenation pattern.



20 : $R^2 = R^6 = H$
21 : $R^2 = H$, $R^6 = CH_3$
22 : $R^2 = R^6 = CH_3$



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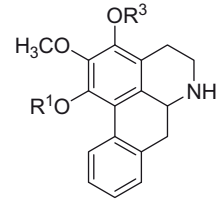
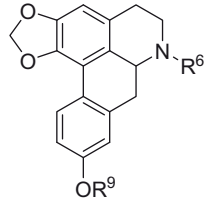
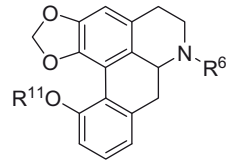


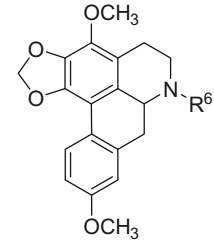
24 : $R^1 = H$, $R^3 = CH_3$
25 : $R^1 = CH_3$, $R^3 = H$
26 : $R^1 = R^3 = CH_3$



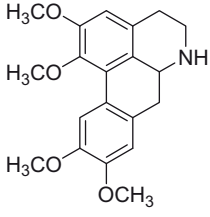
27 : $R^6 = R^9 = H$
28 : $R^6 = H$, $R^9 = CH_3$
29 : $R^6 = R^9 = CH_3$



30 : $R^6 = R^{11} = H$
31 : $R^6 = H$, $R^{11} = H$
32 : $R^6 = H$, $R^{11} = CH_3$



33 : $R^6 = H$,
34 : $R^6 = CH_3$



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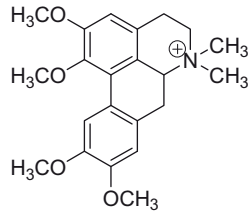

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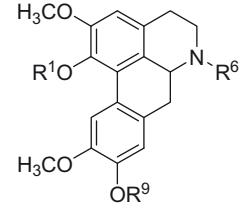
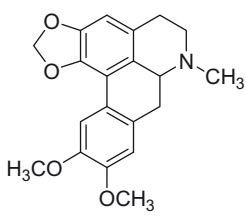


37 : $R^1 = R^6 = CH_3$, $R^9 = H$
38 : $R^1 = R^9 = H$, $R^6 = CH_3$



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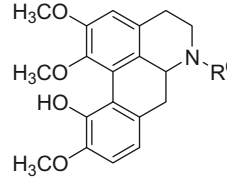
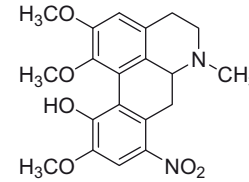


40 : R^6 = H
41 : R^6 = CH_3



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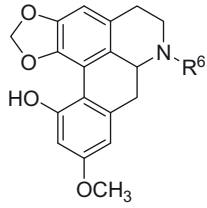
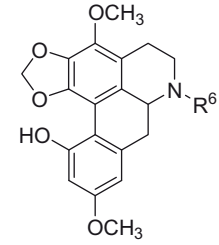


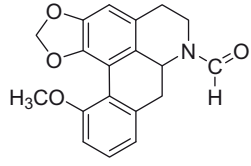
43 : R^6 = H
44 : R^6 = CH_3



45 : R^6 = H
46 : R^6 = CH_3

3. *N*-Formylaporphines

Four *N*-formylaporphines have been reported from the genus *Duguetia*, namely, *N*-formylputerine (**47**) from *D. calycina* (19), and *N*-formylxylopine (**48**), *N*-formylbuxifoline (**49**), and *N*-formylduguevanine (**50**) from *D. obovata* (20).



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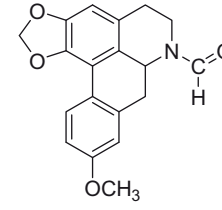

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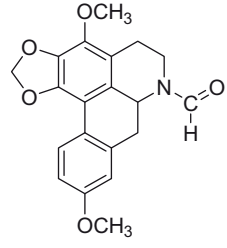

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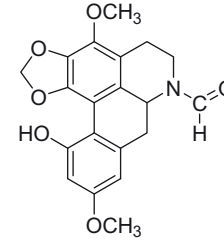
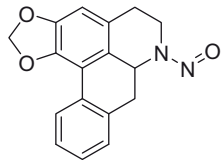


50

4. *N*-Nitrosoaporphines

Two *N*-nitrosonoraporphines, *N*-nitrosoanonaine (**51**) and *N*-nitrosoxylopine (**52**) have been reported from *D. furfuracea*. The structure of *N*-nitrosoanonaine (**51**) was confirmed by X-ray crystallography (12). The same authors have very recently reported the presence of 8-nitroisocorydine (**42**) in the same plant (13).



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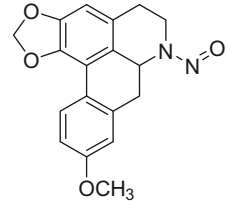

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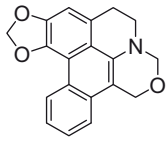
5. 7-Alkyl-6*a*,7-didehydroaporphines

Duguenaine (**53**) and duguecalyne (**54**) were isolated from *D. calycina* (19,20), and duguespixine (**55**) from the bark of the Colombian *D. spixiana* (21,23). The latter alkaloid was also found in *Guatteria sagotiana* (49), but to date duguecalyne (**54**) and duguenaine (**53**) seem to be exclusively *Duguetia* metabolites.



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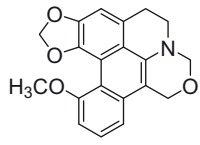

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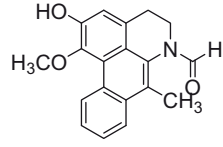
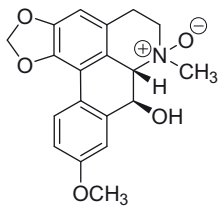


55

6. 7-Hydroxyaporphines

The genus *Duguetia* is remarkably rich in 7-hydroxylated aporphines, of which a small number have also been isolated from *Guatteria* species. Although only found in one half of the species studied, they account for nearly two thirds of the mass of alkaloids isolated from both Colombian and Bolivian *D. spixiana*.



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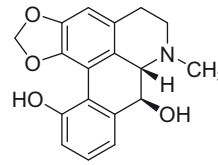

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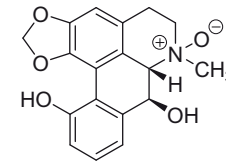

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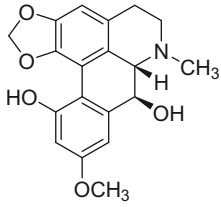

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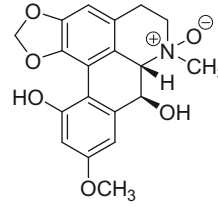

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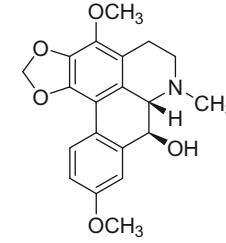

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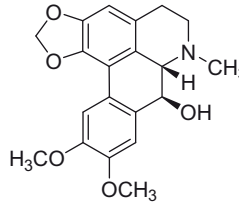

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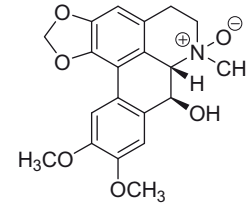

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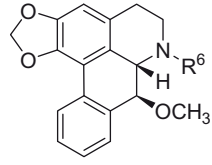
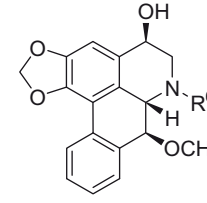
The closely related pachypodanthine (**78**), *N*-methylpachypodanthine (**79**), *N*-acetylpachypodanthine (**80**), pachystaudine (**82**), and norpachystaudine (**81**), all C7 methoxylated, are characteristic of the African species *D. staudtii* and *D. confinis* (formerly designated as *Pachypodanthium*). Although a few other C4–C7 oxygenated aporphines (e.g., stephadiolamine) and oxoaporphines are known, pachystaudine (**82**) and its nor-analog **81** seem to be the only aporphinoids characterized to date with both C4 hydroxy and C7 methoxy substituents.



78: R^6 = H
79: R^6 = CH_3
80: R^6 = $COCH_3$

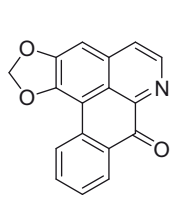


81: R^6 = H
82: R^6 = CH_3

7. Oxoaporphines

Nine 7-oxoaporphine alkaloids (7-oxo-4,5,6,6*a*-tetradehydroaporphines) have been isolated from *Duguetia* species, scattered throughout the genus. Perhaps significantly, all three alkaloids identified as constituents of *Duguetia eximia* belong in this group (28).

So far, duguevalline (**92**) is only the second oxoaporphine known to have the unusual 9,11-dioxygenation pattern. The other, oxoisocalycinine, was isolated from *Guatteria discolor* (50).



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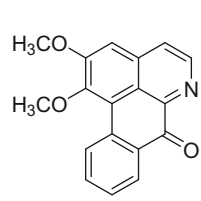

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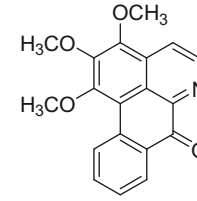

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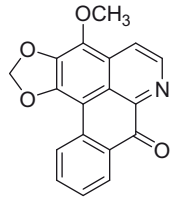

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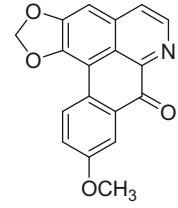

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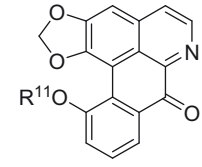
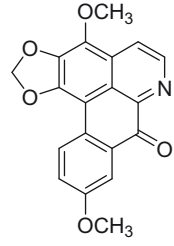


88 : R^{11} = H
89 : R^{11} = CH_3



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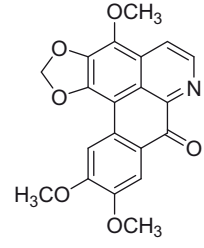

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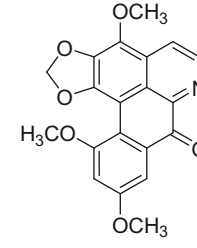

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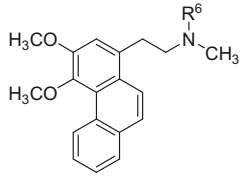
F. Miscellaneous Aporphinoid- and Berbinoid-Related Alkaloids

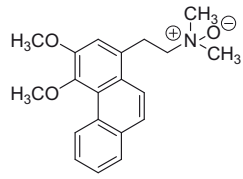
1. Aminoethylphenanthrenes

Four 1-aminoethylphenanthrenes, or 6,6*a*-secoaporphines, have been isolated from *Duguetia* species, these are: atherosperminine (**94**, from

D. spixiana and *D. calycina*), its *N*-oxide (**95**, from *D. spixiana*), noratherosperminine (**93**, from *D. calycina*), and methoxyatherosperminine (**96**, from *D. spixiana*).



93 : R^6 = H
94 : R^6 = CH_3



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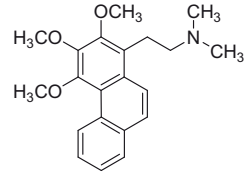
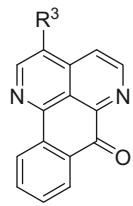


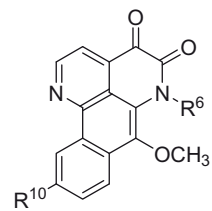
96

2. Copyrine Alkaloids

The relatively rare 1-azaaporphinoids are often referred to as copyrine alkaloids, by analogy with the term isoquinoline alkaloids, as copyrine is the trivial name of the 2,7-diazanaphthalene nucleus. Three 1-aza-4,5-dioxo-7-methoxy-6*a*,7-didehydroaporphines and two 1-aza-7-oxo-4,5,6,6*a*-tetradehydroaporphines were isolated from *Duguetia hadrantha* (16). The fact that these five unusual compounds are the only alkaloids isolated from this particular species, and that they have been found in no other *Duguetia* species, is probably a consequence of the antimalarial/antifungal bioassay-guided fractionation of the plant extract. They are biogenetically related to cleistopholine (**104**), which in this genus has only been recorded as a constituent in *D. vallicola*, and to other annonaceous 1-azaanthra-9,10-quinone derivatives with scattered occurrence in the genera *Annona*, *Cleistopholis*, *Guatteria*, *Meiogyne*, *Porcelia*, *Hornschuchia*, and *Cananga* (51–56).



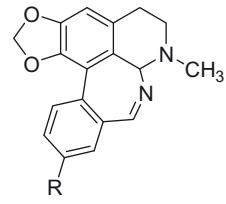
97 : R^3 = H
98 : R^3 = OCH_3



99 : R^6 = CH_3, R^{10} = OCH_3
100 : R^6 = R^{10} = H
101 : R^6 = CH_3, R^{10} = H

3. Azahomoaporphines

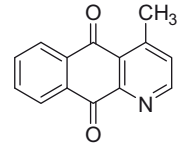
The only two azahomoaporphines found in the genus *Duguetia* are spiguetine (**102**) and spiguetidine (**103**), reported exclusively from a Bolivian accession of *D. spixiana*. They were not isolated from plant material collected in Colombia (24). They are members of a rare alkaloid structural type found only in this species, in *G. sagotiana* (dragabine), and in *Meiogyne virgata* (nordragabine), all in the family Annonaceae.



102 : R = OCH_3
103 : R = OH

4. Azaanthraquinone

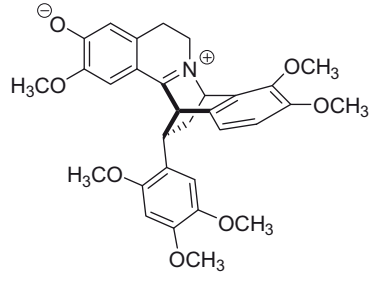
Cleistopholine (**104**), the prototype of the few natural 1-aza-9,10-anthracenedione alkaloids known to date, was isolated from *D. vallicola* (27), and has also been found in several other Annonaceous genera.



104

5. Protoberberine–Styrene Adduct

The structurally unique staudine (**105**) has only been isolated from *D. staudtii* (39,48).



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IV. STRUCTURE AND CHEMISTRY

A. Benzyltetrahydroisoquinolines

Although the configuration of the reticuline (**1**) isolated from *D. trunciflora* was not reported, it seems likely that it is the *S* isomer, as in *D. furfuracea*, and therefore is the immediate precursor of (*S*)-codamine and its *N*-oxide (**2**). The small amount of **2** isolated did not allow its absolute configuration to be determined, but it is depicted here as the more likely (*S*)-reticuline-derived *S* isomer (although in the original reference it is shown with the *R* configuration). The berbines and the 1,2,9,10- and 1,2,10,11-dioxygenated aporphines, of which there are a few in the source plant of *cis*-*N*-oxycodamine, the Colombian accession of *D. spixiana*, are generally derived from (*S*)-reticuline (**1**).

B. Bisbenzyltetrahydroisoquinoline

The complete assignments of the ^{1}H NMR and ^{13}C NMR spectra of isochondodendrine (**3**) have been published (10).

C. Berbinoids

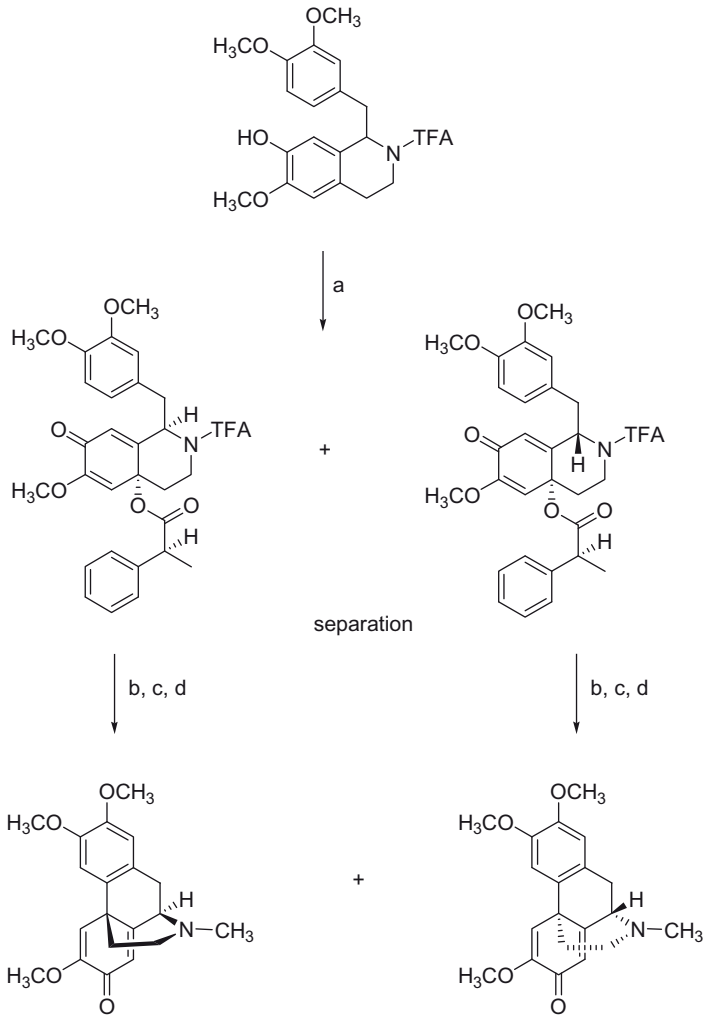
Quite surprisingly, the presence of (*R*)-dicentrine (**39**) and its 7-hydroxy derivative duguetine (**76**) was reported in an unidentified *Duguetia* species (36). This configuration flies in the face of biogenetic theory, but seems to be supported by the negative optical rotation of both alkaloids at 589 nm, and the ORD spectrum of the latter alkaloid. Unfortunately, the only recent report on the reisolation of duguetine from *Duguetia flagellaris* gives no details of its identification or of its physical (including optical rotation) and spectral properties (29,30).

D. Morphinandienone

The stereochemistry of (9*S*)-sebiferine (**18**), which is opposite to that of the morphine alkaloids of *Papaver* species, was demonstrated on the basis of the crystal structure determination of its methiodide (57). Both (9*S*)-sebiferine (**18**) and its enantiomer have been synthesized via *p*-quinol esters starting from the diastereomeric products of the lead tetraacetate oxidation of racemic *N*-trifluoroacetylnorcodamine in (*S*)-2-phenylpropionic acid (58) (Scheme 1).

E. Aporphinoids

The *N*-nitroso, non-phenolic noraporphines **51** and **52** were isolated from a 95% ethanolic extract of the leaves of *D. furfuracea* which was treated with 3% HCl. The authors appropriately state that *N*-nitrosamines can be



Scheme 1 Reagents and conditions: a. $Pb(OAc)_4$, (*S*)-2-phenylpropionic acid; b. TFA, CH_3CN, $-30^{\circ}C$; c. *N*-deprotection; d. *N*-methylation.

carcinogenic and/or mutagenic (59), and also remark that they ''can be regarded as potential NO/NO^+ donors, thus playing an important role in the regulation of many physiological functions'' (60). However, they do not address the possibility that these *N*-nitroso-alkaloids are artifacts of the isolation process.

Nitrates and nitrites commonly accumulate in higher plants. Their occurrence in dietary vegetables has been viewed since at least 1964 as a health hazard (61), and has been the subject of numerous subsequent

publications. Moreover, treatment of some secondary amine alkaloids with nitric acid has been known to lead to the formation of *N*-nitroso derivatives since the end of the 19th century (62), and the *N*-nitrosation of secondary amines occurs readily with inorganic nitrites and acid. It therefore seems possible that the *N*-nitrosoanonaine (**51**) and *N*-nitrosoxylopine (**52**) isolated by Carollo *et al*. were formed on acidification of the ethanol extract of the plant. What concentration of nitrate or nitrite was present in the *Duguetia* sample studied by these authors is a question that would seem to be worth addressing.

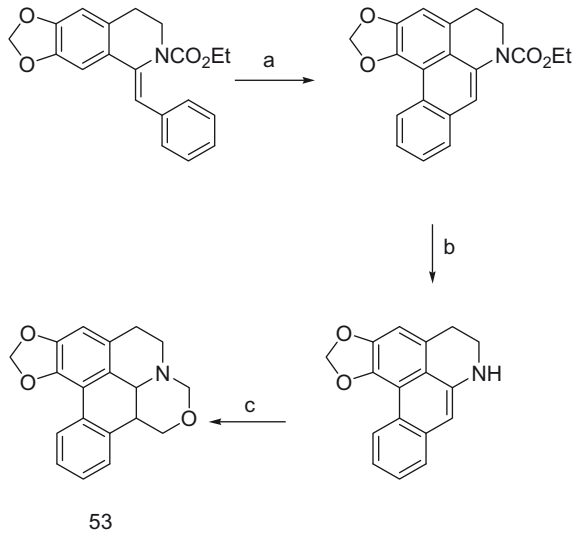
In the opinion of the authors, nitration of isocorydine at the free C8 position, *para* to a phenol function to give 8-nitroisocorydine (**42**), should occur under very mild conditions. This reinforces the hypothesis that these unusual alkaloids are formed either in the living plant or during the extraction procedure by (presumably nonenzymatic) reaction with nitrates or nitrites present in the plant material. The 8-nitroisocorydine structure, however, does not seem to have been established unambiguously. The *N*-methyl ^{1}H resonance is not reported (its ^{13}C resonates at the normal chemical shift value of 43.9 ppm), and the mass-spectral fragmentation shows a possibly suspicious loss of NO from the molecular ion. Is it possible that this isolate is 8-nitrosoisocorydine *N*-oxide, with one or two apparently anomalous *N*-methyl resonances as described by Debourges *et al*. (22). It is probably important to remember that *D. furfuracea* is one of the three *Duguetia* species known to accumulate at least one aporphine *N*-oxide (11).

A biomimetic synthesis of the unusual oxazine-condensed aporphine duguenaine (**53**) and some related analogs has been reported, based on the UV irradiation of an ethanol-tetrahydrofuran solution of 1-benzylidene-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline-2-ethoxycarboxylate in the presence of iodine to produce *N*-ethoxycarbonyldehydro-anonaine. This was followed by *N*-deprotection under basic conditions and quenching with aqueous citric acid to yield the dehydroanonaine salt. The oxazine ring was introduced by treating dehydroanonaine with aqueous formaldehyde at room temperature for 24 h (Scheme 2) (63).

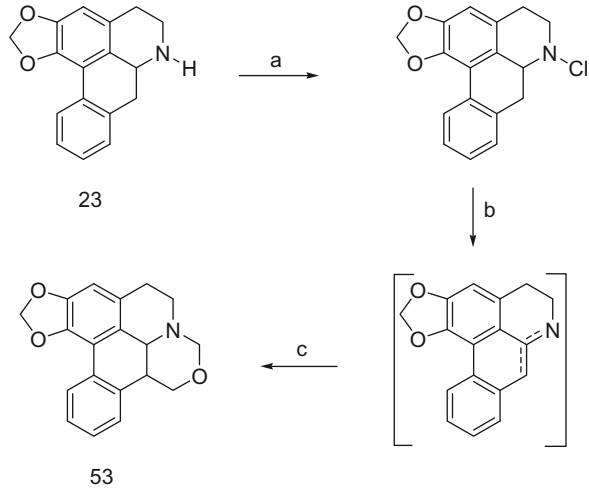
An alternative synthesis of duguenaine (**53**) was published almost simultaneously, using anonaine (**23**) as the starting material. Anonaine was treated with *N*-chlorosuccinimide yielding the corresponding *N*-chloroanonaine. Sodium ethoxide was added to the mixture and the resulting dehydroanonaine was treated with aqueous formaldehyde under reflux for 30 min to furnish **53** (Scheme 3) (23).

F. Miscellaneous Aporphinoid- and Berbinoid-Related Alkaloids

Imbiline 1 (**101**) has been synthesized fairly recently, in seven steps, starting from 4-methoxy-1-naphthylamine, in 9% overall yield (64).



Scheme 2 Reagents and conditions: a. UV, EtOH-THF, I_2, 9.5 h; b. KOH, EtOH, reflux 18 h; c. HCHO, dioxane, rt, 24 h.



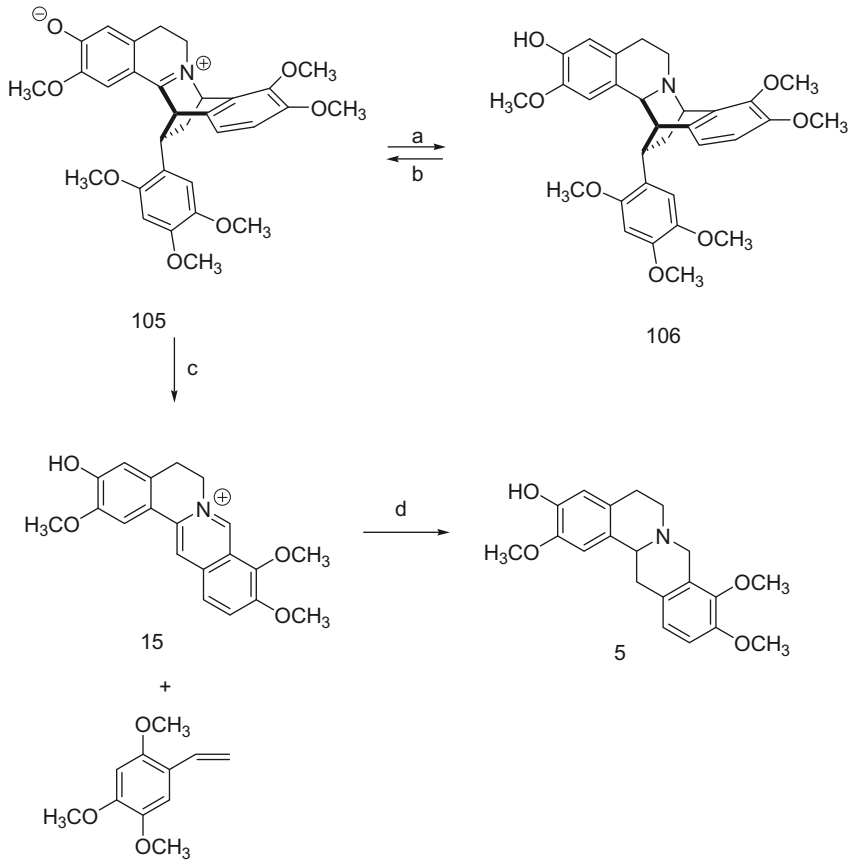
Scheme 3 Reagents and conditions: a. NCS; b. NaOEt; c. HCHO, reflux 0.5 h.

Staudine (**105**, relative configuration shown), isolated from *D. staudtii*, is a unique reverse electron demand Diels–Alder adduct of jatrorrhizine (**15**) and 2,4,5-trimethoxystyrene, which is an abundant metabolite in this plant. Its zwitterionic, rather than phenolic, character, suggested by its high melting point (205–206°C), was revealed by the absence of any change in its UV-VIS spectrum in alkaline solution, and by the failure of

an attempted acetylation with acetic anhydride in pyridine in the presence of 4-dimethylaminopyridine. The presence of a $C{=}N^+$ double bond was apparent from its IR spectrum, which exhibited a strong band at 1605 cm^{-1}. This band disappeared on reduction of staudine (**105**) with sodium borohydride in methanol to afford a dihydro derivative **106** that undergoes facile reoxidation to staudine (**105**) in the presence of air.

The ^1H NMR spectrum of staudine (**105**) showed the presence of six methoxy groups, two single proton multiplets at δ 4.45 and 5.17, and five aromatic proton singlets (one due to two protons, the others to one each). One of the methoxyl resonances (at 3.37 ppm) and one of the aromatic proton signals (at 5.32 ppm) exhibited unusual deshieldings which could be attributed to a structure with closely superimposed aromatic rings. The mass spectrum showed a weak (1%) molecular ion peak, and more abundant fragments at $m/z < 360$. Of particular interest were three peaks at m/z 194 (90%), 179 (40%), and 151 (37%), corresponding to a trimethoxystyrene. The base peak occurred at m/z 337 (M^+-194) with another strong signal at m/z 352 (30%, M^+-179). These data suggested that staudine (**105**) contains a benzylisoquinoline moiety in addition to the trimethoxystyrene moiety, which seem to undergo a retro-Diels–Alder reaction in the mass spectrometer. The ^{13}C NMR spectrum showed all the signals expected for 2,4,5-trimethoxystyrene, with the exception of the ethylene carbon resonances, and all the signals expected for corypalmine (tetrahydrojatrorrhizine, **5**), except for the C14 resonance, plus additional resonances at 32.8, 34.0, and 176.3 ppm. All these data, and further tentative assignments of the sp^3 ^{13}C resonances, showed that the structure of staudine (**105**) incorporates a 2,4,5-trimethoxystyrene moiety bonded through its vinyl side chain to C8 and C13 of corypalmine, but with a C14N double bond. This was confirmed by the pyrolysis of staudine (**105**) under high vacuum at 180°C, which led to the sublimation of 2,4,5-trimethoxystyrene, leaving a highly polar residue. Sodium borohydride reduction of this residue afforded the previously characterized dihydrostaudine (**106**) and corypalmine (**5**) (Scheme 4).

Definitive proof of the structure was provided by an X-ray crystallographic analysis, which showed unambiguously that the benzylic carbon of the styrene residue is bonded to C13 of the corypalmine moiety, and that the more distal styrene carbon atom is bonded to C8. Heating jatrorrhizine (**15**) and 2,4,5-trimethoxystyrene in bromobenzene at 100°C for 10 h produced only a small amount of staudine (**105**), identified by TLC, leading the authors to conclude that this alkaloid is not an isolation artifact (48). Nevertheless, this conclusion is still arguable considering that the same authors reported an $[\alpha]_D{=}0$ for this alkaloid with three stereogenic carbon atoms and, as the crystal structure shows, a highly dissymmetric arrangement of the three benzene chromophores which



Scheme 4 Conditions: a. $NaBH_4$, MeOH; b. Air; c. 180°C, 0.01 Torr, 6 h; d. KBH_4, MeOH.

could be expected to result in a fairly high optical rotation. The crystal packing was not reported and it is therefore not possible to determine if the eight molecules in the unit cell have the same configuration, or if the crystal itself is racemic.

It may be pointed out that 2,4,5-trimethoxystyrene, which is quite toxic to brine shrimp, but only weakly cytotoxic, has been reported as the major bioactive constituent of *Duguetia panamensis* Standley (no studies have been published on the alkaloids of this species) (65), and is also present in *Duguetia colombiana* (31).

V. BIOSYNTHESIS, BIOGENESIS, AND CHEMOSYSTEMATICS

No biosynthetic work has been conducted specifically on plants belonging to the Annonaceae. However, earlier studies of tetrahydrobenzylisoquinoline

alkaloid biosynthesis can be generalized to the more widespread *Duguetia* alkaloids. Regarding biogenetic speculations, some of which have been summarized in an earlier chapter of this series (51), the situation is similar. Some recent developments, both experimental and hypothetical, are reviewed here.

(*S*)-Reticuline (**1**) and codamine *cis-N*-oxide or oxycodamine (**2**) lie near the base of the biosynthetic branch leading to most of the *Duguetia* alkaloids. As the 1,2,9,10- and 1,2,10,11-oxygenated aporphines and the berbines are all derived from (*S*)-reticuline (**1**), but not codamine, the *cis-N*-oxycodamine of *D. furfuracea* can be regarded as a terminal biosynthetic product.

(*S*)-Reticuline (**1**) is the biosynthetic precursor of all known berbines and the 9,10- and 10,11-dioxygenated aporphinoids, and, through the unstable 1,2-dehydroreticuline, is also the precursor of (*R*)-reticuline, the common precursor of most morphinandienone alkaloids. Reasoning biogenetically, (−)-dicentrine (**39**) should originate by direct C8−C6′ coupling of (*R*)-reticuline. It is therefore of interest to note that 1,2-dehydroreticuline synthase, the enzyme at the branching point that separates (*R*)- and (*S*)-reticuline metabolites, has been partially purified and shown to not require a redox cofactor, accepting both (*S*)-reticuline and (*S*)-norreticuline as substrates (66).

The occurrence of isochondodendrine (**3**) as the sole *Duguetia* bisbenzyltetrahydroisoquinoline parallels the limited occurrence of benzyltetrahydroisoquinoline dimers in *Guatteria*. In the largest genus in the Annonaceae, these alkaloids, although many in number, appear to be restricted to *G. boliviana*, *G. guianensis*, and *G. megalophylla* (51,67). *Guatteria gaumeri*, reported to contain a bisbenzylisoquinoline, is a misnomer for *Malmea gaumeri*, now viewed as a synonym of *Malmea depressa* (68). Moreover, cladistic analysis indicates that the split between the branches leading to *Malmea* (the short branch clade of the Annonaceae) and to *Duguetia* and *Guatteria* (the long-branch clade) must have occurred about 60 million years ago, 20 million years before the differentiation of the latter genera (46). Within the long-branch clade, the only other genera for which bisbenzylisoquinolines have been recorded are *Isolona*, *Uvaria*, and *Xylopia*. This suggests that the cytochrome P450 oxidases that presumably catalyze the intermolecular oxidative phenol couplings (two in succession in the case of isochondodendrine) of two coclaurine units (69) are poorly expressed in this group.

In the last few years, particularly important contributions have been made to the knowledge of the berberine bridge enzyme. This protein, incorporating a unique, bi-covalently attached FAD prosthetic group (70), catalyzes the conversion of (*S*)-reticuline to (*S*)-scoulerine by oxidation of the *N*-methyl group and coupling *ortho* to the phenol group of the benzyl ring (71,72). A mechanism has been proposed involving the

removal of hydride from the *N*-methyl group by the FAD cofactor, and concerted carbon−carbon coupling combined with base-catalyzed proton abstraction (73). The enzyme also oxidizes the berbine alkaloid scoulerine to the protoberberine dehydroscoulerine, resembling (*S*)-tetrahydroprotoberberine oxidase (STOX) and canadine oxidase in this regard (74). (*S*)-Tetrahydroprotoberberine oxidase converts (*S*)-tetrahydrocolumbamine to columbamine in the metabolic pathway leading to berberine, jatrorrhizine, and palmatine in *Berberis* species (75). Canadine oxidase catalyzes an alternative route in which formation of the dioxole ring precedes the dehydrogenation leading to berberine (76).

(*S*)-Reticuline (**1**) is not the exclusive berbine precursor. Berberine bridge enzyme of *Eschscholtzia californica*, heterologously expressed in insect cells, transforms other (*S*)- (but not *R*-configured) tetrahydrobenzylisoquinolines with a 2′-hydroxy group into (*S*)-berbines, apparently regardless of the substitution pattern on the benzene ring of the isoquinoline moiety of the precursor (77). In *Corydalis* and *Macleaya* cell cultures both (*S*)-reticuline and (*S*)-protosinomenine (the isomer of reticuline with the positions of the ring A hydroxy and methoxy groups interchanged), but not their enantiomers, undergo the analogous cyclization to (*S*)-scoulerine and tetrahydropalmatrubine (its methoxy derivative at C2) (78). On the other hand, when racemic laudanine (the 7-*O*-methyl ether of reticuline) was fed to the cells, both enantiomers of scoulerine and of the 10,11-dioxygenated berbine corytenchine were formed, in different enantiomeric ratios (78).

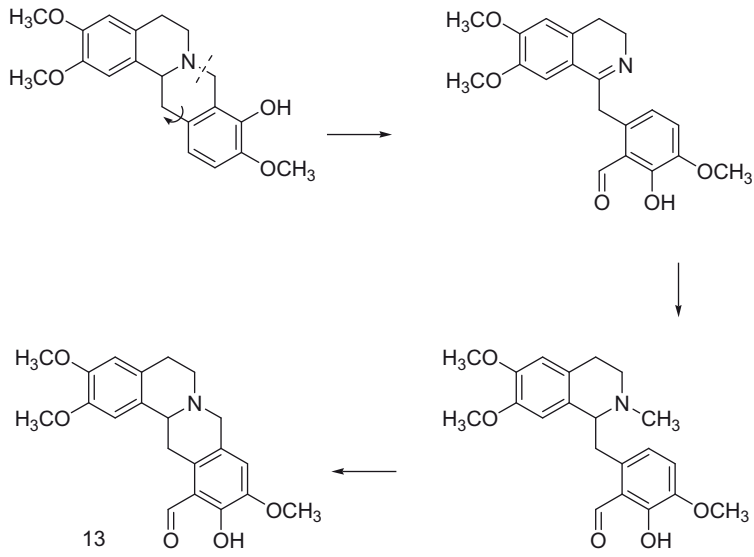
N-Methyltetrahydropalmatine (**8**) and the analogous *N*-methylstylopine and *N*-methylcanadine are synthesized in opium poppy from the corresponding racemic berbines by a recently cloned and characterized *S*-adenosyl-L-methionine:tetrahydroprotoberberine *cis*-*N*-methyltransferase (TNMT) which, however, does not modify (*S*)-scoulerine (79). The stereochemistry of the products was not determined. TNMT activity was detected in several other members of the Papaveraceae, but not in representatives of the Berberidaceae, Menispermaceae, and Ranunculaceae. It remains to be seen if this, or some similar, enzyme is active in the Annonaceae, and specifically in *D. furfuracea*.

It is worth pointing out that no 2-hydroxyberbines or protoberberines have been found in *Duguetia*, although there are a number of occurrences of 3-hydroxyberbines [discretamine (**4**), corypalmine (**5**), and discretine (**10**)] and the oxidation products of **5** and **10** [jatrorrhizine (**15**) and dehydrodiscretine (**16**)]. Assuming that all berbines are formed from (*S*)-norreticuline by a berberine bridge enzyme (73,77,78), this would seem to imply that the formal translocation of a methyl group from the methoxyl at C2 to the C3 hydroxyl group is a practically universal occurrence in this genus. In the rather well-studied genus *Guatteria*, coreximine (2,11-dihydroxy-3,10-dimethoxyberbine, one of the putative precursors of the

whole series) is present in two out of four berbine-accumulating species reviewed two decades ago (51). The fact that only 4 out of 18 *Guatteria* species were shown to contain berbines (and protoberberines were not recorded) suggests that the berberine bridge pathway is considerably less active in *Guatteria* than in *Duguetia*. The presence of spiduxine (**13**) and thaicanine (**14**) in *Duguetia* is another indication of the greater ability of this genus to elaborate the berbine skeleton.

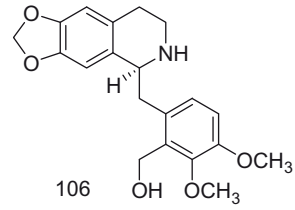
Regarding the (tetrahydro)retroprotoberberine spiduxine (**13**) (21), Shamma proposed in his 1972 treatise on the isoquinoline alkaloids that the related mecambridine, orientalidine, and their oxidation products PO-5 and PO-4 might arise from a berbine by cleavage of the N−C8 bond giving a 1-benzyl-3,4-dihydroisoquinoline that could be reduced to its tetrahydro counterpart, *N*-methylated, and a new ''berberine bridge'' built (80). This scheme is illustrated for the case of spiduxine (**13**) (Scheme 5).

Elegant though this model may appear to be, it lacks experimental support. Considering the ability of *Duguetia* species to introduce one-carbon units in the unexpected C7 position (viz. **53**−**55**), for the sake of parsimony one can also speculate that spiduxine is generated by formylation *ortho* to the phenolic hydroxyl of 2-*O*-methylcoreximine. Nevertheless, a few years ago the unusual structure of a new benzyltetrahydroisoquinoline alkaloid named (+)-argenaxine (**106**) (isolated from *Argemone mexicana*, Papaveraceae) was published (81), with a



Scheme 5 Proposed biogenesis of spiduxine.

regio- and stereochemistry compatible with its hypothetical formation by cleavage of an (*S*)-berbine and the possibility of it being a precursor of a tetrahydroretroprotoberberine (or retroberbine).



106

Interestingly, none of the berbines or protoberberines isolated from *Duguetia* have a methylenedioxy group, suggesting that the enzyme that effects closure of this ring in the many *Duguetia* methylenedioxy-aporphinoids, supposedly a member of the CYP719A subfamily of cytochrome P450s (82), does not accept the geometrically extended berbine skeleton.

The apparently unusual stereochemistry of the morphinandienone (9*S*)-sebiferine (**18**) seems to be justified by the fact that, at least in *Cocculus laurifolius* (Menispermaceae), the biosynthetic conversion of (*S*)- and (*R*)-reticuline (**1**) into sebiferine (**18**) is not stereospecific (83). The C−C phenolic coupling reaction of (*R*)-reticuline (**1**) to salutaridine is the first morphinandienone-forming step, at least in morphine biosynthesis (84), but the enzyme that catalyzes this reaction has not yet been characterized.

Aporphines are believed to be formed by C−C phenolic coupling between C8 and C2′−C6′ of a benzylisoquinoline or, via an intermediate proaporphine, between C8 and C1′. An enzyme catalyzing the first route, CYP80G2, has now been cloned and characterized from *Coptis japonica* (85). This enzyme converts (*S*)-reticuline (**1**) to its direct coupling product (*S*)-corytuberine. If an analogous enzyme is operating in *Duguetia*, it should be responsible for the formation of isocorydine (**41**), an *O*-methylation product of corytuberine and the probably derived norisocorydine (**40**) of *D. vallicola* and *D. furfuracea*. The presence of the proaporphine glaziovine (**19**) in *D. vallicola* is somewhat surprising considering that its likely biogenetic derivatives, (*R*)-aporphines with the 1-hydroxy-2-methoxy, or the 1,10-dihydroxy-2-methoxy, or 1-hydroxy-2,10-dimethoxy substitution patterns seem to be completely absent from the genus.

The 9,11-substitution pattern in the D ring of aporphines is of taxonomic significance in the Annonaceae, as already noted by Roblot *et al*. in 1983 (20). Only one aporphine with this structural feature has been reported in the Ranunculaceae and Menispermaceae (86,87) and these

alkaloids are mainly present in *Guatteria* and *Duguetia* (17,20,21,27, 29,30,50,88). In the review on *Guatteria* alkaloids published in this series (51), it was proposed that one of the ring D substituents might be introduced *meta* to the other, once the aporphine skeleton had been generated from the appropriate proaporphine, stating that either the C11-oxygenated puterine (**31** in this review) or guadiscine (7,7-dimethyl-9-methoxy-1,2-methylenedioxy-6,6*a*-didehydronoraporphine) could be precursors of the 9,11-dioxygenated alkaloids, and that the process might not be very regiospecific. Actually, guadiscine (present in *G. discolor* and *G. melosma*) is only a reasonable precursor of guadiscoline (7,7-dimethyl-9,11-dimethoxy-1,2-methylenedioxy-6,6*a*-didehydronor-aporphine, only found in *G. discolor*), while **31** would be a possible precursor of isocalycinine, discoguattine, oxoisocalycinine, guacolidine, and guacoline, all of which are *Guatteria* alkaloids, and are not isolated from the genus *Duguetia*.

It is intriguing to note that the only American *Duguetia* species known to accumulate a 7-methoxyaporphinoid [pachypodanthine (**78**), in the abundant Amazonian *D. flagellaris*] should grow down to the coast of the Brazilian states of Pará and Maranhão. This part of the South American Gondwana fragment lies opposite to the western reaches of the Gulf of Guinea and Sierra Leone, to which it was formerly attached, and where *D. staudtii* now grows.

In the recent analysis of the anatomical and morphological data of *Duguetia* and closely related genera (6), *D. confinis* and *D. staudtii*, earlier described as *Pachypodanthium*, are placed close to the African species *Duguetia barteri* (Benth.) Chatrou (also formerly *Pachypodanthium*) and *Duguetia dilabens* Chatrou et Repetur (a new species) and to *D. riberensis* of Venezuela, and presumably Colombia. It would be most interesting if the latter plant could be collected and analyzed to determine if it contains 7-methoxylated aporphinoids, like the reasonably well-studied *D. confinis* and *D. staudtii*.

Pachystaudine (**82**) and norpachystaudine (**81**) are said, on the basis of their CD spectra, to have the 6a*S* configuration. This stereochemistry is exceptional for aporphinoids devoid of substituents on ring D, which are generally believed to arise through the dienol−benzene rearrangement of proaporphines derived from (*R*)-coclaurine or norcoclaurine. This apparent anomaly parallels the identification of the (*R*)-9,10-dioxygenated (−)-dicentrine (**39**), from the leaves of an unidentified Amazonian species (36).

It was argued convincingly on the basis of their common 6a*R* configuration (20), that the *N*-formylnoraporphines, found for the first time in *Duguetia* species, cannot be metabolites of *N*-formyl-1-benzyltetrahydroisoquinolines originating from the cleavage of ring C of (14*S*)-berbines as suggested earlier (89). In addition, it was indicated that the

simultaneous presence of *N*-formyl-, *N*-methyl-, and noraporphines, and the accumulation of the latter as major alkaloidal constituents in *D. calycina* and *D. obovata*, pointed to the noraporphines as final biogenetic products (20). Although the precise sequence was not suggested, analogy with the catabolism of *N*-methyl groups in animals allows the sequence aporphine – *N*-formylnoraporphine – noraporphine to be proposed. Noraporphines are therefore likely precursors of the 7- and 4-hydroxynoraporphines, 7-oxo-, and 4,5-dioxoaporphines, and finally the 1-azaaporphinoids (copyrine alkaloids), aristolactams, azaanthraquinones, and their putative derivatives.

The isolated occurrence of duguevalline (**92**) in *D. vallicola* (27) and oxoisocalycinine in *G. discolor* (50) as the only oxoaporphines with the 9,11-dioxygenation pattern is insufficient to suggest any chemosystematic trend. On the other hand, it might be significant that Colombian *D. spixiana* accumulates seven *N*-oxides (five of them aporphine *N*-oxides), while only one each are found in *D. furfuracea*, *D. flagellaris*, and Bolivian *D. spixiana*, and only two in a single *Guatteria* species (*G. sagotiana*) (51).

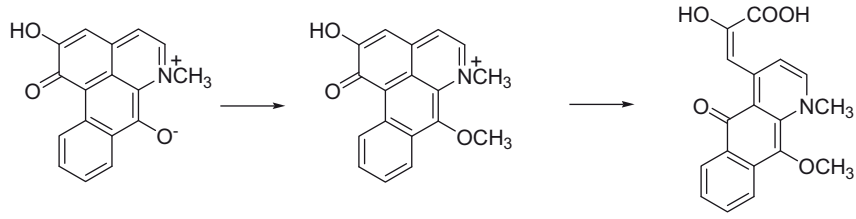
Aminoethylphenanthrenes or secoaporphines are thought to arise by the Hofmann elimination of quaternary aporphine alkaloids (the quaternization and elimination products are commonly termed ''methines''), and this indeed would seem to be the case for atherosperminine (**94**, nuciferine methine) and methoxyatherosperminine (**96**, 3-methoxynuciferine methine). The formation of atherosperminine *N*-oxide (**95**) appears to follow an important catabolic trend for Colombian *D. spixiana*. Noratherosperminine (**93**) would presumably arise through the *N*-demethylation of atherosperminine (**94**), probably catalyzed by a cytochrome P450. An alternative explanation would involve an anomalous Hofmann elimination reaction of the tertiary nuciferine (necessarily in its *N*-protonated form?). Although such a reaction has been documented *in vitro* for boldine (90) in refluxing ammonium acetate solution, it seems extremely unlikely that it should occur nonenzymatically *in vivo*. Therefore, one would have to assume the existence of a ''Hofmannase'' for which there does not seem to be any precedent.

It is interesting that only nuciferine and 3-methoxynuciferine are involved in the biogenesis of these aminoethylphenanthrenes. Nornuciferine (**22**) and 3-hydroxynornuciferine (**25**) have been shown to accumulate only in Bolivian *D. spixiana*, and the former also in *D. flagellaris*, but their tertiary and quaternary analogs, the expected precursors of their ring-opened products, have not been recorded for any *Duguetia* species. This seems remarkable in view of the presence of the close nuciferine congener anonaine (**23**) in Bolivian *D. spixiana* (and also *D. furfuracea*), but not its *N*-methyl homolog roemerine, its quaternary

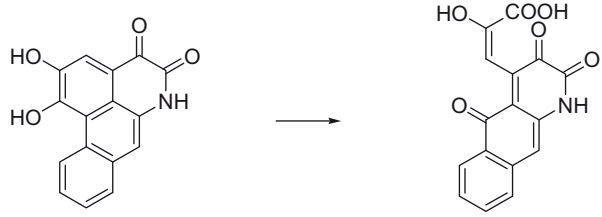
derivative, or its *seco* counterpart. In all, 26 aporphines *sensu stricto*, including several nor- and two quaternary aporphines, are listed above, and only two of them can be envisioned as precursors of the *Duguetia* aminoethylphenanthrenes. On the other hand, the quaternary *N*-methylglaucine (**36**, from *D. furfuracea*) and *N*-methylguatterine (**66**, from *D. odorata*) do not seem to undergo ring opening in this genus. The phytochemical literature records a large number of aminoethylphenanthrenes, many from different Annonaceous genera, apparently derived from aporphines with most of the various substitution patterns. Therefore, the very limited occurrence of these alkaloids in *Duguetia* suggests the hypothesis that they are the products of a metabolic route involving a highly specific enzyme at some key step, possibly the ''Hofmannase'' mentioned above.

The copyrine alkaloids or 1-azaaporphinoids can be viewed as aporphine derivatives in which ring A has been opened (e.g., by extradiol cleavage of a 1,2-catecholic aporphine between C1 and C11b) with subsequent reclosure through condensation with an ammonia molecule (91). Taylor's biogenetic proposal deriving the azafluoranthene, diazafluoranthene, tropoloisoquinoline, 1-azaanthracene, and azafluorenone alkaloids from 1,2-dihydroxy-7-oxoaporphine (liriodendronine) through an initial ring A cleavage (92,93) has been extended to explain the formation of the hadranthines and imbilines via formal 1,4-hydrogenation of the ketoimine function and stabilization by *O*-methylation, either preceded, or followed by, conversion of pyridine ring B to the β-ketolactam function (16). An alternative pathway to the 7-methoxylated 1-aza-4,5-dioxoaporphinoids or the 4,5-dioxocopyrines of *D. hadrantha*, not requiring a reduction step, might start from *N*-methylliriodendronine, in which the C7 oxygen function is already a phenoxy group, particularly in view of the presence of many 7-hydroxy- and two 4-hydroxy-7-methoxyaporphines in *Duguetia* (Scheme 6).

The proposal for the late oxygenation of C4 and C5 could be circumvented by a parallel route to the 4,5-dioxocopyrines starting from 1,2-dihydroxy-4,5-dioxoaporphine, which leaves open the possibility of a monooxygenase-catalysed hydroxylation at C7 (Scheme 7).



Scheme 6 Initial steps of a proposed biogenetic pathway to 4,5-dioxocopyrines starting from *N*-methylliriodendronine.



Scheme 7 First step of a proposed biogenetic pathway to 4,5-dioxocopyrines starting from 1,2-dihydroxy-4,5-dioxoaporphine.

A biogenetic proposal to account for the formation of azahomoaporphines was published 20 years ago in this series (51). According to that hypothesis, spiguetine (**102**) and spiguetidine (**103**) of the Bolivian sample of *D. spixiana* might be derived from the 7-hydroxyaporphines oliveridine (**68**) and roemerolidine (**69**), which are the major alkaloids of the same plant.

It was suggested that α-aroylpyridine derivatives, and more specifically 1-azaanthracene-9,10-diones, such as cleistopholine (**104**), might undergo decarbonylation catalyzed by a metalloenzyme (93). This has now received indirect support from the formation of metal complexes of liriodenine (**83**) which confirm the metallophilicity of the 7-oxoaporphine arrangement of a pyridine nitrogen and a carbonyl oxygen and, presumably, of related systems (94).

Some striking resemblances in the alkaloid chemistry of *Duguetia* and *Guatteria* were pointed out by Cavé in 1984, as indicating the possible proximity of these genera (95). At that time, it was known that both *Duguetia* and *Guatteria* species accumulate 7-alkylated aporphinoids and *N*-formylnoraporphines. It was then suggested that the unusual oxazine-condensed aporphine system of duguenaine (**53**) and duguecalyne (**54**) might arise from ring closure of *N*-formyl-7-methylaporphinoids or, alternatively, their 7-formyl-*N*-methyl counterparts, indicating that such potential intermediates had already been found in *D. spixiana* (duguespixine, **55**) and *Guatteria trichostemon* (trichoguattine, the 1,2-methylenedioxy analog of **55**). In fact, the related 9-hydroxylated belemine and goudotianine have also been isolated from a couple of *Guatteria* species (96,97). Another common feature pointed out by Cavé was the 9,11-dioxygenation pattern of some *Duguetia* and *Guatteria* aporphinoids. At that time (1984), he noted that the phenol function is located at C9 in *Guatteria* and at C11 in *Duguetia*. This is not strictly so, as discoguattine, guacoline and guadiscoline are 9,11-dimethoxylated aporphinoids, but the first two alkaloids could obviously be formed by *O*-methylation of their putative 9-hydroxy precursors isocalycinine and guacolidine.

It is worth mentioning that *D. calycina* and *D. spixiana*, the only *Duguetia* species known to contain 1-aminoethylphenanthrenes, are classed in the section *Sphaerantha*, and thus the occurrence of this small group of alkaloids might be of chemosystematic significance. Interestingly, atherosperminine, *N*-oxyatherosperminine, noratherosperminine, together with the 2-*O*-demethylated atherosperminine analog argentinine (*N*-methylasimilobine methine), are the only 6,6*a*-secoaporphines isolated from the larger Annonaceous genus *Guatteria*, and that from the single species *G. discolor* (50,98). However, *G. discolor* appears to have arisen from fairly recent (Pliocene or Pleistocene) diversification events within *Guatteria* (99), placing it at a considerable evolutionary distance from the Eocene split that presumably originated *Duguetia* (46), and suggesting that aminoethylphenanthrene accumulation is not an ancestral character, but rather one that has appeared in a scattered fashion in plants that synthesize aporphines, either by convergent evolution or by cross-colonization by endophytic fungi with the relevant synthetic abilities. As in *Duguetia*, the *Guatteria* aminoethylphenanthrenes are formally and exclusively derived from ring D-unsubstituted aporphines. As in the case of the copyrine alkaloids, it has been proposed that the azahomoaporphine skeleton arises by oxidative cleavage of the aporphine system, in this case between C6a and C7, and reclosure incorporating an ammonia molecule (100). Finally, if staudine (**105**) is in fact an enzymatic product, one would have to invoke catalysis by a Diels−Alderase to explain its formation.

A striking aspect of the known alkaloid chemistry of *Duguetia* is the apparent lack of correlation between the structures of the isolated alkaloids and the morphologically based classification of the genus into sections. Although the large section *Duguetia*, for example, seems to be well-supported on morphological and genomic grounds, none of the (relatively few) individual alkaloids isolated from *D. odorata* and *D. stelechantha* have been found in the seemingly exhaustive studies of *D. furfuracea*, classed in the same section. One would like to find a more convincing degree of chemosystematic order in such an extensively studied genus, but this will probably be impossible without more exhaustive studies of some species, and adequate quantification of the individual alkaloids in crude extracts rather than the isolated yields, probably using a metabolomic (or metabonomic, or metabolic profiling) approach (101,102). With a significantly more complete picture, it should become possible to reasonably address the fascinating question of how the diverging biosynthetic pathways present in *Duguetia* are regulated.

VI. ETHNOPHARMACOLOGY AND PHARMACOLOGY

Surprisingly little has been published on the ethnopharmacology of *Duguetia* species, as recognized by the authors of one of the most recent papers discussed here (13). A possible explanation is that most of these plants grow in the Amazon region and, if they have medicinal or related uses, are only employed by ethnic groups whose practices have been poorly recorded by outsiders. As is the case with the bulk of ethnopharmacological data, traditional uses are frequently difficult or impossible to ascribe to medical conditions recognized by Western science, and even less so to pharmacological mechanisms. Moreover, in the vast majority of instances, the effectiveness of these practices has not been substantiated scientifically through direct observation. Furthermore, the literature reveals an unfortunate tendency to ascribe a biological activity of a plant or a plant extract, obtained with little regard to the traditional mode of preparation, to whatever can be isolated (and often, but not always, biologically evaluated). Finally, there is an almost complete absence of the quantitative analysis of the active constituents, which can lead to the erroneous conclusion that a substance present in insufficient amounts to produce any effect is responsible in the field for test results obtained with the pure compound.

D. furfuracea has two recorded uses in traditional medicine: its powdered seeds are mixed with water and used to kill lice, and an infusion of the twigs and leaves is used against rheumatism (13). *D. flagellaris* is also used to treat rheumatism as an infusion in sugar cane spirit (30,103). *Duguetia glabriuscula* is said to be used to kill cockroaches, although the report does not mention what part of the plant is insecticidal (104). The insecticidal uses of *Duguetia* species are probably not related to their alkaloid content, but rather to the presence of the so-called ''Annonaceous acetogenins,'' characteristic of many Annonaceae, but not yet reported for the genus *Duguetia*. It is worth noting that the use of powdered Annonaceae seeds as insecticides was first recorded four centuries ago (105).

D. confinis is used in tropical Africa as a cough suppressant and analgesic, particularly for toothache (37). The stem bark of *D. staudtii* is used by some populations in the Ivory Coast as an arrow poison ingredient. The bark is also frequently used in traditional medicine for several indications: ground to a pulp with kola nut it is used to treat gastrointestinal pain and locally, mixed with *Ficus exasperata* leaves, as an anti-inflammatory; it is also considered an analgesic, and some populations in the Congo use it for cough, and for difficulty in breathing. The Pomo tribe, also in the Congo, claims that the bark of this species is a purgative and an aphrodisiac (39).

No ethnopharmacological data seem to have been published for any other *Duguetia* species. In contrast, although information is lacking regarding the pharmacology of most individual *Duguetia* alkaloids, the last two decades have seen an extraordinary number of papers on the biological properties of a few alkaloids that are either abundant, characteristic, or recognized as active principles of other plants, and are also present in *Duguetia*. Additionally, some generalizations can be made safely as to the related pharmacological activities of substances that are close structural congeners.

A. Benzyltetrahydroisoquinolines

(*S*)(+)-Reticuline (**1**) is a dopamine receptor antagonist, blocking the actions of the dopamine agonist apomorphine, causing decreased locomotor activity and producing catalepsy in rats (106,107). These effects seem to be elicited by the blockade of postsynaptic striatal dopamine receptors (108). Dopaminergic antagonism by reticuline (**1**) appears to be rather weak, however, and has not attracted much interest, although it might be involved in the central depressant effects observed in rats and mice (109). Reticuline (**1**) inhibits dopamine uptake and at high concentrations is toxic to dopaminergic and GABAergic neurons. It has therefore been suggested that it might be involved in the genesis of the atypical Parkinsonism of the French West Indies, associated with the consumption of fruit and infusions of the reticuline-containing *Annona muricata* (110). (*S*)(+)-Reticuline (**1**) is also a weak neuromuscular (nicotinic cholinergic) blocker (111). In addition, it reduces the contractile force of guinea pig heart by blocking calcium channels (112). (*S*)(+)-Reticuline-induced uterine relaxation and vasorelaxation by L-type Ca^{2+} channel blockade have also been demonstrated (113,114). Nevertheless, the cardiovascular effects of reticuline (**1**) appear to depend on the blockade of Ca^{2+} entry and on the inhibition of Ca^{2+} release from norepinephrine-sensitive intracellular stores, and by cholinergic (muscarinic) stimulation and nitric oxide synthase activation in the vascular endothelium (115).

(*S*)(+)-Reticuline (**1**) has antiplatelet aggregation activity (116). It shows some antifungal activity (117), and is rather weakly antiplasmodial (118). It is also claimed to accelerate hair growth (119).

Reticuline, at 20 mg/kg, administered intraperitoneally, is significantly antinociceptive in the acetic acid-induced mouse writhing test, and quenches diphenylpicrylhydrazyl (DPPH) radicals with a scavenging concentration (SC_{50}) of 47 μg/mL (143 μM) (120). The latter antioxidant property could well be related to its effects on inflammation and pain.

Nothing is known about the pharmacology of *N*-oxycodamine (**2**) or, in fact, of other benzyltetrahydroisoquinoline *N*-oxides.

B. Bisbenzylisoquinoline

Isochondodendrine (**3**) was mentioned more than 50 years ago as a possible agent for the treatment for dysmenorrhea, but this lead does not seem to have been pursued (121,122). The only recent work found refers to the potent antiplasmodial activity of isochondodendrine (**3**) *in vitro* (IC_{50}=0.10 μg/mL) (123,124), which makes one wonder if *D. furfuracea* might be used to treat fever or, more specifically, malaria, in the area where it grows.

C. Berbinoids

Discretamine (**4**) is a potent α_1-adrenergic blocker, comparable in potency and basic pharmacology to the hypotensive drug phentolamine. It also blocks α_2-adrenoceptors and 5-HT_2 serotonin receptors, at several times higher concentrations, and seems to be devoid of action at acetylcholine, histamine, leukotriene, thromboxane, prostaglandin $F_{2\alpha}$, or angiotensin II receptors (125). Its action on α_1-adrenoceptor subtypes is selective for α_{1D} over α_{1A} and α_{1B} (126). Discretamine (**4**) antagonizes the contraction of human hyperplastic prostate tissue elicited by phenylephrine, electrical stimulation, or high Ca^{2+} (127). Its antiplatelet aggregation effect is another potential beneficial action of this alkaloid (128). Discretamine (**4**) is hypotensive in rats at doses between 0.01 and 10 mg/kg. A series of *in vitro* experiments suggests that the hypotensive effect of discretamine (**4**) is probably due to peripheral vasodilation related to nitric oxide release from the vascular endothelium (34).

Of all the berbine alkaloids recorded as *Duguetia* constituents, THP (**7**) is by far the most studied in relation to its pharmacology, probably because its (*S*)(−)-enantiomer (rotundine) and the racemic mixture are active constituents of the Asian drugs *Stephania rotunda* and *Corydalis racemosa*, respectively. As far back as 1970 (*S*)-THP, with the generic name ''gindarin,'' was evaluated for dermatological use in the treatment of neurodermatitis and alopecia areata, but this study does not seem to have progressed any further (129).

(±)-THP (**7**) is listed in the Chinese Pharmacopoeia as an analgesic with sedative–hypnotic effects. This alkaloid, together with its close analogs tetrahydroberberine and tetrahydrocoptisine, though apparently not tetrahydrojatrorrhizine (**5**), were shown to exhibit central depressant effects in mice and rats similar to those of the well-known neuroleptic chlorpromazine, leading to the suggestion that these berbines might represent ''a new type of tranquilizer'' (130). (±)-THP (**7**) was later

recognized as a dopamine, and, to a lesser extent, noradrenaline and serotonin depletor with an action similar to reserpine (131). In the former Soviet Union, the *S*-enantiomer, ''gindarin,'' was subjected to a preclinical study (in rats) in the framework of its possible use as a tranquilizer (or neuroleptic), and was found to be embryotoxic (132). (*S*)-THP (**7**) was subsequently shown to be a dopamine antagonist, while the *R*-isomer appears to be responsible for dopamine depletion (133,134), acting on both pre- and postsynaptic receptors (135). These dopaminergic actions probably explain the neuroleptic-like activity of both (*S*)- and (±)-THP (**7**). Radioligand displacement studies showed that (*S*)(−)-THP (**7**), but not its enantiomer, has affinity for D_2(-like) receptors (136). Subsequently, *in vivo* data were acquired showing that this alkaloid lacks agonistic effects (137). It has been shown recently that (*S*)(−)-THP (**7**) binds with high affinity (K_i=94 nM) to rat D_1 dopamine receptors, while a 3:1 mixture, in which the *R*-enantiomer predominates, has only micromolar affinity (138).

(±)-THP (**7**) decreases motor activity in rats, producing rigidity (or catalepsy?) at higher doses, apparently due to enhanced turnover of dopamine, although increased turnover is also observed for norepinephrine and, at higher doses, for serotonin (139). The antinociceptive action of (*S*)(−)-THP (**7**) is attributed to its D_2 antagonism in the striatum and nucleus accumbens, thus enhancing the activity of the brainstem descending pain modulation system (140–142). This effect might be reinforced by endogenous opioid release, as chronic administration of the alkaloid increased the Leu-enkephalin content in the rat striatum (143), and lesion of a predominantly β-endorphin pathway abolished the analgesic action of (*S*)-THP (**7**) (144). The hypotensive and heart rate-slowing effects of (±)-THP (**7**) have also been related to D_2 antagonism (145). Nevertheless, other mechanisms are clearly at work in the cardiovascular actions of this alkaloid, whether the *S* isomer or the racemic mixture. Calcium channel blockade and α_1 and α_2 adrenoceptor antagonism were first implicated in 1989 (146). (*S*)-THP (**7**) is also a subtype nonselective α-adrenoceptor antagonist (147). Experiments in rats demonstrated the protective effects of the *S*-enantiomer in experimental myocardial infarction, apparently related to its action on calcium channels (148,149). The first clinical results showing the effectiveness of (*S*)(−)-THP in patients with atrial fibrillation or paroxysmal tachyarrhythmias were published in 1993 (150,151). (±)-THP (**7**) is used for the treatment of pain, but reports have surfaced of severe cardiac and neurological toxic effects from abuse of this drug, and it has been suggested that these problems are also due to calcium channel blockade (152). Although the peripheral effects on calcium channels and adrenergic receptors are supported by later studies, there are strong indications that the cardiovascular effects of (±)-THP (**7**) are due, at least

in part, to hypothalamic dopamine antagonism and/or 5-HT_2 serotonergic agonism (153,154). The racemic mixture also induces hypothermia, which is attenuated by brain serotonin depletion or 5-HT_2 serotonergic receptor activation, again indicating a central serotonin antagonist action of the drug (155).

Pretreatment with (±)-THP (**7**) suppresses behavioral activation by picrotoxin (a noncompetitive $GABA_A$ receptor inhibitor) in rats, suggesting that this alkaloid might suppress epileptic seizures through inhibition of dopamine release (156). In this connection, the alkaloid was tested on the development of seizures in animals with electrically kindled amygdala, and found to be very effective as an antiepileptogenic and anticonvulsant agent in this model (157). It was subsequently shown that THP (**7**) is a positive allosteric modulator of $GABA_A$ receptors, thus sharing some of the pharmacological properties of the antiepileptic barbiturates and benzodiazepines (158). An independent study showed that orally administered (±)-THP (**7**) exhibits anxiolytic-like actions in mice, and that these effects are abolished by coadministration of a benzodiazepine antagonist, suggesting that THP interacts with the benzodiazepine site of the $GABA_A$ receptor (159).

In rats, (*S*)(−)-THP (**7**) inhibits methamphetamine- and cocaine-induced conditioned place preference, a preliminary test of possible antiaddictive activity in humans (160,161). Furthermore, it reduces cocaine self-administration and reinstatement, suggesting that it could also be useful in the treatment of cocaine addiction (162,163). Studies in rodents and in humans suggest that (*S*)(−)-THP (**7**) can ameliorate opioid drug craving and increase abstinence (164−165).

THP (**7**) is a weak inhibitor of the mitochondrial respiratory chain (166), and binds poorly to DNA (dissociation constants of the order of 10^{-4} M, with the *R*-enantiomer binding about twice as strongly as the *S*-enantiomer) (167). In line with these results, THP (**7**) and also xylopinine (**12**) are only weakly cytotoxic (168).

Other miscellaneous effects of THP (**7**) have been examined in relatively little detail. The racemic alkaloid produces significant decreases in thyroid function in hyperthyroid rats, apparently by inhibiting the release of thyrotropin-stimulating hormone (169). (±)-THP (**7**) attenuates several parameters related to neuronal damage caused by heatstroke in rats (170). (*S*)-THP (**7**) has several beneficial actions during acute cerebral ischemia-reperfusion in rats (171−174), and depresses the expression of adhesion molecules induced by lipopolysaccharides, suggesting that it might be useful in the treatment of inflammation (175). In this connection, and considering that free radicals are involved in inflammation, it should be pointed out that THP (**7**) exhibits antioxidative activity of similar potency to phenolic flavonoids in the lipid peroxidation and hemolysis assays (176). The racemic alkaloid

protects against carbon tetrachloride-induced liver damage in mice, which is also related to the formation of free radicals (177). THP (**7**) causes paralysis in the domestic fowl parasitic worm *Raillietina echinobothrida* at 1, 2, and 5 mg/mL, apparently related to disturbance of the nitric oxide signaling pathway (178).

The antiplasmodial activity of thaicanine (**14**) was demonstrated almost two decades ago, at low-to-submicromolar concentrations, against the chloroquine-sensitive *Plasmodium falciparum* D-2 strain and the resistant W-2 strain (120). Discretine (**10**) inhibits the growth of *P. falciparum* (chloroquine-resistant FcB1/Colombia strain) with IC_{50}= 1.6 μM, and is practically noncytotoxic against KB cells (179). THP (**7**) and xylopinine (**12**) are only weakly active against *P. falciparum* (IC_{50}=32 and 52 μM, respectively) (180).

D. Protoberberines

Jatrorrhizine (**15**), only isolated to date, in the Annonaceae, from *D. trunciflora*, is mentioned in a large number of pharmacological papers. Jatrorrhizine (**15**) lowers arterial blood pressure in normotensive dogs (181). It blocks α_1 and α_2 adrenergic receptors with moderate potency and exhibits some antihypertensive and heart rate-slowing activity in rats, although at higher concentrations these effects are reversed (182). Jatrorrhizine (**15**) inhibits both monoamine oxidase isoforms (MAO-A and MAO-B) of rat brain with IC_{50} values of 4 and 62 μM, respectively (183). It also inhibits rabbit platelet aggregation *in vitro* (184), and acetylcholinesterase inhibition by jatrorrhizine (**15**) has also been reported (185).

Antimicrobial activity of jatrorrhizine (**15**) against *Mycobacterium smegmatis* was demonstrated at concentrations of less than 100 μg/mL (184). It was recently tested against a panel of human dermatophytes and yeast-like fungi, exhibiting minimal inhibitory concentrations (MIC) between 62.5 and 125 μg/mL against *Epidermophyton*, *Trichophyton*, and *Microsporum* species, and 250 and 500 μg/mL against *Candida tropicalis* and *Candida albicans*, respectively; all better results than those obtained with berberine. However, it was inactive against *Scopulariopsis brevicaulis* (186). Bifonazole and fluconazole were used as positive controls, the former exhibiting MIC values above 100 μg/mL for all strains, but *Epidermophyton floccosum*, and the latter also, with the additional exception of *Trichophyton rubrum*. Tests against 20 strains of *Staphylococcus* (including 14 of *S. epidermidis*) and 20 strains of *Propionibacterium acnes*, and 20 *Candida* strains (including 17 of *C. albicans*) showed that the antibacterial potency of jatrorrhizine (**15**) is less than that of berberine, and that both alkaloids are inferior to commonly used antibacterial drugs. However, jatrorrhizine (**15**) may be a good lead for the

development of more effective antifungal agents than those in current use (187).

This alkaloid (**15** is active) *in vitro* against two different clones of *P. falciparum* with IC_{50} values of 0.422 and 1.607 μg/mL, potencies comparable to that of quinine, however, in an *in vivo* (mouse) screen against *Plasmodium berghei* it was inactive (188). Against the *P. falciparum* multidrug-resistant strain K1, it exhibited IC_{50}=3.15 μM, (corresponding to 1.1 μg/mL), and showed very modest activity against *Entamoeba histolytica* (189). In cultures of *Babesia gibsoni*, an important parasite in dogs and a member of a genus causing babesiosis in other carnivores, ruminants, and horses, jatrorrhizine (**15**) inhibited growth at low-to-moderate concentrations (190). Dehydrodiscretine (**16**) inhibits the growth of *P. falciparum* with IC_{50}=0.64 μM (multidrug-resistant K1 strain) (189), and 0.9 μM (chloroquine-resistant FcB1/Colombia strain) (179).

Jatrorrhizine (**15**) and dehydrodiscretine (**16**) have negligible cytotoxicity against KB cells (179,189). The interaction of jatrorrhizine (**15**) with DNA resembles that of ethidium bromide, the classical DNA intercalator (191). Binding to calf thymus DNA reveals two different binding sites with dissociation constants of about 25 and 35 μM (192). Binding to the double-stranded oligodeoxynucleotide *d* $(AAGAATTCTT)_2$ shows both 1:1 and 1:2 stoichiometries, with similar affinity to that of palmatine, and greater than those of coptisine or berberine (absolute values were not determined) (193). Similar studies with different sequences indicated that the affinity of jatrorrhizine (**15**) was reduced for *d*$(AAGGATCCTT)_2$ and *d*$(AAGCATGCTT)_2$ relative to the other protoberberine alkaloids tested (194). Finally, using competitive ethidium bromide displacement experiments on calf thymus DNA and synthetic double-stranded polynucleotides, the higher affinity of jatrorrhizine (**15**) relative to palmatine and berberine and their preference for AT-rich DNA were confirmed (195). In an eukaryotic test model (*Euglena gracilis* vs. the direct-acting mutagen acridine orange), jatrorrhizine (**15**) exhibited weak antimutagenic activity (196).

Jatrorrhizine (**15**) was shown to be a weak scavenger of DPPH radicals, and a modest inhibitor of lipid peroxidation in unilamellar dioleyl-phosphatidylcholine liposomes (197). It downregulates tumor necrosis factor alpha (TNFα) and E-selectin expression, and decreases the content of thromboxane B(2) in rat intestinal microvascular endothelial cells, suggesting that it might reduce inflammatory response by affecting cytokines and autacoids (198,199), rather than by virtue of its poor antioxidant properties.

Single doses of 50 and 100 mg/kg jatrorrhizine (**15**) decreased blood glucose in normal and alloxan-diabetic mice and increased succinate dehydrogenase activity in the liver, however, it had no effect on blood

lactic acid or liver lactate dehydrogenase. The alkaloid also decreased liver glycogen in normal mice, suggesting that its hypoglycemic activity can be attributed to increased aerobic glycolysis (184). Several methods have been used to study the binding of jatrorrhizine (**15**) to human serum albumin, concluding that the protein's secondary structure is altered and hydrophobic and electrostatic interactions play a major role (200).

Pseudopalmatine (**17**) does not seem to have been studied pharmacologically.

E. Glaziovine (19)

In the early 1970s the pharmacology of glaziovine (**19**) was explored by an Italian pharmaceutical company that registered it as a tranquilizer under the trademark Suavedol®. Its psychopharmacology was compared with that of diazepam in a double-blind clinical trial (201), and its human pharmacokinetic parameters were studied (202). In addition, it was reported to possess anti-gastric ulcer properties in rodents and in humans (203,204). No studies appear to have addressed its mechanisms of action as either an anxiolytic or antiulcerogenic agent.

More recently, glaziovine (**19**) was evaluated for anti-hepatitis B virus activity. This alkaloid proved to be highly potent, as judged by its IC_{50} value of 8 μM, as an inhibitor of HBV surface antigen production. The corresponding value for the positive control, the anti-HBV drug 3TC or Lamivudine, was 11.7 mM. However, glaziovine (**19**) was more toxic to uninfected that to infected cells (205).

The isolated yield of glaziovine (**19**) from *D. vallicola* leaves was 0.27%, placing this abundant and easily accessible material in a good position as a source of a useful plant drug (26). Glaziovine (**19**) is one of 60 alkaloids listed as having particular pharmaceutical and biological significance (206).

F. Aporphines

Anonaine (**3**) relaxes rat aorta and tail artery precontracted with noradrenaline, predominantly through adrenergic receptors. Since its affinity for L-type Ca^{2+} channels is an order of magnitude less for α_1 adrenoceptors in rat cerebral cortical membranes, it does not contribute to intracellular mobilization of Ca^{2+}, and its effect on phosphodiesterases is negligible. It is also slightly selective for α_{1A} and α_{1D} adrenoreceptors relative to the α_{1B} subtype, as determined by radioligand competition experiments (207,208).

Xylopine (**28**) is a selective α_1 (vs. α_2) adrenergic receptor antagonist with submicromolar functional potency (209). In the rabbit oviduct, isocorydine (**41**) inhibits spontaneous and noradrenaline-elicited

contractions, indicating that this alkaloid is an adrenoceptor antagonist (210). A further study in a rat aorta model suggested that the effect is mediated primarily through α_1 adrenoceptors (211). The effects of isocorydine (**41**) on the action potentials of canine heart muscle cells have also been studied *in vitro* (212).

Asimilobine (**20**) inhibits rabbit aortal contractions induced by 10^{-6} M serotonin with pA_2=5.78, suggesting that this alkaloid is a 5-HT_2 serotonin receptor antagonist (213). Dicentrine (**39**) inhibits the contraction of rat stomach muscle strips induced by serotonin, histamine, K^+, and Ca^{2+} in a noncompetitive manner. In the case of serotonin-induced contractions, the relaxation depends on Ca^{2+} release from intracellular stores, suggesting that 5-HT (presumably 5-HT_{2B}) receptors are involved (214). Asimilobine (**20**), nornuciferine (**22**), and anonaine (**23**) bind to 5-HT_{1A} serotonin receptors with low micromolar IC_{50} values versus [^{3}H] rauwolscine, and were shown to be full agonists (215). In [^{3}H]8-hydroxy-2-(di-*N*-propylamino)tetralin displacement experiments, *N*-methyllaurotetanine (**37**) exhibits high affinity for 5-HT_{1A} receptors (K_i=85 nM, pK_i=7.07) (216).

Isoboldine (**38**) relaxes isolated guinea pig trachea with IC_{50}=710 μM, suggesting a β-adrenoceptor-mediated mechanism (217). Dicentrine (**39**) has been extensively studied as a cardiovascular agent. It was first shown to be a potent α_1-adrenoceptor antagonist (less potent than prazosin, and more potent than phentolamine) with little effect on β-adrenergic receptors (218,219). Its hypotensive effect was demonstrated *in vivo* in rats by the intravenous and oral routes, and in conscious, spontaneously hypertensive animals, oral administration of 5 and 8 mg/kg caused hypotension lasting more than 15 h (220). In rats fed a high-cholesterol diet, oral administration of dicentrine (**39**) decreased the mean arterial pressure (more so in spontaneously hypertensive animals), and reduced the total plasma cholesterol by reducing the low-density lipoprotein fraction, and the total plasma triglyceride by reducing the very low-density lipoprotein fraction (221).

Experiments in isolated cardiac cells and in rabbit heart showed that dicentrine (**39**) blocks sodium and potassium currents, and is a potentially useful antiarrhythmic agent at doses in the same range as quinidine (222,223). The effects of dicentrine (**39**) on the mechanical properties of systemic arterial trees have been studied in dogs (224). Dicentrine (**39**) inhibits serum-stimulated kidney mesangial cell proliferation in the rat, and was therefore viewed, together with other vasodilators, as an agent with the potential to delay the progression of chronic glomerulopathy (225). As an α_1-adrenoceptor antagonist it also inhibits contractions of human hyperplastic prostate elicited by adrenergic stimulation, and might therefore be of use to relieve bladder outlet obstruction in patients with benign prostatic hyperplasia (226).

Anonaine (**23**) and isopiline (**24**) inhibit dopamine uptake by rat striatal synaptosomes with IC_{50}=0.8 and 2.5 μM, respectively. Anonaine (**23**) is a selective uptake inhibitor relative to its affinities for D_1-like and D_2-like dopamine receptors as determined in radioligand displacement experiments (IC_{50} vs. [^{3}H]SCH23390 and [^{3}H]raclopride: 68 and 19 μM, respectively; ratios of uptake to receptor binding IC_{50} values: 85.0 and 23.5), while isopiline (**24**) exhibits much lower selectivity (D_1-like and D_2-like binding IC_{50}: 10 and 34 μM, respectively; IC_{50} ratios 3.0 and 13.6) (227).

Asimilobine (**20**), in the 0.05−0.2 μM range, reduces intracellular dopamine in PC12 cells for 24 h with IC_{50}=0.13 μM. At this concentration it decreases the activities of tyrosine hydroxylase (TH, by 73.2% and for a longer time) and aromatic L-amino acid decarboxylase, and reduces TH mRNA and intracellular cAMP levels. Alone, it does not alter PC12 cell viability at concentrations up to 5 μM. However, in association with L-DOPA asimilobine (**20**) inhibits the L-DOPA-induced increase in dopamine levels and enhances L-DOPA cytotoxicity (228).

N-Methylasimilobine (**21**) is a significant inhibitor of platelet aggregation elicited by collagen, arachidonic acid (AA), and platelet-activating factor (PAF). Xylopine (**28**) and *N*-methyllaurotetanine (**37**) inhibit platelet aggregation with different potencies depending on the substance used as an aggregation inducer in each case (229). Dicentrine (**39**) also inhibits platelet aggregation induced by AA, collagen, adenosine diphosphate (ADP), PAF, thrombin, or the synthetic U46619, and induces ATP release from platelets. Additional experiments indicated that these effects are due to the inhibition of thromboxane B2 formation and increased cAMP levels (218,230,231).

N-Methyllaurotetanine (**37**), administered intravenously, is antihyperglycemic in normal and streptozotocin-induced diabetic rats (232). *N*-Methyllaurotetanine (**37**) and norisocorydine (**40**), at 20 mg/kg i.p., are significantly antinociceptive in the acetic acid-induced mouse writhing test, and quench DPPH radicals with SC_{50}=28 and 14 μg/mL (82 and 43 μM), respectively (25,120). Antinociceptive activity is often associated with free radical inactivation, and in this regard it should be mentioned that anonaine (**23**) was one of the first aporphine alkaloids for which antioxidative activity was demonstrated (233).

Anonaine (**23**) reduces the viability of normal rat hepatocytes, and HepG2 and HeLa tumor cells, with IC_{50} values of 70.3, 33.5, and 24.8 μg/mL, respectively, in 24-h experiments (234). Non-cancer Vero and MDCK cells exposed to 100 μM anonaine (**23**) for 24 h experienced reduced viability by about 25% and 5%, respectively (235). In the case of HeLa cells, the decrease amounted to 77%, and was associated with DNA damage and a dose-related block of the cell cycle before the G_1 phase. These effects were correlated to increased intracellular nitric oxide,

reactive oxygen species, glutathione depletion, disruption of the mitochondrial transmembrane potential, activation of caspases 3, 7, 8, and 9, and poly(ADP-ribose) polymerase (PARP) cleavage with up-regulation of Bax and p53 proteins (235).

Dicentrine (**39**) inhibits the growth of murine leukemia P388 and L1210, melanoma B16, bladder cancer MBC2, and colon cancer Colon 26 cells in culture, and also reduces mitogen-induced lymphocyte proliferation and the growth of IL-dependent CTLL2 cells (236). It slows the growth of the human hepatoma cell line HuH-7 and decreases the efficiency of colony formation by these cells and the MS-G2 line, and strongly inhibits DNA and RNA synthesis. Additional evaluations in 21 tumor cell lines showed that dicentrine (**39**) was particularly cytotoxic to esophageal carcinoma HCE-6, lymphoma Molt-4 and CESS, leukemia HL60 and K562, and hepatoma MS-G2 (237). This alkaloid is active in a DNA unwinding assay, and is a modest inhibitor of topoisomerase II (IC_{50}=27 μM) (238). However, it shows no antiproliferative activity versus several yeast strains (239). Duguetine (**76**) ''caused considerable antitumoral activity'' (240).

An extract of *D. odorata* was found to inhibit the G_2 DNA damage checkpoint, a target that is expected to enhance the effectiveness of DNA-damaging anticancer therapy. Dehydrodiscretine (**16**), pseudopalmatine (**17**), oliveroline (**60**), and *N*-methylguatterine (**66**), were isolated by bioassay-guided fractionation following this bioactivity, however, only oliveroline (**60**) had confirmed, though modest, potency (at concentrations above 10 μM), and was isolated in sufficient amounts for additional testing (14).

Pachystaudine (**82**) interferes with the replicative cycle of herpes simplex virus type 1 (HSV-1) (241).

Anonaine (**23**) and xylopine (**28**) are weakly antibacterial and antifungal (120,242,243), and anolobine (**27**) is only active against Gram-positive bacteria and *Mycobacterium phlei* in the 10^{-4} molar range with MIC_{90}=12−50 and 6−25 μg/mL, respectively (243). Anolobine (**27**) induces chromosomal aberrations in a Chinese hamster lung cell line at concentrations as low as 2.5 μg/mL (244). At 300 μg/mL, dicentrine (**39**) showed ''moderate'' to ''good'' activity against the fungi *Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *E. floccosum*, but was inactive against *C. albicans*, *Aspergillus niger*, and *Penicillium* sp. (245).

Nornuciferine (**22**) and xylopine (**28**) are significantly active against *Leishmania mexicana* and *Leishmania panamensis*, with the latter alkaloid showing LD_{50}=3 μM, vs. *L. mexicana*, and 37-fold higher toxicity towards the parasite than towards the host cells, the macrophages (246). Dicentrine (**39**) is active against *Trypanosoma brucei brucei in vitro* with IC_{50}=3.15 μM (247). Duguetine (**76**) is moderately active against the trypomastigote form of *Trypanosoma cruzi* (IC_{50}=9.32 μM) (120).

Asimilobine (**20**), anonaine (**23**), xylopine (**28**), isolaureline (**29**), and dicentrine (**39**) are antiplasmodial at low-to-micromolar concentrations against the chloroquine-sensitive *P. falciparum* D-2 strain and the resistant W-2 strain, but under the same conditions chloroquine has 1.3 and 11.2 nM ED_{50} values against the sensitive and the resistant strains, respectively (248). Isocorydine (**41**) is moderately active *in vitro* against *P. falciparum*, with IC_{50}=37 μM, and practically noncytotoxic and inactive against *E. histolytica* (193). Oliveroline is active against *P. falciparum* at low micromolar concentrations (27).

Dicentrine (**39**) reduces the motility of *Haemonchus contortus* larvae (the large stomach worm of ruminants), with EC_{90}=6.3 μg/mL, and an oral dose of 25 mg/kg in mice reduced the worm count by 67% (249).

G. Oxoaporphines

Atherospermidine (**86**) relaxes uterine contractions induced by high K^+ and by oxytocin, with a mechanism involving Ca^{2+} entry and release from intracellular stores (250). Liriodenine (**83**), in the 10^{-7}–10^{-4} M range, relaxes rat aorta contracted with potassium chloride or norepinephrine, but in Ca^{2+}-free medium it does not inhibit the response elicited by caffeine, indicating that its vasorelaxant action is mediated by interaction with α_1 adrenergic receptors and voltage-operated calcium channels (251). Dicentrinone (**91**) was also shown to possess weak vasorelaxant activity (252). Liriodenine (**83**) appears to regulate dopamine biosynthesis in the 5–10 μM range by reducing TH gene expression and activity, and is protective against L-DOPA-induced cytotoxicity in PC12 cells (253).

At 100 μM liriodenine (**83**) inhibits platelet aggregation, particularly that elicited by ADP or collagen, and less by AA or PAF, with aggregation falling to 5.4%, 5.3%, 40.5%, and 84.1% of controls, respectively (229,252). Lanuginosine (**87**) shows similar activity to liriodenine (**83**) (254).

Liriodenine (**83**) is cytotoxic to KB, A-549, HCT-8, and L-1210 tumor cells (255,256). It is also a mutagen for *Salmonella typhimurium* TA100 (257). Chromosomal aberrations are induced by liriodenine (**83**) at 5 μg/mL (244). Liriodenine (**83**) is selectively toxic against DNA repair- and recombination-deficient yeast mutants (IC_{12}=16.7 μg/mL vs. the rad 52 mutant), a model in which lysicamine (**84**) and *O*-methylmoschatoline (**85**) are inactive. The selectivity of liriodenine (**83**) suggested that its activity might be mediated by topoisomerase inhibition (258). Topoisomerase II inhibition by liriodenine (**83**) was confirmed in CV-1 cells infected with simian virus 40 (SV40), and it was also shown that this alkaloid is not a substrate for the verapamil-sensitive drug efflux pump (a mechanism underlying drug resistance) in CV-1 cells (248). Liriodenine

(**83**) exhibits moderate antiproliferative activity versus the human breast cancer cell lines MCF-7, the doxorubicin-resistant MCF-7/ADR, and the estrogen receptor-deficient MDA-MB435 and MT-1 lines, with IC_{50}=15.6, 16.7, 16.4, and 18.2 μM, respectively (259). In another study versus MCF-7, NCI-H460, and SF-268 cell lines, IC_{50} values of 3.19, 2.38, and 2.19 μg/mL, respectively, were recorded (260). It should be pointed out that 3.19 μg/mL corresponds to 11.6 μM, in good agreement with the earlier value. In A594 human lung cancer cells, liriodenine suppresses proliferation dose- and time-dependent in the 2−20 μM range, mainly through cell cycle inhibition (G_2/M arrest) and induction of apoptosis (261). Human hepatoma cell lines bearing the wild-type p53 oncogene (Hep G2 and SK-Hep-1) have also been challenged with liriodenine (**83**), which induced cell cycle arrest in the G_1 phase and inhibited DNA synthesis, increasing the expression of p53 and inducible nitric oxide synthase, and the intracellular NO level (262).

Lysicamine (**84**) is a modest inhibitor of the proliferation of two human liver cancer cell lines (Hep G2 and Hep 2,2,15) with IC_{50}=8.4 and 3.4 μg/mL, respectively (56). Dicentrinone (**91**) showed selective antiproliferative activity against some yeast strains, but not others. When tested against recombinant human topoisomerase I it only inhibited the enzyme to a small extent, stabilizing the enzyme−DNA binary complex (239).

Apparently, the earliest recorded biological activities of liriodenine (**83**) are antibacterial and antifungal, which it shares with lysicamine (**84**) (243,263,264). When mice infected with a lethal dose of *C. albicans* were treated with liriodenine (and also its methiodide), the proliferation of the pathogen was reduced significantly (265). The moderate activity of liriodenine (**83**) and *O*-methylmoschatoline (**85**) was demonstrated again more recently against several different fungi and bacteria (266,267).

Liriodenine (**83**) was claimed to be a fairly potent growth inhibitor of *Leishmania major* and *Leishmania donovani*, showing inhibition at 3.12 μg/mL (11.3 μM) (268), although another group reported IC_{50}=26.16 μM for a possibly different strain of *L. donovani* (269). A more recent study using *Leishmania brasiliensis* and *Leishmania guyanensis* promastigotes gave IC_{50}=58.5 and 21.5 μM, respectively, with *O*-methylmoschatoline (**85**) being about five times less active (270). Lysicamine (**84**) is also active against *L. mexicana* (245). Dicentrinone (**91**) is reported to have unusually potent leishmanicidal activity (IC_{50}=0.01 μM) (240). *O*-Methylmoschatoline inhibits the growth of *Trypanosoma brucei* at 6.25 μg/mL (268). Liriodenine (**83**) is active against *P. falciparum* with IC_{50}=15 μM (269,271).

H. Aminoethylphenanthrenes

Atherosperminine (**94**) produces behavioral stereotypy, increased spontaneous motor activity and amphetamine toxicity, reversal of

haloperidol-induced catalepsy, inhibition of conditioned avoidance response, inhibition of morphine analgesia, and potentiation of the anticonvulsant action of diphenylhydantoin, effects associated with dopamine receptor stimulation (272). It also inhibits the contraction of guinea pig trachealis muscle elicited by carbachol, prostaglandin $F_{2\alpha}$, a synthetic thromboxane analogue and leukotriene C4, it potentiates tracheal relaxation and cAMP accumulation elicited by forskolin and, at higher concentrations, by itself raises the content of cAMP, but not cGMP, in the tissue. Thus, its major mechanism of action seems to be the inhibition of cAMP phosphodiesterase (273).

At 100 μg/mL, atherosperminine (**94**) and its *N*-methyl quaternary salt completely inhibited platelet aggregation elicited by ADP, AA, collagen, or PAF, while atherosperminine *N*-oxide (**95**), though inhibiting AA- and collagen-induced aggregation, is less effective against aggregation elicited by ADP or PAF. At this dose, atherosperminine (**94**) and its *N*-oxide are also complete antagonists of high potassium or norepinephrine-induced contractions of rat thoracic aorta, pointing to simultaneous α_1-adrenoceptor and calcium channel inhibition (274).

I. Copyrine Alkaloids

Sampangine (**97**) potently inhibits HL-60 human leukemia cell proliferation by 50% at IC_{50}=2.65 μM, and its (lethal) DC_{50} value is 24.5 μM, suggesting that apoptosis plays a role in the cytotoxicity of this alkaloid, as confirmed by its effect at 20 μM on caspase-3 activity. At 4.0 μM sampangine induces cell cycle arrest in the G_0/G_1 phase, and at 20 μM leads to accumulation of cells with decreased DNA, typical of apoptotic cells. Low and high concentrations of sampangine (**97**) caused opposite effects on the potential of mitochondrial membranes, leading first to hyperpolarization (275). Treatment of HL-60 cells with sampangine (**97**) induced the rapid formation of reactive oxygen species, and quenching these with antioxidants abolished the pro-apoptotic activity of the alkaloid, indicating that sampangine-induced oxidative stress plays a key role in DNA damage (276). Sampangine (**97**) strongly inhibits the proliferation of human malignant melanoma cells (SK-MEL) with IC_{50}=0.37 μg/mL but, as observed previously in the HL-60 model, it is at least ten times less potent than other human cancer cells in culture (KB, BT-549, and SK-OV-3) (16).

Hadranthine A (**99**) was practically inactive against the human cancer cells tested, but hadranthine B (**100**) inhibited the proliferation of SK-MEL, KB, BT-549, and SK-OV-3 cells with IC_{50}=3.0, 6.4, 6.6, and 3.6 μg/mL, respectively. Imbiline-1 (**101**) inhibited SK-MEL and SK-OV-3 cells with IC_{50}=2.0 and 5.0 μg/mL, respectively, but showed IC_{50} values greater than 10 μg/mL in the other cell lines (16).

Sampangine (**97**) and 3-methoxysampangine (**98**) exhibit antifungal and antimycobacterial potencies about one half of those of amphotericin B and rifampicin, with MIC in the 0.78−1.56 μg/mL range against *C. albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Mycobacterium intracellulare*, somewhat higher than the data published previously by these authors for the 3-methoxy derivative (277,278). In *Saccharomyces cerevisiae*, sampangine (**97**) induces oxidative stress, and its antifungal activity is at least partially due to alterations in heme metabolism (279).

Sampangine (**97**), 3-methoxysampangine (**98**), hadranthine A (**99**), and imbiline-1 (**101**), but not hadranthine B (**100**), exhibit antiplasmodial activity *in vitro* against *P. falciparum* (chloroquine-resistant clone W-2 and chloroquine-sensitive clone D-6). Although about ten times less potent than chloroquine against the D-6 clone, hadranthine A (**99**) shows reasonably good selectivity (selectivity index >40) versus Vero cells, while the other alkaloids are even less potent and less selective. On the other hand, sampangine (**97**) and 3-methoxysampangine (**98**) are more potent than chloroquine against the chloroquine-resistant W-2 clone (16).

J. 1-Aza-9,10-anthraquinones

Cleistopholine (**104**) inhibits the proliferation of Hep G2 and Hep 2,2,15 human hepatocarcinoma cell lines, with IC_{50}=0.22 and 0.54 μg/mL, respectively (56). It has modest antifungal and antimycobacterial activities with MIC against *C. albicans*, *C. neoformans*, *A. fumigatus*, and *M. intracellulare* of 12.5, 1.56, 100, and 12.5 μg/mL, respectively (277), and has also shown activity against mutant *S. cerevisiae* strains, *Cladosporium cladosporioides*, and *Cladosporium sphaerospermum* (54). Cleistopholine (**104**) inhibits the growth of *P. falciparum* at low micromolar concentrations (27).

VII. CONCLUDING REMARKS

The foregoing sections illustrate a cyclic trend that has been developing for a long time in natural products research, but which seems to take on specific features in studies on plant families that are traditionally seen as rich sources of alkaloids. In its initial century, from the isolation of morphine and quinine through mescaline, alkaloid chemistry was largely motivated by the desire to understand and to better apply the medicinal or biological properties of plant drugs. Later on, rapid advances in structure elucidation methodology and instrumentation led to an approach akin to the mountaineer's ''Why climb it? Because it's there!,'' while biosynthetic work remained more concerned with a quest for explanations. Over the last few decades a renewed interest in practical uses fired the development of bioassay-guided fractionation and a

preference for biosynthetic studies related to commercially or medically important alkaloids. In the meantime, organic and medicinal chemists developed synthetic methodology, and used alkaloid structural templates to generate new drugs, and fruitful collaborative efforts continue, both in the pharmaceutical industry and in academia.

In the specific case of *Duguetia*, the identification of known alkaloids and the discovery of new structures have slowed considerably, while the pharmacology of some of the more widespread constituents has made surprising progress. But something seems to be lacking. It is most likely that there is an enormous wealth of ethnopharmacological knowledge risking oblivion and still waiting to be recorded. If alkaloid chemistry is to contribute to our understanding of the biology of the genus, it needs to address a wider range of species, particularly those belonging to unexplored or little-explored sections, and metabolic profiling should be applied to many of the plants that have already been studied as well as those that have not. Bioassay-guided fractionation has yielded some spectacular results, but what bioassays should be used? Easy antibacterial assays (unlike antifungal or antiparasitic assays) do not seem to have uncovered anything of interest in higher plants, and natural products chemists are not usually qualified to identify apparently arcane biological targets such as some of those now pursued by the pharmaceutical industry, or to set up the necessary tests, stressing the need to collaborate with pharmacologists. Although much is known about the pharmacology of some *Duguetia* alkaloids commonly found in other plants, the more characteristic alkaloids such as the 7-oxygenated and the 9,11-dioxygenated aporphinoids remain practically untouched. And what about structural modification or analog synthesis?

It is hoped that these comments will stimulate discussion in the alkaloid chemical community and invigorate research, leading to both qualitative and quantitative leaps in productivity, and to novel approaches that will surely have unsuspected, but doubtless very valuable, results.

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CHAPTER 4

Galanthamine from *Galanthus* and Other Amaryllidaceae — Chemistry and Biology Based on Traditional Use

Michael Heinrich*

Contents

I. KNOWLEDGE-DRIVEN DRUG DISCOVERY

Developing new medicines or drug leads from plants, animals, and fungi is based (in many cases unknowingly) on local and traditional knowledge about a species' medical use or toxicological effects. Many scientists have seen this to be one of the key driving forces of ethnobotanical research, even though such research has many other, equally important, strategic goals (1). Some years ago, Corson and Crews (2) provided convincing arguments for knowledge-based drug development programs with the goal of achieving clinical development. Such ''poster children'' of ethnopharmacology-driven drug developments programs

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also include some extracts, which are complex mixtures of active and inactive constituents (3), but more commonly focus on pure chemical entities.

For chemists, it sometimes comes as a surprise that in the context of ethnobotany and ethnopharmacology, great emphasis is put on the traditional holders of knowledge, i.e., on the knowledge and rights of those who provided the original ideas for developing such new leads. For anyone involved in collaborative projects this is an ethical and moral obligation, and part of one's professional role. Ethnobotanists see themselves as scientists who in all ways possible respect the indigenous cultures that host them, and help them in their endeavors to safeguard and further develop their traditional knowledge. The aspects of ethnobotanical research highlighted above are exemplified in the discovery of galanthamine as an Alzheimer's medication.

II. GALANTHAMINE – THE HISTORY OF ITS DEVELOPMENT[1]

Galanthamine (galantamine, **1**) is an alkaloid known from several members of the amaryllis family (Amaryllidaceae), and the idea for developing a medical product from these species seems to be based on the local use of one of these species in a remote part of Europe (4) (ethnobotanical information). Today, galanthamine, especially under its brand names Reminyl® and Nivalin®, is commonly used in the treatment of Alzheimer's disease (AD). This example highlights both the uncertainties of drug development and the problems of linking information about local and traditional uses with a compound's development. Around 2000, the author became interested in the history of the drug, and more broadly the development of galanthamine (1) into a widely used Alzheimer's drug, which can be divided into three main periods:

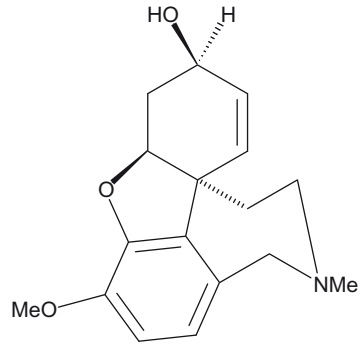
- The early development in Eastern Europe for use in the treatment of poliomyelitis.
- The preclinical development in the 1980s into an Alzheimer's medication.
- The clinical development in the 1990s and the development of a commercially viable full chemical synthesis.

In the context of this review, the first phase is of particular relevance. The early development in Eastern Europe for use in the treatment of poliomyelitis started with the isolation of the alkaloid from snowdrop (*Galanthus* spp., most notably *Galanthus woronowii* Losinsk.). Today, the alkaloid is obtained from other members of the same plant family,

[1] An earlier version of this section was part of an article by Heinrich and Teoh (4)

including daffodil (*Narcissus* spp.) and snowflake (*Leucojum* spp., esp. *Leucojum aestivum* L.), and most importantly synthetically.

Galanthus species are native to many parts of Europe, including Bulgaria, the eastern parts of Turkey, and the Caucasus mountain range. Overall, little is known about the local and traditional uses of this genus in Europe. Plaitakis and Duvoisin (5) hypothesize that Homer's "moly" might have been the snowdrop, *Galanthus nivalis* L. In his epic poem, the Odyssey he described "moly," and its use by Odysseus as an antidote against Circe's poisonous drugs. Thus this description of "moly" as an antidote in Homer's Odyssey may represent the oldest recorded use of *Galanthus*, but the evidence is scanty. The "classical" medico-botanical texts of the 16th century (i.e., Fuchs, Bock, and Brunfels) do not mention snowdrop (*G. nivalis*) and make only cursory reference to *Leucojum*. Interestingly, the German pharmacognosist Madaus (6) does not mention *Galanthus* or *Leucojum*, and only discusses *Narcissus pseudonarcissus* L., giving some isolated uses, which have no direct association with the central nervous system (CNS), while Marzell (7) does not discuss any of these three genera. Additionally, in Koehler's "Arzneipflanzen" (Pabst, 8), practically no medical use is given for species of the three genera. Thus it is certain, that *Galanthus* and other genera of the Amaryllidaceae were not particularly commonly used European medicines. On the other hand, this clearly does not exclude local and traditional uses in rural regions of Europe and Asia.



1 Galanthamine

According to unconfirmed reports, in the 1950s, a Bulgarian pharmacologist noticed the use of the common snowdrop growing in the wild by people who were rubbing it on their foreheads to ease nerve pain. Also, some of the earlier publications indicate the extensive use of snowdrop in Eastern Europe, such as Romania, Ukraine, the Balkan Peninsula, and in Eastern Mediterranean countries. However, we were unable to locate any relevant ethnobotanical literature. In the first

pharmacological publication on galanthamine (**1**), no reference is made by Russian authors (9) to the traditional use of snowdrop in the Caucasian region. An interesting note comes from the English pharmacognosist E. J. Shellard (10) which was published as a ''Letter to the Editor'' of the Pharmaceutical Journal in the United Kingdom. He recalls a presentation in 1965 by ''a Russian pharmacognosist reporting on peasant women living at the foot of the Caucasian mountains (Southern Russia, Georgia) who, when their young children developed symptoms of an illness which, as he described them, was obviously poliomyelitis, they gave them a decoction of the bulbs of the Caucasian snowdrop (*G. woronowii* Los) [*sic*] and the children completely recovered without showing any signs of paralysis'' (10). This is one of the few, second-hand reports currently available recording the use of snowdrop prior to the development of galanthamine as a licensed medicine (see Table I).

Systematic exploration by the author with colleagues from Central Europe and Russia resulted in one additional, but still second-hand report. According to Dr. Teodora Ivanova from the Bulgarian Academy of Sciences (personal communication, 2008), an alcoholic extract of *L. aestivum* L. was used by her grandparents and other older people in Eastern parts of Bulgaria. The plant is alleged to be useful in the prevention or treatment of memory loss, but since this record postdates the introduction of galanthamine as an Alzheimer medication onto the worldwide market, one has to wonder whether this report may not actually be a secondary outcome of the species' use to extract galanthamine for clinical use. However, it is at this point one of the few well-documented local and traditional uses of species, which contains galanthamine and derivatives, and is clearly linked to the modern use.

Most of the early investigations on galanthamine were conducted in Bulgaria and the USSR during the coldest period of the Cold War. In the early 1950s, the Russian pharmacologist Mashkovsky worked with galanthamine isolated from the *G. woronowii*. In 1951, Mashkovsky and Kruglikova–Lvova used an *ex vivo* system (rat smooth muscle) to prove its acetylcholinesterase (AChE) inhibiting properties. *Consequently, this is the first published work that establishes the AChE-inhibiting properties of galanthamine.* In the following year (1952), Proskurnina and Yakovleva established and published the chemical structure of galanthamine as an alkaloid with a tertiary nitrogen atom, based on material isolated from *G. woronowii.* In addition, the physicochemical characteristics of the alkaloid were determined (11). In 1955, Mashkovsky published a second paper on the cholinesterase-inhibiting properties of galanthamine. Unfortunately, he did not indicate the source of the galanthamine used, but most probably the studies were with galanthamine isolated from *G. woronowii*. In 1956, in Sofia, the Bulgarian pharmacologist D. Paskov discovered galanthamine in the European daffodil and in the common

Table I Historical development of galanthamine (**1**) as a clinically used drug (adapted from ref. 4)

Year	Development step of galanthamine
Early 1950s	A Russian pharmacologist discovers that local villagers living at the foot of the Ural mountains use the wild Caucasian snowdrop to treat (what he considers to be) poliomyelitis in children.
1952	Galanthamine was first isolated from *Galanthus woronowii*.
1956	D. Paskov suggested galanthamine can be extracted from the leaves of the *Galanthus*.
Late 1950s	Various preclinical studies on the pharmacology of galanthamine were carried out. For instance: • Galanthamine was found to have antagonistic effects against non-depolarizing neuromuscular blocking agents. This was shown in experiments on neuromuscular preparation of cats *in situ*, in experiments *in vitro* on frog rectus abdominis muscle, etc. • In *vivo* and *in vitro* experiments were performed in rats to determine the effects of galanthamine on the brain. Galanthamine was registered as a medicine under the trade name "Nivalin," and becomes commercially available in Bulgaria.
Early 1960s	The first data on the anticholinesterase activity of galanthamine was reported from an *in vivo* study in an anesthetized cat.
1980s	Preclinical development: Researchers searching for novel treatments of Alzheimer's disease start investigating the therapeutic effects of galanthamine.
1990s	Clinical development of galanthamine into a medication for Alzheimer's disease.
1996	Sanochemia Pharmazeutika obtained the first patent on a synthetic process for galanthamine.
1997	Sanochemia began collaboration with a Belgium-based company (Janssen Pharmaceutica) and an emerging British Company (Shire Pharmaceuticals Group plc).
2000	Galanthamine was licensed in the first countries (Iceland, Ireland, Sweden, and the UK) for the treatment of Alzheimer's disease.
Currently	Galanthamine has been approved for use in the United States, many European countries, and many Asian countries. Controversies remain over the therapeutic benefits of ACE inhibitors, since they "only" delay the onset of more severe symptoms and offer no curative treatment.

snowdrop, *G. nivalis*. Hence, he suggested extracting galanthamine from the leaves of *G. nivalis*. In 1957, this scientist, who was trained in Russia under Mashkovsky, published his results from the study of *L. aestivum* (summer snowflake) and its content of galanthamine, which was to become the main source of the alkaloid. In 1960, a full chemical synthesis was published. This was a biomimetic laboratory process with a yield of below 1%, and had been designed as a proof of structure, not for industrial production (4). New derivatives are continuously isolated highlighting the structural diversity in this class of alkaloids (12).

The indication poliomyelitis, which was the main indication in the Eastern and Central European nations from 1950 until a few years ago, came as a result of data that galanthamine enhances nerve impulse transmission at the synapses. In the form of the hydrobromide salt, it became commercially available as a registered product under the trade name "Nivalin®." Further, galanthamine showed extremely potent antagonizing action against curare (*d*-tubocurarine; Nikolev, personal communication, 2003).

Many preclinical studies were carried out in animals for evaluating the pharmacological activity of galanthamine (**1**). After a few years, some researchers demonstrated the penetration of galanthamine through the blood brain barrier, and thus effects on the CNS became of particular interest. Based on the knowledge of galanthamine in both the peripheral and central nervous systems, many countries in Eastern Europe used it as an acknowledged treatment in myasthenia gravis and muscular dystrophy, residual poliomyelitis paralysis symptoms, trigeminal neurologia, and other forms of neuritis.

Overall, this is an example of the successful ethnobotany-driven development of a natural product into a clinically important drug, and also highlights that it is often difficult to establish the link between local and traditional uses and drug development. Ethnobotany gave an essential, initial hint, but at this point the evidence for the original ethnobotanical information remains scanty.

III. SUSTAINABLE PRODUCTION OF GALANTHAMINE

A crucial step for the success of galanthamine as a medicine is based on the synthesis developed in the mid-1990s. Today, several synthetic routes are available, most recently a total and stereo-selective synthesis of galanthamine (**1**) starting from D-glucose (13). Czollner *et al*. (14) developed a method using 3,4-dimethoxybenzaldehyde as a starting material. This method forms the basis for the industrial production of galanthamine (**1**). Two methods starting from vanillin [Trost *et al*. (15) and Satcharoen *et al*. (16)] are alternatives, but these are currently not used industrially.

As reviewed in the following chapter in this volume, galanthamine is also still obtained through field gathering, often in protected areas (wetlands), and in recent years *in vitro* cultures using especially *L. aestivum* for galanthamine production and for the generation of plantlets were developed on an experimental scale. The callus induction from young fruits highlighted that the amount of accumulated galanthamine strongly depended on the level of the differentiation. Maximum yield of biomass (17.8 g/L) and the maximum amount of accumulated galanthamine (2.5 mg/L) were achieved after day 35 of submerged cultivation of *L. aestivum* 80 shoot cultures, performed under illumination (17). Currently, this is not a viable industrial alternative, but offers longer-term research and development opportunities.

While no detailed information is available, the sustainability of wild harvesting is under serious doubt, which is, of course, due to the size of the plant and the overall yield. *L. aestivum* is collected in the Balkan Region, but only limited measures to maintain these populations are in place. On the other hand, cultivated *Narcissus* hybrids are now widely used for producing the alkaloid, but again the overall yield is limited. In summary, in the foreseeable future the main production of the alkaloid will be based on total synthesis.

IV. THE FUTURE IN THERAPEUTIC TERMS

The scientific rationale for using cholinesterase inhibitors in the management of AD is based on the cholinergic hypothesis. The destructive, primarily degenerative, condition is neuropathologically characterized by the formation of amyloid plaques, neurofibrillary tangles, and loss of neurons and synapses. Early in the disease's course, a degeneration of cholinergic nuclei localized in the basal forebrain is commonly observed. Impairment of this cholinergic system, which projects into large areas of the limbic system and the neocortex, is followed by problems in concentration and cognitive decline. In biochemical–pharmacological terms, in AD there is disproportionate loss of presynaptic cholinergic neurons in the basal forebrain with a marked decrease in the activity of choline acetyltransferase, the synthetic enzyme for the neurotransmitter acetylcholine (ACh). Inhibiting the AChE, which inhibits the enzymatic degradation of ACh, should consequently result in an increase in the CNS's ACh level, and this, in turn, should alleviate some of the symptoms of AD.

Currently (2009), aside from galanthamine, donepezil, and rivastigmine are used clinically. Tacrine, the first AChE inhibitor commercialized as early as 1993, is no longer used since it demonstrated hepatotoxicity. In addition, galanthamine acts as an allosteric modulator of nicotinic receptors, which enhances the release of ACh from the presynaptic

terminal into the synaptic cleft and potentiate the response of nicotinic receptors to ACh (18,19). Consequently, galanthamine, and other choline acetyltransferase inhibitors will ''only'' *delay* the onset of more serious symptoms, and have been classified as a symptomatic treatment. AChE inhibitors are still the state-of-the-art treatment for AD. Due to the controversies about medical risk-benefit, the UK's National Institute of Clinical Excellence (NICE) has, for example, ''recommended'' the use of donepezil, galanthamine, and rivastigmine as options only for the treatment of moderate AD (20). Prior to this, NICE had actually argued that none of the AChE inhibitors is cost-effective.

In terms of research and development, this has important implications, since the focus is shifting away from AChE inhibitors and a large number of other targets are currently being explored. Therefore, intensive research efforts are underway, which would for example, reduce the plaque formation with particular attention being paid to developing potential ''biologicals,'' an area which is outside of the scope of this review.

V. CONCLUSIONS

Without doubt galanthamine is both an example of a ''poster child'' (2) of natural product–driven drug development, and also highlights the opportunities of developing such medications from traditional and local knowledge. In more general terms, it demonstrates the opportunities of ''knowledge-driven'' drug discovery and, at the same time, it is a prime example that local and indigenous groups will only be able to obtain a share of the wealth generated through such research if the local knowledge is documented and – at least for the period after the implementation of the Convention on Biological Diversity – if appropriate agreements for such benefit sharing agreements are put in place (1,21,22). However, in this case, the evidence for any local and traditional use of the relevant species is scanty, and no full ethnopharmacological documentation is available, highlighting the need to have such knowledge recorded for posterity in a permanent way. As in the case of nature conservation, this could be *ex situ* or *in situ*, but the latter is the far better alternative, most importantly since it will also allow the traditional keepers of knowledge to continue such practices, and since this is one of the essential uses of the world's biocultural diversity.

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CHAPTER 5

Galanthamine Production by *Leucojum aestivum* Cultures *In Vitro*

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I. INTRODUCTION

Galanthamine is known for its pharmacological effects and clinical use in the treatment of poliomyelitis, muscle dystrophy, neuromuscular disorders, and many other diseases of the nervous system, as well as in anesthesia (1,2). It facilitates the transmission of nervous impulses: as a reversible acetylcholinesterase inhibitor, it hampers the degradation of acetylcholine at the neuromuscular junction, stimulating at the same time the nicotinic receptors of acetylcholine. Its ability to cross the blood–brain barrier allows its usage in the symptomatic treatment of different forms of dementia. The preparation Nivalin® was created in Bulgaria on the base of galanthamine hydrobromide, mainly for treatment of poliomyelitis (3). Galanthamine was patented for the treatment of alcohol and nicotine dependence (4) and Alzheimer's disease (5). The increasing interest toward galanthamine in the recent decades is mainly due to the possibility to slow down the processes of neurological degeneration in Alzheimer's disease, being the most common form of dementia, and affecting several million people: 4 million in the United States, 1.5 million in Japan, over half a million in the United Kingdom (6).

Galanthamine belongs to the isoquinoline alkaloids, which are typically present in all genera of the Amaryllidaceae family. It is also present in minor concentrations in some species of the closely related families Agavaceae, Haemodoraceae, and Hypoxidaceae (7). Extracts of *Galanthus nivalis* L. (Amaryllidaceae) were widely applied in the folk medicine of many Eastern European and Mediterranean countries. It was supposed that the Moly antidote to the poisons of Circe, from the legend integrated into the Odyssey, was one of the rare plants with cholinergic activity, most probably the snowdrop (8). In Bulgaria, snowdrop brandy was used for the treatment of senile tremor, disturbed vision, and fatigue. Even today, recipes and general information about the external and internal uses of snowdrop and summer snowflake are available on the Internet for the treatment of plexitis, fever, cold, throat pain, and leukemia.

Galanthamine was first isolated from *Galanthus woronowii* Losinsk. from the Caucasus region in 1952 (9). In Bulgaria, galanthamine was isolated from *G. nivalis* var. gracilis (10). However, since 1960 the industrial production of galanthamine originated from the aboveground parts of *Leucojum aestivum* L. (Amaryllidaceae) because of its higher productivity due to both larger size and higher alkaloid concentration. During the first years of development, about 10–15 tons of plant material were collected annually from natural populations in Bulgaria. Subsequently, this decreased to 5 tons because of the depletion of the plant resources and the extinction of the species from some localities (11–14). Indeed, *L. aestivum* was included in the *Red Data Book of Bulgaria* as an

endangered species (15). Different *in situ* measures have been applied to protect the species, according to the Protection of Biological Diversity Act (2002) in Bulgaria. Currently, some of the populations are in reserves or protected areas, while for the economically important populations there are quotas of gathering based on the annual resource estimation requirements as determined by the Ministry of Environment and Waters (16). An Action Plan should be developed for the 16 populations permitted for commercial exploitation, envisaging habitat reclamation. Management plans are needed for each population defining the restoration of the ecohydrological regime, replanting, and resowing with bulbs and seeds of the same origin (17). In addition, *L. aestivum* was proposed to be included in the Annex of the Medicinal Plants' Act (18). Because of the limited control during the gathering campaigns and the subsequent habitat destruction, the status of *L. aestivum* populations in the wild continues to worsen. Conservation of the resources requires *ex situ* cultivation thereby ensuring an adequate and consistent supply of the raw material for pharmaceutical development and marketing. Although a number agricultural techniques were established (19), a high producing variety ''Snejinka'' was created by mass selection, and the first plantation was established near Primorsko (14). Unfortunately, the development of industrial plantations was hindered by the restitution of lands during the last decades and insufficient funding. The variety Snejinka was limited to the maintenance of several individuals, while the method for *in vivo* clonal propagation of *L. aestivum* (20) was not applied for its rapid multiplication. Similarly, the results from the first experiments on the *in vitro* micropropagation of summer snowflake revealed the recalcitrance of this species (21–24) and it remained of only scientific significance.

A new strategy aimed at the rapid multiplication of elite individuals of *L. aestivum* and the *in vitro* biosynthesis of galanthamine has been developed through a 5-year research project ''Alternative approaches of bioproduction of alkaloids and active substances from Bulgarian rare and threatened medicinal plants'' supported by the NATO *Science for Peace* Programme (NATO SfP 974453-Bioproduction, 2001–2006). In order to better understand the factors determining the biosynthetic capacity of the plants, and to clarify some ambiguities existing in the literature, the *in situ* and *ex situ* assessment of the growth and the alkaloid content of the intact plants, soil analyses, and karyological studies were planned. Living *L. aestivum* collection, representative for the species in Bulgaria, was established, consisting of plants collected from 27 populations located in different regions of the country, within them 22 populations containing galanthamine and lycorine in different proportions, one population of lycorine chemo-type, and five populations with other predominating alkaloids. The effects of the genotype and the

culture conditions were compared under field and *in vitro* growing conditions.

Successful *in vitro* bioproduction of secondary metabolites requires cultures with a stable biosynthetic capacity and fast biomass growth. Research directed toward the implementation of such goals in practice, and in particular the manipulation of the parameters concerning the effectiveness of the technology is currently of great pharmaceutical importance. Furthermore, it is desirable to explore all of the factors that could reduce the cost price of the product. In general, liquid cultures are the most suitable systems for the *in vitro* bioproduction of secondary metabolites. However, their establishment is possible only after the effective sterilization of the mother plant organs and is preceded by initiation of the cultures on solid medium. Moreover, it is necessary to determine the most appropriate type of liquid culture experimentally, as it varies from one plant species to another.

II. BIOLOGICAL FACTORS DETERMINING GALANTHAMINE BIOSYNTHESIS

Knowledge of the specific biological features of the species has been of great importance for the success of our work because they characterized the behavior of the *in vitro* cultures, regardless of the permanent cultivation conditions.

L. aestivum is a perennial bulbous plant with a complex physiological cycle comprised of periods of dormancy regulated by phytohormones and forced by environmental temperature. Thus, the aboveground herbage dies with the beginning of summer, while the autumn sprouting of the new leaves lasts until the first winter frosts. The concentration of galanthamine is highest during the blossoming period and decreases sharply with the fruit formation.

The complex approach of our studies, comprising both *in situ* monitoring and *ex situ* cultivation, gave us the rare opportunity to note some plant features that persisted regardless of the quite different growth conditions. For example, the plants gathered from different natural populations with some noncoincidence of the flowering period, strangely have kept these differences for 8 years after their transfer to our experimental field plot. They are also distinguished by their diverse propagation ability, forming clusters with more or less bulbs for the same time period. The propagation rates of the long-term *in vitro* cultures of *L. aestivum* were also influenced by the seasons, *in vitro* seed germination was easier in spring, while *in vitro* rooting occurred more frequently in autumn, despite the permanent growing conditions in the culture room. Still more unexpected were the dynamics of the alkaloid concentrations

that were revealed through the regular analyses of the several-year old cultures: they were specific and dissimilar with those characteristic for the plants growing in the open fields.

Contrary to some reports considering the soil composition as the main reason for determining the alkaloid pattern and content, the results showed clearly that the genotype was of crucial importance for the biosynthetic capacity of the *in vitro* cultures, as well as for the intact plants growing in the living collection.

Finally, we should emphasize that the biosynthetic capacity decreased with tissue dedifferentiation and was lost by cell suspensions. The concentration of galanthamine was different in the bulbs, leaves, and roots of the intact plants, which obviously predetermined the biosynthetic variation of the different types of *in vitro* organ cultures.

A. Genotype Features

1. Alkaloid Biosynthesis

The elucidation of the factors ensuring stable alkaloid biosynthesis was essential for the establishment of *in vitro* cultures able to produce galanthamine in high quantities, suitable for implementation as a larger-scale practice.

Stefanov (14) defined the existence of three chemo-types of *L. aestivum* in the territory of Bulgaria, producing galanthamine (Gal), lycorenine/9-desmethyl-homolycorine (9-DMHL), and lycorine (Lyc) as the main alkaloids, spread in the southeast, northwest, and northeast parts of the country, respectively. All of the 27 Bulgarian populations contained these compounds as the principle or related alkaloids, and two other alkaloids, tazzetine and hippeastrine, were also detected in small concentrations in some of the populations. Consequently, it was considered (14) that the biosynthesis of galanthamine depended on the ecologo-geographical and edaphical factors, though the amplitude of the phenotype plasticity was under genetic control. The direction of the biosynthesis toward one or another alkaloid, the localization of the alkaloids in the plant organs, and the dynamics of alkaloid accumulation were genetically determined. In contrast, the alkaloid content was strongly influenced by the environmental conditions: geographical location, temperature, sun radiation, humus, and presence of some elements in the soil like nitrogen, phosphorus, boron, and molybdenum.

The richest area for galanthamine concentration in the plant population was situated near the most east point of the Black sea coast. A variety registered as ''Snejinka'' (# 25810/1977) was elaborated by a single mass selection of vigorous plants with a high galanthamine content originating from this population, and the southeast part of

Bulgaria was affirmed as the best place for cultivation of the species. The analyses of the populations in some neighboring countries confirmed the relations between the alkaloid profile of *L. aestivum* and the geographical latitude and longitude. Galanthamine predominated in all populations along the Maritsa River and its feeders in south Bulgaria and in Turkey (about 2.3 mg/g DW). Lycorenine and 9-DMHL were the main alkaloids along the Danube River in the most northwest parts of Bulgaria, and their concentrations increased from south to north, in Serbia and Slovenia, lycorenine reaching up to 2.3 mg/g DW in Slovenia. According to other authors, the alkaloid profile of the *L. aestivum* plants depended mostly on the soil composition (25).

The available data was, however, discrepant. More precise studies revealed the heterogeneity of ten Bulgarian *L. aestivum* populations of galanthamine chemo-type according to the content of galanthamine in the herbage (7). Authors reported a normal distribution of hundred plants per population following their galanthamine content, which varied in very large limits: from 0.1 to 4.0 mg/g DW within a single population. Indeed, the populations were distinguished by their average galanthamine contents, but their heterogeneity was incompatible with the hypothesis regarding the decisive role of the environment. We assume the influence of the soil composition and the ecologo-geographical characteristics as factors of secondary importance. The differences between the average galanthamine contents of neighboring populations could be due more probably to the movement of populations. The age of the Bulgarian *L. aestivum* populations was evaluated to be about 60 years (14). Although the vegetative propagation by bulb fragmentation predominated, seed reproduction had been noticed also (14). The population heterogeneity concerning the content of galanthamine of plants growing in the same locality suggested that alkaloid biosynthesis was an innate characteristic conditioned by the genotype.

Another fact conflicting with the eco-geographical determination of the alkaloid profile was the distribution of the lycorine *L. aestivum* chemo-type. The sole Bulgarian population with lycorine as the main alkaloid was located near the northeast Black sea coast. However, the same chemo-type was also noticed for the plant populations in Greece, that is, to the south of Bulgaria (14).

Besides the alkaloid biosynthesis, the morphometric characteristics of the plants from the different populations, such as the leaf length, and the number of flowers per plant, etc., were also assessed, and the mutability was found to be low and transient (14). Conversely, the budding, flowering, and fruit bearing, lasting about 30 days, occurred more or less asynchronously in the different populations, with a fluctuation of about 25 days, due to eco-edaphical peculiarities. Thus, one of the richest galanthamine populations, Arkutino, was in mass flowering by April 30th,

while only about 30% of the plants in the lycorine-type population were flowering at the same time (14).

Logically, the experimental design aimed at stimulation of the galanthamine biosynthesis should target the factors that are of the most decisive importance. Consequently, we needed first a reliable confirmation of the weightings of the genotype and the environmental conditions with respect to their influence on the biosynthetic capacity of the plants. In order to determine the impact of the genotype, it was envisaged initially to multiply and maintain different genotypes *in vitro* under equivalent cultural conditions and nutrient medium, and subsequently to compare their growth and biosynthesis. Once each genotype was adequately multiplied, it should be further tested by parallel cultivation on media with different compositions, and under diverse cultivation conditions. Statistical assessment of the results would then elucidate the basic factors determining the biomass growth and the alkaloid biosynthesis of the *L. aestivum in vitro* cultures. In addition, it was considered important to compare the behavior of the *in vitro* cultures and their clusters of origin cultivated under the controlled conditions of the experimental field plot. For this purpose, plants were needed with diverse galanthamine contents and alkaloid profiles, originating from different localities.

The living collection of *L. aestivum* was established in 2001 near the Institute of Botany, Sofia. The part of the collection destined as the source of donor material for the establishment of *in vitro L. aestivum* germplasm and experiments consisted of 725 plants. There were 538 individuals producing galanthamine, originating from six populations located in the southeast of Bulgaria. Among them, the galanthamine-richest populations Arkutino, Petkaki, and Vesselie, which were also the smallest ones, with a general area of 0.25, 0.5, and 0.8 ha, respectively, were not sanctioned for commercial use (17). Eleven to twenty-six plant clusters were gathered from the populations during flowering in April 2001, after phytochemical screening for galanthamine by a rapid, semiquantitative TLC method (leaves of about 70–80 vigorous clusters per population were tested). We did not analyze the bulbs for galanthamine since for the industrial production of Nivalin® only the aboveground mass is used. Clusters were selected either for the high content of galanthamine, often appearing as the sole alkaloid in the sample, or for the presence of related alkaloids. Thus, the collection offered a good background for the rapid *in vitro* propagation of high galanthamine–producing individuals and for the assessment of environmental factors on the alkaloid profile and content. The analyses of all of the samples were confirmed by a more precise HPLC method validated in our laboratory, as described in Ref. 28.

These results confirmed the heterogeneity of the Bulgarian *L. aestivum* populations with respect to the concentration of galanthamine, and

revealed normal dispersion with a more or less expressed shift from the average in some of the seven *in situ* tested populations (Figure 1). Regardless of the overlap of the dispersion, the differences between the populations concerning the content of galanthamine were established to be significant ($P < 0.001$) (Table I).

The *ex situ* collection was completed with 132 bulbs from the sole lycorine-type Bulgarian population situated in Baltata near the North Black sea coast, and with 54 bulbs from the lycorenine/9-DMHL-type populations near the villages of Antimovo and Archar located in the northwest part of the country along the Danube River. Plants from the populations Baltata, Antimovo, and Archar afforded the opportunity to compare the biosynthetic peculiarities of the *L. aestivum* genotypes with different main alkaloids micropropagated under equal *in vitro* conditions, as well as those specimens cultivated under the controlled conditions of the experimental field plot, over a period of several years.

Clusters growing *in situ* were found to be heterogeneous, consisting of both bulbs separated from the mother bulb, partially connected with it, and less frequently of seedlings with small round bulbs, that confirmed the observations of Stefanov (14). This feature was of great importance for the choice of the initial plant material in all of the *in vitro* studies. Only vegetatively propagated bulbs were planted in the part of the experimental field designed for *in vitro* source material. *In vitro* propagated clones were developed each from a single bulb, in order to maintain their genetic identity. In addition, we initiated several *in vitro* lines, derived from one *in vitro* bulblet each, by consecutive subcultivations. The distinction between the clones and the lines was conventional. Evaluation of the influence of the plant origin on the culture characteristics such as propagation rate, biomass growth, and galanthamine content was made at the clonal, that is, the plant individual level. Further *in vitro* experiments have been planned with respect to the inner features of the clones and the lines, which facilitated the improvement of the culture conditions and finally, the increase of the galanthamine content in the cultures.

Taking into account the importance of the mother plant for the features of the *in vitro* culture, preferably, we initiated cultures from plants with a high galanthamine content. To verify the importance of the cultivation conditions on the alkaloid profile several plants were selected with other main alkaloids as well. Each mother bulb originated a distinct *in vitro* clone by direct plant regeneration, which has been further multiplied by subcultivation. All clones were strictly labeled and maintained as long-term cultures on equal (control) agar-solidified MS-based nutrient medium (27) supplemented with 30 g/L sucrose, 2 mg/L BAP, and 0.15 mg/L NAA and under permanent cultivation conditions: diurnal temperature of $23 \pm 2^{\circ}$C, and an illumination intensity of

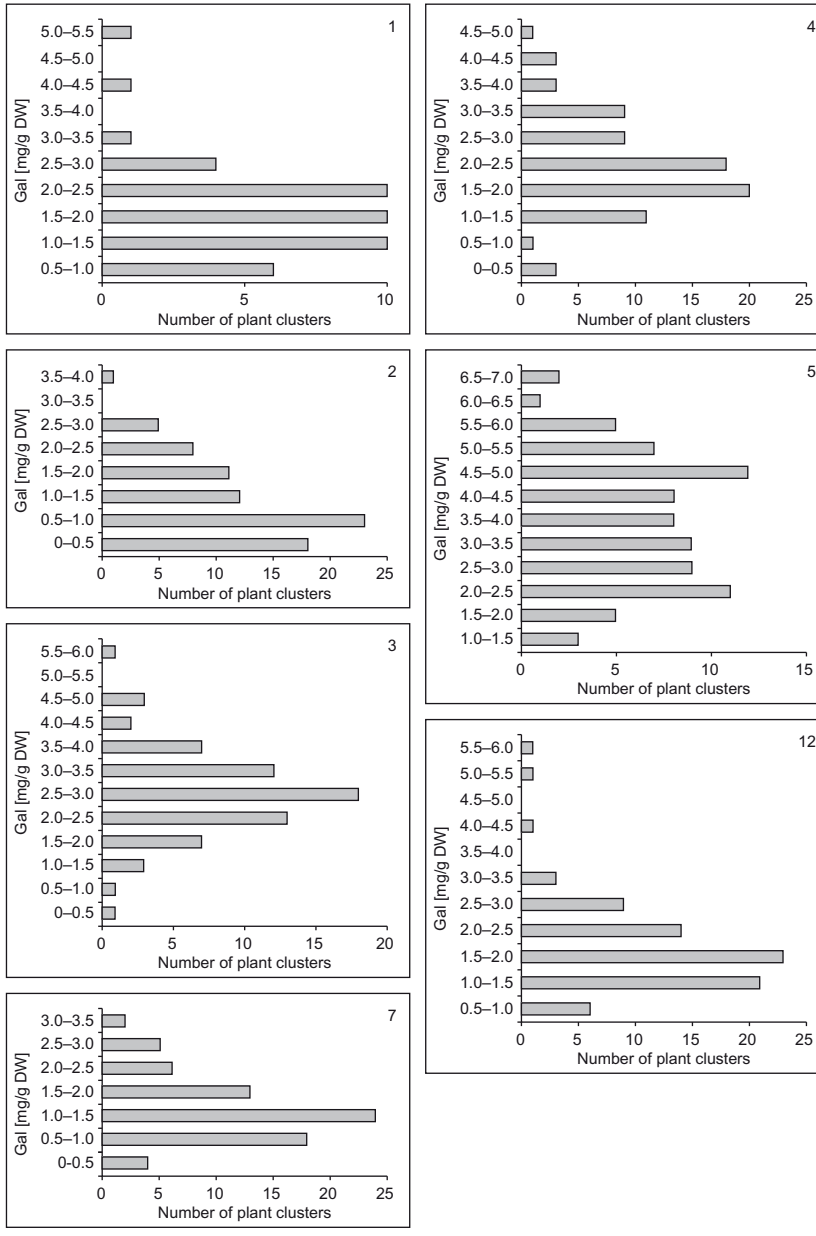

Figure 1 Heterogeneity of seven Bulgarian populations of *Leucojum aestivum* concerning the distribution of the galanthamine content. Populations: 1. Ormana; 2. Blatoto; 3. Petkaki; 4. Vesselie; 5. Arkutino; 7. Kalinata; 12. Karaagach.

Table I Galanthamine content of seven Bulgarian populations of *L. aestivum* located in southeast Bulgaria

Population		Number of analyzed plant clusters	Average Gal (mg/g DW)	SD
No.	Name			
1	Ormana	43	1.88	0.92
2	Blatoto	78	1.19	0.78
3	Petkaki	68	2.81	0.95
4	Vesselie	78	2.26	0.87
5	Arkutino	80	3.69	1.36
7	Kalinata	72	1.39	0.68
12	Karaagach	79	1.90	0.88

3000 lux. The unique code of each plant in the collection allowed for the study of the importance of the genotype on the clone features, comparing the *in vitro* clones, as well as the clusters of origin, and their corresponding *in vitro* clones (Table II).

In vitro cultures were initiated from 24 mother bulbs taken from the living *L. aestivum* collection in 2003, 2 years after its establishment (Table I), and they were multiplied as described previously (28). Genotype was established to be the most important factor influencing the morphological features of the cultures, their biosynthetic capacity, and the micropropagation rate. The first test of 15 *in vitro* clones for alkaloid content was performed in November 2004, after multiplication of the bulblets by direct regeneration. Except for galanthamine and lycorine, the *in vitro* plants were evaluated for the presence of four related alkaloids isolated in the Laboratory of Pharmacognosy in Reims from the aboveground herbage of *L. aestivum* and supplied by the Bulgarian pharmaceutical company Sopharma JSCo, namely, norgalanthamine, homolycorine, ungiminorine, and galanthaminone. Within the clones originating from bulbs of the galanthamine chemo-type, there were five producing only galanthamine and six containing galanthamine and lycorine in different proportions. The *in vitro* bulblets initiated from bulbs originating from the populations of the lycorine and lycorenine−9-DMHL types did not synthesize galanthamine, but contained lycorine. Unfortunately, we did not analyze with standards of the two alkaloids characteristic for the populations along the Danube River.

In order to check the stability of the alkaloid biosynthesis under the long-term *in vitro* conditions, these clones were subcultured over at least 3 years, and the alkaloid content of the shoot-clumps was determined every 3 months. The average content of nine consecutive analyses demonstrated that galanthamine and/or lycorine were the main

Table II Codes of the mother plants and their corresponding *in vitro* clones according to the population of origin

Locality	Geographical latitude (N) longitude (E)	No. of population	Code of the cluster and mother plant[a]	Label of the corresponding *in vitro* clone	Chemo-type: main alkaloids[b]
Ormana	42°32′N 26°31′E	1	C_{FP}-1.15; 1.15/2 C_{FP}-1.19; 1.19/4	La-1.15 La-1.19	Lyc, Gal
Blatoto	42°24′N 27°40′E	2	C_{FP}-2.27; 2.72/3 C_{FP}-2.77; 2.77/1	La-2.72 La-2.77	Lyc, Gal
Petkaki	42°15′N 27°42′E	3	C_{FP}-3.9; 3.9/1	La-3.9	Gal
Vesselie	42°18′N 27°40′E	4	C_{FP}-4.1; 4.1/7 C_{FP}-4.45; 4.45/3	La-4.1 La-4.45	Gal
Arkutino	42°19′N 27°45′E	5	C_{FP}-5.2; 5.2/4 C_{FP}-5.9; 5.9/2 C_{FP}-5.27; 5.27/2 C_{FP}-5.40; 5.40/2 C_{FP}-5.77; 5.77/6	La-5.2 La-5.9 La-5.27 La-5.40 La-5.77	Gal
Dolnata ova	41°49′N 26°08′E	6	C_{FP}-6.31; 6.31/3	La-6.31	Lyc, Gal
Kalinata	42°42′N 27°40′E	7	C_{FP}-7.6; 7.6/2 C_{FP}-7.26; 7.26/2 C_{FP}-7.73; 7.73/2 C_{FP}-7.80; 7.80/3	La-7.6 La-7.26 La-7.73 La-7.80	Gal, Lyc

Table II *(Continued)*

Locality	Geographical latitude (N) longitude (E)	No. of population	Code of the cluster and mother plant[a]	Label of the corresponding *in vitro* clone	Chemo-type: main alkaloids[b]
Lozenski pat	41°46′N 26°10′E	8	C_{FP}-8.24; 8.2/4	La-8.2	Lyc, Gal
Baltata	43°22′N 28°03′E	9	C_{FP}-9.3; 9.3/2 C_{FP}-9.6; 9.6/5 C_{FP}-9.15; 9.15/4	La-9.3 La-9.6 La-9.15	Lyc
Antimovo	44°00′N 22°56′E	10	C_{FP}-10.4; 10.4/2 C_{FP}-10.7; 10.7/2	La-10.4 La-10.7	Lycorenine, 9-DMHL, Lyc
Archar	43°47′N 23°01′E	11	C_{FP}-11.3; 11.3/3	La-11.3	Lycorenine, 9-DMHL, Lyc

[a]First number refers to the population; the second one indicates the cluster in the living collection, and the third number indicates the position of the bulb in the cluster. Bulbs were planted separately on the field plot.
[b]Lyc, lycorine; Gal, galanthamine; 9-DMHL, 9-desmethylehomolycorine.

alkaloids in the cultures (Figure 2). The ANOVA single factor analysis proved that they differed significantly in their galanthamine content ($P < 0.001$). Clones derived from one population produced galanthamine and/or lycorine in different contents and proportions: La-5.2 and La-5.9 from Arkutino; La-7.80, La-7.6, La-7.73, and La-7.26 from Kalinata; La-9.6 and La-9.15 from Baltata.

Several clones produced galanthamine in very high concentrations about or over 1 mg/g DW, which is considered as a borderline for the industrial importance of the biomass. Commercially harvested, wild Bulgarian populations contain this alkaloid between 0.94 and 1.87 mg/g DW (16). Another advantage was the total absence of related alkaloids in four of the *in vitro* clones (La-3.9, La-4.45, La-5.2, and La-5.9) during the 2 years of assessment, which would facilitate the isolation of galanthamine in the case of industrial scale-up of the *in vitro* biomass production.

Other authors aiming at galanthamine *in vitro* production reported recently much lower concentrations of the alkaloid in their *L. aestivum* cultures: up to 0.07 mg/g DW in bulblets regenerated from embryogenic callus (29); up to 0.2 mg/g DW in shoots regenerated from callus (30); and between 0.03 and 0.45 mg/g DW in shoot-clumps obtained from bulbs originating from 18 Bulgarian populations (31). In our opinion, the main reason for these low galanthamine concentrations was the unsuitable initial plant material. Some authors used market bulbs that did not contain galanthamine (29); others collected bulbs from the galanthamine populations, but did not analyze their alkaloid content prior to the culture initiation (31). Our screening of seven *L. aestivum* populations in 2001 showed that even in the populations richest in

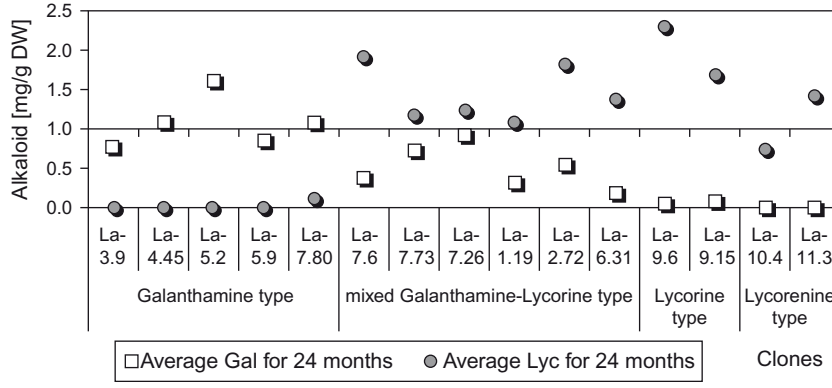

Figure 2 Differences between the *in vitro* clones according to the contents of their main alkaloids galanthamine (Gal) and lycorine (Lyc): an average of nine measurements at intervals of 3 months starting in November 2004, each measurement in two repetitions.

galanthamine there were individual bulbs with galanthamine content barely about 0.5 mg/g DW (Figure 1).

The comparison between the *in vitro* clones of *L. aestivum* and their corresponding clusters of origin proved the crucial importance of the genotype (Figure 3). The *in vitro* clones have kept not only their biosynthetic capacity but also their alkaloid profile under the conditions of long-term culture. Thus, clones producing only galanthamine originated from plant clusters with galanthamine as a sole alkaloid. Similarly, the clusters and the *in vitro* clones originating from the population Baltata synthesized only lycorine. The results showed that after 18 months of *in vitro* cultivation some of the *in vitro* clones produced even more galanthamine than the intact plants on the experimental field. The highest galanthamine content, 3.63 mg/g DW, was determined in clone La-5.2, and was comparable with the average concentration of galanthamine in the herbage of the richest population Arkutino (Table I).

Maintaining the inherent features of the clones producing both alkaloids turned out to be more furtive and complicated to understand. In some cases, it seemed likely that the clones changed their main alkaloid or even their alkaloid profile. Only the continued observation of the contents of the two alkaloids revealed that all these changes were ostensible. This phenomenon will be discussed later, in relation to the dynamics of the biosynthesis that was observed in all of the *L. aestivum* clones.

In general, the galanthamine contents of both the *in vitro* shoot-clumps and the plants from the living collection were lower than those of the initial clusters growing *in situ*. Nevertheless, the plants produced alkaloids in relatively high concentrations and no progressive decrease of

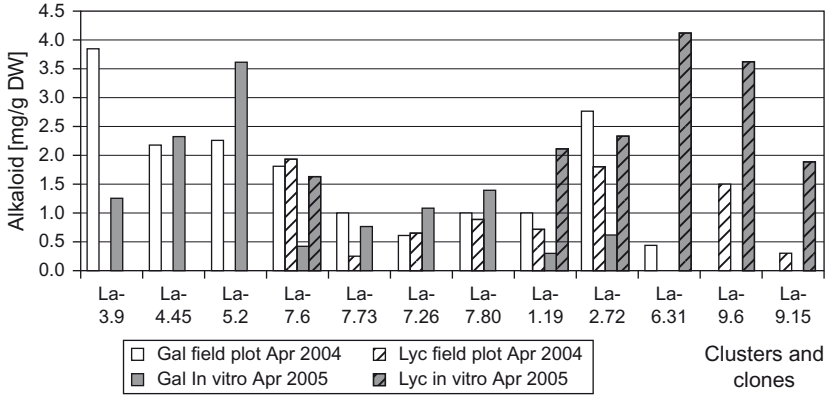

Figure 3 Comparison of galanthamine and lycorine contents of twelve 18-month-old *in vitro* clones (shoot-clumps, April 2005) and their corresponding plant clusters 18 months after the plants' transfer in the living collection (leaves, April 2004).

the alkaloid biosynthesis has been observed. Conversely, the alkaloid contents of many clusters were higher in 2004 compared to 2003; that might be in relation to the plants overcoming of the stress caused by their transfer from the natural populations to the field conditions. This stress was also expressed by scanty flowering in the first year after the establishment of the living collection. Further investigations (in 2006) showed that the contents of galanthamine and lycorine varied in the course of time. However, plants maintained their inherent alkaloid profile (Figure 4). Thus, clusters C_{FP}-3.9, C_{FP}-4.45, C_{FP}-5.2, and C_{FP}-5.9 remained of the galanthamine type after 5 years of field cultivation, clusters C_{FP}-9.6 and C_{FP}-9.15 remained of the lycorine type, while the remaining clusters kept their mixed type, biosynthesizing both alkaloids in similar amounts.

Fluctuations of the galanthamine contents of all the 27 *L. aestivum* natural populations were previously reported for four consecutive years during the flowering, from 1972 to 1975, and were considered as a usual phenomenon (14). Such fluctuations were confirmed not only for galanthamine, but also for lycorine and four related alkaloids (norgalanthamine, homolycorine, ungiminorine and galanthaminone) at population level *in situ* and *ex situ* for sixteen *L. aestivum* populations through the analyses of model plants in two consecutive years, 2003 and 2004 (still unpublished data, obtained within our project NATO SfP-974453-Bioproduction).

Besides the mentioned fluctuations, Stefanov (14) concluded from his convergent and divergent experiments that the concentration of galanthamine in *L. aestivum* plants depended mainly on the location of their populations. Plants from all populations transferred to the field

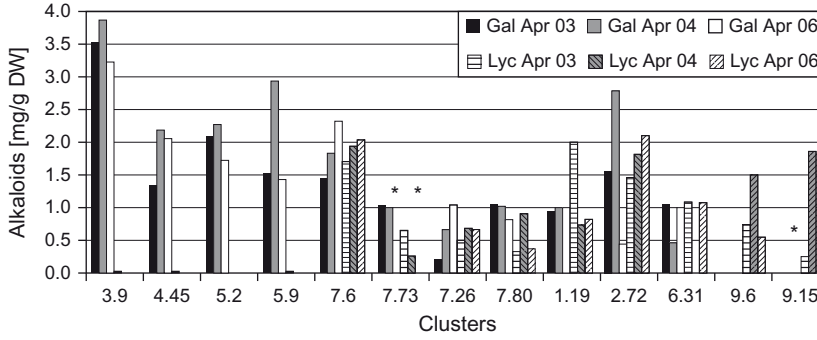

Figure 4 Contents of galanthamine (Gal) and lycorine (Lyc) in the leaves taken from the intact *L. aestivum* plants belonging to the clusters of the living collection, corresponding to the *in vitro* clones. *, no analyses of clusters C_{FP}-7.73 and C_{FP}-9.15 are available for the year 2006 for technical reasons.

near Sofia had the tendency to equalize their galanthamine contents around an average of 2.0–2.4 mg/g DW in the herbage. In our opinion, this conclusion seems to be premature because at the end of the 4 years of assessment the plants originated from the richest of galanthamine populations Yasna polyana, Arkutino, and Vesselie still contained 4.52, 4.47, and 2.77 mg/g DW, respectively. Those samples taken from the lycorine-type population contained only 0.10 mg/g DW galanthamine, declining from 0.3 mg/g DW. Both field-tested populations with lycorenine–9-DMHL as the main alkaloids changed from 0.2 and 0.4 to 0.1 and 0.7 mg/g DW galanthamine, respectively. Indeed, the greatest changes were noticed for the galanthamine-richest populations, which decreased their galanthamine contents by about 20% over the 4-year period, while the changes concerning the other populations rarely exceeded the usual yearly fluctuations of about 5%. Yet, plants originating from all of the populations retained their chemo-type. Summarizing all of the available data, we assumed that only the biosynthetic activity had been affected concerning the populations richest in galanthamine, which might be caused by the different environmental conditions, but only in the frame of the genetically determined biosynthetic capacity. This concept was at the base of the research approach that concentrated efforts on the genotype selection. Trials aimed at the additional enhancement of galanthamine production using different cultivation conditions *in vitro* were planned as a second step in the studies.

We also observed changes of the galanthamine contents at the cluster level, when plant clusters were transferred to the experimental field (Table III). However, this trend cannot be concluded for the behavior of the entire populations on the base of these results because only clusters suitable for our research aims were transferred to the experimental field, that is, those samples rich in galanthamine, with galanthamine as the sole alkaloid, or those with many related alkaloids. Because of this deliberate choice, the average *in situ* assessed galanthamine contents of the clusters collected for the field plot were higher than those of all of the *in situ* analyzed clusters (see Tables I and III for comparison). However, it can be asserted that no tendency toward equalization was noticed under the growing conditions of the experimental field (Table III).

In the case of the clusters originating from the population Kalinata, the average galanthamine content was relatively low, and decreased still further after 3 years of field cultivation. The changes of the galanthamine contents of the clusters coming from the richest populations were not similar: the alkaloid content decreased sharply only in the clusters from Arkutino, while those from Petkaki and Vesselie synthesized a higher level galanthamine than the *in situ* samples. Furthermore, differences between the populations with respect to the distribution of the

Table III Screening of the *L. aestivum* clusters taken from the natural populations during their flowering in April 2001: only for galanthamine *in situ*; for galanthamine, lycorine, and four related alkaloids in April 2004

Population of cluster origin	Number of clusters[a]	Galanthamine of clusters *in situ*, 2001 (mg/g DW)	Alkaloids of the field-growing clusters, 2004 (mg/g DW)[b]				
			Galanthamine	Lycorine	Ungiminorine	Galanthaminone	Homolycorine
Ormana	10	2.33	1.56	0.84	0.03	0.02	0.01
Blatoto	11	1.32	2.06	1.36	0.16	0.01	0.01
Petkaki	16	3.24	3.62	0.04	0.02	0.04	0.01
Vesselie	27	2.58	3.08	0.00	0.00	0.03	0.02
Arkutino	21	4.64	2.60	0.07	0.03	0.03	0.01
Kalinata	14	1.72	1.21	1.20	0.04	0.00	0.00
Baltata	13	–	0.01	1.07	0.00	0.00	0.01

[a]Number of bulbs planted in autumn 2001: 41, 87, 71, 193, 100, 46, and 50 for the seven populations, respectively.
[b]Norgalanthamine was not present in the clusters in 2004.

Table IV Distribution of the clusters according to the percentage of decrease or increase of galanthamine content for a period of 3 years field cultivation (2001–2004)

Population of cluster origin	Number of clusters	% of galanthamine decrease and number of clusters					% of galanthamine increase and number of clusters								Average % of Gal changes
		−100 −80	−80 −60	−60 −40	−40 −20	−20 0	0 +20	+20 +40	+40 +60	+60 +80	+80 +100	+100 +120	+120 +140	+140 +160	
Ormana	10	–	–	4	3	1	1	1	–	–	–	–	–	–	−33.0
Blatoto	11	–	1	2	1	1	3	1	1	–	1	–	–	–	−11.2
Petkaki	16	–	–	–	1	5	4	2	1	2	–	–	–	1	+11.7
Vesselie	27	–	–	–	3	4	5	5	6	–	1	1	1	1	+19.7
Arkutino	21	–	1	12	6	1	–	1	–	–	–	–	–	–	−44.0
Kalinata	14	3	–	3	1	1	–	4	1	1	–	–	–	–	−29.6

galanthamine content emerged clearly through the behavior of the specific clusters (Table IV, Figure 5). It is worth noting that the dispersion increased in two populations. Even if the average percentage of galanthamine changes for Blatoto were relatively slight, the amplitudes of the changes of some clusters were significant and that increased the dispersion. Most rewarding was the behavior of the clusters from Vesselie: 15 of the 27 clusters increased their galanthamine content; and within them, 5 were richer than the average, and also enhanced their alkaloid biosynthesis. The dispersion of the galanthamine content decreased in the other four populations, but for different reasons. Clusters from Petkaki rich in galanthamine decreased slightly, while those poor in galanthamine increased significantly, which resulted in a relatively slight increase for the average content, and a noticeable decrease in the dispersion. For the other three populations, decreases were noticed in both the average galanthamine content and the dispersion of that content. The biosynthetic activity of almost all of the clusters from Arkutino and from Ormana decreased considerably. Six clusters from Arkutino and four from Ormana, for which the galanthamine contents had been below the average for the populations previously decreased still further. For these clusters, the maximum and the minimum values were nearly half compared with those determined *in situ* in 2001. Finally, the decrease of the galanthamine content and its dispersion noticed for the Kalinata clusters was mainly due to several clusters in which biosynthetic activity almost stopped. It is worth mentioning that these clusters did not compensate with an increase of the lycorine content, which is typically in high concentration in this population, but conversely, they did not synthesize any other alkaloid.

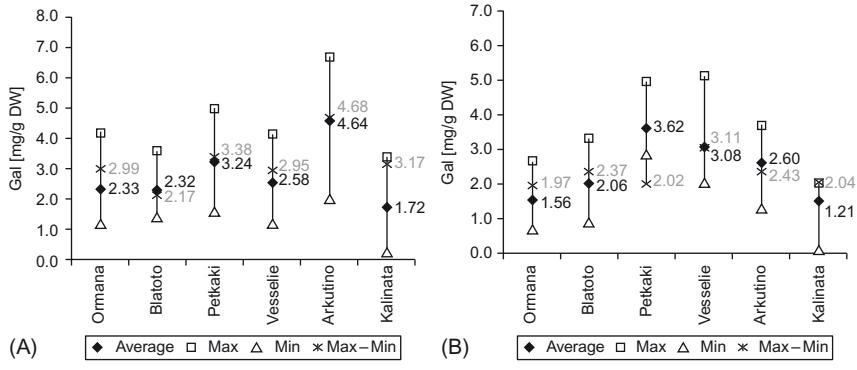

Figure 5 Galanthamine content and range of the clusters taken from the natural populations in 2001 for establishment of the *ex situ* collection: (A) in 2001 *in situ*; (B) in 2004 cultivated on the experimental field of the Institute of Botany, Sofia.

The differences noticed in the biosynthetic responses of the clusters originating from these six natural populations to the environmental changes remained in the frame of their inherent chemo-types. They could be explained bearing in mind the differences between the soil compositions of the corresponding localities. Soil samples from nine natural populations and from the experimental field were compared for 37 parameters: available and total contents of 16 macro- and microelements, pH, humus, and total N, Cl^-, and $CaCO_3$. A mathematical model MST-graph of the mutual distances among the tested soils established that the soil compositions of the Arkutino, Kalinata, and Baltata localities were quite distinct from the others. The soil of the experimental field was found to be suitable for cultivation of *L. aestivum* because it was well balanced, having a composition closest to that of the locality, which represented the limitation element (information still available only in the regular reports of project SfP # 974453 Bioproduction). The results from the soil analyses could explain, in some measure, the results of the divergent experiment of Stefanov (14). There it was reported that the transfer of *L. aestivum* plants from Arkutino to one of the lycorenine–9-DMHL populations near the Danube, and vice versa, led to progressive changes of the alkaloid contents for 10 years, causing a transformation to the chemo-race of the resident locality. However, these experiments neglect the fact that the values of 9-DMHL in the plants originating from the Danube population did not decrease at all, and remained stable at about 1.5 mg/g DW during all of the 10-year period. We did not analyze the soil composition of the localities near the Danube River and did not have standards for the two main alkaloids of these populations. Consequently, we cannot discuss in detail these results. We concur with the statement (14) that the genotype and the environmental conditions are both important factors influencing alkaloid biosynthesis. All of our results obtained *in vivo* and *in vitro* established however, the key role of the genotype as the limiting factor concerning the biosynthetic capacity of the plants.

It transpired that the main *in vitro* clones used in most of the *in vitro* experiments were initiated from bulbs, which belonged to clusters reflecting the overall upward or downward populations' tendencies concerning the content of galanthamine. Thus, cluster C_{FP}-3.9 from Petkaki kept its galanthamine values near the initial 3.78 mg/g DW assessed in 2001 *in situ*, whereas cluster C_{FP}-5.2 from Arkutino decreased from 5.03 mg/g DW *in situ* to less than half the content, and clusters C_{FP}-7.80 and C_{FP}-7.73 from Kalinata also decreased significantly their galanthamine contents starting from 2.06 and 1.87 mg/g DW, respectively, *in situ* (for comparison, see Figure 4). The specific dynamics noticed in the long-term *in vitro* cultures for clones La-7.6 and La-7.73

(see Section II.C.2) were in relation to the different behavior between their corresponding clusters C_{FP}-7.6 and C_{FP}-7.73.

Even if the contents of the main alkaloids galanthamine and lycorine of many of the *in vitro* clones differed from those of their corresponding clusters, as reported previously (28), correlations were found between them (Table V). This confirmed once again the crucial importance of the genotype for the success of the alkaloid bioproduction *in vitro*, as well as in field-grown plants. Galanthamine biosynthesis of *in vitro* clones correlated with that of the clusters of origin growing in the natural populations. In spite of the annual fluctuations, the contents of the two main alkaloids assessed *ex situ* for 5 years of field cultivation correlated with high significance. Galanthamine contents produced *in vitro* by 2- and 3-year-old cultures were also correlated. The lack of correlations between the contents of the two alkaloids produced *in vivo* and *in vitro* was due to the specific dynamics that were occurring *in vitro* (see Section II.C.2 for details). The effect of the dynamics was eliminated when the average contents of galanthamine and lycorine from 9 assessments within 24 months (regular analyses every 3 months) were used for comparison with the spring contents of the *in situ* growing and field-cultivated clusters. A highly significant negative correlation was detected between the biosynthesis of the two alkaloids under long-term *in vitro* conditions. It is worth mentioning however, that no compensatory relationships were found between the contents of galanthamine and lycorine.

Further analyses will elucidate definitively the degree of stability of the galanthamine and lycorine contents of *L. aestivum* plants cultured under the controlled conditions of the field plot. For instance, there is an adequate basis to expect a stable alkaloid production based on the herbage harvest, on the condition that the existing agro technical requirements for the species will be respected. Our 8-year experience, and the available selected high producing individuals are a good prerequisite for the establishment of a plantation designed for alkaloid production.

The related alkaloids ungiminorine, galanthaminone, and homolycorine were present only in some of the clusters, usually in relatively low concentrations (Table III). Ungiminorine was detected in many clusters from all of the populations, except Vesselie, in concentrations between 0.09 and 0.43 mg/g DW; galanthaminone was found in some clusters up to 0.09 mg/g DW, and homolycorine at up to 0.04 mg/g DW. In 2004, norgalanthamine was no longer detected in the field-cultivated clusters. We had no information available regarding the related alkaloids of these clusters when growing *in situ* before the transplantation to the experimental field. However, the analyses allowed for a comparison between the *ex situ* cultivation in the living collection and *in vitro* cultivation.

Table V Correlations between the alkaloid contents of the *in vitro* clones and their corresponding clusters of origin assessed *in situ* (only for galanthamine) and after their transplantation on the experimental field plot (for galanthamine and lycorine)

Z estimations of Gal and Lyc of one cluster or clone		*Z* estimations for significance of correlation coefficients of galanthamine contents in the corresponding clusters and clones					
		FP 2003	FP 2004	FP 2006	*In vitro* 2005	*In vitro* 2006	*In vitro* 24 months
Z estimations of lycorine contents in the corresponding clusters and clones	Nature 2001	1.78*	1.37	0.62	2.08*	1.59	1.70*
	FP 2003	−0.95	4.14***	2.95**	1.24	0.71	1.08
	FP 2004	2.35**	−0.67	2.38**	1.68*	1.17	1.61
	FP 2006	3.36***	3.10***	−0.74	1.24	0.86	1.12
	In vitro 2005	2.03*	1.11	1.50	−2.58**	5.46***	5.44***
	In vitro 2006	1.52	2.09*	2.43**	1.10	−1.30	5.07***
	In vitro 24 months	2.39**	2.35**	2.58**	2.79**	3.78***	−3.37***

*$P<0.05$ (min 1.65); **$P<0.01$ (min 2.35); ***$P<0.001$ (min 3.10).
Z is the value of the standard normal distribution. Correlation coefficients between *in vitro* clones and their corresponding clusters growing on the field plot (FP) were evaluated on the base of 12 genotypes. Correlation coefficients between clusters *in situ* (*Nature*, 2001) and the corresponding field clusters and *in vitro* clones were evaluated on the base of eight genotypes. Lycorine was not determined *in situ*.

It is important to reemphasize the significance of the individual plant features of the mother plants, which should be studied prior to the *in vitro* cultivation. The population of origin, taken alone, is not a guarantee for the alkaloid content of the plants. Biotechnologists often are neglecting the inner features of plants at the individual level, which is affecting the results of otherwise well-planned experiments (29,31,32). Thus, Georgieva *et al.* (31) misunderstood the impact of the genotype, and took in consideration only the different average galanthamine contents assessed for wild *L. aestivum* populations, overlooking their genetic heterogeneity as reported by Poulev *et al.* (7). This could explain the relatively low galanthamine concentrations of the *in vitro* cultures that they obtained, reaching only between 0.03 and 0.45 mg/g DW. Comparison has been made of the alkaloid patterns of 18 Bulgarian populations on the base of two bulbs per population. Furthermore, these authors initiated eight *in vitro* cultures from plants coming from different populations (including the richest in galanthamine, Arkutino) without checking the alkaloid profile and content of the mother plants. The conclusions made at the population level about the similarity or variability of the alkaloid patterns of the intact plants and the *in vitro* shoot-clumps were therefore completely arbitrary. The comparison between the *in vitro* shoot-clumps induced in 2001 from *L. aestivum* plants not checked for alkaloids and a few intact plants collected from the same populations in 2006 was also inadmissible (32). These authors were correct about one aspect: intensive selection is necessary *in vivo* and *in vitro* to obtain cultures that are rich in galanthamine. Our efforts during several years of teamwork covering the resource assessment of the *L. aestivum* populations (16), the establishment and maintenance of *ex situ* collections representative for the biodiversity of the species in Bulgaria, and biotechnological studies (28,33,34) have resulted in the selection of several high galanthamine−producing *in vitro* clones.

2. Plant Propagation

The success of the bioproduction of galanthamine *in vitro* should also depend on the culture propagation rate. The twin-scales excised from the mother bulb proved to be the most appropriate primary explants. They were transferred on fresh medium every month, in Vitro Vent containers (Duchefa, NL), 25 per container. Shoots appeared between the scales after 4 weeks of cultivation and developed in shapely bulblets for about 2 months, but needed between 5 and 10 months to reach a size suitable for subcultivation by a vertical cut to four sectors consisting of several scales. The technique of subcultivation was found to be very appropriate for many reasons. It allowed for the economy of time, labor, and chemicals because the steps of chilling and surface sterilization of the native bulbs prior to new culture initiation were omitted. In addition, the oppressive

effect of the bleach on plant tissues was avoided. The efficiency of the bulb regeneration increased as the shoots appeared more often in clumps between the scales of the bulblet sectors (Figure 6).

It was essential that the age of the culture did not affect the propagation potential. Thus, the number of bulblets originating from one single mother bulb could increase without limit, which was very important for long-term studies, including experiments with a complex design covering numerous variables. Once initiated, the clones with valuable features became eternal and could be multiplied in innumerable quantities. Advantageously, the use of numerous mother bulbs of this threatened plant species could be avoided.

The regeneration potential of 12 *in vitro* clones, initiated from bulbs belonging to different clusters was tested (Figure 7). The propagation coefficient (PC) was calculated as the number of shoots and bulblets

Figure 6 Subcultivation of *L. aestivum* — Left: Shoots formation on bulblet sector, Right: Shapely bulblets and shoots on one explant.

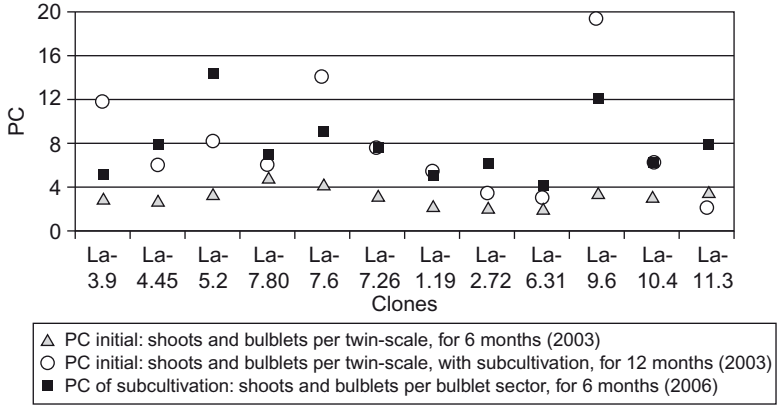

Figure 7 Propagation coefficients (PCs) of 12 clones per initial twin-scale and with subcultivation.

obtained per explant. The *t*-test paired analysis demonstrated significant differences between the PCs of the clones propagated without and with subcultivation ($P < 0.001$). This was due to the smaller meristem tissues of the twin-scales in comparison with the bulblet sectors consisting of more scales.

With the application of the technique of subcultivation, the dispersion of the PCs of the clones increased significantly, which reflected the multiplication of the effect of the different time needed for bulblet growth of the different clones. The dispersion of the clonal PCs decreased in the 3-year, long-term cultures (Figure 8).

Aiming at elucidating the possible effect of the genotype on the effectiveness of plant multiplication, the PC of the field-growing clusters originating from seven populations were compared: six of the galanthamine type (Ormana, Blatoto, Petkaki, Vesselie, Arkutino, and Kalinata) and one of the lycorenine−9-DMHL type (Antimovo). The assessment concerned only the vegetative propagation of the plants. In order to keep the original genetic features of the plants, their generative propagation was avoided by removing of the fruits before their maturation. The PC was calculated at the population and cluster levels, as an average number of plants obtained for the 8 years (2001−2009) per initially planted bulb (Table VI). The ANOVA single factor analysis showed a very high significant difference between the propagation rates of the plants originating from the different populations ($P < 0.001$). Furthermore, we revealed high and specific distribution of the PC of the field-growing plants originating from different populations (Figure 9).

In order to check for correlations between the propagation rates of the genotypes at the population, cluster, and individual levels, the number of the *in vitro* bulblets obtained from the corresponding mother bulbs for a

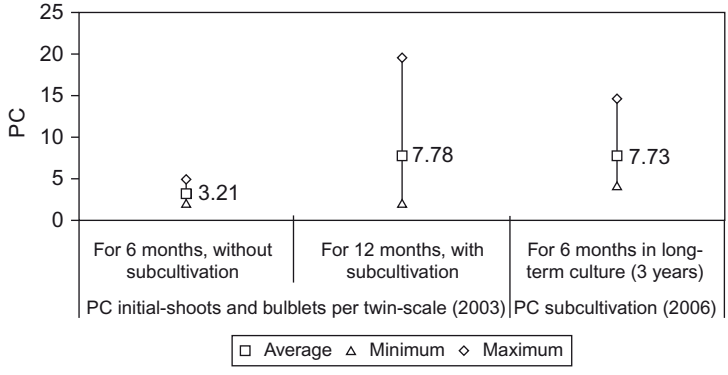

Figure 8 Propagation coefficient (PC) of the initial cultures of *L. aestivum* and long-term *in vitro* cultures.

Table VI Propagation coefficients (PCs) of *L. aestivum* at population, cluster, and individual levels

Population level[a]			Cluster level[a]		Individual level (*in vitro*)[b]			
Population of origin (code and name)	Number of bulbs planted in 2001	Average plants per planted bulb (8 years)	Code of the field cluster	Average plants per planted bulb from the cluster (8 years)	Code of the *in vitro* clone	Average *in vitro* bulblets per twin-scale (1 year)	Average *in vitro* bulblets per mother bulb (1 year)	Prognosis for 24 months subcultivation
1. Ormana	18	4.1	C_{FP}-1.19	4.5	La-1.19/4	3.50	87.5	202
2. Blatoto	69	6.9	C_{FP}-2.72	4.3	La-2.72/3	0.91	22.8	164
3. Petkaki	47	13.3	C_{FP}-3.9	8.5	La-3.9/1	4.47	117.7	3238
4. Vesselie	120	15.9	C_{FP}-4.45	12.3	La-4.45/3	3.33	83.3	1389
5. Arkutino	68	23.8	C_{FP}-5.2	30.0	La-5.2/4	6.59	164.7	4409
7. Kalinata	32	28.1	C_{FP}-7.80	27.0	La-7.80/2	4.75	118.8	4612
11. Antimovo	14	13.2	C_{FP}-11.3	11.8	La-11.3/3	2.08	52.0	60

[a]PC was calculated in 2009 as an average of plants obtained per single survival planted bulb for 8 years cultivation in the experimental field.
[b]PC indicates the average number of *in vitro* bulblets with size appropriate for subcultivation. (First digit corresponds to the population code; second digit to the cluster code; and last digit to the single bulb in the cluster.)

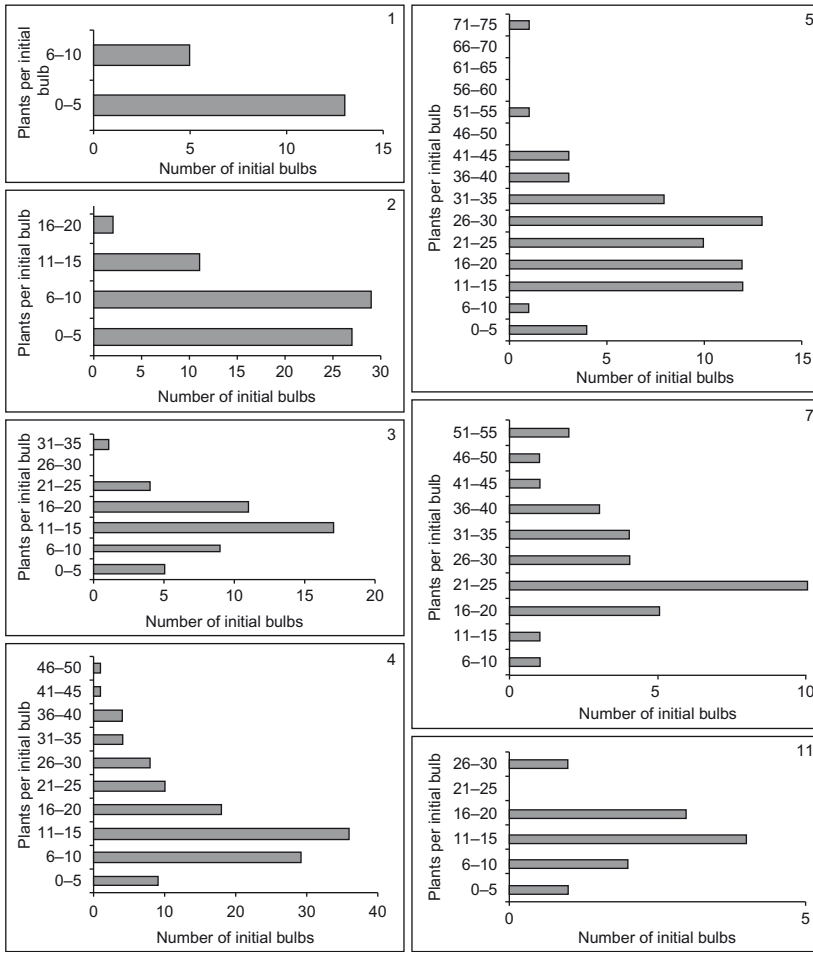

Figure 9 Distribution of the propagation coefficient (PC) of the field-grown plants originating form different populations, for 8 years of cultivation (planted in October 2001, counted in May 2009). Populations: 1. Ormana; 2. Blatoto; 3. Petkaki; 4. Vesselie; 5. Arkutino; 7. Kalinata; 11. Antimovo.

period of 1 year were calculated (Table VI). Correlations were established between the PCs of the field-propagated plants at cluster and population levels ($P < 0.001$), as well as between the PCs of the field-propagated plants and the *in vitro* obtained bulblets from the initial bulbs ($P < 0.05$).

The prognosis we made concerning the long-term cultivation with permanent subcultivation of all bulblets reaching the needed size, showed very fast augmentation of the differences between the clones (Table VI). Indeed, some clones formed large shapely bulblets for 5 months, while other, slow growing clones needed double the time. The

number of bulbs in the clusters growing *in situ* also varied between and within populations, the highest average value being evaluated for Vesselie: 11.46, and the lowest one for Blatoto: 2.97 (unpublished data). This data fits well with our prognosis, bearing in mind the different morphological features of the clones that were reported previously (28). The formless, crispy, bulblet-like structures growing along with the shapely bulblets of high quality in clone La-4.45 could explain the differences observed *in situ* and *in vitro*.

All of our results confirmed the crucial importance of the genotype for the propagation rate of the *L. aestivum* plants. They were in contradiction to the observation of Stefanov (14) concerning the decreasing plant size and biomass quantity of the field-growing plants from all populations. This observation might also be related with the plant watering. Our experimental field was regularly inundated during the vegetative phenophase in the spring, which promoted the growing of exuberant *L. aestivum* clusters.

Besides bulbs, seeds were also suitable for initiation of *in vitro* cultures. They were available during the whole year, and they were characterized by easy sterilization, germination in the dark, and formation of normal seedlings. The bulblets obtained after the removal of the seedling roots and leaves were further multiplied by subcultivation. Initiating *in vitro* culture from seeds also avoided bulb destruction. In spite of these advantages, seeds were considered as risky concerning the alkaloid content of the regenerated plants because of the new genotypes with unknown features. They should be appropriate as initial material for *in vitro* cultures if plant multiplication is aimed at ensuring seedlings for reclamation of the wild populations, because in this case the genetic diversity should be of great importance. Here, we can mention briefly that the *ex vitro* adapted plants needed about 3–4 years to reach the size of the adult *L. aestivum* plants and to form flowers. Even considering the losses caused during the adaptation of the plantlets to open-air conditions, it was established that the PC has been enhanced many-fold using the technique of plant *in vitro* micropropagation (Table VI).

The analysis of the data on the *L. aestivum* plants concerning the PC and the biosynthesis of galanthamine revealed the absence of any correlation between these parameters, that is, between the primary and the secondary metabolism of this species. This means that the selection of elite genotypes with high biomass yields and high galanthamine content should be performed in two distinct, consecutive steps.

The *in vitro* clones La-3.9, La-4.45, La-5.2, La-7.6, La-7.26, La-7.73, and La-7.80 have been selected as most suitable for further experiments, aiming at studying the susceptibility to stimulation of alkaloid biosynthesis under the conditions of long-term *in vitro* cultivation. Their

high alkaloid content and different alkaloid profiles were considered as very promising for elucidation of the clonal specificity.

3. Reliability of the Disinfection

The reliability of the disinfection procedure is of great importance for the success of the *in vitro* multiplication of high-quality individuals. It depends on the plant features, as well as on the sterilizing protocol. Bulbous plants, especially their underground parts, are usually in contact with numerous bacteria and fungi. However, bulbs are the preferred organs for *in vitro* culture initiation because of their availability during almost the whole year, and the meristem tissues concentrated in their base that are suitable for adventitious bulbing.

The heavy microbial contamination of the wild *L. aestivum* plants gathered from different Bulgarian populations was discussed previously. Tchavdarov *et al*. (22) sterilized whole bulbs taken from Sozopol, Arkutino, and Primorsko in 40% $Ca(ClO)_2$ and reported from 86.6 up to 100% contaminated explants. Initiation of *in vitro* cultures was easier when the bulbs were sterilized 6 weeks after picking up, limiting the contaminated explants to 35.7, 67.7, and 80.0%, respectively. Our earlier trials to optimize the process of testing plant storage for 1−6 weeks before culture initiation, combined with washing out the plant material with tap water for several hours to minimize the saprophyte flora, and disinfection with 0.1% $HgCl_2$ for different time periods, had resulted in 36.5% of bulb explants free of contamination (35). However, this sterilizing agent has to be avoided because of its high toxicity, which also causes damage to the plant tissues. Though the field-grown plants are generally less contaminated with fungi and bacteria, the first trials to sterilize bulbs and leaves taken from the living collection during the spring vegetation period confirmed the need for improvement of the sterilization protocol. All of the widely used disinfectants ensured a low percentage of survival explants, and it was impossible to initiate cultures from some of the initial plants. In spite of the gentle effect of the concentrated H_2O_2 on tissues, it turned out to be not convenient because of the heavy fungal contamination of the *L. aestivum* plants. The solutions of commercial bleach Domestos® and $Ca(ClO)_2$ were better for bulb disinfection, while NaDCC (sodium dichloroisocyanurate) seemed more appropriate for surface sterilization of the leaves. Only mature seeds collected in June were easily sterilized (1 min in 70% ethanol followed by 20 min in 50% commercial bleach). As mentioned previously, seeds were not appropriated for our purposes aiming at the multiplication of selected individuals for *in vitro* production of galanthamine.

The improvement of the survival rate of bulb explants was achieved only after some changes in the disinfection procedure. Bulbs were afforded a long chilling treatment primary to sterilization. They were

stored for 2–3 months in a refrigerator at 5°C in paper bags. Additionally, a two-step sterilization procedure was applied: after the consequent soaking of bulbs in 70% ethanol for 1 min and 50% Domestos® (<5% chlorine) for 20 min, they were halved and put in 30% Domestos® for a further 10 min. After the usual three rinses with sterile distilled water, twin-scales joined by a part of the bulb base plate were excised from the inner part of the bulb and used as primary explants (an average of 25 twin-scales per bulb), placed vertically in test tubes, the basal part slightly dipped into the medium. The outer scales were not used because of their higher microbial contamination and lower morphogenetic potential as reported in our preliminary studies (23).

The assessment was performed comparing two groups of bulbs obtained from the living collection in June 2003, at the end of the vegetation phase. The mother bulbs originated from 11 selected populations of different chemo-types (Table II). Chilling treatment was applied to one of the groups, comprising 12 bulbs, while the 13 bulbs of the control group were sterilized immediately after their collection. The sterilization efficiency was evaluated by Excel ANOVA single factor tool, after 4 weeks of cultivation.

The cold storage of bulbs prior to their sterilization was crucial for the efficiency of the procedure (Figure 10). Most explants from the nonchilled bulbs failed due to contamination with various fungi and bacteria, or due to necrosis; one of those bulbs failed (code 3.19/2) and could not be initiated in *in vitro* culture. The cold storage of *L. aestivum* bulbs improved significantly the survival of the primary explants ($P<0.001$). Nine of the chilled bulbs provided twin-scales free of any contamination. Furthermore, the percentage of the necrotic explants after

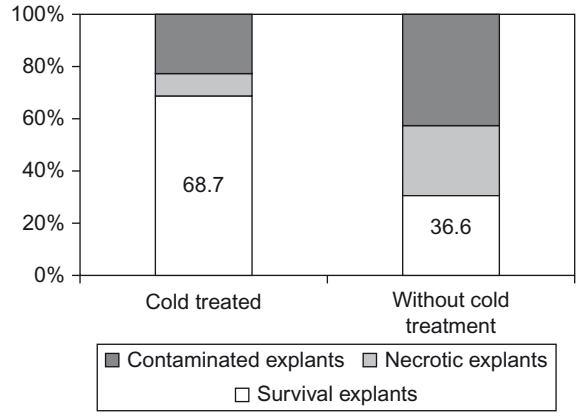

Figure 10 Average axenic, necrotic and contaminated explants of chilled and nonchilled bulbs.

chilling was low (8.86%) and, in the case of two bulbs, all explants survived and manifested morphogenesis. Kohut *et al*. (36) also demonstrated the influence of the low temperature of 2−3 °C applied for a period of 1−5 weeks primary to sterilization, and obtained up to 92.3% sterile explants.

No relation between the sterilization efficiency and the origin of the *L. aestivum* bulbs was observed. Thus, for the bulbs originating from the population Kalinata, the average percentages of axenic explants were 20.0% for the bulbs without cold storage (codes 7.73 and 7.80) and 93.7% for the chilled bulbs (codes 7.6 and 7.26). Similarly, the respective percentages for the bulbs originating from the population Arkutino were: 33.9% for the control bulbs (codes 5.9, 5.27, and 5.40) and 88.2% for the chilled bulbs (codes 5.2 and 5.77).

The significant improvement of the sterilization efficiency was due to the combined influence of the bulb chilling, the two-step procedure, and the use of plants gathered from the field plot instead of the wild populations. The high reliability of the bulb disinfection is of practical importance because it guarantees obtaining *in vitro* culture from any bulb, which is the first prerequisite for the rapid multiplication of high-quality *L. aestivum* individuals by the means of the biotechnology.

B. Tissue Differentiation and Alkaloid Location

Cell suspensions of medicinal plants have been described as the culture of choice for the *in vitro* production of useful metabolites on a large scale, as they are faster growing than tissues and organs cultivated on solid medium, and are easy to manipulate (37). The production of several alkaloids by cell suspension cultures has been successfully scaled-up, some of them to considerable concentrations, others in traces, and with quite different market values: 0.2 g/L ajmalicine from *Catharanthus roseus*, 0.25 g/L morphine from *Papaver somniferum*, 3.3 g/L vincamine from *Vinca*, 2 mg/L reserpine from *Rauvolfia*, and traces of quinine from *Cinchona* (38). Colchicine was produced by callus culture in a concentration of 0.0006% DW (38).

Few compounds were found in cultured cells at higher levels than those observed in the intact plants, examples are artemisin (39) and rosmarinic acid (40). Cell suspension cultures of *Lavandula* produced 60 mg/g DW rosmarinic acid, and a bioreactor callus culture produced 108 mg/g DW, while the intact, field-grown plants produced only 4.8 mg/g DW (41). However, more frequently the yields of the desired metabolites were very low or not detectable in the cell suspensions or in the dedifferentiated callus cells (39). The biosynthetic capacity was preferentially retained by cells organized in tissues and organs. For

example, α-citral and β-citral were produced by *in vitro* shoots of *Cymbopogon* and hypericin by *in vitro* shoots of *Hypericum*. But neither the cell suspensions nor the calli of these species were able to produce any secondary metabolites (41). The alkaloid scopolamine was produced by hairy roots of *Duboisia* and *Hyosciamus* in concentrations of 0.08 g/L and 0.4% DW, respectively (38).

In the case of *L. aestivum*, it was difficult to obtain soft and friable callus necessary for the establishment of cell suspension cultures. Friability was induced only after slight maceration of nonfriable callus by application of the cell wall degrading enzymes Macerozyme R_{10} and Pectinase, followed by consecutive subcultivations on agar and in liquid media (30). These enzymes are also commonly used for the isolation of protoplasts of different species. Although the cell suspensions grew well and achieved a good density of 4.0 g/L, the authors reported a very low content of galanthamine: 0.014 mg/g DW. Furthermore, the biosynthetic capacity of the biomass was found to depend on the tissue differentiation. Shoots regenerated from the callus produced considerably more galanthamine: only 2% of 200 shoot culture lines did not synthesize detectable amounts of the alkaloid; most lines accumulated between 0.001 and 0.1 mg/g DW galanthamine; 22% of the lines – 0.1 to 0.2 mg/g DW, and 10% produced over 0.2 mg/g DW galanthamine (30). The best shoot formation was reported in the presence of 1.15 mg/L NAA and 2 mg/L BAP: up to 70% of the cultured callus pieces developed shoots after 8 weeks. It was important that, during their cultivation in darkness, shoots showed balanced growth and stable morphological characteristics.

In the *in vitro* cultures of *Narcissus confusus*, the alkaloid content also increased with the tissue differentiation: in calli, galanthamine was about 0.15 mg/g DW, whereas in liquid shoot-clump cultures it reached up to 1.42 mg/g DW for the best medium composition (42).

We found much higher concentrations of galanthamine in the directly regenerated shoot-clump cultures, reaching up to 3.63 mg/g DW for the most productive *in vitro* clone La-5.2. It was important that the alkaloid content remained high under the conditions of long-term *in vitro* maintenance. The average galanthamine concentrations of the best clones were over 1 mg/g DW for a period of 24 months (Figure 2). For comparison, the assessment of the plant aboveground mass of several, commercially exploited, *L. aestivum* populations in Bulgaria showed an average galanthamine content from 0.9 to 1.8 mg/g DW (16). The only disadvantage of the directly regenerated shoot-clumps was their relatively slow growth, especially concerning those clones showing a tendency to form crispate shoots and regenerated bulblets with few scales: La-1.19, La-2.72, La-4.45, and La-6.31. Nevertheless, the biosynthetic capacity of such misshapen shoots remained relatively high.

Besides the cell differentiation, the type of organ culture also appeared to be of importance for the success of the *in vitro* biosynthesis. The localization of galanthamine in the main vegetative organs of the *in vitro* obtained plantlets was typical for the plants growing in the field (Table VII). Whenever the galanthamine content was determined separately in the bulbs and the leaves of *L. aestivum* plants, galanthamine was in much higher concentrations in the leaves. Although the ratio between the galanthamine content in the leaves and the bulbs differed, no correlation was found between them. This could be explained by the dynamics of galanthamine accumulation typical for the *in situ* growing plants of the galanthamine chemo-type, as revealed by Stefanov (14). According to these analyses performed every 2 weeks, galanthamine content increased during the spring vegetation period, reaching its maximum during the phases of bud formation and mass flowering in the aboveground plant part and in the bulbs, respectively. With the beginning of the fruit-bearing period, the concentrations of galanthamine in both the herbage and the bulbs decreased sharply by about 40% in only a 2-week period. The lowest content of galanthamine was determined during the summer dormancy. The slight difference between the dynamics of galanthamine accumulation in the main plant organs and the lack of coincidence of the maximal values in them resulted in changing ratios of galanthamine content during the year. Thus, galanthamine was in lower concentrations in the bulbs compared to the leaves from the beginning of March until the middle of April. However, from the first vegetation in autumn until March, it was the opposite situation.

Similar dynamics of galanthamine accumulation in bulbs was also reported for several genera of the Amaryllidaceae family growing in Georgia: *Galanthus woronowii, G. krasnowii, G. caucasicus, G. latifolius, L. aestivum, Narcissus tazetta, Crinum giganteum, Pancratium maritimum*, and *Sternbergia colchiciflora* (43). It was observed that galanthamine levels increased during the spring and reached a maximum at the end of the vegetative phase for all of the tested species, except for *L. aestivum*. Unfortunately, the authors did not report the alkaloid assessment of the aboveground part of the plants prior to their recommendations for the bulbs harvested in the indicated vegetation periods as raw materials for obtaining galanthamine hydrobromide.

The other alkaloids determined in native *L. aestivum* plants were also distributed between the herbage and the bulbs in proportions depending on the seasons (14). Lycorenine and 9-DMHL, main alkaloids in the populations along the Danube River, also reached maximal accumulation in the herbage during the spring vegetation period, 9-DMHL being in a higher concentration in the bud formation phase. In the bulbs, the content of lycorenine was higher than that of 9-DMHL throughout the

Table VII Location of galanthamine and related alkaloids in the main vegetative organs of plants from the living collection, those obtained *in vitro*, and the *ex vitro* adapted plantlets, and the range between their contents (Gal_{leaves} : $Gal_{bulblets}$)

Plants	Organ	Gal (mg/g DW)	Lyc (mg/g DW)	Ung (mg/g DW)	Ratio
La-5.2, *in vitro* on agar medium (Oct 2005, 2-year culture)	Leaves	2.15±0.05	0.00	0.00	1.8
	Bulblets	1.19±0.02	0.00	0.00	
La-5.2, *in vitro* in liquid medium (Dec 2005, 2-year culture)	Leaves	1.62±0.08	0.00	0.00	1.4
	Bulblets	1.15±0.01	0.00	0.00	
La-5.2, *in vitro* in liquid medium (Oct 2006, 4 months in 2 L lab bioreactor)	Leaves	2.39±0.06	0.00	0.00	2.0
	Bulblets	1.17±0.02	0.00	0.00	
Plants from the living collection (May 2005, 4 years after transfer on field)	Leaves	1.54±0.34	0.00	0.00	3.3
	Bulblets	0.46±0.01	0.00	0.00	
Ex vitro adapted plants (Apr 2005, 2 year after transplantation)	Leaves	1.50±0.01	0.00	0.00	1.5
	Bulblets	0.98±0.01	0.00	0.00	

Ex vitro adapted plants (Apr 2005, 1 year after transplantation)	Leaves	2.52 ± 0.31	0.00	0.00	1.9
	Bulblets	1.36 ± 0.04	0.00	0.00	
La-14, *in vitro* in liquid medium (Mar 2006, 4-year-old culture)	Leaves	0.52 ± 0.03	0.00	0.00	1.5
	Bulblets	0.34 ± 0.02	0.00	0.00	
	Roots	0.00	0.00	0.00	
La-14, *in vitro* in liquid medium (Apr 2006, 4-year-old culture)	Leaves	1.22 ± 0.06	0.00	0.00	1.7
	Bulblets	0.73 ± 0.01	0.00	0.00	
La-15, *in vitro* in liquid medium (Aug 2004, 2-year-old culture)	Leaves	1.82 ± 0.64	0.00	0.00	1.7
	Bulblets	1.08 ± 0.15	0.00	0.00	
La-9.6, *in vitro* on agar medium (Apr 2005, 2-year-old culture)	Leaves	0.00	3.75 ± 0.03	0.00	1.1
	Bulblets	0.00	3.52 ± 0.05	0.00	
La-10.4, *in vitro* on agar medium (Apr 2005, 2-year-old culture)	Leaves	0.00	1.06 ± 0.13	1.64 ± 0.15	1.9
	Bulblets	0.00	0.57 ± 0.02	0.25 ± 0.07	

year. The localization of the two alkaloids was reciprocal: 9-DMHL was between 1.1 and 1.6 mg/g DW in the aboveground part and between 0.6 and 1.0 mg/g DW in the bulbs, while for the lycorenine it was the opposite. The main alkaloid for the northeast population, lycorine, differed by its dynamics, decreasing during the vegetation in the spring. The concentration of lycorine in the bulbs was about 20% higher than in the herbage.

The parallel GC/MS analyses of *in vitro* cultures with different cell organization revealed six alkaloids in the bulblets and the shoot-clumps, and four alkaloids in the calli (44). Galanthamine and *N*-formylnorgalanthamine were identified in all of the *in vitro* cultures. Directly regenerated shoot-clumps and bulblets also contained crinine, narwedine, demethylmaritidine, and trisphaeridine, while in the calli, norgalanthamine, and lycorine were additionally identified. These results, however, were not so informative concerning the alkaloid composition of the *in vitro* cultures because they show only a momentary alkaloid pattern. Lycorine and norgalanthamine were determined by routine HPLC analyses in the same long term, shoot-clump cultures during our later tests, sometimes in considerable amounts. These experiments also revealed the transitive disappearance of lycorine in some *in vitro* clones due to the alkaloid dynamics expressed in long-term cultures (see Section II.C.2 for details). Moreover, the *in vitro* dynamics differed from those of the intact plants growing *in situ*.

Diop *et al*. (45) also observed a relationship between the state of tissue differentiation and the accumulation of galanthamine in *L. aestivum* cultures. These authors also succeeded in the induction of hairy roots, which were highly branched and extensively growing on a medium free of growth regulators. However, none of the root cultures, including the hairy roots, produced galanthamine (29). In contrast, the alkaloid was detected in the *in vitro* bulblets in concentrations from 0.01 to 0.07 mg/g DW, depending on the presence and quantities of the growth regulators NAA and BAP in the medium. In our opinion, the low galanthamine concentrations in the bulblets, and its absence in the hairy roots, were most probably due to the genotype features of the mother plants. The authors reported the absence of galanthamine in the initial bulbs supplied from the market. Actually, taking into account the considerable yearly dynamics of galanthamine contents in the bulbs and the leaves of the *L. aestivum* plants revealed by Stefanov (14) for the Bulgarian populations, it is high probable that the mother plants were able to produce some limited amounts of galanthamine. We checked the roots of the *in vitro* plantlets only once and did not detect galanthamine in them, but it is possible that this absence of alkaloids was transitive and related to the dynamics of galanthamine accumulation *in vitro*. The same culture synthesized significantly more galanthamine in the other organs 1 month

later (Table VII, line La-14). The location of galanthamine biosynthesis in the plant is not yet elucidated. Since galanthamine was identified by GS/MS analyses in all of the vegetative organs, including the roots of the *L. aestivum* plants of population Antimovo (44), it is suggested that hairy root cultures obtained from plants with known origin containing high galanthamine content should produce galanthamine.

A very interesting parallel between the biomass growth and the galanthamine content was made concerning the most intensive spring vegetation observed between the phenophases of bud formation and mass flowering (14). Plant herbage increased 30% while the galanthamine concentration decreased by only 0.04%. This observation was evidence of the simultaneous, extremely intensive, primary and secondary metabolism of the plants during a short period of the year. In this regard, it was of great interest for the *in vitro* production of galanthamine to check the stability of both: biomass growth and alkaloid biosynthesis of the *L. aestivum* long-term cultures.

It was very important that the *ex vitro* adapted plants kept their high biosynthetic capacity in the experimental field (Table VII). Similarly, the *ex vitro* plants adapted to the greenhouse and the outdoor plants regenerated from four *Drosera* species maintained *in vitro* at least for 5 years, biosynthesized naphthoquinone in different concentrations, some of them higher than those under *in vitro* conditions (46).

In general, the indirect regeneration increases the chance of somaclonal variation, thus allowing the rise of high producing lines (32,47). However, there is no guarantee with respect to the features of the newly obtained genotypes. The screening for *L. aestivum* plants with a high alkaloid content prior to the initiation of *in vitro* cultures, followed by selection of *in vitro* clones with bulblets of good quality, seems more appropriate and less time consuming due to the strong biological stability of this species. It is well known that plants regenerated by direct organogenesis or somatic embryogenesis are genetically stable, and that the probability of mutation is very low in comparison with those obtained from dedifferentiated callus cells.

C. Impact of the Biological Clock on Long-Term *In Vitro* Cultures

Galanthamine and lycorine are regarded as alkaloids ensuring the protection of the plants in their environment. Indeed, their antiviral effect was confirmed (48,49). In contrast, the *L. aestivum* shoot-clumps were expressing high biosynthetic activity for several years of long-term *in vitro* cultivation, in spite of the comfortable cultivation conditions and the absence of threat. The fact that cell suspensions lost their biosynthetic capacity suggests the existence of a regulation mechanism for alkaloid

metabolism, which seems to be complex and organized at organism level. It appears to determine the dynamics of alkaloid biosynthesis in the intact plants throughout the year, and their unequal distribution between the plant organs, both revealed by Stefanov (14). *In vitro* shoot-clumps representing connected small plantlets composed of bulblets and leaves, and sometimes also developing roots, have kept this mechanism during several years of cultivation. Moreover, the location of galanthamine and the other alkaloids between the main vegetative organs of *in vitro* plantlets was similar to that of the intact plants. Since the roots were not obligatory in the *in vitro* cultures accumulating galanthamine and lycorine, we assumed that they were not related to the control of alkaloid biosynthesis. It was also determined out that the biosynthetic dynamics of the *in vitro* cultures differed from those appropriate to the intact plants and was clone specific. This was of crucial importance for the success of the *in vitro* production and limited the achievable level of biosynthetic stimulation.

1. Dynamics of Bulblet Formation

The morphogenic potential of the bulb explants was not uniform during the year. Comparison between the PCs of cultures initiated in different months allowed us to suggest that the success of *in vitro* multiplication of *L. aestivum* depended on the time of subcultivation, although the temperature and the regime of illumination were permanent. In order to determine the effect of the seasons, five *in vitro* bulblets, which were uniform in size, were subcultured, beginning in November 2004. They were cut into four sectors and the resulting shoots counted once a month during the transfer of the explants to fresh medium. The above-mentioned agar-solidified control MS medium containing BAP and NAA was used. The PC was evaluated as the number of shoots and bulblets obtained per *in vitro* bulblet for 6 months.

The yearly dynamics of shoot formation were clearly revealed. Three periods were well distinguished: the best yield of bulblets was observed when subcultivation was started in the winter or in early spring (December, January, February, or March). Shoot formation began in the first week, and the numbers of obtained bulblets reached up to 23.2 per *in vitro* bulblet for the culture initiated in February (Figures 11 and 12). When subcultivation was performed in April to July, poor results were detected due to necrosis of a part of the explants. Starting in August to November led to retarded formation of shoots during the first months, although the final bulblet yield remained relatively high. The PCs of the three groups of cultures differed significantly ($P < 0.001$) with an average of 21.3, 2.9, and 13.4 regenerated bulblets per *in vitro* bulblet, respectively (Figure 11).

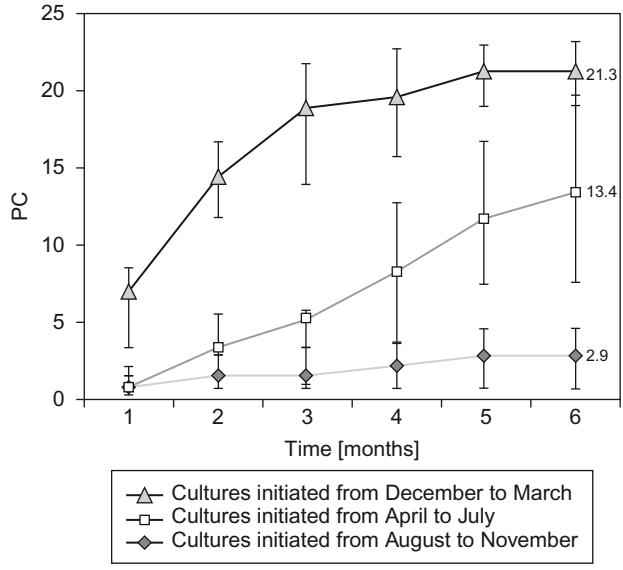

Figure 11 Average propagation coefficients (PCs) for 6-month cultivation of the three groups of cultures initiated along 1 year.

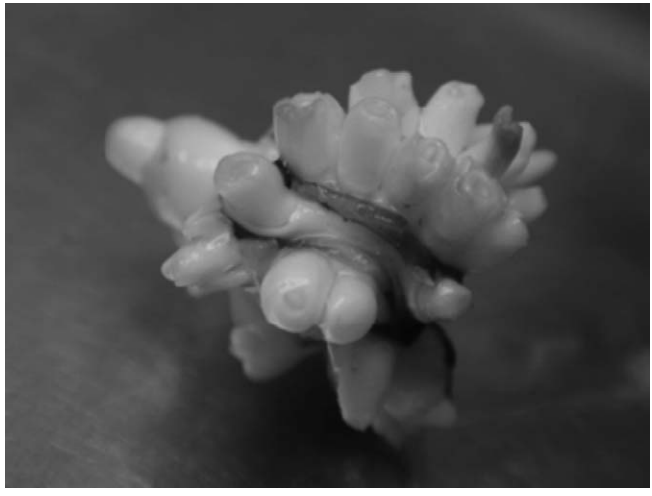

Figure 12 Formation of numerous shoots on bulblet sector (subcultivation started in winter).

These differences were probably related to the annual dynamics of the endogenous plant hormones, such as abscisic acid, that is typical for the bulbous plants growing in open air. New bulblets usually appear *in*

vivo during the dormancy period, for example, in iris, tulip, hyacinth, and lily (50). The 2-year-old cultures of *L. aestivum* expressed different morphogenic potential during the year in spite of the steady medium composition and constant cultivation conditions. It seemed that tissue differentiation occurred in accordance with a form of cell memory. The factors that stimulated the mechanisms of such cell behavior in the absence of temperature and light dynamics are unknown, and need to be further investigated. Whatever the reasons were, this feature could be of practical importance. The morphogenetic response of the tissues, and finally the yield of the *in vitro* regenerated bulblets of the selected individuals, could be significantly increased by choosing the appropriate time for bulblet subcultivation.

The growth periodicity could be due to the carryover effect of endogenous hormones in the relatively short-term cultures, for example, *in vitro* cultures of *Fagus sylvatica* initiated in different seasons, reflected the seasonal behavior of the plant under field conditions when cultured on hormone-free medium (51). In this case, the addition of balanced growth regulators to the medium resulted in similar shoot growth. However, seasonal growth rhythm was reported for 10-year-old *in vitro* cultures of *Populus tremula* (80−120 passages) concerning root formation, shoot elongation, and axillary bud break (52). It was suggested that cell determination and differentiation reflected the expression of specific regulatory genes. The authors succeeded in breaking the growth periodicity only in transgenic plants, thus confirming that periodic behavior may reflect external stimuli and/or endogenous biological clocks of different types with a more or less clear mechanism. Besides the annual periodicity, there are many diurnal rhythms typical for plant physiological processes. Endogenous circadian rhythm of carbon dioxide metabolism was demonstrated in 2-year-old cultures of *Bryophyllum daigremontianum* (53). This rhythm persisted in permanent darkness and the phase was not fixed in time, but was determined by the moment of the darkness beginning.

Treatment with synthetic abscisic acid prior to cultivation or being added to the medium as a plant growth regulator (PGR) was established to enhance the shoot formation of *L. aestivum*. However, the success of the dormancy break was different during the months, and did not compensate for the natural dynamics of the process (unpublished data). More detailed and precise studies should prove useful for acquiring a better understanding of the regulation and appropriate application of the synthetic abscisic acid.

2. Dynamics of the Biosynthetic Activity

In order to investigate the stability of the alkaloid biosynthesis under long-term *in vitro* conditions, 15 clones and 5 one-bulblet derived lines of

different profile were maintained during several years on control nutrient medium, under equal illumination and temperature conditions. Our results proved that the role of the plant genotype was decisive for the biosynthetic capacity of all *L. aestivum* clones maintained in long-term *in vitro* cultures under permanent conditions (Figures 2 and 3). Nevertheless, it was observed that the biosynthetic activity of the *in vitro* cultures was not equal over the course of time. No progressive decrease resulting in biosynthetic loss was, however, observed. In all of the clones, the alkaloid content oscillated between the minimal and the maximal values specific for each genotype (Figure 13). It was surprising because we were unaware of similar dynamics of secondary metabolism *in vitro*. The biosynthesis of alkaloids was expected to be undeviating, reflecting the permanent cultivation conditions *in vitro* comprising temperature, illumination regime, and medium composition.

Variations of secondary metabolites including alkaloids have been reported for many species growing *in situ* or under controlled field conditions, and were attributable to sexual exhaustion and seasonally variable biotic interactions or abiotic parameters. Thus, glaucine was detected in *Croton echinocarpus* only in January and June, and showed a maximum between June and October for *C. hemiargyreus* (54). The concentration of colchicine varied in *Colchicum brachyphyllum* and *C. tunicatum* during different growth stages (55). Crinine, crinamidine, and 1-epi-deacetylbowdensine also showed significant seasonal variation in *Crinum macowanii* (56).

The biosynthetic dynamics that were observed *in vitro* differed from the seasonal variations of galanthamine content revealed by Stefanov (14) for the plants growing *in situ* or under controlled field conditions. In the intact *L. aestivum* plants, clear relationships were found between the alkaloid content and the vegetation phase. Thus, galanthamine was reaching a maximum content during the flowering stage in April, followed by a rapid decrease in May during fruit ripening. High galanthamine content selections from the different populations were detected in different weeks corresponding to the nonsynchronized development of the plants from the tested localities. In contrast, *in vitro* cultures did not bloom and their alkaloid maxima did not coincide necessarily with the flowering season (Figure 13A, B, and E). They expressed clone-specific dynamics of the main alkaloids, often with maxima deviating from the spring or with two maxima yearly. As a result, it was concluded that the flowering might be related to the regulation of the galanthamine content. However, it is not the limiting factor for the biosynthesis. Even if the process is located mostly in the fruit wall and the flower stalks (57), it also obviously takes place in the leaves and bulblets of the *in vitro* plantlets. It was likely the ''missing of seasons'' in the phytostatic room that caused some disorders in the

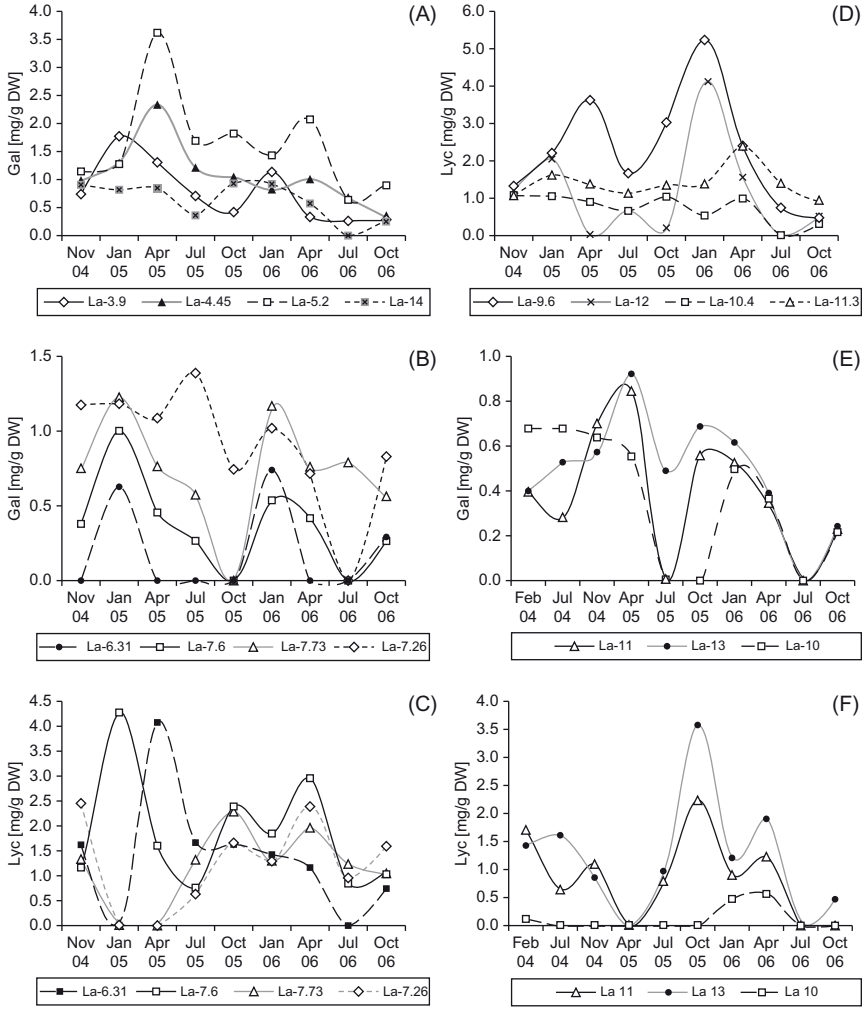

Figure 13 Dynamics of galanthamine and lycorine contents of several *L. aestivum* genotypes culture *in vitro*: clones with origins indicated in Table 2, initiated in autumn 2003, and tested during 24 months; lines, initiated in autumn 2002 by subcultivation of one bulblet each, tested during 33 months. (A) Four galanthamine genotypes; (B, C) four mixed galanthamine–lycorine genotypes, (D) two lycorine and two lycorenine–9-DMHL genotypes; (E, F) three mixed galanthamine–lycorine genotypes.

alkaloid dynamics, although did not annul alkaloid synthesis variability. It is quite possible that the regulation of the biological clocks of the *in vitro* plantlets were disturbed by the absence of both the usual dynamics of the external factors, temperature and light, and the flowering.

Furthermore, it is not clear that all the extremes of alkaloid dynamics have been detected, since our analyses were performed at intervals of 3 months. Some of the one-bulblet derived lines were tested even more rarely at the beginning because of the limited amounts of the cultures. Shoot-clumps were needed for the regular HPLC analyses, and also for the numerous experiments performed on agar and in liquid media. All of the clones and lines were multiplied by regular subcultivations of the large bulblets in order to provide enough shoot-clumps for the major experiments aimed at the stimulation of galanthamine biosynthesis. The percentage of the dry matter of the shoot-clumps, calculated at the same time points, showed also some alteration, suggesting a tendency to increase in winter (Figure 14).

No pronounced fluctuation periodicity of galanthamine changes over the course of time was established because of the quite different harmonic components in the autocorrelation function (ACF) established by *R* (Figure 15). Similarly, nonseasonal temporal variation of alkaloid content with a high degree of intercolony variability was observed in some cases in plants growing at open-air, as had been observed previously for four pyridoacridine alkaloids of the purple morph of the ascidian *Cystodytes* sp. (58). These mechanisms of rhythmic behavior are often complex and not always clear.

Regarding the established dynamics of the two main alkaloids in all tested clones and lines, the role of the genotype factor was characterized by ANOVA analysis at several levels corresponding to the number of the genotypes. In this way, the dynamics can be assessed for each genotype by the variation in the group. The high *P*-values proved that the variations of the average galanthamine and lycorine contents between the genotypes over weighed significantly their variations in the course of time. Thus, the selection of prospective *in vivo* and *in vitro* genotypes was

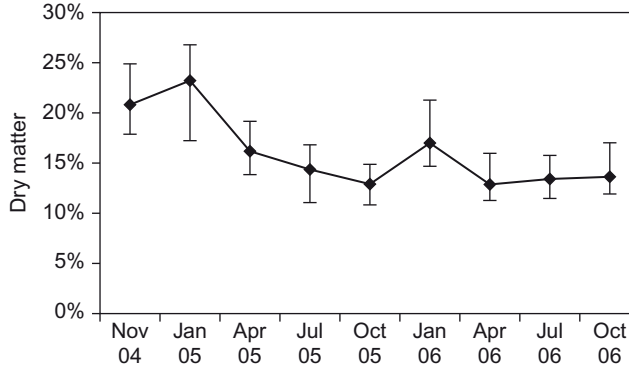

Figure 14 Fluctuations in DW/FW ratio of all tested *L. aestivum* clones.

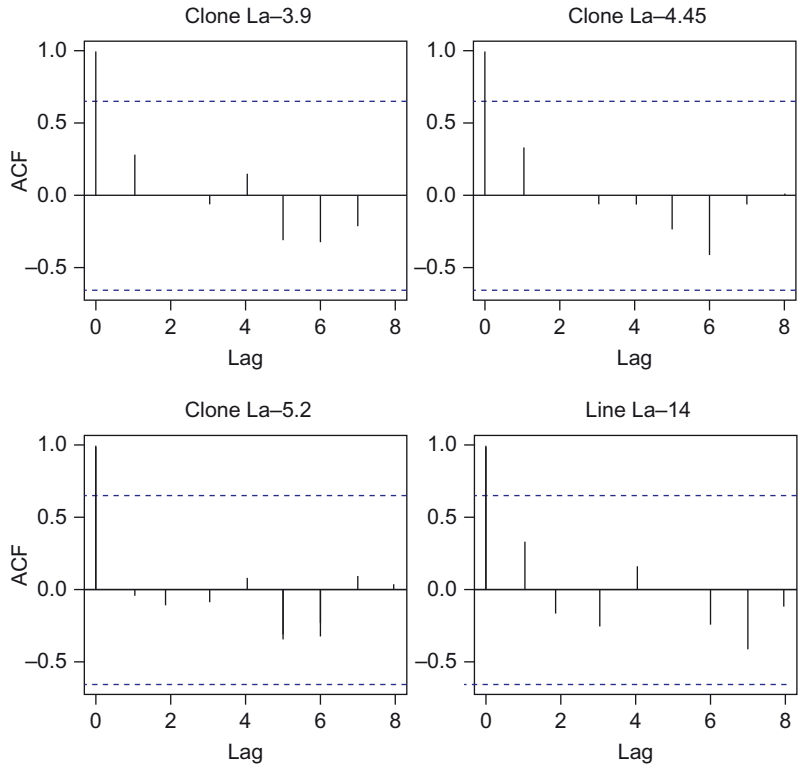

Figure 15 Autocorrelation function (ACF) of galanthamine content in four galanthamine type clones illustrate different behavior regarding periodicity for a period of 2 years. The dashed lines show the level of significance that the harmonic components must reach, so that their presence is regarded as systematic and not as random.

confirmed again as most important step toward a further increase of the biosynthetic activities.

In order to study the degree of the disturbance of the biological clocks of the *in vitro* cultured plantlets, each clone and line was tested for its biosynthetic synchronism, by correlations between galanthamine content, lycorine content, and culture dry matter (Tables VIII–X). Furthermore, each pair of clones, respectively pair of lines, was tested for their detailed monthly correspondence following the same parameters. Some of these, concerning four galanthamine, and four mixed galanthamine–lycorine genotypes, are illustrated by pair-scattered graphics (Figures 16 and 17).

A correlation was found between the fluctuations of the dry matter of most of the clones (Tables VIII and IXA). Linear dependencies were often based on some scattered points: (La-3.9, La-5.2), (La-4.45, La-5.2), and

Table VIII Correlation coefficients and significances of the differences between Gal contents, DW/FW ratios, and Gal contents and DW/FW ratio of four Gal-type clones

Correlations between Gal and DW/FW of one and the same clone	Clone	Correlations between Gal content of each clone pair			
		La-3.9	La-4.45	La-5.2	La-14
Correlations between DW/FW ratio of each clone pair	La-3.9	0.876***	0.604*	0.329	0.566*
	La-4.45	0.991***	0.264	0.887***	0.482
	La-5.2	0.866***	0.861***	−0.186	0.472
	La-14	−0.485	−0.466	−0.299	−0.149

*$P \leq 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table IXA Correlations between the DW/FW ratios of each mixed-type clone pair, and between the dry matter and galanthamine content, and the dry matter and lycorine content of each of the tested clones

Correlations between dry matter and Gal (up) or Lyc (down) content of one and the same clone	Clone	Correlations between DW/FW ratios of each clone pair			
		La-6.31	La-7.6	La-7.73	La-7.26
	La-6.31	0.559* −0.229	0.922***	0.973***	0.973***
	La-7.6		0.814** 0.577*	0.942***	0.854***
	La-7.73			0.600* −0.668*	0.926***
	La-7.26				0.226 −0.263

*$P \leq 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table IXB Correlation coefficients and significances of the differences between galanthamine content, lycorine content, and galanthamine and lycorine content of four mixed-type clones

Correlations between Gal and Lyc content of one and the same clone	Clone	Correlations between Gal contents of each clone pair			
		La-6.31	La-7.6	La-7.73	La-7.26
Correlations between Lyc contents of each clone pair	La-6.31	−0.344	0.675*	0.676*	0.237
	La-7.6	−0.233	0.685*	0.776**	0.561*
	La-7.73	−0.183	−0.194	−0.613*	0.158
	La-7.26	−0.168	−0.174	0.782**	−0.194

*$P \leq 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table X Correlations between the biosynthetic activities of tested one-bulblet derived lines on control medium for 33 months

Correlation coefficients between Gal and Lyc of the same clone		Correlation coefficients between Gal contents of all clone pairs				
	Clone	La-14	La-11	La-13	La-10	La-12
Correlation coefficients between Lyc contents of all clone pairs	La-14	0.91	3.40***	3.31***	1.42	1.11
	La-11	0.22	0.40	2.68*	1.49	1.32
	La-13	−0.22	3.79***	0.70	1.05	0.63
	La-10	−0.50	0.42	0.56	0.51	1.27
	La-12	0.01	0.26	0.38	2.12**	−0.07

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

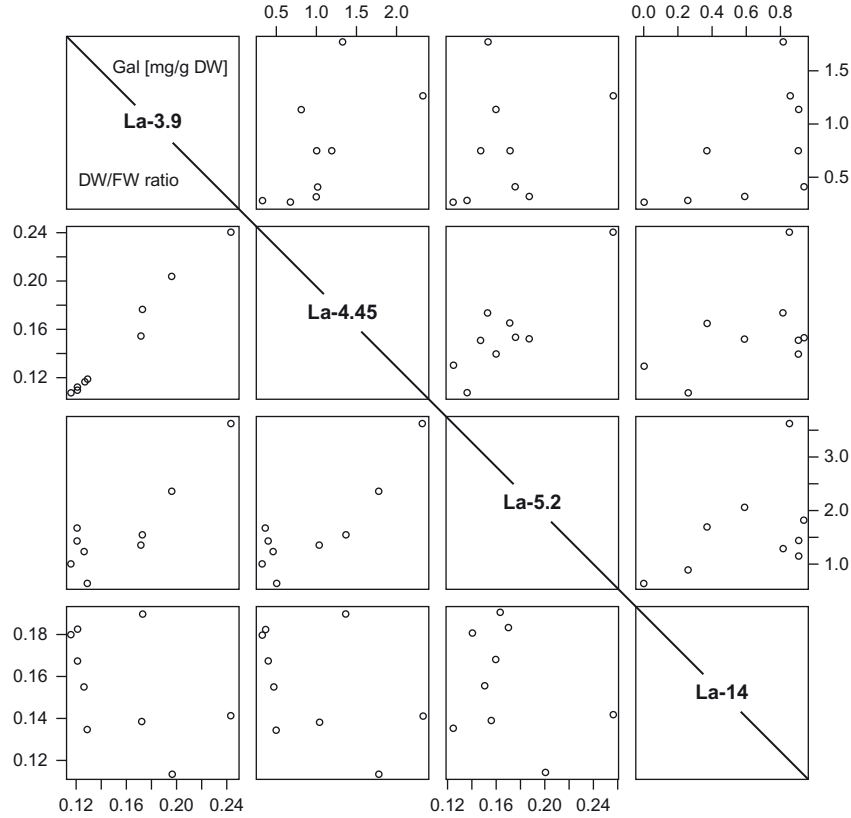

Figure 16 Pair-scattered graphics depicting the relationships between galanthamine contents and DW/FW ratios of four Gal-type *L. aestivum* clones.

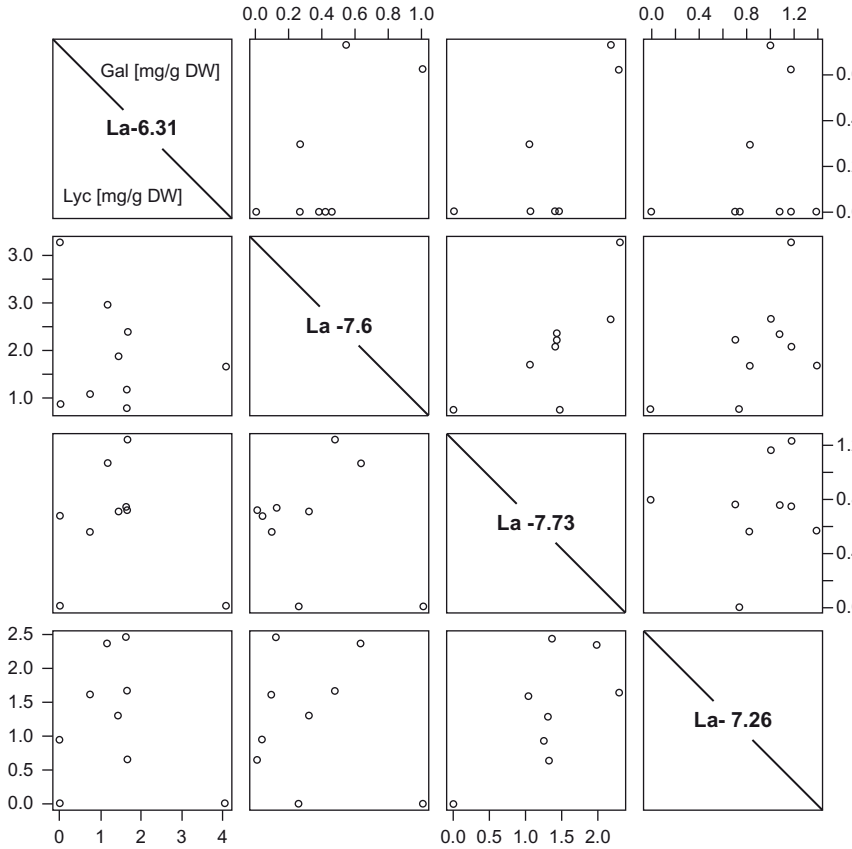

Figure 17 Pair-scattered graphics depicting the relationships between galanthamine and lycorine contents of four mixed *L. aestivum* clones.

(La-4.45, La-14), which explains the absence of periodicity (Figures 16 and 17). Correlations were revealed between the DW/FW ratio and the content of galanthamine or/and lycorine in clones La-3.9, La-6.31, La-7.6, and La-7.73 (Tables VIII and IXA). Correlations in the mixed-type clones were diverse. In clone La-7.6 for example, the dynamics of galanthamine, lycorine, and dry matter correlated positively, while in clone La-7.73, the correlation between galanthamine and dry matter was positive, but those between galanthamine and lycorine and between lycorine and dry matter were negative (Tables IXA and IXB).

The biosynthetic activities in some line pairs also manifested synchronism. Thus, the dynamics of galanthamine content of the Gal-type line La-14 and the mixed-type lines La-11 and La-13 correlated, as well as those of the lycorine contents of lines La-11 and La-13, and of the Lyc-type line La-12 and the mixed-type line La-10 (Table X). Partial

synchronism of the main alkaloids lasting several months was noticed between some of the clones. It also seemed that the maxima of many clones were shifted from April to January (Figure 13). All these observations suggested some kind of disturbance of the inner plant clock.

3. Ostensible Alteration of the Alkaloid Profile

The contents of both main alkaloids decreased to zero at some point in the development in all tested clones and lines (Figure 13). Although no permanent fall was noticed, except for lycorine in the Gal-type line La-14 and for galanthamine in the Lyc-type line La-12, which were detected only in February and November 2004 in small concentrations (up to 0.4 and 0.1 mg/g DW, respectively).

In contrast, some related alkaloids were found only in the first months of the *in vitro* cultivation. Norgalanthamine was detected in genotypes La-5.2 and La-14 (Figure 19), La-9.6 (0.76 mg/g DW in January 2005), La-6.31 (1.14 mg/g DW in April 2005). Similarly, ungiminorine was found in lower quantities (between 0.09 and 0.56 mg/g DW, in January and April 2005) in clones La-10.4 and La-11.3. After the first 2 years of *in vitro* cultivation (spring 2005), the related alkaloids were no longer produced.

In some mixed-type clones, lycorine predominated during the whole test period, even if the ratio of the two alkaloids varied due to the different amplitude of their dynamics (Figure 18A). Clone La-7.6 was a good example of a noninterrupted biosynthetic activity. Other clones were distinguished by alkaloid dynamics that were more complex. Clone La-7.73 appeared the most confused during the initial tests (Figure 18B). Because of the negative correlation between galanthamine and lycorine dynamics, it seemed as though the alkaloid profile changed many times. Initially, it was considered as a mixed-type clone with a predominance of lycorine (November 2004). However, it moved to a Gal-type clone in the winter and spring of 2005, and then back to a mixed-type clone in July 2005, followed by appearing as a Lyc-type clone in October 2005, and then again a mixed-type clone until the end of the experiment, but with different proportions of the two main alkaloids. Continuous studies and comparison with the other mixed-type clones clarified, however, that these changes did not concern the alkaloid profile of the clone, and reflected only the opposite dynamics of galanthamine and lycorine biosynthesis.

No compensatory activity of the biosynthesis was observed and the summary amount of the two main alkaloids differed over the course of time, going to zero at some points of coincident fall, like in July 2006 for all lines and clone La-6.31 (Figure 18C), and in July and October 2005 for La-10 and La-12. The differences in alkaloid dynamics of clones La-7.6, La-7.73, and La-7.26, which originated from the same population,

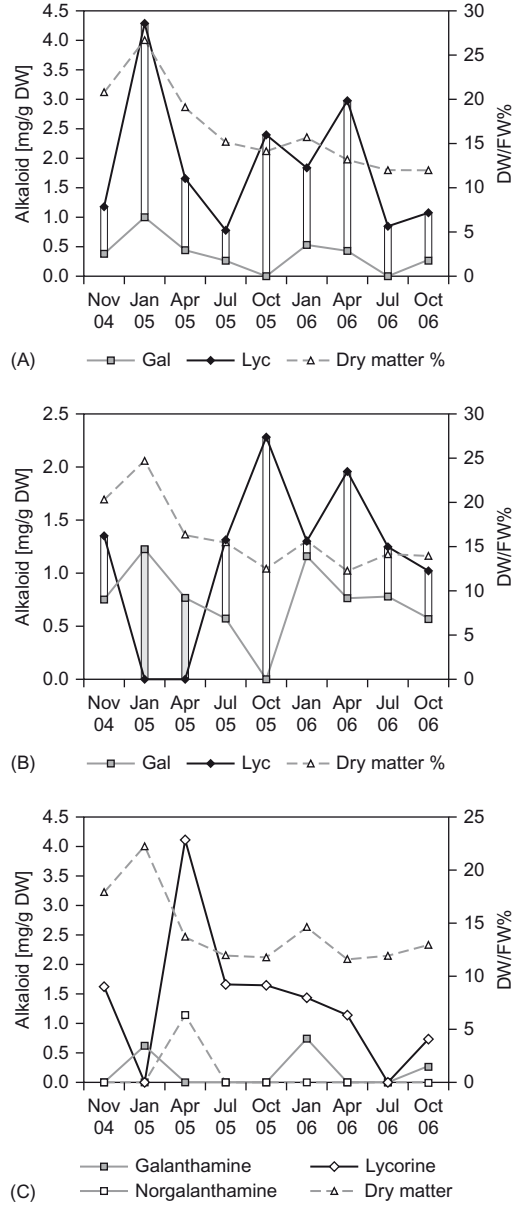

Figure 18 Dynamics of galanthamine, lycorine, and dry matter, with positive correlation: clone La-7.6 (A); negative correlation: clone La-7.73 (B); and lack of correlation: clone La-6.31 (C).

confirmed once more that plant features were strongly individual and stable even under long-term *in vitro* cultivation.

In spite of the alkaloid dynamics, in some clones the alkaloid content seemed to have a tendency to decline, like galanthamine in clones La-3.9, La-4.45, and La-5.2 (Figures 13A and 19A), lycorine in La-6.31 (Figure 18C), and galanthamine and lycorine in La-7.6 (Figure 18A). However, in clones La-9.6, La-12, and La-11.3 (Figure 13D) lycorine reached higher concentrations during the second year. In clones La-6.31 (Figure 18B) and La-7.73 (Figure 18B), the maximum values of galanthamine in the 2 years were similar, as well as the maximum values of lycorine in clone La-10.4 (Figure 13D). Even in the case of an abrupt decrease of the alkaloid content, it would be hasty to conclude that the biosynthetic capacity has been lost. Temporary alkaloid disappearance was followed by accumulation in shoot-clumps in significant quantity, as was observed for lycorine in the clones La-6.31, La-7.73, and La-7.26 (Figures 13C and 18B, C) and for galanthamine in line La-14 (Figure 19B). The interval of 3 months between the analyses is very likely to have been quite long, and some

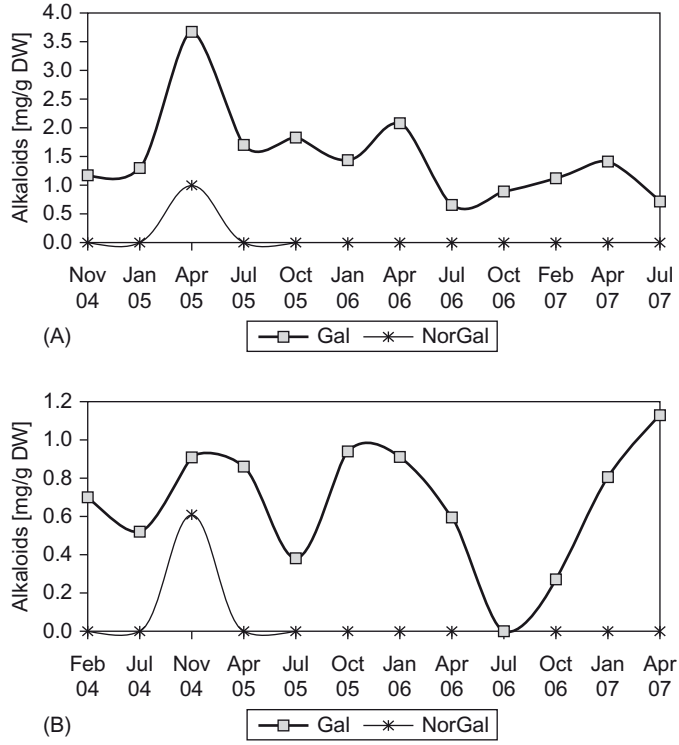

Figure 19 Dynamics of Gal content in clone La-5.2 (A) and line La-14 (B) for a period of 33 months, and related alkaloids in the first months of test.

possible rapid changes of the alkaloids' contents and peaks that might occur between the months of test were probably missed.

Although the reasons of such alkaloid dynamics are not yet elucidated, their existence is without doubt, since the phenomenon was observed in all of the studied clones over several years. If the clone specificity of the alkaloid dynamics had appeared as a result of the disturbance of the biological clock of the plants, the changes could not be predicted for the future. In any case, the dynamics of alkaloid biosynthesis under long-term *in vitro* conditions should be considered in the selection of high producing clones, as well as in all future experiments aimed at an increase in the galanthamine or lycorine content of *L. aestivum* liquid cultures. Galanthamine content in shoot-clumps of *L. aestivum* frequently surpassed the industrial requirement of 1 mg/g DW (Figure 13A and B). An understanding of the regulation of the biosynthetic activity of these alkaloids should be of great practical importance.

Similar data about the dynamics of *in vitro* biosynthesis of secondary metabolites could not be found, although we assume that this could be more common phenomenon, based on the mechanisms that indicate conformity with the environmental conditions. Altman *et al.* (52) proposed that ''specific existing and induced mechanisms contribute to clonal stability in tissue culture, and that these are at least as important as the suggested mechanisms of variation induction *in vitro*.'' Obviously, clonal stability related to the culture propagation features is perceived as being easier, because many researchers are maintaining long-term *in vitro* clones. In contrast, experiments aiming at biosynthesis *in vitro* are usually short, and are analyzed separately. Some of our experiments failed with poor results. However, their understanding became possible later, when considered as a small part of the whole puzzle. We are convinced that the clonal stability should be studied at the anatomical, phytochemical, and molecular levels.

III. STIMULATION OF BIOMASS GROWTH AND GALANTHAMINE BIOSYNTHESIS *IN VITRO*

In vitro production of secondary metabolites is an attractive and important alternative to the gathering of rare medicinal plants from the wild. Nevertheless, the scale-up of the process is often hampered by the low biosynthetic capacity and its gradual attenuation in long-term *in vitro* cultures. This is associated with genetic or epigenetic changes of the regenerated plantlets that might occur under the specific conditions of *in vitro* cultivation systems (59). Usually, secondary metabolism is much more intensive in the intact plants compared to the *in vitro* cultures.

There are a few examples of the higher production of the desired compounds under *in vitro* conditions, for example, 108 mg/g DW rosmarinic acid from the callus of *Lavandula officinalis* compared with 4.8 mg/g DW in field-grown plants (41). *In vitro* long-term cultures of *Drosera madagascariensis* and *D. communis* maintained for at least 5 years, biosynthesized significantly higher concentrations of naphthoquinone compared to the plants *ex vitro* adapted to greenhouse and outdoor conditions (60). These compound levels were much higher than that of the typical market drug.

Actually, the successful manipulation of the bioproduction of secondary metabolites *in vitro* requires elucidation of the rationale for their biosynthesis in the whole plant. Many suggestions have been made concerning the role of the alkaloids for the plant metabolism, however, their functions are still obscure (61). Alkaloids were previously considered as nitrogen waste products, like urea and uric acid in animals, or as nitrogen storage reservoirs, even if many of them are accumulated in the plants and are not further metabolized. The possible protective role of the alkaloids against insect predators or herbivorous animals was widely assumed because of their toxicity. However, this concept was regarded as anthropocentric since many alkaloids that are poisonous to man have no effect on other animals; rabbits, for example, regularly eat belladonna and opium poppy plants. We also saw sheep grazing *L. aestivum* plants in some of their natural habitats. Probably the functions of the alkaloids in the plant organism are diverse, as alkaloids are very heterogeneous group of natural products. Obviously, their metabolism is related to nitrogen and their concentrations in the host plants are not stable over the course of time, for whatever reason. The reasons for alkaloid accumulation, typically in different plant organs, and the dynamics of alkaloid biosynthesis throughout the year are not known.

Currently, different approaches are under development, aiming at enhancing the production of secondary metabolites *in vitro*, including elicitation, feeding with precursors, and metabolic engineering (39). We tested media with different compositions and consistence, as well as diverse explants and culture vessels, in order to stimulate the biomass growth and alkaloid biosynthesis.

A. Cultivation Conditions

During their phenogenetic evolution, plants developed diverse mechanisms in order to adapt to the changing conditions of their typical ambience. The most important factors limiting plant distribution and growth are temperature and the length of the day, specific for the different geographical latitudes and altitudes. Soil structure and

composition, as well as water availability, also influence the expression of the genotype resulting in phenotype forms differing in their size and metabolism.

As far as the temperature and the illumination regime in the phytostatic chambers and the composition of the nutrient medium are liable to change, the manipulation of the *in vitro* culture development is expected to be straightforward. However, it appears that the behavior of the *in vitro* plants had been firmly conditioned by the natural locality. Their biological clocks are determining the metabolic features even in long-term *in vitro* cultures. In the case of bulbous plants, the dormancy period was often considered as a seasonal recalcitrance causing slow growth.

1. Liquid Cultures

In general, liquid cultures enhance the growth of *in vitro* cultures and allow the scale-up and automation of the labor-intensive methods of shoot multiplication, which is very important for the commercial biosynthesis of bioactive compounds offering the possibility to lower the cost of the products. Some problems, like hyperhydricity and leakage of endogenous growth factors, still limit the use of bioreactors for plant biomass production. Advantages and difficulties of the mass propagation of plants were discussed in detail by some authors (62–64). Temporary immersion systems were regarded as an alternative to cultivation under permanent submersion in bioreactors needing an oxygen supply in the liquid medium in order to avoid cell death (65). The propagation of some bulbous species from the genera *Lilium, Ornithogalum, Nerine*, *Narcissus*, and *Cyclamen* in bioreactors has been enhanced through the organogenic pathway (66).

The liquid organ cultures of *L. aestivum* that were established were able to synthesize galanthamine in shaken, as well as in static, flasks and plastic containers. Containers were most suitable because of their square form and lightweight allowing for the arrangement of numerous vessels on the shaker on several levels. Moreover, the shaker was working at a slower pace of 55 rpm, instead of the usual 110 rpm suitable for the flasks, in order to avoid undue agitation causing the shoots to hit against the container walls. Static cultures without agitation also seemed to be suitable for this species. Usually, no hyperhydricity was observed, probably because the natural habitats of *L. aestivum*, which are along rivers are often inundated.

The efficiency of alkaloid biosynthesis *in vitro* could be correctly evaluated only with respect to the biomass growth rate and the alkaloid concentration. In the case of alkaloid accumulation in the biomass, the percentage of dry matter is also of great importance. In addition, the possible release of alkaloids into the medium should be also considered.

Table XI Comparison of the growth parameters of *L. aestivum* shoot-clumps, line La-14, cultured on agar-solidified and in liquid control medium (cultivation in Magenta, 100 mL per container; liquid cultures were static)

Medium	GI	Dry matter (%)	G_eC
Agar	1.02 ± 0.3	14.0 ± 1.8	0.28 ± 0.02
Liquid	1.91 ± 0.1	11.7 ± 0.2	0.34 ± 0.01

The growth rate of the *L. aestivum* shoot-clumps was much higher in static liquid culture than on agar-solidified medium ($P < 0.01$), while the dry matter did not decrease significantly (Table XI). In order to compare the growth rates of the fresh biomass cultured under different conditions, taking in account the small differences between the initial fresh weights (FWs) of the inocula consisting of several shoot-clumps, the coefficient growth index (GI) calculated using the formula: $GI=(FW_{final}-FW_{initial})/FW_{initial}$ was used. To assess the final efficiency of the biomass augmentation, taking into account additionally the percentage of the dry matter, we inserted the growth efficiency coefficient (G_eC): $G_eC=DW/FW_{initial}$. This evaluation needed the biomass to be dried and the dry weight (DW) determined. Finally, the growth efficiency was demonstrated to increase significantly in liquid medium ($P < 0.001$).

The alkaloid concentration of the biomass was also influenced by the consistency of the medium due to several reasons. One of them was the fast, spontaneous elongation of the leaves in liquid medium that altered the initial ratio between the two main vegetative organs of the plantlets (Figure 20A). However, galanthamine remained in much higher concentration in the leaves (Table XII). Thus, even the bulblets contributed to the accumulation of about two-thirds of the phytomass as a result of their twice as high DW, the leaves provided almost half of the galanthamine yield (Figure 20B, C). The related alkaloids were also present in the liquid culture of *L. aestivum*.

Accelerated multiplication of the *in vitro* material of *L. aestivum* was achieved by alternate passages on agar-solidified and in liquid media. Small shoot-clumps obtained on solid medium were detached from the initial explants and grown in liquid medium for the faster development of large bulblets, which were then subcultured on solid medium by a longitudinal cut to produce four new bulblet sectors. Similarly, the period of 4 months needed by the small bulblets of *P. maritimum* to reach a size suitable for subcultivation was shortened to 6 weeks using a liquid medium with the same composition (26).

The plantlets cultured in liquid and on agar-solidified medium differed in their basic physiological parameters. Comparison between leaves taken from field-cultivated plants and plantlets from long-term *in*

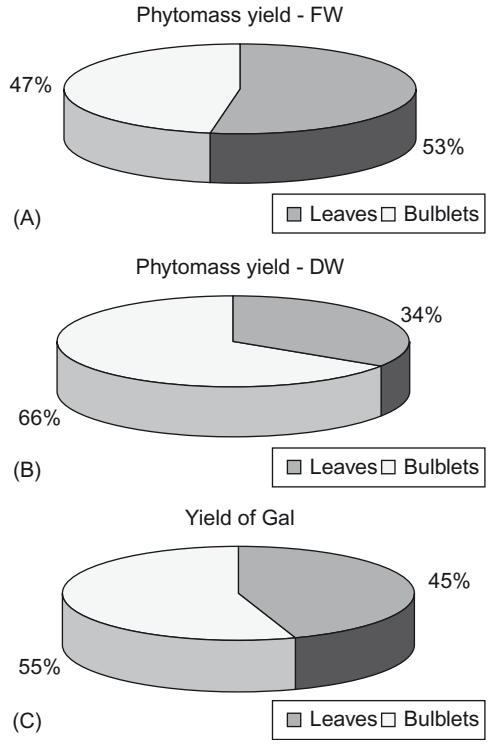

Figure 20 Ratio of the leaves and the bulblets in the yield of *L. aestivum* phytomass and galanthamine, after 3-month cultivation in liquid medium: (A) Fresh phytomass; (B) dried phytomass; and (C) galanthamine (Gal).

Table XII Location of alkaloids in the organs of plantlets grown for 1 month in a control liquid culture (July/August 2004)

Organs	Dry matter (%)	Alkaloids (mg/g DW)[a]					
		Gal	Lyc	NorGal	Ung	G-non	HomoLyc
Leaves	8.9	1.82±0.64	0.30±0.33	0.54±0.55	0.20±0.09	0.04±0.01	0.01±0.02
Bulblets	19.2	1.07±0.15	0.25±0.35	0.80±0.37	0.14±0.02	0.03±0.01	0.02±0.04

[a]Gal, galanthamine; Lyc, lycorine; NorGal, norgalanthamine; Ung, ungiminorine; G-non, galanthaminone; HomoLyc, homolycorine.

vitro cultures demonstrated that photosynthesis depended on the cultivation conditions. The functional activity of photosystems I and II, as well as the oxygen evolution rate of the leaves, were compared. The photosynthetic rate of field-grown plants was double that of *in vitro* plantlets, although *in vitro* plants were distinguished by an intense green

color, like the intact plants. Results were similar for the *ex vitro* plants that were adapted to open-air plants and those plants transplanted from the natural populations in the experimental field in 2001, whereas the photosynthesis was more fully expressed by the *in vitro* plantlets cultured in static liquid medium compared to those grown on the agar solidified medium. These results determined that the *L. aestivum* shoot-clump liquid culture was a suitable system for *in vitro* alkaloid production. The observations of the stomata apparatus of the submerged leaves indicated, however, some structural modifications resulting in abnormal functioning. Numerous anatomical differences were reported between the vegetative organs of *in vivo* and *in vitro* grown *Dianthus callizonus* caused by the high humidity of the air enclosed in the culture vessels (67). The stomata apparatus was blocked, and the trichomes were reduced and with altered morphology, the deposition of epicuticular waxes was reduced, and hypertrophy of the mesophyl was observed. All these changes were concluded to be a stress response to the *in vitro* conditions, as typical symptoms for hyperhydricity. Even if hyperhydricity was rarely observed in the liquid cultures of *L. aestivum*, it will be important to test the cultivation of shoot-clumps in a temporary immersion system for additional enhancement of the growth. Moreover, the combination of bioreactors and temporary immersion systems were recommended as being very potent for the longer cultivation period required for many plant species (65). However, the best conditions should be experimentally determined for each species, according several criteria. Thus, even if root regeneration from nodules of *Charybdis numidica* was improved by cultivation in temporary immersion system (68), the authors reported that the regeneration rate of this species was significantly higher when the nodules were cultured on semisolid medium (60). The shoots of this plant seemed to be sensitive to submerged cultivation and expressed some hyperhydricity, even in a temporary immersion system, however, this system was very attractive as offering significant labor savings.

The cultivation conditions are also able to influence the effectiveness of the *in vitro* biosynthesis of secondary metabolites. Recently, temporary immersion systems were demonstrated to enhance the biomass growth and the biosynthetic activity in many plant species. Thus, *in vitro* shoots of *Cymbopogon* propagated in microcontainers produced 0.27 mg/g DW α-citral and 0.46 mg/g DW β-citral, while shoots propagated in a temporary immersion system increased these concentrations to 0.35 and 0.54 mg/g DW, respectively. For comparison, the intact plants grown on the field produced 3.95 mg/g DW α-citral and 10.25 mg/g DW β-citral (41).

The quality of the inoculum was of great importance for alkaloid biosynthesis. Long-term cultures of *L. aestivum* consisted of bulblets of different sizes, bulblet sectors of subcultured bulblets with small shoots

forming between their scales, small shoot-clumps growing on the explants or around the bulblet periphery, and big shoot-clumps detached from the explants. Usually the shoots were shapely and formed well-distinguished green leaves sticking up at the round shoot base. Some of the cultures were, however, morphologically heterogeneous, containing formless structures of less intensive green along with the bulblets of good quality. The normal organ differentiation was found to be a limiting factor for the biosynthetic capacity (Table XIII). We selected the optimal *L. aestivum* inoculum by separate cultivation of different types of organs of different quality in a control liquid medium containing BAP and NAA. Well-shaped shoot-clumps were further used for all experiments in liquid cultures. Large bulblets were not suitable for the inoculum as they were regularly subcultured to obtain the new sectors necessary for plant material multiplication.

The first alkaloid test of six genotypes in liquid cultures (October 2004) suggested a significant decrease of the galanthamine and lycorine concentrations (Figure 21). Although clones La-3.9, La-4.45, and La-5.2 kept their Gal-type profile, La-9.6 remained of the Lyc-type, and La-7.26 of the mixed Gal−Lyc-type, the high concentration of norgalanthamine in clone La-7.6 was unexpected. At the same time, lycorine was not detected. Only through further regular tests, revealing the characteristic dynamics of the main alkaloids, were these results elucidated.

The percentage of the inoculum was also of importance for the alkaloid yield. Thus, a tendency for the enhancement of alkaloid biosynthesis was observed when equal quantities of shoot-clumps of clones La-5.2 and La-9.6 were cultured in parallel in Vitro Vent containers containing 100 and 50 mL medium, respectively (Figure 22). This effect was further expressed when the cultivation was performed in simple, self-constructed, laboratory bioreactors containing 500 or 1000 mL medium. In this case, the oxygen was supplied either by bubbling sterile air from the bottom of the 4-L large flask (airlift type reactor ensuring also the medium agitation), or by passive exchange of inside and outside air trough a narrow pipe. Besides the ports for air inlet, there was another one open only for medium exit or sampling, using a typical aquarium

Table XIII Content of galanthamine (mg/g DW) in liquid organ cultures of *L. aestivum*

Type of inoculum	Gal (mg/g DW)
Shapely soot-clumps	0.73 ± 0.05
Explants with shapely shoots	0.30 ± 0.17
Formless shoot-clumps	0.00
Explants with formless shoots	0.00

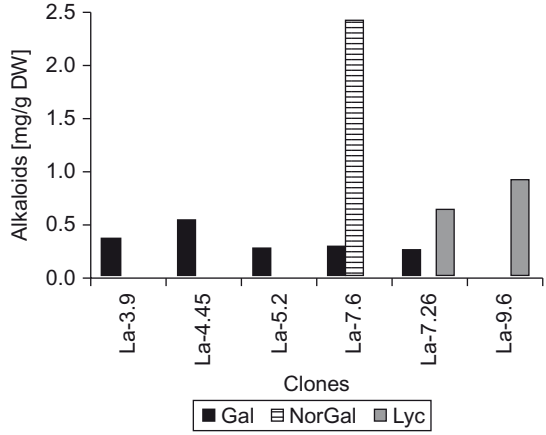

Figure 21 First test of alkaloid contents of six genotypes cultured in liquid control medium (August/October 2004).

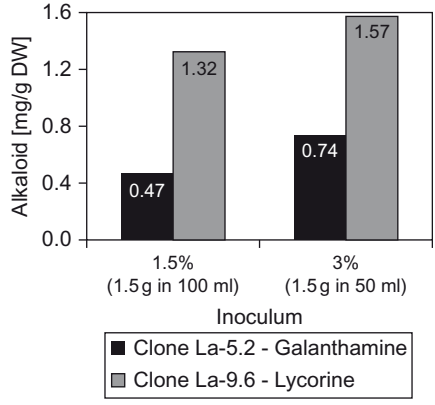

Figure 22 Galanthamine and lycorine contents in shoot-clump cultures of clones La-5.2 and La-9.6, after 2-month cultivation in control liquid medium, differing only in the percentage of the inoculum (June–August 2005).

pump. The other tubules were supplied with sterile filters at the end. Medium filling was performed under the sterile conditions of the laminar hood. Of course, the alkaloid profiles and contents differed according to the genotypes of the tested clones. However, the type of aeration influenced the biosynthetic activity. Shoot-clumps of La-15 yielded higher amounts of both galanthamine and lycorine when the air exchange was passive, with only 1 min per day of active airflow through the liquid medium (Table XIV). Halving the medium volume, thereby doubling the percentage of the inoculum, did not lead to a decrease of the

Table XIV Influence of the inoculum amount and the type of aeration of self-constructed laboratory bioreactors on the productivity of *L. aestivum* line La-15 cultured 2 months in equal liquid media (October 26/December 27, 2005; control medium was replaced by fresh medium containing precursors at the end of the first month)

Inoculum in % (medium volume) Aeration	3% (1000 mL) Permanent	3% (1000 mL) 1 min/day	6% (500 mL) 1 min/day
GI	0.31	0.46	0.54
Dry matter %	13.2	13.0	13.2
Gal (mg/g DW)	0.37	0.43	0.49
Gal yield (mg)	1.90	2.62	2.99
Gal (mg/L)	1.90	2.62	5.98
Gal (mg/L)[a]	0.95	1.31	2.99
Lyc (mg/g DW)	0.71	1.09	1.11
Lyc yield (mg)	3.67	6.31	6.62
Lyc (mg/L)	3.67	6.31	13.24
Lyc (mg/L)[a]	1.83	3.15	6.62

[a]Effectiveness of the biosynthesis according to the total quantity of medium.

alkaloid quantities, but did finally increase significantly the alkaloid yield calculated per liter of medium. The different aeration types and inocula did not affect the dry matter. These results were promising for further experiments with temporary immersion systems, which will allow for further economizing the volume of the used medium. Even if the growth rate of the shoot-clumps was unexpectedly low in the three tested variants, it was improved in the case of increase inoculum percentage and passive air exchange.

Usually, no alkaloids were detected in the medium of the liquid *L. aestivum* cultures of directly regenerated shoot-clumps. Rarely, relatively small amounts of galanthamine, up to 10% of the total, were released into the medium.

The best results obtained using the self-constructed laboratory bioreactor were those of the two galanthamine genotypes La-5.2 (October/December 2005) and La-14 (December/March 2006). Though the cultivation conditions were not the optimal (1.5% w/v shoot-clumps, 2 L medium, with passive air exchange, without medium agitation), the cultures expressed high growth rate and galanthamine content (Table XV). No related alkaloids were detected in the cultures. The entire amount of galanthamine was concentrated in the biomass of La-5.2, while the shoot-clumps of La-14 released 8.8% of the produced galanthamine into the medium. The final concentration of galanthamine was higher than that calculated per liter taking into account the total

Table XV Parameters of the shoot-clump cultures of genotypes La-5.2 and La-14 cultured in a self-constructed bioreactor: 2 months for La-5.2 (October 10/December 10, 2005); 3 months for La-14 (December 15/March 15 2006)

Parameter	Clone	
	La-5.2	La-14
GI	4.66	8.16
Dry matter %	9.7	11.4
Gal (mg/g DW)	1.28	0.44
Gal yield (mg)	22.16	16.96
Gal (mg/L)	11.08	8.48
Gal (mg/L)[a]	5.54	2.83
% Released in medium	None	8.8

[a]Effectiveness of the biosynthesis according to the summary medium volume.

medium volume and the duration of the cultivation (the medium was replaced with a fresh one every month).

The process could be improved using the increase of the inoculum percentage as a tool for a further increase of the galanthamine yield per liter. The lower dry matter weights of the cultures of La-5.2 and La-14, compared to those of La-15 (Table XIV), reflected the ratio of the leaf and bulblet weights. Leaves always had less dry matter than the bulblets. The intensive leaf elongation observed for the cultures of La-5.2 and La-14 was impressive.

2. Physical Factors

Previously, we confirmed that the most frequent illumination regime of 16 h long light was suitable for *L. aestivum* cultures, while the light intensity of 1500 lux at a temperature of 21 °C ensured better growth than the usually reported 3000 lux at 26°C (24). On the base of this knowledge, all further experiments were performed under the same illumination, at an oscillating temperature of 23 ± 2°C, and following the light/dark regime (Figure 23).

Because of the permanent partial air refreshment allowed by the specially built air-conditioning system of the phytostatic room, the fluctuations of the diurnal temperature were more or less expressed, reflecting the amplitudes outdoors as well. This was the reason for the larger fluctuations occurring mainly in winter and spring. It is worth mentioning that the temperature values over 25 °C and less than 21 °C were very transient, lasting only a short time during the 24-h period. The hot summer temperatures, which are typical for Sofia, with a minimal diurnal temperature amplitude, was reflected in a very stable inside

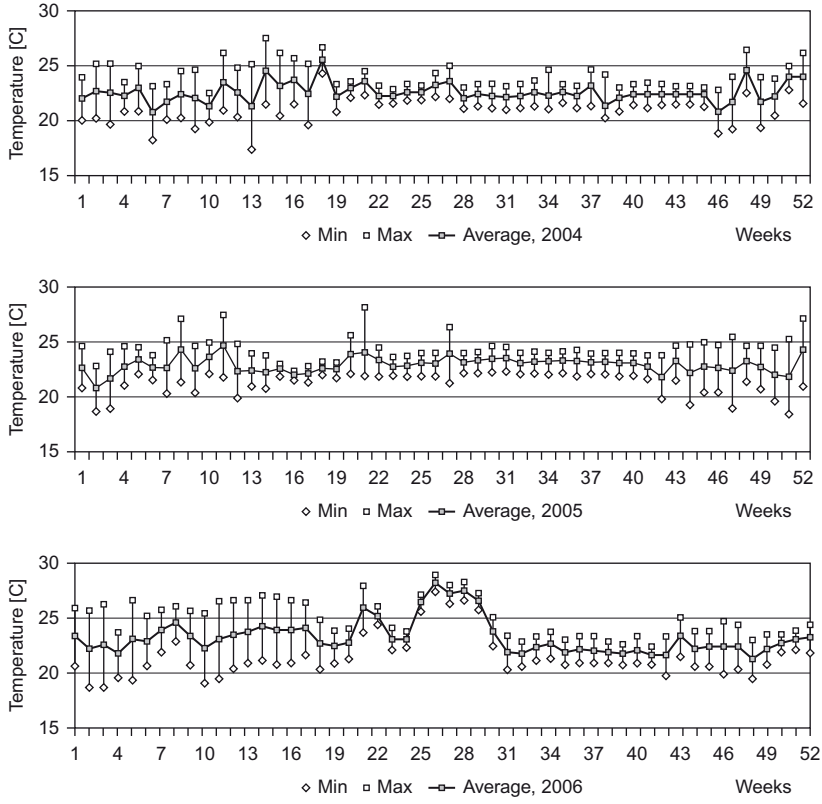

Figure 23 Temperature fluctuations in the phytostatic room during 3 consecutive years, covering the period of studying of the alkaloid biosynthesis dynamics.

temperature shifting within two degrees. The sole problem caused by the air-conditioning system lasted 2 months during the summer of 2006, occasioning a premeditated decrease of the light intensity in order to avoid the additional emission of heat. The stability of the physical factors of the ambiance was of importance for the study of the alkaloid dynamics that were revealed for the long-term *L. aestivum* cultures.

Light appeared to be very important for the augmentation of the biomass, as well as for the alkaloid biosynthesis. The PC (number of bulblets formed per *in vitro* bulblet) in long-term *L. aestivum* cultures was higher under an illumination regime (16 h light daily) than in the dark: 15.3 and 8.6 shoots for a period of 6 months, respectively. The number of the obtained large bulblets suitable for further subcultivation was, however, similar: 3.6 and 4.4, respectively, which could be explained by the exhaustion of the explants. Since most of the shoots reached a size suitable to grow independently from the initial explant, even if they

needed longer time until subcultivation, we chose cultivation under an illumination regime. No comparison was made of galanthamine biosynthesis under light and dark conditions. Only the excessive decrease of galanthamine content in most of the clones cultured under reduced light intensity during the problem of the air-conditioning in the summer of 2006 suggested some possible relation between the biosynthetic activity and the light.

More detailed results were reported by Pavlov *et al.* (30) concerning the influence of light on both biomass growth and galanthamine content. These authors investigated the parameters for the best *L. aestivum* callus-derived shoot line, cultivated in flasks in submerged liquid culture under illumination (16 h light daily) and in darkness. Shoots grew fast both in the dark and under illumination (15.5 and 17.8 g/L, respectively, for 35 days), however, the content of galanthamine calculated per liter was almost doubled under illumination conditions (2.5 mg/L). Light was reported to stimulate the culture growth and the biosynthesis of secondary metabolites of other species as well (69,70).

The higher galanthamine content in the leaves of the *in vitro* plantlets could be related to the location of galanthamine biosynthesis. Some histochemical analyses revealed that galanthamine was mainly localized in the assimilative tissues and the parenchyma around the conductive fascicles of the leaves and the stem of *L. aestivum* (71). These authors reported that the highest galanthamine content was in the stem. Ultrastructural observations confirmed that the cells of the assimilative tissues in both photosynthetic organs were distinguished by very active dictiosoms, suggesting that galanthamine biosynthesis was being realized in the mesophyll. Eichhorn *et al.* (57), using labeled 4′-*O*-methylnorbelladine in different tissues of flowering *L. aestivum* plants, found out that the fruit walls and flower stalks were the plant organs that showed the highest incorporation into galanthamine. Those mentioned organs are green, and since the *in vitro* cultures produced galanthamine without developing flowers, it can be assumed that galanthamine *in vitro* biosynthesis is localized in the mesophil cells of the leaves. Besides the genotype, this could be an additional reason for the lack of galanthamine in the cell suspensions and the root cultures, as well as for the very low concentrations of galanthamine in the calli and the colorless bulblets regenerated from the callus (up to 0.07 mg/g DW) (30).

Owing to the transparent medium, bulblets of the shoot-clumps cultured at 16 h daily light turned green, but, as thick structures, they possessed fewer assimilative cells that could explain their relatively lower galanthamine content. Galanthamine in the bulbs of the intact plants might be transported from the leaves. The elucidation of the organ localization of the galanthamine biosynthesis should be of practical importance in the case of industrial production.

B. Medium Composition

The previously established and fixed illumination regime and temperature allowed for more attention to be concentrated on the medium composition and consistency, testing also different types of inoculum and culture vessels. The effect of the medium composition was vague with respect to both supplemental salts and PGRs (21,24).

The medium used as the control variant in all of the experiments was the same used for maintenance of the long-term cultures. It was MS based (27), with a tenfold increase in thiamine-HCl, supplemented with 30 g/L sucrose and two PGRs: BAP and NAA, in concentrations of 2.0 and 0.15 mg/L, respectively, solidified with 6 g/L plant agar (Duchefa, NL), and a pH adjusted to 5.7 prior to autoclaving. The liquid control variant differed only in the lack of agar. The basic medium modifications related to the sucrose quantity, the inorganic nitrogen source, and the auxin/cytokinin combinations.

Sucrose is the most common carbohydrate source for *in vitro* cultures. Market beet-sugar was used in these experiments, for its lower price, at two concentrations: 30 and 60 g/L. The increase of the sucrose quantity influenced the formation and the growth of the shoots on agar-solidified medium differently. Therefore, for an evaluation of the propagation rate, two different PCs were calculated: one according to the total number of shoots and bulblets formed per *in vitro* bulblet ($PC_{sh\&b}$), and another indicating the number of large bulblets reaching the size for subcultivation (PC_b). Thus, the line La-15, cultured under long-term cultivation conditions on a control medium with 30 g/L sugar, produced, for a period of 6 months, a total of 15.3 bulblets per *in vitro* bulblet, within them 3.6 being large enough to be further subcultured, while the corresponding numbers were 12.3 and 6.4 for the culture medium with double the quantity of sucrose. The propagation rate was lower when the cultivation was performed in the dark. However, the number of the bulblets remained higher on the medium supplemented with less sugar: $PC_{sh\&b}$ 8.6 (PC_b 4.4) against $PC_{sh\&b}$ 5.6 (PC_b 3.8) for the richer sugar medium. It is worth to pointing out that the ratio of the large bulblets was better on the medium supplemented with 60 g/L sugar. The numerous bulblets formed on the medium with 30 g/L required more time to reach a size suitable for subcultivation. The use of 30 or 60 g/L sucrose therefore depended on the purposes of the cultivation. Formation of numerous shoots and fast multiplication of shoot-clumps for the establishment of liquid cultures or for other *in vitro* experiments were performed in a medium containing 30 g/L sugar. Bulblets intended for *ex vitro* adaptation or subcultivation were transferred on a medium supplemented with 60 g/L sugar for faster enlargement.

Doubling of the sucrose quantity in the liquid medium led to an increase in biomass. The growth of the liquid organ cultures was evaluated according to the increase in the fresh biomass weight accumulated for a certain time (GI) and the percentage of its dry matter (DW/FW ratio). The shoot-clumps of La-20 cultured in media with 30 and 60 g/L sucrose for 3 months showed a similar GI, however, they differed significantly in DW/FW ratio ($P < 0.01$) (Figure 24). Although shoot-clumps cultured in liquid medium with 30 g/L sucrose absorbed much water, they did not suffer from hyperhydricity. The dry matter of the shoot-clumps growing in liquid medium supplemented with 60 g/L sucrose was similar to that of the bulblets cultured on agar-solidified medium containing 30 g/L sucrose. Enhancement of the biomass growth was also reported for cultures of *Narcissus* species when cultivated on media with an increased sucrose level up to 9 g/L (72,73).

Taking into account the negative correlation between the content of galanthamine of *L. aestivum* plants growing in the natural populations and the concentration of the potassium cation in the soil (unpublished data), the macro-salt solution was modified by halving the KNO_3 concentration. However, the balance between ammonium and nitrate ions is very important because it acts as a pH buffering system (74). In order to keep the total nitrogen content and the $NO_3^+ : NH_4^-$ ratio as it was in the original MS medium, $Ca(NO_3)_2$ was added in adequate concentration. Calcium was selected for its roles in the plant cell development related to the enhancement of cell mitosis, cell wall thickening, and intracellular coordination (75). This modification was called MS1.

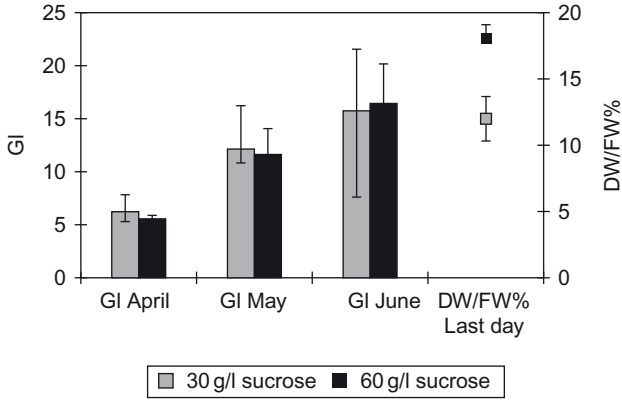

Figure 24 Growth index (GI) and DW/FW ratio of small *L. aestivum* shoot-clumps of line La-20, cultured 3 months in control liquid medium (2 mg/L BAP and 0.2 mg/L NAA) with 30 or 60 g/L sucrose.

Table XVI Initial parameters of four, one-bulblet-derived *L. aestivum* lines, multiplied by direct regeneration (February 2004)

In vitro line	$PC_{sh\&b}$ for 3 months (December 2003–February 2004)	Initial alkaloid levels	
		Gal (mg/g DW)	Lyc (mg/g DW)
La-10	6.5	0.85 ± 0.37	0.06 ± 0.14
La-11	21.7	0.39 ± 0.03	1.72 ± 0.12
La-12	8.6	0.04 ± 0.03	2.10 ± 0.88
La-13	40.0	0.27 ± 0.13	0.86 ± 0.56

The effects of the macro-salt modification were studied in combination with the two concentration levels of sucrose, which resulted in four medium variants: MS 30, MS 60, MS1 30, and MS1 60. The experiment lasted 3 months and evaluated the behavior of four, one-bulblet-derived lines: La-10, La-11, La-12, and La-13, multiplied by several prior subcultivations on control medium. The genotypes differed in their propagation rates and the ratios of galanthamine and lycorine concentration (Table XVI).

The results from the alkaloid determination of the four genotypes were summarized in two ways to compare the differences between the based on their macro-salt composition or their sucrose quantity (Table XVII). The influence of each factor was estimated by the *t*-test paired method, which was most suitable because of the significant variations between the genotypes, as reflected in the large ranges around the average values. The increase of the sucrose in the medium led to a significant augmentation of the biomass. This effect was, however, reduced by the decrease in alkaloid biosynthesis. In contrast, the modified macro-salt composition enhanced the production of alkaloids (Table XVII).

ANOVA two-factor analysis confirmed that the impact of the genotype was decisive for the biosynthesis of galanthamine ($P < 0.001$) and lycorine ($P < 0.01$), while the effect of the medium composition was insignificant, with *P*-values of 0.133 and 0.116, respectively, concerning the two alkaloids (Figure 25). More detailed analysis of the results, allowing the comparison of each pair of medium variants with respect to the genotype specificities (*t*-test paired), revealed significant differences between some of the media (Table XVIII). Thus, medium MS1 30 stimulated considerably the biosynthesis of galanthamine compared to the control medium MS 30. The difference between MS1 30 and MS 60 was most important for the biosynthesis of both alkaloids. However, the content of lycorine depended more on the sucrose quantity, while the content of galanthamine was more influenced by the macro-salt

Table XVII Influence of: (a) sucrose concentration and (b) macro-salt composition on the alkaloid content and the dry matter (DW/FW%) evaluated on the base of the average values of lines La-10, La-11, and La-13 for galanthamine; and La-11, La-12, and La-13 for lycorine

Tested parameter	Sucrose concentration			
	30 g/L	60 g/L	*P*-value	Ratio
A:				
Gal (mg/g DW)	0.60±0.27	0.51±0.34	0.232	85%
Lyc (mg/g DW)	1.42±0.55	1.01±0.44	0.008**	71%
Dry matter %	12.4±2.0	19.7±2.5	1.32E-9***	159%
Tested parameter	**Macroelements**			
	MS	MS1	*P*-value	Ratio
B:				
Gal (mg/g DW)	0.47±0.25	0.63±0.34	0.021*	133%
Lyc (mg/g DW)	1.11±0.51	1.32±0.55	0.075	119%
Dry matter %	16.2±4.8	15.8±4.0	0.478	97%

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

Table XVIII Differences between the contents of the main alkaloids of four *L. aestivum* genotypes cultured on media with different macro-salt compositions and sucrose quantities

	Medium	*p*-values of differences between galanthamine content			
		MS 30	MS1 30	MS 60	MS1 60
p-values of differences between lycorine content	MS 30	–	0.054*	0.095	0.679
	MS1 30	0.082	–	0.002**	0.76
	MS 60	0.012*	0.009**	–	0.124
	MS1 60	0.412	0.139	0.39	–

*$p<0.05$; **$p<0.01$; ***$p<0.001$.
Comparison by the *t*-test paired method, most suitable because of the genotype variations of the alkaloid contents (on the basis of lines La-10, La-11, and La-13 for galanthamine, and La-11, La-12, and La-13 for lycorine).

composition. Decrease of the alkaloid content in cultures grown on media enriched with sugar was also reported for *Narcissus* species (42).

The macro-salt modification MS1 was found to be suitable only for a short period of cultivation. Longer maintenance of the cultures on MS1-based media caused chlorosis, delayed growth, and even necrosis of the

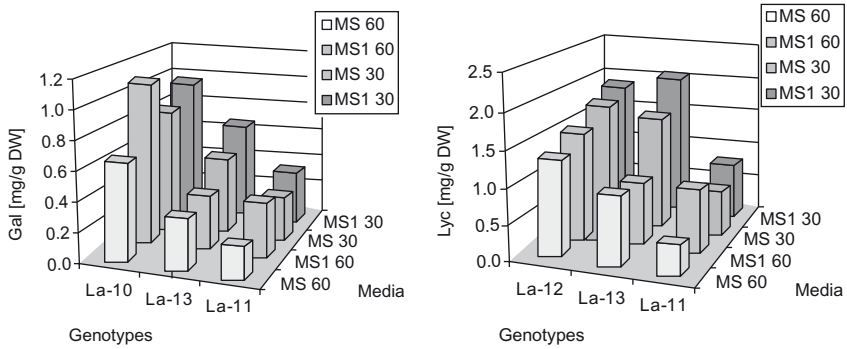

Figure 25 Contents of galanthamine and lycorine of the *in vitro* genotypes La-10, La-11, La-12, and La-13, cultured on agar-solidified media with MS and modified MS1 macro-salts, and sucrose concentrations of 30 and 60 g/L.

tissues. This was probably due to the potassium insufficiency in the medium, which might affect some important parameters as pH, osmosis, and turgor, finally resulting in disturbance of the cellular homeostasis (76).

As far as amino acids contain the ions NO_3^- and NH_4^+, they could be added as a sole source of nitrogen in the nutrient medium. Consequently, the second modification to the macro-salts, MS2, consisted of exclusion of the usual NH_4NO_3 and KNO_3 supplying the *in vitro* plants with inorganic nitrogen. At the same time, the amino acids tyrosine (Tyr) and phenylalanine (Phe) solely at a concentration of 700 µM (medium TP*700) or with 3 g/L casein hydrolysate (CH) that contains many amino acids (medium CH TP*-700) and offering the same concentration of the two amino acids, was added to the medium. The modification MS2 ensured an increase of the alkaloid concentrations (Figure 26). Since these amino acids are known as precursors of galanthamine and lycorine, additional details regarding these experiments will be provided in the next section.

The MS-based media were appropriate for the long-term cultivation of *L. aestivum*. Therefore, the cultures continued to be maintained on the control medium MS 30, which allowed us to study the stability of the genotype features over the course of time.

Optimization of the nutrient medium concerning the concentrations of the sucrose, as well as the ammonium, nitrate, and phosphate ions was carried out for the best, callus-derived, shoot line of *L. aestivum* cultured under submerged conditions (77). These authors modified the MS macro-salts on the basis of the biomass growth, galanthamine accumulation, and utilization of the main nutrient components. They reported a maximum galanthamine yield (96 µg per flask containing 40 mL medium) in a medium containing 60 g/L sucrose, when the standard

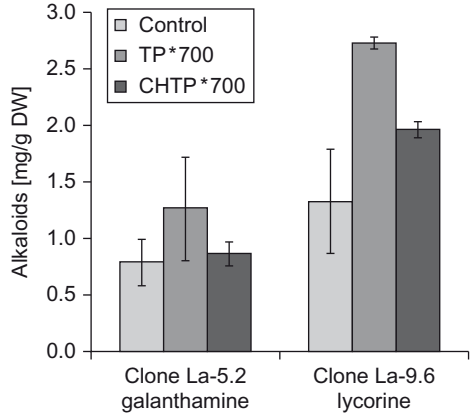

Figure 26 Increase of the main alkaloids on medium modification MS2 containing amino acids and casein hydrolysate (3-month cultivation in liquid medium: May/August 2005).

carbon/nitrogen ratio was increased from 15 to 21, without a change in the NO_3:NH_4^+ ratio. However, under these conditions 45% of the produced galanthamine was released into the cultural medium. Preferred accumulation of galanthamine in one of the culture components, either biomass or liquid medium, should facilitate the commercial scale-up of the process.

Besides modification of the macro-salts, the addition of PGRs in the medium is known to be a powerful tool for the stimulation of biomass growth and the *in vitro* biosynthesis of secondary metabolites. We tested different auxin/cytokinin combinations as possible controlling factors. For the purposes of this experiment, MS medium without any PGRs was used as the control. The effect of the most frequently used auxins: NAA and 2.4-D, and the cytokinins: BAP and kinetin, combined in the proportion 0.2/2.0 mg/L, was tested using shoot-clumps of the line La-20, cultured in flasks with 50 mL liquid media agitated on a shaker (110 rpm). In addition, NAA was tested in combination with two very expensive cytokinins: TDZ and zeatin. As expected, the media containing 2,4-dichlorophenoxyacetic acid (2,4-D) induced callus formation around the bulblet periphery. This auxin, in concentrations of 3 or 4 mg/L and in combination with 2 mg/L BAP, ensured the best callus induction of *L. aestivum* in young fruits (30). Indeed, 2,4-D provoked a faster increase of the fresh biomass ($P \leq 0.001$) (Figure 27A). However, this was due to the excessive water absorption by the plant tissues: the average dry matter of shoot-clumps cultured in media with 2,4-D was 13.3%, compared with 17.1% for those grown in NAA-containing media. Hyperhydricity of these explants was also observed. Moreover, galanthamine concentrations were higher in shoot-clumps cultured in NAA-containing media ($P < 0.05$)

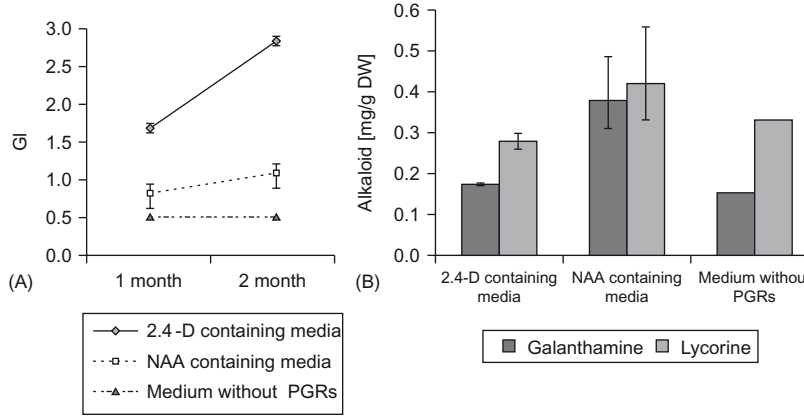

Figure 27 Influence of auxin/cytokinin combinations on: (A) growth of bulblets. (B) Content of the main alkaloids.

(Figure 27B). Within the combinations of NAA, those with BAP contained less dry matter, but were distinguished by the highest galanthamine content. Thus, a medium with 30 g/L sucrose supplemented with BAP and NAA was confirmed to be the most efficient system at the lowest price.

C. Interactions between Plant Biological Clock and External Stimulators

Exposure to biotic or abiotic elicitors as stress factors in plants usually leads to growth retardation, reduction of the FW and seed or fruit production, and frequently induces the synthesis of secondary metabolites. Elicitors like jasmonates, which positively influence the release of active substances, represent a valuable biotechnological strategy, and have been applied to many plant species (78,79). Feeding with precursors has been also widely applied for secondary metabolism enhancement (80). We tested several different approaches in order to stimulate galanthamine biosynthesis *in vitro*: stress with jasmonic acid (JA), feeding with amino acids, and treatment with acoustic waves as an indirect stimulant. Regardless of the factors used, the alkaloid biosynthesis corresponded to the biosynthetic dynamics trend. It seemed that the biological clock of *L. aestivum* remained very strong, even in long-term *in vitro* cultures, and limited the effect of all types of biosynthesis stimulants. That is why the exact time and duration of the experiments concerning stimulation of the galanthamine biosynthesis is so important in our experiments.

1. Elicitation with Jasmonic Acid

JA is a plant hormone that affects negatively the plant growth. Its exogenous addition, in relatively low concentrations, in the nutrient media has been reported to enhance secondary metabolism in cell and organ cultures of different plant species (81,82). This technique is attractive for its low cost and simplicity.

The effect of JA was studied on two of our selected, high producing, galanthamine genotypes: La-5.2 and La-7.80. The medium used for elicitation differed from the liquid control medium only in the presence of 0.5 mg/L JA. Treatments lasted 1 month, and were preceded by 1 or 2 months of cultivation of shoot-clumps in the control liquid medium for the faster accumulation of biomass needed for parallel experiments with precursors.

The results from the two consecutive experiments performed with JA were ambiguous at first sight. The first experiment aimed at comparing the effects of elicitation between the two clones. The second one focused on the dynamics of galanthamine accumulation in the biomass and its release into the medium during the period of elicitation. An understanding of the elicitation was possible only bearing in mind the interaction with the biological clocks of the plants, which had been kept for 3 years through *in vitro* cultivation at the time of the experiments.

The first experiment of elicitation began on January 26, 2006, after 2 months of shoot-clump cultivation in the control liquid medium (1-L bottles with 250 mL medium and 1.5 w/v % inoculum). The great difference between the growth rates of the two clones in the control liquid medium confirmed once again the stable inherent clone specificity of *L. aestivum* cultures. The fresh biomass of clone La-7.80 increased much faster than that of clone La-5.2 while the percentages of their dry matter were similar (Table XIX). The higher galanthamine content of clone La-5.2 did not compensate for its slower growth, and the quantity of the alkaloid was double that in the culture of clone La-7.80 at the beginning of the experiment with JA.

The presence of JA in the experimental medium during the third month of cultivation hampered the biomass growth of both clones, and their GI_1 decreased to 0.56 and 1.37, respectively (Figure 28A). Conversely, the shape and the color of the shoot-clumps remained normal, and the percentages of their dry matter did not change significantly: 10.4% for clone La-5.2 and 10.1% for clone La-7.80.

Shoot-clumps from the two clones cultured under long-term conditions on agar-solidified medium served as the control. By good fortune, their galanthamine contents were practically equivalent at the starting time of the experiment: 1.42 and 1.40 mg/g DW (Figure 29). At the same time (January 26, 2006), the shoot-clumps grown in liquid medium contained 1.09 and 0.79 mg/g DW galanthamine for La-5.2 and La-7.80,

Table XIX Growth index and galanthamine (Gal) accumulation in the cultures of clones La-5.2 and La-7.80 for 2 months cultivation in liquid control medium

Clone	GI_1 December 2005	GI_2 January 2006	DW/FW %	Gal in biomass (mg/g DW)	Gal in medium (mg/L)	Total Gal (mg/L)	% of Gal in biomass
La-5.2	2.47	3.18	10.1	1.09	2.46	9.38	73.7
La-7.80	3.45	9.11	11.9	0.79	4.88	18.91	74.2

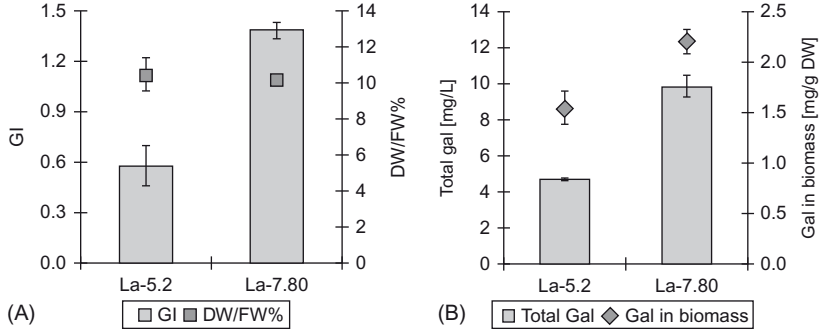

Figure 28 Main parameters of clones during the 3rd month of cultivation, in liquid medium containing JA: (A) Growth index (GI) and dry matter (DW/FW%) of shoot-clumps; (B) concentration of galanthamine (Gal) in the shoot-clumps (mg/g DW), and total galanthamine in the culture: shoot-clumps and medium (mg/L).

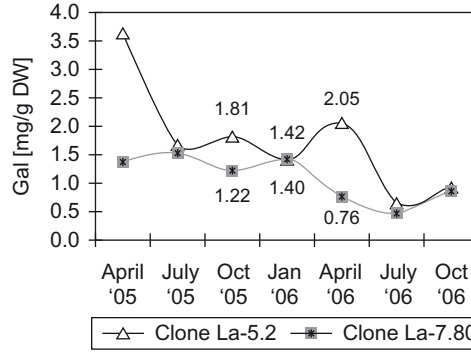

Figure 29 Dynamics of galanthamine (Gal) contents in shoot-clumps of clones La-5.2 and La-7.80 for a period of 18 months (cultivation on control agar medium).

respectively. These differences were due to the release of about one quarter of the alkaloid into the liquid medium (Table XIX). Alkaloid passage from biomass to liquid medium was also reported by other authors (30,42).

The elicitor stimulated the biosynthetic activity in both clones. However, the effects of the treatment were different (Figure 28B). Galanthamine content in the shoot-clumps of clone La-7.80 increased almost three times, reaching 2.21 mg/g DW. The increase of galanthamine in the shoot-clumps of clone La-5.2 was much less (Table XX). The alkaloid release into the medium was, however, similar for both clones, about 20% at the end of the experiment (Table XX and Figure 30). The yield of galanthamine in the biomass should amount to 7.9 mg for clone

Table XX Galanthamine (Gal) content in the biomass of clones La-5.2 and La-7.80 at the end of the first experiment with JA, and galanthamine release into the liquid medium

Clone	Gal in biomass (mg/g DW)	Gal in biomass (mg/L)	Gal in medium (mg/L)	Total Gal (mg/L)	% Gal in biomass	Theoretical yield of Gal in biomass, end of third month (mg)
La-5.2	1.55±0.15	3.66±0.01	1.03	4.69±0.01	77.8	7.9
La-7.80	2.21±0.12	7.97±0.62	1.91	9.88±0.62	80.7	39.9

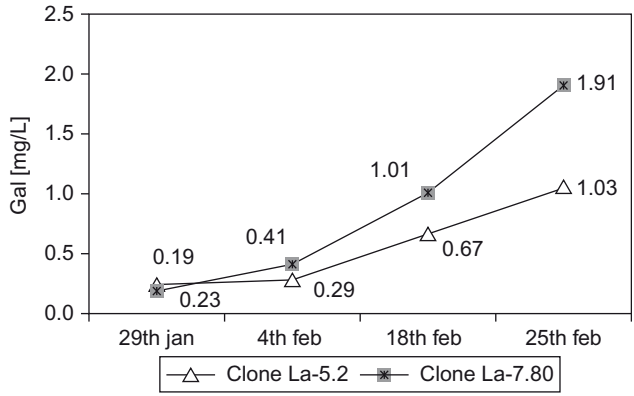

Figure 30 Dynamics of the release of galanthamine (Gal) from the shoot-clumps to the liquid medium supplemented with 0.5 mg/L JA (January 26, 2006–February 25, 2006).

La-5.2 and 39.9 mg for clone La-7.80 if the whole quantity of shoot-clumps obtained at the end of the 2-month cultivation in control medium was used as inoculum. The great difference between the two clones regarding their galanthamine yields was due to both the higher GI of clone La-7.80, and higher galanthamine concentration in its shoot-clumps at the end of the treatment with JA.

Retaining the clone-specific growth rates seemed to be normal. However, the quite different degrees of biosynthetic stimulation caused in the two clones by the elicitor could be understood only in light of the dynamics of galanthamine production presented on Figure 29 for a period of 18 months. A negative correlation was established between the dynamics of galanthamine contents of the two clones for the period July 2005–April 2006 (correlation coefficient −1.51, that corresponds to P=0.065 and is close to statistical significance of P=0.05). The 2-month

cultivation in the control liquid medium corresponded to the period of decline of galanthamine content in clone La-5.2, while alkaloid biosynthesis was in ascension in clone La-7.80. The start of the treatment with elicitor (January 2006) happened to coincide with the moment of equalization of the galanthamine content in the two clones.

Actually, the stimulation effect of JA was pronounced only in clone La-7.80. The content of galanthamine in its shoot-clumps was 0.79 mg/g DW at the beginning of the treatment, and reached 2.21 mg/g DW in February 2006, in spite of the release of about 20% of the alkaloid into the medium. This was the highest concentration of galanthamine ever determined in this clone. In contrast, the elicitor did not influence the biosynthetic activity of clone La-5.2, taking into consideration the relatively slight increase of galanthamine content (from 1.09 to 1.55 mg/g DW), and the fact that at the time of the experiment its alkaloid dynamics, studied on solid control medium, were entering on an ascending phase (Figure 29). Moreover, much higher concentrations of the alkaloid were determined in this clone both before and after the time of this elicitation experiment.

The effectiveness of the elicitation appeared to depend on the clone-specific dynamics of galanthamine biosynthesis. Obviously, the addition of JA at the maximum galanthamine content, as it was in the case of clone La-7.80, led to disturbance of the inner biological clock of the clone and interrupted the dynamics, thus preventing the scheduled decrease of the biosynthetic activity. Conversely, starting the elicitation treatment at the minimum galanthamine content, as in clone La-5.2 appeared to be useless, because the coincidence of the two stimuli: alkaloid dynamics and elicitation did not have additive effects.

The relationship between the effectiveness of the elicitation and the dynamics of the alkaloid biosynthesis was confirmed during an additional experiment carried out with the clone La-5.2. Treatment with JA started on March 12, 2007 after 1 month of shoot-clump cultivation in the control liquid medium (Magenta containers, Phytotechnology Labs, Lenexa, KS, with 125 mL medium, 3 w/v% inoculum). Half of the cultures were transferred to medium containing JA (variant with elicitation) while the remainder were transferred to fresh control medium (control variant) under the same cultivation conditions. The dynamics of both biomass growth and galanthamine accumulation in the biomass and in the medium were studied in parallel by termination of cultures of three containers from each variant on the 10th, 17th, 24th, and 31st day (Table XXI). The initial level of the galanthamine content was determined in the shoot-clumps at the end of the first month, that is, the first day of the elicitation experiment, was 0.84 mg/g DW, equal to 0.80 mg/container (Figure 31). It increased more than twofold in both the control and the variant

Table XXI Biomass accumulation and galanthamine content of shoot-clump cultures of clone La-5.2 with and without treatment with jasmonic acid (initial FW of 3.75 g per container)

Culture	Time	Biomass FW per container (g)	Biomass DW per container (g)	Gal in biomass (mg/g DW)	Gal in biomass per container (mg)[a]	% Gal in biomass
Control variant	10th day	12.6±4.1	1.28±0.34	1.72±0.34	2.27 a	94.5
	17th day	15.5±5.6	1.69±0.58	2.03±0.25	3.51 a	93.6
	24th day	15.7±3.7	1.73±0.42	0.50±0.13	0.90 b	78.3
	31st day	16.4±4.1	1.84±0.46	0.64±0.16	1.23 b	77.8
JA variant	10th day	13.5±3.1	1.41±0.29	2.02±0.28	2.91 a	95.3
	17th day	15.9±2.8	1.68±0.25	1.62±0.14	2.72 a	93.4
	24th day	15.3±3.0	1.72±0.32	0.64±0.10	1.12 b	79.2
	31st day	13.3±2.3	1.57±0.27	0.72±0.34	1.19 b	80.0

[a]Galanthamine content marked with different letters differ significantly.

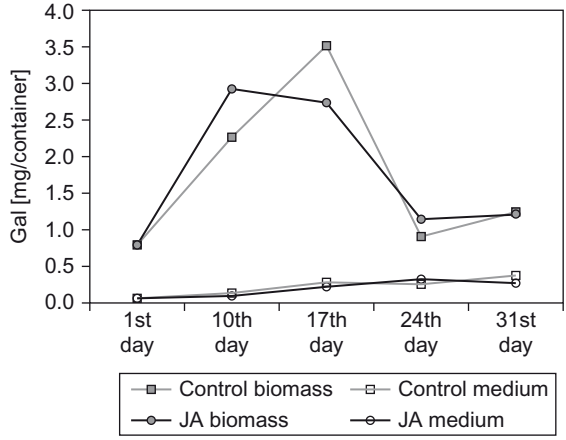

Figure 31 Dynamics of galanthamine accumulation in the culture with JA and in the control culture.

with elicitation during the first half of the experiment, and then decreased about fourfold within 1 week (Table XXI). No significant differences were found between the galanthamine content on the 10th and 17th day, nor between those determined on the 24th and 31st day. However, the differences in galanthamine biosynthesis between the first and the second half of the month were very significant ($P<0.001$).

The galanthamine content remained higher in the biomass compared to those in the media until the end of the experiment. This difference was significant in the cultures with JA ($P=0.01$), as well as in the control cultures ($P<0.05$). The increase of galanthamine in the medium was very slight, and no relationship was found with galanthamine accumulation in the shoot-clumps. The percentage of galanthamine released into the medium increased significantly in the course of the month, which was due to the drastic decrease of the alkaloid level in the shoot-clump cultures during the second part of the month.

The absence of an elicitation effect was confirmed by the correlation between the decrease of galanthamine in the control and in the variant with JA (correlation coefficient 2.09, $P<0.025$). This surprising result suggested the presence of other, more important, factor(s) strongly influencing the biosynthetic capacity of all of the cultures. A similar sharp decrease of galanthamine content was reported for the intact plants *in situ* and in the field during fruit maturation (14). Since the dynamics of galanthamine biosynthesis were observed in clone La-5.2 for 33 months until July 2007 (Figure 19A), the influence of JA was evaluated in relation to the time. ANOVA two-factor with replication analyses confirmed the importance of the factor ''time of experimentation'' ($P=0.001$). In

contrast, the factor ''elicitor'' was not significant and no interaction was found between the two factors.

Galanthamine content of the long-term culture of clone La-5.2 growing on agar-solidified control medium determined in February and in April 2007 were 1.10 and 1.41 mg/g DW, respectively (Figure 19A). At the end of March, on the 17th day of the experiment, galanthamine content in the biomass of the liquid cultures was much higher, although over 5% of the alkaloid was released into the liquid medium. Obviously, the moment of the maximum galanthamine content in the shoot-clumps grown on agar was missed, and the tendency for the slow decline of the alkaloid content over the years was ostensible. During the first part of the experimentation period, the biosynthetic activity of clone La-5.2 was in ascension, as it was in the first elicitation experiment. It appears that the biosynthetic activity oscillates within certain clone-specific limits, and its enhancement with external stimuli is not guaranteed. The biological clock of the species is therefore controlling galanthamine biosynthesis and degradation. The molecular mechanism of this process is not clear, but it also persisted under *in vitro* conditions. The elucidation of the mechanism of the biosynthetic regulation process could open the way toward elimination of the dynamics, which should be a very important prerequisite for the stable *in vitro* production of galanthamine.

Another manifestation of the biological clock of the clones was the dynamics of the biomass growth. The average GI of the cultures during the first month in control liquid medium was 2.36, varying between 1.95 and 2.64 for the 24 containers. Shoot-clumps from the different containers had equal weight, but differed to a certain extent in their size and number of shoots, which resulted in different elongation of their leaves, and eventually in different growth rates. However, the same cultures had incomparably lower growth during the elicitation experiment (Figure 32). No difference between the control and the elicitation variants were

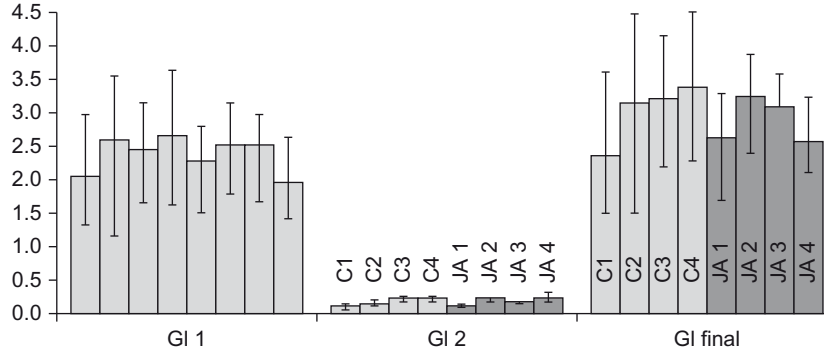

Figure 32 Growth indexes of cultures during the precultivation and the experiment aiming elicitation with JA.

noticed. The GI of the cultures terminated on the 31st day of the experiment (GI_2) was 0.21. Some slight tendency of increase of the dry matter was observed regardless of the presence of JA in the medium with average DW/FW % values of 10.3, 10.9, 11.0, and 11.2 for the controls, and 10.5, 10.6, 11.3, and 11.8 for the cultures with JA, at the four points of analysis, respectively.

Finally, the effectiveness of the galanthamine bioproduction *in vitro* was found to depend on the culture genotype features and the dynamics of both the biosynthetic activity and biomass growth. The addition of JA as an elicitor could be useful in the circumstance where it coincides with the maximum biosynthetic activity. Thus, the investigation on the molecular mechanism of the regulation of alkaloid dynamics, and the possibilities for manipulating the biological clock under long-term *in vitro* conditions appears to be of key importance for successful scale-up of the galanthamine production process.

2. Addition of Galanthamine Precursors to the Media

Feeding with precursors of the desired bioactive compound is a frequently used technique, which was successfully applied for the production of alkaloids from *Catharanthus roseus*, *Camptotheca acuminata*, and *Thalictrum minus* (80). The amino acids tyrosine (Tyr) and phenylalanine (Phe) were defined as precursors of galanthamine and lycorine (83). CH, which consists of many amino acids, including Tyr and Phe, was reported as a suitable, indirect precursor of colchicine, another alkaloid derivative of the two amino acids (84).

It was established that the addition of different alkaloid precursors to the medium could enhance the biosynthesis of galanthamine and lycorine *in vitro*. However, the dynamics of the biosynthetic activity was a more important factor limiting the increase of the alkaloid content during some periods of growth.

Two consecutive experiments were performed aimed at the stimulation of alkaloid biosynthesis by feeding with CH and/or the amino acids tyrosine and phenylalanine as precursors. The initial treatment was started in November 2004 with 12 clones and lasted 2 months. The second treatment was performed with half of the clones for 3 months, from April to July 2005. The effect of the precursors was tested in parallel with modification of the MS macro-salts composition, excluding the compounds containing inorganic nitrogen ions: NO_3^- and NH_4^+. This modification was named as MS2 and the corresponding media indicated with an asterisk to note that the precursors were the sole nitrogen source. During the first treatment, the clones were cultured in control MS medium and in medium TP*-400 containing 400 μM tyrosine and phenylalanine. Six of the clones were further evaluated in the control MS medium, medium TP*-700 with Tyr and Phe at a concentration of

700 μM, and the medium CH TP*-700 supplemented with 3 g/L CH, and Tyr and Phe in amounts ensuring their final concentrations was 700 μM.

Clones were selected for their alkaloid profiles and galanthamine content: La-3.9, La-4.45, and La-5.2 (Gal-type), La-7.6, La-2.72, La-1.19, La-6.31, La-7.73, and La-7.26 (mixed Gal−Lyc-type), La-9.6 and La-9.15 (Lyc-type), and La-10.4 (mixed lycorenine-9-demethylhomolycorine type).

All 20 of the *in vitro* clones and lines of *L. aestivum* were studied for 24 or 33 months under permanent light and temperature conditions, on the control medium, in equal-sized plastic containers, and expressed clone-specific dynamics of the alkaloid biosynthesis (15 of them are represented on Figure 13). Therefore, the stimulant effect of the precursors was evaluated in relation to the dynamics of the biosynthetic activity of each tested clone. The results from the two consecutive treatments differed significantly, and confirmed our hypothesis suggesting that the clone-specific dynamics was of primary importance for the effect of biosynthesis stimulation.

Clones cultured in the control medium differed in their galanthamine content as determined in November 2004 and in January 2005 (P=0.001, t-test paired). The alteration of galanthamine content was synchronized ($P<0.05$). Obviously, the dynamics of galanthamine production was increasing during the first experiment (Figure 33A), while that of lycorine production was more complex (Figure 33B). Only two from the mixed Gal−Lyc-type clones, La-7.6 and La-2.72, expressed similar biosynthetic activity for both alkaloids. The other four, mixed-type clones did not synthesize lycorine in January 2005 when cultured in the control medium. It appeared that feeding with Tyr and Phe led to successful overcoming of the zero point of the biosynthetic activity of clones La-1.19 and La-6.31. However, no lycorine was detected in clones La-7.73 and La-7.26 cultured on the medium TP*-400, which brought the stimulant effect of the precursors in question.

In order to elucidate the relative importance of the genotype and the alkaloid precursors in alkaloid biosynthesis, the results obtained in January 2005 on the control and on TP*-400 media were assessed by applying ANOVA two-factor with replication. Taking into account the peculiarities of the main chemo-types defined for the Bulgarian populations, the clones were compared within each chemo-type independently from the others. The results confirmed the great importance of the genotype for the biosynthetic activity (Table XXII). Galanthamine content of the six genotypes of the mixed Gal−Lyc profile differed very much. The same was true for the lycorine content of the two mixed clones, which produced lycorine during the tested period, as well as for the contents of norgalanthamine in the two Lyc-type clones. The differences between the galanthamine content of the three tested

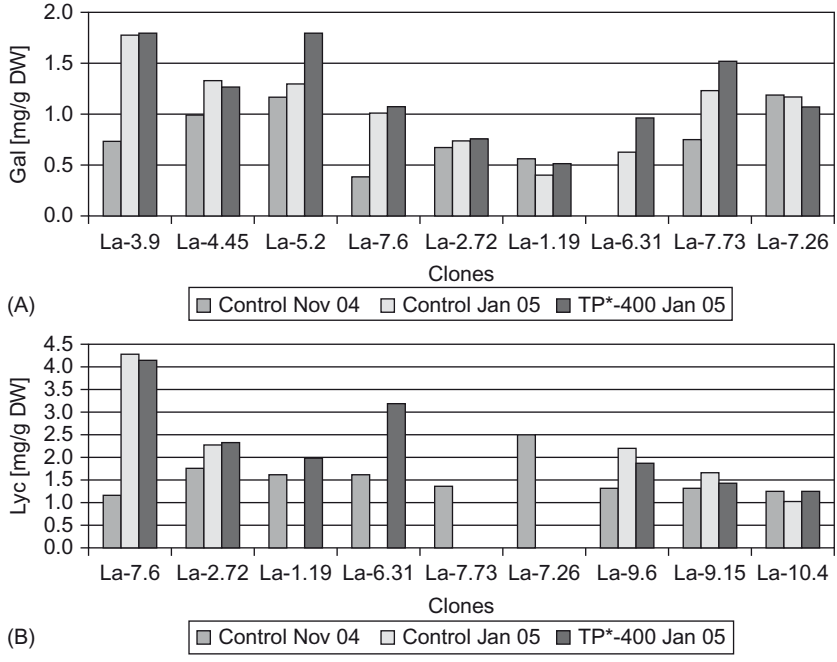

Figure 33 Influence of the biosynthetic dynamics and the alkaloid precursors on the alkaloid contents of 12 *in vitro* clones of *L. aestivum*: (A) Gal contents of three Gal-type and six mixed Gal–Lyc-type clones; (B) lycorine contents of the same six mixed Gal–Lyc-type clones, three Lyc-type, and one lycorenine-type clones.

Gal-type clones, and between the lycorine content of the two Lyc-type clones were very close to the reliable significance. Conversely, the precursors added to the medium did not influence alkaloid biosynthesis. No interaction was found between the genotype and the precursor.

The increase of the alkaloid content of the tested clones was due mainly to the inner dynamics of the biosynthetic activity (Table XXIII). A considerable increase of the concentration of galanthamine and lycorine during the testing period, resulting in a doubling of the alkaloid concentrations, was observed only in the clones whose dynamics expressed maxima in January: La-3.9 and La-7.6 (Figure 13). Similarly, a high increase was detected for the galanthamine content of clone La-6.31, which had a zero point in November 2004 and maximum in January 2005. Significant positive influence of the precursors on the alkaloid content was noticed only in the case of declining trend of the dynamics during the testing period: the galanthamine content of clone La-1.19 and the lycorine content of clone La-10.4. A relatively high increase in the galanthamine content due to both dynamics and precursors was observed in the clones La-5.2 and La-7.73. Unexpectedly, the addition

Table XXII Impact of genotype on alkaloid contents of clones cultured on both control medium and medium containing galanthamine precursors Tyr and Phe

Genotype	Alkaloid	ANOVA *F* ratio	*P*-value
Three Gal-type clones	Galanthamine	3.996	0.0788
Six mixed Gal−	Galanthamine	12.994	0.0001***
Lyc-type clones	Lycorine[a]	106.445	0.0004***
Two Lyc-type clones	Lycorine	5.457	0.0797
	Norgalanthamine	14.95	0.0180*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[a]Lycorine was compared only in two clones because of the zero point of the other four mixed-type clones.

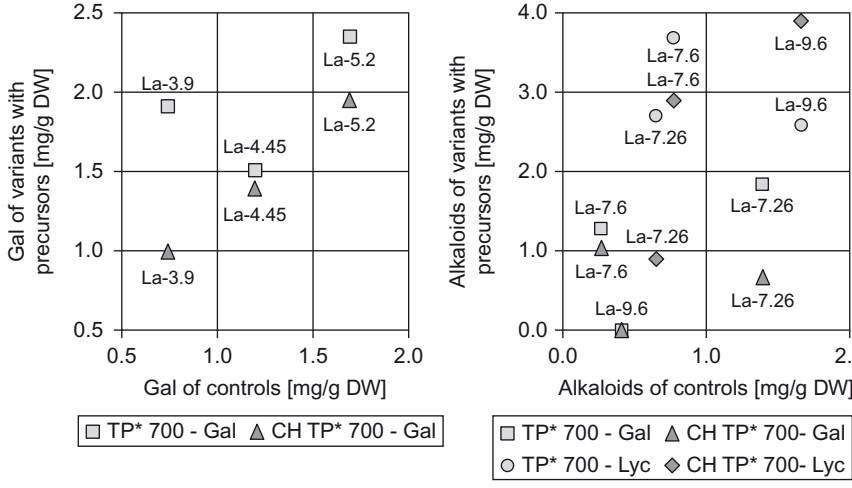

Figure 34 Stimulation of the alkaloid biosynthesis of *L. aestivum* clones by feeding with precursors: (Left) Galanthamine in Gal-type clones; (right) galanthamine and lycorine in mixed Gal−Lyc and Lyc-type clones.

of Tyr and Phe to the medium caused a decrease of lycorine content in the two Lyc-type clones, La-9.6 and La-9.15.

The influence of the alkaloid precursors on the biosynthetic activity was very high during the second treatment. Both alkaloid concentrations increased significantly in all of the tested clones (Figure 34). The ANOVA two-factor with replication confirmed the stimulant effect of the precursors, although the impact of the genotype was stronger (Table XXIV).

According to the content of galanthamine, the genotypes could be arranged in four groups: La-5.2 with the highest galanthamine

Table XXIII Relative weight of the factors "dynamics" and "precursor" on the alteration of the alkaloid contents of 12 *L. aestivum* clones (treatment from November 2004 to January 2005)

Clone	Galanthamine			Lycorine		
	Dynamics ratio (%)	Precursor ratio (%)	Summary dynamics and precursor ratio (%)	Dynamics ratio (%)	Precursor ratio (%)	Summary dynamics and precursor ratio (%)
La-3.9	238 a	101 c	241 a	–	–	–
La-4.45	133 b	95 c	127 b	–	–	–
La-5.2	111 b	139 b	154 b	–	–	–
La-7.6	264 a	107 c	282 a	363 a	97 c	351 a
La-2.72	112 b	102 c	114 b	130 b	101 c	132 b
La-1.19	71 d	127 b	90 c	–	Very high	122 b
La-6.31	Very high	154 b	Very high	–	Very high	198 a
La-7.73	164 b	123 b	202 a	–	–	–
La-7.26	99 c	92 c	92 c	–	–	–
La-9.6	–	–	–	169 b	85 d	143 b
La-9.15	–	–	–	127 b	87 d	110 c
La-10.4	–	–	–	82 d	121 b	99 c

a, double or more increase of alkaloid content; b, increase of alkaloid content of over 10%; c, alteration of alkaloid content less than 10%; d, decrease of alkaloid content over 10%; very high, alkaloid "appearance" after transient absence of biosynthetic activity.

Table XXIV Relative weight of the factors "genotype" and "precursors" on the stimulation of the alkaloid content of six *L. aestivum* clones (treatment from April to July 2005)

Factor	Galanthamine		Lycorine	
	ANOVA *F* ratio	*P*-value	ANOVA *F* ratio	*P*-value
Genotype	21.43106	5.28E-07***	23.60348	2.539E-07***
Precursors	7.76442	0.003704**	7.61060	0.0040245**
Interaction	2.507317	0.043092*	3.05040	0.0190802*

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

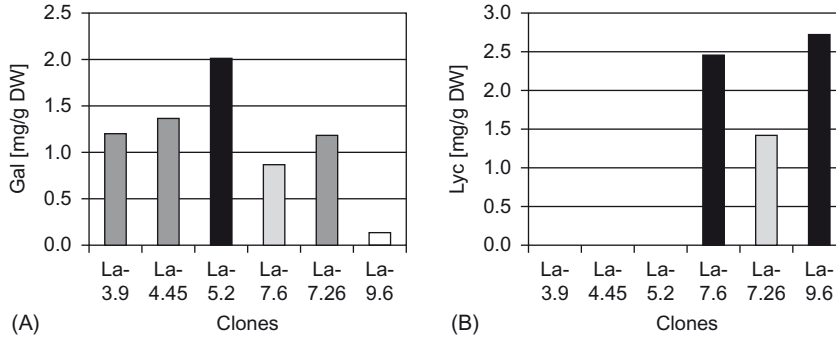

Figure 35 Effect of the genotype: (A) Four groups of clones according to galanthamine content; (B) three groups of clones according to lycorine content.

concentration, followed by the group of the three clones: La-4.45, La-3.9, and La-7.26, and two groups comprising one clone each: La-7.6 containing much less galanthamine, and La-9.6 that was very poor in galanthamine (Figure 35A). Regarding the lycorine content, the genotypes formed into three groups: the first one of clones La-9.6 and La-7.6 with the highest lycorine concentrations, followed by clone La-7.26 with a relatively high lycorine content, and the group of the three Gal-type clones, which did not synthesize lycorine at all (Figure 35B).

The alkaloid content of the six tested clones altered synchronously, but the synchrony was not permanent. Thus, the galanthamine content of the control variant determined in April 2005 correlated with those found in January 2005 ($P<0.05$) and with those in July 2005 ($P<0.01$), but the controls from January and July did not correlate. Regarding lycorine, the controls correlated during whole of this period ($P<0.05$). Correlations were established for the controls and the variants with precursors. They

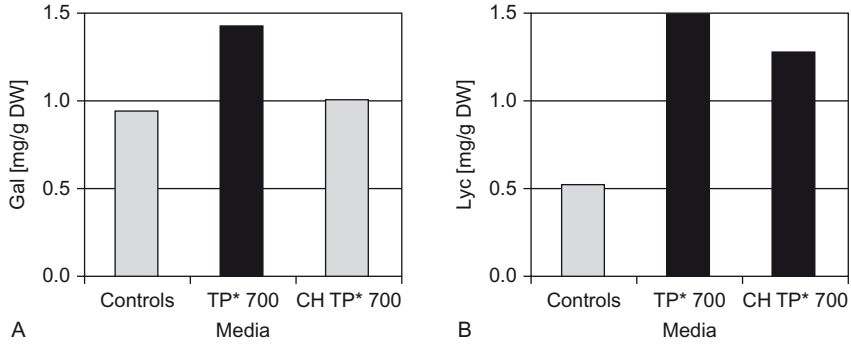

Figure 36 Effect of the medium: (A) Two groups of media according to galanthamine content; (B) two groups of media according to lycorine content.

were more pronounced for lycorine: $P<0.01$ (control and TP*-700) and $P<0.001$ (control and CH TP*-700); regarding galanthamine, the controls correlated with TP*-700 ($P<0.05$) and less with CH TP*-700 ($P=0.07$).

The content of galanthamine in the six clones cultured on the media with precursors highly correlated ($P<0.001$), but differed significantly, from one another ($P<0.01$, *t*-test paired). The biosynthesis of galanthamine was stimulated only by the medium TP*-700 ($P<0.05$, *t*-test paired), while the control medium and the medium CH TP*-700 belonged to one group (Figure 36A). Conversely, lycorine content significantly increased in both variants containing precursors ($P<0.05$, *t*-test paired), and no difference was observed between them, although they correlated highly ($P<0.001$) (Figure 36B). It is worth noting that the content of galanthamine and lycorine of the mixed-type clones cultured on each of the media containing precursors, correlated negatively ($P<0.05$).

Finally, clone specificity was illustrated by interactions between the two tested factors, which were confirmed by ANOVA two-factor analysis with replication (Table XXIV). In spite of all observed correlations, it is impossible to make prognoses about the effect of the precursors because of the unpredictable dynamics of the alkaloid biosynthesis in the clones maintained in the control medium. In general, the stimulant effect of the alkaloid precursors was highly pronounced when they were applied during the declining phase of the biosynthetic dynamics, but their addition was to no avail in the periods of ascending biosynthetic activity. Thus, the first treatment with precursors (November 2004/January 2005) did not hit the suitable biodynamic moment, while the second one (April/July 2005) led to a significant increase of galanthamine content in the clones La-3.9 and La-7.6, and of lycorine content in the clones La-9.6 and La-7.6 (Figure 37). The interaction between the genotype and the feeding with precursors was noticed in some of the clones. Although the

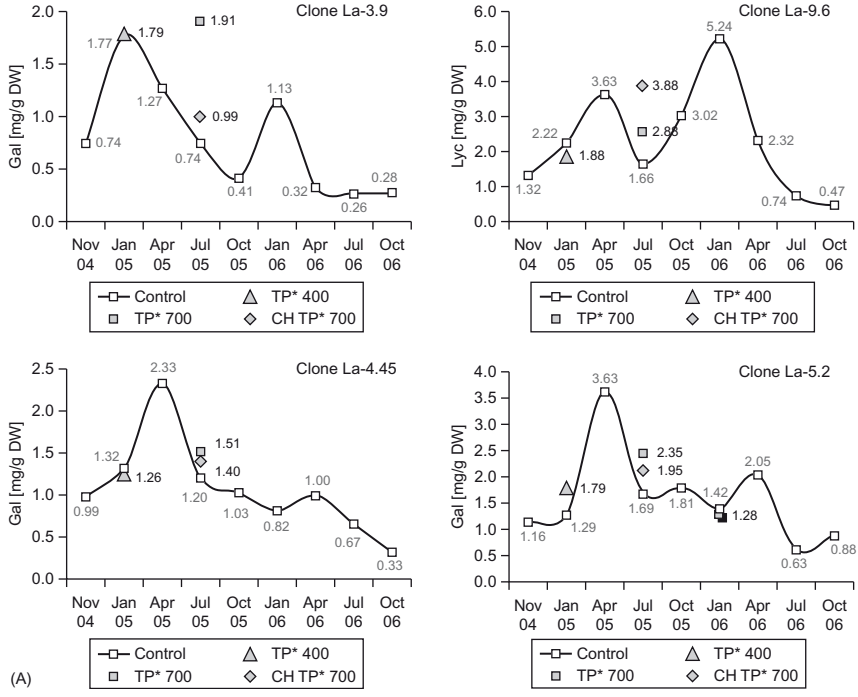

Figure 37(A) Interactions between the biosynthetic dynamics of the four tested genotypes with one alkaloid and the precursors in the media during the two consecutive treatments.

galanthamine content of clones La-4.45 and La-5.2 sharply decreased during the second treatment, the effect of the precursors was less pronounced. These two clones had correlating dynamics (Figure 16). The lycorine ''zero point'' of clone La-7.26 in January 2005 was not overcome by the addition of Tyr and Phe, in spite of the very abrupt decline of its biosynthesis during the treatment (Figure 37B). Obviously, the biological clock of this clone in determining the biosynthetic dynamics was very strong. The effect of the precursors became possible only when the biosynthesis was restored, and the increase of lycorine content was impressive during the second treatment. The different stimulant effects of the two media revealed another interaction between the genotype and the precursors. Medium TP*-700 enhanced much more the biosynthesis of lycorine in clones La-7.26 and La-7.6, whereas clone La-9.6 produced more lycorine in medium CH TP*-700.

The relationship between the level of stimulation of alkaloid production and the concentration of the precursor was not simple. Gal-type clones reached the highest galanthamine levels when cultured on medium TP*-700: 1.91, 1.51, and 2.35 mg/g DW in the clones La-3.9,

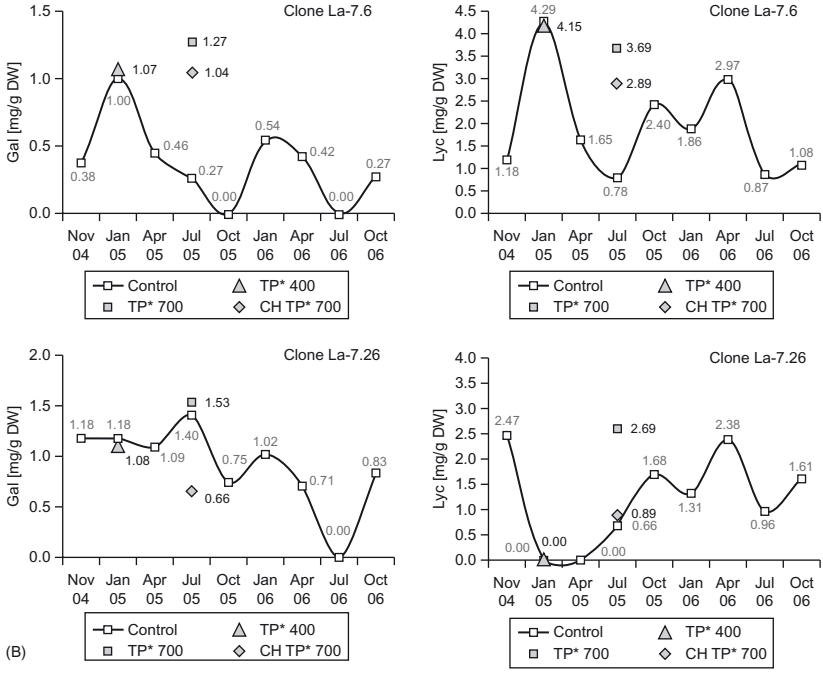

Figure 37(B) Interactions between the biosynthetic dynamics of the two tested genotypes with two main alkaloids and the precursors in the media during the two consecutive treatments.

La-4.45, and La-5.2, respectively (Figure 37A). However, the medium TP*-700 was more appropriate than CH TP*-700 in spite of the equal concentration of the two amino acids (Figure 34, left). Similarly, the Lyc-type clone La-9.6 produced more lycorine in the medium with a higher concentration of the precursors (1.88 mg/g DW on TP*-400 and 2.58 mg/g DW on TP*-700). The difference between the lycorine content of this clone produced in the two medium variants containing equal concentration of Tyr and Phe was very significant, and lycorine content reached the maximum of 3.88 mg/g DW on medium CH TP*-700 (Figure 34, right). Furthermore, the concentration of the amino acids Tyr and Phe seemed to be less important than the dynamics of the biosynthetic activity, which could explain the higher concentration of lycorine obtained in clone La-7.6 cultured in medium TP*-400: 4.15 mg/g DW compared with 3.69 mg/g DW lycorine on TP*-700 (Figure 37B).

The importance of the stimulant effects induced by the precursors was obviously related with the dynamic trends of alkaloid biosynthesis. In our opinion, the alkaloid dynamics could be explained by the persistent biological clock of this bulbous species. Although we were unable to find

similar data concerning the *in vitro* biosynthesis of secondary metabolites, *in vitro* growth periodicity of the regeneration rate and some other parameters was reported for *Populus tremula* and *Fagus sylvatica* (52). These authors revealed somaclonal stability as a phenomenon, which was no less important than the somaclonal variation, and related it to the inner biological clock of the species. It is most probable that the clonal specificity of *L. aestivum* dynamics reflected the different disturbance of the regulatory mechanism caused by the equal cultivation conditions *in vitro*.

Similar results were obtained with the one-bulblet-derived lines. Stimulation experiments were performed in the periods January–April 2005 and July–October 2005. In the first treatment, besides the agar-solidified media CH TP*-700 and TP*-700, liquid TP*-700 was also tested in parallel. The second treatment comprised agar-solidified media with Tyr and Phe in the same concentration (700 μM), which differed only in their macro-salt solution based on MS (TP) or on modification MS2, that is, without the nitrogen-containing macro-salts (TP*). Galanthamine and lycorine reached maximum concentrations on media with different precursors, and seemed to depend on the alkaloid profile of the line, as well as on the dynamics of galanthamine and lycorine biosynthesis (Table XXV).

The most pronounced increase of galanthamine content was in the Gal-type line La-14 during the treatment January–April 2005 (Figure 38). Galanthamine content in the shoot-clumps cultured on the media TP* and CH TP* were 1.94 ± 0.02 and 1.93 ± 0.25 mg/g DW, respectively, which was more than double compared to the control culture. These were the highest concentrations determined ever for this line (see Figure 19B for the dynamics in the control medium). In the biomass of the mixed-type lines La-11, La-13, and La-10, only galanthamine was detected, and the effect of the precursors was not evident. This could be related to the specific dynamics of each line, which seemed more important than the stimulation of the secondary metabolism. At the end of the first treatment (April 2005), galanthamine in the mixed-type lines reached values near to their genetically determined maxima (Figure 13E, A). In contrast, the end of the treatment coincided with the ''zero point'' of the lycorine dynamics for all of the tested lines (Figure 13F, D). Obviously, the inner regulative mechanism of the biosynthesis hampered the assimilation of the precursors. The only exception was noticed for the liquid culture of the Lyc-type line La-12. Shoot-clumps of lines La-14 and La-13 cultured in liquid TP* medium had galanthamine concentrations approximately half those of the corresponding cultures grown on solid TP* medium. In contrast, these differences were insignificant for the lines La-11 and La-10. Since no hyperhydricity was observed, the probable reason for the decrease of galanthamine content in some of the liquid cultures was its partial release into the medium, which was not analyzed in this experiment.

Table XXV Average and extreme values of galanthamine (Gal) and lycorine (Lyc) for 33-month of dynamics test on control medium, and maximal values reached by stimulation of the biosynthesis with precursors

Clone		Average values for 33 months (mg/g DW)		Extreme values (min and max) on control medium for 33 months (mg/g DW)				Maximal and corresponding values of the two main alkaloids on media with precursors (mg/g DW)			
Code	Type	Gal	Lyc	Gal		Lyc		Max Gal	Cor. Lyc	Max Lyc	Cor. Gal
La-14	Gal	0.64	0.02	0	0.94	0	0.19	1.94	0	0	0.86–1.94
La-11	mixed	0.40	0.96	0	0.85	0	2.24	0.69	0	5.68	0.63
La-13	mixed	0.51	1.28	0	0.92	0	3.59	1.16	0	4.57	0.79
La-10	mixed	0.38	0.13	0	0.68	0	0.58	1.43	0	1.20	0
La-12	Lyc	0.06	1.28	0	0.39	0	4.13	0	0–2.24	2.24	0

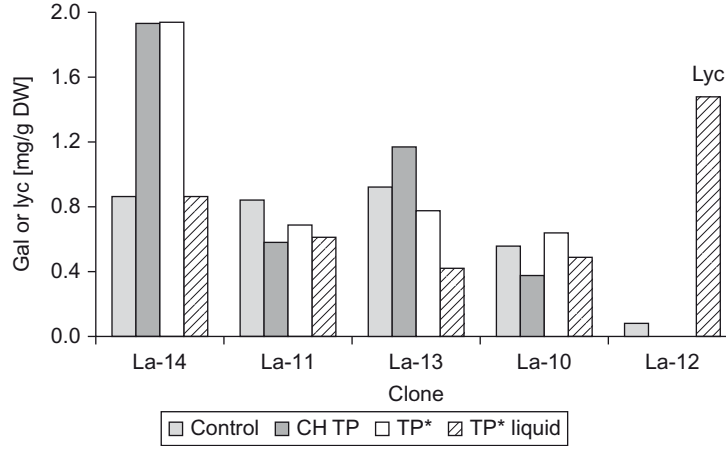

Figure 38 Contents of galanthamine (Gal) and lycorine (Lyc) of *L. aestivum* shoot-clumps cultured on media with different precursors (Treatment January–April 2005).

Table XXVI Factors influencing the dry matter, galanthamine, and lycorine contents of *L. aestivum* shoot-clump *in vitro* cultures (second treatment of the lines: July–October 2005)

Factor	Dry matter		Galanthamine		Lycorine	
	ANOVA *F* ratio	*P*-value	ANOVA *F* ratio	*P*-value	ANOVA *F* ratio	*P*-value
Genotype	110.340	6.2E-11***	55.699	8.01E-09***	188.110	1.27E-12***
Precursors	89.703	4.52E-09***	40.206	9.41E-07***	62.587	5.26E-08***
Interaction	3.202	0.025*	11.683	3.44E-05***	25.067	2.44E-07***

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

The ANOVA two-factor analysis with replication confirmed that both the genotype and the presence of alkaloid precursors in the medium had very high effects on alkaloid biosynthesis, as well as on the dry matter of the shoot-clumps. The interactions between these factors were significant (Table XXVI).

Four of the lines had a similar percentage of dry matter; it was significantly lower only in the shoot-clumps of the line La-12 (Figure 39). The addition of Tyr and Phe to the MS-based medium did not influence the DW/FW ratio. However, when the two amino acids were the only nitrogen source, the dry matter increased (Figure 39). Lines La-12 and La-10 were those that mostly expressed this difference.

The addition of the precursors Tyr and Phe did not influence the alkaloid profile of the lines. La-14 remained of the Gal-type, La-12 of the

Lyc-type, while the other three lines were of the mixed-type, La-11 and La-13 showing a clear predominance of lycorine. Generally, the stimulation of the biosynthesis of both alkaloids was noticed only when the two amino acids were the sole nitrogen source in the medium (Figures 40 and 41). Galanthamine reached the highest content in the shoot-clumps of line La-14 cultured on TP*-700: 1.75 ± 0.06 mg/g DW, which was significantly more than all of the values determined during

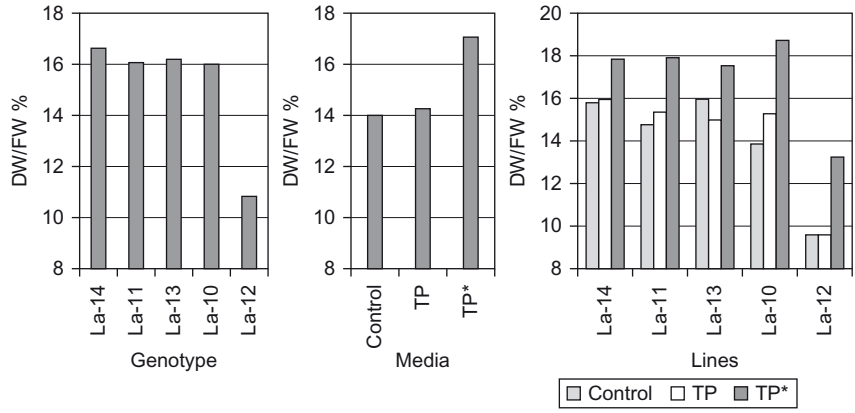

Figure 39 Influence of precursor feeding on dry matter (DW/FW%): Grouping by genotype, grouping by media, and contents of each line cultured on control medium and on media with 700 μM Tyr and Phe (TP – based on MS; TP* – MS2 modification without nitrogen-containing macro-salts).

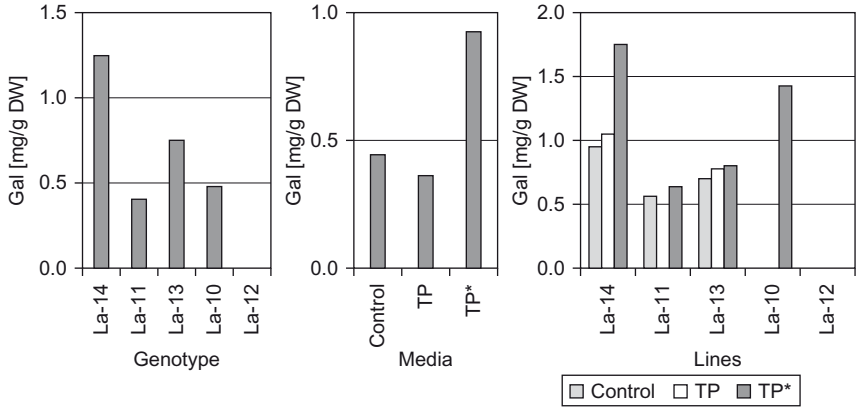

Figure 40 Stimulation of galanthamine with precursors: Grouping by genotype, grouping by media, and contents of each line cultured on control medium and on media with 700 μM Tyr and Phe (TP – based on MS; TP* – MS modification without nitrogen-containing macro-salts).

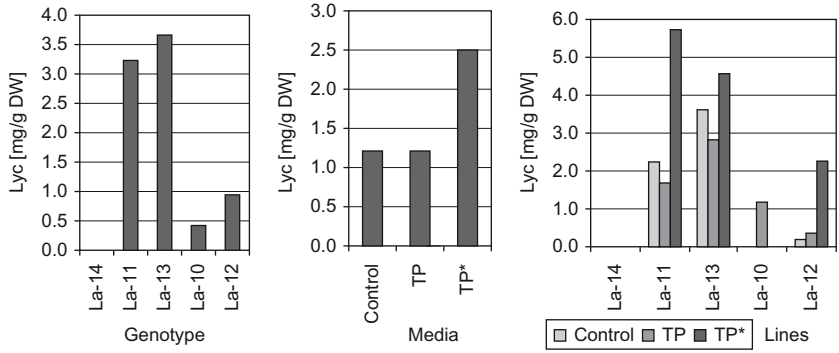

Figure 41 Stimulation of lycorine with precursors: grouping by genotype, grouping by media, and contents of each line cultured on control medium and on media with 700 μM Tyr and Phe (TP – based on MS; TP* – MS modification without nitrogen containing macro-salts).

the 33 months of dynamics test. The best producers of lycorine were the two mixed Gal–Lyc-type lines La-11 with 5.68 ± 0.59 mg/g DW and La-13 with 4.57 ± 0.34 mg/g DW produced on the medium TP*-700. The highest ratio between the lycorine content of the control variant and the variant stimulated with the two amino acids was calculated for line La-12, where the biosynthesis was enhanced 12-fold.

The response of the distinct lines to the presence of the two amino acids in the media was, however, specific. The biosynthetic ''silence'' of line La-10 continued for several months on control medium, including the period of the second treatment with precursors (July–October 2005). Then the two alkaloids were produced again with the usual predominance of galanthamine (Figure 13E, F). The shoot-clumps of this line synthesized galanthamine only on medium TP*-700, thus overcoming the ''zero point'' of its dynamics. Conversely, the ''zero point'' of the lycorine dynamics of the same line was interrupted only on the MS-based TP-700. This medium seemed to promote the prolonged ''zero point'' of galanthamine dynamics of line La-11. The different stimulant effects regarding the two alkaloids could be related to the particular steps in their biosynthetic pathways and requires more precise investigation.

The results from the treatment of July–October 2005 corresponded to our suggestion that the moment for the stimulation alkaloid biosynthesis was of great importance. Thus, the only line with a decline in alkaloid concentration during the period of treatment was La-12, which also explained the greatest enhancement of lycorine biosynthesis in this line. It seemed that the increase of alkaloid content was possible within some specific lines, more probably due to genetically determined limits. Concerning the mixed-type lines, the higher increase of lycorine in line La-11 compared to line La-13 corresponded to its less abrupt increase

during the period of treatment, both lines manifesting correlation in the dynamics of lycorine content ($P < 0.01$).

The dynamics of the galanthamine and lycorine content over the course of time were observed in all of the tested clones and lines. The addition of alkaloid precursors (the amino acids Tyr and Phe, and CH) enhanced alkaloid biosynthesis. However, the level of alkaloid stimulation depended on the timing of their application. Most important for the success of their effect was the course of the alkaloid dynamics during the period of treatment.

3. Treatment with Music

In order to find a less expensive method for the enhancement of galanthamine biosynthesis the influence of music was examined. In practice, different styles of music were used aimed at to modulating the secondary metabolism indirectly by inducing structural changes of the water present in the liquid medium and in the shoot-clumps. During the last few years a lot of information has been published concerning the great importance of the physical properties of water, especially the ability of water to record, keep, and transmit messages coming from the ambiance, called ''water memory'' (85–88). The crystals emerging from the pure water of the natural springs were found to be hexagonal, even though this structure was destroyed under the influence of diverse external forces (87). The revitalizing effect of some styles of music, images, and positive thoughts, consisting in the restoration of the natural hexagonal shape of the water was demonstrated in the IHM Research Institute in Tokyo, Japan. Classical music and songs with positive content like ''Imagine'' sung by John Lennon induced beautiful symmetric ice crystals under special conditions, whereas heavy metal music, especially bad-language songs, caused disorderly figures (87) (www.hado.net). The revitalized water was ideal to help maintaining good health. Thus, the beneficial effects of ''Bio Music 6 in 1 process'' was proved by some of the most eminent researchers in their field using different scientific methods: electrophysionic process which represents an advanced Kirlian method, nutritional microscopy tests, a sensitive crystallization process, formation of ice water crystals, tests on the human aura, etc. (http://www.biomusic-6in1.net/biomusic.php?page=tests_scientifiques; www.waterstar.at).

Kirlian photography demonstrated the rebalancing effect of ''Bio Music 6 in 1 process'' music on plants, since the vibratory field of green leaves exposed to its frequencies expanded harmoniously. Electrophysionic tests using high frequency electron streams to photograph the invisible energy fields radiating from all living things proved a clear enhancement of their vibratory fields under the influence of this music.

The power of intention was also able to restore the natural water structure, which was proved by the collaborative work of the Institute of Noetic Sciences in Petaluma, CA, and the Institute of HeartMath Research in Tokyo, Japan, and also involving the independent assessment of causal volunteers (86).

Our hypothesis was that treatment with different styles of music, causing different changes of water structure in both liquid medium and shoot-clumps, should have an effect on the biosynthesis of alkaloids. Furthermore, it was intended to examine the mostly adopted assumption concerning the function of alkaloids in plant organisms, namely that of defense from microorganisms and other negative factors of the environment. It was suggested that the hexagonal water structure generated by classical music should ensure comfort for the *in vitro* cultures, thus leading to the disappearance of, or a decrease in, their biosynthetic activity. Conversely, songs with bad language were expected to stimulate the production of alkaloids in order to defend the plants.

The same clones were used as in the treatment with JA, namely, La-5.2 and La-7.80, both of the Gal-type. At the beginning of the experiment, the clones had already been subcultured for 5½ years (about 60 passages) under permanent cultivation conditions in the control medium. The treatment lasted 7 weeks, starting on March 27, 2009. All cultures were growing in Magenta containers (Phytotechnology Labs) with 100 mL liquid control medium, and 1.5 w/v% of inoculum. Shoot-clumps ''were listening'' to music one time every second day, namely, the first variant ''Eine kleine nachtmusik'' of Mozart, or the second one was the bad-language song ''Scar U Face'' of *The Prophet Ft Headhunterz* (Figure 42). Precautions were taken not to neglect the shoot-clumps of the control variant, which were not

Figure 42 Liquid culture of *L. aestivum* shoot-clumps "listening" to music.

exposed to music. The simple human intention was proved to affect the chemistry of an array of substances, thus enabling speed up, slow down, or the improvement of many chemical processes (89). The power of intention had been confirmed in Japan by many students and families repeating the ''rice test'' (87); http://www.hado.net/hado/powerword.php). Cooked rice sealed in identical glass jars and labeled with the words ''thank you'' and ''you fool,'' respectively, were exposed to the same spoken words every day. At the end of the third week, the rice of the first jar was nearly fermented and had a nice mellow malted rice aroma, while the other one had turned black and had rotted, with a smell disgusting beyond description. The IHM Research Institute in Tokyo received reports about a similar experiment with ''control'' jar of rice without any label. The status of the rice in this jar was the worst due to disregard. Our shoot-clump cultures were typically static, being shaken by hand once a day for 1 min. To avoid the effect of the ''neglected rice'' each container from the three variants (named for short ''Mozart,'' ''Bad-language song,'' and ''Control'') was carefully shaken with best wishes for growth and health. Furthermore, to avoid the eventual difference of the containers' position on the shelf, taking into account other factors like geomagnetic zones, the places of the variants' ranges were changed every week. Cultivation was maintained under the standard temperature and illumination of the laboratory: $23 \pm 2^{\circ}$, 1500 lux, 16 h daily.

The music influenced alkaloid biosynthesis. However, the genotype remained the most important factor (Figure 43). The GI, as well as the contents of galanthamine and lycorine of the controls of the two clones differed significantly (Table XXVII). It seemed that the music had a

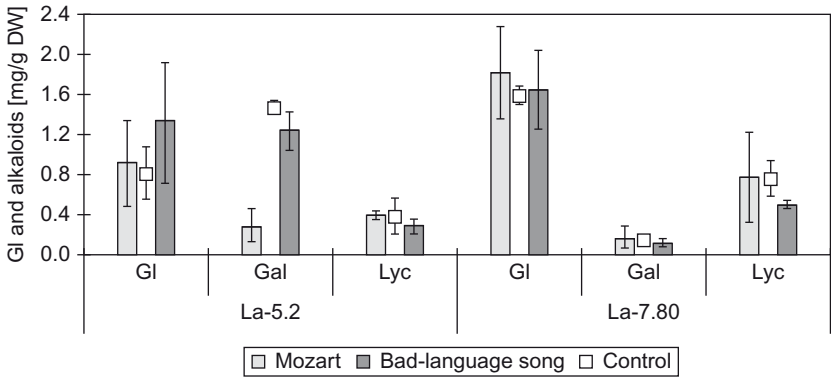

Figure 43 Effect of the music and biomass growth and alkaloid biosynthesis of *L. aestivum* clones La-5.2 and La-7.80 in liquid cultures, treatment from March 27 to May 15, 2009 (GI – growth index; Gal – galanthamine; Lyc – lycorine)

Table XXVII Differences between the growth index (GI) and the contents of galanthamine and lycorine of two *L. aestivum* clones, caused by the tested factors "genotype" and "music"

P-values: differences between the GI of the two clones from the same musical variant		*P*-values: differences between GI of variants of clone La-5.2			
		Control	Mozart	Bad-language song	All variants
P-values: differences between GI of variants of La-7.80	Control	0.0046**	0.7115	0.1684	
	Mozart	0.4467	0.0409*	0.3027	
	Bad-language song	0.8145	0.6560	0.4377	
	All variants				0.0014**
P-values: differences between the Gal of the two clones from the same musical variant		*P*-values: differences between Gal of variants of clone La-5.2			
		Control	Mozart	Bad-language song	All variants
P-values: differences between Gal of variants of La-7.80	Control	6.5E-07***	1.1E-05***	0.0657	
	Mozart	0.7018	0.2906	0.0003***	
	Bad-language song	0.3851	0.5185	0.0002***	
	All variants				0.0002***

Table XXVII *(Continued)*

P-values: differences between the Lyc of the two clones from the same musical variant		*P*-values: differences between Lyc of variants of clone La-5.2			
		Control	Mozart	Bad-language song	All variants
P-values: differences between Lyc of variants of La-7.80	Control	0.0397*	0.9781	0.2986	
	Mozart	0.9731	0.1347	0.0319*	
	Bad-language song	0.0629	0.3464	0.0048**	
	All variants				0.0014**

*$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

tendency to stimulate the growth of the *in vitro* cultures. According to the control variant, clone La-7.80 was faster growing compared to clone La-5.2 ($P<0.01$), which confirmed our previous experiments with JA (Figure 28A). Clones differed also in their growth of the variant ''Mozart'' ($P<0.05$), but the interaction noticed between the factors ''genotype'' and ''music'' led to a lack of statistical difference in the variant ''Bad-language song.'' Since the dry matter of all of the variants were similar, $GI=(FW_{final}-FW_{initial})/FW_{initial}$ and the index of growth efficiency: $G_eI=DW/FW_{initial}$ correlated ($P<0.001$).

In general, the music had a tendency to inhibit the biosynthesis of the alkaloids. Values of galanthamine content were relatively high in the clone La-5.2 reaching a maximum of 1.46 mg/g DW in the control, while the galanthamine content in all of the variants of clone La-7.80 were negligible, and did not differ one from another. ''Eine kleine nachtmusik'' of Mozart strongly inhibited the biosynthesis of galanthamine in clone La-5.2, thus eliminating the difference between the two genotypes in this musical variant. The bad-language song also inhibited the biosynthesis of galanthamine in clone La-5.2, but to a lesser extent ($P=0.06$).

Both clones also produced lycorine that was unexpected. Lycorine was detected on one occasion in the La-7.80 clone, and not a single time in the La-5.2 clone during the whole period of the dynamics test (Figure 44). The production of lycorine by the purely Gal-type clone La-5.2 demonstrated that this clone also possessed the genes responsible for the biosynthesis of this alkaloid. Certainly, the stable alkaloid profile of the clones was due to the strong regulation of the gene expression. Since lycorine was also produced in the control variant, the activation of its biosynthesis cannot be related to the musical treatment. Lycorine was present in low concentrations in all cultures of the clone La-5.2 (up to 0.39 mg/g DW); further tests will elucidate if this presence was transient or not. Obviously, the music of Mozart did not influence the biosynthesis of lycorine, whereas the bad-language song inhibited it. In

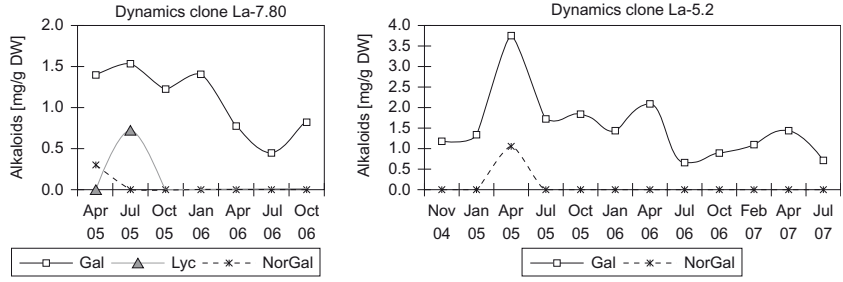

Figure 44 Dynamics of alkaloid biosynthesis of clones La-5.2 (for 33 months) and clone 7.80 (for 19 months).

the case of the clone La-7.80, the difference with the control was statistically almost significant (Table XXVII).

It seemed as though the negative correlation of the dynamics of galanthamine biosynthesis detected previously in the two clones for the period July 2005/April 2006 (Figure 29) was real again. The low galanthamine concentration in clone La-7.80 was somewhat strange (0.12–0.17 mg/g DW in the different variants). Indeed, we had no perspectives regarding the biosynthetic dynamics of this clone during the last years. However, the absence of any influence of the music suggested that the production of galanthamine was limited by the clone-specific biological clock. Obviously, the dynamics of galanthamine biosynthesis of clone La-5.2 was in a different phase, since it was strongly inhibited by the music. Similarly, the influence of the music on the biosynthesis of lycorine was noticed only in clone La-7.80, which suggested that the dynamics of this alkaloid in the two clones was also in negative correlation during the period of the experiment.

The original expectation concerning the influence of the music of Mozart was realized (clone La-5.2). However, we were not convinced that the reason for the decrease in galanthamine content was the comfort of the ambience, even if the water structure was revitalized in variant Mozart. The biosynthesis of galanthamine also decreased in variant Bad-language song, although the negative changes of the water structure were supposed to disturb plant tissues and to stimulate the plant defense, that is, the alkaloid production. Moreover, the biosynthesis of lycorine was mostly inhibited in variant bad-language song compared to variant Mozart. In general, the negative correlation between the biosynthetic activities of the two alkaloids in some of the mixed Gal–Lyc-type clones is in contradiction with the hypothesis that plants are producing alkaloids for defense.

As far as the influence of music on secondary metabolism was not in doubt, another mechanism had certainly been put in motion. This was not the intention because the results differed from the hope to stimulate the biosynthetic activity by the bad-language song.

Since the musical treatment was the only difference between the three variants, attention was focused on the probable direct influence of the acoustic waves on alkaloid biosynthesis. This could also mean control of the biosynthesis of the enzymes related to the alkaloid formation pathway. According to the wave-biocomputer model of DNA, the chromosome quantum nonlocality is a phenomenon of the genetic information, which is of great importance for multicellular organisms, and is applied on various levels (90). The capacity of plant vegetative regeneration is considered as a manifestation of the chromosome

quantum nonlocality at the organism level. At the chromosome-holographic level ''a gene has a holographic memory, which is typically distributed, associative, and nonlocal, where the holograms 'are read' by electromagnetic or acoustic fields.'' A method for the epigenetic regulation of the protein biosynthesis by scale resonance was patented in 1992 (91). The method is based on the existence of quantum waves, which are accompanying sequentially the biosynthesis of proteins. These sequences and their transposition in the audible field were called ''proteodies.'' The distribution of an artificial protein-specific proteody in the audible spectrum near organisms, which are naturally producing this protein, can lead to interaction by scale resonance with the proteody of the quantum scale, which are naturally accompanying the molecular phenomenon of synthesis. The amplification or the interference with the natural signal finally results in the stimulation or inhibition of the concerned synthesis in the organism. Enhancement of protein synthesis by treatment with proteodies has been demonstrated in many plant species (92–95). Thus, treatment of tomato plants with the proteody of the protein GAS14 antidrought resulted in faster growth of the ''musical'' tomatoes compared to the control ones, although they received half of the water. On the basis of this knowledge, it should be possible to enhance the *in vitro* production of galanthamine by playing near the cultures the specific proteodies of the enzymes catalyzing the steps of its biosynthesis. However, some of the enzymes are still unknown. The genome sequences of plants have revealed remarkable duplications of genes involved in natural product biosynthesis, for example, about 290 cytochrome P 450 enzymes were noticed in *Arabidopsis thaliana*, which requires individual biochemical characterization for the correct understanding of the role of each enzyme in the metabolic pathway. Probably, the music that was used in these experiments inhibited by accident the biosynthesis of some of the enzymes responsible for alkaloid biosynthesis. The experiments of Gariaev and coworkers (90) established the existence of an ''essentially new type of radio signal where the information is encoded by polarizations of electromagnetic vectors.'' Such radio waves, when generated under the right conditions by DNA, were found to be genetically very active. For example, artificially produced DNA radiations caused dramatic changes of morphogenesis in potato plants, as well as super-fast growth of the potatoes, reaching up to 1 cm per day. The positive effect of low-strength magnetic field on *in vitro* regeneration of *Paulownia* species was also demonstrated (96). All of these experiments reveal the amazing influence of electromagnetic and acoustic waves on plant metabolism, and suggest their possible application in the future development of agro techniques.

IV. SUMMARY AND CONCLUSIONS

The results described in these studies proved that the successful *in vitro* bioproduction of galanthamine from *L. aestivum* shoot-clumps required mainly the selection of *in vitro* clones with a genetically determined high ability to produce the desired alkaloids, although the expression of this ability could be additionally influenced by diverse exterior factors, such as some components of the nutrient medium, or the cultivation conditions of the ambience.

Tissue differentiation was also of great importance for the biosynthetic capacity of the cultures. The most suitable inocula for *in vitro* biosynthesis of galanthamine in liquid medium were the directly regenerated shoot-clumps, ensuring high alkaloid concentrations between 1 and 2 mg/g DW for the selected clones.

We observed astonishing clone-specific dynamics of the biosynthetic activity of all of the studied *in vitro* clones. The dynamics were obviously related to the strong biological clock of the species, persisting even in several-year old cultures. These dynamics did not coincide with those usual for the plants growing *in situ* and under controlled field conditions. In our opinion, the clone specificity of the biosynthetic dynamics could be due to the disturbance of the plant regulation mechanism under the equal conditions of the ambience in the culture room. The sharp decrease of the alkaloid concentrations were transient, followed by an increase, so that cultures were retaining their biosynthetic capacity.

The biosynthesis of the main alkaloids, galanthamine and lycorine, was influenced by diverse stimulants such as substances causing stress (JA), feeding with alkaloid precursors (the amino acids phenylalanine and tyrosine, and CH), and physical treatment (acoustic waves). However, the course of the biosynthetic dynamics during the period of the treatments was always the most important factor for the success of secondary metabolism stimulation. As far as scaling-up of the *in vitro* biosynthesis of valuable compounds, a stable and predictable yield is required, and additional investigations aimed at the annulment of the effect plant biological clock on alkaloid biosynthesis are needed. The elucidation of the relative influences of the diverse factors modulating alkaloid biosynthesis was of great importance. The high galanthamine concentrations of the selected *in vitro* clones are a promising basis for future studies.

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