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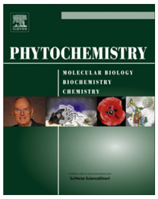

Review

Fifty years of alkaloid biosynthesis in *Phytochemistry* ☆

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ABSTRACT

An overview is presented of the studies related to the biosynthesis of alkaloids published in *Phytochemistry* in the past 50 years.

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☆ This manuscript dedicated to the Memory of Prof. Meinhart H. Zenk.

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1. Introduction

Since *Phytochemistry* was first published in October, 1961, it has evolved to become a flagship international journal for publications in many areas of natural product chemistry and biology, including studies on the isolation, structure elucidation, biosynthesis, and biological evaluation of numerous groups of natural products. One group which has received a great deal of coverage in the annals of *Phytochemistry* over the years is the alkaloids, in all of their structural diversity.

The first article on alkaloids was published in the first issue (Wightman et al., 1961). Subsequently, there have been several thousand articles appearing in the journal relating to various aspects of alkaloids, their origin, occurrence, chemistry, biosynthesis, ecology, and biology. For this contribution, the choice was made to consider only the aspect of alkaloid biosynthesis, and to indicate, briefly, some of the publications that appeared in *Phytochemistry* over the past 50 years. Not all of the published papers can be cited, and not all of the alkaloids studied can be discussed. This article is not intended, and could not be, a comprehensive overview of alkaloid biosynthesis in *Phytochemistry* in 50 years! Some authors have contributed ground-breaking studies to the field of alkaloid biosynthesis through the medium of *Phytochemistry*. These contributions have been an integral aspect of the growth and respect that the journal has engendered over the years. Apologies are tendered to the many authors whose studies on alkaloid biosynthesis were published in *Phytochemistry*, but whose work is not cited here. What is hoped is that a flavor of the coverage of alkaloid biosynthesis in *Phytochemistry* will evolve, and that some sense of the vast changes that have occurred in the strategies and techniques being used will be reflected. It is also hoped that there will be an appreciation of the crucial role that certain specific publications, and certain areas of alkaloid biosynthesis research which have appeared in *Phytochemistry*, have made to the field over these 50 years. The principal groups of alkaloids whose biosynthesis are described are listed, and within each section the discussion is essentially chronological. Sometimes one alkaloid serves as a representative of a group of alkaloids, or as way to introduce how the technologies that have related to alkaloid biosynthesis have changed over these past 50 years.

The first issue of *Phytochemistry* contained two publications on alkaloids (Wightman et al., 1961; Fairbairn and Suwal, 1961). Incredibly, the first dealt with the biosynthesis of tryptophan and gramine in barley, *Hordeum vulgare* L. (Poaceae), and showed that shikimic acid, anthranilic acid, indole acetic acid, and serine were precursors of tryptophan, and that tryptophan was a good precursor of gramine (**1**) (Wightman et al., 1961). The second paper examined the metabolism of the major alkaloids in hemlock, *Conium maculatum* L. (Apiaceae). It demonstrated the rapid changes in alkaloid levels which occurred within a 24 h period, and showed how the increased level of coniine (**2**) corresponded with a decline in the level of γ-coniceine (**3**) (Fairbairn and Suwal, 1961). Another paper on alkaloid biosynthesis also appeared in the first volume of *Phytochemistry*. This article concerned the formation of stachydrine (**4**), and demonstrated that ornithine was incorporated without randomization, and that proline was a direct precursor in mature plants of *Medicago sativa* L. (Fabaceae) (Essery et al., 1962).

With that meaningful start, the reports of alkaloid biosynthesis in *Phytochemistry* grew, and the journal became a popular venue for the leading researchers in the field to publish some of their most important results on the biosynthesis of alkaloids. Two names stand out for their numerous dynamic contributions to *Phytochemistry*, Professor Edward Leete and Professor Meinhart Zenk, both now deceased. The efforts of these groups, which resulted in numerous and diverse studies, revolutionized the thinking in several areas of alkaloid biosynthesis. Leete and co-workers conducted numerous decisive studies to clarify the tropane and nicotine pathways, while Zenk and co-workers carried out innovative studies at the enzyme and gene level which brought new clarity, and overturned many paradigms associated with earlier proposed biosynthetic pathways. Zenk also provided two important overviews of alkaloids and their biosynthesis. The first of these, in 1991 (Zenk, 1991), provided a perspective on the developments of the application of plant cell culture systems for secondary metabolite biosynthesis in the search for the enzymes; a particular emphasis was placed on advances in elucidating the enzymes of alkaloid biosynthesis. The second review was of the evolution and current status of the phytochemistry of nitrogenous compounds (Zenk and Juenger, 2007). Based on an overview of three types of nitrogen-containing plant products (alkaloids, cyanogenic glucosides, and nonprotein amino acids), it was concluded that the integration of the disciplines of chemistry, pharmacognosy, medicine, analytical sciences, cell biology, molecular biology, botany, and chemotaxonomy across traditional borders will lead to new achievements in phytochemistry, and presumably also in *Phytochemistry*.

1.1. Ornithine-derived alkaloids

An early stage in the formation of several simple alkaloid groups is the formation of putrescine from either ornithine or arginine, and the dimerization to spermidine, from which several derivatives are formed, including those which are acylated; among these alkaloids is lunarine (**5**). Zenk's group (Sagner et al., 1997) showed that lunarine (**5**) was formed by stereoselective phenol oxidative coupling of N_1,N_{10}-*bis*(*p*-coumaroyl)spermidine in the seeds of *Lunaria annua* L. (Brassicaceae). *p*-Coumaric acid was formed from L-phenylalanine via *trans*-cinnamic acid, and the spermidine, derived through putrescine, was preferentially synthesized from arginine. Following enzyme studies, it was suggested that a cytochrome P450 might be responsible for the phenol-oxidative coupling of N_1,N_{10}-bis(*p*-coumaroyl)spermidine to afford the hexahydrodibenzofuran ring of lunarine (**5**).

The first committed step in the nicotine and tropane alkaloid pathways is the formation of *N*-methylputrescine (**6**). Putrescine:SAM *N*-methyltransferase (PMT) catalyzes the *N*-methylation of the diamine putrescine to form *N*-methylputrescine (**6**). The *pmt* gene of *Nicotiana tabacum*, under the regulation of the CaMV 35S promoter, was introduced into the genome of a scopolamine-rich *Duboisia* hybrid. Although *N*-methylputrescine levels of the resulting engineered hairy roots increased (2 to 4-fold), there was no significant increase in alkaloid production (Moyano et al., 2002).

A phylogenetic tree of 27 PMT and 14 SPDS proteins (Biastoff et al., 2009) demonstrated the separation of plants in the

Solanaceae from those in the Convolvulaceae. Within the Solanaceae, the relationships based on PMT from *Nicotiana* species paralleled those from earlier chloroplast restriction site analysis. In spite of their plant origin, the plant SPDS sequences cluster together, and are distant from the the PMT enzymes. From an evolutionary perspective, it was considered that a *pmt* gene originated from *spds* at an early stage in the development of higher plants. The *pmt* genes are expressed in the roots in *Nicotiana sylvestris* Speq., *Atropa belladonna* L., *Datura stramonium* L., and the calystegine-producing plants *Solanum lycopersicum* L. (Solanaceae) and *Calystegia sepium* R.Br. (Convolvulaceae) (Biastoff et al., 2009).

1.2. Nicotine

Nicotine (**7**) is derived from a pyridine nucleus and an *N*-pyrrolidine moiety joined at C-3 of the pyridine nucleus. The precursor-metabolite relationships of nicotine and its *N′*-demethyl derivative, nornicotine (**8**) have been well-studied over the years. Root administration of [^{15}N]nornicotine and [methyl-^{14}C]-L-methionine to tobacco (*N. tabacum* L. (Solanaceae)) plants led to incorporation into nicotine (**7**). The latter precursor was incorporated at a higher rate than the former, and the relative incorporations were very similar in the root and shoot (Ladesic and Tso, 1964).

Whether nicotinic acid (pyridine-3-carboxylic acid) was completely decarboxylated as an intermediate step in the process of incorporation into nicotine (**7**) was the subject of speculation. Hydroponic root administration of [2,3,7-^{14}C]nicotinic acid to *N. tabacum* resulted in labeled nicotine (**7**), which was degraded stepwise. Radioactivity was detected at positions 2 and 3 of nicotinic acid, and not at C-4, 5, and 6, showing that the pyridine ring did not become symmetrical during nicotine biosynthesis (Scott and Glynn, 1967). Importantly, it was also demonstrated that attachment of the pyrrolidine moiety occurs exclusively at the carbon from which the carboxylic acid group is lost.

[5-^{14}C]-DL-Δ^1-Pyrroline-5-carboxylic acid was incorporated symmetrically into the pyrrolidine ring of nicotine (**7**) in *Nicotiana rustica* L. (Solanaceae) after 6 h (Krampl et al. 1969). Labeling appeared predominantly at C-2′, 47.8% and C-5′ ⩽40%. However, the low level of incorporation into nicotine (**7**) (0.04%) suggested that it is probably not an intermediate on the main pathway of pyrrolidine ring biosynthesis. The biosynthetic formation of nicotine (**7**) in callus tissue of *N. tabacum* was activated by indole acetic acid (IAA), but suppressed by 2,4-dichlorophenoxyacetic acid (2,4-D) (Furuya et al., 1971).

The biosynthesis of the pyrrolidine ring of nicotine (**7**) was also studied using short-term, steady-state exposures of *N. glutinosa* L. (Solanaceae) seedlings to $^{14}CO_2$. The results, following degradation of the pyrrolidine ring, showed both symmetrical and unsymmetrical labeling (Rueppel et al., 1974), leading to the suggestion of the operation of two biosynthetic pathways, one involving a symmetrical precursor and the other an unsymmetrical one.

A mixture of [2′-^{3}H]-(−)-nicotine (**7**) and [2′-^{14}C]-(±)-nicotine (**7**) was used to study nicotine metabolism in *N. glauca* Graham (Solanaceae) plants. After 3 days, 49.5% of the activity was in nornicotine (**8**), with negligible activity in anabasine (**9**), and a low level (2%) in myosmine (1′,2′-dehydronornicotine) (Leete and Chedekel, 1974). The radioactive nornicotine (**8**) comprised 48% [2′-^{14}C, ^{3}H]-(−)-nornicotine and 52% [2′-^{14}C]-(+)-nornicotine, suggesting loss of H-2′ if (+)-nornicotine is formed from (−)-nicotine (**7**). [2′-^{14}C]Myosmine was not a precursor of nornicotine (**8**) in *N. glauca*, indicating that dehydrogenation is not reversible, but myosmine was degraded to nicotinic acid.

An early experiment using contiguously ^{13}C-labeled precursors was reported by Leete and Yu (1980). Examination of the ^{13}C NMR spectra of nicotine (**7**) and nornicotine (**8**) ten days after administering *N. glutinosa* plants with [2,3-$^{13}C_2$]- and [5-^{14}C]-DL-ornithine showed conclusively the symmetrical incorporation of ornithine into the pyrrolidine ring of these alkaloids. Satellites were observed for the C-2′, 3′, 4′, and 5′ resonances confirming the intact presence of contiguous carbons at C-2′, 3′, and C-4′, 5′ by way of a symmetrical intermediate.

In 1985, for the first time, the pyridine alkaloid content, represented by nicotine (**7**), nornicotine (**8**), anabasine (**9**), and anatabine (**10**), of the leaves and roots of 60 (of the 66 known) *Nicotiana* species was analyzed quantitatively by gas chromatography (Saitoh et al., 1985). The species were identified morphologically, and all contained alkaloids. Root samples usually contained more alkaloids than the leaves, and the alkaloid levels varied by a factor of up to 2000-fold. Nicotine (7) was dominant in the leaves of 33 of the species and in the roots of 51 species. Seeds contained trace alkaloid levels. No correlations were observed between the proposed classification of the genus and the alkaloid content.

Adminstration of asymmetrically-labeled spermidine ([6-^{14}C]-1,5,10-triazadecane) to *N. glutinosa* afforded nicotine (**7**) and nornicotine (**8**) in which the label was equally distributed at C-2′ and C-5′ of their pyrrolidine rings (Leete, 1985). Degradation of spermidine to putrescine therefore occurs prior to incorporation.

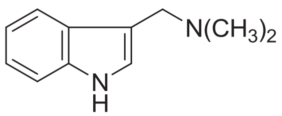
A non-tobacco alkaloid producing cell suspension culture of *Nicotiana plumbaginifolia* Viv. (Solanaceae) demonstrated the capacity to demethylate nicotine (**7**) to nornicotine (**8**) in up to 53.2% yield. Light enhanced the catalytic activity, suggesting that demethylation is a photodependent process (Manceau et al., 1989).

DL-[5-^{14}C]Ornithine afforded radioactive (*S*)-nicotine (**7**) and scopolamine (**11**) in a root culture of *Duboisia leichhardtii* F.Muell. (Solanaceae). Nicotine (**7**) was labeled equally at C-2′ and C-5′, and scopolamine (**11**) equally at C-1 and C-5 (Leete et al., 1990). This experiment supported the common biosynthetic origin for the pyrrolidine ring of both nicotine and scopolamine (**11**) through a symmetrical intermediate. Chemically, an efficient method for the conversion of scopolamine (**11**) to hyoscyamine (**12**) was described.

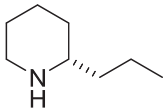
Leete and co-workers (Friesen et al., 1992), using root cultures of *Nicotiana alata* Link & Otto (Solanaceae), showed that in the presence of high levels of nicotinic acid both alkaloid production and the conversion of nicotine (**7**) to nornicotine (**8**) was inhibited, while anatabine (**10**) formation was stimulated. On the other hand, increased levels of 1-methyl-Δ^1-pyrrolinium chloride enhanced nicotine (**7**) production and inhibited *N*-demethylation. Increasing concentrations of Δ^1-piperideine promoted anabasine (**9**) and nornicotine (**8**) production.

The mechanism of nicotine *N*-demethylation was investigated with [pyrrolidine-2′-^{14}C]-nicotine (**7**) to produce (−)-nornicotine (**8**) intracellularly in up to 70% yield (Hao and Yeoman, 1996a). *N*-Formyl-3′-nornicotine was detected as a by-product and was not regarded as an intermediate in nicotine *N*-demethylation. *N*-Demethylation of nicotine (**7**) does not involve pyrrolidine ring-opening. Subsequent studies in a cell-free tobacco system (Hao and Yeoman, 1996b) disclosed an enzyme catalyzing the bioconversion of nicotine (**7**) to nornicotine (**8**). The enzyme had a pH optimum of 9.0–9.5, and a temperature optimum of 25–30 °C. The V_{max} and the apparent K_m were 7.6×10^{-2} pkat and 7.4 μM of ^{14}C-nicotine, respectively; the enzyme may be NADPH dependent. The mechanism of the demethylation of nicotine (**7**) to nornicotine (**8**) was also studied in root cultures of *N. alata* (Solanaceae) using [4′,4′,5′,5′-$^2H_4$]nicotine (**7**) to afford labeled nornicotine (**8**) without loss of label according to ^{2}H NMR (Botte et al., 1997). These data also supported a mechanism in which the C-5′ protons of nicotine are not involved in the demethylation reaction.

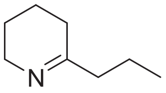
The *N*-demethylation of the piperidine homologs of nicotine was similar, but not identical, to that of the pyrrolidine analogs. (*R,S*)-*N*-Methylanabasine (**13**) and (*R,S*)-*N*-methylanatabine were



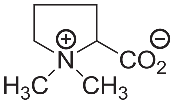
1 Gramine



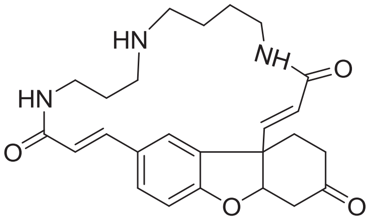
2 Coniine



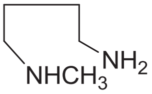
3 γ-Coniceine



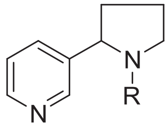
4 Stachydrine



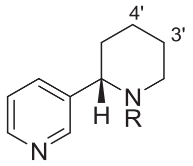
5 Lunarine



6 *N*-Methylputrescine



7 Nicotine, R = CH_3
8 Nornicotine, R = H



9 Anabasine, R = H
10 Anatabine, $\Delta^{3',4'}$-, R = H
13 *N*-Methylanabasine, R = CH_3
14 *N*-Methylanatabine, $\Delta^{3',4'}$-, R = CH_3

effectively demethylated in a cell suspension culture of *N. plumbaginifolia* (Solanaceae). Both substrates accumulated in the medium, and strong stereoselectivity for the (*R*)-isomers was observed. No further degradation of the initial demethylation products occurs (Bartholomeusz et al., 2005a). Furthermore, (*R,S*)-*N*-methylanabasine (**13**) does not compete with (*S*)-nicotine (**7**) demethylation.

The metabolism of nicotine (**7**) and *N*′-nornicotine (**8**) in *N. plumbaginifolia* suspension cell cultures that do not form endogenous nicotine was examined. Nicotine metabolism resulted in the accumulation of *N*′-nornicotine (**8**), and six minor metabolites, four were identified as cotinine (2′-oxo-nicotine), myosmine, *N*′-formylnornicotine, and *N*′-carboethoxynornicotine. Although cotinine was formed from [$^{13}C,^{2}H_3$-methyl]nicotine (**7**) without loss of label, *N*′-formylnornicotine was poorly (6%) labeled, and *N*′-carboethoxynornicotine was unlabeled. [1′-^{15}N]Nornicotine afforded *N*′-formylnornicotine and *N*′-carboethoxynornicotine without loss of label. This indicates that *N*′-nornicotine and cotinine are derived directly from nicotine (**7**), while *N*′-formylnornicotine and *N*′-carboethoxynornicotine are metabolites of nornicotine (**8**); the latter is therefore not an intermediate in nicotine demethylation (Bartholomeusz et al., 2005b).

The next step after the formation of *N*-methylputrescine is oxidation of the primary amine to the aldehyde. A methylputrescine oxidase from *N. tabacum* L. has been cloned and characterized (Heim et al., 2007). Some progress was reported for the identification of the functionality of the genes associated with pyridine alkaloid biosynthesis in *N. tabacum* L. (Solanaceae) plants. The full-length cDNA clones for 34 genes were isolated and some activities of selected genes expressed, including one for a lysine decarboxylase (Häkkinen et al., 2007).

Minimizing the content of nornicotine (**8**) in tobacco is a highly desirable goal, because of the precursor relationship to the potent laboratory carcinogen *N*′-nitrosonornicotine. Typically, in *Nicotiana* species, nornicotine (**8**) is a relatively minor alkaloid (2–5% of the total pyridine alkaloid pool in the mature leaf), and is probably produced almost entirely through the *N*-demethylation of nicotine, catalyzed by nicotine *N*-demethylase (NND). The gene CYP82E4 was identified as the specific NND gene responsible, with CYP82E5v2 as a putative minor NND gene. The discovery and characterization of CYP82E10, another tobacco NND gene, showed the origin of CYP82E10 from *N. sylvestris* Speg. (Solanaceae) (Lewis et al., 2010). Through knockout mutations all three tobacco NND genes were identified. By generating a series of mutant NND genotypes, the relative contribution of each NND gene toward the nornicotine (**8**) content of the plant was assessed. Levels were much lower than in conventional tobacco cultivars. The study suggested a strategy for reducing the levels of this animal carcinogen in tobacco.

To explore the relationships between the arginine and ornithine routes to putrescine, RNAi methodology was used to down-regulate ornithine decarboxylase transcript levels in transgenic *N. tabacum* plants derived from an *Agrobacterium rhizogenes*-derived hairy root culture system, and a disarmed *Agrobacterium tumefaciens* system (DeBoer et al., 2011). Significant ODC transcript down-regulation was observed leading to reduced nicotine (**7**) and increased anatabine (**10**) levels in both cultured hairy roots and intact greenhouse-grown plants. Neither treatment with methyl jasmonate nor wounding (removal of apices) of ODC-RNAi hairy roots restored capacity for normal nicotine synthesis in transgenic tissue, although markedly increased levels of anatabine (**10**) were observed. It was deduced that that the ODC-mediated route to putrescine plays an important role in determining the nicotine:anatabine profile in *N. tabacum*, and is necessary for *N. tabacum* to increase nicotine (**7**) levels in response to wound-associated stress.

1.3. Tropane alkaloids

Many studies on the biosynthesis of the tropane alkaloids hyoscyamine (atropine) (**12**) and scopolamine (**11**) have been described in the past 50 years in *Phytochemistry*. In 1969, Cosson reported that extended periods of light stimulated scopolamine (**11**) production in *Datura metel* L. (Solanaceae) leaves (Cosson, 1969). A simple and elegant, yet powerful, experiment on the biosynthesis of hyoscyamine (**12**) and scopolamine (**11**) was the demonstration by Ahmad and Leete (1970) that DL-δ-[*N*-methyl-^{14}C, 2-^{14}C]ornithine was incorporated intact at the bridgehead carbon C-1, but that the isomer with the α-nitrogen methylated and labeled was not.

Two early studies of tropane alkaloid production in tissue culture were reported by Tabata and co-workers. Callus cultures derived from the roots of *Scopolia parviflora* (Dunn) Nakai (Solanaceae) were maintained for two years. Alkaloid production was much less than the intact rhizome, but could be enhanced slightly by addition of tropic acid [the precursor of the esterifying unit of hyoscyamine (**12**) and scopolamine (**11**)] (Tabata et al., 1972). For *Datura inoxia* Mill. (Solanaceae) cultured on an auxin-free medium supplemented with kinetin, the regenerated roots could be grown to complete, mostly diploid, plants. Scopolamine (**11**) production was observed to begin during root differenetiation, and the alkaloid pattern was restored in the regenerated plants (Hiraoka and Tabata, 1974). An investigation of the stereochemistry of the incorporation of D- and L-hygrine labeled at the 2′-position with ^{14}C into the tropane alkaloids scopolamine (**11**) and atropine (**12**) in *D. inoxia* (Solanaceae) demonstrated that only the D-isomer was incorporated (McGaw and Woolley, 1978). Both isomers were apparently equally incorporated into cuscohygrine in *Physalis*, however (McGaw and Woolley, 1979). Subsequently it was shown that hygrine was not a direct precursor of cuscohygrine in *Erythroxylum coca* (Newquist et al. 1993). Rather, a pyrrolidinium species with a four-carbon side chain was a direct precursor for ethyl (*RS*)[2,3-$^{13}C_2$, 3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate specifically and labeled cuscohygrine, whereas hygrine was poorly incorporated.

In order to examine the sites of tropane alkaloid biosynthesis and distribution, Zenk and co-workers (Weiler et al., 1981) developed a radioimmunoassay for the detection of picomole quantities of scopolamine (**12**) using [*N*-methyl-$^{3}H_3$]-(−)-scopolamine as the tracer. Related alkaloids did not interfere, and the variability in plant and leaf distributions was reported for *Datura sanguinea* Ruiz. & Pav. Cultured roots of *D. leichhardtii* F.Muell. (Solanaceae), *D. myoporoides* R.Br., and *D. hopwoodii* F.Muell. produced both tropane and pyridine-type alkaloids. Root cultures of *D. leichhardtii* showed the highest content of tropane alkaloids (hyoscyamine 0.53%, scopolamine 1.16%) (Endo and Yamada, 1985).

An attempt was made to examine the variation in alkaloid patterns in the petioles of over 1000 plants of the genera *Datura*, *Scopolia*, and *Hyoscyamus*, resulting in the establishment of transformed root cultures of the high and low alkaloid-producing plants. Although relative alkaloid patterns were retained between species, less differentiation within species was observed in tissue culture, suggesting that full expression of alkaloid potential was not occurring (Parr et al. 1990). 5,6-Dichloro-indole acetic acid (at 0.01 mg/L) was a powerful inducer of tropane alkaloid (particularly scopolamine) biosynthesis in a *Duboisia* hybrid root culture (Yoshimatsu et al., 1990).

Tropane alkaloid biosynthesis in hairy roots of *Hyoscyamus albus* L. (Solanaceae) transformed with *A. rhizogenes* was enhanced with added KNO_3 to produce up to 30 mg/100 ml of tropane alkaloids, predominantly 6β-hydroxyhyoscyamine (25%) and hyoscyamine (**12**) (63%) (Sauerwein and Shimomura, 1991). High levels of atropine esterase activity were found at all stages of development of *A. belladonna*, *Datura tatula* L., *D. leichhardtii*, *Hyoscyamus niger* L., and *Scopolia japonica* Maxim. (Kitamura et al., 1992). Evidence was also presented (Kitamura et al., 1996) that recycling of the tropic acid moiety of atropine (**12**) may be occurring in *D. myoporoides* root cultures.

In 1995, Robins and co-workers purified the enzyme tigloyl-CoA:pseudotropine acyl transferase 330-fold from transformed cultures of *D. stramonium*. The transferase had a M_r of 65,000, and maximal activity occurred at pH 9 (Rabot et al., 1995). The enzyme esterifies the 3β-hydroxyl group of pseudotropine (3β-hydroxytropane) with the tiglic acid moiety of tigloyl-CoA. Tropine (3α-hydroxytropane) and norpseudotropine were not acylated.

An early use of ^{15}N NMR spectroscopy in biosynthesis was a study tracking the metabolic fate of [^{15}N]tropinone in transformed root cultures of *D. stramonium* (Ford et al., 1996). Labeling of alkaloids occurred within 2–4 h after administration, particularly to afford the tropine esters.

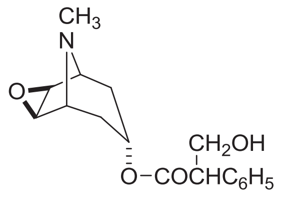
Methyl jasmonate stimulated tropane alkaloid biosynthesis in hairy-root cultures of *D. stramonium* (Solanaceae) more than a yeast elicitor or oligogalactouronides, and was based on higher levels of tropine (Zabetakis et al., 1998).

Contrasting results were observed when a range of analogs of *N*-methylputrescine and tropinone was fed to transformed root cultures of *N. rustica* and/or a *Brugmansia candida* × *aurea* hybrid (Boswell et al., 1999a). Analogs with larger *N*-alkyl substituents were not metabolized. In the *N. rustica* cultures, the analogs at 1 mM caused a 4-fold diminution of nicotine (**7**) in the presence of *N*-*n*-propylputrescine. In the *B. candida* × *aurea* hybrid culture at 1 mM the analogs did not inhibit or substantially interfere with the accumulation of the normal spectrum of alkaloids.

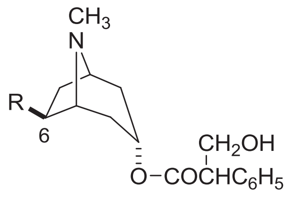
In follow-up experiments, five enzymes: *N*-methylputrescine oxidase (MPO), the tropine-forming tropinone reductase (TRI), the pseudotropine-forming tropinone reductase (TRII), the tropine:acyl-CoA transferase (TAT), and the pseudotropine:acyl-CoA transferase (PAT) derived from transformed root cultures of *D. stramonium* and a *B. candida* × *aurea* hybrid were evaluated for substrate specificity (Boswell et al., 1999b). MPO activity was tested with a series of *N*-alkylputrescines and *N*-alkylcadaverines, and TRI and TRII reduction was evaluated against various *N*-alkylnortropinones, *N*-alkylnorpelletierines and structurally related ketones as substrates. TAT and PAT esterification tested a series of *N*-substituted tropines, pseudotropines, pelletierinols, and pseudopelletierinols as substrates. Some ability of these enzymes to utilize unnatural substrates was observed.

Micellar electrokinetic chromatography was used to study the alkaloids scopolamine (**11**), hyoscyamine (**12**), and littorine (**16**) in hairy root clones of *Hyoscyamus muticus* L. Significant differences were observed in alkaloid content between the clones, and littorine dominated over scopolamine (**11**). Aerial parts of *H. muticus* contained no littorine (Mateus et al., 2000).

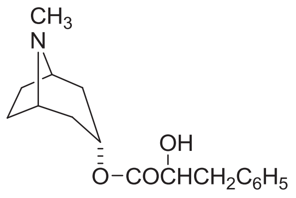
The mechanism for the conversion of littorine (**16**), which contains a phenyllactic acid ester at C-3 of the tropine nucleus, to atropine (**12**), which has a tropic acid ester moiety, has been a subject of controversy over many years, since it was reported to occur through an intramolecular rearrangement (Ansarin and Woolley, 1994). Using (*RS*)-[2-^{2}H]-, [3,3-$^{2}H_2$]-, and [2,3,3-$^{2}H_3$]phenyllactic acids, Patterson and O'Hagan (2002) showed that the resulting atropine (hyoscyamine) (**12**) was not formed as a result of a vicinal interchange taking place during the isomerization. Furthermore, a study with [1′-^{13}C, 3′,3′-$^{2}H_2$]atropine (**12**) indicated the stability of the alkaloid with no reversible oxidation to the aldehyde occurring. The conclusion was that an *S*-adenosyl-L-methionine (SAM)/coenzyme-B_{12} mediated process was not operating for the isomerization of littorine (**16**) to hyoscyamine (**12**), and that the mechanism of the rearrangement remained unknown. Previous work had examined the stereochemical aspects of the biosynthesis



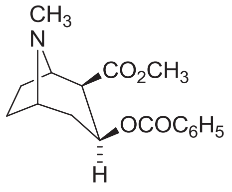
11 Scopolamine



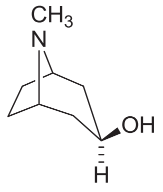
12 Hyoscyamine, R = H
15 6β-Hydroxyhyoscyamine, R = OH



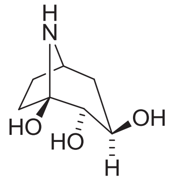
16 Littorine



17 Cocaine



18 Pseudotropine



19 Calystegine A_3

of tropic acid, present as the esterifying unit of both atropine (**12**) and scopolamine (**11**), using specifically labeled phenylalanine precursors (Platt et al., 1984).

Reduction of the 3-keto group in tropinone may occur with delivery of hydride from the *re*- or *si*-face and leads to alkaloids with a 3α- or a 3β-hydroxy group. These are respectively the atropine (**12**), scopolamine (**11**) series with a 3α-orientation, or the cocaine (**17**) and calystegine series which are derivatives of pseudotropine having a 3β-orientation. Dräger (2006) reviewed this area of tropane alkaloid enzymology, including aspects of the isolation, cloning, and heterologous expression of the two reductases, TR-I and TR-II, and their protein crystallization and structure determination. There is about 50% homology between the two enzymes, although this may not be meaningful. Also discussed were their localization in cultured roots, how they regulate the metabolic flux of the tropane alkaloids, and the evolutionary aspects of the enzymes.

The final step in the biosynthetic pathway for the formation of scopolamine (**11**) is epoxidation of 6β-hydroxyhyoscyamine, and an enzyme, 6β-hydroxyhyoscyamine epoxidase, was isolated and characterized from cultured roots of *H. niger* (Hashimoto et al., 1988). The enzyme was a dioxygenase requiring molecular oxygen for activity.

1.4. Calystegines

The calystegines are a group of hydroxylated nortropane alkaloids with specific glycosidase inhibitory activity. Although it was initially thought that their distribution was quite limited, it later became clear that they were also widespread in the Convolvulaceae (Schimming et al., 1998), which is taxonomically close to the Solanaceae, a traditional source of tropane alkaloids.

Using ^{15}N-tropinone it was shown in root cultures of *Calystegia sepium* R.Br. (Convolvulaceae) that pseudotropine (**18**) and calystegine A3 (**19**) were labeled after 2 days, and calystegines in the B group were labeled subsequently. 2,7-Dihydroxy-nortropane was also labeled and maybe a by-product of the tropane pathway which leads to the calystegines (Scholl et al. 2001).

Genes encoding for the formation of the calystegines also occur in plants in the genus *Erythroxylum* P.Browne (Erythroxylaceae), with alkaloid content up to 0.32% in the leaves. At least one of the four main calystegines was found in 38 of 45 herbarium specimens of different *Erythroxylum* species. The family is very distant taxonomically from the Solanaceae and Convolvulaceae (Brock et al., 2007).

1.5. Pyrrolizidine alkaloids

Homospermidine synthase is the first pathway-specific enzyme in pyrrolizidine alkaloid biosynthesis (Böttcher et al., 1993). Root cultures of *Senecio vulgaris* L. (Asteraceae) accumulated labeled homospermidine from putrescine when β-hydroxyethylhydrazine (HEH) was used as a diamine oxidase inhibitor, and was then incorporated into pyrrolizidine alkaloids when HEH was removed. The enzyme was partially purified from root cultures of *Eupatorium cannabinum* L. (Asteraceae). Putrescine is the exclusive substrate, and the enzyme was inhibited by diaminopropane, spermidine, and cadaverine (mixed inhibition). NADH is also an inhibitor, indicating that, even though it is generated in the oxidative deamination of putrescine, it remains enzyme bound. All root cultures of plants in the Asteraceae which produced pyrrolizidine alkaloids showed the presence of homospermidine synthase (Böttcher et al., 1993).

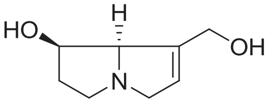
Ober and Kalternegger (2009) reviewed the evolution of the enzyme homospermidine synthase in plants producing pyrrolizidine alkaloids. It has been shown that the enzyme evolved by duplication of a gene encoding deoxyhypusine synthase, and that this occurred several times independently during angiosperm evolution. Only occasionally did these copies function to produce homospermidine synthase, and thus pyrrolizidine alkaloids, indicating that other regulatory elements were involved in the evolutionary process.

1.6. Retronecine

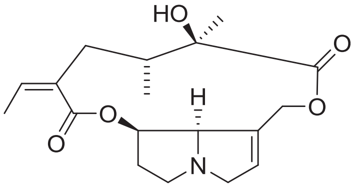
Bottomley and Geissman (1964) demonstrated that labeled putrescine, and [2- and 5-^{14}C]ornithine, were all precursors of the retronecine (**20**) moiety in pyrrolizidine alkaloids in the roots of *Senecio douglasii* DC. (Asteraceae). It was concluded that at least one of the two molecules of ornithine passes through a symmetrical precursor.

An early use of ^{14}C- and ^{3}H-doubly-labeled precursors was an application examining the relative rates of incorporation of arginine and ornithine into retronecine (**20**) in *Senecio magnificus* F.Muell. (Asteraceae). Ornithine was determined to be a significantly more efficient precursor (Bale and Crout, 1975). Both L-arginine and L-ornithine, but not their D-isomers, served as precursors of retronecine (**20**) in *Senecio isatideus* DC. (Asterceae) (Robins and Sweeney 1983).

Senecionine-*N*-oxide (**21**) was found to be the primary pyrrolizidine alkaloid in root cultures of *S. vulgaris* L. (Asteraceae), after administering a variety of precursors including arginine, ornithine, isoleucine, putrescine, and spermidine. Incorporation of the latter precursors was very high (20–30%) (Hartmann and Toppel, 1987). Tertiary pyrrolizidine alkaloids were barely detected.



20 Retronecine



21 Senecionine-*N*-oxide
22 Senecionine

Before the development of metabolomics and principal component analysis, chemical races (chemotypes) within plant species were identified by gas chromatography, and subsequently by gas chromatography coupled with mass spectrometry (GC–MS). Examination by GC–MS of more than 100 populations of *Senecio jacobaea* L., and about 25 populations of *S. erucifolius* L., from different geographical locations for the content of sixteen pyrrolizidine alkaloids established the presence of different chemotypes (Witte et al., 1992). A jacobine chemotype and an erucifoline chemotype were observed for *S. jacobaea*, and an erucifoline chemotype and an eruciflorine chemotype for *S. erucifolius*. Within the respective species there were no morphological differences, however, observed between the chemotypes. Eruciflorine (21-hydroxyintegerrimine) was identified as a new alkaloid.

For the first time, *in vivo* ^{15}N NMR spectroscopic studies were used to study alkaloid metabolism in *D. stramonium* and *N. tabacum* transformed root cultures (Ford et al., 1994). The amount of detectable ^{15}N label in the secondary metabolites was about 1.4 μmol. In *D. stramonium*, the ^{15}N resonance of hyoscyamine (**12**) was observed at −305.5 ppm, along with three unidentified alkaloids, and possibly some conjugated putrescine derivatives. In *N. tabacum*, nicotine (**7**) showed ^{15}N resonances at −75.7 and −309 ppm for the pyridine and pyrrolidine nitrogen atoms, respectively. Aspects of the subcellular distribution of nicotine (**7**) were deduced.

If alkaloids are viewed as protective agents, then the most vulnerable parts should be the most protected. The youngest leaves of *Cynoglossum officinale* L. (Boraginaceae) contained up to 190 times higher levels of pyrrolizidine alkaloids than the older leaves (van Dam et al., 1994). Similar defensive effects were observed for the quinolizidine alkaloids of Spanish broom, *Spartium junceum* L. (Fabaceae), where the highest alkaloid contents were found in young plants, in reproductive organs, such as the seeds, and in primary twigs (Barboni et al., 1994).

Theoretical considerations suggest that incorporation of (*S*)-[1-^{2}H]-putrescine through the symmetrical intermediate homospermidine into the necine unit of the pyrrolizidine alkaloids should proceed with 50% deuterium retention. However, experimentally a level of only 34% to 34.5% retention was found in two different laboratories. Graser and co-workers (1998) confirmed these results and eliminated deuterium isotope effects during homospermidine formation as an explanation. Using *S. vulgaris* root cultures, ^{2}H-retention for putrescine, spermidine, and homospermidine was examined after administration, doubly-labeled [^{2}H-^{14}C]putrescine. Stereospecific loss from (*S*)-[1-^{2}H]-labeled putrescine occurred during the conversion of spermidine into putrescine during its reversible interconversion, accounting for the difference between predicted and determined ^{2}H-retention. The mechanism of the reaction remained to be elucidated.

In *Crotalaria scassellatii* Chiov. (Fabaceae) seeds, pyrrolizidine alkaloids are stored as tertiary amines. At seed germination, conversion to the alkaloid *N*-oxides transportation, metabolism, and storage occurs. A flavin-dependent, mixed function *N*-oxygenase (SNO) was isolated and partially purified. It showed low K_m values for senecionine and monocrotaline (Chang and Hartmann, 1998). The substrate specificity was very similar to an insect SNO characterized from the hemolymph of arctiid larvae (Lindigkeit et al., 1997).

A further study of 24 species of the *Jacobaea* section of the genus *Senecio* for the presence of 26 pyrrolizidine alkaloids (PAs) indicated that, with only one exception, all were derivatives of senecionine (**22**). Based on this, a biosynthetic grid was developed involving two pathways, and an evolutionary history was developed. It was concluded that the differences in PA profiles between the species, and in other sections of *Senecio*, can be attributed to transient switching of the genes encoding the PA pathway-specific enzymes, not their presence or absence (Pelser et al., 2005).

1.7. Lysine-derived alkaloids

The presence of lysine decarboxylase was demonstrated to be positively correlated with leaf alkaloid content in *Lupinus polyphyllus* Lindl. (Fabaceae), and the enzyme was demonstrated to be present in 46 alkaloidal and non-alkaloidal species in 17 plant families (Schoofs et al., 1983).

Quinolizidine alkaloid distribution from seed germination to seed formation was studied in three *Lupinus* species, and discussed in relationship to predation (Williams and Harrison, 1983). Alkaloid levels were highest at the time of seed formation, and declined rapidly after germination.

Quinolizidine alkaloids are mainly distributed in the subfamily Papilionoideae in the family Fabaceae, and a well-established source is the genus *Lupinus*. The first plant-derived lysine decarboxylase activity was reported from *Lathyrus sativus* L. (Fabaceae) (Ramakrishna and Adiga, 1976). There is about a four-fold difference in the alkaloid levels between the sweet and the bitter lupins *Lupinus luteus* L. and *L. albus* L. (Fabaceae). It was deduced that cyclization steps in the quinolizidine pathway were blocked in the sweet lupins, although acyltransferase levels were the same in both lupin varieties (Saito et al., 1993).

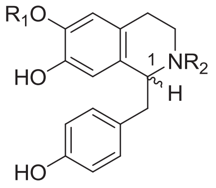
In a subsequent study, the sweet and bitter varieties of three *Lupinus* species (*L. angustifolius* L., *L. albus*, and *L. luteus*) quinolizidine alkaloid levels varied from 0.58 μg/ml (sweet) to 29.6 μg/ml (bitter) in *L. albus* (Aniszewski et al., 2001). Bitter lupin seeds also contained high levels of biogenic polyamines, and low basic amino acid levels. The reverse was noted for sweet lupin seeds.

The subcellular localization of two acyltransferases was studied in *L. albus* and *L. hirsutus* L. The enzymes were not located in the chloroplasts where *de novo* alkaloid synthesis occurs. One was discovered in the mitochondrial fraction and localized in the matrix, and the other was in the cytosol. A proposal for the detailed transport of quinolizidine alkaloids within the cell based on the location of the two enzymes was made (Suzuki et al., 1996).

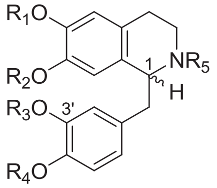
1.8. Phenylalanine–tyrosine-derived alkaloids

1.8.1. Benzylisoquinolines and derivatives

One of the most important alkaloid biosynthesis papers published in *Phytochemistry* in the 50-year period was that by Zenk and co-workers (Stadler et al., 1989) on the role of (*S*)-norcoclaurine (**23**) in benzylisoquinoline alkaloid biosynthesis. Based on long-held biogenetic ideas and precursor administration experiments by others, it was considered that the tetrahydroxylated norlaudanosoline (**24**) was the precursor of the complex benzylisoquinoline alkaloids. However, Zenk and co-workers demonstrated that (*S*)-[1-^{13}C]norcoclaurine (**23**), a trihydroxylated intermediate, was a precursor of pavine, protoberberine, aporphine, and benzophenanthridine alkaloids in selected cell cultures and whole plants. This dramatic result revised the early steps in benzylisoquinoline alkaloid biosynthesis showing that tyrosine is metabolized to dopamine for the upper unit, and then condenses with 4-hydroxyphenylacetaldehyde to form **23**. This result also explains why dopamine, or related 3,4-dihydroxy derivatives, were not incorporated into the lower units of benzylisoquinoline alkaloids.



		R1	R2	
23	(*S*)-Norcoclaurine	H	H	1α-H
25a	(*R*)-Coclaurine	CH_3	H	1β-H
25b	(*S*)-Coclaurine	CH_3	H	1α-H
37a	(*R*)-*N*-Methylcoclaurine	CH_3	CH_3	1β-H
37b	(*S*)-*N*-Methylcoclaurine	CH_3	CH_3	1α-H

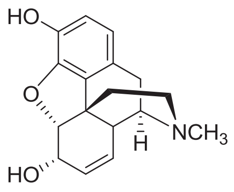


		R1	R2	R3	R4	R5	
24	Norlaudanosoline	H	H	H	H	H	
26	3'-Hydroxy-*N*-methyl -(*S*)-coclaurine	H	H	H	H	CH_3	1α-H
28	(*R*)-Reticuline	CH_3	H	H	CH_3	CH_3	1β-H
29	(*S*)-Reticuline	CH_3	H	H	CH_3	CH_3	1α-H
33	(*S*)-Laudanosine	CH_3	CH_3	CH_3	CH_3	CH_3	1α-H
34a	(*S*)-Nor-reticuline	CH_3	H	H	CH_3	H	1α-H
34b	(*R*)-Nor-reticuline	CH_3	H	H	CH_3	H	1β-H

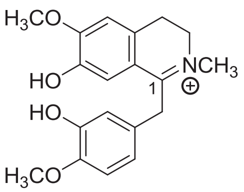
The step after *O*- and *N*-methylation in the benzylisoquinoline biosynthesis pathway is hydroxylation of ring C. Loeffler and Zenk (1990) reported on the isolation of an enzyme purified 488-fold from cultures of *Berberis stolonifera* Koehne & E.L.Wolf (Berberidaceae) which catalyzed the *meta*-hydroxylation of coclaurine (**25**), as well as *N*-methylcoclaurine, tyrosine, and tyramine.

Several isoquinoline-containing species belonging to the families Berberidaceae, Fumariaceae, Papaveraceae, and Ranunculaceae afforded an enzyme complex SAM:3′-hydroxy-*N*-methyl-(*S*)-coclaurine-4′-*O*-methyltransferase (4′-OMT) (Frenzel and Zenk, 1990b). The enzyme was purified 400-fold from *Berberis koehneana* Schneider (Berberidaceae) cell cultures, and consisted of only the 4′-OMT and SAM:norcoclaurine-6-*O*-methyltransferase; further separation was not successful. The 4′-OMT was substrate specific, and, in the presence of SAM, catalyzed methyl transfer to the C-4′ hydroxyl group of benzylisoquinoline alkaloids with the 1*S*-configuration and having *ortho* hydroxy groups at C-3′ and C-4′; the natural substrate is 3′-hydroxy-*N*-methyl-(*S*)-coclaurine (**26**). The pH optimum was 8.3, it had M_r 4.0 kD, and was potently inhibited by the product alkaloid. The half life of the enzyme was three days at room temperature, and it was stable for >1 year at −20 °C.

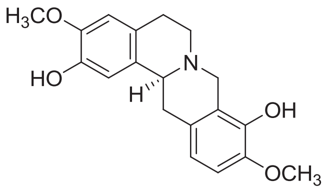
Morphine (**27**) biosynthesis requires a reduction step after the oxidation of (*S*)-reticuline (**29**) to afford (*R*)-reticuline (**28**), and the NADPH-dependent enzyme, 1,2-dehydroreticuline reductase, was found in seedlings of *Papaver somniferum* (De-Eknamkul and Zenk, 1992); it was not found in non-morphine containing plants. The isolated cytosolic enzyme had a M_r of 30 kD, a pH optimum of 8.5 and a temperature optimum of 30 °C. The K_m value for 1,2-dehydroreticuline (**30**) was 10 μM. The enzyme, which transfers the *pro-S*-hydride of NADPH to C-1 of 1,2-dehydroreticuline (**30**), shows high substrate specificity, since neither 1,2-dehydronorreticuline nor 1,2-dehydrococlaurine were substrates; both (*S*)- (**29**) and (*R*)-reticuline (**28**) inhibit the enzyme.



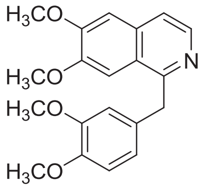
27 Morphine



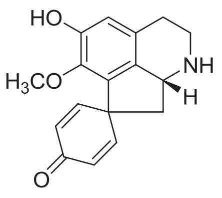
30 1,2-Dehydroreticulinium



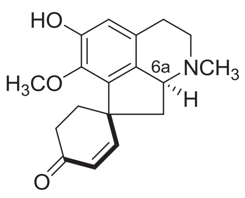
31 Scoulerine



32 Papaverine



35 Crotonosine



36 Linearsine

Peumus boldus Molina (Monimiaceae) and *B. stolonifera* Koehne et E.L.Wolf (Berberidaceae) are sources of aporphines and protoberberines, respectively. When triply-labeled [1-^{13}C,6-O^{13}CH$_3$,N-^{13}CH$_3$]-(*S*)-reticuline (**29**) was administered to their cell cultures unanticipated transmethylations of the methyl groups in both aporphines and protoberberines were observed by ^{13}C NMR spectroscopy. The phenomenon was explained by demethylation, incorporation into the C-1 pool, and remethylation (Schneider and Zenk, 1993a).

When ^{13}C-labeled (*S*)-reticuline (**29**) was utilized in a non-alkaloid producing cell line of *Thalictrum tuberosum* L. (Ranunculaceae), the protoberberine alkaloid scoulerine (**31**) was labeled initially, followed by other protoberberine derivatives (Schneider and Zenk, 1993b). This suggested that early stage events in the pathway were downregulated in the cell line.

From cultures of *Thalictrum minus* L. (Ranunculaceae) *S*-adenosyl-L-methionine:norcoclaurine 6-*O*-methyltransferase was purified and characterized (Hara et al., 1995). In 1995, Zenk and co-workers (Loeffler et al., 1995) showed that norcoclaurine (**23**) in the presence of *S*-adenosyl-L-methionine (SAM) was transformed into *N*-methylnorcoclaurine, coclaurine (**25**), and *N*-methylcoclaurine. Eight plant cell cultures [*Dicentra spectabilis* Lem. (Fumariaceae), *Fumaria capreolata* Leight. (Fumariaceae), *Chondodendron tomentosum* Ruiz & Pav. (Menispermaceae), *Tinospora cordifolia* Miers (Menispermaceae), *Argemone platyceras* Link & Otto (Papaveraceae), *P. somniferum*, *B. stolonifera*, and *Berberis julianae* C.K.Schneid. (Berberidaceae)] were studied. In *T. cordifolia*, (*S*)-norcoclaurine (**23**), but not its (*R*)-enantiomer, was exclusively *O*- and *N*-methylated. The enzyme responsible for the *N*-methylation was purified and shown to catalyze stereoselectively only the methyl transfer from SAM to (*S*)-configured norcoclaurine (**23**) and coclaurine (**25**). Thirteen other benzylisoquinoline alkaloids were unsuccessfully used as substrates. The pH optimum was 8.6, and as a substrate, (*S*)-coclaurine (**25**) showed a K_m of 36 μM; the molecular weight of the enzyme was estimatd as 85 kD. It was deduced that the stereoselective enzyme was present in only certain members of the Menispermaceae and in *D. spectabilis* Lem. (Fumariaceae).

The occurrence of (*S*)-norcoclaurine synthase (NCS) in ninety species of plants suggested the monophyletic origin of benzylisoquinoline alkaloid biosynthesis prior to the emergence of the eudicots. A latent molecular fingerprint for benzylisoquinoline biosynthesis in plants not known to produce such alkaloids was also detected based on analysis for NCS, berberine bridge enzyme, and several *O*-methyltransferases (Liscombe et al., 2005).

As mentioned, for many years, norlaudanosoline (**24**) was regarded as the immediate precursor for many benzylisoquinoline and related alkaloids. Papaverine (**32**) was an early speculated product thought to be derived through complete *O*-methylation of the tetrahydroxylated intermediate [norlaudanosoline (**24**)] and aromatization. Following more detailed precursor administration studies, Zenk and co-workers completely revised this pathway (Han et al., 2010). It was shown using 8-day-old seedlings of *Papaver* followed by direct examination of the stable isotope labeled precursors in the total plant extract, that the trihydroxylated norcoclaurine (**23**) is the primordial benzylisoquinoline alkaloid, and is further converted into (*S*)-reticuline (**29**). Methylation of (*S*)-reticuline (**29**) generates (*S*)-laudanine, and methylation at the 3′-position leads to laudanosine (**33**), known alkaloids from the opium poppy. *N*-Demethylation of laudanosine (**33**) then affords 1,2,3,4-tetrahydropapaverine. Dehydrogenation is step-wise with 1,2-dihydropapaverine as an intermediate, followed by dehydrogenation of the 1,2-bond to papaverine (**32**); the mechanism of the process is not known. The previously claimed nor-reticuline (**34**) was not involved in papaverine (**32**) biosynthesis.

Studies on the relationships of the proaporphine alkaloids, such as crotonosine (**35**), and the dihydroproaporphine alkaloids, such as linearisine (**36**), became somewhat more confused when it was shown that linearisine (**36**) can be oxidized, undergo inversion at C-6a, and *N*-demethylation to afford crotonopsine (**35**) in *Croton linearis* Jacq. (Euphorbiaceae) (Stuart and Graham, 1973b).

1.9. Bisbenzylisoquinoline alkaloids

Very few studies have been reported on the biosynthesis of the bisbenzylisoquinoline alkaloids. In 1980, Bhakuni and co-workers (1980) reported on the incorporation of doubly-labeled (±)-*N*-methylcoclaurine (**37**) into tetrandrine (**38**), and showed that the C-1H was retained.

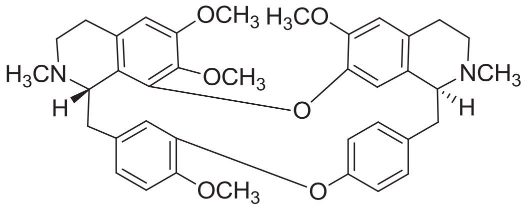
When ^{14}C-labeled tyrosine, tyramine, and several chiral 1-benzyl-1,2,3,4-tetrahydroisoquinolines were administered to bisbenzylisoquinoline alkaloid-producing cell cultures of *B. stolonifera* (Stadler et al., 1988), berbamunine (**39**) showed significantly higher incorporation than 2-norberbamunine, berbamine, aromoline, or isotetrandrine. This suggesed that **39** is possibly the initial dimer formed. Exclusive incorporation of tyramine into the isoquinoline portion of this dimer was observed. Administering various benzylisoquinoline precursors showed that (*R*)-*N*-methylcoclaurine (**37a**) gave the highest relative incorporations (29% into berbamunine) and (*S*)-*N*-methylcoclaurine (**37b**) the lowest (2.4%). Both (*S*)-coclaurine (**25**) and (*S*)-*N*-methylcoclaurine (**37b**) were well incorporated into the protoberberine fraction (21 and 54%, respectively). Consequently, the pathway which promotes dimerization and the C-3′-hydroxylation pathway leading to reticuline are in competition for these substrates. Using [1-^{13}C]-(*R*)- and (*S*)-coclaurine (**25a** and **25b**) showed that the (*R*)-isomer (**25a**) is incorporated similarly into both halves of the (*R*, *R*)-dimer guattegaumerine. Racemization of (*S*)- to (*R*)-coclaurine did not occur.

1.10. Berberines and derivatives

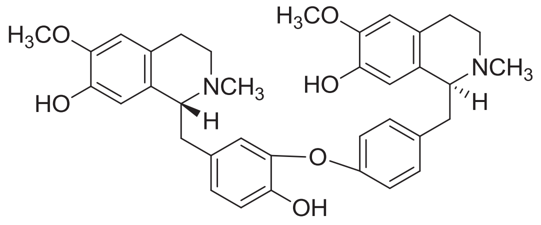
An *N*-methylation step, followed by hydroxylation α to N, is involved in the biosynthesis of the benzazepine alkaloid *cis*-alpinigenine (**40**). When [8,13,14-^{3}H$_3$]-(±)-tetrahydropalmatine (**41**) and [8,13,14-^{3}H$_3$,8-^{14}C]-(±)-tetrahydropalmatine methiodide (**42**) were administered to *Papaver bracteatum* the same loss of ^{3}H was observed, including complete ^{3}H loss from C-14 (Roensch, 1977). The next alkaloid in the biogenetic sequence, [8-^{14}C]muramine (**43**), was efficiently incorporated into alpinigenine (**40**). Using unnatural substrates, such as [2′-^{14}C]hydroxymethylaudanosine and [8-^{14}C]13-hydroxymuramine (**44**), the specificity of alkaloid metabolism was examined. Tracer dilution was used to confirm the occurrence of the three established intermediates.

The most important intermediate enzyme in the formation of many advanced benzylisoquinoline alkaloids is the berberine bridge enzyme (BBE) which catalyzes the oxidation of a *N*-methyl group, the coupling to form the C-ring of protoberberine, and subsequently the benzo[c]phenanthridine alkaloids. Zenk and co-workers (Steffens et al., 1985) found the enzyme in 66 samples of differentiated plants and cell suspension cultures. The enzyme was purified 450-fold from *Berberis beaniana* C.K.Schneid. (Berberidaceae) cell cultures and shown to be homogeneous by gel electrophoresis (mol. wt. 52 kD). The enzyme required the presence of oxygen and catalyzed the transformation of the (*S*)-enantiomers of reticuline (**29**), protosinomenine, and laudanosoline to the corresponding (*S*)-tetrahydroprotoberberines, releasing stoichiometric amounts of H_2O_2 in the process.

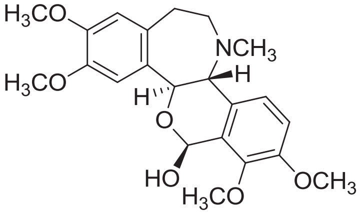
The STOX enzyme, (*S*)-tetrahydroprotoberberine oxidase, was enriched 7.4-fold from a high-yielding cell suspension of *Berberis wilsoniae* Hemsl. var. *subcaulialata* and immobilized (Amann and Zenk, 1987). Compared with immobilized *Berberis* cells and with the soluble enzyme, the pH and temperature optima were slightly shifted by immobilization, but the K_m values for (*S*)-norreticuline (**34a**) were unaffected. Stability was improved 50-fold compared with the free enzyme. A cyclic C-1 inversion process was described



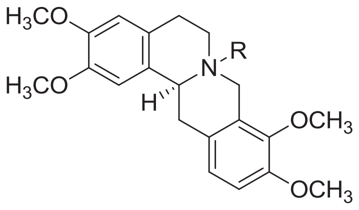
38 Tetrandrine



39 Berbamunine

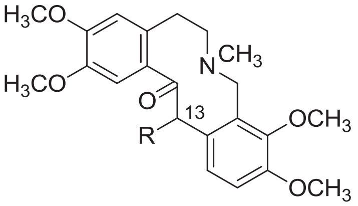


40 *Cis*-Alpinigenine



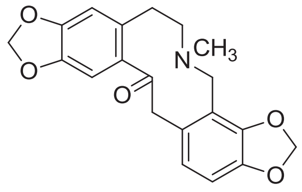
41 Tetrahydropalmatine, No R

42 Tetrahydropalmatine methiodide, $R = CH_3, I^{\ominus}$



43 Muramine, R = H

44 13-hydroxymuramine, R = OH



45 Protopine

using the stereospecific enzymatic oxidation of (*S*)-norreticuline (**34a**) to 1,2-dehydronorreticuline followed by sodium borohydride reduction to the (*R*)-enantiomer (**34b**).

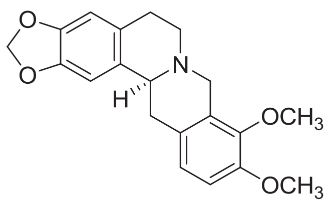
The role of protopine (**40**) in benzo[c]phenanthridine biosynthesis was defined by Tanahashi and Zenk (1990). [6-^{3}H]Protopine (**45**) was synthesized, and in *Eschscholzia californica* suspension cultures induced by a yeast preparation, produced 6-hydroxyprotopine which spontaneously rearranged to [11-^{3}H]-7,8-dihydrosanguinarine. It was deduced that the enzyme was a P450-linked monooxygenase which could be induced by elicitor and was present in different plant species producing benzo[c]phenanthridine alkaloids.

An enzyme was found in different species of isoquinoline alkaloid-producing plant cell cultures which specifically *N*-methylates certain (*S*)-tetrahydroprotoberberine alkaloids, such as (S)-canadine (**46**) and (*S*)-stylopine (**47**), at the expense of *S*-adenosyl-L-methionine (SAM). It was partially purified (90-fold) from *E. californica* Cham. (Papaveraceae) cell suspension cultures and characterized. The enzyme had a pH optimum of 8.9, a temperature optimum of 40 °C, and a molecular weight of 78 kDa. The K_m for (*S*)-canadine (**46** was 6.4 μM, and for (*S*)-stylopine (**47**) was 3.1 μM, and the enzyme was inhibited by *S*-adenosyl-L-homocysteine (SAH) with a Ki of 24 μM (Rueffer et al., 1990).

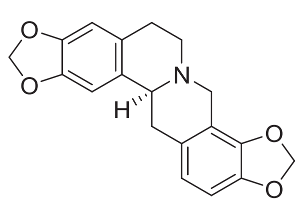
After screening 37 cell suspension cultures and finding *N*-methyltransferase activity in 34 of them, *B. koetineana*, which produced the highest enzyme levels, was selected for further study. Three distinctly different isoforms of *S*-adenosyl-L-methionine-(*R*,*S*)-tetrahydro-benzylisoquinoline-*N*-methyltransferases were isolated and designated as NMT-I, -II, and -III. The three enzymes had different M_r of 60–78 and 103 kD, pH optima (6.8/7.4), kinetic properties, and substrate specificities. NMT-I showed maximal activity with (*R*)-tetrahydropapaverine as substrate, whereas NMT-II and -III were most active against (*R*)-coclaurine (**25a**) (Frenzel and Zenk, 1990a). After immobilization, the enzymes retained their properties, and may serve for the preparation of isotopically labeled *N*-methylated benzylisoquinoline alkaloids.

The enzymatic synthesis of the methylenedioxy moiety in many benzylisoquinoline alkaloid derivatives had long been a subject of discussion and experimentation; Bauer and Zenk (1991) resolved this issue in an elegant manner. A microsomal preparation from *E. californica* cell suspension cultures in the presence of NADPH and O_2 catalyzed the formation of two methylenedioxy bridges in two consecutive steps: (*S*)-[10-OC^{3}H$_3$]scoulerine (**31**) afforded (*S*)-cheilanthifoline and (*S*)-[3-OC^{3}H$_3$]cheilanthifoline produced (*S*)-stylopine (**47**). Nandinine was not an intermediate. The release of HO^{3}H into the aqueous incubation mixture was used as an assay for both enzyme activities. The enzymes were characterized as cytochrome P450 monooxygenases on the basis of substrate specificity, cofactor requirements, and inhibition by cytochrome P450 specific inhibitors. The enzymes were induced (12- and 5-fold) 20 h after challenging the cell suspension culture with elicitor. The methylenedioxy moiety is viewed as derived from the oxidation of an *o*-methoxyphenol.

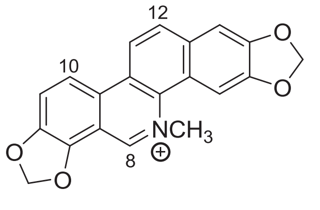
From several different Ranunculaceae and Berberidaceae cell cultures, an enzyme system catalyzing methylenedioxy bridge formation on ring A of (*S*)-canadine [(*S*)-tetrahydroberberine] (**46**) from (*S*)-tetrahydrocolumbamine was found (Rueffer and Zenk, 1994). The cytochrome P450 enzyme complex was partially characterized from a protoberberine-alkaloid producing



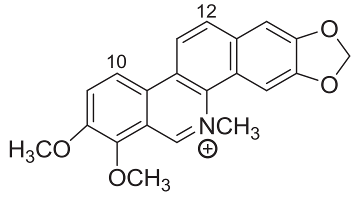
46 (*S*)-Canadine



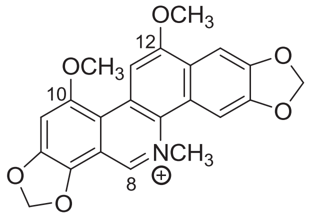
47 (*S*)-Stylopine



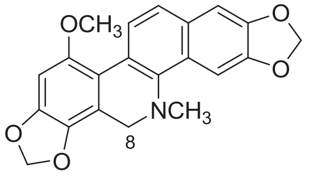
48 Sanguinarine
51 7,8-Dihydrosanguinarine



49 Chelerythrine



50 Macarpine
53 7,8-Dihydromacarpine



52 7,8-Dihydrochelirubine

T. tuberosum L. (Ranunculaceae) cell line, and consisted of a microsomal associated oxidase and a cytochrome P450 reductase with a pH optimum at pH 8.5 and a temperature optimum of 40 °C. The apparent K_m value was 11.5 μM for tetrahydrocolumbamine.

1.11. Benzo[c]phenanthridines

In suspension cultures of *E. californica*, the benzo[c]phenanthridine alkaloids sanguinarine (**48**) and chelerythrine (**49**) reached maximum levels a few hours after elicitation by a yeast extract. Subsequently, their levels declined, and the level of macarpine (5-methoxychelirubine) (**50**), the most highly oxidized benzo[c]phenanthridine alkaloid, increased. Induced tyrosine decarboxylase activity correlated with alkaloid formation (Collinge and Brodelius, 1989).

Temporal studies correlating the metabolism of tyramine with the production of sanguinarine (**48**) and hydroxycinnamic acid amides in *P. somniferum* cultures showed that elicitors tended to induce tyramine hydroxycinnamoyl transferase, which led to high levels of the amides in cell wall fractions, and only a small increase in alkaloid production through induction of berberine bridge enzyme (Facchini, 1998).

Dihydrosanguinarine-10-hydroxylase and 10-hydroxydihydrosanguinarine-10-*O*-methyltransferase, two new enzymes involved in benzo[c]phenanthridine alkaloid biosynthesis, were found in cell-free extracts derived from *E. californica* cell suspension cultures (De-Eknamkul et al. 1992). The hydroxylase is a microsome-associated, cytochrome P450-dependent monooxygenase which acts specifically at C-10 of 7,8-dihydrosanguinarine (**51**). The methyltransferase, which was enriched 60-fold and partially characterized, exclusively methylates the C-10 hydroxyl moiety to form dihydrochelirubine (10-methoxy-7,8-dihydrosanguinarine) (**52**). High substrate specificity was observed for both enzymes.

Cell-free extracts of yeast-elicited *Thalictrum bulgaricum* Velen. (Ranunculaceae) cell cultures afforded two novel enzymes, dihydrochelirubine-12-hydroxylase and SAM:12-hydroxydihydrochelirubine-12-*O*-methyltransferase which are involved in the last two steps in macarpine biosynthesis (Kammerer et al., 1994). The hydroxylase was a microsomal-associated, cytochrome P450-dependent monooxygenase acting exclusively at C-12 of dihydrochelirubine (**52**). The methyltransferase acted highly specifically on the hydroxyl moiety at C-12 to form 7,8-dihydromacarpine (**53**). As a result, the complete sequence of macarpine (**50**) biosynthesis was clarified at the enzymic level, the longest alkaloid pathway at that time.

An elicitor derived from *Penicillium expansum*, or the calcium ionphore A23187, induced benzo[c]phenanthridine alkaloid production in *Sanguinaria canadensis* L. (Berberidaceae) suspension cultures. Adding either a calcium chelatant or a calcium channel inhibitor, such as verapamil, markedly decreased alkaloid production, suggesting that calcium ions were important for stimulating alkaloid biosynthesis (Mahady and Beecher, 1994).

Further studies showed that compounds which modify protein kinase activity, such as phorbol-12-myristate-13-acetate and 1-oleoyl-2-acetyl-glycerol, significantly (65-fold and 25-fold, respectively) increased benzo[c]phenanthridine alkaloid production, whereas a protein kinase C inhibitor, such as staurosporine, markedly decreased alkaloid production in *S. canadensis* cell cultures (Mahady et al., 1998). G-Protein activators, such as mastoparan, mas-7, or melittin also induced alkaloid biosynthesis indicating involvement in signal transduction.

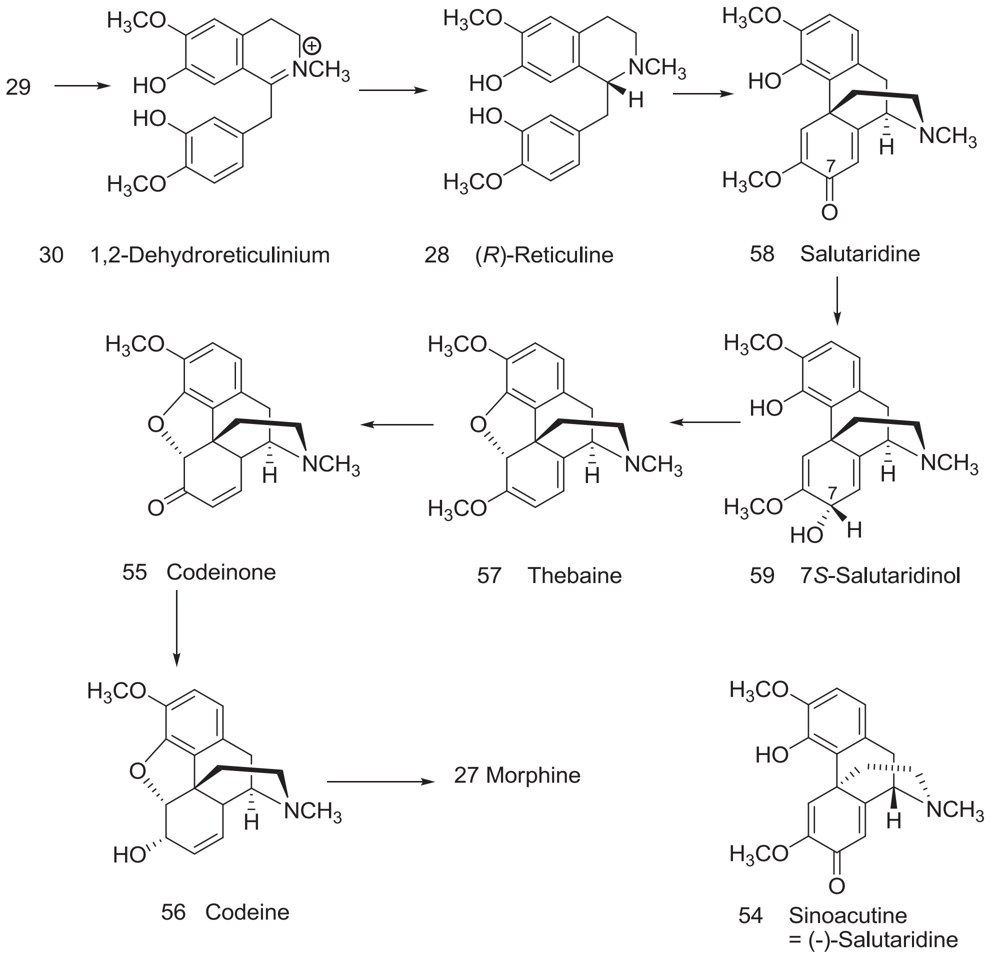
1.12. Morphinan and related alkaloids

An early evaluation of the enzymatic capacity of the latex of *P. somniferum* L. (Papaveraceae) was a demonstration that a centrifuged preparation was capable of converting dihydroxyphenylalanine into morphine (**27**) *in vitro* (Fairbairn and Djote, 1970); further metabolism of morphine (**27**) was noted in the stem latex, but not the capsule latex.

The morphinandienone alkaloid sinoacutine (**54**) is in the enantiomeric series compared with morphine (**27**). An early detailed study of alkaloids in this series using ^{14}C- and ^{3}H-labeled alkaloid precursors was reported by Stuart and Graham (1973a).

baine (**57**), codeine (**56**), and morphine (**27**) were not metabolized (Furuya et al., 1978). Codeine (**56**) was converted to morphine (**27**) in isolated capsules of *P. somniferum*; co-factors such as NAD, ATP, *S*-acetyl CoA, and pyridoxal phosphate were not required (Hsu et al., 1985).

The conversion of (*R*)-reticuline (**28**) to salutaridine (**58**) was catalyzed by microsomal preparations derived from differentiated *P. somniferum* plants or a thebaine-producing cell suspension culture (Gerardy and Zenk, 1992). NADPH is a required co-factor, as is molecular oxygen, and based on inhibition experiments with naphthoquinones, ancymidole, and prochlorax, the enzyme is a cytochrome P450-dependant oxidase which refuted earlier claims about the formation of this key intermediate from (*R*)-reticuline (**28**). The enzyme is present in the roots, shoots and capsules of the poppy plant.



[3-^{14}C]-1,2-Dehydroreticulinium (**30**) and [3-^{14}C]-(±)-reticuline (**28**/**29**) were incorporated into thebaine in *P. bracteatum* Lindl. (Papaveraceae). [16-^{3}H]Codeinone (**55**) was reduced to codeine (**56**), but demethylation reactions were not observed (Hodges et al., 1977).

Roberts and Antoun (1978) detected L-dopa decarboxylase in poppy latex using ^{14}C-labeled dopa derivatives as substrates. Maximum activity was observed at pH 7.2, and L-tyrosine, L-phenylalanine, and L-histidine were also substrates.

In cell cultures of *P. somniferum* (±)-reticuline (**28**/**29**) was stereospecifically converted to (−)-(*S*)-scoulerine (**31**) and (−)-(*S*)-cheilanthifoline. (−)-Codeinone (**55**) was converted in high yield to (−)-codeine (**56**) in both cell culture and enzyme preparations; the-

Gerardy and Zenk (1993) also isolated the cytosolic enzyme salutaridine 7-reductase from *P. somniferum* cell cultures and differentiated plants. The enzyme, of approximate molecular weight 52,000 daltons, and a pH optimum of 6.0–6.5, stereoselectively reduces salutaridine (**58**) to (7*S*)-salutaridinol (**59**) using NADPH as cosubstrate. Screening of isoquinoline-containing plants and cell cultures showed that the enzyme occurs only in *P. somniferum* and *P. bracteatum*.

[*N*-^{14}CH$_3$]Thebaine (**57**), when applied to a low-thebaine yielding cell suspension culture of *P. somniferum*, afforded codeine (**56**) and two new metabolites, tetrahydrothebaine and thebainone, showing that poppy cell cultures contain the enzymes necessary for thebaine enolether cleavage at C-6 (Wilhelm and Zenk, 1997). When the ability of 60 cell suspension cultures of different plant species and 230 field grown plant species was evaluated for their capability to metabolize [*N*-$^{14}CH_3$]thebaine (**57**), only the single cell culture of *Mahonia nervosa* Nutt. (Berberidaceae) cleaved the C-3 phenol methylether to yield oripavine. Field-grown *M. nervosa* could not perform this reaction (Wilhelm and Zenk, 1997).

Thirty years after the initial studies, the biological scene was very different when Huang and Kutchan (2000) reported on the morphinan and benzo[c]phenanthridine alkaloid gene transcript accumulation in *P. somniferum*. Using RNA gel blot analysis, the transcripts of three genes of alkaloid biosynthesis in *P. somniferum* were monitored in developing seedlings, mature plants, and plant cell suspension culture. The genes encoded the early enzyme (*S*)-*N*-methylcoclaurine 3′-hydroxylase (cypb80b1) which that is common to both morphine and sanguinarine biosynthesis, the berberine bridge enzyme (BBE) which lies on the pathway to sanguinarine (**48**), and codeinone reductase (COR), the penultimate enzyme in the biosynthesis of morphine (**27**) biosynthesis. Thebaine (**57**) was present throughout the 20-day germination period, but sanguinarine (**48**) was only detected after day 5, which paralleled the accumulation of the cyp80b1, bbe1, and cor1 gene transcripts. In the mature plant, the cyp80b1, bbe1 and cor1 gene transcripts were detected in the root, the stem, the leaf lamina, and the leaf mid rib. However, only cyp80b1 and cor1 were found in the flower bud and the capsule, paralleling the absence of sanguinarine (**48**). The cyp80b1 and bbe1 gene transcripts were demonstrated in cell suspension cultures induced with methyl jasmonate, consistent with the presence of sanguinarine.

Facchini and Park (2003) demonstrated the significant homology between the clones of three methyltransferases involved in (*S*)-reticuline (**29**) biosynthesis, (*S*)-norcoclaurine-6-*O*-methyltransferase, (*S*)-3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase, and (*S*)-coclaurine-*N*-methyltransferase, in *P. somniferum* and *Coptis japonica* Makino (Ranunculaceae). These cDNAs were used with clones for tyrosine/dopa decarboxylase, (*S*)-*N*-methylcoclaurine-3′-hydoxylase, berberine bridge enzyme, (7*S*)-salutaridinol 7-*O*-acetyltransferase, and codeinone reductase, to examine the accumulation of gene transcripts for alkaloid production in *P. somniferum*. With the exception of codeinone reductase, all of the enzymes were induced by an elicitor or by wounding suggesting a coordination of both developmental and inducible regulation of the genes for alkaloid biosynthesis (Facchini and Park, 2003).

In the morphine biosynthesis pathway, inversion of configuration at C-1 is required in reticuline from (*S*)- (**29**) to (*R*)- (**28**). Zenk's group showed that seedlings of *P. somniferum* contained an unstable synthase in the vesicles which oxidizes (*S*)-reticuline (**29**) to 1,2-dehydroreticuline; (*S*)-norreticuline was also a substrate. It was partially purified and had a pH optimum of 8.75, with an apparent K_m value for reticuline of 117 mM. In combination with the previously discovered 1,2-dehydroreticuline reductase, the 1,2-dehydroreticuline synthase provided for the required inversion of configuration, and completed the pathway of eleven enzymes, beginning with two molecules of L-tyrosine via (*S*)-norcoclaurine (**23**) to (*R*)-reticuline (**28**) (Hirata et al., 2004).

Further studies by the same group, using [ring-$^{13}C_6$]-L-tyrosine, [ring-$^{13}C_6$]-tyramine, and [1,2-$^{13}C_2$], [6-*O*-Me ^{13}C]-(±)-coclaurine (**25**), elucidated all the major fragment ions of labeled codeine (**56**) and morphine (**27**) through a combination of LC-electrospray MS/MS and high resolution FTICR-MS (Poeaknapo et al., 2004).

An important and new approach to studying the range of alkaloids labeled by a given precursor, and an easier method to assess the flux of alkaloids, was reported by Zenk and co-workers (Schmidt et al., 2007). Using [ring-$^{13}C_6$]-tyramine as a precursor of the alkaloids of *P. somniferum*, the labeling into twenty characterized alkaloids of the morphinan, benzylisoquinoline, protoberberine, benzo[c]phenanthridine, phthalide-isoquinoline, and protopine types was described. The alkaloid patterns were determined by direct infusion high-resolution ESI-FTICR mass spectrometry and HPLC/electrospray tandem mass spectrometry.

On the morphine biosynthetic pathway, salutaridine (**58**) is stereospecifically reduced by salutaridine reductase (SalR) to 7*S*-salutaridinol (**59**), which is then acetylated by salutaridinol 7-*O*-acetyltransferase (SalAT). *P. somniferum* was transformed with an RNAi construct designed to reduce transcript levels of the gene encoding the SalAT, which led to the accumulation of salutaridine (**58**) and salutaridinol (**53**) (Kempe et al., 2009). Using quantitative PCR, the SalAT transcript was shown to be diminished, while salR transcript levels were unaffected. Concentrations of thebaine (**57**) and codeine (**56**) in the latex were reduced, while morphine (**27**) levels remained constant.

Ziegler and co-workers (2009) reviewed aspects of the evolution of morphine (**27**) biosynthesis, since this is a rare biosynthetic pathway compared with all the plants producing benzylisoquinoline alkaloids (BIA). Comparative transcriptome analysis of opium poppy and *Papaver* species which do not accumulate morphinan alkaloids showed that known genes encoding BIA biosynthetic enzymes are expressed at higher levels in *P. somniferum*. Salutaridine reductase (SalR) was isolated and heterologously overexpressed. Homologous modeling and substrate docking established the substrate binding site for SalR which was compared to several members of the short chain dehydrogenase/reductase (SDR) family from different plant species.

1.13. Miscellaneous alkaloids

1.13.1. Salsoline

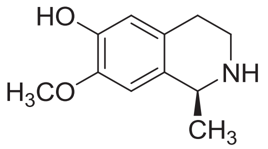
Radiolabeled 3-hydroxy-4-methoxyphenylethylamine and 6-hydroxy-7-methoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid were precursors of salsoline (**60**) in *Echinocereus merkeri* Hildm. ex K.Schum. (Cactaceae) (McFarlane and Slaytor, 1972).

1.13.2. Galanthamine

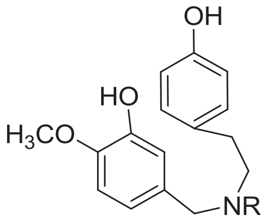
Zenk and co-workers showed that administering ^{13}C-labeled 4′-*O*-methylnorbelladine (**61**) resulted in high levels of incorporation into galanthamine (**62**) (27%) and *N*-demethylgalanthamine (**63**) (31%) in cultivated *Leucojum aestivum* L. (Amaryllidaceae) (Eichhorn et al. 1998). In addition, 4′-*O*-methylbelladine (**64**) was a poor precursor, and it was shown that the final step in galanthamine biosynthesis was *N*-methylation. As a result, a new scheme for galanthamine biosynthesis was proposed in which phenol oxidative coupling of 4′-*O*-methylnorbelladine (**61**) is the first step.

1.13.3. Onychine

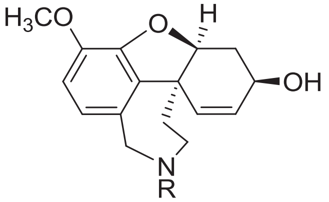
A simple, novel azafluorene alkaloid onychine (**65**) was characterized from the trunk wood of *Onychopetalum amazonicum* R.E.Fr. (Annonaceae) (De Almeida et al., 1976), after the original isomeric structure (1-aza-4-methylfluoren-9-one) was demonstrated by synthesis to be incorrect. The alkaloid was subsequently isolated by Waterman and Muhammad (1985) from *Cleistopholis patens* Engl. & Diels (Annonaceae), together with the 1-aza-4-methylanthraquinone alkaloid, cleistopholine (**66**), and the oxoaporphine alkaloid liriodenine. Cavé and co-workers (Tadic et al., 1987) characterized another member in the series kinabaline (5,8-dimethoxy-6-hydroxyonychine) (**67**), from the trunk bark of *Meiogyne virgata* Miq. (Annonaceae), and extended an earlier proposal regarding the biogenesis of the azaanthracene and the azafluorene alkaloids from oxoaporphinoid precursors.



60 Salsoline



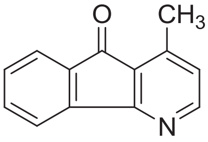
61 4'-*O*-methylnorbelladine, R = H
64 4'-*O*-methylbelladine, R = CH_3



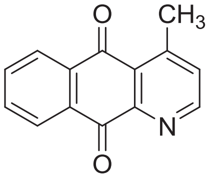
62 Galanthamine, R = CH_3
63 Norgalanthamine, R = H

1.13.4. Securinine

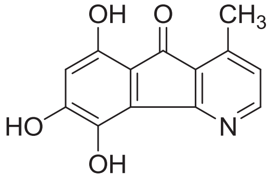
The alkaloid securinine (**68**) represented a new alkaloid skeleton when it was first isolated in 1963. Biogenetically, it was thought to arise in part from lysine, but the origin of the remaining carbons and oxygens was unclear for many years. In 1977, the breakthrough came when Sankawa and co-workers (1977) demonstrated that lysine, cadaverine, and tyrosine were incorporated into **68** in *Securinega suffruticosa* (Pall.) Rehder (Euphorbiaceae). DL-[2-^{14}C]Tyrosine specifically labeled the lactone carbonyl group, and using L-[U-^{14}C]tyrosine and L-[3′,5′-$^{3}H_2$]tyrosine it was shown that the aromatic ring and C-2 and C-3 of tyrosine were incorporated intact into **68**. Similar to the alkaloid profile of the roots, allosecurinine (**69**) (major) and securinine (**68**) were produced in callus cultures derived from budding stem explants of the seeds of *S. suffruticosa*, and the factors affecting alkaloid production (pH, hormones, light/dark) were examined (Ide et al., 1986).



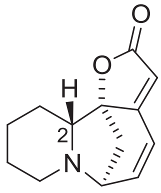
65 Onychine



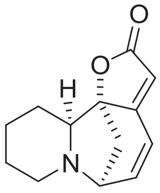
66 Cleistopholine



67 Kinabaline



68 Securinine



69 Allosecurinine

1.13.5. Colchicine

Significantly improved parameters to enhance incorporation rates for the study of colchicine (**70**) biosynthesis in immature seeds of *Colchicum autumnale* L. (Colchicaceae) were developed by Zenk and co-workers (Nasreen et al., 1997). Using ^{3}H- and ^{13}C-labeled phenethylisoquinolines, it was shown that *N*-methylation occurs prior to substitution of ring C, and that a C-13 hydroxy group in autumnaline (**71**) is necessary for phenolic coupling reaction. Autumnaline (**71**) afforded a broad range of metabolic products in the seeds.

1.13.6. Erythrina alkaloids

Zenk's group also developed an improved delivery system of radio- and stable isotope precursors for *Erythrina* alkaloids in *Erythrina crista-galli* L. (Fabaceae), following the demonstration that the fruit wall tissue was the main site of alkaloid biosynthesis (Maier et al., 1999). This ground-work led to the discovery that the previously assumed precursor, (*S*)-norprotosinomenine (**72**), was not incorporated into the *Erythrina* alkaloids. However, (*S*)-coclaurine (**25b**) and (*S*)-norreticuline (**34a**) were metabolized to erythraline (**73**) and erythrinine (**74**), respectively. [1-^{13}C]-(*S*)-Norreticuline (**34a**) exclusively labeled erythraline (**73**) at position C-10, indicating that the participation of a symmetrical intermediate in *Erythrina* alkaloid biosynthesis could be excluded.

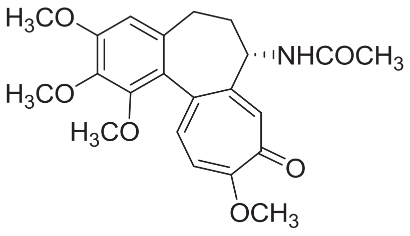
1.13.7. Ipecac alkaloids

The ipecac alkaloids are comprised of a tetrahydroisoquinoline moiety linked to a monoterpenoid unit. De-Eknamkul et al. (1997) found two enzyme activities in cell-free extracts of the leaves of *Alangium lamarckii* Thwaites (Alangiaceae). The first step in the pathway, condensation of dopamine and secologanin, occurred rapidly at pH 7.5 to produce (1*R*)-deacetylipecoside (**75**) and (1*S*)-deacetylisoipecoside (**76**) which spontaneously converted to demethylalangiside (**77**) and demethylisoalangiside (**78**), respectively. Thus, unlike monoterpenoid indole alkaloid biosynthesis, in *A. lamarckii*, the first enzymic condensation yields either the (1*R*)- or (1*S*)-product, and naturally occurring (*S*)- and (*R*)-forms of the tetrahydroisoquinoline-monoterpene alkaloids and related nitrogenous glucosides.

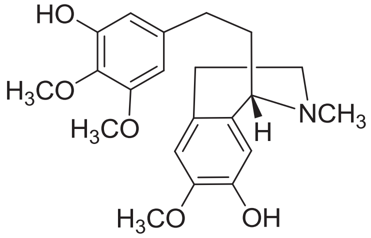
1.14. Tryptophan-derived alkaloids

1.14.1. Simple indole alkaloids

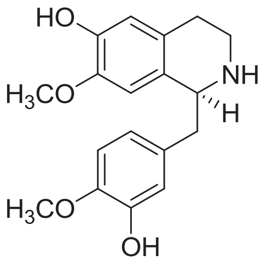
Previously contradictory results regarding the role of L-tryptophan in the biosynthesis of the indigo precursors, indican (**79**)



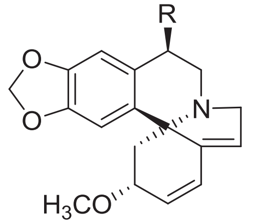
70 Colchicine



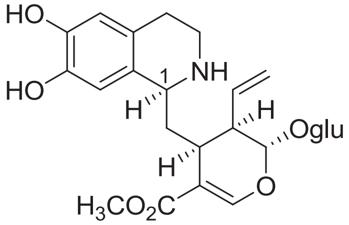
71 *S*-Autumnaline



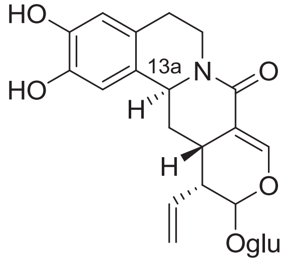
72 Norprotosinomenine



73 Erythraline, R = H
74 Erythrinine, R = OH



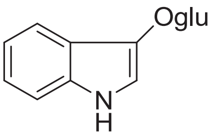
75 1*R*-Deacetylautumnaline, 1β-H
76 1*S*-Deacetylisopecoside, 1α-H



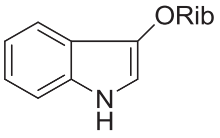
77 Demethylalangiside, 13aβ-H
78 Demethylisoalangiside, 13aα-H

and isatan B (**80**), in four higher plants *Baphicacanthus cusia* (Nees) Bremek. (Acanthaceae), *Calanthe veratrifolia* R.Br. (Orchidaceae), *Isatis tinctoria* L. (Brassicaceae), and *Polygonum tinctorium* Alt. (Polygonaceae) were resolved by Xia and Zenk (1992) using [3-^{13}C]indole and L-[3-^{13}C]tryptophan. Indole and not L-tryptophan was a biosynthetic precursor for the indoxyl derivatives.

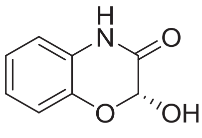
The biosynthesis of the alkaloids gramine (**1**) and 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) (**81**) is mutually exclusive in *Hordeum* species (Poaceae). The cytochrome P450 genes Bx2-Bx5 for DIBOA (**81**) biosynthesis in *H. lechleri* were functionally characterized and demonstrated a monophyletic origin for *H. lechleri* (Gruen et al., 2005). Homologous genes were found in other *Hordeum* species producing DIBOA (**81**), but not in species producing gramine (**1**).



79 Indican



80 Isatan



81 DIBOA

1.14.2. Strictosidine

A number of studies on various aspects of the role of strictosidine (**82**) in monoterpene alkaloid biosynthesis have been reported in *Phytochemistry* over the years. Strictosidine (**82**) from *Rhazya stricta* Decsne. (Apocynaceae) was first described as the key intermediate in indole alkaloid biosynthesis in 1968, and preliminary biosynthetic experiments were conducted by G.N. Smith (University of Manchester) at that time, but not published. Based on personal witness the first publication to affirm that strictosidine (**82**), and not its 3β-isomer vincoside, was a precursor of tetrahydroalstonine, ajmalicine (**83**), catharanthine (**84**), and vindoline (**85**) in *Catharanthus roseus* (L.) G.Don (Apocynaceae) was not reported until later by Brown et al. (1978).

Also in 1978, following the development of a radioimmunoassay for ajmalicine (**83**), a soluble enzyme system from *C. roseus* cell suspension cultures which synthesizes ajmalicine (**83**), 19-epi-ajmalicine, and tetrahydroalstonine from tryptamine and secologanin, was found to function optimally at pH 6.5. Alkaloid biosynthesis was inhibited by γ-D-gluconolactone, a β-glucosidase inhibitor, indicating the involvement of a β-glucosidase in their biosynthesis (Treimer and Zenk, 1978). The enzyme system was not substrate specific, and also utilized ring-substituted tryptamines to catalyze the formation of unnatural ajmalicine derivatives.

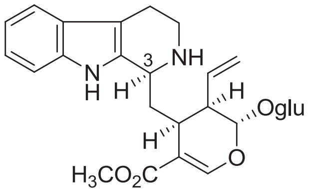
In 1988, Hampp and Zenk (1988) reported on the first purification of strictosidine synthase, the primordial enzyme responsible for the condensation of secologanin and tryptamine to exclusively form 3α(*S*)-strictosidine (**82**). Using suspension cell cultures of *Rauvolfia serpentina* Benth. ex Kurz (Apocynaceae), the enzyme was purified 920-fold and had a specific activity of 184 nkat/mg. The enzyme was a single polypeptide, M_r 30,000, with a pH optimum of 6.5 and a temperature optimum of 45 °C. Apparent K_m values for both tryptamine and secologanin were 4 mM. The immobilized enzyme had a half-life of 100 days at 37 °C.

Verpoorte and co-workers (Stevens et al., 1993) purified to homogeneity four isoforms of strictosidine synthase from a suspension culture of *Cinchona robusta* Howard (Rubiaceae). The molecular weights were 35,000, 33,000, 35,000, and 33,000 daltons, and the pI values were 6.5, 6.5, 7.5, and 7.5, respectively. The K_m values for the four enzymes for tryptamine were in the micromolar range. Inhibition of the enzymes was induced by strictosidine (**82**), and by the subsequently formed quinoline alkaloids. The Ki of quinine for one of the main isoforms was 7 μM. In contrast with strictosidine synthase from *C. roseus*, all four of the isoforms from *C. robusta* also accepted 5-methoxytryptamine as a substrate.

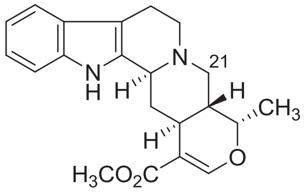
Two cell lines of *T. divaricata* derived from the same suspension culture were compared with respect to their biotransformation capacity (Dagnino et al., 1994). One of the cell lines accumulated indole alkaloids, mainly *O*-acetylvallesamine (**86**), at a high level, whereas the other gave only low yields. When cultured with skeletally diverse isolated alkaloids, such as conopharyngine, coronaridine, vobasine, or tabersonine, the alkaloids were similarly transformed by both cultures, indicating the same biotransformation potential.

[Me-$^2H_3$]Ajmalicine (**83**) degradation was investigated in a low alkaloid-accumulating *C. roseus* cell culture, in comparison with chemical degradation in medium without cells. Degradation was faster in the presence of cells, being high at the beginning of the growth phase and in the stationary phase (dos Santos et al., 1994).

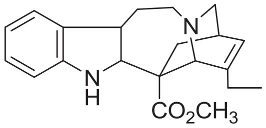
The second enzyme in the monoterpenoid indole alkaloid biosynthetic pathway is strictosidine β-D-glucosidase (SG); and this enzyme was partially purified from suspension cultured cells of *T. divaricata* (Luijendijk et al., 1996). Three different forms were found, each of which specifically catalyzed the deglucosylation of strictosidine (K_m 0.2–1 mM). Some of the characteristics of the enzyme were similar to those of the *C. roseus* SG enzyme. Cathenamine (**87**), 21-hydroxyajmalicine (**88**), and isovallesiachotamine were formed following 3α(*S*)-strictosidine (**82**) hydrolysis.



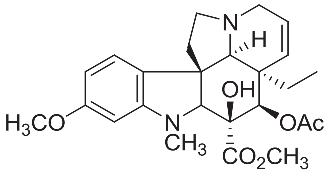
82 Strictosidine



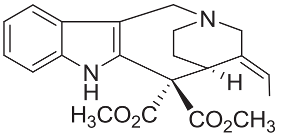
83 Ajmalicine
88 21-Hydroxyajmalicine



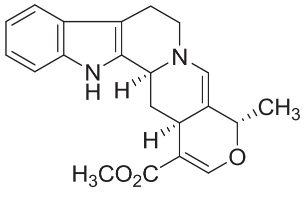
84 Catharanthine



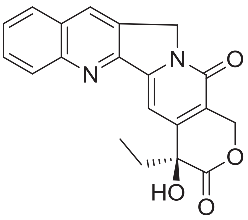
85 Vindoline



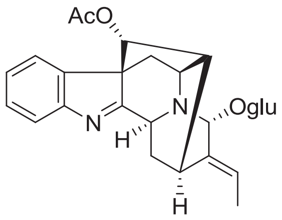
86 *O*-Acetylvallesamine



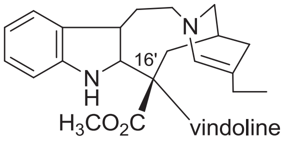
87 Cathenamine



89 Camptothecin



90 Raucaffricine



91 3′,4′-Anhydrovinblastine

Verpoorte and co-workers (Schripsema et al., 1991) administered a cell suspension culture from *Tabernaemontana divaricata* R.Br. ex Roem. & Schult. (Apocynaceae) with ^{15}N-labeled ammonium or nitrate ion as alternative nitrogen sources. Ammonium ion was used more extensively than nitrate in the biosynthesis of the monoterpene indole alkaloids. Considerably lower labeling percentages were found for tryptamine than for *O*-acetylvallesamine (**86**) and the amino acids, suggesting the formation of a vacuolar pool of tryptamine.

Biotransformation of 3α(*S*)-strictosidine (**82**), produced by heterologously expressed strictosidine synthase from *R. serpentina*, with twenty-two bacterial strains demonstrated deglucosylation and rearrangement to vallesiachotamine (Shen et al., 1998). The study indicates the potential for the biotechnological production of alkaloids.

Camptothecin (CPT) (**89**) is a pharmaceutically important monoterpene indole alkaloid derived from strictosidine (**82**) through a series of to-be-defined steps. A hairy root culture of *Ophiorrhiza pumila* Champ. ex Benth. (Rubiaceae) produced high amounts of camptothecin (**89**), and it was shown that the site of biosynthesis is the endoplasmic reticulum. CPT is then transported and localized in the vacuole, or, if the vacuole is blocked, CPT is excreted into the medium (Sirikantaramas et al., 2007).

Compared with other monoterpene indole alkaloids, relatively few studies have been reported on the alkaloids of *Cinchona* species. Levels of two of the early enzymes in the pathway, tryptophan decarboxylase (TDC) and strictosidine synthase (SS) were compared with the production of quinoline alkaloids in C*inchona ledgeriana* Moens ex Trimen (Rubiaceae) suspension cultures transformed with *A. tumefaciens* (Skinner et al., 1987). Dark cultures showed higher levels of alkaloid production than light cultures. Stable levels of SS were up to 80 times higher were observed. The TDC activity declined after 13 days, and was considered potentially rate limiting for alkaloid production.

It is often assumed that alkaloid biosynthesis is a consistent and evolving process from seed to seed. Verpoorte and co-workers showed that for *Cinchona* alkaloids that is not the case (Aerts et al., 1991). Germination of *C. ledgeriana* seeds produces a substantial increase in alkaloid levels which only lasts about 4 days. Although the initial substrates, tryptophan, secologanin, and strictosidine (**82**) are still all present, intracellular compartmentalization occurs which limits alkaloid biosynthesis. The concentration of alkaloids present in the seedlings though was adequate to act as a feeding deterrent to slugs.

The history of strictosidine (**82**), and more particularly the discovery of strictosidine synthase as the key enzyme which catalyzes the stereospecific formation of 3α(*S*)-strictosidine (**82**) from tryptamine and secologanin is discussed in detail in an excellent review by Kutchan (1993). Aspects of the isolation and stereochemical determination of strictosidine (**82**), the biochemical characteristics of the enzyme, and how subsequent research led to new genetic approaches to the study of plant enzymes and their expression in secondary organisms are presented. More than any other paper in the past 50 years in *Phytochemistry*, this review inherently depicts the tremendous progress in the level of understanding of alkaloid biosynthesis in plants.

The levels of five enzymes important in monoterpene indole alkaloid biosynthesis, isopentenyl pyrophosphate isomerase, geraniol 10-hydroxylase, tryptophan decarboxylase, strictosidine synthase, and strictosidine glucosidase, were compared in two cell lines of *Tabernaemontana divaricate* (Dagnino et al., 1995). Only G10H and SG showed a correlation with alkaloid biosynthesis and were considered rate-limiting. Added loganin stimulated the low-alkaloid producing cell line 100-fold, indicating the dependence of alkaloid production on available monoterpene precursor.

1.14.3. Ajmaline

Although *Rauvolfia serpentina* Benth. ex Kurz (Apocynaceae) plants accumulate ajmaline as the main monoterpene indole alkaloid, cell suspension cultures accumulate up to 1.6 g/l of the glucoalkaloid raucaffricine (**90**), because the cell cultures do not contain the enzyme raucaffricine-*O*-β-glucosidase (RG). Molecular cloning and functional expression of this enzyme in *E. coli* showed up to 60% amino acid identity with other glucosidases. Raucaffricine (**90**) was the best substrate for recombinant RG, and strictosidine (**82**) was also a substrate (Warzecha et al., 2000).

1.14.4. Vindoline and related alkaloids

Vindoline (**85**) is a major monoterpene indole alkaloid in the leaves of *C. roseus*, and an important component of the anticancer bisindole alkaloids, vinblastine and vincristine (with the *N*-methyl oxidized). A specific radioimmunoassay for vindoline (**85**) was developed with a detection limit of 5 ng of the alkaloid (Kutney et al., 1980b).

A multiple enzyme complex, involving at least five isozymes, which catalyzed the coupling of vindoline (**85**) and the iboga alkaloid catharanthine (**84**) to afford the bisindole alkaloid 3′,4′-anhydrovinblastine (**91**) was partially purified from cell suspension cultures of *C. roseus* (Endo et al., 1988).

Kutney and co-workers (1980a) examined the formation of monoterpene indole alkaloids in several cell lines of *C. roseus*. The 943 cell line produced a range of alkaloids, including vallesiachotamine, isositsirikine, ajmalicine (**83**), yohimbine, hoerhammerinine, hoerhammericine, vindolinine, 19-epi-vindolinine, and three new alkaloids, including two new tabersonine derivatives, 19-acetoxy-11-methoxy-, and 19-hydroxy-11-methoxy-. No catharanthine (**84**) or vindoline (**85**) was detected.

Cryotechniques coupled with immunogold labeling was used to localize the intracellular storage of vindoline (**85**) in isolated protoplasts derived from the leaves of *C. roseus*. The label was present in small vesicles and the cytoplasmic area bordering the plasmalemma, as well as in the central vacuole and, to a much lesser extent, in the chloroplasts (Brisson et al., 1992). These data support the difficulties encountered in the localization of vindoline (**85**) in leaf tissues.

S-Adenosyl-L-methionine:16-methoxy-2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase (NMT) which catalyzes a late step in vindoline (**85**) biosynthesis is localized in chloroplast thylakoids of *C. roseus*. The solubilized NMT was purified 120-fold and showed an apparent M_r of 60,000. However, it showed altered specificity for substrates compared with the membrane-associated enzyme, and attempts at further purification resulted in loss of activity (Dethier and De Luca, 1993).

Metabolic profiling of 50 flowering cultivars of the ornamental periwinkle, *C. roseus*, by HPLC revealed similar levels of the main monoterpene indole alkaloids, with one cultivar showing a low vindoline (**85**) content, which was traced to low levels of the enzyme tabersonine 16β-hydroxylase (Magnotta et al., 2006).

1.14.5. Purine-derived alkaloids

The biosynthesis of the common purine alkaloids, theophylline (**87**), theobromine (**88**), and caffeine (**89**) in plants has remained a subject of significant investigation for many years. In one of the early studies reported in the journal, leaf disks of *Coffea arabica* L. (Rubiaceae) were infiltrated simultaneously with L-methionine-methyl-^{14}C, and with various possible precursors of caffeine biosynthesis. The results permitted the identification of theobromine (**88**), 7-methylxanthine (**90**), and 7-methylxanthosine (**91**) as precursors of caffeine (**89**) (Looser et al., 1974). 7-Methylguanosine seemed not to be an intermediate in caffeine (**89**) formation.

[8-^{14}C]Adenine (**92**), rather than [8-^{14}C]guanine (**93**), was a more effective precursor of caffeine (**89**) in *Camellia sinensis* (L.) Kuntze (Theaceae). Pulse labeling experiments with [8-^{14}C]adenine (**92**) established that labeling of 7-methylxanthine (**90**) and theobromine (**88**) increased in the first 10 h, and then declined as caffeine (**89**) labeling steadily increased, suggesting a possible precursor relationship (Suzuki and Takahashi, 1976).

Up to 26% of ring-labeled 7-methylxanthosine-^{14}C (**91**) was converted into caffeine (**89**) after injection into leaves of *C. arabica* over 8 days (Baumann et al., 1978). A possible caffeine biosynthetic pathway was discussed.

Cell-free extracts, with pH optima of 8.5, from partially ripe and unripe *C. arabica* fruits catalyzed the transfer of a methyl group from *S*-adenosyl-L-methionine to 7-methylxanthine (**90**) and 7-methylxanthosine (**91**), producing theobromine (**88**). The same system also transferred a methyl group to theobromine (**88**) producing caffeine (**89**); K_m values were determined (Roberts and Waller, 1979). The biogenetic pathway of caffeine formation in *C.*

arabica was proposed to be 7-methylxanthosine (**91**) → 7-methylxanthine (**90**) → theobromine (**88**) → caffeine (**89**).

Cultured cells of *C. sinensis* could produce caffeine (**89**) and theobromine (**88**), but at lower levels than cultured coffee cells (Furuya et al., 1990). [8-^{14}C]Adenine (**92**) was used to show that the biosynthetic pathway to caffeine (**89**) was operative in the stamens and petals of *C. sinensis*, and that the reaction from theobromine (**88**) to caffeine (**89**) was present in *C. sinensis* and deficient in *C. irrawadiensis* Barua (Fujimori and Ashihara 1990). Purine alkaloid biosynthesis was not detectable in *C. japonica* L. and *C. sasanqua* Thunb.

[8-^{14}C]Adenine (**92**) was also used in field-grown leaf disks to examine the annual seasonal variations in the metabolism of adenine nucleotides in the shoots of tea plants (*Camellia sinensis* cv. *Yabukita*) (Fujimori et al. 1991). Incorporation into theobromine (**88**) and caffeine (**89**) was found only in the young leaves. A leaf extract catalyzed the methylation of xanthosine (**94**), 7-methylxanthine (**90**), and theobromine (**88**), but could not be detected in extracts from leaves in which the synthesis of caffeine (**89**) was not observed.

Caffeine (**89**), theobromine (**88**), and theophylline (**87**) levels in aqueous extracts of the endosperm of the immature and mature fruits of *C. arabica* and six other species of *Coffea* were examined by HPLC (Mazzafera et al., 1991). Caffeine (**89**) was the dominant alkaloid, and the highest levels were found in *C. canephora* Pierre ex. A.Froehner (Rubiaceae) at 35.1 and 24.5 mg/g, respectively, in the immature and mature endosperm. Caffeine (**89**) was not detected in the mature fruits of *C. bengalensis* Roxb. The rate of turnover of caffeine (**89**) in various species of *Coffea* was examined using [8-^{3}H]caffeine (**89**). It was metabolized slowly by the immature endosperm of *C. arabica* and *C. canephora*, and more rapidly by *C. dewevrei* De Wild. & T.Durand., *C. eugenioides* S.Moore, *C. stenophylla* G.Don, *C. salvatrix* Wynn. & Philipson, and *C. benghalensis* B.Heyne ex. Schult. Potential sources for a natural decaffeinated coffee were discussed.

Waller and co-workers provided an authoritative review of the metabolism of the purine alkaloids caffeine (**89**) and theobromine (**88**) in coffee and tea plants (Suzuki et al., 1992). The activity of the three *N*-methyltransferases was discussed, together with the different rates of metabolism in the various species of the two genera at different stages of growth. The physiological role of the purine alkaloids in the plant remains elusive,

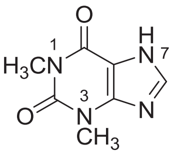
[8-^{14}C]Adenine (**92**), [9-^{14}C]guanosine (**95**), and [8-^{14}C]hypoxanthine (**96**) were incorporated into theobromine (**88**) and caffeine (**89**) in the young, but not the old, leaves of mate [*Ilex paraguariensis* A.St.-Hil. (Aquifoliaceae)] (Ashihara, 1993). The conventional pathway for the degradation of purines via allantoin and allantoic acid was found to operate.

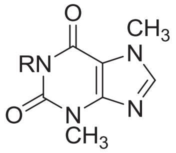
Caffeine (**89**) and theobromine (**88**) were trace components, and theophylline (**87**) was absent, when the seeds and leaves of nine *Herrania* Goudot (Sterculiaceae) and eleven *Theobroma* L. (Sterculiaceae) species were examined. Tetramethyl urate (**97**) was the principal purine alkaloid detected (Hammerstone et al., 1994).

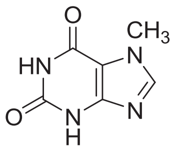
Conversion of [8-^{14}C]adenine (**92**) to theobromine (**88**) and caffeine (**89**) was found only in the first leaves of coffee plants, no theophylline (**87**) was detected (Fujimori and Ashihara, 1994). Secondary leaves produced nucleotides and nucleic acids.

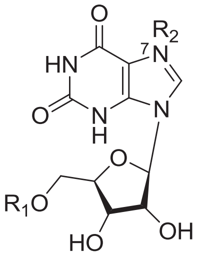
The purification and the *N*-terminal sequence of *S*-adenosyl-L-methionine: theobromine 1-*N*-methyltransferase (STM), the enzyme responsible for the methylation of theobromine (**88**) to caffeine (**89**) in coffee was described (Mazzafera et al., 1994). The enzyme showed an apparent M_r of ca. 54,000 and ca. 60,000 by gel filtration and SDS–PAGE, respectively. Using theobromine (**88**) as substrate, the K_m value for *S*-adenosyl-L-methionine was 10 μM. It is a bifunctional enzyme which also methylates 7-methylxanthine (**90**), the precursor of theobromine (**88**). The *N*-terminal sequence for the first 20 amino acids was obtained for STM, but no similarities were found with other methyltransferases.

Significant challenges were encountered in demonstrating the conversion of xanthosine (**94**) to 7-methylxanthosine (**91**) in suspension cultures of coffee. Cells administered with 5 mM ethephon or 1 mM adenine to enhance purine alkaloid formation increased the incorporation of [^{14}C]adenine (**92**) into the purine alkaloids, as did ethephon (Schulthess and Baumann, 1995a). Neither ethephon nor adenine treatment led to a distinct incorporation of [^{14}C]adenine (**92**) or [methyl-^{14}C]methionine into 7-methylxanthosine (**91**). Kinetic analysis based on [^{14}C]adenine (**92**) indicated that xanthosine (**94**) was not a precursor in caffeine (**89**) biosynthesis. It was postulated that metabolic channeling occurred in purine alkaloid biosynthesis, including the formation of xanthosine


82 Theophylline

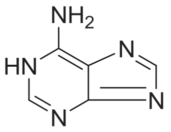

88 Theobromine, R = H
89 Caffeine, R = CH$_3$


90 7-Methylxanthine

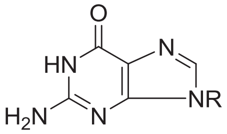

91 7-Methylxanthosine, R$_1$ = H, R$_2$ = CH$_3$
94 Xanthosine, R$_1$= H
98 Xanthosine monophosphate, R$_1$ = P

(**94**), its methylation to 7-methylxanthosine (**91**), and finally enzymatic hydrolysis to 7-methylxanthine (**90**).

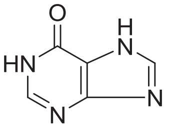
In the dark, both adenine (**92**) and ethephon stimulated purine alkaloid [7-methylxanthine (**90**), theobromine (**88**), and caffeine (**89**)] formation by factors of 4 and 7, respectively in cell suspension-cultures of coffee as determined by HPLC. In light, caffeine (**89**) formation was enhanced by a factor of 21 without affecting theobromine (**88**) and 7-methylxanthine (**90**) pools. Co-feeding adenine feeding resulted in enlarged nucleotide pools (xanthosine, guanosine, and inosine) and 7-glucopyranosyladenine (Schulthess and Baumann, 1995b).



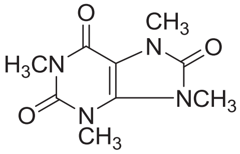
92 Adenine



93 Guanine, R = H
95 Guanosine, R = ribose



96 Hypoxanthine



97 Tetramethylurate

Although young coffee leaflets were actively producing (about 38 or 71% of the recovered radioactivity from [U-^{14}C]adenine (**92**) and [Me-^{14}C]methionine, respectively), 7-methylxanthine (**90**), theobromine (**88**), and caffeine (**89**), no incorporation into 7-methylxanthosine (**91**) or a monomethylated purine other than 7-methylxanthine (**90**), was observed (Schulthess et al., 1996). The result was similar to that obtained with coffee cell suspension cultures, implying that the first methylation step in caffeine (**89**) synthesis is metabolically channeled. When the methyltransferase activity was examined, only xanthosine (**94**) and xanthosine 5′-mono-phosphate (XMP) (**98**) were methylated, and exclusively at the N-7 position. The new XMP *N*-methyltransferase activity was measured under conditions stabilizing the product. As a result, a new hypothesis regarding caffeine (**89**) biosynthesis which favors methylation of XMP (**98**) rather than xanthosine (**94**) as the crucial step which leads from primary metabolism into caffeine (**89**) synthesis was proposed.

The δ^{13}C- and δ^{15}N-values of caffeine (**89**) and theobromine (**88**) permit distinction between natural and synthetic products. The ^{13}C pattern of the natural purine alkaloids indicated an enrichment of ^{13}C in the positions 2 and 8, originating from the THF pool and a depletion in the methyl groups derived from the SAM pool (Weilacher et al., 1996). These results suggest the presence of two separate C1 pools.

The last two steps in caffeine (**89**) biosynthesis in coffee are the conversion of 7-methylxanthine (**90**) to theobromine (**88**), and subsequently of theobromine (**88**) to caffeine (**89**) by two *N*-methyltransferases. Waldhauser et al. (1997a) devised an assay for these enzyme activities using high concentrations of methyl donor and substrate, obviating the need for tracer studies. The enzymes showed maximum stability at pH 7.5, and at around 35 °C. Maximum relative and absolute methyltransferase activities coincided with leaf emergence, when, as expected, chemical defense has the highest priority and the purine alkaloids are at peak concentrations. The transient accumulation of theobromine (**88**) in the young leaflets supported the observed competition of the two NMT activities for SAM.

The first *N*-methylation step in the caffeine biosynthetic pathway is the formation of 7-methylxanthine (**90**). Using young, emerging coffee leaflets, anion exchange chromatography and chromatofocusing separated XMP *N*-7-methyltransferase from 7-methylxanthine *N*-3-methyltransferase, and theobromine *N*-1-methyltransferase (Waldhauser et al. 1997b). The enzymes were separated by photoaffinity labeling with [methyl-^{3}H]SAM, followed by SDS–PAGE, and eventually provided all three *N*-methyltransferase activities in bands at 49, 43, and 40 kDa. Correlations of the 49- and 43-kDa bands with the 2nd and 3rd NMT activities were not established.

Caffeine (**89**) and theobromine (**88**) accumulated in callus and root suspension cultures in MS medium from *C. sinensis* as established by TLC, UV, and GC (Shervington et al., 1998). In preparations from young leaves of *C. sinensis*, the *N*-3-methyltransferase was associated with a purified chloroplast preparation (Kato et al., 1998).

Caffeine (**89**) biosynthesis from [8-^{14}C]adenine (**92**), and its overall metabolism were investigated in anti-sense and RNA interference transgenic plants of *C. canephora* in which the expression of CaMXMT1 was suppressed. Synthesis from adenine (**92**) and conversion of theobromine (**88**) to caffeine (**89**) were both reduced in the transgenic plants, and metabolism of [8-^{14}C]adenine (**92**) shifted from purine alkaloid synthesis to purine catabolism or salvage for nucleotides (Ashihara et al., 2006).

Ashihara et al. (2008) provided a review which details how the biosynthetic pathway of caffeine involves the conversion of xanthosine (**94**) → 7-methylxanthosine (**91**) → 7-methylxanthine (**90**) → theobromine (**88**) → caffeine (**89**). The genes encoding *N*-methyltransferases involved in three of these four reactions have been isolated and their molecular structures investigated. Pathways for the catabolism of caffeine (**89**) have thus far not produced enzymatic or genetic studies. The metabolism of purine alkaloids in *Camellia*, *Coffea*, *Theobroma*, and *Ilex* plants was summarized, and the evidence for the involvement of caffeine (**89**) in chemical defense and allelopathy presented. Studies on metabolic engineering to produce coffee seedlings with reduced caffeine (**89**) content, and transgenic caffeine-producing tobacco plants with enhanced disease resistance were also discussed.

1.15. Miscellaneous alkaloids

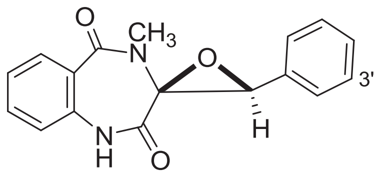
1.15.1. Cyclopenin

In 1976, the enzyme preparation cyclopenin m-hydroxylase was obtained from *Penicillium cyclopium* and transformed cyclopenin (**99**) into the hydroxy derivative cyclopenol (**100**) (Richter and Luckner, 1976). The enzyme required molecular oxygen and NADPH for activity, and was inhibited by both thiocyanide and cyanide ion.

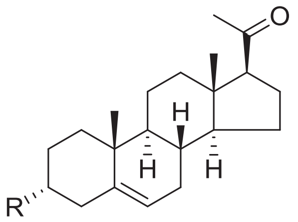
Three of the enzymes, cyclopeptine dehydrogenase, dehydrocyclopeptine epoxidase, and anthranilate adenyltransferase, in cyclopenin (**99**) biosynthesis were induced endogenously in the hyphae of *P. cyclopium* leading to 10-fold increase in alkaloid production (Voigt et al., 1978).

1.15.2. Holaphyllamine

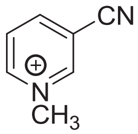
[4-^{14}C]Holaphyllamine (**101**) was converted to pregnenolone (**102**) by the leaves of *Holarrhena floribunda* T.Durand & Schinz (Apocynaceae), and completed a cycle in which pregnenolone (**102**) had been converted to holaphyllamine (**101**) (Bennett et al., 1966).



99 Cyclopenin
100 Cyclopenol, 3'-OH



101 Holaphyllamine, R = NH_2
102 Pregnenolone, R = OH



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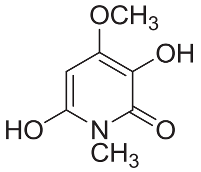

104 Hermidin

1.15.3. Ricinine and Hermidin

One of the earliest plant alkaloid enzyme preparations was developed by Robinson (1965) from *Ricinus communis* L. (Euphorbiaceae) which converted 1-methylnicotinonitrile (**103**) to the corresponding 4- and 6-pyridones.

The biosynthesis of the pyridinone hermidin (**104**) was studied through [U-$^{13}C_6$]glucose and $^{13}CO_2$ pulse labeling experiments in *Mercurialis annua* L. (Euphorbiaceae) (Ostrozhenkova et al., 2007). Based on similar labeling profiles, the results supported the derivation of hermidin (**104**) through the nicotinate pathway, by way of dihydroxyacetone phosphate and aspartate. The pulse/chase application of $^{13}CO_2$ permits experiments under physiological conditions and should be widely applicable.

2. Conclusions

An overview of the studies published in *Phytochemistry* in the past 50 years related to the biosynthesis of alkaloids has been presented. Techniques for the study of alkaloid biosynthesis have evolved dramatically in this period from studies of the utilization of potential precursors to radio- and then stable-isotope studies, to the isolation of crude and then purified enzyme systems. Now studies are routinely providing important information regarding the functionality of the genes which are responsible for the enzymes of alkaloid biosynthetic pathways. Progress on several major alkaloid biosynthetic pathways has appeared in *Phytochemistry* in the past 50 years, and the journal has therefore played a crucial role in advancing the science and the technology behind the determination of how nature produces alkaloids in plants.

Acknowledgments

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Professor Emeritus Geoffrey A. Cordell obtained a Ph.D. in synthetic natural product chemistry at the University of Manchester. After joining the College of Pharmacy at University of Illinois at Chicago he directed drug discovery programs, served as a Department Head and Interim Dean, and held several senior research administrative positions at the College and Campus levels; he retired in 2007.

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After over 45 years studying the isolation, structure elucidation synthesis, and biosynthesis of natural products, his efforts are now focused on developing natural product research programs, where he serves as an advisor and consultant in several countries in various parts of the world. His interests include strategies for the sustainability and quality control of medicinal agents, the detection of biologically active natural products, the chemistry and biosynthesis of alkaloids, and the use of vegetables as chemical reagents.