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TROPANE ALKALOIDS: OLD DRUGS USED IN MODERN MEDICINE

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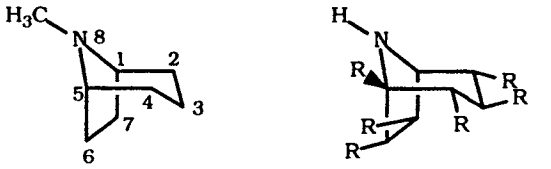
INTRODUCTION

A precise chemical definition of the term alkaloid is somewhat difficult because the word defines structurally the most diverse group of secondary metabolites of plant, microbial or animal origin. Typically, alkaloids contain one or more nitrogen atoms, usually in an heterocyclic ring, have a more or less pronounced basic reaction and generally possess strong and various pharmacological effects when administered to animals and humans. Today, there are over 12'000 known alkaloids and a growing number of new compounds is recorded every year.

Flowering plants, namely the angiosperms are the major source of alkaloids. However, there are increasingly numerous examples of the occurrence of alkaloids in animals, insects and marine organisms, micro-organisms and lower plants. For example, to date nearly 300 alkaloids are known to be found in the skin of amphibians.

For centuries, plants have been a unique source of therapeutically significant alkaloids and they continue to be excellent sources of drugs. Furthermore, alkaloids of natural origin serve as a model for the semisynthesis or the synthesis of derivatives which have improved pharmacokinetic properties, a higher efficacy and/or less toxicity. One of the most recent examples is the isolation of the anticancer agent, called taxol, from the stem bark of the Pacific yew tree *Taxus brevifolia* in 1971 by Wani and co-workers [1] and the development, a few years later, of docetaxel, a semisynthetic derivative obtained from 10-deacetyl-baccatin III [2].

Plants in the *Solanaceae* family produce a variety of alkaloids, some of them having a considerable therapeutic importance. One such group of alkaloids possesses a tropane nucleus. Tropane alkaloids are structurally related natural products having in common the azabicyclo[3.2.1]octane structure and therefore the systematic name for tropane is 8-methyl-8 azabicyclo[3.2.1]octane (Fig. 1). The majority of these alkaloids are esters between organic acids and hydroxytropanes. 3α-Hydroxytropane, called tropine, is the amino alcohol most frequently encountered. In addition, its 3β-isomer (pseudotropine), the di- (3,6-; 3,7- or 6,7-) and trihydroxylated



Tropane skeleton Calystegine skeleton

Fig. (1). Tropane and calystegine skeletons.

3,6,7-tropanes, the 6,7-epoxide and the corresponding *N*-nor derivatives occur in numerous plant species. These bases may be found in free form but are usually esterified with a wide variety of aliphatic, aromatic and heterocyclic acids of various chemical structure such as benzoic, cinnamic, isovaleric, α-methylbutyric, tiglic, tropic, truxillic and veratric acid. Hitherto, approximately 40 different acids have been reported [3], some of which being specific to this class of alkaloids (*e.g.* (S)-tropic acid), while others are regularly distributed in the vegetable kingdom (*e.g.* acetic acid, benzoic acid). Furthermore, 15 dimeric and one trimeric tropane alkaloids have been found so far [3]. The number of known tropanes has increased dramatically over the last twenty years to reach more than 200 compounds today. This class of alkaloids is often accompanied by the pyrrolidine-derived bases hygrine and cuscohygrine (Fig. **4**) since both groups show a common mode of formation.

Despite the fact that a large number of tropanes is known, pharmacologically important alkaloids in which the nitrogenous base is esterified with tropic acid (or a derivative), are apparently unique in the *Solanaceae* family [4].

From a pharmaceutical point of view, three natural-occurring compounds are widely used as chemotherapeutic agents *viz.* (-)-hyoscyamine, (-)-scopolamine (hyoscine) and atropine (Fig. **2**). The latter compound is formed by the racemization of (-)-hyoscyamine during isolation and purification and is thus (±)-hyoscyamine. All three compounds are esters of 3α-tropine with tropic acid.

Hygrine-type alkaloids are very often detected in members of the *Solanaceae* family which contain tropane alkaloids. In particular, cuscohygrine is present in nearly all cases. In a similar way but in a smaller number of genera, hygrine is distributed in plants which contain tropane alkaloids, as for example in the *Erythroxylum* species *(Erythroxylaceae).*

In 1988, Tepfer and co-workers [5] reported the identification of a new group of tropane alkaloids, called calystegines, originally isolated from the roots of the morning glory, *Calystegia sepium (Convolvulaceae),* from which they derive their name. These compounds are characterized by a bicyclic structure, by the absence of an *N*-methyl group and a high degree of hydroxylation. The hydroxyl groups vary in position and

stereochemistry, as shown in Fig. 1. To date, 16 calystegines have been isolated and their structures determined.

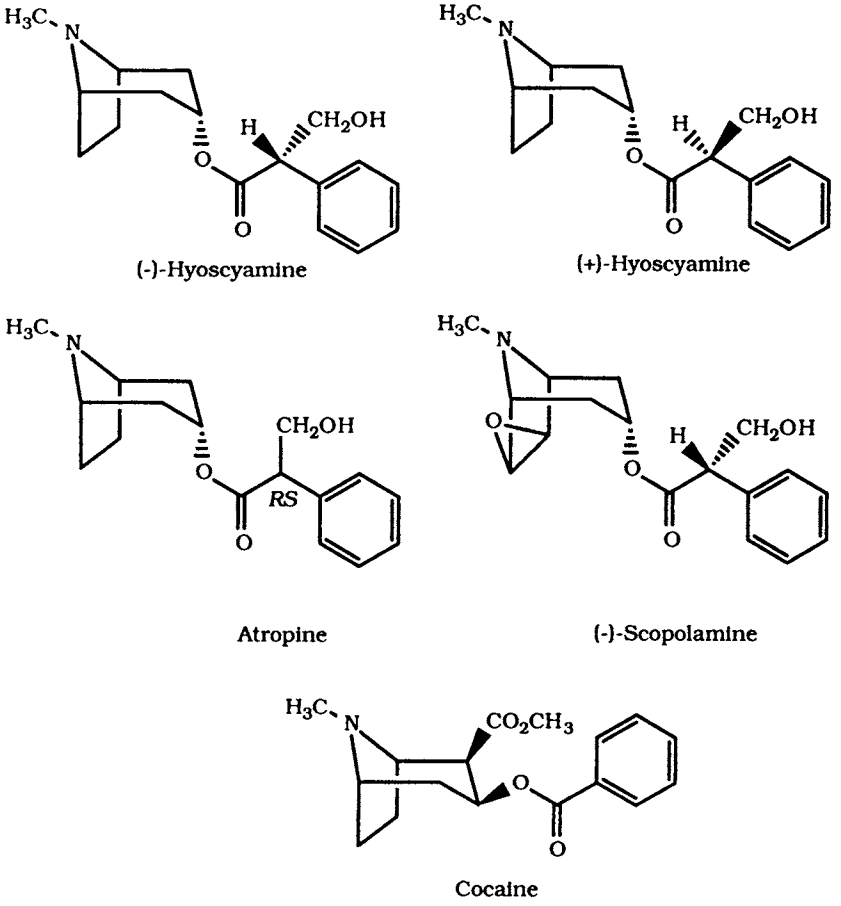


Fig. (2). Tropane alkaloids of pharmaceutical interest.

OCCURRENCE

Tropane alkaloids mainly occur in the *Solanaceae* family but are also found in other families such as *Convolvulaceae, Erythroxylaceae, Proteaceae* and *Rhizophoraceae.* Less frequently, tropane alkaloids have been mentioned in the *Euphorbiaceae, Brassicaceae* and *Olacaceae* families which show no taxonomic relationships with *Solanaceae.* In several species of *Erythroxylum,* the tropane alkaloids are characterized by a 3β-hydroxy function and a carboxyl group at C-2 of the tropane nucleus. The most famous representant of this group is cocaine (Fig. 2). In Table 1 the distribution of tropane alkaloids in the plant kingdom is indicated.

Table 1. Distribution of Tropane Alkaloids in the Plant Kingdom

Families	Genera containing tropane derivatives	Nb of species containing tropane alkaloids	Approx. nb. of alkaloids described
Euphorbiaceae	*Phyllantus*	1	1
Brassicaceae	*Cochlearia*	1	1
Proteaceae	*Agastachys*	1	2
	Bellendena	1	10
	Darlingia	2	7
	Knightia	2	18
Rhizophoraceae	*Bruguiera*	3	7
	Crossostylis	3	6
	Pellacalyx	1	1
Erythroxylaceae	*Erythroxylum*	35	78
Olacaceae	*Heisteria*	1	1
Solanaceae	*Anthocercis*	9	23
	Anthotroche	3	7
	Atropa	6	23
	Crenidium	1	9
	Cyphanthera	7	22
	Cyphomandra	1	5
	Datura	14	62
	Duboisia	3	22
	Grammosolen	1	7
	Hyoscyamus	12	27
	Latua	2	3
	Mandragora	3	13
	Nicandra	1	1
	Physalis	2	7
	Physochlaina	6	13
	Przewalskia	2	6
	Salpichroa	1	3
	Schizanthus	5	20
	Scopolia	9	16
	Solandra	6	13
	Symonanthus	1	8
	Withania	1	3
Convolvulaceae	*Calystegia*	1	3
	Colutea	1	1
	Convolvulus	5	14
	Erycibe	3	3
	Evolvulus	1	3

Solanaceae comprises 2666 species in some 96 genera of herbs, shrubs and a few trees [6]. These plants can be found around the world except in the arctic regions. However, the largest areas of distribution are in South and Central America along the Pacific Coast, where 60 genera have been identified. The *Solanaceae* family is of great economical importance for its

food plants such as the potato *(Solanum tuberosum* L. and related species), the tomato *(Lycopersicum esculentum* Mill.) and the egg-plant *(Solanum melongena* L.) which are among the most popular species. This family is also well known as a source of drugs in medicine but many of them are poisonous when used in excess: *e.g.* deadly nightshade *(Atropa belladonna* L.), henbane *(Hyoscyamus niger* L.), mandrake *(Mandragora officinarum*), thorn apple *(Datura* species).

Tropane alkaloids are particularly numerous in the species of *Atropa, Datura* (incl. *Brugmansia), Duboisia* and *Hyoscyamus.* It is noteworthy that <<classical>> alkaloids such as hyoscyamine, scopolamine, i.e. esters with tropic acid or related acids of alkamines derived from tropane, are restricted to the *Solanaceae* [7].

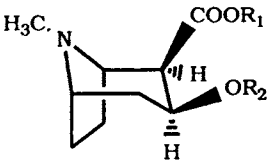
Grafting experiments in which scions from tropane alkaloid-producing species are grafted onto root stock from non-producing species result in plants that do not accumulate alkaloids, whereas grafting in the reciprocal combination produces plants that accumulate tropane alkaloids [8]. These experiments have demonstrated that the main site of tropane alkaloid biosynthesis is the root and that the alkaloids are translocated *via* xylem vessels from the root to the aerial parts of the plant. The mechanism of alkaloid translocation cannot be explained by pH differences only and the regulation of the long-distance transport and the passage of alkaloids from xylem vessels to the accumulating cells are still poorly understood and require further investigations.

The coca leaf *(Erythroxylum coca* Lam. and *E. novogranatense* (Morris) Hieron) contains 0.7-1.5% of total alkaloids, the chief component being (-)-cocaine, a diester of (-)-ecgonine (Fig. 3.). Ecgonine contains four chiral centres and is therefore optically active. Cinnamoylcocaine, α-truxilline, β-truxilline, methylecgonine and tropacocaine are other minor constituents of coca leaves. There are over 200 species of *Erythroxylum* throughout tropical and pantropical regions of the world. Few of the non-cocaine-producing species have been systematically examined but the majority of those that have contain a range of tropane alkaloids [4].

Calystegines are polyhydroxylated *nor*tropane alkaloids with an unusual aminoketal functionality at the bridgehead position. From a general point of view, *nor*tropane alkaloids have not been frequently encountered, although they occasionally occur in association with the corresponding substituted tropane alkaloids. Calystegines have been shown to occur in the *Solanaceae, Convolvulaceae* and *Moraceae* families. As can be seen from Table 2, the greatest number of plant species producing calystegines belong to the *Solanaceae* family, including potato leaves and tubers. From the roots of *Lycium chinense* Mill., 14 calystegines were isolated and among them two polyhydroxytropanes bearing a methyl group on the nitrogen atom, unlike the previously reported *nor*tropane alkaloids [14]. In both compounds, the *N*-methyl group was found to be axially oriented. The only glycoside isolated so far

has been the 3-*O*-β-D-glucoside of 1α,2β,3α,6α-tetrahydroxy-*nor*tropane (calystegine B_1) from the fruits of *Nicandra physalodes* (L.) Gaertn. [15].

Even if there is no doubt that many more alkaloids will be found in diverse plant sources, it is clear that the calystegines appear to be restricted in distribution but it is too early to discuss the significance of the distribution until wider investigations have been conducted.



R_1 : H
R_2 : H — Ecgonine

R_1 : CH_3
R_2 : C_6H_5CO — (-)-Cocaine (2R,3S)

R_1 : CH_3
R_2 : $C_6H_5CH{=}CHCO$ — Cinnamoylcocaine

R_1 : CH_3
R_2 : H — Methylecgonine

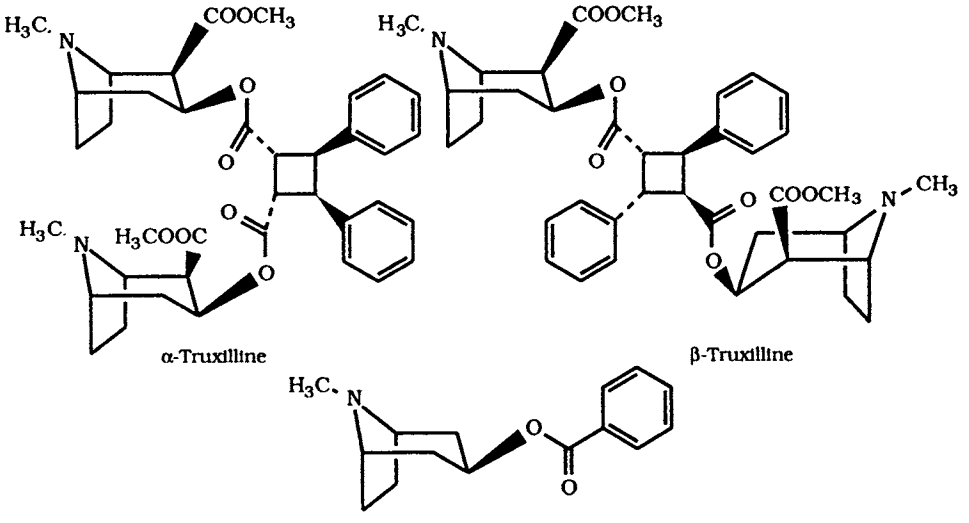


Fig. (3). Tropane alkaloids from *Erythoxylum* sp.

The role of calystegines in plants has not been elucidated, but the fact that they appear in a limited number of species indicates that they might be a source of carbon and nitrogen to soil bacteria that benefit the rhizosphere of the plant [5, 25]. Calystegines may also play a role in plant defence mechanisms and plant-insect interactions as reported by Nash [10].

Table 2. Occurence of calystegines in the plant kingdom

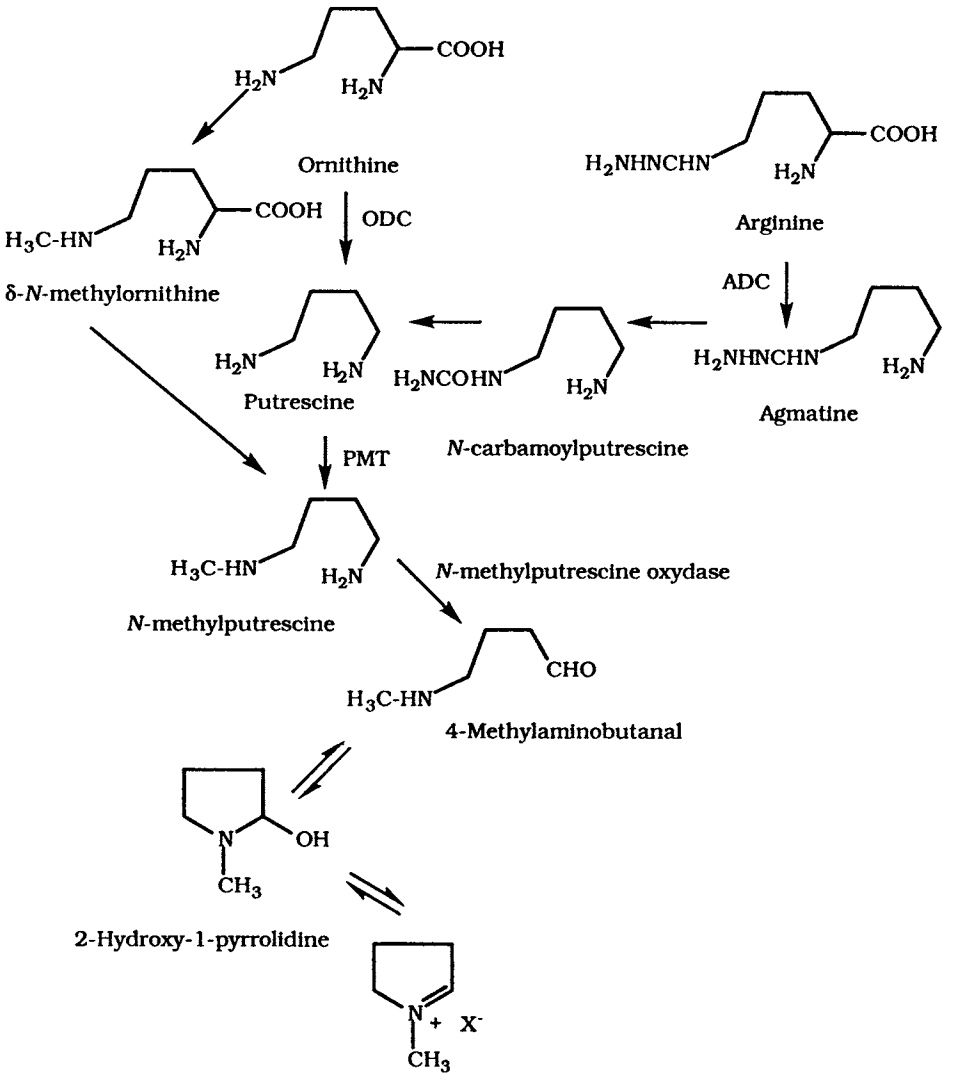
Families	Plant sources	Reference
Solanaceae	*Atropa belladonna L.*	[5]
	Datura stramonium L.	[9]
	D.wrightii Regel	[10]
	Duboisia leichhardtii F. Muell.	[11]
	Hyoscyamus niger L.	[12,13]
	Lycium chinense Mill.	[14]
	Mandragora officinarum L.	[12]
	Nicandra physalodes (L.) Gaertn	[15]
	Physalis alkekengi L.	[16,17]
	Scopolia carniolica Jacq.	[12,9]
	S. japonica Maxim.	[18]
	Solanum dulcamara L.	[10]
	S. melongena L.	[10]
	S. tuberosum L.	[12]
Convolvulaceae	*Calystegia japonica Choisy*	[20]
	S. sepium L.	[5,21]
	Convolvulus arvensis L.	[5,21]
	Ipomoea alba L.	[9]
	I. carnea Jacq.	[9]
	I. polpha	[22]
	I. sp. Q6 (aff. calobra)	[22]
Moraceae	*Morus alba L.*	[23]
	M. bombycis Koidz.	[24]

BIOSYNTHESIS

The biosynthesis of tropane alkaloids has been extensively studied over the last few decades. This is mainly due to the pharmacological importance of compounds such as (-)-hyoscyamine, (-)- scopolamine and (-)-cocaine. An excellent review has been published on that subject by Leete [26].

Major progress has been achieved using labelling and enzymatic methods applied to *in vitro* tissue cultures, in particular with genetically transformed root cultures.

It is now accepted that *N*-methyl-Δ^1-pyrrolinium salt is the common precursor of not only tropane alkaloids but also of the *N*-methylpyrrolidine ring of nicotine, hygrine and cuscohygrine, as shown in Fig. 4. Purification of several enzymes involved in the tropane alkaloid synthesis and the use of radiolabelled precursors have considerably improved our understanding of the biosynthetic pathway.



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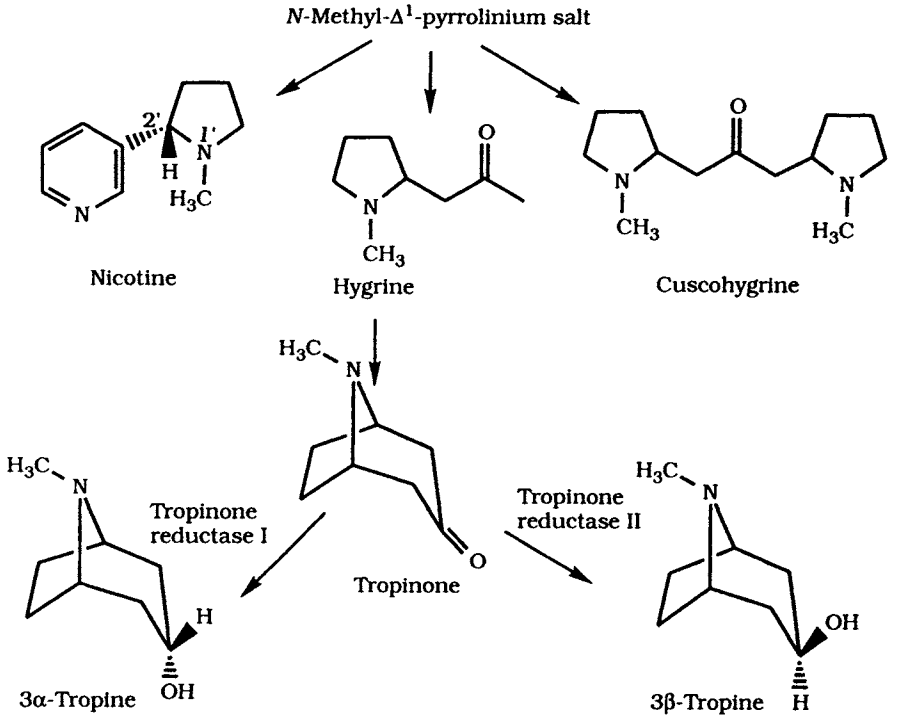

Fig. (4). Biosynthesis of tropine and pseudotropine.

It is now well established that the formation of the tropane ring system derives its pyrrolidine ring from ornithine and/or arginine. The formation of putrescine by the decarboxylation of ornithine has been widely investigated and the enzyme which catalyzes this reaction is called ornithine decarboxylase (ODC). This enzyme has been isolated from tobacco *(Nicotiana tabacum* L.), *Hyoscyamus albus* L. and several other unrelated species. Similarly, arginine is converted into agmatine, the reaction being catalyzed by arginine decarboxylase (ADC). Agmatine is converted to putrescine *via* *N*-carbamoylputrescine. Putrescine is then methylated to *N*-methylputrescine and putrescine *N*-methyltransferase (PMT) appears to be responsible for the *N*-methylation in this pathway. Oxidative deamination of *N*-methylputrescine by the action of a diamine oxidase gives 4-methylaminobutanal. This latter compound is in equilibrium with the *N*-methylpyrrolinium ion.

The subsequent reactions leading from *N*-methylpyrrolinium to tropinone remain doubtful since no enzymes have yet been demonstrated. For a long time, it was believed that the formation of the tropane ring from the *N*-methyl-Δ^1-pyrrolinium cation occurred by condensation with an acetoacetyl unit, with the release of CO_2, to form hygrine. This was

thought to be then oxidized to 5-acetonyl-1-methyl-Δ^1-pyrrolinium, and to undergo a Mannich reaction to form tropinone. However, this hypothesis required that only (2*R*)-hygrine and not (2*S*)-hygrine served as a precursor for the tropane ring. In this context, Leete and Kim [27] suggested an alternative pathway for the formation of the tropane ring of (-)-cocaine in *Erythroxylum coca* as illustrated in Fig. (**5**). According to this hypothesis, *N*-methylpyrrolinum reacts successively with two malonyl CoA units (possibly activated by decarboxylation) instead of acetoacetyl CoA to yield the CoA thioester of 1-methylpyrrolidine-2-acetoacetic acid. Oxidation of the pyrrolidine ring and subsequent Mannich condensation afford the thio ester of 2-carboxytropinone. This latter compound is then converted to the methyl ester (2-carbomethoxytropinone) followed by stereospecific reduction of the carbonyl function in position 3 and benzoylation to yield (-)-cocaine. The benzoyl moiety arises from phenylalanine *via* cinnamic acid and benzoyl-CoA. Abraham and Leete [28] stated that due to several similarities between the biosynthesis of (-)-cocaine and that of (-)-hyoscyamine, it seems plausible that the suggested biosynthetic pathway for (-)cocaine could also operate in the biosynthesis of (-)-hyoscyamine. From this and other results of incorporation experiments [29], it was concluded that both isomers of hygrine are apparently utilized in hyoscyamine biosynthesis.

Tropinone is reduced stereospecifically to either tropine or pseudotropine (Fig. **4**). This reduction is brought about by two independent tropinone reductases, often referred to as TR-I and TR-II [30]. TR-I catalyzes the NADPH-dependent formation of tropine, whereas TR-II reduces tropinone to pseudotropine. The TR-I reaction is reversible but the TR-II reaction is essentially irreversible, the reduction of the ketone being highly favoured over the oxidation of the alcohol pseudotropine [31]. Results of feeding indicate that 3α-tropine and 3β-tropine do not isomerize and that only the former is incorporated into hyoscyamine [32].

The biosynthetic origin of the tropic acid moiety has raised great interest over many years. However, many details of the process remain unclear and little is known about the intermediate steps leading up to tropic acid. It has been recently demonstrated that tropic acid moiety (Fig. **6**) involved in hyoscyamine and scopolamine originates from phenylalanine by way of phenyllactic acid through intramolecular rearrangement [33]. The reduction of the unstable phenylpyruvic acid gives rise to phenyllactic acid. However, to date, no enzyme responsible for the reduction of phenylpyruvic acid to phenyllactic acid has been described. The incorporation of phenyllactic acid into littorine, the phenyllactate ester of 3α-tropine, and hyoscyamine has been clearly established [34]. Labelled experiments have demonstrated that free tropic acid is not an intermediate in hyoscyamine biosynthesis but rather that the rearrangement of phenyllactic acid occurs subsequently to its esterification

[35]. The mechanism of this rearrangement has yet to be proved, though a free radical process with an intermediate cyclopropane-containing radical would fit the available data [36]. The conversion of the phenyllactoyl moiety of littorine into the tropoyl moiety of hyoscyamine or scopolamine involves a mutase reaction [37].

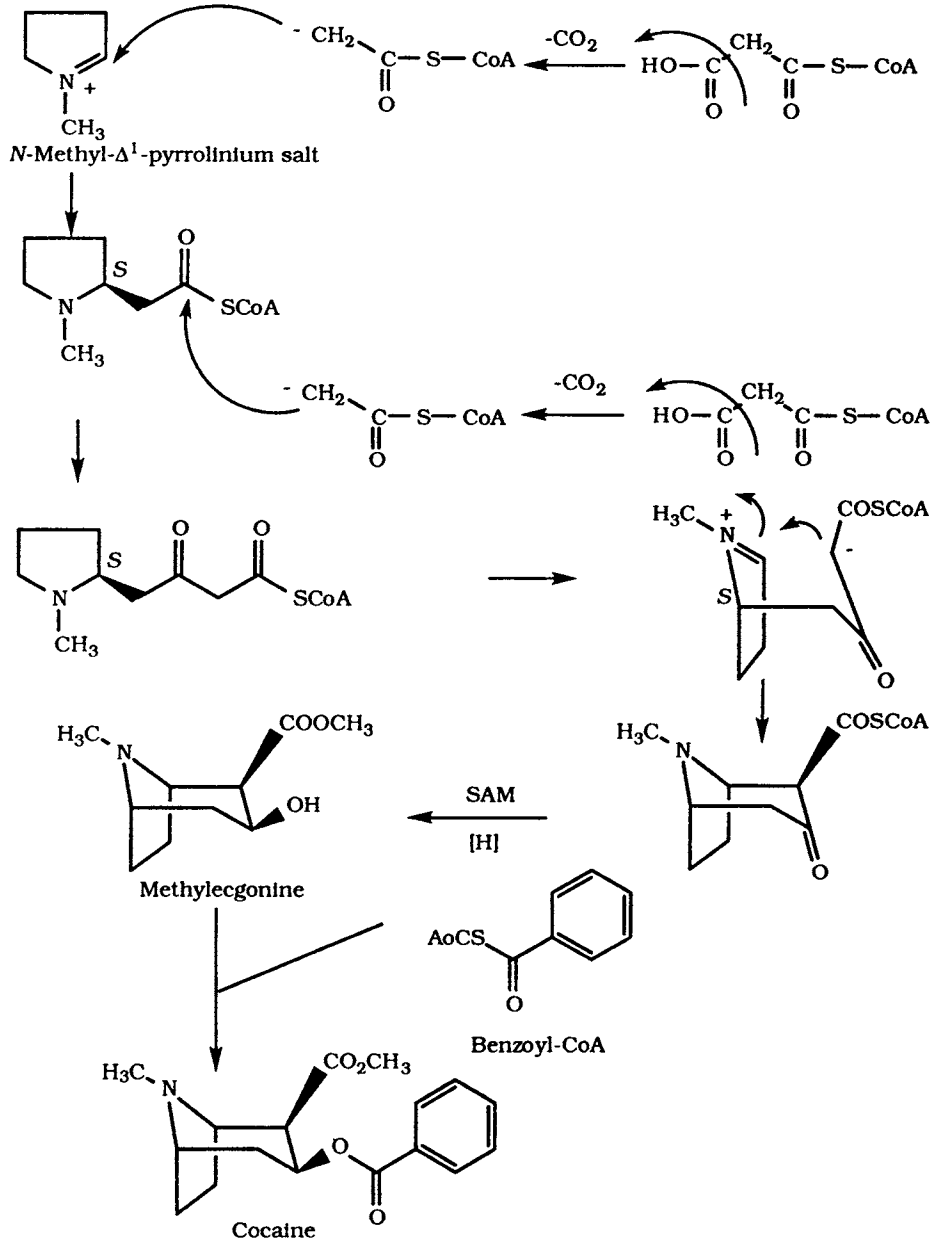

Fig. (5). Hypothetical biosynthetic pathway of cocaine.

Besides tropic and phenyllactic acids, little is known about the biosynthesis of other acids esterifying the tropane nucleus. Leete [26] listed the acids found in tropane alkaloids whose biosynthesis has been studied, usually by feeding labelled precursors to intact plants. Thus, tiglic acid, the acidic moiety of tigloidine and meteloidine, has been shown to be derived from the amino-acid L-isoleucine, probably *via* 2-methylbutanoic acid [38].

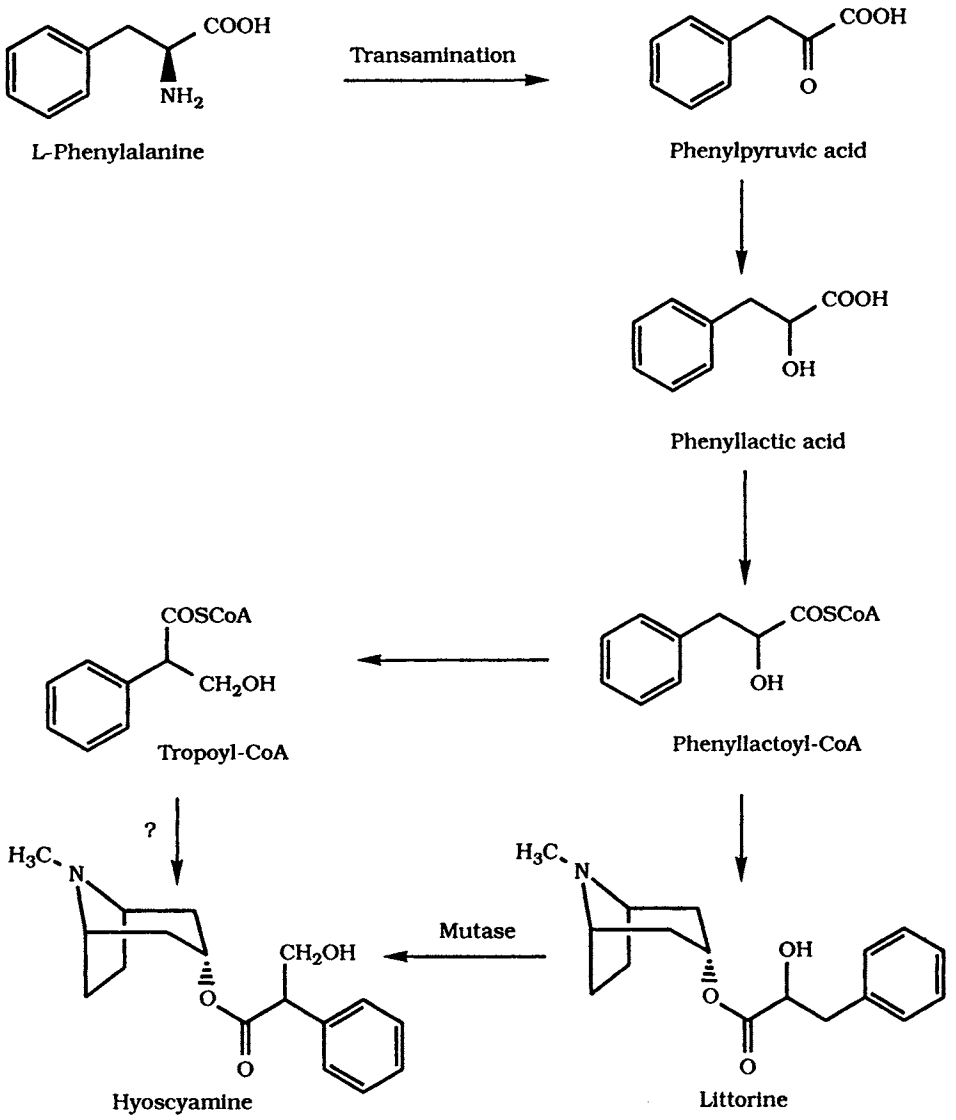

Fig. (6). Origin of the tropic acid moiety involved in hyoscyamine.

Despite the fact that the reaction mechanism, by which the ester bond between the alkamine and the acid moiety is formed, is still not fully understood, it has been recently reported [38] that a number of esters can be formed *in vitro* by acyltransferase reactions involving the transfer of the acidic group from the relevant coenzyme A to tropine or pseudotropine. Thus, tigloyl-CoA: pseudotropine acyl transferase, which esterifies the 3β-hydroxy group of pseudotropine with tigloyl-CoA to give 3β-tigloyloxytropane has been isolated from hairy root cultures of *Datura stramonium* and characterized [39].

Further modifications of the tropane skeleton may occur. One of these is the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine and additional oxidation allowing formation of scopolamine. Initially, it was thought that scopolamine was formed from hyoscyamine *via* 6,7-dehydrohyoscyamine. However this latter intermediate, although incorporated into scopolamine when fed as a precursor, has never been isolated from normal plants. In 1986, Hashimoto's group isolated and partially purified the enzyme responsible for the conversion of hyoscyamine to 6β-hydroxyhyoscyamine [40]. Hyoscyamine 6β-hydroxylase (H6H; EC 1.14.11.11) catalyzes the first oxidative reaction in the biosynthetic pathway leading from hyoscyamine to scopolamine, thus eliminating 6,7-dehydrohyoscyamine from the pathway (Fig. 7). This enzyme requires 2-oxoglutarate, ferrous ion, ascorbate and molecular oxygen for activity. The epoxydase enzyme responsible for the conversion of 6β-hydroxyhyoscyamine to scopolamine appears to be impossible to separate from H6H [41,42]. Molecular cloning and heterologous expression of H6H demonstrated that this enzyme catalyzes both the hydroxylation reaction and the intramolecular epoxidation reaction [43]. Immunohistochemical studies using monoclonal antibody and immunogold-silver enhancement revealed that the scopolamine biosynthesis is specifically localized in the root pericycle [44]. However, this does not imply that the precursors of scopolamine are also synthesized in the pericycle. One could imagine that they are synthesized in root cells other than in the pericycle and then translocated to the pericycle to be converted into scopolamine.

As yet the biosynthesis of the calystegines has not been elucidated. The structure of these compounds suggests that they are biosynthesized, at least partially, by the tropane alkaloid pathway, pseudotropine being the immediate precursor [45]. However, the lack of *N*-methylation in most of the calystegines and the high degree of hydroxylation may indicate that these metabolites are biosynthesized by a divergent route of the tropane alkaloid pathway that does not involve the formation of tropinone or pseudotropine. Recently, the isolation of 1β-amino-2α,3β,5β-trihydroxycycloheptane from *Physalis alkekengi* L. var. *francheti* Hort. [17] and 1β-amino-3β,4β,5α-trihydroxycycloheptane from *Lycium chinense* Mill. may indicate that the calystegines could result from the

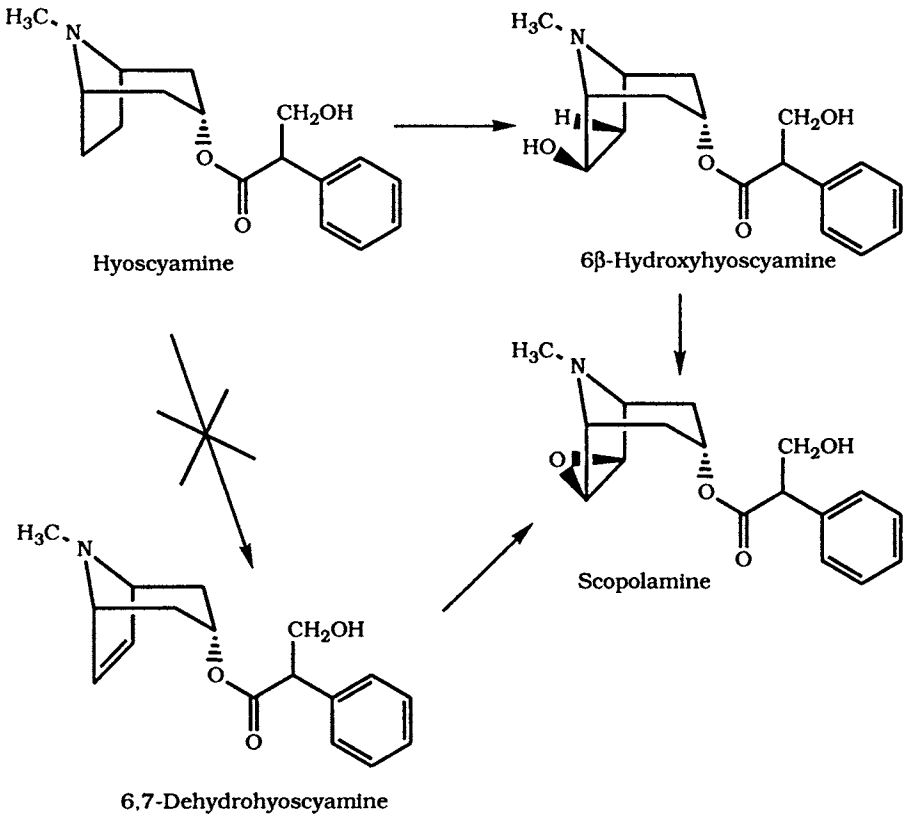

Fig. (7). Conversion of hyoscyamine to scopolamine.

enzymatic oxidation of the 5-OH group of the polyhydroxylated 1-aminocycloheptanes (Fig. **8**). Calystegines, lacking an hydroxyl group at position C3, might be generated from β-elimination by the carbonyl group resulting from this oxidation. Finally, the *N*-methylcalystegines might be derived by the same postulated pathway via *N*-methylputrescine [14].

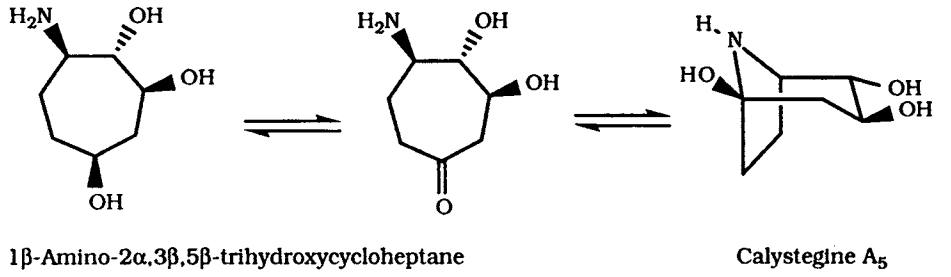

Fig. (8). Hypothetical biosynthesis of calystegine A_5.

CHEMISTRY

The organic synthesis of alkaloids has a long history and numerous synthetic approaches of the tropane skeleton have been developed, from the classical synthesis of tropine by Willstätter at the beginning of the century and comprehensively reviewed by Holmes [46], to the most recent developments dealing with asymmetric deprotonation of tropinone, with chiral lithium amide bases for the enantioselective synthesis of a range of tropanes [47]. New synthetic methods are periodically reviewed and readers interested in this area may refer to specialized literature.

The optical activity of hyoscyamine and scopolamine stems from the chiral center in the acid portion, (*S*)-tropic acid. Tropine itself, although containing chiral centres, is a symetrical molecule optically inactive and can be regarded as a *meso* isomer. Under catalysis of bases, racemization easily takes place at the asymmetric centre of the acyl component. Consequently, depending on the processing conditions, (-)-hyoscyamine or the racemate atropine are obtained during the preparation of the alkaloid. The plant material itself generally only contains the enantiomerically pure alkaloids. Hyoscyamine appears to be much more easily racemized than scopolamine. By means of mineral acids, alkaloids can be converted into corresponding salts with a much better solubility in water than that of free bases. By alkylation with alkyl halides, the corresponding quaternary ammonium compounds are made available. Scopolamine differs chemically from atropine and hyoscyamine in the epoxide bridge between C-6 and C-7 of the tropine cyclic system (Fig. 2). The corresponding heterocycle is called scopine. Scopolamine is a relatively unstable viscous fluid. For this reason, the salts of scopolamine are mainly used for pharmaceutical purposes. As in the case of

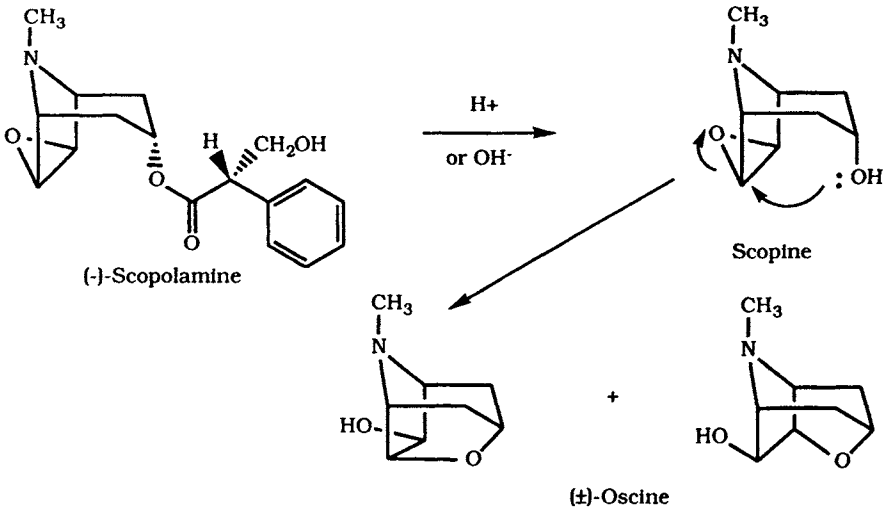

Fig. (9). Hydrolysis of scopolamine.

hyoscyamine, racemization at the asymmetric centre of the tropic acid takes place in an alkaline environment. Furthermore, chemical hydrolysis of scopolamine gives rise to the alcohol (±)-oscine because of the proximity of the 3α-hydroxyl group to the reactive epoxide function (Fig. **9**).

The piperidine ring in the bicyclic tropane system has a chair-like conformation, and there is a ready inversion of configuration at the nitrogen atom so that the *N*-methyl group can equilibrate between equatorial and axial positions (Fig. **10**). The nitrogen configuration in natural tropanes such as hyoscyamine, scopolamine and cocaine, as well as in synthetic compounds such as homatropine and benzatropine, has been studied by ^{1}H-NMR and ^{13}C-NMR [48]. Most derivatives show, at equilibrium, a preponderance for the equatorial position of the methyl group provided there are no substituents on the two-carbon bridge, in which case the axial form may predominate. Furthermore, scopolamine shows a strong solvent dependence with a reversal from axial in D_2O to equatorial in CD_2Cl_2.

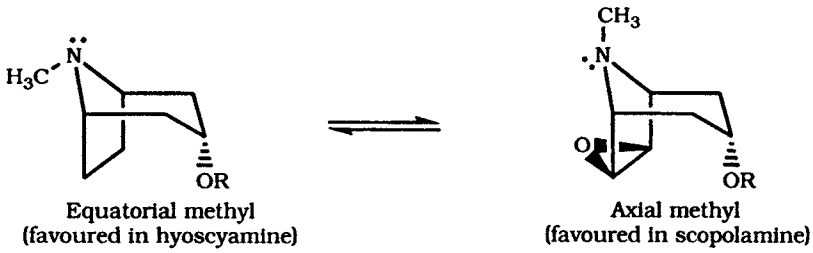

Fig. (10). Axial-equatorial equilibrium of *N*-methyl group in tropane alkaloids.

Tropic acid esters (*e.g.* atropine, scopolamine) give an intense purple colour in the Vitali-Morin reaction, allowing to distinguish them from other tropane alkaloids. The test involves treating 0.1 mg of the alkaloid with a drop of fuming nitric acid, evaporating to dryness at 100°C and adding a drop of freshly prepared ethanolic potassium hydroxide. A bright purple colour develops, which fades slowly to dark red. Tropane alkaloids react readily with picric acid to give crystalline derivatives with characteristic melting points which are valuable for the identification of individual compounds.

Calystegines are classified into three groups: calystegines A, B and C, on the basis of the number of hydroxyl groups attached to the *nor*tropane skeleton, i.e. tri, tetra- and penta-hydroxycalystegines, respectively. Recently, a novel *nor*tropane alkaloid, called calystegine N_1, with a bridgehead amino group was isolated from *Hyoscyamus niger* L.[13].

Due to their high polarity and strong hydrophilicity, extraction of calystegines cannot be performed by the traditional Stas-Otto procedure in which alkaloids can be transferred to either aqueous or organic layer by

changing the pH of the aqueous phase. Hydroalcoholic solvent mixtures, sometimes supplemented with dilute acids, are generally used to extract the calystegines. However these mixtures extract simultaneously many other polar compounds and therefore separation of the alkaloid fraction from the neutral and acidic compounds is frequently carried out by ion-exchange chromatography, usually with Dowex 50 or Amberlite CG 120 resins in their NH_4^+ or H^+ ion forms [49]. The purified calystegine fraction obtained by ion exchange chromatography consists generally of structurally closely related alkaloids. It is then sometimes possible to recrystallize the main alkaloids or to convert the free bases into crystalline salts.

High voltage paper electrophoresis has been applied to determine the presence of calystegines with silver staining [19], using ninhydrin with which they give yellow-brown spots or with nitroprusside reagent, which generates blue colours with most of the alkaloids. However, neither of these reagents is particularly sensitive or specific for the calystegines. Thin layer chromatography with silica gel as stationary phase is frequently used for the analysis of plant extracts containing calystegines. Dragendorff's ($K[BiI_4]$) or iodoplatinate ($Na_2[PtCl_6]$) reagents, the two most commonly used reagents for alkaloid vizualization, do not react with calystegines, except at high concentrations.

HPLC analysis of calystegines is somewhat difficult due to their solubility and high polarity, which limit the selection of column packings and solvent mixtures. Moreover, their lack of chromophore excludes spectroscopic methods of detection without pre- or post derivatization of the samples. Gas chromatography on fused silica capillary column coupled with mass spectrometry is the most suitable analytical technique used for the determination of calystegines. However, the high polarity and the non-volatility of the compounds preclude their analysis without derivatization and numerous derivatization reagents have been tested [9].

Calystegine N_1 undergoes approximately 40% conversion into calystegine B_2 on storage suggesting that *nor*tropane bearing an amino substituent may undergo conversion to the hydroxy derivative during isolation, and that calystegine N_1 may be an artifact formed from calystegine B_2 during the extraction procedure using ammonia as eluent.

Because of the growing interest in calystegines, synthetic studies have been recently reported. The first synthesis of a calystegine skeleton has been carried out by Ducrot et al. [50]. Racemic 1-hydroxy-7-keto*nor*tropane was obtained from cycloheptanone *via* 2,3-epoxy-4 azidocycloheptanone. Total syntheses of racemic calystegines A_3 and three stereoisomers, as well as physoperuvine, have been achieved by Boyer et al. [51] using intramolecular cyclisation of 4-aminocycloheptanones. It is noteworthy that this aminoketal system should exist as a possible equilibrium mixture of 1-hydroxy*nor*tropane and 4-aminocycloheptanone, this equilibrium being shifted toward one of these

forms according to the nature of substituents on the seven membered ring or on the nitrogen atom. Recently, the enantioselective synthesis of both (+)- and (-)-enantiomers of calystegine A_3 has been reported [52]. The stereoselective synthesis of 7(*S*)-hydroxymethylcalystegine B_2, an analogue of calystegine B_2 has been achieved by intramolecular cycloaddition of an olefinic nitrile oxide derived from D-glucose [53], whereas enantiomerically pure (+)- and (-)-calystegine B_2 have been obtained from D-glucose by a ring enlargement of polysubstituted cyclohexanone, followed by introduction of the nitrogen atom as an azide in position 1 or 5 [54]. The same pair of enantiomers had also been synthesized previously using another approach by Duclos *et al.* [55]. Soulié et al. [56] has investigated an alternative route in the preparation of the 4-aminocycloheptanones *via* a Diels-Alder addition between an acylnitroso derivative and a protected polyhydroxy-cyclohepta-1,3-diene. The unusual structures of calystegines and their low abundance in plant material will undoubtedly encourage to explore other strategies for the total synthesis of polyhydroxylated *nor*tropane derivatives.

PHARMACOLOGICAL PROPERTIES

The origin of the use of plants for medical purposes goes back to the dawn of civilization. The effects and uses of solanaceous plants in medicinal practice derive primarily from one of three groups of toxic alkaloids: the tropane-, steroid- or pyridine-types. However, the compounds which play a role as therapeutic or toxic agents are all various tropane alkaloids. The first documented references to solanaceous plants date from at least 2000 B.C. and reveal that the powerful pharmacological properties of *Hyoscyamus niger, Mandragora officinarum* and *Atropa belladonna* were known in ancient Egypt and Mesopotamia [57]. In addition, members of the *Solanaceae* with narcotic and hallucinogenic properties *(Datura, Brugmansia* spp.) have figured prominently in rituals, magic and superstitions associated with healing in the ancient civilizations of both the Old and New World. Even today, *Solanaceae* is one of the top ranking families of drug-yielding plants used not only in modern medicine but also in traditional and herbal medicine for the treatment of a wide range of ailments.

Tropane alkaloids are compounds known as muscarinic receptor antagonists. Atropine, its best-known member, and a number of other compounds, block the action of the neurotransmitter acetylcholine on post-ganglionic cholinergic nerves of the parasympathetic nervous system, essentially by blocking its binding to muscarinic cholinergic receptors, whereas they are much less potent at nicotinic receptor sites.

Table 3. Main Pharmacological Properties of Tropane Alkaloids

Systems	Effects
CNS	Atropine: stimulation of the medulla and higher cerebral centres Scopolamine: drowsiness, amnesia, fatigue but also occasionally excitement, hallucinations or delirium
Respiratory	Inhibition of secretions of the respiratory tract; bronchodilatation
Cardiovascular	Alteration of the heart rate Low doses: bradycardia High doses: tachycardia
Gastrointestinal	Salivary and gastric secretions strongly reduced. Antispasmodic activity by reduction of motility of the gastrointestinal tract
Urinary	Decrease tone and amplitude of contractions of the ureter and bladder
Uterus	Negligible
Sweat glands and temperature	Inhibition of sweating with raise of body temperature
Eye	Pupillary dilatation (mydriasis) and loss of accomodation (cycloplegia)

All the main tropic acid-esterified tropane alkaloids (hyoscyamine, atropine, scopolamine, *N*-butylscopolamine, *N*-ethylscopolamine) show antimuscarinic activity, although their effects differ quantitatively rather than qualitatively. Table 3 reports the main pharmacological properties of tropane alkaloids. Generally, the most active forms are the (-)-isomers, so that atropine ((±)-hyoscyamine) has only about half the potency of (-)-hyoscyamine on a weight basis. With regard to the two enantiomers, the peripheral effects of (-)-hyoscyamine are 10 to 20 times stronger than those of (+)-hyoscyamine. The effects of (-)-hyoscyamine on the central nervous system are supposed to be 8 to 50 times stronger than that of (+)-hyoscyamine. It exerts powerful effects on the major organs of the thorax and abdomen, especially on smooth muscle and exocrine glands, although

Table 4. Dose-dependent Effects of Atropine

Doses	Effects
0.5 mg	Slight bradycardia; some dryness of mouth; inhibition of sweating
1.0 mg	Dryness of mouth; thirst; slight tachycardia, sometimes preceded by bradycardia; mild mydriasis
2.0 mg	Tachycardia; palpitation; marked dryness of mouth; some blurring of near vision
5.0 mg	Restlessness and fatigue; headache; difficulty in micturition; constipation; difficulty in speaking and swallowing
10.0 mg and more	Above symptoms more marked; pulse rapid and weak; vision very blurred; ataxia, restlessness and excitement; hallucinations and delirium; coma

effects on the central nervous system (CNS) activity also occur. Table 4 shows the dose-dependent effects of atropine. The mechanism of action of tropane alkaloids is a competitive antagonism of acetylcholine and other muscarinic agonists such as pilocarpine, physostigmine or arecoline, three major natural alkaloids. Solanaceous drugs are well known in the treatment of a wide variety of medical disorders and have been claimed to be components of a great number of medicinal preparations. The most important chemotherapeutic uses of tropane alkaloids are summarized in Table 5.

Table 5. Main Therapeutic Uses of Tropane Alkaloids

Systems	Effects
CNS	Atropine to prevent vagal reflexes induced by manipulation of visceral organs. Scopolamine to prevent motion sickness
Respiratory	As preanesthetic medication to inhibit excessive salivation and secretion and aid ventilation
Cardiovascular	Atropine in case of acute myocardial infarction or severe bradycardia. As specific antidote for cardiovascular collapse due to erroneous administration of choline esters or inhibitors of cholinesterase
Gastrointestinal	In case of intestinal hypermotility (diarrhoea, diverticulitis)
Genitourinary	In the treatment of renal colic and spasms of the urinary tract
Eye	Examination of retina and optic disc. In the treatment of iridocyclitis, choroditis and keratitis as well as for accurate measurement of refractive errors
Poisoning	Atropine in the treatment of poisoning by anticholinesterase organophosphorus insecticides

Atropine and scopolamine differ quantitatively in their ability to affect the CNS. Whereas atropine has almost no detectable effect on the CNS in doses that are used clinically, scopolamine has prominent central effects already at low doses. This difference may be due to the greater permeation of scopolamine through the blood-brain barrier. Clinical doses of atropine cause mild excitation. At steadily increasing doses, central excitation is increased, but then central depression follows, leading to circulatory collapse, respiratory failure and coma. This, however, is only of toxicological interest. Therapeutic doses of scopolamine cause a CNS depression manifested by drowsiness, amnesia, fatigue. These effects are utilized to prevent motion sickness and as an adjunct for preanesthetic medication. Tropane alkaloids have also long been used in Parkinsonism, especially before the discovery of levodopa.

The *Belladonna* alkaloids are used to reduce salivary and bronchial secretions by smooth muscle relaxation of bronchi and this action is the basis for the use of atropine in preanesthetic medication.

The main effect of atropine on the heart is the alteration of the rate. At low doses, the rate is slowed (bradycardia) without a change in blood pressure or cardiac output. Higher doses cause an increase in pulse rate (tachycardia). Atropine may be used in the initial treatment of a myocardial infarction or high-grade atrioventricular block.

Atropinic drugs dilate the pupil (mydriasis) and paralyse accommodation (cycloplegia). Locally applied, atropine or scopolamine produce ocular effects of considerable duration; accommodation and pupillary reflexes may not be fully recovered for 7 to 12 days. However when applied as a mydriatic, scopolamine acts faster than atropine but for a shorter time.

Because of their antispasmodic effects, tropane alkaloids are also used to relieve spasms of the bowel in the treatment of spastic colitis and gastro-enteritis.

Atropine is also useful in cases of poisoning. In particular, it may be employed in the treatment of anticholinesterase poisoning by organophosphorus insecticides, and of the muscarinic effects due to *Amanita muscaria* ingestion.

Young children are especially exposed to the toxic effects of tropane alkaloids. Serious intoxication may occur by ingestion of berries of *Atropa belladonna.* Furthermore, poisoning from the ingestion and the smoking of jimsonweed or thorn apple is not infrequent. The toxicity symptoms which can occur are skin rash, flushing of skin, dryness of mouth, difficult urination, eye pain, blurred vision and sensitivity to light.

Cocaine is a potent CNS stimulating agent. It has local anesthetic properties and exerts local vasoconstriction. However, the toxicity of cocaine and its potential for abuse have steadily decreased the use of this compound in therapy. Medicinally, cocaine hydrochloride in 0.1-4% aqueous solution is used as a local anesthetic for topical application. It is rapidly absorbed by mucous membranes and paralyses peripheral ends of sensory nerves. It still has applications in ophthalmic, ear, nose and throat surgery where anesthetic and vasoconstriction effects are desired with a single agent. As illicit use, cocaine is frequently sniffed into the nose where it is rapidly absorbed by the mucosa, provoking stimulation and short-lived euphoria. The drug may also be injected intravenously, or the vapour inhaled. For inhalation, the free base, or crack, is employed to increase volatility, speed up and enhance the euphoric lift. Because of its abuse potential, addiction and some tolerance arising from continued use as a central stimulant, the narcotic laws of federal and state governments control the sale of this alkaloid and of its derivatives.

Many semisynthetic or synthetic compounds, including quaternary ammonium derivatives, have been prepared, primarily with the objective of altering gastrointestinal activity without causing dry mouth or pupillary dilation. Homatropine (Fig. **11**) is prepared synthetically by esterification of mandelic acid with 3α-tropine. Its range of action corresponds to

atropine. However, the effect of homatropine is 10 times weaker than that of atropine. At the same time, toxicity decreases correspondingly.

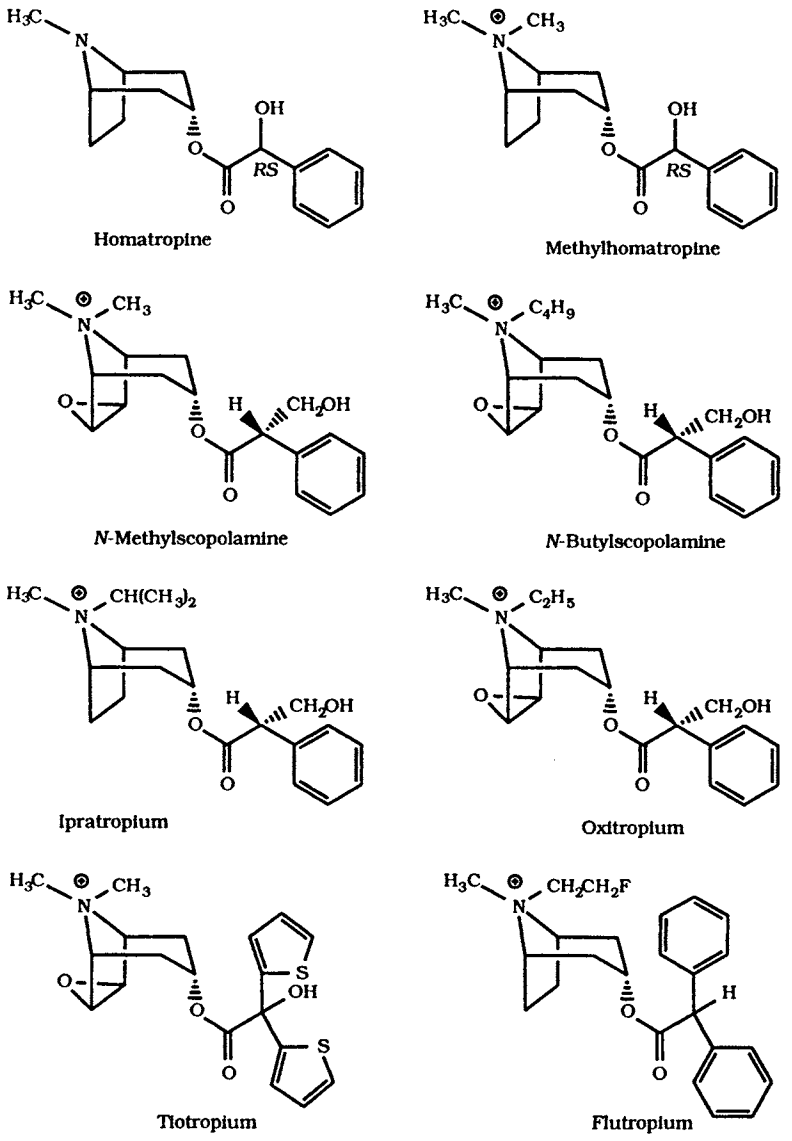

Fig. (11). Semisynthetic and synthetic tropane alkaloids.

Due to their poor lipophilic properties, compounds with a quaternary ammonium structure are poorly absorbed after oral administration. Thus, central effects generally lack, because these alkaloids do not readily cross the blood-brain barrier. Similarly, quaternary ammonium compounds do

not penetrate the conjunctiva and are therefore of poor value in ophthalmology.

Ipratropium is a quaternary ammonium compound formed by introducing an isopropyl group to the *N* atom of atropine, oxitropium and tiotropium, two quaternary derivatives of scopolamine, and flutropium, a fluoroethyl derivative, which are mainly used in the treatment of chronic obstructive pulmonary disease, whereas *N*-butylscopolamine, *N*-methylscopolamine and *N*-methylhomatropine (Fig. **11**) are used to relieve of gastrointestinal spasms.

Calystegines, like other polyhydroxylated derivatives of pyrrolidine, *e.g.* 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine; piperidine, *e.g.* nojirimycin; pyrrolizidine, *e.g.* australine; indolizidine, *e.g.* swainsonine and castanospermine, are currently of great interest as glycosidase inhibitors. Glycosidases are present in all organisms and are necessary for the processing of glycoproteins, which serve as essential regulators of numerous biological mechanisms. Therefore, the general property of glycosidase inhibition has considerable implications with regard to both their biological role in nature and potential chemotherapeutic applications [49]. In this context, the pharmaceutical activity of polyhydroxylated alkaloids as anti-viral agents is of particular interest and appears to result largely from their ability to alter glycoprotein structure by interfering with the processing of the oligosaccharide moiety [20]. Polyhydroxy-*nor*tropane alkaloids have been shown to be potent inhibitors of β-glucosidases and β-galactosidases. However, the extent and specificity of activity is greatly dependent on not only the number of hydroxyl groups present but also upon their stereochemistry. Furthermore, no obvious relationship exists between the hydroxy groups stereochemistry and the glycosidase which is inhibited [49].

It is now established that calystegine B_2 is a competitive inhibitor of β-glucosidases and α-galactosidases and that calystegines B_1 and C_1 are competitive inhibitors of almond β-glucosidase [16; 21; 23; 24]. Calystegine B_2 has been identified in leaves of two species of *Solanum, S. dimidiatum* and *S. kwebense,* which are reported to cause a degenerative neurological disorder in cattle [58]. Furthermore, it is reported [16] that the roots of *Physalis alkekengi* var. *francheti* are used for their antitussive and diuretic properties.

IN VITRO PRODUCTION OF TROPANE ALKALOIDS

For the past 20 years, there has been a growing interest in applying the *in vitro* culture of plant cells, tissues and organs to the study of medicinal plants. Main topics include the development of culture for the large-scale production of valuable chemicals, the discovery of new biologically active metabolites, the selection of plants showing favourable characteristics of productivity, the elucidation of biosynthetic pathways with isolation of

the corresponding enzymes and the improvement of plant species by genetic engineering.

Despite the new syntheses developed, most of the medicinally important tropane alkaloids are still obtained more economically by extraction from plant material. Therefore, tropane alkaloids have been target molecules for plant cell culture and several species belonging to the genera *Anisodus, Atropa, Datura, Duboisia, Hyoscyamus* and *Scopolia* have been extensively studied. From the abundant literature published on the subject, it is clear that the production of tropane alkaloids in cell suspension is low [59]. One of the reasons for cell cultures failing to accumulate alkaloids present in the corresponding whole plant may be the lack of tissue differentiation and the lack of storage facilities, as it is now well established that tropane alkaloids are partly localized in epidermal cell layers, where they are stored in the vacuoles. Moreover, a serious technological problem is the biochemical and genetic instability of the de-differentiated plant cells due to somaclonal variation. A possible approach to increasing yields of tropane alkaloids is therefore to allow the differentiation of tissues by producing shoots or root cultures through manipulating the external hormone balance. However, traditional root organ cultures tend to grow slowly and large scale cultivation is difficult to realize.

About 20 years ago, it became possible to transfer foreign genes into the plant genome [60]. Since then, many attempts have been made to introduce new genes into plants to improve their quality. In this context, an *Agrobacterium*-mediated gene transfer system is widely used and successful results have been obtained in particular with regard to numerous solanaceous species.

The soil pathogenic bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* cause crown gall tumors and hairy root disease, respectively, in the infected plant tissue. The pathogenic responses result from the expression of genetic information, one or both of the two pieces, T_{left} (T_L) or T_{right} (T_R) of the tumor-inducing (Ti) or root-inducing (Ri) plasmid T-DNAs being transferred from the bacteria and incorporated into the host plant nuclear DNA [61]. The resulting transformed plant cells produce specific bacterial metabolites, the opines, which are secreted into the soil, where they are catabolized by free-living *Agrobacteria* [62].

Considerable interest has been shown recently in genetically transformed root cultures for the production of secondary metabolites. In order to develop hairy root cultures in the laboratory, surface-sterilized plant tissue is inoculated with a suspension of *A. rhizogenes* and, after a period of incubation (generally between 1-6 weeks) at 24-28°C, transformed roots emerge at the infection sites. After elimination of the excess bacteria, the excised roots are incubated in liquid culture medium. The genetically transformed roots grow faster than do untransformed roots, they are highly branched and they can be cultivated in hormone-free

medium because genes in the Ri T-DNA regulate the balance of endogenous hormones. In contrast to the instability of cell suspension cultures, hairy roots are genetically and biochemically stable. The synthetic capacity of hairy roots appears generally to mirror closely that of the roots of the parent plant [63]. Furthermore, the yield of secondary metabolites from hairy roots is similar to or even higher than from the whole plant.

Table 6. Advantages and Limitations in The Use of Hairy Root Cultures

Advantages	• High growth rate • Efficient expression of root-specific metabolic pathway • Genetic and biochemical stability • Large-scale culture • Excellent model for studying the biosynthesis of secondary metabolites
Limitations	• Host range of *Agrobacterium rhizogenes* • Production of metabolites synthesized in roots of intact plants

As in the case of cell suspension cultures, an increase in the productivity of hairy roots can be achieved by manipulating the culture conditions such as the nutrient composition, pH, temperature, addition of precursors and/or biosynthetic intermediates. Table 6 summarizes some of the advantages and limitations of transformed root cultures. Major limitations to hairy root cultures are the susceptibility of the plant species to infection by *Agrobacterium rhizogenes* [64] and the fact that transformed root cultures are restricted to the synthesis of products which are formed in the roots of the normal plants. Tepfer [65] has presented a list of plant species from which hairy roots have been obtained. In general, monocotyledonous plants appear to be less sensitive to *A. rhizogenes,* whereas the bacterium is able to infect a wide range of dicotyledonous plants [66]. In this context, the *Solanaceae* family has been particularly studied and hairy roots from different species of *Atropa, Datura, Duboisia, Hyoscyamus* and *Scopolia* have been investigated (Table 7). For the production of hyoscyamine, hairy root cultures of *Atropa belladonna* [68], *Datura innoxia* [75], *D. quercifolia* [76], *D. stramonium* [73], *Hyoscyamus albus* [74], *H. niger* [74] and *Scopolia japonica* [81] demonstrated high biosynthetic activity. For the production of scopolamine, a hybrid of *Datura candida* [70], *Duboisia leichhardtii* [78] and *Scopolia japonica* [81] are particularly suited. Hairy roots of solanaceous species generally present a long-term stable production of alkaloids. For example, some hairy root clones of *Datura stramonium* have shown stable alkaloid production for more than 5 years [77]. Generally, hairy roots produce the same metabolites as those synthesized in non-

transformed plant roots. However, there are some examples in which the pattern of secondary metabolites produced by the hairy roots differs from that of the normal roots [82; 83]. The qualitative changes in the profile of secondary product biosynthesis could therefore be profitable in using hairy roots as a source of new active compounds.

Table 7. Some Examples of Tropane Alkaloid Production by *Agrobacterium rhizogenes*-Transformed Root Cultures

Plant Species	Hyoscyamine [% DW]	Scopolamine [%DW]	Reference
Atropa belladonna L.	0.371	0.024	[67]
	0.950	0.090	[68]
	0.20	0.02	[69]
Datura candida hybrid (Pers.) Saff.	0.11	0.57	[70]
D.candida (Pers.) Saff. x D. aurea (Lagerh.) Saff.	0.47	0.30	[71]
	0.55	0.25	[72]
D. fastuosa L.	0.56	0.01	[73]
D. innoxia Mill.	0.172	0.035	[74]
	1.0	0.30	[75]
D. quercifolia Kunth in H. B. K.	1.33	-	[76]
D. stramonium L.	1.05	-	[73]
	0.11-0.23	0.005-0.077	[77]
D. wrightii Regel	0.82	0.02	[73]
Duboisia leichhardtii F. Muell.	0.07-0.82	2.1	[78]
Duboisia myoporoides R. Br.	0.86	0.15	[79]
Hyoscyamus albus L.	0.52	0.05	[80]
	1.36	0.14	[74]
H. niger L.	1.251	0.086	[74]
Scopolia japonica Maxim.	1.30	0.50	[81]

Even if the alkaloid content is quite high, an important step in the establishment of root cultures is the selection of the best clones which combine good growth and high alkaloid production. The occurrence of clonal variations allows the selection of high-producing lines. Mano et al. [78] were able to select a clone of hairy roots of *Duboisia leichhardtii,* producing 2.1 % DW scopolamine, more than twice the amount found in the leaves of the non-transformed plants.

The use of elicitors can significantly enhance the production of metabolites. The elicitors are divided mainly in two groups. The biotic elicitors which are compounds of biological origin (*e.g.* fungal spores, fungal cell wall fractions, cellulase, chitosane) and the abiotic elicitors which include metal ions, high salt concentrations, UV radiations, sonication. Treatment of *Hyoscyamus muticus* hairy roots with 50-500 μg/ml of chitosane resulted in a 5-fold increase in the accumulation of hyoscyamine [84]. Similar results were obtained by Halpérin and Flores [85] who obtained, with hairy roots of the same species, hyoscyamine up to 6-fold when elicited with mannitol.

Hairy roots are not as readily manipulated by altering culture conditions or pH as are suspension cultures. However, the effect of temperature on growth and hyoscyamine production in transformed root cultures of *Datura stramonium* has been demonstrated by Hilton and Rhodes [86]. Another way to enhance the secondary metabolite accumulation of hairy roots is the addition of precursors and/or metabolic intermediates to the growth medium. The addition of (*R,S*)- phenyllactic acid increased significantly the accumulation of hyoscyamine and scopolamine in the hairy root culture of *Datura candida x D. aurea* [72].

A very promising possibility for enhancing the production of secondary metabolites is to manipulate the cultures genetically. In particular, the transfer and the expression of specific genes which code the enzymes of the biosynthetic pathways of the products of interest may represent good prospects for the development of commercial processes. Extensive studies have been undertaken on the gene encoding for hyoscyamine 6β-hydroxylase catalyzing the conversion of hyoscyamine into scopolamine (Fig. 7). The gene, isolated from *Hyoscyamus niger,* has recently been transferred to *Atropa belladonna* which produces hyoscyamine as the main alkaloid and very little scopolamine. The resulting transgenic plants [87] and hairy roots [88] were found to contain a high level of scopolamine.

Another important factor in the development of an *in vitro* culture process is the release of the metabolites into the medium. The extent of secondary product release in hairy root cultures varies between species. In hairy root cultures of solanaceous species, numerous studies have clearly demonstrated that the secondary products are generally poorly released by the cells [64; 89] and are stored in the cell's vacuoles, except in *Nicotiana rustica* in which 76 % of the nicotine produced by the roots is released into the culture medium [90].

When considering economically feasible production of secondary metabolites by employing *in vitro* cultures, it is worth determining whether the products can be released into the medium and collected without destroying the biomass. Attempts are being made to develop methods for the permeabilization of plant cells for release of intracellularly stored products. The cells should remain viable after the treatment to be

fully biosynthetically active. Muranaka et al. [91] reported that 75 % of the scopolamine produced by *Duboisia leichhardtii* hairy roots was released into the medium within 4 weeks. The authors used a modified Heller's culture medium containing 37 mM of KNO_3 and no NH_4Cl. The use of detergents may also influence significantly the release of tropane alkaloids. The addition of Tween 20 (polyoxyethylenesorbitane monolaurate) to the hairy root culture of *Datura innoxia* resulted in a 3-8 fold increase of tropane alkaloid content per flask, most of the compounds being released into the medium [92]. The use of Amberlite resins (XAD-2, XAD-4, XAD-7) were also demonstrated to be suitable for the recovery of products from hairy root cultures. The addition of Amberlite XAD-4 (1g/flask) to the growth medium, 10 days after subculture of hairy roots of *Datura quercifolia,* resulted in a 4-fold increase of hyoscyamine release into the medium with 80 % of hyoscyamine bound to the resin [76]. The additional advantage of using Amberlite resins may be suppressing feed-back inhibition of alkaloid biosynthesis in hairy root tissues and preventing degradation of the metabolites in the medium.

For production purposes, hairy roots should be cultivated on a large scale. However, despite their outstanding properties, few investigations have been performed in a bioreactor with the solanaceous species. Most of the many bioreactors on the market are developed for microbial fermentations and some of them have been applied to the plant cell technology [66], but are not ideally suited for the growth of transformed roots. From a technical point of view, the main problem is mass transfer. The densely packed mass of roots produces oxygen and nutrient limitations which lead to a reduction in secondary metabolite production, cell necrosis and autolysis. But mechanical agitation is not possible since shear stress causes disorganization and callus formation with a consequently lower productivity. Furthermore, inoculation of the bioreactor and sampling of the roots during the process cause difficulties. Recently, an apparatus for inoculating plant organs into a fermentor has been developed [93] and allows the inoculation of large quantities of plant material. The direct measurement of root growth in the bioreactor is also a difficulty because of the morphology of hairy roots. Therefore, alternative methods for monitoring root growth have to be found. Taya et al. [94] reported that there is a linear relationship between the dry biomass of the hairy roots and the medium conductivity decrease. Thus, it is possible to monitor biomass growth during hairy root cultures by on-line measurement of conductivity in the bioreactor.

Bioreactors for hairy root cultures usually have volumes of a few litres or even less, with the exception of a large fermenter of 500 1 volume [95]. Hilton et al. [96] investigated the growth and hyoscyamine productivity of hairy roots of *Datura stramonium.* Using an impeller-mixed 14 1 vessel with a working volume of 12 1 and a mesh to separate the roots from the stirring mechanism, about 10 g dry wt/1 of biomass was obtained with a

productivity of 1.6 mg of hyoscyamine/l/day in a simple batch fermentation run of 40 days. By operating the fermenter in a continuous mode, the biomass yield was improved 2-fold, with a consequent improvement in productivity. An approximately 3.5-fold increase in both total hyoscyamine accumulation and rate of production was achieved by operating in the continuous mode [97].
A further increase in productivity and hyoscyamine content in roots was observed after changing the culture temperature from 25 to 30°C.

Hairy root cultures of *Atropa belladonna* were cultured in shake flasks, as well as in a 2.5 l airlift bioreactor, both containing MS medium supplemented with 3 % sucrose [98]. An exponential growth of hairy roots was observed. The specific growth rate in shake flasks was 0.28 d^{-1}, corresponding to a doubling time of 60 h. Dry weight in shake flasks increased 285-fold over 28 days. In the airlift reactor, the rate of root growth appeared to be affected by nutrient limitation. Necrotic tissue developing in the bioreactor suggested that the transfer of oxygen and/or carbohydrate was not optimal. Although atropine content in reactor-grown hairy roots (0.37 % dry weight) was higher than in shake flask-grown hairy roots (0.25 % dry weight), the culture performance was much poorer in the bioreactor if one considers the sugar consumption.

Hairy roots of a scopolamine-releasing clone of *Duboisia leichhardtii* was cultured in an Amberlite XAD-2 column-combined bioreactor system for continuous production of scopolamine [99]. The medium used was continuously exchanged during culture to maintain the electrical conductivity of the medium constant. A two-stage culture was carried out by using a turbine-blade reactor with stainless-steel mesh as a support, the first stage in the medium for hairy root growth and the second stage for scopolamine release. Under these conditions, 1.3 g/l of scopolamine was recovered during 11 weeks of culture.

CONCLUSION AND PERSPECTIVES

Because of the proven medicinal value of tropane alkaloids, it has been suggested that much more work could be done, to great human advantage, on both the hyoscyamine/scopolamine-type alkaloids and the plants containing them [100]. As human society develops, there is a concomittant need for new and improved medicines to treat not only new diseases but also well-established but therapeutically-difficult disorders. The *Solanaceae* constitutes a potent reservoir of novel, physiologically active natural products. However, to date, 80 % of solanaceous genera remains phytochemically unexplored. Thus far, 200 tropane alkaloids have been isolated and there is no reason to doubt that the search for new compounds will continue in the coming years.

Important efforts are made to develop economically feasible *in vitro* culture techniques. It is important to study methodologies which ensure

optimal conditions for tissue cultures in order to apply *in vitro* techniques to problems such as phytosanitary control, genetic improvement and high alkaloid yields. Unfortunately, the alkaloid content in cell and suspension cultures of solanaceous species have so far been lower than that in intact plants. However, root cultures, especially when *Agrobacterium*-mediated transformation systems are used, may hold some promise for the tropane alkaloid production. In particular, gene transfer methods can be expected to help complete our knowledge of the biosynthetic pathway of tropane alkaloids and gain insights into metabolic regulation which would be difficult to achieve by biochemical or physiological means. The development of industrial technologies and the commercial production of tropane alkaloids by means of hairy root cultures in bioreactors require much effort. A bioreactor in which hairy root growth may be coupled with an on-line product recovery system is a promising perspective but further studies are to be conducted in the fields of bioreactor design and optimization of cultivation parameters.

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