Cell-Free Synthesis of the Alkaloids Ammodendrine and Smipine

Michael Wink

Genzentrum der Universität München, Pharmazeutische Biologie, Karlstr. 29, D-8000 München 2, Bundesrepublik Deutschland

Ludger Witte

Gesellschaft für Biotechnologische Forschung, D-3300 Braunschweig-Stöckheim, Bundesrepublik Deutschland

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The bipiperidyl alkaloid ammodendrine was detected in 28 plant species as a minor alkaloid besides quinolizidine alkaloids. Cadaverine serves as a precursor for both quinolizidine alkaloids and for ammodendrine, since labelled cadaverine is incorporated into both rings of ammodendrine. Cell-free extracts of *Lupinus arboreus* and of *Pisum sativum,* which contain an active diamine oxidase form ammodendrine from cadaverine and pyruvate. In addition to ammodendrine other alkaloids such as smipine, tetrahydroanabasine and tripiperideine could be detected. Possible reaction schemes are discussed.

In *Lupinus formosus* a number of alkaloids were found [1], such as ammodendrine (**I**), N-methylammodendrine, hystrine N-acetylhystrine and smipine (**II**) (Scheme I), which were thought to be untypical for lupins since species of the genus *Lupinus* usually accumulate alkaloids of the quinolizidine type.

We have studied more than 60 plant species for the occurrence of quinolizidine alkaloids by high resolution capillary GLC and GLC–MS during the last 8 years [2–9]. We could unambiguously identify ammodendrine in 28 species (Table I), in many species for the first time. This indicates that ammodendrine is a common alkaloid in plants which contain quinolizidine alkaloids, but it usually figures as a minor alkaloid.

For quinolizidine alkaloids, the diamine cadaverine serves as the main precursor. It is likely that cadaverine is also the precursor for ammodendrine and smipine [1]. In this communication we report on the biosynthesis of ammodendrine and smipine, based on feeding experiments and on the use of cell-free extracts.

Abbreviations: GLC, gas-liquid chromatography; GLC–MS, gas-liquid chromatography – mass spectrometry; M^+, molecular ion.

Reprint requests to PD Dr. M. Wink.

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Materials and Methods

Plant material

Lupinus polyphyllus and *L. arboreus* were kept in the greenhouse or in the experimental garden. *Pisum sativum* seedlings were used at an age of c. 15 days.

Alkaloids. Plant material was stored at −20 °C. It was homogenized in 0.5 M HCl in a Waring blender and left standing at room temperature for 30 min. After filtering off the particulate material, the extract was made alkaline by addition of 6 M NaOH. 20 ml aliquots were applied to standard Extrelute columns (Merck) and the alkaloids were extracted with CH_2Cl_2. The crude alkaloid mixtures were analyzed by capillary GLC and GLC–MS as described previously [2–8].

Preparation of labelled cadaverines

Carbon 14-labelled cadaverine was prepared from [U-^{14}C]lysine by incubation with 100 mg of lysine decarboxylase (in 0.1 M Na phosphate buffer, pH 6, containing 3 mM pyridoxal phosphate) at 37 °C for 5 h. Deuterium labelled 1-D-cadaverine was prepared from L-lysine by incubation with lysine decarboxylase in D_2O.

Cell-free extracts

Enzymes were isolated from fresh material or from acetone powders and purified on Sephadex G 25 (PD 10 columns, Pharmacia). Incubations were performed in 0.1 M borate buffer (pH 8.5) containing labelled cadaverine at 25 °C for 4 h. The reaction

was terminated by the addition of 6 M NaOH. The alkaloids were extracted as described above.

Results and Discussion

Incorporation of cadaverine into ammodendrine

Since ammodendrine occurs in many species which also contain quinolizidine alkaloids (Table I), we tested whether cadaverine, the precursor for both types of alkaloids, was incorporated into ammodendrine by lupin plants.

Leaves of *Lupinus polyphyllus* contain lupanine and other quinolizidine alkaloids [6] besides small amounts of ammodendrine (c. 10–100 μg/g fresh weight). When entire leaves of *Lupinus polyphyllus* are incubated in a solution containing ^{14}C labelled cadaverine for 48 h, radioactive lupin alkaloids can be detected after chromatography of the alkaloid fraction on thin layer plates (Fig. 1): Lupanine accounts for 84%, 13-hydroxylupanine for 7.5%, angustifoline for 3.6%, 13-tigloyloxylupanine for 2.9% and sparteine for 1.3% of the radioactive alkaloids. Only traces of radioactive ammodendrine were observed under these conditions. When deuterated cadaverine is employed, and the incorporation analyzed by capillary GLC and GLC–MS instead, a significant labelling of ammodendrine (Fig. 2b). can be

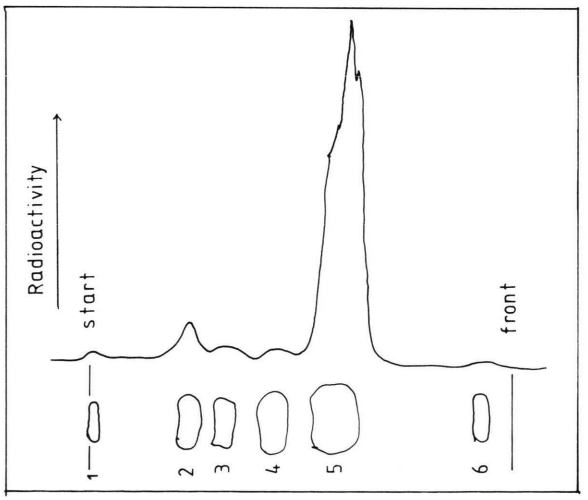


Fig. 1. Incorporation of [U-^{14}C]cadaverine into alkaloids by intact leaves of *Lupinus polyphyllus*. Complete leaves were incubated in 2 μCi cadaverine for 48 h. Alkaloids were extracted and separated by thin layer chromatography using the solvent system cyclohexane/diethylamine (7/3). Radioactivity was recorded by a multichannel analyzer (Berthold). Radioactive peaks correspond to the following alkaloids: 1 = ammodendrine, 2 = 13-hydroxylupanine, 3 = angustifoline, 4 = 13-tigloyloxylupanine, 5 = lupanine, 6 = sparteine.

Table I. Occurrence of ammodendrine. Alkaloid extracts of plants were analyzed by capillary GLC and GLC–MS. Ammodendrine was identified on account of its specific Kovacs-retention index [6] and distinctive mass spectrum. In most instances ammodendrine has not been described previously for the species studied.

Species	Presence of ammodendrine
Lupinus polyphyllus	+
L. albus	+
L. angustifolius	–
L. hartwegii	–
L. pubescens	–
L. mutabilis	+
L. arboreus	+
L. succulentus	–
L. micranthus	–
L. varius	+
L. atlanticus	+
L. palaestinus	+
L. pilosus	–
L. consentinii	+
L. hispanicus	–
L. luteus	+
L. nanus	–
Cytisus scoparius	+
Orobanche rapum-genistae	+
C. beanii	+
C. ingramii	+
C. canariensis	–
C. purpureus	–
Spartium junceum	+
Genista cinerea	+
G. acanthoclada	–
G. tenera	–
G. pilosa	–
G. anglica	–
G. tinctoria	+
G. sagittalis	+
G. hispanica	+
G. lydia	+
Ulex europaeus	–
Anagyris foetida	–
Laburnum anagyroides	+
L. alpinum	+
Thermopsis fabacea	+
Th. chinensis	+
Th. montana	+
Th. divaricarpa	+
Th. lanceolata	+
Th. caroliniana	–
Baptisia australis	+
Sophora tetraptera	+
Pisum sativum	–

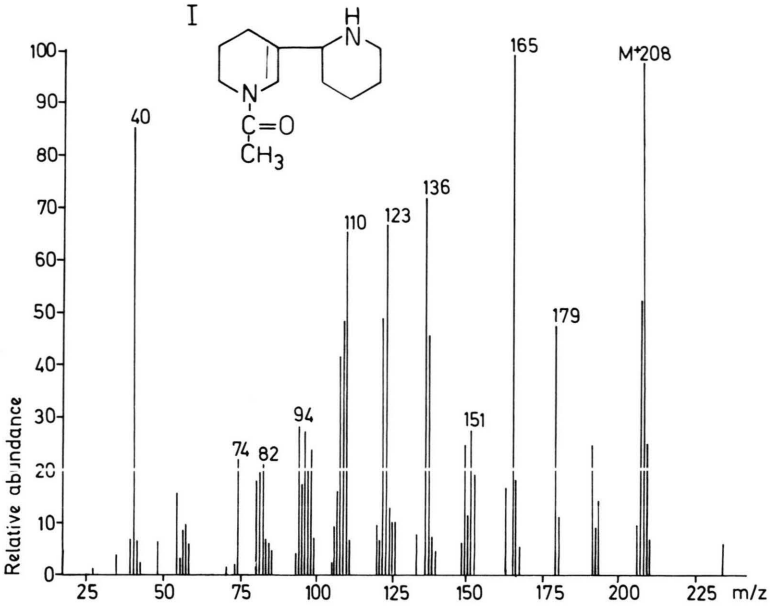
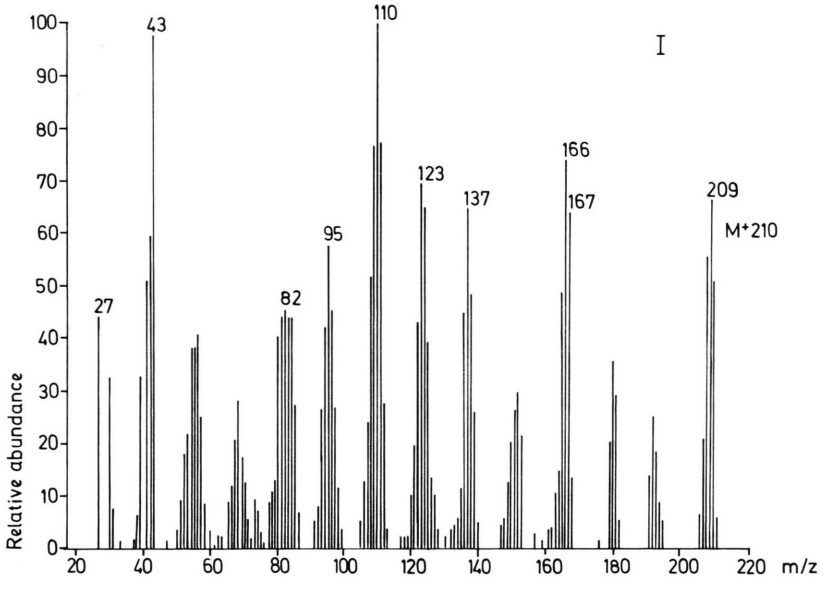
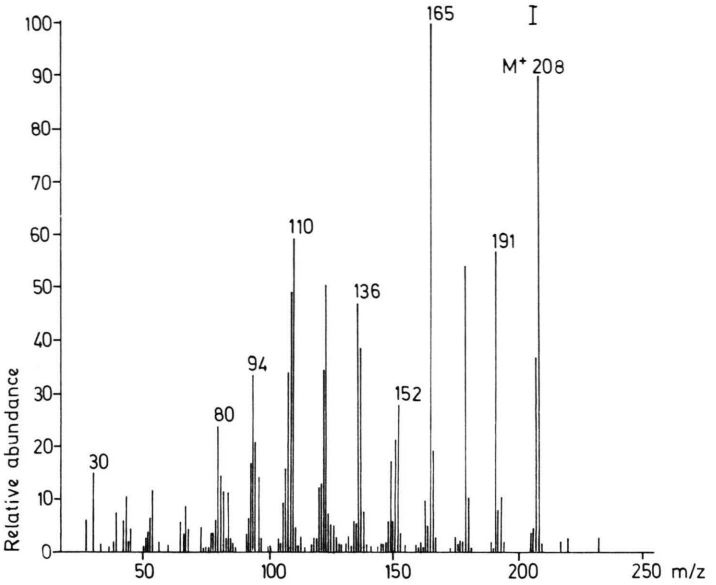


Fig. 2. Incorporation of cadaverine into ammodendrine.
A. Mass spectrum (EI–MS) of endogenous ammodendrine from *Lupinus albus*.

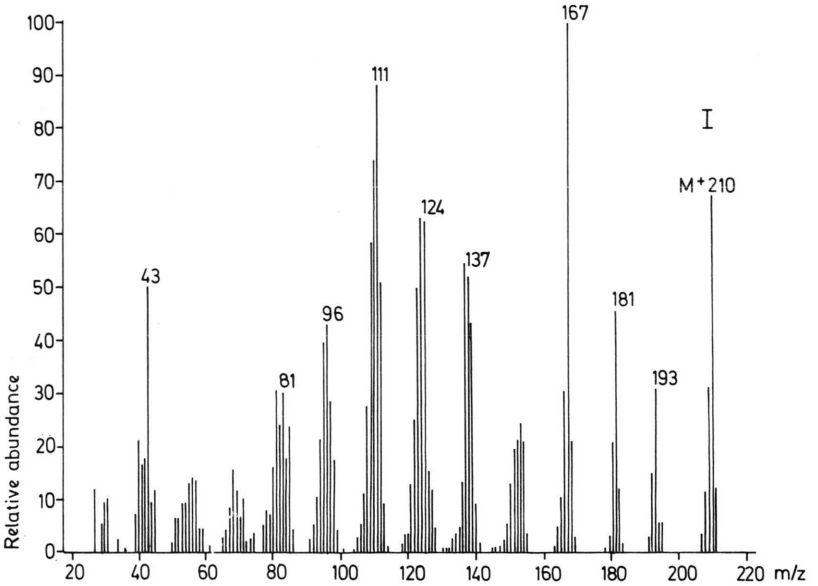


B. Mass spectrum of ammodendrine from *L. polyphyllus* leaves, incubated with 1-D-cadaverine for 48 h.

observed. The endogenous pools of the other alkaloids are obviously too large to allow a labelling of a substantial population of alkaloid molecules. Judging from the respective mass spectra, incorporation of D-cadaverine into lupanine and the other alkaloids accounts for 5–15%. Incorporation of one molecule 1-D-cadaverine into ammodendrine would result in a molecular ion of 209, that of two D-cadaverine molecules in a M^+ of 210. The mass spectrum of ammodendrine shows significant ions both at *m/z* 209 and 210 (Fig. 2b). Therefore, we can assume that both rings of ammodendrine derive from cadaverine.



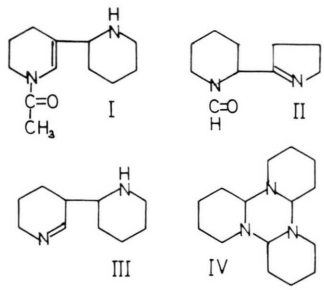
C. Mass spectrum of ammodendrine from cell-free extracts incubated with cadaverine and pyruvate.



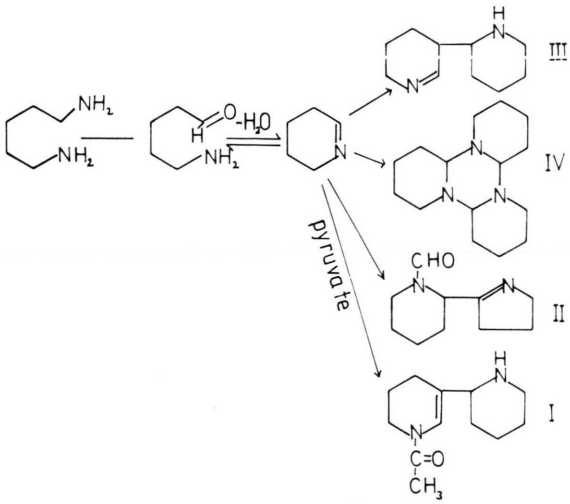
D. As in C, but 1-D-cadaverine was employed.

Cell-free synthesis of ammodendrine and smipine

Ammodendrine can be regarded as an acetyl-derivative of tetrahydroanabasine (Scheme 1). Hasse and Schmidt [10] have reported that tetrahydroanabasine is formed by cellfree extracts of *Pisum sativum*, which is known to contain an active diamine oxidase. Diamine oxidase converts cadaverine into 5-aminopentanal which spontaneously forms Δ^1-piperideine. It is assumed that Δ^1-piperideine polymerizes to tetrahydroanabasine and α-tripiperideine under physiological conditions (Scheme 2).



Scheme 1. Structures of ammodendrine (**I**), smipine (**II**), tetrahydroanabasine (**III**) and α-tripiperideine (**IV**).



Scheme 2. Reaction products of Δ^1-piperideine.

Cell-free extracts of *Lupinus arboreus* and of *Pisum sativum,* which were purified by chromatography on Sephadex G-25 (PD-10 columns), where incubated with cadaverine. After 4 h alkaloids were extracted and analyzed by GLC and GLC–MS. As can be seen from Fig. 3a, only one major peak can be detected besides cadaverine. The corresponding compound could be identified as smipine (Fig. 4), which had been described from *Lupinus formosus.* When we added pyruvate in addition to cadaverine, a second compound was formed, which was identical to ammodendrine (Fig. 2b). When deuterium labelled cadaverine was employed, the corresponding mass spectra showed molecular ions which were two mass units higher than the non-deuterated compounds (Fig. 2D and 4). To our surprise we could not record tetrahydroanabasine or α-tripiperideine which

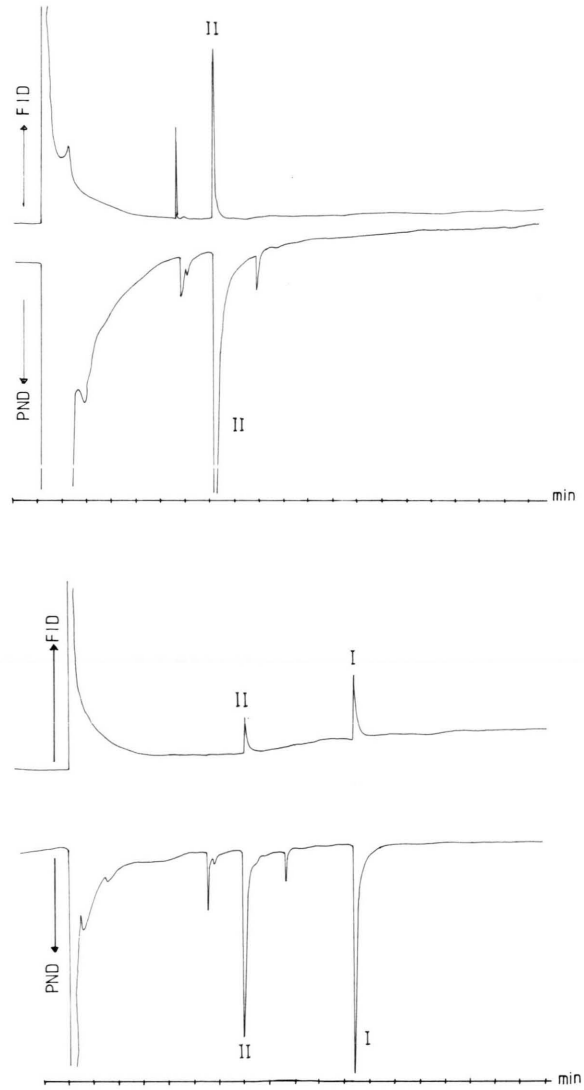


Fig. 3. Cell-free synthesis of smipine and ammodendrine. Alkaloids were extracted from cell-free reaction mixtures *(Pisum sativum),* separated by capillary GLC and analyzed by flame ionization (FID) and nitrogen specific detectors (PND).
A. Incubation with cadaverine. II = smipine.
B. Incubation with cadaverine and 10 mM pyruvate. I = ammodendrine, II = smipine.

should have formed according to Hasse and Schmidt [10]. To check whether these compounds are in fact synthesized, alkaloids were extracted after incubation and were then directly analyzed by fast atom bombardment mass spectrometry (FAB–MS). Δ^1-Piperideine was most abundant followed by am-

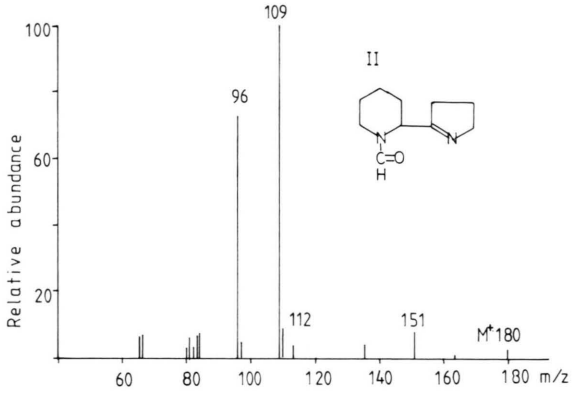
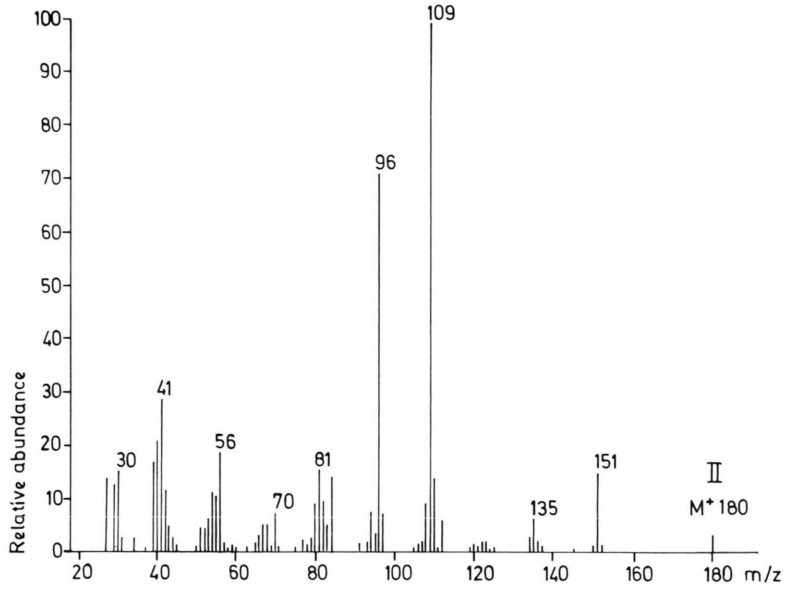
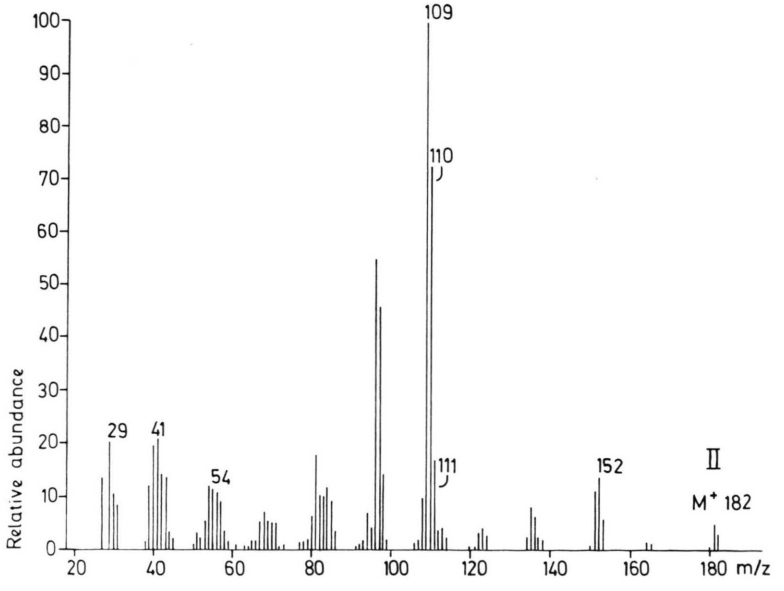


Fig. 4. Incorporation of cadaverine into smipine.
A. Mass spectrum of smipine from *Lupinus formosus* [1].



B. Mass spectrum of smipine from cell-free extracts of *Pisum sativum* incubated with cadaverine.



C. As in B, but 1-D-cadaverine was employed instead.

Table II. Identification of the reaction products from cell-free extracts of *Pisum sativum* incubated with cadaverine and pyruvate by fast atom bombardment – mass spectrometry. A. experiments with unlabelled cadaverine, B. with 1-D-cadaverine.

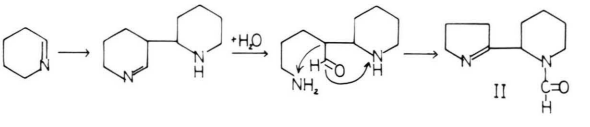
Compound	Expected $(M+H^+)$		Observed $(M+H^+)$		Intensity
	A	B	A	B	
Δ_1-piperideine	84	85	84	85	++++++
Ammodendrine	209	211	209	211	+++
Smipine	181	183	181	183	+
Tetrahydro-anabasine	167	169	167	169	++
Tripiperideine	250	253	250	–	+

modendrine (when pyruvate was included in the assay), tetrahydroanabasine, smipine and tripiperideine (Table II). In a corresponding experiment with 1-D-cadaverine, the compounds were seen again, but their molecular ion was 1 or 2 mass units higher now. We can conclude from this experiment, that tetrahydroanabasine and tripiperideine are indeed reaction products as found by Hasse and Schmidt [10], but both alkaloids are probably discriminated in the GLC-analysis because of their reduced volatility. However, smipine and ammodendrine seem to be more abundant and have probably been overlooked by previous researchers.

Hypothetical reaction mechanisms

The first step in the biosynthesis of the bipiperidyl alkaloids will certainly be the desamination of cadaverine to 5-aminopentanal which is in equilibrium with Δ^1-piperideine (Scheme 2). This step can be either catalyzed by diamine oxidase or by transaminase. Both enzymes are present in lupins [11]. The formation of ammodendrine could be inhibited in this study by addition of the copper chelator diethyldithiocarbamate or by anaerobic incubation. Thus, diamine oxidase was probably the enzyme involved here. Under physiological conditions, a spontaneous dimerization to tetrahydroanabasine or trimerization to α-tripiperideine occurs [10]. Tetrahydroanabasine probably functions as a precursor for ammodendrine and smipine.

In the course of ammodendrine biosynthesis we have to explain the acetylation of N-1. Acetylation reactions usually require acetyl-CoA. Since low molecular weight compounds such as ATP and CoA were not present in the reaction mixture, acetyl-CoA cannot be an intermediate. However, the cell-free extract probably contains the pyruvate dehydrogenase complex. In this enzyme complex pyruvate is decarboxylated after binding to thiamine pyrophosphate and transferred to enzyme bound lipoamide to yield S-acetyl-dihydrolipoamide. The acetyl group forms an energy-rich thioester. This activated acetyl group might then be transferred to the nitrogen of the tetrahydroanabasine molecule to form ammodendrine. The pyruvate dehydrogenase reaction requires NAD^+ for pyruvate oxidation which was not present in the cell-free extracts. Therefore, this reaction scheme is rather speculative and demands further investigation. In nature usually the (+) enantiomer of ammodendrine is present. It is not clear at present, whether the enzymatic reaction product is a racemic mixture or whether it is enantiomerically pure. Smipine could derive from tetrahydroanabasine by the addition of a water molecule, followed by ring opening. After rearrangements smipine would easily form under physiological conditions (Scheme 3). It is possible that this step does not require a specific enzyme but occurs spontaneously. Smipine was found to be optically inactive [1] probably due to enamine imine tautomerism. Thus it is not possible to check whether its formation is enzyme catalyzed or not.



Scheme 3. Hypothecical pathways of smipine (**II**) formation by cell-free extracts.

Ammodendrine or smipine could not be detected in extracts of *Pisum sativum* seeds or leaves using capillary GLC and GLC–MS (Table I), although *Pisum* contains the enzymes required to form these alkaloids. This could mean that the reaction found *in vitro* is an artificial pathway and does not take place *in vivo* in the same fashion. However, the labelling pattern of the enzymatically formed ammodendrine was identical with that synthesized in the intact lupin leaf (Fig. 2), making this assumption less likely. Alternatively, it could be argued that *Pisum* actually makes these alkaloids, but does not store but degrades them instead. Since specific transport molecules are a requirement for alkaloid storage in the vacuole [12, 13] and since these carriers are gene coded, a lack in the expression of the storage genes would also lead to the situation observed.

The data presented in this study provide some evidence how the biosynthesis of ammodendrine and smipine could take place. More data obtained from purified enzymes are necessary to see whether the hypothetical schemes are correct.

Acknowledgements

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