On the Role of Δ1-Piperideine and Tripiperideine in the Biosynthesis of Quinolizidine Alkaloids

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Carbon 14-labelled cadaverine, Δ1-piperideine and α-tripiperideine were administered to leaf disks of *Lupinus polyphyllus* in short term experiments of 12–20 h duration. Whereas cadaverine was readily incorporated into lupanine in good yields, a 7 to 60 times lower incorporation was observed for tripiperideine or Δ1-piperideine. In *L. arboreus* the incorporation of cadaverine into sparteine was 9 times better than that of Δ1-piperideine. These results indicate that Δ1-piperideine and tripiperideine are not direct intermediates of lupanine or of sparteine biosynthesis.

Introduction

Quinolizidine alkaloids (QA) are important secondary compounds of legumes (Fabaceae), especially of lupins (Genus *Lupinus*) and of broom (Genus *Cytisus, Sarothamnus, Genista*). Lupanine and sparteine are considered to be the main alkaloids from which most of the other QA are derived [1–3]. Mothes, Schütte and coworkers established that the amino acid lysine serves as the sole precursor (for review see [1, 4]). Lysine is decarboxylated to cadaverine, a reaction catalyzed by lysine decarboxylase [5, 6]. It was demonstrated also by Mothes, Schütte, Nowacki and coworkers that cadaverine is a direct precursor of QA, such as sparteine, lupanine, 13-hydroxylupanine, matrine, cytisine and lupinine [1]. These results could be confirmed and further elaborated by Golebiewski, Spenser and Robins and coworkers in elegant experiments using deuterium and carbon-13 labelled cadaverines which allowed to study their incorporation into QA by deuterium- and ^{13}C NMR techniques [7–19].

Golebiewski and Spenser [7, 11] have formulated a biogenetic scheme for sparteine and lupanine biosynthesis (Fig. 1) which assumes Δ1-piperideine and tripiperideine as intermediates.

In this communication experimental evidence is presented that Δ1-piperideine and tripiperideine are not or at least not significantly incorporated into QA, indicating that both compounds are probably not intermediates of QA biosynthesis.

Abbreviations: GLC, gas-liquid chromatography; MS, mass spectrometry; EI, electron impact; CI, chemical ionization; FAB, fast atom bombardment; QA, quinolizidine alkaloids; TLC, thin-layer chromatography.

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Materials and Methods

Plants

Leaves of *Lupinus polyphyllus* were taken from a naturalized population near Grafrath/München, those of *L. arboreus* from plants kept in the greenhouse.

Feeding experiments

Several leaflets of *L. polyphyllus* were cut into small pieces of 2–4 mm width as described in [20]. After mixing the leaf "disks", so that they formed a homogenous population, about 400 mg each were suspended in 5 ml tap water in a 100 ml Erlenmeyer flask. After adding a few µl of radioactive tracers, the flasks were incubated at room temperature (20–23 °C) under natural light conditions (8 h darkness) for 12 to 20 h.

For alkaloid extraction the leaf disks were collected and homogenized in 15 ml 0.5 M HCl with mortar and pistil. After leaving the homogenate standing for 30 min at room temperature, it was made alkaline with 6 N NaOH and applied onto Chemelute columns (ICT, Frankfurt). Alkaloids were eluted with methylene chloride and concentrated *in vacuo*. The crude alkaloid mixtures were separated by thin-layer chromatography on silica plates (Merck 60 F 254) using cyclohexane/diethylamine (7/3 or 9/1) as solvents. Radioactivity was monitored with a TLC scanner of Berthold (Wildbad) or by liquid scintillation counting. Alkaloids

were visualized on TLC plates with Dragendorff's reagent.

Preparation of reference alkaloids

Synthesis of 2,3,4,5-tetrahydropyridine trimer (α-tripiperideine)

The following preparation is a slightly modified version of Schöpf *et al.* [21]: 5.7 ml acetic acid (100 mmol) was added dropwise to 9.9 ml piperidine (100 mmol) with ice-cooling and stirring. During the reaction 5 ml water was added to keep the solution fluid. This solution was added dropwise to 250 ml sodium hypochlorite solution (about 13% active chlorine) under cooling and stirring. After 2 h the mixture was extracted with methylene chloride. The organic phase was dried with sodium sulfate and the solvent was evaporated to 50 ml. This solution was added dropwise to stirred and refluxed ethanolic potassium hydroxide (11.2 g; 200 mmol) and the reaction was continued for 2 h. The reaction was allowed to cool to room temperature. The precipitated potassium chloride was filtered off and the ethanol was evaporated. The resulting residue was dissolved in 40 ml 1 N NaOH, extracted with methylene chloride and dried $NaSO_4$. After evaporation of the solvent, the residue was allowed to stand at room temperature for crystallization. The product was recrystallized twice from acetone to give 19.8 g (60%) α-tripiperideine, which was characterized as following:

M.p.: 58–60 °C.

^{13}C NMR ($CDCl_3$): δ (ppm) = 81.8; 46.2; 29.0; 25.6; 22.1; (similar to the data reported in [22]).

Mass spectra (EI, CI, FAB), which have not been documented in detail are illustrated in Fig. 3.

Preparation of isotripiperideine

Synthesis was performed according to [23]: 1 g (4 mmol) tripiperideine was added to 50 ml 0.01 mol/l Tris/HCl (pH 7.5) and stirred at room temperature for 5 h. Then 13 g potassium carbonate was added, completely dissolved and extracted with methylene chloride. The organic solvent was dried with $NaSO_4$ and evaporated, resulting in a crystalline residue. This product was recrystallized twice from acetone to give 0.77 g (77%) isotripiperideine which was identified by ^{13}C NMR ($CDCl_3$) according to [22]: δ (ppm): 81.8; 81.0; 64.0; 48.0; 47.7; 45.5; 43.6; 29.6; 28.5; 27.5; 26.3; 26.0; 25.2; 23.4; 23.3.

Chemical synthesis of radioactive precursors

Synthesis of 2-(3-chloropropyl)-1,3-dioxalane

The following preparations are a modified version of the method of Roffey *et al.* [24]: A solution of 14.1 g (100 mmol) 4-chlorobutyryl chloride in 50 ml toluene was hydrogenated with 10 g palladium (on barium sulfate; reduced palladium content 5%) at room temperature for 10 h. The mixture was then filtered and the filtrate was added to a solution of 25 ml glycol and 0.5 ml concentrated sulfuric acid. After refluxing for 12 h, the toluene was evaporated and 7 g (35%) 2-(3-chloropropyl)-1,3-dioxalane was obtained by fractional distillation.

^1H NMR ($CDCl_3$): δ (ppm) = 4.90 (t, 1H); 3.95 (m, 2H); 3.85 (m, 2H); 3.59 (t, 2H); 1.90 (m, 2H); 1.8 (m, 2H).

^{13}C NMR ($CDCl_3$): δ (ppm) = 103.7; 64.9 (2C); 44.8; 31.0; 26.9.

Synthesis of 2-(3-cyanopropyl)-1,3-dioxalane

A mixture of 1 g (6.6 mmol) 2-(3-chloropropyl)-1,3-dioxalane and 1 g (15.4 mmol) potassium cyanide in 10 ml DMF (dimethylformamid) was stirred at 50 °C for 2 d. The mixture was filtered and 0.5 g (54%) 2-(3-cyanopropyl)-1,3-dioxalane was recovered from the filtrate by fractional distillation.

^1H-NMR ($CDCl_3$): δ (ppm) = 4.9 (t, 1H); 3.95 (m, 2H); 3.85 (m, 2H); 2.45 (t, 2H); 1.82 (m, 4H).

^{13}C NMR ($CDCl_3$): δ (ppm) = 119.2; 103.2; 64.7 (2C); 32.1; 19.7; 16.8.

MS (EI, 70 eV): m/z = 140 (45); 96 (35); 73 (100); 45 (55).

Synthesis of 2-(4-aminobutyl)-1,3-dioxalane

A solution of 0.5 g (3.5 mmol) 2-(3-cyanopropyl)-1,3-dioxalane in 25 ml diethylether was added dropwise to 133 mg (3.5 mmol) lithium aluminum hydride in 10 ml diethylether. After refluxing for 2 h, the mixture was stirred at room temperature for 2 d. The excess hydride was decomposed with 0.5 ml water and then the mixture was filtered. The solvent was evaporated and 0.36 g (71%) 2-(4-aminobutyl)-1,3-dioxalane was obtained by fractional distillation:

^1H NMR ($CDCl_3$): δ (ppm): 4.9 (t, 1H); 3.95 (m, 2H); 3.85 (m, 2H); 2.7 (t, 2H); 1.8–1.5 (m, 8H).

^{13}C NMR ($CDCl_3$): δ (ppm) = 104.5; 64.8 (2C); 42.0; 33.6 (2C); 21.3.

Synthesis of [^{14}C]Δ1-piperideine hydrochloride

A mixture of 0.7 g (4.6 mmol) 2-(3-chloropropyl)-1,3-dioxalane, 0.7 g (10.8 mmol) potassium cyanide and 37 MBq (300 mg) [^{14}C]potassium cyanide in 10 ml DMF was stirred at 50 °C for 2 d. The mixture was filtered and the DMF was evaporated giving 2-(3-cyanopropyl)-1,3-dioxalane (15.9 MBq; 43% radiochemical yield). This reaction product was dissolved in 25 ml diethylether and added dropwise to 100 mg (2.6 mmol) lithium aluminum hydride in 10 ml diethylether. After refluxing for 2 h, the mixture was stirred at room temperature for 2 d. Excess hydride was decomposed with 0.5 ml water, the mixture filtered and the filtrate extracted with 2 N hydrochloric acid (three 30 ml portions). The acidic extract was evaporated at 30 °C to give 110 mg (20%, 1.4 MBq, 4% radiochemical yield) [^{14}C]Δ1-piperideine hydrochloride.

Synthesis of [^{14}C]α-tripiperideine

A solution of 11 mg (93 μmol; 148 KBq) [^{14}C]Δ1-piperideine hydrochloride in 0.15 ml water was added to 112 mg (2 mmol) KOH in 5 ml ethanol. After refluxing for 15 min, the mixture was allowed to cool to room temperature. The precipitated KCl was filtered off and the ethanol was evaporated. The residue was dissolved in 2 ml 1 N NaOH and extracted with methylene chloride. After evaporating the solvent, [^{14}C]α-tripiperideine (3.1 mg; 28% yield; 59 KBq) was obtained by crystallisation from 0.5 ml acetone at −20 °C. Its identity was confirmed by ^{13}C NMR and by chromatographic comparison with authentic material (see above).

Enzymatic synthesis of radioactive precursors

Preparation of [^{14}C]cadaverine

[^{14}C-U]L-lysine (37 MBq; 2.6 μmol) was dissolved in 5 ml phosphate buffer (0.1 mol/l; pH 6.5) and decarboxylated with the aid of lysine decarboxylase (Sigma, München) at 37 °C for 5 h. This solution was then dispensed into 500 μl aliquots and stored at −20 °C.

Preparation of [^{14}C]Δ1-piperideine

[^{14}C]cadaverine (370 KBq; 26 nmol) was added to 500 μl phosphate buffer (0.1 mol/l; pH 7.5) containing diamino oxidase (DAO) purified from *Pisum sativum* (DAO was kindly provided by J. Thuhmann and M. H. Zenk). After 8 h at 37 °C the reaction mixture was transferred to −20 °C. TLC analysis revealed a complete deamination of cadaverine.

Spectroscopy

Mass spectra (EI, CI, FAB) were recorded on a Finnigan MAT 8430; NMR spectra on a Bruker AM 360.

Results and Discussion

According to the hypothesis of Golebiewski and Spenser [7] Δ1-piperideine and its trimer tripiperideine should be precursors of QA biosynthesis. If this assumption is correct we should expect that both compounds are incorporated into QA in a similar or even better way than cadaverine which comes prior to both compounds in the biogenetic sequence (Fig. 1). As experimental system we have

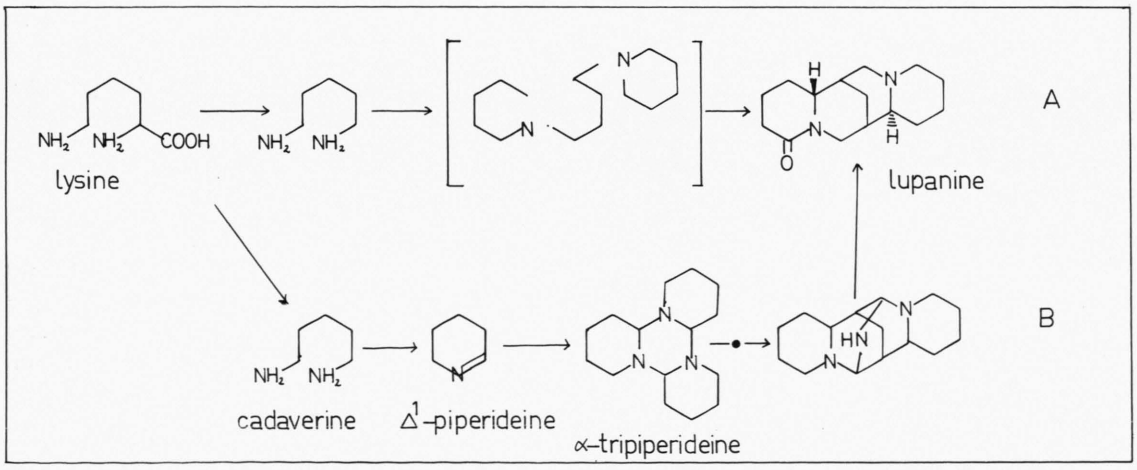

Fig. 1. Schemes of quinolizidine alkaloid biosynthesis A. according to [1], B. [7, 11].

chosen leaf "disks" of *Lupinus polyphyllus*. Because of their small size a precursor can easily be taken up by the cells. Furthermore, we have shown previously that the incorporation of [^{14}C]cadaverine into lupanine proceeds rapidly with a maximum after 8–16 h [25]. Especially these short term experiments in which only lupanine becomes labelled as the first key alkaloid should be best suited for the proposed study.

Since carbon 14-labelled precursors, such as Δ1-piperideine, α-tripiperideine, and isotripiperideine are not available commercially, the respective compounds were synthesized chemically by methods modified from those described in [21, 23]. [^{14}C]Cadaverine was prepared enzymatically from [^{14}C]lysine.

In a first set of experiments leaf disks of *L. polyphyllus* were incubated with either 14 KBq [^{14}C]cadaverine (1 nmol) (Fig. 2a) or 15 KBq [^{14}C]piperideine (9.3 μmol) for 12 h (Fig. 2b). As can be seen from Fig. 2 cadaverine is readily incorporated into lupanine, whereas tripiperideine be-

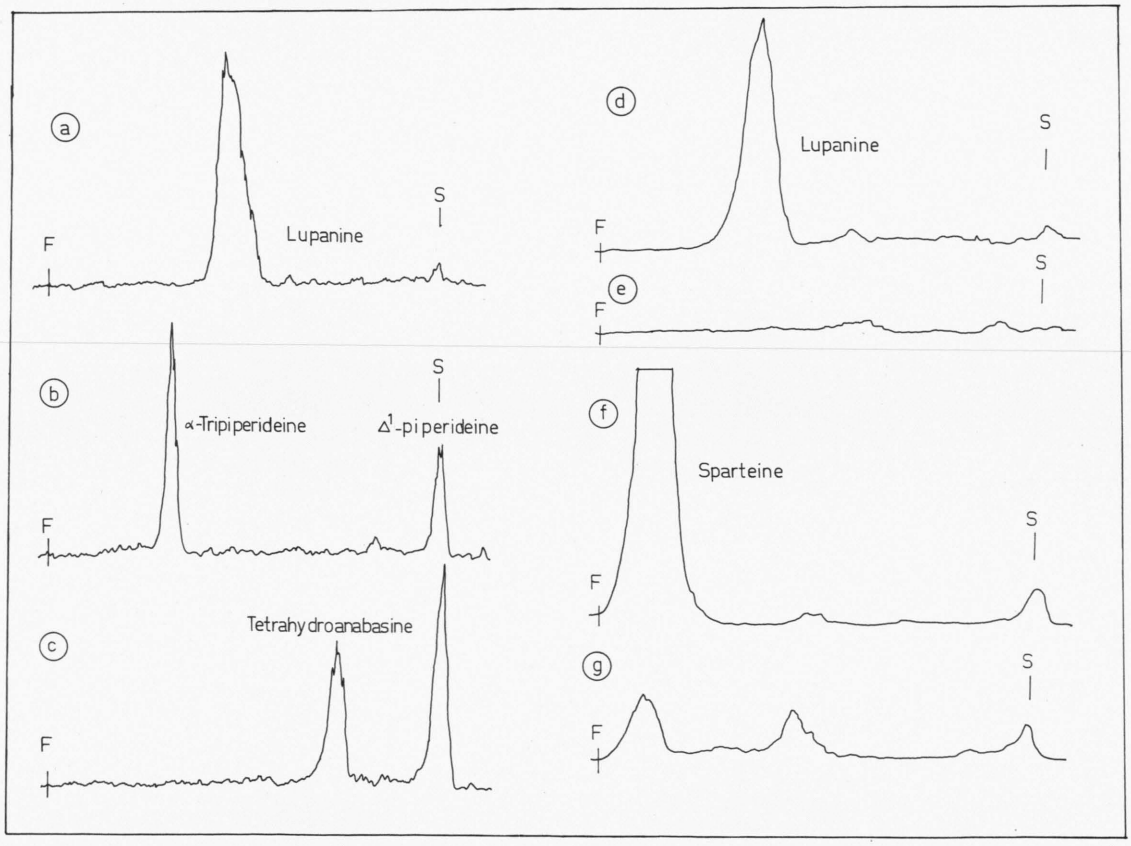

Fig. 2. Biosynthesis of lupanine and of sparteine in leaf disks of *Lupinus polyphyllus* and *L. arboreus*. Leaf disks were incubated with radioactive precursors as described in Materials and Methods. Alkaloid were extracted and separated by TLC. The radioactivity of the alkaloids was recorded by a TLC-scanner. The scanner was operated at the same sensitivity in corresponding experiments (a–c, d, e, and f, g).
a) Precursor: 14 KBq [^{14}C]cadaverine (1 nmol)
b) Precursor: 15 KBq [^{14}C]Δ1-piperideine (9.3 μmol)
c) Precursor: 15 KBq [^{14}C]α-tripiperideine (3.1 μmol)
d) Precursor: 18 KBq [^{14}C]cadaverine (1.3 nmol)
e) Precursor: 18 KBq [^{14}C]Δ1-piperideine (1.3 nmol; enzymatic synthesis)
f) Precursor: 37 KBq [^{14}C]cadaverine (2.6 nmol)
g) Precursor: 37 KBq [^{14}C]Δ1-piperideine (2.6 nmol; enzymatic synthesis)
400 mg leaf disks of *L. polyphyllus* (a–e) or *L. arboreus* (f, g) were used per flasks containing 5 ml tap water. Incubation was 12 h at room temperature over night. TLC conditions: cyclohexane/diethylamine (9/1) (a–c) or (7/3) d–g.

came labelled in the piperideine experiment. These results were reproduced in two further experiments. One could argue that the specific radioactivity of our chemically synthesized Δ1-piperideine was too low to give a good incorporation into lupanine.

This objection was tested by applying 17 KBq [^{14}C]Δ1-piperideine (1.3 nmol) which was prepared enzymatically from [^{14}C]cadaverine with the aid of diamino oxidase (Fig. 2e; for control compare 2 d). In this case both precursors, *i.e.* cadaverine and Δ1-piperideine had almost the same specific and absolute radioactivity. We have compared the incorporation of cadaverine and of Δ1-piperideine into lupanine in 9 further experiments (Table I): The radioactivity in lupanine (which was isolated from the TLC-plate and evaluated by liquid scintillation counting) was always 7 to 50 times higher after the application of cadaverine as compared to that of Δ1-piperideine.

Preliminary data with *Lupinus arboreus*, which converts [^{14}C]cadaverine into sparteine [25] show (Fig. 2f, g), that the application of identical amounts of cadaverine or of Δ1-piperideine resulted in a 9-fold better incorporation in sparteine in case of cadaverine.

In a second set of experiments we applied 15 KBq [^{14}C]α-tripiperideine (3.1 μmol) and for comparison 15 KBq [^{14}C]cadaverine (1 nmol) to leaf disks of *L. polyphyllus*. Almost no incorporation of the α-tripiperideine into lupanine was observed, however a transformation into tetrahydroanabasine and Δ1-piperideine (Fig. 2c). (For mechanisms see [7, 11].)

In a pilot experiment unlabelled α-tripiperideine was applied to cell suspension cultures of *L. polyphyllus*, which convert precursors or intermediates into lupanine [26]. The amount of lupanine or any other QA (as determined by capillary GLC) was unchanged after 6 d *in vitro* as compared to untreated controls, indicating that no conversion of α-tripiperideine took place, *i.e.* that it does not function as precursor of lupanine biosynthesis.

How can we interpret these experimental results, which seem to contradict the assumption of Golebiewski and Spenser [7, 11]? There are some experimental differences to be recalled. Golebiewski and Spenser [7, 9] applied about 2.1 MBq [^{14}C]Δ1-piperideine (48 MBq per mmol) to several plants by the wick method and they waited for 3 days before they analyzed the incorporation into QA. Using this procedure they found an incorporation of piperideine into QA in *Lupinus angustifolius* and *Sophora tetraptera* and *S. microphylla* but they did not compare the incorporation rates with those of cadaverine. From their finding they concluded that Δ1-piperideine was an obligatory intermediate of QA biosynthesis and they formulated the hypothesis outlined in Fig. 1b. In view of our experiments presented in this paper we conclude, that cadaverine is the true precursor and intermediate and that piperideine may serve as *distinct* precursor but not as an intermediate. From enzymic studies it is likely that cadaverine is deaminated to 5-aminopentanal by a transaminase [26] and the biosynthesis proceeds *via* bound intermediates. It is possible that piperideine is channelled into the correct biosynthetic sequence by a side way, *i.e. via* 5-aminopentanal, (with which Δ1-piperideine is in equilibrium), which could than bind to the enzyme complex.

Table I. Incorporation of cadaverine and of Δ1-piperideine into lupanine by leaf disks of *L. polyphyllus*. Experimental procedures as in Fig. 2. Lupanine, which was visualized by Dragendorff's reagent on the TLC-plates was isolated and its radioactivity was determined by liquid scintillation counting. 9 separate charges of leaves were used in these experiments. For each series the incorporation of [^{14}C]cadaverine (cad.) and that of [^{14}C]piperideine (pip.) was determined independently. The absolute and specific radioactivity of both precursors was identical within each series.

Experiment	Radioactivity of precursor [KBq/nmol]	of Lupanine [cpm]
1.	Cad. 37 KBq/2.6 nmol	67056
	Pip. 37 KBq/2.6 nmol	5000
2.	Cad. 37 KBq/2.6 nmol	70826
	Pip. 37 KBq/2.6 nmol	4600
3.	Cad. 18 KBq/1.3 nmol	49000
	Pip. 18 KBq/1.3 nmol	6800
4.	Cad. 18 KBq/1.3 nmol	42814
	Pip. 18 KBq/1.3 nmol	4800
5.	Cad. 18 KBq/1.3 nmol	19016
	Pip. 18 KBq/1.3 nmol	332
6.	Cad. 18 KBq/1.3 nmol	34720
	Pip. 18 KBq/1.3 nmol	1036
7.	Cad. 37 KBq/2.6 nmol	76690
	Pip. 37 KBq/2.6 nmol	7880
8.	Cad. 37 KBq/2.6 nmol	80310
	Pip. 37 KBq/2.6 nmol	7790
9.	Cad. 18 KBq/1.3 nmol	36000
	Pip. 18 KBq/1.3 nmol	3554

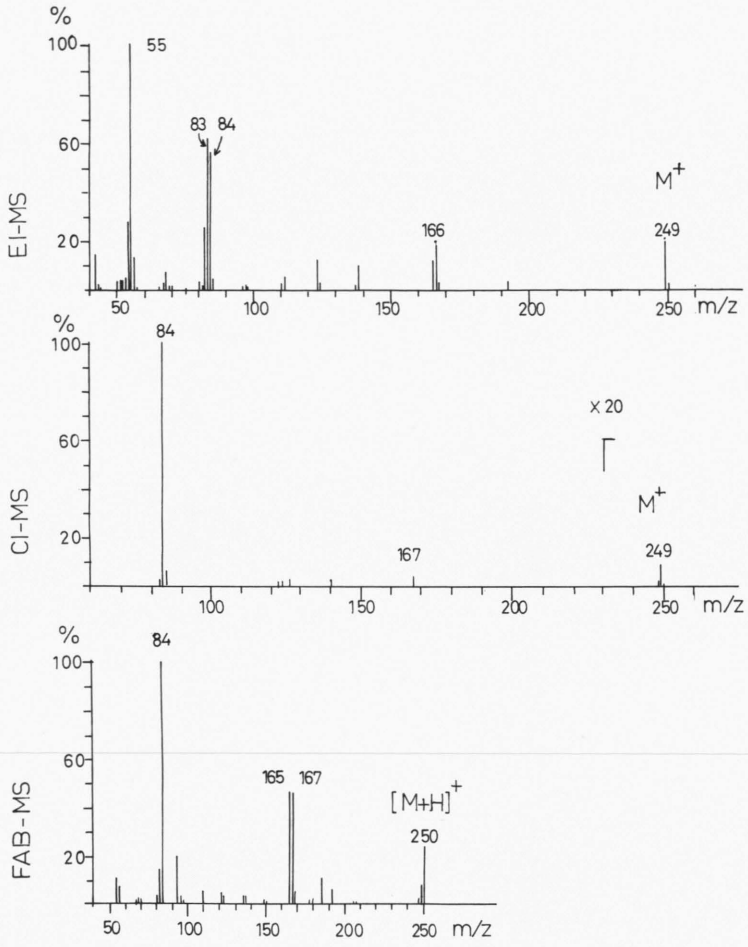

Fig. 3. Mass spectra (EI, CI, FAB-MS) of α-tripiperideine.

It could be argued that the incorporation of piperideine and tripiperideine is so low because both compounds might not be taken up by the cells and thus do not reach the site of biosynthesis. Our experimental data show, however, that all three compounds are taken up by the cells to a similar degree: Aliquots of the incubation mixture were removed after 16 h and their radioactivity was analysed by liquid scintillation counting. Thus, it could be determined that cadaverine, piperideine and tripiperideine were taken up by 52%, 49% and 67%, respectively.

Summarizing the experimental evidence, we think that it is unlikely that Δ1-piperideine and its trimers are direct intermediates of QA biosynthesis but that Δ1-piperideine may serve as a distinct precursor to some degree.

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[1] K. Mothes and H. R. Schütte, Biosynthese der Alkaloide, VEB Berlin, 1969.
[2] A. D. Kinghorn and M. F. Balandrin, in: Alkaloids: Chemical and biological perspectives (W. S. Pelletier, ed.), p. 105, Wiley, New York 1984.
[3] M. Wink, Planta Med. **53,** 509 (1987).
[4] K. Mothes, H. R. Schütte, and M. Luckner, Biosynthesis of alkaloids, Verlag Chemie, Weinheim 1985.
[5] T. Hartmann, G. Schoofs, and M. Wink, FEBS lett. **115,** 35 (1980).
[6] G. Schoofs, S. Teichmann, T. Hartmann, and M. Wink, Phytochemistry **22,** 65 (1982).
[7] W. M. Golebiewski and I. D. Spenser, J. Am. Chem. Soc. **98,** 6726 (1976).
[8] W. M. Golebiewski and I. D. Spenser, J. Chem. Soc. Chem. Comm. **1983,** 1509.
[9] F. J. Leeper, G. Grue-Sorensen, and I. D. Spenser, Can. J. Chem. **59,** 106 (1981).
[10] W. M. Golebiewski and I. D. Spenser, J. Am. Chem. Soc. **106,** 1441 (1984).
[11] I. D. Spenser, Pure Appl. Chem. **57,** 453 (1985).
[12] J. Rana and D. J. Robins, J. Chem. Soc. Chem. Comm. **1983,** 1335.
[13] J. Rana and D. J. Robins, J. Chem. Soc. Chem. Comm. **1984,** 81.
[14] A. M. Fraser and D. J. Robins, J. Chem. Soc. Chem. Comm. **1986,** 545.
[15] J. Rana and D. J. Robins, J. Chem. Res. **1985,** 196.
[16] J. Rana and D. J. Robins, J. Chem. Soc. Perkin Trans. **1986,** 1133.
[17] D. J. Robins and G. N. Sheldrake, J. Chem. Res. (S) **1987,** 159.
[18] T. Hemscheidt and I. D. Spenser, Can. J. Chem. **65,** 170 (1987).
[19] W. M. Golebiewski and I. D. Spenser, Can. J. Chem. **63,** 2707 (1985).
[20] M. Wink, Z. Naturforsch. **38c,** 905 (1983).
[21] C. Schöpf, A. Komzak, F. Braun, and E. Jacobi, Ann. Chem. **559,** 1 (1948).
[22] H. Kessler, H. Möhrle, and G. Zimmermann, J. Org. Chem. **42,** 66 (1977).
[23] C. Schöpf, H. Arm, and F. Braun, Chem. Ber. **85,** 937 (1952).
[24] P. Roffey and M. V. Sargent, J. Chem. Soc. (C) **1967,** 2328.
[25] M. Wink, Z. Naturforsch. **42c,** 868 (1987).
[26] M. Wink and T. Hartmann, in: Natural products chemistry 1984 (R. I. Zalewski and J. J. Skolik, eds.), p. 511, Elsevier, Amsterdam 1985.