Biosynthesis of Anosmine: Incorporation of the Intact Six-Carbon Chain of Lysine and of Pipecolic Acid

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BIOSYNTHESIS OF ANOSMINE: INCORPORATION OF THE INTACT SIX-CARBON CHAIN OF LYSINE AND OF PIPECOLIC ACID[1]

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ABSTRACT.—Label from DL-[1,2-$^{13}C_2$]lysine enters the two carbon atoms, C-7 and C-6, of the orchid alkaloids anosmine [**6**] as an intact C-2 unit. This result provides further evidence for the derivation of the alkaloid from two molecules of lysine [**1**]. One of these lysine units enters via cadaverine [**3**] and Δ^1-piperideine [**5**], supplying the C_5N-unit, C-13 to C-9,N-8, of anosmine, while the other generates the fragment, N-1,C-2 to C-7, by providing a C_6N-chain, which enters by way of pipecolic acid [**4**], since ^{13}C from DL-[carboxyl-^{13}C,(*RS*)-6-$^{2}H_1$]pipecolic acid is incorporated into C-7 of the alkaloid. The complete assignment of the signals in the ^1H and ^{13}C spectra of the alkaloid is discussed.

The alkaloid anosmine (CAS Registry No. 18813-72-4, 1,2,3,4,6,7,8,9-octahydrodipyrido[1,2-*a*: 1′,2′-*c*]imidazol-10-ium bromide) [**6**] was isolated by Leander and Lüning (1) from *Dendrobium anosmum* Lindl. and from *Dendrobium parishii* Rchb. f. (Orchidaceae). The structure of anosmine was confirmed by synthesis (1) and by X-ray crystallography (2).

Recently we presented evidence that anosmine is derived biosynthetically from lysine (3). Whereas label from bond-labelled DL-[6-^{13}C,6-^{15}N]lysine is incorporated into both nuclei of anosmine, label from bond-labelled [1,5-(^{13}C,^{15}N)$_2$]cadaverine enters only one of them. We postulated that the alkaloid skeleton is derived in toto from two molecules of lysine, one of which, after decarboxylation to cadaverine, supplies a C_5N chain, while the other supplies an intact C_6N chain (Scheme 1). We now present further evidence in support of this view.

An aqueous solution of DL-[1,2-$^{13}C_2$]lysine (125 mg, 99% ^{13}C, Isotec Inc., Miamisburg, Ohio) was administered by wick to six green shoots of three plants of *D. parishii* over a period of 3 weeks (July 1992). The fed shoots were cut off, dried, and extracted as described (3), yielding anosmine bromide (91 mg).

The 125 MHz ^{13}C-nmr spectrum of this sample shows satellites (^1J=76 Hz, 0.2% enrichment above natural abundance) at the signals appearing at δ 117.2 and 131.2 ppm (Figure 1), indicating transfer of an intact ^{13}C-^{13}C bond, from C-1–C-2 of DL-lysine into the bond C-6–C-7 of the alkaloid. Signal assignment was based on heteronuclear ^1H-^{13}C and homonuclear ^1H-^1H shift correlation spectra. Two heteronuclear spectra were recorded, one optimized for the detection of one-bond coupling, the other optimized for long-range coupling. An analysis of the ^1H and ^{13}C spectra of anosmine is presented later.

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[1]This paper is dedicated to the memory of Professor Edward Leete, who died in February 1992, after a courageous and resolute battle with cancer. Eddie Leete was one of the pioneers in the study of the biosynthesis of natural products. The hallmarks of his work, over the past 40 years, were imaginativeness, innovation, thoroughness, rigor, and reliability. One of us was privileged in sharing a lab bench with Eddie, 40 years ago, in Leo Marion's laboratory at the National Research Council of Canada Laboratories in Ottawa, where the first studies of alkaloid biosynthesis were carried out. Eddie's work and our friendship have stood the test of time.

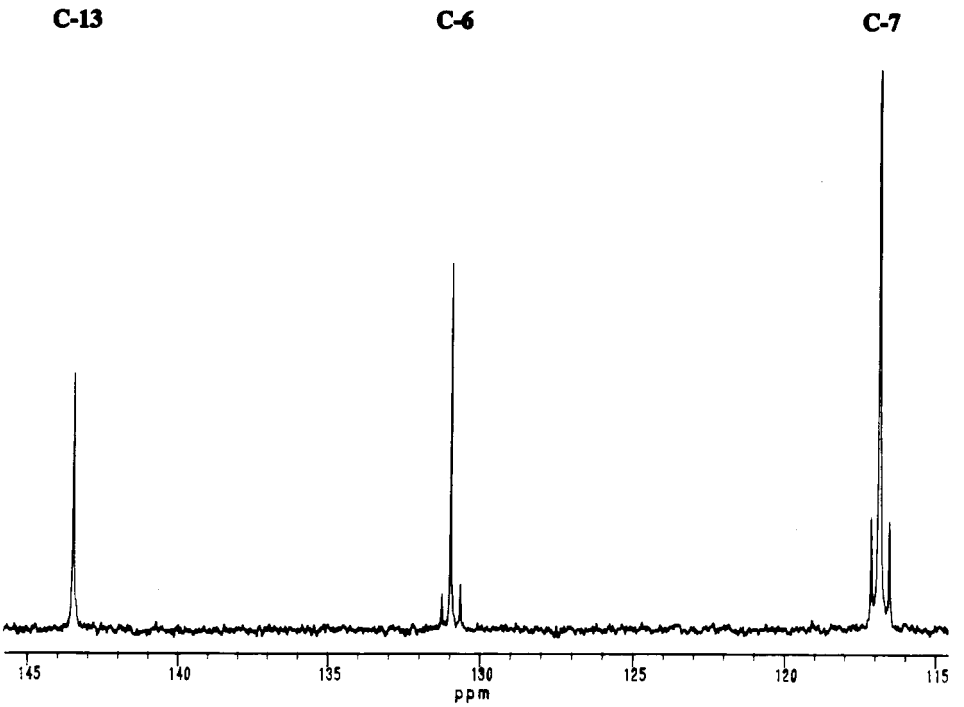


FIGURE 1. The proton noise-decoupled 125 MHz ^{13}C-nmr spectrum of the sample of anosmine bromide (91 mg in 0.6 ml D_2O) obtained from the shoots to which DL-[1,2-$^{13}C_2$] lysine had been administered. For spectral details see text.

This result, together with the earlier observation (3) that the C-6–N-6 bond of DL-[6-^{13}C,6-^{15}N]lysine is transferred intact into C-2–N-1 of anosmine, serves as evidence that the C_6N unit, C-7, -6, -5, -4, -3, -2,N-1, of the alkaloid is derived from the intact C_6N chain, C-1, -2, -3, -4, -5, -6,N-6, of DL-lysine.

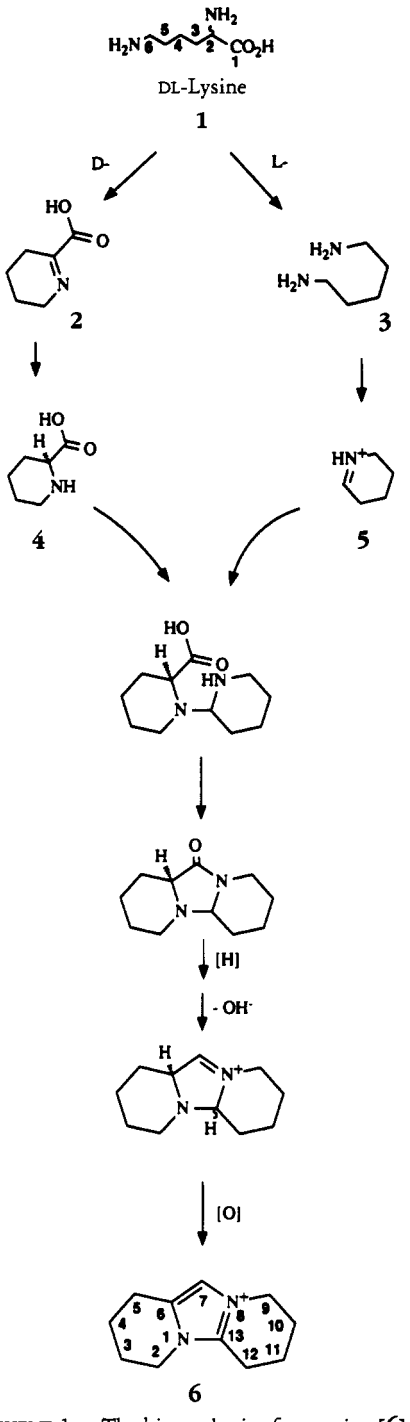
The same intact lysine-derived C_6N unit serves as the progenitor of pipecolic acid [**4**] (4,5), a cyclic amino acid that is a constituent of many higher plants.

It was of interest to investigate whether pipecolic acid served as an intermediate on the route between lysine and the C_6N unit of anosmine. A labelled sample of pipecolic acid was required for this study.

Incorporation, i.e., ^{13}C enrichment in the product, is detected readily on the basis of the appearance of satellites in the nmr signals, when an intact bond from a ^{13}C-^{13}C "bond labelled" substrate is transferred into the product. Detection of enrichment from singly ^{13}C labelled substrates, by observation of signal enhancement of a natural abundance signal, is much less reliable.

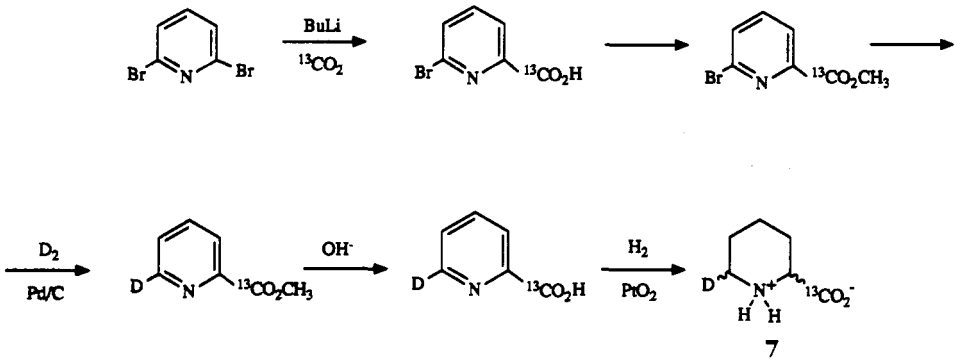
However, since the synthesis of the required ^{13}C-^{13}C "bond labelled" sample, [2,carboxyl-$^{13}C_2$]pipecolic acid, will require a major investment in effort, whereas [carboxyl-^{13}C]pipecolic acid was readily prepared by carboxylation with $^{13}CO_2$ of 2-lithiopyridine followed by hydrogenation, this was the substrate chosen for a first experiment. To enhance the detectability, by nmr spectroscopy, of incorporation at low efficiency, a ^2H label was also introduced into the molecule. Since the natural abundance of deuterium is much lower than that of ^{13}C, even a marginal specific incorporation of, say, 0.1% above natural abundance should be detectable by ^2H nmr. The synthesis of DL-[carboxyl-^{13}C, (*RS*)-6-$^2H_1$]pipecolic acid [**7**] is shown in Scheme 2.

Lithiation of 2,6-dibromopyridine (6), followed by addition of $^{13}CO_2$, yielded a



SCHEME 1. The biosynthesis of anosmine [6].

mixture of [1-^{13}C]valeric acid and [carboxyl-^{13}C]-6-bromopicolinic acid. The former was removed by steam distillation, and the latter was converted into the methyl ester by reaction with TMSi-Cl/MeOH (7). Esterification was accompanied by partial halogen exchange. Hydrogenolysis with deuterium gas, followed by ester hydrolysis, gave [carboxyl-^{13}C,6-^{2}H]picolinic acid. Catalytic hydrogenation then yielded the desired



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SCHEME 2. Synthesis of DL-[carboxyl-^{13}C,(*RS*)-6-$^{2}H_1$]pipecolic acid [7].

product, DL-[carboxyl-^{13}C,(*RS*)-6-$^{2}H_1$]pipecolic acid [**7**], containing >95 atom % carboxyl-^{13}C and >90 atom % 6-$^{2}H_1$. The overall unoptimized yield was 15%. The details of the synthesis are described in the Experimental section.

A solution of DL-[carboxyl-^{13}C,(*RS*)-6-$^{2}H_1$]pipecolic acid (150 mg) in glass-distilled H_2O was administered to six green shoots of three plants of *D. parishii* over a period of 3 weeks (July 1992). The dried plant material was extracted as before to yield anosmine bromide (105 mg).

The ^{13}C- and ^{2}H-nmr spectra of the sample provided evidence for the intact incorportion of the amino acid into the alkaloid: ^{13}C as well as $^{2}H_1$ enrichment at the expected sites was detected. Careful analysis of the high resolution ^{13}C-nmr of the product in D_2O (for details see Experimental) indicated an increase of 18% in the signal intensity for the resonance at δ 117.2 ppm due to C-7, relative to the signal intensity of the corresponding signal in the spectrum of a synthetic reference sample, determined under identical conditions. In each case the signals at δ 44.1 (C-2) and 46.8 ppm (C-9) served as internal standards. The ^{2}H-nmr spectrum of the alkaloid sample showed a signal at δ 3.85 ppm, indicating deuterium enrichment at C -2.

The results that are presented here lead to the conclusion that the C_6N unit, N-1,C-

TABLE 1. Assignment of the Signals in the ^{13}C- and ^{1}H-nmr Spectra of Anosmine [**6**].[a]

Position	Chemical Shift (δ)	
	^{13}C	^{1}H
2	44.1	3.85 (2H)
3	22.5	1.89 (2H)
4	19.9	1.71 (2H)
5	21.0	2.65 (2H)
6	131.2	—
7	117.2	6.80 (1H)
9	46.8	3.95 (2H)
10	22.3	1.90 (2H)
11	19.0	1.87 (2H)
12	21.2	2.75 (2H)
13	143.8	—

[a]Based on homonuclear ^{1}H-^{1}H and heteronuclear ^{1}H-^{13}C shift correlation spectra.

2 to C-7, of anosmine originates from DL-lysine via pipecolic acid. The conversion of lysine into pipecolic acid has been investigated in several other living systems. In this transformation, 6-amino-2-oxohexanoic acid and the corresponding intramolecular Schiff base, Δ^1-piperideine-2-carboxylic acid [**2**], serve as the intermediates (4,5). Furthermore, it has been shown that L-pipecolic acid is derived from D-lysine (5,8,9).

The other fragment of anosmine, the C_5N unit, C-13 to C-9,N-8, is derived from lysine via cadaverine (3). Since cadaverine is generated by stereospecific decarboxylation of L-lysine, catalyzed by L-lysine decarboxylase (E.C. 4.1.1.18) (10), it would appear that one of the two "halves" of anosmine is derived from L-lysine and the other from D-lysine. This hypothesis, summarized in Scheme 1, is currently under investigation.

Assignment of the signals in the ^{13}C- and ^{1}H-nmr spectra of anosimine was based on the following considerations: Of the three aromatic carbon atoms (C-6, -7, -13), showing signals at 143.8, 131.2, and 117.2 ppm, only C-7 can be readily assigned since it bears a hydrogen atom, H-7. Two of the eight remaining carbon resonances (46.8 and 44.1 ppm) can be assigned to the CH_2 carbon atoms adjacent to nitrogen.

The hydrogen atom whose resonance appears at 6.80 ppm is seen (HETCORR) to couple to the carbon atom whose resonance appears at 117.2 ppm. These resonances are thus assigned to H-7 and C-7, respectively. H-7 couples by an allylic coupling of 1.1 Hz to the protons whose resonance appears at 2.65 ppm which are thus H-5. These protons couple to the carbon atom whose resonance appears at 21.0 ppm (C-5) and, via long range coupling, to the carbon whose resonance appears at 131.2 ppm, which is therefore assigned to C-6. The carbon atom giving rise to the remaining aromatic signal, at 143.8 ppm (C-13), shows long range coupling to the aromatic proton at 6.80 ppm (H-7) and to the protons whose signal appears at 2.75 ppm (H-12). These show correlation with the carbon signal at 21.2 ppm (C-12).

The carbon atom whose signal appears at 22.5 ppm shows long-range coupling to the protons whose signal appears at 2.65 ppm (H-5) and to those which resonate at 3.85 ppm. The HETCORR spectrum shows that these two sets of protons are attached to the carbon atoms resonating at 21.0 (C-5) and 44.1 ppm, respectively. It follows that these carbon atoms are part of one and the same ring system, C-2 to C-6. The signal at 44.1 ppm is therefore assigned to C-2, that at 22.5 ppm to C-3, and the proton signal at 3.85 ppm to H-2. The carbon atom whose signal appears at 19.9 ppm shows long-range coupling to the protons whose signal appears at 3.85 ppm (H-2) and to those which resonate at 2.65 ppm (H-5), and is thus C-4. Analogously, the carbon atom whose signal appears at 22.3 ppm shows long-range coupling to the protons that resonate at 2.75 ppm (H-12) and to the protons whose signal appears at 3.95 ppm. These sets of protons are attached to carbon atoms which resonate at 21.2 (C-12) and 46.8, respectively, and must again be part of one and the same ring system, C-9 to C-13. The resonance at 46.8 ppm is therefore assigned to C-9, that at 22.3 ppm to C-10, and the proton resonance at 3.95 ppm to H-9. The carbon atom whose signal appears at 19.0 pm shows long-range coupling to the protons whose signal appears at 3.95 (H-9) and to those that resonate at 2.75 ppm (H-12), and is thus C-11.

The assignments are summarized in Table 1.

EXPERIMENTAL

PLANT MATERIAL.—Plants of *D. parishii* were obtained from "Orchid Haven," 900 Rossland Rd. E., Whitby, Ontario, Canada L1N 5R5. The specimens had been propagated through two growing seasons. Feeding experiments were carried out in July 1992, when the plants had produced vigorous fresh growth. A voucher specimen has been deposited in the Herbarium of the Royal Botanical Gardens, Box 399, Hamilton, Ontario, Canada L8N 3H8.

LABELLED COMPOUNDS.—DL-[1,2-$^{13}C_2$]Lysine was obtained from Isotec Inc., Miamisburg, Ohio 45342.

DL-[Carboxyl-^{13}C,(*RS*)-6-$^{2}H_1$]pipecolic acid [**7**] was synthesized as follows: 2,6-Dibromopyridine (5.92 g, 25 mmol) was dissolved in dry Et_2O (150 ml) and the solution cooled to −30° under He gas. A solution of *n*-butyl lithium in hexane (10 ml, 2.6 M) was added dropwise over 5 min. After stirring 10 min, the cooling bath was removed and stirring continued for 10 min. The mixture was then frozen in liquid N_2, the flask was evacuated to 0.5 mm Hg, and He was admitted to the system to allow the mixture to reach −50°. The freeze-thaw cycle was repeated, and $^{13}CO_2$, prepared by dropwise addition of concentrated H_2SO_4 to dry $Ba^{13}CO_3$ (98.3 atom % ^{13}C, MSD Isotopes, Montreal, Canada) was admitted to the system at −50°. The mixture was stirred at that temperature for 1 h and was then allowed to reach room temperature (2 h), by which time its color had turned from green-black to beige.

An aqueous solution of LiOH (25 ml, 0.5 M) was added; the aqueous phase was washed with Et_2O (3×25 ml) and acidified to Congo Red with HCl (6 N); and the solution was repeatedly evaporated from H_2O until the odor of valeric acid was no longer perceptible. The residue, crude bromopicolinic acid, was dried over KOH and esterified without further purification.

The solid was dissolved in dry MeOH (50 ml), and TMSi chloride (55 mmol) was added dropwise with stirring under N_2. The mixture was refluxed 16 h, cooled to room temperature and repeatedly evaporated from MeOH. The solid residue was suspended in $CHCl_3$ and carefully washed with 5% aqueous $NaHCO_3$ solution. The organic phase was dried over Na_2SO_4, filtered, and evaporated and the crystalline residue recrystallized from Et_2O/petroleum ether (30–60°). Yield 760 mg. Ms indicated that the product was a mixture of the methyl esters of 6-bromo- and 6-chloro-[carboxyl-^{13}C]picolinic acid: eims *m/z* 219(23), 217(23), $[M+H]^+$ (bromo derivative), 175(33), 173(100) $[M+H]^+$ (chloro derivative), 159(10), 157(10), 115(15), 113(48); ^{1}H-nmr (major component) [δ ppm, assignment, multiplicity, coupling constant (Hz)] ($CDCl_3$) 4.0 (OCH_3, d, 3.8), 7.52 (H-5, d, 8.0), 7.82 (H-4, dt, 1.5. 8.0), 8.06 (H-3, dd, 2.2, 8.0).

The mixture of methyl 6-halo-[carboxyl-^{13}C]picolinates (750 mg) was suspended in CH_3OD (40 ml, 99 atom % ^{2}H, MSD Isotopes) and 5% Pd/C (150 mg) was added. The mixture was stirred under $^{2}H_2$ gas (99 atom % ^{2}H, MSD Isotopes) (1 atm) at room temperature for 48 h, during which time the reaction was monitored by gc. When dehalogenation was complete, the mixture was filtered through Celite, the pad was washed with MeOH, and the filtrate was evaporated under reduced pressure. The residue, crude methyl [carboxyl-^{13}C,6-^{2}H]picolinate, was dissolved in MeOH (15 ml) containing KOH (280 mg), and hydrolysis was followed by tlc. Reaction was complete after stirring 3.5 h at room temperature. The mixture was evaporated in vacuo, and the residue was dissolved in glacial HOAc (20 ml). Concentrated HCl (2 ml) was added, followed by PtO_2 (50 mg), and the mixture was stirred at room temperature under H_2 (1 atm) for 24 h. The catalyst was filtered off and the filtrate evaporated repeatedly from H_2O. The residue was dissolved in H_2O and applied to a column of Dowex 50 (0.8×20 cm, 200 mesh, H^+ form). The column was washed with H_2O (50 ml) and eluted with 1 N NH_4OH. The eluate was evaporated in vacuo and the residue recrystallized from EtOH with the aid of charcoal, yielding DL-[carboxyl-^{13}C,(*RS*)-6-$^{2}H_1$]pipecolic acid [**7**] as a powder. Mp>250°, yield (unoptimized) 15% overall.

ADMINISTRATION OF TRACERS AND ISOLATION OF ANOSMINE.—In separate experiments, aqueous solutions of DL-[1,2-$^{13}C_2$]lysine (125 mg in 22 ml) and of DL-[carboxyl-^{13}C,(*RS*)-6-$^{2}H_1$]pipecolic acid [**7**] (150 mg in 22 ml) were administered by wick, each to six green shoots (0.5 ml per day per shoot) of three plants of *D. parishii* over a period of 3 weeks (July 1992). The fed shoots were cut off and dried at room temperature. The dried plant material was continuously extracted with boiling MeOH and the extract worked up as already described (3) to yield anosmine bromide (91 mg and 105 mg, respectively).

NMR SPECTROMETRY.—The proton noise-decoupled ^{13}C-nmr spectrum of a reference sample of anosmine was determined at 125 MHz.

The spectrum of the sample of anosmine bromide (91 mg in 0.6 ml D_2O) obtained from the shoots to which DL-[1,2-$^{13}C_2$]lysine had been administered (Figure 1) was determined at 125 MHz with sodium 3-trimethylsilyl[2,2,3,3-$^{2}H_4$]propanoate (δ 0.0) as internal reference.

The ^{13}C-nmr spectrum of the sample of anosmine bromide (105 mg in 0.6 ml D_2O) from the shoots to which DL-[carboxyl-^{13}C,(*RS*)-6-$^{2}H_1$]pipecolic acid had been administered (64K memory, 3×500 transients) was determined at 125 MHz, with a sweep width of 17,540 Hz, a 2 μsec pulse width and a 2 sec relaxation delay, with a final digital resolution of 0.267 Hz per point and 7–10 data points per peak. The spectrum was obtained in block-averaging mode of 3 blocks of 500 scans each.

The ^{2}H-nmr spectrum of this sample was determined at 76 MHz.

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