Biosynthesis of Anosmine, an Imidazole Alkaloid of the Orchid *Dendrobium parishii*

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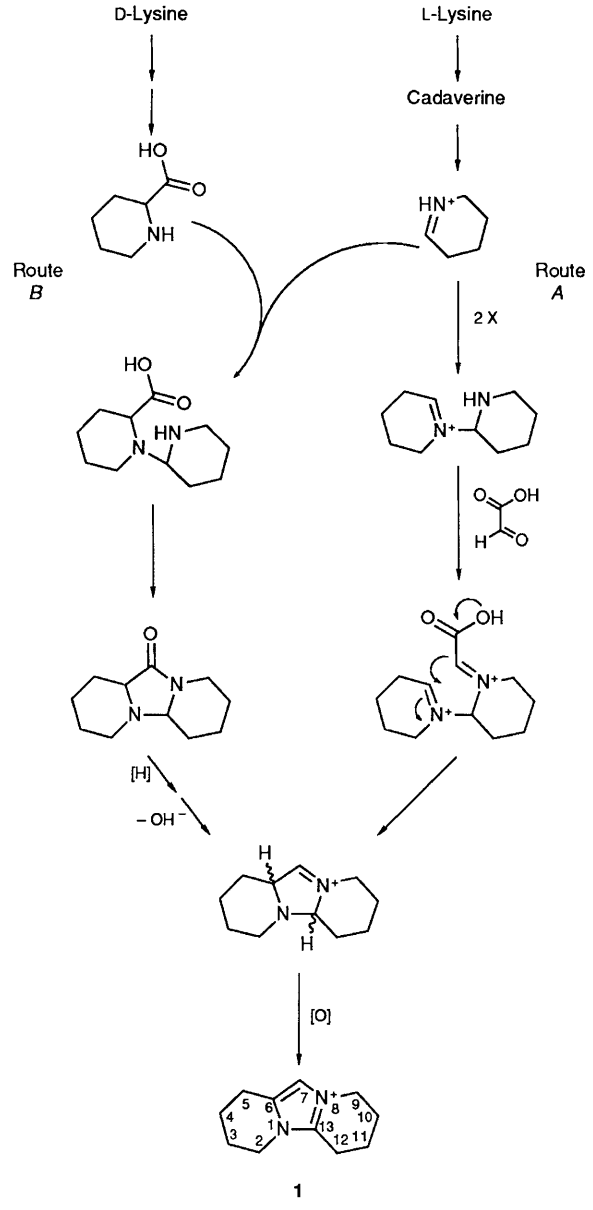
Tracer experiments with $^{13}C,^{15}N$ doubly labelled (*i.e.*, C–N 'bond-labelled') substrates show that the orchid alkaloid anosmine **1** (1,2,3,4,6,7,8,9-octahydrodipyrido[1,2-*a*:1′,2′-*c*]imidazol-10-ium bromide) is derived from two lysine units, one of which is incorporated by way of cadaverine, the other presumably *via* pipecolic acid.

Very few investigations of the biosynthesis of orchid alkaloids have been carried out. The major reasons for this are probably operational. Not many of the alkaloid-bearing species of the family Orchidaceae are readily available, the cultivation of orchids demands specialized knowledge and facilities, the period of growth of the plants is short compared to their period of dormancy and growth is exceedingly slow, unless culture conditions are ideal. Studies have been reported of the incorporation of labelled precursors into three orchid alkaloids, the sesquiterpene alkaloid dendrobine in *Dendrobium*



Scheme 1 Two possible routes for the derivation of anosmine **1** from lysine

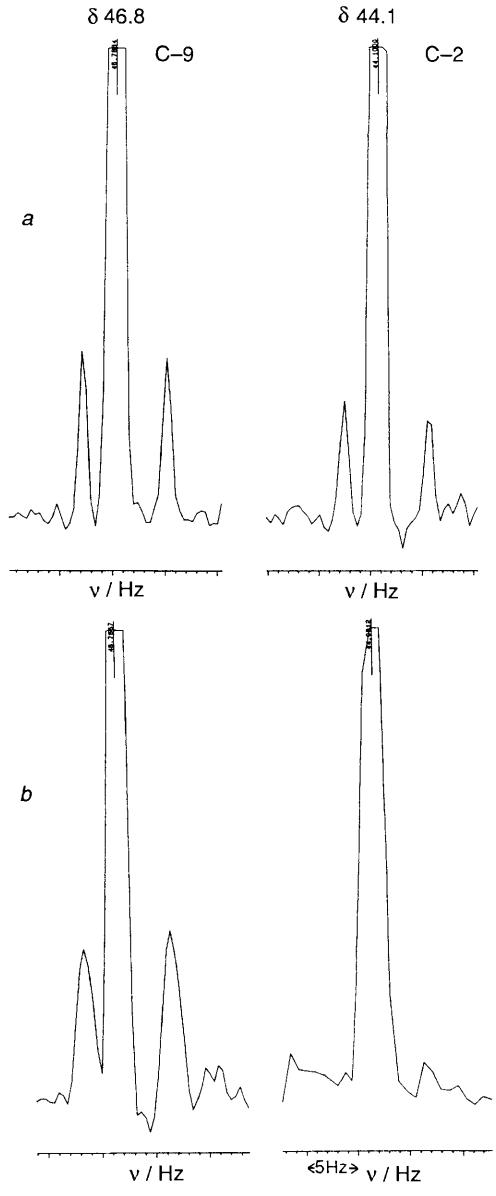


Fig. 1 Signals due to the N–CH_2 groups (C-2 and C-9) in the proton noise decoupled ^{13}C NMR spectra (50 MHz) of the samples of anosmine **1** (in D_2O), obtained from shoots of *D. parishii* to which DL-[6-^{13}C,6-^{15}N]lysine (*a*) and [1,5-(^{13}C,^{15}N)$_2$]cadaverine (*b*), respectively, had been administered. Sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propanoate served as internal reference (δ 0.0).

nobile,[1] the tetrahydroisoquinoline alkaloid cryptostyline I in *Cryptostylis fulva*[2] and the phthalidopyrrolidine alkaloid shihunine in *D. pierardii*.[3] The biosynthesis of orchid alkaloids of other structural types remains unexplored.

We have studied the biosynthesis of the orchid alkaloid anosmine **1**, whose unique octahydrodipyridoimidazolium ring system is not duplicated in any other natural product. The alkaloid was first isolated by Leander and Lüning[4] from *D. anosmum* Lindl. and from *D. parishii* Rchb. f. in the course of their comprehensive screening of orchid species for alkaloidal components.

Among several other possibilities for the biogenesis of anosmine, we considered two modes of origin from the amino acid lysine. The first of these hypotheses envisages the derivation of the ring system by reaction of a Δ^1-piperideine dimer, derived from two cadaverine moieties, with a one-carbon unit (Scheme 1, Route *A*). Δ^1-Piperideine, formed[5] from cadaverine by the action of diamine oxidase (E.C. 1.4.3.6), is implicated in the biosynthesis of several alkaloids containing a piperidine nucleus.[6] Its precursor, cadaverine, is derived[7] by decarboxylation of L-lysine, a process that is catalysed by L-lysine decarboxylase (E.C. 4.1.1.18). This hypothesis thus postulates that the two C_5N nuclei of the alkaloid are of identical origin and demands that label from DL-lysine and from cadaverine should enter both C_5N nuclei.

The second hypothesis postulates that the ring system of anosmine originates by combination of two different lysine metabolites, Δ^1-piperideine and L-pipecolic acid, without the participation of a one-carbon unit (Scheme 1, Route *B*). Pipecolic acid, a non-protein amino acid of wide natural distribution, is derived from lysine *via* the corresponding α-oxo-acid.[18] This hypothesis demands that label from cadaverine should be incorporated into only one of the two C_5N nuclei of anosmine **1**, but that label from DL-lysine should enter both C_5N nuclei.

In each of two separate tracer experiments, ^{13}C–^{15}N 'bond labelled' substrates in aqueous solution were administered by infusion through cotton wicks to two shoots of *D. parishii* over

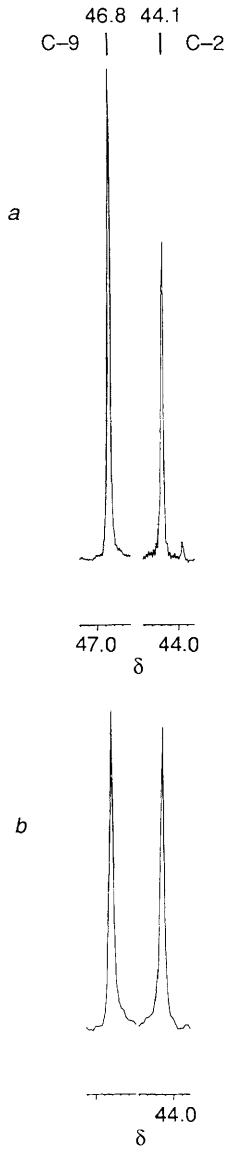


Fig. 2 Signals due to the N–CH_2 groups (C-2 and C-9) in the proton noise decoupled ^{13}C NMR spectra (125.8 MHz) of the sample of anosmine **1** (in D_2O), obtained from shoots of *D. parishii* to which [1,5-$^{13}C_2$]cadaverine had been administered (*a*) and of a natural abundance sample (*b*). Internal reference (δ 0.0) as above.

a period of three weeks. The treated shoots were then cut from the stocks, the harvested plant material was macerated in cold methanol and extracted with methanol in a Soxhlet extractor. The extract was evaporated to dryness, the residue partitioned between water and diethyl ether, and the aqueous phase containing the product was evaporated. The residual solid was triturated with hot methanol (3 × 3 ml) and the methanolic extracts were combined and evaporated. The solid residue was dissolved in ethanol and applied to a column of neutral alumina. The product was eluted with ethanol, fractions containing a Dragendorff positive component were pooled and evaporated, and the residue was dissolved in D_2O for NMR analysis.

The first experiment, with DL-[6-^{13}C,6-^{15}N]lysine monohydrochloride [150 mg, 99% ^{13}C, 99% ^{15}N, prepared[9] by an established method using ^{13}C,^{15}N labelled cyanide (MSD Isotopes)], yielded a sample of anosmine [77 mg, as the bromide salt, determined by UV spectrophotometric assay at λ 221 nm (log ε 3.78)[4]] whose ^{13}C NMR spectrum [Fig. 1(*a*)] showed satellites in two signals: at δ 44.1 (*J* 8 ± 0.8 Hz, Δδ = −0.016 ppm, 0.05% enrichment†) and at δ 46.8 (*J* 8 ± 0.8 Hz, Δδ = −0.016 ppm, 0.09% enrichment†). These two signals represent the two N–CH_2 groups of anosmine **1**, C-2 and C-9, respectively.‡ The presence of the two sets of satellites proves that the N-6,C-6 unit of lysine had been incorporated intact into each of the two C_5N nuclei of the alkaloid and suggests that each one of these nuclei originates from a C_5N chain, derived from C-2,-3,-4,-5,-6,N-6 of DL-lysine.

This result implicates lysine in the biosynthesis of anosmine and eliminates other (*e.g.* polyketide) models. The result does not discriminate between the two biogenetic proposals, *A* and *B* (Scheme 1), both of which are consistent with the entry of lysine into each of the two C_5N nuclei of the alkaloid.§

The result of a second experiment permitted a choice to be made between the two routes, *A* and *B*. This experiment, with [1,5-(^{13}C,^{15}N)$_2$]cadaverine dihydrochloride [150 mg, 99% ^{13}C, 99% ^{15}N, prepared from 1,3-dibromopropane and ^{13}C,^{15}N-labelled cyanide (MSD Isotopes)] as the substrate, yielded a sample of anosmine (30 mg as the bromide) whose ^{13}C NMR spectrum [Fig. 1(*b*)] showed satellites in one signal only, at δ 46.8 (*J* 8 ± 0.8 Hz, Δδ = −0.016 ppm, 0.27% enrichment†). This signal respresents the N–CH_2 group, C-9. The presence of satellites at this signal, and their absence at the signal, δ 44.1 (C-2), proves that an intact C–N unit of cadaverine had been incorporated into only one of the two C_5N nuclei of the alkaloid.

A third experiment, with [1,5-$^{13}C_2$]cadaverine dihydrochloride [150 mg, 99% ^{13}C, prepared from 1,3-dibromopropane and ^{13}C-labelled cyanide (MSD Isotopes)], confirmed this conclusion. The ^{13}C NMR spectrum of anosmine (31 mg as the bromide) from this experiment [Figure 2(*a*)] showed an increase in signal intensity (0.55% enrichment†) for the resonance at δ 46.8 (C-9) but none for that at δ 44.1 (C-2), relative to the intensity of the signals in the ^{13}C NMR spectrum of a natural abundance sample of the alkaloid [Figure 2(*b*)].¶

Thus, whereas both C_5N nuclei of anosmine are derived from DL-lysine, only one of them (N-8, C-9 to C-13) originates from cadaverine. Of the two biogenetic proposals, *A* and *B*, only one, route *B*, is consistent with these results.

In every plant species that has been studied to date, L-pipecolic acid has been found to be derived from D-lysine, and not from L-lysine.[10] The putative derivation of the two 'halves' of anosmine, from pipecolic acid and from cadaverine, respectively, raises the intriguing prospect that one of its 'halves' (N-1, C-2 to C-7) originates from D-lysine and the other (N-8, C-9 to C-13) from L-lysine. Experiments to

† % Enrichment = % ^{13}C above natural abundance.

‡ Assignment of the signals in the ^{13}C and ^{1}H NMR spectra of anosmine **1** is based on homonuclear ^{1}H–^{1}H and heteronuclear ^{1}H–^{13}C shift correlation spectra. Two heteronuclear spectra were recorded, one optimised for the detection of one-bond coupling, the other optimised for long-range coupling. δ_C (δ_H): 44.1 [C-2 (3.85, 2H)], 22.5 [C-3 (1.89, 2H)], 19.9 [C-4 (1.71, 2H)], 21.0 [C-5 (2.65, 2H)], 131.2 [C-6], 117.2 [C-7 (6.80, 1H)], 46.8 [C-9 (3.95, 2H)], 22.3 [C-10 (1.90, 2H)], 19.0 [C-11 (1.87, 2H)], 21.2 [C-12 (2.75, 2H)] and 143.8 [C-13].

§ Route *A* demands equal ^{13}C enrichment within the two sites, C-2 and C-9, whereas route *B* is consistent with either unequal or equal enrichment. The difference in the observed values of the ^{13}C enrichment within the two sites (0.05 and 0.09%, respectively), is too small to be regarded as a reliable indicator for route *B*.

¶ Intact incorporation of a C_5N chain from cadaverine into the nucleus, N-8,C-9,10,11,12,13, of anosmine demands that, as a consequence of the C_{2v} symmetry of the precursor, the alkaloid samples from all three feeding experiments should show ^{13}C enrichment at C-13. Attempts to detect ^{13}C enrichment at this site were not successful, due to an excessively broad line width of the signal at δ 143.8 (C-13) in the spectra of the samples from the first two experiments, and due to an unfavourable signal to noise ratio in the third.

test the incorporation of pipecolic acid and of enantiomerically pure samples of lysine will have to await the next growing season of our specimens of *D. parishii*.

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