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THE BIOSYNTHESIS OF NORNUCIFERINE-I(2-METHOXY-6aα-APORPHINE-1-OL)

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Abstract—The incorporation of (±)-norcoclaurine, (±)-coclaurine, (±)-N-methylcoclaurine, (±)-N-methylnorcoclaurine into nornuciferine-I in *Croton sparsiflorus* morong has been studied, and the specific utilization of the (±)-N-methylcoclaurine demonstrated. The evidence supports the direct oxidative coupling of (+)-(*S*)-N-methylcoclaurine to give N-methylcrotsparine, which in turn is shown to be a specific precursor of nornuciferine-I. The experiments also show that N-methylcrotsparine is reduced to N-methylcrotsparinol and it is N-methylcrotsparinol-I which is preferentially dehydrated and rearranged to nornuciferine-I.

According to the established biogenetic theory,[1] nornuciferine-I[2] (5), a representative of 'abnormal apophine' alkaloids which lack oxygen function in ring D can be biosynthesised from (*S*)-N-methylcoclaurine (1). *Ortho–para* oxidative coupling of 3 gives the proaporphine (2). Reduction of 2 to the dienol (4) followed by dienol–benzene rearrangement finally yields nornuciferine-I (5).

Tracer experiments have shown that the "abnormal aporphine" alkaloids roemerine in *Papaver dubium*[3] and anonaine in *Anona reticulata*[3] are biosynthesised specifically from coclaurine (8). Stereospecific incorporation of (+)-(*S*)-N-methylcoclaurine (1) into roemerine has also been demonstrated.[3] We now present the results of tracer experiments which define the biosynthetic pathways of nornuciferine-I (5) from norcoclaurine (7) in *Croton sparsiflorus* morong.

(±)-Tyrosine was initially fed to young cut branches of *C. sparsiflorus* morong (Euphorbiaceae) and it was found that nornuciferine-I (5) was actively biosynthesised by the plants. In subsequent experiments labelled hypothetical 1-benzylisoquinoline precursors were fed to young cut branches of *C. sparsiflorus*. The results of several feedings are recorded in the Table 1. Feeding of (±)-norcoclaurine (7; experiment 2), (±)-N-methylcoclaurine (9; experiment 4) established that 7, 8 and 9 are efficient precursors of nornuciferine-I (5). (±)-N-

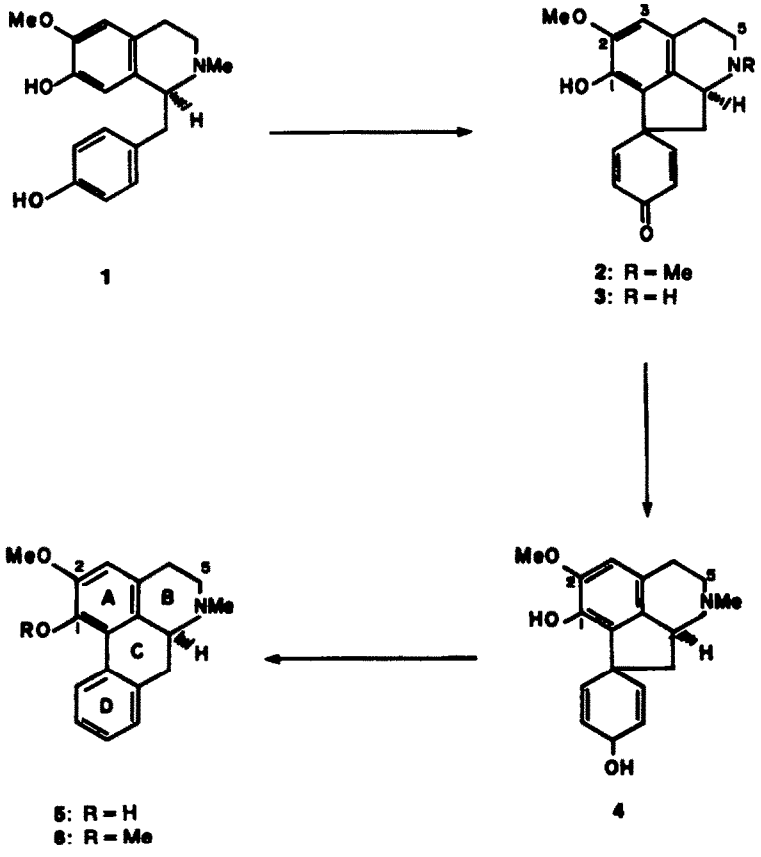


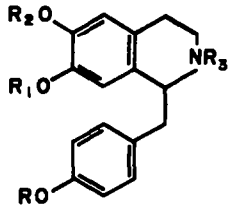
Table 1. Tracer experiments on *C. sparsiflorus*

Expt.	Precursor fed	Incorporation % in nornuciferine-I (5)
1	(±)-[2-^{14}C]Tyrosine	0.08
2	(±)-[1-^{3}H]Nor-colaurine (7)	0.14
3	(±)-[3′,5′,$\beta^{3}H_3$]coclaurine (8)	0.20
4	(±)-N-Methyl[1-^{3}H]coclaurine (9)	0.36
5	(±)-[N-$^{14}CH_3$]N-Methyl-norcoclaurine (11)	0.0004
6	(±)-NOO-Trimethyl[3′,5′,$\beta^{3}H_3$]coclaurine (10)	0.002
7	(±)-N-Methyl[3-^{14}C]coclaurine (9)	0.42
8	(+)-(S)-N-Methyl[3′,5′,$\beta^{3}H_3$]coclaurine (1)	0.72
9	(−)-(R)-N-Methyl[3′,5′,$\beta^{3}H_3$]coclaurine (12)	0.012
10	N-Methyl[5-^{14}C]crotsparine (2)	5.20
11	N-Methyl[5-^{14}C]crotsparinol-I	6.12
12	N-Methyl[5-^{14}C]crotsparinol-II	0.32
13	[5-^{14}C]crotsparine	4.60
14	N-Methyl[5-^{14}C]crotsparine (18)	0.005

Methylnorcoclaurine (11; experiment 5) was not incorporated. The results demonstrated that N-methylation of norcoclaurine (7) does not precede O-methylation in the biosynthesis of nornuciferine-I (5) from norcoclaurine (7). (±)-NOO-Trimethylcoclaurine (10; experiment 6) as expected was not incorporated into 5.

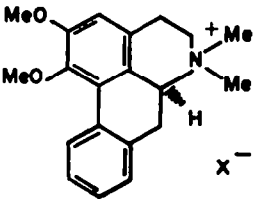
The regiospecificity of the label in the biosynthetic nornuciferine-I (5) derived from (±)-N-methyl-[3-^{14}C] coclaurine (9; experiment 7) was established as follows. Radioactive nornuciferine-I (5) was treated with diazomethane to give nuciferine[4] (6) which was converted into nuciferine methiodide[4] (13) and then into its hydroxide form 14 with no loss of radioactivity. Hofmann degradation of 14 yielded the methine-I (15) which had essentially the same radioactivity as the parent base. Second Hofmann degradation of the methine-I (15) afforded the methine-II (16) with no loss of activity. Ozonolysis of 16 gave formaldehyde, trapped as its dimedone derivative (98% of original activity). The aldehyde 17 was essentially radioinactive. The experiment thus established that nornuciferine-I (5) is specifically biosynthesised from N-methylcoclaurine (9) in *C. sparsiflorus*.

According to biogenetic theory N-methylcrotsparine[2]

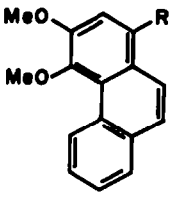


7: R = R1 = R2 = R3 = H
8: R = R1 = H; R2 = Me
9: R = R1 = H; R2 = R3 = Me
10: R = R1 = R2 = R3 = Me
11: R = R1 = R2 = H; R3 = Me

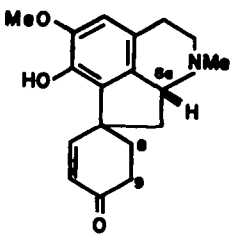
12



13: X = I
14: X = OH



15: R = —[CH2]3—NMe2
16: R = Vinyl
17: R = CHO



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(2) should be an obligatory intermediate in the biosynthesis of nornuciferine-I (5) from N-methylcoclaurine (1). Efficient laboratory synthesis of 5 from 2 strongly supported the idea.[2] When labelled 2 (experiment 10) was fed to young cut branches of *C. sparsiflorus*, it was efficiently incorporated into nornuciferine-I (5). Crotsparine (3; experiment 13) was also converted efficiently into 5. Probably 3 was converted into 2 and then utilized by the plants to form 5.

The regiospecificity of the labelling in the biosynthetic nornuciferine-I (5) derived from N-methyl[5-^{14}C] crotsparine was established as follows. Labelled nornuciferine-I (5) was converted into the methine-II (16) according to the degradation procedure described above. Compound 16 had essentially the same radioactivity as the parent base. Ozonolysis of 16 yielded formaldehyde trapped as its dimedone derivative (97.8% of original activity). The aldehyde 17 was radioinactive. The experiments thus confirmed the intermediacy of N-methylcrotsparine (2) in the biosynthesis of nornuciferine-I (5) in *C. sparsiflorus*.

Conversion of N-methylcrotsparine (2) into nornuciferine-I (5) involves N-methylcrotsparinol[2] (4) as an intermediate which was prepared as follows. Reduction of N-methylcrotsparine (2) with sodium borohydride gave a mixture of epimeric alcohols, N-methylcrotsparinol-I and II which were separated by chromatography. Their relative stereochemistry is being investigated. However, both the alcohols were readily dehydrated in aqueous acidic solution to give nornuciferine-I (5). Parallel feedings of labelled N-methylcrotsparinol-I (experiment 11) and N-methylcrotsparinol-II (experiment 12) to young cut branches of *C. sparsiflorus* demonstrated that the former was preferentially converted into nornuciferine-I (5) by the plants.

The experiments reported above, established that N-methylcoclaurine (9) is a specific precursor of nornuciferine-I (5) in *C. sparsiflorus*. The precursor used, however, was racemic. It would be expected that the enzyme system which carries out the oxidative coupling step would be stereospecific and that only one of the two optical isomers should normally act as a direct substrate. Parallel feeding with (+)-(*S*)-N-methylcoclaurine (1; experiment 8) and (−)-(*R*)-N-methylcoclaurine (12; experiment 9) demonstrated that stereospecificity is maintained in the bioconversion of 1-benzylisoquinoline into (5). The former was incorporated 70 times more efficiently than the latter.

N-Methylcrotsparine (2) and N-methylcrotsparinine (18) have opposite configuration at the asymmetric centre 6a. Whether the plants can metabolise 18 to form nornuciferine-I (5) was next tested. Labelled N-methyl[5-^{14}C]crotsparinine (18; experiment 18) when fed to young cut branches of *C. sparsiflorus* was not incorporated into nornuciferine-I (5). The results showed that the enzyme system present in the plants was not capable of removing H atoms from C8-9 in 18 to form a double bond in that position. The results also demonstrated that the oxidation–reduction system which was very active in poppies[5] and *Cocculus laurifolius*[6] for changing the configuration at the asymmetric centre in the 1-benzyltetrahydroisoquinoline precursors was not active in *C. sparsiflorus*.

The presence of N-methylcoclaurine in *C. sparsiflorus* was demonstrated by trapping experiments. N-methylcrotsparine (2) has actually been isolated from *C. sparsiflorus*. The specific incorporation of both these precursors into nornuciferine-I (5) have also been demonstrated. N-Methylcoclaurine and N-methylcrotsparine (2) are thus, the true precursors of nornuciferine-I (5) in *C. sparsiflorus*.

The foregoing results, thus strongly supported the following sequence for the biosynthesis of nornuciferine-I (5) in *C. sparsiflorus*: tyrosine → norcoclaurine (7) → coclaurine (8) → (+)-(*S*)-N-methylcoclaurine (1) → N-methylcrotsparine (2) → N-methylcrotsparinol-I → nornuciferine (5).

EXPERIMENTAL

Unless otherwise stated UV absorption spectra refer to solns in MeOH, IR absorption spectra to KBr discs, and NMR spectra (recorded with a Varian A-60D spectrometer) in $CDCl_3$ solns. Tlc was carried out, unless specified to the contrary, on silica GF 254.

Counting methods. Liquid scintillation counting was used for the measurement of ^{3}H and ^{14}C activities (Packard 3320 automatic Tricard instrument). Samples were counted in 7 ml of scintillator, after dissolution in MeOH or DMSO (0.2 ml) and values are not corrected for self-absorption. Relative efficiencies were obtained by counting [1,2-$^3H_2$]- and [2-^{14}C]-hexadecane standards.

Synthesis of 1-benzylisoquinoline precursors. The racemates of coclaurine,[7] norcoclaurine[8] and N-methylcoclaurine,[9] were prepared by standard procedures.

Resolution. (±)-Di-O-bisbenzyl-N-methylcoclaurine was resolved by treatment with (−)- and (+)-di-*p*-toluoyltartaric acids. Hydrogenolysis of the benzyl ethers with HCl afforded (−)-(*R*)- and (+)-(*S*)-N-methylcoclaurines.[9]

Labelling of precursors

Tritiation. Tritium was introduced specifically at *ortho* and *para* to a phenolic group by the published procedure.[10] (±)-Coclaurine hydrochloride (100 mg) in tritiated water (0.3 ml; 60 μCi) containing potassium t-butoxide (200 mg) was heated under N_2 (sealed tube) for 100 hr at 100° to give (±)-[3',5',8-$^3H_2$]coclaurine, which was purified as its hydrochloride (64 mg) to constant activity. The other phenolic 1-benzyltetrahydroiso quinoline precursors were tritiated in the same way.

(±)-N-Methyl[1-^{3}H]coclaurine was prepared by reduction of the corresponding dihydroisoquinoline with potassium[^{3}H]borohydride in dry dimethylformamide.

(±)-N-[^{14}C]Methylnorcoclaurine was prepared by treating the corresponding dihydroisoquinoline with [^{14}C]methyl iodide and subsequent reduction with sodium borohydride.

(±)-NOO-Trimethyl[3',5',8-$^3H_3$]coclaurine was prepared by treating (±)-N-methyl[3',5',8-$^3H_3$]coclaurine with diazomethane.

(±)-N-Methyl[3-^{14}C]coclaurine was prepared by complete synthesis. N-Methyl[5-^{14}C]crotsparine and [5-^{14}C]crotsparine were obtained by feeding (±)-N-methyl[3-^{14}C] coclaurine and (±)-[3-^{14}C]coclaurine to *C. sparsiflorus* plants.

N-Methyl[5-^{14}C]crotsparinol-I and N-methyl[5-^{14}C] crotsparinol-II were prepared by sodium borohydride reduction of N-methyl[5-^{14}C] crotsparine.

Feeding experiments. For feeding purposes N-methylcoclaurine and NOO-trimethylcoclaurine were dissolved in water (1 ml) containing tartaric acid (12 mg). Coclaurine hydrochloride, norcoclaurine hydrochloride, N-methylnorcoclaurine hydrochloride, N-methylcrotsparine, crotsparine, N-methylcrotsparinols and N-methylcrotsparinine were dissolved in aqueous dimethyl sulphoxide (1 ml). Freshly cut young branches of *C. sparsiflorus* were dipped into the solutions and allowed to take up the precursor. When uptake was complete water was added for washing. The twigs were then dipped in water, left for 6–8 days, and worked up for nornuciferine-I.

Isolation and purification of nornuciferine-I. The young stems and leaves (typically 110 g wet wt) were macerated in EtOH (250 ml) containing inactive 5 (130 mg) and left for 14 hr. The EtOH was then decanted and the plant material was percolated with fresh EtOH (6 × 150 ml). The combined ethanolic extract was concentrated *in vacuo*. The green viscous mass so obtained

Table 2. Activities of nornuciferine-I degradation products

Compound	Molar activity (disint. min^{-1} $mmol^{-1} \times 10^4$)
Nornuciferine-I (5)	5.52
Nuciferine (6)	5.47
Nuciferine methiodide (13)	5.59
Nuciferine methine-I (15)	5.48
3,4-Dimethoxy-1-vinylphenanthrene (16)	5.44
Formaldehyde-dimethone	5.40

was extracted with 10% tartaric acid (5 × 15 ml). The acidic soln was extracted with hexane (4 × 15 ml) and then basified with Na_2CO_3 aq (ph 8). The liberated bases were extracted with $CHCl_3$ (5 × 20 ml). The combined extract was washed with water, dried (Na_2SO_4) and evaporated to afford crude 5 (110 mg) which was purified by preparative silica gel tlc (solvent; $CHCl_3$:MeOH; 94:6). The region containing the desired alkaloid was cut off and eluated with $CHCl_3$:MeOH (80:20) mixture. Removal of the solvent from the eluate gave the product which was crystallised from EtOH to give pure 5 (85 mg), m.p. 168–170° (lit.[2] 166–168°). Base hydrochloride m.p. 226° dec. (lit.[2] 225°).

Degradation of [5-^{14}C]*nornuciferine-I* (5). Compound 5 (325 mg; molar activity 0.026 μCi $mmol^{-1}$) derived from (±)-N-methyl[3-^{14}C]coclaurine feeding (experiment 7) in MeOH (10 ml) was treated with excess of ethereal diazomethane and left at room temp. for 50 hr to give 6 (308 mg) m.p. 165° (lit.[4] 165-5) (molar activity 0.025 μCi m mol^{-1}). 6 (300 mg) in MeOH (4 ml) was refluxed with MeI (1 ml) to give 13 (310 mg) m.p. 178–179° (lit.[4] 177–178°) (molar activity 0.0264 μCi m mol^{-1}).

Radioactive 13 (300 mg) in MeOH was passed through a column of freshly generated amberlite IR-410 anion exchange resin (OH form) (10 g). The eluate was recycled five times. The column was finally washed with MeOH (100 ml). Removal of the solvent from the combined eluate gave radioactive 14 which was refluxed with KOH (1.5 g in 10 ml aqueous MeOH) for 2 hr to afford radioactive 15 (188 mg; molar activity 0.0246 μCi $mmol^{-1}$).

The radioactive 15 (180 mg) in MeOH (4 ml) was refluxed with MeI (1 ml) for 3 hr to give radioactive *nuciferine methine-I methiodide* (175 mg; molar activity 0.254 μCi $mmol^{-1}$). The methiodide was dissolved in MeOH and treated with amberlite IR-410 anion exchange resin (OH form; 10 g) to give the corresponding methohydroxide which was refluxed with KOH (2 g in 10 ml aqueous MeOH) to yield *3,4-dimethoxy-1-vinylphenanthrene* 16 (95 mg; molar activity 0.024 μCi $mmol^{-1}$).

Ozonised oxygen was passed through a soln of radioactive 16 (85 mg) in EtOAc (6 ml) at −78° for 30 min and the solvent was then removed. The residue so obtained was refluxed for 20 min with a mixture of H_2O (18 ml), Zn dust (200 mg) and $AgNO_3$ (9 mg) and subjected to distillation. The distillate was collected in a soln of dimedone (300 ml) in aqueous EtOH (3:1; 80 ml), left for 1 hr at room temp., concentrated to 10 ml and left overnight. The product so obtained was filtered, washed with H_2O, dried and chromatographed on a column of silica. Elution was affected with C_6H_6 and C_6H_6:$CHCl_3$. Appropriate fractions (tlc basis) containing formaldehyde-dimethone were mixed, solvent removed and the product was crystallised from MeOH–Et_2O to give formaldehyde-dimethone (12 mg) m.p. 188° (molar activity 0.024 μCi $mmol^{-1}$).

Degradation of [5-^{14}C]*nornuciferine-I* (5). Nornuciferine-I (5) derived from 2 (experiment 10) was converted into 6. Double Hofmann degradation of 6 as above gave radioactive 16. Ozonolysis of 16 yielded formaldehyde trapped as formaldehyde-dimethone. The radioactivities of the degradation products are given in Table 2.

Trapping experiment. (±)-[5-^{14}C]-Tyrosine (activity 0.1 μCi) in water (1 ml) was fed to young cut branches of *C. sparsiflorus* plants. The plants were kept alive for 8 days and then macerated with 9 (110 mg) in EtOH (250 ml) containing 1% AcOH and kept for 12 hr. The EtOH was decanted and the plant material was percolated with fresh EtOH (6 × 150 ml) containing 2% AcOH. The combined ethanolic extract was concentrated *in vacuo* to give a green viscous mass which was extracted with water (5 × 10 ml). The combined aqueous acidic soln was defatted with hexane, basified with $NaCO_3$, and extracted with $CHCl_3$. Removal of the solvent from the $CHCl_3$ extract gave the crude base which was purified by preparative tlc on silica gel plates to give N-methylcoclaurine (80 mg; molar activity 1.03 μCi $mmol^{-1}$) incorporation 0.38%).

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