Biosynthesis of Cocsulinin

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The incorporation of (±)-norcoclaurine, (±)-coclaurine, and (±)-*N*-methylcoclaurine into cocsulinin in *Cocculus laurifolius* DC has been studied, and specific utilization of the (±)-*N*-methylcoclaurine has been demonstrated. The evidence supports oxidative dimerisation of two *N*-methylcoclaurine units to give cocsulinin. Experiments with (±)-*N*-[^{14}C]methyl[1-^{3}H]coclaurine have demonstrated that the hydrogen atom at the asymmetric centre in the 1-benzylisoquinoline precursor is retained in the bioconversion into cocsulinin. Parallel feedings of (+)-(*S*)- and (−)-(*R*)-*N*-methylcoclaurines showed that the configuration at C-1 is maintained in the biosynthesis of cocsulinin from the 1-benzylisoquinoline precursor.

A double-labelling experiment with (±)-*N*-methyl[1-^{3}H, 6-O-*methyl*-^{14}C]coclaurine has shown that the 6-*O*-methyl group of an *N*-methylcoclaurine unit is lost in the biotransformation into cocsulinin. Incorporation of (+)-(*S*,*S*)-*O*-methylcocsulinin established that de-*O*-methylation is the terminal step in the biosynthesis of cocsulinin.

COCSULININ [1] (1), the anticancer agent of *Cocculus pendulus* (Menispermaceae) might be biosynthesised by oxidative dimerisation of coclaurine derivatives.[2-4] Intermolecular oxidative coupling of two *N*-methylcoclaurine (13) units could generate first the ether ' bridge ' between the benzylic portions. The two ether

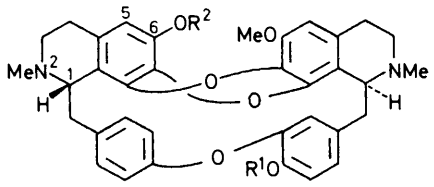
[1] D. S. Bhakuni and P. P. Joshi, *Tetrahedron*, 1975, **31**, 2575; P. P. Joshi, D. S. Bhakuni, and M. M. Dhar, *Indian J. Chem.*, 1974, **12**, 517.

[2] F. Faltis and H. Frauenderfer, *Ber.*, 1930, **63**, 806.

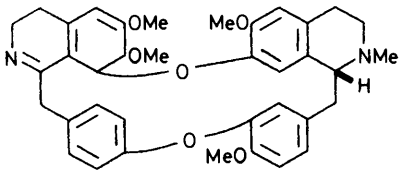
[3] M. Shamma, ' The Isoquinoline Alkaloids,' Academic Press, New York, 1972, p. 138.

[4] A. R. Battersby in ' Oxidative Coupling of Phenols,' eds. A. R. Battersby and W. I. Taylor, Dekker, New York, 1967.

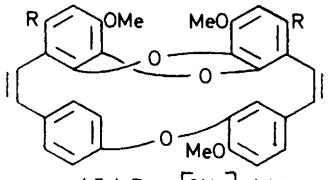
linkages between the isoquinoline portions as present in (1) might then be formed by intramolecular oxidative coupling. 6-De-*O*-methylation of *O*-methylcocsulinin (2) would finally give rise to (1).



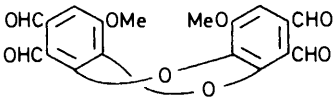
(1) $R^1 = R^2 = H$
(2) $R^1 = H, R^2 = Me$
(3) $R^1 = R^2 = Me$



(4)



(5) $R = [CH_2]_2 NMe_2$
(6) $R = CH{:}CH_2$



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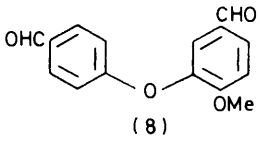

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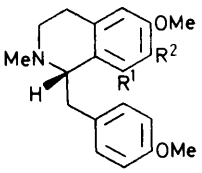
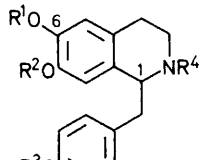
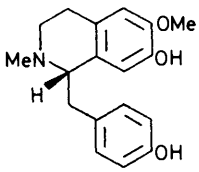


(9) $R^1 = OMe, R^2 = H$
(10) $R^1 = R^2 = H$



(11) $R^1 = R^2 = R^3 = R^4 = H$
(12) $R^1 = Me, R^2 = R^3 = R^4 = H$
(13) $R^1 = R^4 = Me, R^2 = R^3 = H$
(14) $R^1 = R^2 = R^3 = R^4 = Me$



(15)

It has been shown that (±)-coclaurine and (−)-*N*-methylcoclaurine are incorporated into the bisbenzylisoquinoline alkaloid epistephanine [5] (4), which contains two ether linkages. We now present the first report on the biosynthesis of a biscoclaurine alkaloid containing three ether linkages.

(±)-Norcoclaurine (11) (experiment 1) was initially fed to young cut branches of *Cocculus laurifolius* DC (Menispermaceae) and it was found that cocsulinin (1) was being actively biosynthesised by the plants. In subsequent experiments labelled hypothetical precursors were fed to young cut branches of *C. laurifolius*. The results of several feedings are recorded in the Table.

Tracer experiments on *C. laurifolius*

Expt.	Precursor fed	Incorporation (%) into cocsulinin (1)
1	(±)-[1-^{3}H]Norcoclaurine (11)	0.15
2	(±)-[3′,5′,8-$^3H_3$]Coclaurine (12)	0.18
3	(±)-*N*-[^{14}C]Methyl[1-^{3}H]coclaurine (13)	0.28
4	(±)-*NOO*-Trimethyl[3′,5′,8-$^3H_3$]coclaurine	0.000 4
5	(±)-*N*-Methyl[1-^{3}H,6-O-*methyl*-^{14}C]coclaurine (13)	0.20
6	(+)-*N*-Methyl[3′,5′,8-$^3H_3$]coclaurine (15)	0.46
7	(−)-*N*-Methyl[3′,5′,8-$^3H_3$]coclaurine	0.008
8	(+)-*O*-Methyl[*aryl*-^{3}H]cocsulinin (2)	0.87
9	(±)-*N*-Methyl[1-^{3}H]coclaurine (13)	0.26

Feeding of (±)-coclaurine (12) (experiment 2) and (±)-*N*-methylcoclaurine (13) (experiment 3) established that (12) and (13) were efficient precursors of cocsulinin (1). The completely methylated 1-benzyltetrahydroisoquinoline, (±)-*NOO*-trimethylcoclaurine (14) (experiment 4), as expected, was not incorporated into (1).

Feeding of (±)-*N*-[^{14}C]methyl[1-^{3}H]coclaurine (13) (experiment 3) gave cocsulinin (1) labelled with both ^{14}C and ^{3}H. The ^{14}C : ^{3}H ratio was almost the same in the precursor as in the biosynthetic base. The location of the labels in the biosynthetic cocsulinin (1) was established as follows. Labelled cocsulinin (1) was treated with diazomethane to give di-*O*-methylcocsulinin (3) with practically no loss of radioactivity. Compound (3) was converted into its dimethiodide and then into the corresponding methohydroxide. Hofmann degradation then gave the methine (5) which had essentially the same radioactivity as the parent base. Treatment of (5) with dimethyl sulphate–potassium hydroxide gave trimethylamine (trapped as its hydrochloride) which had essentially half the ^{14}C molar activity of the parent base, whereas the methine (6) had essentially all the ^{3}H activity. Treatment of (6) with osmium tetraoxide–sodium periodate gave the aldehyde (7), which had the same ^{3}H molar activity as (6) and the aldehyde (8) which was essentially inactive.

Feeding of (±)-*N*-methyl[1-^{3}H]coclaurine (13) (experiment 9) gave cocsulinin (1) labelled with ^{3}H. The location of the label in the biosynthetic base was shown as follows. Labelled cocsulinin was converted into di-*O*-methylcocsulinin (3) with essentially no loss of radioactivity. Reductive fission of (3) with sodium–liquid

[5] D. H. R. Barton, G. W. Kirby, and A. Wiechers, *J. Chem. Soc.* (*C*), 1966, 266.

ammonia using an improved procedure [6] gave a phenolic product which was purified and then treated with diazomethane to give the methyl ether. Second-stage fission of this compound with sodium–liquid ammonia afforded a mixture of phenolic and non-phenolic bases. Preparative t.l.c. of the non-phenolic bases afforded compounds (9) and (10).

The mixture of phenolic bases was treated with diazomethane to give methyl ethers which were separated by preparative t.l.c. to give compounds (9) and (14). Each product had essentially half the radioactivity of the parent base.

The foregoing experiments established that *N*-methylcoclaurine is a specific precursor of cocsulinin (1) in *C. laurifolius*. The precursors used, however, were racemic. The enzyme system involved in the relevant biotransformation would be expected to be stereospecific. Parallel feedings with (+)-(*S*)- and (−)-(*R*)-*N*-methylcoclaurines showed that the enzyme is stereospecific and that configuration at C-1 is maintained in the oxidative dimerisation of *N*-methylcoclaurine into cocsulinin. The former (15) (experiment 6) was incorporated into cocsulinin (1) about 57 times more efficiently than the latter (experiment 7).

According to the biogenetic theory the 6-*O*-methyl group of one of the *N*-methylcoclaurine (13) units should be eliminated in the biosynthesis of (1) from (13). Feeding of (±)-*N*-methyl[1-^{3}H, 6-*O*-methyl-^{14}C]coclaurine (13) (experiment 5) gave cocsulinin (1) labelled with both ^{14}C and ^{3}H. The ^{14}C : ^{3}H ratio in the precursor was 1 : 29 whereas in the biosynthetic base it was 1 : 60. Ziesel determination of the methoxy-group in the radioactive base gave triethylmethylammonium iodide which had essentially the same activity as the parent base.

The incorporation of (+)-(*S,S*)-*O*-methyl[*aryl*-^{3}H]-cocsulinin (2) (experiment 8) into cocsulinin (1) established that 6-de-*O*-methylation of (2) probably is the terminal step in the biosynthesis of cocsulinin (1).

The foregoing results strongly support the following sequence for the biosynthesis of cocsulinin in *C. laurifolius* DC: norcoclaurine (11) ⟶ coclaurine (12) ⟶ (+)-(*S*)-*N*-methylcoclaurine (15) ⟶ dimerisation ⟶ (+)-(*S,S*)-*O*-methylcocsulinin (2) ⟶ (+)-(*S,S*)-cocsulinin (1).

EXPERIMENTAL

Counting Methods.—Liquid scintillation counting was used for the measurement of ^{3}H and ^{14}C activities (Packard 3320 automatic Tri-carb instrument). Samples were counted in 7 ml of scintillator, after dissolution in methanol or dimethyl sulphoxide (0.2 ml) and values are 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.—(±)-Coclaurine,[7] -norcoclaurine,[8] and -*N*-methylcoclaurine [9] were prepared by standard procedures. (±)-Di-*O*-benzyl-*N*-methylcoclaurine was resolved by treatment with (−)-(*S,S*)- and (+)-(*R,R*)-di-*p*-toluoyltartaric acid. Hydrogenolysis of the benzyl ethers with hydrochloric acid furnished (−)-(*R*)- and (+)-(*S*)-*N*-methylcoclaurines.[9]

Labelling of Precursors.—Tritiation. Tritium was introduced according to the published procedure.[10] (±)-Coclaurine hydrochloride (120 mg) in tritiated water (0.6 ml; 60 mCi) containing potassium t-butoxide (200 mg) was heated (sealed tube) under nitrogen for 110 h at 100 °C to give (±)-[3′,5′,8-$^3H_3$]coclaurine (88 mg), which was purified as its hydrochloride to constant activity. The other 1-benzylisoquinoline precursors were tritiated in the same way.

(±)-*N*-Methyl[1-^{3}H]coclaurine and (±)-[1-^{3}H]norcoclaurine were prepared by reduction of the corresponding dihydroisoquinolines with potassium [^{3}H]borohydride in dry dimethylformamide. (±)-*N*-[^{14}C]Methylcoclaurine was prepared by treating the corresponding dihydroisoquinoline with [^{14}C]methyl iodide and subsequent reduction with sodium borohydride. (±)-*N*-Methyl[6-O-*methyl*-^{14}C]coclaurine was prepared by treatment of (±)-[6-*O*-*methyl*-^{14}C]-coclaurine with formic acid–formaldehyde. (±)-*NOO*-Trimethyl[3′,5′,8-$^3H_3$]coclaurine was prepared by treating (±)-*N*-methyl[3′,5′,8-$^3H_3$]coclaurine with diazomethane.

(±)-*N*-Methyl[1-^{3}H, 6-O-*methyl*-^{14}C]coclaurine was prepared by mixing (±)-*N*-methyl[1-^{3}H]coclaurine and (±)-*N*-methyl[6-O-*methyl*-^{14}C]coclaurine. *N*-[^{14}C]Methyl[1-^{3}H]-coclaurine was prepared by mixing (±)-*N*-methyl[1-^{3}H]-coclaurine and (±)-*N*-[^{14}C]methylcoclaurine.

(+)-O-*Methyl*[aryl-^{3}H]*cocsulinin*. *O*-Methylcocsulinin (2) (55 mg), tritiated water (0.5 ml, 100 mCi), and dimethylformamide (0.4 ml) were heated (sealed tube) under nitrogen at 100 °C for 110 h. The water and dimethylformamide were then removed *in vacuo*. The residue was chromatographed over a column of neutral alumina (7.5 g). Elution with chloroform–methanol (99 : 1) gave (+)-O-*methyl*-[aryl-^{3}H]*cocsulinin* (42 mg).

Feeding Experiments.—For feeding purposes *N*-methylcoclaurine and *NOO*-trimethylcoclaurine were dissolved in water (1 ml) containing tartaric acid (10 mg). Coclaurine hydrochloride and norcoclaurine hydrochloride were dissolved in aqueous dimethyl sulphoxide (1 ml). Freshly cut young branches of *C. laurifolius* were dipped into the solution of the precursor. When uptake was complete the twigs were dipped in water, left for 6—7 days, and then worked up for cocsulinin.

Isolation and Purification of Cocsulinin.—The young stems and leaves (typically 130 g wet) of *C. laurifolius* fed with precursor were macerated in ethanol (250 ml) containing inactive cocsulinin (100 mg) (dissolved in acetic acid) and left for 10 h. The ethanol was decanted and the plant material was percolated with fresh ethanol (6 × 200 ml) containing 0.1% acetic acid. The combined ethanolic extract was concentrated *in vacuo*. The green viscous mass so obtained was extracted with 5% hydrochloric acid (4 × 15 ml). The acidic solution was de-fatted with ether (3 × 10 ml) and basified with aqueous sodium carbonate. The precipitate (A) so obtained was filtered off. The filtrate was extracted with chloroform–methanol (3 : 1 v/v, 6 × 25 ml); the extract was washed with water and evaporated. The residue mixed with the precipitate (A) was chromatographed over a column of neutral alumina.

[6] Y. Inubushi, K. Nomura, and M. Miyawaki, *J. Pharm. Soc. Japan*, 1963, **83**, 282.
[7] K. Kratzl and G. Billek, *Monatsh.*, 1951, **82**, 568.
[8] H. Yamaguchi, *J. Pharm. Soc. Japan*, 1958, **78**, 692.
[9] H. Yamaguchi, *J. Pharm. Soc. Japan*, 1958, **78**, 678.
[10] D. S. Bhakuni, S. Satish, H. Uprety, and R. S. Kapil, *Phytochemistry*, 1974, **13**, 2767.

Elution (t.l.c. control) with chloroform–methanol (99 : 1) gave cocsulinin (75 mg), m.p. 261—262° (lit.,[1] 260—263°), which was crystallised from chloroform–methanol to constant activity.

Feeding of Doubly Labelled Precursors.—(1) (±)-*N*-[^{14}C]Methyl[1-^{3}H]coclaurine hydrochloride (activity ^{14}C 0.79 μCi, ^{3}H 15.8 μCi; ^{14}C : ^{3}H 1 : 20) was fed to freshly cut young branches of *C. laurifolius* and after 4 days the plants were harvested and worked up for cocsulinin (^{14}C activity 0.06 μCi mmol^{-1}, ^{3}H activity 1.27 μCi mmol^{-1}; ^{14}C : ^{3}H 1 : 21).

(2) (±)-*N*-Methyl[1-^{3}H, 6-O-*methyl*-^{14}C]coclaurine hydrochloride (activity ^{14}C 3 μCi, ^{3}H 87 μCi; ^{14}C : ^{3}H 1 : 29) was fed to the young freshly cut branches of *C. laurifolius*. After 4 days the plants were harvested and worked up for cocsulinin (^{14}C activity 0.028 μCi mmol^{-1}; ^{3}H 1.69 μCi mmol^{-1}; ^{14}C : ^{3}H 1 : 60).

Degradation of Doubly Labelled Cocsulinin.—Labelled cocsulinin (300 mg; 44.6 disint. min^{-1} mg^{-1}) derived from (±)-*N*-[^{14}C]methyl[1-^{3}H]coclaurine (experiment 3) in methanol (40 ml) was treated with ethereal diazomethane (from 2 g of nitrosomethylurea) to afford radioactive di-*O*-methylcocsulinin (3) (284 mg), m.p. 147—149° (lit.,[1] 145—146°) (^{14}C activity 0.01 μCi mmol^{-1}).

The preceding methyl ether (3) (280 mg) in methanol (20 ml) was heated to reflux with methyl iodide (9 ml) to give radioactive di-*O*-methylcocsulinin dimethiodide (280 mg), m.p. 260—262° (decomp.) (lit.,[1] 261—263°) (^{14}C activity 0.01 μCi mmol^{-1}).

A solution of the radioactive dimethiodide (275 mg) in methanol (50 ml) was passed through a column of Amberlite IR-410 (OH^- form) resin (8 g) to afford the corresponding methohydroxide, which was heated in methanol (8 ml) with potassium hydroxide (6 g) in water (22 ml) to give the methine (5) (200 mg), m.p. 122° (lit.,[1] 120°) (^{14}C activity 0.011 μCi mmol^{-1}). A solution of the methine (5) (195 mg) in water (10 ml) was adjusted to pH 10 with potassium hydroxide and then stirred at 0 °C with dimethyl sulphate (1 ml) and 10N-potassium hydroxide (0.5 ml) for 1 h. At hourly intervals, more dimethyl sulphate (3 × 0.5 ml) and 10N-potassium hydroxide (3 × 0.25 ml) were added, and after a total of 5 h potassium hydroxide (10 g) was added and the resulting solution was heated to reflux for 2 h. The trimethylamine evolved was collected in 15% hydrochloric acid (^{14}C activity 0.0058 μCi mmol^{-1}).

The remaining alkaline solution was extracted with chloroform (4 × 25 ml). The combined extract was washed with water, dried (Na_2SO_4), and evaporated. The residue in chloroform was passed through a silica gel column. The solvent was removed and the residue was crystallised from chloroform–methanol to give the methine (6) (145 mg), m.p. 250° (decomp.) (lit.,[1] 250—251°) (^{3}H activity 0.252 μCi mmol^{-1}).

To a stirred solution of the methine (6) (120 mg) in t-butyl alcohol (5 ml) and water (4 ml) was added aqueous osmium tetraoxide (1.02 ml; 4%). The orange-coloured complex so obtained was treated with sodium periodate (90 mg). After 2 h more sodium periodate (24 mg) was added. Stirring was continued for another 3 h and the mixture was decomposed with saturated aqueous arsenious oxide (34 ml). It was then extracted with ether–chloroform (3 : 1 v/v; 5 × 40 ml) and the extract was washed with water, dried (Na_2SO_4), and evaporated. The mixture of products was separated by preparative t.l.c. (chloroform–methanol, 99 : 1) to give *4,6-dimethoxydibenzo*[b,e][1,4]*dioxin-1,2,8,9-tetracarbaldehyde* (7) (7 mg), m.p. 110° (^{3}H activity 0.25 μCi mmol^{-1}), and the aldehyde [11] (8) (co-t.l.c. and i.r.), which was essentially radioinactive. A parallel experiment with radioinactive material gave an aldehyde, m.p. 111° (Found: M^+, 356.1574. $C_{18}H_{12}O_8$ requires M, 356.1571) identical with the radioactive aldehyde (7) (m.p., co-t.l.c., and i.r.).

Reductive Fission of Tritium-labelled Cocsulinin.—Cocsulinin (1) (395 mg) (activity 0.26 μCi mmol^{-1}) derived from (±)-*N*-methyl[1-^{3}H]coclaurine (experiment 9) in methanol (100 ml) was treated with an excess of ethereal diazomethane to give di-*O*-methylcocsulinin (3) (388 mg), m.p. 146—148° (lit.,[1] 145—146°) (activity 0.26 μCi mmol^{-1}).

Liquid ammonia (100 ml) (dried over sodium metal) was treated with sodium hydride (1.5 g) and to it was gradually added sodium (280 mg) (the solution acquired a bluish colour). A solution of radioactive (3) (280 mg) in dry toluene (10 ml) was then added dropwise. The blue colour was maintained by adding more sodium (50 mg) and the mixture was left at −68 °C for 3 h. Ammonia was allowed to evaporate off at room temperature. Excess of sodium was decomposed with ethanol, water was added, and the non-phenolic products were extracted with chloroform. The aqueous alkaline solution was saturated with ammonium chloride. The biphenyl derivative from the chloroform solution was extracted with citrate–phosphate buffer (pH 6.5). The solvent from the chloroform solution was removed. The residue in methanol was treated with ethereal diazomethane to give *O*-methyl derivatives, which, in dry toluene (15 ml), were added to liquid ammonia (100 ml) (dried over sodium) containing sodium (200 mg). More sodium was added until a blue colour persisted. After stirring at −60 °C for 3 h the mixture was worked up in the usual manner. The mixture so obtained was separated into phenolic and non-phenolic bases.

The non-phenolic bases were separated by preparative t.l.c. (chloroform–methanol, 9 : 1) to give (9), m.p. 115—117° (lit.,[6] 117—118°) (activity 0.13 μCi mmol^{-1}) and (10) (11 mg), m.p. 63—64° (lit.,[6] 64—65°) (activity 0.128 μCi mmol^{-1}).

The mixture of phenolic bases was treated with diazomethane to give methyl ethers which were subjected to preparative t.l.c. (benzene–ethyl acetate, 24 : 1) to give (9) [6] (activity 0.127 μCi mmol^{-1}) and (14), m.p. 60° (lit.,[12] 61—62°) (activity 0.130 μCi mmol^{-1}).

[7/1438 *Received*, 8*th August*, 1977]

[11] F. V. Bruchhausen, H. Oberembt, and A. Feldhans, *Annalen*, 1933, **507**, 144.

[12] M. Tomita and J. Kunitomo, *J. Pharm. Soc. Japan*, 1962, **82**, 734.