Biosynthesis of Boldine (1,10-Dimethoxy-6aα-aporphine-2,9-diol) [1]

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The incorporation of (±)-norprotosinomenine, (±)-nororientaline, (±)-nor-reticuline, and (±)-reticuline into boldine in *Litsea glutinosa* has been studied, and the specific utilization of the (±)-reticuline demonstrated. The evidence supports the direct oxidative coupling of (+)-reticuline to give isoboldine, which in turn is shown to be a specific precursor of boldine. The plants also converted norboldine into boldine. Double labelling experiments involving the methoxy-group of (±)-reticuline showed that most, but not all, of the methoxy-activity was lost in conversion into boldine.

BOLDINE (1), the choleretic principle of *Peumus boldus* Molina, could be formed in nature from suitable 1-benzyltetrahydroisoquinoline precursors through various biosynthetic routes. Direct oxidative coupling of reticuline [2] (8) could give rise to the boldine; alternatively oxidative coupling of orientaline [3] (10) and protosinomenine [4] (12) involving the dienones (15) and (16), respectively, as intermediates, would furnish the required aporphine system by dienone–phenol rearrangement. We now report the details of studies on these and some other aspects of boldine biosynthesis.

(±)-Tyrosine was initially fed to young shoots of *Litsea glutinosa* (Lauraceae), and it was found that the plants were actively biosynthesizing boldine. In subsequent experiments, labelled hypothetical precursors were fed to young *L. glutinosa* plants. The results of several feedings are recorded in the Table. Feeding (±)-tyrosine

Feeding of *L. glutinosa*

Expt.	Precursor fed	Incorporation (%) into boldine (1)
1	(±)-[2-^{14}C]Tyrosine	0.16
2	(±)-4′-*O*-Methyl[1-^{3}H]norlaudanosoline (6)	0.13
3	(±)-[5′,8-$^{3}H_2$]Nororientaline (9)	0.01
4	(±)-[3-^{14}C]Norprotosinomenine (11)	0.06
5	(±)-[3-^{14}C]Nor-reticuline (7)	0.62
6	(±)-[2′,6′,8-$^{3}H_3$]Reticuline (8)	0.44
7	(+)-[2′,6′,8-$^{3}H_3$]Reticuline (14)	0.43
8	(−)-[2′,6′.8-$^{3}H_3$]Reticuline	0.01
9	(±)-[1-^{3}H,6-*O*-$^{14}CH_3$]Reticuline (8)	0.90
10	(±)-[2′,6′,8-$^{3}H_3$]Laudanosine (13)	0.006
11	(+)-[8-^{3}H]Isoboldine (2)	2.00
12	(+)-[*aryl*-^{3}H]Norboldine (3)	1.86

in parallel with (±)-nororientaline (9) and (±)-norprotosinomenine (11) established that both these 1-benzyltetrahydroisoquinoline derivatives are poorly metabolised by the plants. Feeding with (±)-nor-reticuline (7) and (±)-reticuline (8) showed that both were efficient precursors of boldine (1). The completely methylated 1-benzyltetrahydroisoquinoline, (±)-laudanosine (13), as expected, was not incorporated.

Labelled boldine derived from (±)-reticuline feeding was brominated to afford 3-bromo- and 3,8-dibromoboldines. The former possessed essentially all the radioactivity (97.22% of the original), whereas the latter was virtually inactive (<1.00%).

The label in the biosynthetic boldine derived from (±)-nor-reticuline was located as follows. Radioactive boldine (1) was converted into glaucine (4) (no loss of activity). Degradation of (4) by double Hofmann elimination [5] gave the vinylphenanthrene (17) (no loss of activity). Cleavage of (17) by osmium tetraoxide–periodate gave the aldehyde (18) (inactive); the formaldehyde produced (dimedone derivative) had a molar activity 35% of that of the original base. The reduced activity of the formaldehyde may be a result of dilution with unlabelled formaldehyde arising from oxidation elsewhere in the molecule, *e.g.* *N*- and *O*-methyl groups (*cf.* ref. 6). The radioactive vinylphenanthrene (17) was then degraded alternatively by ozonolysis, giving formaldehyde (dimedone derivative; 98% of original activity).

The foregoing experiments established that reticuline (8) and nor-reticuline (7) are specific precursors of boldine (1) in *L. glutinosa*. The precursors used, however, were 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

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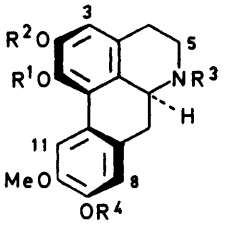
feedings with (+)-reticuline (14) and (−)-reticuline demonstrated that stereospecificity is maintained in the



(1) $R^1 = R^3 = Me, R^2 = R^4 = H$
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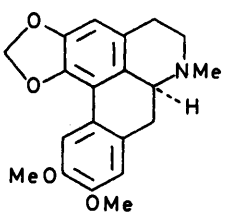
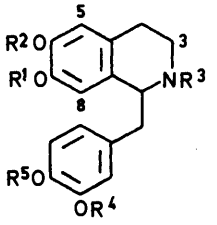
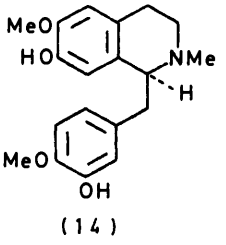


(5)



(6) $R^1 = R^2 = R^3 = R^4 = H, R^5 = Me$
(7) $R^1 = R^3 = R^4 = H, R^2 = R^5 = Me$
(8) $R^1 = R^4 = H, R^2 = R^3 = R^5 = Me$
(9) $R^1 = R^3 = R^5 = H, R^2 = R^4 = Me$
(10) $R^1 = R^5 = H, R^2 = R^3 = R^4 = Me$
(11) $R^1 = R^5 = Me, R^2 = R^3 = R^4 = H$
(12) $R^1 = R^3 = R^5 = Me, R^2 = R^4 = H$
(13) $R^1 = R^2 = R^3 = R^4 = R^5 = Me$



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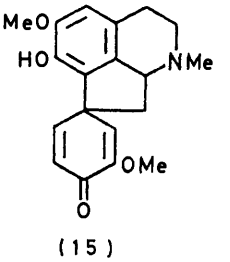

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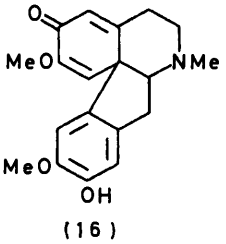

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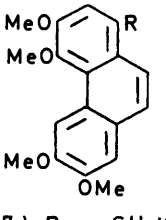

(17) R = $CH_2{:}CH$
(18) R = CHO

bioconversion of 1-benzylisoquinoline precursors into boldine. The former was incorporated 42 times more efficiently than the latter. The regiospecificity of the label in biosynthetic boldine derived from (+)-reticuline (14) feeding was established by the bromination method.

Incorporation of reticuline into boldine implies (+)-isoboldine (2) as an intermediate. Feeding of (+)-isoboldine (2) showed efficient incorporation into (1). The regiospecificity of the label in the radioactive boldine was again shown by bromination. The incorporation of isoboldine into boldine indicated that the change in the methylation pattern takes place, in the biosynthesis of boldine, after the oxidative coupling process.

The feeding experiments described above established that (+)-boldine (1) is biosynthesised from (+)-reticuline (14) *via* (+)-isoboldine (2) in the plant species. The precursors and the biosynthetic base, however, have different methoxy and hydroxy substitution patterns. Two processes could be considered to explain this transformation: [7] (i) demethylation–remethylation and (ii) migration of a methyl group. The loss of activity in the methoxy group (64% of original) in the biosynthesis of boldine from doubly labelled (±)-reticuline (8) provided evidence against methyl migration. Similar results have been obtained by Barton and his co-workers [7] in the biosynthesis of crotonosine in *Croton Linearis* Jacq.

Feeding (+)-norboldine (3) revealed that the plants are capable of converting norboldine into boldine. (±)-Reticuline and (±)-isoboldine have previously been isolated from *L. glutinosa*,[8] and we confirmed the presence of the former by feeding (−)-[2-^{12}C]tyrosine (incorporation 0.16%); (+)-reticuline and (+)-isoboldine are thus true intermediates. The young shoots of *L. glutinosa* were also found to metabolise (±)-4′-*O*-methylnorlaudanosoline (6), and its presence in the plants was shown by a trapping experiment {(−)-[2-^{14}C]tyrosine; incorporation 0·17%}. The foregoing results thus strongly support the following sequence for the biosynthesis of boldine in *L. glutinosa*: 4′-*O*-methylnorlaudanosoline (6) ⟶ nor-reticuline (7) ⟶ (+)-reticuline (14) ⟶ (+)-isoboldine (2) ⟶ (+)-boldine (1).

EXPERIMENTAL

Unless otherwise stated, u.v. absorption spectra refer to solutions in ethanol, i.r. absorption spectra to KBr discs, and n.m.r. spectra to solutions in deuteriochloroform. The n.m.r. spectra were recorded with a Varian A-60 spectrometer. T.l.c. 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 314 Ex instrument). Samples were counted in 7 ml of scintillator, after dissolution in methanol or dimethylformamide (0.2 ml) and values are not corrected for self-absorption except where stated. Relative efficiencies were obtained by counting [1,2-^{3}H]- and [2-^{14}C]-hexadecane standards.

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Synthesis of Benzylisoquinoline Precursors.—The racemates of reticuline,[9] nor-reticuline, norprotosinomenine,[9] and nororientaline [10] were prepared by standard methods. (+)-*OO*-Dibenzylreticuline was resolved by treatment with (+)- and (−)-*OO*-dibenzyltartaric acids.[11] Hydrogenolysis of the products with hydrochloric acid furnished (−)-reticuline and (+)-reticulines, respectively.

Labelling of Precursors—Tritiation. (+)-Reticuline (100 mg) in tritiated water (0.6 ml; activity 60 mCi) containing potassium t-butoxide (200 mg) was heated under nitrogen (sealed tube) for 100 h at 100 °C. The mixture was diluted with water, ammonium chloride was added (pH 7) and the liberated base was extracted with chloroform (3 × 15 ml). The extracts were washed with water and evaporated. The crude product was chromatographed on a column of neutral alumina. Elution with chloroform–methanol (98 : 2) afforded material which was further purified through its perchlorate to give (±)-[2′,6′,8-$^3H_3$]reticuline (8). (±)-[5′,8-$^3H_2$]Nororientaline (9), (+)-[2′,6′,8-$^3H_3$] reticuline (14), and (−)-[2′,6′,8-$^3H_3$]reticuline were prepared similarly. (±)-[2′,6′,8-$^3H_3$]Laudanosine (13) was prepared from (±)-[2′,6′,8-$^3H_3$]reticuline (8) by treatment with diazomethane.

(+)-[aryl-^{3}H]*Norboldine* (3). Thionyl chloride (0.4 ml) was added to tritiated water (0.6 ml), followed by (+)-norboldine (80 mg). The mixture was heated under nitrogen (sealed tube) for 90 h at 100 °C, diluted with water, and basified with sodium carbonate. The liberated base was extracted with chloroform; the extract was washed with water, dried, and evaporated. The crude product was chromatographed on a column of silica. Elution with chloroform–methanol (90 : 10) afforded (+)-[*aryl*-^{3}H]norboldine.

(±)-[3-^{14}C]Nor-reticuline (7) and (±)-[3-^{14}C]norprotosinomenine were prepared by standard procedures.[11] (±)-[1-^{3}H, 6-*O*-$^{14}CH_3$]Reticuline (8) was a mixture of (±)-[1-^{3}H] reticuline and (±)-[6-*O*-$^{14}CH_3$] reticuline. (±)-[1-^{3}H]Reticuline was prepared by reduction of 7-benzyloxy-1-(3-benzyloxy-4-methoxybenzyl)-3,4-dihydro-6-methoxyisoquinoline methiodide in dry dimethyl sulphoxide with sodium [^{3}H]borohydride followed by debenzylation. (±)-4′-*O*-Methyl[1-^{3}H]norlaudanosoline (6) was similarly prepared by reduction of the corresponding dihydroisoquinoline derivative with sodium [^{3}H]borohydride. (±)-[6-*O*-$^{14}CH_3$]Reticuline was prepared by a standard method.[11]

Feeding Experiments.—For feeding purposes tyrosine and reticuline, norprotosinomenine, and 4′-*O*-methyl-laudanosoline hydrochlorides were dissolved in water. Nororientaline, laudanosine, nor-reticuline, and norboldine were dissolved in water (1 ml) containing tartaric acid (10 mg). Isoboldine was dissolved in aqueous dimethyl sulphoxide (1 ml). Into the solution of precursor, freshly cut young *L. glutinosa* plants without roots were dipped and allowed to take up the precursor, then washed with water. The plants were then dipped in water and left for 5–6 days for metabolism, and then worked up for boldine.

Isolation and Purification of Boldine.—Stems and leaves (typically 100 g wet wt.) of the plants were macerated in ethanol (500 ml) with inactive boldine (130 mg) and left for 24 h. The ethanol was then decanted and the plant material was percolated with fresh ethanol (6 × 300 ml). The combined ethanolic extract was concentrated *in vacuo*. The green viscous mass so obtained was treated with N-hydrochloric acid (2 × 5 ml). The acidic solution was defatted with hexane (6 × 50 ml) and then basified with aqueous sodium hydrogen carbonate. The liberated bases were extracted with chloroform (6 × 50 ml); the extracts were washed with water, dried, and evaporated to afford crude boldine (120 mg), which was chromatographed over a column of basic alumina (12 g). Elution was carried out with chloroform and chloroform–methanol (95 : 5). The fractions containing pure boldine (t.l.c.) were mixed and evaporated, and the residue was crystallized from chloroform–hexane to afford boldine (95 mg), m.p. 162—163° (lit.,[12] 162—163°). The base was further converted into its hydrochloride, m.p. 233—235° (decomp.) and was crystallized from methanol–ether to constant activity. The radiopurity of the biosynthetic boldine was checked by the dilution method.

Bromination of [8-^{3}H]*Boldine.*—To a solution of [8-^{3}H]-boldine (60 mg; specific activity 1050 disint. min^{-1} mg^{-1}; molar activity 3.55 × 10^5 disint. min^{-1} mmol^{-1}) in carbon tetrachloride (10 ml) was added dropwise a dilute solution (1%) of bromine in carbon tetrachloride until no more precipitation occurred. After 10 h at 5 °C the precipitate was filtered off, washed with carbon tetrachloride (3 × 5 ml), and dried. The bromide was dissolved in water (3 ml) and basified with aqueous sodium hydrogen carbonate, and the liberated base was extracted with chloroform (5 × 20 ml). The extract was washed with water, dried, and evaporated, and the residue (50 mg) was chromatographed on a column of silica gel (6 g). Elution with chloroform–methanol (95 : 5) afforded inactive 3,8-*dibromoboldine* (20 mg), m.p. 199—200°, as needles (from hot benzene), $\lambda_{max.}$ 207, 255, 257, 283, and 310 nm (log ε 5.59, 5.68, 5.68, 5.72, and 5.75), τ 7.42 (3 H, s, NCH_3), 6.38 (3 H, s, 1-OCH_3), 6.06 (3 H, s, 10-OCH_3), and 2.16 (1 H, s, 11-H) (Found: C, 47.15; H, 4.25; N, 2.9. $C_{19}H_{19}Br_2NO_4$ requires C, 47.0; H, 3.9; N, 2.9%).

Continued elution of the column with chloroform–methanol (95 : 5) (t.l.c. control) yielded 3-*bromoboldine* (15 mg), m.p. 208—209°, as needles (from benzene), $\lambda_{max.}$ 238, 285, and 311 nm (log ε 5.29, 5.37, and 5.41), τ 7.40 (3 H, s, NCH_3), 6.31 (3 H, s, 1-OCH_3), 6.05 (3 H, s, 10-OCH_3), 3.18 (1 H, s, 8-H), and 2.12 (1 H, s, 11-H) (Found: C, 55.8; H, 4.75; N, 3.25. $C_{19}H_{20}BrNO_4$ requires C, 56.15; H, 4.9; N, 3.45%) (specific activity 866 disint. min^{-1} mg^{-1}, molar activity 3.5 × 10^5 disint. min^{-1} mmol^{-1}).

Degradation of [5-^{14}C]*Boldine.*—Labelled boldine (180 mg) in methanol (4 ml) was treated with an excess of ethereal diazomethane to yield labelled glaucine (150 mg), m.p. 120—121° (lit.,[13] 120°) (specific activity 367 disint. min^{-1} mg^{-1}; molar activity 1.30 × 10^5 disint. min^{-1} mmol^{-1}). A solution of the active glaucine (50 mg) in methanol (2 ml) was heated under reflux with methyl iodide (1 ml) for 2 h. The excess of solvent and methyl iodide were removed *in vacuo* and the residue was crystallized from methanol to give labelled glaucine methiodide (65 mg), m.p. 229—230° (lit.,[5] 221°) (specific activity 278 disint. min^{-1} mg^{-1}; molar activity 1.38 × 10^5 disint. min^{-1} mmol^{-1}).

A solution of labelled glaucine methiodide (65 mg) in methanol was passed through a column of freshly regenerated IR-410 anion-exchange resin (2.6 g) to afford labelled

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glaucine methohydroxide. The solution was concentrated to 5 ml and refluxed for 2 h with potassium hydroxide (1.2 g). It was then cooled, diluted with water, and extracted with ether–chloroform (3 : 1 v/v; 5 × 50 ml). The extracts were washed with water, dried, and evaporated to yield the methine base as an oil [5] (49 mg), τ 7.60 (6 H, s, NMe_2), 6.51—7.50 (4 H, m, 2-H_2), 6.05 (3 H, s, OCH_3), 5.96 (6 H, s, 2 OCH_3), 5.90 (3 H, s, OCH_3), 2.78 (1 H, s, 1- or 7-H), 2.71 (1 H, s, 1- or 7-H), 2.32 (2 H, q, 9- and 10-H, J 16 Hz), and 0.77 (1 H, s, 4-H).

The methine base (49 mg), methanol (2 ml), and methyl iodide (1 ml) were refluxed for 2 h to yield labelled glaucine methine methiodide (65.5 mg), m.p. 278—279° (lit.,[5] 276—280°) (specific activity 259 disint. min^{-1} mg^{-1}; molar activity 1.32 × 10^5 disint min^{-1} $mmol^{-1}$). The methiodide (65.5 mg) was converted into its hydroxide form by use of anion-exchange resin as described earlier, and then refluxed with methanolic potassium hydroxide (1.2 g) for 2 h. The resulting solution was cooled, diluted with water, and extracted with ether–chloroform (3 : 1 v/v; 5 × 50 ml). The extracts were washed with water, dried, and evaporated to give 3,4,6,7-tetramethoxy-1-vinylphenanthrene (17) (45 mg), m.p. 141—142° (lit.,[5] 143°) (specific activity 348 disint. min^{-1} $mmol^{-1}$).

Cleavage of the vinylphenanthrene (17) *with osmium tetraoxide–periodate.* To a stirred solution of compound (17) (45 mg) in t-butyl alcohol (3 ml) and water (2.5 ml) was added aqueous osmium tetraoxide (4%; 0.75 ml). To the resulting orange suspension, sodium periodate (59 mg) was added. More periodate (10 mg) was added after 2 h. Stirring was continued for another 3 h and the mixture was decomposed with saturated aqueous arsenic(III) oxide (29 ml). It was then extracted with ether (3 × 25 ml); the extracts were washed with water, dried, and evaporated, and the resulting gum (30 mg) was chromatographed on silica gel (1.5 g). Elution with benzene furnished inactive 3,4,6,7-tetramethoxyphenanthrene-1-carbaldehyde (18) (15 mg), m.p. 173—174° (lit.,[5] 173°).

The aqueous mother liquor was adjusted to pH 10 with potassium carbonate. Dimedone (176.4 mg) was then added and after 10 min the pH was adjusted to 6. The mixture was left for 20 h at room temperature, then the solid was filtered off, washed with water and dried. It was dissolved in chloroform and passed through basic alumina to give labelled formaldehyde dimedone derivative, m.p. 193—194° [specific activity 156 disint. min^{-1} mg^{-1}; molar activity 4.5 × 10^3 disint. min^{-1} $mmol^{-1}$ (35.06% of the original)].

Ozonolysis of the vinylphenanthrene. Ozonized oxygen was passed through a solution of compound (17) (130 mg; specific activity 194 disint. min^{-1} mg^{-1}; molar activity 6.29 × 10^4 disint. min^{-1} $mmol^{-1}$) in ethyl acetate (8 ml) at −78 °C for 10 min. The solvent was removed under reduced pressure and to the residue, water (35 ml), zinc dust (325 mg), and silver nitrate (16 mg) were added. The mixture was refluxed for 20 min and then distilled. The distillate was collected in a solution of dimedone (325 mg) in aqueous ethanol (81 ml). After 1 h, it was concentrated to 10 ml and left for 12 h. The precipitated solid in chloroform was chromatographed over silica. Elution with chloroform (t.l.c. control) afforded formaldehyde dimedone derivative, m.p. 193—194°, as needles (from ethanol) [specific activity 211 disint. min^{-1} mg^{-1}; molar activity 6.16 × 10^4 disint. min^{-1} $mmol^{-1}$ (98% original)].

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