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BIOSYNTHESIS OF THALICARPINE†

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Abstract—The incorporation of (±)-, norlaudanosoline, -nor-reticuline, -reticuline, -N-methylcoclaurine and -norlaudanidine into thalicarpine in *Cocculus laurifolius* DC has been studied and specific utilization of (±)-reticuline demonstrated. The evidence supports that both the "halves" of thalicarpine are derived from reticuline. Parallel feedings of (*S*)-, and (*R*)-, reticulines showed that the stereospecificity is maintained in the biosynthesis of thalicarpine from the 1-benzyl-tetrahydroisoquinoline precursor.

A double-labelling experiment with (±)-[1-^{3}H, 4'-O^{14}CH$_3$] nor-reticuline has shown that the 4'-O-Me group of a nor-reticuline unit is lost in the biotransformation into thalicarpine. Feeding experiments also revealed that the plants can convert (*S*)-bolding and (*S*)-isoboldine into thalicarpine.

Tumor inhibitory[1] base, thalicarpine[2] (**16**) which also exhibited hypotensive activity,[2] is a representative of aporphine-1-benzylisoquinoline alkaloids.[3] Biogenetically thalicarpine (**16**) is unique. It is one of the rare dimeric alkaloid which can derive in nature from two reticuline units. It is suggested[3] that thalicarpine (**16**) type of aporphine - 1 - benzylisoquinoline alkaloids can be formed in nature by oxidative coupling[4] of 1 - benzyl-tetrahydroisoquinoline precursor of reticuline (**1**) type with 1,2,9,10 - tetrasubstituted aporphine of N - methyl-laurotetanine (**2**) type. The aporphine itself can be derived from reticuline.

Thalicarpine (**16**) alternately can derive in nature from (*S*)-reticuline (**1**) as follows: oxidative dimerization[4] of (*S*)-reticuline (**1**) can give the bisbenzylisoquinoline dimer (**12**). Selective O-demethylation of **12** can yield the phenolic base **13**. Intra-molecular oxidative coupling in the "right half" of the molecule of the phenolic base (**13**) can give the key proaporphine - 1 - benzylisoquinoline intermediate (**14**). Dienone-phenol rearrangement as shown in **14** can furnish the aporphine-1-benzylisoquinoline (**15**) system. O-Methylation of the phenolic hydroxylic groups in **15** can finally give thalicarpine (**16**).

(±)-Tyrosine (Exp. 1) was initially fed to young cut branches of *Cocculus laurifolius* DC. (Menispermaceae) and it was found that thalicarpine (**16**) 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 Table 1.

Feeding of (±)-norlaudanosoline (**2**) (Exp. 2) and (±)-reticuline (**7**) (Exp. 3) established that **2** and **7** were efficient precursors of thalicarpine (**16**). (±)-Norlaudanine (**6**) (Exp. 4) was less efficiently utilized by the plant to form **16**. (±)-N-Methylcoclaurine (**9**) (Exp. 5) was not metabolized by the plants to form thalicarpine (**16**). Feeding of (*S*)- boldine (**3**) (Exp. 9) and (*S*)- isoboldine (**4**) (Exp. 10) revealed that plants had the capability to transform **3** and **4** into thalicarpine (**16**).

Biosynthetic thalicarpine (**16**) derived from (±)- reticuline (**7**) (Exp. 3) feeding was subjected to sodium–liquid ammonia reductive fission[5] to give (−) - 6' - hydroxy-laudanosine (**10**) and (+) - 2,10-dimethoxy-aporphine (**11**). The former had essentially 2/3 molar activity and the latter 1/2 molar radioactivity of the parent base (**16**) in accordance with the theory.

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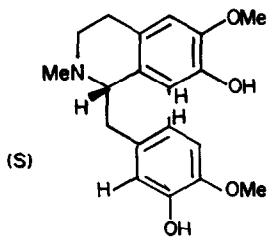
Table 1. Tracer experiment on *C. laurifolius*

Expt.	Precursor Fed	Incorporation % into Thalicarpine(16)
1.	(L)-[U-^{14}C] Tyrosine	0.10
2.	(±)-[1-^{3}H] Norlaudanosoline(5)	0.18
3.	(±)-[2',6', 8-$^{3}H_3$] Reticuline(7)	1.34
4.	(±)-[2',6', -$^{3}H_2$] Norlaudanine(6)	0.30
5.	(±)-[3',5',8-^{3}H] N-Methylcoclaurine(9)	0.008
6.	(S)-[2',6',8-$^{3}H_3$] Reticuline(1)	1.6
7.	(R)-[2',6',8-$^{3}H_3$] Reticuline	0.016
8.	(±)-[1-^{3}H, 4'-O-$^{14}CH_3$] Norreticuline(8)	1.8
9.	(±)-[3,8-$^{3}H_2$] Boldine(3)	0.29
10.	(+)-[8-^{3}H] Isoboldine(4)	0.51



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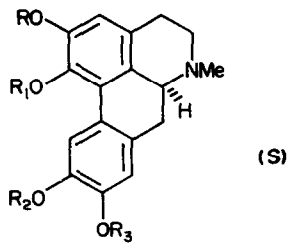
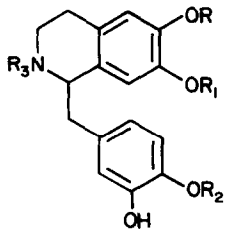
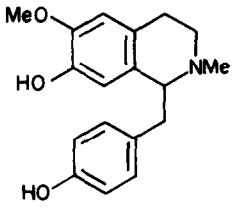


(2) R=R1=R2=Me; R3=H
(3) R=R3=H; R1=R2=Me
(4) R=R2=Me; R1=R3=H



(5) R=R1=R2=R3=H
(6) R=R1=R2=Me; R3=H
(7) R=R2=R3=Me; R1=H
(8) R=R2=Me; R1=R3=H



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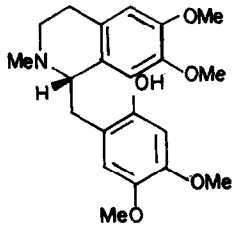

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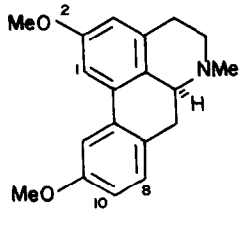

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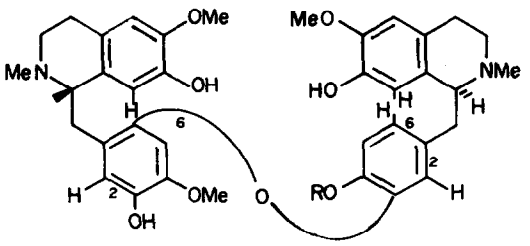
The foregoing experiments established that both the "halves" of thalicarpine (**16**) are derived from reticuline in *C. laurifolius*. The precursors used, however, were racemic. The enzyme system involved in the relevant biotransformation would be expected to be stereospecific. Parallel feedings with (*S*)- and (*R*)- reticulines showed that the enzyme system is stereospecific and that configuration at C-1 is maintained in the oxidative dimerisation of reticuline into **12** and in subsequent transformation into thalicarpine (**16**). (*S*)-Reticuline (**1**) (Exp. 6) was incorporated about 100 times more efficiently than (*R*)-reticuline (Exp. 7) into thalicarpine (**16**).

Bioconversion of the bisbenzylisoquinoline dimer (**12**) into aporphine - 1 - benzylisoquinoline (**15**) system in plants can take place as follows: selective O-demethylation in the benzylic "half" of one reticuline unit of the dimer (**12**) can yield **13**. Intramolecular oxidative coupling in **13** can then provide the key proaporphine - 1 - benzylisoquinoline (**14**) intermediate. Compound **14** can then undergo dienone-phenol rearrangement to give the aporphine - 1 - benzylisoquinoline (**15**) system. O-Methylation of **15** can finally yield thalicarpine (**16**). Feeding of (±) - [1-^{3}H, 4'-O-$^{14}CH_3$] nor-reticuline (**8**) (Exp. 8) gave thalicarpine (**16**) labelled with both ^{14}C and ^{3}H. The ^{14}C:^{3}H ratio in the precursor (**8**) was 1:23 whereas in the biosynthetic base (**16**) it was 1:44.

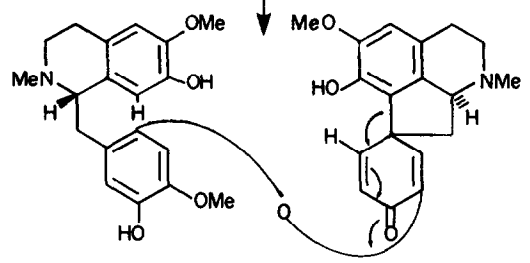
Biosynthetic thalicarpine (**16**) derived from nor-reticuline (**8**) (Exp. 8) feeding was subjected to sodium-ammonia reductive fission to give (−) - 6' - hydroxy-laudanosine (**10**) and (+) - 2,10 - dimethoxy - aporphine (**11**). The former (**10**) had both ^{14}C and ^{3}H activities whereas the latter (**11**) had essentially only tritium activity. Moreover ^{14}C:^{3}H ratios in nor-reticuline (**8**) was 1:23 and in **10** it was 1:20. The results thus confirmed that selective O-demethylation had occurred in one reticuline derived moiety in the bioconversion of **8** into thalicarpine (**16**).

(*S*)- Reticuline (**1**) has been found to be specifically incorporated into thalicarpine (**16**), and it has also been isolated from *C. laurifolius*[6] DC. Thus **1** is a true precursor of **16**. The foregoing results strongly support the following sequence for the biosynthesis of thalicarpine in *C. laurifolius* DC: tyrosine → norlaudanosoline (**5**) → nor-reticuline (**8**) → (*S*)-reticuline (**1**) → (dimerisation) → (selective - O - demethylation) → thalicarpine (**16**).

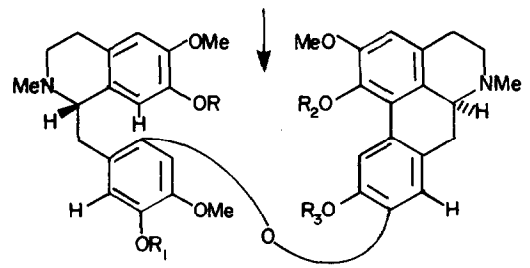
Marekov and Sidjimov[7] have demonstrated that thaliocarpine (**16**) in *Thalictrum minus* is biosynthesised



(12) R=Me
(13) R=H



(14)



(15) R=R₁=R₂=R₃=H
(16) R=R₁=R₂=R₃=Me

from (±)-reticuline. This report came to our notice when we had completed our work on the biosynthesis of thalicarpine.

EXPERIMENTAL

Counting methods. Liquid scintillation counting was used for the measurement of ^3H 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-$^3H_2$], and [2-^{14}C]-hexadecane standards.

Synthesis of benzylisoquinoline precursors. The racemates of reticuline,[8] nor-reticuline,[9] norlaudanosoline,[9] norlaudanine and N-methylcoclaurine[10] were prepared by standard methods. (±)-OO-Dibenzyl reticuline was resolved by treatment with (±)-, and (−)-OO-dibenzyltartaric acids.[11] Hydrogenolysis of the products with HCl furnished (−)-reticuline and (+)-reticuline (**1**), respectively.

Labelling of precursors—Tritiation. Reticuline (120 mg) in tritiated water (0.6 ml; activity 100 mCi) containing t-BuOK (200 mg) was heated under N_2 (sealed tube) for 110 hr at 100°. The mixture was diluted with water, ammonium chloride was added (pH 7) and the liberated base was extracted with $CHCl_3$ (3 × 15 ml). The extract was washed with water, dried and evaporated. The crude product was chromatographed on a column of neutral Al_2O_3. Elution with $CHCl_3$:MeOH (98:2) afforded material which was further purified through its perchlorate to give (±)- **7**. (±)- **6**, (±)[3',5',8-$^3H_3$] **9**, (*R*)-[2',6',8-$^3H_3$]reticuline, (*S*)- **3**, (*S*)- **4** were prepared similarly. (±)-**5** and [1-^{3}H]-**8** were prepared by reduction of the corresponding dihydroisoquinoline in dry dimethyl sulphoxide with sodium [^{3}H]borohydride. (±)-[4'-O-$^{14}CH_3$]Reticuline was prepared by standard method.[10] (±)-[1-^{3}H, 4'-O-$^{14}CH_3$]Reticuline was obtained by mixing of (±)-[1-^{3}H]reticuline and (±)-[4'-O-$^{14}CH_3$]reticuline.

Feeding experiments. For feeding purposes tyrosine, reticuline, nor-laudanine hydrochlorides were dissolved in water. Boldine was dissolved in water (1 ml) containing tartaric acid (10 mg). Norilaudanosoline, N-methylcoclaurine were dissolved in aqueous dimethyl sulphoxide (1 ml). Into the soln of precursor, freshly cut young branches of *C. laurifolius* were dipped and allowed to take up the precursor. The plants were then dipped in water and left for 5 to 6 days for metabolism and worked up for **16**.

Isolation and purification of thalicarpine. Fresh twigs (typically 80–100 g wet wt) of *C. laurifolius* were macerated in EtOH (400 ml) with radioinactive **16** (110 mg) and left for 24 hr. The EtOH was then decanted and the plant material was percolated with fresh EtOH (6 × 300 ml). The combined ethanolic extract was concentrated *in vacuo*. The green viscous mass, so obtained, was extracted with 10% AcOH. The acidic soln was defatted with hexane (2 × 15 ml) and ether (4 × 20 ml), basified with Na_2CO_3 (pH 8) and the liberated bases were extracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O, dried (Na_2SO_4) and solvent was removed. The crude base, so obtained, was subjected to preparative tlc (plate: SiO_2, $CHCl_3$:MeOH, 94:6, double run). The major spot on the plates was cut and extracted with $CHCl_3$:MeOH (85:15). Removal of the solvent from the extract gave a residue which on treatment with acetone–ether gave pure **16** (75 mg), m.p. 160–162° (lit.[2] 160–161°). The ratio purity of the biosynthetic thalicarpine was checked by the dilution method.

Reductive fission of the biosynthetic thalicarpine (**16**). A soln of **16** (200 mg) (molar activity 2.35 μCi m mol^{-1}) derived from (±)-[2',6',8-$^3H_3$] reticuline (Exp. 3) feeding in dry toluene (12 ml) was added dropwise to liquid ammonia (150 ml), pretreated with sodium hydride (1 g) and containing Na (400 mg). The resulting mixture was stirred at −68° and again Na (200 mg) was added till a blue colour persisted. The mixture was left for 3 hr. Ammonia was then allowed to evaporate off at room temp. Water was added to the residue and the product was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O, dried (Na_2SO_4) and solvent removed. The residue, so obtained, was dissolved in 10% HCl. The acidic soln was extracted with hexane, basified with NH_4OH and the liberated bases extracted with ether (5 × 35 ml). The ether extract was washed with H_2O, dried (Na_2SO_4) and solvent removed to give the crude product (140 mg) which was subjected to preparative tlc (plates: SiO_2, solvent: $CHCl_3$:MeOH, 95:5). The two major bands on the plates were cut and extracted with $CHCl_3$:MeOH (80:20). Removal of the solvent from the less polar band gave (±)- **11**[5] (molar activity 0.664 μCi m mol^{-1}). The polar band contained (−)- **10**[5] (molar activity 1.35 μCi m mol^{-1}).

Reductive fission of doubly labelled biosynthetic thalicarpine (**16**). Compound **16** (230 mg) (molar ^{3}H activity 0.17 μCi m mol^{-1}; ^{14}C activity 3.88 × 10^{-3} μCi m mol^{-1}; ^{14}C:^{3}H, 1:44) was subjected to Na/liq. NH_3 reduction as above to give (−)- **10**[5] (molar ^{3}H activity 0.085 μCi m mol^{-1}; ^{14}C activity, 4.27 × 10^{-3} μCi m mol^{-1}, ^{14}C: ^{3}H, 1:20) and **11**[5] (molar ^{3}H activity 0.09 μCi m mol^{-1}).

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