Biosynthesis of Aristolochic Acid †

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The incorporation of tyrosine, (3,4-dihydroxyphenyl)alanine, nororientaline, orientaline, prestephanine, and stephanine into aristolochic acid in *Aristolochia bracteata* has been studied; specific utilisation of nororientaline has been demonstrated. The evidence strongly supports the hypothesis that the oxidative coupling of orientaline gives prestephanine, which is converted into stephanine; oxidative cleavage of stephanine then furnishes aristolochic acid. An experiment with doubly labelled nororientaline showed its incorporation intact into the product, and confirmed the view that the methylenedioxy-group in aristolochic acid originates from an *o*-methoxyphenol precursor. Parallel feedings of (—)- and (+)-orientaline confirmed that stereospecificity is maintained in the biosynthesis of aristolochic acid from the 1-benzyltetrahydroisoquinoline precursors.

ARISTOLOCHIC ACID [1] (6), a representative of the substituted 10-nitrophenanthrene-1-acids which occur in many species of the genus *Aristolochia* and several other members of the family Aristolochiaceae, is a potent tumour inhibitor.[2] Biogenetically it is considered to be derived from 1-benzyltetrahydroisoquinoline precursors *via* aporphine intermediates.[3-5]

The incorporation of tyrosine, (3,4-dihydroxyphenyl)-alanine (dopa), and 3,4-dihydroxyphenethylamine (dopamine) into aristolochic acid in *A. sipho* (Dutchman's pipe) suggested the involvement of 1-benzylisoquinoline precursors,[6] and the specific incorporation of norlaudanosoline (8) into (6) confirmed this hypothesis.[7] Feeding of doubly labelled [3-^{14}C,^{15}N]tyrosine demonstrated that the nitro group of aristolochic acid originated from the amino-function of tyrosine.[1] Further confirmation of the biogenetic route norlaudanosoline (8) ⟶ orientaline (1) ⟶ orientalinone (2) ⟶ orientalinol (3) ⟶ prestephanine (4) ⟶ stephanine (5) ⟶ aristolochic acid (6) is presented in this paper.

TABLE 1

Tracer experiments on *A. bracteata*

Expt.	Precursor fed	Incorporation (%) into aristolochic acid (6)
1	(—)-[U-^{14}C]Tyrosine	0.01
2	(±)-[3-^{14}C]Dopa	0.01
3	(±)-[5′,8-$^{3}H_2$]Nororientaline (9)	0.046
4	(±)-[5′,8-$^{3}H_2$]Orientaline (1)	0.324
5	(±)-[5′,8-$^{3}H_2$; 6-*methoxy*-^{14}C]-Nororientaline (9)	0.029 (^{14}C)
6	(—)-[5′,8-$^{3}H_2$]Orientaline (1)	0.234
7	(+)-[5′,8-$^{3}H_2$]Orientaline	0.06
8	(±)-[*aryl*-^{3}H]Prestephanine (4)	0.036
9	(±)-[*aryl*-^{3}H]Stephanine (5)	0.145

(L)-[U-^{14}C]Tyrosine (experiment 1) was initially fed to *A. bracteata* Linn. It was found that the plants were actively biosynthesising aristolochic acid. In subsequent experiments labelled dopa and various hypothetical 1-benzyltetrahydroisoquinoline and aporphine precursors were fed to *A. bracteata*. The results are recorded in Table 1.

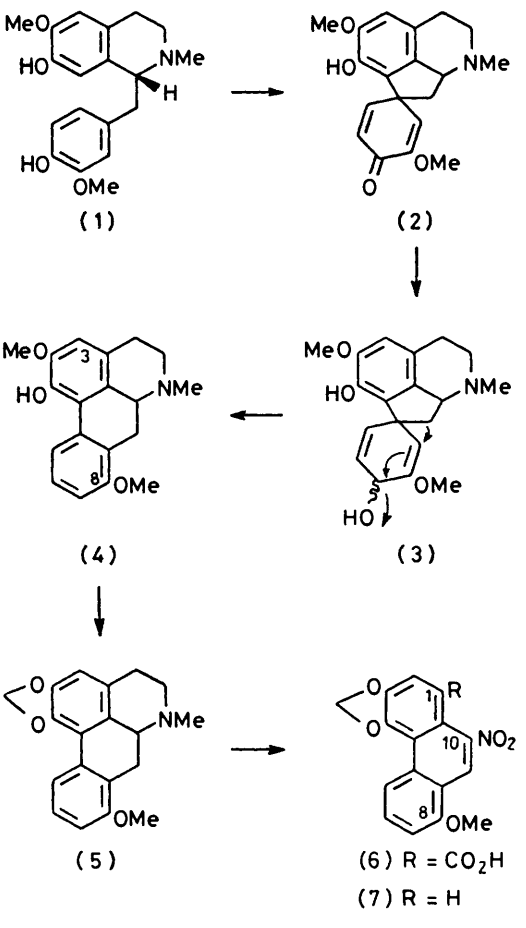
† 8-Methoxy-3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid.

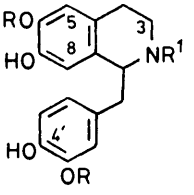
Biosynthetic aristolochic acid derived from DL-[3-^{14}C]dopa (experiment 2) was heated under nitrogen with copper powder in quinoline to yield radioactive CO_2. This was trapped as $BaCO_3$ which had 96.2% of the radioactivity of the parent compound (6); the decarboxylation product (7) was virtually inactive (0.69%). These results established that dopa is the main contributor to the formation of the phenethylamine portion of the 1-benzyltetrahydroisoquinoline precursor, norlaudanosoline, which gives rise to aristolochic acid in *A. bracteata*. Similar results were obtained earlier for the biosynthesis of reticuline [8] and other 1-benzylisoquinoline-derived alkaloids.[9] Spenser and Tiwari,[6] however, reported that all the activity of the biosynthetic aristolochic acid (6) derived from [2-^{14}C]dopa was located at C-10, indicating that dopa is incorporated significantly into the 'lower' part of the molecule (6). We have observed extremely low incorporations of dopa in the 'lower' part of aristolochic acid in *A. bracteata*.

(±)-[5′,8-$^{3}H_2$]Nororientaline (9) (experiment 3) and (±)-[5′,8-$^{3}H_2$]orientaline (1) (experiment 4) were both incorporated into (6) in *A. bracteata*. Specific incorporation of nororientaline into aristolochic acid was demonstrated as follows: (±)-[5′,8-$^{3}H_2$; 6-*methoxy*-^{14}C]nororientaline (experiment 5) was fed to young *A. bracteata*. Biosynthetic aristolochic acid was isolated and found to possess both ^{14}C and ^{3}H activities. Moreover, the ^{14}C : ^{3}H ratio (after allowance for the loss of tritium from position 8 in the precursor) was essentially the same in the precursor as in the product. The methylenedioxy-group of the biosynthetic aristolochic acid was cleaved by treatment with hydrochloric acid, and the radioactive formaldehyde so formed was trapped with dimedone (activity 82%). The results thus established specific incorporation of nororientaline into aristolochic acid and confirmed that the methylenedioxy-group in aristolochic acid, as in other compounds,[10-12] is formed from an *o*-methoxyphenol precursor.

The foregoing experiments established that both nororientaline and orientaline are specific precursors of aristolochic acid in *A. bracteata*. The precursors used were, however, racemic. Enzymic reactions are generally stereospecific and it was expected that only one of the two optical isomers of orientaline would normally

act as a direct substrate. Parallel feedings with (−)-(*R*)-, and (+)-(*S*)-orientalines showed that the stereospecificity is maintained in the biosynthesis of aristolochic acid from orientaline. The *R*-form (experiment 6) was incorporated about 4 times more efficiently than the *S*-form (experiment 7).





The specific incorporation of orientaline into aristolochic acid implies that 1-hydroxy-2,8-dimethoxyaporphine (prestephanine) (4) is an obligatory intermediate in the biosynthesis. Feeding of (±)-[*aryl*-^{3}H]prestephanine (experiment 8) to *A. bracteata* established its intermediacy. Since the methylenedioxy-group originates in nature from an *o*-methoxyphenol precursor, prestephanine (4) was expected to be transformed in the plants into stephanine (5). (±)-[*aryl*-^{3}H]Stephanine (experiment 9) fed to *A. bracteata* was incorporated into aristolochic acid. The result thus demonstrated the involvement of (5) in the biosynthesis of (6).

The presence of nororientaline, orientaline, and stephanine in *A. bracteata* was confirmed by trapping experiments with DL-[3-^{14}C]tyrosine (incorporations, 0.026, 0.011, and 0.135%, respectively). The foregoing experiments strongly support the following sequence for the biosynthesis of aristolochic acid in *A. bracteata*: norlaudanosoline (8) ⟶ nororientaline (9) ⟶ orientaline (1) ⟶ prestephanine (4) ⟶ stephanine (5) ⟶ aristolochic acid (6).

EXPERIMENTAL

For general directions (spectroscopy, counting method, and labelling of precursors) see ref. 8.

(±)-[aryl-^{3}H]*Prestephanine* (4).—Thionyl chloride (0.1 ml) was added to tritiated water (0.3 ml; activity 200 mCi). To this solution was added (±)-prestephanine (57 mg) and the mixture was heated under nitrogen (sealed tube) at 98 °C for 100 h. It was cooled, diluted with water, and basified with aqueous sodium hydrogen carbonate. The liberated base was extracted with chloroform; the extract was washed with water, dried (Na_2SO_4), and evaporated. The residue was chromatographed on alumina to give (±)-[*aryl*-^{3}H]prestephanine (23 mg) (activity 0.46 mCi).

(±)-[aryl-^{3}H]*Stephanine* (5).—Stephanine (15 mg) was tritiated by the method described for (4) except that methanol (0.1 ml) was used in the reaction as diluent so as to prevent cleavage of the methylenedioxy group in (5) (activity 0.03 mCi).

Feeding Experiments.—Tyrosine, dopa, prestephanine, and stephanine were fed in water (1 ml) containing tartaric acid (10 mg); nororientaline and orientaline hydrochlorides were dissolved in aqueous dimethyl sulphoxide (1 ml). The young plants of *A. bracteata* (4—6 months old) were fed with the precursors by the wick technique. When uptake of the radioactive material was complete the tubes were washed with water and the washings left in contact with the wicks. The plants were left for 7 to 8 days for the precursors to be metabolised, and then worked up for aristolochic acid.

Isolation and Purification of Aristolochic Acid.—The plants (typically 40 g wet wt) were macerated in ethanol (200 ml) with radioinactive aristolochic acid (100 mg) and left for 10 h. The ethanol was decanted and the residue was percolated with fresh ethanol (6 × 150 ml). The combined ethanolic extract was concentrated under reduced pressure to afford a greenish viscous mass, which was extracted with aqueous 5% sodium carbonate (5 × 20 ml). It was extracted with light petroleum (4 × 15 ml) and acidified with dilute hydrochloric acid, and the liberated acids were extracted with ethyl acetate (6 × 30 ml). The ethyl acetate extract was washed with water, dried (Na_2SO_4), and evaporated under reduced pressure. The crude aristolochic acid so obtained was subjected to preparative t.l.c. [SiO_2; benzene–methanol–acetic acid (90 : 6 : 4)]. The band corresponding to aristolochic acid was removed and eluted with methanol–acetic acid (90 : 10) to afford pure aristolochic acid (60 mg), m.p. 280 °C (decomp.) [lit.,[13] 281—286 °C (decomp.)], which was crystallised from methanol–ether to constant activity.

Degradation of Biosynthetic Aristolochic Acid Derived from DL-[3-^{14}C]*Dopa Feeding*.—Aristolochic acid (45 mg) (molar activity 3.13×10^4 disint. min^{-1} $mmol^{-1}$) dissolved in freshly distilled quinoline (0.7 ml) was heated under nitrogen with copper powder (16 mg) for 10 min. The radioactive CO_2 evolved was slowly absorbed in aqueous barium hydroxide. The radioactive barium carbonate formed was collected, washed with water, ethanol, and ether, dried, and assayed for ^{14}C activity.

acidic extract was extracted with ether (4×20 ml) and basified with aqueous sodium hydrogen carbonate, and the liberated bases were extracted with chloroform–methanol (90 : 10; 4×25 ml). The organic layer was washed with water, dried (Na_2SO_4), and evaporated. The crude product was subjected to preparative t.l.c. [SiO_2; chloroform–methanol (9 : 1)]. The bands corresponding to nororientaline, orientaline, and stephanine were removed from the plates. Elution of the least polar band with chloroform–

TABLE 2

Degradation of labelled aristolochic acid

Compound	Specific activity (disint. min^{-1} mg^{-1})	Molar activity (disint. min^{-1} $mmol^{-1}$)
Aristolochic acid	91.8	3.13×10^4
$BaCO_3$ (obtained by degradation)	153	3.01×10^4
8-Methoxy-3,4-methylenedioxy-10-nitrophenanthrene	0.73	2.16×10^2

The mixture containing the decarboxylation product was diluted with water and extracted with ether (5×25 ml). The ethereal layer was washed with N-hydrochloric acid, sodium hydroxide, and water, dried (Na_2SO_4), and evaporated. The residue obtained was chromatographed on alumina. Elution with benzene afforded 8-methoxy-3,4-methylenedioxy-10-nitrophenanthrene (11 mg), m.p. 210—212 °C (lit.,[13] 212 °C). The radioactivity of the products is shown in Table 2.

Feeding of (±)-[5′,8-$^3H_2$; 6-methoxy-^{14}C]*Nororientaline*.—(±)-[5′,8-$^3H_2$; 6-*methoxy*-^{14}C]Nororientaline hydrochloride (^{14}C activity 0.004mCi; ^{3}H activity 0.104mCi; after allowing for the loss of one tritium from position 8, the ratio ^{14}C : ^{3}H was 1 : 13) dissolved in aqueous dimethyl sulphoxide (1 ml) was fed to *A. bracteata*. The plants were harvested after 7 days. Radioinactive aristolochic acid (82.3 mg) was then added and the compound was reisolated. Biosynthetic aristolochic acid had both ^{14}C and ^{3}H activity (^{14}C specific activity 3.13×10^2 disint. min^{-1} mg^{-1}; ^{3}H specific activity 3.44×10^3 disint. min^{-1} mg^{-1}, ^{14}C : ^{3}H 1 : 11).

Degradation of Biosynthetic Aristolochic Acid Derived from (±)-[5′,8-$^3H_2$; 6-methoxy-^{14}C]*Nororientaline Feeding*.—A mixture of aristolochic acid (25 mg) (^{14}C molar activity 1.067×10^5 disint. min^{-1} $mmol^{-1}$), dimedone (83 mg), and conc. hydrochloric acid (1.7 ml) was heated at 125 °C for 18 h. The resulting mixture was cooled, diluted with water (18 ml), and extracted with ether. The ethereal layer was washed with aqueous 1% sodium hydrogen carbonate and water, dried (Na_2SO_4), and evaporated to afford crude formaldehyde dimedone derivative, which was purified by chromatography over SiO_2 and crystallised from chloroform–methanol to constant activity (specific activity 3.0×10^2 disint. min^{-1} mg^{-1}; molar activity 0.87×10^5 disint. min^{-1} $mmol^{-1}$).

Isolation of Nororientaline, Orientaline, and Stephanine.—A solution of DL-[3-^{14}C]tyrosine (0.1 mCi) in water (1 ml) was fed to *A. bracteata* by the wick technique. The plants were harvested after 7 days and macerated (70 g wet wt) in ethanol (250 ml) with radioinactive (±)-nororientaline (107 mg), (±)-orientaline (120 mg), and (±)-stephanine (20 mg). After 12 h the ethanol was decanted and the plant material was percolated with fresh ethanol (5×150 ml). The combined percolates were concentrated *in vacuo* and extracted with N-hydrochloric acid (3×10 ml). The aqueous

methanol (80 : 20) furnished (±)-stephanine (5) (12 mg) (specific activity 1.44×10^3 disint. min^{-1} mg^{-1}; incorporation 0.135%).

Elution of the next band with chloroform–methanol (80 : 20) afforded (±)-orientaline (1) (90 mg) (specific activity 2.23×10^2 disint. min^{-1} mg^{-1}; incorporation 0.011%); base perchlorate, m.p. 143—144 °C (lit.,[14] 144—145 °C).

Elution of the most polar band with chloroform–methanol (80 : 20) gave (±)-nororientaline (9) (85 mg) (specific activity 5.33×10^2 disint. min^{-1} mg^{-1}; incorporation 0.026%); base hydrochloride, m.p. 250 °C (lit.,[15] 249—250 °C).

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REFERENCES

[1] F. Comer, H. P. Tiwari, and I. D. Spenser, *Can. J. Chem.*, 1969, **47**, 481.
[2] L. Jackson, *Cancer Chemother. Rep.*, 1964, **42**, 35.
[3] M. Pailer, *Fortschr. Chem. Org. Naturst.*, 1960, **18**, 66.
[4] H.-G. Boit, ' Ergebnisse der Alkaloid—Chemie,' Akademie-Verlag, Berlin, 1961, p. 270.
[5] M. Pailer and G. Pruckmayr, *Monatsh. Chem.*, 1959, **90**, 145.
[6] I. D. Spenser and H. P. Tiwari, *Chem. Commun.*, 1966, 55.
[7] H. R. Schütte, U. Orban, and K. Mothes, *Eur. J. Biochem.*, 1967, **1**, 70.
[8] D. S. Bhakuni, A. N. Singh, S. Tewari, and R. S. Kapil, *J. Chem. Soc., Perkin Trans. 1*, 1977, 1662.
[9] A. R. Battersby, R. C. F. Jones, R. Kazlauskas, C. Poupat, C. W. Thornber, S. Ruchirawat, and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1974, 773.
[10] E. Leete, *J. Am. Chem. Soc.*, 1963, **85**, 473.
[11] A. R. Battersby, J. L. McHugh, J. Staunton, and M. Todd, *Chem. Commun.*, 1971, 985.
[12] D. H. R. Barton, D. S. Bhakuni, G. M. Chapman, and G. W. Kirby, *J. Chem. Soc. C*, 1967, 2134.
[13] M. Pailer, L. Belohlav, and E. Simonitsch, *Monatsh. Chem.*, 1956, **87**, 249.
[14] A. R. Battersby, D. M. Foulkes, and R. Binks, *J. Chem. Soc.*, 1965, 3323.
[15] M. Tomita and J. Kunitomo, *J. Pharm. Soc. Jpn.*, 1960, **80**, 1238.