The Biosynthesis of Annuloline, a Unique Oxazole Alkaloid

By **D. G. O'Donovan** * and **H. Horan**, Chemistry Department, University College, Cork, Ireland

Phenylalanine and tyrosine are incorporated specifically into annuloline in *Lolium multiflorum*. Tyramine, cinnamic acid, *p*-coumaric acid, and caffeic acid are shown to be intermediates in the biosynthesis. Methylation is shown to occur late in the biosynthetic sequence.

ANNULOLINE (IX) was isolated by Axelrod and his co-workers [1] from the annual ryegrass *Lolium multiflorum* in 1958. They later established its structure, by synthesis, as 2-(trans-3,4-dimethoxystyryl)-5-(4-methoxyphenyl)oxazole.[2] This was the first report of a naturally occurring oxazole system.[3] Annuloline, which exhibits a blue fluorescence, occurs in the roots of germinating seedlings and is synthesised between the sixth and fourteenth day.[4] Axelrod [2] pointed out the obvious relationship between annuloline and phenylpropanoid precursors.

We now report tracer studies on the biosynthesis of this alkaloid. DL-[3-^{14}C]phenylalanine (total activity 0·1 mc) was administered to six-day-old seedlings growing on filter paper in trays. The ryegrass was harvested on the fourteenth day. Annuloline was isolated as described later and was purified to constant activity. In an analogous manner the other tracers listed in Table 1 were administered to the ryegrass and annuloline was isolated. Specific activities of the isolated annuloline and percentage incorporation figures for each experiment are reported in Table 1. The active alkaloids from the first six feeds were degraded according to Scheme 2, and the activities of the degradation products are listed in Table 2. In each case the precursor fed was incorporated specifically into

[1] B. Axelrod and J. R. Belzile, *J. Org. Chem.*, 1958, **23**, 919.
[2] R. S. Karimoto, B. Axelrod, J. Wolinsky, and E. D. Schall, *Phytochemistry*, 1964, **3**, 349.
[3] W. D. Crow and J. H. Hodgkin, *Austral. J. Chem.*, 1964, **17**, 119.
[4] W. Schunack and H. Rochelmeyer, *Planta Med.*, 1965, **13**, 1.

annuloline. The active alkaloid from the last three experiments was not degraded. We now propose a route for the biosynthesis of annuloline as outlined in Scheme 1.

TABLE 1

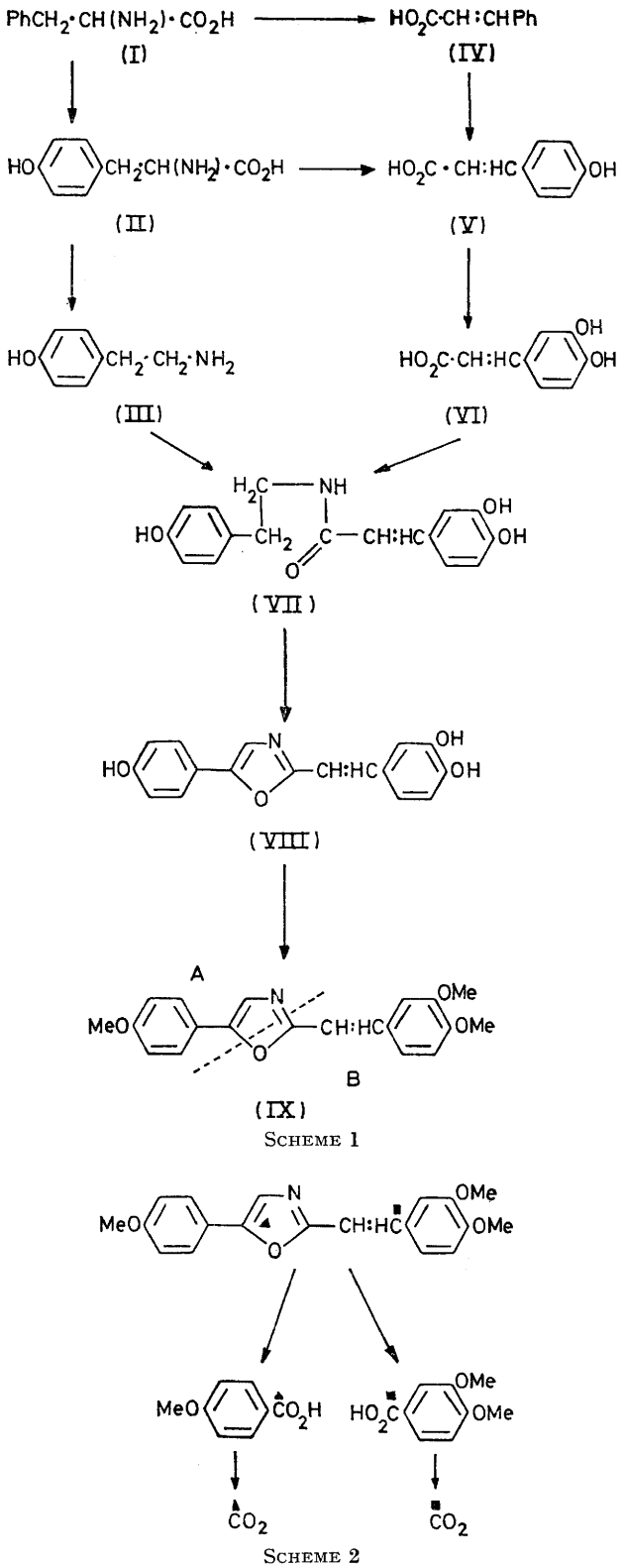
Tracer	Amount fed (counts/min. × 10^{-8})	Activity of annuloline (counts/min./mmole)	Incorporation (%)
DL-[3-^{14}C]Phenylalanine	2·2	1·4 × 10^5	0·037
DL-[3-^{14}C]Tyrosine	2·2	5·39 × 10^5	0·15
DL-[3-^{14}C]-3,4-Dihydroxyphenylalanine	2·2	4·68 × 10^4	0·02
[2-^{14}C]Tyramine	2·2	1·6 × 10^7	4·23
[2-^{14}C]-*p*-Methoxyphenethylamine	2·2	2·2 × 10^5	0·234
[3-^{14}C]Cinnamic acid	2·2	9·96 × 10^5	0·453
[2-^{14}C]-*p*-Methoxycinnamic acid	1·14	1·38 × 10^5	0·11
[2-^{14}C]-*p*-Coumaric acid	1·24	1·17 × 10^6	0·84
[2-^{14}C]Caffeic acid	1·13	1·35 × 10^6	3·56

TABLE 2

Activity of annuloline and its degradation products

	Activity (counts/min./mmole)
(a) Phenylalanine feed	
Annuloline	1·4 × 10^5
Anisic acid	3·92 × 10^4
Barium carbonate	3·90 × 10^4
Veratric acid	1·01 × 10^5
Barium carbonate	1·13 × 10^5
(b) Tyrosine feed	
Annuloline	5·39 × 10^5
Anisic acid	3·44 × 10^5
Barium carbonate	3·40 × 10^5
Veratric acid	1·94 × 10^5
Barium carbonate	1·91 × 10^5
(c) 3,4-Dihydroxyphenylalanine feed	
Annuloline	4·68 × 10^4
Anisic acid	0
Veratric acid	4·64 × 10^4
Barium carbonate	4·62 × 10^4
(d) Tyramine feed	
Annuloline	1·6 × 10^7
Anisic acid	1·58 × 10^7
Barium carbonate	1·53 × 10^7
Veratric acid	0
(e) *p*-Methoxyphenylamine feed	
Annuloline	2·2 × 10^5
Anisic acid	2·03 × 10^5
Barium carbonate	2·1 × 10^5
Veratric acid	0
(f) Cinnamic acid feed	
Annuloline	9·96 × 10^5
Anisic acid	0
Veratric acid	9·91 × 10^5
Barium carbonate	9·85 × 10^5

Both phenylalanine (I) and tyrosine (II) are incorporated into the methoxyphenyl half (A) of annuloline. The conversion of phenylalanine into tyrosine has already been demonstrated in a number of tracer studies.[5] Decarboxylation of tyrosine yields tyramine (III), the amine portion of the proposed amide intermediate (VII). The high percentage incorporation of tyramine confirms its intermediary role in the bio-



SCHEME 1

SCHEME 2

[5] E. Leete and L. Marion, *Canad. J. Chem.*, 1954, **32**, 646; S. Udenfriend and J. B. Wyngarden, *Biochim. Biophys. Acta*, 1956, **20**, 48; H. Rosenberg, J. L. McLaughlin, and A. G. Paul, *Lloydia*, 1967, **30**, 100; D. J. Bennett and G. W. Kirby, *J. Chem. Soc.* (*C*), 1968, 442.

synthesis. A comparison of the efficiencies of incorporation of tyramine (4·23%) and *p*-methoxyphenethylamine (0·23%) into annuloline suggests that methylation of the phenolic group is an end step in the biosynthetic sequence.

The sequence tyrosine (II) ⟶ *p*-coumaric acid (V) ⟶ caffeic acid (VI) ⟶ amide (VII) for the biosynthesis of the dimethoxystyryl portion (B) of annuloline is supported by the increasing efficiency of incorporation of each compound fed. The key step in this sequence, the transformation of tyrosine into *p*-coumaric acid, is visualised as being catalysed by the enzyme tyrosine ammonia lyase.[6] This enzyme occurs in higher plants, particularly the gramineae, of which *L. multiflorum* is a member.[7] The sequence phenylalanine ⟶ *p*-coumaric acid may occur by way of tyrosine [5] or cinnamic acid. The latter is more likely in view of the good incorporation of cinnamic acid itself into annuloline. The required enzyme, phenylalanine ammonia lyase, has been shown to be present in higher plants.[8] An analogous situation is seen in the biosynthesis of colchicine.[9]

That methylation of the phenolic groups in this portion of the annuloline molecule occurs at a late stage in the biosynthesis is shown by the comparison of the incorporation figures for *p*-methoxycinnamic acid (0·11%), *p*-coumaric acid (0·84%), and caffeic acid (3·56%). The observation of incorporation of the methoxy-derivatives into both parts of the annuloline molecule may be due to a prior demethylation to the phenolic compounds.

It has been shown that tyrosine can be converted into 3,4-dihydroxyphenylalanine in plants.[10] The low incorporation of this precursor into annuloline, however, precludes the pathway tyrosine ⟶ 3,4-dihydroxyphenylalanine ⟶ caffeic acid.

The unequal incorporation of phenylalanine and tyrosine into both parts of the annuloline molecule is presumably enzyme-controlled, hydroxylation and decarboxylation occurring more readily than deamination.

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus and are corrected. Radioactive assays were carried out with a Nuclear Chicago Unilux II liquid scintillation counter, by use of the usual scintillators, and the results were processed by an off-line Olivetti programma 101, corrections being made for background and quenching.

Administration of Tracers to L. multiflorum *and Isolation of Annuloline.—Lolium multiflorum* seeds (300 g.) were steeped in distilled water for 8 hr. then spread out on trays on two layers of Whatman filter paper to germinate. The trays were covered and the seeds were kept in a damp condition. After 6 days a solution of DL-[3-^{14}C]phenylalanine (total activity 0·1 mc) was added and allowed to be absorbed uniformly into the filter paper. The seedlings were then exposed to the light More water was added to the filter paper after 24 hr., and the seedlings were allowed to germinate under xerophytic conditions.

After 14 days germination was terminated and the seedlings were harvested. By this time the rootlets had penetrated both layers of the filter paper. The aerial portions were discarded and the remainder was mascerated in a Waring blendor with ethanol–chloroform (1 : 4; 4 l.). Inactive annuloline [2] (300 mg.) was added as carrier and the homogenate was set aside for 48 hr.

The mixture was filtered and evaporated just to dryness on a rotatary evaporator. The residue was dissolved in chloroform (300 ml.) and washed with 1·0N-sodium hydroxide until the washings were colourless. The chloroform solution was dried (Na_2SO_4) and evaporated. The residue was taken up in benzene (20 ml.) and chromatographed on a column (35 × 2 cm.) of Woelm alumina (activity II). Elution with benzene and then chlorofom furnished the fluorescent portion of the extract. The combined fluorescent portions were evaporated and rechromatographed on a column of Woelm alumina (activity II). Elution with benzene and then ether furnished annuloline in the ether fractions. The alkaloid (220 mg.) was recrystallised from methanol (m.p. 114—115°) to constant specific activity (Found: C, 71·2; H, 5·65; N, 4·15. $C_{20}H_{19}NO_4$ requires C, 71·1; H, 5·6; N, 4·15%).

In eight further experiments DL-[3-^{14}C]tyrosine, DL-[3-^{14}C]-3,4-dihydroxyphenylalanine, [2-^{14}C]tyramine, [2-^{14}C]-*p*-methoxyphenethylamine, and [3-^{14}C]cinnamic acid (total activity 0·1 mc in each case), and [2-^{14}C]-*p*-methoxycinnamic acid (total activity 1·14 × 10^8 counts/min.), [2-^{14}C]-*p*-coumaric acid (total activity 1·24 × 10^8 counts/min.), and [2-^{14}C]caffeic acid (total activity 1·13 × 10^8 counts/min.) were administered to the seedlings by the same method and radioactive annuloline was isolated in each case. Specific activities and percentages of incorporation are reported in Table 1.

Degradation of Annuloline.[2]—A mixture of annuloline (300 mg.), potassium permanganate (800 mg.), water (12·5 ml.), and 6N-sodium hydroxide (3·25 ml.) was heated under reflux for 1 hr. The solution was allowed to cool and a few drops of ethanol were added to decompose the excess of permanganate. The manganese dioxide was filtered off and the filtrate was acidified with concentrated sulphuric acid. The solution was heated to boiling and then set aside at 5° for 3 days. The white precipitate which settled was filtered off and dried. Preparative t.l.c. [benzene–ethyl acetate–glacial acetic acid (9 : 2 : 1); 100 × 20 cm. plate coated with silica gel HF_{254} 1 mm. thick] furnished veratric acid (65 mg.) (m.p. 180—182) and anisic acid (23 mg.) (m.p. 181—182). Both acids were purified by sublimation and recrystallisation from water.

Schmidt Degradations on Anisic and Veratric acids.[11]—To the acid (15 mg.), cooled in an ice-bath, was added sodium azide (12 mg.) and chloroform (2 ml.). The flask was connected to a sulphur dioxide trap (5% potassium permanganate in 5% sulphuric acid) and swept out with pure

[6] J. Koukol and E. E. Conn, *J. Biol. Chem.*, 1961, **236**, 2692.
[7] A. C. Neish, *Phytochemistry*, 1961, **1**, 1.
[8] A. C. Neish in 'Plant Biochemistry,' ed. J. Bonner and J. E. Varner, Academic Press, New York, 1965, p. 581.
[9] A. R. Battersby and B. J. T. Harper, *J. Chem. Soc.*, 1964, 4257.
[10] P. Kovacs and A. Jindra, *Experentia*, 1965, **21**, 81.
[11] A. R. Battersby, R. Binks, J. J. Reynolds, and D. A. Yeowell, *J. Chem. Soc.*, 1962, 3526; E. Leete, *J. Amer. Chem. Soc.*, 1963, **85**, 3666; R. D. Hill and A. M. Unrau, *Canad. J. Chem.*, 1965, **43**, 709.

nitrogen. At 0° concentrated sulphuric acid (0·15 ml.) was added and the mixture was warmed at 45° for 1 hr. The evolved carbon dioxide was collected in a trap containing saturated (at 20°) aqueous barium hydroxide which had previously been heated to 80°. Barium carbonate was filtered off and washed.

[2-^{14}C]-p-*Methoxycinnamic Acid*.[12]—Anisaldehyde (20 mg.) and [2-^{14}C]malonic acid (15 mg.) (total activity 0·46 mc) were dissolved in dry pyridine (0·5 ml.) and a trace of analinine was added. The mixture was set aside overnight and then refluxed for 3 hr. The cooled solution was poured into an excess of dilute hydrochloric acid; the [2-^{14}C]-*p*-methoxycinnamic acid (12 mg.) immediately crystallised out. It was recrystallised from ethanol (m.p. 174—176°) to constant specific activity.

[2-^{14}C]-p-*Coumaric Acid*.—*p*-Hydroxybenzaldehyde (31 mg.) and [2-^{14}C]malonic acid (25 mg.) (total activity 0·77 mc) were mixed as before and the solution was set aside for 2 days. It was then heated at 75° for 4 hr. in a water-bath, cooled, and poured into dilute hydrochloric acid (3 ml.). The [2-^{14}C]-*p*-hydroxycinnamic acid (15 mg.) crystallised out almost immediately and was recrystallised from aqueous ethanol (m.p. 215°) to constant specific activity.

[2-^{14}C]-3,4-*Caffeic Acid*.—3,4-Dihydroxybenzaldehyde (34 mg.) and [2-^{14}C]-malonic acid (25 mg.) (total activity 0·77 mc) were treated as before and the mixture was set aside for 2 days. The solution was then heated at 55° for 4 hr. on a water-bath, cooled, and poured into dilute hydrochloric acid (3 ml.). The [2-^{14}C]-3,4-dihydroxycinnamic acid (12 mg.) (m.p. 205°) was recrystallised from water to constant specific activity.

This investigation was supported by a grant from the Irish Agricultural Institute. One of us (H. H.) thanks University College, Cork, for financial support.

[0/1305 *Received*, *July* 29*th*, 1970]

[12] F. Vorsatz, *J. prakt. Chem.*, 1936, **145**, 265.