The Biosynthesis of Arborine.[1]

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Arborine has been shown to be derived, in intact *Glycosmis arborea* Correa plants, from anthranilic acid and phenylalanine. The biosynthesis from phenylalanine has been shown to proceed *via* phenylacetic acid and *N*-methyl-*N*-(phenylacetyl)anthranilic acid.

THE Indian plant *Glycosmis arborea* Correa has been shown to contain a number of quinazolone alkaloids.[2,3] The principal alkaloid present in the leaves, arborine (VIII), 2-benzyl-1-methyl-4-quinazolone, is used extensively as a febrifuge and anthelmintic.

Robinson [4] considered that arborine was formed from anthranilic acid, phenylacetic acid derived from phenyl-

[1] Presented in part at the Fourth International Symposium on the Biochemistry and Physiology of Alkaloids, Halle/Saale, D.D.R., July, 1969.

[2] D. Chakravarti, R. N. Chakravarti, L. A. Cohen, B. Dasgupta, S. Datta, and H. K. Miller, *Tetrahedron*, 1961, **16**, 224 and references cited therein.

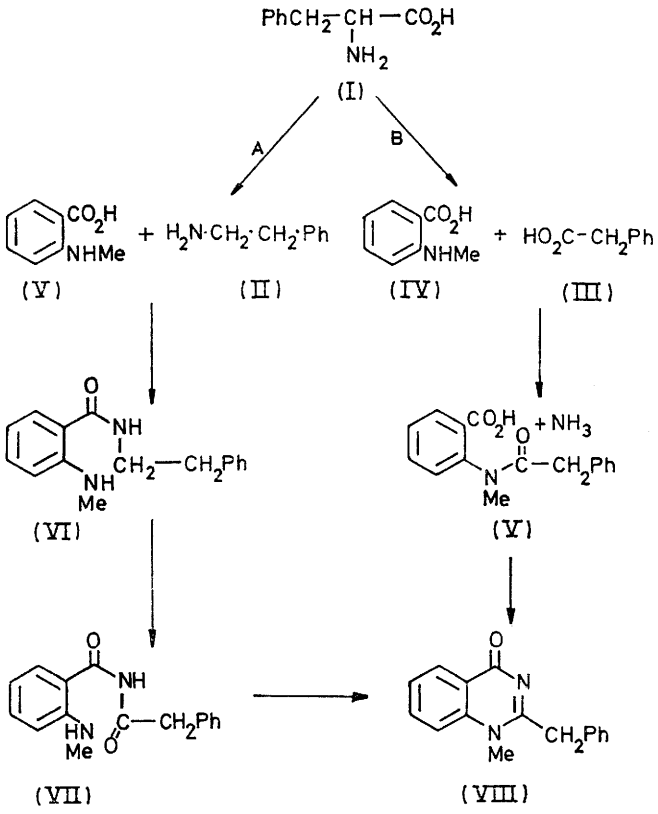
[3] S. C. Pakrashi and J. Bhattacharyya, *J. Sci. Ind. Res., India*, 1962, *B***21**, 49. S. C. Pakrashi, J. Bhattacharyya, L. F. Johnson, and H. Budzikiewiez, *Tetrahedron*, 1963, **19**, 1011.

[4] R. Robinson, 'The Structural Relations of Natural Products,' Clarendon Press, Oxford, 1955.

alanine, and ammonia (Scheme, route B). However, an equally reasonable biosynthetic scheme is that shown as route A. Here the intermediate amide (VI) is formed from anthranilic acid and phenylethylamine (II). A series of unexceptional steps then yields arborine.

Recently it has been reported that 1-[*U*-^{14}C]phenylalanine and [^{3}H]anthranilic acid,[5] and also labelled methionine and *N*-methylanthranilic acid,[6] are incorporated into arborine.

The tracer experiments we now describe show that arborine is derived from phenylalanine and anthranilic acid, and is biosynthesised in agreement with the hypothesis of Robinson.



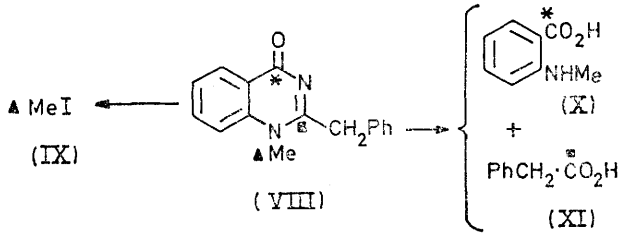
SCHEME

[*carboxy*-^{14}C]Anthranilic acid (total activity 0·05 mc) prepared from [*carbonyl*-^{14}C]phthalic anhydride,[7] and DL-[3-^{14}C]phenylalanine (total activity 0·05 mc), were administered in separate experiments, to six four-month old *Glycosmis arborea* plants growing hydroponically.[8] The plants from the anthranilic acid feed were harvested after 14 days, and after addition of inactive arborine as carrier (150 mg.) the active arborine was isolated by established methods.[9] The arborine was purified as the hydrochloride to constant specific activity (incorporation 0·0035%). In the second experiment, the incorporation was 0·0028%. The active arborine from the two experiments was degraded as follows. Arborine from the anthranilic acid feed was heated with 20% potassium hydroxide. The resulting solution was adjusted to pH 9 and passed through a column of Amberlite IR-120(H^{+}) [10] ion-exchange resin, thus separating phenylacetic acid. The *N*-methylanthranilic acid was isolated by preparative t.l.c. from the residue and purified to constant specific activity. The concordance between the specific activities of arborine and *N*-methylanthranilic acid (Table 2) shows that anthranilic acid was specifically incorporated into arborine. Material from the phenylalanine feed was degraded in a similar way, the isolated phenylacetic acid having essentially all the activity of the arborine (Table 2). Further degradations to establish the activity of the carboxy-group could not be carried out because of the small quantities of the acids isolated. In further experiments 2-phenyl[1-^{14}C]ethylamine (total activity 0·05 mc) and phenyl[1-^{14}C]acetic acid (total activity 0·05 mc) were administered in separate experiments to six four-month old *G. arborea* plants, and active arborine hydrochloride was isolated as before. The incorporation for arborine in the case of the 2-phenylethylamine feed was 0·0038% and in the case of the phenylacetic acid feed, 0·005%. The active arborine from both feeds was degraded as before; the activities of the degradation products are reported in Table 2. Though incorporation levels are low it appears that biosynthesis of the alkaloid was occurring *via* phenylacetic acid. In a second series of experiments the two possible intermediate amides were synthesised in doubly labelled form and administered to the plants. *N*-[^{14}C]-Methyl-*N*-(phenyl[1-^{14}C]acetyl)anthranilic acid (V) was synthesised as follows. Anthranilamide was heated with [^{14}C]methyl iodide [11] to give *N*-[^{14}C]methylanthranilamide, which was hydrolysed to the corresponding acid. Treatment of the acid with phenyl[1-^{14}C]acetyl chloride [12] furnished the doubly labelled acid (V) (specific activity $3{\cdot}35 \times 10^{9}$ counts/min./mmole). A Hertzig–Meyer reaction on this amide liberated [^{14}C]methyl iodide, isolated as triethylmethylammonium iodide, specific activity $1{\cdot}28 \times 10^{9}$ counts/min./mmole. Thus the ratio of [*carbonyl*-^{14}C] to [N-*methyl*-^{14}C] activity was 1·61. *o*-[^{3}H]Methylamino-*N*-(2-phenyl[1-^{14}C]ethyl)benzamide (VI) was prepared as follows. A basic solution of anthranilic acid was shaken with [^{3}H]dimethyl sulphate to give *N*-[^{3}H]methylanthranilic acid. The acid was then esterified with diazomethane and treated with 2-phenyl[1-^{14}C]ethylamine in the presence of ammonium chloride catalyst to yield the amide,[13] which was

[5] D. Groeger and S. Johne, *Z. Naturforsch.*, 1968, **23b**, 1072.
[6] D. Groeger, Discussions at Fourth International Symposium on the Biochemistry and Physiology of Alkaloids, Halle/Saale, D.D.R., July, 1969.
[7] G. Caronna, *Gazzetta*, 1941, **71**, 189; E. Leete, *J. Amer. Chem. Soc.*, 1959, **81**, 3948.
[8] E. Leete, *J. Amer. Chem. Soc.*, 1956, **78**, 3520.
[9] D. Chakravarti, R. N. Chakravarti, and S. Chakravarti, *J. Chem. Soc.*, 1953, 3337.
[10] E. Leete, L. Marion, and I. D. Spenser, *J. Biol. Chem.*, 1955, **214**, 71.
[11] A. Chatterjee and S. G. Majundar, *J. Amer. Chem. Soc.*, 1953, **75**, 4365.
[12] H. de Diesbach, J. Gross, and W. Tschannen, *Helv. Chim. Acta*, 1951, **24**, 1050.
[13] O. C. Dermer and J. King, *J. Org. Chem.*, 1943, **8**, 168.

separated by column chromatography. The $^{14}C:{}^{3}H$ ratio for this amide was 2·57.

The two intermediates (V) and (VI) (4 mg. each) (total activities $4·96 \times 10^7$ and $7·08 \times 10^6$ counts/min., respectively) were fed to four twelve-month old *G. arborea* plants in separate experiments by a wick arrangement. After 21 days the plants were harvested in the



usual manner, inactive arborine (200 mg.) was added as carrier, and the arborine from both feeds was recrystallised to constant specific activity.

The arborine from the first feed, *i.e.* from intermediate (V), incorporation 1·43%, was subjected to a Hertzig–Meyer reaction and the [^{14}C]methyl group was isolated as triethylmethylammonium iodide, specific activity $3·35 \times 10^5$ counts/min./mmole. The phenyl[^{14}C]acetyl group was isolated as before, and the phenyl[^{14}C]acetic acid was subjected to a Schmidt reaction.[14] The isolated barium carbonate had specific activity $5·47 \times 10^5$ counts/min./mmole. The [*carbonyl*-^{14}C] : [N-*methyl*-^{14}C] ratio was 1·62, indicating that the doubly labelled acid (V) was incorporated intact into arborine. The arborine isolated from the intermediate (VI) feed had specific activity $1·08 \times 10^3$ counts/min./mmole, incorporation 0·012%. The $^{14}C:{}^{3}H$ ratio (1·43) indicated that the amide (VI) had been degraded, and that the products had been incorporated independently. These results show that arborine is biosynthesised in *G. arborea* in accordance with route B.

EXPERIMENTAL

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

Administration of Tracers to G. arborea *and Isolation of Arborine.*—Six four-month old *G. arborea* plants were grown hydroponically. After 2 weeks [*carboxy*-^{14}C]anthranilic acid (total activity 0·05 mc) was added to the solution. Within 3 days, essentially all the activity had been absorbed by the plants. These were harvested after 17 days and macerated in a Waring blendor with ethanol (95%). Inactive arborine (150 mg.) was added as carrier and the solution was set aside for 48 hr., then filtered through cloth; the alcoholic extract was evaporated just to dryness on a rotary evaporator. The residue was partitioned between chloroform and 2N-hydrochloric acid (150 ml.). The acid solution was basified with ammonium hydroxide solution and extracted with chloroform (500 ml.). The extract was dried (Na_2SO_4) and evaporated under reduced pressure to yield crude arborine. This was taken up in boiling 2N-hydrochloric acid; the solution was filtered and cooled.

TABLE 1

Incorporation of tracers into arborine

Tracer	Amount fed (counts/min.)	Activity of arborine,HCl (counts/min./mmole)	Activity of arborine (counts/min./mmole)	Incorporation (%)
(*Carboxy*-^{14}C)Anthranilic acid	$1·10 \times 10^8$	$4·83 \times 10^3$	$4·85 \times 10^3$	0·0035
DL-[3-^{14}C]Phenylalanine	$1·10 \times 10^8$	$3·81 \times 10^3$	$3·81 \times 10^3$	0·0028
2-Phenyl[1-^{14}C]ethylamine	$1·10 \times 10^8$	$5·33 \times 10^3$	$5·3 \times 10^3$	0·0038
Phenyl[1-^{14}C]acetic acid	$1·10 \times 10^8$	$6·83 \times 10^3$	$6·85 \times 10^3$	0·005
N-[^{14}C]Methyl-*N*-(phenyl[1-^{14}C]acetyl)-anthranilic acid	$4·96 \times 10^7$ (ratio 1·61)	$8·81 \times 10^5$	$8·83 \times 10^5$	1·43
o-[^{3}H]Methylamino-*N*-(2-phenyl[1-^{14}C]ethyl) benzamide	$7·08 \times 10^6$ ($^{14}C:{}^{3}H$ ratio 2·57)	$1·083 \times 10^3$	$1·08 \times 10^3$	0·012

TABLE 2

Activity * of arborine and its degradation products

Compound	(a) Anthranilic acid feed	(b) Phenylalanine feed	(c) 2-Phenylethylamine feed	(d) Phenylacetic acid feed
Arborine	4·85	3·85	5·3	6·83
Phenylacetic acid	0	3·83	5·28	6·83
N-Methylanthranilic acid	4·81	0	0	0

* In (counts/min./mmole) $\times 10^{-3}$.

Degradation of Intermediate (V)	Activity [a]
Total activity of amide (V)	3·35
Triethylmethylammonium iodide	1·28
Ratio of [*carbonyl*-^{14}C] to [*methyl*-^{14}C] = 1·61	

Arborine degradation; intermediate (V) feed	Activity [b]
Arborine	8·83
Triethylmethylammonium iodide	3·35
Phenylacetic acid	5·45
Barium carbonate	5·47
Ratio of [*carbonyl*-^{14}C] to [*methyl*-^{14}C] = 1·62	

[a] In (counts/min./mmole) $\times 10^{-9}$. [b] In (counts/min./mmole) $\times 10^{-5}$.

[14] R. D. Hill, A. M. Unrau, and D. T. Canvin, *Canad. J. Chem.*, 1966, **44**, 2077.

The resulting precipitate (161 mg.) gave arborine hydrochloride, m.p. 209—210° (from 95% ethanol). The pure hydrochloride was dissolved in 2N-hydrochloric acid (100 ml.), cooled in ice, and basified with ammonium hydroxide. The solution was extracted with chloroform (300 ml.) and the extract was dried (Na_2SO_4) and evaporated to yield pure arborine, m.p. 155—156° (from 95% ethanol and benzene) (Found: C, 76·55; H, 5·5; N, 10·9. Calc. for $C_{16}H_{14}N_2O$: C, 76·8; H, 5·6; N, 11·2%).

In a second experiment DL-[3-^{14}C]phenylalanine (total activity 0·05 mc) was fed to six *G. arborea* plants as before. Inactive arborine (150 mg.) was added as carrier and the alkaloid was isolated and purified as the hydrochloride (153 mg.) which was then converted into free arborine, m.p. 155—156°.

In two subsequent experiments 2-phenyl[1-^{14}C]ethylamine (total activity 0·05 mc) and phenyl[1-^{14}C]acetic acid (total activity 0·05 mc) were administered to six plants each in exactly the same way as before. Arborine (150 mg.) was added as carrier in each case and the hydrochlorides (161 and 159 mg., respectively) were isolated, recrystallised to constant specific activity, and then converted into pure arborine (m.p. 155—156°).

The specific activities of arborine and its degradation products (Table 2) are based on the alkaloid isolated from the plants after the addition of carrier during the plant work-up.

Degradation of Arborine.—The arborine from each feed was degraded in essentially the following way. Arborine (150 mg.) was added to 20% potassium hydroxide (5 ml.) and the mixture was refluxed for 1·5 hr. The solution was adjusted to pH 9—10 with concentrated hydrochloric acid and passed slowly over a column (28 × 1·2 cm.) of Amberlite IR-120 (H^+) ion-exchange resin. The column was washed with distilled water (100 ml.) until the eluate was no longer acidic. The aqueous solution was extracted with chloroform (250 ml.); the extract was dried (Na_2SO_4) and evaporated to yield crude phenylacetic acid (19 mg.), m.p. 77° [from water then light petroleum (b.p. 60—80°)].

The column was then washed with 1N-ammonia (250 ml.). The basic solution was extracted with chloroform and the extract was dried (Na_2SO_4) and evaporated. Preparative t.l.c. in chloroform–methanol (95 : 5) on a 20 × 20 cm. plate coated with silica gel HF_{254} 1·5 mm. thick furnished *N*-methylanthranilic acid (15 mg.), m.p. and mixed m.p. 179—189° (from dilute ethanol).

N-[^{14}C]*Methyl*-N-(*phenyl*[1-^{14}C]*acetyl*)*anthranilic Acid.*—Anthranilamide (220 mg.) was heated with [^{14}C]methyl iodide (0·1 ml.) (total activity 1 mc) in a sealed tube at 100° for 6 hr. The *N*-[^{14}C]methylanthranilamide (180 mg.) had m.p. 159° (from ethanol). The amide (170 mg.) was added to 20% hydrochloric acid (10 ml.) and the mixture was refluxed for 1 hr. The product was diluted with water (20 ml.) and extracted with chloroform (250 ml.); the extract was dried (Na_2SO_4) and evaporated to yield *N*-[^{14}C]methylanthranilic acid (143 mg.) purified by sublimation and recrystallisation from ethanol (m.p. 178—180°). The acid (100 mg.) was then slowly treated with phenyl[1-^{14}C]acetyl chloride (total activity 0·5 mc) (prepared by treating phenyl[1-^{14}C]acetic acid with a two-fold excess of thionyl chloride) in dry ether (35 ml.) with stirring. When precipitation was complete, the solution was filtered and the precipitate was washed with a little dry ether. The combined ethereal solution was washed with 5% hydrochloric acid and water, dried (Na_2SO_4), and evaporated. The *amide* (52 mg.; m.p. 132—133°) was recrystallised from dilute ethanol to constant specific activity and tested for purity on t.l.c. (Found: C, 71·15; H, 5·6; N, 4·8. $C_{16}H_{15}NO_3$ requires C, 71·4; H, 5·55; N, 5·2%).

o-[^{3}H]*Methylamino*-N-(2-*phenyl*[1-^{14}C]*ethyl*)*benzamide* (VI).—Anthranilic acid (200 mg.) was dissolved in N-sodium hydroxide (1·6 ml.), [^{3}H]dimethyl sulphate (0·16 ml.; total activity 2 mc) was added. The mixture was shaken vigorously for 30 min., was cooled in ice-water, and filtered, and the precipitate was thoroughly washed with water and dried. The resulting *N*-[^{3}H]methylanthranilic acid (138 mg.) had m.p. 179—180° (from dilute ethanol). The acid (130 mg.) was esterified with an excess of ethereal diazomethane, and the product was isolated as the hydrochloride (155 mg.), m.p. 137—138° (from methanol) (lit., [13] 137°) (Found: C, 53·4; H, 6·15; N, 7·0. Calc. for $C_9H_{11}NO_2$,HCl: C, 53·6; H, 5·95; N, 6·95%).

N-[^{2}H]Methylanthranilic acid methyl ester (140 mg.) regenerated from the hydrochloride was added to 2-phenyl-[1-^{14}C]ethylamine (200 mg.) (total activity 0·5 mc) and ammonium chloride (20 mg.) and the mixture was refluxed in an oil-bath for 3 hr. A solution of the product in benzene was filtered and passed through a column (50 × 2·5 cm.) of Woelm alumina (activity II). Elution with benzene and then benzene–ether yielded the doubly labelled *amide* (VI) (23 mg.), m.p. 106—107° (Found: C, 75·25; H, 6·95; N, 10·85. $C_{16}H_{18}N_2O$ requires C, 75·6; H, 7·1; N, 11·0%).

Administration of Amides (V) *and* (VI) *to* G. arborea *and Isolation of Arborine.*—The labelled amides (V) (4 mg.) (total activity 4·96 × 10^7 counts/min.) and (VI) (4 mg.) (total activity 7·08 × 10^6 counts/min.) were fed by the wick arrangement to four twelve-month old plants of *G. arborea* in two separate experiments. The solutions were absorbed after 48 hr. Water was fed to the plants through the wick for 4 days. They were then watered normally and grown on for 16 days, after which they were harvested as previously described. Inactive arborine (200 mg.) was added as carrier in both experiments. The isolated arborine hydrochlorides (180 and 193 mg. respectively) were recrystallised to constant specific activity.

Hertzig–Meyer reaction on the Labelled Amide (V).—The amide (30 mg.) was pyrolysed with hydriodic acid under a stream of nitrogen (2 ml./min.). Iodine and hydriodic acid were removed from the gas stream by passing it through a tube of Amberlite IR-120 (Na^+) resin moistened with 0·1M-sodium carbonate. After being dried (silica gel) the nitrogen was passed into a solution of triethylamine in toluene. The triethylmethylammonium iodide which crystallised had m.p. 296—297°.

Degradation of Arborine obtained from the Amide (V) *Feed.*—Arborine (150 mg.) was hydrolysed with 20% potassium hydroxide as before and the isolated phenylacetic acid (25 mg.) was subjected to a Schmidt reaction as follows. A flask containing phenylacetic acid (30 mg.) was cooled in an ice-bath, 100% sulphuric acid (0·3 ml.) was added, and the contents were dissolved by rotating the flask. Sodium azide (40 mg.) was added and the flask was connected to a gas train containing a sulphur dioxide trap. The system was flushed with pure nitrogen and a carbon dioxide trap was added to the system. The flask was heated to 80° for 1 hr. Carbon dioxide was flushed from the system with pure nitrogen. The collected barium carbonate (21 mg.) was washed with ethanol and ether.

Hertzig–Meyer reaction on Arborine.—This reaction was

carried out in the same manner as that on the amide intermediate (V), the methyl iodide being isolated as triethylmethylammonium iodide (m.p. 296—297).

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