Phytochemistry, 1972, Vol. 11, pp. 2475 to 2480. Pergamon Press. Printed in England.

PHENYLALANINE AS A PRECURSOR FOR CRYOGENINE BIOSYNTHESIS IN *HEIMIA SALICIFOLIA*

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(*Received* 19 *January* 1972)

Key Word Index—*Heimia salicifolia*; Lythraceae; biosynthesis; quinolizidine alkaloid; cryogenine; phenylalanine as precursor.

Abstract—The aromatic group of cryogenine, the major alkaloid of *Heimia salicifolia*, is shown to be derived from phenylalanine. Feeding of [3-^{14}C]DL-phenylalanine and degradation of the cryogenine showed that the activity was located specifically at both carbons alpha to the biphenyl ring. Thus, two phenylalanine-derived units take part in the biosynthesis of the alkaloid.

INTRODUCTION

STRUCTURAL similarities of the *Heimia* and *Decodon* alkaloids (Lythraceae) suggest a common biosynthetic pathway. Ferris *et al.* proposed a biogenetic scheme for these alkaloids[1] involving pelletierine and shikimate-derived precursors. Work reported from the laboratories of I. D. Spenser showed that the quinolizidine ring of the *Decodon* alkaloids is partially derived from lysine.[2] Preliminary studies on the biosynthesis of the aromatic groups of both *Heimia*[3] and the *Decodon*[4] alkaloids established the involvement of C_6–C_3 precursors. Data are now presented on the biosynthesis of the aromatic groups of cryogenine (I),[5] a major alkaloid of *Heimia salicifolia* Link et Otto.

RESULTS

[3-^{14}C]DL-Phenylalanine, root-fed to 5–6-month-old *Heimia salicifolia* plantlets, yielded radioactive cryogenine (I) (Table 1, Expt 1). Degradation of I with basic permanganate[6] (Scheme 1) gave the biphenylcarboxylic acid lactone (II) (from rings C + D plus C-4 and 1‴) 3,4-dimethoxyphthalic anhydride (metahemipic anhydride) (III) (ring C + C-4 and 1′), glutaric acid (IV) (carbon atoms 6–10) and succinic acid (V) (carbon atoms 6–9 or 7–10).

The molar activity of compounds II and III relative to the molar activity of the alkaloid was 92% and 31% respectively (Table 2, Expt 1). Thus, 92% of the activity of I derived from [3-^{14}C]phenylalanine was present in the biphenyl group plus C-4 and 1‴; 31% of this activity was located within ring C alone plus C-4 and C-1′. These results indicated that both halves of the biphenyl system were derived from phenylalanine and they implied that the

[1] J. P. FERRIS, C. B. BOYCE and R. C. BRINER, *Tetrahedron Letters* 5129 (1966).
[2] S. H. KOO, R. N. GUPTA, I. D. SPENSER and J. T. WROBEL, *J. Chem. Soc.* D, *Chem. Commun.* 396 (1970).
[3] A. ROTHER and A. E. SCHWARTING, *J. Chem. Soc.* D, *Chem. Commun.* 1411 (1969).
[4] S. H. KOO, F. COMER and I. D. SPENSER, *J. Chem. Soc.* D, *Chem. Commun.* 897 (1970).
[5] It should be noted that cryogenine (MW 435·53) used in this study is the alkaloid isolated from *Heimia salicifolia* and not the trade-name product Cryogénine (phenylsemicarbazide, MW 151·2), a speciality of Lumière of Lyons, France and distributed by Laboratoires Sarbach of Châtillon, France.
[6] A. ROTHER, H. G. APPEL, J. M. KIELY, A. E. SCHWARTING and J. M. BOBBITT, *Lloydia* **28**, 90 (1965).

labelled carbons of I were 4 and 1‴. The activity and yield of compounds II and III obtained from this experiment did not, however, allow for further degradation studies.

To establish the location of the label of I a second [3-^{14}C]DL-phenylalanine feeding, starting with higher activity (Table 1, Expt 2), was conducted. Cryogenine was isolated as before, diluted, permethylated and degraded with basic permanganate[7,8] (Scheme 1). 4-Methoxyisophthalic acid (VI) (from ring D + C-1‴ and 1″) and 3,4-dimethoxyphthalic

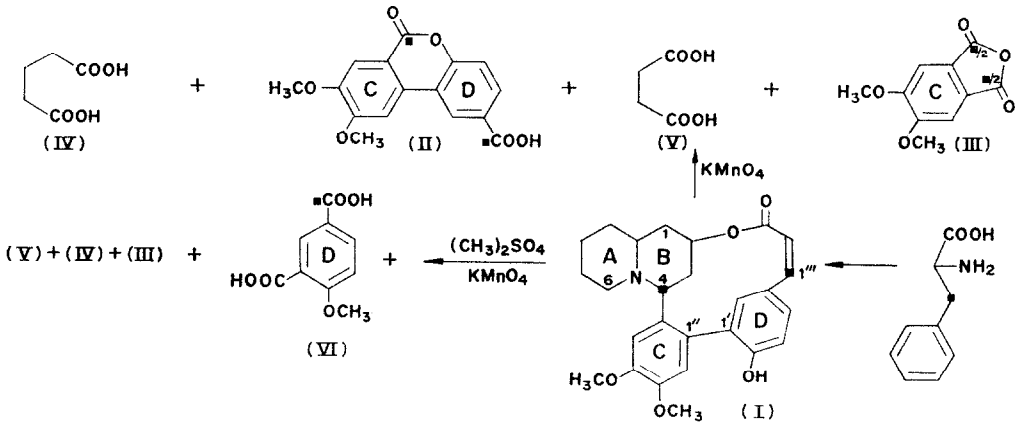
TABLE 1. INCORPORATION OF PRECURSORS INTO CRYOGENINE

Expt No.	yr.	Precursor	Wt (mg)	Total activity* (dpm × 10^{-9})	Sp. act. (dpm/mmol × 10^{-9})	Cryogenine (mg)	Total activity (dpm × 10^{-6})	Sp. act. (dpm/mmol × 10^{-7})	% Incorporation	% Specific incorporation
1	1968	[3-^{14}C]DL-phenylalanine	7·6	0·44	9·9	31·5	0·26	0·36	0·06	0·036
2	1969	[3-^{14}C]DL-phenylalanine	37·4	2·22	9·79	56·84	6·54	5·01	0·3	0·5
3	1968	[1-^{14}C]acetate(Na)	164	8·88	4·44	54·9	2·02	1·6	0·023	0·4

* dpm, disintegrations per minute.

anhydride (III) (ring C + C-4, 1′) were isolated from the mixture of acids obtained. The percent molar activity of compounds VI and III relative to I was 46 and 33 respectively (Table 2, Expt 2).

These results are in accord with the preliminary findings. We did expect, nevertheless, a higher molar activity of VI. In Expt 1 the difference between the molar activities of II and III allotted 61% of the activity to ring D + C-1‴ minus C-1′. Decarboxylation of diluted



SCHEME 1. DEGRADATION OF CRYOGENINE AND LOCATION OF RADIOACTIVITY.

VI (180°) with copper chromite in quinoline (Scheme 2) gave *p*-anisic acid (VII), *p*-hydroxybenzoic acid (VIII) and salicylic acid (IX). The specific activity of both of the para-substituted benzoic acids (VII, VIII) was essentially the same as the specific activity of VI; IX was radioinactive (Table 3). Thus, the carboxyl group of VI that originated from C-1‴ of cryogenine (I) was solely radioactive.

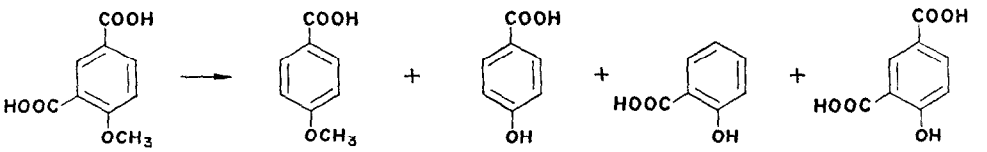
[7] J. P. FERRIS, *J. Org. Chem.* **28,** 817 (1963).
[8] S. H. KOO, Ph.D. thesis, McMaster University (1970).

TABLE 2. RADIOACTIVITY DISTRIBUTION IN CRYOGENINE DERIVED FROM [3-^{14}C]PHENYLALANINE

Expt 1 Compound	Sp. act.* (dpm/mmol × 10^{-4})	Relative sp. act.	Expt 2 Compound	Sp. act.* (dpm/mmol × 10^{-5})	Relative sp. act.
Cryogenine (I)	17·57	100	Cryogenine (I)	25·25	100
5'-Carboxy-2'-hydroxy-4,5-dimethoxy-2-biphenyl carboxylic acid -δ-lactone (II)	16·16	92	4-Methoxyisophthalic acid (VI)	11·68	46
4,5-Dimethoxyphthalic anhydride (III)	5·41	31	4,5-Dimethoxyphthalic anhydride (III)	8·27	33
Glutaric acid (IV)	0·82	4·7			
Succinic acid (V	0·58	3·3			

* Values given were obtained from carrier-diluted cryogenine.

Unfortunately, the small amount of 3,4-dimethoxyphthalic anhydride (III) obtained precluded degradation reactions on the compound. Nevertheless, since salicylic acid (from VI) was radioinactive, it is certain that no activity was located on the carboxyl group of III originating from ring D as well as on the adjacent ring carbon.



(VI) (VII) (VIII) (IX) (X)

SCHEME 2. DEGRADATION OF 4-METHOXYISOPHTHALIC ACID.

Quantitatively, several differences between the phenylalanine experiments were obtained. The incorporation of the amino acid into I was higher in the second feeding. This is especially noticeable in the specific incorporation; the percentage increase was *ca.* twofold that of the increase in radioactivity fed (Table 1). In Expt 1 92% of the molar activity of I was recovered in compound II, but only 79% was found in compounds III + VI in the second

TABLE 3. SPECIFIC ACTIVITIES OF 4-METHOXYISOPHTHALIC ACID* AND ITS DEGRADATION PRODUCTS

Compound	Sp. act. (dpm/mmole × 10^{-4})	Relative sp. act.
4-Methoxyisophthalic acid (VI)	8·29†	100
p-Anisic acid (VII)	8·05	97
p-Hydroxybenzoic acid (VIII)	8·6	104
Salicylic acid (IX)	—	< 1·3

* The 4-methoxyisophthalic acid was obtained from the degradation of [3-^{14}C]DL-phenylalanine-derived cryogenine.

† The value given was obtained from carrier-diluted 4-methoxyisophthalic acid.

experiment (Table 2). A higher yield of I was obtained in Expt 2 and the amount isolated in this experiment was comparable to the amount obtained from an [1-^{14}C]acetate feeding that was run parallel to Expt 1 (Table 1). Plants at approximately the same developmental stage were used in all three studies and the same feeding conditions were employed.

The phenolic acids obtained when VI was heated in quinoline with copper chromite were not expected. Ordinarily, anisole should be produced and, by varying the conditions, it should be possible to obtain also *p*-anisic and *o*-anisic acids. Demethylation was an unexpected occurrence. The major product formed was an acid different from VII–IX. It sublimed at a relatively higher temperature and gave a faint phenol test with diazotized *p*-nitroaniline. It was identified as 4-hydroxyisophthalic acid (X) by m.p.[9] and IR[10] comparison and by methylation. Methylation of X with dimethylsulfate gave VI; with diazomethane, dimethyl 4-methoxyisophthalate was obtained.

DISCUSSION

This study was undertaken to determine the biosynthetic origin of the aromatic system of the alkaloids of *Heimia salicifolia*. The oxygenation pattern of the biphenyl group of the *Heimia* alkaloids and the knowledge of the co-occurrence of the same type of biphenyl and of biphenyl ether alkaloids in other lythraceous species suggest that the biphenyl linkage is formed by oxidative coupling.[1,3,4] A *p*-coumaroyl and a phenolic 4-phenylquinolizidine derivative may be postulated as possible coupling units. Formation of the *p*-coumaroyl group *via* shikimate-prephenate is likely and this pathway could also be operative in the biosynthesis of a component of the 4-phenylquinolizidine unit. Thus, both coupling units could be derivable from phenylalanine. Our results show that phenylalanine is a specific precursor to both aromatic rings of cryogenine.

Without doubt, ring D and C-1‴ of I, in *Heimia*, are specifically derived from phenylalanine. S. H. Koo *et al.* showed that phenylalanine enters as a C_6–C_3 unit into the dihydrocinnamoyl group of the *Decodon* alkaloids,[4] and it is reasonable to assume a similar case for the cinnamoyl group of the *Heimia* alkaloids.

The pathway by which phenylalanine enters the 4-phenylquinolizidine group still requires clarification. The involvement of a C_6–C_1 intermediate has been postulated[1,3,4] but the exact nature of the process is yet to be established. We found an unequal distribution of activity in the two halves of the biphenyl system of [3-^{14}C]phenylalanine-derived I. This points towards a higher endogenous dilution in the path towards the formation of the 4-phenylquinolizidine group.

The data of Tables 1 and 2 show that we obtained a different % incorporation of activity from [3-^{14}C]phenylalanine into I in Expts 1 and 2. These differences remain unexplained.

EXPERIMENTAL

General methods. [3-^{14}C]DL-Phenylalanine, sodium acetate-1-^{14}C and Hyamine hydroxide (one molar soln in MeOH) were purchased from New England Nuclear Corp. Copper chromite was purchased from Pfalz & Bauer, Inc. M.ps were determined in open capillaries on a Thomas–Hoover apparatus and are corrected. Analytical TLC: Al_2O_3–GF_{254}, benzene–MeOH (19:1), was used for alkaloids and alkaloidal extracts; visualizing was with UV and Dragendorff's reagent. SiO_2–GF_{254}, benzene–MeOH–HOAc (23:2:2) and hexane–acetone–HCO_2H (20:15:1) was employed for the carboxylic acids and the products of the degradation procedures; visualizing was with UV and bromcresol green and diazotized *p*-nitroaniline reagents. Radioactivity measurements: A Packard-tri-carb model 3375 and a Packard-tri-carb model 314 EX liquid scintillation spectrometer were used. Samples of compounds II–VI and VIII were solubilized with Hyamine hydroxide (0·2–3 ml/mg compd) and counted in toluene with PPO (0·3%) and POPOP (0·01%). No Hyamine was necessary for I, VII and IX. All samples were counted to a % standard deviation of one or less. Corrections were made for counting efficiency by the internal standardization method. Radiochemical

[9] S. E. HUNT, J. IDRIS JONES and A. S. LINDSEY, *J. Chem. Soc.* 3099 (1965).
[10] SADTLER STANDARD SPECTRA. Infrared Spectrogram 19 977.

dilutions were carried out with chromatographically pure samples. The diluents were recrystallized from the same solvent as the radioactive compounds. The acids II–VI and VIII were recryst from CH_3CN-isopropanol (1:1); $CHCl_3$ was used for VII, IX. Solvents for recrystallizations and preparative chromatography were redistilled.

Radiotracer feeding. Germination and growth of *Heimia salicifolia* was done in soil in the greenhouse. A few days prior to feeding, growth was continued in an environment controlled (Sherer, Model CEL 37-14) chamber (photoperiod, 14 hr; day temp. 27°, and night temp. 15°). The radioactive compounds were dissolved in Hoegland's nutrient solution (50 ml) and divided into two beakers. Approx. 40 5–6-month-old plantlets were removed from the soil, the roots were trimmed under water and immediately placed in the tracer solution (20 plantlets/beaker). The beakers were covered with foil and placed in the growth chamber for 8–9 days. Air was bubbled through the solution. During the first 3 days of metabolism fresh nutrient was added when necessary to maintain a volume of 5–10 ml. During the remaining days the volume was maintained at *ca.* 25 ml. Following incubation, the plants were harvested and immediately dried (air-oven, 40°, 16 hr). Less than 0·4% of the administered activity remained in the aqueous solutions and washings.

Extraction and separation of the alkaloids. The dried and ground plant material was first defatted with hexane (Soxhlet, 48 hr)[11] then treated with an aqueous slurry of $Ca(OH)_2$ (150 mg/g defatted tissue), again dried (*ca.* 40°, air oven) and extracted with methanol (Soxhlet, 25 days). The methanol extract was chromatographed over basic alumina, grade I, to provide the cryogenine enriched fraction. Eluents were hexane, hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$–MeOH, MeOH. The cryogenine fraction was rechromatographed two times employing the same eluent series. Cryogenine was isolated from the $CHCl_3$ eluates, in each case, and purified by fractional crystallization ($CHCl_3$). Finally, I was diluted and recrystallized (MeOH) to constant specific activity (Table 1).

Degradation of labelled samples of [3-^{14}C]DL-*phenylalanine-derived cryogenine* I. (a) *Direct oxidation of cryogenine* (Table 2). Diluted I from Expt 1 (484·3 mg; 403·5 dpm/mg; 175·7 × 10^3 dpm/mmol) was oxidized with basic permanganate.[6] *5′-Carboxy-2′-hydroxy-4,5-dimethoxy-2-biphenylcarboxylic acid -δ-lactone* (II) (6 mg) was separated and recrystallized to constant specific activity and sublimed (Sp. act. 538·2 dpm/mg; 161·6 × 10^3 dpm/mmol). The mixture of acids remaining after the separation of II was resolved by chromatography on silicic acid,[12] to yield compounds III (as the acid), IV, V. *3,4-Dimethoxyphthalic acid* (4·4 mg) was recrystallized, sublimed to yield the anhydride III and then diluted (1:1·5), recrystallized and resublimed. (Sp. act. of undiluted III 260 dpm/mg; 54·1 × 10^3 dpm/mmol). *Glutaric acid* IV (2·5 mg) was sublimed. (Sp. act. 61·5 dpm/mg; 82 × 10^2 dpm/mmol). *Succinic acid* (1·5 mg) was sublimed (Sp. act. 50·5 dpm/mg; 58 × 10^2 dpm/mmol). (b) *Oxidation of permethylated cryogenine* (Table 2). Diluted I from Expt 2 (628 mg; 5797·5 dpm/mg; 252·5 × 10^4 dpm/mmol) was treated with dimethyl sulfate.[6] The combined methyl derivatives obtained were oxidized with basic permanganate[7,8] to a mixture of acids.[13] These were resolved, at first partially, by stepwise sublimation into three fractions. At 85–100° and 0·4 mm, an oily mixture, 56·7 mg, of III, IV and V was obtained; at 100–110° and 0·25 mm, 4·6 mg, a mixture of III, VI, and traces of other compounds was obtained; at 120–130° and 0·003 mm, 11·37 mg of compound VI was obtained. The first two fractions were chromatographed on silicic acid[12] to yield metahemipic acid (2·11 mg), glutaric acid (0·21 mg) and succinic acid (6·75 mg). *4-Methoxyisophthalic acid* (VI). The third fraction, above, was diluted[14] (1:6·68) recrystallized to constant specific activity and sublimed (Sp. act. of undiluted VI 59·4 × 10^2 dpm/mg; 116·8 × 10^4 dpm/mmol). *Metahemipic anhydride* (III). The metahemipic acid obtained above (2·1 mg) was sublimed (760 mm; 150–160°) to separate traces of VI; the anhydride was recrystallized, diluted[15] (1:12) and recrystallized once (Sp. act. of undiluted III 36·5 × 10^2 dpm/mg; 82·7 × 10^4 dpm/mmol). *Succinic acid* (V). The compound was sublimed (0·025 mm, 94°). The samples were inactive by radioanalysis.

p-*Anisic acid* (VII), p-*hydroxybenzoic acid* (VIII) *and salicylic acid* (IX) *from* VI. *4-Methoxyisophthalic acid* (VI) (97·10 mg, 422·7 dpm/mg; 82·9 × 10^3 dpm/mmol) was heated (175–185°; 100 min) with copper chromite (93·2 mg) in quinoline (4·6 ml). The reaction mixture was adjusted to pH 9–10, extracted with EtOAc,[16] acidified and extracted again with EtOAc to yield, after drying (Na_2SO_4) and evaporation of the solvent (vacuum), a mixture of organic acids. These were partially separated by sublimation (80–110°, 0·1–0·06 mm). The sublimate (23·3 mg) consisted of VII, VIII and IX and minor acids. The residue (32·6 mg) was heated again with copper chromite in quinoline and the products were separated as before to yield a small crop of VIII, IX and traces of other acids. The sublimates were separated on silicic acid[12] into VIII (3·5 mg) and a mixture (14·6 mg) of VII and IX. The latter two compounds were separated by TLC (SiO_2–

[11] For the two phenylalanine feedings the hexane extract represented 0·5 and 0·8% of the administered activity respectively. It was 4·2% for the acetate feeding.

[12] J. R. LESSARD and P. MCDONALD, *J. Sci. Food Agric.* **17**, 157 (1966).

[13] A second batch of diluted I (446 mg; 3·30 × 10^3 dpm/mg) was oxidized in a similar manner.

[14] 4-Methoxyisophthalic acid m.p. 277–279°, m.p. of dimethylester 95–96° was obtained by permanganate oxidation of 2,4-dimethylanisole.[9]

[15] Metahemipic anhydride m.p. 179 was obtained from permanganate oxidation of emetine.

[16] No anisole could be detected in this extract [TLC, SiO_2–GF_{254}, hexane–$CHCl_3$ (1:1)].

GF_{254}, 20 × 20 cm × 0·25 mm; benzene–HOAc–MeOH (23:2:2); IX, R_f *ca.* 0·6, VII, R_f *ca.* 0·7). p-*Anisic acid* (VII). The compound was twice recrystallized and sublimed to yield 2·86 mg of VII. After dilution (1:2·4) it was recrystallized once and sublimed (Sp. act. of undiluted VII 80·5 × 10^3 dpm/mmol). *Salicylic acid* (IX). The compound was twice recrystallized (yield 2·13 mg), diluted 1:2·36 and sublimed. The samples (0·455; 0·54; 0·375 mg) were radioinactive. The p-*hydroxybenzoic acid* (VIII) fractions were washed with hexane and $CHCl_3$, recrystallized, diluted (1:4·5) and sublimed (0·065 mm, 100–110°) (Sp. act. of undiluted VIII 86·1 × 10^3 dpm/mmol). 4-*Hydroxyisophthalic acid* (X). 4-Methoxyisophthalic acid (VI) (294 mg) was heated with copper chromite in quinoline in N_2 and extracted as before. The acidic extract was dissolved in hot EtOAc. The precipitate (44 mg) that separated upon cooling was sublimed in a stepwise manner (0·15–0·003 mm; 100–145°). The sublimate collected at 145° and 3 × 10^{-3} mm was recrystallized (EtOAc, yield 21·3 mg). A sample was resublimed to give m.p. 299–302°; reported 310° dec.[9] The IR spectrum was found to be as reported[10] with one additional strong band at 2325 cm^{-1}. Methylation with a tenfold excess of both Me_2SO_4 and NaOH (2·5 N) at reflux temp (8 hr) gave VI (TLC, m.p.). Methylation with CH_2N_2 gave dimethyl 4-methoxyisophthalate[9] (TLC, m.p., m.m.p.). X is the major product of the degradation of VI with heat-copper chromite–quinoline at all the temperatures studied (135–220°). It is almost exclusively obtained at 135° with a shift of the concentration towards the monocarboxylic acids with an increase in reaction temperature. At temperatures above 200° the overall yield is reduced.

Acknowledgement—We thank Professor W. J. Kelleher for constructive criticism and advice during the course of these studies.