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BIOSYNTHESIS OF THE AGLYCONE OF CHARTREUSIN IN *STREPTOMYCES* SP. X-465

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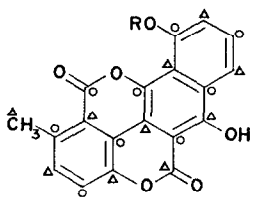
Abstract—The aglycone of the complex coumarin antibiotic chartreusin has been shown to be entirely derived via the acetate pathway. The labelled aglycone, derived from feeding diethylmalonate-^{14}C, has been degraded and a pathway for its biosynthesis is proposed.

INTRODUCTION

CHARTREUSIN (I, Fig. 1) is a glycosidic complex coumarin antibiotic produced by *Streptomyces* spp.[1-4] Although simple coumarins are widespread throughout the plant kingdom relatively few have been isolated from micro-organisms.[5] In general, plant coumarins are derived via the shikimic acid pathway.[6] Of the microbial coumarins however, alternariol[7] and the aflatoxins[8] are derived via the acetate pathway, whereas dicoumarol is a transformation product of *o*-coumaric acid[9] and novobiocin is derived via the shikimic acid pathway.[10] The origin of the aglycone of chartreusin cannot therefore be predicted by consideration of the biogenesis of other coumarins.



I R=*D*-digitalose-*D*-fucose-
II R=H

FIG. 1. DERIVATION OF CHARTREUSIN AND ITS AGLYCONE FROM ACETATE ($\overset{\Delta}{CH_3}-\overset{O}{C}O-$) AS PROPOSED BY SCHMID.[13]

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[10] L. A. KOMINEK, in *Antibiotics* (edited by D. GOTTLIEB and D. P. SHAW), Vol. II, Springer-Verlag, Berlin (1967).

Structural comparison of chartreusin aglycone with other natural products suggests that it could be derived via the acetate pathway. For example ϵ-pyrromycinone,[11] alternariol[7] and curvularin[12] are all products of the acetate pathway. Indeed, Schmid[13] has stated that chartreusin aglycone (II) is formed from ten acetate units as shown in Fig. 1. No experimental results or details were quoted however.

This paper describes experiments carried out to investigate further the route by which chartreusin aglycone is formed.

RESULTS

In order to determine the most suitable time to feed radioactive compounds to *Streptomyces* sp. X-465, cultures used were monitored at regular intervals to find when maximum chartreusin production occurred. The parameters measured were pH, mycelial dry weight and amount of chartreusin. The fermentation was found to occur in the three phases typical of most antibiotic-producing cultures.[14]

Sodium acetate-1-^{14}C and -2-^{14}C, diethylmalonate-1-^{14}C and -2-^{14}C, *trans*-cinnamic acid-1-^{14}C and -3-^{14}C, and methionine-$^{14}CH_3$ were administered to cultures of *Streptomyces* sp. X-465. The specific incorporation of these compounds into chartreusin and its aglycone isolated from these cultures is given in Table 1. (Specific incorporation is the ratio of the specific activities of the metabolite and precursor expressed as a percentage.)

Of the compounds fed to the organism diethylmalonate has the highest specific incorporation into the aglycone, indicating that the aglycone is wholly or partly derived via the acetate pathway. The specific incorporation of *trans*-cinnamic acid-1-^{14}C is 9·27 and

TABLE 1. SPECIFIC INCORPORATION OF POSSIBLE PRECURSORS INTO CHARTREUSIN AND ITS AGLYCONE

Precursor	Specific incorporation ($\times 10^{-2}$%)		Aglycone/chartreusin $\times$ 100
	Chartreusin	Aglycone	
Sodium acetate-1-^{14}C	1·32	0·37	26·68
	6 37	1 58	25·12
	3·49	0·82	23·45
Sodium acetate-2-^{14}C	10·18	1·95	19·37
	9 78	2·01	20·53
	1·17	0·31	25·33
Diethylmalonate-1-^{14}C	139·30	30·16	46·18
	54·64	17·81	30 68
Diethylmalonate-2-^{14}C	134·70	42 45	31·74
	135·30	38·12	35·50
trans-Cinnamic acid-1-^{14}C	48·84	9·27	18·21
	57·19	14 84	27 52
trans-Cinnamic acid-3-^{14}C	11·60	0 25*	2 14
	1·99	0 16*	7·94
L-Methionine-$^{14}CH_3$	8·37	0 25	3·00
	3·00	0 11	3·80

* Approximate value only, due to low activity.

[11] W. D. OLLIS and I. O. SUTHERLAND, in *Recent Developments in the Chemistry of Natural Phenolic Compounds* (edited by W. D. OLLIS), Pergamon Press, Oxford (1961).
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$14{\cdot}84 \times 10^{-2}$ whereas that of *trans*-cinnamic acid -3-^{14}C is 0·16 and $0{\cdot}25 \times 10^{-2}$. If the aglycone were formed via the shikimic acid pathway C-1 and C-3 of cinnamic acid would be expected to be incorporated to the same extent. The results therefore suggest the aglycone is not formed via the shikimic acid pathway as are higher plant coumarins. The specific incorporation of methionine-$^{14}CH_3$ into chartreusin is approximately the same as that of sodium acetate (Table 1). As only 3–4 per cent of this activity is associated with the aglycone none of the carbon atoms of the aglycone are derived from methionine. Therefore the results indicate that the aglycone is totally derived via the acetate pathway.

To investigate the derivation of the individual carbon atoms samples of chartreusin aglycone-^{14}C were degraded according to a scheme (shown in Fig. 2) used in the elucidation of the structure of the aglycone.[15] The aglycone (II) loses CO_2, on heating with alcoholic NaOH in the absence of air, to give the decarboxyaglycone (III). This, on passage of O_2 through the solution, yields a quinone (IV) which can be cleaved by H_2O_2, with loss of CO_2, to 3-hydroxyphthalic acid (V) and 3-hydroxy-6-methylphthalic acid (VI). The two samples of CO_2 were isolated separately as methyl benzoate. The substituted phthalic acids were isolated as the methyl esters of their methyl ethers and were separated and their specific activities determined by radio gas chromatography (RGC).

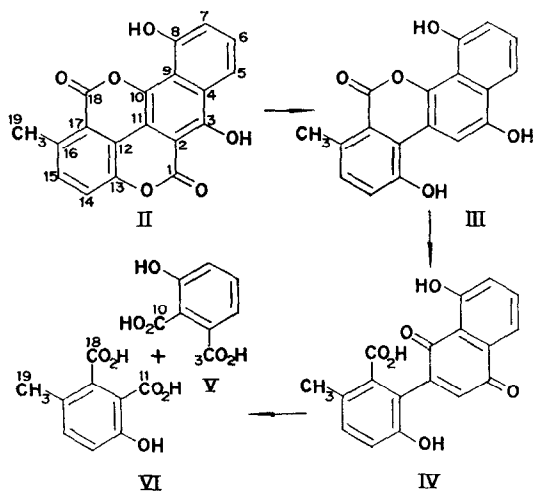

FIG. 2. DEGRADATIONS OF CHARTREUSIN AGLYCONE.
The numbering is not systematic but is used to clarify the results and discussion of the results.

Two samples of chartreusin aglycone-^{14}C derived from feeding diethylmalonate-1-^{14}C and two from diethylmalonate-2-^{14}C were degraded by this method. The results are shown in Table 2. The specific activities of the methyl benzoates show that C-1 of chartreusin aglycone (Fig. 2) is derived from the methylene and C-2 from the carboxyl of malonate. If the aglycone were derived as suggested by Schmid[13] then odd numbered carbon atoms of the aglycone (as shown in Fig. 2) would be derived from the methyl, and even from the carboxyl of acetate. By this scheme, five carbon atoms in the fragment C-11–19 and four in the fragment C-3–10 would be derived from the methyl of acetate. The ratio of the specific activities of C-11–19/C-3–10 on feeding diethylmalonate labelled in the methylene

[15] E. VON SIMONITSCH, W. VON EISENHUTH, O. A. STAMM and H. SCHMID, *Helv. Chim. Acta.* **47**, 1459 (1964).

TABLE 2. SPECIFIC ACTIVITIES OF FRAGMENTS FROM THE DEGRADATION OF CHARTREUSIN AGLYCONE-^{14}C

Degradation product	Specific activity (mc/mM $\times 10^{-4}$)			
	Sample a*	Sample b*	Sample c*	Sample d*
Aglycone	2·547	43 120	3 129	8·507
Methyl benzoate (C1)	0·040	1 021	0 113	0 572
Methyl benzoate (C2)	0·216	3 772	0·019	0 107
Dimethyl 3-methoxy-phthalate (C3-10)	1 010	15·650	0·955	3 352
Dimethyl 3-methoxy-6-methylphthalate (C11-19)	0 987	14·620	1·267	3 932
Ratio of C-11–19/C-3–10	0 977	0 934	1·296	1 173

* Samples a and b were from cultures to which diethylmalonate-1-^{14}C had been administered, and similarly c and d were from diethylmalonate-2-^{14}C.

were found to be 1·296 and 1·173 (Table 2). Similarly the ratio on feeding carboxyl-labelled diethylmalonate were 0·977 and 0·934 compared with a theoretical value of 1·000. The results are therefore consistent with derivation as suggested by Schmid[13] (Fig. 1).

DISCUSSION

The results of feeding acetate-^{14}C and diethylmalonate-^{14}C indicate the acetate pathway as the route of biosynthesis of chartreusin aglycone. The difference in incorporation of C-1 and C-3 of cinnamic acid suggests the shikimic acid pathway is not involved. This difference in incorporation could be due to metabolism of cinnamic acid by β-oxidation, only cinnamic acid-1-^{14}C giving ^{14}C-labelled acetyl Co.A. This does not however explain the higher incorporation of cinnamic acid-1-^{14}C than of sodium acetate-^{14}C. This is possibly due to cinnamic acid not being a normal metabolite and therefore being rapidly metabolized by β-oxidation.

Although methionine is not a precursor of the aglycone, it is incorporated into the glycoside. One of the sugars of the disaccharide chain of chartreusin, digitalose, is a methoxy sugar. By analogy with other such sugars[16] this methyl group would be derived from methionine, accounting for the incorporation of methionine-$^{14}CH_3$.

The results of the feeding experiments therefore suggest the acetate pathway as the sole route of derivation; and the results of the degradations are consistent with the scheme proposed by Schmid.[13] The aglycone however cannot be formed from one chain directly, rearrangement must occur, or alternatively it could be formed from two chains as in citromycetin.[17] If only one chain was involved the aglycone could be formed from a precursor of any of the types shown in Fig. 3 (VII, VIII and IX). Even though VII has been isolated as a metabolite of a mutant strain of *Streptomyces rimosus*,[18] IX is the most likely angular precursor as it would require the least cleavage. However, few angular tetracycles have been isolated whereas many linear tetracycles are known, for example the anthracyclinones[19] and the pretetramids.[20] Precursor VIII is therefore more probable

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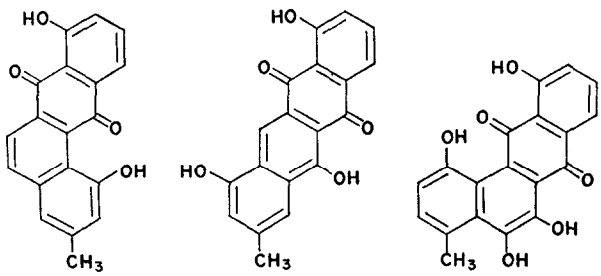

VII VIII IX

FIG. 3. POSSIBLE PRECURSORS OF CHARTREUSIN AGLYCONE.

than IX. Compounds similar to VII, VIII and IX have been suggested as precursors of the fungal coumarins the aflatoxins.[8] However for these postulated precursors the substituent methyl group, which would be expected in compounds derived from the acetate pathway, was absent.

EXPERIMENTAL

Fermentation procedure. A sporulating mycelium was obtained by growth of *Streptomyces* sp. X-465[4] on a solid medium of composition: sucrose, 30 g; $NaNO_3$, 2 g; NaCl, 0·3 g; K_2HPO_4, 0·3 g; $CaCO_3$, 3 g; $MgSO_4.7H_2O$, 1 g; agar, 15 g; and H_2O to 1 l. Spores harvested from this culture were inoculated into a vegetative medium of: dextrose, 30 g; $NaNO_3$, 2·5 g; distiller's solubles,* 7·5 g; NaCl, 5 g; K_2HPO_4, 0·4 g; KH_2PO_4, 1·6 g; $CaCl_2$, 0·4 g; $MgSO_4.7H_2O$, 0·025 g; $FeSO_4.7H_2O$, 0·02 g; $ZnSO_4.7H_2O$, 0·01 g; and H_2O to 1 l. The vegetative cultures were incubated for 48 hr at 250 rev/min and 29 ± 0·5° and aseptically transferred in 2·5 ml aliquots, to sterile powder vials. They were then quick frozen by immersion in liquid N_2, and stored at −40°.

Vegetative cultures were reconstituted by thawing the required amount of frozen vegetative mycelium, at 35°, and inoculation into vegetative medium at a 5 per cent rate. After growth for 2 days, the culture was broken up in a Waring blender and inoculated into a medium which would support production of chartreusin. This production medium had the following composition: lactose, 10 g; $NaNO_3$, 5 g; distiller's solubles, 7·5 g; NaCl, 5 g; KH_2PO_4, 0·5 g; $CaCl_2$, 0·4 g; $MgSO_4.7H_2O$, 0·025 g; $FeSO_4.7H_2O$, 0·025 g; $ZnSO_4.7H_2O$, 0·01 g; H_2O to 1 l. The medium was adjusted to pH 6·0 before autoclaving. Cultures in production medium were incubated at 250 rev/min and 29 ± 0·5°.

Monitoring of production cultures. Aliquots (10 ml) of production cultures were withdrawn at regular intervals. The pH was determined and the aliquot added to acetone (40 ml). After shaking, the mixture was filtered and the mycelium dried at 105° for 1 hr and weighed to give the mycelial dry weight (MDW). The acetone was removed from the filtrate *in vacuo* and the aqueous solution acidified to pH 5·5. It was extracted with 3 × 25 ml of CH_2Cl_2. The extracts were bulked and evaporated. The residue was dissolved in 95% EtOH and assayed for chartreusin by determining the absorption at 424 nm.

Addition of radioactive compounds to cultures. When the curves of pH, mycelial dry weight and chartreusin titre showed that growth was slowing and chartreusin production increasing, the compound was fed as a solution in H_2O or H_2O–EtOH. The radioactive compounds which have been fed are *trans*-cinnamic acid-1-^{14}C and -3-^{14}C, sodium acetate-1-^{14}C and -2-^{14}C, diethylmalonate-1-^{14}C and -2-^{14}C and L-methionine-$^{14}CH_3$. The cinnamic acid-1-^{14}C was available within the laboratory as a gift from Prof. H. G. Floss. The cinnamic acid-3-^{14}C was synthesised from benzaldehyde-1-^{14}C.[21] Other compounds were obtained from the Radiochemical Centre, Amersham, as was benzaldehyde-1-^{14}C.

Extraction and purification of chartreusin. When the titre of chartreusin was no longer increasing, the cultures were centrifuged. The mycelial pellet was extracted with 3 × boiling 80% acetone. The acetone extracts were filtered, and the acetone removed *in vacuo*. The aqueous solution was then added to the supernatant from centrifugation and the bulked solution acidified to pH 5·5. The solution was extracted with 3 × CH_2Cl_2, and the extracts bulked and evaporated to, dryness *in vacuo*. The crude chartreusin obtained was then purified by column and/or paper chromatography. The following solvents were used:

* Scotasol, Thomas Borthwick (Glasgow) Ltd.

[21] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* 33, 948 (1955).

$CHCl_3$–MeOH (95:5); *n*-BuOH saturated with M/25 NH_3; isoPrOH–H_2O (7:3); $CHCl_3$–HOAC–H_2O, (4:1:1), lower phase. The chartreusin was rechromatographed to constant specific activity.

Degradation of chartreusin. Chartreusin was degraded to its aglycone by sublimation at 250° and 0·1 mm Hg.[15] The aglycone was recrystallized from dimethylformamide and dioxan to constant specific activity. 4 samples of aglycone-^{14}C (2 from feeding diethylmalonate-1-^{14}C and 2 from diethylmalonate-2-^{14}C) were degraded further as follows. Aglycone (50 mg) was dissolved in MeOH (5 ml) and NaOH (0·4 g in 1·0 ml CO_2-free H_2O). The solution was heated at 55° for 30 min with a flow of CO_2-free He through the solution It was cooled and HCl (1·0 ml) added. CO_2 from the reaction mixture was carried in the He through a trap containing acetone–dry ice and condensed in a trap in liquid N_2. The CO_2 collected in this trap was converted to methyl benzoate by the method of Lin and Pohlit.[22]

MeOH (10 ml) and NaOH (0·5 g in 1·25 ml CO_2-free H_2O) were added to the reaction flask. CO_2-free air was passed through the reaction mixture for 2 hr. This was replaced by CO_2-free He, and the solution heated to 80°. H_2O_2 (100 vol. 1·0 ml) was added and the mixture heated for a further 5 min. The solution was allowed to cool and acidified with HCl (1·5 ml). The CO_2 was collected and converted to methyl benzoate as above.

The reaction mixture was extracted with Et_2O and the extract evaporated to dryness *in vacuo*. The residue was methylated with Me_2SO_4 and diazomethane.

Determination of radioactivity. Radioactivity measurements were made using an IDL liquid scintillation counter. The scintillators used were Toluene–Triton X-100[23] and a toluene scintillator of composition: PPO, 0·6 g; POPOP, 0 5 g; toluene to 100 ml.

Dimethyl 3-methoxyphthalate and dimethyl 3-methoxy-6-methylphthalate were separated and their specific activities determined by radio gas chromatography (RGC). A Perkin-Elmer F11 gas chromatograph was connected to a Bodenseewerk flow through reactor (RGC 170) the proportional counter of which was connected to a Berthold Esone combination programme counting unit (LB 241). The column used was silicone rubber E301 on AW DMCS Chromosorb G 80–100 mesh, $2\frac{1}{2}$: $97\frac{1}{2}$, 2 m × 6 mm o d. The injection temperature was 300° and the column temperature 210°. He was used as carrier gas at a flow rate of 20 ml/min. The retention times of dimethyl 3-methoxyphthalate and dimethyl 3-methoxy-6-methylphthalate under these conditions are 9 and 11 min respectively.

Dimethyl 3-methoxyphthalate. 3-Hydroxyphthalic anhydride was prepared from 3-nitrophthalic anhydride by the method of Eliel *et al.*[24] and methylated.

Dimethyl 3-methoxy-6-methylphthalate. This was prepared by the method of Birch and Hextall.[25]

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