66. *Studies in Relation to Biosynthesis. Part XIII.* Griseofulvin.*

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Me·$^{14}CO_2H$, when fed to a strain of *Penicillium griseofulvum* Dierckx, leads to griseofulvin with the isotopic distribution shown in (I). This result supports the general validity of the acetic acid hypothesis. A preliminary account of some of this work has already appeared.[1]

Structural evidence reviewed in Part I [2] suggested that the skeletons of many natural phenolic compounds are derived, at least in part, by the head-to-tail linkage of acetic

* Part XII, Birch, Pride, and Smith, *J.*, 1957, 5096.

[1] Birch, Massy-Westropp, Rickards, and Smith, *Proc. Chem. Soc.*, 1957, 98.
[2] Birch and Donovan, *Austral. J. Chem.*, 1953, **6**, 360.

acid units. The complete skeletons of anthraquinones related to emodin and of depsides related to orsellinic acid, and the phloroglucinol ring of plant pigments such as cyanidin and quercetin, probably arise in this way. Two types of ring-closure can be distinguished: an aldol type leading to a variety of structures, notably to orcinol derivatives, and a *C*-acylation type leading notably to phloroglucinol derivatives. This hypothesis was later used to assist structural investigations and has already predicted the correct one of several possible formulæ for eleutherinol,[3] flaviolin,[4] mellein,[5] α- and β-sorigenin,[6] and nalgiovensin.[7] Biochemical evidence in support of the hypothesis was obtained [8] by feeding $Me\cdot{}^{14}CO_2H$ to a strain of *Penicillium griseofulvum* Dierckx; the resulting 2-hydroxy-6-methylbenzoic acid was shown to have the isotopic distribution (II) (an asterisk denotes ^{14}C).

We have now examined the incorporation of $Me\cdot{}^{14}CO_2H$ into griseofulvin (I), produced by another strain of *P. griseofulvum* (L.S.H. Cat. No. P. 38). This substance contains a phloroglucinol ring and a (potential) orcinol ring and its formation from acetic acid would involve both types of ring-closure. The griseofulvin was degraded as outlined below, and the intensities of radioactivity of the products measured by Popjak's method.[9] We found it advantageous to express the activities of the compounds obtained in a series of degradations as the products of the molecular weights and the counts per 100 seconds of 1 cm.^{2} of substance at " infinite " thickness; these quantities are linearly proportional to the molar activities and we term them the relative molar activities. They are proportional to the number of labelled carbon atoms per molecule, which can therefore be calculated from the relative molar activity of griseofulvin on the assumption that it contains seven ^{14}C. These values are shown in the chart. It may be noted that the sum of the relative molar activities of any complete set of degradation products should be identical with that of the original substance. In these experiments the maximum statistical counting error is considered to be ±3%. From the results (see Experimental section) it appears that no other systematic errors are involved. The results prove the isotopic distribution (I) and lead to confidence in the general validity of the acetic acid hypothesis.

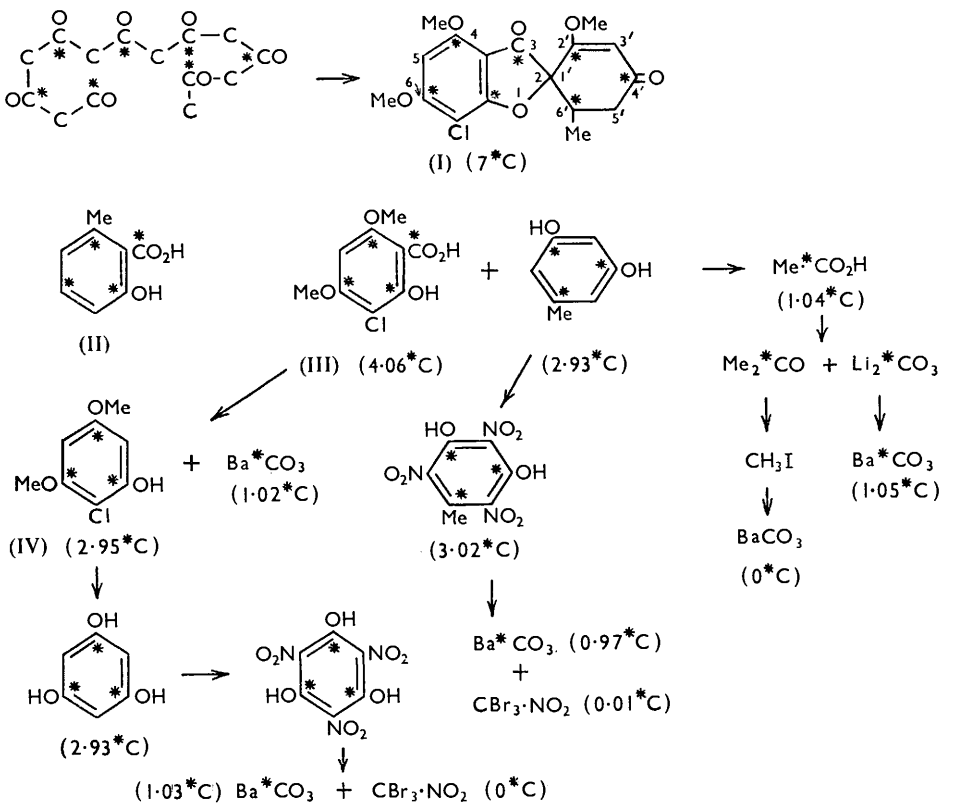
The methoxyl groups have already been shown to arise from the usual biochemical C_1-donor system (experimentally choline was used),[10] so that the sources of all of the carbon atoms in griseofulvin are now known.

Degradations of Griseofulvin.—Attempts to repeat the earlier fission [11] of griseofulvin with 2N-sodium methoxide failed to give the high yield of 3-chloro-2-hydroxy-4 : 6-dimethoxybenzoic acid (III) claimed, and the reaction was found to be sensitive to traces of water. Under our best conditions 35% of the acid (III) was obtained, together with an uncrystallisable gum. Fusion of griseofulvin with potassium hydroxide is known [12, 13] to give orcinol (74%), and this reaction was applied to the gum from the above reaction, producing a further quantity of the acid (52% total) and orcinol (85%). Direct fusion of griseofulvin gave a lower yield of the acid, and the two-stage process is advantageous.

The acid (III) was best decarboxylated by heat in the presence of glass wool, 2-chloro-3 : 5-dimethoxyphenol (IV) being obtained in 81% yield. Attempts were made to obtain phloroglucinol directly by heating the acid with acetic–hydriodic acid, but although decarboxylation was very rapid only a polymer resulted. The acid (III) was readily

[3] Birch and Donovan, *Austral. J. Chem.*, 1953, **6**, 372.
[4] *Idem, Chem. and Ind.*, 1954, 1047; *Austral. J. Chem.*, 1955, **8**, 529; Davies, King, and Roberts, *J.*, 1955, 2782; *Chem. and Ind.*, 1954, 1110.
[5] Blair and Newbold, *Chem. and Ind.*, 1955, **93**; *J.*, 1955, 2871.
[6] Haber, Nikuni, Schmid, and Yai, *Helv. Chim. Acta*, 1956, **39**, 1654.
[7] Birch and Massy-Westropp, *J.*, 1957, 2215.
[8] Birch, Massy-Westropp, and Moye, *Austral. J. Chem.*, 1955, **8**, 539.
[9] Popjak, *Biochem. J.*, 1950, **46**, 560.
[10] Hockenhull and Faulds, *Chem. and Ind.*, 1955, 1390.
[11] Grove, MacMillan, Mulholland, and Rogers, *J.*, 1952, 3977.
[12] Oxford, Raistrick, and Simonart, *Biochem. J.*, 1939, **33**, 240.
[13] Grove, MacMillan, Mulholland, and Rogers, *J.*, 1952, 3949.

dechlorinated by the action of Raney alloy and alkali,[14] giving 2-hydroxy-4 : 6-dimethoxybenzoic acid almost quantitatively. However, again extensive polymerisation occurred on attempted demethylation with 10N-hydrochloric acid.



Dechlorination of the phenol (IV) by the same method gave phloroglucinol dimethyl ether which was also unexpectedly sensitive to concentrated hydrobromic acid. Demethylation in 86% yield to phloroglucinol was accomplished with 5N-hydrochloric acid. The phloroglucinol was nitrosated [15] and the potassium salt of trinitrosophloroglucinol directly oxidised with hydrogen peroxide to trinitrophloroglucinol.[16] Fission of this substance with barium hypobromite gave the hydroxyl-carrying carbon atoms from positions 4, 6, and 7*a* as barium carbonate (2·16 mols.), and those from positions 3*a*, 5, and 7 as bromopicrin (2·46 mols.), further oxidised to barium carbonate. Trinitro-orcinol was degraded similarly, except that only two carbon atoms (positions 4′ and 2′) appear as carbon dioxide (1·84 mols.). It was more convenient not to isolate the acetic acid formed in this experiment but to produce it directly from orcinol itself by a Kuhn–Roth oxidation. The acetic acid was then converted into acetone and lithium carbonate by pyrolysis.[17] Contrary to records,[18] no interchange of carboxyl- and methyl-carbon atoms was observed in this pyrolysis, the barium carbonate from the methyl group being completely inactive.

[14] Schwenk, Papa, Whitman, and Ginsberg, *J. Org. Chem.*, 1944, **9**, 1.
[15] Freudenberg, Fikentscher, and Wenner, *Annalen*, 1925, **442**, 309.
[16] Cf. Travagli, *Atti Accad. sci. Ferrara*, 1949—50, **27**, 3; *Chem. Abs.*, 1951, **45**, 7544
[17] Cornforth, Hunter, and Popjak, *Biochem. J.*, 1953, **54**, 597.
[18] Popjak, Hunter, and French, *ibid.*, 1953, **54**, 238.

Experimental

Radioactive Assay.—Specimens were assayed for radioactivity with an end-window counter as infinitely thick, solid samples of 1 cm.^{2} cross-sectional area,[9] and counting rates were corrected for background and dead time of the instrument. Counting-equipment consisted of an EKCO Automatic Scaler Type N530D, in conjunction with an EKCO Probe Unit Type N558 and an EHM2S Geiger tube. The counts per 100 sec. were determined by recording the time required for 10^4 counts to be aggregated. Hence the statistical counting error is no greater than $3/\sqrt{10^4}$, *i.e.*, 3%. Estimates of absolute activity (where required) were obtained by comparison with a disc of radioactive poly(methyl methacrylate) (Amersham) of nominal specific activity 1 μc/g., counted under identical geometrical conditions. Other errors could be introduced by variations in packing or through the different constitutions of the substances being examined. In practice, no such errors could be distinguished.

Isolation of [^{14}C]*Griseofulvin.*—*Penicillium griseofulvum* Dierckx (London School of Hygiene Catalogue No. P. 38) was grown under the conditions described previously.[12] Preliminary experiments had shown that little mycelianamide and griseofulvin were produced until after about 14 days, production then increasing considerably. Accordingly, after 18 days' growth, a solution of sodium [*carboxy*-^{14}C]acetate (1·0 mc; 7·6 mg.) was distributed evenly among 8 flasks, each containing 350 ml. of medium. After a further 9 days' growth the products were isolated as recorded [12] and the residues chromatographed on acid alumina [13] to obtain a further small quantity of griseofulvin. Griseofulvin (0·2 g.) and mycelianamide (1·2 g.), m. p. 168—170°, resulted on crystallisation. This impure griseofulvin (100 mg.) was diluted 50 times with purified inactive material and recrystallised from ethanol, yielding colourless crystals, m. p. 219—220° (4·64 g.) [Found: relative molar activity × 10^{-3}, 417].

The yield in terms of incorporation of [*carboxy*-^{14}C]acetate was about 1%, 9·6 μc of [^{14}C]-griseofulvin being isolated. The yield has been previously erroneously given by us as 10%.[1]

Alkali Fission of Griseofulvin.—Griseofulvin (500 mg.) in 2N-sodium methoxide (50 ml.) was refluxed for 2·25 hr. under nitrogen. The cooled solution was poured into water (60 ml.) and acidified with hydrochloric acid, and the 3-chloro-2-hydroxy-4 : 6-dimethoxybenzoic acid (106 mg.) was filtered off. The gum (370 mg.) obtained by ether-extraction of the filtrate was mixed with potassium hydroxide (1·4 g.) and water (0·6 ml.) in a nickel crucible and heated under nitrogen at 160°. The temperature was raised to 220° during 15 min. and kept at 220—230° for 70 min. The residue, on cooling, was dissolved in water, and the solution saturated with carbon dioxide and ether-extracted. The precipitate (79 mg.) obtained on acidification of the aqueous layer was combined with the 3-chloro-2-hydroxy-4 : 6-dimethoxybenzoic acid obtained earlier and recrystallised from ethyl acetate, as colourless crystals (52%), m. p. 220—222° (decomp.), which were assayed for ^{14}C [Found: relative molar activity × 10^{-3}, 242; 4*C requires 238].

The ether extract of the bicarbonate solution was dried (Na_2SO_4) and evaporated to a gum, which sublimed at 110—120°/0·05 mm. The sublimate was crystallised from benzene–light petroleum (b. p. 40—60°), then from benzene, and resublimation gave orcinol, m. p. 106—107° (148 mg., 85%) [Found: relative molar activity × 10^{-3}, 175; 3*C requires 179].

2 : 4 : 6-*Trinitro-orcinol.*—To orcinol (164 mg.) dissolved in concentrated sulphuric acid (0·6 ml.) was added with stirring during 5 min. at 0° an ice-cold mixture of concentrated nitric acid (0·4 ml.) and concentrated sulphuric acid (0·8 ml.). Further concentrated nitric acid (0·65 ml.) was added, and after 40 min. at room temperature the mixture was poured into ice and water. 2 : 4 : 6-Trinitro-orcinol was filtered off and recrystallised from water as yellow needles, m. p. 171—172° (decomp.) (274 mg., 80%) (lit., m. p. 162° [19] and 163·5° [20]) [Found: relative molar activity × 10^{-3}, 180; 3*C requires 179]. An inactive specimen was analysed (Found: C, 32·3; H, 2·3; N, 16·0. Calc. for $C_7H_5O_8N_3$: C, 32·4; H, 2·0; N, 16·2%).

Hypobromite Degradation of 2 : 4 : 6-*Trinitro-orcinol.*—(a) *The nitro-carrying carbon atoms.* To trinitro-orcinol (131 mg.) in hot water (10 ml.) was added a hot solution of hydrated barium hydroxide (0·32 g.) in water (8 ml.). The stirred barium salt suspension was cooled to 0°, and an ice-cold solution of barium hypobromite [from bromine (0·4 ml.) and barium hydroxide (2·0 g.) in water (50 ml.)] was added. After 1 hour's stirring at room temperature, the bromopicrin was separated by steam-distillation and collected with ether. Evaporation, after washing

[19] Stenhouse, *J.*, 1871, 357.
[20] Merz and Zetter, *Ber.*, 1879, **12**, 2038.

of the extract with water and drying (Na_2SO_4), left the bromopicrin as a colourless liquid (395 mg., 88%). Van Slyke–Folch oxidation [21] of the bromopicrin gave barium carbonate, which was collected and washed by centrifugation for radioactive assay [Found: relative molar activity $\times 10^{-3}$, 0·6].

(b) *The hydroxyl-carrying carbon atoms.* Carbonate-free solutions were used throughout with a stream of nitrogen free from carbon dioxide and a soda-lime tube to exclude atmospheric contamination. The barium salt formed from trinitro-orcinol (43 mg.) and barium hydroxide (0·32 g.) was stirred with barium hypobromite solution [from bromine (0·16 ml.) and barium hydroxide (1·6 g.) in water (40 ml.)]. After 1 hr. a centrifuge tube containing barium hydroxide solution was connected to the system, which was rapidly evacuated and then closed. Concentrated hydrochloric acid was released into the mixture, which was stirred for 1 hr. and warmed to 40° to assist diffusion of the liberated carbon dioxide. Barium carbonate (60 mg., 92%) was collected by centrifugation and the observed counting rate corrected for a 3% dilution with external carbon dioxide, as indicated by a blank reaction [Found: relative molar activity $\times 10^{-3}$, 57·8; 1*C requires 59·6].

Kuhn–Roth Oxidation of Orcinol.—Kuhn–Roth oxidation of orcinol (270 mg.) gave acetic acid, isolated by titration of the steam-distillate with 0·1N-lithium hydroxide. The *p*-bromophenacyl ester, m. p. 86° [from light petroleum (b. p. 40—60°)], was prepared for ^{14}C assay [Found: relative molar activity $\times 10^{-3}$, 62·2; 1*C requires 59·6], and the remainder of the lithium acetate degraded by pyrolysis *in vacuo* (cf. ref. 17) at 375—385° for 15 min. The residual lithium carbonate was converted into barium carbonate for counting [Found: relative molar activity $\times 10^{-3}$, 62·5; 1*C requires 59·6]. The acetone, condensed in a trap by cooling with liquid nitrogen, was oxidised with hypoiodite, and the iodoform sublimed at 110°/10 mm. before Van Slyke–Folch oxidation to barium carbonate [Found: relative molar activity, 0].

Decarboxylation of 3-Chloro-2-hydroxy-4 : 6-dimethoxybenzoic Acid.—An evacuated tube, containing the acid (65 mg.) beneath pre-heated glass wool, and connected to a centrifuge tube holding barium hydroxide solution, was immersed in a metal-bath at 270° for 5 min. After 20 min. the barium carbonate (54 mg., 98%) was collected and washed by centrifugation [Found: relative molar activity $\times 10^{-3}$, 61·0; 1*C requires 59·6].

The pyrolysis residue was collected with ether and sublimed at 80°/5 $\times 10^{-2}$ mm., to yield 2-chloro-3 : 5-dimethoxyphenol, m. p. 60—61° (42 mg., 81%) [Found: relative molar activity $\times 10^{-3}$, 176; 3*C requires 179].

Phloroglucinol from 3-Chloro-2-hydroxy-4 : 6-dimethoxybenzoic Acid.—2-Chloro-3 : 5-dimethoxyphenol, m. p. 60—61°, was prepared in 91% yield by heating the acid in a sealed tube at 260° for 7 min., and purified by sublimation at 80°/0·05 mm. The phenol (460 mg.), in 5% sodium hydroxide solution (35 ml.), was treated during 2 hr. with Raney nickel–aluminium alloy (2 g.) in small portions, with stirring. After a further hour, the nickel was filtered off and the filtrate poured into concentrated hydrochloric acid (20 ml.). Extraction with ether gave a viscous oil which sublimed at 80—100°/0·02 mm., and the phloroglucinol dimethyl ether (360 mg.) crystallised from benzene–light petroleum (b. p. 40—60°); it had m. p. 37—39° (lit.,[22] 36—38°).

Phloroglucinol dimethyl ether (360 mg.) was warmed for 70 min. on the water-bath with 5N-hydrochloric acid (11 ml.), the excess of acid neutralised with sodium carbonate, and the solution continuously extracted with ether to give a gum, which crystallised on the addition of benzene and sublimed at 190°/0·05 mm., yielding phloroglucinol (253 mg., 86%), m. p. 214—216° [Found: relative molar activity $\times 10^{-3}$, 175; 3*C requires 179].

2 : 4 : 6-*Trinitrophloroglucinol.*—Phloroglucinol (200 mg.) was nitrosated by the method of Freudenberg, Fikentscher, and Wenner [14] and the potassium salt oxidised, without isolation, by 30% hydrogen peroxide (1·5 ml.) (cf. ref. 15) after basification of the reaction mixture. After 2 hr., dilution with alcohol and filtration gave the yellow potassium salt of trinitrophloroglucinol (360 mg., 60%). Decomposition with dilute sulphuric acid gave trinitrophloroglucinol, yellow-orange needles, m. p. 160—161° (decomp.); Benedikt [23] records m. p. 158°.

Hypobromite Degradation of 2 : 4 : 6-Trinitrophloroglucinol.—To trinitrophloroglucinol (40 mg.) in water (4 ml.) at 0° was added a carbonate-free solution of barium hypobromite [from

[21] Cf. Calvin, Heidelberger, Reid, Tolbert, and Yankwich, "Isotopic Carbon," Wiley, New York, 1949, 92.
[22] Heilbron and Bunbury, "Dictionary of Organic Compounds," 1953, Vol. IV, p. 189.
[23] Benedikt, *Ber.*, 1878, **11**, 1374.

barium hydroxide (1·6 g.) and bromine (0·22 ml.) in water (40 ml.)]. A centrifuge tube containing barium hydroxide solution was connected to the system, which was flushed with nitrogen and then closed. After 1 hour's stirring the system was evacuated and the mixture acidified with concentrated hydrochloric acid and warmed to 40°. The liberated carbon dioxide was collected as barium carbonate (66 mg., 72%) for counting [Found: relative molar activity $\times 10^{-3}$, 61·7; 1*C requires 59·6]. The control value under these conditions was negligible.

The residual aqueous mixture was basified and the bromopicrin (112 mg., 82%) isolated and oxidised as before [Found: relative molar activity, 0].

Dechlorination of 3-Chloro-2-hydroxy-4 : 6-dimethoxybenzoic Acid.—Raney alloy (1·0 g.) was added during 1 hr. to the acid (179 mg.) in 5% sodium hydroxide solution (30 ml.) with stirring. After a further hour the nickel was filtered off and the filtrate poured into concentrated hydrochloric acid (20 ml.) with cooling. Filtration of the precipitate and sublimation at 150°/0·02 mm. gave 2-hydroxy-4 : 6-dimethoxybenzoic acid (140 mg., 92%), m. p. 155—157° (lit.,[24] 156°).

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[24] Robertson, Suckling, and Whalley, *J.*, 1949, 1571.