3. *The Biosynthesis of Fungal Metabolites. Part I. Two Different Pathways to β-Ketide Chains in Rotiorin.*

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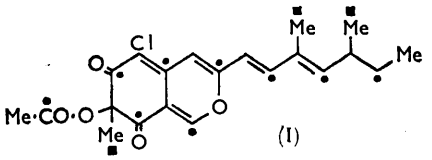
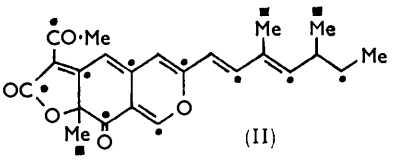
Incorporations of diethyl [1-^{14}C]- and [2-^{14}C]-malonate by *Penicillium sclerotiorum* van Beyma into sclerotiorin (I) and rotiorin (II) have shown that the main poly-β-ketide chain of these metabolites is assembled from a terminal two-carbon unit of acetate origin with chain-building units derived from malonate. Partial decarboxylation of introduced malonate to acetate occurs before incorporation. The acetoacetyl unit in the β-oxo-lactone system of rotiorin is derived from two acetate units without participation of malonate. Sodium [1-^{14}C]butyrate is incorporated intact into the β-oxo-lactone system of rotiorin, but is completely degraded to acetate before incorporation into the main chains of sclerotiorin and rotiorin. An explanation of these results is suggested.

In studies of metabolites derived from poly-β-ketide precursors it has been shown that elaboration of the β-ketide chain normally proceeds by condensation of a terminal unit of acetyl-co-enzyme A with chain-building units of malonyl-co-enzyme A.[1-3] Incorporation of labelled malonate into these metabolites [1-3] yields a product in which each of the chain-building units carries a label but the terminal unit of the chain is inactive.

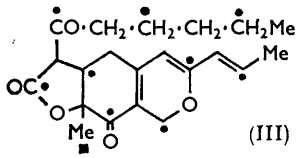
Recent studies [4,5] on the incorporation of sodium [1-^{14}C]acetate and [^{14}C]formate into the fungal metabolites sclerotiorin (I), rotiorin (II), monascin (III), and rubropunctatin (IV) have shown distributions of labels compatible with the patterns (I)—(IV), (• = radioactivity from [1-^{14}C]acetate; ■ = radioactivity from the C_1 pool, contributed by [^{14}C]-formate). Hence, these metabolites are derived by poly-β-ketide pathways. In each compound two chains must be present, the extra one increasing in complexity from acetyl (in I), to an acetoacetyl which has undergone ring closure (in II), and finally to the *n*-hexanoylacetyl residue with similar ring closure (in III and IV) (cf. ref. 6).



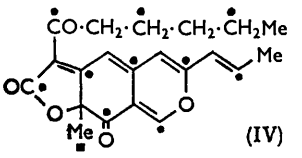
(I)



(II)



(III)



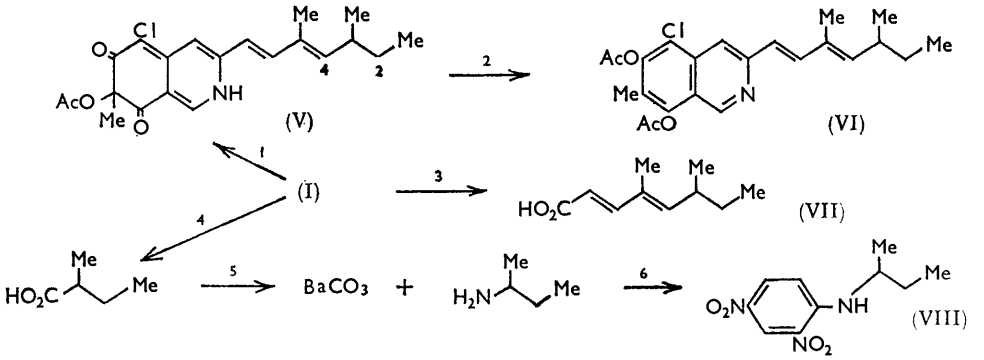
(IV)

Further information concerning the detailed assembly of the β-ketide chains in sclerotiorin (I) and rotiorin (II) has now been obtained from a study of the incorporation of diethyl [1-^{14}C]- and [2-^{14}C]-malonate into the metabolites by *Penicillium sclerotiorum* van Beyma. In view of the unexpected results, sodium [1-^{14}C]butyrate has also been investigated as a precursor. The labelled metabolites were degraded as outlined in schemes 1

[1] Bentley and Keil, *Proc. Chem. Soc.*, 1961, 111.
[2] Birch, Cassera, and Rickards, *Chem. and Ind.*, 1961, 792.
[3] Bu'Lock and Smalley, *Proc. Chem. Soc.*, 1961, 209; Bu'Lock, Smalley, and Smith, *J. Biol. Chem.*, 1962, **137**, 1778.
[4] Birch, Fitton, Pride, Ryan, Smith, and Whalley, *J.*, 1958, 4576.
[5] Birch, Cassera, Fitton, Holker, Smith, Thompson, and Whalley, *J.*, 1962, 3583.
[6] Whalley, " Recent Developments in the Chemistry of Natural Phenolic Compounds," ed. Ollis, Pergamon, London, 1961, p. 20; Haws, Holker, Kelly, Powell, and Robertson, *J.*, 1959, 3588.

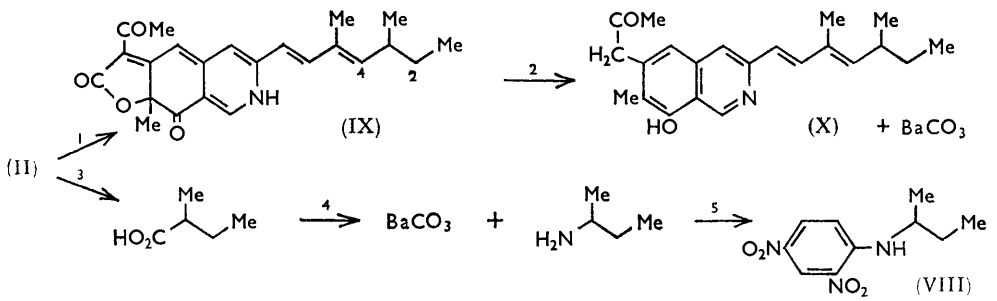
and 2. Thus, treatment of sclerotiorin (I) and rotiorin (II) with ammonia gave respectively sclerotioramine [7] (V) and rotioramine [8] (IX) which were reductively aromatised to the corresponding apo-compounds by treatment with zinc and acetic acid.[7] The carbon dioxide evolved during the formation of aporotioramine (X) was isolated as barium carbonate. Aposclerotioramine was converted into the *OO*-diacetyl derivative [9] (VI) to facilitate purification. Sclerotiorin was also degraded with sodium hydroxide to 4,6-dimethylocta-2,4-dienoic acid [7] (VII). In the studies with diethyl [1-^{14}C]malonate and sodium [1^{14}C]butyrate, comparison of the activities of the terminal and the adjacent two-carbon unit in the main chain is very important. Consequently, both metabolites were ozonised to give α-methylbutyric acid which was degraded by the Schmidt procedure to 1-methylpropylamine [isolated as *N*-(1-methylpropyl)-2,4-dinitroaniline (VIII)] and carbon dioxide (isolated as barium carbonate). These two products contained the radioactivity from C_2 and C_4 respectively [*e.g.*, in (V) and (IX)], being derived from the terminal and the first chain-building unit.

Scheme 1.



Reagents: 1, NH_3. 2, Zn—HOAc; Ac_2O—C_5H_5N. 3, NaOH. 4, O_3; H_2O_2. 5, NaN_3—H_2SO_4. 6, 2,4-$(NO_2)_2C_6H_3Cl$.

Scheme 2.



Reagents: 1, NH_3. 2, Zn—HOAc. 3, O_3—H_2O_2. 4, NaN_3—H_2SO_4. 5, 2,4-$(NO_2)_2C_6H_3Cl$.

Experimental

Relative molar activities of the metabolites and their degradation products were determined by combustion to carbon dioxide, conversion into barium carbonate, and measurement as infinitely thick layers on planchettes by using an end-window counter (cf. ref. 10).

[^{14}C]*Sclerotiorin and* [^{14}C]*Rotiorin.*—*P. sclerotiorum* van Beyma was cultured as described earlier,[8] except that on the fifth day diethyl [1-^{14}C]- or [2-^{14}C]-malonate (100 μc in each case),

[7] Eade, Page, Robertson, Turner, and Whalley, *J.*, 1957, 4913.
[8] Jackman, Robertson, Travers, and Whalley, *J.*, 1958, 1825.
[9] Fielding, Graham, Robertson, Travers, and Whalley, *J.*, 1957, 4931.
[10] Hevesy, " Radioactive Indicators," Interscience Publ. Inc., London, 1948, pp. 48—50.

or sodium [1-^{14}C]butyrate (500 μc), in water was distributed equally between ten flasks (300 ml. of culture fluid in each), under aseptic conditions. After a further 10 days the cultures were harvested, the rotiorin and sclerotiorin isolated,[8] and the compounds purified by dilution with inactive material and crystallisation to constant radioactivity.

Degradations of [^{14}C]*Sclerotiorin.*—Sclerotioramine (V), di-*O*-acetylaposclerotioramine (VI), and 4,6-dimethylocta-2,4-dienoic acid (VII) were prepared as described previously.[7,9]

Formation and Schmidt degradation of α-Methylbutyric Acid from [^{14}C]*Sclerotiorin.*—This metabolite (500 mg.) in ethyl acetate (25 ml.) was treated with a stream of ozonised oxygen for 1 hr. at room temperature and then at −80° until the solution became blue. After warming to room temperature in a stream of oxygen, the reaction mixture was evaporated to dryness *in vacuo* at 40° and the residual ozonide treated successively with water (10 ml.), 2N-sodium carbonate (10 ml.), and hydrogen peroxide (100-vol.; 3 ml.), all at 0°. 12 Hours later the mixture was extracted with ether (4 × 10 ml.) to remove non-acidic material; the aqueous layer was acidified with hydrochloric acid, and the crude mixture of carboxylic acids isolated in ether (4 × 10 ml.). After drying ($MgSO_4$) and removal of ether through a small fractionating column, α-methylbutyric acid was isolated from the residual mixture by Saki's chromatographic technique [11] for the separation of aliphatic carboxylic acids. Thus, a column of " Amberlite I.R.C. 50 " (100 × 1·1 cm.) was standardised against a known mixture of aliphatic carboxylic acids with acetone–ethyl methyl ketone–water (2 : 1 : 9) as initial and developing solvent. The mixed acids from the ozonolysis were then chromatographed on the same column, and the fractions containing α-methylbutyric acid were combined and titrated (pH meter) with 0·05N-sodium hydroxide (Equiv. 55—65 mg. of acid).

The dry sodium α-methylbutyrate obtained on evaporation was submitted to the Schmidt degradation at 40° in sulphuric acid (1·0 ml.) with sodium azide (50 mg.). Carbon dioxide formed in the reaction was carried by a stream of carbon dioxide-free nitrogen into a solution (25 ml.) of barium hydroxide (950 mg.) at 90°: the precipitate of barium carbonate was collected and washed, and its activity was determined. The reaction mixture was then poured into water (100 ml.), and the sulphuric acid removed as barium sulphate. The clear supernatant liquor and washings were combined, acidified with hydrochloric acid, and evaporated to dryness. The residue was extracted with absolute ethanol (3 × 10 ml.) and the filtered solution heated under reflux for 30 min. with 1-chloro-2,4-dinitrobenzene (250 mg.). After dilution of the mixture with water, the product was isolated in ether and purified by adsorption on alumina (20 × 1·5 cm.) from solution in benzene. Eluted with benzene–ether (20 : 1), *N*-(1-methylpropyl)-2,4-dinitroaniline (VIII) separated from light petroleum (b. p. 60—80°) in yellow needles (50—80 mg.), m. p. 55° (Found: C, 50·7; H, 5·8; N, 17·5. Calc. for $C_{10}H_{13}N_3O_4$: C, 50·2; H, 5·5; N, 17·6%).

Degradations of [^{14}C]-*Rotiorin.*—Rotioramine (IX) and aporotioramine (X) were obtained as described previously.[8] Carbon dioxide produced in the formation of aporotioramine was isolated as barium carbonate. The formation and Schmidt degradation of α-methylbutyric acid were carried out as described above.

Results

The observed distributions of labels in sclerotiorin and rotiorin derived from diethyl [1-^{14}C]- and [2-^{14}C]-malonate and sodium [1-^{14}C]butyrate are summarised in columns (1) and (2) of Tables 1, 2, and 3, respectively.

Discussion

The first important finding is that sclerotiorin derived from both [1-^{14}C]- and [2-^{14}C]-malonate carries label in the terminal unit of the poly-β-ketide chain. However, comparison of the R.M.A.'s of the *N*-(1-methylpropyl)-2,4-dinitroaniline and the barium carbonate from sclerotiorin derived from [1-^{14}C]malonate (Table 1) shows that the terminal unit of the chain is less radioactive than the second unit. To explain this observation it is assumed that the biosynthesis of the main chain in sclerotiorin proceeds by the acetate + malonate pathway but that, as adumbrated in previous findings, some of the malonate is

[11] Seki, *J. Biochem. Japan*, 1958, **45**, 855.

TABLE 1.

Distribution of label from diethyl [1-^{14}C]malonate in sclerotiorin and rotiorin.

Diethyl [1-^{14}C]malonate (100 μc) gave sclerotiorin (4·5 μc) and rotiorin (1·8 μc).

Compound	(1) Counts per hr.	(2) R.M.A.	(3) Calc. R.M.A. for distributions in (XI/XII)
Sclerotiorin (I)	6950	146	—
Sclerotioramine (V)	6810	143	146
Di-*O*-acetylaposclerotioramine (VI)	6025	139	142
4,6-Dimethylocta-2,4-dienoic acid (VII)	6531	65·3	64·4
$BaCO_3$ from Schmidt reaction	19,096	19·1	19·4
N-(1-Methylpropyl)-2,4-dinitroaniline (VIII)	612	6·12	6·16
Rotiorin (II)	4050	93·4	—
Rotioramine (IX)	4050	93·4	93·4
Aporotioramine (X)	4140	91·1	89·9
$BaCO_3$ from red. aromatisation	3560	3·56	3·55
$BaCO_3$ from Schmidt reaction	11,000	11·0	11·8
N-(1-Methylpropyl)-2,4-dinitroaniline (VIII)	420	4·20	3·55

TABLE 2.

Distribution of label from diethyl [2-^{14}C]malonate in sclerotiorin and rotiorin.

Diethyl [2-^{14}C]malonate (100 μc) gave sclerotiorin (8·4 μc) and rotiorin (3·7 μc).

Compound	(1) Counts per hr.	(2) R.M.A.	(3) Calc. R.M.A. for distributions in (XIII/XIV)
Sclerotiorin (I)	12,200	256	—
Sclerotioramine (V)	12,200	256	256
Di-*O*-acetylaposclerotioramine (VI)	10,400	239	247
4,6-Dimethylocta-2,4-dienoic acid (VII)	11,300	113	111
$BaCO_3$ from Schmidt reaction	890	0·89	0·00
N-(1-Methylpropyl)-2,4-dinitroaniline (VIII)	4305	43·1	42·9
Rotiorin (II)	20,600	474	—
Rotioramine (IX)	20,500	472	474
Aporotioramine (X)	21,300	468	459
$BaCO_3$ from red. aromatisation	2700	2·70	0·00
$BaCO_3$ from Schmidt reaction	2400	2·40	0·00
N-(1-Methylpropyl)-2,4-dinitroaniline (VIII)	7600	76·0	76·1

TABLE 3.

Distribution of label from sodium [1-^{14}C]butyrate in sclerotiorin and rotiorin.

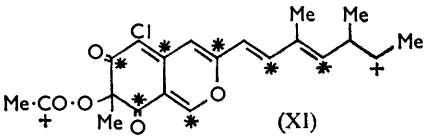
Sodium [1-^{14}C]butyrate (500 μc) gave sclerotiorin (11·4 μc) and rotiorin (5·8 μc).

Compound	(1) Counts per hr.	(2) R.M.A.	(3) Calc. R.M.A. for distributions in (XV/XVI)
Sclerotiorin (I)	22,200	466	—
Sclerotioramine (V)	22,300	468	466
Di-*O*-acetylaposclerotioramine (VI)	18,100	416	414
4,6-Dimethylocta-2,4-dienoic acid (VII)	21,100	210	207
$BaCO_3$ from Schmidt reaction	50,200	50·2	51·8
N-(1-Methylpropyl)-2,4-dinitroaniline (VIII)	5080	50·8	51·8
Rotiorin (II)	10,400	239	—
Rotioramine (IX)	10,400	239	239
Aporotioramine (X)	9100	200	201
$BaCO_3$ from red. aromatisation	38,200	38·2	38·2
$BaCO_3$ from Schmidt reaction	21,800	21·8	22·2
N-(1-Methylpropyl)-2,4-dinitroaniline (VIII)	2240	22·4	22·2

decarboxylated to acetate, which is then incorporated into the terminal unit (cf. refs. 2 and 3). On the assumption that the radioactivity of the terminal unit is a direct measure of this decarboxylation and, further, that the main chain is derived by uniform incorporation

Calculated distribution of label in sclerotiorin and rotiorin derived from diethyl [1-^{14}C]*malonate.*

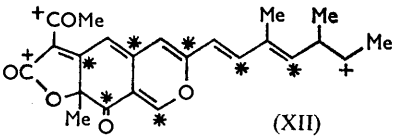
(a) *Sclerotiorin*



(XI)

Carbons labelled + have 4·2% of the total activity.
Carbons labelled * have 13·1% of the total activity.

(b) *Rotiorin*

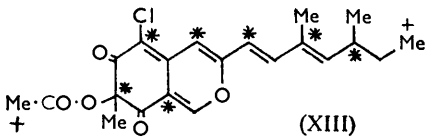


(XII)

Carbons labelled + have 3·8% of the total activity.
Carbons labelled * have 12·7% of the total activity.

Calculated distribution of label in sclerotiorin and rotiorin derived from diethyl [2-^{14}C]*malonate.*

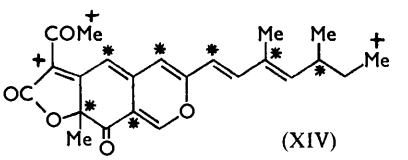
(a) *Sclerotiorin*



(XIII)

Carbons labelled + have 3·45% of the total activity.
Carbons labelled * have 13·3% of the total activity.

(b) *Rotiorin*

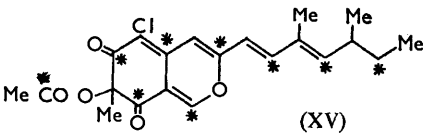


(XIV)

Carbons labelled + have 3·1% of the total activity.
Carbons labelled * have 13·0% of the total activity.

Calculated distribution of label in sclerotiorin and rotiorin derived from sodium [1-^{14}C]*butyrate.*

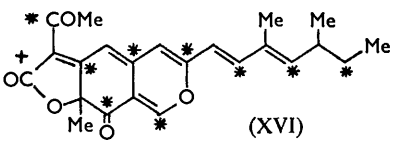
(a) *Sclerotiorin*



(XV)

Carbons labelled * have 11·1% (1/9) of the total activity.

(b) *Rotiorin*



(XVI)

Carbons labelled * have 9·3% of the total activity.
Carbon labelled + has 16% of the total activity.

of malonate into the chain-building units, the percentage distribution of label may be calculated for sclerotiorin; this is summarised for [1-^{14}C]- and [2-^{14}C]-malonate in (XI) and (XIII), respectively. Comparison of columns (2) and (3) in Tables 1 and 2 shows that this distribution is in agreement with experimental findings. It is clear from Table 2 that with [2-^{14}C]malonate a small amount of general randomisation of label occurs. This is undoubtedly due to the participation of [2-^{14}C]acetate in the tricarboxylic acid cycle.[12] Since, in the present case, the degree of randomisation is low it has been neglected in the calculations.

The outstanding observation in the experiments with rotiorin is that the acetoacetate equivalent which forms the β-oxo-lactone system in this metabolite does not appear to be derived from acetate plus malonate. Thus, in rotiorin derived from [1-^{14}C]malonate (Table 1), the R.M.A. of the terminal unit of the main chain is very similar to that of the second unit of the acetoacetate equivalent (determined as barium carbonate from the reductive aromatisation of rotioramine). Since we know that the former unit comes directly from acetate (labelled by decarboxylation of malonate), it is highly likely that the latter unit also arises in this way. The alternative explanation is that the acetoacetate equivalent is biosynthesised from acetate + malonate at a different period in time to that of the main chain, and, therefore, labelled to a different extent. This can be discounted, however, since in this case it would be expected that in [1-^{14}C]acetate-derived material there would be a similar difference in activity between the two chains, and this is not the case.[5] Hence, the acetoacetate equivalent in rotiorin arises directly from two acetate units by a different pathway to that of acetate + malonate operating in the main chain (cf. ref. 3). Calculated on the basis that the acetoacetate equivalent comes from acetate without participation of malonate, the distribution of label in rotiorin derived from [1-^{14}C]- and [2-^{14}C]-malonate is shown in (XII) and (XIV), respectively. The agreement with experimental results is shown by comparison of columns (2) and (3) in Tables 1 and 2.

It has been shown [13] that condensation of acetyl-co-enzyme A with hexanoyl-co-enzyme A to give octanoyl and longer-chain acyl derivatives of co-enzyme A is promoted by an enzyme system from liver mitochondria containing thiolase, together with β-hydroxyacyl dehydrogenase, enol hydrase, DPNH, and TPNH. Thus, liver mitochondria possess an enzyme system capable of increasing the chain length of fatty acids by a process which does not involve malonate and is essentially a reversal of normal fatty-acid oxidation. Consequently, it seems likely that in *P. sclerotiorum* the formation of the acetoactetate equivalent could be initiated by a similar enzymic system.

Evidence to support the presence of a fatty-acid oxidation enzyme system in *P. sclerotiorum* has been obtained. Thus, a study of the incorporation of sodium [1-^{14}C]-butyrate into sclerotiorin (Table 3) has shown a distribution of label (XV) identical with that obtained by incorporation of sodium [1-^{14}C]acetate. The butyrate must therefore be converted into acetate before incorporation, presumably by the normal fatty-acid oxidation pathway involving acetoacetate as an intermediate. The equal distribution of label in the first two units of the main chain precludes the possibility that a four-carbon unit from butyrate forms a terminal unit in the chain. The results (Table 3) clearly show that the main chain of rotiorin formed from [1-^{14}C]butyrate is derived as in sclerotiorin. However, the carboxyl-carbon atom of the β-oxo-lactone system in rotiorin has a much higher activity than any other carbon atom in the molecule. This provides further evidence for the view that the butyrate is oxidised to acetoacetate which is then incorporated into the β-oxo-lactone system as an intact unit (cf. ref. 6). Calculation of the distribution of label on this basis is shown in (XVI) and is in agreement with the experimental results in Table 3. Thus the results of labelling with both malonate and butyrate indicate that there are two pathways operating in the biosynthesis of rotiorin: the main

[12] Krebs and Lowenstein, "Metabolic Pathways," Academic Press, Inc., London, 1960, Vol. I, pp. 129—192.

[13] Seubert, Freull, and Lynen, *Angew. Chem.*, 1957, **69**, 359.

chain is derived by the acetate + malonate pathway, and the β-oxo-lactone system is derived directly from two acetate units, presumably condensed to an acetoacetate equivalent by an enzyme system of the fatty-acid oxidation type.

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UNIVERSITY OF LIVERPOOL. [*Received, April 11th,* 1963.]