The Biosynthesis of Fungal Metabolites. Part IX.[1] Sclerin: Feedings with [1,2-^{13}C]Acetate and [*methyl*-^{13}C]Methionine

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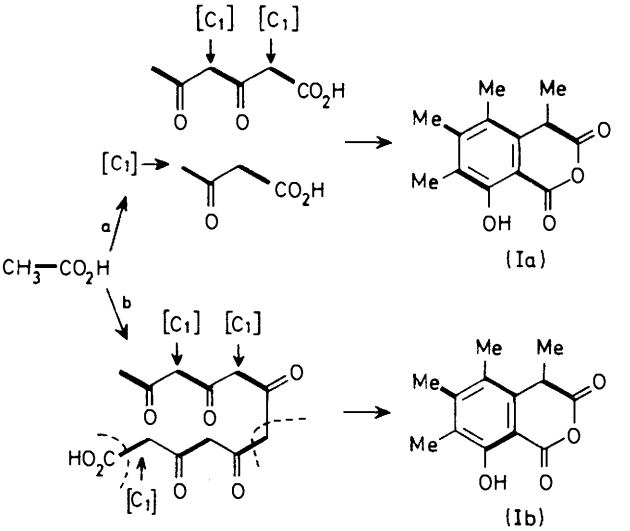
The ^{13}C n.m.r. spectra of methyl 2-(3-hydroxy-2-methoxycarbonyl-4,5,6-trimethylphenyl)propionate (II) derived from the fungal metabolite sclerin (I) enriched with ^{13}C from [1,2-^{13}C]acetate or from [*methyl*-^{13}C]-methionine, establish that the metabolite is derived from two β-ketide precursors with the introduction of three *C*-methyl groups from methionine.

THE poly-β-ketide pathway to natural products has traditionally been investigated by feeding experiments with ^{14}C-labelled acetate and malonate precursors. More recently highly enriched ^{13}C labelled precursors have become available, and incorporations are conveniently assayed by measurements of proton-noise decoupled (p.n.d.) ^{13}C n.m.r. spectra of the metabolites. Although the latter technique has the disadvantage that relatively large amounts of precursor are necessary to give the requisite enrichments, nevertheless there is a great advantage in the ease of recognition of labelling patterns and also in the availability of information not obtainable from ^{14}C studies.[2] In particular, intact acetate residues can be recognised by feedings with [1,2-^{13}C]acetate: the p.n.d. ^{13}C n.m.r. spectra of the metabolites display the signals from adjacent carbon atoms derived from the same two-carbon fragment as ^{13}C-coupled satellites superimposed on the singlet resonances. An added bonus is that individual ^{13}C–^{13}C coupling constants are normally sufficiently different to provide valuable assistance in spectral assignments. Studies of this type have been particularly useful in distinguishing between different foldings of poly-β-ketide chains,[3,4] and in elucidating pathways involving a ring fission of a poly-β-ketide-derived intermediate[5] or molecular rearrangements of either the β-ketide itself[6] or an intermediate.[7]

It seemed to us that the fungal metabolite sclerin (I)[8] would provide an interesting test of the double ^{13}C-labelling technique, since the biosynthesis of the compound cannot be accounted for by a normal β-ketide pathway. Furthermore, although feeding experiments with [1-^{14}C]- and [2-^{14}C]-acetate and [^{14}C]formate have suggested that the compound is derived by condensation of two polyketide chains with the introduction of three *C*-methyl groups from the C-1 pool (Scheme, a),[9,10] this work has been criticised both on experimental grounds and in terms of the almost unprecedented biosynthetic steps involved. Indeed an alternative pathway involving fission of a single hexa-β-ketide chain has been suggested (Scheme, b).[11]

In principle, a distinction between these two pathways and others involving different foldings of a hexa-β-ketide would be possible by feeding experiments with [1,2-^{13}C]-acetate and [*methyl*-^{13}C]methionine. Thus, the two chain hypothesis leads to sclerin containing five intact



SCHEME Alternative biosynthetic pathways to sclerin

acetate residues, whereas fission of a hexa-β-ketide with concomitant decarboxylation would result in only four intact residues in the product (indicated by heavy bonds in the Scheme, a and b, respectively).

As previously described, for other fungal metabolites,[12] feedings of [1-^{14}C]- and [2-^{14}C]-acetate to *Sclerotinia sclerotiorum* were used to establish conditions under which similar feedings of [1,2-^{13}C]acetate would give an excess abundance of *ca.* 1—2% ^{13}C in the individual acetate-derived atoms of sclerin (I). The crude ^{13}C-enriched metabolite thus obtained was characterised as the dimethyl ester (II), prepared by treatment with methanol and hydrogen chloride. An unenriched sample of (II) was converted into compound (III) by hydrolysis, decarboxylation, and re-esterification.

The ^{13}C n.m.r. spectral assignments of the diester (II)

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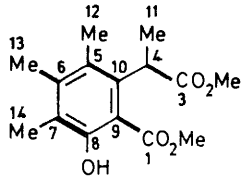
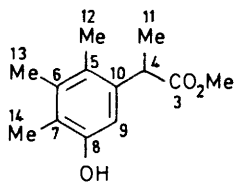
followed from comparisons between resonances of compounds (II) and (III), by use of off-resonance decoupling



(II)



(III)

to determine the number of protons attached at each carbon atom, by comparison with literature values [13] for atoms in similar environments, and most important, from the magnitudes of individual ^{13}C couplings in the [1,2-^{13}C]acetate-enriched sample of compound (II). The values and assignments are listed in the Table.

The obvious starting point for the ^{13}C assignment of compound (II) is the signal at δ 42.2, which is a doublet in the off-resonance spectrum and therefore arises from C-4. Since for the [1,2-^{13}C] acetate-derived sample, the p.n.d. ^{13}C-spectrum shows that this signal is coupled to that at δ 174.6, the latter is due to the carbonyl carbon atom (C-3) of the methoxycarbonyl group. The other carbonyl resonance at δ 171.3 is thus assigned to C-1, and since this signal is coupled to that at δ 109.7 the latter must be due to C-9. From chemical shift considerations, the phenolic carbon resonance C-8 is that at δ 157.3, and since this is coupled to the signal at δ 123.8 the latter is assigned to C-7. The remaining coupled resonances form two pairs, δ 17.6—142.9 and 126.5—136.9, respectively. The only possible interpretation of these data is that the former pair are due to C-13 and C-6 and the latter to C-5 and C-10. Of the three uncoupled *C*-Me resonances, that at highest field, δ 12.3, arises from C-11, by analogy with established chemical shift data. Confirmation of these assignments is provided by the observed chemical shifts of compound (III) and by the use of additivity of substituent chemical shift effects on aromatic rings.[14]

^{13}C Chemical shifts and coupling constants of [1,2-^{13}C] acetate-enriched compound (II) together with ^{13}C chemical shifts of compound (III)

	Compound (II)		Compound (III)
Carbon no.*	δ(±0.05 p.p.m.) †	J(±0.5) Hz	δ(±0.05 p.p.m.) †
11	12.3 (q)		12.1 (q)
12 or 14	16.2 (q)		15.2 (q)
14 or 12	17.0 (q)		16.6 (q)
13	17.6 (q)	44	18.3 (q)
4	42.2 (d)	60	41.6 (d)
OMe	51.4 (q)		52.1 (q)
OMe	51.7 (q)		
9	109.7 (s)	74	110.5 (d)
7	123.8 (s)	67	121.9 (s)
5	126.5 (s) ‡	63	125.5 (s)
10	136.9 (s) ‡	62	136.7 (s) ‡
6	142.9 (s)	45	137.0 (s) ‡
8	157.3 (s)	67	152.1 (s)
1	171.3 (s)	73	
3	174.6 (s)	60	176.3 (s)

* Numbering as in previous work.[10] † Relative to internal Me_4Si. ‡ Assignments may be reversed. Multiplicities refer to off-resonance decoupled spectra.

The presence of five pairs of ^{13}C–^{13}C couplings in the ^{13}C n.m.r. spectrum of [1,2-^{13}C]acetate-derived compound (II), indicated by heavy bonds in the formula, is only compatible with a biogenetic origin for sclerin from two β-ketide fragments as indicated in the Scheme (a). This was confirmed by feedings with [*methyl*-^{13}C]-methionine, which gave sclerin (I), converted as before into the diester (II), in which *ca.* 14-fold enhancement of intensities of the ^{13}C n.m.r. signals at δ 12.3, 16.2, and 17.0 was observed, with no significant enhancement of the signal at δ 17.6. This clearly establishes that the three methyl groups (C-11, -12, and -14) of sclerin arise by biological methylation, whereas C-13 is from acetate.

Prior to completion of the above work, it was considered that propionate might be a precursor of sclerin. Accordingly, tracer studies were initiated with [1-^{14}C]- and [2-^{14}C]-propionate. However, these were relatively inefficiently incorporated, *i.e.* 0.1 and 0.04%, respectively, total incorporation; *cf.* 0.54% for [1-^{14}C]acetate under the same feeding conditions. Moreover, the label from propionate appears to be extensively randomised since comparison of the relative molar activities of compounds (II) and (III) show that 11% of the total activity is present in C-1 from [1-^{14}C]-propionate and 8% from [2-^{14}C]-propionate. Since direct incorporation of propionate into C-1 is unlikely, extensive degradation of the precursor must occur before incorporation.

The demonstration that sclerin is biosynthesised from two β-ketide precursors classifies the compound with the relatively rare group of fungal metabolites for which two-chain pathways have been established, the principal members being the azaphilones rotiorin, rubropunctatin, monascorubrin, and monascin,[15-17] citromycetin,[18] and molliscin.[19]

EXPERIMENTAL

I.r. spectra were measured with a Perkin-Elmer 257 instrument for solutions in chloroform, ^{13}C n.m.r. spectra with a Varian XL-100-15FT instrument for solutions in deuteriochloroform containing tetramethylsilane as internal standard, and mass spectra at 70 eV with an A.E.I. MS12 instrument. Silica gel GF (Merck) was used for preparative layer chromatography (p.l.c.). M.p.s were determined with a Kofler hot-stage instrument. Radioactivity measurements were made by liquid scintillation counting [Packard 3003 Tricarb Scintillation Spectrometer and Butyl-PBD (CIBA) scintillator solution]. Counting efficiencies were determined with [^{14}C]hexadecane as internal standard.

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Sclerin(I) *and Methyl* 2-(3-*Hydroxy*-2-*methoxycarbonyl*-4,5,6-*trimethylphenyl*)*propionate* (II).—*Sclerotinia sclerotiorum* (IMI strain 145.557) was grown in shake culture for 12 days at 25 °C in flasks each containing Czapek-Dox medium (100 ml). Sclerin (I) was isolated in ethyl acetate from the acidified broths (HCl) and assayed by characteristic i.r. bands at 1 800 and 1 690 cm^{-1}.[8] The extract was evaporated and the residue treated with 10% hydrogen chloride in methanol as previously described.[8] The solvent was removed and the residue purified by p.l.c. (methylene chloride). Thus obtained, the diester (II) separated from aqueous ethanol in needles (2—20 mg l^{-1}), m.p. 112—114° (lit., 113—116°) (Found: C, 64.1; H, 7.0%; M^+, 280. Calc. for $C_{15}H_{20}O_5$: C, 64.3; H, 7.2%; *M*, 280).

Methyl 2-(5-*Hydroxy*-2,3,4-*trimethylphenyl*)*propionate* (III).—The diester (II) (20 mg) was heated under reflux for 15 h with aqueous 20% hydrochloric acid (10 ml). The cooled solution was diluted with water (20 ml) and the product was isolated in ether (3 × 10 ml) and esterified with ethereal diazomethane (5 min at room temperature). The excess of diazomethane was then destroyed, the ether evaporated off, and the residue crystallised from aqueous ethanol to give needles (14 mg) of the ester (III), m.p. 112—113° (lit.,[8] 114—115°) (Found: C, 70.4; H, 8.2%; M^+, 222. Calc. for $C_{13}H_{18}O_3$: C, 70.2; H, 8.2%; *M*, 222).

Feeding Experiments.—(*a*) Sodium acetate (240 mg; 6.6 μCi $mmol^{-1}$) was added in portions on days 1, 3, 5, and 7 to 5 shake culture flasks. Sclerin was isolated on day 12 and converted into the dimethyl ester (II) as previously described (10 mg; 0.62 μCi $mmol^{-1}$). On this basis (dilution factor 11) it was anticipated that equivalent feedings of [^{13}C] acetate (90 atom %) would give compound (II) with an excess of 1.7% of ^{13}C label over natural abundance, at each labelled position (assuming a pentaketide origin).

Sodium [1,2-^{13}C]acetate (92 atom % at each position) was fed under similar conditions (480 mg in 10 shake flasks). The dimethyl ester (II) (30 mg) was isolated and purified as described above and the p.n.d. ^{13}C n.m.r. spectrum was determined. Comparisons of the combined heights of the coupled doublets with those of the corresponding singlets indicated excess abundances at each labelled position of between 0.8 and 1.1% ^{13}C (mean 0.9%). The calculation was based on the formula: ratio of satellite to singlet height $(R) = A^2x/(100A - A^2)x + 1\ 110$, where A is the % enrichment of the individual positions of the acetate precursor, and x is the % excess abundance at each labelled position in the metabolite.

(b) Feedings with [*methyl*-^{14}C]methionine (300 mg; 2.5 μCi $mmol^{-1}$), as described above, gave compound (II) (*ca.* 1.1 mg; *ca.* 6 μ Ci $mmol^{-1}$). On this basis (dilution factor *ca.* 0.4) it was anticipated that equivalent feedings of [*methyl*-^{13}C]methionine (90 atom %) would give compound (II) with an excess of *ca.* 70% of ^{13}C label at each of three *C*-Me positions. Accordingly [*methyl*-^{13}C]methionine (92 atom %) was fed under similar conditions (220 mg in 5 shake flasks) to give compound (II) (*ca.* 1.5 mg) which was purified as described above and studied by ^{13}C n.m.r. spectroscopy.

^{14}C *Tracer Feedings*—Sodium [1-^{14}C] acetate (50 μCi), sodium [1-^{14}C]propionate (50 μCi), and sodium [2-^{14}C] propionate (38 μCi) were separately incorporated into 5 shake flasks as described above. The three isolated sclerin samples were converted into the dimethyl ester (II) as described above. The total radioactivity of each sample was measured (0.270, 0.054, and 0.014 μCi, respectively). The [1-^{14}C]- and [2-^{14}C]-propionate-labelled samples of compound (II) were diluted with inactive material and each was crystallised to constant activity (0.038 3 and 0.150 μCi $mmol^{-1}$, respectively). Each was then converted into compound (III) as previously described and the sample were crystallised to constant radioactivity (0.034 0 and 0.137 μCi $mmol^{-1}$, respectively).

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