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BIOSYNTHESIS OF XANTHOFUSIN, A TETRONIC ACID METABOLITE OF *FUSICOCCUM* SP.

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Abstract—Incorporation experiments with [Me-$^{2}H_3$]methionine, [1-^{13}C]- and [1,2-$^{13}C_2$]- acetate showed that the methyl groups of xanthofusin are derived from methionine, and the remaining carbon atoms are derived from three intact acetate units. Incorporation of ^{13}C-labelled 1,3-dihydroxy-2,4-dimethylbenzene shows that this is a probable intermediate. Three possible pathways are discussed, namely a triketide origin of xanthofusin, a route via a tetraketide but with loss of the starter unit and, finally, a route including a triketide with a malonate starter unit.

INTRODUCTION

Xanthofusin (**1**) is a yellow metabolite which has recently been reported from *Fusicoccum* sp. [1]. It exhibits strong antifungal activity *in vitro* against members of Oomycetes, e.g. *Phytophthora infestans*, and belongs to the class of 4-ylidenetetronic acids together with patulin (**2**) and multicolic acid (**3**), both formed *in vivo* via oxidative cleavage of polyketide-derived aromatic intermediates [2]. The structurally related carolic acid (**4**), however, is formed via Krebs cycle intermediates [2] and both pathways seem, in principle, possible for xanthofusin (**1**). In this paper, we report our biosynthetic results from feeding experiments with ^{2}H- or ^{13}C-labelled methionine and acetate. Furthermore, a potential ^{13}C-labelled aromatic precursor has been prepared and administered to the fungus.

RESULTS AND DISCUSSION

Sodium [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate and [Me^{2}-H_3]methionine were separately fed to *Fusicoccum* and after one week the metabolites were isolated by ethyl acetate extraction of the biomass. Pure xanthofusin (**1**) was isolated by preparative TLC.

The ^{13}C NMR spectrum of **1** isolated from the feeding experiment with sodium [1-^{13}C]acetate, showed that C-1, C-3 and C-5 were significantly enriched (three–four times the peak size for that of an unenriched sample) showing their derivation from C-1 of acetate. The results from the feeding experiment with [1,2-^{13}C]acetate are shown in Table 1. In addition to the signals deriving from ^{13}C of natural abundance, C-1 and C-2 each showed doublets deriving from the externally supplied double-labelled acetate. This ascertained that C-1 and C-2 were incorporated as an intact acetate unit. Similarly, doublets were observed for couples C-5/C-6 and C-3/C-4. Neither the C-7 nor the C-8 signals were altered by the administration of doubly labelled acetate. This shows that none of the methyl groups of xanthofusin (**1**) are of acetate origin. We have thus established that C-1 to C-6 originate from three contiguous, intact acetate units. The feeding experiment with [Me–$^{2}H_3$]methionine gave incorporation into both methyl groups; 1.5% into Me-8 and 1% into Me-7. The lower incorporation observed in the 7-methyl group is most likely due to loss of ^{2}H by enolization during the biosynthesis. The established labelling pattern is shown in Fig. 1. Xanthofusin (**1**) thus constitutes an example of a polyketide-derived metabolite in which a MeCO group is not derived from an acetate unit, as is also observed for vermopyrone (**5**) [3].

The labelling pattern fits a biosynthetic pathway with the hydroxy-*m*-xylenes, **6** and **7**, as intermediates. Thus, oxidative cleavage of the latter would lead to xanthofusin (Fig. 2). To test this hypothesis, we decided to synthesize labelled **6** and/or **7** and test them as precursor(s). Base catalysed condensation (Fig. 3) of 2-methylbuten-3-one (**12**) with methyl diethylthioacetate (**13**) yields methyl dithioethylcyclohexandione (**14**). It had been shown [4, 5] that dethioketalization with mercury salts of dithioethylcyclohexanediones leads to trihydroxy benzenes in good yields. Our strategy was thus to prepare **14**, perform a methylation with labelled methyl iodide to obtain **15** and carry out the dethioketalization to obtain labelled **7**. It turned out, however, that methylation of **14**

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Table. 1. ^{13}C NMR spectral data for biosynthetically enriched xanthofusin

Signal	Xanthofusin (1) assignment	Sodium [1,2-$^{13}C_2$]acetate experiment	
		Enrichment*	Coupling constant (Hz)
168.6	1	5	73.3
104.0	2	5	73.6
161.2	3	5	55.9†
159.5	4	5	56.3†
106.9	5	5	56.2
202.9	6	5	56.9
31.4	7		
6.3	8		

*Sum of signal intensities for the doublet derived from the incorporated acetate divided by the signal intensity of the natural abundance signal.
†AB system.

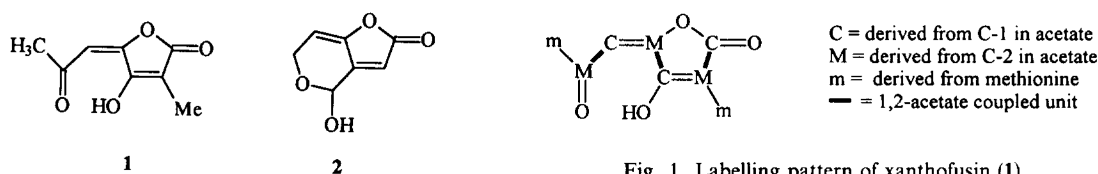
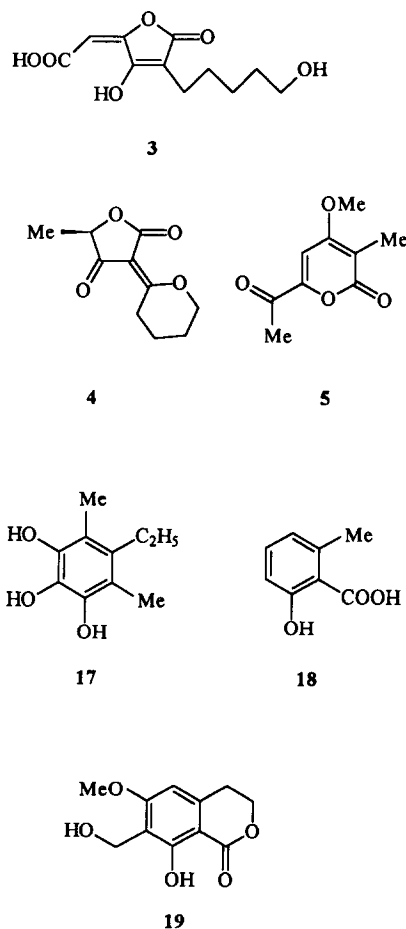


Fig. 1. Labelling pattern of xanthofusin (1).



under several conditions tested led to the aromatized derivative **16**. This is not unexpected, as methylation of **14** could provide a sulphonium salt, which would easily eliminate ethylmethyl sulphide. Fortunately, the aromatic thioether **16** could be reduced to **6** in moderate yield with Raney nickel. Since **6** would serve the biosynthetic purpose just as well as **7**, we refrained from further chemical work and synthesized [2-Me^{13}C]-**6**. This compound was then administered to a culture of *Fusicoccum* and the metabolite **1** isolated. In the ^{13}C NMR spectrum of this sample of xanthofusin (**1**), the intensity of the C-7 signal was 30% higher than in that of an ordinary spectrum of **1**. This shows that labelled **6** has been converted into **1**; the incorporation amounted to 1%.

The formation of the intermediate **6** can be explained in several ways but these require biogenetically unusual steps. (i) If xanthofusin were a triketide (**8**; R = H, Fig. 2), methylation of a terminal methyl group would be necessary. We have not found a precedence for such a reaction, although it has been postulated in the biosynthesis of barnol (**17**) [16]. A transformation similar to the reduction and dehydration of **9** required to form **10** is seen in the biosynthesis of 6-methylsalicylic acid (**18**) [7] while the condensation step from **10** (R = H) to **6** again seems unappealing due to the lack of double activation of the CH_2 group. To overcome these difficulties, an alternative pathway (ii) via a tetraketide (**8**; R = COMe) can be formulated. Here, both the methylation and condensation steps are straightforward. The unusual feature in this pathway is the loss of an entire acetate unit when going from **11** to **6**. Similar situations have been encountered in the biosyntheses of stellatin (**19**) and ethyl 2,4-dihydroxy-3-methylbenzoate, both produced by *Aspergillus* spp.

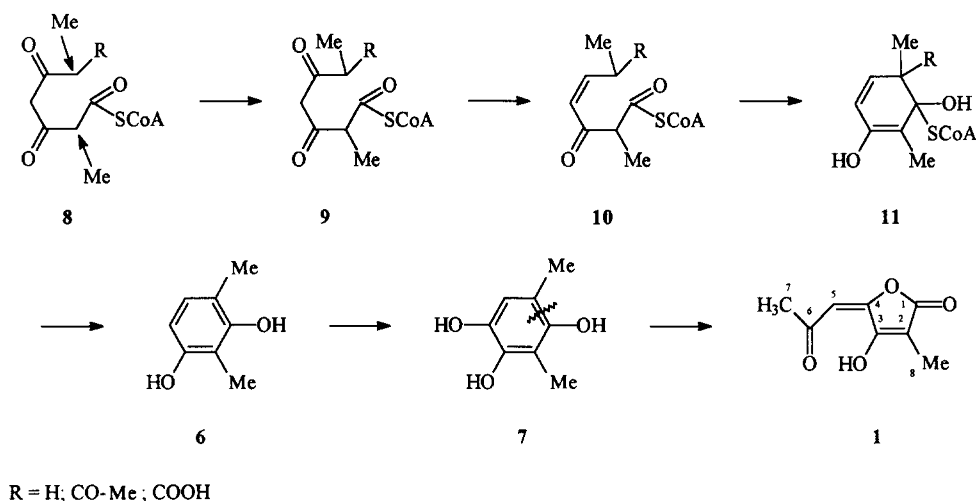


Fig. 2. Possible biosynthetic pathways to xanthofusin (**1**).

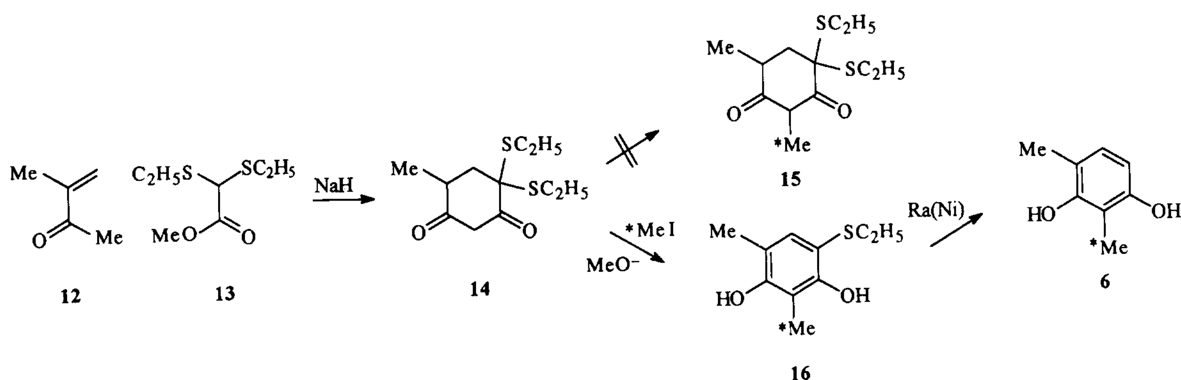


Fig. 3. Synthesis of [2-Me-^{13}C]-**6**.

where a mechanism involving the loss of a starter unit from a pentaketide has been proposed [8] but not proven. Loss of a starter unit also seems to take place during the formation of the aurovertines [9] and of a metabolite from *Aspergillus ustus* [10]. Finally, in pathway (iii), with malonate as an assumed starter unit, the intermediate **8** (R = COOH) would carry the necessary double activation to form **9** and later **11** (R = COOH), respectively, and in addition the activating carboxyl group could be lost in a mechanistically satisfactory way to form **6**. However, the only precedence we would find for such a pathway is that shown for tetracyclines with a malonamyl starter unit [11].

EXPERIMENTAL

General. ^{13}C NMR (123 MHz): $CDCl_3$ ($CHCl_3$ = δ76.9) with a relaxation delay of 5 sec; ^{2}H NMR (77 MHz): $CHCl_3$ ($CDCl_3$ = δ7.27); prep. TLC: 20 cm × 40 cm plates coated with 1 mm layers of Si gel PF_{254} (Merck). The ^{13}C-labelled acetates were purchased from Sigma (99% atom ^{13}C enriched).

Incorporation experiments with labelled acetate and methionine. Fusicoccum sp. (IMI cc No. 351573) were grown in shake flasks as described [1]. Each flask contained 100 ml of culture broth. The precursors were administered 5–6 days after inoculation of the shake flask and after an additional 3 days the mycelium was sepd by centrifugation or filtration. The mycelium was extracted twice with 200 ml EtOAc for 2 hr with vigorous stirring. The combined extracts were filtered and dried by freezing in a CO_2–Me_2CO bath. After removal of ice by filtration, the EtOAc was evaporated and the residue subjected to prep. TLC (EtOAc–pentane, 1:1). In the experiments with labelled acetate, 250 mg of precursor was administered to each flask. [Me-$^{2}H_3$]Methionine was prepared as described [12] and 26 mg used for feeding. In all cases 9–42 mg xanthofusin (**1**) was isolated. All experiments were repeated at least twice.

Synthesis of **6**. NaH (55%, 880 mg) was suspended in dry CH_2Cl_2 (80 ml). The reactants **12** (3.92 g) [13] and **13** (2.65 ml, 93% pure, 1.22 equivalents) [14] were added together with dry dimethoxyethane (2.5 ml). After stirring for 30 min, the soln turned yellow and H_2 was evolved. After a further 5 min, the soln came to reflux and the

almost clear soln was left overnight. NaOH soln (60 ml, 0.5 M) was added to dissolve the crystals in the bright yellow suspension and the phases were sepd. The organic phase was extracted with NaOH (40 ml, 0.5 M). The combined aq. phases were acidified (100 ml, 2 M HCl) and extracted with CH_2Cl_2 (60 + 2 × 30 ml). The combined organic phases were dried and the solvent evapd. After addition of Et_2O (20 ml) to the crude red product (5.4 g) crystals formed. The mixt. was kept at 5° for 2 hr and filtered. The crystals of **14** (2.40 g) had a mp of 97–102° (lit. [5] 102–103). A second crop of crystals with the same mp was obtained from the mother liquor, thereby increasing the yield of **14** to 2.87 g (58%).

The product (**14**, 503.6 mg) was then dissolved in NaOMe–MeOH (2.2 ml, 1.0 M, 2.1 eq.) in a screw-cap ampoule [15] and MeI (300 μl. 2.3 eq.), was added. The mixt. was kept at 60° with stirring for 6 hr. After cooling, NaOH (2 ml, 0.1 M) was added and the aq. phase extracted with CH_2Cl_2 (3 × 2 ml). The aq. phase was acidified with HCl (2 ml, 2 M) and extracted with CH_2Cl_2 (3 × 3 ml). The organic phases were dried and evapd. The crude product (249 mg) was purified by prep. TLC (EtOAc–hexane–HOAc 20:90:1). The upper band ($R_f \sim 0.55$) contained the product **16** (80 mg, 20%). ^{1}H NMR (250 MHz, $CDCl_3$): δ7.10 (*s*, H-5), 5–6 (*br s*, OH), 2.61 (2H, *q*, J = 7.3 Hz, SCH_2), 2.17 (3H, *s*, 2-Me), 2.14 (3H, *s*, 4-Me), 1.80 (3H, *t*, J 7.3 Hz, SCH_2Me); ^{13}C NMR (67 Hz, $CDCl_3$): δ154.3 and 154.1 (C-1 and C-3), 134.4 (C-5), 109.5 (C-4), 109.3 (C-2), 31.1 (SCH_2–), 15.2 and 14.7 (6-Me and SCH_2Me). The above compound (**16**, 231.2 mg) was dissolved in EtOH (15 ml) and H_2O (5 ml) at room temp. Raney nickel W2 [16] (*ca* 1 g) was added and the mixt. refluxed for 60 min and centrifuged. The black ppt. was washed with NaOH (3 × 12 ml, 1 M). The combined aq. phases were acidified with H_2SO_4 (4 M) and extracted with CH_2Cl_2 (3 × 15 ml). The CH_2Cl_2 phases were combined and dried, treated with activated C and the solvent evapd to give a semi-crystalline product (**6**, 140 mg, 88%). After purification by prep. TLC (EtOAc–hexane–HOAc 50:50:1), a crystalline compound (**6**, 64 mg, 41%) was obtained. Mp 104–105° (lit 110° [17] and 112° [18]).

Synthesis of [2-Me-^{13}C]-**6**. The methylation of **14** was carried out with ^{13}Me I to give [2-Me-^{13}C]-**16** in a yield of 15% as described. The ^{1}H NMR data were as above except at δ2.17 (3H, *d*, J_{CH} = 128 Hz, 2-^{13}Me). The reduction of [2-Me-^{13}C]-**16** was carried out as above with similar efficiency. ^{1}H NMR as above except at δ2.13 (3H, *d*, J_{CH} = 127.6 Hz, 2-^{13}Me).

Feeding experiment with [2-Me-^{13}C]-**6**. The precursor (10 mg) was dissolved in H_2O and administered to a shake flask containing 100 ml of culture broth 60 hr after inoculation. After a further 3 days, the xanthofusin (**1**, 40 mg) produced was isolated as described above. The incorporation was measured to be 1% by comparison of the intensities (in 10 spectra) of the C-7 signal in the ^{13}C NMR spectrum of **1** with those of an unlabelled sample.

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