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# APPLICATION OF STABLE ISOTOPE LABELLING METHODOLOGY TO THE BIOSYNTHESIS OF THE MYCOTOXIN, TERRETONIN, BY *ASPERGILLUS TERREUS:* INCORPORATION OF $^{13}C$-LABELLED ACETATES AND METHIONINE, $^{2}H$- AND $^{13}C$, $^{18}O$-LABELLED ETHYL 3,5-DIMETHYLORSELLINATE AND OXYGEN-18 GAS

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**Abstract** - Incorporation of $^{13}C$-labelled acetates and methionine, $^{14}C$ and $^{2}H$-labelled ethyl 3,5-dimethylorsellinate into terretonin (5) by cultures of *Aspergillus terreus* indicated that its biosynthesis proceeds *via* a mixed polyketide-terpenoid (meroterpenoid) pathway. Incorporation of $^{18}O_2$ gas and ethyl 3,5-dimethylorsellinate (7) doubly labelled with $^{13}C$ and $^{18}O$ in the carbonyl of the carboxyl group and at the C-6 position into terretonin (5) and observation of $^{18}O$ isotope-induced shifts in the $^{13}C$ n.m.r. spectra and GC/MS studies of the enriched metabolites determined the origin of all of the oxygen atoms in (5) and provided mechanistic insight into the biosynthetic pathway.

Andibenin B (1)[1], andilesin A (2)[2] and anditomin (3)[3] are members of a group of metabolites isolated from *Aspergillus variecolor.* Along with austin (4) isolated from *Aspergillus ustus,*[4] *Penicillium diversum*[5] and *Emericella dentata*[6] they form a group of metabolites which arise *via* a common pathway. Based on stable isotope incorporation studies involving labelled acetate and methionine, it has been shown that these metabolites originate from a mixed polyketide-terpenoid (meroterpenoid) biosynthetic pathway.[7] The key step in their biosynthesis involves *C*-alkylation of the tetraketide-derived intermediate, 3,5-dimethylorsellinic acid (6), with farnesyl pyrophosphate, giving (8) as shown in Scheme 1. This intermediate is then cyclised and undergoes oxidative modification to produce the above metabolites.

Terretonin (5) is a mycotoxin isolated from *Aspergillus terreus* (NRRL 6273).[8] Although a sesterterpenoid or degraded triterpenoid origin was proposed for terretonin, it appeared likely that terretonin could also be formed *via* the common intermediate (8) shown in Scheme 1. Further modification of this intermediate as indicated in Scheme 2 could account for the formation of terretonin. Aerobic oxidation of the terminal side chain

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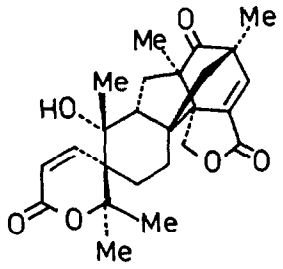
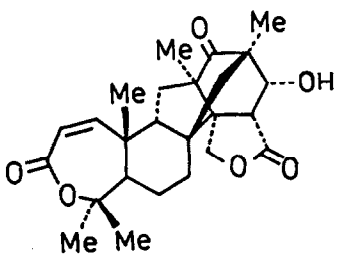


Andibenin B (1)



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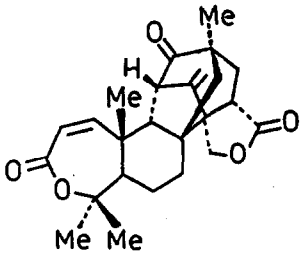
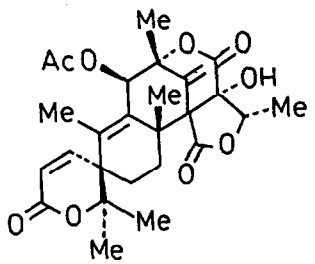
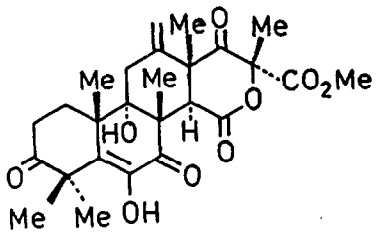


Anditomin (3)

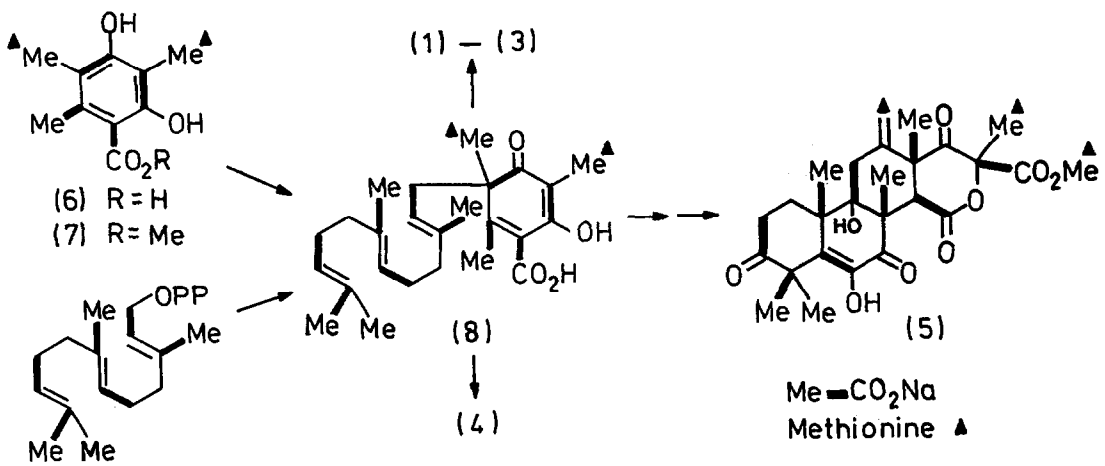


Austin (4)

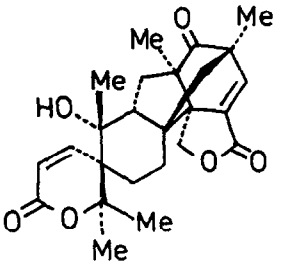


Terretonin (5)

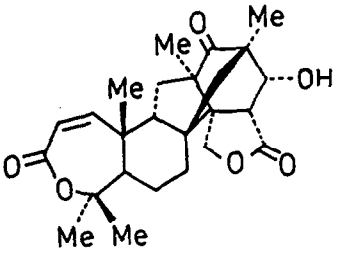
olefin in a fashion similar to that of steroid biosynthesis, followed by acid catalysed opening of the epoxide and cyclisation of the carbon skeleton could give rise to carbocation (9) which contains the desired A/B ring system. The proposed pathway for formation of the C/D rings involves an acyl shift with the loss of a proton to generate the required exocyclic double bond. Aerobic hydroxylation, intramolecular lactonisation and hydrolytic carbon-carbon bond cleavage would afford an intermediate carboxylic acid. Aerobic oxidation to introduce the desired functionality in the A/B ring system and methylation of the free carboxylic acid C-31 by methionine would give (5). Full details of studies with various precursors labelled with $^{13}C$, $^{2}H$ and $^{18}O$ are now presented which provide evidence for this pathway.[9] Both the $^{1}H$ and $^{13}C$ n.m.r. spectra of terretonin have been




Scheme 1



Andibenin B (1)



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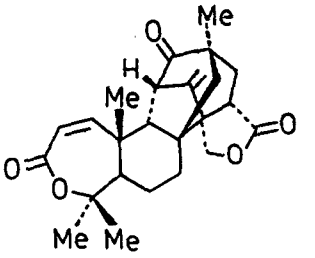

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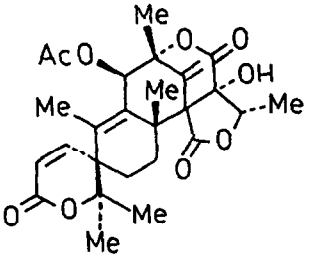

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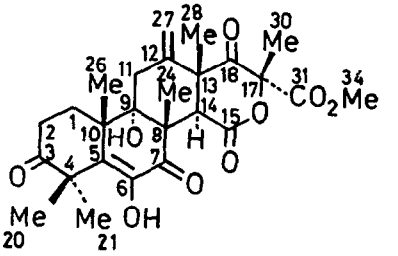
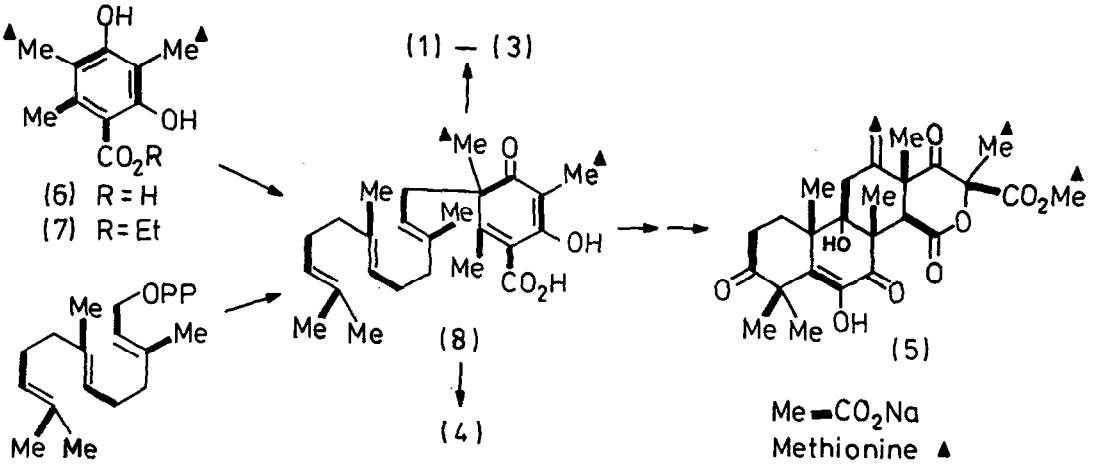
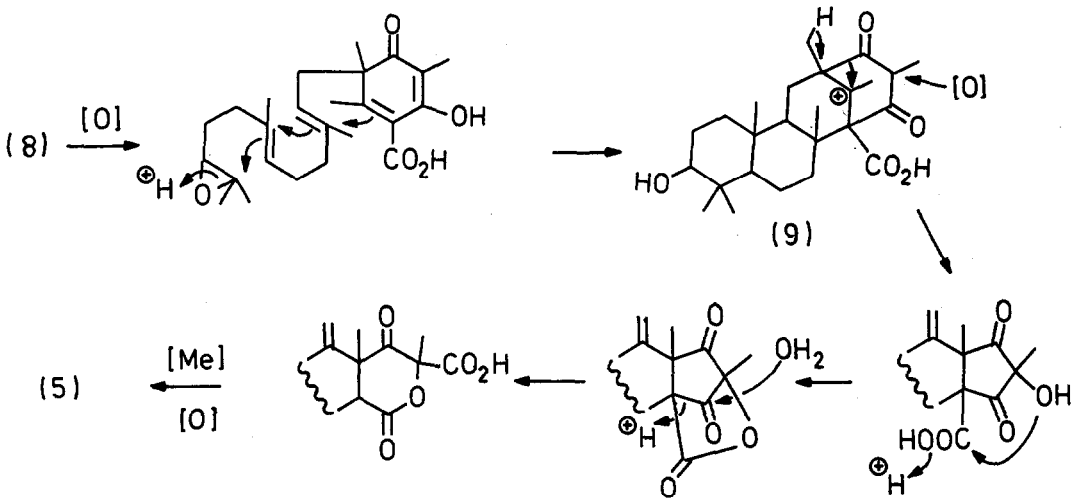


Terretonin (5)




Scheme 1



Scheme 2

rigorously assigned.[10]

Initial experiments with $^{14}C$-labelled acetate indicated that administration of labelled precursor to 3-day old cultures of *A. terreus* grown as previously described[10] gave overall dilution values[11] (*ca* 3.1) which would produce acceptable enrichments (*ca* 2%) with $^{13}C$-labelled acetates. Accordingly [1-$^{13}C$]- and [1,2-$^{13}C_2$]acetates and [*methyl*-$^{13}C$]-methionine were administered to different cultures of *A. terreus*. The resulting three terretonin samples were isolated and their 90.6 MHz $^{1}H$ noise decoupled (p.n.d.) $^{13}C$ n.m.r. spectra were measured. The sample isolated from the [1-$^{13}C$]acetate feed, surprisingly showed no observable $^{13}C$-enrichment at any sites. However, $^{13}C$-$^{13}C$ coupling satellites were observed in the spectrum of terretonin labelled from [1,2-$^{13}C_2$]acetate allowing the coupling constants summarised in Table 1 to be determined. The average level of enrichment was estimated at 0.3% from the relative heights of the coupling satellites and natural abundance signals. This is consistent with the low [1-$^{13}C$]acetate enrichment achieved.

The methionine-labelled sample showed signals resulting from three highly enriched carbons corresponding to C-27, C-30 and C-34. The labelling pattern indicated by these studies is summarised in Scheme 1 and is entirely consistent with the proposed pathway.

Because this pathway requires an unusually large degree of modification of the proposed phenolic precursor (6) further evidence of its intermediacy was sought by incorporation of ethyl 3,5-dimethylorsellinate labelled with $^{14}C$ at C-6 and the carboxyl carbon.[12] The isolated terretonin showed that the labelled 3,5-dimethylorsellinate had been incorporated with a specific dilution value of 38.2 corresponding to a specific incorporation of 2.5%. To further establish that the precursor had been incorporated intact and that the observed incorporation of label was not *via* prior degradation to, and reincorporation of acetate, ethyl [5-*methyl*-$^{2}H_3$]-3,5-dimethylorsellinate (7)[12] was fed to *A. terreus* cultures in a separate experiment.

**Table 1** $^{13}C$ coupling constants (J), enrichments and $^{18}O$ isotope induced shifts in the $^{13}C$ n.m.r. spectra of terretonin (5) enriched from [1,2-$^{13}C_2$]acetate, [*methyl*-$^{13}C$]methionine, $^{18}O_2$ gas and ethyl [$^{13}C$,$^{18}O$]-3,5-dimethylorsellinate

| Carbon | δ (ppm) | J(Hz)[a] | Δδ (ppb)[c] | $^{18}O$:$^{16}O$[c] |
|---|---|---|---|---|
| 1 | 27.9 | - | | |
| 2 | 32.5 | 38 | | |
| 3 | 214.0 | 38 | 51 | 70:30 |
| 4 | 47.9 | 37 | 8 | 72:28 |
| 5 | 131.7 | 84 | 14 | 53:47 |
| 6 | 138.8 | 84 | 14 | 56:44 |
| 7 | 197.1 | - | 44 | 61:39 |
| 8 | 52.5 | 34 | 6 | 55:45 |
| 9 | 77.7 | 35 | 28 | 69:31 |
| 10 | 43.0 | 34 | | |
| 11 | 34.7 | 35 | | |
| 12 | 139.9 | - | | |
| 13 | 49.5 | 32 | | |
| 14 | 44.6 | 55 | | |
| 15 | 167.7 | 55 | 8 | 54:46 |
| | | | 39* | 66:34* |
| 17 | 85.6 | 61 | 25 | 57:43 |
| 18 | 201.6 | - | | |
| 20 | 21.3 | 37 | | |
| 21 | 23.7 | - | | |
| 24 | 19.6 | 33 | | |
| 26 | 18.4 | 34 | | |
| 27 | 117.2[b] | | | |
| 28 | 23.3 | 32 | | |
| 30 | 21.1[b] | | | |
| 31 | 168.7 | | 36* | 42:58* |
| 34 | 53.5[b] | | | |

(a) enriched from [1,2-$^{13}C_2$]acetate; (b) from [*methyl*-$^{13}C$]methionine; (c) enriched by ethyl [$^{13}C$,$^{18}O$]-3,5-dimethylorsellinate (*), others enriched by $^{18}O_2$

The resulting sample of terretonin was examined by 55.3 MHz $^2H$ n.m.r. The spectrum is shown in Figure 1 along with the spectrum which results from growing *A. terreus* in a medium in which 5% of the water has been replaced by $^2H_2O$. This provides a useful method for obtaining the $^2H$ n.m.r. spectrum of a metabolite generally labelled with $^2H$. The methyl signals in these spectra are assigned by comparison with the $^1H$ n.m.r. spectrum.[10] It is apparent that the only position significantly labelled by the specifically deuteriated 3,5-dimethylorsellinate is that of the C-30 methyl group at 1.68 p.p.m. This is consistent with the pathway shown in Scheme 2 and establishes that 3,5-dimethylorsellinate is indeed incorporated intact into terretonin as a specific precursor. A small signal at 1.45 p.p.m. corresponding to the C-20 and C-21 methyls is also visible in Figure 1b and suggests that a small degree of degradation to acetate and reincorporation may have occurred. However, no enrichment of other methyl groups is seen, so it may be that this peak comes from traces of $HO^2H$ or from some other impurity.

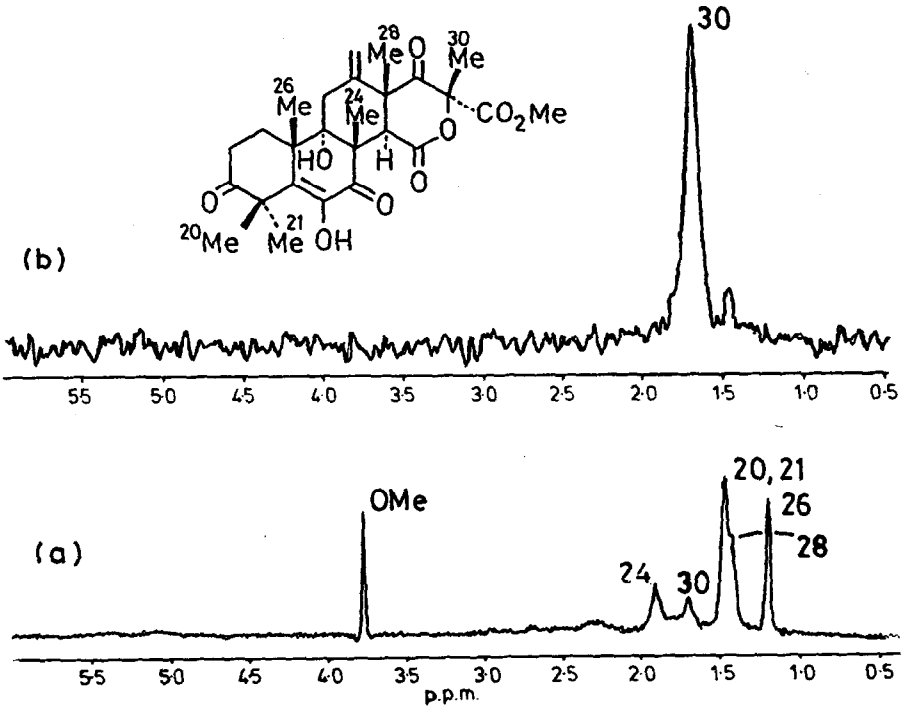



Figure 1 55.3 MHz $^{2}H$ n.m.r. spectra of terretonin labelled from (a) $^{2}H_2O$ and (b) ethyl [3-*methyl*-$^{2}H_3$]-3,5- dimethylorsellinate

Further information on the mechanisms of the oxidative modifications necessary in the biosynthesis of terretonin, particularly with reference to the 3,5-dimethylorsellinate-derived moiety was obtained by oxygen-18 labelling studies. These have previously been shown to provide considerable information on possible intermediate oxidation states and modes of ring formation and cleavage during biosynthesis.[13] *A. terreus* cultures were grown in 500 ml Erlenmeyer flasks connected to a closed system apparatus (Figure 2) for measuring oxygen uptake, under an atmosphere of $^{16}O_2/^{14}N_2$ (20:80) to check that fermentation proceeded normally and metabolite production was maintained. By visual comparison with the control flasks, the growth in the closed system appeared to be retarded by approximately 12 hours, but otherwise the fermentation proceeded as normal, and terretonin production (26 mg $l^{-1}$) was obtained. The experiment was repeated replacing approximately half the $^{16}O_2$ in the controlled atmosphere with $^{18}O_2$. To minimise $^{18}O_2$ consumption, the cultures were grown for four days before being connected to the closed system. Control flasks were checked for terretonin at this stage, and none was detected. The culture was allowed to grow for a further seven days by which time 3.5 litres of oxygen were consumed. A gradual uptake of oxygen was observed, the rate of

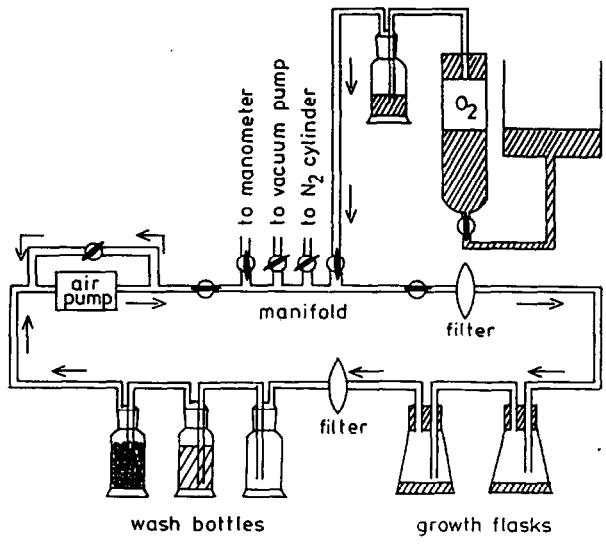



Figure 2 Apparatus for growth of cultures in a controlled atmosphere. The wash bottles are arranged so that the first acts as a suck-back trap, the second contains 5 M KOH to absorb $CO_2$ produced by the cultures and the third contains cotton wool to remove alkaline spray

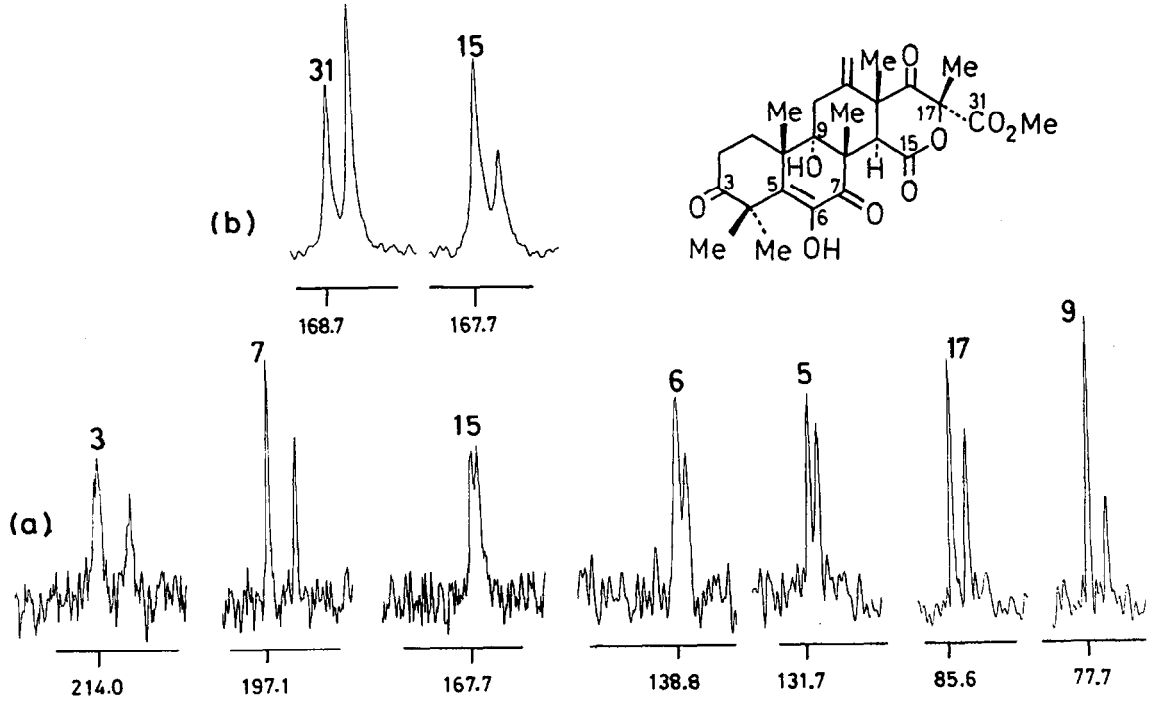


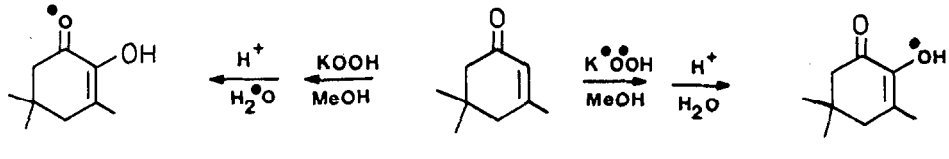

Figure 3 $^{18}O$-Isotopically shifted resonances in the 100.6 MHz proton-decoupled $^{13}C$ n.m.r. spectrum of terretonin (1) enriched by (a) $^{18}O_2$, (b) [$^{13}C$,$^{18}O$]-3,5-dimethylorsellinate

which decreased slowly with time. An unusually large yield of terretonin (52 mg $l^{-1}$) was isolated from the flasks grown in the closed system compared with the remaining control flask (20 mg $l^{-1}$).

The incorporation of oxygen-18 into terretonin was confirmed by its mass spectrum. The positions of incorporation were determined by observation of $^{18}O$ isotope-induced shifts in the 100.6 MHz p.n.d $^{13}C$ n.m.r. spectrum. The anticipated isotope shifts were observed at C-3, C-6, C-9, C-15 and C-17, (Table 1, Figure 3a) and are consistent with the proposed biosynthetic mechanism. The small isotope shift at C-15 of 8 p.p.b. is compatible with an oxygen-18 label occupying only the ether position of the lactone functionality.[14] This agrees with the hypothesis that the lactone ring is formed *via* attack of an aerobically introduced hydroxyl group at C-17 on either a free acid or ester moiety. In addition to the expected isotopically shifted resonances listed above, two smaller shifts of 8 and 6 p.p.b. were observed at C-4 and C-8, respectively. These shifts have small magnitudes and must correspond to $\beta$-isotope shifts[15] originating from oxygen-18 at C-3 and at C-7 or C-9.

A disturbing feature of this spectrum was the occurrence of a considerable shift of 14 p.p.b. at a carbon bearing no oxygen, C-5. The origin of this shift was unclear. It seemed possible that the C-9 hydroxyl group of (5) had added to the $\alpha,\beta$-unsaturated ketone in a Michael type fashion, and that labelling from this oxygen was being observed. However, examination of the published x-ray structure[8] demonstrated that the hydroxyl in question points away from the unsaturated system, ruling out the possibility of bridging. Exchange of the enol proton with deuterium had no effect on the observed shift. Thus, the appearance of two signals in the $^{13}C$ n.m.r. cannot be due to long range coupling of the $\beta$-carbon with the enolisable proton or to deuterium induced isotope shifts from exchange with the deuterated n.m.r. solvent. The remaining possibility, that this effect was due to a large $^{18}O$-induced $\beta$-isotope shift was confirmed by studying model systems.

The model compound, 2-hydroxy-3,5,5-trimethyl-2-cyclohexenone, was prepared bearing an oxygen-18 label at either C-1 or C-2, (10a) and (10b), respectively (Scheme 3). Epoxidation of the corresponding cyclohexenone with hydrogen peroxide according to the procedure of Langin-Lanteri *et al*[16] followed by acid-catalysed opening of the epoxide in the presence of oxygen-18 labelled water gave (10) enriched with only one oxygen-18 atom as determined by its mass spectrum. The 100.6 MHz $^{13}C$ n.m.r. spectrum showed



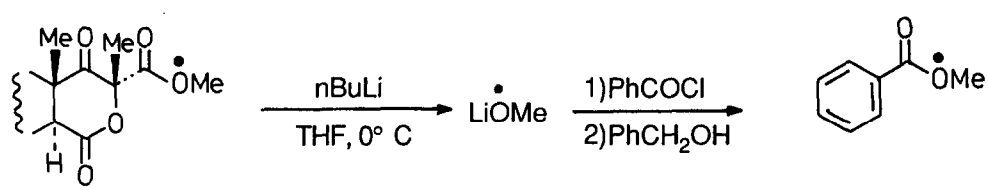

(10a) (10b)

Scheme 3

an isotope induced shift of 41 p.p.b. at C-1 and no observable shift at the carbon signal corresponding to C-2. It is interesting to note that no label was introduced at the C-2 position even after prolonged refluxing in $^{18}O$-labelled water. Treatment of the cyclohexenone with basic hydrogen [$^{18}O$]peroxide[17] followed by aqueous acid rearrangement of the intermediate epoxide afforded (10b) bearing one isotopically substituted oxygen. Examination of the $^{13}C$ n.m.r. spectrum of this compound revealed an isotope shift of 13 p.p.b. at the C-2 position. Carbon 3 displayed a large $\beta$-isotope shift of 11 p.p.b., thereby supporting the contention that terretonin (5) labelled by $^{18}O_2$ gas has an unusually large $\beta$-isotope shift at C-5. Recently, a third example has been found in which an olefinic carbon two bonds away from an enolic oxygen displays a similar very large $\beta$-isotope shift.[18]

In order to study the source of the remaining oxygen atoms, a 1:1 mixture of ethyl 3,5-dimethylorsellinate (7) doubly labelled with $^{18}O$ and $^{13}C$ at either the C-6 alcohol or C-7 carbonyl was prepared.[19] Incorporation of this material into terretonin (5) by *A. terreus* and analysis of the $^{13}C$ n.m.r. spectrum of the enriched metabolite showed isotope shifts for the C-15 and C-31 resonances (Table 1, Figure 3b), thereby clearly demonstrating their origin from the 6-hydroxyl and C-7 carbonyl of (7). The relative intensities of the isotopically shifted peaks indicate that approximately one half of the oxygen atoms derived from C-7 in (7) is lost in the formation of (5). This might be explained by conversion of (7) to the free carboxylic acid (6) before incorporation into (5). However, based on [*methyl*-$^{2}H_3$,$^{13}C$]methionine incorporation experiments which showed that no $^{2}H$ label is lost from methionine, it is most likely that C-31 also passes through a free carboxylic acid stage, which is subsequently *O*- methylated by *S*-adenosylmethionine. This would have the effect of returning the oxygen-18 ratio of the two positions to unity. Thus, it appears that the free carboxylic acid at C-15 must either undergo an additional esterification/de-esterification step (perhaps through an enzyme bound thioester) or can preferentially exchange with water in the medium.

If C-31 does indeed pass through the free carboxylic acid stage as predicted, its $^{13}C$ n.m.r. spectrum would be expected to show a pair of isotopically-shifted peaks consisting of a large shift corresponding to molecules of (5) bearing a label in the carbonyl oxygen of the ester, and a smaller isotope shift from molecules labelled in the alkoxy oxygen.




Scheme 4

However, the smaller of the two expected isotope shifts could not be observed. To demonstrate whether or not the methoxyl oxygen of (5) derived from ethyl [$^{13}C$,$^{18}O$]-3,5-dimethylorsellinate was enriched with $^{18}O$, this labelled terretonin was chemically degraded. Reaction of labelled (5) with n-butyllithium was followed by trapping of the liberated lithium methoxide as its benzoate ester by addition of benzoyl chloride (Scheme 4). GC/MS analysis of the semipurified reaction mixture indicated a 1.1% enrichment in the $^{18}O$ content of the methyl benzoate, which agrees well with the incorporation rate of 1.4% determined from the $^{13}C$ n.m.r. spectrum. Thus, both oxygens at C-31 of terretonin are equally labelled as expected for an intermediate free carboxylic acid. It therefore appears that both the singly and doubly bonded oxygens may be showing the same size of isotopically-induced shift in this case.

The results described above clearly define the sources of the oxygen atoms in terretonin and support the proposed biosynthetic pathway shown in Scheme 2. They also demonstrate the utility of this technique for obtaining information about possible intermediate oxidation states and modes of cyclisation during biosynthesis. However, the abundance of $\beta$-isotope shifts in terretonin emphasises the need for caution when employing the use of oxygen-18 isotope shifts in the detection of labelling.

## Experimental

General Procedures. Labelled precursors were purchased from Amersham and MSD Isotopes. Commercial TLC plates were Merck 60F-254. Flash chromatography employed the method of Still *et al*,[20] with Merck type 60 silica gel, 230-420 mesh. Nuclear magnetic resonance (n.m.r.) spectra were recorded on Bruker WP-80, WH-200, AM-300, WH-360 or WH-400 instruments with tetramethylsilane as internal standard. The $^{18}O$ isotope shifts were determined in the usual fashion[21] by accumulation of the $^{1}H$-decoupled $^{13}C$ n.m.r. spectrum over a narrow window; the FID was then zero-filled once to give a resolution of *ca* 0.1 Hz/pt. Infrared (IR) spectra were obtained on a Nicolet 7199 FT-IR spectrometer. Mass spectra (MS) were recorded at an ionising voltage of 70 eV on an AEI MS-50 instrument for high resolution electron impact (EI) ionisation.

Production and isolation of terretonin

*Aspergillus terreus* (NRRL 6273) was stored in the dark on potato dextrose agar (Oxoid CM 139) slopes. When required the culture was transferred to and grown on potato dextrose agar in medical flats for 8-10 days at 25-27°C, in the light. A spore suspension in distilled water, from the medical flats, was used to inoculate either 500 ml Erlenmeyer flasks or penicillin flasks containing 100 ml or 200 ml, respectively, of malt extract broth: Oxoid malt extract (3% w/v), Oxoid mycological peptone (0.5% w/v), distilled water to 100%. The pH of the medium was *ca* 5.4.

The cultures were normally allowed to grow for 14 days at 25-27°C, by which time a thick pale brown mat of mycelium had formed on the surface of

the medium. The growth medium was decanted from the flasks and filtered. The filtrate (pH ~6) was extracted with chloroform (3 x a third of the liquor volume), the extract dried ($MgSO_4$), and the solvent removed *in vacuo* to give a brown oil. This extract was applied to preparative t.l.c. plates (15-20 mg per plate) and eluted with acetone-chloroform (14:86). The band corresponding to an authentic sample of terretonin was isolated (Rf 0.4) and applied again to preparative t.l.c. plates, eluted with ethyl acetate-methylene chloride (25:75), to afford terretonin (Rf 0.4) as a white crystalline solid. The yield usually varied between 20 and 30 mg $l^{-1}$. $^1H$ n.m.r. spectroscopic data agreed with that of an authentic sample and published data.[8]

Incorporation of [1-$^{13}C$]- and [1,2-$^{13}C_2$]acetates and (*methyl*-$^{13}C$]-L-methionine into terretonin

*Aspergillus terreus* was grown, as previously described, in 15 penicillin flasks. 83.5 hours after inoculation, a sterile solution of sodium [1-$^{13}C$]acetate (1.0 g, 90.6 atom % $^{13}C$) in distilled water (20 ml) was distributed evenly into the culture media of five flasks by injection through the mycelial mat. Parallel experiments, feeding with 20 ml solutions of sodium [1,2-$^{13}C_2$]acetate (1.0 g, 90.0 atom % $^{13}C$), and [*methyl*-$^{13}C$]-L-methionine (200 mg, 90.0 atom % $^{13}C$), also at 83.5 hours, were conducted concurrently. All the flasks were incubated for a total of 336 hours. Terretonin was isolated as usual in yields of 31, 33 and 30 mg $l^{-1}$ respectively.

Incorporation of [*methyl*-$^{13}C^2H_3$]methionine into terretonin

*Aspergillus terreus* was grown as previously described in ten penicillin flasks. [*Methyl*-$^{13}C^2H_3$]methionine (299 mg, 2.01 mmol, 92.2 atom % $^{13}C$, 98 atom % $^2H$) dissolved in distilled water (20 ml) was distributed equally between five flasks, 78 hours after inoculation. After fourteen days growth, the experimental and control flasks were worked up. Isolation and purification in the usual way afforded terretonin 22 mg and 21 mg from the experimental and control flasks respectively. The relative abundance of mass peaks in the molecular ion mregion of the mass spectrum of the enriched terretonin was: 488(100%), 489(32), 490(9), 491(6), 492(10), 493(3), 494(1), 495(3), 496(2), 497(1), 498(1), 499(3), and 500(1).

Incorporation of ethyl [*carboxyl*,6-$^{14}C$]-3,5-dimethylorsellinic acid into terretonin

Ethyl [*carboxyl*,6-$^{14}C$]-3,5-dimethylorsellinate[12] (33 mg, 39.7 $\mu Ci$ $mmol^{-1}$) was dissolved in hot distilled water (25 ml) also containing "Tween 80" detergent (0.5 ml). The sterilised solution was distributed evenly, by injection through the mycelial mat, into the culture media of five penicillin flasks, each containing a 73 hour culture of *Aspergillus terreus*, grown as previously described. After a total of 335 hours growth, terretonin was isolated as normal. On this occasion, however, extra purification was effected by further preparative t.l.c., developing with ethyl acetate-methylene chloride (25:75), which separated terretonin ($R_f$ =

0.49) from a slightly more polar impurity present only in trace quantity. Terretonin (33.8 mg, 2.31 x $10^6$ d.p.m. $mmol^{-1}$) was isolated.

Incorporation of [5-*methyl*-$^2H_3$]-3,5-dimethylorsellinic acid into terretonin

A sterile solution of [5-*methyl*-$^2H_3$]-3,5-dimethylorsellinic acid[12] (50 mg) in distilled water (50 ml), also containing "Tween 80" detergent (0.5 ml) was fed to 5 flasks of a 73 hour culture of *Aspergillus terreus* exactly as described above. After 336 hours, terretonin (35 mg) was isolated as usual. Further purification, effected by preparative t.l.c., developing with ethyl acetate-methylene chloride (15:75), yielded terretonin (27 mg).

Preparation of generally deuterated terretonin

*Aspergillus terreus* was grown, as previously described, in 10 penicillin flasks. In five of these, 5% of the water had been substituted by $^2H_2O$. The flasks were incubated for 336 hours and the terretonin was isolated as usual, 39 and 37 mg respectively from the control and $^2H_2O$ containing flasks.

Incorporation of the ethyl [$^{13}C$,$^{18}O$]-labelled-3,5-dimethylorsellinate into terretonin

*Aspergillus terreus* was grown as previously described in six penicillin flasks. The [$^{13}C$,$^{18}O$]-labelled orsellinate[19] (137 mg, 0.60 mmol, 88 atom % $^{13}C$, 81 atom % $^{18}O$, 73 atom % $^{13}C^{18}O$) was suspended in a solution of "Tween 80" detergent (2 ml) and distilled water (10 ml). This suspension was distributed equally between five penicillin flasks, 3 days after inoculation. After fourteen days of growth the five experimental flasks and the control flask were worked up. Isolation and purification in the usual way afforded terretonin 33.5 mg (33.5 mg $l^{-1}$) and 3.6 mg (18.0 mg $l^{-1}$) from the experimental and control flasks respectively.

Incorporation of $^{18}O_2$ into terretonin

In a preliminary experiment culture flasks of *Aspergillus terreus* were connected to the "constant pressure" apparatus (Figure 3), for growth of cultures in closed atmospheres, immediately after inoculation, and grown in an $^{16}O_2/^{14}N_2$ (20:80) atmosphere. On visual comparison with control flasks, the fermentation appeared to proceed normally but was retarded by approximately 12 hours. The yield of terretonin was 26 mg $l^{-1}$.

Cultures of *Aspergillus terreus* were grown as previously described in 10 Erlenmeyer flasks. After 4 days growth, the five best-growing flasks were selected and linked to the "constant pressure" apparatus and grown in an $^{16}O_2/^{18}O_2/^{14}N_2$ (9.5:10.5:80) atmosphere. On the same day, two of the remaining flasks were checked for terretonin production at this stage in the fermentation. After eleven days growth, the three remaining control flasks and five experimental flasks were worked up. By this time 3.5 l of oxygen had been consumed by the experimental flasks. Terretonin was isolated and purified as usual. The yield of terretonin was 26 mg (52 mg $l^{-1}$) and 6 mg (20 mg $l^{-1}$) from the experimental and control flasks respectively.

Preparation of [1-$^{18}O$]-2-hydroxy-3,5,5,-trimethyl-cyclohex-2-ene-1-one (10a)

Modified literature procedures were followed.[16] A solution of

2,3-epoxy-3,5,5-trimethylcyclohexan-1-one (1.81 g, 11.8 mmol), concentrated $H_2SO_4$ (0.10 mL) and 50% oxygen-18 labelled water (1.9 ml) was heated to reflux for 3 h. The cooled mixture was extracted with ether (3 x 15 ml). The combined organic phases were dried and concentrated *in vacuo* to give 1.6 g of a yellow oil. This material was recrystallised three times from ether/petroleum ether to give [1-$^{18}$O]- (10a) as a fine white solid (0.16 g, 9.2% yield): m.p. 87-89°C (lit[16] m.p. 87°C); IR ($CHCl_3$ cast) 3410 br, 1668, 1649, 1613, 1397, 1181, 679, 626 cm$^{-1}$; $^1$H n.m.r. (80 MHz, $CDCl_3$) 6.25 (1H, s, OH), 2.34 (2H, s, $COCH_2$), 2.27 (2H, s, C=C-$CH_2$), 1.85 (3H, s, C=C-$CH_3$), 1.03 (6H, s, $C(CH_3)_2$); $^{13}$C n.m.r. (100 MHz, $CDCl_3$) 194.0 s, 143.2 s, 127.6 s, 49.4 t, 44.7 t, 33.4 s, 28.3 q, 2 x 17.0 q; m/z 154.0990 (154.0994 calculated for $C_9H_{14}{}^{16}O_2$) 156.1039 (156.1061 calculated for $C_9H_{14}{}^{16}O^{18}O$).

Preparation of Potassium [$^{18}O_2$]-Hydroperoxide

This compound was prepared by adapting the literature procedure.[17] Into a 50 ml 3-neck flask fitted with a gas inlet tube, a 60 ml addition funnel, and a mercury manometer was placed in a suspension of 1.34 g potassium *tert*-butoxide (12 mmol) in 5 ml of benzene. The cooled (0°C) apparatus was evacuated and filled with $^{18}O_2$ gas at 1 atmosphere pressure. A solution of benzhydrol (2.20 g, 12 mmol) in 32 ml benzene was added dropwise over a period of 10 min and the ice bath was removed. $^{18}O_2$ gas was added to the system to maintain a pressure of 1 atmosphere. Upon completion of $^{18}O_2$ uptake the mixture was stirred for 3 more hours during which a precipitate formed. The slurry was then centrifuged and the solvent was removed. The solid was resuspended in 10 ml of benzene, centrifuged, and collected. The solid was dried for 16 h *in vacuo* to give 658 mg of [$^{18}O_2$]potassium hydroperoxide (76% yield) as a white solid.

Preparation of [2-$^{18}$O]-2-Hydroxy-3,5,5-trimethylcyclohex-2-ene-1-one (10b)

Modified literature procedures were followed.[16] To a solution of 3,5,5-trimethylcyclohex-2-ene-1-one (500 mg, 3.62 mmol) in 2 ml of methanol at 0°C was added a solution of 658 mg of potassium [$^{18}O_2$]-hydroperoxide (9.12 mmol) in 2 ml of water. The resulting yellow mixture was stirred for 3 h, extracted with dichloromethane (2 x 30 ml), dried and concentrated *in vacuo*. The residue was refluxed in 2% $H_2SO_4$ (5 ml) for 3 h. The cooled mixture was extracted with dichloromethane (2 x 30 ml) and the combined organic phases were dried and concentrated *in vacuo* to give a brown oil. Purification by flash chromatography (85% hexane/15% ethyl acetate) gave 42 mg of the desired product [2-$^{18}$O]-(10b) (7.5% yield) as a white crystalline solid whose physical constants were similar to those of the corresponding [1-$^{18}$O] labelled compound.

Degradation of $^{18}$O-Labelled Terretonin (5) to Methyl Benzoate and GC/MS Analysis

To a solution of (5) (22.4 μmol) (derived from [$^{13}$C,$^{18}$O] (10) in 0.5 ml tetrahydrofuran at 0°C was added 140 μl of n-butyllithium (1.6 M solution in hexane, 224 μl) with stirring. The solution was warmed to room temperature

and stirred for 30 min. The resulting yellow suspension was cooled to 0 C, 26 $\mu$l of benzoyl chloride (224 $\mu$mol) was added, and stirring was continued for 16 h. Benzyl alcohol (23 $\mu$l, 224 $\mu$mol) was added and the solution was stirred for an additional 3 h. The resulting suspension was partially purified by bulb to bulb distillation (50°C, 0.1 mm Hg) to give 0.1 ml of distillate. This was diluted with 2.5 mL of ether and submitted for GC/MS studies. The GC/MS studies were carried out on a VG 7070E mass spectrometer coupled to a Varian Vista 6000 gas chromatograph fitted with a 30 m x 0.25 cm OV 351 capillary column. A flow rate of 1 ml/min of helium gas was used and a temperature gradient of 10°C/min from 50°C to 250°C was applied. Two peaks were observed, that of benzyl alcohol ($t_r$ = 8.7 min) and methyl benzoate ($t_r$ = 9.97 min). Ratio of $M^+$ : $M^+ + 2$ for unlabelled methyl benzoate was 100:0.77, ratio of $M^+$ : $M^+ + 2$ for methyl benzoate derived from labelled terretonin was 100:1.86.

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