Metabolites of *Aspergillus ustus*. Part 4. Stable-isotope Labelling Studies on the Biosynthesis of the Austalides.[1]

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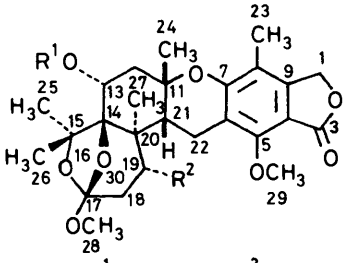
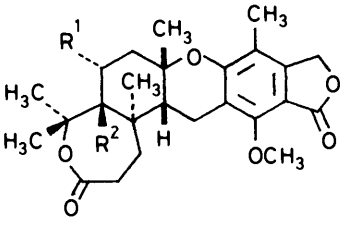
Incorporation of [1-^{13}C]-, [1,2-$^{13}C_2$]- and [1-^{13}C,2,2,2-$^{2}H_3$]-acetate, and (3*RS*)-[2-^{13}C]mevalonolactone into austalide D(**2**), a metabolite of *Aspergillus ustus*, shows that the austalides are derived from 6-[(2*E*,6*E*)farnesyl]-5,7-dihydroxy-4-methylphthalide. The proposed mechanism for the subsequent cyclisation and oxidative modifications of the farnesyl moiety, consistent with the relative stereochemistry of the austalides and the structures of the cometabolites, austalides J (**4**), K (**5**), and L (**6**), is supported by the incorporation of austalide K into austalide D. The addition of ethanol to growing cultures of *A. ustus* severely inhibits normal metabolite production and induces the formation of a new metabolite, ethyl 6-ethyl-2,4-dihydroxy-3-methylbenzoate (**11**). The biosynthetic origin of this metabolite was studied using [1-^{13}C]- and [1-^{13}C,2,2,2-$^{2}H_3$]acetate and (2*S*)-[*methyl*-^{13}C]methionine as precursors.

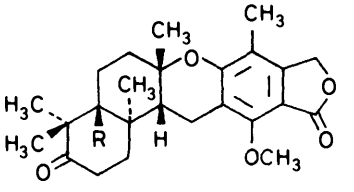
The austalides, *e.g.* austalide A (**1**), D (**2**), I (**3**), and J (**4**), are a group of related meroterpenoid metabolites produced by whole maize cultures of *Aspergillus ustus*, strain MRC 1163.[1-3] Only small amounts of austalide A and D, however, are produced by cultures of *A. ustus* when grown in stationary culture on malt extract medium and instead two new austalides *viz* K (**5**) and L (**6**) were isolated from the mycelial mats.[1,3] A structural analysis of the austalides suggests a biosynthetic pathway which involves 6-[(2*E*,6*E*)farnesyl]-5,7-dihydroxy-4-methylphthalide (**7**), a key intermediate also in the biogenesis of mycophenolic acid.[4] We now report a study of the biosynthesis of austalide D (**2**) using ^{13}C- and ^{2}H-labelled precursors which provides information on the subsequent cyclisation and oxidative modifications of the farnesyl moiety in (**7**).[5]



(1) $R^1 = COCH_3$, $R^2 = H$
(2) $R^1 = H$, $R^2 = OCOCH_3$



(3) $R^1 = OCOCH_3$, $R^2 = H$
(4) $R^1 = H$, $R^2 = OH$



(5) R = H
(6) R = OH

The unambiguous assignment of the different resonances in the ^{13}C n.m.r. spectrum of austalide D (see Table 1), the first step in such a biosynthetic study, is based on the two- and three-bond (C,H) connectivity pattern as determined by heteronuclear $^{13}C\{^{1}H\}$ selective population inversion (SPI)[6] experiments.[3] The method, however, does not allow us to assign the δ_C 25.73 and 29.65 resonances (correlated with the resonances at δ_H 1.771 and 1.488, respectively)[3] to specific carbon atoms of the two prochiral diastereotopic methyl groups, C-25 and C-26. The assignment of the δ_C 25.73 resonance to the *pro-R* methyl group, C-25 and consequently that at δ_C 29.65 to C-26, follows from the relative stereochemistry of the austalides,[2,3] and the observation of an appreciable n.O.e. for the methyl protons at δ_H 1.771 (25-H) upon irradiation of the C-27 protons (δ_H 0.964) in a homonuclear $^{1}H\{^{1}H\}$ n.O.e. experiment. The n.O.e. observed between the C-13 proton and the methyl protons at δ_H 1.488 (26-H) supports the assignments. This assignment is of vital importance to the study of the biosynthesis of the austalides since it allows the formulation of a plausible mechanism for the formation of austalide D, consistent with the relative stereochemistry of these metabolites.

Cultures of *A. ustus* were grown in Petri dishes on cakes of yellow maize meal containing 50% water. Studies of the course of fermentation indicated that austalide D production commenced on day 3 and reached a level of 32 mg per 100 g of maize after 14 days. Preliminary feeding experiments with [1-^{14}C]acetate as precursor established that a good incorporation (0.1%) and satisfactory dilution values (36.3, assuming 11 labelled positions) were obtained by pulse-feeding cultures of *A. ustus* every 24 h from day 3 to day 14 with sodium acetate to a total amount of 800 mg per 100 g of maize.

Incorporation of [1-^{13}C]acetate into austalide D resulted in the enhancement of the resonances assigned to the phthalide moiety carbon atoms, C-3, C-5, C-7, and C-9 (average enrichment factor: 2.1), the farnesyl-derived carbon atoms C-11, C-13, C-15, C-18, C-20, and C-22 (average enrichment factor:

Table 1. ^{13}C N.m.r. data for austalide D (**2**)

Carbon	δ_C/p.p.m.[a,b]	$\Delta\delta$[c]	^1J(C,C)/Hz[d]
1	68.10 T		40.9
3	168.93 S		74.2
4	108.63 S		73.5
5	155.61 S		72.5
6	116.26 S		71.7
7	156.58 S		69.8
8	114.10 S		69.2
9	145.76 S	0.106	40.9
11	78.23 S	*e*	39.2
12	40.84 T		
13	69.92 D		44.8
14	86.10 S		45.3
15	85.69 S	0.051	40.3
		0.050	
		0.050	
17	117.47 S		56.5
18	37.34 T		56.5
19	71.07 D		
20	44.98 S	0.076	35.1
		0.076	
		0.076	
21	38.49 D		35.1
22	19.73 T	0.116	35.4
23	10.61 Q		
24	27.35 Q		39.7
25	25.73 Q		40.4
26	29.65 Q		
27	14.07 Q		34.9
28	48.71 Q		
29	62.16 Q		
31	170.27 S		59.8
32	21.13 Q		59.8

[a] Recorded on a Bruker WM-500 spectrometer for solutions in $CDCl_3$. Chemical shifts relative to Me_4Si. [b] Letters refer to the pattern resulting from directly bonded (C,H)-coupling with S = singlet, D = doublet, T = triplet, and Q = quartet. [c] ^{13}C-^2H Upfield β-shift in p.p.m. for austalide D derived from [1-^{13}C,2,2,2-$^2H_3$]acetate. [d] Intra-acetate coupling. [e] Unresolved signal.

3.5), and the acetate carbonyl carbon atom, C-31 (enrichment factor: 4.1) in the proton-decoupled ^{13}C n.m.r. spectrum.

The arrangement of intact acetate units in austalide D was studied by using [1,2-$^{13}C_2$]acetate as the precursor. The proton-decoupled ^{13}C n.m.r. spectrum of the enriched austalide D exhibited one-bond (C,C) couplings for the resonances of carbon atoms derived from intact acetate units (intra-acetate coupling). The measured ^1J(C,C) values for these couplings are given in Table 1 and prove the presence of 11 intact acetate units arranged as shown in Figure 1. It is of interest to note the value of 56.5 Hz for the one-bond (C,C) coupling between the orthoester carbon atom, C-17 and the methylene carbon atom, C-18, which is relatively large for sp^3 hybridised carbon atoms.[7]

On detailed examination of the ^{13}C n.m.r. spectrum, additional one-bond (C,C) couplings were observed between carbon atoms derived from adjacent acetate units (interacetate and intermevalonate coupling). Similar couplings of this type have been observed previously in biosynthetic studies using ^{13}C-labelled acetate in which high incorporation efficiencies resulted in an increased probability of adjacent acetate units being labelled.[8,9] These additional couplings are readily distinguished from intra-acetate (C,C) couplings by their much lower intensities. The low intensity one-bond (C,C) couplings observed for the C-12 (δ_C 40.84), C-19 (δ_C 71.07), and C-26 (δ_C 29.65) resonances arise from interacetate coupling with C-11, C-20, and C-15, respectively. It was therefore expected that the former group of carbon atoms originated from C-2 of mevalonate. This supposition was corroborated by the proton-decoupled ^{13}C n.m.r. spectrum of (3*RS*)-[2-^{13}C]mevalonolactone-derived austalide D which exhibited three enhanced signals assigned to C-12, C-19, and C-26 (average enrichment factor: 2.8). The pattern and values of the one-bond interacetate and intermevalonate (C,C) couplings is indicated in Figure 1.

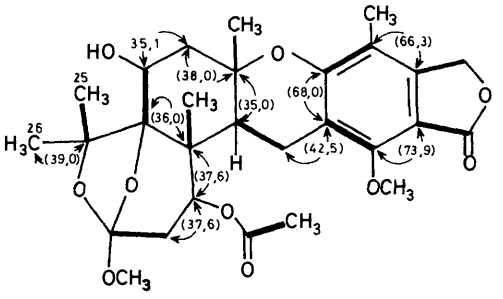


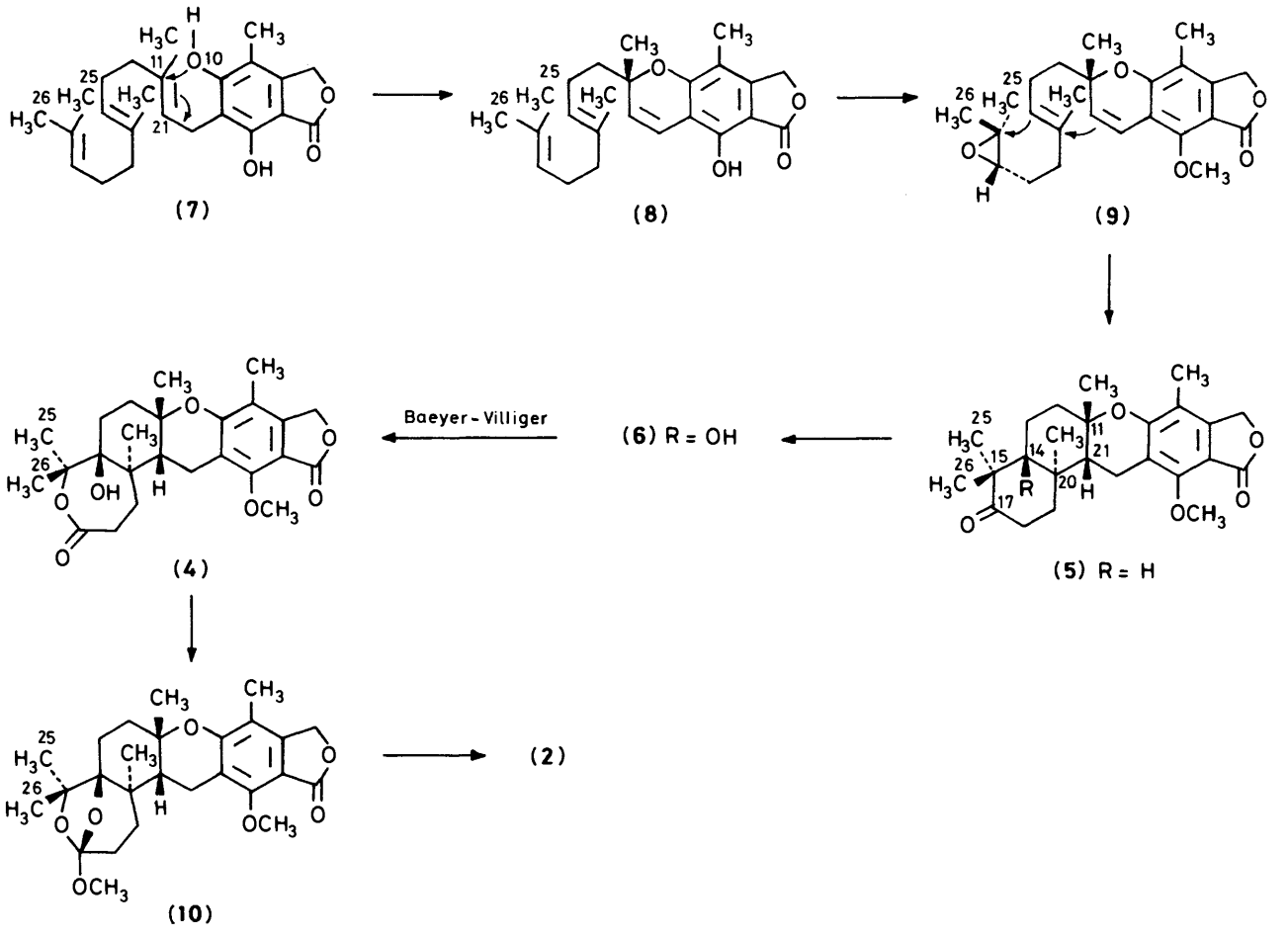
Figure 1. The labelling pattern observed for austalide D enriched with [1,2-$^{13}C_2$]acetate. The intra-acetate (C,C) couplings are indicated by thick lines. The observed interacetate couplings are shown by thin arrowed lines; the magnitude of the coupling constants in Hz is shown on the lines.

A plausible mechanism for the biogenesis of austalide D based on the above results and the known relative configuration of the austalides[1-3] is outlined in the Scheme. The key intermediate 6-[2*E*,6*E*-farnesyl]-5,7-dihydroxy-4-methylphthalide (**7**),[4] cyclises through a stereospecific attack of the phenolic oxygen atom on the 11*si*,21*si*-face* of the double bond to give the chromene (**8**). A similar ring closure occurs in the biosynthesis of siccanin.[10] Cyclisation of a terminal 17*S*-epoxide* intermediate (**9**) produces austalide K (**5**) with the required *trans-transoid-cis* ring fusion of the austalides.† An alternative, concerted oxygen-initiated cyclisation[11] of the precursor (**7**) will lead to a stereoisomer of austalide K with the wrong relative configuration at the ring junctions. Hydroxylation of austalide K (**5**) at C-14 proceeds with retention of configuration to give austalide L (**6**). An enzymatic Baeyer-Villiger oxidation occurs with retention of configuration,[12,13] to produce the seven-membered lactone ring of austalide J (**4**), which has the correct stereochemical orientation of the substituents for *in vivo* cyclisation to give, after *O*-methylation with (*S*)-adenosylmethionine, the orthoester (**10**). The *in vitro* austalide J (**4**) is smoothly transformed to the orthoester (**10**) by treatment with either methyl iodide and potassium carbonate in acetone or by thionyl chloride in methanol.[1] Subsequent hydroxylation at C-13 and C-19, and acetylation would lead to austalide D (**2**). The exact sequence of these events, however, is not known.

The proposed mechanism for the biogenesis of austalide D is in agreement with the known *cis* orientation of intact acetate units in dimethylallyl- and farnesyl-pyrophosphate and the observation that the *pro-R* methyl carbon (C-25) in austalide D is part of an intact acetate unit whereas the *pro-S* methyl carbon atom (C-26) is derived from C-2 of mevalonolactone. The Baeyer-Villiger oxidation of the cyclohexanone ring of austalide K (**5**) or L (**6**) to generate the seven-membered lactone ring of the austalides J and D proceeds with retention of configuration at C-15, and the C-25 *pro-R* methyl group of austalide K is therefore also part of an intact acetate unit.

* The numbering used for the proposed precursors is in accord with the system used for the austalides.[1,3]

† The cyclohexanone ring of austalide K corresponds to the seven-membered ring of austalides A, D, I and J, and is *trans*-fused.



Scheme. Proposed biosynthetic pathway for austalide D

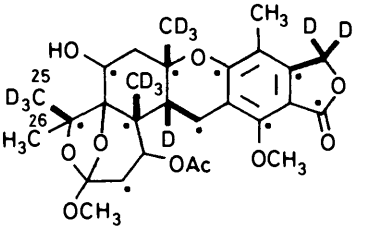
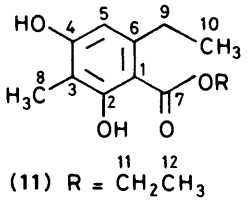


Figure 2. The expected labelling pattern for austalide D enriched with [1-^{13}C,2,2,2-$^{2}H_3$]acetate

The fate of the hydrogen atoms in the biosynthesis of the austalides was studied by incorporation of [1-^{13}C,2,2,2-$^{2}H_3$]acetate into austalide D. The incorporation of ^{2}H located β to a ^{13}C atom can be detected by the small characteristic upfield β-isotope shift in the resonance position of the ^{13}C nucleus in the ^{13}C n.m.r. spectrum.[14] The number of ^{2}H atoms located β to a particular ^{13}C atom can be deduced in turn from the value of the β-isotope shift.[14,15] The expected labelling pattern of austalide D enriched with [1-^{13}C,2,2,2-$^{2}H_3$]acetate is shown in Figure 2. The presence of ^{2}H at C-21, evident from the β-isotope shift of −0.116 p.p.m. for C-22 (see Table 1) indicates that the C-21 proton corresponds to the 4*re* proton of mevalonolactone as this proton is stereospecifically retained in the conversion of mevalonolactone to 3,3-dimethylallyl pyrophosphate. The retention of two ^{2}H atoms at C-1 of the phthalide moiety, indicated by the β-isotope shift of −0.106 p.p.m. for C-9,[16] shows that in the formation of the lactone ring the methyl group of the acetate starter unit is oxidised to a hydroxymethyl function.

The postulated intermediary role of austalides J (**4**), K (**5**), and L (**6**) in the biosynthesis of austalide D (**2**) was investigated as follows. Three-day old cultures of *A. ustus* on malt extract medium were pulse-fed every 24 h with (2*S*)-[*methyl*-^{14}C]-methionine (110 mg; specific activity 517.36 μCi mmol^{-1}) until day 14. The resultant ^{14}C-labelled austalide K (9 mg, specific activity 10.56 μCi mmol^{-1}) in ethanol was added to cultures of *A. ustus* on a potato-dextrose medium to give austalide D (1.5 mg, specific activity 0.42 μCi mmol^{-1}). The dilution value of 25.1 and the absolute incorporation of austalide K (0.5%) are indicative of the intermediacy of this metabolite in the biosynthesis of austalide D.

In the course of the last experiment it was found that the addition of ethanol to growing cultures of *A. ustus* on potato-dextrose medium inhibited the production of the normal array of metabolites,[3,17] and instead induced the formation of a single major metabolite, the ethyl ester (**11**), $C_{12}H_{16}O_4$. The



(**11**) R = $^{11}CH_2\,^{12}CH_3$

(**12**) R = CH_3

Table 2. ^{13}C and ^{1}H N.m.r. data for ethyl 6-ethyl-2,4-dihydroxy-3-methylbenzoate (**11**)

Carbon	δ_C[a]	J(C,H)/Hz	δ_H[a]	J(H,H)/Hz
1	104.63 S			
2	163.03 S			
3	108.74 S			
4	158.26 S			
5	109.23 D	158.2	6.219 s	
6	146.42 S			
7	171.93 S			
8	7.68 Q	128.2	2.085 s	
9	29.61 T	129.2	2.857 q	7.4
10	16.03 Q	126.6	1.163 t	7.4
11	61.24 T	148.6	4.387 q	7.2
12	14.93 Q	126.7	1.396 t	7.2
2-OH			12.026 s	

[a] Recorded on a Bruker WM-500 spectrometer for solutions in $CDCl_3$. Chemical shifts relative to Me_4Si.

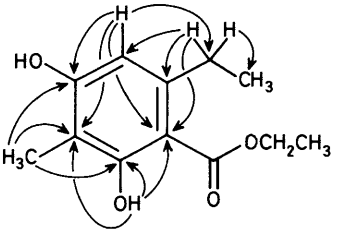


Figure 3. The two-and three-bond (C,H) connectivity pattern determined by SPI experiments for ethyl 6-ethyl-2,4-dihydroxy-3-methylbenzoate (**11**)

maximum production of this metabolite occurred when three-day old cultures of *A. ustus* were treated every 24 h for 12 days with 0.1 ml of ethanol per 100 ml of medium. Similar results were obtained when a malt extract or a solid maize meal medium was used. Substitution of methanol for ethanol resulted in the isolation of the methyl ester analogue (**12**).

The ^{1}H and ^{13}C n.m.r. data for the ethyl ester (**11**) are collated in Table 2. The unambiguous determination of the aromatic substitution pattern in (**11**), and thus the structure, is based on the two- and three-bond (C, H) connectivity pattern established by heteronuclear selective population inversion (SPI) experiments[6] (see Figure 3). The intramolecular hydrogen-bonding between a phenolic hydroxy proton (δ_H 12.026) was also utilised to distinguish the resonances of the two oxygen-bearing aromatic carbon atoms, C-2 and C-4.[18] Thus an upfield two-bond deuterium isotope shift of 0.270 p.p.m. was observed for the δ_C 163.03 resonance (C-2) in the proton-decoupled ^{13}C n.m.r. spectrum when the exchangeable protons were partially exchanged with deuterium upon addition of D_2O:H_2O (1:1). In addition, an upfield four-bond isotope shift of 0.110 p.p.m. was observed for the carbonyl carbon resonance, C-7. Similar isotope effects have been reported for other phenolic compounds with intramolecular hydrogen bonds.[18–20]

The structure of the ethyl ester (**11**) suggests that the metabolite is derived from a tetraketide by methylation of the methyl group of the acetate starter unit. A similar methylation has been postulated to explain the formation of the ethyl side-chain of barnol[21] and is also indicated for the biosynthesis of stellatin,[22] both tetraketide fungal metabolites. However, methylation of a polyketide methyl rather than a methylene group is very unusual, and in all these cases another more plausible mechanism which involves methylation at the methylene position of a pentaketide precursor and subsequent loss of the acetate starter unit, is proposed. Such a biosynthetic pathway is involved in the formation of the ethyl group of both asteltoxin[23] and the aurovertins.[24] In both these cases, however, a second pathway which utilises propionate as a starter unit, is also operative, and the ethyl group could also be derived from propionate.[23,24]

The origin of the *C*-ethyl group of (**11**) was investigated by addition of [1-^{13}C]acetate and (2*S*)-[*methyl*-^{13}C]methionine as well as ethanol to cultures of *A. ustus*. In the proton-decoupled ^{13}C n.m.r. spectrum of (**11**) derived from [1-^{13}C]-acetate the signals assigned to C-2, C-4, C-6, and C-7 were enhanced (average enrichment factor: 6.0). In a similar manner, but using (2*S*)-[*methyl*-^{13}C]methionine as precursor, it was shown that the methyl groups, C-8 and C-10, were derived from methionine (average enrichment factor: 16.9). Experiments using both [1-^{14}C]- and [1-^{13}C]-propionate showed that propionate is not involved in the biosynthesis.

The presence or absence of an acetate starter unit in the ester (**11**) was studied using [1-^{13}C,2,2,2-$^{2}H_3$]acetate as the precursor. Retention of 9-^{2}H in the enriched metabolite, as in the case of austalide D (see earlier) would indicate that C-6—C-9 represents the acetate starter unit of a tetraketide which is subsequently methylated at the terminal methyl group. In the event, no ^{2}H was retained at either C-9 or C-5 in the ester (**11**) derived from [1-^{13}C,2,2,2-$^{2}H_3$]acetate: a result which favours a pentaketide origin and subsequent loss of the acetate starter unit for (**11**).

Experimental

For general directions as well as the description of the isolation and purification of the austalides, see ref. 3.

Incorporation of Labelled Acetate into Austalide D.—Aliquots (0.5 ml) of a sterile, aqueous solution (12 ml) of sodium [1-^{14}C]acetate (800 mg, 25.63 μCi mmol^{-1}) were added every 24 h for 12 days to 3-day old growths of *A. ustus* on cakes of yellow maize meal (2 × 50 g). The cultures were harvested on day 14 to give, after purification as previously described,[3] austalide D (**2**) (16 mg; 7.80 μCi mmol^{-1}).

In similar experiments with other precursors, the following yields of austalide D were obtained:

Precursor	Atom % ^{13}C	Amount (mg)	Yield of (**2**) (mg)
Sodium [1-^{13}C]acetate	91.6	800	17
Sodium [1,2-$^{13}C_2$]acetate	C-1: 91.6 C-2: 90.0	800	30
Sodium [1-^{13}C,2,2,2-$^{2}H_3$]acetate	99.0 (98 atom % ^{2}H)	1 000	32

Incorporation of (3RS)-*Mevalonolactone into Austalide D.*—Aliquots (0.5 ml) of a sterile, aqueous solution (60 ml) of (3*RS*)-[2-^{14}C]-mevalonolactone (130 mg; 50 μCi mmol^{-1}) and sodium hydroxide (40 mg) were added every 24 h for 12 days to each of ten 500 ml flasks containing a 3-day old growth of *A. ustus* on a potato-dextrose medium. The cultures were harvested on day 14, to give, after purification, austalide D (8 mg; 1.50 μCi mmol^{-1}).

In a similar experiment using (3*RS*)-[2-^{13}C]mevalonolactone

(90.0 atom % ^{13}C; 470 mg) as the precursor, austalide D (8 mg) was obtained.

Incorporation of (2S)-[methyl-^{14}C]*Methionine into Austalide K.*—Aliquots (0.5 ml) of a sterile, aqueous solution (120 ml) of (2*S*)-[*methyl*-^{14}C]methionine (100 mg; 517.36 μCi mmol^{-1}) were added every 12 h for 12 days to each of ten 500 ml flasks containing a 3-day old growth of *A. ustus* on a malt extract medium. The cultures were harvested on day 14 to give, after purification,[3] austalide K (**5**) (9 mg; 10.56 μCi mmol^{-1}); dilution value of 98.0 (assuming two labelled positions); absolute incorporation 0.07%.

Incorporation of (2S)-[*methyl*-^{14}C]*Methionine-derived Austalide K into Austalide D.*—Aliquots (0.1 ml) of an ethanol solution (12 ml) of austalide K (9 mg; 10.56 μCi mmol^{-1}), obtained from the experiment described above, were added every 24 h for 12 days to each of ten 500 ml flasks containing a 3-day old growth of *A. ustus* on a potato-dextrose medium. The cultures were harvested on day 14 to give, after purification,[3] austalide D (**2**) (2 mg; 0.42 μCi mmol^{-1}); dilution value of 24.0; absolute incorporation 0.5%.

Isolation of Ethyl 6-*Ethyl*-2,4-*dihydroxy*-3-*methylbenzoate* (**11**).—Ethanol (96%, 0.1 ml) was added every 24 h for 12 days to 3-day old cultures of *A. ustus* on a potato-dextrose medium (10 × 100 ml). The mycelia were macerated with acetone in a Waring blender. The acetone mixture was filtered, evaporated, and the resulting brown gum was partitioned between aqueous methanol (90%) and hexane. Evaporation of the methanol solution gave a residue which was partitioned between chloroform and water. The organic layer was dried (Na_2SO_4), filtered, and evaporated. The residue (250 mg) was subjected to column chromatography on silica gel (50 g) with hexane–ethyl acetate (7:3 v/v) to give ethyl 6-ethyl-2,4-dihydroxy-3-methylbenzoate (**11**) (10 mg), m.p. 95—97 °C (from benzene–hexane), $\lambda_{max.}$ 270 and 304 nm (ε 14 400 and 4 120, respectively); $\nu_{max.}(CHCl_3)$ 3 480, 3 260, and 1 640 cm^{-1} (Found: C, 64.3; H, 7.3%; M^+, 224. $C_{12}H_{16}O_4$ requires C, 64.3; H, 7.2%; *M*, 224).

Substitution of methanol for ethanol in the above experiment resulted in the formation of methyl 6-ethyl-2,4-dihydroxy-3-methylbenzoate (**12**), m.p. 104—106 °C (from benzene–hexane), $\lambda_{max.}$ 270 and 305 nm (ε 14 500 and 4 150, respectively); $\nu_{max.}(CHCl_3)$ 3 580, 3 260, and 1 640 cm^{-1}; $\delta_H(CDCl_3)$ 11.967 (1 H, s, 2-OH), 6.218 (1 H, s, 5-H), 3.904 (3 H, s, CO_2CH_3), 2.810 (2 H, q, *J* 7.4 Hz, 9-H), 2.083 (3 H, s, 8-H), and 1.126 (3 H, t, *J* 7.4 Hz, 10-H); $\delta_C(CDCl_3)$ 172.40 (S, C-7), 162.92 (S, C-2), 158.48 (S, C-4), 146.36 (S, C-6), 109.19 (D, C-5), 108.79 (S, C-3), 104.44 (S, C-1), 51.81 (Q, CO_2CH_3), 29.52 (T, C-9), 15.77 (Q, C-10), and 7.70 (Q, C-8) (Found: C, 62.9; H, 6.8%; M^+, 210. $C_{11}H_{14}O_4$ requires C, 62.8; H, 6.7%; *M*, 224).

Incorporation of Labelled Precursors into Ethyl 6-*Ethyl*-2,4-*dihydroxy*-3-*methylbenzoate* (**11**).—The labelled precursor, together with ethanol (2 ml) was added every 24 h for 12 days to 3-day old cultures of *A. ustus* on cakes of yellow maize meal (2 × 50 g). The cultures were harvested on day 14 to give, after purification,[3] the labelled ester (**11**).

Precursor	Atom % ^{13}C	Amount (mg)	Yield of (**11**) (mg)
Sodium [1-^{13}C]acetate	99.0	1 000	10
(2*S*)-[*methyl*-^{13}C]methionine	90.0	200	7
Sodium [1-^{13}C,2,2,2-$^{2}H_3$]acetate	99.0 (98.0 atom % ^{2}H)	800	19

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Received 17*th October* 1986; *Paper* 6/2032