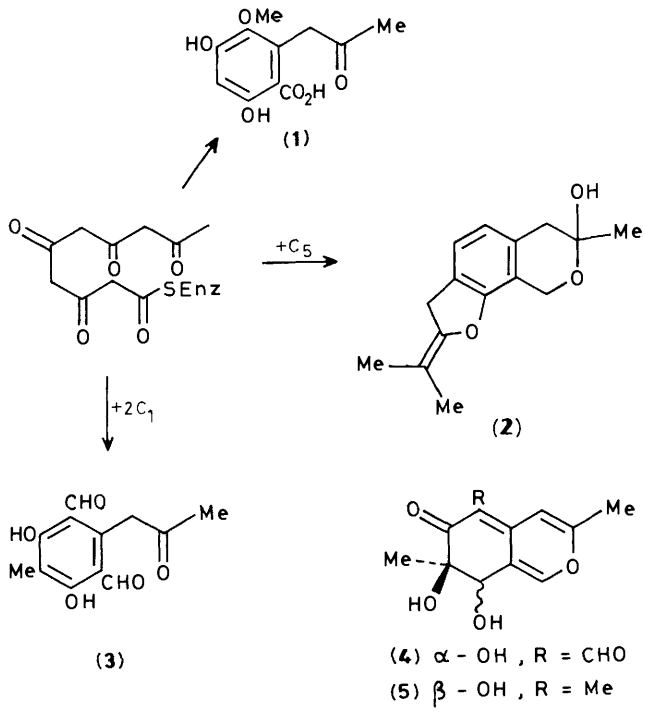
Studies on the Biosynthesis of the Mycotoxin Austin, a Meroterpenoid Metabolite of *Aspergillus ustus*

Salman A. Ahmed, Fiona E. Scott, Desmond J. Stenzel, and Thomas J. Simpson *,†
Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JJ, Scotland
Richard N. Moore, Laird A. Trimble, Kunizo Arai, and John C. Vederas *
Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Incorporations of ^{13}C-labelled acetates and methionine into the mycotoxin austin in cultures of *Aspergillus ustus* give a labelling pattern consistent with a mixed polyketide-terpenoid pathway. Incorporations of ^{14}C and ^{2}H labelled 3,5-dimethylorsellinate confirm the intermediacy of a preformed tetraketide-derived phenolic precursor. Further information on the mechanisms involved in the modifications of both the farnesyl- and tetraketide-derived portions of the molecule are provided by incorporation studies with [1-^{13}C,$^{18}O_2$]acetate, [*methyl*-^{13}C,$^{2}H_3$]methionine, ^{13}C,^{18}O-labelled dimethylorsellinate, $^{18}O_2$ gas and [6-^{13}C,6-$^{2}H_3$]mevalonic acid lactone.

Aspergillus ustus, one of the most prevalent fungi in soil and decaying vegetation, is a known contaminant of stored foodstuffs such as cereals, pulses and cheese and is known to produce a number of toxic secondary metabolites of varied biosynthetic origin.

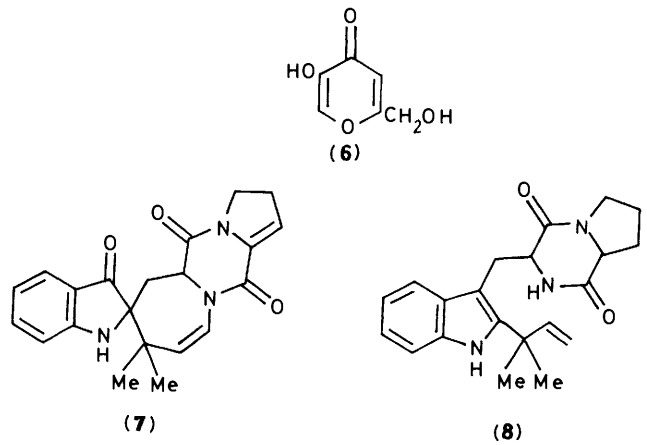
The first secondary metabolite isolated from *A. ustus* in 1951 was ustic acid, which was shown to have the phenolic structure (**1**) by chemical degradation.[1] It is formally a pentaketide, arising from the common mode of pentaketide cyclisation, as shown in Scheme 1. In common with a number of Aspergillus



Scheme 1.

species, *A. ustus* is known[2] to produce kojic acid (**6**). The major pathway to kojic acid is by direct conversion of glucose.[3]

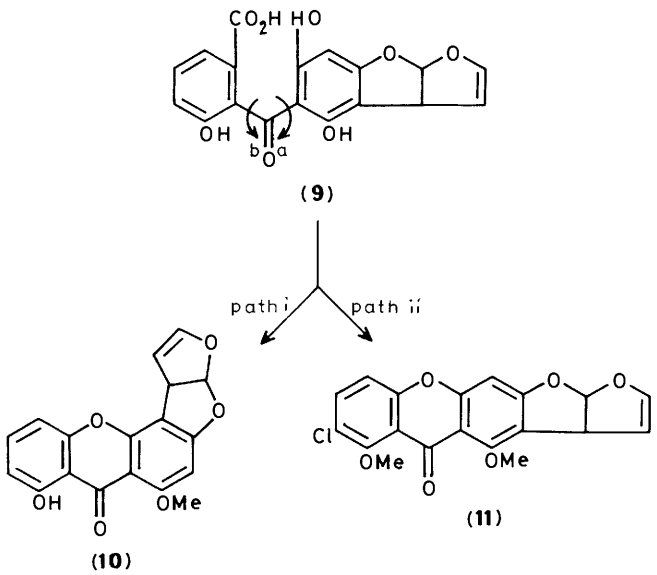
In 1971, extraction and isolation of toxic maize-meal cultures of *A. ustus*, led to the isolation[4] of austamide (**7**). Austamide is an indole alkaloid, formed from tryptophan, isopentenyl pyrophosphate, and proline. Four other related diketopiperazines were later isolated,[5] one of which, (**8**) seems a likely biogenetic precursor to (**7**).



Later work by the same group,[6] resulted in the isolation and structure determination of austdiol (**4**). This metabolite was found to be the major active component of toxic maize-meal cultures of *A. ustus*. The same authors later reported[7] the isolation of dihydrodeoxy-8-*epi*-austdiol (**5**), as a minor co-metabolite of (**4**). The results of incorporation of [$^{13}C_2$]acetate into austdiol, confirmed its biogenesis from a methylated pentaketide analogous to the precursor to ustic acid.[8] In the proton decoupled ^{13}C n.m.r. spectrum of the enriched metabolite, scrambling of label was observed, implying the intermediacy of the symmetrical dialdehyde (**3**) in the biosynthesis of austdiol, as shown in Scheme 1.

In 1974, a group of interrelated xanthone metabolites was isolated from *A. ustus*.[9] The major metabolite (**11**) was named autocystin A, and its structure exhibits a linear fusion between xanthone and bisdihydrofuran systems, reminiscent of sterigmatin, a minor metabolite of aflatoxin producing organisms.[10] In the biosynthetic pathway to the aflatoxins proposed by Buchi,[11] the benzophenone carboxylic acid (**9**) is a key intermediate. As shown in Scheme 2, rotation about (a) leads to an angular system as in sterigmatocystin (**10**), while no rotation, or rotation about (b) could lead to the linear system of the autocystins and sterigmatin. Lending weight to the proposed

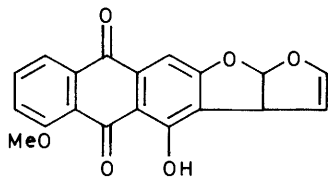
† *Present address:* Department of Chemistry, University of Leicester, University Road, Leicester LG1 7RH.

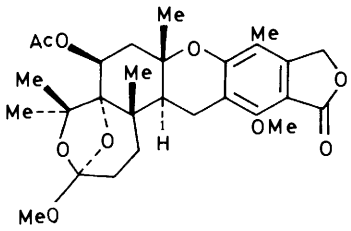


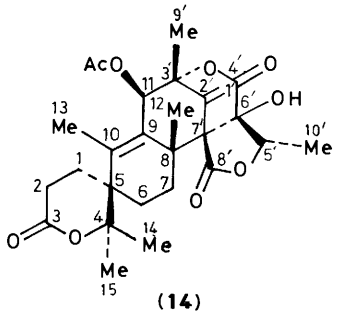
Scheme 2.

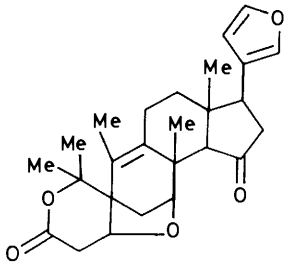
In 1976, the investigation of a strain of *A. ustus* found on stored black-eyed peas, resulted in the isolation of a novel mycotoxin named austin.[16] Its structure was shown to be (**14**) by *X*-ray crystallography and the authors proposed a sesterterpenoid origin. Austin would represent a new structural type of sesterterpene, but could possibly be formed by an initial cyclisation as occurs in chielanthatriol,[17] and subsequent modification. An alternative is that austin could be a degraded triterpene. The liminoids, or meliacins, are triterpenoids produced by trees of the meliacae family, and are the bitter principles of citrus fruits. The lactone (**15**) isolated from *Carapa procera* bark,[18] has a structure which shows some similarity to austin, especially the spiro-lactone ring system and the olefinic methyl of the B ring. The rearranged spiro-lactone could presumably be formed from an α,β-unsaturated ε-lactone, followed by Michael addition of the 7α-hydroxy group to the conjugated system, and indeed, a number of liminoids have this ring A ε-lactone structure *e.g.*, obacunol (**16**).[19]

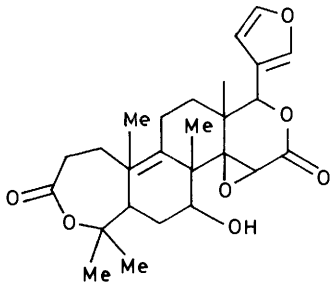
A fungal metabolite which also has this spiro-lactone system, is andibenin B (**17**), isolated from *Aspergillus variecolor*.[20,21] Initially a sesterterpenoid origin was also proposed for andibenin B, but subsequent incorporation experiments[22] with ^{13}C labelled precursors indicated a mixed polyketide-terpenoid

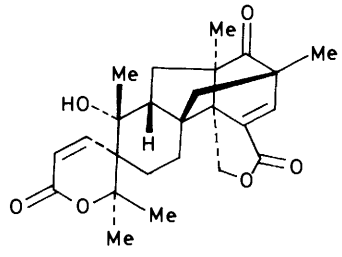










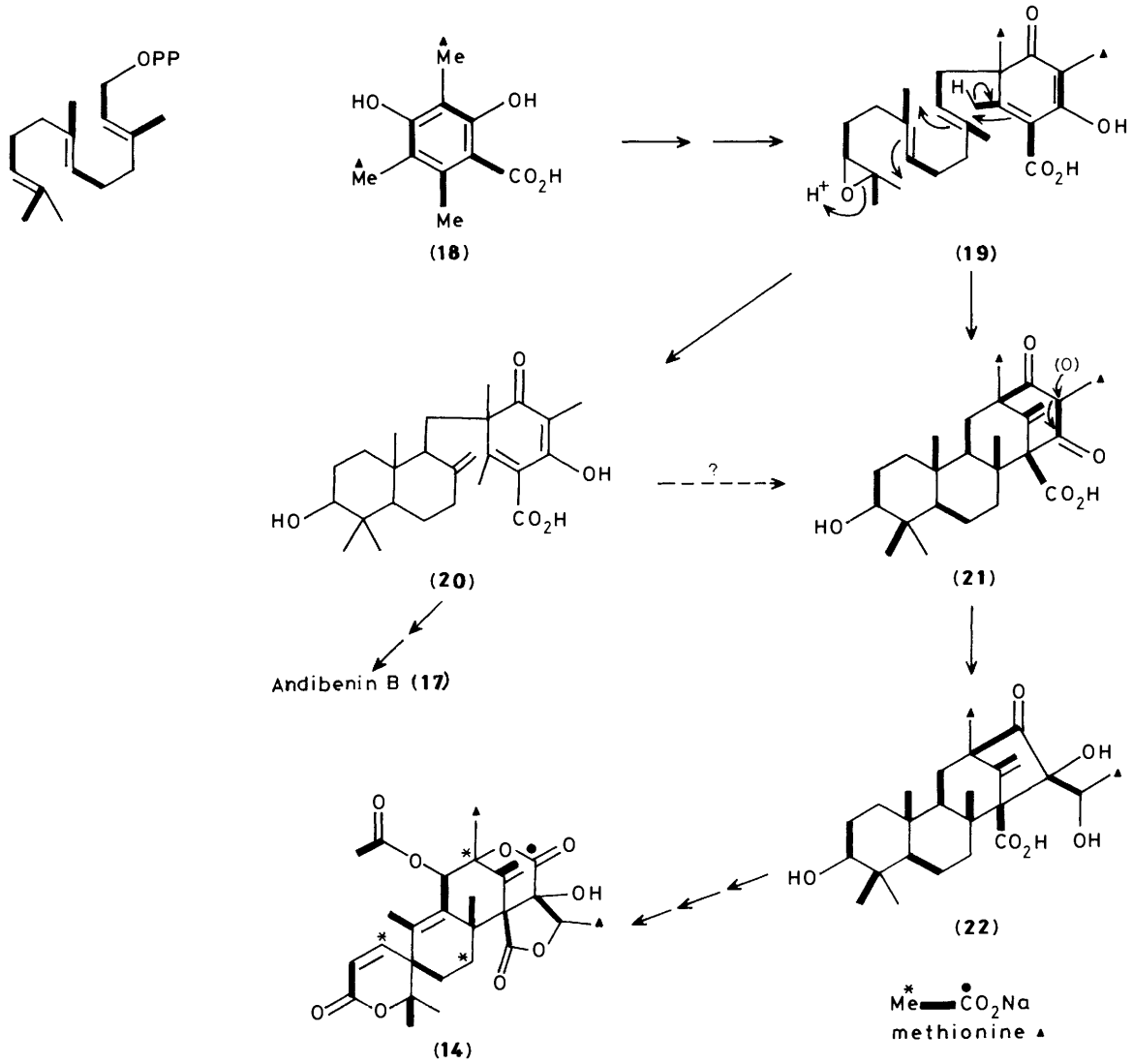


biogenesis of the autocystins from a C_{20} polyketide precursor, is the co-occurrence,[9] albeit in minor amounts, of versicolorin C, averufin, and sterigmatocystin. More recently,[12] Steyn reported the isolation of a further four xanthones, related to autocystin A, and an anthraquinone 8-deoxy-6-*O*-methylversicolorin A (**12**) from a toxigenic strain of *A ustus*.

In 1980, another biologically active metabolite pergillin (**2**) was isolated from a strain of *A. ustus* found on *Pisum sativa*.[13] Pergillin was shown to have moderate plant growth inhibiting properties. The metabolite can be formally biosynthesized by alkylation of a pentaketide by dimethylallyl pyrophosphate, as shown in Scheme 1.

More recently, the austalides, *e.g.* (**13**), have been isolated from *A. ustus*.[14] They represent a new structural type among mycotoxins, and seem to be related to 6-farnesyl-5,7-dihydroxy-4-methylphthalide, which is a known intermediate in the biosynthesis of mycophenolic acid.[15]

(meroterpenoid) origin in which the key step involved alkylation of a bis-*C*-methylated tetraketide *e.g.*, 3,5-dimethylorsellinic acid (**18**) by farnesyl pyrophosphate followed by epoxidation to give the intermediate (**19**) which could cyclise to (**20**) and undergo further elaboration to produce andibenin B as indicated in Scheme 3. As austin has many structural similarities to andibenin B, a common biosynthetic origin seemed feasible. The key intermediate (**19**) in andibenin B biosynthesis could also be transformed into austin as shown in Scheme 3. Cyclisation would produce the tetracyclic structure (**21**) and further addition of oxygen and ring contraction yields the cyclopentanone (**22**). Modification of the tetraketide portion is completed by insertion of oxygen and γ-lactone ring formation. Elaboration of the farnesyl moiety to the spiro-lactone system and introduction of the acetoxy moiety completes the biosynthesis. We now report details of incorporation studies with precursors labelled with a variety of stable isotopes which



Scheme 3.

Table 1. Incorporation of [2-^{14}C]acetate[a] into austin

Addition time (h)	Mass of austin isolated (mg)	Specific activity $\times 10^5$ (d.p.m./mmol)	Dilution
36	43	6.17	83
50	52	7.12	72
60	50	8.48	60
72	56	8.82	58
79	56	7.8	65
Pulse fed at 60, 72 h	59	7.5	68

[a] In all cases 0.5 g (10 μCi, 3.64 × 10^6 d.p.m./mmol) was fed.

confirm these proposals and provide information on the mechanisms of the biosynthetic transformations.[23]

A necessary prerequisite of biosynthetic studies using stable isotopes is the unambiguous assignment of the ^{1}H and ^{13}C n.m.r. spectra; for austin these have been reported already.[24] A second requirement is to establish conditions for the efficient incoporation of labelled precursors. The original isolation of austin was achieved in low yields from a semi-solid medium unsuitable for biosynthetic experiments.[16] Detailed investigations showed that austin could be produced in high and reproducible yields on a medium consisting of malt extract and mycological peptone. In this medium austin production was observed after 4 days growth and reached a maximum (*ca.* 110 mg l^{-1}) after 12 days growth. Preliminary incorporation experiments with [^{14}C]acetate indicated an optimum time for addition of precursor at 72 h after innoculation (Table 1) resulting in a dilution of acetate into austin of *ca.* 60, an acceptable level for observation of ^{13}C-enrichments.[25]

Singly and doubly ^{13}C-labelled acetates were accordingly fed to *A. ustus* and the ^{1}H noise decoupled (p.n.d.) ^{13}C n.m.r. spectra of the resulting austins showed the ^{13}C–^{13}C couplings and ^{13}C enrichments summarised in Table 2. Analysis of the normalised[26] line intensities revealed that only two carbons, C-9′ and C-10′, showed no enrichment, a result in agreement with the proposal of a bis-*C*-methylated tetraketide-derived phenolic precursor. In the phenol (**18**) these methyls would be derived by *C*-methylation *via* *S*-adenosylmethionine. [*Methyl*-^{13}C]Methionine was subsequently fed to *A. ustus* and the ^{13}C n.m.r. spectrum of the isolated austin showed the C-9′ and C-10′ resonances to be highly enriched. These enrichments and the

Table 2. ^{13}C Enrichments and coupling constants observed in p.n.d. ^{13}C n.m.r. spectrum of austin enriched from singly and doubly ^{13}C-labelled acetates and methionine

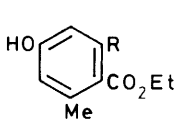
Carbon	δ_C(p.p.m.)	$^1J_{^{13}C-^{13}C}$ (Hz)	Enrichment	Enrichment level (atom %)
1	146.5	—	*	0.4
2	120.2	66	•	1.8
3	163.6	67	*	0.8
4	85.5	42	`	1.1
5	46.6	34	*	0.3
6	27.0	34	•	1.1
7	26.5	—	*	1.1
8	42.1	34	•	1.5
9	132.6	46.5	*	0.7
10	143.8	43	`	1.1
11	74.7	47.5	`	1.5
12	23.5	35	*	0.9
13	15.4	44	*	0.8
14	22.4	43	*	0.8
15	25.9	—	*	0.9
1′	118.0	76	*	0.7
2′	137.5	76	•	0.8
3′	84.1	—	*	0.5
4′	170.1	—	`	0.5
5′	78.7	34	*	0.3
6′	80.6	34	`	0.5
7′	62.8	54	*	0.2
8′	170.8	54	•	0.7
9′	20.2	—	†	20
10′	11.3	—	†	20
CH_3CO	168.4	60	•	0.9
CH_3CO	20.6	61	*	0.6

• [1-^{13}C]Acetate enriched. * [2-^{13}C]Acetate enriched. † [*Me*-^{13}C]Methionine enriched.

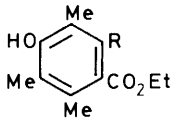
observed ^{13}C–^{13}C couplings are entirely consistent with the overall pathway summarised in Scheme 3.

In contrast to andibenin B (**17**) where the carbon skeleton of the proposed phenolic precursor remains intact, the pathway to austin necessitates an unprecedented degree of modification of the tetraketide-derived precursor, and so further verification of the obligatory intermediacy of such a precursor was sought.

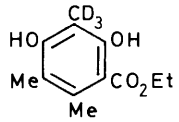
In the case of andibenin (**17**), a number of tetraketide derived



(**23**) R = OH
(**24**) R = H



(**25**) R = OH
(**26**) R = H



(**27**)

phenolic carboxylic acids could be postulated as precursors. In order to gain information on this, a series of ^{14}C labelled compounds (**23**)—(**26**) and the trideuteriomethyl compound (**27**) were synthesized,[27] and their incorporations studied.[28] Analysis of the andibenins isolated from these experiments indicated that significant radioactivity from (**25**) and (**26**) was incorporated into the metabolite, whereas (**23**) and (**24**) showed no significant incorporation. Thus *C*-methylation of the tetraketide occurs prior to aromatisation, in contrast to introduction of the farnesyl moiety, which occurs after aromatisation. However, both the dihydric (**25**) and monohydric (**26**) phenols were incorporated with comparable efficiency (specific incorporations of 1.07 and 0.43% respectively), suggesting that both can be utilised as precursors. In the feeding experiment with (**27**), the ^2H n.m.r. spectrum of the isolated andibenin B, showed only one signal at 1.00 p.p.m., corresponding to the 10′-methyl group.[23] 3,5-Dimethylorsellinic acid (**18**) is thus a specific precursor of andibenin B. It is possible that the observed incorporation of (**26**) could be *via* hydroxylation to give (**25**). However, ^{18}O-labelling studies described below indicate that C-6′ hydroxy group in austin is derived from the C-6 phenolic hydroxy group of 3,5-dimethylorsellinate. Thus, the observed incorporation of label from (**26**) probably proceeds mainly *via* degradation to give labelled acetate and reincorporation.

To gain information on the possible phenolic precursors of austin, ^{14}C-labelled (**25**) and (**26**) were similarly fed to cultures of *A. ustus*. These were incorporated with overall dilution values of 21 and 2 300 respectively. The low dilution of (**25**) indicates its intact incorporation into austin, and its role as a precursor. The very high dilution of (**26**) however, suggests that it is not incorporated intact into austin. These results are also consistent with the proposed pathway, as the presence of the hydroxy group in the position *ortho* to the carboxylate function, is necessary for the subsequent rearrangement of the tetraketide portion of intermediate (**21**) (see below). This hydroxy group might also be involved in the second alkylation of the tetraketide by the farnesyl moiety. The fact that some radioactivity is detectable in the austin isolated from the experiment with (**26**), can be explained as for andibenin B by degradation of the 3,5-dimethylorsellinate to acetate, which can then be incorporated into austin.

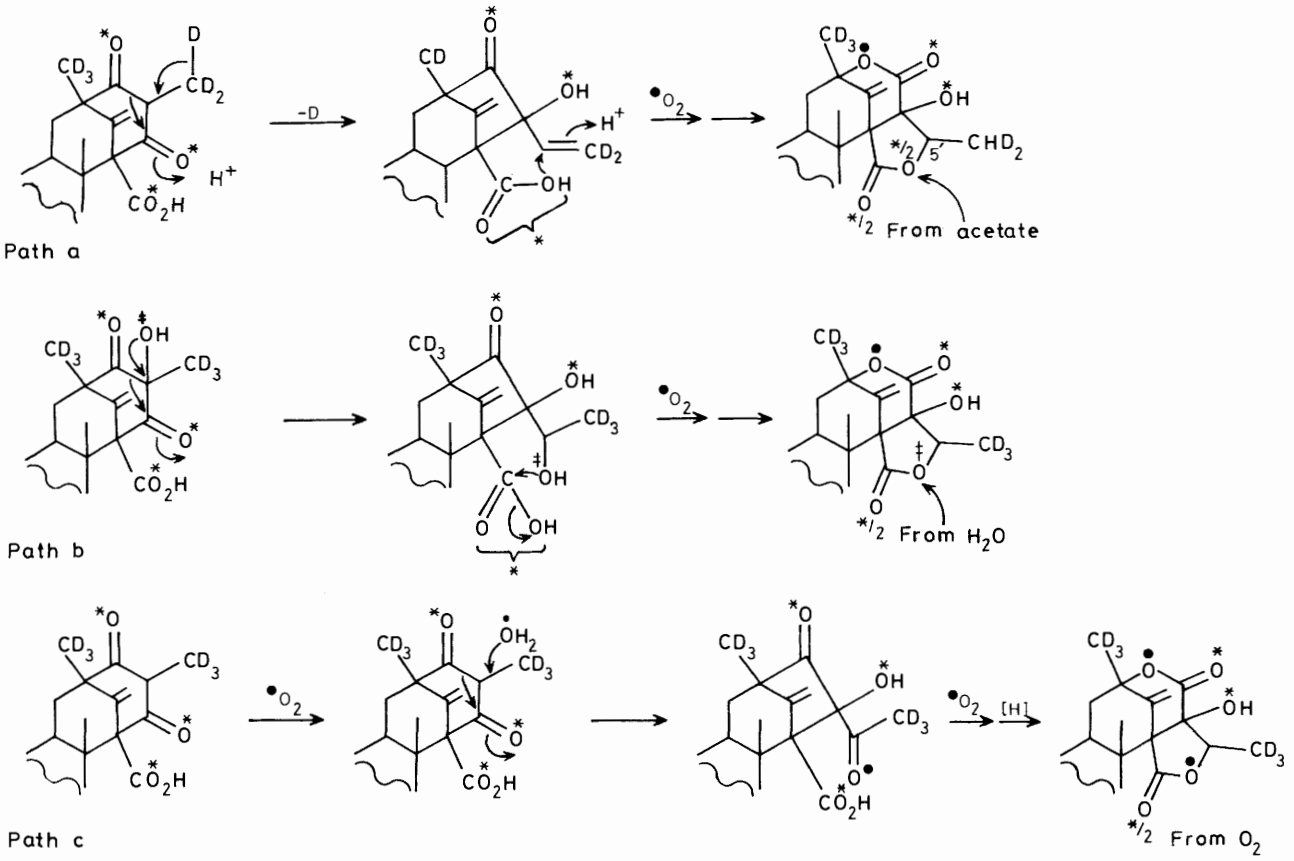
The trideuteriomethyl 3,5-dimethylorsellinate (**27**) was also fed to the culture, and austin subsequently isolated. The ^2H n.m.r. of this austin sample showed only one resonance at 1.29 p.p.m., corresponding to the 10′-methyl group, thus establishing 3,5-dimethylorsellinic acid (**18**) as a specific precursor of austin.

Having established 3,5-dimethylorsellinate (**18**) and farnesyl pyrophosphate as specific precursors, there are still a number of possible mechanisms for elaboration of the final austin structure. In particular, the cleavage and rearrangement of the tetraketide portion of intermediate (**21**) and subsequent cyclisation to the γ-lactone ring, can occur in a number of ways, as shown in Scheme 4.

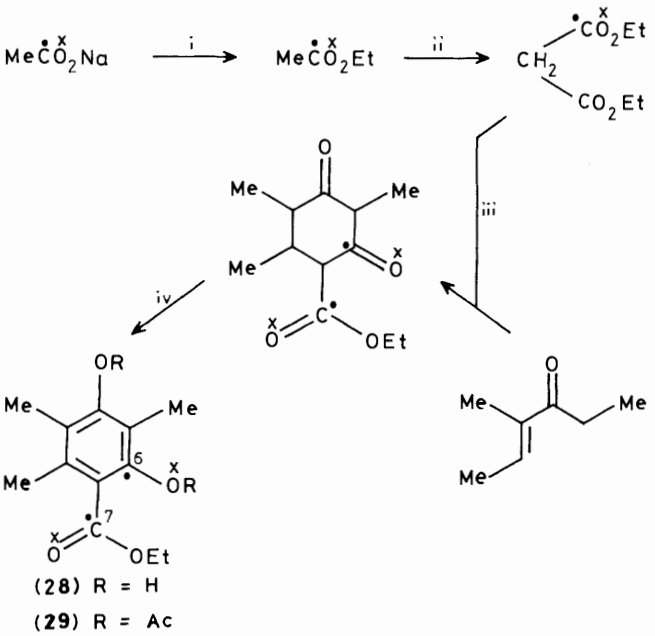
Path a involves an acid-catalysed rearrangement resulting in ring contraction and formation of an ethylidene system, by loss of a proton at C-10′. Formation of the γ-lactone would then proceed by protonation of the double bond, and subsequent attack by the carboxy group on the resulting carbocation. Path b again involves acid catalysed rearrangement, followed by ring contraction, hydration of the intermediate carbocation and lactone formation by attack of the hydroxy oxygen on the carboxyl. Path c proceeds by hydroxylation at C-5′, followed by an α-ketol rearrangement,[29] and reduction of the resultant ketone, with lactone formation occurring as in b by attack of the hydroxy group on the carboxy group.

Formation of the alkene system in path a would necessitate the loss of hydrogen at C-10′, and subsequent reprotonation. A feeding experiment with [*methyl*-$^{13}C^2H_3$]methionine was therefore carried out, and the labelled austin was subjected to mass spectral analysis. Prominent $M + 4$, and $M + 8$ peaks were observed, indicating incorporation of [$^{13}C^2H_3$]-labelled methyl from methionine, without loss of deuterium, thereby ruling out path a as a viable mechanism.

Paths b and c can also be distinguished by determination *inter alia* of the origin of the C-5′ oxygen. If path b operates *in vivo*, then the C-5′ oxygen would originate from the aqueous medium. In path c however, the hydroxylation of C-5′ would be mediated by a mixed function oxygenase, which would incorporate atmospheric oxygen into the metabolite. So, in order to gain information on the origin of the C-5′ oxygen and



Scheme 4.



Scheme 5. *Reagents:* i, $PO(OEt)_3$; ii, $LiN(SiMe_3)_2$, THF, −78 °C, $ClCO_2Et$; iii, NaOEt, EtOH; iv, Br_2, CCl_4

the other eight oxygen atoms in austin, complementary experiments using ^{18}O-labelled precursors were carried out.[30]

According to the mechanisms outlined in Scheme 4, the C-4′ and C-8′ carbonyl and the C-6′ tertiary alcohol oxygens should be derived from acetate. However, attempted incorporation of [1-^{13}C,$^{18}O_2$]acetate into austin to confirm this resulted in low overall incorporation, and the only enrichment that could be reliably observed was at the C-11 acetate carbonyl which showed an isotope induced shift of 0.038 p.p.m. consistent with incorporation of ^{18}O-label into the doubly-bonded oxygen.[30]

In austin and related metabolites, it has been consistently observed that 3,5-dimethylorsellinate is incorporated more efficiently than acetate. Thus, in order to obtain information on the fate of the oxygen atoms in this precursor, its synthesis in double ^{13}C,^{18}O-labelled form was undertaken. The route used (Scheme 5) was based on the previously described synthesis of ^{14}C- and ^{2}H-labelled 3,5-dimethylorsellinate,[27] using ^{13}C,^{18}O-labelled diethyl malonate to introduce label at either the C-7 carbonyl or C-6. Sodium [1-^{13}C,$^{18}O_2$]acetate was converted into ethyl acetate,[31] which on treatment with lithium bistrimethylsilylamide and ethyl chloroformate gave diethyl [1,3-^{13}C; 1,3-^{18}O]malonate.[32] Subsequent condensation with 4-methylhex-4-en-3-one and aromatisation gave a 48% yield of the desired 3,5-dimethylorsellinate. Owing to the symmetrical nature of diethyl malonate the ^{13}C,^{18}O-labelled bond becomes equally randomised between the C-6 phenolic bond and the carbonyl group of the ester. This was clearly seen in the p.n.d. ^{13}C n.m.r. spectrum of the double labelled 3,5-dimethylorsellinate in which C-6 and C-7 show ^{18}O-isotope induced shifts of 0.010 and 0.033 p.p.m. respectively. The corresponding diacetate (**29**) showed ^{18}O-isotope shifts of 0.021 and 0.037 p.p.m. for the corresponding carbons. Mass spectral analysis indicated that 73% of the molecules contained both ^{13}C and ^{18}O.

On incorporation into austin,[23] the ^{13}C n.m.r. spectrum of the enriched metabolite showed isotope shifts for both the C-6′ and C-8′ resonances (Table 3) and established the origin of the 6′-hydroxy and C-8′ carbonyl oxygen from 3,5-dimethylorsellinate.

Table 3. ^{18}O Isotopically shifted resonances observed in the 100.6 MHz ^{13}C n.m.r. spectrum of austin

Carbon	δ_C(p.p.m.)	$\Delta\delta_C$(p.p.m.)	Ratio ^{16}O:^{18}O
8′	170.8	0.012	55:45
		0.038[b]	65:35
4′	170.2	0.013	57:43
MeCO	168.4	0.014	56:44
		0.038[a]	85:15
3	163.6	0.010, 0.037, 0.047	45:13:11:33
4	85.6	0.043	67:33
3′	84.4	0.038	60:40
6′	80.8	0.008	63:37
		0.020[b]	37:63
5′	78.9	0.031	66:34
11	74.9	0.027	67:33

[a] Enriched by sodium [1-^{13}C,$^{18}O_2$]acetate; [b] Enriched from ethyl [^{13}C,^{18}O]-3,5-dimethylorsellinate; all others enriched by $^{18}O_2$.

The relative sizes of the isotopically-shifted and non-shifted signals show that there is no loss of label from C-6 of (**28**) on incorporation. However, approximately half the ^{18}O label is lost from C-7 of (**28**). This is consistent with the formation of a free carboxylate at some stage of the biosynthesis followed by nucleophilic attack of the 5′-hydroxy group on C-8′ to form the γ-lactone as indicated in Scheme 4, path c.

To establish which oxygen atoms were incorporated *via* oxidative processes, *A. ustus* was grown in a closed system containing an atmosphere of 30% $^{18}O_2$ (96% isotopically pure) and 70% of N_2. It was necessary to pump the atmosphere through the system, as a previous culture, with a static, sealed atmosphere had ceased growth after a few days. The cessation of growth, was presumably due to the formation of a 'blanket' of CO_2, produced by the organism's respiration, which led to effective oxygen starvation. As any atmospherically derived oxygen sites in the metabolite would theoretically be subject to 96% ^{18}O incorporation, an equal amount of unlabelled austin was added to the $^{18}O_2$ derived sample, to provide an internal reference for any isotopically shifted signals, in the ^{13}C n.m.r. spectrum. Table 3 lists the observed ^{18}O-isotopically shifted resonances in the ^{13}C n.m.r. spectrum.

In the carbonyl region, all four resonances exhibit ^{18}O shifted signals: C-8′, C-4′, and the acetyl carbonyl show comparable shifts of 0.012, 0.013, and 0.014 p.p.m. respectively, which are of the correct order of magnitude for ^{18}O only in the singly-bonded oxygen atoms. However, C-3 shows three isotopically shifted signals of 0.010, 0.037, and 0.047 p.p.m. These signals are due to the three possible situations, where C-3 has: *a*, an ^{18}O-labelled singly-bonded oxygen; *b*, an ^{18}O-labelled carbonyl oxygen; and *c*, has both attached oxygens ^{18}O-labelled. Both oxygens of the spiro-lactone ring, are therefore derived from atmospheric oxygen.

In the sp^3-oxygen bearing carbon region of the spectrum, again all carbon resonances show isotopically shifted signals. C-4, C-3′, C-5′, and C-11 show shifts of 0.043, 0.038, 0.031, and 0.027 p.p.m., respectively, indicating the presence of directly bonded bridging $^{18}O_2$-labelled oxygens. These results are in agreement with those obtained from the carbonyl region, and confirm the presence of five ^{18}O-labelled bridging oxygens in $^{18}O_2$ derived austin. The additional small isotope shift observed for the C-6′ resonance was unexpected. The 6′-hydroxy has already been shown to be derived from ethyl 3,5-dimethylorsellinate (**28**). The magnitude of the shift (0.008 p.p.m.) in the $^{18}O_2$ experiment, is unusually small for a tertiary alcohol, which is normally of the order 0.035—0.030 p.p.m.[30] The most probable explanation is that it is the result of a β-shift from the C-5′ bridging oxygen, or the C-3′ bridging oxygen, or a double β shift from both.

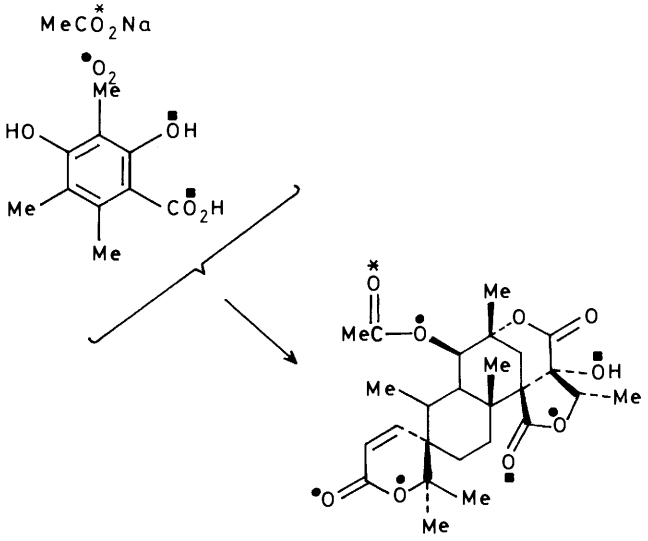
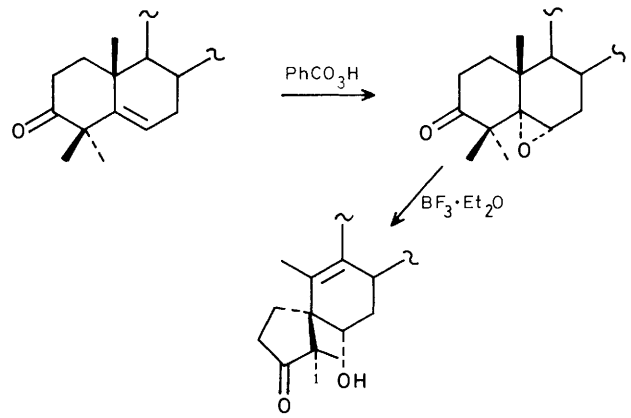


Figure 1. Biosynthetic origins of the oxygen atoms in austin, determined by ^{18}O-labelling studies

The ^{13}C n.m.r. spectrum of $^{18}O_2$-derived austin, therefore indicates the incorporation of five ^{18}O atoms, in the positions indicated in Figure 1. The observation that the C-5′ oxygen is derived from the atmosphere means that of the mechanisms for rearrangement of the tetraketide precursor shown in Scheme 4, path b is no longer feasible and the labelling results are consistent only with path c.

Turning to the farnesyl portion of the metabolite, the major modification of the cyclised decalin system is the elaboration of the spiro-lactone ring. The formation of the spiro-system



Scheme 6.

necessitates a C-1→C-10 bond migration. The most feasible mechanism for this would entail the generation of some formal carbocation ion character at C-5.

Similar bond migrations have been induced, *in vitro*, in steroid derivatives.[33,34] Scheme 6 illustrates one possible mechanism where the 5,6 bond is epoxidised, and subsequent Lewis acid catalysed rearrangement results in the formation of a spiro-cyclopentanone structure. The spiro-lactone system of austin could conceivably be biosynthesized in a similar fashion, through desaturation between C-5 and C-6, epoxide initiated rearrangement, and Baeyer-Villiger oxidation of the resulting cyclopentanone, yielding the spiro-lactone structure.

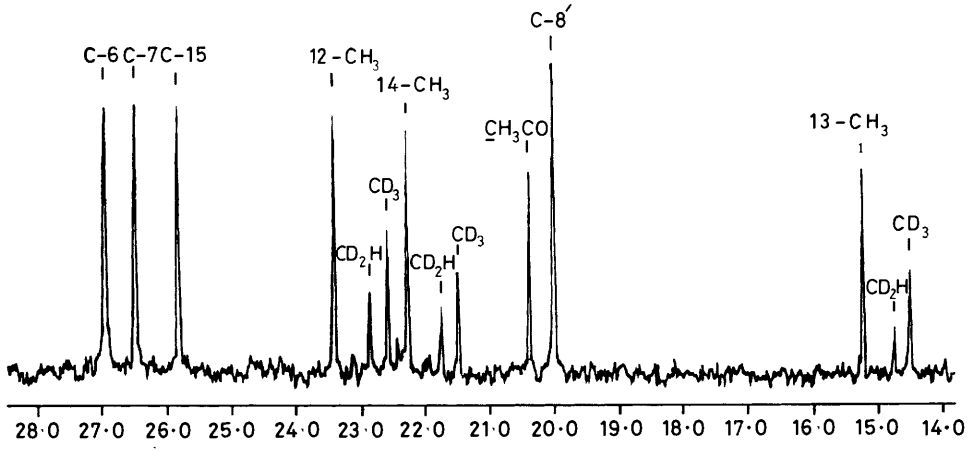
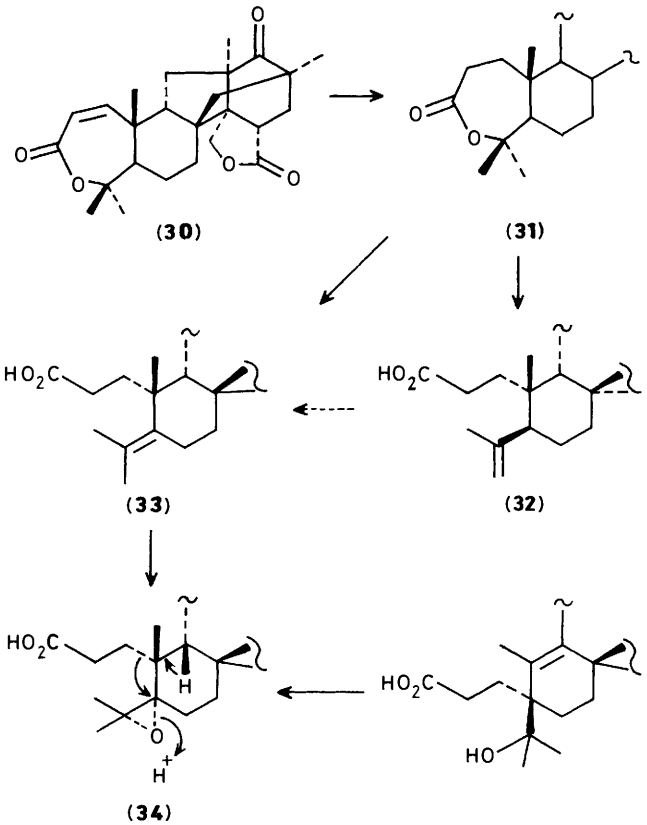


Figure 2. 100.6 MHz ^1H and ^2H noise decoupled ^{13}C n.m.r. spectrum of austin enriched from [6-^{13}C,6-$^2H_3$]mevalonic acid lactone

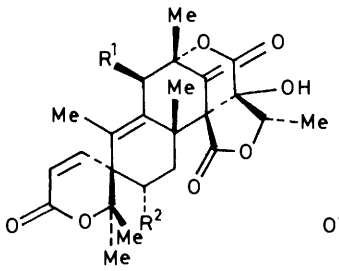
However, a common biosynthetic origin for andibenin and austin, suggests that their spiro-lactone systems are formed in a similar manner. The andilesins, *e.g.*, andilesin C (**30**), are co-metabolites of the andibenins[21] and possible biosynthetic precursors to them. They have an α,β-unsaturated ε-lactone A ring, from which the spiro-lactone system of andibenin is presumably derived. It is known[21] that mild acid treatment of



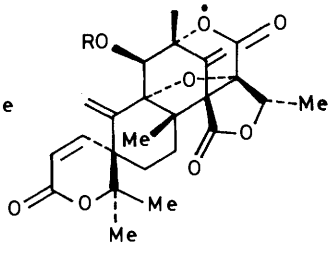
Scheme 7.

dihydroandilesin C (**31**) results in ring opening and formation of the olefinic system of acid (**32**), as shown in Scheme 7. It is conceivable therefore, that *in vivo*, the enzymatic opening of the ε-lactone could result in the formation of a Δ^4 double bond in compound (**33**) as illustrated in Scheme 7. Subsequent acid-catalysed ring opening of a derived 4,5-epoxide (**34**) as shown, would create the necessary carbocation character at C-5 to induce bond migration. Loss of a proton at C-11 would yield the necessary olefinic methyl system on the B ring, while condensation of the acid and hydroxy functions would yield the spiro-lactone ring. Finally, enzymatic desaturation at C-1 and C-2 would yield the α,β-unsaturated spiro-lactone structure. It should be noted that the ortho-ester moiety present in ring A of the austalides could also be formed *via* the diol resulting from an analogous 4,5-epoxide.

The oxygen atom at C-11 is also introduced from the atmosphere, pesumably by allylic oxidation, followed by acetylation with acetyl CoA to give the acetoxy moiety. This may well be a late step in the biosynthesis as the corresponding alcohol (**35**) has been isolated as a co-metabolite of austin (see below).



(**35**) R^1 = OH, R^2 = H
(**38**) R^1 = H, R^2 = OAc



(**36**) R = Ac
(**37**) R = H

In Scheme 3, it has been proposed that the tetracyclic intermediate (**21**) is formed directly from cyclisation of (**19**). An alternative possibility is that (**21**) is formed in a two-step process *via* the same bicyclofarnesyl intermediate (**20**) involved in andibenin biosynthesis. If this was the case then the 12-methyl group could only retain two mevalonate-derived hydrogens whereas the concerted pathway would result in retention of all three mevalonate hydrogens at this position. This has been tested by the synthesis of [6-^{13}C,6-$^2H_3$]mevalonate by a previously described route[35] from [2-^{13}C,$^2H_3$]acetate. The labelled precursor was fed to 5-day old cultures of *A. ustus*, the resultant enriched austin was isolated after a further 7 days growth, and its ^1H, ^2H noise decoupled ^{13}C n.m.r. spectrum (Figure 2) determined. This showed isotopically shifted signals corresponding to the incorporation of two and mainly three deuteriums into each of the 12-, 13-, and 14-methyls. These

results therefore exclude the possibility of the bicyclic intermediate (**20**) in austin biosynthesis.

A number of compounds structurally related to austin have been isolated from *A. ustus* and from a number of other organisms. Austinol (**35**) and dehydroaustin (**36**) are co-metabolites isolated from *A. ustus*. Dehydroaustin has also been isolated[24] from a mutant strain of the andibenin-B producing strain of *Aspergillus variecolor* that no longer produces andibenin B. This provides further evidence for a close biosynthetic relationship between the andibenin and austin-type compounds. Austinol has also been isolated along with isoaustin (**38**) from cultures of *Penicillium diversum*.[24] Finally, dehydroaustinol (**37**) has been isolated from *Emericella dentata* along with austinol.[36] Interestingly, although the absolute configurations of all these compounds remain to be determined, the metabolites of *E. dentata* appear to be enantiomeric to those from the other austin-producing organisms.

The isolation of these closely related metabolites from a variety of different fungal species indicates that this biosynthetic pathway may be more common than was first realised. Another noteworthy feature is the co-occurrence of two different major groups of meroterpenoids, the austins and austalides in the same organism.

Experimental

General Procedures and Instrumentation.—A Varian DMS 90 spectrophotometer was used to obtain u.v.–vis. spectra; baseline correction for solvent absorption was carried out. I.r. spectra were taken on a Perkin-Elmer 781 spectrophotometer and referenced against the polystyrene absorption at 1 601 cm^{-1}. ^1H N.m.r. spectra were obtained from various instruments: Varian EM360 and HA 100 continuous-wave machines and Bruker WP 80 SY, WP 200 SY, WM/WB 300, and WH 360 Fourier-transform machines. ^{13}C N.m.r. spectra were obtained from: Varian XL 100 and CFT 20 and Bruker WP 200 SY, WH 360 and WH 400 Fourier-transform machines. ^2H N.m.r. spectra were obtained on a Bruker WH 360, operating in this case without a frequency lock. In all cases, quoted chemical shifts are relative to tetramethylsilane δ_H and $\delta_C = 0.0$ p.p.m. Mass spectra and exact mass determinations were taken on a A.E.I. MS 902 high-resolution instrument, ionising by electron impact. Peak intensities are expressed as percentages relative to the base peak at 100%.

Radiocounting was carried out using a Beckman LS 7000 liquid scintillation counter, operating on program 4 without automatic quench correction. Counting efficiency was determined by using both standard channels ratio and H-number quench curves. The scintillant was butyl-PBD (10 g l^{-1}) in methanol–toluene (50:50). Samples for radiocounting were purified to constant activity by recrystallisation and dissolved in either methanol or toluene.

Unless otherwise specified, thin layer chromatography was performed using either analytical (5 × 20 cm) or preparative (20 × 20 cm) glass plates coated with a 0.5 mm layer of silica-gel (Merck Art. 7730 Kieselgel 60 GF_{254} or Fluka AG 60765 Kieselgel GF 254). U.v. light of wavelength 254 nm was used to visualise chromatograms.

Solutions for feeding were sterilised by autoclaving at 15 p.s.i. for 15 min. Dry solvents were obtained by standard procedures.[37]

Isolation of Austin.—*Aspergillus ustus* (NRRL 6017) was grown in static culture, at 25 °C, in penicillin pans or 500 ml conical flasks containing 200 ml or 100 ml respectively of medium, consisting of Oxoid malt extract (3.0%) and Oxoid mycological peptone (0.5%) in distilled water (pH = 5.4). The culture was incubated for 14 days, after spore innoculation, then the medium was separated from the mycelium, and extracted with ethyl acetate to give 350 mg of crude organic extract per litre. The extract was then subjected to t.l.c. separation, with 4% MeOH in $CHCl_3$ as the eluant. Removal of the u.v. quenching band at R_F 0.5–0.6 gave *austin* (110 mg l^{-1}).

Table 4. Austin production with time of incubation

No. of days incubated	2	3	4	5	6	7	8	9	10	11	12	13	14
austin (mg)	4	6	9	16	35	42	58	79	92	111	115	109	110

Table 5. Incorporation of ^{13}C-labelled precursors into austin

Precursor	Amount (No of Flasks)	Austin (mg)
[1-^{13}C]Acetate	250 mg (2)	36
[2-^{13}C]Acetate	200 mg (2)	41
[1,2-^{13}C]Acetate	100 mg (2)	40
[*methyl*-^{13}C]Methionine	100 mg (2)	38
[*methyl*-^{13}C, $^2H_3$]Methionine	100 mg (2)	41
[1-^{13}C,$^{18}O_2$]Acetate	600 mg (2)	48

Austin Production—Time Study.—*A. ustus* was incubated for 14 days as described above. Three penicillin flasks were harvested at 2 day intervals and the austin isolated as summarised in Table 4.

Incorporation of [^{14}C]*Acetate into Austin.*—[2-^{14}C]Acetate (10 μCi) was added to 0.5 g unlabelled sodium acetate, dissolved in 18 ml of distilled water, and distributed among 3 penicillin flask cultures, at various times after initial innoculation. The specific activity of precursor in all cases was 3.64×10^6 d.p.m./$mmol^{-1}$, and the results are summarised in Table 1.

Incorporation of ^{13}C, ^2H, *and* ^{18}O *Labelled Acetates into Austin.*—Penicillin flasks each containing malt extract peptone medium (200 ml) were innoculated with a spore suspension of *A. ustus*. Later (72 h), precursors were added in the amounts and to the number of flasks indicated in Table 5. After 14 days of incubation at 25 °C the labelled austins were isolated as described above.

Incorporation of ^{14}C-*Labelled Ethyl* 3,5-*Dimethylorsellinates* (**25**) *and* (**26**) *into Austin.*—Penicillin flasks containing 200 ml medium were innoculated with a spore suspension of *A. ustus*. After 73 h ^{14}C-labelled ethyl 3,5-dimethylorsellinate (**25**) (24 mg, 873×10^7 d.p.m./mol^{-1}) and ethyl 6-deoxy-3,5-dimethylorsellinate [(**26**), 30 mg, 873×10^7 d.p.m./$mmol^{-1}$] were administered as Tween solutions to 2 and 3 flasks respectively. After a total incubation time of 14 days, austins (36 mg, 424×10^6 d.p.m./$mmol^{-1}$) and (24 mg, 38×10^4 d.p.m./$mmol^{-1}$) were isolated. These corresponding dilutions of 20.6 and 23 000 and incorporations of 7.95% and 0.015% respectively.

Incorporation of Ethyl [methyl-$^2H_3$]-3,5-*Dimethylorsellinate into Austin.*—Ethyl [*methyl*-$^2H_3$]-3,5-dimethylorsellinate (53, 50 mg) was fed to 5 penicillin flasks 73 h after innoculation. Austin (90 mg) was isolated after a further 11 days growth.

Ethyl [1-^{13}C,$^{18}O_2$]*Acetate.*—Ethyl [1-^{13}C,$^{18}O_2$]acetate was prepared as previously described[31] from sodium [1-^{13}C,$^{18}O_2$]-acetate (1.04 g, 11.95 mmol; 90 atom % ^{13}C, 81 atom % $^{18}O_2$, 18 atom% $^{18}O_1$) and triethyl phosphate (3.9 ml). Ethyl [1-^{13}C,$^{18}O_2$] acetate (0.95 g, 10.22 mmol, 86%) was isolated after distillation.

Diethyl [1-^{13}C,^{18}O]*Malonate*.—Diethyl [1-^{13}C,^{18}O]-malonate was prepared[32] from ethyl [1-^{13}C,^{18}O$_2$]acetate (0.95 g, 10.22 mmol) and ethyl chloroformate (1.11 g, 10.22 mmol). The product (1.30 g), was analysed by g.l.c. (10% APL column, 136 °C). A purity of 84% was determined, therefore the corrected yield of diethyl [1-^{13}C,^{18}O$_2$]malonate was 1.10 g (6.60 mmol, 65%).

Ethyl [2,7-^{13}C-2-*Hydroxy* 7-*oxo*,^{18}O]-2,4-*Dihydroxy*-3,5,6-*trimethylbenzoate*.—The [^{13}C,^{18}O]-labelled 3,5-dimethylorsellinate was prepared as previously described[27] from diethyl [1-^{13}C,^{18}O$_2$]malonate (1.10 g, 6.67 mmol) and 4-methylhex-4-en-3-one (1.775 g, 6.92 mmol). The crude product was a black tarry residue which was purified by preparative t.l.c., with methanol–chloroform (4:96) as eluant. After isolating the major product, it was subjected to a second preparative t.l.c., with chloroform. Pale yellow crystals of the [^{13}C,^{18}O]-labelled 3,5-dimethylorsellinate (720 mg, 3.17 mmol, 48%, 88 atom % ^{13}C, 81 atom % ^{18}O, 73 atom % ^{13}C,^{18}O) were isolated. I.r. and ^{1}H n.m.r. spectroscopic data agreed with data from unlabelled samples; m.p. 90.5—91.5 °C; δ_H(80 MHz, $CDCl_3$) 1.40 (3 H, t, *J* 7.1 Hz, CH_2CH_3), 2.13 (6 H, s, 3- and 5-ArCH_3), 2.44 (3 H, s, 6-ArCH_3), 4.60 [2 H, q(d), *J* 7.1 Hz, ($^2J_{CH}$ 3.0 Hz) CH_2CH_3], 5.11 (1 H, br s, 4-ArO*H*) and 11.46 [1 H, s(d), $^2J_{CH}$ 4.4 Hz, 2-ArO*H*]; M *m/z* 227 (*M*$^+$, 33%), 181 (100), 153 (38), and 150 (54).

A small quantity of the [^{13}C,^{18}O]-labelled 3,5-dimethylorsellinate (5 mg, 0.022 mmol) was mixed with the unlabelled 3,5-dimethylorsellinate (232 mg, 1.036 mmol) and analysed by ^{13}C n.m.r. spectroscopy; δ_C(100 Hz, $CDCl_3$, ^{1}H decoupled) 8.0 (3-ArCH_3), 11.8 (5- or 6-ArCH_3), 14.3 (5- or 6-ArCH_3), 18.8 (CH_2CH_3), 61.3 (CH_2CH_3), 106.6 (C-1), 107.6 (C-3), 115.0 (C-5), 137.6 (C-6), 156.8 (C-4), and 159.7* (1-Ar$CO_2C_2H_5$).

The mixed sample (111 mg, *ca.* 0.50 mmol) was added to acetic anhydride (3 ml) and pyridine (6 drops), and the resulting solution stirred at room temperature for 66 h. The reaction was monitored by t.l.c., developed with methanol-chloroform (2:98), and by this time all the starting material had been consumed in the reaction. The reaction mixture was poured onto an ice–2M hydrochloric acid mixture, and extracted with chloroform (3 × 10 ml). The chloroform extracts were washed with 5% aqueous sodium hydrogen carbonate (2 × 10 ml), followed by water (2 × 10 ml). The organic extract was dried ($MgSO_4$), and, after removal of the solvent under reduced pressure, a yellow oil remained. On standing the oil crystallised to yield pale yellow crystals (141 mg). After recrystallisation from carbon tetrachloride the [^{13}C,^{18}O]-labelled diacetoxy-3,5-dimethylorsellinate (96 mg, approx. 0.31 mmol, 63%) was isolated. δ_H(80 MHz, $CDCl_3$) 1.33 (3 H, t, CH_2CH_3), 1.91 (3 H, s, 4-ArO_2CCH_3), 2.03 (3 H, s, 2-ArO_2CCH_3), 2.24 (6 H, s, 3- and 5-ArCH_3), 2.32 (3 H, s, 6-ArCH_3), 4.33 (2 H, q, CH_2CH_3); δ_C(100 MHz, $CDCl_3$, ^{1}H decoupled), 168.2 (2- or 4-Ar*O*C)CH_3), 167.0* (1-Ar$CO_2C_2H_5$), 149.4 (C-4), 145.0* (C-2), 133.6 (C-5 or C-6), 127.8 (C-5 or C-6), 125.9 (C-1 or C-3), 121.9 (C-1 or C-3), 61.3 (CH_2CH_3), 20.4 (2-or 4-ArOCOCH_3), 20.3 (2- or 4-ArOCOCH_3), 17.0 (CH_2CH_3), 13.2 (5- or 6-ArCH_3), 12.9 (5-or 6-ArCH_3), 10.3 (3-ArCH_3).

Incorporation of Ethyl 3,5-*Dimethylorsellinate into Austin*.—*Aspergillus ustus* was grown as previously described in six 500 ml conical flasks. A sterile solution of the [^{13}C,^{18}O]-labelled 3,5-dimethylorsellinate (88 mg, 0.39 mmol, 88 atom % ^{13}C, 80 atom % ^{18}O, 73 atom % ^{13}C,^{18}O) in Tween 80 detergent (1.5 ml) and distilled water (12 ml) was prepared. This solution was distributed equally between five flasks, three days after innoculation. After 14 days growth the experimental flasks were worked up and austin was isolated and purified as usual to give 33 mg (66 mg l^{-1}).

Butyl [2-^{13}C,^{2}H$_3$]*Acetate*.[29]— Sodium [2-^{13}C,^{2}H$_3$]acetate (1.5 g, 30 mmol) was mixed with tri-butyl phosphate (10 ml) and the mixture was heated under reflux for 5 h on an oil bath at 200—220 °C. The viscous mixture was cooled to room temperature, the upper end of the reflux condenser was sealed through a liquid nitrogen-cooled trap to a vacuum pump, and the product ester was distilled into the cold trap by heating the reaction flask to 100—160 °C for 2.5 h at 1 mmHg pressure with cold water running through the reflux condenser. Butyl acetate (3.4 g, 96%) was collected.

[6-^{13}C,6-^{2}H$_3$]*Mevalonic Acid Lactone*.—Butyl 2[-^{13}C,^{2}H$_3$]-acetate (1.4 g, 11.6 mmol) was converted into [6-^{13}C,6-^{2}H$_3$]-mevalonic acid lactone by the previously described method.[35] The lactone was isolated as a viscous oil (0.82 g, 6.31 mmol) which was essentially pure by ^{1}H n.m.r.

Incorporation of [6-^{13}C,6-^{2}H$_3$]*Mevalonic Acid Lactone into Austin*.—[6-^{13}C,6-^{2}H$_3$]Mevalonic acid lactone (350 mg) was dissolved in 2M sodium hydroxide (1.3 ml). After 5 min the solution was diluted to 10 ml and added after filter sterilisation to a 3-day culture of *A. ustus* (5 × 500 ml Erlenmeyer flask each containing 100 ml medium). After 14 days the culture filtrate was extracted in the usual way to give crude metabolite which was recrystallised from methanol to give pure austin (9 mg).

* Isotopically shifted signal also observed.

Acknowledgements

We thank the S.E.R.C., N.A.T.O., the Government of Iraq, and the Natural Sciences and Engineering Research Council of Canada for financial support.

References

1 H. Raistrick and C. F. Stickings, *Biochem. J.*, 1951, **48**, 53.
2 W. B. Turner, 'Fungal Metabolites,' Academic Press, London, 1971.
3 H. R. V. Arnstein and R. Bentley, *Biochem. J.*, 1953, **53**, 493.
4 P. S. Steyn, *Tetrahedron Lett.*, 1971, 3331.
5 P. S. Steyn, *Tetrahedron*, 1973, **29**, 107.
6 R. Vleggaar, P. S. Steyn, and D. W. Nagel, *J. Chem. Soc., Perkin Trans. 1*, 1974, 45.
7 P. S. Steyn and R. Vleggaar, *J. Chem. Soc., Perkin Trans. 1*, 1976, 204.
8 L. Colombo, C. Gennari, G. Severiniricca, C. Scolastico, and F. Aragozzini, *J. Chem. Soc., Chem. Commun.*, 1981, 575.
9 P. S. Steyn and R. Vleggaar, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2250.
10 T. Hamasaki, K. Matsui, K. Isono, and Y. Hatsuda, *Agric. Biol. Chem.*, 1973, **37**, 1769.
11 M. Biollaz, G. Buchi, and G. Milne, *J. Am. Chem. Soc.*, 1970, **92**, 1035.
12 R. M. Horak, P. S. Steyn, R. Vleggaar, and C. J. Rabie, S. *Afr. J. Chem.*, 1982, **35**, 60.
13 H. G. Cutler, *J. Agric. Food Chem.*, 1980, **28**, 989.
14 R. M. Horak, P. S. Steyn, R. Vleggaar, and C. J. Rabie, *J. Chem. Soc., Perkin Trans. 1*, 1985, 345.
15 L. Colombo, C. Gennari, and C. Scolastico, *J. Chem. Soc., Chem. Commun.*, 1978, 434.
16 K. K. Chexal, J. P. Springer, J. Clardy, R. J. Cole, J. W. Kirksey, J. W. Dorner, H. G. Cutler, and W. J. Strawter, *J. Am. Chem. Soc.*, 1976, **98**, 6748.
17 H. Kahn, A. Zaman, G. L. Chetty, A. S. Gupta, and S. Dev., *Tetrahedron Lett.*, 1972, 609.
18 A. F. Cameron and J. D. Connolly, *Tetrahedron Lett.*, 1979, 967.
19 D. L. Dreyer, *J. Org. Chem.*, 1968, **33**, 3577.
20 A. W. Dunn, R. A. W. Johnston, B. Sklarz, and T. J. King, *J. Chem. Soc., Chem. Commun.*, 1976, 170.
21 T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, 2118.
22 J. S. E. Holker and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1978, 626.

23 These results have been reported in preliminary form: T. J. Simpson and D. J. Stenzel, *J. Chem. Soc., Chem. Commun.*, 1981, 1042; C. R. McIntyre, T. J. Simpson, D. J. Stenzel, A. J. Bartlett, E. O'Brien, and J. S. E. Holker, *ibid.*, 1982, 781; T. J. Simpson, D. J. Stenzel, R. W. Moore, L. A. Trimble, and J. C. Vederas, *ibid.*, 1984, 1242; F. E. Scott, T. J. Simpson, L. A. Trimble, and J. C. Vederas, *ibid.*, 1986, 214.
24 T. J. Simpson, D. J. Stenzel, A. J. Bartlett, E. O'Brien, and J. S. E. Holker, *J. Chem. Soc., Perkin Trans. 1*, 1982, 2687.
25 T. J. Simpson, *Chem. Soc. Rev.*, 1975, **4**, 497.
26 J. S. E. Holker, R. D. Lapper, and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2135.
27 A. J. Bartlett, J. S. E. Holker, E. O'Brien, and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1983, 667.
28 A. J. Bartlett, J. S. E. Holker, E. O'Brien, and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1981, 1198.
29 S. Selman and J. F. Eastham, *Q. Rev.*, 1960, **14**, 221.
30 J. Diakur, T. T. Nakashima, and J. C. Vederas, *Can. J. Chem.*, 1980, **58**, 1311; J. C. Vederas, *ibid.*, 1982, **60**, 1637; *J. Am. Chem. Soc.*, 1980, **102**, 374; *Nat. Prod. Rep.*, 1987, **4**, 277.
31 G. A. Ropp, *J. Am. Chem. Soc.*, 1950, **72**, 2299.
32 E. Leete and M. E. Mueller, *J. Org. Chem.*, 1981, **46**, 3151.
33 T. G. Nalsall, E. R. H. Jones, E. L. Tan, and G. R. Chaudry, *J. Chem. Soc. C*, 1966, 1374.
34 A. M. Maione, I. Torrini, and A. Romeo, *J. Chem. Soc., Perkin Trans. 1*, 1979, 775.
35 E. Bardshiri, T. J. Simpson, A. I. Scott, and K. Shishido, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1765.
36 Y. Maebayashi, E. Okuyama, M. Yamazaki, and Y. Katsube, *Chem. Pharm. Bull.*, 1982, **30**, 1911.
37 A. I. Vogel, 'A Textbook of Practical Organic Chemistry,' 3rd Edition, Longmans, London, 1967.

Received 9*th June* 1988; *Paper* 8/02312C