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Biosynthesis of Polyketide-Terpenoid (Meroterpenoid) Metabolites Andibenin B and Andilesin A in *Aspergillus variecolor*†

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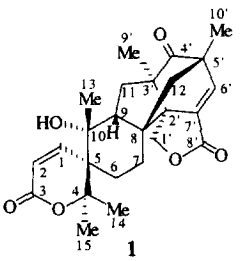
Abstract: Incorporation of ^{13}C-labelled acetates and methionine indicate that andibenin B (**1**) and andilesin A (**5**), C_{25} metabolites of *Aspergillus variecolor*, are biosynthesised via a mixed polyketide-terpenoid pathway. $^{18}O_2$-Labelling studies, and incorporation of aromatic precursors and mevalonic acids variously labelled with ^{13}C, ^{2}H and ^{18}O provide evidence for the extensive oxidative and other modifications involved in the elaboration of the highly oxygenated polycyclic structures found in these metabolites. The biosynthetic interrelationships among these and other complex meroterpenoid metabolites are discussed. © 1997 Elsevier Science Ltd. All rights reserved.

The X-ray structure and absolute stereochemistry of andibenin B (**1**), isolated from a UV-induced mutant strain (212K I69) of *Aspergillus variecolor* (syn. *Aspergillus stellatus*), were first reported in 1976.[1] Two related metabolites, named dihydroandibenin and deoxyandibenin, were also isolated, but were subsequently renamed andilesin A (**5**) and andilesin B (**4**) after X-ray crystallographic studies[2] established the skeletal differences between them and andibenin B, and spectroscopic and chemical studies revealed their structural relationship.[3] Four further compounds with obvious structural relationships were also isolated and characterised. These were andibenins A (**2**) and C (**3**)[3], andilesin C (**6**)[3] and anditomin (**7**).[4] The structures of this group of metabolites



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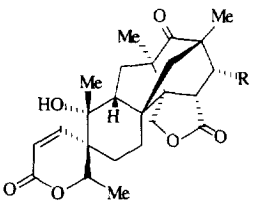

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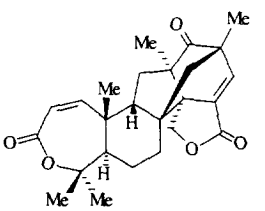

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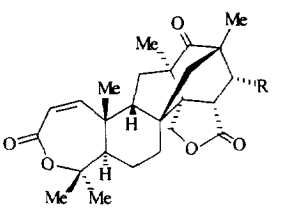

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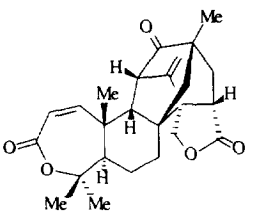

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† Dedicated to the memory of Professor Arthur J. Birch who provided both the original biogenetic principles and personal inspiration which contributed to these studies.

appeared to be consistent with a terpenoid origin and it was proposed[1,2] that they might constitute a new group of sesterterpenoids.[5] Indeed it is possible, allowing for biogenetically reasonable methyl and bond migrations with subsequent oxidative modifications, to rationalise the formation of these compounds from geranylfarnesyl pyrophosphate, and biosynthetic studies were initiated to test this proposal. We now report full details of the results of extensive incorporation studies, mainly with precursors labelled with stable isotopes, which demonstrate that andibenin B and the related metabolites are formed via a mixed polyketide-terpenoid pathway and and are not sesterterpenoid in origin.

Assignment of the ^{1}H and ^{13}C NMR Spectra of Andibenin B

In order to establish the biosynthetic origins of andibenin B it was planned to synthesise and incorporate a range of precursors appropriately labelled with ^{13}C, ^{2}H and ^{18}O. These studies required an exact assignment of both the ^{1}H and ^{13}C nmr spectra which, as far as possible, should be independent of biosynthetic results.

The 360MHz ^{1}H nmr spectrum of andibenin B showed a singlet at 7.04 ppm and doublets at 6.74 and 6.06 ppm which could be readily assigned to H-6', H-1 and H-2 respectively. Mutually coupled doublets at 4.24 and 4.84 ppm could be assigned to the diastereotopic methylene hydrogens at C-1', but the exact assignment to the 1'α and 1'β hydrogens could not be made at this stage. A multiplet at 2.55 ppm had been previously assigned[1] to the 7α hydrogen on the basis of the X-ray structure which showed the proximity of H-7α to the γ-lactone ring. The 9-hydroxyl appeared as a sharp doublet at 2.00 ppm which exchanged with D_2O. Five methyl singlets appeared at 1.44, 1.38, 1.35, 1.16 and 1.02 ppm, but could not be assigned at this stage. The remaining hydrogens appeared as a series of overlapping multiplets between 1.2 and 1.9 ppm which were partially assigned by a series of decoupling experiments. On irradiation of H-7α, a multiplet at 1.70 ppm simplified to a doublet of doublets (14 and 4Hz) due to the removal of a 2.5Hz coupling to H-7α, allowing its assignment to the equatorial H-6α. The signal at 1.50 ppm also changed to a doublet of doublets (14 and 4Hz) due to the removal of the 14Hz coupling at H-7α and so this signal could be assigned to the axial H-6β. The signal at 1.18 ppm due to H-7β also simplified to a doublet of doublets (4 and 2.5Hz) due to removal of the geminal coupling (14Hz) to H-7α. On addition of D_2O or irradiation of the hydroxyl proton, the signal at 1.87 ppm sharpened to a simple triplet (8.5Hz) due to removal of a 4-bond "W" coupling, allowing its assignment to H-9, which additionally shows equal couplings to both H-11α and H-11β (1.59 and 1.80 ppm) which were removed on irradiation of H-9. The remaining signals in this region could now be seen as an AB pattern due to the isolated 12-methylene hydrogens at 1.62 and 1.59 ppm. The final assignment of the methyl signals and the diastereotopic methylene hydrogens at C-11, C-12 and C-1' were achieved from the results of difference nOe studies.[6]

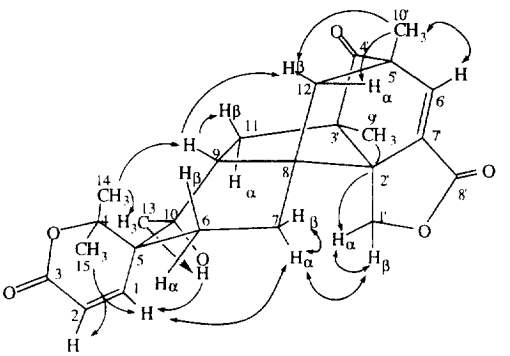


Figure 1. Stereodrawing of andibenin B showing the observed nOes.

Table 1. ^{13} C and ^{1}H NMR Assignments for Andibenin B (**1**); Coupling Constants (Hz) of [1,2-$^{13}C_2$]Acetate-Enriched (**1**); and Enrichments Observed in [1-^{13}C]Acetate (●), [2-^{13}C]Acetate (*) and [*Methyl*-^{13}C]Methionine (□) Enriched (**1**).

Atom	δ_C (ppm)	δ_H (ppm); multiplicity; *J* (Hz)	^1J (^{13}C-^{13}C)	Enrichment[a]
1	151.8	6.74; d; 10	-	*
2	118.3	6.06; d; 10	65	●
3	164.2		66	*
4	85.3		41	●
5	46.7		34	*
6α	35.1	1.70; ddd; 14, 4, 2.5	34	●
6β		1.50; ddd; 14, 14, 2.5		
7α	27.8	2.55; ddd; 14, 14, 2.5	-	*
7β		1.18; ddd; 14, 4, 2.5		
8	48.9		36	●
9	52.7	1.87; ddd; 8.5, 8.5, 1.5	35	*
10	77.2		38	●
11α	28.9	1.80; dd; 12.5, 8.5	34	●
11β		1.59; dd; 11.5, 8.5		
12α	55.6	1.59; d; 12.5	37	*
12β		1.62; d; 11.5		
13	23.6	1.16; s	39	*
14	24.5	1.44; s	40	*
15	27.0	1.38; s	-	*
1'α	68.7	4.24; d; 12	36	*
1'β		4.84; d; 12		
2'	58.5		36	●
3'	52.75		40	*
4'	213.9		40	●
5'	51.0		37	*
6'	140.9	7.04; s	37	●
7'	134.0		73	*
8'	167.4		73	●
9'	17.3	1.02; s	-	□
10'	17.2	1.35; s	-	□

[a] *ca.* 1 atom % for acetate, *ca.* 10 atom % for methionine.

Irradiation of H-6' resulted in enhancement of the methyl singlet at 1.35 ppm which must therefore be the 10'-methyl. Irradiation of the methyl singlet at 1.02 ppm produced an enhancement of the diastereotopic 1'-methylene hydrogen at 4.24 ppm and so these two signals were assigned to the 9'-methyl and H-1α. Irradiation

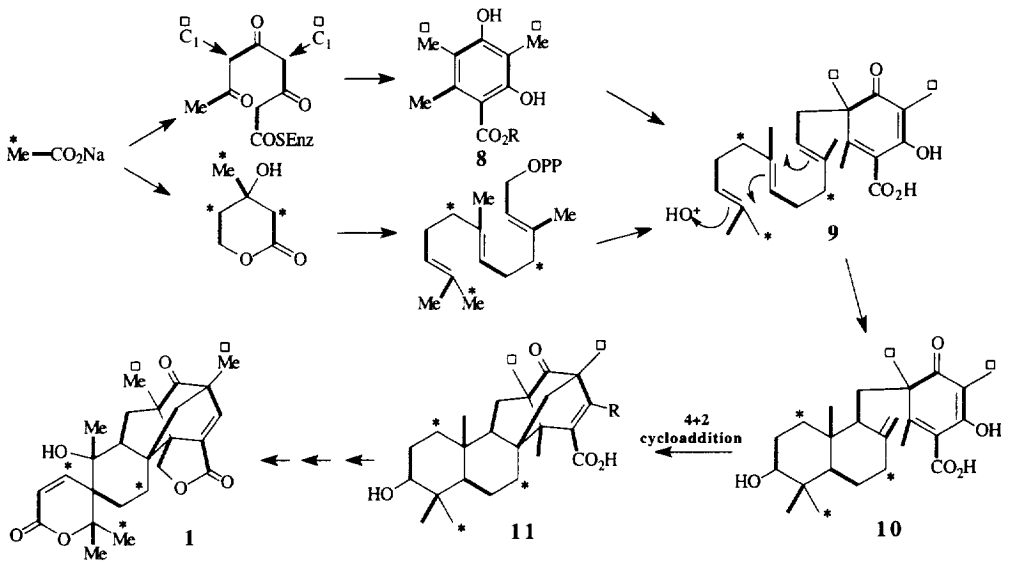
of the methyl singlet at 1.16 ppm enhances the hydroxyl signal and it must be due to the 13-methyl. The signal due to H-7α is also apparently enhanced in this experiment, presumably due to simultaneous irradiation of H-7β which was partially under the methyl resonance. Irradiation of the methyl signal at 1.38 ppm caused a small enhancement of the H-2 and H-1 signals, whereas irradiation of the methyl singlet at 1.44 ppm resulted in a strong enhancement of H-9 and a weak enhancement of the 13-methyl signal. These results are consistent with the assignment of the signals at 1.38 and 1.44 ppm to methyls 15 and 14 respectively. Irradiation of H-7α resulted in strong enhancement of H-1'β, H-1 and H-7β and a small negative nOe at H-1'α. Finally irradiation of H-9 resulted in an enhancement of the lower field portion of the signal due to the 12-methylenes, and so the signal at 1.59 ppm could be assigned to H-12β. These results are summarised in Figure 1 which indicates how the observed nOes allow the spatial relationships among the hydrogens along the top and bottom faces of the molecule to be traced. The nOe between the 13-methyl and 10-hydroxyl signals then connects the top and bottom sequences. The complete ^{1}H nmr assignment is shown in Table 1, which also summarises the assignment of the ^{13}C nmr spectrum which was assigned as follows.

From the observed chemical shifts and proton multiplicities, the signals at 213.9, 134.0 , 68.7 and 52.7 could be confidently assigned to the C-4' ketonic carbon, the C-7' quaternary olefinic carbon and the C-1' methylene and C-9 methine respectively. ^{1}H-^{13}C COSY spectra allowed assignment of all the protonated carbons based on the ^{1}H nmr assignments discussed above. Analysis of long-range couplings in the fully ^{1}H coupled ^{13}C nmr spectrum allowed the assignment of C-5' by selective proton irradiation experiments. Thus irradiation of H-6' and the 10'-methyl each caused a simplification of the multiplet at 51.0 ppm which could therefore be assigned to C-5'. Similar irradiation experiments on the olefinic hydrogens allowed the unambiguous assignment of the signals at 164.2 and 167.4 ppm to the C-3 and C-8' lactone carbonyls respectively. The two oxygen-bearing quaternary carbons at 77.2 and 85.3 ppm were unequivocally assigned to C-10 and C-4 respectively by ^{1}H-^{13}C difference nOe experiments: irradiation of the 15-methyl and 14-methyl protons led to 25% and 26% enhancement of the signal at 85.3 ppm, whereas irradiation of the 13-methyl gave a 15% enhancement at 77.2 ppm. Irradiation of 10'-methyl and 9'-methyl respectively gave a 23% enhancement of the signal at 51.0 ppm and a 20% enhancement of the signal at 52.75 ppm which must therefore be due to the quaternary C-5' and C-3 respectively. The remaining three quaternary signals at 46.7, 48.9 and 58.5 ppm must be due to C-2', C-8 and C-5. They could not be assigned by any of the above methods, but they were assigned by application of the DOUBTFUL technique.[7] This uses a modified INADEQUATE pulse sequence to observe a double quantum coherence between two adjacent carbons. Thus the signals at 58.5 and 46.7 ppm were shown to be adjacent to C-1' and C-4, allowing their assignments to C-2' and C-5 respectively. The remaining signal at 48.9 ppm must therefore be due to C-8. The ^{1}H and ^{13}C nmr spectra of andilesin A were also unambiguously assigned using exactly analogous procedures. The resulting assignments are listed in the experimental section.

Biosynthetic Studies

Preliminary experiments on *A. variecolor* 212 KI69 grown as a surface culture on a malt extract-peptone based liquid medium indicated that good yields of andibenin B (*ca.* 100mg l^{-1}) along with lower yields of andilesin A (20mg l^{-1}) were obtainable, with maximum yields obtained about 10 days after inoculation. Feeding studies with [1-^{14}C]acetate showed that labelled acetate could be incorporated into andibenin B with an overall dilution[8] of 7.7 when the labelled precursor was added 50 hours after inoculation.

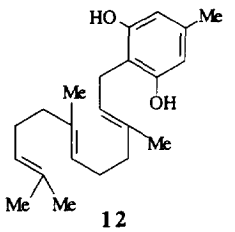
Singly and doubly ^{13}C-labelled acetates were fed to *A. variecolor* and the ^{13}C nmr spectra of the resulting enriched metabolites were determined. The ^{13}C enrichments and ^{13}C-^{13}C couplings observed in andibenin B are summarised in Table 1 and shown in Scheme 1. It was immediately apparent that the 9'- and 10'-methyls were not enriched from either C-1 or C-2 of acetate, and this, along with the observed labelling pattern made the previously proposed sesterterpenoid origin of andibenin B untenable. A subsequent feeding experiment with [*methyl*-^{13}C]methionine resulted in high enrichment of both the 9' and 10' methyls, confirming their origin from the C_1-pool. The labelling pattern shown in Scheme 1 suggested a mixed polyketide-terpenoid origin in which alkylation of a *bis-C*-methylated tetraketide derived phenol (**8**) with farnesyl pyrophosphate would give cyclohexadienone (**9**), which after epoxide initiated cyclisation to the triene (**10**) could undergo an intramolecular 4+2 cycloaddition to generate the required carbon skeleton. Subsequent oxidative modifications (*vide infra*) and elaboration of the spiro-lactone ring system would convert (**11**) to andibenin B.[9]



Scheme 1

According to this proposal, andibenin B and the related metabolites could be described as triprenyl-phenols or meroterpenoids. The latter term was first applied by Cornforth[10] to describe secondary metabolites of mixed, partially terpenoid, biogenesis. Triprenyl-phenols have been isolated from a variety of micro-organisms and marine sources. Simple examples include grifolin (**12**), in which the farnesyl side chain is unaltered[11], and siccanin, (**13**) in which it has undergone cyclisation and modification.[12] More complex examples include the austalides, e.g. (**14**) from *Aspergillus ustus*[13], where the sesquiterpenoid portion of the molecules has undergone extensive oxidative metabolism, analogous to that observed in andilesin A. Another example is mycophenolic acid (**15**), a metabolite of *Penicillium brevicompactum*, the aromatic nucleus in this case being 5,7-dihydroxy-4-methylphthalide (**16**). In classic experiments, Birch demonstrated that the side chain was derived from cleavage of a terpenoid moiety.[14] Originally it was assumed to be a geranyl chain, but it was subsequently shown that the corresponding farnesylphthalide is an intermediate in the biosynthesis.[15] Interestingly, studies of the metabolites isolated from a number of strains of *A. stellatus* have resulted in the isolation of highly oxygenated

sesquiterpenoid metabolites, and astellolides A (**17**) and B (**18**)[16], and stellatin (**19**)[17] which has been shown, like (**8**), to be derived from a *bis*-*C*-methylated tetraketide, albeit with different sites of methylation.[18]



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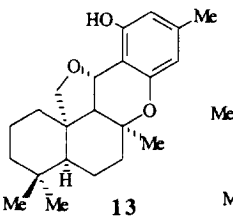

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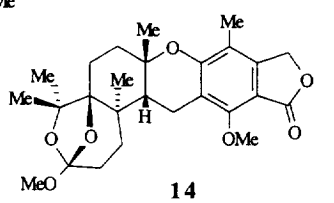

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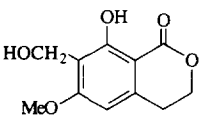

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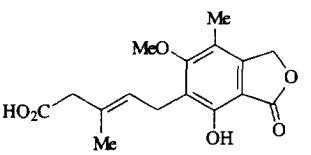

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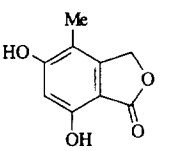

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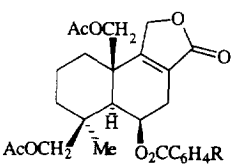

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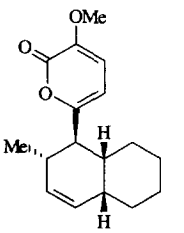
18 R = OH



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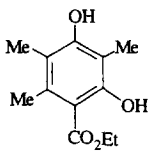
Prior to the preliminary studies on andibenin B, there were no established examples of biosynthetic Diels-Alder reactions. However, it has now been shown quite clearly that the biosynthesis of the phytotoxin, solanapyrone (**20**) involves a cycloaddition reaction and isolation of a corresponding cell-free activity from *Alternaria solani* has been reported.[19] The degree of skeletal modification required to form the meroterpenoids (**1**) - (**7**) is unprecedented and so further evidence was sought to substantiate the proposed pathway.



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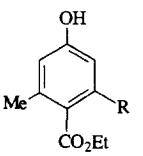

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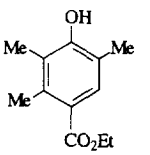
22 R = H



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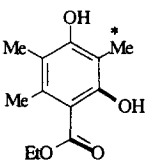
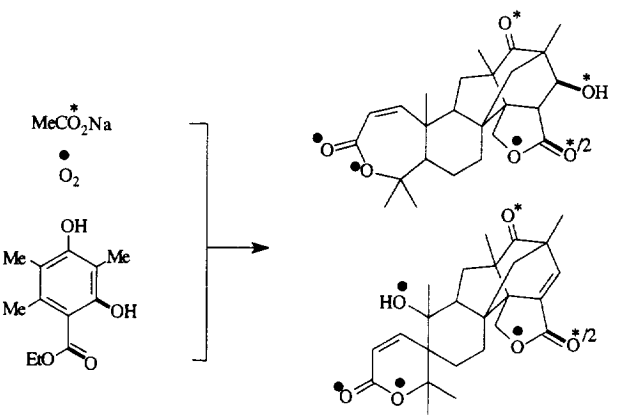


24 * = $^{2}H_3$
25 — = ^{13}C, ^{18}O

Inspection of the structures of the andibenins and andilesins suggests that the orsellinate (**21**), 3,5-dimethylorsellinate (**8**) or the corresponding 6-deoxy-compounds (**22**) or (**23**) could be the key phenolic intermediate which undergoes alkylation. These compounds were synthesised in ^{14}C labelled form as their ethyl esters[20] and fed to *A. variecolor*. Negligible incorporation of the orsellinate (**21**) and deoxyorsellinate was obtained (0.006% and 0.01% respectively), but both 3,5-dimethylorsellinate (**8**) and its deoxyanalogue (**23**) were well incorporated, although the former (1.21%) was utilised some 2.5 times more efficiently that the latter (0.44%).[21] In order to establish the intact incorporation of these phenolic precursors, 3,5-dimethylorsellinate (**24**) was synthesised with the 5-methyl specifically labelled with deuterium and fed to *A. variecolor*. The andibenin B and andilesin A subsequently isolated each showed a single resonance at 1.25 and 1.00 ppm respectively in their ^{2}H nmr spectra, corresponding in each case to the 10'-methyl group. As the unmethylated compounds (**21**) and (**22**) did not label andibenin B to any significant extent, this confirms that the methionine-derived methyl groups must be incorporated into the tetraketide precursor before aromatisation to the phenol. This

lends further support to the view that *C*-methylation processes occur before aromatisation and probably concomitant with polyketide chain assembly, whereas prenylation, despite being a similar electrophilic process, occurs after aromatisation.

The relatively efficient incorporation of isotopic label from the deoxy-compound (**23**) as well as the orsellinate (**8**) was surprising. To obtain more information on its role and the mechanisms of formation of the γ-, δ - and ε-lactone systems in the andibenins and andilesins, the origins of the oxygen atoms were investigated. Sodium [1-^{13}C,$^{18}O_2$]acetate was incorporated into andibenin B and andilesin A. The ^{13}C nmr spectrum of the enriched andibenin B showed isotopically-shifted signals for C-4' and C-8' to confirm the origin of the carbonyl oxygens from acetate (Scheme 2 and Table 2). Andilesin A showed similar shifts for the C-4' and C-8' carbonyls, but no clear isotopically shifted signals could be observed, due to the potentially acetate-derived hydroxyl on C-6', although a small upfield shoulder on the C-6' signal could be observed. However, application of a spin-echo pulse sequence[29] designed to eliminate any long-range ^{13}C-^{13}C couplings arising from adjacent incorporation of labelled acetate units gave a clean signal. No labelling of C-6' was observed in complimentary experiments in which *A. variecolor* was grown under an $^{18}O_2$ atmosphere[22] so the origin of the 6'-hydroxyl remained obscure. This ambiguity was resolved by the synthesis[23] and incorporation of ethyl 3,5-dimethylorsellinate (**25**) doubly labelled with ^{13}C and ^{18}O at the ester carbonyl group and C-6. The ^{13}C nmr spectrum of the resulting enriched andilesin A now showed clear isotopically enriched signals for both C-6' and C-8'. Interestingly, the amount of ^{18}O label incorporated at C-8' is approximately half that present at C-6'. Similar results are found for the acetate incorporation experiments which show that the amount of label at C-8' is approximately half that at C-4' in both andibenin and andilesin A. These results suggest that the γ-lactone is formed by attack of a hydroxyl group on C-1' on to C-8' which has existed as a free carboxylate at some stage during biosynthesis. In agreement with this, the ether oxygen of the γ-lactone is derived from atmospheric



Scheme 2. Incorporation of ^{18}O-labelled substrates into andilesin A and andibenin B.

oxygen. In mycophenolic biosynthesis, it has been clearly demonstrated[15] that the phthalide (**16**) is the substrate for alkylation with farnesyl pyrophosphate, so γ-lactone formation may precede addition of the farnesyl moiety. The derivation of the 6'-hydroxyl group from orsellinate (**25**) confirms its role as an obligatory intermediate to andilesin A. The observed incorporation of ^{14}C label from deoxyorsellinate (**23**) is thus probably via prior degradation to acetyl CoA. The lack of labelling of the 6'-hydroxyl by atmospheric oxygen rules out the

possibility of hydroxylation of deoxyorsellinate (**23**) to orsellinate (**8**). These results therefore indicate a pathway in which andilesin A (**5**) is the first metabolite to be formed and that it is converted by elimination of water into andilesin B (**4**), which is then reduced to andilesin C (**6**). A similar biosynthetic interrelationship can be assumed for andibenins A, B and C. (See Scheme 6 below.)

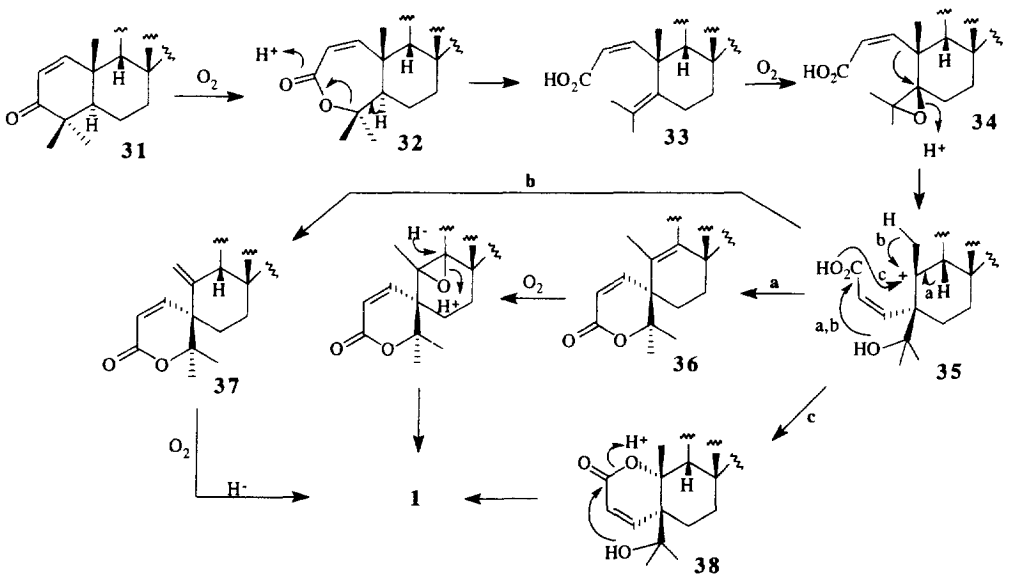
Table 2. ^{18}O And ^{2}H Isotopically Shifted Resonances Observed in the 100.6MHz ^{13}C Nmr Spectra of Andibenin B and Andilesin A.

Andibenin B					Andilesin A			
carbon	δ (ppm)	Δδ (ppm x 100)	Ratio ^{16}O: ^{18}O	Assignment	carbon	δ (ppm)	Δδ (ppm x 100)	Ratio ^{16}O: ^{18}O
					3	166.2	4.7[b]	83:15
3	165.5	4.7[b]	80:20		4	83.5	4.8[b]	87:11
4	86.9	4.3[b]	67:33		1'	69.1	2.9[b]	88:12
10	77.9	3.1[b]	72:28					
11	28.9	3.5[e]		CH_2CD				
12	55.3	80[d]		CD_2	4'	214.9	5.3[a]	84:16
13	23.5	54[d]		CHD_2	6'	71.9	1.2[a]	-
		80[d]		CD_3			1.8[c]	72:28
14	24.0	54[d]		CHD_2	8'	174.5	3.9[a]	82:18
		80[d]		CD_3			1.3[b]	90:11
1'	68.9	2.9[b]	70:30				3.8[c]	88:12
4'	214.2	5.0[a]	81:19					
8'	167.6	3.6[a]	91:9					
		3.4[c]	87:13					

Enriched from: [a]sodium [1-^{13}C, $^{18}O_2$]acetate; [b] $^{18}O_2$; [c]ethyl [^{13}C, ^{18}O]-3,5-dimethylorsellinate; [d][6-^{13}C, 6-$^{2}H_3$]mevalonic acid lactone; [e][5-^{13}C, 4-$^{2}H_2$]mevalonic acid lactone

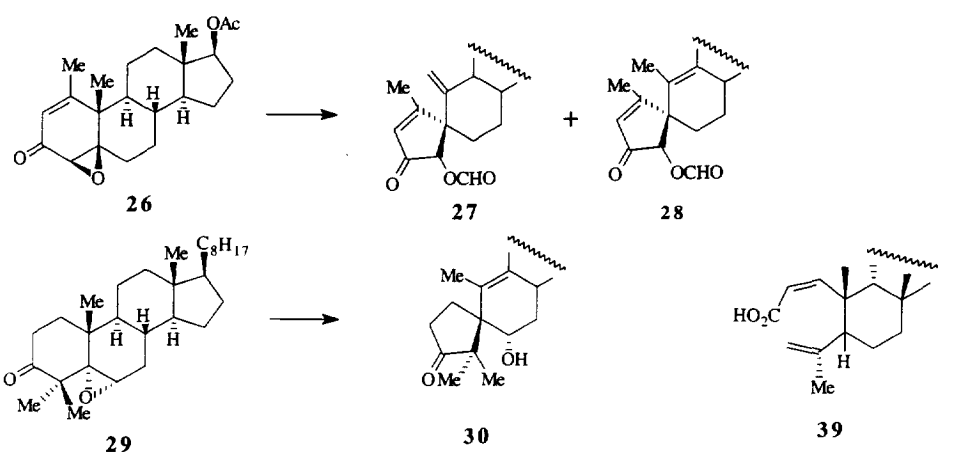
The mass spectrum of andibenin B isolated from the $^{18}O_2$ labelling experiment showed a major parent ion peak at m/z = 434, indicating the simultaneous incorporation of four ^{18}O atoms into andilesin B. The ^{13}C nmr spectrum showed that in addition to the γ-lactone ether oxygen, the oxygens attached to C-3, C-4 and C-10 are all derived from the atmosphere. These results show that the C-3 lactone function must be formed by a biological Baeyer-Villiger type oxidation[24] of a corresponding 3-keto precursor (**31**).

The presence of atmospheric oxygen at both C-4 and C-10 suggests possible mechanisms for the biosynthesis of the spiro-lactone system (Scheme 3). As the andilesins possess a 7-membered lactone ring, it seems reasonable to propose that the required skeletal rearrangement to form the spiro-lactone occurs subsequent to the introduction of the C-4 oxygen. Similar rearrangements of steroids are observed when a good leaving group is present at C-5, e.g. spiro compounds (**27**), (**28**) and (**30**) were formed on treatment of the 4β,5β–epoxide (**26**) with formic acid[25], or of the 5α,6α-epoxide (**29**) with boron trifluoride.[26] A relatively stable



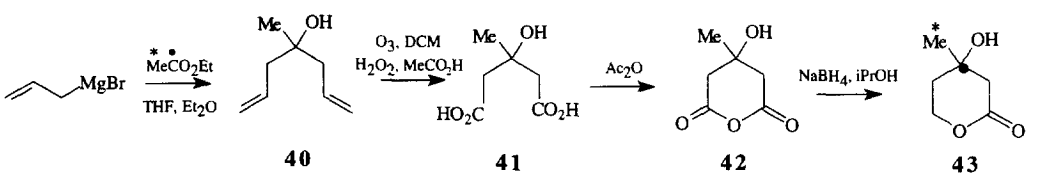
Scheme 3. Possible mechanisms for spiro-lactone formation.

carbocation could be generated from the andilesin lactone ring (**32**) by alkyl-oxygen cleavage, which under acid conditions is the predominant cleavage mechanism for esters of tertiary alcohols.[27] Loss of the C-5 proton would then give the tetrasubstituted alkene (**33**). It is significant that mild acid treatment of dihydroandilesin C results in facile ring opening to give the 4-methyl-4-methylene carboxylic acid (**39**). Subsequent oxidation of (**33**) to epoxide (**34**) followed by rearrangement would give the carbocation intermediate (**35**). Three paths for the conversion of (**35**) to the spirolactone can be envisaged. Elimination to the endocyclic or exocyclic alkenes (**36**) and (**37**) and subsequent epoxidation and hydride reduction (paths **a** and **b**) would account for the origin of the 10-hydroxyl in atmospheric oxygen. Any subsequent mechanism for the formation of the δ-lactone will result in



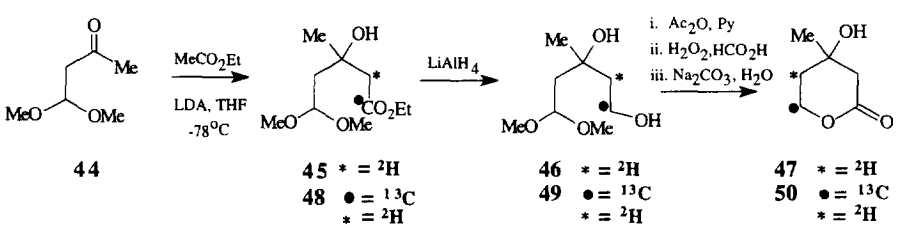
two atoms of atmospheric oxygen in the lactone. The structure of austin (**51**) which contains both the spiro-lactone and 9,10-double bond[28] provides some support for this general route. An alternative route (path **c**) involves trapping of the C-10 carbocation (**35**) by the carboxyl group to form lactone (**38**) which then undergoes transesterification, giving the A and B rings of andibenin B directly. In this case it is not necessary to invoke a further oxidation step. Prior hydrolysis of lactone (**38**) to form the spiro-lactone would imply a loss of half the oxygen label at C-3 which is not consistent with the relatively similar levels of incorporation of atmospheric oxygen observed (Table 2) at C-3, C-4 and C-10.

These possible mechanisms have been distinguished by a series of ^{2}H-labelling experiments with singly (^{2}H) and doubly (^{2}H, ^{13}C) labelled mevalonates. These were prepared by two routes. Treatment of β-hydroxy-β-methylglutaric acid (**41**), readily prepared in high yield from ethyl acetate via diene (**40**) as shown in Scheme 4, with acetic anhydride was reported to give the hydroxy-anhydride (**42**) which on reduction with sodium borohydride in propan-2-ol produced mevalonic acid lactone (**43**).[30] However, problems were reported[31] with this route and systematic examination of the cyclisation step defined the conditions required to produce the hydroxy-anhydride in high and reproducible yield.[32] The overall route is summarised in Scheme 4, and starting with readily available isotopically labelled sodium acetate, mevalonic acid lactone labelled in either position 3 or 6 can be readily synthesised. Thus, [1-^{14}C]-, [2-^{2}H$_3$]- and [2-^{13}C,2-^{2}H$_3$]acetates have been used to synthesise



Scheme 4. Synthesis of mevalonic acid lactone.

[3-^{14}C]-, [6-^{2}H$_3$]- and [6-^{13}C,6-^{2}H$_3$]mevalonic acid lactones respectively. [5-^{2}H$_2$]-Mevalonic acid lactone was readily prepared also by reduction of the hydroxy-anhydride (**42**) prepared from unlabelled acetate with sodium borodeuteride.



Scheme 5. Synthesis of mevalonic acid lactone.

The second route (Scheme 5) was a modification of that reported by Cane and Levin[33] for the preparation of [2-^{13}C]mevalonic acid lactone. Thus, starting with ethyl [2-^{2}H$_3$]acetate, the enolate was condensed with 1,1-dimethoxybutan-3-one (**44**). Lithium aluminium hydride reduction of the resulting hydroxyester (**45**) gave the diol (**46**) which after protection as the acetate was converted in a one-pot reaction to [4-^{2}H$_2$]mevalonic acid lactone (**47**). [5-^{13}C,4-^{2}H$_2$]Mevalonic acid lactone was prepared in a similar fashion starting from ethyl [1-^{13}C, 2-

$^{2}H_3$]acetate[34] to prepare the ^{13}C-labelled hydroxy ester (**48**). Reduction with lithium aluminium hydride gave the doubly labelled diol (**49**) which was converted to the lactone (**50**).

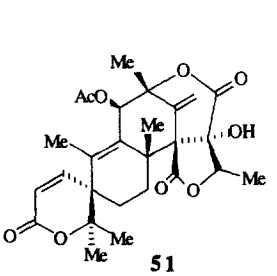
Preliminary experiments with [3-^{14}C]mevalonate showed that satisfactory incorporation levels into andibenin B (overall dilution 28) were obtained by feeding labelled mevalonate at day 5 of fermentation. Accordingly, [5-$^{2}H_2$]- and [6-$^{2}H_3$] mevalonates were incorporated into andibenin B. The ^{2}H nmr spectrum of the metabolite enriched from the former showed good incorporation of label into H-2 and also high incorporation in the region corresponding to the 6- and 11-methylenes. Although the signal integrated for four hydrogens relative to H-2, the individual signals were not resolvable. However, the corresponding andilesin A sample showed equal incorporation at 5 positions with chemical shifts of 5.9, 1.80. 1.77, 1.51 and 1.31 ppm, which correspond well to H-2, H-6α, H-Hα, H-6β and H-11β respectively. Although no certain conclusions can be drawn regarding the number of mevalonate-derived hydrogens at C-6 of andibenin B, it appears unlikely from these results that an intermediate with a 5,6-double bond has been involved in andibenin B biosynthesis. On incorporation of [6-$^{2}H_3$]mevalonate, the ^{2}H nmr spectrum showed signals at 1.15, 1.42, 1.57 and 1.63 ppm consistent with labelling of the 13 and 14 methyls and both 12-methylene hydrogens. This was confirmed by incorporation of [6-^{13}C,6-$^{2}H_3$]mevalonate. Analysis of the ^{13}C nmr spectrum of the enriched andibenin B with simultaneous proton and deuterium noise decoupling showed that two deuterium labels had been incorporated simultaneously at C-12, and three deuteriums at both C-13 and C-14. Small signals due to the retention of only two deuteriums at the two methyl carbons are also apparent. These are presumably due to the loss of a small amount of ^{2}H label ether during the synthesis of the labelled precursor or during biosynthesis. Consistent with the previous results from incorporation of [1,2-$^{13}C_2$]acetate, there is no randomisation of labelling between C-14 and C-15, so the stereochemical integrity of the *gem*-dimethyl group is retained throughout the biosynthetic pathway which perhaps suggests that the alkene (**33**) is formed by a concerted elimination from the ε-lactone system (**32**). The retention of all three hydrogen labels on C-13 clearly rules out the involvement of an intermediate (**37**) with an exocyclic double bond (path **b** in Scheme 3).

The remaining alternative pathways in Scheme 3 were distinguished by the results from incorporation of [5-^{13}C,4-$^{2}H_2$]mevalonate into andibenin B. The ^{13}C nmr spectrum showed a β-isotopically shifted signal at 28.9 ppm assigned to C-11. The shift must be due to incorporation of one ^{2}H at C-9. This rules out intermediate (**36**) containing a 9,10 double bond and so provides strong evidence for path **c**.

Austin (**51**) is clearly biosynthetically related to andibenin B (see below) and previous studies[23] have shown that it is formed from the same key intermediate (**9**) derived by alkylation of 3,5-dimethylorsellinate (**8**) by farnesyl pyrophosphate. An interesting possibility was that the tetracyclic intermediate (**56**) involved in austin biosynthesis (Scheme 8, below) is not formed directly from cyclisation of (**9**) but is formed via the same bicyclofarnesyl intermediate (**10**) involved in andibenin biosynthesis. The growth production characteristics of *A. ustus* and the optimum time for feeding labelled precursors had been determined previously.[23] Accordingly, [6-^{13}C, 6-$^{2}H_3$]mevalonate was fed to 5-day old cultures and the resultant enriched austin isolated and its ^{1}H, ^{2}H noise decoupled ^{2}H nmr spectrum determined. This showed clear isotopically shifted signals corresponding to the incorporation of two and mainly three deuteriums into the 12-, 13- and 14-methyls. The results for the 12-methyl exclude the possibility of the involvement of the bicyclofarnesyl intermediate (**10**) in austin biosynthesis.

Conclusions

The incorporation studies reported above provide definitive evidence for a mixed polyketide-terpenoid biogenesis for the andibenins and andilesins in *A. variecolor*. The formation of the bicyclo[2.2.2]octane system in these metabolites provides a rare example of a biosynthetic Diels-Alder reaction. Mechanisms for the extensive



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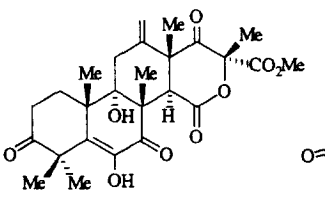

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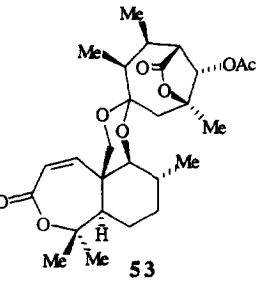

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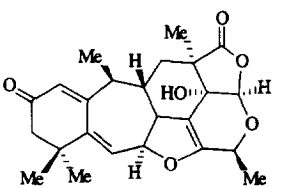

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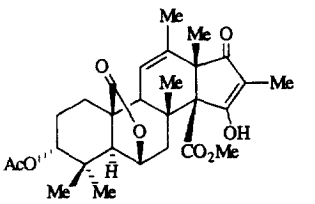

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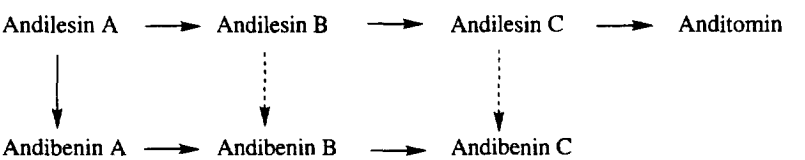
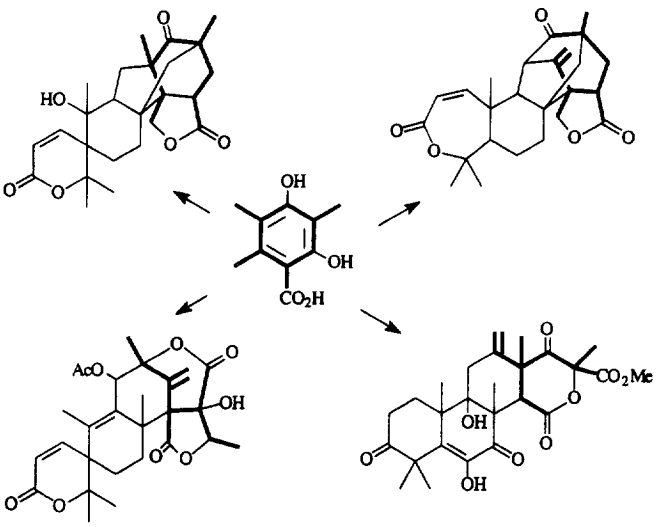
oxidative modifications involved in the formation of the γ-lactone and spiro-δ-lactone systems found in andibenin B have been established. Although the relative timing of the modifications involved is uncertain, there is good precedent which suggests that the γ-lactone will be formed before addition and subsequent modification of the farnesyl-derived moiety, and that the ε-lactone found in the andilesins is the progenitor of the spiro-lactone system of the andibenins. Thus the metabolic pathway shown in Scheme 6 can be proposed. The possibility of a metabolic grid indicated by the dashed arrows cannot be rigorously excluded.

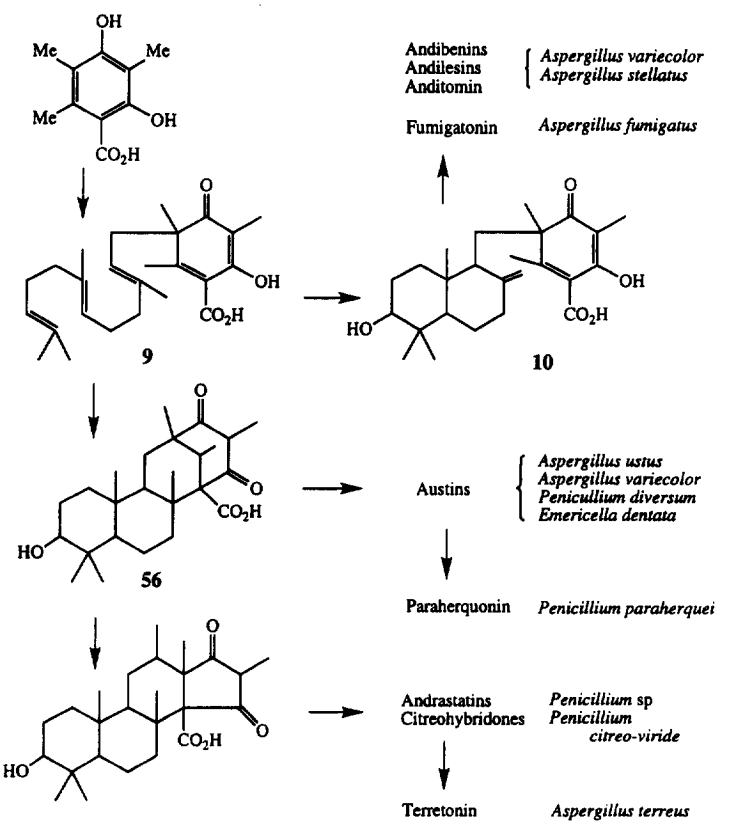


Scheme 6. Biosynthetic interrelationships among the *A. variecolor* meroterpenoids.

Andilesin C (**6**) and anditomin (**7**) have been isolated as co-metabolites in a different strain (CMI 60316) of *A. variecolor*, and ^{13}C-labelling studies are consistent with the formation of anditomin from a carbocation-induced rearrangement of the andilesin C skeleton.[35] Anditomin represents an important modification of the meroterpenoid pathway as it was the first metabolite in which the carbocyclic ring of the tetraketide-derived moiety had been fragmented. Austin (**51**) and terretonin (**52**) which were isolated as toxic metabolites of



Scheme 7. Modifications of the orsellinate moiety in meroterpenoid metabolites.



Scheme 8. The fungal meroterpenoid pathway.

Aspergillus ustus[28] and *Aspergillus terreus*[36] respectively have also been shown[23,38] to be formed from the meroterpenoid pathway via the common intermediate (**9**). The degree of modification of the orsellinate moiety is even more drastic in these metabolites (Scheme 7). The biosynthetic relationship of austin and andibenin was supported by the isolation of austin from another mutant strain of *A. variecolor*.[37] Further metabolites related to austin have been isolated from *Emericella dentata*[38] and *Penicillium diversum*.[37] Other complex metabolites which are almost certainly further products of the meroterpenoid pathway are fumigatonin (**53**) and paraherquonin (**54**) which have been isolated from *Aspergillus fumigatus*[39] and *Penicillium paraherquei* [40]*respectively*. More recently the citreohybridones, e.g. (**55**), and the closely related andrastatins have been isolated from *Penicillium citreoviride*[41] and *Penicillium* sp FO-3929[42] respectively to provide yet further members of the family. These latter metabolites are clearly related to terretonin (**52**) and the likely biogenetic relationships amongst these structurally varied metabolites is summarised in Scheme 8.

EXPERIMENTAL

General Procedures and Instrumentation

A Varian DMS 90 spectrophotometer was used to obtain uv spectra; base-line correction for solvent absorption was carried out. Ir spectra were taken on a Perkin-Elmer 781 spectrophotometer and referenced against the polystyrene absorption at 1 601cm^{-1}. ^{1}H nmr 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 nmr spectra were obtained from: Varian CFT 20 and Bruker WP 200 SY, WH 360 and WH 400 Fourier-transform machines. ^{2}H nmr 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 δ_C = 0.0 ppm). Mass spectra and exact mass determinations were taken on an 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 (10g 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 x 20cm) or preparative (20 x 20cm) glass plates coated with a 0.5mm layer of silica-gel (Merck Art. 7730 Kieselgel 60 GF_{254} or Fluka AG 60765 Kieselgel GF 254). Uv light of wavelength 254 was used to visualise chromatograms.

Solutions for feeding were sterilised by autoclaving at 15 psi for 15 min.

Production and Isolation of Andibenin B and Andilesin A

Aspergillus variecolor (212K I69, a uv-induced mutant strain from NRRL 212) was stored in the dark on Czapek-Dox liquid medium (Oxoid CM 95) containing 2% agar at 4°C. When required the culture was transferred to and grown on potato dextrose agar in medical flats for 7-10 days at 25-27°C in the light. A spore suspension in distilled water, from the medical flats, was used to inoculate 500ml Erlenmeyer flasks containing 100ml of malt extract broth (Oxoid Malt Extract, 3% w/v; Oxoid Mycological Peptone, 0.5% w/v; distilled water, to 100%). The culture was allowed to grow for fourteen days at 25-27°C. The growth medium and mycelium

were separated by filtration. The filtrate was extracted with ethyl acetate (4 x a third of the liquor volume), dried ($MgSO_4$), and the solvent removed *in vacuo* to afford a brown oil. This crude extract was applied to preparative tlc plates and eluted with methanol-chloroform (2:98). The main band (Rf 0.3), due to both andibenin B and andilesin A, was isolated and reapplied to preparative tlc plates, and eluted with petroleum ether-ethyl acetate (50:50). The band corresponding to an authentic sample of andibenin B (Rf 0.2) was isolated as an off-white solid. The area below this band and above the base line was removed, and andilesin A isolated as an off-white solid. (Andilesin A is difficult to observe under uv light.) Typical yields for andibenin B and andilesin A are 80-100mg l^{-1} and 20mg l^{-1} respectively. ^1H nmr spectroscopic data agreed with those of authentic samples and published data. These are summarised in Table 1 for andibenin B. Andilesin A gave:- δ_H (360MHz; $CDCl_3$) 1.12 (3H, s, 10'-Me), 1.13 (3H, s, 9'-Me), 1.13 (1H, dd, *J* 13, 11, H-11β); 1.13 (1H, d, *J* 14, H-12); 1.29 (3H, s, 13-Me), 1.34 (3H, s, 14-Me), 1.44 (3H, s, 15-Me), 1.48 (1H, d, *J* 14, H-12), 1.48 (1H, ddd, *J* 13.0, 11.0, 9.8, H-9), 1.52 (1H, m, H-6β), 1.68 (1H, dd, *J* 13, 5, H-5), 1.73 (1H, ddd, *J* 13.0, 9.8, H-11α), 1.75 (1H, m, H-7), 1.85 (1H, m, H-6α), 1.90 (1H, m, H-7), 2.93 (1H, d, *J* 7, H-7'), 4.05 (1H, d, *J* 7, H-6'), 4.08 (1H, d, *J* 9.5, H-1'β), 4.18 (1H, d, *J* 9.5, H-1'α), 5.75 (1H, d, *J* 10, H-1) and 5.82 (1H, d, *J* 10, H-2); δ_C (90.5MHz, $CDCl_3$) 15.7 (9'-Me), 16.5 (10'-Me), 22.1 (14-Me), 22.5 (C-6), 23.2 (13-Me), 24.6 (C-7), 29.9 (15-Me), 39.1 (C-11), 40.8 (C-7'), 41.9 (C-5), 43.6 (C-10), 45.3 (C-8), 49.0 (C-5'), 50.4 (C-12), 54.9 (C-3'), 55.6 (C-9), 56.5 (C-2'), 68.8 (C-1), 71.7 (C-6'), 83.4 (C-4), 119.1 (C-2), 149.7 (C-2), 166.2 (C-3), 174.4 (C-8') and 215.1 (C-4').

Growth Production Study of Andibenin B and Andilesin A

A. variecolor was grown as described above. At various times after inoculation, 68, 95, 142, 163, 235 hours, two of the culture flasks were worked up and the combined yield of andibenin B and andilesin A was found to be 35, 30, 50, 95, 90 and 110 respectively.

Incorporation of Sodium [1-^{14}C]acetate into Andibenin B

A. variecolor was grown as previously described in 31 flasks. Thirteen hours after inoculation, a sterile solution of sodium [1-^{14}C]acetate (500mg, 11.1 μCi, Sp.Act - 4.04 x 10^6 dpm $mmol^{-1}$) in distilled water (20ml) was distributed evenly by syringe into the culture media of 5 flasks. Parallel experiments, feeding at 25, 36 and 50 hours, were conducted concurrently. A further identical such dose of acetate was made up to 40ml and, at each of the aforementioned times, one quarter of the solution was distributed evenly among the same 5 flasks ("pulsed feed" experiment). A hypodermic needle was used to inject the solutions through the mycelial mat. Six flasks were retained as controls. After a total incubation time of 201 hours, the flasks were refrigerated and the andibenin B was isolated from each batch. The results are summarised below.

Time fed/hours	Yield/mgl^{-1}	Sp.Act./dpm $mmol^{-1}$	Dilution
(control)	98.8		
Pulse fed	59.2	2.59 x 10^5	15.6
13	62.8	2.08 x 10^5	19.4
25	82.6	2.16 x 10^5	18.7
36	72.0	3.16 x 10^5	12.8
50	43.6	5.25 x 10^5	7.7

Incorporation of ^{13}C-Labelled Acetates and Methionine into Andibenin B and Andilesin A

A. variecolor was grown as previously described. Fifty hours after inoculation, sterile solutions of sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate and [*methyl*-^{13}C]methionine (1g of each in 40ml distilled water) were distributed evenly among 10 culture flasks in each case by injection of the solutions through the mycelial mat. After 245 hours the cultures were worked up to yield andibenin B (80-97mg). In the case of the [1-^{13}C, $^{18}O_2$]acetate feed, andilesin A (29mg) was also isolated.

Incorporation of $^{18}O_2$ into Andibenin B and Andilesin A

A. variecolor was grown as above in 16 culture flasks. Four flasks were fitted with foam rubber bungs as usual, and retained as controls. The remaining 12 flasks were attached to a closed volume apparatus[22] for measuring the uptake of oxygen, and grown under an atmosphere composed initially of $^{18}O_2$:N_2 (30:70). Any oxygen debit was replaced by further $^{18}O_2$ at approximately 10 hourly intervals. After 174 hours of incubation, andibenin B (59mg) and andilesin A (17mg) were isolated.

Incorporation of ^{14}C-Labelled Aromatic Precursors in to Andibenin B

Ethyl [*carboxy*, 2-$^{14}C_2$]-2,4-dihydroxy-6-methylbenzoate (**21**, 80mg, 39.75 μCi mmol^{-1}), ethyl[*carboxy*, 6-$^{14}C_2$]-4-dihydroxy-2-methylbenzoate (**22**, 80mg, 39.82 μCi mmol^{-1}), ethyl [*carboxy*, 2-$^{14}C_2$]-2,4-dihydroxy-3,5,6-trimethylbenzoate (**8**, 83mg, 39.70 μCi mmol^{-1}) and ethyl [*carboxy*, 6-$^{14}C_2$]- 4-dihydroxy-2,3,5-trimethylbenzoate (**23**, 85mg, 39.68 μCi mmol^{-1}) were separately emulsified as follows: the aromatic ester was suspended in Tween 80 (1ml), sterile water (5ml) and acetone (10 drops) and was subjected to ultrasonic radiation for three 5-minute periods. Each emulsion was then divided into 4 and injected through the mycelium, under aseptic conditions, into the growth of the 4 flasks of *A. variecolor*. After allowing them to grow for a further 11 days, the 4 growths were worked up as above to give labelled samples of andibenin B: 22mg, 9.04 x 10^{-3} μCi mmol^{-1} from (**21**); 45mg, 86 x 10^{-3} μCi mmol^{-1} from (**22**); 88mg, 423 x 10^{-3} μCi mmol^{-1} from (**8**) and 90mg, 107 x 10^{-3} μCi mmol^{-1} from (**23**).

Incorporation of Labelled Mevalonates into Andibenin B

[5-$^2H_2$]-, [6-$^2H_3$]-, [6-^{13}C, 6-$^2H_3$]- and [5-^{13}C, 4-$^2H_2$]Mevalonic acid lactones (300mg, 2.27mmol) were each dissolved in 2N sodium hydroxide (1.15ml, 2.3mmol). After 5 minutes, the solutions were diluted to 10ml and added under sterile conditions to ten 500ml conical flasks each containing 7-day old 100ml cultures of *A. variecolor* (212169). After 14 days, the cultures were extracted and worked up the usual way to give crude andibenin B. Recrystallisation from methanol gave pure labelled andibenin B (48, 69, 23 and 78mg respectively).

Incorporation of Ethyl [5-$^2H_3$]- and [^{13}C,^{18}O]-Labelled-3,5-Dimethylorsellinates into Andibenin B and Andilesin A

A. variecolor was grown as previously described. Sterile solutions of the [5-$^2H_3$]- and [^{13}C, ^{18}O]-labelled orsellinates (120mg, 99 atom % ^2H and 88 atom % ^{13}C, 81 atom % ^{18}O, 73 atom % $^{13}C^{18}O$) dissolved in acetone (10 drops), Tween 80 detergent (1ml) and distilled water (20ml) were prepared. The cloudy solutions were distributed equally between 8 flasks, three days after inoculation. After 14 days growth the flasks were worked up, and the 2 metabolites isolated and purified to give andibenin B (94mg and 89mg) and andilesin A (25mg and 20mg).

Production and Isolation of Austin (51)

Aspergillus ustus (NRR2 6017) was stored in the dark on Czapek-Dox liquid medium (Oxoid CM95) and 2% agar at 4°C. When required, a spore suspension in distilled water was subsequently used to inoculate the production medium of malt extract/peptone broth, 100ml of which was contained in each of the 500ml Erlenmeyer flasks employed. The fungus was allowed to grow for 14 days, in constant light, at 26°C. The liquid medium and mycelium were separated by filtration. The filtrate was acidified to pH 2 with 2M hydrochloric acid and extracted with ethyl acetate (4 x one-half of liquor volume), dried over anhydrous $MgSO_4$ and the solvent removed *in vacuo* to give a brown gum, the constituents of which were separated by a preparative tlc, developing with chloroform-methanol (96:4), and the band corresponding to an authentic sample of austin (R_f 0.4) was isolated as a white crystalline solid. (A repeat of the preparative tlc was usually necessary to purify austin further.) Typical yields of austin were (*ca* 30mg l^{-1}). Austin recrystallised from methanol to give white needles m.p 298-300°C (lit.[28] m.p 298-300°C). ^1H nmr spectroscopic data were in agreement with published data.

Incorporation of [6-^{13}C, 6-$^2H_3$]Mevalonolate into Austin

[6-^{13}C, 6-$^2H_3$]Mevalonic acid lactone (350mg, 2.6mmol) was dissolved in 1.3ml (2.6mmol) of 2N sodium hydroxide. After 5 minutes the solution was diluted to 10ml and added under sterile conditions to 5 3-day old 100ml cultures of *Aspergillus ustus*. After 14 days the culture filtrate was extracted and worked up in the usual way to give, after recrystallisation from methanol, austin (9mg).

[5-$^2H_2$]-Mevalonic Acid Lactone

3-Hydroxy-3-methylpentane-1,5-dioic anhydride (22, 7.0g) was dissolved in propan-2-ol (100ml) and the solution was added dropwise to sodium borodeuteride (4.0g) suspended in 50ml of propan-2-ol and cooled in an ice bath. The reaction mixture was stirred overnight at room temperature. After removal of the solvent, water (100ml) was added and the mixture was acidified to pH 2 in an ice bath. The solution was extracted continuously with diethyl ether for 3 days. The extract was filtered and dried over magnesium sulphate and the solvent removed on a rotary evaporator to give 5.8g of [5-$^2H_2$]mevalonic acid lactone which was purified by flash column chromatography to give 5.2g of pure lactone. δ_H ($CDCl_3$); 1.36 (3H, s, CH_3), 1.86 (2H, m, CH_2COH), 2.58 (2H, d, *J* 2.8, $CH_2C{=}O$), 3.8 (1H, br, s, O*H*).

n-Butyl [1-^{14}C]acetate[34]

Sodium [1-^{14}C]acetate (50g, 60.97mmol; specific activity 9.102 x 10^6 dpm/mmol) was mixed with 25ml of n-butyl phosphate. The mixture was heated under reflux for 1 hour in an oil bath at 180-200°C. The viscous mixture was cooled at room temperature. The upper end of the reflux condenser was sealed through a liquid nitrogen cooled trap to a vacuum line, and the product ester was distilled into the cold trap by heating for 3 hours to 120-160°C at 0.1mm Hg pressure while cold water was kept running in the vertical condenser. n-Butyl acetate, 6.7g (9% yield) was obtained.

[3-^{14}C]Mevalonic Acid Lactone

A mixture of n-butyl [2-^{14}C]acetate (6.7g, 56.7mmol) and allyl bromide (20.58g, 0.17mol) in a mixture of dry ether and THF (100ml; 70:30) was added during 20 minutes with vigorous stirring to dry magnesium

turnings (14g, 0.58mol) in diethyl ether (50ml). After being heated under reflux for 2 hours, water (30ml) was added followed by 6M H_2SO_4 (100ml) with ice cooling. The solution was extracted with diethyl ether (3 x 80ml) and the extract washed with saturated sodium hydrogen carbonate solution (50ml) and dried over anhydrous $MgSO_4$. Concentration under reduced pressure gave the hydroxy-diene. The crude product distilled at water pump pressure to give [4-^{14}C]-4-hydroxy-4-methylhepta-1,6-diene (6.1g; 84% yield).

The hydroxy-diene was dissolved in dichloromethane-acetic acid (20:1, 100ml) and cooled to -78°C. Ozone was then passed through the solution until a permanent blue colour persisted. The solution was then allowed to warm to room temperature and the solvent was removed under reduced pressure to give a colourless oil. The oil was dissolved in acetic acid-hydrogen peroxide (100%) (80ml, 50:30) and heated under reflux for 24 hours. On removal of the solvent the diacid remained as an oil which slowly solidified to give [3-^{14}C]-3-hydroxy-3-methylpentan-1,5-dioic acid (7.8g, 100%). The acid (7.8g, 47.5mmol) and acetic anhydride (29.1g, 0.28mol) were stirred for 24 hours at room temperature. The suspension had turned into a clear solution. The excess of acetic anhydride was removed under high vacuum to give an off-white solid which was recrystallised from diethyl ether-light petroleum (bp 30-40°C) to give [3-^{14}C]-3-hydroxy-3-methylpentan-1,5-dioic anhydride (5.0g; 72% yield).

The anhydride (5.0g, 34.2mmol) was dissolved in propan-2-ol (40ml) and added dropwise to a suspension of sodium borohydride (3.2g, 85.5mmol) in propan-2-ol (40ml) which had been stirred for 30 minutes. The mixture was then stirred for 24 hours at 4°C and the solvent was removed under reduced pressure. The resulting solid was dissolved in water (20ml) and acidified to pH 2, under ice cooling using 2M MCl. The solution was stirred for 24 hours then continuously extracted with diethyl ether for 72 hours. The diethyl ether extract was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in chloroform and filtered and concentrated under reduced pressure to give [3-^{14}C]mevalonic acid lactone (3.2g, 71% yield) as viscous oil.

[6-$^2H_3$]Mevalonic Acid Lactone

A mixture of ethyl [$^2H_3$]acetate (12.8g, 0.14mol) and allyl bromide (50.8g, 0.42mol) in a mixture of dry ether and THF (3:7, 200ml) was added during 20 minutes with vigorous stirring to dry magnesium turnings (13.0g; 0.54mol) in diethyl ether (200ml). After being heated under reflux for 2 hours, water (150ml) was added followed by 6M H_2SO_4 (200ml) with ice cooling. The solution was extracted with diethyl ether (3 x 150ml) and the extract washed with saturated sodium hydrogen carbonate solution (150ml) and dried over anhydrous $MgSO_4$. Concentration under reduced pressure gave the alcohol diene as an oil. The crude product was distilled at water pump pressure to give 16.3 (90%) of 4-hydroxy-4-[*methyl*-$^2H_3$]hepta-1,6-diene. δ_H ($CDCl_3$); 2.20 (4H, d, *J* 7, CH_2), 5.04-5.1 (4H, m, CH=CH_2), 5.90 (2H, m, C*H*=CH_2).

The hydroxy diene was dissolved in dichloromethane acetic acid (20:1, 200ml) and cooled to -78°C. Ozone was then passed through the solution until a permanent blue colour persisted. The solution was then allowed to warm to room temperature and the solvent removed under reduced pressure to give a colourless oil. The oil was dissolved in acetic acid-hydrogen peroxide (100%) (160ml, 100:60) and heated under reflux for 24 hours. On removal of the solvent, the diacid remained as an oil which slowly solidified to yield 19.3g (92%) of the diacid 3-hydroxy-3-[*methyl*-$^2H_3$]pentan-1,5-dioic acid. δ_H ($^2H_6$-acetone); 2.80, 2.90, 2.98 and 3.06 (4H, m, CH_2), 4.67 (1H, br, s, O*H*).

3-Hydroxy-3-[*methyl*-$^{2}H_3$]pentan-1,5-dioic anhydride (7.5g, 51mmol) was dissolved in propan-2-ol (100ml) and added dropwise to a suspension of sodium borohydride (4.82g; 0.127mol) in propan-2-ol (100ml) which had been stirred for 30 minutes. The mixture was then stirred for 24 hours at 4°C and the solvent was removed under reduced pressure. The resulting solid was dissolved in water (150ml) and acidified to pH 2 under ice cooling using 2M HCl. The solution was stirred for 24 hours then continuously extracted with diethyl ether for 72 hours. The diethyl ether extract was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in chloroform, filtered and concentrated under reduced pressure to give [6-$^{2}H_3$]mevalonic acid lactone as a viscous oil (5.2g, 77%). δ_H ($CDCl_3$); 1.92 (2H, m, C*H*COH), 2.56 (2H, m, CH_2 C=O), 4.4 (2H, m, OCH_2 CH_2), 5.2 (1H, br, s, O*H*).

[6-^{13}C, 6-$^{2}H_3$]Mevalonic Acid Lactone

A mixture of n-butyl [2-^{13}C, $^{2}H_3$]acetate (1.4g, 11.6mmol) and allyl bromide (3.5g, 30mmol) in a mixture of dry ether and THF (3:7, 50ml) was added during 20 minutes with vigorous stirring to dry magnesium turnings (3g) in diethyl ether (20ml). After being heated under reflux for 2 hours, water (20ml) was added followed by 6M H_2SO_4 (50ml) with ice cooling. The solution was extracted with diethyl ether (4 x 50ml) and the extract washed with saturated sodium hydrogen carbonate solution (50ml) and dried over anhydrous $MgSO_4$. Concentration under reduced pressure gave the hydroxy-diene as an oil. The crude product was distilled at water pump pressure to give 4-hydroxy-4-[*methyl*-^{13}C, *methyl*-$^{2}H_3$]hepta-1,6-diene (1.3g, 87% yield). δ_H ($CDCl_3$); 2.2 (4H, m, CH_2), 5.04-5.14 (4H, m, CH=CH_2), 5.9 (2H, m, C*H*=CH_2).

The hydroxy-diene (1.3g) was dissolved in dichloromethane-acetic acid (5ml) and cooled to -78°C. Ozone was then passed through the solution until a permanent blue colour persisted. The solution was then allowed to warm to room temperature and the solvent was removed under reduced pressure to give a colourless oil. The oil was dissolved in acetic acid-hydrogen peroxide (100%) 12:10ml) and heated under reflux for 24 hours. On removal of the solvent the diacid remained as an oil which slowly solidified to give 3-hydroxy-3-[*methyl*-^{13}C, *methyl*-$^{2}H_3$]pentan-1,5-dioic acid (1.6g, 100% yield). δ_H ($^{2}H_6$-acetone); 2.68 (4H, CH_2), 8.5 (3H, br, s, O*H*).

A mixture of 3-hydroxy-3-[*methyl*-^{13}C, $^{2}H_3$]methylpentan-1,5-dioic acid (1.6g, 9.6mmol) and acetic anhydride (7ml) was stirred for 24 hours at room temperature. The suspension had turned into a clear solution. The excess of acetic anhydride was removed under high vacuum to give an off-white solid which was recrystallised from diethyl ether-light petroleum (bp 30-40°C) to give 3-hydroxy-3-[*methyl*-^{13}C, *methyl*-$^{2}H_3$]methylpentan-1,5-dioic anhydride (1g, 71% yield). δ_H ($^{2}H_6$-acetone); 3.09 (4H, m, CH_2), 4.55 (1H, br, s, O*H*).

3-Hydroxy-3-[*methyl*-^{13}C, *methyl*-$^{2}H_3$]methylpentan-1,5-dioic anhydride (1g, 6.7mmol) was dissolved in propan-2-ol (5ml) and added dropwise to a suspension of sodiumborohydride (0.65g) in propan-2-ol (5ml) which had been stirred for 30 minutes. The mixture was then stirred for 24 hours at 4°C and the solvent was removed under reduced pressure. The resulting solid was dissolved in water (10ml) and acidified to pH 2 under ice cooling using 2M HCl. The solution was stirred for 24 hours then continuously extracted with diethyl ether for 72 hours. The diethyl ether extract was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in chloroform and filtered and concentrated under reduced pressure to give 3-[*methyl*-^{13}C, *methyl*-$^{2}H_3$]mevalonic acid lactone as viscous oil (0.82, 90% yield). δ_H ($CDCl_3$); 1.92 (2H, m, CH_2CO), 2.56 (2H, m, CH_2C=O), 4.4 (2H, m, CH_2O), 5.2 (1H, br, s, O*H*).

***Acetoacetaldehyde Dimethyl Acetal*[33]**

A mixture of anhydrous ethyl formate (7.6g, 0.103mol) and acetone (5.0g) in anhydrous ether (20ml) was added dropwise to a stirred suspension of sodium methoxide (4.7g; 86mmol) in anhydrous ether (50ml) under nitrogen. The reaction mixture was refluxed for 1 hour then cooled, and the ether was carefully evaporated at aspirator pressure without stirring. Methanol (20.6ml, 0.515mol) was added to the solid residue and the suspension was stirred for 3 minutes before addition of (13.7ml, 174mmol) of methanolic hydrogen chloride (12.7M). After an additional 2 hours at room temperature the pH was adjusted to 8 by addition of saturated methanolic potassium hydroxide, ether was added and the mixture was filtered. The filtrate was concentrated on a rotary evaporator and the residue was re-dissolved in ether and once again filtered and evaporated. Care was taken to avoid loss of product by prolonged evaporation. The flask containing the residue was connected to a short-path condenser and immersed in liquid nitrogen and the residue was allowed to warm to room temperature. After 45 minutes the distillate was dissolved in ether and the solution was dried over anhydrous sodium sulphate and 4Å molecular sieves (to remove any residual methanol). Filtration and careful evaporation yielded 4.8g (43% yield) of acetoacetaldehyde dimethyl acetal. δ_H ($CDCl_3$); 2.03 (3H, s, CH_3), 2.59 (2H, d, *J* 5.6, CH_2), 3.21 (6H, s, OCH_3), 4.64 (1H, t, *J* 5.6, C*H*).

***Ethyl [1-^{13}C, 2-$^{2}H_2$]-3-hydroxy-3-methyl-5,5-dimethoxypentanoate*[33]**

20ml of dry tetrahydrofuran containing (0.68g, 6.7mmol) of diisopropylamine was charged into a 100ml three-neck flask equipped with a rubber septum fitted with dry nitrogen. The reaction flask was cooled to -78°C before addition of 4ml (6.4mmol) of 1.6M n-butyl lithium. After 10 minutes at -78°C the ether solution of ethyl [1-^{13}C, 2-$^{2}H_3$]acetate (0.57g, 6.3mmol) was added dropwise. The mixture was stirred an additional 15 minutes at -78°C, and (0.88g, 6.3mmol) of acetoacetaldehyde dimethyl acetal in 1.5mol of tetrahydrofuran was added and the mixture was allowed to warm to room temperature. The tetrahydrofuran was evaporated and residual mixture was extracted with ether. The ether extract was dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield 1.1g of ethyl [1-^{13}C, 2-$^{2}H_2$]-3-hydroxy-3-methyl-5,5-dimethoxypentanoate. δ_H (CDCl-3); 1.24 (3H, s, CH_3), 1.25 (3H, t, *J* 7.1, CH_2CH_3), 1.87 (2H, dd, *J* 130, 5.5, CH_2CO), 3.30 (3H, s, OCH_3), 3.31 (3H, s, OCH_3), 3.47 (1H, s, O*H*), 4.12 (2H, q, *J* 7.1, CH_2CH_3), 4.6 (1H, t, *J* 5.6, $CH(OCH_3)_2$).

***[1-^{13}C, 2-$^{2}H_2$]-3-Methyl-5,5-dimethoxypentan-1,3-diol*[33]**

A solution of 0.6g (2.6mmol) of ethyl [1-^{13}C, 2-$^{2}H_2$]-3-hydroxy-3-methyl-5,5-dimethoxypentanoate in tetrahydrofuran (2ml) was added to a stirred suspension of 0.2g (5.26mmol) of lithium aluminium hydride in tetrahydrofuran (10ml) under nitrogen. After 1 hour at room temperature the reaction mixture was refluxed for 1 hour and then cooled, whereupon 0.2ml of water, 0.2ml of 15% sodium hydroxide and 0.6ml of water were added in succession. The granular precipitate was removed by filtration and the filtrate was concentrated under reduced pressure to yield 0.4g of the diol. δ_H($CDCl_3$); 1.26 (3H, s, CH_3), 1.82 (2H, m, CH_2CH), 3.20 (1H, br, s, O*H*), 3.35 (3H, s, OCH_3), 3.37 (3H, s, OCH_3), 3.82 (2H, br, d, *J* 127, CH_2OH), 4.65 (1H, dd, *J* 5.0, $CH(OCH_3)_2$).

[1-^{13}C, 2-$^{2}H_2$]-3-Methyl-5,5-dimethoxy-pentane-1,3-diol-1-acetate

The diol (0.4g) was stirred overnight at 4°C under nitrogen in a mixture of 10ml of dry pyridine and 10ml of acetic anhydride. The reaction mixture was concentrated at 0.1mm (room temperature) to yield 0.46g of the

acetate. δ_H ($CDCl_3$); 1.14 (3H, s, CH_3), 1.72 (2H, d, *J* 6, CH_2CH), 1.95 (3H, s, $OCOCH_3$), 3.26 (3H, s, OCH_3), 3.28 (3H, s, OCH_3), 4.12 (2H, br d, *J* 130, CH_2O), 4.58 (1H, t, $CH(OCH_3)$), 4.65 (1H, br, s, *OH*).

[5-^{13}C, 4-$^{2}H_2$]Mevalonic Acid Lactone

[1-^{13}C, 2-$^{2}H_2$]-3-Methyl-5,5-dimethoxypentane-1,3-diol-1-acetate (0.46g) was refluxed for 1 hour in a mixture of 1.0ml of formic acid, 0.5ml of 30% hydrogen peroxide and 1.0ml of 1% aqueous sulphuric acid. The reaction mixture was cooled to room temperature and 50mg of anhydrous potassium carbonate was added. Following evaporation of the solvent, the residue was dissolved in chloroform and filtered to yield the lactone which was purified by preparative thin layer chromatography using methanol-chloroform (4.96) as eluent to yield 0.2g of the lactone. δ_H ($CDCl_3$); 1.24 (3H, s, CH_3), 2.53 (2H, d, *J* 3.8, $CH_2C{=}O$), 3.24 (1H, br, s, *OH*), 4.32 (2H, d, m, *J* 129, CH_2O).

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