Biosynthesis of Austalide D, a Meroterpenoid Mycotoxin from *Aspergillus ustus*

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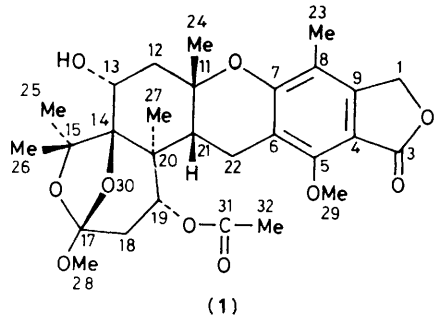
Incorporation of [1-^{13}C]- and [1,2-$^{13}C_2$]-acetate, and (3*RS*)-[2-^{13}C]mevalonate into austalide D, a metabolite of *Aspergillus ustus*, and the isolation of the cometabolites austalide J, K, and L, indicate its formation *via* a mixed polyketide–terpenoid biosynthetic pathway.

Austalide D (**1**) is one of a number of meroterpenoid mycotoxins produced by whole maize cultures of *Aspergillus ustus* strain MRC 1163.[1] A structural analysis of the austalides suggests a biosynthetic pathway which involves 6-*trans*,*trans*-farnesyl-5,7-dihydroxy-4-methylphthalide (**2**), a key intermediate also in the biogenesis of mycophenolic acid.[2] We now report a study of the biosynthesis of austalide D using ^{13}C and ^{14}C labelled precursors which provides information on the subsequent cyclisation and oxidative modifications of the farnesyl moiety in (**2**).

The chemical shift values and multiplicities of the resonances in the natural abundance 125.76 MHz ^{13}C n.m.r. spectrum of austalide D (**1**) as collated in Table 1 were obtained from broad-band proton-decoupled and single frequency nuclear Overhauser enhanced (n.O.e.) spectra. The residual splittings observed in a series of off-resonance proton-decoupled ^{13}C n.m.r. experiments enabled us to correlate the signals of all the proton-bearing carbon atoms with specific proton resonances as shown in Table 1.[3] The assignment of the different ^{13}C n.m.r. resonances is based on the

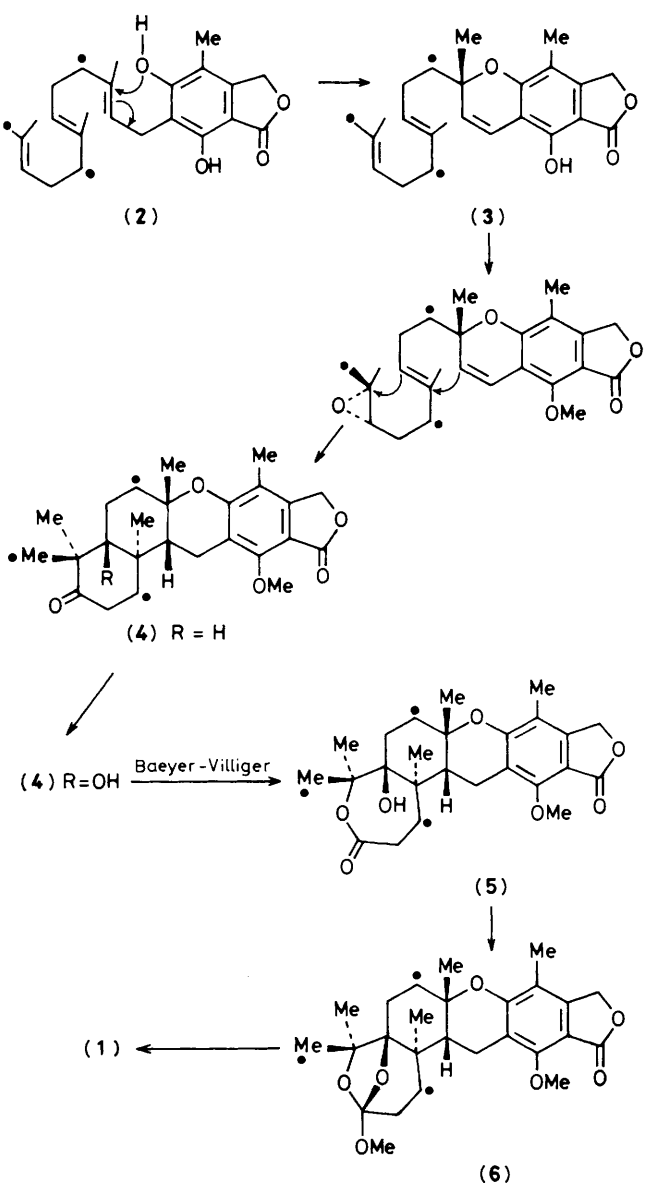


(1)

Table 1. ^{13}C (125.76 MHz) and ^{1}H (500.14 MHz) N.m.r. data for austalide D (**1**).

Carbon atom	δ_C/p.p.m.[a,b]	^1J(CC)/Hz[c]	δ_H[a,d]	^1J(HH)/Hz
1	68.10T	40.9	5.087s	
3	168.93S	74.2		
4	108.63S	73.5		
5	155.61S	72.5		
6	116.26S	71.7		
7	156.58S	69.8		
8	114.10S	69.2		
9	145.76S	40.9		
11	78.23S	39.2		
12	40.84T	—	2.339dd	15.8,2.2
			2.320dd	15.8,4.2
13	69.92D	44.8	4.184m	4.2,2.2,8.0[e]
14	86.10S	45.3		
15	85.69S	40.3		
17	117.47S	56.5		
18	37.34T	56.5	2.274dd	15.0,6.1
			1.935d	15.0
19	71.07D	—	5.433d	6.1
20	44.98S	35.1		
21	38.49D	35.1	2.176d	8.5
22	19.73T	35.4	3.229d	18.9
			2.975dd	18.9,8.5
23	10.61Q	—	2.019s	
24	27.35Q	39.7	1.258s	
25	25.73Q	40.4	1.771s	
26	29.65Q	—	1.488s	
27	14.07Q	34.9	0.964s	
28	48.71Q	—	3.378s	
29	62.16Q	—	4.119s	
31	170.27S	59.8		
32	21.13Q	59.8	2.041s	

[a] Recorded on a Bruker WM-500 spectrometer for solutions in $CDCl_3$. Chemical shifts relative to Me_4Si. [b] Letters refer to the pattern resulting from directly-bonded (C,H)-coupling with S = singlet, D = doublet, T = triplet, Q = quartet. [c] Intra-acetate coupling. [d] s = singlet, d = doublet, m = multiplet. [e] The proton of the C-13 hydroxy-group is a doublet (J 8.0 Hz) at δ_H 2.603.



Scheme 1. Proposed biosynthetic pathway of austalide D. ● = C-2 of mevalonolactone.

results obtained from heteronuclear ^{13}C-{^1H} selective population inversion experiments.[4] The method, however, does not allow us to assign the δ 25.73 and 29.65 p.p.m. resonances to specific methyl carbon atoms of the two prochiral diastereoisotopic methyl groups, C-25 and C-26. The assignment of the δ 25.73 p.p.m. resonance to the *pro-R* methyl group, C-25 and consequently that at δ 29.65 p.p.m. to C-26, follows from the known relative stereochemistry of the austalides,[1] and the observation of an appreciable n.O.e. for the methyl protons at δ_H 1.771 upon irradiation of the C-27 protons (δ_H 0.964) in a homonuclear ^1H-{^1H} n.O.e. experiment. The n.O.e. observed between the C-13 proton and the methyl protons at δ_H 1.488 supports the assignments. The detailed ^1H and ^{13}C n.m.r. study will be described in a subsequent publication.

Cultures of *A. ustus* were grown in Petri dishes on cakes of yellow maize meal containing 50% water. Studies of the course of fermentation indicated that austalide D production commenced on day 3 and reached a level of 30 mg per 100 g maize after 14 days. Preliminary feeding experiments with [1-^{14}C]-acetate as precursor established that a good incorporation (0.1%) and satisfactory dilution values[5] (36.3, assuming 11 labelled positions) were obtained by pulse-feeding cultures of *A. ustus* every 24 h from day 3 to day 14 with sodium acetate to a total amount of 400 mg per 50 g of maize.

The 125.76 MHz broad-band proton-decoupled ^{13}C n.m.r. spectrum of austalide D derived from [1-^{13}C]acetate (91.6 atom% ^{13}C) showed 11 enhanced signals attributed to C-3, C-5, C-7, C-9, C-11, C-13, C-15, C-18, C-20, C-22, and C-31.

The arrangement of intact acetate units in austalide D was studied by addition of [1,2-$^{13}C_2$]acetate (C-1: 91.6, C-2: 90.0 atom% ^{13}C). The broad-band proton-decoupled ^{13}C n.m.r. spectrum of the enriched austalide D exhibited extensive (C,C) spin–spin coupling between carbons derived from adjacent acetate units (interacetate and intermevalonate coupling) in addition to the expected spin–spin coupling between carbon atoms derived from intact acetate units (intra-acetate coupling).

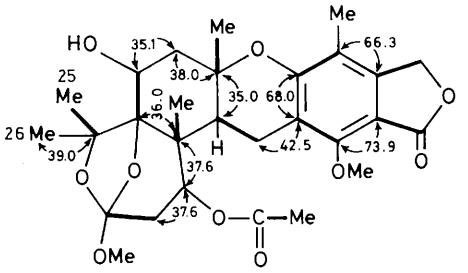


Figure 1. The labelling pattern observed for austalide D enriched with $^{13}CH_3{}^{13}CO_2H$. The intra-acetate (C,C)-couplings are indicated by thick lines. The observed interacetate couplings are shown by thin arrowed lines; the magnitudes of the coupling constants in Hz are shown on the lines.

Additional coupling of this type has previously been observed in several biosynthetic studies using ^{13}C-labelled acetate in which high incorporation efficiencies result in an increased probability of adjacent acetate units being labelled.[6] On detailed examination of the spectrum of the [1,2-$^{13}C_2$]acetate-enriched austalide D, the intra-acetate (C,C)-couplings could be distinguished readily by their greater intensities. The measured ^1J(CC) values of these couplings are given in Table 1 and prove the presence of 11 intact acetate units arranged as shown in Figure 1: C-1–C-9, C-8–C-7, C-6–C-5, C-4–C-3, C-11–C-24, C-13–C-14, C-15–C-25, C-17–C-18, C-20–C-27, C-21–C-22, and C-31–C-32. The lower intensity one-bond (C,C)-couplings observed for the C-12 (δ 40.84), C-19 (δ 71.07), and C-26 (δ 29.65 p.p.m.) resonances arise from interacetate coupling with C-11, C-20, and C-15, respectively, as these three resonances are enhanced in the broad-band proton-decoupled ^{13}C n.m.r. spectrum of austalide D derived from (3*RS*)-[2-^{13}C]mevalonolactone. The pattern of one-bond interacetate and intermevalonate ^{13}C-labelling is indicated in Figure 1.

Only a small amount of austalide D is produced by cultures of *A. ustus* when grown in stationary culture on malt extract medium and instead three new austalides *viz.* K (**4**, R = H) ($C_{25}H_{32}O_5$), L (**4**, R = OH) ($C_{25}H_{32}O_6$; m.p. 207—208 °C), and J (**5**) ($C_{25}H_{32}O_7$, m.p. 284—286 °C) were isolated from the mycelial mats. The structure elucidation of these metabolites is based on a detailed study of their spectral data, particularly their high-field ^1H and ^{13}C n.m.r. spectra, which will be reported in a full paper. The characterization of these apparent precursors of austalide D points to a possible sequence of biosynthetic events which was investigated as follows. Addition of (2*S*)[*Me*-^{14}C]methionine (100 mg, 517.36 μCi mmol^{-1}) to three-day old cultures of *A. ustus* on malt extract medium (1.0 l) gave austalide K (**4**, R = H) (9.1 mg, specific activity 10.56 μCi mmol^{-1}). This ^{14}C-labelled austalide K in ethanol was pulse-fed every 24 h from day 3 to day 14 to cultures of *A. ustus* on potato-dextrose medium (1.0 l) to give austalide D (**1**) (1.5 mg, specific activity 0.42 μCi mmol^{-1}). The dilution value of 25.1 and the absolute incorporation of austalide K (0.5%) are indicative of the intermediacy of this metabolite in the biosynthesis of austalide D.

A plausible mechanism for the formation of austalide D, consistent with the known relative stereochemistry of the austalides,[1] and based on the above results is presented in Scheme 1. The stereospecific oxidative ring closure of (**2**)[2] to give the chromene (**3**) is reminiscent of the corresponding step in the biosynthesis of siccanin.[7] Cyclization of an α-oxirane intermediate generates compound (**4**) with the required *trans-transoid-cis* ring fusion of the austalides *e.g.* K (**4**, R = H) in which the β-methyl of the *gem*-dimethyl moiety is derived from C-2 of mevalonolactone. The proposed enzymatic Baeyer–Villiger oxidation proceeds with retention of configuration[8] and C-26 in austalide D is therefore also derived from C-2 of mevalonolactone. Although the sequence of the C-14 hydroxylation (retention of configuration) in austalide K and the subsequent Baeyer–Villiger oxidation is uncertain, the hydroxy-lactone austalide J (**5**) is formed with the correct stereochemical orientation of the substituents of the seven-membered lactone ring for *in vivo* cyclization to the *ortho*-ester (**6**). *In vitro* austalide J (**5**) was smoothly transformed to the *ortho*-ester (**6**), m.p. 239—241 °C on treatment with either methyl iodide and potassium carbonate in acetone or with thionyl chloride in methanol. The close relationship between (**6**) and austalide D (**1**) is evident from the structures.

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