Stable Isotope Labelling Studies on the Biosynthesis of Asticolorin C by *Aspergillus multicolor*. Evidence for a Symmetrical Intermediate

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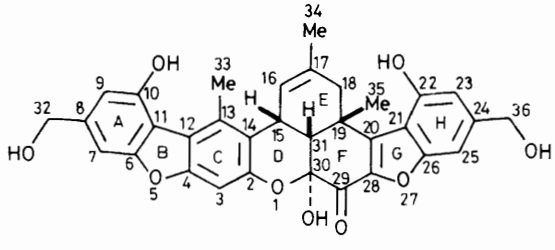
The involvement of mevalonate and four molecules of a symmetrical intermediate, orcinol, in the biosynthetic pathway leading to asticolorin C is evident from incorporation studies with ^{13}C and ^{2}H labelled precursors.

The asticolorins, *e.g.* asticolorin C (**1**), are toxic metabolites produced by cultures of *Aspergillus multicolor*, strain MRC 638.[1] The structures are characterized by the novel way in which a mevalonate-derived 3,3-dimethylallyl group is utilised to link two dibenzofuran moieties. The possible involvement of pannaric acid (3,9-dihydroxy-1,7-dimethyldibenzofuran-2,6-dicarboxylic acid), a compound previously isolated from the lichen *Lepraria membranacea*[2] and formed by oxidative coupling of orsellinic acid, or pannarol (1,7-dihydroxy-3,9-dimethyldibenzofuran) in the biosynthesis of the asticolorins was investigated using primitive ^{13}C and ^{2}H labelled precursors.



(**1**)

Cultures of *A. multicolor* were grown on a yeast extract–sucrose medium. Preliminary feeding experiments with [1-^{14}C]acetate as precursor established that a good incorporation (0.3%) and satisfactory dilution values[3] (20.7, assuming 14 labelled positions) were obtained for asticolorin A[1] by pulse-feeding cultures of *A. multicolor* every 12 h from day 3 to day 14 with sodium acetate to a total amount of 1.0 g l^{-1}.

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Table 1. ^{1}H (500.13 MHz) and ^{13}C (125.76 MHz) n.m.r. data for asticolorin C.[a]

Carbon atom	δ_C [b]	^{1}J(C,C)/Hz [c]	$\Delta\delta$ [c]	δ_H [f]	J(H,H)/Hz
2	149.82 S ●	72.8, 66.2	0.044		
3	97.18 D ★	73.2, 73.2		6.951q	<0.4
4	154.16 S ●	73.3, 58.3	0.046		
6	157.40 S ●	72.0, 60.4	0.049		
7	99.93 D ★	72.2		6.968dt	1.4, 0.7
8	142.35 S ●	46.8	0.053		
			0.052		
9	107.69 D ★	65.9		6.782dt	1.4, 0.6
10	151.37 S ●	75.4, 66.0	0.036		
11	110.67 S ★	75.4, 60.6			
12	118.01 S ★	58.3			
13	131.88 S ●	42.7	0.034		
			0.038		
			0.039		
14	118.31 S ★	65.9			
15	29.56 D ●	42.5	0.082	4.100m	
16	120.60 D ★	42.4		5.533m	
17	130.73 S ●	39.3	0.072		
18	38.89 T ★	38.9		2.559d br	17.5
				2.360d br	17.5
19	34.22 S ●	35.3	0.069		
			0.067		
			0.069		
20	142.50 S ●	[d]			
21	113.15 S ★	73.4, 59.3			
22	152.76 S ●	73.6, 66.5	0.036		
23	107.19 D ★	66.0		6.806dt	1.3, 0.6
24	146.62 S ●	46.3	0.052		
			0.056		
25	100.35 D ★	71.1		7.071dt	1.3, 0.8
26	158.21 S ●	71.3, 58.6	0.044		
28	141.99 S ●	67.2 [d]			
29	180.75 S ★	67.3, 56.1			
30	93.13 S ●	56.2, 41.6	0.089		
31	44.56 D ★	41.5		2.622dd	6.8, 2.0
32	62.73 T ★	47.1		4.543d	4.8
33	18.36 Q ★	42.9		3.039s	
34	32.24 Q ★	42.5		1.641m	
35	25.36 Q ★	35.2		1.829s	
36	62.65 T ★	46.3		4.572d	4.8

[a] Recorded on a Bruker WM-500 spectrometer; solvent $(CD_3)_2SO$. [b] Relative to Me_4Si. Capital letters refer to the pattern resulting from directly bonded (C,H) couplings. S = singlet, D = doublet, T = triplet, and Q = quartet. ● = enriched by [1-^{13}C]acetate; ★ = enriched by [2-^{13}C]acetate. [c] Value obtained from the broad-band proton-decoupled spectrum of asticolorin C derived from [1,2-$^{13}C_2$]acetate. [d] AB spin system obscured by overlapping resonances. [e] ^{13}C–^{2}H Upfield β-shift in p.p.m. [f] Relative to internal Me_4Si. s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. The chemical shifts of the protons of the different hydroxy groups are as follows: C-10, δ 10.26; C-22, 10.84; C-32, 5.193t br; C-36, 4.572t br; C-30, 7.229d (J 2.0 Hz).

The ^{1}H and ^{13}C n.m.r. data for asticolorin C (**1**) are collated in Table 1. The signals of the proton-bearing carbon atoms were correlated with specific proton resonances.[4] Extensive heteronuclear ^{13}C-{^{1}H} selective population inversion experiments,[5] to be described in a subsequent publication, established the two- and three-bond (C,H) connectivity pattern for asticolorin C. The method, however, does not allow us to differentiate between the resonances of the ring A and H carbon atoms. This ambiguity was resolved by the observation of one-bond (C,C) couplings for C-11–C-12 (58.9 Hz) and C-20–C-21 (54.7 Hz) in the broad-band proton-decoupled ^{13}C n.m.r. spectrum of asticolorin C derived from [2-^{13}C]acetate (91.4 atom % ^{13}C). In addition the spectrum showed enhancement of the signals of 19 carbon atoms (average enrichment factor 4.2) whereas that of asticolorin C derived from [1-^{13}C]acetate (91.6 atom % ^{13}C) showed 14 enhanced signals (average enrichment factor 5.0) (see Table 1) so that all the carbon atoms are acetate-derived.

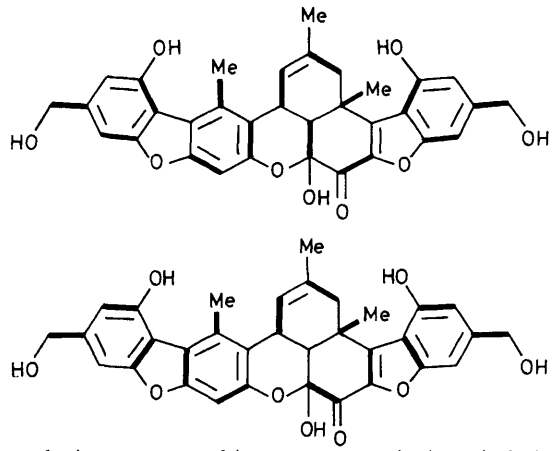

Figure 1. Arrangement of intact acetate units in asticolorin C (**1**) derived from [1,2-$^{13}C_2$]acetate.

The arrangement of intact acetate units in asticolorin C was studied by addition of [1,2-$^{13}C_2$]acetate to the culture medium. The signals of a number of carbon atoms exhibited, as a result of one-bond (C,C) couplings, two pairs of satellite peaks of equal intensity in the broad-band proton-decoupled ^{13}C n.m.r. spectrum of this enriched asticolorin C (see Table 1). This phenomenon, observed for the corresponding carbon atoms in rings A, C, F, and H, indicates the existence of two different arrangements of intact acetate units in each of these rings (see Figure 1). Thus a symmetrical intermediate, orcinol, formed by enzymatic decarboxylation of orsellinic acid, must be involved in the biosynthesis of asticolorin C. Subsequent oxidative coupling of two molecules of orcinol would lead to the formation of pannarol.

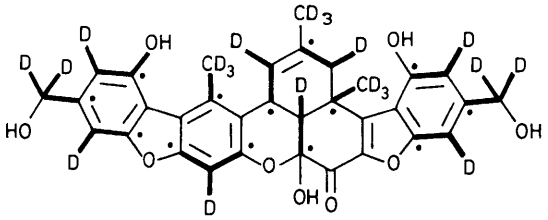

Figure 2. Labelling pattern of asticolorin C enriched by [1-^{13}C,2-$^{2}H_3$]acetate.

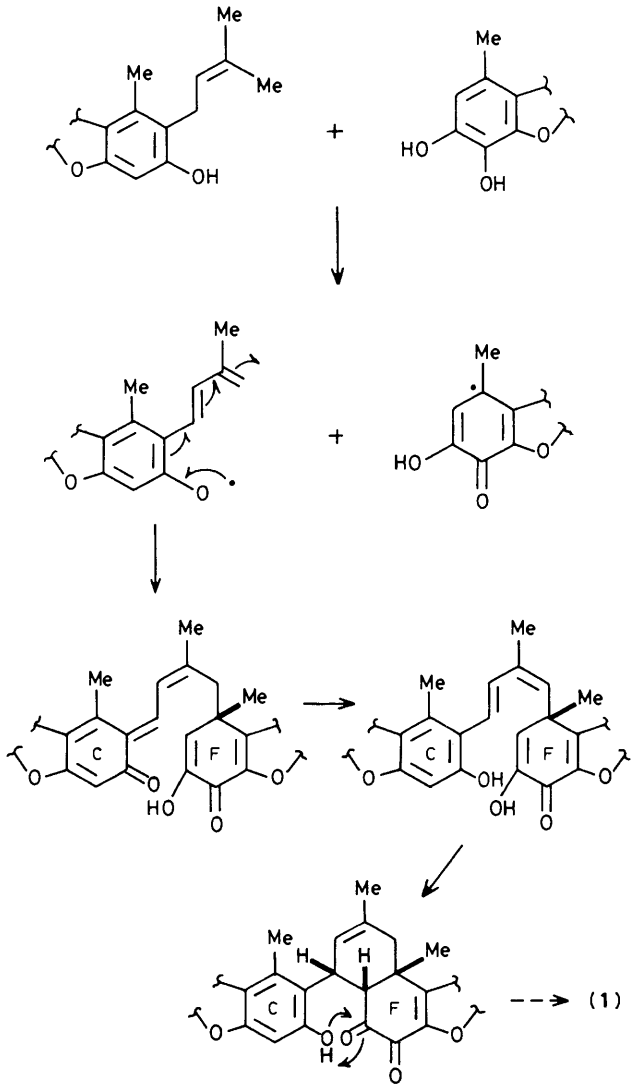

Figure 3. Proposed mechanism for the formation of rings D and E in asticolorin C.

The low-intensity satellite peaks observed for the C-34 signal are due to (C,C) coupling with C-17 and could be the result of multiple labelling as C-34 is derived from C-2 of mevalonate. However, although the C-34 signal is enhanced (enrichment factor 5.0) in the broad-band proton-decoupled ^{13}C n.m.r. spectrum of asticolorin A[1] derived from (3*RS*)-[2-^{13}C]mevalonolactone, an enhancement is also observed for the C-18 signal (enrichment factor 2.2). This result would imply that the stereochemical integrity of the two prochiral enantiotopic methyl groups in 3,9-dihydroxy-1,7-dimethyl-2-(3,3-dimethylallyl)dibenzofuran is lost in the subsequent oxidative coupling reactions leading to asticolorin C. This duality in the origin of C-34 and C-18 is under investigation.

The fate of the hydrogen atoms in the biosynthesis of asticolorin C was studied by incorporation of [1-^{13}C,2-$^{2}H_3$]acetate into the metabolite. The incorporation of ^{2}H located β to a ^{13}C atom can be detected by the small characteristic upfield β-isotope shift in the resonance position of the ^{13}C nucleus in the ^{13}C n.m.r. spectrum.[6] The number of ^{2}H atoms located β to a particular ^{13}C atom can be deduced from the value of the β-isotope shift.[6,7] The labelling pattern of asticolorin C enriched with [1-^{13}C,2-$^{2}H_3$]acetate is shown in Figure 2. The retention of ^{2}H at C-31, evident from the β-isotope shift of −0.089 p.p.m. for C-30 (see Table 1), indicates that C-6 hydroxylation of pannarol occurs at the aromatic stage before oxidative coupling with 3,9-dihydroxy-1,7-dimethyl-2-(3,3-dimethylallyl)dibenzofuran. In the course of this coupling reaction the 4-*pro-R* proton of mevalonate is retained at C-16 in asticolorin C as a β-shift of −0.082 p.p.m. is observed for the C-15 resonance. Significantly, the β-shift observed for the C-17 resonance ($\Delta\delta$ − 0.072 p.p.m.) indicates that only one ^{2}H atom is present at C-18 in asticolorin C. A mechanism for the formation of the central rings D and E of the asticolorins based on the above results is shown in Figure 3.

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