Biosynthesis of Diplosporin by *Diplodia macrospora*. Part 2.[1] Investigation of Ring Formation using Stable Isotopes

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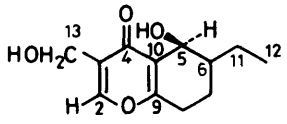
The mode of ring formation in the biosynthesis of diplosporin, a metabolite from *Diplodia macrospora*, has been investigated by incorporation studies with ^{2}H-, ^{13}C-, and ^{18}O-labelled precursors and ^{2}H and ^{13}C n.m.r. spectroscopy.

The biogenesis of diplosporin (1), a mycotoxin elaborated by the maize contaminant *Diplodia macrospora* Earle (MRC 143) has been shown to involve the incorporation of methionine-derived carbon atoms into both a carbocyclic and an heterocyclic ring (Figure 1).[1] In view of the novelty of these events,



(1)

particularly the former, the mechanism of ring formation and the origin and fate of the hydrogen atoms in the biosynthesis of diplosporin were investigated using ^{2}H and ^{18}O labelled precursors.

Cultures of *D. macrospora* were grown on a potato dextrose–yeast extract medium [1] and supplemented in separate experiments with [1-^{13}C, 2-$^{2}H_3$]-, [2-^{13}C, 2-$^{2}H_3$]-, [1-^{13}C, 1-$^{18}O_2$]-, and [2-$^{2}H_3$]acetate, and (2*S*)-[*methyl*-$^{2}H_3$]methionine. The ^{2}H and ^{13}C n.m.r. spectra of the enriched diplosporin samples furnished insight into the biogenetic processes.

The incorporation of ^{2}H into a metabolite can be studied by both direct (^{2}H n.m.r.) and indirect methods (^{13}C n.m.r.).[2] The use of ^{13}C n.m.r. best accommodates precursors in which ^{2}H is attached β to a ^{13}C nucleus. The incorporation of ^{2}H into a metabolite can then be detected by β-^{2}H isotope-induced shifts in the ^{13}C n.m.r. spectrum of the enriched metabolite.[3,4] In the proton-decoupled ^{13}C n.m.r. spectrum of diplosporin derived from incorporation of [1-^{13}C, 2-$^{2}H_3$]acetate, only the resonance due to C-11 shows β-^{2}H isotope shifts indicating the incorporation of ^{2}H at C-12. The presence of three isotopically shifted signals for C-11 (Figure 2a), due to the incorporation of 1—3 ^{2}H atoms into the C-12 methyl group, confirms that this carbon atom is part of the 'starter' acetate unit of the C_{10}-polyketide chain. Experimental evidence has shown that the methyl hydrogens of acetyl-CoA are incorporated into fatty acids in varying degrees; the predominant species (*ca.* 80%) at the terminal methyl group (*i.e.* the methyl group of the starter unit) determined by [2-^{13}C, 2-$^{2}H_3$]acetate, is $^{13}C^{2}H_3$.[5,6] Little loss of ^{2}H from the precursor occurs before the incorporation.[6] The retention of ^{2}H only at C-12, the methyl group of the starter unit, in diplosporin is not surprising as similar results have been obtained in biosynthetic investigations on cytochalasins B and D.[7] The absence of ^{2}H at the other [2-C]-acetate-derived carbon atoms and especially C-8 in diplosporin can be explained as follows. The incorporation of acetate units *via* malonyl-CoA into the progenitor C_{10}-polyketide, followed by a dehydration step as in fatty acid biosynthesis, accounts for

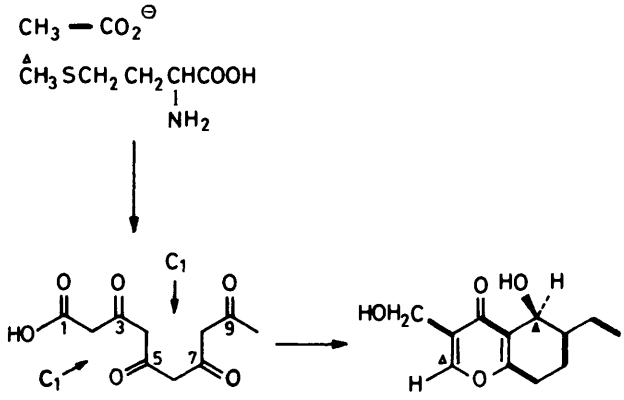


Figure 1. Biosynthesis of diplosporin

the loss of one ^{2}H atom.[5,8] Although ^{2}H can be lost to some degree by non-stereospecific hydrogen exchange at the post-malonate stage [9] it is also likely that further ^{2}H loss from C-6 in the C_{10}-polyketide, *i.e.* C-8 in diplosporin, may be due to involvement of this methylene group in the loss of ketone oxygen from either C-5 or C-7 of the polyketide (C-9 or C-7 in diplosporin).

The proton-decoupled ^{2}H n.m.r. spectrum of diplosporin derived from [2-$^{2}H_3$]acetate is in agreement with the above results. The presence of ^{2}H only at C-12 was evident from the solitary signal at δ 0.93. Final confirmation that hydrogen from acetate is retained only at C-12 in diplosporin was obtained from the proton-decoupled ^{13}C n.m.r. spectrum of [2-^{13}C, 2-$^{2}H_3$]acetate-derived diplosporin. In this instance the only carbon resonance showing an isotope-induced shift was that at δ_c 11.20 which has been assigned to C-12.[1] The signals due to different $^{13}C^{2}H$ species were clearly distinguishable despite the loss in signal to noise ratio in the isotope-shifted resonances caused by poor relaxation and loss of the nuclear Overhauser effect.[3] The presence of $^{13}C^{1}H_2{}^{2}H$, $^{13}C^{1}H^{2}H_2$, and $^{13}C^{2}H_3$ species at C-12 was evident from the triplet, quintet, and heptet signals, respectively [$^{1}J(^{13}C, ^{2}H)$ 19.0 Hz] shifted upfield (Δδ 0.30 p.p.m. per deuterium atom) from the signal due to the $^{13}C^{1}H_3$ species.

Many of the carbon resonances in the p.n.d. ^{13}C n.m.r. spectrum of [2-^{13}C, 2-$^{2}H_3$]acetate-derived diplosporin also showed low intensity satellite resonances due to one-bond (C,C)-couplings (Table). We have previously observed the transformation of [2-^{13}C]- into [1,2-$^{13}C_2$]-acetate by operation of the Krebs' citric acid cycle in the biosynthesis of roquefortine [10] and penitrem A.[11] The enrichment of the carbon atoms derived from (2*S*)-methionine, C-2 and C-5, by [2-^{13}C]-acetate arises from conversion of acetate *via* the Krebs' cycle and pyruvate into serine.[12,13] Subsequent dehydroxymethyl-

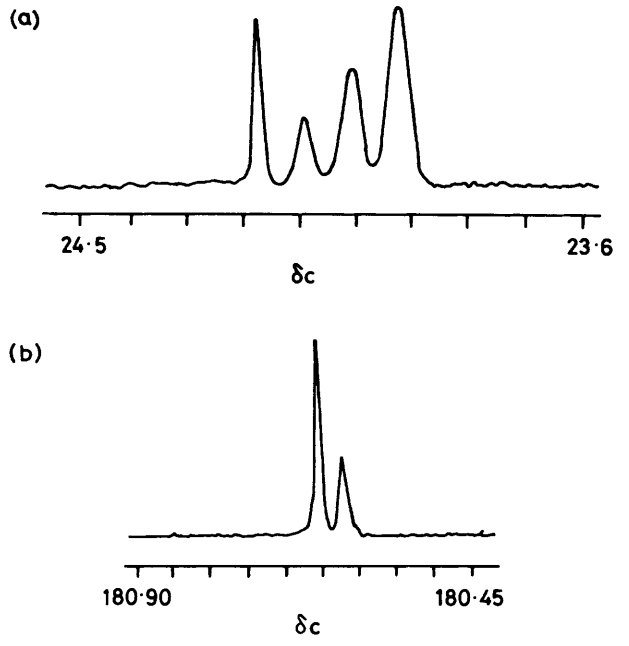


Figure 2. (a) Part of the 50.3 MHz proton-decoupled ^{13}C n.m.r. spectrum of diplosporin derived from [1-^{13}C,2-$^{2}H_3$]acetate showing the C-11 resonance and β-isotope shifts (0.085 p.p.m. per ^{2}H) by mono-, di-, and tri-deuteriated species at C-12. (b) Part of the 50.3 MHz proton-decoupled ^{13}C n.m.r. spectrum of diplosporin derived from [1-^{13}C, $^{18}O_2$]acetate showing the resonance due to C-4 and the isotope shift (0.035 p.p.m.) for the directly bonded $^{13}C-^{18}O$ species

ation of serine would give rise to glycine and a ^{13}C-enriched C_1 pool. The values of the coupling constants $^{1}J(C,C)$ obtained from the proton-decoupled ^{13}C n.m.r. spectrum of [1,2-$^{13}C_2$]acetate-derived diplosporin [1] are in close agreement with those observed (Table).

Further insight into the biosynthetic pathway comes from the incorporation of [1-^{13}C, $^{18}O_2$]acetate (57.9% $^{13}C^{18}O_2$, 25.1% $^{13}C^{18}O$, 4.7% $^{13}C^{16}O$) [14] and (2*S*)-[*methyl*-$^{2}H_3$]methionine into diplosporin. The sites of ^{18}O enrichment are determined by ^{13}C n.m.r. spectroscopy taking advantage of the ^{18}O isotope-induced upfield shifts for the signals of directly attached ^{13}C carbon atoms.[15-17] The presence of [^{18}O]acetate-derived oxygen atoms in diplosporin was detected only at C-4; the resonance at δ 180.7 exhibiting an isotopically shifted signal (Figure 2b). The magnitude of the upfield shift (0.035 p.p.m.) is typical of carbonyl oxygen atoms.[14,16,18]

The p.n.d. ^{2}H n.m.r. spectrum of diplosporin derived from (2*S*)-[*methyl*-$^{2}H_3$]methionine showed two signals at δ 7.80 and δ 4.50, belonging to 2-^{2}H and 5-^{2}H respectively.[19]

The above results do not define an unambiguous biosynthetic pathway leading to diplosporin. It seems likely, however, that C-2 arises from C-methylation of C-3 rather than by *O*-methylation of O-1, as O-1 is not derived from [^{18}O]acetate. There is no evidence to distinguish whether C-5 is derived by C-methylation of C-6 or C-10. The presence of ^{2}H derived from (2*S*)-[*methyl*-$^{2}H_3$]methionine at both C-2 and C-5 implies that oxidation at these two centres does not occur beyond the aldehyde oxidation level during the biosynthesis.

Experimental

^{2}H N.m.r. spectra were recorded on a Varian XL-200 spectrometer (30.7 MHz). ^{13}C N.m.r. were recorded on either a Varian XL-200 (50.3 MHz) or a Bruker WM-400 spectrometer (100.62 MHz).

General conditions for the feeding experiments with the differently labelled precursors to cultures of *Diplodia macrospora* (MRC 143) and isolation and purification of the enriched diplosporin are described in reference 1.

A summary of the yields of diplosporin for the differently labelled precursors is given below.

Table. Values for $^{1}J(C,C)$ observed in the 100.62 MHz proton-decoupled ^{13}C n.m.r. spectrum of [2-^{13}C, 2-$^{2}H_3$]acetate-derived diplosporin

Carbon atom	^{1}J(C,C(/Hz)	Carbon atom	^{1}J(C,C)(Hz)
2	71.5	8	33.2, 48.0
3	71.5, 49.7, 9.4	9	48.0, 69.4
4	54.2	10	70.0, 54.9, 46.7,
5	39.1, 46.3		8.8
6	39.2, 33.9	11	35.6
7	31.7, 33.8	13	49.4

Precursor	Amount (mg)	Volume of medium (ml)	Yield of diplosporin (mg)
Sodium [1-^{13}C, 2-$^{2}H_3$]acetate	133	300	57
Sodium [2-^{13}C, 2-$^{2}H_3$]acetate	200	400	47
Sodium [1-^{13}C, $^{18}O_2$]acetate	200	400	51
Sodium [2-$^{2}H_3$]acetate	1 000	1 000	188
(2*S*)-[*methyl*-$^{2}H_3$]Methionine	100	1 000	101

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References

1 Part 1: A. A. Chalmers, C. P. Gorst-Allman, P. S. Steyn, R. Vleggaar, and D. B. Scott, *J. Chem. Soc., Perkin Trans. 1*, 1979, 1481.
2 M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, 539.
3 C. Abell and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 856.
4 T. J. Simpson, A. E. de Jesus, P. S. Steyn, and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, 1982, 632.
5 R. H. White, *Biochemistry*, 1980, **19**, 9.
6 A. G. McInnes, J. A. Walter, and J. L. C. Wright, *Tetrahedron Lett.*, 1979, 3245.
7 R. Wyss, Ch. Tamm, and J. C. Vederas, *Helv. Chim. Acta*, 1980, **63**, 1538.
8 K. Bloch and D. Vance, *Ann. Rev. Biochem.*, 1977, **46**, 263.
9 B. Sedgwick and J. W. Cornforth, *Eur. J. Biochem.*, 1977, **75**, 465; B. Sedgwick, J. W. Cornforth, S. J. French, R. T. Gray, E. Kelstrup, and P. Willadsen, *ibid.*, 1977, **75**, 481; B. Sedgwick, C. Morris, and S. J. French, *J. Chem. Soc., Chem. Commun.*, 1978, 193; B. Sedgwick and C. Morris, *J. Chem. Soc., Chem. Commun.*, 1980, 96; K. Saito, A. Kawaguchi, Y. Seyama, T. Yamakawa, and S. Okuda, *J. Biochem. (Tokyo)*, 1981, **90**, 1697.
10 C. P. Gorst-Allman, P. S. Steyn, and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, 1982, 652.
11 A. E. de Jesus, W. E. Hull, P. S. Steyn, F. R. van Heerden, R. Vleggaar, and P. L. Wessels, *J. Chem. Soc., Chem. Commun.*, 1982, 837.
12 G. Ehrensvard, L. Reio, E. Saluste, and R. Stjernholm, *J. Biol. Chem.*, 1950, **183**, 93.

13 T. J. Simpson and J. S. E. Holker, *Phytochemistry*, 1977, **16**, 229.
14 A. E. de Jesus, W. E. Hull, P. S. Steyn, F. R. van Heerden, and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, 1982, 902.
15 J. M. Risley and R. L. van Etten, *J. Am. Chem. Soc.*, 1979, **101**, 252; *ibid.*, 1980, **102**, 4609, 6699; *ibid.*, 1981, **103**, 4389.
16 J. C. Vederas, *J. Am. Chem. Soc.*, 1980, **102**, 374.
17 J. C. Vederas and T. T. Nakashima, *J. Chem. Soc., Chem. Commun.*, 1980, 183; T. T. Nakashima and J. C. Vederas, *ibid.*, 1982, 206; M. P. Lane, T. T. Nakashima, and J. C. Vederas, *J. Am. Chem. Soc.*, 1982, **104**, 913; J. G. Hill, T. T. Nakashima, and J. C. Vederas, *J. Am. Chem. Soc.*, 1982, **104**, 1745.
18 J. Diakur, T. T. Nakashima, and J. C. Vederas, *Can. J. Chem.*, 1980, **58**, 1311.
19 A. A. Chalmers, C. P. Gorst-Allman, N. P. J. Kriek, W. F. O. Marasas, P. S. Steyn, and R. Vleggaar, *S. Afr. J. Chem.*, 1978, **31**, 111.

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