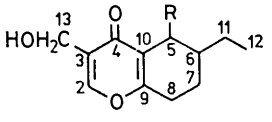
Biosynthesis of Diplosporin by *Diplodia macrospora*. Ring Formation involving Methionine-derived Carbon Atoms

By **Anthony A. Chalmers, Charles P. Gorst-Allman, Pieter S. Steyn,* and Robert Vleggaar,** National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria 0001, Republic of South Africa
De Buys Scott, Faculty of Agriculture, University of Pretoria, Pretoria 0002

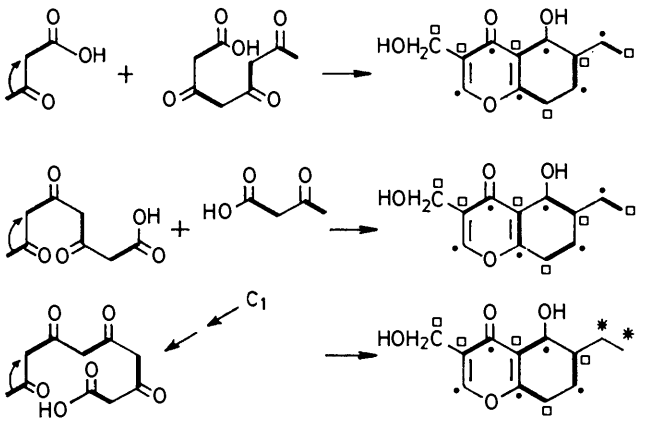
The biosynthesis of diplosporin, a metabolite produced by *Diplodia macrospora*, has been studied utilizing both ^{14}C- and ^{13}C-labelled precursors. The results indicate that diplosporin is formed from methionine and a single acetate-derived polyketide chain. This study presents a rare instance in which methionine-derived carbon atoms are incorporated into a carbocyclic and a heterocyclic ring. The inhibitory effect of methionine on the production of diplosporin was noted. A related metabolite, 5-deoxydiplosporin, was isolated from the culture medium of *D. macrospora*.

In a recent communication the isolation and structure elucidation of diplosporin (1), a mycotoxin elaborated



(1) R=OH
(2) R=H

by the maize contaminant *Diplodia macrospora* Earle, was reported.[1]



Scheme 1 Possible biosynthetic routes to diplosporin

The biosynthetic origins of the metabolite are not obvious from a cursory inspection of the reported structure (1). Three plausible mechanisms are indicated in Scheme 1. Two of these involve the condensation of two separate acetate–polymalonate-derived units; similar mechanisms have been proposed for the bioformation of ochrephilone [2] and for mollisin.[3] The subsequent elaboration of the resultant precursor would involve a 1,2-alkyl migration, a process which is not without precedent.[4,5] A similar type of rearrangement, *viz.* 1,2-migration of a phenyl group, occurs in the biosynthesis of the isoflavones.[6] The third postulate shown in Scheme 1 involves a single polyketide chain and has the ethyl side-chain introduced *via* successive methylations with methionine, as in the case of the phytosterols.[7]

D. macrospora sporulates only sparingly on agar slopes and cultures were, therefore, maintained on sterilized maize. The fungus was cultured on a potato dextrose–yeast extract medium by introducing a *D. macrospora* infected maize kernel into the medium.

Table 1

Diplosporin production

Days after inoculation	Yield of diplosporin/ mg l^{-1}	Total yield of lipid/ mg l^{-1}
3	trace	25
5	trace	20
7	50	120
10	225	475
12	250	500
17	450	790
21	260	565

Initial experiments indicated that production of diplosporin paralleled the production of total lipids, and started on the sixth day after inoculation when the cultures entered the idiophase (Table 1). The results

Table 2

Specific radioactivities and yields of diplosporin derived from ^{14}C-labelled precursors

^{14}C-precursor	Amount of labelled precursor fed/μCi [a]	Amount of carrier fed/ mg [a]	Specific activity of precursor/ μCi mmol^{-1}	Yield of diplosporin/ mg [a]	Incorporation (%)	Specific activity of diplosporin/ μCi mmol^{-1}	Dilution factor
Sodium [1-^{14}C]acetate	250	1 000	59 500	80	2.50	8.79	6 769
2*S*-[methyl-^{14}C]methionine	8.33	0	55 000	108	11.16	1.93	28 497
	8.33	50	24.86	70	13.98	3.73	6.67
	8.33	100	12.43	30	5.51	3.43	3.62
	8.33	150	8.29	28	5.49	3.66	2.27
	8.33	200	6.21	20	3.95	3.69	1.68
	8.33	250	4.97	21	4.43	3.94	1.26

[a] Values relative to 0.5 l of culture medium.

TABLE 3

^{13}C N.m.r. data of diplosporin (1) and 5-deoxy diplosporin (2)

Carbon atom	δ [a] Diplosporin (1)	Enrichment [b] [1-^{13}C]acetate	[2-^{13}C]acetate	2S-[methyl-^{13}C]-methionine	$^{1}J_{CC}$	δ [a] 5-Deoxydiplosporin (2)
2	152.0	1.00	1.00	4.26		151.3d
3	127.0	[c]	4.98	0.52	49.0	125.8s
4	179.9	8.98	0.93	0.82	55.2	179.3s
5	68.2	0.95	0.76	7.01		27.4 †t
6	41.1	0.53	9.15	1.42	33.1	34.6d
7	22.8	13.89	0.79	1.47	33.0	26.6 †t
8	26.3	0.38	5.82	0.91	47.9	27.4t
9	165.6	11.23	0.88	0.82	48.0	164.4s
10	123.3	[c]	5.35	0.87	55.0	122.1s
11	23.9	12.82	0.97	1.00	35.0	28.4t
12	10.8	0.51	9.24	0.86	34.9	11.8q
13	57.2	12.83	1.06	2.87	49.4	58.4t

[a] Relative to internal Me_4Si. [b] Ratios between peak heights of the observed resonances of ^{13}C-enriched and natural abundance diplosporin recorded under identical conditions. Pulse spacing 10 s. [c] Not observed.

† These values may be interchanged. s, d, t, q are s.f.o.r.d. multiplicities; s = singlet, d = doublet, t = triplet, q = quartet.

indicated a possible acetate–polymalonate pathway for the biosynthesis of diplosporin; an analogous relationship was found in the formation of aflatoxin B_1 in *Aspergillus parasiticus*.[8]

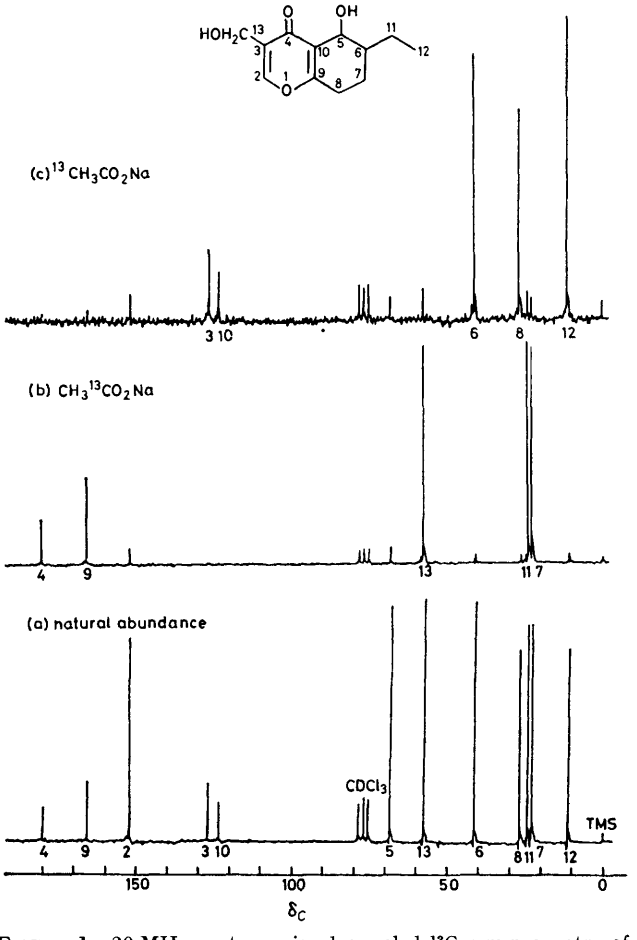
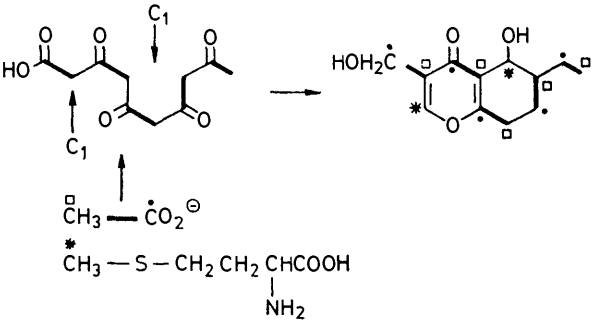


FIGURE 1 20-MHz proton-noise-decoupled ^{13}C n.m.r. spectra of diplosporin; (a) natural abundance; (b) derived from [1-^{13}C]acetate; (c) derived from [2-^{13}C]acetate. Spectral width 4 000 Hz

The addition of sodium [1-^{14}C]acetate (250 μCi) together with carrier sodium acetate (1 g) to growing cultures of *D. macrospora* gave diplosporin in good yield. The high specific activity (8.8 μCi mmol^{-1}) of the diplosporin (Table 2) as well as the absolute incorporation (2.5%) of sodium acetate verified its role as precursor in the biosynthesis.

In subsequent feeding experiments cultures of *D. macrospora* were pulsed with either [1-^{13}C]- or [2-^{13}C]-acetate. The proton-noise-decoupled (p.n.d.) ^{13}C n.m.r. spectrum of diplosporin derived from [1-^{13}C]acetate (Figure 1) showed five enhanced signals due to C(4), C(7), C(9), C(11), and C(13) whereas the spectrum due to [2-^{13}C]acetate-derived diplosporin showed enhanced signals representative of C(3), C(6), C(8), C(10), and C(12). The spread in the value of the individual enrichment factors (*i.e.* the factor by which the natural ^{13}C abundance has been increased) (Table 3) is a problem which has been encountered previously and has no biosynthetic implications in the present case.[4,9] Mass spectrometry indicated an average enrichment factor (*i.e.* the average ratio of excess ^{13}C abundance to the natural ^{13}C abundance) of 15.0 for each labelled position in the [1-^{13}C]acetate-derived diplosporin and an absolute incorporation of 4.9%. The comparable figures for the [2-^{13}C]acetate-derived compound were 14.6 and 4.9%.



SCHEME 2 Biosynthetic route to diplosporin

The above data account for the origin of ten of the twelve carbon atoms in diplosporin, and indicate that the biosynthetic postulates as shown in Scheme 1 have to be discarded. A new biosynthetic pathway involving a single polyketide chain, with the carbon atoms derived from [1-^{13}C]- and [2-^{13}C]-acetate occupying alternating positions is shown in Scheme 2.

Proof for this new hypothesis and the mode of folding of the polyketide progenitor was provided by addition of sodium [1,2-^{13}C]acetate to cultures of *D. macrospora*. All the signals in the p.n.d. ^{13}C n.m.r. spectrum of the [1,2-^{13}C]acetate-derived diplosporin (Figure 2) with the exception of those for C(2) and C(5) exhibited ^{13}C–^{13}C spin–spin coupling. The measured $^{1}J_{CC}$ values are given in Table 3 and prove the presence of the following intact acetate units *viz.* C(13)–C(3), C(4)–C(10), C(9)–C(8), C(7)–C(6), and C(11)–C(12).

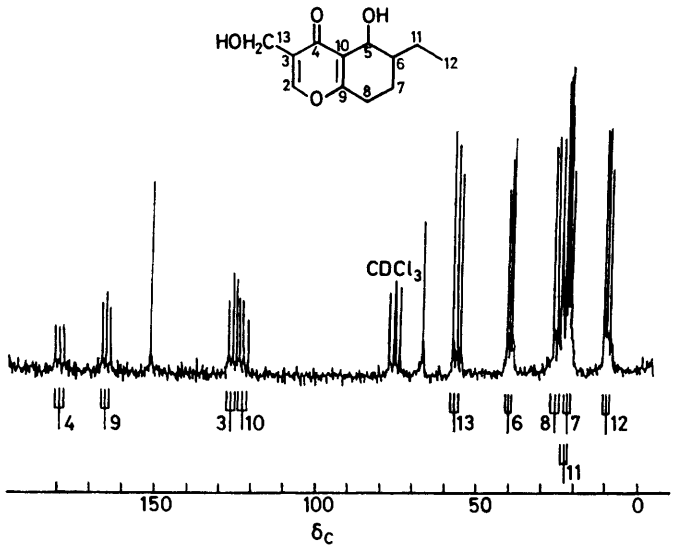


FIGURE 2 Proton-noise-decoupled ^{13}C n.m.r. spectrum of diplosporin derived from [1,2-^{13}C]acetate

The next step in the biosynthetic investigation was to establish the origin of the remaining two carbon atoms, C(2) and C(5). Methionine is an excellent source of one-carbon units in nature and in a preliminary experiment 2*S*-[methyl-^{14}C]methionine (8.33 μCi) was efficiently incorporated into diplosporin (absolute incorporation 11.2%).

The risk of catabolic repression and of inhibition of metabolite production is a major disadvantage associated with the relatively large amounts of stable isotope labelled precursors required in biosynthetic studies. The effect of varying concentrations of added exogenous 2*S*-methionine (spiked in each case with 2*S*-[methyl-^{14}C]-methionine) on the yield of diplosporin was quite dramatic (Table 2). Many biosynthetic pathways of secondary metabolism share intermediates with primary pathways; taken together they constitute branched sequences. In such cases feedback regulation of an early enzyme by the primary end product might be expected to diminish production of the secondary metabolite.[10] An example is the inhibition of β- and α-cyclopiazonic acid production in *Penicillium cyclopium* by 2*S*-tryptophan.[11] The role of methionine in the growth of *D. macrospora* and the production of diplosporin is under investigation. The p.n.d. ^{13}C spectrum of diplosporin derived from 2*S*-[methyl-^{13}C]methionine (Figure 3) showed two enhanced carbon signals due to C(2) and C(5), thus confirming their origin. The absolute incorporation of methionine as determined by mass spectrometry was 7.3% while the average enrichment factor for each labelled carbon atom was 6.8.

C- and *O*-methylation of precursors in a biosynthetic pathway occurs *via* *S*-adenosylmethionine and is a common occurrence. The introduction of a methionine-derived carbon atom into a heterocyclic ring is much less common but has been observed in *e.g.* the biosynthesis of canescin in *Aspergillus malignus* [12] and in that of the rotenones.[13] To our knowledge the presence of a methionine-derived carbon atom in a carbocyclic ring has been demonstrated in two cases *viz.* the biogenesis of the tropolones *via* a rearrangement of 3-methylorsellinic acid [14] and in the production of the cyclopropane ring in lactobacillic acid by *C*-methylation of a *cis*-vaccenic acid.[15] The incorporation of a methionine-derived carbon atom into the carbocyclic ring of diplosporin is thus a highly unusual event. The mechanism probably proceeds by methylation of the C_{10}-polyketide progenitor chain at either the C(10) or C(6) methylene group, followed by oxidative activation of the newly-formed *C*-methyl group to facilitate ring closure. Although the origin of the C(2) carbon atom of diplosporin is shown in Scheme 2 as a *C*-methylation the possibility of *O*-methylation cannot be excluded.

The results obtained from these ^{13}C-labelling experiments provide additional proof for the structure of diplosporin.[1]

A related metabolite, 5-deoxydiplosporin (2), $C_{12}H_{16}O_3$, was isolated from the culture medium of *D. macrospora*. The u.v. ($\lambda_{max.}$ 219 and 258 nm; log ε 3.73 and 3.72) and i.r. data ($\nu_{max.}$ 3 440, 1 660, and 1 610 cm^{-1}) indicate the presence of a γ-pyrone moiety. It was apparent that the substance lacked either the primary or secondary hydroxy-function which is present in diplosporin. Inspection of the p.n.d. and s.f.o.r.d. ^{13}C n.m.r. spectra of the metabolite (Table 3) established the presence of the primary hydroxy-function, and of four methylene carbon atoms. The fragmentation pattern in the mass spectrum of (2) was also consistent with the assigned structure, the

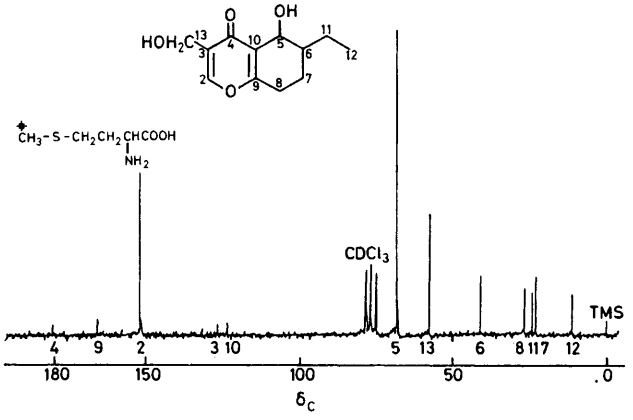


FIGURE 3 Proton-noise-decoupled ^{13}C n.m.r. spectrum of diplosporin derived from 2*S*-[methyl-^{13}C]methionine

ion at *m/e* 152 corresponding to *retro*-Diels–Alder processes in either ring. In diplosporin the ion at *m/e* 150 corresponds to a *retro*-Diels–Alder collapse in the heterocyclic ring with concomitant loss of water from the carbocyclic ring.[1]

EXPERIMENTAL

For instrumental data see reference 1. ^{14}C-Labelled compounds were crystallized to constant activity. Radioactivity was measured on a Packard 3003 liquid scintillation spectrometer and samples were calibrated by internal standardization with n-[1-^{14}C]hexadecane.

Incorporations of Sodium [1-^{14}C]-, [1-^{13}C], [2-^{13}C]-, *and* [1,2-^{13}C]*Acetate*.—Preliminary experiments on cultures of *D. macrospora* (U.P. 370) grown in static culture on a medium consisting of potato dextrose broth (24 g l^{-1}) and yeast extract (1 g l^{-1}) showed that production of diplosporin commenced on day six, and reached a maximum on the eighteenth day after inoculation (Table 1).

Typical Procedure.—To each of ten 250-ml Erlenmeyer flasks containing the 6-day old cultures of *D. macrospora* on the above medium (50 ml) was added 0.25 ml of a solution of [1-^{13}C]acetate (500 mg, 90% enriched) in water (10 ml) every 24 h from day six to day nine. After a further 48 h the culture was filtered, and the filtrate extracted with chloroform (2 × 100 ml). The organic phase was dried (Na_2SO_4), and the diplosporin purified by p.l.c. on silica plates developed with chloroform–acetone–isopropanol (85 : 15 : 20 v/v/v). Crystallization from benzene–n-hexane gave diplosporin (1) (78 mg), m.p. 83—84° (lit.,[1] 83—84°) (average enrichment factor per labelled carbon atom 15.0; absolute incorporation 4.9%).

In separate experiments the following precursors were added as described above: [1-^{14}C]acetate (250 μCi) and sodium acetate (1.0 g) to twenty flasks to yield diplosporin (160 mg) (specific activity 8.8 μCi mmol^{-1}; absolute incorporation 2.5%); [2-^{13}C]acetate (250 mg, 90% enriched) to five flasks to give diplosporin (40 mg) (average enrichment factor per labelled carbon atom 14.6; absolute incorporation 4.9%); [1,2-^{13}C]acetate (125 mg, 90% enriched) and sodium acetate (375 mg) to ten flasks to yield diplosporin (56 mg) (average enrichment factor per labelled carbon atom 4.6; absolute incorporation 4.3%).

Incorporation of 2S-[*Methyl*-^{14}C]- *and* 2S-[*Methyl*-^{13}C]-*methionine*.—The procedure followed is as described above. For the ^{13}C-labelling experiment 2*S*-[methyl-^{13}C]methionine (50 mg; 90% enriched) was added to ten flasks to yield diplosporin (35 mg) (average enrichment factor per labelled carbon atom 6.8; absolute incorporation 7.3%).

For the ^{14}C-labelling experiments 2*S*-[methyl-^{14}C]-methionine (8.33 μCi) and cold methionine (0, 50, 100, 150, 200, and 250 mg) were added to separate batches of ten flasks to yield diplosporin (108, 70, 30, 28, 20, and 21 mg, respectively) (Table 2).

Isolation of 5-Deoxydiplosporin (2).—*D. macrospora* was grown on 5 l of medium as above. Seventeen days after inoculation the cultures were filtered and the filtrate extracted with chloroform (3 × 500 ml). The organic layer was dried (Na_2SO_4), filtered, and evaporated to give a yellow gum which was purified by column chromatography on silica gel (Merck Type H) (500 g) under pressure (100 kPa) using chloroform–acetone–hexane (7 : 2 : 1 v/v/v). The first metabolite to be eluted was *5-deoxydiplosporin* (2) (76 mg) as an oil, $[\alpha]_D^{20}$ +55° (*c* 1.1, MeOH); $\lambda_{max.}$ 258 and 219 nm (log ε 3.73 and 3.72); $\nu_{max.}$ 3 440, 1 660, 1 610, 1 590, and 1 450 cm^{-1} (Found: M^+, 208.110 4. Calc. for $C_{12}H_{16}O_3$: *M*, 208.109 9).

Later fractions gave diplosporin (1) (1.90 g), m.p. 83—84° (lit.,[1] 83—84°).

We thank Darrell Towers for technical assistance.

[8/1233 *Received*, 4*th July*, 1978]

REFERENCES

[1] A. A. Chalmers, C. P. Gorst-Allman, P. S. Steyn, R. Vleggaar, W. F. O. Marasas, and N. P. J. Kriek, *S. Afr. J. Chem.*, 1978, **31**, 111
[2] H. Seto and M. Tanabe, *Tetrahedron Letters*, 1974, 651.
[3] M. L. Casey, R. C. Paulick, and H. W. Whitlock, *J. Amer. Chem. Soc.*, 1976, **98**, 2636.
[4] C. P. Gorst-Allman, K. G. R. Pachler, P. S. Steyn, P. L. Wessels, and D. B. Scott, *J.C.S. Perkin I*, 1977, 2181.
[5] M. Tanabe, M. Uramoto, T. Hamasaki, and L. Cary, *Heterocycles*, 1976, **5**, 355.
[6] E. Wong, *Prog. Chem. Org. Nat. Prod.*, 1970, **28**, 1.
[7] M. Castle, G. Blondin, and W. R. Nes, *J. Amer. Chem. Soc.*, 1963, **85**, 3306; V. Villanueva, M. Barbier, and E. Lederer, *Bull. Soc. chim. France*, 1964, 1423.
[8] C. N. Shih and E. H. Marth, *Biochem. Biophys. Acta*, 1974, **338**, 286.
[9] C. P. Gorst-Allman, P. S. Steyn, P. L. Wessels, and D. B. Scott, *J.C.S. Perkin I*, 1978, 961
[10] S. W. Drew and A. L. Demain, *Ann. Rev. Microbiol.*, 1977, **31**, 343.
[11] R. M. McGrath, P. S. Steyn, N. P. Ferreira, and D. C. Neethling, *Bio-organic Chem.*, 1976, **4**, 11.
[12] A. J. Birch, J. J. Wright, F. Gager, L. Mo, and A. Pelter, *Tetrahedron Letters*, 1969, 1519.
[13] L. Crombie, P. M. Dewich, and D. A. Whiting, *J.C.S. Chem. Comm.*, 1970, 1469; 1971, 1182, 1183.
[14] A. I. Scott, H. Guilford, and E. Lee, *J. Amer. Chem. Soc.*, 1971, **93**, 3534; A. G. McInnes, D. G. Smith, L. C. Vining, and L. Johnson, *J.C.S. Chem. Comm.*, 1971, 325; A. I. Scott and K. J. Wiesner, *ibid.*, 1972, 1075.
[15] E. Lederer, *Quart. Rev.*, 1969, **4**, 453 and references cited therein.