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THE ISOLATION AND STRUCTURE OF A TOXIC METABOLITE FROM *DIPLODIA MAYDIS* (BERK.) SACC.

P. S. STEYN, P. L. WESSELS and C. W. HOLZAPFEL†
National Chemical Research Laboratory, C.S.I.R., Pretoria, South Africa
and
D. J. J. POTGIETER and W. K. A. LOUW
Department of Biochemistry, Faculty of Agriculture, University of Pretoria, Pretoria

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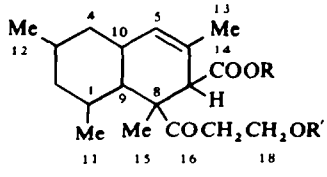
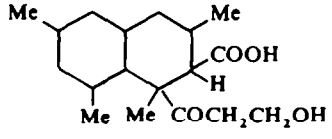
Abstract– Diplodiatoxin is a toxic metabolite of *Diplodia maydis* (Berk.) Sacc. Studies are described which have led to the structure as shown in Ia.

Diplodia maydis (*Diplodia zeae* (Schw.) Lév.) also known as "dry rot" or "cob rot" is a frequent infection of maize and causes a well known disease, diplodiosis, among cattle and sheep in Southern Africa. The first symptoms are lachrymation, salivation and a slight quivering of the muscles of shoulder and flank. The cause of death is muco-enteritis and nephritis.[1] In order to identify the toxic principles a strain of *D. maydis* was cultivated on wet sterile maize. The toxic mouldy maize was dried and continually extracted with chloroform-methanol (72 hr) to yield a crude extract. Bio-assay revealed that this extract accounted for 10% of the total activity of the mouldy maize. The remaining toxic component was neither removed nor destroyed by a similar subsequent extraction. Systematic fractionation of the crude extract, guided by bio-assay indicated that the toxicity was associated with the acidic fraction.[2] The mixture of acids was separated by TLC to give a toxic compound, designated diplodiatoxin. Production of this metabolite starts 18 days after inoculation and reaches a maximum after 30 days.

The proposed structure of diplodiatoxin is unique among fungal metabolites in containing a β-ketol side chain and the rare β, γ-unsaturated acid unit.



Ia: R = R′ = H
Ib: R = Me, R′ = H
Ic: R = Me, R′ = COMe



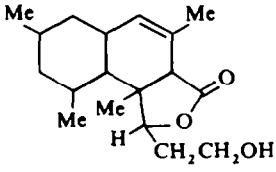
II

† Present address: Department of Chemistry, Rand Afrikaans University, Johannesburg.

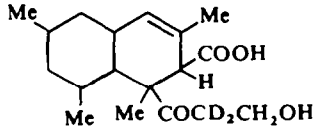
Diplodiatoxin crystallised from chloroform, m.p. 186–187° and had $[\alpha]_D$ + 101°. Elemental analysis and high-resolution mass spectroscopy showed it to have a molecular composition of $C_{18}H_{28}O_4$. The UV spectrum of diplodiatoxin showed a ketone absorption [λ_{max}^{EtOH} 293 nm (ε 50)]. Its IR spectrum exhibited strong OH absorption at 3530 cm^{-1}, a band at 1720 cm^{-1} which together with broad absorption between 2500 and 2800 cm^{-1} indicated a carboxyl group, and a sharp band at 1700 cm^{-1} was attributed to the CO group. The empirical formula of diplodiatoxin indicates that the compound must contain in addition to the two CO groups three double bonds equivalents. The presence of one olefinic bond in this molecule was established by prolonged hydrogenation (60 hr) over PtO_2 in AcOH to yield dihydrodiplodiatoxin $C_{18}H_{30}O_4$ (II). Diplodiatoxin furthermore reacted with only one molar equivalent of bromine and its methyl ester (Ib) gave a mono-epoxide upon epoxidation (see below). The presence of a decalin system in this molecule was established by dehydrogenation over Pd/C to give a tetramethylnaphthalene (M^+, 184) which had typical naphthalene UV characteristics *viz*, λ_{max}^{MeOH} 233, 284, 310, 318 and 323 nm in a ratio of 100:25 < 1 : < 1 : < 1. A very low yield precluded characterization.

Diplodiatoxin behaved like a typical carboxylic acid *e.g.*, solubility in aq $NaHCO_3$. Reaction with ethereal diazomethane yielded a methyl ester $C_{19}H_{30}O_4$ (Ib) (ν_{max} 1743 and 1700 cm^{-1} and no absorption between 2500 and 2800 cm^{-1}). In the presence of morpholine diplodiatoxin showed ν_{max} 1620 and 1585 cm^{-1} (carboxylate) and 1700 cm^{-1} (CO group). The β,γ-unsaturated acid moiety in Ia was indicated by its ready decarboxylation above 190°, while dihydrodiplodiatoxin (II) is stable under these conditions.

The characterization of the other oxygen functions was achieved as follows. Acetylation of the ester (Ib) gave acetoxydiplodiatoxin methyl ester $C_{21}H_{32}O_5$ (Ic) (ν_{max} 1743 and 1699 cm^{-1}). The CO group of diplodiatoxin was inert towards reaction with 2,4-dinitrophenylhydrazine, hydrazine or hydroxylamine under standard conditions. Diplodiatoxin reacted slowly with aq $NaBH_4$ yielding a γ-lactone $C_{18}H_{26}O_3$ (III) (ν_{max} 1762 cm^{-1}), via reduction of the CO group with concurrent lactonization. This experiment established the positional relationship of the CO and carboxyl groups. Exchange of enolisable protons in diplodiatoxin (D_2O/NaOD) gave a dideutero compound $C_{18}H_{26}D_2O_4$ (IV), indicating the presence of two enolisable protons adjacent to the CO group.



III



IV

The CD spectrum of Ia showed a Cotton effect at 293 nm, $\Delta\varepsilon = -0{\cdot}43$ attributable to the ketone group and a strong Cotton effect at 224 nm $\Delta\varepsilon = +11{\cdot}60$ which was assigned to β,γ-unsaturated acid moiety in Ia since it was absent in dihydrodiplodiatoxin (II) which exhibited only the ketone Cotton effect at 293 nm, $\Delta\varepsilon = -1{\cdot}42$. The

enhancement of the Cotton effect associated with the n → π* transition of the carboxyl group is apparently due to homoconjugation of the carboxyl group with the β,γ-double bond. The carboxyl group must adopt a preferred conformation allowing a favourable disposition of the interacting chromophores. In the lactone (III), $\Delta\varepsilon_{224\,nm} = +2{\cdot}09$, the interacting groups are in a fixed arrangement which must be less favourably disposed for interaction.

The literature contained no information on CD data of β,γ-unsaturated acids. The CD spectra of a number of model compounds containing this moiety were determined and the relevant data are summarized in Table 1. Compounds VII and VIII were obtained by submitting the corresponding lactones to acid treatment.[3] Solidagoic acid A (V)[4] and maingayic acid (VI)[5] have the same molecular formulae but differences in stereochemistry at one or more of the chiral centres. These compounds gave Cotton effects below 240 nm which were virtually the mirror image of that of diplodiatoxin. The strong Cotton effects at *ca* 224 nm, Δε 9 to 16 appears to be diagnostic of

TABLE 1. CD DATA OF β,γ-UNSATURATED ACIDS

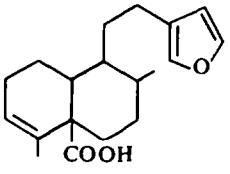
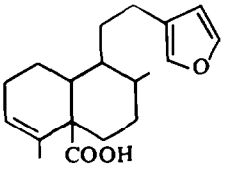
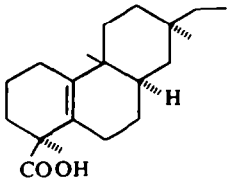
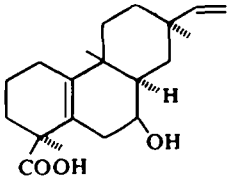
Compound		λ(nm)	Δε
Diplodiatoxin (Ia)		293	−0·43
		224	+11·60
	Solidagoic Acid A (V)	223	−9·4
	Maingayic Acid (VI)	225	−16·6
	VII	224	−10·08
	VIII	223	−9·1

TABLE 2. PMR DATA OF DIPLODIATOXIN AND DERIVATIVES

	Compound									
	Chemical Shift (δ) and Multiplicity (Hz)									
Proton	Ia	Ib	Ic	II	III	IV	IX	Xa	Xb	XI
1								1·39 (see text)		
3								1·69 (see text)		
4								1·75(m) J_{AB} = 13 2·27(m)	1·15(m) J_{AB} = 13 2·28(m)	
5	5·36(br.s.)	5·37(br.s.)	5·36(br.s.)		5·27(br.s.)	5·37(br.s.)	2·76(s)	3·65(d) S = 9·7	3·61(d) S = 9·7	5·31(br q.)
7	2·70(br.s.)	2·68(br.s.)	2·68(br.s.)	2·43(d) S = 3·8	2·61(br.s.)	2·70(br.s.)	2·73(s)	2·93(s)	2·94(s)	2·61(br.s.)
9	2·09(t) S = 9·0	2·04(t) S = 9·0	2·03(t) S = 9·3	2·05(t) S = 9·0		2·10(t) S = 9·0	2·06(t) S = 10·0			
11	0·59(d) J = 5·5	0·55(d) J = 6·1	0·55(d) J = 6·0	0·52(d) J = 6·2	0·87(d) J = 6·2	0·57(d) J = 6·2	0·61(d) J = 6·0	0·88(d) J = 6·0	0·92(d) J = 6·2	0·83(d) J = 6·2
12	0·87(d) J = 5·8	0·88(d) J = 5·7	0·88(d) J = 5·9	0·86(d) J = 6·0	1·02(d) J = 6·0	0·89(d) J = 5·9	0·88(d) J = 5·4	1·00(d) J = 5·2	1·02(d) J = 5·4	0·96(d) J = 6·6
13	1·64(br.s.)	1·59(br.s.)	1·58(br.s.)	0·93(d) J = 7·0	1·93(br.s.)	1·66(s)	1·23(s)	1·54(s)	1·53(s)	1·89(br.s.)
15	1·24(s)	1·24(s)	1·23(s)	1·30(s)	1·04(s)	1·26(s)	1·26(s)	1·25(s)	1·27(s)	1·22(s)
16					4·93(q)S = 4·1, 10·4					
17*	2·90 ABXY	2·86(t) S = 5·2	2·97 ABXY†	2·85 ABXY	3·84	3·84	2·99 ABXY	2·01 ABXY	2·10 ABXY	ABC 4·87
18*	3·88	3·82(t) S = 5·2	4·30	3·86	3·81(t) S = 6·0	3·95 J_{AB} = 11·9	3·79	3·89	4·32	
OMe		3·58(s)	3·56(s)				3·61(s)			
OAc			1·98(s)						2·04(s)	2·04(s)

S = Splittings

* The centre of the multiplets

† J_{AB} = −18·5, J_{AX} = 5·7, J_{AY} = 6·7, J_{BX} = 7·0, J_{BY} = 6·8, J_{XY} = −11·2 Hz
δ_A = 2·93, δ_B = 3·05, δ_X = 4·27, δ_Y = 4·35.

β,γ-unsaturated acids contained in a system where the interacting chromophores have a relative disposition suitable for efficient interaction.

Proton and ^{13}C magnetic resonance have been used extensively in deriving the proposed structure for diplodiatoxin. The values of the different PMR parameters which could be obtained from PMR spectra of diplodiatoxin and its derivatives are given in Table 2. The 100 MHz spectrum of diplodiatoxin methylester (Ib) is reproduced in Fig. 1.

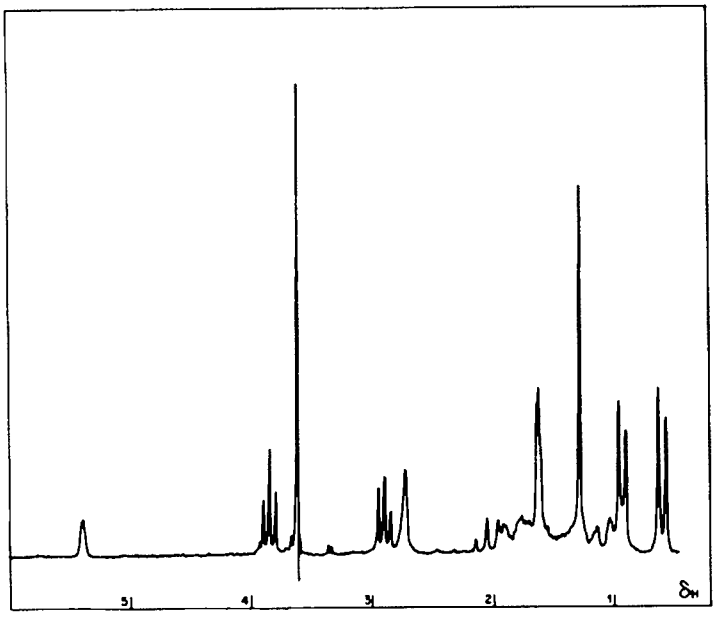


FIG. 1. The 100 MHz PMR Spectrum of Diplodiatoxin Methyl Ester.

One of the most prominent features of the PMR spectra of diplodiatoxin as well as of those derivatives still containing the CO group was the appearance of two well separated 3 proton doublets. In compound Ib at δ 0·88 (J = 5·7 Hz) and δ 0·55 (J = 6·1 Hz) assigned to the secondary Me group at positions 3 and 1, respectively. The mass spectrum of Ia showed no loss of an isopropyl group, this observation was in agreement with the double resonance experiments which proved conclusively that the two Me groups were located at different C atoms. Irradiation at the resonance frequency of these Me groups resulted in a change in the spectrum at different positions. The resonance frequencies of the C_3–H and C_1–H protons were established in the dibromolactone (Xa) (see below) as δ 1·69 and δ 1·39, respectively by noting the value of the second strong radiofrequency when the doublets collapsed to singlets. The very high chemical shift of one of the secondary Me groups (δ 0·55) was thought to be due to the anisotropic effect of the nearby CO group. This effect is also experienced by the proton at position 9 which appeared as a triplet at δ 2·04 (S = 9.0 Hz).

The latter splittings indicated a *trans* fusion of the decalin system and a diaxial relationship of the protons at positions 1 and 9. Inspection of a Dreiding model of diplodiatoxin (Ia) indicated a dihedral angle of close to 90° between C_{10}–H and C_5–H. The dihedral angle is consonant with the lack of observable coupling between the latter protons.

In the γ-lactone (III) the newly formed proton at position 16 gave rise to a quartet at δ 4·93 (S = 4·1, 10·4 Hz) while the adjacent methylene protons shifted upfield. Due to the absence of the CO group in III the Me group at position 8 and the proton at position 9 shifted upfield while the Me group at position 1 shifted to low field δ 0·87 (J = 6·2 Hz). This may be considered as strong evidence for the location of those groups in diplodiatoxin.

In the PMR spectrum of diplodiatoxin methyl ester the resonances resulting from the alicyclic protons (H_1 to H_4 and H_{10}) could not be employed in the structural elucidation due to the complexity of the spectrum between δ 0·5 to 1·9 (Fig 1). The ^{13}C NMR spectrum of the methyl ester (Ib) has therefore been recorded to assist in the structure determination.

The proton noise decoupled 25·1 MHz PFT—CMR spectrum of diplodiatoxin methyl ester (Ib) in $CDCl_3$ is depicted in Fig 2. Chemical shifts are relative to internal TMS indicating down field shifts with positive sign.† The ^{13}C shift data obtained in $CDCl_3$ and CD_3COCD_3 are given in Table 3. According to the chemical shifts[6, 7] diplodiatoxin methyl ester (Ib) contains two CO groups, two olefinic and fifteen aliphatic and cycloaliphatic C atoms. The peak at δ_C = 41·9 contained resonances from two C atoms as has been shown by the proton noise decoupled CMR spectrum of Ib in CD_3COCD_3 where two peaks appeared in this region (Table 3).

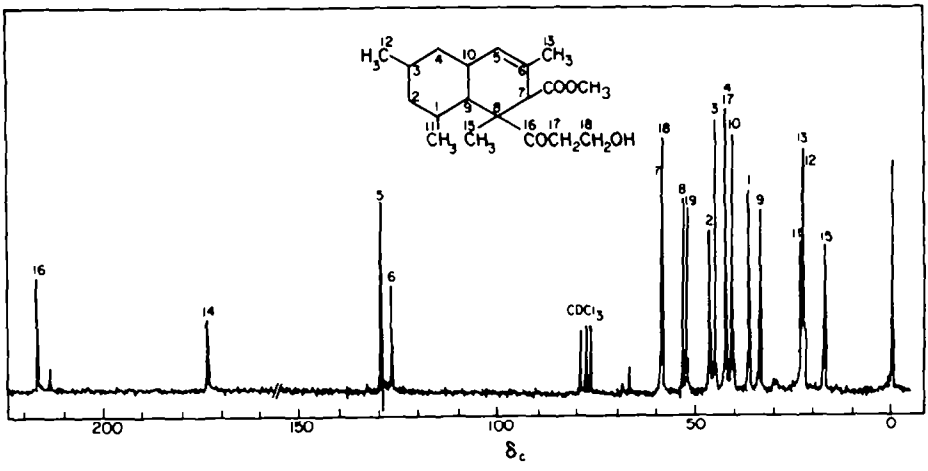


FIG. 2. The 25·1 MHz proton noise decoupled pulsed Fourier ^{13}C NMR Spectrum of Diplodiatoxin Methyl Ester relative to TMS at δ_C = 0.

† In compliance with the decision taken during the ^{13}C NMR Symposium at the 21st Pittsburg Conference on Analytical Chemistry and Applied Spectroscopy Cleveland, Ohio, 1st to 6th March (1970).

TABLE 3. ^{13}C CHEMICAL SHIFTS OF DIPLODIATOXIN METHYL ESTER (Ib)

δ_C^*(p.p.m.)		Multiplicity†	Assignment
$(CD_3)_2CO$	$CDCl_3$		
	216·1	S	16
	173·0	S	14
129·3	129·4	D	5
127·4	126·7	S	6
58·3	58·4	D	7
58·0	58·1	T	18
52·9	52·9	S	8
51·8	52·1	Q	19
46·7	46·1	T	2
45·0	44·8	D	3
42·8	41·9	T	17
42·6	41·9	T	4
40·8	40·4	D	10
36·4	36·2	D	1
33·6	33·4	D	9
23·2	23·1	Q	11
22·6	22·6	Q	13
22·4	22·2	Q	12
16·8	16·9	Q	15

* Relative to internal TMS.

† Obtained from an off-resonance proton decoupling experiment. S singlet, D doublet, T triplet and Q quartet.

The CO resonances at $\delta_C = 216·1$ is attributed to the keto C atom (C—16) and the peak at $\delta_C = 173·0$ to the ester carbonyl C atom (C—14). The latter assignment is confirmed by the down field shift of the peak in the CMR spectrum of diplodiatoxin in which C—14 is an acid carbonyl carbon.

In an off-resonance proton decoupling experiment the olefinic resonances at $\delta_C = 129·3$ and $\delta_C = 126·7$ appeared as a doublet and singlet, respectively. This experiment clearly indicates the presence of a $-CH=C\diagup\diagdown$ grouping present in Ib.

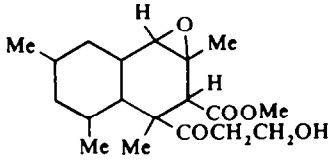
Similar experiments carried out on the aliphatic and acylic region between $\delta_C = 16$ and $\delta_C = 60$ revealed a singlet at $\delta_C = 52·9$ (quaternary C atom), doublets at $\delta_C = 58·4$, 44·8, 40·4, 36·2 and 33·4 (5 methine C atoms) triplets at $\delta_C = 58·1$, 46·9, 41·9 and 41·9 (4 methylene C atoms) and quartets at $\delta_C = 52·1$, 23·1, 22·6, 22·4 and 16·8 (5 methyl C atoms). The peak at $\delta_C = 52·9$ is therefore assigned to C_8. The Me resonances at $\delta_C = 52·1$, attributed to the methoxy C atom,[6,7] was absent in the CMR spectrum of diplodiatoxin. The assignment of the other Me peaks (Table III) has been deduced from the off-resonance proton decoupling experiment only, with the assumption that the $^{13}C—H$ coupling constants in these groups are identical.[8]

From the known chemical shifts of alcohols[9] and ketones[10] and the off-resonance decoupling results the peaks at $\delta_C = 58·1$ and $\delta_C = 41·9$ have been assigned to C—18 and C—17, respectively which formed part of the β-ketol side chain. The remaining

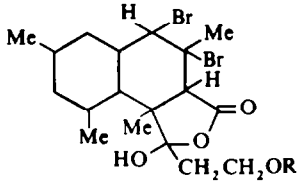
two methylene resonances are assigned to C—4 (δ_C = 41·9) and C—2 (δ_C = 46·1). This assignment is based on data[11] obtained from an extensive study of the ^{13}C shifts of Me substituted cyclohexanes *viz.* C atom β to a Me group: δ_C = 36·6 ± 0·4 C atoms atoms β to two Me groups: δ_C = 45·1 ± 0·5 and C atom further removed from the Me group δ_C = 28·1 ± 0·3. The values of the chemical shifts obtained for C—2 and C—4 are therefore confirmatory for the location of these groups on the cyclohexane ring in diplodiatoxin.

The absorption at δ_C = 58·4 is attributed to C—7 but the assignment of the other methine carbon peaks should be regarded as tentative only. The assignment given in Table 3 for these resonances are based only on the off-resonance proton-decoupled results. For these C atoms identical directly bonded C—H coupling constants cannot be assumed with the same degree of confidence as for Me groups. The assignment of the peaks at δ_C = 33·4 to C_9 is in accord with the observation that a C atom β to a double bond shows a greater shift to high field than one α to the double bond.[12]

Several experiments were carried out on diplodiatoxin to probe the chemical environment of the olefinic proton. Treatment of diplodiatoxin methyl ester (Ib) with *m*-chloroperbenzoic acid gave as a major product the mono-epoxide $C_{19}H_{30}O_5$ (IX). The proton at position 5 shifted to higher field but still appeared as a singlet at δ 2.76.



IX

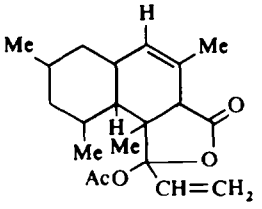


Xa: R = H
Xb: R = $COCH_3$

Bromination of diplodiatoxin with diluted bromine in methanol led immediately to a neutral dibromolactone formulated as Xa. Its IR spectrum shows the absence of any CO absorption (no absorption between 1600 and 1790 cm^{-1}), however, strong absorption at 1806 cm^{-1} (γ-lactol). This bromination reaction was monitored by recording the intensity of the ketone Cotton effect a 293 nm upon the addition of bromine. This absorption was destroyed immediately upon addition of one molar equivalent of bromine; addition of HBr had no influence on this ketone Cotton effect. This experiment establishes very elegantly the collatoral lactolization and masking of the CO group upon bromination of Ia.

The most important feature in the PMR spectrum of Xa is a doublet at δ 3·65 (S = 9·7 Hz) which was assigned to the proton at position 5. This proves that position 10 carried one proton. Two-one-proton multiplets occurred at δ 1·75 and δ 2·27 which exhibited a splitting of 13, 8 and 6 Hz. This pattern is attributed to the methylene protons at position 4. The low field absorption (δ 2.27) of one of these protons may be due to the effect of the bulky nearby Br atom at position 5. Acetylation of Xa furnished a monoacetate (Xb) [ν_{max} 3480 cm^{-1} (OH) 1745 cm^{-1} (acetate CO) and 1806 cm^{-1} (lactol CO)].

Diplodiatoxin was essentially stable towards a variety of experimental conditions which would effect retro-aldol cleavage of a β-ketol. The stability of diplodiatoxin towards alkali treatment is probably due to presence of the proximate carboxylate group which would impede abstraction of a proton from the primary alcohol group. Elimination of water from the β-ketol side chain was readily effected upon treatment with acetic anhydride and sodiumacetate, followed by lactolization and acetylation to yield XI. Its IR spectrum showed $\nu_{max}^{CHCl_3}$ 1736 cm^{-1} (acetate CO) and 1800 cm^{-1} (lactol CO). In the PMR spectrum of XI the acetate protons appeared at δ 2·04 (s, 3H) and the exocyclic double bond gave rise to a degenerate ABC pattern centred at δ 4·87.



XI

The mass spectrum of diplodiatoxin (Ia) showed prominent fragments at 308 (M^+, $C_{18}H_{28}O_4$) (15%) *m/e* 290 ($M^+ - H_2O$) (21%), 235 ($M^+ - COCH_2CH_2OH$) (52%), 209 ($M^+ - C_5H_7O_2$) (53%), 191 (*m/e* 235—CO_2) (100%), 190 (*m/e* 235—HCO_2) (90%), 189 (*m/e* 235—HCO_2H) (98%), 121 (*m/e* 191—C_5H_{10}) (35%), 120 (*m/e* 190—C_5H_{10}) (60%), 119 (*m/e* 189—C_5H_{10}) (90%), 73 ($COCH_2CH_2OH$) (45%) and 69 (C_5H_9) [45%]. Strong metastable peaks were associated with the following fragmentations 235 → 189 (152), 190 → 120 (75·80) and 189 → 119 (74·95).

Confirmatory mass spectral data were obtained for dideuterodiplodiatoxin M^+, 310 $C_{18}H_{26}D_2O_4$ (IV) in showing a peak at *m/e* 235 ($M^+ - COCD_2CH_2OH$) and a peak at *m/e* 75 ($COCD_2CH_2OH$). Diplodiatoxin methyl ester (Ib) had M^+, 322 $C_{19}H_{30}O_4$ (Ib), *m/e* 263 ($M^+ - CO_2CH_3$), 249 ($M^+ - COCH_2CH_2OH$) and 209 ($M^+ - C_6H_9O_2$) and at lower mass units a spectrum virtually identical to that of Ia.

These mass spectral data as well as those of other derivatives were in accordance with the presence of a β-ketol side chain in diplodiatoxin. Furthermore, the loss of $C_5H_7O_2$ from the molecular ion in Ia occurred by cleavage of the 7,8- and 5,10-bonds with a transfer of hydrogen and represented the β, γ-unsaturated acid unit. A distinct characteristic of the mass spectra of Ia, Ib and Ic is the losses of C_5H_{10} from the fragments at *m/e* 191 ($C_{14}H_{23}$), 190 ($C_{14}H_{22}$) and 189 ($C_{14}H_{21}$) to give peaks at *m/e* 121 (C_9H_{13}), 120 (C_9H_{12}) and 119 (C_9H_{11}), respectively. Strong metastable peaks are associated with these fragmentations. It is therefore likely that the fragment C_5H_{10} could have originated from the $CH_3-\underset{H}{\overset{R}{C}}-CH_2-\underset{CH_3}{\overset{R}{C}}-H$ part of diplodiatoxin.

This observation is consonant with the PMR and ^{13}MR data which indicated the location of the secondary methyl groups at position 1 and 3.

EXPERIMENTAL

UV absorption refers to EtOH and IR absorption to $CHCl_3$. UV spectra (Unicam Model S.P. 800 Spectrometer) and IR spectra (Perkin-Elmer Model 237 Spectrometer). Mass spectra were taken on a MS-9 double focusing mass spectrometer. The CD spectra were recorded at 21° with a JASCO ORD UV-5 instrument with attachment for CD measurements and concentrations are given as mg/100 ml MeOH throughout. PMR spectra were recorded on a Varian HA-100 Spectrometer in $CDCl_3$. ^{13}C NMR spectra were recorded on a Varian XL-100-FT spectrometer in $CDCl_3$ and $(CD_3)_2CO$. TLC chromatography was carried out on Merck precoated SiO_2 plates, layer thickness, 0·25 mm and 1·25 mm for analytical and preparative separations, respectively. Chromogenic agent for TLC plates was a soln of 1% $Ce(SO_4)_2$ in 6N H_2SO_4.

Isolation of diplodiatoxin. Diplodia maydis (Berk.) Sacc. was grown in bulk on wet sterilized maize for 30 days. The dried maize (4·9 kg) was ground and extracted with $CHCl_3$–MeOH over a period of 3 days and the solvent removed under reduced press. The toxic residue (355 g) was treated with $CHCl_3$ and partitioned between $CHCl_3$ (4 l) and water (4 l). The $CHCl_3$ layer was extracted with 0·5 M $NaHCO_3$ and the acidified aqueous phase extracted with $CHCl_3$ (4 × 1·5 l) to yield the carboxylic acids (4·0 g). The acids were separated by preparative TLC on twelve SiO_2 plates (40 × 20 cm). Solvent benzene: AcOH 4:1 (v/v). The band at R_f 0·50 represented diplodiatoxin, which after crystallization from $CHCl_3$ gave diplodiatoxin (550 mg) m.p. 187°; $[\alpha]_D^{28°}$ + 101° (*c*, 4·0; $CHCl_3$); λ_{max} 293 nm (ε 50), ν_{max} 3530, 2960, 2800–2500 (broad), 1720, 1700, 1465, 1392, 1078 and 950 cm^{-1}. CD (*c*, 10·0 MeOH): Δε (315 nm) 0, (293 nm) − 0·43, (255 nm) 0, (224 nm) + 11·60, (205 nm) 0.

The high resolution mass spectrum showed: *m/e* 308·198 (M^+, $C_{18}H_{28}O_4$ requires: 308·1987), 235·1701 ($C_{15}H_{23}O_2$ requires: 235·1698), 209·1525 ($C_{12}H_{21}O_2$ requires: 209·1541), 190·1729 ($C_{14}H_{22}$ requires: 190·1721), 120·0937 (C_9H_{12} requires: 120·0938), 73·0295 ($C_3H_5O_2$ requires: 73·0289). [Found: C, 70·11; H, 9·25. $C_{18}H_{28}O_4$ requires: C, 70·10; H, 9·15%].

Diplodiatoxin methyl ester (Ib). Diplodiatoxin (150 mg) in $CHCl_3$ (100 ml) was treated with excess ethereal diazomethane at room temp for 0·5 hr. The excess of reagent was removed in a stream of dry N_2 to yield Ib (155 mg) as an oil, ν_{max} 1743 (ester CO) and 1699 (ketone CO) cm^{-1}; *m/e* 322 (M^+, $C_{19}H_{30}O_4$ requires: 322).

Acetoxydiplodiatoxin methyl ester (Ic). The ester Ib (30 mg) in pyridine: Ac_2O 1:1 (5·0 ml) was left at room temp for 16 hr. The mixture was poured on ice and extracted into $CHCl_3$ to give Ic (32 mg) as an oil, ν_{max} 1743 (ester CO) and 1699 (ketol CO) cm^{-1}; *m/e* 364 (M^+, $C_{21}H_{32}O_5$ requires: 364).

Dihydrodiplodiatoxin (II). Diplodiatoxin (25 mg) was hydrogenated in AcOH (10 ml) over PtO_2 (15 mg). After uptake of 1 mol of H_2 during 5 min the absorption ceased. The experiment was continued for an additional 60 hr. The mixture was filtered and the filtrate evaporated to give II (25 mg), m.p. 185–186° (from $CHCl_3$); CD (*c*, 42, MeOH) Δε (330 nm) 0, (293 nm) − 1·42 and (250 nm) 0; *m/e* 310·214 (M^+, $C_{18}H_{30}O_4$ requires: 310·214).

$NaBH_4$ *reduction of diplodiatoxin.* Diplodiatoxin (80 mg) in water (40 ml) containing $NaBH_4$ (100 mg) was stirred at room temp. Additional portions of $NaBH_4$ (100 mg) were added after 24 hr and 72 hr. The lactone III (60 mg) crystallised from the mixture. It had m.p. 112°; CD (*c*, 27 MeOH): Δε (250 nm) 0, (225 nm + 2·09), (210 nm) 0, ν_{max} 1762 cm^{-1} (lactone CO); *m/e* 292·2046 (M^+, $C_{18}H_{28}O_3$ requires 292·2038).

Deuterium exchange of enolisable protons in diplodiatoxin. Diplodiatoxin (20 mg) in D_2O (15 ml) containing Na (80 mg) was sealed off and kept at room temp for 7 days. The diplodiatoxin was isolated and this experiment repeated to yield IV (18 mg), *m/e* 310 (M^+, $C_{18}H_{26}D_2O_4$ requires: 310).

Epoxidation of dipodiatoxin methyl ester (Ib). The ester Ib (60 mg) in $CHCl_3$ (30 ml) containing *m*-chloroperbenzoic acid (120 mg) was stirred at room temp for 20 hr. Standard workup, followed by separation on preparative SiO_2 TLC in $CHCl_3$:MeOH 98:2 (v/v) yielded IX (144 mg) as an oil, ν_{max}^{CO} 1738 and 1698 cm^{-1}; *m/e* 338·2090 (M^+, $C_{19}H_{30}O_5$ requires: 338·2093).

Preparation of the dibromolactone (Xa). Diplodiatoxin (30 mg) in MeOH (4 ml) was treated with diluted Br_2 in MeOH (15 mg/ml) with exclusion of sunlight at room temp. Crystals of Na_2SO_3 (5 mg) were added when a slightly yellow colour persisted. The MeOH was evaporated at low temp and the residue partitioned between $CHCl_3$ and water. The organic phase was separated on SiO_2 TLC in $CHCl_3$ to yield Xa (28 mg) at R_f 0·20. The oily compound had ν_{max} 1806 cm^{-1} (lactol CO). A molecular ion was not observed by mass spectroscopy, however, a peak at M^+–HBr *viz*: *m/e* 386·1103 ($C_{18}H_{27}O_4Br$ requires 386·1093).

Preparation of the acetate of the dibromolactone. The above Xa (20 mg) in pyridine: Ac_2O 1:1 (6 ml) was left at room temp for 16 hr. The reagents were removed at low temp and the residue partitioned between $CHCl_3$ and water. The organic layer was separated by SiO_2 TLC in $CHCl_3$, yielding Xb (22 mg) at R_f 0·60.

It had $\nu_{max}^{liquid\ film}$ 3480 (OH), 1802 (lactol CO) and 1740 (ester CO) cm^{-1}. A molecular ion was not observed in the mass spectrometer.

Preparation of compound XI. Diplodiatoxin (10 mg) in Ac_2O (1·0 ml) was treated with NaOAc (20 mg) at 95° for 1·5 hr. The mixture was poured onto ice and extracted into $CHCl_3$. The organic layer was separated by SiO_2 TLC in $CHCl_3$ yielding XI (8 mg). Compound XI appeared at R_f 0·60 in this system. It had ν_{max} 1736 (acetate CO) and 1800 cm^{-1} (lactol CO). *m/e* 332 ($C_{20}H_{28}O_4$ requires: 332).

Dehydrogenation of diplodiatoxin. Diplodiatoxin (50 mg) was heated at 250–280° with 30% Pd/C for 30 min in an oxygen free N_2 atmosphere. The mixture was mixed with MeOH (20 ml) and filtered. The filtrate was separated by SiO_2 TLC in benzene-hexane 1:1 (v/v). The major product at R_f 0·90 was isolated and characterized as a tetramethylnaphthalene (< 1 mg). It had λ_{max} 233, 284, 310, 318 and 323 nm in ratio of 100:25: < 1: < 1: < 1; *m/e* 184 (M^+, $C_{14}H_{16}$ requires: 184).

Preparation of model compounds for CD studies. Iso(?) ros-11:12-en-16-oic acid VII (m.p. 134° from aq EtOH) was prepared from dihydrodesoxy-rosenonolactone by the method of Harris *et al.*,[3] lit. m.p. 134°.

Rosenolic acid VIII (m.p. 172–174°) from acetone was similarly prepared[3] from rosenololactone, lit. m.p. 173°.

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