Structure and Biosynthesis of Bipolaramide, a Novel Dioxopiperazine from *Bipolaris sorokiniana*

Catherine M. Maes, 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
Gordon W. Kirby, David J. Robins, and W. Marshall Stark
Department of Chemistry, University of Glasgow, Glasgow G12 8QQ

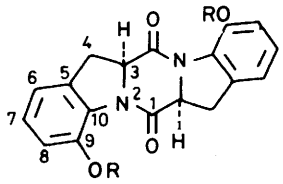
The structure of bipolaramide, a new dioxopiperazine isolated from cultures of *Bipolaris sorokiniana*, is based on ^{1}H and ^{13}C n.m.r. data and *X*-ray crystallography. Incorporation studies with ^{14}C-labelled precursors established that bipolaramide is formed from phenylalanine *via cyclo*-(L-phenylalanyl-L-phenylalanyl).

Investigations on the causal factors of 'yellow thick-head' disease, a major photosensitization syndrome among sheep in the Karoo area of South Africa, led to the isolation of a toxigenic strain of the fungus *Bipolaris sorokiniana* (Saccardo) Shoem. (MRC 93) from the indigenous weed *Tribulus terrestris*. Extraction of bulk cultures of *B. sorokiniana* grown on wet sterilized maize with a mixture of chloroform–methanol, and fractionation of the crude extract, resulted in the isolation of a new, nontoxic dioxopiperazine, bipolaramide (**1**),[1] and the known mycotoxin, sterigmatocystin.

Bipolaramide (**1**) crystallized from acetone as needle-shaped crystals, m.p. 296—297 °C, and analysed for $C_{18}H_{14}N_2O_4$ (M^+, 322); it had $[\alpha]_D$ − 210° (*c* 1.0 in acetone); $\lambda_{max.}$ (MeOH) 218, 260, and 294 nm (ε 44 670, 14 790, and 7 585, respectively); $\nu_{max.}$ (KBr) 1 645 (amide CO) and 1 610 cm^{-1}. The presence of only half the expected resonances in both the ^{1}H and ^{13}C n.m.r. spectra (Table 1) is indicative of a symmetrical dimeric structure for bipolaramide. Acetylation of (**1**) using pyridine and acetic anhydride failed and only starting material was recovered. The phenolic hydroxy groups were acetylated under more forcing conditions with acetic anhydride and catalytic amounts of perchloric acid at −78 °C to give di-*O*-acetylbipolaramide (**2**), m.p. 240—242 °C.

The structure of bipolaramide is based on its ^{1}H and ^{13}C n.m.r. data and a single-crystal *X*-ray crystallographic analysis of the orthorhombic crystals, space group $P2_12_12_1$ with $a = 24.55(1), b = 8.08(1), c = 7.22(1)$ Å, $Z = 4$, which we have reported previously.[1] The crystallographic data indicated the existence of strong intramolecular hydrogen-bonding between the proton of the C-9 hydroxy group and the oxygen atom of the carbonyl group. The O–H • • • O distances are 1.783 and 1.781 Å. The intramolecular hydrogen-bonding is also evident from the chemical shift value (δ_H 11.204) of the C-9 hydroxy-group proton in the ^{1}H n.m.r. spectrum. The vicinal coupling constants, ^3J 9.8 Hz, observed for the C-3 and C-4 protons are in accord with the dihedral angles of 149.8° and 31.6° obtained from *X*-ray crystallography for H(3)–C(3)–C(4)–H(4a) and H(3)–C(3)–C(4)–H(4b), respectively.

The C-3 proton, the X-part of an ABX spin system, appears as a double doublet (^3J 9.8 and 9.8 Hz) at δ_H 5.423. The AB part is formed by the C-4 methylene protons, which resonate at δ_H 3.502 and 3.377, respectively. The low-field resonance exhibits in addition to the two- (^2J 16.3 Hz) and three-bond (^3J 9.8 Hz) couplings, two long-range couplings of 1.2 Hz each. In contrast, each line of the high-field resonance is somewhat broadened. These long-range couplings must arise through spin–spin interaction with the aromatic protons. The double doublet at δ_H 7.049 (*J* 7.4 and 8.1 Hz) is assigned to the C-7 proton. The assignment of the



(**1**) R = H
(**2**) R = Ac

Crystallographic numbering scheme shown[1]

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

Carbon	δ_C(p.p.m.)[b]	*J*(CH)/Hz	δ_H(p.p.m.)[c]	*J*(HH)/Hz
1,1′	164.68 S			
3,3′	61.50 D	146.7	5.423 dd	9.8, 9.8
4,4′	30.38 T	137.0	3.502 dddd	16.3, 9.8, 1.2, 1.2
			3.377 dd	16.3, 10.0
5,5′	132.78 S			
6,6′	115.66 Dd	162.9, 8.4	6.802 dd	7.4, 1.2
7,7′	127.68 D	159.8	7.049 dd	7.4, 8.1
8,8′	116.31 Ddd	160.1, 7.0, 7.0	5.703 br d	8.1
9,9′	145.02 S			
10,10′	126.51 S			

[a] Recorded for solutions in $(CD_3)_2SO$ on a Bruker WM-500 spectrometer. [b] Chemical shifts relative to $(CD_3)_2SO$ at 39.50 p.p.m. Capital letters refer to the pattern resulting from directly bonded (C,H) couplings. S = singlet, D = doublet, and T = triplet. [c] Chemical shifts relative to $(CD_3)_2SO$ at 2.49 p.p.m. The proton of the phenolic hydroxy group appears at δ_H 11.204. d = Doublet and br = broad.

double doublet at δ_H 6.802 (*J* 7.4 and 1.2 Hz) and the broadened doublet at δ_H 6.703 (*J* 8.1 Hz) to 6-H and 8-H, respectively, followed from the results of the heteronuclear ^{13}C{^{1}H} selective population inversion (SPI) experiments (see later).[2] A clearer picture of the proton–proton connectivity pattern of bipolaramide (**1**) emerged from a two-dimensional proton–proton correlation (COSY) experiment[3,4] (Figure 1). For example, it is evident from the cross-peaks in the contour plot that 6-H is coupled to both of the other two aromatic protons, 8-H and 7-H, as well as to the C-4 methylene protons. A similar pattern is observed for 8-H and 7-H although the cross-peaks for the C-7 and the C-4 protons are of much reduced intensity.

The assignment of the resonances in the ^{13}C n.m.r. spectrum of bipolaramide (Table 1) is based on the results obtained from single-frequency n.O.e. (nuclear Overhauser

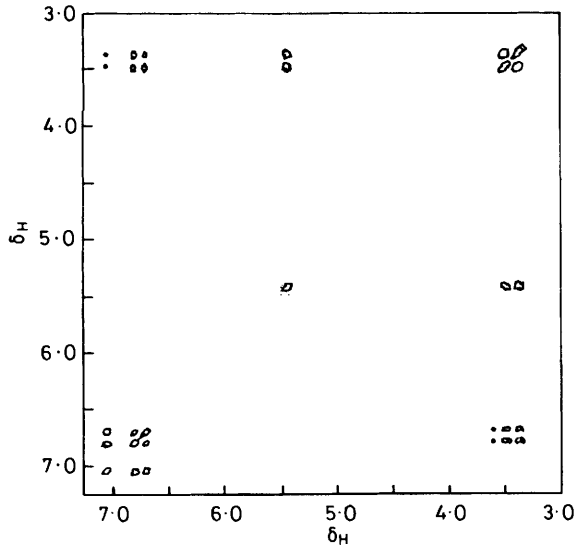


Figure 1. Contour plot of a COSY-45 experiment for bipolaramide (**1**). The matrix used for time-domain data was 512 × 2K (2K in t_2 domain) and for two-dimensional Fourier transformation 1K × 2K, yielding a digital resolution of 2.44 Hz/point in both dimensions. Spectral acquisition: 2 500 Hz sweepwidth in the F_2 dimension, 512 spectra of 32 scans each were accumulated. The signal of the proton at δ_H 11.204 is folded into the spectral window and appears at about δ_H 4.8

effect) and proton-decoupled ^{13}C spectra, SPI experiments,[2] and deuterium isotope shifts.

In the first instance two-dimensional (^{13}C, ^1H) correlation spectroscopy enabled us to correlate the signals of all the proton-bearing carbon atoms with specific proton resonances,[3,5] and in addition allowed us to assign the ^{13}C resonances of C-3 (δ_C 61.50), C-4 (δ_C 30.38) and C-7 (δ_C 127.68 p.p.m.). Chemical-shift considerations dictate that the resonances at δ_C 164.68 and 145.02 p.p.m. be attributed to C-1 and C-9, respectively. This assignment was confirmed by the characteristic two-bond deuterium isotope shift observed for the resonance at δ_C 145.02 p.p.m. ($^2\Delta\delta$ − 0.197 p.p.m.)* upon addition of a mixture of H_2O–D_2O (3:2) to the sample.

Selective irradiation of the proton transitions at δ_H 6.802 (6-H or 8-H) in an SPI experiment[2] affected the resonances at δ_C 132.78, 127.68 (C-7), 126.51, 116.31, and 30.38 (C-4) p.p.m. These resonances must arise from carbon atoms two and/or three bonds removed from the irradiated proton as four-bond (C,H) couplings are normally small (*ca.* 1 Hz)[7] and the irradiating power used in these experiments (γH_2 = 5 Hz) precludes their detection. As the C-4 resonance (δ_C 30.38 p.p.m.) is affected the irradiated proton transitions must be attributed to a proton three bonds removed, *i.e.* to 6-H. This result allows us to assign the resonance at δ_C 116.31 p.p.m. to C-8 and consequently that at δ_C 115.66 p.p.m. to C-6. The singlet resonances at δ_C 132.78 and 126.51 p.p.m. must arise from C-5 and C-10, or *vice versa*. This ambiguity was resolved by selective irradiation of the C-8 proton transitions (δ_H 6.703) in an SPI experiment which affected the resonances at δ_C 115.66 (C-6) and 126.51 p.p.m. The latter resonance is therefore assigned to C-10

* "$\Delta\delta = \delta_C(D) - \delta_C(H)$, *i.e.* the chemical-shift value of the carbon bearing the heavier isotope minus the chemical-shift value of the carbon bearing the lighter isotope; *n* indicates the number of bonds separating the observed nucleus and the isotope in question. The reverse notation is also used (see ref. 6).

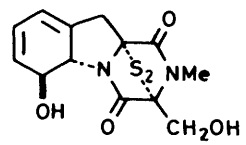
Table 2. ^{13}C Deuterium isotope shifts for bipolaramide[a]

Carbon atom	Δδ(p.p.m.)[b]
C-5	—
C-6	+0.063
C-7	−0.029
C-8	−0.064
C-9	−0.197
C-10	−0.086

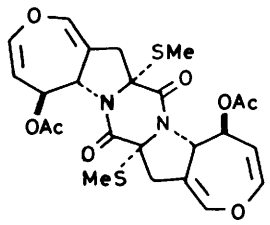
[a] Observed on addition of H_2O–D_2O (3:2). [b] Values obtained from the proton-decoupled 125.76 MHz ^{13}C spectrum. The minus sign indicates an upfield shift.

as this carbon atom is three bonds removed from both 8-H and 6-H. As a consequence the resonance at δ_C 132.78 p.p.m. can be ascribed to C-5.

A number of ^{13}C resonances, in addition to the one assigned to C-9 (δ_C 145.02 p.p.m.), exhibited deuterium isotope shifts on addition of a mixture of H_2O–D_2O to the sample (Table 2). Phenolic compounds show many unusual isotope effects concerning the magnitude, the sign (*i.e.* upfield or downfield shifts), and the long-range nature of the isotope shift.[6] The magnitude of the isotope shift in hydrogen-bonded phenolic compounds, for instance, depends on the strength of the hydrogen bond.[8] In general, $^2\Delta\delta$ and $^3\Delta\delta$ isotope shifts are negative, and $^5\Delta\delta$ is positive,[6,8] and this trend is also observed for the resonances of the affected carbon atoms in bipolaramide. The isotope effects have a large potential for assignment purposes of complex structures containing hydrogen-bonded phenolic hydroxy groups.



(3)



(4)

A comparison of the structure of bipolaramide (**1**) with a number of other fungal dioxopiperazines,[9] *e.g.* gliotoxin (**3**) and the aranotin derivative (**4**), suggests that the metabolite (**1**) is derived from phenylalanine *via* the symmetrical intermediate *cyclo*-(phenylalanylphenylalanyl). An alternative pathway leading to bipolaramide could involve *m*-hydroxyphenylalanine, although the intermediacy of this compound in the biosynthesis of gliotoxin has been discounted.[9] These two possible routes leading to bipolaramide were investigated using ^{14}C-labelled precursors.

Cultures of *B. sorokiniana* were grown on a Czapek medium supplemented with yeast extract (2%). Studies on the course of fermentation indicated that bipolaramide production commenced on day 4 and reached a level of 90 mg l^{-1} 14 days after the inoculation of the medium (Figure 2).

In control experiments, the incorporation of DL-phenylalanine into bipolaramide and the tolerance of *B. sorokiniana* towards dimethyl sulphoxide, a necessary solvent for the more advanced precursor *cyclo*-(L-phenylalanyl-L-phenylalanyl), was investigated using DL-[3-^{14}C]phenylalanine. The results (Table 3) show that the effect of added dimethyl sulphoxide on the yield of bipolaramide (average 28.0 mg l^{-1}), and the absolute incorporation (average 0.93%) of the precursor is minimal at

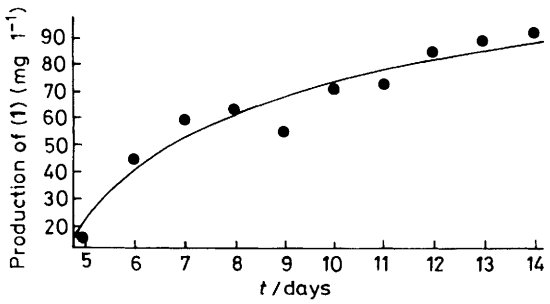


Figure 2. Production of bipolaramide (1) by *B. sorokiniana*

Table 3. Influence of dimethyl sulphoxide on the incorporation of phenylalanine into bipolaramide[a]

Experiment	Amount of dimethyl sulphoxide	Yield of (1)[b]	Specific activity (μCi mmol^{-1})	Dilution	Incorporation (%)
1	1.0 ml	10.2 mg	7.74	13.4	0.97
2	2.0 ml	9.6 mg	7.12	14.5	0.84
3	3.0 ml	13.0 mg	5.96	17.3	0.96
4	4.0 ml	12.0 mg	6.57	15.7	0.96

[a] 40 mg of DL-[3-^{14}C]phenylalanine (25 μCi) was added in each experiment. [b] Relative to 400 ml of medium.

Table 4. Rate of incorporation of phenylalanine into bipolaramide

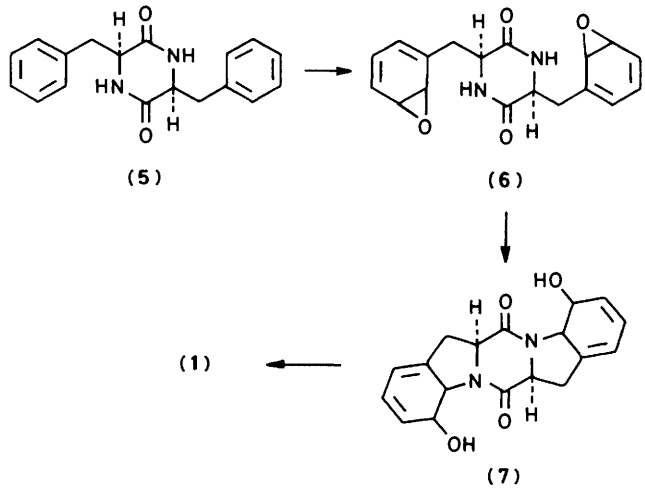
Time interval after addition (minutes)	Specific activity of (1) (μCi mmol^{-1} × 10^2)	Dilution
30	2.3	2 611
60	4.5	1 322
90	13.7	430
120	25.2	234
180	34.8	169
240	210	28
360	446	13

levels of up to 1 ml of dimethyl sulphoxide per 100 ml of medium.

Significant incorporation of phenylalanine into bipolaramide, as measured by the specific activity of (1) and dilution values (Table 4), had occurred only 4 h after the addition of DL-[U-^{14}C]phenylalanine to the medium. A similar, rapid incorporation of phenylalanine into didethiobis(methylthio)-acetylaronotin (4) in *Aspergillus terreus* was observed by Pita Boente *et al.*[10]

Information on the status of *cyclo*-(L-phenylalanyl-L-phenylalanyl) as a natural intermediate in the biosynthesis of bipolaramide (1) was obtained from a trapping experiment. Non-radioactive *cyclo*-(L-phenylalanyl-L-phenylalanyl) (40 mg) in dimethyl sulphoxide (10 ml) was incubated with *B. sorokiniana* and, 2 h later, DL-[2-^{14}C]phenylalanine (50 μCi) was added to the culture medium. The incubation was stopped after 24 h and the culture medium was extracted with chloroform to recover *cyclo*-(L-phenylalanyl-L-phenylalanyl) (20 mg), which had a specific activity of 0.42 μCl mmol^{-1}.

The precursor role of *cyclo*-(L-phenylalanyl-L-phenylalanyl) was confirmed by addition of *cyclo*-(L-[U-^{14}C]phenylalanyl-L-phenylalanyl) (3 mg; 51 μCi mmol^{-1}) to cultures of *B. sorokiniana*. The formed bipolaramide (1) (46 mg) had a specific activity of 0.37 μCi mmol^{-1} which corresponds to an incorporation of 10.2%.



Scheme. Proposed biosynthetic pathway for bipolaramide (1)

From the above experiments it can be concluded that bipolaramide is formed from phenylalanine *via cyclo*-(L-phenylalanyl-L-phenylalanyl) (5). Ring closure of (5) by nucleophilic attack of the nitrogen atom on the bis(arene oxide) (6) would lead to the formation of a hexadienol intermediate (7). Such an arene oxide has also been postulated in the formation of the cyclohexadienol moiety of gliotoxin (3)[9] and the oxepine ring of the aranotins,[10] *e.g.* compound (4). In the final step, oxidation of the hydroxy groups in compound (7) would generate the aromatic rings of the bipolaramide molecule (Scheme).

Experimental

M.p.s were determined on a Kofler hot-stage apparatus. U.v. absorptions were measured for solutions in methanol on a Unicam SP8-100 spectrophotometer. I.r. spectra were recorded on a Perkin-Elmer 237 spectrophotometer and mass spectra on a Varian MAT 212 spectrometer. ^{1}H and ^{13}C N.m.r. spectra were recorded on a Bruker WM-500 spectrometer for solutions in [$^2H_6$]dimethyl sulphoxide. Optical rotations were measured at 24 °C on a Perkin-Elmer 241 polarimeter for solutions in acetone. Radioactive samples were counted on a Packard Tri-Carb 2660 instrument. Merck silica gel (0.063—0.200 mm) was used for column chromatography.

Isolation of Bipolaramide.—*Bipolaris sorokiniana* (MRC 93) was grown on wet sterilized maize for 20 days. The maize (5 kg) was dried, milled, and extracted for 24 h in a Soxhlet apparatus using a mixture of chloroform and methanol (1:1 v/v). The solvent was evaporated off under reduced pressure and the residue (310 g) was partitioned between hexane and 90% aqueous methanol. The 90% methanol solution was evaporated and the aqueous residue was partitioned between chloroform and water. The residue (16 g) obtained from the chloroform solution was separated by column chromatography on silica gel using benzene–acetone (9:1 v/v) to yield sterigmatocystin (1.3 g), identical with an authentic sample, and *bipolaramide* (1) (830 mg). Bipolaramide crystallized from acetone as needles, m.p. 296—297 °C (Found: C, 66.9; H, 4.3; N, 8.6. $C_{18}H_{14}N_2O_4$ requires C, 67.1; H, 4.4; N, 8.7%); $[\alpha]_D$ − 210° (*c* 1.0); $\lambda_{max.}$ 218 (ε 44 670), 260 (14 790), and 294 nm (7 585); $\nu_{max.}$ (KBr) 1 645 and 1 610 cm^{-1}.

Acetylation of Bipolaramide (1).—Bipolaramide (1) (70 mg) was dissolved in acetic anhydride (15 ml), and the solution was

cooled (−78 °C) and treated with perchloric acid (0.05 ml). The mixture was allowed to attain room temperature, and was then poured onto crushed ice and extracted with chloroform (3 × 50 ml). The combined chloroform extracts were washed with water, dried ($MgSO_4$), and evaporated to dryness. The residue was purified by preparative t.l.c. (p.l.c.) on silica gel with benzene–acetone (9:1 v/v) as solvent to give *di*-O-*acetylbipolaramide* (**2**) (75 mg), m.p. 240—242 °C (from acetone) (Found: C, 65.2; H, 4.6; N, 6.95. $C_{22}H_{18}N_2O_6$ requires C, 65.0; H, 4.5; N, 6.9%); $\nu_{max.}$ ($CHCl_3$) 1 710 (acetate CO), 1 685 (amide CO), and 1 605 cm^{-1}; δ_H 2.315 (6 H, $OCOCH_3$).

Production of Bipolaramide on a Liquid Medium.—Conical flasks (50 × 500 ml) containing a Czapek medium (100 ml) enriched with 2% yeast extract were inoculated with spores of *B. sorokiniana*. From the fifth until the fourteenth day, the mycelial mats from four flasks were collected each day and macerated in a Waring blender with acetone. The solvent was evaporated off under reduced pressure and the residue was partitioned between hexane and 90% methanol. The aqueous methanolic solution was evaporated and the aqueous residue was partitioned between chloroform and water. The bipolaramide present in the chloroform solution was determined quantitatively by u.v. absorption analysis at 294 nm. The results are summarized in Figure 2.

Incorporation of Phenylalanine and Influence of Dimethyl Sulphoxide on B. sorokiniana.—In separate experiments, the precursor DL-[3-^{14}C]phenylalanine (40 mg; 25 μCl) was dissolved in a mixture of dimethyl sulphoxide and water (total volume 10 ml) containing 1.0, 2.0, 3.0, or 4.0 ml of dimethyl sulphoxide. These precursor solutions were administered to the culture medium (4 × 100 ml) from the sixth until the tenth day after inoculation with spores of *B. sorokiniana*. The mycelia were worked up on day 11 as usual (see above) and the bipolaramide was isolated and purified by p.l.c. on silica gel with benzene–acetone (9:1 v/v) as solvent. The results are summarized in Table 3.

Rate of Incorporation of Phenylalanine into Bipolaramide.—A solution of DL-[U-^{14}C]phenylalanine (280 mg; 100 μCi) in a mixture of dimethyl sulphoxide (14 ml) and water (70 ml) was fed to a 6-day-old culture of *B. sorokiniana* (28 × 100 ml). The mycelial mats from 400 ml of medium were collected at specific time intervals and worked up as usual to yield bipolaramide (**1**). The results are summarized in Table 4.

Incorporation of Phenylalanine into cyclo-(L-*Phenylalanyl*-L-*phenylalanyl*) (**5**).—A solution of *cyclo*-(L-phenylalanyl-L-phenylalanyl) (40 mg) in dimethyl sulphoxide (10 ml) was added on day 7 to a culture of *B. sorokiniana* (10 × 100 ml). After 2 h, DL-[2-^{14}C]phenylalanine (50 μCi) was added to the culture medium and 24 h later the medium was extracted with chloroform (2 × 500 ml) to recover *cyclo*-(L-phenylalanyl-L-phenylalanyl) (20 mg), specific activity 0.42 μCi $mmol^{-1}$.

Incorporation of cyclo-(L-*Phenylalanyl*-L-*phenylalanyl*) (**5**) *into Bipolaramide.*—Aliquots (0.3 ml) of a solution of *cyclo*-(L-[U-^{14}C]phenylalanyl-L-phenylalanyl) (3 mg; 51 μCi $mmol^{-1}$) in dimethyl sulphoxide (9 ml) were added for three consecutive days to each flask of a 6-day-old culture of *B. sorokiniana* (10 × 100 ml). On day 10 the mycelial mats were collected and extracted in the usual way to yield bipolaramide (**1**) (46 mg), specific activity 0.37 μCi $mmol^{-1}$.

References

1 C. M. Maes, P. S. Steyn, P. H. van Rooyen, and C. J. Rabie, *J. Chem. Soc., Chem. Commun.*, 1982, 350.
2 K. G. R. Pachler and P. L. Wessels, *J. Magn. Reson.*, 1973, **12**, 337; 1977, **28**, 53.
3 A. Bax, 'Two-dimensional Nuclear Magnetic Resonance in Liquids,' Delft University Press, Delft, 1982.
4 A. Bax, R. Freeman, and G. Morris, *J. Magn. Reson.*, 1981, **42**, 164; A. Bax and R. Freeman, *ibid.*, 1981, **44**, 542.
5 G. Bodenhausen and R. Freeman, *J. Magn. Reson.*, 1977, **28**, 471; A. Bax and G. Morris, *ibid.*, 1981, **42**, 501.
6 P. E. Hansen, *Annu. Rep. NMR Spectrosc.*, 1983, **15**, 105; R. A. Newmark and J. R. Hill, *Org. Magn. Reson.*, 1980, **13**, 40.
7 P. E. Hansen, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1981, **14**, 175.
8 P. L. Wessels and D. Erotocritou, unpublished results.
9 G. W. Kirby and D. J. Robins in 'The Biosynthesis of Mycotoxins,' ed. P. S. Steyn, Academic Press, London, 1980, p. 301.
10 M. I. Pita Boente, G. W. Kirby, and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 619.

Received 10*th April* 1985; *Paper* 5/602