Structure Elucidation of Phomopsin A, a Novel Cyclic Hexapeptide Mycotoxin produced by *Phomopsis leptostromiformis*

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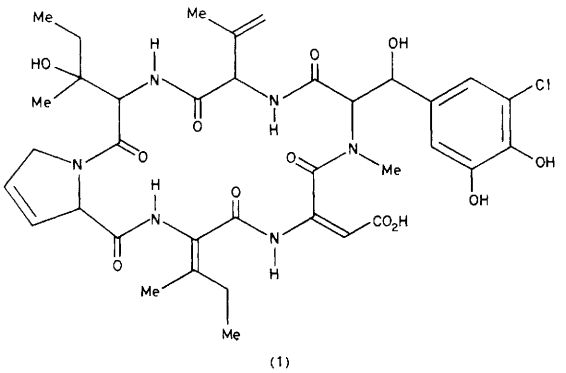
Phomopsin A, the main mycotoxin isolated from cultures of *Phomopsis leptostromiformis* and the cause of lupinosis disease in animals grazing infected lupins, is a cyclic hexapeptide containing 3-hydroxyisoleucine, 3,4-didehydrovaline, *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)-3-hydroxyalanine, *E*-2,3-didehydroaspartic acid, *E*-2,3-didehydroisoleucine, and 3,4-didehydroproline; its ^{13}C n.m.r. spectrum was completely assigned and the amino-acid sequence established unambiguously by extensive heteronuclear ^{13}C-{^{1}H} selective population inversion n.m.r. experiments.

Lupinosis is a mycotoxicosis of sheep, cattle, and horses grazing lupins (*Lupinus* spp.) or post-harvest lupin roughage infested with the fungus *Phomopsis leptostromiformis* (Kühn) Bubak *ex* Lind.[1,2] The condition, characterised by severe liver damage, is of considerable importance in Australia and fieldcases have also been reported in South Africa, New Zealand, Germany, and Poland.[3,4] The strain of *P. leptostromiformis*, MRC 2654 used in the present study was originally isolated from a field outbreak of lupinosis in South Africa during 1969 and was shown to cause lupinosis in sheep[1] and pigs.[5] Phomopsin A (**1**) is the main mycotoxin produced by *P. leptostromiformis* when cultivated on lupin seed,[6] liquid media,[7] or maize kernels (this work). Earlier reports formulate phomopsin A as being probably $C_{36}H_{43}ClN_6O_{11}$ and describe its reactions and mass and n.m.r. spectral features.[8,9] We now report the structure of phomopsin A based on hydrolysis and reduction reactions and a detailed study of its high-field ^{1}H and ^{13}C n.m.r. spectra. The cyclic hexapeptide structure is unique in containing a highly modified phenylalanyl moiety and several 2,3- and 3,4-didehydroamino-acids; the configuration of the two 2,3-didehydroamino-acids was established as *E*.

Phomopsin A and several related metabolites were extracted from a culture of *P. leptostromiformis*, MRC 2654 grown on sterilized whole maize, by high-speed blending in



(1)

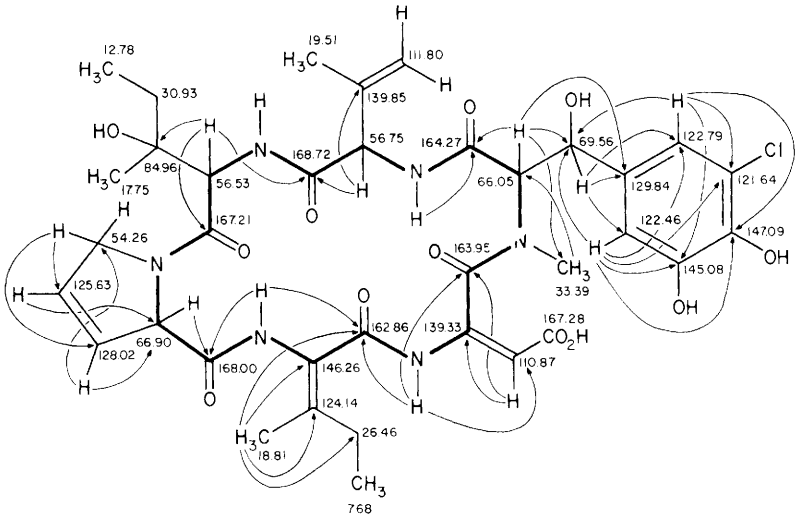
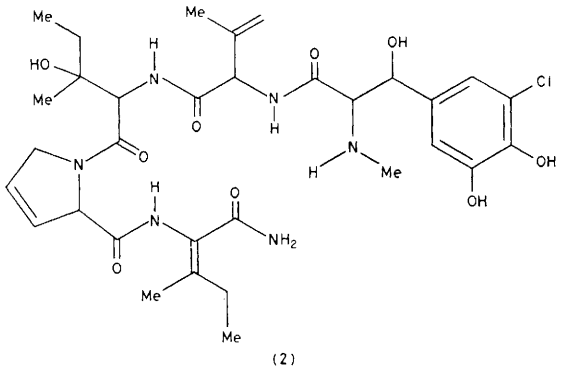


Figure 1. The ^{13}C n.m.r. spectral data for phomopsin A. The (^{1}H, ^{13}C) connectivity pattern as indicated by arrows was determined by heteronuclear ^{13}C-{^{1}H} SPI experiments.



(2)

methanol. The methanol extract was purified by column chromatography on macroreticular polystyrene resin (XAD-2),[6] and the phomopsins isolated by chromatography, first on Sephadex LH-20 (methanol–water, 1 : 1 v/v) and subsequent gradient elution on DEAE cellulose with ammonium hydrogen carbonate buffer, pH 7.9 (0.02 M to 0.2 M). The fractions containing phomopsin A were combined and freeze-dried. Crystallisation from methanol–ethanol–water (5 : 4 : 1 v/v/v) gave phomopsin A (**1**) (decomposition at 205 °C without melting); λ_{max} (MeOH) 209 (ϵ 52300), 222sh (24500), and 288 nm (13900); ν_{max} (KBr) 3340 (amide NH), 1670 and 1645 cm^{-1} (amide CO). The presence of a weak carboxy group in phomopsin A was indicated by electrometric titration and the broad absorption (2550—2800 cm^{-1}) in its i.r. spectrum. Reaction of phomopsin A with ethereal diazomethane or under conditions of permethylation gave intractable mixtures only.

Fast atom bombardment mass spectrometry (f.a.b.-m.s.) gave the molecular ion as *m/z* 789/791 $[M + H]^+$, supported by an ion at *m/z* 811/813 $[M + Na]^+$ and, in negative ion mode, as *m/z* 787/789 $[M - H]^-$. An accurate mass measurement, 789.2823, showed the empirical formula to be $C_{36}H_{45}ClN_6O_{12}$ (calculated for M + H, 789.2862) which was substantiated by the n.m.r. spectral data.

A perusal of the ^{13}C n.m.r. data for phomopsin A, *viz.* seven carbonyl carbon signals in the δ 170—160 p.p.m. region and four methine carbon signals in the δ 67—56 p.p.m. region[10] (see Figure 1) suggested the involvement of several amino-acids, albeit modified, in the construction of phomopsin A. This supposition was substantiated by the incorporation of L-[U-^{14}C]valine,[11] L-[U-^{14}C]isoleucine, L-[U-^{14}C]phenylalanine, and L-[U-^{14}C]proline into phomopsin A.[12] Furthermore in the broad-band proton-decoupled ^{13}C n.m.r. spectrum of phomopsin A biosynthetically derived from L-[3-^{13}C]-phenylalanine the resonance at δ_C 69.56 (see Figure 1) was enhanced.

An acid hydrolysate of phomopsin A (6 M HCl, 110 °C, 20 h), was shown by capillary g.c.–m.s. of the *N*-trifluoroacetyl n-butyl ester derivatives[13] to contain glycine (0.63), sarcosine (0.11), 3,4-didehydrovaline (0.08), valine (0.18), two β,γ-didehydroisoleucines (0.20, 0.44), and 3,4-didehydroproline (1.00) in the indicated ratios. The glycine, sarcosine, valine, and 3,4-didehydroproline[14] were identified by comparison with authentic samples and the dehydrovaline and dehydroisoleucines by their mass spectra as well as by conversion to valine and *allo*-isoleucine/isoleucine, respectively after hydrogenation (PtO_2–H_2) of the hydrolysate. The presence of 2,3-didehydroaspartic acid and 2,3-didehydroisoleucine in phomopsin A was established by sodium borohydride reduction prior to hydrolysis which then yielded, in addition to the above amino-acids, aspartic acid (1.0), *allo*-isoleucine (0.50), and isoleucine (0.50). However, prior catalytic reduction (PtO_2–H_2) of phomopsin A followed by acid hydrolysis led to the formation of valine, isoleucine, proline, and aspartic acid in the ratio 1 : 1 : 1 : 1 and the β,γ-didehydroisoleucines mentioned above in the same relative ratio as before, but with glycine and sarcosine completely absent. Catalytic hydrogenation of the 2,3-didehydroisoleucine moiety in phomopsin A must therefore proceed stereospecifically and as a consequence the *E* configuration is indicated for this dehydroamino-acid. The presence of two β,γ-didehydroisoleucines in the hydrolysates, even after catalytic hydrogenation of phomopsin A, must be due to the loss of water from 3-hydroxyisoleucine, a constituent amino-acid, under the hydrolysis conditions.

Although phenylalanine is efficiently incorporated into phomopsin A (see above) neither this amino-acid nor a derivative was detected in the acid hydrolysates. Instead when phomopsin A was treated with 11.0 M HCl, a chlorodihydroxybenzaldehyde, formed by an acid-catalysed retroaldol fission, was isolated and identified by g.c.–m.s. comparison of its dimethylether (M^+, 200/202; accurate mass

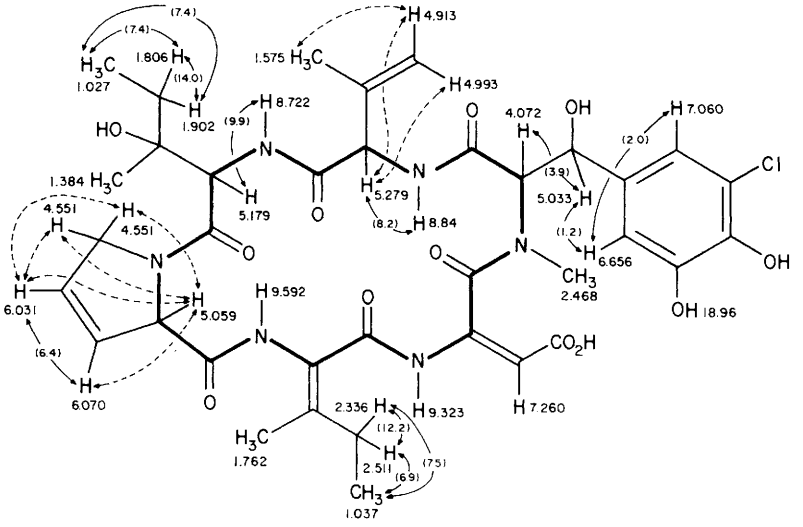


Figure 2. The ^1H n.m.r. chemical shifts and coupling constants (Hz) for phomopsin A. The (^1H, ^1H) connectivity pattern as indicated was determined by homonuclear decoupling experiments. The broken lines show cases where effects were observed during decoupling experiments, although no splittings were measurable.

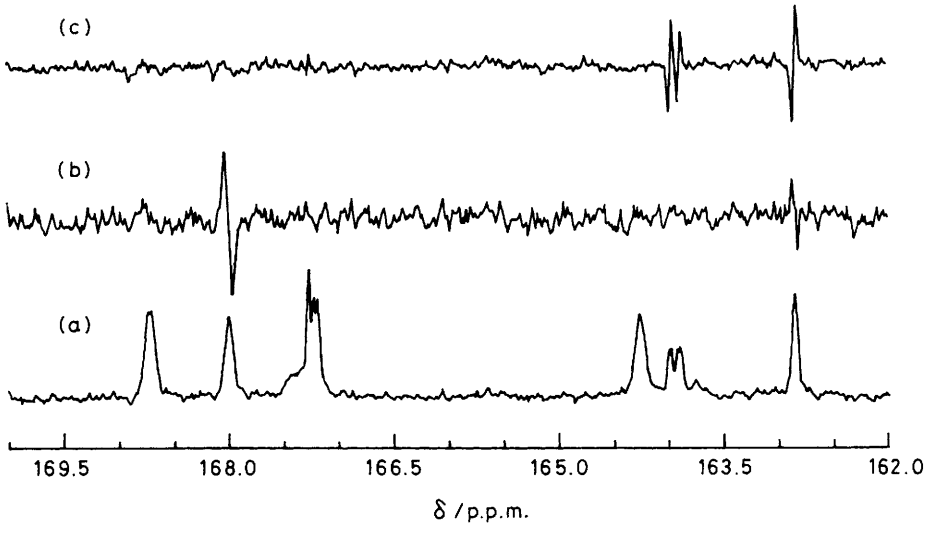


Figure 3. The low-field part of the 125.76 MHz ^{13}C n.m.r. spectrum of phomopsin A (spectral width 26316 Hz; 90° r.f. pulse of 10.0 μsec duration, acquisition time 0.623 s). (a) Single frequency n.O.e. spectrum (11200 transients); (b) after a selective π-pulse is applied to a low-field (C,H) transition of the proton at δ_H 9.592 (γH_2 = 5.0 Hz, 11200 transients); (c) after a selective π-pulse is applied to a high-field (C,H) transition of the proton at δ_H 9.323 (γH_2 = 5.0 Hz, 11200 transients).

determination: *m/z* 200.0234, calculated for $C_9H_9ClO_3$, 200.0240) with authentic 3-chloro-4,5-dimethoxybenzaldehyde (see n.m.r. data below for substitution pattern). Ions at *m/z* 171/173 in the f.a.b. mass spectrum of both phomopsin A (**1**) and phomopsinamine (**2**) are derived from this moiety. A chlorodihydroxyphenylpyruvic acid, derived from the corresponding 2,3-didehydroamino-acid formed by dehydration of the substituted β-hydroxyphenylalanine unit, was also detected in the hydrolysate and identified by the mass spectrum of its tetra(trimethylsilyl) derivative (M^+, 518).

Mild acid hydrolysis of phomopsin A (6 M HCl, 38 °C, 1 h) gave oxaloacetic acid, derived from the constituent 2,3-didehydroaspartic acid moiety, and phomopsinamine (**2**), a moderately strong base. F.a.b.-m.s. of (**2**) showed ions at *m/z* 697/699 which correspond to $[M - H_2O + Na]^+$. The ease of formation of (**2**) is apparently due to the exceptionally strong hydrogen-bonding of the C-5 phenolic hydroxy group (δ 18.96 p.p.m.) of the modified phenylalanine moiety to the carbonyl group of the free carboxy function of the 2,3-didehydroaspartic acid. The ^1H and ^{13}C n.m.r. spectral data support the proposed structure (**2**).

The constituent amino-acids of phomopsin A *viz.* 3,4-didehydroproline, 2,3-didehydroisoleucine, 2,3-didehydroaspartic acid, *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)-3-hydroxyalanine, 3,4-didehydrovaline, and 3-hydroxyisoleucine must be accommodated in a cyclic peptide containing a free carboxy group to account for the empirical formula.

The presence of the above amino-acid structural units, recognized also from a detailed analysis of the resolution enhanced 500.14 MHz ^1H n.m.r. spectrum of phomopsin A recorded in [$^2H_6$]dimethyl sulphoxide, was confirmed by extensive homonuclear ^1H-{^1H} decoupling experiments (Figure 2). The 125.76 MHz ^{13}C n.m.r. data for phomopsin A as shown in Figure 1 were obtained from broad-band

proton-decoupled and single frequency nuclear Overhauser enhanced (n.O.e.) spectra. The residual (C,H) splittings observed in a series of off-resonance proton-decoupled ^{13}C n.m.r. experiments enabled us to correlate the signals of the proton-bearing carbon atoms with specific proton resonances.[15] In the assignment of the different ^{13}C resonances use was made of chemical shift values,[10] (C,H) coupling constants, selective ^{13}C-{^1H} decoupling experiments, and extensive heteronuclear ^{13}C-{^1H} selective population inversion (SPI)[16] experiments. The long-range (C,H) connectivities determined by the SPI technique established unambiguously the amino-acid sequence and the substitution pattern of the modified phenylalanine unit in phomopsin A. The results of the SPI experiments (an example is shown in Figure 3) are indicated in Figure 1 and define the partial sequences 3,4-didehydroproline → 2,3-didehydroisoleucine → 2,3-didehydroaspartic acid and *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)-3-hydroxyalanine → 3,4-didehydrovaline → 3-hydroxyisoleucine. The linkage of 2,3-didehydroaspartic acid→*N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)-3-hydroxyalanine was deduced from the two-bond (C,H) coupling observed for the carbon resonance of the basic secondary *N*-methyl group {δ_C 34.33 p.p.m. [Qd, ^1J(CH) 134.0, ^2J(CH) 3.0 Hz]} in phomopsinamine (**2**), which lacks the 2,3-didehydroaspartic acid moiety. The corresponding resonance in the ^{13}C n.m.r. spectrum of phomopsin A [δ_C 33.39 p.p.m. (Q, ^1J 142.7)] exhibits no long-range (C,H) coupling. By elimination, the remaining linkage between the constituent amino-acids of phomopsin A is defined by the sequence 3-hydroxyisoleucine → 3,4-didehydroproline.

The configuration of the 2,3-didehydroisoleucine moiety was deduced from hydrogenation experiments as *E* (see above) whereas that of the 2,3-didehydroaspartic acid moiety followed from ^{13}C n.m.r. data. The resonance of the amide carbonyl carbon atom of the latter moiety appears as a doublet of doublets at δ_C 163.95 p.p.m. [^3J(CH) 9.5, ^3J(C,NH) 2.3 Hz] (see Figure 3) in the single frequency n.O.e. ^{13}C n.m.r. spectrum of phomopsin A. The magnitude of ^3J(CH) establishes the *E* configuration for 2,3-didehydroaspartic acid.[17] The configuration of the remaining chiral centres and the conformation of phomopsin A are under investigation.

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