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STRUCTURE ELUCIDATION AND ABSOLUTE CONFIGURATION OF PHOMOPSIN A, A HEXAPEPTIDE MYCOTOXIN PRODUCED BY *PHOMOPSIS LEPTOSTROMIFORMIS*

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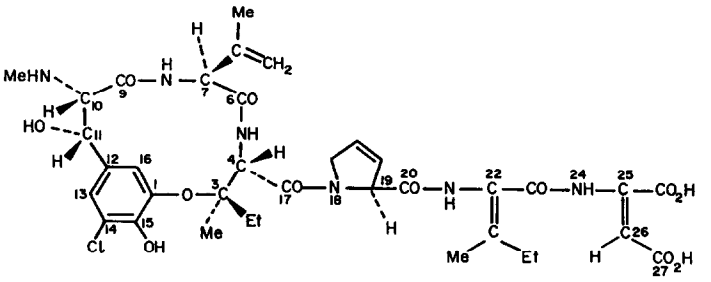
Abstract. Phomopsin A, $C_{36}H_{45}ClN_6O_{12}$, the main mycotoxin isolated from cultures of *Phomopsis leptostromiformis* and the cause of lupinosis disease, is a linear hexapeptide containing 3-hydroxy-L-isoleucine, 3,4-didehydrovaline, *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)serine, *E*-2,3-didehydro-aspartic acid, *E*-2,3-didehydroisoleucine, and 3,4-didehydro-L-proline The L-configuration of the indicated amino acids was established by a comparison of the *N*-trifluoroacetyl n-butylester derivatives of the acid hydrolysis products of phomopsin A with samples prepared from authentic amino acids, using capillary gas chromatography on a chiral stationary phase The *E* configuration of the two 2,3-didehydro amino acids is based on the products obtained by catalytic hydrogenation and sodium borohydride reduction of phomopsin A followed by acid hydrolysis (for 2,3-didehydroisoleucine) or by analysis of the coupled ^{13}C n m r spectrum of phomopsin A (for 2,3-didehydroaspartic acid) Evidence is presented which shows that the glycine formed during the acid hydrolysis of phomopsin A is derived from the 3,4-didehydrovaline moiety The sequence of the amino acids was established by heteronuclear $^{13}C\{^1H\}$ selective population inversion (SPI) experiments and by fast atom bombardment (f a b) mass spectrometry of phomopsin A and its derivatives An *X*-ray crystallographic study of phomopsin A confirmed the amino acid sequence and showed that the linear hexapeptide is modified by an ether bridge in place of the 5-hydroxy group of the *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)serine and the hydroxy group of the 3-hydroxyisoleucine units In addition, the *X*-ray study specified the absolute configuration of phomopsin A as 22*E*, 25*E*, 3*R*, 4*S*, 7*S*, 10*S*, 11*S*, 19*S*

Introduction

The consumption of lupins (*Lupinus* spp) or post-harvest lupin roughage infected with the fungus *Phomopsis leptostromiformis* Kühn Bubak *ex* Lind has been identified as the cause of lupinosis, a mycotoxicosis of sheep, cattle, and horses [1,2] The condition, characterised by severe liver damage, is of considerable economic importance in Australia and field cases have also been reported in South Africa and New Zealand [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)



(1)

We now report the structure and absolute configuration of phomopsin A (**1**) based on the products obtained on hydrolysis and after reduction reactions, ^{1}H and ^{13}C n m r spectral data, fast atom bombardment (f a b) mass spectral evidence and an *X*-ray crystallographic study The linear hexapeptide structure is unique in containing a 13-membered ring involving an ether bridge, a highly substituted phenylserine moiety and several 2,3- and 3,4-didehydro amino acids

Discussion

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 methanol The methanol extract was purified by column chromatography on macroreticular polystyrene resin (XAD-2),[6,7] and the phomopsins isolated by chromatography, first on Sephadex LH-20 using methanol water (1 1 v/v) as eluant and subsequently by gradient elution to 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) that analysed for $2(C_{36}H_{45}ClN_6O_{12})\ 5H_2O$, λ_{max} (MeOH) 209 (ε 53 300), 222 sh (ε 24 500), and 288 nm (ε13 900), ν_{max} (KBr) 3340 (amide NH), 1670 and 1645 cm^{-1} (amide CO) The presence of a carboxyl group in phomopsin A as suggested by the broad absorption (2550-2800 cm^{-1}) in its i r spectrum was also indicated by electrometric titration which provided evidence for the presence of two weak acid moieties and a stronger carboxylic acid

Fast atom bombardment mass spectrometry (f a b -m s) of phomopsin A gave the molecular ion at *m/z* 789/791 $[M+H]^+$, supported by an ion at *m/z* 811/813 $[M+Na]^+$ and, in negative ion mode, at *m/z* 787/789 $[M-H]^-$ An accurate mass measurement, *m/z* 789 2823, showed the empirical formula as $C_{36}H_{45}ClN_6O_{12}$ (calculated for *M*+H, 789 2862), which was substantiated by the elemental analysis and the n m r spectral data ^{252}Cf Plasma desorption mass spectrometry gave a series of sodium and potassium adduct ions corresponding to $M\text{-}CO_2$ which is in accord with the presence of a carboxyl

group The measured mass of the $[M\text{-}CO_2+Na]^+$ ion, *m/z* 767 28 (^{35}Cl isotope peak) was in good agreement with the calculated value *m/z* 767 278

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 suggested the involvement of several amino acids, albeit modified, in the construction of phomopsin A Confirmation of this supposition was obtained by the incorporation of L-[*U*-^{14}C]valine, L-[*U*-^{14}C]isoleucine, L-[*U*-^{14}C)phenylalanine, and L-[*U*-^{14}C]proline into phomopsin A produced on liquid medium [9] Furthermore, in the proton-decoupled ^{13}C n m r spectrum of phomopsin A biosynthetically derived from L-[3-^{13}C]phenylalanine only the resonance at δ_C 69 56, assigned to the β-carbon atom of the substituted phenylserine moiety (see later), was enhanced

An acid hydrolysate of phomopsin A (6M HCl, 110 °C, 20 h) was shown by capillary g c -m s of the *N*-trifluoroacetyl n-butyl ester derivatives[10] 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 (see Figure 1) The glycine, sarcosine, valine, and 3,4-dehydroproline[11] were identified by comparison with authentic samples and the 3,4-didehydrovaline and didehydroisoleucines by their mass spectra as well as conversion to valine and a mixture of *allo*-isoleucine and isoleucine, respectively, after hydrogenation (PtO_2-H_2) of the hydrolysate The presence

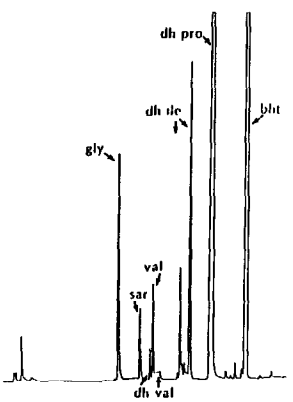


Figure 1 Capillary gas chromatogram (90 m, SCOT OV101) of the N -trifluoroacetyl n-butyl esters of the amino acids obtained on acid hydrolysis of phomopsin A (Abbreviations are gly = glycine, sar = sarcosine, val = valine, dh-val = dehydrovaline, dh-ile = dehydroisoleucine, dh-pro = 3,4-dehydroproline, bht = 3,6-di-t-butylhydroxytoluene)

of 2,3-didehydroaspartic acid and 2,3-didehydroisoleucine in phomopsin A was established by nonstereospecific sodium borohydride reduction prior to acid hydrolysis which then yielded, in addition to the above amino acids, aspartic acid (1 00), *allo*-isoleucine (0 50), and isoleucine (0 50) However, prior catalytic reduction (PtO_2-H_2) of phomopsin A followed by acid hydrolysis and capillary gas

chromatography of the *N*-trifluoroacetyl n-butyl esters on a chiral phase column indicated the presence of DL-valine, DL-isoleucine, L-proline, and DL-aspartic acid in the ratio of 1 1 1 1 and the β,γ-didehydroisoleucines mentioned above in the same relative ratio as before (see Figure 2) It is important to note that both glycine and sarcosine were completely absent Catalytic hydrogenation of the 2,3-didehydroisoleucine moiety must therefore proceed stereospecifically as only isoleucine was detected in this hydrolysate and as a consequence the *E* configuration is indicated for this didehydro amino acid The presence of DL-isoleucine and DL-aspartic acid is to be expected as reduction of the 2,3-didehydro amino acids can occur at either face of the double bond The presence of DL-valine is disconcerting and

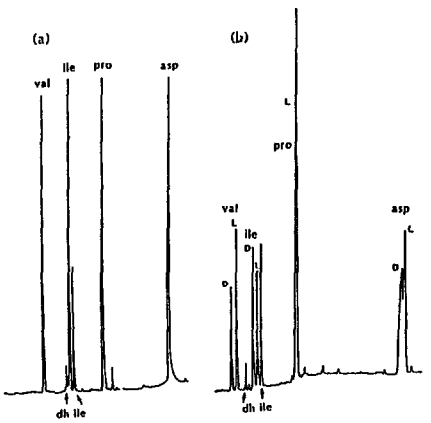


Figure 2 Capillary gas chromatograms of the *N*-trifluoroacetyl *n*-butyl esters of (a) the amino acids obtained on hydrolysis of octahydrophomopsin A chromatographed on a 90 m SCOT OV101 column (b) The same sample on a 25 m WCOT RSL-007 chiral phase column (Abbreviations are val = valine, dh-ile = dehydroisoleucine, ile = isoleucine, pro = proline, asp = aspartic acid)

would imply that a $\Delta^{3,4} \rightarrow \Delta^{2,3}$ isomerization of the 3,4-didehydrovaline moiety occurs on the catalyst surface during reduction The L configuration for the 3-hydroxyisoleucine moiety was established by catalytic reduction of the two β, γ -dehydroisoleucines present in the hydrolysate to L-isoleucine The *E* configuration of the 2,3-didehydroaspartic acid moiety followed from the magnitude of the three-bond ($^{13}C,^{1}H$) coupling observed for the amide carbonyl atom which appears as a doublet of doublets at δ_C 163 95 [^{3}J(CH) 9 5 Hz, ^{3}J(C,NH) 2 3 Hz] in the single frequency n O e ^{13}C n m r spectrum of phomopsin A [12] These results provide firm evidence for the presence of 3,4-didehydro-L-proline, *E*-2,3-didehydroaspartic acid, *E*-2,3-didehydroisoleucine, 3-hydroxy-L-isoleucine and 3,4-didehydrovaline in phomopsin A

An ambiguity in the results obtained from the acid hydrolysis of phomopsin A concerns the origin of the glycine present in the hydrolysate It seems likely that glycine could arise either from the 3-hydroxyisoleucine by a retro-aldol fission, or from the 3,4-didehydrovaline, after Markovnikov addition of water across the double bond followed once again by a retro-aldol reaction Hydrolysis of phomopsin A (6M HCl, 110 ^{O}C, 20h) in a sealed tube, followed by treatment of the hydrolysate with 2,4-

dinitrophenylhydrazine and h p l c analysis of the resultant mixture showed the presence of the 2,4-dinitrophenylhydrazone derivative of acetone The glycine therefore is derived from the 3,4-didehydrovaline moiety in phomopsin A and this deduction was confirmed by hydrolysis of phomopsin A derived biosynthetically from L-[*U*-^{14}C]valine[8] to give glycine which contained 33% of the recovered radioactivity

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, sarcosine and a chlorodihydroxybenzaldehyde are formed by an acid-catalysed retro-aldol fission The latter compound was isolated and identified by g c -m s comparison of its dimethyl ether (M^+, 200/202, accurate mass determination *m/z* 200 0234, calculated for $C_9H_9ClO_3$, 200 0240) with authentic 3-chloro-4,5-dimethoxybenzaldehyde, after the substitution pattern of the aromatic ring had been established by high-field n m r data (see later) Ions at *m/z* 171/173 in the f a b mass spectrum of both phomopsin A 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 phenylserine 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, 1h) 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 $[M\text{-}H_2O+Na]^+$ The origin of the oxaloacetic acid was confirmed by catalytic reduction of phomopsinamine (PtO_2-H_2) followed by acid hydrolysis to give valine, isoleucine, proline and the two β, γ -didehydroisoleucines Aspartic acid was absent

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-didhydroxyphenyl)serine, 3,4-didehydrovaline, and 3-hydroxyisoleucine must be accommodated in a cyclic peptide or a modified linear peptide containing, in each case, at least one free carboxy group to account for the empirical formula

The presence of the above amino acid structural units was also recognized from a detailed analysis of the resolution enhanced 500 MHz ^{1}H n m r spectrum of phomposin A recorded in [$^2H_6$]dimethyl sulphoxide The proton-proton connectivity pattern was confirmed by extensive homonuclear ^{1}H{^{1}H} decoupling experiments (Figure 3) The ^{13}C n m r data for phomopsin A as shown in Figure 4 were obtained from proton-decoupled and single frequency n O e spectra The signals of the proton-bearing carbon atoms were correlated with specific proton resonances by observing the residual (^{13}C,^{1}H) splittings in a series of off-resonance proton-decoupled ^{13}C n m r experiments as well as in a two-dimensional (2-D) (^{13}C,^{1}H) chemical shift correlation experiment [13,14] In the assignment of the different ^{13}C resonances use was made of chemical shift values,[15] (^{13}C,^{1}H)

coupling constants and selective $^{13}C\{^1H\}$ decoupling experiments The long-range ($^{13}C,^1H$) connectivity pattern was determined by extensive heteronuclear $^{13}C\{^1H\}$ selective population inversion (SPI)[16] experiments and by a 2-D chemical shift correlation experiment adjusted to detect correlations *via* long-range ($^{13}C,^1H$) couplings [13,14]

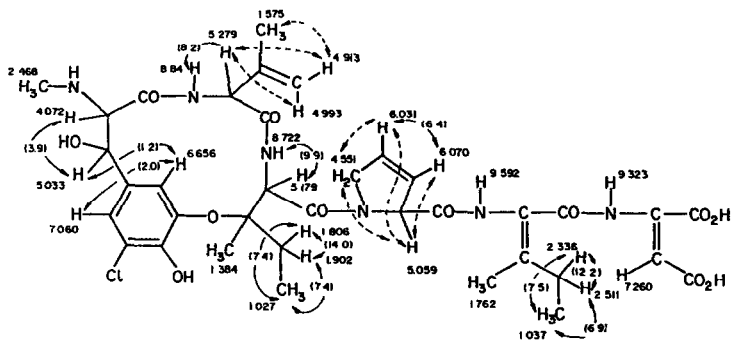


Figure 3. The ^1H chemical shifts and couplings constants (Hz) for phomopsin A (**1**) The ($^1H,^1H$) connectivity pattern as indicated was determined by homonuclear decoupling experiments The broken lines show cases where effects were observed during decoupling experiments, although no splittings were measurable

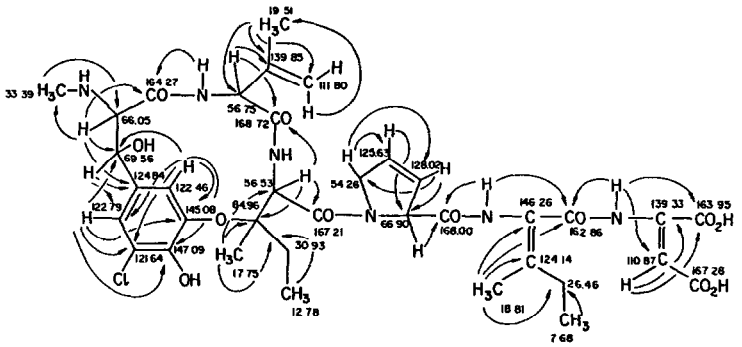


Figure 4. The ^{13}C chemical shifts and long-range ($^1H,^{13}C$) connectivity pattern for phomopsin A (**1**)

The long-range ($^{13}C,^1H$) connectivities determined by these methods partly established the amino acid sequence, the location of the *N*-methyl group, and the substitution pattern of the modified phenylserine moiety in phomopsin A

Evidence for the location of the *N*-methyl group as a constituent of the modified phenylserine moiety was provided by selective irradiation of the *N*-methyl proton transitions (δ_H 2 468) in a SPI experiment The result shows that these protons are coupled to a carbon atom three bonds removed, which resonates at δ_C 66 05 D This carbon chemical shift value, however, has been correlated with the resonance at δ_H 4 072, a terminus of the spin system incorporating the two *meta*-oriented (*J* 2 0 Hz)

aromatic protons of the phenylserine moiety These two resonances (δ_H 4 072, δ_C66 05) are assigned to the nuclei of the α-centre of the amino acid as selective irradiation of the proton transitions in a SPI experiment affected the resonances at δ_C 164 27 S (amide carbonyl carbon), 129 84 S, 69 56 D, and 33 39 Q [^1J(CH) 142 7 Hz, N-CH_3] The resonance at δ_C 69 56 (correlated with δ_H 5 033) is assigned to C-β and that at δ_C 129 84 S to C-γ *i.e.* C-1 of the phenylserine moiety Analysis of the proton-proton connectivity pattern for the phenylserine moiety suggests the presence of either a 3,4,5- or a 2,3,5-trisubstituted phenyl ring in phomopsin A This ambiguity was resolved by irradiation of the β-H proton transition (δ_H 5 033) in a SPI experiment which affected the resonance at δ_C 129 84 S (C-1) and both resonances at δ_C 122 79 D and 122 46 D, correlated with the *meta*-oriented aromatic protons at δ_H 7 060 and 6 656, respectively The nature of the substituents of the 3,4,5-trisubstituted phenyl ring was determined by chemical shift considerations and the results of two additional SPI experiments Selective irradiation of the C-2 proton transitions (δ_H 7 060) affected the resonances assigned to C-6 (δ_C 122 46 D) and C-β (δ_C 69 56 D) as well as those at δ_C 147 09 S and 121 64 S Similar selective irradiation of the C-6 proton transitions (δ_H 6 656) affected the resonances assigned to C-2 (δ_C 122 79 D) and C-β (δ_C 69 56 D) as well as those at δ_C 147 09 S and 145 08 S The chemical shift of the last two resonances is indicative of two oxygen-bearing sp^2 carbon atoms The two- and three-bond (^{13}C,^1H) connectivity pattern demanded by these results is satisfied by a 3-chloro-4,5-dihydroxy substitution pattern for the phenyl ring of the phenylserine moiety and allows the assignment of the resonances for C-3 (δ_C 121 64), C-4 (δ_C 147 09), and C-5 (δ_C 145 08) It is of interest to note that four-bond (^{13}C,^1H) couplings, identified by the value of ^4J(CH), were observed for H-2 and C-5, and H-6 and C-3

The assignment of the resonances in the ^{13}C n m r spectrum of phomopsinamine (**2**) (see Figure 5) is based on the same methodology as that described for phomopsin

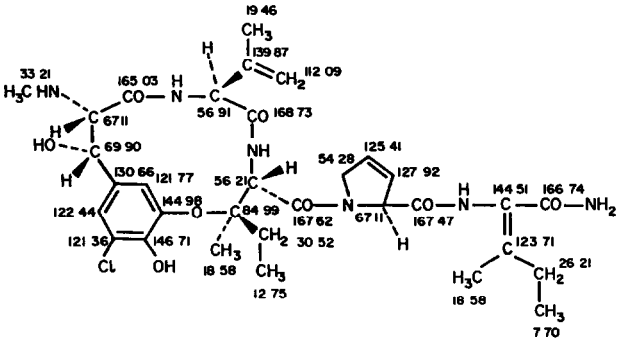


Figure 5. Structure and ^{13}C chemical shifts for phomopsinamine (**2**)

The sequence of the different amino acids in phomopsin A is based on the results obtained from SPI experiments (Figure 4) and the (^1H,^1H) connectivity pattern (Figure 3) For example, selective irradiation of the C-β proton transitions (δ_H 7 260) of the 2,3-didehydroaspartic acid moiety in a SPI experiment affected the resonance at δ_C 139 33 (C-α) and the carbonyl carbon resonance at δ_C 163 95

Selective irradiation of the amide proton (δ_H 9 323) affected the resonances at δ_C 110 87 D and 163 95 S, thus identifying the amide proton as part of the 2,3-didehydroaspartic acid moiety, as well as the carbonyl carbon resonance at δ_C 162 86 S The last resonance is also affected when the amide proton of the 2,3-didehydroisoleucine unit δ_H 9 592 is irradiated in a SPI experiment These results define the linkage 2,3-didehydroisoleucine → 2,3-didehydroaspartic acid In this way, the results of the SPI experiments allow us to formulate the partial sequences 3,4-didehydroproline →2,3-didehydroisoleucine → 2,3-didehydroaspartic acid and *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)serine →3,4-didehydrovaline → 3-hydroxyisoleucine

In a preliminary communication[17] the 2,3-didehydroaspartic acid → *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)serine sequence was deduced from the two-bond ($^{13}C,^{1}H$) coupling observed for the carbon resonance of the basic secondary *N*-methyl group, δ_C 33 21 [Qd, ^{1}J(CH) 134 0, ^{2}J(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 Q, ^{1}J(CH) 142 0 Hz] exhibits no such long-range ($^{13}C,^{1}H$) coupling By elimination, the remaining linkage between the constituent amino acids of phomopsin A was defined by the sequence 3-hydroxyisoleucine → 3,4-didehydroproline and a cyclic hexapeptide structure was proposed for phomopsin A [17]

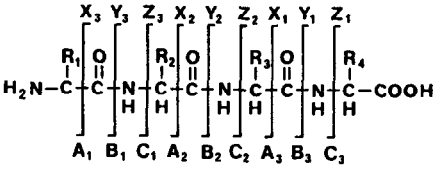
However, such a cyclic hexapeptide structure requires the presence of two *ortho* phenolic hydroxy groups in the substituted phenylserine moiety Chemical evidence for the involvement of the oxygen atom of one of these groups in an ether bond has been reported[18] but was not incorporated into previously reported structures [17,19] Thus phomopsin A fails to complex with borate Methylation of phomopsin A with diazomethane prior to acid hydrolysis and subsequent conversion of the hydrolysis products to their trimethylsilyl derivatives leads to the formation of a monomethyl mono(trimethylsilyl) derivative of 3-chloro-4,5-didhydroxybenzaldehyde (M^+, 258) [18]

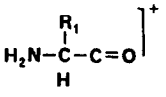
A linear hexapeptide structure lacking the peptide bond between the 2,3-didehydroaspartic acid and *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)serine moieties was, therefore, proposed for phomopsin A on the basis of f a b mass spectral data [19] In an *X*-ray crystallographic study (see later), the linear structure was further modified by an ether bridge replacing hydroxy groups of the substituted phenylserine and 3-hydroxyisoleucine units, as shown in (**1**) [20]

The n m r evidence is equally interpretable on the basis of structure (**1**) for phomopsin A The absence of a two-bond ($^{13}C,^{1}H$) coupling for the *N*-methyl carbon resonance is explained by the observation that the hexapeptide forms a zwitterion involving the carboxy group of the 2,3-didehydroaspartic acid moiety and the *N*-methyl nitrogen atom of the substituted phenylserine residue [20] The 2,3-didehydroaspartic acid forms a hydrogen-bonded 7-membered ring monoanion similar to that formed by maleic acid [21] The stability of the monoanion is enhanced by tautomerism and the resulting high acidity causes phomopsin to retain its zwitterionic character down to *p*H 2 The hydrogen-bonded

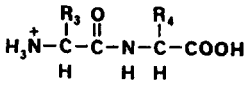
proton which is part of the tautomeric system resonates at unusually low-field, δ_H 18 96 p p m The corresponding proton in the maleic acid monoanion resonates at δ_H 20 1 p p m [19,21]

As stated earlier the complete amino acid sequence data for phomopsin A (**1**) was deduced from f a b mass spectral data [19] The chlorine-containing $[M+H]^+$ ions of phomopsin A (**1**), octahydrophomopsin A (**3**) and their methyl and trideuteromethyl esters (**4**)-(**7**) are prominent features of their positive ion f a b spectra (Figures 6 and 7) The most prominent *N* and *C* terminal sequence ions observed in the positive ion spectra are, in all cases, of the B and Y" types respectively (Scheme 1)[22] with cleavage of the macrocyclic ring occurring by fission of one or other of the ether bonds





B$_1$



Y$_2$

Scheme 1 Nomenclature for the sequence ions in the mass spectra of peptides with examples of B_1 and Y_2" ions (" denotes addition of 2H)

In the Y" series sequence ions the initial loss (243 amu) from the amino termini of phomopsin A (**1**) and its derivatives (**3**)-(**7**) involves fission of the 3-hydroxyisoleucine ether bond and hydrogen transfer to the aromatic amino acid with concomitant loss of hydrogen from the 3-hydroxyisoleucine units to yield didehydroisoleucine residues in the peptide chains The amino terminal *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)serine units generated by this ring opening are then lost (-243) leaving the newly-generated didehydroisoleucine residues present in the Y_5" and Y_4" fragment ions The latter are in turn lost (-111) in producing the Y_3" fragment ions

The B series fragment ions, on the other hand, are characterised by cleavage of the macrocyclic ring between the aryl ring and the ether oxygen with hydrogen transfer in the reverse direction to produce 3-hydroxyisoleucine residues in the peptide chains Thus, generation of the B_2 fragment ions involves a loss of 129 (3-hydroxyisoleucine) from the B_3 ions The B_1 ions have *m/z* 226/228 with no evidence of the (*m/z* 244/246) ions expected if cleavage of the ether bridge had occurred, as in the case of the Y" fragment ions, at the 3-hydroxyisoleucine ether bond

As well as the B and Y" ions, a series of sequence ions (*m/z* 658, 415, 318 and 207), beginning at the B_5 fragment ions, is observed in the spectra of phomopsin A (**1**) and its methyl (**4**) and

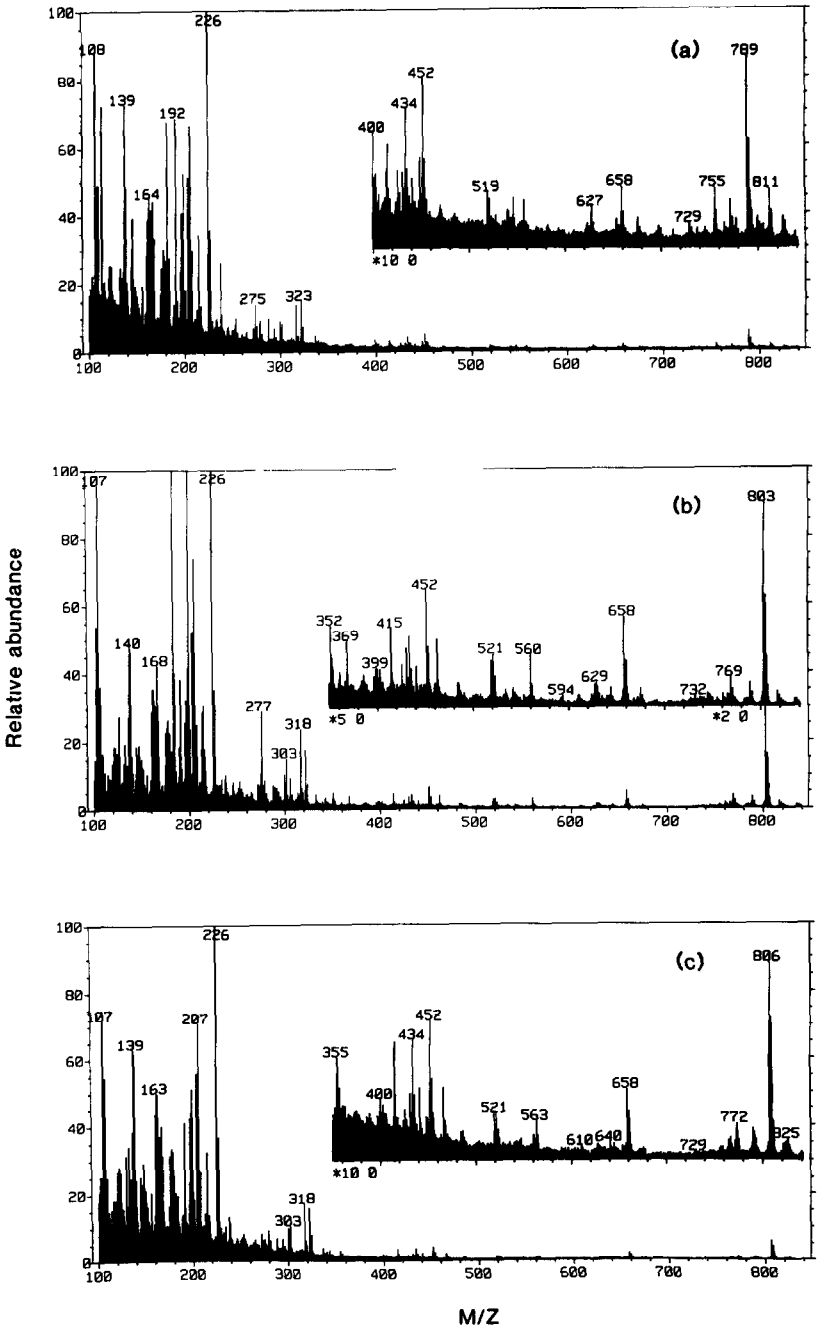


Figure 6. Positive ion f a b mass spectra of (a) phomopsin A (**1**), (b) the methyl ester of phomopsin A (**3**) and (c) the trideuteriomethyl ester of phomopsin A (**6**)

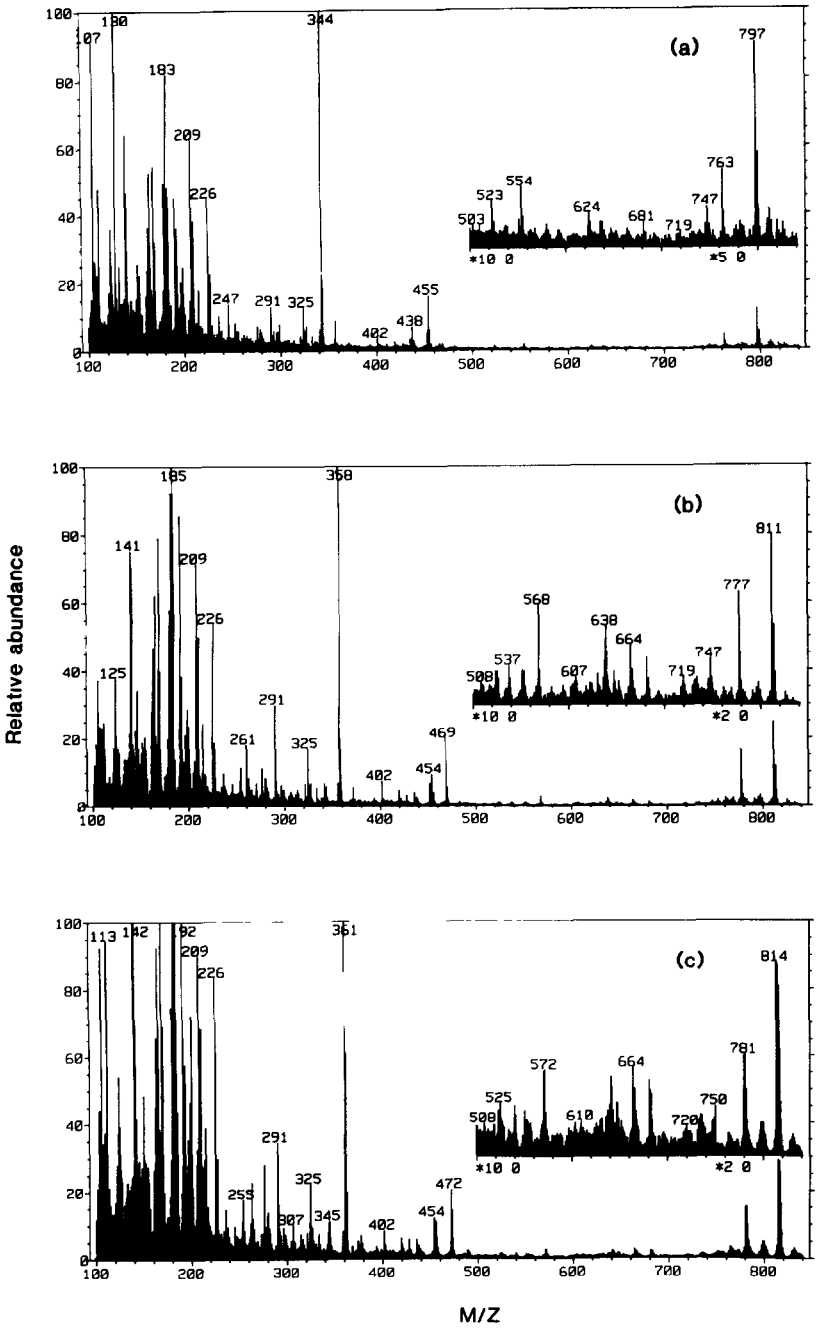


Figure 7 Positive ion f a b mass spectra of (a) octahydrophomopsin A (3), (b) the methyl ester of octahydrophomopsin A (5) and (c) the trideuteromethyl ester of octahydrophomopsin A (7)

Table 1 The *m/z* values of the main sequence ions in the positive ion f a b spectra of phomopsin A (**1**) and its derivatives (**3**) - (**7**)

Compound	$[M+H]^+$	B_1	B_2	B_3	B_4	B_5	$Y_1"$	$Y_2"$	$Y_3"$	$Y_4"$	$Y_5"$
(1)	789	226	323	452	-	658	-	-	338	449	546
(3)	797	226	325	454	-	-	134	247	344	455	554
(4)	803	226	323	452	-	658	-	-	352	463	560
(5)	811	226	325	454	551	664	148	261	358	469	568
(6)	806	226	323	452	547	658	-	-	355	466	563
(7)	814	226	325	454	-	664	151	264	361	472	571

- Indicates not distinguishable from background or absent

trideuteriomethyl (**6**) esters These ions, which involve fission of the aryl-ether bond of the macrocyclic ring, represent amino acid residue losses from the amino terminii of the B_5 acylium ions with transfer of a hydrogen to the acylium species

Dehalogenation reactions have been reported to occur in phenolic and heterocyclic aromatic compounds during f a.b measurement[23] and the f a b positive ion spectra of phomopsin A (**1**) and its derivatives (**3**)-(**7**) also show prominent ions formed by replacement of the aromatic chlorine by hydrogen Ions corresponding to the parent $[M+H]^+$ ions but lacking chlorine are observed at *m/z* 755, 769, 772, 777 and 780 in the spectra of (**1**), (**3**)-(**7**) respectivedly Low intensity Y" and B-series sequence ions of the dechlorinated molecules, corresponding to the ions shown in Table 1 but with *m/z* values of 34 less, are also detectable in the spectra

The mass spectrum of phomopsin A (**1**) in particular also shows evidence of hydrogen addition under f a b conditions leading to a distortion of the expected isotopic intensities from the theoretical distribution This phenomenon has been observed previously with polyunsaturated molecules such as organic dyestuffs[24] and daunomycin derivatives [25]

The chlorine-containing B_1 fragment ions at *m/z* 226/228 are the most intense peaks in the spectra of the unreduced phomopsins (**1**), (**4**) and (**6**), while the $Y_3"$ ions formed by cleavage of the 3-oxyisoleucine-proline peptide bonds are the base peaks in the spectra of the octahydro derivatives (**3**), (**5**) and (**7**)

The f a b mass spectral data provide evidence of, and are fully consistent with the presence of the ether bridge established by the *X*-ray diffraction data,[17] (see later) although this was not recognised in an earlier interpretation of the f a b data [19] The molecular ions and fragment ions observed in the

f a b spectra of (1), (3)-(7) indicated an absence of the elements of water from a linear peptide structure in which the amino terminii and both carboxyl groups at the carboxy terminii are free [19] This suggests an internal ether bridge, most logically between one of the phenolic hydroxy groups and the originally postulated 3-hydroxyisoleucine residues [18] The presence of such a bridge is indicated in the f a b spectra by fragment ions in which the ether oxygen is retained by either the *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl)serine units (Y" series ions) or by the 3-hydroxyisoleucine units (B series ions) but never on both residues simultaneously

Phomopsin A (1) crystallises from ethanol-methanol-water as hydrated prismatic crystals which analyse for $2(C_{36}H_{45}ClN_6O_{12})\ 5H_2O$ The crystals are orthorhombic, space group $P2_12_12_1$, a = 18 756(3), b = 22 321(3), c = 23 940 (6) Å, Z = 4

The crystallographic analysis shows that phomopsin A has the linear peptide configuration shown in (1), with an ether bridge forming a 13-membered macrocyclic ring which incorporates a 3,4,5-trisubstituted phenyl ring Phomopsin thus resembles the cyclopeptide group of alkaloids[26] which are tetra- or pentapeptides with a modified (decarboxylated and dehydrated) 3-(*p*- or *m*-hydroxyphenyl)serine unit linked through an ether bridge to a 3-hydroxy amino acid to form a 13- or 14-membered ring However, in the alkaloids, the peptide chain is connected through the amino group of the phenylserine unit rather than through the carboxyl group as in phomopsin A

The two independent molecules in the crystal (**A** and **B**) adopt different conformations (see Figure 8 and Table 2), the major difference being the relative orientation of the 3-chloro-4-hydroxy-5-oxyphenyl moiety of the macrocyclic ring Compared with its position in molecule **A**, the aromatic ring in molecule **B** has rotated approximately 137° about an axis joining O-2 and C-11 so that in molecule **B** the aromatic ring is face-on to the C-6--C-7 segment of the macrocyclic ring The angle between the aromatic ring and the convenient reference bond C-7--C-32 is 153° in molecule **B** (the angle of rotation of the aromatic ring is the sum of the supplements of these angles) The difference in the two conformations is also reflected in four torsion angles of the macrocyclic ring, *viz* C-16--C-1--O-2-C-3, C-1--O-2--C-3--C-4, C-9--C-10--C-11--C-12 and C-10--C-11--C-12--C-16, which have the respective values -95(2), 91(2), -42(2), 96(2)° in molecule **A** and 95(3), 19(3), 43(3), -93(3)° in molecule **B** Consequently, the side-chain of the *N*-methyl-3-(3-chloro-4-hydroxy-5-oxyphenyl) unit adopts the $[g^+(g^-,g^+)]$ and $[t(g^+,g^-)]$ conformations in molecules **A** and **B** respectively, the respective $\chi_1^{1,2}$, $\chi_1^{2,1}$, $\chi_1^{2,2}$ values being 78(2), -89(2), 96(2)° for molecule **A** and 166(2), 79(3), -93(3)° for molecule **B** Although there is an intramolecular hydrogen bond between the nitrogen atom and the hydroxy group of the protonated phenylserine residue (see Table 3) in molecule **A**, the N-36 O-38 distance being 2 71(2) Å, there is no indication of a similar interaction in molecule **B** The side-chain of the 3-oxyisoleucine moiety is described as $[t(g^+)]$ in molecule **A** with $\chi_3^{1,1}$ = 168(2)° and χ_3^2 = 57(3)° while in molecule **B** its conformation is $[t(t)]$ with $\chi_3^{1,1}$ = 165(2)° and χ_3^2 = 178(2)° as a result of the different macrocyclic ring conformations

Table 2. Selected torsion angles (°) E s d 's range between 2 and 5°

	*	Mol A	Mol B		*	Mol A	Mol B
C1-O2-C3-C4		91	79	N24-C25-C50-O52	ψ_{6T}^{2}	-167	169
O2-C3-C4-N5		-84	-75	C23-N24-C25-C26		-8	20
C3-C4-N5-C6		139	135	N24-C25-C26-C27	χ_{6}^{1}	177	178
C4-N5-C6-C7	ω_2	176	180	C25-C26-C27-O53	$\chi_{6}^{2,2}$	177	-170
N5-C6-C7-N8	ψ_2	111	109	C25-C26-C27-O54	$\chi_{6}^{2,1}$	1	4
C6-C7-N8-C9	ϕ_2	-106	-110	C27-C26-C25-C50		-4	1
C7-N8-C9-C10	ω_1	170	169	C26-C25-C50-O51	$\chi_{6}^{2,1}$	-162	174
N8-C9-C10-C11		-74	-95	C26-C25-C50-O52	$\chi_{6}^{2,2}$	13	-13
C9-C10-C11-C12		-42	43	N36-C10-C11-O38	$\chi_{1}^{1,1}$	-49	45
C10-C11-C12-C16	$\chi_{1}^{2,2}$	96	-93	N36-C10-C11-C12	$\chi_{1}^{1,2}$	78	166
C11-C12-C16-C1	$\chi_{1}^{3,2}$	180	180	N36-C10-C9-N8	ψ_1	-102	-119
C12-C16-C1-O2		-177	173	C37-N36-C10-C9	ϕ_{1T}	-94	-73
C16-C1-O2-C3		-95	95	C37-N36-C10-C11		141	160
O2-C3-C4-C17		160	160	C10-C11-C12-C13	$\chi_{1}^{2,1}$	-89	79
C6-N5-C4-O17	ϕ_3	-104	-96	N5-C4-C3-C28	$\chi_{3}^{1,1}$	168	165
N5-C4-C17-N18	ψ_3	117	116	N5-C4-C3-C30	$\chi_{3}^{1,2}$	38	46
C3-C4-C17-N18		-126	-117	C4-C3-C28-C29	χ_{3}^{2}	57	178
C4-C17-N18-C19	ω_3	-175	-173	C4-C17-N18-C41		-2	-3
C17-N18-C19-C20	ϕ_4	-74	-77	N8-C7-C32-C33	$\chi_{2}^{1,1}$	-9	-6
N18-C19-C20-N21	ψ_4	159	149	N8-C7-C32-C34	$\chi_{2}^{1,2}$	180	176
C19-C20-N21-C-22	ω_4	168	172	N18-C19-C43-C42	χ_{4}^{1}	180	176
C20-N21-C22-C23	ϕ_5	70	53	C19-C43-C42-C41	χ_{4}^{2}	7	2
N21-C22-C23-N24	ψ_5	30	40	N18-C41-C42-C43	χ_{4}^{3}	-8	-2
C-22-C23-N24-C25	ω_5	-178	175	N21-C22-C45-C46	$\chi_{5}^{1,1}$	-173	-165
C-23-N24-C25-C50	ϕ_6	172	-163	N21-C22-C45-C48	$\chi_{5}^{1,2}$	6	-3
N24-C25-C50-O51	ψ_{6T}^{1}	18	-3	C22-C45-C46-C47	χ_{5}^{2}	-102	-119

*The convention for the conformational angles ϕ, ψ, ω, χ, is that proposed by the IUPAC-IUB Commission on biochemical nomenclature (*cf Biochemistry*, 1970, **9**, 3471) The residues R_1 - R_6 are defined as follows

R_1 = *N*-methyl-3-(3-chloro-4-hydroxy-5-oxyphenyl)serine
R_2 = 3,4-didehydrovaline
R_3 = 3-oxyisoleucine
R_4 = 3,4-didehydroproline
R_5 = *E*-2,3-didehydroisoleucine
R_6 = *E*-2,3-didehydroaspartic acid

The segment of the hexapeptide chain comprising the 3,4-didehydroproline, 2,3-didehydroisoleucine and 2,3-didehydroaspartic acid moieties adopts a similar conformation in the two independent molecules The segments, however, are not fully extended but have a turn at the 2,3-didehydroisoleucine residue (ψ_5 = 30(3) and 40(4)° in molecules **A** and **B**, respectively) so that the terminal 2,3-didehydroaspartic acid residue is twisted back towards the *sec*-butyl moiety of the 3-

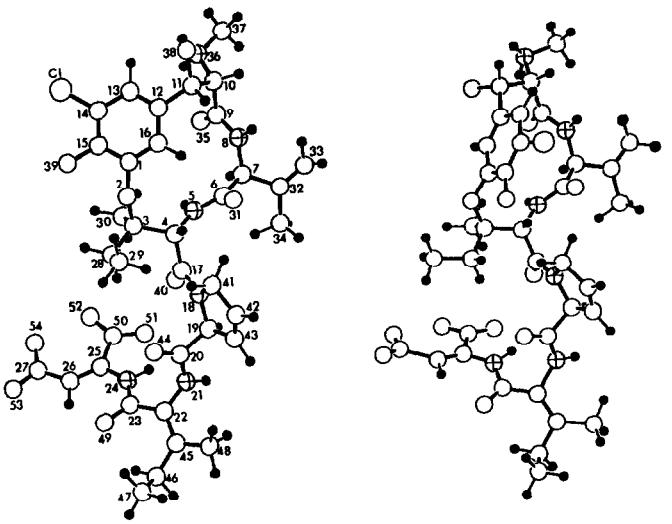


Figure 8. Perspective view of molecules **A** and **B** for phomopsin A

oxyisoleucine The 2,3-didehydroaspartic acid is a monoanion involved in two intramolecular hydrogen bond interactions For one of the interactions, N-24 donates its proton to O-51 of the peptide chain carboxyl group, the N-24 O-51 distances being 2 57(2) and 2 53(2) Å in molecules **A** and **B** respectively For the other interaction, a symmetrical hydrogen bond is formed between the two carboxyl groups The O-52 O-54 distances, 2 43(2) Å in molecule **A** and 2 40(2) Å in molecule **B**, are similar to the value 2 408(1) Å observed for this interaction in the maleate anion in imidazolium hydrogen maleate [27] As in the latter, the four carbon atoms are planar (torsion angle C-27--C-26--C-25--C-50 -4(5) and 1(5)° in molecules **A** and **B**, respectively), but one carboxyl group in molecule **A** and both carboxyl groups in molecule **B** are slightly rotated from the plane (See torsion angles C-25--C-26--C-27--O-53, C-25--C-26--C-27--O-54, C-26--C-25--C-50--O-51, C-26--C-25--C-50--O-52

The peptide bonds in phomopsin A are all approximately *trans* planar, the ω values (Table 2) ranging from 168(1) to 180(2)° in the two independent molecules The largest deviations from planarity of approximately 10° are found for the substituted phenylserine and 3,4-didehydroproline units The orientation of the side-chains of the 3,4-didehydrovaline, 2,3-didehydroisoleucine and 2,3-didehydroaspartic acids units in the two molecules is similar For molecule **A** the respective $\chi^{1,1}$ values are -9(4), -173(2), 177(3)° and for molecule **B** -6(3), -164(3), 178(3)° Atoms of the five-membered ring of 3,4-didehydroproline are planar to within ± 0 08(2) and ± 0 02(2) Å in molecules **A** and **B**, respectively

There is some disordering of the water molecules in the crystal, and as an approximation to allow for this, four of the water oxygen atoms, OW-4--OW-7, have been included with one-half occupancy An intricate system of intermolecular hydrogen bonds links the phomopsin and water molecules into a three-dimensional network (Table 3) Some of the interactions provide a direct linkage between the phomopsin molecules whilst for others, the water molecules provide a bridging between them As

Table 3 Proposed hydrogen bonding for phomopsin A (Distances (Å) and angles (°)

Transformations of the coordinates (*x,y,z*) are given by superscripts

I ($\frac{1}{2}+x, \frac{1}{2}-y, 1-z$), II ($-\frac{1}{2}+x, \frac{1}{2}-y, 1-z$), III ($1-x, \frac{1}{2}+y, \frac{1}{2}-z$), IV ($\frac{1}{2}-x, -y, -\frac{1}{2}+z$), V ($1-x, -\frac{1}{2}+y, \frac{1}{2}-z$), VI ($-x, -\frac{1}{2}+y, \frac{1}{2}-z$), VII ($1-x, -\frac{1}{2}+y, 1\frac{1}{2}-z$), VIII ($-x, -\frac{1}{2}+y, 1\frac{1}{2}-z$), IX ($\frac{1}{2}-x, 1-y, -\frac{1}{2}+z$), X ($\frac{1}{2}-x, 1-y, \frac{1}{2}+z$)

Atoms		X Y	H[a] Y	<X-H Y	Atoms		X Y
N5A	O35B[I]	2 92(2)	2 06	146	O38B	O52A[II]	2 84(3)
N5B	O35A[II]	2 96(3)	2 08	149	O39A	O31B	2 81(2)
N8A	OW3[I]	2 93(4)	1 96	178	O39B	OW4	2 69(5)
N8B	O39A	3 00(2)	2 15	147	O52B	O54B[b]	2 43(2)
N21A	OW1[III]	2 95(3)	1 98	172	O52A	O54A[b]	2 40(2)
N21B	O53A	2 94(3)	1 98	171	OW1	O53B[VI]	2 83(4)
N24A	O51A[b]	2 57(2)	2 04	112	OW2	O51A[VII]	3 11(4)
N24B	O51B[b]	2 53(2)	2 05	108	OW2	O54B[VIII]	2 94(4)
N36A	O38A[b,c]	2 71(2)	2 17	114	OW5	O51B[IX]	3 21(6)
N36A	O51B[Ic]	2 79(3)	2 08	129	OW5	O53A[IX]	3 01(6)
N36A	O40B[I]	3 10(3)	2 42	127	OW6	O44B[X]	2 59(6)
N36B	O40A[II]	2 83(2)	1 95	149	OW3	OW6[d]	3 15(7)
N36B	O51A[IIc]	2 70(3)	1 81	151	OW4	OW7[IXd]	3 21(6)
N36B	OW2[IVc]	3 15(3)	2 56	120	OW6	OW7[d]	2 55(10)
O38A	O44A[V]	2 71(2)			OW4	OW5[d]	2 47(7)

[a] Hydrogen atom coordinates were calculated (N-H 0 97 Å)

[b] Intramolecular H-bond

[c] Bifurcated interaction

[d] Donor atom uncertain

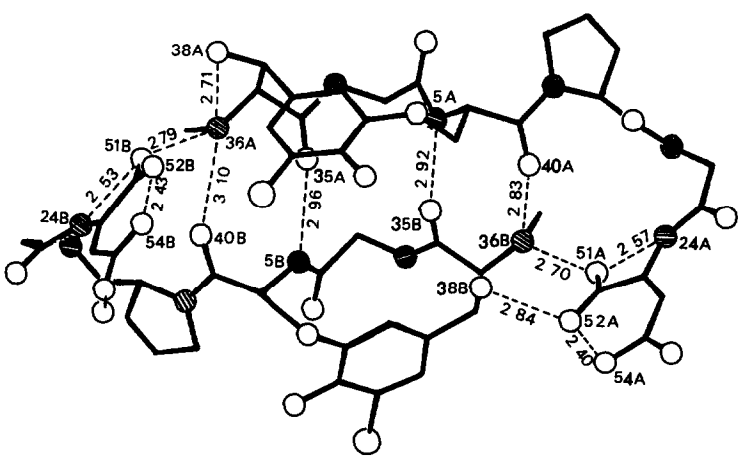


Figure 9 Relative orientations of the **A** and **B** molecules of phomopsin A showing the hydrogen bonding The side-chains of the 3,4-didehydrovaline, 3-oxyisoleucine and 2,3-didehydroisoleucine moieties have been omitted for clarity

illustrated in Figure 9, the **A** and **B** molecules are linked in pairs, with the 2,3-didehydroaspartic acid terminus of molecule **A** adjacent to the substituted phenylserine moiety of molecule **B**, and *vice versa* In addition to the electrostatic bonds formed by these zwitterionic centres, there are seven intermolecular hydrogen bonds linking the two molecules the N-36 nitrogen atoms of the phenylserine moieties are the donor atoms in interactions with the O-51 atoms of the 2,3-didehydroaspartic acid units and the O-40 carbonyl oxygens of the 3-oxyisoleucine units In addition to these interactions, the N-5 atoms of the 3-oxyisoleucine residues donate their protons to the O-35 carbonyl oxygens of the phenylserine moieties, and the hydroxy group oxygen, O-38 of molecule **B**, is the donor atom in an interaction with O-52 of the 2,3-didehydroaspartic acid of molecule **A**

Only fragmentary evidence is available for the conformation of phomopsin A in solution The close similarity of the section of the macrocyclic ring from O-2 to C-10 in the **A** and **B** molecules of the crystal, suggests that this section will retain the same conformation also in solution and that the macrocyclic ring as a whole will approximate the **A** and **B** options or both This may not be true of the acyclic part of the molecule which, in the crystal, is apparently constrained by the favourable energetics of the electrostatically bonded **A-B** pair In solution in water or dimethylsulphoxide, the interpair bonds would be expected to be replaced by solvation and the peptide chain should be free to assume other conformations The most relevant n m r parameter, the vicinal coupling constant of the C*H*OH-C*H*NHMe system *J* 3 9 Hz, is consistent with both the **A** and **B** conformations, the torsion angle, H-10--C-10--C-11--H-11, being -45° in **A** and 43° in **B** An n O e effect observed between the aromatic proton H-16 and the benzylic proton H-11 is indicative of the macrocyclic ring being, to a large extent, in the **A** conformation There is also a 4-bond benzylic coupling between H-16 and H-11, *J* 1 2 Hz, which suggests that it originates in the **B** conformation in which the 4-bond pathway has an extended form Other observed effects such as the sharpening and increase in height of some resonances (*e g* methyl protons of C-30 and C-34) and decrease in height of others (*e g* H-7) when the temperature is raised to 348 K, probably reflect a change in conformer populations but their precise significance is less clear

The temperature gradients for the chemical shift of the hydroxy and amide protons (Table 4) exhibit the wide range of values which are usually indicative of conformational stability, although, in this instance, it probably does not exclude the interconversion of conformers **A** and **B** because of the small change in position of most atoms Both the amide and the carboxyl hydroxy group of the 2,3-didehydroaspartic acid group have gradients of less than 2×10^{-3} ppm K^{-1}, consistent with the tight binding of the carboxyl hydroxy group in the monoanion complex of the 2,3-didehydroaspartic acid and the strong hydrogen-bonding of the amide proton to the adjacent carboxyl [N-24A O-51A, 2 53 Å, N-24B O-51B, 2 51 Å] This amide proton exchanges slowly with deuterium oxide in comparison with that of the 3-oxyisoleucine group with a temperature gradient of $6\ 7\times10^{-3}$ p p m K^{-1}, which exchanges particularly slowly This N-5 proton is hydrogen-bonded in the crystal to the carbonyl (O-35) of the other molecule of the **A-B** pair implying that in an unpaired molecule it should have adequate access to solvent molecules The very slow exchange therefore raises the possibility that the molecular pairing seen in the crystal might actually persist in solution

Table 4 Temperature gradients of the exchangeable protons of phomopsin A (1)

Chemical Shift* (ppm)	Temp Gradient (ppm)K^{-1} ($x10^3$)	D_2O Exchange	Assignment
6 65 br s	4 3	fast	OH
8 55 br h	3 3	fast	OH
8 82 d	6 7	very slow	N-5 proton
8 95 d	7 5	med fast	N-8 proton
9 09 s	5 8	fast	OH
9 36 s	0 7	slow	N-24 proton
9 68 s	4 9	slow	N-21 proton
19 0 s	1 8	fast	OH of 2,3-dide-hydroaspartic acid

*br = broad, d = doublet, h = hump, s = singlet

Although the stereoview (Figure 10) shows that the molecules in the **A-B** pair have juxtaposed hydrophobic regions formed by (1) the side-chains of the substituted phenylserine and 3-oxyisoleucine residues and (2) those of the 3,4-didehydrovaline units, which would tend to hold the molecules together in aqueous solution, the existence of pairing in solution was not supported by ^{13}C relaxation time measurements in [$^2H_6$]dimethyl sulphoxide

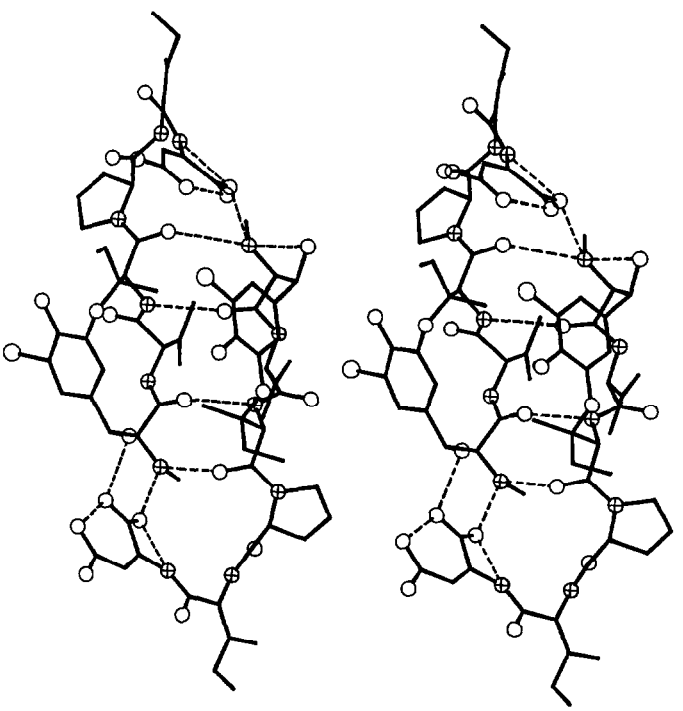


Figure 10. Stereoview of the **A-B** pair in crystals of phomopsin A

The absolute configuration as depicted in (1) is determined by prior evidence that the 3,4-didehydroproline and 3-isoleucine units have the L-configuration (see earlier) The stereochemical specification of phomopsin A is therefore 22*E*, 25*E*, 3*R*, 4*S*, 7*S*, 10*S*, 11*S*, 19*S*, the configuration of the double bonds at C-22 and C-25 also being determined earlier

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 spectrometer, while i r spectra were recorded for KBr discs on a Perkin-Elmer 237 spectrometer N m r spectra of [$^2H_6$]dimethyl sulphoxide solutions were recorded on a Bruker WM-500 spectrometer operating at 500 13 MHz for ^1H and 125 76 MHz for ^{13}C nuclei Fast atom bombardment (f a b) mass spectra were obtained using a JEOL JMS-DCX303 mass spectrometer and JMA-DA5000 data system The f a b gun was operated at 6kV and xenon was used as the bombarding atom beam Analyses were carried out at ambient temperature and 3kV accelerating voltage Samples (5-10 μg) were dissolved in a thioglycerol glycerol (1 1) matrix

Isolation of phomopsin A (1) - *Phomopsis leptostromiformis* (MRC 2654) was grown in bulk on wet, sterilized, whole yellow-maize kernels for 21 days at 25 °C Cultures were dried at 45 °C for 24 h and milled to a fine meal The resulting material was acutely toxic to day-old ducklings The dried, milled, mouldy maize (5 0 kg) was extracted with methanol by high speed blending in a Waring blender The methanol extract was concentrated *in vacuo* and the residue partitioned between aqueous methanol (90%) and hexane The methanol was evaporated and the residual material was partitioned between chloroform and water The toxic material obtained from the water fraction was fractionated by column chromatography on macroreticular polystyrene resin (XAD-2) using (a) water, (b) water methanol (1 1 v/v), and (c) methanol as eluant Bioassay indicated that the toxicity was associated with the residue obtained from the methanol solution (12 6 g)

The active fraction was further fractionated on a Sephadex LH-20 column with methanol water (1 1 v/v) Fractions (150 ml) were analysed for the presence of phomopsin A by t l c on silica gel using *n*-propanol *n*-butanol water ammonium hydroxide (65 15 17 3 v/v/v/v), as the developing solvent Fractions containing phomopsin A were pooled and the solvent evaporated under reduced pressure to give toxic material (9 0 g)

A part of this toxic material (6 0 g) was dissolved in 0 02 M ammonium hydrogen carbonate buffer (*p*H 7 9) and applied to a column of DEAE cellulose (3 8 x 50 cm) The column was developed at a flow rate of 120 ml h^{-1} collecting 20 ml fractions (u v detection at 260 nm) using initially 0 02 M buffer (1 8 l) and subsequently by gradient elution with the same buffer (final concentration 0 2 M) Fractions were analysed for phomopsin A by t l c as described earlier Fractions 220-245 were pooled and freeze-dried for 72h to yield amorphous phomopsin A (660 mg)

Crystallisation from methanol ethanol water (5 4 1 v/v/v) gave phomopsin A (1), m p 205 °C (decomposition without melting), λ_{max} 209 (ε 53 300), 222 sh (ε 24 500) and 288 nm (ε 13 900), ν_{max} (KBr) 3340 (amide NH), 2800 - 2550 (CO_2H), 1670 and 1645 cm^{-1} (amide CO) (Found C, 51 7, H, 5 8, N, 10 3, Cl 4 4% Calculated for 2($C_{36}H_{45}ClN_6O_{12}$) $5H_2O$ C, 51 83, H, 6 04, N, 10 07, Cl 4 25%)

Formation of phomopsinamine (2) - A solution of phomopsin (1) (100 mg) in 6 M hydrochloric acid was stirred for 65 min at 38 °C The solvent was evaporated *in vacuo* and the residue was purified by chromatography on Sephadex LH-20 using methanol as eluant to give amorphous phomopsinamine (2) (56 mg)

Octahydrophomopsin A (3) - A suspension of PtO_2 and phomopsin A (2 mg) in methanol (1 ml) was stirred at room temperature and pressure in a hydrogen atmosphere for 24h to give a mixture of diastereomeric octahydrophomopsin A (3) Longer periods of hydrogenation caused a slow dehalogenation

Phomopsin A methyl (4) and [methyl-$^2H_3$] (6) esters. - The methyl esters were prepared by dissolving phomopsin A in methanol (or [$^2H_3$]methanol) made 0 1 M with respect to hydrochloric acid Complete conversion to the monomethyl ester, monitored by f a b mass spectrometry and paper electrophoresis occurred over a period of a week

Octahydrophomopsin A methyl (5) and [methyl-$^2H_3$] (7) ester. - These esters were prepared as described above by hydrogenation of the respective phomopsin A esters using PtO_2 as catalyst and methanol (or [$^2H_3$]methanol) as solvent

X-ray crystallographic analysis of phomopsin A - Hydrated prismatic crystals of phomopsin A (**1**), $2(C_{36}H_{45}ClN_6O_{12})\ 5H_2O$ were obtained from ethanol methanol water M_W = 1668 5, orthorhombic, space group $P2_12_12_1$, a = 18 756(3), b = 22 321(3), c = 23 940(6) Å V = 10023(5) Å^3, Z = 4, D_c = 1 106 g cm^{-3}, $F(000)$ = 3528, μ(Cu Kα) = 11 0 cm^{-1} A crystal *ca* 0 22 x 0 22 x 0 65 mm was sealed in a thin-walled Lindemann glass capillary with a small amount of mother liquor for the diffraction measurements The intensity data were measured at 288(1)K on a four-circle Rigaku-AFC diffractometer with CuKα radiation (graphite-crystal monochromator, λ = 1 5418 Å) and recorded by an ω - 2θ scan to a $2\theta_{max}$ of 90° The intensities of three standard reflections, measured every 50 reflections, remained constant to within 3% The data were corrected for Lorentz and polarization effects but not for absorption Of the 4495 unique data measured, the 3643 for which $I > \sigma I$ were used for the structure refinement

The structure was solved by direct methods with the MULTAN program Initially, the only sites readily located from *E*-maps were those for atoms of the 3-chloro-4-hydroxy-5-oxyphenyl moiety of one phomopsin molecule, molecule **A** Attempts to initiate phase refinement based on a number of different trial structures (the initial fragment was extended and sites for other plausible molecular fragments included) eventually yielded a reliable set of phases It is of interest to note that the site of the second chlorine atom in the asymmetric unit was located only after molecular fragments corresponding to ~40% of the scattering material were included in the analysis Refinement with anisotropic temperature factors given to the Cl and isotropic for the C, N and O atoms converged at R = 0 125 For the final refinement, seven low order terms (sin θ > 0 10) severely affected by extinction were omitted The parameters were refined in two blocks with the terms given unit weights, the function minimized was $\Sigma(|F_o| - |F_c|)^2$ Hydrogen atoms bonded to C and N were included at idealized positions The non-methyl and methyl H atoms were given common isotropic temperature factors which refined to values of U = 0 07(2) and 0 15(3) Å^2, respectively At convergence the maximum shift-to-error ratio was 0 07 1 and the final difference map showed no unusual features Refinement was carried out with the SHELX76 program on a VAX11/780 computer

Selected torsion angles are given in Table 2 and hydrogen bonding dimensions are given in Table 3 Figure 8 which contains the atom numbering and Figure 9 have been prepared from the output of the ORTEPII program Atomic coordinates for this structure as well as bond lengths, bond angles, torsion angles and intermolecular contacts for the non-hydrogen atoms, structure-factor amplitudes and thermal parameters have been deposited with the Cambridge Crystallographic Data Centre [28]

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