The Isolation, Structure, and Absolute Configuration of the Mycotoxin, Rhizonin A, a Novel Cyclic Heptapeptide containing *N*-Methyl-3-(3-furyl)alanine, produced by *Rhizopus microsporus*

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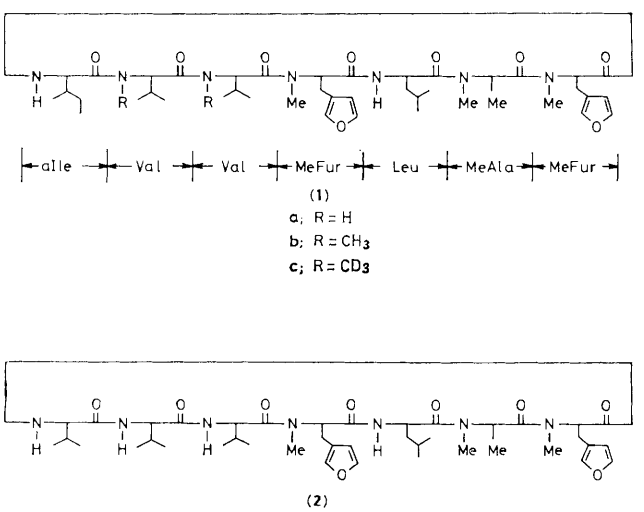
Rhizonin A, the main mycotoxin from *Rhizopus microsporus*, is a cyclic heptapeptide containing valine, *allo*-isoleucine, leucine, *N*-methylalanine, *N*-methyl-3-(3-furyl)alanine (2 : 1 : 1 : 1 : 2), its amino-acid sequence having been established by mass spectrometry; *X*-ray crystallography verified the structure of rhizonin A and afforded its conformation and relative configuration, and its absolute configuration was obtained from high performance liquid chromatographic studies of the dansyl-derivatised amino-acids.

Rhizonin A (**1a**), a novel metabolite from *Rhizopus microsporus* van Tieghem, is the first mycotoxin reported from a fungus which belongs to the order Mucorales of the class Phycomycetes (lower fungi). Most of the known mycotoxin-producing fungi belong to the class Deuteromycetes and to a lesser degree to the class Ascomycetes. Species of *Rhizopus* and the closely related genus *Mucor* are the most common fungi occurring in sorghum malt, *i.e.*, malt produced from *Sorghum bicolor* Moench. Species of *Rhizopus* are furthermore in use as enzymatic sources for the production of fermented foods, such as tempeh. A highly toxinogenic strain of *R. microsporus* (MRC 303), isolated from Mozambican groundnuts was grown on sterilized whole maize and the toxic principles removed by prolonged extraction with chloroform–methanol. Fractionation of the toxic extract, guided by bio-assay in day-old ducklings, involved solvent partition followed by chromatography on silica gel and neutral aluminium oxide to yield rhizonins A and B (0.01 and 0.00024% by weight of mouldered material). Rhizonins A and B were resolved on silica thin layer chromatoplates in the solvent ethyl acetate–hexane (4 : 1 v/v) and appeared at R_F 0.31 and 0.22, respectively. The rhizonins were detected by spraying with 1% cerium sulphate in 3M H_2SO_4. We now report the structure elucidation and absolute configuration of rhizonin A; the structure of rhizonin B is based on only amino-acid analysis and comparison of spectral data. The rhizonins represent the first natural products to contain a derivative of 3-(3-furyl)alanine, an amino-acid which is not a known constituent of peptides or known as a natural product.



(1)

a; R = H
b; R = CH_3
c; R = CD_3

(2)

Rhizonin A (**1a**), $C_{42}H_{65}N_7O_9$, was crystallized from hexane–ethyl acetate, m.p. 243 °C; it had $[\alpha]_D^{24}$ −25° (*c*, 1.15, $CHCl_3$); λ_{max} (MeOH) 206 nm (ϵ 33 000); ν_{max} ($CHCl_3$) 3 418, 3 340, 3 295, and 1 650 cm^{-1}; $\Delta\epsilon$ (263 nm) 0, $\Delta\epsilon$ (237 nm) −21.4, and $\Delta\epsilon$ (224.5 nm) 0. Amino-acid analysis of an acid hydrolysate of rhizonin A indicated the presence of valine (Val), *allo*-isoleucine (aIle), and leucine (Leu) in a ratio of 2 : 1 : 1; whereas an acid hydrolysate of rhizonin B (**2**), $C_{41}H_{63}N_7O_9$,[1] contained Val and Leu in a ratio of 3 : 1. The presence of *N*-methylalanine (MeAla) in the hydrolysate of (**1a**) was verified by high performance liquid chromatographic analysis (h.p.l.c.) of the 2,4-dinitrophenyl derivatives of the amino-acids.

The ^1H and ^{13}C n.m.r. data are in agreement with structure (**1a**) and indicate at least two conformations in a chloroform solution. The proton-noise-decoupled 25.2 MHz ^{13}C n.m.r. spectrum of rhizonin A exhibited 42 main resonances. Chemical shift considerations indicated 7 amido-carbonyl (δ_C 174.9—167.9 p.p.m.), 8 sp^2 (6 methine and 2 quaternary), and 27 sp^3 (12 methyl, 4 methylene, and 11 methine) carbon atoms. The eight olefinic carbon resonances are characteristic of two 3-substituted furyl moieties[2] and appear as pairs *viz.* 142.8 (^1J 201.5 Hz) and 142.5 (^1J 201.4 Hz); 139.9 (^1J 199.7 Hz) and 139.3 (^1J 199.5 Hz); 121.8 (S) and 120.4 (S); and 110.7 (^1J 174 Hz) and 110.4 p.p.m. (^1J 174 Hz). The heptapeptide nature was substantiated by the presence of seven methine carbon atoms, C(2), resonating between δ_C 68 and 47 p.p.m. Three of the amino-acid residues are *N*-methylated [δ_C 40.1 (Q, ^1J 138.2 Hz); δ_C 30.9 (Q, ^1J 138.4 Hz); and δ_C 30.6 p.p.m. (Q, ^1J 138.2 Hz)].

N,*N*-Dimethylrhizonin A (**1b**) and the corresponding *N*,*N*-bis(trideuteriomethyl)rhizonin A (**1c**) were prepared by the Hakomori method,[3] using methyl iodide and trideuteriomethyl iodide, respectively. Amino-acid analysis of (**1b**) proved the indicated sites of bismethylation.

The molecular formula, $C_{42}H_{65}N_7O_9$, of rhizonin A (**1a**) was established by peak matching (*m/z* 811.486) and field

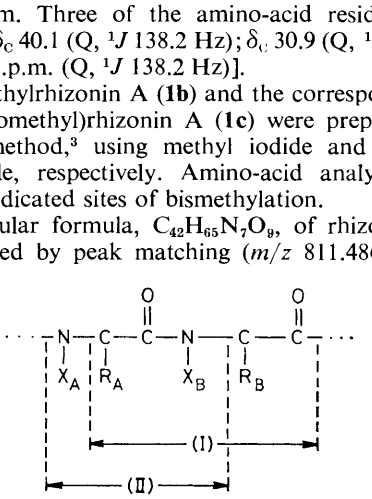


Figure 1. Ion types in the mass spectral fragmentation of peptides.

Table 1. Selected accurate masses of (**1c**) and derived partial sequences for (**1a**).

	Masses		Elementary composition					Ion		Partial sequence
	Found	Calc.	C	H	D	N	O	type[a]	Amino-acid composition[b]	for (**1a**)
1	199.153	199.153	11	15	3	1	2	(I)	$(CD_3Val, CD_3Val) - NCD_3 - H$	Val-Val
2	213.168	213.168	12	17	3	1	2	(I)	$(CD_3Val, Leu) - NH - H$	Leu-Val
3a	563.375	563.376	30	45	3	4	6	(II)	$M - (CD_3Val, MeFur + NH)$	Val-MeFur-Leu or MeFur-Val-Leu
4a	617.380	617.382	33	41	6	4	7	(II)	$M - (Leu, MeAla + NCH_3) - H$	Leu-MeAla-MeFur or MeAla-Leu-MeFur
5	679.478	679.479	36	53	6	5	7	(II)	$M - (MeFur + NH)$	MeFur-Leu
6	697.406	697.405	37	55	0	5	8	(II)	$M - (CD_3Val + NCD_3)$	Val-Val

[a] *Cf.* text for definition of type. [b] These are the only possible assignments using standard *cyclo*-peptide fragmentation patterns.[4-6]

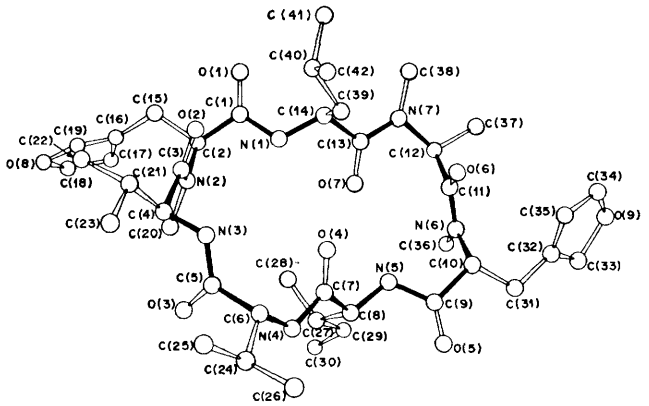


Figure 2. A perspective drawing of rhizonin A showing the numbering scheme of the non-hydrogen atoms. The absolute stereochemistry is implied.

desorption mass spectroscopy. The ion types $NH(X)=CH(R)^+$ and $[M - NX=CH(R)]^+$ are abundant in the electron impact (e.i.) mass spectra of cyclic peptides.[4-6] The high-resolution spectra of (**1a**), (**1b**), and (**1c**) showed abundant ions $C_3H_8N^+$ and $(M - C_3H_7N)^+$, due to MeAla, as well as the ions $C_7H_{10}NO^+$ and $(M - C_7H_9NO)^+$. The u.v.[7] and n.m.r. data[2] of (**1a**), and the constitution of the seven-carbon fragments, indicated the presence of two *N*-methyl-3-(3-furyl)alanine moieties [-NMeCH($CH_2C_4H_3O$)-CO=MeFur]. Therefore, the amino-acid composition of rhizonin A is Val, Leu, aIle, MeFur, MeAla in a ratio 2:1:1:2:1. However, for sequence determination the composition of (**1a**) was treated as Val, Leu, MeFur, MeAla (2:2:2:1), since Leu and aIle are indistinguishable by e.i. mass spectrometry.

N,*N*-Bis(trideuteriomethyl)rhizonin A contains three types of N-substituents, *viz.* 2 × NH (Leu and aIle), 3 × NCH_3 (MeFur and MeAla), and 2 × NCD_3 (CD_3Val). These labelled positions facilitated the identification of ions of types (I) and (II), thereby enabling the determination of both the adjacency of residues A and B, and their sequence, as shown in Figure 1.

Selected ions from the high-resolution mass spectrum of *N*,*N*-bis(trideuteriomethyl)rhizonin A were determined by a computer program[8] to be uniquely assignable to the structures given (Table 1). The partial sequence Leu-Val-Val-MeFur-Leu was deduced from fragments 1, 2, 3a, and 6. Fragment 4a completes the sequence as *cyclo*-Leu-Val-Val-MeFur-Leu-MeAla-MeFur, since 4b is incompatible with the foregoing pentapeptide partial sequence.

Since rhizonin B (**2**) is known (*vide supra*) to have Val substituted for the aIle of rhizonin A, comparison of the low-resolution spectra of these substances allowed differentiation of Leu and aIle in rhizonin A, and thus afforded the final structure (**1a**).

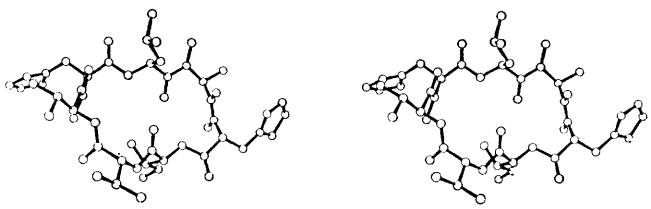


Figure 3. Stereoscopic drawing of rhizonin A.

The structure of rhizonin A was confirmed by *X*-ray crystallography and its solid-state conformation determined. The crystals are orthorhombic, space group $P2_12_12_1$ with $a = 11.37$ (1), $b = 19.41$ (1), $c = 20.65$ (1) Å, $Z = 4$, $D_c = 1.182$ and $D_m = 1.170$ g cm^{-3}. Intensity measurements were made with Cu-K_α radiation ($\lambda = 1.5418$ Å; graphite monochromator) on a Philips PW 1100 four-circle diffractometer in the ω-2θ mode with $3 \leqslant \theta \leqslant 60$. A total of 3 512 reflections were measured of which 948 were regarded as unobserved with $I < 2\sigma(I)$. The measured intensities were corrected for background and Lorentz-polarization effects only. Accurate cell parameters were obtained by least-squares techniques from the setting of 25 reflections. The structure was solved by direct methods using the multiple-solution program MULTAN[9] and selecting the correct phase set with the negative quartet indicator NQEST.[10]†

All the non-hydrogen atoms positions could be found from the first two difference Fourier maps. The structure was refined by blocked-matrix least squares methods using the program SHELX[11] and employing σ_F^{-2} weights. Convergence, with anisotropic thermal factors for all non-hydrogen atoms and a common isotropic thermal factor for the 43 hydrogen atoms included in calculated positions, was reached at $R = 0.118$ and $R_w = 0.094$ using all data. The difference electron density map based on the final atomic parameters showed no maxima greater than 0.34 e Å^{-3}. The resulting structure and numbering system is illustrated in the perspective drawing (Figure 2). The relatively high final R value can be ascribed to the low diffraction quality of the crystal.

The stereodiagram of rhizonin A (Figure 3) shows the

† The atomic co-ordinates for this work are available on request from the Director of the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Rd., Cambridge CB2 1EW. Any request should be accompanied by the full literature citation for this communication.

folding of the peptide backbone and the disposition of the side-chain. It is of importance to note that the two Val units possess opposite absolute stereochemistry; the same phenomenom is observed for the two MeFur units, and for Leu and aIle.

The absolute configuration of (**1a**) was established by converting the acid hydrolysate of rhizonin A into the dansyl derivatives.[12] D- and L-Dansyl leucine were resolved by reversed-phase h.p.l.c. on a Waters μ Bondapak C_{18} column employing an aqueous solution (5 mM L-proline, 2.5 mM $CuSO_4.5H_2O$, 6.5 mM NH_4OAc, pH 7.0) with 20% MeCN as mobile phase (flow-rate: 2 ml/min). The fluorescence of the dansyl derivatives at 480 nm was monitored with excitation at 340 nm. Under these conditions the retention times of L-dansyl leucine and of D-dansyl leucine were 18.9 and 25.3 min, respectively. An h.p.l.c. analysis[12] of the dansyl derivatives of the acid hydrolysate of rhizonin A established the absolute configuration of the leucine residue as L and therefore the absolute configuration as indicated in Figures 2 and 3.

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