

## Trichorzins HA and MA, Antibiotic Peptides from *Trichoderma harzianum*

### II. Sequence Determination

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A series of 18-residue antibiotic-antifungal peptides, trichorzins HA and MA, were isolated from *Trichoderma harzianum* strains exhibiting antagonistic properties against phytopathogenic fungi. The sequences of the nine major pure peptides isolated by HPLC were determined by positive ion FAB-MS data and two-dimensional NMR measurements, including COSY, HOHAHA, ROESY and  $^1\text{H}$ - $^{13}\text{C}$  LRCOSY experiments.

In the preceding paper<sup>1)</sup>, we have described the fermentation, isolation and biological properties of trichorzins HA and MA, two series of original 18-residue peptides biosynthesized by two *Trichoderma harzianum* strains from distinct geographic origins. Trichorzins are peptaibols, linear peptides with  $\alpha,\alpha$ -dialkylated amino acids such as  $\alpha$ -amino isobutyric acid (Aib, U) and isovaline (Iva, J), an acetylated N-terminus and a C-terminal amino alcohol. Based on peculiar characteristics, different subclasses of peptaibols are distinguished: long-sequence peptaibols (18- to 20-residue peptides)<sup>2~6)</sup>, short-sequence peptaibols (11- to 16-residue peptides)<sup>7)</sup> and lipopeptaibols (7- or 11-residue peptides with an N-terminal amino acid acylated by a short lipidic chain instead of an acetyl group)<sup>8)</sup>. Trichorzins have been shown to perturb the permeability properties of phospholipid bilayers, as measured on model membranes and to exert antibiotic activity against Gram-positive bacteria<sup>1)</sup>.

In this paper, we describe the sequence determination of trichorzins HA and MA, original octadecapeptaibols

(Fig. 1), from MS and NMR data.

### Results and Discussion

#### General Characteristics and Amino Acid Composition and Chirality

The major components of the complex peptide mixtures arising from fermentation of *T. harzianum* strain M-903602 and M-922835 were isolated by semi-preparative C18 reversed phase HPLC, giving rise to six trichorzins HA from M-903602 strain and three trichorzins MA from M-922835 strain<sup>1)</sup>.

The presence of sharp singlets at about 2.0 ppm in the  $^1\text{H}$  NMR spectra of HA and MA peptides, together with the absence of free  $\text{NH}_2$  terminal groups revealed by the negative reaction with ninhydrin, were characteristics of the acetylated N-termini of trichorzins. GC analyses on a Chirasil-L-Val capillary column of the total acidic hydrolyzates after derivatization of the given amino acids, led to the amino acid composition and absolute configuration. Comparisons and coinjections of the

Fig. 1. Sequences of trichorzins HA and MA.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
HA I :	Ac	Aib	<b>Gly</b>	Ala	Aib	<b>Aib</b>	Gln	<b>Aib</b>	<b>Val</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Aib</b>	Gln	<b>Leuol</b>
HA II :	Ac	Aib	<b>Gly</b>	Ala	Aib	<b>Aib</b>	Gln	<b>Aib</b>	<b>Val</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Iva</b>	Gln	<b>Leuol</b>
HA III :	Ac	Aib	<b>Gly</b>	Ala	Aib	<b>Iva</b>	Gln	<b>Aib</b>	<b>Val</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Aib</b>	Gln	<b>Leuol</b>
HA V :	Ac	Aib	<b>Gly</b>	Ala	Aib	<b>Iva</b>	Gln	<b>Aib</b>	<b>Val</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Iva</b>	Gln	<b>Leuol</b>
HA VI :	Ac	Aib	<b>Gly</b>	Ala	Aib	<b>Iva</b>	Gln	<b>Iva</b>	<b>Val</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Iva</b>	Gln	<b>Leuol</b>
HA VII :	Ac	Aib	<b>Gly</b>	Ala	Aib	<b>Iva</b>	Gln	<b>Val</b>	<b>Val</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Iva</b>	Gln	<b>Leuol</b>
MA I :	Ac	Aib	<b>Ser</b>	Ala	Aib	<b>Aib</b>	Gln	<b>Aib</b>	<b>Leu</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Aib</b>	Gln	<b>Valol</b>
MA II :	Ac	Aib	<b>Ser</b>	Ala	Aib	<b>Iva</b>	Gln	<b>Aib</b>	<b>Leu</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Aib</b>	Gln	<b>Valol</b>
MA III :	Ac	Aib	<b>Ser</b>	Ala	Aib	<b>Iva</b>	Gln	<b>Iva</b>	<b>Leu</b>	Aib	Gly	Leu	Aib	Pro	Leu	Aib	<b>Aib</b>	Gln	<b>Valol</b>

Leuol: leucinol; Valol: valinol.

hydrolyzates with standards assigned the chirality of all the amino acids and of the amino alcohols Leuol and Valol as L and that of the Iva residues as D. Finally, the L-Gln residues obtained in the total hydrolyzates were assigned to L-Gln from observation of the *syn* and *anti*  $\epsilon$ -protons of the carboxamide groups in the  $^1\text{H}$  NMR spectra.

### Sequence Determination of Trichorzins HA and MA

#### Positive Ion FAB Mass Spectrometry

Positive ion FAB MS was used for the determination of the sequences, from analysis of continuous series of

$b_n$  acylium ions<sup>9~11</sup>). The tertiary amide link Aib-Pro which very often occurs in such peptides, results in an N-terminal acylium ion  $\text{N}^+$  and a diprotonated C-terminal ion  $[\text{HC}, \text{H}]^+$ , each generating independent

Fig. 2. Positive ion FAB mass spectrum of trichorzin HA I.

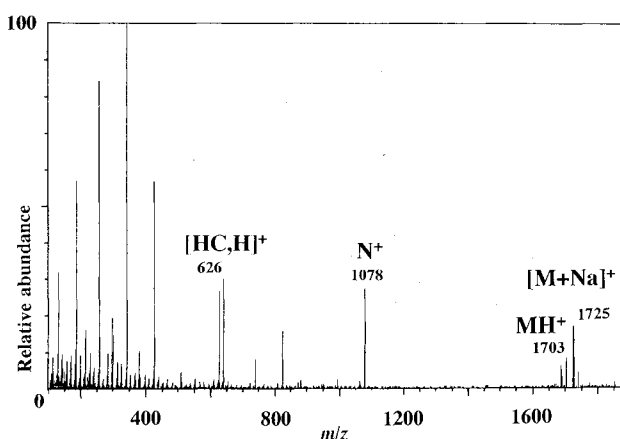


Table 2. Pseudomolecular ion species and sequence-specific fragment ions arising in the (+) ion FAB mass spectra of trichorzins MA.

Ion types	Peptide (MW)					
	MA I 1732		MA II 1746		MA III 1760	
	$\text{C}_{80}\text{H}_{140}\text{O}_{22}\text{N}_{20}$		$\text{C}_{81}\text{H}_{142}\text{O}_{22}\text{N}_{20}$		$\text{C}_{82}\text{H}_{144}\text{O}_{22}\text{N}_{20}$	
	<i>m/z</i>	%	<i>m/z</i>	%	<i>m/z</i>	%
$[\text{M} + \text{Na}]^+$	1755	(100)	1769	(58)	1783	(62)
$\text{MH}^+$	1733	(2)	1747	(4)	1761	(3)
$\text{N}^+$	1122	(10)	1136	(16)	1150	(14)
$[\text{HC}, \text{H}]^+$	612	(11)	612	(21)	612	(15)
<i>N</i>	1037	(1)	1051	(2)	1065	(2)
<i>N</i>	924	(1)	938	(2)	952	(1)
<i>N</i>	867	(5)	881	(10)	895	(8)
<i>N</i>	782	(5)	796	(6)	810	(6)
<i>N</i>	669	(16)	683	(22)	697	(15)
<i>C</i>	509	(1)	509	(2)	509	(2)
<i>N</i>	456	(27)	470	(36)	470	(47)
<i>C</i>	381	(4)	381	(6)	381	(6)
<i>N</i>	371	(83)	371	(90)	371	(100)
<i>C</i>	296	(13)	296	(12)	296	(14)
<i>N</i>	286	(58)	286	(82)	286	(73)
<i>C</i>	211	(12)	211	(12)	211	(12)
<i>N</i>	215	(87)	215	(99)	215	(81)
<i>N</i>	128	(96)	128	(100)	128	(70)

The matrix used was  $\alpha$ -thioglycerol; the origin of the observed ions, from  $\text{N}^+$  or  $[\text{HC}, \text{H}]^+$  is indicated by *N* or *C*, respectively;  $\text{H} = 1.000$ .

Table 1. Pseudomolecular ion species and sequence-specific fragment ions arising in the (+) ion FAB mass spectra of trichorzins HA.

Ion types	Peptide (MW)											
	HA I 1702		HA II 1716		HA III 1716		HA V 1730		HA VI 1744		HA VII 1744	
	$\text{C}_{79}\text{H}_{138}\text{O}_{21}\text{N}_{20}$		$\text{C}_{80}\text{H}_{140}\text{O}_{21}\text{N}_{20}$		$\text{C}_{80}\text{H}_{140}\text{O}_{21}\text{N}_{20}$		$\text{C}_{81}\text{H}_{142}\text{O}_{21}\text{N}_{20}$		$\text{C}_{82}\text{H}_{144}\text{O}_{21}\text{N}_{20}$		$\text{C}_{82}\text{H}_{144}\text{O}_{21}\text{N}_{20}$	
	<i>m/z</i>	%	<i>m/z</i>	%	<i>m/z</i>	%	<i>m/z</i>	%	<i>m/z</i>	%	<i>m/z</i>	%
$[\text{M} + \text{Na}]^+$	1725	(16)	1739	(20)	1739	(15)	1753	(14)	1767	(23)	1767	(22)
$\text{MH}^+$	1703	(8)	1717	(6)	1717	(4)	1731	(7)	1745	(3)	1745	(4)
$\text{N}^+$	1078	(27)	1078	(24)	1092	(10)	1092	(24)	1106	(8)	1106	(13)
$[\text{HC}, \text{H}]^+$	626	(26)	640	(29)	626	(16)	640	(18)	640	(15)	640	(19)
<i>N</i>	993	(2)	993	(2)	1007	(1)	1007	(2)	1021	(1)	1021	(1)
<i>N</i>	880	(2)	880	(2)			894	(2)			908	(1)
<i>N</i>	823	(15)	823	(13)	837	(5)	837	(10)	851	(3)	851	(3)
<i>N</i>	738	(8)	738	(7)	752	(3)	752	(6)	766	(3)	766	(3)
<i>N</i>	639	(30)	639	(26)	653	(12)	653	(20)	667	(10)	667	(9)
<i>N</i>	554	(3)	554	(2)	568	(1)	568	(2)			568	(1)
<i>C</i>	509	(4)	523	(3)	509	(3)	523	(2)	523	(3)	523	(3)
<i>N</i>	426	(57)	426	(56)	440	(32)	440	(47)	440	(34)	440	(35)
<i>C</i>	381	(10)	395	(7)	381	(7)	395	(6)	395	(6)	395	(6)
<i>N</i>	341	(100)	341	(100)	341	(86)	341	(80)	341	(85)	341	(88)
<i>C</i>	296	(19)	296	(19)	296	(18)	296	(14)	296	(16)	296	(17)
<i>N</i>	256	(84)	256	(81)	256	(100)	256	(70)	256	(100)	256	(100)
<i>C</i>	211	(14)	211	(13)	211	(17)	211	(12)	211	(16)	211	(18)
<i>N</i>	185	(57)	185	(57)	185	(88)	185	(47)	185	(88)	185	(87)
<i>N</i>	128	(32)	128	(28)	128	(53)	128	(24)	128	(51)	128	(57)

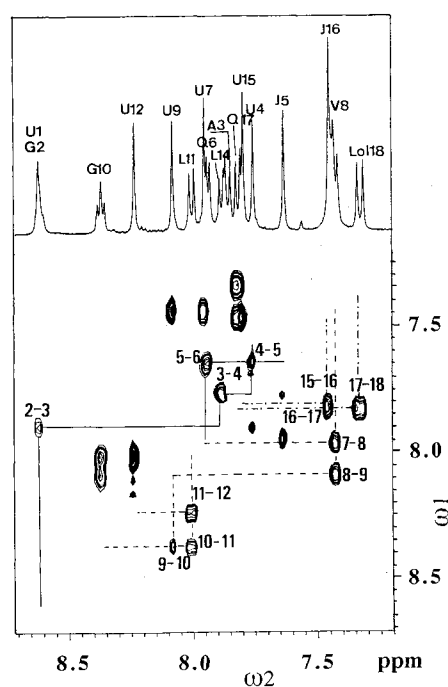
The matrix used was nitrobenzyl alcohol; the origin of the observed ions, from  $\text{N}^+$  or  $[\text{HC}, \text{H}]^+$  is indicated by *N* or *C*, respectively; ( $\text{H} = 1.000$ ).

series of  $b_n$  ions, that superimpose in the spectrum. According to the Roepstorff nomenclature modified by Biemann<sup>12,13</sup>, the  $[HC, H]^+$  ions could be  $y_n$  diprotonated ammonium ions<sup>14,15</sup>.

Assignments of the molecular weights of trichorzins HA and MA could be derived from their molecular ion species  $[M+Na]^+$  which appeared at higher masses on the positive ion FAB mass spectra (Fig. 2; Tables 1 and 2). At lower masses, the spectra exhibited the complexity resulting from the fragmentation pattern described above.

In addition to the pseudomolecular ions  $[M+Na]^+$  ( $m/z$  1725) and  $MH^+$  ( $m/z$  1703), the FAB MS spectrum of HA I (Fig. 2) showed intense  $N^+$  and  $[HC, H]^+$  ions at  $m/z$  1078 and 626 respectively. Continuous fragment ion series arising from such ions were observed. Taking into account the amino acid composition previously determined, the whole sequence was thus described by successive losses of Aib, Leu, Gly, Aib, Val, Aib, Gln, Aib, Aib, Ala, Gly, Ac-Aib from the  $N^+$  ion ( $m/z$  1078, 993, 880, 823, 738, 639, 554, 426, 341, 256, 185, 128) and Leuol, Gln, Aib, Aib, Leu + Pro, from the  $[HC, H]^+$  ion ( $m/z$  626, 509, 381, 296, 211). However, due to the presence of isomeric residues and to the lack of a number of sequence-specific ions, the sequences of the other

Fig. 3. Part of the ROESY spectrum of trichorzin MA II.



The spectrum was acquired in  $CD_3OH$  with a mixing time of 300 ms;  $\omega_2=8.7\sim 7.2$  ppm,  $\omega_1=8.7\sim 7.2$  ppm; U: Aib, J: Iva, Lol: Leuol.

Table 3.  $^1H$  NMR specific assignments ( $\delta$ , ppm) and coupling constants ( $J$ , Hz) of trichorzins HA V and MA II.

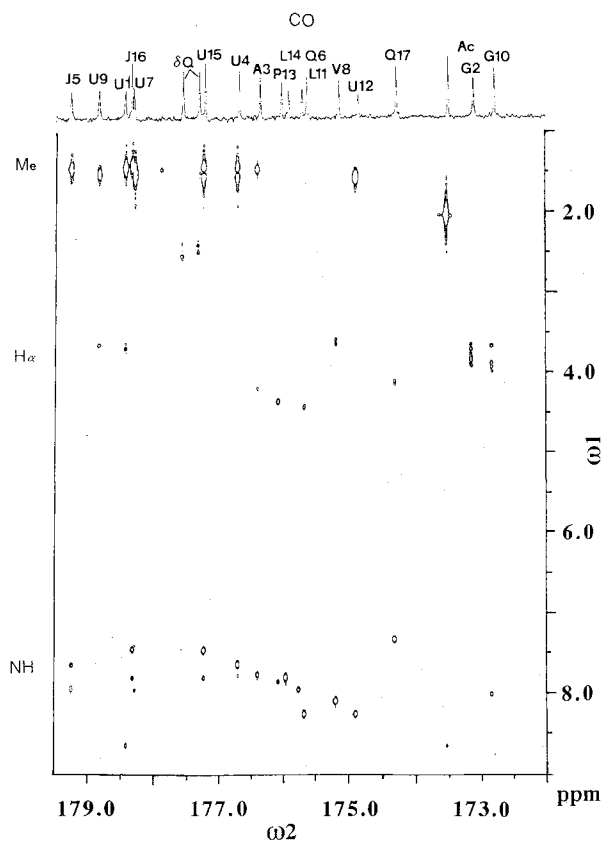
Residue	HA V		MA II	
	$\alpha H$ $\delta$ (J)	Other groups $\delta$ (J)	$\alpha H$ $\delta$	Other groups $\delta$ (J)
Ac		2.038 s		2.034 s
G (S) 2	3.865 d (16.6) 3.707 d (16.6)		4.15	$\beta H$ 3.95, 3.86
A3	4.229 q (7.3)	$\beta Me$ 1.47	4.17	$\beta Me$ 1.46
J5		$\beta H$ 2.40, 1.80; $\gamma Me$ 0.829 t (7.5)		$\beta H$ 2.45, 1.75; $\gamma Me$ 0.839 t (7.5)
Q6	3.903 dd (6.0, 8.9)	$\beta H$ 2.11, 1.95; $\gamma H$ 2.53; 2.39	3.89	$\beta H$ 2.21; $\gamma H$ 2.53, 2.36
V (L) 8	3.642 d (9.6)	$\beta H$ 2.26 $\gamma Me$ 1.077 d (6.5); 0.994 d (6.8)	4.10	$\beta H$ , $\gamma H$ 1.77 $\delta Me$ 0.954 d (6.0); 0.903 d (6.2)
G10	3.934 d (16.4) 3.674 d (16.4)		3.94 3.67	
L11	4.450 dd (3.7, 11.2)	$\beta H$ 1.99, 1.58; $\gamma H$ 1.92 $\delta Me$ 0.945 d (6.4); 0.914 d (6.4)	4.41	$\beta H$ 1.92, 1.62; $\gamma H$ 1.92 $\delta Me$ 0.945 d (6.4); 0.903 d (6.2)
P13	4.382 d (7.9)	$\beta H$ 2.35, 1.75; $\gamma H$ 2.01, 1.96 $\delta H$ 3.94, 3.54	4.38	$\beta H$ 2.33, 1.75; $\gamma H$ 2.01, 1.96 $\delta H$ 3.86, 3.61
L14	4.186 dd (5.1, 10.7)	$\beta H$ 1.90, 1.66; $\gamma H$ 1.86 $\delta Me$ 1.012 d (6.5); 0.901 d (6.4)	4.12	$\beta H$ , $\gamma H$ 1.86 $\delta Me$ 0.998 d (6.2); 0.903 d (6.2)
J16		$\beta H$ 2.40, 1.80; $\gamma Me$ 0.869 t (7.6)		
Q17	4.131 dd (3.7, 11.2)	$\beta H$ 2.17, 2.14; $\gamma H$ 2.53, 2.36	4.11	$\beta H$ 2.28, 2.17; $\gamma H$ 2.54, 2.38
Lol (Vol) 18	4.041	$\beta 1H$ 3.494 dd (5.6, 11.3), 3.534 dd (5.8, 11.3) $\beta 2H$ 1.34, 1.54; $\gamma H$ 1.54 $\delta Me$ 0.914 d (6.4); 0.901 d (6.4)	3.69	$\beta 1H$ 3.69; $\beta 2H$ 1.92 $\gamma Me$ 0.960 d (6.5); 0.933 d (6.8)
U1, U4, J5, U7, U9, U12, U15, U (J) 16		$\beta Me$ 1.603, 1.580 ( $\times 2$ ), 1.573, 1.551, 1.537, 1.529, 1.484, 1.480, 1.467, 1.461 ( $\times 3$ ), 1.435		$\beta Me$ , 1.600, 1.567, 1.553 ( $\times 2$ ), 1.532, 1.520, ( $\times 3$ ), 1.498, 1.481 ( $\times 4$ ), 1.450, 1.434

HA V: 296 K,  $CD_3OD$ , 500.13 MHz; MA II: 296 K,  $CD_3OH$ , 500.13 MHz;  $\delta$  are given to the nearest three decimals when obtained from 1D spectra and to the nearest two decimals when obtained from 2D spectra.

Table 4. Chemical shifts (ppm) and  $^3J_{\text{NH}\alpha\text{H}}$  coupling constants (Hz) of the trichorzin HA amide protons.

Position	Peptide					
	HA I	HA II	HA III	HA V	HA VI	HA VII (a)
U1	8.654	8.656	8.651	8.664	8.650	8.65
G2	8.654 (5.7) (b)	8.656 (5.5) (b)	8.651 n.d.	8.652 (5.5) (b)	8.650 (5.2) (b)	8.66 (5.7) (b)
A3	7.892 (5.0)	7.891 (6.5) (b)	7.888 (5.5)	7.880 (6.5)	7.908 (6.6)	7.90
U4	7.754	7.755	7.769	7.780	7.764	7.80
U5 (J5)*	7.734	7.736	7.654*	7.661*	7.670*	7.84*
Q6	7.939 (4.8)	7.940 (4.8)	7.951 (5.2)	7.948 (4.9)	7.981 (4.6)	7.86
U7 (V7)** (J7)*	7.964	7.964	7.964	7.976	7.880*	7.88**
V8	7.489 (6.2)	7.490 (6.2)	7.442 (6.5)	7.447 (6.1)	7.434 (6.3)	7.50
U9	8.139	8.139	8.098	8.106	8.082	8.21
G10	8.394 (5.6)	8.395 (5.6)	8.387 (5.6)	8.395 (5.7)	8.337 (5.6)	7.928 (5.5) (b)
L11	8.032 (7.9)	8.035 (7.8)	8.014 (7.9)	8.020 (7.9)	8.00 (7.7)	7.92
U12	8.273	8.287	8.245	8.271	8.225	8.16
L14	7.897 (7.6)	7.869 (7.6)	7.894 (7.5)	7.865 (7.6)	7.867 (7.6)	7.88
U15	7.772	7.813	7.769	7.813	7.805	7.80
U16 (J16)*	7.585	7.480*	7.580	7.479*	7.478*	7.50*
Q17	7.810 (7.1)	7.825 (7.1)	7.810 (6.9)	7.831 (7.2)	7.831 (7.3)	7.84
Lol18	7.347 (9.3)	7.342 (9.3)	7.344 (9.3)	7.341 (9.4)	7.341 (9.3)	7.337 (9.3) (b)
ea Q6	7.478	7.480	7.474	7.479	7.478	7.500
ea Q17	7.478	7.480	7.474	7.479	7.478	7.500
es Q6	6.773	6.774	6.770	6.781	6.759	6.766
es Q17	6.773	6.774	6.770	6.781	6.759	6.766

296 K, CD<sub>3</sub>OH, 300.13 MHz; (a) due to severe overlapping of the signals, the chemical shifts arise mainly from the 2D spectra and are given to the nearest two decimal; (b) due to signal superimposition at 296 K, the  $^3J$  coupling constant was determined at a temperature allowing signal separation; n.d. not determined; symbols \* and \*\* specify the replacement of an Aib residue in the HA sequences for an Iva or a Val, respectively.

Fig. 4.  $^1\text{H}$ - $^{13}\text{C}$  LRCOSY spectrum of trichorzin HA V.

The spectrum was acquired in CD<sub>3</sub>OH with a delay of 60 ms;  $\omega_2 = 179 \sim 172$  ppm,  $\omega_1 = 9.0 \sim 1.0$  ppm.

Table 5. Chemical shifts (ppm) and  $^3J_{\text{NH}\alpha\text{H}}$  coupling constants (Hz) of the trichorzin MA amide protons.

Position	Peptide		
	MA I	MA II	MA III
U1	8.598	8.600	8.600
S2	8.026 (5.5)	8.027 (5.3)	8.012 (5.2)
A3	7.928 (5.0)	7.904 (6.3)	7.931 (6.3)
U4	7.734	7.782	7.757
U5 (J5)*	7.688	7.610*	7.615*
Q6	7.928 (5.0)	7.934 (5.0)	7.966 (4.8)
U7 (J7)*	8.039	8.028	7.907*
L8	7.619 (6.1)	7.590 (6.1)	7.589 (6.0)
U9	8.148	8.138	8.115
G10	8.363 (5.8)	8.361 (5.7)	8.327 (5.5)
L11	8.079 (7.8)	8.072 (7.8)	8.057 (7.9)
U12	8.148	8.138	8.115
L14	7.858 (7.5)	7.851 (7.2)	7.854 (7.3)
U15	7.769	7.773	7.767
U16	7.581	7.588	7.579
Q17	7.833 (6.9)	7.833 (6.7)	7.832 (6.8)
Vol18	7.390 (8.9)	7.419 (8.8)	7.390 (8.8)
ea Q6	7.461	7.468	7.461
ea Q17	7.461	7.507	7.461
es Q6	6.776	6.780	6.776
es Q17	6.776	6.808	6.776

296 K, CD<sub>3</sub>OH, 300.13 MHz; symbol \* specifies the replacement of an Aib residue in the MA sequences for an Iva.

trichorzins could not be unequivocally determined by this way (Tables 1 and 2). Particularly, ambiguity remained as regards to the relative position in the sequences of the isomeric Val/Iva residues at positions 5, 7 and 16, and of the Gln and Aib (or Val/Iva) residues at positions 6 and 7.

### NMR Spectroscopy

Complete sequences of trichorzins arose from  $^1\text{H}$  and  $^{13}\text{C}$  NMR data. At first, COSY and HOHAHA experiments were used for assignments of the proton spin system resonances to residue types. Then, sequential

inter-residue dipolar couplings exhibited by the ROESY spectra afforded sequence determination together with sequential  $^1\text{H}$  assignments<sup>6-8,16</sup>. Finally, 2D heteronuclear  $^1\text{H}$ - $^{13}\text{C}$  COSY and  $^1\text{H}$ - $^{13}\text{C}$  LRCOSY optimized for long-range couplings of 5 to 10 Hz enabled sequential carbon assignments to be made and confirmed the above sequences<sup>6,8</sup>.

The amide region of the 300 MHz  $^1\text{H}$  NMR spectra of trichorzins (Fig. 3) had well resolved signals for all the peptides except for HA VII which exhibited strong overlapping. The Gly NHs were readily recognized from their triplet nature, as well as the  $\alpha,\alpha$ -dialkylated residues

Table 6.  $^{13}\text{C}$  NMR chemical shifts for harzianins HA V and MA II.

HA V			MA II		
Residue	Group	$\delta$ (ppm)	Residue	Group	$\delta$ (ppm)
Ac	CH <sub>3</sub>	23.1	Ac	CH <sub>3</sub>	23.1
G2	$\alpha$ CH <sub>2</sub>	45.0	S2	$\alpha$ CH	59.4
				$\beta$ CH <sub>2</sub>	62.0
A3	$\alpha$ CH	53.0	A3	$\alpha$ CH	53.5
	$\beta$ CH <sub>3</sub>	16.5		$\beta$ CH <sub>3</sub>	16.4
J5	$\alpha$ C	60.1	J5	$\alpha$ C	60.1
	$\beta$ CH <sub>2</sub>	26.6		$\beta$ CH <sub>2</sub>	26.5
	$\gamma$ CH <sub>3</sub>	7.6		$\gamma$ CH <sub>3</sub>	7.5
Q6	$\alpha$ CH	58.0	Q6	$\alpha$ CH	58.2
	$\beta$ CH <sub>2</sub>	26.4		$\beta$ CH <sub>2</sub>	27.2
	$\gamma$ CH <sub>2</sub>	32.7*		$\gamma$ CH <sub>2</sub>	32.8*
V8	$\alpha$ CH	65.1	L8	$\alpha$ CH	56.4
	$\beta$ CH	30.5		$\beta$ CH <sub>2</sub>	40.6
	$\gamma$ 1, $\gamma$ 2 CH <sub>3</sub>	20.4, 19.5		$\gamma$ CH	26.0
				$\delta$ 1, $\delta$ 2 CH <sub>3</sub>	22.9, 21.4 <sup>†</sup>
G10	$\alpha$ CH <sub>2</sub>	45.1	G10	$\alpha$ CH <sub>2</sub>	45.0
L11	$\alpha$ CH	54.0	L11	$\alpha$ CH	54.0
	$\beta$ CH <sub>2</sub>	41.4		$\beta$ CH <sub>2</sub>	41.3
	$\gamma$ CH	26.0		$\gamma$ CH	25.7
	$\delta$ 1, $\delta$ 2 CH <sub>3</sub>	23.5; 21.6		$\delta$ 1, $\delta$ 2 CH <sub>3</sub>	23.5, 21.6 <sup>†</sup>
P13	$\alpha$ CH	64.6	P13	$\alpha$ CH	64.7
	$\beta$ CH <sub>2</sub>	29.9		$\beta$ CH <sub>2</sub>	30.0
	$\gamma$ CH <sub>2</sub>	26.9		$\gamma$ CH <sub>2</sub>	27.5
	$\delta$ CH <sub>2</sub>	50.4		$\delta$ CH <sub>2</sub>	50.4
L14	$\alpha$ CH	55.4	L14	$\alpha$ CH	55.5
	$\beta$ CH <sub>2</sub>	40.3		$\beta$ CH <sub>2</sub>	40.2
	$\gamma$ CH	25.5		$\gamma$ CH	25.5
	$\delta$ 1, $\delta$ 2 CH <sub>3</sub>	23.5; 21.3		$\delta$ 1, $\delta$ 2 CH <sub>3</sub>	23.5, 21.7 <sup>†</sup>
J16	$\alpha$ C	61.0			
	$\beta$ CH <sub>2</sub>	26.6			
	$\gamma$ CH <sub>3</sub>	7.6			
Q17	$\alpha$ CH	56.0	Q17	$\alpha$ CH	56.3
	$\beta$ CH <sub>2</sub>	28.4		$\beta$ CH <sub>2</sub>	28.5
	$\gamma$ CH <sub>2</sub>	33.2*		$\gamma$ CH <sub>2</sub>	33.3*
Lo18	$\alpha$ CH	50.9	Vol18	$\alpha$ CH	58.6
	$\beta$ 1 CH <sub>2</sub>	40.7		$\beta$ CH	29.9
	$\beta$ 2 CH <sub>2</sub> (OH)	66.0		$\beta$ 2 CH <sub>2</sub> (OH)	63.7
	$\gamma$ CH	25.6		$\gamma$ 1, $\gamma$ 2 CH <sub>3</sub>	19.4, 20.0
	$\delta$ 1, $\delta$ 2 CH <sub>3</sub>	22.0, 23.8			
U1, 4, 7, 9, 12, 15	$\alpha$ C	58.0, 57.9, 57.7, 57.7 57.6, 57.4	U1, 4, 7, 9, 12, 15, 16,	$\alpha$ C	58.0, 57.9, 57.8, 57.7, 57.7, 57.6, 57.5
U1, 4, 7, 9, 12, 15, J5, J16	$\beta$ CH <sub>3</sub>	27.5 ( $\times$ 2), 27.4 ( $\times$ 2), 26.8 ( $\times$ 2), 24.3 ( $\times$ 2), 23.8, 23.6 ( $\times$ 2), 23.4 ( $\times$ 3)	U1, 4, 7, 9 12, 15, 16, J5	$\beta$ CH <sub>3</sub>	27.5 ( $\times$ 2), 27.4, 27.0, 26.9, 26.8, 26.6 ( $\times$ 2), 23.8, 23.5 ( $\times$ 2), 23.3, 23.2 ( $\times$ 3)

\*† Assignments may be reversed within the same column; 296 K, CD<sub>3</sub>OH, 75.47 MHz.

Table 7. Chemical shifts ( $\delta$ , ppm) of the carbonyl groups for trichorzins HA I, HA II, HA III, HA V, HA VI, MA II and MA III.

Residue	Peptide						
	HA I	HA II	HA III	HA V	HA VI	MA II	MA III
Ac	173.6	173.6	173.6	173.5	173.6	173.6	173.6
U1	178.5	178.5	178.5	178.4	178.5	178.1	178.1
G2 (S2) <sup>s</sup>	173.2	173.2	173.2	173.1	173.1	173.8 <sup>s</sup>	173.8 <sup>s</sup>
A3	176.5	176.4	176.4	176.4	176.2	176.5	176.3
U4	176.8	176.8	176.8	176.7	176.8	176.7	176.8
U5 (J5)*	178.7	178.7	179.3*	179.2*	179.3*	179.2*	179.2*
Q6	175.7	175.8	175.8	175.7	175.7	175.9	175.9
U7 (J7)*	178.3	178.3	178.3	178.3	178.9*	178.5	178.9*
V8 (L8) <sup>†</sup>	175.2	175.3	175.2	175.2	175.2	176.2 <sup>†</sup>	176.2 <sup>†</sup>
U9	178.9	178.9	178.9	178.8	178.9	179.0	179.0
G10	172.9	172.9	172.9	172.8	172.8	172.8	172.8
L11	175.6	175.7	175.6	175.7	175.6	175.6	175.6
U12	175.0	174.9	175.1	174.9	174.9	175.0	174.9
P13	176.1	176.0	176.2	176.0	176.1	176.2	176.1
L14	176.0	175.9	176.0	175.9	176.0	176.1	176.0
U15	177.3	177.3	177.4	177.2	177.3	177.4	177.4
U16 (J16)*	178.9	178.3*	178.0	178.3*	178.3*	178.0	178.0
Q17	174.3	174.3	174.3	174.3	174.3	174.8	174.8
$\delta$ CO Q17	177.6	177.6	177.6	177.6	177.7	177.6	177.6
$\delta$ CO Q6	177.4	177.3	177.4	177.3	177.3	177.4	177.4

296 K, CD<sub>3</sub>OH, 75.47 MHz; symbols, <sup>s</sup>, \* and <sup>†</sup> specify the replacement in the sequences of trichorzins, of a Gly for a Ser, of an Aib for an Iva and of a Val for a Leu, respectively.

Aib and Iva which appeared as singlets from lack of  $\alpha$  proton. The ROESY spectra of trichorzins exhibited continuous series of strong dNN(*i*, *i*+1) (Fig. 3), interrupted by the lack of NH protons in the Pro13 residues and accompanied by weaker  $d\alpha N(i, i+1)$ . These sequential NOE patterns allowed sequence determination together with sequential assignments (Tables 3~5).

The 20 carbonyl groups of trichorzins observed in the <sup>13</sup>C spectra (Fig. 4) were assigned from the long-range <sup>2</sup>J and <sup>3</sup>J heteronuclear couplings between a CO<sub>*i*</sub> group and the NH<sub>*i*+1</sub> and H <sub>$\alpha$ <sub>*i*+1</sub></sub> protons (Fig. 4). The resulting <sup>1</sup>H and <sup>13</sup>C sequential assignments of HA V and MA II, taken as examples, are given in Tables 3~7; full sequential assignments of the NH and CO groups of the other trichorzins are in Tables 4, 5 and 7.

### Conclusion

The octadecapeptaibols, trichorzins HA and MA, illustrate the pronounced microheterogeneity usually observed for peptaibols and, more generally, for peptides biosynthesized by the polyenzymic pathway<sup>17)</sup>. Trichorzins HA differ from trichorzins MA by three substitutions in the sequence, Gly2→Ser2, Val8→Leu8 and Leu118→Val118. In addition, one, two or three Aib→Iva substitutions at positions 5, 7 and 16 in the sequence lead to the diversity which occurs inside each trichorzin group. Such Aib→Iva substitutions were also observed previously in the group of 18-residue peptaibols represented by trichotoxins<sup>18,19)</sup> and trichokindins<sup>20)</sup>. However, this substitution was observed either at position 5 or 16, but

not at both. Finally, the Aib→Val replacement at position 7 is unique in this 18-residue peptaibol series.

### Experimental

#### Amino Acid Analysis

After hydrolysis of HA and MA peptides (HCl 6N, 110°, N<sub>2</sub>), derivatization of the given amino acids and amino alcohols was conducted as previously described<sup>6,8)</sup>. Classically, the GC analyses of the *N*-trifluoroacetyl isopropyl ester derivatives were performed with a Girdel 3000 chromatograph on a Chirasil-L-Val (*N*-propionyl-L-Valine-*tert*-butylamide polysiloxan) quartz capillary column (Chrompack, 25 m length, 0.2 mm i.d.), with He (0.7 bar) as carrier gas and a temperature programme: 50~130°C, 3°C/minute; 130~190°C, 10°C/minute. Rt (separation factor  $\alpha_{L/D}$  for the D-L enantiomers): Aib 10.4, L-Ala 14.8 ( $\alpha=1.16$ ), L-Glu 33.2 ( $\alpha=1.05$ ), L-Gly 17.8, D-Iva 11.2 ( $\alpha=1.02$ ), L-Leu 24.2 ( $\alpha=1.11$ ), L-Leuol 22.2 ( $\alpha=0.98$ ), L-Ser 23.0 ( $\alpha=1.05$ ), L-Val 17.7 ( $\alpha=1.08$ ), L-Valol 18.0 ( $\alpha=0.98$ ). A special temperature programme was used for separation of the proline D,L-enantiomers: 50~110°C, 3°C/minute; plateau at 110°C for 10 minute; 100~190°C, 10°C/minute. Rt ( $\alpha$ ): L-Pro 25.1 ( $\alpha=1.02$ ).

HA I: Aib (8), L-Ala (1), Gly (2), L-Gln (2), L-Leu (2), L-Leuol (1), L-Pro (1), L-Val (1)

HA II: Aib (7), L-Ala (1), Gly (2), L-Gln (2), D-Iva (1), L-Leu (2), L-Leuol (1), L-Pro (1), L-Val (1)

HA III: Aib (7), L-Ala (1), Gly (2), L-Gln (2), D-Iva (1), L-Leu (2), L-Leuol (1), L-Pro (1), L-Val (1)

HA V: Aib (6), L-Ala (1), Gly (2), L-Gln (2), D-Iva (2), L-Leu (2), L-Leuol (1), L-Pro (1), L-Val (1)

HA VI: Aib (5), L-Ala (1), Gly (2), L-Gln (2), D-Iva (3), L-Leu (2), L-Leuol (1), L-Pro (1), L-Val (1)

HA VII: Aib (5), L-Ala (1), Gly (2), L-Gln (2), D-Iva (2), L-Leu (2), L-Leuol (1), L-Pro (1), L-Val (2)

MA I: Aib (8), L-Ala (1), Gly (1), L-Gln (2), L-Leu (3), L-Pro (1), L-Ser (1), L-Valol (1)

MA II: Aib (7), L-Ala (1), Gly (1), L-Gln (2), D-Iva (1), L-Leu (3), L-Pro (1), L-Ser (1), L-Valol (1)

MA III: Aib (6), L-Ala (1), Gly (1), L-Gln (2), D-Iva (2), L-Leu (3), L-Pro (1), L-Ser (1), L-Valol (1).

#### FAB Mass Spectrometry

Positive ion FAB mass spectra were recorded on a ZAB2-SEQ (VG analytical Manchester, UK) mass spectrometer equipped with a standard FAB source and a cesium ion gun operating at 35 kV. Peptide methanolic solutions were mixed with  $\alpha$ -monothio glycerol or 3-nitrobenzyl alcohol used as matrices. The resolution was 2000.

#### NMR Spectroscopy

A 0.5 ml amount of a methanolic ( $\text{CD}_3\text{OH}$ ) solution of HA and MA peptides (15 mm for  $^1\text{H}$  and  $^{13}\text{C}$ - $^1\text{H}$  inverse-detection experiments, 40~70 mm for direct-detection  $^{13}\text{C}$  and  $^{13}\text{C}$ - $^1\text{H}$  experiments) in a 5 mm tube (Wilmad) was used for all the NMR experiments, which unless other specification were conducted at 296 K, either on Bruker AC 300, AM 400 or DMX 500 spectrometers equipped with Aspect 3000 or  $\times 32$  computers or Aspect station 1 Bruker, respectively. Spectra were obtained by solvent presaturation and referenced to the central component of the quintet due to the  $\text{CD}_2\text{H}$  resonance of methanol at 3.313 ppm, downfield from TMS. The HOHAHA (300.13 MHz) and ROESY (400.13 MHz) experiments were acquired with mixing times of 120 and 300 ms respectively; typically, 256 experiments of 96~128 scans each were performed; relaxation delay 1.5 seconds; size 2 K; 9.5 ppm spectral width in F2; zero filling to 1K in F1; sine bell ( $\pi/2$ ) in both dimensions. The reference for  $^{13}\text{C}$  NMR spectra was the central component of  $\text{CD}_3\text{OH}$  at 49.0 ppm downfield from TMS. Direct-detection  $^{13}\text{C}$  NMR data were collected at 75.47 MHz on an AC 300 Bruker spectrometer. Typically,  $^{13}\text{C}$ - $^1\text{H}$ -LR COSY experiments (256 experiments of 128 scans each) were performed with spectral width 674 Hz in F2 (size 1 K) and 1324 Hz in F1 (size 2 K); different delay times  $\tau$  ranging between 60 and 80 ms were used. An inverse-detection HMBC experiment was acquired at 125.13 MHz for HA VI with J filter and Z gradient selection (1024 experiments of 32 transients each) with spectral widths 6000 Hz in F2 and 25000 Hz in F1 and a delay time  $\tau$  of 80 ms.

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