MS-681a, b, c and d, New Inhibitors of Myosin Light Chain Kinase from *Myrothecium* sp.

II. Physico-chemical Properties and Structure Elucidation

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MS-681a, b, c and d are new inhibitors of myosin light chain kinase produced by *Myrothecium* sp. They are novel octapeptides containing α-alkyl-α-amino acids and a polyamine moiety. The structures were determined by NMR and FAB-MS/MS spectral analyses of the intact peptides. Their absolute configurations were elucidated by GC analysis on a chiral column of the constituent amino acids and by chemical synthesis of the polyamine moieties.

In the preceding paper[1], we described the characterization of the producing strain and production, isolation and biological activities of new inhibitors of myosin light chain kinase, MS-681a, b, c and d. In this paper, we describe the structure determination of MS-681a, b, c and d.

Results

The physico-chemical properties of the MS-681 factors are summarized in Table 1. The molecular formula of **2**, which was isolated as the major component, was determined to be $C_{59}H_{90}N_{12}O_9$ based on HRFAB-MS (*m/z* 1111.7037 $(M+H)^+$, $\Delta -0.5$ mmu). The peptidic nature of **2** was suggested by IR absorptions at 1662 cm^{-1} and 3313 cm^{-1} and its ^1H NMR spectrum, and was confirmed by acidic hydrolysis with 6 N HCl for 1 week. Amino acid analysis of the hydrolysate revealed the presence of alanine (Ala), glycine (Gly), phenylalanine (Phe), isovaline (Iva) and α-aminoisobutyric acid (Aib) in the ratio of 1:1:2:2:2. A Phe (7)~Iva (8) residue was resistant to complete hydrolysis (6 N HCl, 110°C, 24 hours), but yielded a partial hydrolysate (**5**).

The structure of **2** was elucidated by ^1H NMR, ^{13}C NMR, DEPT, ^1H-^1H COSY, HSQC, HMBC and NOESY experiments in DMSO-d_6 and CD_3OD. The ^1H and ^{13}C NMR data of **2** are summarized in Table 2. The ^{13}C NMR spectrum showed 59 carbons, which were assigned to 10 methyls, 14 methylenes, 19 methines, 7 quaternary carbons and 9 amide carbonyls by the DEPT experiments. The presence of eight amino acids (Ala, Gly, 2 Phe, 2 Aib and 2 Iva), an acetyl group and an 2-amino-3-phenylpropane unit (APP) was clarified by data of the ^1H-^1H COSY, HSQC and HMBC experiments in DMSO-d_6 (Fig. 2a). The connectivity of a spermidine unit was not observed in DMSO-d_6. The connectivities of the amino acid residues were established from the ^1H-^{13}C correlations between the amide protons and the amide carbonyls and the NOEs between the amide protons and the α-protons. The structure of the *N*-terminus was elucidated to be an acetyl phenylalanine by the ^1H-^{13}C correlation between the acetyl carbonyl group (δ 170.2) and the amide proton (δ 8.23) of Phe (1). On the other hand, the ^1H-^{13}C correlation between the carbonyl group (δ 172.3) of the *C*-terminal Iva (8) and the amide proton (δ 6.93) of APP indicated that the *C*-terminus was linked by the Phe residue with the reduced peptide bond. The structure of the *C*-terminal polyamine moiety was confirmed by NMR experiments in CD_3OD as shown in Fig. 2b. The connectivity of APP and spermidine was assigned by the chemical shift of the methylene (δ 48.0), which was directly bound to the terminal amino group of spermidine. Thus, the *C*-terminus was established as summarized in Fig. 2.

The sequence of **2** was confirmed by a positive ion FAB-MS/MS of its *quasi*-molecular ion (*m/z* 1111 $(M+H)^+$). The fragmentation pattern provided full evidence of the sequence of the amino acids and the

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Fig. 1. Structure of MS-681a (**1**), b (**2**), c (**3**), d (**4**) and hydrolysates (**5**, **6**).

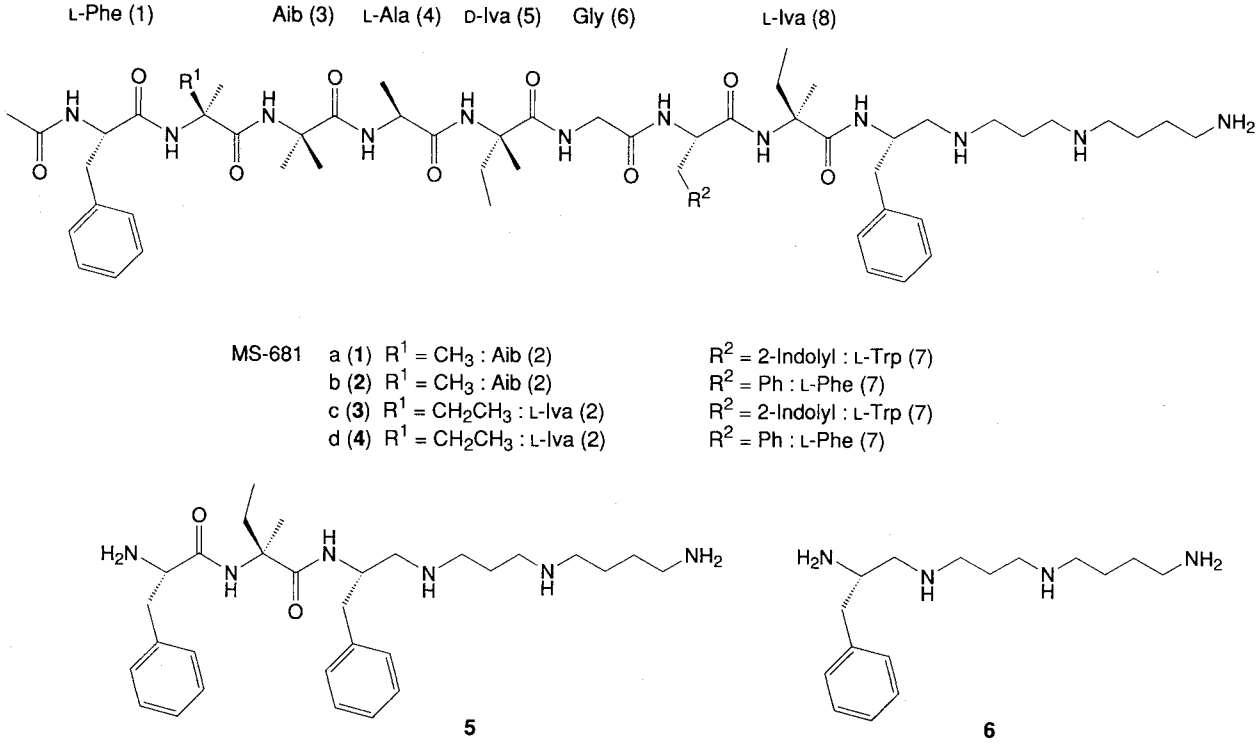


Table 1. Physico-chemical properties of MS-681a (**1**), b (**2**), c (**3**) and d (**4**).

	1	2	3	4
Appearance	Pale yellow solid	Pale yellow solid	Pale yellow solid	Pale yellow solid
Molecular Formula	$C_{61}H_{91}N_{13}O_9$	$C_{59}H_{90}N_{12}O_9$	$C_{62}H_{93}N_{13}O_9$	$C_{60}H_{92}N_{12}O_9$
FABMS	1150 $(M+H)^+$	1111 $(M+H)^+$	1164 $(M+H)^+$	1125 $(M+H)^+$
HRFABMS Found	1150.7106	1111.7037	1164.7268	1125.7189
Calcd	1150.7141	1111.7032	1164.7297	1125.7189
$[\alpha]_D^{26}$ (c 0.2, MeOH)	+20.4°	+28.2°	+24.7°	+24.4°
UV λ_{max}^{MeOH} nm (ε)	217 (26,700), 281 (3,700), 289 (3,100)	260 (1,200)	218 (31,100), 281 (4,100), 289 (3,600)	260 (1,500)
IR ν_{KBr} cm^{-1}	3413, 1668, 1539, 1205, 1180, 1134	3313, 1662, 1539, 1456	3315, 1666, 1539, 1456, 1201, 1176, 1132	3313, 1664, 1539, 1456, 1201, 1174, 1130
Color reaction (positive)	50% H_2SO_4, I_2, Ninhydrin	50% H_2SO_4, I_2, Ninhydrin	50% H_2SO_4, I_2, Ninhydrin	50% H_2SO_4, I_2, Ninhydrin
Solubility Soluble	MeOH, acetone	MeOH, acetone, $CHCl_3$	MeOH, acetone	MeOH, acetone, $CHCl_3$
Slightly soluble	EtOAc, $CHCl_3$	EtOAc	EtOAc, $CHCl_3$	EtOAc
Insoluble	Hexane, H_2O	Hexane, H_2O	Hexane, H_2O	Hexane, H_2O
TLC, Rf Value (Si $60F_{254}$)				
$CHCl_3$-MeOH-H_2O (6:4:0.5)	0.35	0.35	0.35	0.35
$CHCl_3$-MeOH-NH_4OH (6:4:0.5)	0.65	0.65	0.65	0.65
HPLC, Retention time (minutes) [a]	20.1	24.0	27.1	31.9

a) column: YMC-ODS-AM 4.6 x 250 mm, solvent: 70% MeOH/0.1% TFA/H_2O, flow rate: 0.8 ml/min, detection: 210 nm.

C-terminal polyamine moiety and was in agreement with the results of the NMR experiments (Fig. 3). Thus, the structure of the major component **2** was determined as shown in Fig. 3.

The structures of **1**, **3** and **4** were elucidated by comparison with **2**. The molecular formulae of **1**, **3** and **4**

Table 2. ^{1}H and ^{13}C NMR data of MS-681b (**2**) in DMSO-d_6 (500 MHz).

		δ_C	δ_H
Ac			
	C-1	170.2	
	C-2	22.2	1.80 (s)
Phe (1)			
	N-H		8.23 (t)
	C-1	172.4	
	C-2	54.9	4.41 (m)
	C-3	36.4	3.05 (dd), 2.83 (dd)
	C-4	135.5	
	C-5	129.1	7.30 (m)
	C-6	128.1[a)]	7.31 (m)
	C-7	126.3	7.23 (m)
	C-8	128.1[a)]	7.31 (m)
	C-9	129.1	7.30 (m)
Aib (2)			
	N-H		8.69 (s)
	C-1	174.4	
	C-2	55.9	
	C-3	24.8	1.31 (s)
	C-4	24.3	1.32 (s)
Aib (3)			
	N-H		7.71 (m)
	C-1	175.0	
	C-2	55.8	
	C-3	25.3	1.34 (s)
	C-4	24.3	1.34 (s)
Ala (4)			
	N-H		7.69 (m)
	C-1	173.3	
	C-2	50.1	3.98 (m)
	C-2	16.4	1.35 (d)
Iva (5)			
	N-H		7.67 (m)
	C-1	174.8	
	C-2	59.0	
	C-3	21.5	1.38 (s)
	C-4	28.6	1.98 (m), 1.78 (m)
	C-5	7.6	0.78 (t)
Gly (6)			
	N-H		7.95 (dd)
	C-1	169.9	
	C-2	43.1	3.66 (dd), 3.60 (dd)
Phe (7)			
	N-H		7.90 (m)
	C-1	172.5	
	C-2	55.8	4.30 (m)
	C-3	36.1	3.09 (dd), 2.97 (dd)
	C-4	138.0	
	C-5	129.0	7.33 (m)
	C-6	128.0[a)]	7.27 (m)
	C-7	126.2	7.23 (m)
	C-8	128.0[a)]	7.27 (m)
	C-9	129.0	7.33 (m)
Iva (8)			
	N-H		7.43 (d)
	C-1	172.3	
	C-2	59.6	
	C-3	20.9	1.24 (s)
	C-4	30.4	1.65 (m)
	C-5	7.6	0.50 (t)
N^1-(2-amino-3-phenylpropyl)spermidine			
	C-1	54.2	2.49 (m)
	C-2	26.4	1.47 (m)
	C-3	52.8	2.54 (m)
	C-5	47.2	2.60 (m)
	C-6	28.9	1.56 (m)
	C-7	28.8	1.26 (m)
	C-8	40.2	2.65 (m)
	N8-H		6.93 (d)
	C-1'	48.6	2.54 (m)
	C-2'	49.9	4.07 (m)
	C-3'	38.3	2.77 (dd), 2.64 (m)
	C-4'	139.1	
	C-5'	129.0	7.20 (m)
	C-6'	127.9	7.25 (m)
	C-7'	125.7	7.18 (m)
	C-8'	127.9	7.25 (m)
	C-9'	129.0	7.20 (m)

a) These carbons are exchangable.

were determined to be $C_{61}H_{91}N_{13}O_9$, $C_{62}H_{93}N_{13}O_9$ and $C_{60}H_{92}N_{12}O_9$ based on HRFAB-MS data, respectively.

Amino acid analysis of the hydrolysate and ^{1}H NMR and ^{13}C NMR spectra of **1** were very similar to those of **2** expect for the presence of tryptophan (Trp) instead of Phe. The sequence of **1** was determined by a positive ion FAB-MS/MS of its *quasi*-molecular ion (*m/z* 1150 $(M+H)^+$). The fragmentation pattern was similar to that of **2** and clarified the fact that the seventh residue corresponding to Phe of **2** was a Trp (data not shown). The structures of **3** and **4** were determined in a same manner as described above and elucidated as shown in Fig. 1. ^{1}H NMR data of **1**, **3** and **4** are summarized in Table 3.

The presence of minor components in each crude samples of **1**, **2**, **3** and **4**[1)] was indicated in the FAB-MS by the ions observed at molecular weight 12 mass units higher than the molecular ions of **1**, **2**, **3** and **4**, respectively. The molecular formulae of the minor com-

Table 3. ^{1}H NMR data of MS-681a (**1**), b (**2**), c (**3**) and d (**4**) in CD_3OD (400 MHz).

		1	2	3	4
Ac					
	C-2	1.95 (s)	1.96 (s)	1.97 (s)	1.96 (s)
Phe (1)					
	C-2	4.35 - 4.52 (m)	4.45 (dd)	4.10 - 4.52 (m)	4.49 (d)
	C-3	3.10 (dd),2.97 (dd)	3.11 (dd),2.98 (dd)	3.12 (dd),2.57 - 3.06 (m)	3.14 (dd),2.98 (dd)
Aib (2)					
or	C-3	1.37 (s)	1.37 (s)	1.38 (s)	1.38 (s)
Iva (2)	C-4	1.38 (s)	1.38 (s)	1.50 - 1.90 (m)	1.52 - 1.88 (m)
	C-5	—	—	0.84 (t)	0.85 (t)
Aib (3)					
	C-3	1.36 (s)	1.41 (s)	1.37 (s)	1.40 (s)
	C-4	1.45(s)	1.46 (s)	1.49 (s)	1.48 (s)
Ala (4)					
	C-2	3.95 (q)	4.04 (q)	3.97 (q)	4.05 (q)
	C-3	1.42 (d)	1.49 (d)	1.45 (d)	1.50 (d)
Iva (5)					
	C-3	1.48 (s)	1.48 (s)	1.48 (s)	1.49 (s)
	C-4	2.22 (dd),1.47 - 1.90 (m)	2.22 (dd),1.85 (dd)	2.32 (m), 1.50 - 1.90 (m)	2.25 (m), 1.52 - 1.88 (m)
	C-5	0.85 (t)	0.86 (t)	0.84 (t)	0.84 (t)
Gly (6)					
	C-2	3.89 (t), 3.72 (d)	3.87 (d), 3.68 (d)	3.91 (d), 3.70 (d)	3.88 (d), 3.66 (d)
Phe (7)					
or	C-2	4.35 - 4.52 (m)	4.34 (m)	4.10 - 4.52 (m)	4.35 (dd)
Trp (7)	C-3	2.45 - 2.85 (m)	3.20 (dd),3.14 (dd)	2.57 - 3.00 (m)	3.19 (dd),3.12 (dd)
Iva (8)					
	C-3	1.32 (s)	1.32 (s)	1.30 (s)	1.32 (s)
	C-4	1.49 - 1.90 (m)	1.68 (m), 1.59 (m)	1.50 - 1.90 (m)	1.52 - 1.88 (m)
	C-5	0.50 (t)	0.49 (t)	0.47 (t)	0.50 (t)
N^1-(2-amino-3-phenylpropyl)spermidine					
	C-1, 3, 5, 8, 1', 3'				
		2.45 - 2.85 (m)	2.83 (m), 2.81 (m)	2.57 - 3.66 (m)	2.54 - 2.90 (m)
			2.77 (m), 2.71 (m)		
			2.70 (m), 2.69 (m)		
			2.65 (m)		
	C-2, 6, 7				
		1.49 - 1.90 (m)	1.78 (m), 1.60 (m)	1.50 - 1.90 (m)	1.55 - 1.88 (m)
			1.55 (m)		
	C-2'				
		4.35 - 4.52 (m)	4.41 (m)	4.10 - 4.52 (m)	4.11 (m)
aromatic protons					
		7.85 (m), 7.13 - 7.35 (m)	7.13 - 7.35 (m)	7.56 (m), 7.13 - 7.36 (m)	7.13 - 7.35 (m)
		7.09 (m), 7.01 (m)		7.09 (m), 7.01 (m)	

Fig. 2. NMR analysis of **2**.

a) ^{1}H-^{1}H COSY, HMBC and NOESY experiments of **2** (in DMSO-d_6). b) Structure of polyamine moiety (in CD_3OD).

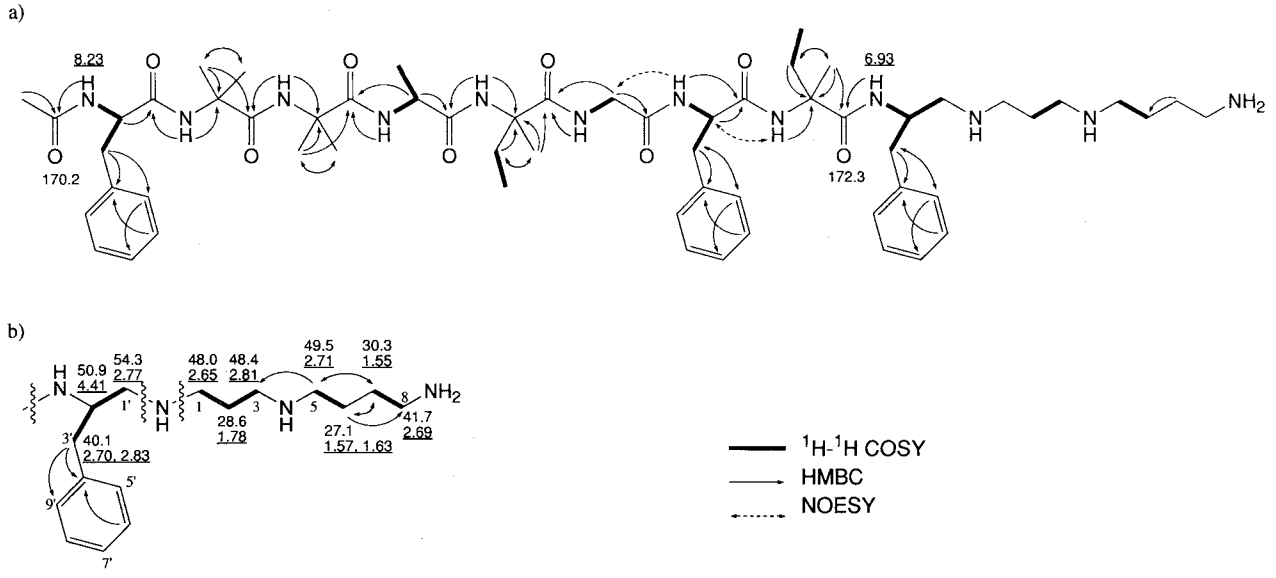
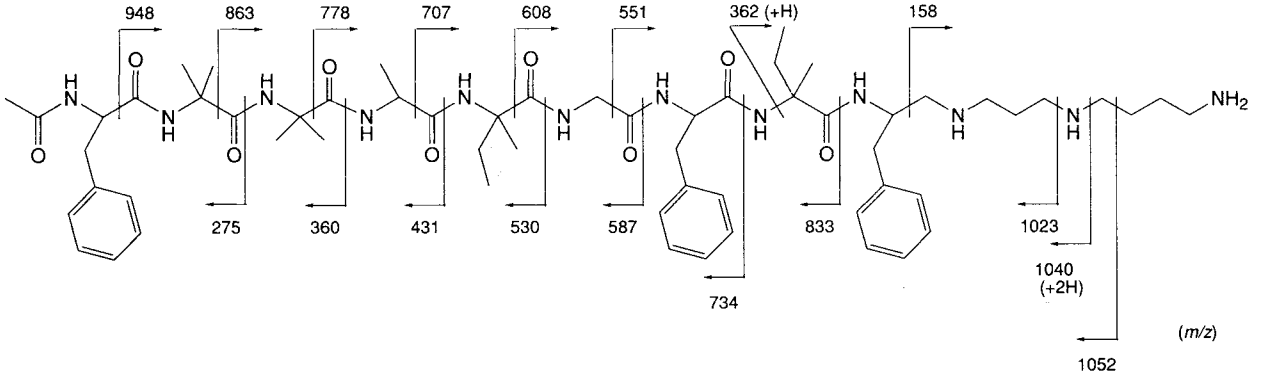


Fig. 3. FAB-MS/MS analysis of **2**.

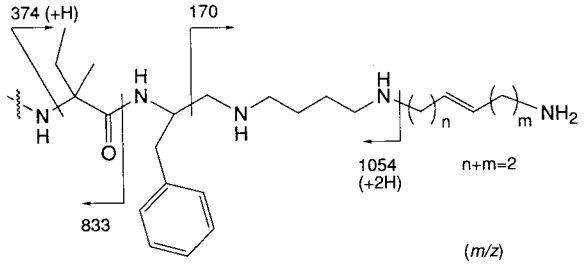


ponents of **1**, **2**, **3** and **4** were established as $C_{62}H_{91}N_{13}O_9$, $C_{60}H_{90}N_{12}O_9$, $C_{63}H_{93}N_{13}O_9$ and $C_{61}H_{92}N_{12}O_9$ based on HRFAB-MS, respectively. The structures of the minor components were confirmed by FAB-MS/MS of each *quasi*-molecular ion.

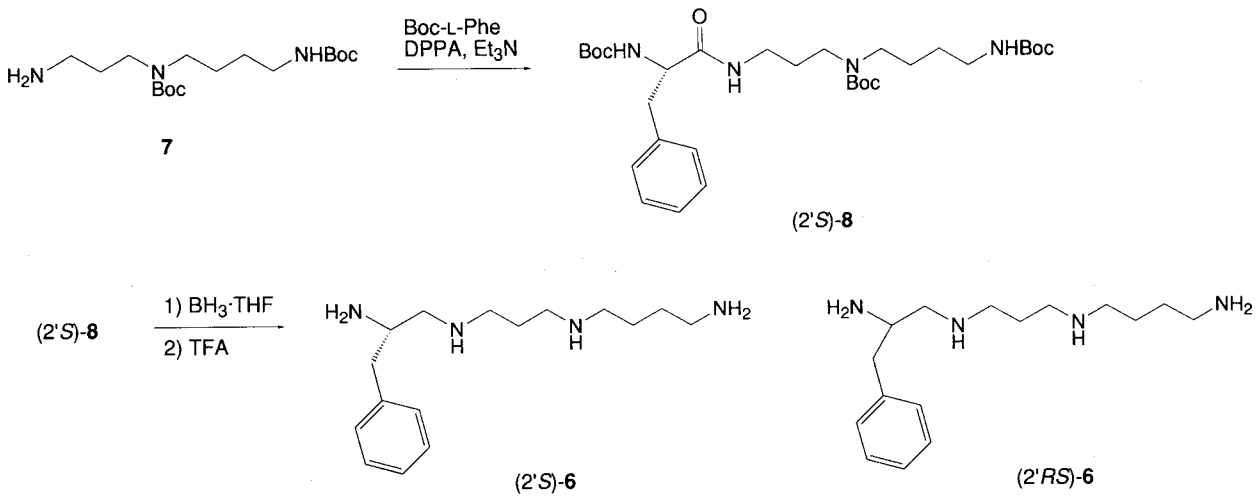
The *N*-terminal fragmentation pattern of the minor component of **2** was similar to that of **2**. On the other hand, all the *C*-terminal ions observed were higher than corresponding ions of **2** by 12 mass units. From these results, the amino acid sequence of the minor component of **2** was consistent with that of **2** and the difference was determined to be the polyamine moiety. Ions at *m/z* 170 and 1054 were attributed to cleavages of the polyamine region and the *C*-terminal polyamine structure of the minor component of **2** was suggested to be as shown in Fig. 4.

Each fragmentation pattern of the minor components of **1**, **3** and **4** was similar to that of the minor component

Fig. 4. FAB-MS/MS analysis of minor component of **2**.



Scheme 1. Synthesis of (2′*S*)- and (2′*RS*)-**6**.



of **2**, indicating that the amino acid sequences of the minor components of **1**, **3** and **4** were identical with those of **1**, **3** and **4**, respectively and their *C*-terminal polyamine moieties were similar to that of the minor component of **2** (data not shown).

The stereochemistry of the constituent amino acids of **1**, **2**, **3** and **4** was determined by GC on a chiral column[2]. Acid hydrolysates of **1**, **2**, **3** and **4** were converted to *N*-trifluoroacetyl *n*-propyl esters, and confirmed in comparison with authentic amino acid derivatives.

All of the protein amino acids, namely Ala, Gly, Phe and Trp, were found to have the *L* configurations. On the other hand, Iva of **1**, **2**, **3** and **4** revealed a mixture of the *L* and *D* forms in the ratio of 1 : 1, 1 : 1, 2 : 1 and 2 : 1, respectively. Each the *C*-terminal Iva (8) was determined to be the *L* configuration based on analyses of acid hydrolysates of **5**, which were derived from mild acid hydrolysis of **2** and **4**. Consequently, the remaining Iva (5) of **2** should have the *D* configuration from the ratio of Iva of the hydrolysate. From these results, Iva (5) and Iva (2) of **4** were suggested to be the *D* and *L* configurations, respectively. Similarly, the stereochemistry of Iva of **1** and **3** was assigned as shown in Fig. 1, and was supported by the fact that ^{1}H NMR data of **1** and **3** were almost identical to those of **2** and **4** expect for the seventh residues, respectively.

The absolute configurations of the tertiary carbons on the polyamine moieties of **1**, **2**, **3** and **4** were determined by comparison of HPLC retention times on a chiral column of tetratrifluoroacetate derivatives of **6** obtained from the acid hydrolysates of **1**, **2**, **3** and **4**, with those of synthetic compounds (2′*S*)- and (2′*RS*)-**6**.

Condensation of a protected spermidine **7**[3] with Boc-*L*-Phe afforded the amide (2′*S*)-**8**. Reduction of the amide group of (2′*S*)-**8** afforded the protected (2′*S*)-**6**, which was deprotected to give (2′*S*)-**6** (Scheme 1). By a similar procedure, (2′*RS*)-**6** was prepared from Boc-*DL*-Phe.

All the HPLC retention times of the tetratrifluoroacetates of **6** obtained from **1**, **2**, **3** and **4** were identical with that of (2′*S*)-**6**. Consequently, the absolute configurations of the polyamine moieties of **1**, **2**, **3** and **4** were determined to be all *S*.

Conclusion

The structures of **1**, **2**, **3** and **4** were elucidated, including the absolute configurations of the amino acids and the polyamine moieties. These compounds are unique octapeptides including an acetyl *N*-terminus, a high amount of α-alkyl-α-amino acids, containing Aib and both enantiomers of Iva, and a *C*-terminal polyamine moiety.

Related peptides containing a high amount of α-alkyl-α-amino acids such as Iva and Aib are the well-known peptaibol antibiotics[4]. However, significant differences reside in the *C*-terminus of the MS-681 compounds, which have the polyamine residues instead of 1,2-aminoalcohol residues such as leucinol[5] and phenylalaninol[6] for the peptaibols.

Experimental

General

NMR spectra were recorded on Bruker AM500, Jeol JMN-α400 and JMN-FX100 spectrometers with TMS as an internal standard. FAB-MS and FAB-MS/MS spectra were measured on a Jeol JMS-HX110/110A spectrometer using 3-nitrobenzyl alcohol as a matrix. Amino acid analyses were performed with a Jeol JLC-300 amino acid autoanalyzer. IR spectra were recorded on a Jeol JIR-RFX3001 spectrometer. HPLC was performed on a Hitachi L-6200 intelligent pump, L-4200 UV-VIS detector and D-2500 chromato-integrator. Preparative HPLC was carried out with a YMC-ODS AM-312 column (YMC Co., Ltd., i.d. 6.0 mm × 150 mm) [eluent MeOH - 0.1% trifluoroacetic acid solution (50 : 50 v/v); flow rate 1.0 ml/minute; column temperature 23°C; UV detector 220 nm]. Analytical HPLC was carried out with a CHIRALCEL OD column (DAICEL CHEMICAL Int., Ltd., i.d. 4.6 mm × 250 mm) [eluent EtOH - *n*-hexane (5 : 95 v/v); flow rate 0.5 ml/minute; column temperature 23°C; UV detector 220 nm]. GC was carried out on a Shimadzu GC-15A spectrometer [carrier gas helium (78 kPa); temperature programme 5 minutes at 70°C, then increased at 3°C/minute to 90°C and at 6°C/minute to 185°C; detection FID or MS] with a WCOT fused-silica column, coated with Chirasil-L-Val (Gasukuro Kogyo Co., Ltd., i.d. 25 m × 0.25 mm) in the split mode (splitting ratio *ca.* 1 : 20). Column chromatography was performed on Wakogel C-200 100 ~ 200 mesh silica gel (WAKO Pure Chemical Int., Ltd.). Preparative TLC was performed on Kieselgel 60 F_{254} (Merck). Organic extracts of reaction mixtures were washed successively with saturated $NaHCO_3$ solution and saturated NaCl solution, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* with a rotary evaporator and a vacuum pump, unless otherwise specified.

Analysis of the Amino Acids

A solution of the MS-681 compounds (0.5 mg) in 6 N HCl solution (1 ml) was heated at 110°C for 24 hours or 1 week in a vacuum-sealed tube. The hydrolysates were concentrated to residues *in vacuo* and dissolved in 0.02 N HCl solution and the solution was analyzed with the amino acid analyzer. **1** and **3** containing Trp were also hydrolyzed for 24 hours in the presence of 2-mercaptoethanesulfonic acid. The hydrolysates of **2** and **4** were subjected to preparative HPLC to give **5**, which were hydrolyzed as described above.

5: FAB-MS *m/z* 525 $(M+H)^+$; HPLC retention time 7.1 minutes.

In order to determine the stereochemistry of amino acids, the hydrolytic residues of the MS-681 compounds (0.1 mg) and **5** and authentic amino acids (2 mg) were derivatized to give the *N*-trifluoroacetyl/*n*-propyl esters in the reported manner[2]. The derivatives were analyzed by the GC spectrometer. The retention times of the authentic amino acids were 8.92 (Aib), 9.73 (*D*-Iva), 9.84 (*L*-Iva), 11.49 (*D*-Ala), 12.88 (*L*-Ala), 15.38 (Gly), 25.62 (*D*-Phe), 25.98 (*L*-Phe), 42.17 (*D*-Trp) and 43.03 minutes (*L*-Trp).

Synthesis of N^1-(2-Amino-3-phenylpropyl)spermidine

(2′*S*)-*N*-(*tert*-Butoxycarbonyl)phenylalanyl-N^4,N^8-bis(*tert*-butoxycarbonyl)spermidine ((2′*S*)-**8**)

To a solution of N^4,N^8-bis(*tert*-butoxycarbonyl)-spermidine (**7**)[3] (402 mg, 1.16 mmol) and *N*-(*tert*-butoxycarbonyl)-*L*-phenylalanine (293 mg, 1.10 mmol) in DMF (15 ml), diphenylphosphoryl azide (DPPA, 0.29 ml, 1.06 mmol) in DMF (5 ml) and triethylamine (0.19 ml, 1.35 mmol) were added at 0°C. After stirring for 21 hours at the same temperature, the reaction mixture was mixed with a 1 N HCl solution and extracted with EtOAc. The organic layer was washed, dried and concentrated to give a crude product, which was chromatographed with *n*-hexane - EtOAc (4 : 1) to afford (2′*S*)-**8** as an oil (497 mg, 72%): FAB-MS *m/z* 593 $(M+H)^+$; HRFAB-MS *m/z* 593.3921 Δ +0.7 mmu for $C_{31}H_{52}N_4O_7+H$; IR (KBr) ν_{max} 3336, 2975, 2931, 1695, 1525, 1365, 1251, 1170; ^1H NMR (100 MHz, $CDCl_3$) δ 7.23 (5H, m), 5.27 (1H, m), 4.82 (1H, m), 4.35 (1H, m), 3.27 ~ 2.92 (10H, m), 1.70 ~ 1.40 (6H, m), 1.44 (9H, s), 1.43 (9H, s), 1.39 (9H, s).

(2′*S*)-N^1-(2-Amino-3-phenylpropyl)spermidine ((2′*S*)-**6**)

To a solution of compound (2′*S*)-**8** (686 mg, 1.16 mmol) in THF (4 ml), borane-THF complex (1.0 M solution in THF, 2.32 ml, 2.32 mmol) was added dropwise at −12°C. After stirring for 24 hours at room temperature, the reaction mixture was quenched with MeOH. After stirring for 17 hours, the reaction mixture was concentrated, diluted with water and adjusted to pH 3 with 1 N HCl solution. The aqueous layer was extracted with EtOAc and the organic layer dried and concentrated to give a crude product, which was chromatographed with $CHCl_3$ - MeOH (20 : 1) to afford (2′*S*)-N^4,N^8-bis(*tert*-butoxycarbonyl)-N^1-{*N*-(*tert*-butoxycarbonyl)-2-amino-3-phenylpropyl}spermidine as an oil (118 mg, 18%).

To a solution of (2′*S*)-N^4,N^8-bis(*tert*-butoxycarbonyl)-N^1-{*N*-(*tert*-butoxycarbonyl)-2-amino-3-phenylpropyl}spermidine (110 mg, 0.19 mmol) in CH_2Cl_2 (6 ml), trifluoroacetic acid (6 ml) was added at 0°C. After stirring for 30 minutes at room temperature, the reaction mixture was concentrated and washed with *n*-hexane. The residue was chromatographed on a column of Dowex 1×4~200 (OH form) with water. The eluate was concentrated to give a crude product, which was chromatographed with CH_2Cl_2-MeOH-28% ammonia solution (2:2:1) to afford (2′*S*)-**6** as an oil (42 mg, 79%).

(2′*S*)-N^4,N^8-Bis(*tert*-butoxycarbonyl)-N^1-{*N*-(*tert*-butoxycarbonyl)-2-amino-3-phenylpropyl}spermidine: FAB-MS *m/z* 579 $(M+H)^+$; IR (KBr) ν_{max} 3342, 2975, 2931, 1695, 1521, 1365, 1270, 1172; ^{1}H NMR (100 MHz, $CDCl_3$) δ 7.23 (5H, m), 5.07 (1H, m), 4.64 (1H, m), 3.89 (1H, m), 3.32~2.97 (6H, m), 2.90~2.40 (7H, m), 1.83~1.47 (6H, m), 1.44 (18H, s), 1.41 (9H, s).

(2′*S*)-**6**: FAB-MS *m/z* 279 $(M+H)^+$; HRFAB-MS *m/z* 279.2528 Δ −2.1 mmu for $C_{16}H_{30}N_4+H$; IR (KBr) ν_{max} 3290, 2920, 1575, 1470, 1300; ^{1}H NMR (100 MHz, CD_3OD) δ 7.22 (5H, m), 3.20~2.20 (13H, m), 1.90~1.40 (6H, m).

(2′*RS*)-*N*-(*tert*-Butoxycarbonyl)phenylalanyl-N^4,N^8-bis(*tert*-butoxycarbonyl)spermidine ((2′*RS*)-**8**)

In a similar manner to that described for the preparation of (2′*S*)-**8**, compound **7** (1.60 g, 4.63 mmol) was treated with *N*-(*tert*-butoxycarbonyl)-*DL*-phenylalanine (1.17 g, 4.41 mmol), DPPA (1.18 ml, 5.46 mmol) and triethylamine (0.75 ml, 5.38 mmol) to give (2′*RS*)-**8** as an oil (2.34 g, 85%): FAB-MS *m/z* 593 $(M+H)^+$; HRFAB-MS *m/z* 593.3942 Δ +2.9 mmu for $C_{31}H_{52}N_4O_7+H$; IR (KBr) ν_{max} 3330, 2976, 2931, 1695, 1525, 1365, 1252, 1171; ^{1}H NMR (100 MHz, $CDCl_3$) δ 7.23 (5H, m), 5.27 (1H, m), 4.82 (1H, m), 4.35 (1H, m), 3.27~2.92 (10H, m), 1.70~1.40 (6H, m), 1.44 (9H, s), 1.43 (9H, s), 1.39 (9H, s).

(2′*RS*)-N^1-(2-Amino-3-phenylpropyl)spermidine ((2′*RS*)-**6**)

In a similar manner to that described for the preparation of (2′*S*)-**6**, compound (2′*RS*)-**8** (2.30 g, 3.88 mmol) was treated with borane-THF complex (1.0 M solution in THF, 7.76 ml, 7.76 mmol) to (2′*RS*)-N^4,N^8-bis(*tert*-butoxycarbonyl)-N^1-{*N*-(*tert*-butoxycarbonyl)-2-amino-3-phenylpropyl}spermidine, which was deprotected with trifluoroacetic acid (24 ml) to afford (2′*RS*)-**6** as an oil (158 mg, 16% from (2′*RS*)-**8**): FAB-MS *m/z* 279 $(M+H)^+$; HRFAB-MS *m/z* 279.2521 Δ −2.8 mmu for $C_{16}H_{30}N_4+H$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.27 (2H, m), 7.19 (2H, m), 7.17 (2H, m), 2.91 (1H, m), 2.65 (1H, dd, *J*=13.2, 5.9 Hz), 2.56 (2H, m), 2.53 (4H, m), 2.47 (1H, m), 2.46 (2H, m), 2.44 (1H, m), 2.28 (1H, dd, *J*=11.4, 8.0 Hz), 1.52 (2H, m), 1.40 (2H, m), 1.39 (2H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ 139.8 (s), 129.1 (d), 128.0 (d), 125.7 (d), 55.6 (t), 52.2 (d), 49.1 (t), 47.8 (t), 47.6 (t), 42.1 (t), 41.0 (t), 30.1 (t), 29.6 (t), 26.8 (t).

Analysis of N^1-(2-Amino-3-phenylpropyl)spermidine (**6**)

In order to determine the absolute configurations of **6** obtained from **1**, **2**, **3** and **4**, the synthetic (2′*S*)- and (2′*RS*)-**6** were derivatized to give the tetratrifluoroacetates. Each solution of synthetic (2′*S*)- and (2′*RS*)-**6** (1 mg) in trifluoroacetic anhydride (0.05 ml) and CH_2Cl_2 (0.2 ml) was heated at 100°C for 1 hour in a sealed tube. The reaction mixture was concentrated to give a crude product, which was purified by preparative TLC with $CHCl_3$-MeOH (10:1) to afford tetratrifluoroacetate. The derivatives and those of **1**, **2**, **3** and **4** were analyzed by HPLC. The retention times of tetratrifluoroacetates were 29.0 (*R*) and 31.5 minutes (*S*).

Tetratrifluoroacetate of (2′*RS*)-**6**; FAB-MS *m/z* 663 $(M+H)^+$; HRFAB-MS *m/z* 663.1833 Δ −0.2 mmu for $C_{24}H_{26}N_4O_4F_{12}+H$; IR (KBr) ν_{max} 1687, 1200, 1148; ^{1}H NMR (100 MHz, $CDCl_3$) δ 7.05~7.40 (5H, m), 6.81 (1H, m), 6.66 (1H, m), 4.49 (1H, m), 4.01 (1H, m), 3.70~2.80 (11H, m), 1.92~1.20 (6H, m).

Tetratrifluoroacetate from **2**; FAB-MS *m/z* 663 $(M+H)^+$.

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