VOL. 54 NO. 2, FEB. 2001 THE JOURNAL OF ANTIBIOTICS pp. 175 — 178

NOTES

Ampullosporins B, C, D, E_1, E_2, E_3 and E_4 from *Sepedonium ampullosporum* HKI-0053: Structures and Biological Activities

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(Received for publication September 18, 2000)

In a previous paper[1] we reported the new peptaibol-type antibiotic ampullosporin A (**1**, Fig. 1), production by the fungal strain of *Sepedonium ampullosporum*, structure elucidation, and biological activities. Ampullosporin A (**1**) was discovered as an inducer of pigment formation by the fungal strain of *Phoma destructiva*. Moreover, strong neuroleptic activity was established for **1** in mice and rats as test animals[1,2].

In order to disclose structure-activity relationships amongst the naturally occurring homologues and isomers of ampullosporin A we investigated the metabolite spectrum of the producer strain *Sepedonium ampullosporum* by HPLC and mass spectrometry (LC-MS, ESI-CID-MS/MS, ESI-CID-MS^n).

These investigations unraveled the presence of minor components such as ampullosporins B (**2**), C (**3**), D (**4**) and E, amounting to 1~5% of the produced ampullosporin A (**1**). Component E was shown to be a mixture of four isomers (E_1 (**5**), E_2 (**6**), E_3 (**7**), E_4 (**8**); Fig. 1). Here we report the isolation, structure elucidation and biological activities of compounds **2**~**8**.

The producer strain *Sepedonium ampullosporum* HKI-0053 was cultivated as was described previously[1]. Surface cultivation was carried out at 25°C in 1 liter Erlenmeyer flasks containing 100 ml of a medium composed of (g/liter): glycerol 30, glucose 10, peptone 5, NaCl 2, molecular sieve (5 nm, Merck) 1, agar 1; pH 7.0 (prior to sterilization).

After 14 days of cultivation as solid culture at 28°C the whole culture broth (40 liters) was extracted twice by 10 liters of ethyl acetate. The dried extract was evaporated and the residue was chromatographed on silica gel 60 (0.063~0.1 mm, $CHCl_3$/MeOH, 9:1, v/v) and, subsequently Sephadex LH-20 (MeOH). The fractions containing ampullosporin A (**1**) and its congeners **2**~**8** were detected by ESI-MS using *m/z* 1622 (**1**: $[M+H]^+$) as a diagnostic tool. The mixture of crude ampullosporins (1.3 g) was separated subsequently by preparative HPLC

Fig. 1. Amino acid sequences of ampullosporins A (**1**), B (**2**), C (**3**), D (**4**), E_1 (**5**), E_2 (**6**), E_3 (**7**) and E_4 (**8**).

Ac-L-Trp^1-L-Ala^2-Aib^3-Aib^4-L-Leu^5-Aib^6-L-Gln^7-X-L-Gln^{11}-L-Leu^{12}-Y-L-Gln^{14}-L-Leuol^{15}

1	X = Aib^8-Aib^9-Aib^{10}	Y = Aib^{13}
2	X = L-Ala^8-Aib^9-Aib^{10}	Y = Aib^{13}
3	X = Aib^8-L-Ala^9-Aib^{10}	Y = Aib^{13}
4	X = Aib^8-Aib^9-L-Ala^{10}	Y = Aib^{13}
5	X = L-Ala^8-Aib^9-Aib^{10}	Y = L-Ala^{13}
6	X = Aib^8-L-Ala^9-L-Ala^{10}	Y = Aib^{13}
7	X = Aib^8-Aib^9-L-Ala^{10}	Y = L-Ala^{13}
8	X = L-Ala^8-L-Ala^9-Aib^{10}	Y = Aib^{13}

Abbreviations: Ac-L-Trp: *N*-Acetyl-L-tryptophane, Aib: α-Aminoisobutyric acid, L-Leuol: L-Leucinol, L-Ala: L-alanine.

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on silica gel RP_{18} (Nucleosil 100-A, 40×250 mm, charged by 15 mg during each run with a gradient of 95% H_2O to 95% acetonitrile, 25 minutes, 210 nm). Ampullosporins A~E were separable under these conditions due to differences in retention times (see Table 1). From 40 liters of the culture broth thus 0.95 g **1**, 35 mg **2**, 8 mg **3**, 5 mg **4** and 3 mg of ampullosporins E (**5**, **6**, **7**, **8**) were isolated as semicrystalline solid (Table 1).

Total hydrolysis of ampullosporins B~E (**2**~**7**) by 6 N hydrochloric acid, derivatization by Marfey's reagent and HPLC analysis[3] suggested that they contained the same amino acids and L-leucinol as **1**[1].

Structure elucidation of **2**~**8** was carried out by tandem mass spectrometry (triple quadrupole mass spectrometer Quattro, VG Biotech, Altrincham, England; LC-coupled ion-trap mass spectrometer LCQ, Finnigan, Bremen, Germany, both instruments equipped with electrospray ion source (ESI)) and NMR spectroscopy (^1H, ^{13}C, DEPT 135, COSY, HSQC, HMBC, NOESY, TOCSY, Bruker AVANCE DRX 500).

The molecular weights of **2**, **3**, **4** and ampullosporin E (**5**, **6**, **7**, **8**) and chemical formulas were readily determined by

Table 1. Physico-chemical properties of ampullosporin B (**2**), C (**3**), D (**4**) and E (**5**, **6**, **7**, **8**).

	2	**3**	**4**	**5/6/7/8**
Appearance	colorless solid	colorless solid	colorless solid	colorless solid
Melting Point (°C)	232-233 (decomposition)	157-159 (decomposition)	182-184 (decomposition)	222-224 (decomposition)
Molecular Weight	1607	1607	1607	1593
HRESI-MS ($[M+Na]^+$)	1630.9284 (calcd. 1630.9272)	1630.9283 (calcd. 1630.9272)	1630.9281 (calcd. 1630.9272)	1616.9137 (calcd. 1616.9133)
Formula	$C_{76}H_{125}N_{19}O_{19}$	$C_{76}H_{125}N_{19}O_{19}$	$C_{76}H_{125}N_{19}O_{19}$	$C_{75}H_{123}N_{19}O_{19}$
R_t (min) on HPLC (gradient H_2O/acetonitrile 1:99 to 99:1 16 min)	15.41	15.29	15.22	14.87

Table 2. Daughter ions observed with ampullosporins B (**2**), C (**3**), D (**4**) and E_1 (**5**), E_2 (**6**), E_3 (**7**) and E_4 (**8**) during CID-MS/MS and CID-MS^n.

Ampullosporins	**Fragments (m/z)**											
	B_1	**B_2**	**B_3**	**B_4**	**B_5**	**B_6**	**B_8**	**B_9**	**B_{10}**	**B_{12}**	**B_{13}**	**$[M+H]^+$**
1	229	300	385	470	583	668	881	966	1051	1292	1377	1622
2	229	300	385	470	583	668	867	952	1037	1278	1363	1608
3	229	300	385	470	583	668	881	952	1037	1278	1363	1608
4	229	300	385	470	583	668	881	966	1037	1278	1363	1608
5	229	300	385	470	583	668	867	952	1037	1278	1349	1594
6	229	300	385	470	583	668	881	952	1023	1264	1349	1594
7	229	300	385	470	583	668	881	966	1037	1278	1349	1594
8	229	300	385	470	583	668	867	938	1023	1264	1349	1594

For comparison the diagnostic daughter ions of ampullosporin A (**1**)[1] are shown.

Table 3. Assignments of the ^{1}H and ^{13}C NMR spectra of ampullosporin B (**2**) (DMSO-d_6, 500 MHz, δ in ppm, TMS as internal standard).

Residue	Proton	δ_H (J in Hz)	δ_C
Ac	CH_3	1.85	22.63
	CO		170.56
Trp[1]	NH	8.32 (d, 7.5)	
	α	4.38 (ddd, 9.5, 7.5, 4.4)	54.81
	β_1	2.97 (dd, 15.0, 9.5)	27.14
	β_2	3.11 (dd, 15.0, 4.4)	
	1'	10.82 (d, 2.8)	
	2'	7.20 (d, 2.8)	123.69
	3'		109.86
	3a'		127.23
	4'	7.53 (d, 7.9)	118.21
	5'	6.93 (t, 7.9)	118.12
	6'	7.03 (t, 7.9)	120.88
	7'	7.31 (d, 7.9)	111.33
	7a'		136.09
	CO		173.15
Ala[2]	NH	8.38 (br, d, 5.3)	
	α	4.02 (dq, 7.2, 5.3)	50.64
	β	1.24 (d, 7.2)	15.96
	CO		174.29
Aib[3]	NH	8.33 (br, s)	
	α		55.71
	β_1	1.35 (s)	22.30
	β_2	1.36 (s)	25.70
	CO		174.90
Aib[4]	NH	8.01 (br, s)	
	α		55.59
	β_1	1.33 (s)	22.39
	β_2	1.37 (s)	26.79
	CO		176.62
Leu[5]	NH	7.69 (d, 5.5)	
	α	3.88 (m)	54.60
	β_1	1.56 (m)	38.92
	β_2	1.77 (m)	
	γ	1.71 (m)	24.51
	δ_1	0.82 (d, 6.6)	21.43
	δ_2	0.91 (d, 6.6)	22.30
	CO		174.08
Aib[6]	NH	7.83 (br, s)	
	α		55.78
	β_1	1.33 (s)	23.23
	β_2	1.45 (s)	26.64
	CO		175.76
Gln[7]	NH	7.37 (br, d, 5.4)	
	α	3.80 (dt, 7.0, 5.4)	56.23
	β_1	1.95 (m)	25.97
	β_2	1.99 (m)	
	γ_1	2.11 (m)	31.03
	γ_2	2.20 (m)	
	δ		173.02
	ε_1	6.72 (br, s)	
	ε_2	7.19 (br, s)	
	CO		173.65
Aib[8]	NH	8.03 (br, s)	
	α		55.56
	β_1	1.32 (s)	22.60
	β_2	1.43 (s)	25.80
	CO		175.42
Aib[9]	NH	8.00 (br, s)	
	α		55.50
	β_1	1.34 (s)	22.39
	β_2	1.39 (s)	26.61
	CO		176.24
Ala[10]	NH	7.56 (br, d, 5.2)	
	α	3.90 (dq, 7.5, 5.2)	51.69
	β	1.42 (d, 7.5)	16.37
	CO		174.94
Gln[11]	NH	7.84 (br, d, 5.6)	
	α	3.96 (m)	55.17
	β_1	1.96 (m)	26.17
	β_2	2.04 (m)	
	γ_1	2.12 (m)	31.91
	γ_2	2.34 (m)	
	δ		173.41
	ε_1	6.72 (br, s)	
	ε_2	7.18 (br, s)	
	CO		173.41
Leu[12]	NH	7.47 (d, 7.7)	
	α	4.06 (m)	52.89
	β_1	1.62 (m)	38.86
	β_2	1.64 (m)	
	γ	1.69 (m)	24.13
	δ_1	0.81 (d, 6.5)	21.07
	δ_2	0.84 (d, 6.7)	22.50
	CO		172.91
Aib[13]	NH	7.48 (br, s)	
	α		56.07
	β_1	1.36 (s)	24.36
	β_2	1.39 (s)	25.38
	CO		173.89
Gln[14]	NH	7.16 (br, d, 7.8)	
	α	3.97 (m)	53.33
	β_1	1.80 (m)	27.14
	β_2	1.99 (m)	
	γ_1	2.06 (m)	31.69
	γ_2	2.19 (m)	
	δ		173.89
	ε_1	6.67 (br, s)	
	ε_2	7.06 (br, s)	
	CO		170.78
Leuol[15]	NH	7.02 (d, 8.1)	
	α	3.75 (m)	48.67
	β_1	1.31 (m)	39.77
	β_2	1.34 (m)	
	β_1'	3.18 (dd, 10.4, 7.0)	63.89
	β_2'	3.30 (dd, 10.4, 5.0)	
	γ	1.63 (m)	23.93
	δ_1	0.79 (d, 6.7)	21.77
	δ_2	0.83 (d, 6.7)	23.47

Abbreviations: s; singlet, d; doublet, t; triplet, q; quartet, m; multiplet, br; broad

HRESI-MS (high-resolution double focussing mass spectrometer Finnigan MAT 95XL).

As shown in Table 1 the ampullosporins B (**2**), C (**3**), and D (**4**) displayed the same molecular weight of 1607 Da suggesting that they are position isomers. Ampullosporin E was distinguishable by lower molecular weight (1593 Da, Table 1).

The sequence of amino acids in **2**, **3** and **4** was determined unambiguously by ESI-CID-MS/MS (argon as collision gas) and FAB-MS (high-resolution sector-field instrument AMD-402, AMD Intectra, Bremen, Germany) furnishing diagnostic B-type fragments[4] (Table 2). Collision-induced fragmentation (CID-MS/MS) of *m/z* 1616.9 ($[M+Na]^+$) of ampullosporin E suggested the occurrence of chromatographically inseparable isomers (**5**, **6**, **7**, **8**; Table 2) due to the appearance of several series of fragments. As was shown for the related roseoferins[5], the sequence of amino acids of peptide isomers such as E_1 (**5**), E_2 (**6**), E_3 (**7**) and E_4 (**8**) was assignable by ESI-MS^n experiments (Finnigan LCQ, ion-trap analyzer, helium as collision gas). For instance, collision-induced dissociation of selected MS^2 daughter ions afforded several series of diagnostic MS^3 ions as a measure for the occurrence of position isomers (Table 2).

Additionally to the above experiments the structure of **2** as the major homologue of ampullosporin A (**1**) was confirmed by one- and two-dimensional NMR measurements. ^1H ^1H-COSY, HSQC, HMBC and NOESY correlations were particularly helpful for the assignment of the ^1H and ^{13}C signals and disclosure of the amino acid sequences (Fig. 1, Table 3).

Ampullosporins B, C, D, E_1, E_2, E_3 and E_4 (**2**~**8**) thus appear as new members of the peptaibol family of fungal peptides. Obviously, the structures of ampullosporins from *Sepedonium ampullosporum* show differences only in positions 8, 9, 10 and 13 whereby Aib is replaced by L-alanine.

The biological activities of **2**, **3** and **4** were compared with that of **1** using *Phoma destructiva* as test organism[5]. In concentration of 50 μg/agar well compounds **2** and **4** induced pigment formation in the same manner and concentration as ampullosporin A (**1**). However, compound **3** was needed in five-times higher concentration to afford a comparable effect.

The neuroleptic activity of **2**~**4** was tested in mice[5]. After a dosage of 1~10 mg **2** or **4** /kg body weight a decrease of body temperature was measured as a characteristic of neuroleptic drugs[1]. The effect was comparable to that of ampullosporin A (**1**). In contrast, compound **3** caused a comparably much lower decrease of body temperature suggesting that sequence and quality of amino acids in positions 8, 9, 10 of the ampullosporins possess a crucial importance for biological activity as morphogenic and neuroleptic drugs.

Acknowledgements

Support of this work by EU project Eurofung and TMWFK of Thuringia is gratefully acknowledged. We thank ING. A. PERNER, Mrs. R. KOCH, C. KARKOWSKI and G. SCHWINGER for skillful assistance.

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