Unguisins A and B: New Cyclic Peptides from the Marine-Derived Fungus *Emericella unguis*

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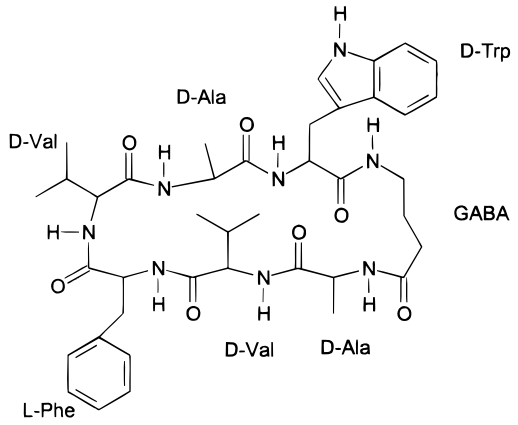
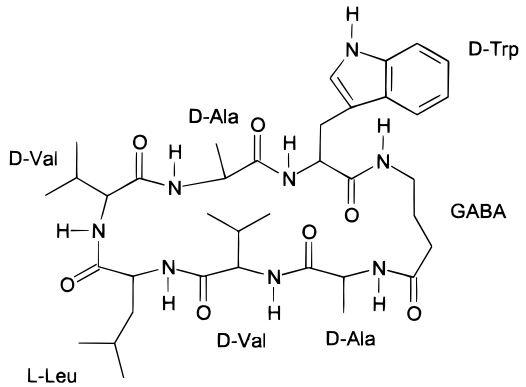
Received November 30, 1998

Unguisin A (**1**) and B (**2**), the first cyclic heptapeptides containing GABA in the ring, were isolated from a marine-derived strain of *Emericella unguis*. The chemical structures of **1** and **2** were elucidated by extensive 2D NMR techniques, and the stereochemistry of the individual amino acids was determined using Marfey's method.

In this paper we report the isolation and structure elucidation of unguisin A (**1**) and B (**2**), two cyclic heptapeptides with GABA incorporated in the ring. These peptides were produced by a fungus, *Emericella unguis*, isolated from a *Stomolopus meliagris* (medusa) collected in Venezuelan waters. Metabolites produced by *E. unguis* include several depsidones, e.g. emeguisins, folipastatin, and yasimin.[1] Previous studies of a marine-derived isolate resulted in the isolation of a depside guisinol, a possible precursor for the depsidones and specific to *E. unguis*.[2] Cyclic peptides containing GABA in the ring have not been isolated before, while those incorporating β-hydroxylated γ-aminobutyric acid (GABOB) are well-known and include the antifungal microsclerodermins A−E isolated from the lithistid marine sponges *Theonella* sp. and *Microsclerodermа* sp.[3] Synthetic cyclic peptides containing GABA have been tested as potential drug carriers[4] and antitumor agents (glutathione homologues).[5]



Unguisin A (**1**)



Unguisin B (**2**)

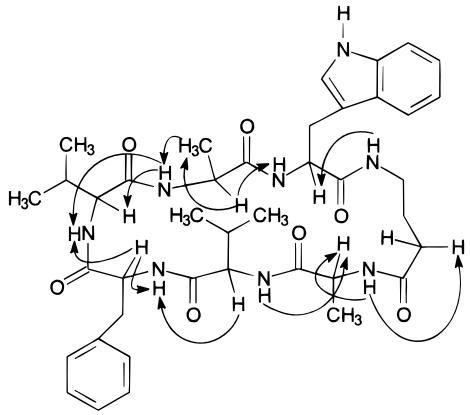


Figure 1. Key NOESY correlations used to determine the structure of unguisin A.

Compound **1** exhibited a positive ion FABMS peak at *m*/*z* 759.5 $(M + H)^+$ and a negative ion FABMS peak at *m*/*z* 757.4 $(M - H)^-$ corresponding to the formula $C_{40}H_{54}N_8O_7$. ^1H and ^{13}C NMR (DMSO-d_6) data suggested a peptide structure and confirmed the elemental composition. The proton NMR spectrum revealed the presence of seven amide NH signals at δ 8.55, 8.43, 8.12, 8.05, 7.89, 7.83, and 7.69 and an additional NH signal at 10.84 ppm, which suggested the presence of an indole ring. The occurrence of seven amino acids was supported by amide carbonyl signals at δ 173.0, 172.6, 171.8, 171.5, 171.4, 171.1, and 170.5. However, in the aromatic region of the carbon NMR spectrum eleven partly overlapping signals were observed, indicating the presence of at least two aromatic amino acids. The remaining signals corresponded to six methyl, five methylene, six α-methine, and two isopropyl methine carbon atoms. 2D NMR experiments (COSY and HMQC in DMSO-d_6) revealed the seven amino acids to be Ala (2 eq), Val (2 eq), Trp (1 eq), Phe (1 eq), and γ-aminobutyric acid (GABA) (1 eq). The peptide sequence was determined from NOESY correlations (Figure 1). The cyclic structure is in agreement with the 18 DBE required by the molecular

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10.1021/np980539z CCC: $18.00
Published on Web 04/08/1999

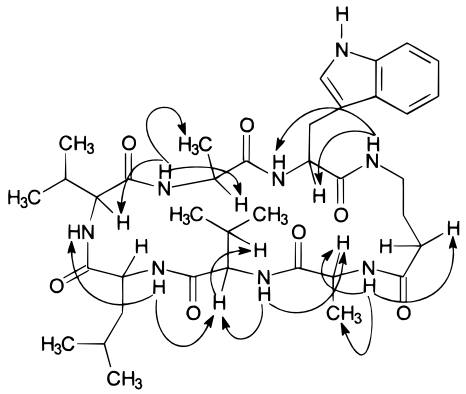

Figure 2. Key NOESY correlations used to determine the structure of unguisin B.

formula. To determine the absolute stereochemistry of the amino acids, **1** was mildly hydrolyzed and derivatized with Marfey's reagent.[6] The configuration of the derivatives was confirmed by comparison of the HPLC retention time with authentic samples. The structure of **1** was therefore determined as *cyclo*-(D-valyl-D-alanyl-D-tryptophyl-GABA-D-alanyl-D-valyl-L-phenylalanyl).

Compound **2** only differed from **1** in the structure of one amino acid. The FABMS exhibited peaks at *m*/*z* 725.3 $(M + H)^+$ and *m*/*z* 723.6 $(M - H)^-$ corresponding to the formula $C_{37}H_{56}N_8O_7$. NMR spectrometry (^1H, ^{13}C, COSY, NOESY) revealed that the seven amino acids corresponded to Ala (2 eq), Val (2 eq), Trp (1 eq), GABA (1 eq), and Leu (1 eq). Thus, phenylalanine in compound **1** is exchanged with leucine in **2** (Figure 2), while the sequence of the six identical amino acids in **1** and **2** was unchanged. The stereochemistry of the individual amino acids was determined by the use of Marfey's reagent and was found to be *cyclo*-(D-valyl-D-alanyl-D-tryptophyl-GABA-D-alanyl-D-valyl-L-leucyl).

The cyclic peptides can either be bio-synthesized by ribosomal or nonribosomal mechanisms with proteinogenic amino acids confined to the former mechanism.[7] The high ratio of D-amino acids in unguisin A and B are thus a strong indication of a nonribosomal origin for these compounds. It is therefore tempting to speculate about the function of GABA in these peptides. Fungi utilize GABA as a C and N source, and it is associated with some of the major facets of the cell cycle, including sporulation, differentiation and development.[8] Hence, pools of GABA are usually present in fungi, making the amino acid easily accessible. The incorporation of GABA in **1** and **2** induces an enhanced conformational mobility relative to cyclic peptides derived solely from α-amino acids. Since the biological activity is strongly dependent on conformation this may present a rationale for the occurrence of GABA in **1** and **2**.

Unguisin A and B were tested for antibacterial activity against *Staphylococcus aureus* and *Vibrio parahaemolyticus*, and only moderate activity was observed against the former.

Experimental Section

General Experimental Procedures. NMR spectra were recorded in DMSO-d_6 on a Varian 400 FT-NMR spectrometer at 400.0 and 100.6 MHz for ^1H and ^{13}C NMR spectra, respectively. The HPLC data were obtained on a HPLC system combined with a Millenium 996 photodiode array detector from Waters. The UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. Circular dichroism (CD) spectra were measured on a JASCO J-710 spectropolarimeter. FABMS were recorded on a JEOL JMS-MX/HX 110 A spectrometer using a m-NBA matrix. Marfey's reagent (N_α-2,4-dinitro-5-fluoro-phenyl-L-alaninamide) was purchased from Sigma.

Table 1. NMR Data for Unguisin A (**1**)[a] in DMSO-d_6

residue/position		δ_C	δ_H (*J*, Hz)
alanine	NH		8.43 (4.6 d)
	Cα	49.8	3.96 (m)
	C*β*	17.4	1.17 (7.0 d)
alanine	NH		7.83 (6.1 d)
	Cα	47.9	4.23 (m)
	C*β*	18.1	1.14 (7.0 d)[b]
valine	NH		8.12 (4.2 d)
	Cα	60.8	3.50 (4.5/8.5 dd)
	C*β*	28.6	1.57 (m)
	C*γ*	18.7	0.75 (6.6 d)[b]
	C*γ*	18.4	0.29 (6.8 d)[b]
valine	NH		7.89 (9.52 d)
	Cα	58.8	4.09 (9.8 t)
	C*β*	30.3	2.01 (m)
	C*γ*	19.6	0.72 (6.6 d)
	C*γ*	18.9	0.67 (6.6 d)[b]
GABA	NH		7.69 (4.8/6.2 t)
	Cα	38.5	3.12 (m), 2.96 (m)
	C*β*	26.0	1.68 (m), 1.57 (m)
	C*γ*	32.9	2.10 (m), 1.96 (m)
tryptophan	NH		8.05 (7.1 d)
	Cα	55.2	4.04 (7.0 q)
	C*β*	25.3	3.19 (7.6 d)
	C-1		10.84 (s)
	C-2	123.7	7.11 (s)
	C-3	110.6	
	C-3a	127.1	
	C-4	118.3	7.53 (7.9 d)
	C-5	121.0	7.06 (7.0 t)
	C-6	118.3	6.98 (7.0 t)
	C-7	111.4	7.33 (8.1 d)
	C-7a	136.2	
phenylalanine	NH		8.55 (8.6 d)
	Cα	55.3	4.33 (12.0, 8.5, 3.4 ddd)
	C*β*	36.5	2.61 (13.1 t), 3.27[c]
	C1	138.5	
	C2-5	128.0−129.1[d]	7.11−7.24[d]
	C6	126.1	7.11−7.24[d]

[a] The seven carbonyl carbon signals appear at *δ* 173.0, 172.6, 171.8, 171.5, 171.4, 171.1, and 170.5. [b] Signals are interchangeable. [c] Water signal interference. [d] Overlapping signals.

Collection, Isolation, and Fermentation. Two strains of *E. unguis* (isolates 1 (M87-2) and 2 (M90B-10)) were collected in the Paria Bay, Venezuela, in January 1997. Isolate 1 originated from a solution of a *S. meliagris* mixed with water, isolate 2 from the soft part of an unidentified mollusc. A comparison of the metabolite profiles obtained from HPLC analysis showed that the two isolates of *E. unguis* (M87-2 and M90B-10) express the same metabolites, although in variable amounts. Isolate 1 was chosen for further studies because of the high concentration of metabolites present in this isolate. The fungus was grown on solid YES medium (yeast extract, 20 g/L; sucrose, 150 g/L) for a period of 14 days at 25 °C.

Extraction and Isolation. Mycelium and agar were harvested and extracted with a mixture of ethyl acetate/chloroform/methanol (3:2:1) containing 1% formic acid. The dried extract was defatted by partition between methanol/water (80:20) and hexane. The polar fraction (7 g) was separated by VLC. The column was packed with RP-18 (40−63 μm), and the fractionation was performed with an increasing gradient of methanol/water (60:40→100:0) containing 1% formic acid. The methanol/water (70:30) fraction was further purified on a Waters RCM Prep Nova-pak HR C18 6 μm column coupled with a Waters HPLC system using methanol/water (55:45) as mobile phase. Of six fractions secured, fractions 4 and 5 were rechromatographed, yielding unguisin B (5.1 mg) and unguisin A (4.0 mg), respectively. Fraction 6 contained a pure compound (30 mg) which proved to be the known depsidone yasimin.[1c]

Table 2. NMR Data for Unguisin B (**2**)[a] in DMSO-d_6

residue/position		δ_C	δ_H (J, in Hz)
alanine	NH		8.36 (4.6 d)
	Cα	49.8	3.92 (m)
	Cβ	17.3[b]	1.16[c]
alanine	NH		7.82 (6.1 d)
	Cα	48.0	4.23 (m)
	Cβ	18.1[b]	1.16[c]
valine	NH		8.13 (4.4 d)
	Cα	60.6	3.67 (4.6/8.2 dd)
	Cβ	28.8	1.82 (m)
	Cγ	19.7	0.95 (6.6 d)
	Cγ	18.8	0.84 (6.4 d)[d]
valine	NH		7.73 (9.7 d)
	Cα	58.5	4.07 (m)
	Cβ	30.2	2.0 (m)
	Cγ	18.6	0.66 (6.6 d)
	Cγ	18.9	0.74[e]
GABA	NH		7.65 (5.3 t)
	Cα	38.5	3.11 (m), 2.95 (m)
	Cβ	25.9	1.65 (m), 1.58 (m)
	Cγ	32.9	2.11 (m), 1.94 (m)
tryptophan	NH		7.98 (6.8 d)
	Cα	55.2	4.05 (m)
	Cβ	25.2	3.20 (8.5 d)
	C-1		10.81 (2.0 d)
	C-2	123.6	7.11 (2.2 d)
	C-3	110.6	
	C-3a	127.1	
	C-4	118.3	7.54 (7.9 d)
	C-5	121.0	7.08 (6.9 t)
	C-6	118.3	6.97 (7.2 t)
	C-7	111.4	7.35 (8.1 d)
	C-7a	136.2	
leucine	NH		8.43 (7.7 d)
	Cα	51.2	4.09 (m)
	Cβ	38.7	1.58 (m)
	Cγ	24.1	1.58 (m)
	Cδ	23.4	0.84 (6.4 d)[d]
	Cδ	20.4	0.74[e]

[a] The seven carbonyl carbon signals appear at δ 173.0, 172.6, 171.9, 171.6, 171.6, 171.5, and 171.1. [b] Signals are interchangeable. [c–e] Overlapping signals.

Unguisin A[1]: colorless solid; UV (EtOH) λ_{max} (log ϵ): 290 (3.58), 281 (3.65), 274 (3.63), 219 (4.49) nm; CD, λ ext (c 0.018, EtOH) ($\Delta\epsilon$) 223 (4.75), 205 (−16.20), FABMS m/z 759.5 (positive), 757.4 (negative); NMR data, see Table 1.

Hydrolysis of Unguisin A. The compound (200 μg) was treated at 165 °C for 25 min with 6 N HCl containing 3% phenol (100 μL).[9] After cooling, the sample was freeze-dried and derivatized with Marfey's reagent.[6] The configuration of Ala and Val, together with the presence of GABA were determined by using a gradient of TEAP (pH 3.0):CH_3CN (start, 90:10; end, 63:37) for 40 min. Retention times (in min) for the standards were Val, L, 22.3; D, 29.5; Ala, L, 14.4, D, 19.7; GABA, 21.5. The configuration of Phe and Thp were determined using a gradient of 0.1% TFA/CH_3CN (start, 85: 15; end, 0:100) for 43 min. Retention times (in min) for the standards were Phe, L, 18.4, D, 19.9; Trp, L, 17.9, D, 18.9.

Unguisin B[2]: colorless solid; UV (EtOH) λ_{max} (log ϵ): 290 (3.53), 280 (3.60), 274 (3.57), 220 (4.42) nm; CD, λ ext (c 0.030, EtOH) ($\Delta\epsilon$) 223 (7.08), 203 (−19.20), FABMS m/z 725.3 (positive), 723.6 (negative); NMR data, see Table 2. The hydrolysis of unguisin B was performed by the same procedure as described for unguisin A.

Acknowledgment. We are grateful to Prof. O. Crescente of Universidad de Oriente, Cumaná, Venezuela, for help in obtaining the fungi. We are indebted to Dr. S. E. Harnung for determination of the CD data measured on a modified Jasco 710 instrument financed by the Danish Natural Science Research Council, Grant No. //-0373-1.

References and Notes

(1) (a) Kawahara, N.; Nozawa, K.; Nakajima, S.; Kawai, K.; Yamazaki, M. *J. Chem Soc., Perkin Trans I* **1988**, 2611. (b) Hamano, K.; Kinoshita-Okami, M.; Hemmi, A.; Sato, A.; Hisamoto, M.; Matsuda, K.; Yoda, K.; Haruyama, H.; Hosoya, T.; Tanzawa, K. *J. Antibiotics* **1992**, *45*, 1195. (c) Kamal, A.; Haider, Y.; Khan, Y. A.; Qureshi, I. H.; Qureshi, A. A. *Pakistan J. Sci. Ind. Res.* **1970**, *13*, 244.
(2) Nielsen, J.; Nielsen, P. H.; Frisvad, J. C. *Phytochemistry* **1999**, *50*, 263.
(3) Bewley, C. A.; Faulkner, D. J. *Angew. Chem., Int. Ed.* **1998**, *37*, 2162.
(4) (a) Sheh, L.; Chang, H.-W.; Ong, C.-W.; Chen, S.-L.; Bailly, C.; Linssen, R. C. M.; Waring, M. J. *Anti-Cancer Drug Design* **1995**, *10*, 373. (b) Chen, S.-L.; Wong, R. N. S.; Lo, V.; Chang, S.; Chiang, C.-D.; Sheh, L. *Anti-Cancer Drug Design* **1998**, *13*, 501.
(5) Sheh, L.; Chen, B.-L.; Chen, C.-F. *Int. J. Peptide Protein Res.* **1990**, *35*, 55.
(6) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591.
(7) Marahiel, M. A.; Stachelhaus, T.; Mootz, H. D. *Chem. Rev.* **1997**, *97*, 2651.
(8) Kumar, S.; Punekar, N. S. *Mycol. Res.* **1997**, *101*, 403.
(9) Muramoto, K.; Kamiya, H. *Anal. Biochem.* **1990**, *189*, 223.

NP980539Z