PERGAMON

Phytochemistry 60 (2002) 869–872

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Unguisin C, a GABA-containing cyclic peptide from the fungus *Emericella unguis*

Joan Malmstrøm[a,*], Anne Ryager[a], Uffe Anthoni[b], Per Halfdan Nielsen[b]

[a]*Department of Structural Chemistry, Pharmaceuticals Development, Research and Development, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Maaloev, Denmark*

[b]*Marine Chemistry Section, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark*

Received 27 November 2001; received in revised form 15 April 2002; accepted 15 April 2002

Abstract

Besides the known unguisins A and B, a new cyclic heptapeptide, unguisin C, containing a GABA-derived moiety in the ring, was isolated from the fungus *Emericella unguis*. The structure was determined by 1D and 2D NMR techniques. Marfey's method was used to determine the absolute stereochemistry. Precursor-directed biosynthesis of the unguisins was performed by supplementation of the culture medium with amino acids (L-Ala, L-Ser, L-Phe and L-Leu). A related cyclic heptapeptide, unguisin D, was detected by HPLC and characterized by sequence analysis using LC-QITMS.

Keywords: *Emericella unguis*; Fungus; Cyclic peptides; GABA; Unguisin C; Unguisin D

1. Introduction

In previous papers, studies of the marine-derived fungus *Emericella unguis*, gave rise to isolation and structure elucidation of a novel depside, guisinol, and two new cyclic peptides, unguisin A and B (Malmstrøm, 1999; Nielsen et al., 1999). We wish to report the isolation of a new cyclic peptide, unguisin C (**1**), analogous to unguisin A (**2**) and B (**3**). Furthermore, precursor-directed biosynthesis (PDB) with exogenously added L-leucine produced the structurally related unguisin D (**4**) characterised by ion trap mass chromatography (LC-QIT-MS).

2. Results and discussion

The defatted *E. unguis* extract was initially fractionated by vacuum liquid chromatography (VLC) with increasing concentrations of MeOH in H_2O. Final C_{18} HPLC purification provided pure **1**–**3**. Unguisin C (**1**) was obtained as a white solid with a molecular formula of $C_{40}H_{54}N_8O_8$ as calculated from the peaks at m/z 775.2 $(M + H)^+$ and m/z 773.6 $(M–H)^-$ in the positive and negative ion FABMS spectra, respectively. The ^{13}C NMR spectrum (Table 1) confirmed the presence of 40 carbon atoms. The observation of seven carbonyl signals between δ 173.0 and 170.3 and seven amide NH signals between δ 8.55 and 7.69 in the ^{13}C and ^1H NMR spectra (Table 1), respectively, indicated a peptide structure. A single bond HMQC experiment confirmed the presence of six α-methine, two isopropyl methine, six methylene and five methyl carbon atoms. The remaining 14 carbon signals appeared partly superimposed in the aromatic region revealing the presence of at least two aromatic amino acids. The ^1H and ^{13}C NMR spectral data of unguisin C (**1**) were very similar to those recorded for unguisin A (**2**), which suggested a close structural relationship between the two compounds.

The amino acid residues were assigned on the basis of a COSY experiment (DMSO-d_6) as Ala (1 eq.), Val (2 eq.), Phe (1 eq.), GABA (1 eq.), Trp (1 eq.) and Ser (1 eq.). The residue sequence for 1 was determined by NOESY data. Correlations from the α-proton in phenylalanine (δ 4.33) to the amide protons of valine-2 (δ 7.94), and from the amide proton of phenylalanine (δ 8.55) to the α-proton of valine-1 (δ 3.52) permitted the establishment

* Corresponding author. Tel.: +45-4443-2119; fax: +45-4443-4590.

E-mail address: joms@novonordisk.com (J. Malmstrøm).

0031-9422/02/$ - see front matter
PII: S0031-9422(02)00150-4

of the partial sequence Val-1→ Phe→Va1-2. Similarly, the cross peaks observed between the amide proton of valine-1 (δ 8.16) and the α-proton of alanine (δ 4.28), and between the amide proton of alanine (δ 7.74) and one of the γ-protons of GABA (δ 2.03) provided evidence for a GABA→Ala→Val-1 sequence. Combined with a correlation between valine-2 (δ 4.11) and serine (δ 8.30), NOESY data supported the partial structure GABA→Ala→Val-1→Phe→Val-2→Ser, indicating the missing link of the cyclic peptide to be Ser→Trp→GABA. Albeit with some difficulty, the correlation between the amide proton (δ 7.69) of GABA and α-proton of tryptophan (δ 4.17) can be identified in the rather complex pattern of overlapping signals in the NOESY spectrum. In order to account for the 18 DBE required by the formula, the peptide must be cyclic, hence, the sequence of the seven amino acid residues is established. A sample of 1 was hydrolysed in 6 N HCl with 3% phenol (165 °C, 25 min) according to Muramoto and Kamiya (1990), and the resulting amino acids were converted to N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide derivatives for analysis by HPLC (Marfey, 1984). Comparison of the HPLC retention time with standards served to identify the configuration of the derivatives. The structure of 1 was established to be *cyclo*-(D-seryl-D-tryptophyl-GABA-D-alanyl-D-valyl-L-phenylalanyl-D-valyl).

Table 1
NMR data for unguisin C (**1**)[a]

Residue/position		^{13}C	^{1}H
Alanine	NH		7.74 (1H, *d*, *J* = 6.4 Hz)
	Cα	47.7	4.28 (1H, *quintet*, *J* = 6.8 Hz)
	Cβ	18.2	1.12 (3H, *d*, *J* = 6.8 Hz)
Serine	NH		8.30 (1H, *d*, *J* = 5.2 Hz)
	Cα	38.3	4.14[b] (1H)
	Cβ	61.4	3.67 (1H), 3.48[c](1H)
	OH		5.16 (1H)
Valine	NH		8.16 (1H, *d*, *J* = 4.0 Hz)
	Cα	60.8	3.52[c] (1H, *dd*, *J* = 4.4/8.6 Hz)
	Cβ	28.6	1.58[e] (1H)
	Cγ	18.7	0.72 (3H, *d*, *J* = 6.8 Hz)
	Cγ	18.4	0.29 (3H, *d*, *J* = 6.8 Hz)
Valine	NH		7.94 (1H, *d*, *J* = 10.0 Hz)
	Cα	58.8	4.11 (1H, *dd*, *J* = 10.0 Hz)
	Cβ	30.3	1.97[d] (1H)
	Cγ	19.6	0.71 (3H, *d*, *J* = 6.4Hz)
	Cγ	18.9	0.66 (3H, *d*, *J* = 6.8Hz)
GABA	NH		7.69 (1H, *t*, *J* = 5.8 Hz)
	Cα	38.6	3.04 (2H, *t*, *J* = 6.4 Hz)
	Cβ	26.1	1.58[e] (2H)
	Cγ	32.9	2.03[d] (1H), 1.93[d] (1H)
Tryptophan	NH		8.11 (1H, *d*, *J* = 6.4 Hz)
	Cα	55.1	4.17[b] (1H)
	Cβ	25.7	3.18 (1H, *dd*, *J* = 4.0/14 Hz), 3.13 (1H, *dd*, *J* = 9.2/14 Hz)
	N-1		10.83 (1H, *s*)
	C-2	123.8	7.20 (1H, *s*)
	C-3	110.3	
	C-3a	127.0	
	C-4	118.3	7.54 (1H, *d*, *J* = 7.6 Hz)
	C-5	121.0	7.07 (1H, *t*, *J* = 7.5 Hz)
	C-6	118.2	6.98 (1H, *t*, *J* = 7.6 Hz)
	C-7	111.4	7.34 (1H, *d*, *J* = 8.0 Hz)
	C-7a	136.2	
Phenylalanine	NH		8.55 (1H, *d*, *J* = 8.4 Hz)
	Cα	55.3	4.33 (1H, *ddd*, *J* = 11.9, 8.4, 3.6 Hz)
	Cβ	36.4	2.62 (1H, *dd*, *J* = 12/13 Hz), 3.28[f] (1H)
	C1	138.5	
	C2–6	128.0[g]	7.16–7.24 (5H, *m*)
		129.1[g]	
		126.1	

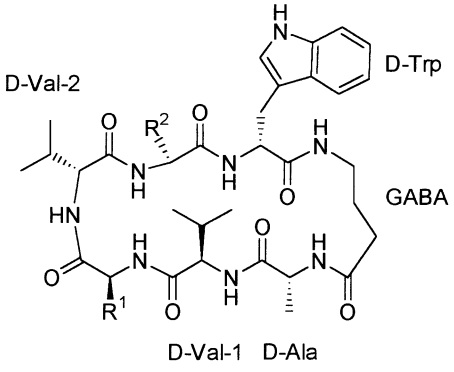
[a] In DMSO-d_6; δ-values referenced to residual DMSO at δ 2.50 (proton) and 39.6 (carbon); spectra taken at 100 and 400 MHz for carbon and proton, respectively. The seven carbonyl carbon signals appear at δ 173.0, 171.7, 171.6, 171.3, 171.2, 170.5, 170.3.

[b–d] Assignments may be reversed.

[e] Overlapping signals.

[f] Water signal interfere.

[g] Four partly overlapping signals.



Unguisin A (**2**): R^1 = Ph (L-Phe); R^2 = Me (D-Ala)
Unguisin B (**3**): R^1 = i-Pr (L-Leu); R^2 = Me (D-Ala)
Unguisin C (**1**): R^1 = Ph (L-Phe); R^2 = CH_2OH (D-Ser)

The two main components unguisin A and B differ in structure by replacement of L-phenylalanine with L-leucine. Isolation of the minor component unguisin C (**1**) revealed the possibility of substituting D-alanine by D-serine, yielding a more hydrophilic peptide. By PDB the secondary metabolite spectrum produced by *E. unguis* using supplemented YES media can be further manipulated although the amount of artificial precursor necessary to override the normal biosynthesis may be very variable (Thiericke and Rohr, 1993). Thus, the production of unguisin A and B were each stimulated considerably by addition of L-phenylalanine and L-leucine, respectively. Exogenous supply of L-alanine and L-serine left the naturally produced unguisin pattern unchanged. Moreover, on addition of L-leucine, besides unguisin B a new unguisin D was formed. The latter is derived from unguisin B by replacement of valine-2 with leucine. A characteristic feature of non-ribosomal

peptide biosynthesis is a low specificity of some modules in the multienzymes envolved (Thiericke and Rohr, 1993). Thus the presence of unguisin A–C in a normal fermentation culture, along with the synthesis of unguisin D upon feeding with L-leucine, supports the hypothesis of a non-ribosomal origin of the unguisines.

All the unguisines A–D contain the key substructure Trp-GABA-Ala-Val suggesting a high specificity of the corresponding part of the parent enzyme. GABA has previously only been found in two other naturally occurring peptides. In the cyclic antibiotics imacidin A–E from *Streptomyces olivaceus* GABA is encountered in the side chain (Laatsch, 1982) and the sequence GABA–GABA is present in some acyclic glutamatergic antagonists from *Nephilengys borbonica* venom (Nakajima et al., 1995). Thus, the unguisines are the first naturally occurring compounds incorporating GABA in a cyclic peptide structure.

3. Experimental

3.1. General

NMR spectra were recorded in DMSO-d_6 on a Varian 400 FT-NMR spectrometer operated at 400.0 MHz and 100.6 MHz for ^{1}H and ^{13}C NMR spectra, respectively. The HPLC data were obtained on a HPLC system combined with a Millenium 996 photo diode array detector from Waters. The UV spectrum was recorded on Hewlett Packard 8452A diode array spectrophotometer. The circular dichroism (CD) spectrum was measured on a JASCO J-710 spectropolarimeter. The FAB MS was recorded on a Jeol JMS-MX/HX 110 A spectrometer using a *m*-nitrobenzoic acid matrix. Marfey's reagent (N_α-(2,4-dinitro-5-fluoro-phenyl)-L-alaninamide), L-alanine and L-leucine was purchased from Sigma. L-Phenylalanine and L-serine were purchased from Fluka.

3.2. Collection, isolation and fermentation

E. unguis (M90A-2) was collected in the Paria Bay, Venezuela, in January 1997. The isolate M90A-2 originated from scrape of the shell of an unidentified mollusc. The fungus was initially grown on solid YES (yeast extract, 20 g/l; sucrose, 150 g/l) and CYA (yeast extract, 5.0 g/l; Czapek Dox Broth, 35.0 g/l) media, for a period of 14 days at 25 °C, however, the highest yield of unguisins were secured from the YES medium.

3.3. Extraction and isolation

Mycelium and agar were harvested and extracted with a mixture of EtOAc:HCl_3:MeOH (3:2:1) containing 1% HCOOH. The dried extract was defatted by partition between methanol:water (80:20) and hexane. The polar fraction (20 g) was separated by VLC. The column was packed with RP-18 (40–63 μm) and the fractionation performed with an increasing gradient of MeOH:H_2O (60:40→100:0) containing 1% HCOOH. The MeOH:H_2O (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 MeOH:H_2O (55:45) as the mobile phase to yield seven fractions. Fractions 4 and fraction 5 contained pure unguisin B (5.1 mg) and A (20.1 mg), respectively, while fraction 3 yielded unguisin C (2.2 mg).

3.4. Feeding experiments

YES solid medium was prepared with an additional supply (2 g/l) of the precursor amino acids (L-alanine, L-serine, L-phenylalanine and L-leucine). The fungus was grown on solid "amino-acid YES" medium for a period of 9 days at 25 °C. Ten plugs were secured from each Petri dish (9 cm) and extracted with a mixture of EtOAc:$CHCl_3$:MeOH (3:2:1) containing 1% HCOOH. The dried extracts were redissolved in methanol and subjected to HPLC analysis using PDA detection as described by Frisvad (1989).

3.5. LC–MS

Analytical separations were acquired on a HP 1100 liquid chromatograph equipped with a TSP UV 6000 LP DAD and a Finnigan LCQ quadrupole ion trap mass spectrometer operating in the positive and negative ion polarity mode. The extract was separated by gradient HPLC on a Nucleosil C18 column (5 μm, 4–124 mm) maintained at 35 °C. The flow rate was 0.2 ml min^{-1} using CH_3CN:H_2O (15:85→100:0 over 43 min) as mobile phase, and spectra were recorded in the interval 2–43 min. Ionisation operating conditions include: ESI source voltage 4.5 kV; capillary voltage 45 V, heated capillary temp.: 200 °C; sheat gas N_2. For mass analyzer collision induced dissociation (CID) experiments the relative collision energy was 30% and the isolation width 3.0 Dalton. Full scan mass spectrometry was performed from 150–1400 Dalton with the DAD detector scanning in the 190–550 nm range.

3.6. Unguisin C (*1*)

Colorless solid; UV λ_{max}^{EtOH}nm (log ϵ): 290 (3.58), 281 (3.65), 274 (3.63), 219 (4.49). CD (EtOH; *c* 0.018) $\Delta\varepsilon_{223}$ 4.75, $\Delta\epsilon_{205}$ −16.20. FAB–MS *m*/*z* 775.2 $[M+H]^+$, 773.6 $[M-H]^-$. NMR data, see Table 1.

Hydrolysis of unguisin C: The compound (200 μg) was treated at 165 °C for 25 min with 6 N HCl containing 3% phenol (100 μl) according to Muramoto and

Kamiya (1990). After cooling, the sample was freeze-dried and derivatized with Marfey's reagent (Marfey, 1984). The configuration of Ala, Val and Ser together with the presence of GABA were determined using a gradient of 50 mM triethylammonium phosphate (pH 3.0):CH_3CN (start: 90:10, end: 63:37) for 40 min. Retention times (min) for the standards: Val (L:23.1, D: 30.1); Ala (L: 14.4, D: 19.7); Ser (L: 9.6, D: 11.0); GABA (20.9). The configuration of Phe and Trp were determined using a gradient of 0.1% TFA:CH_3CN (start: 85:15, end: 0:100) for 43 min. Retention times (min) for the standards: Phe (L: 18.2, D: 19.7); Trp (L: 17.6, D: 18.7).

3.7. Unguisin D (*4*)

Unguisin D was produced when L-leucine was added to the fermentation medium. HPLC analysis of the extract, in addition to unguisin B (R_t 12.8 min) yielded minor amounts of unguisin D (R_t 14.5 min). ESI–MS m/z 739 $[M+H]^+$. Daughter ions on CID (m/z, fragment lost): 668 (Ala), 654 (GABA), 640 (Val), 626 (Leu), 583 (Ala + GABA), 569 (Ala + Val), 555 (Leu + Ala), 553 (Trp), 527 (Val + Leu), 513 (2Leu), 484 (GABA + Ala + Val), 482 (Ala + Trp), 468 (Trp + GABA), 456 (Ala + Val + Leu), 442 (Ala + 2Leu), 414 (Val + 2Leu), 397 (Ala + Trp + GABA), 369 (Leu + Ala + Trp), 343 (2Leu + Val + Ala), 326 (2Ala + Trp + GABA). Comparison of fragmentation pattern and intensities with the corresponding ESI–MS data from unguisin B suggests the structure *cyclo*-(tryptophyl-GABA-alanyl-valyl-leucyl-leucyl-alanyl) for unguisin D (i.e. as unguisin B except that leucine has replaced valine-2).

Acknowledgements

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

Frisvad, J.C., 1989. The use of high-performance liquid chromatography and diode array detection in fungal chemotaxonomy based on profiles of secondary metabolites. Botanical Journal of the Linnean Society 99, 81–87.

Laatsch, H., 1982. Die Struktur von Imacidin C. Liebigs Annalen der Chemie, 28–40.

Malmstrøm, J., 1999. Unguisins A and B. New cyclic peptides from the marine-derived fungus *Emericella unguis*. Journal of Natural Products 62, 787–789.

Marfey, P., 1984. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsberg Research Communications 49, 591–596.

Muramoto, K., Kamiya, H., 1990. Recovery of tryptophan in peptides and proteins by high-temperature and short-term acid hydrolysis in the presence of phenol. Analytical Biochemistry 189, 223–230.

Nakajima, T., Fujita, G., Naoki, H., Matahiro, I., 1995. Extraction of *N*-acylasparagylpolyamine derivatives from *Nephilengys borbonica* venom for treatment of cerebral diseases. Jpn. Kokai Tokkyo Koho. Japanese Patent JP 07304718 A2 19951121 Heisei. Chemical Abstracts 124, 212043.

Nielsen, J., Nielsen, P.H., Frisvad, J.C., 1999. Fungal depside, guisinol, from a marine-derived strain of *Emericella unguis*. Phytochemistry 50, 263–265.

Thiericke, R., Rohr, J., 1993. Biological variation of microbial metabolites by precursor-directed synthesis. Natural Products Reports 10, 265–289.