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Shearamide A: A New Cyclic Peptide from the Ascostromata of *Eupenicillium shearii*

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Abstract: A new antiinsectan cyclic peptide (**3**) was isolated from the ascostromata of *Eupenicillium shearii* (NRRL 3324). The structure was determined by analysis of NMR and FABMS data.

Keywords: biologically active compound; fungi; natural product; cyclic peptide

Our continuing interest in fungal sclerotia as sources of new antiinsectan metabolites[1] prompted us to investigate the chemistry of the sclerotioid ascostromata of *Eupenicillium shearii* (NRRL 3324). Ascostromata are specialized physiological structures which, like sclerotia, are adapted to withstand extreme conditions of temperature, dessiccation, and nutrient depletion.[2,3] Our initial studies of *E. shearii* ascostromata afforded three new janthitrem analogs (e.g., **1**) and several members of the paxilline class (e.g., **2**) with potent activity against the corn earworm *Helicoverpa zea*, but these compounds did not account for all of the activity of the ascostromatal extracts.[4] Further studies of these extracts have resulted in the isolation of a new cyclic octapeptide which we have called shearamide A (**3**). Details of this work are described here.

Shearamide A (**3**) was isolated by bioassay-guided fractionation of organic extracts of ascostromata produced by solid-substrate fermentation of *E. shearii*.[5] Compound **3** has the molecular formula $C_{47}H_{63}N_9O_9$ (21 unsaturations) as deduced from HRFABMS and ^{13}C NMR data. ^1H and ^{13}C NMR spectra recorded in $CDCl_3$ were characteristic of a peptide, but contained doubled signals that suggested the presence of more than one conformer of **3** in solution. When recorded in acetone-d_6, the ^1H and ^{13}C NMR spectra (Table 1) each showed only one set of signals, suggesting rapid conformational interconversion or the presence of a dominant conformer. In the ^{13}C NMR spectrum, eight amide carbonyl signals were observed between 169 and 175 ppm, accounting for all but one nitrogen and one oxygen atom of the molecular formula. Fourteen resonances were present in the aromatic region of the ^{13}C NMR spectrum between 110 ppm and 138 ppm. These signals were attributed to indolyl and phenyl groups, suggesting the presence of tryptophan (Trp) and phenylalanine (Phe) residues, and accounting for the remaining nitrogen atom. A sample of **3** was hydrolyzed in 6N HCl and the resulting amino acids were converted to N-trifluoroacetyl *n*-butyl ester derivatives,[6] which were analyzed by GCMS. Analysis of MS data and retention times for the resulting product mixture revealed the presence of Trp,

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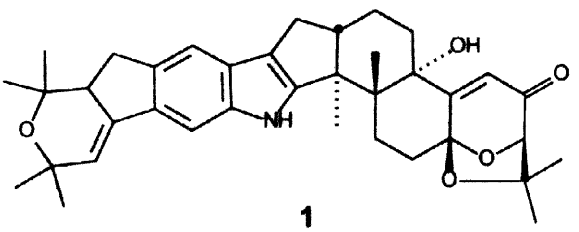

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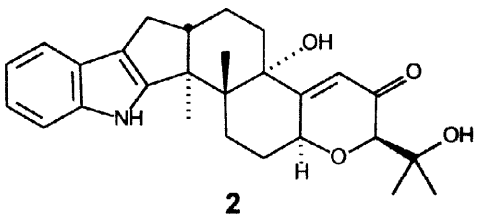

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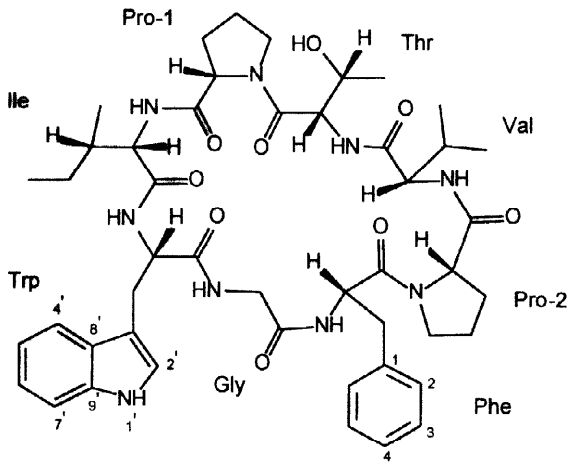

3

Phe, glycine (Gly), threonine (Thr), valine (Val), isoleucine (Ile), and proline (Pro). Chiral capillary GC analysis of the pentafluoropropyl isopropyl ester derivatives,[7] prepared in an analogous fashion, revealed that all of the chiral amino acid residues in **3** possess the L-configuration. Two Pro residues were required to satisfy the observed molecular formula, and the rings of these units accounted for two additional unsaturations. The remaining unsaturation indicated that **3** has a cyclic structure. A series of 2D NMR experiments (HMQC, HMBC, COSY, NOESY, and TOCSY) allowed unambiguous assignment of all of the ^{1}H and ^{13}C NMR signals for the amino acid moieties of **3** except for several of the amide carbonyl carbons.

Two- and three-bond HMBC correlations from the α-, β-, and amide-NH protons to their neighboring carbonyl carbons were useful in assigning the carbonyl signals and establishing the amino acid sequence of **3**. Selective INEPT experiments provided information complementary to that obtained from the HMBC experiment in some instances, and was particularly useful in cases where the ^{13}C NMR signals were minimally resolved. Three-bond correlations from the β-protons of several of the amino acids were useful in matching the carbonyl resonances to individual amino acid units, permitting unambiguous location of several of the amide carbonyls. HMBC correlations alone were sufficient to establish the presence of Gly → Phe and Pro → Ile units. Selective INEPT irradiation of the Trp α-proton at 5.04 ppm and the Gly α-proton at 3.27 ppm revealed correlations from both to the carbonyl signal at 172.88 ppm. The Ile β-proton and the Trp α-proton each correlated with a carbonyl carbon signal at 171.4 ppm. The above information permitted the establishment of the partial sequence Pro → Ile → Trp → Gly → Phe. Both the α-proton of the terminal Pro of this partial sequence and the α- and N-H protons of the Thr residue correlated to the carbonyl carbon signal at 173.5 ppm. These results extended the sequence to Thr → Pro → Ile → Trp → Gly → Phe.

Table 1. NMR Spectral Data for Shearamide A (**3**; acetone-d_6; 300 MHz)

Pos.	^{1}H	^{13}C	HMBC/Selective INEPT (^{13}C δ)
Pro-1			
C=O	---	175.6	
α-CH	4.53 (t; 8.1)	64.3	25.5, 173.5, 175.6
β-CH_2	1.92 (m), 2.24 (m)	30.3	64.3, 175.6
γ-CH_2	2.06 (m)	25.5	
δ-CH_2	3.55 (m), 3.60 (m)	48.6	25.5, 30.3, 64.3
Gly			
N-H	7.87 (br s)	---	172.88
C=O	---	169.2	
α-CH_2	4.11 (m)	43.5	169.2, 172.88
	3.27 (dd; 18, 4.8)		169.2, 172.88
Val			
N-H	6.93 (br s)	---	172.93
C=O	---	172.4	
α-CH	4.11 (m)	61.0	172.4, 172.93
β-CH	2.24 (m)	30.5	61.0, 172.4
γ-CH_3	1.01 (d; 6.8)	19.8	18.9, 30.5, 61.0
γ-CH_3	1.00 (d; 6.8)	18.9	19.8, 30.5, 61.0
Pro-2			
C=O	---	172.93	
α-CH	4.25 (m)	64.7	30.3, 172.93
β-CH_2	2.06 (m), 2.36 (m)	30.3	25.5
γ-CH_2	2.10 (m), 2.20 (m)	25.5	30.3, 64.7
δ-CH_2	3.98 (m)	48.1	25.5
	3.90 (ddd; 8.9, 8.9, 3.0)		25.5, 30.3, 64.7
Ile			
N-H	7.45 (d; 4.9)	---	61.2, 175.6
C=O	---	171.4	
α-CH	3.98 (m)	61.2	26.0, 36.2, 171.4
β-CH	1.62 (m)	36.2	15.5, 171.4
γ-CH_3	0.56 (d; 6.7)	15.5	26.0, 36.2, 61.2
γ-CH_2	1.03 (m), 1.18 (m)	26.0	11.8, 15.5, 36.2
δ-CH_3	0.67 (t; 7.4)	11.8	26.0, 36.2

Pos.	^{1}H	^{13}C	HMBC/Sel. INEPT (^{13}C δ)
Phe			
N-H	7.05 (br s)	---	169.2
C=O	---	174.0	
α-CH	5.15 (m)	52.6	137.5, 169.2, 174.0
β-CH_2	3.30 (m)	39.6	130.3, 137.5
	2.84 (m)		52.6, 130.3, 174.0
1	---	137.5	
2/6	7.25 (m)	130.3	127.5
3/5	7.30 (m)	129.3	137.5
4	7.22 (m)	127.5	130.3
Thr			
N-H	7.15 (d; 3.6)	---	172.4, 173.5
C=O	---	173.5	
α-CH	4.55 (dd; 7.8, 3.6)	55.8	69.0, 172.4, 173.5
β-CH	4.24 (m)	69.0	
γ-CH_3	1.05 (d; 6.4)	20.4	55.8, 69.0
Trp			
N-H	7.80 (d; 9.1)	---	171.4, 172.88
C=O	---	172.88	
α-CH	5.04 (t; 9.3)	53.4	172.88
β-CH_2	3.82 (d; 15)	29.1	112.5, 123.2
	3.15 (dd; 15, 12)		112.5, 123.2, 172.88
1'-NH	10.02 (s)		112.5, 128.7, 137.8
2'	7.17 (br s)	123.2	29.1, 112.5
3'	---	112.5	
4'	7.67 (d; 7.8)	119.2	112.5, 119.4, 122.1, 128.7
5'	7.01 (m)	119.4	119.2, 128.7
6'	7.08 (m)	122.1	112.0, 137.7
7'	7.34 (d; 8.1)	112.0	119.4, 128.7
8'	---	128.7	
9'	---	137.7	

HMBC correlations from the α- and β- protons of Val to the signal at 172.4 ppm, identified this as the Val carbonyl resonance. The resonance at 172.4 ppm, in turn, correlated to the Thr α-proton at 4.55 ppm, indicating that Thr must be acylated by Val. Irradiation of the Val N-H and α-protons in selective INEPT experiments resulted in polarization transfer to the carbonyl signal at 172.93 ppm, assigned by default to the carbonyl of the remaining Pro residue. The sequence was thus extended to Pro → Val → Thr → Pro → Ile → Trp → Gly → Phe, accounting for all of the amino acid units. The second Pro unit must be acylated by Phe to form a cyclic system, thereby completing the structure of shearamide A as shown in **3**. Two-bond correlations from other amide NH protons (Table 1) also supported the connectivity shown for **3**. Further evidence for the proposed sequence was obtained from LRFABMS data. The ten most abundant ions observed in the spectrum could be assigned amino acid compositions fully consistent with subunits of the proposed structure.

Shearamide A (**3**) displayed moderate activity in assays[8] against *H. zea* larvae at a dietary concentration of 100 ppm, causing a 35% reduction in growth rate (RGR) relative to controls, and exhibited similar effects on larvae of the fungivorous beetle *Carpophilus hemipterus* (38% RGR). Although shearamide A (**3**) is less potent in these assays than shearinine A (**1**) and other indole alkaloids isolated previously,[1,2] it does contribute significantly to the overall antiinsectan effects observed for the extracts of *E. shearii* ascostromata. The isolated yield of **3** was limited by the complexity of the extract, but quantitative analysis by HPLC[2] indicated that shearamide A is present in the ascostromata at a concentration of approximately 1000 ppm. Moreover, as in the case of shearinine A (**1**), paxilline (**2**), and related alkaloids,[2,4] comparative HPLC analyses of materials harvested from petri plate cultures revealed that the shearamides are concentrated primarily in the ascostromata of *E. shearii*.

To our knowledge, shearamide A is the first cyclic peptide to be isolated from a *Eupenicillium* sp. Although a considerable number of cyclic peptides are known, cyclic octapeptides are relatively rare, and shearamide A has no known close analogs. The occurrence of these antiinsectan metabolites in *E. shearii* is analogous to the presence of unique antiinsectan compounds in the sclerotia of *Aspergillus* spp.[1], and provides support for the concept that fungal ascostromata are a potentially valuable source of new bioactive metabolites.[1-3]

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5. Powdered ascostromata of *E. shearii* (320 g) were extracted by stirring successively in hexane, CH_2Cl_2, EtOAc, and MeOH. The combined crude CH_2Cl_2, EtOAc, and MeOH extracts (13.4 g) were preadsorbed onto 10 g of silica gel in a solution of 2:1 CH_2Cl_2-MeOH. This mixture was concentrated under vacuum and the resulting powder was subjected to silica gel VLC over a prepacked column bed (6.5 × 5 cm). The column was eluted using a stepwise gradient of EtOAc (0-100%) in hexane, followed by MeOH (0-50%) in CH_2Cl_2, to afford a total of thirteen (0.5 or 1 L) fractions. Fraction 9 (914 mg), which eluted with 10% to 15% MeOH, was further fractionated on a Sephadex LH-20 column (2 × 50 cm) using 3:1:1 hexane-toluene-MeOH. Subfractions of similar composition as determined by TLC were pooled and further purified by preparative C_{18} HPLC (MeOH-H_2O, 80:20) to yield compound **3** (100 mg) as a light yellow solid; mp 185-193 °C (dec.); $[\alpha]_D$ -68° (*c* 0.0015 g/mL, MeOH); IR (AgCl) 3326, 2965, 2932, 1649 (br), 1518, 1452, 1108 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRFABMS (thioglycerol) m/z 898 (100% rel. int.; M + H), 601 (5; Pro-Ile-Trp-Gly-Phe + H), 555 (2; Thr-Pro-Ile-Trp-Gly + H), 502 (4; Gly-Phe-Pro-Val-Thr + H), 454 (16; Pro-Ile-Trp-Gly + H), 391 (8; Trp-Gly-Phe + H), 357 (9; Ile-Trp-Gly + H), 312 (11; Thr-Pro-Ile + H), 298 (43; Pro-Val-Thr + H or Val-Thr-Pro + H), 244 (30; Trp-Gly + H), 211 (12; Pro-Ile + H); HRFABMS obsd. *m/z* 898.4858 $(M + H)^+$, calcd. for $C_{47}H_{64}N_9O_9$, 898.4826.
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