Keenamide A, a Bioactive Cyclic Peptide from the Marine Mollusk *Pleurobranchus forskalii*[1]

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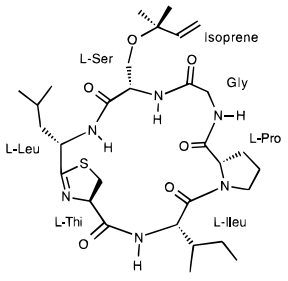
Keenamide A (**1**), a new cytotoxic cyclic hexapeptide, was isolated from the notaspidean mollusk *Pleurobranchus forskalii*. Its structure was determined by NMR spectral data interpretation and chiral amino acid analysis. Keenamide A (**1**) exhibited significant activity against the P-388, A-549, MEL-20, and HT-29 tumor cell lines, but was inactive when evaluated against the D6 and W2 *Plasmodium falciparum* malarial clones.

Marine mollusks are typically protected against predation by hard external shells. Shell-less mollusks (class Gastropoda, subclass Opisthobranchia) that lack physical protection often defend themselves through chemical secretions.[2] Only five of the nine orders of opisthobranchs (Pyramidellacea, Cephalaspidea, Sacoglossa, Acochlidioidea, Anaspidea, Thecosomata, Gymnosomata, Notaspidea, and Nudibranchia)[3] have been examined for secondary metabolites; chemical research has focused on the orders Nudibranchia, Cephalaspidea,[4,5] Anaspidea,[6,7] and Sacoglossa.[8] Few researchers have worked on the order Notaspidea, which includes the species *Umbranculum mediterraneum*[9,10] and *Pleurobranchus membranaceus*; these are the only representatives of the superfamily Pleurobranchacea to have been studied.[4] This paper reports the first chemical study of the notaspidean *Pleurobranchus forskalii*, in which we describe the structure and properties of keenamide A (**1**),[11] the first in a series of cyclic peptides, which occurs in *P. forskalii*.[12] The structures of the other peptides will be reported in a full paper.



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Six animals were collected from the reef tops using SCUBA (−30 ft) after dark, when the animals apparently came out to feed, near Manado, Indonesia. The combined extracts (EtOAc/IPA 1:1 and EtOH/H_2O 1:1) were chromatographed on a Si gel flash column, from which the peptide mixture was eluted with EtOAc/MeOH (9:1). HPLC using a C_{18} reversed-phase column and a gradient from H_2O to MeCN yielded keenamide A (**1**). It was eluted with MeCN/H_2O (4:1) as an off-white amorphous powder (10 mg, 0.004%).

HRFABMS and detailed analysis of the ^{13}C-NMR spectrum provided a molecular formula of $C_{30}H_{49}N_6O_6S$ ($M^+ + 1$, m/z 621.3448, 100%, Δ +2.5 mmu). Amino acid analysis by GC with a Chirasil-Val column revealed five amino acids: glycine, L-leucine, L-isoleucine, L-proline, and L-serine, accounting for $C_{22}H_{36}N_5O_6$.[13] The ^{1}H- and ^{13}C-NMR spectra (Table 1 and supporting information) permitted the assignment of the remaining C_5H_9 isoprene and C_3H_3NS thiazoline residues, leading to the full structural elucidation of keenamide A (**1**). The ^{1}H-NMR spectrum, when measured in $CDCl_3$, revealed four NH protons. One NH proton, a doublet of doublets at 7.75 ppm, suggested a glycine residue. The remaining three NH doublets could be assigned to serine, leucine, and isoleucine with the aid of the COSY, HMQC, and HMBC experiments. The final common amino acid assigned with the use of COSY, HMQC, and HMBC was proline. Elucidation of an isoprene group began with the olefinic methine signal (δ 5.88), the methylene (δ 5.17 and 5.12), and the methyls (δ 1.32 and 1.30), all of which gave HMBC correlations to a quaternary carbon bearing an oxygen at 76.9 ppm. An HMBC correlation between the β-protons at 3.42 ppm of serine and the quaternary carbon (δ 76.9) of the isoprene moiety indicated an ether linkage between these two groups. The ether linkage was further supported by the presence of an IR band at 1068 cm^{-1} and absence of an OH band. Additional evidence for the serine-to-isoprene connection was provided by the ROESY experiment, which showed correlations between the serine β-protons (δ 3.42), the isoprene methyl signals (δ 1.32, 1.30), and olefinic proton at 5.88 ppm.

The amino acid sequence was determined using HMBC and ROESY experiments. The NH (δ 8.20) and the α-proton (δ 4.78) signals of leucine provide HMBC correlations to the carbonyl (δ 171.0) signal of serine. The NH resonance of leucine also provides ROESY correlations to the α- (δ 4.73) and β-proton (δ 3.42) signals of serine. The NH proton signal (δ 8.58) of serine shows an HMBC correlation to the carbonyl resonance (δ 169.2), and ROESY correlation to the NH signal (δ 7.75) of glycine. The NH and α- (δ 3.41, 4.51) proton signals of glycine show HMBC correlations to the carbonyl signal (δ 171.6) of proline. The NH signal of glycine also shows a ROESY correlation to the α-proton (δ 4.12) signal of proline, which then provides an HMBC correlation to the carbonyl resonance (δ 169.7) of isoleucine. Providing further evidence for the proline−isoleucine connection is a ROESY correlation between

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Table 1. ^{1}H-[a] and ^{13}C-NMR[b] Data for Keenamide A ($CDCl_3$)

amino acid	carbon	^{13}C, ppm	mult.	^{1}H, ppm	multiplicity
glycine	1	169.2	s	(NH)7.75	dd, J = 2.6, 9.5
	2	42.9	t	4.51, 3.41	dd, J = 10.0, 17.4, m
proline	1	171.6	s		
	2	62.5	d	4.12	dd, J = 9.7, 6.6
	3	28.8	t	2.17, 2.00	p, J = 6.7, m
	4	25.7	t	2.12, 1.84	m, m
	5	47.5	t	3.67, 3.56	m, m
isoleucine	1	169.7	s	(NH)7.63	d, J = 8.7
	2	55.2	d	4.63	dd, J = 9.0, 3.4
	3	37.2	d	1.87	m
	4	16.1	q	1.00	d, J = 6.8
	5	22.8	t	1.29	m
	6	11.5	q	0.83	t, J = 7.4
thiazoline	1	171.1	s		
	2	78.0	d	5.02	t, J = 9.6
	3	36.0	t	3.60, 3.65	m, m
leucine	1	175.8	s	(NH)8.20	d, J = 8.7
	2	50.5	d	4.78	ddd, J = 9.6, 2.9, 11.1
	3	42.5	t	1.60, 1.51	ddd, J = 14.2, 9.2, 3.7, ddd, J = 14.0, 11.1, 4.7
	4	25.2	d	1.77	m
	5	23.1	q	0.95	d, J = 6.6
	6	21.3	q	0.91	d, J = 6.6
serine	1	171.0	s	(NH)8.58	d, J = 8.7
	2	50.8	d	4.73	q, J = 5.5
	3	62.3	t	3.42	m
isoprene	1	76.9	s		
	2	26.2	q	1.30	s
	3	25.1	q	1.32	s
	4	142.5	d	5.88	dd, J = 17.6, 10.8
	5	114.9	t	5.17, 5.12	dd, J = 17.6, 0.8, dd, J = 10.8, 0.8

[a] At 500 MHz, $CDCl_3$ signal at 7.26 ppm. [b] At 125 MHz, $CDCl_3$ signal at 77.0 ppm.

the δ-proton signals of proline (δ 3.56, 3.67) and the α-proton signal of isoleucine (δ 4.63). The NH (δ 7.63) and the α-proton signals of isoleucine provide HMBC correlations to the carbonyl carbon (δ 171.1), and ROESY correlations to α- and β-protons of the thiazoline containing-residue.

The determination of the thiazoline residue was accomplished by analysis of the HMBC, HMQC, COSY, and HRMS data. The carbon signal at 175.8 ppm could be assigned as part of the leucine residue using HMBC correlations from the α- (δ 4.78) and β-protons (δ 1.60, 1.51) of leucine. The remaining two carbon signals, a methine at δ 78.0 and a methylene at δ 36.0, could be connected by COSY using the methine proton resonance at δ 5.02 and methylene signals at 3.65 and 3.60 ppm. The protons absorbing at δ 5.02, 3.65, and 3.60 show a strong correlation in the HMBC experiment to the leucine C-1 (δ 175.8), which is incorporated into the thiazoline ring. The ability of the thiazoline nitrogen to act as a Schiff base explains the partial epimerization of the leucine residue observed during hydrolysis.[14,15] The absence of L-cysteine and the excess of L-serine obtained after hydrolysis is also best explained by the thiazoline nitrogen acting as a Schiff base. A lone pair of electrons on the sulfur could participate in delocalizing the positive charge obtained on the nitrogen under acidic conditions. The addition of H_2O would then yield serine and a thioamide as an intermediate. Hydrolysis of the thioamide would then provide the observed L-leucine and L-serine residues.

Keenamide A shows activity against several tumor cell lines; IC_{50} values against P-388, A-549, and MEL-20 are 2.5 μg/mL and against HT-29, 5.0 μg/mL. Keenamide A was also examined for antimalarial activity against D6 and W2 clones of *Plasmodium falciparum* and exhibited no significant activity.

Experimental Section

Collection and Extraction. Six mollusks (*P. forskalii*) weighing a total of 280 g (wet wt) were collected during the night from Manado, Indonesia, during October 1994, and extracted two times with EtOAc/IPA (1:1) and two times with EtOH/H_2O (1:1). The combined extracts were then chromatographed using Si gel flash chromatography and a block gradient increasing with 10−20% increments (hexane, EtOAc, MeOH, and H_2O). Keenamide A was eluted with EtOAc/MeOH (9:1). HPLC using a C_{18} column (5 micron Phenomenex Ultracarb 10 × 250 mm) and a gradient from H_2O−MeCN yielded keenamide A with H_2O/MeCN (1:4).

Keenamide A (1): physical data, $[\alpha]_D$ +24° (*c* 0.29, MeOH); ^{1}H NMR (Table 1, 500 MHz, $CDCl_3$); ^{13}C NMR (Table 1, 125 MHz, $CDCl_3$); IR neat (NaCl), 3315 (m, br), 2962 (m, br), 1674 (s), 1629 (s), 1529 (s), 1446 (m) cm^{-1}; HRFABMS *m/z* (fragment) 621.3448 (M^+ + 1, 100), calcd for $C_{30}H_{49}N_6O_6S$, 621.3423, Δ −2.5 mmu); UV ($CHCl_3$), λ_{max} 241 (2,673) nm.

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Supporting Information Available: 125 MHz ^{13}C NMR and 500 MHz ^{1}H NMR, COSY, HMQC, HMBC, and ROESY spectra for **1** in $CDCl_3$ and amino acid analysis by GC−MS on a Chirasil-Val column (8 pages). Ordering information is given on any current masthead page.

References and Notes

(1) Invited paper presented at the Young Investigators Symposium at the 36th Annual Meeting of the American Society of Pharmacognosy, Oxford, MS, July 25, 1995.
(2) Faulkner, D. J. in *Ecological Roles of Marine Natural Products*; Paul, V. J., Ed.; Comstock Publishing, Cornell University Press: Ithaca, NY, 1992; pp 119−163.
(3) Ciavatta, M. L.; Trivellone, E.; Villani, G.; Cimino, G. *Tetrahedron Lett.* **1993**, *34*, 6791−6794.
(4) Spinella, A.; Alvarez, L. A.; Passegio, A.; Cimino, G. *Tetrahedron* **1993**, *49*, 1307−1314.
(5) Yamazaki, M.; Kimura, K.; Kisugi, J.; Kamiya, H. *FEBS Lett.* **1986**, *198*, 25−28.
(6) Iijima, R.; Kisugi, J.; Yamazaki, M. *Dev. Comp. Immunol.* **1995**, *19*, 13−19.
(7) Vardaro, R. R.; Marzo, V. D.; Cimino, G. *Tetrahedron Lett.* **1992**, *33*, 2875−2878.
(8) Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825−5826.
(9) Cimino, G.; Spinella, A.; Scopa, A.; Sodano, G. *Tetrahedron Lett.* **1989**, *30*, 1147−1148.
(10) De Medeiros, E. F.; Herbert, J. M.; Taylor, R. J. K. *J. Chem. Soc., Perkin Trans. 1* **1991**, 2725−2730.
(11) Named to recognize the skill and devotion of the first author who isolated the compound and assigned the NMR data while working as an undergraduate research assistant.
(12) The sample was identified as *Pleurobranchus forskalii*, family Pleurobranchaeidae, by Dr. Marc Slattery, Department of Pharmacognosy, The University of Mississippi; a voucher specimen has deposited at the Department of Pharmacognosy, University, Mississippi (94IND214).
(13) Hydrolysis of 0.5 mg of peptide was accomplished with 6 N HCl for 22 h. The free amino acids were then derivatized as the isopropyl esters and pentafluoropropy amide prior to GC analysis.
(14) Carroll, A. R.; Bowden, B. F.; Coll, J. C.; Hockless, D. C. R.; Skelton, B. W.; White, A. H. *Aust. J. Chem.* **1994**, *47*, 61−69.
(15) McDonald, L. A.; Foster, M. P.; Phillips, D. R.; Ireland, C. M.; Lee, A. Y.; Clardy, J. *J. Org. Chem.* **1992**, *57*, 4616−4624.

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