Eusynstyelamide, a Highly Modified Dimer Peptide from the Ascidian Eusynstyela misakiensis

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EUSYNSTYELAMIDE, A HIGHLY MODIFIED DIMER PEPTIDE FROM THE ASCIDIAN *EUSYNSTYELA MISAKIENSIS*

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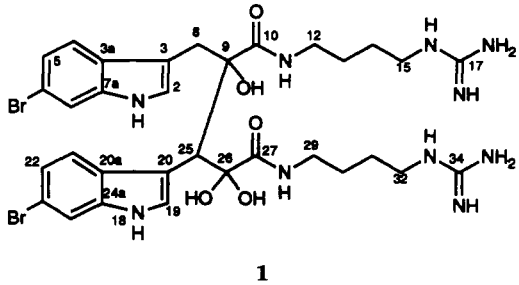
ABSTRACT.—Eusynstyelamide, a novel peptide derivative from the ascidian *Eusynstyela misakiensis*, was isolated and characterized by spectroscopic methods.

The majority of natural products isolated to date from ascidians are nitrogenous (1), exhibiting various degrees of structural modification of their amino acid precursors (2). During routine chemical screening of ascidians collected in the Philippines, we detected an extremely polar constituent from the pink colonial styelid, *Eusynstyela misakiensis* (order Stolidobranchia, family Styelidae) (3). Extracts of this organism were found to be chemically rich, but displayed only weak biological activity, with IC_{50} values of 100 μg/ml for in vitro cytotoxicity against the human colon tumor cell line HCT-116 (4). We report here the isolation and structure elucidation of the major component of an aqueous extract, eusynstyelamide [**1**], a modified tryptophan-arginine dipeptide dimer.

Inspection of the ^{1}H- and ^{13}C-nmr spectra of **1** revealed the presence of a pair of 3,6-disubstituted indole units as well as ^{13}C-nmr signals indicative of two amide functionalities and two guanidino groups. However, there were no signals typical of α-protons or carbons present. Most of the signals in the ^{13}C-nmr spectrum were paired (Table 1). The possibility that the material was an intractable mixture of two closely related congeners was ruled out by HMBC nmr correlations between the two dimer halves of **1**, particularly in the regions of the ^{13}C-nmr spectrum where unpaired signals appeared. The fabms of **1** exhibited a two-bromine cluster at *m/z* 787/789/791 and a one-bromine cluster at *m/z* 394/396 (cleavage of the dimer). Hrfabms of the *m/z* 787 ion, which was ultimately shown to be $MH^+ - H_2O$, suggested a molecular formula of $C_{32}H_{41}{}^{79}Br_2N_{10}O_4$.

The nmr spectra (see Table 1) of **1** displayed the best dispersion in MeOH-d_4. Spectra were also recorded in DMSO-d_6 to allow observation and assignment of crucial ^{1}H-^{1}H and ^{1}H-^{13}C connectivities involving exchangeable protons. Relay-COSY 45 and DQ-COSY experiments established four proton spin-systems. Eight carbon-bound proton signals between δ 6.6 and 7.7, and two exchangeable signals at δ 11.2 and 11.4 observed in DMSO-d_6, were assigned to the 3,6-disubstituted indole units. The two remaining proton spin systems were also



1

TABLE 1. ^1H- and ^{13}C-Nmr Assignments of Eusynstyelamide [**1**].

Position	DMSO-d_6		CD_3OD	
	^{13}C δ (mult.)[a,b]	^1H δ (mult., *J*)[c]	^{13}C δ (mult.)[b,d]	^1H δ (mult., *J*)[e]
1		NH-11.2 (br s)		
2	126.0 (d)	6.83 (br s)	127.1 (s)	6.92 (s)
3	108.3 (s)		110.5 (s)	
3a.....	126.9 (s)		128.3 (s)	
4	120.7 (d)	7.0 (d, 8.5)	121.4 (d)	6.85 (d, 8.5)
5	120.8 (d)	6.75 (dd, 8.2, 1.7)	122.8 (d)	6.67 (dd, 8.5, 2.7)
6	113.4 (s)		115.68 (s)	
7	113.6 (d)	7.47 (d, 2.1)	114.82 (d)	7.43 (d, 2.0)
7a.....	136.7 (s)		138.5 (s)	
8	28.5 (t)	2.65, 3.20 (d, 14)	29.3 (t)	2.96, 3.44 (d, 15)
9	78.9 (s)	OH not observed	81.5 (s)	
10	174.1 (s)		177.2 (s)	
11		NH not observed		
12	38.1 (t)	3.15, 3.12 (m)	39.5 (t)	3.26, 3.27 (d, 6.5)
13*	26.0 (t)	1.38 (m)	27.2 (t)	1.51 (dd, 6.5, 2)
14*	25.12 (t)	1.38 (m)	26.41 (t)	1.50 (m)
15	40.4 (t)	3.05 (m)	42.04 (t)	3.15, 3.19 (m)
16		NH 7.89 (br s)		
17	157.0 (s)		158.6 (s)	
18		NH 11.4 (br s)		
19	125.6 (d)	7.4 (br s)	126.8 (d)	7.61 (s)
20	105.4 (s)		107.0 (s)	
20a.....	127.9 (s)		129.2 (s)	
21	121.3 (d)	7.3 (d, 9)	122.4 (d)	7.22 (d, 9)
22	120.8 (d)	6.94 (dd, 8, 1.5)	122.9 (d)	7.02 (dd, 9, 1.75)
23	113.4 (s)		115.85 (s)	
24	113.6 (s)	7.56 (d, 1.5)	114.86 (d)	7.57 (d, 1.75)
24a.....	135.9 (s)		137.8 (s)	
25	47.4 (d)	3.59 (s)	49.85 (d)	3.58 (s)
26	91.9 (s)	OH 7.14 (br s)	93.7 (s)	
27	171.6 (s)		173.5 (s)	
28		NH 8.17 (dd, 2, 2)		
29	38.4 (t)	2.80, 2.95 (m)	39.7 (t)	2.8, 3.1 (m)
30	25.34 (t)	0.93, 1.10 (m)	26.84 (t)	1.09, 1.12 (m)
31	25.32 (t)	0.93, 1.11 (m)	26.46 (t)	0.95, 0.97 (m)
32	40.2 (t)	2.94 (m)	41.95 (t)	2.89, 2.91 (t, 7.5)
33		NH 7.80 (br s)		
34	156.9 (s)		158.5 (s)	

[a]Measured at 125 MHz; referenced to DMSO (δ 39.5).
[b]Multiplicity determined by a DEPT experiment.
[c]Measured at 500 MHz; referenced to residual DMSO (δ 2.49).
[d]Measured at 125 MHz; referenced to CD_3OD (δ 49.0).
[e]Measured at 500 MHz; referenced to residual CHD_2OD (δ 3.30).
*These assignments may be interchanged.

essentially identical, each consisting of four contiguous methylene groups coupled to one exchangeable proton at each end, consistent with a decarboxylated arginine. In one case, a broad amide NH signal at δ 8.17 (t, 2 Hz) coupled to a pair of geminal methylene signals at δ 2.80 and 2.92, while a broad guanidinium NH signal at δ 7.80 (br s) coupled to a multiplex of methylene signals between δ 3.02–3.14. Both methylene groups adjacent to exchangeable protons further coupled with other complex methylene signals which were readily assignable in

MeOH-d_4 (Table 1). The remaining proton signals observed were a methine singlet at δ 3.6, a pair of isolated diastereotopic methylene signals at δ 3.44 and 2.96 (d, 15 Hz), and, in DMSO-d_6, a broad exchangeable singlet at δ 7.14, and groups of broad exchangeable signals from δ 7.62–7.30 and 7.28–6.96. A positive Sakaguchi test and the relative width of the carbon signals at δ 158.4 and 158.5 supported the assignment of C-17 and C-34 as aliphatic guanidine carbons.

Specific carbon assignments for eusynstyelamide [**1**] were determined using DEPT and reverse-detected ^1H-^{13}C correlation and long-range nmr spectroscopy (HMQC and HMBC). All of the anticipated connectivities were observed for the two indole systems. In particular, coupling from the quaternary carbon at δ 110.5 (C-3) to H-4 and from δ 107.0 (C-20) to H-21 confirmed the proposed 6-substituted nature of each indole unit. The chemical shifts of C-6 (δ 113.4) and C-23 (δ 113.4) were consistent with them bearing bromine (5). Further correlations from C-3a, C-3, and C-2 to the geminal methylenes at δ 3.44 and 2.96 borne by the carbon at δ 29.3 allowed assignment of C-8. Similar connectivities from C-20a, C-20, and C-19 to the methine singlet at δ 3.6 borne by the carbon at δ 49.8 allowed assignment of C-25. Additional correlations derived from a series of HMBC nmr experiments at 7.5, 10, and 13 Hz allowed unequivocal assignment of the only unsymmetric portion of **1**. Proton H-8 showed correlations from δ 81.5, 49.8, and 177.2 assigned to C-9, C-25, and C-10, respectively. The carbon at δ 49.8 was previously assigned at C-25, attached to the 3-position of the other indole. Therefore, the quaternary oxygen bearing carbon at δ 81.5 must be C-9 and the connection between the two halves of the molecule was represented by a bond between C-9 and C-25. Proton H-25 showed correlations from C-8, C-9, and carbons at δ 93.7 and 173.4.

The assignment of C-26 as an α-keto amide hydrate stems from its carbon chemical shift and comparison of spectral data with cyclotheonamide A (6). In MeOH-d_4, the α-keto group of cyclotheonamide A is reported to exist as the sp^3 ketone hydrate in which the amide carbon has a chemical shift of δ 174.7 and the α carbon δ 97.4. However, in DMSO-d_6, cyclotheonamide A dehydrates spontaneously to the α-keto amide, in which the amide carbon shifts to δ 160.9, and the keto group resonates at δ 195.9. Eusynstyelamide [**1**] does not dehydrate in aprotic solvents but apparently does in the mass spectrometer as the highest mass ion observed corresponded to $MH^+ - H_2O$.

Eusynstyelamide [**1**] was nontoxic towards the human colon tumor cell line HCT-116 at concentrations up to 100 μg/ml. Finally, eusynstyelamide [**1**] was found to be chiral, with an ord absorbance, $[\alpha]_{405} + 125.6°$ ($c = 0.226$, MeOH), indicating it is not formed via acid or base catalyzed dimerization during storage or isolation, but rather via a stereospecific process mediated by the ascidian.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Reagent and spectrophotometric grade solvents were all freshly distilled before use. Chemical reagents were used directly from the supplier. Nmr spectra were recorded on IBM AF 200 or Varian Unity 500 spectrometers, operating at 200 MHz and 500 MHz, respectively, for ^1H. Chemical shifts are reported on the δ-scale and are referenced to residual protonated solvent. Uv spectra were recorded on a Beckman DU-8 spectrophotometer in 10 mm cells. Ord spectra were measured in a 50-mm cell with a Jasco DIP-370 polarimeter. Ir spectra were recorded on a Perkin-Elmer 1600 Fourier transform spectrophotometer calibrated with a polystyrene reference at 1601 cm^{-1}, as neat films deposited on NaCl plates. Lr- and hrfabms data were obtained on either a Varian MAT-731 mass spectrometer equipped with an Ion Tech atom gun or a Finnigan MAT 95 instrument.

ANIMAL MATERIAL.—*Eusynstyela misakiensis* was collected from −10 to −20 m by scuba at the northern and northeastern reefs of Siquijor Island, Philippines (April 1991), and frozen until used. A voucher specimen (NCI 759) has been deposited in the National Museum of the Philippines.

EXTRACTION AND ISOLATION.—Frozen animals (56 g dry wt after extraction) were homog-

enized in a blender, extracted four times with MeOH, filtered, and the solvent reduced. Combined aqueous MeOH extracts were extracted successively with hexane, CCl_4, $CHCl_3$, and EtOAc. The aqueous partition was brought to dryness *in vacuo*, and triturated three times with cold MeOH, yielding upon reduction 1.3 g of a tan foam. Flash cc of this material on Bakerbond C18 bonded phase medium with 50% aqueous MeOH, followed by MeOH, yielded 135 mg of a green glassy solid. Repeated C18 flash cc of this material with a stepwise gradient of 40% aqueous MeOH to 100% MeOH gave 89.5 mg of crude **1**. Final purification was achieved on an LH-20 column eluted with 0.2% triethylamine in MeOH to yield eusynstyelamide [**1**] as a pale yellow transparent oil (50.8 mg, 0.091%).

Eusynstyelamide [**1**].—$C_{32}H_{42}Br_2N_{10}O_5$; uv (MeOH) λ max 206 (ε 50900), 224 (69400), 285 (12400) nm; ir (neat, NaCl stage) ν max 3500–3000, 2946, 1694, 1682, 1674, 1668, 1660, 1652, 1645, 1634, 1622, 1615, 1574, 1558, 1538, 1456, 1417, 1337, 1173, 1109, 1048, 1024, 1004, 896 cm^{-1}; ^1H and ^{13}C nmr, see Table 1; fabms (3-nitrobenzyl alcohol matrix) *m/z* 787, 789, 791 (MH^+-H_2O, 22.2, 38.8, 20.1), 629, 631, 633 [$(MH^+-H_2O)-C_6H_{13}N_4O$, 19.5, 39.3, 19.5], 394, 396 [$(MH^+-H_2O)/2+H$, 66.7, 65.3]; fabms (glycerol matrix) *m/z* 787, 789, 791 (MH^+-H_2O, 1.5, 3, 1.5), 709, 711 [$(MH^+-H_2O)-Br+H$, 1.1, 1.1], 629, 631, 633 [$(MH^+-H_2O)-C_6H_{13}N_4O$, 1, 2, 1], 551, 553 [$(MH^+-H_2O)-C_6H_{13}N_4O-Br+H$, 1, 1], 394, 396 [$(MH^+-H_2O)/2+H$, 5, 5]; hrfabms found *m/z* 787.1661 (MH^+-H_2O), $C_{32}H_{41}{}^{79}Br_2N_{10}O_4$ requires 787.1679 (Δ 1.8 mmu), *m/z* 394.0846 [$(M^+-H_2O)/2+H$], $C_{16}H_{21}{}^{79}Br_1N_5O_2$ requires 394.0875, *m/z* 629.0531 [$(M^+-H_2O)-C_6H_{13}N_4O$], $C_{26}H_{27}{}^{79}Br_2N_6O_3$ requires 629.0507; $[\alpha]_{405}$ +125.6° (50 mm, *c*=0.226, MeOH).

CYTOTOXICITY TESTING.—Cytotoxicity was assessed in HCT-116 human colon carcinoma cells by XTT [2,3-*bis* (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide] assay. Cells were plated at 4000 cells/well in 96-well microtiter plates and 24 h later drugs were added and serial diluted. The cells were incubated at 37° for 72 h at which time the tetrazolium dye, XTT, was added. A dehydrogenase enzyme in live cells reduces the XTT to a form that absorbs light at 450 nm which can be quantitated spectrophotometrically. The greater the absorbance, the greater the number of live cells. The results were expressed as an IC_{50} which is the drug concentration required to inhibit cell proliferation (i.e., absorbance at 450 nm) to 50% of that of untreated control cells.

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