Neamphamide A, a New HIV-Inhibitory Depsipeptide from the Papua New Guinea Marine Sponge *Neamphius huxleyi*†

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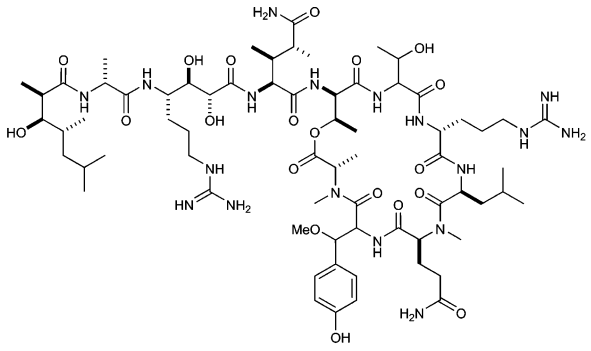
A new HIV-inhibitory cyclic depsipeptide, neamphamide A (**2**), was isolated from a Papua New Guinea collection of the marine sponge *Neamphius huxleyi*. Its structure was established through interpretation of spectroscopic data and by acid hydrolysis, derivatization of the free amino acids, and LC-MS analysis of the derivatives. Neamphamide A (**2**) contains 11 amino acid residues and an amide-linked 3-hydroxy-2,4,6-trimethylheptanoic acid moiety. The amino acid constituents were identified as L-Leu, L-*N*MeGln, D-Arg, D- and L-Asn, two residues of D-*allo*-Thr, L-homoproline, (3*S*,4*R*)-3,4-dimethyl-L-glutamine, *β*-methoxytyrosine, and 4-amino-7-guanidino-2,3-dihydroxyheptanoic acid. In a cell-based XTT assay, **2** exhibited potent cytoprotective activity against HIV-1 infection with an EC_{50} of approximately 28 nM.

Marine organisms are recognized as a rich source of compounds that can inhibit HIV infection or modulate specific molecular targets associated with viral entry or replication.[1,2] Within this group of antiviral marine metabolites is a family of cyclic sponge peptides with potent anti-HIV properties, and the first of these to be identified was the depsipeptide callipeltin A (**1**). Callipeltin A, along with other inactive callipeltin analogues, was initially isolated from the New Caledonian sponge *Callipelta* sp.[3] and later found in a *Latrunculia* sp. collected in Vanuatu.[4] Papuamides A−D were obtained from Papua New Guinea *Theonella mirabilis* and *T. swinhoei* samples,[5] while the Indonesian sponge *Sidonops microspinosa* provided microspinosamide.[6] These peptides are comprised of a number of uncommon amino acid residues, and callipeltin A (**1**) and the papuamides also contain unique N-terminal aliphatic hydroxy acid moieties. All of these sponge peptides inhibit HIV at nM concentrations. The potent anti-HIV activity and novel structural features of callipeltin A (**1**) prompted a number of synthetic efforts to prepare the unusual amino acids[7,8] and the hydroxy acid substituent.[9] As part of the NCI's HIV-inhibitory natural product lead discovery program,[10,11] both the aqueous and organic extracts of a Papua New Guinea collection of the sponge *Neamphius huxleyi* were tested and showed potent activity in the primary in vitro anti-HIV screen. There was only one prior chemical study of *N. huxleyi* in the literature, and it described a cytotoxic sulfur-containing aromatic heterocyclic compound.[12] Anti-HIV bioassay-guided fractionation of the organic extract of *N. huxleyi* resulted in the isolation of a new depsiundecapeptide, designated neamphamide A (**2**), as the principal active constituent.



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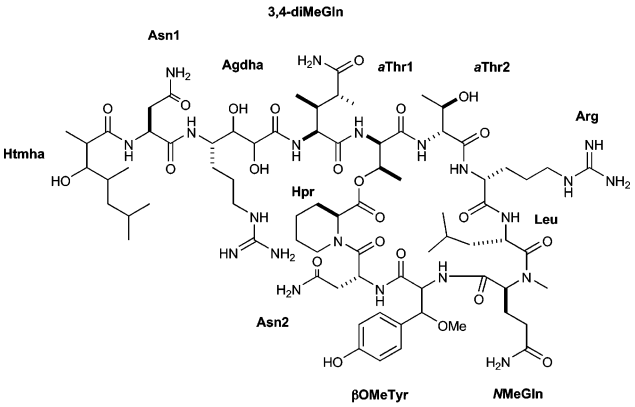

2

The frozen sponge specimens were ground with dry ice, and the resulting powder was extracted with H_2O. After lyophilization, the residue was successively extracted with MeOH−dichloromethane (1:1) and 100% MeOH. The two organic solvent extracts were combined and separated into four fractions by solvent−solvent partitioning. Gel-permeation of the H_2O-soluble material on Sephadex LH-20

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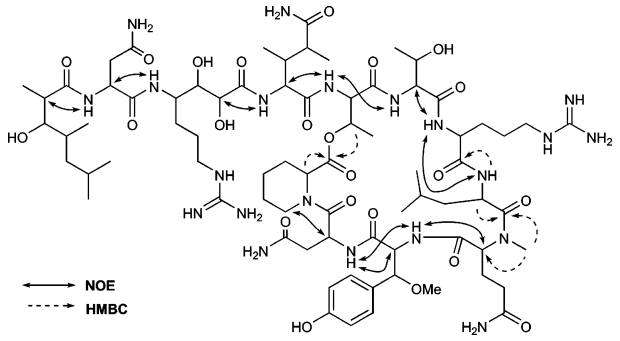

Figure 1. Selected NOE and HMBC correlations for **2**.

followed by HPLC using a reversed-phase polymer support furnished 17 mg of **2**. The nominal molecular weight of neamphamide A (**2**) was established as 1687.9 by low-resolution FAB and ESIMS measurements. A molecular formula of $C_{75}H_{125}N_{21}O_{23}$ for **2** was deduced from a divalent molecular ion $[M + 2H]^{2+}$ observed at *m*/*z* 844.9694 by high-resolution electrospray ionization TOF MS, which corresponds to a molecular weight of 1687.9242 (Δ −0.9 ppm from calcd value, 1687.9257). The facile formation of a doubly charged molecular ion was consistent with the presence of two basic guanidine functionalities in **2**. Its peptidic nature was evident from an abundance of amide NH protons (δ 6.52−9.24) in the ^{1}H and carbonyl carbons (δ 170.66−180.04) in the ^{13}C NMR spectra obtained in CD_3OH. The presence of 2 equiv each of Asn and Thr and 1 equiv each of Arg and Leu were revealed by standard amino acid analysis of the acid hydrolysate of **2**. Extensive interpretation of 2D NMR data obtained by gradient-enhanced versions of COSY45, TOCSY, HSQC, HMBC, NOESY, and ROESY experiments (Table 1) established five unusual amino acid moieties; 4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (Agdha), β-methoxytyrosine (βOMeTyr), 3,4-dimethylglutamine (3,4-diMeGln), *N*-methylglutamine (*N*MeGln), and homoproline (Hpr). Of the 11 amino acid residues identified in **2**, eight were also components of callipeltin A (Arg, Leu, Thr (2×), *N*MeGln, βOMeTyr, 3,4-diMeGln, and Agdha).[3] NMR data also established that the N-terminal acyl group of callipeltin A (**1**), 3-hydroxy-2,4,6-trimethylheptanoic acid (Htmha), was present in **2**.

The amino acid sequence of **2** and placement of the acyl substituent were assigned from a combination of inter-residue NOE interactions and HMBC correlations (Figure 1). The alignment of eight amino acid residues from Agdha to βOMeTyr in **2** was the same as that of callipeltin A (**1**). However, ROESY cross-peaks between the Aghda-4-N*H* (δ 7.64) and the Asn1-H2 (δ 4.68) protons established an amide linkage between these residues, and acylation of Asn1 by the hydroxy acid moiety was evidenced by a NOESY correlation between Asn1-N*H* (δ 8.29) and Htmha-H2 (δ 2.58). Thus, the N-terminus of **2** consisted of Htmha amide linked to Asn1, which in turn was joined to Agdha via a peptide bond. At the C-terminal end of **2**, a ROESY correlation between Asn2-N*H* (δ 8.26) and βOMeTyr-H2 (δ 4.67) and NOESY correlations between the βOMeTyr-N*H* (δ 6.52) and the Asn2-N*H* (δ 8.26) protons and between Asn2-H2 (δ 5.39) and Hpr-H6 (δ 3.69) defined the sequence as βOMeTyr-Asn2-Hpr. Macrocyclic ring formation due to an ester linkage between Hpr and Thr1 was confirmed by HMBC correlations from δ 5.22 (Hpr-H2) to δ 170.93 (Hpr-C1) and from δ 5.73 (Thr1-H3) to δ 170.93 (Hpr-C1), which completed the planar structure of **2**.

The absolute configurations of D-Arg, L-Leu, L-Hpr, and two D-*allo*-Thr (*a*Thr) residues were determined by chromatographic comparison of the acid hydrolysate of **2** (6 N HCl, 110 °C, 6 h) with appropriate amino acid standards by LC-MS after derivatizing with Marfey's reagent (*N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide; L-FDAA).[13] The presence of two D-*a*Thr residues in **2** was established since only a single peak corresponding to D-*a*Thr was observed by ion-selective monitoring for L-FDAA-Thr (*m*/*z* 372). In contrast, both the L- and D-Asp enantiomers were detected in a similar ion-selective analysis for L-FDAA-Asp. To define the chirality of each Asn residue, **2** was cleaved by $NaIO_4$ and the periodate cleavage products were separated by HPLC to provide acylasparginylargininal.[3] This fragment was then oxidized with H_2O_2, hydrolyzed, and subjected to Marfey's analysis as above, which revealed the presence of L-Asp and L-Arg. This allowed the assignment of L-Asn1 and D-Asn2, together with 4*S*-stereochemistry of the Agdha moiety. The L-configuration of *N*MeGln was unambiguously established by acid hydrolysis of **2**, derivatization with GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate),[14] and LC-MS analysis in which the retention time of the *N*MeGlu derivative from **2** matched that for the L-isomer. To establish the absolute configuration of 3,4-diMeGln, an authentic sample of callipeltin A (**1**) was hydrolyzed and derivatized to provide both L-FDAA and GITC standards of (3*S*,4*R*)-3,4-diMe-L-Glu. The L-FDAA and GITC derivatives of 3,4-diMeGlu from neamphamide A (**2**) both coeluted during LC-MS analyses with the equivalent standards derived from callipeltin A, which supported the presence of a (3*S*,4*R*)-3,4-diMe-L-Glu residue in **2**. Assignment of the remaining stereocenters in **2** was hampered by decomposition of the constituents during acid hydrolysis and lack of appropriate stereochemically defined standards.

Neamphamide A (**2**) shares a high degree of structural homology with the callipeltins and less so with the papuamides. The most significant difference between **2** and callipeltin A (**1**) is an expansion of the macrocyclic ring due to the incorporation of a Hpr residue at the C-terminus and the presence of two Asn residues in **2**. Neamphamide A (**2**), the callipeltins, and the papuamides all contain the unusual 3,4-diMeGln and βOMeTyr residues. The papuamides also have a lactonized C-terminal Hpr residue, and to the best of our knowledge the only other marine natural products reported to contain Hpr are the petrosifungins[15] and the thioether derivative pulcherrimine.[16] The anti-HIV activity of neamphamide A (**2**) was evaluated in an XTT-based cell viability assay using the human T-cell line CEM-SS infected with HIV-1_{RF}.[17] After a 6 day incubation period, compound **2** effectively inhibited the cytopathic effect of HIV-1 infection with an EC_{50} of 28 nM. Direct cytotoxicity of **2** against the host cells was observed with a TC_{50} of 260 nM.

Experimental Section

General Experimental Procedures. The optical rotation was measured with a Perkin-Elmer 241 polarimeter, and the UV spectrum was recorded on a Beckman DU 640 spectrophotometer. NMR spectra were obtained with a Varian INOVA NMR spectrometer at 500 MHz for ^{1}H and 125 Hz for ^{13}C using residual solvent peaks at δ_H 3.30 and at δ_C 49.00 ppm as chemical shift reference signals. High-resolution electrospray ionization TOF mass data were acquired on a Waters QTOF Ultima US mass spectrometer. LC-MS analyses were performed on a Hewlett-Packard Series 1100 MSD integrated LC-MS system employing a positive ion ESI mode.

Animal Material. The sponge specimens were collected in November 1993 from a depth of −10 m in the Duke of York Islands, Papua New Guinea, and frozen immediately after

Table 1. ^{1}H and ^{13}C NMR Data for Neamphamide A (**2**) in CD_3OH[a]

residue	position	δ_C	δ_H *J* (Hz)	HMBC (^{1}H to ^{13}C)	NOE[b]
Htmha					
	1	178.85			
	2	44.83	2.58	1, 2-CH_3	5, Asn1-N*H*
	2-CH_3	15.36	1.09, 3H (ovl)	1, 2, 3	
	3	79.17	3.47	1, 2, 5	4
	3-O*H*		na		
	4	34.02	1.68		3
	4-CH_3	17.19	0.94, 3H, d, 7.7	3, 4, 5	
	5	40.07	1.13, 1.19		
	6	26.36	1.63		
	6-CH_3	21.63	0.85, 3H, d, 6.9	5, 6, 7	
	7	24.70	0.92, d, 7.4	5, 6, 6-CH_3	
Asn1					
	1	172.33			
	2	50.34	4.68	1	
	3	37.68	2.73, 2.83	4	
	4	173.46			
	4-NH_2		na		
	N*H*		8.29, brs		Htmha-2
Agdha					
	1[c]				
	2	72.91	3.88		
	2-O*H*		na		
	3	75.36	3.61		4
	3-O*H*		7.04[d]		
	4	50.98	4.16		3
	4-N*H*		7.64		Asn1-2[e]
	5	26.50[f]	1.74, 2H		
	6	26.27[f]	1.60, 1.70		
	7	41.95	3.17, 2H		
	7-N*H*		7.43		
	8	158.65[g]			
	8-NH_x		6.70, 7.21, 7.30[h]		
3,4-diMeGln					
	1[c]				
	2	59.43	4.21, d, 10.0		3, 3-CH_3, 4, 4-CH_3, *a*Thr1-N*H*
	3	37.35	2.27		2, 4, N*H*
	3-CH_3	13.86	1.08, 3H (ovl)	2, 3, 4	2
	4	42.25	2.77	4-CH_3	2, 3, 4-CH_3, N*H*
	4-CH_3	14.93	1.23, 3H, d, 6.5	3, 4, 5	2, 4
	5-*C*O	180.04			
	5-NH_2		6.91, 7.39[i]		
	N*H*		9.24brs		3,[j] 4, Agdha-2[j]
*a*Thr1					
	1[c]				
	2	55.49	5.40		3
	3	71.68	5.73	4, Hpr-1	2, 4, Leu-3
	4	14.54	1.27, 3H, d, 6.3	2, 3	3
	N*H*		9.04, d, 9.3		*a*Thr2-N*H*
*a*Thr2					
	1	172.04			
	2	63.89	3.94s	1, 3	3, 4, N*H*, Arg-N*H*
	3	67.13	4.39, d, 5.4	1	2, 4, N*H*
	3-O*H*		na		
	4	20.11	1.32, 3H, d, 6.4	2, 3	2, 3
	N*H*		8.39		2, 3, *a*Thr1-N*H*
Arg					
	1	174.01			
	2	53.00	4.51		3, Leu-N*H*
	3	28.05	1.59, 2.07		2, 3, 4
	4	30.02	1.60, 1.72		
	5	41.83	3.14, 2H		
	5-N*H*		7.33		
	6	158.68[g]			
	6-NH_2		na		
	N*H*		8.18		*a*Thr2-2, Leu-N*H*
Leu					
	1	176.35			
	2	51.92	4.66	1	3, *N*MeGln-NCH_3
	3	40.49	1.53, 2.25	4	2, 3, 4, N*H*, *a*Thr-3
	4	25.82	1.98		3, 5′, N*H*
	5	21.48	1.08, 3H (ovl)	3, 4, 5′	
	5′	23.91	1.10, 3H (ovl)	3, 4, 5	3
	N*H*		7.58, d, 5.2	Arg-1	3, 4, Arg-N*H*, Arg-2

Table 1. (Continued)

residue	position	δ_C	δ_H *J* (Hz)	HMBC (^{1}H to ^{13}C)	NOE[b]
*N*MeGln					
	1[c]				
	2	57.67	5.36		3, *β*OMeTyr-N*H*
	3	23.74	1.65, 2.24		2, 3
	4	32.32	2.03, 2.10		
	5	177.81			
	5-NH_2		na		
	NCH_3	31.30	2.51s, 3H	2, Leu-1	Leu-2
*β*OMeTyr					
	1	170.66			
	2	60.29	4.67	1	3, 5
	3	81.91	5.03s	1, 2, 3-OCH_3, 4/9, 5,	2, 3-OCH_3
	3-OCH_3	58.76	3.31s, 3H	3	3
	4	129.46			
	5/9	128.14	7.14, d, 8.4	3, 6, 7, 9/5	6/8
	6/8	116.40	6.79, d, 8.4	4, 7, 8/6	5/9, O*H*
	7	158.49			
	O*H*		7.54[k]		6/8
	N*H*		6.52, d, 8.4		*N*MeGln-2, Asn2-N*H*
Asn2					
	1[c]				
	2	47.69	5.39		Hpr-6
	3	37.47	2.45, 2.82	4	3
	4	174.39			
	N*H*		8.26, d, 8.8		*β*OMeTyr-N*H*
Hpr					
	1	170.93			
	2	53.90	5.22	1, 3, 4, 5	3
	3	27.36	1.65, 2.20		2, 3, 4
	4	22.11	1.28, 1.73		3, 4
	5	26.27	1.52, 1.67		6
	6	44.91	3.16, 3.69		5, 6, Asn2-2

[a] Contained a trace amount of trifluoroacetic acid. na: Not assigned. ovl: Signal overlapped. [b] Correlations without annotation were obtained by NOESY with a 200 ms mixing time. [c] Carbonyl resonances at δ 171.39, 173.92, 175.14, and 175.74 were observed but could not be assigned. One carbonyl carbon was not apparent in the ^{13}C NMR. [d] Assignment based on a NOESY cross-peak; δ 7.64/δ 7.04. [e] Observed in a ROESY experiment with a 50 ms mixing time. [f,g] Interchangeable. [h] Assignment based on NOESY cross-peaks; δ 7.21/δ 7.43, δ 7.21/δ 7.30, and δ 7.21/δ 6.70. [i] Assignment based on NOESY cross-peaks; δ 2.77/δ 7.39 and δ 6.91/δ 7.39. [j] Obtained by NOESY with a 100 ms mixing time. [k] Assignment based on a NOESY cross-peak; δ 6.79/δ 7.54.

collection. The taxonomy of the sponge was established as *Neamphius huxleyi* (class Demospongiae, order Choristida, family Jaspidae) by Prof. Rob W. M. van Soest (University of Amsterdam). A voucher specimen (0CDN1923) was deposited at the Smithsonian Institution, Washington, DC.

Extraction and Isolation. The specimens of *N. huxleyi* (141 g wet wt) were ground with dry ice, and the resulting coarse powder was extracted twice with H_2O at 4 °C. The animal residue was then extracted with CH_2Cl_2−MeOH (1:1) and then 100% MeOH. A portion (3.0 g) of the combined organic extracts (4.8 g) was successively partitioned between (i) MeOH−H_2O (9:1) (250 mL) and hexanes (200 mL 3×), (ii) MeOH−H_2O (6:4) (400 mL) and methyl *tert*-butyl ether−hexanes (9:1) (200 mL 2×), and (iii) EtOAc (200 mL, 3×) and H_2O (250 mL). The H_2O-soluble fraction (1.1 g) was gel-filtered on Sephadex LH-20 using 70% aqueous MeOH as the eluant. The HIV-1 inhibitory fractions were combined and subjected to polymeric reversed-phase HPLC (Hamilton PRP-3, 10 × 250 mm, 10 μm particle size, solvent: 20−60% MeCN−0.1% TFA in H_2O over 21 min, 2 mL/min, detection at 220 nm) to furnish neamphamide A (**2**) (17 mg; 0.02% yield based on wet wt) as a white solid.

Neamphamide A (2): $[\alpha]^{21}_D$ −0.6° (*c* 0.2, MeOH); UV-(MeOH) λ_{max} 276 nm (ϵ 1900); ^{1}H and ^{13}C NMR, see Table 1; high-resolution electrospray ionization TOF MS *m/z* 844.9694 $[M + 2H]^{2+}$ corresponding to a molecular weight of 1687.9242, calcd for $C_{75}H_{125}N_{21}O_{23}$, 1687.9257 ($\Delta$ −0.9 ppm).

Acid Hydrolysis of Peptides. A solution of **2** or callipeltin A (**1**) (100 μg) in degassed 6 N HCl (200 μL) was boiled in a sealed hydrolysis tube at 110 °C for 6 h. The solvent was removed in a stream of dry N_2, and the resulting material was subjected to further derivatization for stereochemical assignment.

LC-MS Analysis of Marfey's Derivatives.[13] To a 100 μg portion of an amino acid standard, or the acid hydrolysate of **2** or that of callipeltin A (**1**), was added a 0.1% (w/w) acetone solution of L-FDAA (*N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide) (50 μL) and 0.1 N $NaHCO_3$ (100 μL). The vial was heated at 70 °C for 1 h, and the contents were neutralized with 0.2 N HCl (50 μL) after cooling to room temperature. An aliquot of the L-FDAA derivative was dried under vacuum, diluted with MeCN−5% AcOH in H_2O (1:1), and subjected to LC-MS analysis by separation on Zorbax SB300 C_3 (2.1 × 150 mm), eluting with MeCN−5% AcOH in H_2O at a flow rate of 0.25 mL/min. The elution program was set as follows: 0−5 min (0% MeCN), 5−30 min (0−50% MeCN), 30−35 min (50% MeCN), 35−40 min (50−100% MeCN). FDAA derivatives were detected by absorption at 340 nm, and assignment was secured by ion-selective monitoring. Retention times of authentic FDAA-amino acids (min): L-Arg (15.5), D-Arg (16.9), L-Asp (15.8), D-Asp (17.9), L-Leu (25.5), D-Leu (28.2), (3*S*,4*R*)-3,4-diMe-L-Glu (20.6, from callipeltin A), L-Hpr (24.5), D-Hpr (23.3), L-Thr (15.2), D-Thr (19.6), L-*a*Thr (16.1), D-*a*Thr (17.8). The hydrolysate of **2** contained L-Asp (15.8), D-Asp (17.9), D-Arg (16.8), (3*S*,4*R*)-3,4-diMe- L-Glu (20.6), L-Leu (25.6), L-Hpr (24.5), and D-*a*Thr (17.7).

LC-MS Analysis of GITC Derivatives.[14] Triethylamine (10 μL) and a GITC (2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate) solution (50 μL, made at 3.9 mg/mL in MeCN) were added to the acid hydrolysate (33 μg) of **2** or callipeltin A (**1**), or an authentic amino acid standard (100 μg). The reaction mixture was kept for 30 min at room temperature, and then the reaction was quenched by adding 40 μL of MeCN−5% AcOH in H_2O (1:1). An aliquot was dried under vacuum and then properly diluted with the same solvent mixture and subjected to LC-MS analysis as described above,

except for monitoring absorption at 254 nm. Retention times (min): L-*N*MeGlu (21.1), D-*N*MeGlu (21.6), (3*S*,4*R*)-3,4-diMe-L-Glu from callipeltin A (22.6). The hydrolysate of **2** gave peaks for L-*N*MeGlu (21.0) and (3*S*,4*R*)-3,4-diMe-L-Glu (22.6).

Stereochemistry of Arg Derived from Agdha and the Asn Residues. A 200 μg portion of **2** was stirred in a mixture of EtOH (50 μL) and 1% (w/w) $NaIO_4$ in water (100 μL) for 11 h at ambient temperature. The reaction solution was directly separated by HPLC (Develosil C_{30} RPAQUEOUS-AR-5 column, 10 × 250 mm, Nomura Chemicals) using a gradient elution by aqueous MeCN−5% AcOH (0−50% over 25 min, then 50−100% MeCN over 10 min, 3 mL/min) collected into 15 mL fractions. The fifth fraction contained a pure acylaspartylargininal, whereas the sixth contained both of the $NaIO_4$-cleavage products. Half of the fifth fraction was dissolved in 100 μL of 50% AcOH in H_2O and boiled in a sealed v-bottom vial for 2 h at 102 °C in the presence of 30% H_2O_2 (30 μL) and concentrated HCl (12 μL). The residue was hydrolyzed, derivatized with L-FDAA, and analyzed by LC-MS, which revealed the presence of L-Arg (15.5) and L-Asp (15.8).

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Supporting Information Available: ^1H, ^{13}C, gCOSY45, gTOCSY, gHSQC, gHMBC, gNOESY, and gROESY spectra of compound **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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