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Grassypeptolides F and G, Cyanobacterial Peptides from *Lyngbya majuscula*

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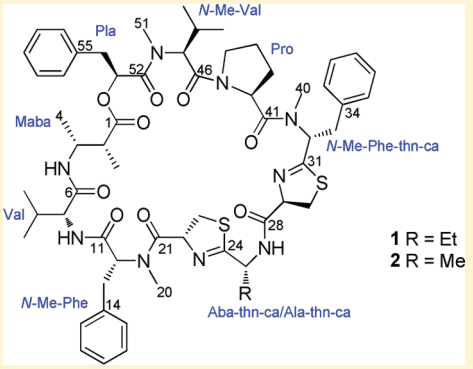
Supporting Information

ABSTRACT: Grassypeptolides F (**1**) and G (**2**), bis-thiazoline-containing cyclic depsipeptides with a rare β-amino acid, extensive *N*-methylation, and a large number of D-amino acids, are reported from an extract of the Palauan cyanobacterium *Lyngbya majuscula*. Both **1** and **2** were found to have moderate inhibitory activity against the transcription factor AP-1 (IC_{50} = 5.2 and 6.0 μM, respectively).



The oncogenic transcription factor activator protein-1 (AP-1) is required for tumor promotion and progression, the rate-limiting steps in carcinogenesis.[1] It has been shown that blocking AP-1 activity inhibits phorbol ester-induced transformation and tumorigenesis without inhibiting cell proliferation or cell survival; therefore identification of novel and specific AP-1 inhibitors may be valuable for the prevention and treatment of cancers.[2−4] A cell-based high-throughput screening protocol was recently developed to identify inhibitors of AP-1. Fluorescence resonance energy transfer (FRET) technology was used to quantify the expression of a β-lactamase reporter driven by a promoter bearing three AP-1 binding sites (TGAGTCA). The AP-1 reporter assay was paired with an XTT assay to evaluate cell viability, allowing the identification and elimination of cytotoxic compounds.[5]

During our screening of extracts from the NCI Natural Product Repository, an extract from the cyanobacterium *Lyngbya majuscula* was identified as an inhibitor of AP-1-mediated transcription. Investigation of this extract led to the isolation of grassypeptolides F (**1**) and G (**2**), bis-thiazoline-containing cyclic depsipeptides with a rare β-amino acid, extensive *N*-methylation, and a large number of D-amino acids. The peptides were found to be close relatives of the cytotoxic bis-thiazoline-containing cyclodepsipeptides grassypeptolides A (**3**), B (**4**), and C (**5**), reported in 2008 and 2010 from Floridian collections of *Lyngbya confervoides*.[6,7]

Grassypeptolide F (**1**) was obtained as a yellow, amorphous solid (5.0 mg). Positive-ion mode HRESIMS and NMR data of **1** gave rise to the molecular formula $C_{60}H_{79}N_9O_9S_2$. The ^1H and ^{13}C NMR data in $CDCl_3$ indicated the peptidic nature of **1** including the presence of at least eight strongly deshielded nonprotonated carbons attributed to the ester/amide functionalities, at least one secondary amide proton doublet (δ_H 7.64), and three *N*-methyl singlets (δ_H 2.70, 2.92, and 3.12) (Table 1).

Detailed analysis of the 1D and 2D NMR data of **1** established two standard α-amino acid units (Val and Pro), two *N*-methylated α-amino acids (*N*-Me-Phe and *N*-Me-Val), one β-amino acid (2-methyl-3-aminobutyric acid, Maba), one α-hydroxy acid (phenyllactic acid, Pla), and two thiazoline carboxylic acid units derived from the condensation of cysteine with 2-aminobutyric acid (Aba-thn-ca) and with *N*-methylphenylalanine (*N*-Me-Phe-thn-ca) (Table 1).

Elucidation of the thiazoline rings posed the greatest challenge. Two identical isolated spin systems were identified from COSY correlations between the proton resonances of C-22 and C-23, and C-29 and C-30. The strongly deshielded chemical shifts of the methine carbons C-22 and C-29 (δ_C 77.6 and 79.4, respectively) were consistent with cyclic secondary ketimine attachment at both centers in addition to the downfield effect of the adjacent carbonyls (C-21 and C-28, respectively).[8]

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Table 1. ^{15}N (60 MHz), ^{13}C (150 MHz), and ^{1}H (600 MHz) NMR Data in $CDCl_3$ for Grassypeptolides F (1) and G (2)

		grassypeptolide F (1)			grassypeptolide G (2)	
amino acids	pos.	δ_C or δ_N, mult.	δ_H (J in Hz)	HMBC[a]	δ_C, mult.	δ_H (J in Hz)
Maba	1	172.0, C			172.1, C	
	2	44.6, CH	2.49, qd (7.1, 3.3)	1, 3, 4, 5, 53,[b] N-1	44.6, CH	2.50, qd (7.0, 3.4)
	3	48.6, CH	4.03, m	1, 2, 4, 5, 6	48.5, CH	4.03, m
	4	19.8, CH_3	1.06, d (6.7)	2, 3, N-1	19.9, CH_3	1.05, d (6.7)
	5	14.6, CH_3	0.96, d (6.9)	1, 2, 3	14.6, CH_3	0.96, d (7.0)
	N-1	117.7, NH	7.15, d (8.2)	2, 3, 6	117.6, NH	7.14, m
Val	6	171.9, C			171.9, C	
	7	60.6, CH	4.36, dd (8.6, 6.4)	6, 8, 9, 10, 11, N-2	60.6, CH	4.36, dd (8.8, 6.2)
	8	30.7, CH	2.25, m	6, 7, 9, 10, N-2	30.8, CH	2.23, sept (6.8)
	9	19.6, CH_3	0.98, dd (6.8)	7, 8, 10	19.7, CH_3	0.99, d (6.3)
	10	18.7, CH_3	0.98, dd (6.8)	7, 8, 9	18.8, CH_3	0.98, d (6.0)
	N-2	115.8, NH	7.15, dd (8.2)	7, 8, 11	115.9, NH	7.13, m
N-Me-Phe	11	169.7, C			169.7, C	
	12	58.5, CH	5.50, t (7.2)	11, 13, 14, 20, 21, N-3	58.6, CH	5.51, t (7.4)
	13	34.7, CH_2	3.44, m	11, 12, 14, 15/19, N-3	34.7, CH_2	3.45, dd (14.0, 8.4)
			2.94, m	11, 12, 14, 15/19		2.91, dd (13.9, 7.5)
	14	137.6, C			137.7, C	
	15/19	129.5, CH	7.24, m	13, 15/19, 17	129.6, CH	7.25, m
	16/18	128.5, CH	7.13, t (6.6)	14, 16/18	128.6, CH	7.13, m
	17	126.6, CH	7.13, t (6.6)	15/19	126.6, CH	7.13, m
	N-3	113.5,[c] N			113.9,[c] N	
	20	31.6, CH_3	3.12, s	12, 21, N-3	31.6, CH3	3.14, s
Aba-thn-ca/	21	169.9, C			169.9, C	
Ala-thn-ca	22	77.6, CH	5.40, t (8.8)	21, 23, 24	77.5, CH	5.39, t (8.1)
	23	33.4, CH_2	3.45, m	21, 22, 24	33.7, CH_2	3.49, m
			3.17, t (10.4)	21, 22, 24		3.18, t (10.4)
	N-4	N[d]			N[d]	
	24	178.5, C			179.0, C	
	25	54.1, CH	4.68, m	24, 26, 27, 28	48.3, CH	4.87, quin (7.1)
	26	25.6, CH_2	2.17, m	24, 25, 27, N-5	18.5, CH_3	1.59, d (7.3)
			1.98, m	24, 25, 27, N-5		
	27	10.8, CH_3	0.97, t (7.6)	25, 26		
	N-5	124.7, NH	7.64, d (8.0)	25, 26, 28	128.6, NH	7.77, d (7.7)
N-Me-Phe-thn-ca	28	171.2, C			170.7, C	
	29	79.4, CH	5.31, dd (7.3, 5.0)	28, 30, 31, N-5, N-6	79.1, CH	5.30, t (6.0)
	30	37.8, CH_2	3.70, m	28, 29, 31	38.0, CH_2	3.71, d (6.7)
	N-6	294.8,[c] N			293.4,[c] N	
	31	177.3, C			177.6, C	
	32	69.0, CH	3.82, dd (10.6, 1.9)	31, 33, 34, 40, 41	69.0, CH	3.83, m
	33	35.3, CH_2	3.59, dd (13.7, 10.8)	31, 32, 34, 35/39	35.3, CH_2	3.62, dd (13.6, 10.9)
			3.41, m	31, 32, 34, 35/39, N-7		3.39, dd (14.1, 3.8)
	34	138.2, C			138.3, C	
	35/39	129.9, CH	7.33, d (7.1)	33, 35/39, 37	129.9, CH	7.33, d (7.0)
	36/38	128.9, CH	7.36, t (7.5)	34, 36/38	128.9, CH	7.37, t (7.4)
	37	126.9, CH	7.25, m	35/39	126.9, CH	7.26, m
	N-7	110.2,[c] N			109.8,[c] N	
	40	39.4, CH_3	2.70, s	32, 41, N-7	39.4, CH_3	2.69, s
Pro	41	172.6, C			172.4, C	
	42	57.5, CH	4.75, dd (8.1, 5.4)	41, 43, 44, 45, 46	57.7, CH	4.74, t (6.7)
	43	27.2, CH_2	2.02, m	41, 42, 44, 45, N-8	27.3, CH_2	2.02, q (6.9)
	44	25.3, CH_2	2.21, m	42, 43, 45, N-8	25.3, CH_2	2.20, m
			1.81, dquin (12.9, 7.1)	42, 43, 45, N-8		1.83, dquin (13.1, 7.1)

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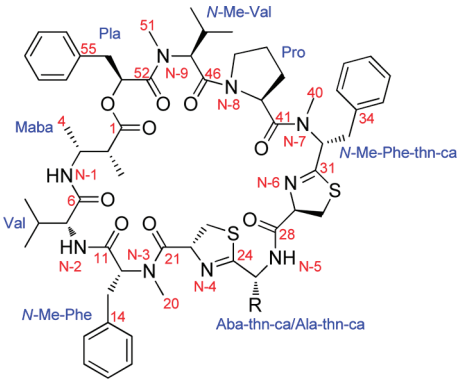
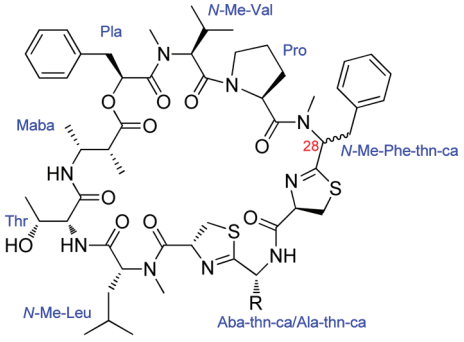
Table 1. Continued

		grassypeptolide F (**1**)			grassypeptolide G (**2**)	
amino acids	pos.	δ_C or δ_N, mult.	δ_H (J in Hz)	HMBC[a]	δ_C, mult.	δ_H (J in Hz)
	45	47.2, CH_2	3.50, dt (10.9, 7.3)	42, 43, 44, 46	47.3, CH_2	3.50, m
			3.28, m	42, 43, 44, 46		3.29, m
	N-8	137.5,[c] N			136.8,[c] N	
N-Me-Val	46	166.9, C			167.0, C	
	47	61.4, CH	4.79, d (10.6)	46, 48, 49, 50, 51, 52, N-9	61.3, CH	4.80, d (10.6)
	48	26.6, CH	2.44, dsept (10.8, 6.4)	46, 47, 49, 50	26.6, CH	2.43, dsept (10.9, 6.5)
	49	20.1, CH_3	0.91, d (6.3)	47, 48, 50	20.0, CH_3	0.88, d (6.3)
	50	18.1, CH_3	0.72, d (6.9)	47, 48, 49	18.1, CH_3	0.71, d (6.9)
	N-9	114.0,[c] N			114.0,[c] N	
	51	30.8, CH_3	2.92, s	47, 52, N-9	30.8, CH_3	2.93, s
Pla	52	171.2, C			171.2, C	
	53	70.4, CH	5.72, dd (8.6, 5.4)	1, 52, 54, 55	70.4, CH	5.72, dd (8.4, 5.5)
	54	38.3, CH_2	3.03, dd (14.4, 8.7)	52, 53, 55, 56/60	38.3, CH_2	3.02, dd (14.4, 8.7)
			2.93, m	52, 53, 55, 56/60		2.91, dd (13.9, 6.4)
	55	135.2, C			135.3, C	
	56/60	129.4, CH	7.20, d (7.1)	54, 56/60, 58	129.5, CH	7.21, d (7.1)
	57/59	128.8, CH	7.29, t (7.5)	55, 57/59	128.8, CH	7.30, t (7.3)
	58	127.5, CH	7.26, m	56/60	127.5, CH	7.26, m

[a] HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon or nitrogen. [b] Four-bond HMBC correlation. [c] Assigned from HMBC data. [d] Not observed.



1 R = Et
2 R = Me



3 R = Et, 28*R*
4 R = Me, 28*R*
5 R = Et, 28*S*

The molecular formula indicated the presence of two sulfur units in the molecule. One-bond carbon−proton coupling constants ($^1J_{CH}$) increase with substitution of electron-withdrawing groups

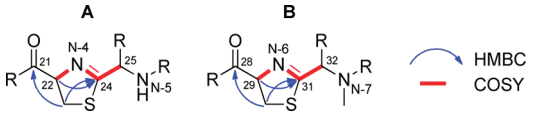


Figure 1. Selected HMBC and COSY correlations establishing the Aba-derived (A) and *N*-Me-Phe-derived (B) thiazoline systems in grassypeptolide F (**1**).

(viz., acetone: 127 Hz; dimethylsulfoxide: 139 Hz).[9−11] This precedent suggested the attachment of sulfur to C-23 and C-30 (δ_C 33.4 and 37.8, respectively), resulting in large $^1J_{CH}$ values for CH_2-23 (146 Hz) and CH_2-30 (146 Hz).

HMBC correlations were observed from both H-22 and H_2-23 to what at first appeared to be the carbonyl of the Aba unit (C-24: δ_C 178.5). Similarly, HMBC correlations were observed from both H-29 and H_2-30 to what appeared to be an *N*-Me-Phe carbonyl (C-31: δ_C 177.3). However, homoallylic COSY correlations observed between H-22 and the methine of Aba (H-25) and between H-29 and the equivalent methine of the *N*-Me-Phe (H-32) required that the isolated spin systems be five-membered-ring thiazoline moieties joined at the carbonyl-derived carbons of Aba and *N*-M-Phe, respectively (Figure 1). The chemical shifts of the Aba-thn-ca and *N*-Me-Phe-thn-ca units are also in agreement to those observed in grassypeptolides A−C (**3**−**5**), further supporting this assignment.

Finally, the amino acid sequence of grassypeptolide F (**1**) was established by HMBC correlations (Figure 2) and supported by observed NOE enhancements, which also established the *trans* conformation of all amide bonds. The *trans* conformation of the Pro was confirmed by the small chemical shift difference (2 Hz) between the β- and γ-carbons ($\Delta\delta_{\beta\gamma}$: *trans* < 6 Hz, *cis* > 8 Hz).[12] ^{15}N-HSQC and ^{15}N-HMBC data were consistent with the proposed structure (Tables 1 and S1).[13]

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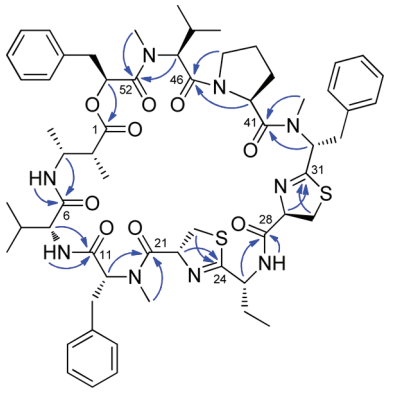

Figure 2. Selected HMBC correlations connecting the amino acid residues in grassypeptolide F (**1**).

The absolute configurations of the amino acid residues in **1** were established through a combination of Marfey's method analysis[14] and the use of chiral-phase HPLC. A small sample of **1** was hydrolyzed with 6 N HCl (110 °C, 22 h) and the hydrolysate then derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA). Analysis by LC-MS revealed the presence of L-Pro, (2*R*,3*R*)-Maba, *N*-Me-L-Val, and D-Val on the basis of comparisons with similarly derivatized standards. Two peaks were observed for Maba corresponding to (2*R*,3*R*)- and (2*S*,3*R*)-Maba at a ratio of 2.7:1. The Maba standards were obtained as the *N*-benzoyl-*O*-methyl ester and showed similar levels of epimerization under the same hydrolysis conditions. This was also consistent with observations reported in the analysis of grassypeptolide A (**3**).[6] Both L-Aba and D-Aba were observed at a ratio of 1:1.3, while *N*-Me-L-Phe and *N*-Me-D-Phe were both detected at a ratio of 1:1.4. Thiazolines are known to readily epimerize in the presence of mild acid or base.[15] To minimize this epimerization and determine the absolute configuration of the Aba and *N*-Me-Phe moieties as well as the cysteic acid (Cya) portion of the thiazoline moieties, the sample was ozonized before acid hydrolysis. The L-FDLA-derivatized hydrolysate was again analyzed by LC-MS to reveal the presence of D-Aba, two *N*-Me-D-Phe, and D- and L-Cya (1:3.8). Similar analysis of grassypeptolide A (**3**) was reported to give an enriched presence of L-Cya, which was concluded to be a result of partial epimerization under the reaction conditions, as the absolute configuration of both thiazolines was confirmed to be L-Cya by X-ray crystal analysis.[6] In this study, the configuration of both thiazolines in **1** was concluded to be *R* (L-Cya) because the ratios of both Cya unit enantiomers obtained after hydrolysis were similar to the ratios of those reported for grassypeptolides A−C (**3**−**5**). The L-Cya assignment is also supported by comparative ^{1}H and ^{13}C NMR data of the thiazoline residues to those reported for **3**−**5**. Finally the remaining hydrolysate was subjected to chiral-phase HPLC, revealing the presence of L-Pla.

Positive-ion mode HRESIMS analysis of the pale yellow, amorphous solid grassypeptolide G (**2**) (3.3 mg) gave rise to two pseudomolecular ions indicative of the molecular formula $C_{59}H_{77}N_9O_9S_2$ (*m*/*z* 1120.5366 $[M + H]^+$ and 1142.5181 $[M + Na]^+$). The ^{1}H and ^{13}C NMR data appeared to be very similar to those of **1** with the exception of the absence of one methylene and presence of a downfield methyl doublet in place of the methyl triplet. Analysis of the NMR data confirmed that nine of the 10 amino acid residues of **2** were identical to those in **1**, with the addition of a new alanine (Ala)-derived thiazoline replacing the Aba-derived unit in **1** as the tenth residue. Once again the amino acid sequence of grassypeptolide G (**2**) was established by HMBC correlations and supported by observed NOE enhancements, which also determined the *trans* conformation of all amide bonds (Table S3).

The absolute configurations of the amino acid units of **2** were determined in an identical fashion to **1**. Acid hydrolysis and L-FDLA derivatization revealed L-Pro, (2*R*,3*R*)-Maba, *N*-Me-L-Val, and D-Val by LC-MS analysis. Again, both thiazoline-derived amino acids epimerized to give a mixture of enantiomers (L-Ala:D-Ala, 1:1.3; *N*-Me-L-Phe:*N*-Me-D-Phe, 1:1.7). Ozonolysis followed by hydrolysis and L-FDLA derivatization minimized this epimerization and gave D-Ala, *N*-Me-D-Phe, and D- and L-Cya (1:3.2). Once again the similar ratio of the Cya epimers and comparison of the NMR data led to the assignment of both thiazolines as *R* (L-Cya). Finally, chiral-phase HPLC revealed L-Pla, completing the absolute configurational assignment of grassypeptolide G (**2**).

The variation between the known grassypeptolides A−C (**3**−**5**) and the new grassypeptolides F (**1**) and G (**2**) occurs at the second and third amino acid residues; **1** and **2** contain valine followed by *N*-methylphenylalanine, while **3**−**5** contain threonine followed by *N*-methylleucine. Both sets of compounds exhibit variation at the fifth residue, with either Aba or Ala adducts. Careful chemical shift analysis by Kwan et al. showed grassypeptolides A (**3**) and C (**5**) to be epimers at the C-28 position (equivalent to C-32 in **1** and **2**) through distinct variation at C-28 and the *N*-methyl signal at C-36 (C-40 in **1** and **2**).[7] The chemical shifts of the equivalent positions in **1** and **2** compared favorably with **3**, supporting the *R* assignment of the *N*-Me-Phe residue (δ_H/δ_C 3.82/69.0 for **1** and 3.83/69.0 for **2** compared to 3.83/69.0 in **3**, and δ_H/δ_C 2.70/39.4 for **1** and 2.69/39.4 for **2** compared to 2.78/39.6 in **3**). Both grassypeptolides F (**1**) and G (**2**) were moderately active in the AP-1 assay (IC_{50} 5.2 and 6.0 μM, respectively); however the cytotoxicity in an XTT assay was found to be on the same order of magnitude.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 241 polarimeter, UV spectra were obtained on a Varian 50 Bio spectrophotometer, and ECD spectra were recorded on a Jasco J-720 spectropolarimeter. NMR spectra were acquired on a Bruker Avance III 600 spectrometer in $CDCl_3$ and CD_3OD. All directly detected ^{1}H and ^{13}C chemical shifts (δ) were internally referenced to the residual solvent peak.[16] Indirectly detected ^{15}N shifts were referenced with a frequency ratio Ξ^{TSP} = 10.132905%.[17] High-resolution mass spectra were obtained with an Agilent 6520 Series Accurate-Mass Q-TOF LC/MS/MS System. HPLC was performed on a Varian Prepstar 218 system with Prostar 325 UV/vis detector. LC-MS was performed on an Agilent 1100 series system with an Agilent 1100 DAD and 1100 MSD.

Extraction and Isolation. The sample (106 g, collection weight) of *Lyngbya majuscula* (order Oscillatoriales, family Oscillatoriaceae) was collected around the Ngerderrak Reef, Palau, in 1995 by P. L. Colin (Coral Reef Research Foundation) at a depth of less than 1 m and identified by G. C. Trono Jr. (University of the Philippines). The cyanobacterial material was frozen immediately upon collection and transported frozen to Frederick, MD, where the extracts were prepared. The frozen material was ground, and two extracts were made: an aqueous extract and an organic solvent mixture CH_2Cl_2/MeOH (1:1).[18] A voucher specimen (OCDN2821) for this collection is maintained at the Smithsonian Institution, Washington DC. A 1.5 g

portion of the organic extract (3.05 g in total) was passed through a normal-phase column (Diol, 16 g), and the column was eluted successively with 500 mL volumes of hexane, CH_2Cl_2, EtOAc, and MeOH. The active EtOAc fraction (212 mg) was further fractionated on Sephadex LH-20 (280 mL; 5:2:1 CH_2Cl_2/hexane/MeOH) followed by a normal-phase column (Si, 50 mL) eluted successively with 100 mL volumes of 1%, 2%, 3%, and 5% MeOH in CH_2Cl_2. Fractions 12−21 of a total of 56 were combined and purified on semipreparative C_{18} RP-HPLC at a flow rate of 2.5 mL/min using a CH_3CN/0.05% TFA linear gradient (70% to 100% over 10 min, then 100% CH_3CN for 10 min). Grassypeptolides F (**1**) (5.0 mg, 0.33% of the extract) and G (**2**) (3.3 mg, 0.22% of the extract) eluted at 17.5 and 16.5 min, respectively.

Grassypeptolide F (1): pale yellow, amorphous solid; $[\alpha]^{25}_D$ +76.9 (*c* 0.43, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.61) nm; ECD (MeOH) λ ($\Delta\varepsilon$) 206 (+11.8), 219 (+23.5), 227 (+14.0), 234 (+16.2), 252 (+8.9); NMR data see Tables 1, S1 ($CDCl_3$), and S2 (CD_3OD); HRESIMS *m*/*z* 1134.5543 $[M + H]^+$ (calcd for $C_{60}H_{80}N_9O_9S_2$, 1134.5515), *m*/*z* 1156.5353 $[M + Na]^+$ (calcd for $C_{60}H_{79}N_9NaO_9S_2$, 1156.5334).

Grassypeptolide G (2): pale yellow, amorphous solid; $[\alpha]^{25}_D$ +35.1 (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.34) nm; ECD (MeOH) λ ($\Delta\varepsilon$) 208 (+5.0), 218 (+9.1), 228 (+4.4), 235 (+5.7), 253 (+3.4); NMR data see Tables 1, S3 ($CDCl_3$), and S4 (CD_3OD); HRESIMS *m*/*z* 1120.5366 $[M + H]^+$ (calcd for $C_{59}H_{78}N_9O_9S_2$, 1120.5358), *m*/*z* 1142.5181 $[M + Na]^+$ (calcd for $C_{59}H_{77}N_9NaO_9S_2$, 1142.5178).

Ozonolysis, Acid Hydrolysis, and Marfey's Analysis of Grassypeptolides F (1) and G (2). A portion (50 μg) of each peptide was subjected to acid hydrolysis (6 N HCl, 110 °C, 22 h) and then evaporated to dryness. The hydrolysates of **1** and **2** were each reconstituted in H_2O (25 μL), and $NaHCO_3$ (10 μL, 1 M) and 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA, 50 μL, 1% w/v in acetone) were added. The mixtures were heated to 35 °C for 1 h with constant mixing, then neutralized with HCl (5 μL, 2 N), concentrated to dryness, and reconstituted with 100 μL of CH_3CN/H_2O (1:1). The *N*-benzoyl-*O*-methyl esters of (2*R*,3*R*)-, (2*R*,3*S*)-, and (2*S*,3*S*)-2-methyl-3-aminobutyric acid (Maba) were treated with 6 N HCl at 110 °C for 22 h.[19] The products of each reaction were evaporated to dryness and made up as 50 mM solutions in H_2O. Stock solutions (50 mM in H_2O) of authentic standards of the other amino acids were also made. To a portion of each stock solution (25 μL) were added $NaHCO_3$ (10 μL, 1 M) and L-FDLA (50 μL, 1% w/v in acetone), and the mixtures were heated to 35 °C for 1 h with constant mixing. The reactions were then neutralized with HCl (5 μL, 2 N), concentrated to dryness, and then reconstituted with 250 μL of CH_3CN/H_2O (1:1). L-FDLA derivatives were analyzed by LC-MS [Phenomenex C_{18}, 5 μm (150 × 2.0 mm), 40 °C; ESIMS detection in positive and negative ion mode; UV detection at 340 nm] at 1 mL/min using a linear gradient from 20% to 50% CH_3CN in 5% CH_3COOH over 30 min. The hydrolysate of **1** gave Pla (1.3 min), L-Pro (9.4 min), L-Aba (10.6 min), (2*R*,3*R*)-Maba (15.1 min), (2*S*,3*R*)-Maba (15.4 min) (Maba: 2*R*,3*R* : 2*S*,3*R*, 2.7 : 1), *N*-Me-L-Val (15.6 min), D-Aba (16.5 min) (Aba: L : D, 1 : 1.3), *N*-Me-L-Phe (18.3 min), *N*-Me-D-Phe (19.2 min) (*N*-Me-Phe: L : D, 1 : 1.4), and D-Val (19.7 min). Similarly, the hydrolysate of **2** gave Pla (1.3 min), L-Ala (9.1 min), L-Pro (9.4 min), D-Ala (13.2 min) (Ala: L : D, 1 : 1.3), (2*R*,3*R*)-Maba (15.1 min), (2*S*,3*R*)-Maba (15.4 min) (Maba: 2*R*,3*R* : 2*S*,3*R*, 2.1 : 1), *N*-Me-L-Val (15.6 min), *N*-Me-L-Phe (18.3 min), *N*-Me-D-Phe (19.2 min) (*N*-Me-Phe: L : D, 1 : 1.7), and D-Val (19.7 min). The retention times of the L-FDLA derivatives of the authentic amino acids were as follows: Pla (1.3 min), L-Ala (9.1 min), L-Pro (9.4 min), L-Aba (10.6 min), L-Val (12.1 min), D-Pro (12.2 min), D-Ala (13.2 min), (2*R*,3*S*)-Maba (13.6 min), (2*S*,3*S*)-Maba (13.7 min), (2*R*,3*R*)-Maba (15.1 min), (2*S*,3*R*)-Maba (15.4 min), *N*-Me-L-Val (15.6 min), D-Aba (16.5 min), *N*-Me-L-Phe (18.3 min), *N*-Me-D-Phe (19.2 min), D-Val (19.7 min), and *N*-Me-D-Val (19.9 min).

Another portion (50 μg) of each peptide was dissolved in 2 mL of CH_2Cl_2, and ozone was bubbled through the solutions for 10 min. The reaction mixtures were evaporated to dryness and subjected to acid hydrolysis (6 N HCl, 110 °C, 16.5 h). The L-FDLA derivatives of the ozonolysis products of **1** and **2** were prepared in a similar way and analyzed by LC-MS [Phenomenex C_{18}, 5 μm (150 × 2.0 mm), 40 °C; ESIMS detection in positive and negative ion mode; UV detection at 340 nm) using two different linear gradient solvent systems.

Solvent System 1. Linear gradient (1 mL/min) from 20% to 50% CH_3CN in 5% CH_3COOH over 30 min. The hydrolysate of the ozonolysis product of **1** gave Pla (1.3 min), Cya (3.1 min, unresolved), L-Pro (9.7 min), (2*R*,3*R*)-Maba (15.4 min), (2*S*,3*R*)-Maba (15.6 min) (Maba: 2*R*,3*R* : 2*S*,3*R*, 3.2 : 1), *N*-Me-L-Val (15.9 min), D-Aba (16.8 min), *N*-Me-D-Phe (19.5 min), and D-Val (19.9 min). Similarly, the hydrolysate of the ozonolysis product of **2** gave Pla (1.3 min), Cya (3.1 min, unresolved), L-Pro (9.7 min), D-Ala (13.5 min), (2*R*,3*R*)-Maba (15.4 min), (2*S*,3*R*)-Maba (15.6 min) (Maba: 2*R*,3*R* : 2*S*,3*R*, 3.1 : 1), *N*-Me-L-Val (15.9 min), *N*-Me-D-Phe (19.4 min), and D-Val (19.9 min). The retention times of the L-FDLA derivatives of the authentic amino acids were as follows: Pla (1.3 min), D-Cya (2.8 min),L-Cya (3.0 min), L-Ala (9.1 min), L-Pro (9.4 min), L-Aba (10.6 min), L-Val (12.1 min), D-Pro (12.2 min), D-Ala (13.2 min), (2*R*,3*S*)-Maba (13.6 min), (2*S*,3*S*)-Maba (13.7 min), (2*R*,3*R*)-Maba (15.1 min), (2*S*,3*R*)-Maba (15.4 min), *N*-Me-L-Val (15.6 min), D-Aba (16.5 min), *N*-Me-L-Phe (18.3 min), *N*-Me-D-Phe (19.2 min), D-Val (19.7 min), and *N*-Me-D-Val (19.9 min).

Solvent System 2. Linear gradient (0.5 mL/min) from 0% to 50% CH_3CN in 5% CH_3COOH over 120 min. The hydrolysate of the ozonolysis product of **1** gave D-Cya (43.4 min), L-Cya (44.2 min) (Cya: D : L, 1 : 3.8), (2*R*,3*R*)-Maba (81.2 min), and (2*S*,3*R*)-Maba (81.8 min) (Maba: 2*R*,3*R* : 2*S*,3*R*, 3.3 : 1). Similarly, the hydrolysate of the ozonolysis product of **2** gave D-Cya (43.6 min), L-Cya (44.3 min) (Cya: D : L, 1 : 3.2), (2*R*,3*R*)-Maba (81.2 min), and (2*S*,3*R*)-Maba (81.8 min) (Maba: 2*R*,3*R* : 2*S*,3*R*, 3.6 : 1). The retention times of the L-FDLA derivatives of the authentic amino acids were as follows: D-Cya (43.1 min), L-Cys (43.9 min), (2*R*,3*S*)-Maba (76.3 min), (2*S*,3*S*)-Maba (76.9 min), (2*R*,3*R*)-Maba (80.4 min), and (2*S*,3*R*)-Maba (81.2 min).

The remainder of the ozonolysis and hydrolysis products of **1** were combined and subjected to chiral-phase HPLC analysis (Phenomenex CHIREX 3126 (D, 150 × 4.6 mm; 15% CH_3CN in 2 mM $CuSO_4$; 1.0 mL/min; detection by UV 254 nm) and compared with commercially available amino acid standards (retention times shown in parentheses): L-Pla (146−150 min) and D-Pla (189−196 min). The hydrolysate of **1** gave L-Pla (139−146 min). All other acid residues eluted in under 25 min. Similarly the remainder of the ozonolysis and hydrolysis products of **2** were combined and subjected to the same chiral-phase HPLC analysis to also give L-Pla (141−147 min).

AP-1 Assay. The AP-1 screen was run as previously described.[5] Briefly, HEK293T cells expressing β-lactamase under AP-1 control were plated (in 384-well black wall, clear bottom, polylysine-coated plates) and preincubated with test samples or controls for 1 h followed by addition of 10 ng·mL^{-1} (16.7 nM) tetradecanoyl phorbol acetate (TPA).[20] After 18 h, β-lactamase activity was measured by ratiometric fluorescence measurement (409 nm excitation, ratio of emission at 460 and 530 nm). After washing, cytotoxicity was assessed using XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt). The fluorescence ratio was normalized to TPA-only controls and reported as % of control activity. A "hit" was defined as one with activity ≤50% of control (i.e., ≥50% inhibition) of β-lactamase activity and ≥50% of control cell number by XTT assay. Staurosporine (0.2 μM) was used as a positive control.

■ ASSOCIATED CONTENT

Supporting Information. NMR tables in $CDCl_3$ and CD_3OD (S1−4) and NMR spectra in $CDCl_3$ for compounds

1 and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) Ruocco et al. incorrectly reported the concentration of TPA as 10 $\mu g \cdot mL^{-1}$ (16.7 μM).