Rolloamides A and B, Cytotoxic Cyclic Heptapeptides Isolated from the Caribbean Marine Sponge *Eurypon laughlini*

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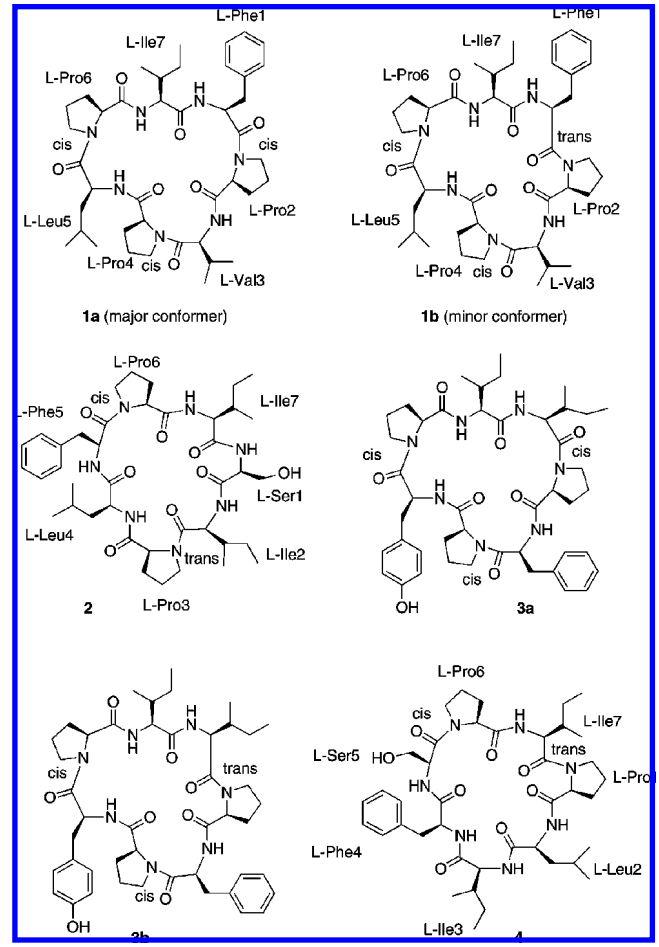
Two cyclic heptapeptides, rolloamides A (**1**) and B (**2**), have been isolated from the Dominican marine sponge *Eurypon laughlini*. The structures of **1** and **2** were elucidated by a combination of spectroscopic analyses and chemical degradation. Rolloamide A (**1**), which was found to exist as two distinct conformers in C_5D_5N, exhibits growth suppressive activity against a panel of histologically diverse cancer cell lines.

Marine sponges continue to attract attention as a rich source of structurally novel bioactive secondary metabolites that are of interest as potential lead compounds for the development of new drugs.[1] As part of an ongoing search for sponge metabolites that might represent viable anticancer drug leads,[2] it was found that extracts of the Dominican sponge *Eurypon laughlini* (Diaz, Alvarez & Van Soest, 1987) (order Poecilosclerida, family Raspailiidae) collected in 1997 exhibited cytotoxic activity. Fractionation of the *E. laughlini* extract led to the isolation of a cyclic peptide. However, at that time of isolation it was not possible to solve the structure of this peptide due to the small amount of sample that was available. A re-collection of the sponge in 2002 led to the identification of the cyclic octapeptide dominicin and the bromopyrrole alkaloid laughine.[3] This second collection of *E. laughlini* did not contain the trace peptide present in the 1997 collection, but instead yielded a different peptide, once again in amounts too small to allow structure elucidation. Subsequent access to a 600 MHz NMR spectrometer equipped with cryoprobe technology made it possible to elucidate the structures of rolloamides A (**1**) and B (**2**), the trace peptides present in the 1997 and 2002 sponge collections. The details of the structure elucidations of **1** and **2** along with the biological activity of **1** are presented below.



Results and Discussion

E. laughlini was harvested by hand using scuba from a rock overhang in 1997 and on the sand flats in 2002 off Rollo Head, south of Portsmouth, Dominica. Freshly collected sponge samples were frozen on site and transported frozen to Vancouver. The sponge samples were extracted with MeOH, and the combined MeOH extracts were concentrated *in vacuo* followed by partitioning the resulting residue between H_2O and EtOAc. Bioassay-guided fractionation of the 1997 EtOAc-soluble materials gave a pure sample of the cyclic heptapeptide rolloamide A (**1**). The 2002 EtOAc extract was partitioned between 4:1 MeOH/H_2O and hexanes. Fractionation of the MeOH/H_2O-soluble materials gave a pure sample of a second cyclic heptapeptide, rolloamide B (**2**), along with dominicin[3] and the alkaloids keramadine,[4] agelongine,[5] and laughine.[3]

Rolloamide A (**1**) was obtained as a clear, optically active glass that gave a $[M + H]^+$ ion in the HRFABMS at *m/z* 764.4723 that was appropriate for a molecular formula of $C_{41}H_{61}N_7O_7$ requiring 15 sites of unsaturation. In numerous NMR solvents (MeOH-d_4, DMSO-d_6, MeCN-d_3, etc.) many or all of the resonances in the ^1H and ^{13}C NMR spectra of **1** were doubled, tripled, or broadened, resulting in poorly resolved spectra that were not suitable for structural studies. Attempts to simplify the NMR spectra by heating (to 50 °C) or adjusting the pH by addition of TFA or Et_3N did not alleviate the problem. Eventually it was found that acceptable spectra with a set of well-resolved resonances could be obtained using C_5D_5N as the NMR solvent.

Rolloamide A (**1**) gave a sharp well-resolved peak when analyzed by reversed-phase HPLC using a variety of solvent systems, and it

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gave a single molecular ion in the HRFABMS. Despite these indications of purity, the ^{1}H NMR spectrum of **1** recorded in C_5D_5N contained two complete sets of signals that integrated for a ratio of 3:2. The ^{13}C/DEPT/HMQC NMR spectra for **1** recorded in C_5D_5N identified 82 carbon resonances. Fourteen of the ^{13}C NMR resonances had chemical shifts appropriate for amide carbonyls, and 14 resonances had chemical shifts appropriate for amino acid α-carbons (Table 1), suggesting a peptide structure. Detailed analysis of the COSY, HMQC, and HMBC data revealed that both the species responsible for the most intense signals (relative intensity 3) and the species responsible for the less intense signals (relative intensity 2) in the NMR spectra of **1** contained one leucine, one isoleucine, one phenylalanine, one valine, and three proline residues. Hydrolysis of **1** with 6 N HCl followed by Marfey's HPLC[6] analysis of the amino acids in the hydrolysate confirmed the presence of these five amino acids and showed that each had the L configuration. The combined molecular weight of the seven amino acid residues identified in the major and minor species was 763, with a molecular formula of $C_{41}H_{61}N_7O_7$, which was also the molecular formula determined by HRFABMS. The chemical shift of the proline γ-carbons and the carbon chemical shift difference of the proline β- and γ-carbons in the ^{13}C NMR spectrum of **1** established five of the proline peptide bonds in the two species as *cis* (δ Cγ < 23.3 ppm, $\Delta\delta_{C\beta-C\gamma}$ > 8.0 ppm) and one as *trans* (Cγ 26.5 ppm, $\Delta\delta_{C\beta-C\gamma}$ = 2.7 ppm),[7−9] suggesting that rolloamide A (**1**) is a heptapeptide existing in two conformations. In one conformer the proline peptide bonds are all *cis*, and in the other conformer two are *cis* and one is *trans*. Since the seven amino acid residues in each conformer accounted for all of the atoms in the molecular formula of rolloamide A (**1**) and 14 of the sites of unsaturation, and there was no evidence for terminal amino or carboxylic acid functionalities, rolloamide A (**1**) was assumed to be cyclic.

The sequence of the amino acids in the two conformers of rolloamide A (**1**) was established to be the same by detailed interpretation of HMBC and NOESY data as outlined in Figure 1. For the major isomer **1a**, the NMR analysis established the complete sequence including the cyclic nature of the peptide. For the minor isomer **1b**, the existence of a final amide bond between Leu^5 and Pro^6 to close the ring was required by the molecular formula. The observation of NOESY correlations for the major conformer **1a** between Phe^1-Hα/Pro^2-Hα, Val^3-Hα/Pro^4-Hα, and Leu^5-Hα/Pro^6-Hα provided evidence for these connectivities and further support that these three proline amide bonds adopted the *cis* geometry.[10,11] In the minor conformer **1b**, the NOESY correlation between Phe^1-Hα/Pro^2-Hδ_A and/or Pro^2-Hδ_B supports the *trans* geometry of this proline peptide bond.[10,11]

The cyclic heptapeptide phakellistatin 2 (**3**) has also been reported to exist as an inseparable mixture of conformers that differ only by the switch of one proline peptide bond from *cis* to a *trans*.[12] Each conformer had different folds, hydrogen-bonding patterns, and solvent-accessible surfaces. It was suggested that not only do these factors contribute to the relative stability of the two conformers, but they may also explain the observed variability in biological activity. There is a marked similarity in the structures of rolloamide A (**1**) and phakellistatin 2 (**3**). Although the constituent amino acids in the two heptapeptides are different, the positions of the proline residues in the cyclic hexapeptide template are the same in both molecules, and the switch from *cis* to *trans* peptide bond geometry occurs at the equivalent proline peptide bond. The doubling, tripling, and/or broadening of resonances in the ^{1}H and ^{13}C NMR spectra of rolloamide A (**1**) recorded in most solvents can be attributed to *cis*−*trans* isomerization of the proline peptide bond(s). The two distinct conformers were still observed in C_5D_5N even at 50 °C.

Rolloamide B (**2**) was obtained as a white, optically active amorphous solid that gave a $[M + H]^+$ ion at *m/z* 768.4677 in the HRFABMS, consistent with a molecular formula of $C_{40}H_{61}N_7O_8$. As with rolloamide A (**1**), rolloamide B (**2**) gave a sharp well-resolved peak when analyzed by reversed-phase HPLC using a variety of solvent systems, and it gave a single molecular ion in the HRFABMS. Despite these indications of purity, the ^{1}H and ^{13}C NMR spectra of **2** also showed broad poorly resolved signals in most solvents, including C_5D_5N. NMR spectra of **2** recorded in MeOH-d_4 gave sharp signals, although there was still doubling or tripling of resonances attributed to the existence of slow confor-

Table 1. NMR Data for Rolloamide A (**1**) (600 MHz, C_5D_5N)

	major conformer **1a**		minor conformer **1b**	
atom #	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
L-Phe^1				
α	52.9	5.20[a]	52.6[c]	5.23[a]
β_A	39.9	3.01[a]	40.5	2.99[a]
β_B		3.23[a]		3.30, bd (11.4)
1	137.7		139.2	
2,6	130.1	7.19, d (7.3)	130.6	7.76, d (7.3)
3,5	129.3	7.26, t (7.3)	129.2	7.41, t (7.3)
4	127.6	7.20, t (7.3)	127.3	7.32, t (7.3)
CO	173.0		171.1	
NH		10.22, d (7.3)		8.23, d (8.6)
L-Pro^2				
α	62.6	3.89, d (8.3)	64.6	4.20, t (8.5)
β_A	32.4	1.46[a]	29.3	1.79[a]
β_B		1.92, m		2.19[a]
γ_A	23.0	1.57, m	26.6	1.77[a]
γ_B		1.84[a]		1.77[a]
δ_A	47.0	3.51, m	46.9	3.23[a]
δ_B		3.81, m		3.23[a]
CO	172.0		173.3	
L-Val^3				
α	56.1	5.19[a]	58.3	4.74, t (8.0)
β	33.7	2.16[a]	34.4	2.26[a]
$\gamma_A CH_3$	18.2	0.83, d (6.7)	18.9	0.77, d (6.7)
$\gamma_B CH_3$	21.0	1.10, d (6.5)	19.8	1.02 d (6.3)
CO	170.0		171.2	
NH		7.14, bs		6.32, d (6.5)
L-Pro^4				
α	59.3	4.87, d (7.7)	60.1	5.08, d (8.2)
β_A	30.7	2.01[a]	31.4	2.09[a]
β_B		2.31[a]		2.55[a]
γ_A	22.7	1.69[a]	23.0[d]	1.71−1.80[a]
γ_B		2.34, m		2.54[a]
δ_A	47.5	3.63, m	47.8	3.74[a]
δ_B		3.96, bt (10.4)		4.07, t (10.6)
CO	173.4		173.8	
L-Leu^5				
α	53.1	4.77, bd (11.2)	52.6[c]	5.15, dd (12.0, 4.9)
β_A	39.6	1.45[a]	40.9	1.45[a]
β_B		1.77[a]		1.81[a]
γ_A	25.6	2.23[a]	25.0	2.71, m
$\delta_A CH_3$	24.3	0.75, d (6.3)	24.6	0.84, d (5.9)
$\delta_B CH_3$	21.9	1.10, d (6.5)	22.8	1.31, d (5.5)
CO	172.3[b]		173.1	
NH		9.99, bs		10.18, d (4.7)
L-Pro^6				
α	62.2	4.61[a]	61.7	4.82, d (7.9)
β_A	32.8	2.19[a]	32.7	2.16[a]
β_B		2.76, bd (8.3)		2.56[a]
γ_A	23.2	1.70[a]	23.0[d]	1.80[a]
γ_B		1.70[a]		1.80[a]
δ_A	48.1	3.74[a]	47.7	3.83[a]
δ_B		3.74[a]		3.83[a]
CO	173.0		172.3[b]	
L-Ile^7				
α	60.0	4.62[a]	61.4	4.57, t (7.7)
β	35.2	2.49, m	36.8	2.03[a]
γ_A	27.0	1.29[a]	26.3	1.25, m
γ_B		1.82[a]		1.68[a]
δCH_3	11.2	0.90, t (7.3)	11.1	0.81[a]
βCH_3	16.7	1.19, d (6.5)	16.5	0.80, d (6.7)
CO	172.2		170.5	
NH		9.73, d (8.2)		7.76[a]

[a] Multiplicity not determined due to overlapping signals/chemical shifts determined from 2D data. [b−d] Assignments are interchangeable.

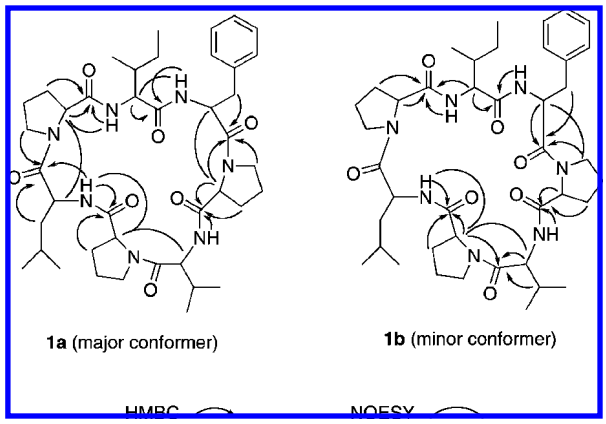

Figure 1. HMBC and NOESY correlations used to sequence **1a** and **1b**.

mational equilibria. One conformer was present in greater than 80% excess in MeOH-d_4, allowing for the structure of this major conformer to be assigned unambiguously.

Analysis of the ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR data for **2** run in MeOH-d_4 (Table 2) established that rolloamide B (**2**) was a heptapeptide consisting of serine, leucine, phenylalanine, two isoleucine, and two proline residues. Marfey's HPLC[6] and chiral GC[13,14] analyses of the hydrolysate of **2** confirmed the presence of the five amino acids and established the absolute configuration of each as L. The seven amino acid residues identified in rolloamide B (**2**) accounted for 13 of the 14 sites of unsaturation required by the molecular formula, and therefore, it was apparent that **2** was also a cyclic peptide.

Stylopeptide 1 (**4**), isolated from both a *Stylotella* and *Phakellia* species, has the same constituent amino acids as rolloamide B (**2**).[15] However, significant differences were observed in the ^{13}C NMR data recorded for **2** and reported in the literature for **4** (see Table 2 and Supporting Information), indicating that they were different. Interpretation of the HMBC and NOESY/ROESY data for rolloamide B (**2**) established the complete sequence of the amide linkages in **2** and confirmed that it was not identical to **4**. As outlined in the Supporting Information, HMBC correlations established amide linkages between the serine residue and two isoleucine residues. In addition, an HMBC correlation between the leucine α-proton at δ 4.68 and a proline carbonyl at δ 173.6 identified a -N-Pro^3-Leu^4-CO- unit. An HMBC correlation between the α-methine proton (δ 3.59) of a second proline residue and the carbonyl carbon (δ 174.1) of the phenylalanine residue established the presence of a -NH-Phe^5-Pro^6-CO- unit, which was confirmed by a NOESY/ROESY correlation that was observed between the same proline α-methine proton and the α-methine proton of the phenylalanine at δ 4.64. A NOESY/ROESY correlation between the Pro^3-δ_A proton resonance at δ 3.53 and the Ile^2-β resonance at δ 2.00 established an amide linkage between the carbonyl of Ile^2 and the amino nitrogen of Pro^3. This amide linkage was confirmed by HMBC correlations observed between both the Pro^3-α and δ_A proton resonances at δ 4.48 and 3.53, respectively, with the carbonyl resonance of Ile^2 at δ 174.4. The above data established the two partial structures NH-Ile^7-Ser^1-Ile^2-Pro^3-Leu^4-CO and -NH-Phe^5-Pro^6-CO-. The two remaining peptide bonds required by the molecular formula to complete the ring had to involve amide linkages between the amino nitrogen of the Phe^5 and the carbonyl of Leu^4 and between the carbonyl of Pro^6 and nitrogen of Ile^7. Weak HMBC correlations observed between the Phe^5 α-methine proton resonance (δ 4.64) and the carbonyl carbon resonance (δ 173.00) of the Leu^4 residue and between the Ile^7 α-methine proton resonance (δ 3.91) and the carbonyl resonance (δ 173.09) of Pro^6 confirmed the presence of these two amide bonds in the structure of rolloamide B (**2**). The ^{13}C NMR data of heptapeptide **2** established the Pro^3 peptide bond as *trans* and the Pro^6 as *cis* via the same arguments outlined for rolloamide A (**1**) above (Pro^3 δ Cγ 26.5 ppm, $\Delta\delta_{C\beta-C\gamma} = 3.8$ ppm; Pro^6 δ Cγ 22.4 ppm, $\Delta\delta_{C\beta-C\gamma} = 9.1$ ppm).[7−9]

Table 2. NMR Data for Rolloamide B (**2**) (600 MHz, MeOH-d_4)

atom #	δ_C	δ_H (J in Hz)
L-Ser^1		
α	54.9	4.51, m
β_A	64.0	3.86, dd (10.4, 2.2)
β_B		4.06, dd (10.4, 2.1)
CO	171.9	
L-Ile^2		
α	65.4	4.05, d (11.0)
β	34.2	2.00, m
γ_A	28.6	1.28, m
γ_B		1.78, m
δCH_3	10.7	0.99, t (7.4)
βCH_3	15.5	0.96, d (7.0)
CO	174.4	
trans L-Pro^3		
α	63.2	4.48, dd (8.8, 5.9)
β_A	30.3	1.78, m
β_B		2.25, m
γ_A	26.5	1.88, m
γ_B		1.94, m
δ_A	47.8	3.53, ddd (9.7, 6.5, 6.5)
δ_B		3.92[a]
CO	173.6	
L-Leu^4		
α	50.9	4.68, dd (8.1, 6.6)
β_A	41.0	1.25, m
β_B		1.74, m
γ	26.0	1.48, m
$\gamma_A CH_3$	23.4	0.88, d (6.7)
$\gamma_B CH_3$	22.9	0.93, d (6.9)
CO	173.0	
L-Phe^5		
α	56.2	4.64, dd (12.0, 4.6)
β_A	38.5	2.93, t (12.0)
β_B		3.33[a]
1	137.4	
2,6	130.8	7.26, m
3,5	130.2	7.33, m
4	128.6	7.30, m
CO	174.1	
cis L-Pro^6		
α	62.6	3.59, d (7.7)
β_A	31.5	0.70, m
β_B		1.87, m
γ_A	22.4	1.39, m
γ_B		1.67, m
δ_A	47.4	3.32[a]
δ_B		3.43, ddd (11.3, 11.3, 7.6)
CO	173.1	
L-Ile^7		
α	62.0	3.91, d (7.9)
β	37.3	2.10, m
γ_A	26.7	1.24, m
γ_B		1.56, m
δCH_3	11.4	0.87, t (7.5)
βCH_3	16.3	0.95, d (7.0)
CO	173.2	

[a] Multiplicity not determined due to overlapping signals/chemical shifts determined from 2D data.

Rolloamide A (**1**) exhibited significant growth suppression against a panel of histologically diverse cancer cells with IC_{50}'s of 0.4−5.8 μM, as shown in Table 3.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Jasco P1010 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a 600 MHz spectrometer with a 5 mm cryoprobe. ^{1}H chemical shifts were referenced to the residual C_5H_5N and MeOH-d_4 signals (δ 8.74, 7.58, 7.22, and 3.30 ppm, respectively), and ^{13}C chemical shifts were referenced to the C_5D_5N and MeOH-d_4 solvent peaks (δ 150.35, 135.91,

Table 3. Antiproliferative Activity of Rolloamide A (**1**) against Diverse Cancer Cell Lines

cell line	IC_{50} (μM)
Prostate	
LNCap	0.8
PC3MM2	4.7
PC3	1.4
DU145	0.85
Breast	
MDA468	0.38
MDA435	0.40
BT549	1.3
MDA361	5.8
MCF7	0.88
MDA231	2.2
Ovarian	
OVCAR3	0.17
SKOV3	1.6
Glioma	
U87MG	0.72
Renal	
A498	1.8

123.87, and 49.0 ppm, respectively). Low- and high-resolution FABMS were recorded with xenon as the bombarding gas and a thioglycerol sample matrix.

Animal Material. Specimens of *Eurypon laughlini* were collected by hand using scuba at a depth of 10−15 m from a rock overhang in June 1997 and sand flats in June 2002 off Rollo Head, 5 km south of Portsmouth, Dominica. Freshly collected sponge samples were frozen on site and transported frozen to Vancouver. Voucher samples have been deposited at the Zoological Museum of Amsterdam (ZMA POR 18519 and ZMA POR 17185, respectively).

Extraction of the 1997 Collection of *E. laughlini* and Isolation of Rolloamide A (1). The 1997 sponge sample (77 g) was cut into small pieces and immersed in and subsequently extracted repeatedly with MeOH (4 × 200 mL) at room temperature. The combined MeOH extracts were concentrated *in vacuo*, and the resultant brown gum was then partitioned between EtOAc (4 × 25 mL) and H_2O (100 mL). The combined EtOAc extract was evaporated to dryness to give 132 mg of pale yellow oil, which was chromatographed on a Sephadex LH-20 column eluing with 4:1 MeOH/CH_2Cl_2 to give a fraction exhibiting cytotoxic activity. The active material was further fractionated using a second Sephadex LH-20 column with 20:5:2 EtOAc/MeOH/H_2O as eluent to give a cytotoxic fraction (13.5 mg). Pure rolloamide A (**1**) (0.7 mg) was obtained from this mixture via reversed-phase HPLC using a C-18 5 μm column with a linear gradient of 4:1 H_2O/MeCN to MeCN over 60 min (flow rate 1 mL min^{-1}).

Extraction of the 2002 Collection of *E. laughlini* and Isolation of Rolloamide B (2). The 2002 sample of sponge (2.6 kg) was cut into small pieces and immersed in and subsequently extracted repeatedly with MeOH (4 × 4 L) at room temperature. The combined MeOH extracts were concentrated *in vacuo*, and the resultant brown gum was then partitioned between EtOAc (4 × 250 mL) and H_2O (1 L). The combined EtOAc extract was evaporated to dryness to give 6.3 g of brown oil, which was partitioned between hexanes (3 × 175 mL) and 4:1 MeOH/H_2O (700 mL). The combined MeOH/H_2O extract was evaporated to dryness to give 3.7 g of an amorphous, brown solid, which was chromatographed on a Sephadex LH-20 column eluted with 4:1 MeOH/CH_2Cl_2 to give an early eluting fraction (264.4 mg). This material was further fractionated using reversed-phase Si gel flash chromatography employing a step gradient from 1:1 MeOH/H_2O to MeOH with a final CH_2Cl_2 wash. A 30.8 mg fraction, eluting with 1:1−7:3 MeOH/H_2O, contained a mixture of dominicin[3] and rolloamide B (**2**). Pure rolloamide B (**2**) (0.8 mg) was obtained from this mixture via C_{18} reversed-phase HPLC using a 5 μm 25 × 0.94 cm column with 1:1 MeCN/H_2O as eluent, followed by a second HPLC purification using the same column but with 13:9 H_2O/MeCN as eluent.

Rolloamide A (1): clear glass; $[\alpha]^{25}_D$ −86.5 (*c* 0.93, MeOH); ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; positive ion HRFABMS $[M + H]^+$ *m/z* 764.4723 (calcd for $C_{41}H_{62}N_7O_7$, 764.4715) and $[M + Na]^+$ *m/z* 786.4532 (calcd for $C_{41}H_{61}N_7O_7Na$, 786.4534).

Rolloamide B (2): white, amorphous solid; $[\alpha]^{25}_D$ −113 (*c* 1.9, MeOH); ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 2; positive ion HRFABMS $[M + H]^+$ *m/z* 768.4677 (calcd for $C_{40}H_{62}N_7O_8$, 768.4664) and $[M + Na]^+$ *m/z* 790.4500 (calcd for $C_{40}H_{61}N_7O_8Na$, 790.4483).

Hydrolysis of Rolloamides A (1) and B (2). Purified peptides [**1** (200 ug, 0.26 μmol) or **2** (200 μg, 0.26 μmol), independently] were hydrolyzed in 0.5 mL of 6 N HCl (freshly distilled, constant boiling HCl) at 108 °C with stirring for 16 h in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolysate by repeated evaporation from H_2O (3 × 0.4 mL). The resultant hydrolysate mixture for **2** was split into two equal portions.

Derivatization of Amino Acids with Marfey's Reagent and HPLC Analysis.[6] To a 0.5 mL vial containing 2.0 μmol of the pure amino acid standard in 40 μL of H_2O was added 2.8 μmol of *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanine amide (FDAA) in 80.0 μL of acetone followed by 20 μL of 1 N $NaHCO_3$. The mixture was heated for 1 h at 40 °C. After cooling to RT, 10 μL of 2 N HCl was added and the resulting solution was filtered through a 4.5 μm filter and stored in the dark until HPLC analysis.

To the hydrolysate mixture of rolloamide A (**1**) in 31 μL of H_2O was added 2.6 μmol of FDAA in 73 μL of acetone, and the mixture was treated as described above to form the Marfey derivatives. Similarly, to half of the hydrolysate mixture of rolloamide B (**2**) in 15 μL of H_2O was added 1.8 μmol of FDAA in 36 μL of acetone, and the mixture was treated as described above to form the Marfey derivatives. In each case a 2 μL aliquot of the resulting mixture of Marfey derivatives was analyzed by reversed-phase HPLC.

For rolloamide A (**1**) a C-18 5 μm, 150 × 2.1 mm column with a linear gradient of (A) 97:3 triethylammonium phosphate (50 mM, pH 1.5)/MeCN and (B) MeCN with 0% B at start to 27% B over 55 min (flow rate 1 mL min^{-1}) was used to separate the FDAA derivatives with UV detection at 340 nm. Each peak in the chromatographic trace was identified by comparing its retention time and photodiode array UV spectrum with that of the FDAA derivative of the pure amino acid standard and by co-injection. In all cases a peak at 34.49 min was observed, which was attributed to excess FDAA. Retention times (min) are given in parentheses: L-Pro (38.77), D-Pro (40.09), L-Val (47.58), D-Val (51.35), L-Ile (54.98), L-*allo*-Ile (55.09), L-Leu (55.55), L-Phe (55.55), D-Phe (57.31), D-Ile (58.47), D-*allo*-Ile (58.62), D-Leu (59.08).

For rolloamide B (**2**) a C-18 5 μm, 250 × 4.6 mm column with a linear gradient of (A) 9:1 triethylammonium phosphate (50 mM, pH 3.0)/MeCN and (B) MeCN with 0% B at start to 40% B over 55 min (flow rate 1 mL min^{-1}) was used to separate the FDAA derivatives with UV detection at 340 nm. Each peak in the chromatographic trace was identified by comparing its retention time and photodiode array UV spectrum with that of the FDAA derivative of the pure amino acid standard and by observation of their coelution, upon co-injection. In all cases a peak at 43.67 min was observed, which was attributed to excess FDAA. Retention times (min) are given in parentheses: L-Ser (32.71), D-Ser (33.07), L-Pro (40.92), D-Pro (42.76), L-Ile (54.04), L-*allo*-Ile (54.04), L-Leu (55.04), L-Phe (55.25), D-Phe (59.05), D-Ile (59.61), D-*allo*-Ile (59.61), D-Leu (60.02).

PFP-IPA Derivatization of Amino Acids and Chiral GC Analysis.[13,14] Acetyl chloride (1.25 mL) was slowly added to 2-propanol in an ice bath. The resulting isopropyl acetate solution (250 μL) was added to half of the dried acid hydrolysate of rolloamide B (**2**) and 1−2 mg of the pure dry amino acid standard, each in a 5 mL screw-capped vial. The vials were heated to 110 °C for 45 min. Excess reagent was removed under a stream of N_2. After cooling the vials in an ice bath, CH_2Cl_2 (250 μL) and pentafluoropropyl anhydride (100 μL) were added and the vials heated to 110 °C for 15 min. Excess reagent was evaporated under dry N_2, and CH_2Cl_2 (200 μL) was added to each vial. A 2 μL aliquot of each sample was analyzed by chiral GC.

Through a combination of both Marfey's HPLC[6] and chiral GC analysis[13,14] it was possible to determine the complete configuration of the isoleucine in the hydrolysate mixture of rolloamide B (**2**) as L (in Marfey's HPLC method used for rolloamide B (**2**) the L- and L-*allo*-isoleucine are unresolvable, as are the D- and D-*allo*-isoleucine, and in the GC method the L-*allo*- and D-isoleucine are unresolvable).

Tumor Cell Growth Inhibition Assay. All human tumor cell lines were obtained from ATCC. The DU145, MDA468, MDA435, BT549, MDA361, MCF7, MDA231, U87MG, and A498 cell lines were maintained on MEM medium (Cellgro cat# 10-010-CV), and the LNCap, PC3MM2, PC3, OVCAR3, and SKOV3R cell lines were

maintained on PMI medium (Cellgro cat# 10-040-CV). Basal media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco cat# 10082) and penicillin−streptomycin (1:100 dilution from stock (Cellgro cat# 30-002-CI). MEM was also supplemented with sodium pyruvate (1:100 dilution from stock, Cellgro cat# 25-000-CI). To determine the cytotoxicity of rolloamide A (**1**), tumor cells were plated in 96 wells at 1000 to 3000 cells per well (predetermined for each line based on respective growth rate) on day 0. Rollamide A (**1**) was dissolved in DMSO as a concentrated stock and then diluted in culture media immediately before it was dosed to cells. Rollamide A (**1**) was tested at eight concentrations: 15, 5, 1.67, 0.56, 0.185, 0.062, 0.021, 0.007 μg mL^{-1} with an $N = 1$. Control cells on the same 96-well plate received vehicle DMSO that was similarly diluted. In all cases, the final DMSO in cells was 0.3% or lower (0.0002 to 0.3%), which did not impact cell growth. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] assays were performed using an assay kit purchased from Promega Corp. (Madison, WI) following the protocol supplied with the kit. On the day of the MTS assay (3 days after rolloamide treatment, 4 days after cell plating), the vehicle-treated cells for all cell lines were subconfluent. This protocol allows for testing of drug effect in log-phase proliferating cells. In the particular assays in which rollamide A was tested, several other known agents were also tested, and these produced the expected growth inhibitory effects. For measuring MTS assay results, plates were read in a 96-well plate reader (THERMO$_{max}$ microplate reader, Molecular Devices). The assay plates were read using the Softmax Pro-program at a single wavelength of 490 nm. In each 96-well assay plate, there were blank wells (no cells), and the A490 value from blank wells was applied for background subtraction. There was a linear range for the MTS assay, and all rollamide A (**1**) assays were performed within this linear range, where they gave normal dose−response curves. IC$_{50}$'s were determined by manual calculation from the experimental dose−response with 8 data points.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compounds **1** and **2**; NOESY and HMBC spectra for compound **1**; table of NMR data for compounds **2** and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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