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Gymnangiamide, a Cytotoxic Pentapeptide from the Marine Hydroid *Gymnangium regae*

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A cytotoxic aqueous extract from the marine hydroid *Gymnangium regae* provided a novel linear pentapeptide, designated gymnangiamide (**1**). The planar structure of **1** was elucidated by interpretation of spectral data as well as chemical degradation and derivatization studies. In addition to the amino acids isoleucine and phenylserine, this peptide contained *N*-desmethyldolaisoleuine, *O*-desmethyldolaproine, and α-guanidino serine, three residues that have not previously been reported in a natural product. The absolute configurations of the constituent amino/guanidino acids were determined by chemical degradation and derivatization, followed by HPLC and LC-MS comparison with authentic standards. Gymnangiamide (**1**) was moderately cytotoxic against a number of human tumor cell lines in vitro.

Introduction

Cnidarians have been the subject of extensive chemical study resulting in the isolation of a large number of unique secondary metabolites. Most of these studies have focused on soft corals and gorgonians, while relatively little attention has been given to marine hydroids (class: Hydrozoa). Compounds that have been reported from marine hydroids include steroids[1−4] and other terpenoids,[5] fatty acid derivatives,[6,7] antimicrobial and cytotoxic aromatic polyketides,[8−12] β-carbolines,[13] and dithiocarbamate[14] and piperidinol[15] containing metabolites with antifeedant activity. It has been suggested that the relatively modest amount of chemical data published on hydroids is due to the difficulty of collection and identification, and not due to a lack of chemical diversity within these organisms.[2,9]

An aqueous extract of the marine hydroid *Gymnangium regae* Jaderholm, collected in the Philippines, was selected for chemical analysis based on the pattern of differential cytotoxicity it produced in the U.S. National Cancer Institue (NCI)'s 60 cell line antitumor screen.[16,17] Cytotoxicity-guided fractionation of the extract led to the isolation of a novel cytotoxic linear pentapeptide, which has been named gymnangiamide (**1**). Of the five residues that comprise this peptide, two were unusual amino acids that are reported for the first time from a natural source, and one is a rare α-guanidino acid that has not previously been found in a naturally occurring peptide. This report represents the first chemical investigation of the genus *Gymnangium* and the first description of a peptide from a hydroid.

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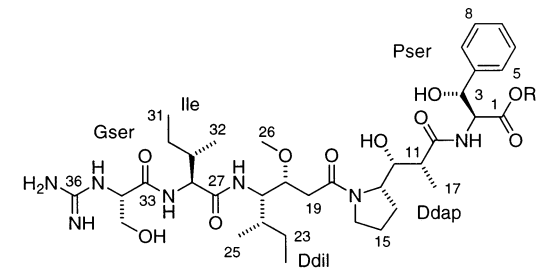
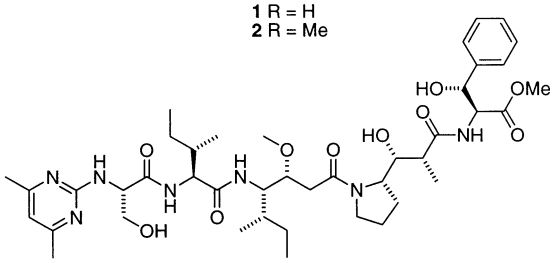


1 R = H
2 R = Me



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Results and Discussion

A 12.8-g portion of the aqueous extract from *Gymnangium regae* was dissolved in H_2O, applied to a wide-pore C_4 vacuum liquid chromatography column, and sequentially eluted with 100% H_2O, H_2O−MeOH (2:1), H_2O−MeOH (1:2), and 100% MeOH. The H_2O−MeOH (1:2) fraction was chromatographed on Sephadex LH-20 eluted with MeOH−H_2O (7:3) and final purification by gradient elution C_{18} HPLC with increasing concentrations of MeCN in H_2O provided 2.4 mg of gymnangiamide (**1**). The positive ion FABMS of **1** exhibited pseudomolecular ions at *m*/*z* 750.5 for $[M + H]^+$ and *m*/*z* 772.5 for $[M + Na]^+$, which indicated a nominal molecular weight of 749. The molecular formula was established as $C_{36}H_{59}N_7O_{10}$ by high-resolution FABMS of a CsI-doped sample ($[M + Cs]^+$ *m*/*z* 882.3374, calcd for $C_{36}H_{59}N_7O_{10}Cs$ 882.3377) in combination with a detailed analysis of the ^1H and ^{13}C NMR data. A cursory examination of the ^1H and ^{13}C NMR spectra indicated that there were approximately twice as many signals present as could be accounted for by the molecular formula. Changing either the solvent composition or temperature of the sample altered the ratio of the signals present, indicating that there were multiple conformers of **1** in solution, similar to that observed for other peptides.[18,19] No experimental conditions were found which could effectively coalesce the NMR signals into a single set of resonances. The data reported below and in Table 1 are for the most abundant form of **1**.

The presence of 5 carbonyl resonances in the ^{13}C NMR spectrum, and 4 amide NH doublets (δ 7.45−8.28) together with a number of upfield methyl doublets and triplets in the ^1H NMR spectrum of **1** (Table 1) suggested it was a peptide. Extensive 1D and 2D NMR analyses of **1** established the presence of the amino acids isoleucine (Ile) and phenyserine (Pser) together with the unusual amino acids, *N*-desmethyldolaisoleuine (Ddil) and *O*-desmethyldolaproine (Ddap), and a single guanidino acid, α-guanidino serine (Gser). The structures of the unusual residues were assigned as follows. The α-methylene of Ddil was established by HMBC correlations between the methylene protons (δ 2.26, 2.18) and the carbonyl at δ 171.62 and methine carbons at δ 54.37 and δ 78.79. COSY correlations were observed between the α-methylenes and the β-oxymethine (δ 4.06), which in turn coupled to the γ-methine proton (δ 4.09), which also correlated with the amide proton at δ 8.28. This established the γ-amino acid Ddil backbone. The side chain was assigned on the basis of 1D-TOCSY, COSY, and HMBC data, while a methoxyl group was placed at the β position in Ddil by an HMBC correlation between the methyl protons (δ 3.28) and the β-oxymethine carbon. The Ddap residue was similarly defined by COSY correlations from the α-methine (δ 2.36) to a methyl group (δ 1.24) and the β-oxymethine (δ 3.79), which in turn was coupled to the γ-methine (δ 3.22). The heterocyclic ring of Ddap was defined by 1D-TOCSY, COSY, and HMBC correlations. These unusual residues are closely related to residues found in the dolastatin family of peptides except that in both there is reduced methylation; Ddil lacks the *N*-methyl group found in dolaisoleuine (Dil), and Ddap lacks the β-methoxy group found in dolaproine (Dap).[18−20] To the best of our knowledge, this is the first isolation of these desmethyl amino acid derivatives from a natural source.

The final residue in **1** was the unusual guanidino acid, α-guanidino serine. HMBC and COSY correlations from the α-methine proton (δ 4.24) to the primary alcohol group (δ 3.81, 3.85) established the presence of a serine moiety. HMBC correlations from both the α-methine proton and the NH proton doublet at δ 7.45 to a carbon at δ 159.2 suggested the presence of a guanidine functionality. Support for this assignment was provided by treatment of the peptide on TLC with either FCNP (ferricyante-sodium nitroprusside) or Sakaguchi spray reagents[21] which both tested positive for the presence of guanidine. Guanidino analogues of common amino acids are quite rare in nature; however, unusual α-guanidino acids have been encountered as constituents of phenganomycin and other related antibiotic peptides isolated from terrestrial fungi.[22,23] Gymnangiamide (**1**) represents the first marine peptide found to contain an α-guanidino acid residue. The five residues established above accounted for all of the unsaturations present in **1**, indicating that it was a linear peptide with a free C-terminus and a guanidine at the N-terminus. This was confirmed by formation of the carboxymethyl ester **2** on treatment with (trimethylsilyl)diazomethane[24] and formation of the

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TABLE 1. NMR Spectral Data for the Gymnangiamide (1) in CD_3OH[a]

	^{13}C[b]	^{1}H[c]	mult (*J*, Hz)	TOCSY[d]	HMBC[d]	ROESY
				phenylserine (Pser)		
1	173.26 (s)					
2	59.31 (d)	4.86	m	H3, NH	C1, C3, C10	H3, H5,9
3	73.15 (d)	5.40	d (2.2)	H2, NH	C4, C5,9	H2, H5,9
4	142.86 (s)					
5,9	126.73 (d)	7.41, 2H	bd (7.7)	H6,8, H7	C3, C7	H2, H3
6,8	129.09 (d)	7.30, 2H	bd (7.7)	H5,9, H7	C4, C6,8	
7	128.16 (d)	7.22	m	H5,9, H6,8	C5,9	
NH		8.25	d (9.2)	H2, H3	C10	H2, H11
				O-desmethyldolaproine (Ddap)		
10	178.02 (s)				C10	
11	45.15 (d)	2.36	m	H12, H17	C12, C17	H14b, H17, NH (Pser)
12	74.80 (d)	3.79	m	H11, H13, H14ab, H17	C11, C13, C14, C17	H13, H17, H19a
13	60.88 (d)	3.22	m	H12, H14ab, H15ab, H16, H17		H12, H17, H19ab
14	26.10 (t)	1.24	m	H13, H15b, H16		
		1.77	m	H13, H15a, H16, H17	C12, C15	H11, H15a, H16
15	24.30 (t)	1.43	m	H13, H14ab, H16	C14, C16	
		1.81	m	H13, H14ab, H16, H17	C14	
16	48.27 (t)	3.12	m	H13, H14ab, H15ab, H17	C14, C15	H14b
17	16.29 (q)	1.24, 3H	d (6.6)	H11, H12, H13, H14b, H15b, H16	C10, C11, C12	H11, H12, H13
				N-desmethyldolaisoleuine (Ddil)		
18	171.62 (s)					
19	35.44 (t)	2.26	dd (~1, 15.9)	H20, H21, H22, NH	C18	H12, H13, H20, H22a
		2.18	dd (8.8, 15.9)	H20, H21, H22, NH	C18, C20	H13
20	78.79 (d)	4.06	m	H19ab, H21, H22, H23ab, H25	C22	H25, H26
21	54.37 (d)	4.09	m	H19ab, H20, H22, H23ab, H25, NH	C19, C20, C22, C23, C27	H22, H25, H26, NH
22	36.80 (d)	1.44	m	H21, H23ab, H24, H25, NH	C23	
23	26.74 (t)	1.58	m	H21, H22, H24	C22, C24, C25	
		1.14	m	H19a, H21, H22	C22, C24, C25	
24	10.98 (q)	0.84, 3H	m	H21, H22, H23ab	C22, C23	
25	16.18 (q)	0.92, 3H	d (7.0)	H21, H22, H23ab, NH	C21, C22, C23	H20
26	57.30 (d)	3.28, 3H	s		C20	H20
NH		8.28	d (9.9)	H19ab, H21, H22, H23ab, H25	C27	H28
				isoleucine (Ile)		
27	174.43 (s)					
28	59.78 (d)	4.29	m	H29, H30ab, H31, H32, NH	C27, C29, C30, C32, C33	H29, H32, NH (Ddil)
29	38.05 (d)	1.83	m	H28, H30ab, H31, H32, NH		H28, H30b, H32
30	25.79 (t)	1.23	m	H28, H29, H31, H32, NH	C29, C31, C32	
		1.59	m	H28, H29, H31, H32, NH	C29, C31, C32	H29
31	11.31 (q)	0.87, 3H	m	H28, H29, H30ab, H32, NH	C29, C30	H30b
32	16.35 (q)	1.03, 3H	d (6.6)	H28, H29, H30ab, H31, NH	C28, C29, C30	H28, H29
NH		7.93	d (8.0)	H28, H29, H30ab, H31, H32	C33	H29, H34
				α-guanidio serine (Gser)		
33	170.03 (s)					
34	58.38 (d)	4.24	m	H35ab, NH	C33, C35, C36	H35ab, NH (Ile)
35	63.92 (t)	3.81	m	H34, NH	C33, C34	H34
		3.85	dd (4.4, 10.6)	H34, NH		H34
36	159.17 (s)					
NH		7.45	d (7.7)	H34, H35ab	C35, C36	

[a] Spectra recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C and referenced to the residual solvent signal (δ_H 3.30, δ_C 49.0); only data for the most abundant conformer are reported. [b] Multiplicities determined by using the DEPT pulse sequence. [c] With geminal protons the upfield resonance is designated "a" and the downfield resonance "b". [d] Geminal couplings are not shown.

dimethylpyrimidine derivative **3** (*m/z* 828.5 $[M + H]^+$) on subsequent treatment of **2** with 2,4-pentanedione.[25]

The linear sequence of **1** was established by a combination of NMR (Figure 1) and MS/MS methods (Figure 2). The α-methine proton (δ 4.24) of the Gser residue showed a correlation to the carbonyl at δ 170.03 as did the amide proton (δ 7.93) of Ile, which placed these two residues in sequence at the N-terminus. Similarly, the amide proton (δ 8.28) of Ddil showed an HMBC correlation to the Ile carbonyl at δ 174.43 placing it next in the sequence. The Ddap residue was connected to Ddil based on a ROESY correlation from the β (δ 3.79) and γ (δ 3.22) protons of the former to the α protons (δ 2.18, 2.26) of the Ddil. Finally, Pser was placed at the C-terminus by

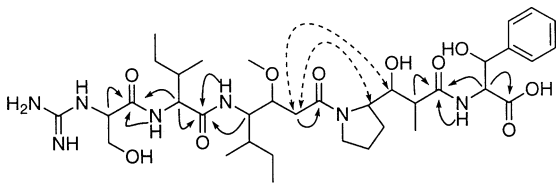

FIGURE 1. Key HMBC (solid arrows) and ROESY (dashed arrows) correlations for **1**.

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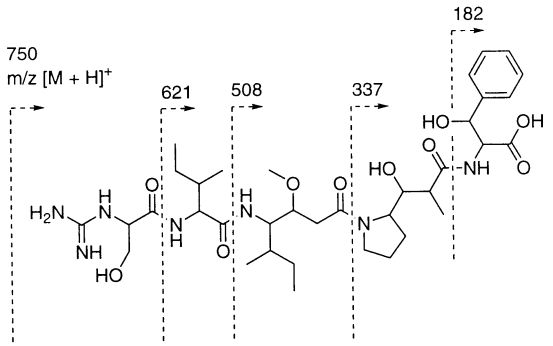

FIGURE 2. Positive ion FAB MS/MS fragmentation pattern for **1**.

an HMBC correlation from the Pser amide proton (δ 8.25) to the Ddap carbonyl at δ 178.02. The linear sequence was confirmed by MS/MS fragmentation analysis as shown in Figure 2.

Unambiguous assignment of the absolute stereochemisty of **1** required different procedures for each of the constituent guanidino or amino acids. Guanidino serine was not detected in the acid hydrolysate of **1** after derivatization of the hydrolysate with either *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA; Marfey's reagent)[26] or 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC).[27] Derivatization of a synthetic standard of Gser also failed to provide an observable derivative. This was overcome by treating the peptide with hydrazine hydrate, either before or after complete acid hydrolysis, to convert the guanidine group to a free amine and thereby allow direct comparison with authentic standards of D- and L-Ser.[28] When **1** was treated with hydrazine prior to acid hydrolysis there was good recovery of Ser but complete racemization was observed. When acid hydrolysis of **1** was performed first, the yield was reduced but L-Ser was recovered in greater than 10:1 excess over D-Ser. Although acid hydrolysis of the intact peptide prior to cleavage of the guanidine moiety resulted in a significant loss of signal, subsequent treatment with hydrazine proceeded with reduced racemization. Similar results were obtained with the carboxymethyl derivative **2**. However, with compound **2** a 6:1 excess of L-Ser over D-Ser was observed when hydrazine treatment was performed after hydrolysis, while hydrazine treatment prior to hydrolysis provided a 3:1 excess of L- relative to D-Ser. These results allowed assignment of the stereochemistry of the α-position as (*S*), which indicated that L-guanidino serine was present in **1**. The reduced recovery when acid hydrolysis occurred before treatment with hydrazine is probably due to decomposition of the guanidino acid to a keto-acid.[23]

Acid hydrolysis of **1** and derivatization with FDAA allowed the stereochemistry at the α position of isoleucine to be determined, but there was insufficient resolution of isoleucine and *allo*-isoleucine to allow unambiguous determination of the stereochemistry at the β position. The four stereoisomers of isoleucine were readily resolved by ligand-exchange chromatography on a (D)-penicillamine column eluted with 2 mM Cu^{2+} and thus the isoleucine residue in **1** was determined to be L-Ile (28*S*,29*S* using the numbering scheme for **1**).

Biosynthetic considerations suggested that Ddap might have the same stereochemisty as the analagous residue Dap, found in the dolastatins. Thus, the Ddap residue in **1** was methylated to allow direct comparison with an authentic sample of Dap. The intact peptide **1** was methylated with NaH/MeI, hydrolyzed, and treated with either FDAA or GITC. Both the FDAA and GITC derivatives of the methylated Ddap from **1** had identical retention times by LC-MS with similarly derivatized authentic Dap, which allowed assignment of the stereochemistry of Ddap as 11*R*,12*R*,13*S*. Under these conditions Ddil did not methylate so **1** was first hydrolyzed, and the free amino acids were acetylated and then methylated as described above. A shortened acid hydrolysis removed the acetate and the product was then derivatized with either FDAA or GITC. LC-MS of methylated Ddil derivatized with either FDAA or GITC showed retention times identical with that of authentic Dil and established the stereochemistry of Ddil as 20*R*,21*S*,22*S*.

Of the four possible phenylserine stereoisomers, the only standard available was a commercial mixture of the D- and L-*threo* diastereomers. Acid-catalyzed racemization of the commercial mixture by extended heating in HCl provided the D- and L-*erythro* isomers. Initial results showed that the FDAA derivatives of the four Pser diastereomers could be clearly resolved by LC-MS and that the isomer present in the hydrolysate of **1** corresponded to one of the *threo* isomers. The commercial DL-*threo*-Pser mixture was derivatized with FMOC-Cl and the FMOC protected D- and L-*threo* diastereomers were separated by chiral HPLC on a Sumipax OA-3100 column. Deprotection and cleanup of the resulting fractions provided the individual D- and L-*threo* compounds which were identified by their optical rotations. Comparison of the acid hydrolyzate of **1** with the FDAA derivatives of the individual *threo*-Pser isomers indicated that L-*threo*-Pser (2*S*,3*R*) is present in **1**.

Gymnangiamide (**1**) was tested for cytotoxic activity by using a panel of 10 human tumor cell lines (see the Experimental Section). Significant differential growth inhibition, with IC_{50} values that ranged from 0.46 to >11 μg/mL, was observed, which was consistent with other cytotoxins that are known to target tubulin. Gymnangiamide (**1**) is structurally related to the potent tubulin interacting pentapeptide dolastatin 10 and its congeners; however, it is significantly less cytotoxic than the dolastatins.[18−20,29,30] The dolastatins that have been isolated to date are either cyclic or have their N-termini, and often their C-termini blocked. The N-termini of linear dolastatins are usually found as *N*,*N*-dimethyl derivatives while the C-termini are variously modified and do not typically exist as the free acid. Compound **1**, with an N-terminal guanidine moiety and a C-terminal carboxylic acid, would exist as a zwitterion in cell culture media. The ionic character of **1** could result in reduced uptake by the cells and therefore reduced cytotoxicity. A comparative study showed that the C-terminally blocked derivative **2** and the C- and N-terminally blocked deriva-

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tive **3** were approximately 7-fold less potent cytotoxins relative to the native peptide **1**. This indicated that the amphoteric nature of **1** alone is not responsible for its modest cytotoxic activity relative to that of the dolastatins. An additional feature of gymnangiamide (**1**) is that both the dolaisoleuine and dolaproine residues that are common among the dolastatins were found for the first time in nature as their desmethyl deriviatives. This difference in methylation pattern between **1** and the dolastatins may account for some of its reduced cytotoxic potency. In a synthetic study, Miyazaki et al.[31] found that the lack of methylation observed in the Ddil or Ddap residues did not lead to significant loss of activity when present individually, but they did not test analogues that were missing methyl groups at both positions simultaneously.

Another point of interest raised by the similarity between the dolastatins and **1** is the biosynthetic origin of **1** in *G. regae*. Although originally isolated from the marine mollusc *Dolabella*, the dolastatins are now believed to be of cyanobacterial origin, accumulating in *Dolabella* as a result of dietary intake. In fact, a number of dolastatins including dolastatin 10 have now been isolated directly from cyanobacteria.[19,29,32,33] The structural similarity between **1** and the dolastatins, suggests that the ultimate origin of gymnangiamide (**1**) may be from cyanobacteria in the diet or those living in association with *Gymnangium regae*.

Experimental Section

Animal Material. Samples of the feathery marine hydroid *Gymnangium regae* (order Conica, family Aglaopheniidae) were collected in 1994 at a depth of −12 m off Luzon in the Philippines. A voucher specimen for this collection (0CDN2622) is maintained at the Smithsonian Institution, Washington, D.C. The genus *Gymnangium* was recently synonymous with *Aglaophenia*. Members of this genus are found in the Indo Pacific, Caribbean, Mediterranean, and North Atlantic regions and are fan or feather-like in appearance.

Extraction and Isolation. The samples were frozen, ground into a coarse powder (369.8 g, dry weight), and extracted with H_2O to yield 45.0 g of crude extract after lyophilization. A 12.8-g portion of the crude aqueous extract was dissolved in H_2O, applied to a wide-pore C_4 column (5 cm × 8 cm), and sequentially eluted with H_2O (100%), H_2O−MeOH (2:1), H_2O−MeOH (1:2), and MeOH (100%) under vacuum. The H_2O−MeOH (1:2) fraction (127.1 mg) was purified by gel permeation chromatography on Sephadex LH-20 (3 cm × 100 cm) eluting with MeOH−H_2O (7:3), followed by C_{18} HPLC (Dynamax, 1 cm × 25 cm, 8 μm) employing a linear gradient from 0 to 100% MeCN in H_2O (+0.1% TFA) over 30 min (3 mL/min) with detection at 220 nm yielding compound **1** (2.4 mg, t_R 17.4 min). Reisolation from associated fractions and extraction of the remaining crude material provided an additional 4.8 mg of **1** (total yield 0.0068% based on dry weight).

Gymnangiamide (1). Amorphous white solid; $[\alpha]_D$ −32.5 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ϵ) 258 (2.99) nm; IR (film, NaCl) υ_{max} 3333, 1655, 1542, 1455, 1204, 1139, 843, 802, 723 cm^{-1}; ^1H NMR and ^{13}C data, see Table 1; FABMS, positive ion, *m/z* 750.5 $[M + H]^+$, *m/z* 772.5 $[M + Na]^+$; HRFABMS, CsI-doped sample, *m/z* 882.3374 $[M + Cs]^+$, calcd for $C_{36}H_{59}N_7O_{10}Cs$ 882.3377 (Δ −0.3 mmu). A positive result for the presence of guanidine was achieved by spotting **1** (5−50 μg) onto TLC plates which were subsequently treated with either FCNP or Sakaguchi spray reagents.[21]

Complete Acid Hydrolysis. The peptide (50−200 μg per hydrolysis) was dissolved in 6 N HCl (constant boiling, 0.5 mL), degassed, and heated at 105−108 °C for 16 h under vacuum. The solvent was removed under N_2, and the residue washed with H_2O, frozen, and lyophilized to give the hydrolysis product.

Preparation and Analysis of FDAA (Marfey's) Derivatives.[26] Acid hydrolysate or appropriate standards were transferred to an LC-MS vial and dried. The residue was dissolved in 15 μL of 6% TEA (in MeCN−H_2O, 1:1) and treated with 7.5 μL of 1% *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA) in acetone at 40 °C for 60 min. The reaction mixture was diluted with 45 μL of ddH_2O and analyzed by LC-MS. A 20-μL aliquot was applied to a C_8 column (Zorbax 300SB, 2.1 mm × 150 mm, 5 μm) eluting with 5% $MeCO_2H$ for 5 min followed by a linear gradient (0−50%) of MeCN in 5% $MeCO_2H$ over 25 min (0.25 mL/min, 50 °C). For analysis of derivatized serine the elution was 15 min with 5% $MeCO_2H$ followed by 0−50% MeCN over 15 min. FDAA derivatized amino acids were detected by absorption at 340 nm and by MSD (positive ion, mass range 100−1000 Da).

Preparation and Analysis of GITC Derivatives.[27] Acid hydrolysate or appropriate standards were transferred to an LC-MS vial and dried. The residue was taken up in 15 μL of 6% TEA (in MeCN−H_2O, 1:1) and treated with 7.5 μL of 1% 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) in acetone at room temperature for 10 min. The reaction mixture was diluted with 45 μL of ddH_2O and analyzed by LC-MS as described for FDAA derivatives above. GITC derivatized amino acids were detected by absorption at 254 nm and by MSD (positive ion, mass range 100−1000 Da).

Preparation of α-Guanidino Serine Standards. A solution of 210 mg of D-serine (2 mmol) and 293 mg of 1*H*-pyrazole-1-carboxamidine HCl (2 mmol) in 2 mL of 1.0 M Na_2CO_3 was stirred for 16 h at room temperature. The reaction mixture was then diluted with 6 mL of H_2O and extracted with 3 × 4 mL of Et_2O to yield α-guanidino D-serine in the aqueous layer. Standards of α-guanidino L-serine were prepared in an analogous manner starting with L-serine.

Stereochemical Determination of Guanidino Serine. Gymnangiamide (**1**) or its carboxymethyl derivative (**2**; 50 μg) was treated with 200 μL of 1:1 hydrazine hydrate−ddH_2O for 60 min at 95 °C.[28] The samples were cooled and dried by passage of a stream of N_2, and then washed with H_2O (0.5 mL), frozen, and lyophilized. The hydrazine-treated samples were then hydrolyzed with 6 N HCl as described above. In a parallel set of reactions acid hydrolysis was performed prior to treatment with hydrazine hydrate. The hydrolysis/hydrazinolysis products (15 μg) and authentic standards of D- and L-serine were derivatized with FDAA and analyzed by LC-MS. The retention times of the standards were t_R = 13.81 min for L-Ser and t_R = 15.36 min for D-Ser. The sample of **1** treated with hydrazine followed by hydrolysis provided racemic Ser [t_R = 13.48 and 15.07 min, ca. 1:1 ratio (area, EIC, *m/z* 358)]. The sample of **1** that was hydrolyzed prior to treatment with hydrazine gave L-Ser (t_R = 13.50) in greater than 10-fold excess over D-Ser but with significantly reduced overall yield. Compound **2** provided a 3:1 excess of L- over D-Ser (t_R = 13.77 and 15.39) when hydrazine treatment was followed by acid hydrolysis and a 6:1 excess of L- over D-Ser when acid hydrolysis preceded treatment with hydrazine (t_R = 13.78 and 15.32).

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Ligand Exchange Chiral HPLC of Isoleucine. The hydrolysis product (50 μg) in ddH_2O was applied to a ligand exchange column (Phenomenex, Chirex 3126 (D)-Penicillamine, 4.6 mm × 250 mm) eluting with 2 mM Cu^{2+} sulfate (1.0 mL/min) with detection at 254 nm. Isoleucine standards with retention times (min) shown in parentheses are as follows: L-*allo*-Ile (50.75), L-Ile (59.74), d-*allo*-Ile (85.81), and D-Ile (102.27). The observed retention time in the hydrolysate of **1** was 59.84 min corresponding to L-Ile.

Methylation and Absolute Stereochemistry of *O*-Desmethyldolaproine. An aliquot of **1** (100 μg) was dried in a small vial to which 100 μL of 0.25 M NaH in DMSO was added. The vial was purged with N_2, capped, and allowed to sit 1.5 h. MeI (5 μL) was then added, and the vial was again purged with N_2 and allowed to sit an additional 16 h in the dark. The reaction was quenched by the addition of 175 μL of ddH_2O and neutralized with 25 μL of 1.2 N HCl. The reaction mixture was dried under N_2 and then hydrolyzed with 6 N HCl as above. The hydrolysate (15 μg per reaction) was derivatized separately with FDAA and GITC and analyzed by LC-MS. The observed retention times for methylated Ddap in the hydrolysate were 27.86 min for FDAA and 24.68 min for GITC. An authentic standard of Dap[30] was similarly derivatized and analyzed by LC-MS, yielding retention times of 27.80 min for FDAA and 24.65 min for GITC.

Methylation and Absolute Stereochemistry of *N*-Desmethyldolaisoleucine. Methylation on the nitrogen of the Ddil residue in the intact peptide was not successful. The free amino acids obtained by acid hydrolysis (45 μg) were acetylated by treatment with acetic anhydride (200 μL) in 800 μL of MeOH and 40 μL of TEA for 60 min at room temperature. The acetylated amino acids were dried under N_2 in a small vial to which 300 μL of 0.05 M NaH in DMSO was added. MeI (5 μL) was added, the vial was purged with N_2 and capped, and the reaction mixture was stirred for 24 h at ambient temperature away from light. The reaction was quenched and neutralized with the addition of 300 μL of ddH_2O and 25 μL of 1.2 M HCl. The resulting solution was then dried under N_2 and hydrolyzed with 0.5 mL of 6 N HCl (constant boiling) for 60 min at 105−108 °C to remove the acetate. The hydrolysate was dried under N_2, brought up in 0.5 mL of H_2O, and lyophilized. The hydrolysate was then derivatized with either FDAA or GITC and analyzed by LC-MS. The observed retention times of methylated Ddil were 26.49 min for the FDAA and 27.53 min for the GITC derivative. An authentic standard of Dil[30] was similarly derivatized and analyzed by LC-MS, yielding retention times of 26.56 min with FDAA and 27.61 min with GITC.

Absolute Stereochemistry of Phenylserine. (a) Preparation of Phenylserine Standards. dl-*threo*-Phenylserine (76 mg) in 1 mL of 9% Na_2CO_3 and 90 mg of FMOC-Cl in 1 mL of dioxane were chilled on ice and combined. The mixture was allowed to warm to room temperature with stirring, which was continued for 60 min. The reaction mixture was then diluted with 8 mL of ddH_2O and extracted with ether (3 × 4 mL), discarding the organic layers. The aqueous layer was acidified (pH 2) with concentrated HCl to afford a white precipitate. The suspension was extracted with EtOAc (4 × 4 mL) and the organic layers combined and dried under N_2 to provide a mixture of FMOC-protected D- and L-*threo*-phenylserines.[31] The FMOC derivatives were separated by chiral HPLC on a Sumipax OA-3100 DNPAC-(S)-V column (Regis, 4.6 mm × 150 mm, 5 μm). Elution was isocratic, using 30 mM ammonium acetate in MeOH at 1 mL/min with detection at 265 nm. Chiral HPLC resulted in the recovery of two components, peak A (t_R = 6.91 min) and peak B (t_R = 7.26 min); peak B was subjected to a second round of purification. The individual derivatives were dried under N_2, 0.5 mL of ddH_2O was added, and the samples were lyophilized. The resulting powders were deprotected with 1 mL of 20% piperidine in DMF for 3 h at ambient temperature. The deprotecting reagents were removed under N_2 and the residue taken up in 1 mL of ddH_2O and extracted with ether (3 × 1 mL), discarding the organic layers. The aqueous layer was acidified with 3 drops of concentrated HCl (pH <2) and extracted with EtOAc (3 × 1 mL), discarding the organic layers. The aqueous layers were dried under N_2, brought up in 0.5 mL of ddH2O, and lyophilzed to provide D- and l-*threo*-phenylserine HCl. Peak A provided 1.4 mg of a relatively pure isomer (ca. 95% based on analysis of FDAA-derivatized sample) with $[\alpha]_D$ +22.1 (*c* 0.14, H_2O), which is in agreement for d-*threo*-phenylserine.[35,36] Peak B provided 3.4 mg of the isomer with $[\alpha]_D$ −20.3 (*c* 0.34, H_2O), which is in agreement for L-*threo*-phenylserine.[37] The *erythro* isomers were obtained by acid-catalyzed racemization of the commercial DL-*threo*-phenylserine. DL-*threo*-Phenylserine (200 μg) was heated 48 h in 6 N HCl (constant boiling) at 125−130 °C, and then dried under N_2 and lyophilized prior to analysis.

(b) Determination of Phenylserine Absolute Stereochemistry in 1. Acid hydrolysate of **1** (20 μg) and phenylserine standards were derivatized with FDAA and analyzed by LC-MS. Retention times (min) of FDAA-derivatized standards are the following: L-*threo*-phenylserine (21.04), d-*threo*-phenylserine (24.16), and *erythro*-phenylserine isomers (19.99, 23.14). The observed retention time for phenylserine in the hydrolysate of **1** was 21.03 min, corresponding to l-*threo*-phenylserine.

Preparation of Methylated Derivative 2.[25] Gymnangiamide (0.5 mg) was brought up in 1.0 mL of benzene and 250 μL of MeOH and treated with 150 μL of (trimethylsilyl)-diazomethane ($TMSCHN_2$). The reaction was stirred for 18 h at room temperature away from light. The mixture was dried under N_2, washed with 0.5 mL of MeOH, and again dried under N_2. The reaction mixture was analyzed by LC-MS: C_8 column (Zorbax 300SB, 2.1 mm × 150 mm, 5 μm) eluting with a linear gradient (0−100%) of MeCN in 5% $MeCO_2H$ over 60 min (0.20 mL/min, 40 °C). The carboxymethyl addition product (**2**) was observed (*m*/*z* 764.7 $[M + H]^+$, t_R = 21.33).

Preparation of the Dimethylpyrimdine Derivative 3.[22] Gymnangiamide (0.5 mg) was methylated as above and then further derivatized by treatment with 500 μL of 2,4-pentanedione containing 12 mg/mL of $NaHCO_3$ for 30 min at 130 °C to give the dimethylpyrimidinyl derivative **3**. The product was chromatographed by LC-MS as above (*m*/*z* 828.5 $[M + H]^+$, t_R = 25.17).

Cytotoxicity Assay. Chromatography fractions and purified **1** were made up in DMSO−H_2O (1:1) and assayed in an in vitro cytotoxicity assay against COLO-205 and OVCAR-3 cell lines to direct purification. Differential cytotoxicity was evaluated utilizing a panel of 10 human tumor cell lines. Following each cell line is the tumor type and IC_{50} observed for **1**: COLO-205 (colon, 4.7 μg/mL), H460 (lung, 0.46 μg/mL), K562 (leukemia, 11.5 μg/mL), MOLT-4 (leukemia, 5.8 μg/mL), A549 (lung, 5.8 μg/mL), and MALME-3M (melanoma, 9.6 μg/mL). With the LOX (melanoma), OVCAR-3 (ovarian), and SNB-19 (cns) cell lines, growth inhibition was observed but an IC_{50} could not be determined, while no inhibition was observed with MCF-7 (breast) at a high test concentration of 15 μg/mL. Relative cytotoxicity for compounds **1**−**3** was determined by using the IC-2^{WT} murine cell line,[38] which provided IC_{50} values for **1** (1.7 μg/mL), **2** (11.2 μg/mL), and **3** (12.5 μg/mL). Experimental details of the 2-day antiproliferation assay employed are published elsewhere.[39]

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Supporting Information Available: General experimental details and NMR spectra for gymnangiamide. This material is available free of charge via the Internet at http://pubs.acs.org.

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