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Namalides B and C and Spumigins K–N from the Cultured Freshwater Cyanobacterium *Sphaerospermopsis torques-reginae*

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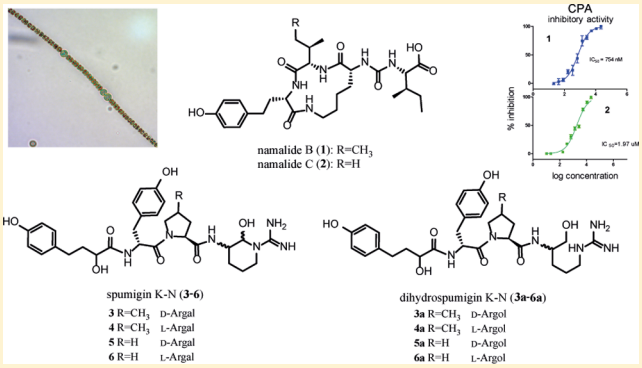
Supporting Information

ABSTRACT: Chemical investigations of the terrestrial cyanobacterium *Sphaerospermopsis torques-reginae* ITEP-024 from northern Brazil afforded namalides B (**1**) and C (**2**), the first analogues of this anabaenopeptide-like metabolite to be described. Four other related peptides (**3**–**6**), termed spumigins K–N, were also identified. Planar structures and absolute configurations for **1**, **2**, and **3a**–**6a** were deduced by a combination of 2D NMR, HRMS analysis, and Marfey's methodology. Spumigins K–N (**3**–**6**) are the first examples of spumigins containing a 2-hydroxy-4-(4-hydroxyphenyl)-butanoic acid (Hhpba) in the N-terminal position. Compounds **1** and **2** inhibited carboxypeptidase A with IC_{50} values of 0.75 and 2.0 μM, respectively.



Cyanobacteria have proved to be an important source of novel bioactive metabolites with unique structural characteristics.[1−4] Antibacterial, antiviral, antifungal, anticancer, immunosuppressive, and protease inhibitory effects among other pharmacological properties have been reported for the great structural diversity of natural products coming from these microorganisms.[1,2,5,6] Many of these secondary metabolites are peptides, typically of nonribosomal biosynthetic origin, and commonly grouped within families according to their structural similarities such as anabaenopeptin, aeruginosin, cryptophycin, cyanopeptolin, cyclamide, microginin, or microviridin.[2,7]

As part of our interest in the search for new bioactive compounds from cyanobacteria we have been investigating the yet underexplored Brazilian microbiota.[8] *Sphaerospermopsis torques-reginae* ITEP-024, a toxic cyanobacterium collected in northeastern Brazil, was one of the selected strains. Morphological and phylogenetic analysis based on 16S rRNA gene sequences allowed the taxonomic identification of the isolate.[9] Previous preliminary studies on *S. torques-reginae* ITEP-024 revealed the production of four new spumigins.[10] These linear peptides, structurally related to aeruginosins, have been isolated from strains of the genera *Nodularia* and *Anabaena*.[11,12] Spumigins are characterized by a hydroxyphenyl lactic acid (Hpla) at position 1, typically homotyrosine (Hty) or homophenylalanine (Hph) at position 2, and an arginine derivative or lysine at the C-terminal end. Unlike aeruginosins, position 3 is occupied by a (2*S*,4*S*)-4-methylproline or L-proline instead of the amino acid 2-carboxy-6-hydroxyoctahydroindole (Choi).[7,12] Spumigins that contain 4-methylproline have been described as important trypsin inhibitors.[13]

Further chemical investigations on this strain have now afforded the discovery of two new namalide variants. Namalide was reported only once in the literature as coming from the marine sponge *Siliquariaspongia mirabilis*.[14] Structurally, this anabaenopeptin-like compound is described as a cyclic tetrapeptide containing a ring of three amino acids and a side chain amino acid linked via a ureido group to the α-amino group of a lysine. As commonly reported for anabaenopeptins, all amino acids with the exception of the conserved lysine in position 2 were found in the L-configuration.[15,16] Namalide was reported to inhibit carboxypeptidase A at submicromolar concentrations.[14] Here, we report the isolation, structure elucidation, absolute configuration, and protease inhibitory activities of the new namalide variants **1** and **2** and of the dihydro derivatives of the four aforementioned spumigin variants **3a**–**6b**.

RESULTS AND DISCUSSION

Freeze-dried cells of *S. torques-reginae* (Figure S1) were extracted with 70% MeOH and subsequently subjected to solid-phase extraction (SPE) on a C18 cartridge with 20–100% MeOH aliquots to yield five fractions. LC-MS analysis of these fractions revealed the presence of a pair of compounds with *m*/*z* 576.3400 (**1**) and *m*/*z* 562.3227 (**2**) in the 40% MeOH

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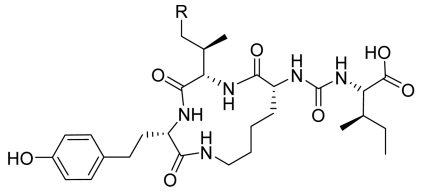
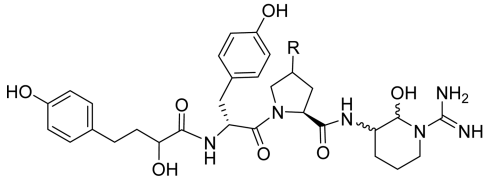
Chart 1

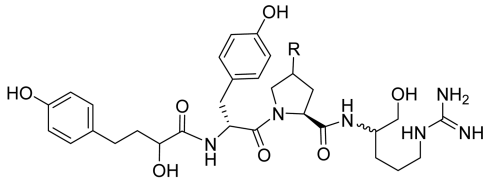


namalide B (**1**): R=CH_3
namalide C (**2**): R=H



spumigin K-N (**3-6**)

3 R=CH_3 D-Argal
4 R=CH_3 L-Argal
5 R=H D-Argal
6 R=H L-Argal



dihydrospumigin K-N (**3a-6a**)

3a R=CH_3 D-Argol
4a R=CH_3 L-Argol
5a R=H D-Argol
6a R=H L-Argol

fraction and further guided their isolation. Dereplication of **1** and **2** from the available literature allowed their identification as newly isolated compounds. Particularly, the interpretation of HRESIMS/MS and NMR data established them as new namalide variants. In addition, four compounds (**3**–**6**) with protonated molecules at *m/z* 597.3014 and 611.31716 were isolated together from the 80% MeOH eluted fraction. The latter had been partially identified as spumigin analogues before.[10]

The chemical structure of **1** was determined using a combination of NMR and MS analysis (Tables 1 and S2, Figures S2–S13). A molecular formula of $C_{29}H_{45}N_5O_7$ and 10 degrees of unsaturation were determined for **1** upon HRESIMS analysis. Initial evaluation of the 2D H–N HSQC, TOCSY, and NOE experiments (in DMSO-d_6) provided clear evidence of a dynamic exchange equilibrium process. This phenomenon was further corroborated by a H–N HSQC experiment slightly modified to allow the exchange of longitudinal magnetization (Figure S9). In face of the limited solubility of **1** in solvents other than DMSO-d_6 and the low signal-to-noise ratio of diluted samples, the complexity of the spectra could not be avoided.

Diagnostic correlations in 2D ^{1}H NMR spectra together with a typical MS fragmentation pattern indicated the peptidic nature for **1**. In this regard, the H–N HSQC spectrum revealed proton-bound nitrogen signals at typical peptidic nitrogen chemical shifts (δ_N ~100–140), and TOCSY and COSY spectra showed characteristic amide NH and α-methine proton signals in the δ_H 6.20–9.50 and 3.85–4.05 regions, respectively (Figures S5 and S6). Pairs of signals in the aromatic region of these spectra also suggested the presence of a para-substituted phenol (Figure S5). Consistent with this interpretation, the ^{13}C signals detected in the H–C HSQC spectrum were in agreement with the existence of four α-amide carbons ($\delta_{C\alpha}$ 52–60) and a phenol moiety (δ_C 128.9, 114.8) (Figures S10 and S11). Similarly, mass fragmentation spectra supported the peptide core. Inspection of the fragment ions at the low *m/z* region in the MS^2 spectrum of **1** (Figure S2) suggested the presence of three different amino acid residues (*m/z* 84.0763, Lys immonium ion; *m/z* 86.0958, Ile immonium ion; *m/z* 107.0483, hydroxytropolinium ion; *m/z* 150.0914, Hty immonium ion). The mass spectrometry data also allowed the proposal of a cyclic tripeptide incorporating Lys, Htyr, and Ile residues with a side chain composed of an additional Ile residue (Table S1). Combined analysis of TOCSY, COSY, ROESY, HMBC, and H–C and H–N HSQC data further confirmed this proposal and provided a complete assignment for ^{1}H, ^{13}C, and ^{15}N NMR signals of the four amino acid residues (Table 1).

The amino acid sequence was established using a combination of HMBC and NOE data (Figure 1, Table 1, and Table S1). HMBC correlations between the 6-NH amide proton of Lys (δ_H 7.48, 6-NH) and the carbonyl group of the Hty (δ_C 170.4, C-1) as well as the amide proton of Hty (δ_H 8.15, NH) and the carbonyl group of Ile(2) (δ_C 171.1, C-1) connected these three substructures. As reported for namalide,[14] no HMBC correlation was observed from the amide NH proton of the amino acid in position 3, the Ile(2). Evidence of the cyclic moiety was hence provided by NOE correlations. Particularly, cross-peaks between the amide NH proton of Ile(2) (δ_H 9.15, NH) and both the amide and α-protons of Lys (δ_H 6.34, 2-NH and δ_H 3.85, H-2) closed the ring. Additional correlations of the amide NH proton of the Hty (δ_H 8.15, NH) and the α-methine proton and amide proton of Ile(2) (δ_H 3.86, H-2 and δ_H 9.15, NH) and the 6-NH proton of Lys (δ_H 7.48) supported the tripeptide cyclic structure (Figures 1 and S7). An exocyclic position was proposed for the remaining Ile(1) amino acid. The connection of the Lys to Ile(1) via a ureido link was proposed on the basis of the HMBC correlations between the ureido carbonyl (δ_C 156.9) and the ^{1}H resonances at δ_H 4.04, 6.26 (Ile(1), H-2 and NH) and 6.34 (Lys, 2-NH) and supported by a NOE correlation between the α-amide NH protons of the Lys (δ_H 6.34, 2-NH) and the Ile(1) (δ_H 6.26, NH). The ureido carbonyl represented the remaining degree of unsaturation. Further evidence of this amino acid sequence was obtained from the

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Table 1. NMR Data for Namalides B (1) and C (2) in DMSO-d_6 (800 MHz for ^{1}H, 200 MHz for ^{13}C, and 81 MHz for ^{15}N)[a]

namalide B (1)						namalide C (2)			
unit	position	δ_N/δ_C, type[b]	δ_H, J (Hz)	selected NOESY correlations	HMBC[c]	unit	position	δ_N/δ_C, type	δ_H, J (Hz)
Ile(1)	1	173.8, C				Ile	1	175.4, C	
	2	56.6, CH	4.04[d]	Lys-3,4	Ile(1)-1,3,4,5, urea-CO		2	58.0, CH	3.94 br
	3	36.8, CH	1.74[d]				3	38.8, CH	1.71, m
	4a	24.4, CH_2	1.40[d]		Ile(1)-2,5,6		4a	25.9, CH_2	1.55, m
	4b		1.14[d]		Ile(1)-2,5,6		4b		1.27[d]
	5	15.4, CH_3	0.85[d]		Ile(1)-4		5	15.2, CH_3	0.85[d]
	6	10.5, CH_3	0.85[d]		Ile(1)-2,3,4		6	12.1, CH_3	0.85[d]
	NH	85.0	6.26, d (8.1)	Lys-2-NH	Ile(1)-1,2,3, urea-CO		NH	91.0	6.99, s
Ile(2)	1	171.1, C				Val	1	170.9, C	
	2	60.4, CH	3.86[d]	Hty-NH			2	62.8, CH	3.63[d]
	3	34.4, CH	1.84[d]				3	29.0, CH	1.95[d]
	4a	25.2, CH_2	1.38[d]	*Hty-NH*[e]	Ile(2)-5,6		4	19.3, CH_3	0.85[d]
	4b		1.11[d]		Ile(2)-3,5,6				
	5	15.0, CH_3	0.85[d]		Ile(2)-3,6		5	19.1, CH_3	0.80, d (3.9)
	6	10.3, CH_3	0.80 d (6.5)	*Hty-NH*	Ile(2)-2,3,4				
	NH	121.2	9.15[d]	Lys-2-NH			NH	128.8	9.30, d (6.7)
Lys	1	172.7, C				Lys	1	173.4, C	
	2	54.9, CH	3.85[d]	Ile(2)-NH			2	55.0, CH	4.10 br
	3	30.4, CH_2	1.57, m		Lys-1		3a	30.3, CH_2	1.65, m
							3b		1.49[d]
	4	19.0, CH_2	1.15[d]		Lys-5,6		4a	20.0, CH_2	1.09, m
							4b		1.44[d]
	5a	27.8, CH_2	1.44, m				5a	27.9, CH_2	1.34[d]
	5b		1.28, m		*Lys-3,6*		5b		1.28[d]
	6a	36.6, CH_2	3.49, m		Lys-4,5, Hty-1		6a	37.3, CH_2	3.43, m
	6b		2.78, m		Lys-1,4, Hty-1		6b		2.55[d]
	2-NH	86.8	6.34, d (4.7)	Ile(1)-NH, Ile(2)-NH, Lys-6-NH	Lys-2,3, urea-CO		2-NH	86.1	6.83, d (9.4)
	6-NH	113.8	7.48, d (7.0)	Hty-2,3a,3b,NH, Lys-2-NH	Lys-6, Ile(2)-1		6-NH	106.8	7.80, s
Hty	1	170.4, C				Hty	1	172.2, C	
	2	52.6, CH	4.07[d]	Lys-2-NH	Hty-1,3		2	53.7, CH	4.20 br
	3a	32.7, CH_2	1.90, m	Lys-6-NH	Hty-2,3a,5, Ile(2)-1		3a	33.9, CH_2	1.92[d]
	3b		1.76[d]	Lys-6-NH	Hty-1,2,3b,5		3b		1.84, m
	4	30.7, CH_2	2.36, m	Hty-6/10	Hty-2,3,6/10		4a	30.8, CH_2	2.42, m
							4b		2.30, m
	5	131.5, C					5	131.5, C	
	6/10	128.9, CH	6.96 d (8.3)	Hty-3a,3b,4, *Ile(2)-4a,4b,6*	Hty-4,6/10,7/9,8		6/10	129.0, CH	6.95, d (7.8)
	7/9	114.8, CH	6.67, d (8.3)		Hty-5,7/9,8		7/9	114.7, CH	6.63, d (7.4)
	8	155.3, C					8	155.1, C	
	NH	120.0	8.15, d (8.3)	Lys-2-NH, Ile(2)-2,4,5,NH	Hty-2, Ile(2)-1		NH	116.8	9.46, d (9.2)
	OH		9.13[d]		Hty-5,7/9		OH		9.13, s
urea	CO	156.9				urea	CO	158.6	

[a]Referenced to residual DMSO-d_6 at δ_H 2.50 and δ_C 39.51 ppm. [b]^{13}C and ^{15}N chemical shifts were obtained from the indirect dimensions of H−C HSQC and H−C HMBC, and H−N HSQC experiments, respectively. [c]Proton showing HMBC correlation to the indicated carbon. [d]Overlapped signal. [e]*Weak correlation.*

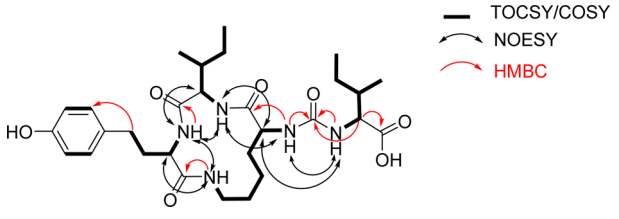

Figure 1. Key 2D NMR correlations for compound **1** in DMSO-d_6.

analysis of MS data. Fragment ions at m/z 463/435, 399/240, and 350/173 corroborated the partial sequences CO-Lys-Ile(1)-Hty, Ile(1)-CO-Lys-Ile(2), and CO-Lys-Hty.

The absolute configurations of the amino acid residues in namalide B were determined by the analysis of N_α-(2,4-dinitro-5-fluorophenyl)-L-alanine amide (FDAA) derivatives of the acid hydrolysate of **1** (Figure S13, Table S3).[17] These residues were confirmed as L-isomers except for lysine (D-isomer).

The MS fragmentation behavior of **1** could be mainly explained by two preferential b-ring opening pathways: the OH rearrangement that involves the loss of the exocyclic Ile(1) residue (C-terminal amino acid) and yields the H_2O adduct of the b-ion and the direct ring opening by cleavage of amide bonds to produce the corresponding b-ions (Figure S3).[18,19] Additionally, these precursor b-ions underwent further

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fragmentations and neutral losses explaining the most significant peaks in the MS spectrum.

An initial examination of the MS and NMR data of **2** suggested a high structural similarity to **1** (Table S1, Figures S15–S24). HRMS analysis of **2** yielded a protonated molecule at *m/z* 562.3229, suggesting the molecular formula of $C_{29}H_{45}N_5O_7$. As for **1**, a complete NMR assignment of **2** was performed through the analysis of 2D NMR experiments, which further established **2** as another namalide analogue (Table 1). The same cyclic structure with the replacement of the Ile unit at position 3 by a Val residue was inferred by comparing the TOCSY, NOE, and HMBC spectra with those of compound **1** (Figures S17, S19, and S23). The MS data were in full agreement with the proposed structure as indicated by the observation of the valine immonium ion at the low *m/z* region of the fragmentation spectra and a 14 amu decrease in the valine-containing fragments (*m/z* 548/534, 445/431, 417/403, 399/385, 291/277, 263/249, 240/226) (Figure 2, Table S1). LC-MS data for the L/D-FDAA derivatives of **2** hydrolysates showed the same absolute configurations found in **1** for the similar residues and established the amino acid in position 3 as L-Val (Figure S24, Table S3).

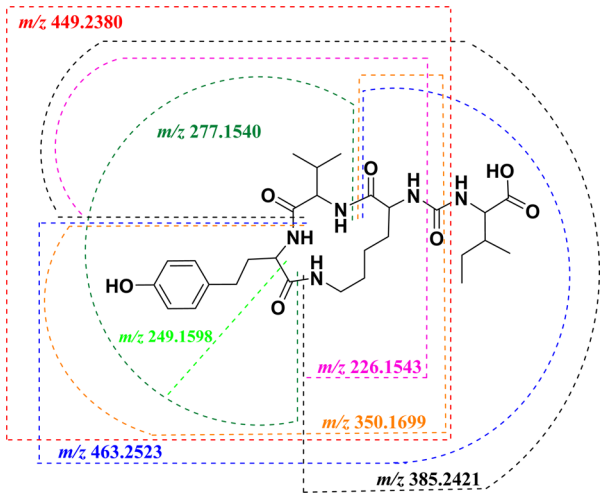

Figure 2. Major MS/MS fragments observed for **2** during positive ion mode ESI-HRMS/MS experiments. Orange and red color annotated fragmentations are explained by the alternative ring opening to form $(b+H_2O)^+$ ions. Other color-annotated fragmentations are explained by the initial cleavage of amide bonds to yield the standard b-ions.

The 80% MeOH eluting fraction contained the second group of identified compounds, the linear peptides spumigins K–N with protonated molecules at *m/z* 611.3 (**5**, **4**) and 597.3 (**5**, **6**) (Figure S25). As evidenced from the fragmentation data, these analogues contain argininal (Argal), proline/methylproline (Pro/MePro), tyrosine (Tyr), and a hydroxyphenyl acid (Table S2, Figures S26 and S27). Argal-containing compounds are well-known to elute as a group of unresolved chromatographic peaks due to the existence of several tautomeric forms, a fact that makes their isolation difficult.[20] Accordingly, the hydrated forms of spumigins K–N were observed together with the protonated forms in the LCMS analysis (*m/z* 629.3, **3**, **4** and *m/z* 615.3, **5**, **6**) (Figure S25). Reduction of the extract with sodium borohydride improved peak resolution.[21] As a result, four chromatographic peaks could be observed in the chromatogram. Interestingly, the two early eluting compounds (**5a**, **6a**) and those eluting later (**3a**, **4a**) showed no mass spectral differences, suggesting that they had the same amino acid composition and sequence (*m/z* 613.3 and *m/z* 599.3, respectively, Figure S25, Table S2). Furthermore, these compounds eluted at significantly different retention times under optimized achiral chromatographic conditions, suggesting that they are diastereoisomers. Proline-containing peptides have been reported to induce peak broadening or splitting when eluting on RP-HPLC.[22,23] This phenomenon is due to the slow *cis/trans* isomerization process around the proline amide bond relative to the chromatographic process. As these spumigins are tetrapeptides that contain a Pro or 4-MePro residue in their core, peaks that showed similar mass spectrometric patterns were attributed to these two conformers in equilibrium and first isolated together. However, extensive NMR analyses ruled out this hypothesis and further separation was performed. LC/MS analysis of the Marfey's derivatives of the isolated dihydrospumigins, after reduction, oxidation, and hydrolysis, demonstrated that **3a**(D)/**4a**(L) and **5a**(D)/**6a**(L) contained argininal in opposite configurations (Figures S30 and S31, Table S3).

Subsequently, the structures of dihydrospumigins K–N (**3a**–**6a**) were determined by NMR and Marfey's analysis (Tables 2, 3, and S3, Figures S25–S59). The amino acid residues Argol, MePro/Pro, and Tyr in **3a**–**6a** could be clearly distinguished by the observation of the characteristic TOCSY and COSY patterns. Complete ^1H, ^{13}C, and ^{15}N NMR assignments of **3a**–**6a** are given in Tables 2 and 3. As expected from the MS and hydrolysis data, the structural similarities among **3a**–**6a** were also evident from the NMR experiments, and the same planar structure could be proposed for compounds **3a**/**4a** and **5a**/**6a**. Replacement of the methylproline in **3a** and **4a** by a proline in **5a** and **6a** was evident from the lack of a TOCSY correlation to a methyl group for this spin system.

The structure of the N-terminus in **3a**–**6a**, further identified as 2-hydroxy-4-(4-hydroxyphenyl)butanoic acid (Hhpba), was proposed on the basis of the NMR data and later corroborated by acid hydrolysis of the peptide and comparison with an authentic standard.[24] This unit was found to contain two isolated spin systems. One spin system was assigned as a *p*-hydroxyphenyl aromatic ring, with signals from two vicinal groups of aromatic protons at δ_H 6.85–6.99 (H-6/H-10) and 6.65–6.70 (H-7/H-9) and HMBC correlations to two nonprotonated carbons (C-5 and C-8). The deshielded ^{13}C chemical shift of C-8 (Tables 2 and 3) is in accordance with the para-hydroxy substitution of the ring. Additionally, one signal at δ_H 5.54 (2-OH) (Figures S33 and S47) was correlated to two consecutive groups of diastereotopic methylene proton signals at δ_H 1.63 and 1.49 (H-3) and δ_H 2.34 and 2.27 (H-4) in the TOCSY spectrum, as well as to a methine proton signal at δ_H 3.75 (H-2) in the COSY spectrum (δ for **3a**, similarly for **4a**, **5a** and **6a**; Figures S33 and S34). This methine proton also exhibited an HMBC correlation to a carbonyl carbon (δ_C ~170), which led us to propose a hydroxy-substituted propyl side chain attached to a carbonyl group for the other spin system of this terminal unit (Figure S38). HMBC correlations between the protons of the aliphatic chain and the aromatic ring (H-3, C5; H4, C5 and C6/10) afforded the connection of these two spin systems (Figures 3 and S38). 2D NOE correlations connecting the two spin systems were also present (H-3, H-6/10 and H-4, H-6/10).

The amino acid sequences proposed by MS/MS fragmentation data for **3a**–**6a** were corroborated by the HMBC and the NOE cross-peaks (Figure 3). HMBC correlations from the

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Table 2. NMR Data for Dihydrospumigins K (3a) and L (4a) in DMSO-d_6 (800 MHz for ^{1}H, 200 MHz for ^{13}C, and 81 MHz for ^{15}N)[a]

		dihydrospumigin K (**3a**)				dihydrospumigin L (**4a**)					
unit	position	δ_N/δ_C, type[b] *cis*	δ_H, J (Hz)	δ_N/δ_C, type[b] *trans*	δ_H J (Hz)	δ_N/δ_C, type[b] *cis*	δ_H, J (Hz)	δ_N/δ_C, type[b] *trans*	δ_H, J (Hz)	NOESY correlations[e]	HMBC[f]
Hhpba	1	173.3, C		173.4, C		173.1, C		173.3, C			
	2	70.0, CH	3.75[c]	70.2, CH	3.84[c]	69.9, CH	3.74, dd (4.6, 8.1)	69.9, CH	3.81[c,b]	Tyr-NH,2, *Hhpba-6/10*	Hhpba-1
	3a	36.3, CH_2	1.63	36.4, CH_2	1.83	36.1, CH_2	1.62	36.1, CH_2	1.63	*Tyr-NH, Hhpba-6/10*	Hhpba-1,2,4,5
	3b		1.49		1.65		1.47		1.65	*Tyr-NH*	Hhpba-1,2,4,5
	4a	29.4, CH_2	2.34	29.7, CH_2	2.48	29.2, CH_2	2.32	29.5, CH_2	2.48		Hhpba-2,3,5,6/10
	4b		2.27				2.23				
	5	131.7, C		131.6, C		131.4, C		131.5, C			
	6/10	129.0, CH	6.87, d (8.9)	128.6, CH	6.98, d (8.9)	128.8, CH	6.86, d (8.8)	128.9, CH	6.96, d (8.8)	Hhpba-2,3,4, *Tyr-2*	Hhpba-4,8,6/10,7/9
	7/9	115.0, CH	6.64[c]	114.7, CH	6.66[c]	114.7, CH	6.64[c,b]	114.6, CH	6.67[c]		Hhpba-5,8,6/10,7/9
	8	155.1, C		155.1, C		155.1, C		155.5, C			
	2-OH		5.54 br		5.83 br		5.57 br[d]		5.92 br[d]	Tyr-NH	
	8-OH		9.20[c]		9.20[c]		9.24[c]		9.24[c]		
Tyr	1	169.3, C		168.9, C		173.2, C		169.0, C			MePro-5
	2	50.6, CH	4.31 br	51.7, CH	4.57, m	50.4, CH	4.31 br	51.7, CH	4.58, m	MePro-5, Tyr-5/9, *MePro-4, 4-CH_3*	Tyr-1,3,4, Hhpba-1
	3a	35.6, CH_2	2.85[c]	36.9, CH_2	2.80[c]	35.5, CH_2	2.85	36.7, CH_2	2.80[c]		Tyr-3,4,5/9
	3b		2.68				2.68				
	4	127.7, C		126.6, C		126.4, C		126.4, C			
	5/9	129.7, CH	6.94, d (8.9)	130.2, CH	6.97, d (8.9)	130.0, CH	6.94[c]	130.2, CH	6.97, d (8.8)	Tyr-2, 3, MePro-2, Hhpba-3	Tyr-3,6/8,7
	6/8	114.6, CH	6.60, d (8.9)	115.0, CH	6.65[c,b]	114.7, CH	6.60, d (8.8)	114.7, CH	6.65[c]	*Tyr-2,3, MePro-2*	Tyr-4,5/9,7
	7	155.8, C		156.1, C		155.2, C		156.0, C			
	7-OH		9.30[c]		9.30[c]		9.33[c]		9.33[c]		
	NH	117.2	7.58 (9.7)	116.9	7.76 (7.8)	116.9	7.51, d (9.1)	116.9	7.75, d (7.7)	Argol-5-NH, Tyr-5/9, Hhpba-2-OH,2,3	Hhpba-1, Tyr-1,2
MePro	1	172.2, C		171.3, C		171.8, C		171.0, C			
	2	59.8, CH	4.77, dd (9.6, 8.8)	60.3, CH	4.11, dd (9.6, 8.8)	59.6, CH	4.78, dd (8.7, 9.6)	60.1, CH	4.09, dd (9.6, 8.8)	Argol-2-NH, **Tyr-2**,*5/9*	MePro-1,3
	3a	39.9, CH_2	2.54	37.1, CH_2	2.19	39.8, CH_2	2.56	37.1, CH_2	2.19	Argol-2-NH	MePro-4,5
	3b		1.53		1.28		1.50		1.31		MePro-2,4,4-CH_3
	4	30.9, CH	2.04	32.6, CH_2	1.78	30.8, CH	2.04	32.3, CH_2	1.77	*Tyr-2, 3*	*MePro-3,5*
	5a	53.2, CH_2	3.86[c]	54.1, CH_2	3.34	53.1, CH_2	3.87	53.6, CH_2	3.35		MePro-2, 3, 4
	5b		2.81[c]		2.90		2.78[c]		2.90	Tyr-2, *Argol-2-NH,* Tyr-NH	MePro-4,4-CH_3
	4-CH_3	17.3, CH_3	0.98, d (7.4)	19.9, CH_3	0.90, d (7.4)	17.0, CH_3	0.99,d (7.2)	15.6, CH_3	0.89, d (7.2)		MePro-3,4,5
Argol[a]	1a	63.5, CH_2	3.28	63.5, CH_2	3.29	63.0, CH_2	3.48	63.2, CH_2	3.34		*Argol-3*
	1b		3.41		3.18		3.30		3.23	*MePro-2*	*Argol-2,3*
	2	50.8, CH	3.75[c]	49.0, CH	3.63	50.2, CH	3.83	48.9, CH	3.67	*MePro-2,* ***Tyr-NH***	*Argol-1*
	3a	27.7, CH_2	1.66	27.8, CH_2	1.59	27.9, CH_2	1.66	27.7, CH_2	1.53		Argol-4
	3b		1.37		1.30		1.37		1.20		Argol-2,4,5
	4a	25.1, CH_2	1.52	24.8, CH_2	1.52	24.7, CH_2	1.51	24.7, CH_2	1.42		Argol-2,3,5

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Table 2. continued

	dihydrospumigin K (**3a**)				dihydrospumigin L (**4a**)					
unit	position	δ_N/δ_C type[b] *cis*	δ_H, *J* (Hz)	δ_N/δ_C type[b] *trans*	δ_H *J* (Hz)	δ_N/δ_C type[b] *cis*	δ_H, *J* (Hz)	δ_N/δ_C type[b] *trans*	δ_H, *J* (Hz)	NOESY correlations[e]	HMBC[f]
	4b				1.47		1.44		1.36		Argol-2,3,5
	5	40.4, CH_2	3.05	40.4, CH_2	3.05	40.3, CH_2	3.06	40.3, CH_2	3.00		Argol-3,4
	1-OH		4.82 br[d]		4.60 br[c,d]		4.93 br[d]		4.71 br[d]		
	2- NH	119.8	8.06, d (9.1)	118.2	7.43, d (9.7)	120.7	8.17, d (9.3)	119.0	7.38, d (9.6)	MePro-2,3,4,5, Tyr-NH_2,2	Pro-1, Argol-2
	5-NH	85.3	8.34 br	85.4	8.17 br	85.4	8.51 br	85.5	8.35[c]		

[a]Referenced to residual DMSO-d_6 at δ_H 2.50 and δ_C 39.51 ppm. [b]^{13}C and ^{15}N chemical shifts were obtained from the indirect dimensions of H−C HSQC and H−C HMBC, and H−N HSQC experiments, respectively. [c]Overlapped signals. [d]Assigned from the TOCSY experiment of the mixture of spumigins K−N and L−M. [e]*Weak correlations*. Strong correlation for **cis isomer** or trans isomer. [f]Proton showing HMBC correlation to the indicated carbon.

amide NH proton of an amino acid to the carbonyl group of the adjacent residue let us propose the partial sequences Hhpba-Tyr and MePro/Pro-Argol (Figure 3, Tables 2 and 3). The HMBC cross-peak from the δ- and α-protons of Pro to the carbonyl group of the Tyr connected the two subunits. The Hα−HN sequential NOE correlations (Hδ for MePro/Pro) between neighboring residues supported the above amino acid sequence.

Duplication of many cross-peaks in the 2D NMR spectra provided evidence of *cis/trans* isomerization of **3a**−**6a**. By examining TOCSY and NOE spectra, it was possible to assign different chemical shifts for the amino acid spin systems in each conformer (Tables 2 and 3). The *cis* and *trans* isomers were differentiated by the observation of the characteristic NOE sequential proline correlations (strong Pro-H-2 to Tyr-H-2 correlation in *cis* conformation and Pro-H-5 to Tyr-H-2 in *trans* conformation) and ^{13}C chemical shifts attributed to each prolyl peptidyl bond configuration.[25,26]

Applying Marfey's methodology,[17] the absolute configurations of the amino acids residues for **3a**−**6a** were determined to be L-Pro and D-Tyr and that of argininol as L for **4a** and **6a** and D for **3a** and **5a**. Small amounts of **3a** and **4a** were treated with Jones' reagent to oxidize argininol residues to arginine before being submitted to Marfey's procedure (Table S3, Figure S27).

Anabaenopeptins and anabaenopeptin-like compounds have been previously reported to exhibit carboxypeptidase-inhibition properties.[14,15,27,28] Particularly, their ability to inhibit carboxypeptidase A (CPA), an enzyme closely related to the thrombin activatable fibrinolysis inhibitor (TAFIa), has recently attracted attention in the pharmacological field, and several studies exploring the ability to inhibit TAFIa by these compounds and structurally related analogues have been conducted.[29,30] On the basis of the structural properties of namalides B (**1**) and C (**2**), CPA inhibition activities were studied for compounds **1** and **2**. It was found that these compounds exhibited potent inhibition of CPA with IC_{50} values of 0.75 and 2.0 μM, respectively. Additionally, as some of the members of the anabaenopeptin family have been described to inhibit serine endopeptidases,[31] the chymotrypsin inhibition activity of **1** and **2** was also evaluated. However, no chymotrypsin inhibition was observed for either **1** or **2** at concentrations up to 0.78 mM.

As members of the spumigin family are found to inhibit serine proteases,[12,13] compounds **3a**−**6a** were tested for thrombin inhibitory activity. However, no significant inhibition activity was observed for **3a** and **4a** at peptide concentrations up to 150−350 μM. Compounds **5a** and **6a**, which differ from **3a** and **4a** by a 4-MePro residue in position 3, did not show remarkable inhibitory activities (IC_{50} of 250 μM). Structurally related argininol-containing compounds have been suggested to display milder trypsin inhibitory activities relative to their argininal analogues due to the impossibility of the alcohol group covalently binding to the trypsin active site.[13,24] Taking into account that compounds **3**−**6** were isolated in their reduced forms (**3a**−**6a**), no trypsin inhibitory activities of the aldehyde forms of spumigins K−N were determined.

This study described the isolation of two new namalide variants (**1** and **2**) and four new spumigin analogues (**3**−**6**) from the freshwater cyanobacterium *S. torques-reginae*. Namalides B (**1**) and C (**2**) belong to a rare subgroup of anabaenopeptin-like compounds,[14,32] which exhibit CPA-inhibition activity. Spumigins K−N (**3**−**6**) are the first examples of spumigins that contain an N-terminal hydroxyphenyl lactic acid residue. Recently, the biosynthetic gene

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Table 3. NMR Data for Dihydrospumigins M (5a) and N (6a) in DMSO-d_6 (800 MHz for ^{1}H, 200 MHz for ^{13}C, and 81 MHz for ^{15}N)[a]

		dihydrospumigin M (**5a**)			dihydrospumigin N (**6b**)				
unit	position	δ_N /δ_C, type[b] *cis*	δ_H (*J* in Hz)	δ_N /δ_C, type[b] *trans*	δ_H (*J* in Hz)	δ_N /δ_C, type[b] *cis*	δ_H (*J* in Hz)	δ_N /δ_C, type[b] *trans*	δ_H (*J* in Hz)
Hhpba	1	173.3,[d] C		173.9,[d] C		173.2, C		173.8, C	
	2	70.0, CH	3.76 br	70.0, CH	3.86 br	69.9, CH	3.75 dd (8.1, 4.5)	69.9, CH	3.81, dd (8.6, 4.1)
	3a	36.3, CH_2	1.64	36.3, CH_2	1.84	36.2, CH_2	1.61	36.2, CH_2	1.81
	3b		1.50		1.67		1.48		1.64
	4a	29.3, CH_2	2.35	29.5, CH_2	2.50	29.3, CH_2	2.32	29.6, CH_2	2.47
	4b		2.27				2.23		
	5	131.5, C		131.5, C		131.6, C		131.6, C	
	6/10	128.9, CH	6.88, d (8.8)	129.1, CH	6.96[c]	128.9, CH	6.87, d (8.8)	129.0, CH	6.96[c]
	7/9	114.8, CH	6.64[c]	114.8, CH	6.67[c]	115.2, CH	6.64[c]	114.9, CH	6.66[c]
	8	155.1, C		155.5, C		155.1, C		154.9, C	
	2-OH	5.56, d (5.5)			5.81br	5.58, d (4.8)		5.88 br	
	8-OH	9.14, br			9.20[c]	9.20[c]		9.20[c]	
Tyr	1	169.5, C		169.5, C		169.2, C		169.6, C	
	2	51.2, CH	4.42 br	52.0, CH	4.54, dd (13.6, 7.2)	51.1, CH	4.41 br	52.0, CH	4.52 dd (13.6, 8.0)
	3a	36.0, CH_2	2.81	36.5, CH_2	2.81	35.8, CH_2	2.78	36.5, CH_2	2.82
	3b		2.68				2.66		
	4	127.5, C		126.5, C		127.4, C		126.5, C	
	5/9	129.9, CH	6.93[c]	130.6, CH	6.97[c]	130.0, CH	6.95[c]	130.2, CH	6.97[c]
	6/8	114.7, CH	6.60, d (8.8)	114.8, CH	6.65[c]	114.6, CH	6.59, d (8.8)	114.6, CH	6.65
	7	155.6, C		155.8, C		155.7, C		156.0, C	
	7-OH		9.30 br		9.30 br		9.33[c]		9.33[c]
	NH	116.5	7.59, d (9.6)	117.4	7.83, d (7.2)	116.1	7.55,d (9.2)	117.4	7.80, d (7.1)
Pro	1	171.4[d], C		170.8, C		171.8, C		170.8, C	
	2	59.7, CH	4.82, d (8.0)[c]	59.7, CH	4.18 d (8.0)	59.6, CH	4.784, d (8.8)	59.8, CH	4.16, d (8.0)
	3a	32.3, CH_2	2.18	28.9, CH_2	1.81	32.3, CH_2	2.22	29.2, CH_2	1.82
	3b		2.00		1.75		1.96		1.77
	4	21.8, CH_2	1.77	23.7, CH_2	1.62	22.1, CH_2	1.78	22.0, CH_2	1.60
	5a	46.7, CH_2	3.45	46.7, CH_2	3.55	46.4, CH_2	3.41	46.4, CH_2	3.56
	5b		3.39[c]		2.98				2.96
Argol[a]	1a	62.6, CH_2	3.40[c]	62.7, CH_2	3.27	63.0, CH_2	3.46	63.3, CH_2	3.32
	1b		3.31		3.17		3.30		3.24
	2	50.8, CH	3.74	50.2, CH	3.63	50.2, CH	3.81	49.8, CH	3.67
	3a	27.8, CH_2	1.61	27.8, CH_2	1.58	24.7, CH_2	1.50	27.7, CH_2	1.51
	3b		1.13		1.28		1.43		1.18
	4a	24.8, CH_2	1.51	24.8, CH_2	1.48	28.0, CH_2	1.65	24.6, CH_2	1.41
	4b		1.46		1.42		1.35		1.36
	5	40.2, CH_2	3.04	40.2, CH	3.05	40.5, CH_2	3.08	40.4, CH	3.00
	1-OH		4.81, s[c]		4.61, s		4.85 br[c]		4.69 br[c]
	2- NH	120.1	8.02[c]	119.0	7.38, d (9.6)	120.3	8.14, d (8.8)	119.2	7.30, d (9.6)
	5-NH	85.6[c]	8.20 br	86.1[c]	8.04[c]	85.6[c]	8.04 br	86.1[d]	7.92 br

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Table 3. continued

[a]Referenced to residual DMSO-d_6 at δ_H 2.50 and δ_C 39.51 ppm. [b]^{13}C and ^{15}N chemical shifts were obtained from the indirect dimensions of H−C HSQC and H−C HMBC, and H−N HSQC experiments, respectively. [c]Overlapped signals. [d]Assigned from the TOCSY experiment of the mixture of spumigins K−N and L−M.

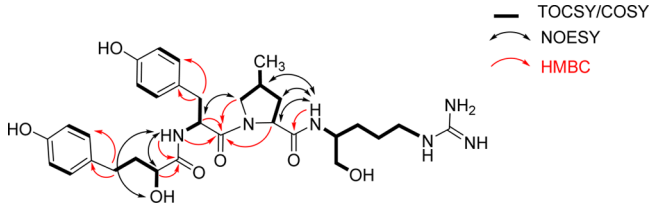

Figure 3. Key 2D NMR correlations for structure elucidation of compounds **3a** and **4a** in DMSO-d_6.

cluster that encodes the production of spumigins from the cyanobacterium *S. torques-reginae* has been described.[33] Because namalides B and C are the only congeners described for this family of metabolites, future efforts to identify their biosynthetic gene cluster would be of great interest. The discovery of namalides B (**1**) and C (**2**) and new variants of spumigins broadens the structural diversity of peptides produced by these microorganisms.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-2000 polarimeter, and UV spectra on an Evolution 260 Bio Thermo Scientific UV−visible spectrophotometer. NMR spectra were recorded in DMSO-d_6 on a Bruker Avance III 800 MHz spectrometer equipped with a 5 mm TCI cryogenic probe using residual solvent signals (δ_H 2.49, δ_C 39.5) as internal standards and a temperature of 298 K. H−C HSQC experiments were optimized for $^1J_{CH}$ 145 Hz, H−N HSQC experiments were optimized for $^1J_{NH}$ 93 Hz, and HMBC experiments were optimized for $^nJ_{CH}$ 8 Hz. Two-dimensional homonuclear (^{1}H−^{1}H) NMR spectra were recorded with weak presaturation ($\gamma_H B_1$ = 50 Hz) of the DMSO H_2O signal. A mixing time of 200 ms was employed for the NOE mixing period, while 70 ms was employed for the TOCSY mixing period with DIPSI-2 as isotropic mixing sequence and a radio frequency field strength of 10.4 kHz. ROESY experiments were recorded with a spin-locking field strength of 3.1 kHz during a mixing time of 200 ms. All NMR spectra were processed using Bruker TopSpin software and analyzed with CCPNMR Analysis.[34] HRMS data were acquired on a Bruker TOF mass spectrometer (micrOTOF II) equipped with an ESI ion source and controlled by a Bruker Compass/HyStar workstation. The ionization source conditions were as follows: positive ionization, capillary potential of 3500 V, temperature and flow of drying gas (nitrogen) of 5 mL/min and 300 °C, respectively, nebulizer pressure of 35 psi. The Q/TOF instrument was operated in scan and AutoMS/MS mode, performing MS/MS experiments on the three most intense ions from each MS survey scan. Three collision-induced dissociation experiments were performed by varying the collision energies from 30 to 70 eV to produce many fragment ions of high abundance. The accurate mass data were processed using Data Analysis 4.0 software (Bruker Daltonics). Elementary composition, deviations from the theoretical value (error), and comparisons of the theoretical and the measured isotope pattern (sigma value) were calculated using the Smart Formula algorithm of the Bruker Daltonics DataAnalysis data processing software. The confirmation of the elemental formula was based on the widely accepted thresholds of 5 ppm and 20 mSigma. LCHRMS profiles of the extracts and fractions were performed in a Shimadzu Prominence HPLC coupled with an electrospray source to the quadrupole time-of-flight instrument as described above. Analytical and semipreparative HPLC purifications were carried out on a Shimadzu Prominence system equipped with an LC-20AT quaternary pump and an SPDM20A photodiode array detector. For MS experiments all solvents were HPLC or LCMS grade. NMR solvents were acquired from Sigma-Aldrich.

Culture Conditions. The cyanobacterium *S. torques-reginae* ITEP 24 was obtained from the culture collection of the Laboratory of Toxins and Natural Products of Algae and Cyanobacteria at the University of São Paulo (LTPNA-USP) and was grown at 25 °C,

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under a 12:12 h light/dark regimen, in ASM1 growth medium. The cultured cells were harvested by centrifugation after a period of 4 weeks of incubation, lyophilized, and kept at −20 °C until extraction.

Extraction and Isolation. The lyophilized material (6 g) was extracted three times with 70% aqueous MeOH (1 g/30 mL) by probe sonication (amplitude of 30%, 2 min, Soni Omni Disruptor) and centrifuged (7000*g*, 4 °C, 10 min, Eppendorf 5804R), and the supernatants were combined. The MeOH was removed in a rotatory evaporator system, and the aqueous suspension split in three aliquots. These aliquots were subjected to SPE extraction (Waters Sep-Pak Vac 35 cm^3 10 g C18 cartridge) and eluted with 20−100% aqueous MeOH to yield five fractions (70 mL). The fractions were concentrated under vacuum, and the residue dissolved in 20% aqueous MeOH and analyzed by LCHRMS. The 40% MeOH fraction was further purified by semipreparative HPLC (column, Phenomenex Luna RP-C18 250 × 10 mm, 5 μm; mobile phases A (H_2O) and B (MeCN), both containing 0.1% formic acid; flow rate 4.5 mL/min) to yield compounds **1** (2.4 mg, 0.040% yield of dry cells weight) and **2** (0.4 mg, 0.007% yield of dry cells weight). The 80% MeOH fraction containing the compounds **3**−**6** was concentrated under a stream of nitrogen (TE-concentrator, Technal), reduced with $NaBH_4$, and separated in the same way as **1** and **2** with slight gradient modifications to yield **3a** (1.0 mg, 0.017% yield of dry cells weight), **4a** (0.8 mg, 0.014% yield of dry cells weight), **5a** (0.8 mg, 0.013% yield of dry cells weight), and **6a** (0.9 mg, 0.015% yield of dry cells weight).

*Namalide B (**1**):* white, amorphous powder; $[\alpha]^{20}_D$ −8 (*c* 0.75, MeOH); UV (MeOH) λ_{max} (log ε) 277 (3.06) nm; ^1H, ^{13}C, and ^{15}N NMR data (800 MHz for ^1H, 201 MHz for ^{13}C, and 81 MHz for ^{15}N, DMSO-d_6) Table 1; HRMS (ESI-QTOF) *m*/*z* 576.3400 $[M + H]^+$ (calcd for $C_{29}H_{46}N_5O_7$, 576.3392; Δ −1.4 ppm).

*Namalide C (**2**):* white, amorphous powder; $[\alpha]^{20}_D$ −34 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 277 (2.94) nm; ^1H, ^{13}C, and ^{15}N NMR data (800 MHz for ^1H, 201 MHz for ^{13}C, and 81 MHz for ^{15}N, DMSO-d_6) Table 1; HRMS (ESI-QTOF) *m*/*z* 562.3227 $[M + H]^+$ (calcd for $C_{28}H_{44}N_5O_7$, 562.3235; Δ 3.8 ppm).

*Dihydrospumigin K (**3a**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −15 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 277 (2.85) nm; ^1H, ^{13}C, and ^{15}N NMR data (800 MHz for ^1H, 201 MHz for ^{13}C, and 81 MHz for ^{15}N, DMSO-d_6) Table 2; HRMS (ESI-QTOF) *m*/*z* 613.3360 $[M + H]^+$ (calcd for $C_{31}H_{45}N_6O_7$, 613.3344; Δ 2.5 ppm).

*Dihydrospumigin L (**4a**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −10 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 277 (2.85) nm; ^1H, ^{13}C, and ^{15}N NMR data (800 MHz for ^1H, 201 MHz for ^{13}C, and 81 MHz for ^{15}N, DMSO-d_6) Table 2; HRMS (ESI-QTOF) *m*/*z* 613.3369 $[M + H]^+$ (calcd for $C_{31}H_{45}N_6O_7$, 613.3344; Δ 4.1 ppm).

*Dihydrospumigin M (**5a**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −19 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 277 (2.85) nm; ^1H, ^{13}C, and ^{15}N NMR data (800 MHz for ^1H, 201 MHz for ^{13}C, and 81 MHz for ^{15}N, DMSO-d_6) Table 3; HRMS (ESI-QTOF) *m*/*z* 599.3205 $[M + H]^+$ (calcd for $C_{30}H_{43}N_6O_7$, 599.3188; Δ 3.9 ppm).

*Dihydrospumigin N (**6a**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −10 (*c* 0.28, MeOH); UV (MeOH) λ_{max} (log ε) 277 (2.85) nm; ^1H, ^{13}C, and ^{15}N NMR data (800 MHz, DMSO-d_6) Table 3; HRMS (ESI-QTOF) *m*/*z* 599.3207 $[M + H]^+$ (calcd for $C_{30}H_{43}N_6O_7$ 599.3188; Δ 3.4 ppm).

Hydrolysis, Oxidation, and Marfey's Analysis for Determination of the Absolute Configuration of the Amino Acids. Hydrolysis (6 N HCl, 105 °C, for 16 h) was performed using 0.1 mg of compounds **1**−**6**. The hydrolysates were dried under a nitrogen stream and resuspended in H_2O (100 μL). FDAA solutions (Sigma-Aldrich) in acetone (0.05 M, 50 μL) and $NaHCO_3$ (1 M, 100 μL) were added to each aqueous solution, and the reaction mixture was heated in a dry block (40 °C, 1 h) (Eppendorf Thermomixer R). The mixture was cooled to room temperature, and HCl (2 M, 40 μL) was added to stop the reaction. The solution was dried under a nitrogen stream, and the resulting residues were reconstituted in MeOH/H_2O (1:1, 100 μL) prior to LC/MS analysis (Phenomenex Luna C18, 150 × 2.1 mm, 0.19 mL/min, 50 °C, A (0.1% aqueous formic acid solution), B (MeCN/MeOH, 90/10) also containing 0.1% formic acid, linear gradient elution of 5−60% B). The masses and retention time of the L- and D-amino acid standards-FDAA derivatives were as follows (*m*/*z*, L-, D-): Arg (427, 16.2 min, 17.2 min); Hty (448, 35.8 min, 39.2 min); Ile (384, 40.4 min, 46.1 min); Leu (384, 41.4 min, 46.7 min); allo-Ile (384, 39.4 min, 45.0 min); Lys (399, 15.6 min, 16.5 min); Pro (368, 29.8 min, 31.5 min); Tyr (434, 32.6 min, 35.5 min).

Compounds(**3a**−**6a** were oxidized using Jones' reagent as previously described.[31] After evaporation the products were hydrolyzed and subjected to Marfey's analyses as described above.

2-Hydroxy-4-(4-hydroxyphenyl)butanoic acid. The acid hydrolysates (1 mL) of compounds **3a**−**6a** were extracted with ethyl ether (1 mL × 3) to separate the hydroxy acid from the amino acids mixture. The ether was removed and the residue dissolved in MeOH (1 mL). The hydroxy acid from the authentic samples and a standard of Hhpba (AnalytiCon Discovery) were subjected to LCMS analysis to confirm their identity using the same chromatographic conditions as described for Marfey's analyses.

Protease Inhibition Assays. Chymotrypsin, thrombin, carboxypeptidase A, and their respective colorimetric substrates were all purchased from Sigma Chemical Co. The thrombin and chymotrypsin inhibitory activities were measured using the methods reported by Zafrir-Ilan and Carmeli,[31] Anas et al.,[12] and Shin et al.[35] with minor modifications. Thrombin was dissolved in a Tris-imidazole buffer (pH 8.2) containing NaCl (30 mM) to prepare a 5.79 U/mL solution. Chymotrypsin was dissolved in 50 mM Tris-HCl (pH 7.6), 1 mM of $CaCl_2$, and 100 mM NaCl to prepare a 50 U/mL solution. Substrates were prepared as follows: *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride/DMSO (6 mg/600 μL)/20-fold volume of Tris-imidazole buffer for thrombin and *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide/50 mM Tris-HC1 (pH 7.6, 1 mg/mL) for chymotrypsin. For the thrombin protease inhibitory activity assay, 90 μL of enzyme solution and 20 μL of sample solution previously dissolved in DMSO were added to each microtiter plate well and preincubated at 37 °C for 5 min. Then, 90 μL of substrate solution was added to start the reaction, and the absorbance was read at 405 nm before and after 30 min of incubation at 37 °C. For chymotrypsin, 30 μL of Tris-HCl buffer, 50 μL of enzyme, and 20 μL of test sample were added to each microtiter plate well and preincubated at 37 °C for 5 min. Then, the substrate solution (100 μL) was added and the absorbance was read at 405 nm before and after 30 min. In both assays the 4-(2-aminomethyl)-benzenesulfonyl fluoride hydrochloride was used as a positive control. Carboxypeptidase A inhibition activity was measured according to the manufacturer's instructions. Briefly, 2 μL of enzyme, 1 μL of test substances, and 97 μL of ultrapure H_2O were added to each microtiter plate well. Then, an aliquot of 100 μL of the substrate solution (*N*-4-(methoxyphenylazoformyl)-Phe-OH) was added to start the reaction. After 5 min of incubation at 25 °C, 100 μL of the sodium carbonate solution was added to stop the reaction and the absorbance was read at 350 nm. A carboxypeptidase inhibitor from potato tuber was used as positive control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00370.

MS/MS spectra and assignment of the corresponding major product ions for the protonated molecules of **1**−**6** and **3a**−**6a**; base peak LC-MS chromatogram and selected LC-MS extracted ion chromatograms for original (**3**−**6**) and reduced (**3a**−**6a**) spumigin-containing fractions. ^1H NMR, ^1H−^{13}C HSQC, HMBC, TOCSY, NOESY, COSY, and ^1H−^{15}N HSQC spectra for **1**, **2**, and **3a**−**6a**; LC/MS data of Marfey derivatives of the hydrolysates of **1**, **2**, and **3a**−**6a**; CPA inhibition curves and estimation of IC_{50} values for **1** and **2** (PDF)

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Notes

The authors declare no competing financial interest.

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