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Thrombin Inhibitors from the Freshwater Cyanobacterium *Anabaena compacta*

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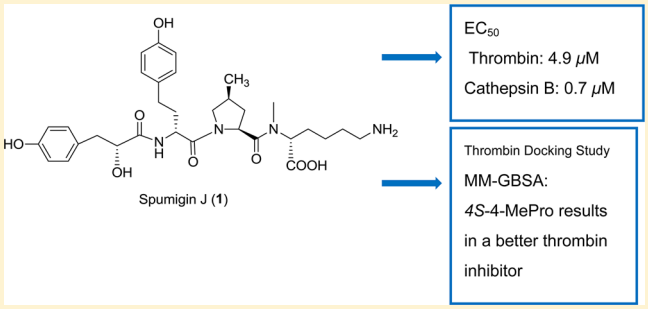
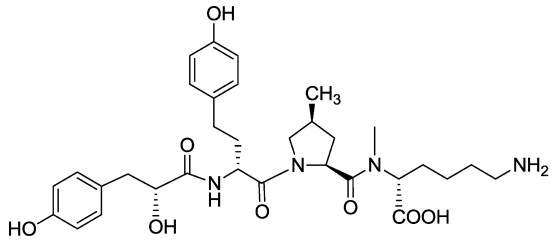
Supporting Information

ABSTRACT: Bioassay-guided investigation of the cyanobacterium *Anabaena compacta* extracts afforded spumigin J (**1**) and the known thrombin inhibitor spumigin A (**2**). The absolute configuration of **1** was analyzed by advanced Marfey's methodology. Compounds **1** and **2** inhibited thrombin with EC_{50} values of 4.9 and 2.1 μM, and 0.7 and 0.2 μM in the cathepsin B inhibitory assay, respectively. The MM-GBSA methodology predicted spumigin A with 2*S*-4-methylproline as the better thrombin inhibitor.



Cyanobacteria grow vigorously during algal blooms, release toxins, and cause problems of eutrophication of lakes, reservoirs, and rivers.[1] Many cyanobacteria produce linear peptides that have protease inhibitory activities.[2] In our continuing search for protease inhibitors from cyanobacteria,[3] we examined cyanobacterial extracts for inhibition of cathepsin B and thrombin. Cathepsin B is a lysosomal cysteine protease, belonging to the papain family, and has been implicated in various pathological conditions linked with malignancy, cancer progression, and tumors.[4] Thrombin, a trypsin-like serine protease, plays a significant role in thrombosis and platelet aggregation. It is the key enzyme in catalyzing the formation of polymerizable fibrin from fibrinogen in the blood coagulation cascade.[5] Cultured *Anabaena compacta* (NIES-835) showed promising cathepsin B and thrombin inhibitory activities. In the present study, spumigin J (**1**) with an *N*-methyllysine moiety was isolated together with the known compound spumigin A (**2**). Spumigins A–C were previously isolated, together with nodularin, from the toxic *Nodularia spumigena* AVI.[6,7] Spumigin A inhibited plasmin and thrombin; both spumigins A and B1 inhibited trypsin in the micromolar range. Spumigin E, a peptide aldehyde, has been reported to inhibit trypsin at approximately the micromolar range.[8] We report the isolation, structure elucidation, chemical assignment, absolute configuration, thrombin and cathepsin B inhibitory activities, and docking studies of **1** and **2**.



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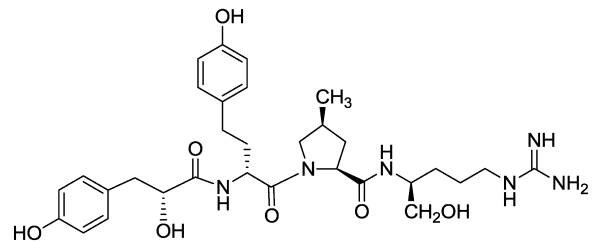

2[a]

Figure 1. Structures of **1** and **2**. [a]Original report of spumigin A (**2**) was by Harada et al.[6]

RESULTS AND DISCUSSION

The freeze-dried cells, 15 g, of *A. compacta* were extracted with 80% MeOH. The 80% MeOH extract was subjected to ODS flash chromatography and eluted with 20–100% MeOH with 20% MeOH increments. The 100% MeOH fraction was repeatedly purified using reversed-phase HPLC to afford spumigin J (**1**) (1.3 mg, yield of 0.01%) along with the

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Table 1. NMR Spectroscopic Data (^{1}H 600 MHz,^{13}C 150 MHz, DMSO-d_6) for Spumigin J (1)[a]

unit	position	δ_C, type	δ_H (mult, J in Hz)	DQF-COSY	HMBC	ROESY
Hpla	1	171.5, C				
	2	72.4, CH	4.03, br	3a, Hpla-OH		3a, 3b, Hpla-OH
	3a	40.0, CH_2	2.63, dd (13.7, 4.4)	2, 3b	2, 5, 9	2, 5, 9
	3b		2.89, dd (13.7, 4.4)	3a	5, 9	2
	4	132.0, C				
	5, 9	130.5, CH	7.00, d (8.2)	6,7, 8	7	3a, 3b
	6, 8	115.3, CH	6.65, d (8.2)	5, 9	4, 5, 7, 9	
	7	155.5, C				
	Ar-OH		9.19, s			
	OH		5.69, br	2		2
Hty	1	167.3, C				
	2	50.6, CH	4.36, m	NH-Hty		
	3a	40.5, CH_2	1.98, m	3b, 4		
	3b		2.49[b]	2, 3a	4, 5, 1-Hty	
	4	30.5, CH_2	2.27, m	3a		6, 10
	5	121.0, C				
	6, 10	129.1, CH	6.86, d (8.2)	7, 9	4, 8, 7, 9	4
	7, 9	114.3, CH	6.62, d (8.2)	6, 10	6, 8, 9	
	8	155.5, C				
	Ar-OH		9.21, s		8	
	NH		8.29, br	2	1-Hpla	2, 2-Hpla
4-MePro	1	169.6, C				
	2	50.6, CH	4.36, m	3a	3, 5	3a, 4
	3a	40.0, CH_2	2.66/2.39, m	2, 3b	4, 6	2
	3b		3.32/2.66, m	4	5	
	4	31.0, CH	2.60, m	3b	3	2, 5a
	5a	49.0, CH_2	3.60, d (12.0)/ 3.35[c]		6	2, 4
	5b		3.70/3.90, d (12.0)		1-Hty	
	6	22.0/24.0, CH_3	0.83, d (7.1)/ 0.84 d (6.6)		3, 4	3
N-Me-Lys	1	169.7, C				
	2	52.6, CH	4.34, m	3b		
	3a	20.0, CH_2	1.55, m	3b	1-*N*-MeLys	
	3b		2.43[c]	2, 3a, 4b		
	4a	24.3, CH_2	1.48, m	4b		OH
	4b		1.68, m	3b, 4a, 5b		
	5a	30, CH_2	1.42, m	6b		6b, OH
	5b		2.25, m	4b		
	6a	40.0, CH_2	3.20, m	6b		
	6b		3.50[c]	5a, 6a		5a, NH_2
	N-CH_3	40.0, CH_3	3.45, s		2, 1-MePro	2/3a-4-MePro
	NH_2		5.69, br			6b
	OH		6.80, br s			4a, 5b

[a]Carbonyls of **1** were detected by band-selective HMBC. [b]Solvent overlap. [c]Broad signal (obscured).

known compound spumigin A (**2**) (3.0 mg, yield of 0.02%). The molecular formula of **1** was deduced as $C_{32}H_{44}N_4O_8$ by HR-ESIMS (m/z 613.3203) and extensive NMR analyses included HSQC, DEPT, DQF-COSY, HMBC, band-selective HMBC, and ROESY. Several features of the ^{1}H NMR spectrum of **1**, specifically amide-type protons at δ 7.60−8.30 and signals in the diagnostic region for α-CH's, suggested its peptidic nature. In addition, three amide carbonyl signals, located between 165 and 175 ppm, were deduced using band-selective HMBC. Analysis of 1D NMR spectra in D_2O, CD_3OD, and DMSO-d_6, together with 2D NMR spectroscopic information, permitted the construction of four partial structures (Table 1): a 4-hydroxyphenyllactic acid unit (Hpla), homotyrosine (Hty), 4-methylproline (4-MePro), and *N*-methyllysine (*N*-MeLys). The sequential relationship of these four residues was established mainly by band-selective HMBC and ROESY and was strengthened by FT-MS/MS data. A total of 13 degrees of unsaturation were accounted for by two aromatic rings, one 4-methylproline ring, and four carbonyls, making **1** a linear peptide. The presence of a fragmentation at m/z 342.1271 $[Hpla - Hty + H]^+$ revealed the Hpla−Hty sequence, and this was verified by the key band-selective HMBC correlation from the amide proton (δ 8.29) of Hty to the carbonyl carbon of Hpla (δ 171.5). The sequence assignment was further supported, as the ion at m/z 342 $[Hpla - Hty + H]^+$ produced fragment ions at m/z 182 $[Hpla - NH_2 + H]^+$ and 314 $[Hpla - Hty - CO + H]^+$ in MS^3. The sequence was extended on the basis of the observation of a peak in the FTMS/MS spectrum with m/z 455.2094 corresponding to $[Hpla - Hty - 4\text{-MePro} + H]^+$. The Hty−4-MePro sequence was strengthened by the band-selective HMBC from H-5 of 4-MePro (δ 3.60, 3.70) to C-1 (δ 167.3) of Hty. The key band-selective HMBC

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correlation from the methyl protons (δ 3.45) of *N*-MeLys to C-1 (δ 169.6) of 4-MePro confirmed the 4-MePro-*N*-MeLys sequence, which was verified in the FTMS/MS spectra with *m*/*z* 613.3262. These data revealed the linear sequence of **1** to be Hpla−Hty−4-MePro−*N*-MeLys.

The ^{1}H NMR spectrum of **1** contained overlapping methyl doublets and doubling of proton signals that may be accounted for by restricted rotation of the proline amide or the *N*-MeLys amide or by the presence of inseparable diastereoisomers of 4-Me-Pro.

The absolute configuration of **1** was decided by advanced Marfey's method[9−12] using L/D-FDLA (1-fluoro-2,4-dinitrophenyl-L/D-leucinamide). Four diastereoisomers of 4-MePro were synthesized, following the approach of Munro et al.[13] with modifications, for Marfey's analysis. Marfey's method disclosed the configurations of the fragment amino acids *R*-Hpla, D-Hty, and *N*-Me-D-Lys. However, four peaks for 4-MePro, $[M + H]^+$ *m*/*z* 424.18, were observed in the Marfey's LC-MS chromatogram with a ratio of 0.42:0.33:0.15:0.1:(2*R*,4*R*):(2*S*,4*S*):(2*R*,4*S*):(2*S*,4*R*). The ∼50:50 mixture of the 4*R*/4*S* configurations observed in the Marfey's analysis can be explained by the occurrence of both C-2 configurations. The 4-position should not be epimerizable under the experimental conditions used, while the known epimerization at C-2 suggests that both the 2*S*,4*S* and 2*R*,4*R* isomers (enantiomers) are present in the natural product. Epimerization at C-2 would result in an equilibrium mixture of each of 2*S*,4*S*:2*R*,4*S* and 2*R*,4*R*:2*S*:4*R* to give the ∼50:50 mixture of the 4*R*/4*S* configurations. However, the complexity of the NMR spectra prevents a definitive assignment of both *cis* and *trans* 4-MePro isomers in spumigin J. The alternate explanation that both *cis* and *trans* 4-MePro isomers occur in spumigin J cannot be ruled out. Therefore, there are still uncertainties about the configuration of the 4-MePro residue, an issue that could be resolved by the synthesis of the individual spumigin J isomers. Interestingly, the modeling experiments described later indicate that spumigin J models containing either 2*S*,4*R* or 2*S*,4*S* 4-MePro have greater binding affinity to thrombin than models with the 2*R* isomers.

Spumigin A (**2**) was isolated as a colorless, amorphous powder from the cultured *A. compacta*. The molecular formula of **2** was deduced as $C_{31}H_{44}N_6O_7$ by LC-ESIMS. Dereplication from available literature elucidated the compound to be spumigin A.[6,8] Due to the complexity of the ^{1}H NMR data and unavailability of prior spectroscopic data from the literature, the reported compounds aeruginosins 102-A, B,[14] and 298-A[15] were used as model compounds for extensive assignments of proton and carbon signals.

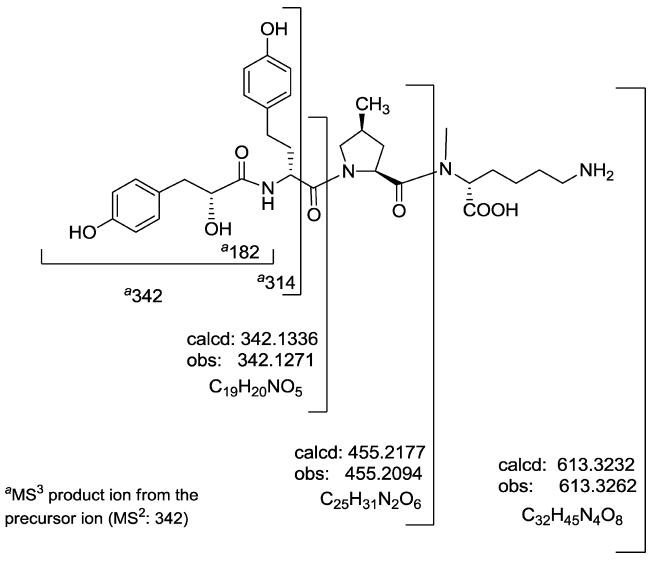

Figure 2. FTMS/MS interpretation of spumigin J (**1**).

The absolute configurations of spumigin A (**2**) residues were reported as *R*-Hpla and D-Hty by Harada et al.,[6] but that of argininol was not determined. The configuration of argininol was first deduced as L in this study. Spumigin A (**2**) was oxidized, hydrolyzed, and subjected to advanced Marfey's to give L-arginine, an oxidized form of L-argininol.

The absolute configuration of 4-MePro in our isolate **2** was deduced using the advanced Marfey's method.[9,10] Although (2*R*,4*R*)-4-MePro was the major isomer in the advanced Marfey's analysis, the presence of (2*S*,4*S*)-4-MePro was also observed in the LC-MS chromatogram with a ratio of 0.28:0.54:(2*S*,4*S*):(2*R*,4*R*) and trace amounts of (2*S*,4*R*)- and (2*R*,4*S*)-4-MePro diastereoisomers, with an observed ratio of 0.1 (2*S*,4*R*) to 0.08 (2*R*,4*S*); spumigin A (**2**) is proposed 2*S*,4*S* on the basis of the same argument as **1**. The specific rotation of spumigin A (**1**), $[\alpha]^{26}_D$ −5.4 (*c* 0.025, MeOH), by Harada et al.,[6] was smaller compared to the isolated spumigin A in this study, with $[\alpha]^{21}_D$ +70 (*c* 0.1, MeOH). As the absolute configuration of argininol in the reported literature was not determined, a possibility exists that the isolated spumigin from this study differs in configuration from the previously reported isolate. The isolated spumigin in this study has an L-argininol, and the lower rotation may be explained if the spumigin argininol isolated by Harada et al. had a D-argininol. However, it is difficult to verify this result. It is inconclusive that the spumigin A isolated previously is different from our isolate.

Previous studies (Table 2) reported spumigins as serine protease inhibitors, active against thrombin, trypsin, and plasmin.[6,8] Compounds **1** and **2** inhibited thrombin with EC_{50} values of 4.9 and 2.1 μM, respectively. Spumigins J (**1**) and A (**2**) inhibited cathepsin B, a cysteine protease, with EC_{50} values of 0.7 and 0.2 μM, respectively. The *N*-methyl-D-lysine residue in **1** is probably less specific to the target enzyme than when L-argininol is present in **2**. The presence of L-argininol in **2** compared to *N*-methyl-D-lysine in **1** gave slight increases in thrombin and cathepsin B inhibition.

Compounds **1** and **2** were initially studied for thrombin inhibitory activity. However, both compounds displayed 7- and 10-fold more potent cathepsin B inhibitory activity, and molecular docking of the spumigins with cathepsin B will be the subject of another paper.

We cannot rule out the possibility that the isolated spumigins are composed of four diastereoisomers of 4-MePro. To investigate the structure−activity of the isolated spumigins, **1** and **2** were subjected to thrombin docking studies. The interactions of the four diastereoisomers of 4-MePro of **1** and **2** were considered in the docking study. The 2*S* diastereoisomers have better binding with thrombin (Table 3). The guanidino-containing argininol in **2** resulted in higher binding affinity than the corresponding *N*-MeLys in **1** in the docking study.

The binding of all four diastereoisomers of spumigins J (**1**) and A (**2**) was examined by docking into thrombin (pdb code 1XM1) using Glide XP (eXtra Precision). Typically, the correlation between experimental K_i values and docking score is unpredictable because many scoring functions (including XP GlideScore) have been designed to predict binding orientations and facilitate database enrichment rather than predicting

Table 2. Protease Inhibitory Activities of the Reported Spumigins[a]

	peptide sequence					inhibitory activity EC_{50} (μM) to serine and cysteine proteases				
spumigin	1	2	3	4	MW	thrombin	trypsin	plasmin	cathepsin B	ref
A	*R*-Hpla	D-Hty	(2*S*,4*S*)-4-MePro	Argol	612	7.5	26.3	4.9		Harada, et al.[6]
A (**2**)	*R*-Hpla	D-Hty	(2*S*,4*S*)-4-MePro	L-Argol	612	2.1			0.2	this study
B1	*R* -Hpla	Hty	(2*S*,4*S*)-4-MePro	L-Arg	626		33.1			Harada, et al.[6]
B2	*R* -Hpla	Hty	(2*S*,4*S*)-4-MePro	D-Arg	626					Harada, et al.[6]
C	*R* -Hpla	Hty	L-Pro	D,L-Arg	612					Harada, et al.[6]
D	Hpla	Hty	Pro	Argol	598					Sivonen et al.[8]
E	Hpla	Hty	(2*S*,4*S*)-4-MePro	Argal	610		0.3−1.0			Sivonen et al.[8]
F	Hpla	Hty	Pro	Argal	596					Sivonen et al.[8]
G	Hpla	Hph	MePro	Argal	594					Sivonen et al.[8]
H	Hpla	Hph	Pro	Argal	580					Sivonen, et al.[8]
I	Hpla	Leu	Pro	Argol	534					Sivonen et al.[8]
J (**1**)	*R*-Hpla	D-Hty	(2*S*,4*S*)-4-MePro	*N*-Me-D-Lys	612	4.9			0.7	this study

[a]Argal, argininal; Argol, argininol; Arg, arginine; Hpla, hydroxyphenyllactic acid; Hty, homotyrosine; Hph, homophenylalanine; Pro, proline; MePro, (2*S*,4*S*)-4-MePro.

Table 3. MM-GBSA Ranking of Spumigins J (1) and A (2)

spumigin	MM-GBSA ΔG_{bind}[a] (kcal/mol)	relative MM-GBSA ΔG_{bind} (kcal/mol)
A (2*S*,4*R*)	−117.22	0
A (2*S*,4*S*)	−110.91	6.31
J (2*S*,4*S*)	−94.78	22.44
A (2*R*,4*S*)	−94.38	22.84
J (2*S*,4*R*)	−92.16	25.06
A (2*R*,4*R*)	−78.29	38.93
J (2*R*,4*R*)	−67.45	49.77
J (2*R*,4*S*)	−63.52	53.7

[a]MM-GBSA $\Delta G_{bind} = E_{complex}(min) - (E_{ligand}(min) + E_{receptor}(min))$.

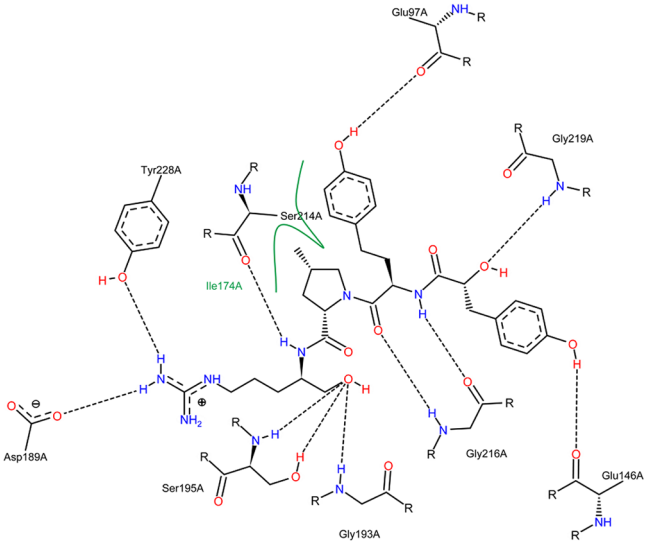

Figure 3. Spumigin A (2*S*,4*S*) key interactions with thrombin predicted by Glide XP docking (highest ranked pose from MM/GBSA rescoring). The 2D diagram was created using PoseViewWeb.

accurate binding free energies or even rank ordering of actives. MM-GBSA rescoring of docking poses has previously been reported to significantly improve the rank ordering of relative binding affinities in congeneric series of inhibitors against several protein targets, including thrombin.[16,17] In the current study, the three highest ranked Glide XP poses of each stereoisomer were subjected to rescoring using the MM-GBSA methodology.

Several trends are apparent in the data presented in Table 3. Spumigins A and J incorporating an L-proline (2*S* configuration) are consistently more highly ranked than the corresponding D-proline (2*R* configuration) isomers. The presence of either a *cis* or *trans* 4-methyl substituent (4*S* or 4*R* configuration) seems to be equally well tolerated within the thrombin binding site. Consistent with the binding data from the current study presented in Table 3, both spumigin A isomers in the preferred 2*S* configuration are more highly ranked than the corresponding spumigin J 2*S* analogues. These general trends are also observed in the less favored 2*R* configuration. Upon examination of the highest ranked pose for spumigin A (2*S*,4*S*), the binding orientation is analogous to that observed in the X-ray crystal structure of melagatran,[18] a well-known potent thrombin inhibitor (Figure 4b). The basic argininol group of the spumigin A is deeply embedded into the S1 pocket containing Asp-189 (Figure 4a), which is similarly occupied by the benzamidine group of melagatran. The hydroxy oxygen atom of the argininol makes hydrogen-bonding interactions with the protein backbone via Ser-195 (the catalytic serine) and Gly-193. These residues constitute the oxyanion hole, which normally stabilizes the tetrahedral intermediate formed upon attack of the substrate sessile bond by Ser-195.

The methyl group of the 2*S*-4-MePro is covered with Trp-60, which is positioned above a relatively small S2 pocket. The homotyrosine group occupies the larger hydrophobic S3 pocket, which is also occupied by the cyclohexyl group of melagatran. Finally, the hydroxyphenyllactic acid up to the hydroxy group occupies a position analogous to the C-terminal acid of melagatran.

The highest ranked pose for spumigin A (2*S*,4*R*) and (2*S*,4*S*) demonstrates that either *cis* or *trans* 4-methyl groups can be accommodated within the restricted S2 binding pocket (Figure 4c, d). As can be seen in Figure 4d, the two L-proline variants of spumigin J bind in a similar manner to spumigin A.

For the first time, we have isolated spumigins from an *Anabaena* species. Spumigins J (**1**) and A (**2**) inhibited thrombin and cathepsin B in the micromolar range. The MM-GBSA docking studies predicted spumigin A with 2*S*-4-MePro as the better thrombin inhibitor. Spumigin J (**1**) is the first spumigin with a lysine moiety.

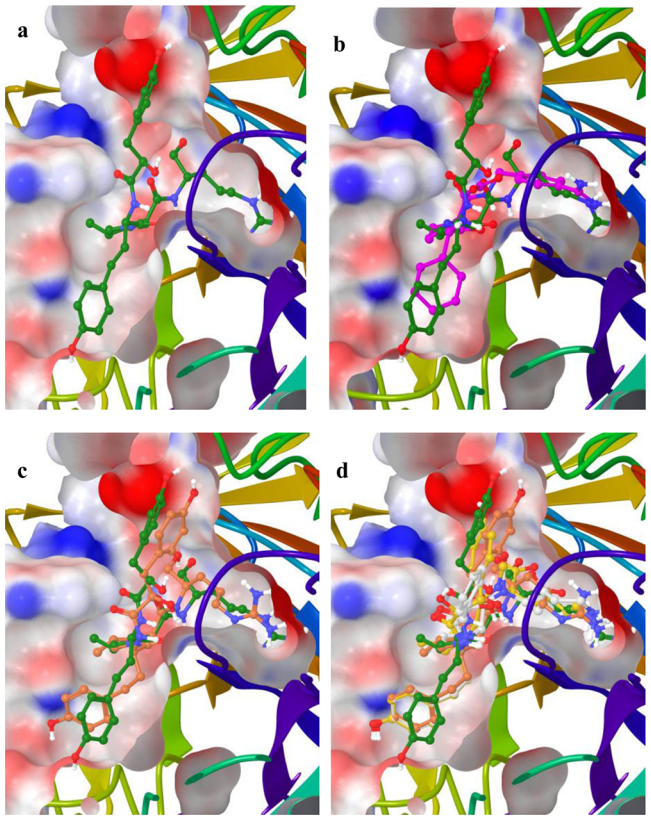

Figure 4. (a) Predicted binding orientation of spumigin A (2*S*,4*S*). (b) Melagatran extracted from the X-ray crystal structure with thrombin (pdb code 1k22) superimposed with spumigin A (2*S*,4*S*). (c) Predicted binding orientation of spumigin A (2*S*,4*S*) (green) compared with spumigin A (2*S*,4*R*) (brown). (d) Overlay of spumigin A (2*S*,4*S*) and (2*S*,4*R*) and spumigin J (2*S*,4*S*) and (2*S*,4*R*).

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations were measured on a Horiba polarimeter, and IR spectra were measured on a Jasco FT/IR-4000 instrument. NMR spectra were recorded on an ECA600 (JEOL) 600 MHz spectrometer using DMSO-d_6 as solvent at 25 °C. ^{1}H and ^{13}C NMR spectra were referenced to solvent peaks: δ_H 2.49 ppm and δ_C 39.6 ppm for DMSO-d_6. The electron spray ionization (ESI) spectra were measured on a Bruker Daltonics micrOTOF-HS. The FT and ITMS analyses were carried out with a Thermo Scientific LTQ Orbitrap Discovery (LTQ XL coupled with LTQ OR) spectrometer. The isolation of the active compounds using HPLC was performed on a Jasco PU-980 system with a UV-970 UV/vis detector with Develosil ODS HG-5, 10.0 × 250 mm and Develosil CN-5, 10.0 × 250 mm columns.

Culture Conditions. The *Anabaena compacta* (NIES-835) was obtained from the NIES collection (Microbial Culture Collection, the National Institute of Environmental Studies, Japan) and cultured in 10 L glass bottles containing CT medium.[19] The cultures were aerated at 25 °C for 22 days under a 12 L:12D cycle. The cells were collected by centrifugation using a Hitachi Himac CR 20 centrifuge at 12 000 rpm. The lyophilized cells were kept at −30 °C until extraction.

Extraction and Isolation. The freeze-dried cells (15 g from 230 L culture) were extracted twice with 80% MeOH, vacuum filtered, and concentrated to give an extract. The 80% MeOH extract was subjected to flash ODS column chromatography, eluting with 20−100% aqueous MeOH, to yield five fractions. The 100% MeOH fraction was purified by reversed-phase HPLC (isocratic elution with 40% MeOH containing 0.05% TFA, Develosil ODS HG-5, 10.0 × 250 mm, 3.0 mL/min, 220 nm). Further purification of the active compounds was conducted by reversed-phase HPLC (isocratic elution with 20% MeCN containing 0.05% TFA, Develosil CN-10 × 250 mm, 3.0 mL/min) to yield spumigin J (**1**, 1.3 mg, 0.01%) and spumigin A (**2**, 3.0 mg, 0.02%).

Spumigin J (1): amorphous powder; $[\alpha]^{21}_D$ +130 (*c* 0.1, MeOH); IR (neat) ν_{max} 3632, 3400, 2961, 2924, 1683, 1558, 1540, 1456, 1260, 1094, 1027 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR, see Table 1; FTMS/MS *m*/*z* 342.1271 [Hpla − Hty + H]$^+$ (calcd for $C_{19}H_{20}NO_5$, 342.1336), 455.2094 [Hpla − Hty − 4-MePro +2H]$^+$ (calcd for $C_{25}H_{31}N_2O_6$, 455.2177), 613.3262 [M + H]$^+$ (calcd for $C_{32}H_{45}N_4O_8$, 613.3232; HRMS (ESI-TOF) *m*/*z* 613.3203 [M + H]$^+$ (calcd for $C_{32}H_{45}N_4O_8$, 613.3232).

Spumigin A (2): colorless, amorphous powder; $[\alpha]^{21}_D$ +70 (*c* 0.1, MeOH); IR (neat) ν_{max} 3444, 2961, 2855, 1733, 1456, 1260, 1099, 1032 cm^{-1}; ^{1}H and ^{13}C NMR data, see Supporting Information; ITMS/MS precursor ion *m*/*z* 645 [M + CH_3OH + H]$^+$, 613 [M + H]$^+$, 595 [M − H_2O + H]$^+$, 555 [Hpla − Hty − 4-MePro − argininol − (CH_2−N=C=NH) + H]$^+$, 535 [M − Ph − OH + H]$^+$, 455 [Hpla − Hty − 4-MePro + 2H]$^{2+}$, 342 [Hpla − Hty + H]$^+$, 314 [Hpla − Hty − CO + H]$^+$, 212 [4-MePro − argininol − (H_2O + HN=C=NH) + H]$^+$, 182 [Hpla − NH_2 + H]$^+$; HRMS (ESITOF) *m*/*z* 613.3325 [M + H]$^+$ (calcd for $C_{31}H_{45}N_6O_7$, 613.3344).

Synthesis of L-*p*-Hydroxyphenyllactic Acid. L-Hpla was obtained by synthesis following the approach of Yamaguchi et al., as previously described.[14]

Oxidation, Hydrolysis, and Advanced Marfey's Analysis. Compound **2** (100 μg) was dissolved with 31% H_2O_2, and one drop of concentrated HCl was added. The solution was left at room temperature (rt) for 24 h, lyophilized, and hydrolyzed as previously described.[14] After evaporation, the hydrolysate was subjected to advanced Marfey's[9,10] analysis using D-Marfey's reagents.[12,14] The resulting FDLA solution was analyzed by FT-MS using a Hypersil Gold column (2.1 × 50 mm, 5 μm); gradient elution from 25% to 65% MeCN with 0.1% HCOOH over 45 min, oven temp 40 °C, flow rate 0.2 mL/min. Retention times (min): D-Arg (3.3), L-Arg (3.9).

Synthesis of Diastereoisomers of 4-Methylproline. The four diastereoisomers of 4-methylproline were synthesized using the method of Munro et al.[13] with modification (Supporting Information). The procedure was modified from *N*-CBz-*trans*-4-hydroxy-L-proline ethyl ester (or *N*-CBz-*cis*-4-hydroxy-D-proline ethyl ester) to *N*-CBz-4-keto-L-proline ethyl ester (or *N*-CBz-4-keto-D-proline ethyl ester). The *N*-CBz-*trans*-4-hydroxy-L-proline ethyl ester (204.4 mg, 0.695 mmol) was dissolved in CH_2Cl_2 (5 mL), and Dess-Martin periodinane (1.2 equiv, 354.5 mg) was added. The solution was allowed to react with stirring and left at rt for 1 h. The reaction mixture was treated with 20% sodium thiosulfate, $Na_2S_2O_3$, and saturated with sodium bicarbonate, $NaHCO_3$. The reaction mixture was stirred for a further 20 min and extracted with EtOAc (2 × 15 mL). The EtOAc layer was dried over anhydrous $MgSO_4$ and filtered, and the solvent was evaporated *in vacuo*. The crude product, 343.7 mg, was purified on silica preparative TLC (gel 60F 254) using 1:1 EtOAc/petroleum ether as mobile phase. The second band (85.5 mg) was *N*-CBz-4-keto-L-proline ethyl ester. Also, purification of *N*-CBz-4-exomethylene-L-proline ethyl ester and *N*-CBz-4-exomethylene-D-proline ethyl ester was carried out using 2−10% EtOAc in hexane.

Advanced Marfey's Analyses of the Standard Amino Acid Residues in the Spumigins Using LC/MS. Spumigins J (**1**) and A (**2**) (0.5 mg each) were subjected to advanced Marfey's[9,10] analysis as described in previous papers.[9−11] The resulting residues were resuspended in 500 μL of 10−50% MeCN, and 10 μL of each solution of FDLA derivatives was analyzed by LC/MS.

The LC/MS analysis of the L- and LD-FDLA (mixture of D- and L-FDLA) was performed on a Hypersil Gold column (2.1 × 50 mm). Aqueous MeCN containing 0.1% formic acid was used as a solvent system under a linear gradient elution of 25−65% MeCN with 0.1% formic acid for 45 min, oven temp 40 °C, at a flow rate of 0.2 mL/min. An LTQ Orbitrap was used for detection in the ESI positive mode. Nitrogen was used as a sheath gas. A mass range of *m*/*z* 50−1000 was scanned. The retention times (t_R, min) of the Marfey's derivatized amino acids are summarized. The retention times (min) of authentic amino acids were as follows: LD-FDLA-Hpla (12.4, 12.9), Hty (11.0, 14.3); DL-FDLA-Arg (3.3, 3.9), *N*-MeLys (7.2, 7.8); L-FDLA-Hpla

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(12.2), Hty (11.0), Arg (3.9), *N*-MeLys (7.8). The retention times (min) of the derivatives prepared from the hydrolysate of spumigin J (**1**) were as follows: L-FDLA-Hpla (13.0), Hty (14.4), *N*-MeLys (7.2). The retention times (min) of the derivatives from the hydrolysate of spumigin A (**2**) were noted as L-FDLA-Hpla (12.8), Hty (14.3), Arg (3.9).

Analysis of the 4-MePro configuration was performed under similar conditions using 25−65% MeCN with 0.1% HCOOH over 40 min, YMC Hydrosphere C18, 2.0 × 150 mm. The synthesized diastereoisomers of 4-MePro were used as standards for the analysis. The retention times (t_R, min) of the Marfey's derivatives of the four synthesized diastereoisomers of 4-methylproline were as follows: 2*S*,4*S* (11.75−11.76), 2*S*,4*R* (11.86−11.88), 2*R*,4*S* (12.84), 2*R*,4*R* (13.00−13.02). The retention times (min) of the derivatives from the hydrolysate of spumigin J (**1**) were as follows: 2*S*,4*S* (11.74), 2*S*,4*R* (11.86), 2*R*,4*S* (12.85), 2*R*,4*R* (13.02). The retention times (min) of spumigin A (**2**) hydrolysate derivatives were as follows: 2*S*,4*S* (11.72), 2*S*,4*R* (11.87), 2*R*,4*S* (12.92), and 2*R*,4*R* (13.02).

Cysteine and Serine Protease Inhibitory Activity Assays. Cathepsin B was purchased from Sigma Chemical Co., and the *Z*-Phe-Arg-MCA substrate from Peptide Institute, Inc. The cathepsin B inhibitory activity was determined using the modified method of Greenspan et al.[20] The cathepsin B enzyme was dissolved in 0.1 M phosphate buffer with 1.33 mM EDTA·2Na, 2.7 mM DTT, and 0.03% Brij-35, adjusted to pH 8.2 for the preparation of a 10 U/mL enzyme solution. A 200 μg/mL solution of *Z*-Phe-Arg-MCA in the buffer was used as the substrate. The buffer (80 μL), enzyme (50 μL), and test solution (20 μL) were added to each 96-plate well and preincubated at 37 °C for 10 min, and substrate (50 μL) was added to start the reaction. The fluorescence was measured directly at 390 nm as excitation wavelength and 460 nm as the emission wavelength at an incubation temperature of 37 °C before and after 30 min.

The thrombin enzyme, from bovine plasma, was purchased from Sigma-Aldrich, while the Bz-Phe-Val-Arg·pNA HCl from Bachem was considered as a substrate in the thrombin inhibitory activity assay. The thrombin inhibitory activity assay was determined by the modified method of Laszlo[21] and Yamaguchi[22] et al. Two stock solutions were prepared before the assay experiment. The 0.15 M, pH 8.2 Tris-imidazole buffer was prepared as follows: (I) 1.211 g of Tris and 0.681 g of imidazole in 200 mL of 0.1 M HCl; (II) 1.211 g of Tris, 0.681 g of imidazole, and 0.585 g of NaCl in 300 mL of distilled water. Solutions I and II were mixed proportionally to make a final pH of 8.2. The enzyme previously dissolved in Tris-imidazole buffer was used to make up 5.32 U/mL thrombin solutions. Substrate (2.0 mg) was diluted with 200 μL of DMSO and a 20-fold buffer. The enzyme (90 μL) and sample (20 μL) solutions were added to each well of a 96-well plate and preincubated for 5 min at 37 °C. A substrate solution (90 μL) was added to start the reaction, and the absorbance was read at 405 nm before and after 30 min with incubation at 37 °C.

Computational Docking Techniques. The most abundant ionization states of the four diastereoisomers of spumigins A and J (2*S*,4*S*; 2*S*,4*R*; 2*R*,4*R*; 2*R*,4*S*) were generated using Epik v2.2 in the Schrödinger Suite 2011.[23] The global minimum energy conformation of each structure was identified using Macromodel v9.9[24] by a mixed torsional/low-mode sampling using the OPLS 2005 force field with a water implicit solvation model. Default parameters were employed elsewhere. Docking experiments were conducted using human thrombin extracted from an X-ray crystal structure of the protein in complex with a peptidic inhibitor at 2.3 Å resolution (pdb code 1xm1). The Schrödinger protein preparation wizard was used to assign atom types, side chain protonation states, disulfide bonds, and hydrogen-bonding network and minimize the resulting protein structure prior to use. Glide receptor grids were created by defining a 12 Å box located at the centroid of the cocrystallized ligand. A hydrogen-bonding interaction to the side chain carboxylate of Asp-189 was added as a docking constraint and default van der Waals scaling factors, and partial charge cutoffs were utilized. Each spumigin stereoisomer was flexibly docked into thrombin using Glide XP and rescored with Prime MM-GBSA using the Schrödinger 2011 Virtual Screening Workflow.[25,26] Multiple input conformations were generated for each compound, and docking experiments were performed using the previously defined hydrogen-bonding constraint with postdocking minimization of the resulting poses. Up to three poses per compound state were retained for rescoring using MM-GBSA. Default options were used elsewhere.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information concerning ^{1}H, ^{13}C, and 2D-NMR spectra of spumigins J (**1**) and A (**2**); ^{1}H NMR spectra of 4-methylproline stereoisomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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