Lyngbyastatin 4, a Dolastatin 13 Analogue with Elastase and Chymotrypsin Inhibitory Activity from the Marine Cyanobacterium *Lyngbya confervoides*

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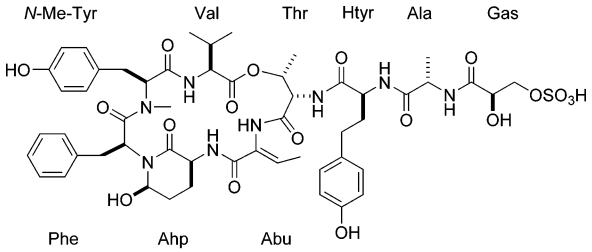
Lyngbyastatin 4 (**1**), a new depsipeptide containing the unusual amino acid homotyrosine and a 3-amino-6-hydroxy-2-piperidone (Ahp) residue, was isolated from a collection of the marine cyanobacterium *Lyngbya confervoides* off the Florida Atlantic coast. Its gross structure was determined by NMR spectroscopy, and the configurations of asymmetric centers were assigned after chiral HPLC analysis of hydrolysis products. Lyngbyastatin 4 (**1**) is an analogue of the sea hare isolate dolastatin 13 and several marine cyanobacterial metabolites, further supporting the notion that many of the dolastatins are of cyanobacterial origin. Lyngbyastatin 4 (**1**) selectively inhibits elastase and chymotrypsin *in vitro* over other serine proteases with IC_{50} values of 0.03 and 0.30 μM, respectively.

Marine cyanobacteria produce a wide array of secondary metabolites, in particular peptides and depsipeptides.[1] Several of these compounds resemble dolastatins, which were originally isolated from the sea hare *Dolabella auricularia*, suggesting that many of the dolastatins are actually derived from the cyanobacterial diet.[2] Cyanobacterial metabolites commonly contain modified or unusual amino acid units, which presumably confer resistance to proteolytic degradation and thus contribute to bioactivity. Concomitantly, these structural features may also allow them to interact with proteases in a substrate-like manner or unconventional ways, leading to protease inhibition, as previously shown.[3,4] In our quest for novel bioactive compounds, including novel protease inhibitors with biomedical utility,[5] we have initiated chemical investigations of cyanobacteria in Florida waters. Here we report the structural elucidation and biological evaluation of a new protease inhibitor we term lyngbyastatin 4 (**1**), due to its structural analogy to dolastatin 13.[6] Lyngbyastatin 4 (**1**) is also closely related to the marine cyanobacterial metabolites symplostatin 2 and somamides A and B (Figure 1).[7,8]



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A sample of the marine cyanobacterium *Lyngbya confervoides* was collected from reef habitats off Fort Lauderdale, Florida, during a cyanobacterial bloom[9] and extracted with organic solvents. The crude extract was fractionated by HP-20 chromatography, where **1** coeluted with several other compounds in a polar fraction (50% aqueous acetone). Subsequent crude fractionation over C_{18} cartridges followed by reversed-phase HPLC afforded lyngbyastatin 4 (**1**) as a colorless, amorphous solid. NMR data combined with a $[M + Na]^+$ peak at *m*/*z* 1159.4199 in the HRESIMS of **1** and a $[M + K]^+$ peak at *m*/*z* 1175.3991 in the MALDI spectrum of **1** suggested a molecular formula of $C_{53}H_{68}N_8O_{18}S$. Analysis of the ^1H NMR, ^{13}C NMR, COSY, TOCSY, HMQC, and HMBC spectra recorded in DMSO-d_6 revealed the presence of alanine, valine, threonine, phenylalanine, *N*-methyltyrosine, glyceric acid (Ga), and the unusual amino acid homotyrosine (Htyr) (Table 1). Additionally, partial

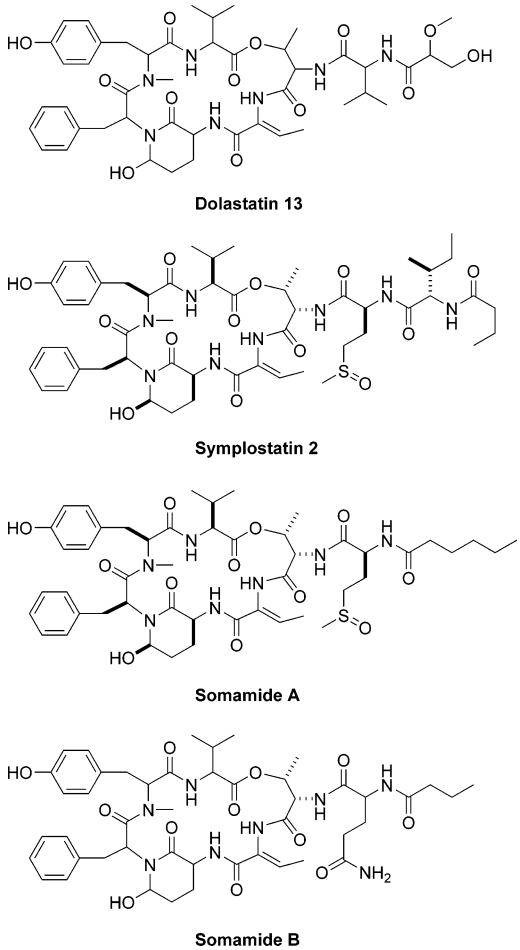

Figure 1. Metabolites related to lyngbyastatin 4 (**1**) isolated from the sea hare *D. auricularia* (dolastatin 13) and from marine cyanobacteria (symplostatin 2, somamides A and B).

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Table 1. NMR Spectral Data for Lyngbyastatin 4 (**1**) in DMSO-d_6 (500 MHz)

C/H no.		δ_H (J in Hz)	δ_C, mult.	COSY	HMBC[a]	ROESY
Val	1		172.5,qC			
	2	4.72, br	55.8, CH	H-3, NH		H-3, H_3-4, H_3-5, NH
	3	2.09, m	30.6, CH	H-2, H_3-4, H_3-5	1, 2, 4, 5	H-2, H_3-4, H_3-5
	4	0.88, d (7.0)	19.1, CH_3	H-3	2, 3, 5	H-2, H-3, H_3-5, *N*-Me (*N*-Me-Tyr), H_3-4 (Thr)
	5	0.75, d (7.0)	17.2, CH_3	H-3	2, 3, 4	H-2, H-3, H_3-4, NH, *N*-Me (*N*-Me-Tyr)
	NH	7.46, br d (7.5)		H-2		H-2, H_3-5, H-2 (*N*-Me-Tyr), *N*-Me (*N*-Me-Tyr), 6-OH (Ahp)
N-Me-Tyr	1		169.3, qC			
	2	4.90, d (11.5)	60.8, CH	H-3a, H-3b	1	H-3a, H-3b, N-Me, H-5/9, H-5/9 (Phe), NH (Val), H-2 (Phe)
	3a	3.10, d (−12.5)	32.9, CH_2	H-2, H-3b	4, 5/9	H-2, H-3b, H-5/9, H-2 (Phe)
	3b	2.71, dd (−12.5, 11.5)		H-2, H-3a	1, 2, 5/9	H-2, H-3a, H-5/9
	4		127.3, qC			
	5/9	6.99, d (8.2)	130.3, CH	H-6/8	3, 4, 9/5, 7	H-2, H-3a, H-3b, H-6/8, *N*-Me, H-2 (Phe)
	6/8	6.76, d (8.2)	115.1, CH	H-5/9	4, 8/6, 7	H-5/9, 7-OH, H-5/9 (Phe)
	7		156.0, qC			
	7-OH	9.33, s			5/9, 7	H-6/8
	N-Me	2.76, s	30.1, CH_3		2, 1 (Phe)	H-2, H-5/9, H_3-4 (Val), H_3-5 (Val), NH (Val)
Phe	1		170.2, qC			
	2	4.74, dd (11.9, 2.0)	50.1, CH	H-3a, H-3b	1, 2 (Ahp)	H-3a, H-3b, H-5/9, H-2 (*N*-Me-Tyr), H-3a (*N*-Me-Tyr), H-5/9 (*N*-Me-Tyr), H-6 (Ahp)
	3a	2.87, dd (−12.8, 11.9)	35.0, CH_2	H-2, H-3b	2, 4, 5/9	H-2, H-3b, H-5/9, H-6 (Ahp), 6-OH (Ahp)
	3b	1.81, dd (−12.8, 2.0)		H-2, H-3a	4, 5/9	H-2, H-3a, H-6 (Ahp)
	4		136.5, qC			
	5/9	6.84, d (7.0)	129.2, CH	H-6/8	3, 9/5, 7	H-2, H-3a, H-6/8, H-2 (*N*-Me-Tyr), H-6/8 (*N*-Me-Tyr)
	6/8	7.19, m	127.5, CH	H-5/9, H-7	4, 8/6	H-5/9, H-7
	7	7.14, m	126.0, CH	H-6/8	5/9	H-6/8
Ahp	2		168.5, qC			
	3	3.79, ddd (11.5, 8, 5.5)	48.0, CH	H-4a, H-4b, NH	2, 4	H-4b, H-5b, NH
	4a	2.43, m	22.0, CH_2	H-3, H-4b, H-5a		H-4b, 6-OH
	4b	1.58, m		H-3, H-4a, H-5a		H-3, H-4a
	5a	1.71, m	29.1, CH_2	H-4a, H-4b, H-5b, H-6		H-5b, H-6, 6-OH
	5b	1.57, m		H-5a, H-6		H-3, H-5a, H-6, 6-OH
	6	5.07, br s	73.5, CH	H-5a, H-5b, 6-OH	2	H-5a, H-5b, 6-OH, H-2 (Phe), H-3a (Phe), H-3b (Phe)
	6-OH	6.05, s		H-6		H-4a, H-5a, H-5b, H-6, NH (Val), H-3a (Phe)
	NH	7.19, d (8)		H-3		H-3
Abu	1		162.5, qC			
	2		129.5, qC			
	3	6.51, q (7.1)	131.5, CH	H_3-4	1, 2, 4	H_3-4
	4	1.46, d (7.1)	12.9, CH_3	H-3, NH	2, 3	H-3, NH[b]
	NH[c]	9.18, br		H_3-4		H_3-4,[b] NH (Thr)[b]
Thr	1		173.5,[d] qC			
	2	4.62, br	55.4, CH	NH[e]		H_3-4
	3	5.52, br	71.6, CH	H_3-4		H_3-4
	4	1.24, d (6.0)	17.6, CH_3	H-3	2, 3	H-2, H-3, H_3-4 (Val)
	NH	7.94, br		H-2[e]		NH (Abu),[b] H-2 (Htyr)
Htyr	1		172.3,[f] qC			
	2	4.49, dd (12.0, 7.5)	52.0, CH	H-3a, H-3b, NH		H-3a, H-3b, H-4, NH, NH (Thr)
	3a	1.91, m	30.9,[g] CH_2	H-2, H-3b, H-4		H-2, H-3b, H-6/10
	3b	1.82, m		H-2, H-3a, H-4		H-2, H-3a, H-4, H-6/10
	4	2.48, m (2H)	30.2, CH_2	H-3a, H-3b	5, 6/10	H-2, H-3b, H-6/10
	5		131.5, qC			
	6/10	6.95, d (8.0)	128.8, CH	H-7/9	4, 10/6, 8	H-3a, H-3b, H-4, H-7/9
	7/9	6.64, d (8.0)	114.9, CH	H-6/10	5, 9/7, 8	H-6/10, 8-OH
	8		155.1, qC			
	8-OH	9.10, s			7/9, 8	H-7/9
	NH	8.22, br d (5.0)		H-2		H-2, H-2 (Ala)
Ala	1		171.6, qC			
	2	4.38, dq (7.5, 7.0)	47.5, CH	H_3-3, NH	1, 3	H_3-3, NH, NH (Htyr)
	3	1.27, d (7.0)	18.5, CH_3	H-2	1, 2	H-2, NH, H-3a (Gas)
	NH	7.86, d (7.5)		H-2	2, 1 (Gas)	H-2, H_3-3, H-2 (Gas)
Gas	1		170.3, qC			
	2	4.10, m	70.8, CH	2-OH, H-3a, H-3b		H-3a, H-3b, 2-OH, NH (Ala)
	2-OH	5.92, d (5.0)		H-2	1, 2, 3	H-2, H-3a, H-3b
	3a	4.02, dd (−11.4, 2.4)	68.2, CH_2	H-2, H-3b	1	H-2, H-3b, 2-OH, H_3-3 (Ala)
	3b	3.74, dd (−11.0, 7.7)		H-2, H-3a	2	H-2, H-3a, 2-OH

[a] Protons showing HMBC correlation to the indicated carbon. [b] Only observed in a selective 1D NOE experiment, irradiating the Abu NH proton signal. [c] Proton showed weak TOCSY correlations to H-3 and H_3-4 of the Abu unit, allowing unambiguous assignment. [d] Signal assigned by comparison with somamide A.[8] [e] Only observed in the TOCSY spectrum and as the only correlation for the NH signal. [f] No HMBC correlation observed and assigned to Htyr unit based on ^{13}C NMR (150 MHz). [g] No HMQC correlation observed. Carbon assigned based on ^{13}C NMR (150 MHz).

structures corresponding to 3-amino-6-hydroxy-2-piperidone (Ahp) and 2-amino-2-butenoic acid (Abu) units were deduced (Table 1), both of which were also found in dolastatin 13, symplostatin 2, and somamides A and B (Figure 1).[6−8]

HMBC analysis in combination with ROESY data unambiguously established two partial sequences as *N*-Me-Tyr-Phe-Ahp and Ga-Ala-Htyr (Table 1). A ROESY correlation between H-2 of *N*-Me-Tyr (δ_H 4.90) and H-2 of Phe (δ_H 4.74) further suggested a *cis* configuration of the amide bond between both amino acid units. Another strong ROESY correlation between H-2 of *N*-Me-Tyr (δ_H 4.90) and the amide NH of Val (δ_H 7.46), characteristic for *trans* amide linkages, allowed us to connect these two units and expand the first sequence to Val-*N*-Me-Tyr-Phe-Ahp. Similarly, on the basis of ROESY correlations between H-2 of Htyr (δ_H 4.49) and the NH of Thr (δ_H 7.94), the second partial sequence was expanded to Ga-Ala-Htyr-Thr. The amide NH signal corresponding to the Abu unit (δ_H 9.18) was unmistakably identified as such on the basis of weak TOCSY cross-peaks with the other protons in this unit (δ_H 6.51, 1.46; Table 1). Irradiation of this signal in a selective 1D NOE experiment enhanced the Ahp NH signal (δ_H 7.19) and the H-2 signal of Thr (δ_H 4.62), suggesting that the Abu unit links Ahp and Thr moieties. Consequently, this prompted us to predict a linear sequence for all nine units of Val-*N*-Me-Tyr-Phe-Ahp-Abu-Thr-Htyr-Ala-Ga. The low-field chemical shift of H-3 of Thr (δ_H 5.52) indicated the presence of an ester bond at this position rather than an alcohol. By default this ester linkage had to be made with the Val carbonyl group, leading to the cyclic core structure as depicted for **1**, which is the same as in symplostatin 2 and somamides A and B (Figure 1). An interresidual NOE between methyl groups of Val (δ_H 0.88) and Thr (δ_H 1.24) supported the connection. The IR absorption at 1733 cm^{-1} in addition to the intense band centered at 1644 cm^{-1} confirmed the presence of both ester and amide functionalities in compound **1**. To account for the molecular formula and the high polarity of **1**, the compound had to be sulfated. Since all 10 exchangeable protons were clearly assigned on the basis of homo- or heteronuclear correlations (Table 1), the sulfate had to be located at the remaining oxygenated atom (δ_C 68.2), C-3 of glyceric acid, establishing the proposed structure for **1**. Chemical shifts for the glyceric acid 3′-*O*-sulfate (Gas) unit were in agreement with values obtained for other compounds containing this moiety.[10,11]

The absolute configuration of amino and glyceric acid-derived units were established by chiral HPLC analysis of the acid hydrolyzate. Except for phenylalanine, which was not clearly identified under those conditions presumably due to incomplete hydrolysis, our analysis revealed an L-configuration for all amino acids and a D-configuration for glyceric acid. CrO_3 oxidation followed by acid hydrolysis additionally liberated L-glutamic acid, thereby establishing the configuration of the Ahp residue at C-3 (3*S*), and this reaction sequence also generated sufficient amounts of L-phenylalanine for unambiguous detection and configurational assignment (2*S*). The ^1H and ^{13}C NMR chemical shifts in the cyclic core structure of **1** could readily be compared with data reported for somamide A (Figure 1), which was also recorded in DMSO-d_6.[8] All resonances were virtually identical, suggesting that the relative configuration of all units of the cyclic core was identical, including the yet unassigned stereocenter of the Ahp unit (6*R*). While NOE data were inconclusive, the observation of two small coupling constants for H-6 of the Ahp unit (δ_H 5.07, br s) and one large and one medium coupling constant for H-3 with vicinal methylene protons (Table 1) suggested that 6-OH and H-3 are located in axial positions of the piperidone ring (presumably in a twisted chair conformation).[3,10] Thus, we assigned the 3*S*,6*R* configuration to the Ahp unit of **1**, as reported for related compounds.[3,8,10] Matching ^{13}C NMR data for the Abu unit in **1** and somamide A as well as an NOE between the Abu NH (δ_H 9.18) and Abu methyl group (δ_H 1.46) indicated the *Z* geometry of the Abu unit in **1**.[8]

Lyngbyastatin 4 (**1**) was evaluated for serine protease-inhibitory activity. Specifically, we tested elastase, chymotrypsin, trypsin, thrombin, and plasmin activity *in vitro* in a dose−response analysis and determined that lyngbyastatin 4 (**1**) selectively inhibits elastase and chymotrypsin activity with IC_{50} values of 0.03 and 0.30 μM, respectively. Remarkably, the other serine proteases were not inhibited by compound **1** up to the highest concentration tested (30 μM), indicating a >1000- and >100-fold selectivity for elastase and chymotrypsin, respectively. Furthermore, lyngbyastatin 4 (**1**) showed no cytotoxicity to various cancer cell lines.

The isolation of yet another close analogue of dolastatin 13 supports the notion that many dolastatins are of cyanobacterial origin. An intriguing structural feature in lyngbyastatin 4 (**1**), in addition to the modified amino acid units also present in dolastatin 13 (Ahp, Abu), is the unusual moiety Htyr. While rare, Htyr units have been an attribute of several metabolites of freshwater cyanobacteria, such as various oscillamides,[12] anabaenopeptins,[13] and oscillapeptins.[14] In fact, many oscillapeptins additionally contain the Ahp and glyceric acid moiety present in lyngbyastatin 4 (**1**).[14,15] Another recently reported Ahp-containing marine cyanobacterial metabolite with Htyr is largamide G.[16] Many of these Ahp-containing cyclodepsipeptides were shown to be serine protease inhibitors, some of which displayed a selectivity profile distinct from that of lyngbyastatin 4 (**1**).[17−19] Structural studies have begun to illuminate the mode of inhibition of these compounds. The related Ahp- and glyceric sulfate-containing serine protease inhibitor A90720A[10] has been demonstrated to bind trypsin in a substrate-like manner.[3] The mode of protease inhibition by **1** is likely similar, although the target selectivity varies. The crystal structure of A90720A bound to trypsin indicated that the arginine residue on the amino group of the Ahp residue occupies trypsin's specificity pocket,[3] while the presence of hydrophobic residues in this position seems to preferentially result in chymotrypsin inhibition.[20] The crystal structure of the Ahp-containing elastase inhibitor scyptolin A[21] bound to elastase revealed that leucine cannot bind favorably in trypsin's specificity pocket built for lysine or arginine.[22] The presence of the Abu unit in **1** in this position and the observed concomitant selectivity profile are supportive of the reported structure−function studies. Several Ahp- and glyceric acid-containing derivatives lacking lysine or arginine in this position exhibit selectivity similar to **1**.[14,23] The selectivity of lyngbyastatin 4 (**1**) for inhibiting elastase and chymotrypsin over other serine proteases is a remarkable biological characteristic that may warrant further exploration.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a Perkin-Elmer 341 polarimeter. ^1H and 2D NMR spectra were recorded in DMSO-d_6 on a Bruker Avance 500 MHz spectrometer operating at 500 MHz, and ^{13}C NMR data were collected on a Bruker Avance 600 MHz spectrometer operating at 150 MHz. Residual solvent signals (δ_H 2.50 ppm, δ_C 39.5 ppm) were used as an internal reference. HMQC experiments were optimized for $^1J_{CH}$ = 145 Hz, and HMBC experiments were optimized for $^nJ_{C,H}$ = 5, 10, and 15 Hz. HRESIMS were recorded on a Bruker APEX II FTICR spectrometer and MALDI spectra on a DE-STR instrument in the positive mode.

Extraction and Isolation. Samples of *Lyngbya confervoides* were collected off the coast of Broward County (Fort Lauderdale and Pompano Beach, Florida) (26°01.1414′ N, 80°05.9973′ W; 26°15.134′ N, 80°03.908′ W) at a depth of 7−15 m in July 2004 and August 2005.[9] A voucher specimen is retained at the Smithsonian Marine Station. The freeze-dried organism was extracted with EtOAc−MeOH (1:1) to afford the lipophilic extract, VP56L (3.4 g). VP56L was applied to a diaion HP-20 polymeric resin and subsequently fractionated with H_2O and increasing concentrations of acetone. The fraction eluting with 50% aqueous acetone (80 mg) was applied to a C_{18} SPE cartridge and elution initiated with H_2O followed by aqueous solutions containing 25, 50,

75, and 100% MeOH. The fraction eluting with 25% aqueous MeOH was then purified by semipreparative reversed-phase HPLC (YMC-Pack ODS-AQ, 250 × 10 mm, 2.0 mL/min; UV detection at 220 and 240 nm) using a MeOH−H_2O linear gradient (20−100% over 70 min and then 100% MeOH for 10 min), to furnish compound **1**, t_R 35.0 min (3.6 mg).

Lyngbyastatin 4 (1): colorless, amorphous powder; $[\alpha]^{20}_D$ +8.4 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (4.57), 280 (sh) (3.60) nm; IR (film) ν_{max} 3364 (br), 2963, 2929, 1733, 1644 (br), 1540, 1518, 1451, 1262, 1204 cm^{-1}; ^1H NMR, ^{13}C NMR, COSY, HMBC, and ROESY data, see Table 1; HRESIMS *m/z* $[M + Na]^+$ 1159.4199 (calcd for $C_{53}H_{68}N_8O_{18}SNa$, 1159.4270); MALDI *m/z* $[M + K]^+$ 1175.3991 (calcd for $C_{53}H_{68}N_8O_{18}SK$, 1175.4004).

Absolute Configuration of Amino Acids and Glyceric Acid in Lyngbyastatin 4 (1). Compound **1** (200 μg) was treated with 6 N HCl (200 μL) for 18 h at 110 °C. The hydrolyzed product was evaporated to dryness, and the residue resuspended in H_2O and passed over a C_{18} SPE cartridge eluting with H_2O and H_2O−MeCN (85:15). The combined eluted material was subjected to chiral HPLC analysis [column, Phenomenex Chirex phase 3126 *N*,*S*-dioctyl-(D)-pencillamine, 4.60 × 250 mm, 5 μm; solvents, 2 mM $CuSO_4$−MeCN (95:5 or 85:15); flow rate 1.0 mL/min; detection at 254 nm]. The absolute configuration of the amino acids and glyceric acid in the hydrolyzate was determined by direct comparison with the retention times or by co-injection with those of authentic standards. The retention times (t_R, min) for standard amino acids and glyceric acids were as follows: L-Ala (7.7), D-Ala (9.0), L-Thr (7.9), D-Thr (8.5), L-*allo*-Thr (10.2), D-*allo*-Thr (10.5), L-Val (20.0), D-Val (26.0) (solvent mixture 95:5); L-Htyr (27.5), D-Htyr (36.1), L-glyceric acid (17.0), D-glyceric acid (25.5), *N*-Me-L-Tyr (16.5), *N*-Me-D-Tyr (18.5) (solvent mixture 85:15). The retention times (t_R, min) of the hydrolyzate components were 7.7, 7.9, and 20.0 (solvent mixture 95:5) and 16.5, 25.5, and 27.5 (solvent mixture 85:15), confirming the presence of L-Ala, L-Thr, L-Val, *N*-Me-L-Tyr, D-glyceric acid, and L-Htyr, respectively, in **1**.

Oxidation of **1** was carried out similarly as described previously.[14] A sample of compound **1** (300 μg) was dissolved in glacial AcOH (300 μL) and added to CrO_3 (2 mg). After stirring at room temperature for 5 h, the reaction mixture was applied onto a C_{18} SPE cartridge eluting with H_2O and MeOH. The resulting oxidized material from the MeOH fraction was hydrolyzed with 6 N HCl for 18 h at 110 °C and subjected to chiral HPLC as described above, using 2 mM $CuSO_4$−MeCN (90:10 or 85:15) as the mobile phase. L-Glu liberated from the reaction mixture was detected at t_R 29 min (standard L-Glu, t_R 29 min; standard D-Glu, t_R 30 min; solvent mixture 90:10). A larger peak (compared to the acid hydrolysis profile without prior oxidation) at t_R 48 min was attributed to L-Phe (standard L-Phe, t_R 48 min; standard D-Phe, t_R 49 min; solvent mixture 85:15).

Protease Inhibition Assays. Inhibitory activities against chymotrypsin and trypsin were determined as previously described[19] with only slight modifications, using α-chymotrypsin from bovine pancreas (Sigma C4129) and trypsin from porcine pancreas (Sigma T0303), with 2 mM *N*-succinyl-Gly-Gly-Phe-*p*-nitroanilide as a substrate solution for chymotrypsin and 2 mM *N*α-benzoyl-DL-arginine 4-nitroanilide hydrochloride for trypsin. Briefly, 80 μL of the buffer solution (50 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.8), 10 μL of enzyme solution (1 mg/mL), and 10 μL of test solution were added to each microtiter plate well followed by addition of 50 μL of substrate solution. The increase in absorbance was measured for 30 min at 405 nm.

Elastase-inhibitory activity was tested using elastase from porcine pancreas (Sigma E0258).[21] After preincubation of 150 μL of Tris-HCl (pH 8.0), 10 μL of elastase solution (75 μg/mL), and 10 μL of compound **1** in a microtiter plate at 30 °C for 20 min, 30 μL of substrate solution (2 mM *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide; Sigma S4760) was added to the mixture. The increase in absorbance was measured for 3−30 min at 405 nm.

Inhibitory activities against thrombin and plasmin were assayed as described[21] using thrombin from bovine plasma (Sigma T4648) and plasmin from human plasma (Sigma P1867). Phenylmethylsulfonyl fluoride (PMSF) and 3,4-dichloroisocoumarin were used as positive controls in the enzyme assays.

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