Cyclotheonamides E2 and E3, New Potent Serine Protease Inhibitors from the Marine Sponge of the Genus *Theonella*[1]

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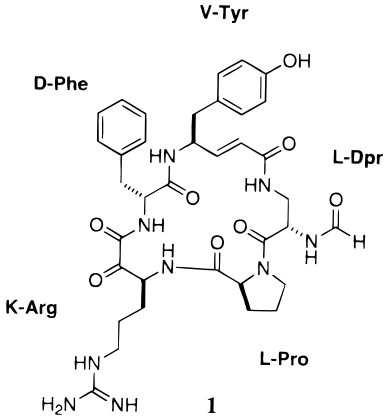
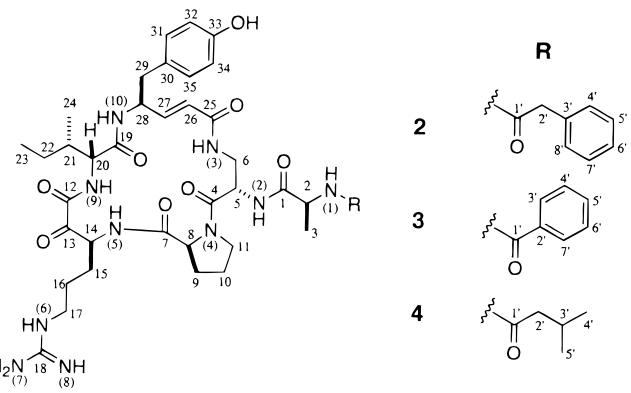
Two new potent serine protease inhibitors, cyclotheonamides E2 (**3**) and E3 (**4**), have been isolated from a marine sponge of the genus *Theonella*. Their structures were determined by interpretation of spectral data and chemical degradation studies. They are closely related to the previously reported cyclotheonamide E, from which they differ in the *N*-acyl group of the alanyl side chain. Cyclotheonamides E, E2, and E3 were more active against thrombin than against trypsin.

Cyclotheonamide A (**1**) is a potent serine protease inhibitor isolated from the marine sponge *Theonella swinhoei*.[2] It is a cyclic pentapeptide containing unusual amino acid residues, i.e., vinylogous tyrosine (V-Tyr), α-ketohomoarginine (K-Arg), and *β*-linked-diaminopropionic acid (Dpr). X-ray crystallography of the complex between human α-thrombin and cyclotheonamide A unambiguously disclosed that the α-keto group in the K-Arg residue forms a covalent bond with the hydroxyl group of Ser195 in the enzyme, one of a catalytic triad, which results in inhibition of the enzyme.[3] A similar mode of action against trypsin was also demonstrated by an X-ray study.[4]

Variants of cyclotheonamide A have also been isolated:[2,5] cyclotheonamide B is an acetamide derivative of **1**; cyclotheonamide C has an additional unsaturation in the V-Tyr residue; in cyclotheonamide D, the D-Phe residue in cyclotheonamide A is replaced by a Leu residue; cyclotheonamide E has a phenylacetylalanyl side chain. Cyclotheonamides A−D were isolated from the same specimens of *T. swinhoei*, while cyclotheonamide E (**2**) was isolated from a morphologically different *Theonella swinhoei*.[5] To obtain further information on the structure−activity relationships for the cyclotheonamides, we have examined the EtOH extract of a *Theonella* sp. collected from Tanegashima Island, which resulted in the isolation of two new cyclotheonamides, E2 and E3.

The sponge *Theonella* sp. was collected by hand using scuba (−10 m) off Tanegashima Island, 1400 km southwest of Tokyo. Frozen samples (4.2 kg) were homogenized and extracted with EtOH. The extract was partitioned between Et_2O and H_2O. The aqueous layer was further extracted with *n*-BuOH. Potent thrombin inhibitory activity was found in the *n*-BuOH layer, which was successively fractionated by ODS flash chromatography, gel filtration on Sephadex LH-20, flash chromatography on ODS, and reversed-phase HPLC. Final purification was performed by ODS HPLC to yield cyclotheonamide E2 (**3**, 1.6 mg) and cyclotheonamide E3 (**4**, 4.2 mg) along with cyclotheonamide E (**2**, 8.3 mg).





Cyclotheonamide E2 (**3**) had a molecular formula of $C_{42}H_{56}N_{10}O_9$ as determined by HRFABMS, differing from cyclotheonamide E (**2**) by the elements CH_2. The ^1H and ^{13}C NMR spectra of **3** were similar to those of **2**. Interpretation of HOHAHA, HMQC,[6] and HMBC[7] data (Table 1) led to the assignment of the component units, i.e., Ala, Ile, Pro, Dpr, K-Arg, V-Tyr, and a monosubstituted benzene. A characteristic benzylic methylene carbon observed in **2** was missing. Sequencing of the above units was done by ROESY[8] and HMBC experiments, thus assigning a gross structure identical with that of **2** except for the presence of a benzoyl group instead of the phenylacetyl group.

Marfey analysis[9] of the acid hydrolysate revealed that the absolute stereochemistries of Ala, Pro, and Dpr were

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Table 1. NMR Data for Cyclotheonamides E2 (**3**) and E3 (**4**)[a,b]

	3			**4**		
no.	^{13}C	^{1}H	HMBC	^{13}C	^{1}H	HMBC
1′	169.7			175.5		
2′	134.6			45.8	2.08 m	C: 1′, 3′, 4′, 5′
3′	128.0	7.84 d 7.3	C: 1′, 5′, 7′	27.8	2.02 m	C: 1′, 2′, 4′, 5′
4′	129.0	7.45 t 7.3	C: 2′, 6′	22.6	0.92 d 6.2	C: 2′, 3′, 5′
5′	132.3	7.53 t 7.3	C: 3′, 7′	22.6	0.92 d 6.2	C: 2′, 3′, 4′
6′	129.0	7.45 t 7.3	C: 2′, 4′			
7′	128.0	7.84 d 7.3	C: 1′, 3′			
1	174.6			174.9		
2	50.3	4.55 dq 6.2, 7.3	C: 1′, 1, 3	49.9	4.33 dq 6.5, 6.9	C: 1, 3
3	17.3	1.42 d 7.3	C: 1, 2	17.6	1.28 d 6.9	C: 1, 2
NH-1		8.47 d 6.2	C: 1′, 3		8.18 d 6.5	C: 1′ , 2, 3
4	172.5			171.5		
5	51.3	4.65 m	C: 6	51.4	4.60 dd 11.0, 5.4	C: 3, 4, 6
6a	40.5	4.28 m	C: 4, 5, 25	40.9	4.23 m	C: 4, 5, 25
6b		2.84 ddd 12.3, 11.0, 3.5	C: 5, 25		2.81 bdd 11.0, 10.4	C: 5, 25
NH-2		8.32 d 5.4 (8.30 d 5.8)	C: 1		8.23 d 5.4	C: 1
NH-3		8.57 d 11.0 (8.81 d 11.2)			8.57 d 10.4 (8.82 d 10.1)	
7	173.6			174.1		
8	61.3	4.50 m	C: 9, 10	61.7	4.49 m	C: 7, 9, 10
9a	30.7	2.21 m	C: 7, 10, 11	31.3	2.23 m	C: 7, 10, 11
9b		1.97 m	C: 7, 8, 10		1.97 m	C: 7, 10, 11
10	25.5	1.96 m	C: 8, 9	26.0	1.93 m	C: 8, 9
11a	49.0	3.84 m	C: 10	49.6	3.81 m	C: 9, 10
11b		3.52 m	C: 10		3.51 m	C: 9, 10
12	171.2			171.5		
13	99.2[c]			99.5[c]		
14	54.6	4.11 m (4.04 m)		55.2 (55.8)	4.11 ddd 11.2, 10.0, 2.5 (4.03 m)	
15a	24.7	1.98 m		25.0	1.98 m	
15b		1.55 m			1.56 m	
16a	25.5	1.68 m		26.1	1.68 m	C: 15, 17
16b		1.55 m	C: 15		1.55 m	C: 15, 17
17	41.6	3.13 m (3.11 m)	C: 15, 18	42.1	3.12 m	C: 15, 18
18	158.3			158.6		
NH-5		7.97 d 9.2 (8.00 d 10.4)	C: 7		8.00 d10.0 (8.03 d 10.4)	C: 7
NH-6		7.26 m			7.29 m	C: 17
19	171.7			172.1 (170.3)		
20	57.9 (57.6)	4.28 m (4.24 m)	C: 12, 24	58.4 (57.9)	4.23 m (4.27 m)	C: 19
21	41.0 (40.2)	1.37 m (1.35 m)		41.4 (40.7)	1.38 m (1.34 m)	
22	26.8 (26.5)	1.28 m (1.08 m)		27.3 (27.0)	1.28 m (1.04 m)	
23	11.7	0.81 m (0.79 m)	C: 21, 22	12.2	0.80 m (0.78 m)	C: 21, 22
24	14.2 (14.0)	0.55 d 6.5 (0.61 d 6.9)	C: 20, 21, 22	14.6 (14.4)	0.54 d 6.9 (0.61 d 6.9)	C: 20, 21, 22
NH-9		7.31 d 8.5 (7.33 d 9.2)	C: 12		7.31 d 8.5 (7.34 d 9.6)	C: 12
25	167.4			167.9		
26	124.6	6.14 d 15.0	C: 25, 28	124.9	6.14 d 15.8	C: 25, 28
27	143.3	6.78 bd 15.0	C: 25, 28	143.7	6.78 dd 15.8, 1.3	C: 25, 28
28	52.8	4.71 m		53.4	4.69 m	
29a	38.8	3.05 dd 14.1, 5.0	C: 28, 30, 31, 35	39.4	3.05 dd 14.1, 5.0	C: 28, 30, 31, 35
29b		2.58 dd 14.1, 11.9	C: 28, 30, 31, 35		2.58 dd 14.1, 11.5	C: 28, 30, 31, 35
30	129.3			129.7		
31	130.4	7.06 d 8.5	C: 29, 32, 33, 35	130.9	7.06 d 8.5	C: 29, 32, 33, 35
32	115.8	6.69 d 8.5	C: 30, 33, 34	116.2	6.69 d 8.5	C: 30, 33, 34
33	156.8			157.3		
34	115.8	6.69 d 8.5	C: 30, 32, 33	116.2	6.69 d 8.5	C: 30, 32, 33
35	130.4	7.06 d 8.5	C: 29, 31, 33, 34	130.9	7.06 d 8.5	C: 29, 31, 33, 34
NH-10		8.21 d 8.1 (8.27 d 8.1)	C: 19		8.23 d 6.9 (8.30 d 8.5)	C: 19

[a] In CD_3OH. [b] Chemical shift values of the minor conformers are shown in parentheses. [c] Exist as a hemiacetal in CD_3OH.

L. The stereochemistry of the K-Arg residue was assigned as 3*S* by a modification of Marfey's method[10] using L-FDAA, D-FDAA, and a standard (3*S*)-K-Arg. To determine the stereochemistry of the V-Tyr residue, cyclotheonamide E2 was subjected to ozonolysis followed by acid hydrolysis; the stereochemistry of the liberated Asp was L as analyzed by Marfey's method. Therefore, the V-Tyr residue has the 4*S*-stereochemistry. Since discrimination between Ile and *a*Ile could not be accomplished by Marfey analysis, the acid hydrolysate was analyzed by chiral GC after treatment with HCl/MeOH and TFAA, which disclosed that **3** contained D-*a*Ile. Additionally, *a*Ile was isolated from the acid hydrolysate of cyclotheonamide E(**2**), and its stereochemistry was unambiguously determined to be D both by ^{1}H NMR spectroscopy and chiral GC. Thus, the structure of **2** must be as shown.

Cyclotheonamide E3 (**4**), having a molecular formula of $C_{40}H_{60}N_{10}O_9$, exhibited a ^{1}H NMR spectrum similar to that of cyclotheonamide E2; in place of the monosubstituted benzene ring were present two doublet methyls coupled to a methine, which was in turn correlated with a methylene. Interpretation of 2D NMR data revealed that the benzoyl in **3** was replaced by an isovaleryl group. The absolute stereochemistry of the component amino acid residues was determined as for **3**.

Doubled signals in the ^{1}H NMR spectra of **2**−**4** were observed for all protons in the Ile residue, the β-H of

Table 2. Inhibitory Activity of Cyclotheonamides against Thrombin and Trypsin[a]

	A (**1**)	E (**2**)	E2 (**3**)	E3 (**4**)
thrombin	23	2.9	13	9.5
trypsin	16	30	55	52

[a] IC_{50} values (nM).

K-Arg, and all amide protons within the macrocyclic ring, but this was not the case for cyclotheonamide A.[11] To clarify the reason for the doubled signals, further ^{1}H NMR studies were carried out with cyclotheonamide E3 (**4**). The most obvious doubling was observed for the CH_3-24 of the Ile residue, which appeared as an isolated doublet in each case. The possibility of the presence of diastereomeric methyl acetals in CD_3OH was ruled out because the doubling was also observed in D_2O. The doubling was most likely due to the presence of two conformers; the intensity ratio of the CH_3-24 signal changed from 2:1 at 300 K to 3:1 at 253 K. The commonly observed cis−trans isomerism of the Pro residue was also ruled out, because the Pro residue appeared exclusively as the trans-isomer (Table 1). Although most coupling constants and ROESY cross-peaks in the two conformers were identical throughout the entire molecule, there was a difference in the ROESY cross-peak between NH-(9) and H-14; in the major conformer an intense cross-peak was observed, thus suggesting that N-(9) and C-14 were in a gauche relationship, while in the minor conformer only a faint cross-peak was observed between the relevant protons, which indicated that N-(9) and C-14 were anti to the C12−C13 bond. Although not rigorously proven, the doubled signals in **2** and **3** were also ascribed to C-12−C-13 rotamers.

The enzyme inhibitory activity of cyclotheonamides E2 and E3 together with cyclotheonamides A and E is summarized in Table 2, which indicates that compounds **2**−**4** have enhanced specificity toward thrombin. On the basis of X-ray crystallographic studies of the complex between cyclotheonamide A and either human α-thrombin or bovine β-trypsin,[12] cyclotheonamide A was rationalized to be more potent against trypsin than thrombin[13] because of the more favorable (1) aromatic interaction of the D-Phe residue in **1** with Tyr39 and Phe41 in trypsin than with Glu39 and Leu41 in thrombin and (2) interaction of *N*-formyl Dpr residue with Gly174 and Gln175 in trypsin than with Ile174 and Arg175 in thrombin. Substitution of D-Phe by D-*a*Ile decreases the aromatic hydrophobic interaction of cyclotheonamides E, E2, and E3 with trypsin, while replacement of formamide by a bulky acylated alanyl residue increases the hydrophobic interaction with Ile174 in thrombin. Interestingly, desformylcyclotheonamide A exhibits less selectivity toward thrombin,[5] revealing that the interaction between the substituent of the Dpr residue and Arg175 residue in thrombin is operating. Noting that substitutions of the D-Phe residue and the side chain group reverse the selectivity of the inhibitor toward trypsin and thrombin,[14] it will be possible to design inhibitors retaining the cyclotheonamide skeleton with higher selectivity by choosing a correct array of side-chain structures.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a JEOL A600 NMR spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. ^{1}H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ 3.30 and 49.0 for CD_3OH. FAB mass spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using glycerol as matrix. Amino acid analysis was carried out with a Hitachi L 8500-A amino acid analyzer. Chiral GC analysis was carried out on a Shimadzu GC-9A gas chromatograph. Optical rotations were determined on a JASCO DIP-1000 digital polarimeter in CH_3OH. UV spectra were recorded on a Hitachi 330 spectrophotometer.

Animal Material. Specimens of *Theonella* sp. were collected using scuba at a depth of 10 m off Tanegashima Island, 1400 km southwest of Tokyo, in July 1993, frozen immediately, and preserved at −20 °C until extraction. The sponge was identified as *Theonella* sp. by Dr. Rob van Soest, University of Amsterdam. Actually, the sponge skeleton looked very much like *T. swinhoei*, but its morphology was different. It was rather round in shape, and tubes were not developed. A voucher specimen (ZMA POR 11509) was deposited at the Zoological Museum of the University of Amsterdam.

Extraction and Isolation. The frozen sponge (4.2 kg, wet wt) was exhaustively extracted with EtOH (3 L × 5), and the combined extracts were concentrated and partitioned between Et_2O (2.5 L × 4) and H_2O (2.7 L). The aqueous phase was further extracted with *n*-BuOH (2.5 L × 5); the *n*-BuOH phase (31.6 g) was separated by ODS flash column chromatography with aqueous MeOH. The 75% aqueous MeOH fraction (2.3 g), which was most active against thrombin, was separated by gel filtration on Sephadex LH-20 with $CHCl_3$−MeOH (1:1), followed by ODS flash chromatography with aqueous MeCN containing 0.05% TFA. The fraction eluted with MeCN−H_2O−TFA (23:77:0.05) was subjected to reversed-phase HPLC [Cosmosil 5C_{18}-AR (20 × 250 mm); 25% MeCN in 0.05% TFA; 8.0 mL/min; UV detection at 220 nm] to furnish seven fractions. Rechromatography of the fourth, fifth, and sixth fractions afforded cyclotheonamides E3 (**4**; 4.2 mg, 1.0×10^{-4}% yield), E2 (**3**; 1.6 mg, 3.9×10^{-5}% yield), and E (**2**; 8.3 mg, 2.0×10^{-4}% yield), respectively.

Enzyme Inhibition Assays. Inhibitory activity against thrombin was determined essentially according to the method of Sevendsen et al.,[15] whereas that against trypsin by a modified method of Cannell et al.[16]

Cyclotheonamide E2 (3): pale brown amorphous solid; $[\alpha]^{26}_D$ −37.3° (*c* 0.10, MeOH); UV (MeOH) λ_{max} 274 nm (ϵ 4020); ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS (glycerol matrix) *m*/*z* 877.4571 (M + MeOH + H)$^+$ (calcd for $C_{42}H_{56}N_{10}O_9$, 877.4572).

Cyclotheonamide E3 (4): pale brown amorphous solid; $[\alpha]^{26}_D$ −40.2° (*c* 0.10, MeOH); UV (MeOH) λ_{max} 272 nm (ϵ 2140); ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS (glycerol matrix) *m*/*z* 857.4874 (M + MeOH + H)$^+$ (calcd for $C_{40}H_{60}N_{10}O_9$, 857.4885).

Determination of the Stereochemistry of Amino Acids by Marfey's Method. Cyclotheonamide E2 and E3 (100 μg each) were placed in 1 mL conical vials containing 5 N HCl (0.5 mL), and the sealed vials were

heated at 105 °C for 12 h. After evaporation of the solvent under N_2, 50 μL of 0.1% 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA) in acetone and 100 μL of 0.1 M $NaHCO_3$ were added, and the sealed vials were heated at 80 °C for 5 min. To the reaction mixture were added 50 μL of 0.2 N HCl and 100 μL of 50% aqueous MeCN containing 0.05% TFA, and the mixture was subjected to HPLC analysis [Cosmosil 5C_{18}-MS (4.6 × 250 mm); aqueous MeCN containing 50 mM NH_4OAc].

Determination of the Stereochemistry of K-Arg Residue in Cyclotheonamides E (2), E2 (3), and E3 (4). Cyclotheonamide A (**1**; 10 mg) was hydrolyzed with 6 N HCl at 105 °C for 12 h and dried in a stream of N_2. The residue was passed through a short column of Dowex 1 × 8 (1.3 × 4 cm, H^+ form) with H_2O. The eluate was derivatized with L- and D-FDAA, and the reaction mixture was analyzed by HPLC, which exhibited peaks at retention times 14.40 and 11.06 min, respectively. L-FDAA derivatives of K-Arg derived from compounds **2**−**4** showed a peak at retention times of 14.40, 14.34, and 14.36 min, respectively.

Determination of the Stereochemistry of V-Tyr Residue in Cyclotheonamide E2 (3) and E3 (4). Ozone in oxygen was bubbled through a cooling solution of cyclotheonamide E2 and E3 (100 μg of each) in MeOH (10 mL) at 0 °C for 10 min. The reaction was stopped by addition of 1 mL of AcOH. The mixture was concentrated to 200 μL and treated with 0.5 mL of AcOH, 0.5 mL of H_2O, 0.3 mL of H_2O_2, and 12 μL of H_2SO_4 at 90 °C for 1.5 h. After cooling to room temperature, the reaction mixture was neutralized with 45 mg of $BaCO_3$ and filtered. The filtrate was kept over Pd/C for 16 h to decompose excess H_2O_2 and then was filtered through a Celite layer, desalted on a short ODS column, and subjected to acid hydrolysis. The acid hydrolysate was derivatized with L-FDAA and analyzed by ODS HPLC [Cosmosil 5C_{18}-MS (4.6 × 250 mm); 15% MeCN (50 mM NH_4OAc)]. In this manner, L-Asp was identified in both **3** and **4**.

Chiral GC Analysis of the *a*Ile Residue. The acid hydrolysate of the peptide (0.1 mg) obtained as described above was heated in 10% HCl in MeOH (0.3 mL) at 100 °C for 30 min in a pressure vial. After removal of the methanolic HCl in vacuo, CH_2Cl_2 (0.3 mL) and trifluoroacetic anhydride (0.3 mL) were added to the residue, and the mixture was kept at 100 °C for 5 min. The reaction mixture was evaporated to dryness and dissolved in CH_2Cl_2, aliquots (0.5 μL) were injected onto a Chirasil-L-Val capillary column (0.39 mm × 25 m), and the temperature was raised from 60 to 200 °C over a period of 37.5 min. Retention times (minutes): D-*a*Ile (8.22), D-Ile (8.91), L-Ile (9.24), L-*a*Ile (9.75), *a*Ile from **2** (8.25), *a*Ile from **3** (8.29), and *a*Ile from **4** (8.28). A coinjection experiment confirmed the identification of the peaks.

Isolation of *a*Ile. A 0.5 mg portion of **2** was hydrolyzed with 5 N HCl at 105 °C for 12 h, and the hydrolysate was chromatographed by RP-HPLC [Cosmosil 5C_{18}-MS (4.6 × 250 mm); mobile phase, 1% MeCN in H_2O containing 0.05% TFA] to yield *a*Ile: ^{1}H NMR (D_2O) δ 3.67 (1H, m, α-H), 1.97 (1H, m, β-H), 1.28 (1H, m, γ-H), 1.20 (1H, m, γ-H), 0.81 (overlapped), 0.81 (overlapped). The ^{1}H NMR spectrum was superimposable on that of an authentic *a*Ile.

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