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Three More Cyclotheonamides, C, D, and E, Potent Thrombin Inhibitors from the Marine Sponge *Theonella swinhoei*[1]

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Abstract—Three new thrombin and trypsin inhibitors, cyclotheonamides C (**3**), D (**4**), and E (**8**) were isolated from the marine sponge *Theonella swinhoei*. Their structures were determined by spectral and chemical methods.

Introduction

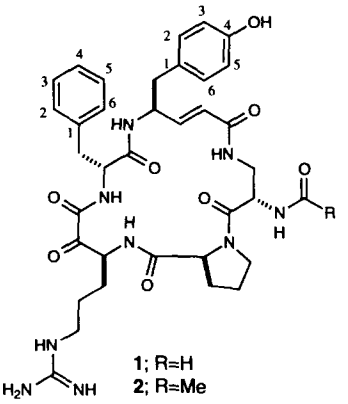
Cyclotheonamides A (**1**) and B (**2**) are potent thrombin inhibiting cyclic peptides isolated from the Japanese marine sponge *Theonella swinhoei*.[2] They contain two unusual amino acids, α-ketoarginine (K-Arg) and vinylogous tyrosine (V-Tyr), which are involved in binding to the thrombin molecule. Cyclotheonamides are also strong inhibitors of other serine proteases.

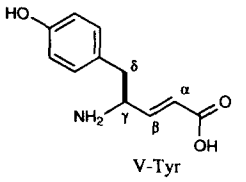
Further investigation of the extract of *T. swinhoei*, which had a bright yellow interior led to the isolation of cyclotheonamides C (**3**) and D (**4**) along with three artifacts (**5**–**7**), while a morphologically-different *T. swinhoei* afforded cyclotheonamide E (**8**). This paper deals with the isolation, structure elucidation, and enzyme inhibitory activity of these compounds.

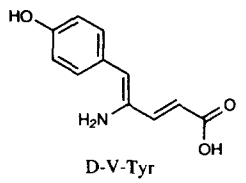
Results

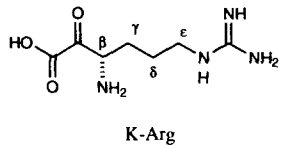
Isolation of cyclotheonamides

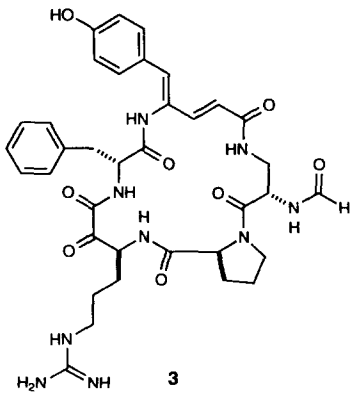
The EtOH extract of *T. swinhoei* collected off Hachijo-jima Island in 1992, which had a bright yellow interior and contained cyclotheonamide A, was partitioned be-

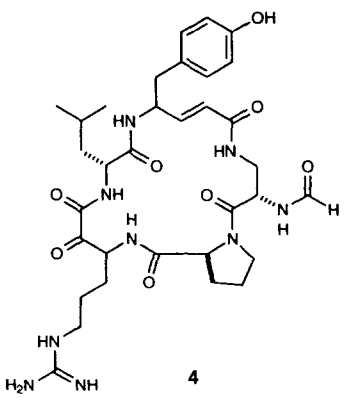


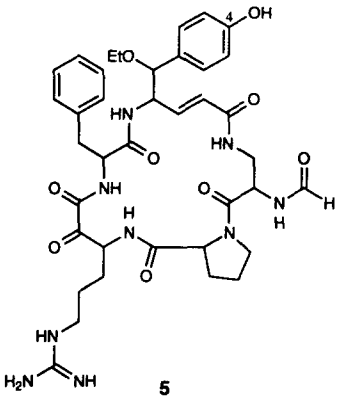












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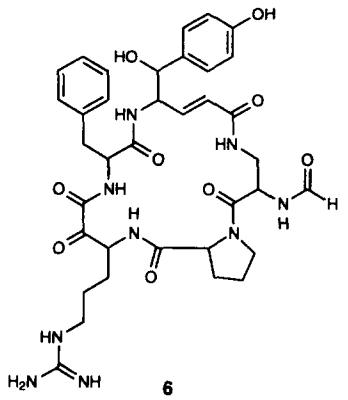

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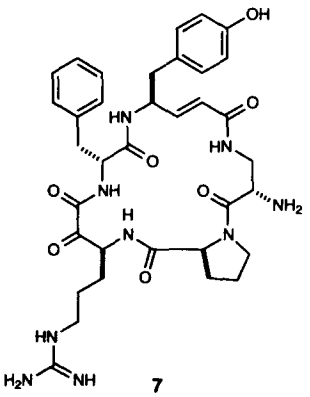

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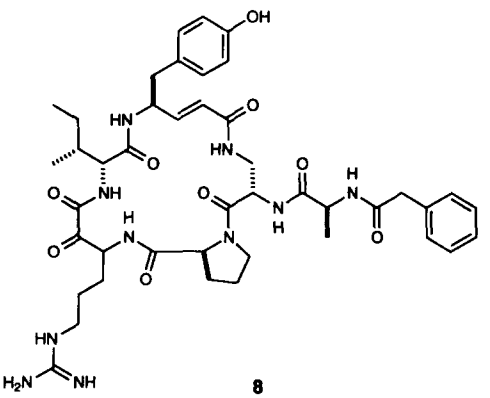

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tween Et_2O and H_2O. The aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH extract was separated by ODS flash chromatography, gel-filtration, ODS column chromatography, and ODS HPLC to afford cyclotheonamide C (**3**; 1.7×10^{-4}% based on wet weight) and D (**4**). Cyclotheonamide D (**4**) was also obtained from the 1993 collection (1.8×10^{-5}% based on wet weight), following the same procedure, but finally purified by HPLC on an Asahipak GS 320 column. Three other active metabolites (**5–7**) were obtained from the 1991 collection by similar isolation procedures.

The MeOH extract of a morphologically different *T. swinhoei* was partitioned between H_2O and Et_2O; the aqueous layer was extracted with *n*-BuOH. The *n*-BuOH extract was fractionated by gel filtration, and chromatographies on an NH_2 column: the active fraction was finally purified by ODS HPLC to yield cyclotheonamide E (**8**; 2.25×10^{-4}% yield based on wet weight) as the major inhibitor of serine proteases. Incidentally, we could not detect cyclotheonamides A–D in this sponge.

Cyclotheonamide C (3)

Cyclotheonamide C (**3**) had a molecular formula of $C_{36}H_{43}N_9O_8$ [HR-FABMS m/z 730.3265 $(M + H)^+$, Δ –4.9 mmu], suggesting a dehydro derivative of cyclotheonamide A. Amino acid analysis of the acid hydrolysate showed the presence of 1 mol each of Pro, Phe, diaminopropionic acid (Dpr), and α-ketoarginine (K-Arg). The ^1H NMR spectrum of cyclotheonamide C was almost superimposable on that of cyclotheonamide A, except for a V-Tyr unit; γ- and δ-protons were missing and a singlet olefinic proton was observed at δ 6.89, indicating the presence of one more double bond. Therefore, the V-Tyr unit in **1** was replaced by a dehydrovinylogous tyrosine (D-V-Tyr) unit in **3**. This was supported by a bathochromic shift of the UV maximum from 278 nm to 332 nm. *E*-Geometry of the disubstituted double bond was deduced on the basis of a vicinal coupling constant of 15.8 Hz, while *Z*-geometry for the trisubstituted double bond was inferred from a ROESY[3] crosspeak between α- and δ-protons.

Sequence analysis was carried out by HMBC[4] and ROESY data. In addition to HMBC crosspeaks Dpr (NH)/formyl (CO), Phe (NH)/K-Arg (CO), and K-Arg (NH)/Pro (CO), an HMBC crosspeak was observed between the β-proton of Dpr (δ 2.78) and the carbonyl carbon of D-V-Tyr. The connection of Pro and Dpr residues was established by the ROESY spectrum in which the α-proton of Dpr was correlated with δ-protons of Pro. However, connectivity between D-V-Tyr and Phe units could not be established by NMR data due to low intensity of the NH signal of the D-V-Tyr unit. Nevertheless, in order to satisfy the molecular formula, the two units must be connected through an amide bond.

The stereochemistry of Pro, Phe, and Dpr residues was determined by Marfey's method[5] to be L, D, and L, respectively, which were identical with those in cyclotheonamide A (**1**). The stereochemistry of the K-Arg residue was determined by chemical derivatisation. Cyclotheonamide C was hydrogenated to two diastereomeric tetrahydro derivatives, one of which exhibited the identical ^{1}H NMR spectrum with dihydrocyclotheonamide A prepared from cyclotheonamide A by catalytic hydrogenation. Therefore, the stereochemistry of the K-Arg residue in cyclotheonamide C was L.

Cyclotheonamide D (4)

The molecular formula of cyclotheonamide D was $C_{33}H_{47}N_9O_8$ from HR-FABMS [m/z 698.3611 $(M + H)^+$, Δ −1.5 mmu]. The amino acid analysis confirmed 1 mol each of Pro, Leu, Dpr, and K-Arg in **4**. The geometry of the double bond in the V-Tyr unit, whose spin system was readily identified from the HOHAHA and HMBC spectra, was *E* from the vicinal coupling constant value of 15.5 Hz.

Sequencing of each residue by HMBC experiments resulted in the following connectivities: Dpr (αNH)/formyl (CO), K-Arg (αNH)/Pro (CO), Leu (NH)/K-Arg (CO), V-Tyr (NH)/Leu (CO). An HMBC crosspeak was also observed between the β-proton of Dpr (δ 2.78) and the carbonyl carbon of V-Tyr. Connection of Pro and Dpr residues was established by the ROESY spectrum in which the α-proton (δ 4.72) of Dpr and δ-protons of Pro were correlated.

The stereochemistry of Pro, Leu, and Dpr residues was determined by Marfey's method to be L, D and L, respectively.

Artifacts 5, 6 and 7

The molecular formula of **5** was larger than that of cyclotheonamide A by C_2H_4O as determined by HR-FABMS [m/z 776.3724 $(M + H)^+$, Δ −0.7 mmu]. The compound exhibited NMR spectra quite similar to those of cyclotheonamide A, and gave rise to 1 mol each of Pro, Phe, Dpr, and K-Arg in standard amino acids analysis of the acid hydrolysate, thereby suggesting that modification of the V-Tyr unit had occurred. The ^{1}H NMR spectrum in CD_3OH revealed a sharp triplet methyl signal at δ 1.18, which was correlated with oxymethylene proton signals (δ 3.49 and 3.37) in the COSY spectrum. This oxymethylene was in turn coupled to an oxymethine at δ 82.6 in the HMBC spectrum, thereby indicating the presence of a δ-ethoxy vinylogous tyrosine unit (E-V-Tyr). The geometry of the double bond of the E-V-Tyr unit was determined as *E* on the basis of a geminal coupling constant value of 16.1 Hz. The sequencing of these units was carried out by HMBC and ROESY experiments in CD_3OH, and the gross structure was determined as shown.

A second component **6** had the molecular formula of $C_{36}H_{45}N_9O_9$ as determined by HR-FABMS [m/z 748.3450 $(M + H)^+$, Δ +3.2 mmu] i.e. one more oxygen atom than cyclotheonamide A. Amino acid analysis showed the presence of 1 mol each of Pro, Phe, Dpr, and K-Arg. As in the case of **5**, the V-Tyr unit in cyclotheonamide A was replaced by a δ-hydroxy vinylogous tyrosine unit (H-V-Tyr) in **6**. The sequencing of these units by HMBC and ROESY experiments in CD_3OH led to the structure as shown. Four conformers were observed in the ^{1}H NMR spectrum in CD_3OH.

The molecular fomula of **7** was $C_{35}H_{45}N_9O_7$ by HR-FABMS [m/z 704.3575 $(M + H)^+$, Δ +5.5 mmu]. Amino acid analysis indicated the presence of Pro, Phe, Dpr, and K-Arg. In the ^{1}H NMR spectrum of **7**, the formyl proton signal was missing. Therefore, **7** was deformylcyclotheonamide A.

Components **5** and **6** were isolated in small amounts from the sponge collected in 1992; larger amounts (3.8×10^{-5} and 8.9×10^{-5}% yield based on wet weight) were recovered from the 1991 collection, which did not contain cyclotheonamide C. It is likely that **5** and **6** were artifacts arising from cyclotheonamide C (**3**) during isolation. Although **7** was obtained from both 1991 and 1992 collections, the formyl group of cyclotheonamide A could easily be hydrolysed by trace amounts of TFA in HPLC solvents.

Cyclotheonamide E (8)

Cyclotheonamide E (**8**) had a molecular formula of $C_{43}H_{58}N_{10}O_9$ as determined by HR-FABMS [m/z 859.4521, $(M + H)^+$ Δ +5.4 mmu]. Amino acid analysis of the hydrolysate gave rise to 1 mol each of Ala, Pro, Ile, Dpr, and K-Arg. Although the ^{1}H NMR spectrum revealed signals for two conformers in a 3:2 ratio, all NMR signals could be assigned unambiguously to each conformer by means of 2D NMR techniques including COSY, HOHAHA, HMQC,[6] and HMBC, which not only confirmed above amino acid residues, but also the presence of a V-Tyr unit. In addition, the ^{1}H NMR spectrum exhibited signals for two aliphatic protons at δ 3.52 (2H, s) and five aromatic protons (δ 7.29 × 2, 7.25 × 2, and 7.22) assignable to an isolated methylene and a monosubstituted benzene ring, respectively. The HMBC spectrum indicated that the singlet methylene signal at δ 3.52 was correlated with a carbonyl carbon (δ 173.5), C-1 (δ 136.3), and C-2 (δ 129.6) of the monosubstituted benzene ring, thereby implying the presence of a phenyl acetyl group (Pha). Connection of all these units through amide bonds matched with the molecular formula obtained by HR-FABMS.

Sequence analysis was done by interpretation of the HMBC and ROESY spectra. In the HMBC spectrum, sequential correlations were observed between K-Arg (NH)/Pro (CO), Ile (NH)/K-Arg (CO), V-Tyr (NH)/Ile (CO), Dpr (NH)/V-Tyr (CO), Dpr (NH)/Ala (CO), and Ala (NH)/Pha (CO). Connectivity between Dpr and Pro residues was inferred from ROESY crosspeaks between δ-protons of Pro and α-proton of Dpr. Thus, the gross

structure was determined as shown.

The stereochemistry of the amino acid residues was determined by Marfey's method to be L-Pro, L-Ala, L-Dpr, and D-Ile. The stereochemistry of the V-Tyr residue was determined by chemical degradation. Cyclotheonamide D was subjected to ozonolysis followed by acid hydrolysis. The hydrolysate was derivatised with Marfey's reagent followed by HPLC analysis, which revealed the presence of L-Asp. The stereochemistry of K-Arg unit has not been determined.

Biological activity

Cyclotheonamides C (**3**), D (**4**), E (**8**), **5**, **6**, and **7** inhibited thrombin with IC_{50}s of 8.4, 5.2, 28, 7.0, 3.5, and 80 nM, while they were inhibitory against trypsin with IC_{50}s of 7.4, 63, 370, 61, 29, and 100 nM, respectively, as shown in Table 4.

Discussion

Cyclotheonamides C (**3**) in which the vinylogous tyrosine unit was replaced by a dehydrovinylogous tyrosine unit showed thrombin inhibiting activity comparable to that of cyclotheonamide A (**1**). Cyclotheonamide E (**8**) which includes D-Ile and phenyl acetyl-Ala amide groups in place of D-Phe and formyl groups in cyclotheonamide A was 20-fold less active. The X-ray crystal structure of a complex between cyclotheonamide A and α-thrombin showed that Ser-195 of the catalytic triad forms a network of hydrogen bonds with K-Arg of cyclotheonamide A.[7] V-Tyr of cyclotheonamide A is also involved in the bonding mechanism. Therefore, it is reasonable that cyclotheonamides A and C show similar inhibitory activity. The hydrophilic formamide group in **1** was replaced by the hydrophobic phenyl acetyl-alanyl group in cyclotheonamide E, which may affect the activity, since the phenyl acetyl group will come close to Trp-215 of α-thrombin. The formyl group also seems important for activity; deformylcyclotheonamide A (**7**) was 10 times less active than cyclotheonamide A. Cyclotheonamide D which containes D-Leu instead of D-Phe in **1** showed comparable activity against thrombin. Thus, a hydrophobic amino acid can replace D-Phe.

We have isolated three more cyclotheonamides from the marine sponge *T. swinhoei*: cyclotheonamides C and D from *T. swinhoei* with a bright yellow interior from which we had previously isolated cyclotheonamides A and B, and cyclotheonamide E from *T. swinhoei* with a white interior. Interestingly, cyclotheonamide A was not detected in the white variety. It should also be noted that cyclotheonamide B was present only in the 1990 collection of the yellow variety. We never encountered cyclotheonamide B thereafter. These observations may indicate the involvement of microbial symbionts in the synthesis of cyclotheonamides.

Experimental

General methods

NMR spectra were recorded on a Bruker AM 600 NMR spectrometer operating at 600 MHz for ^1H and 150 MHz for ^{13}C. ^1H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 3.3 and δ_C 49 ppm 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 835 amino acid analyser. Optical rotation was determined on a Jasco DIP-371 digital polarimeter in CH_3OH. UV spectra were recorded on a Hitachi 330 spectrometer.

Biological material

Sponges were collected by SCUBA at depths of 10–20 m off Hachijo-jima Island, 300 km south of Tokyo, in 1991, 1992, and 1993, while the white variety was procured at a depth of 8 m in Kaminato Harbor on the same Island in 1986. The samples were immediately frozen after collection and preserved at –20 °C until extraction.

Thrombin inhibition assay

Thrombin inhibition assay was performed essentially according to the method of Sevendsen *et al.*[8] To each test solution (20 μL) in microtiter plates 90 μL of thrombin solution was added, and the mixture preincubated at 37 °C for 30 min. Then, 90 μL of substrate solution was added to start the reaction. Absorbance at 405 nm was measured after 30 min incubation at 37 °C.

Bovine plasma thrombin was purchased from Sigma Chemical Co. The substrate, benzoyl-phenylalanyl-valyl-arginine-*p*-nitroanilide was obtained from Bachem Feinchemikalien AG. Thrombin (1.1 U mL^{-1}) was dissolved in Tris–imidazole buffer (pH 8.2, Ip 0.15) which had been prepared by mixing a 1:1 mixture of 0.1 M imidazole–HCl and 0.1 M Tris–HCl with a 1:1 mixture of 0.1 M imidazole and 0.1 M Tris both in 0.1 M NaCl. Ionic strength of the buffer was adjusted by addition of an equal volume of 0.2 M NaCl. Five milligram of the substrate dissolved in 1 mL of DMSO and 10 μL of this solution was diluted with 990 μL of the Tris–imidazole buffer.

Three test samples for each concentration were tested and the average IC_{50} value was used.

Trypsin inhibition assay

Trypsin inhibition assay was performed by a modification of the method of Cannell *et al.*[9] To the test solution (20 μL) in microtiter plates, 45 μL of trypsin solution and 45 μL of 0.4 M Tris–HCl buffer (pH 7.6) were added and preincubated at 37 °C for 30 min. After the preincubation, 90 μL of substrate solution was added to begin the reaction. Absorbance at 405 nm was measured after 30 min incubation at 37 °C.

Bovine pancreas trypsin and substrate were purchased from Sigma Chemical Co. Trypsin was dissolved in 50 mM Tris–HCl (pH 7.6) to prepare 150 U mL^{-1} solution. *N*-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) was used as substrate of trypsin: 43.3 mg of BAPNA in 1 mL of DMSO was diluted with 99 mL of 50 mM Tris–HCl (pH 7.6).

Three test samples for each concentration were tested and the average IC_{50} value was used.

Extraction and isolation

The EtOH extract of *T. swinhoei* with yellow interior (25 kg) collected in 1992 was partitioned between Et_2O and H_2O. The aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH extract was separated by ODS flash chromatography with aqueous MeCN containing 0.05% TFA. The fraction eluted with 30% MeCN–0.05% TFA, which was inhibitory against thrombin, was separated by gel-filtration on Sephadex LH-20 (MeOH), ODS column chromatography (23% MeCN containing 0.05% TFA), and ODS HPLC on Cosmosil $5C_{18}$-AR (23% MeCN containing 0.05% TFA) to yield cyclotheonamide C (**3**; 1.7×10^{-4}% based on wet weight). Artifacts **5**, **6** and **7** were obtained in the same way from the sponge collected in 1991.

The EtOH extract of the same sponge (30 kg) collected in 1993 was partitioned between Et_2O and H_2O; the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH extract was separated by ODS flash chromatography first with aqueous MeOH containing 0.05% TFA, then with aqueous MeCN containing 0.05% TFA. The resulting active fraction was gel-filtered on Sephadex LH-20 (MeOH), followed again by ODS flash chromatography with aqueous MeCN containing 0.05% TFA. The fraction eluted with 20-25% MeCN–0.05% TFA was separated by ODS column chromatography (24% MeCN containing 0.05% TFA), followed by ODS HPLC on Cosmosil $5C_{18}$-MS (18% MeCN containing 0.05% TFA) and HPLC on Asahipak GS320 to afford cyclotheonamide D (**4**; 1.8×10^{-5}% based on wet weight).

The MeOH extract of *T. swinhoei* with white interior (800 g) was partitioned between H_2O and Et_2O, and the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH extract was gel-filtered on Sephadex LH-20 (MeOH) and Toyopearl HW-40SF (MeOH containing 2% AcOH). The active fractions were combined and fractionated by column chromatographies on an NH_2 column, first with $CHCl_3$–MeOH (stepwise gradient) and then with H_2O–MeOH (stepwise gradient). The active fraction was finally purified on ODS HPLC on Cosmosil $5C_{18}$-AR (30% MeCN, 0.1% TFA) to afford cyclotheonamide E (**6**) as a colorless amorphous solid (2.25×10^{-4}% yield based on wet weight).

Cyclotheonamide C (3). Yellowish amorphous solid; $[\alpha]^{23}_D$ +42.4° (*c* 1.0, MeOH); UV (MeOH) λ_{max} 271 nm (ε 7000), 332 (12,000); $C_{36}H_{43}N_9O_8$; HR-FABMS m/z 730.3265 $(M + H)^+$ (Δ –4.9 mmu); ^1H and ^{13}C NMR (CD_3OH) see Table 1.

Table 1. NMR data of cyclotheonamide C (**3**)[a]

		^1H	^{13}C[b]			^1H	^{13}C
formyl	CO	8.06 *bs* [8.05 *bs*][c]	163.2	K-Arg	ε	3.12 *m*	41.5
					C=N		158.5
Dpr	CO		171.0		αNH	8.02 *m* [7.98 *d* (8.9)]	
	α	4.72 *m*	49.5		εNH	7.27 *m*	
	β	4.29 *m*	40.6				
		2.82 *m*		Phe	CO		172.0
	αNH	8.44 *brd* (5.3) [8.42 *brd* (6.3)]			α	5.03*m*	54.2
	βNH	8.85 *m* [8.87 *m*]			β	3.04 *dd* (13.9, 8.2)	40.0
						3.27 *dd* (13.9, 4.1)	
Pro	CO		173.5		1		137.2
	α	4.51 *m*	61.2		2,6	7.16 *m*	129.7
	β	2.22 *m*	30.7		3,5	7.21 *m*	128.3
		2.00 *m*			4	7.18 *m*	126.9
	γ	1.93 *m*	25.4		NH	7.63 *bd* (7.4)	
	δ	3.74 *m*	49.0				
		3.54 *m*		D-V-Tyr	CO		168.2
					α	6.21 *d* (15.8) [6.17 *d* (15.8)]	121.0 [120.6]
K-Arg	CO		172.5		β	7.26 *m* [7.31 *m*]	139.5
	α		[d]		γ		129.7
	β	4.13 *q* (8.2) [4.03 *q* (8.2)]	54.5 [55.3]		δ	6.89 *s*	136.3
					1		126.0
	γ	1.88 *m*	24.3		2,6	7.39 *d* (8.4) [7.44 *d* (8.4)]	131.2 [130.2]
		1.54 *m*			3,5	6.75 *d* (8.9) [6.80 *d* (8.9)]	115.5
	δ	1.68 *m*	25.8		4		159.7
		1.55 *m*			NH	7.11 *m*	

[a] In CD_3OH.
[b] ^{13}C chemical shifts were determined by tracing the HMQC and HMBC spectra.
[c] 3:1 signal pair was observed. The minor signals are shown in brackets.
[d] Not observed.

Table 2. NMR data of cyclotheonamide D (4)[a]

		^{1}H	^{13}C[b]			^{1}H	^{13}C
formyl	CHO	8.04 *s*	168.1	K-Arg	ε	3.13 *q* (6.5)	41.9
					C=N		158.5
Dpr	CO		171.1		αNH	7.98 *d* (10.0)	
	α	4.72 *m*	50.0		εNH	7.25 *t* (4.6)	
	β	4.24 *ddd* (11.9, 10.5, 5.8)	40.8				
		2.81 *t* (11.3)		Leu	CO		173.0
	αNH	8.41 *d* (5.8) [8.40 *d* (5.8)][c]			α	4.33 *td* (6.8, 8.6)	53.4
	βNH	8.68 *d* (10.5) [8.82 *d* (10.5)]			β	1.25 *t* (6.9)	44.7
					γ	1.02 (6.6)	25.2
Pro	CO		173.4		δ	0.73 *d* (6.5) [0.79 *d* (6.2)]	22.7
	α	4.44 *dd* (8.6, 5.5)	61.5		δ'	0.77 *d* (6.5) [0.85 *d* (6.4)]	22.9
		2.26 *m*	31.1		NH	7.37 *d* (8.8) [7.32 *d* (8.8)]	
	β	1.94 *m*					
	γ	1.94 *m*	25.8	V-Tyr	CO		167.6
	δ	3.82 *dt* (10.0, 6.4)	49.4		α	6.13 *dd* (15.2, 2.3)	124.5
		3.48 *dt* (10.0, 7.0)			β	6.81 *dd* (15.5, 2.5)	143.8
					γ	4.72 *m*	52.4
K-Arg	CO		171.1		δ	3.05 *dd* (14.2, 4.8)	39.4
	α		[d]			2.57 *dd* (14.2, 11.6)	
	β	4.14 *dt* (3.0, 10.5)	54.8		1		129.4
	γ	1.99 *m*	24.8		2,6	7.06 *d* (8.4)	130.8
		1.54 *m*			3,5	6.69 *d* (8.4)	116.1
	δ	1.57 *m*	25.9		4		157.1
		1.68 *m*			NH	8.23 *d* (8.2) [8.24 *d* (8.2)]	

[a] In CD_3OH.
[b] ^{13}C chemical shifts were determined by tracing the HMQC and HMBC spectra.
[c] 3:1 signal pairs were observed. The minor signals are shown in brackets.
[d] Not observed.

Cyclotheonamide D (4). Colorless amorphous solid; $[\alpha]^{23}_D$ −16.7° (*c* 0.275, MeOH); $C_{33}H_{47}N_9O_8$; HR-FABMS m/z 698.3611 (M + H)$^+$ (Δ −1.5 mmu); ^{1}H and ^{13}C NMR (CD_3OH) see Table 2.

Compound (5). Colorless amorphous solid; $[\alpha]^{23}_D$ −5.04° (*c* 0.5, MeOH); UV (MeOH) λmax 270 nm (ε 4100); $C_{38}H_{49}N_9O_9$; HR-FABMS m/z 776.3724 (M + H)$^+$ (Δ −0.7 mmu); ^{1}H NMR (CD_3OH): (minor signals are shown in brackets) formyl 8.04 (*bs*); Dpr 4.72 (*m*, α), 4.22 (*dt*, *J* = 5.3, 10.7 Hz, β), 2.78 (*bt*, *J* = 11.4 Hz, β), 8.39 (*d*, *J* = 8.8 Hz, αNH), [8.42 (*d*, *J* = 5.2 Hz, αNH)], 8.45 (*d*, *J* = 10.4 Hz, βNH), [8.72 (*d*, *J* = 9.6 Hz, βNH)]; Pro 4.48 (*m*, α), 2.24 (*m*, β), 1.98 (*m*, β), 1.95 (2H, *m*, γ), 3.78 (*m*, δ), 3.53 (*m*, δ); K-Arg 4.03 (*dt*, *J* = 2.6, 10.6 Hz, β), [3.96 (*bt*, *J* = 10.6 Hz, β)], 1.93 (*m*, γ), 1.51 (*m*, γ), 1.65 (*m*, δ), 1.53 (*m*, δ), 3.09 (*q*, *J* = 6.0 Hz, ε), 7.96 (*d*, *J* = 9.3 Hz, αNH), [7.92 (*d*, *J* = 9.3 Hz, αNH)], 7.23 (*m*, εNH); Phe 4.70 (*m*, α), 3.04 (*dd*, *J* = 13.6, 5.3 Hz, β), 2.88 (*dd*, *J* = 13.6, 4.7 Hz, β), 6.77 (*d*, *J* = 6.5 Hz, 2, 6), [7.03 (*d*, *J* = 6.5 Hz, 2, 6)], 7.12 (*m*, 3, 5), 7.14 (*m*, 4), 7.24 (*m*, NH); E-V-Tyr 6.09 (*dd*, *J* = 16.1, 2.7 Hz, α), [6.08 (*dd*, *J* = 16.1, 2.7 Hz, α)], 6.74 (*dd*, *J* = 16.1, 3.2 Hz, β), [6.77 (*dd*, *J* = 16.1, 3.2 Hz, β)], 4.68 (*m*, γ), 4.52 (*d*, *J* = 5.3 Hz, δ), 3.49 (*m*, $OC\underline{H}_2CH_3$), 3.37 (*m*, $OCH_2C\underline{H}_3$), 1.18 (*t*, *J* = 6.7 Hz, 2'), 7.21 (*d*, *J* = 8.3 Hz, 2, 6), [7.12 (*m*, 2, 6)], 6.80 (*d*, *J* = 8.3 Hz, 3, 5), [6.81 (*d*, *J* = 8.3 Hz, 3, 5)], 8.36 (*d*, *J* = 8.4, NH); ^{13}C NMR (CD_3OH): (minor signals are shown in brackets) formyl 162.8; Dpr 171.5 (CO), 49.7 (α), 40.6 (β); Pro 173.4 (CO), 61.3 (α), 30.8 (β), 25.6 (γ), 49.2 (δ); K-Arg 170.9 (CO), 99.3 (α), 55.0 [55.5] (β), 24.6 (γ), 25.6 (δ), 41.7 (ε), 158.4 (N=C); Phe 171.5 (CO), 55.0 (α), 40.1 (β), 137.6 (1), 131.0 [130.8] (2, 6), 129.0 (3, 5), 127.7 (4); E-V-Tyr 167.1 (CO), 126.0 (α), 142.3 (β), 57.3 (γ), 82.6 (δ), 65.3 ($O\underline{C}H_2CH_3$), 15.0 ($OCH_2\underline{C}H_3$), 131.0 (1), 129.5 (2, 6), 116.3 (3, 5), 158.1 (4).

Compound (6). Colorless amorphous solid; $[\alpha]^{23}_D$ +0.16° (*c* 0.5, MeOH); UV (MeOH) λmax 273 nm (ε 2000), 328 (300); $C_{36}H_{45}N_9O_9$; HR-FABMS m/z 748.3450 (M + H)$^+$ (Δ +3.2 mmu); ^{1}H NMR (CD_3OH): (minor signals are shown in brackets) formyl 8.04 (*bs*); Dpr 4.72 (*m*, α), 4.22 (*dt*, *J* = 6.5, 11.8 Hz, β), 2.80 (*bt*, *J* = 11.1 Hz, β), 8.47 (*d*, *J* = 5.6 Hz, αNH), [8.44 (*d*, *J* = 5.6 Hz, αNH)], 8.49 (*d*, *J* = 11.3 Hz, βNH), [8.77 (*d*, *J* = 11.3 Hz, βNH)]; Pro 4.48 (*bdd*, *J* = 3.9, 4.6 Hz, α), 2.23 (*m*, β), 1.97 (*m*, β), 1.95 (2H, *m*, γ), 3.78 (*m*, δ), 3.51 (*m*, δ); K-Arg 4.05 (*q*, *J* = 9.4 Hz, β), [3.97 (*t*, *J* = 9.8 Hz, β)], 1.93 (*m*, γ), 1.52 (*m*, γ), 1.67 (*m*, δ), 1.54 (*m*, δ), 3.10 (quint., *J* = 6.5 Hz, ε), 7.96 (*d*, *J* = 10.1 Hz, αNH), [7.91 (*d*, *J* = 10.1 Hz, αNH)], 7.27 (*m*, εNH); Phe 4.55 (*q*, *J* = 6.5 Hz, α), 2.84 (*dd*, *J* = 13.1, 6.5 Hz, β), [2.98 (*dd*, *J* = 13.1, 6.5 Hz, β)], 2.70 (*dd*, *J* = 13.1, 4.6 Hz, β), [2.82 (*m*, β)], 6.67 (*m*, *J* = 6.5 Hz, 2, 6), [6.64 (*d*, *J* = 6.8 Hz, 2, 6), 6.92 (*d*, *J* = 6.8 Hz, 2, 6), 6.96 (*d*, *J* = 6.8 Hz, 2, 6)], 7.07 (*m*, 3, 5), [7.15 (*m*, 3, 5)], 7.13 (*m*, 4), 7.29 (*m*, NH), [7.22 (*d*, *J* = 6.8 Hz, NH)]; H-V-Tyr 6.12 (*d*, *J* = 15.8, α), [6.09 (*d*, *J* = 15.8 Hz, α)], 7.10 (*m*, β), [6.85 (*m*, β), 7.17 (*m*, β)], 4.61 (*bt*, *J* = 7.9 Hz, γ), [4.65 (γ), 4.67 (γ), 4.69 (γ)], 4.42 (*d*, *J* = 8.5 Hz, δ), [4.44 (*d*, *J* = 8.5 Hz, δ)], 7.27 (*d*, *J* = 7.9 Hz, 2, 6), 6.80 (*d*, *J* = 7.9 Hz, 3, 5), [6.77 (*d*, *J* = 7.9 Hz, 3)], 8.25 (*d*, *J* = 7.9 Hz, NH), [8.28 (*d*, *J* = 7.9 Hz, NH), 8.31 (*d*, *J* = 9.6 Hz,

NH), 8.33 (*d*, *J* = 9.6 Hz, NH)]; ^{13}C NMR (CD_3OH): (minor signals are shown in brackets) formyl 163.0; Dpr 171.0 (CO), 49.9 (α), 40.9 (β); Pro 173.7 (CO), 61.6 (α), 30.9 [30.4] (β), 25.6 (γ), 49.1 (δ); K-Arg 171.2 (CO), 55.0 [55.6] (β), 24.5 (γ), 25.7 (δ), 41.9 (ε), 158.4 (N=C); Phe 171.6 (CO), 55.3 (α), 40.4 (β), 136.6 [137.1] (1), 131.1 [131.0] (2, 6), 129.3 (3, 5), 128.0 (4); H-V-Tyr 167.6 (CO), 126.1 (α), 142.0 [143.0, 142.3] (β), 57.5 (γ), 75.5 [75.8 (δ)], 134.0 [133.7] (1), 129.3 (2), 116.4 (3), 158.2 (4).

Compound (7). Colorless amorphous solid; $C_{35}H_{45}N_9O_7$; HR-FABMS m/z 704.3575 $(M + H)^+$ (Δ +5.5 mmu); ^{1}H NMR (CD_3OD) Dpr 4.38 (α), 4.27 (β), 2.86 (β); Pro 4.58 (α), 2.24 (β), 2.05 (β), 2.04 (γ), 1.94 (γ), 3.64 (δ), 3.47 (δ); K-Arg 4.08 (β), 1.96 (γ), 1.54 (γ), 1.68 (δ), 1.60 (δ), 3.13 (ε); Phe 4.60 (α), 2.93 (β), 2.83 (β), 7.15 (2-6); H-V-Tyr 6.03 (α), 6.78 (β), 4.61 (γ), 2.88 (δ), 2.52 (δ), 7.10 (2, 6), 6.76 (3, 5).

Cyclotheonamide E (8). Colorless amorphous solid; $[\alpha]^{23}_D$ −17.6° (*c* 0.075, MeOH); UV (MeOH) λ_{max} 273 nm (ε 2700), 323 (ε 400); $C_{43}H_{58}N_{10}O_9$; HR-FABMS m/z 859.4521 $(M + H)^+$ (Δ +5.4 mmu); ^{1}H and ^{13}C NMR (CD_3OH) see Table 3.

Determination of stereochemistry of amino acids by Marfey's method

A 0.1 mg portion of cyclotheonamide C was hydrolysed with 6 N HCl at 110 °C for 17 h. The hydrolysate was lyophilised; to the residue 50 μL of 0.1% 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) in acetone and 100 μL of 0.1 M $NaHCO_3$ were added, and the mixture was kept at 80 °C for 3 min. To this reaction mixture was added 50 μL of 0.2 N HCl, and a portion of the solution was subjected to HPLC analysis [column, Cosmosil $5C_{18}$-MS; mobile phase, linear gradient from 0.1 N TFA to MeCN–50 mM KH_2PO_4 (1:1)].

Table 3. NMR data of cyclotheonamide E[a] (**8**)

		^{1}H	^{13}C[b]			^{1}H	^{13}C
Pha	CO		173.5	K-Arg	γ	1.97 *m*	24.7
	α	3.52 *bs*	43.0			1.50 *m*	
	1		136.3		δ	1.59 *m*	26.0
	2,6	7.25 *m*	129.6			1.70 *m*	
	3,5	7.29 *m*	129.0		ε	3.10 *q* (7.0)	41.7
	4	7.22 *m*	127.5		C=N		158.2
					αNH	8.04 *d* (10.1) [8.00 *d* (10.1)]	
Ala	CO		174.3		εNH	7.20 *m*	
	α	4.32 quint. (7.2)	49.7				
	β	1.30 *d* (7.2)	17.3	Ile	CO		171.8 [172.3]
	NH	8.36 *d* (7.2) [8.35 *d* (7.2)][c]			α	4.29 *dd* (5.9, 8.5) [4.24 *m*]	58.2 [57.8]
					β	1.36 *m* [1.39 *m*]	40.5 [41.2]
Dpr	CO		170.9		γ	1.3 *m*	26.9 [27.0]
	α	4.59 *m*	51.2		δ	0.78 *m* [0.80 *m*]	12.2
	β	4.19 *m*	40.7		γ'	0.60 *d* (6.8) [0.54 *d* (6.8)]	14.4 [14.2]
		2.72 *dt* (12.0, 10.7)			NH	7.32 *d* (8.5)	
	αNH	8.12 *d* (8.5) [8.16 *d* (8.5)]					
	βNH	8.78 *d* (10.3) [8.53 *d* (10.3)]		V-Tyr	CO		167.3
					α	6.13 *d* (15.3)	124.2
Pro	CO		173.7		β	6.80 *dd* (15.3, 2.4) [6.77 *dd* (15.3, 2.4)]	143.2
	α	4.49 *t* (6.2) [4.48 *t* (6.2)]	61.4				
	β	2.22 *m*	30.8		γ	4.7 *m*	53.3
		1.96 *m*			δ	3.05 *m*	39.3
	γ	1.95 *m*	25.7			2.59 *dd* (11.8, 13.9) [2.55 *dd* (11.6, 14.0)]	
	δ	3.8 *m*	48.9		1		129.4
		3.5 *m*			2,6	7.05 *d* (8.5)	130.5
					3,5	6.69 *dd* (8.5, 2.4)	115.8
K-Arg	CO		170.9		4		157.0
	α		[d]		NH	8.29 *d* (8.5) [8.21 *d* (8.5)]	
	β	4.05 *m* [4.10 *m*]	54.6				

[a] In CD_3OH.
[b] ^{13}C chemical shifts were determined by tracing the HMQC and HMBC spectra.
[c] 3:2 signal pairs were observed in CD_3OH. The minor signals are shown in brackets.
[d] Not observed

Table 4. Protease inhibitory activity (IC_{50}; nM)

	1	3	4	5	6	7	8
thrombin	1.5	8.4	5.2	7.0	3.5	8.0 × 10	2.8 × 10
trypsin	1.6 × 10	7.4	6.3 × 10	6.1 × 10	2.9 × 10	1.0 × 10^2	3.7 × 10^2

Hydrogenation of cyclotheonamide C

To a solution of cyclotheonamide C (2.4 mg) in MeOH was added 15 mg of 5% Pd/C; the mixture was stirred under 1 atm of H_2 at room temperature for 10 h. The reaction mixture was filtered through a membrane filter which was further washed with $CHCl_3$:MeOH:H_2O (6:4:1), and separated by ODS HPLC (Cosmosil $5C_{18}$-AR; 23% MeCN + 0.05% TFA) to give two tetrahydrocyclotheonamide C derivatives.

Determination of stereochemistry of V-Tyr in 8

Cyclotheonamide E (**8**; 0.1 mg) was ozonised for 5 min at –78 °C in MeOH (5 mL). The mixture was warmed to room temparature and evaporated to dryness. The residue was treated with 2 mL of AcOH and 30% H_2O_2 (1:1) at room temperature for 12 h. The reaction mixture was evaporated to dryness; the residue was hydrolysed with 6 N HCl at 110 °C for 17 h. The hydrolysate was analysed by Marfey's method.

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