Cyclotheonamide E4 and E5, New Potent Tryptase Inhibitors from an *Ircinia* Species of Sponge

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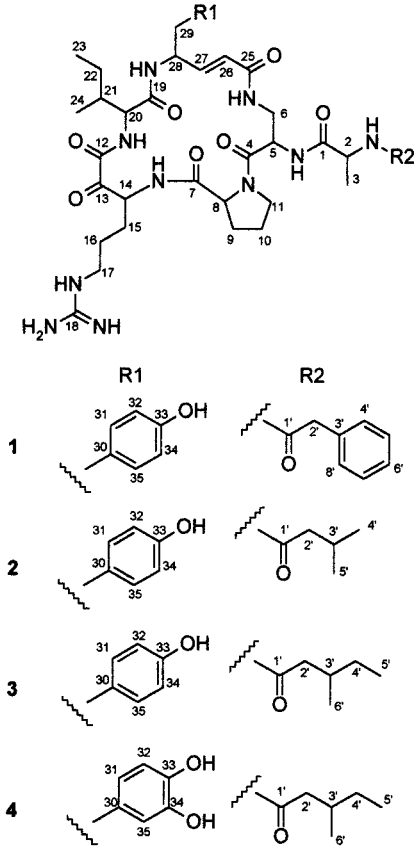
Tryptase is a protease released from mast cells and is believed to contribute to the inflammatory process in allergic diseases including asthma. In the course of screening to find tryptase inhibitors, we isolated two new tryptase inhibitors, cyclotheonamide E4 (**3**) and E5 (**4**), from a marine sponge of the genus *Ircinia*. The structures of these molecules were determined by interpretation of ^{1}H and ^{13}C NMR spectra, and they were shown to be closely related to the previously reported cyclotheonamides E (**1**), E2, and E3 (**2**). These molecules contain two unusual amino acids, vinylogous tyrosine and α-ketohomoarginine, which are involved in strong activities against serine proteases. Cyclotheonamide E4 showed potent inhibitory activity against human tryptase (IC_{50} 5.1 nM). Therefore, cyclotheonamide E4 may be useful as a therapeutic agent in the treatment of allergic diseases including asthma.

Mast cells are key cells in allergic reactions. Interactions of mast cells with multivalent antigens for the membrane-bound IgE antibodies, or various nonspecific stimuli, lead to the activation of mast cells. The release of mediators from mast cells activated by allergens has long been acknowledged to be the initiation step in the early phase response. Many compounds that inhibit the activation of mast cells have been reported to be therapeutically useful agents for treatment of allergic diseases.[1]

Tryptase is a serine protease released from the secretary granules of human mast cells along with other mediators during mast cell degranulation.[2] In animal models, the injection of purified human tryptase can induce microvascular leakage[3] and stimulate the accumulation of eosinophils at the site of injection.[4] Tryptase concentrations in the airways and in skin have been reported to be increased in biological fluids immediately after acute antigen challenge, reaching maximal levels within 1 h and declining over the next 5 to 6 h in the skin.[5,6] Furthermore, administration of tryptase inhibitor APC-366 can reduce allergen-induced microvascular leakage and eosinophilia in a sheep model of asthma, as well as help to protect against both early and late phase bronchoconstriction and airway hyperesponsiveness.[7] In view of the significance of tryptase in induction of allergic diseases, we have searched for tryptase inhibitors among natural products. In the course of our screening, we found two new tryptase inhibitors cyclotheonamides E4 and E5 in a marine sponge. Here, we describe the isolation, structure, and inhibitory activities of cyclotheonamides against several serine proteases.



Results and Discussion

In the course of our screening for tryptase inhibitors, we found potent activity in the extracts of a marine sponge collected at Miyako Island in 1997 and identified as an *Ircinia* sp. The methanol−chloroform extract of the sponge (13 kg) was partitioned between EtOAc and H_2O. The aqueous layer was applied to a Diaion HP-20 column. Active fractions were subjected to HPLC on Asahipak GS320 and on CAPCELL PAK SCX to afford cyclotheonamides E (**1**, 1.0 mg), E4 (**3**, 1.1 mg), and E5 (**4**, 0.8 mg).

^{1}H and ^{13}C NMR spectra of **3** and **4** were similar to those reported for cyclotheonamides E (**1**), E2, and E3 (**2**).[8,9] Cyclotheonamides were initially isolated from a marine sponge of the genus *Theonella* and shown to be potent thrombin inhibitors.[10] Cyclotheonamides are cyclic penta-

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Table 1. 125 MHz ^{13}C NMR and 500 MHz ^{1}H NMR Data for Cyclotheonamides E4 (**3**) and E5 (**4**)[a,b]

	cyclotheonamide E4 (**3**)		cyclotheonamide E5 (**4**)	
no.	^{13}C	^{1}H	^{13}C	^{1}H
1′	175.7		175.7	
2′a	43.9	1.98[c]	43.9	1.98[c]
2′b		2.22[c]		2.22[c]
3′	33.6	1.82 m	33.6	1.82 m
4′a	30.5	1.22[c]	30.5	1.22[c]
4′b		1.36[c]		1.36[c]
5′	11.6	0.91[c]	11.6	0.91[c]
6′	19.4	0.90[c]	19.4	0.90[c]
1	174.9		174.9	
2	49.8	4.34 q, 6.7	49.8	4.33 q, 7.1
3	17.6	1.29 d, 6.7	17.6	1.28 d, 7.1
4	171.6		171.5	
5	51.4	4.62 d, 11.0, 5.4	51.4	4.62 dd, 11.0, 5.4
6a	40.9	4.23[c]	40.9	4.23[c]
6b		2.81 d, 11.5, 11.5		2.81 dd, 11.0, 10.4
7	174.1		174.1	
8	61.7	4.49 m	61.7	4.48 m
9a	31.4	2.23[c]	31.4	2.23[c]
9b		1.97[c]		1.97[c]
10	26.1	1.93[c]	26.1	1.93[c]
11a	49.6	3.81 m	49.6	3.81 m
11b		3.52 m		3.51 m
12	171.5		171.4	
13	99.5[d]		99.5[d]	
14	55.6 (55.0)	4.08 dd, 11.2, 10.0	55.0	4.11 ddd, 11.2, 10.0, 2.5
15a	25.0	1.98[c]	25.1	1.98[c]
15b		1.56[c]		1.56[c]
16a	26.1	1.68[c]	26.2	1.68[c]
16b		1.55[c]		1.55[c]
17	42.1	3.12[c]	42.1	3.12[c]
18	158.6		158.5	
19	172.1 (170.3)		172.1 (170.3)	
20	58.3 (57.9)	4.23[c]	58.4 (57.9)	4.23[c]
21	41.3 (40.7)	1.38[c]	41.5 (40.7)	1.38[c]
22	27.2 (27.1)	1.28[c]	27.2 (27.1)	1.28[c]
23	12.3 (12.1)	0.80[c]	12.3 (12.1)	0.80[c]
24	14.6 (14.4)	0.55 d, 6.9 (0.62 d, 6.9)	14.6 (14.4)	0.61 d, 6.9 (0.54 d, 6.9)
25	167.9		167.9	
26	125.0	6.15 d, 15.8	125.0	6.14 dd, 15.8, 1.3
27	143.8	6.78 br.d, 15.8	143.8	6.78 dd, 15.8, 1.3
28	53.4	4.89[c]	53.4	4.89[c]
29a	39.4	3.08 dd, 14.1, 5.0	39.7	3.05 dd, 14.1, 5.0
29b		2.58 dd, 14.1, 11.5		2.53 dd, 14.1, 5.0
30	129.8		132.5	
31	131.0	7.06 d, 8.5	121.3	6.67 d, 7.0
32	116.2	6.69 d, 8.5	116.9	6.55 d, 7.0
33	157.3		145.1	
34	116.2	6.69 d, 8.5	146.3	
35	131.0	7.06 d, 8.5	116.2	6.67

[a] In CD_3OD. [b] Chemical shift values of the minor conformers are shown in parentheses. [c] Resonances in one-dimensional spectra obscured by overlapping signals. [d] Exist as a hemiacetal in CD_3OD.

peptides, and they contain two unusual amino acids, α-ketohomoarginine (K-Arg) and vinylogous tyrosine (V-Tyr). It was reported that the α-ketogroup in the α-ketohomoarginine residue is important for the inhibition of thrombin and trypsin activities.[11,12]

The molecular formula of **3** was determined to be $C_{41}H_{62}N_{10}O_9$ by HRFABMS, differing from **2** by CH_2. $^{1}H-^{1}H$ COSY, HOHAHA, and HMQC data of **3** showed that V-Tyr, Ile, K-Arg, Pro, Ala, and *β*-linked diaminopropionic acid units in **2** were also present in **3**. The connectivities of these units were confirmed by HMBC experiment. From the experiment, the preservation of the connectivities from C-1 to C-35 between **2** and **3** was proved. The NMR spectral data indicated that a 3-methyl pentanoyl moiety was present in **3**, rather than the isovaleryl group (C-1′−C-5′) in **2**. From these findings, the structure of **3** was determined as shown.

The results of HRFABMS for **4** indicated that the formula-difference between **3** ($C_{41}H_{62}N_{10}O_9$) and **4** ($C_{41}H_{62}N_{10}O_{10}$) was one oxygen atom. Comparison of $^{1}H-^{1}H$ COSY and HMQC data between **3** and **4** indicated the presence of a 3,4-hydroxybenzene in **4**; see Table 1.

Table 2. In Vitro IC_{50} Values[a] (nM) of Cyclotheonamides against Human Tryptase, Mouse Tryptase, Human Thrombin, and Bovine Tryptase

	E (**1**)	E4 (**3**)	E5 (**4**)
human tryptase	6.9 ± 0.4	5.1 ± 0.5	84.7 ± 4.3
mouse tryptase	17.0 ± 1.3	6.5 ± 1.0	54.1 ± 6.6
human thrombin	16.1 ± 1.2	7.4 ± 0.9	67.9 ± 5.7
bovine trypsin	10.6 ± 0.2	5.9 ± 0.3	37.3 ± 1.4

[a] Data are mean± SEM of three independent experiments.

As shown in Table 2, cyclotheonamides **1** and **3** showed potent inhibitory activities against human tryptase, with **3** showing more potent activity, with IC_{50} values of 5.1 nM. In addition to inhibiting tryptase, **1** and **3** potently inhibited trypsin and thrombin activities. These observations indicated that cyclotheonamides may nonspecifically inhibit

a variety of serine proteases. The secretory leukocyte protease inhibitor (SLPI) has been reported to be a broad spectrum serine protease inhibitor.[13] Treatment with SLPI and heparin completely blocked the antigen-induced development of hyper-responsiveness in the sheep model.[14] Since cyclotheonamides also showed broad spectrum inhibitory effects against serine proteases, these compounds are expected to show effective responses. Further studies are needed to evaluate the effect of cyclotheonamides in animal models of allergic diseases.

In conclusion, the newly identified compounds cyclotheonamide E4 and E5 inhibited tryptase activity in cultured human mast cells. These results suggested that cyclotheonamides may be useful for treatment of asthma and other disorders associated with inflammation of the respiratory tract.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a JEOL α500 NMR spectrometer in CD_3OD. FAB-mass spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using a glycerol matrix. UV spectra were recorded on a Hitachi U-3200 spectrophotometer. Human thrombin, bovine trypsin, and *N*-*p*-tosyl-gly-pro-arg *p*-nitroanilide acetate salt were all purchased from Sigma Chemical Co. (Poole, UK).

Animal Material. The marine organisms were collected in Miyako Island, Okinawa, in 1997 and kept frozen until used. When observed in situ, the color of the sponge was light grayish brown. Under atmospheric conditions, the color changed to dark gray-brown. Growth form of the sponge was spherical-bulbous with prominent conulose surface. Large sieve-plates were scattered over the surface containing clusters of small oscules. The texture was tough and compressible. Surface ornamentation was opaque, membranous, uneven, with regular and large conus interconnected by a tangential weblike fiber arrangement on the external surface. Ectosomatic skeleton was membranous, arenaceous, with a fine crust of minute sand grains, but this did not represent a true sand cortex. It was identified as *Ircinia* sp. by John N. A. Hooper, Queensland Museum, South Brisbane, Australia. A voucher specimen (QMG315199) has been deposited at the museum.

Extraction and Isolation. The methanol−chloroform extract of the sponge (13 kg) was partitioned between EtOAc and H_2O. The aqueous layer was concentrated to a small volume and applied to a Diaion HP-20 column. After washing with 20% acetone, the active fractions were obtained by eluting with 70% acetone. The eluate was evaporated to dryness and subjected to reversed-phase HPLC [Asahipak GS320 column (15% aqueous acetonitrile, 100 mM phosphate buffer, pH 4.0)]. The active fractions were concentrated and further chromatographed by HPLC [CAPCELL PAK SCX column (20% aqueous acetonitrile, 100 mM NaCl, 10 mM phosphoric acid)]. In this chromatography, the inhibitory activity was separated into three fractions. These fractions were desalted using a Diaion HP-20 column to give cyclotheonamide E (**1**, 1.0 mg), cyclotheonamide E4 (**3**, 1.1 mg), and cyclotheonamide E5 (**4**, 0.8 mg).

Cyclotheonamide E4 (**3**): pale yellow, amorphous solid; $[\alpha]^{25}_D$ −101° (*c* 0.04, MeOH); UV (MeOH) λ_{max} 287 nm (ϵ 1367); ^{1}H and ^{13}C NMR data, see Table 1; HRFABMAS (glycerol matrix) *m*/*z* 871.5012 $(M + MeOH + H)^+$ (calcd for $C_{41}H_{62}N_{10}O_9$, 871.5046).

Cyclotheonamide E5 (**4**): pale yellow, amorphous solid; $[\alpha]^{25}_D$ −64.5° (*c* 0.035, MeOH); UV (MeOH) λ_{max} 285 nm (ϵ 3780); ^{1}H and ^{13}C NMR data, see Table 1; HRFABMAS (glycerol matrix) *m*/*z* 887.4969 $(M + MeOH + H)^+$ (calcd for $C_{41}H_{62}N_{10}O_{10}$, 887.4995).

Cell Culture of Human Mast Cells (HCMC). Mononuclear cells were obtained from umbilical cord blood and cultured in α-minimum essential medium (Gibco, Grand Island, NY) containing 20% (v/v) fetal calf serum (HyClone, Logen, UT), 10 μg/mL deoxyadenosine, deoxyguanosine, deoxycytidine, adenosine, guanosine, cytidine, thymidine, and uridine (Gibco) in the presence of 100 ng/mL stem-cell factor and 10 ng/mL interleukin-6 (Kirin Brewery, Gunma, Japan). The purity of mast cells was determined by staining with May-Grunwald and Giemsa reagents every 2 weeks. Mast cells (≥12 weeks) of 100% purity were used for the experiments.[15−17]

Culture of Mouse Bone Marrow Mast Cells (BMMC). Bone marrow cells were obtained from the femurs of CBA/J mice (Jackson Laboratory, Bar Harbor, ME) and cultured for 5 weeks in the presence of mouse IL-3 (Kirin Brewery, Gunma, Japan). More than 99% of the total cells recovered from the culture were mast cells.[18]

Enzymatic Efficiency of Serine Protease Inhibitor. Human cultured mast cell (HCMC) and mouse bone marrow mast cell (BMMC) lysates were prepared using a detergent and hypotonic buffer as described elsewhere.[19] These lysates were used as sources of human or mouse tryptase, respectively. Human tryptase, mouse tryptase, human thrombin, and mouse trypsin activities were measured by the cleavage of 0.2 mM N-*p*-tosyl-gly-pro-arg *p*-nitroanilide acetate salt in Tris-buffer containing 0.1 M Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 M glycerol, and 0.1% Triton X adjusted to pH 8.0 with HCl. Each lysate (50 μL; 8×10^3 HCMC or 8×10^4 BMMC equiv/mL) for tryptase activities, human thrombin or bovine trypsine, was incubated with various concentrations of each inhibitor, and total volume was adjusted to 190 μL. After 10 min of incubation at 37 °C, 10 μL of the substrate was added to the mixture. Then, the mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 405 nm.

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