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Tokaramide A, a New Cathepsin B Inhibitor from the Marine Sponge *Theonella* aff. *mirabilis*[1]

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Abstract : A new cathepsin B inhibitor, tokaramide A (**1**) has been isolated from the marine sponge *Theonella* aff. *mirabilis*. Its structure was determined by spectroscopic and chemical methods. Tokaramide A inhibits cathepsin B with an IC_{50} value of 29.0 ng / mL.

Keywords : Enzyme inhibitors; Marine metabolites; Natural products; Peptides and polypeptides

Introduction

The cathepsins are the lysosomal cysteine proteases which play a role in a large number of physiological processes, e.g. in activation and deactivation of enzymes and hormones.[2] Cathepsin B, the most extensively studied enzyme of the group, is also known to be involved in various disease stages, such as inflammation, trauma, muscular dystrophy, and tumors. In particular, its possible roles in cancer metastasis are of major concern in cancer chemotherapy as cathepsin B inhibitors are potential anticancer drugs. In search for such bioactivity, we found that the MeOH extract of the marine sponge *Theonella* aff. *mirabilis* collected in the Tokara Archipelago inhibited cathepsin B. Bioassay-guided separation of the extract afforded a new cathepsin B inhibitor named tokaramide A (**1**).[3] In this paper we describe isolation, structure elucidation, and enzyme inhibitory activity of this compound.

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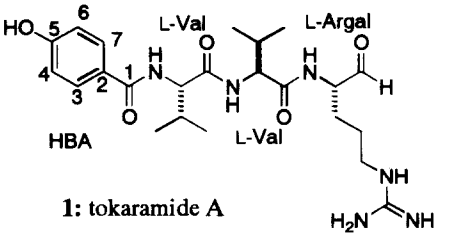
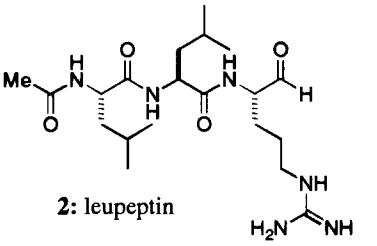
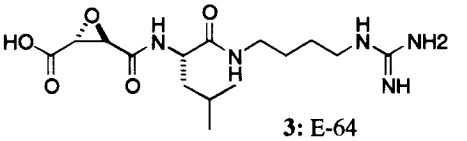


1: tokaramide A



2: leupeptin



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Results and Discussion

Frozen sponge (11.5 kg) was extracted with MeOH and $CHCl_3$/MeOH (1:1); the combined extracts were concentrated and extracted with $CHCl_3$ and then with *n*-BuOH. The organic layers were combined and fractionated by modified Kupchan solvent partitioning[4] to yield hexane, $CHCl_3$ and 60% MeOH layers. The 60% MeOH and H_2O layers were separately fractionated by ODS flash and ODS column chromatographies. Active fractions from both layers were combined and separated by HPLC on Asahipak GS-320 with aqueous MeCN containing 250 mM $NaClO_4$. The final purification was done by two HPLC runs on an ODS column to afford 1.2 mg of tokaramide A (**1**) as a pale-yellow solid.

Tokaramide A (**1**) showed ion peaks at *m/z* 477, 495 and 512 in FABMS which corresponded to $(M+H)^+$, $(M+H+H_2O)^+$, and $(M+H+MeOH)^+$, respectively, which suggested facile formation of a hemiacetal with H_2O or MeOH. Interpretation of 2D NMR data including HOHAHA,[5] HMQC,[6] and HMBC[7] spectra confirmed the presence of a methyl hemiacetal (δ_C 99.2, δ_H 4.45) in CD_3OH (Table 1). Presumably, tokaramide A (**1**) exists in an equilibrium mixture of the aldehyde, hemiacetal, and cyclic carbinolamine, which is the reason why it elutes in a broad band in HPLC.[8] Further analysis of NMR data led to all structual units in **1**; i.e. two Val, a methyl hemiacetal of argininal (Argal), and *p*-hydroxybenzoyl (HBA) residues. These units were connected on the basis of ROESY[9] and HMBC data as shown in Fig. 1.

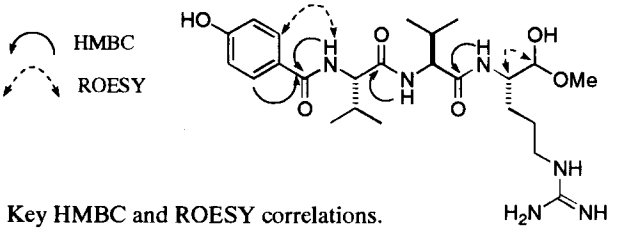


Fig. 1. Key HMBC and ROESY correlations.

Table 1. NMR Data for Tokaramide A (1)[a]

	#	^{13}C	^{1}H	HMBC
HBA	CO	170.2		
	2	125.8		
	3,7	130.4	7.71 d (J = 8.85 Hz)	130.4, 162.0, 170.2
	4,6	116.2	6.85 d (8.85)	116.2, 125.8, 162.0
	5	162.0		
Val 1	CO	174.2		
	α	61.3	4.31 t (8.47)	19.2, 19.7, 31.6, 170.2, 174.2
	β	31.6	2.14 m	61.3
	γ	19.2	1.00 d (6.9)	19.7, 31.6, 61.3
		19.7	0.98 d (6.5)	19.2, 31.6, 61.3
	NH		8.01 d (8.5)	170.2
Val 2	CO	173.7		
	α	60.8	4.08 dt (8.1, 2.7)	18.9, 19.5, 31.6, 173.7
	β	31.6	2.03 m	60.8
	γ	19.5	0.95 d (6.16)	18.9, 31.6, 60.8
		18.9	0.96 d (6.15)	19.5, 31.6, 60.8
	NH		8.09 dd (8.1, 5.4)	174.2
Argal[b]	acetal	99.2	4.45 m	
	α	54.0, 54.2[c]	3.87 m	
	β	26.7	1.74 m, 1.49 m	26.0
	γ	26.0	1.61 m, 1.51 m	42.2
	δ	42.2	3.17 m, 3.11 m	
	C=N	158.5		
	αNH		7.88 d, 7.90 d[c]	173.7
	εNH		7.35 br	
	OH		6.72 br	

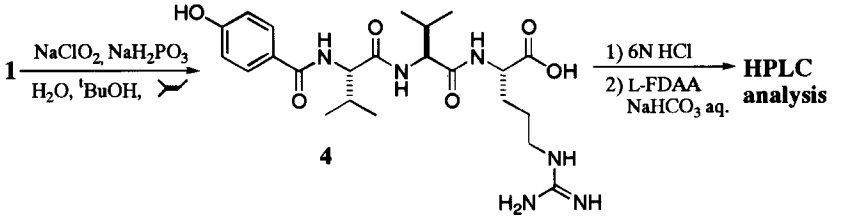
a. in CD_3OH.

b. exist as a hemiacetal in CD_3OH.

c. due to hemiacetal stereoisomer

The absolute stereochemistry of **1** was determined as follows. Tokaramide A was oxidized with $NaClO_2$[10] to furnish **4** which was subjected to acid hydrolysis.[11] (Scheme 1.) HPLC analysis of the hydrolysate after derivatization with L-FDAA[12] indicated L-configuration of Val and Arg.

Scheme 1.



Two types of cathepsin B inhibitors have been known from terrestrial microorganisms.[2] Leupeptin (**2**)[13] represents a number of peptidyl aldehydes isolated from *Streptmyces* spp., which is believed to inhibit the enzyme by forming a tetrahedral hemithioacetate between its aldehyde and the thiolate of the enzyme's active site.[2] However, these aldehydes are not selective inhibitors. The epoxysuccinyl peptides[2] are another class of natural inhibitors isolated from fungi; E-64 (**3**) was reported from *Aspergillus japonicus*.[14] In this case, the *trans*-L-(*S*, *S*)-epoxysuccinic acid is the reactive group essential for inhibition. Obviously our peptide falls in the peptidyl aldehyde class.

Tokaramide A inhibited cathepsin B with an IC_{50} value of 29 ng/mL, while leupeptin and E-64 had IC_{50} values of 9.2 and 4.9 ng/mL, respectively. (Fig. 3.)

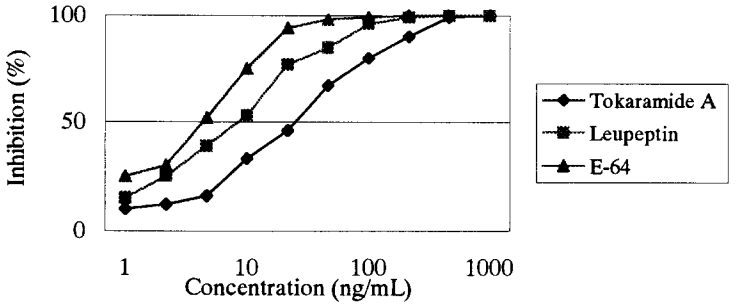


Fig. 3. Inhibiton of cathepsin B by **1**, **2**, and **3**.

Experimental

General Procedures : NMR spectra were recorded on a JEOL alpha 600 NMR spectrometer. ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks: δ_H 3.30 and δ_C 49.0 for CD_3OH. FAB mass spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using glycerol as a matrix. Optical rotations were determined on a JASCO DIP-1000 digital polarimeter in CH_3OH. UV spectra were recorded on a Hitachi 330 spectrophotometer. Fluorescence for enzyme inhibition assay was determined with a SHIMAZDU CS-9300PC densitometer.

Animal Material : Sponge samples were collected using SCUBA at depths of 15-20 m off Nakano-shima Island in the Tokara Islands (129°51'0''N, 29°50'18''E). The specimens were frozen immediately and preserved at -20 °C until extraction. The sponge was identified as *Theonella* aff. *mirabilis* (Desmospongiae, Lithistida, Theonellidae). The sponge forms a mass of volcano-shaped lobes with apical oscules. Individual lobes are 3 cm high and have a diameter of 1.5-2 cm. Color dark blue. The surface is somewhat wrinkled but otherwise smooth, although rough to the touch. In between the lobes at their base there are algae and accumulated debris. The surface skeleton is well-developed, consisting of a crust of tangentially intercrossing strongyles carrying a dense mass of acanthose microrhabds and a cover of calcareous micronodules. The calcareous nodules may be foreign, but are known to occur as structural elements in

other sponges. The crust is carried by a subectosomal layer of desmas. The choanosomal skeleton is a largely irregular reticulation of bundles of strongyles, 80-120 μm in diameter with up to 15 spicules per cross section. Desmas are mixed in with the strongyles at the periphery, gradually becoming more numerous and eventually replacing the strongyles towards the interior. No firmly interlocked desma skeleton is formed. The spicules include peculiar calthrops-like desmas with equal-shaped rays branching dichotomously and proliferating at the end into sharp conical points, cladomes 180-215 μm, cladi 90-100 x 15-25 μm. Strongyles robust, curved, 310-405 x 5-10 μm. Microscleres acanthorhabds of 9-15 x 1-1.5 μm. The sponge is closely similar to *Theonella mirabilis* in spiculation, but differs in live color. The crust of calcareous nodules has not been noted previously. *T. mirabilis* and the present material are atypical *Theonella* because of the lack of special ectosomal phyllotriaenes and possession of the peculiar calthrops-like desmas. They are likely to belong to a separate undescribed genus of Theonellidae and are only provisionally assigned to *Theonella* on account of the shared possession of acanthose microrhabds and strongyles. A voucher specimen (ZMA POR. 14406) was deposited at the Institute for Systematics and Ecology (Zoological Museum), University of Amsterdam.

Extraction and Isolation : The frozen sponge (11.5 kg, wet wt) was extracted with MeOH (6 L x 3) and $CHCl_3$/MeOH (1:1) (4 L x 2). The combined extracts were partitioned between $CHCl_3$ and H_2O. The aqueous layer was further extracted with *n*-BuOH. The combined organic layers were successively partitioned between hexane and 90% MeOH, and the latter phase was diluted with H_2O to 60% MeOH and extracted with $CHCl_3$. Enzyme inhibitory activity was observed in 60% MeOH and H_2O layers, which were separately fractionated by ODS flash column chromatography by stepwise elution with aqueous MeOH containing 0.1% TFA. The fractions eluted with 50% MeOH from both layers were combined (61.2 g) and further separated by ODS column chromatography (YMC ODS-A; ϕ 5 x 100 cm) with aqueous MeCN containing 0.05% TFA. Active fractions were purified by HPLC on an Asahipak GS-320 column with aqueous MeCN containing 250 mM $NaClO_4$. The active fraction was purified twice by HPLC on an ODS column (COSMOSIL AR-II) with 18% MeCN afforded tokaramide A (1.2 mg, 1.0 x 10^{-4} % yield based on wet weight).

Enzyme Inhibition Assay : Inhibition activity against cathepsin B was determined essentially according to the method of Hiwasa *et al.*[15]

Tokaramide A : pale-yellow solid; $[\alpha]^{29}_{D}$ -19.0° (*c* 0.06, MeOH); UV (MeOH) λ_{max} 255 nm (ε 5950); HR-FABMS (glycerol matrix) *m/z* 477.2823 $(M+H)^+$ (calcd for $C_{23}H_{37}N_6O_5$, 477.2828); ^1H and ^{13}C NMR data, see Table 1.

Determination of the Stereochemistry of Amino Acids by Marfey's Method : Tokaramide A (100 μg) was dissolved in a mixture of 490 μL of 2-methyl-2-butene, 125 μg of NaH_2PO_3, 2 μL of H_2O, and 15 μL of ^{t}BuOH. To the mixture $NaClO_2$ powder

(1.0 mg) was added, and the mixture was stirred for 2 h at 30 °C. After addition of 100 μL of 0.5 N HCl to the reaction mixture, the solution was dried in a stream of N_2. The residue was hydrolyzed with 6 N HCl at 105 °C for 12 h. After removal of HCl in a stream of 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 to the hydrolysate, and the mixture heated at 80 °C for 10 min. To the reaction mixture were added 50 μL of 0.2 N HCl and 100 μL of 50% aq. MeCN containing 0.05% TFA, and 20 μL of aliquots of the mixture subjected to HPLC analysis [COSMOSIL $5C_{18}$-MS (ϕ 4.6 x 250 mm); mobile phase, aq. MeCN containing 50 mM NH_4OAc].

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