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# Shishicrellastatins, inhibitors of cathepsin B, from the marine sponge *Crella* (*Yvesia*) *spinulata*

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ABSTRACT

Two dimeric steroid derivatives, shishicrellastatin A (**1**) and B (**2**), have been isolated as cathepsin B inhibitors from the marine sponge *Crella* (*Yvesia*) *spinulata*. Their structures were determined by interpretation of spectroscopic data. Shishicrellastatins inhibit cathepsin B with an $IC_{50}$ value of 8 μg/mL each.



## 1. Introduction

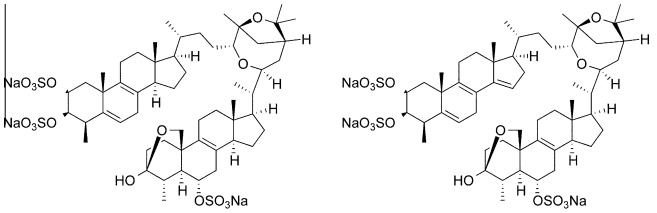
Invasion and metastasis are fundamental properties of malignant tumor cells which have a tendency to infiltrate the surrounding tissues and gain access to the vessels. Some cysteine cathepsins are highly upregulated in cancer, and their roles in tumor invasion and metastasis are well-documented.[1] Cathepsin B plays two separate roles in tumor invasion and metastasis, that is, degradation of the surrounding tissues and activation of other proteases such as MMP2 which also degrade surrounding tissues. Inhibition of cathepsin B resulted in decreased invasiveness of tumor cells.[2] Therefore, small-molecule cathepsin B inhibitors are promising candidates for antitumor agents.

During our screening of the extracts of marine invertebrates for cathepsin B inhibitors, the marine sponge *Crella (Yvesia) spinulata* exhibited significant activity. Bioassay guided fractionation afforded two dimeric steroids named shishicrellastatins A (**1**) and B (**2**) which were related to crellastatins.[3–5]

## 2. Results and discussion

### 2.1. Isolation of shishicrellastatin A and shishicrellastatin B

The sponge *Crella (Yvesia) spinulata* was extracted with MeOH, and the extract was partitioned between $H_2O$ and $CHCl_3$. The aqueous phase was then partitioned between $H_2O$ and *n*-BuOH. The two organic layers were combined and fractionated by silica gel flash column chromatography followed by silica gel column chromatography. The active fractions were repeatedly purified by reversed-phase HPLC in the presence of $NaClO_4$ to furnish shishicrellastatin A (**1**) and shishicrellastatin B (**2**) each as colorless solids.




1 2

### 2.2. Structure elucidation of shishicrellastatin A

#### 2.2.1. Partial structures and their connection

Shishicrellastatin A (**1**) was optically active and had the molecular formula of $C_{56}H_{83}Na_3O_{16}S_3$ as determined by HRESIMS in conjunction with the 1D and 2D NMR data (Table 1). The molecular formula indicated a high degree of unsaturation. UV spectrum showed an absorption at $\lambda_{max}$ 205 nm (ε 4000). The $^1H$ NMR spectrum exhibited six singlet and four doublet methyl signals. The edited-HSQC spectrum showed the presence of 18 methylenes and 15 methines including five oxymethines. One olefinic proton signal was observed. The presence of seven non-protonated $sp^3$ carbons, including one acetal, and five non-protonated $sp^2$ carbons was

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**Table 1**
$^1$H and $^{13}$C NMR data for **1** and **2** in $CD_3OD$

| | 1 | | | 2 | |
|---|---|---|---|---|---|
| | $^{13}$C | $^1$H | HMBC (#C) | $^{13}$C | $^1$H |
| 1 | 39.8 | 1.54 m | | 39.4 | 1.68 |
| | | 2.67 dd (3.4, 14.7) | 5 | | 2.71 |
| 2 | 78.0 | 4.94 q (3.4) | | 77.9 | 5.00 |
| 3 | 78.6 | 4.38 dd (3.8, 6.9) | 28 | 78.0 | 4.54 |
| 4 | 43.7 | 3.02 quint (7.2) | 2, 3, 5, 6, 10, 28 | 43.7 | 3.03 |
| 5 | 145.0 | | | 144.0 | |
| 6 | 122.9 | 5.60 t (3.8) | | 122.4 | 5.66 |
| 7 | 29.8 | 2.52 m | | 28.0 | 2.54 |
| | | 2.57 m | | | 2.90 |
| 8 | 127.5 | | | 122.7 | |
| 9 | 135.0 | | | 139.8 | |
| 10 | 38.2 | | | 38.2 | |
| 11 | 22.8 | 2.13 m | | 22.3 | 2.25 |
| | | 2.28 m | | | 2.40 |
| 12 | 37.9 | 1.45 m | | 37.8 | 1.42 |
| | | 2.03 m | 9, 14 | | 2.07 |
| 13 | 43.1 | | | 45.8 | |
| 14 | 52.8 | 2.14 m | | 150.9 | |
| 15 | 24.9 | 1.38 m | | 118.4 | 5.33 |
| | | 1.65 m | | | |
| 16 | 30.0 | 1.41 m | | 36.9 | 2.15 |
| | | 1.94 m | | | 2.39 |
| 17 | 55.8 | 1.22 m | | 57.2 | 1.57 |
| 18 | 11.5 | 0.68 s | 12, 13, 14, 17 | 15.5 | 0.84 |
| 19 | 28.1 | 1.45 s | 1, 5, 9, 10 | 28.4 | 1.48 |
| 20 | 36.4 | 1.50 m | | 34.1 | 1.79 |
| 21 | 18.8 | 0.97 d (6.5) | 17, 20, 22 | 19.1 | 0.99 |
| 22 | 34.6 | 1.21 m | | 35.6 | 1.38 |
| | | 1.60 m | | | 1.59 |
| 23 | 24.5 | 1.27 m | | 24.9 | 1.37 |
| | | 1.68 m | | | 1.63 |
| 24 | 84.5 | 3.26 dd (3.5, 7.9) | 25, 22′ | 85.1 | 3.23 |
| 25 | 87.6 | | | 87.9 | |
| 26 | 36.2 | 2.04 m | 24, 25, 27, 23′, 24′ | 36.1 | 2.04 |
| | | 2.17 m | 23′, 24′, 25′ | | 2.20 |
| 27 | 27.4 | 1.18 s | 24, 25, 26 | 27.0 | 1.17 |
| 28 | 19.0 | 1.43 d (7.9) | 3, 4, 5 | 19.0 | 1.43 |
| 1′ | 31.7 | 1.45 m | 19′ | 31.7 | 1.45 |
| | | 2.21 m | 5′, 10′ | | 2.23 |
| 2′ | 27.7 | 1.67 m | | 27.8 | 1.69 |
| | | 2.01 m | | | 2.02 |
| 3′ | 98.7 | | | 98.7 | |
| 4′ | 42.0 | 2.18 m | 3′, 5′, 6′ | 41.9 | 2.15 |
| 5′ | 51.3 | 1.45 m | 6', 28' | 51.0 | 1.45 |
| 6′ | 78.2 | 4.58 m | | 78.4 | 4.53 |
| 7′ | 33.5 | 2.05 m | | 35.2 | 1.97 |
| | | 2.72 dd (5.9, 16.8) | 5′, 6′, 8′, 9′ | | 2.63 |
| 8′ | 131.6 | | | 132.1 | |
| 9′ | 128.4 | | | 128.1 | |
| 10′ | 39.7 | | | 39.2 | |
| 11′ | 23.4 | 1.94 m | | 23.4 | 1.92 |
| | | 2.12 m | | | 2.11 |
| 12' | 37.8 | 1.39 m | | 37.4 | 1.36 |
| | | 1.99 m | 9′, 14′ | | 1.98 |
| 13′ | 42.8 | | | 42.5 | |
| 14′ | 53.2 | 2.13 m | | 52.7 | 2.14 |
| 15′ | 23.6 | 1.34 m | | 24.8 | 1.33 |
| | | 1.65 m | 13′ | | 1.61 |
| 16′ | 30.0 | 1.37 m | | 30.3 | 1.32 |
| | | 2.06 m | 17′ | | 2.09 |
| 17′ | 52.5 | 1.59 m | | 52.2 | 1.54 |
| 18′ | 11.1 | 0.61 s | 12′, 13′, 14′, 17′ | 11.0 | 0.59 |
| 19′ | 71.2 | 3.89 dd (2.7, 9.1) | 1′ | 71.2 | 3.86 |
| | | 3.92 d (9.1) | 2′, 5′, 10′ | | 3.92 |
| 20′ | 43.6 | 1.37 m | | 43.5 | 1.35 |
| 21′ | 13.7 | 0.99 d (6.9) | 17′, 20′, 22′ | 13.3 | 0.98 |
| 22′ | 70.9 | 3.73 dd (4.8, 11.4) | | 71.2 | 3.68 |
| 23′ | 33.8 | 1.30 m | | 33.6 | 1.28 |
| | | 2.00 m | 24′ | | 2.00 |
| 24′ | 45.4 | 2.12 m | 25, 26, 22′, 23′, 27′ | 45.3 | 2.13 |
| 25′ | 85.1 | | | 85.2 | |
| 26′ | 25.3 | 1.32 s | 24′, 25′, 27′ | 25.3 | 1.32 |
| 27′ | 32.0 | 1.22 s | 24′, 25′, 26′ | 32.0 | 1.22 |
| 28′ | 18.2 | 1.16 d (6.9) | 3′, 4′, 5′ | 18.5 | 1.18 |

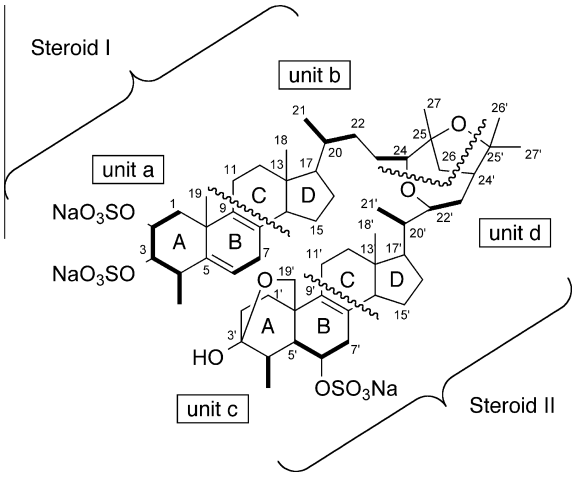



**Figure 1.** Partial structures of shishicrellastatin A (**1**). Assigned COSY correlations are shown in thick lines.

demonstrated by the $^{13}$C NMR spectrum. The presence of three sulfur atoms and numerous oxygen atoms together with the presence of deshielded oxymethines in the $^{13}$C NMR spectrum suggested that shishicrellastatin A had three sulfate groups. Fourteen degrees of unsaturation for the rest of the molecule was accounted for by three double bonds and 11 rings (Table 1).

The $^1$H and $^{13}$C NMR data, in particular, the number of methyl groups, suggested the presence of two steroid units. Interpretation of the high-field region of the $^1$H NMR data by analysis of COSY and TOCSY spectra was hindered by the presence of exceedingly overlapped signals. It was necessary to sort out signals by analyzing HMBC cross peaks from methyl proton signals, which were sufficiently separated, and use them as the starting points to deduce partial structures. Unit a comprised A and B rings of the steroid I in which C2 and C3 were oxygenated, C4 was methylated, and C5–C6 and C8–C9 double bonds were introduced (Fig. 1). Unit b constituted the C and D rings and the side chain of steroid I, in which oxygenation took place at C24 and C25. Importantly $H_2$26 were correlated to H24′ in unit d (vide infra) in the COSY spectrum, demonstrating the formation of a covalent bond between the side chains of two steroid units. Unit c composed A and B rings of steroid II, in which C19′ was oxidized and linked to C3′ through an ether bond, C4′ was methylated, C6′ was oxygenated and $\Delta^{8,9}$ double bond was introduced. Unit d corresponded to the remaining portion of steroid II, in which C22′ and C25′ were oxidized and C24′ was covalently linked to C26. Above-mentioned features are reminiscent of crellastatins, steroid dimers joined between the side chains.[3–5]

ROESY cross peaks, H19/H11b and $H_2$7/$H_2$15 suggested that partial structures a and b were connected to each other. ROESY cross peaks, H19′b/H11′b and H7′b/H15′a indicted that partial structures c and d were connected to each other (Fig. 2). Deuterium-induced chemical shifts in the $^{13}$C NMR spectrum displayed that a hydroxyl group was present at C3′.[6] An HMBC cross peak between H24 and C22′ revealed an ether linkage between C22′ and C24. Because shishicrellastatin A had one more unsaturation and C2, C3, and C6′ were considered to be sulfated on the basis of $^1$H and $^{13}$C NMR data, C25

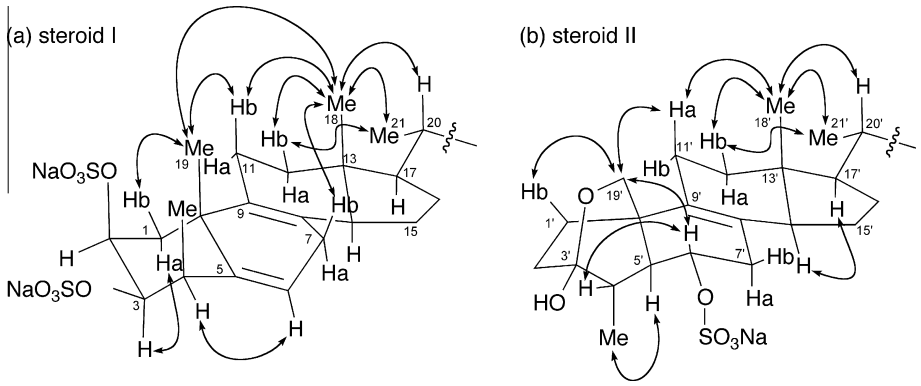



**Figure 2.** Relative configurations of shishicrellastatin A (**1**) as assigned by the ROESY data.

and C25′ were connected by an ether bond, allowing us to assign a side chain identical with that of crellastatin A.[3]

### 2.2.2. Relative configuration of steroid skeletons in shishicrellastatin A

The relative configuration of each steroid skeleton was assigned on the basis of $^1$H–1H coupling constants, ROESY data (Fig. 2), and comparison of chemical shift values with those in the literature.[3–5] H2 was equatorial (α-oriented), because it did not exhibited a large coupling with either $H_2$1 or H3. H3 was assigned as axial (α-oriented) on the basis of an intense ROESY cross peak with H1a. A small coupling and intense ROESY cross peak between H3 and H4 showed that H4 was equatorial and α-oriented. There was a ROESY cross peak between Me18 and Me19, demonstrating that the two methyls were on the same face of the tetracyclic skeleton. The absence of ROESY cross peak between Me18 and H14 indicated that the CD ring junction was *trans*. A ROESY cross peak between Me18 and H20 suggested that H17 was α-oriented. An intense COSY cross peak between H17 and H20 suggested that the two hydrogen atoms were in an anti-periplanar relationship. A distinct ROESY cross peak between Me21 and H12b suggested the 20R* configuration.[5] The relative configuration of steroid II was similarly assigned (Fig. 2). A ROESY cross peak between H19′a and H6′ suggested that these protons were on the same face of the ring system. H5′ and H6′ were in the anti-periplanar relationship on the basis of a large coupling constant of 9.8 Hz between them. A ROESY cross peak between Me28′ and H5′ showed that they were *syn*. ROESY cross peaks between Me18′ and $CH_2$19′, between Me18′ and H20′ and between Me21′ and H12′b as well as a large coupling between H17′ and H20′ and carbon chemical shift of C18′ ($\delta_H$ 11.5 ppm) suggested that steroid II was a derivative of conventional $\Delta^8$-steroid with 4α-methyl substitution (Fig. 2).

### 2.2.3. Relative configuration of the bicyclic system

The relative configuration of the bicyclic system in the side chain was assigned on the basis of the ROESY data (Fig. 3). H22′ was correlated with H26b and H23b, suggesting that H22′, H26b, and C23 were on the same face of the oxepane ring and all axially oriented. Because Me27′ was correlated to H26α C25′ and 25-O were placed on the other face of the oxepane ring.

### 2.2.4. Correlation of the relative configuration of a steroid skeleton with that of the bicyclic system

Then we attempted to correlate the configuration of C22′ with that of C20′. H20′ and H22′ were gauche because the coupling constant between these protons was small as suggested from the absence of a COSY correlation. With this information in mind, we inspected the possibility of 22′*R** and 22′*S** structures (Fig. 4).

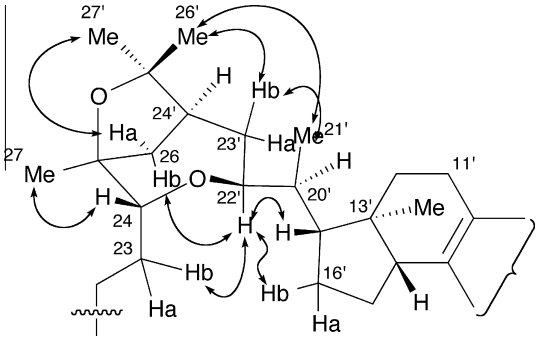


**Figure 3.** ROESY cross peaks for the side chain portion in **1**.

In the 22′*R** isomer, there are two stable conformers with H20′–H22′ gauche orientation (Fig. 4, A1 and A2). Similarly, two stable conformations are possible for the 22′*S** isomer (Fig. 4, B1 and B2). In the ROESY spectrum, a diagnostic cross peak was observed between H16′b and H22′ (H16′b is syn to H17′ on the basis of the ROESY data). The presence of the cross peak suggested the vicinity of H22′ and H16′b in agreement with conformers A2 and B2 but not A1 nor B1. On the other hand conformers A2 and B2 can be distinguished as follows. In conformer A2, H17′ and H23′a are close, whereas in conformer B2, H17′ and H23′a are remote and instead H16′b and H23′a is close. Additionally, in conformers A2, H17 is remote from C22′–O, whereas in conformer A2, these atoms are close. In the ROESY spectrum, the cross peak between H17′ and H23′a was not present and the cross peak between H16′b and H23′a was not distinguishable due to overlapped signals. It is noteworthy that H17′ ($\delta$ 1.59) is significantly deshielded compared to H17 ($\delta$ 1.26), indicating that H17′ is spatially close to C22′–O.[7] Therefore, we reasoned that shishicrellastatin A assumes conformer B2. However, the evidence presented here is not strong enough and our assignment should be considered as tentative. Although our attempts to hydrolyze the sulfate esters to assign the absolute configurations by the modified Mosher's method were unsuccessful, it was suggested from the biogenetic point of view that the absolute configurations of the two steroid skeletons in shishicrellastatin A are of conventional steroids.

## 2.3. Structure elucidation of shishicrellastatin B

Shishicrellastatin B (**2**) had two less hydrogen atoms than shishicrellastatin A (**1**) as shown by HRESIMS. The NMR data (Table 1) showed the presence of one additional double bond ($\delta_H$ 5.33; $\delta_C$ 118.4 and 150.9) in **2**. A comparison of the HSQC spectra of **1** and **2** suggested that the structural units a, c, and d were conserved

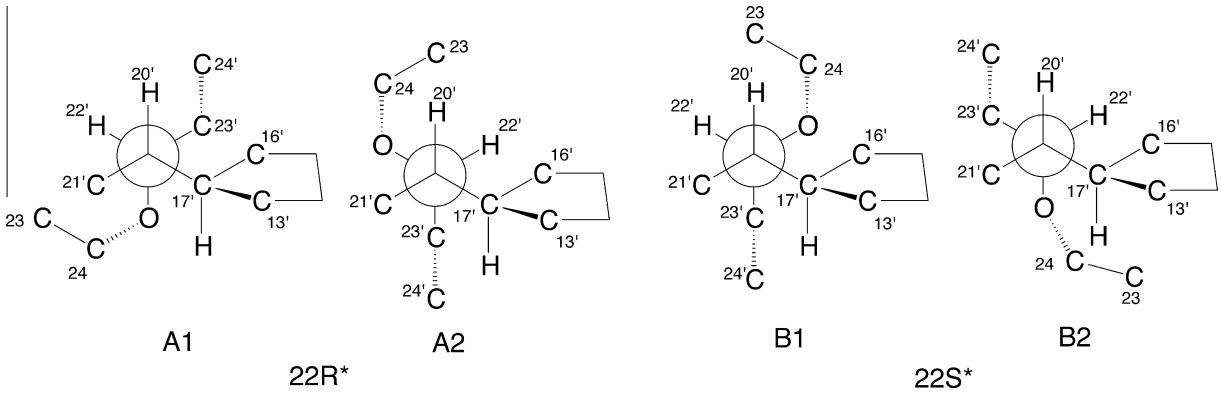



**Figure 4.** Newman projections and possible conformations of shishicrellastatin A with respect to the C20′–C22′ bond.

in **2**, but signals in ring D in steroid I were perturbed. Interpretation of 2D NMR data demonstrated an introduction of $\Delta^{14,15}$-olefin. This was in agreement with the UV absorption at $\lambda_{max}$ 250 nm (ε 1600). The relative configurations of the two steroid ring systems and the side chain portion in **2** were assigned on the basis of the ROESY data, which were essentially identical with that of **1**.

### 2.4. Cathepsin B inhibitory activities of shishicrellastatins

Shishicrellastatin A (**1**) and B (**2**) inhibit cathepsin B with an $IC_{50}$ value of each 8 μg/mL.

## 3. Discussion

Shishicrellastatins A and B were isolated from a marine sponge *Crella (Yvesia) spinulata* as inhibitors of cathepsin B. They are closely related to crellastatins (crellastatin A; **3**) isolated from a Vanuatu marine sponge *Crella* sp.,[3–5] hamigerols (hamigerol A; **4**) from the Mediterranean sponge *Hamigera hamigera*,[8] and amaroxocanes (amaroxocane B; **5**) from the Caribbean sponge *Phorbas amaranthus*.[9] They all have the common feature of side chain to side chain dimerization of polyoxygenated and sulfated steroids. They have the 3,8-dioxabicyclo[4.2.1]nonane ring system in common. The absolute configuration of the dioxabicyclo-ring system of crellastain A was assigned by a combination of molecular mechanics/dynamics calculation and the ROESY data.[3] The absolute configuration of the dioxabicyclo-ring system was not reported for hamigerols,[8] whereas that of amaroxocane B was proposed on the basis of NOESY data.[9]

In our study we have noticed that the establishment of the configurational relationship between C-22′ and C-20′ was not easy because of excessively crowded $^1$H NMR signals in the aliphatic region. From the biosynthetic point of view, it is likely that the congeners isolated from the four sponge species share the same absolute configuration for the relevant portion. We need to wait for a total synthesis of both configurational forms before we can be confident about the absolute configuration of the dioxabicyclononane ring system. It is interesting to note that this class of compounds display a wide range of biological activities.[3,8,9]

## 4. Experimental

### 4.1. General remarks

NMR spectra were recorded on a JEOL delta 600 NMR spectrometer at 600 MHz for $^1$H and 150 MHz for $^{13}$C. $^1$H and $^{13}$C chemical shifts were referenced to the solvent peak ($CD_3OD$) at $\delta$ 3.31 and 49.15 ppm, respectively. Standard parameters were used for the measurements of 2D NMR spectra. HRESI mass spectra were measured on a JEOL JMS-T100LC time-of-flight mass spectrometer. Fluorescence for enzyme inhibition assay was determined with a Molecular Devices SPECTRA MAX GEMINI fluorescence spectrometer. UV spectra were recorded on a Shimadzu BioSpec-1600 spectrophotometer. Optical rotations were recorded on a JASCO DIP-1000 digital parameter.

### 4.2. Animal material

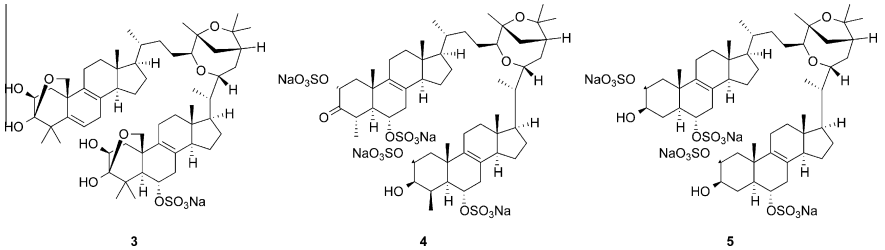
A specimen of *Crella (Yvesia) spinulata* was collected by scuba diving (12–15 m) off Shishi Island (32–15.94N; 130–15.75E), Kagoshima Prefecture. The animal was frozen after collection and kept at −20 °C until extraction. A voucher specimen was deposited in the collections of Zoological Museum of Amsterdam (Registration No. ZMAPOR 20128).

### 4.3. Cathepsin B inhibition assays

Cathepsin B inhibition assay was carried out following the method of Hiwasa et al.[10] with a slight modification. Test samples were added to each well containing buffer. Activated bovine cathepsin B solution was added to each well, and pre-incubated, followed by the addition of substrate solution. The fluorescence was measured at an excitation of 345 nm and an emission of 440 nm after 1 h-incubation at 37 °C.

### 4.4. Extraction and isolation

The sponge (2.0 kg, wet weight) was extracted with MeOH (3 L × 3), and the extract was concentrated under vacuum to yield



a dark brown oil. This material was partitioned between $H_2O$ (500 mL) and $CHCl_3$ (500 mL × 3). The aqueous fraction was partitioned between $H_2O$ (500 mL) and *n*-BuOH (500 mL × 2). All organic fractions were combined and separated by silica gel flash column chromatography with a step-wise gradient using $CHCl_3$, MeOH, and $H_2O$. The fraction eluted with $CHCl_3$–MeOH–$H_2O$ (6:4:1) was separated by silica gel column chromatography with a step-wise gradient using $CHCl_3$, MeOH, and $H_2O$. The $CHCl_3$–MeOH–$H_2O$ (6:4:1) fraction (497 mg) was separated by $C_{18}$ HPLC with a mixture of 1-PrOH–$H_2O$ containing 1.5 M $NaClO_4$ (3:7) to yield fractions 3A and 3B. Fraction 3A was purified by $C_{18}$ HPLC (1-PrOH–$H_2O$ containing 0.15 M $NaClO_4$ (3:7)) followed by C18 HPLC (1-PrOH–$H_2O$ (3:7)) to yield shishicrellastatin A (**1**, 1.3 mg). Fraction 3B was purified in the same manner to yield shishicrellastatin B (**2**, 2.7 mg).

#### 4.4.1. Shishicrellastatin A

Shishicrellastatin A (**1**): white solids; $[\alpha]_D^{22}$ +11 (*c* 0.05 MeOH); UV (MeOH) $\lambda_{max}$ 205 nm (ε 4000); $^1H$ and $^{13}C$ NMR data, see Table 1; HRESIMS *m*/*z* 1199.4429 (calcd for $C_{56}H_{83}Na_3O_{16}S_3$ $[M+Na]^+$ 1199.4434, Δ −0.5 mmu).

#### 4.4.2. Shishicrellastatin B

Shishicrellastatin B (**2**): white solid; $[\alpha]_D^{22}$ +11 (*c* 0.10 MeOH); UV (MeOH) $\lambda_{max}$ 205 nm (ε 2000), 250 nm (ε 1600); $^1H$ and $^{13}C$ NMR data, see Table 1; HRESIMS *m*/*z* 1197.4304 (calcd for $C_{56}H_{81}Na_3O_{16}S_3$ $[M+Na]^+$ 1197.4278, Δ −2.6 mmu).

## Acknowledgements

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.052.

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