Cristaxenicin A, an Antiprotozoal Xenicane Diterpenoid from the Deep Sea Gorgonian Acanthoprimnoa cristata

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Cristaxenicin A, an Antiprotozoal Xenicane Diterpenoid from the Deep Sea Gorgonian *Acanthoprimnoa cristata*

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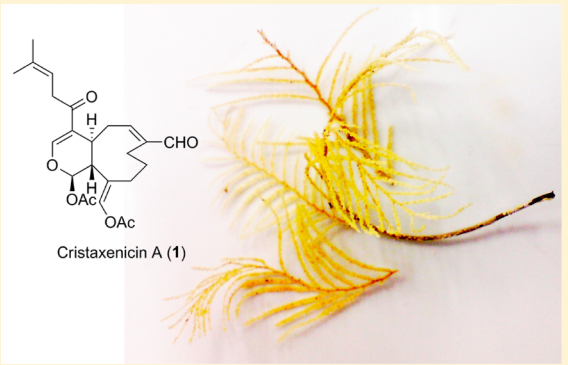
Supporting Information

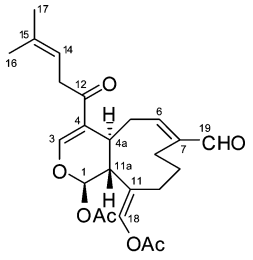
ABSTRACT: A new xenicane diterpenoid, cristaxenicin A (**1**), has been isolated from the deep sea gorgonian *Acanthoprimnoa cristata*. The structure of **1** was elucidated on the basis of spectral analysis including NMR and MS. The absolute configuration of **1** was determined on the basis of quantum chemical calculation of CD spectra. Cristaxenicin A (**1**) showed antiprotozoal activities against *Leishmania amazonensis* and *Trypanosoma congolense* with IC_{50} values of 0.088 and 0.25 μM, respectively.



More than one billion people are affected by neglected tropical diseases,[2] of which the leishmaniasis is thought to be the most difficult to control.[3] This disease is caused by the intracellular protozoa belonging to the genus *Leishmania* and is a threat to 350 million people, with 1.5−2 million new cases annually. Pentavalent antimony compounds have been used as the first line drugs, while amphotericin B and other antifungal agents have been used for the second line. However, toxicity and the high cost for the treatments of antileishmanial drugs pose serious problem for controlling leishmaniasis. Thus, new drugs should be urgently developed. More than 90 marine natural products have been reported so far, but none of them have reached clinical trials.[4]

In the course of our efforts to discover potential drug leads from marine invertebrates, we have tested 1565 extracts of marine organisms against the recombinant *L. amazonensis* doped with green fluorescence protein (*La/egfp*).[5] In this screening, we found promising activity in the lipophilic extract of the deep-sea gorgonian *Acanthoprimnoa cristata* collected in southern Japan. Bioassay-guided isolation yielded a highly antileishmanial new xenicane diterpenoid, cristaxenicin A (**1**).

The frozen specimen of *A. cristata* (90 g, wet weight) was extracted with MeOH. The extract was concentrated and then extracted with $CHCl_3$ and *n*-BuOH. The antileishmanial $CHCl_3$ and *n*-BuOH layers were combined and fractionated by the Kupchan procedure[6] to yield *n*-hexane, $CHCl_3$, and aqueous MeOH layers. The active $CHCl_3$ layer was subjected to ODS flash chromatography and reversed-phase HPLC with the linear gradient solvent system of aqueous MeOH to afford cristaxenicin A (**1**) as a yellowish amorphous solid (19.7 mg, 0.022% yield based on wet weight).



(**1**)

Cristaxenicin A (**1**) had a molecular formula of $C_{24}H_{30}O_7$ as determined by HRFABMS at *m/z* 431.2089 $[M + H]^+$ [calcd for $C_{24}H_{31}O_7$ *m/z* 431.2070 (Δ −1.9 mmu)]. The ^1H and ^{13}C NMR spectra in CD_3OD showed the presence of two methyls (CH_3-16 and 17) connected to the olefinic carbons, two acetyl groups (OAc-1 and 18), four methylenes with geminal nonequivalent protons (CH_2-5, 8, 10, and 13), a methylene

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with overlapping protons (CH_2-9), two methines (CH-4a and 11a), an acetal methine (CH-1), four olefinic methines (CH-3, 6, 14, and 18), four quaternary sp^2 carbons (C-4, 7, 11, and 15), an α,β-conjugated aldehyde (CHO-19), and an α,β-conjugated ketone (CO-12).

Analysis of the COSY spectrum revealed the presence of three spin systems **a**-**c** (Figure 1). Connectivities of these spin

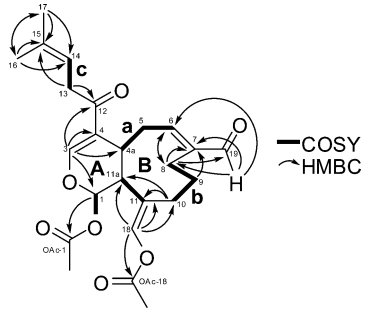

Figure 1. Key COSY and HMBC correlations of **1**.

systems were established on the basis of HMBC spectral analysis. HMBC correlations observed from the aldehyde proton (H-19) to C-6, C-7, and C-8, indicated that units **a** and **b** were linked via an α,β-unsaturated aldehyde group, while those from H-18 to C-10, C-11, C-11a, and the carbonyl carbon of OAc-18 indicated units **a** and **b** were also connected via an enol acetate group on the other sides to make a nine-membered ring **B**. The lower chemical shift of C-18 (δ_C 136.0 ppm) compared to that of the *exo*-methylene in the related compound[7] showed good agreement with that of the acetoxylated olefinic carbon (δ_C 135.2 ppm) in the sesquiterpene from the soft coral *Paralemnalia thyrsoides*.[8] HMBC correlations from H-3 to C-1, C-4, and C-4a indicated the presence of a dihydropyran ring **A** in which C-4a and C-11a were also involved in ring **B**. A correlation from H-1 to the carbonyl carbon of OAc-1 indicated that C-1 was acetoxylated. Connection of unit **c** and ring **A** at C-4 via C-12 ketone was confirmed by HMBC correlations from H-3 and H-13 to C-12. Attachment of an isopropenyl group at C-14 on the basis of HMBC correlations from H-16 and H-17 to C-14 and C-15 completed the planar structure of cristaxenicin A (**1**) as the highly functionalized xenicane-type diterpenoid.[7]

A NOESY correlation between the aldehyde proton (H-19) and H-6 indicated *E* geometry of $\Delta^{6,7}$. The *E* geometry of $\Delta^{11,18}$ was deduced by NOESY correlations among H-18/H-1, H-4a, and H-11a. The relative stereochemistry at C-1, C-11a, and C-4a was determined on the basis of $J_{H,H}$ coupling constants and NOEs. The large coupling constants among H-4a/H-11a ($J_{H,H}$ = 11.4 Hz) and H-1/H-11a ($J_{H,H}$ = 9.5 Hz) suggested that all these protons are axially oriented, thus indicating the relative configuration of 1*R**,4a*S**,11*R** which was supported by NOESY correlations among H-1/H-4a and H-5′/H-11a (Figure 2).

The absolute configuration of cristaxenicin A (**1**) was determined by quantum chemical calculation of the circular dichroism (CD) spectra. The energy optimized structures were calculated for 1*R*,4a*S*,11a*R*- and 1*S*,4a*R*,11a*S*-configuration by Gaussian 09 with the density functional theory (DFT) at B3LYP/6-31++G** level.[9] The simulated coupling constant values among H-4a/H-11a ($J_{H,H}$ = 8.9 Hz) and H-1/H-11a ($J_{H,H}$ = 7.5 Hz) showed good agreement with those measured [H-4a/H-11a ($J_{H,H}$ = 11.4 Hz) and H-1/H-11a ($J_{H,H}$ = 9.5 Hz),

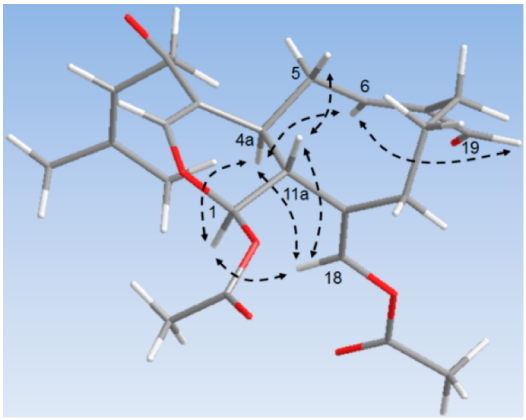

Figure 2. Key NOEs observed for **1**.

respectively]. Comparison of the measured CD spectrum of cristaxenicin A (**1**) with those simulated on the basis of the calculated structures for 1*R*,4a*S*,11a*R*- and 1*S*,4a*R*,11a*S*-configuration revealed that the calculated spectrum for the former configuration was in good agreement with the measured spectrum (Figure 3). To exclude the possibility of the diastereomeric configuration, CD spectra were simulated for additional six stereoisomers of 1*R*,4a*R*,11a*R*, 1*S*,4a*S*,11a*S*, 1*S*,4a*R*,11a*R*, 1*R*,4a*S*,11a*S*, 1*S*,4a*S*,11a*R*, and 1*R*,4a*R*,11a*S*. As a result, none of these calculated CD spectra showed agreement with the measured spectrum (Figure S8, Supporting Information). Therefore, the absolute configuration of cristaxenicin A (**1**) was concluded as 1*R*,4a*S*,11a*R* which was coincided with that of the related xenicin-type diterpenoids whose configuration was determined by the modified Mosher's method or X-ray analysis.[10,11]

Antiprotozoal activities of cristaxenicin A (**1**) were evaluated against *L. amazonesis* (*La/egfp*), *Trypanosoma congolense*, and *Plasmodium falciparum*. Cristaxenicin A (**1**) showed antileishmanial activity with an IC_{50} value of 0.088 μM (Figure 4). It also showed antitrypanosomal activity (IC_{50} 0.25 μM), while it showed only moderate antimalarial activity with an IC_{50} value of 11 μM. The cytotoxicity of **1** against P388 and HeLa cells remained lower levels with IC_{50} values of 4.7 and 2.1 μM, respectively. Thus, the selectivity index (Supporting Information) of cristaxenicin A (**1**) was 23−53-fold against *La/egfp*.

An antiprotozoal xenicane-type diterpenoid, cristaxenicin A (**1**), was isolated from the gorgonian *Acanthoprimnoa cristata* collected from the deep sea in southern Japan. Cristaxenicin A (**1**) was as equivalently potent as amphotericin B against *La/egfp* and *T. congolense* (IC_{50} of amphotericin B: IC_{50} 0.021 and 0.88 μM, respectively).

Cristaxenicin A (**1**) is a new xenicane-type diterpenoid highly functionalized with an enol acetate, an α,β-unsaturated aldehyde, a ketone conjugated with a dihydropyran ring, and an acetylated acetal. Xenicane-type diterpenoid was originally isolated from the soft coral *Xenia elongate*.[7] These compounds are characterized by the dihydropyran ring fused to the nine-membered ring and are classified mainly into four families: xenicins, xenialactols, and xeniolides A and B.[12] Cristaxenicin A (**1**) belongs to the xenicin family, of which other examples were reported from gorgonians *Acalycigorgia inermis*[13] and *Acanthogorgia turgida*.[14] Although there are many enol acetate containing compounds from marine algae, those are rare from marine invertebrates. There are two compounds from the sponges *Aplysilla pallida*[15] and *Ircinia felix*[16] and five terpenoids

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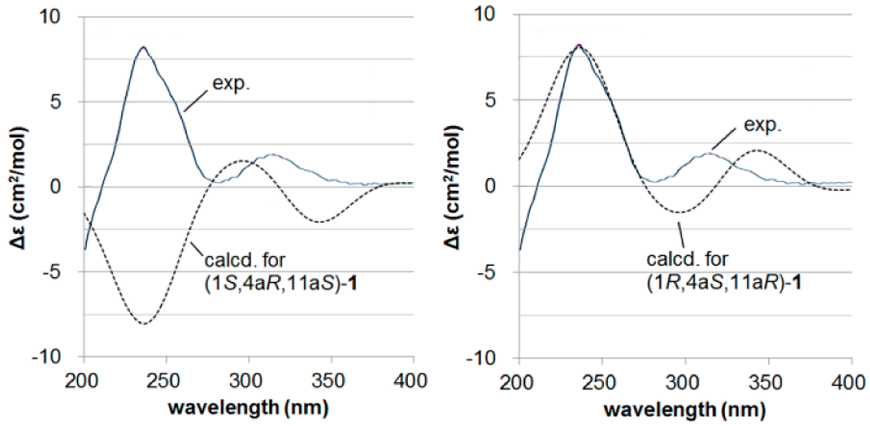

Figure 3. Comparison of the measured CD spectrum of **1** with that for 1*R*,4a*S*,11a*R* and 1*S*,4a*R*,11a*S* configurations.

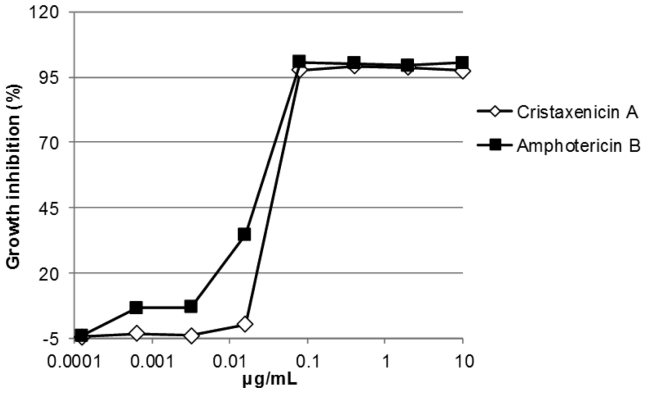

Figure 4. Inhibition of *La/egfp* by cristaxenicin A (**1**).

from the cnidarians *Paralemnalia thyrsoides*[8,17] and *Pseudopterogorgia elisabethae*.[18] A closely related structure was suggested as the biogenetic precursor of plumisclerin A.[19]

Absolute configuration of xenicane-type compounds was determined by Cimino et al.[20] applying the octant rule for the CD spectra. This is the first example of the application of ab initio calculation of CD spectra to determine the absolute configuration of xenicane compounds.

It should be noted that this is the first report of a xenicane-type diterpenoid exhibiting antiprozoan activity. The unique structural motif of this compound is suggesting the novel mode of action, which is necessary for the development of new treatments for leishmaniasis.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded on a 400 MHz spectrometer. ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks of δ_H 3.30 and δ_C 49.0 for CD_3OD. FAB mass spectra were measured on a reversed-geometry double-focusing mass spectrometer (for HRFABMS) using 3-NBA as a matrix. Optical rotation was determined using a digital polarimeter in MeOH. UV spectrum was recorded by spectrophotometer using MeOH as the solvent. IR spectrum was measured by IR spectrometer using ATR plate. Fluorescence was measured on a fluorescence plate reader. The CD spectrum was recorded on a circular dichroism spectrometer in MeOH.

Extraction and Isolation. *Acanthoprimnoa cristata* (No.C07110), identified by Dr. Yukimitsu Imahara, was collected by dredging at the depth of 138 m, Yakushima-Shinsone (N 29°46.55′, E 130°21.92′), Kagoshima prefecture, Japan, on June 24, 2007. The sample was immediately frozen and kept at −30 °C until chemical investigation. The frozen *A. cristata* (90 g, wet weight) was extracted with MeOH (2 × 500 mL) and evaporated in vacuo. The extract was suspended in H_2O (100 mL) and extracted with $CHCl_3$ (2 × 100 mL) and *n*-BuOH (2 × 100 mL). The $CHCl_3$ and *n*-BuOH layers were combined, evaporated, and partitioned between 90% MeOH (100 mL) and *n*-hexane (2 × 100 mL). The 90% MeOH layer was adjusted to 60% MeOH by addition of H_2O (50 mL) and extracted with $CHCl_3$ (2 × 150 mL). The $CHCl_3$ layer which showed antileishmanial activity was subjected to ODS flash chromatography [50 and 70% MeOH, 70 and 85% MeCN, MeOH, and $CHCl_3$/MeOH/H_2O (6:4:1)] to yield six fractions. The antileishmanial activity was found in the fraction eluting with 70% MeOH. Therefore, this fraction was further separated by the reversed-phase HPLC (COSMOSIL 5C_{18}-AR II, 250 × 20 mm, 70 to 90% MeOH linear gradient) to afford cristaxenicin A (**1**, 19.7 mg, 0.022% yield based on wet weight) as the yellowish amorphous solid.

CD Spectrum Calculation. All conformers were optimized at the B3LYP/6-31++G** level for the solvent (MeOH). The CD spectrum was simulated for the stereoisomers calculated at the same level. All calculations were performed with Gaussian 09.[9]

NMR Coupling Constant Calculation. All conformers were optimized at the B3LYP/6-31++G** level for the solvent (MeOH). The NMR coupling constants was simulated for the stereoisomers calculated at the same level. All calculations were performed with Gaussian 09.[9]

Antileishmanial Assay. *La/egfp* promastigotes (1 × 10^5 cells) were cultured in 199 medium (NISSUI Pharmaceutical, Tokyo, Japan) in 96-well plates with various concentrations of cristaxenicin A (**1**) for 72 h. Fluorescence was measured with excitation at 485 nm and emission at 538 nm.

Antitrypanosoma Assay. Procyclic form parasites (2 × 10^5 cells per well) of *Trypanosoma congolense* IL 3000 were cultured in TVM-1 medium (Sakurai, T. et al., Mol Biochem Parasitol, 2008) in 96-well plates with various concentrations of cristaxenicin A (**1**) for 48 h. To each well was added 10 μL of TetraColor ONE (Seikagaku Biobusiness, Tokyo, Japan) solution. After 4 h, the plates were read for absorbance at 450 nm using a microplate reader.

Antimalarial Assay. In vitro activity of cristaxenicin A (**1**) against *Plasmodium falciparum* FCR-3 was examined by using SYBR Green I as previously described.[21]

Evaluation of Cytotoxicity. HeLa human cervical cancer cells (1 × 10^5 cells) were cultured at 37 °C under 5% CO_2 in D-MEM medium (low glucose, No. 041-29775, Wako) containing 10% fetal bovine serum (Lot No. S1820, BioWest), 18 μg/mL of gentamicin, and 1% antibiotic−antimicotic, with various concentrations of cristaxenicin A (**1**) in each well of 96-well plates for 72 h. P388 murine leukemia cells (1 × 10^5 cells) were cultured at 37 °C under 5% CO_2 in RPMI medium (No. 189−02025, Wako), supplemented with 10% fetal bovine serum (Lot No. S1820, BioWest), 6.0 × 10^{-5}% HEDS solution (2,2′-dithiodiethanol), and 40 μg/mL kanamycin sulfate with various concentrations of cristaxenicin A (**1**) in each well of 96-well plates for 72 h. Fifty μL of the saline solution of 3-(4,5-

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Table 1. ^{1}H and ^{13}C NMR Data of Cristaxenicin A (1) in CD_3OD

position	δ_H mult, (J, Hz)	δ_C	COSY	HMBC	NOESY
1	5.86 d (9.5)	94.5	H-11a	1-OAc	H-4a, H-18
3	7.68 s	154.6		C-1, C-4, C-4a, C-12	H-13
4		120.2			
4a	3.04 m	35.9	H-5, H-5′, H-11a		H-1, H-5, H-6, H-18
5	2.86 ddd (8.5, 3.6, 13.0)	30.0	H-4a, H-5′, H-6		H-4a, H-6, H-5′
5′	2.65 ddd (8.5, 6.5, 13.0)		H-4a, H-5, H-6		H-6, H-5, H-11a
6	6.65 t (8.5)	153.4	H-5, H-5′	C-8, CHO	H-4a, H-5, H-5′, H-19
7		144.9			
8	2.39 dd (14.0, 6.8)	21.2	H-8′, H-9	C-6, C-7, CHO	H-9
8′	2.32 dd (14.0, 5.9)		H-8, H-9		H-9
9	1.83 m	22.5	H-8, H-8′, H-10, H-10′	C-7	H-8, H-10
10	2.54 dd (14.3, 6.7)	28.8	H-9, H-10′	C-11a, C-11, C-8	H-9, H-10′
10′	2.00 dd (14.3, 6.0)		H-9, H-10		H-10, H-11a
11		120.6			
11a	2.48 dd (11.4, 9.5)	49[a]	H-1, H-4a	C-18	H-5′, H-10′, H-18
12		198.2			
13	3.40 dd (7.2, 16.1)	37.6	H-13′, H-14	C-12, C-15	H-3, H-13′, H-14
13′	3.33 dd (7.2, 16.1)		H-13, H-14		H-3, H-13, H-14
14	5.30 t (7.2)	116.9	H-13, H-13′		H-13, H-17
15		134.9			
16	1.68 s	16.7		C-14, C-15, C-17	
17	1.75 s	24.5		C-14, C-15, C-16	H-14
18	6.98 s	136.0		C-10, C-11, C-11a, 18-OAc	H-1, H-4a, H-11a
19	9.27 s	195.8		C-6, C-7, C-8	H-6
OAc-1	2.05 s	19.1			
		168.9			
OAc-18	2.10 s	19.0			
		167.8			

[a]Overlap in the solvent signal.

dimethyl-2-thiazoyl)- 2,5-diphenyl-2*H*-tetrazolium bromide (MTT, 1 mg/mL) was added to each well, and the plate was incubated for 4 h. Medium was removed by aspiration and 150 μL DMSO was added to each well. The absorption for 570 nm was measured to estimate IC_{50} values.

Characteristic data for cristaxenicin A (1): yellowish amorphous solid; ^{1}H and ^{13}C NMR data, see Table 1; $[\alpha]^{21.7}_{D}$ +90.5 (*c* 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 231 (4.28) nm; IR (film) ν_{max} 2931, 1755, 1681, 1616, 1454, 1371, 1340, 1209, 1176, 1108, 1076, 1015 cm^{-1}; CD (*c* 0.13, MeOH) $\Delta_{\varepsilon 314}$ +1.9, $\Delta_{\varepsilon 282}$ +0.02, $\Delta_{\varepsilon 236}$ +8.2; FABMS *m*/*z* 431 $[M + H]^+$, 453 $[M + Na]^+$; HRFABMS *m*/*z* 431.2089 (calcd for $C_{24}H_{31}O_7$ 431.2070).

■ ASSOCIATED CONTENT

Supporting Information

MS, ^{1}H, and ^{13}C NMR spectra of compound **1** and the calculated CD spectra for the stereoisomers of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

In memory of Professor Ernesto Fattorusso.

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