The discovery and anti-colorectal cancer activities of cembranolides from the South China Sea soft coral Sinularia pendunculata (Postprint)

Authors: Hong-Ru Wang, Yang Jin, Ruo-Nan Sun, Li-Gong Yao, Jian-Hua Xia, Yun Huang, San-Hong Liu, Wei-Dong Zhang, Xu-Wen Li, San-Hong Liu, Wei-Dong Zhang, Xu-Wen Li

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Abstract

A new cembranolide, namely, sinupendunculide A (1), along with eight known related compounds (2-9), was isolated from the South China Sea soft coral *Sinularia pendunculata*. The structure of sinupendunculide A (1) was established by extensive spectroscopic analysis and X-ray diffraction experiments. In a bioassay, anti-colorectal cancer (CRC) activity was evaluated, and the results showed that several compounds exhibited cytotoxicity against RKO cells, with a preliminary structure-activity relationship analysis conducted. Meanwhile, the most effective compound 7 was proven to increase reactive oxygen species levels, which promoted cell apoptosis and inhibited cell proliferation.

Full Text

Preamble

Discovery of Anti-Colorectal Cancer Cembranolides from the South China Sea Soft Coral *Sinularia pendunculata*

Hong-Ru Wanga,1, Yang Jinb,c,1, Ruo-Nan Sunb,c, Li-Gong Yaob, Jian-Hua Xiaa, Yun Huanga, San-Hong Liua,, *Wei-Dong Zhanga,d,*, Xu-Wen Lib,c,*

a Shanghai Frontiers Science Center of TCM Chemical Biology, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China; wdzhangy@hotmail.com; liush@shutcm.edu.cn

b Natural Products Chemistry Department, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China; xwli@simm.ac.cn c Shandong Laboratory of Yantai Drug Discovery, Bohai Rim Advanced Research Institute for Drug Discovery, Yantai, Shandong 264117, China

d Department of Phytochemistry, School of Pharmacy, Second Military Medical University, Shanghai 200433, China

1 These authors contributed equally to this work.

Abstract

A new cembranolide, sinupendunculide A (1), along with eight known related compounds (2–9), was isolated from the South China Sea soft coral *Sinularia pendunculata*. The structure of sinupendunculide A (1) was established through extensive spectroscopic analysis and X-ray diffraction experiments. In bioassays evaluating anti-colorectal cancer (CRC) activity, several compounds exhibited cytotoxicity against RKO cells, and a preliminary structure-activity relationship was analyzed. Meanwhile, the most effective compound, 7, was demonstrated to increase reactive oxygen species levels, which promoted cell apoptosis and inhibited cell proliferation.

Keywords: *Sinularia pendunculata*; marine natural products; cembranolide; antitumor; cell apoptosis

Introduction

Colorectal cancer is a malignant tumor originating from the colorectal mucosal epithelium and represents one of the most common malignancies in clinical practice [**?**, **?**]. Due to immune escape and malignant proliferation of cancer cells, approximately 35% of patients present with metastatic disease at diagnosis, and as many as 50% of patients with nonmetastatic colorectal cancer eventually develop metastases [**?**].

Marine natural products are characterized by rich chemical diversity and broad biological activities, particularly antitumor effects [**?**]. Many prominent antitumor drugs on the market are derived from marine organisms, such as Halaven (eribulin), Yondelis (ecteinascidin 743), Zepzelca (lurbinectedin), and Aplidin (plitidepsin).

Soft corals of the genus *Sinularia* (order Alcyonacea, family Alcyoniidae) have proven to be rich sources of secondary metabolites [**?**, **?**], especially cembrane-type diterpenes [**?**], which exhibit complex chemical diversity and a wide spectrum of biological activities, including antitumoral, anti-inflammatory, and neuroprotective effects [**?**, **?**]. Cembranolides from *Sinularia* soft corals have previously been found to exhibit cytotoxicity against several cancer cell lines by our group [**?**]. To further explore the chemical diversity of antitumoral cembranolides for structure-activity relationship (SAR) studies, we conducted a chemical investigation of *Sinularia pendunculata*, resulting in the isolation of a highly oxidized cembranolide, sinupendunculide A (1), together with eight related known cembranolides (2–9). This paper reports the isolation, structural determination, anti-CRC activity evaluation, and SAR analysis of all isolates, as well as an in-depth antitumoral mechanism study of the most effective compound, 7.

[Figure 1: see original paper]. Structures of compounds 1–9.

Results and Discussion

Isolation and Identification

The frozen animals were processed using the same flow as previously described [?], yielding pure compounds 1 (5.2 mg), 2 (7.4 mg), 3 (2.6 mg), 4 (19.1 mg), 5 (344.0 mg), 6 (86.5 mg), 7 (7.6 mg), 8 (54.3 mg), and 9 (130.2 mg). The known compounds 2–9 were readily identified as sinulaflexiolide E (2) [?], flexibilisin A (3) [?], (1R,13S,12S,9S,8R,5S,4R)-9-acetoxy-5,8:12,13-diepoxycembrane-15(17)-en-16,4-olide (4) [?], 7,8-epoxy-11-epi-sinulariolide acetate (5) [?], 11-epi-sinulariolide acetate (6) [?], flexilarin D (7) [?], 11-dehydrosinulariolide (8) [?], and (+)-sinulariolide (9) [?] by comparison of their NMR data and optical rotation values with literature values.

Structural Elucidation of Sinupendunculide A (1)

Sinupendunculide A (1) was obtained as a colorless oil and assigned the molecular formula C20H28O5 based on HR-EI-MS at m/z 348.1931 (calcd 348.1931), indicating seven double bond equivalents (DBEs). The IR spectrum showed diagnostic absorption bands for an α,β-unsaturated ester (1717 cm−1) functionality. The 13C NMR, DEPT, and HSQC spectra of 1 revealed 20 carbon signals, including three methyls, seven sp3 methylenes, three sp3 methines, three sp3 quaternary carbons, one sp2 methylene, and three sp2 quaternary carbons (one ketone carbonyl at δC 209.6). Furthermore, the presence of two epoxides was inferred from four oxygenated carbon signals at δC 62.8 (CH), 59.9 (qC), 55.7 (CH), and 59.9 (qC). An α,β-unsaturated ester, one ketone carbonyl, two tricyclic ring systems, and a typical cembrane skeleton with a 14-membered macrocycle accounted for six of the seven DBEs, implying the presence of an exo-methylene conjugated lactone ring, which established the planar structure of 1.

The relative configuration of 1 was established by analysis of its NOESY spectrum (Figure 2 [Figure 2: see original paper]). For the configuration of the two tricyclic rings, clear NOE correlations of H-2b (δH 2.01)/H-3 (δH 3.05), H-3/H-5a (δH 1.23), H-5a/H-6a (δH 1.59), H-6a/H3-19 (δH 1.37), H-7 (δH 2.71)/H-1 (δH 2.32), H-1/H-2a (δH 1.50), and H-2a/H3-18 (δH 1.30), as well as the lack of correlation between H-3/H-2a, indicated trans configurations for both epoxides. However, it remained difficult to deduce the relative configuration of C-1, C-3, C-4, C-7, C-8, and C-12 from the NOESY spectrum due to the absence of correlation between H3-20 and other protons.

[Figure 2: see original paper]. 1H–1H COSY, selected key HMBC, and NOESY correlations of 1.

Fortunately, a suitable single crystal was obtained by recrystallization of 1 from a methanol/ethyl ether (1:1) solvent system. X-ray crystallography analysis using Cu Kα radiation (λ = 1.54178 Å) clearly revealed its absolute configuration as 1R, 3S, 4S, 7S, 8S, 12R [Flack parameter = 0.00(7)] (Figure 3 [Figure 3: see original paper]), confirming the structure as sinupendunculide A (Figure 1).

[Figure 3: see original paper]. Perspective ORTEP drawing of the X-ray structure of 1 (displacement ellipsoids drawn at 50% probability level).

. 1H [δH, mult. (J, Hz)]a and 13C [δC (mult.)]b NMR data of compound 1 in CDCl3.

Position	δH, mult. (J, Hz)	δC (mult.)
1	2.32 (m)	35.2 (d)
2a	1.50 (ddd, 13.4, 11.3, 1.9)	33.3 (t)
2b	2.01 (t)	-
3	3.05 (dd, 11.2, 2.4)	62.8 (d)
4	-	59.9 (s)
5a	1.23 (m)	34.9 (t)
5b	2.11 (ddd, 13.2, 5.3, 2.5)	-
6a	1.59 (m)	22.6 (t)
6b	1.81 (m)	-
7	2.71 (m)	55.7 (d)
8	-	59.9 (s)
9a	1.99 (t)	26.7 (t)
9b	2.40 (t)	-
10a	2.43 (m)	30.6 (t)
10b	2.72 (m)	-
11	-	209.6 (s)
12	1.92 (ddd, 15.4, 12.9, 5.7)	92.0 (s)
13a	2.44 (m)	33.7 (t)
13b	1.25 (m)	-
14a	2.52 (m)	31.0 (t)
14b	-	-
15	-	144.2 (s)
16	5.52 (s)	125.3 (t)
17	6.32 (s)	-
18	1.30 (s)	168.4 (s)
19	1.37 (s)	15.8 (q)
20	1.47 (s)	22.0 (q)
-	-	29.8 (q)

a Recorded at 600 MHz. b Recorded at 125 MHz. Assignments were deduced by analysis of 1D and 2D NMR spectra.

Biological Activity and Structure-Activity Relationship

All isolated compounds were evaluated for antitumor and cytotoxic effects in bioassays. In the RKO cell viability assay, compound 7 showed the most potent bioactivity, reducing cell viability to 30.7% at 20 M, with an IC50 value of 8.5 M. For the SAR analysis, we propose that the lactone structure represents a critical fragment for biological activity; compounds 2 and 3, which lack this moiety, showed no biological activity. For compounds 7 and 8, the hydroperoxyl group at C-8 contributed to biological activity. Additionally, comparison of compounds 5 and 6 revealed that substitution at C-7 and C-8 also had a slight effect on activity. The activity of the epoxide ring was slightly lower than that of the double bond. The differences among compounds 5–8 primarily involved the epoxide configuration at C-3 and C-4 and substitution at C-11. Reduction at C-11 to either a hydroxyl group or formation of an epoxide was detrimental to activity. For sinupendunculide A (1), the epoxide at C-7 and C-8 reduced activity compared with compounds 7 and 8. Interestingly, compound 5, which also contains an epoxide, showed better activity than compound 1, suggesting that the conformation of the epoxide at this position is an additional factor affecting activity. In summary, compound 7 was selected for further evaluation of its cytotoxic activities against CRC in vitro.

In Vitro Antitumor Mechanism of Compound 7

We initiated our chemical screen by detecting cell viability to identify compounds with obvious cytotoxic effects on RKO cells. RKO cells were treated with various compounds (20 M) for 24 h (Figure 4A [Figure 4: see original paper]), and cell viability was measured by CCK-8 assay. Compound 7 efficiently killed RKO cells, with an IC50 of 8.5 M (Figure 4B [Figure 4: see original paper]). To further assess its efficacy in vitro, a colony formation assay demonstrated that 7 treatment significantly suppressed cell growth (Figure 4C–D [Figure 4: see original paper]). EdU is a thymidine analogue that incorporates into replicating DNA during cell proliferation. DNA replication activity is detected based on the specific reaction between EdU and fluorescent dye, allowing accurate assessment of cell proliferation through EdU markers. EdU staining (green fluorescence) showed that 7 inhibited RKO cell proliferation in a dose-dependent manner (Figure 4E–F [Figure 4: see original paper]). To further validate 7-mediated growth inhibition, cell cycle analysis was performed by flow cytometry. The results (Figure 4G–H [Figure 4: see original paper]) showed that 7 treatment significantly induced G2/M phase arrest in RKO cells in a concentration-dependent manner.

[Figure 4: see original paper]. Compound 7 exhibits significant antitumor activity. (A) Cell viability detected by CCK-8 assay after treatment with 20 M of different compounds on RKO cells. (B) Cell viability measured by CCK-8 assay with indicated concentrations (1, 2.5, 5, 10, 20 M) of compound 7 for 24 h in RKO cells. (C–D) Cell growth capacity detected by colony formation assay to validate the effect of 7 on RKO cells. Data are shown as mean ± SD (**P** <

0.02, *$P < 0.01$). (E–F) Cell proliferation capacity suppressed after treatment with compound 7. Green represents intracellular DNA content. Data are shown as mean ± SD (*$\boldsymbol{P < 0.02}$**,** $P < 0.01$). (G–H) Cell cycle distribution analyzed by flow cytometry. Data are shown as mean ± SD.

Compound 7 Increases ROS Levels and Promotes RKO Cell Apoptosis

We measured ROS levels after 7 treatment, as shown in Figure 5D [Figure 5: see original paper]. ROS levels in RKO cells were significantly elevated following 7 treatment. Concurrently, Annexin V-FITC/PI double-staining assay performed by flow cytometry showed that only higher concentrations of 7 increased the proportion of apoptotic RKO cells (Figure 5A–B [Figure 5: see original paper]). Furthermore, high-content screening with calcein-AM/PI dye was used to detect intracellular esterase activity and plasma membrane integrity. As shown in Figure 5C [Figure 5: see original paper], very few red fluorescence signals representing dead cells were observed in the control group; however, as the concentration of 7 increased, living cells represented by green fluorescence were markedly reduced.

[Figure 5: see original paper]. Compound 7 increases ROS levels and promotes RKO cell apoptosis. (A–B) Percentage of apoptotic cells analyzed by flow cytometry. Data are shown as mean ± SD (*$\boldsymbol{P < 0.01}$**,** $P < 0.02$). (C) Cells suppressed in a dose-dependent manner after treatment with indicated concentrations of 7. Green indicates calcein-positive cells (living), and red indicates PI-positive cells (dead). Data are presented as mean ± SD. (D) Cellular ROS levels measured by flow cytometry after RKO cells were treated with 7.

Conclusions

In summary, further investigation of *Sinularia pendunculata* has enriched research on cembrane-type diterpenoids with anti-colorectal cancer activity. One new compound, sinupendunculide A (1), and eight known compounds (2–9) were isolated and fully characterized. The stereochemistry of the new compound was determined by X-ray diffraction analysis. The discovery of compounds 1–9 expands the diversity and complexity of marine diterpenes. For the SAR analysis, in addition to the lactone structure being essential for anti-CRC activity, substitution at C-7 and C-8 represents another fragment worth investigating. Although the detailed antitumor mechanism of compound 7 remains to be fully elucidated, its anti-colorectal cancer bioactivity was confirmed through a series of in vitro cell experiments, suggesting that exploration of coral-derived active ingredients may provide new ideas and directions for cancer treatment. Marine organisms are an important component of ecosystems with rich species diversity and bioactive constituents, and continuous efforts should be made to explore physiologically active substances from marine natural products.

Experimental Section

General Experimental Procedures

Optical rotations were measured on a Perkin-Elmer 241-MC polarimeter (PerkinElmer, Fremont, CA, USA). IR spectra were recorded on a Nicolet 6700 spectrometer (Thermo Scientific, Waltham, MA, USA). NMR spectra were measured on Bruker DRX-500 and Bruker DRX-600 MHz NMR spectrometers (Bruker Biospin AG, Fällanden, Germany) using residual CDCl3 (δH 7.26 ppm, δC 77.16 ppm) as an internal standard. Chemical shifts are expressed in δ (ppm) and coupling constants (J) in Hz. Structural assignments were made with additional information from 1H–1H COSY, HSQC, NOESY, and HMBC experiments. HR-EIMS data were recorded on a Finnigan-MAT-95 mass spectrometer (Finnigan-MAT, San Jose, CA, USA). Commercial silica gel (200–300 and 300–400 mesh; Qingdao, China) and Sephadex LH-20 gel (Amersham Biosciences) were used for column chromatography (CC). Precoated silica gel plates (Yantai Zi Fu Chemical Group Co., Yantai, China, G60 F-254) were used for analytical TLC. Semi-preparative HPLC was performed on an Agilent-1260 system equipped with a DAD G1315D detector at 210 and 254 nm using an ODS-HG-5 column (250 mm × 9.4 mm, 5 m) with a CH3CN–H2O system at 3 mL/min. All solvents used for CC and HPLC were of analytical grade (Shanghai Chemical Reagents Co., Ltd.) and chromatographic grade (Dikma Technologies Inc.), respectively.

Biological Material

The soft coral *S. pendunculuiata* was identified by Prof. Xiu-Bao Li from Hainan University and collected off the coast of Ximao Island, Hainan Province, China, in 2019 at a depth of –15 m. A voucher specimen (No. 19XD-1) is deposited at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Extraction and Isolation

The frozen animals (1198 g, dry weight) were cut into pieces and exhaustively extracted with dichloromethane/methanol (1:1). The organic extract was partitioned between H2O and Et2O. The Et2O-soluble portion was evaporated to yield a brown residue, which was separated into 10 fractions (1–10) by gradient silica gel column chromatography (200–300 mesh, 0% to 100% Et2O in light petroleum ether). Fraction 6 (2311.2 mg) was subjected to Sephadex LH-20 (PE/CH2Cl2/MeOH, 2:1:1) to obtain four subfractions (6A–6D). Subfraction 6B (446.9 mg) was further purified by silica gel CC eluted with PE–Et2O (15:1 to 1:2) to give five subfractions (6B1–6B5), yielding compound 6 (86.5 mg). Subfraction 6B3 was purified by silica gel CC (300–400 mesh) eluting with PE-Et2O (10:1) to afford compound 4 (19.1 mg). Subfraction 6C (1581.7 mg) was separated by silica gel CC (300–400 mesh) to give five subfractions (6C1–6C5), yielding compound 5 (344.0 mg). Compound 8 (54.3 mg) was obtained from fraction 6C1 by silica gel CC (300–400 mesh). Compound 2 (7.4 mg; tR = 4.3

min) was obtained from fraction 6C2 by RP-HPLC [CH3CN/H2O (8.5:1.5), 3.0 mL/min]. Subfraction 6C3 was further purified by RP-HPLC [CH3CN/H2O (7.5:2.5), 3.0 mL/min], yielding compounds 1 (5.2 mg, tR = 4.4 min) and 3 (2.6 mg, tR = 7.3 min). Subfraction 6D (401.6 mg) was subjected to silica gel CC eluted with PE-Et2O (15:1 to 1:4) to give five subfractions (6D1–6D5), yielding compound 9 (130.2 mg). Subfraction 6D3 afforded compound 7 (7.6 mg; tR = 10.4 min) by RP-HPLC [CH3CN/H2O (8:2), 3.0 mL/min].

Sinupendunculide A (1)

Colorless oil; [α]20D +5.3 (c 0.6, MeOH); IR (KBr) max/cm−1: 3445, 2924, 1717, 1385, 1232, 1127, 1104, 1064; for 1H NMR (CDCl3, 600 MHz) and 13C NMR (CDCl3, 125 MHz) data, see Table 1; HR-EIMS m/z 348.1931 [M]+ (calcd for C20H28O5, 348.1931).

X-ray Crystal Structure Analysis of Sinupendunculide A (1)

Sinupendunculide A (1) was crystallized from CH2Cl2/MeOH (1:1) at room temperature. C20H28O5, Mr = 348.42, monoclinic, crystal size 0.12 × 0.08 × 0.05 mm3, space group P21, a = 14.4674(3) Å, b = 10.7543(2) Å, c = 17.8659(4) Å, V = 2769.36(10) Å3, Z = 6, calcd = 1.253 g/cm3, F(000) = 1128.0, 21,978 collected reflections, 10,721 independent reflections (Rint = 0.0363, Rsigma = 0.0449), final R1 = 0.0346 (wR2 = 0.0766) for reflections with $I \geq 2\sigma(I)$, R1 = 0.0388, wR2 = 0.0799 for all unique data. X-ray measurements were performed on a Bruker D8 Venture X-ray diffractometer with Cu Kα radiation (λ = 1.54178 Å) at 170.0 K. Data integration and reduction were processed with SAINT V8.37A software, and multiscan absorption corrections were performed using the SADABS program. The structure was solved with the ShelXT [**?**] structure solution program using intrinsic phasing and refined with the ShelXL [**?**] refinement package using least squares minimization. Crystallographic data for 1 were deposited at the Cambridge Crystallographic Data Centre (deposition number CCDC 2234283). Copies of these data can be obtained free of charge via www.ccdc.cam.ac.uk/data_{request}/cif or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

Bioassay Procedures

Cell Culture RKO cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in MEM (Meilunbio, Dalian, China) supplemented with 10% fetal bovine serum (Biological Industries, Cromwell, CT, USA) and streptomycin-penicillin (Meilunbio, Dalian, China) and incubated in a humidified atmosphere with 5% CO2 at 37 °C.

Cell Viability Assay RKO cells (5,000–6,000 cells/well) were seeded in 96-well plates. After overnight incubation, cells were treated with different concentrations of compound 7 for 24 h. The CCK-8 kit (Meilunbio, Dalian, China) was used to evaluate the effect of compound 7 on cell viability according to the manufacturer' s instructions, and optical density was measured by Cytation 5 at 450 nm. Half-maximal inhibitory concentration values and cell viability were calculated using the formula: Cell viability (%) = [(OD value of experimental group -OD value of blank group)/(OD value of control group -OD value of blank group)] × 100%.

Cell Colony Formation Assay RKO cells were seeded in 12-well plates at 1 × 103 cells per well and treated with compound 7 (4, 8, and 16 M) for 7 days. Colonies were then fixed with 4% paraformaldehyde for 15 min and observed and counted with crystal violet. Images were captured and cell colony numbers were counted.

Apoptosis and Cell Cycle Analysis An Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) was used to detect cell apoptosis. RKO cells were seeded in 6-well plates at 4 × 105 cells per well, cultured overnight, and treated with different concentrations of compound 7 (4, 8, and 16 M) for 24 h. Floating and adherent cells were collected and washed twice with cold PBS. According to the manufacturer' s instructions, 100 L of 1× binding buffer containing 5 L Annexin V-FITC dye and 5 L PI dye was added to each sample and incubated with cells in the dark at room temperature for 30 min. Finally, 300 L of 1× binding buffer was added to each tube for flow cytometric analysis. For cell cycle analysis, collected cells were fixed in 70% ethanol and stored at 4 °C overnight. The Cell Cycle and Apoptosis Analysis Kit (Beyotime, China) was used according to the manufacturer' s instructions.

ROS Level Measurement RKO cells were seeded in 6-well plates at 4 × 105 cells per well and cultured for 24 h. Cells were collected and washed twice with PBS. A reactive oxygen species assay kit (Beyotime) was used to detect ROS levels. According to the manufacturer' s instructions, cells were stained with 10 M DCFH-DA at 37 °C for 30 min. Each sample was then washed gently with PBS and treated with different concentrations of compound 7 (4, 8, 16 M) for 1.5 h. ROS levels were finally detected by flow cytometry.

EdU-488 Staining Cells were seeded in 12-well plates at 2 × 105 cells per well. After overnight incubation, cells were treated with compound 7 for 22 h. A 10 M EdU working solution diluted in cell culture medium was preheated and added to each well for 2 h. The medium was then removed, and cells were fixed with 4% paraformaldehyde for 15 min. After removal of paraformaldehyde, cells were washed with 1 mL BSA solution three times per well. Next, 0.3% Triton X-100 was added to each well and incubated at room temperature for 15 min. Click reaction solution was prepared and incubated for 30 min in the dark according to the manufacturer' s instructions. Fluorescence detection was finally performed at 488 nm using a high-content cell imaging analysis system.

Calcein AM/PI Staining Cells were seeded in 96-well plates at 6,000 cells per well (100 L/well) and cultured overnight in a 5% CO2 incubator at 37 °C. Cells were treated with different concentrations of compound 7 for 24 h. According to the instructions, 2 L of PI solution and 2 L of calcein-AM solution were added to 2 mL of working solution. Then, 100 L of dye solution was added to each well and incubated continuously at 37 °C for 30 min. Cells were finally observed and photographed using Cytation 5.

Supplementary Material

The HR-EIMS, IR, ORD, 1D, and 2D NMR spectra for sinupendunculide A (1) are available in the Supporting Information.

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Author Contribution Statement

Hong-Ru Wang and Jian-Hua Xia performed cell biology experiments. Yang Jin, Ruo-Nan Sun, and Li-Gong Yao conducted extraction, isolation, and structure elucidation experiments. Hong-Ru Wang and Yang Jin prepared the manuscript. Xu-Wen Li, Wei-Dong Zhang, and San-Hong Liu conceived and designed the experiments. All authors have read and approved the published version of the manuscript.

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Note: Figure translations are in progress. See original paper for figures.