Lissoclibadin 1, a Polysulfur Aromatic Alkaloid from the Indonesian Ascidian Lissoclinum cf. badium, Induces Caspase-Dependent Apoptosis in Human Colon Cancer Cells and Suppresses Tumor Growth in Nude

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Lissoclibadin 1, a Polysulfur Aromatic Alkaloid from the Indonesian Ascidian *Lissoclinum* cf. *badium*, Induces Caspase-Dependent Apoptosis in Human Colon Cancer Cells and Suppresses Tumor Growth in Nude Mice

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Supporting Information

ABSTRACT: Lissoclibadins, polysulfur aromatic alkaloids, were isolated from the Indonesian ascidian *Lissoclinum* cf. *badium*. Lissoclibadins 1 (**1**), 3 (**2**), 4 (**3**), 7 (**4**), 8 (**5**), and 14 (**6**) inhibited the growth of four human solid cancer cell lines: HCT-15 (colon adenocarcinoma), HeLa-S3 (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma), and NCI-H28 (mesothelioma). Lissoclibadin 1 (**1**) exerted the most potent cytotoxic effects *in vitro* and mainly promoted apoptosis through an intrinsic pathway with the activation of a caspase-dependent pathway in HCT-15 cells. *In vivo* studies demonstrated that **1** suppressed tumor growth in nude mice carrying HCT-15 cells without significant secondary adverse effects. In conclusion, the results obtained in the present study demonstrate that **1** has potential as a chemotherapeutic candidate for preclinical investigations.

Marine natural products possess unique structural features and various biological activities.[1−3] A number of marine-derived compounds have exhibited potent inhibitory activities against human tumor cells in *in vitro* and *in vivo* experiments. Some marine substances have entered clinical trials for the treatment of cancers, and cytarabine, trabectedin (ET743), eribulin (a synthetic derivative of halichondrin B), and monomethylauristatin E (MMAE) as the warhead of a monoclonal antibody (Adcetris) have been approved as anticancer agents.[4−7]

In the course of our studies on bioactive metabolites from marine organisms, we have reported the isolation and structures of three novel sulfur-containing alkaloids, lissoclibadins 1−3, together with four known congeners from the Indonesian ascidian *Lissoclinum* cf. *badium*, which have rare polysulfur aromatic amine structures.[8] Further studies on this ascidian led to the discovery of 11 related compounds, lissoclibadins 4−14.[9] We previously reported their antimicrobial activities, inhibition of cell proliferation in murine and human cancer cell lines, and effects on IL-8 production in PMA-stimulated human promyelocytic leukemia (HL-60) cells.[10]

We have now further evaluated the antitumor properties of lissoclibadins *in vitro* and *in vivo*, and the findings obtained indicated that lissoclibadin 1 induced caspase-dependent apoptosis in the human colon cancer cell line HCT-15 and exerted suppressive effects on HCT-15 cells in antitumor experiments using xenograft model nude mice.

RESULTS AND DISCUSSION

Lissoclibadins 1−3 were obtained as cytotoxic components against HL-60 cells[8] and exhibited inhibitory activities against colony formation by Chinese hamster V79 cells and cell proliferation in nine human solid-tumor cell lines.[10a] The effects of lissoclibadins 4−14 have also been investigated against V79 and murine leukemia L1210 cells.[9]

Further cytotoxicity studies were performed using lissoclibadins 1 (**1**), 3 (**2**), 4 (**3**), 7 (**4**), 8 (**5**), and 14 (**6**). The cytotoxicities of compounds **1**−**6** were initially investigated in four human cancer cell lines: HCT-15 (colon adenocarcinoma), HeLa-S3 (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma), and NCI-H28 (mesothelioma). These cancer cells were treated with each test compound at various concentrations for 24 h, and cell viabilities were measured using the WST-1 assay.[11] The effects of compounds **1**−**6** on the viabilities of four cancer cell lines are summarized in Table 1. Among these lissoclibadins, compounds **1**, **5**, and **6** exhibited stronger

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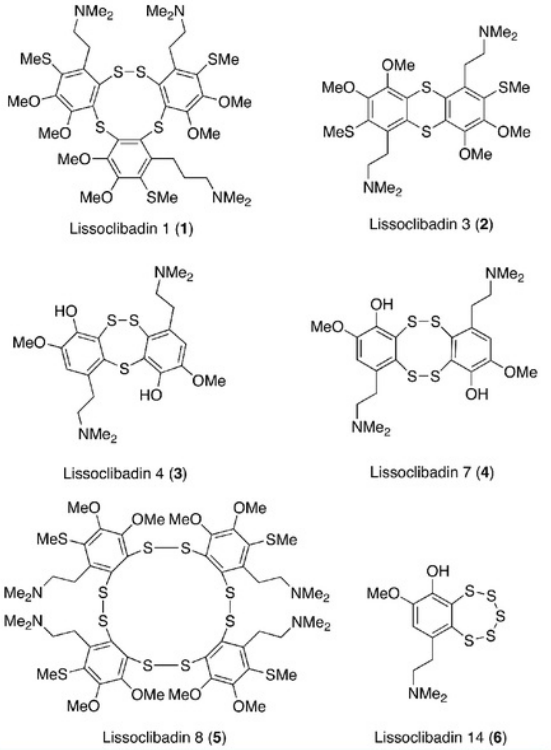

Table 1. Cytotoxicities of Compounds 1–6 against Four Human Cancer Cell Lines: HCT-15, HeLa-S3, MCF-7, and NCI-H28[a]

	cytotoxicity (IC_{50}, μM)			
compound	HCT-15	HeLa-S3	MCF-7	NCI-H28
lissoclibadin 1 (1)	4.0 ± 1.8	6.3 ± 1.4	7.6 ± 4.0	7.1 ± 2.3
lissoclibadin 3 (2)	13.2 ± 1.4	16.0 ± 1.8	n.d.[b]	n.d.
lissoclibadin 4 (3)	17.2 ± 5.2	17.8 ± 5.3	n.d.	n.d.
lissoclibadin 7 (4)	15.7 ± 1.2	14.2 ± 1.3	n.d.	n.d.
lissoclibadin 8 (5)	4.9 ± 1.9	6.3 ± 0.5	11.8 ± 3.1	7.1 ± 1.0
lissoclibadin 14 (6)	4.2 ± 2.4	5.9 ± 1.6	6.4 ± 2.7	6.4 ± 1.6
etoposide	19.7 ± 5.7	43.1 ± 6.5	n.d.	n.d.

[a]Each value indicates the mean ± SD of three different experiments performed in triplicate. [b]Not determined.

cytotoxicity against all cancer cell lines and were more potent than etoposide, an anticancer drug. Moreover, compounds **1**, **5**, and **6** showed the lowest IC_{50} values of 4.0, 4.9, and 4.2 μM, respectively, against HCT-15 cells (Table 1). On the other hand, compounds **2**–**4** exhibited weaker activities than **1**, **5**, and **6** (Table 1).

Among the six lissoclibadins tested, lissoclibadins 1 (**1**) and 14 (**6**) were roughly equipotent, but compound **1** was selected for further studies. Thus, morphological changes in cancer cells treated with **1** (0–10 μM) were examined using light microscopy. Lissoclibadin 1 (**1**) influenced cell adhesion and morphology in a dose-dependent manner in the four cell lines (Figure S1). These results suggest that compound **1** possesses potent cytotoxic effects.

Some anticancer agents are known to eliminate cancer cells through apoptotic processes.[12] Apoptosis is essential for tissue development and homeostasis and also functions as a defense system to avoid the effects of many toxic substances.[12] Therefore, nuclear morphological observations by fluorescent staining and a flow cytometric analysis by double staining were performed in order to investigate whether the cytotoxicity of lissoclibadin 1 (**1**) is triggered by an apoptotic pathway. HCT-15 cells treated with 5 μM **1** for 24 h were stained with Hoechst-33258, and nuclear morphological effects were observed using a fluorescence microscope. Nuclear morphological observations of 5 μM **1** revealed the condensation of chromatin and fragmentation of nuclei in a few cells, as shown in Figure 1. Moreover, similar nuclear morphological changes were detected in HeLa-S3, MCF-7, and NCI-H28 cells treated with 10 μM **1** for 24 h (Figure 1).

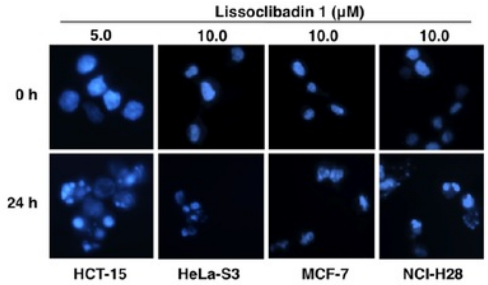

Figure 1. Nuclear morphological changes induced by lissoclibadin 1 (**1**) in HCT-15, HeLa-S3, MCF-7, and NCI-H28 cells. Each cell line (5×10^4 cells/mL) was precultured for 24 h, and compound **1** was then added at the indicated concentrations and cultured for 24 h. Cells were stained with Hoechst-33258 and observed using a fluorescent microscope.

The occurrence of apoptosis was analyzed using flow cytometry. HCT-15 cells were treated with 5 μM **1** for 3, 6, 12, and 24 h and then stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (annexin V) and propidium iodide (PI) in order to confirm the apoptotic actions induced by compound **1**. Annexin V staining (lower right) signified early apoptotic events, while dual annexin V and PI staining (upper right) indicated the late phase of apoptosis (Figure 2). On the other hand, PI staining (upper left) showed necrosis, and nonstained cells (lower left) were viable cells (Figure 2). As shown in Figure 2, the proportion of early apoptotic cells (lower right) significantly increased to 11%, 37%, 43%, and 40% after the incubation with **1** at 5 μM for 3, 6, 12, and 24 h, respectively. The late apoptotic cell proportion (upper right) was also increased after the 24 h incubation with 5 μM **1** in a time-dependent manner (Figure 2). These results indicate that the cytotoxic effects of lissoclibadin 1 are attributable to the induction of apoptosis.

The apoptotic response in HCT-15 cells to **1** was significantly decreased by the addition of the pan-caspase inhibitor z-VAD-fmk (Figure 3). The percentage of apoptotic cells (upper right and lower right) decreased from 64% to 11%. Therefore, compound **1** induced apoptotic death in HCT-15 cells through a caspase-dependent pathway.

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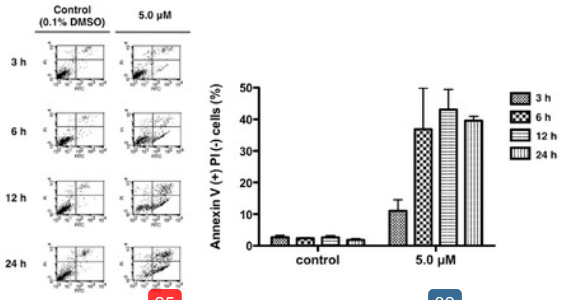

Figure 2. Analysis of apoptosis stages in HCT-15 cells assessed by flow cytometry with double staining (annexin V and PI). HCT-15 cells (5 × 10^4 cells/mL) were precultured for 24 h in 1 mL, lissoclibadin 1 in DMSO (5.0 μM) or DMSO alone (final concentration 0.1%) was then added, and the cells were incubated for the indicated time. Analyses of annexin V binding and PI incorporation were performed by FACSCalibur. The percentages of annexin V-positive cells are presented in the right panel. Each value is the mean ± SD of three independent experiments.

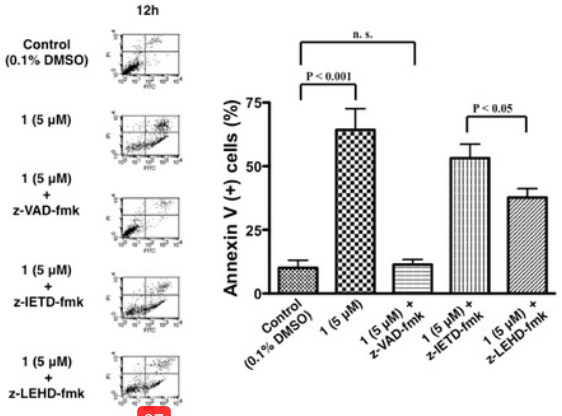

Figure 3. Effects of pan-caspase, caspase-8, and caspase-9 inhibitors on apoptosis induced by lissoclibadin 1 (**1**). HCT-15 cells (5 × 10^4 cells/mL) were precultured for 24 h in 1 mL. Each caspase inhibitor was added at 50 μM, and the cells were incubated for 30 min. Lissoclibadin 1 in DMSO (5.0 μM) or DMSO alone (final concentration 0.1%) was then added, and the cells were incubated for the indicated time. Analyses of annexin V binding and PI incorporation were performed by FACSCalibur. The percentages of annexin V-positive cells are presented in the right panel. Each value is the mean ± SD of three independent experiments.

The caspase cascade is transmitted through two kinds of pathways: intrinsic and extrinsic pathways.[13] Therefore, the pathway activated by the treatment with **1** was investigated in the annexin V−PI dual-staining analysis using specific caspase-8 (z-IETD-fmk) and caspase-9 (z-LEHD-fmk) inhibitors. As shown Figure 3, the prevention of annexin V-positive staining by z-LEHD-fmk was more potent than that by z-IETD-fmk, which indicated that apoptosis induced by **1** was caused by the intrinsic pathway rather than the extrinsic pathway. z-LEHD-fmk could not completely prevent the apoptosis in contrast to z-VAD-fmk, indicating that **1** triggers the apoptotic signal cascade so strongly that inhibition of caspase-9 alone is not sufficient for complete prevention of **1**-induced apoptosis. The treatment with caspase inhibitors did not suppress the effects of **1** on cell adhesion and morphology (Figure S2). It may be demonstrated that the treatment with **1** causes an irreversible lethal stress in cancer cells upstream of caspase cascade activation or prevention of **1**-induced caspase activation triggers induction of other cell death signaling. Taken together, we have concluded that compound **1** induces potent cytotoxicity attributed to strong activation of caspase cascade originating in caspase-9 activation.

BALB/c nu/nu nude mice were used as a xenograft model to evaluate the *in vivo* antitumor effects of lissoclibadin 1 (**1**) on the HCT-15 cell line. Tumor growth on day 28 in the group treated with **1** at 25 mg/kg per day (n = 6) was approximately 60% less than that in the control group, whereas the administration group at 5 mg/kg per day (n = 6) showed no effect (Figure 4A). Neither obvious toxicities nor body weight changes were observed during the experimental period (Figure 4B). These results show that compound **1** suppressed tumor growth in nude mice.

The present study revealed that lissoclibadin 1 (**1**) induced cell death via apoptosis due to the mitochondrial cytochrome *c*-dependent activation (intrinsic pathway) of the caspase-9 and caspase-3 cascade pathway. Moreover, compound **1** exhibited antineoplastic activity in HCT-15 cells *in vivo* at 25 mg/kg per day with relative safety. This is the first study on the *in vivo* antitumor efficacy of **1**, and, accordingly, lissoclibadin 1 has potential as a lead compound for further development as a cancer chemotherapy agent.

EXPERIMENTAL SECTION

General Experimental Procedures. Lissoclibadins 1 (**1**), 3 (**2**), 4 (**3**), 7 (**4**), 8 (**5**), and 14 (**6**) were isolated from the ascidian *L.* cf. *badium* collected in Indonesia as described previously.[8,9] A cell counting kit (WST assay) was purchased from Dojindo Molecular Technologies, Inc. Caspase inhibitors (z-VAD-fmk, z-IETD-fmk, and z-LEHD-fmk) were purchased from Medical & Biological Laboratories Co., Ltd. (MBL).

Cell Cultures. HCT-15 (colon adenocarcinoma), HeLa-S3 (cervix adenocarcinoma), and MCF-7 (breast adenocarcinoma) cell lines were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. The NCI-H28 cell line (malignant mesothelioma) was purchased from the American Type Culture Collection. Cells were routinely kept in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd.) supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a 95% air and 5% CO_2 atmosphere.

WST Assay. WST-1 assays were performed in accordance with the manufacturer's instructions. Briefly, cells were plated at 5 × 10^3 cells/well on 96-well plates and grown for 24 h. Various concentrations of lissoclibadins (0.1−40 μM) and etoposide (1.0−100 μM) were added in triplicate to the cultures and incubated for 24 h before adding the WST-1 solution. The absorbance of the resulting product was measured 4 h later at a wavelength of 450 nm with background subtraction at 650 nm. The cytostatic/cytotoxic effects of compounds were expressed as relative cell viability (% of control). The experiments were conducted three times, and the IC_{50} value showing the compound concentration required for the 50% inhibition of cell viability was calculated employing GraphPad Prism 3.0 software.

Observation of Nuclear Morphology. Cells (5 × 10^4 cells/mL) were cultured in 5 mL on six-well plates and grown for 24 h. After treatment, cells were trypsinized, collected by centrifugation, and washed with PBS. Cells were then fixed with 100 μL of 1.0% paraformaldehyde at 4 °C for 15 min and stained with 50 μL of 1 mg/mL Hoechst-33258 at 4 °C for 15 min. After three washes with PBS, cells were mounted on glass slides using Prolong gold antifade reagents (Molecular Probes). Fluorescence was visualized with a Zeiss Axioscope 2 fluorescence microscope (Carl Zeiss).

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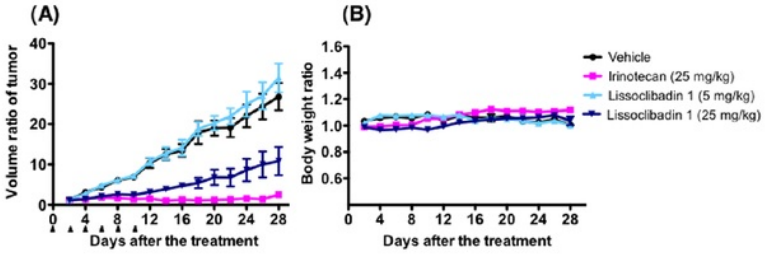

Figure 4. Antitumor effects of lissoclibadin 1 (**1**) in a tumor xenograft model bearing HCT-15 cells. HCT-15 cells (5×10^6 cells) were implanted into each nude mouse. When the tumor size reached 50−80 mm^3, mice were injected intratumorally with 5 mg/kg of **1**, 25 mg/kg of **1**, and 25 mg/kg of irinotecan (as a positive control) six times every second day from day 0 (indicated by arrowhead). Tumor sizes for calculating the volume (A) and body weight (B) were measured every second day. The body weight was monitored for toxicity.

Annexin V/PI Staining. Annexin V binding and PI incorporation were detected with a MEBCYTO apoptosis kit (MBL) according to the manufacturer's directions. Briefly, cells (5×10^4 cells/mL) were cultured in 1 mL on 24-well plates. After the treatment with 5 μM lissoclibadin 1 (**1**), adherent cells were trypsinized, pooled, and stained with annexin V-FITC and PI. The fluorescence intensities of FITC-annexin V and PI were measured using a FACSCalibur flow cytometer (Becton Dickinson).

The role of caspase activation in this process was examined by the addition of the pan-caspase inhibitor z-VAD-fmk, caspase-8-specific inhibitor z-IETD-fmk, and caspase-9-specific inhibitor z-LEHD-fmk. Each caspase inhibitor (50 μM) was added to cultured cells 30 min before the addition of **1** (5 μM). The analysis of annexin V binding and PI incorporation was then completed, as described above. The results from three independent experiments were expressed as the mean ± SD. Statistical analyses were conducted using GraphPad Prism 3.0, and comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* tests.

***In Vivo* Antitumor Experiment.** All animal experiments were performed in accordance with the Guidelines for Animal Experiments of the Tohoku Medical and Pharmaceutical University (permission number 08029). HCT-15 cells (1×10^8/mL) were mixed with an equal volume of ice-cooled Matrigel (Becton Dickinson Labware), and an aliquot of the cell suspension (100 μL) was injected subcutaneously into the lower backs of 6-week-old female athymic nude mice (Balb/c nu/nu, Charles River Lab. Inc.). One week after the inoculation (day 0), mice bearing tumor volumes from 50 to 100 mm^3 were randomly divided into 4 groups with 6 mice in each group. Lissoclibadin 1 (**1**) and irinotecan, used as a reference antitumor drug, were dissolved in sterile PBS containing 5% DMSO and 5% glucose (solvent). Groups 1, 2, and 3 were injected intratumorally with 5 and 25 mg/kg of **1** and 25 mg/kg of irinotecan, respectively, on days 2, 4, 6, 8, and 10. Group 4 was administered vehicle (solvent only). Body weights and tumor sizes were measured every other day from day 2 until 28. Solid tumor volumes were calculated as follows: length (mm) × width (mm) × depth (mm) × π/6. Tumor growth and body weight changes were evaluated by the ratio of each value against the initial values on day 0.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b01051.

Experimental data for antitumor effects of **1** and ^{1}H NMR spectra for **1**−**6** (PDF)

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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