

## Isolation of Sphingoid Bases of Sea Cucumber Cerebrosides and Their Cytotoxicity against Human Colon Cancer Cells

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**Sea cucumber is a health-beneficial food, and contains a variety of physiologically active substances including glycosphingolipids. We show here the sphingoid base composition of cerebrosides prepared from sea cucumber and the cytotoxicity against human colon cancer cell lines. The composition of sphingoid bases prepared from sea cucumber was different from that of mammals, and the major constituents estimated from mass spectra had a branched C17–19 alkyl chain with 1–3 double bonds. The viability of DLD-1, WiDr and Caco-2 cells treated with sea cucumber sphingoid bases was reduced in a dose-dependent manner and was similar to that of cells treated with sphingosine. The sphingoid bases induced such a morphological change as condensed chromatin fragments and increased the caspase-3 activity, indicating that the sphingoid bases reduced the cell viability by causing apoptosis in these cells. Sphingolipids of sea cucumber might therefore serve as bioactive dietary components to suppress colon cancer.**

**Key words:** apoptosis; cerebroside; sea cucumber; sphingolipid; sphingoid base

Sphingolipids are a family of compounds that have a sphingoid base (long-chain base) with an amide-linked fatty acid and a polar head group such as phosphorylcholine (for sphingomyelin) or carbohydrates (for cerebrosides, gangliosides and other complex glycolipids). Sphingolipids and their metabolites, including ceramide, sphingosine and sphingosine-1-phosphate, have been recognized as being intracellular mediators of cell differentiation and apoptosis.<sup>1–3)</sup> Recent works have indicated that the intake of sphingolipids incorporating sphingomyelin from milk and cerebroside (glucosylceramide) from milk, rice, maize and yeast significantly reduced the incidence of colonic aberrant crypt foci in mice treated with tumorigenic reagents.<sup>4–8)</sup> The generation of sphingolipid metabolites has been linked to the prevention of colon tumorigenesis. A

possible mechanism for this suppression by dietary sphingolipids is their hydrolysis to bioactive ceramide and sphingoid bases, because these hydrolysates have induced apoptosis in human colon cancer cells.<sup>9–11)</sup>

The physiologically active substances including glycosylceramides and related compounds have been extracted from a variety of sea cucumber species irrespective of order. Glycosphingolipids in sea cucumbers have recently been systematically analyzed, and the neurotogenic activity of the lipids proved *in vitro*, which may lead to the development of therapeutic products for neurological disorders.<sup>12,13)</sup> The sugar moiety of the gangliosides found in several types of sea cucumber differed from those in mammals in that a sialic acid of the ganglioside was bound directly to the glucose component of cerebroside. Another interesting feature is that two or three sialic acids are tandemly connected to each other and, in some cases, are sandwiched between sugar chains.<sup>12)</sup> The sialic acid moiety appeared to play an important role in the development of activity. Although these works focused on the structures of the glycosyl chains, the sphingoid base structures of sphingolipids are complicated in nature (Fig. 1). However, the structures of the sphingoid bases of sphingolipids in sea cucumbers are still not well understood.

The aims of the present study were to analyze the structures of the sphingoid bases of cerebroside in a sea cucumber and to assess the apoptotic effect of the sphingoid bases on human colon cancer cells.

### Materials and Methods

*Preparation of sphingoid bases from sea cucumber powder.*<sup>14–16)</sup> Total lipids were extracted with chloroform/methanol (2:1, v/v) from sea cucumber powder (*Stichopus variegates*) kindly supplied by H.E.C. Group International (Osaka, Japan) and were saponified with 0.4N KOH in methanol at 37 °C for 2 h. Cerebrosides were prepared by TLC (silica gel 60 F254, 20 × 20 cm,

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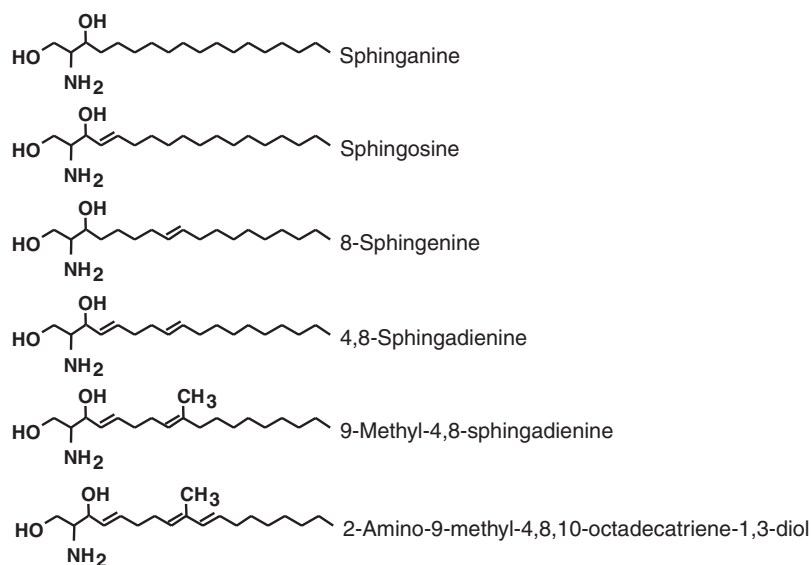


Fig. 1. Structures of Naturally Occurring Sphingoid Bases.

Merck, Darmstadt, Germany) developed in chloroform/methanol/water (65:16:2, by vol.).<sup>14</sup> Under UV light irradiation, the visualized lipid band corresponding to cerebroside was collected and extracted from the silica according to the method of Bligh and Dyer.<sup>17</sup> To isolate the sphingoid bases, the prepared cerebroside was subjected to strong alkaline hydrolysis (10% aqueous Ba(OH)<sub>2</sub>/dioxane, 1:1, 24 h at 110 °C).<sup>18</sup> The liberated sphingoid bases were then extracted with diethyl ether and purified by silica TLC developed in chloroform/methanol/ammonia (40/10/1, by vol.). The composition of the sphingoid bases was determined by oxidation with sodium periodate and subsequent GC-MS of the resulting fatty aldehydes.<sup>19</sup> The GC-MS analysis was performed with a QP5050A instrument (Shimadzu, Kyoto, Japan) equipped with a CP-Sil 88 column (0.25 mm i.d. × 50 m, 0.2 μm; Chrompak, Middelburg, The Netherlands). The column temperature was programmed from 80 °C to 160 °C at 10 °C/min and then to 200 °C at 2 °C/min. The injector and detector were held at 250 °C. The mass spectra were identified by referring to the data reported by Karlsson *et al.*<sup>20</sup> The sphingoid bases were also analyzed by GC-MS in a DB-1 capillary column at 220 °C after converting to *N*-acetylated-*O*-trimethylsilylated derivatives.<sup>21</sup> Some isomers of the sphingoid bases were isolated by HPLC as described previously.<sup>15,16</sup>

**Cell culture.** Caco-2 cells (Riken Gene Bank, Tsukuba, Japan) were cultured in DMEM containing 10% FBS and supplemented with antibiotics (100 unit/ml of penicillin and 100 μg/ml of streptomycin) and 1% non-essential amino acids. DLD-1 cells and WiDr cells (Human Science Research Resources Bank, Osaka, Japan) were respectively cultured in an RPMI 1460 medium and DMEM supplemented with 10% FBS and

antibiotics. The cells were kept at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Cell proliferation assays.**<sup>10</sup> The cells were seeded in 96-well plates (100 μl/well at a density of 1 × 10<sup>5</sup> cells/ml). After incubating for 24 h, the medium was removed and the cells were cultured in a medium containing different doses of sphingoid bases prepared from the sea cucumber and commercial sphingosine (Sigma, St. Louis, MO, USA). The sphingoid bases were dissolved in ethanol and diluted into the culture medium (the final ethanol concentration was 0.1%). Control experiments were done with 0.1% ethanol as the vehicle. The cell viability was determined by assaying the residual mitochondrial activity of the treated cells with the highly sensitive the WST-1 assay.<sup>22</sup> Briefly, 10 μl of the WST-1 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to each well. The cells were incubated for an additional 1 h, and the soluble formazan dye produced by the metabolically active cells was detected at 450 nm in a 96-well spectrophotometric plate reader.

**Microscopic assay.** The apoptotic cells were evaluated by their morphological changes, the condensed chromatin fragments being visualized under a fluorescence microscope after being stained with 4',6-diamidino-2-phenylindole (DAPI). DLD-1 cells were seeded at 5 × 10<sup>4</sup> cells/well in 8-well chamber slides (Nunc, Naperville, IL, USA) and cultured for 24 h, before changing the medium to one containing 20 μM sphingoid bases and culturing for a further 24 h, and then the cells were stained with 0.05% DAPI.<sup>23</sup>

**Caspase-3 activity.** DLD-1 cells were seeded at 2.0 × 10<sup>5</sup> cells/well in 6-well plates and cultured for 24 h, before exposing the cells to 30 μM sphingoid bases



**Fig. 2.** Thin-Layer Chromatogram of Sphingoid Bases Prepared from Sea Cucumber Cerebrosides.

TLC was developed in chloroform/methanol/ammonia (40/10/1, by vol.) and each spot was visualized by the ninhydrin reagent. Sa, sphinganine; Sph, sphingosine; Phy, phytosphingosine; SC, sphingoid bases prepared from sea cucumber.

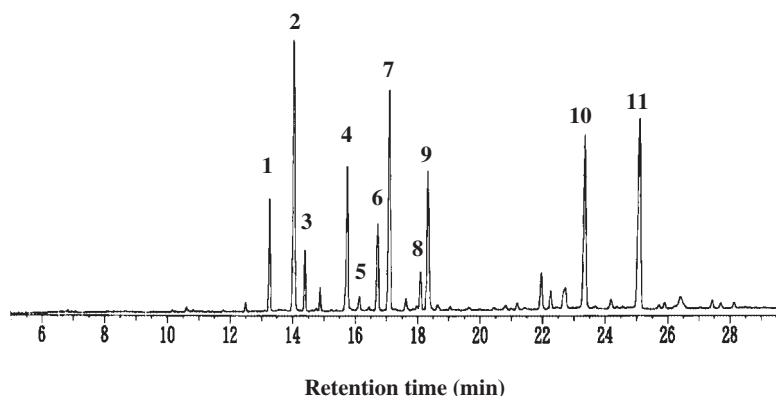
for 24 h. The caspase-3 activity was measured with a colorimetric assay kit (Bio Vision, Mountain View, CA, USA) according to the manufacturer's instructions.

**Statistical analysis.** Each data value is presented as the mean  $\pm$  SD. Statistical analyses were performed by a one-way analysis of variance (ANOVA) with Dunnett's test or Fisher's PLSD to identify the level of significance between groups.

## Results

The sphingoid bases from sea cucumber were separated by TLC (Fig. 2). The TLC profile indicated the absence of trihydroxy bases in the cerebroside preparation. Some of the bases showed the same mass spectrum by GC-MS of the *N*-acetylated-*O*-TMS derivatives, these being considered as possible isomers of each other with normal and branched structures. The sphingoid bases were also analyzed by GC-MS as aldehyde derivatives (Fig. 3). The retention times and mass spectra enabled the C16 to C19 unsaturated aldehydes with normal, *iso* and *anteiso* chains to be identified. The isomers were characterized by the fragment ions, M-15 for *iso* and M-29 for *anteiso*, neither of which has been found in the normal aldehydes.<sup>20)</sup> The results of the GC-MS analysis are summarized in Table 1. The major constituents estimated from the mass spectra had a branched C17-19 alkyl chain with 1-3 double bonds. The content of sphingosine, the most common sphingoid base of mammalian sphingolipids, was less than 5%. This result shows that the composition of the sphingoid bases prepared from sea cucumber was quite different from that of mammals.

Sphingoid bases prepared from the sea cucumber cerebroside reduced the viability of three human colon cancer cell lines, Caco-2, DLD-1, and WiDr cells, in a dose-dependent manner (Fig. 4). It is well known that Caco-2 cells spontaneously differentiate into cells with structural and biochemical properties of small intestinal epithelial cells, although in this study, we used undifferentiated Caco-2 cells as the colon cancer cell model. The reduction of cell viability by the sea cucumber sphingoid bases was similar to that by sphingosine. Apoptotic cells with characteristic nuclear condensation and fragmented nuclei were observed by a fluorescence microscope after being treated with 20  $\mu$ M sea cucumber sphingoid bases for 24 h (Fig. 5). To elucidate the apoptotic mechanism induced by the sphingoid bases, we evaluated its effect on the caspase-3 activity. The results show that caspase-3 was

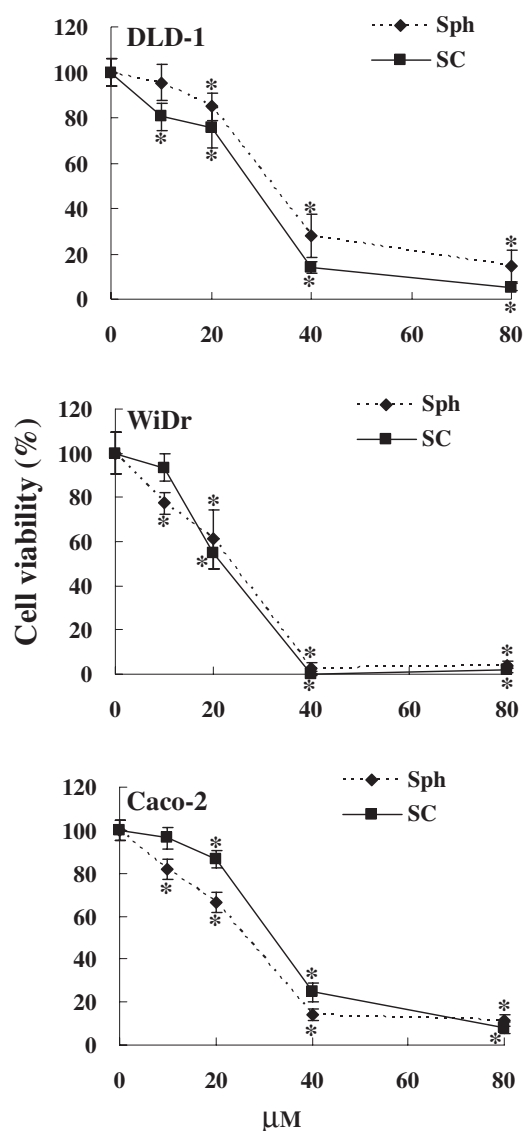


**Fig. 3.** Total Ion Chromatogram of Aldehydes Derived from the Sphingoid Bases in Sea Cucumber Cerebrosides. The sample was analyzed in a CP-Sil 88 column as described in the Materials and Methods section.

**Table 1.** Sphingoid Base Composition of Cerebrosides in Sea Cucumber

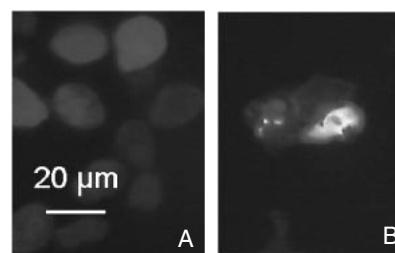
Peak no.	M <sup>+</sup>	Aldehyde	Parent base	%	
1	210	14:1	d16:1	4.24	
2	224	<i>iso</i> 15:1	<i>iso</i> d17:1	14.80	
3	224	<i>anteiso</i> 15:1	<i>anteiso</i> d17:1	2.43	
4	238	<i>iso</i> 16:1	<i>iso</i> d18:1	7.90	
5	238	<i>anteiso</i> 16:1	<i>anteiso</i> d18:1	0.99	
6	238	16:1	d18:1	4.75	sphingosine
7	236	16:2	d18:2	13.81	sphingadienine
8	252	<i>anteiso</i> 17:1	<i>anteiso</i> d19:1	2.05	
9	250	17:2	d19:2	8.13	methyl-sphingadienine
10	234	16:3	d18:3	13.08	
11	248	17:3	d19:3	14.72	
Others				13.10	

The aldehyde and parent bases are designated by the chain length, this being followed by the number of double bonds, and d means dihydroxy base.



**Fig. 4.** Effects of Sphingosine and Sea Cucumber Sphingoid Bases on the Viability of Colon Cancer Cells.

The number of viable cells was estimated by a WST-1 assay and is expressed as a percentage of the control value. Each value is the mean  $\pm$  SD ( $n = 8$ ). \*Significantly different from the control value,  $P < 0.05$ . Sph, sphingosine; SC, sphingoid bases prepared from sea cucumber.



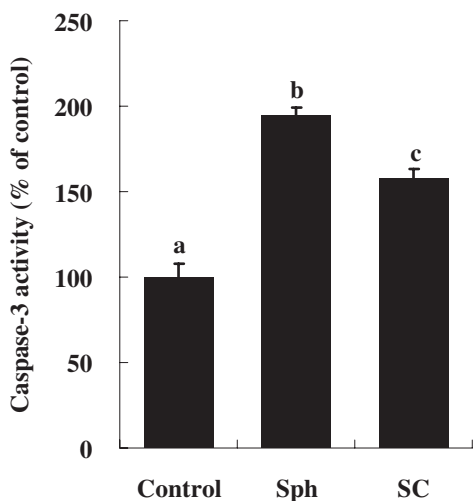
**Fig. 5.** Nuclear Morphology of DLD-1 Cells Added with Sea Cucumber Sphingoid Bases.

The cells were incubated for 24 h in the presence or absence of sea cucumber sphingoid bases ( $20 \mu\text{M}$ ), and then stained with 4',6-diamidino-2-phenylindole. The nuclear morphology was assessed by fluorescence microscopy. A, cells without sphingoids; B, cells cultured with sea cucumber sphingoid bases.

significantly increased after a 24-h incubation of DLD-1 cells with the sphingoid bases (Fig. 6). These results indicate that the sea cucumber sphingoid base backbones not found in mammals also induced apoptosis in human colon cancer cells. The triene bases (peaks 10 and 11 in Fig. 3) have never been identified in mammals. These two isomers of the sphingoid bases purified by HPLC reduced the cell viability of DLD-1 cells in a dose-dependent manner, the reductions being similar to that by sphingosine (Fig. 7).

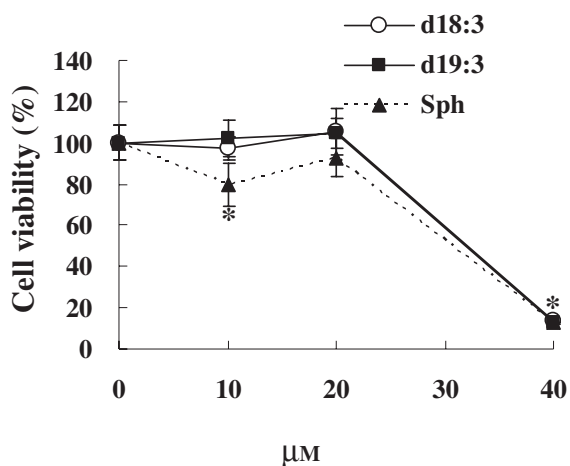
## Discussion

The most common sphingoid base of mammalian sphingolipids is sphingosine (*trans*-4-sphingenine, d18:1), and smaller amounts of other sphingoid bases such as dihydrosphingosine (sphinganine, d18:0) and phytosphingosine (4-hydroxysphinganine, t18:0) are frequently present (Fig. 1);<sup>24)</sup> for example, human plasma cerebrosides contained about 90% sphingosine.<sup>25)</sup> The sphingoid base structures in higher plants and fungi are more complicated than those in mammals, because the sphingoid bases can be desaturated at the C8-position.<sup>26)</sup> 4,8-Sphingadienine and 9-methyl-4,8-sphingadienine represented 70–80% of the total sphin-



**Fig. 6.** Caspase Activity Enhanced by Treating DLD-1 Cells with Sphingoid Bases.

The cells were exposed to 30  $\mu\text{M}$  sphingoid bases for 24 h. Each value is the mean  $\pm$  S.D. of three wells. Values with different letters are significantly different ( $P < 0.05$ ). Sph, sphingosine; SC, sphingoid bases prepared from sea cucumber.



**Fig. 7.** Effect of Sphingoid Bases Having Three Double Bonds from Sea Cucumber Cerebrosides on the Viability of DLD-1 Cells.

Viable cells were counted by a WST-1 assay, and the cell viability is expressed as a percentage of the control value. Each value is the mean  $\pm$  SD ( $n = 8$ ). The bases are designated by the chain length, this being followed by the number of double bonds, and d means dihydroxy base. \*Significantly different from the control value,  $P < 0.05$ .

goid bases in the cerebrosides of maize and yeast (*Saccharomyces kluyveri*), respectively.<sup>7)</sup> We found in the present study that sea cucumber cerebrosides had unique sphingoid bases having three double bonds (Table 1), although the positions of the double bonds were not determined. The triene bases with conjugated diene such as 2-amino-4,8,10-octatriene-1,3-diol (d18:3) and 2-amino-9-methyl-4,8,10-octatriene-1,3-diol (d19:3) have been identified from marine inverte-

brates including ascidians,<sup>27)</sup> starfish<sup>28,29)</sup> and squid.<sup>30)</sup> We speculate that the triene bases in sea cucumber cerebrosides may have the same structures as these bases with a conjugated diene, because both sea cucumber and starfish belong to same phylum, echinoderm.

Ceramide and sphingosine, the hydrolyzed products from sphingolipids, have induced apoptosis in several cancer cells.<sup>1,9)</sup> We have previously confirmed that the sphingoid bases in higher plants and fungi, which are more complicated than those in mammals, also induced apoptosis in colon cancer cells.<sup>10,11)</sup> The present results indicate that sea cucumber sphingoid bases had strong cytotoxicity against several colon cancer cell lines *via* the induction of apoptosis. It has been reported that the apoptotic effect of sphingosine on cancer cells was related to the activation of caspases<sup>31)</sup> and the regulation of the Bcl-2 gene family.<sup>32,33)</sup> Hung *et al.* have indicated that other long-chain bases (including sphinganine, dimethylsphingosine, and stearylamine) induced apoptosis in Hep3B hepatoma cells, but that octylamine (a short-chain analog of sphinganine) did not.<sup>34)</sup> A long-chain structure, as is present in the bases of sea cucumber, may be essential for this apoptotic effect of sphingoid bases on cancer cells. It has been reported that exogenously added sphingolipids exerted low toxicity toward normal cells at the concentration that would trigger apoptosis in cancer cell lines,<sup>35)</sup> but we did not examine the effect of sphingoid bases prepared from sea cucumber on normal cells in the present study.

The fate of dietary marine invertebrate-origin sphingolipids is still not well understood. Orally fed mammalian-origin sphingolipids can be hydrolyzed by intestinal enzymes and taken up by mucosal cells.<sup>36-38)</sup> Our previous findings of hydrolytic activity in the small intestine toward sphingolipids prepared from higher plants are consistent with the findings of such activity toward mammalian-origin sphingolipids.<sup>15)</sup> Thus, it seems that the dietary sphingolipids of sea cucumber could also be hydrolyzed in the intestines. To clarify the biological and food function, the absorption and metabolism of sea cucumber sphingolipids that have specific sphingoid bases with three double bonds deserve future study.

A recent paper has reported the anti-proliferative effect of a branched-chain fatty acid from sea cucumber on prostate cancer cells.<sup>39)</sup> This effect may be attributed to a change in the 5-lipoxygenase activity. Our study shows that sphingoid bases from sea cucumber induced significant apoptosis in several colon cancer cell lines. The sphingolipids of sea cucumber might therefore serve as bioactive dietary components to suppress colon cancer. Sea cucumber used in the present study contained about 200 mg of cerebrosides per 100 g of dry powder. Cerebrosides used as food and cosmetic ingredients have been isolated from some plant sources, but their content is very low (1–40 mg/100 g dry weight).<sup>14)</sup> Thus, sea cucumber might be suitable as a dietary source of cerebrosides.

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