Inconspicamide, New N-Acylated Serinol from the Marine Sponge Stelletta inconspicua

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A new N-acylated serinol, inconspicamide (1), was isolated from the marine sponge, Stelletta inconspicua, together with a glyceryl ether (2). Their structures were determined on the basis of spectroscopic data and the modified Mosher analysis. They exhibited moderate cytotoxic activity against HeLa human cervical cancer cells.

Key words: cytotoxic metabolite; marine sponge; amide

Marine invertebrates, especially marine sponges are a rich source of bioactive substances. With the objective of finding new anticancer lead compounds from them, we carried out a screening for cytotoxic activity against HeLa human cervical cancer cells. The cytotoxicity assay has played a pivotal role in the discovery of anticancer agents that are clinically used today.1) In the course of the screening, the marine sponge, Stelletta inconspicua, collected at Oshima-shinsone exhibited activity. We isolated from the sponge a new N-acylated serinol, inconspicamide (1), together with a glyceryl ether (2).2) Their structures were elucidated on the basis of NMR and MS/MS data. Several serinol ethers have been reported from tunicates;3) to the best of our knowledge, this is the first report of an N-acylated serinol from nature. In this report, we describe the isolation, structural elucidation, and biological activities of these compounds.

The frozen sponge (1.6 kg wet weight) was extracted with MeOH and EtOH, and the extracts were combined and concentrated in vacuo. The residue was suspended in H2O and extracted with CHCl3 and n-BuOH. The CHCl3 layer was subjected to a modification of the Kupchan procedure4) to yield 60% MeOH, CHCl3 and n-hexane layers. The CHCl3 layer was separated by ODS flash chromatography and silica gel open-column chromatography, and the cytotoxic fraction was purified by repetitive reversed-phase HPLC to give inconspicamide (1) and a glyceryl ether (2).

Inconspicamide (1) had the molecular formula of C22H36NO3 which was inferred from HR-FT-ICR MS data [m/z 394.3292, (M + Na)⁺, Δ +0.0 mmu]. An analysis of the 1H-NMR data in conjunction with the HSQC spectrum5) revealed the presence of two oxy-methylene, one N-substituted methine, one triplet methyl, one doublet methyl and many methylene carbons (Table 1). Partial structures a–d were deduced from the COSY data and confirmed by HMBC correlations6) (Fig. 1). The connection of units a and b via an amide linkage was determined by HMBC cross peaks between (H-2, H-2' and H-3')/C-1 (Fig. 1).

The location of the branched methyl group in the alkyl chain was determined by an analysis of the FT-ICR MS data for inconspicamide (1). Notable fragment ions generated from the [M + Na]⁺ ion were observed at m/z 266 and 294, but the ion at m/z 280 was not apparent, which allowed us to locate the methyl group at C-11' (Fig. 1). The stereochemistry of C-11 was not determined.

The glyceryl ether (2) had the molecular formula of C20H32O4 which was inferred from HR-FT-ICR MS data [m/z 353.3028, (M + Na)⁺, Δ +0.2 mmu]. The 1H- and 13C-NMR data for 2 were very close to those of known compound 2,2) which had been isolated from a marine sponge by Lee et al. as a cytotoxic compound. The FT-ICR MS data revealed the location of the branched methyl group in the alkyl chain. Fragment ions from the [M + Li]⁺ ion observed at m/z 223 and 251 and the absence of an ion at m/z 237 showed that the methyl group was positioned at C-10' (Fig. 2), this being identical with that of the known compound.2)

In the paper reported by Lee et al.,2) the configuration

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at C-2 in 2 was determined from the positive optical rotation ([α]$_D^{21}$ +1'). However, our compound gave a negative optical rotation ([α]$_D^{22}$ $-1.2'$). Since the values are so small, we attempted to determine the configuration by applying the modified Mosher method to the diol. Treatment of 2 with R-(-) or S-(-)-MTPACl yielded S-(-) and R-(-)-MTPA bisesters 2a and 2b, respectively. The $\Delta\delta$ values indicated the 2S configuration which was the same as that of the known compound (Fig. 2). Since we, as well as Lee et al., were not able to determine the stereochemistry at C-10, we cannot conclude whether the two compounds are identical or not.

Inconspicamide (1) and 2 exhibited moderate cytotoxicity against HeLa human cervical cancer cell lines at 22 µg/ml, respectively.

**Table 1.** $^1$H- and $^{13}$C-NMR Data for Inconspicamide (1) in CD$_3$OD

<table>
<thead>
<tr>
<th>No.</th>
<th>$^1$H (ppm)</th>
<th>$^{13}$C (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.59 (d, 5.5)</td>
<td>61.7</td>
</tr>
<tr>
<td>2</td>
<td>3.91 (quint, 5.5)</td>
<td>54.0</td>
</tr>
<tr>
<td>3</td>
<td>3.59 (d, 5.5)</td>
<td>61.7</td>
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<tr>
<td>$1'$</td>
<td>176.2</td>
<td></td>
</tr>
<tr>
<td>$2'$</td>
<td>2.21 (t, 7.5)</td>
<td>37.0</td>
</tr>
<tr>
<td>$3'$</td>
<td>1.60 (quint, 7.5)</td>
<td>26.7</td>
</tr>
<tr>
<td>$4'$</td>
<td>1.31 m</td>
<td>30.1</td>
</tr>
<tr>
<td>$5'$-$15'$</td>
<td>1.30 m</td>
<td>30.5</td>
</tr>
<tr>
<td>16'</td>
<td>1.28 m</td>
<td>32.7</td>
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<tr>
<td>17'</td>
<td>1.30 m</td>
<td>23.5</td>
</tr>
<tr>
<td>18'</td>
<td>0.89 (t, 6.9)</td>
<td>14.1</td>
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<tr>
<td>$1''$</td>
<td>0.85 (d, 6.2)</td>
<td>19.9</td>
</tr>
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<td>$2''$</td>
<td>1.37 m</td>
<td>33.6</td>
</tr>
<tr>
<td>$3''$</td>
<td>1.09 m</td>
<td>38.0</td>
</tr>
<tr>
<td>$4''$</td>
<td>1.09 m</td>
<td>38.0</td>
</tr>
</tbody>
</table>

Ceramides are lipids consisting of a sphingosine base and a fatty acid. They are well known as the second messenger in the cell-growth mechanism. A variety of ceramides and sphingosines have been reported from marine organisms. The sphingosine base is biosynthesized by the conjugation of serine and palmitic acid. Instead of a sphingosine base, inconspicamide contains a serine moiety. Therefore, inconspicamide can be considered as a truncated ceramide.

In a previous study, synthetic N-acylated serinol (C16-serinol) was shown to induce apoptosis partially mediated by the activation of PKCζ in neuroblastoma cells. It is likely that inconspicamide exerts a similar effect toward cultured cells.

**Experimental**

General experimental procedures. Optical rotation data were measured by a Jasco DIP-1000 digital polarimeter in MeOH. FT-ICR MS spectra were measured by a Bruker Daltonics apex-Qe 9.4T instrument. NMR spectra were recorded by a Jeol delta 600 NMR.
The residue was suspended in H$_2$O and the extracts were combined and concentrated in vacuo (wet weight) was extracted with MeOH and EtOH, and the residue was suspended in H$_2$O and extracted with CHCl$_3$ and n-BuOH. The CHCl$_3$ layer was partitioned between 90% MeOH and n-hexane. To the 90% MeOH layer add was water to yield 60% MeOH which was then extracted with CHCl$_3$. The CHCl$_3$ layer was concentrated and separated by ODS flash chromatography to afford six fractions (A–F). Fraction F (the 100% MeOH group are omitted). Cytotoxic fraction D (CHCl$_3$/MeOH 19:1) was separated by reversed-phase HPLC (COSMOSIL 5C$_3$-AR-II, 20 $\times$ 250 mm) with 65% 1-PrOH, and purified by reversed-phase HPLC (COSMOSIL 5C$_3$-MS-II, 10 $\times$ 250 mm) with 55% 1-PrOH to give 3.4 mg of inconspicamide (1). Fraction F (the 100% MeOH eluate) was further separated by silica gel column chromatography to give 10 fractions (A–F). Fraction F (the 100% MeOH fraction D was collected by dredging at a depth of 150 m at Oshima-shinsone (28’52’N; 129’33’E) Kagoshima pre-fecture (Japan) in 2004, immediately frozen, and kept at −20 °C until needed for analysis. A voucher specimen has been deposited at the Zoological Museum, University of Amsterdam (ZMAPOR 20605).

Extraction and isolation. The frozen sponge (1.6 kg wet weight) was extracted with MeOH and EtOH, and the extracts were combined and concentrated in vacuo. The residue was suspended in H$_2$O and extracted with CHCl$_3$ and n-BuOH. The CHCl$_3$ layer was partitioned between 90% MeOH and n-hexane. To the 90% MeOH layer was added H$_2$O to yield 60% MeOH which was then extracted with CHCl$_3$. The CHCl$_3$ layer was concentrated and separated by ODS flash chromatography to give six fractions (A–F). Fraction F (the 100% MeOH eluate) was further separated by silica gel open-column chromatography to give 10 fractions (A–F). Cytotoxic fraction D (CHCl$_3$/MeOH 19:1) was separated by reversed-phase HPLC (COSMOSIL 5C$_3$-AR-II, 20 $\times$ 250 mm) with 65% 1-PrOH, and purified by reversed-phase HPLC (COSMOSIL 5C$_3$-MS-II, 10 $\times$ 250 mm) with 55% 1-PrOH to give 3.4 mg of inconspicamide (1). Fraction F (the 100% MeOH eluate) was further separated by silica gel column chromatography to give 10 fractions (A–F). Cytotoxic fraction D (CHCl$_3$/MeOH 19:1) was separated by reversed-phase HPLC (COSMOSIL 5C$_3$-AR-II, 20 $\times$ 250 mm) with 65% 1-PrOH, and purified by reversed-phase HPLC (COSMOSIL 5C$_3$-AR-II, 20 $\times$ 250 mm) with 65% 1-PrOH to give 11.7 mg of a glyceryl ether (2).

Inconspicamide (1): colorless powder; [α]$_D^{25}$ = −6.3° (c 0.17, MeOH); 1H- and 13C-NMR, see Table 1. HR-FT-ICR MS $m/z$ (M + Na)$^+$: calcd. for C$_{22}$H$_{45}$NO$_3$Na, 394.3292; found, 394.3292.

Glyceryl ether (2): light yellow oil; [α]$_D^{22,5}$ −1.2° (c 0.53, MeOH); 1H- and 13C-NMR, same as ref. 10. HR-FT-ICR MS $m/z$ (M + Na)$^+$: calcd. for C$_{26}$H$_{42}$O$_3$Na, 535.3026; found, 535.3028.

Assay for the cytotoxicity against HeLa cells. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) containing 10% of fetal bovine serum, 2 μg/ml of gentamycin, 2 μg/ml of antibiotic-antimotic, and 0.3 M NaHCO$_3$ (adjusted to pH 7.0–7.4 with 2N HCl) at 37 °C under an atmosphere of 5% CO$_2$. To each well of a 96-well microplate containing 200 μl of the tumor cell suspension (1 $\times$ 10$^5$cells/ml), the test solution was added after a 24-h preincubation, and the plate was incubated for 72 h. After adding 50 μl of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazoli-um bromide (MTT) saline solution (1 mg/ml) to each well, the plate was incubated for 3 h under the same condition to stain the live cells. After this incubation, the plate was centrifuged, the supernatant removed, and the cells were dissolved in 150 μl of DMSO to determine the IC$_{50}$ values.

Preparation of MTPA esters 2a and 2b. The glyceryl ether (2, 500 μg) was reacted with (R)-MTPACl (5 μl) in 100 μl of CH$_2$Cl$_2$ containing 1 mg of DMAP for 5 minutes. The mixture was partitioned between 0.1 M NaHCO$_3$ and CHCl$_3$, and the CHCl$_3$ layer was successively washed with 0.1 N HCl and H$_2$O. The organic layer was concentrated and separated by silica gel column chromatography to afford (S)-MTPA ester 2a. Compound 2 was similarly treated with (S)-MTPACl to afford (R)-MTPA ester 2b.

2a $\delta_{1H}$ (CD$_3$OD): 5.49 (1H, m), 4.63 (1H, br dd), 4.37 (1H, dd, $J$ = 4.4, 12.1 Hz), 3.61 (1H, dd, $J$ = 6.9, 10.2 Hz), 3.56 (1H, dd, $J$ = 4.9, 10.2 Hz), 3.39 (2H, m), 1.57 m, 1.52 m, 1.36 m, 1.26 m, 1.08 m, 0.89 (3H, t, $J$ = 6.9 Hz), 0.84 (3H, d, $J$ = 6.4 Hz) (signals for the MTPA group are omitted).

2b $\delta_{1H}$ (CD$_3$OD): 5.44 (1H, m), 4.74 (1H, br dd), 4.43 (1H, dd, $J$ = 6.4, 12.4 Hz), 3.51 (2H, m), 3.31 (2H, t, $J$ = 6.7 Hz), 1.57 m, 1.46 m, 1.36 m, 1.25 m, 1.08 m, 0.89 (3H, t, $J$ = 6.6 Hz), 0.84 (3H, d, $J$ = 6.4 Hz) (signals for the MTPA group are omitted).

Acknowledgments

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