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Chemical Investigation of the Sea Cucumber *Stichopus japonicus*

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Abstract – A chemical investigation of the polar extract of the sea cucumber *Stichopus japonicus*, collected from Jeju Island, Korea, has led to isolation of five new fatty acid derivatives (**1**, **4** - **7**) along with known compounds (**2** - **3**, **8** - **14**). Their structures were elucidated by a combination of MS and NMR spectroscopy.
Keywords – Sea cucumber, *Stichopus japonicus*, isolation, fatty acid derivatives, lyso-PAF analogue, nucleosides

Introduction

Triterpene glycosides are the predominant secondary metabolites of sea cucumbers (holothurians) and are responsible for their general toxicity. These glycosides have been reported to possess a wide spectrum of biological effects, including cytotoxic, antifungal, hemolytic, and immunomodulatory activities. More than 100 of these glycosides have been described, and the majority are lanosterol type triterpenes with an 18(20) lactone and a sugar chain linked to the C-3 of the aglycone (Zhang *et al.*, 2006). In the course of our continuing research aimed at the discovery of biologically active secondary metabolites from marine organisms (Mansoor *et al.*, 2007a; Mansoor *et al.*, 2007b; Shinde *et al.*, 2007), investigation of a sea cucumber, *Stichopus japonicus*, resulted in the isolation of fatty acid derivatives, nucleosides and a glycerol derivative. The sea cucumber *Stichopus japonicus* is reported to contain saponins (Kitagawa *et al.*, 1976; Elyakov *et al.*, 1980; Maltsev *et al.*, 1984) and cerebrosides (Kisa *et al.*, 2005; Kaneko *et al.*, 2003). Herein, we report the isolation and structure elucidation of three fatty acid alcohols (**1** - **3**), four fatty acid esters (**4** - **7**), a fatty acid (**8**), a lyso-PAF analogue (**9**), a pyrimidine derivative (**10**), and four nucleosides (**11** - **14**) from the BuOH extract of the sea cucumber *Stichopus japonicus*.

Experimental

General procedures – Optical rotations were measured with a Jasco P-1020 polarimeter using a 1 dm path length cell. The ^{1}H and 2D NMR spectra were recorded at 500 MHz and 400 MHz using Varian INOVA 500 and Varian UNITY 400 spectrometers. FABMS data were obtained on a JEOL JMS SX-102A spectrometer. Chemical shifts were reported with reference to the respective and residual solvent peaks (δ_H 3.30 and δ_C 49.0 for CD_3OD). MPLC was carried out on Combiflash Retrieve using Redisep C-18 column (130 gm). HPLC was performed on a Gilson 370 pump with a Shodex C18M 10E (preparative, 250 × 10 mm, 5 μm, and 100 Å) column using Shodex RI-101 detector.

Animal material – The sea cucumber was collected in February 2005, off the coast of Jeju Island, South Korea. The samples were frozen immediately after collection and stored at –20 °C until extraction. This organism was identified as *Stichopus japonicus* by Prof. Sook Shin of Sahmyook University. A voucher specimen was deposited in Department of Life Science, Sahmyook University, Korea.

Extraction and isolation – The body walls and Cuvierian tubules of the frozen sea cucumber were separated. The freeze dried Cuvierian tubules of the sea cucumber were extensively extracted with *n*-BuOH till became colorless and then extracted with MeOH at room temperature. The *n*-BuOH/H_2O extract was further partitioned between H_2O and *n*-BuOH. The *n*-BuOH fraction was subjected to a reversed-phase flash column chromatography (YMC Gel ODS-A, 60 Å 500/400 mesh), eluting with gradient solvent system of 50 to 100% MeOH to yield nineteen fractions (CIB1-CIB19). Fraction CIB-1 was subjected to MPLC using Combiflash Retrieve

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on column Redisep C-18 (130 gm), eluting with gradient solvent system of 0 to 50% MeOH/H_2O to yield twenty fractions (CIB1-1 to CIB1-20). Compounds **1** - **14** were isolated from selected fractions by repeated chromatographic separation using RP-HPLC. Compounds **1**, **10**, and **11** were obtained by purification of sub-fraction CIB1-18. Compounds **2** and **13** were obtained by purification of subfraction CIB1-16. Compounds **3** and **14** were isolated by purification of subfraction CIB1-14. Compounds **4-9** were obtained by purification of fraction CIB16. Compound **12** was isolated by purification of subfraction CIB1-10.

Compound 1 – Pale yellow oil; ^{1}H-NMR (CD_3OD, 500 MHz) δ : 5.81 (1H, ddt, J = 10.5, 6.5, 6.5 Hz, H-9), 5.45 (1H, m, H-3), 5.43 (1H, m, H-4), 5.42 (1H, m, H-6), 5.40 (1H, m, H-7), 5.05 (1H, dq, J = 17.0, 2.0 Hz, H-10a), 4.95 (1H, dq, J = 17.0, 2.0 Hz, H-10b), 3.96 (2H, t, J = 6.5 Hz, H-1), 2.82 (2H, m, H-5), 2.81 (2H, m, H-8), 2.42 (2H, q, J = 6.5 Hz, H-2); ^{13}C-NMR (CD_3OD, based on HSQC and HMBC experiments, 500 MHz) δ : 138.0 (C-9), 131.2 (C-3), 129.8 (C-6), 128.0 (C-7), 126.0 (C-4), 114.8 (C-10), 68.0 (C-1), 32.0 (C-8), 28.2 (C-2), 26.2 (C-5); LRFABMS *m/z* 153 $[M+H]^+$.

Compound 4 – Pale yellow oil; ^{1}H-NMR (CD_3OD, 500 MHz) δ : 5.35 (8H, m, H-7, H-8, H-10, H-11, H-13, H-14, H-16, H-17), 4.06 (2H, t, J = 7.0 Hz, H-1'), 2.83 (6H, m, H-9, H-12, H-15), 2.31 (2H, t, J = 7.5 Hz, H-2), 2.16 (2H, m, H-6), 2.12 (2H, m, H-18), 1.66 (2H, quin, J = 7.5 Hz, H-3), 1.60 (2H, quin, J = 6.5 Hz, H-3'), 1.38 (2H, m, H-2'), 1.28 (4H, m, H-4, H-5), 0.96 (3H, t, J = 7.5 Hz, H-19), 0.93 (t, J = 7.5 Hz, H-4'); ^{13}C-NMR (CD_3OD, based on HSQC and HMBC experiments, 500 MHz) δ : 174.2 (C-1), 128.2 (C-7, C-8, C-10, C-11, C-13, C-14, C-16, C-17), 64.0 (C-1'), 33.2 (C-2), 30.5 (C-2'), 29.2 (C-4, C-5), 26.2 (C-6, C-18), 25.1 (C-9, C-12, C-15), 24.5 (C-3), 19.0 (C-3'), 12.8 (C-19, C-4'); LRFABMS *m/z* 369 $[M+Na]^+$.

Compound 5 – Pale yellow oil; ^{1}H-NMR (CD_3OD, 500 MHz) δ : 5.35 (6H, m, H-4, H-5, H-7, H-8, H-10, H-11), 4.06 (2H, t, J = 7.0 Hz, H-1'), 2.83 (4H, m, H-6, H-9), 2.35 (2H, t, J = 7.5 Hz, H-2), 2.07 (2H, m, H-3, H-12), 1.60 (2H, quin, J = 6.5 Hz, H-3'), 1.38 (2H, m, H-2'), 1.28 (18H, m, H-13-H-21), 0.96 (3H, t, J = 7.5 Hz, H-22), 0.93 (t, J = 7.5 Hz, H-4'); LRFABMS *m/z* 413 $[M+Na]^+$.

Compound 6 – Pale yellow oil; ^{1}H-NMR (CD_3OD, 500 MHz) δ : 5.35 (8H, m, H-6, H-7, H-9, H-10, H-12, H-13, H-15, H-16), 4.06 (2H, t, J = 7.0 Hz, H-1'), 2.83 (6H, m, H-8, H-11, H-14), 2.31 (2H, t, J = 7.5 Hz, H-2), 2.16 (2H, m, H-5), 2.12 (2H, m, H-17), 1.66 (2H, quin, J = 7.5 Hz, H-3), 1.60 (2H, quin, J = 6.5 Hz, H-3'), 1.38 (2H, m, H-2'), 1.28 (2H, m, H-4), 0.96 (3H, t, J = 7.5 Hz, H-18), 0.93 (t, J = 7.5 Hz, H-4'); LRFABMS *m/z* 333 $[M+H]^+$.

Compound 7 – Pale yellow oil; ^{1}H-NMR (CD_3OD, 500 MHz) δ : 4.06 (2H, t, J = 7.0 Hz, H-1'), 2.29 (2H, t, J = 7.5 Hz, H-2), 1.60 (4H, m, H-3, H-3'), 1.52 (1H, m, H-14), 1.38 (2H, m, H-2'), 1.28 (18H, m, H-3-H-12), 1.20 (2H, m, H-13), 0.94 (t, J = 7.5 Hz, H-4'), 0.86 (6H, d, J = 6.5 Hz, H-15, H-16); LRFABMS *m/z* 334 $[M+Na-H]^+$.

Results and Discussion

The *n*-BuOH extract of the sea cucumber was subjected to solvent partition and reversed-phase flash column chromatography, MPLC and HPLC to yield compounds **1** - **14** (Fig. 1). The structures of these metabolites were deduced using NMR (^{1}H, ^{13}C, COSY, HSQC, and HMBC) and MS analysis.

Compound **1** was isolated as a pale yellow oil. Its molecular formula was established as $C_{10}H_{16}O$ by FABMS and ^{13}C-NMR data. The FABMS spectrum showed $[M+H]^+$ ion at *m/z* 153. The ^{13}C-NMR data showed six olefinic carbons, one allylic carbon, two diallylic carbons and one oxymethylene carbon. In the HSQC spectrum, signals at δ 5.05 (H-10a) and 4.95 (H-10b) showed correlation to the carbon at δ 114.8 (C-10), strongly suggesting the presence of a terminal methylene group, which was coupled with protons at δ 5.81 (H-9), which in turn were coupled with the diallylic protons at δ 2.81 (H_2-8). The ^{1}H-NMR spectrum showed the presence of four diallylic protons gauged from the integration, indicating the presence of two double bonds in the molecule. Furthermore, olefinic protons showed coupling with a methylene group at δ 2.42 (H_2-2) which in turn was coupled with the oxymethylene signal at δ 3.96 (H_2-1). The geometry of Δ^3 and Δ^6 was defined as *cis* on the basis of chemical shifts of the allylic carbon at δ 28.2 (C-2) and diallylic carbon at δ 26.2 (C-5). Thus, the structure of **1** was assigned as (3*Z*,6*Z*)-deca-3,6,9-trien-1-ol.

Compound **2** was identified as (3*Z*,6*Z*)-deca-3,6-dien-1-ol, which was previously reported from the plant *Jasminum sambac* (Kaiser, 1988). Compound **3** was identified as (3*Z*)-non-3-en-1-ol, which was previously reported as a synthetic product (Zheng *et al.*, 2005).

Compound **4** was isolated as a pale yellow oil and its molecular formula was established as $C_{23}H_{38}O_2$ on the basis of the ^{13}C-NMR and MS analyses. The FABMS spectrum of **4** showed $[M+Na]^+$ ion at *m/z* 369. The ^{13}C-NMR data indicated the presence of eleven carbons which included an ester carbon (δ 174.2), an olefinic carbon (δ 128.2), diallylic carbon (δ 25.1), allylic carbon (δ 26.2), five methylene carbon (δ 33.2, 30.5, 29.2, 24.5,

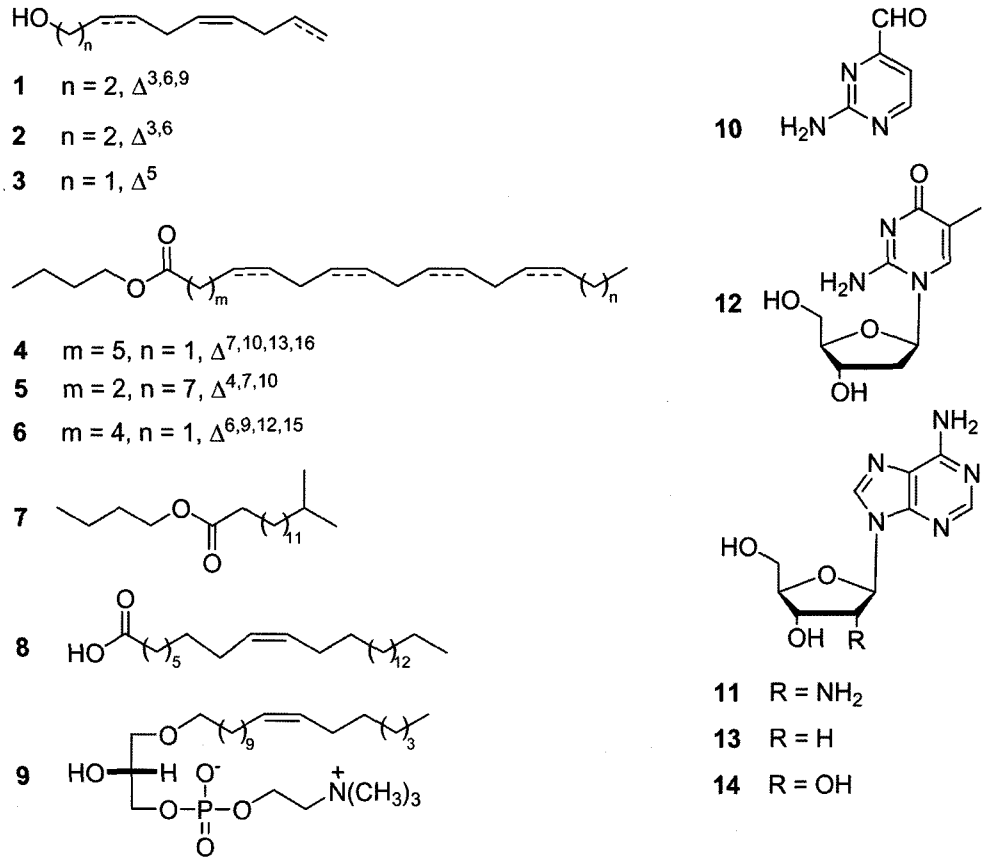

Fig. 1. Structures of compounds **1** - **14**.

19.0), a oxymethylene group (δ 64.0) and a terminal methyl group (δ 12.8). The ^{1}H NMR spectrum showed the presence of six diallylic protons gauged from the integration, indicating the presence of four double bonds in the molecule. Also observed were signals for oxymethylene protons (δ 4.06), two terminal methyls (δ 0.96, 0.93), four allylic protons (δ 2.16, 2.12), six diallylic protons (δ 2.83) and six methylene groups (δ 2.31, 1.66, 1.60, 1.38, 1.28, 1.28). The ^{1}H-^{1}H COSY spectrum helped to identify two different spin systems, one comprising a chain of four carbons and another consisting of a long unsaturated chain. In the HMBC spectrum, correlations were found from protons (d 2.31/H_2-2 and δ 4.06/H_2-1') to the ester carbon (δ 174.2/C-1). Double bond positions were determined on the basis of HMBC correlation from the terminal methyl proton (δ 0.96/H_3-19) to the olefinic carbon (δ 128.2/C-17). This was further supported by the ^{1}H-^{1}H COSY spectrum, where strong coupling was observed between allylic protons (δ 2.12/H_2-18) and terminal methyl protons (δ 0.96/H_3-19). The geometry of Δ^7, Δ^{10}, Δ^{13}, and Δ^{16} was defined as *cis* on the basis of chemical shifts of the allylic carbons at δ 26.2 (C-6, C-18) and diallylic carbons at δ 25.1 (C-9, C-12, C-15). Therefore, the structure of compound **4** was proposed as (7*Z*,10*Z*,13*Z*,16*Z*)-butyl nonadeca-7,10,13,16-tetraenoate.

Compound **5** was isolated as a pale yellow oil with the molecular formula $C_{26}H_{46}O_2$, which was determined on the basis of MS analysis. The $[M + Na]^+$ ion was observed at *m/z* 413 in the FABMS spectrum of **5**. The ^{1}H-NMR data were identical with those of **4** except the absence of methylene signals (δ_H 1.66) and slightly downfield shifted signal for H_2-2 (δ_H 2.35 as compared to δ_H 2.31 in **4**). This suggested the presence of double bonds near to the ester group, which was confirmed by the HMBC correlation from allylic protons (δ_H 2.16/H_2-3) to the ester carbon (δ_C 174.2/C-1). Hence, compound **5** was determined to be (4*Z*,7*Z*,10*Z*)-butyl docosa-4,7,10-trienoate.

Compound **6** was isolated as a pale yellow oil. In the FABMS spectrum, the $[M + H]^+$ ion was observed at *m/z* 333, suggesting the molecular formula to be $C_{22}H_{36}O_2$, which was 14 mass units lesser than **4**. The ^{1}H-NMR data of **6** were exactly identical with those of compound **4**. Thus, the structure of **6** was elucidated as (6*Z*,9*Z*,12*Z*,15*Z*)-butyl octadeca-6,9,12,15-tetraenoate.

Compound **7**, obtained as a pale yellow oil, showed the molecular formula $C_{20}H_{40}O_2$, as deduced from the

FABMS ($[M + Na - H]^+$ at *m/z* 334). The main difference between ^1H NMR spectra of **6** and **7** was the absence of unsaturation in **7**, while **6** contained four double bonds. The ^1H-NMR spectrum of **7** exhibited additional signals for an isopropyl group (Mansoor *et al.*, 2005), indicated by a two-methyl doublet at δ 0.86 (J = 6.5 Hz, H_3-15, H_3-16), which in turn showed coupling with methine proton signal at δ 1.52 (H-14), which was coupled with methylene protons at δ 1.18 (H_2-13) in the COSY spectrum. Thus, the structure of compound **7** was elucidated as butyl 14-methylpentadecanoate.

Compound **8** was identified as (9*Z*)-hexacos-9-enoic acid, which was previously isolated from marine sponge (Rezanka *et al.*, 2002). Compound **9** was identified as a known lyso-PAF analogue previously isolated from the marine sponge *Spirastrella abata* (Shin *et al.*, 1999). Compound **10** was identified as 2-amino-pyrimidine-4-carbaldehyde as a first report from natural source, although it was used as an intermediate in synthesis. Compound **11** was identified as 2'-amino-2'-deoxyadenosine, which was previously reported from the actinomycete *Actinomadura* sp. (Iwai *et al.*, 1979). Compounds **12** (2'-deoxy-5-methylisocytidine), **13** (2'-deoxyadenosine), **14** (adenosine) are common nucleosides.

Isolation of fatty acid alcohols (**1** - **3**) from the Cuvierian tubules is important in context of the origin of these alcohols. These compounds might be derived from the food consumed by sea cucumber and further study may throw light on the food cycle. Butyl esters of fatty acids (**4** - **7**) could be artifacts produced during the extraction and isolation process. It is interesting to isolate long-chain fatty acid (**8**) from sea cucumber, since long-chain fatty acids are characteristic metabolites of sponges.

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