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New Antibacterial Sulfated Alkenes in the Pleated Sea Squirt, *Styela plicata*

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오만둥이(*Styela plicata*)로 부터 신 항균성 sulfated alkenes의 분리 및 구조

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Abstract

The objective of this study was to investigate antibacterial compounds in pleated sea squirt (*Styela plicata*, Omandungi in Korean language). Three antibacterial compounds against the *Bacillus subtilis* were isolated from acetone extract of the cultured pleated sea squirt using reversed phase liquid chromatography. Structures of those compounds were determined through 1D and 2D NMR spectral data analysis and mass spectra. Structure of one of those compounds was elucidated as known 2,6-dimethylheptyl sodium sulfate (**1**) and another two new compounds were confirmed as (3*Z*)-3-decenyl sodium sulfate (**2**) and (3Z,6Z)-3,6-decadienyl sodium sulfate (**3**), respectively. As the results, chemical structures of two new antibacterial sulfated alkenes were cleared.

Key words : *Styela plicata*, Pleated sea squirt, Antibacterial activity, 3-decenyl sodium sulfate, 3,6-decadienyl sodium sulfate

Ⅰ. Introduction

Styela plicata (pleated sea squirt, Omandungi in Korean language) is a species of sea squirts (Ascidian), having ovular, greyish to tannish white solitary benthic tunicate and distribute from subtropics to temperate seawaters (http://en.wikipedia.org/wiki/Sea_pineapple). It has been culturing together other two species such as *Halocynthia roretzi* and *Styela clava* in the southern coast of Korea as a resource of food material and produced about 6,400 tons in 2017(https://www.mof.go.kr, 2018). They are using as a major ingredient with another tunicates for some cooked seafood due to their peculiar flavor and taste.

Many antimicrobial sulfated alkane and alkenes have been isolated from the ascidians. C-11 alkene (4,8-dimethyl nonenyl sulfate) was found from the cultured *S. clava* (Yun et al., 2007). And, C-9 alkane (2,6-dimethylheptyl sodium sulfate) and sulfated C-10 alkenes (4,7-decadienyl sulfate and 3,6,9-decatrienyl sulfate) were isolated as the antimicrobial active compounds from the hepatopancreas of the Japanese tunicate (ascidian), *H. roretzi* and *H. aurantium* (Tsukamoto et al., 1994). They presumed those similar compounds had

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been contained in the *S. plicata*, but, not confirmed. 2,6-dimethylheptyl sulfate, was also found in the ascidian, *Policitor adriaticus*, collected at Croatia (De Rosa et al., 1997) and from the Mediterranean ascidian, *H. papillosa* (Aiello et al., 2000). 6-methylheptyl sulfate and 5-octenyl sulfate were also found in the same *P. adriaticus* with cytotoxic activity *in vitro* (De Rosa et al., 1997).

Differently from alklyl or alkenyl sulfates, many peptides were reported from tunicates (Tincu et al., 2004). Plicatamide was found as anti microbial octapeptide from the *S. plicata* (Tincu et al., 2003) and halocyamines having bromo-tetrapeptides from *H. roretzi* (Azumi et al., 1990), dicynchaurin and halocidin from *H. aurantium* (Lee et al., 2001a), clavanins, clavaspirin and styelins from *H. styela* (Lee et al., 1997, Lee et al., 2001b).

In the course of our studies evaluating potential bio-active materials in the cultured pleated sea squirt (*S. plicata*), we found that those methanol extract showed antibacterial activity against *Bacillus subtilis*. We isolated a known polar antibacterial sulfated alkane (**1**) and new two unknown antibacterial compounds. In this paper, we described the isolation and structural determination of those compounds as sulfated alkenes (**2**, **3**).

Ⅱ. Materials and Methods

1. Material

The cultured pleated sea squirt (*S. plicata*, Omandungi in Korean language), was collected at Jido Bay, Tongyeong, Gyeongnam Province, Korea.

2. Instruments and reagents

Measurement of 1-dimensional (1D) and 2D nuclear magnetic resonance (NMR) spectra were conducted in dimethylsulfoxide-*d*6 (DMSO-*d*6, 99.8%, Sigma-Aldrich) for the antibacterial compound **1** on the JEOL JNM-LA 600 NMR spectrometer (600 MHz, JEOL, Japan), and in CD_3OD (99.8%, Sigma-Aldrich) for antibacterial compound **2** and **3** on a JEOL JNM-LA 400 NMR spectrometer (400 MHz, JEOL, Japan). The chemical shifts were referenced with residual solvent signals. Molecular weights were measured by fast atom bombardment mass spectroscopy (FAB-MS) with glycerol as matrix at the positive and negative mode. Electron impact mass spectroscopic (EI-MS) data were obtained on the JEOL JMS-700 MS spectrometer (JEOL, Japan).

3. Antimicrobial assay

During the isolation procedures, the paper disc (0.8 mm diameter) method was adopted for the screening of antibacterial activity as reported by Yun et al.(2007). Active fractions or peaks were collected from the column guided by the formation of growth inhibition zone against the *Bacillus subtilis* around the paper disc on the plate count agar. Minimum inhibitory activity (MIC) was measured by nutrient broth dilution method against *B. subtilis* (Ikegawa et al., 1989).

4. Extraction and isolation of antibacterial compounds

After washing the whole body of pleated sea squirt(*S. plicata,* 5 kg wet weight) were minced and extracted with acetone and filtrated. The residue was further extracted 2 times more and those extracts were concentrated in vacuum under 40ºC. The concentrated extract was dissolved in 80% MeOH solution (350 mL) and then partitioned

with n-hexane solution (2×350 mL) to give hexane layer (oily residue). Aqueous MeOH solution was diluted to 40% MeOH solution and partitioned with $CHCl_3$ solution (3×500 mL) to afford a $CHCl_3$ soluble fraction. After this, aqueous MeOH solution was concentrated and partitioned between BuOH and water layer and each fraction was tested for antibacterial activity against *B. subtilis* by paper disc method.

$CHCl_3$ and BuOH soluble fractions that showed antibacterial activity were combined and subjected to a basic alumina (70~230 μm, Merck) column (2.0 cm, i.d. x 25 cm) and eluted with 1% NH_4OH-MeOH solution (1:1) after washing with $CHCl_3$-MeOH (1:1) and MeOH successively. The 1% NH_4OH-MeOH (1:1) fraction was concentrated and charged on the silica gel (200~400 μm, Merck) column (2.0cm, i.d. x 25cm) and eluted with $CHCl_3$, $CHCl_3$-MeOH (95:5), $CHCl_3$-MeOH (9:1), $CHCl_3$-MeOH(7:3), $CHCl_3$-MeOH(1:1), and MeOH, successively. $CHCl_3$-MeOH(9:1) and $CHCl_3$-MeOH (7:3) fractions were combined and concentrated. The concentrate was loaded on the ODS column (1.0 cm, i.d. x 20 cm, ODS-Q3, Fuji Gel, Japan) and was eluted stepwisely with 50, 70, 85, and 100% MeOH. The 50% MeOH fraction was concentrated and eluted on the same column with 30, 50, and 100% MeOH. 30% MeOH fraction (60 mg) was chromatographed on the HPLC attached ODS column (Develosil ODS-7, 1× 25 cm, Nomura Chemical Co., Japan) with 65% MeOH, monitoring at 215 nm and re-chromatographed to give active component **1** (19 mg), **2** (8 mg), and **3** (5 mg).

Ⅲ. Results and Discussion

Compound **1** was obtained amorphous colorless powder and the structure was elucidated by comparing ^1H, ^{13}C and MS spectral data with known 2,6-dimethylheptyl sodium sulfate (Tsukamoto et. al., 1994). Negative FAB-MS of compound **1** exhibited an $[M - Na]^-$ ion peak at *m/z* 223, corresponding to a molecular formula $C_9H_{19}O_4S$. The ^1H NMR spectrum showed three doublet methyl signals at δ = 0.73 ppm (3H, d, J = 6.4 Hz, CH_3-2), δ = 0.74 ppm (6H, d, J = 6.4 Hz, CH_3-7 and CH_3-9), two methylene protons of CH_2-1 bearing as sulfate group appeared at δ = 3.45 and δ = 3.55 ppm, a methine proton of CH-2 at 1.62 ppm, CH-6 at δ = 1.55 ppm, and six methylene protons at δ = 1.01 ~ 1.29 ppm <Table 1>. The connectivity of protons and neighboring carbon atoms on the Heteronuclear Multiple Bond Correlation (HMBC) spectrum for the compound **1** led us the known structure, 2,6-dimethylheptyl sodium sulfate. Indubitably, all of those assignments were well coincided with those of 2,6-dimethyl heptyl sodium sulfate reported by Tsukamoto et al.(1994).

Compound **2** was isolated amorphous pale yellowish powder with ascidian smell.

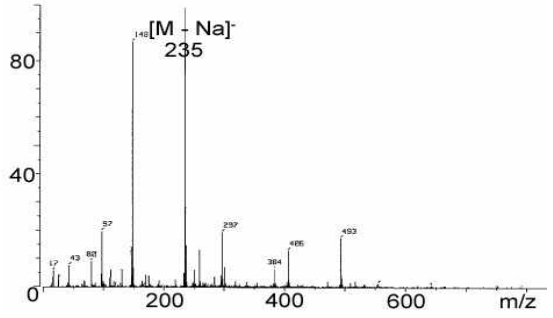
Negative FAB-MS showed $[M - Na]^-$ ion at *m/z* 235 [Fig. 1], and positive FAB-MS gave an $[M + Na]^+$ *m/z* 281, HRFAB-MS *m/z* $[M - Na]^-$ *m/z* 235.0891 (obsd. -11.3 mmu) corresponding to the molecular formula $C_{10}H_{19}O_4S$.

The structure of compound **2** was elucidated by ^1H, ^{13}C, 2D NMR spectra such as HMBC, Heteronuclear Multiple-Quantum Correlation (HMQC), Nuclear Overhauser Spectroscopy (NOESY) spectral data and comparing the structure obtained from the 5-octenyl sulfate (Aiello et al., 2000). On the ^1H NMR spectrum, a terminal methyl proton observed at δ = 0.84 ppm (3H, t, J = 6.2Hz, CH_3-10), two methylene proton signals

<Table 1> ^{1}H and ^{13}C Nuclear Magnetic Resonance(NMR) Data for Antibacterial Compounds **1**, **2** and **3** from *Styela plicata*.

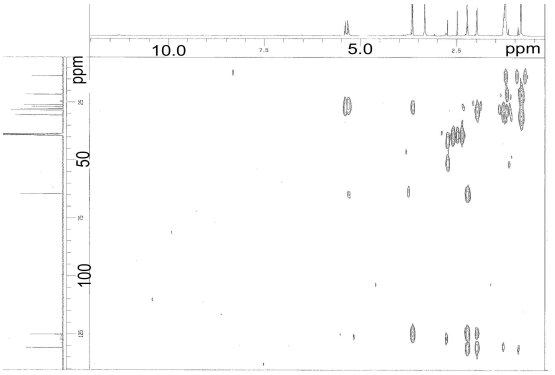
Compound 1*			Compound 2**			Compound 3**		
position	$^{1}H(\delta)$	$^{13}C(\delta)$	position	$^{1}H(\delta)$	$^{13}C(\delta)$	position	$^{1}H(\delta)$	$^{13}C(\delta)$
1-CH_2-	3.46(1H, d,d,*J*=8.0, 4.8) 3.55(1H, d,d,*J*=8.0, 4.0)	70.0	1-CH_2-	3.65(2H, t, *J*=6.2)	64.6	1-CH_2-	4.01(2H, t, J=8.0)	68.6
2-CH-	1.62(1H, m)	32.1	2-CH_2-	2.22(2H, q, *J*=8.2)	26.9	2-CH_2-	2.36(2H, m)	26.5
3-CH_2-	1.01(1H,m),1.29(1H, m)	32.6	3-CH-	5.31(1H, q, *J*=9.2)	125.2	3-CH-	5.46(1H, m)	126.4
4-CH_2-	1.23(1H,m),1.28(1H, m)	23.5	4-CH-	5.39(1H, q, *J*=9.2)	130.8	4-CH-	5.39(1H, q, *J*=9.2)	129.7
5-CH_2-	1.12(2H, m)	38.6	5-CH_2-	1.98 (2H, q, *J*=6.2)	26.2	5-CH_2-	2.48(2H, m)	30.8
6-CH-	1.51(1H,septet, *J*=5.6)	26.9	6-CH_2-	1.25 (2H, m)	28.5	6-CH-	5.46(1H, m)	131.7
7-CH_3-	0.74(3H, d, *J*=6.4)	22.0	7-CH_2-	1.25 (2H, m)	27.8	7-CH-	5.35(1H, m)	128.4
8-CH_3-	0.73(3H, d, *J*=6.4)	16.4	8-CH_2-	1.25 (2H, m)	30.6	8-CH_2-	2.11(2H, m)	28.7
9-CH_3-	0.74(3H, d, *J*=6.4)	22.1	9-CH_2-	1.25 (2H, m)	21.6	9-CH_2-	1.33(2H, m)	21.6
			10-CH_3-	0.84 (3H t, *J*=6.2)	13.4	10-CH_3-	1.00(3H, t, *J*=6.0)	14.8

*^{1}H Measured in DMSO-d_6 at 600 MHz and ^{13}C125 MHz, δ = chemical shift(ppm), *J* = coupling constant(Hz), d = doublet, m = multiplet, q = quartet.
**Measured in CD_3OD at 400 MHz(^{1}H) and 125 MHz(^{13}C).



[Fig. 1] FAB-MS(negative mode) spectrum of compound **2**.

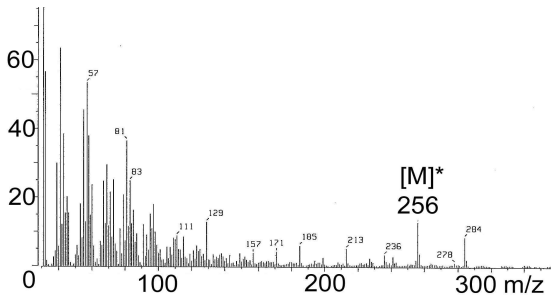
neighboring a sulfate group (CH_2-1) at δ = 3.65 ppm, four methylene protons connected methane (CH_2-3 and CH_2-4) at δ = 1.98 ppm (2H, q, *J* = 6.2 Hz CH_2-5) and δ = 2.22 ppm (2H, q, *J* = 8.2 Hz, CH_2-2). Four methylene protons(CH_2-6, CH_2-7, CH_2-8 and CH_2-9) gave overlapped signals at δ = 1.25 ppm and two methine protons of CH-3 and CH-4 were observed at δ = 5.31 ppm and δ = 5.39 ppm, respectively <Table 1>. Those assignment of proton signals with corresponding carbon signals and correlations between each proton and carbon on the HMBC spectrum [Fig. 2, 5] allowed us to establish compound **2** as 3-decenyl sulfate [Fig. 6].



[Fig. 2] HMBC spectrum of antibacterial compound **2** (400 MHz, CD_3OD).

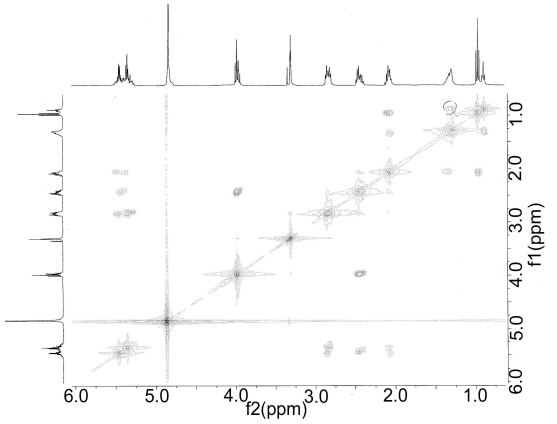
The *Z* configuration of the double bond was assigned on the basis of the value of the H-3/H-4

coupling constant (12 Hz) and of the ^{13}C NMR chemical shifts of the allylic methylenes CH_2-2 and CH_2-5 and compared with those of 5-octenyl sulfate.



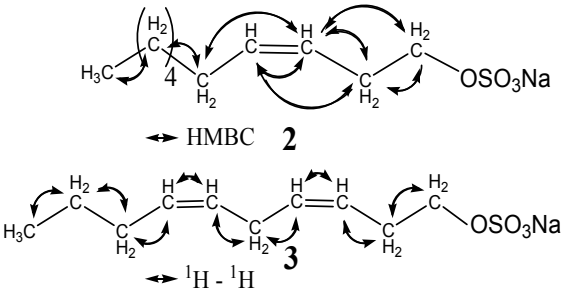
[Fig. 3] EI-MS spectrum of antibacterial compound **3**.

Compound **3** was also gained as amorphous pale yellow powder. EI-MS of compound **3** exhibited molecular ion peak at *m/z* 256 [Fig. 3]. The molecular weight of compound **3** was same as known 4,7-decadienyl sulfate (Tsukamoto et al., 1994).

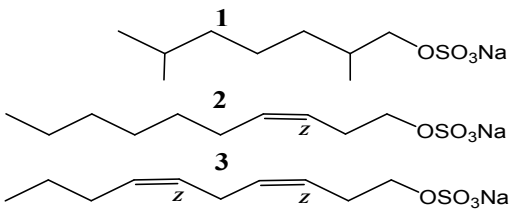


[Fig. 4] ^1H- ^1H COSY spectrum of antibacterial compound **3** (400 MHz, CD_3OD).

All the proton and carbon signals of compound **3** on the NMR spectra were strictly compared with those of 4,7-decadienyl sulfate. ^1H-NMR (400 MHz, CD_3OD) revealed a methyl group at δ = 1.00 ppm (3H, t, J = 6.0 Hz, CH_3-10), 4 methine protons at δ 5.35 ppm and 5.46 ppm, two methylene protons connected sulfate group at δ = 4.01 ppm (2H, t, J=8.0 Hz, CH_2-1) and 8 methylene protons such as δ = 2.36 ppm (2H, m, CH_2-2), δ 2.48 (2H, m CH_2-5), δ = 2.11 ppm (2H, m, CH_2-8), δ = 1.33 ppm (2H, m, CH-9) as <Table 1>. ^1H-^1H COSY spectrum of compound **3** [Fig. 5] showed those assignments were suitable to the structure as 3,6-decadienyl sulfate [Fig. 6], differently the position of double bond at C-4 and C-7 at 4,7-decadienyl sulfate.



[Fig. 5] HMBC correlations of antibacterial compound **2** and ^1H-^1H COSY correlations of **3**.



[Fig. 6] Structures of antibacterial compound **1, 2** and **3** isolated from *S. plicata.*

The stereochemistry of both double bonds in compound **3** assumed *Z* configurations due to those upfield shifted chemical shifts of allylic methylenes observed on the ^{13}C NMR spectrum (Ciminiello et al., 1991, Gunstone, 1993), similarly to the two double bonds of 4,7-decadienyl sulfate (Tsukamoto

et al.,1994).

During the isolation procedure, the antimicrobial placatamide (Tincu et al., 2003) was not detected from our species. MIC of purified **1**, **2**, and **3** against to the Gram positive *B. subtilis* showed 200 μg/mL. However, in the same concentration, they exhibited very weak antimicrobial activities against to the gram negative *Escherichia coli*, pathogenic yeast, *Candida albicans* and the mold, *Epidermophyton floccosum*. They showed different antimicrobial activity depends on the bacterial strain, Some sulfated alkenes have cytotoxicity (Aiello et al., 2000, Rosa et al., 1997) or ability to inhibit the matrix metalloproteinase 2 (MMP2, Fujita et al., 2002). Therefore, sulfated alkane and alkene have significant value for drug development. Details about in vitro antimicrobial activity to pathogenic bacteria, yeasts, fungi and another biological activities such as anti-oxidant and antitumor including physico-chemical properties will be published elsewhere.

Many alkyl sulfates having similar structure are using important material for surfactant. Sodium Lauryl Sulfate (SLS), as one of the Linear Alkyl Sulfate (LAS), made by sulfatation of lauryl alcohol and neutralization with sodium hydroxide, industrially. SLS is an lower toxic anionic surfactants used widely in cosmetics as cleansing agents (http://ijt.sagepub.com/content /2/7/127) and also, effective as microbicide against enveloped and non enveloped viruses (Piret et al., 2002) and HIV (Piret et al., 2000).

Some kind of alkyl sulfate sulfactants such as sodium octyl sulfate (SOS), sodium decyl sulfate (SDecS) and sodium dodecyl sulfate (SDS), were known as effective to the chemical shark repellent (Joseph and Nelson, 2001).

After 7-decene-1-ol was firstly reported from the unsafonifiable fraction of the *H. roretzi* (Kita, 1957), this unique flavor of ascidian were known that it was originated from the presence of many C-8 to C-10 saturated and unsaturated alcohols and named 2,7 decadienol as cynthiaol by Suzuki (1960) and the formation of peculiar ascidian flavors by octanol was reported(Fujimoto et al., 1982a). Furthermore, Fujimoto et al., (1982b) reported as those precursors of peculiar ascidian flavor in *H. roretzi* was C-8 to C-10 alkyl sulfates because they gave C-8 to C-10 alcohols by hydrolysis. From these fact, those alkyl sulfates may convert to those alcohols by some enzyme such as sulfatase in the body of ascidian.

From these results, sodium alkyl sulfates contained in ascidian show not only the role of peculiar flavor but also have other biological activities. It is need further study on the Industrial and medicinal utilization of those alkyl sulfates.

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