Isolation and Absolute Configuration Determination of Aliphatic Sulfates as the *Daphnia* Kairomones Inducing Morphological Defense of a Phytoplankton

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2,6-Dimethylheptyl sulfate (1) and 6-methyloctyl sulfate (3) were isolated from *Daphnia pulex* as the *Daphnia* kairomones that induced morphological defense of a freshwater phytoplankton *Scenedesmus gutwinskii* var. *heterospina* (NIES-802). The absolute stereochemistry at C2 of 1 was determined by ^{1}H-NMR analysis of the (*R*)-MTPA ester of alcohol 2. The absolute configuration at C6 of 3 was determined by Ohrui's method applied to alcohol 4.

Key words *Daphnia* kairomone; *Scenedesmus*; aliphatic sulfate; absolute configuration

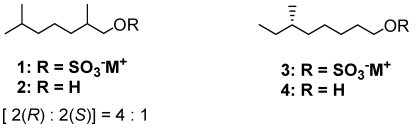
In aquatic environments, phytoplankton is the bottom creature playing an important role in the food chain. It has been soundly believed that phytoplankton is docile and never resists against its fate. Recently, it became clear that many phytoplankton resist their predators using various strategies. *Scenedesmus*, a unicellular fresh-water phytoplankton, resists its grazer by changing its morphology. Addition of filtered medium of *Daphnia*, a grazer of the plankton, to unicellular *Scenedesmus subspicatus* achieves morphological change into 2, 4, and 8 colonies within a few days. Such a change of morphology increases resistance of the colonies against grazer.[1)] This metamorphosis was supposed to be a self-defense mechanism acquired by the phytoplankton and triggered by a kairomone secreted from *Daphnia*.[1)]

Recently, we reported identification of the *Daphnia* kairomones that cause the morphological change in a unicellular green alga *Scenedesmus gutwinskii* var. *heterospina* (NIES-802) at 10^{-1}—10^3 ng/ml concentrations.[2)]

Here we report isolation and absolute configuration determination of new aliphatic sulfates **1** and **3** as the *Daphnia* kairomones. The absolute stereochemistrys at C2 of **1** and C6 of **3** were determined by ^{1}H-NMR analysis of the (*R*)-MTPA ester of alcohol **2** and Ohrui's method applied to alcohol **4**, respectively.

Frozen *Daphnia* (10 kg; Aso Tropical Fish Co. Ltd., Osaka) was soaked with methanol (20 l×3), and the methanol solution was evaporated, the residue being treated with water (9 l). The mixture was successively extracted with hexane, dichloromethane, and butanol, and the most active butanol extract was separated by HPLC monitoring the activity[2)] to afford **1** (9 mg) and **3** (5 mg) (Fig. 1).

The molecular formulae of **1** and **3** were established as $C_9H_{19}O_4S$ on the basis of HR-FAB-MS. The presence of a sulfate group was suggested by a fragment ion peak at *m/z* 97 (HSO_4^-) in the negative ion FAB-MS of **1** and **3**. The ^{1}H-NMR spectrum of **1** exhibited three doublet methyls at δ 0.98 (3H, d, J=6.7 Hz, Me-2) and 0.91 (6H, d, J=6.6 Hz, H-7, Me-6), two protons of a methylene bearing a sulfate group at δ 3.90 (1H, dd, J=9.4, 5.8 Hz, H-1) and 3.81 (1H, dd, J=9.4, 6.7 Hz, H-1) and eight methine/methylene protons at δ 1.12—1.88. Interpretation of the ^{1}H–^{1}H COSY spectrum of **1** led to a gross structure as 2,6-dimethylheptyl sulfate.[3—5)]



1: R = $SO_3^-M^+$
2: R = H
[2(*R*) : 2(*S*)] = 4 : 1

3: R = $SO_3^-M^+$
4: R = H

Fig. 1. The Structure of *Daphnia* Kairomones **1** and **3**
The countercations were not identified and expressed as M^+.

The absolute stereochemistry at C-2 of **1** was determined by the ^{1}H-NMR analysis of (*R*)-α-methoxy-α-(trifluoromethy)phenylacetic acid (MTPA) ester of **2**, a hydrolysis product. It has been established that,[6)] in the ^{1}H-NMR of an MTPA ester of a 2(*S*)-methyl aliphatic alcohol, the chemical shift difference of the methylene protons at C1 (appearing as an ABX pattern) is larger in the (*S*)-MTPA ester than in the (*R*)-MTPA ester. The reverse holds true for a 2(*R*)-methyl alcohol.

Sulfate **1** was hydrolyzed by heating with 3 M HCl, and the resulting alcohol **2** was treated with an excess of (+)-(*S*)-α-methoxy-α-(trifluoromethy)phenylacetyl chloride (MTPA-Cl) in pyridine-d_5 to yield the (*R*)-MTPA ester (**5**). In its ^{1}H-NMR spectrum, two sets of the AB signals of ABX patterns due to the methylene protons at C1 appear at δ 4.33 (1H, dd, J=10.7, 5.8 Hz) and 4.16 (1H, dd, J=10.7, 6.6 Hz) [major: 2(*R*)-**5** (=**10**)], and at 4.26 (1H, dd, J=10.7, 6.1 Hz) and 4.23 (1H, dd, J=10.7, 5.9 Hz) [minor: 2(*S*)-**5** (=**9**)] (Fig. 2). These ^{1}H-NMR data for the MTPA esters (**5**) indicated that natural **1** was a 4 : 1 mixture of 2(*R*) and 2(*S*) enantiomers (Fig. 2).

We were interested which enantiomar had stronger activity. Therefore, we synthesized 2(*R*)- and 2(*S*)-**1** by the reactions shown in Chart 1. The olefin of aldehyde **6** was hydrogenated over Pd-C catalyst, followed by reduction of the subsequent aldehyde to primary alcohol with $LiAlH_4$. The racemic alcohol (*rac*-**2**)[7)] was esterified with (*S*)-methoxy-(1-naphthyl) acetic acid ((*S*)-1NMA).[8,9)] The (*S*)-1NMA esters were separated to **7** and **8** by recycling HPLC. Hydrolysis of **7** and **8** afforded 2(*S*)- and 2(*R*)-**2**,[7)] respectively, the absolute configuration of which was confirmed by the MTPA method: (*R*)-MTPA ester (**9**) of 2(*S*)-**2** shows an overlapped AB pattern of H_2-1, while (*R*)-MTPA ester (**10**) of 2(*R*)-**2** shows a well-separated AB pattern (Experimental). Interestingly, the

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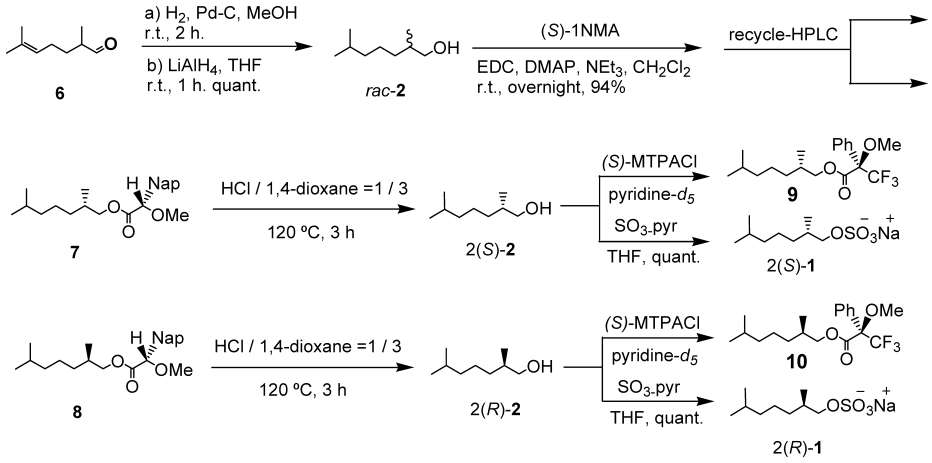

Chart 1. Synthesis of (*R*)-MTPA Esters **9** and **10**, and Sulfates 2(*S*)-**1** and 2(*R*)-**1**

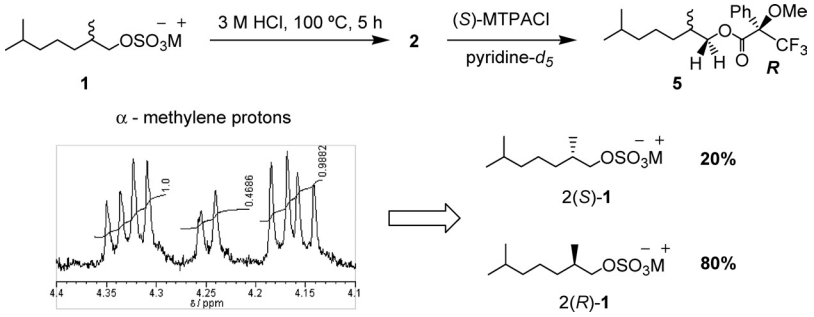

Fig. 2. Determination of the Absolute Configuration of **1**

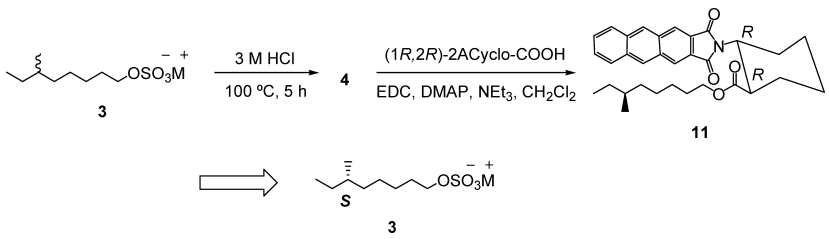

Fig. 3. Determination of the Absolute Configuration of **3**

(*S*)-1NMA derivatives **7** and **8** show the ^{1}H-NMR patterns of H_2-1 opposite to those of the MTPA esters: the (*S*)-1NMA ester of 2(*S*)-**2** exhibits a narrow AB pattern of H_2-1 and (*S*)-MTPA ester of 2(*S*)-**2** is a well separated pattern (Experimental). 2(*S*)- and 2(*R*)-**2** were sulfated, giving 2(*S*)-**1** and 2(*R*)-**2**, respectively. Then, we examined that the morphological change activity of the compounds. But, there were no difference in the activity between 2(*R*)- and 2(*S*)-sulfates.

The ^{1}H-NMR spectrum of **3** reveals two methyl groups at δ 0.92 (3H, t, J=7.8 Hz, H-8), 0.91 (3H, d, J=7.4 Hz, Me-6), methylene protons bearing a sulfate group at δ 4.03 (2H, t, J=6.6 Hz, H-1), and 11 methine/methylene protons at δ 1.73—1.10. The ^{1}H–^{1}H COSY, HSQC and HMBC NMR spectra of **3** were consistent with the structure of 6-methyloctyl sulfate. To the best of our knowledge, **3** is a new compound. The absolute stereochemistry at C-6 of **3** was determined by the ^{1}H-NMR analysis of a (1*R*,2*R*)-2-(2,3-anthracenedicarboximido)cyclohexanecarboxylic acid ((1*R*,2*R*)-2ACyclo-COOH, Ohrui reagent) ester.[10]

The alcohol derivative **4**, obtained by hydrolysis of **3** with 3 M HCl, was condensed with (1*R*,2*R*)-2ACyclo-COOH to yield ester **11**. The methyl proton signals of the ester appeared at δ 0.70 (3H, t, J=7.2 Hz, H-8) and 0.62 (3H, d, J=6.6 Hz, Me-6). These chemical shifts are the same with those of the methyl protons of (1*R*,2*R*)-2ACyclo-COOH ester of 6(*S*)-methyloctan-1-ol,[10] leading to the 6(*S*)-configuration of **3**. This experiment also indicated that natural 6(*S*)-**3** was free from its antipode.

Experimental

High-resolution MS were recorded on a JEOL JMS-SX102A spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker Avance-400 (^{1}H and ^{13}C at 400 and 100 MHz, respectively). Assignments of the proton and carbon signals were established by COSY, HSQC and HMBC spectra. Optical rotations were determined on a JASCO DIP-370 polarimeter.

Bioassay Each 200 ml of C medium of *S. gutwinskii* (5.0×10^2 cells/ml) is delivered into the central 30—50 wells of 96-well polystyrene tissue culture plate (CELLSTAR, Greiner Bio-one Co., Ltd) containing the test samples (1000—0.01 ng/ml), and the outer wells are filled with distilled water to avoid dehydration of the system. The plate is covered with a plastic lid, and

incubated at 20 °C (12 light/12 dark) for 10 d. A drop of the medium is placed on a Thoma's hemacytometer, and the numbers of 1-, 2-, 4-, and 8-cell types were counted under a microscope (×200).

Extraction and Isolation Frozen *Daphnia* (10 kg; Aso Tropical Fish Co. Ltd., Osaka) was soaked with methanol (20 l×3), and the methanol solution was evaporated, the residue being treated with water (9 l). The mixture was successively extracted with hexane (9 l), dichloromethane (9 l), and butanol (9 l), and the most active butanol extract (18 g) was chromatographed on a Cosmosil 75C_{18}-OPN (25 g), eluting with MeOH–H_2O in a gradient manner (1 : 1→10 : 0). The active fractions were further purified by HPLC (CAPCELLPAK C_{18} column, 5 μm, 10×250 mm, MeCN–H_2O (40 : 60) containing 250 mM $NaClO_4$ as mobile phase with the flow rate 1.0 ml/min (using an RI detector)) to afford **1** (9 mg) and **3** (5 mg).

2,6-Dimethylheptyl Sulfate (1) The spectral data (^{1}H-, ^{13}C-NMR and MS) were in excellent agreement with those previously reported.[3,4]

2,6-Dimethylheptyl (*R*)-α-Methoxy-α-(trifluoromethyl)phenylacetate (5) **1** (2.7 mg) was dissolved in a 3 M HCl solution (0.5 ml) and the solution was heated at 100 °C for 5 h. Dichloromethane (CH_2Cl_2) and water were added to the solution. The organic phase was dried over Na_2SO_4 and evaporated *in vacuo* to give alcohol (**2**). This alcohol was dissolved in pyridine-d_5 (100 μl) and to the solution was added (+)-(*S*)-MTPA-Cl (15 μl). The mixture was allowed to stand at room temperature for 30 min, diluted with pyridine-d_5 (400 μl), and the ^{1}H-NMR spectrum was recorded. The spectrum showed complete formation of the (*R*)-MTPA ester (**5**) without the starting alcohol. ^{1}H-NMR (C_5D_5N) δ: (The phenyl protons were obscured by the solvent signals.) 4.33 (1H, dd, J=10.7, 5.8 Hz, major H-1), 4.26 (1H, dd, J=10.7, 6.1 Hz, minor H-1), 4.23 (1H, dd, J=10.7, 5.9 Hz, minor H-1), 4.16 (1H, dd, J=10.7, 6.6 Hz, major H-1), 1.77 (1H, octet, J=6.8 Hz, H-2), 1.42 (1H, nonet, J=6.6 Hz, H-6), 1.35—0.98 (6H, m, H-3, 4 , 5), 0.86 (3H, d, J=6.6 Hz, Me-2), 0.81 (6H, d, J=6.6 Hz, H-7, Me-6).

2,6-Dimethylheptan-1-ol (*rac*-2) To 255 mg (1.8 mmol) of 2,6-dimethyl-5-hepten-1-al (**6**: commercially available) in MeOH (5 ml) was added Pd-C catalyst (24 mg, 10% w/w). The reaction mixture was stirred at room temperature for 2 h under hydrogen atmosphere. The mixture was filtered and the aldehyde (265 mg, 1.8 mmol) was obtained after concentration. To a solution of 265 mg (1.8 mmol) of the aldehyde in THF (6 ml) was added 212 mg (5.5 mmol) of $LiAlH_4$. After 1 h, the reaction was quenched by adding 200 μl of H_2O, 15% NaOH, and then 500 μl of H_2O. The mixture was diluted with 10 ml EtOAc and filtered through Celite. The filtrate was concentrated under reduced pressure to furnish 265 mg (1.8 mmol, 99% yield) of the racemic alcohol (*rac*-**2**). ^{1}H-NMR ($CDCl_3$) δ: 3.45 (1H, dd, J=10.4, 5.9 Hz, H-1), 3.35 (1H, dd, J=10.4, 6.7 Hz, H-1), 1.57 (1H, m, H-2), 1.49 (1H, nonet, J=6.6 Hz, H-6), 0.97—1.38 (6H, m, H-3, 4, 5), 0.87 (3H, d, J=6.6 Hz, Me-2), 0.83 (6H, d, J=6.6 Hz, H-7, Me-6). ^{13}C-NMR ($CDCl_3$) δ: 68.2 (C-1), 39.2 (C-5), 35.7 (C-2), 33.4 (C-3), 27.9 (C-6), 24.7 (C-4), 22.7 and 22.6 (C-7, Me-6), 16.6 (Me-2).

(*S*)- and (*R*)-2,6-Dimethylheptyl (*S*)-Methoxy-(1-naphthyl)acetates (7) and (8) To a CH_2Cl_2 solution (5 ml) of *rac*-**2** (116 mg, 0.81 mmol) were added 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (470 mg, 2.4 mmol), 4-dimethylaminopyridine (DMAP) (300 mg, 2.4 mmol), NEt_3 (510 μl, 3.6 mmol) and (*S*)-methoxy-(1-naphthyl)acetic acid (1NMA) (261 mg, 1.2 mmol). After the mixture was stirred for 12 h, 10 ml of CH_2Cl_2 was added, and the organic layer was washed with 10% aqueous citric acid, 5% sodium hydrogen carbonate, H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo* to yield a crude ester. The crude ester was purified by SiO_2 column chromatography with hexane/EtOAc solvent system to afford the (*S*)-1NMA ester (261 mg, 0.76 mmol, 94%). The diastereomeric (*S*)-1NMA ester (261 mg, 0.76 mmol) was separated by 11 times recycling HPLC with 80% aqueous methanol on CAPCELLPAK C_{18} to yield the first-eluting ester (**7**) (100 mg, 0.29 mmol) and the second-eluting ester (**8**) (90 mg, 0.26 mmol). **7**: ^{1}H-NMR ($CDCl_3$) δ: 8.29 (1H, d, J'8.3 Hz), 7.84 (2H, t, J=8.8 Hz), 7.61 (1H, d, J=6.8 Hz), 7.49 (3H, m), 5.39 (1H, s), 3.94 (1H, dd, J=10.5, 6.6 Hz, H-1), 3.89 (1H, dd, J=10.5, 5.8 Hz, H-1), 3.45 (3H, s), 1.58 (1H, octet, J=6.6 Hz), 1.36 (1H, nonet, J=6.4 Hz), 1.14—0.82 (6H, m), 0.79 (6H, d, J=6.6 Hz), 0.67 (3H, d, J=6.8 Hz). ^{13}C-NMR ($CDCl_3$) δ: 170.8, 133.8, 132.3, 131.1, 129.3, 128.6, 126.6, 126.4, 125.7, 125.1, 124.0, 81.1, 69.9, 57.4, 38.9, 33.1, 32.5, 27.8, 24.4, 22.6, 22.6, 16.6. **8**: ^{1}H-NMR ($CDCl_3$) δ: 8.29 (1H, d, J=8.6 Hz), 7.84 (2H, t, J=8.6 Hz), 7.62 (1H, d, J=7.1 Hz), 7.49 (3H, m), 5.39 (1H, s), 3.96 (1H, dd, J=10.5, 6.3 Hz, H-1), 3.87 (1H, dd, J=10.5, 6.6 Hz, H-1), 3.46 (3H, s), 1.60 (1H, octet, J=6.4 Hz), 1.39 (1H, nonet, J=6.6 Hz), 1.13—0.85 (6H, m), 0.80 (6H, d, J=6.6 Hz), 0.65 (3H, d, J=6.6 Hz). ^{13}C-NMR ($CDCl_3$) δ: 170.8, 133.8, 132.2, 131.0, 129.3, 128.6, 126.5, 126.3, 125.7, 125.1, 124.0, 81.0, 69.9, 57.4, 39.0, 33.2, 32.4, 27.8, 24.3, 22.6, 22.6, 16.5.

Hydrolysis of (*S*)- and (*R*)-2,6-Dimethylheptyl (*S*)-Methoxy-(1-naphthyl)acetate The first-eluting ester (**7**) (100 mg, 0.29 mmol) was dissolved in a dioxane–conc. HCl mixture 3 : 1 (1.5 ml) and the mixture was heated at 110 °C for 3 h. Water was added to the cooled solution before extraction with hexane. The organic layer was washed with 5% sodium hydrogen carbonate and H_2O, dried over Na_2SO_4, and concentrated *in vacuo* to yield (*S*)-2,6-dimethylheptan-1-ol (2(*S*)-**2**) (40 mg, 0.27 mmol, 95%). Hydrolysis of the second-eluting ester (**8**) (90 mg, 0.26 mmol) was performed under the same condition, affording (*R*)-2,6-dimethylheptan-1-ol (2(*R*)-**2**) (35 mg, 0.24 mmol, 92%).

(*S*)-2,6-Dimethylheptyl (*R*)-α-Methoxy-α-(trifluoromethyl)phenylacetate (9) To a pyridine-d_5 solution (100 μl) of 2(*S*)-**2** (1 mg) was added (+)-(*S*)-MTPACl (15 μl) and the mixture was allowed to stand at room temperature for 30 min, the solution diluted with pyridine-d_5 (400 μl), and the ^{1}H-NMR spectrum was recorded. The ^{1}H-NMR spectrum indicated complete formation of the (*R*)-MTPA ester (**9**). ^{1}H-NMR (C_5D_5N) δ: (The phenyl protons were obscured by the solvent signals.) 4.26 (1H, dd, J=10.7, 6.1 Hz, H-1), 4.23 (1H, dd, J=10.7, 5.9 Hz, H-1), 1.77 (1H, octet, J=6.8 Hz, H-2), 1.41 (1H, nonet, J=6.8 Hz, H-6), 1.34—0.97 (6H, m, H-3, 4, 5), 0.87 (3H, d, J=6.8 Hz, Me-2), 0.81 (6H, d, J=6.6 Hz, H-7, Me-6).

(*R*)-2,6-Dimethylheptyl (*R*)-α-Methoxy-α-(trifluoromethyl)phenylacetate (10) To a pyridine-d_5 solution (100 μl) of 2(*R*)-2 (1 mg) was added (+)-(*S*)-MTPA-Cl (15 μl) at room temperature for 30 min, the solution diluted with pyridine-d_5 (400 μl), and the ^{1}H-NMR spectrum was recorded for the (*R*)-MPTA ester (**10**). ^{1}H-NMR (C_5D_5N) δ: (the phenyl protons were obscured by the solvent signals) 4.33 (1H, dd, J=10.7, 5.8 Hz, H-1), 4.16 (1H, dd, J=10.7, 6.6 Hz, H-1), 1.77 (1H, octet, J=6.8 Hz, H-2), 1.41 (1H, nonet, J=7.1 Hz, H-6), 1.35—0.98 (6H, m, H-3, 4, 5), 0.86 (3H, d, J=6.8 Hz, Me-2), 0.81 (6H, d, J=6.6 Hz, H-7, Me-6).

(*S*)-2,6-Dimethylheptyl Sulfate (2(*S*)-1) The alcohol 2(*S*)-**2** (36 mg, 0.25 mmol), obtained by hydrolysis of the 1NMA ester (**7**) was converted to the sulfate by treatment with pyridine–SO_3 complex (159 mg, 1.00 mmol) in tetrahydrofuran (3 ml) at room temperature for 24 h. The resulting mixture was neutralized with 1 M sodium hydroxide solution and the aqueous solution was extracted with hexane to remove the residual alcohol. The aqueous layer was passed through an ODS column. Mineral salt was removed by washing the column with distilled water and elution with methanol gave 2(*S*)-**1** (58 mg, 0.25 mmol). The ^{1}H- and ^{13}C-NMR spectra of this product were identical with those of natural **1**. HR-FAB-MS *m*/*z*: 223.1004 (Calcd for $C_9H_{19}O_4S$: 223.1004). $[\alpha]_D^{25}$ +2.6° (c=0.9, MeOH).

(*R*)-2,6-Dimethylheptyl Sulfate (2(*R*)-1) The sulfate (2(*R*)-**1**) was obtained in the same manner as described for (2(*S*)-**1**). HR-FAB-MS *m*/*z*: 223.0978 (Calcd for $C_9H_{19}O_4S$: 223.1004). $[\alpha]_D^{25}$ −0.9° (c=1.0, MeOH).

(*S*)-6-Methyloctyl Sulfate (3) Negative HR-FAB-MS: *m*/*z*: 223.0975 (Calcd for $C_9H_{19}O_4S$: 223.1004). ^{1}H-NMR (CD_3OD) δ: 4.03 (2H, t, J=6.6 Hz, H-1), 1.70 (2H, quint, J=6.6 Hz, H-2), 1.48—1.29 (7H, m, H-3, 4, 5a, 6, 7a), 1.24—1.13 (2H, m, H-5b, 7b), 0.92 (3H, t, J=7.8 Hz, H-8), 0.91 (3H, d, J=7.4 Hz, Me-6). ^{13}C-NMR (CD_3OD) δ: 61.9 (C-1), 37.7 (C-5), 35.6 (C-6), 30.6 (C-7), 30.5 (C-2), 27.8 (C-4), 27.2 (C-3), 19.6 (C-9), 11.7 (C-8). $[\alpha]_D^{25}$ −1.9° (c=0.15, MeOH).

(*S*)-6-Methyloctyl (1*R*,2*R*)-2-(2,3-Anthracenedicarboximido)cyclohexanecarboxylate (11) Sulfate **3** (1.8 mg, 7.5 μmol) was dissolved in 3 M HCl (0.5 ml) and the solution was heated at 100 °C for 5 h. Dichloromethane and water were added to the solution. The organic phase was dried over Na_2SO_4, and evaporated *in vacuo* to give the alcohol (**4**). To a CH_2Cl_2 solution of the alcohol (**4**), were added EDC (1 mg, 5.2 μmol), DMAP (0.6 mg, 4.9 μmol), NEt_3 (1 μl, 6.3 μmol) and (1*R*,2*R*)-2-(2,3-anthracenedicarboximido)cyclohexanecarboxylic acid (0.6 mg, 1.5 μmol). After the mixture was stirred for 16 h at room temperature, the crude ester was purified by SiO_2 column chromatography with hexane/EtOAc solvent system to afford the ester **11**.

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