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Synthesis and identification of an endogenous sperm activating and attracting factor isolated from eggs of the ascidian *Ciona intestinalis*; an example of nanomolar-level structure elucidation of novel natural compound

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Abstract—A sperm-activating and attracting factor (SAAF) was isolated from the eggs of the ascidian *Ciona intestinalis*, and its structure was deduced with only approximately 4 μg of the specimen. Based upon the proposed structure, two epimers were synthesized from chenodeoxycholic acid in 16 steps. Comparison between synthetic and natural compounds led to the unambiguous structure determination of SAAF to be (3*R*,4*R*,7*R*,25*S*)-3,4,7,26-tetrahydroxycholestane-3,26-disulfate. The synthetic pure specimen was also utilized to confirm that both sperm-activation and attraction were elicited by a single compound.

1. Introduction

Sperm chemotractants, ubiquitously found in the animal kingdom, play an essential role in fertilization.[1,2] Chemotaxis of sperm toward an egg is a crucial event particularly in aquatic environment, where sperm frequently travels long distance to run into an egg. The relevant factors may control the chance of intra- or inter-species mating, which may take part in securing biodiversity, and thus have been a target of biological research for a long time. To investigate the molecular basis of fertilization, the pure specimen of a sperm attractant is indispensable, particularly for tracking signal transduction from chemoreception to flagellar movement. Although numerous peptides and small organic compounds have been so far proposed as a candidate of chemoattractant,[3,4] only a few of them have been unambiguously identified as an endogenous factor,[5] such as those for the sea urchin *Arbacia punctulata*,[6] the coral *Montipora digitata*,[7] and the frog *Xenopus laevis*. The difficulty in these studies lies in the quantification of chemotactic activity of sperm. Two of the authors at Misaki Marine Biological Station have extensively examined the chemotaxis in ascidian and clearly demonstrated that sperm activation and attractant are caused by different mechanism.[8,9] In the present study, we report the structural elucidation and synthesis of the sperm-activating and attracting factor (SAAF) from a species of acidean.[10,11]

2. Results and discussion

2.1. Structure determination

SAAF was extracted and purified from the eggs of the ascidian *Ciona intestinalis* as previously described.[12,13] During the purification procedure, the sperm-activating and attracting activities were always found in same fractions, indicating that both activities were elicited by a single compound. The chemical structure of SAAF was deduced based on NMR and mass spectrometry. The molecular-related ions were observed in negative ion FAB MS at *m/z* 617 and 595, which corresponded to $(X-H)^-$ and $(X-Na)^-$, respectively. In addition, a prominent ion peak at *m/z* 515 corresponded to a fragment lost typical for a sulfate ester ($-SO_3Na+H$). These data indicate that SAAF has two sulfate ester groups and, after one of them is lost, it still possesses a negatively charged group (=sulfate) to give rise to a prominent ion at *m/z* 515. High-resolution ESI-TOF MS further supported the presence of two sulfate esters by giving rise to the exact mass of *m/z* 297.1273 for a divalent anion, corresponding to $C_{27}H_{48}O_{18}S_2$ (calculated mass, *m/z* 297.1266). Two-dimensional ^1H NMR (2D TOCSY) spectrum in Figure 3 was successfully determined with

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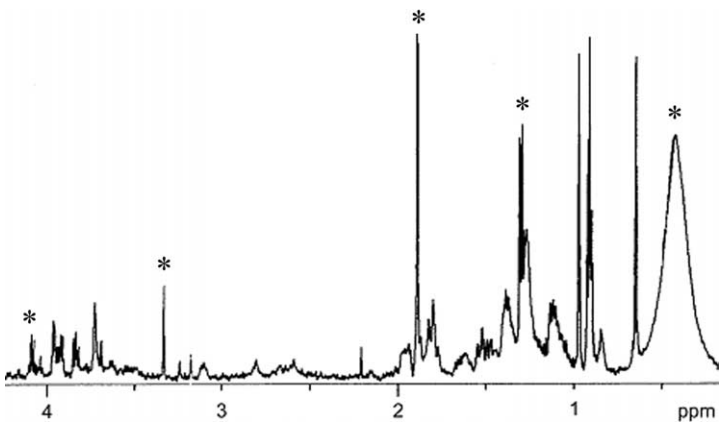


Figure 1. ^{1}H NMR spectrum of SAAF (**1**, ca. 4 μg); D_2O, 500 MHz. The spectrum was measured with using a special probe (Nanolac, Z-Spec-SMIDG500) for 1.7-mm diameter sample tube on a JEOL L-500 spectrometer. *Signals due to impurities. The broad peak at δ 0.4 is due to silicon grease outside of the tube used for sliding the tube into a spinner.

approximately 4 μg based upon an NMR spectrum in comparison with that of a synthetic specimen. The spectra revealed two singlet methyl signals at δ 0.65 and 0.97 characteristic of steroid skeletons. Additional two methyl doublets at δ 0.90 and 0.92 and complex signals at 0.8–2.0 ppm further support the presence of a steroid backbone. The down-field signals at δ 4.41, 3.96, 3.92, 3.83, and 3.72 correspond to five protons on four oxygen-bearing carbon atoms (those at δ 3.92/3.83 were apparently derived from a methylene group). Among these, the connectivity of signals at δ 4.41, 3.92, and 3.72 was elucidated by 2D TOCSY (Fig. 3). This structural moiety fits the C3–C7 part of a steroid skeleton. Signals at δ 3.92 and 3.83, which revealed geminal coupling, were assigned to a methylene group of C26 on the basis of relayed coupling to one of the methyl doublets. The location of sulfate groups was deduced from low-field chemical shifts of signals at δ 4.41 and 3.92/3.83 in comparison with those of hydroxyl-bearing methine and methylene groups. Moreover, their chemical shifts agreed well with reported values for starfish sterol sulfates.[12] Those spectral data indicated that SAAF was a novel sulfated steroid, 3,4,7,26-tetrahydroxy-cholestane-3,26-disulfate (Fig. 1).

High-energy collision-induced dissociation (CID) MS/MS is a versatile tool for structural elucidation of ionic compounds.[13] We adopted the tandem MS instrument of EB–EB geometry with the negative-ion fast atom bombardment (FAB) ionization method. The product ion spectrum was recorded for precursor ions at *m/z* 515, which corresponds to a desulfated product of SAAF presumably generated through the first MS section. The structural basis of the assignment for major product ions is shown in Figure 2. These fragmentations are reasonably explained by the

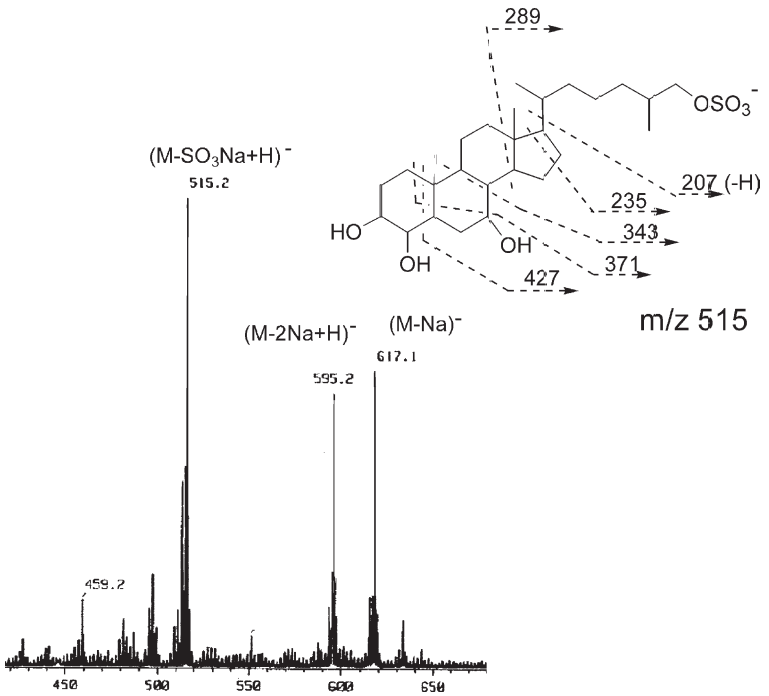
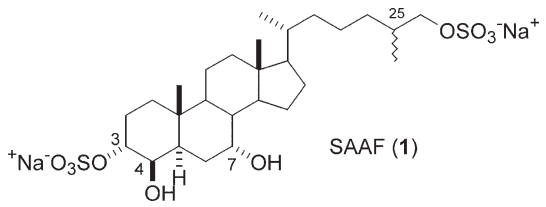


Figure 2. Normal FAB spectrum of SAAF (1) and fragment patterns for 3-*O*-defulfated ion of SAAF. Product ion spectra were obtained by CID MS/MS experiments. See text for details.[10]

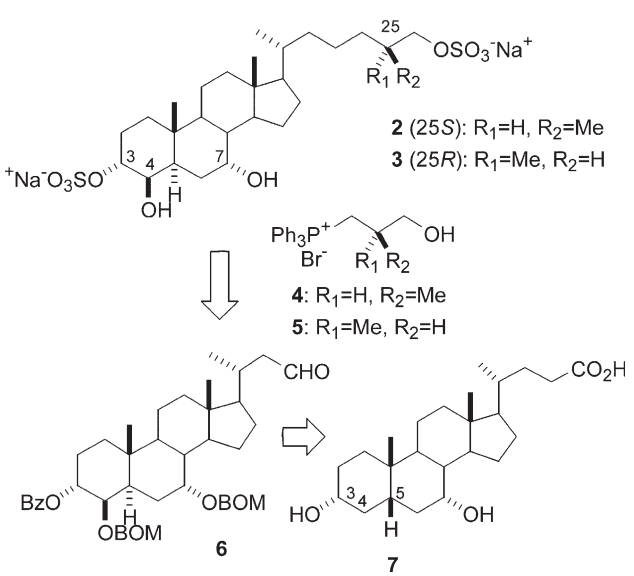
proposed structure, particularly for the part of three oxygen functionalities, hence confirming the planner structure of SAAF.



This working structure of SAAF was derived from limited NMR and MS data, hence leaving some ambiguities to be addressed. For the sterol skeleton, where ^{1}H NMR signals heavily overlap, no clear evidence was obtained regarding stereochemistry. Chemical synthesis was thus necessary for the complete structure identification. For this purpose, we had to deduce the configuration of SAAF. First, the skeletal stereostructure was deduced to be cholestan by the following two reasons; most of sterols isolated from tunicates possess a cholestan skeleton and a large coupling constant between H-5 and H-6 suggested the *trans* fusion of rings A and B. The stereochemistry of oxygenated carbon atoms was deduced from the small coupling constants for the signals at δ 4.41, 3.96, and 3.72 as one can observe in the attached 1D spectrum of Figure 3. The data clearly reveal that no diaxial coupling is involved in these spin systems, which indicates that all the three oxygen atoms were axially oriented. The stereochemistry at C25, which could not be assigned by NMR, remained to be elucidated by synthesizing both epimers at this positions.

2.2. Synthesis of SAAF and 25-*epi*-SAAF

Synthesis plan of SAAF and its C-25 epimer is shown in Scheme 1. We planed a versatile route that could provide both diastereomers by introducing the side chain using **4** and **5** at the latest steps via a common intermediate **6**, which would be synthesized from chenodeoxycholic acid **7**.



Scheme 1. Synthesis plan of SAAF and 25-*epi*-SAAF.

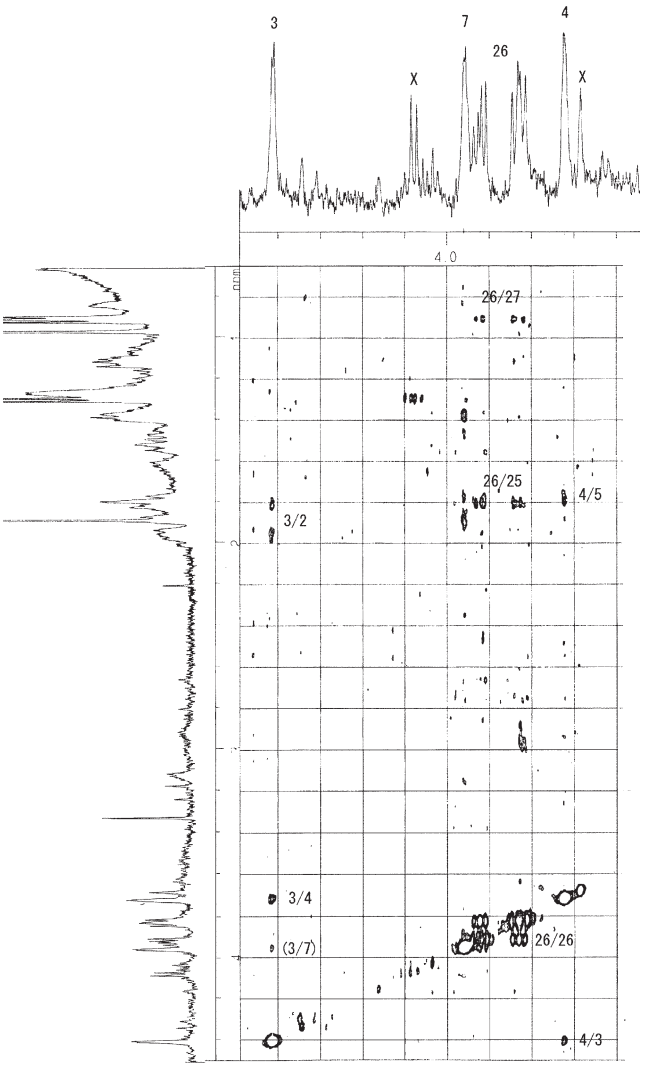
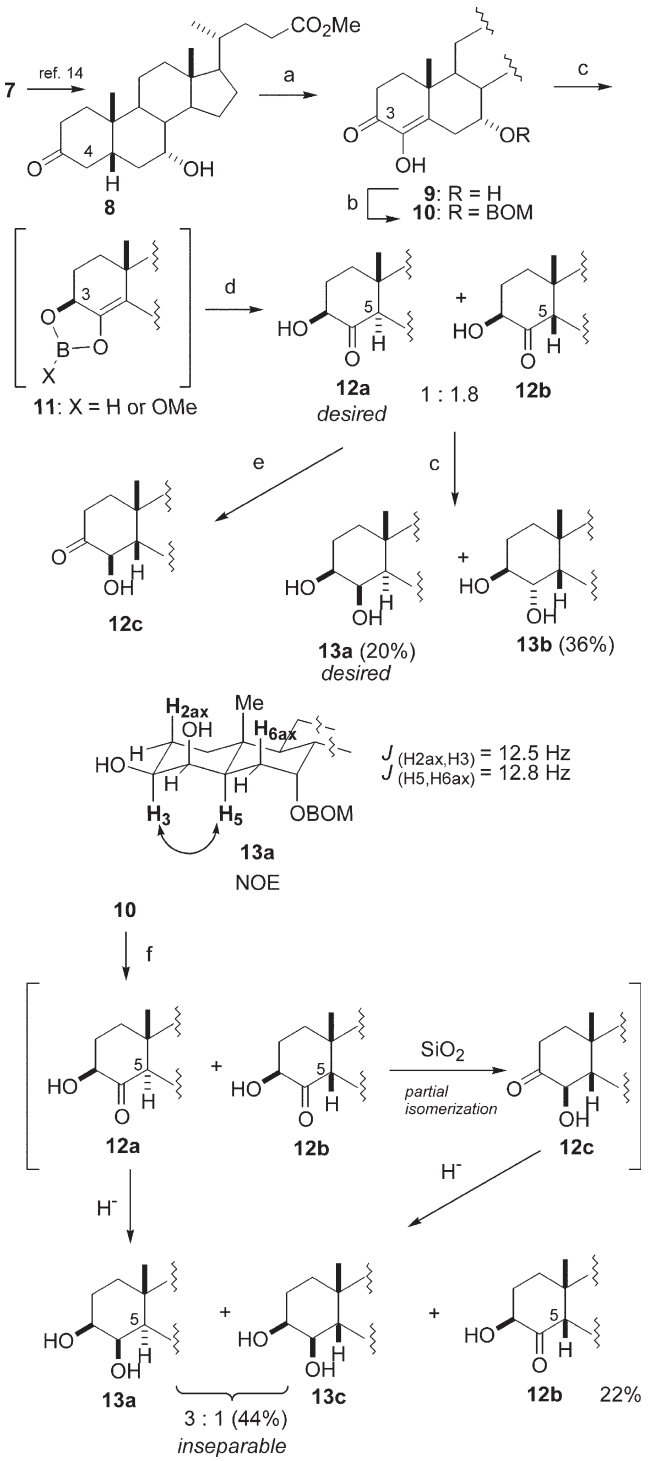


Figure 3. TOCSY spectrum of SAAF (**1**) of an extremely limited amound (ca. 4 μg). The spectrum was measured as the same conditions as those in Figure 1.

Synthesis of SAAF commenced with 7α-hydroxy-3-oxo-5β-cholan-24-oate (**8**), prepared from **7** in two steps (Scheme 2).[14] Oxidation of **8** with molecular oxygen in the presence of *t*-BuOK proceeded regioselectively via the C-4 enolate, and following esterification of the concomitantly hydrolyzed product afforded 3-keto-4-enol **9**. Selective protection of the C-7 alcohol of **9** as its benzyloxymethyl (BOM) ether in the presence of C-4 enol yielded **10**. Reduction of **10** with $NaBH_4$ proceeded stereoselectively at C-3 to give cyclic enol borate **11**, and hydrolysis of **11** with 1 M HCl yielded 4-keto-3-ol **12a** concomitant with its C-5 epimer **12b** as an inseparable mixture in a 1:1.8 ratio. Treatment of the mixture with $NaBH_4$ gave the desired diol **13a** from **12a** and diastereomer **13b** from **12b** in 20 and 36%, respectively. The stereochemistry of **12a** was unambiguously determined by ^{1}H NMR analysis. Isomerization of **12b** to **12a** under the basic conditions failed to give 3-keto-4-ol **12c** as a single isomer. Attempts to reduce **9** using other conventional methods were unsuccessful due to the formation of undesired diastereomers preferentially with low reproducibility. Finally, a solid-phase reduction of **10** with $NaBH_3CN$ on



Scheme 2. Reagents and conditions: (a) *t*-BuOK, O_2, *t*-BuOH, then CH_2N_2, Et_2O, MeOH, $CHCl_3$ (71%); (b) BOMCl, *i*-Pr_2NEt, CH_2Cl_2 (60%); (c) $NaBH_4$; $CHCl_3$, MeOH, 0 °C; (d) 1 M HCl; (e) DBU, THF, 60 °C; (f) $NaBH_3CN$, SiO_2, $CHCl_3$, MeOH, then evaporated.

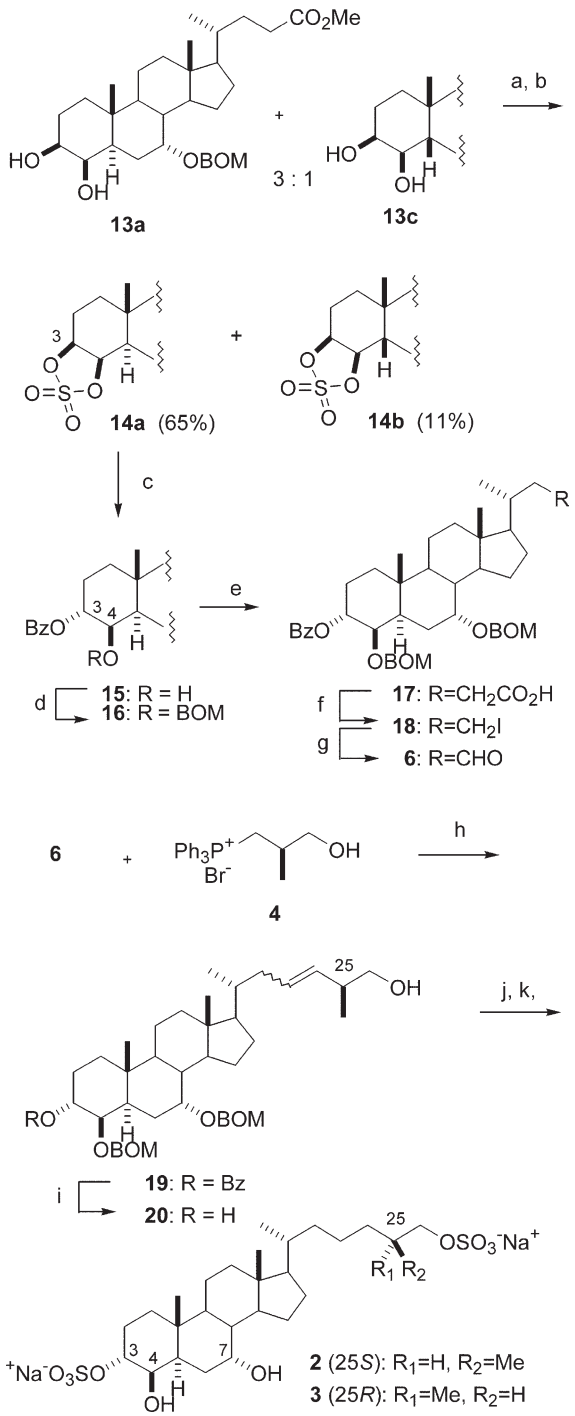
silica gel was found to afford desired diol **13a** in moderate yield. A solution of **10** in chloroform and methanol was added $NaBH_3CN$ (5 equiv.) and silica gel (**10**–silica gel=1:3, w/w). After stirring for 10 min the solvent was removed under reduced pressure, and the residue was stirred without solvent. Under the reaction conditions, hydrolysis of the borate **11** proceeded smoothly to give a mixture of **12a** and **12b**. Further reduction of **12a** proceeded smoothly to give **13a**, whereas **12b** remained intact and easily separated from **13a**. However, partial isomerization of **12b** occurred to give **12c**, which was further reduced to **13c** resulting in the formation of inseparable mixture of **13a** and **13c** in a ratio of 3:1 (44%). Even though the yield of **13a** should be improved, the contiguous stereogenic centers at C-3, C-4, and C-5 on the steroid framework were properly installed for the next operations.

Treatment of the mixture of diols **13a** and **13c** with thionyl chloride followed by oxidation with RuO_2–$NaIO_4$ gave cyclic sulfate **14a** in 65% yield for two steps,[15] which was easily separated from 5β-isomer **14b** (11%) by silica gel chromatography (Scheme 3). Regioselective opening of the cyclic sulfate at C-3 position was successfully achieved by treating with benzoic acid in the presence of Cs_2CO_3 in DMF (75%) followed by hydrolysis of the resulting sulfate.[16] Protection of the resulting C-4 alcohol (**15**) as BOM ether afforded benzoate **16** as a single isomer (71%). Exposure of **16** to *t*-BuOK in *t*-BuOH resulted in the selective hydrolysis of the methyl ester in the presence of benzoate ester to yield carboxylic acid **17** (95%), which was further converted to iodide **18** through the decarboxylation by treating with $Pb(OAc)_4$ in the presence of iodine under irradiation using a tungsten lamp (84%).[17]Oxidation of **18** with DMSO in the presence of 2,4,6-collidine gave an aldehyde **6** (94%), a common precursor of the two diastereomers at C-25. Elongation of the side chain was achieved through a Wittig reaction between the aldehyde **6**, and an ylide generated from (*R*)-phosphonium salt **4**[18] and *n*-BuLi–TMSCl[19] to give olefin **19** (*E*/*Z*=1:8) in 69% yield. Reductive removal of the benzoyl group of **19** with $LiAlH_4$ gave diol **20**, which was converted to the corresponding sodium bis-sulfate through the successive treatment with SO_3·Py and ion-exchange resin (IR-120B, Na^+ form). Hydrogenation of the double bond and concomitant removal of the BOM groups afforded 25*S*-isomer (**2**), and the resulting polar material was purified by reverse-phase HPLC. The C-25 epimer **3** was synthesized by the identical procedure as **2** except for the use of (*S*)-phosphonium salt **5** in place of **4**.

2.3. Comparison with the natural product

The ^1H NMR spectrum of the natural product was compared with those of the synthetic samples, **2** (25*S*) and **3** (25*R*), as shown in Figure 4.[10] The areas other than those in Figure 4 were indistinguishable among the natural SAAF and the synthetic pair. While the chemical shifts of the 25-methyl group and H-26a in **3** do not match those of the natural product ($\Delta\delta$ values for H-26a and 25-methyl are −0.014 and −0.007 ppm, respectively), those of **2** are identical with SAAF as are other resonances of the steroid framework.[4] Thus, the configuration of the side chain was confirmed as 25*S*, resulting in the first synthesis of SAAF. When the bioactivity and biosynthetic rationality are taken into account, the absolute stereochemistry is assigned to be 3*R*, 4*R*, 7*R*, and 25*S*.

Both the sperm-activating and attracting activity of synthetic SAAF were bioassayed using methods previously reported.[4] Synthetic SAAF (**2**) activated sperm of the ascidian *C. intestinalis* at 3.7 nM and concurrently exhibited



Scheme 3. Reagents and conditions: (a) $SOCl_2$, Et_3N, THF, 94%; (b) $RuCl_3{\cdot}nH_2O$, $NaIO_4$, **14a** (65%), **14b** (11%); (c) $PhCO_2H$, Cs_2CO_3, DMF; then conc. H_2SO_4, THF, 75%; (d) BOMCl, *i*-Pr_2NEt, CH_2Cl_2 (84%); (e) *t*-BuOK, *t*-BuOH, 95%; (f) $Pb(OAc)_4$, I_2, hν, CCl_4, hν (300 W tungsten lamp), 84%; (g) DMSO, 2,4,6-collidine, 94%; (h) *n*-BuLi, TMSCl, THF, then 0.5 M HCl, 69%; (i) $LiAlH_4$, THF, 80%; (j) $SO_3{\cdot}Py$, Py, then Amberite IR-120B Na^+ form; (k) Pd/C, H_2, MeOH.

the attracting activity at <10 nM;[11] these quantitative evaluations were first accomplished with the synthetic specimen. The concentration of SAAF (10 nM) is for an aqueous gel in a capillary from which SAAF diffuses to a sperm-containing medium. Thus, the minimum active concentration is thought to be the subnano–pico molar range. It is noteworthy that 25-*epi*-SAAF (**3**) possesses comparative activities as those with SAAF (activated at ~3.7 nM and attracted at <10 nM).

We succeeded in elucidating the structure of SAAF with only ca. 4 μg of a specimen. This may be partly due to the sensitivity improvement of modern NMR instruments as demonstrated by the special probe for a capillary tube. We rather think that the high purity of the specimen is a key point. Usually, it is very difficult to prepare the microgram amount of an NMR sample without contaminations, which may come from both HPLC and NMR solvents or from containers. In this study, the sample was mostly treated as a aqueous solution in plastic vessels, which helped reducing the contamination with detergents or plasticizers; the only prominent contaminant seen in Figure 1 is lactic acid, one of common impurities under aqueous conditions. Another problem encountered in 2D NMR measurements is that an enormous solvent peak often results in an uneven base plane. In this study, the volume of the solution was only 20 μL, which effectively reduced the contamination and secure the flatness of the base line or base plane in NMR

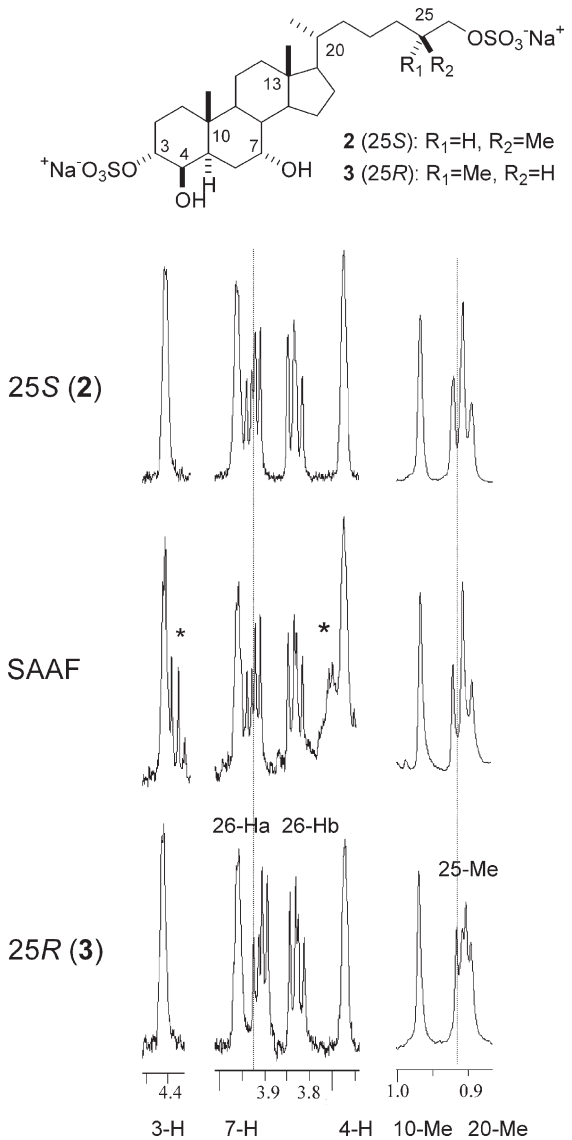


Figure 4. Partial ^1H NMR spectra of SAAF, **2**(25*S*), and **3**(25*R*). Asterisks indicate the signals attributed to contaminants.

measurements. The other methodology useful for the structure analysis in nanomolar scale is MS/MS; in particular, high energy collision experiments are helpful since C–C bond cleavages provides the information about skeletal structures. Key steps in this study was to obtain the reliable working structures and to synthesize these effectively.

3. Conclusion

In conclusion, the sperm-activating and attracting factor (SAAF) was synthesized from **7** in 17 steps, which led to the unambiguous structure determination of SAAF to be (3*R*,4*R*,7*R*,25*S*)-3,4,7,26-tetrahydroxycholestane-3,26-disulfate (**2**). The synthetic pure specimen was also used to confirm the dual sperm-activating and attracting activity. Currently, we are preparing molecular probes to be used for identification of the receptor and the relevant signal transduction pathway(s).

4. Experimental

All reactions sensitive to air or moisture were carried out under argon atmosphere in dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. THF was distilled sodium/benzophenone, diethyl ether (Et_2O) from $LiAlH_4$, diisopropylamine, diisopropylethylamine (*i*-Pr_2NEt), pyridine, from calcium hydride, and DMF, DMSO and HMPA from calcium hydride under reduced pressure. Dichloromethane (CH_2Cl_2) and toluene was dried over activated MS4A. All other reagents were used as supplied unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed using E. Merck Silica gel 60 F254 pre-coated plates. Column chromatography was performed using 100–210 μm Silica Gel 60N (Kanto Chemical), and for flash column chromatography 40–63 μm Silica Gel 60N (Merck) was used. ^1H and ^{13}C NMR spectra were recorded on a JEOL EX-270 (270 MHz), a JEOL GSX-500 (500 MHz), or JEOL JMN-LA500 (500 MHz) spectrometer. Chemical shifts are reported in δ (ppm) using residual $CHCl_3$ as an internal standard of δ 7.26 and δ 77.00 for ^1H and ^{13}C NMR, respectively. Signal patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad peak. IR spectra were recorded on a Jasco FT/IR-300E spectrometer. Mass spectra (ESI) were recorded on a ThermoQuest LCQ DECA instrument. Optical rotations were recorded on a Perkin–Elmer 241 polarimeter.

4.1. Structure determination

Materials. The ascidians, *C. intestinalis* was collected at the Onagawa Bay (Miyagi prefecture), Aburatsubo Bay (Kanagawa prefecture), and Ootsuchi Bay (Iwate prefecture) in Japan. SAAF was extracted and purified as reported previously.[10]

Structure analysis of SAAF. Electrospray-ionization time-of-flight (ESI-TOF) MS was performed in the negative-ion mode with a Mariner (ABI) mass spectrometer. ^1H NMR spectra of SAAF in 20 μL of a D_2O solution were recorded at 23 °C on a JEOL L-500 spectrometer (500 MHz) with a 1.7-mm probe (Nanolac, Z-Spec-SMIDG500). 2D TOCSY spectra were measured for a spin locking time of 20 ms with data matrices of 2k×512 (a total of 82,000 acquisitions for 35 h) while a peak of solvent-derived deuterium being presaturated. Fast atom bombardment (FAB) MS/MS experiments were carried out in the negative-ion mode using a HX-110/HX-110 tandem mass spectrometer (JEOL) equipped with a variable dispersion array detector (MS-ADS11). Collision-induced dissociation (CID) was effected by introducing helium at a pressure that reduced the intensity of precursor ions to 30%.

4.2. Synthesis of SAAF and 25-*epi*-SAAF

4.2.1. Methyl 7α-hydroxy-3,4-dioxo- cholan-24-oate 9. To a stirred solution of **8** (2.57 g, 6.35 mmol) in ^{t}BuOH (15 mL) was added ^{t}BuOK (2.10 g, 19.1 mmol) and the resulting suspension was stirred for 1.5 h at rt under oxygen atmosphere (under the reaction conditions, the methyl ester was hydrolyzed to give the corresponding carboxylate). The reaction was carefully quenched with 1.87 M NH_4Cl (130 mL). The reaction mixture was extracted with ethyl acetate, and the combined organic layer was concentrated. The residue was dissolved in chloroform (10 mL) and methanol (10 mL), which was treated with a solution of diazomethane in ether at 0 °C and the mixture stirred for 1 h. Evaporation followed by silica gel column chromatography (hexane/ethyl acetate=8:1–2:1) gave **9** (1.81 g, 68% from **8**) as colorless solid.

Compound **9**. R_f 0.20 (hexane/ethyl acetate=2:1); IR (KBr) 3448, 2940, 1736, 1670, 1636, 1381, 1169 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 6.11 (1H, s, 4-OH), 3.96 (1H, s, 7-H), 3.64 (3H, s, CO_2CH_3), 3.14 (1H, dd, *J*=15.5, 3.5 Hz, 6-Ha), 2.16 (1H, dd, *J*=15.5, 3.5 Hz, 6-Hb), 1.16 (3H, s, 10-Me), 0.90 (3H, d, *J*=7.0 Hz, 20-Me), 0.68 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 192.9, 174.5, 143.3, 135.9, 67.8, 55.7, 51.5, 50.3, 45.6, 42.4, 39.4, 39.2, 37.6, 35.4, 34.4, 31.8, 31.6, 31.0, 31.0, 28.1, 23.6, 20.8, 18.3, 16.8, 11.9.

4.2.2. Methyl 7α-benzyloxymethoxy-3,4-dioxocholan-24-oate 10. To a solution of **9** (2.63 g, 6.29 mmol) in dichloromethane (18.9 mL) was added diisopropylethylamine (2.19 mL, 12.6 mmol) and BOMCl (1.31 mL, 9.44 mmol), and the mixture was stirred for 18 h at rt. The reaction was quenched with water and extracted with dichloromethane. The combined organic layer was washed with saturated NaCl, dried over anhydrous $MgSO_4$, and filtered. Concentration followed by purification by flash column chromatography (hexane/ethyl acetate=10:1–5:1) gave **10** (2.03 g, 60%) as colorless amorphous, and 17% of **9** (464 mg) was recovered.

Compound **10**. Colorless amorphous; R_f 0.55 (hexane/ethyl acetate=2:1); $[\alpha]_D^{22}$ −2.4° (*c* 1.58, $CHCl_3$); IR (KBr) 3456, 2960, 1740, 1664, 1636, 1380, 1172, 1052 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.36–7.24 (5H, m, Ph), 6.03 (1H, s, 4-OH), 4.88 (1H, d, *J*=7.0 Hz, OCH_2OBn), 4.74 (1H, d, *J*=7.0 Hz, OCH_2OBn), 4.65 (1H, d, *J*=11.5 Hz, OCH_2Ph), 4.57 (1H, d, *J*=11.5 Hz, OCH_2Ph), 3.88 (1H, s, 7-H), 3.65 (3H, s, CO_2CH_3), 3.39 (1H, dd, *J*=16.0, 3.0 Hz, 6-Ha), 2.00 (1H, dd, *J*=16.0, 3.0 Hz, 6-Hb), 1.16 (3H, s, 10-Me), 0.91

(3H, d, J=6.5 Hz, 20-Me), 0.68 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 193.1, 174.5, 142.8, 137.9, 136.7, 128.2, 127.6, 127.5, 93.7, 73.7, 70.0, 55.6, 51.5, 49.9, 45.9, 42.4, 39.6, 39.1, 37.6, 35.4, 34.5, 31.9, 31.1, 31.0, 28.5, 28.1, 24.0, 20.8, 18.4, 16.9, 11.8; MS (ESI) m/z 561 $(M+Na)^+$.

4.2.3. Methyl 7α-benzyloxymethoxy-3β,4β-dihydroxy-5α-cholan-24-oate 13a. To a stirred solution of **10** (1.01 g, 1.88 mmol) in chloroform (4 mL) and methanol (4 mL) was added $NaBH_3CN$ (589 mg, 9.37 mmol) and silica gel (3.00 g), and the reaction mixture was stirred for 10 min at rt. The solvent was evaporated and the residue was stirred for 1 h at rt. The residue was added chloroform (4 mL) and methanol (4 mL) again and stirred for 10 min. The solvent was removed by evaporation and the resulting residue was stirred for 3 h at rt. The same procedure was repeated again and the residue was stirred for 13 h at rt. The residue was washed with a mixed solvent of chloroform and methanol (chloroform/methanol=2:1), and the precipitates were removed by filtration. The residue was washed with the same solvent three times. The combined filtrate was concentrated, and the residue was dissolved in ethyl acetate. The organic layer was washed with saturated NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/ethyl acetate=8:1–2:1) to give a mixture of **13a** and **13c** (443 mg, 44% **13a/13c**=3:1), which was separated from **12b** (220 mg, 22%).

Compound **13a**: R_f 0.15 (hexane/ethyl acetate=2:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.24 (5H, m, Ph), 4.83 (1H, d, J=7.5 Hz, OCH_2OBn), 4.76 (1H, d, J=7.5 Hz, OCH_2OBn), 4.60 (1H, d, J=12.0 Hz, OCH_2Ph), 4.57 (1H, d, J=12.0 Hz, OCH_2Ph), 3.75 (1H, d, J=1.5 Hz, 7-H), 3.64 (3H, s, CO_2Me), 3.59 (1H, s, 4-H), 3.54 (1H, m, 3-H), 1.59 (1H, d, J=13.0 Hz, 5-H), 1.01 (3H, s, 10-Me), 0.90 (3H, d, J=7.0 Hz, 20-Me), 0.62 (3H, s, 13-Me).

Compound **13c**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36–7.24 (5H, m, Ph), 4.93 (1H, d, J=5.5 Hz, OCH_2OBn), 4.63 (1H, d, J=5.5 Hz, OCH_2OBn), 4.62 (1H, d, J=12.0 Hz, OCH_2Ph), 4.57 (1H, d, J=12.0 Hz, OCH_2Ph), 4.26 (1H, m, 4-H), 3.95 (1H, d, J=2.5 Hz, 3-H), 3.64 (3H, s, CO_2Me), 3.57 (1H, d, J=2.5 Hz, 7-H), 3.35 (1H, d, J=6.0 Hz, 4-OH), 0.94 (3H, s, 10-Me), 0.90 (3H, d, J=7.0 Hz, 20-Me), 0.62 (3H, s, 13-Me).

4.2.4. Methyl 7α-benzyloxymethoxy-3β,4β-cyclic sulfate-5α-cholan-24-oate 14a. To a stirred solution of **13a** and **13c** (437 mg, 0.801 mmol) and triethylamine (446 μL, 3.22 mmol) in THF (3 mL) was added a solution of thionyl chloride (94.6 μL, 1.21 mmol) in THF (1 mL) and stirred for 10 min at 0 °C. The reaction mixture was quenched with water and extracted with ethyl acetate. The combined organic layer was washed with saturated NaCl, dried over anhydrous $MgSO_4$. Concentration followed by flash column chromatography (hexane/ethyl acetate=50:1–10:1) gave a mixture of cyclic sulfites (441 mg, 94%) as colorless oil. The mixture of cyclic sulfites (552 mg, 0.938 mmol) was dissolved in a mixed solvent of acetonitrile (4.6 mL), CCl_4 (3.0 mL), and 50 mM phosphate buffer (3.0 mL). To the solution was added $RuCl_3$ (19.5 mg, 94 μmol), $NaIO_4$ (241 mg, 1.13 μmol) in a mixed solvent of acetonitrile (2.3 mL), CCl_4 (1.5 mL), and 50 mM phosphate buffer (1.5 mL). The reaction mixture was stirred for 20 min, then quenched with saturated $Na_2S_2O_3$ and extracted with ethyl acetate. The organic layers were combined, washed with saturated NaCl, dried over dried over $MgSO_4$, filtered. Concentration followed by florisil column chromatography (hexane/ethyl acetate=30:1–6:1) gave cyclic sulfate **14a** (367 mg, 65%) as colorless amorphous, and side product **14b** (62 mg, 11%).

Compound **14a**. Colorless amorphous; R_f 0.32 (hexane/ethyl acetate=5:1, 3 times development); $[\alpha]_D^{23}$ +6.4° (c 0.12, $CHCl_3$); IR (thin film) 2945, 2869, 1736, 1381, 1208, 1039 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36–7.27 (5H, m, Ph), 4.82 (1H, d, J=7.0 Hz, OCH_2OBn), 4.74 (1H, d, J=7.0 Hz, OCH_2OBn), 4.63–4.56 (4H, m, OCH_2Ph, 3,4-H), 3.74 (1H, d, J=2.0 Hz, 7-H), 3.64 (3H, s, CO_2CH_3), 1.03 (3H, s, 10-Me), 0.90 (3H, d, J=7.0 Hz, 20-Me), 0.63 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 174.5, 137.7, 128.4, 127.8, 127.3, 94.7, 85.8, 83.0, 75.2, 70.3, 55.6, 51.5, 49.9, 46.5, 42.5, 40.0, 39.6, 39.0, 35.4, 34.9, 34.1, 31.1, 31.0, 30.6, 28.0, 23.7, 20.4, 18.3, 12.9, 11.9; MS (ESI) m/z 627 $(M+Na)^+$.

Compound **14b**. Amorphous material; R_f 0.43 (hexane/EtOAc=5:1, 3 times development); $[\alpha]_D^{23}$ +32.1° (c 1.44, $CHCl_3$); IR (thin film) 2945, 2870, 1736, 1382, 1208, 1035 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.26 (5H, m, Ph), 5.76 (1H, dd, J=10.0, 4.5 Hz, 4-H), 5.12 (1H, q, J=4.5 Hz, 3-H), 4.81 (1H, d, J=6.5 Hz, OCH_2OBn), 4.74 (1H, d, J=6.5 Hz, OCH_2OBn), 4.62 (1H, d, J=12.0 Hz, OCH_2Ph), 4.59 (1H, d, J=12.0 Hz, OCH_2Ph), 3.76 (1H, d, J=2.5 Hz, 7-H), 3.64 (3H, s, CO_2CH_3), 1.03 (3H, s, 10-Me), 0.90 (3H, d, J=7.0 Hz, 20-Me), 0.63 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 174.4, 137.5, 128.3, 127.6, 127.5, 94.2, 86.4, 81.5, 74.7, 70.6, 55.6, 51.5, 49.7, 42.7, 42.6, 39.1, 39.0, 36.2, 35.4, 35.0, 31.1, 31.0, 29.3, 28.1, 25.6, 23.9, 23.0, 21.6, 20.7, 18.4, 11.8; MS (ESI) m/z 627 $(M+Na)^+$.

4.2.5. Methyl 3α-benzoyloxy-4β,7α-dibenzyloxy- methoxy-5α- cholan-24-oate 15. A mixture of **13a** (109 mg, 0.180 mmol), benzoic acid (55 mg, 0.450 mmol), and Cs_2CO_3 (147 mg, 0.450 mmol) in a 10 mL flask was dried under the reduced pressure. To the mixture was added DMF (2 mL) and stirred for 6 h at rt. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (hexane/ethyl acetate=5:1–2:1, then chloroform/methanol=1:0–1:2) to give 3-O-benzoyl-4-O-sulfate (R_f 0.30, 5:1—chloroform/methanol) (110 mg) as a mixture of benzoic acid, and 21 mg (19%) of **14a** was recovered. To the solution of 3-O-benzoyl-4-O-sulfate (110 mg) in THF (2.4 mL) was added a solution of conc. sulfuric acid (54 μL) in THF (0.1 mL). The mixture was stirred for 15 min at rt, then quenched with saturated $NaHCO_3$ and extracted with ethyl acetate. The combined organic layer was washed with saturated $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give **15** (71 mg, 75% from **14a**).

Compound **15**. Amorphous material; R_f 0.43 (hexane/ethyl acetate=2:1); IR (thin film) 3487, 2940, 1734, 1717, 1270,

1111, 1041 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 8.01 (2H, d, J=8.0 Hz, $PhCO_2$), 7.53–7.06 (8H, m, $PhCO_2$, Ph), 5.13 (1H, d, J=2.5 Hz 3-H), 4.81 (1H, d, J=7.0 Hz, OCH_2OBn), 4.72 (1H, d, J=7.0 Hz, OCH_2OBn), 4.47 (1H, d, J=12.0 Hz, OCH_2Ph), 4.43 (1H, d, J=12.0 Hz, OCH_2Ph), 3.74 (1H, s, 7-H), 3.67 (1H, s, 4-H), 3.64 (3H, s, CO_2CH_3), 2.08 (1H, dt, J=13.5, 3.0 Hz, 5-H), 1.08 (3H, s, 10-Me), 0.91 (3H, d, J=7.0 Hz, 20-Me), 0.64 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 174.6, 165.5, 137.6, 132.9, 130.3, 129.5, 128.3, 128.2, 127.4, 127.1, 95.0, 73.2, 73.2, 69.5, 55.7, 51.5, 50.2, 47.3, 42.6, 39.8, 39.2, 38.2, 35.9, 35.4, 32.9, 31.1, 31.1, 30.8, 28.1, 23.8, 22.1, 20.1, 18.4, 13.5, 11.9; MS (ESI) m/z 669 $(M+Na)^+$.

4.2.6. Methyl 3α-benzoyloxy-4β,7α-dibenzyloxy-methoxy-5α-cholan-24-oate 16. To a solution of **15** (71 mg, 0.110 mmol) in dichloromethane (1 mL) was added diisopropylethylamine (345 μL, 1.98 mmol) and BOMCl (122 μL, 0.880 mmol) and the mixture was stirred for 22 h at rt. The reaction mixture was quenched with water and extracted with dichloromethane. The combined organic layer was washed with saturated NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=15:1–4:1) to give **16** (56 mg, 67%) as colorless oil and 11 mg (15%) of **15** was recovered.

Compound **16**. Colorless oil; R_f 0.63 (hexane/ethyl acetate=2:1); $[\alpha]_D^{22}$ −6.4° (*c* 0.64, $CHCl_3$); IR (thin film) 2943, 1734, 1717, 1270, 1099, 1038, 1026 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 8.00 (2H, d, J=8.0 Hz, $PhCO_2$), 7.52–7.09 (13H, m, $PhCO_2$, Ph), 5.28 (1H, d, J=3.0 Hz 3-H), 4.82, 4.80, 4.71 and 4.70 (4H, d×4, J=7.0 Hz, OCH_2O), 4.64, 4.60, 4.49 and 4.47 (4H, d×4, J=12.0 Hz, OCH_2Ph), 3.74 (1H, s, 7-H), 3.65 (3H, s, CO_2CH_3), 3.57 (1H, s, 4-H), 1.07 (3H, s, 10-Me), 0.91 (3H, d, J=7.0 Hz, 20-Me), 0.65 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 174.6, 165.4, 137.7, 137.6, 132.8, 130.4, 129.4, 128.3, 128.2, 127.7, 127.5, 127.4, 127.2, 94.7, 94.5, 79.2, 76.4, 70.5, 69.7, 69.5, 55.7, 51.5, 50.1, 47.2, 42.6, 39.8, 39.2, 38.1, 35.9, 35.4, 32.9, 31.1, 28.1, 23.8, 22.4, 20.3, 18.4, 13.3, 11.9; MS (ESI) m/z 789 $(M+Na)^+$.

4.2.7. 3α-Benzoyloxy-4β,7α-dibenzyloxymethoxy-5α-cholan-24-oic acid 17. To a solution of **16** (4.9 mg, 6.39 μmol) in ^{t}BuOH (0.1 mL) was added ^{t}BuOK (2.2 mg, 19.2 μmol), and the mixture was stirred for 2 h at rt. The reaction mixture was quenched with saturated NH_4Cl, and extracted with ethyl acetate. The combined organic layer was washed with saturated NaCl. Concentration followed by silica gel column chromatography (hexane/ethyl acetate=8:1–4:1) gave **17** (4.6 mg, 95%) as colorless amorphous.

Compound **17**. Amorphous material; R_f 0.27 (hexane/ethylacetate=2:1); IR (thin film) 1714, 1451, 1270, 1101, 1038 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 8.00 (2H, d, J=8.0 Hz, $PhCO_2$), 7.53–7.09 (13H, m, $PhCO_2$, Ph), 5.27 (1H, d, J=3.0 Hz 3-H), 4.82, 4.79, 4.71 and 4.70 (4H, d×4, J=7.0 Hz, OCH_2O), 4.64, 4.60, 4.49 and 4.47 (4H, d×4, J=12.0 Hz, OCH_2Ph), 3.73 (1H, s, 7-H), 3.56 (1H, s, 4-H), 1.06 (3H, s, 10-Me), 0.92 (3H, d, J=6.5 Hz, 20-Me), 0.65 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 179.9, 165.4, 137.7, 137.6, 132.8, 130.4, 129.4, 128.3, 128.2, 127.7, 127.5, 127.4, 127.2, 94.6, 94.5, 79.2, 76.4, 70.5, 69.7, 69.5, 55.7, 50.2, 47.2, 42.6, 39.8, 39.2, 38.1, 35.9, 35.4, 32.8, 31.0, 30.8, 28.1, 23.8, 22.4, 20.3, 18.3, 13.3, 11.9; MS (ESI) m/z 776 $(M+Na)^+$.

4.2.8. 24-Nor-5α-cholane-4β,7α-dibenzyloxymethoxy-23-iodo-3α-ol, benzoate 18. A solution of **17** (43 mg, 57.1 μmol), $Pb(OAc)_4$ (38 mg, 86 μmol), and iodine (43 mg, 171 μmol) in CCl_4 (2 mL) was refluxed for 20 min under irradiation of tungsten lamp (300 W). The reaction mixture was quenched with saturated $Na_2S_2O_3$, and extracted with dichloromethane. The combined organic layer was washed with saturated NaCl, and dried over $MgSO_4$. Concentration followed by silica gel column chromatography (hexane/ethyl acetate=20:1–2:1) gave **18** (40 mg, 84%) as colorless oil.

Compound **18**. Colorless oil; R_f 0.43 (hexane/ethyl acetate=5:1); IR (thin film) 3424, 1715, 1452, 1270, 1109, 1039 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 8.00 (2H, d, J=7.5 Hz, $PhCO_2$), 7.53–7.09 (13H, m, $PhCO_2$, Ph), 5.27 (1H, q, J=2.5 Hz 3-H), 4.82, 4.79 and 4.70×2 (4H, d×4, J=7.0 Hz, OCH_2OBn), 4.64, 4.60, 4.49 and 4.47 (4H, d×4, J=12.0 Hz, OCH_2Ph), 3.73 (1H, d, J=1.5 Hz 7-H), 3.57 (1H, s, 4-H), 3.29 (1H, td, J=9.0, 5.5 Hz, 23-Ha), 3.08 (1H, q, J=9.0 Hz, 23-Hb), 1.07 (3H, s, 10-Me), 0.91 (3H, d, J=6.5 Hz, 20-Me), 0.66 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 165.4, 137.7, 137.6, 132.8, 130.4, 129.4, 128.3, 128.2, 127.7, 127.5, 127.4, 127.2, 94.6, 94.5, 79.2, 76.4, 70.5, 69.7, 69.5, 55.6, 50.2, 47.2, 42.7, 39.7, 39.2, 38.1, 37.3, 35.9, 32.8, 31.0, 28.1, 23.8, 22.4, 20.2, 17.9, 13.3, 11.9, 5.4; MS (ESI) m/z 857 $(M+Na)^+$.

4.2.9. 3α-Benzoyloxy-4β,7α-dibenzyloxymethoxy-24-nor-5α-cholan-23-al 6. To a solution of iodide **18** (14 mg, 17 μmol) in DMSO (0.5 mL) was added 2,4,6-collidine (11 μL, 81 μmol). After stirring for 10 min at 150 °C, the reaction mixture was cooled to rt, then quenched with water, and extracted with dichloromethane. The combined organic layer was washed with 0.5 M HCl and saturated NaCl, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=15:1–8:1) to give **6** (11 mg, 94%) as colorless oil.

Compound **6**. Colorless oil; R_f 0.25 (hexane: ethyl acetate=5:1); $[\alpha]_D^{23}$ −11.3° (*c* 0.23, $CHCl_3$); IR (thin film) 2933, 2719, 1718, 1270, 1108, 1038, 1026 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 9.73 (1H, dd, J=3.5, 1.0 Hz, CHO), 8.00 (2H, d, J=8.5 Hz, $PhCO_2$), 7.56–7.09 (13H, m, $PhCO_2$, Ph), 5.27 (1H, q, J=3.5 Hz 3-H), 4.82, 4.78, 4.70 and 4.69 (4H, d×4, J=7.0 Hz, OCH_2O), 4.64, 4.60, 4.49 and 4.46 (4H, d×4, J=12.0 Hz, OCH_2Ph), 3.72 (1H, s, 7-H), 3.57 (1H, s, 4-H), 1.07 (3H, s, 10-Me), 1.01 (3H, d, J=6.0 Hz, 20-Me), 0.70 (3H, s, 13-Me); ^{13}C NMR (500 MHz, $CDCl_3$) δ 203.3, 165.4, 160.7, 137.7, 137.6, 132.8, 130.4, 129.4, 128.3, 128.2, 127.7, 127.5, 127.4, 127.2, 94.6, 94.6, 79.2, 76.4, 70.5, 69.8, 69.5, 55.8, 50.9, 50.2, 47.2, 42.7, 39.8, 39.1, 38.1, 35.9, 32.9, 31.7, 31.0, 28.4, 23.8, 22.4, 20.2, 20.1, 13.3, 11.9; MS (ESI) m/z 777 $(M+MeOH+Na)^+$.

4.2.10. 104β,7α-Dibenzyloxymethoxy-5α-cholest-23-ene-3α,26-diol,3α-benzoate, (25*S*) 19. To a stirred suspension of phosphonium salt **4** (101 mg, 0.243 mmol) in (1 mL) was added in dropwise a solution of 1.6 M *n*-BuLi in hexane (304 μl, 0.486 mmol) at −78 °C, then stirred for 10 min at 0 °C. TMSCl (31 μl, 0.243 mmol) was added to the reaction mixture at 0 °C, and stirred for 30 min at rt. A portion of the resulting solution of the ylide (324 μL, 60.9 μmol) was added to the solution of **6** (11.0 mg, 15.2 μmol) in THF (0.54 mL) at −78 °C, and stirred for 15 min, then stirred at 0 °C for 1 h, and stirred for 2 h at rt. The reaction mixture was quenched with 0.5 M HCl (0.2 mL) and stirred for 10 min at rt, and extracted with ether. Combined organic layer was washed with saturated NaCl. Concentration followed by silica gel column chromatography (hexane/ethylacetate=10:1–5:1) gave **19** (8.2 mg, *E*/*Z*=1:8, 69%) as colorless oil.

Compound **19**. Colorless oil; R_f 0.45 (1:2—ethyl acetate/hexane); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.00 (2H, d, *J*=7.5 Hz, $PhCO_2$), 7.52–7.09 (13H, m, $PhCO_2$, Ph), 5.53–5.44 (1H, m, 23-H), 5.27 (1H, d, *J*=2.5 Hz 3-H), 5.22 (1H, dd, *J*=15.5, 8.0 Hz, 24-H,E), 5.15 (1H, t, *J*=10.5 Hz, 24-H,Z), 4.82, 4.80, 4.71 and 4.70 (4H, d×4, *J*=6.5 Hz, OCH_2O), 4.64, 4.60, 4.49 and 4.47 (4H, d×4, *J*=12.0 Hz, OCH_2Ph), 3.73 (1H, s, 7-H), 3.57 (1H, s, 4-H), 3.46 (1H, dd, *J*=10.0, 6.0 Hz 26-Ha), 3.36–3.31 (1H, m, 26-Hb), 1.07 (3H, s, 10-Me), 0.93 (3H, d, *J*=6.0 Hz, 20-Me or 25-Me), 0.92 (3H, d, *J*=6.0 Hz, 20-Me or 25-Me), 0.66 (3H, s, 13-Me).

4.2.11. 4β,7α-Dibenzyloxymethoxy-5α-cholest-23-ene-3α,26-diol, (25*S*) 20. To a solution of **19** (8.2 mg, 10.5 μmol) in THF (0.6 mL) was added $LiAlH_4$ (4.0 mg, 105 μmol) at 0 °C. After stirring for 15 min, the reaction was quenched with water and diluted with ether, and the resulting precipitates were removed by filtration through the Celite pad. The filtrate was concentrated and purified by silica gel column chromatography (hexane/ethyl acetate=5:1–2:1) to give **20** (6.2 mg, 87%) as colorless oil.

Compound **20**. Colorless oil; R_f 0.20 (1:2—hexane/ethyl acetate=2:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.24 (10H, m, Ph), 5.53–5.44 (1H, m, 23-H), 5.21 (1H, dd, *J*=15.5, 8.0 Hz, 24-H,*E*), 5.16 (1H, t, *J*=10.5 Hz, 24-H,*Z*), 4.83, 4.75 and 4.72 (3H, d×3, *J*=7.5 Hz, OCH_2O), 4.65–4.55 (5H, m, OCH_2O×1 and OCH_2Ph×4), 3.93 (1H, d, *J*=2.5 Hz, 3-H), 3.74 (1H, s, 7-H), 3.48–3.43 (1H, m, 26-Ha), 3.36–3.31 (2H, m, 26-Hb and 4-H), 0.99 (3H, s, 10-Me), 0.92 (3H, d, *J*=7.0 Hz, 20-Me or 25-Me), 0.91 (3H, d, *J*=5.0 Hz, 20-Me or 25-Me), 0.64 (3H, s, 13-Me).

4.2.12. (3α,4β,7α,25*S*)-3,4,7,26-Tetrahydroxy-5α-cholestane-3,26-disulfate 2. A mixture of **20** (4.1 mg, 6.07 μmol) and SO_3·Py (19.3 mg, 0.121 mmol) in a 10 mL flask was dried under the reduced pressure. To the mixture was added pyridine (0.6 mL) and the resulting mixture was stirred for 50 min at 60 °C. The pyridine was removed and dried in vacuo for 5 h. To the residue was added ether, and decanted to remove excess SO_3·Py. The precipitates were dissolved in chloroform, and insoluble impurities were removed by decantation. The solvent was removed under the reduced pressure to give the pyridinium salt. To the solution of the residue in methanol (2.0 mL) was added Amberite IR-120B (1.7 g), and stirred for 3 h at rt. The resin was removed by filtration, and the filtrate was concentrated. The residue was dissolved in methanol (2.0 mL) again, and the solution was added Amberite IR-120B (1.7 g), and stirred for 4.5 h at rt. The resin was removed by filtration, and the filtrate was concentrated. The residue was dissolved in methanol (2.0 mL) and added 10% Pd on charcoal (52 mg, 61 μmol). The mixture was stirred under the hydrogen atmosphere for 2.5 h at rt. The catalyst was removed by filtration through Celite pad, and the filtrate was concentrated in vacuo. The residue was purified by HPLC to give **2** as colorless solid.

Compound **2**. Colorless solid; R_f 0.13 (chloroform/methanol=2:1); HPLC RT 5.2 min; HPLC conditions {column, TSK-GEL ODS-120T (φ 4.6×250 mm); flow rate, 1.0 mL/min; solvent A, H_2O; solvent B, CH_3CN; gradient, solvent B 21% (0–20 min)}; ^{1}H NMR (500 MHz, D_2O) δ 4.41 (1H, d, *J*=2.5 Hz, 3-H), 3.96 (1H, d, *J*=2.0 Hz 7-H), 3.92 (1H, dd, *J*=9.0, 5.5 Hz, 26-Ha), 3.83 (1H, dd, *J*=9.0, 7.0 Hz, 26-Hb), 3.72 (1H, s, 4-H), 1.81 (1H, d, *J*=15.0 Hz, 5-H), 0.97 (3H, s, 10-Me), 0.92 (3H, d, *J*=6.5 Hz, 25-Me), 0.90 (3H, d, *J*=6.5 Hz, 20-Me), 0.65 (3H, s, 13-Me); MS (ESI) *m*/*z* 297 $(M-2Na)^{-2}$.

4.2.13. (3α,4β,7α,25*R*)-3,4,7,26-Tetrahydroxy-5α-cholestane-3,26-disulfate 3. *Compound* **3**. Colorless solid; R_f 0.13 (chloroform/methanol=2:1); HPLC conditions {column, TSK-GEL ODS-120T (φ 4.6×250 mm); flow rate, 1.0 mL/min; solvent A, H_2O; solvent B, CH_3CN; gradient, solvent B 21% (0–20 min)}; ^{1}H NMR (500 MHz, D_2O) δ 4.41 (1H, d, *J*=2.5 Hz, 3-H), 3.96 (1H, d, *J*=2.5 Hz 7-H), 3.91 (1H, dd, *J*=9.0, 5.5 Hz, 26-Ha), 3.83 (1H, dd, *J*=9.0, 7.0 Hz, 26-Hb), 3.72 (1H, s, 4-H), 1.81 (1H, d, *J*=15.0 Hz, 5-H), 0.97 (3H, s, 10-Me), 0.91 (3H, d, *J*=7.0 Hz, 25-Me), 0.90 (3H, d, *J*=6.0 Hz, 20-Me), 0.65 (3H, s, 13-Me); MS (ESI) *m*/*z* 297 $(M-2Na)^{-2}$.

4.3. Estimation of the amount of natural SAAF

A standard solution of synthetic SAAF in D_2O was prepared and its concentration was estimated by ^{1}H NMR using DMF as internal standard (1.26 mM). ^{1}H NMR spectra of natural SAAF (x μg) and synthetic SAAF (4 μg and 40 μg) in 40 μL of D_2O solution were recorded on a JEOL L-500 spectrometer (500 MHz) with a 1.7-mm probe (Nanolac, Z-Spec-SMIDG500) under the same conditions (receiver gain: 28, acquisition times: 6176, temperature, 29.9 °C). The signal intensity of the natural SAAF was identical with that of the synthetic SAAF containing 4 μg of sample, and approximately tenth part of 40 μg of sample.

4.4. Biological activities

A sample in an aqueous solution was mixed with the same volume of 2% agar, and the mixture was enclosed in the tip of glass capillary with a diameter of ca. 50 μm. The capillary was inserted in sea water including an appropriate number of sperm, and images of sperm around the capillary tip were recorded onto a personal computer every 20 msec

using a high-speed CCD camera (HAS-200, Ditect) and a video card (HAS-PCI, Ditect). The position of each sperm was analyzed using the image analyzing program (Dip-motion 2D, Ditect), and parameters (the chemotaxis index, D, dD/dt, velocity, θ) were calculated from the position of the data. See a previous report for details of the bioassay.[10]

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