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Synthesis of endogenous sperm-activating and attracting factor isolated from ascidian *Ciona intestinalis*

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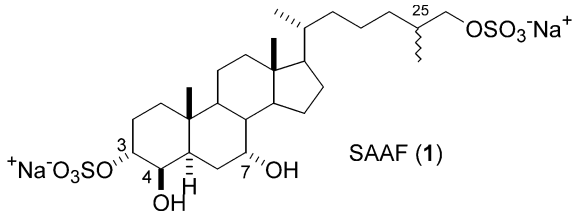
Abstract—A chemoattractant candidate named sperm-activating and attracting factor (SAAF) from the eggs of ascidian *Ciona intestinalis*, was synthesized from chenodeoxycholic acid in 16 steps. The present synthesis led to the unambiguous structure determination of SAAF to be (3*R*,4*R*,7*R*,25*S*)-3,4,7,26-tetrahydroxycholestane-3,26-disulfate. The synthetic pure specimen was also used to confirm the dual sperm-activating and attracting activity.

Chemotaxis of sperm toward eggs during fertilization is a crucial event for species conservation, particularly for animals living in aquatic environments.[1] Relevant chemical attractants have been found from a few marine organisms such as sea urchins and corals.[2,3] Recently, we have reported a non-peptidic chemoattractant candidate named sperm-activating and attracting factor (SAAF) from the eggs of ascidian *Ciona intestinalis*.[4] SAAF is the first steroid possessing chemotaxis activity, as well as the first example of a single agent concomitantly revealing both sperm activation and attraction, which are reportedly elicited by different mechanisms.[5,6] Thus, SAAF may serve as a key ligand for future biological studies on signal transduction pathways leading to sperm's flagellum movement. We proposed the structure of SAAF to be a novel polyhydroxysterol sulfate (**1**) by means of 2D-^{1}H NMR and FAB-MS/MS analysis using approximately 4 μg of sample.[4] However, we could not rule out the possibility that the biological activity might be attributed to a minor constituent since spectral measurements were carried out with a very limited amount of the specimen, which was not completely pure as can be observed in Figure 1. Synthesis is, therefore, essential for the unequivocal identification of **1** as the active principle, for the complete structure elucidation including the stereochemistry at C-25, and for providing a specimen for further biological studies. In this communication we attempted to synthesize of SAAF and its C-25 epimer, which allowed us not only to determine the complete structure, but also to confirm the dual activity of SAAF.



SAAF (**1**)

Synthesis of SAAF and its C-25 epimer commenced with methyl 7α-hydroxy-3-oxo-5β-cholan-24-oate (**2**)[7] as shown in Scheme 1. We planed a versatile route that could provide both diastereomers by introducing the side chain at the latest steps via a common intermediate. Oxidation of the ketone **2** 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 **3**. Selective protection of the C-7 alcohol of **3** as its benzyloxymethyl (BOM) ether in the presence of C-4 enol yielded **4**. Although many attempts to reduce the 3-keto-4-enol **4** using conventional methods were unsuccessful due to the formation of undesired diastereomers preferentially with low reproducibility, a solid-phase reduction of **4** with $NaBH_3CN$ on silica gel was found to afford desired diol **5a** as an inseparable mixture with 5β-epimer **5b** in a 3 to 1 ratio (44%);

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major side product was 4-keto-3-ol **6** (20%). Even though the yield of **5a** 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 **5a** and **5b** with thionyl chloride followed by oxidation with RuO_2–$NaIO_4$ gave cyclic sulfate **7a** in 65% yield for two steps,[8] which was easily separated from 5β-isomer **7b** (11%) by silica gel chromatography. 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%),[9] and successive protection of the resulting C-4 alcohol as BOM ether afforded benzoate **8** as a single isomer (71%). Exposure of **8** 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 **9** (95%), which was further converted to iodide **10** through the decarboxylation by treating with $Pb(OAc)_4$ in the presence of iodine under irradiation using a tungsten lamp (84%).[10] Oxidation of **10** with DMSO in the presence of collidine gave an aldehyde **11** (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 **11**, and an ylide generated from (*R*)-phosphonium salt **12**[11] and *n*-BuLi–TMSCl[12] to give olefin **13** (*E*:*Z*= 1:8) in 69% yield. Reductive removal of the benzoyl group of **13** with $LiAlH_4$ gave diol **14**, 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

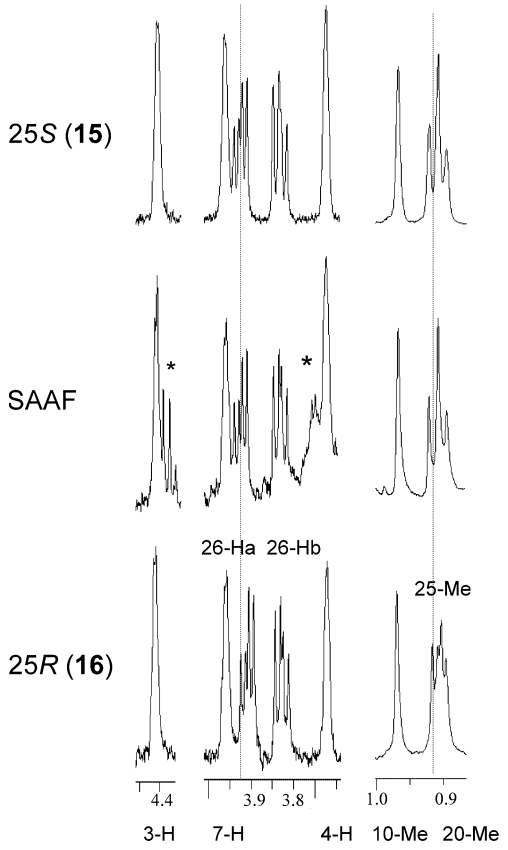
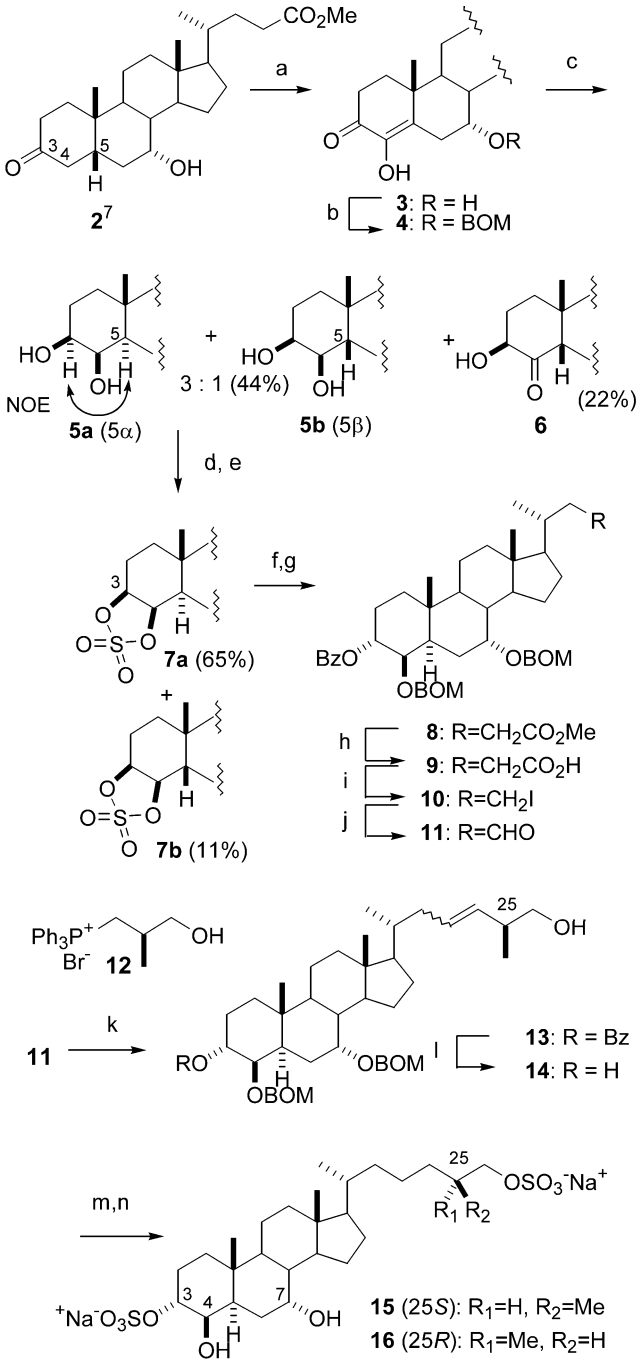


Figure 1. Partial ^1H NMR spectra of SAAF, **15** (25*S*), and **16** (25*R*). Asterisks indicate the signals attributed to contaminants.



Scheme 1. *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 (83%); (c) $NaBH_3CN$; $CHCl_3$, MeOH, then evaporated; (d) $SOCl_2$, Et_3N, THF, 93%; (e) $RuCl_3$·nH_2O, $NaIO_4$; (f) $PhCO_2H$, Cs_2CO_3, DMF, 75%; (g) BOMCl, *i*-Pr_2NEt, CH_2Cl_2 (84%); (h) *t*-BuOK, *t*-BuOH, 95%; (i) $Pb(OAc)_4$, I_2, *hv*, CCl_4, 84%; (j) DMSO, 2,4,6-collidine, 94%; (k) *n*-BuLi, TMSCl, THF, 69%; (l) $LiAlH_4$, THF, 87%; (m) SO_3·Py, Py, then Amberite IR-120B Na^+ form; (n) Pd/C, H_2, MeOH.

the double bond and concomitant removal of the BOM groups afforded 25*S*-isomer (**15**), and the resulting polar material was purified by reverse-phase HPLC. The C-25 epimer **16** was synthesized by the identical procedure as **15** except for the use of (*S*)-phosphonium salt in place of **12**.

The ^{1}H NMR spectrum of the natural product was compared with those of the synthetic samples, **15** (25*S*) and **16** (25*R*), as shown in Figure 1.[13] While the chemical shifts of the 25-methyl group and H-26a in **16** 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 **15** 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 based on methods previously reported.[4] Synthetic SAAF (**15**) activated sperm of ascidian *Ciona intestinalis* at 3.7 nM and concurrently exhibited the attracting activity at <10 nM;[14] these quantitative evaluations were first accomplished with the synthetic specimen. It is noteworthy that 25-*epi*-SAAF (**16**) possesses comparative activities as those with SAAF (activated at $\sim$3.7 nM and attracted at <10 nM).

In conclusion, the sperm-activating and attracting factor (SAAF) was synthesized from **2** in 16 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 (**15**). 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).

Acknowledgements

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13. **15**: ^{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 $(M-2Na)^{2-}$; **16**: ^{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 $(M-2Na)^{2-}$.
14. The concentration of SAAF (10 nM) is for an aqueous gel in a capirally from which SAAF diffuses to a sperm-containing medium. Thus, the minimum active concentration is thought to be the subnano-pico molar range.