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A Novel Sperm-Activating and Attracting Factor from the Ascidian *Ascidia sydneiensis*

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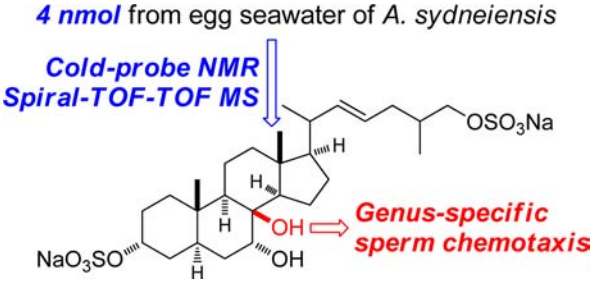
ABSTRACT



A novel SAAF was isolated from the title ascidian. The structure was elucidated using the entire sample of 4 nmol, suggesting that the position of the OH group confers genus-specificity to sperm chemotaxis in ascidians. This study not only provides insight into the chemical tactics in sperm chemotaxis but demonstrates that the innovative techniques allow structure determination of natural products in trace amounts.

Sperm activation and chemotaxis that ubiquitously occur in animal species, including humans, play an important role in fertilization.[1−3] The species specificity of sperm chemotaxis toward eggs was frequently observed in externally fertilizing marine invertebrates such as hydrozoa,[4] echinoderm,[5] and abalone.[6] Ascidians also show genera-level sperm chemotaxis specificity.[7] Species- or genera-specificity of sperm chemotaxis is considered to prevent crossbreeding and ensure fertilization; however, its molecular

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(1) Morisawa, M.; Yoshida, M. *Reprod. Med. Biol.* **2005**, *4*, 101–114.
(2) Metz, C. B.; Monroy, A.; Miller, R. L. In *Biology of Fertilization, Sperm Chemo-orientation in Metazoa*; Metz, C. B., Monroy, A., Eds.; Academic Press: New York, 1985; Vol. 2, pp 275−337.
(3) Eisenbach, M. *Rev. Reprod.* **1999**, *4*, 56–66.
(4) Miller, R. L. *Mar. Biol.* **1979**, *53*, 99–114.
(5) Miller, R. L. *J. Exp. Zool.* **1985**, *234*, 383–414.
(6) Riffell, J. A.; Krug, P. J.; Zimmer, R. K. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4501–4506.
(7) Miller, R. L. *Amer. Zool.* **1982**, *22*, 827–840.

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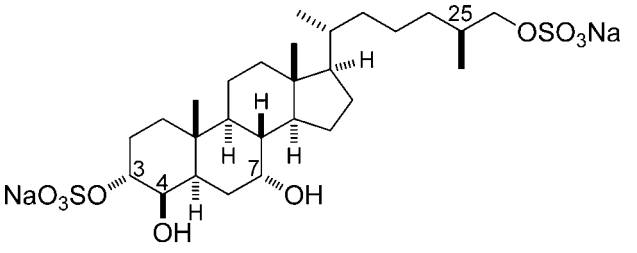
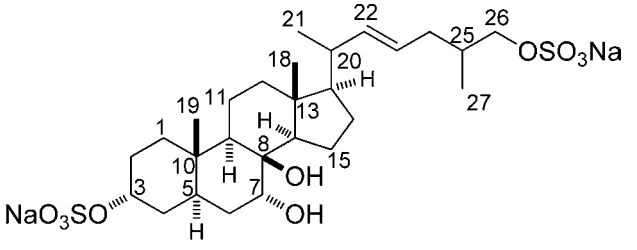
mechanism remains totally unknown. Spermatozoa of the ascidians *Ciona intestinalis* and *Ciona savignyi* exhibit clear chemotaxis toward their eggs,[8,9] and the chemoattractant released by the eggs was identified and designated as a sperm-activating and attracting factor (*Ciona*-SAAF **1**, Figure 1).[10,11] This was the first example of a single agent concomitantly inducing sperm activation and attraction. To understand the molecular mechanism underlying the genus-specificity of sperm chemotaxis of ascidians, further structure analysis of SAAF molecules from different genera is indispensable. In this paper, we report the structure of a novel SAAF (*Ascidia*-SAAF **2**, Figure 1) isolated from the eggs of the ascidian *Ascidia sydneiensis* belonging to a genus different from that of *C. intestinalis* and *C. savignyi*.



Ciona-SAAF (**1**) from *Ciona intestinalis*



Ascidia-SAAF (**2**) from *Ascidia sydneiensis*

Figure 1. Structures of SAAF **1** from *Ciona intestinalis* and SAAF **2** from *Ascidia sydneiensis*.

Ascidia-SAAF **2** was isolated from the egg seawater of the ascidian *Ascidia sydneiensis* that is known to induce the activation of the *Ascidia* sperm (Figure 2a). The egg seawater was first fractioned with an oasis HLB column and then purified by performing reversed-phase HPLC. Each HPLC fraction was subjected to sperm-attracting and -activating assays (Figure 2b), which were performed by previously reported methods.[10,12,13] Notably, both activities were consistently eluted in the same fractions (Figure 2b), indicating that the same molecule caused both sperm activation and chemotaxis, as is the case for *Ciona*-SAAF **1**.[10] We obtained 4 nmol (2.6 μg) of *Ascidia*-SAAF **2** from the active HPLC fractions. *Ciona*-SAAF **1** acts not only on *Ciona intestinalis* but also on another species of the same genus, *Ciona savignyi*;[10] however, it does not activate sperms of organisms from a different genus, such as *A. sydneiensis* (Figure 2c), demonstrating the genus-specificity of SAAF.[14]

The chemical structure of *Ascidia*-SAAF **2** (Figure 1) was elucidated by performing NMR spectroscopy and MS. The molecular formula of *Ascidia*-SAAF **2**, $C_{27}H_{44}O_{10}S_2Na_2$, was obtained from the negative-ion high-resolution MS (m/z 296.1188, $[M - 2Na]^{2-}$, calculated m/z 296.1193), indicating a dehydrogenated or oxygenated ($-H_2$) form of *Ciona*-SAAF **1** ($C_{27}H_{46}O_{10}S_2Na_2$). The gDQF-COSY and gHMBC spectra (see Figures S1–10 in the Supporting Information) obtained using the cold-probe technology[15] unambiguously demonstrated the presence of a double bond between C-22 and C-23. Although chemical shifts of H-22 and H-23 almost overlapped to provide second-order signals, its *E* configuration was identified on the basis of a large $^3J_{HH}$ (17 Hz) between H-22 and H-23, deduced from a spectral simulation of the signals (see Figure S11 in the Supporting Information). The absence of ROE between H-21 and H-24 could lend support for the presence of this configuration.

Unexpectedly, the connectivity of signals elucidated by gDQF-COSY and gTOCSY indicated an absence of the 4-OH group, which was present in *Ciona*-SAAF **1**. In contrast, a hydroxy group located on C8 was deduced from the ^{1}H and ^{13}C NMR assignments of *Ascidia*-SAAF **2** (Table 1) that were interpreted on the basis of the gDQF-COSY, gTOCSY, gHSQC, and gHMBC spectra (see Figures S1–10 in the Supporting Information); among 27 carbons, chemical shifts of 26 carbons except a quaternary carbon located at 8 position were assigned, which allowed us to reasonably assign/allocate the remaining hydroxy group at C8.

The stereochemistry of the sterol ring moiety could be deduced from the coupling constants and ROEs (Figure 3). Small *J*-couplings for H-3 and H-7 indicated that 3-OSO_3 and 7-OH were axially substituted on rings A/B, whereas a relatively large coupling for H-5/H-4, together with ROE between H-5 and H-9, pointed to the trans-fused configuration of rings A/B. A confirmation of the trans-fused arrangement of B and C rings of *Ascidia*-SAAF **2** was obtained from an ROE correlation between H-9 and H-14, and an ROE correlation between H-7 and H-15 indicated the β configuration of the 8-OH. An ROE between H-18 and H-20 suggested an *R* configuration of the methine group at C-17. The proposed planar and partial stereo structure of *Ascidia*-SAAF **2** thus obtained was further verified by synthesizing model compounds of SAAF **2** along with all the possible diastereomers at

(8) Miller, R. L. *Nature* **1975**, *254*, 244–245.
(9) Yoshida, M.; Inaba, K.; Morisawa, M. *Dev. Biol.* **1993**, *157*, 497–506.
(10) Yoshida, M.; Murata, M.; Inaba, K.; Morisawa, M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14831–14836.
(11) Oishi, T.; Tsuchikawa, H.; Murata, M.; Yoshida, M.; Morisawa, M. *Tetrahedron* **2004**, *60*, 6971–6980.
(12) Shiba, K.; Márián, T.; Krasznai, Z.; Baba, S. A.; Morisawa, M.; Yoshida, M. *Cell Motil. Cytoskeleton* **2006**, *63*, 623–632.
(13) Yoshida, M.; Shiba, K.; Yoshida, K.; Tsuchikawa, H.; Ootou, K.; Oishi, T.; Murata, M. *FEBS Lett.* **2008**, *582*, 3429–3433.
(14) Minganti, A. *Pubbl. Staz. Zool. Napoli* **1951**, *23*, 58–65.
(15) Styles, P.; Soffe, P. N. F.; Scott, C. A.; Cragg, D. A.; White, D. J.; White, P. C. J. *J. Magn. Reson.* **1984**, *60*, 397–404.

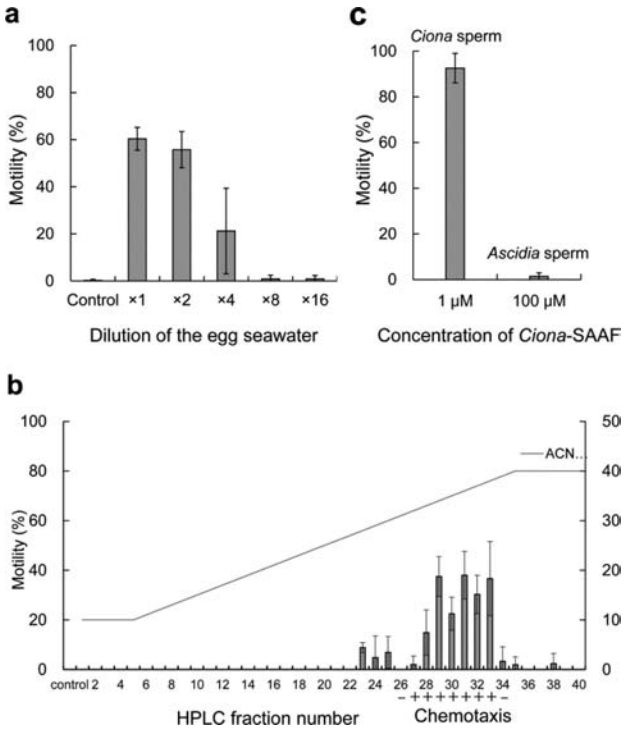


Figure 2. SAAF-induced activation of sperm motility. Sperm motility is the rate of motile sperms in each solution. (a) The egg seawater of the ascidian *Ascidia sydneiensis* induces the activation of *Ascidia* sperm motility. Control is the artificial seawater. (b) The motility of *Ascidia*-sperm for HPLC fractions of the *Ascidia* egg seawater. Fractions 27−33 induced not only sperm activation but also chemotaxis. (c) *Ciona*-SAAF **1** does not activate *Ascidia* sperm motility even at 100 μM. The bars in panels a−c show the standard deviations (n = 3).

C7 and C8 (details of the synthetic process will be published in due course).

Given the complexity of the structure of *Ascidia*-SAAF **2** elucidated from NMR measurements and the extremely low sample amount, other supporting structural data was indispensable for confirming the structure. So far, charge-remote fragmentation patterns obtained from high-energy collision-induced dissociation experiments on a tandem FABMS spectrometer have been effectively used for structural elucidation of natural products, including *Ciona*-SAAF **1**.[10] Recently, it was shown that MALDI-TOF-TOF using a Spiral TOF technique[16−18] provides similar fragmentation patterns with a sample amount of less than 1% of that necessary for performing tandem FABMS measurement. In the product ion spectrum obtained from the MALDI-TOF-TOF experiment of *Ascidia*-SAAF **2** (Figure 4), the precursor ion at m/z 513 corresponding to a desulfated product $[M - 2Na - SO_3 + H]^-$ of *Ascidia*-SAAF **2**

Table 1. NMR Data (ppm) for *Ascidia*-SAAF **2** in D_2O at 300 K[a]

no.	^{1}H	^{13}C	DQF-COSY	HMBC[b]
1	1.18, 1.55	33.3	2	
2	1.80, 1.84	26.5	1, 3	
3	4.65	78.5	4	
4	1.55, 1.60	18.0	3, 5	
5	1.90	32.6	4, 6	
6	1.18, 1.90	31.5	5, 7	
7	3.56 (brt <3 Hz)	72.0	6	
8		n.d.		
9	1.25	50.2	11	
10		36.0		
11	1.59	32.9	9, 12	
12	1.21, 1.99	40.7	11	
13		43.2		
14	1.54	53.9	15	
15	1.38, 1.47	18.6	14, 16	
16	1.28, 1.73	28.4	15	
17	1.14	56.6	16, 20	
18	0.90	13.5		12, 13, 14, 17
19	0.90	11.5		1, 5, 9, 10
20	2.07	39.7	21	
21	0.98	20.3	20	17, 20, 22
22	5.39	140.5	20	
23	5.39	125.5	24	
24	1.93, 2.02	36.0	23, 25	22, 23
25	1.87	33.5	27	
26	3.83, 3.95	74.1	25	
27	0.92	16.4	25	24, 25, 26

[a] Chemical shifts were referred to trace methanol (3.34 ppm for ^{1}H NMR, and 49.5 ppm for ^{13}C NMR). [b] Correlations are from H to C.

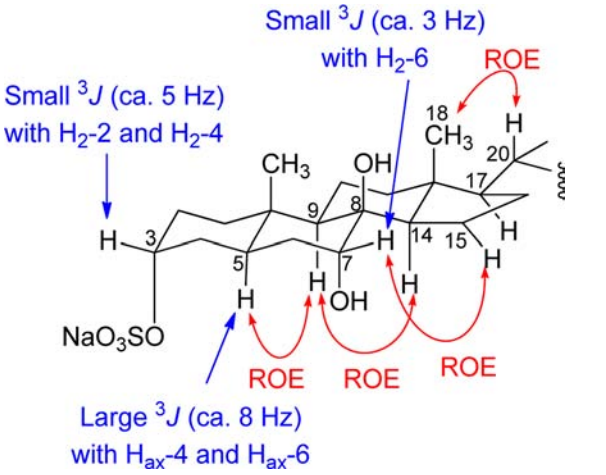


Figure 3. NMR elucidation of partial stereostructure of *Ascidia*-SAAF **2**.

provides a typical charge-remote fragmentation pattern. The structural basis for the assignment of prominent product ions is presented in Figure 4, and the fragmentations can be explained reasonably by the proposed structure, particularly for the three oxygen functionalities including

(16) Satoh, T.; Sato, T.; Tamura, J. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 1318–1323.
(17) Satoh., T.; Sato, T.; Kubo, A.; Tamura, J. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 797–803.
(18) Hamamoto, Y.; Tachibana, K.; Holland, T. P.; Shi, F.; Beuzenberg, V.; Itoh, Y.; Satake, M. *J. Am. Chem. Soc.* **2012**, *134*, 4963–4968.

the C8-OH, thus further supporting the proposed structure of *Ascidia*-SAAF **2**.

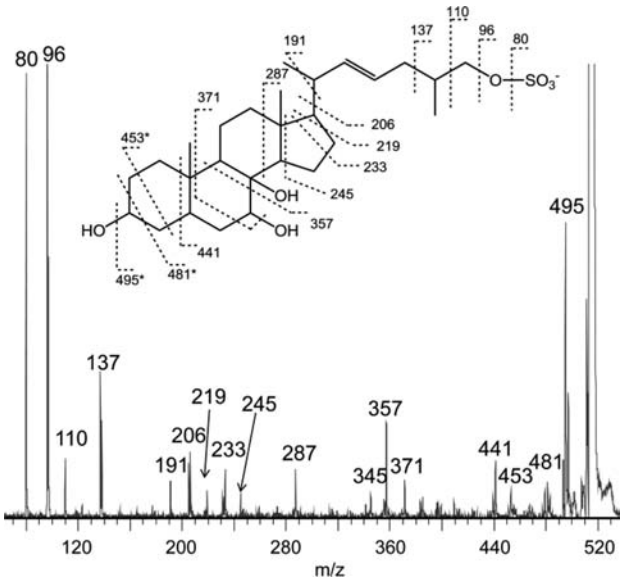


Figure 4. Spiral-TOF-TOF MS spectrum and plausible fragmentation patterns of *Ascidia*-SAAF **2**. The spectrum was derived from precursor ions at *m*/*z* 513, which was generated by the removal of a sulfate group at C3. Asterisks show dehydrated ions.

In conclusion, we successfully determined the structure of a new SAAF **2** isolated from *Ascidia sydneiensis* with only 4 nmol (2.6 μg) of the sample. The structural differences between SAAFs **1** and **2** are in the double bond of the side chain and the position of a hydroxy group, the latter of which is more likely to confer the genus-specificity because the Spiral-TOF-TOF MS analysis of a different HPLC fraction having a similar activity toward the *Ascidia* sperm further detected a congener of *Ascidia*-SAAF **2** differing from **2** in the side chain structure, thus indicating the tolerance of the side chain recognition. Here it should be emphasized that, to the best of our knowledge, there has been no prior study on the structural comparison of species- or genus-specific sperm attractants, and therefore the molecular mechanism underlying the specificity of sperm chemotaxis is a total mystery. In this context, this study provides the first clue on the molecular strategy in genus-specific mating of aquatic animals (that is, *chemical tactics* in sperm *chemotaxis*). To gain further insight into strategies and mechanism of sperm chemotaxis in ascidians, isolation of new SAAFs from different genus as well as identification of their receptors are indispensable, and such studies are now in progress. Finally, we demonstrate in this study that highly sensitive cold-probe-equipped NMR[15] enables assignment of not only ^{1}H but also the ^{13}C signals of only a 4 nmol sample. Combination with recently developed Spiral-TOF-TOF MS[16−18] will further accelerate discovery and structure determination of natural products in vanishingly small quantities.

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Supporting Information Available. Experimental details of purification and structure analysis of *Ascidia*-SAAF **2**, NMR spectra of *Ascidia*-SAAF **2**, and spectral simulation of ^{1}H NMR of the olefin (H-22 and H-23) signals. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.