A chemoattractant for ascidian spermatozoa is a sulfated steroid

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Edited by William J. Lennarz, State University of New York, Stony Brook, NY, and approved September 26, 2002 (received for review August 5, 2002)

Sperm chemotaxis toward eggs before fertilization has been demonstrated in many animals and plants, and several peptides and small organic compounds acting as chemoattractants have been identified. We previously showed that sperm of the ascidians *Ciona intestinalis* and *Ciona savignyi* are activated and then attracted toward the egg by a common factor released from the egg. In this study, we purified sperm-activating and -attracting factor (SAAF) from the egg-conditioning medium of *C. intestinalis* by using several steps of column chromatography. Determination of the molecular structure by NMR and MS/MS analysis revealed that SAAF is a previously uncharacterized sulfated steroid: 3,4,7,26-tetrahydroxycholestane-3,26-disulfate. Furthermore, it was shown that the SAAF of *C. savignyi* was indistinguishable from that of *C. intestinalis* in terms of the chromatographic behavior and molecular weight, indicating that the same compound might be responsible for sperm activation and chemotaxis in both the species. Furthermore, we established a method for quantitative analysis of sperm chemotaxis and showed that the chemotactic behavior of *Ciona* sperm is controlled by the "chemotactic turn" associated with decrease in the concentration of SAAF.

Chemotactic behavior is an important communication system among cells. Chemotaxis of spermatozoa toward eggs during fertilization is known in most animals and lower plants (1, 2). The chemical nature of the sperm attractants has been determined in the bracken fern to be a bimalate ion (3) and in brown algae to be unsaturated hydrocarbons (4–6). In animal species also, some candidates of sperm attractants have been reported (7, 8), and the chemical structures of the sperm chemoattractants in three species, the sea urchin *Arbacia punctulata* (9), the coral *Montipora digitata* (10), and *Xenopus laevis*, have been identified (11). Despite much effort having been devoted to clarification of the mechanism underlying the chemotaxis, the absence of reliable bioassay methods has hampered the quantitative evaluation of sperm chemotaxis.

Spermatozoa of the ascidian *Ciona intestinalis* are either immotile or only slightly motile when suspended in seawater. However, when an unfertilized egg is set in the sperm suspension, the spermatozoa near the egg are intensely activated and begin to show chemotactic behavior toward the egg (12–14). We showed in a previous study that the eggs probably release a sperm-activating and -attracting factor (SAAF) from their vegetal pole (14, 15). SAAF induces entry of extracellular Ca^{2+} and an increase in intracellular cAMP in the sperm (15, 16), which induces protein kinase A-dependent phosphorylation of 21- and 26-kDa axonemal proteins and activation of sperm motility (17). On the other hand, the chemotactic behavior of the ascidian sperm also requires extracellular Ca^{2+} , but theophylline-activated sperm, in which the drug induces increase in the $[\text{cAMP}]_i$ by virtue of being a phosphodiesterase inhibitor, show similar chemotactic behavior to that of normal sperm (14, 15). Therefore, changes in the $[\text{cAMP}]_i$ probably are not required for sperm chemotaxis, and sperm chemotaxis is probably induced by a mechanism different from that inducing sperm activation, although SAAF induces both phenomena.

To understand the mechanism underlying sperm activation and chemotaxis, we attempted to determine the molecular structure of the chemoattractant SAAF. Purification and structural analysis showed that SAAF is a previously uncharacterized sulfated steroid: 3,4,7,26-tetrahydroxycholestane-3,26-disulfate. Furthermore, to distinguish sperm chemotactic responses from sperm activation, sperm motility was selectively activated by theophylline. The chemotactic behavior of the activated sperm was analyzed by using a systematic method. The results clearly show that the quick turn of sperm direction was induced by a decrease in concentration of SAAF. The quick turn controls the chemotactic behavior of the sperm toward the egg.

Methods

Materials. The ascidians *C. intestinalis* and *Ciona savignyi* were collected from Aburatsubo Bay (Kanagawa prefecture), Onagawa Bay (Miyagi prefecture), and Otsuchi Bay (Iwate prefecture) in Japan and maintained in circulating seawater aquaria under constant light for the accumulation of gametes without spontaneous spawning. Eggs and semen were obtained by dissection. Semen was stored at 4°C, and eggs were washed with artificial seawater (ASW, 462 mM NaCl/9 mM KCl/10 mM CaCl_2 /48 mM MgCl_2 /10 mM Hepes, pH 8.2) and maintained in ASW until use at room temperature.

Purification of SAAF. The eggs were washed with ASW, suspended in ≈ 40 volumes of ASW, and incubated at 4°C for 14–20 h. The egg suspension was centrifuged at $1,600 \times g$ for 15 min, and the supernatant was centrifuged further at $22,000 \times g$ for 30 min at 4°C. The clear supernatant was designated as egg seawater (ESW). The ESW was treated with ethanol and chloroform as described (15) to obtain the crude SAAF fraction containing the sperm-activating and -attracting activities (the crude SAAF fraction). This fraction was applied to a Sep-Pak C18 reversed-phase column (20 $\times$ 100 mm; Millipore) equilibrated with deionized water. The adsorbed substances were eluted with 200 ml of a 0 (deionized water) to 100% linear methanol gradient. Fractions (5 ml each) were collected, dried in a centrifugal vaporizer (CVE-200D; Tokyo Rikagaku, Tokyo), and dissolved in 1 ml of deionized water. An aliquot of each fraction was used for the assay of sperm-activating and -attracting activities. Fractions exhibiting these activities were pooled and lyophilized. The residue was dissolved in 2.5 ml of deionized water and loaded onto a Shim-pack PRC-ODS HPLC column (20 mm $\times$ 25 cm; Shimadzu, Kyoto) equilibrated with 20% acetonitrile. The adsorbed substances were eluted with 60 ml of 20% acetonitrile and then with 190 ml of a linear gradient of 20–30% acetonitrile at

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: SAAF, sperm-activating and -attracting factor; ASW, artificial seawater; ESW, egg seawater; ESI/TOF, electrospray ionization/time of flight; LECI, linear equation chemotaxis index.

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5 ml/min. Fractions (10 ml each) were collected, dried in the centrifugal vaporizer, and dissolved in 1 ml of deionized water. The fractions that showed sperm-activating and -attracting activities were pooled and loaded onto a TSKgel ODS-120T HPLC column (4.6 mm $\times$ 25 cm; Tosoh, Tokyo). The column was eluted with 21% acetonitrile at 1 ml/min. The fractions (1 ml each) were collected, dried in the centrifugal vaporizer, and dissolved in 100 μ l of deionized water. All the HPLC procedures were performed on an LC-10 HPLC system (Shimadzu).

Measurements of Sperm Motility. Each sample for examination of the chemotactic activity (ASW, ESW, SAAF, fractions of columns, etc.) was mixed with the same volume of 2% agar, and the mixture was enclosed in the tips of glass capillaries having a diameter of 50–100 μ m. To examine the chemotactic activity of the sperm, stored semen was diluted in ASW containing 1 mM theophylline and incubated for 1 min at room temperature for induction of sperm motility. The theophylline-activated sperm suspension was placed on a glass slide coated with BSA, and the capillary was inserted into the sperm suspension. Just after insertion of the capillary, images of sperm around the capillary tip were recorded on a personal computer every 20 msec by using a high-speed charge-coupled device camera (HAS-200; Ditect, Tokyo) and a video card (HAS-PCI; Ditect). The position of each sperm was analyzed by using an image-analysis program (DIP-MOTION 2D; Ditect), and parameters of chemotactic activity (the chemotaxis index, D , dD/dt , velocity, and θ) were calculated from the data. The precise definition of each parameter is given in *Results*.

Structure Analysis of SAAF. Electrospray-ionization/time-of-flight (ESI/TOF) MS was performed in negative-ion mode with a Mariner (Applied Biosystems) mass spectrometer. The ^1H NMR spectra of SAAF in 20 μ l of a $^2\text{H}_2\text{O}$ solution were recorded at 23°C on a JEOL L-500 spectrometer (500 MHz) by using a 1.7-mm probe (Z-Spec-SMIDG500; Nalorac, Martinez, CA). 2D total correlation spectroscopy spectra were measured for a spin-locking time of 20 msec with data matrices of $2,024 \times 512$ (a total of 82,000 acquisitions for 35 h). Fast atom-bombardment MS/MS experiments were carried out in negative-ion mode by using a HX-110/HX-110 tandem mass spectrometer (JEOL) equipped with a variable dispersion array detector (MS-ADS11). Collision-induced dissociation was effected by introducing helium at a pressure that reduced the intensity of the precursor ions to 30%.

Results

Purification and Characterization of SAAF. The crude SAAF fraction was obtained by ethanol and chloroform treatments from the ESW of the ascidian *C. intestinalis* as described (15). In the present study, the crude SAAF fraction was purified further by sequential application to Sep-Pak C18, Shim-pack PRC-ODS, and the TSKgel ODS-120T columns. The sperm-attracting activity was manifested as a typical sperm trajectory toward the tip of the capillary containing SAAF (Fig. 1). During the purification procedure, the sperm-activating and -attracting activities consistently comigrated in the same fractions, indicating that both activities originated from a single molecular species, SAAF. In other words, the same molecule probably caused both sperm activation and chemotaxis.

The chemical structure of SAAF was elucidated by NMR spectroscopy and MS. The SAAF molecule-related ions appeared at an m/z of 297 $[\text{M}-2\text{Na}]^{-2}$ in ESI/TOF MS (Fig. 2A). High-resolution ESI/TOF-MS analysis of this peak showed that the exact mass was at m/z = 297.1273, corresponding to the structure of $\text{C}_{27}\text{H}_{46}\text{O}_{10}\text{S}_2\text{Na}_2$ (calculated mass, m/z = 297.1266). The presence of two sulfur atoms and the divalent anion observed in the MS suggested that SAAF possesses two sulfate

Fig. 1. Photograph of sperm trajectories near a capillary tube containing purified SAAF. The photo was integrated from 100 images taken every 20 msec. White lines show the axes for analysis. Ticks of the axes are labeled every 50 μ m.

ester groups within its molecule. 2D ^1H NMR spectra were determined successfully even with an extremely limited amount of the specimen (probably a few micrograms) as shown in Fig. 2B. The spectra revealed two singlet methyl signals **g** and **i**, characteristic of steroid skeletons. The presence of additional methyl doublets **h** and complex signals at 0.8–2.0 ppm implied that SAAF possesses a cholestan skeleton, in which the low-field signals **a**, **b**, **c**, **d**, and **e** indicated the presence of four oxygen-bearing carbon atoms. Among these, the connectivity of signals **a**, **b**, and **e** was elucidated by 2D total correlation spectroscopy spectra as the partial structure shown in Fig. 2B *Inset*. This structural moiety fits the description of the C3–C7 part of the steroid skeleton. Signals **c** and **d**, which revealed geminal coupling patterns, were assigned to a methylene group of C26 on the basis of relayed coupling from signals **c/d** to one of the signal **h** methyl doublets (the circled cross-peaks in Fig. 2B are due to the interaction between CH_2 at C26 and CH_3 at C27). The location of the sulfate groups were deduced from the low-field chemical shifts of signals **a** and **c/d** compared with those of hydroxyl-bearing methine and methylene groups. Moreover, their chemical shifts agreed well with the reported values for sulfated sterols from starfish (18). The spectral data indicated that SAAF is a sulfated steroid: 3,4,7,26-tetrahydroxycholestane-3, 26-disulfate (molecular weight, 640.758 as a disodium salt; Fig. 2C). The stereochemistry of the three hydroxyl groups in the sterol ring moiety could be deduced mainly from the interproton coupling constants. Namely, weakly coupling signals for the oxygenated methine proton (Fig. 2B) demonstrated that 3- OSO_3^- , 4-OH, and 7-OH were axially substituted on rings A/B, and relatively large coupling between 5-H and 6-H revealed that rings A/B adopted the trans-fused configuration. A synthetic analog of SAAF that possessed the same ring structure but a shorter side chain exhibited virtually identical ^1H NMR signals for the ring moiety including those for the 3,4,7-oxygenated methine groups (T. Oishi and H. Tuchikawa, personal communication; details of the synthetic process are published elsewhere).

The results of tandem MS/MS analysis were consistent with the NMR-derived structure. High-energy collision-induced dissociation MS/MS experiments were carried out by the negative-ion fast atom-bombardment ionization method. Fig. 3 shows a product ion spectrum obtained from 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 prominent product ions is shown in Fig. 3B. These fragmentations are explained reasonably by the proposed structure, particularly for the part of the three oxygen functionalities. These spectral data and those of the synthetic

Fig. 2. Structure analysis of SAAF. (A) Spectrum of ESI/TOF MS for SAAF at negative-ion mode. One major peak of sample at an m/z of 297.1 and a few accompanying peaks were observed. The intervals between the major peak and the accompanying peaks were $m/z = 0.5$; therefore, the sample of the peak was a divalent anion. (B) 2D total correlation spectroscopy spectrum of SAAF with partial signal assignments (see *Results* for details). (C) Deduced planar structure for SAAF.

analog unambiguously confirmed the structure of SAAF including its stereochemistry in the ring part (the configuration of C25 remains unknown).

Analysis of Sperm Chemotaxis. The ascidian sperm are quiescent in seawater, but SAAF induces both sperm activation and chemotaxis (14). In relation to these phenomena, although activation of sperm motility requires both Ca^{2+} and cAMP, induction of chemotaxis of the sperm does not require any changes in the cAMP levels (15). To distinguish induction of chemotaxis from activation of the sperm motility, the cells were treated first with 1 mM theophylline for 1 min, which causes an increase in the intracellular cAMP level and results in activation of sperm motility with circular movements (15). When the capillary containing ASW was inserted into the sperm suspension, the sperm swam in circular trajectories, and none showed chemotactic behavior toward the capillary tip (Fig. 4A Left). However, when a capillary containing ESW or purified SAAF was inserted into the sperm suspension, the sperm moved in spiral trajectories toward the capillary tip and showed the “chemotactic turn” (arrowheads in Fig. 4B and C) mentioned previously (ref. 19; Fig. 4B and C).

Analysis of these trajectories using two parameters, namely the distance between the capillary tip and the sperm head (D), and the average speed of the sperm approaching the capillary tip (the linear equation chemotaxis index, LECI), clearly demonstrated the physiological function of SAAF in the ascidian sperm. LECI is a parameter that is derived from the negative value of the coefficient ($-a$) in the linear equation ($y = ax + b$) of time (abscissa in the right column of Fig. 4A–D) vs. D (ordinate in the right column of Fig. 4A–D). When the trajectories of sperm swimming around the capillary containing ESW (Fig. 4B Right) or purified SAAF (Fig. 4C Right) were analyzed, D decreased with periodic oscillation, whereas no such decrease was observed

in the case of ASW (Fig. 4A Right). The turns occurred at the peak of the periodical oscillation in the presence of ESW or SAAF (arrows in Fig. 4B and C). The values of LECI were 30.97 ± 15.16 , 37.28 ± 13.54 , and $-0.30 \pm 1.50 \mu\text{m}/\text{sec}$ in the presence of SAAF, ESW, and ASW in the capillaries, respectively (Fig. 4E). These results suggest that the chemotactic turn and LECI may represent the strength of the sperm-attracting activity and offer relevant aspects in the discussion of quantification of sperm chemotaxis.

Detailed Analysis of Sperm Chemotaxis. In addition to LECI, three parameters were used for detailed analysis of the chemotaxis of the *Ciona* sperm as shown in Fig. 5C. The first parameter was the velocity of the sperm (v); the second was the differential quotient of the distance between the capillary tip and the sperm head (D) over time (dD/dt), which represents the velocity of the sperm approaching the capillary tip; and the third parameter was the change in sperm direction moving around a capillary (θ). The v was almost constant regardless of the presence ($212.45 \pm 29.47 \mu\text{m}/\text{sec}$) or absence ($214.03 \pm 20.18 \mu\text{m}/\text{sec}$) of SAAF (Fig. 5), indicating that the sperm velocity was independent of the sperm chemotactic behavior. On the other hand, the change of sperm direction (θ) was almost constant in the 0 – 30° range in which the sperm did not show any chemotaxis in the absence of SAAF (Fig. 5A), indicating that the *Ciona* sperm usually move with a constant curvature. However, when the sperm showed chemotaxis in the presence of SAAF, the swimming direction of the sperm quickly changed, and the θ periodically increased to values of $>60^\circ$ (Fig. 5B, arrowhead). The increase in θ was correlated with the chemotactic turn (arrow in Fig. 5B), which is one of the characteristic movements associated with sperm chemotaxis (19, 20). These results indicate that the chemotactic turn can be characterized quantitatively as quick changes in the direction of sperm movement, that is, a quick increase of θ . Furthermore, θ

Fig. 3. (A) Tandem MS/MS spectrum derived from precursor ions at $m/z = 515$, which was generated by the removal of a sulfate group at C3. (B) A plausible fragmentation pattern is proposed for the precursor ions. Asterisks show dehydrated ions.

always started to increase just after the peak of dD/dt and decreased coincidentally with a decrease in dD/dt (Fig. 5). Because a positive value of dD/dt indicates that the sperm is moving away from the capillary tip, these results suggest that the chemotactic turn occurs when the sperm swims away from the capillary tip. In other words, the cell-signaling pathway for chemotaxis is triggered when the concentration of the attractant around the sperm decreases.

The Roles of SAAF in Sperm Chemotaxis. The sperm-attracting activity of SAAF was no longer observed when SAAF was treated with 1 M HCl at 110°C for 30 min (Fig. 4D), and the LECI of HCl-treated SAAF ($5.85 \pm 7.81 \mu\text{m}/\text{sec}$) was almost the same as that of the control without SAAF ($-0.30 \pm 1.50 \mu\text{m}/\text{sec}$; Fig. 4F). These results suggest that the sulfate groups of SAAF possess an important role in chemotaxis. During ESI/TOF-MS analysis, the peak of m/z at 297 in HCl-treated SAAF disappeared, suggesting that the complete structure of 3,4,7,26-tetrahydroxycholestan-3,26-disulfate is required for complete manifestation of the chemotactic behavior of the ascidian sperm.

Because there was no species-specific difference in the chemotactic behavior of the sperm between *C. intestinalis* and *C. savignyi* (14), we attempted to purify the sperm attractant from the eggs of *C. savignyi* using the same procedure as that for the case of *C. intestinalis*. The sperm attractant of this species also showed the molecular ion peak at an m/z of 297 $[\text{M}-2\text{H}]^{-2}$ in ESI/TOF MS, suggesting that the sperm attractant of *C. savignyi* is identical to the SAAF of *C. intestinalis*. The identical molecular structure of SAAF in both the species may explain the

Fig. 4. Trajectories and chemotactic indices of sperm approaching the tip of capillaries containing ASW (A), ESW (B), purified SAAF (C), and HCl-treated SAAF (D). The sperm were preactivated with 1 mM theophylline. (Left) Typical trajectories of two sperm (black and red dots) in each figure. The capillary tip was set as the origin of the coordinates (0). Arrowheads indicate chemotactic turns. (Right) Change in the distance (D) between the capillary tip (origin) and the head of the swimming sperm. The line and formula represent the linear equation and coefficient of time vs. D , respectively. The LECI is represented by a negative value of the coefficients of the equation. The arrow shows the point of the chemotactic turn shown in Right. (E) Average LECI of purified SAAF, ESW, ASW, and HCl-treated SAAF. The bar shows the standard deviation ($n = 4-8$).

absence of any species-specific differences between the two species in relation to sperm activation and chemotaxis.

Discussion

Previous studies demonstrated that sperm chemotaxis in *Ciona* is caused by SAAF derived from the vegetal pole of the egg. In

Fig. 5. Quantitative analysis of sperm chemotaxis. The parameters (D , dD/dt , velocity, and θ) were calculated from the data as described below. The parameters for sperm moving toward ASW (A) and those for sperm approaching purified SAAF (B) are shown. The same sperm shown in Fig. 4 were used for the analysis. (Top) dD/dt is represented by a red circle, and θ is represented by a blue circle. (Middle) D is shown. Peaks of θ over 60° (arrowhead in upper row) were observed in the sperm showing a chemotactic response (arrow). (Bottom) Sperm velocity is shown. (C) Definition of each parameter. The capillary tip was located at the origin (O). The method for calculation of each parameter on the point $P(x_t, y_t)$ is: the distance between the sperm and the capillary tip (D); $(x_t^2 + y_t^2)^{1/2}$, differential quotient of D with respect to time (dD/dt); $(D_{P2} - D_{P1})/\Delta t$, change of sperm direction (θ), which is shown in C and displayed as an angle. $P_1(x_{t-\Delta t}, y_{t-\Delta t})$ and $P_2(x_{t+\Delta t}, y_{t+\Delta t})$ represent the points of the sperm before and after Δt time, respectively.

the present study, we show that the factor responsible for chemotaxis of the sperm of the ascidians *C. intestinalis* and *C. savignyi* is not a protein or a peptide but a steroid. Detailed analysis of the chemical structure of the sperm attractant SAAF of the ascidian *C. intestinalis* by NMR and MS revealed that it is a sulfated steroid: 3,4,7,26-tetrahydroxycholestane-3,26-disulfate. Some of its chemical characteristics are unique; the hydroxylation pattern at 3, 4, 7, and 26 positions of a cholestan skeleton has never been reported in any other natural substance. In addition, the 3 and 26 positions of the sulfate esters are also unique among sulfated polyhydroxysterols of marine origin.

Sperm chemoattractants in plants were identified as low-molecular-weight organic compounds, e.g., bimalate ion in bracken fern (3, 21) and unsaturated cyclic or linear hydrocarbons, such as ectocarpene, in algae (5). On the other hand, in animals most of the known chemoattractants and candidates are proteins or peptides. The chemoattractant of the sea urchin *A. punctulata*, termed resact, is a 14-aa peptide (9), and that of the amphibian *X. laevis* is a 21-kDa protein (11). The sperm-attracting activity of the hydrozoan *Hippopodius hippopus* and the starfish *Pycnopodia helianthoides* was found to be lost after treatment with some proteases (7, 8). Only the chemoattractant derived from the eggs of the coral *M. digitata* was determined to be an unsaturated fatty alcohol, dodeca-2,4-diynol (10). We show here that the chemoattractant of the ascidian *C. intestinalis* is

neither a peptide nor a protein but a sulfated steroid. Interestingly, a steroid, progesterone, was demonstrated in one study to be a chemoattractant for human sperm (22), although the exact opposite results were obtained by another group (23). Progesterone is one of the components of follicular fluid in mammals and has been shown also to induce the acrosome reaction (24). Thus, although the role of progesterone in the chemotaxis of human sperm is still obscure, steroids released from the female gametes may frequently play important roles in sperm motility among chordates including ascidians and mammals.

Although the molecular structures of the attractants have been determined in several species, no method for quantitative evaluation of sperm chemotaxis has been well established, which makes investigation of the mechanisms of sperm chemotaxis incomplete. Furthermore, in the ascidian, it has proved impossible to distinguish between the chemotaxis and activation of sperm motility, because the ascidian sperm suspended in seawater exhibits little motility, and SAAF induces both activation of motility and chemotaxis. Previously, we demonstrated that theophylline activates motility of the *Ciona* sperm but does not induce chemotactic behavior, and that the theophylline-activated sperm still show normal chemotactic response to SAAF (15). Therefore, theophylline-activated sperm can be used for investigating sperm chemotaxis while excluding the effect of SAAF on the activation of sperm motility.

We established here a method for quantitative analysis of sperm chemotaxis using the LECI besides three parameters, namely velocity (v), quotient of D with time (dD/dt), and change of sperm direction (θ). From the analyses, it was demonstrated clearly that the ascidian sperm are attracted toward the capillary tip containing purified SAAF as well as toward the egg of the species with quick turns of swimming direction. The chemotactic turn occurs when the sperm moves away from the capillary tip. These results suggest that the chemotactic behavior of sperm is controlled by the chemotactic turn that is triggered when sperm detects a decrease in the concentration of the chemoattractant. Repetitions of the turns would enable the sperm to approach the egg to complete fertilization. From the observations of sperm trajectories around a capillary containing an ethanol extract of hydrozoan eggs, similar conclusions could be drawn (19). Therefore, quick turns of the sperm direction with decrease in the concentration of the chemoattractant surrounding the sperm may be one of general mechanisms stimulating sperm chemotaxis.

In terms of the prevention from crossbreeding, species specificity of sperm chemotaxis is interesting, especially in the animals that show external fertilization. Species specificity of sperm chemotaxis was observed in hydrozoa (25) and echinoderm (26). The existence of not species-level but genera-level specificity has been shown in ascidians (13). In our previous study, we showed that there is no species specificity in relation to the activation of sperm motility and chemotaxis between *C. intestinalis* and *C. savignyi* (14). We showed here that the SAAF of *C. intestinalis* and *C. savignyi* have an identical molecular weight. These findings suggest that the lack of species specificity in relation to activation of sperm motility and chemotaxis is ascribable to the molecular identity of the SAAF in both species. Identification of the chemoattractant steroid and its receptor in the ascidians may yield insights into the strategies and mechanisms of sperm chemotaxis in animals.

Steroid hormones usually act on nuclear receptors and activate gene expression (27). However, the chemotactic behavior of sperm occurs within a few seconds, and the sperm nucleus is so condensed that genes cannot be expressed in the sperm. These facts may rule out the involvement of gene expression in sperm chemotaxis. Furthermore, SAAF has four hydroxyl groups, two of which are sulfated esters (Fig. 2C), indicating the hydrophilic nature of the molecule. Therefore, SAAF may bind to receptors

located on the sperm plasma membrane, which may mediate chemotactic behavior via an unknown signal transduction pathway. Identification of the SAAF receptor and elucidation of its signal transduction pathway may be of general significance to the mechanism of chemotaxis in eukaryotes.

We thank Drs. M. Ikeda and H. Ohtake (Dokkyo University School of Medicine, Tochigi, Japan) for help with the ESI/TOF-MS measurements; Dr. H. Naoki (Suntory Institute for Bioorganic Research, Osaka) for help with MS/MS measurements; Mr. S. Adachi (Osaka University)

for help with the NMR measurements; Dr. T. Oishi and Mr. H. Tsuchikawa (Osaka University) for preparation of the synthetic analogs; Dr. H. Tsutsui (University of Tokyo) for advice; and Ms. M. Ishikawa, Mr. H. Iryu, and Mr. M. Sekifuji (University of Tokyo) for technical work. We also thank the directors and staff of the Education and Research Center of Marine Bioresources (Tohoku University) and Otsuchi Marine Research Center (University of Tokyo) for supplying the materials. The work was supported in part by Grant-in-Aids for Scientific Research on Priority Area (A) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to M.Y. and M. Murata), and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to M. Morisawa).

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