Biosci. Biotechnol. Biochem., **72** (7), 1764–1771, 2008

Anti-Fertilization Activity of a Spirocyclic Sesquiterpene Isocyanide Isolated from the Marine Sponge *Geodia exigua* and Related Compounds

Emi OHTA,[1] Mylene M. UY,[2,*] Shinji OHTA,[1] Mihoko YANAI,[3]
Toshifumi HIRATA,[2] and Susumu IKEGAMI[1,†]

[1]*Nagahama Institute of Bio-Science and Technology, 1266 Tamura-cho, Nagahama, Shiga 526-0829, Japan*
[2]*Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*
[3]*Natural Science Center for Basic Research and Development, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*

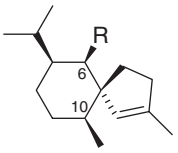
Received January 30, 2008; Accepted April 1, 2008; Online Publication, July 7, 2008
[doi:10.1271/bbb.80071]

(−)-10-*epi*-Axisonitrile-3, a spirocyclic sesquiterpene isocyanide obtained from the marine sponge *Geodia exigua*, immobilized sperm of sea urchin and starfish to block fertilization at the minimum effective concentration of 0.4 μg/ml. On the other hand, fertilized eggs developed normally to the gastrula stage in the presence of a 250-times higher concentration of the isocyanide. Analysis by ^{31}P NMR revealed an accumulation of phosphocreatine and a depletion of inorganic phosphate in the isocyanide-treated sperm, suggesting that (−)-10-*epi*-axisonitrile-3 inhibited the phosphocreatine shuttle participating in the high-energy phosphate metabolism, thereby immobilizing sperm to block fertilization. No analogs of (−)-10-*epi*-axisonitrile-3 containing different functionalities or isocyanides with different carbon skeleton exhibited such activity.

Key words: (−)-10-*epi*-axisonitrile-3; fertilization inhibition; sperm; ^{31}P NMR; structure-activity relationship

Marine organisms are a rich source of biologically active terpene isocyanides.[1–3] Some of them exhibit antimalarial,[4–7] antifouling,[8,9] antimicrobial,[10] antifungal,[11] and antiparasitic[12] activities.

In our continuing search for selective inhibitors of echinoderm fertilization and of embryonic development from marine organisms,[13–16] we have isolated new spirocyclic sesquiterpenes, (−)-10-*epi*-axisonitrile-3 (**1**), exiguamide (**2**), exicarbamate (**3**), and exigurin (**4**), from the marine sponge *Geodia exigua* Thiele, and have reported the inhibitory activity of **2** on the spicule formation of sea urchin.[17,18] Further investigation of the biological activity of spirocyclic sesquiterpenes revealed that **1** potently inhibits fertilization of sea urchin and starfish gametes by immobilizing sperm without affecting the embryogenesis of the fertilized eggs. In this paper, we report the selective anti-fertilization activity of **1** and its mode of action. The effects of **2**–**4** and several isocyanides and formamides bearing different carbon skeletons on echinoderm fertilization and embryogenesis are also described.



1 R = NC
2 R = NHCHO
3 R = $NHCO_2CH_3$
4 R = $NHCOCH_2N(CH_3)CH_2COOCH_3$

Materials and Methods

General procedures. IR spectra were recorded on a Jasco FT/IR-5300 spectrometer (Jasco, Tokyo). NMR spectra were recorded on JEOL GSX500 and LA500 spectrometers. Mass spectra were obtained with a JEOL SX102A spectrometer. Elemental analyses were performed on a Perkin Elmer 2400II CHNS/O analyzer (Perkin Elmer, Waltham, MA).

Bioassay using sea urchin gametes and embryos. Winter sea urchin (*Hemicentrotus pulcherrimus*) and summer sea urchin (*Anthocidaris crassispina*) were used in these experiments during the winter (from February to March) and summer seasons (from July to September) respectively. Experiments were performed at 20 °C, and filtered seawater was used throughout. Eggs and sperm

† To whom correspondence should be addressed. Tel: +81-749-64-8160; Fax: +81-749-64-8140; E-mail: s_ikegami@nagahama-i-bio.ac.jp
* Current address: *Department of Chemistry, College of Sciences and Mathematics, Mindanao State University-Iligan Institute of Technology, Andres Bonifacio Avenue, 9200 Iligan City, Philippines*
Abbreviations: EIMS, electron impact mass spectrometry; FABMS, fast-atom bombardment mass spectrometry

were collected by injection of 0.5 M KCl solution to the mature female and male sea urchin respectively, allowing them to shed into beakers filled with filtered seawater. Sample solutions were prepared by adding DMSO solutions of test compounds to seawater to give final DMSO concentrations of less than 0.2%. DMSO had no effect on the biological phenomena observed at the concentrations tested. For the fertilization-inhibition assay, 1 drop of a diluted sperm suspension was added to serially diluted sample solutions, and 10 min later, 1 drop of an egg suspension was added. Thirty min later, the presence of moving sperm and the fertilization envelope were examined under a light microscope. For the development-inhibition assay, 2-cell stage embryos were placed into serially diluted sample solutions. After they were cultured to the 16-cell stage, the embryos were transferred to seawater. They were periodically examined for cytological changes.

Bioassay using starfish gametes and embryos. Oocytes and sperm of the starfish *Asterina pectinifera* were taken from ovarian and testicular fragments respectively. All experiments were carried out at 20 °C using 90% filtered natural seawater throughout. Sample solutions were prepared as described above. Maturation of oocytes was induced by the addition of 1-methyladenine to give a final concentration of 1 μM. Maturing oocytes were inseminated 1 h after the start of 1-methyladenine treatment. In the fertilization-inhibition assay, mature eggs and sperm suspensions were used. The assay was performed in the same manner as described for the sea urchin. In the development-inhibition assay, fertilized eggs were added in serially diluted sample solutions within 30 min of insemination, after which they were examined periodically for cytological changes.

Measurement of ^{31}P NMR spectra of sperm. Testicular fragments of the starfish *A. pectinifera* were used as intact quiescent dry sperm (approximately 2×10^{10} cells/ml). Dry sperm were diluted with 3-fold Ca^{2+}-free seawater to prepare a suspension of actively motile sperm (approximately 5×10^9 cells/ml). DMSO solution of **1** was added to the motile sperm suspension to give a final **1** concentration of 40 μg/ml (approximately 5×10^9 cells/ml). These sperm suspensions were prepared at 4–10 °C. In NMR measurements, 3 ml of a sperm suspension with or without **1** was placed into a 10-mm diameter NMR tube. Measurements were performed at 10 °C in a JEOL GSX500 spectrometer operating at 202 MHz for ^{31}P observation using 45° pulses and a repetition time of 1.0 s without proton decoupling for 10 min (600 scans). Chemical shifts were measured relative to H_3PO_4 dissolved in D_2O (0 ppm) in a capillary tube, which was added as an external standard in a 10-mm diameter NMR tube.

Natural products. Compounds **1–4** were obtained from the marine sponge *Geodia exigua* Thiele, as described in our previous paper.[18] Compound **13** was isolated from a marine sponge, *Acanthella* sp., and identified as kalihinol F on the basis of spectral data, as described in our previous paper.[19]

Synthetic isocyanides. Compound **5** was synthesized from cyclohexanone and 1,4-dibromobutane *via* spiro[4.5]decan-6-one (**5a**) and spiro[4.5]dec-6-ylamine (**5b**). Compounds **7**, **9**, and **12** were prepared by coupling of commercially available amine derivatives with $CHCl_3$. Compounds **6**, **8**, **10**, and **11** were purchased from Sigma-Aldrich (St. Louis, MO).

*Spiro[4.5]decan-6-one (**5a**).* Following the procedure described by Mousseron *et al.*,[20] to a solution of *t*-BuOK (2.2 g, 20 mmol) in *t*-BuOH (20 ml) and dry benzene (15 ml) were added cyclohexanone (1.0 g, 10 mmol) and 1,4-dibromobutane (2.2 g, 10 mmol). The reaction mixture was refluxed for 5 h at 120 °C. After cooling and the addition of 5% HCl to the reaction mixture, the benzene layer was separated and the aqueous layer was extracted with ether. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane/EtOAc = 99:1 as an eluent) to give **5a** (0.80 g, 25%) as a colorless oil. IR ν_{max} (film) cm^{-1}: 2,942, 2,864, 1,707, 1,447; NMR δ_H (500 MHz, $CDCl_3$): 1.25–1.71 (m, 12H), 1.94 (m, 2H), 2.29 (t, 2H, $J = 6.5$ Hz); NMR δ_C (125 MHz, $CDCl_3$, a selected signal): 214.0 (C=O); EIMS m/z (rel. intensity) 152 M^+ (60), 111 (100).

*Spiro[4.5]dec-6-ylamine (**5b**).* Following the procedure described by Borch *et al.*,[21] a solution of **5a** (0.40 g, 2.6 mmol), ammonium acetate (2.5 g, 33 mmol) and $NaBH_3CN$ (0.18 g, 2.8 mmol) in absolute MeOH (10 ml) was stirred for 48 h at room temperature. Concentrated HCl was added to acidify to pH 2 or lower, and the MeOH was removed *in vacuo*. The residue was taken up in water and extracted with ether. The aqueous solution was brought to alkalinity over pH 10 using a KOH solution, saturated with NaCl, and extracted with ether. The combined organic extracts were dried over Na_2SO_4 and concentrated *in vacuo* to give crude **5b** (0.29 g, 73%). IR ν_{max} (film) cm^{-1}: 3,364, 2,928, 2,861, 1,449; NMR δ_H (500 MHz, $CDCl_3$): 1.04–1.56 (m, 16H), 2.51 (dd, 1H, $J = 8.5$, 3.7 Hz, C*H*NH_2), 2.54 (br s, 2H, NH_2); NMR δ_C (125 MHz, $CDCl_3$, a selected signal): 56.3 ($CHNH_2$); FABMS m/z 154 $[M + H]^+$.

*6-Isocyanospiro[4.5]decane (**5**).* Following the procedure described by Sasaki *et al.*,[22] a mixture of crude **5b** (100 mg, 0.58 mmol), benzyltriethylammonium chloride (10 mg), benzene (5 ml), and 50% aqueous KOH (8 ml) was vigorously stirred for 30 min at room temperature. To the resulting emulsion was added $CHCl_3$ (80 mg, 0.67 mmol) in benzene (3 ml) on ice for 1 h. Stirring was continued for 2 h at room temper-

ature. The benzene layer was separated, washed with water, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was passed through a silica gel column and eluted with hexane to give **5** (11 mg) as a colorless volatile oil from crude **5b** in 11% yield. IR ν_{max} (film) cm^{-1}: 2,940, 2,865, 2,135, 1,449; NMR δ_H (500 MHz, $CDCl_3$): 1.20–1.82 (m, 16H), 3.42 (br t, 1H, $J = 3.7$ Hz, CHNC); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 60.7 (t, $J_{N\text{-}C} = 5.7$ Hz, *C*HNC), 154.4 (t, $J_{N\text{-}C} = 5.7$ Hz, NC); EIMS m/z (rel. intensity) 162 $[M-H]^+$ (38), 134 (100). It has been reported that some isocyanide compounds show an $[M-H]^+$ ion abundantly in their EIMS.[23] HREIMS m/z 162.1279 $[M-H]^+$ (calcd. for $C_{11}H_{16}N$, 162.1282).

*2-Adamantylisocyanide (**7**).* This compound was prepared as colorless crystals (mp 178–180 °C) from 2-adamantylamine hydrochloride in 63% yield according to the procedure described for the conversion from **5b** to **5**. IR ν_{max} (film) cm^{-1}: 2,909, 2,857, 2,133; NMR δ_H (500 MHz, $CDCl_3$): 1.62–2.16 (m, 14H), 3.80 (br s, 1H, CHNC); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 58.9 (t, $J_{N\text{-}C} = 5.7$ Hz, *C*HNC), 154.5 (t, $J_{N\text{-}C} = 5.7$ Hz, NC); EIMS m/z (rel. intensity) 161 M^+ (10), 160 $[M-H]^+$ (20), 134 (100). *Anal.* Found: C, 81.97; H, 9.23; N, 8.50%. Calcd. for $C_{11}H_{15}N$: C, 81.94; H, 9.38; N, 8.69%.

*Stearylisocyanide (**9**).* Following the procedure described by Gokel *et al.*,[24] NaOH pellets (6 g) were added in portions to H_2O (6 ml) under constant vigorous stirring. A solution of stearylamine (1.4 g, 5.0 mmol), $CHCl_3$ (2.4 g, 20 mmol), and benzyltriethylammonium chloride (0.18 g) in CH_2Cl_2 (6 ml) was added dropwise to the alkaline solution over a period of 20 min. After initiation of the addition, the reaction mixture began to boil spontaneously, and subsided within 1 h. Stirring was continued for an additional 1 h and the mixture was diluted with ice-cooled water. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic extracts were successively washed with water and 5% NaCl and dried over K_2CO_3, and the solvent was removed *in vacuo*. The residue was purified by silica gel column chromatography (hexane/diethyl ether = 98:2 as an eluent) to furnish **9** (0.136 g, 10%) as a yellow oil. IR ν_{max} (film) cm^{-1}: 2,924, 2,853, 2,145, 1,466; NMR δ_H (500 MHz, $CDCl_3$): 0.88 (t, 3H, $J = 6.7$ Hz), 1.26 (m, 32H), 3.37 (br t, 2H, $J = 6.1$ Hz, CH_2NC); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 41.5 (t, $J_{N\text{-}C} = 6.7$ Hz, *C*H_2NC), 155.8 (t, $J_{N\text{-}C} = 5.7$ Hz, NC); EIMS m/z (rel. intensity) 279 M^+ (24), 278 $[M-H]^+$ (49), 96 (100). *Anal.* Found: C, 81.52; H, 13.32; N, 4.99%. Calcd. for $C_{19}H_{37}N$: C, 81.65; H, 13.34; N, 5.05%.

*(R)-(+)-1-(1-Naphthyl)ethylisocyanide (**12**).* This compound was prepared as a yellow oil from (*R*)-(+)-1-(1-naphthyl)ethylamine in 35% yield according to the procedure described for **7**. IR ν_{max} (film) cm^{-1}: 2,137, 1,514, 1,445; NMR δ_H (500 MHz, $CDCl_3$): 1.85 (d, 3H, $J = 6.7$ Hz), 5.59 (br q, 1H, $J = 6.7$ Hz), 7.52–7.93 (m, 7H, naphthyl); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 51.0 (t, $J_{N\text{-}C} = 5.7$ Hz, *C*HNC), 156.8 (t, $J_{N\text{-}C} = 5.7$ Hz, NC); EIMS m/z (rel. intensity) 181 M^+ (49), 180 $[M-H]^+$ (67), 166 (88), 153 (100). *Anal.* Found: C, 86.10; H, 6.06; N, 7.63%. Calcd for $C_{13}H_{11}N$: C, 86.15; H, 6.12; N, 7.73%.

Formamides. The formamides were generally prepared as follows: to the corresponding isonitrile analog was added acetic acid, and the solution was stirred at room temperature for 20 h. After the solvent was removed, the residue was passed through a short silica gel column, which was then eluted with varying ratios of hexane-ethyl acetate mixture to give the corresponding formamide as a mixture of s-*cis* and s-*trans* isomers, the s-*cis* isomer being the major product. Compound **15** was purchased from Sigma-Aldrich.

*6-Formylaminospiro[4.5]decane (**14**).* IR ν_{max} (film) cm^{-1}: 3,263, 1,663; NMR δ_H (500 MHz, $CDCl_3$): 1.23–1.75 (m, 16H), 3.17 (dt, 0.3H, $J = 3.7$, 9.5 Hz), 3.94 (dt, 0.7H, $J = 3.7$, 9.2 Hz), 5.51 (br, 0.7H, NH), 5.75 (br, 0.3H, NH), 8.08 (d, 0.3H, $J = 12.2$ Hz, CHO), 8.20 (br s, 0.7H, CHO); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 52.4, 57.4 (*C*HNHCHO), 160.7, 164.0 (NHCHO); FABMS m/z 182 $[M+H]^+$, 137 [M-NHCHO]$^+$; HRFABMS m/z 182.1538 $[M+H]^+$ (calcd. for $C_{11}H_{19}NO$, 182.1545).

*2-Adamantylformamide (**16**).* IR ν_{max} (film) cm^{-1}: 3,299, 1,688, 1,657; NMR δ_H (500 MHz, $CDCl_3$): 1.64–1.93 (m, 14H), 3.62 (d, 0.3H, $J = 9.2$ Hz), 4.15 (d, 0.7H, $J = 8.5$ Hz), 5.94 (br, 0.7H, NH), 6.23 (br, 0.3H, NH), 8.15 (d, 0.3H, $J = 12.2$ Hz, CHO), 8.18 (br s, 0.7H, CHO); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 52.2, 56.0 (*C*HNHCHO), 160.3, 163.8 (NHCHO); FABMS m/z 180 $[M+H]^+$, 135 [M-NHCHO]$^+$; HRFABMS m/z 180.1387 $[M+H]^+$ (calcd for $C_{11}H_{18}NO$, 180.1388).

*1,1,3,3-Tetramethybutylformamide (**17**).* Compound **17** was identified as 1,1,3,3-tetramethylbutylformamide from its IR, ^1H NMR, ^{13}C NMR, and FABMS spectral data. IR ν_{max} (film) cm^{-1}: 3,293, 1,682; NMR δ_H (500 MHz, $CDCl_3$): 0.84 (s, 9H, *t*-Bu), 1.21 (s, 3H, Me), 1.26 (s, 3H, Me), 1.41 (s, 1H, H-2), 1.61 (s, 1H, H-2), 6.20 (br s, 0.5H, NH), 7.38 (br d, 0.5H, $J = 12.2$ Hz, NH), 7.83 (d, 0.5H, $J = 1.5$ Hz, CHO), 8.04 (d, 0.5H, $J = 12.2$ Hz, CHO); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 54.7, 55.4 (*C*NHCHO), 160.6, 163.2 (NHCHO); FABMS m/z 158 $[M+H]^+$, 113 [M-NHCHO]$^+$.

*Stearylformamide (**18**).* IR ν_{max} (film) cm^{-1}: 3,295, 1,645; NMR δ_H (500 MHz, $CDCl_3$): 0.85 (t, 3H, $J =$ 7.0 Hz, Me), 1.20–1.35 (m, 30H), 1.45–1.55 (m, 2H),

3.18 (dd, 2H, $J = 13.4$, 6.7 Hz), 3.18 (q, 0.4H, $J =$ 6.7 Hz), 3.26 (q, 1.6H, $J = 6.7$ Hz), 5.89 (br, 0.8H, NH), 5.95 (br, 0.2H, NH), 8.00 (d, 0.2H, $J = 12.2$ Hz, CHO), 8.12 (br s, 0.8H, CHO); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 38.1, 41.7 (CH_2NHCHO), 161.2, 164.6 (NHCHO); FABMS m/z 298 $[M + H]^+$; HRFABMS m/z 298.3112 $[M + H]^+$ (calcd for $C_{19}H_{40}NO$, 298.3109).

*Benzylformamide (**19**).* Compound **19** was identified as benzylformamide from its IR, ^1H NMR, ^{13}C NMR, and FABMS spectral data. IR ν_{max} (film) cm^{-1}: 3,281, 1,651, 1,535, 1,499, 1,454; NMR δ_H (500 MHz, $CDCl_3$): 4.33 (d, 0.2H, $J = 6.1$ Hz), 4.44 (d, 1.8H, $J = 6.1$ Hz), 6.46 (br, 0.1H, NH), 6.79 (br, 0.9H, NH), 7.22–7.38 (m, 5H, phenyl), 8.05 (d, 0.1H, $J = 11.6$ Hz, CHO), 8.14 (br s, 0.9H, CHO); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 41.8, 45.5 (CH_2NHCHO), 161.2, 164.7 (NHCHO); FABMS m/z 136 $[M + H]^+$, 91 $[M\text{-}NHCHO]^+$.

*α-Methylbenzylformamide (**20**).* Compound **20** was identified as α-methylbenzylformamide from IR, ^1H NMR, ^{13}C NMR, and FABMS spectral data. IR ν_{max} (film) cm^{-1}: 3,275, 1,661, 1,535, 1,495, 1,452; NMR δ_H (500 MHz, $CDCl_3$): 1.50 (d, 2.4H, $J = 6.7$ Hz, Me), 1.55 (d, 0.6H, $J = 6.7$ Hz, Me), 4.67 (quin, 0.2H, $J = 6.7$ Hz), 5.19 (quin, 0.8H, $J = 6.7$ Hz), 6.03 (br, 0.8H, NH), 6.23 (br, 0.2H, NH), 7.25–7.37 (m, 5H, phenyl), 8.12 (d, 0.2H, $J = 11.6$ Hz, CHO), 8.13 (br s, 0.8H, CHO); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 47.6, 51.6 ($CHNHCHO$), 160.2, 164.1 (NHCHO); FABMS m/z 150 $[M + H]^+$, 105 $[M\text{-}NHCHO]^+$.

*(R)-(+)-1-(1-Naphthyl)ethylformamide (**21**).* IR ν_{max} (film) cm^{-1}: 3,275, 1,655, 1,532; NMR δ_H (500 MHz, $CDCl_3$): 1.69 (d, 2.4H, $J = 6.7$ Hz), 1.70 (d, 0.6H, $J = 6.7$ Hz), 5.47 (quin, 0.2H, $J = 6.7$ Hz), 5.86 (br, 0.8H, NH), 6.01, (quin, 0.8H, $J = 6.7$ Hz m), 6.16 (br, 0.2H, NH), 7.44–8.10 (m, 7H, naphthyl), 8.12 (br s, 0.8H, CHO), 8.20 (d, 0.2H, $J = 12.2$ Hz, CHO); NMR δ_C (125 MHz, $CDCl_3$, selected signals): 43.4, 47.9 ($CHNHCHO$), 160.0, 164.1 ($NHCHO$); FABMS m/z 200 $[M + H]^+$; HRFABMS m/z 200.1069 $[M + H]^+$ (calcd for $C_{13}H_{14}NO$, 200.1076).

Results and Discussion

*Inhibitory activity of (−)-10-*epi*-axisonitrile-3 (**1**) on echinoderm fertilization*

Sea urchin (*Hemicentrotus pulcherrimus*) gametes were treated with **1**–**4** to investigate their effects on fertilization. At concentrations of 0.4 μg/ml and greater, **1** prevented fertilization. On the other hand, compounds **2**, **3**, or **4** did not affect fertilization even at a concentration of 100 μg/ml (Table 1). Although embryogenesis of fertilized eggs was unaffected by **1**, **3**, or **4**, only exiguamide (**2**) inhibited gastrulation, thereby produced spicule-deficient larvae at the minimum effective concentration of 0.1 μg/ml, as described below.

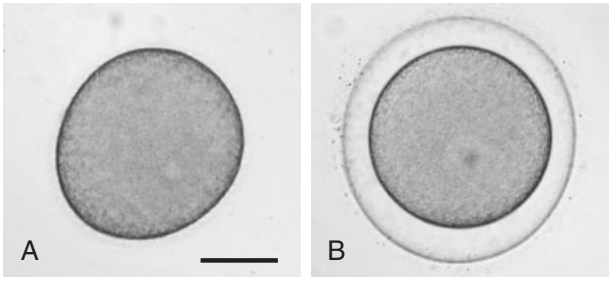


Fig. 1. Inhibition of Fertilization of Starfish by (−)-10-*epi*-Axisonitrile-3 (**1**).

Starfish sperm were incubated in the presence (panel A) and absence (panel B) of 10 μg/ml **1** for 10 min before the addition of an egg suspension. The photographs were taken 10 min after the addition of the egg suspension. Bar indicates 50 μm.

Table 1. Inhibitory Effects of **1**–**4** on the Fertilization and Gastrulation of Winter Sea Urchin (*Hemicentrotus pulcherrimus*) and Starfish (*Asterina pectinifera*)

Compound	Minimum inhibitory concentration (μg/ml)			
	Fertilization		Gastrulation	
	Sea urchin	Starfish	Sea urchin	Starfish
1	0.4	0.4	>100	>100
2	>100	>100	0.1	25
3	>100	>100	>100	>100
4	>100	>100	>100	6.3

When starfish (*Asterina pectinifera*) gametes were treated with **1**–**4**, only **1** exhibited an inhibitory effect on fertilization at a concentration of 0.4 μg/ml, as shown in Fig. 1, while **2**–**4** did not affect fertilization even at concentrations higher than 100 μg/ml. (−)-10-*epi*-Axisonitrile-3 (**1**) did not affect the embryogenesis of fertilized eggs, while **2** and **4** inhibited gastrulation at the minimum effective concentrations of 25 and 6.3 μg/ml respectively (Table 1). These findings indicate that (−)-10-*epi*-axisonitrile-3 (**1**) is a selective inhibitor of echinoderm fertilization.

When starfish or sea urchin unfertilized eggs were admixed with sperm of the corresponding species in the presence of **1** at 0.4 μg/ml, the sperm around the eggs were found to be immotile, indicating that the inhibition of fertilization by **1** was due to inhibition of sperm motility.

Upon dilution in seawater, the sperm of the sea urchin and starfish released from testicular fragments initiated respiration and became motile. Dynein ATPase initiates flagellar movement by driving ATP hydrolysis to produce ADP. Energy utilization by the flagellum of motile sperm is tightly coupled to the rate of energy production by the mitochondria. This coupling depends on the transport of high-energy phosphate compounds

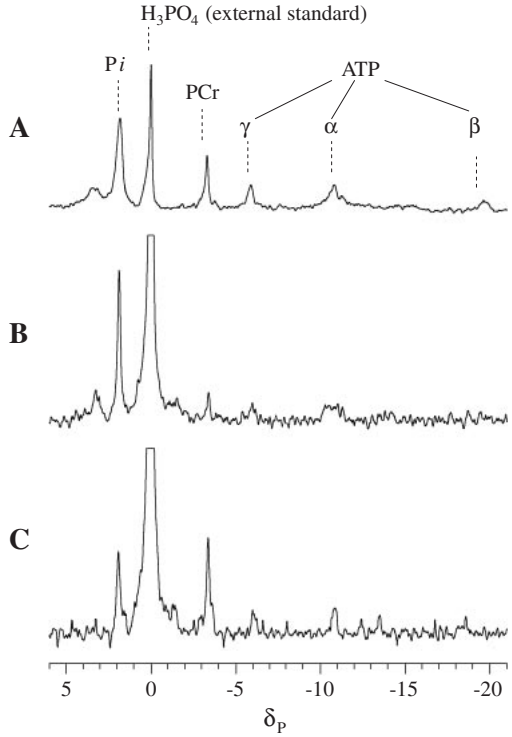
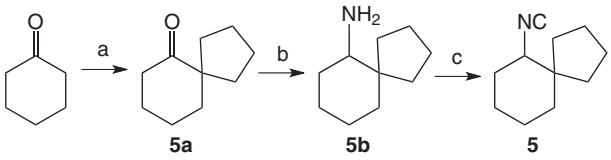


Fig. 2. ^{31}P NMR Spectra of Starfish Sperm Measured at 10 °C.
A, Dry sperm; B, sperm diluted 1:4 in Ca^{2+}-free sea water (pH 8.2) (600 scans); C, after the addition of **1** (40 μg/ml) to the sperm in Ca^{2+}-free sea water (pH 8.2) (600 scans).

from the mitochondria to a flagellar axoneme, mediated by the phosphocreatine energy shuttle.[25,26] To analyze phosphate metabolites of the **1**-immobilized starfish sperm, we assigned ^{31}P signals due to inorganic phosphate (P_i) and high-energy phosphate compounds in intact starfish sperm in the ^{31}P NMR spectra. As shown in Fig. 2A, quiescent dry sperm accumulated phosphocreatine (PCr), but had low levels of P_i.[25,27] When the sperm were diluted to 25% in seawater, a decrease in PCr and an increase in P_i were observed (Fig. 2B), accompanied by sperm mobilization. The ratio of ^{31}P signal areas of P_i to PCr was 1:0.2. The addition of **1** to motile sperm caused an arrest of sperm motility, and concomitantly a decrease in P_i and an increase in PCr were observed in the ^{31}P NMR spectrum (P_i: PCr = 1:1), as shown in Fig. 2C. On the other hand, when a simple spirocyclic isocyanide **5**, whose synthesis is described below, was added to the motile sperm, the ratio of ^{31}P signal areas of P_i to PCr was unchanged (P_i: PCr = 1:0.2; spectral data not shown). These observations suggest that **1** inhibits the phosphocreatine energy shuttle.[25,26] To the best of our knowledge, **1** is the first isocyanide compound found to exhibit selective inhibitory activity on sperm motility.

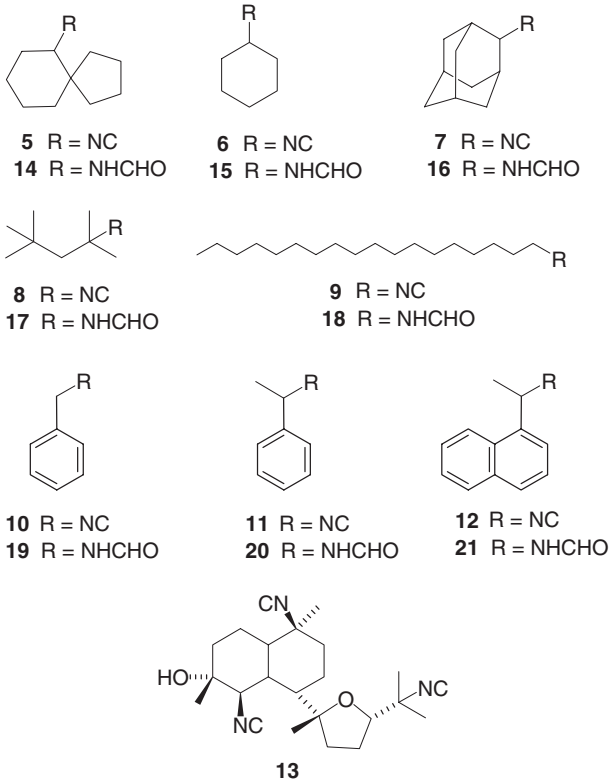
Inhibitory activity of various isocyanides on echinoderm fertilization

Among spirocyclic sesquiterpenes **1**–**4**, only **1** exhibited anti-fertilization activity. These findings indicate



Scheme 1. Synthetic Route for **5**.
Reagents and conditions: a, 1,4-dibromobutane, *t*-BuOK, *t*-BuOH, benzene, reflux; b, ammonium acetate, $NaBH_3CN$, MeOH, r.t.; c, $CHCl_3$, benzyltriethylammonium chloride, aq KOH, benzene, r.t.

that the isocyanide group attached at C-6 of **1** plays a critical role in the inhibition of echinoderm fertilization. In order to determine the structural requirements for the inhibitory activity, we prepared some isocyanides, as follows: Synthesis of a simple spirocyclic isocyanide, 6-isocyanospiro[4.5]decane (**5**), was achieved by previously reported methods,[20–22] as outlined in Scheme 1. Spiro[4.5]decan-6-one (**5a**) was prepared by alkaline-catalyzed coupling of cyclohexanone with 1,4-dibromobutane.[20] Compound **5a** was reduced to an amine derivative (**5b**),[21] followed by alkaline-catalyzed coupling of the amino group with chloroform to yield **5**.[22] Other isocyanide compounds which have an aliphatic ring (**6** and **7**), a linear chain (**8** and **9**), or an aromatic moiety (**10**, **11**, and **12**) were commercially available or were derived from the corresponding amines. Compound **13**, kalihinol F,[28] a diterpene with three isocyanide groups, was isolated from the marine sponge *Acanthella* sp. as described previously.[19]



When sea urchin gametes were used as the test material, compounds **5**–**13** exhibited weak or no

Table 2. Inhibitory Effects of **5**–**13** on the Fertilization and Gastrulation of Summer Sea Urchin (*Anthocidaris crassispina*) and Starfish (*Asterina pectinifera*)

Compound	Minimum inhibitory concentration (μg/ml)			
	Fertilization		Gastrulation	
	Sea urchin	Starfish	Sea urchin	Starfish
5	50	50	>100	13
6	>100	>100	50	>100
7	50	25	50	13
8	>100	>100	>100	100
9	>100	>100	>100	>100
10	100	>100	>100	100
11	>100	100	>100	100
12	100	13	25	3.1
13	>100	>100	100	1.6

inhibition on fertilization, as summarized in Table 2. When starfish gametes were used as the test material, **5**, **7**, and **12** weakly inhibited fertilization, but **6**, **8**, **9**, **10**, **11**, and **13** did not. Notably, the inhibition of sea urchin and starfish fertilization by **5**, **7**, and **12** was not due to the blockade of sperm motility as was observed for **1**, suggesting that they act on gamete fusion or later stages of fertilization.

When fertilized eggs of sea urchin or starfish were used as the test material, kalihinol F (**13**), an inhibitor of topoisomerase I,[19] inhibited the gastrulation of starfish. Compounds **5**–**12** exhibited relatively weak or no inhibitory activity on embryogenesis (Table 2).

These findings indicate that **1** is a highly selective inhibitor of sperm motility, and that the structural requirements for the activity are stringent. Although several natural inhibitors of echinoderm fertilization have been obtained from marine organisms, *e.g.*, jaspisin,[13,15] callyspongins A and B,[16] and halenaquinol sulfate,[29,30] all of them inhibit sperm-egg fusion, but do not affect sperm motility. There are very few, if any, selective inhibitors of sperm motility.

Inhibitory activity of various formamides on spicule formation of sea urchin

Exiguamide (**2**), a spirocyclic sesquiterpene analogue with a formylamino group at C-6, inhibited the micromere formation of sea urchin embryos and subsequent spicule formation at inhibitory concentrations of 0.1–3.0 μg/ml (Fig. 3), as previously reported.[5,6] On the assumption that the formylamino group plays a critical role in the inhibition of spicule formation of the sea urchin embryo, we prepared several formamides (**14**–**21**) by hydrolysis of the corresponding isonitriles (**5**–**12**) and examined their effects on spiculogenesis and fertilization.

As shown in Table 3, only **18** inhibited spicule formation but did not affect fertilization. The other synthetic formamides **14**–**17** and **19**–**21**, affected neither spicule formation nor fertilization. These findings suggest that the structural requirements for the inhibitory activity on spicule formation of sea urchin are stringent.

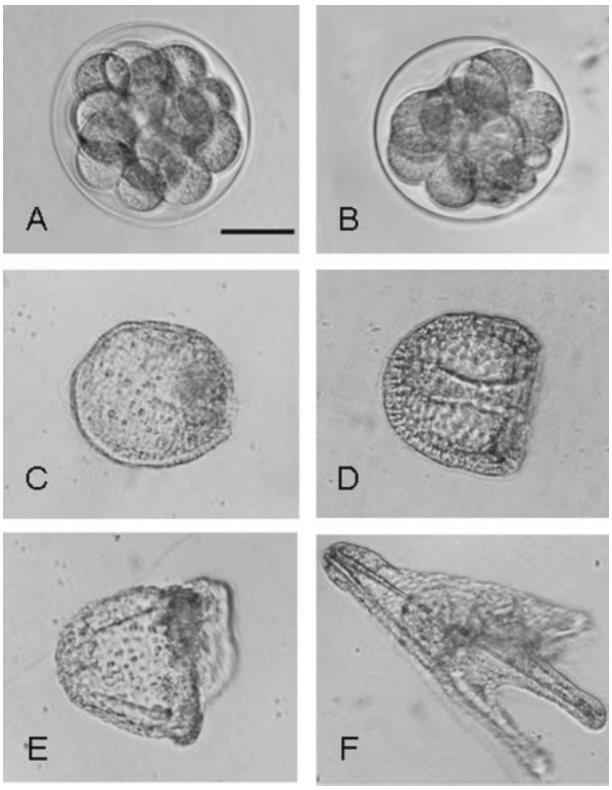


Fig. 3. Effect of Exiguamide (**2**) on the Development of Sea Urchin Embryos.

Embryos were cultured in the presence (panels A, C, and E) and absence (panels B, D, and F) of 0.2 μg/ml of **2** from the 2-cell stage. After they were cultured to the 16-cell stage, the embryos were transferred to seawater. The photographs were taken 2.5 h (panels A and B), 24 h (panels C and D), and 48 h (panels E and F) after insemination. Bar indicates 20 μm.

Table 3. Inhibitory Effects of Formamides **2** and **14**–**21** on the Spicule Formation and Fertilization of Winter Sea Urchin (*Hemicentrotus pulcherrimus*)

Compound	Minimum inhibitory concentration (μg/ml)	
	Spicule formation	Fertilization
2	0.1	>100
14	>100	>100
15	>100	>100
16	>100	>100
17	>100	>100
18	50	>100
19	>100	>100
20	>100	>100
21	>100	>100

In conclusion, the spiro[4.5]decene skeleton of (−)-10-*epi*-axisonitrile-3 (**1**) and exiguamide (**2**) plays an important role in exhibiting the selective biological activity, and the functionality attached to the skeleton is also crucial to the biological activity. Since the sesquiterpene isocyanide (−)-10-*epi*-axisonitrile-3 (**1**) is a major metabolite of the marine sponge *Geodia exigua*

Thiele, it is suggested that its potent anti-fertilization property brings about a the sponge-living marine environment unsuitable for echinoderm reproduction.

Acknowledgments

We thank Mr. Hitoshi Fujitaka of Hiroshima University for NMR measurements. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1) Chang, C. W. J., Naturally occurring isocyano/isothiocyanato and related compounds. *Fortschr. Chem. Org. Naturst.*, **80**, 1–186 (2000).
2) Garson, M. J., and Simpson, J. S., Marine isocyanides and related natural products: structure, biosynthesis and ecology. *Nat. Prod. Rep.*, **21**, 164–179 (2004).
3) Blunt, J. W., Copp, B. R., Hu, W., Munro, M. H. G., Northcote, P. T., and Prinsep, M. R., Marine natural products. *Nat. Prod. Rep.*, **24**, 31–84 (2007), and previous reviews in the series.
4) Angerhofer, C. K., Pezzuto, J. M., Konig, G. M., Wright, A. D., and Sticher, O., Antimalarial activity of sesquiterpenes from the marine sponge *Acanthella klethra*. *J. Nat. Prod.*, **55**, 1787–1789 (1992).
5) Wright, A. D., Konig, G. M., Angerhofer, C. K., Greenidge, P., Linden, A., and Desqueyroux-Faundez, R., Antimalarial activity: the search for marine-derived natural products with selective antimalarial activity. *J. Nat. Prod.*, **59**, 710–716 (1996).
6) Wright, A. D., Wang, H., Gurrath, M., Konig, G. M., Neumann, G., Loria, P., Foley, M., and Tilley, L., Inhibition of heme detoxification processes underlies the antimalarial activity of terpene isonitrile compounds from marine sponges. *J. Med. Chem.*, **44**, 873–885 (2001).
7) Singh, C., Srivastav, N. C., and Puri, S. K., *In vivo* active antimalarial isonitriles. *Bioorg. Med. Chem. Lett.*, **12**, 2277–2279 (2002).
8) Hirota, H., Tomono, Y., and Fusetani, N., Terpenoids with antifouling activity against barnacle larvae from the marine sponge *Acanthella cavernosa*. *Tetrahedron*, **52**, 2359–2368 (1996).
9) Okino, T., Yoshimura, E., Hirota, H., and Fusetani, N., New antifouling sesquiterpenes from four nudibranchs of the family Phyllidiidae. *Tetrahedron*, **52**, 9447–9454 (1996).
10) Chang, C. W. J., Patra, A., Baker, J. A., and Scheuer, P. J., Kalihinols, multifunctional diterpenoid antibiotics from marine sponges *Acanthella* spp. *J. Am. Chem. Soc.*, **109**, 6119–6123 (1987).
11) Trimurtulu, G., and Faulkner, D. J., Six new diterpene isonitriles from the sponge *Acanthella cavernosa*. *J. Nat. Prod.*, **57**, 501–506 (1994).
12) Alvi, K. A., Tenenbaum, L., and Crews, P., Anthelmintic polyfunctional nitrogen-containing terpenoids from marine sponges. *J. Nat. Prod.*, **54**, 71–78 (1991).
13) Ikegami, S., Kobayashi, H., Myotoishi, Y., Ohta, S., and Kato, K. H., Selective inhibition of exoplasmic membrane fusion in echinoderm gametes with jaspisin, a novel antihatching substance isolated from a marine sponge. *J. Biol. Chem.*, **269**, 23262–23267 (1994).
14) Ohta, S., Kobayashi, H., and Ikegami, S., Isojaspisin: a novel styryl sulfate from a marine sponge, *Jaspis* sp., that inhibits hatching of sea urchin embryos. *Tetrahedron Lett.*, **35**, 4579–4580 (1994).
15) Ohta, S., Kobayashi, H., and Ikegami, S., Jaspisin: a novel styryl sulfate from the marine sponge, *Jaspis* species. *Biosci. Biotechnol. Biochem.*, **58**, 1752–1753 (1994).
16) Uno, M., Ohta, S., Ohta, E., and Ikegami, S., Callyspongins A and B: novel polyacetylene sulfates from the marine sponge *Callyspongia truncata* that inhibit fertilization of starfish gametes. *J. Nat. Prod.*, **59**, 1146–1148 (1996).
17) Uy, M. M., Ohta, S., Yanai, M., Ohta, E., Hirata, T., and Ikegami, S., Exiguamide, a new spirocyclic sesquiterpene from the marine sponge *Geodia exigua* that inhibits cell fate specification during sea urchin embryogenesis. *Biorg. Med. Chem. Lett.*, **12**, 3037–3039 (2002).
18) Uy, M. M., Ohta, S., Yanai, M., Ohta, E., Hirata, T., and Ikegami, S., New spirocyclic sesquiterpenes from the marine sponge *Geodia exigua*. *Tetrahedron*, **59**, 731–736 (2003).
19) Ohta, E., Ohta, S., Hongo, T., Hamaguchi, Y., Andoh, T., Shioda, M., and Ikegami, S., Inhibition of chromosome separation in fertilized starfish eggs by kalihinol F, a topoisomerase I inhibitor obtained from a marine sponge. *Biosci. Biotechnol. Biochem.*, **67**, 2365–2372 (2003).
20) Mousseron, M., Jacquier, R., and Christol, H., Rearrangements acido-catalyses (1er memoire). Nouvelles syntheses d′α-spirocetones. *Bull. Soc. Chim. Fr.*, 346–356 (1957).
21) Borch, R. F., Bernstein, M. D., and Durst, H. D., Cyanohydridoborate anion as a selective reducing agent. *J. Am. Chem. Soc.*, **93**, 2897–2904 (1971).
22) Sasaki, T., Eguchi, S., and Katada, T., Synthesis of adamantane derivatives. XXV. Synthesis and reactions of 1- and 2-adamantyl isocyanides. *J. Org. Chem.*, **39**, 1239–1242 (1974).
23) Gillis, R. G., and Occolowitz, J. L., Isocyanides. III. Electron impact study of aliphatic isocyanides. *J. Org. Chem.*, **28**, 2924–2925 (1963).
24) Gokel, G. W., Widera, R. P., and Weber, W. P., Phase-transfer Hofmann carbylamine reaction: *tert*-butyl isocyanide (2-Methylpropane, 2-isocyano). *Org. Syn.*, **55**, 96–99 (1976).
25) van Dorsten, F. A., Wyss, M., Wallimann, T., and Nicolay, K., Activation of sea-urchin sperm motility is accompanied by an increase in the creatine kinase exchange flux. *Biochem. J.*, **325**, 411–416 (1997).
26) Tombes, R. M., and Shapiro, B. M., Metabolite channeling: a phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. *Cell*, **41**, 325–334 (1985).
27) Winkler, M. M., Matson, G. B., Hershey, J. W. B., and Bradbury, E. M., ^{31}P-NMR study of the activation of the sea urchin egg. *Exp. Cell Res.*, **139**, 217–222 (1982).
28) Patra, A., Chang, C. W. J., Scheuer, P. J., Duyne, G. D. V., Matsumoto, G. K., and Clardy, J., An unprecedented

triisocyano diterpenoid antibiotic from a sponge. *J. Am. Chem. Soc.*, **106**, 7981–7983 (1984).

29) Kobayashi, M., Shimizu, N., Kitagawa, I., Kyogoku, Y., Harada, N., and Uda, H., Absolute stereostructures of halenaquinol and halenaquinol sulfate, pentacyclic hydroquinones from the Okinawan marine sponge *Xestospongia sapra*, as determined by theoretical calculation of CD spectra. *Tetrahedron Lett.*, **26**, 3833–3836 (1985).

30) Ikegami, S., Kajiyama, N., Ozaki, Y., Myotoishi, Y., Miyashiro, S., Takayama, S., Kobayashi, M., and Kitagawa, I., Selective inhibition of membrane fusion events in echinoderm gametes and embryos by halenaquinol sulfate. *FEBS Lett.*, **302**, 284–286 (1992).