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A FEMALE GAMETE-CHARACTERISTIC (3Z,6Z,9Z)-DODECATRIENOIC ACID FROM *ANALIPUS JAPONICUS*

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Key Word Index—*Analipus japonicus*; brown alga; sex attractant; (3Z,6Z,9Z)-dodecatrienoic acid; ectocarpene; hormosirene.

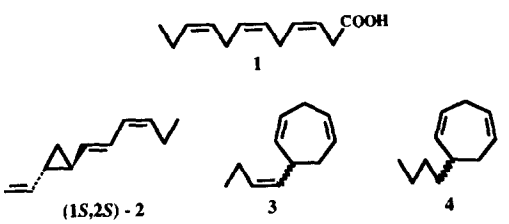
Abstract—(3Z,6Z,9Z)-Dodecatrienoic acid, proposed as a possible precursor of algal sex attractants, has been identified from female gametes of *Analipus japonicus* by comparison of HPLC analyses and mass spectral data with those of a synthetic sample.

INTRODUCTION

Up to this time, eight algal C_{11}-hydrocarbon sex attractants have been identified from marine brown algae [1]. These compounds can be classified into two groups of non-terpenoid hydrocarbons with molecular formulae of $C_{11}H_{18}$ and $C_{11}H_{16}$. In our preceding paper, the $C_{11}H_{16}$ compounds, (1*R*,2*R*)-hormosirene 1-[(1*E*,3*Z*)-hexadienyl]-2-vinylcyclopropane, (1*R*,2*R*)-**2** and ectocarpene 6-[(1*Z*)-butenyl]-1,4-cycloheptadiene, **3** were identified as sex pheromones of the Japanese marine brown algae, *Scytosiphon lomentaria*, *Colpomenia bullosa* and *Analipus japonicus* [2–5]. Based on evidence obtained by incorporation experiments of a synthetic precursor in a terrestrial plant, a hypothetical biogenesis of (1*R*,2*R*)-hormosirene [(1*R*,2*R*)-**2**] and (6*S*)-ectocarpene [(*S*)-**3**] via (3*Z*,6*Z*,9*Z*)-dodecatrienoic acid (**1**) has been proposed by Boland *et al.* [6]. However, the C_{12}-trienoic acid (**1**) has not been detected in marine brown algae so far. We now report its identification in female gametes of *A. japonicus*.

RESULTS AND DISCUSSION

Mature thalli of *A. japonicus* are abundant along the Charatsunai coast at Muroran, the Pacific coast of Hokkaido, northern Japan, from May to June. The sexually mature plants are isomorphic and heterothallic. The mature gametophytes release motile gametes which are anisogamous [7]. In a preliminary experiment, the compositions of free fatty acids in male and female gametes of *A. japonicus* were examined in the form of 9-anthrylmethyl (ADAM) esters by HPLC (Zorbax C_8, MeCN–H_2O, 19:1) equipped with a fluorescence detector (Ex. 365 nm; Em. 412 nm) [8]. Identity of the long-chain fatty acids was established by comparison with the authentic ADAM esters. The major fatty acids were 16:0 and 18:0 in both male and female gametes. A comparison of the compositions of long-chain free fatty acids did not show any striking differences between male and female gametes. To examine polyunsaturated medium chain fatty acids, including the C_{12} acid in both the gametes, appropriate fractions (R_t 5–8 min) were separated by HPLC under the above conditions. From the fraction of the female gametes, a characteristic fatty acid (R_t 24.4 min) was separated on the same column using MeCN–MeOH–H_2O (20:1:5). In order to identify the characteristic fatty acid, a large amount of female gamete suspension was prepared from the female gametophytes (22 bodies) of *A. japonicus* and was immediately treated with hot ethanol to stop the biogeneration of sex attractants and subsequently extracted with methylene chloride. An acidic fraction in the extract was esterified with 1% ADAM solution in ethyl acetate. The ADAM ester fraction was then separated through a Sep-Pak C_{18} cartridge using MeCN–H_2O (4:1). The separated ester was further purified by a repeated chromatography on Zorbax C_8. The purified ADAM ester was estimated to be *ca* 100 ng by a quantitative HPLC analysis. HR-EI mass spectrometry of the ADAM ester of the characteristic fatty acid in female gametes showed a molecular formula $C_{27}H_{28}O_2$ (found: *m/z* 384.2119; calcd for $C_{27}H_{28}O_2$, 384.2090). This corresponds to the molecular formula $C_{12}H_{18}O_2$ of the C_{12}-unsaturated fatty acid (**1**)



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(1*S*,2*S*)-**2** **3** **4**

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proposed by Boland *et al.* [6]. Thus, the authentic trienoic acid (**1**) was prepared by stereo selective hydrogenation of 3,6,9-dodecatriynol [9,10] over Lindlar's catalyst, followed by Jones oxidation [11]. Synthetic **1** was purified by a silica gel column chromatography (hexane–Et_2O, 7:3). The ADAM ester of synthetic (3*Z*,6*Z*,9*Z*)-dodecatrienoic acid (**1**) (HR-EIMS: *m/z* 384.2056 $C_{27}H_{28}O_2$), coincided with the ADAM derivative of the isolated female gamete-characteristic fatty acid. This is the first detection of the C_{12}-trienoic acid proposed as a possible biogenetic precursor of sex attractants in the female gametes of marine brown algae [6].

In order to examine the possible biogenesis of (1*S*,2*S*)-hormosirene and ectocarpene via the C_{12}-trienoic acid (**1**) in female gametes of *A. japonicus*, the sex attractant from the female gamete suspensions (1.5×10^7 cell ml^{-1}) at various times after release of gametes was extracted with CH_2Cl_2–MeOH (2:1). GC analysis of the extract showed that the attractant consists of hormosirene (**2**), ectocarpene (**3**) and dictyotene (**4**) (11:87:2). The amount of (1*S*,2*S*)-**2** (66% e.e.) in the attractants was determined by a quantitative HPLC (Zorbax ODS MeCN–H_2O, 4:1; UV 247 nm) using synthetic (±)-hormosirene. From the GC-compositions of the attractant, total amounts were calculated and plotted (Fig. 1). The concentration of sex attractants was estimated to be 5.3×10^{-17} mol $cell^{-1}$ [recovery efficiency 48.5% for synthetic (±)-**2** from sea water] in the swimming gametes (incubation time 60 min after release). After 6 hr incubation of the gametes (settling gametes), the concentration increased to 14.5×10^{-17} mol $cell^{-1}$ compared with 13.2×10^{-17} mol $cell^{-1}$ after 3 hr incubation (a mixture of swimming and settling gametes). On the other hand, with heat-treated female gametes, there was no increase in the amount of C_{11}-compounds. Thus, the sex attractant was found to be biogenerated at least during the release and settling of the female gametes. However, a significant alternation in the concentrations of **1** and the sex attractant in female gametes was not observed during attractant formation and the concentration of **1** (*ca* 10^{-20} mol $cell^{-1}$) was consistently much lower than that of the attractant (**2**,**3**). These results suggest that the biosynthetic pathway of the sex attractant in the female gametes of *A. japonicus* might be different from that of the higher plants. Very recently, Stratmann *et al.* reported that ectocarpene (**3**) in the female gametes of *Ectocarpus siliculosus* should be derived from ω12-hydroperoxyeicosanoides but not from the C_{12}-trienoic acid [12].

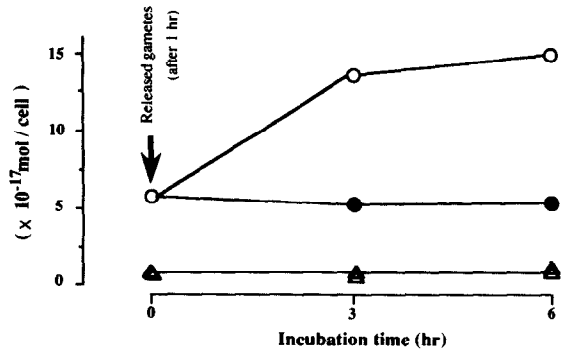

Fig. 1. Time-course of production of sex attractant and its possible precursor (3*Z*,6*Z*,9*Z*)-dodecatrienoic acid (**1**) in female gametes of *A. japonicus*. The concentrations of attractant (○ intact, ● heat-treated) and acid (**1**) ADAM ester (△ intact, ▲ heat-treated) in female gametes were determined by HPLC analyses [Zorbax ODS, MeCN–H_2O (4:1), UV 247 nm for attractant; and Zorbax C_8 4.6 mm × 25 cm, MeCN–MeOH–H_2O (20:1:5), fluorescence detector, Ex. 365 nm, Em. 412 nm for the acid (**1**) ADAM ester].

EXPERIMENTAL

Plant materials. Analipus japonicus was collected along the Charatsunai coast in Muroran, the Pacific coast of Hokkaido, northern Japan, in May–June at low tide in 1988–1992.

Preparation of gametes. Mature gametophytes were rinsed with filtered sea water and blotted on a sheet of filter paper. One cleaned thallus was placed in a Petri dish and kept at 5° for 3 hr in the dark. Then, cold sea water was poured on to the thallus. This resulted in the release of many gametes within 5–10 min. Mating reactions distinguished female or male gametes from the released gametes under a microscope (× 200).

Extraction and analyses of volatile compounds. Living male or female gametes were kept in sea water (100 ml) in a 1 l vessel at room temp. Volatile compounds were trapped on active carbon (5 mg) in a closed-loop-stripping system [13]. After looping for 18 hr, adsorbed compounds on the carbon were eluted with CH_2Cl_2 (50 μl) and analysed by GC and GC-MS. GC was performed on a 0.28 mm × 50 m fused silica glass capillary column coated with SF-96, inj. temp. 120° and column temp. 100°. GC-MS was carried out on a 0.25 mm × 50 m fused silica capillary column coated with DB-1, inj. temp. 120°, column temp. 100° and ionization energy 20 eV. Ectocarpene (**3**): RI 1141; *m/z* (%) 148 (10, $[M]^+$), 133 (13), 91 (100), 77 (27). Hormosirene (**2**): RI 1181; *m/z* (%) 148 (12, $[M]^+$), 133 (2), 79 (100), 77 (53). Dictyotene (**4**) [14]: RI 1168; *m/z* (%) 150 (41, $[M]^+$), 121 (23), 93 (89), 79 (100), 57 (25).

Absolute configuration of hormosirene. Volatile compounds obtained by closed-loop-stripping system of female gametes were subjected to HPLC [Zorbax ODS, 4.6 mm × 15 cm; MeCN–H_2O (17:3), flow rate 1 ml min^{-1}, UV 247 nm] and the peak of **2** (R_t 6.7 min) was sepd. The absolute configuration and enantiomeric excess of sepd **2** were determined by HPLC [Daicel Chiralcel-OB; MeOH–H_2O (17:3), flow rate 0.8 ml min^{-1}, UV 247 nm] using synthetic (±)-**2** and (−)-**2** from *Dictyopteris prolifera* [15]. The R_t of (1*S*,2*S*)-**2** and (1*R*,2*R*)-**2** were 26.1 and 34.6 min, respectively. Enantiomeric sepn of **2** was also achieved by HPLC on Ceramospher Chiral RU-1 (4.6 mm × 25 cm, Shiseido, Japan) using MeOH, flow rate, 0.6 ml min^{-1}; (1*R*,2*R*)-**2** R_t 8.22 min; (1*S*,2*S*)-**2** R_t 10.26 min.

Time-course of attractant production. Mature gametophytes (52 bodies) were rinsed with filtered sea water and blotted on to filter paper. One cleaned thallus was

placed in a Petri dish and kept at 5° for 3 hr in the dark. Then, cold sea water (50 ml) was poured on to the thallus and allowed to stand at 5° for 30 min. From a mating reaction of released gametes, 7 bodies of female and 6 bodies of male gametophytes were selected. A female gamete suspension (1.5×10^7 cell ml^{-1}) was incubated at 5° for 0 hr (60 min after releasing from gametangia), 3 hr (a mixt. of swimming and settling gametes) and 6 hr (settling gametes) in the dark, respectively. To 16 ml of the female gamete suspension, 8 ml of CH_2Cl_2–MeOH (2:1) was added and stirred vigorously (5 min). The mixt. was centrifuged at 2000 rpm for 5 min and the CH_2Cl_2 layer concd (0°, 20 mm Hg). The concentrate was taken up to MeCN (100 μl) and an aliquot was quantitatively analysed by HPLC [Zorbax ODS, 4.6 mm × 15 cm MeCN–H_2O, (17:3), flow rate 1 ml min^{-1}, UV 247 nm]. Recovery efficiency was estimated to be 48.5% using synthetic (±)-hormosirene.

Analysis of free fatty acids and a possible precursor of attractants in female or male gametes. Male or female gamete suspensions were centrifuged at 2500 rpm for 10 min. The resulting ppt. was treated with hot EtOH (2 ml) under N_2. The mixt. was centrifuged at 2000 rpm for 5 min and the supernatant concd to leave a green oil. This oil was suspended in EtOAc (500 μl) and esterified with a 0.5% Me_2CO soln of ADAM (25 μl) for 1 hr at room temp. The esters were analysed by HPLC [fluorescence detector: Ex. 365 nm; Em. 412 nm, Zorbax C_8 4.6 mm × 25 cm, system 1: MeCN–H_2O (19:1), system 2: MeCN–MeOH–H_2O (20:1:5), flow rate 1 ml min^{-1}]. R_ts of ADAM ester of **1** were 6.8 min (system 1) and 24.4 min (system 2), respectively.

Synthesis of (3Z,6Z,9Z)*-dodecatrienoic acid* (**1**). 2-(3-butynyloxy)-Tetrahydropyrane (5.4 g, 35 mmol) in THF (10 ml) was rapidly added into a soln of EtMgBr at 0°, which was prepd from EtBr (7.6 g, 70 mmol) and Mg (0.84 g, 35 mmol) with a catalyst of I_2 in THF (30 ml). After heating at 40° for 5 hr with stirring, the mixt. was cooled to 20° and then CuCN (0.4 g, 4.4 mmol) was added and stirred for 30 min at 0°. Then a soln of 1-bromo-2,5-octadiyne (5.6 g, 30.4 mmol) in THF (20 ml) was slowly added to the mixt. at 0–5°, and the reaction mixt. stirred for 12 hr at 20°. Work-up (cold 2 N HCl, aq. NH_4Cl and brine) gave 2-(3,6,9-dodecatriynyloxy)-tetrahydropyrane (crude 7.6 g). To a soln of 2-(3,6,9-dodecatriynyloxy)-tetrahydropyrane in MeOH (80 ml) was added H_3PO_4 at room temp. and the reaction mixt. stirred for 10 hr at room temp. Work-up (aq. $NaHCO_3$ and brine) gave 3,6,9-dodecatriynol (4.5 g), which was used for the next reaction without further purification. A soln of the residue (1 g) in MeOH (50 ml) was semihydrogenated using Lindlar catalyst (100 mg). The crude product was purified by a silica gel CC (hexane–Et_2O, 7:3) to yield (3Z,6Z,9Z)-dodecatrienol (0.9 g). A soln of CrO_3 (300 mg, 3 mmol) in H_2O (0.86 ml) and conc. H_2SO_4 (0.27 ml) was slowly added to a soln of the dodecatrienol (202 mg, 1.02 mmol) in Me_2CO (15 ml) at 0° with stirring. After stirring for 1.5 hr at 0°, H_2O (30 ml) was added to the mixt. and the greenish soln extracted with pentane (5 × 20 ml). The combined organic layers were washed with brine. After drying, evapn and purification by silica gel CC (pentane–Et_2O, 7:3), 124 mg (57%) of (3Z,6Z,9Z)-dodecatrienoic acid (**1**) was obtained. IR ν_{max} cm^{-1}: 3500, 3020, 2960, 2860, 1710, 1650, 930 and 720. ^1H NMR (400 MHz, $CDCl_3$): δ0.98 (3H, *t*, *J* = 7.5 Hz), 2.07 (2H, *m*), 2.82 (4H, *m*), 3.18 (2H, *d*, *J* = 6.4 Hz), 5.32–5.42 (4H, *m*), 5.58–5.62 (2H, *m*). ^{13}C NMR (100.54 MHz, $CDCl_3$): δ177.7, 132.2, 132.0, 129.2, 128.3, 126.9, 120.5, 32.6, 25.8, 25.5, 20.6, 14.3. EI-MS (Me ester; 20 eV) *m/z* (%): 208 (1, $[M]^+$), 177 (1, $[M-OMe]^+$), 147 (8), 134 (29), 119 (22), 105 (54), 95 (100), 79 (83), 67 (46), 55 (25), 41 (27). HR-EIMS (ADAM ester) *m/z*: 384.2056 and 191.0842. Calcd for $C_{27}H_{28}O_2$ and $C_{15}H_{11}$, 384.2090 and 191.0861, respectively.

Identification of (3Z,6Z,9Z)*-dodecatrienoic acid in female gametes.* A female gamete suspension prepd from 22 bodies was centrifuged at 3000 rpm for 5 min. The ppt. was treated with hot EtOH (100 ml) under N_2. The mixt. was centrifuged at 2000 rpm for 5 min and the resultant supernatant concd to leave a green oil (1.2 g). The oil was partitioned between Et_2O (20 ml) and 1% Na_2CO_3 (10 ml). After the alkaline layer was adjusted to pH 3 with 1 N HCl, the mixt. was extracted × 3 with Et_2O (10 ml) to give a free fatty acid fr. (1.5 mg). This fr. was treated with an Me_2CO soln of ADAM as described above. The ester soln was subjected to reverse-phase chromatography on a Sep-Pak C_{18} cartridge with system 3, MeCN–H_2O (4:1) (8 ml). The ADAM ester was further purified by HPLC on Zorbax C_8 using systems 3 and 2 to yield 98 ng of the ADAM ester of the female gamete-characteristic fatty acid. The isolated ester was identical with the synthetic ADAM ester of **1**. HR-EIMS (ADAM ester) *m/z*: 384.2119 and 191.0871. Calcd for $C_{27}H_{28}O_2$ and $C_{15}H_{11}$, 384.2090 and 191.0861.

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