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AN ALLELOPATHIC POLYUNSATURATED FATTY ACID FROM RED ALGAE

MINORU SUZUKI,* ISAMU WAKANA,†‡ TAKASHI DENBOH‡ and MASAKAZU TATEWAKI†

Division of Material Science, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060, Japan; †Institute of Algological Research, Faculty of Science, Hokkaido University, Muroran 051, Japan

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Key Word Index—Red algae; allelochemicals; growth-inhibitory substance; spore-settlement suppressive substance; unsaturated fatty acid; eicosapentaenoic acid.

Abstract—An allelopathic substance, which displays growth-inhibitory activity and spore-settlement suppressive activity, was isolated from some red algae and identified as (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoic acid on the basis of spectroscopic and chemical evidence. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

During our studies on morphogenetic substances from the foliaceous green alga, *Monostroma oxyspermum* (MK-001) [1], we found that a fraction obtained by column chromatography of extracts from the red alga, *Neodilsea yendoana*, inhibited growth of this alga. On the other hand, we previously reported [2] that in the spore-washing medium which contained extracellular substances from fertile tetrasporophytes or gametophytes of red algae, the rapid settlement to substratum of spores in most species was delayed for a few hours without any adverse effects by the mechanical washing and isolation of non-motile spores in cultures. This suggested that extracellular substances originating from sporulating plants may ensure spore dispersal from the site of the same species as a survival strategy. Thus, we investigated these allelopathic substances. Bioassay-guided separation of the methanol extracts from some red algae led to the isolation of an active substance, which was identified as (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoic acid. In the present paper, we describe the isolation and identification of this compound.

RESULTS AND DISCUSSION

Freshly collected *N. yendoana* was extracted with methanol and the extracts fractionated by column chromatography on silica gel. The fraction eluted with benzene–ethyl acetate (1:1), which showed growth-inhibitory activity, was further subjected to silica gel column chromatography, elution with benzene–ethyl acetate (10:1) yielding an active fraction. This active fraction was then separated by preparative TLC with benzene–ethyl acetate (10:1) to afford an active substance, which was found to consist of a mixture of fatty acids. Further separation was carried out *via* *p*-bromophenacyl ester derivatives.

Treatment of the mixture of fatty acids with *p*-bromophenacyl bromide and *N*-ethyldi*iso*propylamine in acetonitrile yielded crystalline compounds which were then submitted to preparative TLC to give two *p*-bromophenacyl esters **1** and **2**. After saponification of **1** and **2** with 5% potassium hydroxide in methanol, the free acid from the minor ester **1** showed growth-inhibitory activity, while that from the major ester **2** was inactive. The less polar major ester **2** was identified as the *p*-bromophenacyl ester of palmitic acid by comparison of the spectral data.

The more polar minor ester **1** had the molecular formula of $C_{28}H_{35}BrO_3$ established from its HR-mass spectrum. The ^1H NMR spectrum of **1** showed signals due to a methyl group at δ 0.97 (3H, *t*, $J = 7.7$ Hz), a methylene group adjacent to a carbonyl group at δ 2.50 (2H, *t*, $J = 7.3$ Hz), four doubly allylic methylene groups at δ 2.79–2.90 (8H, *m*) and a *p*-bromophenacyl group at δ 5.29 (2H, *s*), 7.63 (2H, *d*, $J = 8.4$ Hz) and 7.78 (2H, *d*, $J = 8.4$ Hz). These data strongly suggested that compound **1** is the *p*-bromophenacyl ester of eicosapentaenoic acid. The ^1H NMR spectrum of **1** was identical to that of the *p*-bromophenacyl ester of commercially available (5Z,8Z,11Z,14Z, 17Z)-eicosapentaenoic acid. Moreover, saponification of **1** with 5% methanolic potassium hydroxide gave a free acid which was identical with authentic (5Z,8Z,11Z,14Z, 17Z)-eicosapentaenoic acid. This acid was also found in the other red algae examined, e.g. *Palmaria palmata*, *Chondrus yendoi* and *Ptilota filicina*.

Growth-inhibitory activities of five commercially available unsaturated fatty acids were tested; minimum growth-inhibitory concentrations were: 5,8,11,14,17-

*Author to whom correspondence should be addressed.

‡Present address: Akan Town Board of Education, Akan 085-02, Japan.

eicosapentaenoic acid (1 μg ml^{-1}), 5,8,11,14-eicosatetraenoic acid (Arachidonic acid) (1 μg ml^{-1}), 8,11,14-eicosatrienoic acid (dihomo-γ-linolenic acid) (10 μg ml^{-1}), 6,9,12,15-octadecatetraenoic acid (1 μg ml^{-1}) [3] and 9,12,15-octadecatrienoic acid (linolenic acid) (10 μg ml^{-1}). Furthermore, 5,8,11,14,17-eicosapentaenoic acid acted harmfully or beneficially according to species of red algae, influencing germination of spores and further development as well as spore settlement (unpublished data).

Eicosanoids, eicosanoid-like compounds and related fatty acids have been discovered in marine organisms. Although these metabolites are known to possess various physiological and biological functions for mammals, ecological roles for organisms which produced these metabolites are little known [4]. More recently free fatty acids containing eicosapentaenoic acid, which were extracted from the articulated coralline red alga *Corallina pilulifera*, were found to induce larval settlement and metamorphosis of the sea urchins, *Pseudocentrotus depressus* and *Anthocidaris crassispina* [5]. We have not yet been able to detect eicosapentaenoic acid in sea water but our present study provided additional evidence that polyunsaturated fatty acids may play important ecological roles in the marine environment.

EXPERIMENTAL

^{1}H NMR: 270 MHz, $CDCl_3$, TMS as int. standard. LRMS and HRMS: 70 eV. CC: silica gel (Merck, Kieselgel 60, 70-230 mesh). Prep. TLC: silica gel (Merck, Kieselgel 60 F_{254S}).

Bioassay for growth-inhibitory substance. Samples (100, 10, 1 and 0.1 μg ml^{-1}) were prep. as follows. Various quantities of each fr. obtained by CC dissolved in a small vol. of DMSO were added to screw-cap test tubes containing 10 ml of culture medium (ASP_7 + SII), which was modified by addition of SII metals [6] to the basal medium ASP_7 by 5 ml l^{-1}. Then, the green alga, *M. oxyspermum* (Kütz.) Doty (strain MK-001), was added to the test tubes. The culture was maintained for 1 week under axenic condition at 14° on a 14:10 hr light–dark cycle with illumination from cool-white fluorescent light. Activity was evaluated as the minimum concn which caused rupture of cells.

Collection. Red algae were collected from the Charatsunai coast, Muroran, Hokkaido; *P. palmata* (Linnaeus) O. Kuntze (March 30, 1989), *N. yendoana* Tokida (June 3, 1989), *C yendoi* Yamada et Mikami (June 3, 1989), *P. filicina* J. Agardh (June 3, 1989) and *Porphyra variegata* (Kjellman) Kjellman (May 25, 1990).

Extraction of Neodilsea yendoana. Fresh algae (2 kg) were immersed in MeOH and the MeOH soln concd *in vacuo* to leave a residue. The residual MeOH extracts were partitioned between EtOAc and H_2O. The EtOAc layer was washed with H_2O, dried (Na_2SO_4) and evapd to give a brown oil (2 g).

Sepn of active substance. MeOH extracts (2 g) were fractionated by CC over silica gel using gradient elution (hexane, benzene and EtOAc). The fr. (350 mg) eluted with benzene–EtOAc (1:1), which showed growth-inhibitory activity, was further chromatographed by silica gel CC (benzene–EtOAc (10:1)) to yield an active fr. (90 mg). This was then subjected to prep. TLC (benzene–EtOAc (10:1)) to afford a mixt. of fatty acids (15 mg), which showed growth-inhibitory activity at 10 μg ml^{-1}.

p-*Bromophenacyl esters of fatty acids from* N. yendoana. To a soln of the mixt. of fatty acids (9 mg) in MeCN (2 ml) were added *p*-bromophenacyl bromide (10 mg) and *N*-ethyldi*iso*propylamine (100 μl). The mixt. was stirred for 1 hr at room temp. and evapd to leave crystalline substances, which were then subjected to prep. TLC (benzene) to give two *p*-bromophenacyl esters **1** (3 mg) and **2** (10 mg). After saponification of each ester with 5% KOH in MeOH, a free acid from the minor ester **1** showed growth-inhibitory activity at 1 μg ml^{-1}, while that from the major ester **2** did not show a growth-inhibitory activity.

Compound **1**. Amorphous solid. ^{1}H NMR: δ 0.97 (3H, *t*, J = 7.7 Hz), 1.78 (2H, *quintet*, J = 7.3 Hz), 2.07 (2H, *br quintet*, J = 7.7 Hz), 2.18 (2H, *m*), 2.50 (2H, *t*, J = 7.3 Hz), 2.79–2.90 (8H, *m*), 5.29 (2H, *s*), 5.30–5.47 (10 H, *m*), 7.63 (2H, *d*, J = 8.4 Hz), 7.78 (2H, *d*, J = 8.4 Hz). LREIMS *m/z* (rel. int.): 500, 498 (0.9:0.9) $[M]^+$, 431, 429 (1.9:1.8), 404, 402 (1.8:1.7), 364, 362 (3.8:3.7), 301 (23), 185, 183 (53:55) $[M - C_{20}H_{29}O_2 - CH_2]^+$, 175 (22), 148 (25), 133 (26), 131 (33), 119 (45), 117 (31), 108 (54), 107 (27), 106 (49), 105 (51), 95 (31), 93 (56), 91 (71), 79 (100), 67 (67), 55 (44) and 41 (62); HREIMS *m/z*: 500.1733 and 301.2183. Calc. for $C_{28}H_{35}{}^{81}BrO_3$, 500.1749 [M] and $C_{20}H_{29}O_2$, 301.2168 $[M - C_8H_6BrO]$.

Compound **2**. Needles, mp 82–84° (hexane). ^{1}H NMR: δ 0.88 (3H, *br t*, J = 7.0 Hz), 1.26 (24H, *s*), 1.69 (2H, *br quintet*, J = 7.3 Hz), 2.48 (2H, *t*, J = 7.3 Hz), 5.28 (2H, *s*), 7.63 (2H, *d*, J = 8.4 Hz), 7.78 (2H, *d*, J = 8.4 Hz). LREIMS *m/z* (rel. int.): 454, 452 (0.5:0.4) $[M]^+$, 373 (3.1), 280 (0.5), 255 (1.1), 239 (22), 185, 183 (100:98) $[M - C_{16}H_{31}O_2 - CH_2]^+$, 157, 155 (8:8), 69 (24), 57 (42), 55 (46), 43 (69), 41 (46); HREIMS *m/z*; 452.1897, 255.2334 and 182.9428. Calc. for $C_{24}H_{37}{}^{79}BrO_3$, 452.1926 [M], $C_7H_4{}^{79}BrO$, 182.9446 $[M - C_{17}H_{33}O_2]$ and $C_{16}H_{31}O_2$, 255.2324 $[M - C_8H_6BrO]$.

Synthesis of p-*bromophenacyl ester of* (5Z,8Z, 11Z,14Z,17Z)-*eicosapentaenoic acid.* To a soln of commercially available (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoic acid (10 mg) in MeCN (2 ml) were added *p*-bromophenacyl bromide (8 mg) and *N*-ethyldi*iso*propylamine (100 μl). The mixt. was stirred for 30 min at room temp. After removal of solvent, the resulting crystalline substance was subjected to prep. TLC (benzene) to yield the corresponding *p*-bromophenacyl ester (6 mg) which was identical to *p*-bromophenacyl ester **1**.

Synthesis of p-*bromophenacyl ester of palmitic acid.* This was prepared from commercial palmitic acid using the method described above and found to be identical to *p*-bromophenacyl ester **2**.

Saponification of p-*bromophenacyl ester* **1**. A soln of **1** (6 mg) in 5% KOH in MeOH (500 μl) was stirred for 2 hr at room temp. under N_2 and then H_2O added. The reaction mixt. was acidified with 1M HCl and then extracted with Et_2O. The Et_2O soln was washed with H_2O, dried (Na_2SO_4) and evapd to give a crystalline substance, which was subjected to prep. TLC (benzene–EtOAc (10:1)) to afford an oily compound (4 mg). ^{1}H NMR: δ 0.98 (3H, *t*, J = 7.7 Hz), 1.72 (2H, *quintet*, J = 7.3 Hz), 2.02–2.17 (4H, *m*), 2.37 (2H, *t*, J = 7.3 Hz), 2.79–2.87 (8H, *m*), 5.27–5.46 (10H, *m*). The ^{1}H NMR spectrum was identical to that of authentic (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoic acid.

Isolation of (5Z,8Z,11Z,14Z,17Z)-*eicosapentaenoic acid from other red algae.* Extraction of the red algae, *P. palmata*, *C. yendoi*, *P. filicina* and *P. variegata*, was carried out according to the procedure described for *N. yendoana*. The MeOH extracts were percolated with EtOAc and the EtOAc soln shaken with 0.5 M aq. KOH. The alkaline layer was acidified with 1 M HCl and then extracted with Et_2O. The Et_2O soln was washed with H_2O and then dried (Na_2SO_4). Solvent was evapd to give acidic substances, which were subjected to silica gel CC to afford an active fr. consisting of a mixt. of fatty acids. Growth-inhibitory substances were also separated as described above *via* *p*-bromophenacyl derivative. The four species of red algae contained (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoic acid.

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