Isolation and Structural Analysis of Stimulating Substances for Morphogenesis in Marine Green Alga, *Monostroma oxyspermum*[1),2)]

By

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Introduction

Recent progress in phycological research has shown that growth and development of many marine algae are dependent on some of organic materials derived from other marine organisms (FRIES 1973, PROVASOLI and CARLUCCI 1974, BUGGELN 1981). In green algae, having foliaceous morphology, such as *Ulva* and *Enteromorpha*, their axenic (bacteria free) cultures lose the natural morphology and develop into pincushion- or colony-like masses in defined synthetic media. The atypical morphology is partially restored to normal by a re-infection of epiphytic marine bacteria isolated from natural plants of the alga or by dosage of plant growth regulators (plant hormones), like IAA and kinetin (PROVASOLI 1958, 1969, PROVASOLI and PINTNER 1964, 1966, 1972, 1980, KAPRAUN 1970, FRIES 1975, FRIES and ABERG 1978). These bacteria and many of marine algae produce plant growth regulators (BUGGELN 1981, MARUYAMA *et al.* 1986, MOONEY and STADEN 1986), which seem to be effective on algal morphogenesis. In most of marine red algae investigated, normal growth of these plants requires vitamin B_{12} probably released from marine bacteria or green and brown algae (FRIES 1973, PROVASOLI and CARLUCCI 1974). Growth and morphogenesis in some brown algae are similarly stimulated by exogenous sources of vitamins or plant growth regulators (PEDERSÉN 1968, FRIES 1984a, b). These exogenous chemical factors are thought acting important role on a marine ecosystem, except other environmental factors (light, temperature, inorganic nutrients, water action, *etc.*) and biological interrelationship (competition, predation, parasitism, symbiosis, *etc.*).

One of the most prototypic phenomena on dependent morphogenesis of marine algae seems to be the case of foliaceous green alga, *Monostroma oxyspermum*. This alga, like *Ulva* and *Enteromorpha*, loses its typical morphology when cultured under axenic conditions with artificial media (PROVASOLI and PINTNER 1964, TATEWAKI and PROVASOLI 1977). Germlings

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derived from swarmers of the cultures develop first into uniseriate filaments composed of several cells, and these plants finally grow into colony-like masses consisting of loosely aggregated cells. In these cultures, the normal morphology is drastically restored by the following experimental treatments; infection of appropriate marine bacteria, bialgal (double members) cultures with other axenic strains of various species of red and brown algae except the species of green algae, dosage of sterilized supernatant obtained from culture medium of the specific bacteria or several axenic red and brown algae, and dosage of water and alcohol soluble materials extracted from the bacteria or the red and brown algae (PROVASOLI and PINTNER 1964, PROVASOLI *et al.* 1977, TATEWAKI and KANEKO 1977, TATEWAKI *et al.* 1983, TATEWAKI 1985). The uniseriate filaments and colony-like masses of the axenic *Monostroma* are induced to develop into typical expanded foliaceous or saccate fronds composed of cells closely connected together by those treatments. These facts imply that seawater soluble active factors supplied by the marine organisms are also required for normal morphogenesis of *M. oxyspermum*.

TATEWAKI and KANEKO (1977) have reported that methanolic extracts from red alga *Neodilsea yendoana* contained at least two kinds of active factors stimulating the normal morphogenesis of axenic *Monostroma*, and that these substances were tentatively identified as a peptide and saturated hydrocarbon. PROVASOLI *et al.* (1977) have partially purified an active material, which positively responded to the ninhydrin reagent, from the culture medium of a specific marine bacteria *Caulobacter*. However each material of the factors, called "morphogenetic substances", is still not understood despite repeated efforts because of their unstableness. Some investigations have furthermore shown that various known substances, such as vitamins, phenolic compounds including plant growth regulators, amino acids, nucleic acids, yeast or liver extract, peptone, *etc.* are not effective on the morphogenesis of *Monostroma* (TATEWAKI in PROVASOLI and CARLUCCI 1974, TATEWAKI *et al.* 1983).

In this paper, I have reported that diacylglycerides stimulating early development of the normal morphogenesis in the axenic strain of *M. oxyspermum* were obtained from red alga *N. yendoana*, as well as structural elucidation of these compounds.

Acknowledgments

I am grateful to Prof. Masakazu TATEWAKI, the Institute of Algological Research, Faculty of Science, Hokkaido University, for his kind guidance and critical reading of the manuscript. I wish to thank Dr. Minoru SUZUKI, Department of Chemistry, Faculty of Science, Hokkaido University, for his facilities and instruction in the chemical study, and staffs and graduate students of the Institute of Algological Research for their supports in my works.

Materials and Methods

Plant materials for extraction of morphogenetic substances

Neodilsea yendoana TOKIDA was collected at Charatsunai, Muroran, Hokkaido, in July 3 and August 3, 1989. Twenty kg of the fresh-collected alga were soaked in 88 *l* of methanol containing butylated hydroxytoluene (BHT, 0.1 g/*l*) and dry ice (1 kg/*l*), and were statically allowed to stand for 2 weeks at 0 °C in darkness. The methanolic extracts obtained were filtrated with a paper filter and used in the experiment.

Plant materials for bioassay of the morphogenetic substance

Axenic cultures of *Monostroma oxyspermum* (KÜTZ.) DOTY were established by TATEWAKI in 1962 (TATEWAKI *et al.* 1983), and have been maintained aseptically as a culture collection (strain MK-001) at the Institute of Algological Research, Hokkaido University[4]. At regular intervals, fragments of thalli (colony-like masses) or swarmers of the materials were transferred into screw-cap test tubes (1.8−2.0 cm×13.0−13.5 cm) containing 10 m*l* of ASP_7+SII medium[5], and were cultured in a growth chamber maintained at 14±1 °C under 14 hr light: 10 hr dark photoperiod with illumination provided by cool-white fluorescent lamps at 12 W/m^2. Sterility of the materials was examined by transferring some plants of the cultures into ST_3 or STP sterility test medium (TATEWAKI and PROVASOLI 1964) and maintaining them for at least 20 days at approximately 20 °C

Bioassay

Preparation method A —— Samples isolated from the extract of *N. yendoana* were dried *in vacuo*, and each was dissolved in dimethyl sulfoxide (DMSO) at a required concentration. One hundred μl of this solution were dropped into the screw-cap test tubes containing 10 m*l* of the culture medium, and were provided for the bioassay after being sterilized by autoclaving.

Preparation method B —— To prevent the purified samples from their acidification, fractions obtained by chromatography were often prepared for the bioassay as the following method: the samples were concentrated and dried by a N_2 gas stream, and then, dissolved in chloroform/methanol (1 : 1) at various concentrations without weighing; fixed volumes of them were transferred into the screw-cap test tubes containing 100 μl of the lipid mixture[6]; these solutions were dried under a N_2 gas stream, and then, dissolved in 80 μl of DMSO; to

4) The detailed life cycle and developmental morphology of the axenic strain of *M. oxyspermum* grown in artificial medium ASP_7 have already been reported by TATEWAKI *et al.* (1983).
5) Modified by an addition of the SII metals (5 m*l*/*l*) to the basal medium, ASP_7 (PROVASOLI 1963).
6) The lipid mixture contained 50 μg of cholesterol, 50 μg of egg lecithin. 25 μg of DL-α-tocopherol and 10 μl of Tween 80 per 1m*l* of chloroform/methanol (3 : 1).

each solution 10 m*l* of the culture medium was added, and was sonicated for 30 sec; and these prepared assay media were sterilized by autoclaving before used. Addition of DMSO or the lipid mixture did not affect algal growth and morphology.

Inoculation of alga —— About 100 fragments of the stock cultures of *M. oxyspermum* were aseptically inoculated into the assay media, and were incubated under the culture conditions mentioned above. After 2-4 weeks, the presence or absence of the restoration of foliaceous morphology in the growing plantlets was observed under a stereoscopic microscope.

Results

Isolation and purification of morphogenetic substances

Early fractionation —— The methanolic extracts obtained from *N. yendoana* were concentrated under reduced pressure at less than 40°C (bath temperature) with a rotary evaporator. An aqueous concentrate was obtained, which induced axenic *M. oxyspermum* to develop into typical foliaceous plants at 10 μg/m*l*. When the concentrate was fractionated by a conventional partition method with ethyl acetate, the morphogenetic activity was obviously found in the fraction of the ethyl acetate layer, but not of the residual aqueous layer (Table 1). The active ethyl acetate fraction (67.3 g) was divided into four portions and stored in a freezer at −20°C until used for subsequent experiments. It was found that the ethyl acetate fraction inhibited algal growth at high concentrations (over 10 μg/m*l*), and this inhibitory material was purified and identified as 5, 8, 11, 14, 17-icosapentaenoic acid (SUZUKI *et al.* 1990).

DEAE cellulose chromatography —— A part of the ethyl acetate fraction (21.6 g) was dissolved in a small amount of chloroform/methanol (1 : 1), placed on a column of DEAE cellulose (120 g, CH_3COO^- form, 4.0 cm × 75 cm, Wako Pure Chemical Industries Ltd., Osaka), and successively eluted with chloroform/methanol (35 : 65), methanol, chloroform/

Table 1 Effects of methanolic extract of *N. yendoana* and fractions separated from the extract on the developmental morphology in axenic strain of *M. oxyspermum*

Fractions	Concentrations (g/m*l*)							
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Methanolic extract	gi	+	+	−	−	−	−	nt
Water soluble fraction	gi	+/−	−	−	−	−	−	nt
Ethyl acetate soluble fraction	gi	gi	gi	+	+	−	−	−

gi: Growth inhibition. nt: Not tested. +: Effective on the induction of foliaceous morphology. +/−: Partially effective. −: Not effective.

Table 2 Separation of the active ethyl acetate fraction (21.6 g) by DEAE cellulose chromatography

Eluates*	Elution volume	Dry weight of fraction
Chloroform/Methanol (35: 65)	2.0 *l*	20.5722 g
Methanol	2.0 *l*	141.0 mg
Chloroform/Acetic acid (80: 20)	2.0 *l*	88.6 mg
Acetic acid	2.5 *l*	591.2 mg

* Each eluate contained 10 mg/*l* of BHT.

Table 3 Effects of the fractions separated by DEAE cellulose chromatography (shown in Table 2) on the induction of foliaceous morphology in axenic strain of *M. oxyspermum*

Fractions	Concentrations (g/m*l*)				
	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Ethyl acetate fraction (control)	+	+	−	−	−
Chloroform/Methanol fraction	+/−	−	−	−	−
Methanol fraction	+	+/−	−	−	−
Chloroform/Acetic acid fraction	+	+	+/−	−	−
Acetic acid fraction	+	+	+	+	+/−

+: Effective. +/−: Partially effective. −: Not effective.

acetic acid (80 : 20), and acetic acid (Table 2). After being evaporated to remove the solvents, each of the residual fractions was dissolved in 10 m*l* of chloroform/methanol (1 : 1). The activity was concentrated in the fraction eluted with acetic acid, which induced typical morphology at 1-0.1 ng/m*l* (Table 3).

High performance liquid chromatography (HPLC) —— A part of the active acetic acid fraction (about 200 mg) was dissolved in 2 m*l* of methanol containing a small amount of chloroform, and then, HPLC separated. This solution was put onto an ODS column (Shimpack PREP-ODS [H], Shimadzu Co., Kyoto), and eluted with methanol/*iso*-propanol/acetonitrile (40 : 35 : 25) at a flow rate of 8.2 m*l*/min for 80 min, and then, with 300 m*l* of chloroform/methanol (1 : 1) at a flow rate of 5.5 m*l*/min. Based on the result from monitoring of the refractive index, the eluates were divided into 17 fractions, and each fraction was rapidly mixed with 1 m*l* of chloroform containing 1 mg of BHT. Figure 1 shows an elution profile of the HPLC and the activities of the separated fractions, indicating that the eluates from HPLC contained at least five kinds of active substances. Although most of the activity

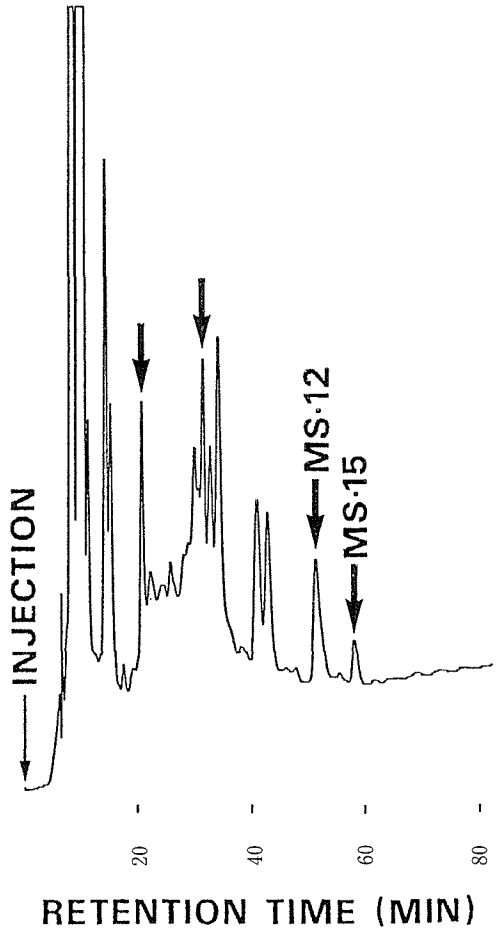

Fig. 1 Elution profile of reverse phase HPLC separation of the active acetic acid fraction obtained by DEAE cellulose chromatography. Column: Shim-pack PREP-ODS [H]. Mobile phase: methanol/*iso*-propanol/acetonitrilie (40 : 35 : 25). Flow rate: 8.2 m*l*/min for 80 min. Detection: refractive index. Allows indicate the fractions showing activity after bioassay.

remained with the chloroform/methanol fraction (minimum active concentration; 10 pg/m*l*), further separation of these active substances is still in progress.

Purification —— Two of the active fractions named MS-12 and MS-15 were divided into 13 and 7 portions, respectively, and each portion was furthermore purified by HPLC with an ODS column (Shim-pack CLC-ODS [M], Shimadzu Co.). An elution profile of the MS-12 monitored by absorption of UV at 220 nm is shown in Fig. 2. Two major compounds, "MS-12" and "MS-15", were purified by further HPLC. The purity of the both compounds was checked by HPLC analyses using different solvent systems, i.e., methanol, acetonitrile/*iso*-propanol (85 : 15) and methanol/*iso*-propanol (80 : 20)(Fig. 3).

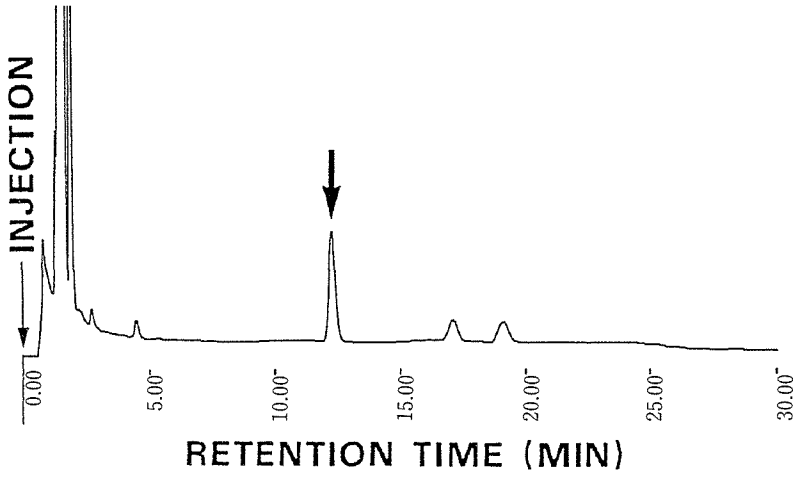

Fig. 2 Elution profile of reverse phase HPLC separation of the active fraction MS-12 obtained by the preparative HPLC shown in Figure 1. Column: Shim-pack CLC-ODS [M]. Mobile phase: methanol/*iso*-propanol/acetonitrile (20 : 15 : 65). Flow rate: 2.5 m*l*/min. Detection: UV (220 nm). Allow indicates the fraction purified as a major active compound "MS-12".

Effects of isolated compounds "MS-12" and "MS-15" on the morphogenesis of axenic *M. oxyspermum*

When the axenic plants of *M. oxyspermum* were cultured in the presence of the crude fraction of MS-12 or MS-15, these cultures grew into cellular masses or filaments as rudiments of typical fronds within 2 weeks. Cells of these plantlets were tightly connected together, and developed into foliaceous or saccate fronds by the 4 to 6 week. In the presence of the purified "MS-12" or "MS-15", the alga also formed the cellular masses and filaments. However, the connection among the cells constituting the plantlets was gradually weakened by the long-term culture, and those plantlets finally disintegrated into colony-like masses by the 4 to 8 week as cultured in the plain medium.

Structural analysis of the "MS-12" and "MS-15"

Structure of "MS-12" —— One of two major compounds, "MS-12", oil, $[\alpha]_D^{15}$ −1.63°(c 0.40; methanol), was analyzed for $C_{39}H_{66}O_5$ by low-resolution electron-ionization mass spectroscopy (EI-MS)*m/z* 614 and field-desorption mass spectroscopy (FD-MS) *m/z* 614 (Fig. 4) as well as high-resolution electron-ionization mass spectroscopy (HR-EIMS) *m/z* 614.4924 (calculated for $C_{39}H_{66}O_5$, MW = 614.4910 [M]). "MS-12" had the following additional spectral characteristics:

(1)^{1}H NMR (270 MHz, $CDCl_3$): δ0.88 (3H, broad triplet, J = 7 Hz), 0.98 (3H, triplet, J = 7.7 Hz), 1.26 (28H, broad singlet), 1.70 (2H, multiplet), 2.0-2.2 (4H, multiplet), 2.34 (4H,double

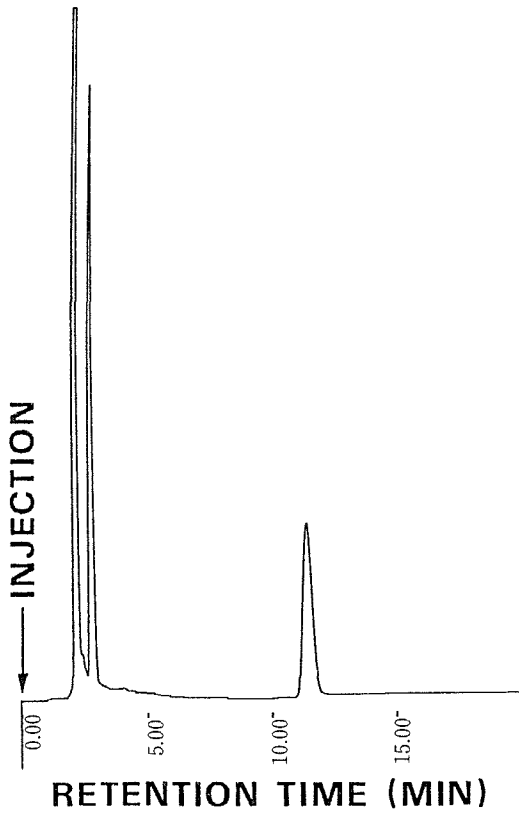

Fig. 3 Elution profile of reverse phase HPLC analysis of the purified fraction "MS-12" obtained the preparative HPLC shown in Figure 2. Column: Shim-pack CLC-ODS [M]. Mobile phase: methanol. Flow rate: 1.5 m*l*/min. Detection: UV (220 nm).

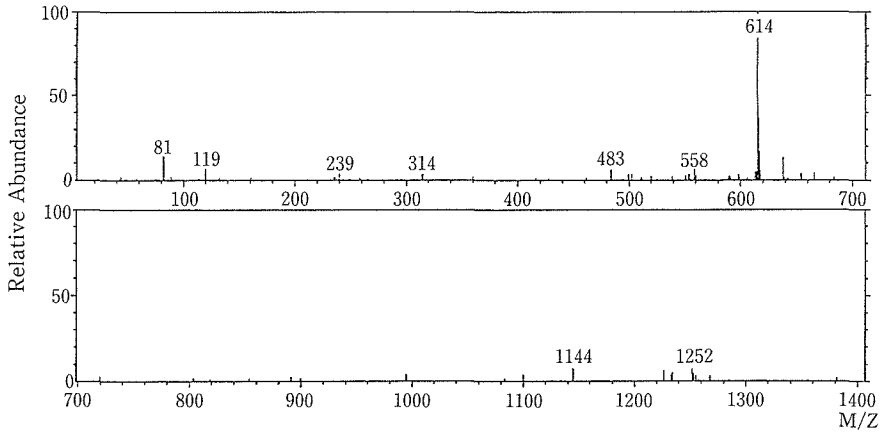

Fig. 4 FD mass spectrum of compound "MS-12". Ionization voltage: 70 eV.

doublet, J = 7.3 and 7.3 Hz),2.8 (8H, multiplet), 3.73 (2H, broad doublet, J = 4.8 Hz), 4.23 (1H, double doublet, J= 5.5 and 11.7 Hz), 4.32 (1H, double doublet, J = 4.4, 11.7 Hz), 5.05 (1H, multiplet) and 5.38 (10H, multiplet)

(2)^{13}C NMR (67.9 MHz, $CDCl_3$): CH_3, δ14.1 and 14.2; CH_2, δ20.6, 22.7, 24.8, 25.0, 25.6, 25.7×2, 26.6, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7×5, 31.9, 33.5, 34.3, 61.7 and 62.1; CH, δ72.2, 127.1, 127.9, 128.1, 128.2, 128.3, 128.4, 128.6, 128.8, 129.1 and 132.1; and C, not detectable by this integration

(3) EI-MS (70 eV): *m/z* (relative intensity) 614 (14; M^+),359 (7; M^+-$C_{16}H_{31}O_2$),314 (24), 313 (100; M^+-$C_{20}H_{29}O_2$), 285 (6; $C_{20}H_{29}O^+$), 239 (38; $C_{16}H_{31}O^+$), 201 (23), 175 (40), 148 (32), 131 (33), 129 (33), 119 (46), 117 (30), 109 (28), 108 (66), 105 (45), 95 (49), 93 (60), 91 (61), 79 (93), 67 (71), 57 (87), 55 (66), 43 (80) and 41 (63)

The presence of 5 double bonds was evident from the spectral data of ^{1}H NMR (δ5.38, 10

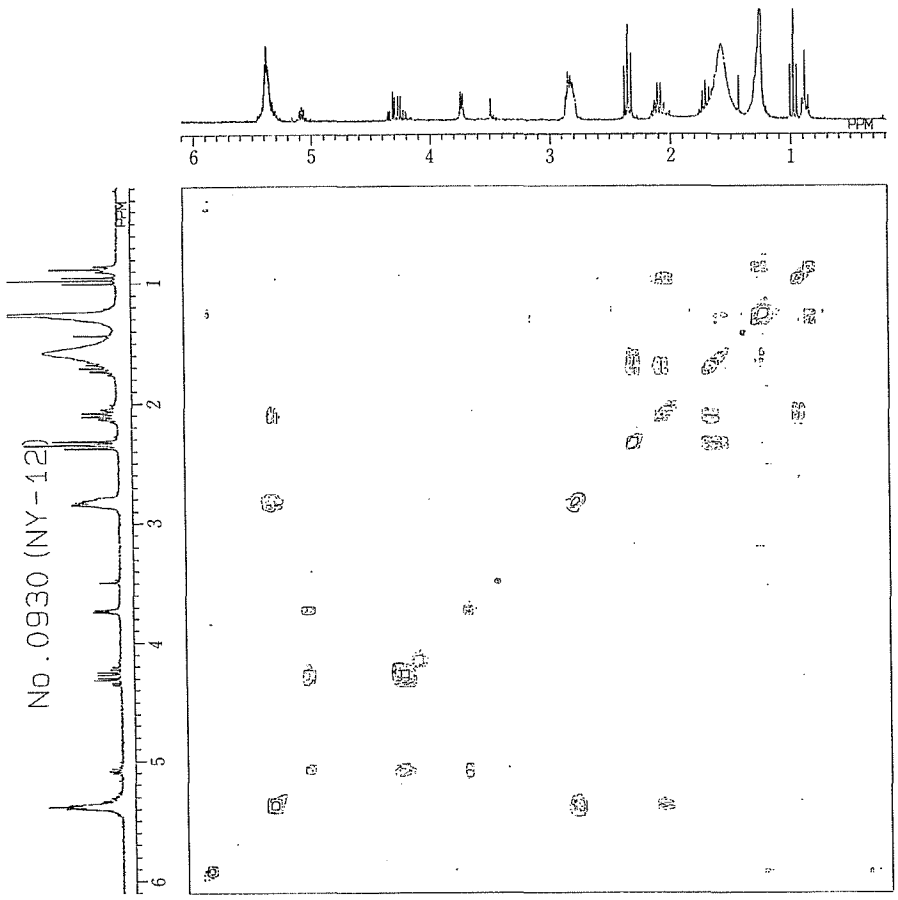

Fig. 5 ^{1}H-^{1}H 2D-COSY spectrum of compound "MS-12". Frequency: 270 MHz. Solvent: $CDCl_3$. Internal reference: tetramethylsilane.

protons) and ^{13}C NMR (δ127.1-132.1, 10 sp^2 carbons). The ^1H-^1H shift correlated two-dimensional NMR (^1H-^1H 2D-COSY) spectrum (Fig. 5) indicated that one of the terminal methyl group at δ0.98 was coupled with the methylene protons at δ2.0-2.2, which were further coupled to the olefinic proton at δ5.38. In the 2D-COSY spectrum, the 8-protons multiplet (4 methylenes) at δ2.8 showed only a cross peak to the olefinic protons at δ5.38, thus indicating that this multiplet was ascribed to 4 doubly allylic methylene protons. The methylene protons at δ1.71 spin-coupled to the allylic protons at δ2.0-2.2, which further showed a cross peak to the olefinic proton at δ5.38. The data presented here unambiguously prove the presence of such spin systems as the following partial structural unit A:

$$\blacksquare\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH_3$$

■: quaternary carbon atom

Partial structural unit A of "MS-12"

Moreover, in the $^1H-^1H$ 2D-COSY spectrum, one-proton multiplet at δ5.05 showed cross peaks to the methylene protons at δ3.73 and the methylene protons at δ4.23 and 4.32. Both of the methylene protons revealed only a cross peak to the proton at δ5.05. These data consequently indicate the presence of such spin systems as the following partial structural unit B:

$$\begin{array}{l} {}^1CH_2\text{-}X \\ \quad | \\ {}^2CH\text{-}Y \\ \quad | \\ {}^3CH_2\text{-}Z \end{array}$$

	Chemical shifts (δ)	
	^1H NMR	^{13}C NMR
C-1	4.23 and 4.32	62.1
C-2	5.05	72.2
C-3	3.73 and 3.73	61.7

Partial structural unit B of "MS-12"

In addition to the above ^1H NMR chemical shifts, the ^{13}C NMR chemical shifts due to the C-1 (δ62.1), C-2 (δ72.2), and C-3 (δ61.7) showed that each of the two substituents X and Y at C-1 and C-2 had to be an acyloxy group and substituent Z had to be a hydroxyl group, thus, extending the partial structures A and B to the following partial structure C with 1, 2-diacylglycerol moiety for the structure of "MS-12":

$$\begin{array}{l} {}^{1}CH_2-O-CO-R_1 \\ \quad | \\ {}^{2}CH-O-CO-R_2 \\ \quad | \\ {}^{3}CH_2-OH \end{array} \qquad R_1 \text{ and } R_2 = \text{alkyl or alkenyl}$$

Partial structural unit C of "MS-12"

Judging from the chemical shifts [δ_H 2.34 (2H, double doublet, J = 7.3 and 7.3) and δ_C 34.3] for the protons and carbon of the methylene group, which was adjacent to a quaternary carbon atom in partial structure A, this methylene group must be connected to the carbonyl group. This suggests that "MS-12" possesses an icosapentaenoyl group in the molecule. The fact was further supported by the presence of a fragment ion at *m/z* 285 [$C_{19}H_{29}CO^+$, HR-EIMS; 285.2210 (Δmmu-0.8)] in the mass spectrum. The presence of a palmitoyl group was indicated by the ^1H and ^{13}C NMR spectra coupled with a fragment ion as *m/z* 239 [$C_{15}H_{31}CO^+$,HR-EIMS; 239.2367(Δmmu-0.8)].

In the mass spectra of 1, 2-diacylglycerol derivatives, the fragment ion generated by cleavage of the acyloxy group at C-2 shows relatively a more intense peak than that generated by cleavage of the acyloxy group at C-1[7]. The mass spectrum of "MS-12" revealed the fragment ions at *m/z* 313 [relative intensity 100; $C_{19}H_{37}O_3^+$ (M^+-$C_{19}H_{29}COO$), HR-EIMS 313.2745 (Δ mmu 0.2)] and *m/z* 359 [relative intensity 7; $C_{23}H_{35}O_3^+$ (M^+-$C_{15}H_{31}COO$), HR-EIMS 359.2616 (Δmmu 2.9)]. These data, therefore, prove that the acyl groups at C-1 and C-2 in "MS-12" are palmitoyl and icosapentaenoyl group, respectively. As a whole, the structure of "MS-12" was represented as the following 1-palmitoyl-2-5, 8, 11, 14, 17 -icosapentaenoyl-*sn*-glycerol:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad CH_2-O-CO-CH_2-(CH_2)_{13}-CH_3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ CH_3-CH_2-(CH=CH-CH_2)_5-(CH_2)_2-CO-O-CH \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad CH_2-OH \end{array}$$

Structural formula of "MS-12"

Structure of "MS-15" —— Another major compound, "MS-15", oil, had a molecular formula of $C_{39}H_{68}O_5$ (EI-and FD-MS *m/z* 616; M^+) and the following spectral characteristics:

(1) ^1H NMR (270 MHz, $CDCl_3$): δ0.88 (3H, broad triplet, J = 7.0 Hz), 0.89 (3H, broad triplet,

7) BARBER *et al.* 1968 in Biochemistry Data Book, Vol. I, Tokyo Kagakudojin, p. 929.

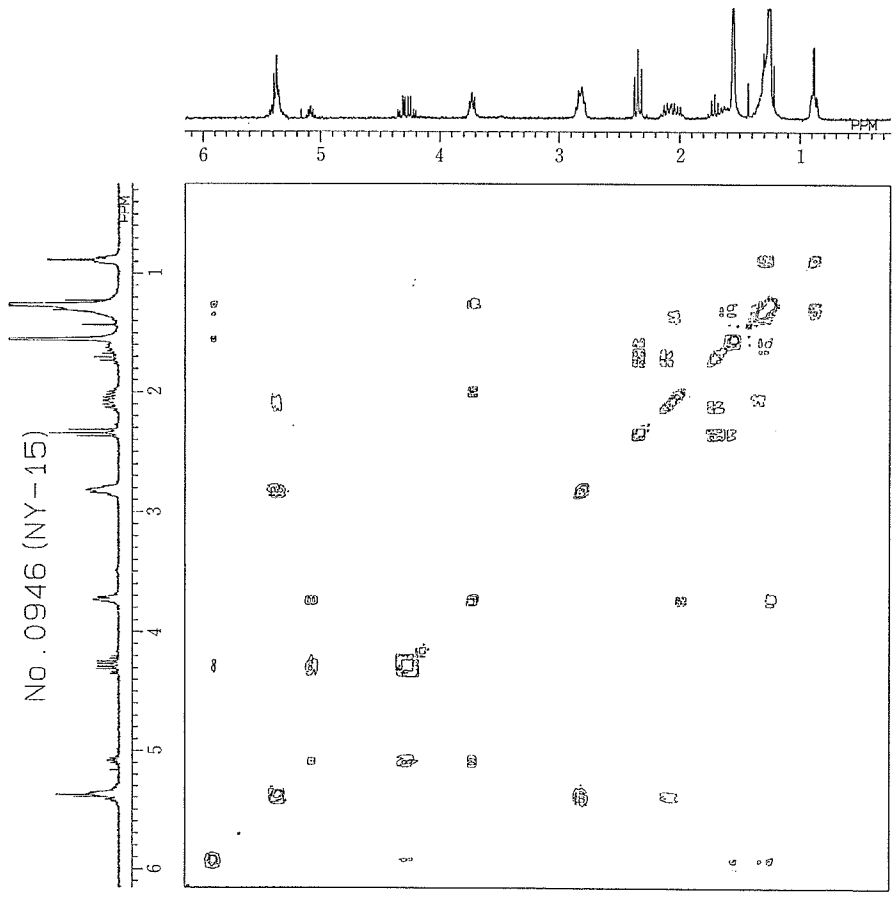

Fig. 6 ^{1}H-^{1}H 2D-COSY spectrum of compound "MS-15". Frequency: 270 MHz. Solvent: $CDCl_3$. Internal reference: tetramethylsilane.

J = 7.0 Hz), 1.26 (32H, broad singlet), 2.0-2.2 (4H, multiplet), 2.34 (4H, double doublet, J = 7.7 Hz), 2.8 (6H, multiplet), 3.73 (2H, broad double doublet, J = 5.0 and 5.0 Hz), 4.24 (1H, double doublet, J = 5.5, 11.7 Hz), 4.32 (1H, double doublet, J = 4.7, 11.7 Hz), 5.08 (1H, multiplet) and 5.37 (8H, multiplet)

(2) ^{13}C NMR (67.9 MHz, $CDCl_3$): CH_3, δ14.0 and 14.1; CH_2, δ22.6, 22.7, 24.8, 25.0, 25.7×2, 26.6, 27.3, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7×5, 31.6, 32.2, 33.5, 34.3, 61.7, and 62.2; CH, δ72.2, 127.6, 127.9, 128.2, 128.4, 128.7, 128.8, 129.1 and 130.6; and C, δ173.5×2

(3) EI-MS (70 eV): m/z (relative intensity) 616 (5; M^+),361 (2; $M^+-C_{16}H_{31}O_2$), 313 (36; $M^+-C_{20}H_{31}O_2$), 287 (2; $C_{20}H_{31}O^+$), 239 (20; $C_{16}H_{31}O^+$), 150 (23), 129 (20), 98 (34), 97 (31), 95 (34), 93 (33), 91 (28), 85 (31), 84 (27), 83 (34), 81 (43), 79 (46), 71 (52), 69 (59), 67 (52), 57 (100), 55 (79), 43 (95) and 41 (69)

The ^{1}H and ^{13}C NMR data of "MS-15" were very similar to those of "MS-12". The ^{1}H and ^{13}C NMR spectra of "MS-15" showed the presence of four double bonds (δ_H 5.37, 8 protons and δ_C 127.6-130.6, 8 sp^2 carbons) and esteric carbonyl carbons (δ_C 173.5×2). The ^{1}H-^{1}H 2D-COSY spectrum of "MS-15" (Fig. 6) revealed the presence of the partial structural units B and the following D:

$$\blacksquare-CH_2-CH_2-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH$$
$$=CH-CH_2-CH_2-CH_2-CH_2-CH_3$$

■: quaternary carbon atom

Partial structural unit D of "MS-15"

The structure of "MS-15" was established based on the chemical shift arguments in the ^{1}H and ^{13}C NMR spectra and the fragment ion analyses in the mass spectrum as in the case of the structural elucidation of "MS-12", and was represented as the following 1-palmitoyl-2-arachidonoyl-*sn*-glycerol:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad CH_2-O-CO-CH_2-(CH_2)_{13}-CH_3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ CH_3-(CH_2)_4-(CH=CH-CH_2)_4-(CH_2)_2-CO-O-CH \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad CH_2-OH \end{array}$$

Structural formula of "MS-15"

Discussion

In the present study, two kinds of highly unsaturated diacylglycerides were isolated from *N. yendoana* as stimuli for morphogenesis in *M. oxyspermum*, and their structures were determined as 1-palmitoyl-2-5, 8, 11, 14, 17-icosapentaenoyl-*sn*-glycerol and 1-palmitoyl-2-arachidonoyl-*sn*-glycerol. However, their action on the restoration of morphogenesis in the axenic *Monostroma* was imperfect, and the early stage belonging to the normal development was only induced. The results of preparative HPLC showed that some active substances, other than isolated compounds, were present in the same eluate obtained by DEAE cellulose chromatography, and that impurities of the fraction MS-12 and MS-15 were necessary to induce normal morphogenesis. Thus, the typical morphogenesis of *Monostroma* seems to be stimulated by other materials and/or by their support as co-factors. The activity behavior regarding DEAE cellulose chromatography showed that the morphogenetic substances were acidic materials, and accordingly indicated that the isolated diacylglycerides were degraded

compounds because of the absence of acidic function(s) in their structures. In general, polar acidic phospholipids are separated from a mixture of liposoluble materials by DEAE cellulose chromatography (YAMAMOTO *et al.* in FUJINO 1978), and phosphatidyl serine and phosphatidic acid has been mainly obtained from some marine red algae by this separation (ANDO and KANEDA 1968, ARAKI *et al.* 1986). Although these phospholipids are not still identified from *N. yendoana*, the fact that the isolated "MS-12" and "MS-15" were derived from the similar liposoluble fraction, suggests native molecules of the isolated materials having acidic function, such as phosphate or sulfate of phospholipids or sulpholipids.

The diacylglycerides are well known as mediators of the second messenger system of endogenous regulation in many eucaryotes (MIYAMOTO 1986). These lipids activate the Ca^{2+}/phospholipids dependent protein kinase (C-kinase) of cytosol, and the activated C-kinase phospholilates various substrates and regulates a variety of cell functions; such as cell growth, cell differentiation, metabolic control, secretion and exocytosis (NISHIZUKA 1984, KAJIKAWA and ASE 1986). In higher plants, there is now sufficient evidence to suggest that the second messenger system operates in the membrane transduction processes involving primary exogenous signals by plant growth regulators, light and other environmental stimuli (ELLIOTT 1985, HEPLER and WAYNE 1985, ELLIOTT *et al.* 1988, ETTLINGER and LEHLE 1988, BLATT *et al.* 1990, GILROY *et al.* 1990). Although the importance of the Ca^{2+} and Ca^{2+} binding proteins (like calmodulin) or the cyclic AMP as mediators of intracellular regulation has been reported regarding cell differentiation of zygotes in brown alga *Fucus* (WEISENSEEL 1979, EVANS *et al.* 1982, BROWNLEE and PULSFORD 1988, BRAWLEY and ROBERTS 1989) or on phototropic bending in xanthophycean alga *Vaucheria* (KATAOKA 1977), there is little information about the second messenger system mediated by diacylglycerides in algae. The data presented in this paper have not been capable of explaining about the effect of diacylglycerides on stimulation of the morphogenesis in *M. oxyspermum*. Further work is therefore needed to determine native molecules of the morphogenetic substances and to afford proof of the presence of C-kinase in this alga. We are presently studying the effects of phospholipids and several compounds related to the second messenger system on the morphogenesis in axenic *Monostroma* and the result from these experiments will be reported on another occasion.

Summary

The marine green alga *Monostroma oxyspermum* loses its native foliaceous morphology when grown aseptically in defined synthetic media. Earlier investigations have shown that the morphogenesis of this alga is induced by exogenous organic compounds produced by other marine organisms, red and brown algae, and bacteria. However, the nature of these inducing factors, called the morphogenetic substances, remains unknown because of their unstableness. In this report, the active substances that extracted from marine red alga *Neodilsea yendoana* with methanol were separated by conventional partition method, DEAE cellulose

chromatography and preparative HPLC, and were followed up their activities by newly developed bioassay method. As a result, two major compounds were obtained as materials stimulating early development of the normal morphogenesis in *M. oxyspermum*, though they seemed to be degraded compounds. Their structures were analyzed by ^{1}H NMR, ^{13}C NMR and mass spectrometry, and were identified as 1-palmitoyl-2-5, 8, 11, 14, 17-icosapentaenoyl-*sn*-glycerol and 1-palmitoyl-2-arachidonoyl-*sn*-glycerol which were well known as relational compounds of mediators of the second messenger system in many eucaryotes.

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