Non-methylene-interrupted Fatty Acids with $\Delta 5$ Unsaturation in *Sargassum* Species

Gwang-Woo Kim and Yutaka Itabashi*

Faculty of Fisheries Sciences, Hokkaido University (Hakodate 041-8611, Japan)

Abstract: Detailed fatty acid compositions of five species of the brown algae *Sargassum* (*S. fulvellum*, *S. horneri*, *S. boreale*, *S. thunbergii*, and *S. yezoense*) were determined using silver ion solid phase extraction, gas chromatography (GC), and GC-mass spectrometry (GC-MS) techniques. In addition to a high number of typical saturated and unsaturated fatty acids, the GC-MS spectra of the 4,4-dimethyloxazoline derivatives of fatty acids revealed the occurrence of small amounts of unusual non-methylene-interrupted (NMI) fatty acids with $\Delta 5$ unsaturation, namely, 5,9-eicosadienoic (5,9-20:2), 5,11,14-eicosatrienoic (5,11,14-20:3), and 5,11,14,17-eicosatetraenoic (5,11,14,17-20:4) acids. Of these three NMI acids, the 5,9-20:2 acid was found to be the most abundant (0.4%–2.3% of the total fatty acids) and was detected for the first time in algae.

Key words: brown algae, *Sargassum* species, $\Delta 5$ unsaturation, 5,9-eicosadienoic acid, non-methylene-interrupted fatty acids

1 INTRODUCTION

Fatty acids with unique structures that contain non-methylene-interrupted (NMI) double bonds in their molecules occur widely as minor components in marine organisms such as mollusks, algae, echinoderms, sponge, tropical rays, and many other invertebrates^{1,2)}, while NMI acids with $\Delta 5$ unsaturation occur systematically in gymnosperm seed oils³⁾. The content of NMI fatty acids in algae is generally very low (<0.5% of the total fatty acids)^{4–7)}, but a considerable amount of 5,11,14-20:3 acid (3.0% – 4.2%) was found in *Ascophyllum nodosum*, order Fucales⁴⁾. Several researchers have reported on the fatty acid composition of the total lipids derived from different species of *Sargassum* (Fucales, Phaeophyta), and the major components have been recognized as palmitic acid (16:0), oleic acid (18:1n-9), and C₁₈ and C₂₀ polyunsaturated fatty acids of n-6 and n-3 series (18:2n-6, 18:3n-3, 18:4n-3, 20:4n-6, and 20:5n-3)^{8–14)}. In addition to these fatty acids, appreciable amounts of 20:2 NMI and 24:1n-9 acids were found in seven *Sargassum* species isolated from different parts of the world (*S. miyabei*, *S. pallidum*, *S. herklotsii*, *S. baccularia*, *S. microcystum*, *S. turbinarioides*, and *S. cristaefolium*)⁸⁾. Khotimchenko⁸⁾ suggested that the ratio of 20:4n-6 and 20:5n-3, the content of n-6 fatty acids, and the occurrence of appreciable amounts of rare fatty acids (20:2 NMI and 24:1n-9) were characteristic features of *Sargassum* species. The 20:2 NMI acid in these *Sargassum* species (0.1% – 2.2% of the total fatty acids) was ten-

tatively identified as 5,11-20:2, based on the equivalent chain length (ECL) and retention time of the acid obtained from a mollusk⁸⁾. On the other hand, Bhaskar *et al.*^{9,10)} reported that the 20:2 NMI acid in three *Sargassum* species (*S. marginatum*, *S. thunbergii*, and *S. confusum*) was 4,9-20:2 by gas chromatography-mass spectrometry (GC-MS) of the 4,4-dimethyloxazoline (DMOX) derivatives. These discrepancies prompted us to elucidate the structure of the 20:2 NMI acid in *Sargassum* species. Our careful GC-MS analysis has revealed that the 20:2 NMI acid in the five *Sargassum* species examined (*S. fulvellum*, *S. horneri*, *S. boreale*, *S. thunbergii*, and *S. yezoense*) is 5,9-20:2, neither 5,11-20:2 nor 4,9-20:2. This report describes the structural determination of the 5,9-20:2 NMI acid found in the five *Sargassum* species examined as well as their detailed fatty acid compositions.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

Five species of brown seaweed belonging to the genus *Sargassum* (Fucales, Phaeophyta), namely, *S. fulvellum*, *S. horneri*, *S. boreale*, *S. thunbergii*, and *S. yezoense* were examined in this study (Table 1). All the seaweed samples were transported to the laboratory on ice from the collection site. They were kept frozen and freeze-dried at –30°C until analysis. 2-Amino-2-methyl-1-propanol and

*Correspondence to: Yutaka Itabashi, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, JAPAN

E-mail: yutaka@fish.hokudai.ac.jp

Accepted December 25, 2011 (received for review November 9, 2011)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs

Table 1 List of the *Sargassum* species studied.

No.	Species	Time of sampling	Site of sampling
1	<i>Sargassum fulvellum</i>	April 2010	Akita (Japan), Sea of Japan
2	<i>S. horneri</i>	April 2010	Ishikawa (Japan), Sea of Japan
3	<i>S. boreale</i>	June 2011	Hakodate (Hokkaido, Japan), Pacific Ocean
4	<i>S. thunbergii</i>	July 2011	Hakodate (Hokkaido, Japan), Tsugaru Strait
5	<i>S. yezoense</i>	August 2011	Hakodate (Hokkaido, Japan), Tsugaru Strait

trifluoroacetic anhydride (Fluka, Germany) were used to prepare DMOX derivatives. Silver ion solid phase extraction (Ag-ion SPE) cartridges (DiscoveryTM AG-ION, 750 mg/6 mL) were purchased from Supelco Inc. (Bellafonte, PA, USA). All other chemicals and solvents (Wako Pure Chemicals, Osaka, Japan) were of reagent grade or better quality.

2.2 Lipid extraction and fatty acid analysis

Lipids were extracted using the Bligh and Dyer method¹⁵⁾, and then converted to fatty acid methyl esters (FAMES) by heating at 95°C for 1 h in 5% HCl in methanol. The resulting FAMES were confirmed by thin-layer chromatography (TLC) on silica gel 60 F₂₅₄ aluminum sheets (Merck, Darmstadt, Germany), using hexane-diethyl ether-acetic acid (70:30:1, v/v) as the developing solvent¹⁶⁾.

GC analysis of FAMES was performed on a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and an Omegawax 320 column (30 m × 0.32 mm i.d., 0.25-μm film thickness; Supelco Inc., USA). The carrier gas was helium, which was administered at a flow rate of 1 mL/min, with a split ratio of 1:50. The injector and FID temperatures were 250°C and 260°C, respectively. The column temperature was programmed to increase from 160°C to 220°C at a rate of 2°C/min. Peaks were monitored on a Chromatopac C-R6A integrator (Shimadzu) and identified by using authentic standards (GLC-462; Nu-Chek Prep., Elysian, MN, USA), ECL values¹⁷⁾, and GC-MS, as described below.

2.3 Fractionation of FAME using Ag-ion SPE cartridges

FAMES were fractionated by Ag-ion SPE according to the number and position of double bonds¹⁸⁾. Before use, the SPE cartridges were conditioned with 4 mL of acetone to remove any moisture, followed by 4-mL of hexane. Total FAMES (about 1 mg in 1-mL hexane) were applied to the cartridge, and eluted with 6-mL volumes of hexane containing increasing amounts of acetone: 99:1 (v/v) eluted saturates (Fr. 1); 96:4 (v/v) eluted monoenoates (Fr. 2); 90:10 (v/v) eluted methylene-interrupted (MI) dienoates (Fr. 3); 0:100 (v/v) eluted NMI dienoates and MI trienoates (Fr. 4). This was followed by 3% acetonitrile in acetone to elute NMI trienoates and MI tetraenoates (Fr. 5), and 6% acetonitrile in acetone to elute NMI tetraenoates and MI pentae-

noates (Fr. 6). All the fractions were dried in a stream of N₂, and reconstituted in an appropriate volume of hexane for GC or CCl₄ for infrared spectrometry (IR). IR spectra were taken on an IRPrestige-21 FT-IR spectrometer (Shimadzu).

2.4 Preparation of 4,4-dimethyloxazoline (DMOX) derivatives

DMOX derivatives from the FAMES fractionated by Ag-ion SPE were prepared by the modified method of Svetashev¹⁹⁾. Briefly, 300 μL of 50% (w/v) 2-amino-2-methyl-1-propanol in toluene were added to 3–5 mg of the FAMES. After mixing this solution, 50 μL of 1% (w/v) NaOCH₃ in methanol was added to it. The mixture was incubated overnight in the dark at room temperature, and then, the intermediate was isolated by partitioning between 1 mL of water and 5 mL of hexane-diethyl ether mixture (9:1, v/v). After solvent evaporation, 300 μL of trifluoroacetic anhydride (TFAA) was added to the extract and incubated at 50°C for 45 min. After the reaction was completed, TFAA was evaporated under nitrogen. The resulting DMOX derivatives were purified by TLC on silica gel 60 F₂₅₄-coated glass plates (Merck), using hexane-diethyl ether-acetic acid (50:50:1, v/v, R_f 0.41) as the developing solvent. The derivatives were dissolved in hexane for GC-MS analysis.

2.5 GC-MS

For GC-MS, a Hewlett-Packard model 6890 GC system (Agilent Technologies, Wilmington, DE, USA) linked to a JEOL JMS-700Tz mass spectrometer (JEOL, Tokyo, Japan) was used. The latter was operated in the electron impact (EI) mode at an ionization voltage of 70 eV with a source temperature of 230°C. The DMOX derivatives were separated on a fused silica DB-23 capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness; Agilent Technologies). The injector in split mode (50:1), and the interface temperatures were maintained at 230°C, and helium was used as the carrier gas at a flow rate of 1 mL/min. The temperature-programming mode was as follows: 40°C isothermal for 1 min, increase to 200°C at 40°C/min, and then to 240°C at 10°C/min.

2.6 Mass spectral data of the DMOX derivatives of NMI acids with $\Delta 5$ unsaturation

2.6.1 5,9-20:2

MS m/z (rel. int.): 361 [M^+] (7.7), 346 (4.5), 332 (1.5), 318 (1.8), 304 (1.0), 290 (2.0), 276 (1.8), 262 (2.3), 248 (2.0), 234 (4.8), 220 (1.7), 206 (1.2), 194 (1.0), 180 (100), 166 (2.5), 153 (3.6), 126 (13.8), 113 (62.1), 98 (3.7), 81 (5.5), 67 (4.5), 55 (3.5), 41 (3.2).

2.6.2 5,11,14-20:3

MS m/z (rel. int.): 359 [M^+] (13.9), 344 (3.2), 330 (1.5), 316 (3.1), 302 (13.8), 288 (3.2), 274 (2.2), 262 (1.5), 248 (12.2), 234 (1.7), 222 (1.6), 208 (8.0), 194 (5.7), 180 (16.7), 166 (8.0), 153 (12.6), 136 (3.8), 126 (18.3), 113 (100), 98 (7.4), 81 (4.4), 67 (7.2), 55 (6.3), 41 (2.3).

2.6.3 5,11,14,17-20:4

MS m/z (rel. int.): 357 [M^+] (19.5), 342 (2.4), 328 (2.2), 314 (3.3), 302 (2.8), 288 (11.0), 274 (3.3), 262 (2.6), 248 (11.6), 234 (3.5), 222 (2.1), 208 (7.2), 194 (9.0), 180 (12.5), 166 (10.2), 153 (11.3), 136 (2.1), 126 (30.4), 113 (100), 98

(19.3), 81 (10.0), 67 (8.2), 55 (5.0), 41 (5.5).

3 RESULTS AND DISCUSSION

3.1 GC elution profiles

Figure 1 shows the chromatograms of the FAMES from the total lipids of *S. boreale* (A) and *S. fulvellum* (B) on an Omegawax 320 column. In addition to the usual peaks of saturated and unsaturated fatty acids, unusual peaks (No. 36, 39, and 44) appeared on the chromatograms. Peaks 39 and 44 were supposed to be 5,11,14-20:3 and 5,11,14,17-20:4 FAMES, respectively, by comparison of the retention times of methyl esters of the known fatty acids from gymnosperm seed oils²⁰ and sea urchin lipids²¹. Peak 36 was supposed to be a C_{20} unsaturated FAME from its ECL value (see Table 2). The FAMES from *S. boreale* were fractionated into 6 fractions by Ag-ion SPE according to the number and position of double bonds. Peak 36 ap-

Fig. 1 GC profiles of the total fatty acids from *Sargassum boreale* (A) and *Sargassum fulvellum* (B) on Omegawax 320.

Peak identification: 1 = 14:0; 7 = 16:0; 9 = 16:1n-7; 11 = 13-16:1; 13 = 16:2n-4; 20 = 18:0; 22 = 18:1n-9; 26 = 18:2n-6; 29 = 18:3n-6; 31 = 18:3n-3; 32 = 18:4n-3; 34 = 20:1n-11; 36 = 5,9-20:2; 38 = 20:2n-6; 39 = 5,11,14-20:3; 40 = 20:3n-6; 41 = 20:4n-6; 43 = 20:3n-3; 44 = 5,11,14,17-20:4; 45 = 20:4n-3; 46 = 20:5n-3; 48 = 22:1n-13; 56 = 22:6n-3. The column temperature was programmed from 160°C to 220°C at 2°C/min. Other GC conditions as given in text.

Table 2 Fatty acid composition of *Sargassum* algae (wt%)^a

Peak no.	Fatty acid	ECL ^c	Sample no. ^b				
			1	2	3	4	5
1	14:0	14.00	7.44	2.40	5.38	3.24	3.03
2	9-14:1 (14:1n-5)	14.33	0.50	0.37	0.16	0.27	0.21
3	iso15:0	14.51	0.38	0.31	0.12	0.19	0.16
4	15:0	15.00	0.35	0.16	0.38	0.32	0.27
5	iso16:0	15.30	0.20	0.20	0.09	0.13	0.22
6	anteiso16:0	15.63	0.42	0.41	0.23	0.29	0.39
7	16:0	16.00	20.97	16.85	22.14	25.47	25.91
8	7-16:1 (16:1n-9)	16.31	tr	0.06	tr	0.35	0.29
9	9-16:1 (16:1n-7)	16.41	4.03	2.97	4.70	5.48	1.85
10	11-16:1 (16:1n-5)	16.48	0.14	0.12	0.23	0.20	0.15
11	13-16:1 (16:1n-3)	16.55	1.10	1.23	0.44	0.38	0.76
12	iso17:0	16.70	0.68	0.66	0.37	0.06	0.52
13	9,12-16:2 (16:2n-4)	16.92	0.74	0.41	0.53	0.53	0.27
14	6,9,12-16:3 (16:3n-4)	17.30	0.16	0.06	0.08	0.10	tr
15	9-17:1 (17:1n-8)	17.40	0.19	1.13	0.28	0.58	0.35
16	9,12,15-16:3 (16:3n-1)	17.52	0.29	0.18	0.13	0.15	0.07
17	4,7,10,13-16:4 (16:4n-3)	17.71	0.17	tr	tr	0.30	tr
18	9,12-17:2 (17:2n-15)	17.81	tr	—	0.09	tr	0.19
19	6,9,12,15-16:4 (16:4n-1)	17.89	0.30	0.28	0.29	0.43	0.08
20	18:0	18.00	0.40	0.22	0.93	1.00	0.62
21	5-18:1 (18:1n-13)	18.23	0.05	tr	tr	0.10	tr
22	9-18:1 (18:1n-9)	18.34	5.49	4.68	4.62	6.68	5.93
23	11-18:1 (18:1n-7)	18.40	0.41	0.16	0.58	1.19	0.41
24	12-18:1 (18:1n-6)	18.46	0.05	0.09	0.13	0.24	tr
25	13-18:1 (18:1n-5)	18.52	0.11	tr	0.15	0.14	0.08
26	9,12-18:2 (18:2n-6)	18.81	4.58	4.50	5.40	4.81	6.09
27	3,9,12-18:3	18.95	0.07	0.12	0.06	0.08	—
28	11,14-18:2 (18:2n-4)	19.00	0.15	0.15	0.15	0.05	0.05
29	6,9,12-18:3 (18:3n-6)	19.11	1.78	0.72	0.91	0.39	0.41
30	9-19:1 (19:1n-10)	19.31	0.14	tr	0.07	—	—
31	9,12,15-18:3 (18:3n-3)	19.45	4.72	6.31	4.51	11.60	11.46
32	6,9,12,15-18:4 (18:4n-3)	19.77	6.55	18.25	4.78	2.53	7.45
33	20:0	20.00	0.16	0.13	0.30	0.27	0.31
34	9-20:1 (20:1n-11)	20.29	1.50	1.54	1.42	1.54	2.02
35	11-20:1 (20:1n-9)	20.38	0.16	0.08	0.21	0.17	0.10
36	5,9-20:2	20.47	0.59	1.03	2.34	0.93	0.38
37	9,12-20:2 (20:2n-8)	20.64	0.13	0.07	tr	tr	0.13
38	11,14-20:2 (20:2n-6)	20.77	1.66	0.11	0.16	0.25	0.28
39	5,11,14-20:3	20.90	0.68	tr	tr	0.07	0.08
40	8,11,14-20:3 (20:3n-6)	21.02	1.66	0.41	0.92	0.41	0.51

Peak no.	Fatty acid	ECL ^c	Sample no. ^b				
			1	2	3	4	5
41	5,8,11,14-20:4 (20:4n-6)	21.25	18.41	14.83	15.41	13.43	13.41
42	10,13,16-20:3 (20:3n-4)	21.31	0.17	0.15	0.13	0.09	0.10
43	11,14,17-20:3 (20:3n-3)	21.38	0.61	0.13	0.15	0.25	0.23
44	5,11,14,17-20:4	21.51	0.57	0.12	tr	0.15	0.13
45	8,11,14,17-20:4 (20:4n-3)	21.62	0.77	0.56	1.05	0.40	0.94
46	5,8,11,14,17-20:5 (20:5n-3)	21.83	5.95	15.45	13.70	10.24	10.05
47	22:0	22.00	0.38	0.15	0.23	0.49	1.00
48	9-22:1 (22:1n-13)	22.14	1.49	0.92	0.93	1.45	1.56
49	11-22:1 (22:1n-11)	22.37	0.09	tr	0.05	—	—
50	13,16-22:2 (22:2n-6)	22.62	0.35	—	—	—	—
51	7,13,16-22:3	22.74	0.15	—	tr	—	—
52	10,13,16-22:3 (22:3n-6)	22.82	0.13	—	tr	—	—
53	7,10,13,16-22:4 (22:4n-6)	23.02	0.39	—	tr	0.15	—
54	7,10,13,16,19-22:5 (22:5n-3)	23.57	0.10	—	0.12	0.34	tr
55	24:0	24.00	0.09	tr	0.10	0.05	0.05
56	4,7,10,13,16,19-22:6 (22:6n-3)	24.05	0.26	0.12	2.54	0.82	0.35
57	9-24:1 (24:1n-15)	24.14	0.08	0.12	0.15	0.17	0.21
	Σ Saturates		31.97	21.84	30.43	31.77	32.70
	Σ Monoenes		14.87	13.02	13.75	18.51	13.61
	Σ Polyenes		53.01	64.31	53.72	49.41	52.81
	Σ NMI		2.06	1.27	2.40	1.23	0.59
	Others ^d		0.92	1.11	2.15	1.04	0.93

^a Mean of triplicate determinations. tr: Trace <0.05% of total fatty acids. -: Not detected.

^b Numbers designate the algal species given in Table 1.

^c Equivalent chain length at 180°C.

^d 12:0, iso13:0, 13:0, iso14:0, ai14:0, 17:0, 18:1n-4, 18:3n-4.

peared in Fr. 4 (acetone-eluting fraction) along with usual MI trienoates (mainly 18:3n-3), showing 28.2% of the total peak areas (chromatogram not shown). This elution profile suggested that Peak 36 was an NMI dienoate. A slower mobility of NMI dienoates on Ag-ion SPE than the usual MI dienoates has also been observed on Ag-ion TLC²⁰⁾. The FT-IR spectrum of Fr. 4 showed the absence of an absorption band between 960 and 970 cm⁻¹ (*trans* C-H out-of-plane deformation) (spectrum not shown), which demonstrated that the peak 36 component had only *cis* configuration. Fatty acids from *S. horneri*, *S. thunbergii*, and *S. yezoense* were also examined and showed similar GC elution profiles as those shown in Fig. 1.

3.2 Structural determination of 20:2 NMI acid

To ascertain the positions of double bonds in Peak 36 by GC-MS, a part of Fr. 4 was converted to the DMOX derivative. Figure 2 illustrates the mass spectrum of the DMOX derivative of Peak 36. The fragmentation pattern is very

similar to that of 5,9-18:2 and different from that of 5,11-20:2²²⁾. The double bond at the $\Delta 9$ position can be confirmed by a gap of 12 amu between *m/z* 194 and 206 (carbons 8 and 9), which means that the double bond is between carbons 9 and 10. A more abundant ion at *m/z* 234 (cleavage beta to the double bond) gives an additional diagnostic information for the double bond at the $\Delta 9$ position²²⁾. The ions produced by cleavage at the double bond *per se* are not easily distinguished when the double bond is closer to the carboxyl group than the $\Delta 7$ position in the chain²²⁾. Thus, in this study, it was not possible to confirm a $\Delta 5$ unsaturation for Peak 36 with a gap of 12 amu. However, this hardly matters as the prominent ion at *m/z* 180 (base peak), which represents cleavage at the center of bis-allylic system, is sufficiently characteristic for a 5,9-NMI diene system in the molecule²²⁾. The odd-numbered ion at *m/z* 153 and the intense ion at *m/z* 166 also support the double bond at the $\Delta 5$ position^{22, 23)}. When the double bond is located at the $\Delta 4$ position, this diagnostic

Fig. 2 GC-MS spectrum of the DMOX derivative of 5,9-eicosadienoic acid from *Sargassum boreale*.

Fig. 3 Possible biosynthetic pathways for fatty acids in *Sargassum* species.

odd-numbered ion appears at m/z 139²³). The prominent ion at m/z 113 and the intense ion at m/z 126 (Fig. 2) are invariably seen with such derivatives, and are produced by McLafferty rearrangement (cleavage between carbons 2 and 3) and a cyclization reaction, respectively²⁴). The spectrum also shows a molecular ion at m/z 361, suggesting a

C₂₀ FAME (Fig. 2). These results clearly demonstrate that the 20:2 NMI acid found in the five *Sargassum* species examined in this study is 5,9-20:2, but neither 5,11-20:2^{6,8)} nor 4,9-20:2^{9,10)}. Khotimchenko⁸⁾ reported that the 20:2 NMI acid obtained from seven *Sargassum* species (*S. miyabei*, *S. pallidum*, *S. herklotsii*, *S. baccularia*, *S. mi-*

crocystum, *S. turbinarioides*, and *S. cristaefolium*) was identical to 5,11-20:2 by comparing the ECL value and retention times of the acid obtained from a mollusk. Kaneniwa *et al.*⁶⁾ also identified 5,11-20:2 in several seaweeds, including Chlorophyta, Phaeophyta and Rhodophyta, by using the ECL value and retention time of the known acid obtained from sea urchin lipids. Both the studies did not note the presence of 5,9-20:2. On the other hand, Bhaskar *et al.*^{9, 10)} reported that the 20:2 NMI acid found in three *Sargassum* species (*S. marginatum*, *S. thunbergi*, and *S. confusum*) was 4,9-20:2 by performing GC-MS analysis of the DMOX derivative, which showed almost the same mass spectrum as that in Fig. 2. We cannot explain their results regarding the structural analysis of the 20:2 NMI acid. In this study, in addition to 5,9-20:2, two more NMI acids from the algae examined, *i.e.*, 5,9,12-20:3 and 5,11,14,17-20:4, were successfully identified in the above-described manner (for key fragments, see 2.6).

3.3 Fatty acid composition

The fatty acid compositions of the total lipids from the five *Sargassum* species studied are given in Table 2. Their major fatty acids were 16:0 (16.9%–25.9%), 18:1n-9 (4.7%–6.7%), 18:2n-6 (4.5%–6.1%), 18:3n-3 (4.7%–11.6%), 18:4n-3 (2.5%–18.3%), 20:4n-6 (13.4%–18.4%), and 20:5n-3 (6.0%–15.5%). *Sargassum* algae typically have high concentrations of C₁₈ and C₂₀ polyunsaturated fatty acids (PUFAs)^{8–14)}. This characteristic feature was also observed for all *Sargassum* species examined in our study, but the levels of individual n-3 PUFA were very different among the species, that is, 18:4n-3 was much higher in *S. horneri* (18.3%) than in other species (2.5%–7.5%); 20:5n-3 was much lower in *S. fulvellum* (6.0%) than in other species (10.2%–15.5%), while the amounts of n-6 PUFA (18:2n-6 and 20:4n-6) were similar among species (Table 2). These results suggest that the fatty acid composition of *Sargassum* species is species specific, and the contents of C₁₈ (18:3n-3 and 18:4n-3) and C₂₀ (20:5n-3) PUFAs vary in the algae collected from different places (*S. horneri* and *S. fulvellum* were collected at different places from three other species in Japan), although Khotimchenko⁸⁾ has reported that the content of C₁₈ PUFA (18:2n-6, 18:3n-3, and 18:4n-3) in *Sargassum* algae probably does not depend on the site of collection.

Several monounsaturated fatty acids such as 9-14:1, 9-16:1, 9-17:1, 9-18:1, 9-20:1, 9-22:1 and 9-24:1 were detected in all *Sargassum* species examined. The predominance of 9-16:1 and 9-18:1 suggests the presence of $\Delta 9$ desaturase that primarily acts on 16:0 and 18:0 fatty acids⁸⁾. Interestingly, all *Sargassum* species studied, especially *S. boreale* contained a high amount of 22:6n-3, accounting for 2.5% of the total fatty acid content. The content of 22:6n-3 in algae, including the other genus *Sargassum*, has been reported to be less than 1%^{4, 6, 7, 9, 13)}.

In addition to an appreciable amount of 5,9-20:2 (0.4%–2.3%), tri- and tetraenoic NMI acids with $\Delta 5$ -unsaturation, that is, 5,11,14-20:3 (0.1%–0.7%) and 5,11,14,17-20:4 (0.1%–0.6%), were detected in all *Sargassum* species studied (Table 2). Of these NMI acids, 5,9-20:2 was found to be the most abundant, especially rich in *S. boreale* (2.3%). The low level of 5,11,14-20:3 and 5,11,14,17-20:4 has also been observed in other marine algae⁶⁾. Thus, these findings indicate that bis- and polymethylene-interrupted dienoic fatty acids are more common in nature than assumed previously^{25, 26)}. In particular, fatty acids with a 5,9-double bond system or their chain elongation products are common in seed oils from Gymnosperms³⁾ or in certain marine invertebrates such as echinoderms, mollusks, and sponges^{1, 2)}. The 5,9-20:2 NMI acid has been identified in the phospholipids from the sea anemones *Stoichactis helianthus*²⁷⁾ and *Condylactis gigantea*²⁸⁾, and the zoanthid *Palythoa caribaeorum*²⁸⁾, accounting for 2.3%, 3.0%, and 2.0% of the total phospholipid fatty acids, respectively. Female gonads of the limpet *Cellana grata* also contain this acid, but its content is very low (less than 0.1% of the total fatty acids from triacylglycerols)²⁹⁾. Thus, *S. boreale* examined in our study would be one of the potential sources of 5,9-20:2.

It has been established that C₂₀ NMI dienoic acids of mollusks and other invertebrates represent a mixture of 5,11-20:2 and 5,13-20:2 isomers³⁰⁾. Zhukova³¹⁾ demonstrated that 5,11-20:2 and 5,13-20:2 in mollusks were formed by the action of $\Delta 5$ desaturase from 11-20:1 and 13-20:1, respectively, which were derived from saturated and monoenic acids, synthesized from the acetate, but did not result from the elongation of stored fatty acids.

Figure 3 gives possible biosynthetic pathways of the major or characteristic fatty acids in *Sargassum* species. It seems likely that similar biosynthetic pathways occur in mollusks and *Sargassum* species. The $\Delta 5$ -NMI acids, namely, 5,9-20:2, 5,11,14-20:3, and 5,11,14,17-20:4, would be produced by the action of $\Delta 5$ desaturase from their precursor acids, namely, 9-20:1, 11,14-20:2, and 11,14,17-20:3, respectively. Meyer *et al.*³²⁾ reported that both n-6 and n-3 pathways involved in the production of 20:4n-6, 20:5n-3, and 22:6n-3 were found in algae. In addition, some algae can use an alternative pathway, in which linoleic acid (18:2n-6) and α -linolenic acid (18:3n-3) are elongated to the corresponding $\Delta 11$ -C₂₀ polyunsaturated fatty acids, necessitating subsequent $\Delta 8$ desaturation³³⁾.

No biological activity has been reported to date for the most encountered dienoic 20:2 and 22:2 NMI fatty acids, except for $\Delta 5,9$ -NMI fatty acids, which exhibit interesting antiparasitic activity³⁾. The most promising fatty acid topoisomerase I inhibitors to date seem to be the long-chain $\Delta 5,9$ -NMI dienoic acids such as $\Delta 5,9$ -23:2³⁴⁾. The activity of the $\Delta 5,9$ -20:2 identified in this study is unknown.

4 CONCLUSION

Using GC, Ag-ion SPE, and GC-MS techniques, we investigated in detail the fatty acid composition of the brown algae *Sargassum* species, and found, for the first time, 5,9-20:2 NMI acid in all the species examined. To our knowledge, this acid has not been found in other algal species. Further study on the distribution of 5,9-20:2 NMI acid in other species of algae and its biological activities is necessary to exploit the potential of algae as a source of pharmaceutical materials.

ACKNOWLEDGEMENTS

This work was partly supported by a Grant-in-Aid for Scientific Research (No. 21658074) and Regional Innovation Cluster Program (Global Type), Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT). The first author thanks MEXT for the award of the scholarship during the study period. The authors thank Prof. Hajime Yasui (Faculty of Fisheries Sciences, Hokkaido University) for collection and identification of the sample seaweeds. Thanks are also due to Ms. Seiko Oka (Instrumental Analysis Division, Hokkaido University) for obtaining the GC-MS spectra.

References

- 1) Barnathan, G. Non-methylene-interrupted fatty acids from marine invertebrates: Occurrence, characterization and biological properties. *Biochimie* **91**, 671-678 (2009).
- 2) Kornprobst, J.-M.; Barnathan, G. Demospongiac acids revisited. *Mar. Drugs* **8**, 2569-2577 (2010).
- 3) Wolff, R. L.; Pédrone, F.; Pasquier, E.; Marpeau, A. M. General characteristics of *Pinus* spp. Seed fatty acid compositions, and importance of Δ^5 -olefinic acids in the taxonomy and phylogeny of the genus. *Lipids* **35**, 1-22 (2000).
- 4) Jamieson, G. R.; Reid, E. H. The component fatty acids of some marine algal lipids. *Phytochemistry* **11**, 1423-1432 (1972).
- 5) Ackman, R. G.; McLachlan, J. Fatty acids in some Nova Scotian seaweeds: a survey for octadecapentaenoic and other biochemically novel fatty acids. *Proc. N. S. Inst. Sci.* **28**, 47-64 (1977).
- 6) Kaneniwa, M.; Itabashi, Y.; Takagi, T. Unusual 5-olefinic acids in the lipids of algae from Japanese waters. *Nippon Suisan Gakkaishi* **53**, 861-866 (1987).
- 7) Johns, R. B.; Nichols, P. D.; Perry, G. J. Fatty acid composition of ten marine algae from Australian waters. *Phytochemistry* **18**, 799-802 (1979).
- 8) Khotimchenko, S. V. Fatty acid composition of seven *Sargassum* species. *Phytochemistry* **30**, 2639-2641 (1991).
- 9) Bhaskar, N.; Miyashita, K.; Hosokawa, M. Comparative evaluation of fatty acid composition of different *Sargassum* (Fueles, Phaeophyta) species harvested from temperate and tropical waters. *J. Aqu. Prod. Technol.* **13**, 53-70 (2004).
- 10) Bhaskar, N.; Hosokawa, M.; Miyashita, K. Growth inhibition of human pro-myelocytic leukemia (HL-60) cells by lipid extracts of marine alga *Sargassum marginatum* (Fueles, Phaeophyta) harvested off Goa (west coast of India) with special reference to fatty acid composition. *Indian J. Mar. Sci.* **33**, 355-360 (2004).
- 11) Zhukova, N. V.; Svetashev, V. I. A high level of dihomogammalinolenic acid in brown alga *Sargassum pallidum* (Turn.). *Phytochemistry* **50**, 1209-1211 (1999).
- 12) Hamdy, A. E. A.; Dawes, C. J. Proximate constituents and lipid chemistry in two species of *Sargassum* from the west coast of Florida. *Bot. Mar.* **31**, 79-81 (1988).
- 13) El-Sayed, M. M. Fatty acid composition of some algal lipids from the Red Sea. *J. Fac. Mar. Sci.* **3**, 141-148 (1983).
- 14) Xiang-chun, W. U.; Bao-ren, L. U.; Tseng, C. K. Comparative fatty acid composition of four *Sargassum* species (Fueles, Phaeophyta). *Chin. J. Oceanol. Limnol.* **13**, 370-373 (1995).
- 15) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917 (1959).
- 16) Christie, W. W.; Han, H. *Lipid Analysis*. 4th ed. The Oily Press. Bridgwater, England (2010).
- 17) Stransky, K.; Jursik, T.; Vitek, A.; Skorepa, J. An improved method of characterizing fatty acids by equivalent chain length values. *J. High Res. Chromatogr.* **15**, 730-740 (1992).
- 18) Kramer, J. K. G.; Hernandez, M.; Gruz-Hernandez, C.; Kraft, J.; Dugan, M. E. R. Combining results of two GC separations partly archives determination of all *cis* and *trans* 16:1, 18:1, 18:2 and 18:3 except CLA isomers of milk fat as demonstrated using Ag-ion SPE fractionation. *Lipids* **43**, 259-273 (2008).
- 19) Svetashev, V. I. Mild method for preparation of 4,4-dimethyloxazoline derivatives of polyunsaturated fatty acids for GC-MS. *Lipids* **46**, 463-46 (2011).
- 20) Takagi, T.; Itabashi, Y. *cis*-5-Olefinic unusual fatty acids in seed lipids of gymnospermae and their distribution in triacylglycerols. *Lipids* **17**, 716-723 (1982).
- 21) Takagi, T.; Kaneniwa, M.; Itabashi, Y.; Ackman, R. G. Fatty acids in Echinoidea: Unusual *cis*-5-olefinic acids as distinctive lipid components in sea urchins. *Lipids* **21**, 558-565 (1986).
- 22) Wolf, R. L.; Christie, W. W. Structures, practical sources (gymnosperm seeds), gas-liquid chromatographic

- data (equivalent chain lengths), and mass spectrometric characteristics of all-*cis*- $\Delta 5$ -olefinic acids. *Eur. J. Lipid Sci. Technol.* **104**, 234-244 (2002).
- 23) Fay, L.; Richli, U. Location of double bonds in polyunsaturated fatty acids by gas chromatography-mass spectrometry after 4,4-dimethyloxazoline derivatization. *J. Chromatogr.* **541**, 89-98 (1991).
- 24) Zhang, J. Y.; Yu, Q. T.; Liu, B. N.; Huang, Z. H. Chemical modification in mass spectrometry IV. 2-Alkenyl-4,4-dimethyloxazolines as derivatives for the double bond location of long-chain olefinic acids. *Biomed. Environ. Mass Spectrom.* **15**, 33-44 (1988).
- 25) Kawashima, H. Structural varieties of novel non-methylene-interrupted fatty acids in marine gastropods and bivalves. *Oleoscience* **10**, 255-261 (2010).
- 26) Saito, H. Unusual novel n-4 polyunsaturated fatty acids in cold-seep mussels (*Bathymodiulus japonicus* and *Bathymodiulus platifrons*), originating from symbiotic methanotrophic bacteria. *J. Chromatogr. A* **1200**, 242-254 (2008).
- 27) Carballeira, N. M.; Medina, J. R. New $\Delta^{5,9}$ fatty acids in the phospholipids of the sea anemone *Stoichactis helianthus*. *J. Nat. Prod.* **57**, 1688-1695 (1994).
- 28) Carballeira, N. M.; Reyes, M. Identification of a new 6-bromo-5,9-eicosadienoic acid from the anemone *Condylactis gigantea* and the zoanthid *Palythoa caribaeorum*. *J. Nat. Prod.* **58**, 1689-1694 (1995).
- 29) Kawashima, H.; Ohnishi, M.; Ogawa, S.; Matsui, K. Unusual fatty acid isomers of triacylglycerols and polar lipids in female limpet gonads of *Cellana grata*. *Lipids* **43**, 559-567 (2008).
- 30) Paradis, M.; Ackman, R. G. Occurrence and chemical structure of nonmethylene-interrupted dienoic fatty acids in American oyster *Crassostrea virginica*. *Lipids* **10**, 12-16 (1975).
- 31) Zhukova, N. V. The pathway of the biosynthesis of non-methylene-interrupted dienoic fatty acids in molluscs. *Comp. Biochem. Physiol.* **100B**, 801-804 (1991).
- 32) Meyer, A.; Kirsch, H.; Domergue, F.; Abbadi, A.; Sperling, P.; Bauer, J.; Cirpus, P.; Zank, T. K.; Moreau, H.; Roscoe, T. J.; Zähringer, U.; Heinz, E. Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *J. Lipid Res.* **45**, 1899-1909 (2004).
- 33) Qi, B.; Beaudoin, F.; Fraser, T.; Stobart, A. K.; Napier, J. A.; Lazarus, C. M. Identification of a cDNA encoding a novel C18- $\Delta 9$ polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing microalga, *Isochrysis galbana*. *FEBS Lett.* **510**, 159-165 (2002).
- 34) Carballeira, N. M. New advances in fatty acids as antimalarial, antimycobacterial and antifungal agents. *Prog. Lipid Res.* **47**, 50-61 (2008).