Algicidal hydroxy monounsaturated fatty acids of the red alga *Tricleocarpa jejuensis*: Isolation, structure determination, synthesis, and biological activities

（紅藻ガラガラ *Tricleocarpa jejuensis*　由来の殺藻活性ヒドロキシ不飽和脂肪酸の単離・同定・合成及び生理活性に関する研究）

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Contents

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種々の海藻（大型藻類）が，他の海藻の侵入や生育を抑制する多感作用（アレロパシー）物質を生産･放出しているが，これらの多感作用物質が赤潮の原因となるラフィド藻や渦鞭毛藻などの微細藻に対しても毒性を示すことが知られており，環境負荷が少ない赤潮防除物質としての利用が期待される。

著者は，紅藻ガラガラ *Tricleocarpa jejuensis* のメタノール抽出物が，0.1 mg/mL 濃度で赤潮プランクトン *Chattonella antiqua* を完全に死滅させることを見出し，活性物質の単離と同定を行った（第 2 章）。*C. antiqua* に対する殺藻活性を指標に，ガラガラのメタノール抽出物を，液液分配，次いで各種クロマトグラフィー（Diaion® HP20, silica gel 60, 逆相分配 HPLC）で分画し，活性画分 **f5** (4.4 mg)を得た。この画分をさらに分離することは困難であったため，混合物のまま機器分析（NMR, IR, MS）による構造解析を行った。その結果, **f5** は, (*E*)-9-hydroxyoctadec-10-enoic acid (**1**), (*E*)-10-hydroxyoctadec-8-enoic acid (**2**), (*E*)-11-hydroxyoctadec-12-enoic acid (**3**)及び(*E*)-12-hydroxyoctadec-10-enoic acid (**4**)の混合物であると決定し，キラル HPLC 分析により，化合物 **1** ～ **4** は全てラセミ体であることを確認した。さらに, 化合物 **1** ～ **4** それぞれを化学合成することにより標準サンプルを得, 天然物のスペクトルデータと比較したところ完全に一致した。これにより，構造決定に誤りがないことが確認された。

化合物 **1** ～ **4** 及びこれらの合成中間体を含む全 12 化合物の *C. antiqua* に対する殺藻活性を調べたところ, ①ヒドロキシ基の存在が活性発現に必須であるが, 結合位置は活性に影響しない, ②二重結合は三重結合に変更可能, ③二重結合の幾何異性は活性に影響しない, ④カルボキシル基はヒドロキシ基で代用可能など, 構造と活性の関係に関する基礎

的な知見が得られた。また，三重結合をもつ 12-hydroxyoctadec-10-ynoic acid (**10**)に天然物をしのぐ急性毒性を見出した。

第 3 章では，赤潮防除物質としての有効性の検討，及び作用機構解明への手掛かりを得るために，化合物 **4** と **10** を用いて詳細な生理活性を検討した。まず，ラフィド藻 *C. antiqua* と *C. marina* 及び渦鞭毛藻 *Heterocapsa circularisquama* と *Karenia mikimotoi* に対する殺藻活性を調べたところ，両者ともに 4 種全ての赤潮プランクトンに対して 10 □g/mL 以下の LC_{50} 値を示した。一方，いずれの化合物も動物プランクトンのワムシ *Brachionys plicatilis* には比較的無毒で，赤潮プランクトン暴露実験においてワムシへの保護効果が見られた。化合物 **4** と **10** は，がん細胞株（HeLa, XC, U937）に対する細胞毒性及びグラム陽性菌 *Staphylococcus aureus* に対する殺菌活性を示した。さらに化合物 **10** は，強い溶血活性を示すことにより，これらヒドロキシ不飽和脂肪酸類は，微細藻の細胞膜に作用し毒性を発現することが示唆された。また，行ったいずれの生理活性試験においても化合物 **10** の方がより高活性であった。

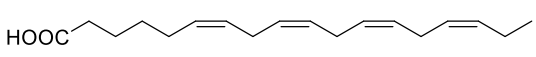
前章において，化合物 **4** が，がん細胞株に対して細胞毒性を示すことが明らかになったが，その作用機構は不明であった。ある種の不飽和脂肪酸がヒトリンパ腫由来 U937 細胞株においてアポトーシスを誘導することが知られているので，第 4 章では，化合物 **4** の細胞毒性機構解明の手掛かりとして，アポトーシス誘起活性の有無を調べた。その結果，化合物 **4** が U937 細胞に対して数十□g/mL 濃度で，細胞溶解や DNA の断片化等のアポトーシス様形態変化を引き起こすことが明らかになった。

本研究の結果，ガラガラの殺藻活性物質として 4 種類の C18 ヒドロキシ *trans*-モノ不飽和脂肪酸を同定するとともに，二重結合の三重結合への変換は活性を増大させることなど，構造と活性の関係に関する重要な知見が得られた。また，赤潮防除剤としてだけでなく，抗ガン剤や抗菌剤のリード化合物としても有望な誘導体 12-hydroxyoctadec-10-ynoic acid (**10**)を見出した。

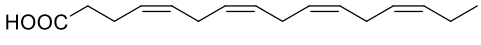
Chapter I General introduction

Macroalgae have been studied for many years. Researches on macroalgae are mostly focused on the following aspects including effects of ecology, energy, pharmaceutical value as well as food sources. Chemistry and biological activity of the compounds produced by macroalgae is also a major field in algal research. Secondary metabolites of marine macroalgae involve fatty acids, halogenated compounds, sterols, terpenoids, and phenolics. Many of such secondary metabolites might be produced under stressed condition such as infection by pathogens, oxidative stress, and exposure to other competing organism. Thus, marine macroalgae have received much attention as potential rich sources with a wide variety of biological activities including anti-bacterial [1], anti-tumor [2], antioxidant [3], anti-inflammatory [4], and anti-algal [5] activities.

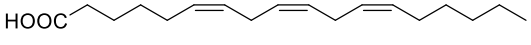
Harmful algal blooms (HABs), commonly known as red tides, due to eutrophication of coastal waters are one of the most serious threat to marine environment, which can cause tremendous damage on coastal areas associated with massive mortality of natural and aquacultured fish and shellfish, and even with public health problem depending on the causative species [6-8]. Various physical, chemical, physico-chemical, and biological methods to control HABs have been developed so far [9]; however, many of them are unacceptable for practical use in marine environments due to the second pollution, high cost, or difficulty of handling. Regarding HAB control strategy, macroalgae have many advantages such as the abundant natural sources, low cost, and relatively environmentally friendly features in addition to the allelopathic effects on HABs [5,10,11,12]. In fact, diverse chemical compounds with growth inhibitory effects on HAB species have been found in several marine macroalgal species. The algicidal (antialgal) compounds isolated so far include polyunsaturated fatty acids (PUFAs) from *Cladosiphon okamuranus* [13], *Botryococcus braunii* [14], *Ulva fasciata* [15] *Lithophyllum yessoense* [16], and *Sargassum thunbergii* [17]; glycerolipids from *Ishige sinicola* [18] and *Ulva prolifera* [19, 20]; terpenoids from *Dictyota dichotoma* [21], *Gracilaria lemaneiformis* [22, 23], *Dictyopteris undulata* [24], and *Ulva pertusa* [25]; and phenolics [23, 25] (Fig. 1.1). Many of these compounds are reported to have potent algicidal activity at concentrations of low μg/mL range against some of the raphidophytes



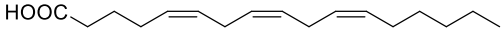
Cladosiphon okamuranus



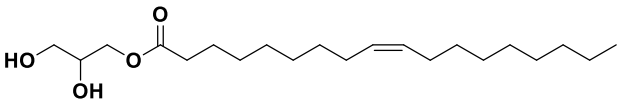
Ulva fasciata



Ulva fasciata
Botryococcus braunii

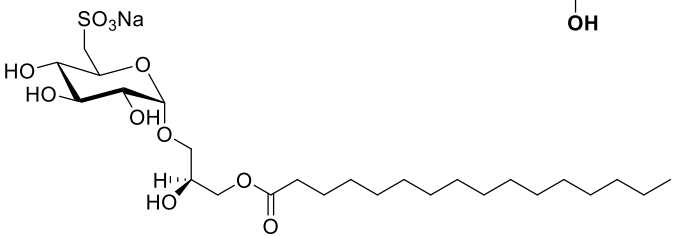


Lithophyllum yessoense

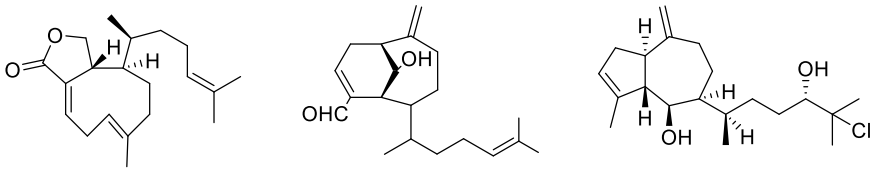


Ishige sinicola

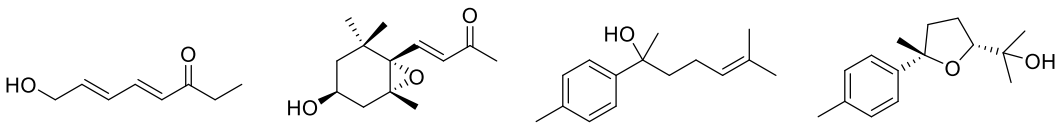
Ulva prolifera



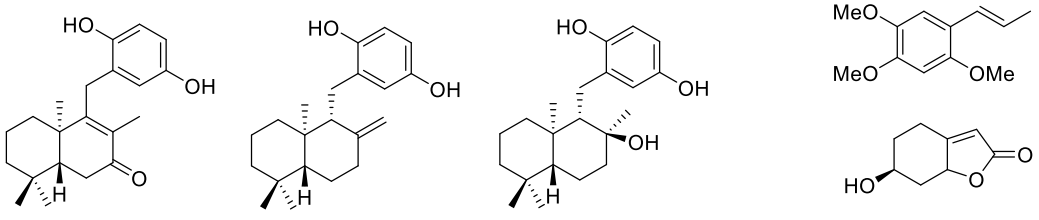
Ishige sinicola



Dictyota dichotoma



Gracilaria lemaneiformis



Dictyopteris undulata

Ulva pertusa

Fig. 1.1. Representative algicidal compounds isolated from macroalgae

and dinoflagellates responsible for red tides. In this context, I started a project to explore novel algicidal compounds from macroalgae.

In Chapter II, I separated an algicidal fraction comprising four isomeric hydroxy monounsaturated fatty acids which showed lethal (cell lysis) activity against the red-tide plankton *Chattonella antiqua* at 20 μg/mL from the red alga *Tricleocarpa jejuensis*. The structures of the algicidal compounds were determined to be (±)-(*E*)-9-hydroxyoctadec-10-enoic acid, (±)-(*E*)-10-hydroxyoctadec-8-enoic acid, (±)-(*E*)-11-hydroxyoctadec-12-enoic acid, and (±)-(*E*)-12-hydroxyoctadec-10-enoic acid (compound **4**) on the basis of spectroscopic information. The structures were further confirmed by comparison of the NMR and MS data with their authentic compounds obtained by unambiguous syntheses. A propargyl derivative of compound **4**, (±)-12-hydroxyoctadec-10-ynoic acid (compound **10**), which is a precursor of the synthesis of compound **4**, was found to induce cell lysis of *C. antiqua* with even more immediate action of mode than the parental compound **4**. Fundamental structure-activity relationship using 11 related compounds is also described.

In Chapter III, I evaluated further detailed biological activities of compound **4** and **10** involving algicidal activity against other HAB species *Chattonella marina, Heterocapsa circularisquama* and *Karenia mikimotoi*, protective effect on the rotifer *Brachionus plicatilis*, cytotoxic effects on cancer cell lines (HeLa, XC, and U937), haemolytic activity against rabbit erythrocytes, and antibacterial effects on gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*. Compound **10**, having triple bond instead of double bond, was found to be much stronger than compound **4** in terms of algicidal, cancer cell cytotoxic, and bactericidal activities. LC_{50} values of compound **4** and **10** against HAB species were much lower than those against cell lines and bacteria. The hydroxy unsaturated fatty acids may be specific to marine microalgae, and thus are considered to be promising agents to control HAB without affecting other organisms.

In Chapter IV, I investigated the cytotoxic mechanism of Compound **4** on U937 cells focusing on apoptosis induction. My preliminary results indicate that compound **4** could induce apoptosis of U937 cells.

Chapter II Algicidal compounds from the red alga *Tricleocarpa jejuensis*

1. Introduction

I screened 17 species of macroalgae including 9 Rhodophyta, 6 Phaeophyta, and 2 Chlorophyta collected from the coastal region of Nagasaki Prefecture, Japan, for their algicidal activity against the red tide phytoplankton *Chattonella antiqua* and found that a methanol extracts of the green alga *Codium cylindricum*, the brown alga *Undaria*

Table 2.1 Algicidal activiity of the methanolic extract of macroalgae against *Chattonella antiqua* at a dose of 0.1 mg/mL (mortality, %).

Macroalga		mortality (%)
Rhodophyta	*Lomentaria catenata*	0
	Grateloupia filicina	0
	Chrysymenia wrightii	0
	Gelidium elegans	0
	Laurencia undulata	0
	Portieria hornemannii	0
	Tricleocarpa jejuensis	100
	Gracilaria bursa-pastoris	0
	Dudresnaya japonica	0
Phaeophyta	*Sargassum alternato*	0
	Undaria pinnatifida	100
	Golpomenia sinuisa	0
	Dictyopteris prolifera	0
	Padina arborescens	0
	Sargassum piluliferum	0
Chlorophyta	*Codium cylindricum*	100
	Codium latum	0

pinnatifida, and the red alga *Tricleocarpa jejuensis* had cell lysis activity at concentrations of 0.1 mg/mL (Table. 2.1). Since the chemical composition of green and brown alga had been extensively studied in my laboratory, I interested in the algicidal principle(s) of the red alga *Tricleocarpa jejuensis*.

2. Materials and methods

2.1 Plant materials

Specimens of *T. jejuensis* was collected from coast of Teguma of Nagasaki Prefecture, Japan, in June 2018, and from Ishigaki Island, Okinawa prefecture, Japan, in June 2016. The samples from Teguma were collected in laundry net and keep in a box, then brought to laboratory quickly and washed with tap water to remove potential contaminants. The samples from Ishigaki Island were collected and frozen quickly, then brought to laboratory with an ice-cool preservation box. The frozen samples were melted under room temperature and washed as describe above. Macroalgae were dried on draft chamber overnight at room temperature.

2.2 Cultivation of phytoplankton

Chattonella antiqua, isolated from Shimabara Bay, Japan in 2010 by Dr. Tatsuya Oda, Nagasaki University, was cultured aseptically in PES medium at 20 °C under 40 μmol/m^2/s using 40 W fluorescent lamps with a 12 h day cycle and 12 h night cycle and sub-cultured after approximately 14 days.

2.3 Algicidal assay

The algicidal assay was performed according to Kakisawa's procedure [13], with a slight modification. In brief, a methanol solution of the extract or sample at varying concentrations was added to the cell suspension (cell density ca. 2×10^4 cells/mL) of *C. antiqua* in a 48-well microplate to make the final concentrations of 5, 20, or 80 μg/mL (methanol concentration ≤ 1%). After incubation at 20 °C for 24 h, the cell mortality was calculated under microscope observation (×400). The assay was performed in triplicate.

Algicidal activity (AA) was calculated using a formula: AA (%) = (1-T/C) x 100, where T and C represent number of the living cells in the presence and absence of the compound tested, respectively. Swollen and burst cells were considered dead cells.

2.4 General experimental procedure

NMR spectra were recorded on a Varian System 500PS SN spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C), a JEOL JNM AL400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) or a Varian Gemini 300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) in $CDCl_3$ using tetramethylsilane and $CDCl_3$ as the internal standards for ^{1}H and ^{13}C nuclei, respectively. High resolution (HR) electron impact mass spectroscopy (EIMS) was carried out on a JEOL JMS-700N spectrometer. Electron spray ionization (ESI) and direct analysis in real time (DART) mass spectra (MS) were obtained on a JEOL JMS-T100TD spectrometer. IR spectra were recorded on a ThermoFisher Scientific Nicolet Nexus 670NT spectrophotometer. Optical rotation was measured on a JASCO P-2200 polarimeter using a 10-cm microcell. GC-EIMS analysis was performed using an Agilent Technologies GC7890A-MS7000A system equipped with an HP-1MS capillary column (length 30 m, inside diameter 0.250 mm, film thickness 0.25 μm) in EI mode at 70 eV. GLC conditions: carrier gas, He; flow rate, 1.8 mL/min; oven, 120 °C, 5 min isothermal, 120 °C~300 °C with 10 °C/min.

Silica gel gravity and medium pressure column chromatography separations were performed using Kanto Chem. Co. Ltd. Silica Gel N (spherical neutral) 100-210 μm and 40-60 μm, respectively. Preparative TLC was performed using Merck Silica Gel 60 F254 (20 × 20 cm, layer thickness 1.0 mm).

2.5 Extraction and isolation of algicidal compounds

T. jejuensis (240 g dry wt) was powdered using a blender, soaked in 2.0 L methanol for 3 days and filtrated through a pack of Celilet. The extraction process was repeated again. Then the crude extracts were combined, concentrated and partitioned between hexane and 80% aqueous MeOH. After almost of the MeOH had been removed

in *vacuo*, the aqueous layer was partitioned between water and EtOAc. The EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated to afford an EtOAc extract (1.5 g). The crude EtOAc extract was separated over HP20 resin (V 108 cm3) by successive elutions with 20%, 40%, 60%, 80%, and 100% MeOH, and finally with acetone to afford **TC1** (52.1 mg), **TC2** (13.7 mg), **TC3** (42.9 mg), **TC4** (382.1 mg), **TC5** (733.8 mg) and **TC6** (106.0 mg), respectively. Algicidal activity of each fraction was measured, According to the algicidal assay results, fraction **TC5** (100% MeOH) was separated by silica gel (100-200 μm, 37.60 g) column chromatography with gradient elution of hexane : EtOAc (9:1; 5:1; 2:1; 1:1; 0:1) and EtOAc : MeOH (1:1) to afford **B1** (2.6 mg), **B2** (178.8 mg), **B3** (47.4 mg), **B4** (51.1 mg), **B5** (51.8 mg) and **B6** (443.8 mg), respectively. Fraction **B5** was separated by silica gel TLC (0.50 mm thickness; 10 × 20 cm; solvent, hexane-EtOAc (1:1)), affording two fractions **B5-1** and **B5-2**, respectively. Each of these fractions was further separated by preparative HPLC (column, Capcell Pak C18, 10-mm i.d. × 250 mm; solvent, 80% MeOH; flow rate, 2 mL/min) to obtain an active fraction **f5** (4.4 mg).

2.6 Structural determination of algicidal compounds f5

Fraction **f5**: a colorless oil, $[\alpha]_D^{20}$ –0.67° (*c* 0.1, MeOH). ESIMS *m/z* 321 $[M+Na]^+$, EIMS (bis-TMS derivative) *m/z* 442 (M^+), 427, 357, 329, 227, 199. HR-EIMS (bis-TMS derivative) calcd for $C_{24}H_{50}O_3Si_2$: 442.3298, found 442.3299. ^{1}H NMR (500 MHz) δ 0.879 and 0.881 (3H, t × 2, *J*=7.0 Hz), 1.22-1.41 (14H, m), 1.42-1.45 (1H, m), 1.45-1.59 (1H, m), 1.64 (2H, m), 2.57 (2H, m), 2.35 (2H, t, *J*=7.5 Hz), 3.45-3.65 (1H, br), 4.03 (1H, m), 5.443 and 5.445 (1H, dd × 2, *J*=7.1, 1.0 Hz), 5.62 (1H, m). ^{13}C NMR (125 MHz) δ 14.05, 14.10, 14.10, 14.11, 22.60, 22.66, 24.58, 24.66, 25.39, 25.43, 25.49, 28.52, 28.75, 28.79, 28.96, 29.11, 29.14, 29.18, 29.26, 29.3, 29.55, 31.36, 31.82, 31.84, 31.87, 32.00, 32.18, 33.54, 37.25, 33.59, 33.59, 37.32, 73.20, 73.25, 73.26, 73.30, 131.97, 132.20, 132.28, 132.31, 132.93, 132.94, 132.97, 133.16, 177.09, 177.12, 177.16, 177.18. IR (KBr) ν_{max} 980, 1260, 1445, 1710, 2870, 2920 cm^{-1}.

*p-Bromophenacyl esterification of **f5***

A mixture of **f5** (1 mg) and K_2CO_3 (spray dried, 8 mg) in dry acetone (1 mL) was stirred at rt for 15 min. A 0.1 M acetone solution of *p*-bromophenacyl bromide (0.090 mL, 9.0 mmol) was added and the whole was stirred for 5 h. The mixture was diluted with CH_2Cl_2 (1 mL) and filtered. The filtrate was concentrated and the residue was purified by silica gel TLC (0.25 mm thickness; 10 × 20 cm; solvent, hexane-EtOAc (2:1)) to afford fraction **f5a** (Rf 0.53, 0.2 mg) and **f5b** (Rf 0.48, 0.2 mg).

Fraction **f5a**. ^{1}H NMR (500 MHz) δ 0.880 and 0.884 (3H, t × 2, J=6.8 Hz), 1.20-1.65 (21H, m), 1.69 (1H, m), 2.00-2.05 (2H, m), 2.48 (2H, deformed-t, J=7.5 Hz), 3.67 (1H, s), 4.03 (1H, m), 5.28 (2H, s), 5.41-5.48 (1H, m), 5.59-5.67 (1H, m), 7.64 (2H, d, J=8.6 Hz), 7.78 (2H, d, J=8.6 Hz).

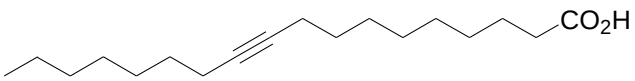
Fraction **f5b**. ^{1}H NMR (500 MHz) δ 0.877 and 0.881 (3H, t × 2, J=7.1 Hz), 1.20-1.65 (21H, m), 1.66-1.75 (2H, m), 1.99-2.07 (2H, m), 2.479 and 2.483 (2H, t×2, J=7.5 Hz), 4.03 (1H, m), 5.29 (2H, s), 5.41-5.48 (1H, m), 5.59-5.67 (1H, m), 7.64 (2H, d, J=8.6 Hz), 7.78 (2H, d, J=8.6 Hz).

2.7 Chemicals

(*E*)-Octadec-9-enoic acid (elaidic acid) was prepared by nitrous acid mediated isomerization of oleic acid [26]. (*R*)-(+)-Ricinoleic acid was purchased from Tokyo Kasei, Tokyo.

2.8 Synthesis

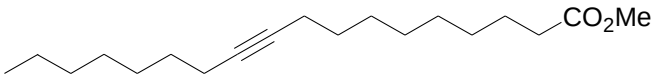
*2.8.1 Octadec-10-ynoic acid (**5**)*



To a cooled (−78 °C) solution of 10-undecynoic acid (1.00 g, 5.49 mmol) in

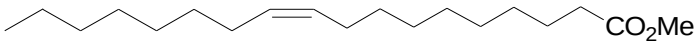
anhydrous THF (40 mL) and HMPA (10 mL), was added dropwise via a syringe a 2.5 M cyclohexane solution of BuLi (5.27 mL, 13.2 mmol) over a period of 30 min. The mixture was wormed up to 0 °C and kept at this temperature for 2 h. The mixture was cooled again to −78 °C and 1-bromoheptane (0.95 mL, 6.04 mmol) was injected. The whole was stirred at rt for 18 h before being quenched with 10% NH_4Cl and 1M HCl solutions. The THF was removed *in vacuo*, and the residue was acidified to pH 1 with 1 M HCl and extracted twice with EtOAc. The organic layer washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified with flash chromatography on silica gel eluted with hexane-EtOAc (4:1) to give **5** (0.421 g, 1.50 mmol, 27 %) as white crystals, mp 43 °C, with 50% recovery of 10-undecynoic acid. ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=6.7 Hz), 1.25-1.40 (16H, m), 1.40-1.54 (4H, m), 1.57-1.70 (2H, m), 2.14 (4H, t, *J*=7.0 Hz), 2.35 (2H, t, *J*=7.62 Hz), 9.96-10.42 (1H, br.). ^{13}C NMR (100 MHz) δ 14.00, 18.65, 22.55, 24.56, 28.69, 28.74, 28.76, 28.87, 28.93, 29.02, 29.07, 29.08, 29.70, 31.70, 33.97, 80.12, 80.29, 180.28. DART-MS *m/z* (rel intensity) 282 (26), 281 (100), 215 (17), 180 (16). HR-DART-MS $[M+H]^+$ *m/z* 281.24840 (calcd for $C_{18}H_{33}O_2$: 281.24806).

*2.8.2 Methyl octadec-10-ynoate (**6**)*



To a solution of **5** (123 mg, 0.440 mmol) in a mixture of CH_2Cl_2 (6 mL) and MeOH (6 mL), was added 2M ethereal solution of $TMSCH_2N_2$ (0.9 mL, 1.8 mmol) and the mixture was stirred at rt until TLC revealed the disappearance of the acid. The reaction was then quenched with one drop AcOH and the solvent was removed *in vacuo* to afford **6** (129 mg, 0.439 mmol, 100%) as a colorless oil. This was used for the next step without further purifications. ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=7.0 Hz), 1.23-1.41 (16H, m), 1.41-1.54 (4H, m), 1.58-1.70 (2H, m), 2.14 (4H, t, *J*=7.0), 2.30 (2H, t, *J*=7.6 Hz), 3.67 (3H, s).

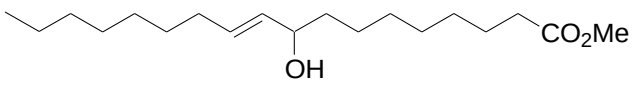
2.8.3 (Z)-Methyl octadec-10-enoate (7)



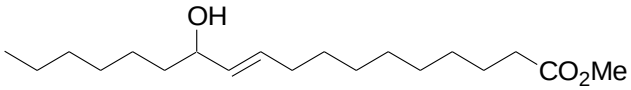
The acetylenic fatty acid ester **6** (107 mg, 0.363 mmol) was hydrogenated over 5% $Pd/CaCO_3$ poisoned with Pb (67.8 mg) in EtOAc (10 mL) under H_2 (balloon pressure) for 35 min at rt. The mixture was filtered through a short column on silica gel and concentrated *in vacuo* to give olefin **7** (125 mg, 0.420 mmol, 96%) as a pale yellow oil. This was used for the next step without further purifications. ^{1}H NMR (500 MHz) δ 0.88 (3H, t, *J*=7.0 Hz), 1.22-1.40 (20H, m), 1.55-1.68 (2H, m), 1.96-2.07 (4H, m), 2.30 (2H, t, *J*=7.6 Hz), 3.66 (3H, s), 5.32-5.37 (2H, m). ^{13}C NMR (125 MHz) δ 14.10, 22.67, 24.95, 27.18, 27.20, 29.13, 29.22 (×2), 29.27, 29.33, 29.72, 29.76, 31.86, 34.10, 51.42, 129.80. 129.94, 174.33. DART-MS *m/z* (rel intensity) 298 (20), 297 (100). HR-DART-MS $[M+H]^+$ *m/z* 297.28021 (calcd for $C_{19}H_{37}O_2$: 297.27936).

*2.8.4 (E)-Methyl 9-hydroxyoctadec-10-enoate (**8**) and (E)-Methyl 12-hydroxyoctadec-10E-enoate (**9**)*

A mixture of **7** (87.3 mg, 0.294 mmol), SeO_2 (28.0 mg, 0.252 mmol), and *t*-BuOOH (5 M in decane, 0.213 mL, 1.18 mmol) in dry CH_2Cl_2 (2 mL) was stirred at rt for 50 h. The reaction was then quenched by addition of 10% $Na_2S_2O_3$ solution (5 mL) and extracted three times with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, and concentrated *in vacuo.* The residue was purified by a column chromatography on silica gel eluted with hexane-EtOAc (4:1~3:1) to give a mixture of **8** and **9** (67.6 mg, 0.216 mmol, 74%). Further elution of the column with EtOAc gave methyl (*E*)-9,12-dihydroxyoctadec-10-enoate (13.9 mg, 0.0424 mmol, 14%) as a mixture of diastereomers. The mixture of **8** and **9** was separated by MPLC on silica gel eluted with hexane-EtOAc (9:1).

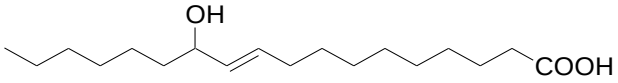


Compound **8**; a colorless oil. ^{1}H NMR (500 MHz) δ 0.88 (3H, t, *J*=7.0 Hz), 1.15-1.38 (18H, m), 1.45-1.75 (5H, m), 1.97-2.07 (2H, m), 2.30 (2H, t, *J*=7.6 Hz), 3.67 (3H, s), 4.02 (1H, q, *J*=6.5 H), 5.38-5.48 (1H, m), 5.63 (1H, dt, *J*=15.2, 6.5 Hz). ^{13}C NMR (75 MHz) δ 14.11, 22.64, 24.89, 25.39, 29.04, 29.09, 29.13, 29.16, 29.16, 29.31, 31.82, 32.16, 34.06, 37.23, 51.44, 73.18, 132.26, 132.92, 174.30. DART-MS *m/z* (rel intensity) 311 (20), 296 (32), 295 (100), 177 (11). HR-DART-MS $[M+H-H_2O]^+$ *m/z* 295.26377 (calcd for $C_{19}H_{35}O_2$: 295.26371).



Compound **9**; a colorless oil. ^{1}H NMR (500 MHz) δ 0.88 (t, 3H, *J*=7.0 Hz), 1.23-1.40 (18H, m), 1.42-1.67 (5H, m), 2.01 (2H, q, *J*=6.7 Hz), 2.30 (2H, t, *J*=7.0 Hz), 3.67 (3H, s), 4.03 (1H, q, *J*=6.7 Hz), 5.44 (1H, ddt, *J*=15.3, 7.0, 1.2 Hz), 5.62 (1H, dt, *J*=15.3, 7.0 Hz). ^{13}C NMR (75 MHz) δ 14.11, 22.58, 24.89, 25.43, 29.00, 29.07, 29.11, 29.15, 29.20, 29.20, 31.80, 31.13, 34.07, 37.32, 51.43, 73.20, 132.07, 133.05, 174.32. DART-MS *m/z* (rel intensity) 312 (12), 311 (26), 295 (100), 284 (20), 282 (23), 256 (20). HR-DART-MS $[M+H-H_2O]^+$ *m/z* 295.26214 (calcd for $C_{19}H_{35}O_2$: 295.26371).

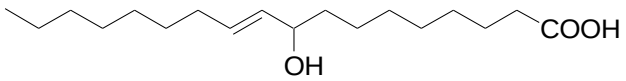
*2.8.5 (E)-12-Hydroxyoctadec-10-enoic acid (**4**)*



A solution of **9** (21.7 mg, 0.0694 mmol) in a mixture of 10% NaOH (1 mL) and MeOH (4 mL) was heated at reflux for 7.5 h. After cooling, the MeOH was removed *in vacuo,* the aqueous residue was diluted with water, acidified with 3 M HCl, extracted twice with ether, washed with brine and concentrated. The crude product was purified by silica gel TLC developed with hexane-EtOAc (1:1) to give **4** (18.4 mg, 0.0616 mmol, 89%) as white crystals, mp 47.5~49.5 °C. ^{1}H NMR (500 MHz) δ 0.88 (3H, t, *J*=7.0 Hz), 1.22-1.41 (18H, m), 1.42-1.57 (2H, m), 1.57-1.66 (2H, m), 2.02 (2H, q, *J*=7.0 Hz), 2.34

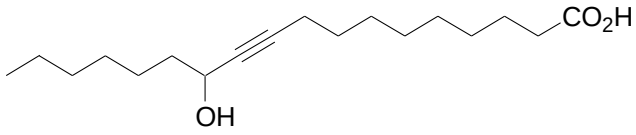
(2H, t, *J*=7.3 Hz), 4.04 (1H, q, *J*=6.7 Hz), 4.50-6.50 (2H, br), 5.42-5.47 (1H, m), 5.62 (1H, dt, *J*=15.2, 6.6 Hz). ^{13}C NMR (125 MHz) δ 14.07, 22.58, 24.58, 25.42, 28.83 (×2), 29.01, 29.04, 29.06, 29.20, 31.80, 32.10, 33.95, 37.26, 73.30, 132.19, 132.94, 179.22. DART-MS *m/z* (rel intensity) 298 (38), 297 (35), 282 (22), 281 (100), 187 (22). HR-DART-MS *m/z* $[M+H-H_2O]^+$ 281.24701 (calcd for $C_{18}H_{33}O_2$: 281.24806).

*2.8.6 (E)-9-Hydroxyoctadec-10-enoic acid (**1**)*



The title compound was obtained from **8** in 85% yield in a similar procedure used for the synthesis of **4**. Mp 49~50.5 °C. ^{1}H NMR (500 MHz) δ 0.88 (3H, t, *J*=7.1 Hz), 1.22-1.41 (18H, m), 1.12-1.50 (1H, m), 1.51-1.57 (1H, m), 1.58-1.66 (2H, m), 2.02 (2H, q, *J*=7.1 Hz), 2.34 (2H, t, *J*=7.4 Hz), 4.03 (1H, q, *J*=6.7 Hz), 4.67-5.60 (2H, br), 5.41-5.48 (1H, m), 5.62 (1H, dt, *J*=15.4, 6.7 Hz). ^{13}C NMR (125 MHz) δ 14.08, 22.64, 24.65, 25.37, 28.96, 29.10, 29.12, 29.15, 29.17, 29.29, 31.82, 32.16, 33.95, 37.21, 73.23, 132.34, 132.85, 179.22. DART-MS *m/z* (rel intensity) 298 (31), 297 (22), 282 (42), 281 (100). DART-MS *m/z* 298, 287, 282, 281, 263. HR-DART-MS *m/z* $[M+H-H_2O]^+$ 281.24717 (calcd for $C_{18}H_{33}O_2$: 281.24806).

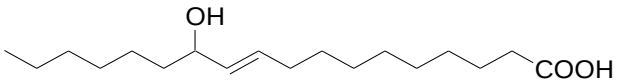
*2.8.7 12-Hydroxyoctadec-10-ynoic acid (**10**)*



To a cooled (−78 °C) and stirred solution of 10-undecynoic acid (424 mg, 2.33 mmol) in dry THF (24 mL), was added dropwise a 2.5 M solution of BuLi in hexane (2.05 mL, 5.12 mmol). After 10 min at that temperature, the cooling bath was removed and the whole was stirred at rt for 45 min. The mixture was cooled again to −78 °C and heptanal

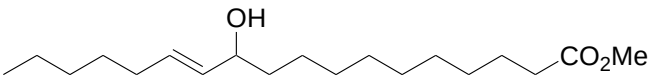
(293 mg, 2.56 mmol) dissolved in THF (2 mL) was injected. The cooling bath was removed and the mixture was stirred at rt for 1.5 h. The reaction was then quenched with 2 M HCl solution and extracted twice with ether. The ethereal extracts were combined, washed with brine, dried over Na_2SO_4, and concentrated. The crude product was purified by silica gel column chromatography eluted with hexane-EtOAc (2:1) to give **10** (332 mg, 1.12 mmol, 48%) as white crystals, mp 35~36.5 °C. ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=6.5 Hz), 1.22-1.55 (18H, m), 1.58-1.72 (4H, m), 2.20 (2H, dt, *J*=6.8, 1.8 Hz), 2.34 (2H, t, *J*=7.6 Hz), 4.36 (1H, dt, *J*=6.5, 1.8 Hz), 5.50-6.52 (2H, br). ^{13}C NMR (125 MHz) δ 14.02, 18.57, 22.53, 24.53, 25.11, 28.50, 28.53, 28.69, 28.77, 28.89, 28.92, 31.72, 33.97, 38.05, 62.68, 81.21, 85.36, 179.47.

*2.8.8 (E)-12-Hydroxyoctadec-10-enoic acid (**4**) from compound **10***



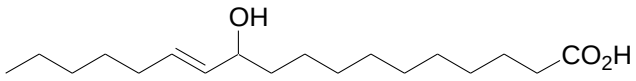
Clean cut Li (42 mg, 6.0 mmol) was added in small portions to liquid NH_3 (ca. 3 mL) at −78 °C. After 10 min, a solution of **10** (35.4 mg, 0.120 mmol) in dry THF/*t*-BuOH (3:1, 1.5 mL) was added as drops to the deep blue solution of Li metal in liquid NH_3 and the mixture was stirred at this temperature for 2 h. The reaction was quenched by addition of solid NH_4Cl (0.5 g) and the cooling bath was removed. After the NH_3 was evaporated, the residue was acidified with 3 M HCl solution, extracted twice with ether, washed with brine, dried over Na_2SO_4, and evaporated. The crude product was purified by reversed-phase HPLC (Capcell Pak C18, 10 mm x 250 mm) eluted with 85% CH_3CN to give **4** (13.7 mg, 0.0459 mmol, 38%) as white crystals, mp 49~51 °C.

*2.8.9 (E)-Methyl 11-hydroxyoctadec-12-enoate (**13**)*



A 1.0 M toluene solution of DIBAL (1.36 mL, 1.36 mmol) was injected via a syringe to a stirred solution of 1-heptyne (153 mg, 1.59 mmol) in dry hexane (4 mL) at rt under Ar atmosphere. After the mixture had been stirred at 60 °C for 5 h, it was cooled to −78 °C (dry ice-acetone bath) and a solution of **11** (195 mg, 0.909 mmol) in toluene (2 mL) was added as drops. After 20 min, the cooling bath was replaced with an ice-salt bath and the mixture was stirred for 1 h. The reaction was then quenched by addition of a saturated solution of Rochelle's salt (0.2 mL), stirred overnight, dried over $MgSO_4$, and filtered through a pad of Celite, washed well with EtOAc, and concentrated. The crude product was purified by a column chromatography on silica gel eluted with hexane-EtOAc (5:1) to give **13** (79.3 mg, 0.254 mmol, 28%) as a pale yellow oil with 77.0 mg (40%) recovery of aldehyde **11**. ^{1}H NMR (300 MHz) δ 0.89 (3H, deformed t, *J*=7.0 Hz), 1.20-1.55 (19H, m), 1.55-1.68 (4H, m), 2.02 (2H, q, *J*=7.0 Hz), 2.30 (2H, t, *J*=7.6 Hz), 3.67 (3H, s), 4.02 (1H, q, *J*=6.5 Hz), 5.44 (1H, dd, *J*=15.4, 7.0 Hz), 5.63 (1H, dt, *J*=15.4, 6.8 Hz). ^{13}C NMR (75 MHz) δ 14.02, 22.47, 24.91, 25.44, 28.83, 29.09, 29.18, 29.32, 29.47 (×2), 31.32, 32.11, 34.07, 37.27, 51.42, 73.18, 132.17, 132.97, 174.33. DART-MS *m/z* (rel intensity) 312 (15), 296 (22), 295 (100), 293 (38), 282 (21). HR-DART-MS *m/z* $[M+H-H_2O]^+$ 295.26347 (calcd for $C_{19}H_{35}O_2$: 295.26371).

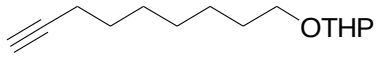
*2.8.10 (E)-11-hydroxyoctadec-12-enoic acid (**3**)*



The title compound was obtained by alkaline hydrolysis of **13** at rt in 78% yield in a similar procedure used for the synthesis of **4**. White crystals, mp 49 °C. ^{1}H NMR (500 MHz) δ 0.89 (3H, t, *J*=7.1 Hz), 1.23-1.41 (19H, m), 1.41-1.57 (2H, m), 1.57-1.668 (2H, m), 2.03 (2H, q, *J*=7.1 Hz), 2.34 (2H, t, *J*=7.3 Hz), 4.04 (1H, q, *J*=6.8 Hz), 5.44 (1H, dd, *J*=15.4, 7.1 Hz), 5.63 (1H, dt, *J*=15.4, 6.7 Hz), 9.75-9.77 (1H, br.). ^{13}C NMR (125 MHz) δ 14.03, 22.49, 24.65, 25.43, 28.85, 29.00, 29.16, 29.28, 29.46 (×2), 31.34, 32.13, 33.93, 37.26, 73.27, 132.29, 132.89, 179.23. DART-MS *m/z* (rel intensity) 298 (36), 297 (46), 282 (76), 281 (100). HR-DART-MS *m/z* $[M+H-H_2O]^+$ 281.24863 (calcd for $C_{18}H_{33}O_2$:

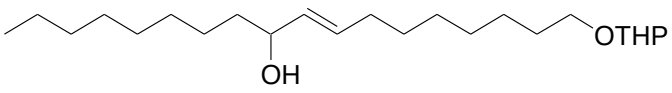
281.24806).

*2.8.11 9-(Tetrahydropyran-2-yl)oxy-1-nonyne (**15**)*



A solution of **14** (1.10 g, 7.83 mmol), dihydro-2*H*-pyran (1.24 g, 14.7 mmol), and *p*-TsOH·H_2O (0.05 g) in dry CH_2Cl_2 (60 mL) was stirred at rt for 18 h. The mixture was then washed with 5% $NaHCO_3$ solution (30 mL) and the aqueous layer was extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated. The oily residue was chromatographed on silica gel eluted with hexane-Et_2O (19:1) to give **15** (1.52 g, 6.76 mmol, 69 %) as a colorless oil. ^{1}H NMR (300 MHz) δ 1.21-1.46 (6H, m), 1.46-1.67 (8H, m), 1.67-1.90 (2H, m), 1.94 (1H, t, *J*=2.6 Hz), 2.18 (2H, dt, *J*=6.9, 2.6 Hz), 3.38 (1H, dt, *J*=9.5, 6.7 Hz), 3.45-3.56 (1H, m), 3.73 (1H, dt, *J*=9.5, 7.0 Hz), 3.81 3.94 (1H, m), 4.58 (1H, dd, *J*=4.1, 2.8 Hz). ^{13}C NMR (75 MHz) δ 18.33, 19.66, 25.44, 26.07, 28.36, 28.63, 28.89, 29.64, 30.72, 62.30, 67.55, 68.06, 84.66, 98.80. DART-MS *m/z* (rel intensity) 225 (9), 169 (12), 102 (19), 85 (100). HR-DART-MS *m/z* $[M+H]^+$ 225.18602 (calcd for $C_{14}H_{25}O_2$: 225.18546).

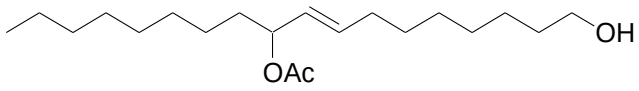
*2.8.12 (E)-18-(Tetrahydropyran-2-yl)oxyoctadec-10-en-9-ol (**16**)*



To a solution of **15** (0.758 g, 3.38 mmol) in dry hexane (5 mL), a 1 M toluene solution of DIBAL (3.71 mL, 3.71 mmol) was added dropwise at rt under Ar atmosphere, and the mixture was stirred at 60 °C for 2h. The mixture was then cooled to –78 °C and nonanal (0.577 mg, 4.06 mmol) dissolved in toluene (4 mL) was added dropwise. After 2 h at –60 °C, the reaction mixture was warmed up to rt, quenched with water, and acidified with 1 M HCl. The whole was extracted twice with EtOAc, washed with brine, dried over Na_2SO_4, and concentrated. The oily residue was chromatographed on silica gel eluted

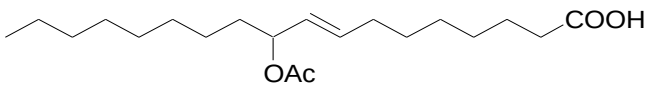
with hexane-EtOAc (7:1 to 2:1) gave crude **16** (0.548 g, 1.49 mmol, 44%) as a mixture of diastereomers. DART-MS *m/z* (rel intensity) 367 (5), 352 (33), 351 (100), 333 (37), 283 (37), 281 (33), 85 (94).

*2.8.13 (E)-10-(Acetoxy)octadec-8-en-1-ol (**18**)*



A solution of the crude **16** (0.548 mg, 1.49 mmol) in Ac_2O (0.5 mL) and pyridine (1 mL) was stirred at rt for 40 h. The reaction was quenched with water, acidified with 2 M HCl, and extracted twice with ether. The ethereal extracts were combined, washed with 5% $NaHCO_3$, dried over Na_2SO_4, and concentrated. The crude product **17** was dissolved EtOH (12 mL) and a catalytic amount of *p*-TsOH·H_2O (0.05 g) was added. After the mixture had been stirred at rt for 25 min, it was concentrated and purified by a column chromatography on silica gel eluted with hexane-EtOAc (3:1) to give **18** (160 mg, 0.491 mmol, 33%) as a pale yellow oil. ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=6.7 Hz), 1.19-1.44 (20H, m), 1.46-1.68 (4H, m), 1.61 (1H, br. s), 1.96-2.09 (2H, m), 2.04 (3H, s), 3.64 (2H, t, *J*=6.7 Hz), 5.17 (1H, q, *J*=7.0 Hz), 5.36 (1H, m), 5.68 (1H, dt, *J*=15.5, 6.8 Hz). ^{13}C NMR (75 MHz) δ 14.07, 21.38, 22.62, 25.15, 25.60, 28.78, 28.95, 29.14, 29.18, 29.32, 29.44, 31.81, 32.11, 32.66, 34.46, 62.93, 75.12, 128.33, 134.31, 170.50. DART-MS *m/z* (rel intensity) 326 (22), 267 (100), 429 (61), 177 (56). HR-DART-MS $[M+H-AcOH]^+$ *m/z* 267.26766 (calcd for $C_{18}H_{35}O$: 267.26879).

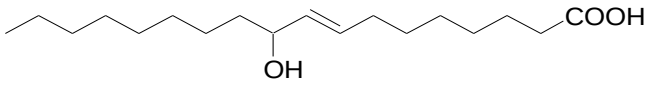
*2.8.14 (E)-10-(Acetoxy)octadec-8-enoic acid (**19**)*



A mixture of **18** (66.5 mg, 0.204 mmol) and PDC (268 mg, 0.713 mmol) dry DMF (2 mL) was stirred at rt for 17 h. The mixture was poured into water, extracted twice with ether, washed with brine, dried over Na_2SO_4, and concentrated. The crude product was

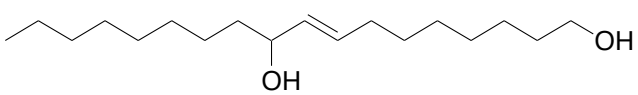
purified by a column chromatography on silica gel eluted with hexane-EtOAc (3:1) to give **19** (31.6 mg, 0.0928 mmol, 45%) as a pale oil. ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=7.0 Hz), 1.16-1.41 (18H, m), 1.48-1.69 (4H, m), 1.95-2.11 (2H, m), 2.04 (3H, s), 2.34 (2H, t, *J*=7.3 Hz), 5.17 (1H, q, *J*=7.0 Hz), 5.36 (1H, m), 5.67 (1H, dt, *J*=15.7, 7.0 Hz), 9.75-9.77 (1H, br.). ^{13}C NMR (75 MHz) δ 14.09, 21.42, 22.63, 24.56, 25.18, 28.65, 28.80, 29.07, 29.21, 29.33, 29.46, 31.82, 32.07, 33.99, 34.48, 75.14, 128.45, 134.16, 170.58, 179.93. DART-MS *m/z* (rel intensity) 281 $[M+H-AcOH]^+$ (42), 89 (100), 61 (38). HR-DART-MS $[M+H-AcOH]^+$ *m/z* 281.24687 (calcd for $C_{18}H_{33}O_2$: 281.24805).

*2.8.15 (E)-10-Hydroxyoctadec-8-enoic acid (**2**)*



The title compound was obtained by alkaline hydrolysis of **19** at rt in 36% yield in a similar procedure used for the synthesis of **1**. Pale yellow crystals, mp 49 °C. ^{1}H NMR (500 MHz) δ 0.88 (3H, t, *J*=6.9 Hz), 1.18-1.58 (19H, m), 1.59-1.72 (3H, m), 2.04 (2H, t, *J*=7.3 Hz), 2.34 (2H, t, *J*=7.3 Hz), 3.45-3.73 (2H, br.), 4.04 (1H, q, *J*=6.7 Hz), 5.41-5.48 (1H, m), 5.61 (1H, dt, *J*=15.4, 6.9 Hz). ^{13}C NMR (125 MHz) δ 14.09, 22.65, 24.57, 25.47, 28.26, 28.57, 28.76, 28.82, 29.25, 29.54, 31.86, 32.01, 33.88, 37.28, 73.27, 131.97, 133.12, 179.08. DART-MS *m/z* (rel intensity) 299 (31), 298 (34), 282 (38), 281 (100), 279 (58). HR-DART-MS $[M+H-H_2O]^+$ *m/z* 281.24670 (calcd for $C_{18}H_{34}O_3$: 281.24805).

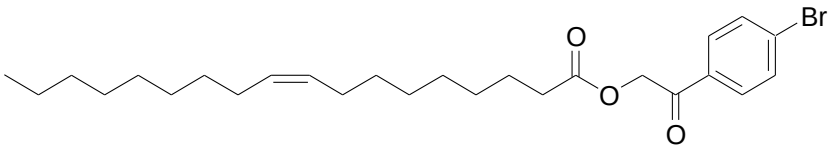
*2.8.16 1,10-Dihydroxyoctadec-8-ene (**20**)*



A solution of **16** (167.7 mg, 0.455 mmol) in MeOH (3 mL) containing a catalytic amount of PPTS was allowed to stand at rt for 20 h. After the MeOH had been removed *in vacuo*, the residue was chromatographed on silica gel eluted with hexane-EtOAc (2:1) to give **20** (59.5 mg, 0.209 mmol, 46%) as a colorless oil. ^{1}H NMR (300 MHz) δ 0.88

(3H, t, *J*=7.0 Hz), 1.22-1.62 (25H, m), 1.64-1.71 (1H, m), 2.02 (1H, q, *J*=7.0 Hz), 2.21 (1H, dt, *J*=7.0, 1.8 Hz), 3.64 (2H, t, *J*=6.7 Hz), 4.03 (1H, q, *J*=6.8 Hz), 5.44 (1H, m), 5.62 (1H, dt, *J*=15.2, 6.7 Hz). DART-MS *m/z* (rel intensity) 284 (18), 283 (33), 281 (25), 267 (71), 265 (100), 249 (38), 247 (31). HR-DART-MS $[M+H-H_2O]^+$ *m/z* 267.26888 (calcd for $C_{18}H_{35}O$: 267.26879).

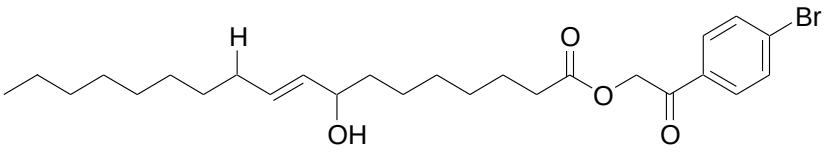
*2.8.17 p-Bromophenacyl ester of oleic acid (**25**)*



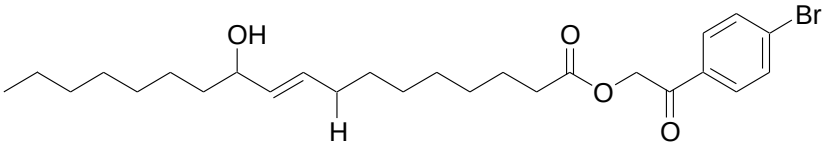
A mixture of oleic acid (2.82 g, 10.0 mmol) and K_2CO_3 (2.28 g, 16.0 mmol) in dry acetone (25 mL) was stirred at rt for 30 min. *p*-Bromophenacy bromide (3.06 g, 11.0 mmol) was then added and the whole was stirred overnight. The reaction mixture was filtrated and the filtrate was evaporated. The residue was then extracted with diethyl ether, washed with 5% $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and concentrated. The crystalline product was recrystallized from methanol, washed with hexane to remove unreacted oleic acid, giving **25** as a pale yellow powder. ^{1}H NMR (400 MHz) δ 0.88 (3H, t, *J*=7.3 Hz), 1.17-1.45 (20H, m), 1.70 (2 H, m), 1.96-2.08 (4H, m), 2.48 (2H, t, *J*=7.3 Hz), 5.28 (2H, s), 5.32-5.38 (2H, m), 7.64 (2H, d, *J*=8.8 Hz), 7.78 (2H, d, *J*=8.8 Hz). ^{13}C NMR (100 MHz) δ 14.09, 22.67, 24.85, 27.14, 27.18, 29.03, 29.06, 29.14, 29.29 (×2), 29.49, 29.67, 29.74, 31.87, 33.84, 65.61, 129.05, 129.21 (×2), 129.73, 129.95, 132.18 (×2), 132.94, 173.15, 191.44.

*2.8.18 Selenium dioxide oxidation of oleate **25** (**26**, **27**, and **28**)*

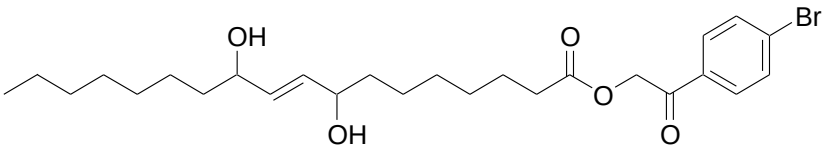
Compounds **26**, **27**, and **28** were synthesized in a similar procedure to that of **8** and **9** in 22%, 20%, and 13% yields, respectively.



Compound **26**: ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=6.9 Hz),1.15-1.18 (23H, m), 1.97-2.13 (2H, m), 2.49 (2H, t, *J*=7.7 Hz), 4.04 (1H, q, *J*=6.6 Hz), 5.29 (2H, s), 5.45 (1H, dt, *J*=15.4, 7.2 Hz), 5.58-5.69 (1H, m), 7.64 (2H, *J*=8.8 Hz), 7.78 (2H, d, *J*=8.8 Hz).

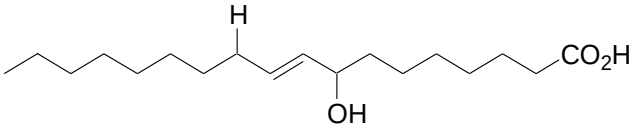


Compound **27**: ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=6.6 Hz), 1.18-1.79 (23H, m), 2.02 (2H, q, *J*=6.8 Hz), 2.48 (2H, t, *J*=7.7 Hz), 4.04 (1H, q, *J*=6.7 Hz), 5.29 (2H, s), 5.45 (1H, *J*=15.4, 7.3 Hz), 5.57-5.68 (1H, m), 7.64 (2H, dt, *J*=8.8, 1.9 Hz), 7.78 (2H, dt, *J*=8.8, 1.9 Hz).



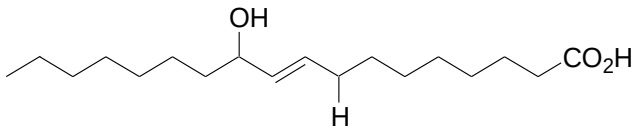
Compound **28**: ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=6.6 Hz), 1.15-1.85 (24H, m), 2.49 (2H, t, *J*=7.4 Hz), 4.09-4.16 (2H, m), 5.29 (2H, s), 5.66-5.74 (2H, m), 7.64 (2H, d, *J*=8.8 Hz), 7.78 (2H, d, *J*=8.8 Hz).

*2.8.19 (E)-8-Hydroxyoctadec-9-enoic acid (**21**)*



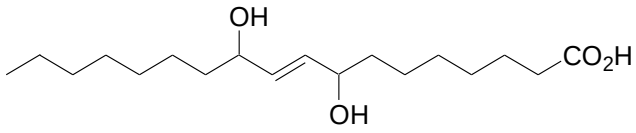
The title compound was obtained by an alkaline hydrolysis of **26** at 50 ºC in 38% yield in a similar procedure used for the synthesis of **4**. White crystals, mp 52-54 ºC (lit. mp 54-55 ºC) [27]. ^{1}H NMR (500 MHz) δ 0.88 (3 H, m, *J*=7.0 Hz) 1.18-1.40 (18H, m), 1.43-1.68 (4H, m), 2.02 (2 H, q, *J*=6.9 Hz), 2.33 (2H, t, *J*=7.5 Hz), 4.04 (1H, q, *J*=6.7 Hz), 5.44 (1H, dd, *J*=15.28, 7.21 Hz, 1 H), 5.63 (1 H, dt, *J*=15.2, 6.6 Hz), 6.20 (2H, br. s). ^{13}C NMR (125 MHz) δ 14.07, 22.63, 24.62, 25.45, 28.81, 28.93, 28.97, 29.02, 29.24, 29.48, 31.79, 32.07, 34.05, 37.23, 73.28, 132.11, 132.93, 179.56. EIMS (bis TMS derivative) *m/z* (rel intensity) 442 (M^+, 6), 427 (9), 274 (13), 242 (21), 241 (100).

*2.8.20 (E)-11-Hydroxyoctadec-9-enoic acid (**22**)*



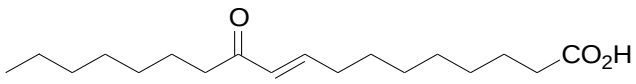
The tile compound was obtained from **27** in 34% yield in the same procedure used for the synthesis of **4**. Mp 43~46 ºC (lit. mp 43~44 ºC) [27]. ^{1}H NMR (500 MHz) δ 0.88 (3H, t, *J*=7.0 Hz), 1.18-1.42 (18H, m), 1.42-1.68 (4H, m), 2.02 (2H, q, *J*=7.1 Hz), 2.32 (2H, t, *J*=7.6 Hz), 4.04 (1H, q, *J*=6.9 Hz), 5.43 (1H, m), 5.46 (1H, dt, *J*=15.2, 6.9 Hz), 5.80-6.97 (2H, br. s). ^{13}C NMR (125 MHz) δ 14.09, 22.65, 24.64, 25.48, 28.83, 28.95, 28.99, 29.04, 29.26, 29.50, 31.81, 32.09, 34.07, 37.25, 73.30, 132.13, 132.95, 179.58. HR-EI MS $[M-H_2O+H]^+$ *m/z* 281.24672 (calcd for $C_{18}H_{33}O_2$: 281.24806). EIMS (bis TMS derivative) *m/z* (rel intensity) 442 (M^+, 5), 427 (9), 345 (14), 344 (40), 343 (100), 227 (7).

*2.8.21 8,11-Dihydroxyoctadec-9-enoic acid (**23**)*



The tile compound was obtained from **28** in 19% yield in the same procedure used for the synthesis of **4**. A colorless oil. ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=7.2 Hz), 1.20-1.43 (17H, m), 1.43-1.70 (6H, m), 2.35 (2H, t, *J*=7.5 Hz), 3.38-3.57 (1H, m), 3.65-3.83 (1H, m), 4.05-4.18 (2H, m), 5.63-5.73(2H, m).

*2.8.22 (E)-11-Oxooctadec-9-enoic acid (**24**)*



To a stirred solution of **22** (57.3 mg, 0.192 mmol) in CH_2Cl_2 (1 mL), Dess-Martin periodinane (169.7 mg, 0.400 mmol) was slowly added at rt. After stirring overnight, the reaction was quenched by adding 10% $Na_2S_2O_3$ solution. The CH_2Cl_2 layer was separated and the aqueous layer was extracted with CH_2Cl_2. Organic layers were combined and dried over anhydrous Na_2SO_4. Purification by a column chromatography on silica gel eluted with hexane-EtOAc (2:1) and then reversed phase HPLC (COSMOSIL 5C18-MS-II, 90% methanol) gave **24** (36.0 mg, 0.121 mmol, 63%) as white crystals, mp 52~54 ℃. ^{1}H NMR (300 MHz) δ 0.88 (3H, t, *J*=7.0 Hz), 1.18-1.72 (21H, m), 2.20 (2H, q, *J*=7.1 Hz), 2.36 (2H, t, *J*=7.0 Hz), 2.53 (2H, t, *J*=7.3 Hz), 6.09 (1H, d, *J*=15.8 Hz), 6.82 (1H, dt, *J*=15.8, 7.0 Hz). ^{13}C NMR (75 MHz) δ 14.04, 22.57, 24.31, 24.57, 27.98, 28.86, 28.91, 28.94, 29.06, 29.24, 31.65, 32.36, 33.97, 40.06, 130.30, 147.27, 172.16, 201.21. HR-DART-MS $[M+H]^+$ *m/z* 297.24295 (calcd for $C_{18}H_{33}O_3$: 297.24297).

3. Results and discussion

3.1 First isolation of algicidal compounds form T. jejuensis

A specimen of *T. jejuensis* was collected from Ishigaki Island of Okinawa Prefecture, Japan, in June 2016. *T. jejuensis* (240 g dry wt) was powdered using a blender, extracted twice with MeOH (2 L × 2) for 3 days, where upon the MeOH was evaporated under reduced pressure. The crude extract was partitioned between hexane and 80% aqueous MeOH. The algicidal activity was found in 80% MeOH fraction. After almost of the MeOH had been removed in vacuo, the aqueous layer was then partitioned between water and EtOAc. The EtOAc fraction was found to be active (Fig. 2-1).

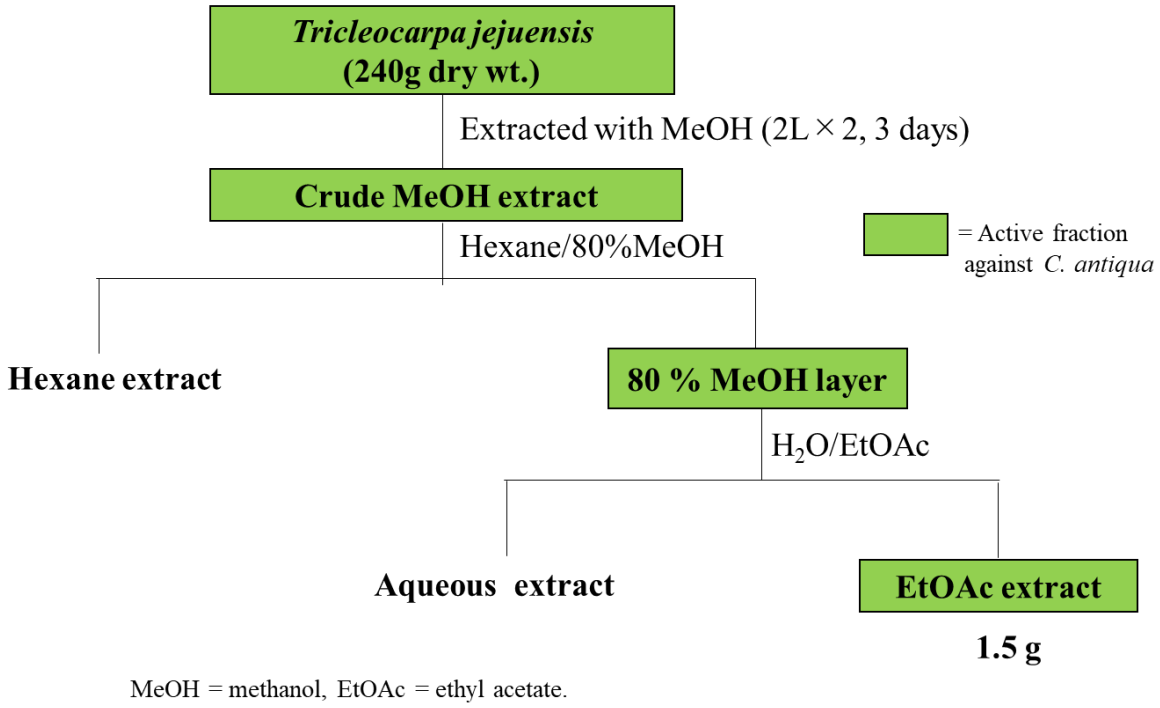
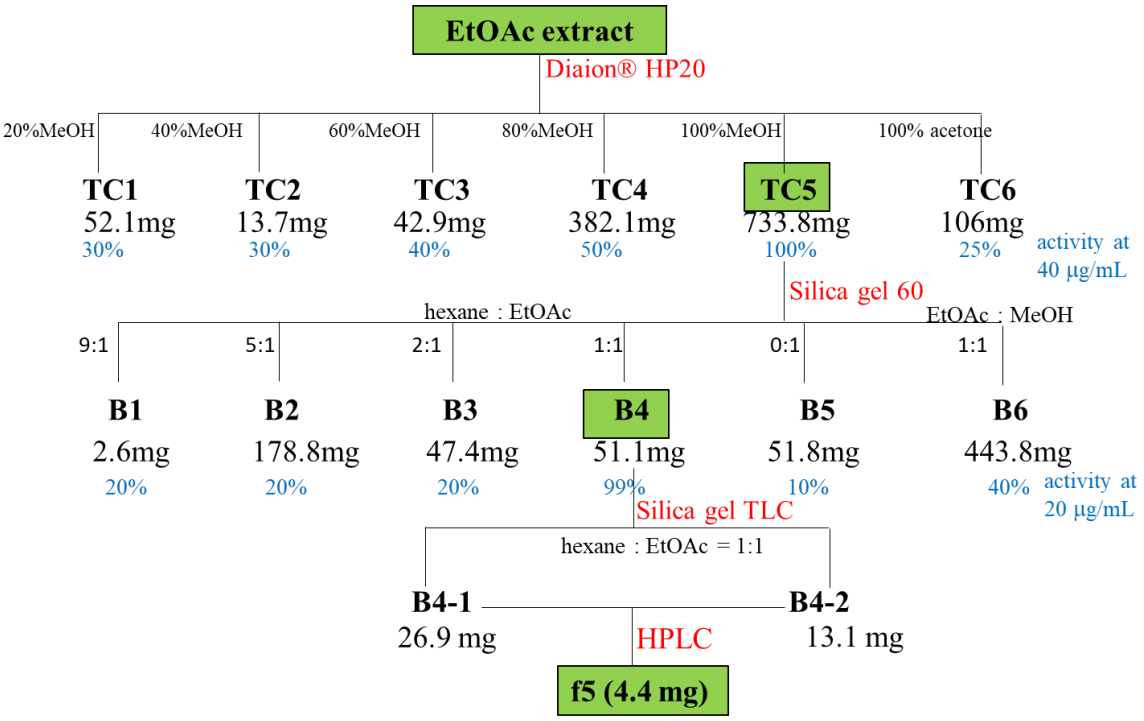


Fig. 2-1 Extraction and solvent-solvent partitioning

The EtOAc layer (1.5 g) was first separated using HP20 resin into 6 fractions (**TC1**~**TC6**) by successive elutions with 20%, 40%, 60%, 80%, and 100% MeOH, and finally with acetone (Fig.2), in which **TC5** eluted with 100% MeOH showed 100% mortality to *C. antiqua*. The fraction **TC5** was then separated by silica gel column chromatography into 6 subfractions (**B1**~**B6**) using hexane/EtOAc gradient, among which **B4** (51.1 mg) eluted with hexane-EtOAc (1:1) was active (99% mortality). The active fraction **B4** was further separated by TLC using hexane-EtOAc (1:1) as the solvent,

to give two active fractions, **B4-1** (26.9 mg) and **B4-2** (13.1 mg), both of which was finally separated by reversed-phase HPLC (Capcell Pak C18, 10 mm × 250 mm, 90% MeOH) to give an active fraction **f5** (4.4 mg) (Fig. 2-2).



HPLC conditions: column, Capcell Pack C18, 10 x 250 mm; solvent, 90% MeOH; follow rate, 3 mL/min

Fig. 2-2 Chromatographic separation of EtOAc extracts of *T. jejuensis*

3.2 Structural determination of algicidal compounds

The unseparated fraction **f5**, showed a single molecular ion peak at *m/z* 321 $[M+Na]^+$ by ESI-MS, indicating that the active compounds were isomeric to each other (Fig. 2-3). A molecular formula of $C_{18}H_{34}O_3$ was established by HR-EI-MS of the bistrimethylsilyl derivative of the mixture.

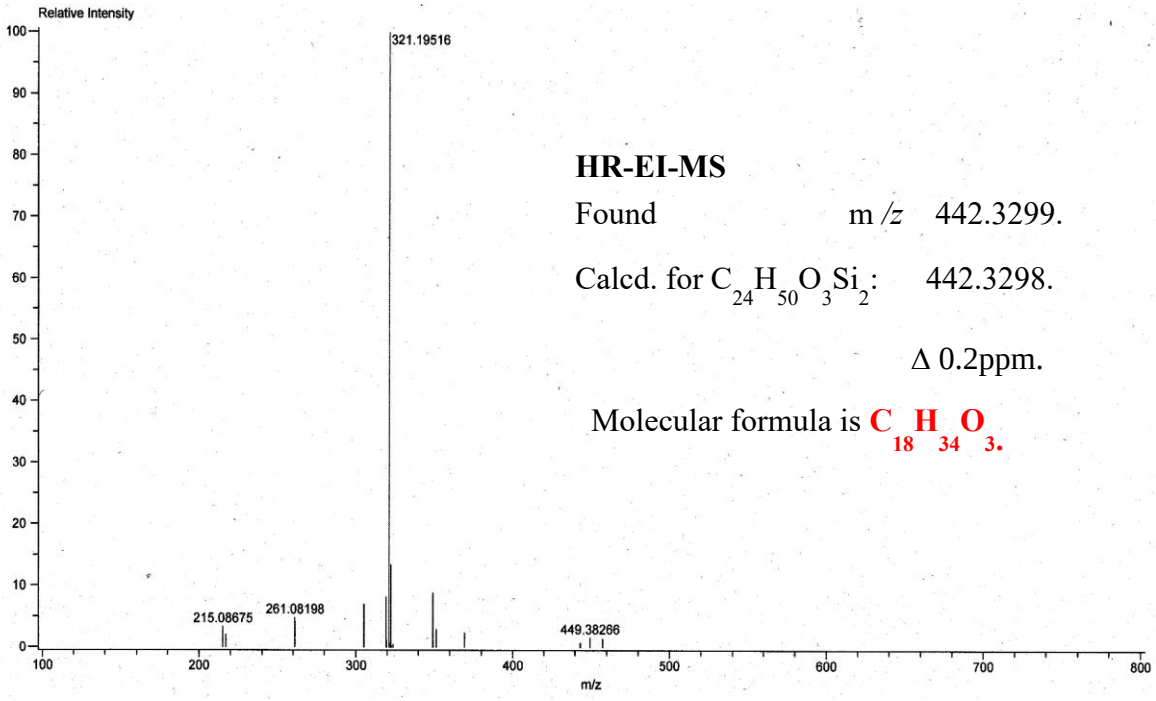


Fig. 2-3 ESI-MS Spectrum and HR-EIMS Data of **f5**

^{13}C NMR spectrum showed signals for carboxylic carbons at δ 177.09, 177.12, 177.16, and 177.18, olefinic carbons at δ 131.97, 132.20, 132.28, 132.31, 132.93, 132.94, 132.97, and 133.16, hydroxymethine carbons at δ 73.20, 73.25, 73.26, and 73.30, many methylene carbons, and overlapping methyl carbons in the sp3 carbon region. The ^{1}H NMR spectrum showed overlapping signals of multiplets at δ 5.59-5.66 (1H), two doublet of doublets at δ 5.443 (*J*=15.4, 7.1 Hz) and δ 5.445 (*J*=15.3, 7.2 Hz), and quartets at δ 4.036 (*J*=6.7 Hz) and 4.031 (*J*=6.7 Hz), indicating the presence of substructure –CH=CH-CH(OH)- having *E*-configuration (Fig. 2-4).

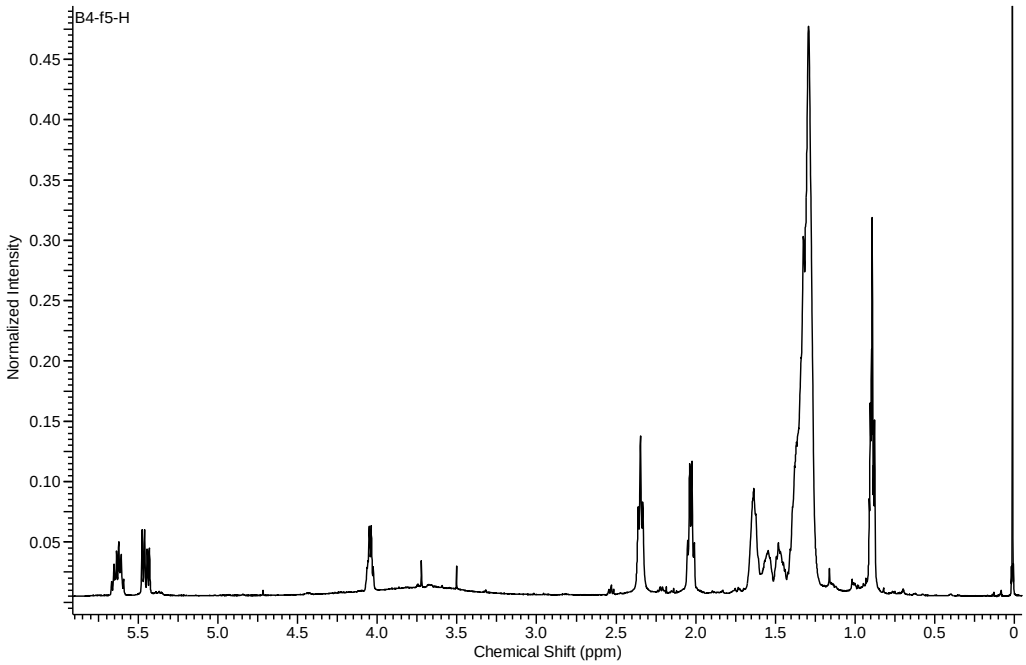


Fig. 2-4 ^{1}H NMR Spectrum of **f5** (500 MHz, $CDCl_3$)

At this stage, the active compounds were assumed to be four isomeric hydroxylated C18 *trans*-monounsaturated fatty acids. The positions of the double bonds and hydroxyl groups were determined by the EI-MS fragmentation pattern (Fig. 2-5). The EI-MS of the bistrimethylsilyl derivatives of the mixture showed four distinct fragment ion peaks at *m/z* 227, 329, 199 and 357, which corresponded to the fragment ions of $CH_3(CH_2)_6CH{=}CHCHOTMS$, $TMSOCHCH{=}CH(CH_2)_6COOTMS$, $CH_3(CH_2)_4CH{=}CHCHOTMS$, and $TMSOCHCH{=}CH(CH_2)_8COOTMS$, respectively, from cleavage at the allylic positions adjacent to the hydroxyl groups [28,29]. From these spectroscopic data, the active compounds were deduced to be (*E*)-9-hydroxyoctadec-10-enoic acid (**1**), (*E*)-10-hydroxyoctadec-8-enoic acid (**2**), (*E*)-11-hydroxyoctadec-12-enoic acid (**3**), and (*E*)-12-hydroxyoctadec-10-enoic acid (**4**) (Fig. 2-6).

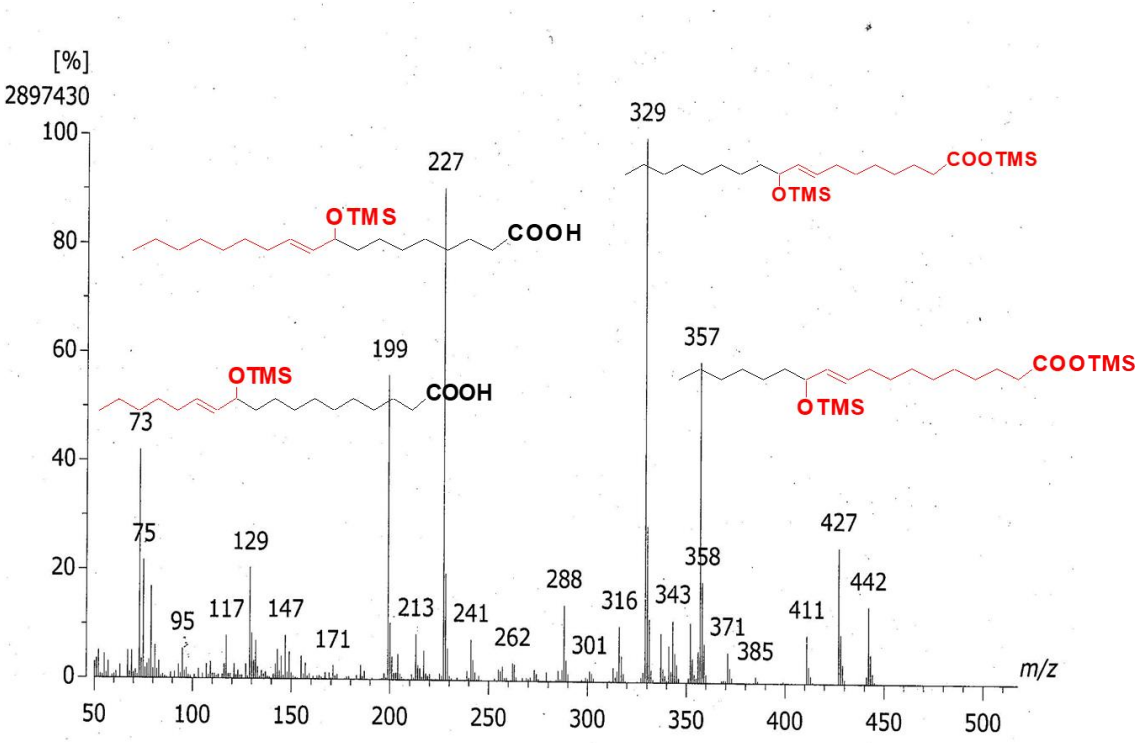


Fig. 2-5 EIMS of the Bis-trimethylsilyl Derivative of **f5** (70 eV)

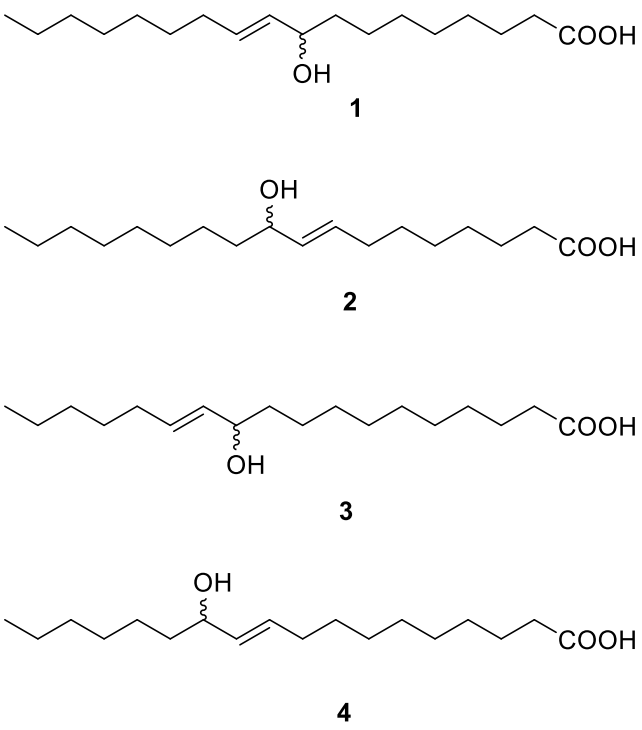


Fig. 2-6 Structures of compound **1**~**4**

It is necessary to separate the mixture and to obtain the spectroscopic data of individual pure compounds for determination of the exact structure of the active compounds, however, the amount of **f5** (4.4 mg) was not enough for further separation. To obtain more of the active fraction, I repeated the isolation procedure again using another specimen of *T. jejuensis*.

3.3 Second isolation of algicidal fraction f5 from T. jejuensis

This time, *T. jejuensis* for isolation of algicidal compounds was collected at Teguma beach, Nagasaki prefecture, in June 2017 (Fig. 2-7). The methanol extract of *T. jejuensis* was separated according the procedure used for the first isolation to obtain fraction **B4** (18.8 mg) and **B5** (21.8 mg), however, the fraction did not exhibit the existence of the hydroxy unsaturated fatty acid both in TLC analysis and ^{1}H NMR measurement. Thus, I purified **B5** with silica gel TLC to afford three fractions, **B5-1**, **B5-2** and **B5-3**, and their HPLC profiles were compared with those of **f5**. However, only trace peaks corresponding to the active compounds were detected (Fig. 2-8). Thus, I left from pure isolation of algicidal compounds and decided to verify the proposed structures of algicidal compounds by comparison of their spectral data with those of authentic samples obtained by synthesis.

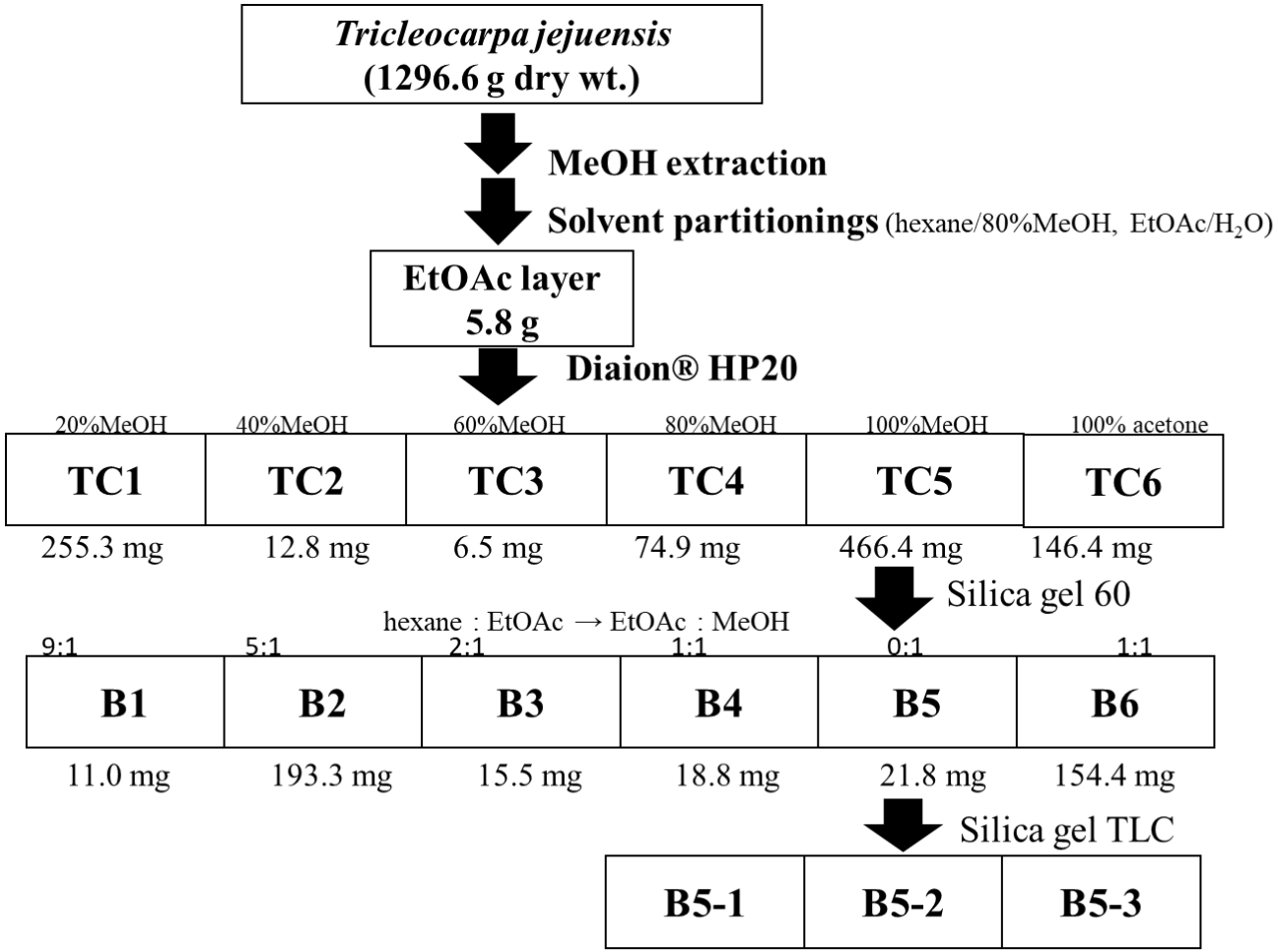


Fig. 2-7 Second isolation of *T. jejuensis*

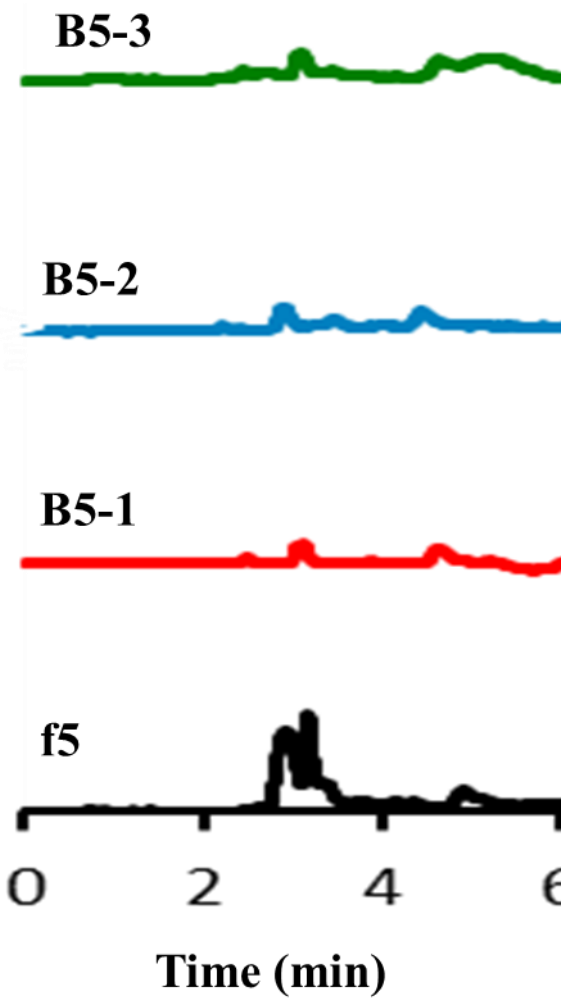


Fig. 2-8 Comparison of HPLC chart of **f5** with **f5** with those of **B5-1**, **B5-2** and **B5-3**. HPLC conditions: column, Capcell Pack C18, 4.6 × 250 mm; solvent, 80% MeOH; follow rate, 1 mL/min; wavelength, 202 nm.

3.4 Synthesis of the hydroxy monounsaturated fatty acids 1~4

To confirm the structure elucidation as well as to obtain pure samples for evaluation of the algicidal activity of each acid and its related compounds, I synthesized each of the acids **1**~**4** by unambiguous routes. Considerable methods for the synthesis of

trans-allylic alcohols have been developed so far, among which following three different methods were adopted for the synthesis of **1**~**4** (Fig. 2-9). The most convenient method is selenium oxide allylic oxidation of alkene (method A). This oxidation reaction directly produces two diastereomeric allylic alcohols by simple operation. *Cis*-hydromethalation and subsequent addition reaction to aldehyde (method B) is an alternative of the one-pot preparation of *trans*-allylic alcohols. Hydrozirconocene chloride (Schwartz's reagent) is known as the most effective hydromethalation reagent to produce variety of allylic alcohols in high yield. However, this reaction requires quite expensive dimethyl zinc to activate alkenyl zirconium species. Thus, I decided to use inexpensive diisobutyl aluminum hydride for this purpose. Two steps reaction sequence with alkenyl lithium addition and subsequent Birch reduction (method C) is also employed in the synthesis of compound **4**.

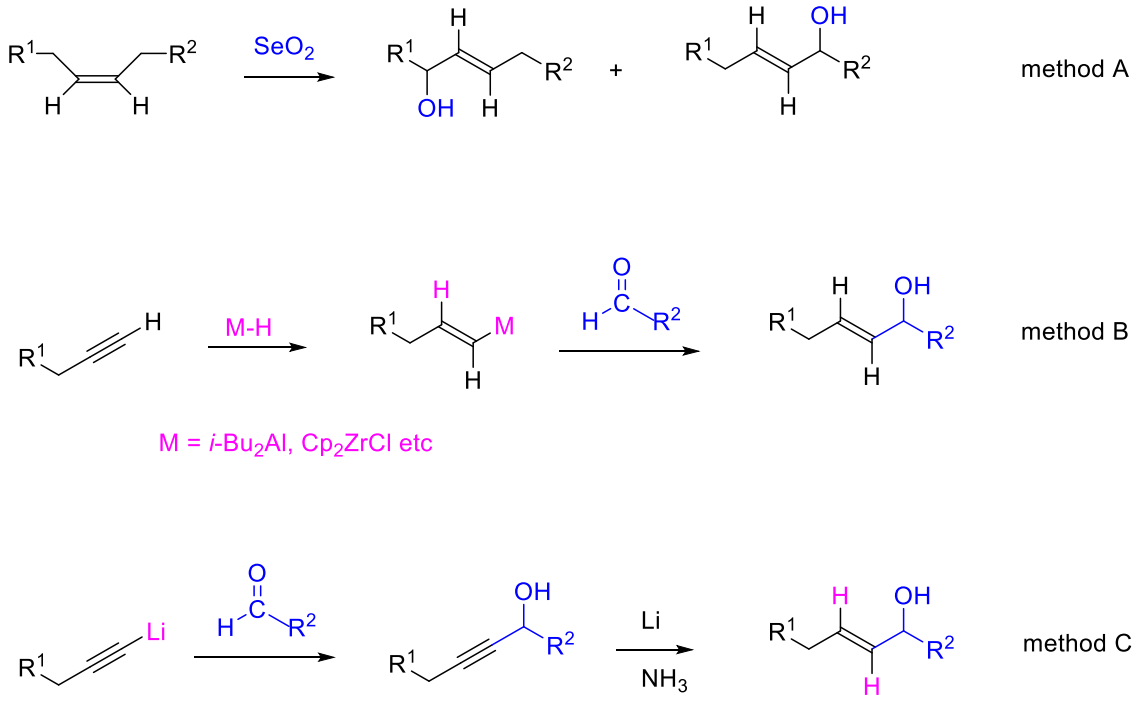
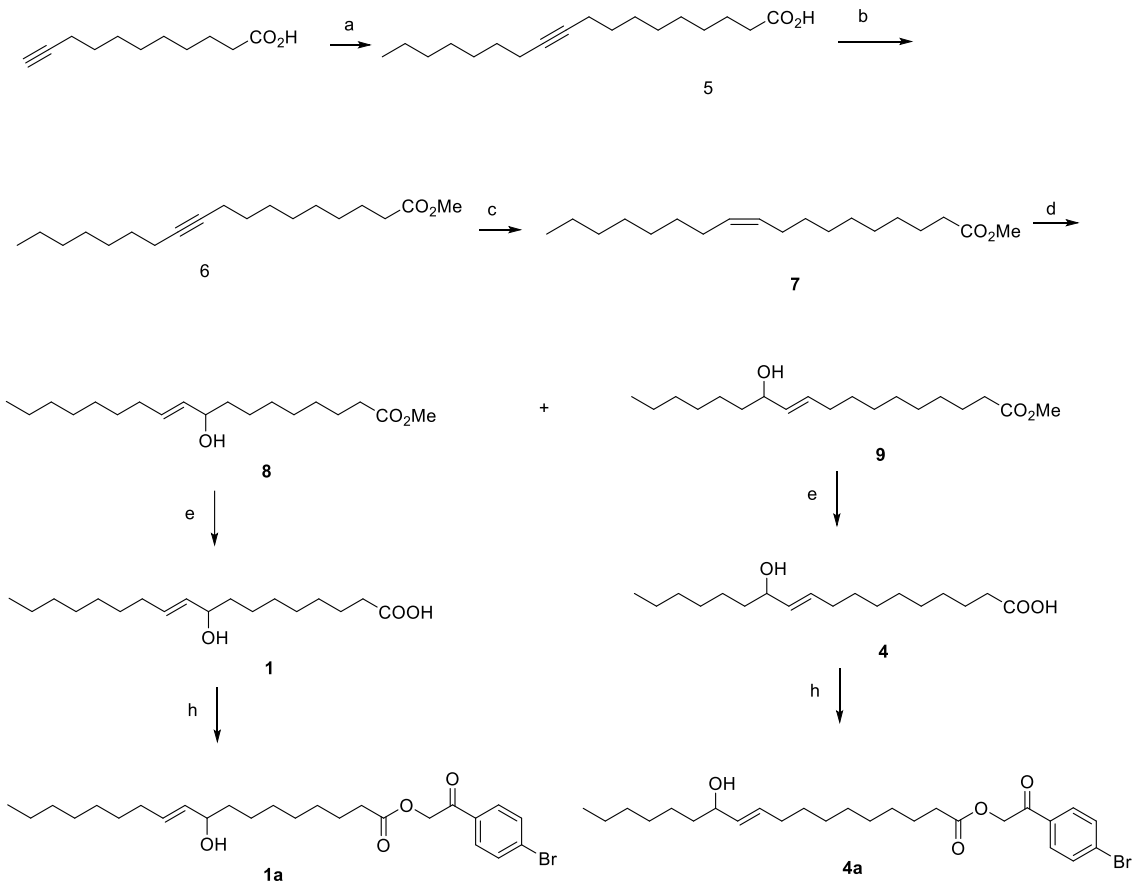


Fig. 2-9 Three different methods for the synthesis of *trans*-allylic alcohol.

3.3.1 Synthesis of (*E*)-9-Hydroxyoctadec-10-enoic acid (**1**) and (*E*)-12-hydroxyoctadec-10-enoic acid (**4**)

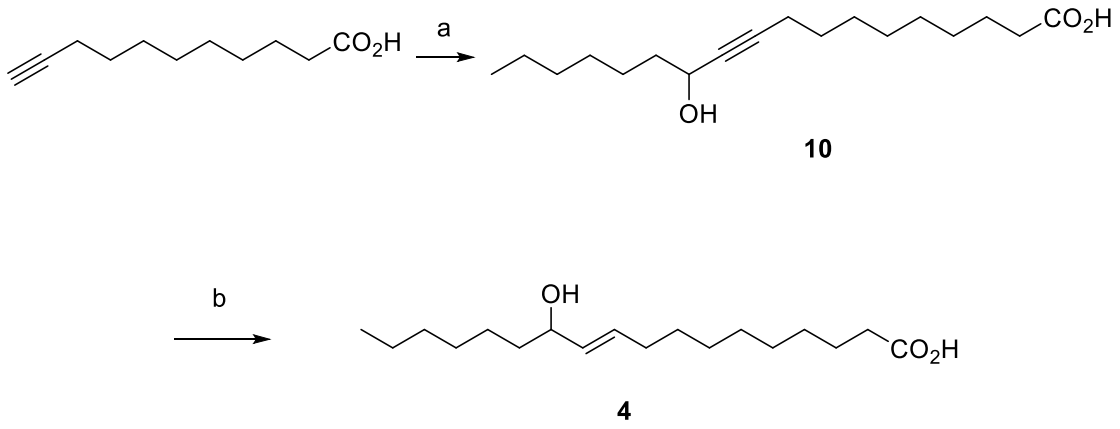
Compound **1** and **4** are regioisomeric in the hydroxyl group position; thus both could be obtained from the same intermediate, (*Z*)-octadec-10-enoate (**7**), using Method A (Scheme 1). Alkylation of the lithium acetylide of 10-undecynoic acid with 1-bromoheptane followed by methyl esterification gave C18 acetylenic acid ester **6** in 27% yield. Partial hydrogenation of the triple bond of **6** with Lindlar's catalyst followed by selenium dioxide oxidation [27] of the resulting (*Z*)-olefin **7** afforded an equimolar mixture of alcohols **8** and **9** in 74% combined yield along with a trace of the 9,12-dihydroxylated compound (14% yield). After separation of the regioisomeric monoalcohols by silica gel chromatography, each methyl ester was hydrolyzed to obtain (*E*)-9-hydroxyoctadec-10-enoic acid (**1**) and (*E*)-12-hydroxyoctadec-10-enoic acid (**4**).



Scheme 1. Synthesis of (*E*)-9-Hydroxyoctadec-10-enoic acid (**1**) and (*E*)-12-hydroxyoctadec-10-enoic acid (**4**)

Reagents: (a) BuLi, HMPA-THF, 0 °C, 2 h, then 1-bromoheptane, r.t., 18 h (27%); (b) $TMSCHN_2$, DCM-MeOH, rt (100%); (c) H_2, 5% Pd/Ca-Pb, EtOAc, rt (96%); (d) SeO_2, TBHP, DCM, rt, 50 h (74%); (e) NaOH, MeOH, reflux, **1** (89%), **2** (85%); (h) 4-bromophenacyl bromide, K_2CO_3, acetone, rt, **1a** (66%), **4a** (43%).

Compound **4** was also synthesized by Method C. A Birch reduction of alkynol **10**, which was obtained by acetylenic addition of 10-undecynoic acid to heptanal, gave **4** in 38% yield. (Scheme 2).

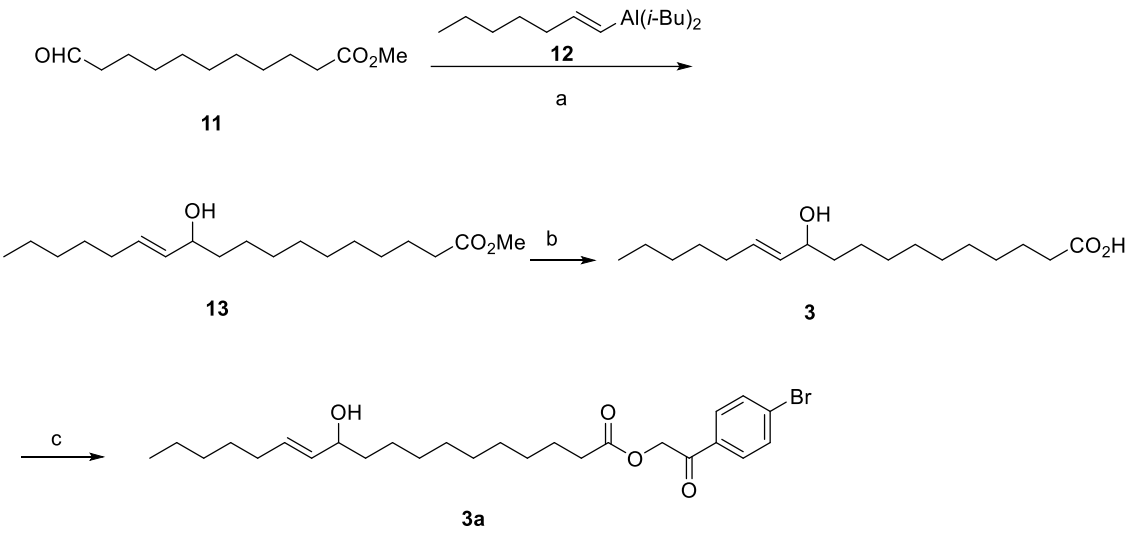


Scheme 2. Alternative synthesis of (*E*)-12-hydroxyoctadec-10-enoic acid (**4**)
Reagents: (a) BuLi, THF, rt, 20 min, then heptanal, -78 °C to rt, 1.5 h (48%); (b) Li, NH_3, *t*-BuOH, THF, -78 °C, 2 h (38%)

3.3.2 Syntheses of (*E*)-11-hydroxoctadec-12-enoic acid (**3**) and (*E*)-10-hydroxyoctadec-8-enoic acid (**2**)

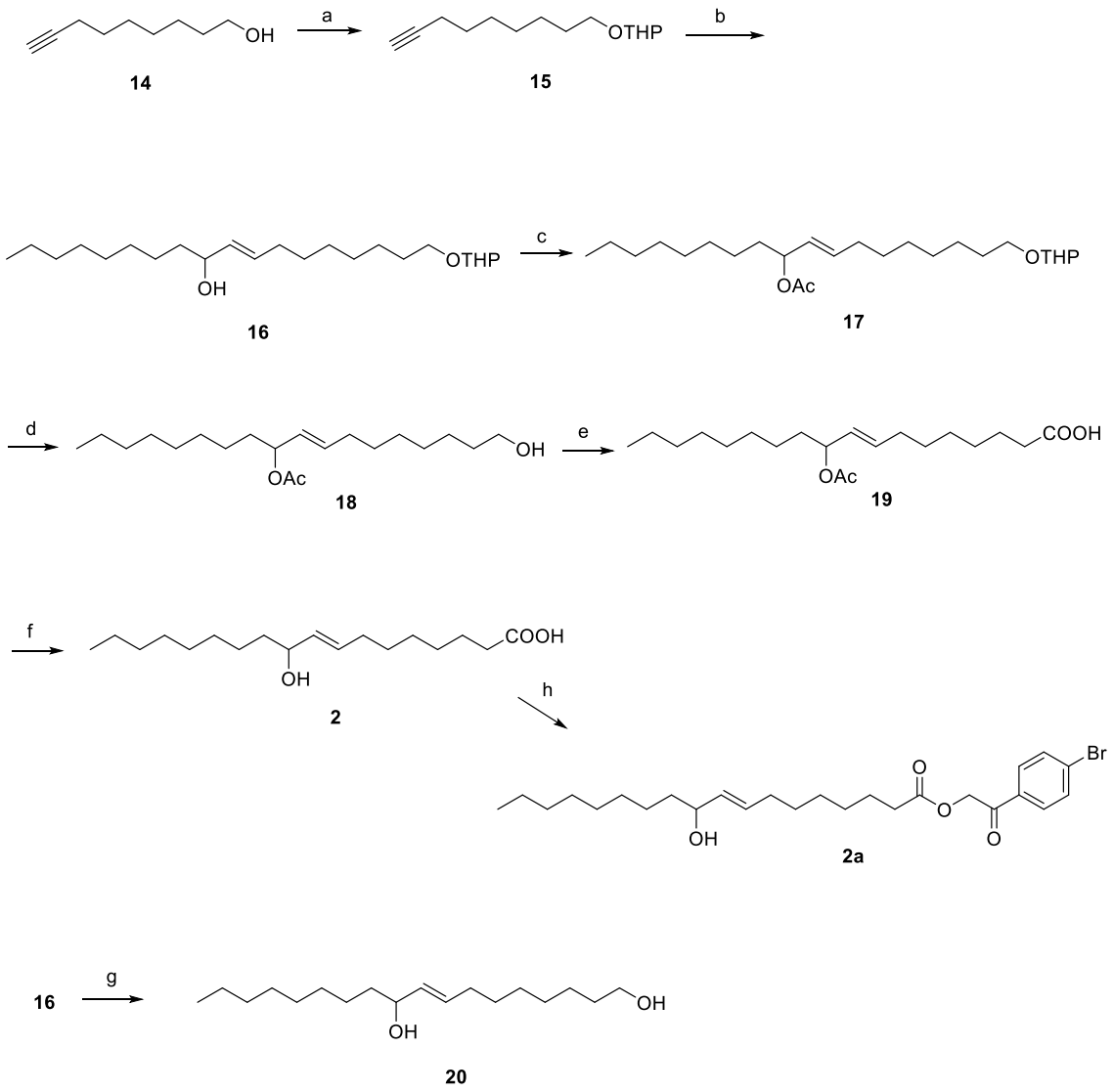
Syntheses of 11- and 10-hydroxyoctadecenoic acids (**3** and **2**) were achieved via addition reactions of alkenyl aluminum reagents (Method B). Aldehyde **11**, prepared by a Kornblum oxidation of methyl 11-bromoundecanoate using a reported procedure [30], was reacted with alkenyl aluminum **12** prepared *in situ* from heptyne and DIBAL to give (*E*)-11-hydroxy-12-octadecanoate (**13**) in 28% yield. One of the target compounds (*E*)-11-hydroxyoctadec-12-enoic acid (**3**) was obtained by alkaline hydrolysis of **13** (Scheme

3).



Scheme 3. Synthesis of (*E*)-11-hydroxyoctadec-12-enoic acid (**3**)
Reagents; (a) hexane-toluene, -10 ^{o}C, 1 h (28%); (b) NaOH, MeOH, rt, **3** (72%); (c) 4-bromophenacyl bromide, K_2CO_3, acetone (52%).

In this strategy, synthesis of another hydroxyoctadecenoic acid, **2**, required alkyne **15** as the source of alkenyl aluminum, which was prepared through an acetylene zipper reaction of commercially available 2-nonyn-1-ol according to a reported procedure [31]. After THP protection of the hydroxyl group of **14**, alkyne **15** was reacted with DIBAL to generate alkenyl aluminum, which was then trapped with nonanal to afford the *trans*-allylic alcohol **16** in 44% yield. The secondary hydroxy group was protected as the acetate, and then the primary hydroxy group was oxidized to furnish (*E*)-10-hydroxyoctadec-8-enoic acid (**2**) after hydrolytic removal of the acetyl group (Scheme 4). For comparison of the biological activity, the alcohol derivative **20** was synthesized by deprotection of **16**.



Scheme 4. Syntheses of (*E*)-10-hydroxyoctadec-8-enoic acid (**2**) and (*E*)-1,10-dihydroxyoctadec-8-ene (**20**)

Reagents; (a) DHP, *p*-TsOH, DCM, rt, 18 h (69%); (b) DIBAL, hexane-toluene, 60 ºC, 2 h then nonanal, -60 ºC, 2 h (44%); (c) Ac_2O, pyridine, rt 40 h; (d) *p*-TsOH, EtOH, rt, 25 min (33%, 2 steps); (e) PDC, DMF, rt 17 h (45%); (f) NaOH, MeOH, rt, (36%); (g) PPTS, MeOH, rt, 20 h (46%); (h) 4-bromophenacyl bromide, K_2CO_3, acetone (59%).

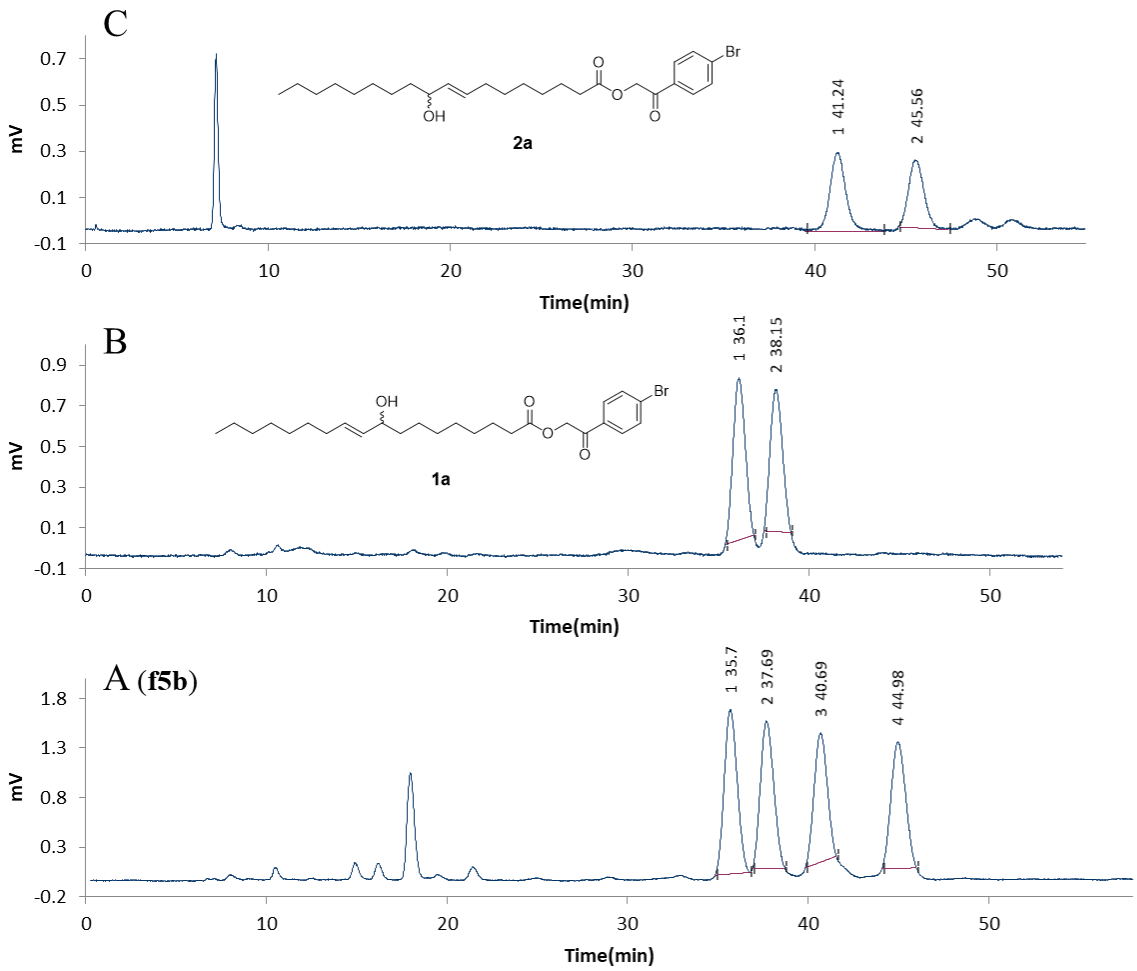
3.5 verification of the proposed structures of 1~4 and stereochemistry

Chemical shift values of selected carbons of the natural products **f5** and synthesized compounds **1~4** are listed with chemical shift difference values (Δ) in Table 2-2. The chemical shift values of all the carbons of **f5** exactly matched those of the corresponding carbons of synthesized compounds **1~4** within a 0.36-ppm difference with the exception of the carboxyl carbons. The slight change in the chemical shift values of the carboxyl carbons between the natural products and synthesized compounds might be due to a considerable difference in the concentration of the sample solutions prepared for NMR measurements. Indeed, 10-fold dilution of the NMR sample solution of **3** from 30 mg/mL to 3 mg/mL resulted in a 1.59-ppm upfield shift in the carboxyl carbon signal.

Recorded specific rotation value of **f5** was close to zero (–0.67°). In order to clarify if the isolated compounds are racemic, scalemic, or enantiomerically pure, the *R*/*S* ratios of **1~4** were determined by chiral HPLC analysis. Esterification of **f5** with *p*-bromophenacyl bromide (K_2CO_3, acetone, rt) gave two separable fractions on silica gel TLC (hexane:EtOAc=2:1), named as **f5a** (Rf 0.50) and **f5b** (Rf 0.43), the former being a mixture of the *p*-bromophenacyl esters of **3** (**3a**, Rf 0.49) and **4** (**4a**,Rf 0.49), and the latter being the esters of **1** (**1a**, Rf 0.46) and **2** (**2a**, Rf 0.43). HPLC analysis of **f5b** using a chiral column, Chiralpak AD-H (solvent, 2-propanol : hexane =15 : 85; flow rate, 0.5 mL/min) showed two pairs of peaks of almost equal intensities corresponding to the respective enantiomers of **1a** (t_R 35.7 and 37.7 min) and **2a** (t_R 40.7 and 45.0 min), indicating **1** and **2** were isolated as racemates (Fig. 2-10). On the other hand, **f5a** showed two peaks at t_R 41.6 min and 45.0 min in an area ratio of 1:3. In the same HPLC conditions, synthesized (±)-**3a** was separated into two peaks at t_R 41.6 min and 44.8 min, suggesting the isolated **3** was a racemate (Fig. 2-11). However, (±)-**4a** was unable to separate by this chiral column and appeared as a single peak at nearly 45.0 min (46.1 min). Finally, separation of (±)-**4a** was achieved by using Chiralpak IA (solvent, MeOH, flow rate 0.5 mL/min) and analysis of **f5a** revealed that the isolated compound **4** was a racemate (Fig. 2-12).

Table 2-2 Chemical shift values (δ_C, ppm) of the selected carbons of fraction **f5** and compounds **1**~**4** (125 MHz in $CDCl_3$)

^{13}C atom	fraction/compound					chemical shift
	f5	**1**	**2**	**3**	**4**	difference
<u>C</u>H$_3$	14.05			14.03		0.02
	14.10				14.07	0.03
	14.10	14.08				0.02
	14.11		14.09			0.02
<u>C</u>H$_2$-CH=CH -CH(OH)	31.36			31.34		0.02
	31.82				31.80	0.02
	31.84	31.82				0.02
	31.87		31.86			0.01
CH$_2$-<u>C</u>H=CH-CH(OH)	131.97		131.97			0.00
	132.20				132.19	0.01
	132.28			132.29		0.01
	132.31	132.34				0.03
CH$_2$-CH=<u>C</u>H-CH(OH)	132.93	132.85				0.05
	132.94			132.89		0.05
	132.97				132.94	0.03
	133.16		133.12			0.04
CH$_2$-CH=CH-<u>C</u>H(OH)	73.20	73.23				0.03
	73.25			73.27		0.02
	73.26		73.27			0.01
	73.30				73.30	0.00
<u>C</u>H$_2$-COOH	33.54		33.88			0.34
	33.57			33.93		0.36
	33.59				33.95	0.36
	33.59	33.95				0.36
CH$_2$-<u>C</u>OOH	177.09		179.08			1.99
	177.12				179.22	2.11
	177.16	179.22				2.06
	177.18			179.23		2.05

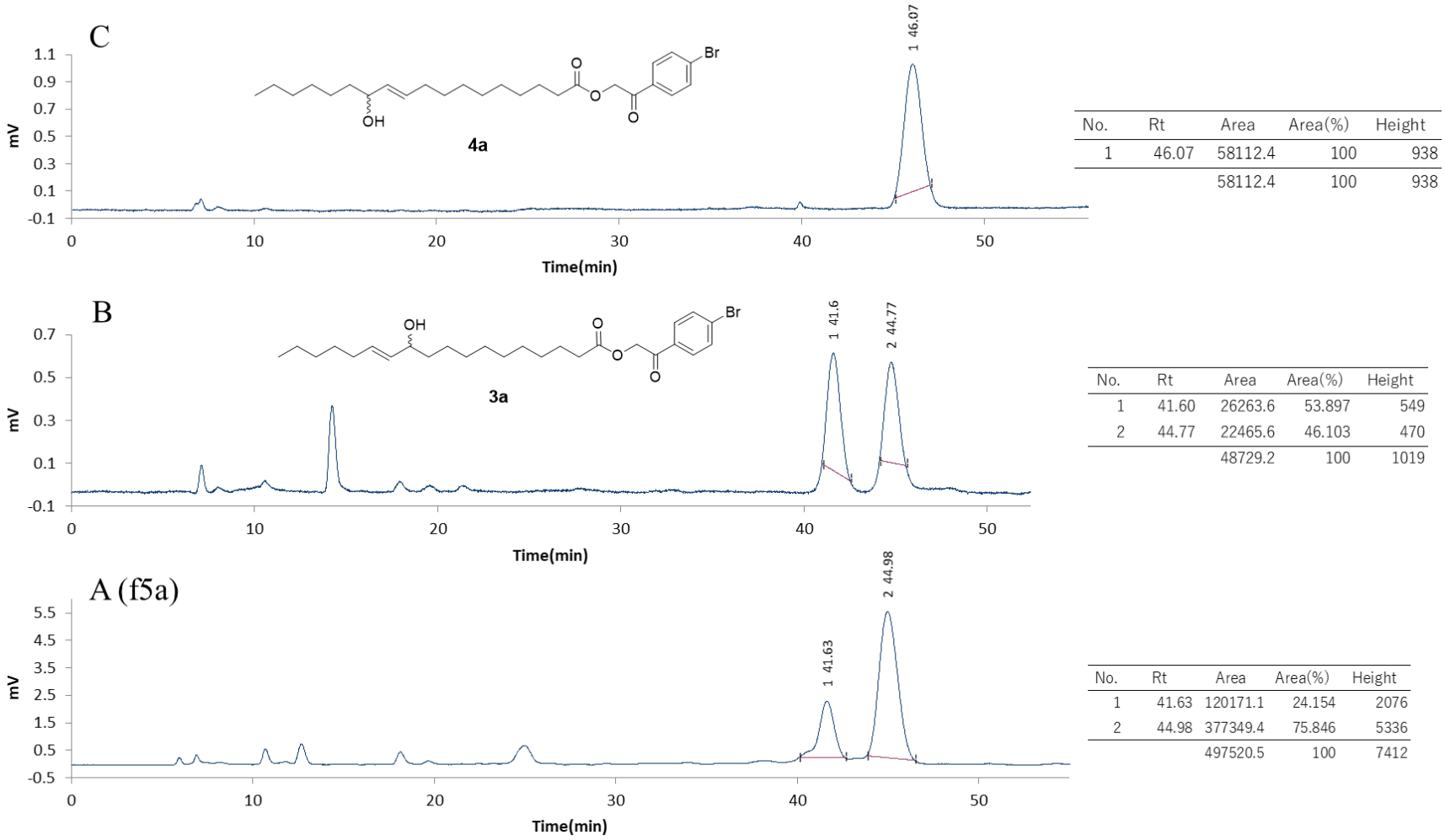


No.	Rt	Area	Area(%)	Height
1	41.24	20916.15	54.5314	344
2	45.56	17440	45.4686	299
		38356.15	100	643

No.	Rt	Area	Area(%)	Height
1	36.1	37276.5	53.1103	796
2	38.15	32910.4	46.8897	703
		70186.9	100	1499

No.	Rt	Area	Area(%)	Height
1	35.7	82904	28.0513	1658
2	37.69	75728.9	25.6235	1485
3	40.69	65967.65	22.3207	1306
4	44.98	70943.6	24.0044	1273
		295544.2	100	5722

Fig. 2-10 Chiral HPLC Analysis of Fraction **f5b** (A), (±)-**1a** (B), and (±)-**2a** (C)
HPLC condition: column, Chiralpak AD-H; solvent, IPA:hexane=15:85; flow rate, 0.5 mL/min; detection, 254 nm.



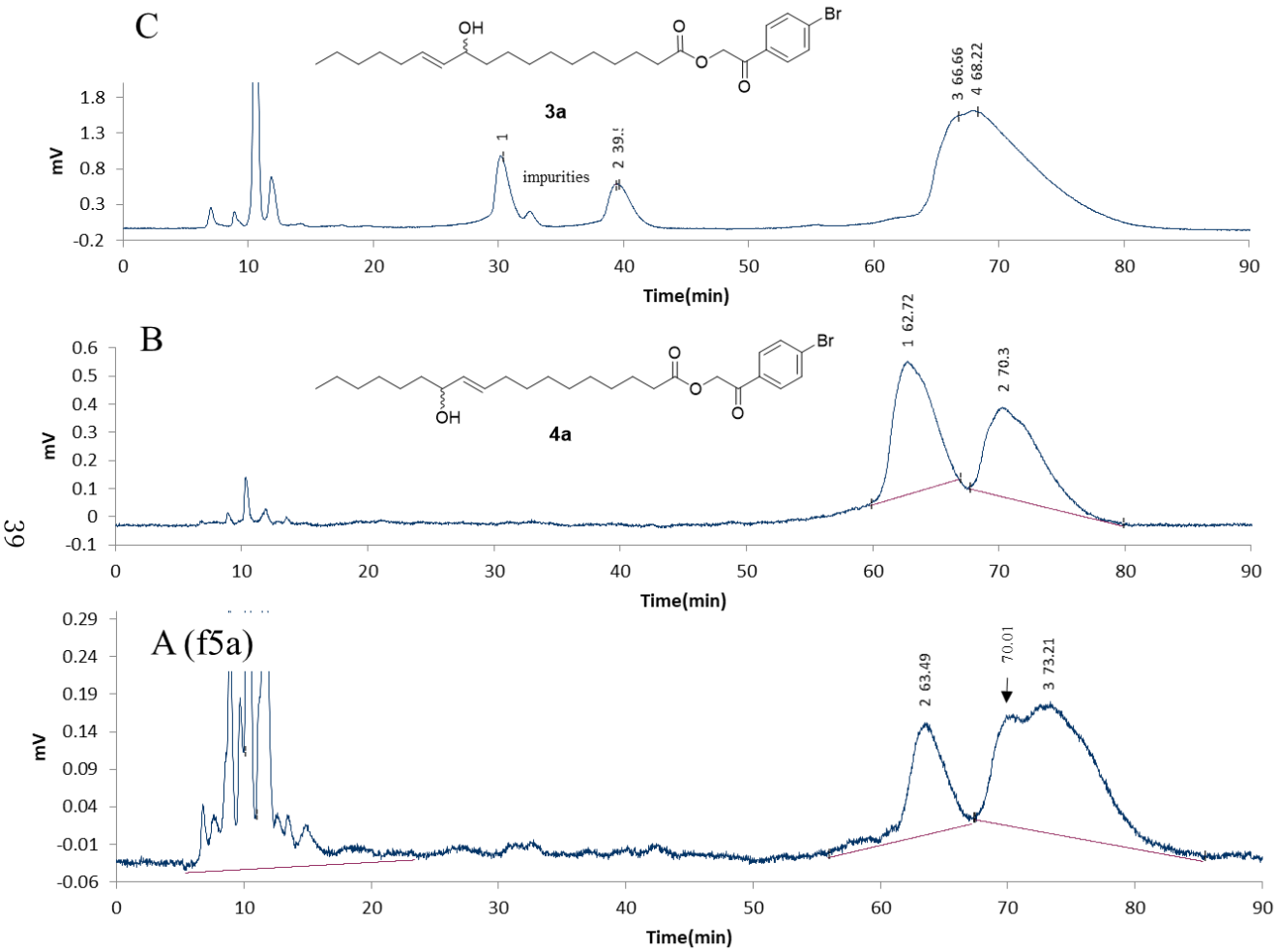
No.	Rt	Area	Area(%)	Height
1	46.07	58112.4	100	938
		58112.4	100	938

No.	Rt	Area	Area(%)	Height
1	41.60	26263.6	53.897	549
2	44.77	22465.6	46.103	470
		48729.2	100	1019

No.	Rt	Area	Area(%)	Height
1	41.63	120171.1	24.154	2076
2	44.98	377349.4	75.846	5336
		497520.5	100	7412

Fig. 2-11 Chiral HPLC Analysis of Fraction **f5a** (A), (±)-**3a** (B), and (±)-**4a** (C)

HPLC condition: column, Chiralpak AD-H; solvent, IPA:hexane=15:85; flow rate, 0.5 mL/min; detection, 254 nm.



No.	Rt	Area	Area(%)	Height
1	62.72	100406.9	51.3151	477
2	70.3	95260.4	48.6849	320
		195667.3	100	797

No.	Rt	Area	Area(%)	Height
1	63.49	30864.2	26.1247	152
2	73.21	87277.3	73.8753	177
		118141.5	100	329

Fig. 2-12 Chiral HPLC Analysis of Fraction **f5a** (A), (±)-**4a** (B) and (±)-**3a** (C)
HPLC condition: column, Chiralpak IA; solvent, MeOH; flow rate, 0.5 mL/min; detection, 254 nm.

3.6 Algicidal activity of hysroxylated trans-monounsaturated fatty acids 1~4 and their derivatives

Each of the synthesized hydroxy acids **1**~**4** as well as their synthetic intermediates **10** and **20** were evaluated for algicidal activity against *C. antiqua* (Fig. 2-13a and Fig. 2-13b). For comparison, autoxidation products of oleic acid, (*E*)-8-hydroxyoctadec-9-enoic acid (**21**) and (*E*)-11-hydroxyoctadec-9-enoic acid (**22**) [32], their oxidized derivatives, diol **23** and ketone **24** (Scheme 4), (*Z*)-12-hydroxyoctadec-9-enoic acid (ricinoleic acid), and (*E*)-octadec-9-enoic acid (elaidic acid) were tested for algicidal activity. All the compounds isolated from *T. jejuensis* except for compound **1** showed complete toxicity to the phytoplankton at a concentration of 20 μg/mL. Among the compounds tested, compound **2** had the highest activity. The autoxidation products of oleic acid **(21** and **22**) and 8,11-dihydroxy derivative **23** also showed high activity. Oxidation of the hydroxy group of **22** as ketone **24** maintained the activity, whereas elaidic acid, which lacks the 11-OH of **22**, had no activity at concentrations less than 80 μg/mL. Ricinoleic acid having *cis*-double bond with a hydroxy group at the homoallylic position displayed the same level of the activity as the *trans*-allylic alcohols. Taken together, presence of oxygen functional group(s) such as hydroxy and carbonyl group is necessary for the activity, but the positions of the hydroxy group and the geometry of the double bond are less important. Reduction of the carboxyl group to alcohol **20** caused somewhat decrease in activity compared with carboxylic acid **2**, but still maintained a moderate level of activity, indicating that the carboxyl group may be replaced with other polar functional groups. Compound **10** having triple bond had the same level of activity as **4**. Fig. 3. shows the cell of *C. antiqua* treated with 5 μg/mL of compound **10** (A) and compound **2** (B) after 0.5- and 4-hour incubations. Interestingly, this propargylic alcohol **10** caused acute lysis of planktonic cells within 30 min (Fig. 2-14, A), at which period no other allylic alcohols affected the planktonic cells (Fig. 2-14, B).

(*E*)-9-Hydroxyoctadec-10-enoic acid (**1**) and (*E*)-10-hydroxyoctadec-8-enoic acid (**2**) have previously been isolated as the biotransformation products of oleic acid by *Pseudomonas* sp. [33 - 37]. The oxidation of unsaturated fatty acids proceeds via three different pathways; autoxidation, photo-oxidation and enzymatic oxidation such as that

of lipoxygenases. Autoxidation of oleic acid involves allylic oxidation and allylic rearrangement of the resulting hydroperoxide, and is characterized by the formation of both cis and trans isomers of 8-hydroxyoctadec-9-enoic acid (8-OH$\Delta_{9,10}$) and 11-hydroxyoctadec-9-enoic acid (11-OH$\Delta_{9,10}$), and the trans isomers of 9-OH$\Delta_{10,11}$ (**1**) and 10-OH$\Delta_{8,9}$ (**2**) [30]. Photo-oxidation of oleic acid involves concerted ene reactions with a singlet oxygen, in which the oxidation proceeds at one end of the double bond to predominantly produce *trans*-9-OH$\Delta_{10,11}$ (**1**) and *trans*-10-OH$\Delta_{8,9}$ (**2**) [32]. (*E*)-11-hydroxyoctadec-12-enoic acid (**3**) and (*E*)-12-Hydroxyoctadec-10-enoic acid (**4**) may arise from *cis*-vaccenic acid by the same mechanism as that for **1** and **2**. Since oleic acid is widely distributed in nature, hydroxy acids **1** and **2** have been isolated from several plants and microorganisms; in some cases, both compounds were co-isolated from the same natural source. Compounds **1** and **2** isolated from stroma of the timothy plant *Epichloe typhina* showed antifungal activity against plant-pathogenic *Cladosporium herbarum* [39], and those isolated from the medicinal plant *Alternanthera brasiliana* and its endophytic bacteria had antimicrobial activity against some human pathogenic bacteria [40]. These hydroxy acids have also been found in macroalgae. Compound **2** isolated from the red alga *Gracilaria verrucosa* is reported to have moderate anti-inflammatory activity [41] and compound **1** isolated from the green alga *Caulerpa racemosa* exhibited potent protein tyrosine phosphatase 1B (PTP1B) inhibitory activity [42]. In contrast, (*E*)-11-hydroxyoctadec-12-enoic acid (**3**) and (*E*)-12-hydroxyoctadec-10-enoic acid (**4**) derived from *cis*-vaccenic acid have rarely been found in nature. Compound **3** was isolated from the green alga *Ulva fasciata* Delile and shown to have moderate and weak antibacterial activity against *Streptomyces aureus* and *Escherichia coli*, respectively [43]. Compounds **1**~**4** have been detected in particulate matter and sediment samples collected in the northwestern Mediterranean Sea in GC/EIMS [44]. Nevertheless, to our knowledge, this is the first isolation of (*E*)-12-hydroxyoctadec-10-enoic acid (**4**) from living organisms. It has also been reported that the hydroxy lipids are the photo-oxidation products of oleic and *cis*-vaccenic acids generated in senescent phytoplanktonic cells [44]. Thereafter, Rontani et al. [45] investigated the origin of the *cis*-vaccenic acid photo-oxidation products in marine environment and concluded that heterotrophic bacteria that are attached to senescent phytoplanktonic cells most likely constitute the source of *cis*-

vaccenic acid oxidation products **3** and **4** detected in the particulate matter samples.

Although the exact ratio of the four compounds was not determined, a GC/EI-MS spectrum of the mixture fraction **f5** displayed two peaks at t_R 18.14 min and t_R 18.21 min in a ratio of 59:41, the former being attributed to a mixture of compounds **3** and **4** and the latter to a mixture of compounds **1** and **2** (Fig. 2-15). It is interesting that the hydroxy fatty acids derived from *cis*-vaccenic acid are dominant over those from oleic acid in this alga.

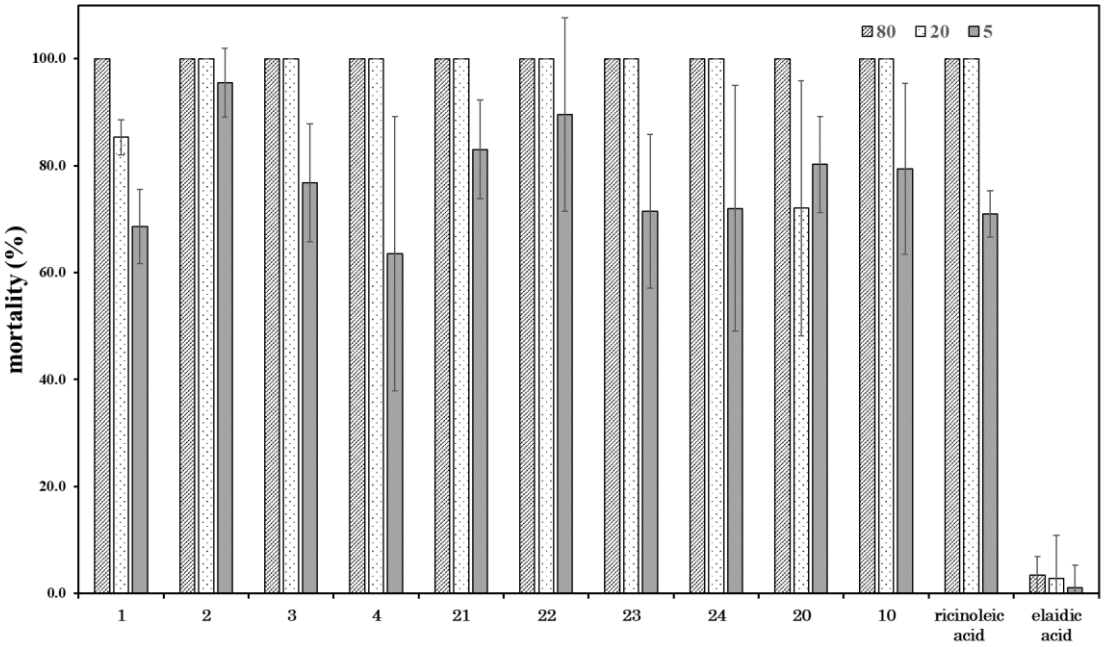


Fig. 2-13a Algicidal activity [mortality (%)] of compound 1~4 and its related compounds at concentrations of 80, 20 and 5 μg/mL for 24 h against *C. antiqua*. Values are the mean ± SD from three independent experiments.

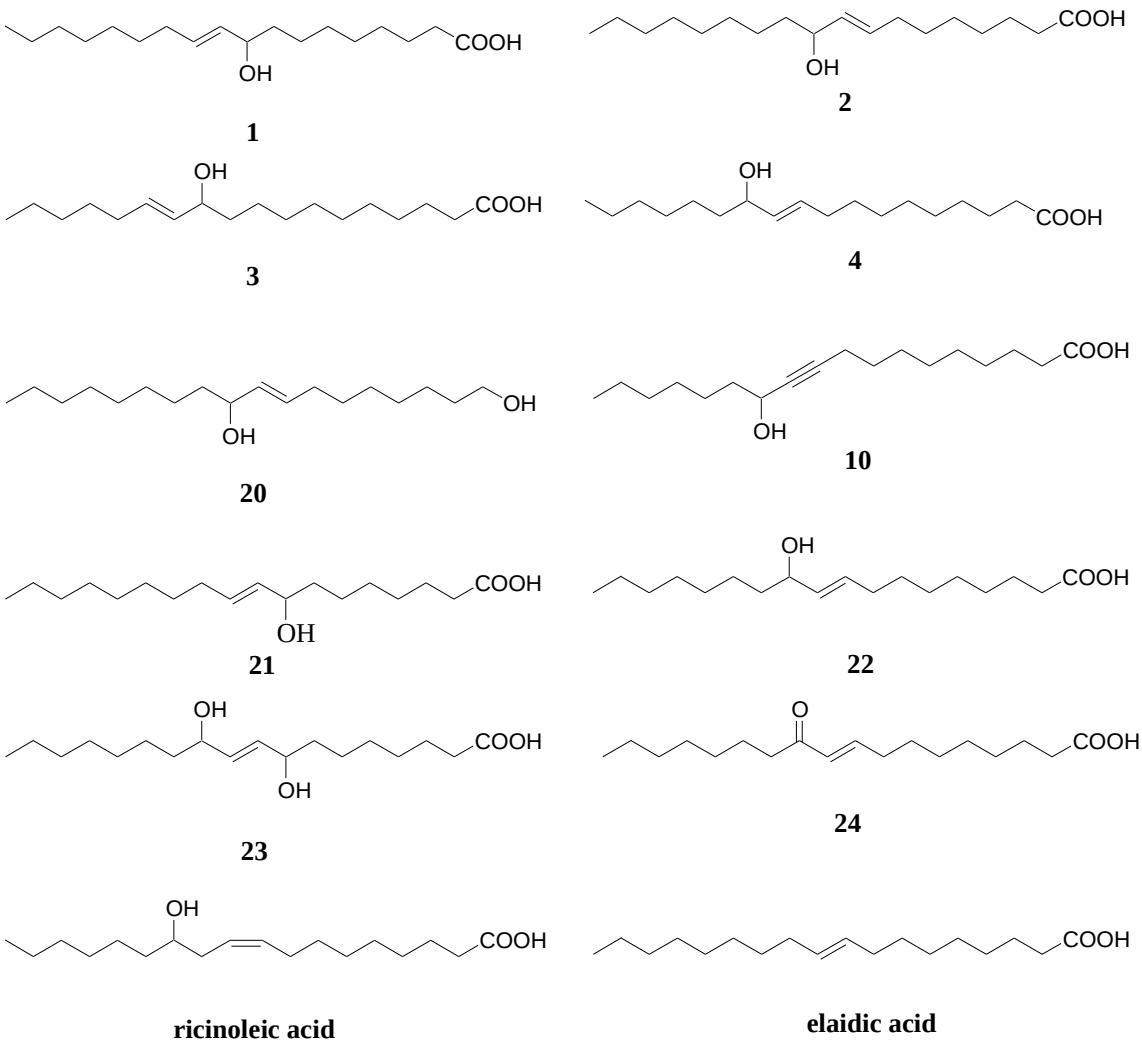


Fig. 2-13b Chemical structures of algicidal compounds

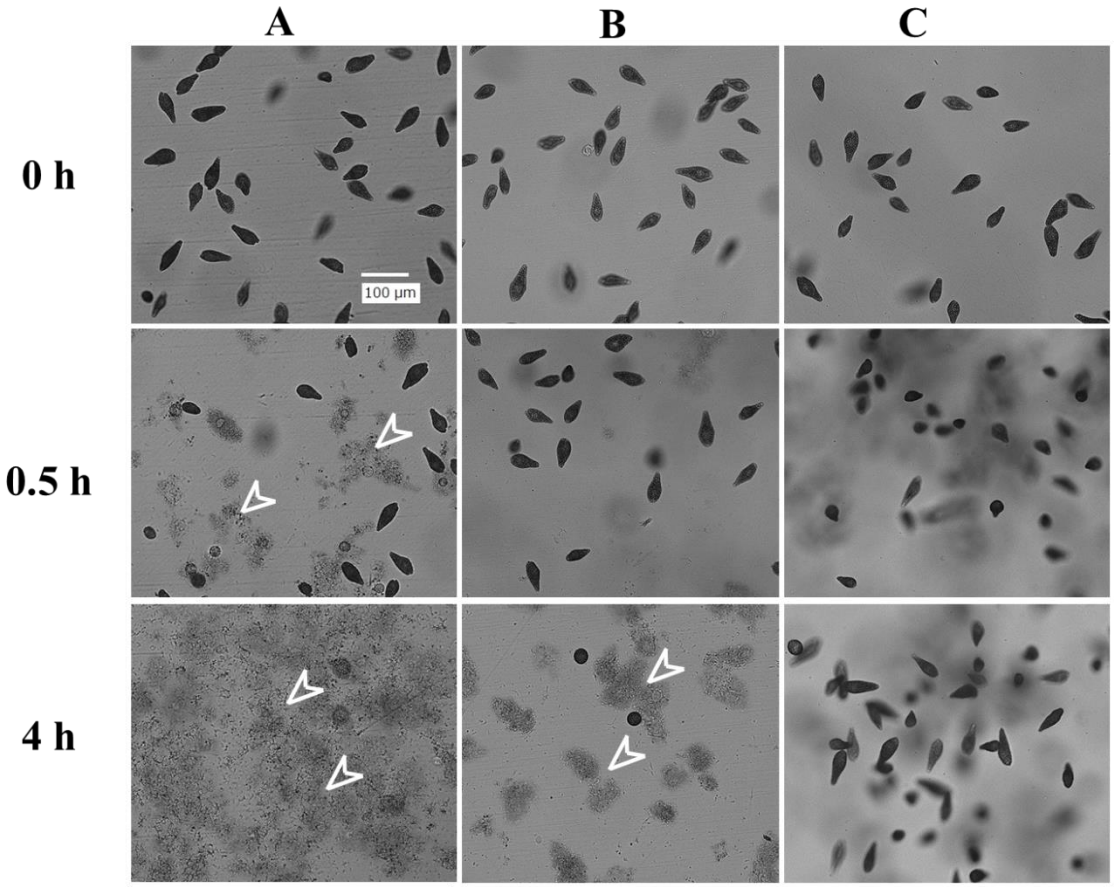
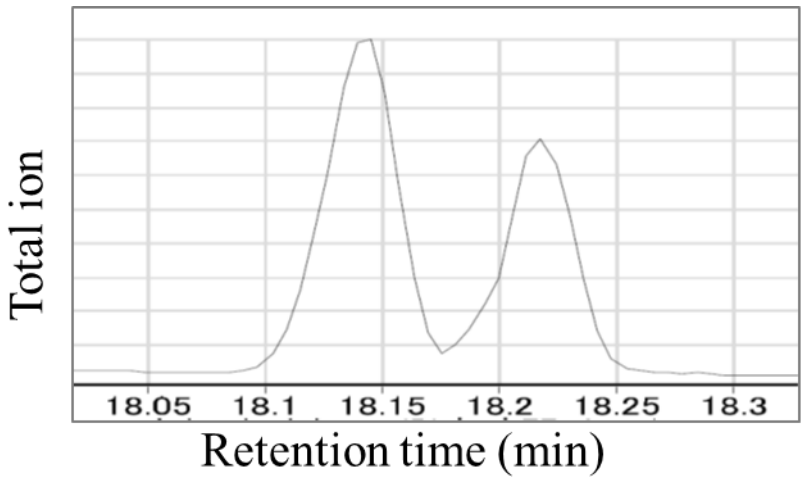


Fig. 2-14 The cell of *Chattonella antiqua* treated with compound **10** (A) and compound **2** (B) at a concentration of 5 μg/mL each, and untreated cells (C) just after treatment (0 h) and after 0.5- and 4-hour incubations. Arrowheads indicate debris of dead cells of *C. antiqua* cells. Bar indicates 100 μm.



Column, HP-1MS (Aglient J&W), 0.25 mm x 30 m; carrier gas, He; flow rate, 1.8 mL/min; oven temp., 120 °C (5min) to 300 °C, 10 °C/min

Fig. 2-15 GC chart of bisTMS Derivative of **f5**

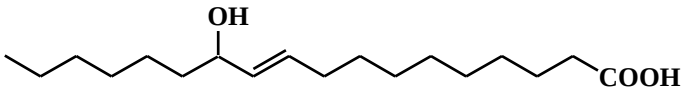
3.7 Conclusions

I isolated a highly algicidal fraction **f5** comprising four C18 hydroxy unsaturated fatty acids, (*E*)-9-hydroxyoctadec-10-enoic acid (**1**), (*E*)-10-hydroxyoctadec-8-enoic acid (**2**), (*E*)-11-hydroxyoctadec-12-enoic acid (**3**) and (*E*)-12-hydroxyoctadec-10-enoic acid (**4**), from a methanol extract of *T. jejuensis*. Their structures were confirmed by comparison of their spectral data with those of synthesized compounds. Among them, compound **2** was found to have the highest algicidal activity, showing >95% mortality against *C. antiqua* at a concentration of 5 μg/mL after 24 h. I also found that propargylic derivative **10** had high acute toxicity to the phytoplankton. Further detailed biological activity study to evaluate the effectiveness of these hydroxy lipids as anti-red tide agents and to obtain an insight on the mode of action will be discussed in the next chapter.

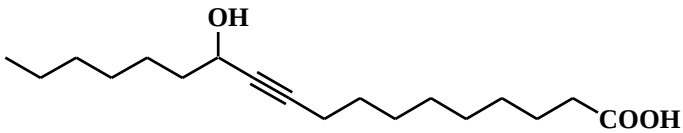
Chapter III Bioactivities of (*E*)-12-hydroxyoctadec-10-enoic acid from the red alga *Tricleocarpa jejuensis* and its synthesized propargylic derivative

1. Introduction

I identified the algicidal principles of the red alga *Tricleocarpa jejuensis* as four isomers of C18 hydroxy unsaturated fatty acids; (±)-(*E*)-9-hydroxyoctadec-10-enoic acid, (±)-(*E*)-10-hydroxyoctadec-8-enoic acid, (±)-(*E*)-11-hydroxyoctadec-12-enoic acid, and (±)-(*E*)-12-hydroxyoctadec-10-enoic acid (compound **4**). The structures were confirmed by comparison of the NMR and MS data with their authentic compounds obtained by unambiguous syntheses in chapter II. During the processes of the chemical syntheses, several intermediate derivatives were obtained. Interestingly, one of such derivatives (±)-12-hydroxyoctadec-10-ynoic acid (compound **10**) induced cell lysis of *C. antiqua* with even more immediate action of mode than the parental (*E*)-12-hydroxyoctadec-10-enoic acid (compound **4**), suggesting that replacing of a double bond in the hydroxy unsaturated fatty acid with a triple bond leads to the augmentation of the bioactivity (Fig. 3-1) (Chapter II). Hence, it is considered that compound **4** and **10** are good pair molecules, which may provide new insight into structure-activity relationship of bioactive fatty acids isolated from marine macroalga. To further evaluate the bioactivities and the specificity of these fatty acids, in this chapter, I performed comparative studies regarding the effects of compound **4** and **10** on four HAB species, three mammalian tumor cell lines, rabbit erythrocytes, and Gram positive and negative bacteria.



Compound 4: (*E*)-12-Hydroxyoctadec-10-enoic acid



Compound 10: 12-Hydroxyoctadec-10-ynoic acid

Fig. 3-1 The chemical structure of compound **4** and compound **10**.

2. Materials and methods

2.1 Chemical compounds

Compound **4** ((±)-*E*-12-hydroxyoctadec-10-enoic acid) and **10** ((±)-12-hydroxyoctadec-10-ynoic acid) were synthesised as described previously [Chapter II]. The structures of compound **4** and **10** are shown in Fig. 3-1. The solutions of these compounds in dimethyl sulfoxide (DMSO) at 4 mg/mL were used throughout the experiments. All the DMSO solutions of these compounds used for cell cytotoxicity experiment were filtrated through a 0.22-μm Millipore membrane filter to avoid contamination.

2.2 Plant culture

Heterocapsa circularisquama (NIES-3621), *Karenia mikimotoi* (NIES-2411), and *Chattonella antiqua* (NIES-1) were obtained from the National Institute for Environmental Studies, Environmental Agency, Japan. *Chattonella marina was* isolated from Kagoshima Bay, Japan. These phytoplankton species were maintained at 26 °C in 100 mL Erlenmeyer flasks containing 50-60 mL of modified seawater medium (SWM-3) at a salinity of 25 [46] under a 12:12 h photoperiod using a cool-white fluorescent lamp (200 ± 5 μmol m^{-2} s^{-1}). The modified SWM-3 containing Tris-HCl buffer system was autoclaved for 15 min at 121 °C before use. The cell numbers of the cultures were counted microscopically using a haemocytometer (Erma Inc., Tokyo, Japan). The cultures at late exponential growth phase were used throughout the experiments. The rotifers *Brachionys plicatilis* were kindly provided by Dr. A. Hagiwara (Faculty of Fisheries, Nagasaki University, Japan) and were maintained with *Nannochloropsis oculata* (*Ochrophyta, Eustigmatophyceae*) as described previously [47]. All the cultures were conducted using sterilized instruments.

2.3 Algicidal activity

To each of phytoplankton cell suspension in 96-well flat bottom plates, varying concentrations of compound **4** and **10** were added (100 μL/well). After of 4 h cultivation, swimming phytoplankton in culture condition was counted under microscope observation.

The survival rate (SR) of each phytoplankton was defined using the following equation:

$$SR\ (\%) = \frac{N}{N0} \times 100$$

where N and N0 represent the numbers of swimming cells in the treatment and control cultures, respectively.

2.4 Rotifer exposure experiment

The rotifer exposure to HAB species or compound **4** and **10** were carried out in 48-well flat bottom plates (Becton-Dikinson) according to the procedure of Zou et al. [48]. Ten rotifers in each well were incubated with varying concentrations of compound **4** and **10** or HAB species in modified SWM-3 (500 μL/well) at 26 ℃ in light for 4 h. Then, the surviving individuals, which were defined as those actively swimming in each well, were counted using a stereomicroscope.

2.5 Cytotoxicity

HeLa (human epithelia carcinoma), XC (rat sarcoma), and U937 (human histiocytic lymphoma) cells were obtained from American type culture correction. HeLa and XC cells were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 10 μg each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 IU/mL), and streptomycin (100 μg/mL). U937 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Cytotoxicities of the samples were evaluated by MTT assay, which detects mitochondrial succinate dehydrogenase present in living cells. In brief, 1~4 x 10^4 cells/well in a 96-well plate in the medium were incubated with varying concentrations of the samples in the culture medium for 24 h at 37 °C, and then conducted MTT assay [48].

2.6 Haemolytic activity

Fresh rabbit blood was obtained from Nippon Bio-Test Laboratories (Tokyo, Japan). Erythrocytes were washed three times with PBS and the final erythrocyte pellet was suspended in PBS to make 2% (v/v) suspension. Triplicate 50 μL aliquots of serial

two-fold dilutions of the samples were added to round-bottom 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA). To the wells containing 50 μL of samples in PBS, 50 μL of a 2% (v/v) suspension of erythrocytes were added, after which the well plates were gently shaken. After incubation for 4 h at 26 °C in the light, the plates were centrifuged at 900 × *g* for 10 min at 4 °C. Aliquots (50 μL) of the supernatant were withdrawn from the wells and transferred to flat-bottom 96-well plates (Becton-Dickinson). The amount of haemoglobin released was determined by measuring absorbance at 560 nm using a microplate reader (MPR-A4i, TOSOH Co., LTD., Tokyo, Japan). Negative controls (zero haemolysis) and positive controls (100% haemolysis) were included as erythrocytes suspended in PBS alone and in PBS containing 1% v/v Triton X-100, respectively.

2.7 Antibacterial activity

Staphylococcus aureus (NBRC12732) and *Escherichia coli* (NBRC13898) were obtained from NITE Biological Resource Centre (Tsukuba, Japan). Nutrient agar was used to maintain the strains. Bacterial strains were cultured for 18 h at 37 °C in nutrient broth medium. The cells were harvested and washed with phosphate-buffered saline (PBS) by centrifugation at 15,000 × g for 10 min at 4 °C. The cell pellets were diluted to the appropriate cell density with PBS, and immediately used for the experiments. Bacterial cultures were incubated with varying concentrations of samples for 4 h at 37 °C in triplicate. An aliquot of each reaction mixture was withdrawn and suitably diluted with PBS and inoculated in triplicates into nutrient agar medium. After 48 h of incubation at 37 °C, the numbers of colonies formed were counted to determine the colony forming unit (CFU). The survival rate was calculated by dividing the CFU at time 4 h by the CFU at time 0 h.

2.8 Statistical analysis

All the experiments were repeated at least three times. The results were expressed as a mean ± standard deviation (SD), and the data was analyzed by a paired t-test to determine significant differences. A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Effects of compound 4 and 10 on four HAB species

Compound **4** is one of the four isomeric C18 hydroxy unsaturated fatty acids found in a methanol extract of *Tricleocarpa jejuensis*, which exerts algicidal activity against the red tide phytoplankton *C. antiqua* with >80% mortality after 24 h incubation at a concentration of 20 μg/mL in chapter II. To further evaluate the algicidal activity of compound **4**, its concentration-dependent effects on *C. marina*, *K. mikimotoi*, and *H. circularisquama* in addition to *C. antiqua* were examined. From the dose response curves (Fig. 3-2), the 50% lethal concentration (LC_{50}) of compound **4** against *C. antiqua*, *C. marina*, *K. mikimotoi*, and *H. circularisquama* were estimated to be 3.31, 5.64, 5.70, and 1.68 μg/mL, respectively under the experimental conditions used in this study (Table 3-1). Interestingly, the cytotoxicity of compound **10** on these HAB species was evidently more potent than compound **4** (Fig. 3-2), and its LC_{50} against *C. antiqua*, *C. marina*, *K. mikimotoi*, and *H. circularisquama* were estimated to be 0.8, 2.98, 1.63, and 0.69 μg/mL, respectively (Table 3-1). These results suggest that replacing a double bond in the compound **4** with a triple bond can lead to enhanced algicidal activity. Previous numerous studies have reported that certain free fatty acids produced by algae exhibit toxic effects on various aquatic organisms including phytoplankton [13, 15-17, 49-51]. Regarding toxic mechanism of free fatty acids, Wu et al. have found that leakage of intracellular potassium (K^+) occurred as a result of plasma membrane damage after exposure to deleterious concentrations of fatty acids, and unsaturated fatty acids induced higher level of K^+ leakage than saturated ones [52]. Among the fatty acids tested (lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and α-linolenic

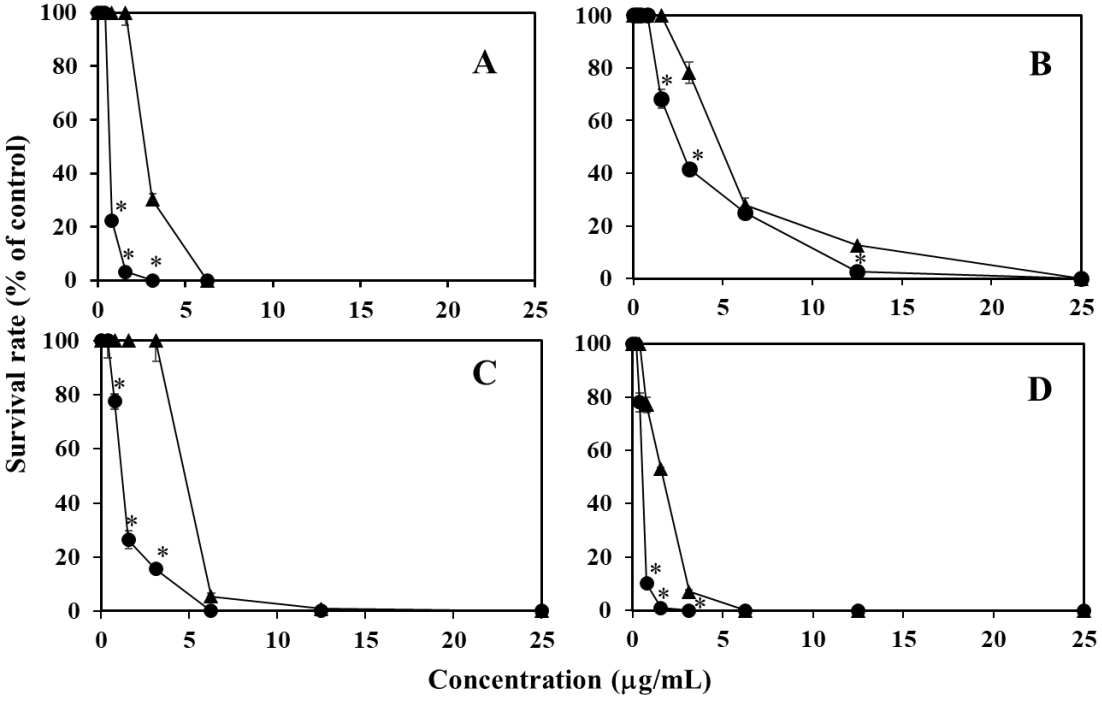


Fig. 3-2 The effects of compound **4** (▲) and compound **10** (●) on *C. antiqua* (A), *C. marina* (B), *H. circularisquama* (C), and *K. mikimotoi* (D). To the cell suspension of each phytoplankton species in 96-well plates, the indicated concentrations of compound **4** or **10** were added. After 4 h incubation under the phytoplankton culture conditions, the cell viabilities were determined as described in the text. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound **4** and **10** ($p < 0.05$).

Table 3-1 The LC_{50} (50% lethal concentration) of compound **4** and compound **10** on various species after 4 h of treatment.

Species	LC_{50} (μg/mL)	
	Compound **4**	Compound **10**
Phytoplankton		
C. antiqua	3.31	0.80
C. marina	5.64	2.98
H. circularisquama	5.70	1.63
K. mikimotoi	1.68	0.69
Zooplankton		
B. plicatilis	56.99	39.76
Tumor cell lines		
XC cells	84.07	46.31
U937 cells	45.02	40.52
HeLa cells	75.86	42.51
Bacteria		
E. coli	>100	>100
S. aureus	79.31	11.01

acid), α-linolenic acid induced the highest level of K^+ leakage from chlorophyte *Monoraphidium contortum*, and the cytotoxicity and K^+ leakage were well correlated [52]. Other studies also found that the toxicity of fatty acids increased with an increase in the degree of unsaturated double bonds [13, 53]. In the previous study by Alamsjar et al., linoleic acid and α-linolenic acid were isolated from green alga *Ulva lactuca* during the course of screening various macroalgae for the algicidal agents against *Heterosigma akashiwo* (*Ochrophyta,Raphidophyceae*) [54]. Comparative study on the cytotoxic effects of linoleic acid and α-linolenic acid on various red tide phytoplankton species (*C. marina*, *H. akashiwo*, *Alexandrium tamarense*, *Alexandrium taylori*, *Gymnodinium impudicum, and H. circularisquama* (*Miozoa, Dinophyceae*)) demonstrated that the LC_{50} values of α-linolenic acid were lower than those of linoleic acid, suggesting that number

of unsaturated double bond of fatty acid is important structural element involved in the cytotoxic effect in agreement with other study [13]. The cytotoxic activities of these unsaturated fatty acids were highly species-specific. The LC_{50} values of linoleic acid and α-linolenic acid against *C. marina* were estimated to be 22.35 and 3.22 μg/mL, respectively, whereas no significant toxic effects were observed on *H. circularisquarma* up to 1000 μg/mL [54]. In contrast to linoleic acid and α-linolenic acid, *C. marina* and *H. circularisquarma* showed nearly equal susceptibility to compound **4** and **10** (Fig. 3-2 and Table 3-1). Probably, hydroxylated unsaturated fatty acids like compound **4** and **10** may be able to exert potent cytotoxicity on broader phytoplankton species. This notion may be supported by the study in which Aliotta et al. found that unsaturated fatty acids were oxidized to form hydroxy compounds and exhibited anti-algal activity [55]. Taken together with our results obtained in this study and previous findings suggest that unsaturated double bond and hydroxy group are important structural elements influencing the cytotoxic activity of fatty acids against microalgae, and replacing the double bondwith a triple bound can lead to further potentiation of the activity. To the best of myknowledge, this is the first report indicating that propargyl group can contribute to the enhancement of the algicidal activity of bioactive fatty acids.

3.2 Protective effects of compound 4 and 10 on rotifer exposed to H. circularisquarma and K. mikimotoi

Herbivorous zooplankton, such as rotifer and copepod, have been used to evaluate toxic potential of HAB species [56-58] and several dinoflagellates exhibited lethal effects on the rotifer *Brachionus plicatilis* [59-60]. Oda and co-workers found that the rotifer is highly susceptible to *H. circularisquarma* and *K. mikimotoi* [47, 60]. The toxic effects of the strains of *H. circularisquarma* and *K. mikimotoi* used in this study were confirmed, and 10 or 8 out of 10 rotifers exposed to *H. circularisquarma* at 2×10^4 cells/mL or *K. mikimotoi* at 2×10^3 cells/mL died after 4 h incubation, respectively (Table 3-2). As compared to *H. circularisquarma* and *K. mikimotoi*, rotifer was relatively resistant to compound **4** and **10** (Fig. 3-3), and the LC_{50} values were estimated to be 56.99 and 39.76 μg/mL, respectively (Table 3-1), which were more than 10 times higher than those against *H. circularisquarma* and *K. mikimotoi* (Table 3-1). In the presence of

compound **4** (20 μg/mL) or **10** (10 μg/mL) at the concentration toxic enough to the dinoflagellates but not so much detrimental to rotifer, significant increases in the survival rates of rotifers exposed to *H. circularisquarma* and *K. mikimotoi* were observed (Table 3-2). The results suggest that compound **4** or **10** can be used to suppress or mitigate the toxicity of these HAB species.

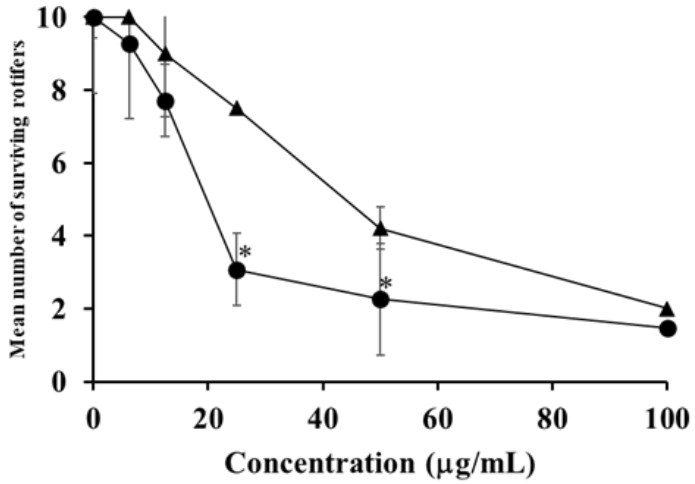


Fig. 3-3 The effects of compound **4** (▲) and compound **10** (●) on rotifer (*Brachionus plicatilis*). To the rotifers in 48-well plates (10 rotifers/well), the indicated concentrations of compound **4** or **10** were added. After 4 h incubation under the phytoplankton culture conditions, the viabilities of rotifers were determined as described in the text. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound **4** and **10** ($p < 0.05$).

Table 3-2 Effects of *K. mikimotoi* (2.0×10^3 cells/mL) and *H. circularisquama* (2.0×10^4 cells/mL) on rotifers in the presence or absence of compound **4** (20 μg/mL) or **10** (10 μg/mL). After 4 h of cultivation, the number of living rotifers were counted as described in the text.

Exposure condition	Number of live rotifers/exposed	Exposure condition	Number of live rotifers/exposed
Control (medium only)	10/10	Control (medium only)	10/10
Exposure to *H. circularisquma*	0/10	Exposure to *K. mikimotoi*	2/10
+ Compound **4**	8/10	+ Compound **4**	8/10
+ Compound **10**	9/10	+ Compound **10**	4/10

3.3 Cytotoxic effects of compound 4 and 10 on three cancer cell lines

Certain fatty acids show cytotoxic effects on cancer cells with different degrees depending on the structures [61], and some of them have been recognized as effective bioactive molecules in the treatment of tumors [62-63]. To gain further insight into the specificity of the cytotoxic mechanism of compound **4** and **10** and their potential as effective anti-tumor agents, the effects of compound **4** and **10** on three tumor cell lines were examined. As shown in Fig. 3-4, both compounds showed cytotoxic effects on HeLa, XC, and U937 cells in a concentration-dependent manner with different extents depending on the cell lines, and the cytotoxicity of compound **10** was stronger than compound **4**, and was similar to the toxicity to HAB species. In contrast to hydroxy unsaturated fatty acids like compound **4** and **10**, it has previously found that α-linolenic acid and linoleic acid isolated from green alga *U. lactuca* as potent algicidal agents showed no significant cytotoxic effects on mammalian cell lines including tumor cell lines such as HeLa cells and U937 cells [54]. Although further studies are necessary to

clarify the exact cytotoxic mechanism, it is considered that hydroxy group in compound **4** and **10** may be an important structural group commonly involved in the cytotoxicity against phytoplankton and tumor cells. Since the cytotoxic effects of compound **10** on these tumor cell lines were greater than compound **4** as observed in the toxicities on microalgae, the triple bond in compound **10** may contribute to the increase in the cytotoxic activity against mammalian tumor cells as well as to unicellular phytoplankton cells.

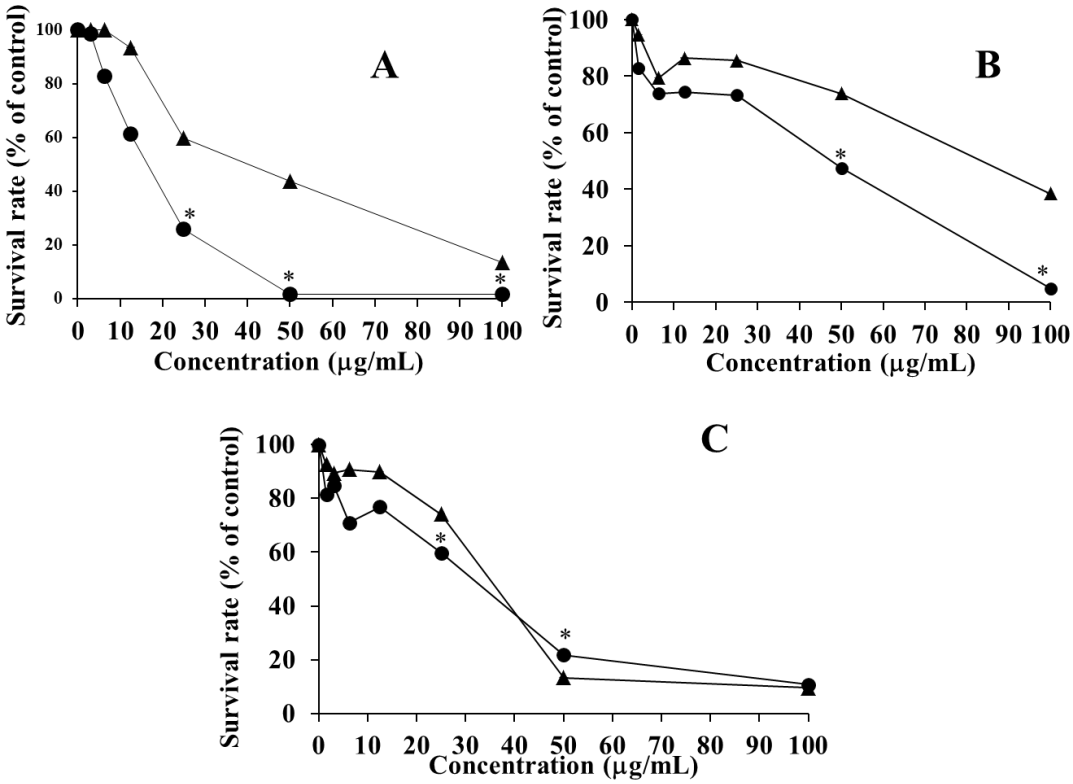


Fig. 3-4 The effects of compound **4** (▲) and compound **10** (●) on HeLa cells (A), XC cells (B), and U937 cells (C). To the cells in 96-well plates, the indicated concentrations of compound **4** or **10** were added. After 24 h incubation under culture conditions, the survival rates of the cells were determined by MTT assay. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound **4** and **10** ($p < 0.05$).

3.4 Haemolytic activity of compound 4 and 10

One possible cellular level action mechanisms of compound **4** and **10** may be an

effect on the membrane integrity of target cells. The damaging effects on cell membrane are easily detected as a haemolytic activity, and cytotoxicity and haemolytic activity of such compounds are well correlated. A mixture of two unsaturated fatty acids isolated from sponge *Geodinella robusta* show cytotoxicity on mouse Ehrlich carcinoma cells and causes haemolysis toward mouse erythrocytes with nearly equal 50% effective dose (ED_{50}) [64]. As expected, compound **10** showed haemolytic activity toward rabbit erythrocytes, but no significant activity of compound **4** was observed up to 100 μg/mL (Fig. 3-5). Although further studies are necessary to clarify the exact action mechanisms of compound **4** and **10**, the potent haemolytic activity of compound **10** may partly contribute to the stronger cytotoxicity than compound **4**. Probably compound **10** may be able to induce membrane damage or perturbation through the triple bond-mediated specific interaction with the membrane of the target cells.

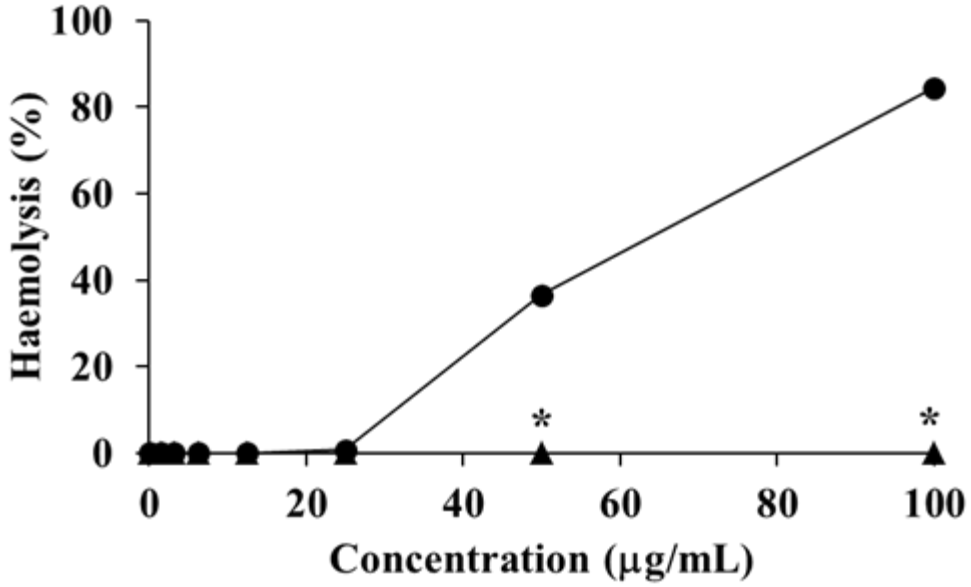


Fig. 3-5 Haemolytic activities of compound **4** (▲) and compound **10** (●) against rabbit erythrocytes. To the indicated concentrations of compound **4** or **10** in PBS in 96-well plates, the rabbit erythrocytes in PBS were added. After incubation for 4 h at 26°C, the extents of haemolysis were measured as described in the text. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound **4** and **10** ($p < 0.05$).

3.5 Antibacterial effects of compound 4 and 10

Compound **4** is one of the four isomers of C18 hydroxy unsaturated fatty acids. Some isomers of compound **4** were isolated from other natural sources have been reported to show antimicrobial activities. (*E*)-9-hydroxyoctadec-10-enoic acid and (*E*)-10-hydroxyoctadec-8-enoic acid found in a higher plant (*Epichloe typhina*) showed antifungal activity against plant-pathogenic *Cladosporium herbarum* (*Fungi, Ascomycota*) [65]. These isomers were also isolated from the medicinal plant *Alternanthera brasiliana* and its endophytic bacteria, and they exhibited antimicrobial activity against some human pathogenic bacteria [66]. (*E*)-11-hydroxyoctadec-12-enoic acid isolated from the green alga *U. lactuca* showed antibacterial activity against *Streptomyces aureus* and *Escherichia coli* with different extent depending on the bacterial species [67]. Moreover, the antibacterial activities of various fatty acids have been well documented, and there are studies focused on therapeutic potential of fatty acids against infectious diseases [67, 68]. Especially certain long-chain unsaturated fatty acids are even bactericidal to important pathogenic microorganisms such as *Helicobacter pylori* [69] and mycobacteria [70]. To see the fundamental aspect of antibacterial activity of compound **4** and **10**, in the current study, colony forming assay against gram-positive *S. aureus* and gram-negative *E. coli* were conducted (Fig. 3-6). Compound **4** and **10** exerted bactericidal activity against *S. aureus* in a concentration-dependent manner, and compound **10** was obviously more potent than compound **4**, whereas no significant toxicity of these compounds on *E. coli* was observed up to 100 μg/mL (Fig. 3-6). The results suggest that compound **4** and **10** may have specificity to gram-positive bacteria. Regarding antibacterial activity of unsaturated fatty acids, it has been reported that gram-positive bacteria are more susceptible than gram-negative bacteria [71-72]. The differences in the sensitivities toward fatty acids between gram-positive and gram-negative bacteria may result from the impermeability of the outer membrane of gram-negative bacteria since the outer membrane of gram-negative bacteria can function as an effective barrier against hydrophobic substances such as fatty acids [73-74]. More importantly, my results suggest that having triple bond instead of double bond in the hydroxy unsaturated fatty acid can lead to the increase in the bactericidal activity in

addition to the algicidal and the tumor cell cytotoxicity.

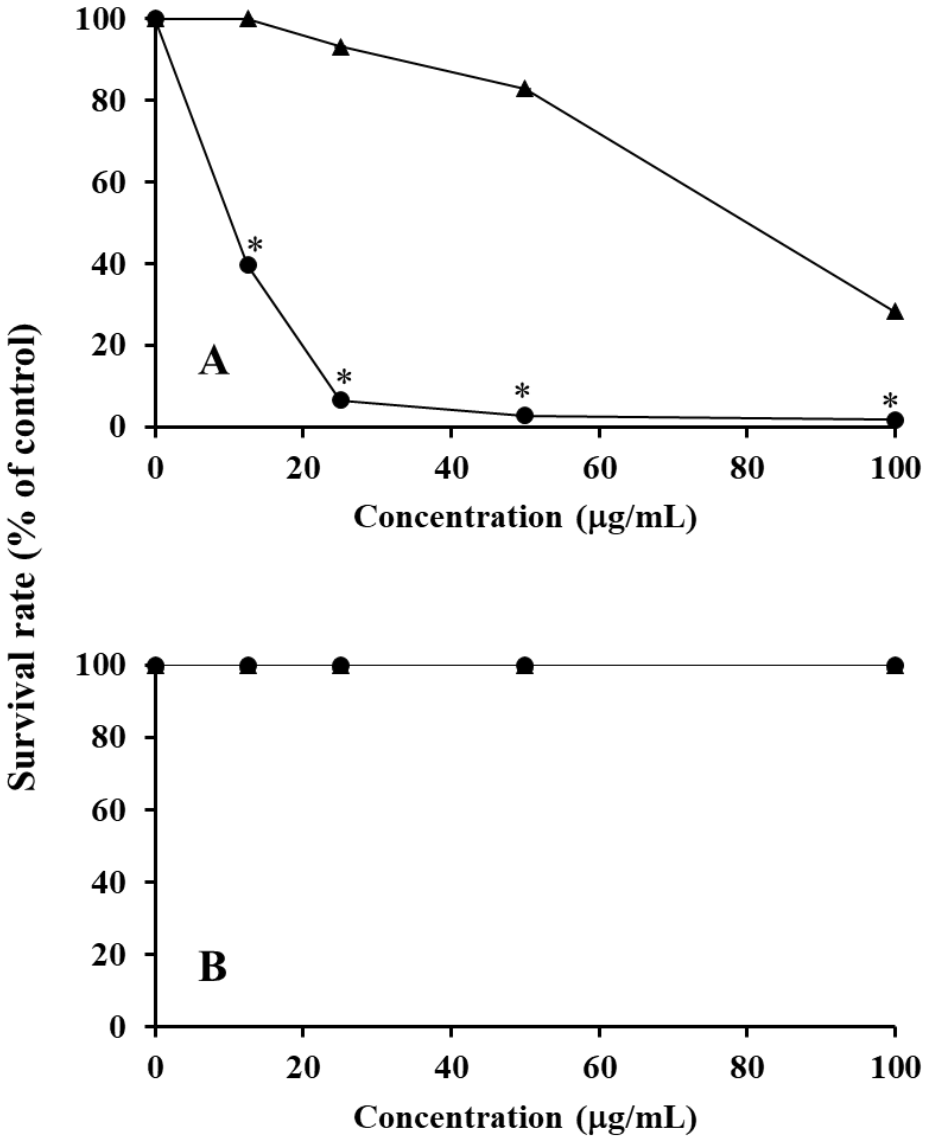


Fig. 3-6 The effects of compound **4** (▲) and compound **10** (●) on *Staphylococcus aureus* (A) and *Escherichia coli* (B). The indicated concentrations of compound **4** or **10** were added to the cell suspensions of the bacterial strains in PBS, and incubated at 37°C. After incubation for 4 h, the CFU of each assay mixture was measured as described in the text. Points are average of triplicate measurements, and bars indicate the standard deviation. Asterisks indicate significant differences between compound **4** and **10** ($p < 0.05$).

3.6 Conclusions

Comparative studies on the bioactivities of C18 hydroxy unsaturated fatty acid (compound **4**) and its synthesized propargylic derivative (compound **10**) revealed that compound **10** was much stronger than compound **4** in terms of the algicidal, tumour cell-killing, and bactericidal activities. This is the first finding that replacing a double bond in hydroxy unsaturated fatty acid with a triple bond can result in significant increase in the multiple bioactivities. Regarding structure-activity relationship of unsaturated fatty acids, our results suggest that triple bond is an important structural element influencing the bioactivities in addition to the chain length, the degree of unsaturation, and the functional group such as hydroxy group. Since the LC_{50} values of compound **4** and **10** against HAB species are much lower than those against tumor cells and bacteria, the hydroxy unsaturated fatty acids may be able to exhibit highly specific cytotoxicity toward marine microalgae, and are promising agents to control HABs or mitigate their impacts.

Chapter IV Induction of Apoptotic Cell Death in Human Leukemia U937 Cells by Compound 4

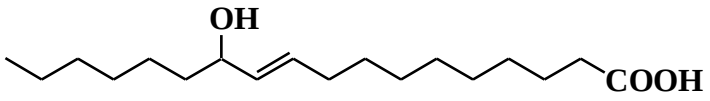
1. Introduction

Apoptosis is a programmed cell death, which was first reported by Kerr et al. (1972) [75]. Apoptosis is generally functioning in multicellular organisms as an important defense system, and extracellular or intracellular stresses can initiate specific cell death program [76-81]. For example, certain levels of DNA damage caused by radiation or mutagenic chemicals can lead to apoptosis, and apoptotic cells are effectively and immediately removed by phagocytic cells without any detrimental effects to surrounding tissues or cells. If the damaged cells can continue to live without apoptosis, they eventually develop to cancer cells and result in dysfunction of the normal tissues where the cancer cells propagated. On the other hand, effective apoptosis inducer can be promising cancer chemotherapeutic agents [82-84]. In fact, it is well-known that some clinically used antitumor agents induce DNA damage concomitant with apoptosis, and many screening studies seeking for new compounds with the ability to induce apoptosis in cancer cells have been conducted. A recent successful example is the anticancer drug trabectedin (ET 743), which had been isolated from the ascidian *Ectenascidia turbinata*. This marine natural product causes apoptotic cancer cell death through DNA damage by alkylation of the N2 position of guanine residue in the minor groove of DNA. Trabectedin (brand name Yondelis) is used as an FDA approved orphan drug for the treatment of ovarian cancer. Thus, discovery of novel compounds with apoptosis-inducing ability is a significantly valuable study. The human myeloid leukemia U937 cell line is often used for searching such compounds, and several natural agents have been discovered using U937 cells [85-88].

Although apoptosis is a complicated process and multiple intracellular pathways are involved, two main pathways are identified. The extrinsic pathway is initiated via the binding of death ligands such as TNF α and Fas on specific receptors, which transmit intracellular death signal toward apoptosis. Another intrinsic pathway is initiated by the nuclear stress signal. Then this signal is recognized by the cells as internal damage. If cells can unrepair this damage, the cells transmit its signal to the mitochondrial and no-

return phase leading to apoptosis.

In chapter III, I investigated the cytotoxicity of (*E*)-12-hydroxyoctadec-10-enoic acid (compound **4**) against various species including Gram negative and positive bacteria, harmful marine microalgae, and mammalian tumor cells, however, mechanism of the cytotoxic action of compound **4** remained unknown. It has been reported that some unsaturated fatty acids induce apoptosis in U937 cells [86-87]. Therefore, in this chapter, I investigated the cytotoxic mechanism of compound **4** against U937 cells to ascertain whether apoptotic cell death pathways are involved or not.



Compound 4: (*E*)-12-Hydroxyoctadec-10-enoic acid

Fig. 4-1 Structure of (*E*)-12-Hydroxyoctadec-10-enoic acid (compound **4**).

2. Materials and methods

2.1 Cell culture

The human myeloid leukemia U937 cells obtained from Riken Cell Bank (Tsukuba, Japan). The cells were cultured at 37 °C in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 and 95% air.

2.2 Chemicals and reagents

(*E*)-12-Hydroxyoctadec-10-enoic acid (**4**) was prepared as described in the previous chapter. Their structures are displayed in Fig. 4-1. The compound was dissolved in dimethyl sulfoxide (DMSO) at 4 mg/mL and filtered through 0.2 μm pore-size filter, and then used throughout the experiments as a stock solution.

2.3 Cytotoxicity assay

Cells (2×10^4 cells/well) in the growth medium in a 96-well plate with a vary concentrations of compound **4**. After 24 h of incubation at 37°C, the viability of the cells in the wells were measured by cell counting kit-8 (Cell Counting kit-8 Dojindo, Japan)

according to the manufacturer's instruction. The value of the control without test compounds was taken as 100%, and the results were expressed as % of viability.

2.4 Cytolytic assay

The cytolytic effects of compound **4** were determined by lactate dehydrogenase (LDH) assay. To the cell suspension in the growth medium in 96 well plate (2×10^4 cells/well), varying concentrations of compound **4** was added. After 24 h of incubation at 37°C, the plate was centrifuged at 1500 rpm for 20 min. The supernatants (50 μL) of the wells were transferred to the wells of a new 96-well plate and LDH assay was conducted according to the manufacturer's instruction (Cytotoxicity LDH Assay Kit – WST® Dojindo, Japan). The value obtained by complete cell lysis with detergent was taken as 100% cell lysis, and the results were expressed as % cell lysis.

2.5 Observation of cellular morphological changes

200 μL of cell suspension (2×10^6 cells/mL) in the growth medium in 1.5 mL centrifuge tubes with the indicated concentrations of compound **4**. After incubation at 37°C for 1 or 3 h, 50 μL aliquot of the cell suspension was withdrawn and mixed with or without Hoechst 33342 in PBS (final conc. 10 μg/mL). After the incubation at 37°C for 15 min, the cells were observed by using a fluorescence microscope (Keyence BZ-X710).

2.6 Detection of DNA fragmentation

Quantitative detection of DNA fragmentation was conducted by diphenylamine assay. 2 mL of the cell suspension in the growth medium in *Φ* 35 mm dish (2×10^6 cells/mL) was incubated with the indicated concentrations of compound **4** at 37°C for 3 h. The cells were harvested by centrifugation (2000 rpm for 10 min). The cell pellets were lysed in 300 μL of ice-cold lysis buffer (10 mM pH 7.4 Tris-HCl, 10 mM pH 8.0 EDTA· 2Na, 0.5% Trton X-100) on crash ice for a while. The mixture was centrifuged at 15000 rpm, 4 ℃ for 20 min. The cell pellets were subjected to diphenylamine assay as described previously [90].

3. Results and discussion

3.1 Cytotoxicity of compound 4 on U937 cells

As shown in Fig. 4-2, compound **4** shows cytotoxicity against U937 cells determined by CCK-8 assay after 24 h incubation in a concentration-dependent manner.

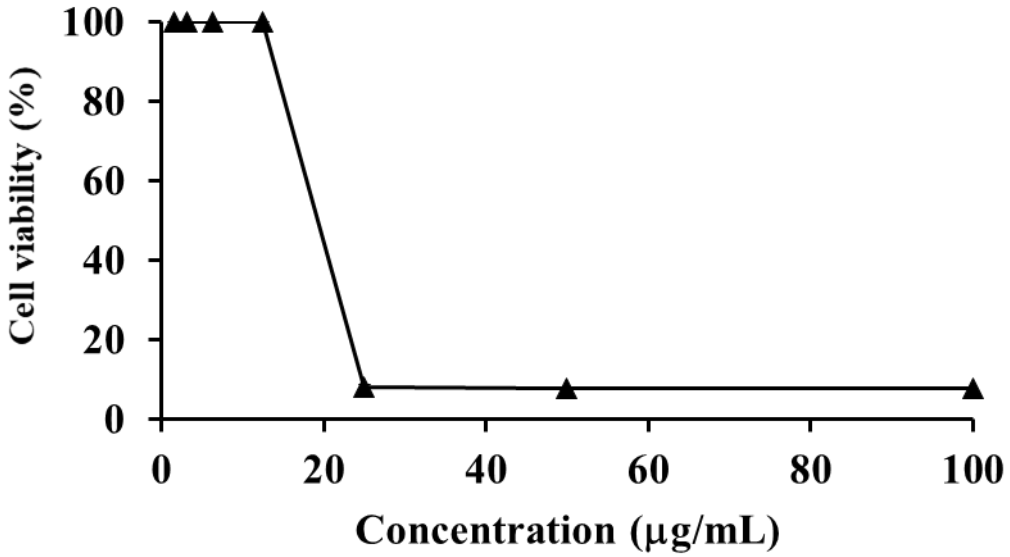


Fig. 4-2 Concentration-dependent cell viability of compound **4** (▲). Cells were treated with a diluted concentration of compound **4** in growth medium. After 24 h of incubation, cell viability was determined as described in the text. The point means an average of triplicate measurements.

3.2 Concentration-dependent cytolytic activity of compound 4 against U937 cell

Lactate dehydrogenase (LDH) is an enzyme required during the interconversion of pyruvate and lactate under Cori cycle to provide energy to cells. The LDH release of cell reflect integrity of the cell membrane. Thus, I investigated concentration dependence of cytolytic activity of compound **4** against U937 cells in Fig. 4-3. Compound **4** showed a concentration-dependent tendency on cytolytic activity.

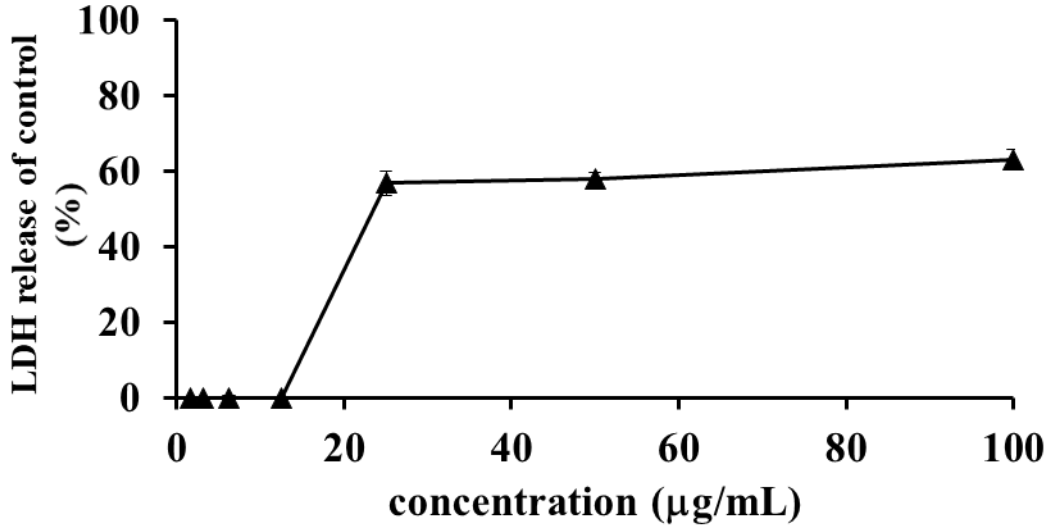
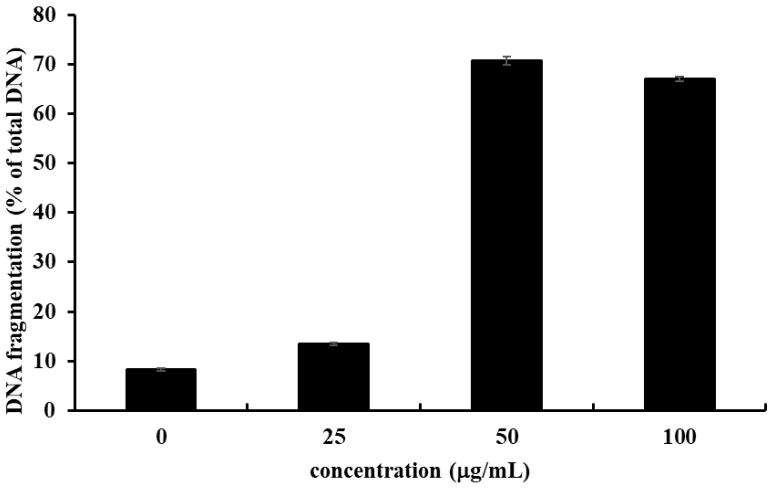


Fig. 4-3 Concentration-dependent cell cytolytic activity of compound **4**. Cells (2×10^4 cells/well) with a diluted concentration of compound **4** (max conc. 100 μg/mL) were incubated at 37 ℃ for 24 h in a 96-well plate. The cell suspension was centrifuged to separate supernatant and pellets. The supernatant was collected and conducted by LDH assay as described on the text. The cell cytolytic activity was determined by the LDH release of total cellular LDH contents. The point means an average of duplicate measurements and the bar indicates the standard deviation.

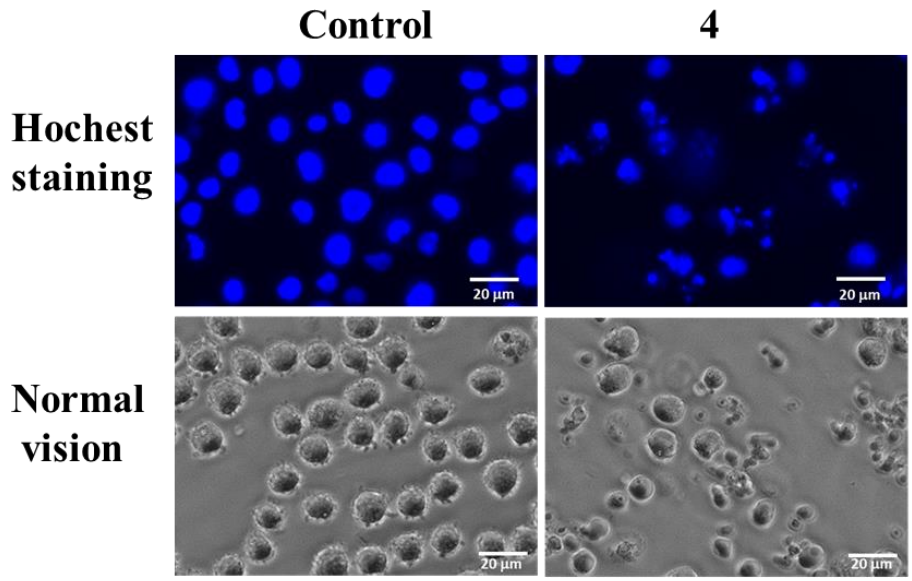
3.3 Effect of compound 4 on cell morphology change and DNA fragmentation.

When apoptosis is initiated, the cell nuclear change is always accompanied by degradation of DNA and the formation of DNA ladder. I delved DNA fragmentation by using diphenylamine assay (Fig. 4-4a). These results suggest that compound **4** induce DNA fragmentation in U937 cells at 50 and 100 μg/mL.

On the other hand, I examined cell nuclear and cell morphology changes (Fig. 4-4b) caused by treatment with compound **4**. The Hoechst 33342 stained cells displayed the control cells present intact nuclear structure, while the cells treated with compound **4** show chromosomal condensation. Direct observation of non-stained cell demonstrated that the treatment of compound **4** exhibited morphology changed in the majority of the cells.



(a)



(b)

Fig. 4-4 (a) ***Detection of DNA fragmentation by diphenylamine assay*.** U937 cells (2 × 10^6 cells/mL) was incubated with a variety of compound **4** in growth medium at 37 ℃ for 3 h, the cells were harvested and analyzed with diphenylamine assay. The data indicate the mean of triplicate measurements and the bar indicates the standard deviation. (b). ***Cell morph*ology *change***. Cells (2 × 10^6 cells/mL) was incubated with or without compound **4 (**50 μg/mL) in growth medium at 37 ℃ for 3 h. After Hoechst 33342

staining, the cells were immediately observed by microscope under fluorescent and path-contrast condition. The bar indicates 20 μm.

3.4 Conclusion

My previous studies have found that (±)-(*E*)-12-hydroxyoctadec-10-enoic acid (compound **4**) isolated from the red alga *Tricleocarpa jejuensis* showed cytotoxic effects on various species including harmful microalgae, Gram-positive bacteria, and mammalian tumour cells. Since natural products with apoptosis-inducing ability can be promising anti-cancer agents, in this study, I investigated the cytotoxic mechanism of compound **4** on U937 cells focusing on apoptosis induction. The effects of compound **4** on U937 cells were analyzed in terms of apoptotic features such as cell-lysis, DNA-fragmentation and nuclear-morphological changes. CCK-8 cytotoxicity assay revealed that compound **4** showed cytotoxicity on U937 cells with the LD_{50} of 20 μg/mL. Treatment with compound **4** at 50 μg/mL for 3 h resulted in significant apoptotic nuclear morphological changes, DNA fragmentation. Our results indicate that compound **4** could induce apoptosis of U937 cells.

Chapter V General conclusions

In this study, I investigated algicidal hydroxyl monounsaturated fatty acids of the red alga *Tricleocarpa jejuensis*: Isolation, Structure determination, synthesis, and biological activities.

Firstly, I identified (±)-(*E*)-9-hydroxyoctadec-10-enoic acid (**1**), (±)-(*E*)-10-hydroxyoctadec-8-enoic acid (**2**), (±)- (*E*)-11-hydroxyoctadec-12-enoic acid (**3**) and (±)-(*E*)-12-hydroxyoctadec-10-enoic acid (compound **4**) as the algicidal principles of the red alga *Tricleocarpa jejuensis*. These C18 hydroxy *trans*-monounsaturated fatty acids may arise from *cis*-vaccenic acid and oleic acid via photooxydation process.

As the result of the algicidal activity assay using synthesized **1**~**4** and their related compounds, following structural-activity relationships were observed: (1) hydroxy group is essential but replaceable with keto group, (2) the double bond can be replaced with a triple bond, (3) *cis/trans* geometry of the double bond is not important for the activity, (4) the carboxylic group can be replaced with a polar group such as hydroxy group. These findings may serve as important information in designing a novel anti-HAB agent.

Secondly, in the comparative studies on the bioactivities of compound **4** and its synthesized propargylic derivative compound **10**, it was revealed that compound **10** was much stronger than compound **4** in terms of the algicidal, tumour cell-killing, and bactericidal activities. Therefore, compound **10** can be regarded as a potential candidate for development of not only anti-HAB but also anticancer and antibacterial agent. The lethal activity of compound **4** and **10** against HAB species are much stronger than those against tumor cells and bacteria. Thus, these hydroxy unsaturated fatty acids might be promising lead compounds for creating safe, environmentally benign, microalgae-specific anti-HAB agent.

Finally, I investigated the apoptosis-inducing effect of compound **4** to gain an insight into the mechanism of action of the cytotoxic activity. Although this study is currently in progress, the preliminary results showed that compound **4** caused typical apoptotic morphological changes in U937 cells involving DNA fragmentation and cell-lysis.

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