# Acetylene Sesquiterpenoid Esters from the Green Alga *Caulerpa prolifera*

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Sixteen secondary metabolites of the green alga *Caulerpa prolifera* have been isolated, and their chemical structures elucidated by analysis of their spectroscopic data. Two groups of metabolites have been established, with either a 1,2-dihydro- (**2a**−**2i**) or a 1,2,3,3′-tetrahydro-2,3-didehydro (**3a**−**3f**) caulerpenyne carbon backbone. The terminal vinyl acetoxy group of caulerpenyne was substituted by various fatty acid residues. The antifouling activity of the algal extract was tested in laboratory assays against two of the major groups of fouling organisms (bacteria, microalgae).

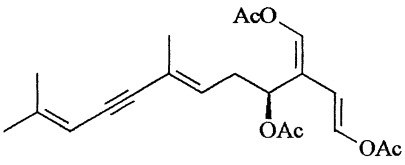
Green algae of the family *Caulerpaceae*, represented by the single genus *Caulerpa*, are found worldwide, generally in shallow water tropical and subtropical marine habitats. Mediterranean species of *Caulerpales* are also known. The genus *Caulerpa* has been widely studied, and the structures of many new compounds, such as triterpenoids and squalene derivatives, the nitrogen-containing compounds caulerpin and caulerpicin, and the diterpenoid alcohol caulerpol, have been described.[1−4] More recently, linear terpenoids possessing (or derived from) terminal *E*,*E*-1,4-diacetoxybutadiene moieties, like caulerpenyne, have been isolated from several *Caulerpa* species.[5−9] Unlike the former compounds, these latter metabolites, analogues of which are also isolated from phylogenetically related algae of the family *Udoteaceae*, appear to possess ichthyotoxic, cytotoxic, and feeding deterrent properties, thus aiding these algal species in enduring the great pressure in the tropics for food, space, and control of epibiont overgrowth.[5,6,10−13]

Marine fouling and corrosion caused by the growth of organisms on immersed surfaces generate serious and expensive problems in several maritime economic domains. Fouling development prevented by means of paints containing toxic compounds is associated with environmental and human health problems. Marine secondary metabolites might comprise a potential alternative for the prevention of fouling. As *Caulerpa* species are usually found in abundance and highly exposed in areas of significant herbivore populations, it seems likely that these uncalcified algae possess chemical deterrents to reduce predation.[1]

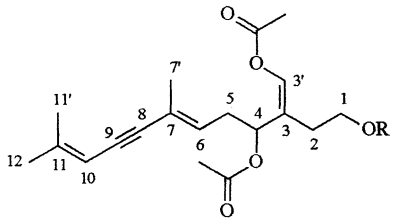
In the course of our investigations toward the isolation of bioactive metabolites from marine sources,[14−16] we describe here the isolation and structural elucidation of 16 metabolites of the *E*,*E*-1,4-diacetoxybutadiene class and the antifouling efficacy of *C. prolifera* extract. Metabolites **2c**, **2d**, **2f**−**2i**, and **3a**−**3f** are reported for the first time.

## Results and Discussion

The terpene esters isolated are grouped according to their structural similarity with respect to caulerpenyne (**1**). Caulerpenyne (**1**), which has been previously isolated from several *Caulerpa* species from the Mediterranean Sea (*C. prolifera*, *C. taxifolia*),[6,8,17] the Pacific Ocean, and the Caribbean Sea (*C. prolifera*, *C. racemosa*, *C. taxifolia*, *C. lanuginosa*, etc.),[4,5] was identified on the basis of its NMR and MS spectral data that were identical with those originally reported.[6,8]




**1**

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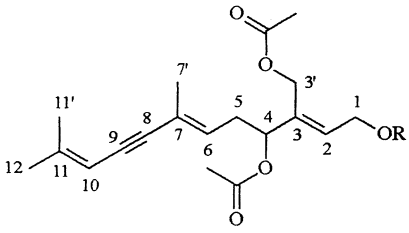


**3**

**2a**, **3d** R= $CH_3$-$(CH_2)_4$-(CH=CH-$CH_2)_2$-$(CH_2)_6$CO-

**2b**, **3c** R= $CH_3$-$(CH_2)_{14}$CO-

**2c** R= $CH_3$-$CH_2$-(CH=CH-$CH_2)_3$-$(CH_2)_6$CO-

**2d** R= $CH_3$-$CH_2$-(CH=CH-$CH_2)_5$-$(CH_2)_2$CO-

**2e** R= $CH_3$-$(CH_2)_{16}$CO-

**2f** R= $CH_3$-$(CH_2)_7$-CH=CH-$(CH_2)_9$CO-

**2g** R= $CH_3$-$(CH_2)_4$-(CH=CH-$CH_2)_4$-$(CH_2)_2$CO-

**2h**, **3e** R= $CH_3$-$CH_2$-(CH=CH-$CH_2)_3$-$(CH_2)_4$CO-

**2i** R= $CH_3$-$CH_2$-(CH=CH-$CH_2)_4$-$(CH_2)_3$CO-

**3a** R= $CH_3$-$(CH_2)_{12}$CO-

**3b** R= $CH_3$-$(CH_2)_5$-CH=CH-$(CH_2)_7$CO-

**3f** R= $CH_3$-$(CH_2)_7$-CH=CH-$(CH_2)_7$CO-

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**Table 1.** NMR Data[a] of 1,2-Dihydro-1-caulerpenyne (**2**) and 1,2,3,3′-Tetrahydro-2,3-didehydro-1-caulerpenyne (**3**)

| | 1,2-dihydro-1-caulerpenyne moiety | | 1,2,3,3′-tetrahydro-2,3-didehydro-1-caulerpenyne moiety | | |
|---|---|---|---|---|---|
| position | $\delta_H$[b] | $\delta_C$ | $\delta_H$ | $\delta_C$ | HMBC (C)[c] |
| 1 | 4.18, 4.08 (dt, 10.2, 10.7, 7.3) | 62.7 | 4.69 (br d, 6.6) | 59.9 | carbonyl of the lipid ester |
| 2 | 2.33 (m) | 27.0 | 5.82 (br t, 6.6) | 128.1 | 3′, 4 |
| 3 | | 118.4 | | 136.3 | |
| 3′ | 7.03 (br s) | 133.8 | 4.66 (br s) | 59.3 | 2, 3, 4, Ac-3′ (CO) |
| 4 | 5.83 (t, 7.3) | 69.5 | 5.24 (t, 6.8) | 74.3 | 2, 3, Ac-4 (CO) |
| 5 | 2.56, 2.35 (ddd, 14.6, 7.3, 7.3) | 32.0 | 2.46 (m) | 31.9 | 4, 6, 7 |
| 6 | 5.63 (br t, 7.3) | 129.8 | 5.65 (br t, 7.3) | 129.9 | 7′, 8 |
| 7 | | 121.4 | | 121.3 | |
| 8 | | 94.0 | | 94.0 | |
| 9 | | 85.2 | | 85.2 | |
| 10 | 5.32 (br s) | 105.2 | 5.32 (br s) | 105.2 | 8, 11′, 12 |
| 11 | | 148.2 | | 148.2 | |
| 7′ | 1.81 (br s) | 17.7 | 1.79 (br s) | 17.8 | 6, 7, 8 |
| 11′ | 1.86 (br s) | 20.9 | 1.87 (br s) | 20.8 | 9, 10, 11, 12 |
| 12 | 1.78 (br s) | 24.8 | 1.79 (br s) | 24.8 | 9, 10, 11, 11′ |
| Ac-3′ (CO)[d] | | 167.3 | | 170.5 | |
| Ac-3′ (Me)[e] | 2.14 (s) | 20.7 | 2.04 (s) | 21.0 | Ac-3′ (CO) |
| Ac-4 (CO)[d] | | 169.9 | | 169.9 | |
| Ac-4 (Me)[e] | 2.03 (s) | 21.1 | 2.03 (s) | 21.1 | Ac-4 (CO) |

[a] Solvent $CDCl_3$. [b] $^1H$ chemical shift values ($\delta$ ppm from $SiMe_4$) followed in parentheses by multiplicity and then coupling constants ($J$ in Hz). [c] HMBC correlations from H to C. [d,e] Resonances may be interchanged

**Table 2.** Growth Inhibition Zone (mm) Activity of the *C. prolifera* Extract against Six Marine Bacteria

| | bacterial strains | | | | | |
|---|---|---|---|---|---|---|
| sample | $B_1$ Gram (+) | $B_2$ Gram (−) | $B_3$ Gram (+) | $B_4$ Gram (−) | $B_5$ Gram (+) | $B_6$ Gram (−) |
| control | 0 | 0 | 0 | 0 | 0 | 0 |
| TBTO | 65 | 45 | 18 | 15 | 18 | 12 |
| *C. prolifera* extract | 11 | 12 | 15 | 0 | 0 | 0 |

Nine esters (**2a**−**2i**), sharing the same 1,2-dihydro caulerpenyne (**2**) sesquiterpene skeleton previously described,[6,7] comprised the first group of metabolites isolated. Characteristic features in the $^1H$ NMR spectra of **2a**−**2i** (Table 1) denote the presence of fatty acid chains, including a nonsymmetrical triplet at $\delta$ 0.85 ($\delta_C$ 14.1 q), assigned to the terminal methyl group of the fatty acid residue, and an intense broad resonance at $\delta$ 1.23 ($\delta_C$ 29.0 t) assigned to the shielded aliphatic chain methylenes. The triplet at $\delta$ 2.27 was assigned to the α-methylene of the fatty acid chain ($\delta_C$ 34.2 t).

The second group of compounds is characterized by the sesquiterpenoid moiety of 1,2,3,3′-tetrahydro-2,3-didehydro caulerpenyne (**3**), which has been previously reported only as an aldehyde from *C. taxifolia*.[6] The $^1H$ NMR resonances for the C(6)−C(11) conjugation system of metabolites **3a**−**3f** are nearly superimposable to those of caulerpenyne (**1**) (Table 1). The presence of the vinylic (Ac-3′) and the allylic (Ac-4) acetoxy-methyl groups is confirmed by the $^1H$ NMR singlets at $\delta$ 2.04 (3H) and 2.03 (3H), which were correlated by HMBC with the carbonyls resonating at $\delta$ 170.5 (s) and 169.9 (s), respectively. Characteristic in the $^1H$ NMR spectrum are the H-7′, H-11′, and H-12 methyl groups, noted as broad singlets, due to long-range coupling with H-6 and H-10, respectively, deduced from the COSY, HMQC, and HMBC experiments.

The HMBC spectrum revealed correlations of H-6 with C-7′ and H-10 with C-11′ and C-12, while the COSY spectrum revealed correlations of H-5 with the triplets at $\delta$ 5.65 (H-6) and 5.24 (H-4). Characteristic are the resonances at $\delta$ 4.66 (br s), 4.69 (br d), and 5.82 (br t), which were not present in the $^1H$ NMR spectra of **2**, and suggest the presence of two oxygenated vinyl methylenes and one olefinic methine, respectively. The broad singlet at $\delta$ 7.03 of the deshielded H-3′ observed in both **1** and **2** is missing. The *E*-configuration of the C-2, C-3 double bond is supported by an intense NOE between H-2 and H-4 and H-5, while no correlation is observed between H-3′ and H-2.

Six esters of **3** were recognized by analysis of the $^1H$ and $^{13}C$ NMR spectra of **3a**−**3f**, in which the presence of fatty acid chains is denoted as previously described. The identity of the fatty acid was established and confirmed following GC/MS and HRMS analyses.[18] Analyses of the 2D NMR data lead to the assignments of $^1H$ and $^{13}C$ signals summarized in Table 1.

The antibacterial activity of the *C. prolifera* extract was tested against six unidentified marine bacterial strains (Table 2). The extract exhibited moderate to significant activity against two Gram-positive and one Gram-negative marine bacteria. The measured inhibition zone varied from 17 to 83% of that observed with the biocide TBTO [bis-(tributylin) oxide)].

The growth inhibitory effect of the *C. prolifera* extract was evaluated against the marine microalga *Phaedactylum tricornutum*, a representative of the primary surface colonizers.[19] The extract proved to be as effective as TBTO against the development of the fouling microalga, as shown in Figure 1, thus rendering the *C. prolifera* extract a potential biocide candidate.

## Experimental Section

**General Experimental Procedures.** $^1H$ and $^{13}C$ NMR spectra were recorded using Bruker AC 200, DRX 400, and Oxford 300 spectrometers. Chemical shifts are given in $\delta$ (ppm) scale using TMS as internal standard (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). High-resolution FAB mass spectral data were recorded on a JEOL AX505HA mass selective detector and were provided by the University of Notre

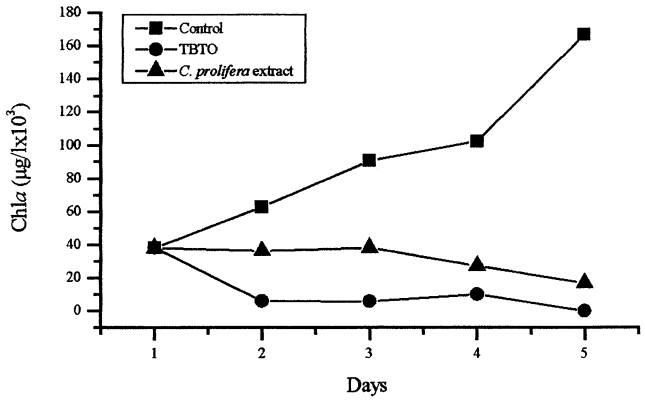


**Figure 1.** Inhibition effect on the growth of *P. tricornutum* (as presented by chorophyll concentration).

Dame, Department of Chemistry and Biochemistry, Notre Dame, Indiana. Low-resolution EI mass spectral data were recorded on a HP 5973 mass selective detector. Column chromatography was performed with Kieselgel 60 (Merck). HPLC was conducted using a Hewlett-Packard Agilent 1100 Pharmacia LKB 2248 model and an EBC LC-1240 equipped with a refractive index detector, with Spherisorb S10W and Supelcosil SPLC-Si CC1593 25 cm × 10 mm, 5 $\mu$m, columns. TLCs were performed with Kieselgel 60 $F_{254}$ (Merck aluminum support plates). Absorbance measurements were performed in a Unicam Helios $\gamma$ spectrophotometer.

**Plant Material.** *C. prolifera* collected from shallow habitats in Saronicos Gulf, Greece, was kept frozen until analyzed, and specimens are deposited at the herbarium of the Laboratory of Pharmacognosy and Chemistry of Natural Products (ATPH/MO/112). Bacterial strains $B_1$−$B_6$, isolated from the surface of various submerged objects, are kept in the culture collection of the Laboratory of Pharmacognosy and Chemistry of Natural Products.

**Extraction and Isolation.** The organism was initially freeze-dried (351.0 g dry weight) and then exhaustively extracted at room temperature with mixtures of $CH_2Cl_2$/MeOH (3:1, v/v). Part of the organic extract (37.4 g), after evaporation of the solvents, was subjected to vacuum column chromatography (VCC) on silica gel using cyclohexane with increasing amounts (10%) of EtOAc and finally MeOH. Part (2.3 g) of the cyclohexane−EtOAc (60:40) eluate (3.3 g) was further chromatographed on a silica gel column using cyclohexane with increasing amounts (2%) of EtOAc. Part of the cyclohexane−EtOAc (84:16) eluate (110 mg out of 0.3 g total) was further purified by HPLC using cyclohexane−EtOAc (85:15) as the eluent to afford **1** (15.9 mg). Part of the cyclohexane−EtOAc (88:12) eluate (183.8 mg out of 0.2 g) was further purified by HPLC using cyclohexane−EtOAc (95:5) as the eluent to afford **2a** (22.8 mg), **2b** (18.3 mg), **2c** (14.1 mg), **2d** (9.8 mg), **2e**−**2f** (1.8 mg), **2g** (4.5 mg), **2h**−**2i** (7.5 mg), **3a**−**3c** (7.8 mg), and **3d** (7.2 mg). Part of the cyclohexane−EtOAc (86:14) eluate (59.8 mg out of 0.2 g) afforded **3e** (1.8 mg) and **3f** (0.8 mg), after purification by HPLC using cyclohexane−EtOAc (92:8) as the eluent. The structure identification was based on analyses of their spectral characteristics in comparison with previously reported spectral data.[6−8]

The fatty-acid methyl esters (FAMEs) were analyzed by gas chromatography−mass spectrometry (GC/MS) using a fused silica capillary column (HP-5, 30 m × 0.25 mm, film thickness 0.25 $\mu$m). Initial oven temperature was set to 140 °C and increased at a rate of 2 °C/min up to 220 °C, at which it was held for 5 min. The injection port temperature was set to 250 °C. Helium was used as the carrier gas. Individual FAMEs were identified by comparison of their retention times and mass spectra with those of authentic standards (Supelco 37 component FAME Mix, Bellefonte, PA).

**Chemical Modifications.** The caulerpenyne esters (2 mg) were dialyzed in 10 mL of 1 N HCl in MeOH. The mixture was stirred overnight with reflux. After the completion of the hydrolysis, the solvent was evaporated and the residue was dialyzed in water and extracted with $CH_2Cl_2$. The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated in vacuo. The methyl esters of the fatty acids were analyzed as previously described.

**Biological Assays. Antibacterial Activity.** Antibacterial testing of the extract was performed by the disk diffusion technique in agar-plated Petri dishes.[20] Whatman filter paper disks (6 mm diameter) were used. A sample consisting of algae extract (300 $\mu$g) diluted in $CH_2Cl_2$ was loaded on the disks and allowed to dry at room temperature. Bacterial cultures of six unidentified bacterial strains were grown in liquid marine broth (2216 DIFCO) overnight, and 0.1 mL samples of the culture ($10^6$ CFU/mL) were spread over the agar. After incubation for 2 days at 20 °C, the activity was evaluated on the basis of the inhibition zones around the disks. Control tests with the solvents were also performed and showed no inhibition of the microbial growth. In addition, the biocide TBTO (1 $\mu$g) was used as a positive control to check the sensitivity.[21] All inhibition assays were carried out in duplicate.

**Growth Inhibition on the Microalga *Phaedactylum tricornutum*.** *P. tricornutum* of the class Bacillariophyceae was obtained from the UTEX Culture Collection of Algae, University of Texas, Austin (UTEX 646).

An inoculum of cultivated microalgae was introduced into 1 L of autoclave-sterilized (25 min at 121 °C) Jones medium (pH 7.6) to a final concentration of $7 \times 10^4$ cells/mL and was cultivated for 5 days until the exponential growth phase ($5 \times 10^5$ cells/mL) was reached. Sterile conditions were maintained at all stages of transfer and culturing, although the cultures were not axenic. A 1.2 mg portion of the algal extract was then introduced in a conical flask (100 mL), and 40 mL of the microalgal culture was added to a final concentration of 30 $\mu$g of extract per milliliter of culture. The flasks were then incubated in a constant-temperature-controlled growth room at 20 ± 1 °C. "Cool white" fluorescent Sylvania Grolux tubes provided illumination levels of 2200 erg/cm$^2$ s, at 14:10 light: dark cycle. Flasks containing TBTO (1 $\mu$g/mL) were set up as positive controls. The assays were carried out in duplicate, and the mean values are shown in Figure 1.

Cell growth was estimated for 5 days by daily measurements of Chl *a*. An aliquot (1 mL) of culture was filtered through a GF-F Whatman filter (47 mm diameter) under vacuum ($P$ < 0.5 bar). Filters were extracted with 5 mL of acetone (90%), and the extract was centrifuged at 4000 rpm for 10 min. Chl *a* concentration was determined at the supernatant according to the protocol described by McKinney.[22]

Cell numbers were determined by microscopical counting using a Neubauer "improved" haematocytometer.

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