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Minor Bioactive Dihydrophenanthrenes from *Juncus effusus*

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Seven new minor dihydrophenanthrenes have been isolated from *Juncus effusus* L. Structures have been defined by spectroscopic means. An antialgal assay, using the green alga *Selenastrum capricornutum*, showed good activity for six of them. The presence of both polar and nonpolar substituents in these molecules seems to be important for the bioactivity.

The presence in many macrophytes of metabolites that inhibit microalgal growth in vitro has been documented in many papers,[1] and recently we have proved that bioactive compounds of *Pistia stratiotes* are released into the environment.[2] The allelopathic implications of such observations could play an important role in the defense of aquatic ecosystems, and, in pursuing our studies, we are currently investigating *Juncus effusus* L. (Juncaceae), a wetland plant widely distributed in the Mediterranean area.

In previous papers we reported the isolation of 18 free[3,4] and nine glucosylated[5,6] 9,10-dihydrophenanthrenes from the aereal parts of the plant, many of them having strong inhibitory activity on *Selenastrum capricornutum*,[7] the algal species selected for studies in aquatic environments.[8] In a study on phytoalexins, Stoessl et al.[9] identified dihydrophenanthrenes in bulbs of various Orchidaceae produced in response to infection by phytopathogenic fungi. Our unpublished observations show that *J. effusus* is infected by fungi, and Kohlmeyer et al. have recently reported[10] that new species of fungi are present in *Juncus roemerianus*. Thus, the high content of dihydrophenanthrenes in Juncaceae could represent their response to fungal infections.

Results and Discussion

Eighteen dihydrophenanthrenes were previously isolated from the ethyl ether extract of *J. effusus*,[3,4] while nine glucosyl derivatives were obtained from the MeOH extract.[5,6] A reexamination of the less polar components of the latter extract has led to the isolation of seven new dihydrophenanthrenes **1**−**7**, identified on the basis of their spectroscopic features. The ^{1}H-NMR and the ^{13}C-NMR data, respectively, are reported in Tables 1 and 2.

The EIMS spectrum of **1** had an apparent molecular ion peak at *m/z* 266 and showed 18 carbon signals in the ^{13}C-NMR spectrum consistent with a molecular formula of $C_{18}H_{18}O_2$. The DEPT experiment showed four aromatic methine carbons and eight quaternary aromatic carbons, a methylene and a methine olefinic carbons, an oxygenated methylene carbon, two aliphatic methylene carbons, and a methyl carbon. The ^{1}H-NMR spectrum showed two ortho-coupled protons at δ 6.70 and 7.41; two meta-coupled protons at δ 7.21 and 7.43; three vinyl protons as double doublets at δ 6.99, 5.75, and 5.29; two methylene protons as a singlet at δ 4.72; four protons of two methylenes as a broad singlet at δ 2.76, and a methyl singlet at δ 2.28. ^{1}H−^{13}C one-bond COSY showed the correlations of these protons with the corresponding carbons. The locations of the methyl at C-1, the hydroxyl at C-2, and the protons at C-3 and C-4 were derived from ^{1}H−^{13}C long-range COSY heterocorrelations: the H-3 proton had cross peaks with the C-1 and the C-4a carbons at δ 120.6 and 126.8, respectively. Both these carbons were correlated to the methylene protons at δ 2.76, while the first also had correlation with the H-11 methyl at δ 2.28. The H-4 proton, along with the methylene protons at δ 2.76, gave cross peaks with the C-1a carbon at δ 139.1 and had further correlations with the C-2 and C-5a carbons at δ 152.7 and 132.0 Carbon C-5a was also correlated to the H-12 vinyl proton at δ 6.99 and both the aromatic protons at δ 7.43 and 7.21. The coupling of these two protons agreed with the presence of the hydroxymethyl group at C-7 (δ 140.1). The H-6 and H-8 protons did not show further cross peaks, so that the assignment of chemical shifts to them was based upon the higher downfield shift of the H-6 proton already observed in other similar 7-substituted compounds.[3,4] Thus, structure 2-hydroxy-7-(hydroxymethyl)-1-methyl-5-vinyl-9,10 dihydrophenanthrene was assigned to compound **1**.

The isomeric structure 2-hydroxy-6-(hydroxymethyl)-1-methyl-5-vinyl-9,10-dihydrophenanthrene was assigned to compound **2**. Compound **2** also showed an apparent molecular ion peak at *m/z* 266 in the EIMS spectrum and 18 carbon signals in the ^{13}C-NMR spectrum. The chemical shifts of H-3, H-4, and H-11, as well as those of C-1−C-4, C-1a, and C-4a, similar to those of **1**, justified the same substitution pattern in the A ring. In addition to the H-3 and H-4 protons in the ^{1}H-NMR spectrum, two ortho-coupled protons at δ 7.30 and 7.58 were present, indicating that the hydroxymethyl group could either be at C-6 or at C-8. It was located at C-6

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Table 1. ^{1}H-NMR Data of Dihydrophenanthrenes **1**−**7** [δ ppm (J = Hz)]

H	**1**[a]	**2**[b]	**3**[b]	**4**[c]	**5**[c]	**6**[c]	**7**[c]
3	6.70 d (8.4)	6.80 d (8.4)	6.80 d (8.3)	6.72 d (8.3)	6.71 d (8.5)	6.70 d (8.3)	6.73 d (8.2)
4	7.41 d (8.4)	7.48 d (8.4)	7.44 d (8.3)	7.41 d (8.3)	7.31 d (8.5)	6.93 d (8.3)	7.95 d (8.2)
6	7.43 d (1.6)	7.58 d (8.0)	7.62 d (1.6)	6.70 s	7.05 s	6.90 s	
7		7.39 d (8.0)					
8	7.21 d (1.6)		6.94 d (1.6)				6.97 s
9	2.76 br s	2.73 m	2.69 br s	2.69 m	2.68 br s	2.89 m	2.4−2.9
10	2.76 br s	2.88 m	2.69 br s	2.69 m	2.68 br s	2.89 m	2.4−2.9
11	2.28 s	2.19 s	2.13 s	2.20 s	2.21 s	2.21 s	2.19 s
12	6.99 dd (10.8 17.4)	6.88 dd (11.4 17.9)	4.58 s	4.67 s	4.73 s	4.88 q (5.3)	4.92 s
13	5.75 dd (1.5 17.4) 5.29 dd (1.5 10.8)	5.57 dd (2.3 11.4) 5.29 dd (2.3 17.9)				1.58 d (5.3)	5.65 s
14	4.72 s	4.63 s	2.23 s	2.33 s	2.24 s	2.25 s	2.21 s
OMe					3.89 s		

[a] $CDCl_3$. [b] Acetone-d_6. [c] CD_3OD.

Table 2. ^{13}C-NMR Chemical Shifts of Dihydrophenanthrenes **1**−**7** (δ ppm)

C	**1**[a]	**2**[b]	**3**[b]	**4**[c]	**5**[c]	**6**[c]	**7**[c]
1	120.6	120.6	121.4	121.3	121.6	121.9	122.6
2	152.7	155.8	155.8	154.2	154.1	154.6	156.3
3	111.7	113.9	113.7	111.9	111.8	111.6	113.9
4	128.0	126.5	129.9	126.2	126.3	127.3	126.9
4a	126.8	127.3	126.6	128.6	128.8	124.5	127.5
1a	139.1	139.2	142.0	138.0	137.9	140.0	140.2
5	136.0	136.5	133.2	136.1	135.8	139.0	119.3
6	125.3	138.7	122.7	114.4	109.8	112.4	159.3
7	140.1	122.9	122.0	153.4	152.3	159.5	123.1
8	128.0	123.1	122.4	120.9	120.4	121.1	132.0
8a	138.6	138.5	134.2	138.3	138.1	139.0	131.7
5a	132.0	135.2	130.5	127.6	125.4	127.9	133.6
9	29.9	26.6		25.5	25.4	27.2	30.5
10	25.5	26.2	25.7	25.0	25.1	26.3	27.3
11	11.7	11.5	11.5	11.6	11.6	11.7	12.0
12	138.6	135.2	62.6	63.4	62.1	75.6	79.0
13	114.0	120.6				23.6	107.8
14	65.2	63.1	16.1	11.6	11.6	11.7	15.3
OMe					55.7	55.5	

[a] $CDCl_3$. [b] Acetone-d_6. [c] CD_3OD.

on the basis of the chemical shifts of the H-13 protons. In all of the isolated 5-vinyl-9,10-dihydrophenanthrenes bearing a substituent at C-6, the H-13 trans appears at higher field than cis, while the opposite was observed in compounds with a hydrogen at that position.[3−5] In the ^{1}H−^{13}C long-range COSY experiment, the H-12 proton at δ 6.88 and the H-14 methylene protons at δ 4.63 were correlated to the same carbon at δ 138.7 attributed to C-6. The heterocorrelations of the proton at δ 7.39 with the C-6 and C-5a carbons at δ 138.7 and 135.2 and those of the proton at δ 7.58 with the C-5 and C-8a carbons at δ 136.5 and 138.5 allowed assignment of the former to C-8 and the latter to C-7.

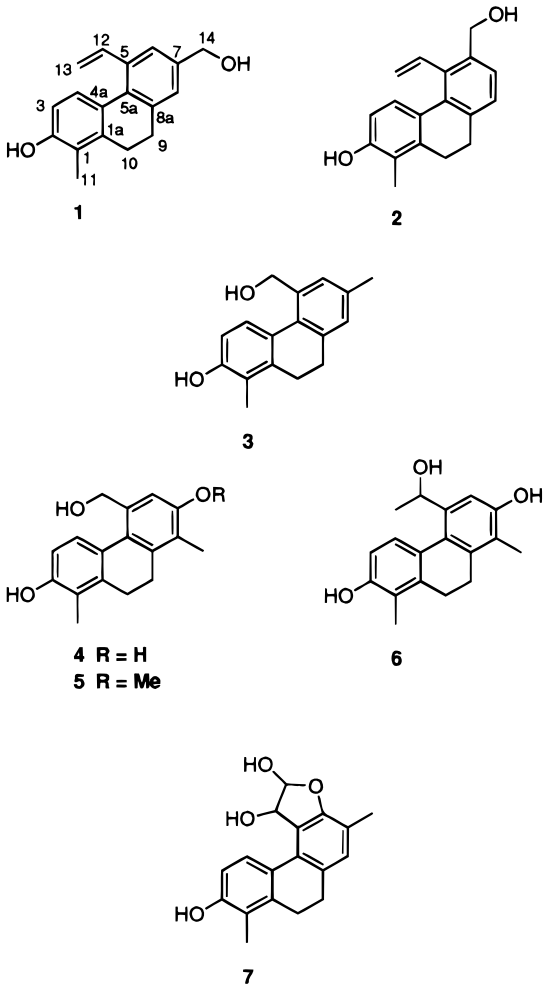
Compounds **3**−**5** had a hydroxymethyl group at C-5 instead of the vinyl chain. 2-Hydroxy-5-(hydroxymethyl)-1,7-dimethyl-9,10-dihydrophenanthrene (**3**) had only 17 carbon signals in the ^{13}C-NMR spectrum and gave an apparent molecular ion at m/z 254 in the EIMS spectrum consistent with a molecular formula of $C_{17}H_{18}O_2$. In the ^{1}H-NMR spectrum, the ortho-coupled protons H-3 and H-4 were at δ 6.80 and 7.44, and the meta-coupled protons H-6 and H-8 were at δ 7.62 and 6.94. In the spectrum the H-12 methylene singlet at δ 4.58, the H-11 and H-14 methyls at δ 2.13 and 2.23, and the H-9 and H-10 methylene protons as a singlet at δ 2.69 were also apparent. The heterocorrelations of the H-12, H-6, and H-8 protons with the C-5a carbon at δ 130.5 in the ^{1}H−^{13}C long-range COSY experiment were consistent with the location of the hydroxymethyl group at C-5.

Structures 2, 7-dihydroxy-5-(hydroxymethyl)-1,8-dimethyl-9,10-dihydrophenanthrene and 2-hydroxy-5-(hydroxymethyl)-7-methoxy-1,8-dimethyl-9,10-dihydrophenanthrene were easily assigned to compounds **4** and **5**, respectively, as they were previously isolated from the plant as glucosyl derivatives.[6] Dihydrophenanthrene **4** had the H-3, H-4, and H-6 aromatic protons at δ 6.72, 7.41, and 6.70; the H-12 methylene at δ 4.67; the H-9 and H-10 methylenes at δ 2.69; and the H-11 and H-14 methyls at δ 2.20 and 2.33 in the ^{1}H-NMR spectrum. The methoxy derivative **5** had the H-6 proton shifted downfield to δ 7.05 and the C-6 carbon shifted upfield to δ 109.8 consistent with the presence of a methoxyl group at C-7. Furthermore, a NOE experiment showed interaction between the methoxyl methyl at δ 3.89 and the H-6 proton.

Compound **6** was an isomer of a previously isolated dihydrophenanthrene 5-(1-ethoxy)-2,7-dihydroxy-1,8-dimethyl-9,10-dihydrophenanthrene. The EIMS spectrum gave an apparent molecular ion at m/z 284 consistent with the molecular formula $C_{18}H_{20}O_3$. The side chain, in the ^{1}H-NMR spectrum, contained a carbinol proton as a quartet at δ 4.88 and a methyl doublet at δ 1.58. The spectrum also showed the H-3 and H-4 protons at δ 6.70 and 6.93, the H-6 proton at δ 6.90, the H-9 and H-10 methylenes at δ 2.89, and the H-11 and H-14 methyls at δ 2.21 and 2.25, respectively. In the ^{1}H−^{13}C long-range COSY spectrum, the H-3 proton was correlated to C-1 and C-4a at δ 121.9 and 124.5; the H-4 proton was correlated to C-2, C-1a, and C-5a at δ 154.6, 140.0, and 127.9; the H-6 and the H-14 methyl protons at δ 2.25 were correlated to C-5a and C-8 at δ 127.9 and 121.1.

Dihydrophenanthrene **7** showed 18 carbon signals in the ^{13}C-NMR spectrum, which, along with the molecular ion at m/z 298 in the EIMS spectrum, justified assignment of molecular formula $C_{18}H_{18}O_4$. In addition to 12 aromatic carbons, three of them protonated, the ^{13}C-NMR data showed the presence of two methine carbons at δ 79.0 and 107.8, two methylene carbons at δ 30.5 and 27.3, and two methyl carbons at δ 12.0 and 15.3. The ^{1}H-NMR spectrum contained signals for three aromatic protons as two coupled doublets at δ 6.73 and 7.95, and a singlet at δ 6.97, two broad singlets at δ 4.92 and 5.65, two methyl singlets at δ 2.19 and 2.21, and four protons in the 2.4−2.9 ppm range. The ^{1}H−^{1}H one-bond COSY showed a cross peak between the protons at δ 4.92 and 5.65, and in the ^{1}H−^{13}C one-bond COSY they were correlated to the carbons at δ 79.0 and 107.8, respectively. The ^{1}H−^{13}C long-range COSY

spectrum showed the heterocorrelations of the H-3 proton with C-1 and C-4a and those of the H-4 proton with C-2, C-1a, and C-5a. Furthermore, the H-12 proton at δ 4.92 and the H-8 proton at δ 6.97 were correlated to C-5a at δ 133.6, and the latter proton was also correlated to C-6 (δ 159.3). This carbon also gave a cross peak with the H-13 proton at δ 5.65 and the H-14 methyl protons at δ 2.21. Structure **7**, with a hemiacetal ring, was assigned on the basis of the above data and the shape of the H-12 and H-13 signals which suggest a trans orientation of the hydroxyl group.



All the dihydrophenanthrenes isolated from *J. effusus* have a hydroxyl function at C-2 supporting the hypothesis that these metabolites arise from L-phenylalanine through *m*-hydroxycinamic acid.[11]

Dihydrophenanthrenes **1**−**7** were assayed in broth at four different concentrations (10^{-4} M, 5×10^{-4} M, 10^{-5} M, and 5×10^{-6} M) against the green unicellular alga *S. capricornutum* (strain UTEX 1648). The antialgal activity was calculated as percentage of growth inhibition caused by the compounds and is reported in Table 3. All of the dihydrophenanthrenes were active, causing a decrease of the algal growth rate during the exponential phase of growth. Dihydrophenanthrenes **3**−**5** were the most active (70−80% inhibition at 10^{-4} M and 18−20% at 10^{-5} M). Compounds **1** and **2**, with the vinyl side chain, were slightly active at 10^{-4} M and did not inhibit algal growth at 10^{-5} M. The least active was hemiacetal **7**, causing only 20% of inhibition at 10^{-4} M. The activity observed is in agreement with that previously reported for the other dihydrophenanthrenes isolated from *J. effusus*.[7] The presence on the C ring of both polar and nonpolar groups seems to be important for the antialgal activity of these compounds. Structure−activity studies previously carried out on aromatic phenylpropanoid compounds[12,13] led to the same result.

Table 3. Inhibition (%) of Growth of *S. capricornutum* with Respect to the Blank[a]

M	1	2	3	4	5	6	7
10^{-4}	57c	64c	80d	70d	80d	75d	20c
5×10^{-5}	38b	40b	50c	40c	55c	52c	10b
10^{-5}	0a	5a	20b	10b	18b	20b	0a
5×10^{-6}	0a	0a	7a	4a	0a	5a	0a

[a] Values followed by different letters are statistically significant. Test LSD $-p \leq 5\%$.

Experimental Section

General Experimental Procedures. EIMS were obtained with a Kratos MS 80 apparatus. NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker AC 400 spectrometer. One-bond and long-range ^{1}H−^{13}C COSY experiments were performed with the XHCORR microprogram using delays corresponding to $J_{C,H}$ 160 and 8 Hz, respectively. IR spectra were determined in $CHCl_3$ solutions on a FT-IR Perkin-Elmer 1740 spectrometer. Reversed-phase HPLC was performed using LiChrosorb RP18 columns [MeCN−MeOH−H_2O (4:9:7) for **1** and MeOH−H_2O (7:3) for **3**, **5**, and **6**] and LiChrosorb RP8 columns [MeCN−MeOH−H_2O (4:9:7)] for **4** and **7**. Sephadex LH-20 was used for column chromatography.

Plant Collection and Extraction. Plants of *J. effusus* were collected during spring and summer 1991, near Naples. A voucher specimen is on deposit at the Botanical Garden of Naples. The plants were dried at room temperature and sequentially extracted with Et_2O and MeOH. The extracts were frozen and stored at −20 °C until used.

Methanolic Extract Fractionation. The MeOH extract (350 g), after removal of the solvent, was partitioned between EtOAc and H_2O. The organic layer was chromatographed on Si gel to give fractions A−E. Fraction E, eluted with $CHCl_3$−MeOH (9:1) gave glucosides, identical to those previously isolated. Fraction A, eluted with $CHCl_3$ was rechromatographed on neutral alumina to give crude **2**, which was purified by preparative TLC [benzene−Et_2O (4:1), 9 mg]. Fraction B, eluted with $CHCl_3$−Me_2CO (9:1), was rechromatographed on Sephadex [hexane−$CHCl_3$−MeOH (3:1:1)] to give crude **1**, purified by C18 HPLC (6 mg). Fraction C, eluted with $CHCl_3$−Me_2CO (17:3), was chromatographed on Sephadex [hexane−$CHCl_3$−MeOH (1:1:1)] to give a mixture of **4** and **7**, which were separated by C8 HPLC (12 and 7 mg). Fraction D, eluted with $CHCl_3$−Me_2CO (4:1), was rechromatographed on Sephadex [hexane−$CHCl_3$−MeOH (1:1:1)] to give a mixture of **3**, **5**, and **6**, which were separated by C18 HPLC (5, 8, and 6 mg, respectively).

2-Hydroxy-7-(hydroxymethyl)-1-methyl-5-vinyl-9,10-dihydrophenanthrene (1): UV (EtOH) λ_{max} (ϵ) 281 (14 000) nm; IR ($CHCl_3$) ν_{max} 3350, 3020, 2358, 1602 cm^{-1}; HREIMS *m/z* [M]$^+$ 266.1312 ($C_{18}H_{18}O_2$ requires 266.1307); EIMS *m/z* [M]$^+$ 266 (100), 251 (87), 236 (49), 205 (25); ^{1}H-NMR and ^{13}C-NMR data in Tables 1 and 2, respectively.

2-Hydroxy-6-(hydroxymethyl)-1-methyl-5-vinyl-9,10-dihydrophenanthrene (2): UV (EtOH) λ_{max} (ϵ) 282 (13 600) nm; IR ($CHCl_3$) ν_{max} 3382, 3030, 2360, 1602 cm^{-1}; HREIMS *m/z* $[M]^+$ 266.1321 ($C_{18}H_{18}O_2$ requires 266.1307); EIMS *m/z* $[M]^+$ 266 (100), 251 (81), 236 (59), 205 (20); ^{1}H-NMR and ^{13}C-NMR data in Tables 1 and 2, respectively.

2-Hydroxy-5-(hydroxymethyl)-1,7-dimethyl-9,10-dihydrophenanthrene (3): UV (EtOH) λ_{max} (ϵ) 279 (13 900) nm; IR ($CHCl_3$) ν_{max} 3687, 3324, 3043, 2360, 1603 cm^{-1}; HREIMS *m/z* $[M]^+$ 254.1285 ($C_{17}H_{18}O_2$ requires 254.1307); EIMS *m/z* $[M]^+$ 254 (78), 239 (100), 224 (41); ^{1}H-NMR and ^{13}C-NMR data in Tables 1 and 2, respectively.

2,7-Dihydroxy-5-(hydroxymethyl)-1,8-dimethyl-9,10-dihydrophenanthrene (4): UV (EtOH) λ_{max} (ϵ) 279 (14 000) nm; IR ($CHCl_3$) ν_{max} 3689, 3043, 2360, 1603 cm^{-1}; HREIMS *m/z* $[M]^+$ 270.1275 ($C_{17}H_{18}O_3$ requires 270.1256); EIMS *m/z* $[M]^+$ 270 (100), 255 (92), 240 (61); ^{1}H-NMR and ^{13}C-NMR data in Tables 1 and 2, respectively.

2-Hydroxy-5-(hydroxymethyl)-7-methoxy-1,8-dimethyl-9,10-dihydrophenanthrene (5): UV (EtOH) λ_{max} (ϵ) 277 (13 700) nm; IR ($CHCl_3$) ν_{max} 3601, 3346, 1600 cm^{-1}; HREIMS *m/z* $[M]^+$ 284.1426 ($C_{18}H_{20}O_3$ requires 284.1412); EIMS *m/z* $[M]^+$ 284 (100), 269 (61), 239 (84); ^{1}H-NMR and ^{13}C-NMR data in Tables 1 and 2, respectively.

5-(1-ethoxy)-2,7-dihydroxy-1,8-dimethyl-9,10-dihydrophenanthrene (6): $[\alpha]^{25}_D$ ±0° (*c* 0.25, MeOH); UV (EtOH) λ_{max} (ϵ) 282 (14 100) nm; IR ($CHCl_3$) ν_{max} 3680, 3314, 3033, 2350, 1602 cm^{-1}; HREIMS *m/z* $[M]^+$ 284.1433 ($C_{18}H_{20}O_3$ requires 284.1412); EIMS *m/z* $[M]^+$ 284 (88), 276 (100), 261 (70); ^{1}H-NMR and ^{13}C-NMR data in Tables 1 and 2, respectively.

2-Hydroxy-1,7-dimethyl-9,10-dihydrophenanthro-[5,6-*b*]-4′,5′-dihydro-4′,5′-dihydroxyfuran (7): $[\alpha]^{25}_D$ ±0° (c 0.4, MeOH), UV (EtOH) λ_{max} (ϵ) 316 (9900), 278 (13 600) nm; IR ($CHCl_3$) ν_{max} 3401, 3040, 1705, 1603 cm^{-1}; HREIMS *m/z* $[M]^+$ 298.1219 ($C_{18}H_{18}O_4$ requires 298.1205); EIMS *m/z* $[M]^+$ 298 (28), 280 (87), 265 (100); ^{1}H-NMR and ^{13}C-NMR data in Tables 1 and 2, respectively.

Bioassay. The strain UTEX 1648 *S. capricornutum* was maintained on Bold basal medium[14] solidified with agar 1.5% in continuous light at 23 °C. Fresh axenic cultures for the experiments were grown in 100-mL cylinders on the same culture medium. For the growth tests, the dihydrophenanthrenes were dissolved in Me_2CO. Each solution (20 μL) was added to the test tubes containing 6 mL of inoculated medium, giving final concentrations of 10^{-4}, 7.5×10^{-5}, 5×10^{-5}, 2.5×10^{-5}, 10^{-5} and 5×10^{-6} M. Blanks, containing only Me_2CO, were also tested.

The test tubes were incubated at 23 °C on a shaking apparatus previously described.[15] The total irradiation of 150 μE s^{-1} m^{-2} was provided by daylight fluorescent lamps (Philips TLD 30 w/55). The photoperiod was 16 h light/8 h dark. Growth of cultures was followed daily, either by measuring the absorbance increase at 550 nm with a Bausch & Lomb Spectronic 20 colorimeter or by counting the cell numbers with a Thoma bloodcounting chamber. The cell numbers of the initial inocula ranged from 10^6 to 1.5×10^6 mL^{-1}, corresponding to 0.05−0.06 units of absorbance. Growth experiments were carried out in triplicate. To test statistical significance of results, one-way ANOVA was performed at 0.05 *p*. For each compound, a comparison among means was performed using Student−Newman−Keuls test, at 0.05 *p*. Statistical package SPSS was used. The index of inhibition was calculated as: $(1 - X_a/Y_a) \times 100$, where X_a = growth rate of the alga in the presence of the compound tested, and Y_a = growth rate of control.

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