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Phenanthrenoids from the wetland *Juncus acutus*

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Dedicated to Professor Lorenzo Mangoni on the occasion of his 70th birthday.

Abstract

Nine 9,10-dihydrophenanthrenes, three phenanthrenes and a related pyrene have been isolated from the wetland plant *Juncus acutus*. The structures have been attributed by means of their spectral data and chemical correlation. 5-(1-Ethoxy-ethyl)-2-hydroxy-7-methoxy-1,8-dimethyl-9,10-dihydrophenanthrene and 5-(1-phytoxy-ethyl)-2-hydroxy-7-methoxy-1,8-dimethyl-9,10-dihydrophenanthrene, 2,7-dihydroxy-1-methyl-5-vinylphenanthrene, 2,7-dimethoxy-1,6-dimethyl-5-vinylphenanthrene and 2,7-dihydroxy-1,6-dimethylpyrene are described for the first time. Many of the compounds showed in vitro phytotoxicity against *Selenastrum capricornutum*, a microalga used in aquatic tests. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Juncus acutus; Juncaceae; *Selenastrum capricornutum*; Phytotoxic compounds; 9,10-Dihydrophenanthrenes; Phenanthrenes; Spectroscopic analysis; Toxicity test

1. Introduction

The search of eco-friendly algaecides for the control of algal bloom in eutrophic habitats is an interesting field of natural products chemistry. Among natural compounds which exert an allelochemical activity against algal pests must be included the 9,10-dihydrophenanthrenes isolated from the wetland plant *Juncus effusus*. A study of these metabolites led to the identification of more than 30 compounds, many of them with strong antialgal activity (DellaGreca et al., 1996 and ref. cit.). A toxicity evaluation of these compounds on aquatic species from various taxonomic groups and their possible selective toxicity in water ecosystems has been also reported (DellaGreca et al., 2001). In continuing our studies we have investigated *Juncus acutus*, another wetland Juncacea of the mediterranean area, and we here report the isolations of nine 9,10-dihydrophenanthrenes (**1–9**), three phenanthrenes (**10–12**) and a related pyrene (**13**).

2. Results and discussion

The aerial part of the plant, collected near Cagliari during the Summer, was air dried and extracted with solvents of increasing polarity. Preliminary assays on the green alga *Selenastrum capricornutum* evidenced an algal growth inhibition of the petrol and EtOAc extracts while the MeOH extract was inactive. Repeated chromatographic processes of the petrol fraction gave the 9,10-dihydrophenanthrenes. Compounds **1**, **2** and **7** (Della Greca et al., 1992), **3** (Sarkar et al., 1988), **6** (Shima et al., 1991), **8** and **9** (DellaGreca et al., 1993), have been identified by comparison of their physical data with those of the compounds previously isolated.

The structure of 5-(1-ethoxy-ethyl)-2-hydroxy-7-methoxy-1,8-dimethyl-9,10-dihydrophenanthrene was attributed to compound **4**. It had the molecular formula $C_{21}H_{26}O_3$ according to the molecular ion at m/z 326 in its EIMS spectrum and the presence of 21 carbon signals in the ^{13}C NMR spectrum (Table 1). The ^1H NMR spectrum (Table 1) showed three aromatic protons, a carbinol proton, a methoxyl, three methylenes and four methyls. In the HMBC experiment the H-3 proton was correlated to the C-4a carbon and the C-1 carbon. Both

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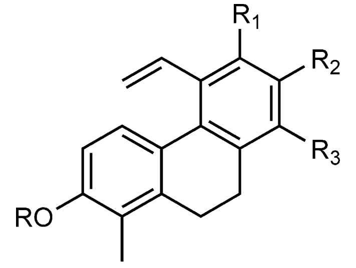
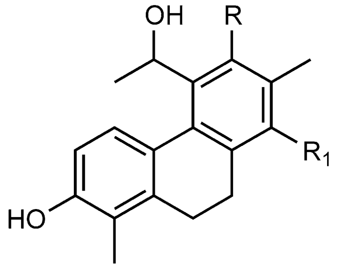
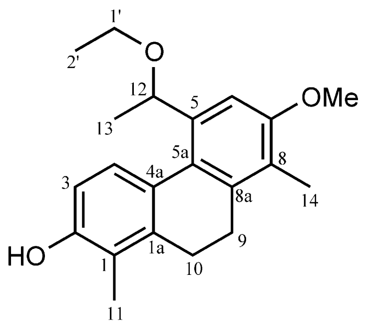


1 R = Me R_1 = Me R_2 = H R_3 = OH
2 R = H R_1 = H R_2 = OMe R_3 = Me
3 R = H R_1 = H R_2 = Me R_3 = H
6 R = H R_1 = Me R_2 = OH R_3 = H
7 R = H R_1 = H R_2 = OH R_3= Me



8 R = H R_1 = OH
9 R = OH R_1 = H



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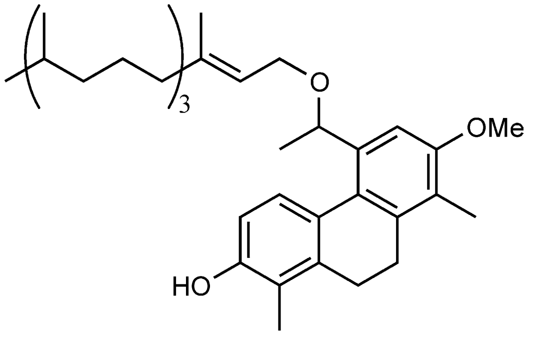

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these carbons were correlated to the H-10 methylene protons, while the latter gave a further cross peak with the H-11 methyl protons. The H-4 proton gave cross peaks with the C-2 carbon bearing a hydroxyl group, the C-1a carbon and the C-5a carbon. This carbon was also correlated to the H-9 protons, the H-12 carbinol proton and the H-6 proton. The C-12 carbinol carbon was correlated to the methylene C-1′, according to the ethyl ether function, while the H-6 proton was also correlated to the C-8 carbon. Finally, the methyl protons at δ 2.22 were correlated to the C-8 carbon. Accordingly to the structure, in a NOE experiment the methoxyl had relations with both the singlet at δ 7.05 and the methyl at δ 2.22.

Compound **5** was identified as structure 5-(1-phytoxyethyl)-2-hydroxy-7-methoxy-1,8-dimethyl-9,10-dihydrophenanthrene. GC–MS analysis evidenced the presence of three peaks. The first peak showed a molecular ion at *m/z* 576 corresponding to the molecular formula $C_{39}H_{54}O_3$, the second peak an ion at *m/z* 295 corresponding to a phytoxyl group $[C_{20}H_{39}O]^+$, while the third peak showed an ion at *m/z* 281 corresponding to $[M–C_{20}H_{39}O]^+$. The ^{1}H NMR spectrum (Table 1) was rather similar to that of **4**. Instead of the ethyl signals, there were present an olefinic proton as a triplet at δ 5.19, a methylene as a doublet at δ 3.56, a methyl singlet at δ 1.36, four methyl doublets at δ 0.94, 0.93, 0.89 and 0.87, beside other signals in the 1.9–1.1 ppm range.

Table 1

^{1}H and ^{13}C NMR spectral data for compounds **4** and **5** (400/100.25 MHz, $CDCl_3$)

Compound Position	**4**		**5**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	120.2		120.3	
2	152.1		152.1	
3	111.6	6.69 *d* (8.4)	111.6	6.67 *d* (8.4)
4	126.7	7.02 *d* (8.4)	126.8	7.00 *d* (8.4)
5	138.6		138.6	
6	106.3	7.05 *s*	106.4	7.07 *s*
7	156.5		156.6	
8	121.9		121.8	
9	26.5	2.88 *d* (10.6)	26.6	2.45 *d* (10.6)
10	25.6	2.47 *d* (10.6)	25.7	2.91 *d* (10.6)
1a	139.8		140.6	
4a	127.5		127.7	
5a	128.3		128.3	
8a	138.4		138.5	
11	11.7	2.29 *s*	11.6	2.27 s
12	73.3	4.99 *q* (6.0)	72.4	5.01 *q* (6.0)
13	23.8	1.64 *d* (6.0)	24.1	1.63 *d* (6.0)
14	11.6	2.22 *s*	11.6	2.22 *s*
OCH_3	55.5	3.89 *s*	55.5	3.89 *s*
1′	63.1	3.11 *q* (7.2)	64.0	3.56 *q* (5.8)
2′	15.4	1.05 *t* (7.2)	120.6	5.19 *t* (5.8)

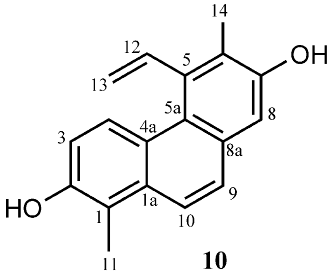
5: δ_H 1.99 (*m*, H-4′), 1.0–1.9 (overlapped, H-5′–H-15′), 0.85 (*d*, 6.4, H-16′ and H-17′), 0.87 (*d*, 6.4, H-18′ and 19′), 1.67 (*s*, H-20′). δ_C 139.9 (C-3′), 39.9 C-4′), 25.3 (C-5′), 36.8 (C-6′), 32.8 (C-7′), 37.9 (C-8′), 24.5 (C-9′), 37.5 (C-10′), 32.8 (C-11′), 37.4 (C-12′), 24.8 (C-13′), 39.5 (C-14′), 28.5 (C-15′), 22.7 (C-16′ and C-17′), 19.8 (C-18′ and C-19′) and 16.7 (C-20′).

In spite of the great number of 9,10-dihydrophenanthrenes, only three phenathrenes have been reported until now as constituents of Juncaceae. The first two compounds, called dehydroeffusol (**14**) and dehydroeffusal (**15**) have been isolated from the medullae of *Juncus effusus* by Shima et al. (1991) while the third, called dehydrojuncusol (**10**) has been isolated from *Juncus roemerianus* (Miles et al., 1977). Such kinds of compounds have been now identified in the ethyl acetate extract of *J. acutus*. This extract was divided into an acid and a neutral fraction by conventional procedures and both the fractions were subjected to repeated chromatographic processes. Compounds **10** and **13** were obtained from the acid fraction and were identified by their physical features and by chemical correlation.

Phenanthrene **10** showed in the ^{1}H NMR spectrum (Table 2) the sharp singlets of the H-14 and H-11 methyls, the three double doublet of the H-13 and H-12 vinyl protons and the five aromatic protons of the H-8 singlet and the H-3, H-4, H-9 and H-10 doublets. In addition to the molecular ion at *m*/*z* 264 for $C_{18}H_{16}O_2$, the EI mass spectrum showed significant peaks at *m*/*z* 249 $[M-Me]^+$ and 234 $[M-2Me]^+$. These data matched those reported by Sarkar et al. (1988) for dehydrojuncusol. The unreported ^{13}C NMR data (Table 2) have been attributed by HMQC and HMBC experiments.

The pyrene structure was attributed to compound **13**. The EI–MS spectrum showed the molecular peak at *m*/*z* 262 and a significant peak at *m*/*z* 247 $[M-Me]^+$. These data and the elemental analysis agreed with the molecular formula $C_{18}H_{14}O_2$. The ^{13}C NMR spectrum (Table 2) showed only nine signals which were defined by a DEPT experiment as one methyl, three methine and five quaternary carbons. In the ^{1}H NMR spectrum (Table 2) signals of a methyl singlet, an aromatic proton as singlet and two aromatic *ortho*-coupled protons were present. The HMBC experiment showed that the proton at δ 7.67 was correlated to the carbons at δ 120.1, 121.2 and 128.0, the proton at δ 7.88 to the carbons at δ 112.4, 121.2, 124.0 and 131.2, the proton at δ 8.10 to the carbons 120.1, 121.2 and 128.0, the methyl to the carbons at δ 120.1, 131.2 and 153.9. These data indicated a highly symmetric molecule and were consistent with both the 2,7-dihydroxy-1,6-dimethyl-pyrene and the 2,7-dihydroxy-1,8-dimethyl-pyrene structure. However, the etherotopic character of the H-9/H-13 and H-10/H-12 protons was in agreement with the presence of a symmetry center in the molecule and, consequently, with the structure **13**. Accordingly, pyrene **13** was obtained by semisynthesis from dehydrojuncusol **10**. Acetylated **10** was irradiated with a 450 Hanovia lamp (Padwa et al., 1977) and the photocyclization product was deacetylated with ethanolic 10% KOH to obtain a compound spectroscopically identical to the natural one. Structures 2-hydroxy-1,6-dimethyl-5-vinyl-phenanthrene and 2,7-dimethoxy-1,6-dimethyl-5-vinyl-phenathrene were respectively attributed to compounds **11** and **12**, derived from the neutral fraction.

The molecular peak at *m*/*z* 248 in the EI mass spectrum and the elemental analysis defined the molecular formula $C_{18}H_{16}O$ of compound **11**. The ^{1}H NMR (Table 2) and ^{1}H–^{1}H COSY spectra exhibited six aromatic protons *ortho*-coupled, three vinyl protons as double doublets and two methyls. In the ^{13}C NMR spectrum (Table 2) 18 carbon signals were present, which were defined by a DEPT experiments as two methyls, one methylene, seven methine and eight quaternary carbons. The HMBC spectrum showed cross-peaks of the H-11 methyl protons with the carbons C-1, C-2 and C-1a.



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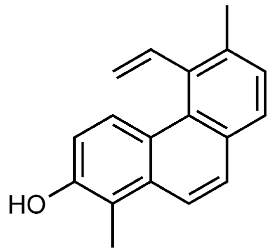

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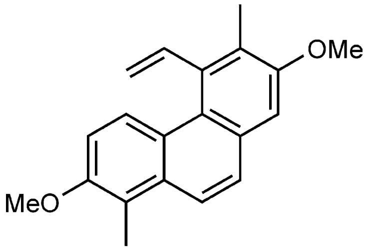

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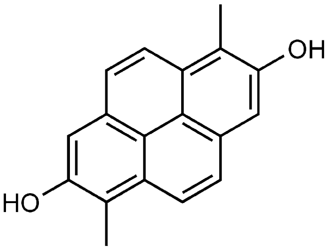

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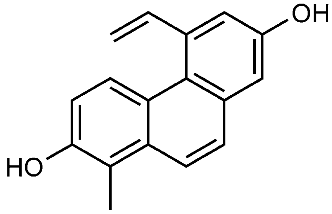

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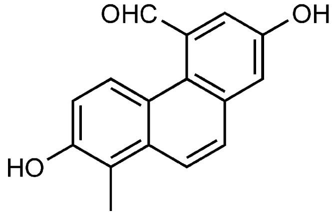

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Table 2
^1H and ^{13}C NMR spectral data for compounds **10–13** (400/100.25 MHz)

	Compound Position							
	10[a]		**11**[b]		**12**[b]		**13**[c]	
	δ ^{13}C	δ ^1H (J in Hz)	δ ^{13}C	δ ^1H (J in Hz)	δ ^{13}C	δ ^1H (J in Hz)	δ ^{13}C	δ ^1H (J in Hz)
1	118.9		116.8		120.2		120.1	
2	153.6		151.0		154.3		153.9	
3	115.9	6.99 *d* (9.2)	116.0	7.16 *d* (9.5)	110.4	7.19 *d* (9.1)	112.4	7.67 *s*
4	129.0	8.83 *d* (9.2)	121.9	8.45 *d* (9.5)	127.7	9.05 *d* (9.1)	121.2	
5	138.9		132.4		137.4		131.2	
6	126.9		135.4		126.6		120.1	
7	155.3		128.7	8.45 *d* (8.2)	156.0		153.9	
8	112.2	7.12 *s*	124.5	7.46 *d* (8.2)	106.2	7.17 *s*	112.4	7.67 *s*
9	128.5	7.78 *d* (9.0)	120.9	8.13 *d* (9.6)	127.6	7.89 *d* (9.0)	128.0	7.88 *d* (9.2)
10	123.9	7.50 *d* (9.0)	121.4	7.88 *d* (9.6)	122.7	7.65 *d* (9.0)	124.0	8.10 *d* (9.2)
1a	134.9		131.2		133.8		131.2	
4a	127.1		124.8		127.1		130.3	
5a	125.6		128.2		124.0		130.3	
8a	132.9		128.9		131.0		121.2	
11	12.1	2.52 s	10.5	2.59 *s*	11.4	2.62 *s*	11.9	2.76 *s*
12	142.3	7.30 *dd* (18.2, 13.4)	134.3	7.08 *dd* (17.8, 10.0)	140.0	7.33 *dd* (18.2, 13.4)	124.0	8.10 *d* (9.2)
13	119.7	5.21 *dd* (18.2, 2.2) 5.67 *dd* (13.4, 2.2)	120.8	5.43 *dd* (17.8, 2.2) 5.80 *dd* (10.0, 2.2)	119.2	5.30 *dd* (18.2, 2.2) 5.73 *dd* (13.4, 2.2)	128.0	7.88 *d* (9.2)
14	15.5	2.38	20.4	2.50 *s*	14.6	2.44	11.9	2.76 *s*
OCH_3					56.4	3.99		
OCH_3					55.7	3.97		

[a] Recorded in CD_3OD.
[b] Recorded in $CDCl_3$.
[c] Recorded in acetone-d_6.

The H-3 proton was heterocorrelated with the carbons C-1, C-2 and C-4a. According to the ^1H–^1H COSY one of the protons at δ 8.44 was attributed to the H-4. It gave interactions with the carbons C-2, C-1a and C-5a. The interaction of the H-12 vinyl proton with the C-5a carbon located the vinyl chain at C-5 and, accordingly, this carbon was correlated with the H-13 protons. In the same experiment both the H-7, second proton at δ 8.44, and the H-14 methyl protons were correlated to the C-5 carbon according to the location of the methyl at the C-6 position. The H-8 proton gave cross peaks with the carbon C-6. Finally the H-9 proton gave cross peaks with the C-1a and C-5a and the H-10 proton gave cross peaks with the C-1 and C-4. According to the structure a NOE correlation between the H-14 methyl and the H-7 proton was observed in a NOESY experiment.

Compound **12** had molecular formula $C_{20}H_{20}O_2$ according to the molecular ion at m/z 292 in the EIMS spectrum and the presence of 20 carbon signals in the ^{13}C NMR spectrum (Table 2). The ^1H NMR spectrum showed the H-3 and H-4 doublets, the H-8 singlet, the H-9 and H-10 doublets, the H-12 and H-13 double doublets, the H-11 and H-14 methyls. In addition, the signals of two methoxyl groups were present. The chemical shifts well resembled those of dehydrojuncusol so that the structure of dimethylether of **10** was attributed to compound **12**. In agreement, treatment of **10** with Me_2SO_4 gave **12**. The phytoxicity of all the compounds was tested on the freshwater green alga *Selenastrum capricornutum*. The already known compounds **1–3** and **6–9** were previously assayed (DellaGreca et al., 1996) using the classical algal growth inhibition method in a 4-day static test with laboratory cultures of algae. In this work, a 3-day microbiotest independent of the live culture stocks of the alga was used. Both methods measured the response of the algal population in terms of changes in cell density (cell counts per ml) when exposed in static systems to five concentrations for each pure chemical and three replicates included the controls. The major advantage of a microbiotest in comparison to conventional algal assay is that it is miniaturized. In fact, the bioassays can be performed with smaller quantities of test soln (25 ml versus 100 ml) and therefore, chemicals that generally are extracted from plants in few milligrams. A further advantage is that after 72 h incubation, the growth of the microalgae exceeds the minimum multiplication factor (16 times) imposed by OECD (guideline 201) so that the test is performed in a short time.

The previous data were tabulated as a percentage of algal growth inhibition. In this study, the values were reported against $\log_{10}$-transformed data of concentrations (μmol/l) to determine the IC_{50}, the concentration estimated to cause a 50% decrease in algal cell density relative to the control, using a linear interpolation

Table 3
Median inhibition concentration (IC_{50}) for *Selenastrum capricornutum*

Compound	IC_{50} (μmol)
1	11.1 (8.6–15.3)
2	28.0 (24.5–32.4)
3	45.6 (37.6–58.0)
4	49.7 (43.2–58.3)
5	30% Biostimulation at the highest concentration (43.4)
6	19.9 (12.8–35.7)
7	69.0 (43.6–185.6)
8	35.6 (29.9 – 41.6)
9	16.8 (7.5–25.5)
10	72.9 (67.9–78.4)
11	83.1 (63.9–119.1)
12	90% Biostimulation at the highest concentration (410.9)
13	126.8 (114.2–139.5)

95% Confidence interval in parentheses.

method (Norberg-King, 1993). Confidence limits were calculated by standard deviation and *t* student.

The values obtained by testing compounds both with the 4-day static test and with the microbiotest were compared. A comparable response was found with a sensitivity of the same order of magnitude and data are reported in Table 3. The antialgal activity of the two new 9,10-dihydrophenanthrenes showed that compound **4**, with an ethoxyl group in the side chain, is weakly active compared with other dihydrophenanthrenes, while compound **5** with a phytoxyl group, is biostimulant at the highest tested concentration. Phenanthrene **10** has an activity comparable to that of the corresponding 9,10-dihydrophenanthrene **6**. As already observed in the 9,10-dihydrophenanthrenes series, a reduction of the polarity causes a decreasing of the activity. Phenanthrene **11** lacking hydroxyl group at C-7 is about three times less active than **10**. Furthermore, the cyclization of **10–13** halves the toxicity and the methylation of **10** to **12** makes the compounds biostimulating.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C on a Bruker AC400 spectrometer with 0.05 M solns in $CDCl_3$ at 25 °C. Proton-detected heteronuclear correlations were measured using HMQC (optimised for $^1J_{HC} = 145$ Hz) and HMBC (optimised for $^1J_{HC} = 7$ Hz). MS spectra were obtained with a HP 6890 spectrometer equipped with a MS 5973 N detector. HPLC was performed on a Varian 5500 by using an UV detector. TLC was performed on a Merck Kiesegel 60 F_{254} with 0.2 mm layer thickness. Preparative HPLC were performed using SiO_2 (LiChrospher Silica 10 μm, 250×10 mm i.d., Merck) and RP-18 (LiChrospher 10 μm, 250×10 mm i.d., Merck) columns. Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm film thickness. Spots were visualized by UV light or by spraying with H_2SO_4–AcOH–H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Prep. TLC was performed on a Merck Kiesegel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Flash column chromatography was performed on Merck Kiesegel 60 (230–400 mesh) at medium pressure.

3.2. Plant material

Aerial part of plants of *Juncus acutus* were collected near Cagliari (Italy) during the summer and identified by Professor Giovanni Ballero of the Dipartimento di Biologia Vegetale of University of Cagliari. A voucher specimen (HERBNAPY140) is deposited at the Dipartimento di Biologia Vegetale of University Federico II of Naples.

3.3. Extraction and isolation

The air-dried plant material (2.4 kg) was sequentially and exhaustively extracted with light petrol and ethyl acetate at room temperature for 7 days. The extracts were frozen and stored at −80 °C until used. The phytotoxicity of these extracts were determined by the bioassay, described in the experimental section, with the unicellular green alga *Selenastrum capricornutum* (CCAP 278/4).

3.3.1. Light petrol extract fractionation

An aliquot of the light petrol extract (700 mg), after removal of the solvent, was chromatographed on silica gel to give fractions A and B. Fraction A, eluted with petrol, gave a mixture of **1–3**, which was separated by flash column chromatography [petrol–Et_2O (9:1), 21, 30 and 24 mg, respectively]. Fraction B, eluted with $CHCl_3$, was rechromatographed on silica gel. The fraction eluted with petrol–Me_2CO (4:1) gave crude compounds **4** and **5**. Compound **4** was purified by prep TLC [petrol–Et_2O (3:1), 8 mg]; elemental analysis: found: C, 77.2; H, 8.1. $C_{21}H_{26}O_3$ requires: C, 77.3; H, 8.0%. Compound **5** was purified by prep. TLC [petrol–Et_2O (4:1), 9 mg]; elemental analysis: found: C, 81.1; H, 10.6. $C_{39}H_{60}O_3$ requires: C, 81.2; H, 10.5%. The fraction eluted with $CHCl_3$–Me_2CO (9:1) gave a mixture of **6** and **7** which was separated by flash column chromatography [$CHCl_3$–EtOAc (19:1), 16 and 18 mg, respectively]. Further elution with the same eluent gave **8**, purified by flash column chromatography with $CHCl_3$–EtOAc (9:1) (12 mg) and **9**, purified with $CHCl_3$–EtOAc 1:1 (15 mg).

3.3.2. Ethyl acetate extract fractionation

The EtOAc extract was fractionated into acidic and neutral fractions with aq 2 M NaOH. The aqueous alkaline solution was acidified in the cold with conc. HCl and liberated solids were extracted with Et_2O, washed with H_2O, dried and solvent removed. An aliquot (2 g) of the residue (72 g) was chromatographed on acid washed silica gel using $CHCl_3$–AcOEt solns as eluent. The fraction eluted with $CHCl_3$–AcOEt (6:1) was rechromatographed on prep. RP18 eluting with MeOH–H_2O–CH_3CN (2:2:1) to give a mixture of **10** and **13** which was separated on prep. HPLC (silica gel) with hexane–AcOEt (4:1) to give pure **10** (55 mg) and **13** (32 mg). Elemental analysis of **13**: found: C, 82.0; H, 5.2; $C_{18}H_{14}O_2$ requires: C, 82.4; H, 5.4%. An aliquot (3 g) of the neutral fraction (35 g) was subjected to silica gel CC. The hexane–AcOEt (9:1) eluate gave a mixture of **11** and **12** which was separated on prep. HPLC (silica gel column) with hexane–EtOAc (9:1) to give pure **11** (23 mg), elemental analysis: found: C, 87.2; H, 6.3; $C_{18}H_{16}O$ requires: C, 87.1; H, 6.4% and **12** (15 mg) elemental analysis: found: C, 82.0; H, 6.8; $C_{20}H_{20}O_2$; requires: C, 82.2; H, 6.9%.

3.3.3. Preparation of 13

Pure 2,7-dihydroxy-1,6-dimethyl-5-vinylphenanthrene (**10**) (20 mg) was acetylated with Ac_2O (0.5 ml) and dry pyridine (0.5 ml) at room temperature overnight. The crude acetate obtained was dissolved in benzene (10 ml) and irradiated with a 450 W Hanovia lamp equipped with Pyrex filter sleeve for 2 h. The product was deacetylated with KOH 10% in ethanol for 1 h and purified on prep. TLC [hexane–AcOEt (4:1)] to give **13** (30% yield).

3.4. Bioassay

The microbiotest Algaltoxkit F^{TM} (Creasel, 1996) using the alga *Selenastrum capricornutum* was applied for the antialgal activity. This test is a 0–72 h algal growth inhibition assay and is independent of the live culture stocks of the alga. Pure chemicals (99%) were dissolved in DMSO and then diluted in bidistilled water to make the final stock solns. The highest DMSO concentration in the test solns. did not exceed 0.01% (v/v). Blanks were carried out to detect whether the DMSO concentrations might cause toxicity. The dilution water used for the test solns. was the OECD medium (OECD, 1994). The alga was inoculated (1×10^4 cells/ml) in 25 ml of test soln. prepared in five concentrations for each chemical and three replicates included the control. Compound concentrations used in the definitive tests were based on results from range-finding tests. In the previous experiment the concentrations tested were 1×10^{-4}, 7.5×10^{-5}, 5×10^{-5}, 2.5×10^{-5} and 1×10^{-5} M, while in the present experiment the concentrations ranged from 2×10^{-4} to 0.625×10^{-5} M with a dilution factor of 0.5. Details on the toxicity test procedures are given in DellaGreca et al. (2001). The cell density was measured at 0 time and every 24 h for 3 days by an electronic particle dual threshold counter (Coulter Counter Z2, 100 μm capillary) and from these data the algal growth inhibition was calculated by integrating the mean values from t_0 to t_{72} h (area under the curve), calculating the median inhibition (IC_{50}) in μmol/l (OECD, 1994).

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