ELSEVIER

Fitoterapia 76 (2005) 433–438

FITOTERAPIA

www.elsevier.com/locate/fitote

A new dihydroxysterol from the marine phytoplankton *Diacronema* sp.

Amélia P. Rauter[a,*], Manuela M. Filipe[a], Carla Prata[a], João P. Noronha[b], Maria A.M. Sampayo[c], Jorge Justino[d], Jaime Bermejo[e]

[a]*Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, Ed. C8, 5° piso, 1749-016 Lisboa, Portugal*
[b]*Departamento de Química, REQUIMTE–CQFB, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal*
[c]*INIAP, Rua Barata Salgueiro, 37-3°, 1250-042 Lisboa, Portugal*
[d]*Escola Superior Agrária–Instituto Politécnico de Santarém, Complexo Andaluz, Apartado 279, 2001-904 Santarém, Portugal*
[e]*Instituto de Productos Naturales y Agrobiologia–C.S.I.C.–Instituto Universitário de Bio-Orgánica "António González", Avenida Astrofísico F. Sanchez 3, 38206 La Laguna, Tenerife, Spain*

Received 10 May 2004; accepted 17 March 2005
Available online 12 May 2005

Abstract

Diacronema sp. was cultured and its sterols were separated by column chromatography on silica gel. The new sterol 24-ethyl-4α-methyl-cholestane-3,20-diol (**1**) was characterised by NMR and MS spectrometry, as well as (22*E*)-24-ethyl-4α-methyl-5α-cholest-22-en-3β-ol (**2**) and β-sitosterol, the major components of the sterol fractions. Neither the biosynthetic origin of the new dihydroxysterol nor its role in the biochemistry of *Diacronema* is known.

Keywords: Sterols; Dihydroxysterol; *Diacronema* sp.; Microalgae

* Corresponding author. Tel.: +351 21 7500952; fax: +351 21 7500088.
E-mail address: aprauter@fc.ul.pt (A.P. Rauter).

0367-326X/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.fitote.2005.03.003

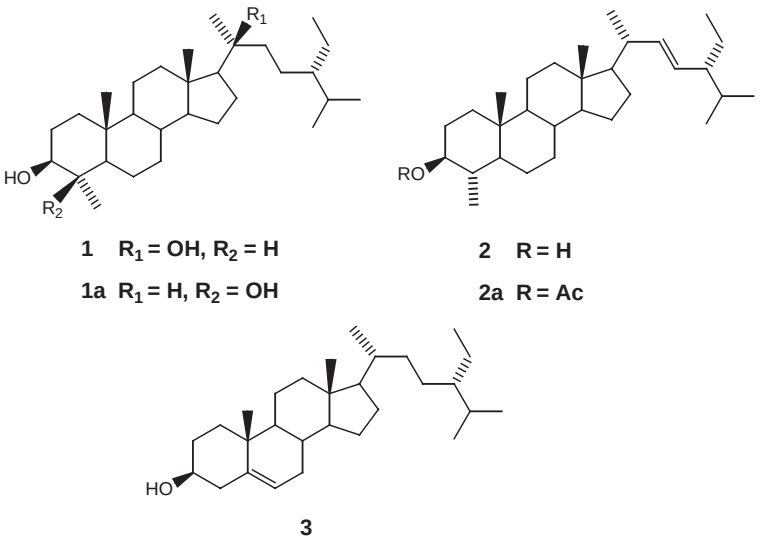
1. Introduction

Marine algae are important sources of a wide variety of chemical products [1–5] used in food and in pharmaceutical industries [6] as well as useful biomarkers for identifying the origin of organic matter in seawater and marine sediments [7,8]. Proteins, amino acids, vitamins, polysaccharides and other carbohydrates extracted from algae have been used as food supplement. Microalgae also produce lipids such as linoleic and γ-linoleic acids, which are precursors of medicinal compounds with hypotensive properties. Antibacterial and antifungal activities of compounds isolated from algae have also been described [9]. Microalgae sterols have been used as starting materials for the synthesis of hormone steroids [10].

The sterols found in microalgae display a fascinating diversity of sterolic patterns as might be expected from the great number of microalgal classes, genera and species combined with the long evolutionary history of most classes, but their metabolism and roles are still poorly understood [11,12]. The presence of methyl groups at positions C-4, C-14 or C-23, of methyl, ethyl or propyl groups at position C-24, and of an additional hydroxyl group, is common in the structure of marine sterols.

Double bonds may be found at C-5, C-7, C-8, C-22, C-24 or C-25(27). Modified side-chains occur infrequently as in 27-nor- and 27-nor-24-methyl sterols [13]. Some sterols are widespread, but others appear to be restricted to just a few algal classes.

Microalgae of the class Haptophyceae are common in the marine environment and important nutritional feedstocks in aquaculture [14]. Their sterols have been subjected to many analyses because of their value as biomarkers of the origin of organic matter in sediments [7]. Some groups within the Haptophyta contain compounds whose



1 $R_1 = OH, R_2 = H$ **2** $R = H$

1a $R_1 = H, R_2 = OH$ **2a** $R = Ac$

3

Fig. 1. Structure of the major compounds 1–3 isolated from *Diacronema* sp., of ethylpavlovol (1a) and of the acetyl derivative 2a.

structures are sufficiently distinctive that they can be used as biomarkers in support of taxonomic classification [15]. One of these groups consists of the order Pavlovales, which contains a wide range of 4-desmethylsterols as well as uncommon 5α(H)-stanols, 4α-methylsterols and pavlovols [7]. These dihydroxysterols have been reported in microalgae of both the genus Pavlova and Diacronema [16]. Their identification in *Diacronema vlkianum* shows that they are not unique to the genus Pavlova. However, they are probably restricted to species from the order Pavlovales. The modes of biosynthesis and functions of pavlovols remain unknown. Thus, the recognition of novel compounds may be used as a tool to aid in the classification of a particular species. Several 4α-methylsterols have been identified in microalgae from *Pavlova* spp. confirming that they are not unique to dinoflagellates. The major 4α-methylsterol found is (22*E*)-24-ethyl-4α-methyl-5α-cholest-22-en-3β-ol (**2**), which has a conventional C-24 alkylated side-chain rather than a 23,24-dimethyl structure [8,12,16]. We now report the isolation and structural elucidation of a new dihydroxysterol, 24-ethyl-4α-methyl-cholestane-3,20-diol (**1**), together with (22*E*)-24-ethyl-4α-methyl-5α-cholest-22-en-3β-ol (**2**) and β-sitosterol (**3**) as major components of the dichloromethane/methanol (1:1) extract of *Diacronema* sp. (Fig. 1).

2. Experimental

2.1. General

Melting points were determined with a digital melting point apparatus (Electrothermal) and are uncorrected. ^{1}H NMR (300 MHz) and ^{13}C NMR (22.5 MHz) spectra were run in a Bruker CXP 300 and Bruker WP-200 SY spectrometers, respectively, in $CDCl_3$ using tetramethylsilane as internal standard. IR spectra (cm^{-1}) were obtained on an Hitachi 270-50 spectrophotometer in KBr pellets. Mass spectra EIMS were recorded at 70 eV and 200 °C on an updated AEI MS9 spectrometer.

Column chromatography was carried out using silica gel 60G (0.040–0.063 mm, E. Merck). Analytical and preparative TLC were conducted on Merck 60 GF_{254} silica gel plates (absorbent thickness: 0.25 and 0.75 mm, respectively). The standard compound β-sitosterol was supplied by Extrasynthese, France.

2.2. Microalgal cultures

The microalga *Diacronema* sp. belongs to the division Chrysophytae, class Haptophyceae, order Pavlovales, family Pavlovaceae and genera *Diacronema*. It was isolated from the Portuguese coast by Sampayo in 1980 and belongs to the AQ/INIAP collection of living microalgae provided from a single clone since then. For the present study *Diacronema* sp. was grown as clonal batch cultures in 2 l, 6 l and 60 l of mineral salts "Miquel's Medium" [17] enriched sea water and cultivated until the exponential growth phase. Growth was maintained at 20–22 °C under cool white fluorescent light 24 h/day. The microalgae obtained were then collected by centrifugation at 4000 rev./min for 25 min (40 g).

2.3. Extraction and isolation

The biomass obtained (40 g) was extracted with MeOH–dichloromethane (1:1) and concentrated to give a residue (4 g), which was CC under low pressure with EtOAc–toluene mixtures from 1:15 to 1:1. Preparative TLC was eluted with chloroform–Et_2O 9:1 to give 24-ethyl-4α-methyl-cholestane-3,20-diol (**1**) (16 mg; 0.4%), (22*E*)-24-ethyl-4α-methyl-5α-cholest-22-en-3β-ol (**2**) (105 mg, 2.6%) and β-sitosterol (3) (117 mg, 2.9%), whose physical and spectroscopic data were compared to the standard compound and literature [18].

24-Ethyl-4α-methyl-cholestane-3,20-diol (**1**), mp 221–222 °C; IR bands (KBr) cm^{-1}: 3416 cm^{-1}; ^1H NMR: δ 3.18 (1 H, *m*, H-3α), 1.16 (3H, *s*, H-21), 0.94 (3H, *s*, H-19), 0.84–0.77 (6H, *m*, H-29, H-30), 0.71 and 0.69 (each 3H, *d*, H-26, H-27, *J* 4.8 Hz, 5.4 Hz), 0.57 (3H, *s*, H-18); EIMS *m/z* (rel. int.): 446 $[M]^+$(2), 445 $[M-H]^+$(8), 431 $[M-Me]^+$(30), 430 $[M-Me-H]^+$(21), 416 $[M-2Me]^+$(54), 398 $[M-2Me-H_2O]^+$(24), 386 $[M-4Me]^+$(24), 372 $[M-3Me-Et]^+$(49), 356 $[M-H_2O-Et-CHMe_2]^+$(65), 300 $[M-H_2O-Me-C_8H_{17}]^+$(10), 262 $[M-C_{10}H_{21}O-H_2O-C_2H_4]^+$(28), 202 $[M-C_{10}H_{21}O-H-C_5H_{10}O]^+$(25), 95 $[C_7H_{11}]^+$(100).

(22E)-24-Ethyl-4α-methyl-5α-cholest-22-en-3β-ol (**2**), mp 187 °C, 187–188 °C [19]; IR bands (KBr): 3445, 1630 cm^{-1}; ^1H NMR: δ 5.17 (1 H, *dd*, H-22, $J_{20,22}$ 8.2 Hz, $J_{22,23}$ 15.2 Hz), 5.03 (1H, *dd*, H-23, $J_{23,24}$ 8.0 Hz and $J_{22,23}$ 15.2 Hz), 3.04 (1 H, *m*, H-3α), 1.05 (3 H, *d*, H-21, $J_{20,21}$ 6.0 Hz), 0.99–0.75 (12 H, *m*, H-26, H-27, H-29, H-30), 0.82 (3H, *s*, H-19), 0.66 (3H, *s*, H-18); EIMS *m/z* (rel. int.): 428 $[M]^+$(4), 367 $[M-Me-H_2O]^+$(2), 316 $[M-C_8H_{16}]^+$(7), 287 $[M-C_{10}H_{19}$ (side chain)$-2H]^+$(4), 229 (4), 161 (7), 105 (14), 55 (100).

(22E)-24-Ethyl-4α-methyl-5α-cholest-22-en-3β-yl acetate (**2a**). Syrup; IR bands (KBr) cm^{-1}: 1732 cm^{-1}; ^1H NMR: δ 5.16 (1 H, *dd*, H-22, $J_{20,22}$ 8.2 Hz, $J_{22,23}$ 15.2 Hz), 5.03 (1H, *dd*, H-23, $J_{23,24}$ 8.0 Hz), 4.37 (1 H, *m*, H-3α), 2.05 (3 H, *s*, MeCO), 1.05 (3 H, *d*, H-21, $J_{20,21}$ 6.0 Hz), 0.99 (3H, *s*, H-19), 0.89–0.77 (12 H, *m*, H-26, H-27, H-29, H-30), 0.65 (3H, *s*, H-18); EIMS *m/z* (rel. int.) 470 $[M]^+$(28), 455 $[M-Me]^+$(2), 428 $[M+H-Ac]^+$(1), 410 $[M-AcOH]^+$(3), 395 $[M-Me-AcOH]^+$(4), 369 $[M+2H-C_3H_7-AcOH]^+$(40), 358 $[M-C_8H_{16}]^+$(49), 343 $[M-Me-C_8H_{16}]^+$(12), 329 $[M-C_{10}H_{19}$ (side chain)$-2H]^+$(40), 271 $[M-C_{10}H_{19}$ (side chain)$-AcOH]^+$(37), 175 (16), 121 (36), 55 (100).

3. Results and discussion

The structure proposed for compound **1** is based on its sterol pattern being a dihydroxy-4α-methylsterol with a saturated nucleus. These unusual structural features are known in sterols composition of microalgae [20]. An example of such type of compounds is ethylpavlovol, a constitutional isomer of compound **1**, previously isolated from *Pavlova* spp., which are constituents of the phytoplankton used in bivalves diet. Assignment of structure **1** was accomplished by means of NMR spectroscopy and mass spectrometry. Comparison of the ^{13}C NMR spectrum of **1** with that of ethylpavlovol (**1a**) [21] (Table 1) shows that significant changes in the chemical shifts of the resonances of C-4 and C-20 were detected, confirming the absence of the hydroxyl group at C-4. ^{13}C NMR signals of C-21, C-22 and C-23 also demonstrate that this moiety does not have the same structure exhibited by the side chain of ethylpavlovol. The chemical shift

Table 1
^{13}C NMR chemical shifts (δ in ppm) of compounds **1**, **1a**, **2**, **2a**, **3** in $CDCl_3$

Carbon	**1**	**1a**	**2**	**2a**	**3**
1	37.2	36.6	36.8	36.5	37.2
2	32.1	27.2	28.8	28.2	31.7
3	76.1	75.7	76.6	78.9	71.8
4	38.6	74.2	39.2	40.1	42.3
5	52.5	52.9	50.9	51.0	140.7
6	27.8	20.6	31.0	31.9	121.8
7	30.2	32.5	32.2	32.1	31.9
8	35.8	34.9	34.8	34.9	31.8
9	55.2	55.6	54.5	54.4	51.2
10	33.5	36.2	35.6	36.0	36.5
11	20.2	20.6	21.1	21.1	21.1
12	39.5	40.0	40.0	40.0	39.8
13	42.1	42.5	42.4	42.4	42.4
14	56.2	56.6	56.6	56.6	56.8
15	22.6	24.2	24.2	24.2	24.3
16	26.7	28.3	29.0	29.0	28.2
17	55.7	56.1	56.0	56.0	55.9
18	11.9	12.1	12.2	12.2	11.9
19	11.6	14.0	13.3	13.2	19.4
20	73.7	36.3	40.5	40.5	36.1
21	24.9	18.8	21.1	21.2	18.8
22	39.5	33.9	138.3	138.4	33.9
23	33.3	26.4	129.2	129.2	26.4
24	45.6	46.1	51.2	51.2	45.8
25	31.0	28.9	31.8	31.7	19.1
26	20.1	19.6	20.9	21.1	19.8
27	17.2	19.0	19.0	19.0	19.2
28	23.7	23.0	25.4	25.4	23.0
29	13.1	12.3	25.4	25.4	12.1
30	15.0	25.4	15.2	15.2	
CH_3CO–				21.1	

changes observed for the C-2, C-6 and C-30 signals of compound **1**, when compared to those of ethylpavlovol **1a**, are also in agreement with the proposed structure for **1**. Similar differences are also observed when comparing the calculated ^{13}C NMR spectra for both isomers. ^{1}H NMR spectrum confirms the presence of OH-3 due to the signal of H-3 at δ 3.18 as a multiplet, being identified the resonances of Me-26 and Me-27 at δ 0.69 and δ 0.72 as doublets, and those of Me-18, Me-19 and Me-21 as singlets at δ 0.57, 0.97 and 1.16, respectively.

The fragmentation detected by EIMS is in agreement with the structure proposed for this new dihydroxysterol. The molecular ion appeared at *m/z* 446 corresponding to molecular formula $C_{30}H_{45}O_2$. The fragments at *m/z* 431(30) $[M\text{-}Me]^+$, 416(54) $[M\text{-}2Me]^+$, 398(24) $[M\text{-}2Me\text{-}H_2O]^+$, 386(24) $[M\text{-}4Me]^+$, 372(49) $[M\text{-}3Me\text{-}Et]^+$and 356(65) $[M\text{-}Et\text{-}H_2O\text{-}CHMe_2]^+$as well as those at *m/z* 262(28) $[M\text{-}C_{10}H_{21}O$ (side chain)$\text{-}H_2O\text{-}C_2H_4$ (ring D)$]^+$and *m/z* 202(25) $[M\text{-}C_{10}H_{21}O\text{-}H\text{-}C_5H_{10}O$ (ring A)$]^+$confirm the proposed structure for this 24-ethyl-4α-methyl-cholestane-3,20-diol.

The mass spectrum of compound **2** exhibited fragments at *m/z* 428 corresponding to the molecular ion, at *m/z* 367 $[M\text{-}C_3H_7\text{-}H_2O]^+$, 316 $[M\text{-}C_8H_{16}]^+$, 289 $[M\text{-}C_{10}H_{19}(\text{side chain})]^+$, and 161 $[M\text{-}C_{10}H_{19}\text{-}C_3H_6\text{-}C_5H_{10}O]^+$, indicating the structure of a C_{30} sterol with a C_{10} monounsaturated chain. The fragmentation pattern observed is in agreement with that reported [19] as well as its ^1H NMR spectrum [22].

References

[1] Martinez SME, Molina GE, Garcia CF. Quim lnd 1994;41:19.
[2] Elyakov GB, Stonik VA. Russ Chem Bull (Int Ed) 2003;52:1.
[3] De Rosa S, Kamenarska Z, Stefanov K, Dimitrova-Konaklieva S, Najdenski C, Tzvetkova I. Z Naturforsch 2003;58c:325.
[4] Barrett SM, Volkman JK, Dunstan GA. J Phycol 1995;31:360.
[5] De Rosa S. Marine natural products—analysis, structure elucidation, bioactivity and potential use as drugs. Mediterranean marine organisms as source of new potential drugs. In: Rauter AP, Palma FB, Araújo ME, Justino J, Santos SP, editors. Natural products in the new millennium: prospects and application industrial. The Netherlands: Kluwer Academic Publishers; 2002. p. 441.
[6] Borowitzka MA. J Appl Phycol 1995;7:3.
[7] Volkman JK, Barrett SM, Blackburn SI, Mansour MP, Sikes EL, Gelin F. Org Geochem 1998;29:1163.
[8] Véron B, Dauguet J-C, Billard C. Eur J Phycol 1996;32:211.
[9] Lembi CA, Waaland JR. Algae and human affairs. Cambridge: Cambridge University Press; 1990.
[10] Borowitzka MA, Borowitzka L. Vitamins and fine chemicals. In: Borowitzka MA, Borowitzka LJ, editors. Microalgal biotechnology. Cambridge: Cambridge University Press; 1988. p. 153.
[11] Véron B, Dauguet J-C, Billard C. J Phycol 1998;34:273.
[12] Volkman JK, Rijpstra WIC, de Leeuw JW, Mansour MP, Jackson AE, Blackburn SI. Phytochemistry 1999;52:659.
[13] Volkman JK. Appl Microbiol Biotechnol 2003;60:495.
[14] Jeffrey SW, Brown MR, Volkman JK. Haptophytes as feedstocks in mariculture. In: Green JC, Leadbeater B, editors. The haptophyte algae. Oxford: Clarendon Press; 1994. p. 287.
[15] Conte MH, Volkman JK, Eglinton G. Lipid biomarkers of the Haptophyta. In: Green JC, Leadbeater B, editors. The haptophyte algae. Oxford: Clarendon Press; 1994. p. 351.
[16] Volkman JK, Farmer CL, Barrett SM, Sikes EL. J Phycol 1997;33:1016.
[17] Brotas V, Sampayo MAM. 1° Simpósio Nacional NOPROT-81; 1983.
[18] Kojima H, Sato N, Hatano A, Ogura H. Phytochemistry 1990;29:2351.
[19] Knapp Jr FF, Schroepfer Jr GJ. Steroids 1975;26:339.
[20] Gladu PK, Patterson GW, Wikfors GH, Lusby WR. Lipids 1991;26:656.
[21] Patterson GW, Gladu PK, Wikfors GH, Parish EJ, Livant PD. Lipids 1993;28:771.
[22] Tam Ha TB, Kokke WCMC, Djerassi C. Steroids 1982;40:433.