Steroids 74 (2009) 927–930

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

3-Keto-22-epi-28-nor-cathasterone, a brassinosteroid-related metabolite from *Cystoseira myrica*

Abdel-Hamid A. Hamdy[a], Elsayed A. Aboutabl[b], Somayah Sameer[a], Ahmed A. Hussein[c,*], Ana R. Díaz-Marrero[d], José Darias[d], Mercedes Cueto[d,**]

[a] *Chemistry of Natural and Microbial Products Department, NRC, Cairo, Egypt*
[b] *Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt*
[c] *Chemistry of Medicinal Plants Department, NRC, Cairo, Egypt*
[d] *Instituto de Productos Naturales y Agrobiología del CSIC, Avda. Astrofísico F. Sánchez, 3, Apdo. 195, 38206 La Laguna, Tenerife, Spain*

ARTICLE INFO

Article history:
Received 18 May 2009
Received in revised form 23 June 2009
Accepted 24 June 2009
Available online 2 July 2009

Keywords:
Cystoseira
C_{27}-brassinosteroid-related
Cytotoxicity

ABSTRACT

Bioassay-guided purification of an ethanolic extract of *Cystoseira myrica* against HEPG-2 (liver) and HCT116 (colon) human cancer cell lines led to the isolation of 3-keto-22-epi-28-nor-cathasterone, **1** and cholest-4-ene-3,6-dione, **2**. This finding allowed us to report for the first time that a brassinosteroid-related metabolite occurs in seaweed. These compounds showed activity in the range of 12.38–1.16 μM with selective activity of compound **2** to liver cancer cell lines.

1. Introduction

Despite that plants and animals have diverged more than 1 billion years ago, they both use polyoxygenated steroidal molecules as hormones. Brassinosteroids (BRs), the most recently discovered class of plant-specific steroid hormones, essential for growth and development, are polyoxygenated plant steroids with structural similarity to ecdysteroids, growth-regulating steroid hormones found in vertebrates and insects [1]. More than 50 naturally occurring BRs have been identified in a wide range of plant species being C_{28}-BRs (i.e., casthasterone (CS), brassinolide (BL)) and C_{27}-BRs (i.e., 28-norcasthasterone) the most abundant and widely occurring [2] (Fig. 1). Other brassinosteroids are distributed in a limited number of plant species but two of them, epicasthasterone and (24*S*)-24-ethylbrassinone, have been found in a freshwater green alga [3] (Fig. 1).

Isolation of compound **1** from *Cystoseira myrica* (Fig. 2), allowed us to report for the first time that a brassinosteroid-related metabolite occurs in seaweed. The brown alga *C. myrica* was selected for chemical study among several algal species of the Red Sea by the cytotoxic activity of its ethanolic extract against HEPG-2 and HCT116, IC_{50} 16.10 and 6.96 μg/mL, respectively. Bioassay-guided fractionation of the extract led to the isolation of the novel compound **1** and the known **2** [4].

2. Experimental

2.1. General procedures

Optical rotations were measured on a Perkin-Elmer model 343 Plus polarimeter using a Na lamp at 25 °C. IR spectra were obtained on a Fourier Transform Infrared Spectrometer (JASCO FT/IR–6100 Japan). Analysis was performed using KBr method. ^{1}H NMR and ^{13}C NMR, HSQC, HMBC, COSY and NOESY spectra were measured employing a Bruker AMX 500 instrument operating at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. Two-dimensional NMR spectra were obtained with the standard Bruker software. EIMS and HREIMS data were taken on a Micromass Autospec spectrometer. Merck Si gel (70–230 mesh and 230–400 mesh, for gravity flow and flash chromatography, respectively) was used for column chromatography. Merck 5554 Kieselgel 60 F_{254} sheets were used for TLC analysis.

2.2. Biological material

C. myrica was collected from the region of Fayed, Egypt, in April 2006 and was identified by Dr. Adel Fahmi, Faculty of Science, Ein

* Corresponding author. Tel.: +20 2 33371499; fax: +20 2 33077931.
** Corresponding author. Tel.: +34 922 252 144; fax: +34 922 260 135.
E-mail addresses: hussein-ahmed@lycos.com (A.A. Hussein), mcueto@ipna.csic.es (M. Cueto).

0039-128X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2009.06.008

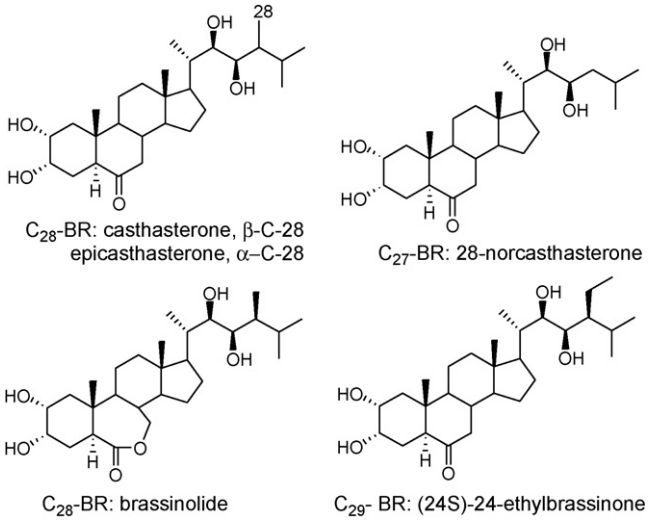

Fig. 1. Selected examples of C_{27}-, C_{28}- and C_{29}-BRs.

Shams University. The alga was washed with water, dried in the shade, and ground to a fine powder with a blender.

2.3. Extraction and isolation

2.5 kg of air-dried *C. myrica* was soaked in ethanol 95% at room temperature for 3 days, the process was repeated three times. The combined ethanol extracts were filtered and evaporated under reduced pressure to yield 115.0 g total extract. The total crude extract was applied to a flash silica column (15 cm × 25 cm) and eluted with a solvent gradient of increasing polarity from hexane to ethyl acetate. Fractions of 250 ml were collected and those exhibiting similar TLC profiles were combined. Fraction C-9 (522.0 mg, IC_{50} = 0.8, 4.36 μg/mL) was eluted through silica gel using hexane–EtOAc (75:25) from which sub-fraction 9-1 (60.0 mg) was purified by prep-TLC, solvent system $CHCl_3$–MeOH (99:1), to yield compound **1** (37.0 mg). Fraction C-6 (2.12 g, IC_{50} = 10.7, 13.6 μg/mL) was chromatographed on silica gel and eluted with hexane–EtOAc mixtures (3%, 5%, 10%, 20%, 50% and EtOAc 100%). Sub-fraction 6-3 (165.0 mg) was eluted with hexane–EtOAc (9:1), and purified on prep-TLC ($CHCl_3$–hexane–MeOH (77:22:1)) to yield compound **2** (15.0 mg).

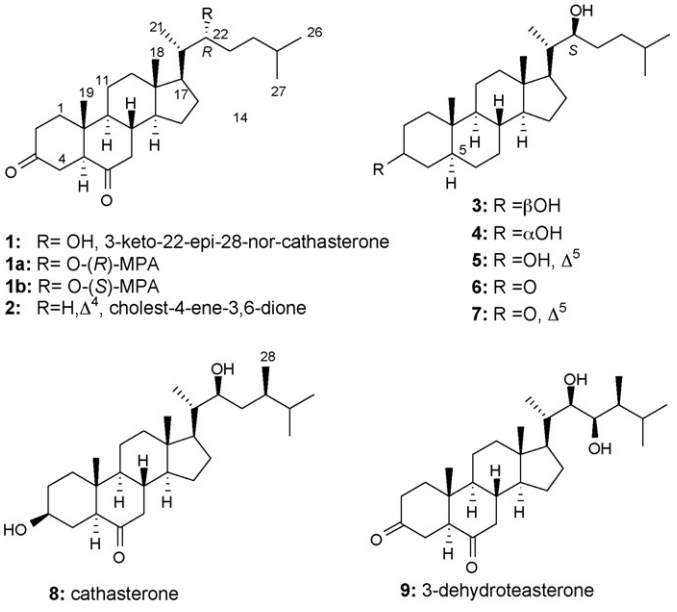

Fig. 2. Occurring 22-hydroxylated BRs-related compounds in *C. myrica* and Arabidopsis seedlings.

Table 1
NMR data of compound 1 [500 MHz, δ ppm, (*J*) Hz, $CDCl_3$].

#	δ_H	δ_C
1	1.60 m; 2.08 m	38.1
2	2.33 m; 2.40 m	37.4
3	–	211.3
4	2.30 m; 2.57 m	36.9
5	2.58 m	57.5
6	–	208.9
7	1.98 dd (12.9; 12.9); 2.36 m	46.6
8	1.83 m	37.9
9	1.32 m	53.5
10	–	41.2
11	1.44 m; 1.65 m	21.7
12	α: 1.24 m; β: 2.04 m	39.4
13	–	43.3
14	1.26 m	56.2
15	1.11 m; 1.56 m	24.1
16	1.36 m; 1.72 m	27.2
17	1.17 m	53.1
18	0.70 s	12.0
19	0.94 s	12.5
20	1.70 m	42.2
21	0.92 d (6.6)	12.4
22	3.60 bd (9.8)	73.9
23	1.18 m; 1.23 m	27.5
24	1.15 m; 1.40 m	36.0
25	1.24 m	28.1
26	0.87 d (6.6)	22.4
27	0.89 d (6.6)	22.9

2.3.1. Compound *1*

White powder; $[\alpha]^{20}$ −30 (*c* 0.33, CH_2Cl_2); IR (KBr) ν_{max} 3545, 3406, 2925, 2857, 1709, 1631, 1462, 1385, 1271, 1242, 1163, 1116, 1078, 1027, 798, 648, 611, 464 cm^{-1}; ^1H NMR and ^{13}C NMR in $CDCl_3$, see Table 1; EIMS 70 eV *m/z* (rel. int.): 416 $[M]^+$ (1), 398 $[M-H_2O]^+$ (11), 316 $[M-C_6H_{12}O]^+$ (100), 287 $[M-C_8H_{17}O]^+$ (46), 83 (66); HREIMS *m/z* 416.3306 (calcd. for $C_{27}H_{44}O_3$, 416.3290), 398.3190 (calcd. for $C_{27}H_{42}O_2$, 398.3185), 316.2388 (calcd. for $C_{21}H_{32}O_2$, 316.2402), 287.2027 (calcd. for $C_{19}H_{27}O_2$, 287.2011).

2.4. (R)- and (S)-MPA ester derivatives *1a* and *1b*

A solution of compound **1** (4.1 mg, 9.8×10^{-3} mmol) in 1.0 mL of CH_2Cl_2 was treated with *N,N′*-dicyclohexylcarbodiimide (9.9 mg, 4.8×10^{-2} mmol), 4-dimethylaminopyridine (5.3 mg, 4.3×10^{-2} mmol) and (*R*)-α-methoxy-α-phenylacetic acid (9.9 mg, 6.0×10^{-2} mmol) and stirred at room temperature for 5 h. After filtration, the reaction mixture was purified by silica gel chromatography (hexane–EtOAc (1:1)) to give the (*R*)-MPA ester derivative **1a** (2.6 mg, 4.7×10^{-3} mmol, 48.3% yield). The same experimental procedure was followed to obtain the (*S*)-MPA ester derivative **1b** (2.4 mg, 4.4×10^{-3} mmol, 44.9% yield).

2.5. Cytotoxicity

The method used was previously described [5]. Human cancer cells were seeded into 96-multiwell plates at 104 cells per well. After 24 h, the cells were washed and maintained with different concentrations of isolate for 48 h, at 37 °C and 5% CO_2. Triplicate wells were prepared for each individual dose. Cytotoxicity was determined using the colorimetric SRB assay and scored as percentage reduction of treated culture *versus* untreated control culture.

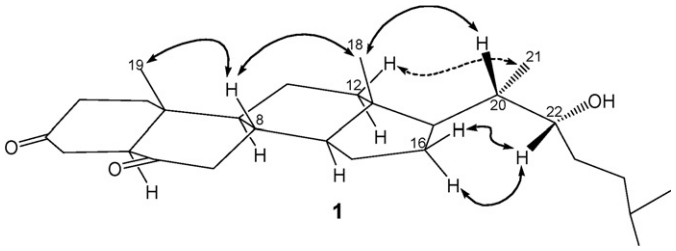

Fig. 3. Selected NOEs of compound **1**.

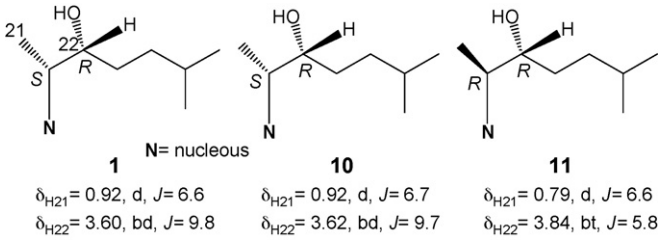

Fig. 4. Selected ^{1}H NMR chemical shifts of 1, 10 and 11.

The IC_{50} values on the cell growth were obtained from the drug dose–response curves using the software Origin 6.1.

3. Results and discussion

3-Keto-22-epi-28-nor-cathasterone **1** is a C_{27}-polyoxygenated steroid with the following features: (a) like natural brassinosteroids it possesses a common 5α-cholestane skeleton; (b) it contains only one hydroxyl group in the side chain like compounds **3**–**7** [6], and cathasterone **8** [7], both identified in cultured cells of *Catharanthus roseus*; (c) A/B rings of **1** bear a 3,6-diketo functionality, which is uncommon in C_{27}-steroids. At present, only a few compounds, in addition to **1** and **2**, have been reported with that functionality [8]. Among BRs only one compound bearing a 3,6-diketo group, 3-dehydroteasterone **9**, has been identified [9].

Compound **1** was isolated as a white powder. NMR data coupled with a molecular ion at *m*/*z* 416 (HREIMS) suggested a molecular formula of $C_{27}H_{44}O_3$, indicating six degrees of unsaturation. The ^{13}C NMR data and a DEPT NMR experiment (Table 1) were consistent for a C-27 sterol having two ketone and one hydroxyl groups. Comparison of ^{1}H and ^{13}C chemical shifts of **1** with those reported for steroids possessing a diketo-3,6 nucleus such as stigmastane-3,6-dieneone [10] indicated that **1** must be a 3,6-diketocholestane. 2D NMR experiments, HSQC, HMBC, and COSY, allowed us to confirm the position of the ketone groups at C-3 and C-6. The presence of a hydroxyl group at C-22 was established by COSY correlations and by the HMBC correlation of H_3-21 (δ_H 0.92) with the carbon that bears the hydroxyl group (δ_C 73.9, C-22).

The relative stereochemistry of **1** was assigned on the basis of a 2D NOESY experiment. The C-5/C-10; C-8/C-9 and C-13/C-14 *trans* fused ring junctions were established by NOE correlations of H-8 with H_3-18 and H_3-19. NOEs observed between H_3-18 and H-20 indicated a β disposition for the side chain (Fig. 3). The absolute configuration of C-22 was established by derivatization with (*R*)- and (*S*)-α-methoxy-α-phenylacetic acids (MPA). NMR analysis [11] of the $\Delta\delta$ values for the two MPA esters **1a** and **1b** gave clear evidence to assign the absolute stereochemistry at C-22 as *R* (Table 2). The observed NOEs between H-22 and H_2-16 and between H_3-21 and H-12β indicated C-21 must be in α disposition, therefore, considering that the absolute stereochemistry of C-20 has been established as *R*, the absolute stereochemistry of C-20 must be *S*. This is in good agreement with the chemical shift of C-20 (δ 42.6) [12] and with those reported for H_3-21 and H-22, for the synthetic steroids **10** [13] and **11** [14] whose stereochemistries are 20*S*, 22*R* and 20*R*, 22*R*, respectively (Fig. 4). Thus, the absolute configuration of **1** can be established as 5*S*, 8*S*, 9*S*, 10*R*, 13*S*, 14*S*, 17*R*, 20*S* and 22*R*.

Investigation on the natural occurrence of 22-hydroxylated steroids in wild-type Arabidopsis seedlings allowed the identification, for the first time in the Plant Kingdom, of the C_{27}-BRs **3**–**6** (Fig. 2). Both **3** and **6** partially rescued the *det2* mutant phenotype in the dark which supports the presence of an early C-22 oxidation pathway [6]. Although little is known about the biosynthetic pathways of C_{27}-BRs such as 28-norcastasterone (Fig. 1), the finding of compounds **3**–**6** supports the idea that C_{27}-sterols are direct precursors of the corresponding C_{27}-BRs, and suggests that **3**–**6** derive from cholesterol *via* a biosynthetic pathway similar to C_{28}-BRs biosynthesis [15]. In fact, cholesterol is the best substrate for cytochrome P450, CYP90B1, that catalyses C-22 hydroxylation and, according to kinetic analysis, the substrate specificity of CYP90B1 indicated that sterols with a C-5–C-6 double bond are preferred substrates when compared with stanols, which have no double bond at that position [16].

Compound **1** was active against human liver (HEPG-2) and colon (HCT116) cancer cells with an IC_{50} 2.96 μM and IC_{50} 12.38 μM, respectively. Compound **2** was found to be significantly cytotoxic to both cell lines, with an IC_{50} of 5.63 and 1.16 μM, respectively. It exhibited a particularly high cytotoxicity to liver cancer cells, where the cell number of HEPG-2 fell below 10% that of the control at 3.79 μM (IC_{10}).

Table 2
^{1}H NMR $\Delta\delta$ ($\delta_R - \delta_S$) values ($CDCl_3$, ppm, recorded at 500 MHz) of the diastereomeric mPA esters **1a** and **1b**.

#	δ_R	δ_S	$\Delta\delta^{RS}$ ($\delta_R - \delta_S$)
H-20	1.53	1.81	−0.28
Me-21	0.64	0.89	−0.25
H-25	1.46	1.24	+0.22
Me-26[a]	0.82	0.64	+0.18
Me-27[a]	0.83	0.65	+0.18

[a] Interchangeable values.

Acknowledgments

This work was supported by the Academy of Science and Technology (Egypt) and Ministerio de Educación y Ciencia (Spain) (BIO2007-61745, SAF2006-03004). A.R.D.-M. acknowledges financial support from Programa Juan de la Cierva (Ministerio de Educación y Ciencia of Spain). Mr. E. Palmer and Mr. D. Liles provided technical support. The Government of Egypt granted permission for the collection of the samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2009.06.008.

References

[1] Clouse SD. Brassinosteroids plant counterparts to animal steroid hormones? Vitam Horm 2002;65:195–223.
[2] Bajguz A, Tretyn A. The chemical characteristic and distribution of brassinosteroids in plants. Phytochemistry 2003;62:1027–46.
[3] Yokota T, Kim SK, Fukui Y, Takahashi N, Takeuchi Y, Takematsu T. Brassinosteroids and sterols from a green alga, *Hydrodictyon reticulatum*: configuration at C-24. Phytochemistry 1987;26:503–6.
[4] Kontiza I, Abatis D, Malakate K, Vagias C, Roussis V. 3-Keto steroids from the marine organisms *Dendrophyllia cornigera* and *Cymodocea nodosa*. Steroids 2006;71:177–81.

[5] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107–12.
[6] Fujioka S, Takatsuto S, Yoshida S. An early C-22 oxidation branch in the brassinosteroid biosynthetic pathway. Plant Physiol 2002;130:930–9.
[7] Fujioka S, Inoue T, Takatsuto S, Yanagisawa T, Yokota T, Sakurai A. Identification of a new brassinosteroid, cathasterone, in cultured cells of *Catharanthus roseus* as a biosynthetic precursor of teasterone. Biosci Biotechnol Biochem 1995;59:1543–7.
[8] (a) Migliuolo A, Piccialli V, Sica D. Steroidal ketones from the sponge *Geodia cydonium*. J Nat Prod 1990;53:1262–6;
(b) Ktari L, Blond A, Guyot M. 16β-Hydroxy-5α-cholestane-3,6-dione, a novel cytotoxic oxysterol from the red alga *Jania rubens*. Biorg Med Chem Lett 2000;10:2563–5;
(c) Prakash O, Roy R, Bhakuni DS, Wahidulla S, Kamat SY. 2D-NMR Studies of a novel steroid from the red alga, *Acantophora spicifera*. J Nat Prod 1989;52:686–92;
(d) Bultel-Ponce V, Etahiri S, Guyot M. New ketosteroids from the red alga *Hypnea musciformis*. Biorg Med Chem Lett 2002;12:1715–8.
[9] Yokota T, Nakayama M, Wakisaka T, Schmidt J, Adam G. 3-Dehydroteasterone, a 3 6-diketobrassinosteroid as a possible biosynthetic intermediate of brassinolide from wheat grain. Biosci Biotechnol Biochem 1994;58:1183–5.
[10] Wei K, Wei L, Koike K, Pei Y, Chen Y, Nikaido T. Complete ^{1}H and ^{13}C NMR assignments of two phytosterols from roots of *Piper nigrum*. Magn Reson Chem 2004;42:355–9.
[11] Seco JM, Quiñoá E, Riguera R. A practical guide for the assignment of the absolute configuration of alcohols, amines and carboxylic acids by NMR. Tetrahedron Asymm 2001;12:2915–25.
[12] Letourneux Y, Khuong-Huu Q, Gut M, Kuckacs G. Carbon-13 nuclear magnetic resonance of natural and related products XXII. Identification of C-22 epimers in steroids by carbon-13 nuclear magnetic resonance spectroscopy. J Org Chem 1975;40:1674–5.
[13] Amann A, Ourisson G, Luu B. Stereospecific synthesis of the 4 epimers of 7 22-dihydroxycholesterol. Synthesis 1987:1002–5.
[14] Kurek-Tyrlik A, Wicha J, Zarecki A. Methylation and hydroxymethylation of allylic alcohols via radical cyclization. Methodology for stereoselective construction of an aliphatic chain in application to sterol synthesis. J Org Chem 1990;55:3484–92.
[15] Sakurai A, Biosynthesis. In: Sakurai A, Yokota T, Clouse SD, editors. Brassinosteroids—steroidal plant hormones. Tokyo, Japan: Springer; 1999. p. 91–111.
[16] Fujita S, Ohnishi T, Watanabe B, Yokota T, Takatsuto S, Fujioka S, et al. Arabidopsis CYP90B1 catalyses the early C-22 hydroxylation of C27, C28 and C29 sterols. Plant J 2006;45:765–74.