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A new cytotoxic stigmastane steroid from *Pistia stratiotes*

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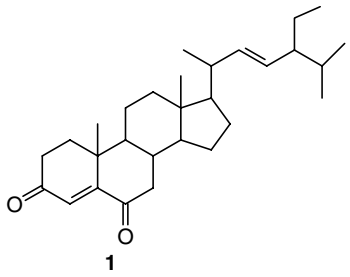
A new cytotoxic stigmastane, 7β-hydroxy-4,22-stigmastadien-3-one (**3**), together with two known cytotoxic stigmastanes (**1**, **2**) and a norisoprenoid (**4**), have been isolated from the aquatic plant *pistia stratiotes*. The structure determination was accomplished by spectroscopic methods.

1. Introduction

In pursuing our chemical study of aquatic plants distributed in Egypt, I report here the results of chemical examined of *Pistia stratiotes* L. Two reports have described the isolation of a stigmastane derivative, a sitosterol glycoside characterized by the presence of two acetyl groups at C-2′ and C-4′ in addition to a stearyl residue at the C-6′ position and two sitosterol xylopyranosides bearing a stearyl residue at the C-2′ and C-4′ positions from this plant [1, 2].
In this paper I describe the isolation and the characterization of a new stigmastane derivative (**3**) together with the known a stigmastane derivative (**1**, **2**) and norisoprenoid (**4**).

2. Investigations, results and discussion

In methylene chloride-methanol (1 : 1) extract of the aquatic plant *Pistia stratiotes* was fractionated on a silica gel column using a gradient of n-hexane/ethyl acetate as gradient solvent. The fractionation were monitored by cytotoxic bioassay using two proliferating mouse cell lines, NIH3T3 and KA3IT to yield four compounds (**1**–**4**).
The structures of the known compounds **1**, **2** and **4** were established by comparing their physical and spectral data with those in the corresponding products reported in the literature [1, 3–6].
Compound **3**, $[\alpha]_D^{24}$ − 50°, showed in its EI MS a molecular ion at m/z 426 which, together with ^{13}C NMR data, and HREI MS suggested a molecular formula of $C_{29}H_{46}O_2$ (m/z 426.3504; calcd. for 426.3349). Two double doublets at δ 5.17 and 5.03, attributable to two olefinic protons, three methyl doublets at δ 1.02, 0.85, 0.79 and a methyl triplet at δ 0.80 were displayed in the ^1H NMR spectrum, these data, together with the presence in the MS of a fragment at m/z 287 due to the loss of the side-chain $C_{10}H_{19}$, suggest a stigmastane skeleton with unsaturation at C-22. The ^{13}C NMR data (Table 1) supported this. Moreover the chemical shifts of the side-chain protons and carbons were in good agreement with the absolute configuration S at C-24 [7, 8].
The IR spectrum showed strong bands at 3400 and 1670 cm^{-1}. The above data, together with the presence of



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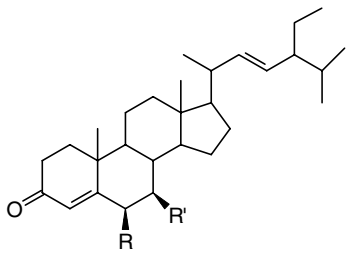

R	R'
2 = OH	H
3 = H	OH

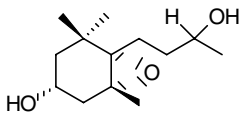

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Table 1: ^{13}C NMR data of compounds 1–4 (75 MHz, $CDCl_3$)

Compd. No.	1	2	3	4
1	35.5	37.1	38.6	34.9
2	34.2	34.3	36.3	47.0
3	202.3	200.5	202.3	66.5
4	125.5	126.3	126.1	40.8
5	161.1	168.6	165.2	64.2
6	199.5	73.3	31.2	68.2
7	46.8	38.6	70.6	124.9
8	39.8	29.7	45.4	137.8
9	51.3	53.6	50.0	69.4
10	33.9	38.0	38.3	19.9
11	21.1	21.1	21.2	24.7
12	40.4	39.5	41.8	29.5
13	42.4	42.4	43.0	23.7
14	55.7	55.9	51.3	
15	25.4	25.4	26.4	
16	29.7	28.9	29.1	
17	56.7	56.0	54.7	
18	12.1	12.2	12.2	
19	17.4	19.5	17.3	
20	39.0	40.5	40.3	
21	17.5	20.9	21.0	
22	137.9	138.2	138.1	
23	129.8	129.5	129.5	
24	50.9	51.3	50.0	
25	31.9	31.9	31.9	
26	20.9	21.2	21.4	
27	19.0	19.0	19.0	
28	24.0	24.2	25.4	
29	12.3	12.3	12.3	

Table 2: Cytotoxic activity of alcohol extract and the compounds 1–3 from *Pistia stratiotes* in cultured mammalian cell lines

Compd.	IC_{50} (mg/ml)	
	NIH3T3	KA3IT
Alc. Ext. of *P. stratiotes*	20	10
1	40	20
2	40	10
3	40	10

signals at δ 202.34, 165.17, 126.12 and 70.55 in the ^{13}C NMR spectrum and a signal at δ 5.69 and 3.68 in the ^{1}H NMR spectrum, suggested the presence of an α,β-unsaturated carbonyl group and a secondary hydroxyl function in the molecule.

The hydroxyl function was located at C-7 on the basis of the H−H COSY cross peaks related to the geminal proton at α 3.68 and the signals at α 1.58, 1.94 and 2.39. The signal at δ 1.94, correlated in the H−C COSY to the methine carbon at δ 51.25, was attributed to H-8 owing to its cross-peak with the signal at δ 1.62, already attributed to H-9. On the other hand, the signals at δ 1.58 and 2.39, attributed to the H-6 protons and correlated to the methylene carbon at δ 41.84 did not show further correlations according to the presence of the quaternary carbon C-5 at the α-position.

Cytotoxic assay [9, 10] for the total alcohol extract of *Pistia stratiotes* as well as the isolated compounds **1–3** (Table 2) were carried out *in vitro* using two proliferating mouse cell lines, a normal fibrolast line NIH3T3 and virally transformed forms KA3IT. Two compounds exhibited substantial activities on the cancer cell line KA3IT (IC50 = 10 μg/ml) and fortunately they showed less cytotoxicity towards the normal cells NIH3T3. These results suggest the potential of the plant metabolites as a source of anticancer drugs.

3. Experimental

3.1. Plant material, apparatus and methods

^{1}H- and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively (VI-500 Spectrometer, USA) and chemical shifts are given in δ (ppm) relative to TMS as internal standard. IR: thin films of $CHCl_3$, recorded on a Protégé-400 (S.S.P.) Spectrophotometer. EIMS: 70 eV on a Kratof MS-25.

The aquatic plant *Pistia stratiotes* was collected from the irrigation channels of the Nile Delta in Dammietta, Egypt. A voucher sample was identified by Prof. Abdel-Hamed Cheder, Dept. of Botany, Dammietta Faculty of Science, Mansoura University, and deposited at the Botany Department, Dammietta Faculty of Science, Mansoura University.

3.2. Extraction and isolation

The plant was dried in the shade at room temperature and powdered. The powdered dried plant 1 kg was extracted to exhaustion with CH_2CH_2 : MeOH (1 : 1) at room temperature. The extract was evaporated under reduced pressure to give a dark brown viscous oily residue (20 g 2% dry weight). The latter analysed on a silica packed column using n-hexane-EtOAc gradient. Fractions of 50 ml were collected. The fractions containing a single compound were combined and further purified by preparative TLC to give the following compounds in the following order.

3.2.1. Compound **1**

Identified as Stigmast-4,22-diene-3,6-dione. Fractions 13,14 were combined and rechromatographed on silica gel plates using a mixture of n-hexane-EtOAc (7 : 3) to give compound **1** (37 mg, 0.0037% dry wt.).
$[\alpha]_D^{24}$ + 10.4° (c 1.25, $CHCl_3$); IR (thin film, cm^{-1}) 2984, 1681 (C=O), 1640 (C=C); HREIMS: m/z 424.3341 (calcd. 424.335112) $C_{29}H_{44}O_2$; EIMS m/z (rel. int.) 424 (55)$[M]^+$ $[C_{29}H_{44}O_2]$, 381 (70) $[M\text{-}C_3H_7]^+$, 312 (20), 285 (100), 243 (40), 189 (20), 137 (95), 83 (60), 55 (96). ^{1}H NMR ($CDCl_3$) δ ppm: 6.17 (1 H, br, s, H-4), 5.15 (1 H, dd, j = 15,9 Hz, H-22), 5.04 (1 H, dd, j = 15, 8.5 Hz, H-23), 1.16 (3 H, s, Me-19), 1.04 (3 H, d, j = 6 Hz, Me-21), 0.85 (3 H, t, j = 6.5 Hz, H-29), 0.80 (6 H, d, j = 7.5 Hz, Me-26 and Me-27), 0.74 (3 H, s, Me-18); ^{13}C NMR: see Table 1. The physical and spectral properties identical to the values quoted in the literature [4].

3.2.2. Compound **2**

Identified as 6B-hydroxystigmasta-4,22-diene-3-one. Fractions 18–20 were combined and rechromatographed on silica gel plates using a mixture of n-hexane-EtOAc (6 : 4) to give compound **2** (25 mg, 0.0025% dry wt.).
$[\alpha]_D^{24}$ − 0.88° (c 1.35, $CHCl_3$); IR (thin film, cm^{-1}) 3501 (OH), 3000, 2825, 1673 (C=O), HREIMS: m/z 426.3513 (calcd. 426.3349762) $C_{29}H_{46}O_2$; EIMS m/z (rel. int.) 426 (40) $[M]^+$ $[C_{29}H_{46}O_2]$, 408 (20) $[M\text{-}18]^+$, 365 (75), 314 (37), 285 (40), 269 (95), 227 (45), 97 (60), 81 (65), 55 (100). ^{1}H NMR ($CDCl_3$) δ ppm: 5.81 (1 H, br s, H-4), 5.14 (1 H, dd, j = 15, 8.5 Hz, H-22), 5.03 (1 H, dd, j = 15, 8 Hz, H-23), 4.35 (1 H, t, j = 2.5 Hz, H-6), 1.38 (3 H, s, Me-19), 1.02 (3 H, d, j = 7 Hz, Me-21), 0.85 (3 H, t, j = 6.5 Hz, H-29), 0.81 (3 H, d, j = 7.5 Hz, Me-26), 0.80 (3 H, d, j = 7.5 Hz, Me-27), 0.76 (3 H, s, Me-18); ^{13}C-NMR: see Table 1. The physical and spectral properties identical to the values quoted in the literature [5].

3.2.3. Compound **3**

Fractions 26–28 which exhibited a major spot on TLC were combined and purified by CC (silica gel, $CHCl_3$−MeOH, 19 : 1) to give 20 mg of 7B-hydroxy-stigmata-4,22-diene-3-one (3). $[\alpha]_D^{24}$ − 50.4° (c 1.34, $CHCl_3$); IR (thin film, cm^{-1}) 3400 (OH), 3017, 2960, 2825, 1670 (C=O), HREIMS: m/z 426.3504 (calcd. 426.3349762) $C_{29}H_{46}O_2$; EIMS m/z (rel. int.) 426 (62) $[M]^+$ $[C_{29}H_{46}O_2]$, 383 (43) $[M\text{-}43]^+$, 314 (53), 287 (100), 107 (38), 81 (50), 55 (60). ^{1}H NMR ($CDCl_3$) δ ppm: 5.69 (1 H, br s, H-4), 5.17 (1 H, dd, j = 15, 8.5 Hz, H-22), 5.03 (1 H, dd, j = 15, 8.5 Hz, H-23), 3.68 (1 H, m, H-7), 1.20 (3 H, s, Me-19), 1.02 (3 H, d, j = 6.5 Hz, Me-21), 0.85 (3 H, d, j = 6.5 Hz, H-26), 0.80 (3 H, t, j = 7.5 Hz, Me-29), 0.79 (3 H, d, j = 6.5 Hz, Me-27), 0.69 (3 H, s, Me-18); ^{13}C-NMR: see Table 1.

3.2.4. Compound **4**

Fractions 40–42 which exhibited a major spot on TLC were combined and purified by CC (silica gel, $CHCl_3$−MeOH, 19 : 1) to give 10 mg of 5,6-epoxy-3-hydroxy-B-ionol (**4**); EIMS m/z (rel. int.) 208 (30) $[M\text{-}18]^+$ $[C_{13}H_{20}O_2]$, 193 (5), 125 (100), 109 (40), 95 (20), 82 (50), 55 (25). ^{1}H NMR ($CDCl_3$) δ ppm: 5.89 (1 H, d, j = 15 Hz, H-7), 5.74 (1 H, dd, j = 15, 6 Hz, H-8), 4.38 (1 H, p, j = 6 Hz, H-9), 3.88 (1 H, m, H-3), 1.28 (3 H, d, j = 6 Hz, Me-10), 1.18 (3 H, s, Me-10), 1.12 (3 H, s, Me-11), 0.97 (3 H, s, Me-12); ^{13}C-NMR: see Table 1. The physical and spectral properties identical to the values quoted in the literature [6].

3.3. Cytotoxicity

Cytotoxic assay [9, 10] was performed using two proliferating mouse cell lines, a normal fibroblast line NIH3T3 and a virally transformed form KA3IT. Samples of the extract or pure compound (5 mg) were dissolved in 62.2 μl of DMSO, and working solutions made by diluting 20 μl of DMSO solution into 2 ml of sterile medium (Dulbecco's modified Eagle's medium, Sigma Chemical Co. St. Louis, MO, USA). Two-fold or 2.5-fold dilutions of the extracts of pure compounds from 200 μl/ml to 0.5 μl/ml were prepared in triplicate in the wells of 96-well culture trays (Falcon Micro test III, #3072, Becton Dickinson Labware, Lincoln Park, NJ, USA) in 200 μl of medium containing 5% (v/v) calf serum (Hyclone Laboratories, Logon, Utah, USA). An inoculum of 2×10^3 cells was added to each well in a 100 μl aliquot of 10% calf serum in medium. The 96-well trays of cells were cultured under standard conditions until uninhibited cultures (control) became confluent. The contents of the wells were decanted, and each cell layer washed with a small amount of the medium. The wells were filled with formal saline (3.7% w/v formaldehyde in 0.15 M NaCl), and allowed to stand at room temperature for at least 30 min. The trays were washed with tap water, and attached cells stained by adding two drops of 0.5% (w/v) crystal violet solution in 20% (v/v) aqueous methanol added to each well. The trays were washed with tap water, and the IC_{50} estimated visually as the approximate concentration that causes 50% reduction in the number of stained cells adhering to the bottom of the wells.

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