Chemistry of Natural Compounds, Vol. 47, No. 5, November, 2011 [Russian original No. 5, September–October, 2011]

CYTOTOXIC EFFECT OF SOME PENTACYCLIC TRITERPENES AND HEMISYNTHETIC DERIVATIVES OF STIGMASTEROL

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UDC 547.918

*Two different oxidation reactions (PCC and $KMnO_4$) of stigmasterol gave three products, stigmasta-4,22-dien-3-one (**1a**), stigmasta-4,22-diene-3,6-dione (**1b**), and 3-O-acetyl-5β,6β-epoxystigmast-22-ene-3-ol (**1c**). The cytotoxic activities of hemisynthetic compounds, stigmasterol, and four pentacyclic triterpens **2–5** previously isolated from cultivated and wild* Triumfetta cordifolia *and identified were investigated against human fibrosarcoma cell line (HT1080). Most of the drugs showed moderate cytotoxic activity. It was also notice that the triterpene skeleton had a range of number of OH functions in which activity was observed. Spectroscopic analyses (^1H and ^{13}C NMR) and mass were used to elucidate the structure of hemisynthetic compounds.*

Keywords: oxidation reaction, stigmasterol derivatives, pentacyclic triterpenes, cytotoxic activity.

Steroids and triterpenes are two classes of secondary metabolites widespread in vegetables possessing a wide spectrum of biological activities. Some previous studies showed that many pentacyclic triterpenoids such as betulinic, oleanolic, and ursolic acids have antitumor activity [1]. Near this class, we have their biosynthetic descents that show important pharmacological properties. Thus, stigmasterol has shown anticancer activities [2] and analgesic [3] and hypoglycemic effect [4]. In continuation of our study and from the above-mentioned information, we have found it interesting to evaluate the cytotoxicity properties of three steroids, **1a**, **1b**, **1c**, obtained from oxidation reactions of stigmasterol (**1**), and four pentacyclic triterpenes (lupeol (**2**), oleanolic acid (**3**), maslinic acid (**4**), and tormentic acid) isolated from the stems of wild and cultivated *Triumfetta cordifolia* (Tiliaceae). We hereby describe the valorization of natural products and its derivatives.

Chemistry. Stigmasterol and pentacyclic triterpenes were previously isolated from *Triumfetta cordifolia* (Tiliaceae) and identified on the basis of their spectroscopic data. Some of them were also compared by TLC with the authentic samples in our laboratory. Stigmasterol (**1**), obtained in the largest amount, was used as starting material for oxidation reactions.

After its acetylation in pyridine and acetic anhydride, the product obtained was subjected to oxidation with $KMnO_4$, yielding the 5β,6β-epoxide derivative **1c** (Scheme 1). This reaction, which was carried out in the heterogeneous phase, followed the procedure described by Syamala et al. [5]. It was already noticed that this reaction was regioselective [5], according to which the trisubstituted olefinic function was more easily transformed to epoxide than di- and mono- substituted compound. Comparison between the carbons C-5 (δ_C 63.0) and C-6 (63.6) of compound **1c** from the APT spectrum with the data of the literature [6] confirmed also that this transformation is also stereoselective.

Compound **1** was also subjected to two oxidation reactions with PCC (pyridinium chlorochromate), yielding two compounds **1a**, and **1b** (Scheme 1) [7]. The main factor was the time of reaction according to which after 24 h, the product was different from those obtained after 36 h.

In the IR spectra of **1a** and **1b**, no band of the hydroxyl function was observed, and one band appeared at 1678 (1670) cm^{-1} corresponding to a conjugated ketone. The ^{13}C NMR spectrum of **1a** exhibited the signal of carbonyl at δ_C 200.4 (C-3), while those of **1b** showed two carbonyls at δ_C 202.3 (C-3) and 199.5 (C-6).

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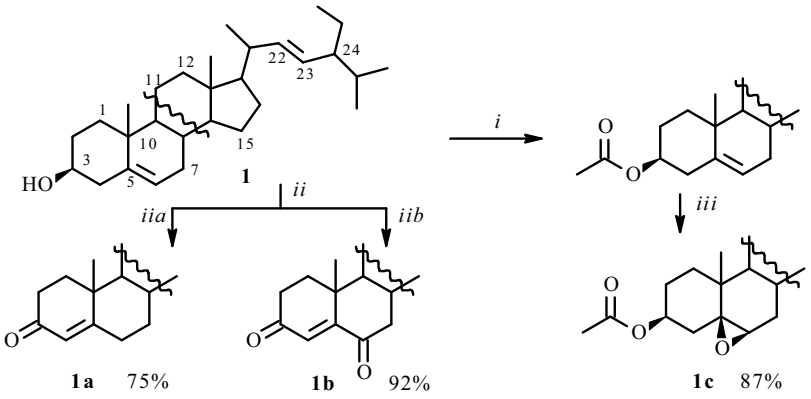
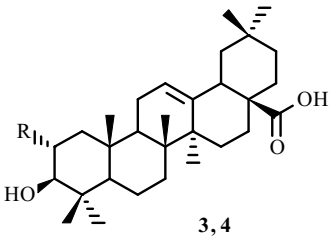
0009-3130/11/4705-0731



i. $C_5H_5N/(H_3CCO)_2O$ 1:1 (1.5 eq), room temperature, 3 h; *ii*. PCC–Al_2O_3 (10 eq)/CH_2Cl_2, room temperature; *iia*) 24 h, room temperature, R_f 31/40 (hexane–ethyl acetate 9:1); *iib)* 36 h, R_f 1/2 (hexane–ethyl acetate 9:1); *iii*. $KMnO_4/CuSO_4 \cdot 5H_2O$ (2:1), CH_2Cl_2/*tert*-ButOH/H_2O (20:0.3:1), R_f 20/37 (hexane–ethyl acetate 85:15), room temperature, 2 h.

Scheme 1

Cytotoxicity Assay. The cytotoxic activities of eight compounds were investigated on the human HT1080 tumoral cell line. These cells are able to undergo physiological cell death, a process termed apoptosis [8], and are sensitive to a wide variety of cytotoxic drugs such as etoposide, cisplatin, staurosporine, or TNF-α. In order to avoid a putative toxicity of the solvents, the maximum used volume of the compound solution never exceeded 1/100 of the total volume of culture medium. Controls with analogous concentrations of solvents were always carried out in parallel to check their lack of toxicity. Three compounds induced less than 50% cell death after 48 hours of treatment at maximum concentrations, rendering impossible the determination of an IC_{50}. This was the case of stigmasterol (**1**) (C_{max} 500 mM), stigmasta-4,22-diene-3,6-dione (**1b**) (C_{max} 100 mM), and tormentic acid (**5**) (C_{max} 100 mM). Among the eight compounds, it was possible to determine the IC_{50} value of six: stigmasta-4,22-dien-3-one (**1a**) (IC_{50} 0.3 mM), stigmasta-4,22-diene-dione (**1b**), 3-*O*-acetyl-5β,6β-epoxystigmast-22-en-3-ol (**1c**) (IC_{50} 0.54 mM), lupeol (**2**) (IC_{50} 1.1 mM), oleanolic acid (**3**) (IC_{50} 0.7 mM), and maslinic acid (**4**) (IC_{50} 0.075 mM). Thus, stigmasta-4,22-dien-3-one showed high specificity ($IC_{50} \leq 0.3$ mM) in inducing efficient cellular toxicity. The epoxide ring and the α,β-unsaturated ketone appear to be the biologically active sites. Additionnaly, considering the compounds **1**, **1c**, **2**, **3**, **4**, and **5**, it was noticed that cytotoxic activity increased with the number of hydroxyl group until a maximal number of OH functions. These means that triterpene skeletons have a range of number of OH functions in which some could have cytotoxic activity against these cells line. Even if the OH group is an alcohol function or in a carboxylic group, it is one of the great parameters of lipophilicity and hydrophobicity; accordingly, the cell membrane permeability depends on these two descriptors [9].



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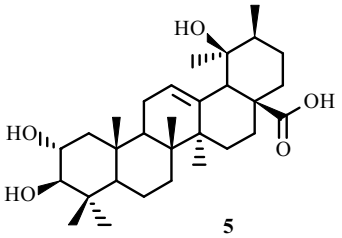
3: R = H; **4:** R = OH



5

EXPERIMENTAL

IR spectra were recorded on a Perkin–Elmer FT-IR system spectrum BX spectrometer using KBr disks. 1D NMR spectra were carried out on a Bruker DRX-400 MHz spectrometer. Melting points were measured in a Stuart Scientific Melting Point apparatus SMP_3 and are uncorrected. Silica gel GF254 and 60A (size 70–200 μm) were used to perform thin layer chromatography and column chromatography, respectively. The matrix used for the MALDI-TOF-MS was a 1 M solution of

2,5-dihydroxybenzoic acid (2,5-DHB) in 50% acetonitrile, 50% ultrapure water, and 0.1% trifluoroacetic acid (TFA); its measurements were possible using a Bruker Reflex IV time-of-flight mass spectrometer (TOF-MS) (Bruker-Daltonic, Bremen, Germany) equipped with a Scout 384 probe ion source, using a nitrogen pulsed laser (337 nm, model VSD-337ND, Laser Science Inc., Boston, MA) with energy output of 400 μJ/pulse.

We dissolved 0.034 g (0.082 mmol) of stigmasterol and 0.75 g of PCC/Al_2O_3 in DCM under magnetic stirring at room temperature for 24 h. The product (0.025 g, 75%) was obtained as a brown solid after filtration under vacuum on Celite.

Stigmasta-4,22-dien-3-one (1a). White solid; mp 123°C. IR (KBr, ν_{max}, cm^{-1}): 1678, 1640. MALDI-TOF *m/z* 433.347 $[C_{29}H_{46}O + Na]^+$. ^{13}C NMR: 37.8 (C-1), 32.2 (C-2), 200.4 (C-3), 125.4 (C-4), 168.5 (C-5), 46.9 (C-6), 31.2 (C-7), 31.4 (C-8), 50.9 (C-9), 36.5 (C-10), 21.7 (C-11), 39.8 (C-12), 42.4 (C-13), 56.4 (C-14), 24.2 (C-15), 28.2 (C-16), 56.2 (C-17), 11.9 (C-18), 20.1 (C-19), 40.4 (C-20), 21.4 (C-21), 138.1 (C-22), 129.3 (C-23), 51.2 (C-24), 31.8 (C-25), 21.1 (C-26), 18.3 (C-27), 25.4 (C-28), 11.9 (C-29).

We treated 0.034 g (0.082 mmol) of stigmasterol under the same conditions as the reaction mentioned above. The difference was the reaction time, which was 36 h in this case. Compound **1b** (0.032 g, 92%) was obtained by filtration under vacuum on Celite.

Stigmasta-4,22-diene-3,6-dione (1b). Amorphous solid. IR (KBr, ν_{max}, cm^{-1}): 2985, 1670, 1639. MALDI-TOF *m/z* 447.331 $[C_{29}H_{50}O_2 + Na]^+$. ^{13}C NMR: 34.2 (C-1), 34.0 (C-2), 202.3 (C-3), 125.4 (C-4), 165.5 (C-5), 199.5 (C-6), 46.8 (C-7), 34.2 (C-8), 51.0 (C-9), 39.1 (C-10), 20.9 (C-11), 39.8 (C-12), 42.5 (C-13), 56.5 (C-14), 24.0 (C-15), 28.0 (C-16), 55.8 (C-17), 12.0 (C-18), 17.5 (C-19), 36.0 (C-20), 18.7 (C-21), 149.5 (C-22), 129.8 (C-23), 51.2 (C-24), 28.7 (C-25), 21.1 (C-26), 21.1 (C-27), 25.4 (C-28), 17.5 (C-29).

Oxydation by $KMnO_4$. We acetylated 0.153 g (0.3713 mmol) of stigmasterol in 1.0 mL of pyridine and 1.0 mL acetic anhydride for 3 h under stirring at room temperature. The acetylated compound was obtained after rotary evaporation under vacuum. This compound was dissolved in a mixture of 20 mL of DCM, 1 mL of *tert*-butanol, and 0.3 mL of H_2O. The medium was stirred at room temperature. We added 1.6 g of $KMnO_4$ and 0.8 of $CuSO_4 \cdot 5H_2O$ to the medium, and the reaction was carried out under N_2 for 24 h. After the reaction stop, 25 mL of DCM was added, and the medium was filtered under vacuum on Celite. The DCM solution was concentrated under vacuum with a rotary evaporator, yielding compound **1c** (0.152 g, 87%).

3-*O*-Acetyl-5β,6β-epoxystigmast-22-en-3-ol (1c). White solid; mp 140–141.3°C. IR (KBr, ν_{max}, cm^{-1}): 1738, 1635. MALDI-TOF *m/z* 471.385 $[C_{31}H_{50}O_3 + H]^+$. ^{13}C NMR: 37.8 (C-1), 32.2 (C-2), 71.3 (C-3), 42.4 (C-4), 63.0 (C-5), 63.6 (C-6), 31.2 (C-7), 31.4 (C-8), 50.9 (C-9), 36.5 (C-10), 21.7 (C-11), 39.8 (C-12), 42.4 (C-13), 56.4 (C-14), 24.2 (C-15), 28.2 (C-16), 56.2 (C-17), 11.9 (C-18), 20.1 (C-19), 40.4 (C-20), 21.4 (C-21), 138.1 (C-22), 129.3 (C-23), 51.2 (C-24), 31.8 (C-25), 21.1 (C-26), 19.0 (C-27), 25.4 (C-28), 11.7 (C-29), 170.6 (C=O), 21.4 (CH_3).

Cellular Viability. Human HT1080 fibrosarcoma adherent cell line was cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco′s modified Eagle′s medium (DMEM/F12) supplemented with 10% fetal bovine serum together with penicillin (100 μg/mL), streptomycin (100 U/mL), and glutamax (1% v/v) from Invitrogen. The cells were seeded in 12-well plates (5.10^4 cells/well). After 24 h, the medium was replaced in each well by 1 mL of complete medium with the appropriate concentrations of the tested drugs in THF and stored at –20°C. The cells were then incubated for 48 h, and cellular viability was determined by flow cytometric analysis. It was shown that type I (apoptosis), type II (autophagy), and type III (necrosis) cell death triggers permeabilization of mitochondria [10]. Global cell death was then determined with the cationic lipophilic $DiOC_6$ (3) dye (Invitrogen), which specifically probes mitochondrial membrane potential (ΔΨm) [11]. The decrease in forward light scattering (FSC) was also checked to confirm the cell death process [12]. After drug treatment, the media from each well were kept in centrifuge tubes. The adherent cells were detached using trypsin, pooled with the corresponding media, centrifuged, and resuspended in complete medium. The cells were then loaded with 100 nM $DiOC_6$ (3) and incubated for 30 min at 37°C. Flow cytometric measurements were performed using an XL3C flow cytometer (Beckman-Coulter). Fluorescence was induced by the blue line of an argon ion laser (488 nm) at 15 mW. Green fluorescence of $DiOC_6$ (3) was collected with a 525 nm band pass filter. The percentage of dead cells was determined by measuring the percentage of cells harboring low $DiOC_6$ (3) fluorescence. Analyses were performed on 10^4 cells.

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