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Unusual cytotoxic sulfated cadinene-type sesquiterpene glycosides from cottonseed (*Gossypium hirsutum*)

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ABSTRACT

Two new sulfated cadinene-type sesquiterpene glycosides, 13-hydroxy-7-*O*-(6′-*O*-sulfate-β-D-glucopyranosyl)-desoxyhemigossypol (**1**) and 13,15-dihydroxy-7-*O*-(6′-*O*-sulfate-β-D-glucopyranosyl)-desoxyhemigossypol (**2**), have been isolated from whole cottonseed (*Gossypium hirsutum*). Their structures, which possess an unusual 6-*O*-sulfate-glucopyranosyl moiety, were determined through the interpretation of 2D NMR spectral data and H/D exchange ESI-MS experiments. Compounds **1** and **2** were screened for their toxicity on Jurkat cells. Both compounds inhibited cellular proliferation with IC_{50} values of 8.1 and 4.2 μg, respectively.

1. Introduction

Cotton (*Gossypium* spp.) produces a large group of sesquiterpenes with the cadinane carbon skeleton that includes desoxyhemigossypol (dHG), hemigossypol (HG), gossypol (G), hemigossypolone (HGQ),[1] and the heliocides H_1, H_2, H_3, and H_4 (Fig. 1).[2] Unstressed *Gossypium* plants accumulate many of these compounds in subepidermal glands of all green tissues, which deter herbivores, and in epidermal cells of roots, probably as defense against soilborne pathogens.[3] Various sesquiterpenoid cadinanes are also elicited by fungal and bacterial infection, by toxic chemicals, and by cold stress.[4,5] Of the phytoalexins, which accumulate in stem stele of *Verticillium* wilt-resistant cotton in response to *Verticillium dahliae infection*, Mace et al. have shown that dHG has the highest antifungal activity.[6] Recently, however, it has been reported that gossypol and its metabolite, gossypolone, have anticancer effects in animal models.[7,8]

Whole cottonseed (WCS) is the unprocessed and unadulterated oilseed, which has been separated from the cotton fiber. Cottonseeds are fed to high-producing dairy cows as a source of fat and highly digestible fiber. They are also used as a forage replacer. Secondary metabolites in WCS have been studied because some components have been blamed for anti-nutritive or toxic effects:[9,10] gossypol is the main anti-nutrient limiting the use of cottonseed in monogastric animals and humans, where it acts by reducing the oxygen-carrying capacity of the blood and results in shortness of breath and of edema of the lungs.[11] Also, flavonoids have recently been reported in WCS.[12,13] The occurrence of this type of secondary metabolite in food and feed is highly desirable because they contribute to their nutritional value.

In the course of our continuing search for novel secondary metabolites of biomedical and ecological importance we investigated the terpenoids of whole cottonseed. Two new compounds, **1** and **2**, with an unusual 6-*O*-sulfate-glucopyranosyl group were isolated as minor components (Fig. 2). The structures of these compounds were elucidated by extensive spectroscopic methods including 1D (^{1}H and ^{13}C) and 2D NMR experiments (DQF-COSY, HSQC, HMBC, NOESY) as well as ESI-MS analysis and H/D exchange MS experiments. Compounds **1** and **2** were tested for cytotoxicity against Jurkat cells a human lymphoblastoid T cell line usually utilized for studies of immunotoxicity in vitro.

To the best of our knowledge this is the first report of the occurrence of glycosylated cadinene-type sesquiterpene in *Gossipium*. Compounds **1** and **2** are structurally related to desoxyhemigossypol (dHG), a key intermediate in the biosynthesis of cotton terpenoid,[1] and are probably derived through the same biosynthetic pathway.

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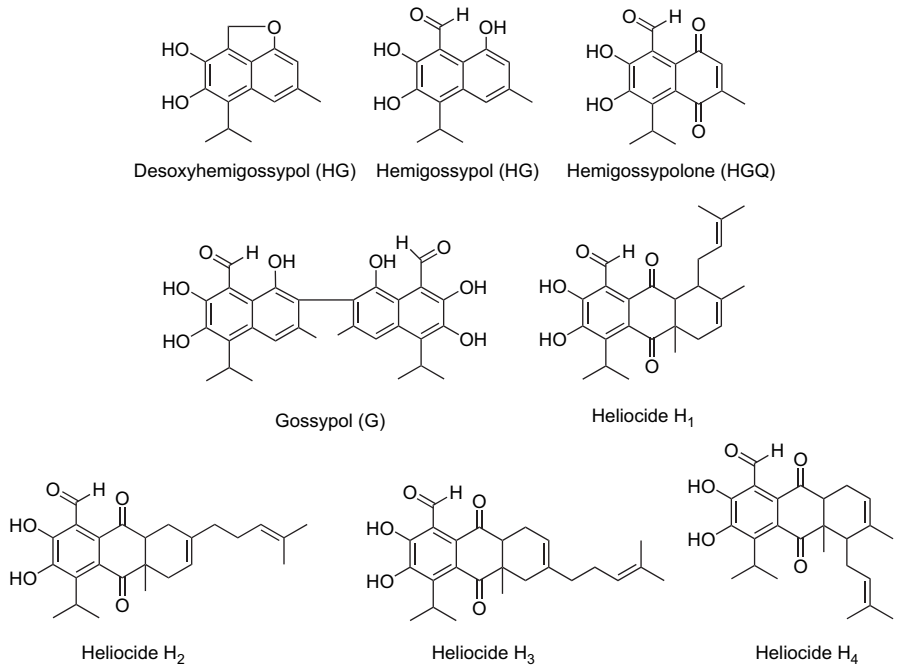

Figure 1. Sesquiterpenes with the cadinane carbon skeleton found in cotton tissue.

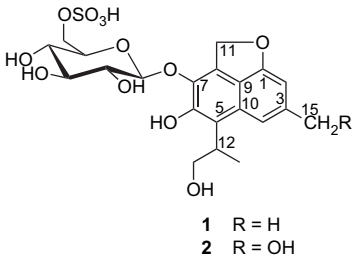

Figure 2. Cadinene-type sesquiterpene **1** and **2** from cottonseed.

There has been only two publications with reference to the structural characterization of dHG and these two studies did not report ^{13}C NMR data for dHG.[14,15] Compounds **1** and **2**, which, respectively, bear one and two oxygenated methylene substituent on naphthalene ring, instead of methyl groups present in dHG, were fully characterized by NMR and spectral data reported herein could be useful for the future NMR assessment of cotton terpenoids.

2. Results and discussion

2.1. NMR structure elucidation of 1 and 2

Compound **1** showed absorption bands for hydroxyl (3390 cm^{-1}), aromatic (1681 cm^{-1}), and sulfate (1211 cm^{-1}) groups in its IR spectrum. The (−)-ESI-MS spectrum of **1** showed as base peak the pseudomolecular ion $[M-H]^-$ at *m/z* 501 and its MS/MS spectrum gave ions at *m/z* 421 $[M-H-80]^-$ and *m/z* 259 $[M-H-80-162]^-$, which indicated the presence of a sulfate ($-OSO_3H$) or phosphate ($-OPO_3H_2$) group and a hexose. The HRESI-MS of compound **1** showed an $[M-H]^-$ ion peak at *m/z* 501.1100, consistent with the molecular formula $C_{21}H_{26}O_{12}S$.

The ^{13}C NMR spectrum of **1** showed 21 carbon signals (Table 1), 6 of which could be assigned to a hexose moiety. Aglycon signals including 10 carbons in the range δ_C 101–163 ppm, ascribable to a naphthalene ring with two directly attached hydrogen atoms, according to HSQC and HMBC spectra, an oxygenated methylene (δ_C 77.19 ppm), a methyne (δ_C 36.87 ppm), and a tertiary and a secondary methyl group. All the information mentioned above was in support of compound **1** being a sesquiterpene glycoside. Inspection of the ^1H NMR spectrum of **1** led to the identification of the following representative signals: a methyl doublet signal protons at δ 1.46 (d, J=6.9 Hz), a methyl singlet protons at δ 2.84, a methyne proton at δ 3.73 (m), two oxymethylene protons at δ 4.03 (m), two protons of a further oxymethylene groups linked directly at the naphthalene ring (δ 5.82 and 5.90; J=15.3 Hz), and two *meta*-related doublets (δ 6.34 and 7.06; J=1.5 Hz). The ^1H NMR spectrum

Table 1
^1H and ^{13}C NMR (600 MHz) data for compounds **1** and **2** in CD_3OD[a]

Position	**1**		**2**	
	δ ^1H (J_{HH} in Hz)	δ ^{13}C	δ ^1H (J_{HH} in Hz)	δ ^{13}C
1		163.29		163.30
2	6.34 (d, 1.5)	101.20	6.52 (d, 1.5)	98.70
3		139.96		143.6
4	7.06 (d, 1.5)	112.44	7.28 (d, 1.5)	111.30
5		138.12		130.10
6		138.13		138.74
7		150.10		150.11
8		126.09		125.90
9		123.21		124.39
10		121.83		122.64
11a	5.90 (d, 15.3)	77.19	5.86 (d, 15.3)	77.28
11b	5.82 (d, 15.3)		5.94 (d, 15.3)	
12	3.73 (br s)	36.87	3.75 (br s)	36.92
13a	4.03 (m)	66.47	4.05 (m)	66.46
13b	3.98 (m)		4.00 (m)	
14	1.46 (d, 6.9)	15.77	1.53 (d, 6.9)	15.78
15	2.84 (s)	23.15	4.69 (s)	66.49
1′	4.71 (d, 7.5)	105.90	4.74 (d, 7.5)	105.85
2′	3.61 (dd, 9.5, 7.5)	74.80	3.61 (dd, 9.5, 7.5)	74.80
3′	3.59 (t, 9.5)	77.32	3.58 (t, 9.5)	77.32
4′	3.54 (t 9.5)	71.13	3.53 (t, 9.5)	71.13
5′	3.70 (m)	76.61	3.71 (m)	76.61
6″a	4.43 (dd, 12.2, 4.5)	68.18	4.44 (dd, 12.2, 4.5)	68.12
6″b	4.21 (dd, 12.2, 4.5)		4.21 (dd, 12.2, 4.5)	

[a] Chemical shift values are in parts per million from TMS, and values in hertz are presented in parentheses. All signals were assigned by DQF-COSY, HSQC, and HMBC experiments.

also indicated an anomeric proton at δ 4.71 (1H, d, J=7.5 Hz) and signals in the δ 3.54–4.43 region attributable to a sugar moiety. The ^{1}H and ^{13}C NMR data of the key hydrogens and carbons (C-2′, C-3′, and C-5′) indicated a β-configuration at the anomeric position ($J_{H\text{-}1'\text{-}H\text{-}2'}$=7.5 Hz). The sugar substituent was identified as β-glucopyranosyl from the ^{1}D-TOCSY, DQF-COSY, and HSQC spectra. The ^{13}C NMR signal of C-6′ (δ 68.18) of the glucopyranosyl group was downfield with respect to those in unsubstituted glucose ($\Delta\delta_{C\text{-}6'}$=ca.+6 ppm) as expected for a 6-*O*-substituted glucosyl moiety. The inorganic nature of this substituent was deduced from the lack of further carbon resonances. The IR, MS, and NMR (absence of ^{31}P–^{13}C coupling in ^{13}C NMR spectra) data strongly suggested that this substituent is a sulfate ester of OH-6′. A further confirmation of C-6′–OSO_3H substitution was obtained with H/D exchange ESI-MS experiments (see Section 2.2).

The ^{1}H–^{1}H COSY spectrum of **1** showed proton correlations between δ_H 1.46 (Me-14) and δ_H 3.73 (H-12), between H-12 and protons at δ_H 3.98 and 4.03 (H_2-13), indicating the presence of a –CH_3–CH–CH_2OH chain together with the spin system belonging to the glucose moiety. The HSQC spectrum of **1** indicated all of the protonated carbon correlations, thereby leading to the elucidation of the structural skeleton. These results clearly showed that **1** was closely related to desoxyhemigossypol, except for the oxidation of isopropyl group and the presence of the sugar residue. HMBC correlations (H-12/C-6 and C-10, H_2-11/C-1, C-7 and C-9, H-4/C-2, C-5 and C-9, Me-15/C-4 and C-2 and H-1′/C-7) confirmed the position of the isopropanol group at C-5 and the glycosidic linkage at the C-7 position of the naphthalene ring and indicated that the methylene C-11 was connected via oxygen at C-1 position. In addition, the correlation of H-4 with H-12 in the ROESY spectrum showed them to be in the *peri* position and allowed us to discriminate between the two *meta* coupled protons, in consequence, the remaining proton at δ_H 6.34 could be assigned as H-2. On the basis of these spectral data, the structure of **1** was identified as 13-hydroxy-7-*O*-(6′-*O*-sulfate-β-D-glucopyranosyl)-desoxyhemigossypol (Fig. 2).

Compound **2** showed similar IR absorption bands and ESI-MS behavior ($[M-H]^-$ at m/z 517 and fragment ions at m/z 437 $[M-H-80]^-$ and m/z 275 $[M-H-80-162]^-$) of compound **1**. It was assigned a molecular formula of $C_{21}H_{26}O_{13}S$ as deduced by HRESI-MS ($[M-H]^-$ ion peak a m/z 517.1057) and ^{13}C NMR and DEPT analysis. In the ^{1}H and ^{13}C NMR spectra, all of the proton and carbon signals of **2** were almost superimposable on those of **1** (Table 1), except for the appearance of a further oxymethylene group (δ 4.69 and 66.49 in the ^{1}H and ^{13}C NMR spectra, respectively) and loss of one methyl group. The ^{13}C NMR signals of C-4 and C-2 were shifted upfield by 1.14 and 2.50 ppm, respectively, and C-3 was shifted downfield by 3.64 ppm, taking into account **1** as reference compound. These data suggested that the oxymethylene group is located at C-3. The sugar moiety of compound **2** exhibited a good coincidence with those of **1** in the ^{1}H and ^{13}C NMR spectra. From all these data compound **2** was identified as 13,15-dihydroxy-7-*O*-(6′-*O*-sulfate-β-D-glucopyranosyl)-desoxyhemigossypol (Fig. 2).

Compounds **1** and **2** appeared to be the first example of sulfated cadinene-type sesquiterpene glycosides in *Gossipium*.

2.2. H/D exchange ESI-MS experiments on 1 and 2

Some natural products and metabolites contain one or more sulfate or phosphate groups. Sulfo compounds have been identified from high-resolution mass spectrometry (HRMS). Recently, many reports have confirmed the presence of the sulfate ester only from the results of hydrolysis by sulfatase and low-resolution mass spectrometry (LRMS) analysis with a fragment loss of 80 mass units.[16] However, phosphate esters also give the same fragment loss and the difference between sulfate and phosphate in their exact masses is only 0.009 mass units, which is in the range of technical errors (m/z±0.01) of conventional high-resolution mass spectrometer.

Kanakubo et al. reported that daidzein-7-*O*-phosphate and genistein-7-*O*-phosphate were surprisingly hydrolyzed by sulfatase,[17] they developed a method to distinguish phosphate from sulfate group by H/D exchange MS with a low-resolution mass spectrometer (FABMS and Q-TOF).[18] This method was based on the different numbers of exchangeable protons between sulfate and phosphate groups. After these protons are exchanged for deuterium atoms, the mass difference between phosphate and sulfate should shift from 0.009 to 1.016 mass units,[18] and, in the negative ion mode, anionic phosphate species should have one deuterium atom but sulfate none. The difference of 1 nominal mass unit is very easy to distinguish by LRMS.

We applied this method to distinguish between sulfate and phosphate by LRMS using ion trap mass spectrometers. Compounds **1** and **2** with sulfate as substituent contain five and six exchangeable protons and should give a 5 and 6 mass units increase, respectively, with a neutral loss of 80 mass units, while, the same compounds with a phosphate group (six and seven exchangeable protons) should give a 6 and 7 mass units increase, respectively, and a 81 mass units loss. When compounds **1** and **2** were analyzed in the deuterium-exchange, they showed the $[M-D]^-$ peaks at m/z 506 and 523, respectively (Fig. 3). The (−)-ESI-MS spectra displayed also peaks at m/z 505 and 504, for **1**, and at m/z 522, 521 and 520 for **2**, which were due to the fact that the H/D exchange was not completed. This data suggested the presence of a sulfate group, which was confirmed by fragment ions in the MS/MS spectra of peaks at m/z 506 and 523 corresponding at neutral loss of 80 mass units (Fig. 3). Thus, in accordance with the spectroscopic data reported above, the C-6′–OSO_3H substitution of compounds **1** and **2** was ulteriorly confirmed.

These results demonstrate that H/D exchange MS with a low-resolution mass spectrometer is a convenient method to distinguish between phosphate and sulfate at the picomole level and may provide an alternative to classical and not rigorous hydrolysis by sulfatase.

2.3. Cell proliferation assay

In our study, we assessed the effects of compounds **1** and **2** on the proliferation of Jurkat T cells. We incubated cells with different doses (from 0.5 to 420 μg/ml) of both compounds and we found that both compounds were cytotoxic at highest doses. To assess the effects of methanol, in which compounds **1** and **2** were dissolved, on cellular proliferation, we incubated Jurkat cells with different concentrations of alcohol (0.5–420 μg/ml). We also calculated the IC_{50} values for both compounds and we found that IC_{50} of compound **1** was 8.1 μg while the IC_{50} of compound **2** was 4.2 μg.

3. Experimental

3.1. General methods

Unless specified, solvents were reagent grade. They were purchased from Aldrich or Fluka or Carlo Erba and were used without further purification. Optical rotations were measured on a Jasco DIP-370 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. UV spectra were obtained with a Beckman DU 670 spectrophotometer and IR spectra with a Biorad FTS 155 FT-IR spectrophotometer. A Bruker DRX-600 spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 MHz for ^{13}C, using the UXNMR software package was used for NMR experiments in $CDCl_3$. ^{1}H–^{1}H DQF-COSY (Double Quantum Filtered COSY), ^{1}H–^{13}C HSQC, HMBC, and ROESY experiments were obtained using conventional pulse sequences. Electrospray ionization mass spectrometry (ESI-MS) was performed using a LCQ Advantage ion trap mass spectrometer

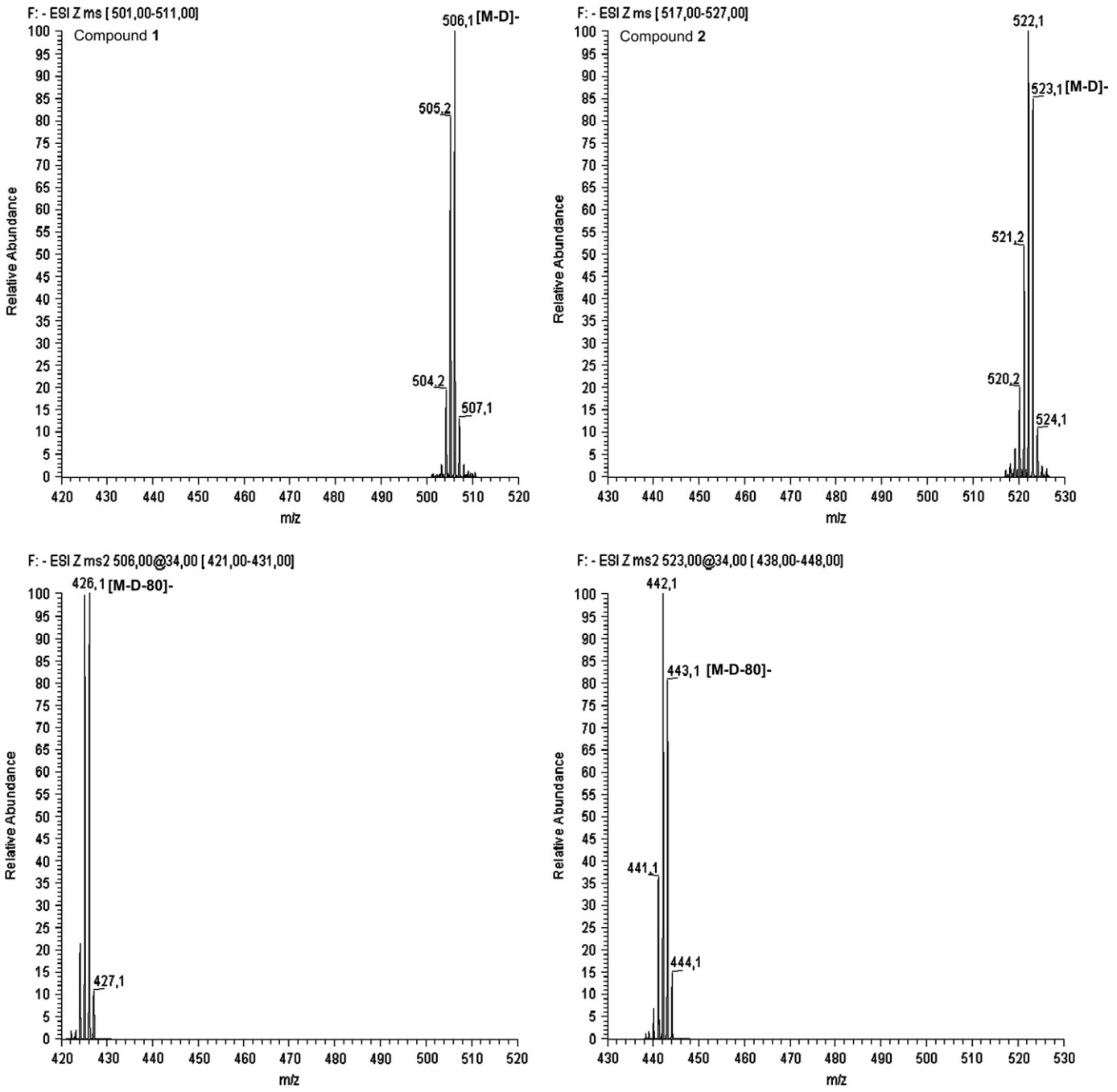

Figure 3. H/D exchange IT-ESI-MS spectra of compounds **1** and **2**. Above are reported the (−)-ESI-MS spectra and below the (−)-ESI-MS/MS spectra acquired in zoom scan mode.

(Thermo Finnigan, San Jose, CA) equipped with Xcalibur 3.1 software. Samples were dissolved in MeOH/H_2O 1:1 and infused in the ESI source by using a syringe pump at a flow rate of 3 μL/min. The ionization conditions were optimized, and the parameters were maintained as follows: capillary temperature 160 °C, capillary voltage −36 V, spray voltage 5.0 kV, tube lens voltage −65 V, sheath gas flow rate 30 (arbitrary units), scan range of *m*/*z* 150–1000 amu. N_2 was used as the sheath and auxiliary gas. Data were acquired in the full MS and MS/MS scan modes. Exact masses were measured by a Q-TOF premier (Waters, Maniford, MA, USA) high-resolution mass spectrometer. Column chromatographies were performed over Sephadex LH-20 (1 m×3 cm i.d; Pharmacia, Uppsala, Sweden). HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Kromasil C18 10 μm (300×10 mm) column.

3.2. Plant material

Unstressed *Gossypium hirsutum* was grown in the field at Cereal Comm Feed Company (Brescia, Italy). Whole cottonseed is a by-product of cotton production and acreage is expanding in the North Italy. Whole cottonseed (WCS) was furnished by Cereal Comm Feed Company.

3.3. Extraction and isolation procedure

The dried and powdered WCS (405 g) were defatted with hexane and $CHCl_3$ and then extracted with MeOH to give 19 g of residue. The MeOH extract was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH soluble portion (5.8 g), which was chromatographed twice on a Sephadex LH-20. The fraction with the

sesquiterpene mixture was purified by RP-HPLC (flow rate 2.5 ml/min) using MeOH/H_2O (40:60) as the eluent to yield pure compounds **1** (3.7 mg, t_R=8 min) and **2** (4.4 mg, t_R=12 min).

*3.3.1. 13-Hydroxy-7-O-(6′-O-sulfate-β-D-glucopyranosyl)-desoxyhemigossypol (**1**)*

Yellow sticky solid; $[\alpha]_D^{24}$ −57.6 (*c* 1.3, MeOH); UV (MeOH) λ_{max} (log ε) 256 nm (5.1); IR (film) ν_{max}: 3390, 2930, 1681, 1211 cm^{-1}; ^1H NMR and ^{13}C NMR (CD_3OD, 600 MHz), see Table 1; (−)-HRESI-MS, *m/z* 501.1100 $[M-H]^-$, calcd for $C_{21}H_{25}O_{12}S$ 501.1067 (3.3 mDa); ESI-MS (negative mode) *m/z* 501.1 $[M-H]^-$; MS/MS *m/z* 421.1, 259.1, 241.1.

*3.3.2. 13,15-Dihydroxy-7-O-(6′-O-sulfate-β-D-glucopyranosyl)-desoxyhemigossypol (**2**)*

Yellow sticky solid; $[\alpha]_D^{24}$ −49.1 (*c* 1.5, MeOH); UV (MeOH) λ_{max} (log ε) 258 nm (4.8); IR (film) ν_{max}: 3366, 2927, 1651, 1213 cm^{-1}; ^1H NMR and ^{13}C NMR (CD_3OD, 600 MHz), see Table 1; (−)-HRESI-MS, *m/z* 517.1057 $[M-H]^-$, calcd for $C_{21}H_{25}O_{13}S$ 517.1016 (4.1 mDa); ESI-MS (negative mode) *m/z* 517.1 $[M-H]^-$; MS/MS *m/z* 437.1, 275.1, 241.1.

3.4. H/D exchange MS of 1 and 2

Compounds **1** and **2** were dissolved in deuterium oxide (D_2O) and kept at 20 °C for 4 h and lyophilized. This process was repeated two times. The samples for MS experiments were dissolved in D_2O (5 μg/ml) and directly sprayed at 3 μl/min. An ion trap mass spectrometer equipped with an ESI source was used for H/D exchange experiments and the ionization conditions were the same reported in the general methods. Before analysis the mass spectrometer was conditioned with D_2O.

3.5. Cells proliferation assay

3.5.1. Reagents

RPMI-1640 medium, L-glutamine, streptomycin, penicillin, fetal bovine serum (FBS), and non-essential aminoacids were purchased from Cambrex Bioproducts Europe (Verviers, Belgium).

3.5.2. Cell culture

Jurkat T cells (ATCC, Manassas, VA) were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2/95% air in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% (w/v) non-essential aminoacids. Cells were kept in the exponential growth phase by passages at 2–3 days interval.

3.5.3. Cell proliferation assay

Cells were cultured in flat bottom 96-well plates (4×10^4/well) for 72 h in the presence of different concentrations of two compounds **1** and **2**. The compounds are both dissolved in methanol. Eighteen hours prior to harvesting, cells were pulsed with 1 μCi/well $[^3H]$-thymidine. Cultures were harvested on filters using a semiautomatic cell harvester (Filtermate, Packard, Danvers, MA). $[^3H]$-Thymidine incorporation was assessed by a microplate liquid scintillator (Top Count NXT™, Packard, Danvers, MA). Results were expressed as cpm (counts per minute).

3.5.4. Statistical analysis

Student's *t*-test was used to compare results from three different experiments. Results were expressed as mean±SD. Statistical significance between treated and non-treated cells with gossypol derivates was reached when *P* values were ≤0.05.

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