# Cytotoxic Steroidal Saponins from *Agave sisalana*

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- Agavaceae
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## Abstract

Two new steroidal saponins, **8** and **10,** along with 7 known steroidal sapogenins and saponins (**1**–**7**) and a furostanol saponin (**9**) were isolated from *Agave sisalana* Perrine ex Engelm. The structures of these two new compounds were identified and characterized by 1D and 2D NMR spectroscopy and mass spectrometry. In addition, acid hydrolysis and GC-FID were used to confirm the sugar moieties of **8** and **10**. The cytotoxic effects of **1**–**10** on MCF-7, NCI-H460, and SF-268 cancer cells were evaluated, and among them, compound **10** proved to be the most cytotoxic with $IC_{50}$ values of 1.2, 3.8, and 1.5 µM, respectively.

**Supporting information** available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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## Introduction

Steroidal saponins are secondary metabolites commonly found in several botanical species of the family Agavaceae [1,2]. The basic chemical structure of steroidal saponins is a glycosylated $C_{27}$ steroidal aglycone skeleton. On the basis of the ring structural features, $C_{27}$ steroidal aglycones are further categorized into spirostanes and furostanes. Furostanol saponins, characterized by a pentacyclic system, are the biosynthetic precursors of spirostanes, which have a 6–ring structure [3]. In most steroidal saponins, a hydroxyl group located at the C-3 or C-26 has a glycosidic linkage to a sugar moiety. Steroidal saponins have been reported to possess many useful properties, such as antifungal, antibacterial, and cytotoxic activities [1].

The family Agavaceae includes more than 300 species and is common in tropical and subtropical regions [4]. In southern Taiwan, two species of the genus, *Agave–Agave americana* and *Agave sisalana*, have been cultivated since 1918 for the fiber industry. Previously, it was shown that methanolic extracts of *A. sisalana* containing homoisoflavonoids and flavonoids have immunomodulatory effects [5]. Besides, steroidal saponins are reported to be rich in *A. sisalana* [6], and they exhibited potent cytotoxicities [1,7]. In our preliminary study, the methanolic extracts of *A. sisalana* were also evaluated for their cytotoxicity against MCF-7, NCI-H460, and SF-268 cancer cells. Our results were consistent with those reported previously (unpublished data). In this study, we purified the polar fraction of the methanolic extract to isolate two new steroidal saponins, **8** and **10**, along with seven known steroidal sapogenins and saponins, **1**–**7**, and a furostanol saponin, **9**. Herein, we report the isolation and identification of these compounds and assess their cytotoxic effects on three human cancer cell lines.

## Materials and Methods

### General experimental procedures

Optical rotations were measured in methanol (MeOH) on a JASCO P-1020 digital polarimeter. IR spectra were measured on a JASCO FT/IR 4100 spectrometer. $^1$H- and $^{13}$C-NMR spectra were acquired on a Bruker DRX-500 SB spectrometer. LC/MS/MS spectra were recorded on an Agilent 1100 HPLC system and an ABI API 4000Q Trap. Mass spectra were recorded on a JEOL JMS-700 mass spectrometer using the positive ion mode and high-resolution fast atom bombardment (FAB). Semipreparative HPLC was performed on a Hitachi L-7110 HPLC with a refractive index detector (Thermo Separation Products). A Phenomenex Luna silica column (5 µm, 10 × 250 mm, 3 mL · min$^{-1}$

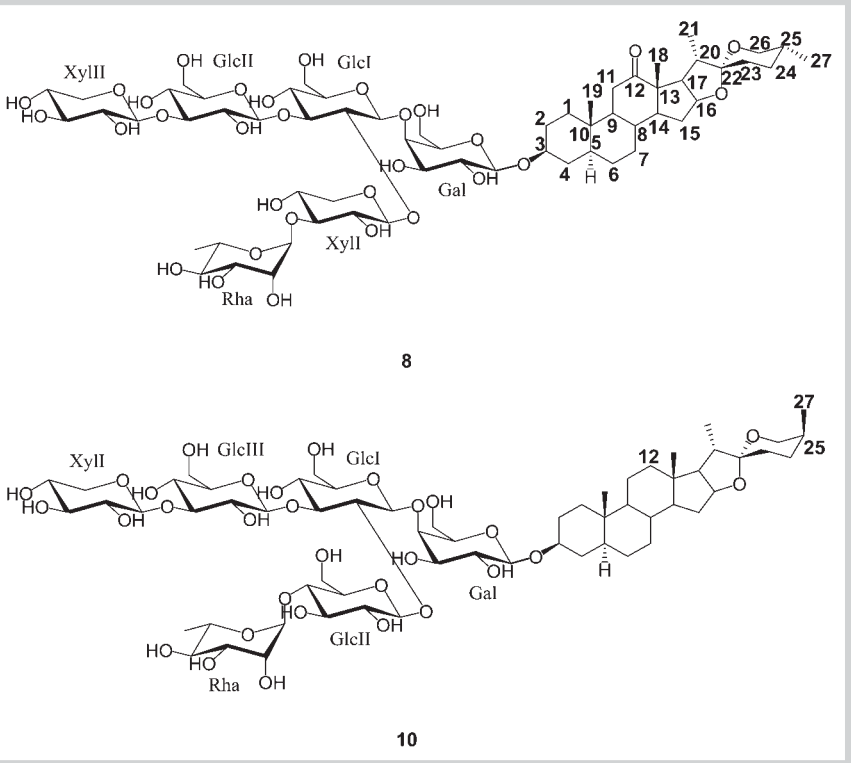


**Fig. 1** Chemical structures of compounds **8** and **10**.

flow rate) and a Merck Lichrospher 100 RP-8 reverse-phase column (10 µm, 10 × 250 mm, 3 mL·min$^{-1}$ flow rate) were used for normal-phase and reverse-phase chromatography, respectively. Silica gel (Merck, Geduran® Si 60 0.063–0.2 mm) and octadecylsilanized (ODS)-silica gel (BioSil, ODS-W 45–55 µm) were used for column chromatography. GC analysis was performed on an Agilent GC 7890A gas chromatograph equipped with a flame ionization detector. An Agilent DB-5 GC column was chosen for GC analysis.

## Plant material

Leaves of *Agave sisalana* Perrine ex Engelm (Agavaceae) were collected in Heng-Chun Town, Ping-Tung County, Taiwan, in July 2004. Dr. Chi-I Chang (Graduate Institute of Biotechnology, National Pintung Science and Technology University, Taiwan) identified the material. A voucher specimen (LCK9306) was deposited in the Graduate Institute of Pharmacy, Taipei Medical University, Taipei, Taiwan.

## Extraction and isolation

The air-dried leaves (61.1 kg) of *A. sisalana* were cut into small pieces and extracted with 100 L methanol at room temperature for seven days. After removal of the solvent under reduced pressure, the methanolic extract was dissolved in 4 L of water and then partitioned between ethyl acetate and water (3 × 4 L, 30 min each). Then, the extracted water layer was partitioned with *n*-butanol (3 × 4 L, 30 min each). Silica gel column chromatography of the ethyl acetate layer (69.6 g) was eluted stepwise with *n*-hexane-ethyl acetate (85 : 15, 80 : 20, 75 : 25, 65 : 35, 55 : 45, 40 : 60, and 20 : 80, v/v) and finally with pure ethyl acetate. Fractions of 500 mL each were collected and monitored by TLC using *n*-hexane-ethyl acetate (1 : 1, v/v) and observed at 254 nm. Fractions with similar components were pooled into 8 portions. Portion 2 (fractions 54–65) was separated by normal-phase HPLC on a semipreparative normal phase column (Luna silica, 5 µm, 10 × 250 mm; Phenomenex) at a flow rate of 3 mL·min$^{-1}$, with a mixture of *n*-hexane : ethyl acetate (7 : 1, v/v) followed by *n*-hexane : ethyl acetate : acetone (14 : 2 : 1, v/v/v) to obtain compound **1** (5.2 mg, $t_R$ = 11.62 min) and compound **2** (3.5 mg, $t_R$ = 11.65 min). Compound **3** (8.4 mg, $t_R$ = 12.15 min) was obtained from portion 7 (fractions 89–99) by HPLC with an isocratic mixture of *n*-hexane-acetone (16 : 7, v/v).

Silica gel column chromatography of the *n*-butanol layer (546.0 g) was eluted stepwise with chloroform-methanol (14 : 1, 7 : 1, 7 : 2, 7 : 3, 7 : 4, 7 : 5, 7 : 6, 7 : 7, and 7 : 14, v/v) and finally with pure methanol. Fractions of 500 mL each were collected and monitored by TLC using chloroform-methanol-water (14 : 6 : 1, v/v/v) and were observed at 254 nm. Similar fractions were pooled into 10 portions. Portion 1 (fractions 1–9) was separated by normal-phase HPLC with a mixture of *n*-hexane-chloroform-ethyl acetate-acetone (18 : 2 : 5 : 1, v/v/v/v) to obtain compound **4** (3.5 mg, $t_R$ = 30.85 min) and compound **5** (1.1 mg, $t_R$ = 32.21 min). Portion 4 (fractions 65–76) was separated on a Sephadex LH-20 column (3 × 50 cm) with chloroform-methanol (5 : 2, v/v) and collected in 200 mL of each subfraction. The precipitate in subfraction 6 was washed with methanol to obtain compound **6** (143.5 mg). Then, the supernatant from subfraction 6 was separated further by reverse-phase HPLC on a semipreparative reverse-phase column (Lichrospher® 100 RP-8 column, 10 µm, 10 × 250 mm; Merck) at a flow rate of 3 mL·min$^{-1}$, with a mixture of water-methanol (1 : 4, v/v) to obtain compound **7** (16.6 mg, $t_R$ = 19.65 min). Portion 8 (fractions 85–95) was separated by silica gel column chromatography with a mixture of chloroform-methanol-water (7 : 3 : 0.5, v/v/v) to obtain compound **8** (22.7 mg). Portion 9 (fractions 96–105) was subjected to (ODS) silica gel column chromatography and eluted with water-methanol-acetonitrile (2 : 1 : 1, v/v/v) to give compound **9** (793.2 mg). Finally, the wash subfraction of portion 9 was subjected again to silica gel column

chromatography and eluted with chloroform-methanol-water (10 : 3 : 0.5, v/v/v) to obtain compound **10** (22.2 mg).

*Compound **8***: colorless, amorphous powder; $[\alpha]_D^{23}$ −50.0 (*c* 0.10; MeOH); IR (film) $\nu_{max}$ 3410, 2925, 1700, 1645, 1374, 1042 $cm^{-1}$; $^1H$ NMR ($C_5D_5N$, 500 MHz) and $^{13}C$ NMR ($C_5D_5N$, 125 MHz) assignments (**Tables 1** and **2**); ESI-MS (neg. ion mode) *m/z* 1325.4 $[M - H]^-$, 1193.6, 1031.5, 885.5, 753.5, 591.4, HR FAB-MS (pos. ion mode) *m/z* 1349.5981 $[M + Na]^+$, (calcd. for $C_{61}H_{98}O_{31}Na$, 1349.5990).

*Compound **10***: colorless, amorphous powder; $[\alpha]_D^{23}$ −63.0 (*c* 0.10; MeOH); IR (film) $\nu_{max}$ 3449, 2928, 1631, 1062 $cm^{-1}$; $^1H$ NMR ($C_5D_5N$, 500 MHz) and $^{13}C$ NMR ($C_5D_5N$, 125 MHz) assignments (**Tables 1** and **2**); ESI-MS (neg. ion mode) *m/z* 1341.4 $[M - H]^-$, 1209.6, 1047.6, 901.5, 739.5, 577.4, HR FAB-MS (pos. ion mode) *m/z* 1365.6293 $[M + Na]^+$ (calcd. for $C_{62}H_{102}O_{31}Na$, 1365.6303).

## Acid hydrolysis and sugar analysis

Compounds **8** and **10** (2.0 mg each) were hydrolyzed with 4 N aqueous HCl (0.5 mL) at 60 °C for 1 h. The reaction mixture was partitioned between ethyl acetate and water. The water layer was neutralized by passing it through an ion exchange solid phase extraction (SPE) cartridge (Strata X-AW 33 µm polymeric Weak Anion; Phenomenex) [8]. After removal of the solvent under reduced pressure, the evaporation residue of the aqueous layer was dissolved in anhydrous pyridine (1 mL), and L-cysteine methyl ester hydrochloride (2.3 mg) was added. The mixture was stirred at room temperature for 24 h. Then 300 µL of HMDS-TMCS (hexamethyldisilazane-trimethylchlorosilane, 2 : 1) was added, and the mixture was stirred at room temperature for 30 min. The precipitate was centrifuged off, and 20 µL of supernatant was added into ethyl acetate (950 µL). After being well mixed and filtered, the solution was analyzed by GC-FID [9]. The program of oven temperature was 180 to 300 °C at 4 °C/min; injection temperature 250 °C; the carrier had a heat flow rate of 0.8 mL/min. The silylated standards of D-galactose, D-glucose, D-xylose, and L-rhamnose were detected at 19.75, 19.32, 15.27, and 16.56 min, respectively. Identification of D-galactose, D-glucose, D-xylose, and L-rhamnose was carried out for compounds **8** and **10**, giving peaks respectively at 19.76, 19.32, 15.27, and 16.56 min for compound **8**, and at 19.80, 19.36, 15.32, and 16.60 min for compound **10**.

## Growth inhibition assay

Human MCF-7 breast cancer and NCI-H460 non-small cell lung cancer cell lines were purchased from the American Type Culture Collection (ATCC). Human SF-268 glioblastoma cell line was purchased from the National Cancer Institute (NCI). These three cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acids (Life Technologies, Inc.) at 37 °C in a humidified incubator with 5% $CO_2$ and utilized by the Developmental Therapeutics Program of the NCI for anticancer compound prescreen. Cells in logarithmic growth phase were cultured at a density of 10 000 cells · $mL^{-1}$ in a 24-well plate. The cells were treated with various concentrations of compounds **1**–**10** for 72 h. Subsequently, the cells were fixed and stained with 50% ethanol containing 0.5% methylene blue for 30 min. The plates were washed 5 times with water and then air-dried. The resulting residue was dissolved in 1% *N*-lauroyl-sarcosine, and then the optical density was measured at 570 nm using a microplate reader. Cell number data were normalized to the percentage of vehicle-treated control and then graphed. $IC_{50}$ values were determined by graphing cell number data as percentage of control versus compound concentration, and the $IC_{50}$ was the point on the graph where 50% growth inhibition occurred. Each data point represented the mean of at least 3 independent experiments run in triplicate [10]. Actinomycin D (98%, Sigma) was used as the positive control. The purity of the test samples was greater than 95% as verified by HPLC and NMR.

**Table 1** $^1H$ and $^{13}C$-NMR chemical shift assignments for the aglycone moiety of compounds **8** and **10** in $C_5D_5N$.

| | 8 | | 10 | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ |
| 1 | 36.7 | 0.69, 1.30 | 37.2 | 0.77, 1.49 |
| 2 | 29.8 | 1.55, 1.97 | 30.0 | 1.61, 2.04 |
| 3 | 77.3 | 3.89 | 77.5 | 3.93 |
| 4 | 34.7 | 1.22, 1.76 | 34.9 | 1.33, 1.77 |
| 5 | 44.6 | 0.84 | 44.7 | 0.87 |
| 6 | 28.7 | 1.11 | 29.0 | 1.03, 1.10 |
| 7 | 31.5 | 1.56, 2.10 | 32.5 | 0.77, 1.50 |
| 8 | 34.4 | 1.76 | 35.3 | 1.38 |
| 9 | 55.7 | 0.88 | 54.5 | 0.48, t, *J* = 10.7 |
| 10 | 36.4 | | 35.9 | |
| 11 | 38.1 | 2.21, brd, *J* = 13.6<br>2.36, t, *J* = 13.6 | 21.3 | 1.19, 1.38 |
| 12 | 213.2 | | 40.2 | 1.01, 1.64 |
| 13 | 55.5 | | 40.8 | |
| 14 | 56.0 | 1.34 | 56.5 | 1.00 |
| 15 | 31.9 | 0.75, 1.56 | 32.2 | 1.38, 2.00 |
| 16 | 79.8 | 4.47 | 81.3 | 4.50 |
| 17 | 54.4 | 2.74, t, *J* = 7.4 | 62.9 | 1.77 |
| 18 | 16.2 | 1.07, s | 16.7 | 0.80, s |
| 19 | 11.8 | 0.65, s | 12.4 | 0.63, s |
| 20 | 42.7 | 1.91, t, *J* = 6.7 | 42.5 | 1.88 |
| 21 | 14.0 | 1.33, d, *J* = 6.7 | 15.0 | 1.13, d, *J* = 6.7 |
| 22 | 109.5 | | 109.8 | |
| 23 | 31.8 | 1.60, 1.70 | 26.4 | 1.44, 1.89 |
| 24 | 29.3 | 1.56 | 26.3 | 1.34, 2.12 |
| 25 | 30.7 | 1.56 | 27.6 | 1.57 |
| 26 | 67.1 | 3.47, 3.59 | 65.2 | 3.35, d, *J* = 10.8<br>4.06 |
| 27 | 17.5 | 0.68, d, *J* = 5.4 | 16.4 | 1.06, d, *J* = 7.1 |

## Supporting information

$^1H$, $^{13}C$ NMR, and selective TOCSY spectra of compounds **8** and **10** are available as Supporting Information.

## Results and Discussion

Chromatographic fractionation of a methanolic extract of *A. sisalana* leaves yielded 2 new compounds, **8** and **10** (**Fig. 1**), and 8 known compounds, **1**–**7** and **9**. The structures of the compounds were identified by using spectroscopic analysis, 2D NMR, and acid hydrolysis. Compounds **1**–**7** and **9** were identified by comparing their spectroscopic data with published results for tigogenin (**1**) [11], neotigogenin (**2**) [11], hecogenin (**3**) [12], neohecogenin (**4**) [13], rockogenin (**5**) [14], cantalasaponin-1 (**6**) [15], hecogenin 3-*O*-*β*-D-glucopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (**7**) [7], and polianthosides E (**9**) [16].

Compound **8** was obtained as a colorless amorphous powder. Its molecular formula was assigned as $C_{61}H_{98}O_{31}$ on the basis of the

**Table 2** $^1$H- and $^{13}$C-NMR chemical shift assignments for the sugar moieties of compounds **8** and **10** in $C_5D_5N$.

| | | 8 | | | | 10 | |
|---|---|---|---|---|---|---|---|
| **Position** | | **$\delta_C$** | **$\delta_H$** | **Position** | | **$\delta_C$** | **$\delta_H$** |
| Gal | 1 | 102.6 | 4.84, d, $J$ = 7.4 | Gal | 1 | 102.5 | 4.86, d, $J$ = 7.5 |
| | 2 | 73.2 | 4.37, m | | 2 | 73.2 | 4.41, m |
| | 3 | 75.6 | 4.10, m | | 3 | 75.6 | 4.11, m |
| | 4 | 80.0 | 4.47, m | | 4 | 80.0 | 4.56, m |
| | 5 | 75.1 | 3.99, m | | 5 | 75.4 | 3.96, m |
| | 6a | 60.9 | 4.63, m | | 6a | 60.8 | 4.65, m |
| | 6b | | 4.19, m | | 6b | | 4.21, m |
| GlcI | 1 | 104.8 | 5.11, d, $J$ = 8.0 | GlcI | 1 | 104.8 | 5.08, d, $J$ = 7.3 |
| | 2 | 80.8 | 4.29, m | | 2 | 80.9 | 4.29, m |
| | 3 | 86.9 | 4.05, m | | 3 | 88.0 | 4.12, m |
| | 4 | 70.4 | 3.70, m | | 4 | 70.6 | 3.72, m |
| | 5 | 77.4 | 3.89, m | | 5 | 78.2 | 3.81, m |
| | 6a | 62.6 | 4.38, m | | 6a | 63.0 | 4.41, m |
| | 6b | | 4.04, m | | 6b | | 3.96, m |
| GlcII | 1 | 104.0 | 5.55, d, $J$ = 7.9 | GlcII | 1 | 104.2 | 5.17, m |
| | 2 | 75.3 | 4.10, m | | 2 | 75.6 | 4.11, m |
| | 3 | 87.1 | 4.09, m | | 3 | 76.6 | 4.06, m |
| | 4 | 69.3 | 4.01, m | | 4 | 78.0 | 4.29, m |
| | 5 | 78.3 | 4.05, m | | 5 | 75.4 | 3.98, m |
| | 6a | 62.2 | 4.48, m | | 6a | 61.2 | 4.21, m |
| | 6b | | 4.29, m | | 6b | | 4.03, m |
| Xyl I | 1 | 104.7 | 5.20, d, $J$ = 7.5 | Rha | 1 | 102.7 | 5.74, brs |
| | 2 | 75.4 | 3.92, m | | 2 | 72.6 | 4.61, m |
| | 3 | 74.5 | 4.00, m | | 3 | 72.8 | 4.50, m |
| | 4 | 76.0 | 4.10, m | | 4 | 74.0 | 4.31, m |
| | 5a | 64.2 | 4.16, m | | 5 | 70.5 | 4.91, m |
| | 5b | | 3.44, m | | 6 | 18.6 | 1.67, d, $J$ = 5.9 |
| Rha | 1 | 99.7 | 5.41, brs | GlcIII | 1 | 104.0 | 5.56, d, $J$ = 6.5 |
| | 2 | 72.5 | 4.47, m | | 2 | 75.1 | 4.05, m |
| | 3 | 72.6 | 4.48, m | | 3 | 87.1 | 4.06, m |
| | 4 | 74.0 | 4.27, m | | 4 | 70.8 | 4.08, m |
| | 5 | 70.0 | 4.79, m | | 5 | 77.5 | 3.91, m |
| | 6 | 18.7 | 1.62, d, $J$ = 6.0 | | 6a | 62.1 | 4.46, m |
| Xyl II | 1 | 106.2 | 5.07, d, $J$ = 7.5 | | 6b | | 4.29, m |
| | 2 | 75.4 | 3.92, m | Xyl | 1 | 106.2 | 5.09, m |
| | 3 | 77.6 | 4.05, m | | 2 | 75.6 | 3.96, m |
| | 4 | 70.4 | 4.05, m | | 3 | 77.8 | 4.07, m |
| | 5a | 67.2 | 4.17, m | | 4 | 69.2 | 4.06, m |
| | 5b | | 3.52, m | | 5a | 67.3 | 4.19, m |
| | | | | | 5b | | 3.54, m |

$^{13}$C-NMR and HR-FAB-MS data (*m/z* 1349.5981 [M + Na]$^+$, calcd. 1349.5990). The $^1$H-NMR spectrum of **8** showed 2 methyl singlet signals at $\delta$ 0.65 and 1.07 as well as 2 methyl doublet signals at $\delta$ 0.68 ($J$ = 5.4 Hz) and 1.33 ($J$ = 6.7 Hz), which is characteristic of a steroidal skeleton. In the $^{13}$C-NMR spectrum of **8** (**Table 1**), a quaternary carbon at $\delta$ 109.5 indicated the distinctive hemiacetal C-22 of the spirostanol skeleton. The heteronuclear multiple bond coherence (HMBC) correlations of the proton signals at $H_2$-11 ($\delta_H$ 2.21 and 2.36) and $H_3$-18 ($\delta_H$ 1.07) with the carbonyl signal at $\delta_C$ 213.2 indicated that a carbonyl was located at C-12. The $^1$H-NMR spectrum also showed 6 anomeric proton signals at $\delta_H$ 4.84, 5.07, 5.11, 5.20, 5.41, and 5.55 indicating that there are 6 monosaccharide moieties in **8**. Acid hydrolysis of compound **8** with 4 N HCl yielded hecogenin (**3**), the aglycone of **8**, which was identified by comparing its $^1$H- and $^{13}$C-NMR spectra with published results [12]. The water layer of hydrolysis products of compound **8** was silylated and then analyzed by GC-FID. GC analyses showed that the sugar composition of **8** includes L-rhamnopyranose, D-xylopyranose, D-glucopyranose, and D-galactopyranose. In the HMBC spectrum, the anomeric proton signals of galactose at $\delta_H$ 4.84 correlated with the C-3 ($\delta_C$ 77.3) of **8**. The sequence of the oligosaccharide chain was deduced from HMBC correlations of $\delta_H$ 5.11 (Glc I H-1)/$\delta_C$ 80.0 (Gal C-4), $\delta_H$ 5.20 (Xyl I H-1)/$\delta_C$ 80.8 (Glc I C-2), $\delta_H$ 5.41 (Rha H-1)/$\delta_C$ 74.5 (Xyl I C-3), $\delta_H$ 5.55 (Glc II H-1)/$\delta_C$ 86.9 (Glc I C-3) and $\delta_H$ 5.07 (Xyl II H-1)/$\delta_C$ 87.1 (Glc II C-3). Furthermore, the $\delta_H$ of each proton of the monosaccharides was fully assigned by a selective TOCSY experiment (shown in Supporting Information). In the TOCSY spectrum, the nearby protons would be enhanced when exciting the H-1 of the monosugar. As a result, **8** was determined to be hecogenin 3-*O*-*α*-L-rhamnopyranosyl-(1 → 3)-*β*-D-xylopyranosyl-(1 → 2)-[*β*-D-xylopyranosyl-(1 → 3)-*β*-D-glucopyranosyl-(1 → 3)]-*β*-D-glucopyranosyl-(1 → 4)-*β*-D-galactopyranoside.

Compound **10** was obtained as a colorless amorphous powder. Its molecular formula was assigned as $C_{62}H_{102}O_{31}$ on the basis of $^{13}$C-NMR and HR-FAB-MS data (*m/z* 1365.6293 [M + Na]$^+$, calcd. 1365.6303). In the $^1$H-NMR spectrum of **10**, the 2 methyl singlets at $\delta_H$ 0.63 and 0.80 as well as the 2 methyl doublets at $\delta_H$ 1.06 ($J$ = 7.1 Hz), 1.13 ($J$ = 6.8 Hz) were in agreement with the characteristic signals of the aglycone moiety of neotigogenin (**2**) [11]. This result was also reflected in the $^{13}$C-NMR data of **10**, in which the carbonyl group at $\delta_C$ 213.2 of **8** was absent. The type of monosaccharide of **10** was the same as that of **8** as shown by $^1$H-NMR and GC-FID data. However, the oligosaccharide linkages of **10** were quite different from those of **8**. In the HMBC spectrum of **10**, the long range correlations of $\delta_H$ 4.86 (Gal-1)/$\delta_C$ 77.5 (C-3), $\delta_H$ 5.08 (Glc I-1)/$\delta_C$ 80.0 (Gal-4), $\delta_H$ 5.17 (Glc II-1)/$\delta_C$ 80.9 (Glc I-2), $\delta_H$ 5.74 (Rha-1)/$\delta_C$ 78.0 (Glc II-4), $\delta_H$ 5.56 (Glc III-1)/$\delta_C$ 88.0 (Glc I-3), and $\delta_H$ 5.09 (Xyl-1)/$\delta_C$ 87.1 (Glc III-3) were used to deduce the sequence of the oligosaccharide chain. In addition, the $\delta_H$ of each proton of the monosaccharide was determined by the selective TOCSY spectrum (shown in Supporting Information). Compared with the reference of Ding et al. [17], the sugar moiety of compound **10** was the same as dongnoside A. The chemical shifts of C-22 to C-27 of compound **10** were more up field than the signals of dongnoside A, and that was the characteristic difference between C-25*R* and C-25*S*. Besides, the proton data matched those of the 25*S* spirostane-type steroidal saponins (H-26a-H26b = 0.71 > 0.35) [11,18]. The $^{13}$C-NMR data of the aglycone of compound **10** was the same as the compound **2**, neotigogenin [11]. Consequently, **10** was determined to be neotigogenin 3-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 2)-[*β*-D-xylopyranosyl-(1 → 3)-*β*-D-glucopyranosyl-(1 → 3)]-*β*-D-glucopyranosyl-(1 → 4)-*β*-D-galactopyranoside.

The growth inhibition assays of purified compounds **1**–**10** using MCF-7, NCI-H460, and SF-268 cancer cells showed that 4 compounds (**7**–**10**) were significantly cytotoxic compared to the positive control actinomycin D (**Table 3**). In particular **10** was the most cytotoxic compound; its $IC_{50}$ values against MCF-7, NCI-H460, and SF-268 cells were 1.2, 3.8, and 1.5 µM, respectively. In addition, steroidal saponins with a sugar moiety (compounds **7**–**10**) were more cytotoxic than their aglycones (**1**–**5**) (**Table 3**).

**Table 3** $IC_{50}$ values (μM ± SD) of compounds **1–10** and actinomycin D in human NCI-H460, MCF-7, and SF268 cancer cells[a].

| Compound | NCI-H460 | MCF-7 | SF268 |
|---|---|---|---|
| **1** | > 20 | > 20 | > 20 |
| **2** | > 20 | > 20 | > 20 |
| **3** | > 20 | > 20 | > 20 |
| **4** | > 20 | > 20 | > 20 |
| **5** | > 20 | > 20 | > 20 |
| **6** | > 20 | > 20 | > 20 |
| **7** | 5.3 ± 1.8 | 11.9 ± 2.6 | 4.0 ± 2.2 |
| **8** | 6.5 ± 1.1 | 9.5 ± 4.8 | 8.2 ± 1.2 |
| **9** | > 20 | > 20 | 7.5 ± 1.4 |
| **10** | 3.8 ± 2.7 | 1.2 ± 0.1 | 1.5 ± 0.8 |
| Actinomycin D[b] | 2.6 ± 1.6 | 31.1 ± 2.9 | 7.5 ± 5.2 |

[a] Cells were treated with various concentrations of test drugs for 72 hours. Cell growth was determined with the methylene blue dye assay. The $IC_{50}$ value resulting from 50% inhibition of cell growth was calculated. Each value represents the means ± SD of three independent experiments. [b] Actinomycin D was used as the positive control

These results confirm that the sugar moiety plays an important role in the bioactivity of steroidal saponins.

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