Supporting Information

Cytotoxic Asterosaponins Capable of Promoting Polymerization of Tubulin from the Starfish *Culcita novaeguineae*

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Preparation of the 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives. The following solutions were added to the monosaccharides mixture obtained from acid hydrolysis: (*S*)-1-amino-2-propanol/ MeOH (1:8) 20 μL, AcOH/MeOH (1:4) 17 μL, and 3% $Na[BH_3CN]$ in MeOH 13 μL. The mixture was reacted at 65 °C for 1.5 h, cooled, adjusted with 3 mol/L CF_3COOH to pH 1–2, and evaporated under reduced pressure. The residue was acetylated with Ac_2O (0.5 mL) and pyridine (0.5 mL) at 100 °C for 1 h. The derivatives were partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 extract was washed with saturated $NaHCO_3$ solution (0.5 mL) and H_2O (2×0.5 mL) and concentrated. The resulting 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives of the monosaccharides were analyzed by GC, with standard sugar derivatives prepared under the same conditions as reference samples.

Methanolysis of 3 and *p*-bromobenzoylation. A solution of asterosaponin **3** (10 mg) in anhydrous 2 mol/L HCl/MeOH (0.5 mL) was heated at at 100 °C for 4 h. The solution was neutralized with Ag_2CO_3 and centrifuged. The supernatant was evaporated to dryness and the residue was treated with *p*-bromobenzoyl chloride (15 mg) and 4-dimethylaminopyridine (1 mg) in dry pyridine (0.5 mL). The mixture was stirred overnight at 60 °C under nitrogen, and partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ extract was washed with saturated $NaHCO_3$ solution and H_2O (2×) and concentrated. The dark brown polar material was removed by dissolving the residue in $CHCl_3$ and passage of the solution through a column filled with a slurry of silica gel in hexane/Et_2O (7:3). The fastest moving portions was eluted with hexane/Et_2O (7:3), and the eluate was evaporated to yield a residue containing the resulting *p*-bromobenzoate mixture. The mixture was separated by HPLC to yield methyl 2,4-di-*O*-(*p*-bromobenzoyl)-3-*O*-methyl-α-D-quinovopyranoside.

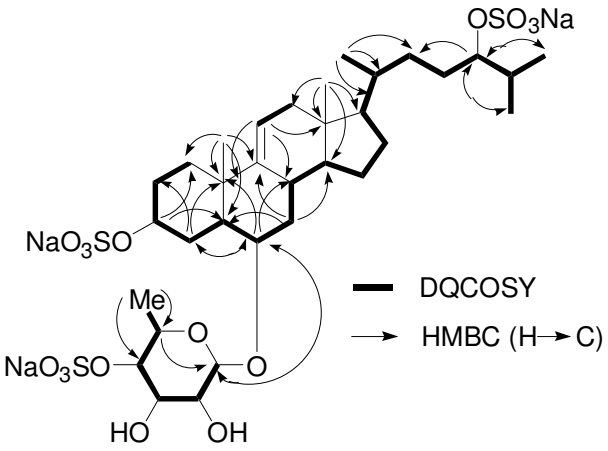

Figure S1. Key DQCOSY and HMBC Correlations of Novaeguinoside A (**1**).

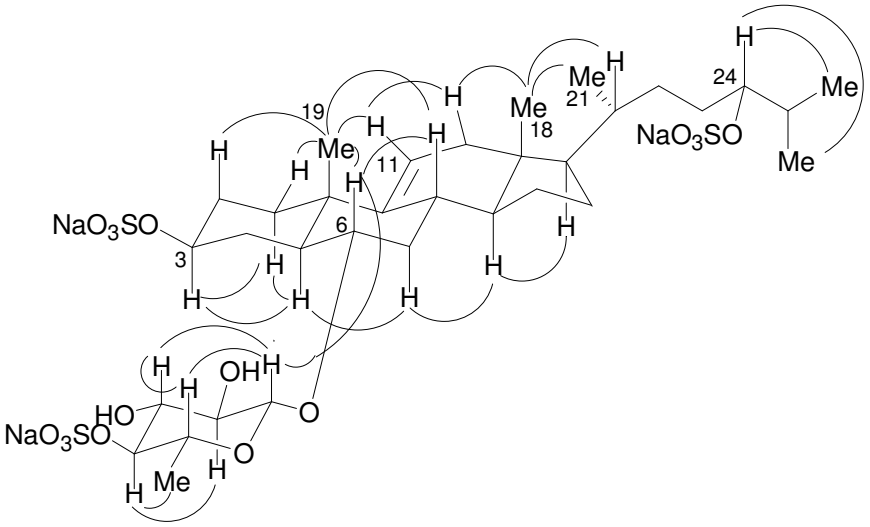

Figure S2. Key NOESY Correlations of Novaeguinoside A (**1**).

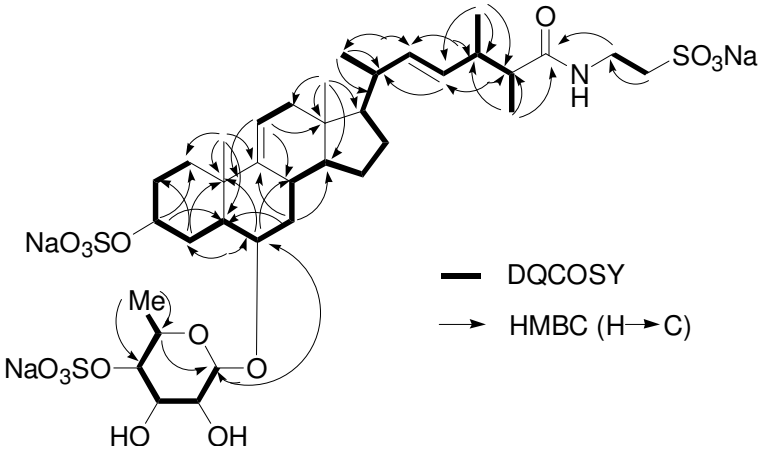

Figure S3. Key DQCOSY and HMBC Correlations of Novaeguinoside B (**2**).

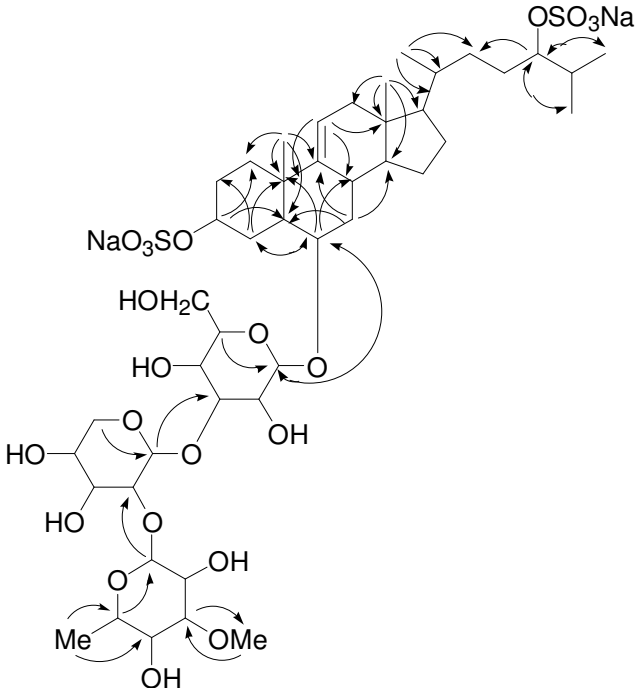

Figure S4. Key HMBC Correlations of Novaeguinoside C (**3**).

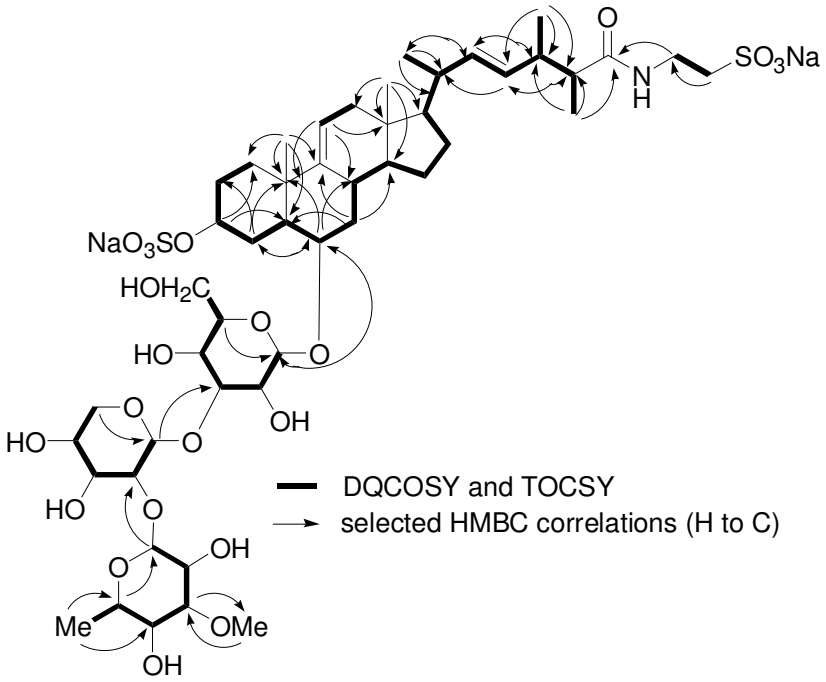

Figure S5. Gross Structure of Novaeguinoside D (**4**) with 2D NMR Correlations.

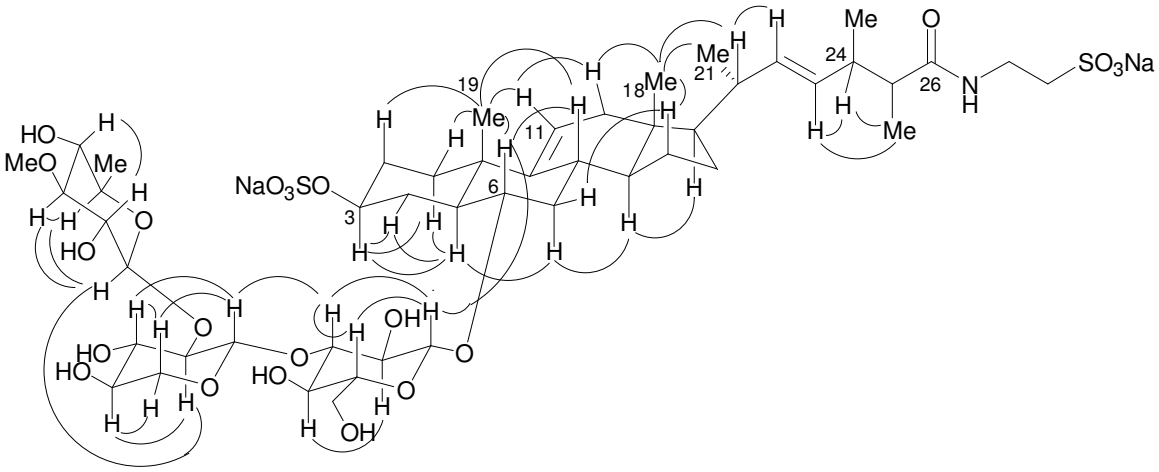

Figure S6. Key NOESY Correlations of Novaeguinoside D (**4**).

Description of Tubulin-polymerization Assay

Asterosaponins **1**–**4** were screened by a high-throughput model for screening antitumor agents capable of promoting polymerization of tubulin *in vitro*. Tubulin was prepared from pig brain tissue. Briefly, Fresh pig brains were homogenized with the solution containing 1 mol/L monosodium glutamate and 0.1 mmol/L GTP. The homogenate was centrifuged at 100000×*g* for 1 h, and the supernatant was subjected to chromatography on a DEAE-Sephadex column eluting with a solution containing 0.8 mol/L glutamate and 0.1 mmol/L GTP, and a solution containing 1 mol/L NaCl, 1 mol/L glutamate and 0.1 mmol/L GTP to yield DEAE-tubulin. The DEAE-tubulin was mixed with 1 mmol/L GTP, warmed to 37 °C, incubated for 45 min, and centrifuged at 100000×*g* for 1 h (37 °C) to obtain warm polymerized microtubule sediment. The sediment was suspended in a solution containing 1 mol/L glutamate and 0.1 mmol/L GTP and iced at 0 °C for 30 min, centrifuged at 100000×*g* for 40 min (4 °C) to obtain cold supernatant (purified tubulin). The tubulin was used in end-point assay and stored in liquid nitrogen. The GDP/GTP-free tubulin was prepared by Sephadex G-50 chromatography eluting with 1 mol/L glutamate and then used in kinetic assay. Tubulin polymerization was monitored by turbidimetry with measurement of the change in absorbance at 350 nm. The anticancer agent paclitaxel was used as positive control.

First high-throughput sceening assay (end-point assay). An assay mixture containing 1 g/L tubulin, 0.1 mmol/L GTP, 0.1 mmol/L $CaCl_2$, and 10 μg/mL test or control sample in a total volume of 0.1 mL of 4% Me_2SO was added to each well of a 96-well plate at 2 °C. The optical density of each well was immediately measured at 2 °C with a microplate reader at 350 nm. Then the plate was moved to an incubator at 37 °C for 20 min, and transferred to the microplate reader. The optical density of each well was measured at 37 °C and 350 nm again. Data were calculated as percent end-point promote coefficient (P_e) by the formula: $P_e = [(OD_t^{37} - OD_t^2)/ (OD_s^{37} - OD_s^2) - 1] \times 100\%$, OD_t^{37}, OD_t^2, OD_s^{37} and OD_s^2 being the mean optical densities of the test compounds at 37 °C and 2 °C, and of the solvent control at 37 °C and 2 °C, respectively. The data represented the means of three independent experiments in

which each compound was tested in eight replicate wells and were expressed as means ± SD. The positive control paclitaxel displayed a P_e value of (32 ± 3) % at 10 μg/mL, and a P_e value of >20% at 10 μg/mL was considered active.

Second confirmation screening assay (kinetic assay). In the kinetic assay, the assay mixture containing 1 g/L tubulin without GTP, 0.1 mmol/L $CaCl_2$, and 10 μg/mL test or control sample in a total volume of 0.1 mL of 4% Me_2SO was added to each well of a 96-well plate at 2 °C. The plate was transferred to the microplate reader, and the optical density of each well was immediately measured at 37 °C and 350 nm. The change of optical density at 350 nm was monitored for 25 min, and the slope of the change from 6 to 16 min was recorded. Data were calculated as percent kinetic promote coefficient (P_k) by the formula: $P_k = [(k_t - k_s)/ (k_p - k_s)] \times 100\%$, k_t, k_s and k_p being the mean slopes of the test compounds, the solvent control and the positive control, respectively. The data represented the means of three independent experiments in which each compound was tested in eight replicate wells and were expressed as means ± SD. The positive control paclitaxel was considered as 100% activity and displayed a P_k value of 100% at 10 μg/mL.

Description of MTT Assay

The cytotoxicity of asterosaponins **1**–**4** against human leukemia K-562 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay, with the anticancer agent 10-hydroxycamptothecine (HCP) as positive control. Briefly, target tumor cells were grown to log phase in DMEM medium containing 10% fetal bovine serum and transferred to serum free medium in 96-well plates at a density of 4×10^3 cells/well. Cultures were preincubated for 24 h in a humidified 5% CO_2–95% O_2 atmosphere at 37 °C. Then, control or test solution was put into each well, and the plates were incubated for an additional 72 h. At the end of exposure, MTT solved in PBS was added to each well at a final concentration of 5 mg/mL, and then incubated for 4 h. The water-insoluble dark blue formazan crystals formed during MTT cleavage in actively metabolizing cells were dissolved in DMSO. The optical density of each well was measured with a Bio-Rad 680 microplate reader at 490 nm. The activities of asterosaponins **1**–**4** and HCP were determined at 100, 10, 1, 0.1, and 0.01 μmol/L (each concentration was tested in triplex wells), respectively. Data were calculated as percentage of inhibition by the formula: % Inhibition = $[100 - (OD_t/OD_s) \times 100]\%$, OD_t and OD_s being the mean optical densities of the test compounds and the solvent control, respectively. The concentration inducing a 50% inhibition of cell growth (IC_{50}) was determined graphically for each experiment using the curve-fitting routine of the Prism software (GraphPad Software, Inc.; San Diego, CA, USA). The IC_{50} value represented the mean of three independent experiments and was expressed as mean ± SD.

Description of SRB Assay

The cytotoxicity of asterosaponins **1**–**4** against human hepatoma BEL-7402 cells was evaluated by sulforhodamine B (SRB) protein assay, with the anticancer agent 10-hydroxycamptothecine (HCP) as positive control. Briefly, target tumor cells were grown to log phase in RPMI 1640 medium containing 10% fetal bovine serum. After diluting to 4×10^4 cells/mL with the complete medium, 90 μL of the obtained cell suspension was added to each well of 96-well culture plates. Cultures were preincubated for 24 h in a humidified 5% CO_2 atmosphere at 37 °C. Then, 10 μL control or test solution was put into each well, and the plate was incubated for an additional 72 h. At the end of exposure, the cells were fixed by addition of 50 μL of cold 50% trichloroacetic acid at 4 °C for 1 h, and the plates were washed five times with tap water, and air-dried. Then, SRB solution (50 μL, 0.4% in 1% AcOH) was added, and staning was performed for 30 min. The residual dye was washed out four times with 1% AcOH, and the plates were air-dried. To each well, 10 mM unbuffered Tris solution (150 μL) was added. The optical density of each well was measured with a microplate reader at 520 nm. The activities of asterosaponins **1**–**4** and HCP were determined at 100, 10, 1, 0.1, and 0.01 μmol/L (each concentration was tested in triplex wells), respectively. Data were calculated as percentage of inhibition by the formula: % Inhibition = $(100-(OD_t/OD_s)\times100)\%$, OD_t and OD_s being the mean optical densities of the test compounds and the solvent control, respectively. The concentration inducing a 50% inhibition of cell growth (IC_{50}) was determined graphically for each experiment using the curve-fitting routine of the Prism software (GraphPad Software, Inc.; San Diego, CA, USA). The IC_{50} value represented the mean of three independent experiments and was expressed as mean ± SD.