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

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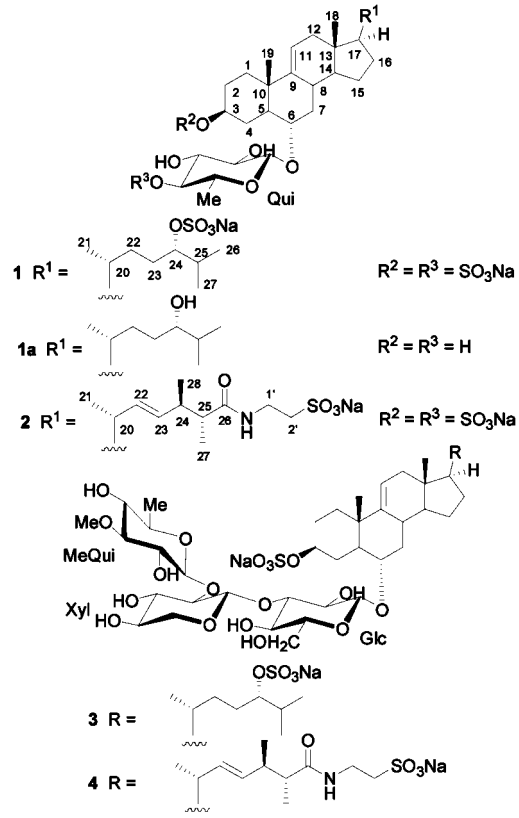
Four new asterosaponins, novaeguinosides A (**1**), B (**2**), C (**3**), and D (**4**), were isolated from the bioactive fraction of the starfish *Culcita novaeguineae*, as active compounds capable of promoting polymerization of tubulin. Their structures were elucidated by extensive spectroscopic studies and chemical evidence. Compounds **1** and **3** are characterized by sulfated side chains not previously found in asterosaponins, and **1** is the first example of a trisulfated asterosaponin. In the side chains of **2** and **4**, the 26-carboxylic acid function is found as an amide derivative of taurine, which is a rare feature and first encountered among asterosaponins. All the asterosaponins showed cytotoxicity against two human tumor cell lines.

Microtubule is an important component of the eukaryotic cytoskeleton that is essential for separation of the duplicated chromosome pairs during mitosis. The antimitotic drug paclitaxel (Taxol) has undergone extensive clinical development as a result of its efficiency in the treatment of many cancers. Paclitaxel promotes hyperstabilization of microtubule, which is resistant to depolymerization conditions, such as calcium and cold *in vitro*, arrests cells in mitosis, eventually leading to cell death, and is called a microtubule-stabilizing agent. The success of paclitaxel in therapies of human tumors and the disadvantages lead to renewed interests in searching for and developing novel compounds that mimic the activity of paclitaxel, and thus tubulin has become a molecular target amenable to small-molecule drug discovery.[1] Recently, a high-throughput model for screening antitumor agents capable of promoting polymerization of tubulin *in vitro* has been established. Tubulin was prepared from pig brain tissue for two screening steps, and the polymerization was monitored by turbidimetry with measurement of the change in absorbance at 350 nm. The first step was performed using the end-reading method and the second step by a kinetic assay. The promoting activity was expressed as P_e (percent end-point promote coefficient) and P_k (percent kinetic promote coefficient), respectively.[2] In the course of our search for new anticancer compounds from echinoderms, we found that a fraction prepared from the starfish *Culcita novaeguineae* Müller et Troschel (Oreasteridae) showed significant promoting activity of tubulin polymerization ($P_e = 28\%$ at 10 μg/mL, paclitaxel as positive control with $P_e = 31\%$ at 11.7 μM). *C. novaeguineae* is an abundant starfish distributed in the South China Sea. Asterosaponins characterized as sulfated steroidal penta- or hexaglycosides based on a $\Delta^{9(11)}$-3β,6α-dioxysteroidal aglycone with a sulfate group attached at C-3 and the oligosaccharide moiety at C-6[3] and polyhydroxysteroid glycosides are the predominant metabolites of the starfish.[4] We have reported the isolation of 12 asterosaponins from this species.[5] However, none of them displayed tubulin polymerization promoting activity in the screening assay. A reinvestigation of the bioactive fraction led to the isolation of four new asterosaponins, **1**−**4**, as active compounds capable of promoting polymerization of tubulin *in vitro*. We report herein the isolation, structural elucidation, and biological activity of novaeguinosides A (**1**), B (**2**), C (**3**), and D (**4**).



An ethanolic extract of *C. novaeguineae* was suspended in H_2O and partitioned successively with petroleum ether and *n*-BuOH. The *n*-BuOH extract was subjected to several chromatographic purification steps to afford **1**−**4**.

The molecular formula of novaeguinoside A (**1**) was established as $C_{33}H_{53}O_{16}S_3Na_3$ from the $[M + Na]^+$ ion at *m*/*z* 893.2090 in the positive ion mode HRESIMS and $[M - Na]^-$ ion at *m*/*z* 847 in the negative ion mode ESIMS. Fragment ions at *m*/*z* 773 $[M - NaHSO_4]^+$, 653 $[M + Na - 2 \times NaHSO_4]^+$, and 533 $[M + Na - 3 \times NaHSO_4]^+$ indicated the presence of three sulfate groups, and the fagment at *m*/*z* 645 $[M + Na - 248]^+$ showed the loss of a sulfated deoxyhexose unit. The ^{1}H NMR, ^{13}C NMR, and DEPT

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Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of Novaeguinosides A (**1**) and B (**2**) and the Desulfated Saponin **1a** in CD_3OD[a]

	δ_H (J in Hz)			δ_C		
position	**1**	**1a**	**2**	**1**	**1a**	**2**
1	1.75 m, 1.48 m	1.70 m, 1.44 m	1.76 m, 1.46 m	36.9	37.0	36.9
2	2.20 m, 1.65 m	2.02 m, 1.56 m	2.24 m, 1.68 m	29.7	31.4	29.6
3	4.22 m	3.48 m	4.22 m	79.7	71.9	79.7
4	2.62 d (10.2), 1.31 m	2.58 d (10.2), 1.30 m	2.62 d (10.2), 1.34 m	30.8	32.9	31.0
5	1.28 m	1.25 m	1.29 m	50.1	50.3	50.2
6	3.60 td (10.2, 4.2)	3.59 td (10.2, 4.2)	3.58 td (10.8, 4.8)	80.8	81.1	81.0
7	2.40 m, 0.97 m	2.40 m, 0.99 m	2.42 m, 0.98 m	42.4	42.3	42.4
8	2.10 m	2.12 m	2.12 m	36.8	36.8	36.6
9				146.8	146.9	146.6
10				39.3	39.2	39.5
11	5.36 br d (5.4)	5.34 br d (5.4)	5.37 br d (5.4)	117.8	117.9	117.7
12	2.22 m, 2.08 m	2.18 m, 2.06 m	2.26 m, 2.10 m	42.9	42.9	43.1
13				42.3	42.3	42.6
14	1.32 m	1.32 m	1.33 m	55.1	55.2	55.0
15	1.78 m, 1.36 m	1.77 m, 1.35 m	1.80 m, 1.38 m	26.3	26.3	26.5
16	1.92 m	1.90 m	1.95 m	29.5	29.5	30.1
17	1.30 m	1.31 m	1.32 m	57.7	57.8	58.0
18	0.70 s	0.68 s	0.73 s	11.9	11.9	12.1
19	1.02 s	1.01 s	1.03 s	19.7	19.8	19.7
20	1.40 m	1.41 m	2.21 m	36.2	36.3	41.0
21	0.98 d (6.6)	0.97 d (6.6)	0.99 d (6.6)	19.0	19.0	21.3
22	1.68 m	1.65 m	5.31 dd (15.2, 7.8)	32.3	33.2	137.8
23	1.70 m, 1.34 m	1.54 m, 1.22 m	5.16 dd (15.2, 7.8)	28.5	31.6	132.6
24	4.10 q (6.6)	3.22 m	2.40 m	86.0	78.0	41.4
25	1.72 m	1.63 m	2.29 m	32.0	34.6	48.5
26	0.95 d (6.6)	0.94 d (6.6)		18.5	19.5	178.4
27	0.94 d (6.6)	0.92 d 96.6)	1.09 d (6.6)	18.0	17.5	18.3
28			1.05 d (6.6)			15.3
taurine 1′			3.64 t (6.6)			36.7
2′			3.00 t (6.6)			51.9
Qui 1	4.42 d (7.8)	4.60 d (7.8)	4.44 d (7.8)	105.5	106.1	105.6
2	3.30 dd (9.0, 7.8)	3.31 dd (9.0, 7.8)	3.31 dd (9.0, 7.2)	75.8	76.4	75.9
3	3.64 t (9.0)	3.36 t (9.0)	3.67 t (9.0)	76.7	77.2	76.7
4	3.92 t (9.0)	3.16 t (9.0)	3.95 t (9.0)	83.1	76.0	83.2
5	3.50 m	3.40 m	3.51 m	71.3	73.7	71.4
6	1.35 d (6.0)	1.39 d (6.0)	1.36 d (6.0)	18.2	18.3	18.2

[a] Assignments aided by DQCOSY, TOCSY, NOESY, DEPT, HMQC, and HMBC experiments.

spectra displayed resonances due to two tertiary methyl groups (δ_H 0.70, 1.02; δ_C 11.9, 19.7), one olefinic bond (δ_H 5.36; δ_C 146.8, 117.8), one oxomethine (δ_H 3.60; δ_C 80.8), and one sulfated oxomethine (δ_H 4.22; δ_C 79.7) and suggested that the aglycone of **1** has a $\Delta^{9(11)}$-3β,6α-dioxysteroidal nucleus with a sulfate group attached at C-3, characteristic for asterosaponins.[3,6] Glycosidation at C-6 was supported by the downfield shift of the C-6 signal in the ^{13}C NMR spectrum with respect to the corresponding value in asterosaponin aglycones with 3β-sulfated and 6α-hydroxy groups, such as 3-*O*-sulfomarthasterone from *Asterias rathbuni*.[7] The assignment of the NMR signals associated with the aglycone moiety (Table 1) was derived from DQCOSY, TOCSY, HMQC, and HMBC experiments, as shown in Figure S1, Supporting Information. These data were similar to those observed for 3-*O*-sulfomarthasterone, except for the signals due to the side chain.[7] Comparison of the NMR signals associated with the side chain of **1** with those reported for 5α-cholestan-3β,6α,8,15β,24-pentaol 24-sulfate from *Astropevten scoparius* and 3-*O*-β-xylopyranosyl-5α-cholest-4-ene-3β,6β,8,15α,24-pentaol 24-sulfate from *Pisaster giganteus*[8] clearly indicated a 24-sulfated cholestane-type side chain for **1**. On solvolysis in dioxane/pyridine, **1** afforded a desulfated derivative, **1a**. The location of the sulfate group at C-24 of **1** was confirmed by the upfield shifts of H-24 to δ_H 3.22 (δ_H 4.10 in **1**) and C-24 to δ_C 78.0 (δ_C 86.0 in **1**) in the NMR spectra of **1a**. Acidic hydrolysis of **1** afforded the desulfated aglycone **1b**, which was esterified with (+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride in dry pyridine. The ^{1}H NMR spectrum of the resulting (+)-(*R*)-MTPA ester displayed signals for the isopropyl methyl protons at δ 0.83 and 0.86, which matched with the signals (δ 0.84 and 0.86) found in the spectrum of the (+)-(*R*)-MTPA ester of the (24*S*)-24-OH model steroid, whereas the corresponding signal was observed as one 6H doublet at δ 0.92 for the (+)-(*R*)-MTPA ester of the 24*R* isomer.[8a] The 20*R* configuration was assigned on the basis of the signal of CH_3-21 at δ 0.98 in the ^{1}H NMR of **1**, which appeared at approximately δ_H 0.90−0.97 for the 20*R* isomers and δ_H 0.8 for the 20*S* isomers in CD_3OD.[9] Thus, the aglycone of **1** was determined to be sodium (20*R*,24*S*)-5α-cholest-9(11)-en-3β,6α,24-triol 3,24-disulfate, whose relative configuration was confirmed from the NOESY spectrum as shown in Figure S2, Supporting Information. The ^{1}H NMR spectrum showed a signal for an anomeric proton at δ 4.42 as a doublet with $^3J_{H\text{-}1/H\text{-}2}$ = 7.8 Hz, indicative of β-configuration, which was correlated in the HMQC experiment with the corresponding carbon at δ 105.5. On acid hydrolysis **1** gave D-quinovose (Qui) determined by preparation of the corresponding 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivative, which was analyzed by GC.[10] DQCOSY and HMQC experiments allowed the complete assignment of the protons and carbons of the monosaccharide residue. The presence of a sulfate group at C-4 of quinovose was indicated by the downfield esterification shift observed for the signal of Qui C-4 (from δ_C 76.0 in the desulfated derivative **1a** to δ_C 83.1 in **1**). The attachment of the quinovopyranose-4-sulfate moiety to C-6 of the aglycone was confirmed by the correlation between Qui H-1 and C-6 in the HMBC spectrum, as well as the cross-peak Qui H-1/H-6 of the alycone in the NOESY spectrum. Hence, the structure of novaeguinoside A (**1**) was elucidated as sodium (20*R*,24*S*)-6α-*O*-(4-*O*-sodium sulfato-β-D-quinovopyranosyl)-5α-cholest-9(11)-en-3β,24-diyl disulfate. Compound **1** is the first example of a trisulfated asterosaponin and the first asterosaponin with a sulfated side chain.

Morever, only a few asterosaponins with a monosaccharide chain have been identified previously.[3]

The molecular formula of novaeguinoside B (**2**) was determined as $C_{36}H_{56}NO_{16}S_3Na_3$ by ^{13}C NMR and HRESIMS. The positive ion mode ESIMS showed fragment ion peaks at *m/z* 826 [M + Na − $NaHSO_4$]$^+$ and 706 [M + Na − 2 × $NaHSO_4$]$^+$, indicating the presence of two sulfate groups. The fragment at *m/z* 698 [M + Na − 248]$^+$ showed the loss of a sulfated deoxyhexose unit. On acid hydrolysis, **2** gave D-quinovose. The NMR data of **2** bore a close resemblance to those of **1** and indicated that both have the same monosaccharide residues and steroidal nuclei but with a different side chain. The ^1H NMR spectrum of **2** showed three methyl doublets at δ 0.99 (CH_3-21, coupled with a multiplet at δ 2.21, H-20), 1.09 (CH_3-27, coupled with a multiplet at δ 2.29, H-25), and 1.05 (CH_3-28, coupled with a multiplet at δ 2.40, H-24) and two double doublets at δ 5.31 and 5.16 both with J = 15.2, 7.8 Hz, characteristic for a 22*E* double bond. The partial structure of the side chain depicted with the bold bonds in Figure S3 (Supporting Information) was determined by the DQCOSY cross-peaks H_3-21/H-20, H-20/H-22, H-22/H-23, H-23/H-24, H-24/H_3-28, H-24/H-25, and H-25/H_3-27 and confirmed by the analysis of the HMBC data. Two extra methylene triplets at δ 3.64 and 3.00 coupled to each other by 6.6 Hz in the ^1H NMR spectrum of **2**, together with the intense amide bands at 1652 and 1555 cm^{-1} and the sulfonic acid salt bands at 1218 and 1046 cm^{-1} in the IR spectrum, suggested the presence of a taurine residue linked to the steroidal moiety through an amide functionality. An HMBC experiment established the connection between the taurine residue and the carbonyl carbon at δ 178.4 (C-26). Acid hydrolysis of **2** gave taurine, identified by comparison of its spectroscopic data with a reference sample. All the evidence suggest structure **2** with a Δ^{22E},26-amide ergostane side chain. For studies of the configurations at C-24 and C-25 of 24-methyl-26-oate steroids, all the possible model stereoisomers, i.e., ethyl (22*E*, 24*R*, 25*S*; 22*E*, 24*R*, 25*R*; 22*E*, 24*S*, 25*R*; and 22*E*, 24*S*, 25*S*)-6β-methoxy-3α,5-cyclo-5α-ergost-22-en-26-oate, have been synthesized. In the ^1H NMR spectra of the two erythro isomers the olefinic protons appeared as two double doublets at δ 5.33−5.31 and 5.16, while in that of the two threo isomers they resonated as one multiplet at δ 5.27. Significant differences dealing with the ^1H NMR signals due to the three side chain methyl protons were observed between the two erythro isomers: three well-separated doublets at δ 0.99, 1.05, and 1.09 for the 24*R*, 25*R* isomer, whereas one 3H doublet at δ 1.00 and a 6H apparent triplet at δ 1.07 for the 24*S*, 25*S* isomer.[11] The olefinic proton and methyl doublets pattern of **2** were in good agreement with those of the 24*R*, 25*R* erythro isomer. The side chain of **2** has been identified in two polyhydroxylated steroids from *Myxoderma platyacanthum*[11] and triseramide from *Astropecten triseriatus* and *Trofodiscus über*[12] with 24*R*, 25*S* configurations, but reported with 24*R*, 25*R* configurations as a natural product for the first time. Therefore, novaeguinoside B (**2**) was elucidated as (20*R*,22*E*,24*R*,25*R*)-6α-*O*-(4-*O*-sodiumsulfato-β-D-quinovopyranosyl)-24-methyl-3β-*O*-sodium sulfato-5α-cholest-9(11),22-dien-26-oic acid (2-sodium sulfethyl)amide. It is the first example of an asterosaponin with a methyl group oxidized to a carboxy function.

Novaeguinoside C (**3**) was obtained as colorless crystals, slightly soluble in MeOH. An examination of its ^1H and ^{13}C NMR spectra in C_5D_5N revealed signals due to aglycone protons and carbons consistent with those observed in **1**, containing the same typical $\Delta^{9(11)}$-3β,6α-dihydroxysteroidal nucleus with a 24-sulfated side chain. Acidic hydrolysis of **3** afforded the desulfated aglycone **3b** with an identical t_R value to that of **1b** when separated by HPLC under the same conditions. The same 24*S* configuration was assigned to **3** based on analysis of the ^1H NMR spectrum of the (+)-(*R*)-MTPA ester of **3b**. The molecular formula of **3** was determined as $C_{45}H_{74}O_{22}S_2Na_2$ by ^{13}C NMR as well as from HRESIMS (positive ion mode). Ion peaks at *m/z* 979 [M + Na − $NaHSO_4$]$^+$ and 859 [M + Na − 2 × $NaHSO_4$]$^+$ confirmed the presence of two sulfate groups. The negative ion mode ESIMS showed a series of fragmentations with the following sugar losses: *m/z* 1053 [M − Na]$^-$; 893 [1053 − 160]$^-$ loss of a methoxylated deoxyhexose; 761 [893 − 132]$^-$ loss of a pentose; 599 [761 − 162]$^-$ loss of a hexose unit. Acid hydrolysis of **3** and GC analysis with reference sugars afforded D-glucose (Glc) and D-xylose (Xyl) in the ratio 1:1. Because the standard 3-*O*-methyl-D-quinovose was unobtainable, the third sugar could not be identified by GC. After methanolysis, **3** was *p*-bromobenzoylated and the reaction mixture was separated by HPLC. The major UV-absorbing HPLC peaks were then subjected to ^1H NMR analysis, and methyl 2,4-di-*O*-(*p*-bromobenzoyl)-3-*O*-methyl-α-D-quinovopyranoside was identified by comparison of its ^1H NMR data with literature data.[13] The ^1H NMR spectrum showed signals for three anomeric protons at δ 4.75, 4.84, and 5.01, each a doublet with $^3J_{H\text{-}1/H\text{-}2}$ = 7.2 Hz, indicative of a β-configuration, which were correlated in the HMQC experiment with the corresponding carbons at δ 105.6, 103.9, and 105.1, respectively. The ^1H and ^{13}C NMR signals attributable to the various sugar units were assigned by the application of 2D NMR experiments including DQCOSY, TOCSY, HMQC, and HMBC (Table 2). The structure of the carbohydrate chain of **3** was determined by an HMBC spectrum, which showed a cross-peak between C-6 of the aglycone and H-1 of glucose, indicating that Glc was connected to C-6 of the aglycone, and the linkages of the terminal 3-*O*-methylquinovose (MeQui) at C-2 of xylose in turn linked to C-3 of Glc were indicated by cross-peaks MeQui H-1/Xyl C-2 and Xyl H-1/Glc C-3. Thus, novaeguinoside C (**3**) was defined as sodium (20*R*,24*S*)-6α-*O*-[3-*O*-methyl-β-D-quinovopyranosyl-(1→2)-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl]-5α-cholest-9(11)-en-3β,24-diyl disulfate. Trisaccharide chains were previously found only in a limited number of asterosaponins,[3] and 3-*O*-methyl-D-quinovose was only identified in three asterosaponins from *Goniopecten demonstrans*.[13]

Novaeguinoside D (**4**) was analyzed for $C_{48}H_{77}NO_{22}S_2Na_2$ by combined HRESIMS and ^{13}C NMR analyses. Ion peaks at *m/z* 1032 [M + Na − $NaHSO_4$]$^+$ and 1007 [M + Na − $NCH_2CH_2SO_3Na$]$^+$ confirmed the presence of a sulfate group and a taurine residue. Comparison of the NMR spectra of **4** with those of **3** indicated that both possess the same trisaccharide chain and steroidal skeleton, but differ in their side chains. Because of the slight solubility in MeOH, only the ^1H NMR spectrum of **4** was measured in CD_3OD. The side chain in the aglycone moiety of **4** was shown to be identical with that of **2**, by comparison of the ^1H NMR spectra of their corresponding side chain parts, i.e., the Δ^{22E}, 26-amide ergostane side chain. The three asymmetric carbons in the side chain were also presumed to have the same configurations as those of **2**, because the olefinic proton and methyl doublets pattern in the ^1H NMR spectrum (CD_3OD) of **4** were in good agreement with those of **2**. The structure of the side chain was corroborated by comparison of the NMR data of **4** in C_5D_5N with those of triseramide, possessing the same side chain with 24*R*, 25*S* configuration.[12a] The ^1H and ^{13}C NMR signals were completely assigned by extensive 2D NMR studies (Supporting Information), and the data confirmed the structure of novaeguinoside D (**4**) as (20*R*,22*E*,24*R*,25*R*)-6α-*O*-[3-*O*-methyl-β-D-quinovopyranosyl-(1→2)-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl]-24-methyl-3β-*O*-sodiumsulfato-5α-cholest-9(11),22-dien-26-oic acid (2-sodium sulfethyl)amide.

When tested for promoting activity of tubulin polymerization *in vitro*, **1**, **3**, and **4** exhibited significant activity with P_e values of (28 ± 2)%, (42 ± 3)%, and (23 ± 2)% and P_k values of (94 ± 5)%, (149 ± 8)%, and (85 ± 4)% at 11.5, 9.3, and 8.9 μM, respectively. Because samples with higher than 20% P_e values were considered active,[2] **2** was marginally active with P_e = (18 ± 3)% and P_k = (76 ± 5)% at 10.8 μM. The *in vitro* cytotoxicity of **1**−**4** against human leukemia K-562 and human hepatoma BEL-7402 cells was also evaluated. The IC_{50} of each compound was measured

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of Novaeguinosides C (**3**) and D (**4**) in C_5D_5N[a]

	δ_H (J in Hz)		δ_C	
position	**3**	**4**	**3**	**4**
1	1.51 m, 1.25 m	1.54 m, 1.26 m	36.0	36.0
2	2.59 m, 1.71 m	2.60 m, 1.77 m	29.5	29.4
3	4.70 m	4.72 m	77.8	77.7
4	3.24 br d (10.2), 1.54 m	3.33 br d (11.4), 1.55 m	30.8	30.7
5	1.35 m	1.37 m	49.4	49.4
6	3.67 m	3.66 m	80.9	80.5
7	2.57 br d (11.4), 1.13 m	2.57 br d (11.4), 1.15 m	41.6	41.5
8	1.97 m	2.02 m	35.3	35.4
9			145.7	145.6
10			38.3	38.3
11	5.12 br d (4.8)	5.12 br d (4.8)	116.7	116.6
12	2.17 dd (15.2, 4.0), 1.95 m	2.16 dd (15.2, 5.4), 1.96 br d (15.2)	42.3	42.5
13	-	-	41.8	41.6
14	1.20 m	1.19 m	53.8	54.1
15	2.08 m, 1.65 m	2.09 m, 1.66 m	24.8	24.9
16	1.68 m, 1.16 m	1.69 m, 1.17 m	28.5	29.2
17	1.27 m	1.30 m	57.3	57.1
18	0.75 s	0.77 s	11.4	11.6
19	0.90 s	0.91 s	19.3	19.3
20	1.38 m	2.23 m	35.5	40.4
21	1.11 d (6.6)	1.09 d (6.6)/0.98 d (6.6)[b]	18.9	20.7
22	2.09 m, 1.34 m	5.39 dd (15.2, 7.2)/5.30 dd (15.2, 7.2)[b]	31.8	136.7
23	1.90 m, 1.64 m	5.24 dd (15.2, 7.8)/5.15 dd (15.2, 7.2)[b]	28.2	131.7
24	4.47 m	2.61 m	84.9	40.6
25	1.96 m	2.38 m	31.3	47.2
26	1.15 d (6.6)		18.3	175.9
27	1.14 d (6.6)	1.20 d (6.6)/1.08 d (6.6)[b]	17.8	18.2
28		1.12 d (6.6)/1.04 d (6.6)[b]		15.3
taurine 1′		4.22 m		36.2
2′		3.48 t (6.6)		51.6
NH		8.50 t (4.8)		
Glc 1	4.75 d (7.2)	4.78 d (7.2)	105.6	105.5
2	3.82 m	3.85 m	73.9	73.8
3	3.66 m	3.69 m	91.9	91.8
4	3.87 m	3.88 m	69.9	69.7
5	3.68 m	3.71 m	77.6	77.5
6	4.29 br d (11.4), 4.14 br d (11.4)	4.32 br d (11.4), 4.15 br d (11.4)	62.4	62.3
Xyl 1	4.84 d (7.2)	4.86 d (7.2)	103.9	103.9
2	3.77 dd (9.0, 7.2)	3.78 dd (9.0, 7.2)	81.2	81.1
3	3.81 m	3.83 m	76.6	76.6
4	3.89 m	3.90 m	70.1	70.1
5	4.10 dd (11.4, 4.2), 3.43 br d (11.4)	4.10 dd (11.4, 4.2), 3.44 br d (11.4)	66.6	66.5
MeQui 1	5.01 d (7.2)	5.02 d (7.2)	105.1	105.0
2	3.92 m	3.94 m	76.0	76.0
3	3.64 t (9.0)	3.65 m	86.3	86.2
4	3.84 m	3.86 m	75.3	75.2
5	3.55 m	3.54 m	75.6	75.6
6	1.63 d (6.6)	1.65 d (6.0)	17.8	17.8
3-OMe	3.74 s	3.77 s	60.8	60.7

[a] Assignments aided by DQCOSY, TOCSY, NOESY, DEPT, HMQC, and HMBC experiments. [b] Recorded in CD_3OD.

Table 3. Cytotoxicity of Asterosaponins **1**−**4** against Two Cancer Cell Lines *in Vitro* (IC_{50}, μM)[a]

cell line	**1**	**2**	**3**	**4**	HCP[b]
K-562	3.0 ± 0.6	7.9 ± 1.5	1.3 ± 0.2	4.6 ± 0.5	0.18 ± 0.05
BEL-7402	2.4 ± 0.3	9.5 ± 1.1	0.7 ± 0.1	4.1 ± 1.0	0.40 ± 0.08

[a] IC_{50} values are means from three independent experiments (average ± SD). [b] 10-Hydroxycamptothecine (HCP) as positive control.

on the basis of cell viability after 72 h treatment. The results are listed in Table 3 and showed that all the asterosaponins exhibited cytotoxicity against the two cell lines, while **3** was more potent and **2** was only marginally cytotoxic. Our previous studies have shown that the 12 asterosaponins isolated from this species all displayed cytotoxicity against the two cell lines except the asterosaponins with asterone as the aglycone moiety.[5] However, none of them exhibited tubulin polymerization promoting activity in a screening assay. In view of their distinct structures compared with novaeguinosides A−D, it revealed that the structural feature, i.e., the $\Delta^{9(11)}$-3β,6α-dioxysteroidal aglycone with a sulfate group attached at C-3 and an oligosaccharide moiety at C-6, is responsible for the remarkable cytotoxic activity. Our results suggested that the structural differences such as the 24-sulfated side chain and the trisaccharide moiety in these asterosaponins play an important role in terms of tubulin polymerization promoting activity. However, more extensive studies are needed before a clear structure−activity relationship can be reached. Considering only six types of compounds that mimic the effects of paclitaxel on tumors have been found from numerous sources and they mostly possessed a common pharmacophore responsible for their microtubule-stabilizing effects,[2,16] the potent activity and relatively different structures of these asterosaponins merit further studies.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. Melting points were determined on an XT5-XMT apparatus and are uncorrected. IR spectra were recorded on a Bruker Vector 22 infrared spectrometer. NMR spectra were recorded on a Bruker AV-600 spectrometer, and the 2D NMR spectra were obtained using standard pulse sequences. ESIMS and

HRESIMS were recorded on a Micromass Quattro mass spectrometer. GC were performed on a Finnigan Voyager apparatus with an ULTRA-2 column (50 m × 0.2 mm i.d.). HPLC was carried out on a Dionex P680 liquid chromatograph equipped with a UV170 UV/vis detector using a Sino Chrom ODS-BP column (25 cm × 10 mm i.d., Elite Inc., China) and monitored at 206 nm for the semipreparation or a Phenomenex Luna 3 silica column (15 cm × 4.6 mm i.d.) and monitored at 260 nm for the separation of the *p*-bromobenzoate mixture. Column chromatographies were performed on Si gel H (10−40 μm, Qingdao Marine Chemical Inc.), Sephadex LH-20 (Pharmacia), and reversed-phase Si gel (Lichroprep RP-18, 40−63 μm). Fractions were monitored by TLC, and spots were visualized by heating Si gel plates sprayed with 10% H_2SO_4 in EtOH.

Animal Material. Specimens of *C. novaeguineae* were collected at a depth 2−20 m by hand using scuba or by a fishery bottom trawler from offshore waters of the Sanya Bay in the South China Sea in May 2006 and stored in EtOH at room temperature. The organisms were identified by Dr. Hans Uwe Dahms of the Oldenburg University, Germany. A voucher specimen was deposited in the Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi'an, China, under the registration code number XJ-HNHX-05.

Extraction and Isolation. The starfishes (101 kg, wet weight) were cut into pieces and extracted three times with refluxing 95% EtOH. The combined extracts were concentrated to leave a rufous residue, which was suspended in H_2O (15 L) and then partitioned successively using petroleum ether (15 L × 3) and *n*-BuOH (15 L × 5). The *n*-BuOH fraction (170 g) was chromatographed on Si gel eluting with a $CHCl_3$/*n*-BuOH (saturated with H_2O)/MeOH (2:1:0 to 0:6:1) (lower phase) gradient to give 15 major fractions (1−15) based on TLC analysis. Fraction 12 (2.0 g) was proved to be capable of promoting polymerization of tubulin *in vitro* (P_e = 28% at 10 μg/mL) and subjected to size exclusion chromatography on a Sephadex LH-20 column equilibrated with MeOH/H_2O (1:1) to give three major fractions, A, B, and C. By TLC comparison with authentic samples, asterosaponins with penta- or hexasaccharide moieties, such as novaeguinosides I and II, marthasteroside A_1, and regularoside B, which were isolated previously,[5] were identified as the main constituents of fractions A and B. Fraction C (0.5 g) was subjected to MPLC on a Lobar column (Lichroprep RP-18) eluting with MeOH/H_2O (1:1) to give two major fractions, C1 and C2. C1 was further purified by repeated semipreparative HPLC (ODS-BP column) to afford pure compounds **3** (170 mg, t_R = 34.8 min) and **4** (135 mg, t_R = 33.3 min) using MeOH/H_2O (48.5:51.5) as the mobile phase and a flow rate of 2 mL/min. C2 was separated by HPLC eluting with MeOH/H_2O (1:1) at a flow rate of 2 mL/min to yield compounds **1** (20 mg) and **2** (30 mg) in 35.6 and 32.9 min, respectively.

Novaeguinoside A (1): colorless crystals ($CHCl_3$/MeOH, 5:1); mp 225−226 °C; $[\alpha]^{22}_D$ +10.2 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3440, 1640, 1240, 1208, 1062 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS (positive) *m/z* 893 $[M + Na]^+$, 791 $[M + Na - SO_3Na + H]^+$, 773 $[M + Na - NaHSO_4]^+$, 653 $[M + Na - 2 \times NaHSO_4]^+$, 645 $[M + Na - 248$ (Qui-4 − $OSO_3Na)]^+$, 533 $[M + Na - 3 \times NaHSO_4]^+$, 525 $[645 - NaHSO_4]^+$, 405 $[525 - NaHSO_4]^+$; ESIMS (negative) *m/z* 847 $[M - Na]^-$, 745 $[M - Na - SO_3Na + H]^-$, 643 $[M - Na - 2 \times SO_3Na + 2H]^-$, 599 $[M - Na - 248]^-$; HRESIMS (positive) *m/z* 893.2090 $[M + Na]^+$ (calcd for $C_{33}H_{53}O_{16}S_3Na_4$, 893.2086).

Novaeguinoside B (2): colorless crystals ($CHCl_3$/MeOH, 5:1); mp 202−203 °C; $[\alpha]^{22}_D$ + 2.4 (*c* 0.12, MeOH); IR (KBr) ν_{max} 3430, 1652, 1640, 1555, 1242, 1218, 1210, 1062, 1046 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS (positive) *m/z* 946 $[M + Na]^+$, 844 $[M + Na - SO_3Na + H]^+$, 826 $[M + Na - NaHSO_4]^+$, 801 $[M + Na - NCH_2CH_2SO_3Na]^+$, 706 $[M + Na - 2 \times NaHSO_4]^+$, 698 $[M + Na - 248$ (Qui-4 − $OSO_3Na)]^+$, 681 $[801 - NaHSO_4]^+$, 578 $[698 - NaHSO_4]^+$, 561 $[681 - NaHSO_4]^+$, 553 $[801 - 248]^+$, 433 $[553 - NaHSO_4]^+$; ESIMS (negative) *m/z* 900 $[M - Na]^-$, 798 $[M - Na - SO_3Na + H]^-$, 696 $[M - Na - 2 \times SO_3Na + 2H]^-$, 652 $[M - Na - 248]^-$; HRESIMS (positive) *m/z* 946.2355 $[M + Na]^+$ (calcd for $C_{36}H_{56}NO_{16}S_3Na_4$, 946.2352).

Novaeguinoside C (3): colorless crystals ($CHCl_3$/MeOH/H_2O, 2:1:0.1); mp 231−232 °C; $[\alpha]^{22}_D$ +19.8 (*c* 0.85, pyridine); IR (KBr) ν_{max} 3445, 1638, 1238, 1210, 1060 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; ESIMS (positive) *m/z* 1099 $[M + Na]^+$, 997 $[M + Na\text{-}SO_3Na + H]^+$, 979 $[M + Na - NaHSO_4]^+$, 939 $[M + Na - MeQui]^+$, 859 $[M + Na - 2 \times NaHSO_4]^+$, 819 $[979 - MeQui]^+$, 699 $[859 - MeQui]^+$, 687 $[819 - Xyl]^+$, 477 $[MeQui + Xyl + Glc + Na]^+$, 315 $[MeQui + Xyl + Na]^+$; ESIMS (negative) *m/z* 1053 $[M - Na]^-$, 951 $[M - Na - SO_3Na + H]^-$, 893 $[M - Na - MeQui]^-$, 761 $[893 - Xyl]^-$, 599 $[761 - Glc]^-$; HRESIMS (positive) *m/z* 1099.3809 $[M + Na]^+$ (calcd for $C_{45}H_{74}O_{22}S_2Na_3$, 1099.3806).

Novaeguinoside D (4): colorless crystals ($CHCl_3$/MeOH/H_2O, 2:1:0.1); mp 209−210 °C; $[\alpha]^{22}_D$ +10.8 (*c* 0.60, pyridine); IR (KBr) ν_{max} 3435, 1655, 1641, 1558, 1240, 1218, 1210, 1064, 1048 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; ESIMS (positive) *m/z* 1152 $[M + Na]^+$, 1050 $[M + Na - SO_3Na + H]^+$, 1032 $[M + Na - NaHSO_4]^+$, 1007 $[M + Na - NCH_2CH_2SO_3Na]^+$, 992 $[M + Na - MeQui]^+$, 887 $[1007 - NaHSO_4]^+$, 872 $[1032 - MeQui]^+$, 847 $[1007 - MeQui]^+$, 740 $[872 - Xyl]^+$, 727 $[887 - MeQui]^+$, 477 $[MeQui + Xyl + Glc + Na]^+$, 315 $[MeQui + Xyl + Na]^+$; ESIMS (negative) *m/z* 1106 $[M - Na]^-$, 1004 $[M - Na - SO_3Na + H]^-$, 946 $[M - Na - MeQui]^-$, 814 $[946 - Xyl]^-$, 652 $[814 - Glc]^-$; HRESIMS (positive) *m/z* 1152.4076 $[M + Na]^+$ (calcd for $C_{48}H_{77}NO_{22}S_2Na_3$, 1152.4071).

Desulfation of 1. A solution of compound **1** (8 mg) in 1 mL of dioxane/pyridine (1:1) was heated at 120 °C for 2.5 h. After the solution had cooled, H_2O (5 mL) was added and the solution was extracted with *n*-BuOH (3 mL × 3). The combined extracts were washed with H_2O, evaporated under reduced pressure, and purified by HPLC (ODS-BP column) eluting with MeOH/H_2O (4:1) to yield the pure desulfated saponin **1a** (3.5 mg) as a colorless, amorphous powder: ^1H and ^{13}C NMR, see Table 1; ESIMS (positive) *m/z* 587 $[M + Na]^+$, 441 $[M + Na - Qui]^+$; ESIMS (negative) *m/z* 563 $[M - H]^-$, 417 $[M - H - Qui]^-$.

Acid Hydrolysis of 1−3. Each compound (3 mg for **1** and **3**, 10 mg for **2**) was heated with 2 M CF_3COOH (2 mL) at 120 °C for 2 h. The reaction mixture was evaporated to dryness, and the residue was partitioned between CH_2Cl_2 and H_2O. The organic phases (for **1** and **3**) were evaporated under reduced pressure and purified by HPLC (ODS-BP column) eluting with MeOH/H_2O (9:1) at a flow rate of 2 mL/min to yield **1b** and **3b** (the desulfated alycones of **1** and **3**) both in 17.2 min. Each desulfated aglycone was treated with freshly distilled (+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μL) in dry pyridine (50 μL) for 1 h at room temperature. After removal of the solvent, the resulting 3,24-di-(+)-(*R*)-MTPA ester was analyzed by ^1H NMR. 3,24-Di-(+)-(*R*)-MTPA ester of **1b**: ^1H NMR (600 MHz, $CDCl_3$) δ 0.62 (3H, s, Me-18), 0.83 (3H, d, J = 6.6 Hz, Me-27), 0.86 (3H, d, J = 6.6 Hz, Me-26), 0.92 (3H, d, J = 6.6 Hz, Me-21), 0.95 (3H, s, Me-19), 3.50 (1H, m, H-6), 4.50 (1H, m, H-24), 5.24 (1H, m, H-3), 5.31 (1H, brd, J = 5.4 Hz, H-11). 3,24-Di-(+)-(*R*)-MTPA ester of **3b**: ^1H NMR (600 MHz, $CDCl_3$) δ 0.63 (3H, s, Me-18), 0.84 (3H, d, J = 6.6 Hz, Me-27), 0.87 (3H, d, J = 6.6 Hz, Me-26), 0.93 (3H, d, J = 6.6 Hz, Me-21), 0.95 (3H, s, Me-19), 3.52 (1H, m, H-6), 4.51 (1H, m, H-24), 5.26 (1H, m, H-3), 5.32 (1H, brd, J = 5.4 Hz, H-11).

The aqueous phase was concentrated to furnish the monosaccharides mixture (for **2**, the aqueous phase was divided into two equal portions and only one portion was used). Then, the 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives were prepared following the reported method[10] (Supporting Information) and analyzed by GC, with standard sugar derivatives prepared under the same conditions as reference samples. The derivatives of D-glucose, D-xylose, and D-quinovose were detected with t_R of 40.05, 29.22, and 28.40 min, respectively. Compounds **1** and **2** gave peaks of the derivatives of D-quinovose, while D-glucose and D-xylose were identified in a ratio of 1:1 for compound **3**.

The other portion of the dried residue for compound **2** was chromatographed on Si gel eluting with *n*-BuOH/EtOAc/H_2O (5:1:1) to give taurine (0.5 mg) as a colorless, amorphous powder: ESIMS (positive) *m/z* 148 $[M + Na]^+$, 132 $[M + Na - NH_2]^+$, 126 $[M + H]^+$; ^1H NMR (600 MHz, D_2O) δ 3.85 (t, J = 6.4 Hz, H-1′), 3.66 (t, J = 6.4 Hz, H-2′); ^{13}C NMR (150 MHz, D_2O) δ 37.3 (C-1′), 49.2 (C-2′).

Methanolysis of 3. Compound **3** (10 mg) was methanolyzed and *p*-bromobenzoylated according to a procedure previously reported[13] (see Supporting Information), and the *p*-bromobenzoate mixture was separated by HPLC (Luna 3 silica column) eluting with Et_2O/*n*-hexane (1:4) to yield methyl 2,4-di-*O*-(*p*-bromobenzoyl)-3-*O*-methyl-α-D-quinovopyranoside: ^1H NMR (600 MHz, $CDCl_3$) δ 1.33 (3H, d, J = 6.0 Hz, Me-6), 3.40 (OMe), 3.45 (OMe), 4.00 (1H, m, H-5), 3.98 (1H, t, J = 9.0 Hz, H-3), 5.01−5.10 (3H, m, H-1, H-2, and H-4), 7.59−7.98 (8H, m, ArH).

Bioassays. Compounds **1**−**4** were screened by a high-throughput model for screening antitumor agents capable of promoting polymerization of tubulin *in vitro* as previously reported.[2] The P_e and P_k values

represented the means of three independent experiments in which each compound was tested in eight replicate wells. The anticancer agent paclitaxel was used as a positive control with $P_e = (32 \pm 3)\%$ and $P_k = 100\%$ at 11.7 μM (description of tubulin-polymerization assay, see Supporting Information). The cytotoxicity against human leukemia K-562 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay described in a previous paper.[14] The cytotoxicity against human hepatoma BEL-7402 cells was evaluated by the sulforhodamine B (SRB) protein assay following a reported method.[15] Dose−response curves were plotted for the samples, and the IC_{50} were calculated as the concentrations of the test asterosaponins resulting in 50% reduction of absorption compared to the control cells. The data represented the means of three independent experiments in which each compound concentration was tested in three replicate wells. The anticancer agent 10-hydroxycamptothecine (HCP) was used as the positive control with $IC_{50} = 0.18 \pm 0.05$ μM against K-562 cells and $IC_{50} = 0.40 \pm 0.08$ μM against BEL-7402 cells (description of MTT and SRB assay, see Supporting Information).

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Supporting Information Available: Details for the preparation of the 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives, methanolysis of **3** and *p*-bromobenzoylation, key DQCOSY and HMBC correlations of novaeguinosides A (**1**), B (**2**), and D (**4**) (Figures S1, S3, and S5), key NOESY correlations of novaeguinosides A (**1**) and D (**4**) (Figures S2 and S6), key HMBC correlations of novaeguinoside C (**3**) (Figure S4), descriptions of tubulin-polymerization assay, MTT assay, and SRB assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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