Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Synthesis and Cytotoxic Property of Annonaceous Acetogenin Glycoconjugates

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Background: Annonaceous acetogenins (ACGs) are secondary metabolites produced by the Annonaceae family and display potent anticancer activity against various cancer cell lines. Squamocin and bullatacin are two examples of ACGs that show promising antitumor activity; however, preclinical data are not sufficient partly due to their being highly lipophilic and poorly soluble in water. These compounds also display high toxicity to normal cells. Due to these disadvantageous properties, the therapeutic potential of squamocin and bullatacin as antitumor agents has not been fully evaluated.

Methods: In order to enhance their water solubility and potentially improve their cancer targeting, squamocin and bullatacin were conjugated to a glucose or galactose to yield glycosylated derivatives by direct glycosylation or the Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reaction (the click reaction). The synthesized compounds were evaluated for their anticancer property against HeLa, A549 and HepG2 cancer cell lines using MTT assay.

Results: Nine glycosyl derivatives were synthesized and structurally characterized. Most of them show comparable in vitro cytotoxicity against HeLa, A549 and HepG2 cancer cell lines as their parent compounds squamocin and bullatacin. It appears that the type of sugar residue (glucose or galactose), the position at which the sugar residue is attached, and whether or not a linking spacer is present do not affect the potency of these derivatives much. The solubility of galactosylated squamocin **13** in phosphate buffer saline (PBS, pH = 7) is greatly improved (1.37 mg/mL) in comparison to squamocin (not detected in PBS).

Conclusion: The conjugation of a glucose or galactose to squamocin and bullatacin yields glycosyl derivatives with similar level of anticancer activity in tested cell lines. Further studies are needed to demonstrate whether or not these compounds show reduced toxicity to normal cells and their therapeutic potential as antitumor agents.

Keywords: annonaceous acetogenins, squamocin, bullatacin, glycosylated, cytotoxicity, anticancer, solubility

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Introduction

Annonaceous acetogenins (ACGs) have been isolated exclusively from species of the annonaceae family, exhibiting potent growth inhibitory activity against various cancer cell lines.[1–7] Bullatacin and squamocin (Figure 1) are two of the most cytotoxic acetogenins bearing adjacent tetrahydrofuran (THF) rings, which have been reported mainly in the seeds of these plants.[8–11] The anticancer potency of bullatacin has been reported to be much higher than some of the anticancer drugs currently used in the clinic. For example, bullatacin was found to be 10^4–10^5 times more potent than doxorubicin against both A549 and MCF-7 cell lines,[12] and 250 times more potent than doxorubicin against MCF-7/ADR, the human breast

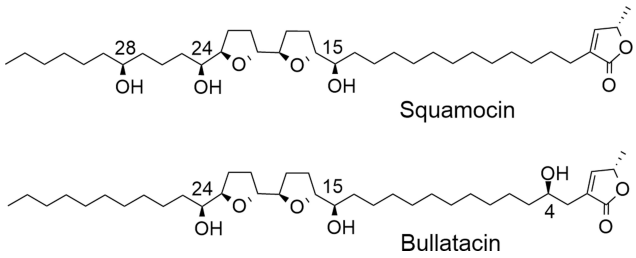


Figure 1 Structures of squamocin and bullatacin.

adenocarcinoma multidrug-resistant cell line.[13] In addition, bullatacin was found to be 300 times more effective than taxol in treating L1210 murine leukemia in mouse model.[14] Bullatacin and squamocin are promising anticancer agents worthy of further investigation. However, they display high toxicity toward normal cells as well[10] and show poor solubility in water (less than 1 μg/mL).[15] These properties prevent them from full evaluation of their therapeutic potential for cancer treatment. The preparation of derivatives for tumor-specific targeting by incorporation of different ligands could improve their activity and yield more suitable drugs.

Conjugation of carbohydrates with drugs has become increasingly important in bioorganic chemistry and chemical biology.[16,17] Glycosylation of drug molecules has been employed to modify their physico-chemical properties, including polarity, solubility, and stability, without affecting their biological activities.[18,19] For example, glycosylated porphyrins have been shown as promising photosensitizers in photodynamic therapy (PDT) for cancer owing to their good solubility and specific membrane interactions.[20] Moreover, glycosylation has been widely explored as a targeting strategy to selectively deliver anticancer drugs to cancer cells. This strategy is based on the findings that cancer cells require more glucose due to their rapid growth and altered metabolism than normal cells do, which results in the expression of higher levels of glucose transporters on plasma membrane of cancer cells to facilitate the uptake of glucose. For example, glucose transporter-1 (GLUT1), which also transports other hexoses such as galactose, mannose, and glucosamine,[21] is expressed at a level 100–300 times higher in malignant cells than in normal cells.[22] Anticancer agents conjugated to a glucose (or another sugar that is also a glucose transporter substrate) may be taken up by cancer cells more rapidly via facilitation by glucose transporters and be more selective toward cancer cells than normal cells. A number of anticancer agents such as doxorubicin,[23] daunorubicin,[24] paclitaxel and docetaxel,[25] 8-hydroxyquinoline and derivatives,[26] and benzodiazepins[27] have been redesigned to improve their cancer targeting and selectivity.

Previously, we reported a series of biotin-conjugated squamocin/bullatacin derivatives that showed tumor cell growth inhibitory activity with higher selectivity toward biotin receptor (+) tumor cells than their parent squamocin/bullatacin.[28] In this study, squamocin and bullatacin are conjugated to a glucose or galactose to improve their solubility in water and potentially their cancer targeting. The synthesis and preliminary studies of the anticancer activity of these new glycoconjugates are described.

Materials and Methods

Reagents and Instrumentation

Squamocin and bullatacin were obtained in our previous chemical investigation on the seeds of *Annona squamosa*.[42] 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl azide (**6**) and 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl azide (**7**) were purchased from J&K scientific Ltd. (Beijing, China). Sodium ascorbate, 4-dimethylaminopyridine (DMAP) and N,N′–dicyclohexylcarbodiimide (DCC), copper sulfate pentahydrate, D-galactose, 5-hexynoic acid, acetic anhydride, trichloroacetonitrile, hydrazine acetate, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and trimethylsilyl trifluoromethanesulfonate (TMSOTf) were purchased from Aladdin Chemical Co., Ltd (Shanghai, China). Anhydrous sodium sulfate and N,N-dimethylformamide (DMF) were purchased from Guangzhou Reagent Factory (Guangzhou, China). Pyridine was refluxed with CaH_2 for 3 h, then distilled and immediately stored over activated 4Å molecular sieves. CH_2Cl_2 was refluxed with CaH_2 for 4 h, then distilled and immediately stored over activated 4Å molecular sieves.

Optical rotations were obtained on a Perkin-Elmer 341 polarimeter with MeOH as solvent. The ^1H and ^{13}C NMR spectra were collected on a Bruker Avance-600 instrument at 600 (^1H) and 150 (^{13}C) MHz. The 2D NMR (^1H-^1H COSY, HSQC, and HMBC) spectra were recorded on a Bruker Avance-600 instrument. Chemical shifts are reported as ppm (δ units) relative to tetramethylsilane (TMS) as an internal standard and the coupling constants as J in hertz. The splitting pattern abbreviations are as follows: s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet. ESIMS data were obtained on an MDS SCIEX API 2000 LC/MS instrument. HRESIMS data were obtained on a Bruker maXis Q-TOF mass spectrometer. Preparative

HPLC was run on a Shimadzu LC-6A pump and a Shimadzu RID-10A refractive index detector and all separations were carried out with an XTerra Prep MS C_{18} column (19 × 300 mm, 10 μm) at a flow rate of 5 mL/min except where otherwise stated.

Preparation of Galacosylated Squamocin 4 and 5

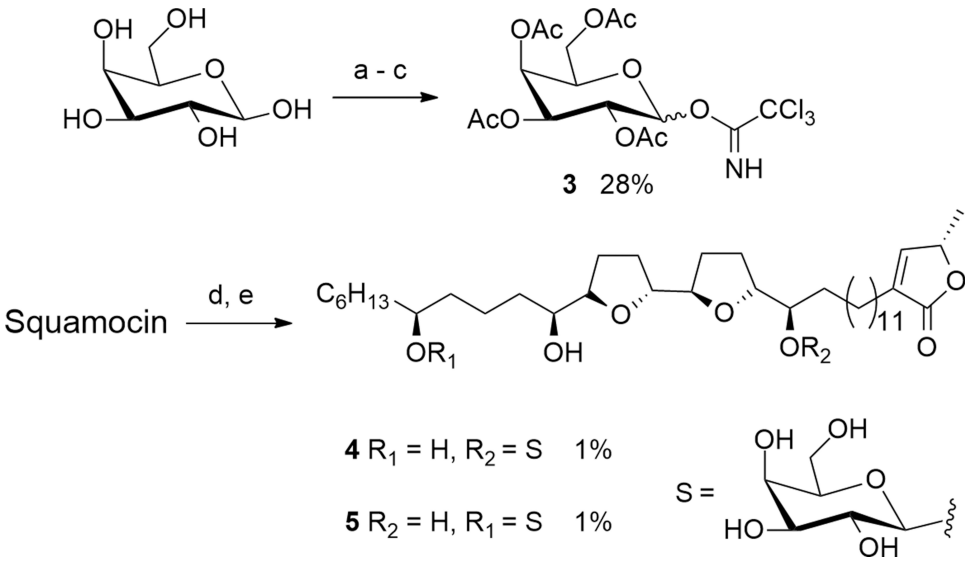
D-galactose (1 g, 5.56 mmol) was dissolved in dried pyridine (10 mL). Acetic anhydride (5.33 mL, 55.6 mmol) was added and the mixture was stirred overnight at room temperature (Scheme 1). The solvent was removed under reduced pressure and then the residue was dissolved in EtOAc (100 mL). The EtOAc solution was extracted with 1 N HCl (2 x 50 mL), saturated aqueous $NaHCO_3$ (2 x 50 mL) and saturated aqueous NaCl solution (50 mL), respectively. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to provide D-galactose pentaacetate (2.186 g, 100%) as yellow syrup.

D-galactose pentaacetate (2.186 g, 5.56 mmol) was dissolved in DMF (16 mL). Hydrazine acetate (614 mg, 6.67 mmol) was added and then the mixture was stirred at 40 °C for 4 h (Scheme 1). The reaction was monitored by TLC (petroleum ether: acetone, 2:1). Upon completion, the solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed with saturated aqueous NaCl solution. The organic layer was then dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to afford 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose (1.756 g, 91%) as a white solid.

2,3,4,6-Tetra-*O*-acetyl-D-galactopyranose (1.756 g, 5.05 mmol) was dissolved in dried CH_2Cl_2 (7 mL) under nitrogen atmosphere. Trichloroacetonitrile (7.2 mL, 50.5 mmol) and 1.8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.8 mL, 5.55 mmol) was slowly added, respectively, when the solution was cooled to 0°C. The mixture was stirred at 0°C for 7 h (Scheme 1) and monitored by TLC (petroleum ether: acetone, 2:1). When the reaction was completed, the mixture was filtered over celite, and was concentrated to give product **3** (761 mg, 31%) as an amorphous solid.

Squamocin (124 mg, 0.2 mmol), **3** (147 mg, 0.3 mmol) were dissolved in dried CH_2Cl_2 (5 mL) at 0°C. 4Å molecular sieves (200 mg) and TMSOTf (0.02 mL, 0.1mmol) was added at nitrogen atmosphere and the mixture was stirred at 0°C for 30 min (Scheme 1). The reaction mixture was quenched with triethylamine and then concentrated under reduced pressure. The residue was dissolved in EtOAc and extracted with 1 N HCl, saturated aqueous $NaHCO_3$ and saturated aqueous NaCl solution. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to give crude product 245 mg.

The deprotection was performed by treatment with Et_3N: MeOH: H_2O (1:8:1) at 35°C for 30 h (Scheme 1). The reaction mixture was concentrated in vacuo and the crude product was separated by Sephadex LH-20 gel permeation chromatography using MeOH, followed by preparative HPLC using 88% MeOH to afford **4** (2 mg, 1%, t_R = 43.6 min) and **5** (2 mg, 1%, t_R = 65.1 min).



Scheme 1 Synthesis of galactosyl squamocin **4** and **5**. Reagents and conditions: (a) Ac_2O, pyridine, rt, N_2, 16 h, 100%. (b) hydrazine acetate, DMF, 40 °C, 4 h, 91%. (c) trichloroacetonitrile, DBU, 0°C, 7 h, 31%. (d) **3**, TMSOTf, CH_2Cl_2, rt, 16 h. (e) Et_3N:MeOH:H_2O (1:8:1, v/v), 35°C, 30 h.

Characterization Data of Compound 4

A yellowish waxy solid; $[\alpha]^{20}_{D}$ +4.6 (*c* 0.06, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): 7.00 (1H, m, H-35), 5.00 (1H, m, H-36), 4.37 (1H, d, *J* = 7.6 Hz, H-1′), 4.03 (3H, m, H-15, 16, 4′), 3.86 (3H, m, H-20, 2′, 6′a), 3.80 (2H, m, H-19, 6′b), 3.74 (1H, m, H-24), 3.62 (2H, m, H-23, 3′), 3.57 (1H, m, H-28), 3.53 (1H, m, H-5′), 2.26 (2H, t, *J* = 7.7 Hz, H-3), 1.95 (3H, m), 1.88 (1H, m), 1.82 (1H, m), 1.69 (1H, m), 1.45–1.58 (5H, m), 1.38–1.44 (4H, m), 1.41 (3H, d, *J* = 6 Hz, H-37), 1.20–1.37 (30H, m), 0.88 (3H, t, *J* = 6.9 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): 174.2 (C-1), 149.2 (C-35), 134.5 (C-2), 102.3 (C-1′), 82.8 (2C, C-16, C-19), 82.2 (C-20), 81.3 (C-23), 81.2 (C-15), 74.8 (C-5′), 77.5 (C-36), 73.7 (C-3′), 71.8 (C-28), 71.7 (C-2′), 71.2 (C-24), 69.2 (C-4′), 61.5 (C-6′), 37.7 and 37.4 (C-27, C-29), 32.1 (C-25), 31.6 (C-32), 30.2 (C-14), 30.0, 29.9 (3C), 29.8 (2C), 29.7, 29.5, 29.4, 29.3, 29.0, 28.9, 27.6, 26.0, 25.4, 25.1, 24.9, 22.9 (C-33), 21.7 (C-26), 19.4 (C-37), 14.3 (C-34); HR-ESIMS *m/z*: 785.5413 $[M + H]^+$ (calcd for $C_{43}H_{77}O_{12}$, 785.5410).

Characterization Data of Compound 5

A yellowish waxy solid; $[\alpha]^{20}_{D}$ −1.2 (*c* 0.07, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): 7.00 (1H, m, H-35), 4.99 (1H, m, H-36), 4.30 (1H, d, *J* = 6.7 Hz, H-1′), 4.04 (1H, m, H-4′), 3.95 (2H, m, H-19, 20), 3.82 (5H, m, H-16, 23, 2′, 6′), 3.63 (3H, m, H-24, 28, 3′), 3.54 (1H, m, H-5′), 3.39 (1H, m, H-15), 2.28 (2H, t, *J* = 7.7 Hz, H-3), 1.93 (3H, m), 1.81 (1H, m), 1.72 (1H, m), 1.61 (1H, m), 1.45–1.56 (5H, m), 1.38–1.44 (4H, m), 1.40 (3H, d, *J* = 6 Hz, H-37), 1.20–1.35 (30H, m), 0.88 (3H, t, *J* = 6.9 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): 174.2 (C-1), 149.2 (C-35), 134.5 (C-2), 103.5 (C-1′), 83.6 (C-16), 83.1 (C-19), 82.7 (C-20), 82.2 (C-23), 81.4 (C-28), 77.7 (C-36), 74.6 (C-15), 74.4 (C-5′), 73.6 (C-3′), 71.8 (C-24, C-2′), 69.9 (C-4′), 61.4 (C-6′), 35.6 and 34.1 (C-27, C-29), 33.2 (C-14), 32.6 (C-25), 32.1 (C-32), 30.0, 29.9 (4C), 29.8, 29.7, 29.5, 29.4, 29.1, 29.0, 28.9, 27.6, 25.8, 25.7, 25.4 (2C), 22.9 (C-33), 21.7 (C-26), 19.4 (C-37), 14.4 (C-34); HR-ESIMS *m/z*: 785.5415 $[M + H]^+$ (calcd for $C_{43}H_{77}O_{12}$, 785.5410).

Preparation of β-D-Galactopyranosyl Azide 8 and β-D-Glucopyranosyl Azide 9

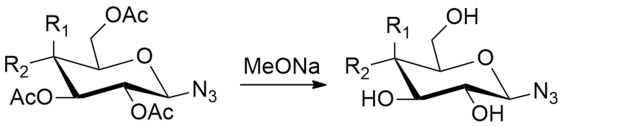
Eighty microliters of sodium methoxide solution in methanol (5 M) was added to 2 mL dried MeOH, then 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl azide (186 mg, 0.5 mmol) was added to the solution and the mixture was stirred for 4 h at room temperature. 0.5 N HCl solution was then added to the reaction mixture until pH 6 (Scheme 2). The mixture was concentrated under reduced pressure and the residue re-dissolved in 0.5 mL MeOH and solid filtrated off. The filtrate was concentrated under reduced pressure to afford β-D-galactopyranosyl azide **8** (93 mg, 90.7%).

Eighty microliters of sodium methoxide was added to 2 mL dried MeOH, then 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl azide (261 mg, 0.7 mmol) was added to the solution (Scheme 2). Following the same procedure, as described for the preparation of **8**, β-D-glucopyranosyl azide **9** (141 mg, 100%) was obtained.

Preparation of Galactosyl Squamocin Derivatives 11–13

Squamocin (187 mg, 0.3 mmol), 5-hexynoic acid (40 mg, 0.36 mmol) and DMAP (3 mg, 0.03 mmol) were dissolved in 3 mL dried CH_2Cl_2. DCC (123 mg, 0.6 mmol) was added and the mixture was stirred under N_2 atmosphere at room temperature for 16 h (Scheme 3). The resulting precipitate (DCU) was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was dissolved in EtOAc and sequentially washed with 0.5% aqueous HCl solution, saturated aqueous $NaHCO_3$ solution and saturated aqueous NaCl. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude products were separated by Sephadex LH-20 size exclusion chromatography using MeOH to give crude product **10A** (204 mg).

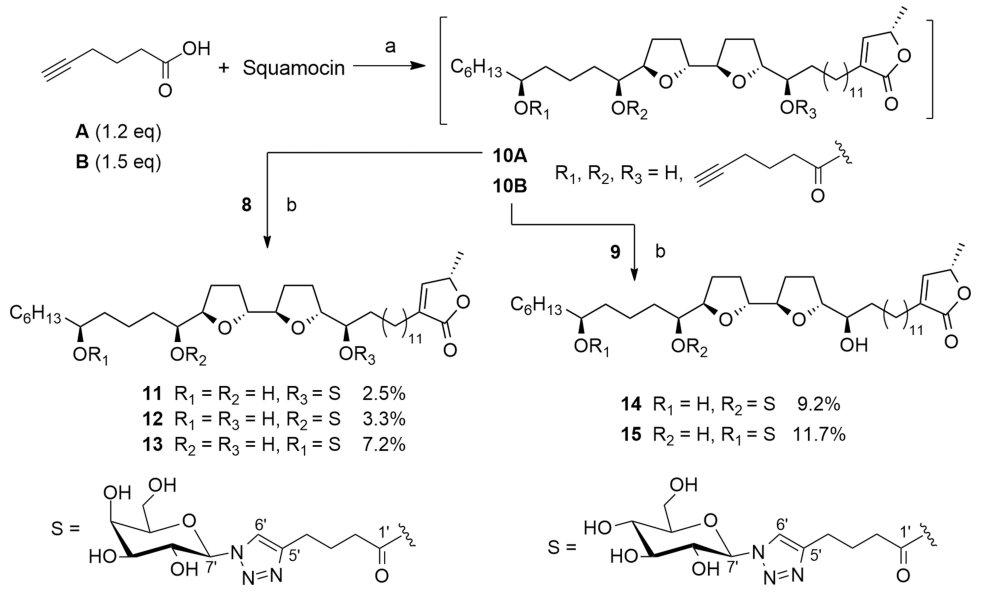
The crude product **10A** (204 mg) and β-D-galactopyranosyl azide **8** (93 mg) were suspended in MeOH. Then, $CuSO_4 \cdot 5H_2O$ (30 mg, 0.12 mmol) and sodium ascorbate (47 mg, 0.24 mmol) in 2 mL of distilled water were added. The suspension was stirred at 50°C for 16 h and monitored by TLC ($CHCl_3$: MeOH, 85:15) until the reaction was finished (Scheme 2). The suspension was concentrated under reduced pressure and then purified by silica gel column chromatography using $CHCl_3$-MeOH (97:3, and 93:7; v/v) to afford three fractions with F-II as the fraction of target



6 R_1 = OAc, R_2 = H **8** R_1 = OH, R_2 = H 91%

7 R_1 = H, R_2 = OAc **9** R_1 = H, R_2 = OH 100%

Scheme 2 Synthesis of glycosyl azides **8** and **9.**



Scheme 3 Synthesis of **11**–**15** by click chemistry. Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2, rt, N_2, 16 h. (b) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, MeOH/H_2O, 50 °C, 16 h.

products. F-II was further separated by HPLC using MeCN-H_2O (78:22, v/v) at a flow rate of 5 mL/min to afford **11** (7 mg, t_R = 61.47 min, 2.5%), **12** (9 mg, t_R = 55.53 min, 3.3%), and **13** (20 mg, t_R = 50.06 min, 7.2%).

Characterization Data of Compound 11

A colorless waxy solid; $[\alpha]^{20}_D$ +19.6 (*c* 0.2, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.92 (1H, s, H-6′), 7.00 (1H, m, H-35), 5.55 (1H, d, *J* = 8.0 Hz, H-7′), 5.00 (1H, m, H-36), 4.83 (1H, m, H-15), 3.94 (1H, m, H-16), 4.29 (1H, m, H-8′), 4.14 (1H, m, H-10′), 3.80 (7H, m, H-19, 20, 23, 9′, 11′, 12′), 3.59 (1H, m, H-24), 3.52 (1H, m, H-28), 2.75 (2H, m, H-4′), 2.36 (2H, m, H-2′), 2.26 (2H, m, H-3), 1.85–2.02 (4H, m), 1.74 (2H, m), 1.48–1.59 (6H, m), 1.38–1.47 (6H, m), 1.41 (3H, d, *J* = 6.0 Hz, H-37), 1.16–1.35 (30H, m), 0.87 (3H, t, *J* = 6.6 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.2 (C-1), 173.6 (C-1′), 149.2 (C-35), 147.2 (C-5′), 134.5 (C-2), 122.4 (C-6′), 88.3 (C-7′), 82.7 (C-20), 82.2 (C-23), 81.9 (C-19), 80.7 (C-16), 77.9 (C-11′), 77.7 (C-36), 75.7 (C-15), 74.0 (C-9′), 72.2 (C-24), 71.9 (C-28), 70.3 (C-8′), 69.0 (C-10′), 61.2 (C-12′), 37.8 and 37.3 (C-27, C-29), 33.8 (C-2′), 33.0 (C-25), 32.1 (C-32), 30.9 (C-14), 29.8 (3C), 29.7 (5C), 29.5, 29.4, 28.9, 28.6, 28.6, 27.6, 26.0, 25.5, 25.4 (2C), 24.7, 24.6 (C-4′), 22.9 (C-33), 22.4 (C-26), 19.4 (C-37), 14.3 (C-34); HR-ESIMS *m/z*: 922.6015 $[M + H]^+$ (calcd for $C_{49}H_{84}N_3O_{13}$, 922.5999).

Characterization Data of Compound 12

A colorless waxy solid; $[\alpha]^{20}_D$ +16.0 (*c* 0.2, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.82 (1H, s, H-6′), 7.00 (1H, m, H-35), 5.58 (1H, br s, H-7′), 5.00 (2H, m, H-36, H-24), 4.32 (1H, m, H-8′), 4.18 (1H, m, H-10′), 4.05 (1H, m, H-23), 3.85 (2H, m, H- 9′, 11′), 3.83 (1H, m, H-16), 3.80 (4H, m, H-19, 20, 12′), 3.53 (1H, m, H-28), 3.39 (1H, m, H-15), 2.67 (2H, m, H-4′), 2.35 (2H, m, H-2′), 2.26 (2H, m, H-3), 1.85–2.02 (5H, m), 1.76 (2H, m), 1.48–1.62 (5H, m), 1.37–1.47 (6H, m), 1.41 (3H, d, *J* = 6.0 Hz, H-37), 1.16–1.36 (30H, m), 0.87 (3H, t, *J* = 7.0 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.3 (C-1), 174.2 (C-1′), 149.2 (C-35, C-5′), 134.5 (C-2), 88.3 (C-7′), 83.7 (C-16), 82.7 (C-20), 81.9 (C-19), 80.9 (C-23), 77.7 (C-11′, C-36), 75.1 (C-24), 74.7 (C-15), 73.9 (C-9′), 71.4 (C-28), 70.3 (C-8′), 69.3 (C-10′), 61.1 (C-12′), 37.8 and 37.1 (C-27, C-29), 33.9 (C-2′), 33.1 (C-14), 32.1 (C-32), 31.2 (C-25), 30.0, 29.9 (3C), 29.8 (2C), 29.7, 29.5, 29.4, 29.1, 28.8, 28.4, 27.6, 25.9, 25.7, 25.4 (2C), 24.9 (C-4′), 22.9, 22.9 (C-33), 22.0 (C-26), 19.4 (C-37), 14.4 (C-34); HR-ESIMS *m/z*: 922.6020 $[M + H]^+$ (calcd for $C_{49}H_{84}N_3O_{13}$, 922.5999).

Characterization Data of Compound 13

A colorless waxy solid; $[\alpha]^{20}_D$ +15.6 (*c* 0.2, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.85 (1H, s, H-6′), 7.00 (1H, m, H-35), 5.57 (1H, br s, H-7′), 5.00 (1H, m, H-36), 4.88 (1H, m, H-28), 4.28 (1H, m, H-8′), 4.16 (1H, m, H-10′), 3.91 (2H, m, H-19, 20), 3.83 (6H, m, H-16, 23, 9′,

11′, 12′), 3.75 (1H, m, H-24), 3.39 (1H, m, H-15), 2.68 (2H, m, H-4′), 2.33 (2H, m, H-2′), 2.26 (2H, m, H-3), 1.85–2.03 (6H, m), 1.79 (2H, m), 1.44–1.61 (10H, m), 1.41 (3H, d, J = 6.0 Hz, H-37), 1.20–1.37 (30H, m), 0.88 (3H, t, J = 7.0 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.2 (C-1), 173.5 (C-1′), 149.2 (C-35, C-5′), 134.5 (C-2), 88.3 (C-7′), 84.0 (C-16), 83.2 (C-19), 83.0 (C-20), 82.8 (C-23), 77.8 (C-11′), 77.7 (C-36), 74.6 (C-15), 74.3 (C-9′), 74.2 (C-28), 71.7 (C-24), 70.6 (C-8′), 69.2 (C-10′), 61.7 (C-12′), 34.6 and 34.5 (C-27, C-29), 34.0 (C-2′), 32.9 (C-14), 32.5 (C-25), 31.9 (C-32), 29.9 (4C), 29.8 (2C), 29.7, 29.5, 29.4 (2C), 29.2 (2C), 28.7, 27.6, 25.8, 25.6, 25.4 (2C), 24.8 (C-4′), 24.7, 22.8 (C-33), 22.4 (C-26), 19.4 (C-37), 14.3 (C-34); HR-ESIMS *m/z*: 922.6017 $[M + H]^+$ (calcd for $C_{49}H_{84}N_3O_{13}$, 922.5999).

Preparation of Glucosylated Squamocin Derivatives **14** and **15**

Squamocin (249 mg, 0.4 mmol), 5-hexynoic acid (67 mg, 0.6 mmol) and DMAP (7 mg, 0.06 mmol) were dissolved in 5 mL dried CH_2Cl_2. DCC (247 mg, 1.2 mmol) was added and the mixture was stirred under N_2 atmosphere at room temperature for 16 h (Scheme 3). Following the same procedure as described for the preparation of **10A** in the Experimental Section 3.4, intermediate product **10B** (288 mg) was obtained.

The intermediate product **10B** (288 mg) and β-D-glucopyranosyl azide **9** (82 mg, 0.4 mmol) were suspended in 4 mL MeOH. Then, $CuSO_4{\cdot}5H_2O$ (40 mg, 0.16 mmol) and sodium ascorbate (63 mg, 0.32 mmol) in 2 mL of distilled water were added. The suspension was stirred at 50°C for 16 h. The suspension was then concentrated in vacuo and the residue purified by silica gel column chromatography using $CHCl_3$-MeOH (97:3 and 93:7; v/v) to afford five fractions. F-II was further separated by HPLC using MeOH-H_2O (80:20, v/v) at a flow rate of 5 mL/min to afford **14** (34 mg, t_R = 75.74 min, 9.2%). F-III and F-IV were combined and further separated by HPLC using MeOH-H_2O (80:20, v/v) at a flow rate of 5 mL/min to afford **15** (43 mg, t_R = 56.68 min, 11.7%).

Characterization Data of Compound **14**

A yellowish waxy solid; $[\alpha]^{20}_D$ +1.4 (*c* 1.1, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.82 (1H, s, H-6′), 7.00 (1H, m, H-35), 5.59 (1H, d, J = 7.9 Hz, H-7′), 5.00 (1H, m, H-36), 4.96 (1H, m, H-24), 4.01 (2H, m, H-23, 8′), 3.82 (1H, m, H-20), 3.77 (6H, m, H-16, 19, 9′, 10′, 12′), 3.58 (1H, m, H-11′), 3.51 (1H, m, H-28), 3.35 (1H, m, H-15), 2.68 (2H, m, H-4′), 2.35 (2H, m, H-2′), 2.25 (2H, m, H-3), 1.94 (5H, m), 1.72 (2H, m), 1.52 (11H, m), 1.41 (3H, d, J = 6.0 Hz, H-37), 1.19–1.37 (30H, m), 0.87 (3H, t, J = 7.0 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.1 (C-1), 173.6 (C-2′), 149.2 (C-35), 134.4 (C-2), 129.0 (C-6′), 87.9 (C-7′), 83.6 (C-16), 82.6 (C-20), 81.8 (C-19), 80.8 (C-23), 79.2 (C-11′), 77.6 (C-36), 76.9 (C-9′), 75.1 (C-24), 74.4 (C-15), 72.7 (C-8′), 71.4 (C-28), 69.1 (C-10′), 61.1 (C-12′), 37.7 and 37.0 (C-27, C-29), 33.9 (C-2′), 33.1 (C-14), 32.0 (C-32), 31.3 (C-25), 29.9 (2C), 29.8 (3C), 29.7, 29.6, 29.5, 29.4, 29.0, 28.6, 28.5, 27.6 (2C), 25.9, 25.7, 25.3, 24.8 (2C), 24.5, 22.8 (C-33), 21.9 (C-26), 19.4 (C-37), 14.3 (C-34); HR-ESIMS *m/z*: 922.5996 $[M + H]^+$ (calcd for $C_{49}H_{84}N_3O_{13}$, 922.5999).

Characterization Data of Compound **15**

A yellowish waxy solid; $[\alpha]^{20}_D$ +0.8 (*c* 1.1, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.71 (1H, s, H-6′), 7.00 (1H, m, H-35), 5.57 (1H, d, J = 8.0 Hz, H-7′), 4.99 (1H, m, H-36), 4.86 (1H, m, H-28), 4.03 (1H, m, H-8′), 3.91 (2H, m, H-19, 20), 3.82 (3H, m, H-16, 12′), 3.78 (4H, m, H-23, 24, 9′, 10′), 3.61 (1H, m, H-11′), 3.37 (1H, m, H-15), 2.68 (2H, m, H-4′), 2.32 (2H, m, H-2′), 2.25 (2H, m, H-3), 1.93 (4H, m), 1.86 (1H, m), 1.77 (1H, m), 1.52 (12H, m), 1.40 (3H, d, J = 6.8 Hz, H-37), 1.19–1.35 (30H, m), 0.88 (3H, t, J = 7.0 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.1 (C-1), 173.4 (C-2′), 149.1 (C-35), 146.9 (C-5′), 134.3 (C-2), 121.6 (C-6′), 87.8 (C-7′), 83.6 (C-16), 83.0 (C-19), 82.7 (C-20), 82.2 (C-23), 79.1 (C-11′), 77.6 (C-36), 76.9 (C-9′), 74.4 (C-15), 74.3 (C-28), 72.7 (C-8′), 71.5 (C-24), 69.0 (C-10′), 61.1 (C-12′), 34.4 and 34.3 (C-27, C-29), 33.9 (C-2′), 33.0 (C-14), 32.4 (C-25), 31.8 (C-32), 29.9, 29.8, 29.7 (2C), 29.6, 29.4, 29.3 (2C), 29.0, 29.0, 28.6, 27.5, 25.7, 25.4, 25.3, 24.9, 24.8, 24.6, 23.6, 22.7 (C-33), 22.2 (C-26), 19.3 (C-37), 14.2 (C-34); HR-ESIMS *m/z*: 922.5986 $[M + H]^+$ (calcd for $C_{49}H_{84}N_3O_{13}$, 922.5999).

Preparation of Galactosylated Bullatacin **17**

Galactosylated bullatacin derivative was synthesized using the same method as described for galactosylated squamocin derivatives **11**–**13**. Briefly, bullatacin (93 mg, 0.15 mmol), 5-hexynoic acid (20 mg, 0.18 mmol) and DMAP (2 mg, 0.02 mmol) were dissolved in 3 mL dried CH_2Cl_2. DCC (62 mg, 0.3 mmol) was added and the mixture was stirred under N_2 atmosphere at room temperature for 16 h (Scheme 4). The

resulting precipitate (DCU) was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was dissolved in EtOAc and sequentially washed with 0.5% aqueous HCl solution, saturated aqueous $NaHCO_3$ solution and saturated aqueous NaCl. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude products were separated by Sephadex LH-20 gel permeation chromatography using MeOH to provide intermediate product **16A** (93 mg).

The intermediate product **16A** (93 mg) and β-D-galactopyranosyl azide **8** (30 mg, 0.15 mmol) were suspended in 2 mL MeOH. Then, $CuSO_4{\cdot}5H_2O$ (15 mg, 0.06 mmol) and sodium ascorbate (47 mg, 0.24 mmol) in 1 mL of distilled water were added. The suspension was stirred at 50° C for 16 h and monitored by TLC ($CHCl_3$:MeOH, 85:15) until the reaction was finished (Scheme 2). The suspension was concentrated under reduced pressure and then purified by silica gel column chromatography using $CHCl_3$-MeOH (97:3, and 93:7; v/v) to afford three fractions with F-II as the fraction of target products. F-II was further separated by HPLC using MeCN-H_2O (77:23, v/v) at a flow rate of 5 mL/min to afford **17** (48 mg, t_R = 65.24 min, 34.7%).

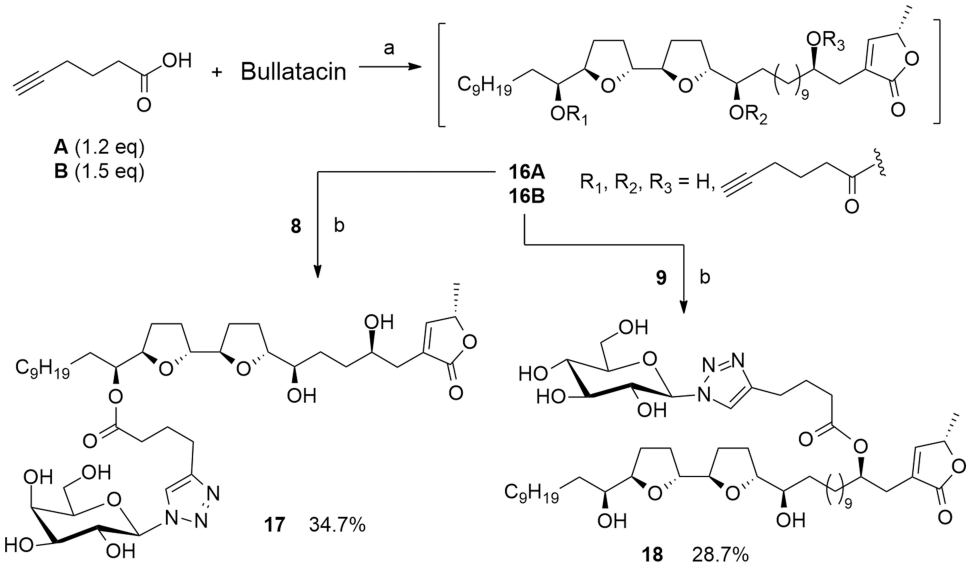
Characterization Data of Compound 17

A colorless waxy solid; $[\alpha]^{20}_D$ +20.3 (*c* 0.1, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.83 (1H, s, H-6′), 7.20 (1H, m, H-35), 5.49 (1H, m H-7′), 5.03 (1H, m, H-36), 4.86 (1H, m, H-15), 4.28 (1H, m, H-8′), 4.10 (1H, m, H-10′), 3.99 (1H, m, H-16), 3.85 (3H, m, H-19, 20, 23), 3.81 (5H, m, H-4, 9′, 11′, 12′), 3.73 (1H, m, H-24), 2.70 (2H, m, H-4′), 2.48 (1H, d, H-3a), 2.40 (1H, d, H-3b), 2.36 (2H, m, H-2′), 1.88–1.99 (6H, m), 1.77 (2H, m), 1.58 (2H, m), 1.51 (2H, m), 1.43 (3H, m), 1.39 (3H, d, *J* = 6.7 Hz, H-37), 1.20–1.33 (33H, m), 0.86 (3H, t, *J* = 7.0 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): δ 175.0 (C-1), 173.5 (C-2′), 152.3 (C-35), 147.1 (C-5′), 131.1 (C-2), 122.0 (C-6′), 88.4 (C-7′), 82.8 (C-19), 82.3 (C-20), 82.2 (C-23), 80.3 (C-16), 78.3 (C-11′), 77.9 (C-36), 74.1 (C-9′), 75.5 (C-15), 70.2 (C-8′), 70.0 (C-4), 71.7 (C-24), 69.0 (C-10′), 61.2 (C-12′), 37.5 (C-5), 33.8 (C-2′), 33.3 (C-3), 32.8 (C-25), 32.1 (C-32), 30.7 (C-14), 30.0, 29.8 (4C), 29.7 (2C), 29.6 (2C), 29.5 (2C), 28.8, 28.5, 28.2, 26.2, 25.7, 25.5, 24.9, 24.8, 24.5, 22.8 (C-33), 19.2 (C-37), 14.3 (C-34); HR-ESIMS *m/z*: 922.6013 $[M + H]^+$ (calcd for $C_{49}H_{84}N_3O_{13}$, 922.5999).

Preparation of Glucosylated Bullatacin 18

Bullatacin (87 mg, 0.14 mmol), 5-hexynoic acid (22 mg, 0.21 mmol) and DMAP (2.4 mg, 0.02 mmol) were dissolved in 3 mL dried CH_2Cl_2. DCC (86 mg, 0.42 mmol) was added and the mixture was stirred under N_2 atmosphere at room temperature for 16 h (Scheme 4). The crude product was purified in a similar way as described for the preparation of **16A** to provide the intermediate product **16B** (115 mg).

The intermediate product **16B** (115 mg) and β-D-glucopyranosyl azide **9** (82 mg, 0.42 mmol) were suspended in 3 mL MeOH. Then, $CuSO_4{\cdot}5H_2O$ (15 mg, 0.06 mmol) and sodium ascorbate (24 mg, 0.12 mmol) in 1 mL



Scheme 4 Synthesis of **17** and **18** by click chemistry. Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2, rt, N_2, 16 h. (b) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, MeOH/H_2O, 50 °C, 16 h.

of distilled water were added. The suspension was stirred at 50°C for 16 h. The mixture was concentrated and then purified by silica gel column chromatography using $CHCl_3$-MeOH (97:3, and 93:7; v/v) to afford five fractions. The product containing fraction was further separated by HPLC using MeOH-H_2O (83:17, v/v) at a flow rate of 5 mL/min to afford **18** (37 mg, t_R = 55.23 min, 28.7%).

Characterization Data of Compound 18

A yellowish waxy solid; $[\alpha]^{20}_D$ +4.5 (*c* 1.1, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.75 (1H, s, H-6′), 7.15 (1H, m, H-35), 5.58 (1H, d, *J* = 7.9 Hz, H-7′), 5.06 (1H, m, H-36), 5.00 (1H, m, H-4), 4.01 (1H, m, H-8′), 3.94 (1H, m, H-19), 3.90 (1H, m, H-20), 3.83 (5H, m, H-16, 23, 24, 12′), 3.74 (2H, m, H-9′, 10′), 3.59 (1H, m, H-11′), 3.39 (1H, m, H-15), 2.63 (2H, m, H-4′), 2.55 (1H, m, H-3a), 2.47 (1H, m, H-3b), 2.31 (2H, m, H-2′), 1.95 (3H, m), 1.88 (2H, m), 1.78 (1H, m), 1.41–1.61 (6H, m), 1.43 (3H, m), 1.32 (3H, d, *J* = 6.7 Hz, H-37), 1.20–1.33 (33H, m), 0.88 (3H, t, *J* = 7.0 Hz, H-34); ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.1 (C-1), 173.3 (C-2′), 152.2 (C-35), 147.0 (C-5′), 129.8 (C-2), 122.0 (C-6′), 87.8 (C-7′), 83.5 (C-16), 83.0 (C-20), 82.8 (C-19), 82.4 (C-23), 79.1 (C-11′), 78.1 (C-36), 76.9 (C-9′), 74.4 (C-15), 72.6 (C-8′), 72.3 (C-4), 71.6 (C-24), 69.1 (C-10′), 61.1 (C-12′), 34.1 (C-2′), 33.8 (C-14), 33.3 (C-5), 32.6 (C-25), 32.1 (C-32), 29.9, 29.8 (5C), 29.7 (2C), 29.6, 29.5 (3C), 29.1 (2C), 28.6, 26.3, 25.8, 25.4, 24.9, 24.6, 24.5, 22.9 (C-33), 19.1 (C-37), 14.3 (C-34); HR-ESIMS *m/z*: 922.6012 $[M + H]^+$ (calcd for $C_{49}H_{84}N_3O_{13}$, 922.5999).

Aqueous Solubility of Squamocin and Galactosyl Derivative 13

HPLC method with refractive index detector was employed to measure the aqueous solubility of squamocin and its galactosyl derivative **13**. Standard curve of compound **13** was found to be $y=1.45*10^6x + 8.64*10^5$ (R^2 = 0.9970) by using six gradient concentrations (10, 5, 2.5, 1.25, 0.625, and 0.3125 mM) in methanol. Excess amount of squamocin/compound **13** was added to 1 mL of phosphate-buffered saline (PBS, pH = 7.0) and sonicated for 5 min to provide saturated solution. The mixture was centrifuged (12000 rpm) for 2 min and the supernatant was analyzed by HPLC for the content of squamocin/compound **13**. The concentration of compound **13** was found to be 1.49 mM (1.37 mg/mL) in PBS (pH 7.0) at ambient temperature (around 28°C) while squamocin was not detected in PBS (pH 7.0).

Cell Culture

A549, HeLa and HepG2 cell lines were obtained from Kunming Cell Bank, Chinese Academy of Sciences. The cell lines were cultured in RPMI1640 (Invitrogen) cell culture medium supplemented with 10% FBS and 1% penicillin and streptomycin. All cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Cell Viability Assay

A549, HeLa and HepG2 cells (5×10^4/well) were seeded in flat-bottomed 96 well microplates. Then, squamocin/bullatacin and glycosylated squamocin/bullatacin derivatives dissolved in DMSO (10, 5, 2.5, 1.25, 0.625, 0.3125 μM as concentration gradient) were added to wells, respectively. The cells were incubated for 72h at 37°C in CO_2 gas incubator and then treated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (20 μL/well, 5 mg/mL). After another 4 h incubation, the medium was removed and 150 μL of DMSO was added to each well. Absorbance in each well, including the blanks, was measured at 570 nm in a microtiter plate reader after the samples were swirled gently. Experiments were repeated at least three times. Growth inhibition rate was calculated as follows: inhibition rate (%) =x{1 - [ΔOD (compound) - ΔOD (blank)]/[ΔOD (control) - ΔOD (blank)]}× 100%.

Results and Discussion

Synthesis and Structure Characterization

Queiroz et al reported the synthesis of several glycosyl derivatives of squamocin in which the squamocin scaffold was modified through either acetylation of the hydroxyl group(s) during the glycosylation reaction or reduction of the α,β-unsaturated γ-lactone moiety during the deprotection of benzyl groups on the sugar residue.[29] We aim to prepare glycosylated acetogenin derivatives wherein the acetogenin scaffold remains intact. The three hydroxyl groups in squamocin and bullatacin (Figure 1) play less important roles in their anticancer activity[13,28] and sugar residue(s) may be attached to these hydroxyl groups without affecting their activities much.

We first tried the trichloroacetimidate as the glycosylation donor. Galactose trichloroacetimidate (**3**) was readily prepared from D-galactose in three steps (Scheme 1) according to literature procedure.[30] Treatment of squamocin with imidate **3** in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) as the catalyst resulted in complicated product mixture comprising of mono- and di-

glycosylated products (as indicated by mass spectrometry data). The glycosylation yield was also found to be very low partly due to the decomposition of the imidate. The glycosylated product was isolated and treated with Et_3N-MeOH-H_2O (1:8:1, v/v) to remove the acetyl groups on the sugar.[31] Unfortunately, the γ-lactone ring of squamocin was not stable to allow for the complete removal of all acetyl groups. In the end, we managed to obtain two mono-glycosylated products **4** (glycosylated at C-15) and **5** (glycosylated at C-28) after purification by HPLC, albeit at only 1% yield. Due to the complications associated with glycosylation reaction and the removal of the acetyl protection groups on the sugar, alternative methods of attaching sugars to acetogenins are desirable.

The Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reaction, popularly known as the "click reaction", is an efficient method to covalently link two molecular entities together and has been widely used to prepare various glycoconjugates[32] and carbohydrate macrocycles alike.[33] Here we use the CuAAC reaction to conjugate glucose or galactose with squamocin/bullatacin through an alkyne-functionalized linker. Thus, β-D-galactopyranosyl azide (**8**) and β-D-glucopyranosyl azide (**9**) were prepared from the corresponding commercially available per-acetylated sugar derivatives **6** and **7** (Scheme 2). Squamocin was first treated with 5-hexynoic acid (1.2 or 1.5 eq.) in the presence of dicyclohexylcarbodiimide (DCC) and 4-*N,N*-dimethylaminopyridine (DMAP) to provide alkyne functionalized intermediate (**10A** or **10B**) after purification through Sephadex LH-20 gel permeation chromatography (Scheme 3). Treatment of **10A** with galactosyl azide **8** in the presence of $CuSO_4$ and sodium ascorbate afforded mono-galactosylated squamocin derivatives **11–13**. The 28-*O*-substituted product (**13**) was obtained in relatively higher yield, which coincided with our earlier observation that the 28-OH was most reactive among the three hydroxyl groups in squamocin.[28] Similarly, reaction of intermediate **10B** with glucosyl azide **9** afforded mono-glucosylated squamocin derivatives **14** and **15**. Following the same reaction sequence, bullatacin was first treated with 5-hexynoic acid (1.2 or 15 eq.) to give the ester intermediate (**16A** or **16B**) which was then coupled with the glycosyl azide **8** or **9** to provide the mono-glycosylated bullatacin derivative **17** or **18** in 34.7% and 28.7% yield, respectively (Scheme 4). Compound **17** is 24-*O*-substituted while **18** 4-*O*-substituted. This level of regio-selectivity is not expected for bullatacin.[28] Multiple chromatographic procedures involved in the purification of the reaction intermediate products (**16A**/**16B**) and the final products might have resulted in the loss of other minor products.

Typically, reaction products were purified by silica gel chromatography and preparative HPLC. All products were structurally confirmed by the analysis of 1D (^1H, ^{13}C and DEPT) and 2D (COSY, HSQC and HMBC) NMR spectra, and HR-ESIMS data. The selected ^1H and ^{13}C chemical shifts of the glycosylated derivatives were listed in Tables 1 and 2, respectively, and compared with those of squamocin or bullatacin. The changes in ^1H and ^{13}C chemical shifts of these glycosylated derivatives with an ester linkage (**11–15, 17** and **18**) were similar with those observed for biotinylated acetogenins we reported earlier.[28] When a secondary hydroxyl group was acylated, the chemical shift of the carbon (C-4, C-15, C-24, and C-28) bearing an oxygen atom moved downfield (Table 2). Up to 3.4–3.6 ppm downfield shift was observed for C-24 in compound **12** and compound **14** while the shift for C-15 was smaller (1.6 ppm) in compound **11**. On the other hand, the chemical shifts of the neighboring carbons (C-5, C-14, C-16, C-23, C-25, C-27, and C-29) moved upfield (in a magnitude of 1.1–4.1 ppm) upon acylation of the hydroxyl group. When a hydroxyl group was glycosylated, the carbon bearing that hydroxyl group exhibited a significant downfield shift (7.1 ppm for C-15 in **4** and 9.8 ppm for C-28 in **5**). Similarly, upfield shifts (1.9–3.2 ppm) for the neighboring carbons (C-14, C-16, C-27, and C-29) were observed as a result of direct glycosylation of that hydroxyl group (Table 2). In the case of ^1H NMR data, a significant downfield shift (>1.5 ppm) was observed for the proton directly attached to the carbon bearing the hydroxyl group that was acylated while a smaller downfield shift

Table 1 Selected ^1H NMR Data (600 MHz, $CDCl_3$) for Squamocin (**1**), Bullatacin (**2**) and Their Glycosylated Derivatives

Compound	δ_H (ppm)			
	H-4	H-15	H-24	H-28
Squamocin (**1**)[a]		3.33	3.76	3.52
4		4.03	3.74	3.57
5		3.39	3.63	3.63
11		4.83	3.59	3.52
12		3.39	5.00	3.53
13		3.39	3.75	4.88
14		3.35	4.96	3.51
15		3.37	3.78	4.86
Bullatacin (**2**)[b]	3.80	3.38	3.83	
17	3.81	4.86	3.73	
18	5.00	3.39	3.83	

Notes: [a]Data from Fujimoto et al.[34] [b]Data from Hui et al.[35]

Table 2 Selected ^{13}C NMR Data (150 MHz, $CDCl_3$) for Squamocin (**1**), Bullatacin (**2**) and Their Glycosylated Derivatives

Compound	δ_C (ppm)											
	C-3	C-4	C-5	C-14	C-15	C-16	C-23	C-24	C-25	C-27	C-28	C-29
Squamocin (**1**)[a]				33.1	74.1	83.4	82.2	71.5	32.5	37.5[c]	71.6	37.2[c]
4				30.2	81.2	81.3	82.8	71.2	32.1	37.7[c]	71.7	37.4[c]
5				33.2	74.6	83.7	82.2	71.8	32.6	35.6[c]	81.4	34.1[c]
11				30.9	75.7	80.7	82.2	72.2	33.0	37.8	71.9	37.3
12				33.1	74.7	83.7	80.9	75.1	31.2	37.8	71.4	37.1
13				32.9	74.6	84.0	82.8	71.7	32.5	34.6	74.2	34.5
14				33.1	74.4	83.6	80.8	75.1	31.3	37.7[c]	74.1	37.0[c]
15				33.0	74.4	83.6	82.2	71.5	32.4	34.4[c]	73.9	34.3[c]
Bullatacin (**2**)[b]	33.2	69.9	37.3	33.2	74.1	83.2	82.8	71.3	32.4			
17	33.3	70.0	37.5	30.7	75.5	80.3	82.2	71.7	32.8			
18	29.7	72.3	33.3	33.8	74.4	83.5	82.4	71.6	32.6			

Notes: [a]Data from Fujimoto et al.[34] [b]Data from Hui et al.[35] [c]Assignments interchangeable in the same row.

(0.1–0.7 ppm) resulted from *O*-galactosylation of that hydroxyl group (Table 1). The ^1H and ^{13}C NMR spectra of all synthesized glycosyl derivatives of squamocin and bullatacin (**4, 5, 11**–**15, 17** and **18**) are provided in the Supplementary Material of this article.

Solubility

Squamocin and bulattacin are poorly soluble in water, which is a major drawback in the further exploitation of their potential uses. The attachment of a sugar residue is expected to increase their water solubility. Squamocin derivative **13** bearing a galactose residue was suspended in the phosphate buffer saline (PBS, pH = 7.0) and the mixture sonicated for 5 min. The supernatant was analyzed by HPLC for the content of **13**. The solubility of **13** in PBS was found to be 1.37 mg/mL (1.49 mM) at ambient temperature (around 28°C). On the other hand, the solubility of squamocin in PBS was close to zero because no squamocin was detected in the supernatant of the PBS saturated with squamocin (Table 3). The data clearly showed that the attachment of one sugar residue to squamocin significantly improved its solubility in water.

Biological Evaluation

The cytotoxicity of the synthesized glycoconjugate derivatives against three tumor cell lines (Table 4) were evaluated using MTT assay. The IC_{50} values of these compounds were calculated based on three parallel experiments and presented in Table 4. Overall, when compared to squamocin (**1**) and bullatacin (**2**), the majority of these derivatives show similar level of anticancer activity against all three tumor cell lines with their IC_{50} values in μM range. Compound **15** bearing a glucose residue is slightly more active than squamocin. A few derivatives (eg, **11, 17** and **18**) show up to 8–10 times higher IC_{50} than squamocin or bullatacin against certain cell lines (HeLa or A549), suggesting that these compounds exhibit some selectivity toward certain type of cancer cells. The data also suggest that all glycosyl derivatives either via glycosidic linkage (**4** and **5**) or triazolyl-ester linkage retain the anticancer activity. Furthermore, the position at which the glycosyl residue is attached does not affect their potency much, eg, compounds **4** and **5** having similar level of potency. The glucosyl derivatives (**14, 15** and **18**) are similarly active as the galactosyl derivatives (**12, 13** and **17**).

Table 3 Solubility of Squamocin and Its Derivative **13**

Compound	Solubility (mg/mL)
Squamocin	Not detected
13	1.37

Numerous studies have been conducted to elucidate the mechanism of action of Annonaceous acetogenins (ACGs) for their potent cytotoxic activity. ACGs are potent inhibitors of mitochondrial complex I in the electron transport system, with squamocin having the lowest IC_{50} value reported for this class of compounds.[36] They are also powerful inhibitors of the NADH oxidases peculiar to the plasma membranes of cancer cells.[37] Acetogenins cause cell cycle arrest at different phases in different cancer cells and are strong apoptosis inducers. Squamocin arrested T24 bladder cancer cells at the G1 phase, enhanced caspase-3 activity, cleaved the

Table 4 Cytotoxic Activity (IC_{50}) of Glycosylated Derivatives of Squamocin and Bullatacin

Compound	IC_{50} (μM)		
	A549	HeLa	HepG2
Squamocin (**1**)	1.99 ± 0.49	0.93 ± 0.079	1.70 ± 0.14
4	6.62 ± 1.23	1.96 ± 0.23	2.93 ± 0.44
5	4.60 ± 0.69	2.24 ± 0.049	2.82 ± 0.31
11	3.88 ± 0.005	7.16 ± 1.45	1.52 ± 0.40
12	4.67 ± 0.69	3.24 ± 0.57	1.97 ± 0.18
13	2.99 ± 0.39	0.98 ± 0.13	4.09 ± 0.68
14	6.33 ± 0.96	1.56 ± 0.16	1.68 ± 0.29
15	1.80 ± 0.25	0.77± 0.0092	0.98 ± 0.042
Bullatacin (**2**)	1.74 ± 0.34	1.42 ± 0.14	2.48 ± 0.29
17	1.46 ± 0.38	13.77 ± 1.51	1.51 ± 0.09
18	15.44 ± 2.93	1.42 ± 0.12	1.13 ± 0.17

functional protein of PARP and caused cell apoptosis.[38] Squamocin also inhibited the proliferation of chronic myeloid leukemia (K562) cells via G2/M arrest in association with the induction of p21, p27 and the reduction of Cdk1 and Cdc25C kinase activities.[39] Three other THF-containing acetogenins (squamostatin A, squamocin M and corossolone) also showed potent antiproliferative activity against human nasopharyngeal carcinoma (NPC) cell lines and induced G2/M phase arrest, mitochondrial damage and apoptosis, and increased cytosolic and mitochondrial Ca^{2+} in NPCs.[40] Furthermore, Dzhemilev et al recently showed that muricadienin, a linear acetogenin containing a 1*Z*,5*Z*-diene unit, induced apoptosis in the HEK293 kidney cancer cells and exhibited a moderate inhibitory activity against topoisomerases I and IIα.[41] ACGs are versatile anticancer agents causing tumor cell death by different mechanisms. For these glycosylated acetogenins described here, more studies are required to demonstrate their cancer selectivity and mechanism(s) of action.

In conclusion, we have prepared nine glycosyl derivatives of squamocin and bullatacin and evaluated their growth inhibitory activity with three cancer cell lines, including A549, HeLa and HepG2. Most squamocin/bullatacin glycoconjugates show similar in vitro cytotoxicity against the tested cell lines as squamocin and bullatacin, respectively. Among the synthesized compounds, compound **15** displays slightly higher activity than squamocin while some other derivatives show up to 8–10 times lower activity in A549 or HeLa cell line than squamocin and bullatacin, respectively. The type of sugar residue (glucose or galactose), the position of attachment, and whether or not a linking spacer is present do not seem to affect the potency much. The solubility of galactosylated squamocin **13** in phosphate buffer saline (PBS, pH = 7) at ambient temperature is greatly improved (1.37 mg/mL) in comparison to squamocin (not detectable). Further studies are needed to show whether or not these compounds exhibit reduced toxicity to normal cells, the mechanism of action, and their therapeutic potential for cancer.

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Disclosure

The authors declare no conflicts of interest.

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