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GLYCOSYLATION OF GLAUCOCALYXIN A AND EVALUATION OF ITS CYTOTOXIC ACTIVITY

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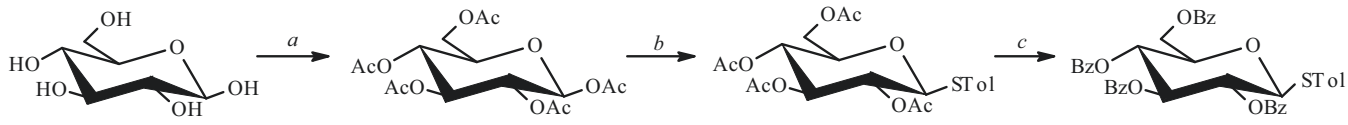
The 7,14-diglucoside of glaucocalyxin A was prepared by a five-step reaction. Its structure was confirmed by spectroscopic methods, and its cytotoxic activity was tested by the MTT method.

Keywords: glaucocalyxin A, 7,14-diglucoside of glaucocalyxin A, cytotoxic activity.

Glaucocalyxin A (GLA) [7α,14β-dihydroxy-*ent*-kaur-16-en-3,15-dione] is a natural *ent*-kaurane diterpenoid with an α-methylene cyclopentanone structure isolated from *Isodon japonica* (Burm.f) Hara var. *galaucocalyx* (Maxim.) Hara, which is especially abundant in the Northern part of P. R. China. It has been used as anticancer, antimastitis, and antiarthralgia agents in folk medicine [1]. GLA has attracted considerable attention in recent years because of its pharmacological activities such as cytotoxicity and antitumor activity [2, 3], anticoagulative activity [4], antithrombotic activity [5, 6], immune-enhancing activity [7], antineuroinflammatory activity [8], and antioxidant activity [9]. Among them, studies on its cytotoxicity is of vital significance [10] because of the prevalence of tumor diseases [11].

Carbohydrates are an essential structural component of living cells and participate in many biological activities: the immunological response, infection mechanisms, signal transduction, inammation, cell differentiation, and so on [12]. In natural products, the activities of the active ingredients are related to the existence of glucose. During the process of glycosylation, the conformation of the molecule is modified to improve its solubility and orientation and ultimately to increase its affinity and capacity as a biological receptor, such as glucose-aspirin, steviolbioside, stearylamide, and glycosylated cucurbitacins [13]. However, glycosidation of natural compounds is very difficult. At present, little research has been done on the enhancement of antitumor activity of *ent*-kaurane diterpenoid by glycosidation. In order to compare the antitumor activity of diterpenoid and its glycoside derivatives, glycosylation of glaucocalyxin A was accomplished and its cytotoxic activity examined.

p-Tolyl thio-2,3,4,6-tetra-*O*-benzoyl-3-D-glucopyranoside (**4**) was obtained by the procedure shown in Scheme 1; the percentage after the number of the substance is the yield.



1 **2**, 96% **3**, 85% **4**, 86%

a. Ac_2O; *b*. TolSH–BF_3·EtO, DCM, 0°C; *c*. 1. NaOMe–MeOH, 2. BzCl–Py–DAMP

Scheme 1

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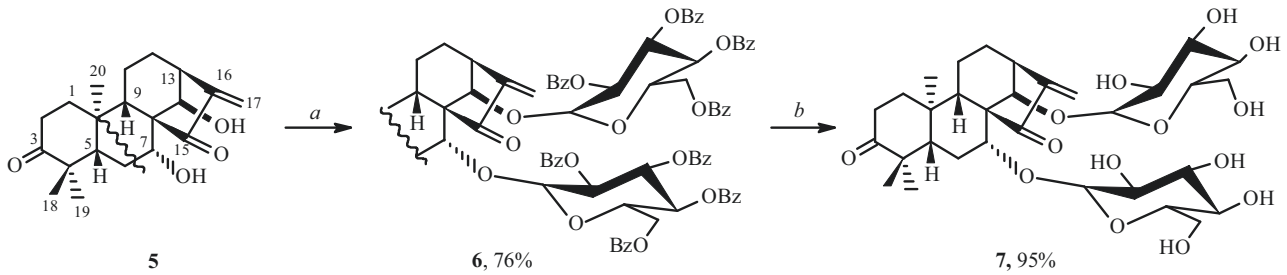
TABLE 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of **7** (DMSO-d_6, δ, ppm, J/Hz)

C atom	δ_H	δ_C	C atom	δ_H	δ_C
1	1.17 (m); 1.85 (m)	37.8 (CH_2)	18	0.93 (s)	32.3 (CH_3)
2	2.29 (m); 2.53 (m)	33.5 (CH_2)	19	1.09 (s)	21.6 (CH_3)
3	–	216.4 (C)	20	1.22 (s)	13.9 (CH_3)
4	–	46.7 (C)	7-Glc		
5	1.31 (m)	51.7 (CH)	1′	5.05 (d, J = 7.5)	103.5 (CH)
6	1.76 (m); 2.13 (m)	27.0 (CH_2)	2′	4.02 (m)	75.3 (CH)
7	4.25 (m)	79.2 (CH)	3′	4.10 (m)	77.2 (CH)
8	–	57.1 (C)	4′	3.84 (m)	72.1 (CH)
9	1.49 (m)	52.8 (CH)	5′	3.53 (m)	78.2 (CH)
10	–	38.8 (C)	6′	4.34 (dd, J = 11.5, 5.0); 4.20 (d, J = 11.5)	62.4 (CH_2)
11	1.53 (m); 1.56 (m)	18.1 (CH_2)	14-Glc		
12	1.84 (m); 1.96 (m)	30.7 (CH_2)	1″	5.18 (d, J = 7.5)	102.2 (CH)
13	3.16 (s)	43.0 (CH)	2″	4.45 (m)	75.5 (CH)
14	4.49 (s)	80.7 (CH)	3″	4.18 (m)	78.3 (CH)
15	3.57 (s)	72.3 (CH)	4″	3.95 (m)	71.8 (CH)
16	–	155.9 (C)	5″	3.60 (m)	78.0 (CH)
17	6.06 (s); 5.33 (s)	106.3 (CH_2)	6″	4.41 (m); 4.27 (d, J = 11.5)	62.1 (CH_2)

TABLE 2. Cytotoxicity of Compounds **5**, **7**

Compound	MIC, (IC_{50}, μg/mL)			
	A549	LOVO	HL-60	6T-CEM
5	3.05 ± 0.12	2.47 ± 0.11	0.88 ± 0.04	0.27 ± 0.02
7	0.75 ± 0.04	0.56 ± 0.03	0.21 ± 0.01	0.13 ± 0.01
Doxorubicin	0.036 ± 0.002	0.42 ± 0.01	0.0071 ± 0.0001	0.035 ± 0.002

Values represent means ± standard deviation of three independent experiments.



a. **4**, TfOH–NIS, –30°C, 3h; *b*. $MeNH_2$, MeOH, 0–5°C, 48h

Scheme 2

Glycosylation of GLA was carried out by the procedure shown in Scheme 2; the percentage after the number of the substance is the yield.

7,14-Diglucoside of GLA (**7**), a gray powder with optical rotation $[\alpha]_D^{20}$ –45.2° (*c* 0.2, MeOH), exhibited a quasi-molecular ion peak at *m/z* 657.3 $[M + H]^+$ by ESI-MS, and the molecular formula $C_{32}H_{48}O_{14}$ was determined by HR-ESI-MS (*m/z* 656.3033, calcd 656.3044). Compared with GLA in the NMR spectra, the two groups of glucose signals increased in the spectra of 7,14-diglucoside of GLA. The ^{1}H and ^{13}C NMR data are showed in Table 1.

Glycosylation of GLA was achieved by a five-step reaction under simple reaction conditions and gave a good yield. It has been universally regarded that an *exo*-methylene cyclopentanone or an α-methylene lactone moiety are essential groups for the cytotoxic activity of *ent*-kaurane diterpenoid. Meanwhile, the activities of active compounds are related to the existence

of glucose. A glycosylated derivative of GLA containing an *exo*-methylene cyclopentanone or an α-methylene lactone moiety was synthesized in this study for the first time.

The IC_{50} values, which are presented in Table 2, were used to determine the growth inhibition against the A549, LOVO, HL-60, and 6T-CEM human cancer cell lines. The 7,14-diglucoside of GLA exhibited better cytotoxic activity than GLA. It has been proven that glycosylation can improve the cytotoxic activity of *ent*-kaurane diterpenoid without changing the *exo*-methylene cyclopentanone moiety.

EXPERIMENTAL

General. The ^1H and ^{13}C NMR spectra were recorded on Bruker spectrometers (Bruker, Germany) operating at 500 and 125 MHz, respectively, in $CDCl_3$ or DMSO-d_6 using TMS as internal standard. The chemical shift values are reported in ppm (δ) units, and the coupling constants (J) are in Hz. ESI-MS was performed with a Mat-212 Auto Spec Q-TOF spectrometer (Agilent, USA). Optical rotations were measured with a Perkin-Elmer 341 automatic polarimeter (PE, USA). The optical density at 570 nm was taken using a Labsystems-WellscanMK-2 automated immunoanalyser (Labsystems, Finland). Gel filtration was done on Sephadex LH-20, a product of GE Healthcare Bio-Sciences AB (GE, Sweden). Silica gel for column chromatography and silica gel GF254 for TLC were obtained from Qingdao Marine Chemical Company (Qingdao, China).

All chemicals and reagents used were of analytical grade.

Synthesis of *p*-Tolyl Thio-2,3,4,6-tetra-*O*-benzoyl-3-D-glucopyranoside (4). The total acetylated glucose (**2**, 8.1 g, 20.7 mmoL) and *p*-toluenethiol (2.3 g, 20.6 mmoL) were dissolved in dichloromethane, After stirring for 2 h, boron trifluoride ether solution was added dropwise continuously for 30 min. Then, after stirring for 10 h at 0°C in dark, the reaction was transferred to an ice bath. TLC was used to monitor the reaction process. After the reaction of *p*-toluenethiol was completed, the reactant was filtered by suction, washed with sodium bicarbonate solution, dried with anhydrous sodium sulfate, filtered by suction, and concentrated by rotation; 12.2 g of the crude product is obtained.

The reaction was monitored by TLC after the methanol solution of sodium methoxide was added into the crude product and stirred for 7 h. When the acetyl group was removed, the solution was rotated and evaporated to obtain a yellow foamy substance. Then 10 mL pyridine was added, and the whole was shaken until dissolution. Then 3 mL benzoyl chloride was added, the whole stirred overnight, and the reaction process monitored by TLC. After the reaction was completed, the reaction solution was filtered by suction. The filtrate was then washed with ethyl acetate solution, 50% hydrochloric acid solution, distilled water, and saturated sodium bicarbonate solution four times until the benzoyl chloride was removed. The filtrate was then dried over anhydrous sodium sulfate. After sodium sulfate was removed by suction filtration, the filtrate was concentrated by rotary evaporation to obtain a crude product. 10.5 g of the product was obtained by silica gel chromatography.

***p*-Tolyl Thio-2,3,4,6-tetra-*O*-benzoyl-3-D-glucopyranoside (4)**, white powder. ESI-MS, *m/z* 718.77. ^1H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 8.11 (2H, d, J = 7.0), 8.04 (2H, d, J = 7.5), 7.97 (2H, d, J = 7.5), 7.89 (2H, d, J = 7.5), 7.79 (2H, d, J = 7.5), 7.64–7.22 (12H, m), 6.93 (2H, d, J = 8.0), 5.90 (1H, t, J = 9.5), 5.60 (1H, t, J = 10.0), 5.46 (1H, t, J = 10.0), 4.98 (1H, d, J = 10.0), 4.68 (1H, dd, J = 12.5, 3.0), 4.47 (1H, dd, J = 12.5, 5.5), 4.20–4.14 (1H, m), 2.27 (3H, s).

Glycosylation of GLA. GLA (**5**, 50 mg, 0.15 mmoL) and *p*-tolyl thio-2,3,4,6-tetra-*O*-benzoyl-3-D-glucopyranoside (**4**, 228 mg, 0.32 mmoL) were dissolved in dichloromethane. After 30 min, NIS (192 mg, 0.35 mmol) was added. The mixture was transferred to a low-temperature reaction bath and stirred for 30 min at 30°C in the dark; after TfOH was added dropwise, the reaction was continued for 2 h under the same condition. The crude product was obtained by rotary evaporation concentration. The crude product was dissolved in MeOH, and $MeNH_2$ was added. The mixture was kept in the refrigerator (0–5°C) for 48 h. The crude product was obtained by rotary evaporation concentration. The product **7** obtained was 72 mg by Sephadex LH-20.

MTT Assay. HL-60, 6T-CEM, LOVO, and A549 (4×10^4 cells) were plated on 96-well plates (100 µL) and maintained at 37°C in a 5% CO_2 incubator for 24 h. Then sample (7,14-diglucoside of GLA and GLA) solutions at various concentrations were added in 10 µL/well in triplicate and maintained at 37°C in a 5% CO_2 incubator for 72 h. After incubation, 20 µL of MTT (5 mg/mL) was added to each well, and the cells were incubated for 4 h. The MTT was converted to a blue formazan product by mitochondrial succinate dehydrogenase. The resulting product was solubilized by addition of 100 µL of DMSO per well. The OD values of 570 nm were determined with an MK-2 fully automatic enzyme analyzer [14]. Doxorubicin was used for comparison, and the results are shown in Table 2.

All the experiments were conducted in triplicate. The results are expressed as mean ± standard deviations. Significant differences were determined by the Duncan post hoc test at 5% confidence level using DPS (version 9.50, Jiangsu, China).

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