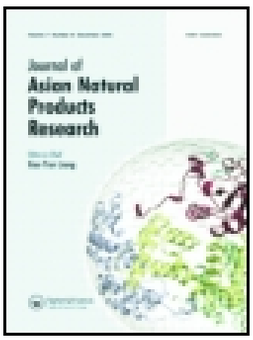

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Synthesis and *in vitro* anticancer activities of biotinylated derivatives of glaucocalyxin A and oridonin

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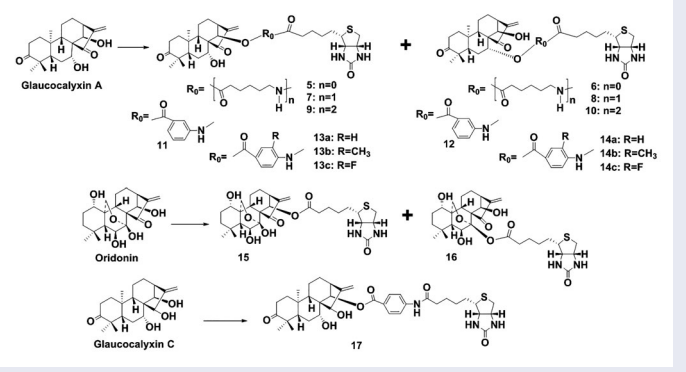
ABSTRACT

Fourteen glaucocalyxin A biotinylated derivatives, one glaucocalyxin C biotinylated derivative, and two oridonin biotinylated derivatives were designed and synthesized. Their structures were confirmed from ^{1}H NMR, ^{13}C NMR and HRMS data. The derivatives were evaluated for cytotoxic activities against lung (A549), cervical cancer cell line HeLa derivative (KB), multidrug-resistant KB subline (KB-VIN), triple-negative breast (MDA-MB-231), and estrogen receptor-positive breast (MCF-7) cancer cell lines.



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1. Introduction

Glaucocalyxin A (GLA), glaucocalyxin B (GLB) and glaucocalyxin C (GLC) are *ent*-kaurane type diterpenoids isolated and purified from the aerial parts of *Rabdosia japonica* (Burm. f.) var. *glaucocalyx* (Maxim.) Hara by us [1]. During the last decade, we performed several studies on GLA and GLB [2–7]. GLA showed various biological activities, such as cytotoxic effects, as well as inhibition of platelet activation and thrombus formation [2–6]. GLA showed strong cytotoxic effects against HL60, Jurkat, BRL, SK-BR-3, SW480, Bel-7402, SGC-7901, HUT-78 cancer cells [2, 3]. Mannich base-type derivatives of GLA were prepared and evaluated for cytotoxic activities against six tumour cell lines [5]. GLA nanosuspensions, prepared with precipitation-combined ultrasonication, showed higher antitumor efficacy against HepG2 cells and H22 bearing mice than free GLA [6]. GLA and GLB strongly decreased lipopolysaccharides (LPS) induced NO generation in BV-2 microglia cells; furthermore, GLB exerted anti-inflammatory effects mainly by targeting p38 mitogen-activated protein kinase, nuclear factor-κB and the heme oxygenase (HO)-1 signaling pathway [7]. Studies by other researchers on GLA revealed the following findings: GLA γ-cyclodextrin clathrate improved the water solubility and anticancer activity of GLA; GLA induced G2/M cell cycle arrest and apoptosis through the PI3K/Akt pathway in human bladder cancer cells; GLA reversed EMT and TGF-β1-induced EMT by inhibiting TGF-β1/Smad2/3 signaling pathway in osteosarcoma [8–10]. Oridonin is a related highly oxygenated 7, 20-epoxy-*ent*-kaurane type diterpenoid, and its derivatives also exhibited strong antitumor activities [11–14]. However, the mechanisms underlying GLA and oridonin induced cytotoxicities remain unclear.

Biotin (vitamin H or B-7) promotes growth at the cellular level and is found in substantially higher levels in tumors than in normal tissues [15]. Also, biotin receptors are over expressed in many cancer cells, such as L1210 FR, A549, and MCF-7 [16, 17]. Consequently, biotin receptors could be explored for targeted delivery of biotinylated derivatives of cytotoxic drugs to cancer cells and the biotin-avidin labeling method could be used to study the anti-tumor mechanism.

In this study, we designed and synthesized biotinylated derivatives of GLA, GLC and oridonin in order to find the biomarkers which could be used to study the anti-tumor mechanism. The cytotoxic activities of these derivatives against lung (A549), cervical cancer cell line HeLa derivative (KB), multidrug-resistant KB subline (KB-VIN), triple-negative breast (MDA-MB-231), and estrogen receptor-positive breast (MCF-7) cancer cell lines were evaluated. In addition, the stabilities of biotinylated GLA in trichloromethane ($CHCl_3$), methanol (MeOH) and dimethyl sulfoxide (DMSO) were analysed by high performance liquid chromatography (HPLC).

2. Results and discussion

2.1. Synthesis of biotin derivatives

Following literature methods [18, 19], biotin was linked to one and two units of 6-aminocaproic acid as well as to various aminobenzoic acids to give six biotin extended-chain reactants (**1-4c**, Figure 1). Then, GLA-14-biotin derivatives (**5**, **7**, **9**,

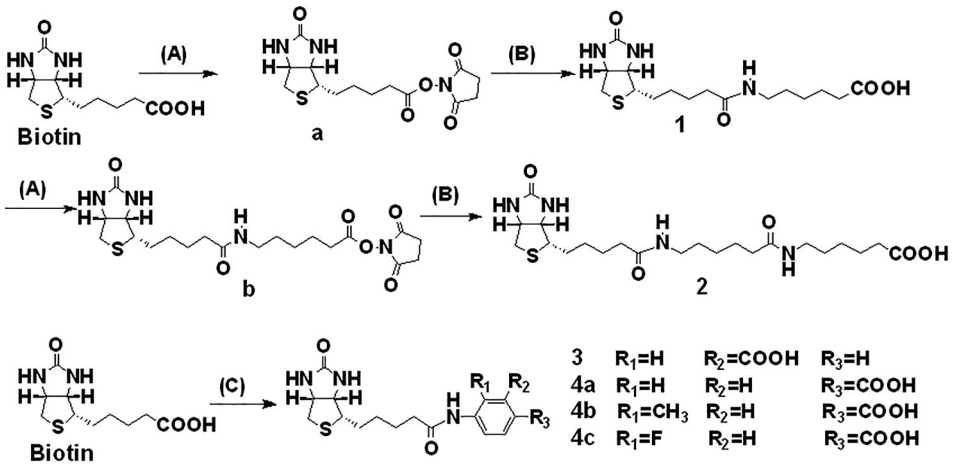

Figure 1. Synthesis of biotin derivatives **1-4c**. Reagent and conditions: (A) DMF, NHS, DCC, DMAP at room temperature, overnight. (B) 6-Aminocaproic acid, DMF, Triethylamine, overnight. (C) a: Tributylamine, DMF, Isobutyl chloroformate, at room temperature; b: 3-Aminobenzoic acid or 4-Aminobenzoic acid or 4-Amino-3-methylbenzoic acid or 4-Amino-3-fluorobenzoic acid, 0 °C, 2 h.

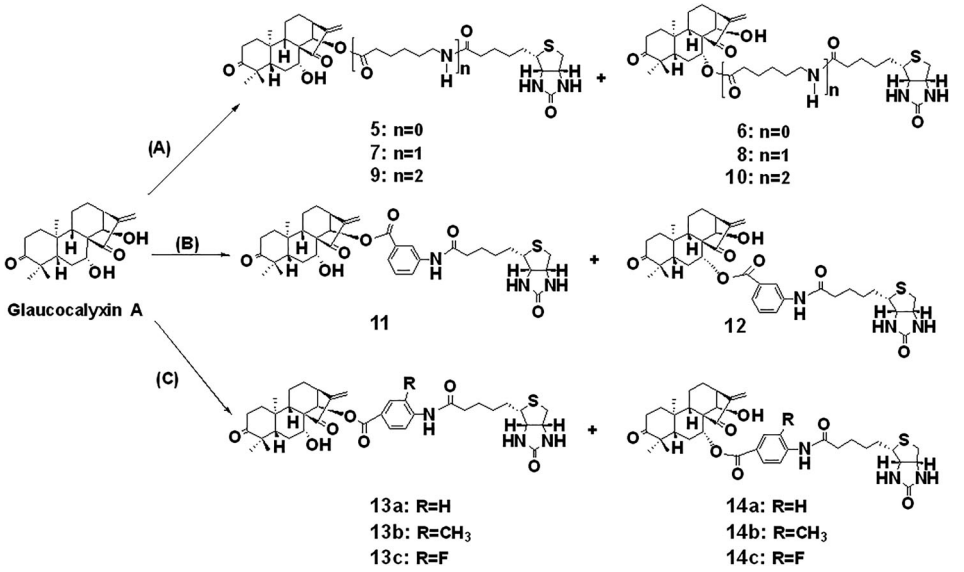

Figure 2. Synthesis of GLA biotin derivatives **5-14c**. Reagent and conditions: (A) GLA, DMF, biotin or compound 1 or 2, EDCI, DMAP, at room temperature, 24 h; (B) GLA, DMF, compound 3, EDCI, DMAP, at room temperature, 24 h; (C) GLA, DMF, compound 4a or 4 b or 4c, EDCI, DMAP, at room temperature, 24 h.

11, **13a-c**, Figure 2) and GLA-7-biotin derivatives (**6**, **8**, **10**, **12**, **14a-c**, Figure 2), as well as oridonin-7-biotin, oridonin-14-biotin and GLC-14-biotin derivative (**15-17**, Figure 3) were obtained by esterification between the kaurene diterpenoid and biotin or **1-4c**. The products were purified by extraction and column chromatography; their structures were confirmed from NMR and HRMS data (supplemental material).

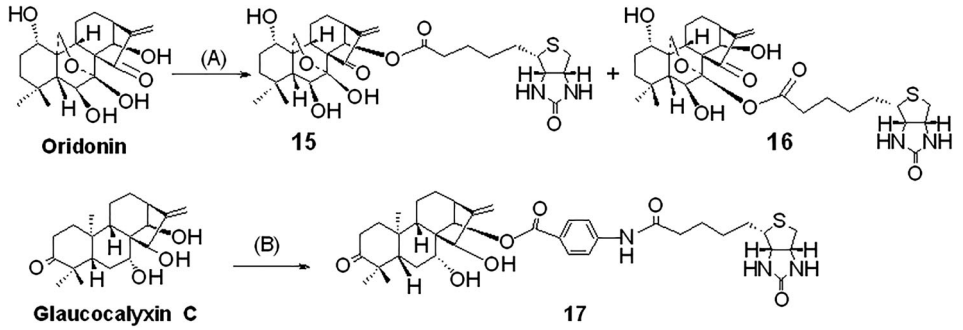

Figure 3. Synthesis of oridonin biotin derivatives and GLC biotin derivative. Reagent and conditions: (A) oridonin, DMF, biotin, EDCI, DMAP, at room temperature, 24 h; (B) GLC, DMF, compound 4a, EDCI, DMAP, at room temperature, 24 h.

Among the 14 new biotin-extended-chain-linked GLA biotin markers, the products of the reactions of GLA with biotin or 6-(biotinylamido)caproic acid were obtained in significantly higher yields than those of the reactions between GLA and biotinylamido benzoic acids. This phenomenon might be explained by steric hindrance. The esterification reactions between GLA and the (biotinylamido)benzoic acids were difficult and, thus, the yields of the target products were relatively low.

Esterification could occur at either the 7- or 14-hydroxyl group of GLA, and the GLA-14-biotin derivatives were generally obtained in higher yields compared with the GLA-7-biotin derivatives. Hydrogen bonding between the 7-hydroxyl and the 15-carbonyl group of GLA might hinder the esterification between the hydroxyl group of the diterpenoid and the carboxylic acid of the biotin reactants. Similarly, the yield of the oridonin-14-biotin derivative (**15**) was significantly higher than that of the oridonin-7-biotin derivative (**16**). In this reaction, LC-MS revealed two additional products; these possible di-esterified compounds were formed in only small amounts and were not isolated. The reaction between GLC and 4-(biotinylamido)benzoic acid led to a complex mixture; only the main product (**17**) from esterification of the 14-hydroxyl group was isolated.

2.2. The stability of biotinylated GLA

GLA-7-biotin (**6**) and GLA-14-biotin (**5**) were most stable in DMSO, slightly less stable in MeOH, and least stable in $CHCl_3$. GLA-14-biotin (**5**) was more stable than GLA-7-biotin (**6**) in these solvents, as shown in Figure 4. GLA-biotin was stable in DMSO and MeOH, presumably due to strong hydrogen bond between the compound and the solvent. On the other hand, the lower stability in $CHCl_3$ might result from acyl group transfer. GLA-7-biotin and GLA-14-biotin could interconvert to reach equilibrium. We speculated that the 7-hydroxyl group, at a β-position to the carbonyl group at C-15 of GLA-biotin, might show an inductive effect in the Lewis acid $CHCl_3$. Similar phenomena occurred between oridonin-7-biotin (**16**) and oridonin-14-biotin (**15**). However, the GLC-14-biotin derivative (**17**) was stable in all three solvents. The only structural difference between GLA and GLC is the presence of a

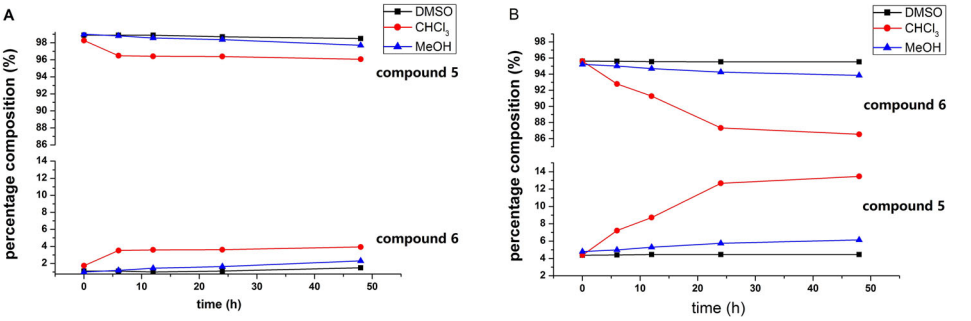

Figure 4. Time course for compounds **5** and **6** in different solutions. (A) compound **5** in different solutions (B) compound **6** in different solutions.

carbonyl group at C-15 in GLA rather than a hydroxyl group at the same position in GLC.

2.3. The anticancer activities of biotinylated derivatives in vitro

The target compounds were evaluated for their activities against A549, MCF-7, KB, MDA-MB-231, KB-VIN cancer cell lines [20]. As shown in Table 1, the cytotoxic potencies of GLA-biotinylated derivatives **5** and **6** were similar. Among the five tested human cancer cell lines, MDA-MB-231 was the most sensitive. All IC_{50} values of the remaining tested compounds (**7 – 10**, **13 b**, **13c**, **14a**, and **14 b**) were greater than 10 μM. Although compounds **5** and **6** were less potent than GLA, they could be used as biomarkers for studying the anti-tumor mechanism of GLA especially in MDA-MB-231 cells using a biotin-avidin labeling method.

The methylenecyclopentanone moiety in GLA is certainly an essential cytotoxic pharmacophore. The biotin esters attached to hydroxyl groups at positions 7 or 14 would likely hinder binding of methylenecyclopentanone to corresponding protein receptors; thus, the cytotoxic potencies of all tested GLA-biotinylated derivatives were lower than that of GLA itself.

3. Experimental

3.1. General experimental procedures

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian UNITY INOVA - 600 with tetramethylsilane (TMS) as an internal standard. High resolution electrospray ionisation mass (HR-ESI-MS) spectra were recorded on Agilent 6520 B Q-TOF spectrometer (Agilent Technologies, Santa Clara, CA, USA). Silica gel (200-300 mesh, Qingdao Marine Chemical Co., Qingdao, China) and RP-C18 (50-70 μm, Daiso, Japan) were used for column chromatography.

3.2. General procedure for the synthesis of biotin derivatives

A solution of Biotin (488 mg, 2.0 mmol, 1.0 equiv) in N, N-dimethylformamide (DMF, 20 ml) was added N-hydroxysuccinimide (NHS, 276 mg, 2.4 mmol, 1.2 equiv)

Table 1. The anticancer activities of biotinylated derivatives *in vitro*.

Compound	tumor cells, IC_{50}, μM				
	A549	KB	KB-VIN	MDA-MB-231	MCF-7
GLA	2.063 ± 0.350	4.582 ± 0.993	2.807 ± 1.162	1.417 ± 0.252	4.490 ± 1.516
5	10.267 ± 0.041	11.991 ± 1.647	13.494 ± 1.689	6.077 ± 0.420	10.993 ± 0.146
6	8.705 ± 1.322	10.480 ± 1.647	10.196 ± 0.110	6.074 ± 0.217	10.196 ± 0.705
7	>10	>10	>10	>10	>10
8	>10	>10	>10	>10	>10
9	>10	>10	>10	>10	>10
10	>10	>10	>10	>10	>10
14a	>10	>10	>10	>10	>10
13b	>10	>10	>10	>10	>10
14b	>10	>10	>10	>10	>10
13c	>10	>10	>10	>10	>10
taxol	0.924 ± 0.063	1.372 ± 0.016	1.241 ± 0.028	7.246 ± 0.913	8.231 ± 1.006

and dicyclohexylcarbodiimide (DCC, 824 mg, 4.0 mmol, 2.0 equiv) and 4-dimethylaminopyridine (DMAP, 610 mg, 5.0 mmol, 2.5 equiv) at room temperature. The solution was stirred overnight, and then the solvent was removed under reduced pressure. The residue was dissolved in isopropanol (40 ml) with ultrasonic. The solution was cool to 4 °C and stood for 1 h. The resulted mixture was filtered to afford intermediate a. Intermediate a (341 mg, 1.0 mmol, 1.0 equiv) and 6-aminocaproic acid (157 mg, 1.2 mmol, 1.2 equiv) were dissolved in 20 ml DMF. Triethylamine (0.3 ml, 2.16 mmol, 2.16 equiv) was added. The solution was stirred overnight, and then the solvent was removed under reduced pressure. The residue was dissolved in water (25 ml) and formic acid (2 ml) with ultrasonic. The solution was cooled to 4 °C. The resulted mixture was filtered to afford compound **1**. Intermediate b was obtained according to the similar procedure of preparing intermediate a staring from **1**. Compound **2** was obtained according to the similar procedure of preparing compound **1** staring from intermediate b. To a solution of biotin (400 mg, 2.0 mmol, 1.0 equiv) and tributylamine (0.64 ml, 2.7 mmol, 1.3 equiv) in DMF (40 ml) was added isobutyl chloroformate (0.32 ml, 2.5 mmol, 1.2 equiv) at room temperature. The reaction mixture was stirred 10 min, and then 3-aminobenzoic acid (548 mg, 4.0 mmol, 2.0 equiv) in 40 ml DMF was added slowly to the mixture at 0 °C and left to stir for 2 h. The solvent was removed under reduced pressure. The residue was dissolved in warm ethanol solution (50%, 36 ml). The pH of the mixture was adjusted to 2.0 by hydrochloric acid. The mixture was cooled to 0 °C and stood for 12 h. The resulted mixture was filtered to give compound **3**. Compounds **4a, 4 b** and **4c** were obtained according to the similar procedure of preparing **3** starting from 4-aminobenzoic acid, 4-amino-3-methylbenzoic acid and 4-amino-3-fluorobenzoic acid. The biotin derivatives were all white solids. Compound **1**, 270 mg, yield 75.6%. Compound **2**, 340 mg, yield 72.2%. Compound **3**, 465 mg, yield 62.4%. Compound **4a**, 483 mg, yield 64.9%. Compound **4 b**, 600 mg, yield 77.6%. Compound **4c**, 465 mg, yield 62.4%.

Compound **1**, a white powder, 270 mg, yield 75.6%, HRESIMS: *m/z* 358.1791 $[M+H]^+$ (calcd for $C_{16}H_{28}N_3O_4S$, 358.1756). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 7.71 (1 H, s), 6.39 (1 H, s), 6.33 (1 H, s), 4.29 (1 H, t, $J = 6.4$ Hz), 4.10-4.11 (1 H, m), 3.06-3.08 (1 H, m), 3.00 (2 H, dd, $J = 12.5$, 6.3 Hz), 2.81 (1 H, dd, $J = 12.4$, 4.9 Hz), 2.56 (1 H, d, $J = 12.4$ Hz), 2.18 (2 H, t, $J = 7.3$ Hz), 2.03 (2 H, t, $J = 7.3$ Hz), 1.66-1.22 (12 H,

m). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 174.9, 172.2, 163.1, 61.5, 59.6, 55.9, 38.7, 35.6, 34.1, 29.3, 28.7, 28.5, 26.4, 25.8, 24.7.

3.3. General procedure for the synthesis of GLA-biotin derivatives

A solution of glaucocalyxin A (332 mg, 1.0 mmol, 1.0 equiv) in DMF (20 ml) was added biotin (293 mg, 1.2 mmol, 1.2 equiv) and 1-ethyl-3(3-dimethylpropylamine) carbodiimide (EDCI, 383 mg, 2.0 mmol, 2.0 equiv) and DMAP (122 mg, 1.0 mmol, 1.0 equiv) at room temperature. The reaction mixture was stirred at room temperature for 24 h. The reaction mixture was then diluted with water and extracted with ethyl acetate. The extract was washed with saturated NaCl (aqueous) solution, dried over anhydrous Na_2SO_4, filtered, and evaporated to give a white solid residue. The crude residue was purified by silica gel column, eluted with EtOAc/MeOH (12:1, v/v) to afford a mixture. Then the crude residue was further purified by preparative reversed phase liquid chromatography, elution with MeOH-H_2O (70:30, v/v) at 3 ml/min to afford the desired compounds **5** and **6**. Compounds **7, 8, 9, 10, 11, 12, 13a, 14a, 13 b**, **14 b**,**13c** and **14c** were obtained according to the similar procedure of preparing **5** and **6** starting from **1, 2, 3**, **4a**, **4 b** and **4c**. GLA-biotin derivatives were all white solids. Compound **5**, 280 mg, yield 50.2%. Compound **6**, 101 mg, yield 18.1%. Compound **7**, 251 mg, yield 18.0%. Compound **8**, 121 mg, yield 37.4%. Compound **9**, 110 mg, yield 13.4%. Compound **10**, 105 mg, yield 14.0%. Compound **11**, 94 mg, yield 13.9%. Compound **12**, 30 mg, yield 4.4%. Compound **13a**, 102 mg, yield 15.1%. Compound **14a**, 65 mg, yield 9.6%. Compound **13 b**, 169 mg, yield 21.0%. Compound **14 b**, 45 mg, yield 6.5%. Compound **13c**, 96 mg, yield 13.8%. Compound **14c**, 64 mg, yield 9.2%.

Compound **5**: a white powder, 280 mg, yield 50.2%. HRESIMS: *m/z* 559.2843 $[M+H]^+$ (calcd for $C_{30}H_{43}N_2O_6S$, 559.2842). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 6.40 (1 H, s), 6.35 (1 H, s), 5.96 (1 H, s), 5.85 (1 H, s), 5.46 (1 H, s), 4.41 (1 H, d, $J=4.3$ Hz), 4.31 (1 H, t, $J=5.8$ Hz), 4.10-4.13 (2 H, m), 3.93-3.95 (1 H, m), 3.08-3.10 (1 H, m), 3.03 (1 H, s), 2.83 (1 H, dd, $J=12.4$, 5.1 Hz), 2.58 (1 H, d, $J=12.4$ Hz), 2.47-2.49 (1 H, m), 2.41-2.43 (1 H, m), 1.12 (3 H, s), 1.01 (3 H, s), 0.96 (3 H, s). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 216.1, 205.5, 172.6, 162.7, 147.0, 116.3, 74.2, 70.9, 61.3, 61.0, 59.2, 55.4, 53.9, 50.6, 48.6, 46.1, 43.7, 39.8, 38.4, 37.3, 33.8, 33.4, 31.9, 28.9, 28.0, 26.9, 24.0, 20.7, 17.8, 17.5.

3.4. General procedure for the synthesis of oridonin-biotin derivatives

Compounds **15** and **16** were obtained according to the similar procedure of preparing **5** and **6** starting from oridonin. Compound **15**, a white solid, 212 mg, yield 35.9%. Compound **16**, a white solid, 78 mg, yield 13.2%.

Compound 15: a white powder, 212 mg, yield 35.9%. HRESIMS: *m/z* 591.2715 $[M+H]^+$ (calcd for $C_{30}H_{43}N_2O_8S$, 591.2695). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 6.38 (1 H, s), 6.33 (1 H, s), 5.98 (1 H, s), 5.88 (1 H, d, $J=10.3$ Hz), 5.82 (1 H, s), 5.82 (1 H, s), 5.59 (1 H, s), 4.38 (1 H, d, $J=4.9$ Hz), 4.11 (1 H, t, $J=4.9$ Hz), 4.06 (1 H, d, $J=10.2$ Hz), 3.82 (1 H, d, $J=10.2$ Hz), 3.50 (1 H, dd, $J=10.2$, 7.0 Hz), 3.05-3.07 (1 H,

m), 2.95 (1 H, d, $J = 9.7$ Hz), 2.81 (1 H, dd, $J = 12.5$, 5.1 Hz), 2.56 (1 H, d, $J = 12.4$ Hz), 0.98 (3 H, s), 0.97 (3 H, s). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 207.6, 172.0, 163.2, 151.3, 119.8, 96.2, 74.5, 73.6, 72.0, 62.9, 62.2, 61.5, 59.6, 59.5, 55.8, 54.3, 41.8, 40.9, 40.3, 38.8, 34.2, 33.8, 33.2, 30.7, 29.8, 28.4 × 2, 24.5, 22.1, 20.2.

3.5. General procedure for the synthesis of GLC-biotin derivative

Compound **17** was obtained according to the similar procedure of preparing **5** and **6** starting from **GLC** and **4a**. 7-substitued compound was not found in this reaction.

Compound **17**: a white powder, 51 mg, yield 8.9%. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.2 (1 H, s), 7.94 (2 H, d, $J = 8.7$ Hz), 7.68 (2 H, d, $J = 8.7$ Hz), 6.42 (1 H, s), 6.35 (1 H, s), 5.20 (1 H, d, $J = 7.2$ Hz), 5.00 (1 H, s), 4.88 (1 H, s), 4.51 (1 H, s), 4.41 (1 H, s), 4.34 (1 H, s), 4.28 (1 H, t, $J = 7.4$ Hz), 4.10-4.12 (1 H, m), 3.07-3.09 (1 H, m), 2.81 (1 H, dd, $J = 12.4$, 5.0 Hz), 2.60-2.62 (1 H, m), 2.31-2.33 (2 H, m), 1.01 (3 H, s), 0.92 (6 H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 216.8, 171.8, 165.2, 162.8, 157.4, 143.6, 130.6 × 2, 124.5, 118.2 × 2, 106.2, 75.7, 73.7, 71.2, 61.1, 59.2, 55.5, 54.1, 50.7, 48.8, 47.1, 45.9, 40.5, 38.5, 37.8, 36.3, 33.5, 32.8, 28.3, 28.1, 27.6, 25.0, 20.6, 18.6, 17.6.

3.6. The stability of biotinylated GLA

HPLC analyses were performed on an Agilent 1260 HPLC system equipped with a photodiode array detector (DAD). A 100-5C18 column (250 mm × 4.6 mm, 5 μm, Kromasil) was used. The mobile phase was methanol and water (65: 35, v/v). The flow rate was 1 ml/min. Monitoring was performed at 230 nm and the injection volume was 20 μl. The stabilities of compounds **5** and **6** in different solvents (DMSO, MeOH, $CHCl_3$) were studied at room temperature (25 °C). At different time intervals, the remaining compound was measured according to the above-mentioned HPLC method.

3.7. Cytotoxic activity assay

Cytotoxic activity was determined by the sulforhodamine B (SRB) colorimetric assay. In brief, the cells (4-11 × 103 cells/well) were seeded in 96-well plates filled with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) containing various concentrations of samples, and incubated for 72 h. At the end of the exposure period, the attached cells were fixed with cold 50% trichloroacetic acid for 30 min followed by staining with 0.04% SRB (Sigma Chemical Co.) for 30 min. The bound SRB was solubilized in 10 mM Tris-base and the absorbance was measured at 515 nm on a Microplate Reader ELx800 (Bio-Tek Instruments, Winooski, VT) with a Gen5 software. All results were representative of three or more experiments and IC_{50} is expressed as the average with standard deviation.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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