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Synthesis of a targeting drug for antifibrosis of liver; A conjugate for delivering glycyrrhetin to hepatic stellate cells

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The neoglycoproteins that consist of human serum albumin (HSA) modified with mannose 6-phosphate ([M6P]*x*-HSA) were synthesized, and they showed high binding property to hepatic stellate cells (HSC) by immunohistochemical analysis. In addition, an increased substitution (X) of 6-phosphated mannose (M6P) was associated with an increased accumulation in HSC. So the [M6P]*x*-HSA might be a carrier to deliver drugs to HSC. The antifibrotic drug, glycyrrhetin, was chosen to conjugate to $M6P_{26}$-HSA. The result suggests there were 6∼7 glycyrrhetin molecules having been conjugated to the carrier. Targeting glycyrrhetin to HSC might reduce its adverse affects and increase the efficacy.
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Keywords: **hepatic stellate cells, antifibrosis, mannose 6-phosphate, glycyrrhetin, neoglycoprotein**

Introduction

Inflammatory processes are involved in a large number of liver diseases and may lead to liver fibrosis and cirrhosis [1–3]. The hepatic stellate cells (HSC) play an important role in the initiation and propagation of inflammatory reactions. HSC may cause an increased extracellar matrix deposition. So this cell type is an important target for pharmacotherapeutic intervention [4,5]. However, antifibrotic drugs may not be efficiently taken up by HSC or may produce unwanted side effects outside the liver. Drug targeting may be exploited to elicit cell-specific uptake of drugs. To obtain a cell-specific drug delivery to HSC, relevant target receptors should be identified. The mannose 6-phosphate/insulin-like growth factor II (M6P/IGF-II) receptor is expressed in particular upon HSC during fibrosis. So, we want to examine whether the M6P/IGF-II receptor could serve as a target for specific HSC targeting [6–8]. We synthesized human serum albumin (HSA) modified with mannose 6-phosphate (M6P) and determined whether this neoglycoprotein (M6P-HSA) was taken up by HSC.

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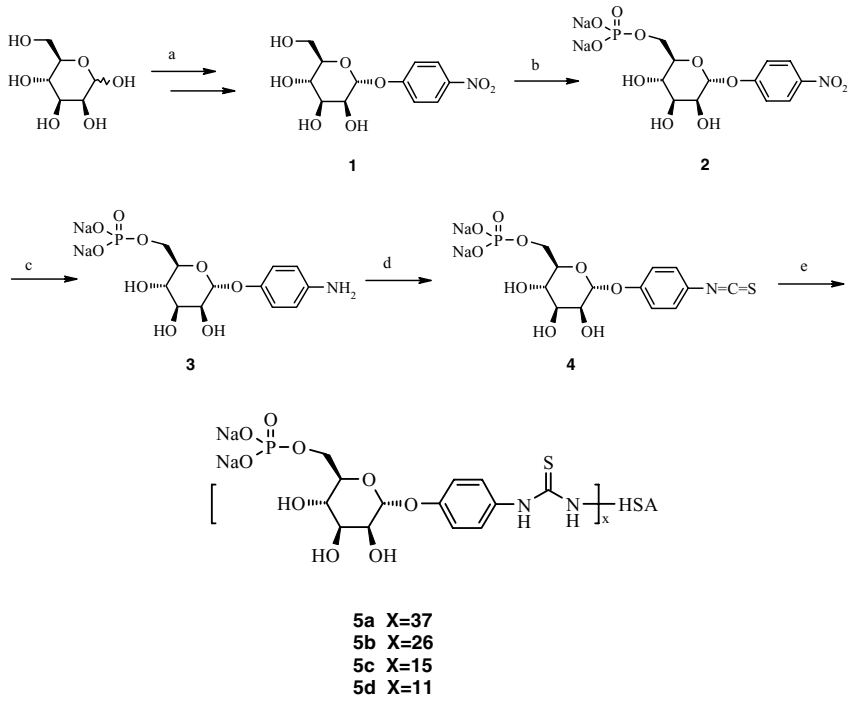
Glycyrrhetin was extracted from a traditional Chinese herb *Glycyrrhiza Uralensis Fisch*. Its preparation of complex prescription has been used to treat chronic hepatitis in the clinic. Glycyrrhetin treats the rat experimental hepatic fibrosis by inhibiting the initiation and propagation of HSC on the cellular level [9,10], inhibiting the activity of nuclear factor (NF-κB), and transforming growth factor β (TGF-β). But in the clinic, its application is limited because of its adrenocortical hormone-like action. So if we use the M6P-HSA as carrier for delivering glycyrrhetin to HSC, the drug concentration on the receptor of HSC will increase; and at the same time, the distribution in other organs will decrease. It will provide a new method for treating hepatic fibrosis in the clinic.

Results and discussion

Synthesis of $[M6P]_X$-HSA (**5a–5d**)

The neoglycoprotein (**5a–5d**) was synthesized as shown in Scheme 1. Compound **1** was synthesized in three steps as previously described for mannose. Compound **1** was phosphorylated according to Roche et al. [11]. Product **2** was identified with ^{13}C NMR and ^{31}P NMR. The signal corresponding to C-6 in **2** was shified 2.1 ppm downfield from that in **1**, and peaks



5a X=37
5b X=26
5c X=15
5d X=11

Scheme 1. (a) (1) Ac_2O, Py; (2) *p*-nitrophenol, $ZnCl_2$; (3) NaOMe/MeOH, 26% over three steps; (b) $POCl_3$, Py, H_2O, CH_3CN, 63.7%; (c) (1) Pd/C, H_2, 76.9%; (2) Dowex 50 X2 (H^+); (d) $CSCl_2$, 2:1 EtOH/H_2O; (e) HSA, borate buffer, pH 9.0, 49.2% over two steps.

assigned to C-5 and C-6 were doublets, with values for coupling constants of 8.6 and 4.8 Hz, respectively. The signal of ^{31}P was a singlet at 2.20 ppm, which suggested only one phosphoryl coupled to the primary alcohol. Subsequently, the nitro group was reduced with 10% Pd/C under hydrogen to give compound **3**. The p-aminophenylglycoside **3** then reacted with thiophosgene leading to compound **4** as shown by TLC, which gave the very characteristic broad band centered at 2125 cm^{-1} in the infrared region. Then compound **4** was reacted with HSA to give neoglycoproteins ($[M6P]_X$-HSA). By varying the molar ratio of HSA and **3**, we obtained M6P-HSA with a different degree of substitution (X).

The reaction and results are shown in Table 1. The more the M6P was coupled to HSA, the higher was the molecular weight as reflected by the decreased retention time after size exclusion chromatography (Figure 1).

Conjugation of glycyrrhetin to compound **5b** to target HSC

Drugs can be coupled to the carrier either directly or through a spacer. The bond between the carrier and the drug has to be stable in plasma but biodegradable when the drug has reached the target site [12,13]. We chose glycine or glycyl-glycine as spacer, because the peptide spacers are susceptible to degradation by endopeptidases, which either disrupt the bond between the spacer and drug molecular or, alternatively, the internal peptide bonds of the spacer. As shown in Scheme 2, glycine was coupled to glycyrrhetin by DCC (dicyclohexylcarbodiimide) [14]. The deprotected products **11**,**12** were then coupled to $[M6P]_{26}$-HSA (**5b**) using the mixed anhydride method [15] to give conjugates **13**,**14**. We determined the molecular weight and purity of the conjugate by SDS-PAGE (Figure 2). The molecular weights of **13** and **14** are about 73.8 and 74.4 kDa, respectively.

Table 1. Characterization of the different preparation of neoglycoproteins with an increase in the molar ratio of M6P/HSA

Compound	*Sugar/protein ratio in the reaction mixture (mol/mol)*	*Yield (%)*	*Sugar/protein ratio in the product (x) (mol/mol)*	*Concentration of protein (%)*	*HPLC*[1] t_R *(min)*
5a	280	49.2	36.7	62.7	11.899
5b	180	56.3	25.5	66.7	11.990
5c	120	60.9	15.2	70.6	12.009
5d	53	69.7	11.1	77.7	12.021

[1] t_R of HSA: 12.085 min.

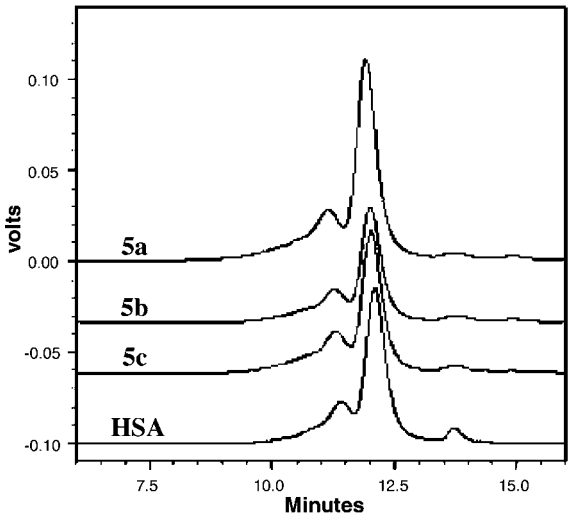


Figure 1. HPLC of compounds **5a–5c** and HSA on a Bio-Sep-SEC-S4000 column (300 × 7.80 mm) in 0.1 M phosphate buffer, pH 7.4 at a flow rate of 1 mL/min.

So about 6–7 glycyrrhetin molecules (*n*) were conjugated to the carrier.

Immunohistochemical detection of M6P-HSA

Immunohistochemical analysis showed that $[M6P]_{37}$-HSA (**5a**) and $[M6P]_{26}$-HSA (**5b**) were taken up more efficiently by HSC than $[M6P]_{15}$-HSA (**5c**) (Figure 3). So we conclude that the more the M6P was linked to HSA, the greater the efficiency of the carrier would be for drug delivery to HSC.

Experimental procedures

Materials

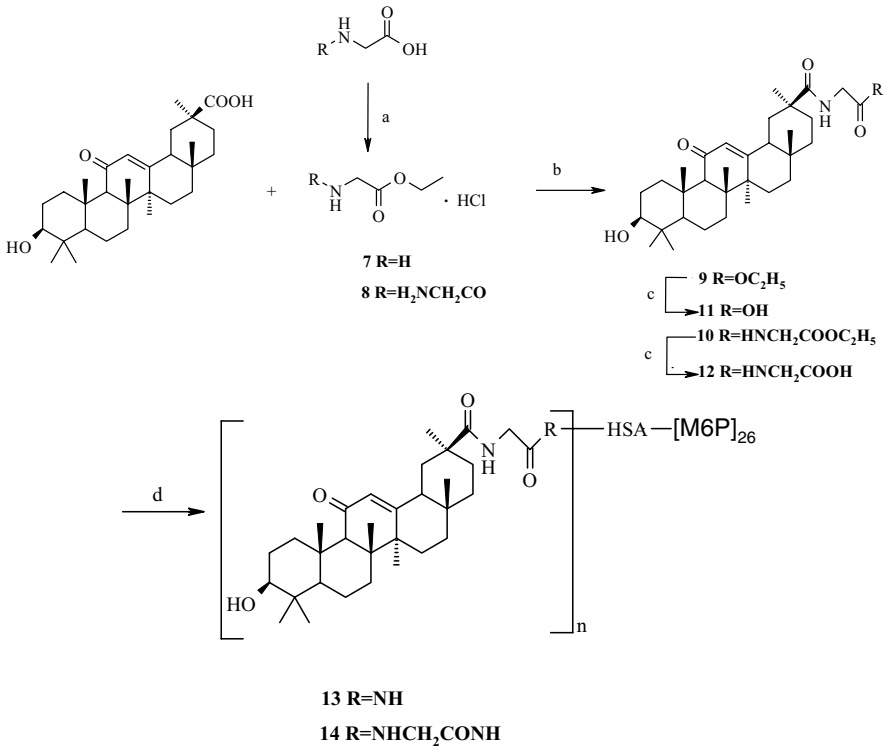
^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 spectrometer (300 and 75.5 MHz, respectively). Thin-layer chromatography (TLC): E. Merck silica gel 60 F254, and compounds were detected under UV or by spraying 5% Concd. H_2SO_4 in MeOH and then by heating the plates. Silica gel 60 was used for flash chromatography (230–400 mesh). HPLC analysis was performed on a Biosep-SEC-S4000 column (300 × 7.80 mm) with 0.1 M phosphate buffer (pH 7.4), 1 mL/min as mobile phase.

*p-nitrophenyl-α-*D*-mannopyranoside (**1**)*

Compound **1** was synthesized as in [16]. Yield 26.0% (Three steps). ^{1}H NMR (D_2O): δ 8.08 (d, 2H, J = 7.5 Hz, H-meta), 7.13 (d, 2H, H-ortho), 5.61 (s, 1H, H-1), 3.48–4.06 (m, 6H, H-2, H-3, H-4, H-5, H-6a, H-6b). ^{13}C NMR (D_2O): δ 163.6, 145.0, 128.7, 119.4, 100.5 (C-1), 76.5 (C-5), 73.0 (C-2), 72.4 (C-3), 69.2 (C-4), 63.4 (C-6).

*6-phospho-p-nitrophenyl-α-*D*-mannopyranoside (**2**)*

p-nitrophenyl-α-D-mannopyranoside **1** (2.1 g, 7.0 mmol) was dissolved in pyridine (2.8 mL, 35.0 mmol), acetonitrile (7 mL,



Scheme 2. (a) EtOH, $SOCl_2$; (b) DCC, CH_2Cl_2; (c) KOH, 3:1 MeOH/H_2O; (d) (1) C_2H_5OCOCl, Et_3N, dioxane; (2) **5b**, 1:1 dioxane/H_2O, 1N NaOH.

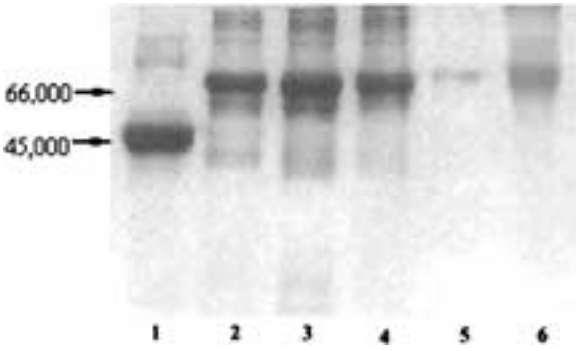


Figure 2. SDS-PAGE analysis of compounds **5b, 13, 14**. Molecular weight markers were indicated on line 1–line 3. line 1, Mw = 45000; line 2, BSA, Mw = 66000; line 3, HSA, Mw = 66270; line 4, $[M6P]_{26}$-HSA (**5b**), Mw = 70370; line 5, **13**, Mw = 73831; line 6, **14**, Mw = 74434.

133 mmol), and water (0.28 mL, 15.4 mmol). The mixture was cooled to 0°C. To this solution phosphoryl chloride (2.8 mL, 30.8 mmol) was added dropwise. The temperature of the reaction was maintained at 0°C. The mixture was stirred for 2 h at 0°C. The reaction was poured into 80 g ice and adjusted to pH 7.0 by slowly adding 2.5 M NaOH. The neutralized solution was evaporated to dryness and purified by chromatograph (MeOH-DCM, 1:1). The crude product was dissolved in 0.5 M NaOH (15 mL). The pH of the solution was adjusted to 8∼9 with Dowex 50 X2 (H^+ form) resin. The mixture was filtered and resin was washed with water. The combined filtrate was lyophilized. The residue was desalted with Sephadex G-10 gel chromatography, eluted with distilled water. The sugar fractions were concentrated to 5 mL and lyophilized to give **2** (solvent $CHCl_3$/MeOH/H_2O, 13:8:2, Rf = 0.35; 1.7 g, 63.7%) as a white solid. ^{1}H NMR (D_2O): δ 8.11 (d, 2H, J = 9.3 Hz, H-meta), 7.13 (d, 2H, J = 9.3 Hz, H-ortho), 5.72 (s, 1H, H-1), 3.59–4.05 (m, 6H, H-2, H-3, H-4, H-5, H-6a, H-6b). ^{13}C NMR (D_2O): δ 163.6, 145.0, 143.8, 130.1, 128.7, 119.5, 100.6 (C-1), 75.4 (C-5, J = 8.6 Hz), 72.8 (C-2), 72.3 (C-3), 68.6 (C-4), 66.5 (C-6, J = 4.8 Hz). ^{31}P NMR (D_2O): δ 2.20 (s).

*p-aminophenyl-6-phospho-α-D-mannopyranoside (**3**)*

To a solution of **2** (1.4 g, 3.67 mmol) in a 4:1 (V/V) methanol-water mixture (100 mL) 10% palladium was added on charcoal (0.15 g). The suspension was stirred under H_2(1 atm) for 2 h. After filtration the solvent was evaporated under reduced pressure. The residue was redissolved in water and lyophilized to give **3** (solvent $CHCl_3$/MeOH/H_2O, 13:8:2, Rf = 0.20; 1.0 g, 76.9%) as a pale yellow solid. ^{1}H NMR (D_2O): δ 6.87 (d, 2H, J = 9.0 Hz, H-meta), 6.69 (d, 2H, H-ortho), 5.29 (s, 1H, H-1), 3.36–3.99 (m, 6H, H-2, H-3, H-4, H-5, H-6a, H-6b). ^{13}C NMR (D_2O): δ 155.7, 124.5, 124.4, 118.5, 98.5 (C-1), 75.4 (d, J = 8.6 Hz, C-5), 70.3 (C-2), 69.8 (C-3), 66.1 (C-4), 64.0 (d, J = 8.6 Hz, C-6). ^{31}P NMR (D_2O): δ 1.23 (s). ESI-MS (negative-ion mode): 350.1 ($[M+H]^-$, 100%).

Preparation of neoglycoprotein $M6P_x$-HSA (**5a–5d**)

Thiophosgen (27 μL, 0.35 mmol) was added with stirring to the solution containing **3** (21.7 mg, 0.062 mmol) in 67% ethanol (5 mL). The reaction was stirred for 1.5 h at room temperature. Nitrogen was bubbled through the solution until it nearly became odorless. The pH was adjusted to 6.0 with 0.1 N NaOH and the solution was evaporated under reduced pressure to give compound **4** (solvent $CHCl_3$/MeOH/H_2O, 13:8:2, Rf = 0.36),

Figure 3. Double staining of HSC sections was performed to identify the cellular localization of M6P-HSA. Double positive cells were indicated with arrows (original magnification 40×). (A) no M6P-HSA; (B) $[M6P]_{37}$-HSA **(5a)**, 0.1 mg/mL, 2 h; (C) $[M6P]_{26}$-HSA **(5b)**, 0.1 mg/mL, 2 h; (D) $[M6P]_{15}$-HSA **(5c)**, 0.1 mg/mL, 2 h.

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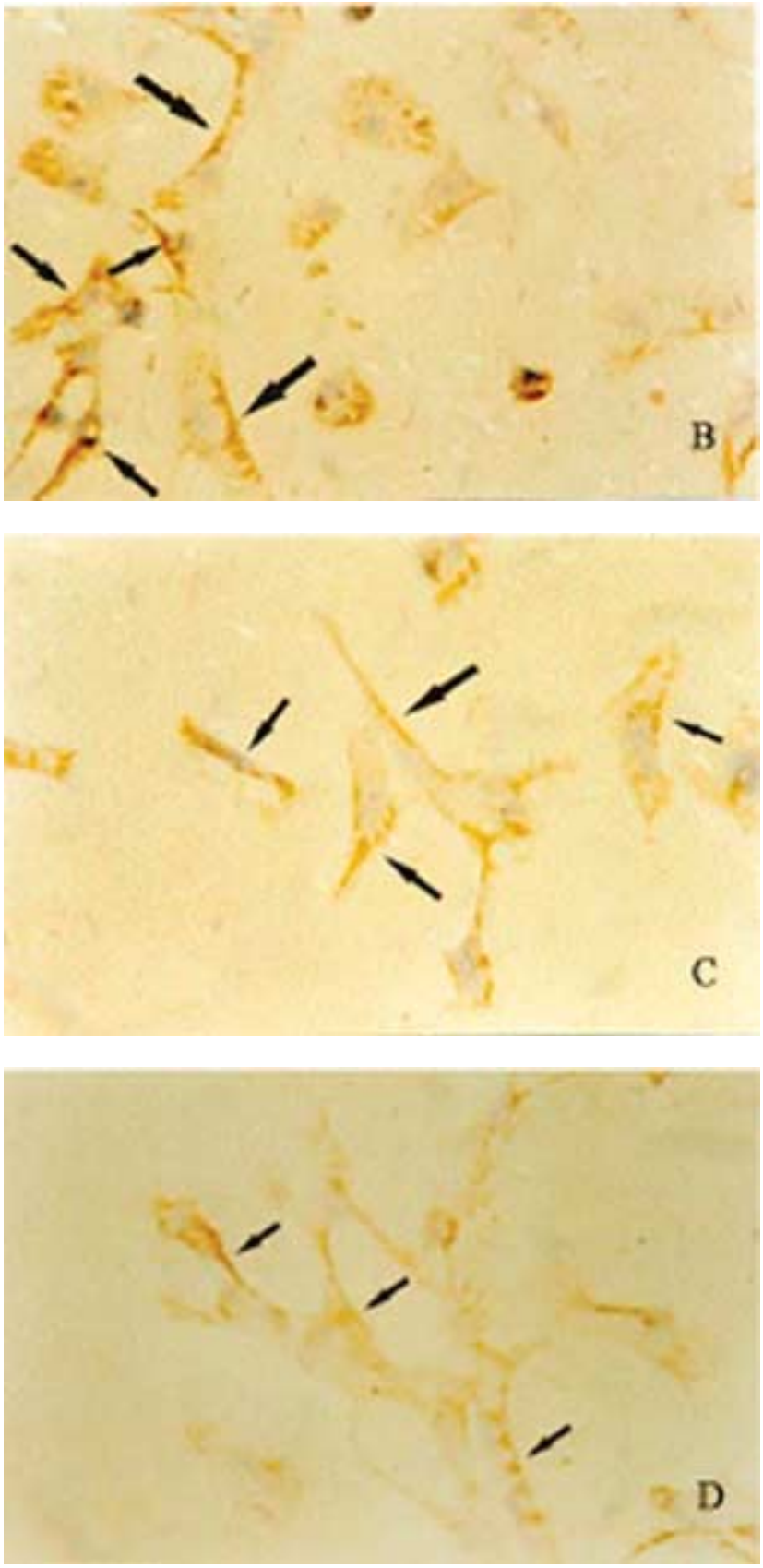

Figure 3. (*Continued*).

IR: 2125 cm^{-1}. ^1H NMR (D_2O): δ 7.16 (d, 2H, $J = 8.7$ Hz, H-meta), 6.99 (d, 2H, H-ortho), 5.44 (s, 1H, H-1), 3.55–3.98 (m, 6H, H-2, H-3, H-4, H-5, H-6a, H-6b). Distilled water (5 mL) was added to dissolve the solid. The aqueous solution of sugar derivative was then added slowly to a solution of HSA (0.22 μmol, 14.5 mg) in 0.01 M borate buffer (7.5 mL), pH = 9.0. The reaction mixture was stirred at room temperature for 18 h, during which time the pH was kept at 9.0. This solution was then dialyzed against 0.15 M NaCl solution (2 × 500 mL) and distilled water (4 × 500 mL, 1000 MW cutoff), respectively, lyophilized to afford a white solid. The crude product was purified with Sephadex G-25 gel chromatography, eluted with 0.15 M NaCl to give **5a** (17.8 g, 49.2%).

Compounds **5b–5d** were synthesized following the same procedure of **5a** (Table 1).

Preparation of glycine ethyl ester hydrochloride (**7**)

A solution of glycine (0.57 g, 7.6 mmol) in absolute alcohol (18 mL) was cooled to –5°C. A mixture of $SOCl_2$(0.68 mL, 9.52 mmol) in absolute alcohol (2 mL) was added dropwise over 20 min and the mixture was allowed to warm at room temperature over 30 min. The mixture was then heated to reflux for 3–4 h. The solution was cooled and evaporated. The residue was recrystallized from ethanol to give **7** (0.75 g, 95.0%). ^1H NMR (D_2O): δ 4.24 (q, 2H, $J = 6.9$ Hz, OCH_2), 3.84 (S, 2H, NCH_2), 1.22 (t, 3H, $J = 7.2$ Hz, CH_3).

The procedure used for the synthesis of **7** was also applied to obtain glycyl-glycine ester hydrochloride (**8**), yield 72.6%. ^1H NMR (D_2O): δ 4.06 (q, 2H, $J = 7.5$ Hz, OCH_2), 3.91 (s, 2H, $NHCH_2$), 3.73 (s, 2H, H_2NCH_2), 1.10 (t, 3H, $J = 6.9$ Hz, CH_3).

Preparation of compounds **9**, **10**, **11**, **12**

To a solution of glycyrrhetin (47 mg, 0.1 mmol) in CH_2Cl_2 (2 mL) **7** (21 mg, 0.15 mmol) and DCC (31.5 mg, 0.15 mmol) was added and the reaction mixture was stirred at room temperature for two days. After filtration, the solution was washed with water, dried over $MgSO_4$, concentrated and the residue was purified by flash chromatography (hexane/EtOAc, 2:1 to 1:1) to give **9**. To the solution of compound **9** in a 3:1 (v/v) methanol–water mixture (2 mL) KOH powder was added (60 mg) and the reaction solution was stirred for 3 h at room temperature. Then the methanol was evaporated, the aqueous solution was extracted with $CHCl_3$ to remove traces of unchanged compound **9**. Then the aqueous solution was acidified with 1N HCl to pH 2, extracted with EtOAc (three times). The combined organic solvent was dried with $MgSO_4$. The residue obtained from the solvent gave **11** (solvent MeOH/$CHCl_3$, 1:5, Rf = 0.16; 43 mg, 81.6%, two steps). ^1H NMR (DMSO-d_6): δ 12.33 (s, 1H, COOH), 7.92 (t, 1H, $J = 5.1$ Hz, NH), 5.59 (s, 1H, C_{12}-H), 4.34 (d, 1H, $J = 5.4$ Hz, OH), 3.77 (q, 2H, $J =$ 17.4 Hz, NCH_2CO), 3.02 (m, 1H, C_3-H), 2.62 (m, 1H, C_{18}-H), 2.23 (s, 1H, C_9-H), 2.10 (m, 2H, C_{19}-H), 1.92 (m, 2H, C_{21}-H), 1.72 (m, 6H), 1.52 (m, 4H), 1.36 (s, 6H, C_{25}-H, C_{26}-H), 1.21 (m, 4H), 1.05 (s, 6H, C_{27}-H, C_{28}-H), 0.92 (s, 3H, C_{29}-H), 0.74 (s, 3H, C_{24}-H), 0.70 (s, 3H, C_{23}-H). ESI-MS: 528.3 ($M + H^+$, 100 %), 529.3 ($M + 2H^+$, 54%).

The procedure used for the synthesis of **11** was also applied to obtain compound **12** (25.7%, two steps). ^1H NMR (DMSO-d_6): δ 8.45 (s, 1H, COOH), 7.91 (m, 1H, NH), 7.28 (m, 1H, NH), 5.57 (s, 1H, C_{12}-H), 4.33 (d, 1H, $J = 3.3$ Hz, OH), 3.68 (m, 2H, NCH_2CO), 3.52 (m, 2H, NCH_2CO), 3.01 (m, 1H, C_3-H), 2.57 (m, 1H, C_{18}-H), 2.32 (s, 1H, C_9-H), 2.15 (m, 2H, C_{19}-H), 1.88 (m, 2H), 1.64 (m, 4H), 1.54 (m, 4H), 1.36 (s, 3H, C_{26}-H), 1.24 (m, 4H), 1.08 (s, 3H, C_{25}-H), 1.04 (s, 6H, C_{27}-H, C_{22}-H), 0.91 (s, 3H, C_{29}-H), 0.74 (s, 3H, C_{24}-H), 0.70 (s, 3H, C_{25}-H). ESI-MS: 607.5 ($M + Na^+$, 28%), 629.5 ($M +$ 2 Na^+, 12%), 686.7 ($M + Na^+ + 2K^+$, 100%).

Preparation of conjugates **13**, **14**

To a solution of **13** (5.3 mg, 0.01 mmol) in dioxane (1 mL) Et_3N was added (0.0042 mL, 0.030 mmol). After the solution was cooled to 10°C, ethyl chloroformate (0.003 mL, 0.0031 mmol) was added, and the reaction was allowed to proceed for 2 h at room temperature, after which the mixture was added in one portion to a stirred, cooled solution of **5b** (15.0 mg, 0.2 μ mmol) in 1:1 (v/v) dioxane-water (4 mL) and 1N NaOH (0.1 mL) mixture. The pH fell to 7.5. After 1 h, another portion of 1 N NaOH (0.05 mL) was added to bring the pH to 8.5. Stirring and cooling were continued for 4 h. The solution was then dialyzed against water at 4°C (4 × 500 mL, 10,000 MW cutoff) for two days, and lyophilized to afford a white solid. The crude product was purified with Sephadex G-25 gel chromatography, eluted with 0.01 M PBS (pH 8.0) to give conjugate **13** (10.4 mg, 55%).

The same procedure used with **13** was applied to conjugate **14** (40%).

Characterization of $[M6P]_x$-HSA and the drug conjugate

The degree of modification was assessed by sugar determination according to the phenol-sulfuric acid method [17]. The protein content was measured by microbiuret method [18]. The molecular weight of conjugates **13** and **14** was determined by SDS-PAGE electrophoresis, which was performed as follows. Separation gel: 11.5% acrylamide gel (Arc) containing 0.31% of *N*,*N*′-methylene bisacrylamide (Bis), 0.1% sodium dodecyl sulfate (SDS), and 375 mM Tris-HCl buffer (pH 8.8); stacking gel: 3.9% Arc containing 0.08% of Bis, 0.1% of SDS, and 125 mM Tris-HCl buffer (pH 6.8); loading buffer: 57% sucrose containing 2.9% SDS, 0.14% bromophenol blue, 7.1% 2-mercaptoethanol, and 70 mM Tris-HCl buffer (pH 6.8). Two μg of sample in 200 μL loading buffer and 0.1 mL water was heated to 95°C for 3 min. Two μL of this solution was running for 1.5 h at 120 V in running buffer containing 0.1% SDS and 125 mM glycine/Tris buffer (pH 8.3). Gel was stained by 0.25% Coomassie Brilliant Blue R-250 in 45% methanol, 20% acetic acid, and 35% water for 1 h. Destaining was accomplished by

gentle agitation in the same acid-methanol solution but in the absence of the dye.

Immunohistochemical detection of $[M6P]_X$-HSA in HSC

The isolation, culture, and identification of HSC were performed according to the method of Hu et al. [19]. Desmin immunohistochemical method was used to assess whether the modified albumin was taken up in HSC [20,21].

First, HSC was incubated at 4°C for 2 h in 0.1–1 mg/mL $M6P_X$-HSA solution. The sections were fixed with acetone for 30 min, washed with 0.1 M phosphate buffered saline (pH= 7.4, PBS), and then incubated with one of the rabbit primary antibodies directed against human albumin for one night at 4°C. Subsequently, the sections were washed with 1 M PBS, incubated with secondary antibodies, namely goat anti-rabbit at 37°C for 2 h, and washed with 0.1 PBS and 0.05 M Tris-HCl buffer (pH = 7.6), respectively. Finally, the sections were visualized with DAB solution (20 mg 3,3-diaminobiphenyl amine in 100 mL 0.05 mol/L Tris-HCl [pH = 7.6]) for 5–10 min, washed with 0.5 mol/L Tris-HCl and double-distilled water. After nuclear staining with Mayer's hematoxylin, the sections were embedded in Kaiser's glycerine–gelatine.

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