Chinese Chemical Letters Vol. 17, No. 2, pp 173-176, 2006 173
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Synthesis and Characterization of a Novel Galactosylated Cholesterol

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Abstract: A novel galactosylated cholesterol with mono-galactoside moiety, (5-cholesten-3-yl)-4-oxo-4-[2-(lactobionylamido)ethylamido]butanoate **6** was synthesized. Its chemical structure was characterized by IR, ESI-MS, ^{13}C NMR, ^{1}H NMR. Doxorubicin entrapped in the liposomes containing 10% mol/mol **6** was rapidly accumulated in liver and mainly uptake by parenchymal cells in mice.

Keywords: Galactosylated cholesterol, (5-cholesten-3-yl)-4-oxo-4-[2-(lactobionylamido)ethyl-amido]butanoate, hepatocyte-selective targeting, doxorubicin.

Mammalian hepatocytes (parenchymal cells) exclusively possess large number of asialoglycoprotein receptors (ASGPr), which can recognize terminal D-galactose or N-acetylgalactosamine residues. Lactobionic acid **1**, bearing a galactosyl group, is usually used as a recognizing moiety of the hepatocyte-targeting carrier[1]. Cholesterol, one of the lipid components used to form liposomes, is usually selected as the lipophilic anchor moiety for stably introducing the galactosyl moiety in liposomes[2,3]. In the present study, **1** and cholesterol were chemically attached *via* the esterification and amidation reactions to form a novel galactosylated cholesterol (**Scheme 1**), (5-cholesten-3β-yl)4-oxo-4-[2-(lactobionylamido)ethylamido]butanoate **6**, which had a great potential as drug delivery carriers for hepatocyte-selective targeting.

Experimental

A solution of lactobionic acid calcium salt was passed through a cation-exchange resin column (Dowex 50WX8) to convert to free lactobionic acid **1**. The eluted free acid solution was lyophilized and converted to lactobionic lactone **2** by repeated evaporation from methanol and ethanol[4].

A solution of **2** (5 g, 14.7 mmol) in 50 mL methanol was added dropwise to the solution of ethylenediamine (8.8 g, 147 mmol) in 20 mL methanol. The reaction mixture was stirred at reflux for 6 h, then the solvent was removed. 300 mL dichloro-methane was added to the residue and the precipitated white crystals were filtered and

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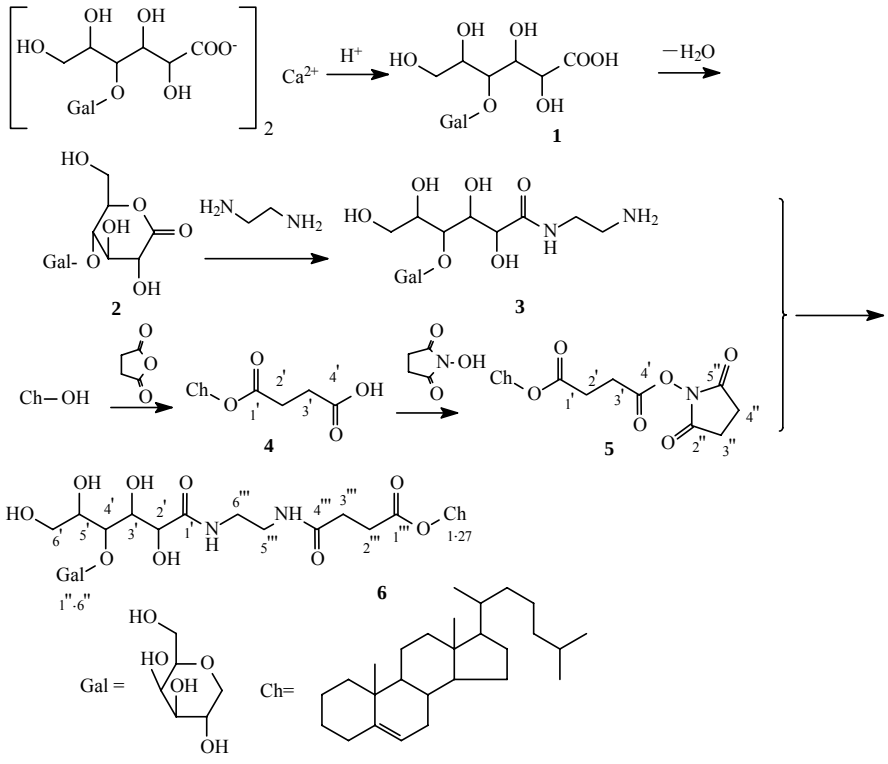
washed with ether and small amount of cold methanol, yielding 5.03 g (12.6 mmol, 85.7%) of 1-N-[*O*-β-D-galactopyranosyl-(1,4)-D-gluconamide]-2-N'-methylamine **3**.

N-Hydroxysuccinimidyl 5-cholesten-3β-yloxy succinate **4** was synthesized according to the method previously described by Kempen *et al.*[5]. Briefly, cholesterol (3.87 g, 10 mmol) was dissolved in 20 mL dichloromethane and 5 mL pyridine, and then succinic anhydride (1.03 g, 10 mmol) was added. After stirring at reflux for 8 h, the mixture was evaporated to dryness. The residue was dissolved in warm acetone and crystallized by cooling to -20°C overnight to yield 4.05 g (8.32 mmol, 83.2%) of cholesteryl hydrogen succinate **4**. And then, N, N'-dicyclohexylcarbodiimide (5 g, 24.2 mmol) was added to the mixture of the solution of **4** (4.0 g, 8.2 mmol) and N-hydroxysuccinimide (1.9 g, 16.4 mmol) in 50 mL tetrahydrofuran (cooled to -20 °C). The mixture was stirred for 60 min at -20°C, then left at 4 °C overnight. The precipitated N, N'-dicyclohexylurea was removed by filtration, the filtrate was evaporated to dryness, and the residue was crystallized from THF/2-propanol (1:2, V/V) to yield 4.5 g (7.7 mmol, 93.0%) of **5**.

0.8 g (2 mmol) of **3** was dissolved in DMSO/dichloromethane (1:1, V/V). A solution of **5** (1.2 g, 2 mmol) in the same solvent was added to the above solution, the mixture was stirred at 4 °C for 24 h. Dichloromethane was evaporated, and the precipitate was removed by filtration. Then 20 mL water was added to the residue.

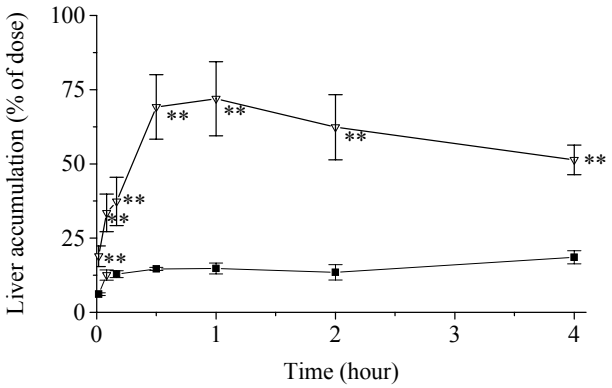
Scheme 1 Synthesis of **6**



The resulted suspension was dialyzed against distilled water for 48 h (10-12 KD cutoff dialysis membrane) to remove DMSO and **3**. The dialysate was lyophilized to yield 1.43 g (1.6 mmol, 80%) of **6**, which was a novel compound. The structure of **6** was determined by the aids of ESI-MS, ^{1}H NMR, ^{13}C NMR and 2D-NMR spectra[7].

Doxroubicin (DOX) was uptake in conventional liposomes (CL DOX) and galactosylated liposomes (incorporated with 10% mol/mol of **6**, GalL DOX) in response to sulfate ammonium gradients, and the entrapped efficiency was more than 95%[6]. The liver accumulation and intrahepatic distribution in mice of CL DOX and Gal DOX was studied just as Kawakami described[3]. Kunming mice were injected with CL DOX or GalL DOX through tail vein at a dose of 10 mg /kg, the profile of time course of DOX concentration in liver was shown in **Figure 1**. GalL DOX was rapidly accumulated to the liver (up to 70.29 ±11.66 % of the dose within 1 h). The amount of GalL DOX in liver was significantly higher than that of CL DOX. Moreover, the results of intrahepatic distribution experiment demonstrated that GalL DOX was mainly taken up by parenchymal cells (88% of the total hepatic uptake). These results suggested that liposomes containing **6** could be a useful drug delivery carrier for hepatocyte-selective targeting.

Figure 1 Liver accumulation of DOX after intravenous injection of GalL DOX (-▽-) and CL DOX (-■-).



Each point represents the mean percentage dose ± SD. (** P<0.05)

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7. **4**: yield 83%, ^{1}H NMR (C_5D_5N, δ ppm): 5.33 (m, 1H, H-6), 4.85 (m, 1H, H-3), 2.93 (m, 2H, H-3'), 2.88 (m, 2H, H-2'), 2.48 (m, 2H, H-4), 1.97 (m, 1H, H-8), 0.97 (s, 3H, H-19), 0.96 (d, 3H, J=6.5, H-21), 0.90 (d, 6H, J=6.5, H-26, 27), 0.65 (s, 3H, H-18); ^{13}C NMR (C_5D_5N, δ ppm):

174.8 (C-4'), 172.3 (C-1'), 140.0 (C-5), 122.8 (C-6), 74.2 (C-3), 56.8 (C-14), 56.4 (C-17), 50.2 (C-9), 42.5 (C-13), 39.9 (C-16), 39.8 (C-24), 38.5 (C-4), 37.2 (C-1), 36.8 (C-10), 36.5 (C-22), 36.1 (C-20), 32.1 (C-8), 32.0 (C-7), 30.2 (C-3'), 29.9 (C-2'), 28.5 (C-2), 28.3 (C-12), 28.1 (C-25), 24.5 (C-15), 24.2 (C-23), 23.0, 22.7 (C-26, 27), 21.3 (C-11), 19.3 (C-19), 19.0 (C-21), 12.0 (C-18); ESI-MS *m/z*: 509.4 $(M+Na)^+$, calcd. for $C_{31}H_{50}O_4$ 509.3607.

5: yield 93%, ^{1}H NMR (C_5D_5N, δ ppm): 5.35 (m, 1H, H-6), 4.81 (m, 1H, H-3), 3.12 (t, 2H, J=6.4, H-3'), 2.84 (t, 2H, J=6.4, H-2'), 2.82 (s, 4H, H-3'',4''), 2.47 (m, 2H, H-4), 1.98 (m, 1H, H-8), 1.01 (s, 3H, H-19), 0.97 (d, 3H, J=6.5, H-21), 0.90 (d, 6H, J=6.5, H-26, 27), 0.65 (s, 3H, H-18); ^{13}C NMR (C_5D_5N, δ ppm): 170.68 (C-1'''), 169.92 (C-1'), 168.95 (C-4'''), 139.83 (C-5), 122.77 (C-6), 74.73 (C-3), 56.68 (C-14), 56.28 (C-17), 50.14 (C-9), 42.4 (C-13), 39.84 (C-16), 39.65 (C-24), 38.22 (C-4), 37.08 (C-1), 36.7 (C-10), 36.41 (C-22), 35.94 (C-20), 32.04 (C-8), 31.94 (C-7), 29.25 (C-2'''), 28.4 (C-2), 28.15 (C-12), 27.85 (C-25), 26.69 (C-3'''), 26 (C-5''', 6'''), 24.39 (C-15), 24.06 (C-23), 22.84, 22.59 (C-26, 27), 21.17 (C-11), 19.25 (C-19), 18.86 (C-21), 11.9 (C-18); ESI-MS *m/z*: 606.4 $(M+Na)^+$, calcd. for $C_{35}H_{53}O_6$ N 606.3771.

6: yield 80%, IR (KBr) υ/cm^{-1}: 3377 (br, OH), 2937 (-CH2-), 1732 (ester, C=O), 1651(amide, C=O), 1543 (C=C); ^{1}H NMR (C_5D_5N, δ ppm): 8.68 (brt, 2H, J=6.7, -NH×2), 5.32 (m, 1H, H-6), 5.26 (m, 1H, J=3.18, H-3'), 5.20 (d, 1H, J=6.7, H-1''), 5.19 (d, 1H, J=3.7, H-2'), 4.82 (m, 1H, H-3), 4.78 (m, 1H, H-4'), 4.70 (m, 1H, H-5'), [4.41 (dd, 1H, J=2.7, 11.2) & 4.26 (dd, 1H, *J*=4.4, 11.2), H-6''], 4.12 (dd, 1H, *J*=3.0, 9.5, H-3''), 4.07 (m, 1H, H-5''), 3.70 (m, 2H, H-6'''), 3.67 (m, 2H, H-5'''), 2.87 (m, 2H, H-3'''), 2.76 (m, 2H, H-2'''), [2.45 (dd, 1H, *J*=12.2, 3.5) & 2.40 (brd, 1H, *J*=12.2) , H-4], 1.94 (m, 1H, H-8), 0.96 (d, 3H, J=6.5, H-21), 0.95 (s, 3H, H-19), 0.89 (d, 6H, J=7.7, H-26, 27), 0.63 (s, 3H, H-18); ^{13}C NMR (C_5D_5N, δ ppm): 174.82 (C-1'), 172.70 (C-4'''), 172.24 (C-1'''), 140.03 (C-5), 122.75 (C-6), 106.58 (C-1''), 84.11 (C-4'), 77.54 (C-5''), 75.26 (C-3''), 74.14 (C-3), 73.66 (C-2'), 73.23 (C-5'), 72.99 (C-2''), 72.76 (C-3'), 70.26 (C-4''), 64.44 (C-6'), 62.57 (C-6''), 56.75 (C-14), 56.33 (C-17), 50.18 (C-9), 42.47 (C-13), 39.92 (C-16), 39.74 (C-5''', 6'''), 39.60 (C-24), 38.46 (C-4), 37.19 (C-1), 36.78 (C-10), 36.49 (C-22), 36.05 (C-20), 32.13 (C-8), 31.99 (C-7), 31.04 (C-2'''), 30.27 (C-3'''), 28.52 (C-2), 28.27 (C-12), 28.08 (C-25), 24.50 (C-15), 24.16 (C-23), 22.97, 22.71 (C-26, 27), 21.25 (C-11), 19.36 (C-19), 18.95 (C-21), 11.99 (C-18); ESI-MS *m/z*: 891.6 $(M+Na)^+$, calcd. for $C_{45}H_{76}O_{14}N_2$ 891.5194.

Received 2 June, 2005

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刊名： 中国化学快报（英文版） ISTIC SCI

英文刊名： CHINESE CHEMICAL LETTERS

年，卷(期)： 2006,17(2)

参考文献(6条)

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