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Design and Synthesis of Cholestane Derivatives Bearing a Cascade-type Polyol and the Effect of Their Property on a Complement System in Rat Serum

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Abstract: The ß-cholestane derivatives **1a~c** bearing a cascade-type polyol, were newly synthesized. The release of fluorescent marker 6-CF [5(6)-carboxyfluorescein] encapsulated in the modified liposomes prepared from **1** was dramatically faster than that in the conventional liposomes prepared from cholesterol.

Keywords: complement system; cholesterol; liposome

Interaction of liposomes with a blood complement system has been studied to elucidate the important biological mechanism.[1] It is well-known that a thioester moiety of ***C3b***, *the degradative fragment of the third component of a complement system*, is attacked by nucleophilic portions on the surface of foreign particles.[2] We previously reported the significant relationship between the releasing rate of the fluorescent marker encapsulated in the liposomes and their diameter or content of cholesterol,[3] and demonstrated that the marker releases faster when more cholesterol is contained in the liposomes. It is also well accepted that cholesterol is vertically embedded from the D-ring side chain into a number of straight alkyl chains of liposomal lipids (**Fig 1**). Therefore, we postulate a hypothesis that the thioester of **C3b** is attacked directly by the nucleophilic hydroxyl group of cholesterol.

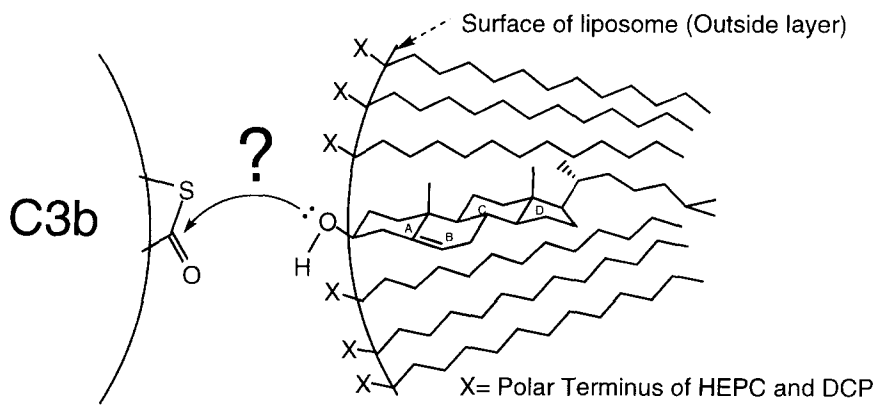

Fig 1. Interaction of **C3b** to Liposome

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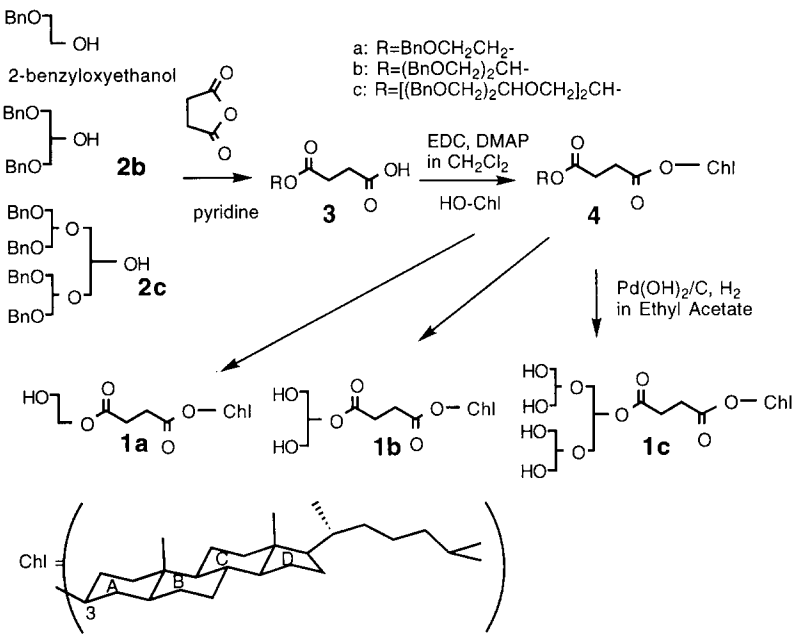
We report here the synthesis of several new cholestane derivatives, preparation of the modified liposomes by using the new derivatives, and their ability to activate the complement system compared with conventional liposomes.

At the beginning of this research, we prepared conventional liposomes (**L-con**) from HEPC (hydrogenated egg phosphatidylcholine) and DCP (dicetyl phosphate) with *cholesterol* as well as unusual liposomes (**LH2**) from HEPC and DCP with *ß-cholestanol* and compared the releasing rate of the encapsulated marker 6-CF. As shown in **Fig 2**, the result of the property of **LH2** in rat serum is quite similar to that of **L-con**. Thus, we initiated our synthesis of various compounds without a double bond in the B-ring.



Scheme 1. Preparation of New ß-Cholestanol Derivatives

We designed a series of compounds **1b** and **1c** bearing the *cascade-type polyols* that we have previously developed[4] as well as **1a** for the purpose of comparison (**Scheme 1**). The polyol moiety could have a number of hydroxyl groups but *no asymmetric center*. Therefore, we could concentrate on considering the numerical value of the hydroxyl groups and ignore the complicated asymmetric factor.

Treatment of commercially available 2-benzyloxyethanol, the polybenzyl ethers **2b** and **2c**[4a] with succinic anhydride in pyridine gave the half-esters **3a, 3b** and **3c** respectively in quantitative yields. The esterification of the carboxylic acid moiety of **3a**, **3b** and **3c** with ß-cholestanol gave the esters **4a, 4b** and **4c** in 95%, 95%, and 68% yields respectively by using EDC (*N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide) and DMAP (4-dimethylaminopyridine) in methylene chloride. The benzyl moieties of **4a, 4b** and **4c** were deprotected by palladium hydroxide on carbon under hydrogen atmosphere to give our designed derivatives **1a**, **1b**, and **1c**[5] in 89%, 60%, and 83% yields, respectively.

The modified liposomes (**La**) were prepared from **1a** with HEPC, DCP, and ß-cholestanol as well as **Lb** and **Lc** from **1b** and **1c**, respectively. A mixture of HEPC, DCP and ß-cholestanol or **1a-c** (molar ratio = 5.0 : 1.0 : 4.0) was dissolved in chloroform and the resulting solution was evaporated to give a lipid film. A solution of 6-CF in water was added to the lipid film and the mixture was shaken vigorously to encapsulate 6-CF in liposomes. Using polycarbonate membrane filter, extrusion was carried out at least eight times to obtain a narrow distribution of liposomes in diameter (~800 nm).

The liposomes **La**, **Lb**, **Lc** an **LH2** were incubated with fresh rat serum and time courses of the released 6-CF were measured continuously (final concentration of liposomes at 1 *μmol/ml*). The results are summarized in **Fig 2**. The releasing rate of 6-CF from **Lb** was found to be slightly higher than that from **La**, and much higher than that from **LH2.** Furthermore, it is noteworthy that the rate as well as extent of release of 6-CF from **La** and **Lb** are much higher than that from **LH2** at this lipid concentration since such a dramatic increase of the releasing rate has not been observed previously. Unfortunately, **Lc** is too labile to allow measurement of the releasing rate.

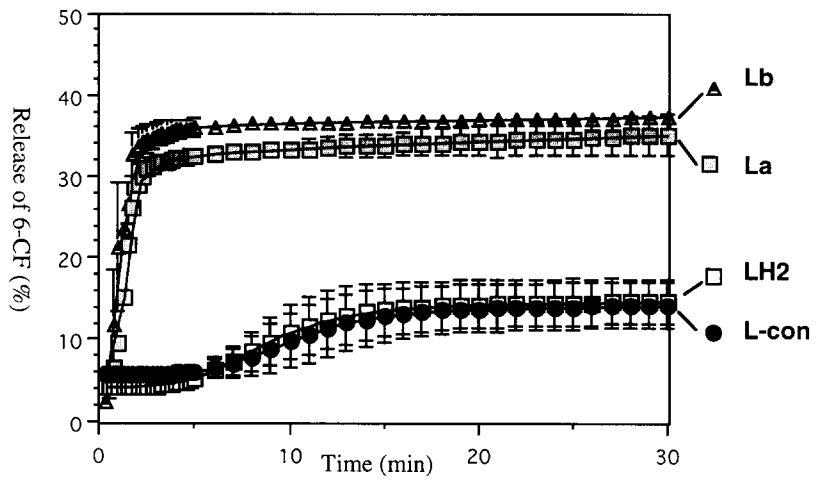


Fig. 2 Effects of side-chain length and number of the hydroxyl group of ß-cholestanol derivatives on the release of 6-CF from liposomes in rat serum.

In the case of HEPC-based liposomes used in this study, the release of 6-CF from liposomes was the result from the activation of the rat complement system *via* the alternative pathway[6], where not antibodies but the ***C3*** (*the third component of the complement system*) molecule itself is generally considered to initiate this pathway.[2] The hydroxyl group is the only putative reactive site against **C3b**, since these liposomes have no amino group. Thus, the remarkable increase in the rate as well as the extent of 6-CF release from liposomes **La** and **Lb** indicates the central role of the hydroxyl group of cholesterol in the recognition of liposomes by the complement system. The rapid release of 6-CF even at this lipid concentration (1 *μmol/ml*) has not been observed in the conventional liposomes, which indicates the increased reactivity of the modified liposomes to the complement system. There was no remarkable difference in the effect of the number of hydroxyl group on the activation of the complement system. Further study is now underway to estimate the affinity of these liposomes to the complement system by changing the concentration of liposomes. Preparation of a variety of cholestane derivatives and the newly modified liposomes is now in progress and a

further report will be presented soon.

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5. **1a:** colorless powder; IR (neat) 3490, 1731, 1708, and 1186cm^{-1}; ^{1}H-NMR (200MHz, $CDCl_3$): δ 4.70 (1H, m), 4.25 (2H, t, J = 4.6), 3.82 (2H, dt, J = 4.2, 4.6), 2.64 (4H, s), 2.18 (1H, brt, J = 6.4), 1.99-0.94 (31H, m), 0.90 (3H, d, J = 6.6), 0.86 (6H, d, J = 6.8), 0.81(3H, s), 0.64 (3H, s); ^{13}C-NMR (100MHz, $CDCl_3$): δ 172.6, 172.1, 74.4, 66.3, 61.1, 56.4, 56.3, 54.2, 44.6, 42.6, 40.0, 39.5, 36.7, 36.2, 35.8, 35.5, 35.4, 34.0, 32.0, 29.7, 29.3, 28.6, 28.2, 28.0, 27.4, 24.2, 23.8, 22.8, 22.6, 21.2, 18.7, 12.2, 12.1; EI-MS m/z: 532 (M^+).

   1b: colorless amorph; IR (neat) 3435, 1732, and 1166cm^{-1}; ^{1}H-NMR (200MHz, $CDCl_3$): δ 4.95 (1H, m), 4.70 (1H, m), 3.74-3.90 (4H, m), 2.95 (2H, brs), 2.65 (4H, s), 2.20-0.96 (31H, m), 0.89 (3H, d, J = 6.8), 0.86 (6H, d, J = 6.6), 0.81 (3H, s), 0.64 (3H, s); ^{13}C-NMR (100MHz, $CDCl_3$): δ 172.5, 172.4, 75.6, 74.7, 74.5, 62.1, 56.4, 56.3, 54.2, 44.6, 42.6, 40.0, 39.5, 36.7, 36.2, 35.8, 35.5, 35.4, 33.9, 32.0, 29.8, 29.6, 28.6, 28.2, 28.0, 27.4, 24.2, 23.8, 22.8, 22.6, 21.2, 18.7, 12.2, 12.1; EI-MS m/z: 562 (M^+)

   1c: colorless amorph; IR (neat) 3409, 1730, and 1049cm^{-1}; ^{1}H-NMR (200MHz, $CDCl_3$): δ 5.13 (1H, m), 4.69 (1H, m), 3.83-3.48 (14H, m), 3.41 (4H, brs), 2.62 (4H, s), 1.99-0.95 (31H, m), 0.90 (3H, d, J = 6.6), 0.86 (6H, d, J = 6.6), 0.82 (3H, s), 0.65 (3H, s); ^{13}C-NMR (100MHz, $CDCl_3$): δ 172.2, 172.2, 81.3, 74.5, 72.0, 68.1, 62.1, 61.9, 56.4, 56.3, 54.2, 44.6, 42.6, 40.0, 39.5, 36.7, 36.2, 35.8, 35.5, 35.4, 34.0, 32.0, 29.4, 29.3, 28.6, 28.2, 28.0, 27.4, 24.2, 23.9, 22.8, 22.6, 21.2, 18.7, 12.2, 12.1; FAB-MS m/z: 733.6 ($M+Na^+$).
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