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Synthesis of the Fatty Sterol Bound Protein for a New Sterol Antibody

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Abstract—For the purpose of applying the particular antibodies as a new diagnostic procedure for atherosclerosis and related diseases, we successfully achieved the synthesis of the fatty sterol with a linker, then linked the target protein to this sterol. Synthesis was started from pregnenolone and achieved by the Grignard reaction with pentenyl magnesium bromide, regioselective photoaddition of thiolacetic acid toward the 25-double bond, esterification of 3-OH with linoleic anhydride, in situ conjunction of the cross-linker (MBS) to the thiol group after selective deprotection from its acetyl ester, and finally by the reaction with protein such as KLH or albumin through this linker.

Cholesterol and its fatty acid ester are the most abundant sterol derivatives in higher animals, and are present in the plasma membranes, cell membranes, and nervous tissue, etc. They play an important role serving as components of cell growth, and acting as a precursor of bile acids, steroid hormones, vitamins, and lipoproteins.[1] However, under certain specialized circumstances, excess quantities of sterol accumulate in cells in the human body. The initial pathology of atherosclerosis is considered to occur by the deposition of a lipid such as fatty streak and fibrous plaque at the inner artery wall which is composed of cholesterol and cholesteryl ester (fatty sterol) and oxidized sterols from endogenous and exogenous conditions.[1–4] In the medical care of this disease the demand for therapeutic approaches has been extensively promoted by a clinical development of progressive agents together with other therapies and diagnostic methods.[1,5–7] As one of the possibilities for the diagnostic approach, we considered the use of antibody agents which may inherently involve disease-specificity and simplicity to assay. Thus, to elucidate the mechanism of the lesion of atherosclerosis probably induced by cholesteryl ester and its oxidized derivatives, and to further develop the immuno-diagnostic method toward these sterols, we have started the synthesis of the modified proteins bound to fatty sterol through a proper linker at the steroidal terminus as the antigen for production of the monoclonal antibodies.

Herein, we report the syntheses of a fatty sterol-binding protein antigen which are achieved through a chemical junction between a manipulated fatty sterol with a linker and KLH (keyhold limpet hemocyanin) or albumin as a carrier protein.

The synthesis of the target oxidized sterol bound to a linker **9**[†] was started with 3β-hydroxy-pregn-5-en-20 one **1** (Scheme 1). The silyl ether **2** led to the terminus alkenyl sterol **3** in 67% yield by the Grignard condensation with *n*-pentenyl magnesium bromide in ether at −78 °C. The C-20 tertiary alcohol **3** so obtained was found to be almost pure α-OH isomer.[8] Methylation of the hindered hydroxy group was achieved by treating with KH, and subsequent addition of MeI in dry THF to give the ether **4** in 92% yield.[9] Only one epimer of **4** was successfully obtained in a pure crystalline form by recrystallization in CH_3CN. The spatial arrangement at C-20 is considered to be crucial for the recognition at the receptor binding site. Therefore, to confirm the

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[†]Spectral data of **9**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.78 (s, 3H), 0.89 (t, J = 6.8 Hz, 3H), 1.02 (s, 3H), 1.17 (s, 3H), 2.70 (dd, J = 18.8, 3.6 Hz, 1H), 2.92 (bs, 4H), 3.12 (s, 3H), 3.34 (dd, J = 18.8, 9.2 Hz, 1H), 3.62–3.72 (m, 1H), 3.89 (dd, J = 9.2, 3.6 Hz, 1H), 4.56–4.66 (m, 1H), 5.27–5.45 (m, 5H), 7.62–7.70 (m, 2H), 8.11–8.15 (m, 1H), 8.16–8.22 (m, 1H); LRMS (FAB$^+$) m/z 1010.

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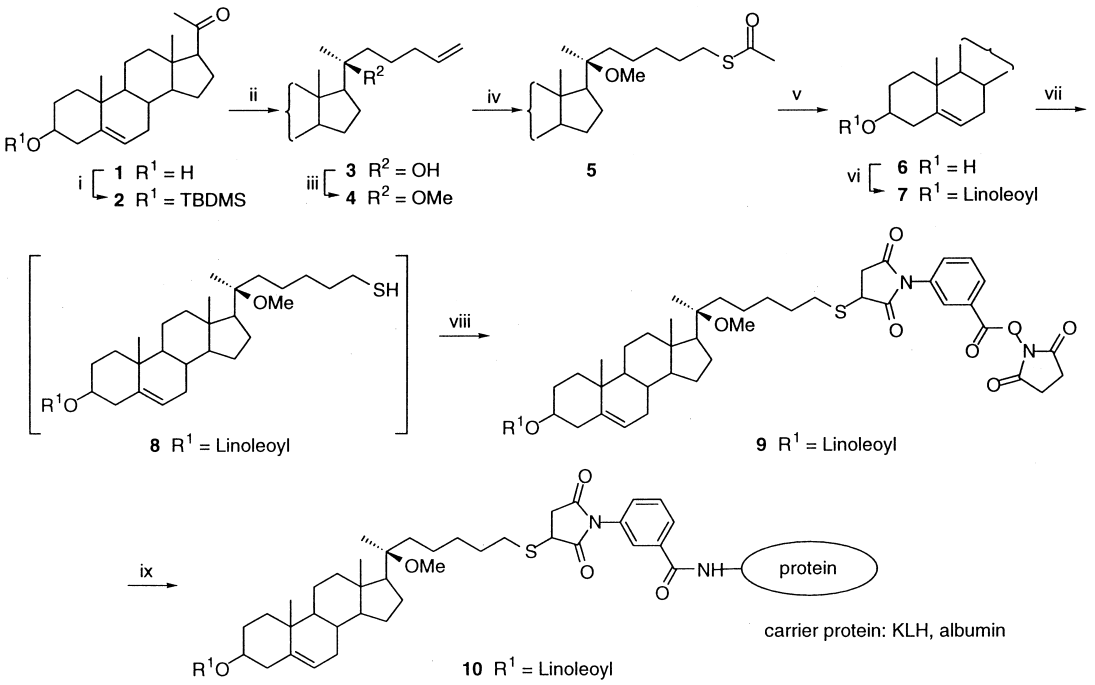

Scheme 1. Reagents and conditions (i) TBDMSCl, Im, DMF, 0 °C→rt, 97%; (ii) $H_2C{=}CH(CH_2)_3$ MgBr, ether −78 °C, 67%; (iii) KH, MeI, THF, rt, 92%; (iv) AcSH, Ch_2Cl_2, hν (>300 nm), in a Pyrex tube, rt, 85%; (v) THF:EtOH (1:1), HCl(1), rt, 90%; (vi) linoleic anhydride, 4-pyrrolidinopyridine, benzene, 99%; (vii) NaOEt:EtOH–DME (1:1), then AcOH, rt; (viii), MBS, **7**→**9**, overall yield, 49%; (ix) KLH or albumin in buffer.

stereochemistry on this carbon the X-ray crystallographic analysis was performed and revealed the β-Me form, the same as the natural sterols.[8]

The key step in the synthetic sequence is the introduction of an acetylthio group and the subsequent deprotection to thiol, to bind with a proper linker, i.e. *m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester (MBS). Thiolacetic acid addition to olefins has been well established by the reaction of olefins with thiolacetic acid under acidic, AIBN-initiated, and visible light irradiation conditions.[10–14] All these conditions were tried in the addition reaction of **4**, however, they resulted in the recovery of starting material or a very poor yield of the desired product. Finally, we found a mild photolytic protocol, that is, the reaction of **4** with an equimolar amount of thiolacetic acid under irradiation with a high pressure mercury lamp with a Pyrex filter (hν: >300 nm), resulted in a good yield (85%) of **5**. After TBDMS deprotection of **5**, the acetylthio sterol **6** was treated with linoleic anhydride in the presence of 4-pyrrolidinopyridine in benzene to give the fatty sterol **7** quantitatively.[15] The selective hydrolysis of **7** to **8** with controlled addition of NaOEt, acidification with acetic acid, and in situ addition of MBS[16–18] under N_2 successfully led to the fatty sterol parts **9** equipped with the protein linker in 49% overall yield. The target protein **10**, bound to the fatty sterol through the linker was obtained by the treatment of **9** with KLH or albumin in phosphate buffer solution[16–18] and used directly as a mouse antigen.[19] At present, our efforts are directed toward the syntheses of the other manipulated sterols with the MBS-linker and the preparation of the proteins to bind with these sterols.

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