PRODUCTION OF HIGHLY SPECIFIC ANTIBODIES TO 1α,25-DIHYDROXYVITAMIN D_3 UTILIZING A NOVEL HAPTENIC DERIVATIVE HAVING A CHEMICAL BRIDGE AT 11α-POSITION

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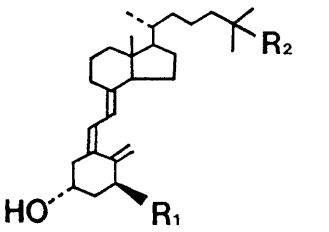
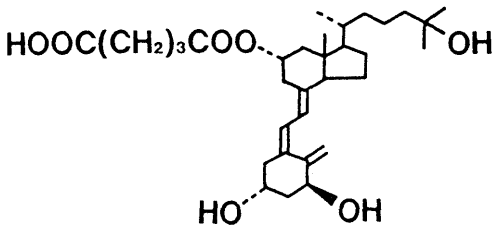
The production of highly specific antibodies to 1α,25-dihydroxyvitamin D_3 **1a** utilizing a novel haptenic derivative **2**, which was synthesized from 11α,25-dihydroxycholesterol via 21 steps, has been reported. The properties of the obtained antibodies are also described.

KEYWORDS 1α,25-dihydroxyvitamin D_3; RIA; specific antibody; haptenic derivative; 11α-hemiglutaryloxy-1α,25-dihydroxyvitamin D_3; 11α,25-dihydroxycholesterol

The production of specific antibodies to 1α,25-dihydroxyvitamin D_3 [**1a**, $1,25(OH)_2D_3$], the active metabolite of vitamin D_3 (D_3) **1b**, is required for the development of a simple and reliable immunoassay as an alternative methodology to conventional radioreceptor assays. In recent years, some antibodies have been raised against the haptens linked to carrier proteins through C-3 or a position on the side chain. But the antibodies obtained from the former haptens usually lacked the specificity for the A ring of this steroid, while those obtained from the latter haptens have little ability to recognize the side chain structure of $1,25(OH)_2D_3$.[1] Consequently, complicated pretreatments are necessary to apply these antibodies to biological fluids. It is anticipated that the use of hapten-carrier conjugates exposing both the A-ring and side chain of the metabolite would provide antibodies having much higher specificity, and thus the 11α-position of the metabolite seems promising as a coupling site for the carrier protein. We report here the production of highly specific antibodies to $1,25(OH)_2D_3$, which was generated utilizing a novel haptenic derivative 2 [11α-hemiglutaryloxy-$1,25(OH)_2D_3$]. The properties of the obtained antibodies are also described.



1a : $R_1 = R_2 = OH$
1b : $R_1 = R_2 = H$

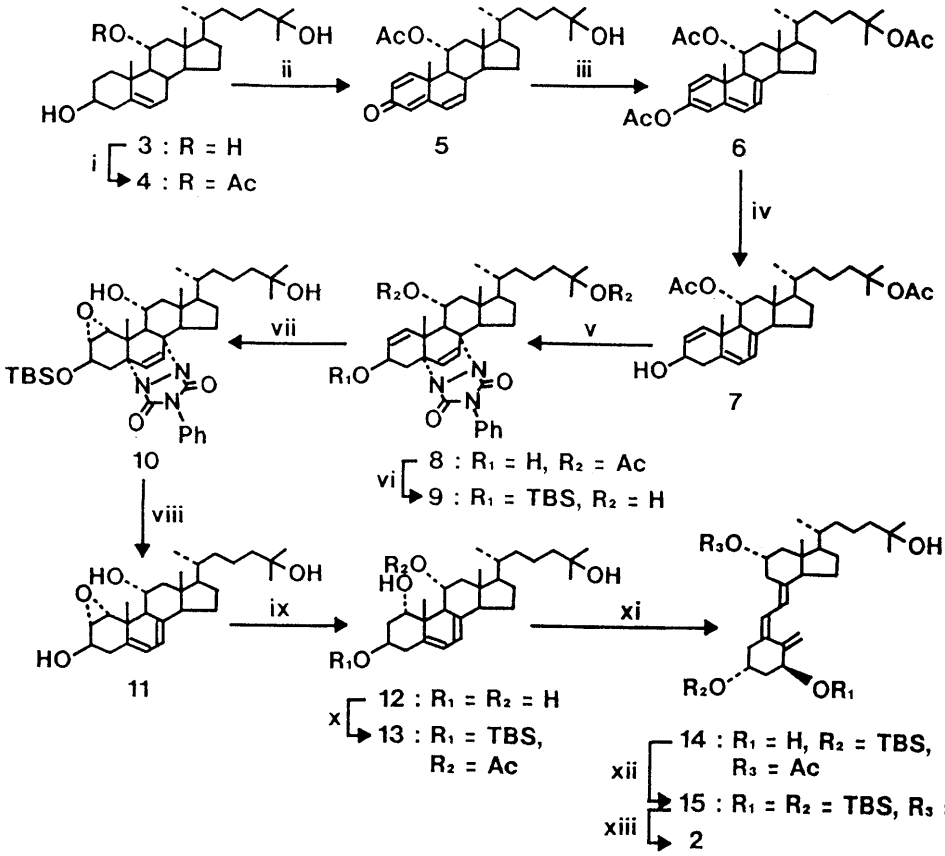


2

Initially, the haptenic derivative **2** was synthesized from 11α,25-dihydroxycholesterol **3**[2] via 21 steps. Compound **3** was converted into the 11-acetate **4** via three steps: selective silylation of the 3β-hydroxy group with t-$BuMe_2SiCl$ (TBSCl) and the usual acetylation of the 11α-hydroxy group followed by desilylation with n-Bu_4NF (91%). Reaction of **4** with DDQ afforded the 1,4,6-trien-3-one **5** (61%),[3] which was then converted into 1,3,5,7-tetraenyl acetate **6** by the enol acetylation using isopropenyl acetate.[4] The reduction of **6** with $Ca(BH_4)_2$ at a low temperature provided 1,5,7-trien-3β-ol **7** (40%).[4,5] After the 5,7-diene structure of **7** was protected with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD)[5] as a Diels-Alder adduct **8** (88%), the 1α-hydroxy group was introduced by the following reaction sequence.[5] Thus, **8** was converted into the 3-silyl ether **9** (99%) via two steps for performing the selective α-epoxidation of the double bond at C-1.[5b] The reaction of **9** with m-CPBA proceeded smoothly at room temperature, and

the 1α,2α-epoxide **10** was obtained in 75% yield. Desilylation of **10** followed by removal of the PTAD group by heating in 1,1,3,3-tetramethylguanidine[6] gave the 5,7-diene **11** (81%). Reductive cleavage of the epoxide with excess $NaBH_4$ (ca. 70 eq) in diglyme[7] gave the desired 5,7-diene-tetraol **12** (70%).

The 3β- and 11α-hydroxy groups of **12** were selectively protected with TBS and an acetyl group, respectively, to give **13** (75%). Irradiation of **13** with a high-pressure mercury lamp (400 W, Vycor filter) followed by thermal isomerization gave a mixture from which the $1,25(OH)_2D_3$ derivative **14** was separated by preparative TLC (26%). Silylation of the 1-hydroxy group and deacetylation gave the suitably protected compound **15** (82%). The introduction of the hemiglutaryl group at the 11α-position by the reaction of **15** with glutaric anhydride followed by desilylation afforded the desired hapten **2** (80%), whose structure was confirmed by various spectral data.[8,9]



i) a, TBSCl, imidazole, DMF, r.t., b, Ac_2O, pyridine, r.t., c, TBAF, THF, r.t.; ii) DDQ, dioxane, reflux; iii) isopropenyl acetate, p-TsOH, BuOAc, reflux (recycling 3 times); iv) $Ca(BH_4)_2$, MeOH-EtOH, 0℃→4℃; v) PTAD, CH_2Cl_2, r.t.; vi) a, TBSCl, imidazole, DMF, r.t., b, 15% KOH in MeOH-THF, r.t.; vii) m-CPBA, $CHCl_3$, r.t.; viii) a, TBAF, THF, r.t., b, 1,1,3,3-tetramethylguanidine, 170℃ (bath temp.); ix) $NaBH_4$, diglyme, 80℃ (bath temp.); x) a, TBSCl, imidazole, DMF, r.t., b, Ac_2O, pyridine, r.t.; xi) a, hν, Et_2O, 0℃, b, hexane-THF, r.t.; xii) a, TBSCl, imidazole, DMF, r.t., b, 5% KOH in MeOH, 0℃; xiii) a, glutaric anhydride, pyridine, r.t., b, TBAF, THF, r.t.

Next, the hapten **2** was coupled with bovine serum albumin (BSA) using N-hydroxysuccinimidyl ester method. Repeated immunization of rabbits with the obtained hapten-carrier conjugate (hapten/BSA molar ratio 17) afforded four kinds of polyclonal antibodies whose properties were then examined in a RIA procedure. The RIA was carried out using [26,27-methyl-^{3}H]-$1,25(OH)_2D_3$ as a labeled antigen, and the bound and free fractions were separated by a dextran-charcoal method. All of the antibodies showed high

titers (optimum dilution 1:1300-1:220000) and affinity constants[10] (Ka=0.34-3.3x10^{10} M^{-1}) and gave sensitive dose-response curves (detection limit ca. 2-10 pg/tube). Cross-reactivities[11] of these antibodies with related vitamin D derivatives were as follows: D_3 (<0.02%), 25(OH)D_3 (0.69-1.5%), 24,25$(OH)_2D_3$ (<0.05-0.16%), 25R,26$(OH)_2D_3$ (0.01-0.09%), 25S,26$(OH)_2D_3$ (0.02-0.12%), 1,24,25$(OH)_3D_3$ (0.30-1.9%) and 1,25$(OH)_2D_3$ 26,23-lactone (<0.08-0.72%). These data demonstrate that the antibodies easily recognize both the A-ring and side chain structure of 1,25$(OH)_2D_3$, and are highly specific to the metabolite compared with conventional antibodies.[1] The application of the present antibodies for the development of simple and practical immunoassay of 1,25$(OH)_2D_3$ is now in progress in our laboratories.

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8) ^1H NMR (270 MHz, acetone-d_6 + D_2O) δ: 0.64 (3H, s, H-18), 0.97 (3H, d, J=5.3 Hz, H-21), 1.16 (6H, s, H-26,27), 4.17 (1H, m, H-3), 4.39 (1H, m, H-1), 4.87 (1H, m, H-19E), 4.95 (1H, m, H-11β), 5.33 (1H, m, H-19Z), 6.19, 6.30 (2H, ABq, J=11.2 Hz, H-7,6). UV (EtOH) λ_{max} nm: 264, λ_{min} nm: 229. MS (FAB) m/z: 545 $[M-H]^-$.
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