

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

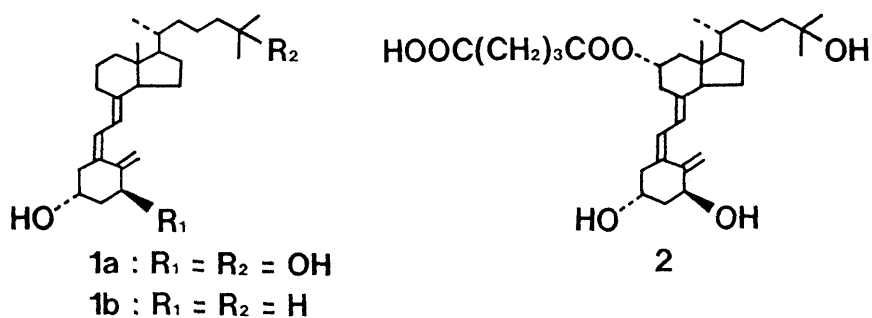
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The production of highly specific antibodies to $1\alpha,25$ -dihydroxyvitamin D_3 **1a** utilizing a novel haptenic derivative **2**, which was synthesized from $11\alpha,25$ -dihydroxycholesterol via 21 steps, has been reported. The properties of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

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

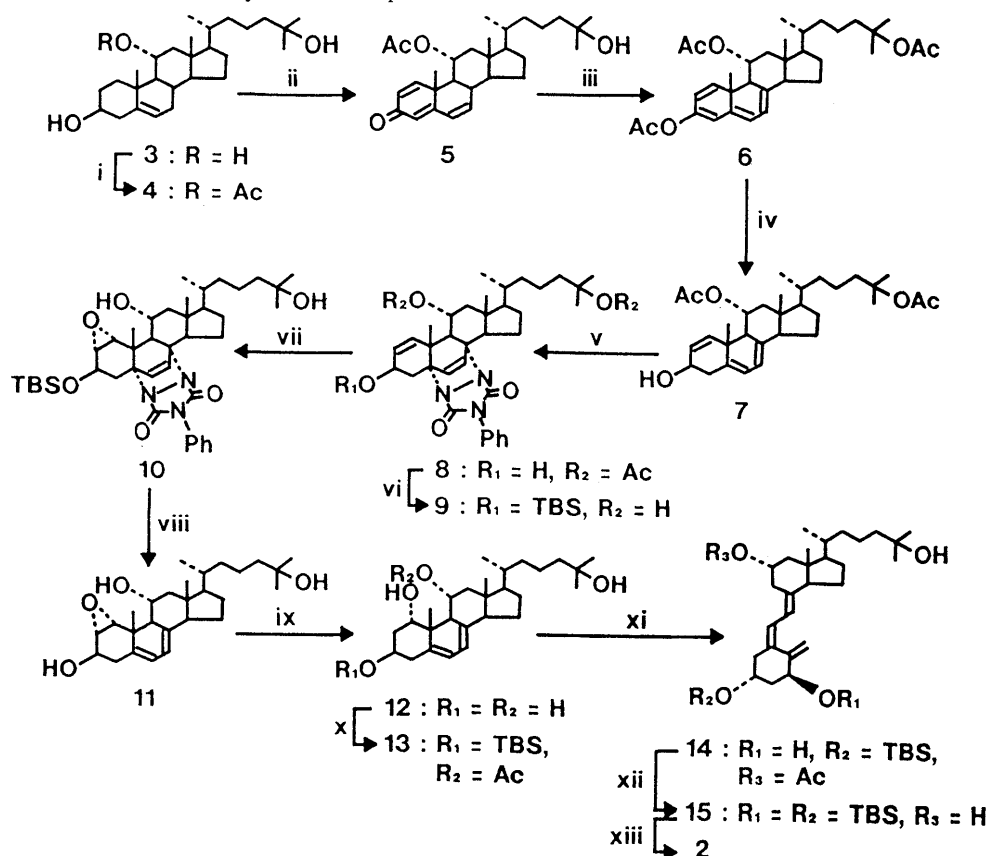
The production of specific antibodies to $1\alpha,25$ -dihydroxyvitamin D_3 [**1a**, $1,25(OH)_2D_3$], the active metabolite of vitamin D_3 (D_3) **1b**, is required for the development of a simple and reliable immunoassay as an alternative methodology to conventional radioreceptor assays. In recent 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.



Initially, the haptenic derivative **2** was synthesized from $11\alpha,25$ -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 -BuMe₂SiCl (TBSCl) and the usual acetylation of the 11α -hydroxy group followed by desilylation with n -Bu₄NF (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 1 α ,2 α -epoxide 10 was obtained in 75% yield. Desilylation of 10 followed by removal of the PTAD group by heating in 1,1,3,3-tetramethylguanidine⁶⁾ gave the 5,7-diene 11 (81%). Reductive cleavage of the epoxide with excess NaBH₄ (ca. 70 eq) in diglyme⁷⁾ gave the desired 5,7-diene-tetraol 12 (70%).

The 3 β - and 11 α -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)₂D₃ 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 11 α -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, Ac₂O, pyridine, r.t., c, TBAF, THF, r.t.; ii) DDQ, dioxane, reflux; iii) isopropenyl acetate, p-TsOH, BuOAc, reflux (recycling 3 times); iv) Ca(BH₄)₂, MeOH-EtOH, 0°C→4°C; v) PTAD, CH₂Cl₂, r.t.; vi) a, TBSCl, imidazole, DMF, r.t., b, 15% KOH in MeOH-THF, r.t.; vii) m-CPBA, CHCl₃, r.t.; viii) a, TBAF, THF, r.t., b, 1,1,3,3-tetramethylguanidine, 170°C (bath temp.); ix) NaBH₄, diglyme, 80°C (bath temp.); x) a, TBSCl, imidazole, DMF, r.t., b, Ac₂O, pyridine, r.t.; xi) a, hv, Et₂O, 0°C, b, hexane-THF, r.t.; xii) a, TBSCl, imidazole, DMF, r.t., b, 5% KOH in MeOH, 0°C; 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-³H]-1,25(OH)₂D₃ 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 constants¹⁰⁾ ($K_a=0.34\text{--}3.3\times 10^{10}\text{ M}^{-1}$) and gave sensitive dose-response curves (detection limit ca. 2–10 pg/tube). Cross-reactivities¹¹⁾ 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-19), 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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