Production and Specificity of Anti-22-oxacalcitriol Antisera

Norihiro KOBAYASHI,[a] Tomoko ASANO,[a] Junichi KITAHORI,[a] Kazutake SHIMADA,*,[a] Noboru KUBODERA,[b] and Hiroyoshi WATANABE[b]

Faculty of Pharmaceutical Sciences, Kanazawa University,[a] 13–1 Takara-machi, Kanazawa 920, Japan and Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd.,[b] 1–135 Komakado, Gotemba 412, Japan. *Received November 27, 1991*

22-Oxacalcitriol 3-hemiglutarate, a haptenic derivative of 22-oxacalcitriol, was synthesized to obtain a specific antibody for use in radioimmunoassay. Three antisera were elicited in rabbits against the hapten conjugated with bovine serum albumin, and their specificity was examined by cross-reaction study. One of the antisera was found to be satisfactorily specific, and expected to provide a radioimmunoassay useful for the pharmacokinetic study of 22-oxacalcitriol.

Keywords vitamin D_3 analog; 22-oxacalcitriol; hapten synthesis; 22-oxacalcitriol 3-hemiglutarate; antibody production; radioimmunoassay; cross-reaction study

Since 1α,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] was shown to induce differentiation on myeloid leukemia cells in addition to its regulatory activity of calcium metabolism,[1] a number of attempts have been made to separate these physiological actions by modifying its chemical structure. 22-Oxacalcitriol (OCT, Chart 1) is an analog of 1,25(OH)$_2$D$_3$ having an oxygen atom at 22-position in the side chain, which has been synthesized for this purpose.[2] Although OCT exhibits negligible calcemic activity, its differentiation-inducing activity on myeloid cells is much stronger than that of 1,25(OH)$_2$D$_3$.[3] Recently, additional novel properties of OCT have been identified, and much interest is now focused on the possibility that OCT may be a valuable agent for the treatment of breast cancer[4] and secondary hyperparathyroidism accompanied by chronic renal failure[5] without inducing hypercalcemia.

To clarify the effectiveness of OCT from pharmacokinetic aspects, we attempted to develop a method of determining its concentration in various biological fluids. Since the effective doses of OCT would be extremely low (less than μg/body) and its levels in plasma and urine are predicted to be much lower than those of various endogenous vitamin D (D) metabolites, a highly selective and sensitive radioimmunoassay (RIA) seemed most promising for this purpose.

We therefore synthesized a haptenic derivative, OCT 3-hemiglutarate (OCT-3-HG), to produce a specific anti-OCT antibody. Antisera were then elicited in three rabbits against the hapten linked to bovine serum albumin (BSA), and their specificity was examined by cross-reaction study.

Materials and Methods

Melting points were determined on a Yanaco micromelting point apparatus. Proton nuclear magnetic resonance (^{1}H-NMR) spectra (200 MHz, $CDCl_3$) were recorded on a JEOL FX-200 spectrometer using tetramethylsilane as an internal standard. Ultraviolet (UV) (EtOH) and mass (MS; electron impact) spectra were obtained on a Shimadzu UV-240 spectrophotometer and a Shimadzu GCMS-QP 1000 spectrometer, respectively. OCT, 24(OH)OCT (isomeric mixture of 24-hydroxy group), 1,20(OH)$_2$hexanor-D$_3$ (Chart 1), 1,25(OH)$_2$D$_3$, 24,25(OH)$_2$D$_3$, 25*R*,26(OH)$_2$D$_3$, 25*S*,26(OH)$_2$D$_3$ and [26-methyl-^{3}H]-OCT (86.5 Ci/mmol) were synthesized in our laboratories. D_3 and 25(OH)D_3 were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and Duphar B.V. Co. (Amsterdam, the Netherlands), respectively. BSA and complete Freund's adjuvant (FCA) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Difco Laboratories (Detroit, MI, U.S.A.), respectively. Flash chromatography and preparative thin layer chromatography (TLC) were carried out using Silica gel 60 (230—400 mesh) and Silica gel 60 pre-coated TLC plate (F_{254}, 20 × 20 cm), respectively, obtained from E. Merck AG (Darmstadt, Germany).

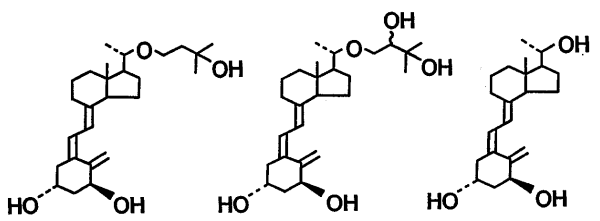

Chart 1. Structures of OCT and Its Predicted Metabolites

22-Oxacholesta-5,7-diene-1α,3β,25-triol (3) A mixture of the silyl ether (**2**, 18.4 g)[2] and tetrabutylammonium fluoride (0.29 mol) in tetrahydrofuran (THF; 285 ml) was refluxed for 18 h. The resulting solution was concentrated *in vacuo*, diluted with AcOEt, washed (H_2O and brine), and then dried ($MgSO_4$). After removal of the solvent, the crude product was recrystallized (acetone) to give **3** (10.1 g, 85%) as colorless prisms. mp 186.5—187.5 °C. ^{1}H-NMR δ: 0.62 (3H, s, 18-H), 0.94 (3H, s, 19-H), 1.22 (3H, d, J=7.2 Hz, 21-H), 1.24 (6H, s, 26,27-H), 3.20—3.32, 3.42—3.56 (each 1H, m, 23-H), 3.72—3.91 (2H, m, 1,20-H), 4.06 (1H, m, 3-H), 5.35—5.43 (1H, m, 7-H), 5.72 (1H, d, J=5.7 Hz, 6-H). UV λ_{max} nm: 270, 281, 293. MS *m/z*: 418 (M^+). *Anal.* Calcd for $C_{26}H_{42}O_4 \cdot 1/3H_2O$: C, 73.55; H, 10.13. Found: C, 73.75; H, 10.53.

1α,25-Dihydroxy-22-oxacholesta-5,7-dien-3β-yl (2′,2′,2′-trichloroethyl-glutarate) (4) A solution of 2,2,2-trichloroethylglutaryl chloride (prepared from glutaric anhydride according to the reported method[6]) (2.65 g) in THF (13 ml) was added dropwise over 1 h to a solution of **3** (3.15 g) in pyridine (19 ml)–THF (190 ml) at 0 °C under N_2. After stirring for 15 h, the reaction mixture was extracted with CH_2Cl_2. The organic layer was washed (dil. HCl and brine), dried ($MgSO_4$), and evaporated. The crude product was purified by successive flash chromatographies [CH_2Cl_2–EtOH (10 : 1), then *n*-hexane–AcOEt (1 : 1)] to give **4** (1.33 g, 27%) as a yellow oily substance. ^{1}H-NMR δ: 0.62 (3H, s, 18-H), 0.94 (3H, s, 19-H), 1.21 (3H, d, J=6.1 Hz, 21-H), 1.24 (6H, s, 26,27-H), 2.35—2.45, 2.48—2.60 (each 2H, m, CH_2CO), 3.20—3.33, 3.43—3.53 (each 1H, m, 23-H), 3.78 (1H, br s, 1-H), 3.84 (1H, m, 20-H), 4.75 (2H, s, CH_2CCl_3), 5.12 (1H, m, 3-H), 5.35—5.38 (1H, m, 7-H), 5.69 (1H, d, J=5.7 Hz, 6-H). UV λ_{max} nm: 270, 281, 293. MS *m/z*: 662 (M^+).

(5*Z*,7*E*)-(1*S*,3*R*)-1,25-Dihydroxy-22-oxa-9,10-secocholesta-5,7,10(19)-trien-3-yl (2′,2′,2′-trichloroethylglutarate) (5) A solution of **4** (0.67 g) in THF (310 ml) was irradiated at 0 °C for 15 min under argon with a 400 W high pressure mercury lamp through a Vycor filter. The procedure was repeated once more and the resulting solutions were combined, and then refluxed for 4 h. The crude product was purified by flash chromatography [*n*-hexane–AcOEt (11 : 9)] followed by preparative TLC [CH_2Cl_2–EtOH (100 : 3), developed twice] to give **5** (182 mg, 14%) as a yellow oily substance. ^{1}H-NMR δ: 0.53 (3H, s, 18-H), 1.19 (3H, d, J=6.1 Hz, 21-H), 1.23 (6H, s, 26,27-H), 2.35—2.43, 2.48—2.56 (each 2H, m, CH_2CO), 3.18—3.32, 3.42—3.53 (each 1H, m, 23-H), 4.75 (2H, s, CH_2CCl_3), 5.00

(1H, br s, 19-H), 5.20 (1H, m, 3-H), 5.36 (1H, br s, 19-H), 6.02 (1H, d, $J=12.0$ Hz, 7-H), 6.30 (1H, d, $J=12.0$ Hz, 6-H). UV λ_{max} nm: 260, λ_{min} nm: 228.

(5*Z*,7*E*)-(1*S*,3*R*)-1,25-Dihydroxy-22-oxa-9,10-secocholesta-5,7,10(19)-trien-3-yl Hemiglutarate (OCT-3-HG, 6) To a solution of **5** (180 mg) in THF (22.5 ml)–H_2O (9.0 ml), 1 M NaH_2PO_4 (4.5 ml) and Zn (4.5 g) were added and the mixture was stirred at 0 °C for 4.5 h under N_2. After filtration, the filtrate was extracted with CH_2Cl_2. The organic layer was washed (H_2O), dried ($MgSO_4$), and evaporated down. The crude product was purified by flash chromatography [CH_2Cl_2–EtOH (12 : 1)] to give **6** (90 mg, 62%). ^{1}H-NMR δ: 0.53 (3H, s, 18-H), 1.19 (3H, d, $J=6.1$ Hz, 21-H), 1.23 (6H, s, 26,27-H), 2.33—2.42 (4H, m, 2 × CH_2CO), 3.18—3.31, 3.42—3.53 (each 1H, m, 23-H), 3.84 (1H, m, 20-H), 4.39 (1H, m, 1-H), 4.98 (1H, br s, 19-H), 5.17 (1H, m, 3-H), 5.33 (1H, br s, 19-H), 5.98 (1H, d, $J=11.0$ Hz, 7-H), 6.29 (1H, d, $J=11.0$ Hz, 6-H). UV λ_{max} nm: 263, λ_{min} nm: 227. MS *m/z*: 532 (M^+).

Antiserum Production The hapten (**6**, 14.2 mg) was coupled with BSA (30 mg) by *N*-succinimidyl ester method[7] to give the desired conjugate (27.5 mg). The number of hapten molecules linked to a BSA molecule was determined to be 8 by UV spectrometric analysis [OCT: ε value (16981) at 264 nm]. The suspension of conjugate (1.0 mg) in saline (0.5 ml) was emulsified with FCA (0.5 ml), and the emulsion was injected into three domestic female albino rabbits (3 months old) subcutaneously at multiple sites along the back. The procedure was repeated biweekly for 5 months using the conjugate (0.5 mg). Blood was collected 10 d after the last immunization and allowed to stand overnight at 4 °C. Centrifugation at 4 °C (1000 × *g* for 20 min) afforded the antisera (As-1—3), which were stored at −20 °C until use.

Radioimmunoassay Procedure A sodium phosphate buffer (0.05 M, pH 7.3) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 was used in the RIA. A series of standard OCT (0—1000 pg) and [^{3}H]-OCT (*ca.* 15000 dpm) dissolved in EtOH (each 25 μl) was added to the diluted antiserum with the buffer (0.5 ml), and the mixture was incubated at 4 °C for 4 h. After addition of the suspension containing dextran (0.1%)–charcoal (0.5%) in the buffer (0.5 ml), the mixture was vortex-mixed, allowed to stand at 0 °C for 20 min, and then centrifuged at 4 °C (1000 × *g* for 10 min). The supernatant was transferred by decantation into a vial containing the tT-21 scintillation cocktail (10 ml),[8] and the radioactivity was measured.

Cross-Reaction Study The cross-reactions of antisera were tested with 4—8 kinds of closely related secosterols. The results were expressed according to the method reported by Abraham.[9]

Results and Discussion

The specificity of antibodies to small molecules is known to be significantly influenced by the position on the hapten used for the conjugation with a carrier protein. The C-3 position of OCT is thought to be suitable site for the conjugation, because it is essentially remote from the 22-oxa-side chain which is characteristic of the OCT structure. Therefore, we synthesized a haptenic derivative, OCT-3-HG (**6**) from the silyl ether (**2**) which was obtained from 1α-hydroxydehydroepiandrosterone (**1**) by the reported method[2] (Chart 2). First, compound **2** was converted into the triol (**3**) by usual desilylation. Selective acylation of the 3-hydroxy group on compound **3** at a low temperature[6] gave the monoester (**4**). Irradiation of compound **4** followed by thermal isomerization afforded a mixture from which the OCT derivative (**5**) was separated by successive chromatographies. Finally, the trichloroethyl group of compound **5** was removed by zinc and 1 M NaH_2PO_4 to give the desired hapten (**6**). The structures of all these compounds were confirmed by various spectral data.

A BSA conjugate of the hapten having a satisfactory molar ratio as immunogen, was prepared by *N*-succinimidyl ester method.[7] Three antisera (As-1—3) were then produced by the immunization of rabbits with the conjugate, and their specificity in RIA was examined.

RIA was carried out using [^{3}H]-OCT as a labeled antigen, and the separation of bound and free fractions was performed by a dextran-charcoal method. Every antiserum showed a satisfactorily high titer (Fig. 1), high affinity constant[10] (Table I), and sensitive dose-response curve (Fig. 2). The minimal detectable amount, that is twice the standard deviation of the zero determination ($n=10$), was 9.0, 1.5, and 2.5 pg/tube with As-1, 2, and 3, respectively.

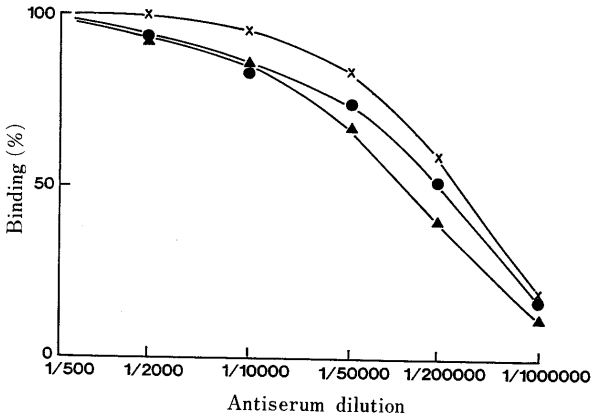

Fig. 1. Antibody Dilution Curves Obtained with As-1—3

The bound radioactivity obtained with the antiserum diluted at 1 : 500 was arbitrarily taken as 100%. ●, As-1; ▲, As-2; ×, As-3.

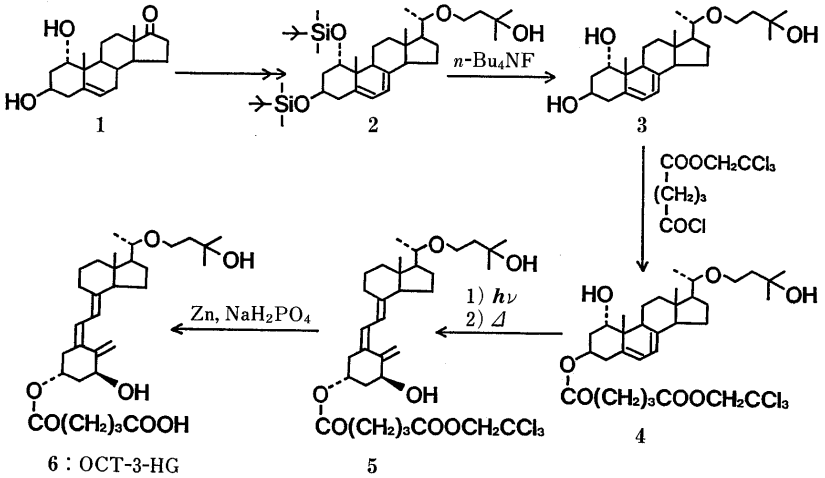

Chart 2. Synthesis of OCT Hapten

TABLE I. Per Cent Cross-Reaction of Anti-OCT Antisera

Compound	Antiserum (Titer[a])		
	As-1 (1:220000)	As-2 (1:132000)	As-3 (1:385000)
OCT	100	100	100
D_3	<0.02	<0.01	<0.01
25(OH)D_3	22	4.0	0.39
1,25(OH)$_2D_3$	28	7.5	0.49
24,25(OH)$_2D_3$	0.45	0.93	0.05
25*R*,26(OH)$_2D_3$	—	—	0.02
25*S*,26(OH)$_2D_3$	—	—	0.06
24(OH)OCT	—	—	4.9
1,20(OH)$_2$hexanor-D_3	—	—	0.02
K_a ($M^{-1} \times 10^{-10}$)[b]	0.63	0.65	1.8

a) Final dilution of antiserum needed to bind 50% of [^{3}H]-OCT. *b*) Affinity constant obtained by Scatchard analysis.[10]

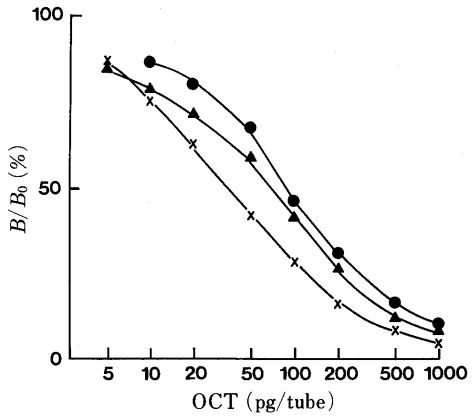

Fig. 2. Dose–Response Curves for OCT Radioimmunoassays
●, As-1; ▲, As-2; ×, As-3.

The specificity of antisera was assessed by a cross-reaction study with various secosterols having related structures to OCT. First, the cross-reactivities with four kinds of endogenous compounds, D_3 and its metabolites, were examined (Table I). As-3 was found to have the highest ability to discriminate these compounds, showing rather large variation of the values among the three antisera. It is noteworthy that the cross-reactivity with 1,25(OH)$_2D_3$ was markedly low (0.49%), demonstrating that the antiserum recognizes the oxygen atom at 22-position. The other compounds having the 1α-deoxygenated A ring structure gave even lower values. Especially, the result with 25(OH)D_3 (0.39%) which is one of the major D metabolites circulating in blood is believed excellent from the view of practical use, because the metabolite has been anticipated to interfere with the OCT assay due to its much higher plasma level.[11] With 24,25(OH)$_2D_3$ and D_3, only negligible values (0.05% and <0.01%) were found for the antiserum. Therefore, the cross-reactivity of As-3 was further examined with four additional compounds and the results are shown in Table I. We found fairly low cross-reactivity with (25*R*)- and (25*S*)-25,26(OH)$_2D_3$, both of which are considered to be a D_3 metabolite (0.02% and 0.06%). Furthermore, the cross-reactivities with two kinds of predicted OCT metabolites, 24(OH)OCT and 1,20(OH)$_2$-hexanor-D_3 (Chart 1), was satisfactorily low (4.9% and 0.02%) showing that the antiserum well recognizes the modification on the 22-oxa-side chain.

In conclusion, the obtained anti-OCT antiserum, As-3, has satisfactorily high specificity as well as high titer and affinity and will be applicable to the pharmacokinetic study of OCT without complicated pretreatment such as successive high performance liquid chromatography. The details will be reported elsewhere in future.

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