Synthesis and Assignment of Novel [^{125}I]-Labeled $1\alpha,25$ -Dihydroxyvitamin D_3 Derivatives

Miyuki TANABE, Masaya HARADA, Fumitomo ODAWARA,
Shigeru IKUTA, Nobuaki NAKAGAWA,
and Masaru OTANI

*Research Laboratories, Toyo Jozo Co., Ltd., Mifuku 632-1,
Ohito-cho, Tagata-gun, Shizuoka-ken 410-23, Japan*

(Received September 1, 1990)

Summary This paper describes the synthesis and structural assignment of novel ^{125}I - $1\alpha,25$ -dihydroxyvitamin D_3 derivatives (**1a**) and (**1b**) labeled with ^{125}I -Bolton-Hunter reagent [*N*-succinimidyl 3-(4-hydroxy-3-iodo[^{125}I]phenyl)propionate] (*I*), which is known as a protein-labeling reagent, as tracers for radioimmunoassay (RIA). The radiospecific activities of these tracers (**1a**) and (**1b**) were calculated as 2,200 Ci/mmol (81.4 TBq/mmol).

Key Words $1\alpha,25$ -dihydroxyvitamin D_3 , iodo[^{125}I]-labeled $1\alpha,25$ -dihydroxyvitamin D_3 , ^{125}I -Bolton-Hunter reagent, anti- $1\alpha,25$ -dihydroxyvitamin D_3 sheep serum, radioimmunoassay (RIA)

It is already recognized that vitamin D_3 is metabolized to 25-hydroxyvitamin D_3 (25-OH- D_3) in the liver, and subsequently to $1\alpha,25$ -dihydroxyvitamin D_3 ($1\alpha,25$ -(OH) $_2$ - D_3) in the kidney.

$1\alpha,25$ -(OH) $_2$ - D_3 is a hormone-like substance with very high biological activity. The known radioactive vitamin D_3 derivatives are known as ^3H - and ^{14}C -labeled compounds which have played an important part in the determination and estimation of vitamin D metabolites in blood (2–6). The measurement for $1\alpha,25$ -(OH) $_2$ - D_3 has been undertaken by RIA or radio-receptor assay (RRA) using a ^3H -tracer (7). On the other hand, radioactive iodo[^{125}I]-residues have been used in labeling small molecules such as iodo[^{125}I]histamine, iodo[^{125}I]tyramine, iodo[^{125}I]tyrosine, and 3-(4-hydroxy-3-iodo[^{125}I]phenyl)propionate-residues.

^{125}I -Bolton-Hunter reagent (^{125}I -BHR), which possesses the active ester moiety of 3-(4-hydroxy-3-iodo[^{125}I]phenyl)propionic acid with *N*-hydroxy succinimide, is known as an available protein-labeling reagent.

This non-direct labeling method using ^{125}I -BHR under mild conditions is very suitable for labeling unstable small molecules such as vitamin D. We have tried to label vitamin D_3 derivative by using chloramine T method of direct labeling or

Scheme 1. The synthesis of **1a** and **1b**. Reaction conditions: *i*) Me₃C-CO-Cl, Fmoc-β-Ala, DMAP, in anhydrous THF, at -15°C for 15 min → at 0°C for 1.5 h; *ii*) in morpholine, at room temp. for 2 h; *iii*) ¹²⁵I-BHR, Et₃N, in anhydrous THF at room temp. for 24 h.

Fig. 1. The structure of **1a** and **1b**.

exchanging halogen (chlorine or bromine) to iodo[¹²⁵I], while this reaction produced little ¹²⁵I-vitamin D₃ derivative together with complex mixtures of products or low radioactivity product.

Therefore, we investigated the synthesis method for highly radioactive derivatives to estimate 1α,25-(OH)₂-D₃ in serum with high sensitivity, and we successfully synthesized iodo[¹²⁵I]-labeled 1α,25-(OH)₂-D₃ derivatives (**1a** and **1b**) by the use of ¹²⁵I-BHR. Here, we report the synthesis (Scheme 1) and structural

assignment of derivatives **1a** and **1b** (Fig.1) in detail.

EXPERIMENTAL

General. Proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectra were recorded with a JEOL GSX-400 and a JEOL FX-90A spectrometer in CDCl_3 ; chemical shifts are reported in parts per million downfield from tetramethylsilane, and coupling constants are reported in Hz.

Ultraviolet (UV) spectra were recorded on a Hitachi U-3200 spectrophotometer in ethanol solution. High-performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-6A equipped with a Shimadzu SPD-6A spectrophotometer and/or an Aloka RLC-551 radioactive analyzer and an Aloka FGD-101 gamma-flow detector. All solvents were distilled and/or dried prior to use by standard methods. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. All air-sensitive reactions were run under an argon atmosphere. $1\alpha,25$ -Dihydroxyvitamin D_3 ($1\alpha,25\text{-(OH)}_2\text{-D}_3$), $24R,25$ -dihydroxyvitamin D_3 ($24R,25\text{-(OH)}_2\text{-D}_3$), and 25 -hydroxyvitamin D_3 (25-OH-D_3) were purchased from Kali-Dupha Co., Ltd. $25,26$ -Dihydroxyvitamin D_3 ($25,26\text{-(OH)}_2\text{-D}_3$), 25 -hydroxy- 24 -oxo-vitamin D_3 ($25\text{-OH-}24\text{-oxo-D}_3$), 25 -hydroxyvitamin D_3 - $26,23$ -lactone (25-OH-D_3 - $26,23$ -lactone) were kindly donated by Dr. Yamada, Institute for Medical and Dental Engineering, Tokyo Medical and Dental University. $^3\text{H-}1\alpha,25\text{-(OH)}_2\text{-D}_3$ used was TRK.656 of Amersham Co., Ltd. ^{125}I -Bolton-Hunter reagent (^{125}I -BHR) used was from New England Nuclear (NEX-120-10, 2,200 Ci/mmol, 1 Ci = 37 GBq).^{*} Non-radioactive Bolton-Hunter Reagent (I-BHR) was synthesized in our laboratory using methods reported in the literature.^{**}

Synthesis of Fmoc protected amino- $1\alpha,25\text{-(OH)}_2\text{-D}_3$ derivatives, (3a) and (3b). Trimethylacetyl chloride, $3.07\ \mu\text{l}$ ($25\ \mu\text{mol}$), and N,N -dimethylaminopyridine (DMAP) (**8**), $3.05\ \text{mg}$ ($25\ \mu\text{mol}$), were added to 3-(N -fluorenylmethyloxycarbonyl)amino propionic acid: Fmoc- β -Ala $7.75\ \text{mg}$ ($25\ \mu\text{mol}$) dissolved in anhydrous THF ($2\ \text{ml}$), and the mixture was kept at -15°C for 15 min. $1\alpha,25\text{-(OH)}_2\text{-D}_3$

^{*} We used ^{125}I -BHR of NEX-120-10(NEN) in this experiment. In another experiment with ^{125}I -BHR of IM.5861 (Amersham), it was ascertained that this reagent also gave **1a** and **1b**.

^{**} Non-radioactive I-BHR was synthesized by the reaction with N -succinimidyl 3-(4-hydroxyphenyl)propionate and benzyltrimethyl ammonium dichloroiodate (BTMA Cl_2I) (**9**) in our laboratories. On reaction for 2 h at room temperature, a mixture of 3-monoiodate (I-BHR) and 3,5-diiodate (I_2 -BHR) was obtained, which were separated by HPLC and the structures were assigned by NMR. Using 3-monoiodate (I-BHR), **5a** and **5b** were synthesized. $^1\text{H-NMR}$ ($\text{DMSO-}d_6$, 400 MHz) ppm I-BHR: 2.80–3.05 (8H, m, $\text{N-CO-CH}_2 \times 2$, O-CO-CH_2 , Ph-CH_2), 6.78 (1H, d, Ph-H , $J=8.3$), 7.12 (1H, dd, Ph-H , $J=1.95$, and 8.3), 7.60 (1H, d, Ph-H , $J=1.95$), 10.15 (1H, b, Ph-OH); I_2 -BHR: 2.78–2.82 (6H, m, $\text{N-CO-CH}_2 \times 2$, O-CO-CH_2), 2.97 (2H, m, Ph-CH_2), 7.69 (2H, s, Ph-H), 9.35 (1H, s, Ph-OH).

D₃ (**2**), 10.4 mg (25 μ mol), dissolved in anhydrous THF (0.5 ml) was then added and the mixture allowed to react at -15°C for 5 min and at 0°C for 1.5 h.

Methanol (0.5 ml) was added and the mixture was evaporated *in vacuo*. The residue was separated by silica-gel column chromatography (Wakogel C-200, Wako Pure Chemical Industries, Ltd.), 150 g, ethyl acetate/*n*-hexane (4:1) to obtain three fractions (a mixture of **3a**, 15%, and **3b**, 5.3%, **3c**, 19.1%, and unreacted **2**, 41.2%).

The mixture of **3a** and **3b** was separated by HPLC (DuPont: Zorbax-ODS $\phi 4.6 \times 250$ mm, 10% H₂O-methanol, 1 ml/min).

3a: ¹H-NMR (400 MHz) 0.55 (3H, s, CH₃-18), 0.93 (3H, d, CH₃-21, $J=6.3$), 1.22 (6H, s, CH₃-26, and -27), 2.38–2.43 (1H, m, H-4 β), 2.53 (2H, m, O-CO-CH₂), 2.59–2.63 (1H, m, H-4 α), 2.76–2.80 (1H, m, H-9 β), 3.44–3.48 (2H, m, N-CH₂), 4.22 (1H, t, FMOC-CH), 4.37–4.41 (3H, m, FMOC-CH₂, and H-1 β), 5.03 (1H, d, H-19), 5.27–5.30 (2H, m, H-3 α , and NH), 5.35 (1H, m, H-19), 6.01 (1H, d, H-7, $J=11.2$), 6.34 (1H, d, H-6, $J=11.2$), 7.29–7.77 (8H, m, FMOC-Ph); UV_{max}, 300.5, 266.3, 214.6 nm; HPLC-Rt (retention time), 17.5 min.

3b: ¹H-NMR (400 MHz) 0.49 (3H, s, CH₃-18), 0.92 (3H, d, CH₃-21, $J=6.3$), 1.20 (3H, s, CH₃-26), 1.27 (3H, s, CH₃-27), 2.50–2.54 (3H, m, O-CO-CH₂, and H-4 α), 2.71–2.80 (1H, m, H-9 β), 3.37–3.48 (2H, m, N-CH₂), 4.05–4.15 (1H, m, H-3 α), 4.21 (1H, m, FMOC-CH), 4.41–4.46 (2H, m, FMOC-CH₂), 5.05 (1H, m, H-19), 5.27 (1H, t, NH), 5.32 (1H, m, H-19), 5.50 (1H, m, H-1 β), 5.90 (1H, d, H-7, $J=11.2$), 6.30 (1H, d, H-6, $J=11.2$), 7.28–7.79 (8H, m, FMOC-Ph); UV_{max}, 300.4, 266.4, 214.3 nm; HPLC-Rt, 14.9 min.

3c: ¹H-NMR (90 MHz) 0.50 (3H, s, CH₃-18), 0.90 (3H, d, CH₃-21), 1.20 (3H, s, CH₃-26), 1.26 (3H, s, CH₃-27), 3.30–3.55 (4H, m, N-CH₂ $\times 2$), 4.10–4.50 (4H, m, FMOC-CH₂ $\times 2$), 5.05–5.60 (6H, m, H-19, H-3 α , H-19, H-1 β , NH $\times 2$), 5.90 (1H, d, H-7), 6.32 (1H, d, H-6), 7.20–7.83 (16H, m, FMOC-Ph).

Synthesis of amino-1 α ,25-(OH) $_x$ D₃ derivatives, (4a) and (4b). Ethanol (2.5 ml) containing **3a**, 13.4 mg (0.019 mmol), was added to morpholine (2.5 ml), and the mixture was stirred at room temp. for 2 h. The mixture was concentrated *in vacuo*, and the residue was purified by LH-20 (Pharmacia Co., Ltd.) and Amprep-NH₂ (Amersham Co., Ltd.) column chromatography. LH-20 (10 ml volume with H₂O) was used to remove morpholine, after washing with H₂O (20–30 ml), and the product was eluted with methanol. The eluted product was charged onto Amprep-NH₂ column (500 mg with *n*-hexane-CH₂Cl₂ (1:1)), and washed with the same solvent (30 ml), then eluted with ethanol to obtain **4a**: isolated yield, 46.3%; ninhydrin reaction: positive; UV_{max}, 264.2 nm.

The same treatment of **3b** with morpholine gave corresponding **4b**: isolated yield, 62.5%; ninhydrin reaction: positive; UV_{max}, 265.4 nm.

Synthesis and purification of ¹²⁵I-labeled 1 α ,25-(OH) $_x$ D₃ derivatives, (1a) and (1b). ¹²⁵I-BHR 50 μ Ci/22.7 pmol (NEN, NEX-120-10, 2,200 Ci/mmol, 3.3 mCi/ml), triethylamine (Et₃N) 300 pmol diluted with anhydrous THF, and anhydrous THF (100 μ l) were added to ethanol solution (25 μ l) containing **4a** 72.8 nmol. The

mixture was reacted at room temp. for 24 h. The product was purified by HPLC (conditions (a): DuPont, Zorbax-ODS $\phi 4.6 \times 250$ mm, 15% H_2O -methanol, 1 ml/min, or (b): DuPont, Zorbax-SIL $\phi 4.6 \times 250$ mm, 20% isopropanol-*n*-hexane, 1 ml/min) to obtain **1a**. HPLC-Rt: (a) 13.5–15.6 min; (b) 16–22 min, radioactivity recovery by method (b), 27.4 μCi (54.8%).

The same treatment of **4b** with ^{125}I -BHR gave the corresponding **1b**. HPLC-Rt: (a) 14.2–16.8 min; (b) 13.6–16.1 min, radioactivity recovery by chromatography (b), 30.2 μCi (60.4%).

Synthesis of non-radioactive derivatives corresponding to 1a and 1b, (5a) and (5b). Non-radioactive BHR (I-BHR), 1.1 mg (2.9 μmol), and Et_3N , 0.4 μl (2.9 μmol), were added to a solution of **4a**, 1.41 mg (2.9 μmol), maintained at 5°C for 16 h. The reaction mixture was distilled *in vacuo*, and the product (**5a**) was separated by HPLC (Mitsui Toatsu: Zorbax Pro-10 (SIL) $\phi 4.6 \times 250$ mm, 20% isopropanol-*n*-hexane, 1 ml/min); yield, 60.4%.

5a: ^1H -NMR (400 MHz) 0.52 (3H, s, CH_3 -18), 0.94 (3H, d, CH_3 -21, $J=6.35$), 1.23 (6H, s, CH_3 -26, and -27), 2.29–2.47 (5H, m, O-CO- CH_2 , N-CO- CH_2 , and H-4 β), 2.58 (1H, m, H-4 α), 2.75–2.85 (3H, m, Ph- CH_2 , and H-9 β), 3.37–3.55 (2H, m, N- CH_2), 4.39 (1H, m, H-1 β), 5.04 (1H, m, H-19), 5.21 (1H, m, H-3 α), 5.37 (1H, m, H-19), 5.89 (1H, m, NH), 5.99 (1H, d, H-7, $J=11.2$), 6.32 (1H, d, H-6, $J=11.2$), 6.86 (1H, d, Ph-H, $J=8.30$), 7.01 (1H, dd, Ph-H, $J=8.30$ and 1.95), 7.48 (1H, d, Ph-H, $J=1.95$); UV_{max} , 266.2 nm.

The same treatment of **4b** gave the corresponding **5b**; yield, 79.1%. **5b**: ^1H -NMR (400 MHz) 0.48 (3H, s, CH_3 -18), 0.93 (3H, d, CH_3 -21, $J=6.35$), 1.22 (6H, s, CH_3 -26, and -27), 2.28–2.47 (5H, m, O-CO- CH_2 , N-CO- CH_2 , and H-4 β), 2.62 (1H, m, H-4 α), 2.78–2.87 (3H, m, Ph- CH_2 , and H-9 β), 3.38–3.53 (2H, m, N- CH_2), 4.14 (1H, m, H-3 α), 5.05 (1H, m, H-19), 5.28 (1H, m, H-19), 5.48 (1H, m, H-1 β), 5.90 (1H, d, H-7, $J=11.2$), 5.96 (1H, m, NH), 6.37 (1H, d, H-6, $J=11.2$), 6.87 (1H, d, Ph-H, $J=8.30$), 7.05 (1H, dd, Ph-H, $J=8.30$ and 1.95), 7.50 (1H, d, Ph-H, $J=1.95$); UV_{max} , 268.7 nm.

The identification of **1a** and **5a** (**1b** and **5b**) was confirmed by HPLC (analytical conditions: DuPont, Zorbax-ODS $\phi 4.6 \times 250$ mm; H_2O -MeOH-MeCN (1:4.5:4.5); flow rate 1 ml/min; UV detection, 265 nm).

Affinity of the antibody (S-2) to vitamin D compounds. Competition using ^3H -1 α ,25-(OH) $_2$ -D $_3$ to vitamin D compounds was carried out in the following operation. H_2O solution containing 50% ethanol 50 μl of ^3H -1 α ,25-(OH) $_2$ -D $_3$ (ca. 30,000 dpm), 50% ethanol solution 20 μl of 1 α ,25-(OH) $_2$ -D $_3$ or the other vitamin D compound (1–1,000 pg/tube), and 0.1 M Tris-HCl buffer, pH 7.4 (500 μl) containing the antibody (S-2) were mixed and incubated at 4°C for 4 h. Dextran-charcoal solution (300 μl : 0.1 M glycine buffer, pH 8.6, containing 0.25% Norit SX-3 (Wako Pure Chemical Industries, Ltd.) and 0.025% dextran T-70 (Pharmacia Co.) was added to the mixture, after which it was incubated at 4°C for 30 min.

After centrifugation at 4°C for 15 min ($1,500 \times g$), scintillation cocktail reagent (2.5 ml; Insta-Gel: Packard Co.) was added to the supernatant (500 μl), and

the cocktail mixture was counted by a liquid-scintillation counter for 10 min.

*Typical calibration curve of $1\alpha,25-(OH)_2D_3$ as a standard curve using **1a** or **1b** with the antibody (S-2).* A mixture of 50% ethanol solution (20 μ l) of $1\alpha,25-(OH)_2D_3$ (4–512 pg), 50% ethanol solution (50 μ l) of **1a** or **1b** (ca. 18,000 cpm) and 0.1 M Tris-HCl buffer, pH 7.4, (500 μ l) containing the antibody (S-2) was incubated at 4°C for 4 h. After addition of the above buffer (300 μ l) containing dextran-charcoal, the mixture was incubated at 4°C for 30 min, and centrifuged at 4°C for 10 min (1,500 $\times$ g).

The supernatant (500 μ l) was counted for a minute by auto-well γ -counter.

RESULTS AND DISCUSSION

The identification of **1a** and **5a** as well as **1b** and **5b** was confirmed by HPLC (Fig. 2). **5a** and **5b** are cold-compounds corresponding to iodo[^{125}I]-labeled $1\alpha,25-(OH)_2D_3$ derivatives (**1a** and **1b**). In labeling with ^{125}I -BHR, it is considered that the shift of **1a** and **1b** on HPLC retention time (Rt) derives from the presence of more polar moiety involving the amide and phenol residues in these molecules (Rt of $1\alpha,25-(OH)_2D_3$ under the same conditions: 7.38 min). The specific activity of

Fig. 2. The identification of **1a** and **1b**. Figure 2a indicates identification of **1a** with **5a**. Figure 2b indicates identification of **1b** with **5b**. HPLC conditions in Fig. 2a and Fig. 2b are the same. Analytical conditions: Zorbax-ODS (DuPont) $\phi 4.6 \times 250$ mm, H_2O -MeOH-MeCN (1:4.5:4.5), flow rate 1 ml/min, UV detection 265 nm.

Table 1. The cross-reactivity of vitamin D metabolites for the antibody (S-2).

	Cross-reactivity (%)
1 α ,25-(OH) ₂ -D ₃	100
25-OH-D ₃	35.4
25-OH-24-oxo-D ₃	14.3
24R,25-(OH) ₂ -D ₃	3.5
25,26-(OH) ₂ -D ₃	1.8
25-OH-D ₃ -26,23-lactone	1.8
1 α -OH-D ₃	< 0.1
D ₃	< 0.1
D ₂	< 0.1
5a	24.7
5b	24.7

As the value (A) of the 50 (%) displacement of maximal binding of 1 α ,25-(OH)₂-D₃ and the value (B) of the 50 (%) displacement of maximal binding each vitamin D metabolites, the cross-reactivity percent is calculated as (A/B) $\times$ 100(%).

1a or **1b** was 2,200 Ci/mmol (81.4 TBq/mmol) and in ethanolic solution of **1a** or **1b** was stable for 60 days at -20°C .

In affinity between the antibody and **1a** or **1b** and native 1 α ,25-(OH)₂-D₃ the cross-reactivity was based on the competition to 1 α ,25-(OH)₂-D₃ with **5a** or **5b** or other vitamin D compounds using the antibody (S-2) obtained by immunizing sheep*** (Table 1).

From the result it is indicated that the antibody recognizes 1 α - and 25-OH groups, especially 25-OH moiety more than 1 α - or 3 β -OH moiety; the reason is that the antibody is an antiserum obtained by immunizing with an antigen modified 1 α - or 3 β -OH site. This result is consistent with experienced law that the antibody recognizes the far site as against the site of attachment to the ligand. No differences between **5a** and **5b** is caused by using a mixture of an antigen modified 1 α - and 3 β -OH site.

The affinity of **1a** and **1b** is approximately 4-times less than that of 1 α ,25-(OH)₂-D₃; it is suggested that introduction of iodo phenyl moiety causes a decrease of

*** The antibody (S-2) was produced by the following procedure. A mixture of **3a** and **3b** linking covalently to BSA by mixed anhydride reaction was used as antigen of immunizing. Four sheep were immunized with complete Freund's adjuvant at an interval of 2 weeks for 6 months, and 10 ml of serum from a sheep was collected to test a titer at an interval of 1 month. The serum was purified to IgG fraction by HPLC (column: mono-Q HR5/5 (Pharmacia Co.); eluent A: 50 mM Tris-HCl, pH 8.6; eluent B: eluent A with 0.5 M NaCl; gradient: 0–100% eluent B in 20 min; flow rate: 2 ml/min; UV detection: 280 nm). On the above conditions IgG fraction was eluted at 8.5–10.5 min, and albumin fraction was eluted at 13.0–14.0 min. This mono-Q eluted IgG fraction, which has binding activity to ³H-1 α ,25-(OH)₂-D₃, **1a** and **1b**, was identified as an antibody of IgG by electrophoresis.

Fig. 3. The standard curves of **1a** and **1b** by RIA. $\circ$, **1a**; $\triangle$, **1b**. 0.1 M Tris-HCl buffer, pH 7.4; antiserum dilution, $\times 7,000$. Total count (T) of **1a** or **1b** is each 30,000 cpm. Bound (B)/T using **1a** or **1b** is each *ca.* 35%. B_0 is the count of a maximum binding.

recognition. For measurement of vitamin D metabolites in serum, a combination of an antibody with high specificity and an iodo [^{125}I]-labeled tracer with high radioactivity will favor a high-sensitive assay.

Figure 3 demonstrates that the standard curve by RIA using **1a** or **1b** is able to measure in the range of 4–512 pg/test. We are investigating the preparation of more specific antibodies for the purpose of more sensitive assays of $1\alpha,25-(OH)_2-D_3$ with these tracers (**1a** or **1b**).

We thank Dr. Sachiko Yamada (Institute for Medical and Dental Engineering, Tokyo Medical and Dental University) for the present of vitamin D metabolites and valuable advice on nomenclature matters.

REFERENCES

- 1) Bolton, A. E., and Hunter, W. M. (1973): The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I -containing acylating agent. *Biochem. J.*, **133**, 529–539.
- 2) Suda, T., DeLuca, H. F., and Hallick, R. B. (1971): Synthesis of $[26,27-^3\text{H}]$ -25-hydroxycholecalciferol. *Anal. Biochem.*, **43**, 139–146.
- 3) Lawson, D. E. M., Fraser, D. R., Kodicek, E., Morris, H. R., and Williams, D. H. (1971): Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling metabolism. *Nature*, **230**, 228–230.
- 4) Yamada, S., Schnoes, H. K., and DeLuca, H. F. (1978): Synthesis of 25-hydroxy- $[23,24-^3\text{H}]$ vitamin D_3 . *Anal. Biochem.*, **85**, 34–41.
- 5) Yamada, S., Shimizu, M., Fukushima, K., Niimura, K., and Maeda, Y. (1989): Synthesis of 24R,25-dihydroxy- $[6,19,19-^3\text{H}]$ vitamin D_3 and 24R,25-dihydroxy- $[6,19,19-^2\text{H}]$ vitamin D_3 . *Steroids*, **54/2**, 145–157.

- 6) Tanabe, M., Harada, M., Ikuta, S., Nakagawa, N., and Otani, M. (1989): Synthesis of a ^{125}I -labelled derivative of 25-hydroxy vitamin D_3 . *J. Chem. Soc., Chem. Commun.*, 1220–1221.
- 7) Porteous, C. E., Coldwell, R. D., Trafford, R. D. H., and Makin, H. L. J. (1987): Recent development in the measurement of vitamin D and its metabolites in human body fluids. *J. Steroid Biochem.*, **28**(6), 785–801.
- 8) Hassner, A., Krepsi, L. R., and Alexanian, V. (1978): Aminopyridines as acylation catalysts for tertiary alcohols. *Tetrahedron*, **34**, 2069–2076.
- 9) Kajigaeshi, S., Kakinami, T., Yamasaki, H., Fujisaki, S., Kondo, M., and Okamoto, T. (1987): Iodination of phenols by use of benzyltrimethylammonium dichloroiodate. *Chem. Lett.*, 2109–2112.