Synthesis and Biological Activities of 1α,4α,25- and 1α,4β,25-Trihydroxyvitamin D_3 and Their Metabolism by Human CYP24A1 and UDP-Glucuronosyltransferase

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A previous report has demonstrated the existence of a C4-hydroxylated vitamin D_2 metabolite in serum of rats treated with pharmacological doses of vitamin D_2. However, the biological significance and metabolic fate of this metabolite have not been described. To explore its potential biological activities, we therefore synthesized 1α,4α,25-trihydroxyvitamin D_3 and its diastereoisomer, 1α,4β,25-trihydroxyvitamin D_3, using Trost Pd-mediated coupling reaction, and studied their vitamin D receptor (VDR) binding affinity, osteocalcin promoter transactivation activity, and their further metabolism by human CYP24A1 as well as by human liver microsomal fraction based on CYP- and UDP-glucuronosyltransferases (UGTs)-reactions.

Key words trihydroxyvitamin D_3; vitamin D receptor; metabolism; human CYP24A1; human liver microsome; UDP-glucuronosyltransferase

Vitamin D_3 is converted to 25-hydroxyvitamin D_3 [25(OH)D_3], the major circulating form of vitamin D_3, by 25-hydroxylases (CYP2R1 and CYP27A1) in the liver, which was then oxidized by 1α-hydroxylase (CYP27B1) at the C1α-position to produce the physiologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2$$D_3$, **1**], in the kidney and in a variety of target tissues in an autocrine/paracrine manner. 1α,25(OH)$_2$$D_3$ (**1**) acts through its nuclear receptors, vitamin D receptors (VDRs) to regulates cellular growth, differentiation, and apoptosis, in addition to its classical roles in calcium homeostasis and bone mineralization.[1–4] The ubiquitous distribution of VDRs in the body makes this hormone a potentially useful therapeutic agent for certain cancers, skin diseases, and immune disorders. In fact, **1** and some synthetic analogs of **1** have been used clinically in the treatment of bone diseases, secondary

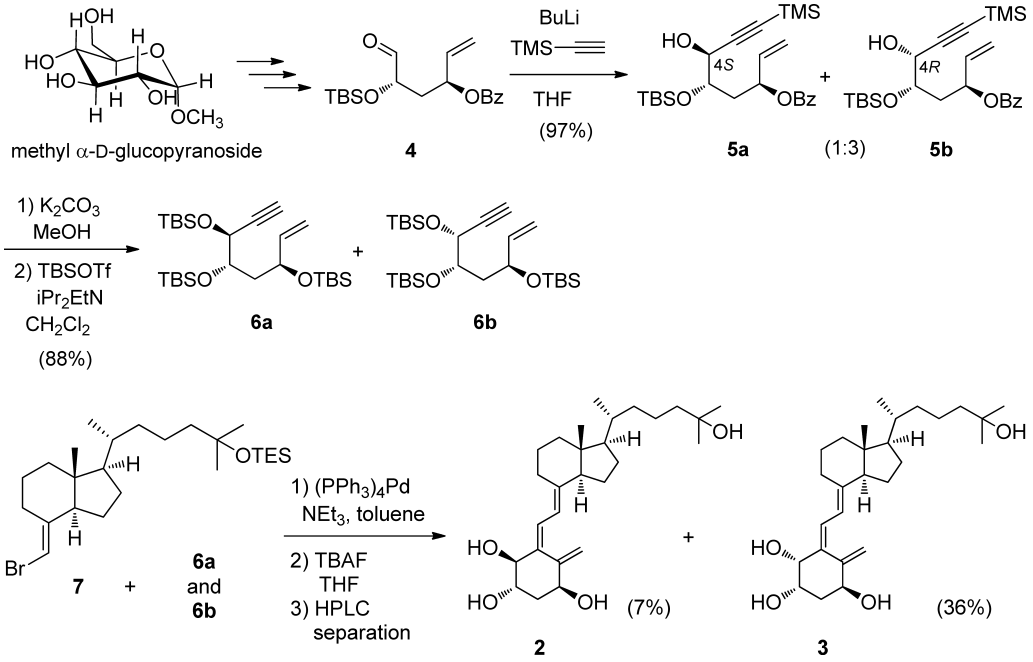

Chart 1. Synthesis of 1α,4α,25- and 1α,4β,25-Trihydroxyvitamin D_3 (**2** and **3**)

The authors declare no conflict of interest.

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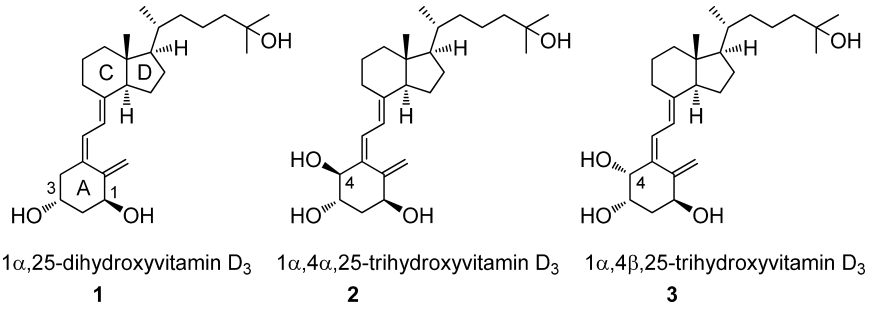

Fig. 1. Structures of 1α,25-Dihydroxyvitamin D_3 (**1**) and Synthetic Trihydroxy Analogs with the 4-Hydroxy Groups **2** and **3**

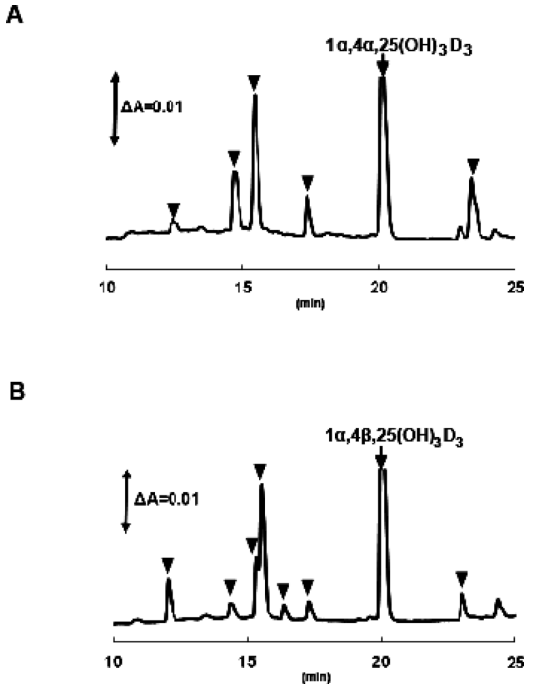

Fig. 2. HPLC Profiles of 1α,4α,25- (A) and 1α,4β,25-Trihydroxyvitamin D_3 (B) and Their Metabolites by Human CYP24A1

After incubation with 5.0 μM of each substrate at 37°C for 60 min in the presence of 20 nM hCYP24A1, 2 μM ADX, 0.2 μM ADR, and 1 mM NADPH, the reaction mixture was extracted, and analyzed by HPLC. The peaks with arrow heads show putative metabolites by human CYP24A1.[12]

Table 1. Relative Binding Affinity for hVDR and Osteocalcin Promoter Transactivation Activity in HOS Cells of **1**–**3**

Compound	hVDR binding affinity[a]	Osteocalcin transactivation activity (EC_{50} (nM))
1α,25$(OH)_2D_3$ (**1**)	100	0.03
1α,4α,25$(OH)_3D_3$ (**2**)	0.9	17.4
1α,4β,25$(OH)_3D_3$ (**3**)	2.9	0.22

a) The potency of 1α,25$(OH)_3D_3$ is normalized to 100.

hyperparathyroidism, psoriasis, and osteoporosis.[5] Both 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ are subject to CYP24A1 degradation *via* C-24 hydroxylation at the side chain. In addition to a number of hydroxylated vitamin D metabolites found in the blood of mammals, Reddy *et al.* have identified 4,25-dihydroxyvitamin D_2 from the serum of rats intoxicated with pharmacological amounts of vitamin D_2 in 1999.[6] However, its biological significance of the 4-hydroxy group remains unclear. Recently, we have synthesized 1α,4β,25-trihydroxyvitamin

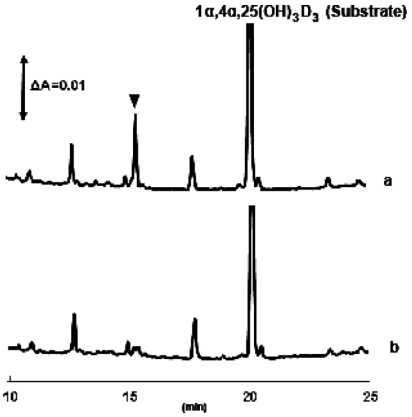

Fig. 3. HPLC Profiles of 1α,4α,25-Trihydroxyvitamin D_3 and Its Metabolite in Human Liver Microsomes

The reaction mixture containing 10 μM substrate, 2 mM UDP-GlcUA, 1 mM $MgCl_2$, 0.5 mg/mL human liver microsomal fraction was incubated at 37°C for 60 min (a), and they were further incubated with β-glucuronidase for 60 min (b). After addition of methanol to the reaction mixture, metabolites were analyzed by HPLC. The peak with arrow head shows a putative metabolite by UGTs.[14]

D_3 (**3**) and evaluated its VDR binding affinity and osteocalcin promoter transactivation activity.[7] Now, we report here the preparation of 1α,4α,25-trihydroxyvitamin D_3 (**2**) and the studies of its biological activities and further metabolism by human CYP24A1 and human liver microsomal fraction to compare with those of 1α,4β,25-trihydroxyvitamin D_3 (**3**) and the natural hormone **1**.

The synthetic route is shown in Chart 1. The A-ring precursors **6a** and **6b** for Trost coupling was prepared from methyl α-D-glucoside *via* aldehyde **4**.[7] TMS-ethynylation of **4** afforded enyne products **5a** and **5b** in a 1 : 3 diastereomeric ratio, and each diastereomer was able to be separated by HPLC. Stereochemistry at the 4-position (steroidal numbering) of the major and minor isomers was determined by the modified Mosher's method as 4*R* and 4*S*, respectively.[7] Deprotection under basic conditions and subsequent TBS-protection afforded the A-ring precursors **6a** and **6b**. The mixture of the diastereomers of enyne **6a** and **6b** was coupled with CD-ring bromoolefin **7** using Pd-catalyst to give the coupling products,[8] and tetrabutylammonium fluoride (TBAF)-deprotection followed by HPLC-separation gave the target molecules **2**[9] with 4α-orientation and **3** with 4β-orientation,[7] respectively.

The binding affinity of 1α,4α,25- and 1α,4β,25-trihydroxyvitamin D_3 for the human VDR was 0.9% and 2.9% of the natural hormone **1**, respectively (Table 1).[10] Osteocalcin promoter transactivation activities expressed as the EC_{50} values were evaluated in the human osteosarcoma cell line HOS

cells, and are shown in Table 1.[11] The data demonstrate that neither stereochemistry of the 4-OH group on the active vitamin D skeleton is effective in enhancing the binding to VDR and inducing VDR transactivation.

Metabolism of 1α,4α,25- and 1α,4β,25-Trihydroxyvitamin D_3 by CYP24A1 and Drug Metabolizing Enzymes in Liver To investigate the metabolism of 1α,4α,25- and 1α,4β,25-trihydroxyvitamin D_3 by the human CYP24A1, a reconstituted system containing adrenodoxin reductase (ADR), adrenodoxin (ADX), and the human CYP24A1 was utilized.[12] Figure 1 shows the HPLC profiles of the substrates 1α,4α,25- and 1α,4β,25-trihydroxyvitamin D_3 and their metabolites produced by the human CYP24A1. Both substrates were metabolized to several metabolites probably by CYP24A1-dependent multi-step oxidation pathways similar to that for 1α,25-dihydroxyvitamin D_3 or its A-ring diastereomers.[13] However, no metabolites were observed from each analog when the heat-inactivated human CYP24A1 was used (data not shown). It should be noted that HPLC profile of the metabolites of 1α,4α,25-trihydroxyvitamin D_3 is quite similar to that of 1α,25-dihydroxyvitamin D_3, while that of 1α,4β,25-trihydroxyvitamin D_3 is quite similar to that of 3-epimers of 1α,25-dihydroxyvitamin D_3.[13] After 60 min incubation, the conversion ratios of 1α,4α,25- and 1α,4β,25-trihydroxyvitamin D_3 were about 45% and 44%, respectively, which were nearly the same conversion ratio of 1α,25-dihydroxyvitamin D_3 (data not shown).

Next, human liver microsomal fraction including drug-metabolizing enzymes was examined for the metabolism of both 4-hydroxyvitamin D_3 analogs. No metabolite from either analog was detected under the experimental conditions with 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mg/mL human liver microsomal fraction, suggesting that both analogs were poor substrates for human hepatic cytochrome P450s (data not shown). However, addition of 2 mM UDP-glucuronic acid produced a metabolite of 1α,4α,25-trihydroxyvitamin D_3 whereas no metabolite of 1α,4β,25-trihydroxyvitamin D_3 was observed (Fig. 2). β-Glucuronidase treatment converted the metabolite of 1α,4α,25-trihydroxyvitamin D_3 back to 1α,4α,25-trihydroxyvitamin D_3 itself (Fig. 2). These results strongly suggest that the metabolite is a glucuronide of 1α,4α,25-trihydroxyvitamin D_3. Based on our findings that neither 1α,4β,25-trihydroxyvitamin D_3 nor 1α,25-dihydroxyvitamin D_3 was converted to its glucuronide, the 4α-hydroxy group of 1α,4α,25-trihydroxyvitamin D_3 appeared to be glucuronidated by some hepatic UGT(s).

Conclusion

We have synthesized the diastereoisomers of 4-hydroxyvitamin D_3 analogs **2** and **3** to study their biological activities and metabolism. Both isomers showed much weaker VDR binding affinity and osteocalcin promoter transactivation activity than the natural hormone. In particular, 4α-hydroxylated analog **2** was found to have the least activities in both assays. Although both isomers were metabolized by hCYP24A1 almost the same level as the natural hormone, their patterns were different from each other. Furthermore, only the 4α-hydroxylated analog was metabolized by UGTs to produce a glucuronate conjugate. Therefore, the stereochemistry of the 4-OH group may affect not only the VDR-mediated biological activities but also their metabolism in human.

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References and Notes

1) Feldman D., Pike J. W., Adams J. S., "Vitamin D," 3rd ed., Elsevier Academic Press, New York, 2011.
2) Brown A. J., Slatopolsky E., *Mol. Aspects Med.*, **29**, 433–452 (2008).
3) Laverny G., Penna G., Uskokovic M., Marczak S., Maehr H., Jankowski P., Ceailles C., Vouros P., Smith B., Robinson M., Reddy G. S., Adorini L., *J. Med. Chem.*, **52**, 2204–2213 (2009).
4) DeLuca H. F., *Nutr. Rev.*, **66** (Suppl. 2), S73–S87 (2008).
5) Kubodera N., *Heterocycles*, **80**, 83–98 (2010).
6) Rao D. S., Dayal R., Siu-Caldera M.-L., Horst R. L., Uskokovic M. R., Tserng K.-Y., Reddy G. S., *J. Steroid Biochem. Mol. Biol.*, **71**, 63–70 (1999).
7) Sawada D., Tsukuda Y., Saito H., Takagi K., Horie K., Ochiai E., Takenouchi K., Kittaka A., *Chem. Pharm. Bull.*, **57**, 1431–1433 (2009). Revised data of compound **3** (4β-OH analog): $[\alpha]_D^{20}$ +108.1 (c=0.2, $CHCl_3$); UV (EtOH) λ_{max} 265.5 nm, λ_{min} 215.0 nm; ^{13}C-NMR (150 MHz, $CDCl_3$) δ 12.1, 18.9, 20.9, 22.3, 23.7, 27.7, 29.3, 29.5, 36.1, 36.5, 37.5, 40.5, 44.5, 46.2, 56.5, 56.6, 68.9, 71.2, 71.5, 76.3, 115.7, 116.6, 125.9, 134.5, 144.8, 146.3.
8) Trost B. M., Dumas J., Villa M., *J. Am. Chem. Soc.*, **114**, 9836–9845 (1992).
9) Spectroscopic data for **2**: $[\alpha]_D^{22}$ +77.8 (c=0.1, $CHCl_3$); UV (EtOH) λ_{max} 267.0 nm, λ_{min} 230.5 nm; IR (neat) 3375, 2926, 1724, 1644, 1467, 1377, 1264, 1054, 909 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.53 (s, 3H), 0.93 (d, J=6.4 Hz, 3H), 1.03–1.09 (m, 1H), 1.22 (s, 6H), 1.17–1.94 (m, 15H), 1.96–2.04 (m, 3H), 2.24 (ddd, J=3.9, 5.4, 13.4 Hz, 1H), 2.84–2.93 (m, 1H), 3.92–4.01 (m, 2H), 4.40 (dd, J=4.2, 4.6 Hz, 1H), 5.06 (d, J=2.0 Hz, 1H), 5.38 (s, 1H), 6.02 (d, J=11.5 Hz, 1H), 6.76 (d, J=11.5 Hz, 1H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 12.1, 18.8, 20.8, 22.2, 23.5, 27.6, 29.2, 29.4, 36.1, 36.4, 38.4, 40.5, 44.4, 46.0, 56.4, 56.5, 71.1, 71.2, 71.6, 78.1, 115.8, 116.5, 123.2, 134.4, 145.4, 145.5; EI-HR-MS Calcd for $C_{27}H_{44}O_4$ $[M+Na]^+$ 455.3132, Found 455.3112.
10) Binding affinity for hVDR was evaluated using a 1α,25(OH)$_2$D$_3$ assay kit (Polarscreen Vitamin D Receptor Competitor Assay, Red, Cat. No. PV4569) purchased from Invitrogen.
11) Transactivation activities were tested according to our previous paper: Kumagai G., Takano M., Shindo K., Sawada D., Saito N., Saito H., Kakuda S., Takagi K., Takimoto-Kamimura M., Takenouchi K., Chen T. C., Kittaka A., *Anticancer Res.*, **32**, 311–318 (2012).
12) The metabolism of **2** and **3** by CYP24A1 were analyzed using the membrane fraction prepared from the recombinant *E. coli* cells expressing human CYP24A1.[13] The reconstituted system contains 0.02 μM of human CYP24A1, 2.0 μM of adrenodoxin (ADX), 0.2 μM of NADPH-adrenodoxin reductase (ADR), 5.0 μM of each of **2** and **3**, 1.0 mM of NADPH, 100 mM Tris–HCl (pH 7.4) and 1 mM EDTA. The reaction was initiated by addition of NADPH. After 60 min of incubation at 37°C, the reaction mixture was extracted with 4 vol. of $CHCl_3$–CH_3OH (3 : 1). The organic phase was recovered and dried up *in vacuo*. The resultant residue was dissolved in acetonitrile and applied to HPLC under the following conditions: column, YMC-Pack ODS-AM (5 μm) (4.6 mm×300 mm) (YMC Co., Kyoto, Japan); UV detection, 265 nm; flow-rate, 1.0 mL min^{-1}; column temperature, 40°C; mobile phase, CH_3CN: a linear gradient of 20–100% CH_3CN aqueous solution per 25 min and 100% CH_3CN for 12 min.
13) Kusudo T., Sakaki T., Abe D., Fujishima T., Kittaka A., Takayama H., Hatakeyama S., Ohta M., Inouye K., *Biochem. Biophys. Res.*

Commun., **321**, 774–782 (2004).

14) The metabolism of **2** and **3** by CYPs and UGTs in human liver microsomes were analyzed as described below. In CYP-dependent oxidation of each analog, the reaction mixture containing 0.5 mg protein/mL of the liver microsomes, 10 μM substrate, 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) was incubated for 60 min at 37°C, and the metabolite was analyzed as described above (ref. 12). In UGT-dependent glucuronidation of each analog, the reaction mixture containing 0.5 mg protein/mL of the liver microsomes,[15] 10 μM substrate, 2 mM UDP-GlcUA, 1 mM $MgCl_2$ in 100 mM potassium phosphate buffer (pH 7.4) was incubated for 60 min at 37°C, and the reaction was terminated by addition of equal volume of cold methanol. After centrifugation at 14500 rpm for 15 min, the supernatant was analyzed with HPLC as described above except for the mobile phase: a linear gradient of 20–95% CH_3CN aqueous solution containing 0.05% trifluoroacetic acid (TFA) per 25 min and 95% CH_3CN containing 0.05% TFA for 12 min. To confirm that the metabolite was glucuronide, the aliquot of the reaction mixture was further incubated for 60 min at 37°C in the presence of 0.1 mg/mL β-glucuronidase in 20 mM potassium phosphate buffer (pH 7.4).

15) Kasai N., Sakaki T., Shinkyo R., Ikushiro S., Iyanagi T., Ohta M., Inouye K., *Drug Metab. Dispos.*, **33**, 102–107 (2005).