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Synthesis of 2α-propoxy-1α,25-dihydroxyvitamin D_3 and comparison of its metabolism by human CYP24A1 and rat CYP24A1

Nozomi Saito [a,†], Yoshitomo Suhara [a,‡], Daisuke Abe [b,§], Tatsuya Kusudo [b,¶], Miho Ohta [c], Kaori Yasuda [d], Toshiyuki Sakaki [d,*], Shinobu Honzawa [a,‖], Toshie Fujishima [a,††], Atsushi Kittaka [a,*]

[a] Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Sagamihara, Kanagawa 229-0195, Japan
[b] Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
[c] Department of Food and Nutrition Management Studies, Faculty of Human Development, Soai University, Osaka 559-0033, Japan
[d] Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, Toyama 939-0398, Japan

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ABSTRACT

A new vitamin D_3 analogue, 2α-propoxy-1α,25-dihydroxyvitamin D_3 (C3O1), was synthesized starting from D-glucose as a chiral template of the A-ring with a CD-ring bromoolefin unit using the Trost coupling method. We studied the metabolism of the new analogue by human CYP24A1 and rat CYP24A1 to learn of species-based differences and found that the former has multiple metabolic pathways, but the latter has only a single pathway.

1. Introduction

The physiologically active metabolite of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (**1**), is a nuclear hormone that regulates cellular growth, differentiation, and apoptosis, in addition to its classical role in calcium homeostasis and bone mineralization.[1–3] Most of the biological effects of **1** are considered to be mediated via binding to the specific intracellular receptor, vitamin D receptor (VDR), which belongs to the nuclear receptor superfamily and acts as a ligand-dependant transcription factor with coactivators.[4] The ubiquitous distribution of VDR in the body makes this hormone a potentially useful therapeutic agent for certain cancers, skin diseases, and immune disorders, and in fact, **1** and some synthetic analogues of **1** are used clinically in the treatment of bone diseases, secondary hyperparathyroidism, and psoriasis.[1] In order to investigate the structure–activity relationship of the natural hormone, we systematically synthesized A-ring modified analogues such as 2α-methyl-, 2α-alkyl- (**2**), 2α-(ω-hydroxyalkyl)- (**3**), and 2α-(ω-hydroxyalkoxy)-1α,25-dihydroxyvitamin D_3 (**4**) (Fig. 1).[5–10]

In regard to modification with the 2α-(ω-hydroxyalkyl) group, it was found that the 2α-(3-hydroxypropyl) group on **1** best fits the cavity of the ligand-binding domain (LBD) of VDR among the 2α-(ω-hydroxyalkyl) analogues of **3**, and the binding activity is threefold higher than that of **1**.[7] Recently, an X-ray crystallographic analysis showed the structure of the complex of the hVDR LBD with **3c** and **4b**.[11] The role of the 2α-(3-hydroxypropyl) group in **3c** was clarified: this moiety enters the water channel around the A-ring moiety at the LBD of the VDR, and the terminal OH group of **3c** replaces one of the water molecules constituting the water channel to maintain hydrogen bond networks, thus making the complex up to threefold more stable than the VDR-**1** complex. Chugai Pharmaceutical Co. Ltd, developed 2β-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 (ED-71, in Fig. 1) as a promising candidate for the treatment of osteoporosis in Japan.[1,12] Although ED-71 shows high calcemic activity and a long half-life in plasma due to its strong affinity for vitamin D binding protein (DBP, twice the affinity of **1**), its affinity for bovine thymus VDR is weaker than that

* Corresponding authors. Tel.: +81 766 56 7500x601; fax: +81 766 56 2498 (T.S.), tel./fax: +81 42 685 3713 (A.K.).
E-mail addresses: tsakaki@pu-toyama.ac.jp (T. Sakaki), akittaka@pharm.teikyo-u.ac.jp (A. Kittaka).
† Present address: Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan.
‡ Present address: Department of Environmental Sciences, Yokohama College of Pharmacy, Yokohama 245-0066, Japan.
§ Present address: Process Engineering Section, Nagase ChemteX Corporation, Kyoto, 620-0853, Japan.
¶ Present address: Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai 487-8501, Japan.
‖ Present address: Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata 956-8603, Japan.
†† Present address: Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima-Bunri University, Kagawa 769-2193, Japan.

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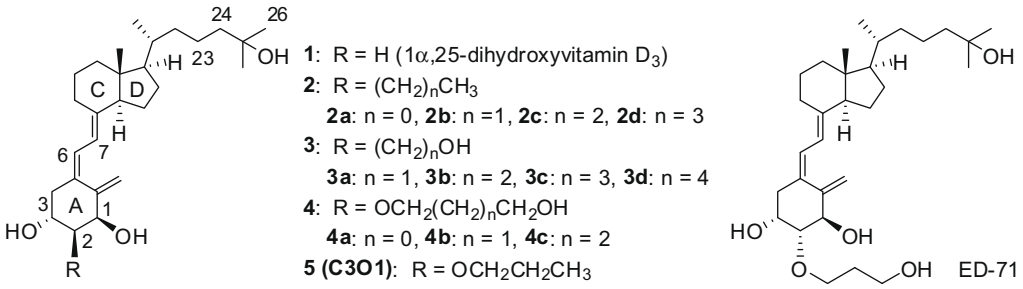


Figure 1. Structures of 1α,25-dihydroxyvitamin D_3 (**1**), its 2α-substituted analogues (**2**–**4**), 2α-propoxy-1α,25-dihydroxyvitamin D_3 (**5**, C3O1), and ED-71.

of the natural hormone (13–93%).[12,13] It was found that one of our synthetic analogues, 2α-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 (**4b**), that is, the 2α-epimer of ED-71, showed greater affinity for VDR (180%) than did **1**.[8] We demonstrated that the biologically active **4b** was produced from the less active 2α-propoxy-1α,25-dihydroxyvitamin D_3 (**5**, C3O1) through metabolic ω-hydroxylation by CYP27A1.[14] We report here the concise synthesis, and the metabolism by human CYP24A1 and rat CYP24A1, of C3O1.

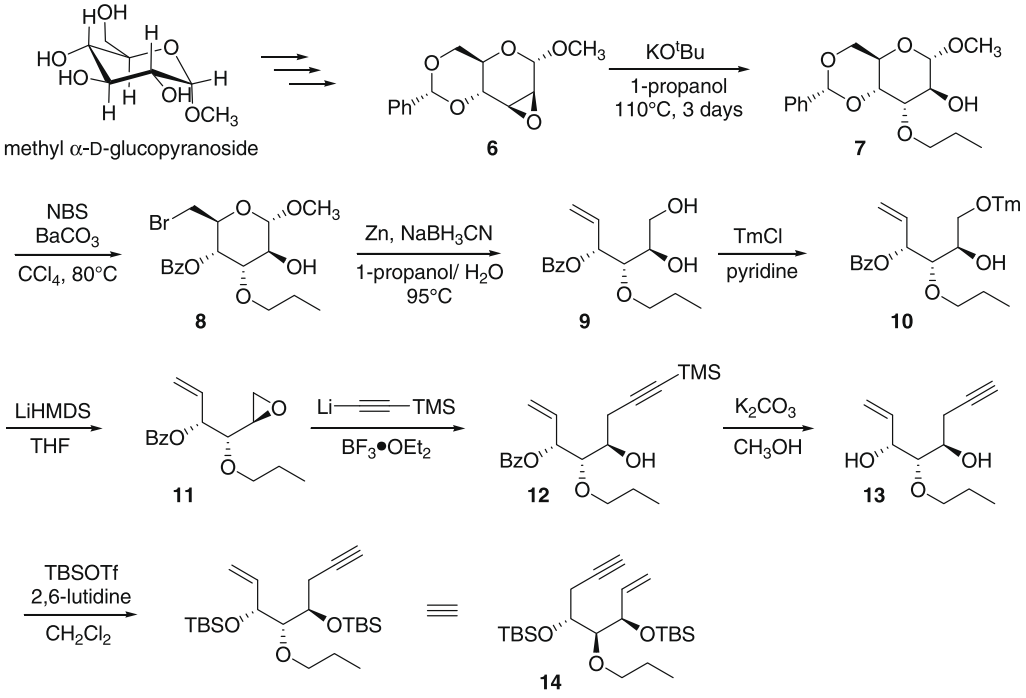
2. Results and discussion

In 2000, we reported that 2α-propyl-1α,25-dihydroxyvitamin D_3 (**2c**) showed strong calcemic activity in vivo, although its affinity for VDR and HL-60 cell differentiation activity were weak in vitro.[7a] We hypothesized that **2c** was hydroxylated at the ω-position of the 2α-side chain of **2c** through metabolism in the rat liver, and consequently, inactive **2c** was converted to active **3c**, which showed strong calcemic activity in vivo. We synthesized **5** to investigate whether this kind of metabolism actually occurs, and proved that **5** was activated through hydroxylation by CYP27A1 and **4b** could be detected by LC–MS.[14a]

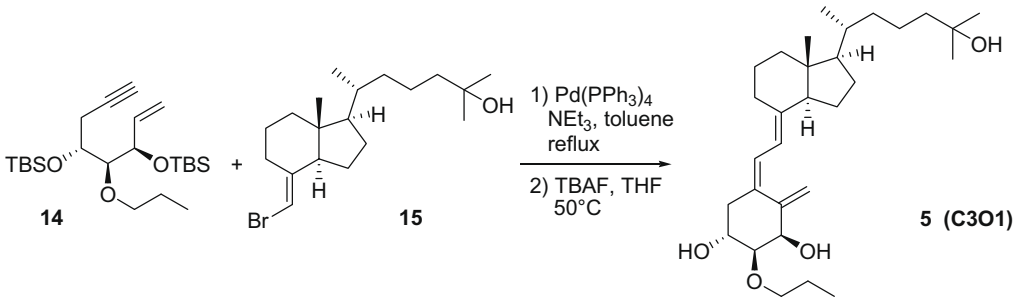
The route by which we synthesized **5** is shown in Scheme 1. To create 1α,2α,3β-stereochemistry on the A-ring of the target vitamin D analogue (**5**), the known crystalline epoxide **6**, which was readily available from methyl α-D-glucoside, was chosen as the chiral template.[8,15] Regiospecific ring opening by 1-propanol at the C3 position established the altrose configuration of **7**, in which the C2, C3, and C4 asymmetric centers satisfy the corresponding desired 3β, 2α, and 1α stereochemistries of **5**, respectively. Treatment of the benzylidene acetal **7** with NBS gave the bromide **8**. The reaction of **8** with activated zinc powder in the presence of $NaBH_3CN$ gave the alcohol **9**. Sulfonylation of the primary hydroxy group and LiHMDS treatment afforded the epoxide **11**, into which was subsequently introduced the acetylene unit with the epoxide-ring opening to give **12**. Removal of the terminal TMS and benzoyl groups under basic conditions afforded the enyne **13**, and protection of the secondary hydroxy groups with the TBS groups provided the A-ring precursor **14**.

Palladium-mediated alkylative cyclization with vinyl bromide of the CD fragment **15**[16] from Grundmann's ketone and subsequent deprotection furnished C3O1 (**5**) (Scheme 2).

The affinity of C3O1 (**5**) for calf-thymus VDR was 64% as compared with the affinity of the natural hormone **1**. The metabolism of C3O1 by human CYP27A1 produced O2C3 (**4b**), which showed 180–204% binding affinity for the VDR.[14] However, inactivation of C3O1 by CYP24A1 also occurs in the body. This time we focused on the metabolism of C3O1 by human CYP24A1 and rat CPY24A1.



Scheme 1. Synthesis of the A-ring precursor **14** for C3O1.



Scheme 2. Trost coupling of the enyne **14** and bromoolefin **15**.

3. Metabolism of C3O1 by CYP24A1

A reconstituted system containing adrenodoxin reductase (ADR), adrenodoxin (ADX), and human CYP24A1 or rat CYP24A1 was examined for the metabolism of C3O1. Figure 2 shows HPLC profiles of the substrate C3O1 and its metabolites produced by human CYP24A1 or rat CYP24A1. The rat CYP24A1-dependent metabolism of C3O1, giving three metabolites M2, M3, and M4, was quite similar to that of **4b** designated as O2C3 in our previous report.[14a] However, in the human CYP24A1-dependent metabolism, we noticed a clear difference between C3O1 and O2C3. M6 was a major metabolite in the metabolism of C3O1, but M6 was not observed in the metabolism of O2C3.[14a] Based on our previous studies, the metabolites M1–M5 are assumed to be 23,26(OH)$_2$–C3O1 (M1), 24-oxo-23(OH)–C3O1 (M2), a mixture of 23(OH)–C3O1 (M3) and 24(OH)–C3O1 (M3), 24-oxo-C3O1 (M4), and 25,26,27-trinor-24-ene-C3O1 (M5), respectively.[14a] On the other hand, the metabolite M6 is assumed to be 23-oxo-C3O1 based on the metabolism of 1β,25(OH)$_2$-3-*epi*-D$_3$.[14b] To confirm these assumptions, we collected M3 and M6 produced by human CYP24A1, and subjected them to mass spectrometric analysis.

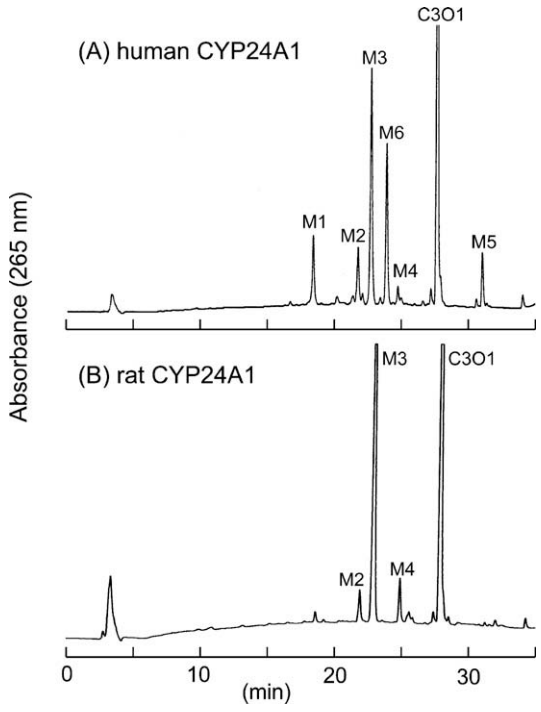


Figure 2. HPLC profiles of C3O1 and its metabolites with human CYP24A1 (A) and rat CYP24A1 (B). After incubation with 4.0 μM of C3O1 for 60 min, the reaction mixture was extracted, and analyzed by HPLC as described in Section 5.

As shown in Figure 3, the mass spectrum of M3 showed a molecular ion at *m*/*z* 491 (M+H) and fragment ions at 473 (M+H−H$_2$O), 455 (M+H−2H$_2$O), 437 (M+H−3H$_2$O), 413 (M+H−60−H$_2$O), 395 (M+H−60−2H$_2$O), and 377 (M+H−60−3H$_2$O). The fragment ion at *m*/*z* 413 indicates losses of a 2α-propoxy group and a water molecule. In addition, fragment ions at 339 (M+H−60−74−H$_2$O), 321 (M+H−60−74−2H$_2$O), and 303 (M+H−76−74−3H$_2$O) which result after cleavage between C-23 and C-24 are characteristic of the 23-hydroxylated compound as described previously.[14a] These results indicate that M3 contains 23(OH)–C3O1. However, based on the facts that the putative metabolites of the C-24 oxidation pathway, 24-oxo-C3O1 (M4) and 24-oxo-23(OH)–C3O1 (M2) were detected, it appeared that M3 contained both 23(OH)–C3O1 and 24(OH)–C3O1. On the other hand, the mass spectrum of M6 showed a molecular ion at *m*/*z* 489 (M+H) and fragment ions at 471 (M+H−H$_2$O), 453 (M+H−2H$_2$O), 411 (M+H−60−H$_2$O), 393 (M+H−60−2H$_2$O). The fragment ion at *m*/*z* 411 indicates losses of a 2α-propoxy group and a water molecule. In addition, fragment ions at 353 (M+H−60−58−H$_2$O), 335 (M+H−60−58−2H$_2$O) which result after cleavage between C-24 and C-25 are characteristic of the 23-oxo compound as described previously.[14b]

Thus, human CYP24A1-dependent metabolism of C3O1 contains a unique C-23 oxidation pathway from C3O1 to 23-oxo-C3O1 (M6) via 23(OH)–C3O1 (M3), indicating that a significant species-based difference in the CYP24A1-dependent metabolism of C3O1 between humans and rats.[14b]

4. Conclusion

We synthesized a new 2α-modified vitamin D$_3$ analogue (C3O1, **5**) that showed moderate affinity for VDR, and then investigated metabolic pathways for both human CYP24A1 and rat CPY24A1. Human CYP24A1 showed multiple metabolic pathways, but rat CPY24A1 has a single metabolic pathway. In previous report, we proved that C3O1 was converted to active O2C3 (**4b**) by human CYP27A1, and the catalytic efficiency of human CYP24A1 for C3O1 and O2C3 was only 2% of the natural hormone **1**.[14a] C3O1 would possess long half-life in vivo, and these results will help us to develop C3O1 as a therapeutic agent.

5. Experimental

5.1. Synthesis

5.1.1. General

All manipulations were performed under an argon atmosphere unless otherwise mentioned. All solvents and reagents were purified when necessary using standard procedures. Column chromatography was performed on silica gel 60 N (Kanto Chemical Co., Inc., 100–210 μm), and flash column chromatography was per-

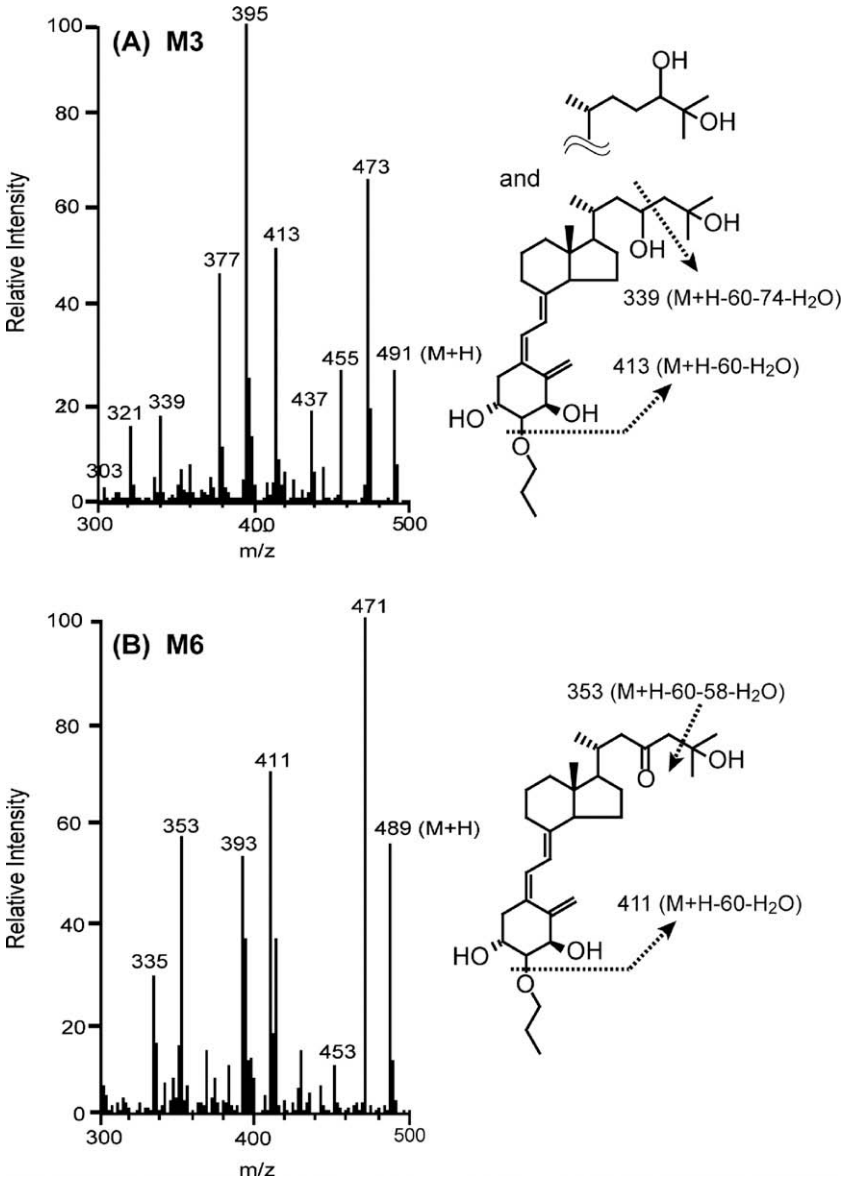


Figure 3. Mass spectra of the metabolites M3 (A) and M6 (B) produced by human CYP24A1. The metabolites were isolated by HPLC, and then analyzed by mass spectrometry as described in Section 5.

formed on silica gel 60 (Merck, 40–63 μm). NMR spectra were measured on a JEOL AL-400 magnetic resonance spectrometer. Infrared spectra were recorded on a JASCO FTIR-8000 spectrometer. Mass spectra were measured on a JEOL JMX-SX 102 mass spectrometer. Specific optical rotations were measured on a JASCO DIP-370 digital polarimeter.

5.1.2. Methyl 4,6-*O*-benzylidene-3-*O*-propyl-α-D-altropyranoside (7)

To a stirred solution of epoxide **6** (3.40 g, 12.9 mmol) in 1-propanol (70 mL) was added KO^{t}Bu (4.76 g, 42.5 mmol) at room temperature. The solution was stirred at 110 °C for 3 days. The reaction mixture was cooled to room temperature, and the solution was partitioned between EtOAc (500 mL) and saturated aqueous NH_4Cl (200 mL). The organic layer was washed with saturated aqueous NH_4Cl, H_2O, and brine (200 mL each), successively, and dried over $MgSO_4$. After filtration and concentration, the residue was purified on silica gel column chromatography (10–33% EtOAc in hexane) to give 2.39 g of **7** as a colorless oil (57%). $[\alpha]_D^{25} + 89.5$ (*c* 1.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.91 (3H, t, *J* = 7.3 Hz), 1.62 (2H, m), 1.93 (1H, br d, *J* = 5.6 Hz), 3.40 (3H, s), 3.58 (1H, dt, *J* = 6.6, 9.5 Hz), 3.69 (1H, dt, *J* = 6.8, 9.5 Hz), 3.75 (1H, t, *J* = 9.8 Hz), 3.80 (1H, br t, *J* = 2.9 Hz), 3.96 (1H, dd, *J* = 2.9, 9.5 Hz), 4.01 (1H, br dd, *J* = 2.9, 5.6 Hz), 4.28–4.36 (2H, m), 4.59 (1H, s), 5.56 (1H, s), 7.33–7.40 (3H, m), 7.45–7.52 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.4, 23.1, 55.6, 58.6, 69.4, 70.2, 73.4, 76.1, 102.0, 102.3, 126.2, 128.2, 129.0, 137.7. IR (neat) 3462, 2963, 2934, 1456, 1383 cm^{-1}; EIMS *m*/*z* 324 (M^+); HREIMS Calcd for $C_{17}H_{24}O_6$ (M^+) 324.1573, found 324.1570.

5.1.3. Methyl 4-*O*-benzoyl-6-bromo-3-*O*-propyl-6-deoxy-α-D-altropyranoside (8)

Benzylidene acetal **7** (2.10 g, 6.48 mmol) was dissolved in CCl_4 (70 mL), and NBS (1.38 g, 7.78 mmol) and $BaCO_3$ (1.28 g, 6.48 mmol) were added at room temperature. The mixture was stirred at 80 °C for 35 min, and then cooled to room temperature. The mixture was diluted with EtOAc (300 mL), and unsolved materials were filtered off with a Celite pad. The filtrate was washed

with 1 M aqueous $Na_2S_2O_3$, saturated aqueous $NaHCO_3$, and brine (100 mL each), successively. The organic layer was dried over $MgSO_4$, filtration and concentration followed by flash chromatography on silica gel (20% EtOAc in hexane) gave 1.90 g of **8** as a colorless oil (73%). $[\alpha]_D^{25} + 51.0$ (*c* 1.1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.82 (3H, t, *J* = 7.4 Hz), 1.52 (2H, m), 2.49 (1H, br d, *J* = 2.9 Hz), 3.47 (1H, dt, *J* = 6.6, 9.2 Hz), 3.51 (3H, s), 3.53 (1H, dt, *J* = 6.6, 9.2 Hz), 3.60 (1H, dd, *J* = 6.8, 11.0 Hz), 3.64 (1H, dd, *J* = 3.9, 11.0), 3.76 (1H, dd, *J* = 3.9, 7.2 Hz), 3.99 (1H, br m), 4.38 (1H, dt, *J* = 3.9, 6.8 Hz), 4.71 (1H, d, *J* = 3.4 Hz), 5.47 (1H, dd, *J* = 3.9, 6.8 Hz), 7.44–7.47 (2H, m), 7.57 (1H, m), 8.04–8.06 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.5, 23.1, 32.8, 56.1, 69.1, 69.8, 69.9, 72.5, 76.7, 102.6, 128.3, 129.3, 129.7, 133.2, 165.5; IR (neat) 3474, 1718, 1603, 1275, 1033 cm^{-1}; EIMS *m*/*z* 402 (M^+); HREIMS Calcd for $C_{17}H_{23}O_6{}^{79}Br$ (M^+) 402.0678, found 402.0686.

5.1.4. (2*R*,3*S*,4*R*)-4-(Benzoyloxy)-3-(propoxy)hex-5-en-1,2-diol (9)

Bromide **8** (1.27 g, 3.15 mmol) was dissolved in 1-propanol (25 mL) and H_2O (2.5 mL) was added. The solution was heated at 95 °C, and then Zn powder (6.11 g, 93.5 mmol) and $NaBH_3CN$ (403.0 mg, 6.41 mmol) were added. The mixture was refluxed at the same temperature for 20 min, and additional Zn powder (4.10 g, 62.7 mmol) and $NaBH_3CN$ (398.0 mg, 6.33 mmol) were added. After refluxing for 35 min, the reaction mixture was allowed to cool to room temperature and filtered by a Celite pad. The filtrate was concentrated in vacuo, and the residue was purified by a silica gel column chromatography (10% EtOAc in hexane) to give 781.0 mg of **9** as a colorless oil (84%). $[\alpha]_D^{20} + 29.6$ (*c* 1.38, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (3H, t, *J* = 7.1 Hz), 1.62 (2H, tq, *J* = 7.1 Hz), 3.50 (1H, dt, *J* = 6.6, 9.1 Hz), 3.60 (1H, t, *J* = 4.7 Hz), 3.74–3.82 (4H, m), 5.34 (1H, dt, *J* = 1.4, 10.4 Hz), 5.43 (1H, dt, *J* = 1.4, 17.3 Hz), 5.71–5.73 (1H, m), 6.05 (1H, ddd, *J* = 6.3, 10.4, 17.3 Hz), 7.26–7.47 (2H, m), 7.57–7.60 (1H, m), 8.05–8.07 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.6, 23.2, 63.8, 71.0, 74.2, 74.8, 80.4, 118.1, 128.3 (2C), 129.5 (2C), 129.8, 132.5, 133.1, 165.4; IR (neat) 3432, 2963, 2936, 2878, 1719, 1647, 1271, 1111, 713 cm^{-1}; EIMS *m*/*z* 276 ($M^+ - H_2O$); HREIMS calcd for $C_{16}H_{20}O_4$ ($M^+ - H_2O$) 276.1362, found 276.1369.

5.1.5. (3*R*,4*S*,5*R*)-3-(Benzoyloxy)-4-(propoxy)-5,6-epoxyhex-1-ene (11)

To a solution of diol **9** (220.2 mg, 0.748 mmol) in pyridine (0.75 mL) was added 2,4,6-trimethylbenzenesulfonyl chloride (TmCl, 180.0 mg, 0.823 mmol) at 0 °C, and the mixture was stirred at room temperature for 14 h. To the mixture was added water at 0 °C, and the aqueous layer was extracted with Et_2O. The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The residue was dissolved in THF (7.5 mL). To the solution was added a solution of LiHMDS in THF (1.0 M, 0.82 mL, 0.82 mmol) at −78 °C, and the resulting mixture was warmed to 0 °C over 1 h. To the mixture was added brine at 0 °C, and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The residue was purified by flash column chromatography on silica gel (5% EtOAc in hexane) to give epoxide **11** (148.8 mg, 72% in two steps) as a colorless oil. $[\alpha]_D^{20} + 47.8$ (*c* 0.48, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (3H, t, *J* = 7.4 Hz), 1.61 (2H, m), 2.63 (1H, dd, *J* = 2.7, 4.9 Hz), 2.78 (1H, dd, *J* = 4.2, 4.9 Hz), 3.11 (1H, ddd, *J* = 2.7, 4.2, 6.8 Hz), 3.19 (1H, dd, *J* = 5.1, 6.8 Hz), 3.55 and 3.71 (2H, each as dt, *J* = 6.9, 9.5 Hz), 5.32 (1H, dt, *J* = 1.2, 10.5 Hz), 5.42 (1H, dt, *J* = 1.2, 17.2 Hz), 5.67 (1H, ddd, *J* = 1.2, 5.1, 6.4 Hz), 6.06 (1H, ddd, *J* = 6.4, 10.5, 17.2 Hz), 7.44–7.49 (2H, m), 7.51–7.56 (1H, m), 8.05–8.08 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.5, 23.0, 43.5, 52.2, 72.6, 74.6, 82.2, 118.1, 128.3, 129.4, 129.8, 132.8, 133.0, 165.1; IR (neat) 2969, 2940, 1726, 1176, 1109 cm^{-1}; EIMS *m*/*z* 276 (M^+); HREIMS Calcd for $C_{16}H_{20}O_4$ (M^+), 276.1362, found 276.1369.

5.1.6. (4*R*,5*S*,6*R*)-5-(Propoxy)oct-7-en-1-yn-4,6-diol (13)

To a solution of ethynyltrimethylsilane (0.17 mL, 1.20 mmol) in THF (1 mL) was added a solution of BuLi in hexane (1.5 M, 0.69 mL, 1.04 mmol) at −78 °C, and the mixture was stirred at the same temperature for 30 min. To the mixture were added a solution of epoxide **11** (124.7 mg, 0.451 mmol) in THF (2.5 mL) and $BF_3{\cdot}OEt_2$ (65 μL, 0.513 mmol) at −78 °C, and the resulting mixture was warmed to room temperature over 2 h. After additional stirring at room temperature for 1 h, the reaction was quenched by addition of saturated aqueous NH_4Cl at 0 °C. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was dissolved in MeOH (3 mL). To the MeOH solution was added K_2CO_3 (187.0 mg, 1.35 mmol) at 0 °C, and the mixture was stirred at room temperature for 2 h. To the mixture was added saturated aqueous NH_4Cl at 0 °C, and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The residue was purified by flash column chromatography on silica gel (25% EtOAc in hexane) to give diol **13** (74.2 mg, 83% in two steps) as a colorless oil. $[\alpha]_D^{20} - 0.74$ (*c* 1.08, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (3H, t, *J* = 7.4 Hz), 1.60–1.69 (2H, m), 2.02 (1H, t, *J* = 2.7 Hz), 2.47 (1H, dd, *J* = 2.7, 16.7 Hz), 2.53 (1H, ddd, *J* = 1.2, 2.7, 16.7 Hz), 2.69 (1H, d, *J* = 4.9 Hz), 2.90 (1H, d, *J* = 6.8 Hz), 3.39 (1H, dd, *J* = 2.2, 4.9 Hz), 3.49 and 3.70 (2H, each as dt, *J* = 6.6, 9.0 Hz), 4.05 (1H, dt, *J* = 2.2, 6.8 Hz), 4.50 (1H, br q, *J* = 4.9 Hz), 5.28 (1H, br d, *J* = 10.5 Hz), 5.44 (1H, br d, *J* = 17.1 Hz), 5.95 (1H, ddd, *J* = 4.9, 10.5, 17.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.6, 23.2, 23.6, 69.8, 70.3, 72.3, 73.2, 80.4, 80.8, 116.2, 137.0; IR (neat) 3395, 2965, 2936, 2120, 1429, 1111, 1076, 930 cm^{-1}; EIMS *m*/*z* 198(M^+); HREIMS calcd for $C_{11}H_{18}O_3$ (M^+) 198.1256, found 198.1266.

5.1.7. (3*R*,4*S*,5*R*)-3,5-Bis-[(*tert*-butyldimethylsilyl)oxy]-4-(propoxy)oct-1-en-7-yne (14)

To a cooled (0 °C) and stirred solution of the diol **13** (300 mg, 1.52 mmol) in CH_2Cl_2 (20 mL) were added 2,6-lutidine (811 μL, 7.58 mmol) and TBSOTf (1.04 mL, 4.55 mmol). After stirring at 0 °C for 1 h, the solution was diluted with EtOAc (50 mL), and then washed with water and brine (30 mL each). The organic layer was separated and dried over $MgSO_4$. Filtration and concentration followed by flash chromatography on silica gel (10% EtOAc in hexane) gave 580 mg of **14** as a colorless oil (90%). $[\alpha]_D^{20}$0.070 (*c* 1.43, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.03, 0.07, 0.09, and 0.10 (12H, each as s), 0.90 (3H, t, *J* = 7.4 Hz), 0.89 and 0.90 (18H, each as s), 1.56 (2H, m), 1.95 (1H, t, *J* = 2.7 Hz), 2.36 (1H, ddd, *J* = 2.7, 5.6, 16.8 Hz), 2.50 (1H, ddd, *J* = 2.7, 5.6, 16.8 Hz), 3.36 (1H, dd, *J* = 3.7, 5.6 Hz), 3.52 (1H, dt, *J* = 6.6, 8.9 Hz), 3.62 (1H, dt, *J* = 7.0, 8.9 Hz), 3.87 (1H, q, *J* = 5.6 Hz), 4.31 (1H, dd, *J* = 3.7, 6.8 Hz), 5.13 (1H, dt, *J* = 1.2, 10.3 Hz), 5.21 (1H, dt, *J* = 1.2, 17.3 Hz), 5.97 (1H, ddd, *J* = 6.8, 10.3, 17.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ −4.6, −4.2, −4.1, −4.0, 10.7, 18.1, 18.2, 23.4, 24.2, 25.9 (3C), 26.0 (3C), 69.8, 71.7, 74.3, 74.5, 82.1, 84.9, 115.8, 138.8; IR (neat) 2957, 2932, 2888, 2859, 1472, 1254, 1094, 835, 777 cm^{-1}; EIMS *m*/*z* 369 ($M - {}^t$Bu)$^+$; HREIMS Calcd for $C_{19}H_{37}O_3Si_2$ ($M - {}^t$Bu)$^+$ 369.2281, found 369.2275.

5.1.8. 2α-Propoxy-1α,25-dihydroxyvitamin D_3 (C3O1, 5)

The silyl ether **14** (40 mg, 93.7 μmol) and vinyl bromide **15** (40 mg, 112 μmol) were dissolved in TEA/toluene (3:1, 2.0 mL), and tetrakis(triphenylphosphine)palladium(0) (8.0 mg, 7.72 μmol) was added. After stirring at room temperature for 15 min, the resulting yellow solution was refluxed for 2 h. The reaction mixture was filtered through a pad of silica gel. Concentration followed

by preparative thin-layer chromatography on silica gel (20% EtOAc in hexane) gave a crude coupling product as a white solid, which was used in the next step without further purification.

To a cold (0 °C) and stirred solution of the crude protected vitamin D in THF (2.0 mL) was added 1 M TBAF in THF (1 mL, 1 mmol). The reaction mixture was stirred at 50 °C for 1 h. The solution was diluted with EtOAc (20 mL), and the solution was washed with H_2O and brine (2.0 mL each). The aqueous layer was extracted with EtOAc (6 × 2.0 mL), and the combined organic layer was dried over Na_2SO_4. Filtration and concentration followed by preparative thin-layer chromatography on silica gel (20% MeOH in CH_2Cl_2) gave 28 mg of **5** as a white powder (42% in two steps). Further purification for biological evaluation was conducted by using reversed-phase recycle HPLC (YMC-Pack ODS column, 20 × 150 mm, 9.0 mL/min, CH_3CN/H_2O = 85:15). $[\alpha]_D^{20}$ + 56.1 (*c* 1.21, $CHCl_3$); UV (EtOH) λ_{max} 267.0 nm, λ_{min} 228.4 nm; ^1H NMR (400 MHz, $CDCl_3$) δ 0.54 (3H, s), 0.93 (3H, d, *J* = 5.9 Hz), 0.96 (3H, t, *J* = 7.0 Hz), 1.05 (1H, m), 1.21 (6H, s), 1.24–1.72 (17H, m), 1.89 (1H, m), 1.92–2.02 (2H, m), 2.17–2.32 (3H, m), 2.69 (1H, dd, *J* = 4.4, 13.4 Hz), 2.83 (1H, m), 3.34 (1H, dd, *J* = 3.3, 7.4 Hz), 3.50 (1H, dt, *J* =9.3, 6.6 Hz), 3.63 (1H, dt, *J* = 6.7, 9.3 Hz), 4.05 (1H, ddd, *J* = 4.6, 7.7, 8.6 Hz), 4.41 (1H, d, *J* = 2.9 Hz), 5.10 (1H, d, *J* = 1.3 Hz), 5.40 (1H, d, *J* = 1.3 Hz), 6.03 (1H, d, *J* = 11.2 Hz), 6.42 (1H, d, *J* = 11.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.6, 12.1, 18.8, 20.8, 22.2, 23.2, 23.5, 27.7, 29.1, 29.2, 29.4, 36.1, 36.4, 40.5, 40.7, 44.4, 45.9, 56.4, 56.6, 68.3, 71.1, 71.7, 71.8, 84.3, 116.3, 117.2, 125.5, 131.6, 143.5, 144.3; EIMS *m*/*z* 474 (M^+); HREIMS calcd for $C_{30}H_{50}O_4$ (M^+) 474.3710, found 474.3701.

5.2. Metabolism

The activity of CYP24A1 towards C3O1 was measured using the membrane fraction prepared from the recombinant *Escherichia coli* cells expressing human CYP24A1 or rat CYP24A1.[14a] The reconstituted system contains 0.02 μM of human CYP24A1 or rat CYP24A1, 1.0 μM of adrenodoxin (ADX), 0.1 μM of NADPH–adrenodoxin reductase (ADR), 4.0 μM of C3O1, 0.5 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 CH_3CN 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.

5.2.1. LC–MS analysis of the metabolites

The metabolite M3 and M6 produced by human CYP24A1 was isolated from HPLC effluents, and subjected to mass spectrometric analysis using a Finnegan Mat TSQ-70 with atmospheric pressure chemical ionization, positive mode, as described previously.[14] The conditions of LC were described below: column, reverse phase ODS column (μBondapak C18, 5 μm, Waters) (6 mm × 150 mm); mobile phase, 80% CH_3OH aqueous solution per 25 min; flow-rate, 1.0 mL min^{-1}; UV detection, 265 nm.

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