Synthesis of 24,24-Difluoro-1,3-cis-25-dihydroxy-19-norvitamin D₃ Derivatives and Evaluation of Their Vitamin D Receptor-Binding Affinity

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Two vitamin D₃ derivatives, namely 24,24-difluoro-1β,3β,25-dihydroxy-19-norvitamin D₃ (6a) and 24,24-difluoro-1α,3α,25-dihydroxy-19-norvitamin D₃ (6b), were synthesized via a convergent route employing Julia–Kocienski olefination as a key step. Compounds 6a and b bound to vitamin D receptor (VDR) with IC₅₀ values of 64.8 and 57.6 nM, respectively, exhibiting about 400- and 30-fold greater binding affinity than the corresponding non-fluorinated derivatives 5a and b.

Key words 24,24-difluoro-19-norvitamin D₃; vitamin D receptor binding; cis-A ring synthon; vitamin D; Julia–Kocienski coupling

1α,25-Dihydroxyvitamin D₃ (1α,25-(OH)₂D₃, 2, Fig. 1; also known as calcitriol) is the active hormonal form of vitamin D₃ (1); it regulates calcium and phosphorus homeostasis and exhibits potent antiproliferative activity.⁴⁻⁵ Its activity is regulated by the cytochrome oxidase P450 family member CYP24A1, which oxidizes 2 at the C24 and C23 positions to afford biologically inactive, side-chain-truncated, water-soluble end products.⁶⁻⁷ However, the metabolic stability of 2 can be increased by substitution of side-chain hydrogen with fluorine, which is very similar in size to hydrogen, but has different chemical bonding properties. The highly electron-withdrawing nature of fluorine means that the C–F bond has a marked ionic character, and this blocks the oxidation by CYP24A1. Indeed, 24,24-difluorinated 1α,25-dihydroxyvitamin D₃ showed high metabolic stability and almost 100% absorption in rats, although this enhanced metabolic stability did not yield increased biological activity.³⁵ Many fluorinated derivatives of 2 have already been reported. Among them, falcacalciotriol⁸⁻¹² is used to treat hypercalcemia, osteomalacia, and rickets, and is more efficient than calcitriol (2). Recently, DeLuca and colleagues reported 24,24-difluoro-1α,25-(OH)₂-19-norvitamin D₃ (4), which showed stronger vitamin D receptor (VDR) binding affinity than compound 2 and promoted bone formation more efficiently.¹³ Thus, although 24-difluoro substitution has little effect on the biological potency of the natural hormone (2), it does alter the biological activity profile of 1α,25-(OH)₂-19-norvitamin D₃ (3).

With this background, we decided to extend our previously reported synthetic studies of 1,3-cis-25-dihydroxy-19-norvitamin D₃ (5a, b)¹⁴ to obtain the 24-difluoro series (6a, b), to investigate the effects of fluoro substitution at the side chain on the biological activities of those ligands. Here, we describe the synthesis of 24,24-difluoro-1β,3β,25-dihydroxy-19-norvitamin D₃ (6a) and 24,24-difluoro-1α,3α,25-dihydroxy-19-norvitamin D₃ (6b), and a comparison of their VDR-binding affinity with that of compounds 5a and b.

RESULTS AND DISCUSSION

In our previous study, we synthesized 1β,3β,25-dihydroxy-19-norvitamin D₃ (5a) and 1α,3α,25-dihydroxy-19-norvitamin D₃ (5b) by means of the Julia–Kocienski coupling with a 1,3-cis type A-ring synthon, followed by separation of the isomers 5a and b.¹⁴ Here we adopted similar methodology, using ketone 11 and sulfone 10. The sulfone 10 was synthesized from the Grundmann's ketone 7, which was reported by DeLuca and colleagues¹⁵ (Chart 1). Compound 7 was subjected to Horner–Wittig olefination using triethyl phosphonoacetate in the presence of sodium hydride in tetrahydrofuran (THF) to give the α,β-unsaturated ester 8, and the ester was then reduced with disobutylaluminium hydride (DIBAL)-H in toluene to give the allylic alcohol 9 in 68% yield (2 steps). The allylic alcohol 9 was subjected to the Mitsunobu reaction using 2-mercaptobenzothiazole in the presence of disopropyl azodicarboxylate (DIAD) and triphenylphosphine in CH₂Cl₂, followed by hydrogen peroxide oxidation of the resulting sulfide in the presence of ammonium molybdate tetrahydrate catalyst¹⁶ to give the sulfone 10 (Chart 1).

With sulfone 10 and ketone 11 in hand, we performed Julia–Kocienski coupling to obtain a mixture of 12a and b, which was separated by silica gel column chromatography. The relative stereochemistries were assigned by ¹H-NMR and nuclear Overhauser effect (NOE) correlations, as reported previously in the literature.¹⁴,¹⁷,¹⁸ Each compound was subjected to two-step deprotection using K₂CO₃ in methanol, followed by hydrogen fluoride pyridine (HF-Py) in THF, to afford 6a

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and b, respectively, in 64% yield (Chart 2).

**Biological Evaluation** The vitamin D receptor (VDR) affinities of the 24-difluoro analogues 6a and b and their corresponding non-fluorinated derivatives 5a and b were evaluated. Compounds 5a and b showed very feeble binding affinity, with IC$_{50}$ values of 24.5 and 2.1 µM, respectively, compared to that of the control 19-nor analogue 3 (Table 1, entries 2, 4, 5). However, 24-difluoro substitution markedly increased the VDR-binding affinity. Compound 6a showed nearly 400-fold higher binding affinity than that of 5a while 6b exhibited almost 30-fold greater binding affinity than that of its non-fluoro derivative 5b. Although, the binding affinities of 6a and b are still several orders of magnitude less than those of the native hormone (2) and 24,24-difluoro-1α,25-dihydroxy-19-norvitamin D$_3$ (4). Although 6a and b carries different geometry of A-ring at C1, it is very interesting finding that their VDR affinities are almost the same, which indicates that their VDR binding is apparently independent on the C1-hydroxy group’s geometry. These results suggest that 24-difluoro substitution significantly alters the side chain geometry in the VDR pocket in case of 19-norvitamin D$_3$ analogues.

**CONCLUSION**

Two new 24,24-difluorinated-1,3-cis-25-dihydroxy-19-norvitamin D$_3$ derivatives (6a, b) were synthesized using previously reported methodology. The VDR-binding affinities of these compounds were almost the same irrespective of their different C1 hydroxy group’s geometry and were increased...
considerably, compared to that of the non-fluorinated derivatives (5a, b). This result suggests that binding of 19-nor type vitamin D₃ ligands to VDR is more dependent on the side-chain substitution than on the A ring geometry. Further study will be needed to establish the precise binding mode of 24,24-difluorinated-19-norvitamin D₃ derivatives to the VDR pocket.

MATERIALS AND METHODS

All reactions have been carried out in dry solvents, and under inert atmosphere unless otherwise mentioned. Reagents were purchased from Sigma-Aldrich, TCI and Wako Pure Chemical Industries, Ltd. Flash chromatography was performed on silica gel 60 (spherical, particle size 40–100 mm; Kanto). ¹H-NMR spectra have been recorded in deuteriochloroform at 300 and 400 MHz using JEOL instruments, JMT 300 and JMN-ECX 400 spectrometers, whereas ¹³C-NMR spectra have been recorded at 75 and 100 MHz using the same spectrometers. The spectra are referenced internally according to residual solvent signal of CDCl₃ (¹H-NMR: δ = 7.26 ppm; ¹³C-NMR: δ = 77.0 ppm). Data for ¹H-NMR are recorded as follows: chemical shift (δ ppm), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad) and coupling constant (Hz). Data for ¹³C-NMR are reported in terms of chemical shift (δ ppm). Mass spectra were recorded on a JMS-T100X (JEOL) spectrometer in electrospray ionization (ESI)-MS mode using methanol as solvent.

Ethyl 2-((1R,7aR,E)-1-((R)-5,5-Difluoro-6-methyl-6-((trime-thylsilyl)oxy)heptan-2-yl)-7a-methyl-octahydro-4H-inden-4-ylidene)acetate (8) To a mixture of compound 7 (126.1 mg, 0.32 mmol) in THF (3 mL) was added sodium hydride (NaH) (60% mineral oil dispersion, 45.5 mg, 1.88 mmol) at 0°C under argon. The resulting mixture was allowed to stir at that temperature for 30 min, then triethyl phosphonoacetate (481.1 mg, 0.4 mL, 2.14 mmol) was added to the mixture dropwise, upon completion of addition the mixture was stirred for additional 30 min. To this mixture, saturated Rochelle’s salt (1.8 mL) was added and stirred for another 30 min. The aqueous phase was then extracted with ethyl acetate for three times. Combined organic layer was washed with saturated Rochelle’s salt, saturated NH₄Cl and brine, then dried over MgSO₄, filtered and concentrated in vacuo. The residue was purified on silica gel column (16–18% ethyl acetate–hexanes), to give pure alcohol 9 (175 mg, 76%). Spectral data for 9: ¹H-NMR (300 MHz, CDCl₃): δ = 5.22 (1H, t, J = 7.2 Hz), 4.19 (1H, d, J = 6.8 Hz), 2.61 (1H, dd, J = 4.5, 12.6 Hz), 2.13–1.37 (16 H, m), 1.28 (12H, s), 0.90 (3H, d, J = 6.2 Hz), 0.56 (3H, s); ¹³C-NMR (75 MHz, CDCl₃) δ = 143.74, 125.32 (t, JCD = 251.4 Hz), 119.38, 76.09 (t, JCD = 28.2 Hz), 58.77, 56.48, 55.67, 45.42, 40.43, 35.92, 28.77, 27.66, 27.18 (t, JCD = 23.8 Hz), 24.60, 24.56, 23.57, 22.24, 18.71, 11.94, 2.43; HR-MS ESI: m/z Calcd for C₂₅H₄₁F₂NaO₅Si: 439.2819 [M+Na]+. Found 439.2778.

2-((1R,7aR,E)-1-((R)-5,5-Difluoro-6-methyl-6-((trimethylsilyl)oxy)heptan-2-yl)-7a-methyloctahydro-4H-inden-4-ylidene)ethyl)sulfonylbenzod[thi]azole (10) To a solution of alcohol 9 (148.5 mg, 0.36 mmol) in CH₂Cl₂ (1.2 mL) was added 2-mercapto benzothiazole (95.5 mg, 0.21 mmol) and PPh₃ (149.8 mg, 0.21 mmol) at 0°C under argon. To the resulting mixture was added diisopropyl azodicarboxylate (115.5 mg, 0.11 mL, 0.21 mmol) dropwise, and the mixture was stirred for 15 min at 0°C. Then, the reaction was concentrated in vacuo, and the residue was passed through a short silica gel column, the eluent was concentrated in vacuo to give crude product, which was subjected to the oxidation reaction at the next step. The crude sulfide was dissolved in EtOH (3 mL) at 0°C. Ammonium molybdate tetrahydrate (88.2 mg, 71 µmol) was added to the mixture, followed by a slow addition of 30% H₂O₂ (1 mL). The resulting mixture was then raised at room temperature and was allowed to stir for 3 h. To the reaction mixture was added saturated Na₂S₂O₃ at 0°C, and the aqueous layer was extracted with ethyl acetate for three times. The extracts were washed with water and brine, dried over MgSO₄, filtered and concentrated in vacuo. The residue was purified on silica gel column (8% ethyl acetate–hexanes) to give 10 (214.8 mg, 2 steps 94%). Spectral data for 10: ¹H-NMR (400 MHz, CDCl₃): δ = 8.22 (1H, d, J = 7.8 Hz), 7.99 (1H, d, J = 7.8 Hz), 7.65–7.58 (3H, m), 5.01 (1H, t, J = 8.2 Hz), 4.26 (1H, dd, J = 9.2, 14.2 Hz), 4.20 (1H, dd, J = 6.4, 14.2 Hz), 2.55 (1H, d, J = 14.2 Hz), 2.02–1.36 (11H, m), 1.31–1.19 (12H, m), 0.93 (9H, t, J = 7.8 Hz), 0.86 (6H, d, J = 6.4 Hz), 0.58 (6H, q, J = 7.8 Hz), 0.27 (3H, s); ¹³C-NMR (100 MHz, CDCl₃): δ = 152.91, 152.13, 127.96, 127.66, 125.45 (t, JCD = 248.2 Hz), 122.32, 104.31, 77.1 δ = 21.1 Hz), 9.26, 29.66, 27.38, 27.33 (t, JCD = 24.9 Hz), 26.98, 25.44, 25.40, 23.90, 22.21, 18.65, 2.40. High resolution (HR)-MS ESI: m/z Calcd for C₂₅H₃₅F₂Na₂O₅Si: 481.2925 [M+Na]+. Found 481.2917.

(1S,3R,E)-3-(((tert-Butyl dimethylsilyl)oxy)-5-(2-((1R,7aR,E)-1-((R)-5,5-difluoro-6-methyl-6-((trimethylsilyl)oxy)heptan-2-yl)-7a-methyloctahydro-4H-inden-4-ylidene)ethan-1-ol (11) To a solution of compound 8 (228 mg, 0.5 mmol) in toluene (2.5 mL) was added DIBAL-H (1.49 mL, 1.5 mmol) dropwise at −78°C under argon. The resultant mixture was then raised at room temperature and was allowed to stir for 2 h. MeOH (1 mL) was added to the mixture and stirred for additional 30 min. To this mixture, lithium bis(trimethylsilyl)amide (LiHMDS) (1.3 M in hexane, 52 µL, 68 µmol) was slowly added to a mixture of CD-ring synthon 10 (28.7 mg, 48 µmol) and ketone 11 (12 mg, 34 µmol) in THF (0.7 mL) at −78°C under argon,
and the mixture was stirred for 2.5 h, then the mixture was raised at room temperature and allowed to stand for 10 min. To the reaction mixture was added water, and extracted with ether for three times. The combined organic layer was washed with brine, dried over MgSO$_4$, filtered and concentrated in vacuo. The residue was passed through a small silica gel column to give 12 as two diastereomers mixture (31 mg, 92%). These diastereomers were separated on a silica gel column (0.5% ether–hexanes) to give 12a (15 mg, 46%) and 12b (16 mg, 46%). Spectral data for 12a: $^1$H-NMR (300 MHz, CDCl$_3$) $\delta$: 8.04 (2H, d, $J=7.9$ Hz), 7.58–7.53 (1H, m), 7.44 (2H, d, $J=7.22$ Hz), 6.22 (1H, d, $J=11.0$ Hz), 5.81 (1H, d, $J=11.0$ Hz), 4.96–4.85 (1H, m), 3.75–3.65 (1H, m), 3.13 (1H, dd, $J=4.1$, 12.6 Hz), 2.80 (1H, dd, $J=3.1$, 11.5 Hz), 2.48–2.36 (2H, m), 2.14 (1H, t, $J=11.4$ Hz), 2.03–1.87 (5H, m), 1.75–1.47 (10H, m), 1.29 (9H, s), 0.93 (3H, d, $J=6.2$ Hz), 0.88 (9H, s), 0.57 (3H, s), 0.08 (6H, d, $J=6.2$ Hz). $^{13}$C-NMR (75 MHz, CDCl$_3$) $\delta$: 165.98, 143.15, 133.01, 130.60, 129.71 (t, $J_{CF}=249.3$ Hz), 129.45, 122.99, 115.66, 76.07 (t, $J_{CF}=28.2$ Hz), 70.44, 68.72, 56.48, 56.39, 46.44, 45.90, 41.83, 40.58, 35.94, 33.71, 28.94, 27.32 (t, $J_{CF}=22.4$ Hz), 25.93, 24.57, 23.57, 22.36, 18.71, 18.21, 12.81, 2.45, −4.48, −4.55. HR-MS ESI: m/z Calcd for C$_5$H$_7$F$_2$O$_2$N$_2$: 753.4521 [M+Na$^+$]. Found: 753.4528. Spectral data for 12b: $^1$H-NMR (300 MHz, CDCl$_3$) $\delta$: 8.03 (2H, d, $J=7.9$ Hz), 7.55 (2H, t, $J=7.6$ Hz), 7.43 (2H, t, $J=6.6$ Hz), 6.29 (1H, d, $J=11.4$ Hz), 5.85 (1H, d, $J=11.4$ Hz), 4.92–4.84 (1H, m), 3.64–3.57 (1H, m), 2.98 (1H, dd, $J=4.1$, 12.6 Hz), 2.79 (1H, dd, $J=3.8$, 10.5 Hz), 2.66 (1H, dd, $J=4.5$, 12.0 Hz), 2.37–2.35 (2H, m), 2.23 (1H, t, $J=11.7$ Hz), 2.05–1.82 (6H, m), 1.68 (4H, t, $J=11.7$ Hz), 1.29 (12H, s), 0.97 (3H, d, $J=6.2$ Hz), 0.87 (9H, s), 0.56 (3H, s), 0.08 (6H, d, $J=6.2$ Hz). $^{13}$C-NMR (75 MHz, CDCl$_3$) $\delta$: 165.98, 143.21, 142.95, 132.99, 130.60, 129.69 (t, $J_{CF}=249.3$ Hz), 129.45, 123.02, 115.64, 76.08 (t, $J_{CF}=28.9$ Hz), 71.04, 68.59, 56.48, 56.35, 45.84, 42.07, 41.76, 40.54, 38.33, 35.96, 28.94, 27.31 (t, $J_{CF}=20.9$ Hz), 25.98, 24.57, 23.61, 22.20, 18.72, 18.32, 2.46, −4.38, −4.46. HR-MS ESI: m/z Calcd for C$_{42}$H$_{30}$F$_2$Na$_2$O$_4$Si$_2$: 753.4521 [M+Na$^+$]. Found: 753.4536.

(1R,3S,5)-5-((1R,7aR,8E)-1-((R)-5,5-Difuoro-6-hydroxy-6-methylheptan-2-yl)-7a-methyloctahydro-4H-inden-4-ylidene)ethylenecyclohexane-1,3-diol (6a) To a solution of 12a (15 mg, 20 µmol) in MeOH (0.4 mL) and THF (1.6 mL) was added K$_2$CO$_3$ (4.2 mg, 36 µmol) at room temperature under argon, and the mixture was stirred overnight. The combined organic layer was washed with water and brine, dried over MgSO$_4$, filtered and concentrated in vacuo. The residue was first eluted using 3% MeOH–CH$_2$Cl$_2$ on a silica gel column, and then again purified on a preparative TLC plate using 5% MeOH–CH$_2$Cl$_2$ as the mobile phase to give 6a (5.7 mg, 64%). Spectral data for 6a: $^1$H-NMR (300 MHz, CDCl$_3$) $\delta$: 6.34 (1H, d, $J=11.0$ Hz), 5.86 (1H, d, $J=11.0$ Hz), 4.02 (2H, br), 2.82 (2H, dd, $J=4.1$, 10.3 Hz), 2.57 (1H, dd, $J=5.8$, 15.3 Hz), 2.51–2.43 (2H, m), 2.33–2.18 (3H, m), 2.08–1.65 (13H, m), 1.31 (9H, s), 1.24 (3H, $t=7.2$ Hz), 0.94 (3H, d, $J=6.2$ Hz), 0.55 (3H, s). $^{13}$C-NMR (75 MHz, CDCl$_3$) $\delta$: 142.70, 130.15, 126.44 (t, $J_{CF}=249.8$ Hz), 124.12, 115.52, 73.48 (t, $J_{CF}=27.4$ Hz), 68.96, 68.78, 56.34, 56.19, 48.53, 45.12, 40.51, 40.29, 36.75, 35.80, 29.79, 28.98, 27.40 (t, $J_{CF}=24.5$ Hz), 26.87, 23.67, 22.33, 18.74, 12.18. HR-MS ESI: m/z Calcd for C$_{26}$H$_{22}$F$_2$Na$_2$O$_4$: 463.2999 [M+Na$^+$]. Found: 463.3048.

Measurement of Binding Affinity of the Derivatives 5a, b, 6a, and b for VDR (Table 1) The binding affinity of 1a,25-dihydroxyvitamin D$_3$ 1a,25-dihydroxy-19-norvitamin D$_3$, 24,24-difluoro-1a,25-dihydroxy-19-norvitamin D$_3$, and their derivatives 5a, b, 6a, and b for VDR was examined using a nuclear receptor cofactor assay system (Enbio RCAS for VDR, EnBioTec Laboratory) according to the manufacturer's instructions. First, the kit components were brought down at room temperature. The standard was reconstituted with 1.0 mL of standard diluent and was kept for 10 min at room temperature with occasional shaking. The initial concentration of the stock solution was 200 ng/mL, which was then diluted to yield 7 standard solutions of different concentration. The highest concentration of stock solution being 20 ng/mL and the lowest being 0.312 ng/mL, with one blank solution. The working concentrations were diluted with detection reagent A and B, respectively (1:100).

Seven wells were determined for standard and 1 well for the blank. A hundred microliters of each dilution of standard, blank and samples were added to appropriate wells. Then
those wells were covered with plate sealer and was incubated for 2 h at 37°C. After that liquid was removed from each well, and 100 µL of working solution with detection reagent A was added to each well and was incubated for 1 h at 37°C after covering with plate sealer. Then the wells were washed with 350 µL of Wash solution and was allowed to sit for 1–2 min. Remaining liquids were removed from all wells by snapping the plates on absorbent paper. This wash process was repeated for 3 times, and after the last wash, any remaining buffer was completely removed by aspirating or decanting the wells. A hundred microliters of detection reagent B was added to each well and incubated for 10 min at 37°C, the liquid was turned blue by the addition of substrate solution. Then 50 µL of the substrate solution was added to each well and was covered by a new plate sealer, which was then allowed to incubate for 1 h at 37°C after covering the plates with plate sealer. The aspiration process was repeated for 2 h at 37°C. After that liquid was removed from each well, LXXIX. Studies on steroids. LXXIX. synthesis of 1α,25-dihydroxy-26,26,27,27,27-hexafluorovitamin D₃, Chem. Pharm. Bull. 30, 4297–4303 (1982).

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