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Structure–activity relationship studies on vitamin D lactam derivatives as vitamin D receptor antagonist

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ABSTRACT

Structure–activity relationship studies on 1α,25-dihydroxyvitamin D_3-26,23-lactams (DLAMs), antagonists of vitamin D, were conducted, focusing on the substituents of the phenyl group. One of the derivatives (23*S*,25*S*)-DLAM-1P-3,5$(OEt)_2$, showed potent antagonistic activity with an IC_{50} of 90 nM.

1α,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the active metabolite of vitamin D_3, has critical roles in various biological activities, including calcium and phosphorus homeostasis, cell proliferation, and differentiation.[1] Most of these biological activities are considered to be mediated via binding to the specific receptor, VDR (vitamin D receptor), which is a member of the nuclear receptor superfamily. The VDR contains a DNA-binding domain (DBD), which is formed by two zinc-finger motifs (characteristic of the nuclear receptor superfamily), and a ligand-binding domain (LBD), which consists of 12 α-helical structures, containing a short trans-activation function 2 (AF-2) domain.[2] Helix 12 plays an important role in regulation of the transcriptional activity of the receptor. Ligands with agonistic activity induce a conformational change of the LBD, that is, folding of helix 12 to form a lid over the LBD pocket. On the other hand, antagonistic activity is thought to be exhibited by ligands that inhibit the folding or that induce mis-folding of helix 12.[3]

Vitamin D antagonists are expected to be therapeutically effective for metabolic bone disease and hypercalcemia. They should also be useful as tools for studying the mechanisms of vitamin D functions. Although literally thousands of vitamin D derivatives have been synthesized, only a few families of vitamin D antagonists, that is, TEI-9647,[4] ZK168281,[5] and ADMI-3[6] have been reported so far.

We have recently developed novel vitamin D antagonists, DLAMs (1α,25-dihydroxyvitamin D_3-26,23-lactams), which have a lactam structure on the side chain (Fig. 1).[7] DLAMs inhibit the activation of human and rat VDRs at the transcriptional level. The substituent on the nitrogen in the lactam moiety of DLAMs was suggested to be important for the antagonistic activity, based on a docking study of DLAM-1P (**1**) with VDR, that is, steric hindrance occurs between the Phe422 residue in helix 12 and the phenyl substituent in DLAM-1P (**1**), which leads to inhibition of folding or mis-folding of helix 12.[7c] A series of DLAM derivatives with different alkyl chain lengths on the nitrogen of the lactam ring (DLAM-1P (**1**)–4P (**4**), $n = 1$–4) and their stereoisomers (16 compounds; Fig. 1) were synthesized, and their biological activities were evaluated. The antagonistic activities of these derivatives depended upon the alkyl chain length and the stereochemistry at C23 and C25. Among them, (23*S*, 25*S*)-DLAM-2P (**2a**, $n = 2$) showed the highest VDR binding affinity (1/12.5 of that of 1,25-$(OH)_2D_3$) and the strongest antagonistic activity.[7c]

Since the phenyl group in DLAM was revealed to play an important role for the antagonistic activity, we were interested in substituent effects on the phenyl group of these DLAMs. In this communication, we describe structure–activity relationship (SAR) studies, focusing on substitution of the phenyl group in DLAMs.

Initially, we examined the steric and electronic effects of substitution on the phenyl group of DLAM-1P–3P. Thus, novel DLAM derivatives having methoxy group and/or trifluoromethyl group at the 3 and 5 positions on the phenyl group, i.e., DLAM-1P–

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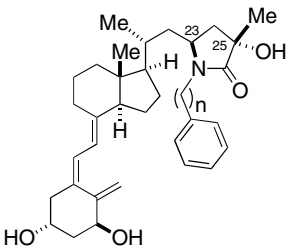
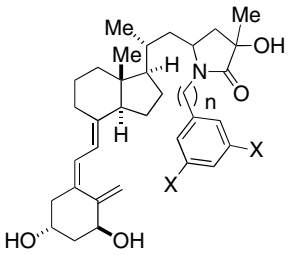




1a: (23*S*,25*S*)-DLAM-1P (n = 1, IC_{50} = 700 nM)
2a: (23*S*,25*S*)-DLAM-2P (n = 2, IC_{50} = 194 nM)
3a: (23*S*,25*S*)-DLAM-3P (n = 3, IC_{50} = 2200 nM)
4a: (23*S*,25*S*)-DLAM-4P (n = 4, IC_{50} = 390 nM)

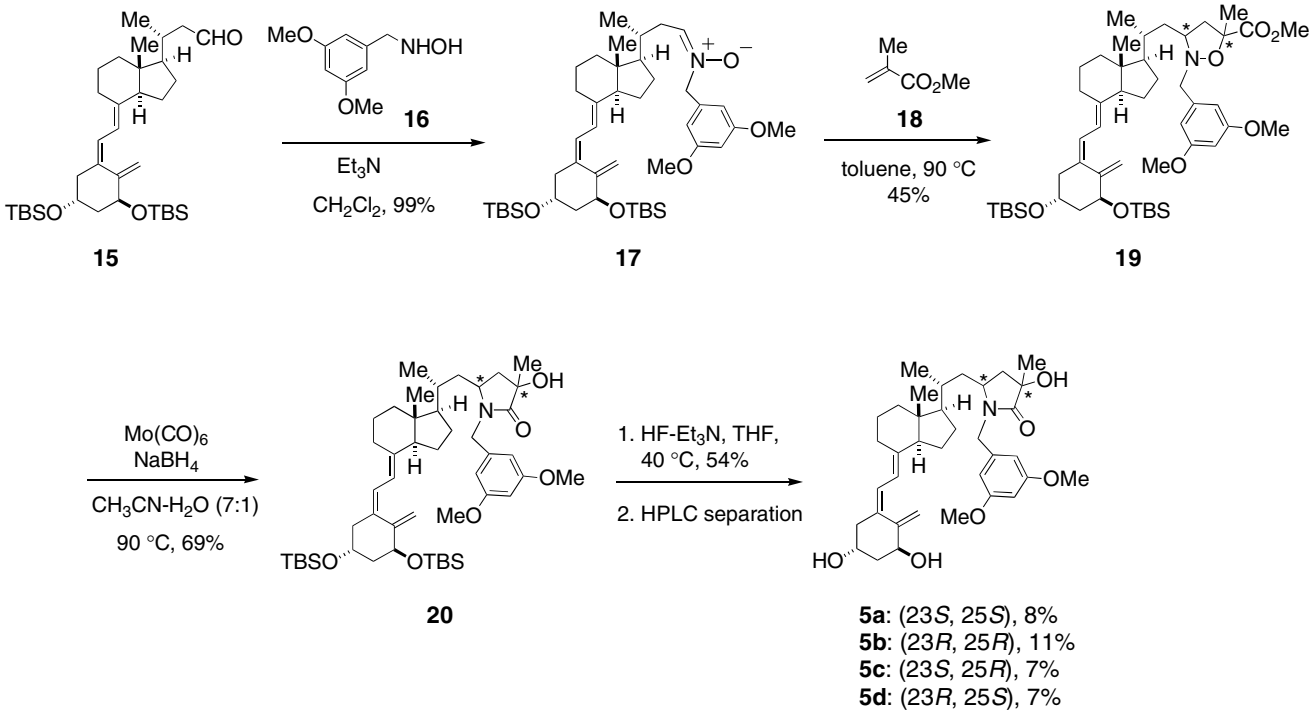
5: DLAM-1P-3,5$(OMe)_2$ (n = 1, X = OMe)
6: DLAM-2P-3,5$(OMe)_2$ (n = 2, X = OMe)
7: DLAM-3P-3,5$(OMe)_2$ (n = 3, X = OMe)
8: DLAM-1P-3,5$(CF_3)_2$ (n = 1, X = CF_3)
9: DLAM-2P-3,5$(CF_3)_2$ (n = 2, X = CF_3)
10: DLAM-3P-3,5$(CF_3)_2$ (n = 3, X = CF_3)

11: DLAM-1P-3,5$(OH)_2$ (n = 1, X = OH)
12: DLAM-1P-3,5$(OEt)_2$ (n = 1, X = OEt)
13: DLAM-1P-3,5$(O^nPr)_2$ (n = 1, X = O^nPr)
14: DLAM-1P-3,5$(O^nBu)_2$ (n = 1, X = O^nBu)

Figure 1. Structures of DLAM-1P 4P and their derivatives.

3P-3,5$(OMe)_2$ (**5**)–(**7**) and DLAM-1P–3P-3,5$(CF_3)_2$ (**8**)–(**10**) (Fig. 1), were synthesized based upon our previously reported strategy.[7c,8] The synthesis of **5** is illustrated in Scheme 1. Briefly, reaction of aldehyde **15** and hydroxylamine **16** in the presence of Et_3N gave nitrone **17** quantitatively. Then, 1,3-dipolar cycloaddition between nitrone **17** and methyl methacrylate (**18**) provided isoxazolidine **19** as a mixture of four possible diastereomers at C23 and C25. Reduction of the N–O bond of the isoxazolidine **19** with $Mo(CO)_6$–$NaBH_4$, with simultaneous cyclization of the resulting amine, gave lactam **20**. After deprotection of the TBS groups, the four diastereomers were separated with HPLC to give **5a–d**. In a similar way, **6a–d**–**10a–d** were synthesized by changing the hydroxylamine.[9] The stereochemistries at C23 and C25 of the new DLAM derivatives were determined by comparison of the spectral data with those of DLAM-1P (**1**).

With the new DLAM derivatives in hand, we next evaluated the relative binding affinity for VDR and the antagonistic activity of these compounds **5–10**.[10,11] The VDR binding affinity of the synthetic compounds was examined by the use of VDR from chick intestine. Chick intestinal 1,25-$(OH)_2D_3$ receptor was dissolved in 0.05 M phosphate buffer (pH 7.4) containing 0.3 M KCl and 5 mM dithiothreitol just before use. The receptor solution (500 μL, 0.35 mg protein) was pre-incubated with 5 μL of ethanol solution of 1,25-$(OH)_2D_3$ or an analog at various concentrations for 60 min at 25 °C. Then, the mixture was left to stand for 24 h with 0.1 nM [^{3}H]-1,25-$(OH)_2D_3$ (Amersham) at 4 °C. Bound and free [^{3}H]-1,25-$(OH)_2D_3$ were separated by treatment with dextran-coated charcoal for 30 min at 4 °C, followed by centrifugation at 3000 rpm for 10 min. The radioactivity of the supernatant (500 μL) was then counted using Atomlight (Perkin Elmer). Antagonistic activities of synthetic compounds were evaluated as follows. HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS. Exponentially proliferating cells were collected, suspended in fresh medium, and



Scheme 1. Synthesis of DLAM-1P-3,5$(OMe)_2$ (**5**).

seeded in 24-well culture plates (2×10^4 cells/mL, 1 mL/well). An ethanol solution of 1,25-$(OH)_2D_3$ and a synthetic compound was added to the culture medium at 0.1% by volume and culture was continued for 96 h at 37 °C in a humidified atmosphere of 5% CO_2 in air without changing the medium. The same amount of vehicle was added to the control culture. NBT-reducing assay was performed according to the method of Collins. Briefly, cells were collected, washed with PBS, and suspended in serum-free medium, then NBT/TPA solution was added. Final concentrations of NBT and TPA were 0.1% and 100 nm/mL, respectively. The cell suspensions were incubated at 37 °C for 25 min, then the cells were collected by centrifugation and resuspended in FCS. Cytospin smears were prepared, and nuclear counter-staining was done with Kemechrot solution. At least 500 cells per preparation were observed. The results obtained for **5**–**10** are summarized in Table 1.

Among **5**–**7**, (23*S*,25*S*)-stereoisomers showed higher VDR binding affinity and antagonistic activity than other stereoisomers, which is consistent with the results obtained for DLAMs **1**–**4**.[7c] (23*S*,25*S*)-DLAM-1P-3,5(OMe)$_2$ (**5a**) showed the highest VDR binding affinity (1/1.9 compared with 1,25-$(OH)_2D_3$) and the strongest antagonistic activity, with an IC_{50} of 300 nM. In a series of DLAM-1P derivatives **8**–**10** having electron-withdrawing character on the phenyl group, (23*S*,25*S*)-stereoisomers also showed higher VDR binding affinity; however, no antagonistic activity was observed. Electronic character was suggested to be critical as well as steric effects for eliciting the antagonistic activity of DLAMs. Thus, we next focused on the other alkyloxy substituents in DLAM-1P.

According to the synthetic scheme described above, new alkyloxy-type DLAM-1P derivatives **11**–**14** were synthesized,[9] and the biological activities of these (23*S*,25*S*)-stereoisomers **11a**–**14a** were evaluated (Table 2). We found that **12a**–**14a** showed high VDR binding affinities and potent antagonistic activities. Among them, (23*S*,25*S*)-DLAM-1P-3,5(OEt)$_2$ (**12a**) showed the most potent antagonistic activity (IC_{50} = 90 nM), being 7.8 times more potent than the original DLAM-1P (**1a**). The alkyloxy substituents on the phenyl group in DLAM thus have a marked effect on both VDR binding affinity and antagonistic activity, presumably as a result of steric hindrance. Interestingly, hydroxyl group-substituted derivatives of **11a** showed high binding affinity for VDR, but lacked antagonistic activity. The reasons are not clear at this stage, although some interactions between the phenolic alcohol in **11a** and amino acid residues of helix 12 of VDR very likely prevent the inhibition of folding of helix 12.[12]

In summary, SAR studies for DLAM derivatives were conducted, focusing on substituents of the phenyl group. Alkyloxy substitution of the phenyl group in DLAM-1P derivatives was found to be increase the activity, and (23*S*,25*S*)-DLAM-1P-3,5(OEt)$_2$ (**12a**) showed the most potent antagonistic activity, with an IC_{50} of 90 nM. On the other hand, antagonistic activity of DLAM-1P was disappeared by the substitution with electron-withdrawing character of trifluoromethyl group. Further SAR studies on DLAMs are in progress.

Table 1
VDR Binding affinity and antagonistic activity of DLAM derivatives **5**–**10**

DLAMs	VDR Binding affinity[a]	Antagonistic activity[b] (IC_{50}, nM)
1a	1/36.4	700[c]
5a	1/1.9	300
5b	1/185	>3000
5c	1/131	>3000
5d	1/60	>3000
6a	1/12	860
6b	1/284	NA[d]
6c	<1/1515	NA[d]
6d	<1/1515	NA[d]
7a	1/57	780
7b	<1/1515	NA[e]
7c	<1/1515	NA[e]
7d	<1/1515	NA[e]
8a	1/138	NA[e]
8b	<1/1515	NA[e]
8c	<1/1515	NA[e]
8d	<1/1515	NA[e]
9a	1/42	NA[e]
9b	1/236	NA[e]
9c	1/1182	NA[e]
9d	1/757	NA[e]
10a	1/181	NA[d]
10b	<1/5400	NA[d]
10c	<1/5400	NA[d]
10d	<1/5400	NA[d]

[a] The potency of 1,25-$(OH)_2$-D_3 is normalized to 1.
[b] The antagonistic activity was assessed in terms of IC_{50} for the differentiation of HL-60 cells induced by 10 nM of 1,25-$(OH)_2$-D_3.
[c] See Ref. 7c.
[d] NA, not antagonist below 1 μM.
[e] NA, not antagonist below 3 μM.

Table 2
VDR Binding affinity and antagonistic activity of DLAM derivatives **11a**–**14a**

DLAMs	VDR Binding Affinity[a]	Antagonistic Activity[b] (IC_{50}, nM)
11a	1/9.6	NA[c]
12a	1/3.5	90
13a	1/11.9	180
14a	1/18.2	540

[a] The potency of 1,25-$(OH)_2$-D_3 is normalized to 1.
[b] The antagonistic activity was assessed in terms of IC_{50} for the differentiation of HL-60 cells induced by 10 nM of 1,25-$(OH)_2$-D_3.
[c] NA, not antagonist below 1 μM.

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8. In our preliminary SAR studies on DLAM, methoxy substitution at 4 position of phenyl group (DLAM-MPM) did not affect both VDR binding affinity and antagonistic activity.[7c]
9. Spectral data for VDR antagonists **5a**–**7a** and **12a**–**14a**. Underlined values show the typical chemical shifts for the (23*S*,25*S*) configurations. **5a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.37 (m, 3H), 6.36 (d, J = 2.1 Hz, 1H), 6.01 (d, J = 11.5 Hz, 1H), 5.32 (s, 1H), 4.99 (s, 1H), 4.92 (d, J = 15 Hz, 1H), 4.44–4.42 (m, 1H), 4.24–

4.22 (m, 1H), 3.88 (d, *J* = 15 Hz, 1H), 3.76 (s, 6H), *3.54* (m, 1H), 2.82 (dd, *J* = 4.7, 13.3 Hz, 1H), 2.59 (dd, *J* = 3.0, 13.3 Hz, 1H), 2.31 (dd, *J* = 6.4, 13.3 Hz, 1H), 2.28 (dd, *J* = 7.7, 13.3 Hz, 1H), 2.04–1.12 (m, 20 H), 1.48 (s, 3H), 0.81 (d, *J* = 6.0 Hz, 3H), 0.53 (s, 3H); **6a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.39–6.32 (m, 4H), 6.02 (d, *J* = 11.6 Hz, 1H), 5.33 (s, 1H), 4.99 (s, 1H), 4.48–4.40 (m, 1H), 4.28–4.20 (m, 1H), 3.78–3.74 (m, 9H), *3.44* (m, 1H), 3.22–3.14 (m, 1H), 2.88–2.70 (m, 3H), 2.60 (d, *J* = 12.0 Hz, 1H), 2.34–1.17 (m, 23H), 0.87 (d, *J* = 5.6 Hz, 3H), 0.55 (s, 3H); **7a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.39–6.31 (m, 4H), 6.02 (d, *J* = 11.2 Hz, 1H), 5.33 (s, 1H), 5.00 (s, 1H), 4.99 (br s, 1H), 4.43 (br s, 1H), 3.82–3.56 (m, 9H), *3.60* (m, 1H), 3.05–2.98 (m, 1H), 2.90–2.78 (m, 1H), 2.62–2.53 (m, 3H), 2.35–2.26 (m, 3H), 2.03–1.19 (m, 22H), 0.95 (d, *J* = 5.2 Hz, 3H), 0.57 (s, 3H); **12a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.38–6.35 (m, 4H), 6.01 (d, *J* = 10.8 Hz, 1H), 5.33 (s, 1H), 4.99 (s, 1H), 4.91 (d, *J* = 15.2 Hz, 1H), 4.43 (br s, 1H), 4.23 (br s, 1H), 3.97 (q, *J* = 7.0 Hz, 4H), 3.86 (d, *J* = 14.9 Hz, 1H), *3.54* (m, 1H), 2.83 (d, *J* = 12.8 Hz, 1H), 2.60 (d, *J* = 12.8 Hz, 1H), 2.34–2.25 (m, 3H), 2.05–1.15 (m, 28H), 0.81 (d, *J* = 6.0 Hz, 3H), 0.54 (s, 3H); **13a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.37 (d, *J* = 11.5 Hz, 4H), 6.01 (d, *J* = 11.0 Hz, 1H), 5.32 (s, 1H), 4.99 (s, 1H), 4.91 (d, *J* = 14.9 Hz, 1H), 4.42 (br s, 1H), 4.23 (br s, 1H), 3.88–3.84 (m, 5H), *3.54* (m, 1H), 2.82 (dd, *J* = 12.0, 3.7 Hz, 1H), 2.60 (d, *J* = 12.4 Hz, 1H), 2.34–2.24 (m, 3H), 2.03–0.88 (m, 32H), 0.82 (d, *J* = 6.1 Hz, 3H), 0.54 (s, 3H); **14a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.38–6.32 (m, 4H), 6.01 (d, *J* = 11.0 Hz, 1H), 5.33 (s, 1H), 4.99 (s, 1H), 4.90 (d, *J* = 14.9 Hz, 1H), 4.43 (br s, 1H), 4.23 (br s, 1H), 3.96–3.88 (m, 5H), *3.51* (m, 1H), 2.82 (dd, *J* = 12.1, 4.0 Hz, 1H), 2.60 (dd, *J* = 13.5, 3.3 Hz, 1H), 2.38–2.29 (m, 3H), 2.07–0.96 (m, 36H), 0.89 (d, *J* = 6.6 Hz, 3H), 0.50 (s, 3H).

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12. Compound **11a** did not show any agonistic activity (below 10 μM) under the luciferase reporter gene assay system.[4b]