Design, Synthesis, and Antifibrosis Activity in Liver of Nonsecosteroidal Vitamin D Receptor Agonists with Phenyl-pyrrolyl Pentane Skeleton

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§Supporting Information

ABSTRACT: Liver fibrosis is characterized by excessive deposition of extracellular matrix (ECM) components and results in impaired liver function. Vitamin D plays a critical role in the development of liver fibrosis as it inhibits transforming growth factor β1 (TGF/β1)-induced excessive deposition of ECM in activated hepatic stellate cells (HSCs). Here, a series of novel nonsecosteroidal vitamin D receptor (VDR) agonists with phenyl-pyrrolyl pentane skeleton was designed and synthesized. Among them, seven compounds including 15a exhibited more efficient inhibitory activity in collagen deposition and fibrotic gene expression. Histological examination results displayed that compound 15a treatment prevented the development of hepatic fibrosis that induced by carbon tetrachloride (CCl4) injections in mice. In addition, compound 15a, unlike the positive control calcipotriol and 1,25(OH)2D3, did not cause hypercalcemia that is toxic to nerve, heart, and many other organs. These findings provide novel insights into drug discoveries for hepatic fibrosis using nonsecosteroidal VDR modulators.

INTRODUCTION

Liver fibrosis, characterized by excessive accumulation of extracellular matrix (ECM) and loss of pliability and liver function, is the result of wound-healing responses that were triggered by either chronic or acute liver injury, such as alcohol abuse, chronic hepatitis virus (hepatitis B virus/hepatitis C virus) infection, and, increasingly, nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver disease (NAFLD). With continuous injury, the fibrillar collagens were progressively deposited and parenchymal nodules were surrounded by collagen bands, eventually leading to the histological signature of hepatic cirrhosis, which represents a major global health concern. At present, the only way to treat the end-stage cirrhosis is liver transplantation. However, the condition of the potential recipients, especially the number of available donor organs, limited the applicability of this technique even in the developed world. Moreover, the Food and Drug Administration has not yet approved antifibrotic therapies for chronic liver disease. Hepatic stellate cells (HSCs) are established as a major cellular source of ECM and the main driver of liver fibrogenesis. In healthy liver cells, HSCs remains quiescent, and the main function is storing vitamin A. Once being activated following liver injury, HSCs would enter into a α-smooth muscle actin (α-SMA) positive phenotypic transformation and differentiate into ECM-secreting cells. The activated HSCs produce a considerable amount of collagen I, which is the main component of ECM, resulting in the loss of liver pliability and function.

Transforming growth factor β1 (TGF/β1) is one of the most potent pro-fibrotic modulators. In paracrine and autocrine fashion, TGF/β1 promotes HSC activation and contributes to fibrotic processes in liver. Therefore, the inhibition of TGF/β1 pathway to reduce ECM production in HSCs is recognized as an effective antifibrotic strategy. While the precise mechanisms of regulating ECM synthesis via TGF/β1 pathway have yet to be elucidated, vitamin D has been recognized as an effective antifibrotic strategy. While the precise mechanisms of regulating ECM synthesis via TGF/β1 pathway have yet to be elucidated, vitamin D has been established in a close relationship with TGF/β1 and liver fibrosis development. Previous studies demonstrated a beneficial effect for 1,25(OH)2D3 (1, Figure 1), the most active form of vitamin D, to attenuate liver fibrosis. It is widely recognized that 1,25(OH)2D3 plays a pivotal role in the homeostasis of calcium and phosphorus, cell
proliferation and differentiation, as well as immunomodulation.\textsuperscript{17,18} \(1,25(\text{OH})_2\text{D}_3\) exerts actions through promoting gene transcription by binding to vitamin D receptor (VDR), which belongs to the superfamily of nuclear receptor. VDR is robustly expressed in HSCs and fully functional in these cells.\textsuperscript{19} In 2013, Ding et al. reported that calcipotriol, an analog of \(1,25(\text{OH})_2\text{D}_3\), could inhibit the collagen I and \(\alpha\)-SMA expression via reducing the occupancy of SMAD3 at these sites and antagonizing a wide range of transcriptional responses on profibrotic genes that depend on TGF\(\beta\)/SMAD signaling pathway.\textsuperscript{20} These findings suggest that VDR is a checkpoint to modulate the liver wound-healing response and that VDR ligands may serve as a potential therapy for the treatment of liver fibrosis.

VDR ligands have already been attractive therapeutics against psoriasis, osteoporosis, and cancer.\textsuperscript{21−23} At present, more than 3000 VDR modulators with secosteroid skeleton have been synthesized and biologically evaluated as drug candidates,\textsuperscript{24} such as calcipotriol (2) and paricalcitol (3). Almost all of the VDR ligands with high activity have the same secosteroidal skeleton as \(1−3\), structurally consisting of the A-ring that borne two hydroxyl groups, a triene moiety or conjugated diene, a side chain, and the CD ring. Although many compounds exhibit efficient VDR activity in \textit{in vitro} and \textit{in vivo} studies, their synthetic inconvenience, structural complexity, chemical instability, and hypercalcemia limit the clinical application in the treatment of liver fibrosis. Recently, a lot of attention has been drawn to nonsecosteroidal vitamin D mimics,\textsuperscript{25−28} such as bisphenol derivative (4),\textsuperscript{29} tris-aromatic derivatives (5),\textsuperscript{30} and carborane derivatives (6),\textsuperscript{31−33} due to their less calcium mobilization and simpler structures. Previously, we have reported phenyl-pyrrolyl pentane skeleton as a novel nonsecosteroidal VDR ligand skeleton, which possessed the potential to inhibit proliferation of cancer cells without inducing hypercalcemia effect.\textsuperscript{34−36} However, we found that some compounds had no effect on cancer cells but show significant inhibitory effect on HSCs activation, indicating that VDR agonists may affect HSCs more strongly than cancer cells. Therefore, it is noteworthy to verify whether these nonsecosteroidal vitamin D ligands can act as effective as \(1,25(\text{OH})_2\text{D}_3\) or calcipotriol that with secosteroidal skeleton for preventing the progression of liver fibrosis but have smaller side effects like hypercalcemia.

To explore the relationship between structure and antifibrotic activity for these nonsecosteroidal compounds based on the phenyl-pyrrolyl pentane skeleton, 22 new compounds have been designed, synthesized, and examined with various biological assays. Seven compounds showed much better properties than positive control calcipotriol in the anticollagen I synthetic activities assay. Among them, compound 15a exhibited more potent inhibitory activity against both fibrotic gene expression and collagen deposition by Q-PCR and Western blot assays. Results of histological examination displayed that the treatment of compound 15a prevented hepatic fibrosis induced by carbon tetrachloride (CCl\(_4\)) injection in mice. Moreover, compound 15a had no significant change on serum calcium that can be raised by positive control calcipotriol or \(1,25(\text{OH})_2\text{D}_3\).

\section*{RESULTS AND DISCUSSION}

\subsection*{Design of Target Compounds.}
Boehm et al. reported the first nonsecosteroidal analogs of vitamin D\(_3\), LG190178 (4), and found that propane-1,2-diol of 4 are important for the binding affinity. Based on the phenyl-pyrrolyl pentane skeleton and the structure feature of 4, we designed derivatives 13 using a scaffold hopping strategy and introducing different \(R_1\) substituents to identify antifibrotic VDR ligands. Then, the phenyl-pentane group on the pyrrole ring C-4 position instead of the C-5 position was designed to investigate the influence of the substitution positions of the pyrrole ring, and we obtained...
Finally, we further investigated the A ring part of target compounds and designed compounds 17, 19, and 20 by substitution of the A ring with other hydrophilic groups, such as the butanoic acid, pentanoic acid, alanine, β-alanine, and succinic acid (Figure 2).

**Synthetic Procedures of Target Compounds.** The synthetic pathway of target compounds 13a–h is outlined in Scheme 1. The intermediate 7 was prepared using a previously reported approach, then it reacted with ethyl pyrrole-2-carboxylate at 0 °C, producing intermediate 8a. After the management with iodoethane in DMF, intermediate 9a was obtained. The reduction reaction of 9a gives the intermediate 10a, which was hydrolyzed by KOH to produce 11a in high yield. By reaction with the corresponding amines, 11a gave intermediates 12a–h, respectively. Finally, target compounds 13a–h were obtained by electrophilic substitution of glycidol with intermediates 12a–h in the presence of NaH.

![Scheme 1. Synthesis of Compounds 13a–h](image)

Reagents and conditions: (a) ethyl 1H-pyrrole-2-carboxylate, BF₃·Et₂O, 0 °C, 1 h, 73%; (b) C₂H₅I, NaH, DMF, 0−25 °C, 2 h, 82.4%; (c) Pd/C, HCOONH₄, CH₃OH/EtOAc (10:1), 25 °C, 1 h, 98%; (d) 2 mol/L KOH, EtOH, rt, 1 h, 94%; (e) EDCI, HOBt, TEA, RNH₂, DCM, rt, overnight, 35−96%; (f) glycidol, NaH, DMF, 80 °C, 5 h, 54−82%.

![Scheme 2. Synthesis of Compounds 15a–i](image)

Reagents and conditions: (a) ethyl 1H-pyrrole-2-carboxylate, BF₃·Et₂O, 25 °C, 1 h, 44%; (b) C₂H₅I, NaH, DMF, 0−25 °C, 2 h, 85%; (c) Pd/C, HCOONH₄, CH₃OH/EtOAc (10:1), 25 °C, 1 h, 97%; (d) 2 mol/L KOH, EtOH, rt, 1 h, 95%; (e) EDCI, HOBt, TEA, RNH₂, DCM, rt, overnight, 32−89%; (f) glycidol, NaH, DMF, 80 °C, 5 h, 47−78%.

Compounds 15. Finally, we further investigated the A ring part of target compounds and designed compounds 17, 19, and 20 by substitution of the A ring with other hydrophilic groups, such as the butanoic acid, pentanoic acid, alanine, β-alanine, and succinic acid (Figure 2).

![Figure 2. Design of the novel nonsecosteroidal VDR ligands.](image)
Scheme 3. Synthesis of Compounds 17a−b, 19a−b, and 20

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Reagents and conditions: (a) ethyl 4-bromobutyrate or ethyl 5-bromopentanoate, NaH, DMF, 80 °C, 5 h, 58−67%; (b) 2 mol/L KOH, EtOH, rt, 1 h, 95−97%; (c) Boc-AA, EDCI, DMAP, DCM, rt, overnight, 43−52%; (d) TFA, DCM, rt, 1 h, 87−92%; (e) succinic anhydride, NaH, DMF, 80 °C, 2 h, 43%.
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In moderate yield. By the same method as described for the synthesis of 9a-13, target compounds 15a−i were obtained. The synthetic pathway of compounds 17a−b, 19a−b, and 20 is outlined in Scheme 3. Intermediate 14a was subjected to nucleophilic substitution with the corresponding halohydrocarbon to give intermediates 16a−b, which were further treated by hydrolysis of ester groups to afford target compounds 17a−b in high yield. However, intermediates 18a−b were synthesized by treatment of intermediate 14a with corresponding amino acids. Deprotection of 18a−b using CF3COOH yielded target compounds 19a−b. Compound 20 was obtained by the same method as described in the synthesis of intermediates 16a−b.

**VDR Binding Affinity.** The VDR binding affinity of synthesized compounds was tested with VDR competitor assay, and 1,25(OH)2D3 was applied as the positive control. All compounds were evaluated for their binding affinity in triplicates at 100 nM. The binding affinity of compounds was exhibited by a reference value to 1,25(OH)2D3, which is assigned as 100%. Tables 1 and 2 showed the structure−activity relationships (SARs) for these compounds. First, we focused on the important pharmacophore side chain of the pyrrole ring at the C-5 position bearing a phenyl-pentane group; the results showed that compounds with the terminal hydrophobic groups in the side chain section, such as tert-butoxide (13a), tert-butyl (13b), trifluoromethyl (13e), displayed significant binding affinities. However, introducing flexible hydrophobic substitution of the n-pentyl group to give compound 13h resulted in decreased affinity, which suggests that flexible substitution is not preferred in the VDR ligand binding pocket (LBP). Moreover, substitutions of hydrophilic groups, such as 1-hydroxy (13d) and aminos (13f−g), dramatically decreased the binding affinities. By removing the substitution on the pyrrole group C-5 position to C-4 position, compounds 15a−i were synthesized to explore the influence of substitution position on the binding affinity. Although most compounds displayed decreased binding affinities compared to the pyrrole group C-5 position, compound 15a bearing tert-butoxide group showed better activity than the corresponding compound 13a and turned out to be the most potent molecule. Subsequently, we further investigated the A ring part of target compounds by substitution of the A ring with other hydrophilic groups, such as butanoic acid (17a), pentanoic acid (17b), alanine (19a), β-alanine (19b), and butanedioic acid (20). As a result, no improvement of the binding affinities was detected compared with that of compound 15a. Meanwhile, carboxylic acid as the terminal hydrophilic group, such as in compounds 17a−b and 20, displayed better binding affinities than that of amine groups (19a−b).

**Transactivation.** To estimate agonistic abilities of the nonsecosteroidal ligands bearing phenyl-pyrrolyl pentane skeleton, a transactivation assay was performed in HEK293 cells using pGL4.27-SPP × 3-Luci reporter plasmid. Compounds 13a−b, 15a, and 15e with strong binding affinities were selected, and calcipotriol and 1,25(OH)2D3 were used as positive controls. As shown in Figure 3, all four compounds acted as potent agonists with concentration-dependent transcriptional activity. Among them, compound 15a was the most potent compound and displayed transcriptional activity at 100 nM, while compounds 13a−b and 15e did not reach the optimal transcription level even at 1000 nM. This may be due to the interaction of heterodimer partners with VDR, such as nuclear receptor corepressor 1 and steroid receptor coactivator 1, which has an effect on the biological activity of VDR. In addition, a significantly higher increase of transcripts encoding 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) by compounds 15a was shown (Figure S1).

**Anticollagen I Synthetic Activities in Vitro.** Liver fibrosis is characterized by the replacement of functional hepatic tissue with highly cross-linked collagen I-rich ECM, and TGFβ1 is recognized as one of the most potent pro-fibrotic modulators responsible for collagen I synthesis.
Consequently, inhibiting the production of collagen I induced by TGF/β1 is an effective strategy against fibrotic progress. To examined the antifibrotic effects of all target compounds, a stable and unlimited source of human HSCs, LX-2 cells, was employed as a valuable cell model to study human hepatic fibrosis. Calcipotriol and 1,25(OH)2D3 were applied as positive control (Table 2). Compared with calcipotriol and 1,25(OH)2D3, seven of the synthesized analogues (13a–b, 13e, 15a–b, 15e, and 17a) at the concentration of 100 nM, which was little cytotoxic to LX-2 (Table S1), demonstrated more effective inhibitory properties against collagen I synthesis, with the values at the range of 112–210%, and six compounds (13c, 13h, 17b, 19a–b, and 20) displayed an equivalent inhibitory potency. This discrepancy between binding affinity and agonistic activity could be interpreted by the interactions between the cofactors and VDR ligand complex. It is required that the AF-2 transactivation motif of VDR interacts with cofactors such as VDR interacting proteins (DRIPs) for VDR transcriptional activation.37–39 Table 2 highlights the important SAR features of inhibitory potencies. Similar to the SARs of binding affinities, 13a–b and 13e bearing terminal hydrophobic groups in the side chain section also displayed significant inhibitory activities. In addition, compound 13h, which showed decreased binding affinity compared with other compounds bearing terminal hydrophobic groups as described above, was also only moderately active. Replacement of hydrophobic groups with hydrophilic groups, such as 1-hydroxy (13d) and aminos (13f–g), dramatically weakened the inhibitory activities. On this point, it could be proved that the inhibitory activities of synthesized compounds are positively correlative with VDR binding affinities. Varying the position of the substitution from the pyrrole group on the C-5 position to the C-4 had dramatic effects on inhibitory activities. As likely as the binding affinities,
compound 15a showed better activity than the corresponding compound 13a and turned out to be the most potent molecule. In addition, all compounds varying propane-1,2-diol to other hydrophilic groups displayed moderate inhibitor activities against collagen I synthesis and had no better results than 15a. Meanwhile, carboxylic acid as the terminal hydrophilic group, such as in compounds 17a–b and 20, displayed better inhibitory activities than that of amine groups (19a–b).

**Effects on the Expression of Collagen I and α-SMA in LX-2 Cells.** The antifibrotic activities of selected compounds 13a, 13b, 15a, and 15e, which displayed optimal property on binding affinities and antifibrocollagen I synthetic activities, were analyzed using Western blot and Q-PCR assays. The increased expression of collagen I and α-SMA is shown by the markers of activated HSCs. As described above, liver fibrosis, regardless of its cause, is featured by progressive accumulation of ECM proteins, and the main component is collagen I. Moreover, the α-SMA-positive myofibroblasts are considered as the key promoters in the progression of liver fibrosis. Collagen I alpha 1 (COL1A1) is the direct target of VDR. In addition, α-SMA and collagen I are both upregulated by TGFβ1 in HSCs. Therefore, the expression of α-SMA and collagen I were selected to determine the antifibrotic activities of selected compounds. As shown in Figure 4C,D, the activity of these molecules was significantly affected by the pyrrole group substitution position: compounds 15a and 15e (C-4 substitution) at the concentration of 100 nM significantly reduced α-SMA and collagen I protein levels in TGFβ1-treated LX-2 cells, while compounds 13a and 13b (C-4 substitution) exhibited no significant influence on ACTA2 expression. Compared with the hydroxyl group of analog 15a, 15e showed less activity, which suggests that a hydroxyl group in the side chain terminal is essential. Q-PCR results (Figure 4A,B) showed that compound 15a significantly down-regulated COL1A1 and ACTA2 mRNA expression. Moreover, compared with positive control calcipotriol and 1,25(OH)2D3, compound 15a showed more effective inhibitory potency against COL1A1 mRNA expression. The results impel us continuously to test the antifibrotic effect of these compounds.

**Compound 15a Inhibited Activation of LX-2 Cells through VDR.** VDR is recognized as the potential therapy target for liver fibrosis, and the above-mentioned results suggested the compounds may be VDR agonists. To confirm that compound 15a repressed fibrotic gene expression via VDR, RNA interference (RNAi) was used in LX-2 cells. Loss of VDR-abolished 15a-mediated repression of collagen I and α-SMA expression was shown in Figure 5. These data demonstrated that compound 15a exerts its repressing effect on HSCs activation through interaction with VDR.

**Effects on Suppressing the Expression of α-SMA in CCL4-Induced Hepatic Fibrosis Mice.** Based on *in vitro* results, compound 15a was selected for further studies in *vivo*. To explore whether compound 15a could repress the expression of fibrotic gene and inhibit hepatic fibrogenesis in *vivo*, CCL4 was used to induce liver fibrosis by intraperitoneal (IP) injection in C57BL/6j mice. The fibrotic property of compound 15a was determined by histological examination. As shown in Figure 6, consistent with the above studies in *vitro*, oral administration of compound 15a to CCL4-treated mice reduced α-SMA levels in the liver tissues according to Q-PCR and IHC staining. In addition, compound 15a also increased the mRNA levels of Cyp24a1, suggesting 15a inhibits fibrotic progress through agitating VDR (Figure S2).

**Effects on Suppressing the Expression of Collagen in CCL4-Induced Hepatic Fibrosis mice.** In addition, we measured collagen content to further examine the antifibrotic

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**Table 2. Affinities of VDR Binding and Activities of Anticollagen I Synthetic at 100 nM**

<table>
<thead>
<tr>
<th>compd</th>
<th>relative VDR binding ability (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>anticollagen I at 100 nM (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>compd</th>
<th>relative VDR binding ability (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>anticollagen I at 100 nM (%)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>13a</td>
<td>43 ± 3.2</td>
<td>150 ± 13.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>15e</td>
<td>39 ± 4.8</td>
<td>133 ± 14.5&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>13b</td>
<td>45 ± 5.3</td>
<td>159 ± 9.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>15f</td>
<td>17 ± 3.9</td>
<td>112 ± 7.8</td>
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<tr>
<td>13c</td>
<td>27 ± 4.1</td>
<td>71 ± 7.2</td>
<td>15g</td>
<td>17b ± 3.2</td>
<td>90 ± 9.2</td>
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<tr>
<td>13d</td>
<td>18 ± 2.5</td>
<td>135 ± 15.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>15h</td>
<td>16 ± 1.3</td>
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<tr>
<td>13e</td>
<td>37 ± 2.5</td>
<td>70 ± 7.9</td>
<td>15i</td>
<td>17a ± 3.9</td>
<td>112 ± 7.8</td>
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<tr>
<td>13f</td>
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<td>210 ± 20.1&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>13g</td>
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<td>119 ± 7.3&lt;sup&gt;*&lt;/sup&gt;</td>
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<td></td>
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<sup>a</sup>The values represent the mean ± SD of three independent experiments. 1,25(OH)2D3 (1) is assigned as 100%. 1,25(OH)2D3 (1) and calcipotriol (2) are the positive controls. *P < 0.05 vs 1,25(OH)2D3 (1).
Effect of compound 15a. Histopathologically, compound 15a treatment resulted in the inhibition of collagen accumulation in CCl4 mice liver based on H&E and Masson's trichrome staining (Figure 7A). The amounts of hepatic hydroxyproline in liver tissue were estimated, which was a major component of the collagen. As shown in Figure 7B, treatment of compound 15a had a significant reduction in hydroxyproline content, with slightly stronger potency than positive control calcipotriol and 1,25(OH)2D3. Moreover, mRNA levels of Col1α1 were elevated in the liver fibrosis models, and the results showed that the expression of Col1α1 was also reduced by compound 15a treatment (Figure 7C). These results suggest that compound 15a treatment prevents CCl4-induced liver injury and hepatic fibrosis.

Effects on Liver Function and Serum Calcium of Fibrotic Models. Serum alanine transaminase (ALT), aspartate transaminase (AST), and total bile acid (TBA) levels are commonly measured clinically as biomarkers for liver health.40 Significantly elevated levels of AST, ALT, and TBA often suggest the existence of liver damage. As shown in Figure 8A−C, the levels of AST, ALT, and TBA were significantly decreased in compound 15a-treated mice as compared with control animals. Moreover, compound 15a displayed better results than positive control calcipotriol and 1,25(OH)2D3, which are very promising for the reduction of liver damage. To determine the effect of novel designed nonsecosteroidal analogs on inducing hypercalcemic, calcium concentration was measured by calcemic activity assay in vivo. Ma et al. reported that nonsecosteroidal VDR modulators showed poor activity in intestinal cells.29 Moreover, the nonsecosteroidal compound has the ability to activate VDR and is weak in binding to vitamin D-binding proteins, so that it does not accumulate excessively in the intestine, resulting in no excessive calcium absorption.29 In our study, compound 15a displayed small impact on the expression of intestinal Trpv6, which is a VDR target gene involved in calcium metabolism (Figure S3). As shown in Figure 8D, compared with calcipotriol and 1,25(OH)2D3, there was no significant effect on serum calcium when treated with compound 15a in mice, which suggests that nonsecosteroidal analog 15a results in a high dissociation of antifibrotic potency from calcemic effects.

In Vivo Pharmacokinetics Study. Pharmacokinetic studies of compound 15a and 1,25(OH)2D3 were performed in rats. The results were shown in Table S3. Oral bioavailability of compound 15a was 29.32% and t1/2 value was 6.57 h after oral administration. Compound 15a displayed similar bioavailability compared with 1,25(OH)2D3, whose bioavailability was 30.83% after oral administration. However, the t1/2 value of 15a was a little smaller compared with 1,25(OH)2D3, whose t1/2 value was 7.55 h after oral administration. This maybe because nonsecosteroidal VDR agonist did not combined with vitamin D binding protein,29 so the metabolism of 15a is slightly faster. Still, the results suggested that 15a could possess therapeutic potentials for treatment of liver fibrosis.

Docking Analysis. In this study, we have performed VDR binding and transactivation assays, as well as knocking down VDR gene to prove that compound 15a represses fibrotic gene expression via VDR. To clarify the detailed interactions of VDR and the most promising compound 15a, molecular docking study was made on the basis of the complexation of...
the crystallographic structure of LG190178 and VDR (PDB code: 2ZFX). Using software Discovery Studio 3.0, compound 15a was manually docked into the crystal structure of VDR. Figure 9A showed the conformation superposition of compound 15a and the natural ligand 1,25(OH)2D3. Figure 9B showed the conformation superposition of compound 15a and YR301. The results demonstrated that the A ring part and side chain of compound 15a exhibited similar conformations to those detected in the presence of YR301 and 1,25(OH)2D3.

As shown in Figure 9C, the hydroxyl group of compound 15a in the side chain could form the hydrogen-bonding interactions with His 301 and His 393, which are the same for 1,25(OH)2D3 bound to the hVDR LBD complex. However, the A ring part of 1,25(OH)2D3 was bound with Ser 233, Arg 270, Tyr 139, and Ser 274, while compound 15a only forms a hydrogen-bonding interaction with Arg 270 by 2-
OH. This may affect the interactions of compound 15a to VDR and result in reducing binding affinity.

**CONCLUSION**

In conclusion, the design, synthesis, and biological assessment of nonsecosteroidal derivatives with phenyl-pyrrolyl pentane skeleton have been described in this Article. The selected compounds act as VDR agonists for effectively preventing the progression of liver fibrosis. The analysis of SARs directed the synthesis of derivative 15a, which may be a strong inhibitor for collagen 1 synthesis. Further exploration demonstrated that compound 15a had higher inhibitory activity on fibrotic gene expression and collagen deposition. Histological examination results displayed that compound 15a treatment prevented...
hepatic fibrosis induced by CCl₄ injections in mice. More importantly, compound 15a can display better results for the reduction of liver damage without significant change on serum calcium, which can be induced by positive control calcipotriol and 1,25(OH)₂D₃. This work supports that using non-secosteroidal VDR modulators may be applied for the treatment of hepatic fibrosis because, presently, there are still no effective therapeutic strategies.

**EXPERIMENTAL SECTION**

**Chemistry. General Information.** Commercially available reagents and solvents were used without further purification. Column chromatography was carried out on Merck silica gel 60 (200–300 mesh). ¹H and ¹³C NMR spectra were recorded with 300 MHz spectrometers in the indicated solvents (TMS as internal standard). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), double doublet (dd), multiplet (m), and broad (br). Purity of all tested compounds was ≥95%, as estimated by HPLC analysis. The major peak of the compounds analyzed by HPLC accounted for ≥95% of the combined total peak area when monitored by a UV detector at 254 nm. Low-resolution mass spectra (LR-MS) and high-resolution mass spectra (HR-MS) were measured on Agilent QTOF 6520.

**General Procedures.** Ethyl-5-(3-(4-benzyloxy)-3-methylphenyl)pentan-3-yl)-1H-pyrole-2-carboxylate (8a). BF₃·Et₂O (13 mL, 105 mmol) was added dropwise to a solution of intermediate 7 (13 g, 46 mmol) and ethyl-1H-pyrole-2-carboxylate (7.1 g, 51 mmol) in dichloromethane (20 mL) at 0 °C. The mixture was stirred for 1 h at 25 °C. Then to the solution was added H₂O (20 mL), and the organic phase was separated. The organic phases were washed with brine, dried over anhydrous Na₂SO₄, and evaporated to give compound 8a as yellow solid (13.5 g, 73% yield).

Ethyl-4-(3-(4-benzyloxy)-3-methylphenyl)pentan-3-yl)-1H-pyrole-2-carboxylate (8b). By the same manner as described for the preparation of 8a, the intermediate 8b was prepared from the intermediate 4 and purified by silica gel chromatography with petroleum ether/ethyl acetate (12/1, v/v). Yield: 44%.

Ethyl-5-(3-(4-(benzyloxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-1H-pyrole-2-carboxylate (9a). To a solution of compound 8a (4.05 g, 10 mmol) in DMF (5 mL), NaH (288 mg, 12 mmol) was added portion-wise at 0 °C. After stirring for 0.5 h, ethyl iodide (1.25 g, 8 mmol) was added. The reaction mixture was stirred at 25 °C for 2 h, and then H₂O (20 mL) was added dropwise followed by ethyl acetate (10 mL). The organic phase was separated, and the aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with H₂O and brine and then dried over anhydrous Na₂SO₄, and evaporated. The residue was purified by column chromatography with petroleum ether/ethyl acetate (20/1, v/v) to give compound 9a as yellow oil (3.57 g, 82.4% yield).

Ethyl-4-(3-(4-(benzyloxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-1H-pyrole-2-carboxylate (9b). By the same manner as described for the preparation of 9a, the intermediate 9b was prepared from the intermediate 8b and purified by silica gel chromatography with petroleum ether/ethyl acetate (20/1, v/v). Yield: 85%.

Ethyl-1-ethyl-5-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-1H-pyrole-2-carboxylate (10a). To a solution of intermediate 9a (2.0 g, 4.6 mmol) in methanol (20 mL), Pd/C (0.2 g) and ammonium formate (2.9 g, 46 mmol) were added. The reaction mixture was stirred at 25 °C overnight. The precipitate was filtered off, and H₂O (100 mL) and ethyl acetate (50 mL) was added to the solution. The organic phase was separated, and the aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous Na₂SO₄, and evaporated to give compound 10a as white solid (1.55 g, 98% yield).

Ethyl-1-ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-1H-pyrole-2-carboxylate (10b). By the same manner as described for the preparation of 10a, the intermediate 10b was prepared from the intermediate 9b. Yield: 97%.

1-Ethyl-5-(3-(4-(hydroxy-3-methylphenyl)pentan-3-yl)-1H-pyrole-2-carboxylic acid (11a). The intermediate 10a (351 mg, 1 mmol) was dissolved in ethanol (6 mL) and treated with KOH (168 mg, 3 mmol) in H₂O, and the reaction mixture was stirred at 80 °C for 5 h. The solution was diluted with H₂O (20 mL), and the pH value was adjusted to about 3–4 using 1 M HCl. Then, it was...
extracted with ethyl acetate. The aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with brine and then dried over anhydrous Na2SO4 and evaporated. The residue was purified by column chromatography with petroleum ether/ethyl acetate (10:1, v/v/v) to give the intermediate 11a as yellow oil (307 mg, 94%).

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-1H-pyrrole-2-carboxylic acid (11b). By the same manner as described for the preparation of 11a, the intermediate 11b was prepared from the intermediate 10b. Yield: 95%.

1-Ethyl-N-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (12a). To a solution of compound 11a (250 mg, 0.61 mmol) in CH2Cl2 (10 mL) was added Et3N (255 μL, 1.83 mmol), followed by 1-amino-2-methylpropan-2-ol (153 mg, 1.22 mmol), EDCI (175 mg, 0.92 mmol), and HOBT (124 mg, 0.92 mmol). The reaction mixture was stirred at 25 °C overnight and then poured into H2O. The solution was extracted with CH2Cl2, and the aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with brine and then dried over anhydrous Na2SO4 and evaporated. The residue was purified by column chromatography with petroleum ether/ethyl acetate (10/1, v/v) to give the intermediate 12a as white solid (100 mg, 96% yield).

N-(Cyclopropylmethyl)-5-(3-(4,2-dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1H-pyrrole-2-carboxamide (13a). By the same manner as described for the preparation of 13a, compound 13b was prepared from the intermediate 12b. Yield: 87%.

Methyl(1-ethyl-5-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(2,2,2-trifluoroethyl)-1H-pyrrole-2-carboxamide (13e). By the same manner as described for the preparation of 13a, compound 13e was prepared from the intermediate 12e. Yield: 96%.

N-(Cyclopropylmethyl)-5-(3-(4,2-dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1H-pyrrole-2-carboxamide (13d). By the same manner as described for the preparation of 13a, compound 13d was prepared from the intermediate 12d. Yield: 35%.

4-(3-(4,2-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1H-pyrrole-2-carboxamide (13f). By the same manner as described for the preparation of 13a, compound 13f was prepared from the intermediate 12f. Yield: 68%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-neopenetyl-1H-pyrrole-2-carboxamide (12b). By the same manner as described for the preparation of 12a, the intermediate 12b was prepared from the intermediate 11a. Yield: 78%.

By the same manner as described for the preparation of 12a, the intermediate 12c was prepared from the intermediate 11a. Yield: 57%.

Methyl(1-ethyl-5-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-1H-pyrrole-2-carboxyl)glycinate (12d). By the same manner as described for the preparation of 12a, the intermediate 12d was prepared from the intermediate 11a. Yield: 57%.

1-Ethyl-5-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (12d-0H). To a solution of 12d (114 mg, 0.23 mmol) in ethyl acetate (10 mL), LiAlH4 (13 mg, 0.35 mmol) was added portion-wise at 0 °C. The reaction mixture was stirred at 25 °C for 1 h, and then H2O (10 mL) was added. The solution was extracted with ethyl acetate, and aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with brine and then dried over anhydrous Na2SO4, and evaporated. The residue was purified by column chromatography with petroleum ether/ethyl acetate (1:1, v/v) to give compound 12d-0H as white solid (100 mg, 96% yield).

1-Ethyl-5-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(2,2,2-trifluoroethyl)-1H-pyrrole-2-carboxamide (12e). By the same manner as described for the preparation of 12a, the intermediate 12e was prepared from the intermediate 11a. Yield: 45%.

1-Ethyl-5-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(2,2,2-trifluoroethyl)-1H-pyrrole-2-carboxamide (12f). By the same manner as described for the preparation of 12a, the intermediate 12f was prepared from the intermediate 11a. Yield: 68%.

Methyl(1-ethyl-5-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-1H-pyrrole-2-carboxyl)glycinate (12g). By the same manner as described for the preparation of 12a, the intermediate 12g was prepared from the intermediate 11a. Yield: 62%.

1-Ethyl-5-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-pentyl-1H-pyrrole-2-carboxamide (12h). By the same manner as described for the preparation of 12a, the intermediate 12g was prepared from the intermediate 11a. Yield: 87%.

5-(3-(4,2-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-N-(2-hydroxy-2-methylpropyl)-1H-pyrrole-2-carboxamide (13a). To a solution of intermediate 12a (386 mg, 1 mmol) in DMF, NaH (80 mg, 2 mmol) was added portion-wise at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, and then glycidol (0.1 mL, 1.5 mmol) was added. The reaction mixture was moved to 80 °C for 5 h and then H2O (10 mL) was added. The solution was extracted with ethyl acetate, and aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with brine and then dried over anhydrous Na2SO4, and evaporated. The residue was purified by column chromatography (CH2Cl2/CH3OH = 50/1) to give compound 13a as white solid (245 mg, 53% yield).

1-Ethyl-N-neopenetyl-1H-pyrrole-2-carboxamide (13b). By the same manner as described for the preparation of 13a, compound 13b was prepared from the intermediate 12b. Yield: 87%.

1-Ethyl-N-(2-(Dimethylamino)ethyl)-1H-pyrrole-2-carboxamide (13c). By the same manner as described for the preparation of 13a, compound 13c was prepared from the intermediate 12c. Yield: 44%.

1-Ethyl-N-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (13d). By the same manner as described for the preparation of 13a, compound 13d was prepared from the intermediate 12d. Yield: 35%.

1-Ethyl-N-(2,2,2-trifluoroethyl)-1H-pyrrole-2-carboxamide (13e). By the same manner as described for the preparation of 13a, compound 13e was prepared from the intermediate 12e. Yield: 68%.

1-Ethyl-N-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (13f). By the same manner as described for the preparation of 13a, compound 13f was prepared from the intermediate 12f. Yield: 96%.

By the same manner as described for the preparation of 13a, compound 13h was prepared from the intermediate 12h. Yield: 96%.

1-Ethyl-N-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (14a). By the same manner as described for the preparation of 12a, the intermediate 14a was prepared from the intermediate 11b. Yield: 53%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-neopenetyl-1H-pyrrole-2-carboxamide (14b). By the same manner as described for the preparation of 12a, the intermediate 14b was prepared from the intermediate 11b. Yield: 76%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(2,2,2-trifluoroethyl)-1H-pyrrole-2-carboxamide (14c). By the same manner as described for the preparation of 12a, the intermediate 14c was prepared from the intermediate 11b. Yield: 42%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (14d-0H). By the same manner as described for the preparation of 12a, the intermediate 14d-0H was prepared from the intermediate 11b. Yield: 89%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (14d). By the same manner as described for the preparation of 12a, the intermediate 14d was prepared from the intermediate 11b. Yield: 87%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(2,2,2-trifluoroethyl)-1H-pyrrole-2-carboxamide (14e). By the same manner as described for the preparation of 12a, the intermediate 14e was prepared from the intermediate 11b. Yield: 32%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(1-hydroxy-2-propyl)-1H-pyrrole-2-carboxyl)glycinate (14f). By the same manner as described for the preparation of 12a, the intermediate 14f-0H was prepared from the intermediate 14f. Yield: 46%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-1H-pyrrole-2-carboxyl)valinate (14g). By the same manner as described for the preparation of 12a, the intermediate 14g was prepared from the intermediate 11b. Yield: 49%.
1-Ethyl-N-(1-hydroxy-3-methylbutan-2-yl)-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-1H-pyrrole-2-carboxamide (14g—OH). By the same manner as described for the preparation of 12d—OH, the intermediate 14g—OH was prepared from the intermediate 14g. Yield: 61%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-pentyl-1H-pyrrole-2-carboxamide (14h). By the same manner as described for the preparation of 12a, the intermediate 14h was prepared from the intermediate 11b. Yield: 81%.

1-Ethyl-4-(3-(4-hydroxy-3-methylphenyl)pentan-3-yl)-N-(3-hydroxypropyl)-1H-pyrrole-2-carboxamide (14i). By the same manner as described for the preparation of 12a, the intermediate 14i was prepared from the intermediate 11b. Yield: 72%.

4-(3-(4-(2,3-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-N-(2-hydroxy-2-methylpropyl)-1H-pyrrole-2-carboxamide (15a). By the same manner as described for the preparation of 13a, compound 15a was prepared from the intermediate 14a. Yield: 67%.

4-(3-(2,3-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-N-neopentyl-1H-pyrrole-2-carboxamide (15b). By the same manner as described for the preparation of 13a, compound 15b was prepared from the intermediate 14b. Yield: 58%.

N-(Cyclopentylmethyl)-4-(3-(4-(2,3-dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-1H-pyrrole-2-carboxamide (15c). By the same manner as described for the preparation of 13a, compound 15c was prepared from the intermediate 14c. Yield: 47%.

4-(3-(4-(2,3-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-4-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (15d). By the same manner as described for the preparation of 13a, compound 15d was prepared from the intermediate 14d—OH. Yield: 54%.

4-(3-(2,3-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-N(2,2,2-trifluoroethyl)-1H-pyrrole-2-carboxamide (15e). By the same manner as described for the preparation of 13a, compound 15e was prepared from the intermediate 14e. Yield: 78%.

4-(3-(2,3-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-N(1-hydroxypropan-2-yl)-1H-pyrrole-2-carboxamide (15f). By the same manner as described for the preparation of 13a, compound 15f was prepared from the intermediate 14f—OH. Yield: 72%.

4-(3-(2,3-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-N-(1-hydroxy-3-methylbutan-2-yl)-1H-pyrrole-2-carboxamide (15g). By the same manner as described for the preparation of 13a, compound 15g was prepared from the intermediate 14g—OH. Yield: 66%.

4-(3-(2,3-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-N-pentyl-1H-pyrrole-2-carboxamide (15h). By the same manner as described for the preparation of 13a, compound 15h was prepared from the intermediate 14h. Yield: 76%.

4-(3-(2,3-Dihydroxypropoxy)-3-methylphenyl)pentan-3-yl)-1-ethyl-N-(3-hydroxypropyl)-1H-pyrrole-2-carboxamide (15i). By the same manner as described for the preparation of 13a, compound 15i was prepared from the intermediate 14i. Yield: 69%.

Ethyl-4-(4-(1-ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)butanoate (16a). To a solution of the intermediate 14a (386 mg, 1 mmol) in DMF, NaH (80 mg, 2 mmol) was added portion-wise at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, and then ethyl 4-bromobutyrate (291 mg, 1.5 mmol) was added. The reaction mixture was moved to 25 °C for 5 h, and then H2O (10 mL) was added. The solution was extracted with ethyl acetate, and the aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with brine and then dried over anhydrous Na2SO4 and evaporated. The residue was purified by column chromatography (CH2Cl2/CH3OH, 25 nm bandwidth) to give compound 16a as white solid (290 mg, 58% yield).

Ethyl-5-(4-(1-ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)pentanoate (16b). By the same manner as described for the preparation of 16a, compound 16b was prepared from the intermediate 14a. Yield: 67%.

Ethyl-4-(3-(1-ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)butanoic Acid (17a). To a solution of the intermediate 16a (200 mg, 0.4 mmol) in ethanol, KOH (67 mg, 1.2 mmol) in H2O was added, and the reaction mixture was stirred at 80 °C for 5 h. The solution was diluted with H2O (20 mL), and the pH value was adjusted to about 3–4 using 1 M HCl. Then it was extracted with ethyl acetate. The aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with brine and then dried over anhydrous Na2SO4 and evaporated. The residue was purified by column chromatography with petroleum ether/ethyl acetate (180 mg, 95%). 5-(4-(1-Ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)pentanoic Acid (17b). By the same manner as described for the preparation of 17a, compound 17b was prepared from the intermediate 16b. Yield: 87%.

5-(4-(1-Ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)tert-butoxy carbonylalaninate (18a). To a solution of the intermediate 14a (386 mg, 1 mmol) in CH2Cl2 (10 mL) was added Et3N (418 μL, 3 mmol), followed by Boc-alanine (153 mg, 1.5 mmol), EDCI (288 mg, 1.5 mmol), and DMAP (12 mg, 0.1 mmol). The reaction mixture was stirred at 25 °C overnight and then poured into H2O. The solution was extracted with CH2Cl2 and aqueous phase was extracted with CH3Cl2. The combined organic phases were washed with H2O and brine and then dried over anhydrous Na2SO4 and evaporated. The residue was purified by column chromatography with petroleum ether/ethyl acetate (4/1, v/v) to give compound 18a as white solid (197 mg, 43% yield).

5-(4-(1-Ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)tert-butoxy carbonylaminopropanoate (18b). By the same manner as described for the preparation of 18a, intermediate 18b was prepared from the intermediate 14a. Yield: 52%.

5-(4-(1-Ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)tert-butoxy carbonylalaninate (19a). Yield: 67%.

5-(4-(1-Ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)tert-butoxy carbonylaminopropanoate (18b). By the same manner as described for the preparation of 18a, intermediate 18b was prepared from the intermediate 14a. Yield: 67%.

5-(4-(1-Ethyl-5-(2-hydroxy-2-methylpropyl)carbamoyl)-1H-pyrrol-3-yl)pentan-3-yl)-2-methylphenoxy)tert-butoxy carbonylalaninate (19a). Yield: 67%.
**Transcription Assay.** Luciferase activity assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. HEK293 cells of 85–90% confluence were seeded in 48-well plates. Transfections were composed of 140 ng of TK-SPP X 3-Luci reporter plasmid, 20 ng of pCMX-Renilla, 30 ng of pENTER-CMV-hRXRα, and 100 ng of pCMX-VDR for each well using Lipofectamine2000 Reagent (Invitrogen). Eight hours after transfection, test compounds were added. Luciferase activity assay was performed 24 h later using the Dual-Luciferase Assay System. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. All the experiments were performed three times.

**Anticollagen I Synthetic Activity Assay.** Anticollagen I synthetic activity assay was performed using the human collagen I ELISA Kit (Elabscience, Wuhan). LX-2 cells obtained from American Type Culture Collection were maintained in Gibco 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Approximately 1 × 10⁵ cells, suspended in medium, were plated into each well of a 24-well plate and grown at 37 °C in humidified atmosphere with 5% CO₂ for 24 h. The following day, tested compounds at the concentration of 100 nM were added to the culture medium and incubated for 24 h. Supernate was centrifuged for 20 min to remove insoluble impurity and cell debris at 10000g at 2–8 °C. The clear supernate was suspended and the assay was immediately carried out following the description of the Kit. Finally, the optical density (OD) was measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value is proportional to the concentration of human collagen I.

**Small Interfering RNA (siRNA) Transfection.** A VDR-directed siRNA and a scrambled siRNA were purchased from RIBOBIO Biotechnologies (Guangzhou, China). Transfection was carried out at a concentration of 50 nM using Lipofectamine2000 Reagent (Invitrogen). Transfected cells were cultured 24 h prior to terminal assays.

**CCL4-Induced Mouse Hepatic Fibrosis Model.** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Experimentation Ethics Review Committee of China Pharmaceutical University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Male C57BL/6j mice (8 weeks old) were purchased from the Medical School of Yangzhou University (Yangzhou, China). All mice were maintained under standard conditions with free access to water and laboratory rodent food. To set up CCL4-induced mouse hepatic fibrosis model, mice were IP injected with 0.5 mL/kg bodyweight CCL4 (1:50 v/v in corn oil from Sigma) three times a week for 4 weeks. Control mice received vehicle (DMSO in corn oil) instead. The effect of vitamin D analogues on CCL4-induced mouse hepatic fibrosis was evaluated 20 days after the first dose of CCL4 (Dawley, calciumtriol, 1,25(OH)₂D₃ or compound 15a (20 μg/kg weight) was administered by oral gavage five times a week. Mice were sacrificed 72 h after the final CCL4 injection. Mouse livers and serum were obtained for histopathology, collagen assay, biochemical, and molecular analyses.

**Lab Data Detection of Serum Sample.** Serum was collected from blood after centrifugation at 3000 rpm for 15 min at 4 °C. Serum alanine amino transferase (ALT), aspartate transaminase (AST), and total bile acid (TBA) were detected using commercial kits from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China) according to the manufacturer’s instructions.

**Histology.** Livers were fixed in 4% (w/v) neutral phosphate-buffered paraformaldehyde for 24 h, dehydrated, transparentized, and embedded in paraffin. Liver tissues were cut into 5 μm sections, which were stained with hematoxylin-eosin (H&E) for structured observation, or with Masson’s trichrome stain for detection of collagen deposition. Determination of hydroxyproline content was carried out using a kit from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China) according to the instruction by the manufacturer.

**Immunohistochemistry Analysis.** Immunohistochemistry staining for detection of HSC activation in vivo was performed as previously described. Briefly, paraffin was removed from the slides, subjected to antigen retrieval, and quenched of endogenous peroxidase activity using 3% (v/v) H₂O₂ for 10 min. Immune complexes were visualized using suitable peroxidase-coupled secondary antibodies, according to the manufacturer’s protocol (SP-9000 D 2-step plus poly-HP Parini-mouse/rabbit IgG detection system, ZSGB-BIO, Beijing, China). Mouse anti-α-SMA was employed as the primary antibody (Boster, Wuhan, China). The secondary antibodies incubated were horseradish peroxidase-conjugated goat antimouse IgG (Boster, Wuhan, China).

**RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (Q-PCR).** cDNA was generated from RNA extracts derived from cultured LX-2 cells and liver tissues using a reverse transcription kit (Transgen, Beijing, China). β-actin (mouse) or U36B4 (human) was used as an internal control. Q-PCR was performed using the SYBR Green Master Mix (Vazyme). Primer pairs of mRNA used are as shown in Table S2.

**Western Blot.** Proteins were purified from LX-2 cells. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and were electrothermally transferred to polyvinylidene fluoride (PVDF) membranes using standard procedures. The following primary antibodies were employed: mouse anti-α-SMA (Boster, Wuhan, China), rabbit anti-collagenI (Boster, Wuhan, China), mouse anti-VDR (Santa Cruz, Inc.), and mouse anti-β-actin (Boster, Wuhan, China). Horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (Boster, Wuhan, China) was used as the secondary antibody. Immunoreactive protein bands were detected using an Odyssey Scanning System (LI-COR Inc.).

**Pharmacokinetics Study.** Compound 15a and 1,25(OH)₂D₃ were dissolved in ethanol/EL/saline (1:1:18). Male Sprague–Dawley (SD) rats (n = 3) weighing 180–220 g were injected with these compounds intravenously (5 mg/kg) or intragastrically (20 mg/kg). Blood plasma samples were collected at 5, 15, and 30 min and 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h after administration of compounds and then immediately centrifuged (12000 rpm, 10 min) to obtain plasma samples. The concentration of compounds in plasma was measured by HPLC. The pharmacokinetic parameters were calculated using Kinetic 4.4 software.

**Statistical Analysis.** Data were expressed as means ± SD from at least three independent experiments. The differences between groups were analyzed for significance by t test when only two groups were compared or by one-way analysis of variance (ANOVA). All statistical analysis was performed using SPSS for Windows version 11.0 (SPSS, Chicago, IL).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01165.

Cytotoxicities of synthesized compounds against LX-2 cells. Primers used for PCR analysis. Pharmacokinetic parameters of compounds 15a and 1,25(OH)₂D₃ in rats. Relative expression of CYP24A1 mRNA in LX-2 cells incubated with compound for 24 h measured by Q-PCR. Relative expression of Cyp24a1 mRNA in liver measured by Q-PCR. Relative expression of Trp6 mRNA in mouse intestine measured by Q-PCR. H NMR and 13C NMR spectra of compounds (PDF)

**Molecular formula strings (CSV)**

**Compound 15a (PDB)**

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DOIs: 10.1021/acs.jmedchem.8b01165.
Author Contributions

These authors contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Public platform of State Key Laboratory of Natural Medicine. This work was supported by the National Natural Science Foundation of China (81273468, 81473153, 81703585), Basic National Research Program of China (2015CB755900), Fundamental Research Funds for the Central Universities of China (2632017PY10), 111 Project from the Ministry of Education of China and the State Administration of Foreign Expert Affairs of China (No. 111-2-07), the fund of Fujian Provincial Key Laboratory of Hepatic Drug Research (KFLX2018002), and the Open Project of State Key Laboratory of Natural Medicine (No. SKLNMZ2CX201811).

ABBREVIATIONS USED

ALT, alanine transaminase; AST, aspartate transaminase; CCl4, carbon tetrachloride; DRIPs, VDR interacting proteins; ECM, extracellular matrix; HSCs, hepatic stellate cells; IP, intraperitoneal; LBP, ligand binding pocket; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RNAi, RNA interference; SARs, structure–activity relationships; TBA, total bile acid; TGF, transforming growth factor; VDR, vitamin D receptor

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