Journal of Medicinal Chemistry

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Structure–Activity Relationship of Novel Menaquinone-4 Analogues: Modification of the Side Chain Affects their Biological Activities

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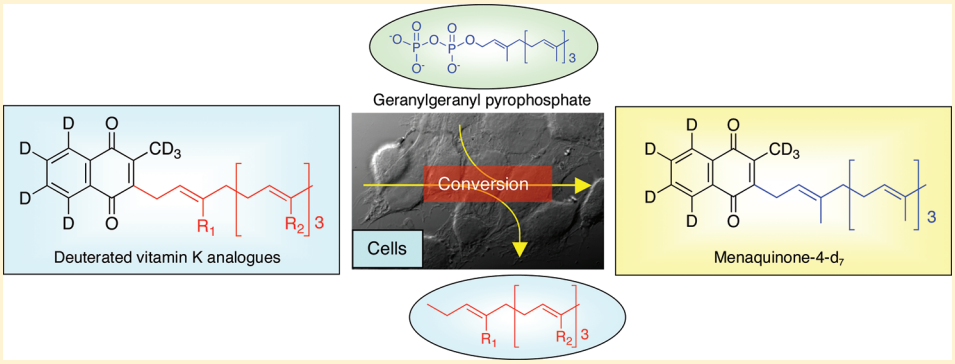
Supporting Information



ABSTRACT: We synthesized new vitamin K analogues with demethylation or reduction of the double bonds of the side chain of menaquinone-4 (MK-4) and evaluated their SXR-mediated transcriptional activity as well as the extent of their conversion to MK-4. The results indicated that the analogue with the methyl group deleted at the 7′ site of the side chain part affected conversion activity to MK-4. In contrast, a decrease in the number of the double bonds in the side chain moiety appeared to decrease the SXR-mediated transcriptional activity.

INTRODUCTION

Vitamin K is an essential cofactor of the γ-carboxylase (GGCX), which converts glutamic acid residues of specific substrate proteins into γ-carboxyglutamic acid (Gla) residues[1] and is involved in the activation of the cascade of blood coagulation proteins. In addition, vitamin K is required for the synthesis of other calcium-binding proteins such as bone Gla protein (osteocalcin), matrix Gla-protein, protein S, and the growth arrest specific 6 protein (gas6).[2−4] Natural vitamin K has two molecular forms of homologues. One is plant-derived vitamin K_1 (**1**) (phylloquinone), which contains the phytyl group as the side chain, and the other is bacterial-derived vitamin K_2 (**2**) (menaquinone-*n*, MK-*n*), with a polyisoprenyl side chain (Figure 1). These homologues have been considered to act only as cofactors for a number of years, however, it has been revealed that MK-4 (**3**), one of menaquinone's homologues, has additional biological actions (Figure 1). For example, effects of **3** on gene transcription through a steroid and xenobiotic receptor (SXR) have been evaluated.[5−8] Furthermore, our group and other groups confirmed that **3** was converted from dietary vitamin K homologues in the body by exchange of the side chain part and was accumulated into various tissues. Levels of **3** were especially high in brain.[9] These findings suggested that **3** may be a preferred form among vitamin K homologues and may play an important, but as yet unknown, role in the body. Therefore, we predicted that the conversion reaction that produces **3** and the SXR-mediated transcriptional action could be essential targets for explanation of new drugs for some diseases. To clarify the structure–activity relationship of the side chain of vitamin K, we synthesized new analogues and evaluated their biological activities.

Received: October 2, 2011
Published: January 17, 2012

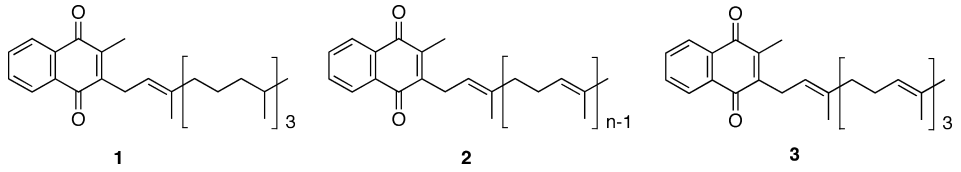


Figure 1. Structures of natural vitamin K homologues.

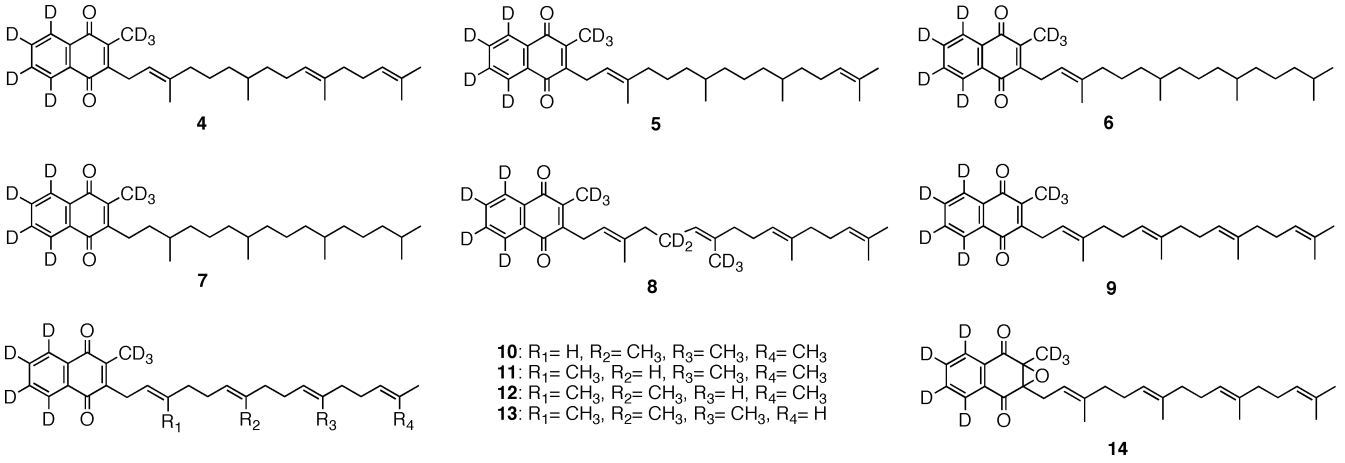
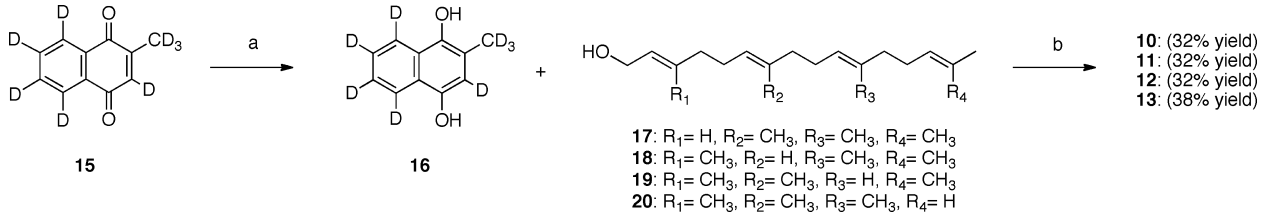


Figure 2. Deuterated vitamin K analogues with different alkyl side chains.

Scheme 1. Synthesis of 10−13[a]



[a]Reagents and conditions: (a) 2-methyl-1,4-naphthoquinone, $Na_2S_2O_4$, Et_2O, quant; (b) $BF_3{\cdot}Et_2O$.

We previously reported that modification of the side chain of vitamin K influenced the conversion reaction that produced **3** using cultured human cell lines.[10] In that study, we used deuterated analogues to distinguish the fraction of **3** converted by cells from the original pool of **3** contained in the cells using liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) system. Our results with analogues **4**−**7** and MK-4-d_{12} (**8**) indicated that the amount of MK-4-d_7 (**9**) was proportionate to the number of double bonds in the side chain. As a further modification of the side chain moiety, we have synthesized additional vitamin K analogues **10**−**13** by removing the 3′, 7′, 10′, or 13′-methyl group from the side chain part (Figure 2). Here, we report the synthesis of these new deuterated vitamin K analogues and examine their structure−activity relationship in terms of conversion to **9** and their SXR-mediated transcriptional activity.

CHEMISTRY

Synthesis of Vitamin K Analogues with Modification of Side Chain Part. The compounds **4**−**7**, **8**, **9**, and MK-4-epoxide-d_7 (**14**) were prepared by our reported method.[10] Very recently, we have reported the efficient synthesis and biological evaluation of "demethylated" geranylgeranoic acid derivatives.[11] We used a part of the intermediates for the side chain of **17**−**20**. The syntheses of the desired compounds **10**−**13** were carried out by our previously reported method,[10] that is, **17**−**20** were respectively treated with hydroquinone **16**, reduced from **15**, in the presence of $BF_3{\cdot}Et_2O$ (Scheme 1).

RESULTS

Conversion of Vitamin K Derivatives into 9. The conversion of vitamin K analogues into **9** was examined in vitro using human osteosarcoma-derived MG-63 cells using the method reported by us.[10] The synthesized compounds were added at a final concentration of 1.0 μM, and the cells incubated at 37 °C for 24 h. After reactants were collected, the vitamin K compounds contained in the cells were extracted with a hexane solvent. The amount of converted **9** and epoxide **14** in each sample was measured by LC-APCI-MS/MS.

We compared the conversion of compound **8** and the analogues **10**−**13** into **9** and **14** in MG-63 cells (Figure 3). The epoxide **14** was produced from **9** by the vitamin K cycle in the cells. The extent of conversion of most of the demethyl compounds to **9** and **14** by MG-63 cells was not significantly different from that observed with **8**. Only **11** exhibited a significant increase in the total amount of converted MK-4 (**9** + **14**). Interestingly, the amount of **14**, generated via the vitamin K cycle in MG-63 cells, appeared to depend on the structure of the compounds. Therefore, the location of the site lacking the

dx.doi.org/10.1021/jm2013166 | J. Med. Chem. 2012, 55, 1553−1558

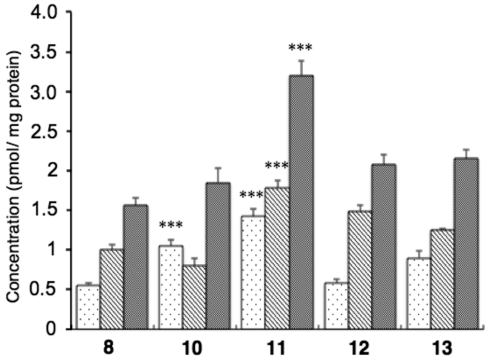


Figure 3. Conversion of **8** and **10**–**13**, to **9** (dotted bars), **14** (striped bars), and **9** + **14** (gray bars) in MG-63 cells. Each analogue (1 μM) was incubated with MG-63 cells for 24 h. The resulting vitamin K analogues were extracted from each reactant, and concentrations of **9** and **14** were measured with LC-APCI-MS/MS as reported previously.[10] The histogram data are expressed as the means obtained from three independent experiments; the error bars indicate the SD. Significant difference between **8** group and compounds **10**–**13** group is as follows: ***, $p < 0.01$ (by Dunnett's t test).

methyl group in the side chain may have a specific effect on coenzyme activity of GGCX.

SXR-Mediated Transcriptional Activity. SXR is a master gene orchestrating the expression of a large family of genes involved in uptake, metabolism, and disposal of a number of endo- and xenobiotics, including drugs, bile acids, steroid hormones, and metabolic intermediates in mammalian cells.[12] Following ligand binding, SXR forms a heterodimer with the retinoid-X-receptor (RXR) that binds to SXR responsive elements (SXREs), located in the 5′-flanking regions of SXR target genes, resulting in their transcriptional activation.[13]

We evaluated the transcriptional activity of the new analogues containing modified side chains by two luciferase-based assay methods using HepG2 cells. The one-hybrid luciferase assay with SXR-GAL4 permitted us to examine only the effects of ligand-binding affinity to SXR on the transcription and excluded any influence of heterodimerization with RXR and binding to SXREs. The second luciferase-based assay included SXRE with the CYP3A4 promoter and allowed us to examine the influences of heterodimerization with RXR and binding to SXREs. We used rifampicin (RIF), a known SXR agonist, as a positive control in this experiment.

Consistent with our earlier studies, the SXR-GAL4 assay indicated that **9** increased transcriptional activity ~7-fold (Figure 4). The demethylated analogues **10**–**13** also enhanced transcription at levels that exceeded control values. Compound **11** was comparable in effect to **9**, while **10**, **12**, and **13** were somewhat less effective. Virtually no enhancement of transcriptional activity occurred with the hydrogenated analogues **5**–**7**, while compound **4**, with three double bonds in the side chain, afforded increased induction of transcriptional activity, although it was significantly less than that produced by **9**. With respect to **4** and **9**, the increase in activity appeared to be related to the number of double bonds in the side chain.

Similar tendencies were exhibited in the induction of transcriptional activity of the CYP3A4 promoter (Figure 5). Compound **9**, hydrogenated compound **4**, and analogues **10**–**13** all exhibited increased activity. The demethylated analogues **10**, **11**, and **13** promoted formation of heterodimers to a degree comparable to **9**, while **12** was significantly less effective ($p < 0.001$). A comparison of the activity of the hydrogenated

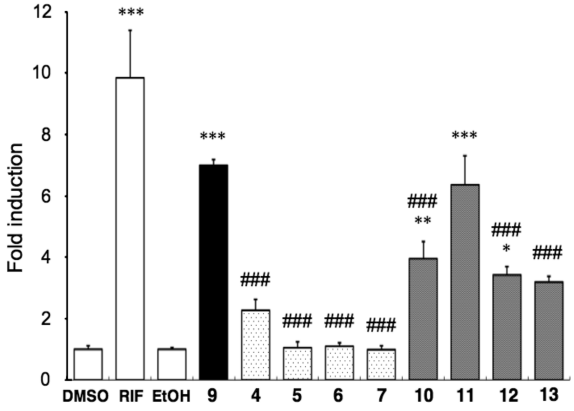


Figure 4. Transcriptional activity with a one-hybrid luciferase assay with SXR-GAL4. HepG2 cells were treated with new vitamin K_2 analogues as well as natural homologues at 5.0×10^{-6} M. The histogram data are expressed as the means obtained from three independent experiments; the error bars indicate the SD. Significant difference between control group and each compound group is as follows: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (by Dunnett's t test). Significant differences between the **9** group and the demethylated analogues **10**–**13** and hydrogenated group **4**–**7** are as follows: ###, $p < 0.001$ (by Student's t test).

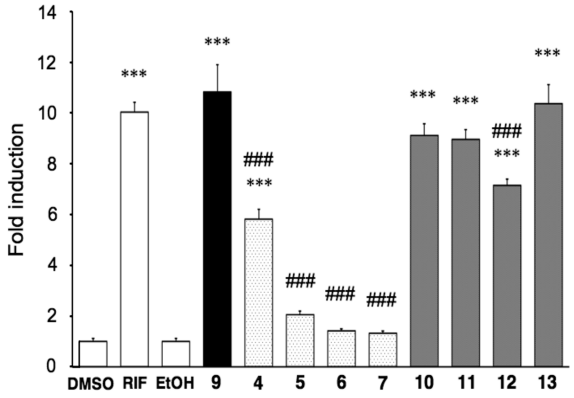


Figure 5. Transcriptional activity with luciferase assay including SXRE with CYP3A4 promoter. HepG2 cells were treated with new vitamin K_2 analogues and natural homologues at 1.0×10^{-6} M. The histogram data indicate the mean obtained from three independent experiments. The error bars indicate the SD. Significant differences between control group and each compounds group is as follows: ***, $p < 0.001$ (by Dunnett's t test). Significant differences between **9** and **4**–**7** and **10**–**13** are as follows: ###, $p < 0.001$ (by Student's t test).

analogues **4**–**7** and **9** suggested that enhancement of the induction of transcription was correlated with the increase in the number of double bonds in the side chain.

Docking Studies. We performed in silico analysis of "hydrogenated" compounds to the SXR-LBD based on the MOE-Dock method[14] using the Molecular Operating Environment (MOE) program package (see Computational Details). The different values of binding score (BS) from **7** clearly show the number of double bonds of the analogues is proportionate to the transcriptional activity mediated by SXR as shown in Figure 6.

■ DISCUSSION

We have described the synthesis and the pharmacological profile related to the conversion of a series of vitamin K analogues into **3**, and we have examined the SXR-mediated

dx.doi.org/10.1021/jm2013166 | J. Med. Chem. 2012, 55, 1553–1558

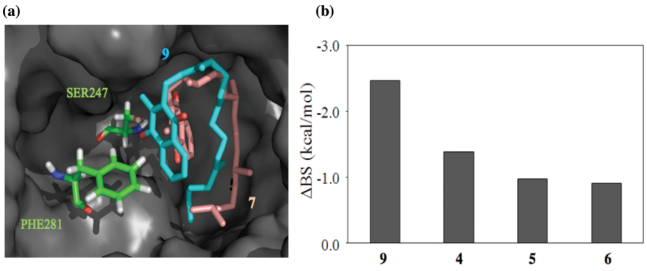


Figure 6. (a) docking pose of **7** (pink) and **9** (blue) with SXR, and (b) the difference of their binding scores of **4**–**6** and **9** from **7**, which has no double bond. The ΔBS values show the stability of binding states between the ligands and SXR.

transcriptional activities of these analogues. In a recent study, we determined that the conversion exzyme was UBIAD1.[15] Thus, conversion of vitamin K analogues to **3** appears to play an important, but as yet undefined, role in the body. In our earlier work, we demonstrated that the extent of conversion of compounds **4**–**7** to **9** was dependent on the number of double bonds in the side chains of **4**–**7**. In the present study, the rate conversion of most of the demethyl analogues to **9** and **14** did not show significant differences as compared to **8** as shown in Figure 3.

With respect to ligand binding to SXR (Figure 4), the activity of **4**–**7** and **9** increased in a manner dependent on the number of saturated double bonds of the side chain moiety, with greatest being **9** bearing the same "geranylgeranyl" side chain as **3**. On the other hand, compound **6** having the same "phytyl" side chain as **1**, and compound **7** bearing no double bond in the side chain, showed no changes in transcriptional activity. All the demethylated compounds promoted ligand binding, with **11** being as active as **9** and the remaining demethylated analogues showing a somewhat lesser effect.

Similar tendencies were noted in the assay that evaluated heterodimerization and binding to SXREs (Figure 5). Compounds **4**–**7** significantly decreased the expression of a well-characterized SXR target gene, CYP3A4, in a human hepatocyte cell line, as compared with **9**. On the other hand, most demethyl compounds showed similar activity to **9**.

We theoretically analyzed the docking state of the representative analogues **7** and **9** with MOE in detail. The comparative docking studies for **7** or **9** and SXR revealed that each binding state was different. In the binding state between **9** and SXR, an oxygen atom of the naphthoquinone moiety exhibited hydrogen bonding with hydroxyl group of Ser247, and delocalized π–electrons on naphthoquinone ring showed CH–π stabilizing interaction with the CH group of Phe281. In addition to these effects, the geranylgeranyl side chain moiety of **9** showed potent hydrophobic interactions with many hydrophobic amino acids of SXR such as Leu209 and Met243. In contrast, **7** did not exhibit effective hydrophobic interactions except with the naphthoquinone residue.

Thus, in the overview, demethlylation of the side chain part did not provide a notable effect on the biological activity. On the other hand, selective reduction of the double bonds in the side chain of **3** dramatically reduced the activities. These findings indicated that loss of a double bond would have a greater effect on the conformation of the side chain part than would loss of a methyl group. In fact, the "flat" conformation of the side chain, originating from the sp^2 orbital of the double bond, would be maintained even though one of the methyl groups was deleted from the side chain. In contrast, introduction of single bonds would increase the flexibility of the side chain part and may impede effective binding. On the basis of the structure of **3**, more potent ligands may arise if new analogues were constructed with the following structural features: (i) maintenance of the double bonds of the side chain and (ii) substitution of one of the methyl groups with other functional groups.

CONCLUSION

In this study, we synthesized vitamin K derivatives and examined their conversion to **9** and SXR-mediated transcriptional activity in cells. We clarified that the activities differed significantly with the structural characteristics of the side chain. Therefore, it would be possible to greatly improve the amount of conversion to **3** or SXR-mediated potent transcriptional activity by modifying the side chain. A more detailed examination is underway to clarify the physiological significance of **3** and the precise mechanism of conversion.

EXPERIMENTAL SECTION

The synthesis of and the data on vitamin K analogues are shown in Scheme 1. ^{1}H NMR spectra were recorded on a Varian VXR-500 spectrometer at 500 MHz, and ^{13}C NMR spectra were recorded at 125 MHz using deuterized chloroform ($CDCl_3$) (Merck, Germany). Chemical shifts are given in ppm (δ) using tetramethylsilane (TMS) as an internal standard. Mass spectra were 220 recorded on a JMS SX-102A. Preparative thin layer chromatography (TLC) was carried out on Silica Gel 60 F254 (Merck). Unless otherwise noted, all reagents were purchased from commercial suppliers and were used as received.

Synthesis of 10. To a solution of **15** (70 mg, 389 μmol) in ether (10 mL) was added a 10% $Na_2S_2O_4$ aqueous solution (10 mL), and the mixture stirred vigorously at 30 °C for 1 h under argon. After the yellow ether layer turned colorless, the mixture was extracted with AcOEt (30 mL × 3). The combined organic layer was washed with brine (30 mL × 3), dried over $MgSO_4$, and concentrated to afford crude hydroquinone **16**. The residue was immediately dissolved in AcOEt (1 mL) and dioxane (1 mL), and then 3-demethyl geranylgeraniol analogue **17** (50 mg, 181 μmol) and boron trifluoride ether complex (20 μL) were added. The mixture was stirred at 70 °C for 3 h under argon and cooled to room temperature. The reaction mixture was poured into ice–water and extracted with AcOEt (50 mL × 3). The combined organic layer was washed with water (100 mL) and brine (100 mL), dried over $MgSO_4$, and concentrated. The residue was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1) to afford **10** (25 mg, 32%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.55 (3H, s), 1.57 (3H, s), 1.60 (3H, s), 1.67 (3H, s), 1.93–2.09 (12H, m), 3.34 (2H, d, J = 6.5 Hz), 5.06–5.12 (3H, m), 5.38–5.51 (2H, m). ^{13}C NMR (125 MHz) δ 16.0, 17.7, 25.7, 26.0, 26.6, 26.7, 27.8, 29.9, 32.7, 39.68, 39.72, 123.7, 124.2, 124.4, 124.6, 132.7, 134.9, 135.4, 143.6, 145.2, 184.4, 185.4. D NMR (77 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.71 (2D, s), 8.10 (2D, s): EI-LRMS *m*/*z* 437 (M^+). EI-HRMS calcd for $C_{30}H_{31}D_7O_2$ 437.3310; found 437.3309.

Synthesis of 11. Similar to the synthesis of **10** from **15** and **17**, a crude product **16**, which was obtained from **15** (70 mg, 181 μmol), 7-demethyl geranylgeraniol analogue **18** (43 mg, 156 μmol), and boron trifluoride ether complex (15 μL) in AcOEt (0.5 mL) and dioxane (0.5 mL), was purified by preparative TLC on silica gel (hexane/AcOEt = 20:1), giving **11** (21 mg, 32%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (3H, s), 1.59 (3H, s), 1.67 (3H, s), 1.78 (3H, s), 1.94–2.07 (12H, m), 3.36 (2H, d, J = 7.0 Hz), 5.00–5.11 (3H, m), 5.32–5.40 (2H, m). ^{13}C NMR (125 MHz) δ 16.0, 16.4, 17.7, 25.7, 26.0, 26.7, 28.0, 31.1, 32.8, 39.71, 39.74, 119.2, 124.0, 124.4, 129.7, 130.3, 131.3, 132.10, 132.12, 135.1, 137.3, 143.3, 146.2, 184.5, 185.5. D NMR (77 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.71 (2D, s), 8.10 (2D, s). EI-LRMS

dx.doi.org/10.1021/jm2013166 | *J. Med. Chem.* 2012, 55, 1553–1558

m/z 437 (M^+). EI-HRMS calcd for $C_{30}H_{31}D_7O_2$ 437.3310; found 437.3311.

Synthesis of 12. Similar to the synthesis of **10** from **15** and **17**, a crude product **16** which was obtained from **15** (70 mg, 181 μmol), 11-demethyl geranylgeraniol analogue **19** (43 mg, 156 μmol), and boron trifluoride ether complex (15 μL) in AcOEt (0.5 mL) and dioxane (0.5 mL), was purified by preparative TLC on silica gel (hexane/AcOEt = 20:1), giving **12** (26 mg, 38%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.55 (3H, s), 1.59 (3H, s), 1.68 (3H, s), 1.79 (3H, s), 1.94−2.08 (12H, m), 3.37 (2H, d, J = 6.5 Hz), 5.00−5.11 (3H, m), 5.27−5.42 (2H, m). ^{13}C NMR (125 MHz) δ 16.0, 16.4, 17.7, 25.7, 26.0, 26.4, 27.5, 28.2, 29.7, 31.2, 32.1, 32.8, 37.3, 39.7, 42.9, 119.2, 123.9, 124.2, 129.9, 130.0, 131.3, 132.10, 135.0, 137.5, 143.2, 146.2, 184.5, 185.5. D NMR (77 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.71 (2D, s), 8.10 (2D, s): EI-LRMS m/z 437 (M^+). EI-HRMS calcd for $C_{30}H_{31}D_7O_2$ 437.3310; found 437.3299.

Synthesis of 13. Similar to the synthesis of **10** from **15** and **17**, a crude product **16**, which was obtained from **15** (70 mg, 181 μmol), 15-demethyl geranylgeraniol analogue **20** (43 mg, 156 μmol), and boron trifluoride ether complex (15 μL) in AcOEt (0.5 mL) and dioxane (0.5 mL), was purified by preparative TLC on silica gel (hexane/AcOEt = 20:1), giving **13** (23 mg, 34%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.54 (3H, s), 1.55 (3H, s), 1.56 (3H, s), 1.79 (3H, s), 1.92−2.07 (12H, m), 3.36 (2H, d, J = 6.5 Hz), 5.00−5.08 (3H, m), 5.31−5.46 (2H, m). ^{13}C NMR (125 MHz) δ 15.9, 16.4, 17.9, 25.7, 26.0, 26.5, 26.7, 31.3, 39.6, 39.7, 42.9, 119.1, 123.9, 124.6, 127.3, 130.4, 131.2, 132.1, 133.3, 134.3, 135.2, 137.6, 143.3, 146.2, 184.5, 185.5. D NMR (77 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.71 (2D, s), 8.10 (2D, s): EI-LRMS m/z 437 (M^+). EI-HRMS calcd for $C_{30}H_{31}D_7O_2$ 437.3310; found 437.3315.

Cloning and Construction of cDNAs. Human SXR expression vector (pcDNA3.1-FLAG-SXR) was generated by polymerase chain reaction (PCR) using human genomic DNA as templates, respectively, and then inserted in-frame to pcDNA3.1(+) vector (Invitrogen) at *Eco*RI and *Xho*I sites. The CYP3A4 luciferase reporter plasmid, pGL3-CYP3A4pro, was constructed in our laboratory. The CYP3A4 promoter, from base pairs −362 to +53, and a distal enhancer module of CYP3A4 promoter, from base pairs −7876 to −7208, was generated by PCR using the DNA template isolated from the human genome DNA. These products were then subcloned into the pGL4.10 vector, a promoter-less luciferase reporter vector, at the *Nhe*I and *Bam*HI sites. Finally, pGL4.10-CYP3A4pro, a CYP3A4 luciferase reporter plasmid containing −7876 to −7208 and −362 to +53 bp of the CYP3A4 proximal promoter, was prepared.[16]

Luciferase Assay. Human hepatoma cell line HepG2 cells were maintained in Eagle's modified medium (Nakalai Tesque) supplemented with 1% penicillin, 1% streptomycin, and 10% fetal calf serum (FCS) (Gibco BRL). Luciferase-based assay of SXR-GAL4 was performed using HepG2 cells (2×10^5 cells/well on 6-well plates) transfected with 0.1 μg of pM-SXR (constructed in our laboratory), 1.0 μg of pGVP2-GAL2 (obtained from Prof. Keiichi Ozono, Osaka University), and 0.05 μg of pRL-CMV (Promega) using Lipofectamine (Invitrogen). In a similar way, luciferase assay of CYP3A4 promoter was conducted using HepG2 cells transfected with 0.25 μg of pcDNA3.1-FLAG-SXR, 0.25 μg of pGL4.10-CYP3A4pro luciferase reporter vector (Toyo Ink. Co., Ltd.), and 0.1 μg of pRL-CMV using lipofectamine. Twenty-four hours after transfection, cells were treated with rifampicin (Nakalai Tesque, Kyoto, Japan), vitamin Ks, or vehicle (ethanol) for 48 h in fresh media, and luciferase activities were determined by a Lumat LB9507 luminometer (Berthold Technologies) using the dual-luciferase assay system (Toyo Ink.). Firefly luciferase activity was normalized to that of Renilla luciferase, which was used as a transfection control. The experiments were repeated three times with similar results.

Computational Details. Calculations were performed on a Dell Precision T3500 workstation. Before docking simulations, conformational analysis of menaquinones and their analogues were calculated using MOE program package[17] with the standard MMFF94x molecular mechanics force field. In the docking simulations, **9** and **4**−**7** were superimposed on the original hyperforin position of the crystal structure of the human pregnane X receptor complex (PDB accession code 1M13)[18] using the MOE-Dock procedure.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **10**−**13**, cells and cell culture, and incubation of cultured human cell lines with vitamin K analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Dr. Atsuko Takeuchi and Dr. Chisato Tode for the spectroscopic measurements at Kobe Pharmaceutical University, Japan. This study was supported in part by a Grant-in-aid for Scientific Research <KAKENHI> (C) (grant number 20590113 to Y.S.) from the Japan Society for the Promotion of Science.

■ ABBREVIATIONS USED

GGCX, γ-carboxylase; Gla, γ-carboxyglutamic acid; gas6, growth arrest specific 6 protein; MK-*n*, menaquinone-*n*; LC-APCI-MS/MS, liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry; SXR, steroid and xenobiotic receptor; CYP3A4, cytochrome P450 3A4; RXR, retinoid-X- receptor; SXREs, SXR response elements; MOE, Molecular Operating Environment; EI-HRMS, electrospray ionization−high-resolution mass spectroscopy; TMS, tetramethylsilane; TLC, thin-layer chromatography; PCR, polymerase chain reaction; PDB, Protein Data Bank

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