Bioorganic & Medicinal Chemistry 18 (2010) 3116–3124

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure–activity relationships in the conversion of vitamin K analogues into menaquinone-4. Substrates essential to the synthesis of menaquinone-4 in cultured human cell lines

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ARTICLE INFO

Article history:
Received 19 February 2010
Revised 15 March 2010
Accepted 16 March 2010
Available online 19 March 2010

Keywords:
Vitamin K
Menaquinone-4
Conversion
Structure–activity relationships
Analogues
Deuterated compound

ABSTRACT

To reveal an essential biological role of menaquinone-4, we have clarified that dietary PK was converted to menaquinone-4 (MK-4) in animal tissues using deuterated vitamin K analogues. However, the kinds of analogue converted into MK-4 have not been elucidated. In this study, we examined structure–activity relationships in the conversion of several vitamin K analogues, with a substituted side chain, into MK-4 using cultured human cell lines. The results differed with the side chain of the analogues, that is, (1) the length of the isoprene unit and (2) the number of double bonds in the side chain. These findings would be useful for clarifying the mechanism of conversion of other vitamin K homologs into MK-4 as well as related enzymes.

1. Introduction

Vitamin K is a fat-soluble vitamin and a well-known cofactor for γ-glutamyl carboxylase (GGCX), an enzyme that converts specific glutamic acid residues in several proteins to γ-carboxyglutamic acid (Gla) residues related to blood coagulation[1] and bone formation in the vitamin K cycle.[2–4] It has two major homologs, the plant-derived vitamin K_1 (**A**) (phylloquinone: PK) and the bacterium-derived vitamin K_2 (**B**) (menaquinone-*n*: MK-*n*) (Fig. 1).[5] Among these homologs, MK-4 has attracted attention because of its interesting biological activities. For example, MK-4 showed additional biological actions related to gene transcription through the steroid and xenobiotic receptor (SXR),[6] suppression of cancer cell proliferation,[7–9] and so on. Recently we confirmed that MK-4 was converted from dietary PK, and then accumulated in various tissues in mice.[10] Notably, certain tissues with a high lipid content, such as brain tissue, contain high concentrations of MK-4. In a study of the brain in vitro, MK-4 was shown to prevent oxidative injury to oligodendrocyte precursors and immature fetal cortical neurons, independent of the vitamin K-dependent γ-carboxylative reaction.[11] These findings suggest that MK-4 is the preferred form among vitamin K homologs and plays an important role in the function of the brain. However, it is not known why this conversion occurs because MK-4 has long been thought to be only a cofactor for the vitamin K-dependent γ-carboxylative reaction. As a first step toward clarifying the biological importance of MK-4, we investigated the kinds of vitamin K derivatives easily converted to MK-4 in vitro. No report has focused on the side chains of vitamin K analogues, and examined structure–activity relationships for conversion to MK-4.[12–14] The development of new analogues with high rates of conversion to MK-4 would help to elucidate the mechanism of conversion as well as the enzymes involved. Furthermore, it can be expected to lead to new drugs once the biological importance of MK-4 is clarified. We report here a method of

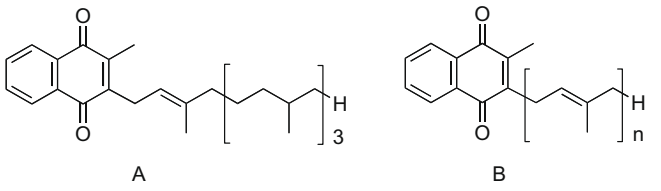

Figure 1. Structure of vitamin K homologs: phylloquinone (A) and menaquinones (B).

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doi:10.1016/j.bmc.2010.03.035

synthesizing vitamin K analogues and results of conversion to MK-4 using cultured human cell lines.

2. Results and discussion

2.1. Synthesis of vitamin K analogues

We synthesized several analogues of deuterated vitamin K to examine the conversion to MK-4 in vitro. In this study, we focused on the side-chain moiety of vitamin K and synthesized two general groups of compounds. Each compound was modified in the side chain as follows, (1) the length of the isoprene unit was changed and (2) the number of double bonds in the side chain was decreased, as shown in Figure 2. MK-4 was focused on because it potentially plays an important role in the living body. Deuterated menaquinones were synthesized because they can be distinguished from the native vitamin K homologs originally contained in cells and tissues with LC–APCI–MS/MS.

The deuterated menaquinones in which the length of the isoprene unit was changed MK-2-d_7 (**1**) to MK-7-d_7 (**6**), MK-4-d_{12} (**7**), and PK-d_7 (**10**) (Fig. 2). Compound **7** contains deuterium in the side chain as well as menadione for observing its conversion to MK-4-d_7. As shown in Scheme 1, the analogues **1**–**6** and **10** were obtained from isoprene side-chain moieties such as prenol, geraniol, farnesol, geranylgeraniol, geranylfarnesol, farnesylfarnesol, geranylgeranylfarnesol, and phytol, kindly provided by Eisai Co., Ltd. The deuterated MK-4 analogue **7** and its side-chain moiety were prepared as reported.[15] Meanwhile, the naphthoquinone moiety used was a commercially available 'menadione-d_8 (K_3-d_8)' (**13**).[15] After the reduction of K_3-d_8 (**13**) with sodium hydrosulfite in Et_2O and water to form the hydroquinone **14**, several side-chain moieties were successively coupled with **14** in the presence of a catalytic amount of $BF_3{\cdot}Et_2O$ before the hydroquinone was oxidized to the quinone **13** under atmospheric conditions. Thus, MK-2-d_7 (**1**)–MK-7-d_7 (**6**), MK-4-d_{12} (**7**), and PK-d_7 (**10**) were all obtained in good yields. We did not use 'MK-1-d_7', supposedly consisting of prenol and K_3-d_8, because it was difficult to obtain and the chemical yield was not satisfactory with our method.

The vitamin K analogues (**8**)–(**11**), in which the number of double bonds in the side chain of MK-4 was decreased, are shown in Figure 2. The synthesis of the 6′,7′-dihydro analogue (6,7-DH-MK-4-d_7 (**8**)) is shown in Scheme 2. We chose farnesol (**15**) as a starting material to produce the side chain of the analogue. After the 2,3-double bond nearest to the hydroxyl group had been saturated by selective catalytic reduction, the hydroxyl group of **16** was converted into the iodide **17** through methane sulfonate in good yield in two steps. The introduction of a carbonyl group into **17** under basic conditions in two steps, followed by a Horner–Wadsworth–Emmons reaction, gave the methyl ester **19** in 75% yield. Reduction of **19** with Vitride reagent[16] gave 6,7-dihydrogeranylgeraniol (**20**) in 57% yield. Finally, 6,7-DH-MK-4-d_7 (**8**) was obtained from the alcohol **20** and K_3-d_8 (**13**) as mentioned in 43% yield.

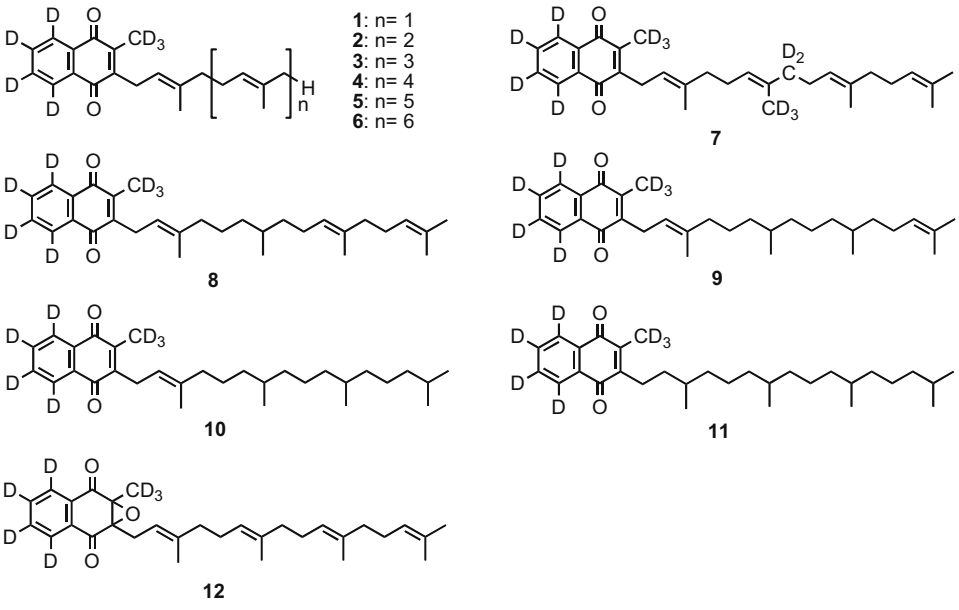
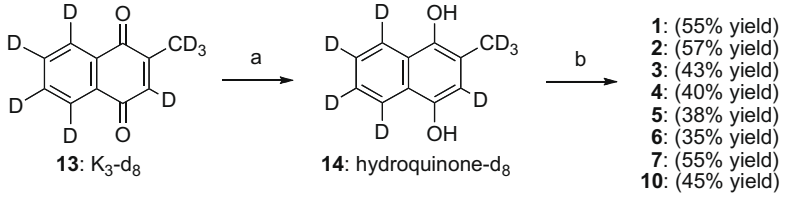
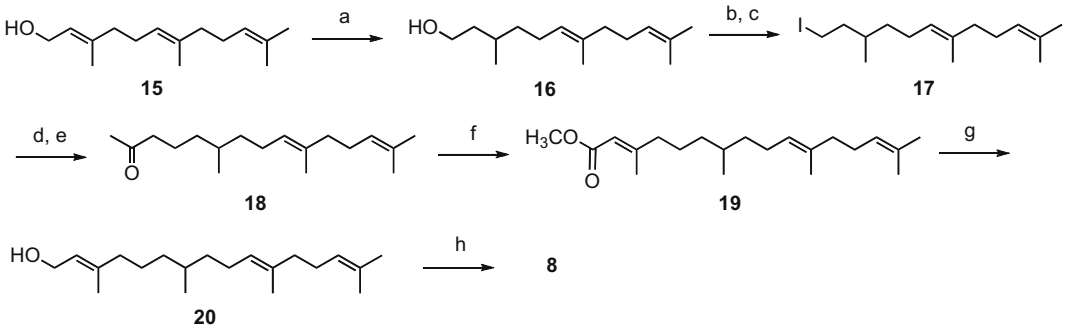


Figure 2. The newly synthesized deuterated vitamin K analogues with different alkyl side chains.



Scheme 1. Synthesis of vitamin K-d_7 analogues. Reagents and conditions: (a) 10% $Na_2S_2O_4$ aqueous, Et_2O; (b) isoprenyl alcohols, $BF_3{\cdot}Et_2O$, EtOAc/dioxane (1:1).



Scheme 2. Synthesis of vitamin K analogues. Reagents and conditions: (a) 2% PtO_2, H_2 (quant.); (b) CH_3SO_2Cl, Et_3N; (c) NaI (73% in two steps); (d) $CH_3COCH_2COOC_2H_5$, 28% MeONa, THF; (e) KOH (70% in two steps); (f) $PO(OC_2H_5)_2CH_2COOC_2H_5$, 28% MeONa (81%); (g) Vitride reagent (90%); (h) 13, $BF_3{\cdot}Et_2O$, EtOAc/dioxane (1:1) (43%).

Scheme 3 shows the synthesis of 6′,7′,10′,11′-tetrahydro-MK-4 (6,7,10,11-TH-MK-4-d_7 (**9**)). After the hydroxyl group of β-citronellol (**21**) was converted to the iodide **22** in 87% yield in two steps, the introduction of a carbonyl group into the molecule was followed by conversion of the ketone **23** into the methyl ester **24** with a Horner–Wadsworth–Emmons reaction in the same manner as above. **24** was reduced to the alcohol **25** with Vitride reagent in 92% yield, then the 2,3-double bond nearest the hydroxyl group of **25** was reduced by selective catalytic reduction with monitoring using TLC to give **26** in quantitative yield. With the same reaction leading from **21** to **26**, 6,7,10,11-tetrahydrogeranylgeraniol (**30**) was obtained in good yield. The synthesis of 6,7,10,11-TH-MK-4-d_7 (**9**) was carried out with the side chain of **30** and K_3-d_8 (**13**) in 43% yield. The 6′,7′,10′,11′,14′,15′-hexahydro-MK-4 was identical compound with PK-d_7 (**10**). The vitamin K analogue **11**, in which all double bonds of the side chain are saturated, was obtained by hydrogenation as described in the Section 4. '2′,3′-Dihydrophylloquinone (2,3-DH-PK-d_7 (**11**))' is frequently used in the manufacturing of foods because of its stability.[17]

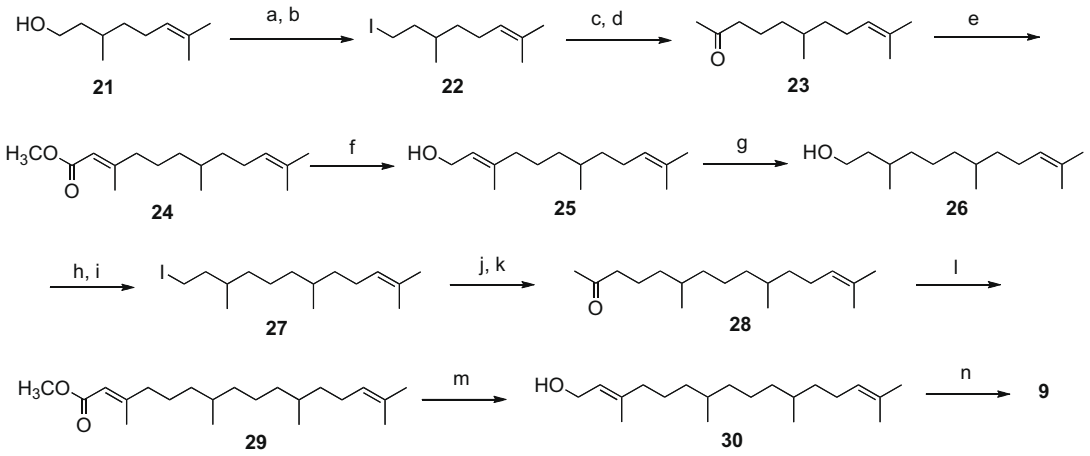
Besides these vitamin K analogues, we synthesized menaquinone-4-epoxide-d_7 (MK-4-epoxide-d_7) (**12**) as shown in Figure 2 to detect turnover of the vitamin K cycle in cells. The method used was reported previously.[18]

Regarding the two general groups of compounds, we investigated the conversion of analogues to MK-4-d_7 as well as MK-4-epoxide-d_7, one of the metabolites of the vitamin K cycle, with LC–APCI–MS/MS as reported,[13] then elucidated the effect of substitution of the side chain moiety on conversion to MK-4 and turnover of the vitamin K cycle.

2.2. Evaluation of the rate of conversion of vitamin K derivatives into MK-4

The conversion of vitamin K analogues into MK-4 was examined in vitro using human osteosarcoma-derived MG-63 cells. First, the synthesized compounds were added at 1 μM, and the cells incubated at 37 °C for 24 h. After reactants were collected, the vitamin K compounds contained in cells were extracted with a *n*-hexane solvent. The amount of converted MK-4-d_7 in each sample was measured by LC–APCI–MS/MS.

The results for each vitamin K analogue added to MG-63 cells are shown in Figures 3 and 4. Figure 3 shows the results for the compounds in which the isoprene unit of the side chain was changed as well as K_3-d_8. The vertical and horizontal axes in the graph show the concentration of MK-4-d_7 and MK-4-epoxide-d_7 converted from deuterated vitamin K analogues with the vitamin K cycle, and the kinds of vitamin K derivatives, respectively. The rate of conversion to MK-4 increased as the side chain became shorter. Consequently, MK-2 was the best substrate for conversion to MK-4 among menaquinones in MG-63 cells. The amount of K_3-d_8 obtained was largest, however, K_3 was reported to have serious toxicity.[19] The conversion might depend on the amount taken up into cells or ease with which the side chain of vitamin K analogues was cleaved. Interestingly, a deuterated MK-4-epoxide, generated



Scheme 3. Synthesis of vitamin K analogues. Reagents and conditions: (a) CH_3SO_2Cl, Et_3N; (b) NaI (87% in two steps); (c) $CH_3COCH_2COOC_2H_5$, 28% MeONa, THF; (d) KOH (99% in two steps); (e) $PO(OC_2H_5)_2CH_2COOC_2H_5$, 28% MeONa (91%); (f) Vitride reagent (92%); (g) 2% PtO_2, H_2, (quant.); (h) CH_3SO_2Cl, Et_3N; (i) NaI (94% in two steps); (j) $CH_3COCH_2COOC_2H_5$, 28% MeONa, THF; (k) KOH (quant.); (l) $PO(OC_2H_5)_2CH_2COOC_2H_5$, 28% MeONa (63%); (m) Vitride reagent (57%); (n) **13**, $BF_3{\cdot}Et_2O$, EtOAc/dioxane (1:1) (43%).

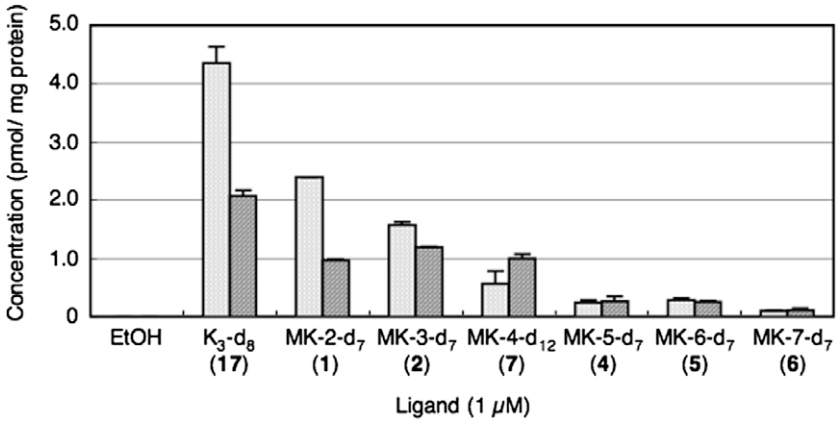


Figure 3. The conversion of deuterated vitamin K analogues (**1**)–(**7**) as well as **13**, in which an isoprene unit of the side chain was changed, to MK-4-d_7 (**3**) in MG-63 cells. The vertical and horizontal axes show the concentrations of MK-4-d_7 (**3**) (□) and MK-4-epoxide-d_7 (**12**) (■), and ligands of vitamin K derivatives, respectively. Each analogue was incubated at 1 μM with MG-63 cells for 24 h. The resulting vitamin K analogues were extracted from each reactant, and concentrations of MK-4-d_7 (**3**) and MK-4-epoxide-d_7 (**12**) were measured with LC–APCI–MS/MS as described in Section 4.

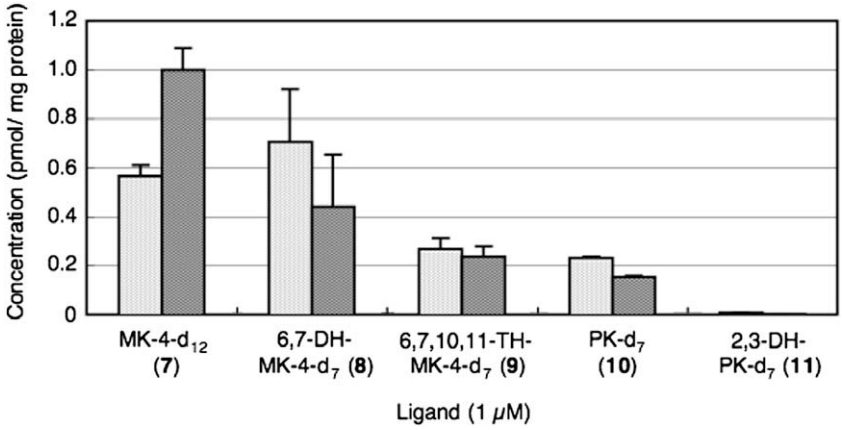


Figure 4. The conversion of deuterated vitamin K analogues (**8**)–(**11**) as well as **7**, some double bonds of which were hydrogenated, to MK-4-d_7 (**3**) in MG-63 cells. The vertical and horizontal axes show the concentrations of MK-4-d_7 (**3**) (□) and MK-4-epoxide-d_7 (**12**) (■), and ligands of vitamin K derivatives, respectively. Experimental conditions were similar to those in Figure 3.

in the vitamin K cycle, was also detected in cells. This finding indicates that the converted MK-4-d_7 was utilized in the vitamin K cycle in cells.

Regarding the derivatives in which some double bonds were hydrogenated in the side chain, the rate of conversion to MK-4 was proportional to the number of double bonds as shown in Figure 4. Notably, 2,3-dihydro-phylloquinone (2,3-DH-PK) (**11**), a hydrogenated form of PK produced during the hydrogenation of PK-rich plant oils,[17] was not converted into MK-4 in our 'in vitro' experiment. MK-4-epoxide-d_7 was also generated and utilized in cells the same as in Figure 3. The total amount of MK-4-d_7 and MK-4-epoxide-d_7 obtained from MK-d_{12} (**7**) was largest, therefore, MK-4-d_{12} might be much easier to convert to MK-4 than other hydrogenated analogues. This finding suggests the double bonds of the side chain to be important for conversion to MK-4.

Similar results were obtained with human hepatocarcinoma HepG2 cells. This means that the conversion to MK-4 occurred in sequence regardless of cell species. The mechanism of conversion has been reported by us[20] and another group.[21] We can speculate further based on the present results. Briefly, once absorbed by cells, the analogues would be transferred to intracellular compartments, then the side chain would be cleaved and the resulting naphthoquinone derivative would be bound to GGPP, biosynthesized in the mevalonate pathway, with an enzymatic reaction to produce MK-4.

3. Conclusions

In this study, we synthesized various vitamin K derivatives, and examined their conversion to MK-4 in cells. We clarified that the rate of conversion differed significantly with the side chain, therefore, the structure of the side chain part of vitamin K is important for the conversion to MK-4. These findings should help to reveal the system converting vitamin K to MK-4. Moreover, it may be possible to greatly improve the rate of conversion to MK-4 by modifying the side chain. A more detailed examination is underway to clarify the physiological significance of MK-4 and the precise mechanism of conversion.

4. Experimental

4.1. Synthesis

4.1.1. General

The synthesis of and the data on deuterated vitamin K homologs are shown in Schemes 1–3. ^{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 recorded on a JMS SX-102A. Preparative thin layer chromatography (TLC) was carried out on Silica Gel 60 F_{254} (Merck). Unless otherwise noted, all reagents were purchased from commercial suppliers and used as received.

4.1.2. Menaquinone-2-d_7 (MK-2-d_7) (1)

To a solution of menadion-d_8 (K_3-d_8) (**13**) (150 mg, 833 μmol) in ether (20 mL) was added a 10% $Na_2S_2O_4$ aqueous solution (20 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 (50 mL × 3). The combined organic layer was washed with brine (50 mL × 3), dried over $MgSO_4$, and concentrated to afford crude hydroquinone (**14**). The residue was immediately dissolved in AcOEt (1 mL) and dioxane (1 mL), then, geraniol (139 mg, 900 μmol) and boron trifluoride ether complex (50 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 **1** (145 mg, 55%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.57 (3H, s), 1.63 (3H, s), 1.80 (3H, s), 2.00 (2H, t, J = 7.5 Hz), 2.06 (2H, t, J = 7.5 Hz), 3.38 (2H, d, J = 7.0 Hz), 5.01–5.16 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.4, 17.7, 25.6, 26.0, 26.5, 39.7, 119.2, 124.0, 131.5, 132.2, 137.5, 143.3, 146.3, 184.6, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.72 (2D, s), 8.11 (2D, s): EI-LRMS *m/z* 315 (M^+). EI-HRMS calcd for $C_{21}H_{17}D_7O_2$: 315.2214. Found: 315.2221.

4.1.3. Menaquinone-3-d_7 (MK-3-d_7) (2)

Similar to the synthesis of **1** from **13**, a crude product (**2**), which was obtained from **14** (50 mg, 278 μmol), farnesol (69 mg, 310 μmol), and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1), giving **2** (61 mg, 57%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (6H, s), 1.65 (3H, s), 1.80 (3H, s), 1.91 (2H, t, J = 7.5 Hz), 1.99 (4H, t, J = 8.0 Hz), 2.06 (2H, t, J = 8.0 Hz), 3.37 (2H, d, J = 6.5 Hz), 5.01–5.06 (3H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.0, 16.4, 17.6, 25.7, 26.0, 26.5, 26.7, 39.67, 39.71, 119.1, 123.9, 124.3, 131.2, 132.1, 135.2, 137.5, 143.3, 146.2, 184.5, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.72 (2D, s), 8.11 (2D, s): EI-LRMS *m/z* 383 (M^+). EI-HRMS calcd for $C_{26}H_{25}D_7O_2$: 383.2840. Found: 383.2837.

4.1.4. Menaquinone-4-d_7 (MK-4-d_7) (3)

Similar to the synthesis of **1** from **13**, a crude product (**3**), which was obtained from **13** (50 mg, 278 μmol), geranylgeraniol (90 mg, 310 μmol), and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1), giving **3** (54 mg, 43%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.557 (3H, s), 1.561 (3H, s), 1.59 (3H, s), 1.67 (3H, s), 1.79 (3H, s), 1.91–2.07 (12H, m), 3.37 (2H, d, J = 7.0 Hz), 5.00–5.11 (4H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.9, 16.0, 16.4, 17.7, 25.7, 26.0, 26.5, 26.6, 26.7, 39.6, 39.69, 39.71, 119.1, 123.9, 124.1, 124.4, 131.2, 132.09, 132.12, 134.9, 135.2, 135.5, 143.2, 146.2, 184.5, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.71 (2D, s), 8.10 (2D, s): EI-LRMS *m/z* 451 (M^+). EI-HRMS calcd for $C_{31}H_{33}D_7O_2$: 451.3460. Found: 451.3466.

4.1.5. Menaquinone-5-d_7 (MK-5-d_7) (4)

Similar to the synthesis of **1** from **13**, a crude product (**4**), which was obtained from **13** (50 mg, 278 μmol), geranylfarnesol (111 mg, 310 μmol), and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1), giving **4** (58 mg, 40%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (6H, s), 1.587 (3H, s), 1.593 (3H, s), 1.67 (3H, s), 1.79 (3H, s), 1.98–2.09 (16H, m), 3.37 (2H, d, J = 6.5 Hz), 5.01–5.11 (5H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.96, 16.01, 16.4, 17.7, 25.7, 26.0, 26.5, 26.6, 26.8, 39.7, 119.1, 123.8, 123.9, 124.2, 124.3, 124.4, 131.2, 132.1, 134.8, 134.9, 135.2, 137.5, 143.2, 146.2, 184.5, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.13 (3D, s), 7.70 (2D, s), 8.10 (2D, s): EI-LRMS *m/z* 519 (M^+). EI-HRMS calcd for $C_{36}H_{41}D_7O_2$: 519.4091. Found: 519.4100.

4.1.6. Menaquinone-6-d_7 (MK-6-d_7) (5)

Similar to the synthesis of **1** from **13**, a crude product (**5**), which was obtained from **13** (50 mg, 278 μmol), farnesylfarnesol (132 mg, 310 μmol), and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1), giving **5** (61 mg, 38%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (6H, s), 1.591 (6H, s), 1.595 (3H, s), 1.68 (3H, s), 1.79 (3H, s), 1.91–2.09 (20H, m), 3.37 (2H, d, J = 7.0 Hz), 5.00–5.12 (6H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.99, 16.01, 16.02, 16.04, 16.4, 17.7, 25.7, 26.0, 26.5, 26.7, 26.8, 39.7, 119.1, 123.9, 124.2, 124.3, 124.4, 131.2, 132.1, 134.9, 135.2, 137.6, 143.2, 146.2, 184.5, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.13 (3D, s), 7.71 (2D, s), 8.10 (2D, s): EI-LRMS *m/z* 587 (M^+). EI-HRMS calcd for $C_{41}H_{49}D_7O_2$ 587.4717. Found 587.4723.

4.1.7. Menaquinone-7-d_7 (MK-7-d_7) (6)

Similar to the synthesis of **1** from **13**, a crude product (**6**), which was obtained from **13** (50 mg, 278 μmol), farnesylfarnesol (153 mg, 310 μmol), and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1), giving **6** (64 mg, 35%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (6H, s), 1.59 (12H, s), 1.67 (3H, s), 1.79 (3H, s), 1.91–2.07 (24H, m), 3.37 (2H, d, J = 7.0 Hz), 5.01–5.12 (7H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.01, 16.03, 16.4, 17.7, 25.7, 26.0, 26.5, 26.7, 26.8, 39.7, 119.1, 123.9, 124.2, 124.3, 124.4, 131.2, 132.1, 134.9, 135.2, 137.6, 143.3, 146.2, 184.5, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.70 (2D, s), 8.10 (2D, s): EI-LRMS *m/z* 656 (M^+). EI-HRMS calcd for $C_{46}H_{57}D_7O_2$: 655.5343. Found: 655.5352.

4.1.8. Menaquinone-4-d_{12} (MK-4-d_{12}) (7)

Similar to the synthesis of **1** from **13**, a crude product (**7**), which was obtained from **13** (50 mg, 278 μmol), geranylgeraniol-d_5 (91 mg, 310 μmol)[6], and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1), giving **7** (70 mg, 55%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (3H, s), 1.59 (3H, s), 1.67 (3H, s), 1.79 (3H, s), 1.91–2.07 (10H, m), 3.37 (2H, d, J = 6.5 Hz), 5.00–5.10 (4H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.9, 16.4, 17.7, 25.7, 26.0, 26.6, 26.8, 39.57, 39.61, 39.7, 119.1, 123.8, 124.2, 124.4, 131.2, 132.12, 132.15, 134.9, 135.2, 137.6, 143.3, 146.2, 184.5, 185.5; D NMR (500 MHz, $CHCl_3$) δ 1.51 (3D, s), 2.02 (2D, s), 2.14 (3D, s), 7.72 (2D, s), 8.11 (2D, s): EI-LRMS *m/z* 456 (M^+). EI-HRMS calcd for $C_{31}H_{28}D_{12}O_2$: 456.3780. Found: 456.3777.

4.1.9. 6′,7′-Dihydro-menaquinone-4-d_7 (6,7-DH-MK-4) (8)

Similar to the synthesis of **1** from **13**, a crude product (**8**), which was obtained from **13** (50 mg, 278 μmol), **20** (90 mg, 310 μmol), and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel

(*n*-hexane/AcOEt = 20:1), giving **8** (54 mg, 43%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.83 (3H, d, J = 6.5 Hz), 0.84–1.48 (6H, m), 1.57 (3H, s), 1.59 (3H, s), 1.60–1.61 (1H, m), 1.67 (3H, s), 1.78 (3H, s), 1.90–2.07 (8H, m), 3.35 (2H, d, J = 7.0 Hz), 4.99–5.13 (3H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.9, 16.3, 17.7, 19.6, 25.2, 25.4, 25.7, 26.0, 26.7, 32.2, 36.5, 37.0, 39.7, 40.0, 119.1, 124.4, 124.8, 131.2, 132.08, 132.12, 134.6, 135.2, 137.9, 143.2, 146.3, 184.5, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.72 (2D, s), 8.11 (2D, s): EI-LRMS *m/z* 453 (M^+). EI-HRMS calcd for $C_{31}H_{35}D_7O_2$: 453.3622. Found: 453.3610.

4.1.10. 6′,7′-10′,11′-Tetrahydro-menaquinone-4-d_7 (6,7,10, 11-TH-MK-4-d_7) (9)

Similar to the synthesis of **1** from **13**, a crude product (**9**), which was obtained from **13** (50 mg, 278 μmol), **30** (90 mg, 310 μmol), and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1), giving **9** (54 mg, 43%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.81 (3H, d, J = 7.0 Hz), 0.86 (3H, d, J = 7.0 Hz), 1.00–1.41 (14H, m), 1.59 (3H, s), 1.67 (3H, s), 1.78 (3H, s), 1.91–1.97 (4H, m), 3.37 (2H, d, J = 7.0 Hz), 4.99–5.11 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.2, 17.6, 19.6, 22.6, 22.7, 24.4, 25.2, 25.4, 25.6, 25.7, 26.0, 28.0, 32.2, 36.6, 37.3, 39.3, 40.0, 118.8, 125.1, 130.9, 132.08, 132.12, 137.9, 143.2, 146.3, 184.5, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.72 (2D, s), 8.11 (2D, s): EI-LRMS *m/z* 455 (M^+). EI-HRMS calcd for $C_{31}H_{37}D_7O_2$: 455.3781. Found: 455.3788.

4.1.11. Phylloquinone-d_7 (PK-d_7) (10)

Similar to the synthesis of **1** from **13**, a crude product (**10**), which was obtained from **13** (50 mg, 278 μmol), phytol (92 mg, 310 μmol), and boron trifluoride ether complex (30 μL) in AcOEt (1 mL) and dioxane (1 mL), was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1), giving **10** (57 mg, 45%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.79–0.85 (total 12H, m), 0.99–1.34 (18H, m), 1.49 (1H, m), 1.76 (3H, s), 1.92 (2H, m), 3.35 (2H, d, J = 7.0 Hz), 4.98 (1H, dt, J = 1.0, 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.3, 19.70, 19.71, 22.6, 22.7, 24.4, 24.8, 25.3, 26.0, 28.0, 32.6, 32.8, 36.6, 37.3, 37.36, 37.39, 39.4, 40.0, 118.8, 119.3, 132.1, 137.9, 138.3, 143.2, 184.6, 185.5; D NMR (500 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.71 (2D, s), 8.10 (2D, s): EI-LRMS *m/z* 457 (M^+), 193. EI-HRMS calcd for $C_{31}H_{39}D_7O_2$: 457.3935. Found: 457.3935.

4.1.12. 2′,3′-Dihydro-phylloquinone-d_7 (2,3-DH-PK-d_7) (11)

To a solution of **10** (20 mg, 44 μmol) in AcOEt (10 mL) was added a catalytic amount of PtO_2 (2 mg), and the mixture stirred vigorously under a hydrogen atmosphere for 12 h at room temperature. After filtration through Celite, the filtrate was concentrated. The residue was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1) to give **11** (20 mg, quantitative yield) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.84–0.87 (12H, m), 0.98 (3H, dd, J = 1.5, 11.5 Hz), 1.05–1.38 (22H, m), 1.48–1.55 (2H, m), 2.56–2.68 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.47, 19.53, 19.7, 19.8, 22.6, 22.7, 24.4, 24.5, 24.8, 28.0, 32.8, 33.4, 35.7, 35.8, 37.0, 37.1, 37.3, 37.4, 37.5, 39.4, 132.1, 132.2, 142.8, 148.1, 184.7, 185.4; D NMR (500 MHz, $CHCl_3$) δ 2.14 (3D, s), 7.72 (2D, s), 8.11 (2D, s): EI-LRMS *m/z* 459 (M^+). EI-HRMS calcd for $C_{31}H_{41}D_7O_2$: 459.4092. Found: 459.4090.

4.1.13. Menaquinone-4-epoxide-d_7 (MK-4-epoxide-d_7) (12)

A mixture of 43 mg (95 μmol) of MK-4-d_7 (**3**) and 100 μL of 30% hydrogen peroxide solution in 2.5 mL of ethanol was combined with an aqueous solution of 100 mg of sodium carbonate in 0.3 mL of H_2O and heated at 75 °C for 1 h. The mixture was poured into 10 mL of H_2O and extracted with three 10 mL portions of ether. The reaction mixture was extracted with ether (3 × 10 mL) and the organic layer was combined. The ether layer was washed with brine (10 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by preparative TLC on silica gel (*n*-hexane/AcOEt = 20:1) to afford **12** (41 mg, 92%) as a pale yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (3H, s), 1.57 (3H, s), 1.59 (3H, s), 1.67 (3H, s), 1.76 (3H, s), 1.91–2.09 (12H, m), 2.43 (1H, dd, J = 7.0, 15.0 Hz), 3.25 (1H, dd, J = 7.0, 15.0 Hz), 5.05–5.14 (4H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.0, 16.1, 16.6, 17.7, 25.6, 25.7, 26.5, 26.6, 26.8, 39.68, 39.73, 39.79, 67.5, 116.7, 123.8, 124.2, 124.4, 131.3, 131.9, 132.2, 134.9, 135.3, 139.1, 192.1, 193.1; D NMR (500 MHz, $CHCl_3$) δ 1.73 (3D, s), 7.77 (2D, s), 8.03 (2D, s): EI-LRMS *m/z* 467 (M^+). EI-HRMS calcd for $C_{31}H_{33}D_7O_3$: 467.3414. Found: 467.3422.

4.1.14. (*E*)-3,7,11-Trimethyldodeca-6,10-dien-1-ol (16)

Farnesol (**15**) (5.0 g, 22.5 mmol) was hydrogenated with PtO_2 (100 mg) in AcOEt (100 mL) for 40 min. The completion of the hydrogenation was checked by ^{1}H NMR spectroscopy with part of the reaction mixture. The reaction mixture was filtrated and evaporated in vacuo. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to give **16** (5.0 g, quantitative yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.91 (3H, d, J = 6.5 Hz), 1.18–1.43 (5H, m), 1.56–1.65 (1H, m), 1.60 (6H, s), 1.68 (3H, s), 1.95–2.09 (6H, m), 3.64–3.73 (2H, m), 5.07–5.12 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.9, 17.7, 19.5, 25.3, 25.7, 26.7, 29.2, 37.2, 39.7, 39.9, 61.2, 124.4, 124.6, 131.3, 134.9: EI-LRMS *m/z* 224 (M^+). EI-HRMS calcd for $C_{15}H_{28}O$: 224.2139. Found: 224.2140.

4.1.15. (*E*)-12-Iodo-2,6,10-trimethyldodeca-2,6-diene (17)

To a stirred solution of **16** (2.0 g, 8.9 mmol) in dichloromethane (23.5 mL) was added methanesulfonyl chloride (1.2 g, 10.5 mmol) and Et_3N (1.2 g, 11.9 mmol) at 0–5 °C, and the resulting mixture was stirred for 30 min. The mixture was washed with a saturated aqueous $NaHCO_3$ solution and brine, dried over $MgSO_4$, filtered, and concentrated. Acetone (20 mL) and NaI (5.0 g, 33.3 mmol) were added to the residue and the mixture was refluxed for 1.5 h. The reaction mixture was quenched with water, extracted with AcOEt, and subjected to a standard work up. The residue was purified by column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to afford **17** (2.18 g, 73% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (3H, d, J = 6.5 Hz), 1.15–1.49 (4H, m), 1.56–1.65 (1H, m), 1.60 (6H, s), 1.68 (3H, s), 1.85–2.09 (6H, m), 3.14–3.27 (2H, m), 5.08–5.12 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.0, 17.7, 18.7, 25.2, 25.7, 26.7, 33.5, 36.3, 39.7, 40.9, 124.30, 124.35, 131.3, 135.1: EI-LRMS *m/z* 334 (M^+). EI-HRMS calcd for $C_{15}H_{27}I$: 334.1156. Found: 334.1167.

4.1.16. (*E*)-6,10,14-Trimethylpentadeca-9,13-dien-2-one (18)

To a solution of **17** (2.18 g, 6.5 mmol) in THF (20 mL) were added a 28% sodium methoxide in methanol solution (3.3 mL) and $CH_3COCH_2COOC_2H_5$ (4.5 g, 34.6 mmol), and then refluxed for 20 h. After addition of water to the mixture and extraction with AcOEt, the organic layer was dried and concentrated. The residue was dissolved in isopropyl alcohol (4.5 mL) and a 50% aqueous KOH solution (4.0 mL), then heated at 85 °C and subjected to a standard work up. The residue was purified by column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to afford **18** (1.2 g, 70% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (3H, d, J = 6.5 Hz), 1.10–1.16 (2H, m), 1.25–1.43 (3H, m), 1.52–1.62 (2H, m), 1.60 (6H, s), 1.68 (3H, s), 1.93–2.07 (6H, m), 2.13 (3H, s), 2.40 (2H, t, J = 7.5 Hz), 5.08–5.11 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.0, 17.6, 19.4, 21.4, 25.4, 25.7, 26.7, 29.8, 32.2, 36.4, 36.9, 39.7, 44.1, 124.4, 124.7, 131.2, 134.7, 209.2: EI-LRMS *m/z* 264 (M^+). EI-HRMS calcd for $C_{18}H_{32}O$: 264.2453. Found: 264.2469.

4.1.17. (2*E*,10*E*)-Methyl 3,7,11,15-tetramethylhexadeca-2,10, 14-trienoate (19)

To a solution of **18** (0.9 g, 3.4 mmol) in *n*-hexane (6.0 mL) were added $PO(OCH_2CH_3)_2CH_2COOC_2H_5$ (1.3 g, 5.8 mmol) and a 28% sodium methoxide in methanol solution (1.35 g), and the mixture was stirred for 2 h at room temperature. Following the addition of water to the reaction mixture followed by extraction with *n*-hexane, the organic layer was washed with 60% methanol, dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 95:5) to give **19** (0.89 g, 81% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (3H, d, J = 6.5 Hz), 1.08–1.18 (2H, m), 1.25–1.33 (3H, m), 1.40–1.44 (2H, m), 1.60 (6H, s), 1.68 (3H, s), 1.93–2.13 (8H, m), 2.15 (3H, s), 3.67 (3H, s), 5.08–5.11 (2H, m), 5.65–5.67 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.9, 17.7, 18.7, 19.5, 24.8, 25.4, 25.7, 26.7, 32.2, 36.4, 37.0, 39.7, 41.2, 50.7, 115.0, 124.4, 124.7, 131.2, 134.7, 160.7, 167.3: EI-LRMS *m/z* 320 (M^+). EI-HRMS calcd for $C_{21}H_{36}O_2$: 320.2713. Found: 320.2693.

4.1.18. (2*E*,10*E*)-3,7,11,15-Tetramethylhexadeca-2,10,14-trien-1-ol (20)

To a solution of **19** (0.89 g, 2.8 mmol) in *n*-hexane (10 mL) was added Vitride reagent (1.2 g), and stirred for 2 h. After the addition of acetone (1 mL) and 50% acetic acid (10 mL), the mixture was dehydrated with a Dean–Stark apparatus. The reaction mixture was washed with water, saturated aqueous $NaHCO_3$, and brine, and the organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to give **20** (0.72 g, 90% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (3H, d, J = 6.5 Hz), 1.08–1.18 (3H, m), 1.25–1.44 (5H, m), 1.60 (6H, s), 1.67 (3H, s), 1.68 (3H, s), 1.93–2.08 (8H, m), 4.15–4.16 (2H, m), 5.08–5.12 (2H, m), 5.39–5.42 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.9, 16.2, 17.7, 19.6, 25.1, 25.4, 25.7, 26.7, 32.3, 36.6, 37.0, 39.7, 39.9, 59.4, 123.1, 124.4, 124.9, 131.2, 134.6, 140.3: EI-LRMS *m/z* (M^+) 292. EI-HRMS calcd for $C_{20}H_{36}O$: 292.2765. Found: 292.2760.

4.1.19. 8-Iodo-2,6-dimethyloct-2-ene (22)

To a solution of β-citronellol (**21**) (8.5 g, 54.4 mmol) and methanesulfonyl chloride (7.5 g, 65.4 mmol) in CH_2Cl_2 (100 mL) was added triethylamine (10 g, 169 mmol) dropwise at 0 °C under argon, and the mixture stirred at room temperature for 2 h. After extraction with AcOEt (50 mL × 3), the combined organic layer was washed with saturated sodium bicarbonate aqueous solution (100 mL) and then brine (100 mL), dried over $MgSO_4$, and concentrated to afford a crude product. The residue was dissolved in acetone (100 mL), and sodium iodide (16.5 g, 110 mmol) was added. The mixture was heated to reflux for 3 h under argon. After the reaction mixture had cooled to room temperature, it was poured into ice-water, and extracted with AcOEt (50 mL × 3). The combined organic layer was washed with brine (100 mL), dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 6:1) to give **22** (12.5 g, 87% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.89 (3H, d, J = 6.5 Hz), 1.14–1.21 (1H, m), 1.27–1.38 (2H, m), 1.53–1.71 (2H, m), 1.61 (3H, s), 1.68 (3H, s), 1.85–2.01 (2H, m), 3.14–3.19 (1H, m), 3.22–3.27 (1H, m), 5.09 (1H, t, J = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 5.1, 17.7, 18.6, 25.3, 25.7, 33.5, 36.3, 40.9, 124.4, 131.4: EI-LRMS *m/z* 266 (M^+). EI-HRMS calcd for $C_{10}H_{19}I$: 266.0530. Found: 266.0543.

4.1.20. 6,10-Dimethylundec-9-en-2-one (23)

To a solution of **22** (7.0 g, 26.3 mmol) in THF (70 mL) were added a 28% sodium methoxide methanol solution (10 g) and ethyl acetoacetate (15 g, 115 mmol), and the mixture was heated to reflux for 12 h. After cooling to room temperature, the mixture was poured into water. The reaction mixture was extracted with AcOEt (100 mL × 3), and the combined organic layer was washed with brine (100 mL), dried over $MgSO_4$, and concentrated. The residue was dissolved in isopropyl ether (10 mL), added to a 50% KOH solution (12 mL), and stirred at 85 °C for 3 h. After addition of water to the solution, the mixture was extracted with AcOEt (100 mL × 3), washed with brine (100 mL), dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 6:1) to give **23** (5.1 g, 99% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (3H, d, J = 6.5 Hz), 1.08–1.17 (2H, m), 1.25–1.36 (2H, m), 1.37–1.43 (1H, m), 1.52–1.63 (2H, m), 1.60 (3H, s), 1.68 (3H, s), 1.92–2.00 (2H, m), 2.13 (3H, s), 2.40 (2H, t, J = 7.0 Hz), 5.07–5.10 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.4, 21.3, 25.4, 25.7, 29.8, 30.9, 32.2, 36.4, 36.9, 44.1, 124.8, 131.0, 209.2: EI-LRMS *m/z* 196 (M^+). EI-HRMS calcd for $C_{13}H_{24}O$: 196.1826. Found: 196.1832.

4.1.21. (*E*)-Methyl 3,7,11-trimethyldodeca-2,10-dienoate (24)

To a solution of **23** (5.1 g, 26.0 mmol) in *n*-hexane (50 mL) were added $PO(OC_2H_5)_2CH_2COOC_2H_5$ (9.0 g, 40.1 mmol) and 28% sodium methoxide in methanol (10.0 g), and the mixture was stirred for 2 h at room temperature. Addition of water to the reaction mixture was followed by extraction with *n*-hexane (100 mL × 3), and the organic layer was washed with 60% methanol (100 mL), dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 95:5) to give **24** (6.0 g, 91% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (3H, d, J = 6.5 Hz), 1.10–1.51 (7H, m), 1.60 (3H, s), 1.68 (3H, s), 1.90–2.00 (2H, m), 2.10–2.13 (2H, m), 2.16 (3H, s), 3.68 (3H, s), 5.10 (1H, m), 5.66 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.4, 22.6, 24.8, 25.5, 25.7, 31.6, 32.2, 36.4, 36.9, 41.2, 50.7, 115.0, 124.9, 131.1, 160.7, 167.3: EI-LRMS *m/z* 252 (M^+). EI-HRMS calcd for $C_{16}H_{28}O_2$: 252.2088. Found: 252.2092.

4.1.22. (*E*)-3,7,11-Trimethyldodeca-2,10-dien-1-ol (25)

To a solution of **24** (6.0 g, 23.7 mmol) in *n*-hexane (60 mL) was added Vitride reagent (10 g), and the mixture stirred for 2 h. After the addition of acetone (2 mL) and 50% acetic acid (10 mL), the mixture was dehydrated with the Dean–Stark apparatus. The reaction mixture was washed with water, saturated aqueous $NaHCO_3$, and brine, and the organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to give **25** (5.3 g, 92% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (3H, d, J = 6.5 Hz), 1.06–1.16 (2H, m), 1.25–1.43 (6H, m), 1.60 (3H, s), 1.67 (3H, s), 1.68 (3H, s), 1.93–2.05 (4H, m), 4.14 (2H, d, J = 5.5 Hz), 5.08–5.11 (1H, m), 5.39–5.42 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.1, 17.6, 19.5, 25.1, 25.5, 25.7, 32.2, 36.6, 37.0, 39.8, 59.4, 123.1, 125.0, 131.0: EI-LRMS *m/z* 224 (M^+). EI-HRMS calcd for $C_{15}H_{28}O$: 224.2140. Found: 224.2152.

4.1.23. 3,7,11-Trimethyldodec-10-en-1-ol (26)

Compound **25** (5.2 g, 23.2 mmol) was hydrogenated with PtO_2 (100 mg) in AcOEt (100 mL) for 40 min. Completion of the hydrogenation was checked by ^{1}H NMR spectroscopy with part of the reaction mixture. The reaction mixture was filtrated and evaporated in vacuo. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to give **26** (5.2 g, quantitative yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (3H, d, J = 6.5 Hz), 0.90 (3H, d, J = 6.5 Hz), 1.07–1.40 (11H, m), 1.50–1.63 (2H, m), 1.60 (3H, s), 1.68 (3H, s), 1.92–2.02 (2H, m), 3.64–3.72 (2H, m), 5.08–5.12 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.6, 22.6, 22.7, 24.3, 24.8, 25.6, 25.7, 28.0, 39.3, 39.96, 40.0, 61.2, 125.0, 130.9: EI-LRMS *m/z* 226 (M^+). EI-HRMS calcd for $C_{15}H_{30}O$: 226.2295. Found: 226.2310.

4.1.24. 12-Iodo-2,6,10-trimethyldodec-2-ene (27)

To a stirred solution of **26** (5.2 g, 23.2 mmol) in dichloromethane (50 mL) were added methanesulfonyl chloride (3.2 g, 27.9 mmol) and Et_3N (3.5 g, 34.6 mmol) at 0–5 °C, and the resulting mixture was stirred for 30 min. The mixture was washed with a saturated aqueous $NaHCO_3$ solution and brine, dried over $MgSO_4$, filtered, and concentrated. Acetone (100 mL) and NaI (9.0 g, 60.0 mmol) were added to the residue and the mixture was refluxed for 1.5 h. The reaction mixture was quenched with water, extracted with AcOEt, and subjected to a standard work up. The residue was purified by column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to afford **27** (7.3 g, 94% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (3H, d, J = 3.0 Hz), 0.87 (3H, d, J = 3.0 Hz), 1.07–1.32 (10H, m), 1.50–1.67 (2H, m), 1.61 (3H, s), 1.69 (3H, s), 1.85–2.00 (2H, m), 3.14–3.19 (1H, m), 3.23–3.28 (1H, m), 5.09–5.12 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 5.4, 17.6, 18.7, 18.8, 22.6, 24.8, 25.6, 25.7, 28.0, 34.0, 36.5, 39.3, 41.0, 125.0, 131.0: EI-LRMS m/z 336 (M^+). EI-HRMS calcd for $C_{15}H_{29}I$: 336.1312. Found: 336.1322.

4.1.25. 6,10,14-Trimethylpentadec-13-en-2-one (28)

To a solution of **27** (5.8 g, 17.2 mmol) in THF (60 mL) were added 28% sodium methoxide in methanol (7 mL) and $CH_3COCH_2COOC_2H_5$ (12 g, 92.2 mmol), and the mixture was refluxed for 20 h. After addition of water to the mixture and extraction with AcOEt, the organic layer was dried and concentrated. The residue was dissolved in isopropyl alcohol and a 50% aqueous KOH solution, heated at 85 °C, and subjected to a standard work up. The residue was purified by column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to afford **28** (6.0 g, quantitative yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (3H, d, J = 4.5 Hz), 0.86 (3H, d, J = 4.5 Hz), 1.06–1.42 (14H, m), 1.60 (3H, s), 1.68 (3H, s), 1.88–2.01 (2H, m), 2.13 (3H, s), 2.40 (2H, t, J = 7.0 Hz), 5.09–5.12 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.6, 19.5, 21.4, 22.6, 24.4, 25.6, 28.0, 29.8, 32.4, 36.5, 37.2, 39.4, 44.1, 125.1, 130.9, 209.4: EI-LRMS m/z 266 (M^+). EI-HRMS calcd for $C_{18}H_{34}O$: 266.2610. Found: 266.2622.

4.1.26. (*E*)-Methyl 3,7,11,15-tetramethylhexadeca-2,14-dienoate (29)

To a solution of **28** (6.0 g, 22.5 mmol) in *n*-hexane (40 mL) were added $PO(OC_2H_5)_2CH_2COOC_2H_5$ (9.8 g, 43.7 mmol) and 28% sodium methoxide in methanol (8.5 g), and the mixture was stirred for 2 h at room temperature. After quenching with water, and extraction with *n*-hexane, the organic layer was washed with a 60% methanol solution, and concentrated. The residue was purified by column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to give **29** (4.6 g, 63% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.84–0.87 (6H, m), 1.06–1.52 (14H, m), 1.60 (3H, s), 1.68 (3H, s), 1.90–2.00 (2H, m), 2.10–2.13 (2H, m), 2.15 (3H, s), 3.69 (3H, s), 5.09–5.12 (1H, m), 5.65–5.67 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.6, 18.7, 19.6, 22.6, 24.4, 24.9, 25.7, 28.0, 32.6, 39.4, 41.2, 50.7, 115.0, 125.1, 131.0, 160.8, 167.3: EI-LRMS m/z 322 (M^+). EI-HRMS calcd for $C_{21}H_{38}O_2$: 322.2872. Found: 322.2874.

4.1.27. (*E*)-3,7,11,15-Tetramethylhexadeca-2,14-dien-1-ol (30)

To a solution of **29** (4.6 g, 14.3 mmol) in *n*-hexane (50 mL) was added Vitride reagent (6.1 g), and the mixture stirred for 1 h. After the addition of acetone (1 mL) and 50% acetic acid (10 mL), the mixture was dehydrated with the Dean–Stark apparatus. The reaction mixture was washed with water, saturated aqueous $NaHCO_3$, and brine, and the organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (*n*-hexane/AcOEt = 10:1) to give **30** (3.7 g, 57% yield) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.84–0.87 (6H, m), 1.05–1.41 (15H, m), 1.61 (3H, s), 1.67 (3H, s), 1.69 (2.7H (trans), s), 1.74 (0.3H (cis), s), 1.94–2.06 (4H, m), 4.12–4.22 (2H, m), 5.09–5.12 (1H, m), 5.40–5.43 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.1, 17.6, 19.6, 22.6, 24.4, 25.1, 25.7, 28.0, 30.9, 32.7, 37.1, 39.4, 39.9, 59.4, 123.1, 125.1 130.9, 140.3: EI-LRMS m/z 294 (M^+). EI-HRMS calcd for $C_{20}H_{38}O$: 294.2923. Found: 294.2922.

4.2. Cells and cell culture

The human hepatoblastoma cell line (HepG2) and human osteosarcoma cell line (MG-63) were obtained from Riken cell bank (Tsukuba Science City, Japan). Minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM) (low-glucose type), Dulbecco's phosphate-buffered saline (PBS), penicillin–streptomycin (10,000 U/mL penicillin), sodium pyruvate (100 mM), and MEM non-essential amino acid solution (10 mM) were all purchased from Gibco (Grand Island, NY). Trypsin (2.5% solution) and trypsin-EDTA (2.5% trypsin, 1 mM EDTA-solution) were purchased from Nacalai Tesque (Kyoto, Japan). Fetal Bovine Serum (FBS) was purchased from Termo Trace (Melbourne, Australia). HepG2 was maintained in MEM supplemented with 1 mM sodium pyruvate, 100 μM MEM non-essential amino acid solution, 10% heat-inactivated FBS, and 1% penicillin–streptomycin. MG-63 was maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin. Cells were cultured in T-75 flasks (BD Falcon, MA) at 37 °C in a humidified 5% CO_2 atmosphere.

4.3. Incubation of cultured human cell lines with vitamin K analogues

To compare the conversion to MK-4-d_7, and utilization of MK-4-d_7 in cultured human cell lines, concentrations of vitamin K metabolites in cells were measured after the addition of the analogues. The experimental medium consisted of culture medium containing 1 μM of each analogue. In the case of HepG2 cells, 8×10^5 cells/well were seeded onto 6-well plates on the first day, and the culture medium was changed every 2 days. Six days later, when cells were confluent, the medium was replaced with the experimental medium. In the case of MG-63 cells, 4×10^5 cells/well were seeded onto six-well plates on the first day. When cells were confluent 3 days later, the medium was changed to the experimental medium. After cells were incubated in the presence of each analogue for 24 h, they were washed with cold PBS three times and then refrigerated at −30 °C. After being warmed to room temperature, cells were lysed in 1 mL of PBS. With this procedure, 10 μL of cell lysate was analyzed for protein determination with a BCA protein assay kit (Pierce, IL, USA).

4.4. Preparation of standard solutions

A stock solution (100 μg/mL) of each standard (MK-4-d_7 (**3**) and MK-4-epoxide-d_7 (**12**)) as a reference was prepared in ethanol according to the solubility of the solute and stored in the dark at −30 °C prior to use. For the analytical curves, working solutions of the standard mixture, ranging from 25 to 400 ng/mL, were prepared by dilution of the stock solution with ethanol. The stock solution (10 μg/mL) of the MK-4 analogue containing heavy oxygen (MK-4-^{18}O)[18,20] for the internal standard was prepared by dilution in ethanol and stored in the dark at −30 °C prior to use. Dilution of this solution with ethanol gave working internal standard solutions of 3.6 and 100 ng/mL MK-4-^{18}O, respectively.[18,20] The 3.6 ng/mL mixture was used for the determination of vitamin K homologs in cells and medium. An equal amount of the standard solution (25–400 ng/mL) and internal standard solution (100 ng/mL) gave the solution used for the standard curve. The final con-

centration ranged from 12.5 to 200 ng/mL in the case of the standard and contained 50 ng/mL of internal standard.

4.5. Sample preparation (extraction of vitamin K and its metabolites from cells)

The medium (1 mL) or cell lysate (PBS solution) (1 mL) in a brown screw-capped Pyrex tube was supplemented with 1 mL of 3.6 ng/mL MK-4-^{18}O in ethanol as an internal standard. The addition of extra ethanol (1 mL) to denature the protein and 3 mL of *n*-hexane was followed by shaking for 5 min. The solution was centrifuged at 3000 rpm for 5 min, and the upper layer was separated. After the organic layer was evaporated under reduced pressure, the dried sample was dissolved in 60 μL of ethanol and sonicated for 10 s. The solutions were transferred to microvials, capped, and placed in a SIL-10AD vp autosampler rack. Aliquots (30 μL) were automatically injected into the HPLC system.

4.6. Apparatus and HPLC conditions

The HPLC analyses were conducted with a Shimadzu HPLC system (Simadzu, Kyoto, Japan) consisting of a binary pump (LC-10AD liquid chromatography), an automatic solvent degasser (DGU-14A degasser), and an autosampler (SIL-10AD autoinjector). Separations were carried out using a reversed-phase C_{18} analytical column (Capcell PAK C_{18} UG120, 5 mm; 4.6 mm id × 250 mm) (Shiseido, Tokyo, Japan) with a solvent system consisting of an isocratic solvent A (0–25 min) and then a linear gradient from 0% to 20% ethanol (25–50 min). Solvent A contained methanol/0.1% acetic acid aqueous (95:5, v/v) and was delivered at 1.0 mL/min. This mobile phase was passed through the column at 1.0 mL/min. The column was maintained at 35 °C with a column oven (CTO-10AC column oven). The HPLC system was controlled by a SCL-10A System Controller (Shimadzu). Acetic acid both functioned as an ion pair reagent during reversed-phase HPLC and facilitated the formation of protonated vitamin K, $[M+H]^+$, in the positive ion mode with an APCI. The autosampler was maintained at 25 °C.

4.7. Quantitation

A quantitative analysis was carried out using MS/MS-MRM (multiple reaction monitoring) of the precursor ion of vitamin K homologs (*m/z* 451 (MK-4-d_7 (**3**)), 468 (MK-4-epoxide-d_7 (**12**)), and 449 (MK-4-^{18}O),[18,20] and their product ion (*m/z* 194 (d_7-labeled vitamin K analogues), 168 (d_7-labeled vitamin K epoxide analogues) and 191 (^{18}O-labeled vitamin K analogues)) with a dwell time of 300 ms. Calibration, using internal standardization, was done by linear regression analysis using five different concentrations, 12.5, 25, 50, 100, and 200 ng/mL. The points were given by the calculated peak area ratio of the standard and internal standard.

Acknowledgments

We are grateful to Dr. Makiko Sugiura, Dr. Atsuko Takeuchi, and Dr. Chisato Tode for the spectroscopic measurements at Kobe Pharmaceutical University. We also thank Eisai Co., Ltd for generously providing the intermediate of the vitamin K analogues. Y.S. acknowledges Takeda Science Foundation. This study was supported in part by a Grant-in-aid for Scientific Research <KAKENHI> (C) (Grant number 18590112 to Y.S.) from the Japan Society for the Promotion of Science.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.035.

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