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Elucidation of the mechanism producing menaquinone-4 in osteoblastic cells

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ABSTRACT

Vitamin K is an essential nutrient and a cofactor for the carboxylation of specific glutamyl residues of proteins to γ-glutamyl residues, which activates osteocalcin related to bone formation. Among vitamin K homologues, menaquinone-4 (MK-4) is the most active biologically, up-regulating the gene expression of bone markers, and thus has been clinically used in the treatment of osteoporosis in Japan. Recently, we confirmed that MK-4 was converted from dietary phylloquinone (PK), and then accumulated in various tissues at high concentrations. This system should play an important role in biological functions including bone formation, however, the pathway by which MK-4 is converted remains unclear. In this study, we studied the mechanism of MK-4's conversion with chemical techniques using deuterated analogues.

Vitamin K is a well-known cofactor for the vitamin K-dependent γ-carboxylative reaction that converts specific glutamic acid residues to γ-carboxyglutamic acid residues in certain proteins related to blood coagulation and bone formation.[1] Vitamin K has two major homologues, the plant-derived vitamin K_1 (**A**) (phylloquinone: PK) and the bacterium-derived vitamin K_2 (**B**) (menaquinone-*n*: MK-*n*) (Fig. 1).[2] It has been revealed that menaquinone-4 (MK-4), one of the menaquinone homologues, has additional biological actions related to gene transcription through steroid and xenobiotic receptor (SXR),[3] and suppression of cancer cell proliferation.[4] Furthermore, MK-4 as well as PK was shown to protect oligodendrocyte precursors and immature fetal cortical neurons from oxidative injury, independent of the vitamin K-dependent γ-carboxylation reaction.[5] There is consistent evidence the MK-4 is converted from other dietary vitamin K homologues.[6] We recently confirmed that MK-4 was converted from dietary phylloquinone (PK), and then accumulated in various tissues at high concentrations.[7] In certain tissues, such as brain tissue, containing high concentrations of lipids, MK-4 would be a preferable form of vitamin K. Although this system should play an important role in biological functions, the pathway of MK-4 synthesis remains unclear. As a first step toward clarifying the biological importance of MK-4, we investigated the mechanism by which dietary PK is converted to MK-4 using chemical techniques.

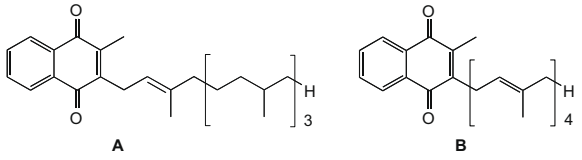

Figure 1. Structure of vitamin K homologues: phylloquinone (**A**) and menaquinones (**B**).

A mechanism for the conversion of dietary PK to MK-4 is proposed in Figure 2. After a quinone moiety of dietary PK is reduced to hydroquinone with a reductase such as NAD(P)H dehydrogenase, quinone (NQO) 1, NQO2, or glutathione reductase, the side chain moiety is cleaved by a specific enzyme. The resulting intermediate, vitamin K_3 (menadione) hydroquinone, is subsequently converted to MK-4 hydroquinone by a bond-forming reaction with geranylgeranylpyrophosphate (GGPP) derived from the mevalonate pathway. Finally, MK-4 hydroquinone is changed to MK-4 with oxidation under atmospheric conditions. Although this mechanism seems plausible, it has not been clarified whether the conversion of PK to MK-4 proceeds directly, or through an "intermediate" menadione. On this background, we first elucidated the conversion reaction using deuterated menadione (K_3-d_8) and geranylgeranylpyrophosphate (GGPP-d_5) with LC–APCI–MS/MS.[8] We used deuterated compounds so as to distinguish them from the native MK-4, menadione, and GGPP originally contained in the cells and to observe the synthetic reaction itself.

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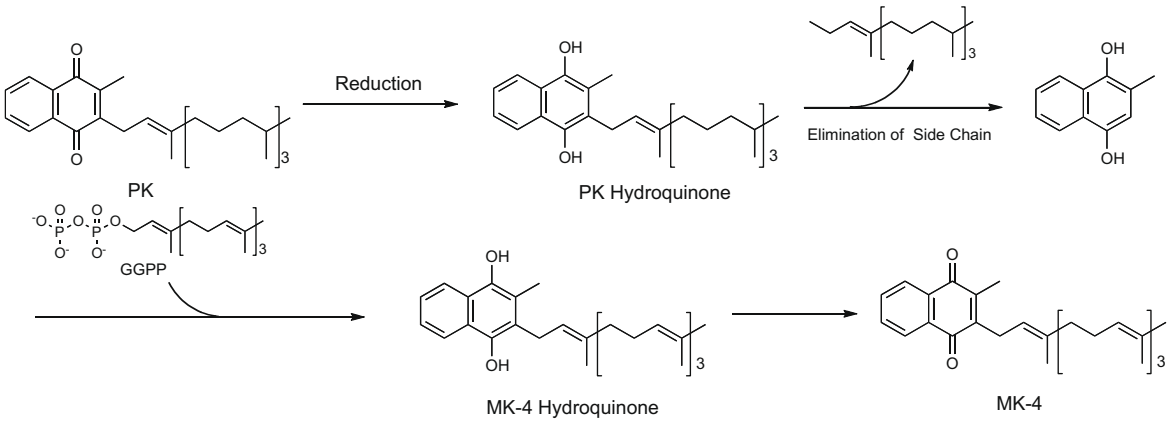
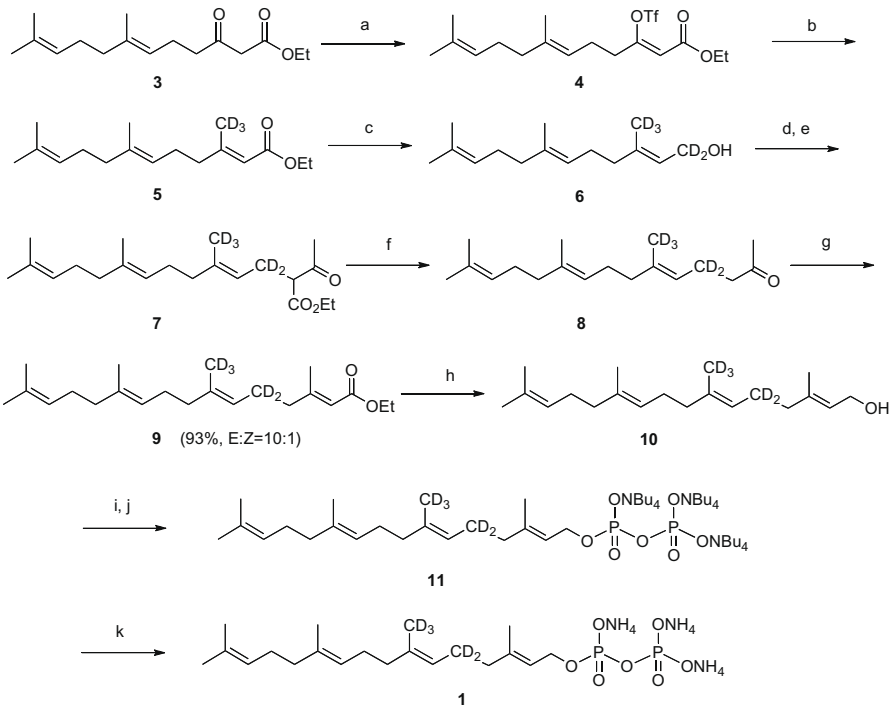


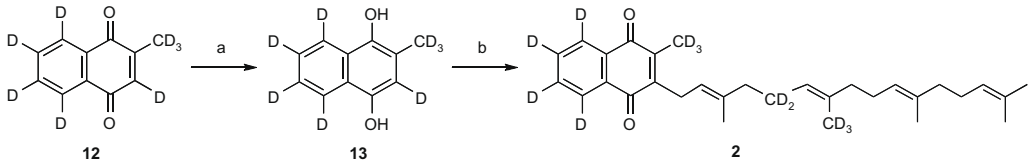
Figure 2. Proposed mechanism of conversion of PK to MK-4.



Scheme 1. Synthesis of geranylgeraniol-d_5. Reagents and conditions: (a) KHMDS, *N*-phenyl-bis(trifluoromethane-sulfonimide), THF, 58%; (b) CD_3Li, CuI, ether, 94%; (c) $LiAlD_4$, ether, quant.; (d) pyridine, PBr_3, ether; (e) NaH, ethyl acetoacetate, ether, 60% in two steps; (f) 10% KOH aq, 10% HCl, 89%; (g) NaH, triethylphosphonoacetate, THF, 93%; (h) DIBAL-H, hexane, 87%; (i) LiCl, collidine, CH_3SO_2Cl, DMF; (j) $HOPP(NBu_4)_3$, CH_3CN, 33%; (k) ion-exchanger, lyophilization, 20%.

The aim of our study is to confirm the synthesis of MK-4 in cells using deuterated menadione and GGPP. The method of producing GGPP-d_5 is shown in Scheme 1. A β-ketoester (**3**)[9] as a starting material was converted to a triflate (**4**) with potassium bis(trimethylsilyl)amide (KHMDS) and *N*-phenyl-bis(trifluoromethanesulfonimide) at −78 °C.[10] To introduce a deuterium, **4** was treated with CD_3Li and CuI at 0 °C to obtain deuterated **5** in 94% yield. The introduction of an additional deuterium into **5** by reduction with $LiAlD_4$ gave farnesol-d_5 (**6**) in quantitative yield. The formation of a C–C bond for elongation of the alkyl chain was achieved using PBr_3 and ethyl acetoacetate to obtain an ethyl ester (**7**) in 60% yield in two steps. Compound **7** was converted to a ketone (**8**) with 10% NaOH aq and 10% HCl in good yield. A conventional Horner–Emmons reaction using NaH and triethylphosphonoacetate gave an unsaturated alkyl compound (**9**, *E*:*Z* = 10:1) in 93% yield. Reduction of the *E*-form with DIBAL-H led to geranylgeraniol-d_5 (**10**) in 87% yield. The introduction of a methanesulfonyl group into the hydroxyl group of **10** with methanesulfonyl chloride and collidine in CH_2Cl_2, followed by phosphorylation with $HOPP(NBu_4)_3$, gave a tetrabutylammonium pyrophosphate derivative (**11**) in 33% yield in two steps.[11] Finally, the tetrabutylammonium salt of **11** was converted to an ammonium salt using ion-exchange resin and the lyophilization of the resulting solution gave GGPP-d_5 ammonium salt (**1**) in 20% yield.[12]

Meanwhile, MK-4-d_{12} (**2**) was synthesized from a deuterated vitamin K_3 analogue (K_3-d_8) available commercially, and geranylgeraniol-d_5 was synthesized as shown in Scheme 2. K_3-d_8 was vigorously stirred with a 10% sodium hydrosulfite aq solution and Et_2O to form K_3-d_8 hydroquinone, to which geranylgeraniol-d_5 was then coupled in the presence of a catalytic amount of $BF_3 \cdot Et_2O$. Finally,



Scheme 2. Synthesis of the deuterated vitamin K analogue (MK-4-d_{12}). Reagents and conditions: (a) 10% NaS_2O_4 aq ether, quant.; (b) 1, $BF_3 \cdot OEt_2$, dioxane/AcOEt, 55%.

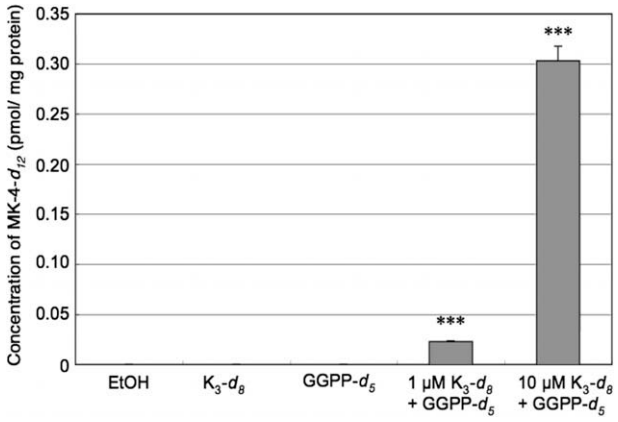


Figure 3. Result of the conversion of K3-d_8 and GGPP-d_5 into MK-4-d_{12}. Columns, means obtained from three independent experiments; bars, SD. Significant difference: $^{***}P < 0.001$, compared with cells untreated with ligands.

K_3-d_8 hydroquinone was oxidized to K_3-d_8 under atmospheric conditions.[13] Thus the desired MK-4-d_{12} (**2**)[14] was obtained in 55% yield.

To evaluate the synthesis of MK-4 from these synthetic ligands, we used a human osteoblastic cell line, MG-63. Briefly, MG-63 cells were seeded in 6-well plates at 2×10^5 cells/well and incubated for 2 days with DMEM. After confirmation of confluency, an ethanol solution of each ligand was added to the cells. The cells were incubated for 24 h, then collected from the 6-well plates. Vitamin K analogues were extracted from the cells and the concentrations of MK-4-d_{12} and its epoxide analogue were measured with LC–APCI–MS/MS to obtain the rate of conversion to MK-4 in MG-63 cells.

Figure 3 shows a concentration of MK-4-d_{12} converted from synthesized ligands in cells. In case of addition of ethanol, K_3-d_8, and GGPP-d_5 alone, MK-4-d_{12} was not detected at all. When 1 μM or 10 μM of K_3-d_8 and GGPP-d_5 were added at the same time, MK-4-d_{12} was produced with the dose-dependent manner. This result indicates that MK-4 could be converted through menadione, followed by a prenylation with GGPP.

Next we evaluated whether MK-4 would be indeed synthesized through a pathway involving hydroquinone derivatives when converted from other vitamin K homologues. The commonly accepted notion about the conversion of MK-4 in living body is that the side chain moiety is exchanged after dietary vitamin K is reduced to hydroquinone derivatives. However, the "intermediate" hydroquinone derivatives have not been detected so far. To prove that hydroquinone is an intermediate in the conversion, we tried to detect hydroquinone derivatives using chemically synthesized compounds as shown in Figure 4. Regarding these compounds, one or both phenol groups of the naphthoquinone moiety was protected to maintain the hydroquinone form. For example, we used 1-acetoxy[15] or 1-methoxy 2-methyl-1,4-naphthalenediol[15] and 1,4-dimethoxy-2-methyl-1,4-naphthalenediol as substrates for the conversion.

We added these compounds to cells in the same manner as GGPP-d_5, and investigated the conversion to MK-4 using a HPLC-fluorescent method,[16] however, the desired intermediates **17**–**19** were not obtained at all. Both the mono acetate **14** and mono methyl ether **15** were converted to MK-4 in cells because the amount of MK-4 was remarkably increased in comparison to the control. Presumably, the acetyl group of **14** was hydrolyzed to produce menadione. Meanwhile, **15** was oxygen-sensitive, therefore, methyl groups would be easily removed and oxidized to produce menadione in culture medium. The dimethyl ether **16** was not converted to MK-4. This result indicates that a C-4 "free" phenol group was important for the interaction with geranylgeranylpyrophosphate and conversion to MK-4. There is much evidence that hydroquinone is involved in the transfer of electrons.

In this study, we studied the mechanism by which MK-4 is converted in osteoblastic cells using deuterated compounds and vitamin K derivatives. The results indicated that MK-4 was synthesized from vitamin K_3 generated from dietary vitamin K homologues and GGPP biosynthesized in the mevalonate pathway. Furthermore, the hydroxyl group of the vitamin K_3 hydroquinone was important for the conversion to MK-4. These findings should be useful in helping to clarify the biosynthetic mechanism in vivo and the essential biological role of MK-4.

Acknowledgments

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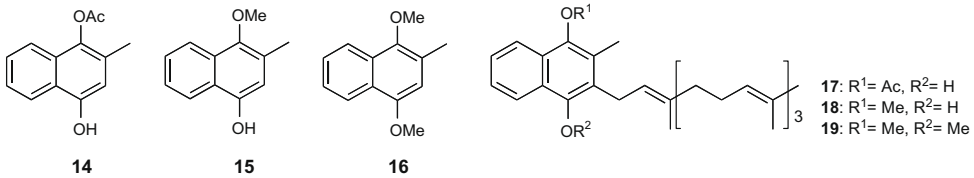

Figure 4. Chemically synthesized compounds used to form the hydroquinone structure and desired intermediates.

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12. Data for compound **1**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (3H, s), 1.59 (3H, s), 1.67 (3H, s), 1.79 (3H, s), 1.92–2.08 (10H, m), 4.95 (2H, d, J = 6.5 Hz), 5.00–5.12 (4H, m); D NMR (500 MHz, $CHCl_3$) δ 1.51 (3D, s), 2.03 (2D, s).
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14. Data for compound **2**: ^{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); 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 456.3477.
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