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Efficient synthesis and biological evaluation of ω-oxygenated analogues of vitamin K_2: Study of modification and structure–activity relationship of vitamin K_2 metabolites

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Abstract—Novel ω-oxygenated vitamin K_2 analogues, which are candidates for metabolites of vitamin K_2 homologues, were efficiently synthesized and their apoptosis-inducing activity was evaluated. We revealed that some of those analogues were biologically active and the side-chain part played an important role in apoptosis-inducing activity. Our results can provide useful information to develop the structure–activity relationship of vitamin K_2 analogues for new drugs based on vitamin K.
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Vitamin K is an essential nutrient and a cofactor for the carboxylation of specific glutamyl residues of proteins to γ-glutamyl residues, which are vital to the blood coagulation system.[1,2] Vitamin K-dependent carboxylase was originally observed in the liver, and it has subsequently been detected in other tissues.[3] In bone, γ-carboxylated osteocalcin is known to be involved in the bone remodeling system, and consequently, vitamin K insufficiency may cause related diseases such as osteoporosis.[4–6] There are two major forms of vitamin K analogues. Examples include the plant-derived vitamin K_1 (**1**) (phylloquinone, PK) and the bacterium-derived vitamin K_2 (**2**) (menaquinone, MK) (Fig. 1). Among these homologues, MK-4 is the most potent coagulation cofactor for γ-carboxylase with hemostatic activity in comparison with PK and other menaquinones.

Until recently, the metabolism of vitamin K has been studied in humans and rats. For example, the administration of radiolabeled phylloquinone to healthy male volunteers led to the detection of urinary metabolites as K acid I (**10**) and K acid II (**11**).[7] In addition, rats fed a diet containing [^{14}C]-labeled menaquinone-4 excreted glucuronides of **10** and **11** as well as those of ω-carboxylic acid (**9**) (Fig. 2).[8,9] However, ω-alcohol and ω-aldehyde metabolites, which are the first step to glucronide conversion, have not been detected yet, although it was suggested that metabolites were formed by side-chain degradation via ω- and β-oxidation as aforementioned.

In this study, we focused on metabolites of vitamin K_2 homologues because MK-4 has unique biological activities among menaquinones. In fact, some groups recently reported that MK-4 showed anti-arteriosclerosis activity[10] and anti-tumor actions in various cancer cells.[11–13] Furthermore, it was revealed that MK-4 works as an SXR-specific ligand and regulates gene transcription.[14] Quite recently, MK-4 binding protein was identified; in short, 17β-hydroxysteroid dehydrogenase 4 concerning estradiol production might be a

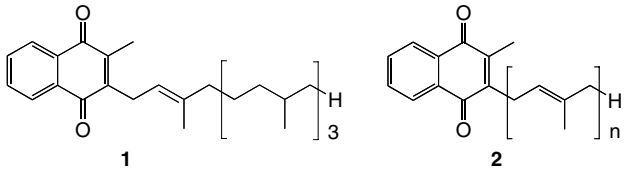

Figure 1. Structure of vitamin K homologues: phylloquinone (**1**) and menaquinones (**2**).

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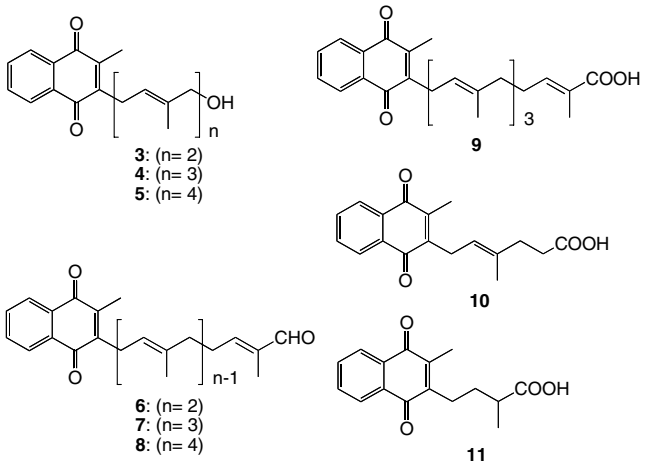
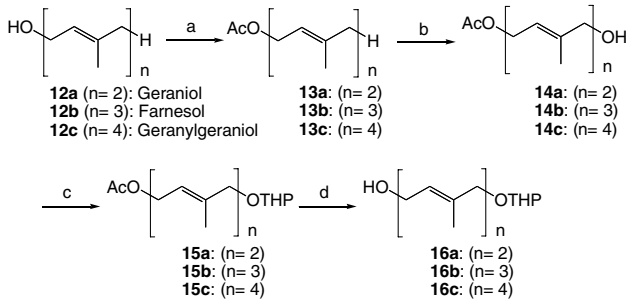


Figure 2. Our synthesized compounds **3**–**8** and chemical structures of the known metabolites of menaquinone-4, ω-COOH (**9**), K acid I (**10**), K acid II (**11**).

candidate;[15] however, the biological activities of these metabolites have hardly been evaluated so far. We anticipated that if those metabolites were studied in detail, they would provide insight into the biological significance of vitamin K and valuable information for the development of new drugs. We were interested in ω-carboxylate **9** as a metabolite of MK-4 at first, because the terminal functional group of an unsaturated alkyl side-chain would induce various biological functions such as geranylgeraniol, farnesol, and acyclic retinoid, but we found that **9** was quite unstable and degraded unless the conjugate was formed. For this reason, we took particular note of 'stable' ω-alcohol and ω-aldehyde menaquinones, which were precursors of ω-carboxylated menaquinones. In this paper, we present the synthesis of six kinds of ω-oxygenated menaquinone analogues **3**–**8** and initial studies on the metabolite of vitamin K homologues.

We first planned the synthesis of ω-oxygenated menaquinones. The requisite analogues were synthesized by coupling the naphthoquinone derivative and side-chain moiety. Scheme 1 outlines the synthesis of side-chain synthons of ω-oxygenated MK-2, MK-3, and MK-4 analogues. We chose geraniol (**12a**), farnesol (**12b**), and geranylgeraniol (**12c**) as the starting material.

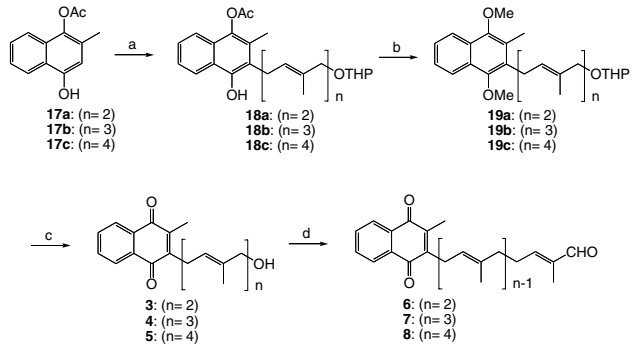


Scheme 1. Reagents: (a) pyridine, Ac_2O, 92–95%; (b) SeO_2, 70% *tert*-BuOOH, salicylic acid, CH_2Cl_2, 40–75%; (c) DHP, TsOH, CH_2Cl_2, 75–81%; (d) 1 N NaOH aq, MeOH, 78–82%.

The primary hydroxyl group of **12a**–**c** was first protected with an acetyl group to give **13a**–**c** in quantitative yield. Then, the selective ω-oxygenation of **13a** and **13b** with SeO_2, 70% *tert*-BuOOH solution, and salicylic acid in CH_2Cl_2 afforded ω-oxygenated alcohol **14a** and **14b** with ω-aldehyde analogue in good yield.[16] Hence, **13c** in the same condition was converted to ω-oxygenated **14c** as well as ω-1-oxygenated and diol derivatives. A significant reason for the low chemical yield was that selective reactivity of SeO_2 was reduced due to a longer alkyl chain length. Then, separation of the mixture by silica gel column chromatography gave the desired **14c** in 20% yield. Protection of the hydroxyl group of **14a**–**c** with 3,4-dihydro-2*H*-pyran (DHP) and TsOH in CH_2Cl_2 gave **15a**–**c** in excellent yield. Removal of the protecting group in **15a**–**c** gave the corresponding alcohol **16a**–**c** in 78–82%.

Scheme 2 shows the synthesis of ω-oxygenated vitamin K analogues. Vitamin K_4 monoacetate (**17**)[17] was used as a naphthoquinone synthon, which condensed with side-chain analogues. Treatment of the monoacetate **17** with an alkyl side-chain alcohol **16a**–**c** in the presence of boron trifluoride etherate yielded **18a**–**c** in 45–51% yield. We first tried to obtain ω-alcohol compounds **3**–**5** directly by alkaline hydrolysis, but the chemical yield was extremely low. Presumably, the quinone form of **18a**–**c** might be degraded under alkaline conditions; therefore, we employed a two-step synthesis by way of dimethyl ether analogues **19a**–**c**. In short, monoacetate derivatives **18a**–**c** were treated with an excess amount of potassium hydride in anhydrous conditions followed by the addition of methyl iodide to give **19a**–**c** in good yield. Finally, oxidation and deprotection of the THP group of naphthohydroquinone methyl ether **19a**–**c** with $Ce(NH_4)_2(NO_3)_6$ in water gave the desired ω-oxygenated vitamin K homologues **3**–**5** in good yield. These analogues were further converted to ω-aldehyde analogues **6**–**8** with PDC treatment in good yield. Thus, six kinds of ω-oxygenated analogues were prepared.[18]

To investigate the biological activities of those analogues, we especially focused on the apoptosis-inducing activity against cancer cell lines among their various biological activities. MK-4 is used as an anti-hepatic cancer drug in clinical practice; therefore, we believe



Scheme 2. Reagents: (a) **16a**–**c**, $BF_3{\cdot}Et_2O$, EtOAc/dioxane (1:1), 45–51%; (b) KH, CH_3I, THF, 60–65%; (c) CAN, CH_3CN–H_2O, 71–76%, (d) PDC, CH_2Cl_2 35–55%.

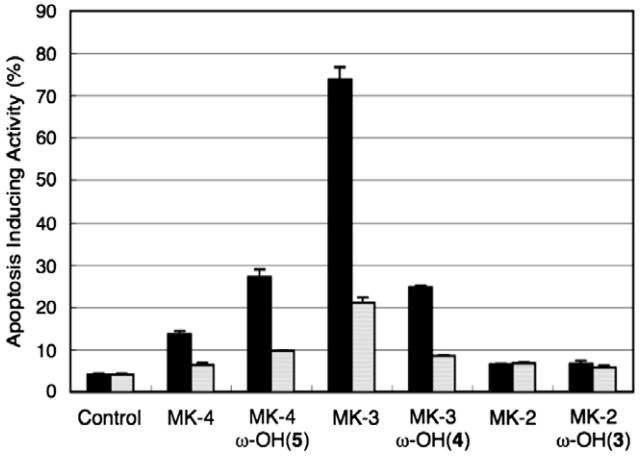


Figure 3. Apoptosis-inducing activity of menaquinone derivatives in HL-60 cells. HL-60 cells were treated with menaquinone analogues at 10 μM (■) and 5 μM (▩) for 3 days. Apoptosis-inducing activity was identified with flow cytometric analysis of DNA content. Results represent the mean and standard errors of three separate experiments. ω-Aldehyde analogues **6**–**8** did not exhibit apoptosis-inducing activity.

that vitamin K analogues can be applied to anti-cancer drugs. Regarding the cancer cell lines, we chose HL-60 cells (human leukemia cells) for preliminary study since they are the most common and convenient to evaluate the apoptosis-inducing activity of biologically active agents.[19] Samples of 5 and 10 μM analogues were added to HL-60 cells. After 72 h, the cells were collected and treated with pyridinium iodide, and then apoptosis activity was determined with a fluorescence-activated cell sorter (FACS). As shown in Figure 3, the activity of most analogues increased in a dose-dependent manner. MK-3 particularly showed the most potent activity, around 75%, among vitamin K_2 homologues at 10 μM. On the other hand, the potency of MK-4 was approximately 15%, which equaled 20% of MK-3 at the same dose; however, MK-2 and ω-aldehyde analogues **6**–**8** did not exhibit such activity. In terms of ω-oxygenated alcohol **3**–**5**, ω-oxygenated MK-3 (**4**) decreased its potency compared to MK-3; however, it retained about 50% potency of MK-3. Interestingly, only ω-oxygenated MK-4 (**5**) increased the activity more than substrate MK-4 at 10 μM.

We synthesized six kinds of vitamin K metabolites and evaluated their biological activities in vitro. Regarding MK-4 analogues, ω-hydroxylation of the side-chain part increased apoptosis-inducing activity for HL-60 cells compared to MK-4. Until recently, only the apoptosis-inducing activity of vitamin K analogues as well as their unsaturated alkyl side-chain moiety such as geranylgeraniol and farnesol had been investigated in vitro. Both action mechanisms of vitamin K and alkyl side-chain analogues are similar in the point of inducing caspase 3 through mitochondria;[20] however, the activity of the unsaturated alkyl side-chain moiety appeared within a few hours, whereas that of vitamin K took 3 days. Presumably, the time of their uptake to cells and transfer to mitochondria were affected. Our ω-hydroxylated compound **5** was exhibited about 2–3 days after the addition to cells; therefore, our compound would preserve the nature of vitamin K analogues rather than alkyl side-chain analogues. We previously reported the synthesis of new vitamin D analogues, which introduced the ω-hydroxyl alkyl group to the molecule.[21] Some had potent binding affinity to vitamin D receptor and this depended on the length of the alkyl group. Similar to this case, the interaction of compound **5** with the target protein increased. Our results indicated that metabolites of vitamin K might have potential as biologically active compounds and can provide useful information to develop new drugs based on vitamin K with modification of the terminal alkyl group.

Acknowledgments

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18. Compound **3**: ^{1}H NMR (500 Mz, $CDCl_3$): δ 1.63 (3H, s), 1.79 (3H, s), 2.03 (2H, t, $J = 7.5$ Hz), 2.12

(2H, t, J = 7.5 Hz), 2.19 (3H, s), 3.36 (2H, d, J = 6.5 Hz), 3.94 (2H, br s), 5.01 (1H, t, J = 7.0 Hz), 5.32 (1H, t, J = 7.0 Hz), 7.68–7.70 (2H, m), 8.07–8.09 (2H, m); ^{13}C NMR (125 Mz, $CDCl_3$): δ 12.7, 13.7, 16.3, 25.9, 26.1, 39.2, 68.9, 119.6, 125.6, 126.2, 126.3, 132.1, 133.3, 133.4, 135.0, 137.0, 143.4, 146.1, 184.6, 185.4; EI-LRMS *m/z* 324 (M^+). EI-HRMS calcd for $C_{21}H_{24}O_3$ 324.1725. Found 324.1748. Compound **4**: ^{1}H NMR (500 Mz, $CDCl_3$): δ 1.57 (3H, s), 1.63 (3H, s), 1.79 (3H, s), 1.93–2.09 (8H, m), 2.17 (3H, s), 3.67 (2H, d, J = 7.5 Hz), 3.97 (2H, br s), 5.00–5.07 (2H, m), 5.32–5.35 (1H, m), 7.68–7.70 (2H, m), 8.07–8.09 (2H, m); ^{13}C NMR (125 Mz, $CDCl_3$): δ 12.7, 13.6, 16.0, 16.4, 26.0, 26.2, 26.3, 39.2, 39.6, 69.0, 119.2, 124.1, 126.0, 126.2, 126.3, 132.1, 132.2, 133.28, 133.34, 134.7, 134.8, 137.4, 143.3, 146.1, 184.5, 185.5; EI-LRMS *m/z* 392 (M^+). EI-HRMS calcd for $C_{26}H_{32}O_3$ 392.2351. Found 392.2376. Compound **5**: ^{1}H NMR (500 Mz, $CDCl_3$): δ 1.53 (3H, s), 1.56 (3H, s), 1.66 (3H, s), 1.79 (3H, s), 1.91–2.13 (12H, m), 2.19 (3H, s), 3.37 (2H, d, J = 7.5 Hz), 3.99 (2H, br s), 5.00–5.08 (3H, m), 5.38 (1H, dt, J = 1.5, 7.5 Hz), 7.68–7.70 (2H, m), 8.07–8.09 (2H, m); ^{13}C NMR (125 Mz, $CDCl_3$): δ 12.7, 13.7, 15.96, 16.0, 16.4, 26.0, 26.2, 26.5, 26.6, 39.3, 39.6, 39.7, 69.0, 119.1, 123.9, 124.5, 126.16, 126.22, 126.3, 132.2, 133.3, 133.4, 134.5, 135.1, 137.5, 143.4, 146.2, 184.5, 185.4; EI-LRMS *m/z* 460 (M^+). EI-HRMS calcd for $C_{31}H_{40}O_3$ 460.2977. Found 460.2965. Compound **6**: ^{1}H NMR (500 Mz, $CDCl_3$): δ 1.72 (3H, s), 1.83 (3H, s), 2.18 (2H, dd, J = 7.5, 15.0 Hz), 2.19 (3H, s), 2.45 (2H, dd, J = 7.5, 15.0 Hz), 3.39 (2H, d, J = 7.0 Hz), 5.08 5.09 (1H, dt, J = 1.5, 7.5 Hz), 6.41 (1H, dt, J = 1.5, 7.5 Hz), 7.69–7.71 (2H, m), 8.07–8.10 (2H, m), 9.33 (1H, s); ^{13}C NMR (125 Mz, $CDCl_3$): δ 9.2, 12.7, 16.3, 26.1, 7.3, 38.0, 120.5, 121.3, 126.27, 126.31, 132.1, 133.41, 133.44, 136.0, 143.5, 145.7, 153.8, 184.5, 185.4, 195.1; EI-LRMS *m/z* 322 (M^+). EI-HRMS calcd for $C_{21}H_{22}O_3$ 322.1569. Found 322.1554. Compound **7**: ^{1}H NMR (500 Mz, $CDCl_3$): δ 1.59 (3H, s), 1.70 (3H, s), 1.79 (3H, s), 2.00 (2H, t, J = 7.5 Hz), 2.06–2.11 (4H, m), 2.19 (3H, s), 2.39(2H, dd, J = 7.5, 15.0 Hz), 3.37 (2H, d, J = 7.5 Hz), 5.02 (1H, dt, J = 1.5, 7.5 Hz), 5.09 (1H, dt, J = 1.5, 7.5 Hz), 6.40 (1H, dt, J = 1.5, 7.5 Hz), 7.68–7.70 (2H, m), 8.07–8.09 (2H, m), 9.35 (1H, s); ^{13}C NMR (125 Mz, $CDCl_3$): δ 9.19, 12.7, 15.9, 16.4, 26.1, 26.4, 27.4, 37.9, 39.5, 119.4, 125.2, 126.3, 126.3, 132.17, 132.21, 133.37, 133.41, 133.6, 137.3, 139.4, 143.4, 146.2, 154.4, 184.6, 185.5, 195.3; EI-LRMS *m/z* 390 (M^+). EI-HRMS calcd for $C_{26}H_{30}O_3$ 390.2195. Found 390.2201. Compound **8**: ^{1}H NMR (500 Mz, $CDCl_3$): δ 1.56 (3H, s), 1.59 (3H, s), 1.74 (3H, s), 1.79 (3H, s), 1.92 (2H, t, J = 7.0 Hz), 1.98–2.07 (6H, m), 2.14 (2H, t, J = 7.5 Hz), 2.19 (3H, s), 2.43 (2H, dd, J = 7.0, 14.5 Hz), 3.37 (2H, d, J = 7.5 Hz), 5.00–5.12 (3H, m), 6.45 (1H, dt, J = 1.5, 7.5 Hz), 7.68–7.70 (2H, m), 8.07–8.09 (2H, m), 9.37 (1H, s); ^{13}C NMR (125 Mz, $CDCl_3$): δ 9.23, 12.7, 15.9, 16.4, 26.0, 26.5, 26.6, 27.4, 38.0, 39.5, 39.7, 119.2, 124.1, 125.6, 126.2, 126.3, 132.2, 133.3, 133.4, 135.0, 137.5, 139.4, 143.4, 146.2, 154.5, 184.6, 185.5, 195.3; EI-LRMS *m/z* 458 (M^+). EI-HRMS calcd for $C_{31}H_{38}O_3$ 458.2821. Found 458.2812.

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