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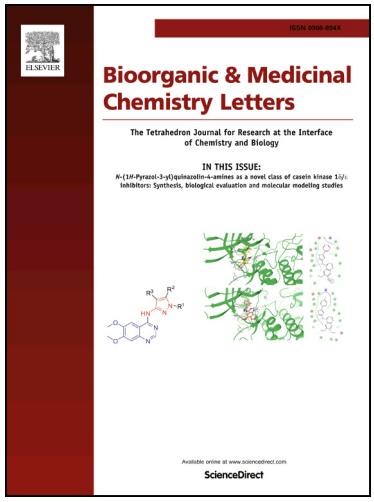


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# Synthesis of novel vitamin K derivatives with alkylated phenyl groups introduced at the ω-terminal side chain and evaluation of their neural differentiation activities

Rie Sakane[a, #], Kimito Kimura[a, #], Yoshihisa Hirota[b, #], Michiyasu Ishizawa[c], Yuta Takagi[b], Akimori Wada[d], Shigefumi Kuwahara[e], Makoto Makishima[c], and Yoshitomo Suhara[a, *]

[a]*Laboratory of Organic Synthesis and Medicinal Chemistry, Faculty of Bioscience and Engineering, College of Systems Engineering and Science, Shibaura Institute of Technology, 307 Fukasaku, Minuma-ku, Saitama 337-8570, Japan*
[b]*Laboratory of Biochemistry, Faculty of Bioscience and Engineering, College of Systems Engineering and Science, Shibaura Institute of Technology, 307 Fukasaku, Minuma-ku, Saitama 337-8570, Japan*
[c] *Department of Biomedical Sciences, Nihon University School of Medicine, 30-1 Oyaguchi-kamicho, Itabashi-ku, Tokyo 173-8610, Japan*
[d]*Department of Life Science for Organic Chemistry, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan*
[e]*Laboratory of Applied Bioorganic Chemistry, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai, 981-8555, Japan*

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ABSTRACT

Vitamin K is an essential cofactor of γ-glutamylcarboxylase as related to blood coagulation and bone formation. Menaquinone-4, one of the vitamin K homologues, is biosynthesized in the body and has various biological activities such as being a ligand for steroid and xenobiotic receptors, protection of neuronal cells from oxidative stress, and so on. From this background, we focused on the role of menaquinone in the differentiation activity of progenitor cells into neuronal cells and we synthesized novel vitamin K derivatives with modification of the ω-terminal side chain. We report here new vitamin K analogues, which introduced an alkylated phenyl group at the ω-terminal side chain. These compounds exhibited potent differentiation activity as compared to control.



Vitamin K is an essential cofactor of γ-glutamyl carboxylase of Gla proteins related to blood coagulation and bone formation.[1] There are two different kinds of natural vitamin K homologues: vitamin $K_1$ (also called phylloquinone (PK)), which has a phytyl group for a side chain, and vitamin $K_2$ (also called menaquinone-n (MK-n)), which has an isoprene unit (Fig. 1).[2, 3]

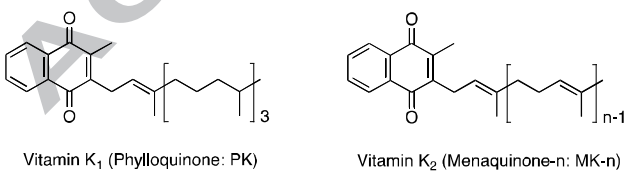



**Figure 1.** Natural vitamin K homologues

We have reported that MK-4 is biosynthesized in the body by conversion from other dietary vitamin K homologues such as PK or MK-7, and is accumulated in various tissues, especially in brain.[4,5] Therefore, we anticipated that MK-4 would play an important role in the body. In fact, it has been clarified that MK-4 showed various biological activities except the γ-glutamyl carboxylation reaction, such as SXR-mediated transcriptional activity, protective effects from oxidative stress, and so on.[6-8] We especially focused on the differentiation activity from neural progenitor cells (NPCs) to neuronal cells, and synthesized some vitamin K analogues for the purpose of increasing the biological activities. In the previous study, we found that the biological activities of vitamin K analogues were increased by introduction of a phenyl group at the ω-terminal position,[9] and we have already succeeded in obtaining some analogues more potent than MK-4.[10,11] The most potent analogue **1** among our analogues contained a *m*-methylphenyl group at the ω-terminal side chain of MK-3 (Fig. 2).[10]

Based on these previous observations, we predicted that an alkyl group, including the methyl group, would play an important role for differentiation activities, and the potency might parallel the number or position of alkyl groups, which were bound to the terminal phenyl group. Therefore, we synthesized new vitamin K analogues, introduced a phenyl group modified with 2 or 3 methyl groups or a *tert*-butyl group at the ω-terminal position of

isoprene unit (Fig. 2). At the same time, the ability of the compounds to induce cell differentiation was evaluated. We report here the synthetic method of the analogues and the results of the biological activity assays.

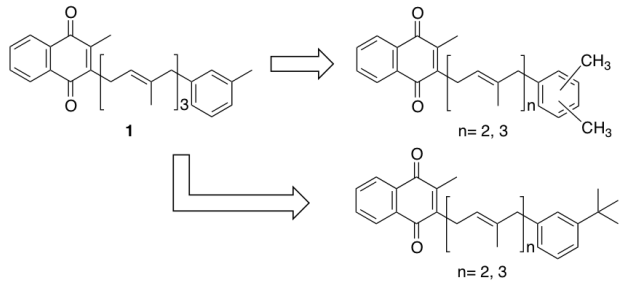


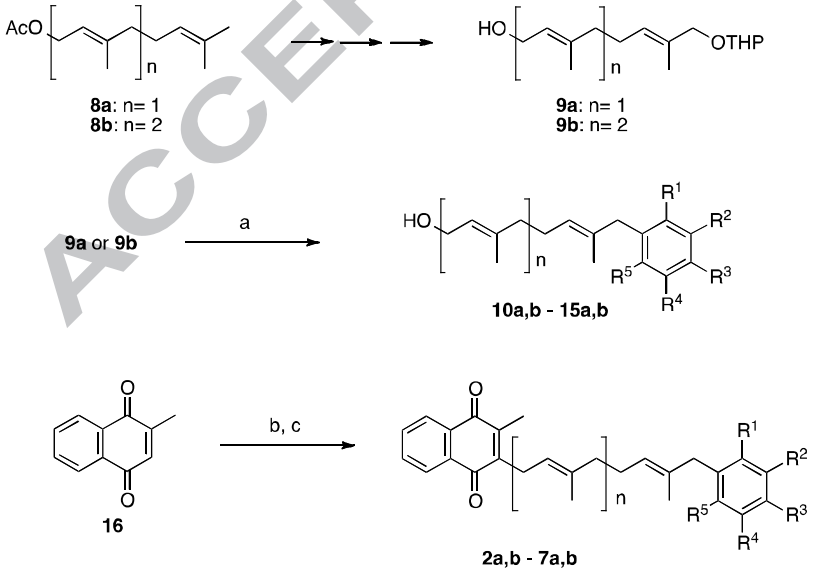

**Figure 2.** Vitamin K analogues with methylphenyl group or *tert*-butylphenyl group introduced at the side chain.

The synthetic scheme of the desired compounds is shown in Scheme 1. We chose geranyl acetate (**8a**) or farnesyl acetate (**8b**) as a starting material for the synthesis of the side chain part. The intermediates of the side chain parts **9a**,**b** were obtained from **8a**,**b** in 3 steps by our reported method.[9] Then, the intermediates **9a**,**b** were converted to **10a**,**b** – **15a,b** with introduction of an alkylated phenyl group by Grignard reaction.[12] Regarding the amount of Grignard reagents, which were successively prepared from commercially available alkylated phenyl bromide reagents and magnesium tips, we used a ten-fold molar excess with respect to the side chain part **9a**,**b**. The chemical yields of the reaction ranged from 19-86%. The yields depended on the kinds of Grignard reagents so that the reactivity differed with respect to the nature of the reagents. Finally, the side chain part (**10a**,**b** – **15a,b**) was coupled with menadione (**16**) according to the Friedel-Crafts reaction.

Menadione (**16**) was converted to hydroquinone form by reduction with sodium dithionate. Then the coupling reaction with side chain part **10a**,**b** – **15a,b** in the presence of the catalytic amount of $BF_3$•$OEt_2$ gave coupling product **2a**,**b** – **7a**,**b** in 11%-48% yield.[13] Totally, 12 kinds of compounds were synthesized in this study. The physiochemical stability of the compounds tended to decrease as the number of methyl groups attached to the phenyl group increased, therefore requiring that resulting compounds be preserved under refrigeration.

Before evaluating the biological activities of the compounds, we examined the cytotoxicity by MTT cell proliferation assay. No toxicity was observed about any of the compounds. We first tried to investigate neurite elongation activity of the compounds by evaluation of mRNA expression of growth associated protein 43 and tubulin βIII as neural differentiation markers after addition of 1 $\mu$M MK-4 to PC12 cells, which is a cell line widely used as a model for neural differentiation, in the presence of nerve growth factor. However, no differences were observed between control and MK-4. Possibly, the reactivity of the cell line was low against MK-4.

Then, we investigated the neural differentiation-inducing activity of the vitamin K analogues using NPCs. We prepared the NPCs from embryonic mouse brain. Our findings showed that the reactivity of the NPCs toward MK-4 was higher than in cultured cell lines. The general method for preparation was according to our previous way. In short, (i) neural stem cells were dissociated from embryonic day 14 mouse cerebrum and cultured according to the method previously described.[10, 11, 14] (ii) After the cells were seeded and cultured for 24 h, they were treated with 1 $\mu$M of the vitamin K analogues every second day for 4 days. We confirmed the differentiation of the NPCs by an immunofluorescence staining method. If the specific antigen microtubule-associated protein 2 (Map2) was expressed on the surface of neuronal cells that had successfully differentiated from progenitor cells, the sample emitted red fluorescence. The differentiation could be evaluated from the resulting fluorescence with fluorescence microscopy (Fig. 3). Our analogues including **7a** and **7b** selectively differentiated NPCs to neuronal cells since the differentiated cells were mostly observed with red fluorescence. With compounds **2a** and **4a**, we did not observe neurite elongation as compared to other compounds.




| | n | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ |
|---|---|---|---|---|---|---|
| **2a**, **10a**: | 1 | $CH_3$ | $CH_3$ | H | H | H |
| **3a**, **11a**: | 1 | $CH_3$ | H | $CH_3$ | H | H |
| **4a**, **12a**: | 1 | H | $CH_3$ | $CH_3$ | H | H |
| **5a**, **13a**: | 1 | H | $CH_3$ | H | $CH_3$ | H |
| **6a**, **14a**: | 1 | $CH_3$ | H | $CH_3$ | H | $CH_3$ |
| **7a**, **15a**: | 1 | H | $^t$Bu | H | H | H |
| **2b**, **10b**: | 2 | $CH_3$ | $CH_3$ | H | H | H |
| **3b**, **11b**: | 2 | $CH_3$ | H | $CH_3$ | H | H |
| **4b**, **12b**: | 2 | H | $CH_3$ | $CH_3$ | H | H |
| **5b**, **13b**: | 2 | H | $CH_3$ | H | $CH_3$ | H |
| **6b**, **14b**: | 2 | $CH_3$ | H | $CH_3$ | H | $CH_3$ |
| **7b**, **15b**: | 2 | H | $^t$Bu | H | H | H |

**Scheme 1.** Reagents and conditions: (a) Grignard reagent, CuI, THF, 50 °C, 1 h, 19-86%; (b) $Na_2S_2O_4$, $Et_2O$, quant; (c) **10a**,**b**-**15a**,**b**, $BF_3$•$Et_2O$, dioxane: EtOAc (1:1), 70 °C, 3 h, 11-48%.

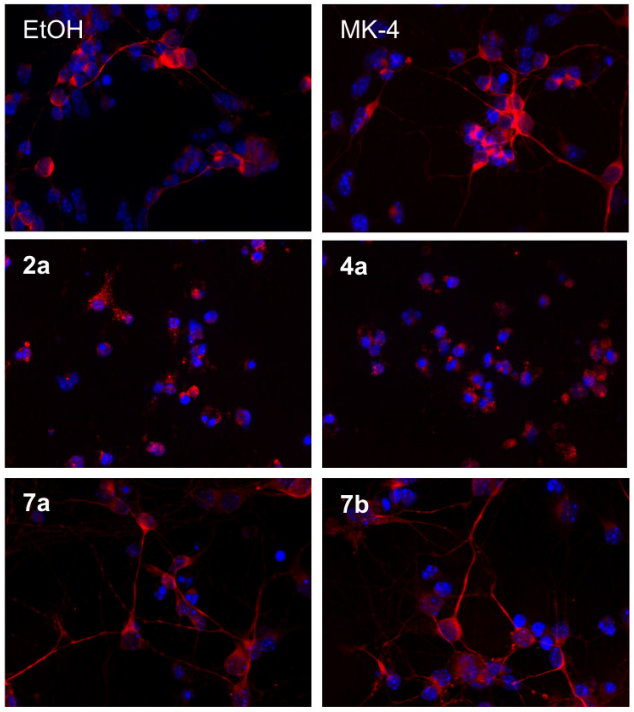



**Figure 3.** Vitamin K derivatives induced neuronal differentiation of NPCs isolated from an embryonic mouse cerebrum. The cells were treated with the indicated compounds at 1 $\mu$M. After 48 h, cells were immunostained with markers for neurons (Map2, red) and with DAPI (blue) for nuclei. Mature neurons were observed with Map2 (red) after treatment with MK-4 and the synthesized analogues induced neuronal differentiation.

We next undertook the quantitation of the mRNA of *Map2* and *β-actin* of the cells using real-time PCR methodology to measure differentiation activity into neuronal cells (Fig. 4).

For purposes of comparison, the sample treated with EtOH served as the baseline, untreated control, while the samples treated with MK-4 served as positive controls. The MK-4 was twice as effective as the EtOH control. Compound **7b** showed the most potent activity among the analogues and the potency was almost the same activity as MK-4. Compound **7a** also significantly exhibited neural differentiation activity. However, most compounds did not show significant activity compared to the control group. Interestingly, the compounds **2a** and **4a** significantly suppressed expression of mRNA of *Map2* at levels less than the EtOH control. From the results of the fluorescent immunostaining as shown in Fig. 3, it is possible that these compounds might have an inhibitory effect on neural differentiation. We have not clarified why compounds **2a** and **4a** suppressed expression of the mRNA, but MK-2 and MK-3 analogues with a *m*-methylphenyl group introduced at the side chain showed potent neural differentiation activities.[10] We therefore predicted that the number and the position of methyl groups attached to terminal phenyl group as well as the length of the isoprene unit would influence the neural differentiation activity.

Our results indicated that the cell differentiation activity of the vitamin K derivatives depended on the structure of the side chain part and the number and the position of the alkyl groups bound to the phenyl group at the ω-terminal position. The ω-terminal *tert*-butylphenyl group, as a "bulky" functional group, showed the most potent activity, while the methylphenyl groups did not increase or decrease differentiation activity. We previously reported that the QSAR analysis of the vitamin K derivatives suggested that introduction of bulky and highly lipid soluble substituents to the ω-terminal phenyl group of vitamin K would increase the differentiation activity.[10] This results obtained here proved our prediction to be correct.

In conclusion, we successfully obtained novel vitamin K derivatives based on natural MK-4, and found that some of them had potent differentiation activity toward neuronal progenitor cells. In contrast, we also obtained compounds that inhibited neural differentiation. These derivatives are the only chemical compounds which have an ability to selectively affect neural differentiation. We clarified that significant differences in differentiation activity could be obtained by altering the functional group of the side chain. A more detailed examination is underway to clarify the functional mechanism of the vitamin K analogues regarding accelerating or suppressing of neural differentiation and to synthesize compounds which are much more potent.

## Acknowledgments

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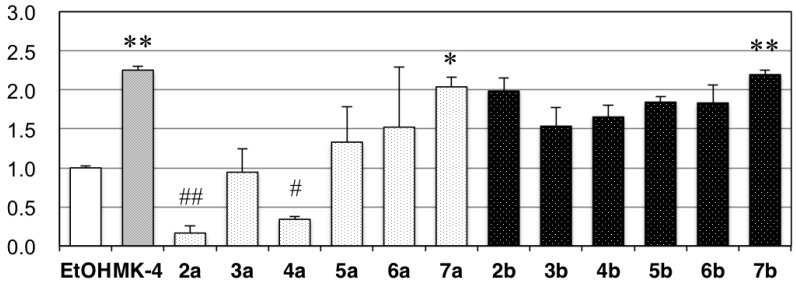


**Figure 4.** Differentiation-inducing activity of vitamin $K_2$ analogues (MK-2 analogues **2a**-**7a**, MK-3 analogues **2b**-**7b**). Neuronal progenitor cells were transformed into neuronal cells as determined by quantitation of mRNA synthesis for *Map2* and *β-actin* using PCR methodology. Cells were treated with the indicated vitamin $K_2$ analogues as well as MK-4 at $1.0 \times 10^{-6}$ M. The histogram data are expressed as the means obtained from three independent experiments; the error bars indicate the SD. Significant difference: (**) $p < 0.05$, (*) $p < 0.1$, between EtOH and compounds (by Dunnett's *t*-test); (##) $p < 0.05$, (#) $p < 0.1$, between EtOH and **2a** and **4a** (by Student's *t*-test). Data are reported with respect to the EtOH control which was expressed as 1.0.

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13. Data for compound **2a**: $^1$H NMR (400MHz, $CDCl_3$) δ 8.09-8.05 (m, 2H), 7.69-7.67 (m, 2H), 6.95-6.88 (m, 3H), 4.98 (dd, 2H, J= 7.6, 15.2 Hz), 3.36 (d, 2H, *J*= 7.6 Hz), 3.17 (s, 2H), 2.26 (s, 3H), 2.18 (s, 3H), 2.16 (s, 3H), 2.09 (dd, 2H, *J*= 7.4, 14.6 Hz), 1.99 (dd, 2H, *J*= 7.4, 14.6 Hz), 1.78 (s, 3H), 1.54 (s, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 185.5, 184.6, 146.2, 143.4, 137.5, 136.7, 135.5, 135.2, 133.8, 133.44, 133.39, 132.26, 132.22, 130.9, 129.6, 126.4, 126.3, 125.5, 119.2, 42.9, 39.7, 26.6, 26.1, 21.0, 19.4, 16.5, 16.4, 12.8; HRMS ($M+H^+$) m/z calcd for $C_{29}H_{33}O_2$ 413.2481; found 413.2484. Data for compound **4a**: $^1$H NMR (400MHz, $CDCl_3$) δ 8.10-8.06 (m, 2H), 7.69-7.67 (m, 2H), 7.01-6.88 (m, 3H), 5.19-4.95 (m, 2H), 3.39-3.35 (m, 2H), 3.23 (s, 1H), 3.14 (s, 1H), 2.24-1.96 (m, 13H), 1.80 and 1.78 (2s, 3H), 1.55 and 1.49 (2s, 3H); $^{13}$C NMR (100 Mz, $CDCl_3$) δ 185.6, 184.6, 146.22, 146.19, 143.4, 138.1, 137.8, 137.57, 137.51, 136.7, 136.3, 134.9, 134.0, 133.9, 133.43, 133.38, 132.3, 132.2, 130.2, 129.5, 127.89, 127.81, 126.4, 126.3, 126.2, 125.8, 125.5, 125.2, 119.29, 119.23, 45.8, 43.8, 39.76, 39.71, 26.65, 26.59, 26.1, 20.8, 19.8, 19.4, 16.53, 16.49,16.47, 15.9, 15.2, 12.8; HRMS ($M+H^+$) m/z calcd for $C_{29}H_{33}O_2$ 413.2481; found 413.2482. Data for compound **7a**: $^1$H NMR (400MHz, $CDCl_3$) δ 8.09-8.06 (m, 2H), 7.69-7.67 (m, 2H), 7.20-7.14 (m, 3H), 6.94-6.92 (m, 1H), 5.20-5.16 (m, 1H), 5.06-5.02 (m, 1H), 3.38 (d, 2H, *J*= 6.8 Hz), 3.22 (s, 2H), 2.19 (s, 3H), 2.14-2.09 (m, 2H), 2.05-2.01 (m, 2H), 1.80 (s, 3H), 1.51 (s, 3H), 1.29 (s, 9H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 185.6, 184.6, 151.0, 146.2, 143.5, 140.0, 137.6, 134.8, 133.45, 133.39, 132.3, 132.2, 127.9, 126.4, 126.3, 126.01, 125.96, 125.88, 122.8, 119.3, 46.5, 39.8, 34.6, 31.5, 26.7, 26.1, 16.5, 15.9, 12.8; HRMS ($M+Na^+$) m/z calcd for $C_{31}H_{36}O_2Na$ 463.2613; found 463.2612. Data for compound **7b**: $^1$H NMR (400MHz, $CDCl_3$) δ 8.09-8.06 (m, 2H), 7.683-7.675 (m, 2H), 7.21-7.17 (m, 3H), 6.97-6.94 (m, 1H), 5.18 (t, 1H, *J*= 6.8 Hz), 5.08-5.00 (m, 2H), 3.37 (d, 2H, *J*= 6.8 Hz), 3.24 (s, 2H), 2.19 (s, 3H), 2.10-1.94 (m, 8H), 1.80 (s, 3H), 1.57 (s, 3H), 1.50 (s, 3H), 1.30 (s, 9H); 13C NMR (100 MHz, $CDCl_3$) δ 185.5, 184.6, 151.0, 146.2, 143.4, 140.1, 137.6, 135.2, 134.5, 133.42, 133.36, 132.3, 132.2, 127.9, 126.4, 126.3, 126.0, 125.9, 124.1, 122.8, 119.2, 46.5, 39.8, 39.7, 34.6, 31.5, 26.8, 26.6, 26.1, 16.5, 16.1, 15.9, 12.8; HRMS ($M+Na^+$) m/z calcd for $C_{36}H_{44}O_2Na$ 531.3239; found 531.3244.
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* Corresponding author Tel: +81 48 720 6043.

E-mail address: suhara@shibaura-it.ac.jp (Y. Suhara).

#R.S., K.K., and Y.H. contributed equally.

## Graphical Abstract

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**Synthesis of novel vitamin K derivatives with alkylated phenyl groups introduced at the ω-terminal side chain and evaluation of their neural differentiation activities**

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