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Synthesis of 2-methyl-1,4-naphthoquinones with higher gamma-glutamyl carboxylase activity than MK-4 both *in vitro* and *in vivo*

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ABSTRACT

Vitamin K is the collective term for compounds that share a 2-methyl-1,4-naphthoquinone ring, but differ in the side-chain at the 3-position. We synthesized novel 2-methyl-1,4-naphthoquinone derivatives with different side chain length at the 3-position. Derivatives with C-14 and C-16 tails showed the highest *in vitro* bioactivity resulting in 2.5 and 2-fold higher carboxylated osteocalcin synthesis in MG63 cells than menaquinone-4 (MK-4, form of vitamin K2). Longer side chain lengths resulted in lower bioactivity. The *in vivo* vitamin K activity of the C-14 tail derivative was further tested in WKY rats receiving a vitamin K-deficient diet that resulted in a 40% decrease of prothrombin activity. The C-14 tail derivative was able to counteract the effects on vitamin K deficiency induced by the diet and resulted in the complete restoration of prothrombin activity. Compared to naturally occurring forms of vitamin K, synthetic vitamin K derivatives may have higher bioactivity and different pharmacological characteristics that are more favorable for use as supplements or in clinical settings.

Vitamin K is the common designation for compounds sharing a 2-methyl-1,4-naphthoquinone ring, but differing in side-chain structure at the 3-position.[1] Natural vitamin K exists in different forms: it is present in plants as vitamin K1 (phylloquinone, structure **1** in Fig. 1) and produced by bacteria as vitamin K2 (menaquinones, MK-n, structure **2**).[2–4]

Phylloquinone has a phytyl side-chain containing four isoprenoid residues (one unsaturated) and is mainly found in green leafy vegetables. Menaquinones have a side-chain containing multiple unsaturated isoprenoid residues (*n*), where the length of the side-chain governs important physicochemical properties, including lipophilicity[1–5] and kinetic constants for cofactor function.[6] The main sources of menaquinones are meat (MK-4, structure **3**), fermented foods like cheese (MK-8 and MK-9) and fermented soybeans known as natto (MK-7, structure **4**). Synthetic forms of vitamin K include menadione (structure **5** in Scheme 1), a water-soluble compound lacking vitamin K activity by itself but which – like phylloquinone – may be converted into active MK-4 in several tissues.[7]

Vitamin K drives the posttranslational carboxylation of glutamate (Glu) residues into γ-carboxyglutamate (Gla), which are present in the Gla-protein family, conferring them functionality.[8] Beyond their central role in blood coagulation, Gla-proteins have a diversity of regulatory functions in important physiological processes, including bone mineralization (osteocalcin (OC), synthesized by osteoblasts) and vascular calcification (matrix Gla-protein, synthesized by vascular smooth muscle cells).[9,10]

Chemical synthesis may yield entirely new compounds with vitamin K activity and pharmacological properties different from those of the natural homologues **1** and **2**, that may in turn lead to novel commercially interesting biologically active drugs. A limited number of papers have described the synthesis of vitamin K analogues and their biological activities. Suhara et al. synthesized two analogues with hydroxyl or phenyl groups at the ω-terminal of the side-chain.[11] The same group also synthesized vitamin K analogues with demethylation or reduction of the double bonds of the side-chain of menaquinone-4 (MK-4) (structure **3** in Fig. 1).[12] The authors concluded that more potent ligands may arise if new analogues are constructed with the following structural features: maintenance of the double bonds of the side-chain and substitution of one of the methyl groups with other functional groups.

In this study we provide proof-of-concept for a novel synthesis route enabling researchers to create a broad spectrum of 2-methyl-1,4-naphthoquione derivatives with high vitamin K activity. We report the synthesis of several examples of such derivatives and demonstrate their biological activity both *in vitro* and *in vivo*.

Structures of compounds referred below are indicated in Schemes 1 and 2 and detailed synthesis steps are provided in Supplementary data. Briefly, ethyl bromoacetate and pyridine

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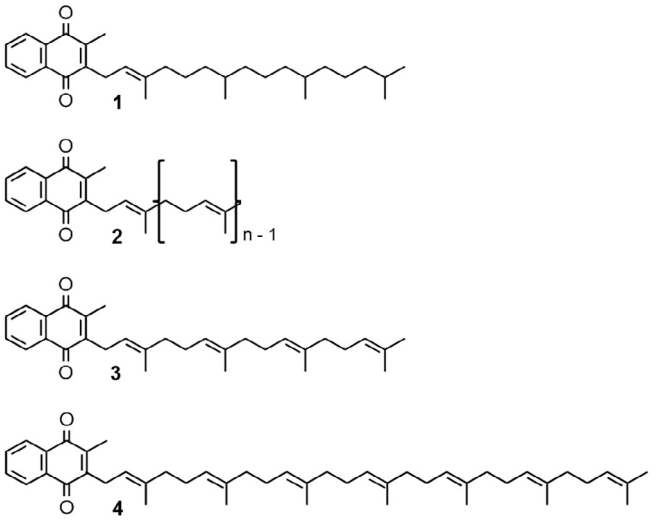


Fig. 1. Structures of natural vitamin K homologues. 1: phylloquinone (K1); 2: menaquinones (K2, general structure); 3: menaquinone-4 (MK-4); 4: menaquinone-7 (MK-7).

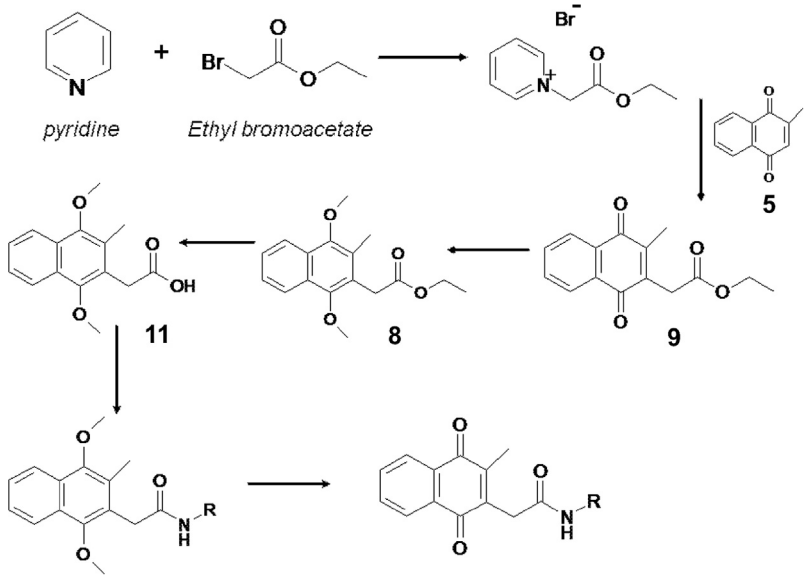
served as starting materials providing an ylide. Next, the ylide reacted with compound **5** to give compound **9**. Subsequently, compound **9** was further reduced with tetrabutylammonium iodide producing compound **8**. Then, the ester was hydrolyzed yielding compound **11**. Finally, compound **11** can react with any primary amine. Re-oxidation can follow through standard treatment with ceric ammonium nitrate. The structures of the final products were verified by NMR spectroscopy (see Supplementary data).

The bioactivity of compounds **5**–**15** was defined by their capability to serve as agonists for the endoplasmic enzyme γ-glutamyl carboxylase, and thus to promote the formation of γ-carboxy glutamate residues in proteins belonging to the Gla-protein family. We used the human osteosarcoma cell line MG63 and determined the levels of uncarboxylated (ucOC) and carboxylated (cOC) osteocalcin in the culture media. No cytotoxicity, measured as cell viability, of the vitamin K derivatives **5**–**15** was observed with concentrations up to 500 nM (data not shown). In controls, where no vitamin K or vitamin K analogue was added, no measurable amounts of cOC were produced, which is consistent with minimal vitamin K activity. As previously found[6], menadione (compound **5** in Scheme 2) was not biologically active. In line with this, **6** and its reduced form **7** showed no activity in the *in vitro* experiments. The ethylester of **7**, i.e. **8**, exhibited almost no activity (data not shown). However, as shown in Fig. 2, its oxidized form **9**, and the carboxylic acid forms **10**–**11** showed substantially reduced ucOC levels and significantly increased cOC levels compared to MK-4 (compound **3**). These data demonstrate that vitamin K bioactivity of compounds **9**–**11** was higher than that of MK-4.

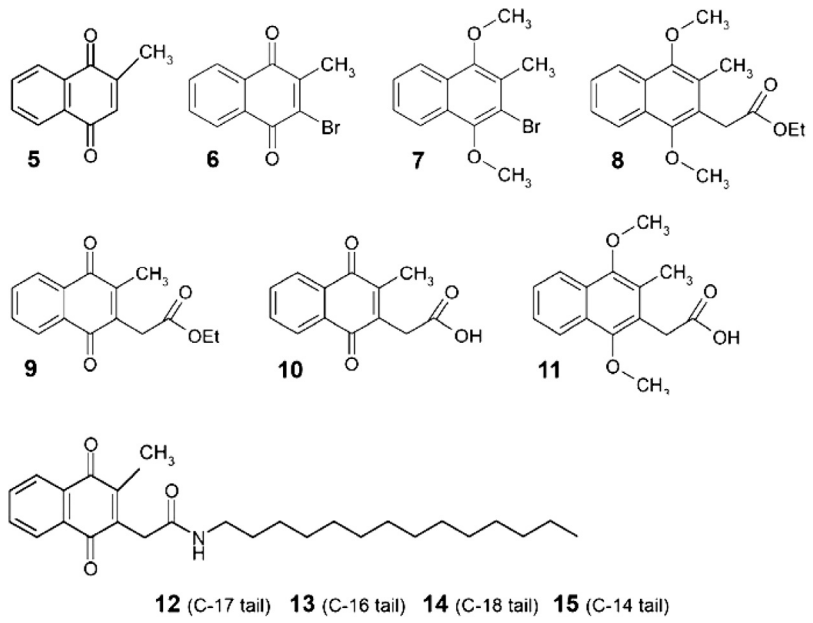
Longer side-chain derivatives may possess even higher vitamin K activity, and therefore, we developed and tested compounds **12**–**15** (depicted in Scheme 2) in an analogous follow-up experiment. Levels of ucOC in supernatants of cells were lower than controls (where no vitamin K or vitamin K analogue was added), in all compounds tested (**3** and **12**–**15**) (Fig. 3) with a stronger effect seen for compounds **13** (C-16 tail) and **15** (C-14 tail) (lines of **3** and **15** are superimposed in the graph). Comparably, compounds **13** and **15** resulted in the most efficient production of cOC, with concentrations after 72 h that were approximately 2-fold higher for compound **13** and 2.5-fold higher for compound **15**, respectively, compared to compound **3**. Cells supplemented with **12** (C-17 tail) and **14** (C-18 tail) produced minimal levels of cOC.

These results indicate that while the shortest tail (compound **15**, C-14) resulted in the highest potency with 2.5 times higher activity than MK-4 (compound **3**), more than 16 C-atoms appeared to be detrimental to activity.

Based on these results, we selected compound **15** in order to further characterize it in WKY rats. Two compound **15** enriched diets were prepared: a high-dose diet containing 5 μg of compound **15**/g of food and a low-dose diet containing 0.5 μg of compound **15**/g of food. The low-dose was selected in order to provide the minimal amount of vitamin K required for normal coagulation activity in rats. At baseline (after one-week of vitamin K-deficient diet (VKDD) feeding), four rats were sacrificed (t = 0; baseline, Fig. 4). The remaining 20 rats were randomly assigned to either receive the VKDD alone for 2 or 5 days (controls, n = 8), the low-dose compound **15** diet for 5 days (n = 4) or the high-dose compound **15** diet for either 2 or 5 days (n = 8). Rats were sacrificed after two or five days to study short-term or long-term effects, respectively. Body weights were determined before sacrifice and



Scheme 1. Schematic representation of the steps for synthesis of the 2-methyl-1,4-naphthoquinone derivatives.



Scheme 2. Structures of novel synthesized 2-methyl-1,4-naphthoquinone derivatives with different side-chains at the 3-position. The lower structure shows compound 15 (the C-14 tailed derivative), while compounds 12, 13 and 14 refer to structures with a C-17, a C-16 and a C-18 tail, respectively.

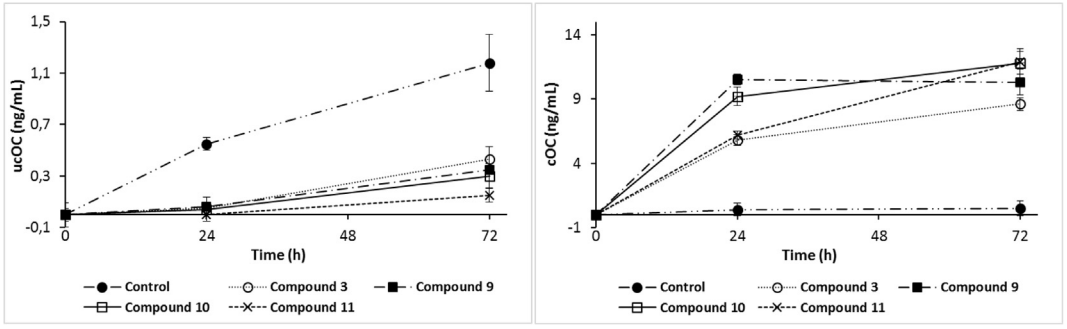


Fig. 2. Effect of vitamin K derivatives on the synthesis of uncarboxylated (ucOC) and carboxylated (cOC) osteocalcin by human MG63 cells. In a first experiment, the effect of compounds **3** (MK-4) and **9**–**11** on the synthesis of ucOC (left) and cOC (right) osteocalcin was studied. Experiments were performed in triplicates.

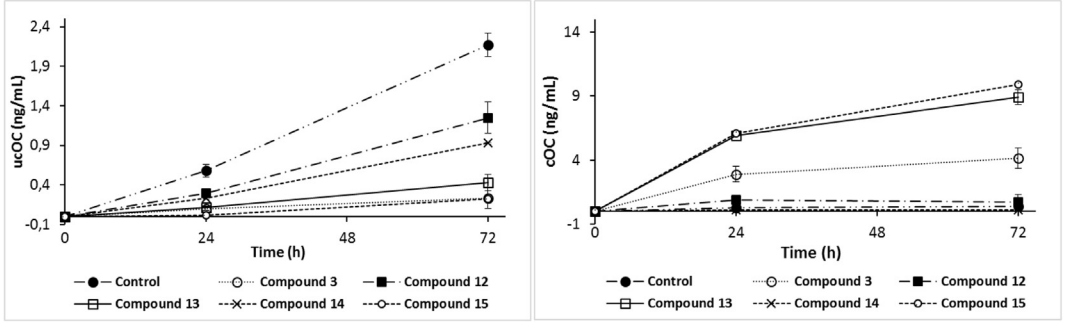


Fig. 3. The effect of compounds **3** and **12**–**15** on the production of uncarboxylated (ucOC) (left) and carboxylated (cOC) (right) osteocalcin was investigated. Experiments were performed in triplicates.

no substantial differences were observed between the different treatments (data not shown).

Vitamin K bioactivity was determined by assessing circulating levels of prothrombin, since vitamin K is known to promote prothrombin biosynthesis is the rat model system.[13] In male WKY rats, plasma prothrombin levels decreased by approximately 40% after one-week of VKDD feeding (Table 1; baseline). Supplementing the low-dose compound **15** diet for 5 days, increased plasma prothrombin levels back to normal. Therefore, even at the lowest dose equaling the minimal dose of vitamin K necessary for normal blood coagulation, **15** was able to quickly restore prothrombin activity. These results indicate that **15** may serve as a cofactor for γ-carboxylation of the vitamin K-dependent coagulation factors synthesized in the liver. Administration of the high-dose compound **15**

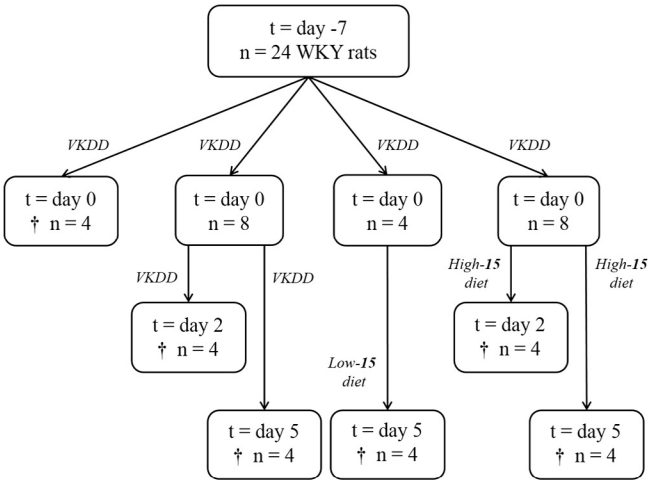


Fig. 4. Study design of a rat study for the determination of the *in vivo* biological activity of derivative **15**.

Table 1
Plasma prothrombin levels. Values are medians with ranges. Values are expressed in % as compared to normal plasma pooled from approximately 30 rats and that has been set to 100%.

Day-7 (Ref)			Day 0 (baseline)	Day 2	Day 5
99 (95–102)	*VKDD*	–	60 (57–62)	67 (64–69)	53 (50–56)
	VKDD	*Low-15 diet*		–	103 (100–105)
	VKDD	*High-15 diet*		107 (106–108)	106 (104–108)

diet for only 2 days, also increased plasma prothrombin back to normal levels. Supplementation for 3 extra days with the high-dose diet had no additional effects. Female rats did not reach a vitamin K-deficient state (no change in plasma prothrombin levels) after one week of feeding the VKDD (results not shown). This is consistent with previous observations that estrogen can cause – when vitamin K is low – a more rapid and efficient production of prothrombin[14], and this may be the explanation for the lack of effect in female rats.

In conclusion, in this study we report the synthesis of novel 2-methyl-1,4-naphthoquinone derivatives and demonstrate their biological activity as carboxylase agonists. Most of the vitamin K derivatives possessed vitamin K activity and this differed depending on the structural characteristics of the side-chain: while compounds **13** (C-16 tail) and **15** (C-14 tail) showed strong bioactivity, compounds **12** (C-17 tail) and **14** (C-18 tail) showed only weak activity.

The derivative **15** is a potent carboxylase agonist that may be useful in uncovering unknown or novel biological roles of vitamin K and that may be commercially interesting as a new biologically active drug. The data presented here are, however, only proof-of-principle and further studies are required to compare the activity of the compound **15** or other vitamin K analogues to the natural vitamin K homologues like vitamin K1, MK-4 and MK-7 in *in vitro* and *in vivo* models.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.11.073.

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