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Synthesis and development of biologically active fluorescent-labeled vitamin K analogues and monitoring of their subcellular distribution

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ABSTRACT

New fluorescent analogues of menaquinone-4 and phylloquinone were prepared and their subcellular distribution monitored using a confocal laser scanning microscope. These analogues incorporate an FITC group anchored to the naphthoquinone skeleton through an amide bond expected to be resistant to metabolism. On their addition to the culture medium, fluorescence was readily observed inside a human osteosarcoma cell line. This result indicates that the fluorescent analogues penetrate into cells the same as vitamin K, and therefore, would be useful for achieving insight into the action mechanism of vitamin K.

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1. Introduction

Vitamin K is an essential nutrient and 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).[1] Vitamin K is a cofactor for γ-glutamyl carboxylase, which is required for blood clotting and also activates osteocalcin related to the formation of bone.[2] Among its homologues, menaquinone-4 (MK-4) up-regulates the expression of bone markers, increases bone density in vivo, and is used clinically in the management of osteoporosis.[3] The mechanism of action of MK-4 in bone formation was thought to involve its normal role as an essential cofactor for γ-carboxylation of bone matrix proteins, however, it has been clarified that MK-4 possesses post-transcriptional activity according to binding to the orphan nuclear receptor SXR. The treatment of osteosarcoma cells with MK-4 increased mRNA levels for the osteoblast markers' bone alkaline phosphatase, osteoprotegerin, osteopontin, and matrix Gla protein.[4] Meanwhile, it was also revealed that vitamin K plays a role in preventing oxidative damage to developing oligodendrocytes and neurons in the brain.[5] Against this background, we have reported a procedure for measuring vitamin K homologues in human plasma[6] and the uptake, metabolism, and utilization of vitamin K in cultured human cell lines,[7] however, the cellular pharmacokinetics of vitamin K after its absorption is not well understood. It is quite important to observe the uptake and distribution of vitamin K analogues to clarify the action mechanism of vitamin K. Although one possible approach is to use an isotope-labeled vitamin K analogue, the sensitivity and measurement time would be problematic. To solve these problems and gain better insight into the mechanism of vitamin K's action, we have developed the first pharmacologically relevant fluorescent-labeled vitamin K analogues.

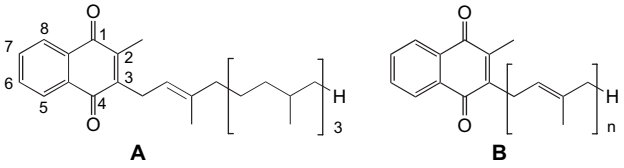

Figure 1. Structure of vitamin K homologues: phylloquinone (**A**) and menaquinones (**B**).

2. Results and discussion

We first tried to observe the intracellular distribution of the analogues using a fluorescence microscope since vitamin K exhibits fluorescence generated from its naphthoquinone ring under UV

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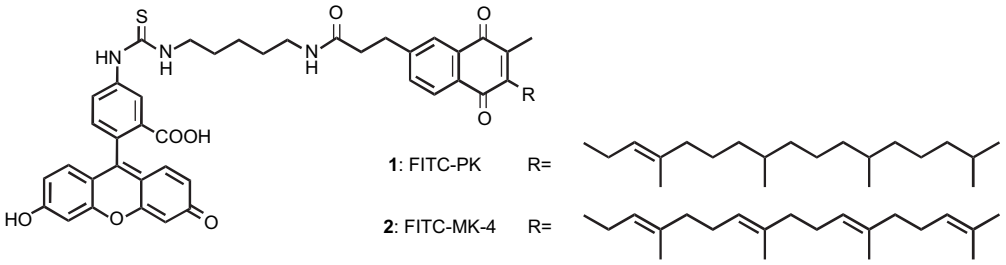


Figure 2. FITC-labeled vitamin K analogues: FITC–PK (**1**) and FITC–MK-4 (**2**).

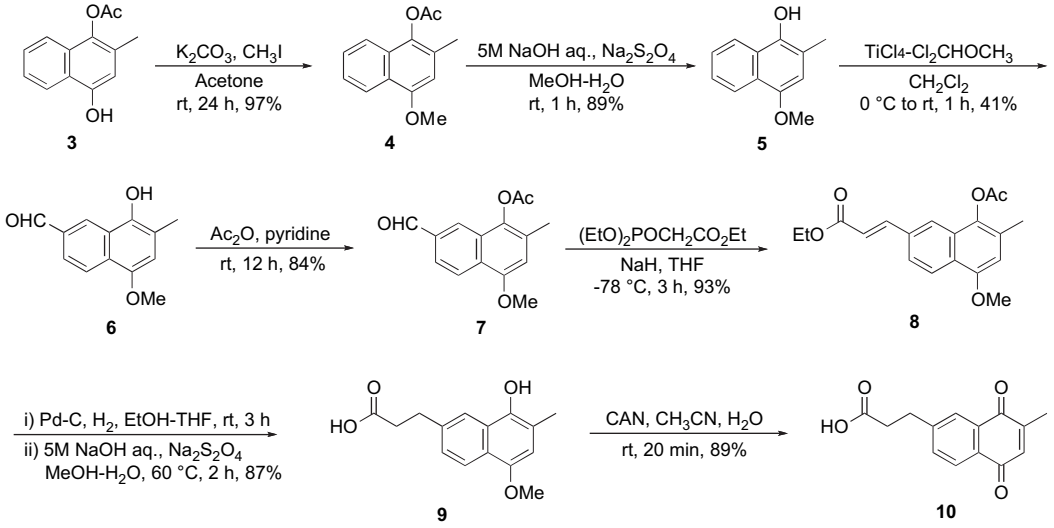
light, however, the fluorescence was too weak to detect even in a high-density solution. Furthermore, it sometimes overlapped with the 'native' fluorescence of the cells.[8] Therefore, we designed and synthesized new fluorescent-labeled analogues of vitamin K.

Regarding the introduction of a fluorescent substance, we proposed to introduce a small luminescent material such as BODIPY (borondipyrromethene difluoride, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) or FITC (fluorescein isothiocyanate) into the vitamin K molecule because the fluorescence of such compounds can be distinguished from that native to the cells. Various biologically active fluorescent-labeled analogues have been reported to date. For example, Barsony et al. synthesized BODIPY-labeled vitamin D analogues[9] and Kim et al. synthesized fluorecent isoprenoid pyorophosphate analogues to observe their distribution inside human cell lines.[10] In our case, the issue was which position in the vitamin K molecule is the most appropriate to link to the fluorescent substance. According to some findings, the side chain is not suitable because it is important for biological action. For example, some reports revealed that the level of γ-glutamyl carboxylase (GGCX) activity of vitamin K homologues depends on the side-chain length, in short, MK-4 had the most potent activity among homologues.[11] Therefore, we decided to modify the C6-position of the naphthoquinone ring moiety, which is farthest from the side chain, to avoid influencing the biological activities of vitamin K. Then, the vitamin K analogue and fluorescent substance were linked via an amide bond as shown in Figure 2. We chose a linker 'C5–NHCO–C2' between fluorescein and naphthoquinone. The length of the linker would be appropriate for compounds because it was reported that the biotynylated vitamin K, which had the same linker, was used for characterization of vitamin K-binding proteins by pull-down experiment.[12] It means the linker would not affect the activity and cellular localization of compounds. We report here the synthesis and comparative localization of fluorescent-labeled vitamin K analogues in cells.

To introduce a fluorescent-label into vitamin K analogues such as PK and MK-4, we first synthesized an intermediate, which had ethyl carboxylic acid at position C-7 of the naphthoquinone ring moiety. Then, the combining of FITC with PK or MK-4 via an amide linkage gave the desired FITC–PK and FITC–MK-4.

The method used is shown in Scheme 1. We chose VK_4 monoacetate (**3**) as a starting material to obtain intermediates **12** and **13**.[13] To introduce a functional group at C-7 of the naphthoquinone ring, compound **3** was converted to phenol **5**[14] with methylation of the hydroxyl group at C-4 of **3** followed by deprotection of the acetyl group of **4** in a conventional manner. Then, compound **5** was treated with Cl_2CHOCH_3 and $TiCl_4$ in CH_2Cl_2 to give aldehyde **6** in 41% yield. After acetylation of the hydroxyl group of **6**, elongation of the alkyl chain at C-7 in **7** with the Wittig reaction gave carboxylate **8** in 93% yield. Reduction of unsaturated bonds in **8** with palladium/carbon and H_2 followed by alkaline hydrolysis afforded alcohol **9** in 87% yield. Deprotection of methyl groups with ceric ammonium nitrate gave quinone **10** in good yield.

After the reduction of **10** with sodium hydrosulfite in Et_2O and water to form hydroquinone **11**, phytol and geranylgeraniol were successively coupled with **11** in the presence of a catalytic amount of $BF_3{\cdot}Et_2O$ before hydroquinone **11** was oxidized to quinone **10** under atmospheric condition. Thus, the intermediates **12** and **13** were successively obtained in 35% and 37% yields. To couple **12** with FITC through an amide linkage, **12** and FITC cadaverine were treated with BOP reagent[15] and *N*,*N*-diisopropylethylamine in DMF as shown in Scheme 2. Although the chemical yields were approximately 30% because of steric hindrance, the desired compound **1** or **2** was obtained.



Scheme 1. Synthesis of intermediate **10** for vitamin K analogues.

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