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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.



## 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.

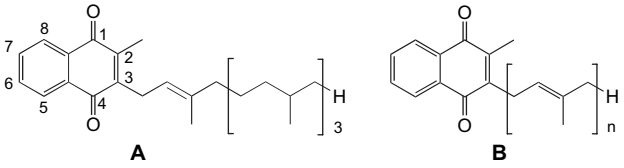


**Figure 1.** Structure of vitamin K homologues: phylloquinone (**A**) and menaquinones (**B**).

## 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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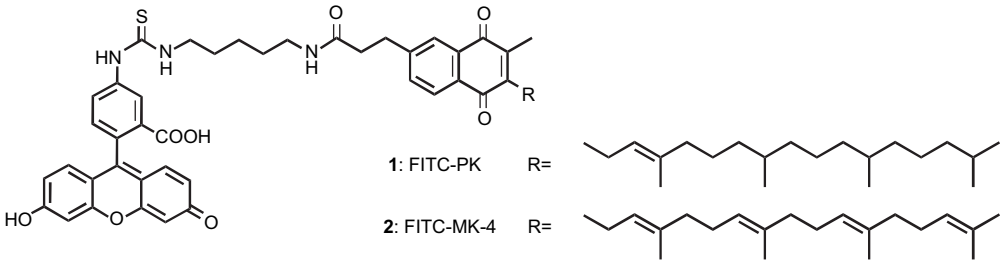



**Figure 2.** FITC-labeled vitamin K analogues: FITC–PK (**1**) and FITC–MK-4 (**2**).

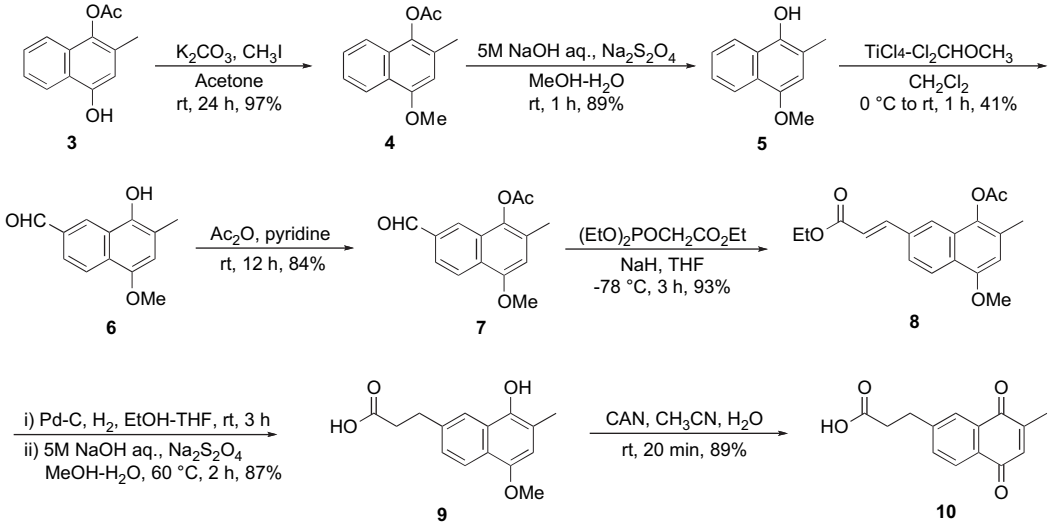
light, however, the fluorescence was too weak to detect even in a high-density solution. Furthermore, it sometimes overlapped with the 'native' fluorescence of the cells.[8] Therefore, we designed and synthesized new fluorescent-labeled analogues of vitamin K.

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.

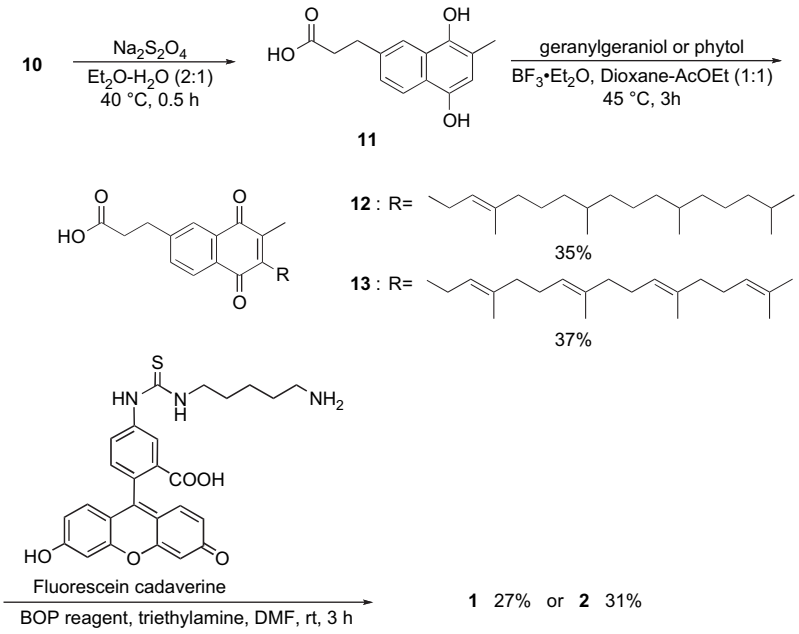
To introduce a fluorescent-label into vitamin K analogues such as PK and MK-4, we first synthesized an intermediate, which had ethyl carboxylic acid at position C-7 of the naphthoquinone ring moiety. Then, the combining of FITC with PK or MK-4 via an amide linkage gave the desired FITC–PK and FITC–MK-4.

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.




**Scheme 2.** Synthesis of FITC–PK (**1**) and FITC–MK-4 (**2**).

We investigated the coenzyme activity for GGCX according to a reported method.[16] The biological activity increased in a dose-dependent manner, the same as vitamin K. This result means that our fluorescent-labeled analogues would retain the nature of vitamin K and function as vitamin K in cells.

Next we observed the intracellular localization of the vitamin K analogues using osteosarcoma MG-63 cells. Our method of sample preparation was as follows. After the addition of fluorescent-labeled analogues to culture medium of the cells, the cells were fixed on slide glass to make samples for the time-course experiment. Then, the samples were subjected to confocal laser scanning microscopy. We first treated MG-63 cells with fluorescent-labeled PK (FITC–PK), and monitored the subcellular distribution using a confocal laser scanning microscopy with appropriate excitation lines and emission filters for fluorescein.

To find the appropriate concentration of fluorescent-labeled analogues to add to the cells, several solutions (100 nM, 500 nM, 1 μM, 5 μM and 10 μM) were prepared. The fluorescent intensity in cells was then compared. It increased in a time-dependent manner and the clearest image was obtained with the 1 μM solution. Therefore, we used the 1 μM solution to observe the intracellular localization of vitamin K analogues.

We observed the comparative uptake of FITC cadaverine and FITC–PK for 24 h. It was found that FITC cadaverine itself was not absorbed by the cells, however, FITC–PK was gradually taken up (Fig. 3). This result indicated that the FITC-label did not affect the uptake of the parental vitamin K.

Then, we conducted competitive assays of FITC–PK with PK and FITC–MK-4 with MK-4 for uptake into cells. The concentration of each compound was 1 μM, however, no remarkable inhibition was observed for 24 h (data not shown). Although we need to add a large amount of vitamin K analogue to cells in this assay, we could not elucidate the conditions because a high concentration such as 10 μM induced cell cycle arrest and led to apoptosis.[17]

We then examined in which part of the cells the FITC analogue accumulated using ER-Tracker Red, which fluorescently and specifically stains the endoplasmic reticulum and Golgi apparatus. On addition of the analogue to the culture medium of MG-63 cells and incubation for 24 h, as shown in Figure 4, the area of fluorescence and part of the endoplasmic reticulum completely matched when the photographic images were merged. Therefore, it was suggested that PK accumulated in the endoplasmic reticulum after it was absorbed.

To compare the intracellular distribution of FITC–PK and FITC–MK-4, we added them simultaneously to the culture medium of MG-63 cells. Figure 5 shows that FITC–MK-4 was absorbed and accumulated faster than FITC–PK. We previously investigated the time course of the uptake of $^{18}O$-labeled vitamin K derivatives in MG-63 cells.[7] In that study, the total amount of MK-4 derivatives was greatest at 12 h, whereas that of PK derivatives kept increasing for 48 h. These results suggested that our synthesized FITC–PK and FITC–MK-4 retained the nature of vitamin K homologues. A more detailed examination is underway based on these findings.

## 3. Conclusions

In summary, we developed new fluorescent-labeled vitamin K analogues, FITC–PK and FITC–MK-4, and examined their distribution in living cells. Compounds would be useful not only at the cell level but also at the tissue level to evaluate still unknown biological mechanisms of vitamin K analogues.

## 4. Experimental

### 4.1. General

$^1$H NMR spectra were recorded at 500 MHz and $^{13}$C NMR spectra were recorded at 125 MHz using $CDCl_3$ as a solvent unless otherwise specified. Chemical shifts are given in parts per million ($\delta$) using tetramethylsilane (TMS) as the internal standard. MS data were obtained by EI with low-resolution MS spectra (EIMS) and high-resolution MS spectra (HREIMS). Melting points were determined with a micro-melting point apparatus. IR spectra were recorded on an FT-IR Spectrometer. Column chromatography was carried out on silica gel 60 (70–230 mesh) and preparative thin layer chromatography (TLC) was run on silica gel 60$F_{254}$. Unless otherwise noted, all reagents were purchased from commercial suppliers and used as-received. Nomenclature of organic compounds was based on vitamin K structure.

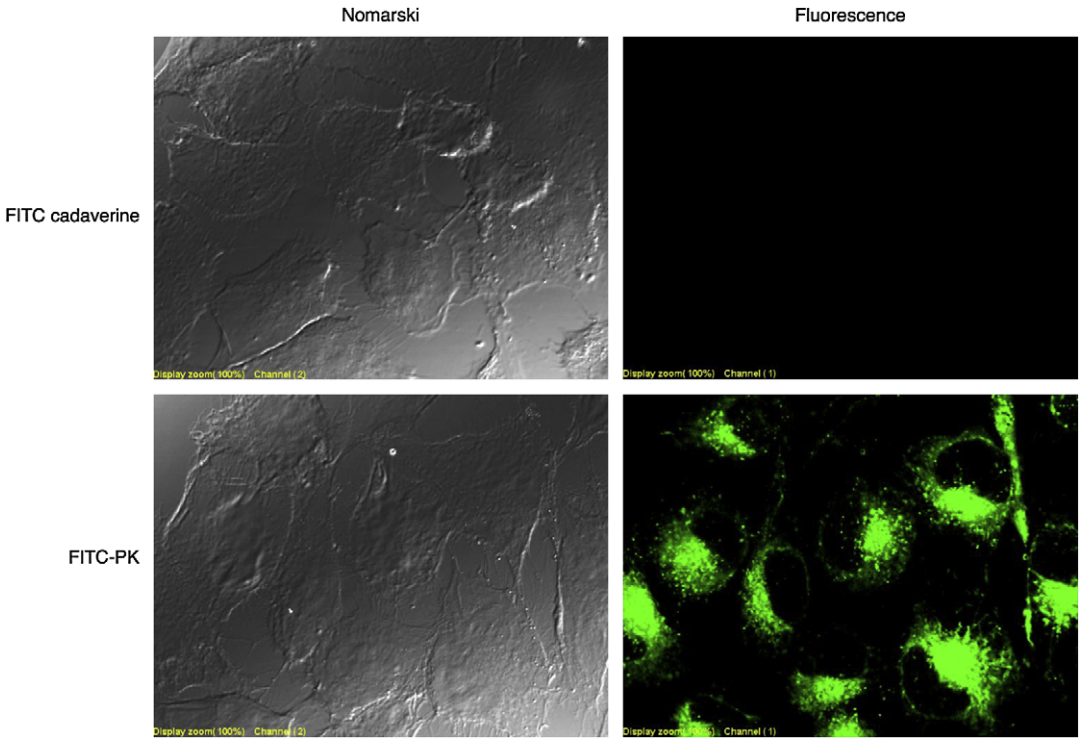



**Figure 3.** Comparative uptake of FITC cadaverine and FITC–PK in MG-63 cells. Cells were incubated for 24 h with vehicle (1 μM FITC cadaverine) or with 1 μM FITC–PK. Removal of the culture medium and washing of cells with phosphate-buffered saline (PBS) three times were followed by fixation on the glass slide using 4% formaldehyde for 2 min at 37 °C. The cover glass was removed from the six-well plate and cells were fixed onto a glass slide using antifade reagent. Finally, the samples were subjected to confocal laser scanning microscopy with appropriate excitation lines and emission filters for fluorescein. FITC–PK, but not FITC cadaverine, was retained by the cells.

## 4.2. Synthesis of fluorescent-labeled analogues 1 and 2

### *4.2.1. 1-Acetoxy-4-methoxy-2-methylnaphthalene (**4**)*

To a suspension of 2-methyl-1,4-naphthalenediol, 1-acetate (**3**) (5.5 g, 25.4 mmol) and potassium carbonate (7.0 g, 50.6 mmol) in acetone (30 mL) was added methyl iodide (4.0 mL, 64.3 mmol), and the mixture was stirred at room temperature for 24 h under argon. The reaction mixture was diluted with $Et_2O$ (100 mL) and filtered through silica gel. After addition of $Et_2O$ (200 mL), the organic layer was washed with water (100 mL) and brine, dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography on silica gel (hexane/AcOEt=10:1 to 5:1) to give **4** (5.7 g, 97% yield) as a colorless oil: IR ($CHCl_3$) 3072, 3022, 2940, 2858, 1752, 1636, 1601 $cm^{-1}$; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 2.34 (3H, s),

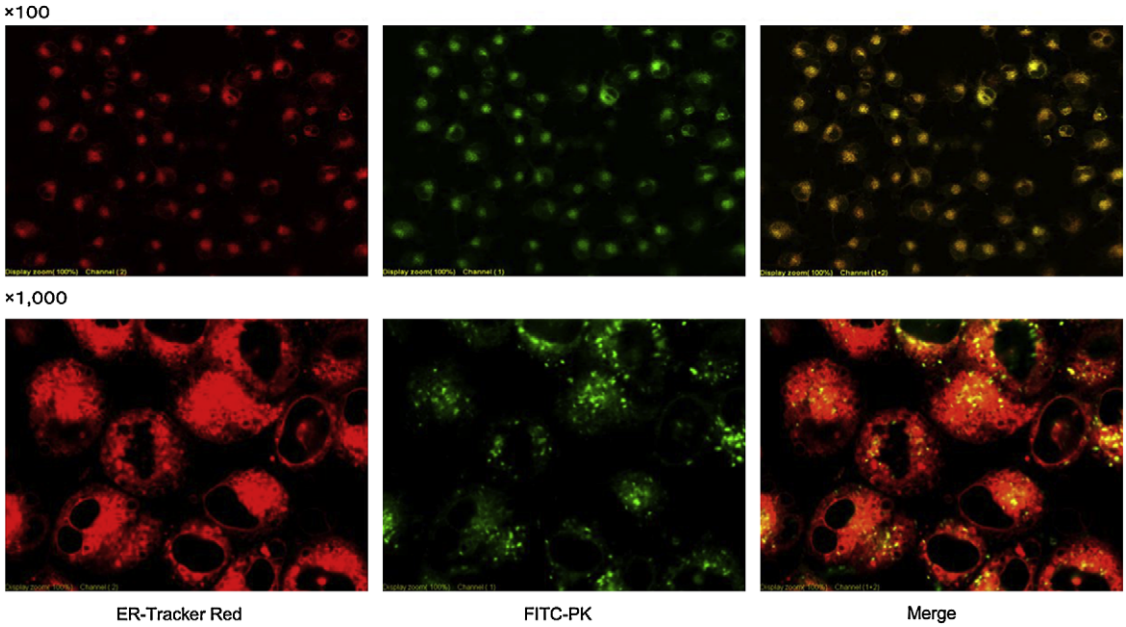



**Figure 4.** Subcellular distribution of FITC–PK in MG-63 cells. Cells were exposed to 1 μM FITC–PK for 24 h and 100 nM ER-Tracker™ Red for 30 min. The red signal shows ER and Golgi membranes, green signal shows the distribution of FITC-PK, and yellow signal shows the colocalization of the two signals. Pictures were taken of fixed cells using a confocal laser scanning microscopy with appropriate excitation lines and emission filters for rhodamine or for fluorescein. Merging of digitized image files was done by Fluoview software.

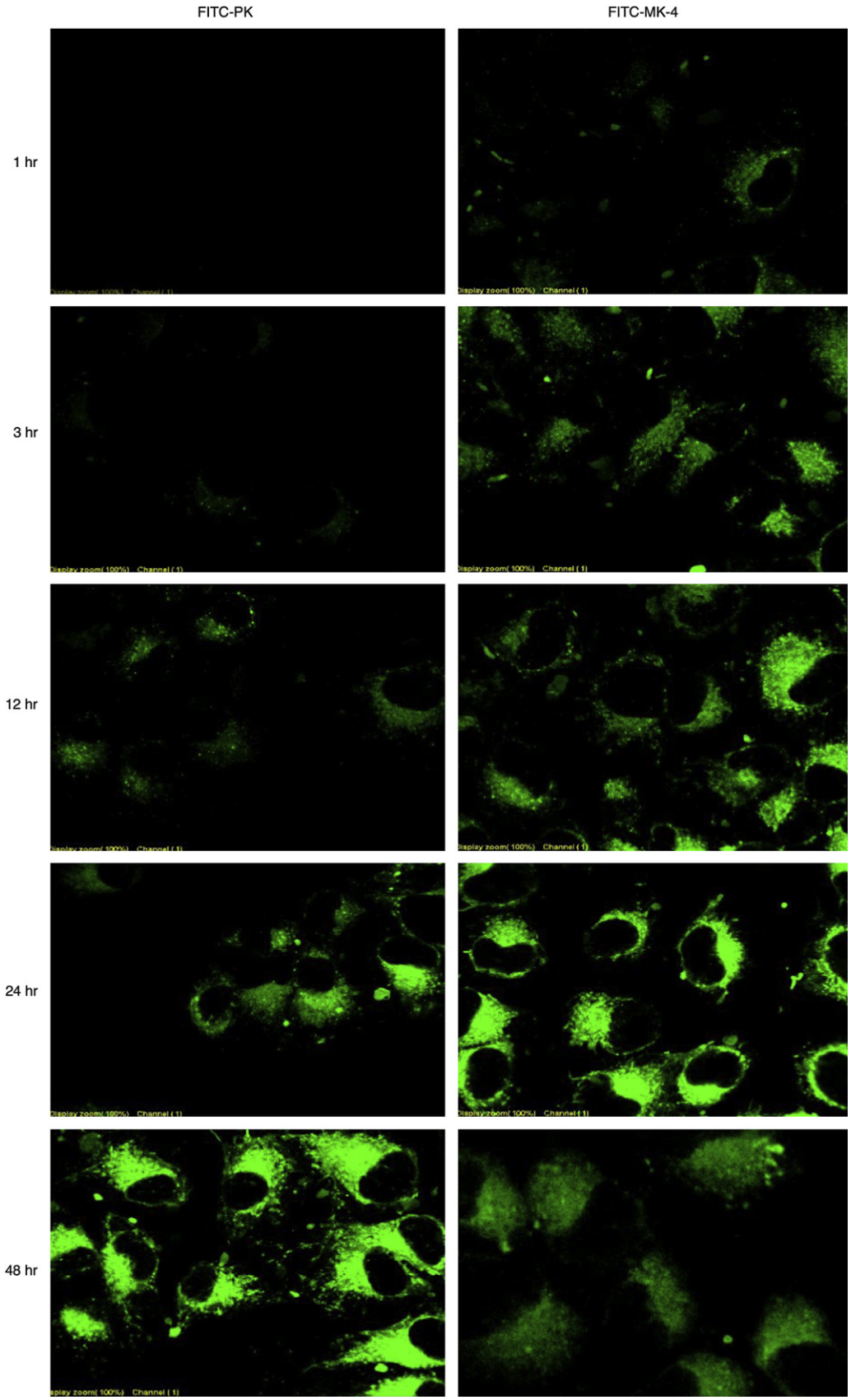



**Figure 5.** Comparative uptake of FITC–PK or FITC–MK-4 in MG-63 cells with time; 1 μM of fluorescent-labeled vitamin K analogue was added to the culture medium. Removal of the culture medium and washing of the cells with PBS three times were followed by fixation on the glass slide using 4% formaldehyde for 2 min at 37 °C in a time-dependent manner (3–48 h). After fixation of the cells, the samples were subjected to confocal laser scanning microscopy with appropriate excitation lines and emission filters for fluorescein.

2.47 (3H, s), 3.97 (3H, s), 6.62 (1H, s), 7.47 (1H, dd, *J*=7.0, 8.5 Hz), 7.53 (1H, dd, *J*=7.0, 8.5 Hz), 7.72 (1H, d, *J*=8.5 Hz), 8.27 (1H, d, *J*=8.5 Hz); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 16.7, 20.4, 55.5, 106.1, 120.3, 122.2, 124.7, 124.8, 126.1, 127.0, 127.6, 137.6, 153.1, 169.3; EI-LRMS *m*/*z* 230 ($M^+$), 188, 173. EI-HRMS calcd for $C_{14}H_{14}O_3$ 230.0942; found 230.0941.

#### 4.2.2. 4-Methoxy-2-methyl-1-naphthalenol (**5**)

To a suspension of **4** (12.0 g, 52.1 mmol) and $Na_2S_2O_4$ (1.0 g) in MeOH (200 mL) and water (20 mL) was added 5 M NaOH aqueous solution (14 mL), and the mixture was stirred at room temperature for 1 h. The reaction mixture was poured into ice-water (300 mL) and then 5 M HCl (30 mL) was added to the solution. The resulting solution was extracted with AcOEt (200 mL×3) and the combined organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography on silica gel (hexane/AcOEt=10:1 to 5:1) and recrystallized from hexane to afford **5** (9.3 g, 89% yield) as white needles. It was reported that this half-ether compound was oxygen-sensitive, therefore, we could not obtain the elemental analysis: IR ($CHCl_3$) 3388, 3029, 3015, 2940, 1635, 1599, 1464 cm$^{-1}$; mp 99–100 °C; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 2.40 (3H, s), 3.95 (3H, s), 4.67 (1H, s), 6.58 (1H, s), 7.41–7.45 (1H, m), 7.48–7.51 (1H, m), 8.06 (1H, d, *J*=8.5 Hz), 8.17 (1H, d, *J*=8.5 Hz); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 16.2, 55.8, 107.0, 116.2, 120.7, 121.9, 124.7, 124.9, 125.4, 126.0, 142.0, 149.3; EI-LRMS *m*/*z* 188 ($M^+$), 173, 105. EI-HRMS calcd for $C_{12}H_{12}O_2$ 188.0836; found 188.0834.

#### 4.2.3. 7-Formyl-4-methoxy-2-methyl-1-naphthalenol (**6**)

To a solution of **5** (3.0 g, 15.9 mmol) in $CH_2Cl_2$ (20 mL) was added dropwise $TiCl_4$ (2.6 mL, 23.7 mmol) at 0 °C for 10 min and stirred for 30 min. Addition of $Cl_2CHOCH_3$ (1.15 mL, 12.7 mmol) was followed by stirring with a mechanical stirrer at room temperature for 1 h. The excess $TiCl_4$ was destroyed by the addition of water (100 mL) with cooling, and the mixture was stirred for 10 min and extracted with AcOEt (100 mL×3). The extracts were washed with water and brine, dried over $MgSO_4$, and concentrated. The residue was chromatographed on silica gel (hexane/AcOEt=10:1 to 5:1) and recrystallized from hexane and AcOEt to afford **6** (1.4 g, 41% yield) as yellow needles: IR ($CHCl_3$) 3380, 3030, 2939, 2828, 1693, 1629, 1602, 1464 cm$^{-1}$; mp 174–175 °C; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 2.45 (3H, s), 4.00 (3H, s), 4.74 (1H, s), 6.68 (1H, s), 7.96 (1H, dd, *J*=1.5, 8.5 Hz), 8.17 (1H, d, *J*=8.5 Hz), 8.69 (1H, d, *J*=1.0 Hz), 10.14 (1H, d, *J*=0.5 Hz); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 16.6, 55.8, 108.0, 121.0, 122.1, 122.7, 124.1, 127.9, 129.5, 132.9, 142.3, 150.4, 192.5; EI-LRMS *m*/*z* 216 ($M^+$), 201. EI-HRMS calcd for $C_{13}H_{12}O_3$ 216.0786; found 216.0801. Elemental Anal. Calcd for $C_{13}H_{12}O_3$: C, 72.21; H, 5.59. Found: C, 72.19; H, 5.43.

#### 4.2.4. 7-Formyl-4-methoxy-2-methyl-1-naphthyl acetate (**7**)

To a cooled solution of **6** (1.5 g, 6.9 mmol) in pyridine (10 mL) was added acetic anhydride (5 mL), and the mixture was stirred at room temperature for 12 h. The reaction mixture was diluted with AcOEt (100 mL), and was successively washed with saturated $CuSO_4$ aqueous solution, water, and brine. The organic layer was dried over $MgSO_4$ and concentrated. The residue was chromatographed on silica gel (hexane/AcOEt=10:1 to 5:1), and recrystallized from hexane and AcOEt to afford **7** (1.5 g, 84% yield) as white needles: IR ($CHCl_3$) 3025, 2966, 2960, 2825, 1757, 1697, 1632, 1606 cm$^{-1}$; mp 148–150 °C; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 2.36 (3H, s), 2.48 (3H, s), 4.04 (3H, s), 6.75 (1H, s), 7.75 (1H, d, *J*=9.0 Hz), 7.98 (1H, dd, *J*=2.0, 9.0 Hz), 8.72 (1H, d, *J*=1.0 Hz), 10.13 (1H, d, *J*=0.5 Hz); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 17.3, 20.6, 55.8, 107.6, 121.7, 123.9, 124.1, 129.6, 130.5, 131.0, 133.0, 137.7, 154.2, 169.3, 192.1; EI-LRMS *m*/*z* 258 ($M^+$), 216, 201. EI-HRMS calcd for $C_{15}H_{14}O_4$ 258.0891; found 258.0902. Anal. Calcd for $C_{15}H_{14}O_4$: C, 69.76; H, 5.46. Found: C, 69.19; H, 5.83.

#### 4.2.5. (E)-Ethyl 3-(1-acetoxy-4-methoxy-2-methylnaphthalen-7-yl)acrylate (**8**)

To a stirred solution of 70% NaH in oil (400 mg, 11.6 mmol) in THF (40 mL) was added ethyl diethylphosphonoacetate (5.1 g, 22.8 mmol) at −78 °C under argon, and the mixture was stirred at −78 °C for 1 h. After addition of **7** (2.0 g, 7.7 mmol) in THF (5 mL), the solution was stirred at −78 °C for 3 h and then warmed to room temperature. The reaction mixture was poured into ice-water and extracted with AcOEt (100 mL×3). The combined organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated. The residue was chromatographed on silica gel (hexane/AcOEt=10:1 to 5:1) and recrystallized from hexane and AcOEt to give **8** (472 mg, 93% yield) as white powder: IR ($CHCl_3$) 3018, 1755, 1703, 1630, 1578, 1463 cm$^{-1}$; mp 141–144 °C; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 1.35 (3H, t, *J*=7.0 Hz), 2.32 (3H, s), 2.47 (3H, s), 4.00 (3H, s), 4.28 (2H, dd, *J*=7.0, 14.0 Hz), 6.53 (1H, d, *J*=16.0 Hz), 6.67 (1H, s), 7.64–7.68 (2H, m), 7.84 (1H, d, *J*=16.0 Hz), 8.33 (1H, s); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 14.3, 17.0, 20.6, 55.7, 60.5, 107.2, 118.1, 121.3, 124.3, 124.7, 125.0, 128.2, 128.3, 130.9, 137.7, 144.7, 153.6, 167.1, 169.4; EI-LRMS *m*/*z* 328 ($M^+$), 286. EI-HRMS calcd for $C_{19}H_{20}O_5$ 328.1309; found 328.1319.

#### 4.2.6. 3-(1-Hydroxy-4-methoxy-2-methylnaphthalen-7-yl)propanoic acid (**9**)

Compound **8** (450 mg, 1.37 mmol) was hydrogenated over 10% Pd/C (40 mg) in THF (8 mL)/EtOH (2 mL) at room temperature for 3 h. The reaction mixture was filtered through a Celite pad and the filtrate was concentrated. Then, the resin and $Na_2S_2O_4$ (500 mg) were suspended in MeOH (10 mL), and then added to 5 M NaOH aqueous solution (1.1 mL). The mixture was stirred at 60 °C for 2 h under argon. After further addition of water (1 mL) and 5 M NaOH aqueous solution (0.2 mL), the mixture was stirred at 60 °C for 30 min. The suspension was poured into ice-water and neutralized with 5 M HCl aqueous solution (1.5 mL). The solution was extracted with AcOEt (50 mL×3), and the combined organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH=10:1 to 3:1) to give **9** (275 mg, 87% yield) as purple powder: IR (KBr) 3234, 1695, 1604 cm$^{-1}$; mp 144–148 °C; $^1$H NMR (500 MHz, $CDCl_3$/$CD_3OD$) $\delta$ 2.40 (3H, d, *J*=1.0 Hz), 2.71 (2H, t, *J*=8.0 Hz), 3.10 (2H, t, *J*=8.0 Hz), 3.94 (3H, d, *J*=1.0 Hz), 6.59 (1H, s), 7.34–7.36 (1H, m), 7.97 (1H, s), 8.05 (1H, d, *J*=9.0 Hz); $^{13}$C NMR (125 MHz, $CDCl_3$/$CD_3OD$) $\delta$ 16.0, 31.0, 35.6, 55.5, 107.6, 117.3, 120.1, 121.4, 124.8, 125.0, 126.5, 136.5, 142.4, 148.6, 175.5; EI-LRMS *m*/*z* 260 ($M^+$), 245, 199. EI-HRMS calcd for $C_{15}H_{16}O_4$ 260.1047; found 260.1055.

#### 4.2.7. 3-(2-methyl-1,4-naphthoquinon-7-yl)propanoic acid (**10**)

To a solution of **9** (323 mg, 1.24 mmol) in $CH_3CN$ (5 mL)/ether (1 mL) was added ceric ammonium nitrate (CAN) (2.0 g, 3.65 mmol) in water (1 mL) and extra $CH_3CN$ (3 mL), and the mixture was stirred at room temperature for 20 min. After the reaction mixture was diluted with ether (10 mL), it was washed with water and brine, dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH=10:1 to 5:1) to afford **10** (271 mg, 89% yield) as yellow powder: IR (KBr) 3423, 1709, 1660, 1599 cm$^{-1}$; mp 200–203 °C; $^1$H NMR (500 MHz, $CDCl_3$/$CD_3OD$) $\delta$ 2.19 (3H, d, *J*=1.5 Hz), 2.69 (2H, t, *J*=8.0 Hz), 3.08 (2H, t, *J*=8.0 Hz), 6.82 (1H, dd, *J*=1.5, 3.0 Hz), 7.60 (1H, dd, *J*=1.5, 8.5 Hz), 7.90 (1H, d, *J*=1.5 Hz), 8.02 (1H, d, *J*=8.5 Hz); $^{13}$C NMR (125 MHz, $CDCl_3$/$CD_3OD$) $\delta$ 16.2, 30.8, 34.6, 125.6, 126.8, 130.4, 132.2, 133.7, 135.4, 147.4, 148.3, 174.5, 185.3, 185.4; EI-LRMS

*m*/*z* 244 ($M^+$), 199. EI-HRMS calcd for $C_{14}H_{12}O_4$ 244.0735; found 244.0746.

#### 4.2.8. 3-(2-Methyl-3-phytyl-1,4-naphthoquinon-7-yl)propanoic acid (**12**)

To a solution of **10** (100 mg, 0.41 mmol) in ether (10 mL) was added 10% $Na_2S_2O_4$ aqueous solution (5 mL) and stirred at 40 °C for 30 min under argon. The pH of the solution was adjusted to 1–2 by the addition of 1 M HCl aqueous solution and the mixture was extracted with AcOEt (50 mL×3). The combined organic layer was washed with brine, dried over $MgSO_4$, and concentrated to afford a crude hydroquinone derivative (**11**). The residue was immediately dissolved in AcOEt (1 mL)–dioxane (1 mL), and then phytol (243 mg, 0.82 mmol) and boron trifluoride ether complex (50 μL) were added. The mixture was stirred at 45 °C for 3 h under argon and then cooled to room temperature. The reaction mixture was poured into ice-water and extracted with AcOEt (50 mL×3). The combined organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated. The residue was purified by preparative TLC (hexane/AcOEt=10:1) to afford **12** (75 mg, 35% yield) as yellow paste: IR ($CHCl_3$) 3018, 2929, 2869, 1711, 1655, 1589, 1463 $cm^{-1}$; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 0.81 (3H, s), 0.83 (3H, s), 0.85(3H, s), 0.86 (3H, s), 0.99–1.35 (19H, m), 1.76 (3H, s), 1.88–1.93 (2H, m), 2.15 (3H, s), 2.70 (2H, br s), 3.02 (2H, br s), 3.25 (2H, d, *J*=5.5 Hz), 4.97 (1H, t, *J*=6.0 Hz), 7.47 (1H, br s), 7.84 (1H, br s), 7.93 (1H, br s); $^{13}C$ NMR (125 MHz) $\delta$ 12.7, 16.3, 19.7, 22.6, 22.7, 24.5, 24.8, 25.3, 28.0, 32.7, 37.4, 39.4, 40.1, 118.8, 125.9, 126.6, 130.5, 132.3, 133.3, 137.9, 143.4, 146.0, 146.5, 177.5, 184.6, 185.1; EI-LRMS *m*/*z* 522 ($M^+$), 297, 246. EI-HRMS calcd for $C_{34}H_{50}O_4$ 522.3706; found 522.3699.

#### 4.2.9. 3-(2-Methyl-3-((2E,6E,10E,14E)-3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraenyl)-1,4-naphthoquinon-7-yl)propanoic acid (**13**)

In a manner similar to that for the synthesis of **12** from **10**, a crude product, which was obtained from **10** (120 mg, 0.49 mmol), geranylgeraniol (287 mg, 0.98 mmol), and boron trifluoride ether complex (60 μL) in AcOEt (1 mL)/dioxane (1 mL), was purified by preparative TLC (hexane/AcOEt=10:1), which gave **12** (94 mg, 37% yield) as yellow paste: IR ($CHCl_3$) 3018, 2926, 1711, 1655, 1589, 1449 $cm^{-1}$; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 1.54 (3H, s), 1.55 (3H, s), 1.58 (3H, s), 1.65 (3H, s), 1.74 (3H, s), 1.90–2.05 (12H, m), 2.08 (3H, s), 2.59 (2H, br s), 2.90 (2H, br s), 3.25 (2H, d, *J*=5.5 Hz), 4.96 (2H, t, *J*=7.0 Hz), 5.02–5.08 (3H, m), 7.34 (1H, br s), 7.69 (1H, br s), 7.77 (1H, br s); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 12.6, 16.0, 16.4, 17.7, 25.7, 26.6, 26.7, 26.8, 39.7, 39.8, 119.0, 123.9, 124.2, 124.4, 125.7, 126.4, 130.2, 131.2, 132.0, 133.2, 134.9, 135.2, 137.5, 143.5, 145.7, 179.2, 184.5, 184.8; EI-LRMS *m*/*z* 516 ($M^+$), 297. EI-HRMS calcd for $C_{34}H_{44}O_4$ 516.3238; found 516.3218.

#### 4.2.10. FITC-labeled PK (**1**)

To a solution of **13** (10 mg, 19 μmol) in DMF (0.5 mL) were added 5-((5-aminopentyl)thioureidyl)fluorescein, hydrobromide salt (fluorescein cadaverine) (10 mg, 21 μmol), BOP reagent (12 mg, 28 μmol), and DIEA (5 μL, 37 μmol), and the mixture was stirred at room temperature for 3 h. The reaction mixture was poured into ice-water and extracted with AcOEt (10 mL×3). The combined organic layer was washed with water (20 mL) and brine (20 mL), dried, and concentrated. The residue was purified by preparative chromatography (AcOEt only to $CHCl_3$/MeOH=20:1) to afford **1** (5 mg, 27%) as yellow powder: IR ($CHCl_3$) 2929, 1762, 1654, 1604, 1458, 1376 $cm^{-1}$; $^1H$ NMR (500 MHz, DMSO-$d_6$) $\delta$ 0.75 (3H, s), 0.76 (3H, s), 0.81 (3H, s), 0.82 (3H, s), 0.96–1.63 (25H, s), 1.71 (3H, s), 1.91 (2H, t, *J*=7.0 Hz), 2.06 (3H, s), 2.35–2.40 (2H, m), 2.48–2.52 (2H, m), 3.01 (2H, d, *J*=5.5 Hz), 3.28–3.32 (5H, m), 3.40–3.45 (3H, m), 4.99 (1H, t, *J*=6.5 Hz), 6.46 (2H, br s), 6.53 (1H, br s), 6.63 (2H, d, *J*=8.5 Hz), 7.09 (1H, d, *J*=8.0 Hz), 7.58 (1H, d, *J*=7.5 Hz), 7.66 (1H, t, *J*=7.5 Hz), 7.71–7.73 (1H, m), 7.76 (1H, br s), 7.90 (1H, d, *J*=7.5 Hz), 8.21 (1H, d, *J*=7.0 Hz); $^{13}C$ NMR (125 MHz, DMSO-$d_6$) $\delta$ 11.9, 19.6, 22.4, 25.5, 24.1, 27.3, 32.0, 36.6, 36.7, 43.7, 102.3, 115.4, 116.1, 122.3, 127.8, 129.3, 137.7, 142.8, 142.9, 171.3, 180.3; SIMS-LRMS *m*/*z* 998 ($[M+3H]^+$). SIMS-HRMS calcd for $C_{60}H_{76}N_3O_8S$ 998.5353; found 998.5348.

#### 4.2.11. FITC-labeled MK-4 (**2**)

To a solution of **14** (9 mg, 18 μmol) in DMF (0.5 mL) were added 5-((5-aminopentyl)thioureidyl)fluorescein, hydrobromide salt (fluorescein cadaverine) (8 mg, 16 μmol), BOP reagent (11 mg, 24 μmol), and DIEA (4 μL, 32 μmol), and the mixture was stirred at room temperature for 3 h. The reaction mixture was poured into ice-water and extracted with AcOEt (10 mL×3). The combined organic layer was washed with water (20 mL) and brine (20 mL), dried, and concentrated. The residue was purified by preparative chromatography (AcOEt only) to afford **2** (5 mg, 31%) as yellow powder: IR ($CHCl_3$) 2930, 1759, 1654, 1607, 1457, 1377 $cm^{-1}$; $^1H$ NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.22–1.37 (6H, m), 1.48 (3H, s), 1.51 (3H, s), 1.52 (3H, s), 1.59 (3H, s), 1.72 (3H, s), 1.79–2.06 (12H, m), 2.05 (3H, s), 2.37 (2H, t, *J*=7.5 Hz), 2.48–2.52 (2H, m), 2.95–3.03 (2H, m), 3.27–3.32 (5H, m), 3.40–3.48 (3H, m), 4.93–5.08 (4H, m), 6.47 (2H, br s), 6.53 (1H, br s), 6.62 (2H, d, *J*=9.0 Hz), 7.08 (1H, d, *J*=8.5 Hz), 7.58 (1H, d, *J*=7.5 Hz), 7.65 (1H, t, *J*=7.5 Hz), 7.69–7.76 (1H, m), 7.83 (1H, d, *J*=7.5 Hz), 7.90 (1H, d, *J*=6.5 Hz), 8.23 (1H, d, *J*=7.0 Hz); $^{13}C$ NMR (125 MHz, DMSO-$d_6$) $\delta$ 15.7, 17.5, 23.5, 23.8, 25.4, 26.2, 28.8, 30.3, 37.8, 43.7, 102.3, 112.1, 117.6, 118.9, 120.2, 121.7, 124.0, 124.9, 127.0, 130.2, 131.1, 132.0, 133.2, 134.9, 135.2, 137.5, 143.5, 145.7, 162.3, 170.5, 184.5, 184.8; SIMS-LRMS *m*/*z* 992 ($[M+3H]^+$). SIMS-HRMS calcd for $C_{60}H_{70}N_3O_8S$ 992.4879; found 992.4867.

### 4.3. Cell culture

MG-63 cells were cultured at 37 °C in the presence of 5% $CO_2$ in DMEM containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin.

### 4.4. Fixation of MG-63 cells

MG-63 cells were seeded onto micro-cover glasses (circle 15 mm, thickness 0.12–0.17 mm), which were put in six-well plates. After incubation for 24 h, 1 μM of fluorescent-labeled vitamin K analogue was added to the culture medium. Removal of the culture medium and washing of cells with phosphate-buffered saline (PBS) three times were followed by fixation on the glass slide using 4% formaldehyde for 2 min at 37 °C (3–48 h). The cover glass was removed from the six-well plates and cells were fixed onto a glass slide using 1 drop of Prolong Gold® antifade reagent. Finally, the samples were subjected to confocal laser scanning microscopy.

### 4.5. Fixation for ER-Tracker™ Red dye

MG-63 cells were seeded onto micro-cover glasses, which were put in six-well plates. After incubation for 24 h, 1 μM of fluorescent-labeled vitamin K analogue was added to the culture medium. After removal of the medium from a six-well plate, cells were rinsed with Hank's Balanced Salt Solution. Then, ER-Tracker™ Red in a DMSO solution was added to cells at a working concentration of 100 nM. Incubation of the cells for 30 min at 37 °C was followed by washing with PBS. Fixation of stained cells was done using 4% formaldehyde for 2 min at 37 °C. After the fixation, two 5-min washes in PBS were performed. The cover glass was removed from the six-well plate and cells were fixed onto a glass slide using 1 drop of Prolong Gold®

antifade reagent. The resulting specimen was examined and photographed using a confocal laser microscope.

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## Supplementary data

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