2-(Fluoromethyl)-3-phytyl-1,4-naphthoquinone and Its 2,3-Epoxide. Inhibition of Vitamin K Epoxide Reductase

Richard B. Silverman* and John S. Oliver

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208-3113. *Received January 20, 1989*

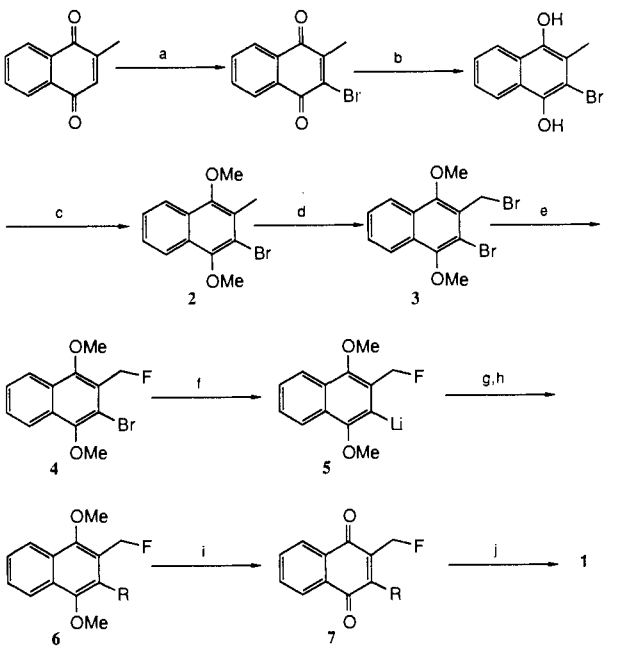
2-(Fluoromethyl)-3-phytyl-1,4-naphthoquinone (**7**) was synthesized from the known compound 2-bromo-3-methyl-1,4-dimethoxynaphthalene by *N*-bromosuccinimide bromination of the 3-methyl group, conversion to the corresponding 3-fluoromethyl compound with silver fluoride, attachment of the 3-phytyl substitutent via the lithium diaryl cuprate and phytyl bromide, and then silver oxide oxidation to **7**. Epoxidation with basic hydrogen peroxide gave the corresponding 2,3-oxide (**1**) in a very low yield. Compound **1** was not a time-dependent inhibitor of beef liver microsomal vitamin K epoxide reductase, but it was a competitive, reversible inhibitor. It was not possible to determine if **1** was a substrate for the enzyme because the expected product of reduction, namely **7**, rapidly decomposed under the assay conditions.

Vitamin K (2-methyl-3-phytyl-1,4-naphthoquinone; phylloquinone) is required for the biosynthesis of plasma clotting factors II, VII, IX, and X and several other vitamin K dependent proteins.[1-3] The first step in the vitamin K cycle involves reduction of vitamin K to its hydroquinone form, catalyzed by certain NAD(P)H-dependent dehydrogenases[4,5] as well as a dithiothreitol-dependent reductase that is sensitive to the oral anticoagulant warfarin.[6,7] In the presence of molecular oxygen and carbon dioxide, the hydroquinone is converted to vitamin K 2,3-oxide with concomitant γ-carboxylation of various glutamyl residues in the proteins. This vitamin K dependent γ-glutamate carboxylation is responsible for the formation of active prothrombin and the clotting factors. It is believed that both epoxidation of reduced vitamin K and carboxylation are catalyzed by the same enzyme.[8,9] The vitamin K 2,3-oxide generated is converted back to vitamin K by vitamin K epoxide reductase,[6,10] a thiol-requiring enzyme that is strongly inhibited by warfarin and other oral anticoagulants.[11-13] Dithiothreitol, a nonphysiological thiol, is the standard reducing agent for the latter enzyme; recently, however, it was found that reduced thioredoxin is an even more potent reducing agent and may be the physiological cofactor.[14]

Oral anticoagulants such as warfarin are some of the most important drugs for the prevention and treatment of a variety of venous, and to a lesser extent, arterial thromboembolic disorders.[15,16] Most of these compounds, however, produce side effects and exhibit some toxicity. Warfarin is known to inhibit vitamin K epoxide reductase,[6,17,18] and this inhibits the formation of the plasma clotting factors.[19] We have found that warfarin is noncompetitive against vitamin K epoxide for beef liver microsomal vitamin K epoxide reductase, but is competitive with dithiothreitol.[20]

We have been interested in mechanism-based enzyme inactivators over the years[21] and set out to design such an

Scheme I. Synthetic Route to 2-(Fluoromethyl)-3-phytyl-1,4-naphthoquinone (**7**) and Its 2,3-Epoxide (**1**)[a]



[a] R = phytyl. (a) Br_2/HOAc; (b) $SnCl_2$/HCl; (c) $(CH_3)_2SO_4$/KOH; (d) *N*-bromosuccinimide; (e) AgF; (f) *n*-BuLi; (g) CuBr·SMe_2; (h) RBr; (i) AgO; (j) H_2O_2/Na_2CO_3.

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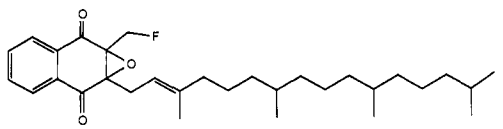
inactivator for vitamin K epoxide reductase on the basis of the proposed mechanism of this enzyme.[22,23] Because the activity of a mechanism-based inactivator depends on the catalytic mechanism of the enzyme, these compounds also are useful for obtaining information regarding the enzyme mechanism. Also, since mechanism-based inactivators are, by definition, compounds that interact with the active site of the enzyme, *competitive* inhibition of vitamin K epoxide reductase would be involved. The compound designed was 2-(fluoromethyl)-3-phytyl-1,4-naphthoquinone 2,3-oxide (1); however, it was found not to be an inactivator, but it was a potent, competitive, reversible inhibitor of vitamin K epoxide reductase.

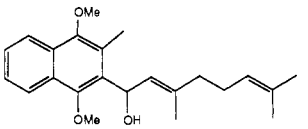


1

Results and Discussion

Synthesis. After several unsuccessful attempts at the synthesis of 1, the route shown in Scheme I proved fruitful. The synthesis of bromide **2** was reported previously.[24] *N*-Bromosuccinimide treatment gave the dibromide **3**, which slowly decomposed upon standing; consequently, it was used directly in the silver fluoride reaction to give **4**. The key step in the synthesis was the prenylation of the aromatic nucleus. Model reactions were carried out on **2**. The lithium anion formed by transmetalation with *n*-butyllithium in diethyl ether at room temperature was reported.[25,26] However, treatment of this anion with phytyl bromide or phytyl iodide gave only trace amounts of phylloquinone; warming or extending the time of the reaction had no effect on the yield. Addition of n-butyllithium to **4** at room temperature led to immediate decomposition; at −78 °C the solubility of the compound in ether was too low to allow efficient anion generation. It was reported[25,26] that transmetalation of **2** in THF at room temperature resulted in intramolecular proton transfer, but transmetalation of **4** at −78 °C in THF (to increase its solubility) proved successful. No intramolecular proton transfer was observed at this temperature, despite the greater acidity of the fluoromethyl protons. However, treatment of **5** with phytyl bromide at −78 °C gave only protonated product (**6**, R = H) after workup. It has been reported[27] that related aryllithiums were too unreactive with allylic halides. In order to verify that lithiation had occurred, the transmetalation product of **2** was allowed to react with citral; after 15 min at room temperature, an 80% yield of alcohol **8** was obtained. However, condensation with phytyl did not give the analogous product; instead, reduced **2** (**2** with H in place of Br) was obtained in a 70% yield.

Lithium dialkyl cuprates are known[28,29] to be quite efficient at coupling with allylic halides. The lithium compound **5** reacted with copper(I) bromide-dimethyl sulfide complex to give a highly stable species that could be warmed to −23 °C without decomposition. Treatment of the lithium diaryl cuprate of **5** with phytyl bromide followed by silver oxidde oxidation gave **7** in moderate yield. Epoxidation of the quinone with basic hydrogen peroxide, however, was a troublesome reaction, and on a 0.2-mmol scale, the desired product 1 was obtained in only 2% yield.



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Enzymology. It was thought that 1 might inactivate vitamin K epoxide reductase by the hypothetical inactivation mechanism shown in Scheme II (pathway b). This mechanism mimics the proposed catalytic mechanism for the enzyme[22,23] up to **9**. If this is a discreet intermediate, there should be competition for elimination of fluoride ion (pathway b) versus water (pathway a), the latter of which was proposed[22,23] to occur in the substrate mechanism. Elimination of fluoride ion would generate **10**, which is a good Michael acceptor. Active site nucleophilic addition followed by dehydration would give inactivated enzyme (**11**). This inactivation mechanism is a variation on the proposed mechanism for alkylation of solid tumor DNA by the potential prodrugs 2-(halomethyl)-1,4-quinones.[30] Compound 1, however, exhibited no time-dependent inhibition of beef liver microsomal vitamin K epoxide reductase at a concentration of 43 μM over a 5-h period. Unlike the oral anticoagulant warfarin, which is a noncompetitive inhibitor of vitamin K epoxide reductase, 1 was found by Lineweaver–Burk analysis to be competitive with vitamin K epoxide. At pH 8.2, the K_i (average of three experiments) for 1 was 16 μM and the K_m for vitamin K 2,3-epoxide was 24 μM.

This compound represents the first halomethyl vitamin K epoxide analogue. The fact that 1 does not inactivate vitamin K epoxide reductase suggests that either the catalytic mechanism of the enzyme should be reconsidered or, perhaps, the fluoride ion elimination from intermediate **9** in Scheme II (pathway b) is not important. If that is the case, then 1 should act as a substrate, producing **7**. However, 1 could not be tested as a substrate for the enzyme because the expected product **7** decomposes very quickly under the assay conditions. Time-dependent and microsome-dependent consumption of 1 was observed, but pretreatment of the microsomes with warfarin did not block the metabolism of 1, suggesting that there is at least one other enzyme in microsomes that can degrade 1, possibly the warfarin-insensitive vitamin K epoxide reductase.[31]

Experimental Section

Analytical Methods. Proton magnetic spectra were recorded on either a Varian EM-390 90-MHz or a Varian XLA-400 400-MHz spectrometer. Chemical shifts are reported as δ values in parts per million downfield from internal tetramethylsilane. IR

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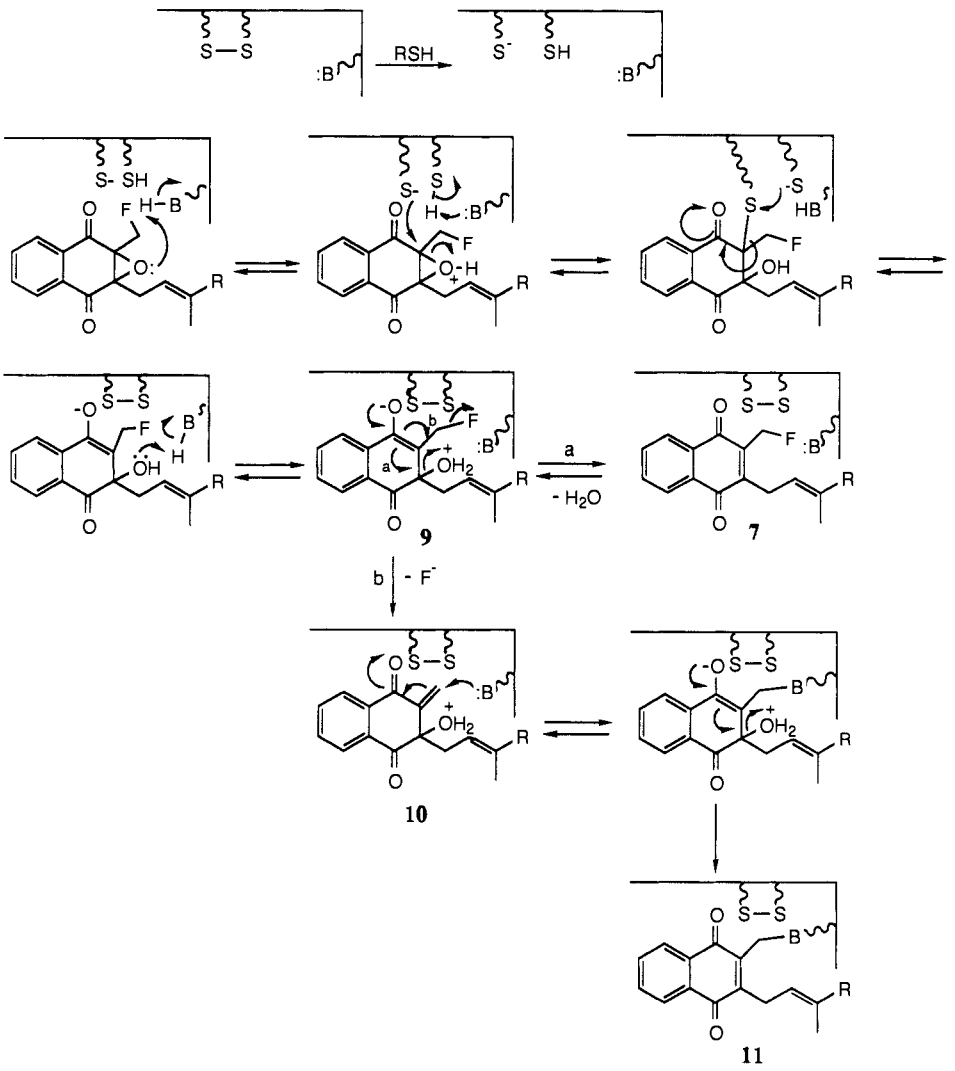
Scheme II. Hypothetical Mechanism of Inactivation of Vitamin K Epoxide Reductase by 1 and Reduction of 1[a]



[a] R = $[(CH_2)_3CHCH_3]_3CH_3$.

spectra were recorded on either a Perkin-Elmer Model 283 or a Mattson Instruments Alpha Centauri spectrophotometer. Mass spectra were obtained with a Hewlett-Packard 5985 spectrometer. High-resolution mass spectra were obtained from the Midwest Center for Mass Spectrometry, Lincoln, NE. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN. Melting points were obtained on a Fisher-Johns melting point apparatus and are uncorrected. HPLC was performed with Beckman Model 110A pumps, Model 330 UV detector, and Model 420 system controller. HPLC columns were purchased from Alltech Associates, Deerfield, IL. Solvents were Mallinkrodt HPLC grade. TLC was done with EM Reagents precoated silica gel 60 F-254 sheets. Column and flash chromatography were performed with Merck silica gel 60 (230–400 mesh).

Reagents. THF and diethyl ether were distilled from sodium. Other chemicals were purchased from Aldrich Chemical Co., Milwaukee, WI, or Sigma Chemical Co., St. Louis, MO.

2-Bromo-3-(bromomethyl)-1,4-dimethoxynaphthalene (3). To a solution of 2-bromo-3-methyl-1,4-dimethoxynaphthalene[24] (2) (2.27 g, 8.06 mmol) in dry CCl_4 (50 mL) was added *N*-bromosuccinimide (1.52 g, 8.52 mmol). Benzoyl peroxide (3 mg) was added, and the reaction mixture was refluxed under an inert atmosphere for 2 h. After being cooled to room temperature, the reaction mixture was filtered, and the yellow filtrate was rotary evaporated to give a yellow solid. The crude solid was suitable for use in the next reaction. Pure material could be obtained by recrystallization from acetonitrile, but the material degraded upon standing: mp 84–87 °C; ^1H NMR ($CDCl_3$) δ 4.0 (s, 3 H), 4.1 (s, 3 H), 4.9 (s, 2 H), 7.5 (m, 2 H), 8.0 (m, 2 H); mass spectrum (70 ev), *m/z* (relative intensity) 362 (8), 360 (16), 358 (8), 281 (42), 279 (43), 200 (52), 185 (100), 170 (13), 157 (9), 155 (8); high-resolution mass spectrum calcd for $C_{13}H_{12}{}^{79}Br_2O_2$ 357.9204, found 357.9205.

2-Bromo-3-(fluoromethyl)-1,4-dimethoxynaphthalene (4). To a suspension of silver fluoride (2.11 g, 16.6 mmol) protected from light in dry CH_3CN (20 mL) was added a solution of **3** (3.0 g, 8.3 mmol) in dry CH_3CN (30 mL) over 1 h. The reaction mixture was stirred at room temperature overnight and filtered through a silica pad, and the clear, yellow filtrate was rotary evaporated to give a yellow solid. Pure product was obtained by sublimation or flash chromatography over silica gel (2 × 26 cm column) eluting with 10% ether in hexane. The first UV-active band off of the column gave pure product as a white solid (1.8 g, 75% from **2**): mp 82–83 °C; IR (CCl_4) 2950, 1580, 1460, 1405, 1360, 1270, 1200, 1170, 1090 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.9 (s, 3 H), 4.0 (s, 3 H), 5.7 (d, 2 H, J = 48 Hz), 7.5 (m, 2 H), 8.1 (m, 2 H); mass spectrum (70 eV), *m/z* (relative intensity) 300 (67), 298 (72), 285 (96), 283 (100), 223 (12), 221 (11), 204 (20), 189 (22), 161 (43), 133 (45). Anal. Calcd for $C_{13}H_{12}BrFO_2$: C, 52.20; H, 4.04; Br, 26.72; F, 6.35. Found: C, 52.11; H, 4.22; Br, 26.71; F, 6.38.

2-(Fluoromethyl)-3-phytyl-1,4-naphthoquinone (7). Compound 4 (85 mg, 0.284 mmol) was dissolved in dry THF (4.0 mL), and the solution was cooled to -78 °C. *n*-Butyllithium (0.178 mL, 1.6 M, 0.285 mmol) was added dropwise over 10 min by syringe, and then the solution was stirred at −78 °C for 10 min before the addition of $CuBr{\cdot}S(CH_3)_2$ (69 mg, 0.33 mmol). The reaction mixture was stirred for 1 h at −78 °C, during which time the color gradually changed from bright yellow to brown-yellow. Phytyl bromide (102 mg, 0.283 mmol) in dry THF (1 mL) was added by syringe and the solution was allowed to warm to -23 °C. The

reaction mixture was stirred at −23 °C for 1.5 h before being quenched with acetic acid in THF. After warming to room temperature and the addition of water (5 mL), the reaction mixture was extracted with hexane until no UV-active material was found in the extracts. The combined organic extracts were washed with water and rotary evaporated. The crude material was applied to a silica gel column (1.5 × 13 cm) and eluted with 5% ether in hexane. The product was dissolved in dioxane (3.75 mL) and water (0.25 mL). The solution was protected from light, then silver oxide (78 mg, 0.63 mmol) was added followed by 6.2 N HNO_3 (104 μL). After 30 min of stirring, the suspension was filtered to give a yellow solution. The solution was extracted with ether, and then the combined ether extracts were washed with water and brine before being rotary evaporated to give a yellow solid. Column chromatography over silica gel, eluting with 5% ether in hexane, gave the desired material (59 mg, 45% from 4): IR (CCl_4) 2956, 2898, 2872, 1668, 1597, 1465, 1377, 1293 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.73 (d, 3 H, J = 6.5 Hz), 0.75 (d, 3 H, J = 6.6 Hz), 0.79 (d, 6 H, J = 8.5 Hz), 0.9–2.0 (br, 2 H), 1.70 (s, 3 H), 3.43 (d, 2 H, J = 8 Hz), 4.95 (t, 1 H, J = 8 Hz), 5.44 (d, 2 H, J = 47 Hz), 7.67 (m, 2 H), 8.05 (m, 2 H); mass spectrum (70 eV), m/z (relative intensity) 468 (26), 448 (19), 265 (5), 252 (4), 243 (14), 223 (100), 209 (23); high-resolution mass spectrum calcd for $C_{31}H_{45}FO_2$ 468.3406, found 468.3399 (−1.0 ppm dev.).

2-(Fluoromethyl)-3-phytyl-1,4-naphthoquinone 2,3-Oxide (1). 2-(Fluoromethyl)-3-phytyl-1,4-naphthoquinone (99 mg, 0.211 mmol) was dissolved in EtOH (5.0 mL), and the solution was warmed to 70 °C. Hydrogen peroxide (30%, 99 μL) was added, and after the mixture was swirled, a saturated aqueous sodium carbonate solution (198 μL) was added. The reaction mixture was stirred with a glass rod at 70 °C for 20 min then water (1 mL) was added, and the reaction mixture was extracted with hexane (4 × 15 mL). The combined organic layers were washed with water, concentrated to 800 μL, and purified on a C-18 semiprep HPLC column (10 mm × 50 cm), eluting with 100% methanol at a flow rate of 4 mL/min. The peak with a retention time of 23 min was collected. Final traces of MeOH could not be removed from the colorless oil under vacuum, so the material was rechromatographed over a silica gel semiprep HPLC column (10 mm × 25 cm), eluting with 5% EtOAc in hexane at a flow rate of 3 mL/min (t_R = 11 min) to give the product as a colorless oil (1.2 mg, 2%): ^1H NMR ($CDCl_3$) δ 0.74 (d, 3 H), 0.76 (d, 3 H), 0.80 (d, 6 H), 0.9–1.5 (br, 2 H), 1.69 (s, 3 H), 1.90 (t, 2 H), 2.55 (dd, 1 H), 3.25 (dd, 1 H), 4.57 (dd, 1 H, J_1 = 47 Hz, J_2 = 10 Hz), 5.07 (t, 1 H), 5.25 (dd, 1 H, J_1 = 47 Hz, J_2 = 1 Hz), 7.69 (m, 2 H), 7.96 (m, 2 H); mass spectrum (70 eV), m/z (relative intensity) 484 (28), 451 (13), 423 (19), 259 (100), 220 (94), 159 (44); high-resolution mass spectrum calcd for $C_{31}H_{45}FO_3$ 484.3355, found 484.3360 (1.5 ppm dev.).

Enzyme and Assays. Beef liver microsomes were prepared by the homogenization of well-minced liver with 3 times its volume of 50 mM potassium phosphate buffer, pH 7.4, containing 250 mM sucrose and 0.5 mM EDTA in a Waring blender (six 20-s pulses) at 4 °C and made into a smooth suspension with 20 additional strokes of a Teflon-cap hand homogenizer. The homogenate was centrifuged at 12 000 rpm (17000 g) for 30 min to remove the cell debris. The supernatant was centrifuged for 90 min at 105000 g. The pellets were washed by resuspension in 50 mM Tris-HCl buffer, pH 8.0, and recentrifuged at 105000 g for 90 min. The pellets were stored at −80 °C for further use.

Vitamin K epoxide reductase activity was assayed by incubating the microsomal preparation (added last) at 24 °C in a total volume of 0.5 mL of 50 mM Tris-phosphate buffer, pH 8.2, containing 0.5 M sucrose, 0.5 mM EDTA, 5 mM dithiothreitol, and 40 μM vitamin K 2,3-epoxide (added in aqueous Emulgen 911 so that the final concentration of detergent is 0.05%) for 20 min, during which time the reaction was linear. The reaction was quenched by the addition of 2.0 mL of 25 mM $HgCl_2$ to minimize the nonenzymatic reaction products.[32] The reaction mixture was extracted with 3 mL of 2-propanol–n-hexane (1:1) by vortex mixing (20 s) and centrifugation, and then the aqueous layer was extracted again with 1.0 mL of n-hexane. The organic extracts were combined, and the solvent was removed in a Speed-Vac (Savant). The residue was dissolved in 140 μL of HPLC-grade methanol and centrifuged, and a 100-μL aliquot was analyzed by HPLC (Beckman Model 330 with a UV detector and a Hewlett-Packard 3390A integrator) using a 10 μ C_{18} reverse-phase column and eluting with 100% methanol at 2 mL/min.

Time-Dependent Inhibition of Vitamin K Epoxide Reductase. Beef liver microsomes were incubated in the presence (43 μM) and absence of 1 under the same conditions as the assay except without vitamin K epoxide. At various times aliquots (480 μL) were removed from the tubes containing inhibited or control microsomes and assayed as described above.

Competitive Inhibition of Vitamin K Epoxide Reductase by 1. The activity of vitamin K epoxide reductase (0.8 mg of microsomes) was determined with varying concentrations (8, 10, 13.3, 20, and 40 μM) of vitamin K epoxide in the absence or presence of 1 (0, 10, 20, and 30 μM). After incubation at each concentration for 25 min, enzyme reactions were worked up and analyzed as described above. Double-reciprocal plots of activity versus vitamin K epoxide concentration of 1 were constructed. A replot of $K_{m,app}$ versus [1] gave the K_i value for 1.

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