Derivatives of 2-Methyl-1,4-naphthoquinone as Substrates and Inhibitors of the Vitamin K Dependent Carboxylase

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A series of peptides that contain an N-terminal 2-methyl-1,4-naphthoquinone group or analogues of this structure have been prepared as potential substrates or inhibitors of the rat liver microsomal vitamin K dependent carboxylase. The parent compound, γ-2-(methyl-1,4-naphthoquinonyl-3)butyryl-Glu-Glu-Leu-OMe, is a good substrate for the carboxylase at low concentrations and has a K_m of about 50 μM. This is roughly 2 orders of magnitude lower than the K_m of most simple peptide substrates that have been synthesized. Replacement of the 2-methyl-1,4-naphthoquinone group with its desmethyl analogue, a naphthyl, or a stearyl group decreased substrate effectiveness. At higher concentrations, the parent compound and its desmethyl analogue were potent inhibitors of the vitamin K dependent carboxylation reaction. The degree of inhibition exhibited by these peptides was dependent on the vitamin KH_2 concentration of the incubation.

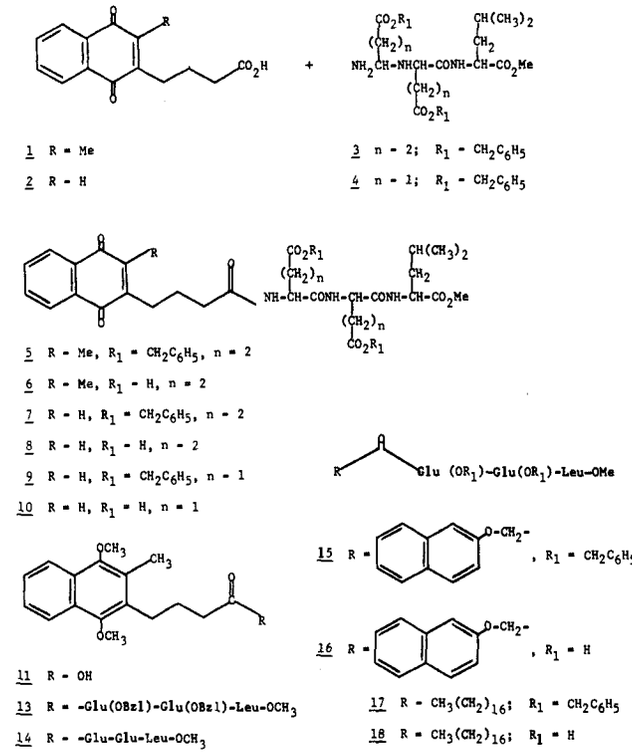
Vitamin K is a required cofactor for a microsomal enzyme that converts glutamyl residues in precursor proteins to γ-carboxyglutamyl (Gla) residues in the vitamin K dependent clotting factors and a number of other proteins.[1,2] Although the mechanism of this oxygen-dependent carboxylase is not clear, knowledge of substrate specificity of this enzyme has been gained through studies of synthetic substrates. Following the demonstration[3] that the peptide Phe-Leu-Glu-Glu-Val, which is homologous to residues 5–9 of the bovine prothrombin precursor, would serve as a substrate for the enzyme, a number of similar peptides have been demonstrated to have substrate activity.[4-9] An extensive study[10] of analogues of this region of the bovine prothrombin precursor has shown that a large number of low-molecular-weight peptides containing a Glu-Glu sequence will serve as substrates for the enzyme but that some specificity is imparted by adjacent residues.

With the exception of a report of Soute et al.[11] that a peptide isolated from a digest of plasma abnormal prothrombin had an apparent K_m in the μM range, other carboxylase substrates have been reported to have apparent K_ms of a few mM. In an effort to produce a multisubstrate analogue inhibitor of this enzyme, we have reported[12] that a compound containing a carboxylatable peptide linked to the 3-position of 2-methyl-1,4-naphthoquinone was not an active form of the vitamin but was carboxylated by the enzyme. This compound was a substrate of the enzyme at low concentrations but a poor substrate at high concentrations and an inhibitor of the carboxylation of both endogenous proteins and added peptide substrates. This article describes the synthesis of a number of substrates for the vitamin K dependent carboxylase that have relatively low K_ms but are also inhibitors of the enzyme at higher concentrations.

Results

Peptide Synthesis. γ-2-(Methyl-1,4-naphthoquinonyl-3)butyric acid (1) was prepared by the reaction of 2-methyl-1,4-naphthoquinone with digluteroyl peroxide following a procedure developed by Fieser and Turner.[13] The desmethyl analogue, γ-(1,4-naphthoquinonyl-3)butyric acid (2),[14] and the dimethylated analogue, γ-2-(methyl-1,4-dimethoxynaphthyl-3)butyric acid (11) were prepared from the corresponding starting materials following essentially the same reaction procedures. No attempt was made to optimize the yield of either reaction.

The quinone acid 1 was coupled with the free tripeptide, H-Glu(OBzl)-Glu(OBzl)-Leu-OMe (3)[10] by using the mixed anhydride procedure (isobutyl chloroformate, *N*-methylmorpholine, THF, −15 °C) to give the naphthoquinone



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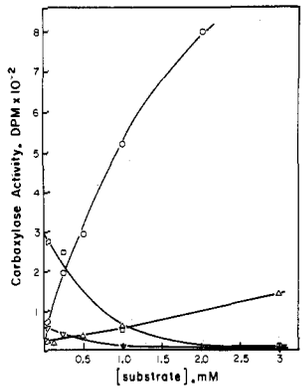

Figure 1. Carboxylation of substrates as a function of substrate concentration: (□) **6**, (▽) **8**, (○) **16**, and (△) **18**.

tripeptide **5** in 55% yield. In a similar fashion, the desmethyl derivative **2** was treated with tripeptide **3** to give the desmethylated naphthoquinone tripeptide **7** in 68% yield. Reaction of **11** with tripeptide **3** using DCC–HOBt as the coupling reagent[15] gave the corresponding tripeptide **13** in 75% yield. Two other acyl tripeptide derivatives (**15**, **17**) were prepared by the reaction of tripeptide **3** with the corresponding mixed anhydrides prepared from (2-naphthyloxy)acetic acid and from stearic acid. Reaction of naphthoquinone acid **2** with the free base of H-Asp(OBzl)-Asp(OBzl)-Leu-OMe (**4**) gave the corresponding aspartic acid containing naphthoquinone tripeptide **9**.

The benzyl groups were removed from the glutamic and aspartic acid side chains in the tripeptide derivatives by hydrogenolysis at atmosphere pressure with use of palladium on carbon as the catalyst. Peptides **6**, **8**, **10**, **16**, and **18** were obtained in nearly quantitative yields. The structure of **14**, assigned by amino acid analysis and spectral data, also was confirmed by conversion into **6**. Demethylation of the bis-ether derivative **14** with silver oxide followed by oxidation[16] gave the naphthoquinone tripeptide **6** in 50% yield.[12]

Biological Activity. The data in Figure 1 illustrate the activity of compound **6**, γ-2-(methyl-1,4-naphthoquinonyl-3)butyryl-L-glutamyl-L-glutamyl-L-leucine methyl ester, as a substrate for the vitamin K dependent carboxylase. The activity of this vitamin K analogue is compared to that exhibited by the corresponding desmethyl compound **8**, γ-(1,4-naphthoquionyl-2)butyryl-L-glutamyl-L-glutamyl-L-leucine methyl ester, a naphthalene containing compound **16**, (2-naphthyloxy)acetyl-L-glutamyl-L-glutamyl-L-leucine methyl ester, and to compound **18**, stearyl-L-glutamyl-L-glutamyl-L-leucine methyl ester. These data demonstrate that although all four potential substrates contained a large hydrophobic group, there were significant differences in their substrate activity. The two non-naphthoquinoid compounds (**16**, **18**) exhibited typical substrate–activity curves. That is, increasing carboxylation was observed as the concentration of the substrate was increased. However, substrates **6** and **8** containing a 2-methyl-1,4-naphthoquinone group, or the desmethyl analogue of this compound, exhibited highest activity at the lowest concentration tested (0.063 mM), and this activity decreased as the concentration was increased. This observation suggested that these compounds were acting as very low K_m substrates for the enzyme but that they were also acting as an inhibitor of the reaction as their concentration was increased.

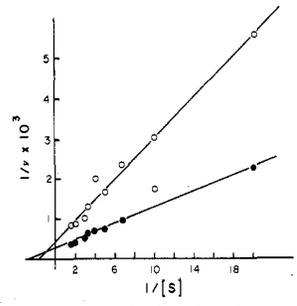

Figure 2. Double-reciprocal plot for the carboxylation of two substrates. $1/[S]$ values are $\mu M \times 10^{-2}$. (○) **8**, (●) **6**.

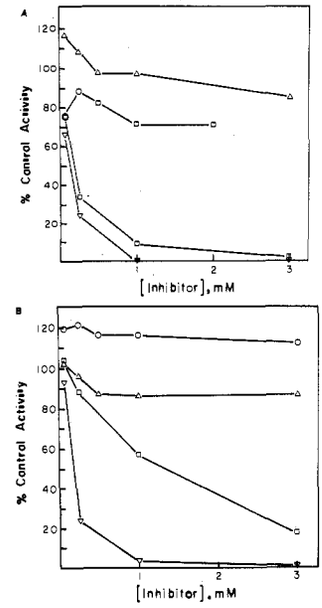

Figure 3. (A) Effect of other peptides on the carboxylation of Phe-Leu-Glu-Glu-Leu. The values plotted are the total carboxylation from 0.5 mM Phe-Leu-Glu-Glu-Leu and the indicated concentration of the other compounds and are expressed relative to the carboxylation of Phe-Leu-Glu-Glu-Leu alone. (B) Effect of other peptides on the carboxylation of endogenous microsomal proteins. Values are expressed relative to carboxylation of endogenous protein observed in the presence of 0.5 mM Phe-Leu-Glu-Glu-Leu alone. (□) **6**, (▽) **8**, (△) **18**, and (○) **16**.

The activities of these two compounds were therefore assessed at lower substrate concentrations, and the data in Figure 2 illustrate typical double-reciprocal plots obtained. Because of the inhibition noted in Figure 1, only substrate concentrations of 60 μM or lower were used. An average of data obtained from a number of experiments indicated an apparent K_m of 44 ± 4 μM (means ± SEM, $n = 6$) for the phylloquinone-containing tripeptide, **6**, and 85 ± 11 μM (mean ± SEM, $n = 4$) for the desmethyl analogue **8**. These values are 50–100 times lower than the values of 1.5–14 mM that have been reported[17] for the apparent K_m of six simple peptide substrates containing the Glu-Glu sequence. Peptide **10**, which contains aspartyl rather than glutamyl residues, was a substrate only at low concentrations and was carboxylated only 5–10% as efficiently as the Glu analogue compound **6**. This is consistent with the previously reported[10,18,19] low activity of

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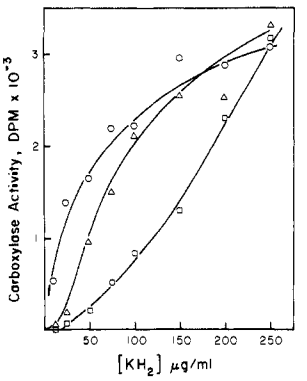

Figure 4. Effect of compound **6** on the carboxylation of Boc-Glu-Glu-Leu-OMe at various concentrations of vitamin KH_2. Incubations were carried out with 0.5 mM Boc-Glu-Glu-Leu-OMe alone (○) or with this substrate in the presence of 0.015 mM (△) or 0.25 mM (□) **6**. The data plotted represent the total incorporation in the incubation and include carboxylation of both substrates.

the carboxylase toward Asp-containing substrates. Compound **14** has been previously reported[12] to be a very poor substrate for the enyzme.

Inhibitory Properties. The ability of these peptides to inhibit the carboxylation of standard peptide substrate, Phe-Leu-Glu-Glu-Leu, and of microsomal endogenous protein substrates was also assessed. The data in Figure 3A illustrate that peptides **6** and **8** were effective inhibitors of the carboxylation of a good carboxylase substrate but that the non-naphthoquinoid substrates had little influence on the reaction. The data plotted (Figure 3A) represent the carboxylation of both the low-molecular-weight peptide substrate and compounds **6**, **8**, **16**, and **18** in the incubation mixture. Carboxylation of the added inhibitor is not a complication when the effect of these peptides on the carboxylation of endogenous microsomal proteins is assessed, and the data in Figure 3B again illustrate that the two compounds that contain a naphthoquinone ring are effective inhibitors of carboxylation, while the other two are not. Compound **8**, the desmethyl analogue of **6**, appeared to be significantly more effective as an inhibitor than the parent compound.

These data raised the question of the nature of the inhibition. Vitamin K analogues with polar groups on the 3-position are known[20,21] to be inactive forms of the vitamin, and it seemed likely that these compounds were interfering with the vitamin K utilization by the enzyme. If this were the case, inhibition should be dependent on the vitamin K concentration of the incubation medium, and the data in Figure 4 are suggestive of such an effect. These data indicate that the degree of inhibition of carboxylation of Boc-Glu-Glu-Leu-OMe by compound **6** is strongly dependent on the concentration of vitamin K in the incubation. The inhibition was substantial at low vitamin concentrations but was essentially overcome by increasing the concentrations of vitamin K in the incubation mixture. The data in Figure 4 again represent the combined carboxylation of both substrates, and further analyses depended on the ability to separate the two products of the incubation. A high-performance liquid chromatography system that would resolve compound **6** and Boc-Glu-Glu-Leu-OMe was developed, and an example of the separation achieved is shown in Figure 5.

With use of this procedure, the effect of vitamin K concentration on the inhibition of Boc-Glu-Glu-Leu-OMe carboxylation by compound **6** is shown in Figure 6. Qualitatively this demonstrates the same effect as Figure 4, but it can be seen that the apparent lack of any inhibition at high vitamin K concentrations was an artifact due to a significant carboxylation of the inhibitor itself. Double-reciprocal plots of the data in Figure 6 were not linear. The data suggested that, at least at the higher vitamin concentrations, the inhibition was competitive with regard to the concentration of reduced vitamin K. However, this relationship did not hold at the lower vitamin concentrations, and it is likely that other interactions are also important. When the concentration of Boc-Glu-Glu-Leu-OMe rather than that of vitamin K was varied, the degree of inhibition of carboxylation by the phylloquinone tripeptide was not greatly influenced, and double-reciprocal plots of these data produced curves that were not typical of any simple inhibition pattern. Compound **10** produced inhibition patterns similar to those of **6** and **8**, and compound **14** exhibited no significant inhibition of carboxylation.

Discussion

The synthesis and study of this new class of peptide substrates for the vitamin K dependent carboxylase has led to three significant results. A peptide substrate with a very low K_m has been synthesized, new methods for separating products of the carboxylation reaction have been developed, and a new class of inhibitors of the enzyme has been discovered.

The phylloquinone tripeptide has an apparent K_m of less than 50 μM, which is about 2 orders of magnitude lower than other peptide substrates that have been investigated. The peptide comprising residues 13–29 of bovine abnormal prothrombin has been reported to be an excellent substrate with a K_m of about 1 μM.[11] All other reported substrates for this enzyme have K_ms in the range of a few mM. The basis for the low K_m of compounds **6** and **8** was not determined but does not appear to depend on simple hydrophobic or aromatic interactions, as substrate **16** containing a naphthyl and **18** containing a stearyl group were much less active. The naphthoquinone ring appeared to be important as both the compounds containing this ring had good activity.

The type of chromatographic procedures described here are essential for any studies of this enzyme that involved more than one potentially carboxylated substrate. Modification of this basic procedure should enable the separation of almost any peptide substrate and will be of particular importance in the study of Glu site inhibitors. The demonstration that compounds **6** and **8** are potent inhibitors of the carboxylase reaction at concentrations much above their K_m as a substrate has made available a new class of inhibitors of the enzyme that appear to be dependent on the vitamin K concentration of incubation. The available data are not sufficient to classify these compounds as simple competitive inhibitors of the vitamin binding site of the enzyme. It has been suggested[20] that a number of vitamin K analogues with polar constituents at the 3-position inhibit carboxylation through the ability to oxidize the hydroquinone form of the vitamin, and this interaction may be involved in the response noted. These compounds have utility in that, in contrast to other recently developed inhibitors,[22,23] they are not directed to-

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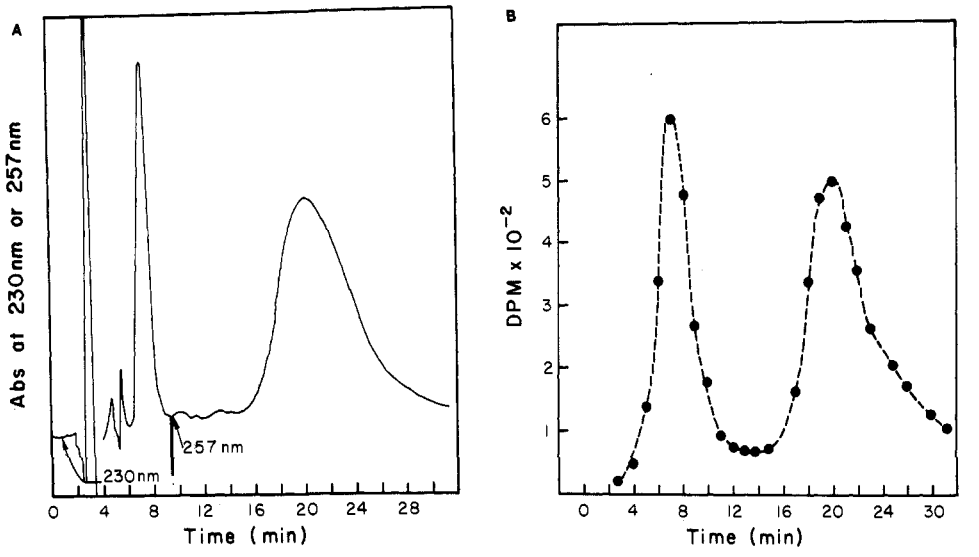

Figure 5. Separation of Boc-Glu-Glu-Leu-OMe and compound **6**. (A) Chromatography of 10 nmol of Boc-Glu-Glu-Leu-OMe (early peak) and 15 nmol of **6** (later peak). (B) Radioactive profile obtained from an incubation containing 0.5 mM Boc-Glu-Glu-Leu-OMe and 0.25 mM **6**. Details of separation procedure are in the Materials and Methods section.

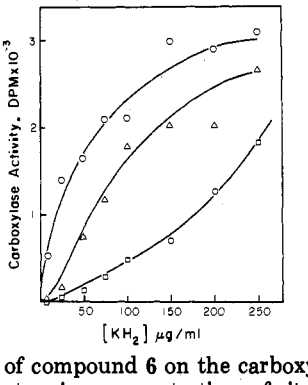

Figure 6. Effect of compound **6** on the carboxylation of Boc-Glu-Glu-Leu-OMe at various concentrations of vitamin KH_2. The data represented are a replot of the same data shown in Figure 4 after the carboxylated products have been separated by the procedure shown in Figure 5. Only the carboxylation associated with the substrate Boc-Glu-Glu-Leu-OMe has been plotted. Incubations were carried out with 0.5 mM Boc-Glu-Glu-Leu-OMe alone (○) or this substrate in the presence of 0.15 mM (△) or 0.25 mM (□) compound **6**.

ward the Glu binding site. The low K_m of compound **6** suggests that replacing the Glu residues of Glu-Glu-Leu-OMe with an *O*-phosphoserine[22] or a γ-methyl-Glu residue[23] should result in a compound that is a low K_i competitive inhibitor of the Glu binding sie at elevated vitamin K concentrations. If incubations are carried out under normal conditions, such a compound would probably also interfere with vitamin K utilization by the enzyme.

Experimental Section

The protected amino acids and the coupling reagents used in this study were obtained from commercial sources and used without further purification. Tetrahydrofuran was distilled prior to use from sodium benzophenone ketyl. Dichloromethane was distilled from phosphorus pentoxide and stored over Linde 3-Å molecular sieves. Ten percent Palladium on charcoal catalyst was obtained from Alfa Division.

Melting points were determined on a Fischer-Johns melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on Varian Model EM-390, Bruker Model HX90E pulse Fourier-transform NMR interfaced with a Nicolet 1080 computer disk unit, and Bruker WH270 spectrometer. Chemical shifts were reported as δ units relative to tetramethylsilane as an internal standard. Optical rotations were recorded on a Perkin-Elmer 241 automatic polarimeter. Amino acid analyses were determined on a Durum D500 amino acid analyzer following hydrolysis in degassed 6 N HCl at 100 °C for 24 h. Elemental analyses were determined by Galbraith Laboratories, Inc., Knoxville, TN. Analytical thin-layer chromatography was carried out with use of precoated TLC plates (silica gel 60F-254, catalog no. 5765, E. Merck) and polygram plastic sheet (Silg/UV254, Brinkmann). Spots were visualized by UV, ninhydrin, and chlorox–tolidine reagents. The following solvent systems were used: A, chloroform/methanol/acetic acid (9:0.8:0.2); B, chloroform/methanol/acetic acid (8.5:1:0.5); C, chloroform methanol (8:2); D, chloroform/methanol (9.5:0.5).

Synthesis. γ-(2-Methyl-1,4-naphthoquinonyl-3)butyric Acid (1). The quinone acid was prepared following the reported procedure[13] and recrystallized from a mixture of chloroform and *n*-hexane (yield 38%): mp 144–145 °C.

γ-(1,4-Naphthoquinonyl-2)butyric Acid (2). The title compound was prepared by a modification of the Fieser procedure.[13] 1,4-Naphthoquinone (27.1 g, 171.7 mmol) was dissolved in warm glacial acetic acid (60 mL) and heated with stirring to 115 °C in an Erlenmyer flask. Digluteroyl peroxide[25] (18 g, 68.7 mmol) was added slowly over a period of 1.5 h and then the reaction mixture was stirred for an additional 5 min and worked up as for compound **1** to give **2** as a bright yellow solid (3.9 g, 23.3%): mp 151–152 °C (lit.[14] mp 151–153 °C); TLC (solvent A) R_f 0.40.

[γ-(2-Methyl-1,4-naphthoquinonyl-3)butyryl]-L-γ-*O*-benzylglutamyl-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (5). A solution of Boc-Glu(OBzl)-Glu(OBzl)-Leu-OMe (0.50 g, 0.73 mmol)[10] in 4 N HCl–dioxane was stirred at room temperature for 50 min. The solvent was removed and the residue taken up in ethyl acetate (30 mL) and washed with saturated $NaHCO_3$ solution (3 × 20 mL) and water (2 × 15 mL), dried (Na_2SO_4), and concentrated to give 0.37 g (88%) of amine tripeptide **3**.

To a stirred solution of **1** (0.1 g, 0.38 mmol) in dry THF (3 mL) at −15 °C was added isobutyl chloroformate (0.052 g, 0.38 mmol) followed by *N*-methylmorpholine (0.042 mL, 0.39 mmol). The reaction mixture was stirred at ambient temperature for 5 min and then a precooled solution of the deprotected tripeptide **3** (0.22 g, 0.39 mmol) in dry THF (4 mL) was added dropwise (10 min). The reaction mixture was stirred for 1 h at −15 °C and 1.5 h at

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room temperature and then concentrated. The residue was taken up in ethyl acetate (20 mL). The organic layer was washed with 1 N HCl (2 × 10 mL), saturated $NaHCO_3$ solution (2 × 10 mL), and water (2 × 10 mL), dried (Na_2SO_4), and concentrated. The residue was crystallized from ethyl acetate/*n*-hexane (twice) to give 0.17 g (55%) of **5**: mp 143–145 °C; TLC (solvent A) R_f 0.70; $[\alpha]^{24}_D$ −23.5° (*c* 0.57, MeOH). The ^1H NMR was consistent with the expected product. This material was used directly in the next reaction.

γ-[(2-Methyl-1,4-naphthoquinonyl-3)butyryl]-L-glutamyl-L-glutamyl-L-leucine Methyl Ester (6). Ester **5** (338 mg, 0.41 mmol) was mixed with 10% Pd/C (120 mg) in 95% ethanol. After the mixture was purged with nitrogen for 15 min, hydrogen gas was bubbled through the reaction mixture for 5–6 h at atmospheric pressure and ambient temperature. The reaction mixture was filtered and concentrated and the residue was solidified by triturating with ether/*n*-hexane to give 0.26 g (98%) of **6**: mp 204–206 °C; TLC (solvent B) R_f 0.44; ^1H NMR (MeOH-d_4) δ 0.89–0.95 (br d, 6 H), 1.62–1.78 (m, 5 H), 2.01–2.19 (m, 6 H), 2.29 (s, 3 H), 2.37–2.55 (m, 6 H), 4.35–4.52 (m, 3 H), 7.72–7.87 (m, 2 H), 8.02–8.20 (m, 2 H); MS, *m/e* (% intensity) 643 (M^+ + 1, 0.01), 608 (0.68), 549 (0.55), 387 (1.14), 369 (2.75), 309 (1.57), 257 (4.64), 241 (18.72), 212 (22.97), 146 (21.23); UV (MeOH) λ_{max} (log ε) 246 (4.16), 266 (4.10), 329 (3.38). Amino acid analysis: Glu 1.95, Leu 1.00.

γ-[(1,4-Naphthoquinonyl-2)butyryl]-L-γ-*O*-benzylglutamyl-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (7). To a stirred solution of **2** (0.058 g, 0.23 mmol) in dry THF (3 mL) at −15 °C was added isobutyl chloroformate (0.031 g, 0.23 mmol) followed by *N*-methylmorpholine (0.026 mL, 0.23 mmol). The reaction mixture was stirred at ambient temperature for 5 min and then a precooled solution of tripeptide **3** (0.14 g, 0.24 mmol) in dry THF (4 mL) was added slowly. The reaction was carried out and the product worked up as described for **5**. The crude product was recrystallized twice from ethyl acetate/*n*-hexane to give a yellow solid in 0.13 g (68.4%) yield: mp 147–149 °C; TLC (solvent A) R_f 0.66; $[\alpha]^{24}_D$ −25.6° (*c* 0.6, MeOH). The ^1H NMR was consistent with the product. Anal. ($C_{45}H_{51}N_3O_{11}$) C, H, N.

γ-[(1,4-Naphthoquinonyl-2)butyryl]-L-glutamyl-L-glutamyl-L-leucine Methyl Ester (8). A solution of **7** (0.05 g, 0.06 mmol) in 95% ethanol containing 10% Pd/C (25 mg) was hydrogenated as described for **6**. After workup the product was obtained by triturating with ether/*n*-hexane to yield 32 mg (84%) of **8**: mp 172–174 °C; TLC (solvent B) R_f 0.47; ^1H NMR (Me_2SO-d_6) δ 0.7–0.97 (m, 6 H), 1.39–2.00 (br m, 9 H), 2.11–2.37 (br s, 6 H), 2.50 (br d, 2 H), 3.59 (s, 3 H), 4.12–4.40 (m, 3 H), 6.92 (s, 1 H), 7.27–7.53 (br m, 2 H), 7.80–8.35 (br m, 7 H). Anal. ($C_{31}H_{39}N_3O_{11}H_2O$) C, H, N.

γ-[(1,4-Naphthoquinonyl-2-)butyryl]-L-β-*O*-benzylaspartyl-L-β-*O*-benzylaspartyl-L-leucine Methyl Ester (9). Boc-Asp(OBzl)-Asp(OBzl)-Leu-OMe was prepared according to the reported procedure,[10] and the Boc group was removed by using 4 N HCl in dioxane and the hydrochloride salt neutralized with sodium bicarbonate solution. The free tripeptide (0.29 g, 0.52 mmol) was condensed with the mixed anhydride of **2** (0.12 g, 0.51 mmol), generated from isobutyl chloroformate (0.07 g, 0.51 mmol) and *N*-methylmorpholine (0.057 mL, 0.52 mmol). The reaction was carried out and worked up as described (vide supra). The crude product was crystallized from ethyl acetate/*n*-hexane to give **9** as a yellow solid (0.26 g, 63.4%): mp 132–134 °C; TLC (solvent A) R_f 0.70; $[\alpha]^{24}D$ −26.3° (*c* 0.54, MeOH). The ^1H NMR was consistent with the product. Anal. ($C_{43}H_{47}N_3O_{11}$) C, H, N.

γ-[(1,4-Naphthoquinonyl-2-)butyryl-L-aspartyl-L-aspartyl-L-leucine Methyl Ester (10). Compound **9** (0.1 g, 0.12 mmol) was hydrogenated at atmospheric pressure in 95% ethanol (15 mL) over 10% Pd/C (25 mg) as described for compound **8**. The product, obtained after removal of solvent and catalyst, was precipitated by triturating with dry ether to give **10** as a brown solid (63 mg, 90%): mp 135–137 °C dec; TLC (solvent B) R_f 0.35; ^1H NMR (Me_2SO-d_6) δ 0.7–0.95 (m, 6 H), 1.07–1.19 (br m, 3 H), 1.37–1.68 (br m, 4 H), 1.72–1.86 (m, 2 H), 2.24 (t, 2 H), 2.55–2.75 (br m, 2 H), 3.58 (s, 3 H), 3.97–4.12 (br m, 1 H), 4.40–4.64 (m, 2 H), 6.89 (s, 1 H), 7.95–8.27 (br m, 5 H), 8.31 (s, 1 H), 8.86 (d, 1 H). Anal. ($C_{29}H_{35}N_3O_{11}$) C, H, N.

γ-[(1,4-Dimethoxy-2-methylnaphthyl-3)butyryl]-L-γ-*O*-benzylglutamyl-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (13). A solution of Boc-Glu(OBzl)-Glu(OBzl)-Leu-OMe (**12**; 346 mg, 0.52 mmol)[10] was deprotected with 4 N HCl–dioxane and coupled with (1,4-dimethoxy-2-methylnaphthyl-3)butyric acid[12] (**11**; 123 mg, 0.43 mmol) in methylene chloride by using HOBt (0.08 g, 0.55 mmol) and DCC (0.11 g, 0.55 mmol) as the coupling reagents. The reaction mixture was stirred for 6 h at 0 °C and then overnight at room temperature. The solution was cooled, filtered to remove dicyclohexylurea, and worked up as for **5**. The crude product was recrystallized from ethyl acetate/*n*-hexane to give 0.45 g (60%) of **13**: TLC (solvent C) R_f 0.89. The NMR was consistent with the expected product. This material was used directly in the next reaction.

γ-[(1,4-Dimethoxy-2-methylnaphthyl-3)butyryl]-L-glutamyl-L-glutamyl-L-leucine Methyl Ester (14). The protected dimethyl quinol ether tripeptide (**13**; 90 mg, 0.11 mmol) was mixed with 10% Pd/C (20 mg), and the benzyl esters were removed as described for **6**. The product was triturated with ether/*n*-hexane to give 55 mg of **14** obtained as a white solid (78%): mp 199–201 °C; TLC (solvent C) R_f 0.11; *MS*, *m/e* (% intensity) 671 (0.19), 638 (3.02), 526 (0.46), 399 (1.70), 288 (21.25), 271 (10.68), 255 (23.14), 241 (9.22), 228 (27.99), 211 (24.64). Amino acid analysis: Glu 1.98, *Leu* 1.00.

[(2-Naphthyloxy)acetyl]-L-γ-*O*-benzylglutamyl-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (15). To a stirred solution of (2-naphthyloxy)acetic acid (0.15 g, 0.77 mmol) in dry THF (5 ml) at −15 °C was added isobutyl chloroformate (0.1 g, 0.77 mmol) followed by *N*-methylmorpholine (0.084 mL, 0.77 mmol). The reaction mixture was stirred for 5 min and then a precooled solution of tripeptide **3** (0.44 g, 0.75 mmol) in dry THF (4 mL) was added dropwise. The reaction was carried out and worked up as described for **5**. The crude product obtained was recrystallized from ethyl acetate/*n*-hexane to give **15**: 0.48 g (83%); mp 121–122 °C; TLC (solvent D) R_f 0.57; $[\alpha]^{24}_D$ −18.42° (*c* 0.57, MeOH), ^1H NMR ($CDCl_3$) δ 0.90 (d, 6 H), 1.42–1.76 (br m, 3 H), 1.75–2.64 (m, 8 H), 3.69 (s, 3 H), 4.35–4.75 (m, 5 H), 5.05 (s, 4 H), 6.88 (d, 1 H), 7.02–7.47 (br s, 17 H), 7.48–7.89 (m, 2 H). Anal. ($C_{43}H_{49}N_3O_{10}$) C, H, N.

[(2-Naphthyloxy)acetyl]-L-glutamyl-L-glutamyl-L-leucine Methyl Ester (16). Compound **15** (0.2 g, 0.26 mmol) was hydrogenated at atmospheric pressure in 95% ethanol (15 mL) over 10% Pd/C (60 mg) as described. The product obtained after removal of solvent and catalyst was precipitated with dry ether. Recrystallization from ethyl acetate/*n*-hexane gave a brownish gel, which was filtered and dried to give **16**: 0.12 g (80%); mp 175–176 °C; TLC (solvent B) R_f 0.50. Anal. ($C_{29}H_{37}N_3O_{10}$) C, H, N.

Stearyl-L-γ-*O*-benzylglutamyl-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (17). The mixed anhydride of stearic acid (0.2 g, 0.72 mmol) in THF (4 mL) was generated from isobutyl chloroformate (0.098 g, 0.72 mmol) and *N*-methylmorpholine (0.08 mL, 0.72 mmol), as described for compound **7**. To this anhydride was added dropwise a precooled solution of tripeptide **3** (0.41 g, 0.7 mmol) in dry THF (4 mL). The reaction was carried out and worked up as described for **5**. The crude product was recrystallized from ethyl acetate/*n*-hexane to yield 0.47 g (80%) of **17**: mp 92–93 °C; TLC solvent D) R_f 0.40; $[\alpha]^{24}_D$ −23.9° (*c* 0.57, MeOH); ^1H NMR ($CDCl_3$) δ 0.90 (d, 6 H), 1.24 (br m, 33 H), 1.42–1.76 (m, 3 H), 1.76–2.30 (br m, 6 H), 2.31–2.67 (m, 4 H), 3.68 (s, 3 H), 4.31–4.76 (br m, 3 H), 5.10 (s, 4 H), 6.56 (d, 1 H), 6.99 (d, 1 H), 7.32 (m, 11 H). Anal. ($C_{49}H_{75}N_3O_9$) C, H, N.

Stearyl-L-glutamyl-L-glutamyl-L-leucine Methyl Ester (18). Compound **17** (0.19 g, 0.22 mmol was hydrogenated at atmospheric pressure in a mixture of *tert*-butyl alcohol (8 mL) and methanol (10 mL) over 10% Pd/C (100 mg) as described earlier. The product obtained after removal of the catalyst and solvent was precipitated with dry ether to give **18**: 0.14 g (93%); mp 146–148 °C; TLC (solvent B) R_f 0.47. Anal. ($C_{35}H_{63}N_3O_9$) C, H, N.

Carboxylase Assays. Peptides were assayed as substrates for the vitamin K dependent carboxylase by methods previously described.[5,10]

Peptide Separation. Utilization of two potential substrates for the enzyme in the same incubation mixture required development of techniques to separate the carboxylated products. Separation was achieved by chromatography in the presence of tetrabutylammonium phosphate (PIK A) obtained from Waters

Associates, Milford, MA, by a procedure similar to that previously described.[24] Following incubation, the sample was quenched with 3 vol of methanol and centrifuged to remove precipitated proteins. An aliquot of the supernatant was applied to a C-18 Sep Pak (Waters Associates, Milford, MA), which had been washed with 2 mL of MeOH and 2 mL of SIK/PIC A buffer (0.25 M sucrose, 0.025 M imidazole, 0.5 M KCl, 0.005 M PIK A, and pH 7.2). After application, the Sep Pak was washed with SIK/PIC A buffer and the sample eluted with 2 mL of MeOH.

The two carboxylated products were separated by ion-exchanged HPLC on a Dupont Zorbax analytical column. The sample was applied to the column and eluted for 10 min with 25% buffer A (5 mM NH_4OAc, pH 6.0/20% MeOH) and 75% buffer B (500 mM NH_4OAc, pH 6.0/20% MeOH). At 25 min the elution was altered to 10% buffer A and 90% buffer B and at 27 min to 100% buffer B. The eluate was monitored at 230 nm for the first 10 min (to detect Boc tripeptide) and at 257 nm after 10 min (to detect quinone tripeptide). Fractions of about 600 μL were collected at 0.5-min intervals, and radioactivity was determined by liquid scintillation spectrometry.

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Registry No. 1, 82376-80-5; 2, 90764-20-8; 3, 77302-67-1; 4, 77302-88-6; 5, 82376-81-6; 6, 82376-79-2; 7, 90764-21-9; 8, 90764-22-0; 9, 90764-23-1; 10, 90764-24-2; 11, 82376-82-7; 13, 82376-83-8; 14, 82376-85-0; 15, 90764-25-3; 16, 90764-26-4; 17, 90790-64-0; 18, 90790-65-1; 1,4-naphthoquinone, 130-15-4; digluteroyl peroxide, 10195-54-7; vitamin K dependent carboxylase, 81181-72-8.

Nonquaternary Cholinesterase Reactivators. 2. α-Heteroaromatic Aldoximes and Thiohydroximates as Reactivators of Ethyl Methylphosphonyl–Acetylcholinesterase in Vitro

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We prepared six pairs of α-heteroaromatic aldoximes, RC(=NOH)H, and thiohydroximates, RC(=NOH)S-$(CH_2)_2N(C_2H_5)_2$, where R represents various oxadiazole and thiadiazole rings. Each compound was characterized with respect to the following: structure, (hydroxyimino)methyl acid dissociation constant, nucleophilicity toward trigonal carbon and tetrahedral phosphorus, octanol–buffer partition coefficient, reversible inhibition of eel acetylcholinesterase (AChE), and in vitro reactivation of AChE inhibited by ethyl *p*-nitrophenyl methylphosphonate. Eight of the twelve compounds significantly reactivate ethyl methylphosphonyl–AChE, but inherent reactivities are moderate to low: the most potent nonquaternary reactivator, 3-phenyl-5-[(hydroxyimino)methyl]-1,2,4-oxadiazole, is 17 times less reactive than the well-known reactivator 2-[(hydroxyimino)methyl]-1-methylpyridiniun iodide (2-PAM). One of the nonquaternary compounds, 3-phenyl-1,2,4-oxadiazole-5-thiohydroximic acid 2-(diethylamino)ethyl *S*-ester, is a powerful reversible inhibitor of AChE (I_{50} = 7.5 μM). The observed relationships between nonquaternary compound structure, reactivation potency, and anti-AChE activity reveal important molecular requirements for high reactivity toward phosphonylated AChE.

Many organophosphorus (OP) compounds irreversibly inhibit acetylcholinesterase (AChE).[1-7] Therapy of intoxication by these nerve poisons relies on coadministration of cholinolytics (e.g., atropine) and so-called cholinesterase reactivators.[8-11] The latter drugs combine strong reversible binding to inhibited AChE with high inherent nucleophilicity to effect rapid displacement of inhibitor from the enzyme. For all practical purposes, pyridinium aldoximes, such as 2-[(hydroxyimino)methyl]-1-methylpyridinium halide (2-PAM), are the only cholinesterase reactivators currently available for clinical or emergency first-aid application. As quaternary ammonium salts, the pyridinium aldoximes penetrate poorly from the serum into hydrophobic cell membranes. Limited tissue distribution (especially within the central nervous system[12-14]) for pyridinium oximes is a potentially serious disadvantage that could be improved upon, in principle, by developing nonquaternary cholinesterase reactivators.

In our search for improved anticholinesterase agent antidotes, we previously prepared and evaluated[15,16] a series of α-keto thiohydroximic acid (dialkylamino)alkyl *S*-esters, 1. Our objective was to incorporate into a single molecule

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