compound was prepared by general procedure D from HCl·Val-(*S,S*)-Sta-Ala-Ipa and isovaleric anhydride with DMF as solvent. Crystallization yielded pure product: NMR (Me_2SO-d_6) δ 0.83–1.17 (m, 24 H), 1.20–1.50 (m, 8 H), 2.0–2.33 (m, 4 H), 3.55–4.44 (m, 5 H), 4.84 (d, J = 6 Hz, 1 H), 7.22 (m, 1 H), 7.60–7.67 (m, 3 H). See Table I for other physical and microanalytical data.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (AM 20100).

Registry No. **3**, 72155-64-7; **4**, 84850-91-9; **5**, 84850-92-0; **6**, 84850-93-1; **7**, 84850-94-2; **8**, 84850-95-3; **9**, 84850-96-4; **10**, 84850-97-5; **11**, 84850-98-6; **12**, 84850-99-7; **13**, 50903-48-5; **14**, 84851-00-3; **15**, 84851-01-4; **16**, 84851-02-5; **17**, 84851-03-6; **18**, 84851-04-7; **19**, 84851-05-8; **20**, 84851-06-9; **21**, 84851-07-0; **22**, 72155-75-0; **23**, 84851-08-1; **24**, 84851-09-2; **25**, 84851-10-5; **26**, 84851-11-6; **27**, 84851-12-7; **28**, 84863-67-2; **29**, 84851-13-8; **30**, 84851-14-9; **31**, 84851-15-0; **32**, 84851-16-1; **33**, 84851-17-2; **34**, 84851-18-3; **35**, 84851-19-4; **36**, 84851-20-7; **37**, 84851-21-8; **38**, 84851-22-9; **39**, 84863-68-3; **40**, 84851-23-0; **41**, 84851-24-1; **42**, 84851-25-2; **43**, 84851-26-3; **44**, 84851-27-4; **45**, 84062-22-6; **46**, 84851-28-5; **47**, 84851-29-6; **48**, 84851-30-9; Ala-Phe-OMe·HCl, 2280-75-3; (3*S*,4*S*)-Boc-Sta-OH, 58521-49-6; (*S,S*)-Sta-Ala-Phe-OMe·HCl, 84851-31-0; Boc-L-Val anhydride, 33294-55-2; Val-(*S,S*)-Sta-Ala-Phe-OMe·HCl, 84851-32-1; Ala-Leu-OMe·HCl, 59515-79-6; (*S,S*)-Sta-Ala-Leu-OMe·HCl, 84851-33-2; Val-(*S,S*)-Sta-Ala-Leu-OMe·HCl, 84851-34-3; Ala-Gly-OMe·HCl, 23404-09-3; (*S,S*)-Sta-Ala-Gly-OMe·HCl, 84851-35-4; Val-(*S,S*)-Sta-Ala-Gly-OMe·HCl, 84851-36-5; Boc-Leu-OH, 13139-15-6; Leu-Iaa·HCl, 84851-37-6; (*S,S*)-Sta-Leu-Iaa·HCl, 84851-38-7; Val-(*S,S*)-Sta-Leu-Iaa·HCl, 84863-69-4; Boc-Gly-OH, 4530-20-5; Gly-Ias·HCl, 84851-39-8; (*S,S*)-Sta-Gly-Iaa·HCl, 84851-40-1; Val-(*S,S*)-Sta-Gly-Iaa·HCl, 84851-41-2; (*S,S*)-Sta-Ala-Iaa·HCl, 84894-08-6; Gly-(*S,S*)-Sta-Ala-Iaa·HCl, 84851-42-3; Boc-Ala anhydride, 33294-53-0; Ala-(*S,S*)-Sta-Ala-Iaa·HCl, 84851-43-4; Boc-Leu anhydride, 51499-91-3; Leu-(*S,S*)-Sta-Ala-Iaa·HCl, 84851-44-5; Boc-Phe anhydride, 33294-54-1; Phe-(*S,S*)-Sta-OEt·HCl, 84851-45-6; (*S,S*)-Sta-OEt·HCl, 84851-46-7; Val-(*S,S*)-Sta-OEt·HCl, 84851-47-8; Ala-OMe·HCl, 2491-20-5; Val-Val-(*S,S*)-Sta-OEt·HCl, 84851-48-9; Val-Val-(*S,S*)-Ala-OMe·HCl, 84851-49-0; (*S,S*)-Sta-Ipa·HCl, 84851-50-3; Val-(*S,S*)-Sta-Ipa·HCl, 84851-51-4; Iva-Val-(*S,S*)-Sta-OH, 84851-52-5; Ala-NH-CH_3·HCl, 61275-22-7; Ala-Ipa·HCl, 84851-53-6; (*S,S*)-Sta-Ala-Ipa·HCl, 84851-54-7; Val-(*S,S*)-Sta-Ala-Ipa·HCl, 84851-55-8; isovaleric anhydride, 1468-39-9; isoamylamine, 107-85-7; isopropylamine, 75-31-0; methylamine, 74-89-5; pepsin, 9001-75-6.

Synthesis and Biological Properties of New Hexapeptide Substrates for Vitamin K Dependent Carboxylase. Evidence for X-Pro Cis/Trans Amide Bond Interconversions in Prothrombin Precursor Fragment 18–23

Daniel H. Rich,*[†] Megumi Kawai,[†] Hedda L. Goodman,[‡] and John W. Suttie[‡]

School of Pharmacy and Department of Biochemistry, College of Agriculture and Life Science, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received August 23, 1982*

Three hexapeptide analogues, corresponding to sequence 18–23 of bovine prothrombin precursor [-Cys-Leu-Glu-Glu-Pro-Cys-] have been synthesized and evaluated as substrates for vitamin K dependent carboxylase. These new hexapeptides are moderately good substrates for the carboxylase but do not significantly inhibit carboxylation of Phe-Leu-Glu-Glu-Leu, a good substrate for the enzyme. Based on proton and carbon-13 NMR experiments, it is established that the conformation of sequence 18–23, which contains proline at position 22, has a trans amide bond for the Glu-Pro[22] sequence in chloroform-*d*. This amide bond is converted to the cis amide geometry in Me_2SO-d_6. It is proposed that good substrates for the carboxylase require a trans amide bond between residues 21 and 22.

Vitamin K is required for the posttranslational carboxylation of L-glutamyl (Glu) residues to γ-carboxyglutamyl residues (Gla)[1] in microsomal precursors of prothrombin,[2–4] clotting factors VII, IX, and X,[5] plasma proteins C, Z, and S,[5,6] and a limited number of other proteins.[6,7] This transformation from Glu to Gla converts these inactive prothrombin precursors to their biologically active forms.[7,8] The enzyme catalyzing this reaction, vitamin K dependent carboxylase,[9] requires oxygen, reduced vitamin K, and carbon dioxide in order to carboxylate the γ-carbon of Glu.[6,8] Location of Gla residues in three clotting proteins is shown in Figure 1.

We have reported that the synthetic pentapeptides H-Phe-Leu-Glu-Glu-Leu-OH (**1**), a pentapeptide corresponding to prothrombin precursor sequence 5–9 (see Figure 1), and the shorter Boc-Glu-Glu-Leu-OMe (**2**) are substrates for the carboxylase and have been utilized to characterize the substrate requirements of the enzyme.[10,11] The carboxylase appears to act selectively at L-glutamyl residues because peptides containing L-aspartyl, L-homoglutamyl, L-glutaminyl, and D-glutamyl residues were not measurably carboxylated in our assay system.[12a] (Recent data suggest L-aspartyl may be carboxylated in certain systems.)[12b] The peptide sequence adjacent to the site of

[†] School of Pharmacy.
[‡] Department of Biochemistry.

(1) Abbreviations used follow IUPAC–IUB tentative nomenclature described in *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations used are: Gla, γ-carboxyglutamic acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Acm, acetamidomethyl.
(2) Stenflo, J.; Fernlund, P.; Egan, W.; Roestorff, P. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 1730.
(3) Magnusson, S.; Sottrup-Jensen, L.; Peterson, T.; Morris, H.; Dell, A. *FEBS Lett.* **1974**, *44*, 189.
(4) Nelsestuen, G.; Zytkovicz, T.; Howard, J. *J. Biol. Chem.* **1974**, *249*, 6347.
(5) Jackson, C. M.; Nemerson, Y. *Annu. Rev. Biochem.* **1980**, *49*, 765.
(6) Suttie, J. W. In "The Enzymology of Post-translational Modification of Proteins"; Freedman, R. B.; H. C. Hawkins, Eds.; Academic Press: London, 1980; pp 213–258.
(7) Suttie, J. W., Ed. "Vitamin K Metabolism and Vitamin K Dependent Protein"; University Park Press: Baltimore, 1980.
(8) Suttie, J. *CRC Crit. Rev. Biochem.* **1980**, *8*, 191.
(9) Esmon, C.; Sadowski, J.; Suttie, J. *J. Biol. Chem.* **1975**, *250*, 4744.
(10) Suttie, J.; Hageman, J.; Lehrman, S.; Rich, D. *J. Biol. Chem.* **1976**, *251*, 5827.
(11) Suttie, J.; Lehrman, R.; Geweke, L.; Hageman, J.; Rich, D. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 500.

0022-2623/83/1826-0910$01.50/0

Table I. Analogues to Prothrombin Sequence 18–23

no.	substrate	A. Substrate Activity at Varying Concentrations 0.25 mM	1 mM	4.25 mM	5.5 mM
7	H-Ala-Leu-Glu-Glu-Pro-Ala-OH	14%	16%	24%	
6b	H-Cys(S—S)-Leu-Glu-Glu-D-Ala-Cys-OH	63%	60%	59%	
6a	H-Cys(S—S)-Leu-Glu-Glu-L-Ala-Cys-OH	50%	48%	56%	
3a[a]	H-Cys(S—S)-Leu-Glu-Glu-Pro-Cys-OH	7.5%	7.0%		8.7%
3b[a]	Ac-Cys(S—S)-Leu-Glu-Glu-Pro-Cys-OMe	3.3%	2.4%		2.2%

B. Carboxylation of 0.25 mM Phe-Leu-Glu-Glu-Leu in the Presence of Hexapeptide Analogues at Varying Concentrations: substrate	1 mM	4.25 mM	5.5 mM
H-Ala-Leu-Glu-Glu-Pro-Ala-OH	99%	134%	
H-Cys(S—S)-Leu-Glu-Glu-D-Ala-Cys-OH	94%	83%	
H-Cys(S—S)-Leu-Glu-Glu-L-Ala-Cys-OH	103%	85%	
H-Cys(S—S)-Leu-Glu-Glu-Pro-Cys-OH	98%[b]		78%[b]
Ac-Cys(S—S)-Leu-Glu-Glu-Pro-Cys-OMe	51%[b]		54%[b]

[a] Data are expressed as the percent of CO_2 incorporation exhibited by Phe-Leu-Glu-Glu-Leu (1) at the same concentration. [b] Data taken from ref 12.

```
                5                  10
Prothrombin -Phe-Leu-Gla-Gla-Val-Arg-Cys-Gly-

Factor X    -Phe-Leu-Gla-Gla-Val-Lys-Gla-Gly-

Protein C   -Phe-Leu-Gla-Gla-Leu-Arg-Pro-Gly-

                    15                  20
            Asn-Leu-Gla-Arg-Gla-Cys-Leu-Gla-Gla-Pro-Cys-

            Asn-Leu-Gla-Arg-Gla-Cys-Leu-Gla-Gla-Ala-Cys-

            Asn-Val-Gla-Arg-Gla-Cys-Ser-Gla-Gla-Val-Cys-

                25                  30                  35
            Ser-Arg-Gla-Gla-Ala-Phe-Gla-Ala-Leu-Gla-Ser-Leu

            Ser-Leu-Gla-Gla-Ala-Arg-Gla-Val-Phe-Gla-Asp-Ala

             ? -Phe-Gla-Gla-Ala-Arg-Gla-Leu-Phe- ? -Asn-Thr
```

Figure 1. Amino acid sequences of vitamin K dependent plasma proteins (bovine). Gla = γ-carboxyglutamic acid.

carboxylation also affects the conversion of glutamyl to γ-carboxyglutamyl. This finding is most clearly illustrated by comparing sequences 5–9 (**1**) and 18–23 (**3**), both of which contain the segment Leu-Glu-Glu; peptide **1** is efficiently carboxylated,[10] while the cyclic disulfide peptides **3a,b** are partial substrates–inhibitors of the carboxylase.[13] Furthermore, Soute et al.[14] reported that segment 13–29 of prothrombin precursor (**4**) is efficiently carboxylated at 1000-fold lower concentrations ($K_m \simeq 1$ μM) than segments 18–23 (**3a,b**) or 24–29 (**5**),[15] as well as segment 5–9 (**1**) (K_m = 8 mmol), implying that some favorable structural or conformational features are present in segment 13–29 that are not present in the smaller segments.

We report here the synthesis and biological properties of three analogues of prothrombin precursor 18–23. Peptides **6a,b** which correspond to the replacement of Pro[22] by L- or D-Ala, were designed to evaluate the effect of a trans amide bond at this position, while linear analogue **7** was synthesized to evaluate the effect the cyclic disulfide ring system has on carboxylation of glutamyl residues in the cyclic system. Preliminary conformational studies of the ring system are also described that indicate the X-Pro amide bond in **3** is very sensitive to the solvent system: in Me_2SO, the amide bond is cis; in $CDCl_3$, it is trans.

Results

Synthesis of Peptides. The synthesis of new cyclic peptides **6a,b** and the linear hexapeptide **7** (Table I) were carried out by the procedures described in our previous paper.[13] The linear hexapeptides **8a,b** and **10** were prepared stepwise in solution starting with Boc-Cys(Acm)-OBzl[1] and Boc-Ala-OBzl,[17] respectively, by coupling with

X-Leu-Glu(OBzl)-Glu(OBzl)-Y-Z

8a, X = Boc-Cys(Acm); Y = L-Ala; Z = Cys(Acm)-OBzl
8b, X = Boc-Cys(Acm); Y = D-Ala; Z = Cys(Acm)-OBzl
10, X = Cbz-Ala; Y = L-Pro; Z = Ala-OBzl

X-Cys(S—S)-Leu-Glu(OBzl)-Glu(OBzl)-Y-Cys(S—S)-OBzl

9a, X = Boc; Y = L-Ala
9b, X = Boc; Y = D-Ala
3c, X = Ac; Y = L-Pro
3d, X = H; Y = L-Pro

(12) (a) Rich, D.; Lehrman, S.; Kawai, M.; Goodman, H.; Suttie, J. *J. Med. Chem.* **1981**, *24*, 706. (b) Hamilton, E. S.; Tesch, D.; Zerner, B. *Biochem. Biophys. Res. Commun.* **1982**, *107*, 246.
(13) Rich, D.; Kawai, M.; Goodman, H.; Suttie, J. *Int. J. Pept. Protein Res.* **1981**, *18*, 41.
(14) Soute, B.; Vermeer, C.; DeMetz, M.; Hemker, H.; Liinen, H. *Biochim. Biophys. Acta* **1981**, 676, 101.
(15) Dhaon, M., unpublished results.
(16) Konig, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788. *Ibid.* **1970**, *103*, 2024. *Ibid.* **1970**, *103*, 2041.

DCC and 1-hydroxybenzotriazole (HOBt).[16] The intermediate peptides were obtained in acceptable yield. They were characterized by NMR and TLC and used without further characterization. The acetamidomethyl (Acm)[18] thiol-protecting groups were removed, and the thiol was simultaneously oxidized by reaction with iodine in methanol[19] to yield the cyclic hexapeptides **9a,b**. Treatment with anhydrous hydrogen bromide in trifluoroacetic acid (HBr/F_3AcOH) removed the remaining protecting groups. The cyclic hexapeptides **6a,b** contained two very minor impurities, which were easily removed by recrystallization.

In contrast, deprotection of the linear hexapeptide **10** by catalytic hydrogenolysis did not go to completion. Reaction in the presence of a large amount of 10% palladium on charcoal catalyst (10% Pd/C) at 40–50 psi for 60 h[20] gave at least six different products in addition to the desired compound. However, all protecting groups were removed within 2 h at room temperature by treatment with ammonium formate–10% Pd/C in methanol.[21] Catalytic transfer hydrogenation with formic acid–10% Pd/C did not work.[22]

Biological Assays. The new cyclic hexapeptides **6a,b** and linear hexapeptide **7** were evaluated as substrates for vitamin K dependent carboxylase; the results are shown in Table I. Both cyclic peptides **6a** and **6b** are carboxylated by the enzyme, are reasonably good substrates when compared with pentapeptide **1**, and are much better substrates than the Pro-containing peptides **3a,b**. It is also remarkable to note that the chirality of the amino acid in the fifth position of the peptide chain does not affect carboxylation. The D-Ala peptide **6b** is a slightly better substrate than the L-Ala peptide **6a**. Surprisingly, linear hexapeptide **7** turned out to be a poor substrate for the carboxylate relative to the two cyclic hexapeptides **6a,b**. The new peptides **6a,b** and **7** were also tested to determine if any would inhibit carboxylation of pentapeptide **1** by the carboxylase (Table I). No significant inhibition was detected for either of the Ala-peptides **6a,b**. In addition, the linear peptide **7** appears to stimulate carboxylation of pentapeptide **1**.

Conformational Studies of the Cyclic Hexapeptides. The ^{13}C NMR data for protected cyclic hexapeptides **9a,b** and **3c** in Me_2SO-d_6 are presented in the supplementary material (Table II). Assignments are based upon comparisons with other model peptides and with synthetic precursors.

The ^{13}C NMR data for the dibenzyl ester Pro-containing cyclic hexapeptide **3c** clearly indicate the presence of one predominant conformation that contains a cis X-Pro amide bond. The Pro β-carbon resonates at 31.09 ppm and the Pro γ-carbon resonates at 22.22 ppm in Me_2SO-d_6 solution, which is consistent with a cis X-Pro bond.[23]

The conformation of the L-Pro-peptide **3c** changes in chloroform solution. The ^{13}C NMR data for cyclic hexapeptides **9a,b** and **3c** are presented in the supplementary material (Table III). The most significant change is the shift of the Pro γ-carbon resonance from 22.2 to 25.3 ppm, consistent with a change to a trans X-Pro amide bond in chloroform.[22]

Chemical shifts, coupling constants, and temperature dependencies for the amide protons of protected cyclic peptide **3c** are shown in Table IV. All assignments were carried out by double resonance experiments. Although we could not assign individual Cys and Glu resonances to particular residues with the data available, it is apparent that one of the Glu amide protons is shielded from the solvent, indicating an intramolecular hydrogen bond. The temperature coefficient ($\Delta\delta/\Delta T$) for one of the Glu NH's is relatively small ($+1.08 \times 10^{-3}$ ppm/°C), and the chemical shift of one of the Glu amide protons is almost solvent independent (Figure 2). The NMR spectrum of the un-

Table IV. Summary of 270-MHz ^1H NMR of

Ac-Cys-Leu-Glu(OBzl)-Glu(OBzl)-Pro-Cys-OMe
(S—S)

residue NH	chem shift, ppm	J_{NH-CH},[a] Hz	$d\delta/dT$,[b] ppm/°C	δ S[c]
Cys	8.90	8.5	-4.87×10^{-3}	+0.95
Leu	8.74	8.9–9.0	-4.54×10^{-3}	+0.95
Glu	8.49	7.2–7.3	-5.78×10^{-3}	+0.94
Glu	7.28	8.0	$+1.08 \times 10^{-3}$	−0.07
Cys	8.38	8.5	-6.75×10^{-3}	+2.24

[a] Concentration: 3.68 mg to 5.47 mg/0.4 mL of Me_2SO-d_6. [b] Concentration range: 3.24–3.69 mg/0.4 mL of mixed solvent. Temperature range: 0–40 °C. [c] $\delta(CHCl_3) - \delta(Me_2SO\text{-}d_6)$.

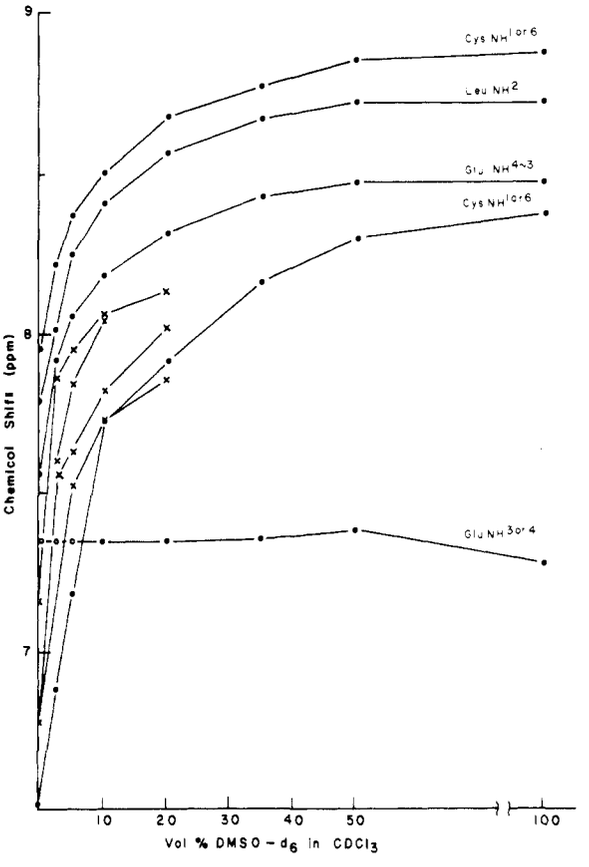

Figure 2. Solvent dependence of Ac-Cys-Leu-Glu(OBzl)-Glu(OBzl)-Pro-Cys-OMe. Concentration range: 3.24–3.69 mg/0.4 mL of solvent (270 MHz).

(17) Laufer, D.; Chapman, T.; Marlborough, D.; Vaidya, V.; Blout, E. *J. Am. Chem. Soc.* **1968**, *90*, 2696.
(18) Veber, D.; Mikowski, J.; Denkewalter, R.; Hirschmann, R. *Tetrahedron Lett.* **1968**, *26*, 3057.
(19) Kamber, B. *Helv. Chim. Acta* **1971**, 927.
(20) We have used 500 mg of Pd/C catalyst for 1.0 g of the compound. Hydrogenation was carried out at 50 psi for 20 h, and the solution was filtered. Fresh catalyst was added, and the hydrogenation was repeated twice.
(21) Anwer, M.; Spatola, A. *Synthesis* **1980**, 929.
(22) ElAmin, B.; Anantharamaiah, G.; Royer, G.; Means, G. *J. Org. Chem.* **1979**, *44*, 3442.
(23) Deber, C.; Madison, V.; Blout, E. *Acc. Chem. Res.* **1976**, *9*, 106, and references therein.

protected hexapeptide **3d** shows the same chemical-shift pattern in the NH region. This indicates that the hydrogen bond is retained in the molecule even though the N-terminal NH is free. Thus, the shielded Glu NH is not hydrogen bonded to the carbonyl of the *N*-acetyl group in **3c**.

A possible conformation for the Pro-containing cyclic hexapeptide **3c** posits a hydrogen bond between the Glu[4] NH and the carbonyl group of Cys[1]. The torsional angles for this conformation are consistent with the observed coupling constants. A schematic diagram of this conformation is shown in Figure 3; approximate torsional angles at $i + 1$ and $i + 2$ of the β-turn are listed below. An alternative γ-turn conformation involving the Glu[3] NH is not consistent with the coupling constants.

	Leu[2]	Glu[3]
ϕ	+60°	−80°
ψ	−100°	−10°

Preliminary NMR studies (solvent titrations, temperature-dependency studies) indicate that the L-Ala-containing cyclic hexapeptide **6b** does not have a solvent-shielded amide proton. It is very interesting that even though the Pro residue in peptide **3c** does not participate in the β-turn structure, the Pro residue facilitates the hydrogen-bonded conformation through the cis X-Pro conformer.

Discussion

The hexapeptides reported in this paper are analogues of the sequence 18–23 of bovine prothrombin precursor (Figure 1). Carboxylation of this sequence when it is present in the endogenous protein substrate for the carboxylase proceeds to completion; all 10 Glu residues in precursor protein are carboxylated,[6,8] and the reaction is fast.[11] It therefore was surprising to find that hexapeptides **3a,b** not only are poor substrates for the carboxylase but also inhibited it. Thus, **3b** binds to the carboxylase but is only poorly carboxylated by it. Subsequently, Soute et al. reported that the prothrombin precursor fragment **4**, assigned to sequence 13–29, is a remarkedly good substrate for the carboxylase and is carboxylated at a rate approaching that of the endogenous precursor.[14] These results strongly suggested that some property of the 18–23 sequence differs in the small peptide. One possible difference could be in the conformation of this 20-membered heterodectic ring system in **3a,b** vs. **4**.

The NMR data reported here establish that the hexapeptide disulfide ring system 18–23 is conformationally labile about the X-Pro amide bond in peptide **3c**. In chloroform this amide bond is trans, but in Me_2SO this amide bond is cis. Furthermore, the Me_2SO conformation of peptide **3c** exhibits a solvent-shielded amide proton, assigned to either Glu[3] or Glu[4]. These results suggest a hydrogen-bonded conformation involving a Glu amide proton plus the cis X-Pro bond. The conformation shown in Figure 3 is consistent with the observed coupling constants, but until the tentative assignment of the invariant NH to Glu[4] has been confirmed by other experiments, the proposed conformation is only one of many possibilities. The most important feature of the proposed conformation is the presence of the cis amide bond in Me_2SO.

It must be stressed that the proposed ring conformation (Figure 3) is for the protected hexapeptide in which the Glu residues are functionalized as benzyl esters. Undoubtedly, the presence of free acids rather than benzyl esters at these positions will influence the overall conformation of the hexapeptides, especially as the ionic state of the carboxy group changes with pH. There is evidence that the X-Pro[22] bond in prothrombin or in F_1 fragment 1–42 adopts a cis conformation, as binding to phospholipids follows a first-order activation process consistent with a cis to trans isomerization of the X-Pro bond.[24,25] The direction of isomerization is probably cis to trans because secondary amides are preferentially trans and because coagulation peptides lacking Pro[22] do not exhibit the time-dependent binding.

The Ala[22] and D-Ala[22] peptides **6a** and **6b** were synthesized to determine if the inhibitory properties of **3b** were influenced by the presence of a cis amide bond between positions 21 and 22 in cyclic hexapeptide **3b**. The assumption here, that the secondary amide bond, X-Ala, will favor a trans X-Ala amide bond over a cis amide bond, is based on numerous theoretical energy calculations that predict the trans amide bond is more stable by 8 kcal.[26] While cis secondary amide bonds have been observed in cyclic peptides, these are usually found in relatively strained systems, such as cyclic tetrapeptides.[27,28]

When tested in the carboxylase assay, both Ala peptides **6a** and **6b** were found to be good substrates for the carboxylase. Generally, they are carboxylated about half as well as standard pentapeptide **1** or tripeptide **2** but also about 10-fold better than the corresponding Pro[22] analogues **3a,b**. Thus, the replacement of Pro[22] with Ala[22] converts a partial substrate into a significantly better substrate. In view of the energetic conformational preferences of X-Ala for a trans amide[26] bond and the NMR data described here, which suggest the Ala peptides have different conformations than the Pro peptide **3b**, it is reasonable to propose that the substrate conformations of peptide sequence 18–23 have a trans amide bond between residues 21 and 22, and the inhibitor conformation has a cis X-Pro amide bond. If the report of Soute et al.[14] is correct, then it follows that the added peptide fragments in sequences 13–17 and 24–29 stabilize the 21–22 amide bond in the trans conformation as part of their contribution to the improved substrate binding of the 13–29 segment. Other factors, such as additional binding sites or favorable partition properties, may also contribute to the improved substrate properties. The synthesis of larger precursor segments related to the 13–29 sequence **4** is in progress.

Experimental Section

General Methods. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TX. Melting points and the thin-layer chromatography were carried out as previously described. Solvent systems used are: A, 6% methanol in chloroform; B, 10% methanol in chloroform; C, 15% methanol in chloroform; D, 7% ethanol in ethyl acetate; E, 1-butanol/pyridine/acetic acid/water (15:10:3:12); F, 1-butanol/water/acetic acid (4:1:1). Proton NMR spectra were recorded on a Bruker HX-90E (90 MHz) or WH-270 (270 MHz) spectrometer operated in the Fourier-transform mode. Carbon-13 NMR spectra were recorded on a JEOL FX 90 Q (22.5 kHz) spectrometer. Chemical shifts (δ) were reported as parts per million (ppm) relative to tetramethyl silane as an internal reference. Amino acid analyses were determined on a Durrum D500 amino acid analyzer. Chloroform-*d* (99.8% atom % D, Gold Label) and dimethyl-d_6 sulfoxide (99.9%

(24) Marsh, H.; Scott, M.; Hiskey, R.; Koehler, K. *Biochem. J.* **1979**, *183*, 513.
(25) Marsh, H.; Sarasua, M.; Madar, D.; Boggs, N.; Pedersen, L.; Hiskey, R.; Koehler, K. In "Peptides: Synthesis, Structure, Function"; Proceedings of the American Peptide Symposium, 7th, Rich, D. H.; Gross, E., Eds.; Pierce Chemical Co.: Rockford, IL, 1981; pp 277–286.
(26) Zimmerman, S.; Scheraga, H. *Macromolecules* **1976**, *9*, 408.
(27) Dale, J.; Titlestad, K. *Tetrahedron Lett.* **1978**, 379.
(28) Meyer, W.; Kuyper, L.; Phelps, D.; Cordes, A. *J. Chem. Soc., Chem. Commun.* **1974**, 339.

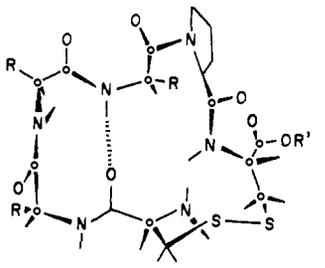

Figure 3. Proposed conformation of cyclic hexapeptide.

atom % D, Gold Label) were purchased from Aldrich Chemical Co. Inc. Boc-protected amino acid derivatives were obtained from Bachem Inc. and Penninsula Laboratories, Inc.

General Procedure A. Removal of the *tert*-Butyloxycarbonyl Group. The Boc-protected amino acid or peptide ester (1 mmol) was dissolved in 4 N HCl in dioxane (about 5 mL), and the solution was stirred at room temperature for 45–60 min. After excess reagent was removed under reduced pressure, the residue was reevaporated twice from the anhydrous ether and then dried over potassium hydroxide in a desiccator in vacuo (oil pump) for at least 3 h. It was not characterized unless stated otherwise.

General Procedure B. Coupling Reactions via DCC–HOBt. Amino acid ester or peptide ester hydrochloride (1 mmol) was dissolved in methylene chloride or in mixed solvent and cooled in an ice bath with stirring. *N*-Methylmorpholine (NMM) (1 mmol) was added. After 5–10 min, Boc-protected amino acid (1 mmol), HOBt (1.1 mmol), and DCC (1 mmol) were added. The reaction was carried out at 0 °C for 5 h, at 4 °C overnight, and at room temperature for 3–5 h. The reaction mixture was filtered to remove DCU, and the filtrate was evaporated to dryness under reduced pressure.

The residue was dissolved in ethyl acetate, washed successively with water, saturated $NaHCO_3$ (3 times), water, 1 N citric acid (3 times), and water, and then dried over anhydrous sodium sulfate or magnesium sulfate. It was then recrystallized or purified by silica gel chromatography.

Boc-Ala-Cys(Acm)-OBzl (11a). Boc-Cys(Acm)-OBzl (1.148 g, 3 mmol) was treated with 30 mL of 4 N HCl in dioxane under general procedure A and coupled with Boc-Ala-OH (0.568 g, 3 mmol) in the presence of NMM (0.33 mL, 3 mmol), HOBt (0.505 g, 3.3 mmol), and DCC (0.619 g, 3 mmol) in 50 mL of CH_2Cl_2/DMF (3:2) mixture. It was treated as in general procedure B and then recrystallized from 15 mL of ethyl acetate and 40 mL of Skellysolve B to yield 1.108 g (81.4%) of the title compound: mp 89–90 °C; R_f (A) 0.18, F_f (D) 0.42; ^{1}H NMR (90 MHz in $CDCl_3$) was consistent with the structure.

Boc-D-Ala-Cys(Acm)-OBzl (11b). The title compound was prepared from Boc-D-Ala-OH (1.514 g, 8 mmol), HCl·H-Cys-(Acm)-OBzl (2.534 g, 8 mmol), NMM (0.88 mL, 8 mmol), HOBt (1.348 g, 8.8 mmol), and DCC (1.650 g, 8 mmol) in 40 mL of CH_2Cl_2/DMF (1:1) mixture, following general procedure B. The crude product was recrystallized from 20 mL of ethyl acetate and 50 mL of Skellysolve B: yield 2.890 g (79.6%); mp 122–123 °C; R_f (A) 0.27, R_f (D) 0.47; ^{1}H NMR (90 MHz in $CDCl_3$) consistent with structure 11b.

Boc-Pro-Ala-OBzl (11c). *p*-Tos-OH·H-Ala-OBzl (5.271 g, 15 mmol), NMM (1.650 mL, 15 mmol), Boc-Pro-OH (3.229 g, 15 mmol), HOBt (2.527 g, 16.5 mmol), and DCC (3.095 g, 15 mmol) in 50 mL of CH_2Cl_2 were used to prepare the title compound. Recrystallization from 30 mL of ethyl acetate gave 3.963 g (70.2%) of pure compound: mp 113–114 °C; R_f (A) 0.76, R_f (D) 0.73; ^{1}H NMR (90 MHz in $CDCl_3$) was consistent with the structure 11b. Another 0.362 g (6.4%) of pure compound (Ic) was obtained from the filtrate.

Boc-Glu(OBzl)-Ala-Cys(Acm)-OBzl (12a). Compound 11a (1.043 g, 2.3 mmol) was deprotected and coupled with Boc-Glu-(OBzl)-OH (0.776 g, 2.3 mmol) in the presence of NMM (0.253 mL, 2.3 mmol), HOBt (0.387 g, 2.5 mmol), and DCC (0.475 g, 2.3 mmol) in 5 mL of DMF and 20 mL of CH_2Cl_2. The obtained product was recrystallized from 15 mL of ethyl acetate and 60 mL of Skellysolve B: yield 1.154 g (74.6%); mp 119 °C; R_f (B) 0.59, R_f (D) 0.51; ^{1}H NMR (90 MHz in $CDCl_3$) consistent with structure 12a.

Boc-Glu(OBzl)-D-Ala-Cys(Acm)-OBzl (12b). Compound 11b (2.721 g, 6 mmol) was treated with 4 N HCl in dioxane and coupled with Boc-Glu(OBzl)-OH (2.024 g, 6 mmol) in the presence of NMM (0.66 mL, 6 mmol), HOBt (1.011 g, 6.6 mmol), and DCC (1.238 g, 6 mmol) in 30 mL of mixed solvent (DMF/CH_2Cl_2, 1:1). After recrystallization from 20 mL of ethyl acetate and 40 mL of Skellysolve B, it was purified further by chromatography over silica gel eluting with 2% methanol in chloroform: yield 2.620 g (64.9%); mp 142–143 °C; R_f (B) 0.53, R_f (A) 0.29; ^{1}H NMR (90 MHz in $CDCl_3$) consistent with structure 12b.

Boc-Glu(OBzl)-Pro-Ala-OBzl (12c). Compound 11c (3.765 g, 10 mmol) was deprotected and coupled with Boc-Glu(OBzl)-OH (3.374 g, 10 mmol) in the presence of NMM (1.10 mL, 10 mmol), HOBt (1.685 g, 11 mmol), and DCC (2.063 g, 10 mmol) in 40 mL of CH_2Cl_2. The obtained compound was purified on a silica gel column (solvent, 2% methanol in chloroform; column size, 3.5 × 47 cm); 5.182 g (87.0%) of pure 12c was obtained as an oil: R_f (A) 0.72; R_f (D) 0.73; ^{1}H NMR consistent with structure 12c.

Boc-Glu(OBzl)-Glu(OBzl)-Ala-Cys(Acm)-OBzl (13a). Compound 12a (1.009 g, 1.5 mmol) was deprotected and coupled with Boc-Glu(OBzl)-OH (0.506 g, 1.5 mmol) in 20 mL of mixed solvent (DMF/CH_2Cl_2, 1:3), in the presence of NMM (0.165 mL, 1.5 mmol), HOBt (0.253 g, 1.65 mmol), and DCC (0.309 g, 1.5 mmol). It was recrystallized from 35 mL of ethyl acetate–2 mL of methanol and 20 mL of ether: yield 1.087 g (81.2%); mp 155–156 °C; R_f (B) 0.45; R_f (D) 0.51; ^{1}H NMR (270 MHz in $CDCl_3$) consistent with structure 13a.

Boc-Glu(OBzl)-Glu(OBzl)-D-Ala-Cys(Acm)-OBzl (13b). Compound 12b (2.610 g, 3.88 mmol) was treated with 4 N HCl in dioxane and coupled with Boc-Glu(OBzl)-OH (1.309 g, 3.88 mmol) in the presence of NMM (0.427 mL, 3.88 mmol), HOBt (0.654 g, 4.27 mmol), and DCC (0.800 g, 3.88 mmol) in 40 mL of mixed solvent (DMF/CH_2Cl_2, 1:1). The product obtained was purified by chromatography over silica gel (solvent, 2.5% methanol in chloroform): yield 2.510 g (72.5%); mp 139–140 °C; R_f (B) 0.55, R_f (A) 0.47, R_f (D) 0.60; ^{1}H NMR consistent with 13b.

Boc-Glu(OBzl)-Glu(OBzl)-Pro-Ala-OBzl (13c). Compound 12c (2.978 g, 5 mmol) was deprotected and coupled with Boc-Glu(OBzl)-OH (1.687 g, 5 mmol) by using NMM (0.55 mL, 5 mmol), HOBt (0.842 g, 5.5 mmol), and DCC (1.032 g, 5 mmol) in 35 mL of CH_2Cl_2. The obtained product was dissolved in 20 mL of ether, cooled in an ice bath, and filtered. The filtrate was evaporated to dryness and kept in a refrigerator for 3 days to yield crystals, which were recrystallized from 15 mL of ether and 50 mL of Skellysolve B to yield 3.861 g (97.4%) of 13c: mp 95–97 °C; R_f (A) 0.70 R_f (D) 0.75; ^{1}H NMR consistent with structure 13c.

Boc-Leu-Glu(OBzl)-Glu(OBzl)-Ala-Cys(Acm)-OBzl (14a). Compound 13a (1.070 g, 1.2 mmol) was converted to the hydrochloride and coupled with Boc-Leu-OH·H_2O (0.299 g, 1.2 mmol) by using NMM (0.132 mL, 1.2 mmol), HOBt (0.202 g, 1.32 mmol), and DCC (0.248 g, 1.2 mmol) in 5 mL of DMF/15 mL of CH_2Cl_2. It was worked up as described in general procedure A, except 100 mL of CH_2Cl_2 was used instead of ethyl acetate. Recrystallization from 15 mL of methanol and 75 mL of ether gave pure compound 14a: yield 824.6 mg (68.4%); mp 182–183 °C; R_f (B) 0.44, R_f (D) 0.50; ^{1}H NMR (270 MHz in $CDCl_3$) consistent with the structure.

Boc-Leu-Glu(OBzl)-Glu(OBzl)-D-Ala-Cys(Acm)-OBzl (14b). Deprotection of compound 13b (2.408 g, 2.7 mmol) was carried out, and the amine was coupled with Boc-Leu-OH·H_2O (0.673 g, 2.7 mmol) by using NMM (0.297 mL, 2.7 mmol), HOBt (0.455 g, 2.7 mmol), and DCC (0.557 g, 2.7 mmol) in 35 mL of CH_2Cl_2. The obtained product was recrystallized from 30 mL of methanol–30 mL of ether and 40 mL of Skellysolve B to yield 2.158 g (79.5%) of 14b: R_f (A) 0.45, R_f (D) 0.60; ^{1}H NMR (90 MHz in $CDCl_3$) consistent with the structure of 14b.

Boc-Leu-Glu(OBzl)-Glu(OBzl)-Pro-Ala-OBzl (14c). Compound 13c (2.445 g, 3 mmol) was deprotected and coupled with Boc-Leu-OH·H_2O (0.748 g, 3 mmol) in 25 mL of CH_2Cl_2 by using NMM (0.33 mL, 3 mmol), HOBt (0.505 g, 3 mmol), and DCC (0.619 g, 3 mmol). The obtained oil was dissolved in 20 mL of ether, cooled in an ice bath for 2 h, and filtered. The filtrate was evaporated to dryness to yield 2.613 g (93.9%) of gummy residue, containing, according to TLC results, three very minor impurities [R_f (A) 0.83, 0.17, and 0.00. Compound 14c: R_f (A) 0.67; R_f (D) 0.84, 0.41, and 0.00. For compound 14c: R_f (C) 0.78]. This was

used to next step without further purification.

Boc-Cys(Acm)-Leu-Glu(OBzl)-Glu(OBzl)-Ala-Cys-(Acm)-OBzl (8a). Compound **14a** (804.2 mg, 0.8 mmol) was deprotected and coupled with Boc-Cys(Acm)-OH (257.3 mg, 0.88 mmol) in 20 mL of mixed solvent (DMF/CH_2Cl_2, 1:1) by using NMM (0.0968 mL, 0.8 mmol), HOBt (134.8 mg, 0.88 mmol), and DCC (181.5 mg, 0.88 mmol). It was purified by chromatography over silica gel (solvent, 2% methanol in chloroform; column size, 1.6 × 50 cm) and then crystallized from 25 mL of methanol and 50 mL of ether to give 616.0 mg (66.6%) of **8a**: mp 209–210.5 °C; R_f (D) 2.47; ^1H NMR (90 MHz in Me_2SO-d_6) δ 0.84 (br, 6 H), 1.17 (d, 3 H, J = 6.8 Hz), 1.31–1.59 [m, 12 H, includes δ 1.82 (s, 3 H), 1.84 (s, 3 H)], 2.33–2.56 (m, 4 H), 2.74–2.96 (m, 4 H), 4.02–4.58 (br m, 10 H), 5.07 (s, 4 H), 5.11 (s, 2 H), 6.97–7.13 (br, 1 H), 7.34 (s, 15 H), 7.79–8.11 (br m, 4 H), 8.42–8.56 (m, 3 H). Anal. Calcd for $C_{56}H_{75}N_8O_{15}S_2$: C, 57.72; H, 6.57; N, 9.62. Found: C, 57.84; H, 6.72; N, 9.51.

Boc-Cys(Acm)-Leu-Glu(OBzl)-Glu(OBzl)-D-Ala-Cys-(Acm)-OBzl (8b). Compound **14b** (1.922 g, 1.9 mmol) was deprotected and coupled with Boc-Cys(Acm)-OH (0.559 g, 1.9 mmol) in 30 mL of mixed solvent (DMF/CH_2Cl_2, 2:1) by using NMM (0.209 mL, 1.9 mmol), HOBt (0.320 g, 2.1 mmol), and DCC (0.392 g, 1.9 mmol). The product obtained was purified by chromatography over silica gel (solvent, 4% methanol in chloroform; column size, 2.5 × 70 cm) to give 1.70 g (75.9%) of **8b** as a powder. About 1 g was crystallized from 10 mL of methanol and 80 mL of ether for analysis: mp 172–173 °C; R_f (C) 0.65, R_f (A) 0.36, R_f (D) 0.35; ^1H NMR (90 MHz in $CDCl_3$) δ 0.91 (d, 3 H, J = 5.4 Hz), 0.97 (d, 3 H, J = 5.4 Hz), 1.27–1.67 [m, 15 H, includes δ 1.42, (s, 9 H)], 1.90–2.21 [m, 10 H, includes δ 1.97 (s), 2.01 (s)], 2.33–2.67 (br, m, 4 H), 2.74–3.09 (m, 4 H), 3.89–4.64 (m, 7 H), 4.90 (q, 1 H, J = 7.8 Hz), 5.08 (s, 4 H), 5.12 (s, 2 H), 6.05 (d, 1 H, J = 5.9 Hz), 6.90 (br t, 1 H), 7.04–7.48 (m, 19 H), 7.56–7.78 (br m, 3 H). Anal. Calcd for $C_{56}H_{78}N_8O_{15}S_2$: C, 57.72; H, 6.57; N, 9.62. Found: C, 57.91; H, 6.74; N, 9.55.

Cbz-Ala-Leu-Glu(OBzl)-Glu(OBzl)-Pro-Ala-OBzl (10). The title compound was prepared from Cbz-Ala-OH (0.491 g, 2 mmol), and the hydrochloride salt was prepared from compound **14c** (1.856 g, 2 mmol) in 30 mL of CH_2Cl_2 by using NMM (0.22 mL, 2 mmol), HOBt (0.337 g, 2 mmol), and DCC (0.454 g, 2 mmol). The obtained solid was purified over a silica gel column (solvent, 1.5% methanol in chloroform) and was crystallized from 10 mL of ethyl acetate and 50 mL of ether: yield 1.548 g (77.5%); mp 104–106 °C; R_f (A) 0.55, R_f (D) 0.69; ^1H NMR (90 MHz in $CDCl_3$) δ 0.82 (br d, 6 H), 1.12–1.27 (br m, 6 H), 1.51 (br, 3 H), 1.99 (br, 8 H), 2.34 (m, 4 H), 3.65 (br, 2 H), 4.41–4.64 (m, 4 H), 4.95 (s, 2 H), 5.07 (s, 6 H), 5.22 (br, 1 H), 6.11 (d, 1 H, J = 6.6 Hz), 7.11 (d, 1 H, overlapped with impurity peak partially), 7.31 (s, 20 H), 7.56 (d, 1 H), 8.26 (br, 2 H). Anal. Calcd for $C_{56}H_{68}N_6O_{13}$: C, 65.10; H, 6.63; N, 8.13. Found: C, 64.99; H, 6.80; N, 8.20.

Boc-Cys-Leu-Glu(OBzl)-Glu(OBzl)-Ala-Cys-OBzl (9a). Into a solution of iodine (1.269 g, 5 mmol) in 400 mL of dry methanol was added dropwise hexapeptide **8a** (589.1 mg, 0.5 mmol) in 250 mL of dry methanol, over 5 h at room temperature with mechanically stirring. The solution was then stirred for 3 h at room temperature and then cooled in an ice bath. Aqueous sodium thiosulfate (1 N, 18.9 mL) was added dropwise until the iodine color disappeared. After evaporation to dryness, 150 mL of ethyl acetate was added to the residue, which was washed with water (50 mL × 2), saturated NaCl solution (30 mL × 2), and water (50 mL × 2) and then dried over anhydrous magnesium sulfate. The crude product was purified by silica gel chromatography (solvent, 2% methanol in chloroform; 50 g of silica gel was used). The fractions [R_f (A) 0.48] were pooled and evaporated to dryness to give a powder: yield 348.2 mg (67.3%).

Crystallization from 3 mL of CH_2Cl_2 and 30 mL of ether gave 303.2 mg (58.6%) of **9a**: mp 200–203 °C; R_f (A) 0.48, R_f (D) 0.62; ^1H NMR (90 MHz in $CDCl_3$) δ 0.94 (br, 6 H), 1.44 (br s, 12 H), 1.62–1.78 (br, 3 H), 2.09–2.30 (m, 4 H), 2.39–2.58 (m, 4 H), 2.93–3.26 (m, 4 H), 3.93 (q, 1 H, J = 6.4 Hz), 4.16–4.47 (m, 3 H), 4.58 (q, 1 H, J = 6.8 Hz), 4.90 (q, 1 H, J = 8.4 Hz), 5.09 (s, 2 H), 5.12 (s, 2 H), 5.15 (s, 2 H), 5.54 (d, 1 H, J = 7.8 Hz), 6.97–7.43 (m, 17 H), 7.53–7.72 (m, 3 H). Anal. Calcd for $C_{50}H_{64}O_3S_{12}$: C, 58.81; H, 6.32; N, 8.23. Found: C, 59.01; H, 6.49; N, 8.16.

Boc-Cys-Leu-Glu(OBzl)-Glu(OBzl)-D-Ala-Cys-OBzl (9b). Hexapeptide **8b** (589.1 mg, 0.5 mmol) was cyclized by using iodine (1.269 g, 5.0 mmol) in 750 mL of dry methanol following the same procedure for the preparation of **9a**. The obtained crude product was purified by chromatography over silica gel (solvent, 2% methanol in chloroform), and the powder (389.6 mg, 75.3%) was crystallized from 3 mL of CH_2Cl_2 and 30 mL of ether to give 354.2 mg (68.5%) of **9b**: mp 112–113 °C; R_f (A) 0.64, R_f (D) 0.81; ^1H NMR (90 MHz in $CDCl_3$) δ 0.99 (d, 3 H, J = 4.9 Hz), 1.02 (d, 3 H, J = 4.8 Hz), 1.40–1.53 [m, 15 H, includes δ 1.48 (s)], 2.06–2.61 (m, 8 H), 2.91–3.65 (m, 4 H), 4.03–4.79 (m, 6 H), 5.02–5.14 (m, 6 H), 5.71 (d, 1 H, J = 6.8 Hz), 6.96–7.64 [m, 19 H, includes δ 7.29 (s), 7.33 (s)], 7.88 (d, 1 H, J = 5.9 Hz). Anal. Calcd for $C_{50}H_{64}N_8O_{13}S_2$: C, 58.81; H, 6.32; N, 8.23. Found: C, 59.05; H, 6.48; N, 8.20.

H-Cys-Leu-Glu-Glu-Ala-Cys-OH (6a). Anisole (1.01 mL, 10 mmol) was added to a solution of compound **9a** (103.4 mg, 0.1 mmol) in 25 mL of freshly distilled trifluoroacetic acid. A moderately rapid stream of hydrogen bromide gas was passed through the reaction mixture for 2 h at room temperature. After stirring at room temperature for 30 min, the solution was evaporated to remove F_3AcOH. The anisole-rich residue was then poured into 100 mL of anhydrous ether to precipitate the fine white crystals. These were filtered, and the solid was washed with anhydrous ether and dried in a desiccator. The obtained product (53 mg, 79.6%) was recrystallized twice from 2 mL of methanol and 8 mL of ether without cooling to afford 44.0 mg (66.1%) of compound **6a**: mp 196–199 °C dec; R_f (F) 0.11, R_f (E) 0.28; ^{13}C NMR (5.27 mg/0.3 mL of Me_2SO-d_6) CO (173.90, 173.74, 172.00, 171.68, 171.30, 171.03, 170.54, 169.95 ppm), C^α (53.09, 52.93, 52.77, 52.33, 48.65 ppm); ^{13}C NMR (2 Cys C^β Leu C^β overlap with Me_2SO-d_6 peak), Glu C^γ (30.18, 29.96 ppm), Glu C^β (26.44, 26.11 ppm), Leu C^γ (24.16 ppm), Leu C^δ (22.81, 21.45 ppm), Ala C^β (17.23 ppm). Amino acid analysis after hydrolysis in 12 N HCl/propionic acid (1:1) for 24 h at 105–107 °C: $^1/_2$-Cys, 1.74; Glu, 2.01; Ala, 1.00; Leu, 1.03.

H-Cys-Leu-Glu-Glu-D-Ala-Cys-OH (6b). The title compound was prepared from compound **9b** (103.4 mg, 0.1 mmol), anisole (1.01 mL, 0.01 mmol), F_3AcOH (25 mL), and anhydrous hydrogen bromide gas following the procedure described for the preparation of **9a**. The obtained white solid (71 mg) was recrystallized twice from 1 mL of methanol and 8 mL of ether without cooling: yield 58.0 mg (87.1%); mp 191–195 °C dec; R_f (F) 0.12, R_f (E) 0.29; ^{13}C NMR (10.54 mg/0.4 mL of Me_2SO-d_6) CO (173.90, 173.52, 172.17, 171.68, 171.35, 171.25, 170.81, 169.95 ppm), C^α (53.63, 53.31, 52.55, 52.06, 51.68, 48.05 ppm); ^{13}C NMR (2 Cys C^β, Leu C^β; overlapped with Me_2SO-d_6 peak), Glu C^* (30.07, 29.76 ppm), Glu C^β (25.62, 27.68 ppm), Leu C^γ (24.05 ppm), Leu C^δ (21.18, 22.97 ppm), Ala C^β (17.23 ppm). Amino acid analysis after hydrolysis in 12 N HCl/propionic acid (1:1) for 24 h at 105–107 °C: $^1/_2$-Cys, 1.38; Glu, 2.00; Ala, 1.00; Leu, 1.16.

H-Ala-Leu-Glu-Glu-Pro-Ala-OH (7). To a solution of compound **10** (999.2 mg, 1.0 mmol) and ammonium formate (756.5 mg, 12.0 mmol) in 50 mL of methanol was added 10% Pd/C catalyst (1.0 g). The mixture was stirred at room temperature for 2 h. After filtration through a Celite 545 column, the solvent was then evaporated to dryness to yield a white solid. This was dissolved in 10 mL of water and filtered, and the filtrate was then lyophilized. The obtained white powder was recrystallized twice from 15 mL of methanol and 75 mL of ether to give 413 mg (65.7%) of **7c**: mp 179–181 °C; R_f (E) 0.22, R_f (F) 0.11; ^1H NMR (90 MHz in Me_2SO-d_6) δ 0.87 (br, 6 H), 1.13–1.29 (m, 6 H), 1.56 (br, 3 H), 1.71–2.27 (m, 12 H), 3.70 (br, 2 H), 4.25 (br, 2 H), 4.28–4.80 (br, 3 H overlapped with water peak), 7.50 (br, 2 H), 8.10 (br, 2 H), 9.05 (br, 2 H).

Biological Assay. Detailed experimental procedures for the biological assay were described previously.[12]

Acknowledgment. This work was supported by grants from the National Institutes of Health (AM-21472 and AM-14881).

Registry No. 6a, 85250-21-1; **6b**, 85281-30-7; **7**, 85250-22-2; **8a**, 85250-23-3; **8b**, 85281-31-8; **9a**, 85250-24-4; **9b**, 85281-32-9; **10**, 85250-25-5; **11a**, 85250-26-6; **11b**, 85250-30-2; **11c**, 52616-95-2; **12a**, 85250-27-7; **12b**, 85250-31-3; **12c**, 85250-32-4; **13a**, 85250-28-8; **13b**,

85250-33-5; **13c**, 85250-34-6; **14a**, 85250-29-9; 14b, 85281-33-0; **14c**, 85250-35-7; 14c·HCl, 85250-36-8; Boc-Cys(Acm)-OBzl, 79396-91-1; Boc-Ala-OH, 15761-38-3; Boc-D-Ala-OH, 7764-95-6; H-Cys-(Acm)-OBzl·HCl, 79396-92-2; H-Ala-OBzl·*p*-Tos-OH, 42854-62-6; Boc-Pro-OH, 15761-39-4; Boc-Glu(OBzl)-OH, 13574-13-5; Boc-Leu-OH, 13139-15-6; Cbz-Ala-OH, 1142-20-7; prothrombin, 9001-26-7; vitamin K dependent carboxylase, 64641-76-5.

Supplementary Material Available: Full ^{13}C NMR data for compounds **9a**,b and **3c** in Me_2SO-d_6 (Table II) and $CDCl_3$ (Table III) (2 pages). Ordering information is given on any current masthead page.

Inhibition by Carboxamides and Sulfoxides of Liver Alcohol Dehydrogenase and Ethanol Metabolism

Vijay K. Chadha, Kevin G. Leidal, and Bryce V. Plapp*

Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242. *Received September 30, 1982*

Sulfoxides and amides were tested as inhibitors of liver alcohol dehydrogenase and ethanol metabolism in rats. With both series of compounds, increasing the hydrophobicity resulted in better inhibition, and introduction of polar groups reduced inhibition. Of the cyclic sulfoxides, tetramethylene sulfoxide was the best inhibitor as compared to the tri- and pentamethylene analogue and other compounds, and it may be a transition-state analogue. The most promising compounds, tetramethylene sulfoxide and isovaleramide, were essentially uncompetitive inhibitors of purified horse and rat liver alcohol dehydrogenases with respect to ethanol as substrate. These compounds also were uncompetitive inhibitors in vivo, which is advantageous since the inhibition is not overcome at higher concentrations of ethanol, as it is with competitive inhibitors, such as pyrazole. The uncompetitive inhibition constants for tetramethylene sulfoxide and isovaleramide for rat liver alcohol dehydrogenase were 200 and 20 μM, respectively, in vitro, whereas in vivo the values were 340 and 180 μmol/kg. The differences in the values may be due to metabolism or distribution of the compounds. Further studies will be required to determine if isovaleramide or tetramethylene sulfoxide is suitable for therapeutic purposes.

Liver alcohol dehydrogenase (EC 1.1.1.1) catalyzes the first step in alcohol metabolism and would be a rational target for inhibiting alcohol metabolism.[1,2] Inhibitors of the dehydrogenase would be useful for studying the metabolism of alcohols[3] and for therapeutically preventing poisoning by methanol[4,5] and ethylene glycol.[6,7]

Pyrazole and its 4-substituted derivatives are effective competitive inhibitors of alcohol dehydrogenation in vitro and in vivo[8] since they bind tightly to the alcohol dehydrogenase–NAD^+ complex.[9] Pyrazole itself is toxic in doses that are required to significantly inhibit alcohol metabolism in vivo,[10] but 4-methylpyrazole is less toxic and more effective as an inhibitor of alcohol dehydrogenase and may be useful for treatment of humans.[5,11] However, 4-alkylpyrazoles depress the central nervous system in rats and mice.[8]

Other organic compounds, such as fatty acid amides[12-16] and aromatic amides,[17] have been tested as inhibitors of the enzyme. *n*-Butyraldoxime and other oximes are about as effective as pyrazole in rats,[18] but in man the oxime induced a reaction like that with disulfiram, in which blood acetaldehyde levels increased after ingestion of ethanol.[19] *n*-Butyramide is almost as effective as pyrazole in blocking ethanol oxidation in rats and is less toxic.[18] Thus, it is worthwhile to test other carboxamides to find potent, nontoxic inhibitors of alcohol dehydrogenase.

Dimethyl sulfoxide forms a highly fluorescent ternary complex[20] with the enzyme and reduced nicotinamide adenine dinucleotide and is an inhibitor in vitro of alcohol dehydrogenase.[21] The three-dimensional structure of the complex has been determined by X-ray crystallography to high resolution.[22] Dimethyl sulfoxide (Me_2SO) is used

(1) Li, T.-K.; Theorell H. *Acta Chem. Scand.* **1969**, *23*, 892–902.
(2) Plapp, B. V. in "Biochemical Pharmacology of Ethanol"; Majchrowicz, E., Ed.; Plenum Press: New York, 1975; pp 77–109.
(3) Rognstad, R.; Grunnet, N. In "Biochemistry and Pharmacology of Ethanol"; Majchrowicz, E.; Noble, E. P., Eds.; Plenum Press: New York, 1979; Vol. 1, pp 65–77.
(4) McMartin, K. E.; Makar, A. B.; Martin-Amat, G.; Palese, M.; Tephly, T. R. *Biochem. Med.* **1975**, *13*, 319–333.
(5) McMartin, K. E.; Hedström, K.-G.; Tolf, B.-R.; Östling-Wintzell, H.; Blomstrand, R. *Arch. Biochem. Biophys.* **1980**, *199*, 606–614.
(6) Beasley, V. R.; Buck, W. B. *Vet. Hum. Toxicol.* **1980**, *22*, 255–263.
(7) Chou, J. Y.; Richardson, K. E. *Toxicol. Appl. Pharmacol.* **1978**, *43*, 33–44.
(8) Deis, F. H.; Lester, D. In "Biochemistry and Pharmacology of Ethanol"; Majchrowicz, E.; Noble, E. P., Eds.; Plenum Press: New York, 1979; Vol. 2, pp 303–319.
(9) Theorell, H.; Yonetani, T. *Biochem. Z.* **1963**, *338*, 537–553.
(10) Magnusson, G.; Nyberg, J.-A.; Bodin, N.-O.; Hansson, E. *Experientia* **1972**, *28*, 1198–1200.
(11) Blomstrand, R.; Ellin, Å.; Löf, A. and Östling-Wintzell, H. *Arch. Biochem. Biophys.* **1980**, *199*, 591–605.
(12) Winer, A. D.; Theorell, H. *Acta Chem. Scand.* **1960**, *14*, 1729–1742.
(13) Porter, C. C.; Titus, D. C.; DeFelice, M. J. *Life Sci.* **1976**, *18*, 953–960.
(14) Woronick, C. L. *Acta Chem. Scand.* **1961**, *15*, 2062–2064.
(15) Woronick, C. L. *Acta Chem. Scand.* **1963**, *17*, 1789–1791.
(16) Sigman, D. S.; Winer, A. D. *Biochim. Biophys. Acta* **1970**, *206*, 183–186.
(17) Sarma, R. H.; Woronick, C. L. *Biochemistry* **1972**, *11*, 170–179.
(18) Lester, D.; Benson, G. D. *Science* **1970**, *169*, 282–284.
(19) Lewis, W.; Schwartz, L. *Med. Ann. D.C.* **1956**, *25*, 485–490.
(20) Perlman, R. L.; Wolff, J. *Science* **1968**, *160*, 317–319.
(21) Sharkawi, M. *Toxicol. Lett.* **1979**, *4*, 493–497.
(22) Eklund, H.; Samama, J.-P.; Wallén, L.; Brändén, C.-I.; Åkeson, Å.; Jones, T. A. *J. Mol. Biol.* **1981**, *146*, 561–587.

0022-2623/83/1826-0916$01.50/0