Synthetic Antagonists of in Vivo Antidiuretic and Vasopressor Responses to Arginine-vasopressin

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Received December 29, 1980

Four analogues of [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),4-valine,8-D-arginine]vasopressin [d-$(CH_2)_5$VDAVP] and four analogues of its L-arginine isomer d$(CH_2)_5$VAVP with *O*-methyl-, *O*-ethyl-, *O*-isopropyl, and *O*-*n*-propyltyrosine substituents at position 2 were prepared by the solid-phase method using a slightly modified reoxidation procedure following deblocking with sodium in liquid ammonia to overcome losses due to insolubility. These analogues are the following: **1**, d$(CH_2)_5$Tyr(Me)VDAVP; **2**, d$(CH_2)_5$Tyr(Et)VDAVP; **3**, d$(CH_2)_5$Tyr(*i*-Pr)VDAVP; **4**, d$(CH_2)_5$Tyr(*n*-Pr)VDAVP; **5**, d$(CH_2)_5$Tyr(Me)VAVP; **6**, d$(CH_2)_5$Tyr(Et)VAVP; **7**, d$(CH_2)_5$Tyr(*i*-Pr)VAVP; **8**, d$(CH_2)_5$Tyr(*n*-Pr)VAVP. These analogues were tested for agonistic and antagonistic activities in rat antidiuretic and rat vasopressor assay systems. All eight analogues cause a transient antidiuresis when injected intravenously and effectively antagonize antidiuretic responses to subsequent injections of arginine-vasopressin (AVP). They exhibit the following antiantidiuretic pA_2 values: **1**, 6.68 ± 0.11; **2**, 7.10 ± 0.08; **3**, 6.88 ± 0.07; **4**, 6.67 ± 0.05; **5**, 7.35 ± 0.06; **6**, 7.57 ± 0.06; **7**, 7.32 ± 0.10; **8**, 7.29 ± 0.07. They are also highly effective antagonists of the vasopressor responses to AVP, with antivasopressor pA_2 values in the range 7.86 to 8.44. These findings indicate that in this series *O*-ethyl substitution on the tyrosine at position 2 is optimal for antiantidiuretic potency and that L-arginine is far superior to D-arginine in this regard also. Thus, d$(CH_2)_5$Tyr(Et)VAVP with an antiantidiuretic pA_2 of 7.57 ± 0.06 is the most potent of these eight antidiuretic antagonists. These are the first known effective antagonists of in vivo antidiuretic responses to AVP. They are, thus, potentially useful pharmacological tools for studies on the roles of AVP in regulating water balance in normal and pathophysiological states in animals and in humans. They also serve as excellent lead compounds for the design of even more potent antagonists for potential therapeutic use for the treatment of hyponatremia secondary to inappropriate secretion of the antidiuretic hormone (SIADH or the Schwartz–Bartter syndrome).

The design and synthesis of antagonists of the vasopressor and antidiuretic responses to arginine-vasopressin (AVP) have been long-standing goals of structure-activity studies on the neurohypophysial peptides.[1,2] In recent years many effective antagonists of the vasopressor response to AVP have been reported.[3-7] To date, however, attempts to design effective antagonists of the antidiuretic responses to AVP have yielded disappointing results. Thus, [4-leucine]oxytocin[8] and [4-phenylalanine]oxytocin[9] have been reported to reverse the antidiuresis produced by infusing a submaximal dose of AVP intravenously into water-loaded rats. Others, however, have failed to confirm these findings.[10] Both of these analogues can increase water excretion when infused into water-loaded rats. It remains unclear whether they inhibit AVP-induced antidiuresis by competitive antagonism of AVP at the renal AVP receptors or by delivering more fluid into the distal nephron. In any event, attempts to modify further these 4-substituted oxytocins have not resulted in more effective antagonists.[11]

Larsson et al. reported that three *O*-alkyltyrosine substituted analogues of deaminolysine-vasopressin inhibited antidiuresis caused by infusing lysine-vasopressin into water-loaded rats.[12] Deamino[2-(*O*-ethyl)tyrosine]-lysine-vasopressin was the most active. These analogues did not act as antagonists in all rats and only at a limited range of low doses. At higher doses they were, in themselves, antidiuretic, and their antidiuretic actions were additive to that of lysine-vasopressin. Such analogues offer little promise as useful pharmacological or therapeutic agents.

We now report the design and synthesis of a series of eight analogues which are effective antagonists of the in vivo antidiuretic responses to exogenous and endogenous AVP. In earlier attempts to design an antagonist of the antidiuretic response to arginine-vasopressin (AVP), we synthesized two analogues of the highly active and specific antidiuretic peptide [1-deamino,4-valine,8-D-arginine]vasopressin (dVDAVP)[13] in which the two hydrogens on the β carbon at position 1 are replaced by methyl groups and by a cyclopentamethylene group, $(CH_2)_5$, respectively. These two analogues were [1-deaminopenicillamine,4-valine,8-D-arginine]vasopressin (dPVDAVP)[4] and [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),4-valine,8-D-arginine]vasopressin [d$(CH_2)_5$VDAVP].[5] These

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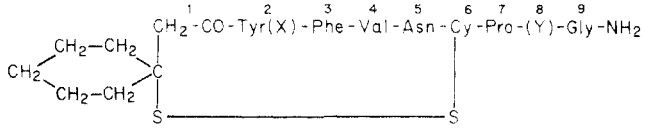
0022-2623/81/1824-0701$01.25/0

two peptides were found to be potent antagonists of the vasopressor responses to AVP. Although they were not antagonists of the antidiuretic responses to AVP, they exhibited substantially reduced antidiuretic agonistic potencies. Thus, dPVDAVP retained one-tenth and d-$(CH_2)_5$VDAVP retained only $^1/_{12\,000}$ the antidiuretic activity of dVDAVP. Furthermore, d$(CH_2)_5$VDAVP was subsequently found to act as a competitive antagonist of the binding of AVP to rat renal medullary membranes and of AVP-stimulated adenylate cyclase activation in vitro.[14] These two findings, its weak in vivo antidiuretic activity coupled with the in vitro antagonistic properties, pointed to d$(CH_2)_5$VDAVP as an excellent lead compound for the possible desing of an in vivo antagonist of the antidiuretic responses to AVP.

We had found that O-alkylation of the tyrosine residue in some of our oxytocic[15] and vasopressor[7] antagonists brought about enhancements in their respective antagonistic potencies. These findings, coupled with the aforementioned effects of *O*-alkyltyrosine substitutions in deaminolysine-vasopressin,[12] indicated that similar *O*-alkyltyrosine substitutions in d$(CH_2)_5$VDAVP might lead to an antagonist of the antidiuretic responses to AVP. Following initial encouraging findings, we wished to explore the effects of these same substituents in the L-arginine-containing isomer of d$(CH_2)_5$VDAVP. The eight analogues designed according to this rationale are the following: **1**, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine,4-valine,8-D-arginine]vasopressin [d$(CH_2)_5$Tyr(Me)VDAVP]; **2**, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl)tyrosine-,4-valine,8-D-arginine]vasopressin [d$(CH_2)_5$Tyr(Et)-VDAVP]; **3**, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl)tyrosine,4-valine,8-D-arginine]vasopressin [d$(CH_2)_5$Tyr(*i*-Pr)VDAVP]; **4**, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-*O*-*n*-propyl)tyrosine,4-valine,8-D-arginine]vasopressin [d-$(CH_2)_5$Tyr(*n*-Pr)VDAVP]; **5**, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine,4-valine]arginine-vasopressin [d$(CH_2)_5$Tyr(Me)VAVP]; **6**, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl)tyrosine,4-valine]arginine-vasopressin [d-$(CH_2)_5$Tyr(Et)VAVP]; **7**, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl)tyrosine,4-valine]arginine-vasopressin [d$(CH_2)_5$Tyr(*i*-Pr)VAVP]; **8**, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-*n*-propyl)tyrosine,4-valine]arginine-vasopressin [d$(CH_2)_5$Tyr(*n*-Pr)VAVP].

These analogues have the following general structures:



1-4, X = Me, Et, *i*-Pr, *n*-Pr; Y = D-Arg
5-8, X = Me, Et, *i*-Pr, *n*-Pr; Y = L-Arg

We now present the synthesis and some pharmacological properties of these eight analogues—all of which have been found to antagonize effectively antidiuretic responses by rats to exogenous AVP. A preliminary communication on analogues **1**, **2**, **5**, and **6** has been reported.[16]

Peptide Synthesis. The protected peptide precursors required for the synthesis of all eight peptide antagonists were prepared by the solid-phase method[17,18] of peptide synthesis either by 8+1 couplings in solution[7] (analogues **1**, **2**, and **5**) or entirely on the resin.[19,20] Boc-Tyr(Me) and Boc-Tyr(Et) were obtained commercially. Boc-Tyr(*i*-Pr) and Boc-Tyr(*n*-Pr) were synthesized.[21] *p*-Nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate[22] was used in each final coupling step. All active ester couplings[23,24] were facilitated by the addition of *N*-hydroxybenzotriazole (HOBT).[25] All of the protected peptide amides were obtained by ammonolytic cleavage[19] from the respective Boc octapeptide or acyl octapeptide resins. Each protected precursor was deblocked with Na in NH_3.[26] Unusually low yields (<20%) due to insolubility and excessive polymerization of the *O*-Et, *O*-*i*-Pr and *O*-*n*-Pr analogues in the initial syntheses necessitated the following modification of our previously described procedure.[7,13,20] Following removal of the NH_3, the flask was flushed with N_2 for 5 min, and the residue was taken up in 10% acetic acid to ensure total solution and immediately diluted ~60-fold with ice-cold water. With one exception (analogue **4**), the above modification brought about substantial improvements in the yield of each analogue. The remainder of the procedure was essentially the same as previously described.[7,13,20] The deblocked disulfhydryl compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$.[27] The analogues were desalted and purified by gel filtration on Sephadex G-15 as previously described.[28]

Bioassay Methods. Agonistic and antagonistic potencies of these analogues were estimated by previously described methods.[4,7,16,29] These included intravenous antidiuretic assays in rats under ethanol anesthesia and vasopressor assays in phenoxybenzamine-treated rats under urethane anesthesia. The USP posterior pituitary reference standard was used in all assays for agonistic and antagonistic activities. Agonistic activities are expressed in units per milligram. Antagonistic potencies were determined and expressed as "effective doses" and as pA_2 values.[30] The "effective dose" is defined as the dose (in nanomoles per kilogram) that reduces the response seen from 2*x* units of agonist to the response with 1*x* units of agonist. Estimated in vivo "pA_2" values represent the negative logarithms of the effective doses divided by the estimated volume of distribution (67 mL/kg).[3] For the measurement of antiantidiuretic potencies, the agonist was

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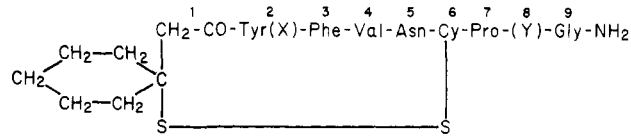
Table I. Antiantidiuretic and Antivasopressor Potencies of Eight New Antagonists of Arginine-vasopressin (AVP)



$d(CH_2)_5Tyr(X)V(Y)VP$

no.	antagonists[d]	X	Y	antiantidiuretic effective dose,[a] nmol/kg	antiantidiuretic pA_2[b]	antivasopressor effective dose,[a] nmol/kg	antivasopressor pA_2[b]
1	$d(CH_2)_5Tyr(Me)VDAVP$	Me	D-Arg	15 ± 3 (4)[c]	6.68 ± 0.11	0.28 ± 0.05 (8)	8.44 ± 0.07
2	$d(CH_2)_5Tyr(Et)VDAVP$	Et	D-Arg	5.7 ± 0.5 (4)	7.10 ± 0.08	0.34 ± 0.04 (8)	8.31 ± 0.05
3	$d(CH_2)_5Tyr(i\text{-}Pr)VDAVP$	*i*-Pr	D-Arg	8.5 ± 1.7 (4)	6.88 ± 0.07	0.28 ± 0.07 (4)	8.41 ± 0.08
4	$d(CH_2)_5Tyr(n\text{-}Pr)VDAVP$	*n*-Pr	D-Arg	14 ± 2 (4)	6.67 ± 0.05	1.1 ± 0.2 (8)	7.86 ± 0.10
5	$d(CH_2)_5Tyr(Me)VAVP$	Me	L-Arg	3.1 ± 0.4 (4)	7.35 ± 0.06	0.29 ± 0.06 (4)	8.32 ± 0.08
6	$d(CH_2)_5Tyr(Et)VAVP$	Et	L-Arg	1.9 ± 0.2 (4)	7.57 ± 0.06	0.49 ± 0.11 (4)	8.16 ± 0.09
7	$d(CH_2)_5Tyr(i\text{-}Pr)VAVP$	*i*-Pr	L-Arg	3.6 ± 0.9 (6)	7.32 ± 0.10	0.31 ± 0.06 (4)	8.36 ± 0.09
8	$d(CH_2)_5Tyr(n\text{-}Pr)VAVP$	*n*-Pr	L-Arg	3.5 ± 0.6 (4)	7.29 ± 0.07	0.40 ± 0.04 (4)	8.22 ± 0.04

[a] The effective dose is defined as the dose (in nanomoles per kilogram) that reduces the response seen with $2x$ units of agonist to equal the response seen with x units of agonist administered 20 min after antagonist. [b] Estimated in vivo pA_2 values represent the negative logarithms of the "effective dose" divided by the estimated volume of distribution (67 mL/kg). [c] Means ± SE; number of assay groups in parentheses. [d] The abbreviations and their full names are as follows: $d(CH_2)_5Tyr(Me)VDAVP$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine,4-valine,8-D-arginine]vasopressin; $d(CH_2)_5Tyr(Et)VDAVP$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl)tyrosine,4-valine,8-D-arginine]vasopressin; $d(CH_2)_5Tyr(i\text{-}Pr)VDAVP$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl)tyrosine,4-valine,8-D-arginine]vasopressin; $d(CH_2)_5Tyr(n\text{-}Pr)VDAVP$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-propyl)tyrosine,4-valine,8-D-arginine]vasopressin; $d(CH_2)_5Tyr(Me)VAVP$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine,4-valine]arginine-vasopressin; $d(CH_2)_5Tyr(Et)VAVP$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl)tyrosine,4-valine]arginine-vasopressin; $d(CH_2)_5Tyr(i\text{-}Pr)VAVP$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl)tyrosine,4-valine]arginine-vasopressin; $d(CH_2)_5Tyr(n\text{-}Pr)VAVP$, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-propyl)tyrosine,4-valine]arginine-vasopressin.

injected 20 min after the antagonist. Each antagonist was administered in two doses, a high dose which reduced the response to $2x$ units of agonist to less than the response to $1x$ units of agonist, and a low dose which did not fully reduce the response to that given by $1x$ units of agonist. The effective dose in each case was obtained by interpolation on a logarithmic scale between the two doses of antagonist.[30]

Results and Discussion

The antiantidiuretic and antivasopressor potencies of the eight *O*-alkyltyrosine analogues of $d(CH_2)_5VDAVP$ and of $d(CH_2)_5VAVP$ are presented as effective doses and as pA_2 values in Table I. None of the analogues exhibit any pressor activity. All eight analogues possess weak antidiuretic activities (0.004–0.05 U/mg). They cause an initial transient and submaximal antidiuresis when injected intravenously into hydrated rats under ethanol anesthesia. Subsequent doses of AVP are reversibly antagonized for 1–3 h, depending on the dose of antagonist.

Relative Effects of the *O*-Alkyltyrosine Substituents on Antidiuretic Antagonism to Exogenous AVP. $d(CH_2)_5VDAVP$ was found not to be an in vivo antagonist of the antidiuretic responses to AVP.[5] Alkylation of the tyrosine residue at position 2 of both $d(CH_2)_5VDAVP$ and of $d(CH_2)_5VAVP$ with methyl, ethyl, isopropyl, and *n*-propyl groups resulted in antagonists of in vivo antidiuretic responses to AVP. In both series of antagonists, the *O*-ethyltyrosine-containing analogue appears to be the most potent.

Effects of L-Arginine vs. D-Arginine on Antidiuretic Antagonism to Exogenous AVP. It is clear that the replacement of D-arginine by L-arginine in each of the four *O*-alkyltyrosine analogues of $d(CH_2)_5VDAVP$ led to an enhancement of antiantidiuretic potency in each case. Thus, all four *O*-alkyltyrosine analogues of $d(CH_2)_5VAVP$ are more potent than $d(CH_2)_5Tyr(Et)VDAVP$, the most potent analogue of the $d(CH_2)_5Tyr(O\text{-alkyl})VDAVP$ series. With an antiantidiuretic pA_2 of 7.57, $d(CH_2)_5Tyr(Et)VAVP$ appears to be the most potent antidiuretic antagonist of the entire series.

Other Structural Requirements for Antidiuretic Antagonism. The structural requirements in these analogues for antagonism appear quite rigid. In addition to *O*-alkylation of tyrosine, it is clear from studies on many closely related analogues[31] that the β,β-cyclopentamethylene group at position 1 and the valine residue at position 4 are essential for antidiuretic antagonism. Thus, neither the β,β-dimethyl or the β,β-diethyl analogues of $d(CH_2)_5Tyr(Et)VAVP$ exhibit detectable antagonistic activity in the intravenous rat antidiuretic assay.[31] Also, replacement of the valine residue in $d(CH_2)_5Tyr(Et)VAVP$ by a glutamine residue leads to loss of antagonistic potency.[31]

Antagonism of Antidiuretic Effects to Endogenous AVP. The *O*-methyl- and *O*-ethyltyrosine analogues of both the L-Arg and D-Arg containing analogues have been found to antagonize the antidiuretic responses to endogenous AVP.[16] When administered by intraperitoneal injection to normally hydrated rats, they bring about a rapid increase in urine flow and a fall in urinary osmolality. They exhibit the same order of relative potencies in this assay as that estimated from their antagonistic effects to exogenous AVP.[16]

Antagonism of Vasopressor Responses to Exogenous AVP. All eight analogues are potent antagonists of vasopressor responses to AVP. In this respect, they are among the most potent vasopressor antagonists reported to date.[6,7] In view of the structural similarity of these peptides to a number of our earlier antagonists, such findings were not unexpected. It may be recalled that [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)]-arginine-vasopressin [$d(CH_2)_5AVP$] and [1-(β-mercapto-

(31) Lammek, B.; Manning, M.; Sawyer, W. H., unpublished.

β,β-cyclopentamethylenepropionic acid),2-(O-methyl)tyrosine]arginine-vasopressin [d$(CH_2)_5$Tyr(Me)AVP][7] were found to possess antivasopressor pA_2 values of 8.32 and 8.67, respectively. Neither of these peptides are antidiuretic antagonists. However, it is of interest to note that the replacement of the glutamine at position 4 in d-$(CH_2)_5$Tyr(Me)AVP by valine converted this weak antidiuretic agonist into the presently reported antidiuretic antagonist d$(CH_2)_5$Tyr(Me)VAVP (Table I) with a pA_2 of 7.35 while at the same time retaining much of its antivasopressor potency, pA_2 = 8.32.

Conclusion

The eight analogues reported here appear to be the most effective antagonists of the antidiuretic responses to AVP yet reported. Should these analogues exhibit similar actions in humans they could be useful pharmacological tools for clarifying the role of AVP in clinical syndromes involving excessive water retention. They could also be the first specific agents for the treatment of hyponatremia secondary to the inappropriate secretion of AVP (SIADH or the Schwartz–Bartter syndrome).[32] The antivasopressor properties of these antagonists should not pose any problems as far as their potential clinical usefulness is concerned. However, for pharmacological and receptor studies, specific antagonists of the antidiuretic response, i.e., with no vasopressor antagonism, are desirable. The findings presented here have obvious potential for the design of more selective and more potent antagonists of the antidiuretic response to AVP. Such studies are presently underway in these laboratories.

Experimental Section

The procedure of "solid phase" synthesis conformed to that previously published.[17-20] Chloromethylated resin (Bio-Rad, Bio Beads SX-1) was esterified[33] with Boc-Gly to an incoporation of 0.5 mmol/g. Amino acid derivatives, including Boc-Tyr(Me) and Boc-Tyr(Et), were supplied by Bachem Inc. or Chemalog Inc. Boc-Tyr(*i*-Pr)[21] Boc-Tyr(*n*-Pr),[21] and *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate[22] were synthesized. Triethylamine (TEA) and *N*-methylmorpholine (NMM) were distilled from ninhydrin. Dimethylformamide (DMF) was distilled under reduced pressure immediately prior to its use. Other solvents and reagents were analytical grade. Thin-layer chromatography (TLC) was on silica gel (0.25 mm, Brinkman Silplate). The following solvent systems were used: A, butan-1-ol–acetic acid–water (4:1:5, v/v upper phase); B, chloroform–methanol (7:3, v/v); C, butan-1-ol–acetic acid–water–pyridine (15:3:3:10, v/v); D, butan-1-ol–acetic acid–water (4:1:1, v/v). Loads of 10–50 μg were applied and chromatograms were a minimum length of 10 cm. Iodine vapor was used for detection. For amino acid analysis,[34] peptides (~0.8 mg) were hydrolyzed with constant-boiling hydrochloric acid (500 μL) containing phenol (10 μL) in evacuated and sealed ampules for 18 h at either 110 or 120 °C.[35] The analyses were performed using a Beckman automatic amino acid analyzer Model 121. Molar ratios were referred to Gly = 1.00. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, or by Integral Microanalytical Laboratories, Inc, Raleigh, NC. The analytical results for elements indicated by their symbols were within ±0.4% of the theoretical values. Optical rotations were measured with a Rudolph polarimeter Model 80.

Boc-Phe-Val-Asn-Cys(Bzl)-Pro-D-Arg(Tos)-Gly-resin (A). Boc-Gly-resin (8 g, 4 mmol, of Gly) was subjected to six cycles of deprotection, neutralization, and coupling[17-20] to yield the protected heptapeptidyl resin A (12 g, 4 mmol).

Boc-Phe-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-resin (B). The protected heptapeptidyl resin B (12 g, 4 mmol) was prepared from 8 g (4.0 mmol) of Boc-Gly-resin using solid-phase methodology.[17-20]

β-(*S*-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(Me)-Phe-Val-Asn-Cys(Bzl)-Pro-D-Arg-(Tos)-Gly-NH_2 (I). The heptapeptidyl resin A (1.5 g, 0.5 mmol) was converted to Boc-octapeptidyl-resin in one cycle of solid-phase synthesis with Boc-Tyr(Me) as the carboxy component. This resin was ammonolyzed[19] and the product was extracted with warm DMF. The product was precipitated by the addition of water and reprecipitated from DMF–ethanol–ethyl ether to give Boc-Tyr(Me)-Phe-Val-Asn-Cys(Bzl)-Pro-D-Arg(Tos)-Gly-NH_2 as a white powder (0.581 g, 88.5% based on initial Gly content of the resin): mp 239–240 °C; $[\alpha]^{24}_D$ −14.9° (*c* 1, DMF); TLC R_f (A) 0.54, R_f (B) 0.73. Anal. ($C_{63}H_{85}N_{13}O_{14}S_2$) C, H, N. Amino acid analysis:[34,35] Tyr, 1.02; Phe, 0.98; Val. 1.02; Asp, 1.00; Cys(Bzl), 0.98; Pro, 1.01; Arg, 0.97; Gly, 1.00; NH_3, 2.1. An aliquot (0.270 g, 0.206 mmol) of this Boc octapeptide amide was dissolved in TFA (3 mL) and left to stand at room temperature for 20 min. Cold ether was added and the precipitated material was filtered and washed with ether (5 × 10 mL). The product was dried in vacuo over sodium hydroxide pellets. This material (250 mg) was dissolved in DMF (0.8 mL), and *N*-methylmorpholine was added to give a solution of pH 7–8 to moist pH paper.[24] This neutralized solution was stirred at room temperature for 20 min. A solution of *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate[22,23] (0.135 g, 0.37 mmol) and *N*-hydroxybenzotriazole monohydrate[25] (57 mg, 0.37 mmol) in DMF (1.0 ml) was added. The reaction mixture was stirred at room temperature overnight and TLC (in system A) showed that the reaction was complete. Methanol (80 mL) and ether (20 mL) were added with vigorous mixing. The precipitated material was filtered, washed with a mixture of methanol–ether (8:2), and dried in vacuo. The crude product (270 mg) was reprecipitated from DMF–methanol to give the acyl peptide amide I (263 mg, 75.2%): mp 220–221 °C; $[\alpha]^{24}_D$ −25.7° (*c* 1, DMF); TLC R_f (A) 0.55, R_f (B) 0.83. Anal. ($C_{73}H_{95}N_{13}O_{13}S_3$) C, H, N. Amino acid analysis:[34,35] Tyr, 0.98; Phe, 1.01; Val, 1.02; Asp, 1.02; Cys(Bzl), 0.97; Pro, 1.03; Arg, 1.0; Gly, 1.00; NH_3, 2.06.

β-(*S*-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(Et)-Phe-Val-Asn-Cys(Bzl)-Pro-D-Arg-(Tos)-Gly-NH_2 (II). The heptapeptidyl resin A (1.5 g, 0.5 mmol) was converted to Boc octapeptide resin in one cycle of solid-phase synthesis with Boc-Tyr(Et) as the carboxy component. Ammonolysis and workup as described for I yielded Boc-Tyr(Et)-Phe-Val-Asn-Cys(Bzl)-Pro-D-Arg(Tos)-Gly-NH_2 (0.535 g, 80.7% based on initial Gly content of the resin): mp 211–213 °C; $[\alpha]^{24}_D$ −16.4° (*c* 1, DMF); TLC R_f (A) 0.61, R_f (B) 0.83. Anal. ($C_{64}H_{87}N_{13}O_{14}S_2$) C, H, N. Amino acid analysis: Tyr, 0.99; Phe, 1.00; Val, 1.01; Asp, 1.02; Cys(Bzl), 0.98; Pro, 1.00; Arg, 0.98; Gly, 1.00; NH_3, 2.13. An aliquot (0.398 g, 0.3 mmol) of this Boc-octapeptide amide was deprotected and coupled with *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate (0.232 g, 0.6 mmol)

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(35) We had found[6,7,15] that hydrolysis of protected peptides which have *O*-methyltyrosine at the N-terminal position with hydrochloric acid in the usual way[34] at 110 °C gave a quantitative recovery of tyrosine. However, hydrolysis of protected and free peptides which have *O*-methyltyrosine in an internal position under the same conditions were found in previous studies[6,15,36] to result in a nonquantitative recovery of tyrosine due to the incompleteness of demethylation.[36] In this study, hydrolysis of all the *O*-alkyltyrosine-containing peptides at 120 °C with constant-boiling hydrochloric acid as described under Experimental Section resulted in a quantitative recovery of tyrosine.
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(37) This is the critical point in determining the yield and purity of the desired peptide. It is essential to obtain complete solution of the residue in as short a time as possible, hence, the use of 10% acetic acid instead of 0.2% normally used.[7,13,20] With the higher *O*-alkyltyrosine-containing analogues, glacial acetic acid (1–5 mL) was added to the 10% acetic acid solution to ensure complete solubility. Dilution with ice-cold water rather than with room temperature water also afforded a substantial improvement in both the yield and the purity of the final product.

in the manner described above for I and yielded the acyl octapeptide amide II (0.361 g, 81.7%): mp 222–224 °C; $[\alpha]^{20}_D$ −22.8° (c 0.5, DMF); TLC R_f (A) 0.5, R_f (B) 0.83. Anal. ($C_{74}H_{97}N_{13}O_{13}S_3$) C, H, N. Amino acid analysis: Tyr, 1.00; Phe, 1.02; Val, 1.03; Asp, 1.02; Cys(Bzl), 0.98; Pro, 1.03; Arg, 0.99; Gly, 1.00; NH_3, 2.11.

β-(S-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(*i*-Pr)-Phe-Val-Asn-Cys(Bzl)-Pro-D-Arg(Tos)-Gly-NH_2 (III). The Boc heptapeptidyl resin A (1.5 g, 0.5 mmol) was converted to the acyl octapeptidyl resin (1.70 g, 0.5 mmol) in two cycles of solid-phase synthesis using Boc-Tyr(*i*-Pr)[21] and *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate in each coupling step. The protected acyl octapeptidyl resin was ammonolyzed, and the amide was extracted with hot DMF and precipitated by addition of water. The crude product was reprecipitated from DMF–ethanol–ethyl ether to give III (0.53 g, 71.3% based on initial Gly content of the resin): mp 223–225 °C; $[\alpha]^{22}_D$ −24.6° (c 1.0, DMF); TLC R_f (B) 0.86; R_f (D) 0.63. Anal. ($C_{75}H_{99}O_{13}N_{13}S_3$) C, H, N. Amino acid analysis: Tyr, 1,00; Phe, 1.03; Val, 1.02; Asp, 1.00, Cys(Bzl), 0.99; Pro, 1.01; Arg, 0.97; Gly, 1.00; NH_3, 2.01.

β-(S-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(*n*-Pr)-Phe-Val-Asn-Cys(Bzl)-Pro-D-Arg(Tos)-Gly-NH_2 (IV). The Boc heptapeptidyl resin A (1.2 g, 0.4 mmol) was converted to the acyl octapeptidyl resin (1.36 g, 0.4 mmol) in two cycles of solid-phase synthesis using Boc-Tyr(*n*-Pr)[21] and *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate in each coupling step. The protected acyl octapeptidyl resin was ammonolyzed, and the amide was extracted with hot DMF and precipitated by the addition of water. The crude product was reprecipitated from DMF–ethanol–ethyl ether to give IV (0.45 g, 75.6% based on initial Gly content of the resin): mp 227–228 °C; $[\alpha]^{22}_D$ −26.7° (c 1.0, DMF); TLC R_f (B) 0.86; R_f (D) 0.63. Anal. ($C_{75}H_{99}O_{13}N_{13}S_3$) C, H, N. Amino acid analysis: Tyr, 1.02; Phe, 1.02; Val, 1.03; Asp, 1.00; Cys(Bzl), 1.01; Pro, 1.02; Arg, 0.98; Gly, 1.00; NH_3, 1.98.

β-(S-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(Me)-Phe-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (V). The heptapeptidyl resin B (1.5 g, 0.5 mmole) was converted to Boc octapeptidyl resin in one cycle of deprotection, neutralization, and coupling with Boc-Tyr(Me). Ammonolysis and workups as described for I yielded Boc-Tyr(Me)-Phe-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (0.597 g, 91.0% based on initial Gly content of the resin): mp 216–217 °C dec; $[\alpha]^{24}_D$ −34.8° (c 1, DMF); TLC R_f (A) 0.54, R_f (B) 0.73. Anal. ($C_{63}H_{85}N_{13}O_{14}S_2$) C, H, N. Amino acid analysis: Tyr, 0.99; Phe, 1.00; Val, 1.02; Asp, 1.01; Cys(Bzl), 0.98; Pro, 1.01; Arg, 0.98; Gly, 1.00; NH_3, 2.09. An aliquot (0.394 g, 0.3 mmol) of this Boc octapeptide amide was deprotected and coupled with *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate (0.32 g, 0.6 mmol) as detailed above for I and yielded the acyl octapeptide amide V (0.388 g, 88.7%): mp 211–214 °C; $[\alpha]^{21}_D$ −39.2° (c 1, DMF); TLC R_f (A) 0.47; R_f (B) 0.85. Anal. ($C_{73}H_{95}N_{13}O_{13}S_3$) C, H, N. Amino acid analysis: Tyr, 0.99; Phe, 1.02; Val, 1.03; Asp, 1.01; Cys(Bzl), 0.99; Pro, 1.02; Arg, 0.99; Gly, 1.00; NH_3, 2.04.

β-(S-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(Et)-Phe-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (VI). The heptapeptidyl resin B (1.5 g, 0.5 mmol) was converted into acyl octapeptidyl resin (1.70 g, 0.5 mmol) in two cycles of solid-phase peptide synthesis using as the carboxy component, respectively, Boc-Tyr(Et) and *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate. The protected acyl octapeptidyl resin was ammonolyzed, and the amide was extracted with hot DMF and precipitated by the addition of water. The crude product was reprecipitated from DMF–ethanol–ethyl ether to give VI (0.49 g, 66.5% based on initial Gly content of the resin): mp 211–213 °C; $[\alpha]^{24}_D$ −39.8° (c 1.0, DMF); TLC R_f (A) 0.59, R_f (B) 0.75. Anal. ($C_{74}H_{97}N_{13}O_{13}S_3$) C, H, N. Amino acid analysis: Tyr, 0.99; Phe, 1.01; Val, 1.01; Asp, 1.01; Cys(Bzl), 0.99; Pro, 1.02; Arg, 1.02; Gly, 1.00; NH_3, 2.07.

β-(S-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(*i*-Pr)-Phe-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (VII). The Boc heptapeptidyl resin B (1.2 g, 0.4 mmol) was converted to protected acyl octapeptidyl resin (1.36 g, 0.4 mmol) in two cycles of solid-phase synthesis using Boc-Tyr(*i*-Pr) and *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate. The protected acyl octapeptidyl resin was ammonolyzed, and the amide was extracted with hot DMF and precipitated by addition of water. The crude product was reprecipitated from DMF–ethanol–ethyl ether to give VII (0.34 g, 57.1% based on initial Gly content of the resin): mp 214–216 °C; $[\alpha]^{23}_D$ 92.5° (c 1.0, DMF); TLC R_f (B) 0.82; R_f (D) 0.57. Anal. ($C_{75}H_{99}O_{13}N_{13}S_3$) C, H, N. Amino acid analysis: Tyr, 1.02; Phe, 1.03; Val, 1.01; Asp, 1.02; Cys(Bzl), 1.02; Pro, 1.03; Arg, 0.97; Gly, 1.00; NH_3, 1.96.

β-(S-Benzylmercapto)-β,β-cyclopentamethylenepropionyl-Tyr(*n*-Pr)-Phe-Val-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (VIII). The Boc heptapeptidyl resin B (1.2 g, 0.4 mmol) was converted to protected acyl octapeptidyl resin (1.36 g, 0.4 mmol) in two cycles of solid-phase synthesis using Boc-Tyr(*n*-Pr) and *p*-nitrophenyl β-(*S*-benzylmercapto)-β,β-cyclopentamethylenepropionate. The protected acyl octapeptidyl resin was ammonolyzed, and the amide was extracted with hot DMF and precipitated by the addition of water. The crude product was reprecipitated from DMF–ethanol–ethyl ether to give VIII (0.37 g, 62.2% based on initial Gly content of the resin): mp 217–218 °C; $[\alpha]^{23}_D$ −34.6° (c 1.0, DMF); TLC R_f (B) 0.82; R_f (D) 0.57. Anal. ($C_{75}H_{99}O_{13}N_{13}S_3$) C, H, N. Amino acid analysis: Tyr, 1.03; Phe, 1.03; Val, 1.01; Asp, 1.00; Cys(Bzl), 1.01; Pro, 1.04; Arg, 0.98; Gly, 1.00; NH_3, 2.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine,4-valine,8-D-arginine]vasopressin (IX). A solution of the protected acyl octapeptide amide I (168 mg, 0.115 mmol) in sodium-dried and redistilled ammonia (400 mL) was treated at the boiling point and with stirring with sodium[26] from a stick of the metal contained in a small-bore glass tube until a light blue color persisted in the solution for 30 s.[13,20] Dry glacial acetic acid (0.4 mL) was added to discharge the color. The solution was evaporated and N_2 was passed through the flask. After 5 min the residue was dissolved in degassed aqueous acetic acid (10%, 50 mL)[37] and quickly poured into ice-cold water (~1200 mL). The pH was adjusted to ~7 with concentrated ammonium hydroxide solution. An excess of a solution of potassium ferricyanide[27] (0.01 M, 16 mL) was added gradually with stirring. The yellow solution was stirred for a further 20 min and for 10 min with the anion exchange resin (Bio-Rad AG-3, Cl^- form, 10 g damp weight). The suspension was slowly filtered through a bed of resin (50 g damp weight). The bed was washed with aqueous acetic acid (0.2%, 200 mL), and the combined filtrate and washings were lyophilized. The resulting powder (1.63 g) was desalted on a Sephadex G-15 column (110 × 2.7 cm) eluting with aqueous acetic acid (50%)[28] with a flow rate of 5 mL/h. The eluate was fractioned and monitored for absorbance at 280 nm. The fractions comprising the major peak were checked by TLC (A), pooled, and lyophilized, and the residue was further subjected to gel filtration on a Sephadex G-15 column (100 × 1.5 cm) eluting with aqueous acetic acid (0.2 M)[28] with a flow rate of 4 mL/h. The peptide was eluted in a single peak (absorbance 280 nm). Lyophilization of the pertinent fractions yielded the vasopressin analogue IX as a white powder (49.5 mg, 35.5%): TLC R_f (A) 0.30, R_f (C) 0.61; $[\alpha]^{23}_D$ −46.4° (c 0.4, 1 M AcOH). Amino acid analysis: Tyr, 0.98; Phe, 1.01; Val, 0.98; Asp, 0.99; Pro, 1.03; Arg, 0.98; Gly, 1.00; NH_3, 2.1. Analysis following performic acid oxidation prior to hydrolysis[38] gave a Cys(O_3H)–Gly ratio of 1.03:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl)tyrosine,4-valine,8-D-arginine]vasopressin (X). The analogue X was prepared from the intermediate II (167 mg, 0.113 mmol) in the manner detailed above for IX: yield 50 mg (36%); TLC R_f (A) 0.29, R_f (C) 0.57; $[\alpha]^{23}_D$ −41.4° (c 0.3, 1 M AcOH). Amino acid analysis: Tyr, 0.98; Phe, 1.01; Val, 1.03; Asp, 0.99; Pro, 1.03; Arg, 1.02; Gly, 1.00; NH_3, 1.98. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl)tyrosine,4-valine,8-D-arginine]vasopressin (XI). The protected acyl octapeptide amide III (130 mg, 0.087 mmol) was reduced by sodium in liquid NH_3, reoxidized, deionized, and purified as for IX to give XI: yield 33 mg (33.6%); $[\alpha]^{22}_D$ −35.7° (c 0.7, 50% AcOH); TLC R_f (C) 0.53; R_f (D) 0.32. Amino acid analysis: Tyr, 1.00; Phe, 1.03; Val, 1.02; Asp, 1.00; Pro, 1.01;

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Arg, 0.97; Gly, 1.00; NH_3, 2.01. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-*n*-propyl)tyrosine,4-valine,8-D-arginine]vasopressin (XII). The analogue XII was prepared from intermediate IV (115 mg, 0.076 mmol) in the manner detailed above for IX: yield 11.1 mg (13%); $[\alpha]^{22}_D$ -29.7° (*c* 0.2, 50% AcOH); TLC R_f (C) 0.49, R_f (D) 0.23. Amino acid analysis: Tyr, 1.00; Phe, 1.03; Val, 1.01; Asp, 1.02; Pro, 0.99; Arg, 0.97; Gly, 1.00; NH_3, 1.96. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine,4-valine]arginine-vasopressin (XIII). Treatment of the protected acyl octapeptide V (174 mg, 0.119 mmol) as detailed for IX yielded the analogue XIII (51.5 mg, 35.6%): TLC R_f (A) 0.28, R_f (C) 0.60; $[\alpha]^{23}_D$ -66.3° (*c* 0.4, 1 M AcOH). Amino acid analysis: Tyr, 0.99; Phe, 1.01; Val, 1.02; Asp, 1.01; Pro, 1.00; Arg, 1.01; Gly, 1.00; NH_3, 2.11. Analysis following performic acid oxidation prior to hydrolysis have a Cys(O_3H)-Gly ratio of 1.03:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl)tyrosine,4-valine]arginine-vasopressin (XIV). The peptide intermediate VI (115 mg, 0.078 mmol) was reduced by sodium in liquid ammonia, reoxidized, deionized, and purified as for IX to give XIV: yield 44 mg (40%); TLC R_f (A) 0.31, R_f (C) 0.62; $[\alpha]_D$ -65.1° (*c* 0.2, 1 M AcOH). Amino acid analysis: Tyr, 1.00; Phe, 1.01; Val, 1.01; Asp, 1.01; Pro, 1.01; Arg, 1.00; Gly, 1.00; NH_3, 1.97. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl)tyrosine,4-valine]arginine-vasopressin (XV). Treatment of the protected acyl octapeptidyl amide VII (140 g, 0.094 mmol) as detailed above for IX gave the analogue XV: yield 46 mg (43.3%); TLC R_f (C) 0.63, R_f (D) 0.29; $[\alpha]^{23}_D$ -49.5° (*c* 1.0, 50% AcOH). Amino acid analysis: Tyr, 1.02; Phe, 1.03; Val, 1.01; Asp, 1.02; Pro, 1.03; Arg, 0.97; Gly, 1.00; NH_3, 1.96. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.02:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-*n*-propyl)tyrosine,4-valine]arginine-vasopressin (XVI). The analogue XVI was prepared from intermediate VIII (140 mg, 0.094 mmol) in the manner detailed above for IX: yield 28 mg (26.4%); $[\alpha]^{22}_D$ -46.7° (*c* 0.8, 50% AcOH); TLC R_f (C) 0.63; R_f (D) 0.30. Amino acid analysis: Tyr, 1.00; Phe, 1.02; Val, 1.02; Asp, 1.01; Pro, 1.00; Arg, 0.97; Gly, 1.00; NH_3, 1.96. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

Acknowledgment. This work has supported in part by research grants from the National Institutes of General Medical Sciences (GM-25280), the National Institute of Arthritis, Metabolism and Digestive Diseases (AM-01940), and the National Heart and Lung Institute (HL-12738). The authors thank Dr. T. C. Wuu for generous use of amino acid analysis facilities, Drs. Aleksandra Olma and Wieslaw Klis for repeat synthesis of a number of the analogues using the modified reoxidation procedure, and Ms. Beverly Lockwood and Ms. Carlene Thomas for assistance in the preparation of the manuscript.

Synthesis of Peptide Analogues of Prothrombin Precursor Sequence 5-9. Substrate Specificity of Vitamin K Dependent Carboxylase

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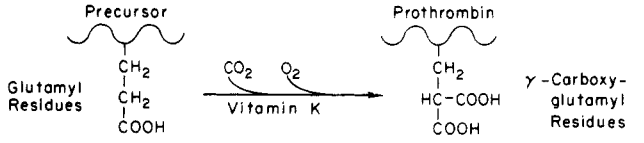
Thirty-five analogues of Phe-Leu-Glu-Glu-Leu, the pentapeptide sequence 5-9 of bovine prothrombin precursor, were synthesized and assayed as potential substrates or inhibitors of rat liver vitamin K dependent carboxylase. Carboxylation of substrate was determined by measuring the incorporation of carbon-14 labeled bicarbonate into product. Changes in substrate carboxylation produced by changing peptide chain length, amino acid chirality, or the distance separating the peptide chain backbone from the carboxyl group were measured. The data suggest that the carboxylase carboxylates L-glutamic acid residues and does not carboxylate L-aspartic acid, L-homoglutamic acid, glutamine, or D-glutamic acid residues; tri- through pentapeptides are better substrates than mono- or bis(amino acid) derivatives, and hydrophobic groups added to the N-terminus can produce better substrates for the enzyme. None of the synthetic substrates is carboxylated as effectively as the endogenous protein substrates for the enzyme. The effect of structure on additional parameters affecting carboxylation is discussed.

Prothrombin (factor II) and the other vitamin K dependent plasma clotting factors (VII, IX, and X) are synthesized in the liver by a reaction involving the vitamin K dependent conversion of specific glutamic acid residues of microsomal precursor proteins to γ-carboxyglutamic acid (Gla)[1] residues of finished proteins.[2-4] In prothrombin, ten glutamyl residues in positions 7, 8, 15, 17, 20, 21, 26, 27, 30, and 33 of the protein chain are carboxylated. The enzyme that catalyzes this reaction, vitamin K dependent carboxylase,[5] requires reduced vitamin

Scheme I



(1) Abbreviations used follow IUPAC-IUB tentative rules as described in *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations used are: Gla, γ-carboxyglutamic acid; K-H_2, reduced vitamin K; Aad, homoglutamic acid, or α-aminoadipic acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; Boc, *tert*-butyloxycarbonyl; Bz, benzoyl; Bzl, benzyl.

(2) Suttie, J. W. "*Handb. Lipid Res.* **1978**, *2*, 211-277.

(3) Suttie, J. W. *CRC Crit. Rev. Biochem.*, in press.

(4) Suttie, J. W., Ed. "Vitamin K Metabolism and Vitamin K-dependent Proteins"; University Park Press: Baltimore, 1980; pp 592.

0022-2623/81/1824-0706$01.25/0