Synthesis of Nonreducible Bicyclic Analogues of Somatostatin

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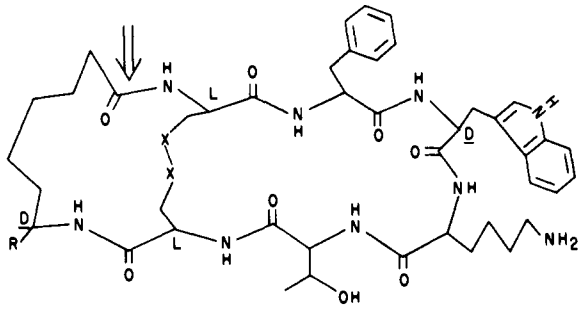
Abstract: The bis-carba analogues (**Ib** and **Ic**) of *cyclo*-(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys) (Ia) and *cyclo*-(ω-D-Asu-Cys-Phe-D-Trp-Lys-Thr-Cys) were prepared by a combination of solid-phase and solution peptide synthesis methods. Tactics involving three types of amino-protecting groups and two types of carboxyl protection were applied. The 20-membered ring portion formed in high yield under standard peptide cyclization conditions, using the azide method. The 16-membered ring portion formed well only with catalysis of the azide acylation reaction by *N*-hydroxybenzotriazole. The analogues are more resistant to hydrolysis catalyzed by trypsin but do not show increased duration of action for inhibition of growth hormone release relative to the disulfide. Disulfide reduction in vivo does not appear to be the rate-limiting step for inactivation of the sulfur containing bicyclic somatostatin analogues.

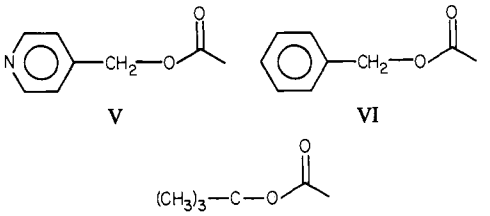
A bicyclic analogue of somatostatin, *cyclo*-(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys) (**Ia**), has been reported to show high biological



I a.) R=H, X=S b.) R=H, X=CH_2 c.) R=CO_2H, X=CH_2

potency and long duration of action.[2] It was shown to be relatively resistant to the proteolytic action of trypsin but more easily cleaved in the presence of mercaptoethanol. This observation presents the possibility that the rate-limiting step in metabolism (inactivation) of Ia in vivo might be the reduction of the disulfide bridge. It was therefore of interest to us to prepare the "bis-carba" analogue Ib wherein the sulfurs are replaced by methylene groups. This analogue would be incapable of reductive ring opening in vivo and might show even greater duration of action than Ia. In view of the special biological selectivity reported for small ring somatostatin analogues[3] having D-Cys, we felt it to be of interest to prepare also the analogue Ic having a carboxyl group in the same configuration as reported in the analogues of ref 3. Ic, therefore, incorporates a residue of D-α-aminosuberic acid in place of the ω-aminoheptanoic acid of Ib.

The unique macrobicyclic system of Ib and Ic requires a totally different synthetic strategy from that previously used for the synthesis of Ia since the final ring-forming step for synthesis of Ia had been formation of the sulfur–sulfur bond.[2] We chose the amide bond marked by ⇓ as the new final ring forming bond since it is the least sterically hindered amide bond. A retrosynthetic analysis allows one to arrive at compounds II, III, and IV (Figure 1) as basic building blocks for the synthesis of Ic. The synthesis of Ic required careful planning in the choice of protecting groups for the various amine and carboxyl functions along with careful choice of the order of formation of the amide bonds. We chose a route of synthesis which required three types of amino group protection and two types of carboxyl group protection. The "permanent" protection of the ε-amino of lysine was accomplished by the isonicotinyloxycarbonyl (iNOC) group[4] (V)[5] which is stable



V VI

VII

to acidic conditions but is removed reductively by catalytic hydrogenation. The two other amine-protecting groups chosen were the Cbz (VI) and Boc (VII) groups, which were removed by acid catalysis. The selective removal of Boc in the presence of Cbz is a result of a kinetic difference[6] and is dependent on reaction conditions. The order of bond formation was chosen to minimize the number of times kinetic selectivity would be required. The two carboxyl protecting groups chosen were methyl and *tert*-butyl esters. The presence of the benzyl ether protection for threonine was a consequence of the solid phase synthesis of IV and was not important to the subsequent tactical considerations. The choice of protecting groups for the synthesis of Ic described above requires the order of amide bond formation indicated in Figure 1.

An important consideration in this synthesis as outlined in Figure 1 was the preparation of selectively protected derivatives of D-α-aminosuberic acid (II) and LL-α,α′-diaminosuberic acid (III) in optically pure form since they are not available from natural sources. A stereocontrolled route of synthesis for these derivatives by Kolbe electrolysis of protected glutamic acid derivatives was devised and has been reported elsewhere.[7] The synthesis of Ib is less complex and requires only that II be replaced by methyl ω-aminoheptanoate in the synthetic scheme.

Results and Conclusions

The common intermediate XII for the synthesis of Ib and Ic was synthesized according to Scheme I. Solid-phase synthesis

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(2) Veber, D. F.; Holly, F. W.; Nutt, R. F.; Bergstrand, S. J.; Brady, S. F.; Hirschmann, R.; Glitzer, M. S.; Saperstein, R. *Nature (London)* **1979**, *280*, 512–514.

(3) Vale, W.; Rivier, J.; Ling, N.; Brown, M. *Metabolism* **1978** *27* (Suppl 1), 1391–1401.

(4) The abbreviations used are standard. In addition: Aha = 7-aminoheptanoic acid, Asu = α-aminosuberic acid. Nomenclature rules for the inclusion of diaminosuberic acid have not been worked out. We propose, and have used, the symbol 1/2Dsu for the radical –NHCH(CH_2CH_2)CO–. A line is then used to connect the two 1/2Dsu residues in analogy to the convention for cystine.

(5) Veber, D. F.; Paleveda, W. J., Jr.; Lee, Y. C.; Hirschmann, R. *J. Org. Chem.* **1977**, *42*, 3286.

(6) Hirschmann, R.; Veber, D. F. In "The Chemistry of Polypeptides", Katsoyannis, P. G., Ed.; Plenum: New York, 1973; p 125.

(7) Nutt, R. F.; Strachan, R. G.; Holly, F. W.; Veber, D. F. *J. Org. Chem.* **1980**, *45*, 3078–3080.

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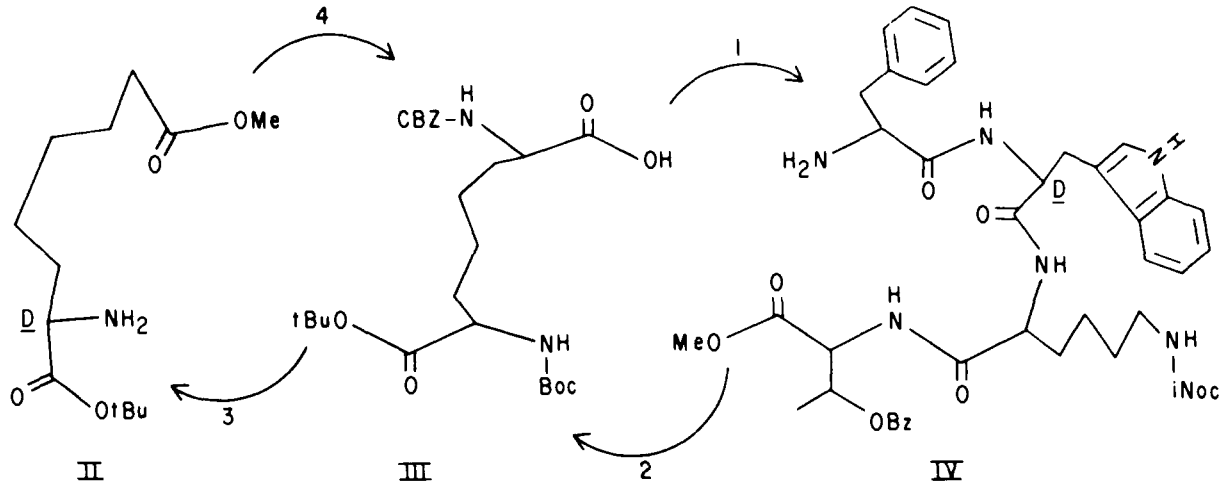

Figure 1. Intermediates and order of coupling.

of VIII followed by base-catalyzed transesterification gave the tetrapeptide methyl ester IX which was purified by silica gel chromatography. Removal of the Boc protecting group was catalyzed by HCl in ethyl acetate to give IV. Acylation of IV by III was accomplished through dicyclohexylcarbodiimide activation in the presence of *N*-hydroxybenzotriazole[9] to give X. Removal of the Boc and *tert*-butyl ester groups from X to give XI requires considerable attention to experimental detail because the difference in rate of removal of the *tert*-butyl ester and Cbz groups is not great. Careful control of temperature, times, and work-up are required to give complete removal of the *tert*-butyl ester without loss of Cbz. The methyl ester of XI is converted to the acyl azide via the hydrazide and the peptide cyclized under usual conditions[10] to give XII. The structure of XII was confirmed by amino acid analysis, NMR, molecular weight determination by gel chromatography,[11] along with the absence of a terminal amine group as indicated by a negative ninhydrin test. XII serves as a common intermediate for the synthesis of both Ib and Ic supplying the 20-membered ring of the bicyclic system.

Formation of the 16-membered ring portion was accomplished as indicated in Scheme II. Condensation of either IIa or IIb with XII was mediated by diphenylphosphoryl azide.[12] IIb was prepared by selective removal of the Boc group from α-*tert*-butyl ω-methyl *N*-Boc-D-α-aminosuberate. The benzyl ether, benzylurethane, and *tert*-butyl ester of XIIIb were simultaneously cleaved by the action of HF using anisole as carbonium ion scavenger without loss of the iNoc or methyl ester groups. The methyl ester of XIVb was converted to the acyl azide via the hydrazide (XIVd) and the ring was allowed to form under dilute conditions (1 μmol/mL) upon neutralization with diisopropylethylamine (DIPEA). Under these conditions XVb formed only slowly with the major products being dimer and higher molecular weight products. Cyclic dimer was recognized through its earlier elution from Sephadex G-50, a negative ninhydrin reaction, and the formation of a product having both a free and a protected lysine on partial hydrogenolysis. We made no attempt to optimize the formation of XVb but studied the comparable reaction in more detail for the synthesis of XVa. The cyclization to form XVa proceeded even slower than the cyclization to form XVb with only minor amounts of XVa being formed after 24 h at −20 °C followed by 4 h at 5 °C. It was discovered that this cyclization reaction is catalyzed by *N*-hydroxybenzotriazole (HOBT). Thus, a cyclization at a peptide concentration of 0.2 μmol/mL in the presence of 6 μmol/mL of HOBT was nearly complete after 24 h at −20 °C. A reaction identical in every way except for a lack of HOBT showed only a trace of XVa after the same time. Both reactions were adjusted to an apparent "pH" of 7.2 by the addition of DIPEA, indicating the catalysis to be by HOBT, not just an acid. This is, to our knowledge, the first example of the catalysis of an azide coupling by HOBT. In this instance, the catalysis not only increased the reaction rate to a practical level, it also, therefore, increased product yield by reducing the various known competing side reactions of the azide method including Curtius rearrangement.[13] High dilution was also found to be more critical in this cyclization than has been the case for other examples studied in our laboratories. The usual concentration of 1 μmol/mL normally is expected to give predominantly monomeric product. In this instance a high yield of monomer required a concentration of about 0.2 μmol/mL (Table I).

Table I. Product Distribution as a Function of Concentration in the Formation of XVa[a]

	concentration, μmol/mL		
	1	0.5	0.2
monomer	0.10	0.59	0.76
dimer	0.54	0.36	0.23
trimer	0.36	0.05	0.01

[a] Given as the fraction of the three products. Determined after separation on Sephadex G-50 using 50% acetic acid as eluent. The ratios are based on UV analysis of the eluent and therefore include linear peptides which represented only a small part of each peak.

Perhaps a different choice for final bond formation would have given a higher yield of product since the slow rate probably reflects a problem with the conformation of the monocyclic precursor.[11]

The bicyclic products XVa and XVb were chromatographically purified before the hydrogenation step required for removal of the iNoc protecting group from the ϵ-amino group of lysine. The final products after the hydrogenation step were then purified by a combination of chromatographic techniques. The purity of the final products (Ib and Ic) was estimated to be greater than 99% by TLC and LC analyses. The molecular weight was confirmed by gel filtration.[11] The composition was confirmed by amino acid analysis. The NMR spectrum at 300 MHz was consistent with the assigned structure. The biological potencies of Ib and Ic are given in Table II along with those of Ia previously reported.[2] Both carba analogues show reduced activity compared to Ia, on all biological parameters studied, with Ic being consistently more potent than Ib.

Ib and Ic are hydrolyzed between lysine and threonine under the catalytic influence of trypsin. The rate of cleavage is less than $^1/_{100}$th that observed for Ia.[2] This increased stability to a model

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(9) Konig, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788.
(10) Veber, D. F.; Holly, F. W.; Paleveda, W. J.; Nutt, R. F.; Bergstrand, S. J.; Torchiana, M.; Glitzer, M. S.; Saperstein, R.; Hirschmann, R. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *75*, 2636–2640.
(11) Brady, S. F.; Varga, S. L.; Freidinger, R. M.; Schwenk, D. A.; Mendlowski, M.; Holly, F. W.; Veber, D. F. *J. Org. Chem.* **1979**, *44*, 3101–5.
(12) Shioiri, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* **1972**, *94*, 6203. Kopple, K. D.; Schamper, T. J.; Go, A. *Ibid.* **1974**, *96*, 2597.
(13) Klausner, Y. S.; Bodanszky, M. *Synthesis* **1974**, *8*, 549.

Table II. Inhibition of Release[a] (Relative Potency of Somatostatin = 1)[b]

	compound	glucagon	insulin	growth hormone (in vitro)
Ia	*cyclo*-(Aha-Cys-Phe-D-Trp-Lys-Thr-Cys)	2.66 (1.32–6.10)	3.50 (2.31–6.38)	1.24 (0.81–1.88)
Ib	*cyclo*-(Aha-$^1/_2$Dsu-Phe-D-Trp-Lys-Thr-$^1/_2$Dsu)	0.17 (0.03–0.56)	0.21 (0.12–0.35)	0.09 (0.06–0.11)
Ic	*cyclo*-(ω-D-Asu-$^1/_2$Dsu-Phe-D-Trp-Lys-Thr-$^1/_2$Dsu)	1.2 (0.04–3.12)	0.5 (0.11–1.85)	0.26 (0.12–0.56)

[a] The biological effects were measured using methods described previously.[2] Inhibition of glucagon and insulin release was measured in urethane anesthetized rats. In vitro inhibition of growth hormone release was measured using dispersed pituitary cells. [b] 95% confidence limits given in parentheses.

Table III. Duration of Action of Bicyclic Somatostatin Analogues on the Inhibition of Growth Hormone Release

treatment	dose, μg/kg	RGH, ng/mL (95% confidence limit)
control		1085.9 (381.2, 3093.1)
Ia	75	307.7 (127.1–744.9)[b]
	250	37.0 (15.2–90.2)[c]
	750	33.9 (26.3–43.6)[c]
Ib	75	904.5 (275.5–2969.6)[a]
	250	938.6 (214.8–4095.4)[a]
	750	938.6 (270.6–3209.2)[a]
Ic	75	1379.7 (481.3–3955.5)[a]
	250	809.8 (183.1–3580.9)[a]
	750	343.5 (96.3–1210.3)[a]

[a] Not statistically different from control. [b] $p < 0.05$. [c] $p < 0.001$. Male Sprague-Dawley rats (180–200 g) were injected with saline or compound subcutaneously. After 4 h, sodium pentobarbital (17 mg/kg intravenously) was administered. The rats were bled 15 min later from the orbital sinus. The plasma was collected and assayed for growth hormone by radioimmunoassay. The data are presented as the geometric mean (95% confidence limits) (six rats/treatment). Logarithmic transformation was used in the statistical analysis of the data.

enzyme system is not reflected in an increased duration of action, however. Table III shows the effect of varied doses of Ia, Ib, and Ic on growth hormone levels 4 h after subcutaneous administration. At each dose level, after the fixed time period, Ia is more effective than either Ib or Ic in lowering growth hormone levels. Clearly, Ib and Ic cannot be evaluated as longer acting than Ia and indeed are probably shorter acting than Ia. All three analogues are longer acting than somatostatin.[2] The CD spectra of Ib and Ic (Figure 2) suggest a difference in conformation from that of Ia. The strong CD band in the 200–220-mμ region for Ia is consistent with a relatively fixed peptide backbone geometry while Ib and Ic show weaker absorption indicative of a different and perhaps less rigid conformation in the peptide backbone. This apparent change in conformation may be the reason for the loss of activity in the carba analogues compared to Ia. The changes in conformation may be small since there are no obvious differences in the NMR. The upfield shift[4] of the γ-CH_2 of lysine, when following D-tryptophan, is present in Ib and Ic indicating the proximity of the lysine and tryptophan side chains, an important conformational feature also of Ia.

In conclusion, our studies demonstrate the feasibility of the synthesis of complex carba analogues of macro-bicyclic peptides. Our studies also point up the difficulties in predicting those factors which will influence proteolysis and in vivo duration of action of biologically active peptides. It is not likely that the rate-limiting step in the hydrolysis of Ia in vivo involves disulfide reduction.

Experimental Section

Amino acid analyses and results of TLC and HPLC analyses are summarized in Table IV. The following solvents were used for TLC using Analtech silica gel plates: (A) 95–5, $CHCl_3$–*i*-PrOH; (B) 90–10–0.5, $CHCl_3$–*i*-PrOH–H_2O; (C) 90–10–1, $CHCl_3$–MeOH–H_2O; (D) 90–10–1, $CHCl_3$–MeOH–concn NH_4OH; (E) 95–5–0.2, $CHCl_3$–*i*-PrOH–H_2O; (F) 70–30–3, $CHCl_3$–MeOH–H_2O; (G) 70–30–3, $CHCl_3$–MeOH–concn NH_4OH; (H) 80–20–1.5, $CHCl_3$–*i*-PrOH–H_2O;

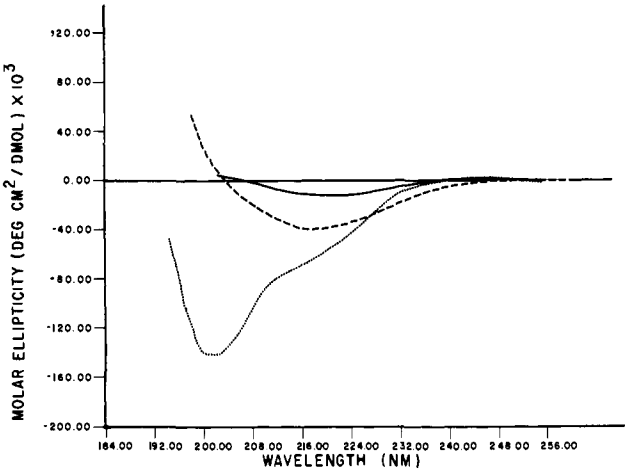


Figure 2. CD spectra of (···) **Ia**, (—) **Ib**, (– – –) **Ic** in 0.1 ionic strength phosphate buffer at pH 7.4.

(I) 70–30–2.5, $CHCl_3$–*i*-PrOH–H_2O; (J) 80–20–2, $CHCl_3$–MeOH–NH_4OH; (K) 80–20–2, $CHCl_3$–MeOH–H_2O; (L) 60–30–5, $CHCl_3$–MeOH–H_2O; (M) 10–5–1–3, EtOAc–pyridine–AcOH–H_2O; (N) 65–10–25, BuOH–HOAc–H_2O; (O) 60–40–10, $CHCl_3$–MeOH–H_2O; (P) 60–30–4–6, $CHCl_3$–MeOH–H_2O–concn NH_4OH.

The following solvent systems were used for reversed phase LC using LiChrosorb RP8 10μ as stationary phase: (A) 0.01 M NH_4Ac, pH 4.6: CH_3CN (50:50); (B) 0.01 M NH_4Ac, pH 4.6: CH_3CN (35:65); (C) 0.01 M NH_4Ac, pH 4.6: CH_3CN (60:40); (D) 0.01 M NH_4Ac, pH 4.6: CH_3OH (60–40); (E) 0.01 M NH_4Ac, pH 4.6; CH_3CN (70:30); (F) 0.01 M NH_4Ac, pH 4.6: CH_3CN (80:20).

The following abbreviations are used in the Experimental Section: HOBt·H_2O, hydroxybenzotriazole hydrate; DCCI, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DIPEA, diisoproylethylamine; TEA, triethylamine; DPPA, diphenylphosphorylazide; AAA, amino acid analysis. ^{1}H NMR spectra were taken at 300 MHz using a Varian SC 300 spectrometer.

Boc-Phe-D-Trp-Lys(iNoc)-Thr(OBz)-OMe (IX). The polymer-bound tetrapeptide Boc-Phe-D-Trp-Lys-(iNoc)-Thr(OBz)-O—Ⓟ (8.9 g, 4 mmol) was prepared using the relatively standard procedure for solid-phase synthesis described previously.[15] IX was formed by transesterification with MeOH (360 mL) catalyzed by TEA (29 mL). After 4 days at room temperature, the mixture was filtered to remove the resin and evaporated to dryness in vacuo to give IX (5.23 g). Purification was accomplished by chromatography using silica 60 (E. Merck), 600 g, and system A as eluent. Fractions containing product with R_f 0.6 (B) were combined to give 3.0 g (81% yield) of IX (Table IV for characterization). An additional 700 mg (19%) of product containing a 10% impurity as determined by HPLC was obtained by combining side fractions.

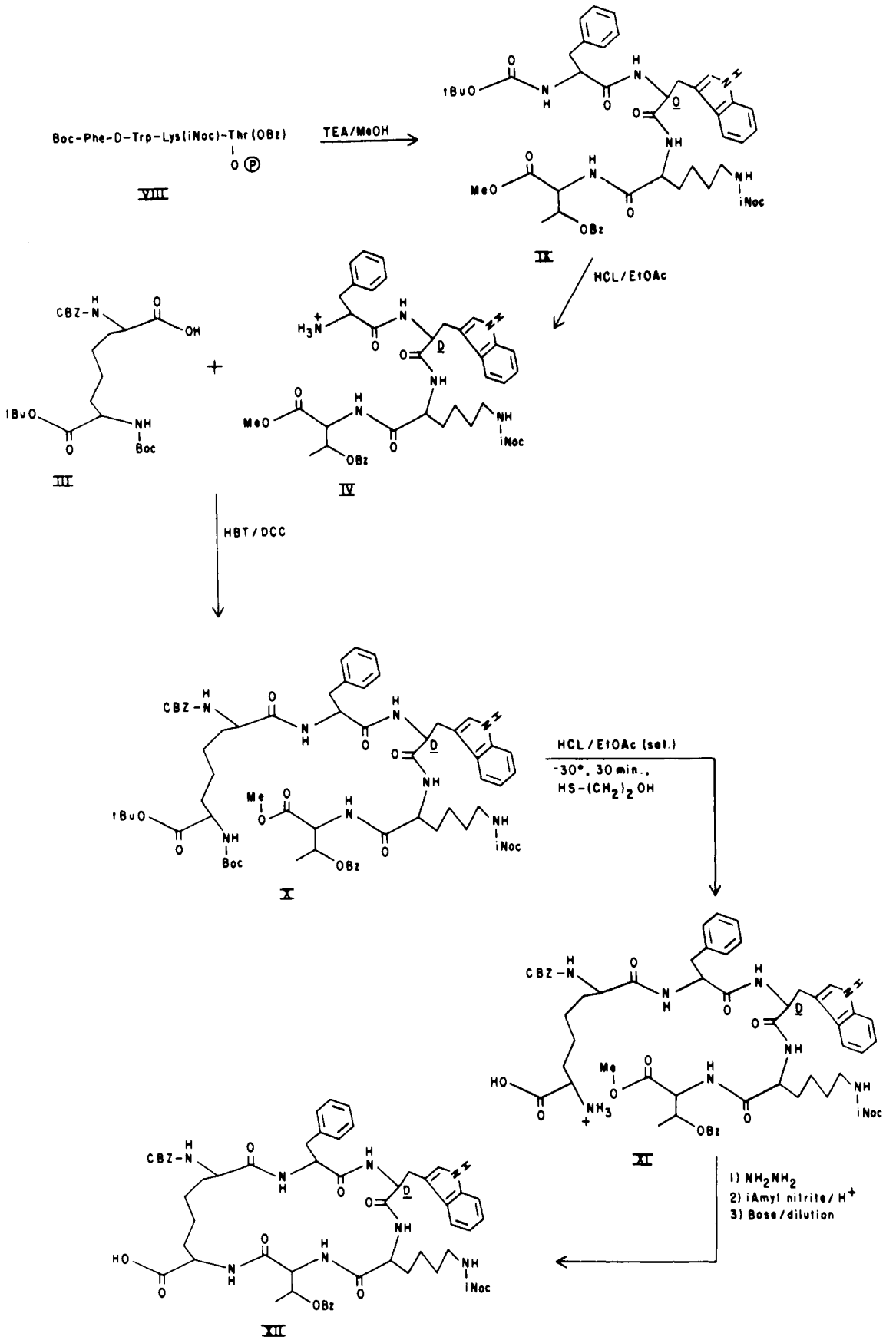
Phe-D-Trp-Lys(iNoc)-Thr(OBz)-OMe·2HCl (IV). A vigorous stream of HCl gas was introduced below the surface of a solution of Boc-Phe-D-Trp-Lys(iNoc)-Thr(OBz)-OMe (3 g) in EtOAc (75 mL) for 5 min while the temperature was maintained at −30 °C. The solution was purged with N_2 for 5 min at 0 °C during which period product partially precipitated. Ether (30 mL) and petroleum ether (100 mL) were added to fully precipitate the product, which was collected by filtration and dried to give IV (2.9 g), single spot by TLC, R_f 0.5 (D), R_f 0.3 (C).

Boc-1/2Dsu-O-*t*-Bu Cbz-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr(OBz)-OMe (X). To a suspension of 2.9 g (3.14 mmol) of IV and 1 g (6.5 mmol) of HOBt·H_2O in 40 mL of CH_2Cl_2 was added III[5] (1.55 g, 3.14

(14) Arison, B. H.; Hirschmann, R.; Veber, D. F. *Biorg. Chem.* **1978**, *7*, 447-451.

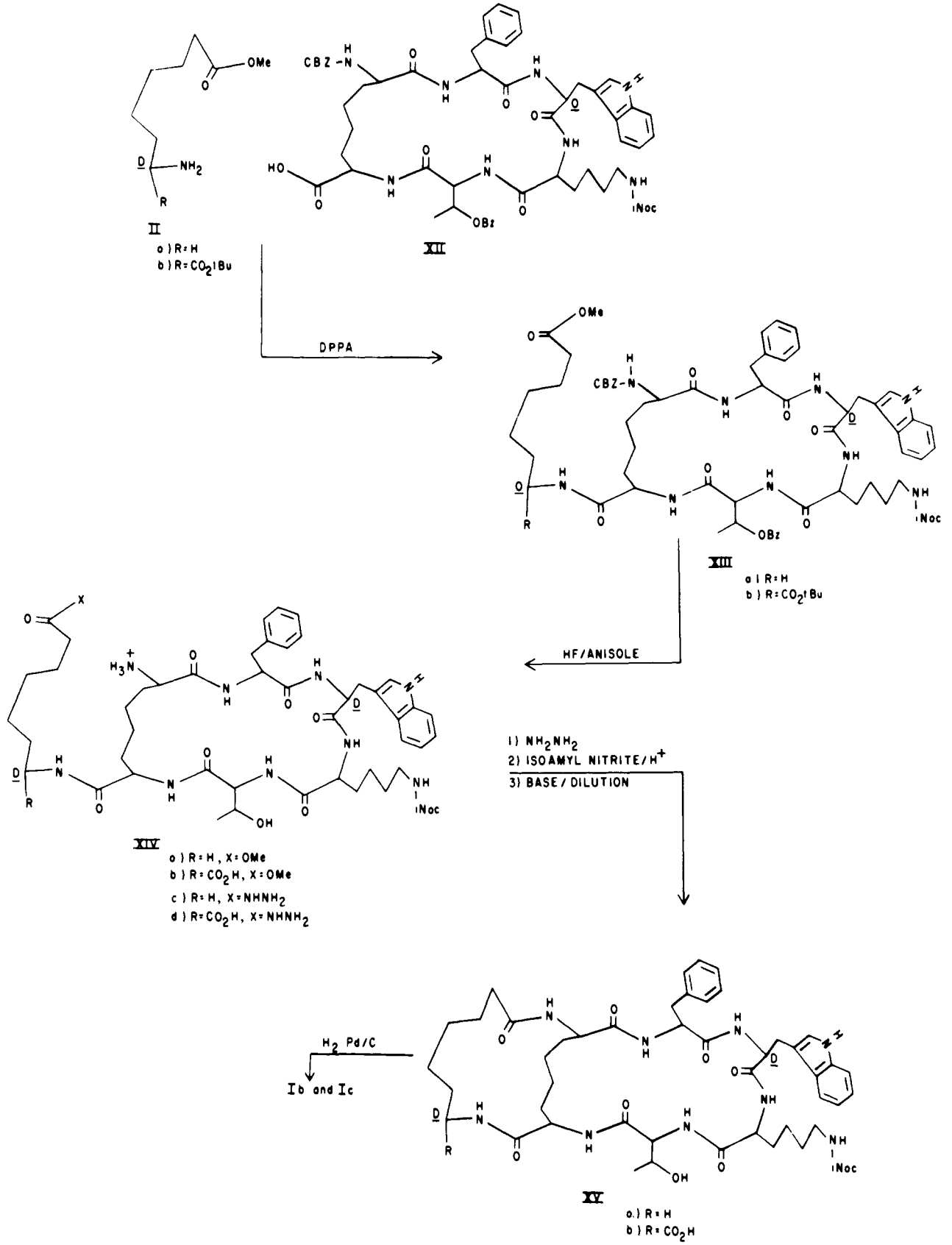
(15) Strachan, R. G.; Paleveda, W. J.; Bergstrand, S. J.; Nutt, R. F.; Holly, F. W.; Veber, D. F. *J. Med. Chem.* **1979**, *22*, 586.

Scheme I



mmol) in 20 mL of CH_2Cl_2. To this mixture was added 700 mg (3.40 mmol) of DCCI and the suspension was neutralized to an apparent pH of 5.5 (measured by using moist pH paper, range 3–5.5) by addition of TEA (0.675 mL, 4.7 mmol). After 2 h at room temperature, DCU was removed by filtration. The filtrate was extracted with three 75-mL portions of H_2O, dried over $MgSO_4$, and evaporated in vacuo to a foam (4.07 g) which was purified by chromatography using a 500-g silica 60 column with system E as eluent. Fractions containing product were identified by TLC, combined, and evaporated to dryness in vacuo to give X (3.51 g, 87% yield); single spot by TLC (Table IV). Side fractions

Scheme II



gave 190 mg (4.6%) of X containing minor impurities.

H-1/2Dsu-OH Cbz-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr(OBz)-OMe·2HCl (XI). A vigorous stream of HCl was introduced below the surface of a solution of X (3.4 g, 2.62 mmol) in EtOAc (87 mL) containing 0.87 mL of ethanedithiol at −30 °C for 35 min. The solution was then purged with a stream of N_2 at 0 °C until the product started to precipitate (8 min). Ether (30 mL) and petroleum ether (150 mL) were added to fully precipitate product which was collected by filtration and dried to give XI (2.88 g, 91% yield), R_f 0.05 (C and D), R_f 0.55 (F), R_f 0.45 (G).

Cbz-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr(OBz)-1/2Dsu-OH (XII). XI (2.86 g, 2.36 mmol) was dissolved in 75 mL of a mixture of DMF and NH_2NH_2 (2:1). After 1 h at room temperature, the solvents were

Table IV. Physical Properties and Analytical Data for Peptides Synthesized

	amino acid analysis[a]							TLC R_f (solvent system)			LC t_{min} (system)		LC purity,[d] %
	Lys	Thr	Phe	Trp	Dsu	Asu	Aha						
IX	0.97	1.03	0.95	1.05				0.2 (A)	0.6 (B)	0.6 (D)		5.23 (A)	99
X	1.00	1.00	0.98	0.66	1.01			0.25 (E)	0.7 (D)	0.6 (C)		7.03 (B)	98
XII	1.00	1.00	0.98	1.03[b]	1.03			0.44 (J)	0.25 (I)	0.4 (K)		7.53 (C)	99
XIIIb	1.00	0.98	0.99	0.66	1.01	1.00		0.64 (D)	0.48 (B)	0.54 (C)		8.67 (B)	99
XIVb	1.03	0.94	1.00	0.97	0.97	0.95		0.62 (L)	0.21 (F)	0.38 (G)			
XVb	1.00	1.00	0.99	1.05[b]	0.99	1.02		0.39 (G)	0.53 (L)	0.62 (M)			
Ic	0.99	1.01	0.99	0.97[b]	0.98	1.02		0.46 (N)	0.45 (P)	0.27 (M)	0.51 (O)	10.54 (D)	99
XIIIa	1.01	0.94	0.98	0.64	1.04		1.02	0.68 (D)					
XIVc	0.99	1.00	0.99	0.89	1.03		1.00	0.2 (K)	0.55 (J)				
Ib	1.01	1.02	1.00	1.00[c]	1.00		0.96	0.54 (N)	0.41 (G)	0.59 (M)	0.35 (L)	12.20 (E)	99

[a] 20 h hydrolysis (6 N HCl), 110 °C. [b] By UV. [c] 20 h hydrolysis (4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole), 110 °C. [d] Estimated from area under curve of the UV trace at 280 mμ.

removed by evaporation in vacuo and the residue was triturated with 300 mL of EtOAc. The resulting peptide hydrazide was isolated by filtration, washed with three additional 100-mL protions of ether and dried in vacuo for 20 h. Additional washing of the crude hydrazide with H_2O to remove formylhydrazide gave, after drying in vacuo, 2.56 g (95.2% yield) of XI hydrazide, R_f 0.45 (F). A solution of 2.43 g (2.13 mmol) of XI hydrazide in 50 mL of degassed DMF at −25 °C was acidified to an apparent pH 1.5 (measured by moist pH paper, range 1–2.5) with 2.3 mL (12.6 mmol) of freshly prepared 5.5 M HCl in THF. Isoamyl nitrite (0.378 mL) was added at −25 °C and the solution stirred for 1 h. The resulting solution of peptide azide was added to 2.4 L of degassed DMF previously cooled to −40 °C and the solution neutralized to an apparent pH of 7.0–7.2 (moist pH paper, range 6–8) by addition of 3.45 mL of DIPEA. The reaction solution was kept at −20 °C for 24 h and at 5 °C for 72 h, during which period an additional 0.4 mL of DIPEA was added as required to maintain the apparent pH 7.0–7.2. The solvent was removed by evaporation in vacuo and the residual oil triturated with a mixture of ether (20 mL) and petroleum ether (80 mL). The solid was isolated by filtration, washed with three 30-mL portions of water, and dried in vacuo (2.64 g). The product was purified by chromatography on silica gel 60 (300 g). The column was packed using system H and elution was accomplished with system I. Fractions containing product were identified by TLC, combined, and evaporated to give XII (1.17 g, 50% yield), single spot by TLC. Side fractions gave 0.7 g (30%) of XII containing minor impurities. Product XII was found to have ^1H NMR and IR spectra consistent with the desired structure. XII was found to be monomeric, MW 1124 (calcd 1108), as determined on a calibrated Sephadex G-25F column, 50% HOAc eluent.[9] UV in 50% HOAc showed λ_{max} 254 (7883), 281 (5745), 289 (4875), and $[\alpha]_D^{20}$ −14.1° (*c* 0.15, 50% HOAc); R_f HPLC, and amino acid analysis in Table IV.

ω-Methyl α-*tert*-Butyl D-α-Aminosuberate (IIb). To a solution of 1.25 g of ω-methyl α-*tert*-butyl Boc-D-α-aminosuberate[5] in 13 mL of EtOAc was added 26 mL of 4 N HCl in EtOAc. After standing for 20 min at 20 °C, the solution was poured into cold saturated sodium bicarbonate solution and the product extracted into ethyl acetate (3 × 110 mL). The combined EtOAc extracts were dried over $MgSO_4$, filtered, and evaporated in vacuo to give IIb (760 mg, 85% yield), single spot by TLC, R_f 0.46 (C), 0.67 (D); IR $CHCl_3$ (μ) 5.82 (C=O), 3.0 (NH), 6.25 (NH); δ $CDCl_3$ (ppm) 1.47 (s, 9 H, OBu^t), 2.32 (t, 2 H, CH_2C=O, *J* = 6 Hz), 3.68 (s, 3 H, OMe), total absence of signal at 1.45 ppm which was a singlet of 9 protons in the precursor (Nα-Boc).

Cbz-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr(OBz)-1/2Dsu-D-Asu(ω-OMe)-O-*t*-Bu (XIIIb). DPPA (0.35 mL, 1.62 mmol) was added to a solution of XII (1.49 g, 1.34 mmol) and IIb (432 mg, 1.32 mmol) in 10 mL of degassed DMF at −20 °C, and the solution was maintained at that temperature for 24 h and then allowed to stand at 5 °C for an additional 24 h. During this period, 0.25 mL of DIPEA was added as required to maintain an apparent pH of 7.2 (moist pH paper, range 6–8). The mixture was filtered to remove precipitated $(PhO)_2PO_2H$ and the filtrate was evaporated to a thick oil which, upon trituration with ether, gave 1.62 g of crude product. Purification was accomplished by chromatography on a 170 g silica gel column, packed in system B and eluted at a rate of 100 mL/3 min with the same solvent. Fractions containing product were identified by TLC, combined, and evaporated to dryness in vacuo to give XIIIb (1.4 g, 81% yield); TLC, LC, and AAA in Table IV and ^1H NMR spectrum consistent with the structure of XIIIb.

H-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr-1/2Dsu-D-Asu(ω-OMe)-OH (XIVb). The blocked peptide XIIIb (1.43 g, 1.06 mmol) was suspended in 3 mL of anisole in a Teflon vessel and liquid HF (30 mL) was added by distillation (−78 °C). The solution was allowed to warm to 0 °C and stirred for 30 min. The HF was removed by evaporation in vacuo at 0 °C, and the oily residue was triturated and washed by decantation with two portions of 20% EtOAc–petroleum ether. The granular solid was collected by filtration to give XIVb (1.18 g, 100% yield); TLC and AAA data in Table IV.

H-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr-1/2Dsu-D-Asu(ω-$NHNH_2$)-OH (XIVd). XIVb (1.1 g, 0.99 mmol) was converted to the hydrazide by dissolution in a 2:1 mixture of DMF–NH_2NH_2 (33 mL) for 1 h at room temperature. The solvents were removed by evaporation in vacuo, and the residue was triturated and washed by decantation with three 50-mL portions of EtOAc, isolated by filtration, and dried in vacuo. The solid was washed by slurrying with nine 10-mL portions of H_2O to remove formylhydrazide to give XIVd (700 mg). The H_2O washes were lyophilized to give an additional 510 mg of hydrazide contaminated with formylhydrazide. The two crops were purified separately by chromatography using a ratio of silica gel 60 to product of 150:1. The products were applied to the column in system L containing 2–5 drops of NH_2NH_2 and eluted with the same solvent containing no NH_2NH_2. Fractions containing product were identified by TLC analysis, combined, evaporated to a small volume, and lyophilized to give the hydrazide (0.755 g, 80%), R_f 0.19 (L); AAA after acid hydrolysis (20 h) gave: $Asu_{1.03}Phe_{1.00}D\text{-}Trp_{0.7}Lys_{0.99}Thr_{1.01}Dsu_{1.00}$.

***cyclo*-(ω-D-Asu-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr-1/2Dsu) XVb.** A solution of 750 mg (0.7 mmol) of hydrazide XIVd in 15 mL of degassed DMF was acidified by addition of 5.41 M HCl in THF (0.78 mL, 4.2 mmol) at −25 °C. Isoamyl nitrite (0.115 mL) was added and the solution stirred for 1 h. The resulting solution of acyl azide was added to 750 mL of cold (−40 °C), degassed DMF. The solution was neutralized to an apparent pH of 7.0 (moist pH paper, range 6–8) by addition of DIPEA (1.15 mL) and stored at −20 °C for 18 h and 5 °C for 72 h, during which period 0.3 mL of DIPEA was added to maintain pH 7.0. The solvent was evaporated in vacuo and the oily residue triturated with 30 mL of EtOAc–petroleum ether (1:4). The semisolid was filtered and washed with three 20-mL portions of H_2O to give 690 mg of crude product after drying in vacuo. The product was purified by filtration through a 5 × 100 cm column of Sephadex G50F using 50% HOAc as eluent (17.5 mL fractions). Fractions 60–82 gave 188 mg of trimer (R_f 0.04 (G), 0.46 (L), 0.46 (M)), fractions 83–96 gave 382 mg of dimer (R_f 0.15 (G), 0.47 (L), 0.55 (M)), and fractions 99–107 yielded 115 mg of monomeric product XVb. An analytically pure sample (Table IV) of monomer XVb was obtained by adsorption chromatography on Sephadex G25F column (5 × 100 cm) using 2 N HOAc as eluent (19-mL fractions). Fractions 121–5 gave monomeric cyclic product whereas fractions 110–120 contained linear byproducts.

***cyclo*-(ω-D-Asu-1/2Dsu-Phe-D-Trp-Lys-Thr-1/2Dsu) (Ic).** XVb (99 mg) suspended in 7 mL of 50% HOAc and 5 mL of EtOH was hydrogenated at 960 mmHg pressure for 1 h using 10% Pd/C catalyst (82 mg). The reaction mixture was filtered to remove catalyst and the filtrate evaporated to dryness. The residue was disolved in 5 mL of 2 N HOAc and filtered through a Sephadex G25F column (5 × 100 cm) using 2 N HOAc as eluent (22-mL fractions). Fractions 104–116 were combined to give 36.3 mg of IIc, $[\alpha]^{23}_D$ −16.2 (*c* 0.1, 50% HOAc), having a molecular weight of 955 (calcd 902) as determined by a calibrated Sephadex G-25 column[9] and showing characteristic chemical shifts for each residue in the NMR spectrum (300 MHz); TLC, LC, and AAA are given in Table IV.

Methyl 7-Aminoheptanoate Hydrochloride (IIa). $SOCl_2$ (40 mL) was added dropwise to 200 mL MeOH at 0 °C. 7-Aminoheptanoic acid (20

g) was added and the suspension stirred at 23 °C for 5 h. The solvents were removed by evaporation in vacuo. Toluene was added and removed by evaporation and the product was crystallized from MeOH-ether to give IIa (24.2 g, 90% yield), mp 142-145.5 °C; R_f 0.49 (J). Anal. Calcd for $C_8H_{18}ClNO_2$: C, 49.10; H, 9.27; N, 7.16; Cl, 18.14. Found: C, 48.85; H, 9.28; N, 7.16; Cl, 18.00. δ $CDCl_3$ (ppm) 1.3-1.9 (m, 8 H), 2.3 (t, 2 H, $CH_2C{=}O$), 3.05 (t, 2 H, CH_2N), 3.66 (s, 3 H, OMe); IR $CHCl_3$ 5.75 μ.

Cbz-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr(OBz)-1/2Dsu-Aha-OMe (XIIIa). A solution of 1.72 g (1.55 mmol) of XII and 370 mg (1.71 mmol) of IIa in 10 mL of degassed DMF was cooled to -20 °C and DPPA (0.40 mL, 1.86 mmol) was added followed by DIPEA (0.31 mL). The solution was maintained at -20 °C for 96 h followed by 48 h at 5 °C. During this period additional DPPA (0.08 mL) and IIa (33 mg) were added followed by DIPEA (0.4 mL) to keep the pH at 7.0 (moist pH paper, range 6-8). The resultant mixture was filtered to remove the precipitated $(PhO)_2PO_2H$ and the filtrate evaporated in vacuo to an oil which was triturated with two 30-mL portions of ether, air-dried, and washed in portions with a total of 150 mL of H_2O which after drying in vacuo gave XIIIa (1.83 g, 95% yield) and amino acid analysis in Table IV.

H-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr-1/2Dsu-Aha-OMe (XIVa). XIIIa (1.9 g, 1.5 mmol) was moistened with 4 mL of anisole in a Teflon vessel into which HF (40 mL) was introduced by distillation and the mixture held at 0 °C for 30 min. The HF was removed by evaporation in vacuo at 0 °C. The oily residue was triturated with two portions of 20% EtOAc-petroleum ether and the granular solid was collected by filtration to give XIVa (1.6 g, 100%), R_f 0.45 (K), 0.4 (D), 91.3% pure by LC, 7.80 min (F).

***cyclo*-(Aha-1/2Dsu-Phe-D-Trp-Lys(iNoc)-Thr-1/2Dsu) (XVa).** To a solution of XIVa (1.6 g, 1.5 mmol) in degassed DMF (32 mL) was added 95% NH_2NH_2 (16 mL), and the solution allowed to stand for 100 min at 23 °C. After the solvents were evaporated in vacuo, two 20-mL portions of DMF were added and evaporated in vacuo. The residue was triturated three times with 50-mL portions of EtOAc to give crude hydrazide which was purified by chromatography on silica gel 60 (200 g) packed in solvent K. Elution with 2 L of solvent K was followed by solvent F. Fractions containing product were identified by TLC and combined to give single spot hydrazide XIVc (820 mg, 54%), R_f 0.2 (K), 0.55 (J); $Aha_{1.00}Phe_{0.99}D\text{-}Trp_{0.89}Lys_{0.99}Thr_{1.00}Dsu_{1.03}$.

To a solution of XIVc (800 mg, 0.78 mmol) in 16 mL of degassed DMF and 5.92 M HCl in THF (1.0 mL, 5.92 mmol) was added isoamyl nitrite (0.13 mL, 0.96 mmol) at -25 °C and the solution stirred for 1 h. The solution was added to 4.1 L of degassed, cold DMF. HBT·H_2O (4 g) was added; the solution was neutralized with DIPEA (7 mL) to an apparent pH 7.0 (moist pH paper, range 6-8) and stored at -20 °C for 24 h followed by 24 h at 5 °C, during which period additional DIPEA (2 mL) was added to maintain pH 7.0. The solvent was evaporated in vacuo, and the residue was triturated with a total of 200 mL of 5% $NaHCO_3$ solution to give, after drying, 500 mg (65%) of crude product XVa.

***cyclo*-(Aha-1/2Dsu-Phe-D-Trp-Lys-Thr-1/2Dsu) (Ib).** A suspension of 400 mg (0.4 mmol) of crude XVa and 313 mg of 10% Pd/C in a mixture of 30 mL of 50% HOAc and 30 mL of EtOH was hydrogenated for 2 h (40 psig) in a Parr shaker. The mixture was filtered and the filtrate evaporated in vacuo. The crude product was purified by filtration through a Sephadex G-50F column (5 × 100 cm) eluted with 50% HOAc, collecting 16-mL fractions. Monomeric product was eluted in fractions 91-106 and dimeric product was present in fractions 80-91. Fractions containing monomeric product were combined and passed through a Sephadex G25F column (5 × 100 cm) eluted with 2 N HOAc. Fractions 95-100 (20 mL each) were shown to contain product by TLC and combined to give 105 mg (29%) of Ib, $[\alpha]^{23}_D$ -12.6 (*c* 0.1, 50% HOAc), having a molecular weight of 977 (calcd 858). An additional 55 mg (15%) of product (97.6% pure, LC) was obtained from side fractions.

Trypsin Cleavage of Ia, Ib, and Ic. To a suspension of 1 mg of peptide in 0.5 mL of 0.046 M, pH 8.1, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride buffer containing 0.015 M $CaCl_2$ was added an aqueous solution of Trypsin-TPCk (0.1 mL (666 units)); the mixture was stirred at 37 °C. Aliquots (20 μL) were removed at designated time intervals, acidified with 5 μL of acetic acid, and analyzed by HPLC using a Waters μBondapak C-18 column having a Whatman Co. PELL ODS C-18 guard column using 0.0085 M H_3PO_4/CH_3CN as the mobile phase. Ia and Ib were eluted at 8.90 and 9.40 min and their hydrolysis products at 2.27 and 4.00 min, respectively, using a buffer: CH_3CN ratio of 72:28. Ic and its hydrolysis product were eluted at 12.57 and 5.37 min using a buffer: CH_3CN ratio of 80:20. Ia was 50% hydrolyzed after 45 min. Ib and Ic showed 12 and 13% hydrolysis after 24 h.

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Kinetic Studies on Reactions of Iron-Sulfur Proteins. 3. Oxidation of the Reduced Form of *Clostridium pasteurianum* 8-Iron Ferredoxin with Inorganic Complexes. Observation of Single-Stage Kinetics for a Difunctional Protein Reactant

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Abstract: The kinetics of the 2-equiv oxidation of reduced *C. pasteurianum* 8-Fe ferredoxin, which contains one-electron active $Fe_4S_4{*}(SR)_4^{3-}$ clusters (average Fe oxidation state 2.25), with a range of oxidants $(NH_3)_5CoNH_2Co(NH_3)_5^{5+}$, $Pt(NH_3)_6^{4+}$, $Co(NH_3)_6^{3+}$, $Co(en)_3^{3+}$, $Co(NH_3)_5Cl^{2+}$, $Co(acac)_3$, $Co(edta)^-$, $Co(cydta)^-$, and $Co(C_2O_4)_3^{3-}$, have been studied at pH 8.0 (Tris buffer), I = 0.10 M (NaCl). All the reactions give a single kinetic step which can be accounted for in terms of statistically related biphasic schemes, in which the difunctional fully reduced 8-Fe(rr) protein reacts at twice the rate of the monofunctional half-reduced 8-Fe(or) form. Rate constants listed are for the oxidation of 8-Fe(or) to 8-Fe(oo). The oxidants $Pt(NH_3)_6^{4+}$, $Co(NH_3)_6^{3+}$, and $Co(en)_3^{3+}$ exhibit limiting kinetic behavior, consistent with a mechanism involving association (K) followed by outer-sphere electron transfer (k_{et}). With $Co(NH_3)_6^{3+}$ and $Co(en)_3^{3+}$ the temperature dependences for K give $\Delta H°$ and $\Delta S°$ values which suggest a predominantly electrostatic interaction. Overall rate constants $k(=Kk_{et})$ are reported for all other oxidants, including $(NH_3)_5CoNH_2Co(NH_3)_5^{5+}$, which reacted too rapidly for a full study.

Ferredoxins isolated from bacteria are known to contain $Fe_4S_4{*}(SR)_4$ clusters,[1] where S* represents inorganic sulfide and SR a cysteine amino acid residue of the polypeptide chain. Such clusters also occur in complex enzymes, including xanthine oxidase

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