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SYNTHESIS OF A CHELATED CORE RELATED TO RUBREDOXIN

Amino Acids and Peptides. XXXV*

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Two protected pentapeptides corresponding to the amino acid sequences 6–10 and 38–42 of rubredoxin were synthesized and coupled to yield a decapeptide. This hybrid compound, as well as the corresponding deblocked derivative, formed soluble iron complexes, whose visual absorption colors were similar to the native protein.

The rubredoxins are non-heme iron proteins of low molecular weight that participate in various oxidation-reduction schemes, although their exact role in anaerobic bacteria is not understood at the present time (3, 4). However, rubredoxin can replace ferredoxin as an electron carrier, while a more complex rubredoxin from an aerobic bacterium is known to function in a ω-hydroxylation scheme (5). An archtype correlation between the rubredoxins and the ferredoxins has been proposed (6), which is of considerable interest in view of the fundamental, evolutionary nature of the ferredoxins (7).

Rubredoxin from *Micrococcus aerogenes* is a linear polypeptide of 53 residues and contains an iron that is coordinated to the cysteine residues at positions 6, 9, 38, and 41 in the molecule. An idealized two dimensional arrangement for this protein is presented in Fig. 1 (8). The chelate structure has been studied by various physical techniques (9, 10), but considerably more work exists on the related compound from *Clostridium pasteurianum* (11–15), which includes the preparation of several simple inorganic models (16–18). A detailed X-ray study of this last rubredoxin showed recently that the metal was in a strained tetrahedral configuration and served to connect two separate, secondary hairpin turns of the main chain involving the regions between residues 5–10 and 37–42 (19). Most importantly, the large, middle peptide section consisting of residues 11–36 was not in bonding contact with the metallo-organic region. Such information suggested that the synthesis of a small, model peptide area might produce an "active-site" or, at least, give evidence as to the stability of the existing central core of rubredoxin.

This idea has been partially verified in terms of the rubredoxin from *M. aerogenes* by two related chemical approaches. In terms of organic synthesis, it was initially necessary to prepare a protected peptide having the same amino acid sequence as that found in the 38–42 region. Thus, methyl glycinate hydrochloride (I) was coupled to N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteine (II) by either the mixed anhydride or the *N,N'*-dicyclohexylcarbodiimide (DCCI) procedures (20, 21) to yield methyl N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (III). Addition of trifluoroacetic acid to the dipeptide III cleaved the *N*-terminal protecting group and produced the corresponding trifluoroacetate salt (IV). The reaction of compound IV with N^{α}-*tert*-butyloxycarbonyl-L-leucine (V) in the presence of isobutyl chloroformate formed

* For the previous paper in this series see Reference (1). For a preliminary communication on this subject see Reference (2).

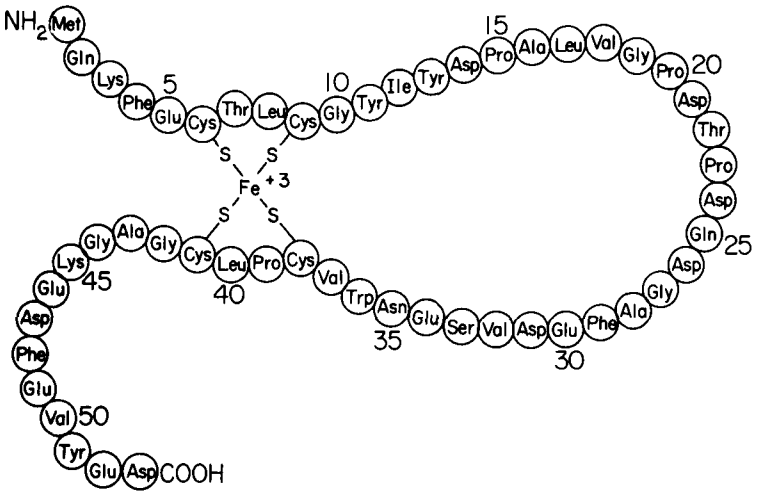

FIGURE 1
The amino acid sequence of the rubredoxin from *Micrococcus aerogenes*.

methyl N^{a}-*tert*-butyloxycarbonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (VI). Treatment with trifluoroacetic acid gave the salt (VII), which was joined to N^{a}-*tert*-butyloxycarbonyl-L-proline (VIII) by a mixed anhydride reagent to afford methyl N^{a}-*tert*-butyloxycarbonyl-L-prolyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (IX). The N^{a}-protecting group was removed in the usual fashion and the resulting salt (X) was combined with compound II to give the desired methyl N^{a}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-S-*p*-methoxy benzyl-L-cysteinylglycinate (XI). Another route involved the coupling of methyl L-leucinate hydrochloride (XII) to N^{a}-benzyloxycarbonyl-L-proline (XII) by a mixed anhydride to yield methyl N^{a}-benzyloxycarbonyl-L-prolyl-L-leucinate (XIV). Hydrogenolysis of the N^{a}-protecting group formed the dipeptide amine acetate (XV), and a coupling to compound II by an anhydride afforded methyl N^{a}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucinate (XVI). Hydrolysis of the tripeptide XVI gave the corresponding acid (XVII), which was merged with the dipeptide IV by a mixed anhydride reaction to produce the pentapeptide XI, identical in all aspects to the compound made exclusively by the mixed anhydride scheme. On standing with trifluoroacetic acid, compound XI yielded the deblocked salt (XVIII).

The synthesis of the protected pentapeptide R_{6-10}, methyl N^{a}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (XIX) has been recently described, as well as the corresponding hydrazide (XX) (22). Alternatively, hydrolysis of compound XIX formed the acid (XXI). The coupling of the salt XVIII with either the hydrazide XX *via* the azide procedure (23) or with the acid XXI gave the same decapeptide, methyl N^{a}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinyl-glycinate (XXII). The addition of sodium to a liquid ammonia solution of XXII cleaved the S-*p*-methoxybenzyl protecting groups (24) and generated the partially deblocked peptide, methyl N^{a}-*tert*-butyloxycarbonyl-L-cysteinyl-L-threonyl-L-leucyl-L-cysteinylglycyl-L-cysteinyl-L-prolyl-L-leucyl-L-cysteinylglycinate (XXIII). If the decapeptide XXIII was hydrolyzed to the acid (XXIV), then the remaining blocking groups could be removed by warming with trifluoroacetic acid-anisole to afford the salt, L-cysteinyl-L-threonyl-L-leucyl-L-cysteinylglycyl-L-cysteinyl-L-prolyl-L-leucyl-L-cysteinylglycine trifluoroacetate (XXV). Idometric titration of decapeptides XXIII and XXV showed the presence of 3.80 and 3.60 free cysteinyl groups, respectively (25). All of the above work is summarized in Fig. 2.

The formation of iron-cysteinate complexes has been observed (26) and studied (27) by a number of groups. The principal routes appear to involve the mixing of ferric ion with an inorganic sulfide ion source and an organic cysteine derivative, or the addition of ferrous ion to a similar mixture, followed by an oxidation step (16, 18). The ap-

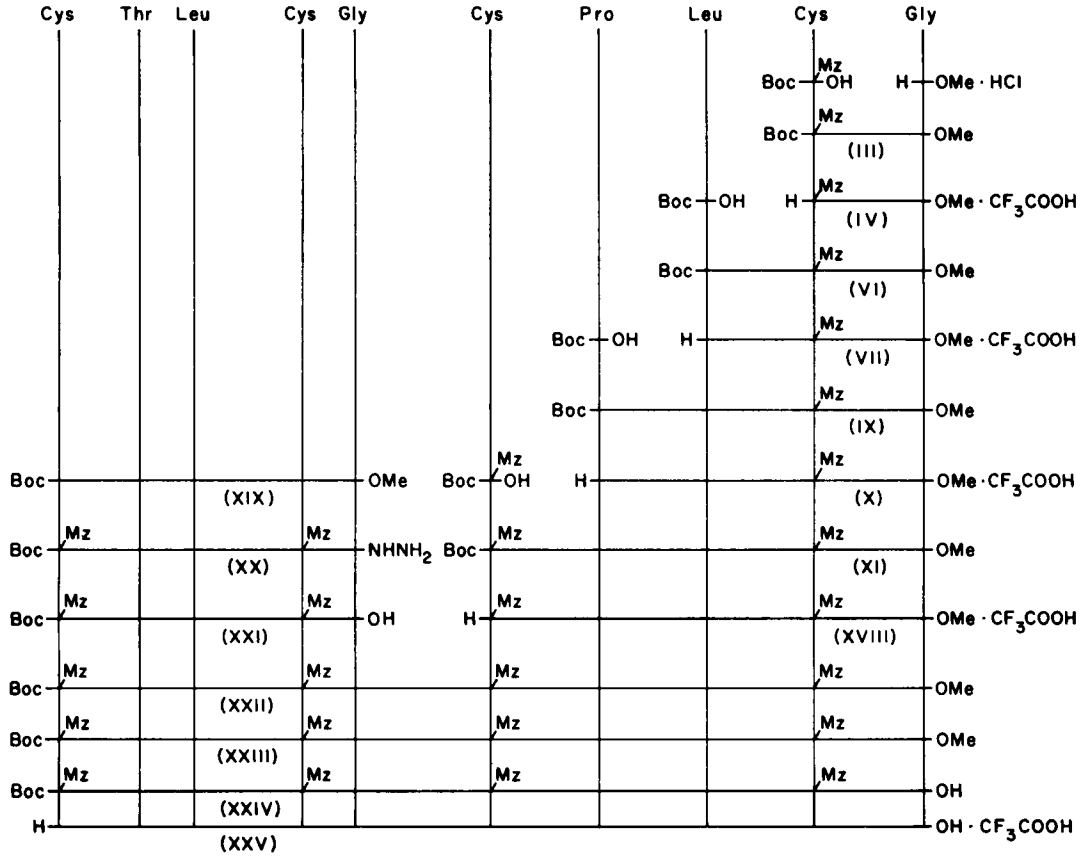

FIGURE 2

Schematic diagram of the synthesis of the decapeptides (XXIV) and (XXV) related to rubredoxin: Boc, *tert*-butyloxycarbonyl; HCl, hydrochloride; TFA, trifluoroacetate; MBzl, *p*-methoxybenzyl; OMe, methyl ester; $NHNH_2$, hydrazide.

pearance of a dark-brown color has been taken in the past to imply the existence of a chelate, whose exact structure is still subject to considerable speculation. Moreover, the exact shape of the resulting absorption curve is known to vary as a function of pH, time, oxygen content, reactant concentration, and the presence of so-called labile sulfide (28). It is important to note that in no case has the resulting organic complex been isolated in a solid form or characterized by the usual techniques.

With these thoughts in mind, an attempt was made to form a complex between the artificial rubredoxin cysteine core and soluble iron. Both compounds XXIV and XXV were suspended separately in water, mercaptoethanol was added, and the pH was adjusted to 10 by addition of triethylamine. The clear liquid was deaerated with nitrogen and an aqueous solution of ferrous ammonium sulfate was introduced, after which the reaction was cooled to 0°. The admission of air into the flask produced an immediate dark red-brown coloration. The failure to see any precipitate under these conditions meant that the ferrous ion had been incorporated into a chelate structure and subsequently was oxidized to the ferric state. Each solution was passed through a Sephadex LH-20 column, previously equilibrated to a pH value of 9.5 ($\mu m = 0.06$), to yield two pale yellow and one brown fractions. The latter possessed a continuous band spectrum, which was different in shape from those produced by mixing the various reactants in any two combinations. It should be noted that a match to the exact spec-

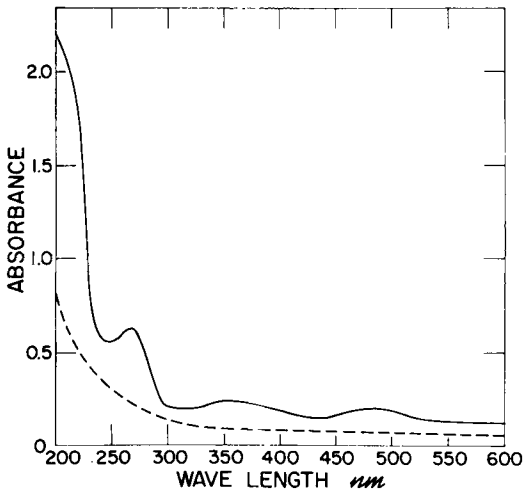

FIGURE 3

Absorption spectrum of native rubredoxin (—) and the iron complex derived from compound XXIII (---) in water.

trum of rubredoxin would not be expected (Fig. 3), as no aromatic residues are present in compounds XXIV and XXV. Lyophilization of either product produced a pale-green powder, which was partially insoluble in water and contained starting peptide and iron salts. The failure to obtain a stable, solid adduct implies the existence of a strained geometry for these two synthetic substrates or a gradual conversion of the complexed ferric ion to the ferrous state.

The conclusions reached are as follows: first, it is possible to form a complex between the ferric ion and the cysteines of the two peptides, whose visible colors are similar to that of rubredoxin; and, second, electron-transfer models are attainable using existing, simple structural features found in native proteins (6, 29). We plan to extend this work to the synthesis of such peptides as cys-x-x-cys-gly-gly-gly-gly-cys-x-x-cys, since this unit represents an optimum distance between the cysteinyl residues, as measured on an actual molecular model. Alternatively, the preparation of hybrid peptides similar to cys-x-x-cys-$(CH_2)_n$-cys-x-x-cys would permit the study of subtle geometric features involved in the formation of these chelates.

Finally, after this work was completed in early 1971, a related model pentapeptide, whose sequence corresponds to positions 7–11 of clostridial ferredoxin, was prepared by a Japanese group (30). Later, their compound was reacted with ferric ion in the presence of sodium sulfide so as to yield a similar complex (31). Thus, it seems likely that the active-site in both the ferredoxins and rubredoxins may be approached along fairly parallel lines. The ease of these synthetic operations should permit the construction of many molecular models in the near future and aid in an understanding of the precise role played by electron-transfer proteins in biological systems.

EXPERIMENTAL PROCEDURES*

Methyl N$^{\alpha}$-tert-*Butyloxycarbonyl-S*-p-*methoxybenzyl-L-cysteinylglycinate* (*III*). *A. Mixed anhydride.* A solution of *N*$^{\alpha}$-*tert*-butyloxycarbonyl-S-*p*-methoxybenzylcysteine (19.0 g, 0.05 mol) in tetrahydrofuran (300 ml) was cooled to −15° and treated in turn with *N*-methylmorpholine (5.6 g, 0.055 mol) and isobutyl chloroformate (7.5 g, 0.055 mol). After 5 min, a solution of methyl glycinate, freshly prepared by mixing a suspension of the corresponding salt (8.3 g, 0.066 mol) in tetrahydrofuran (60 ml) with *N*-methylmorpholine (6.7 g, 0.066 mol) in water (20 ml), was added and stirred for 15 min at −10°, followed by allowing the reaction mixture to warm to room temperature over 2 h. The solvent was evaporated and the residue was redissolved in ethyl acetate (300 ml). The solution was washed with 10% citric acid solution (two 60-ml portions), and water (two 60-ml portions), then dried and evaporated. The residue was crystallized from ethyl acetate-petroleum ether (18.0 g, 79%): mp 93°; $[\alpha]_D^{25}$ +18.9° (*c*, 1.50, chloroform); R_f 0.62 (solvent H).

Anal. Calcd. for $C_{19}H_{28}H_2O_6S$ (412.52): C, 55.32; H, 6.84; N, 6.79; S, 7.77. Found: C, 55.34; H, 7.07; N, 6.90; S, 7.81.

* All melting points were determined on a Reichert "Thermopan" unit and are uncorrected. Evaporations were performed under reduced pressure (water pump) with a rotatory apparatus at minimum temperature, while high-boiling solvents were removed at vacuum pressure (0.2–0.5 mm). Magnesium sulfate was used for drying purposes. Acetonitrile and *N*,*N*′-dimethylformamide were spectroscopic quality; other solvents were reagent grade and petroleum ether had bp 30–60°. Microanalyses were furnished by Galbraith Laboratories, Knoxville, Tenn., and amino acid data were obtained from AAA Laboratories, Seattle, Wa. Thin-layer chromatographic solvent systems were as follows: A=chloroform-methanol (95:5); B=pyridine-*n*-butanol-water (1:2:1); C=*sec*-butanol-30% ammonia (100:44).

B. Dicyclohexylcarbodiimide. A solution of N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzylcysteine (13.7 g, 0.040 mol) and methyl glycinate hydrochloride (5.0 g, 0.040 mol) in dichloromethane (150 ml) was treated with triethylamine (4.1 g, 0.04 mol) and the solution was cooled to −10°. *N,N'*-Dicyclohexylcarbodiimide (8.3 g, 0.04 mol) was added and the resulting mixture was stirred vigorously overnight, while slowly warming to room temperature. The solvent was evaporated and the residue was dissolved in ethyl acetate (200 ml). After filtration of the *N,N'*-dicyclohexylurea, the solution was worked up in the usual fashion to obtain an oil, which was crystallized from ethyl acetate-petroleum ether (10.7 g, 65%): mp 93°, identical in all aspects to the previous material.

*Methyl S-*p*-Methoxybenzyl-L-cysteinylglycinate Trifluoroacetate (IV).* Compound III (18.8 g, 0.046 mol) was dissolved in trifluoroacetic acid (75 ml) and the solution was allowed to stand for 30 min at room temperature. The solvent was evaporated and the residue was dried in a vacuum desiccator over sodium hydroxide for 5 h to afford an oil.

Methyl N^{α}-tert-*Butyloxycarbonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (VI).* A solution of N^{α}-*tert*-butyloxycarbonyl-L-leucine (10.5 g, 0.046 mol) in tetrahydrofuran (250 ml) was cooled to −15° and treated in turn with *N*-methylmorpholine (4.7 g, 0.046 mol) and isobutyl chloroformate (6.3 g, 0.046 mol). After 4 min, a solution of methyl S-*p*-methoxybenzyl-L-cysteinylglycinate in tetrahydrofuran (70 ml), freshly prepared by dissolving the corresponding trifluoroacetate salt IV in tetrahydrofuran (20 ml) and trimethylamine (8 ml) and evaporating to dryness, was added and stirred for 30 min at −15°, followed by allowing the reaction mixture to warm to room temperature over 2 h. The solvent was evaporated and the residue was dissolved in ethyl acetate (300 ml) and worked-up in the usual fashion to obtain an oil, which was crystallized from ethyl acetate-petroleum ether (16.5 g, 71%): mp 94–96°; $[\alpha]_D^{25}$ +29.0° (*c* 1.00, chloroform); R_f 0.84 (solvent B).

Anal. Calcd. for $C_{25}H_{39}N_3O_7S$ (525.68): C, 57.12; H, 7.48; N, 7.99; S, 6.10. Found: C, 57.00; H, 7.44; N, 7.75; S, 5.82.

*Methyl L-Leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate Trifluoroacetate (VII).* The deprotection of compound VI (11.4 g, 0.022 mol) with trifluoroacetic acid was carried out as described for the preparation of IV to give an oily product.

Methyl N^{α}-tert-*Butyloxycarbonyl-L-prolyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (IX).* A coupling between N^{α}-*tert*-butyloxycarbonyl-L-proline (4.7 g, 0.022 mol) and freshly prepared methyl L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate, derived from compound VII, was done by the mixed anhydride procedure described for the preparation of VI (11.2 g, 82%): mp 103–106°; $[\alpha]_D^{25}$ +83.3° (*c* 1.00, chloroform); R_f 0.54 (solvent A).

Anal. Calcd. for $C_{30}H_{46}N_4O_8S$ (622.77): C, 57.89; H, 7.45; N, 8.99; S, 5.14. Found: C, 58.60; H, 7.57; N, 9.69; S, 4.64.

*Methyl L-Prolyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate Trifluoroacetate (X).* The deprotection of compound IX (11.1 g, 0.018 mol) with trifluoroacetic acid was achieved as described for the preparation of IV to produce an oil.

Methyl N^{α}-tert-*Butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (XI).* A coupling between N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteine (6.1 g, 0.018 mol) and freshly prepared methyl L-prolyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate, derived from compound X, was done by the mixed anhydride procedure as described for the preparation of VI to yield a foam (8.0 g, 53%): $[\alpha]_D^{25}$ −65.0° (*c*, 1.00 methanol); R_f 0.70 (solvent A).

Anal. Calcd. for $C_{41}H_{59}N_9O_{10}S_2$ (846.08): C, 58.20; H, 7.03; N, 8.27; S, 7.58. Found: C, 57.72; H, 6.90; N, 7.84; S, 7.35.

A similar reaction between N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl L-leucine and methyl S-*p*-methoxybenzyl-L-cysteinylglycinate formed the same product.

Methyl N^{α}-*Benzyloxycarbonyl-L-prolyl-L-leucinate (XIV).* A coupling between N^{α}-benzyloxycarbonyl-L-proline (7.5 g, 0.030 mol) and freshly prepared methyl L-leucinate derived from the corresponding hydrochloride (5.4 g, 0.030 mol), was done by the mixed anhydride procedure as

described for the preparation of VI (6.0 g, 55%): mp 86–87°; $[\alpha]_D^{25}$ −38.4° (*c* 1.00, methanol).

Anal. Calcd. for $C_{20}H_{28}N_2O_5$ (376.44): C, 63.81; H, 7.50; N, 7.44. Found: C, 64.01; H, 7.58; N, 7.21.

Methyl L-Prolyl-L-leucinate Acetate (*XV*). A solution of methyl N^{α}-benzyloxycarbonyl-L-prolyl-L-leucinate (3.8 g, 0.010 mol) in methanol-acetic acid (1:1, 100 ml) containing 10% palladium-on-charcoal catalyst (0.50 g) was hydrogenated for 3 h. The catalyst was removed by filtration and evaporation of the solvent left a thick oil.

Methyl N$^{\alpha}$-tert-*Butyloxycarbonyl-S*-p-*methoxybenzyl-L-cysteinyl-L-prolyl-L-leucinate* (*XVI*). The aforementioned compound in the form of the free amine was joined to N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteine (3.8 g, 0.011 mol) by the mixed anhydride procedure as described for the preparation of VI. The impure residue was chromatographed over silica gel using chloroform-methanol (97:3) as the eluent to yield a viscous oil (3.7 g, 65%): $[\alpha]_D^{25}$ −43.8° (*c* 1.00, methanol).

Anal. Calcd. for $C_{28}H_{43}N_3O_7S$ (566.71): C, 59.45; H, 7.66; N, 7.43; S, 5.66. Found: C, 59.04; H, 7.56; N, 7.23; S, 5.67.

N$^{\alpha}$-tert-*Butyloxycarbonyl-S*-p-*methoxybenzyl-L-cysteinyl-L-prolyl-L-leucine* (*XVII*). A solution of methyl N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucinate (3.7 g, 0.066 mol) in methanol (25 ml) was treated with 1 *N* sodium hydroxide (7.2 ml) and the reaction mixture was stirred at room temperature for 1 h. Water (50 ml) was added, the solution was extracted with ether (two 50-ml portions), and the aqueous phase was acidified with saturated citric acid solution, followed by an ethyl acetate extraction (two 50-ml portions). The latter were combined, washed with water, dried, and evaporated to produce a solid (3.2 g, 90%): mp 60°.

Methyl S-p-*Methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-S*-p-*methoxybenzyl-L-cysteinylglycinate Trifluoroacetate* (*XVIII*). The deprotection of compound XI (1.7 g, 0.002 mol) with trifluoroacetic acid was done as described for the preparation of IV to yield an oil.

N$^{\alpha}$-tert-*Butyloxycarbonyl-S*-p-*methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S*-p-*methoxybenzyl-L-cysteinylglycinate* N^2-*Hydrazide* (*XX*). A solution of methyl N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (2.5 g, 0.003 mol) in methanol (20 ml) was treated with hydrazine hydrate (90%, 0.3 ml, 0.006 mol). After 2 h ether (50 ml) was added to the gelatinous reaction mixture and the precipitated product was filtered, washed, and purified from methanol-ether (1.7 g, 68%): mp 185–187°; $[\alpha]_D^{25}$ −36.5° (*c* 1.00, *N*,*N*′-dimethylformamide); R_f 0.60 (solvent A).

N$^{\alpha}$-tert-*Butyloxycarbonyl-S*-p-*methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S*-p-*methoxybenzyl-L-cysteinylglycine* (*XXI*). The hydrolysis of methyl N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (2.5 g, 0.003 mol) (22) was carried out as described for the preparation of XVII to give a foam (1.9 g, 76%): $[\alpha]_D^{25}$ −29.9° (*c* 1.00, *N*,*N*′-dimethylformamide); R_f 0.60 (solvent C).

Methyl N$^{\alpha}$-tert-*Butyloxycarbonyl-S*-p-*methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S*-p-*methoxybenzyl-L-cysteinylglycyl-S*-p-*methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-S*-p-*methoxybenzyl-L-cysteinylglycinate* (*XXII*). *A. Azide.* A stirred solution of N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate N^2-hydrazide (1.7 g, 0.002 mol) in *N*,*N*′-dimethylformamide (10 ml) was cooled to −30° and treated in turn with 2 *N* hydrogen chloride in tetrahydrofuran (3 ml) and *tert*-butyl nitrite (0.45 ml, 0.004 mol). After 15 min, triethylamine (1.1 ml, 0.008 mol) was added, followed by a solution of methyl S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-S-*p*-methoxy benzyl-L-cysteinylglycinate in *N*,*N*′-dimethylformamide (10 ml), freshly prepared by dissolving the pentapeptide salt XVIII in tetrahydrofuran (10 ml), adding trimethylamine (1 ml), and evaporating to dryness. After 2 h at −30°, the reaction was left for 1 d at −25°, for 2 d at 0°, and then worked up in the usual fashion. The residue was chromatographed over silica gel with chloroform-methanol (95:5) as the eluent to give a foam (1.8 g, 58%): mp 165–168°; $[\alpha]_D^{25}$ −81.4° (*c* 1.00, chloroform); R_f 0.30 (solvent B).

Anal. Calcd. for $C_{75}H_{106}N_{10}O_{18}S_4$ (1,563.94):

C, 57.59; H, 6.83; N, 8.95; S, 8.20. Found: C, 57.10; H, 6.56; N, 8.61; S, 10.06; amino acid ratios: 3.80 cys, 2.0 gly, 1.85 leu, 1.0 pro, 0.96 thr.

B. Mixed anhydride. N^{α}-*tert*-Butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (1.7 g, 0.002 mol) was reacted with compound XVIII using the mixed anhydride procedure as described for the preparation of VI. The resulting foam (1.3 g, 42%) was identical in all aspects to the previous material.

Methyl N$^{\alpha}$-tert-*Butyloxycarbonyl-L-cysteinyl-L-threonyl-L-leucyl-L-cysteinylglycyl-L-cysteinyl-L-prolyl-L-leucyl-L-cysteinylglycinate (XXIII).* A solution of methyl N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycinate (0.150 g, 0.1 mmol) in dry, liquid ammonia (50 ml) was treated with small pieces of freshly cut sodium metal (total amount, 25 mg) until a permanent blue persisted for more than 10 min. Solid ammonium chloride was added to discharge the color, the solvent was allowed to evaporate slowly, the residue was dissolved in water (20 ml), and the solution was filtered, neutralized with 1 *N* hydrochloric acid, acidified with solid citric acid, and extracted with ethyl acetate (three 30-ml portions). The combined organic phases were washed with water, evaporated to dryness, and the residue washed with benzene to remove products derived from the *p*-methoxybenzyl group, which exhibited a maximum in the ultraviolet spectrum at 278 nm. The remaining white solid (0.085 g, 80%) was titrated with iodine-sodium thiosulfate solution for free sulfhydryl groups: 3.8 (95%).

N$^{\alpha}$-tert-*Butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycine (XXIV).* The hydrolysis of compound XXIII (1.1 g, 0.0007 mol) was done as described for the preparation of XVII to afford a solid (1.0 g, 91%): $[\alpha]_D^{25}$ −51.7° (*c* 1.00, *N,N'*-dimethylformamide); R_f 0.45 (solvent C).

L-Cysteinyl-L-threonyl-L-leucyl-L-cysteinylglycyl-L-cysteinyl-L-prolyl-L-leucyl-L-cysteinylglycine Trifluoroacetate (XXV). A solution of N^{α}-*tert*-butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteinyl-L-threonyl-L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycyl-S-*p*-methoxybenzyl-L-cysteinyl-L-prolyl L-leucyl-S-*p*-methoxybenzyl-L-cysteinylglycine (0.15 g, 0.1 mmol) in anisole (0.025 ml) and trifluoroacetic acid (30 ml) was gently refluxed for 2 h. The solvent was evaporated and the residue was dissolved in methanol (3 ml). The addition of ether (200 ml) gave a precipitate, which was centrifuged, collected, washed with benzene and dried. The white solid (0.060 g, 55%) was titrated with iodine-sodium thiosulfate solution for free sulfhydryl groups: 3.6 (90%).

Formation of the iron complex. A solution of N^{α}-*tert*-butyloxycarbonyl-L-cysteinyl-L-threonyl-L-leucyl-L-cysteinylglycyl-L-cysteinyl-L-prolyl-L-leucyl-L-cysteinylglycine (0.1 g, 0.1 mmol) in water (5 ml) and mercaptoethanol (2.3 ml, 30 mmol) was adjusted to a pH value of 10 by the addition of trimethylamine. Nitrogen gas was bubbled through the solution for 10 min, then an 0.1 *M* solution of ferrous ammonium sulfate (5 ml, 0.5 mmol) was added, and the reaction was cooled to 0°. On the admission of air to the flask, the faintly red solution turned dark immediately. After 30 min, the liquid was passed through a Sephadex LH-20 column, which had been previously equilibrated with triethylamine-acetic acid with a pH value of 9.5 ($\mu m = 0.06$). The main brown band was separated from two minor light yellow ones, and it gave an absorption spectrum, as shown in Fig. 3. Lyophilization produced a pale-green powder composed of iron salts and peptide.

Alternatively, compound XXV (0.15 g, 0.1 mmol) was treated in the same manner to produce a similar result.

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