comitant with nucleophilic attack of sulfite ion on C-6 of the pyrimidine ring system can occur. This would seem quite analogous to nucleophilic attack by a thiol at the active site of an enzyme on C-6 concomitant with methyl donation to C-5 from an electrophile such as either *S*-adenosylmethionine or the cationic imine ($N^+=CH_2$) shown by Kallen and Jencks[25] to be an intermediate in the condensation of formaldehyde with tetrahydrofolate. Also the collapse of an intermediate such as **4** to yield the methylated pyrimidine involves proton abstraction from C-5. Since enzymes must catalyze reactions under constant and physiological conditions of pH, the enzyme most likely catalyzes the breakdown of the enzyme–dihydropyrimidine intermediate **4** *via* a general base catalyzed mechanism. This presumption is also in accord with our data with the sodium bisulfite–1,3-dimethyluracil system.

Another important biological aspect of the reaction of bisulfite ion with pyrimidines is the finding that the 5,6-dihydrocytosine-6-sulfonate is rapidly deaminated under physiological conditions of temperature and pH to yield uracil.[4,6] Thus, bisulfite can be considered mutagenic as has been demonstrated by Hayatsu and Miura who have shown that bisulfite induces mutations in phage λ.[26]

(25) R. H. Kallen and W. P. Jencks, *J. Biol. Chem.*, **241**, 5851 (1966).

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Studies on Polypeptides. L. Synthesis of a Protected Tritricontapeptide Hydrazide Corresponding to Positions 48–80 of the Primary Structure of Ribonuclease T_1[1-4]

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Abstract: Syntheses are described of two *N*-benzyloxycarbonylpeptide *tert*-butoxycarbonylhydrazides which correspond to positions 48–65 and 66–80 of the proposed primary structure of the enzyme ribonuclease T_1. Evidence is presented to indicate that these materials are sequentially homogeneous. These fragments were condensed to form an *N*-benzyloxycarbonyltritricontapeptide *tert*-butoxycarbonylhydrazide corresponding to positions 48–80 of the sequence of the enzyme. Advantages and disadvantages of the stepwise synthesis of peptides in solution are discussed.

For reasons discussed in previous communications,[1,3] we have subdivided the peptide chain of ribonuclease T_1 (ribonucleate guanine nucleotido-2′-transferase (cyclizing) 2.7.7.26) into a series of fragments (Figure 1) which serve as the starting materials for attempts to construct the entire primary structure of the enzyme *via* fragment condensation. Thus far, we have described details pertaining to the synthesis of fragments B, C, and D and to their condensation to form the partially protected hexatricontapeptide BCD (Figure 2) and have provided evidence for the sequential and stereochemical homogeneity of this compound.[1] The present investigation concerns the preparation of the partially protected tritricontapeptide EF (Figure 2) which corresponds to positions 48–80 of the enzyme's peptide chain.

Four protected peptide hydrazides, subfragments E_1, E_2, F_1, and F_2 (Figure 1), were prepared mainly by stepwise methods in solution[5,6] according to the routes illustrated in Schemes I–IV. Following suitable deprotection, subfragments E_1 and E_2 were linked to form fragment E; subfragments F_1 and F_2 were similarly

(1) See J. Beacham, G. Dupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **93**, 5526 (1971), for paper XLIX in this series.

(2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation. The early phases of this investigation were supported by the Research Laboratories, Edgewood Arsenal, Contract No. DA-18-035-AMC-307 (A). The opinions expressed are those of the authors and do not reflect endorsement by the contractor.

(3) Preliminary communications of some of the results presented in this article have appeared: N. Yanaihara, C. Yanaihara, G. Dupuis, J. Beacham, R. Camble, and K. Hofmann, *J. Amer. Chem. Soc.*, **91**, 2184 (1969); K. Hofmann, *Peptides, Proc. Eur. Peptide Symp., 10th, 1969*, 130 (1971); H. T. Storey and K. Hofmann, *ibid., 12th, 1971*, in press.

(4) The amino acid residues except glycine are of the L configuration. The following abbreviations are used: AP-M = aminopeptidase M; DCC = *N,N′*-dicyclohexylcarbodiimide; DMSO = dimethyl sulfoxide; DMF = dimethylformamide; EC = ethylcarbamoyl; EtOH = ethanol; F = formyl; *i*-PrOH = isopropyl alcohol; MeOH = methanol; N_3 = azide; NMM = *N*-methylmorpholine; OCP = 2,4,5-trichlorophenyl ester; ONHS = *N*-hydroxysuccinimido ester; *O*-*t*-Bu = *tert*-butyl ester; TEA = triethylamine; TFA = trifluoroacetic acid; THF = tetrahydrofuran; tlc = thin layer chromatography; X = *tert*-butoxycarbonylhydrazide; Y = benzyloxycarbonylhydrazide; Z = benzyloxycarbonyl. In order to simplify the designation of complex peptide derivatives, the following nomenclature is used: subfragments E_1, E_2, F_1, and F_2 and fragments E, F, and EF = the *N*-benzyloxycarbonyl *tert*-butoxycarbonylhydrazides of subfragments E_1, E_2, F_1, and F_2 and fragments E, F, and EF. Subfragments E_1, E_2, F_1, and F_2 and fragments E, F, and EF *tert*-butoxycarbonylhydrazides = the amino-deprotected *tert*-butoxycarbonylhydrazides. Subfragments E_1, E_2, F_1, and F_2 and fragments E, F, and EF hydrazides = the unprotected hydrazides of the *N*-benzyloxycarbonyl subfragments and fragments.

(5) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).

(6) J. S. Morley, *J. Chem. Soc. C*, 2410 (1967).

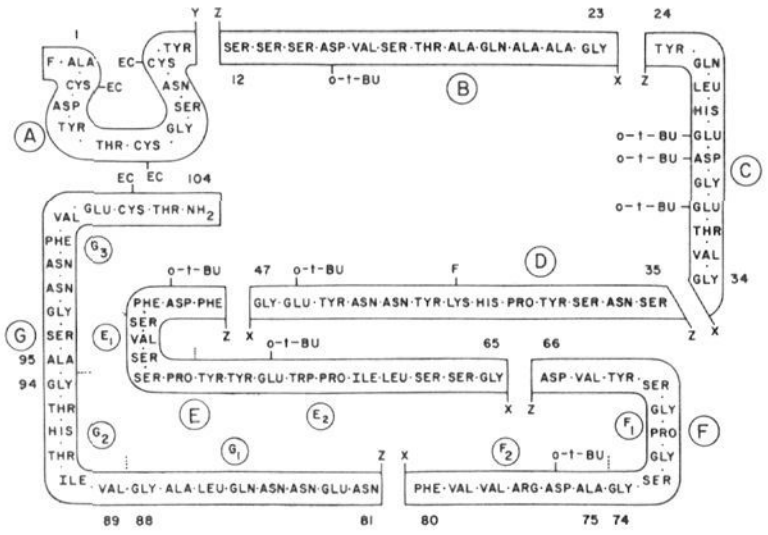


Figure 1. Protected peptide hydrazides corresponding to the sequence of ribonuclease T_1.

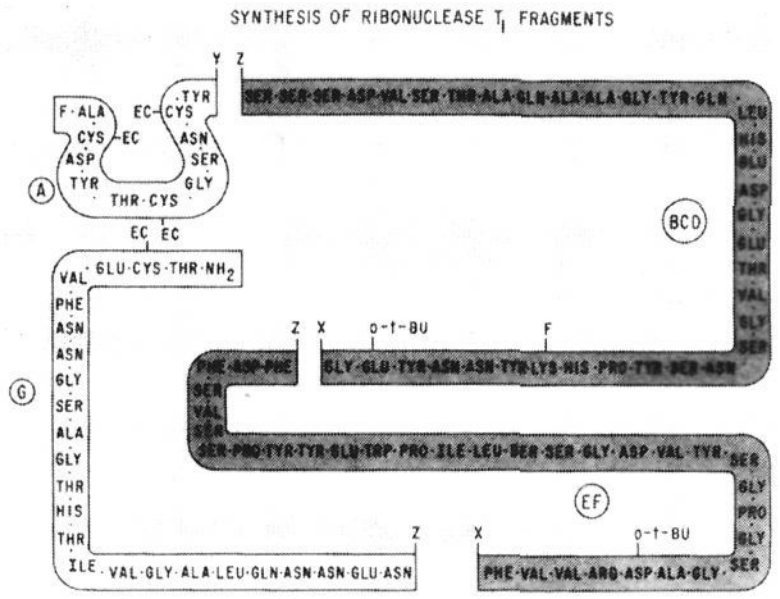
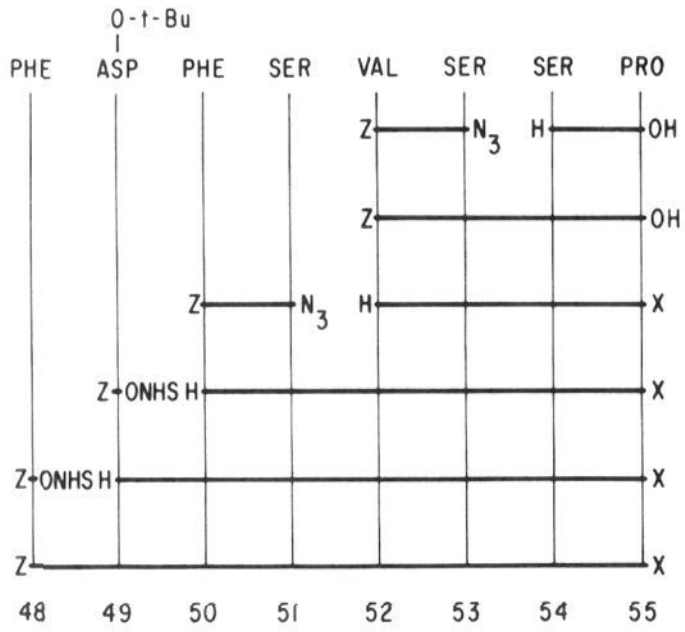


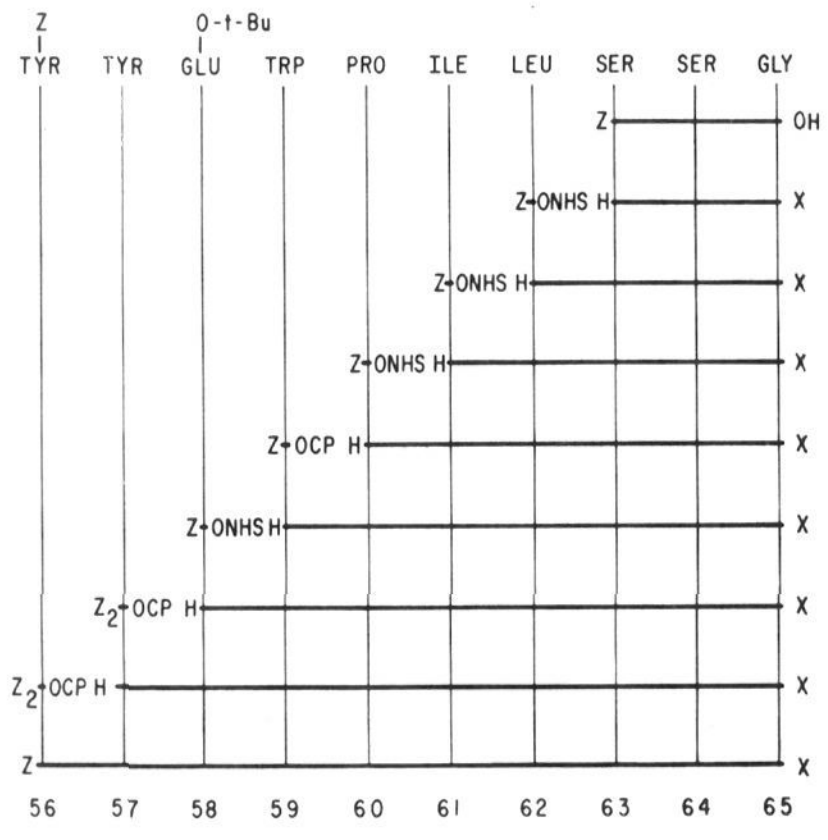
Figure 2. Location of fragments BCD and EF within the sequence of ribonuclease T_1.

condensed to afford fragment F. The hydrazide obtained from fragment E by treatment with trifluoroacetic acid was converted to the azide[7] and the latter coupled to the *tert*-butoxycarbonylhydrazide derived from fragment F by hydrogenolysis. This overall synthetic scheme is based on the protected hydrazide procedure.[8] The following protecting groups

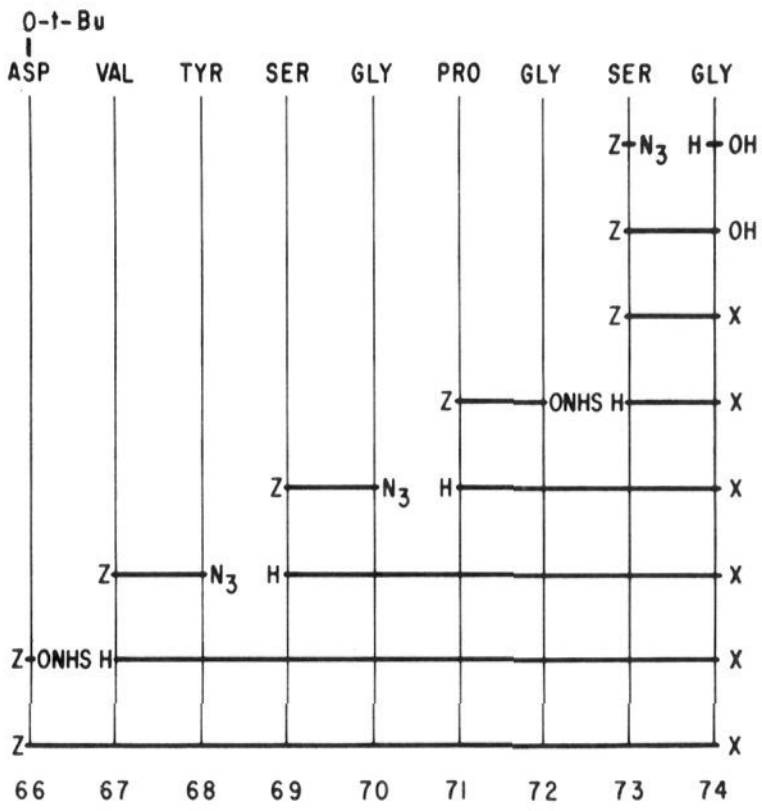
Scheme I Synthesis of Subfragment E_1 (Mol Wt 1189)



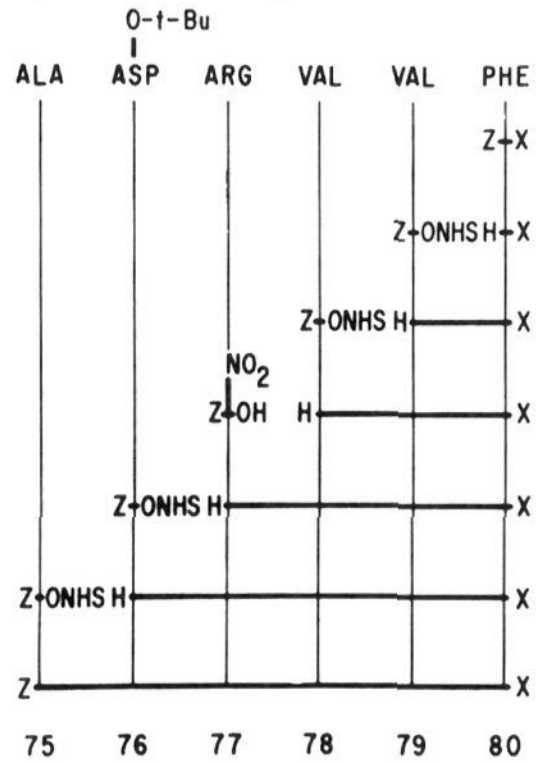
Scheme II Synthesis of Subfragment E_2 (Mol Wt 1653)



Scheme III. Synthesis of Subsequent F_1 (Mol Wt 1142)



Scheme IV. Synthesis of Subfragment F_2 Mol Wt 1070)



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(8) K. Hofmann, A. Lindenmann, M. Z. Magee, and N. H. Khan, *J. Amer. Chem. Soc.*, **74**, 470 (1952).

were used: the benzyloxycarbonyl group for α-amino groups and the phenolic hydroxyl of tyrosine, the *tert*-butoxycarbonyl group for the hydrazide function,[9] the *tert*-butyl ester group for the β-carboxyl of aspartic acid and the γ-carboxyl of glutamic acid,[10] and the nitro group for the guanido function of arginine.[11] The serine hydroxyl group was not protected. Benzyloxycarbonyl groups were removed by hydrogenolysis, *tert*-butoxycarbonyl and *tert*-butyl ester groups by exposure to trifluoroacetic acid.

Preparative Aspects

Synthesis of Fragment E. For the synthesis of subfragment E_1 (Scheme I), serylproline[12] (positions 54–55) was coupled with the azide of benzyloxycarbonylvalylserine[1] and the ensuing benzyloxycarbonyltetrapeptide was converted to the *tert*-butoxycarbonylhydrazide by reaction with *tert*-butoxycarbonylhydrazine[13] in the presence of DCC. The benzyloxycarbonyl group was removed and the resulting peptide *tert*-butoxycarbonylhydrazide condensed with benzyloxycarbonylphenylalanylserine azide[14] to give benzyloxycarbonylphenylalanylserylvalylserylserylproline *tert*-butoxycarbonylhydrazide. This compound was decarbobenzoxylated and the chain elongated by two single steps using α-*N*-hydroxysuccinimido β-*tert*-butyl benzyloxycarbonylaspartate[15] and *N*-hydroxysuccinimido benzyloxycarbonylphenylalaninate[16] for acylation.

Benzyloxycarbonylserylserylglycine, the starting material for the synthesis of subfragment E_2 (Scheme II), was converted to the crystalline *tert*-butoxycarbonylhydrazide and the benzyloxycarbonyl group was removed. The remaining amino acid residues were added by the stepwise method using appropriate benzyloxycarbonylamino acid 2,4,5-trichlorophenyl[17] or *N*-hydrosuccinimido esters[16] for acylation. The 2,4,5-trichlorophenyl ester of *N*,*O*-dibenzyloxycarbonyltyrosine served to introduce the tyrosine residues in positions 56 and 57. The reaction of 2,4,5-trichlorophenyl *N*,*O*-dibenzyloxycarbonyltyrosinate with the nonapeptide *tert*-butoxycarbonylhydrazide (positions 57–65) in the presence of 2 equiv of triethylamine yielded a material exhibiting a number of high running contaminants on thin-layer chromatography in the solvent system methanol–chloroform (10:90). It was suspected that these impurities were the result of O-acylation of the tyrosine residue in position 57. This side reaction is known to occur during active ester couplings in the presence of excess base[18] and apparently was responsible for some of the difficulties encountered in the synthesis of fragment D.[1] Various attempts to purify the product by precipitation or trituration were unsuccessful. The procedure chosen for the final step in the synthesis of fragment E_2 involved acylation of the nonapeptide *tert*-butoxycarbonylhydrazide acetate (positions 57–65) with 2,4,5-trichlorophenyl *N*,*O*-dibenzyloxycarbonyltyrosinate in the absence of triethylamine. Under these conditions, a product of good chromatographic quality was obtained in satisfactory yield.

The synthesis of fragment E involved conversion of subfragment E_1 to the hydrazide and azide[7] and coupling to subfragment E_2 *tert*-butoxycarbonylhydrazide. The yield of chromatographically homogeneous fragment E was 48%.

Synthesis of Fragment F. Scheme III illustrates the route employed to prepare subfragment F_1 in crystalline form. This synthesis proceeded smoothly and requires no additional comments. However, the preparation of subfragment F_2 created problems. This synthesis (Scheme IV) was carried out entirely *via* the stepwise procedure starting with phenylalanine *tert*-butoxycarbonylhydrazide and using the *N*-hydroxysuccinimido esters of the required benzyloxycarbonylamino acids, excepting arginine-77 which was incorporated as N^{α}-benzyloxycarbonyl-N^{ω}-nitroarginine by the Anderson modification[19] of the mixed anhydride procedure. Difficulties arose during the last two active ester couplings. Originally, an excess of active ester was employed in these reactions in order to promote maximal yields, but the ensuing products were contaminated by a higher running component on thin-layer chromatography which could not be removed by distribution between 1-butanol and 5% acetic acid. Addition of 3-dimethylaminopropylamine after completion of the coupling reaction followed by distribution in the abovementioned solvents failed to improve the quality of the products whose acid hydrolysates showed the presence of an excess of the N-terminal amino acids aspartic acid and alanine. Chromatographically homogeneous samples of subfragment F_2 were obtained when equimolar proportions of active ester and amino component were employed. Subfragment F_2 was found to be heat labile and exhibited a lower running component on thin-layer chromatography when work-up evaporations were carried out at temperatures in excess of 40°. Acid hydrolysates of samples which were heavily contaminated by this component showed essentially the same amino acid composition as the desired material suggesting that the *tert*-butoxycarbonylhydrazide was partially deprotected. Further work will be required to clarify this point, but the formation of the contaminant was avoided when evaporations were performed at temperatures not exceeding 36°. A similar problem was not encountered during extensive studies with peptide *tert*-butoxycarbonylhydrazides C terminating in glycine in this and a previous investigation.[1]

The final step in the synthesis of fragment F involved coupling of subfragment F_1 azide with subfragment F_2 *tert*-butoxycarbonylhydrazide. The yield of chromatographically homogeneous fragment F was 77% of theory.

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(18) (a) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **28**, 173 (1963); (b) R. Paul, *ibid.*, **28**, 236 (1963).

(19) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **88**, 1338 (1966).

Synthesis of Fragment EF. Exposure of fragment E to the action of trifluoroacetic acid afforded the free hydrazide which was converted to the azide.[7] Fragment F was converted to fragment F *tert*-butoxycarbonylhydrazide by hydrogenolysis and this compound was coupled with the above azide in dimethyl sulfoxide–dimethylformamide solution to give fragment EF. The yield of chromatographically homogeneous material was 39%.

Purification and Assessment of Homogeneity

Countercurrent distribution, solvent precipitation, ion-exchange chromatography, gel filtration, and in some instances crystallization were employed for purification of intermediates and final products. Details pertaining to the procedures used are described in the Experimental Section; only a few pertinent comments will be offered at this point. For removal of unreacted amino component, the crude coupling products were frequently distributed between ethyl acetate and aqueous citric acid. Ethyl acetate insoluble materials were distributed between 1-butanol and dilute aqueous acetic acid for the same purpose. These procedures were successful in most instances but failed in the synthesis of benzyloxycarbonyltryptophylprolylisoleucylleucylserylserylglycine *tert*-butyoxycarbonylhydrazide (positions 59–65). In this situation, unreacted amino component was removed from the coupling mixture by treating the product in 80% aqueous methanol with Dowex 50W-X2 (H^+ cycle) at 0–5°. This technique was also used for removal of unreacted amino component (fragment E_2 *tert*-butoxycarbonylhydrazide) from crude fragment E with dimethylformamide–methanol–water (1:7:2) serving as the solvent. Countercurrent distribution between 1-butanol and 5% acetic acid was employed for purification of fragment F_2 and its *tert*-butoxycarbonyl hydrazide. Chromatography on the ion-exchange resin AG 1-X2 served to remove excess acylating component (rearrangement products of subfragment F_1 azide) from crude fragment F. Fragment E was obtained in chromatographically homogeneous form by gel filtration on Sephadex LH-20 using dimethylformamide for column development. Gel filtration on Sephadex G-100 served to purify fragment EF. The solvent in these experiments was 50% aqueous acetic acid and columns were operated at 4° to minimize deblocking of the *tert*-butoxycarbonylhydrazide function.

Protected intermediates were characterized by thin-layer chromatography in at least two solvent systems and in certain instances by paper chromatography. Melting points, optical rotations, and elemental analyses were also performed. Since they provide no information regarding homogeneity, elemental analyses were not performed on the precious complex peptides. However, amino acid analyses of acid hydrolysates of these protected peptides were carried out with particular emphasis on the ratios of the "diagnostic" amino acid residues. We have defined the "diagnostic" residues as those amino acids stable to acid hydrolysis, which occur in one but not in the other of the reaction partners in a coupling reaction.[1] The ratio of the "diagnostic" residues in fragment E, valine derived from fragment E_1 and isoleucine contributed by fragment E_2, was unity as predicted by theory. The ratio Gly/3 derived from fragment F_1 to alanine from fragment F_2 was 0.94 in fragment F and 1.0 in fragment F *tert*-butoxycarbonylhydrazide. Theory predicts a ratio of 1. The ratio of isoleucine (fragment E) to alanine (fragment F) was unity in fragment EF, in excellent agreement with prediction.

The tryptophan content of fragment EF, assessed by the method of Liu and Chang,[20] showed the presence of one residue. It was observed, however, that the recovery of valine in *p*-toluenesulfonic acid hydrolysates of this compound was low. Similar low valine recoveries were realized in *p*-toluenesulfonic acid hydrolysates of fragment F *tert*-butoxycarbonylhydrazide. It appears likely that the low valine recovery is a reflection of a slow hydrolysis rate of the valylvaline bond present in both compounds.

Optical rotations, thin-layer chromatography in at least two solvent systems, and digestibility by aminopeptidase M (AP-M)[21] served to assess the homogeneity of the various peptide *tert*-butoxycarbonylhydrazides. Buffer-insoluble peptide *tert*-butoxycarbonylhydrazides were deblocked with trifluoroacetic acid and the ensuing soluble peptide hydrazide trifluoroacetate salts were subjected to AP-M digestion. Average recoveries of amino acid residues in acid hydrolysates and enzymic digests provide a satisfactory measure of peptide content of a given sample, and such figures, based on formula weight, are given in the Experimental Section.

We conclude from these results that fragment EF is sequentially homogeneous but realize that the analytical procedures employed are not sensitive enough to detect minor degrees of racemization.

Conclusions

Based on experience which is presented in this and an earlier communication,[1] we conclude that the assembly of homogeneous subfragments in the decapeptide range or smaller, which can be synthesized rapidly and cleanly *via* the stepwise method, appears to be the method of choice for production of complex homogeneous protected peptide hydrazides. Our experience with the stepwise active ester method when applied to the synthesis of larger partially protected peptide hydrazides and using a minimum of side-chain protection has not been encouraging. A gradual accumulation of side products accompanies chain elongation and purification becomes increasingly difficult. This behavior was observed during the synthesis of fragment D[1] and in this study with fragment E. A preparation of fragment E synthesized exclusively by the stepwise active ester procedure contained many components and could not be purified satisfactorily.

Experimental Section[22]

Benzyloxycarbonylphenylalanine *tert*-Butoxycarbonylhydrazide. A mixed anhydride was prepared, in the usual manner, from benzyl-

(20) T.-Y. Liu and Y. H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).

(21) G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964).

(22) Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with a mercury lamp at 546 and 576 nm and extrapolated to the 589-nm sodium line. Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.; oxygen values were actually determined and not computed by difference. Analytical samples were dried *in vacuo* over P_2O_5 at 50–60°. The amino acid compositions of acid hydrolysates and AP-M digests were determined with a Beckman-Spinco Model 120 amino acid analyzer according to the

oxycarbonylphenylalanine[23] (6.0 g) in THF (35 ml) with TEA (2.8 ml) and ethyl chloroformate (1.92 ml). This solution was added with stirring to an ice-cold solution of *tert*-butoxycarbonylhydrazide[13] (2.6 g) in THF (15 ml). The mixture was stirred at ice-bath temperature for 1 hr and at room temperature for 12 hr and was then filtered. The filtrate was evaporated, the residue was dissolved in ethyl acetate (50 ml), and the solution was washed in the usual manner and dried over sodium sulfate. The residue which ensued after removal of the solvent was recrystallized from ether: 5.2 g (63%); mp 81–83°; $[\alpha]^{23}D$ −26.6° (*c* 2.20, MeOH); R_f^{I} 0.8; R_f^{IV} 0.8.

Anal. Calcd for $C_{22}H_{27}O_5N_3$: C, 63.9; H, 6.6; N, 10.2; Found: C, 63.7; H, 6.7; N, 10.2.

Phenylalanine *tert*-Butoxycarbonylhydrazide. Benzyloxycarbonylphenylalanine *tert*-butoxycarbonylhydrazide (2.1 g) was hydrogenated in MeOH (50 ml). The catalyst was removed by filtration, the solvent was evaporated, and the residue was recrystallized from ethyl acetate–petroleum ether: 1.3 g (93%); mp 117–119°; $[\alpha]^{22}D$ +48.8° (*c* 2.40, 1 *N* HCl); R_f^{I} 0.6; R_f^{IV} 0.6.

Anal. Calcd for $C_{14}H_{21}O_3N_3$: C, 60.2; H, 7.6; N, 15.0. Found: C, 60.6; H, 7.6; N, 14.6.

Preparation of Subfragment E_1 (Positions 48–55). (Positions 54–55) Serylproline. Benzyloxycarbonylserylproline[12] (16.8 g) dissolved in MeOH (30 ml) and 10% acetic acid (150 ml) was hydrogenated. The catalyst was removed by filtration and the solvents were evaporated. Addition of EtOH gave crystals which were recrystallized from a small volume of water by addition of EtOH: needles; 9.9 g (99%); mp 196–198° dec; $[\alpha]^{24}D$ −109.3° (*c* 2.83, acetic acid); R_f^{1} 0.6; R_f^{2} 0.6; R_f^{I} 0.2; amino acid ratios in AP-M digest: $Ser_{0.8}$ $Pro_{1.1}$ (100%).

Anal. Calcd for $C_8H_{14}O_4N_2$: C, 47.5; H, 7.0; N, 13.9. Found: C, 47.7; H, 7.0; N, 13.6.

(Positions 52–55) Benzyloxycarbonylvalylserylserylproline. Sodium nitrite (2.10 g) dissolved in ice-cold water (10 ml) was added slowly to an ice-cold stirred solution of benzyloxycarbonylvalylserine hydrazide[1] (10.56 g) in 2 *N* hydrochloric acid (45 ml), THF (50 ml), and dioxane (75 ml). Ice-cold THF (100 ml) was added to dissolve the crystalline azide. The mixture was stirred at −5° for 5 min and TEA (8.4 ml) was added. To this azide solution was added a solution of serylproline (4.04 g) in water (40 ml) and THF (20 ml) containing TEA (4.2 ml). The mixture was stirred at 4° for 48 hr and the bulk of the solvents was removed. The residue was extracted with three portions of ethyl acetate and the organic layers were extracted with 1 *N* ammonium hydroxide. The combined aqueous phases were acidified with 4 *N* hydrochloric acid and extracted three times with ethyl acetate. The ethyl acetate extracts were washed in the usual manner and dried over sodium sulfate. Evaporation of the solvent gave a viscous oil which solidified on addition of acetonitrile. The compound was recrystallized from acetonitrile: 5.82 g (55%); mp 111–116°; $[\alpha]^{27}D$ −74.7° (*c* 3.80, MeOH); R_f^{1} 0.9; R_f^{2} 0.7; R_f^{I} 0.6.

Anal. Calcd for $C_{24}H_{34}O_9N_4$: C, 55.2; H, 6.6; N, 10.7. Found: C, 55.1; H, 6.8; N, 10.5.

(Positions 52–55) Benzyloxycarbonylvalylserylserylproline *tert*-Butoxycarbonylhydrazide Monohydrate. DCC (2.04 g) was added to an ice-cold solution of benzyloxycarbonylvalylserylserylproline (5.23 g) and *tert*-butoxycarbonylhydrazide (1.45 g) in DMF (10 ml) and dioxane (40 ml) and the mixture was stirred at 4° for 15 hr. The suspension was filtered and the filtrate was concentrated to a small volume. The residue was dissolved in ethyl acetate (200 ml) and the solution washed in the usual manner and dried over sodium sulfate. Evaporation of the solvent gave an oil which solidified on addition of acetonitrile and ether. The compound was recrystallized from MeOH: 4.70 g (72%); mp 128–131° (sintering at 124°); $[\alpha]^{24}D$ −94.6° (*c* 1.39, MeOH); R_f^{I} 0.6.

Anal. Calcd for $C_{29}H_{44}O_{10}N_6 \cdot H_2O$: C, 53.2; H, 7.1; N, 12.8. Found: C, 53.8; H, 6.9; N, 12.6.

(Positions 52–55) Valylserylserylproline *tert*-Butoxycarbonylhydrazide Acetate. Benzyloxycarbonylvalylserylserylproline *tert*-butoxycarbonylhydrazide (5.82 g) was hydrogenated in MeOH (60 ml) and 10% acetic acid (20 ml). The catalyst was removed by filtration and the solvents were evaporated. The residue was dissolved in a small volume of EtOH and ether was added. The precipitate was collected and dried: 4.52 g (90%); $[\alpha]^{22}D$ −89.6° (*c* 3.00, MeOH); R_f^{1} 0.6; R_f^{2} 0.9; R_f^{I} 0.3; amino acid ratios in AP-M digest, $Val_{0.9}Ser_{2.1}Pro_{1.0}$ (92%).

(Positions 50–55) Benzyloxycarbonylphenylalanylserylvalylserylserylproline *tert*-Butoxycarbonylhydrazide. Sodium nitrite (1.1 g) in ice-cold water (10 ml) was added slowly to an ice-cold stirred solution of benzyloxycarbonylphenylalanylserine hydrazide[14] (5.76 g) in 1 *N* hydrochloric acid (43.2 ml) and THF (100 ml). The mixture was stirred at −5° for 5 min and TEA (4.0 ml) was added. To this solution containing ZPheSer azide was added a solution of valylserylserylproline *tert*-butoxycarbonylhydrazide acetate (4.06 g) in DMF (8 ml) and THF (30 ml) containing TEA (1.0 ml). The mixture was stirred at 4° for 20 hr, then additional azide (prepared from 2.88 g of benzyloxycarbonylphenylalanylserine hydrazide) was added. Stirring was continued at 4° for 20 hr when the bulk of the solvents was removed. Ethyl acetate (150 ml) was added and the mixture was kept in a refrigerator for 12 hr. The resulting amorphous product was collected, washed with ice-cold ethyl acetate, and dried. The compound was dissolved in hot MeOH and the solution was left to cool slowly. The ensuing gelatinous mass was collected and dried: 4.28 g (68%); mp 215–216° dec (sintering at 205°); $[\alpha]^{24}D$ −32.2° (*c* 3.01, DMF); R_f^{I} 0.6.

Anal. Calcd for $C_{41}H_{58}O_{13}N_8$: C, 56.5; H, 6.7; N, 12.9. Found: C, 56.3; H, 7.0; N, 12.7.

(Positions 50–55) Phenylalanylserylvalylserylserylproline *tert*-Butoxycarbonylhydrazide Acetate. Benzyloxycarbonylphenylalanylserylvalylserylserylproline *tert*-butoxycarbonylhydrazide (4.63 g) was hydrogenated in MeOH (60 ml) and 10% acetic acid (15 ml). The catalyst was removed by filtration and the solvent evaporated. The residue was dissolved in a small volume of MeOH and ether was added. The precipitate was collected and dried: 3.74 g (88%); $[\alpha]^{25}D$ −73.9° (*c* 2.09, MeOH); R_f^{1} 0.6; R_f^{2} 0.9; R_f^{I} 0.4; amino acid ratios in AP-M digest, $Phe_{1.1}Ser_{3.0}$-$Val_{1.1}Pro_{0.8}$ (86%).

(Positions 49–55) Benzyloxycarbonyl-β-*tert*-butylaspartylphenylalanylserylvalylserylserylproline *tert*-Butoxycarbonylhydrazide. α-*N*-Hydroxysuccinimido β-*tert*-butyl benzyloxycarbonylasparate[15] (1.98 g) was added to a solution of phenylalanylserylvalylserylserylproline *tert*-butoxycarbonylhydrazide acetate (3.75 g) in DMF (15 ml) and THF (40 ml) containing TEA (0.66 ml). The mixture was stirred at room temperature for 5 hr; then the THF was removed. The residual solution was diluted with water (100 ml); the precipitate was collected and dried. For purification, the compound was dissolved in hot MeOH, precipitated with water, and dried: 4.00 g (81%); mp 200–201° dec; $[\alpha]^{25}D$ −35.2° (*c* 1.16, DMF); R_f^{I} 0.7.

Anal. Calcd for $C_{49}H_{71}O_{16}N_9$: C, 56.5; H, 6.9; N, 12.1. Found: C, 55.9; H, 6.8; N, 12.1.

(Positions 49–55) β-*tert*-Butylaspartylphenylalanylserylvalylserylserylproline *tert*-Butoxycarbonylhydrazide Acetate. Benzyloxycarbonyl-β-*tert*-butylaspartylphenylalanylserylvalylserylserylproline *tert*-butoxycarbonylhydrazide (2.40 g) was hydrogenated over palladium in MeOH (60 ml) and 10% acetic acid (15 ml). The catalyst was removed by filtration and the filtrate evaporated. The residue was dissolved in a small volume of EtOH and ether was added. The precipitate was collected and dried: 2.08 g (93%); $[\alpha]^{24}D$ −57.0° (*c* 2.40, MeOH); R_f^{1} 0.8; R_f^{I} 0.5; amino acid

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method of S. Moore, D. H. Spackman, and W. H. Stein (*Anal. Chem.* **30**, 1185 (1958)). The figures in parentheses are average recoveries of amino acids based on formula weight. Nle and α-amino-β-guanidopropionic acid served as internal standards. Acid hydrolyses were performed in constant boiling HCl at 110° for 24 hr in evacuated tubes except with peptides containing valine or isoleucine which were hydrolyzed for 48 hr; values are not corrected for amino acid destruction. AP-M digests were performed as described by K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti (*J. Amer. Chem. Soc.*, **88**, 3633 (1966)) except when noted otherwise. Designations of solvent systems for descending paper chromatography on Whatman no. 1 filter paper are: R_f^{1} 1-butanol–glacial acetic acid–water (4:1:5) top layer; R_f^{2} 2-butanol–3% ammonium hydroxide (3:1); R_f^{3} 1-butanol–pyridine–water–glacial acetic acid (45:30:36:9); with this system R_f values are expressed as multiples of the distance traveled by a histidine marker. Designation of solvent systems for ascending tlc on silica gel G (E. Merck and Co., Darmstadt, West Germany) are: R_f^{I} 1-butanol–glacial acetic acid–water (60:20:20); R_f^{II} 95% EtOH–concentrated NH_4OH (100:27); R_f^{III} 1-butanol–pyridine–glacial acetic acid–water (30:20:6:24); R_f^{IV} MeOH–chloroform (1:1); R_f^{V} MeOH–chloroform (5:95); R_f^{VI} MeOH–chloroform (10:90); R_f^{VII} MeOH–chloroform (20:80). Sparingly soluble compounds were prepared for spotting by dissolving them in a few drops of glacial acetic acid with slight warming and adding a few drops of water. Solvents were freshly distilled and, except when noted otherwise, evaporations were carried out *in vacuo* at a temperature of 40–45° in rotary evaporators. Hydrogenations were performed at room temperature and atmospheric pressure. Routinely catalyst was removed by filtering the hydrogenation mixture through a bed of Filter Cel. Yields are based on weight of vacuum dried (over P_2O_5 and KOH) substance.

(23) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 462 (1958).

ratios in AP-M digest, $(O\text{-}t\text{-BuAsp} + \text{Asp})_{1.0}\text{Phe}_{0.9}\text{Ser}_{3.0}\text{Val}_{0.9}\text{Pro}_{1.1}$ (91%).[24]

(Positions 48–55) Benzyloxycarbonylphenylalanyl-β-*tert*-butylaspartylphenylalanylserylvalylserylserylproline *tert*-Butoxycarbonylhydrazide (Subfragment E_1). *N*-Hydroxysuccinimido benzyloxycarbonylphenylalaninate[16] (2.08 g) was added to a solution of β-*tert*-butylaspartylphenylalanylserylvalylserylserylproline *tert*-butoxycarbonylhydrazide acetate (5.03 g) in DMF (20 ml) and THF (40 ml) containing TEA (0.73 ml). The mixture was kept at room temperature for 5 hr, then water (300 ml) was added. The precipitate was collected and dried. For purification, the compound was dissolved in hot MeOH and precipitated by addition of water: 5.42 g (88%); mp 214–217° dec; $[\alpha]^{25}\text{D}$ −36.3° (*c* 1.87, DMF); R_f^1 0.9; R_f^2 0.9; R_f^I 0.7.

Anal. Calcd for $C_{58}H_{80}O_{17}N_{10}$: C, 58.6; H, 6.8; N, 11.8. Found: C, 58.4; H, 7.0; N, 11.6.

Subfragment E_1 *tert*-Butoxycarbonylhydrazide Acetate. Subfragment E_1 (100 mg) was hydrogenated in MeOH (20 ml) and 10% acetic acid (2 ml). The catalyst was removed by filtration and the solvents were evaporated. The residue was dissolved in a small volume of EtOH and ether was added. The precipitate was collected and dried: 85 mg (91%); $[\alpha]^{25}\text{D}$ −65.8° (*c* 0.96, MeOH); R_f^1 0.9; R_f^2 0.9; R_f^I 0.6; amino acid ratios in 72-hr AP-M digest: $(O\text{-}t\text{-BuAsp} + \text{Asp})_{1.2}\text{Phe}_{2.0}\text{Ser}_{3.1}\text{Val}_{1.0}\text{Pro}_{0.8}$ (88%).[24]

Subfragment E_1 Hydrazide Trifluoroacetate. Subfragment E_1 (2.97 g) was dissolved in chilled 90% TFA (20 ml) and the solution kept at room temperature for 30 min. The solvent was evaporated and the residue triturated with ether. The white precipitate was collected, washed with ether, reprecipitated twice from DMF with ether, and dried: colorless powder; 2.61 g (91%); mp dec above 198°; $[\alpha]^{27}\text{D}$ −32.5° (*c* 1.75, DMF); R_f^I 0.6 with faint traces at 0.3 and 0.2; R_f^{III} 0.7; ninhydrin negative, chlorine and hydrazide positive spot.

Preparation of Subfragment E_2 (Positions 56–65). (Positions 63–65) Benzyloxycarbonylserylserylglycine. Sodium nitrite (3.7 g) in ice-cold water (15 ml) was added slowly with stirring to an ice-cold solution of benzyloxycarbonylserylserine hydrazide[15] (17 g) in THF (100 ml) and 2 *N* hydrochloric acid (75 ml). After 10 min TEA (14 ml) was added slowly at a temperature of 0–2°. This solution containing ZSerSer azide was added to an ice-cold solution of glycine (5.3 g) in water (70 ml), THF (35 ml), and TEA (10 ml). The mixture was stirred for 20 hr at 4°; then the bulk of the solvents was evaporated. The residual solution was extracted with three portions of ethyl acetate and the ethyl acetate layers were in turn extracted twice with 1 *N* ammonium hydroxide. The combined aqueous phases were acidified with 4 *N* hydrochloric acid and extracted with five portions of 1-butanol. The butanol layers were washed five times with 5% acetic acid and evaporated. The resulting solid was triturated with ether and dried. The compound was recrystallized from MeOH–ether: prisms; 12.8 g (64%); mp 169–171° dec; $[\alpha]^{24}\text{D}$ −12.2° (*c* 3.52, MeOH); R_f^I 0.7; single chlorine positive spot.

Anal. Calcd for $C_{16}H_{21}O_8N_3$: C, 50.1; H, 5.5; N, 11.0. Found: C, 50.4; H, 5.5; N, 10.8.

(Positions 63–65) Benzyloxycarbonylserylserylglycine *tert*-Butoxycarbonylhydrazide. DCC (4.12 g) was added to a solution cooled at −5° of benzyloxycarbonylserylserylglycine (7.67 g) and *tert*-butoxycarbonylhydrazine (2.64 g) in DMF (10 ml) and dioxane (40 ml). The mixture was stirred for 1 hr at 0° and for 20 hr at room temperature. The *N*,*N*′-dicyclohexylurea was removed by filtration, the bulk of the solvents was evaporated, and the residue was dissolved in ethyl acetate (200 ml). The solution was washed in the usual manner and dried. The solvent was evaporated, the ensuing viscous oil was dissolved in ethyl acetate, and ether was added. The precipitate was collected and recrystallized from acetonitrile: needles; 6.0 g (61%); mp 111–112°; $[\alpha]^{25}\text{D}$ −10.7° (*c* 2.86, MeOH); R_f^I 0.7.

Anal. Calcd for $C_{21}H_{31}O_9N_5$: C, 50.7; H, 6.3; N, 14.1. Found: C, 51.0; H, 6.3; N, 14.2.

(Positions 63–65) Serylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tripeptide hydrazide (7.46 g) in MeOH (50 ml) and 10% acetic acid (15 ml) was hydrogenated. The catalyst was removed by filtration and the solvent was evaporated. The oily residue was lyophilized from a small volume of water–dioxane: colorless powder; 6.3 g (100%); $[\alpha]^{25}\text{D}$ −6.5° (*c* 3.11, MeOH); R_f^1 0.6; R_f^2 0.8; R_f^I 0.4; trace impurity at 0.5; amino acid ratios in AP-M digest, $\text{Ser}_{2.1}\text{Gly}_{1.0}$ (81%).

(24) β-*tert*-Butyl aspartate undergoes partial deblocking during amino acid analysis.

(Positions 62–65) Benzyloxycarbonylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylleucinate[16] (4.0 g) was added to a solution of serylserylglycine *tert*-butoxycarbonylhydrazide acetate (4.65 g) and TEA (1.6 ml) in DMF (20 ml) and THF (70 ml). The mixture was stirred for 5 hr at room temperature when the bulk of the solvents was evaporated. The residue was dissolved in ethyl acetate (200 ml) and the solution was washed three times with 1 *N* citric acid, once with saturated sodium bicarbonate, and once with water. The solvent was removed to give a solid, which was recrystallized from a mixture of MeOH and acetonitrile (1:5): needles; 4.84 g (72%); mp 189–191° dec; $[\alpha]^{25}\text{D}$ −22.1° (*c* 2.00, MeOH); R_f^I 0.7.

Anal. Calcd for $C_{27}H_{42}O_{10}N_6$: C, 53.1; H, 6.9; N, 13.8. Found: C, 53.4; H, 6.8; N, 14.0.

(Positions 62–65) Leucylserylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tetrapeptide hydrazide (3.91 g) dissolved in MeOH (50 ml) and 10% acetic acid (15 ml) was hydrogenated. The catalyst was removed and the solvent was evaporated. The crystalline residue was recrystallized from EtOH: 3.31 g (96%); $[\alpha]^{26}\text{D}$ −6.9° (*c* 3.09, MeOH); R_f^1 0.7; R_f^2 0.9; R_f^I 0.5; amino acid ratios in AP-M digest, $\text{Leu}_{1.0}\text{Ser}_{2.0}\text{Gly}_{1.0}$ (82%).

(Positions 61–65) Benzyloxycarbonylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylisoleucinate[16] (2.17 g) in dioxane (20 ml) was added to a solution of leucylserylserylglycine *tert*-butoxycarbonylhydrazide acetate (2.95 g) in DMF (10 ml) containing TEA (0.84 ml). The mixture was stirred for 6 hr at room temperature and water (70 ml) was added. The precipitate was collected, dissolved in dioxane, and precipitated by addition of water. For purification the material was triturated twice with 250 ml each of hot acetonitrile and then again precipitated from dioxane with water: 3.10 g (78%); mp 232–234° dec; $[\alpha]^{24}\text{D}$ −41.0° (*c* 3.09, MeOH); R_f^I 0.8; R_f^{III} 0.7; R_f^{IV} 0.8.

Anal. Calcd for $C_{33}H_{53}O_{11}N_7$: C, 54.8; H, 7.4; N, 13.6. Found: C, 54.5; H, 7.7; N, 13.7.

(Positions 61–65) Isoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected pentapeptide hydrazide (3.11 g) in MeOH (50 ml) and 10% acetic acid (10 ml) was hydrogenated over palladium. The catalyst was removed, the solvents were evaporated, and the residue was dissolved in a small volume of EtOH. Addition of ether gave a precipitate which was collected and dried: 2.72 g (97%); $[\alpha]^{25}\text{D}$ −23.5° (*c* 2.56, MeOH); R_f^1 0.8; R_f^2 0.9; R_f^I 0.5; R_f^{III} 0.6; amino acid ratios in AP-M digest, $\text{Ile}_{1.0}\text{Leu}_{1.0}\text{Ser}_{2.0}\text{Gly}_{1.0}$ (89%).

(Positions 60–65) Benzyloxycarbonylprolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylprolinate[16] (1.45 g) was added to a solution of isoleucylleucylserylserylglycine *tert*-butoxycarbonylhydrazide acetate (2.73 g) in DMF (10 ml), THF (20 ml), and TEA (0.58 ml). The mixture was stirred for 6 hr at room temperature and water (100 ml) was added. The precipitate was collected and reprecipitated from MeOH by addition of water: 3.05 g (88%); mp 238–239° dec; $[\alpha]^{26}\text{D}$ −69.4° (*c* 2.01, MeOH); R_f^I 0.7; contaminated with traces of Z-Pro-ONHS.

Anal. Calcd for $C_{38}H_{60}O_{12}N_8$: C, 55.6; H, 7.4; N, 13.7. Found: C, 55.4; H, 7.5; N, 13.7.

(Positions 60–65) Prolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected hexapeptide hydrazide (3.0 g) was hydrogenated in MeOH (50 ml) and 10% acetic acid (10 ml). The catalyst was removed, the solvents were evaporated, the residue was dissolved in a small volume of EtOH, and ether was added. The precipitate was collected and dried: 2.71 g (99%); $[\alpha]^{26}\text{D}$ −56.1° (*c* 1.09, MeOH); R_f^1 0.8; R_f^2 0.9; R_f^I 0.4 with trace impurity at 0.2; R_f^{III} 0.7 with trace impurity at 0.5; amino acid ratios in AP-M digest $\text{Pro}_{1.1}\text{Ile}_{1.0}\text{Leu}_{1.0}\text{Ser}_{2.0}\text{Gly}_{0.9}$ (94%).

(Positions 59–65) Benzyloxycarbonyltryptophylprolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl benzyloxycarbonyltryptophanate[17] (3.75 g) was added to a partial suspension of prolylisoleucylleucylserylserylglycine *tert*-butoxycarbonylhydrazide acetate (5.40 g) in DMF (80 ml) containing TEA (1.0 ml); a clear solution was slowly formed. The mixture was stirred for 20 hr at room temperature and was diluted with ethyl acetate (900 ml). The solution was washed in the usual manner and was concentrated to a gel. The amorphous product was dissolved in hot EtOH (150 ml), the solution was concentrated at 40° to about 50 ml, and the product precipitated with ether: 6.20 g; R_f^I 0.8 with some streaking; R_f^{III} 0.7 with trace impurity at 0.8. This material was dissolved in 80% MeOH and treated twice with Dowex 50W-X2 (H^+ cycle) (10 ml of settled resin) at

0–5°. After evaporation and reprecipitation of the residue from EtOH–ether a white solid was obtained: 5.5 g (76%); mp 160–162° dec; $[\alpha]^{27}D$ −52.5° (*c* 2.23, MeOH); R_f^{I} 0.8; single, chlorine, hydrazide, and Ehrlich positive spot with no tailing.

Anal. Calcd for $C_{49}H_{70}O_{18}N_{10}$: C, 58.4; H, 7.0; N, 13.9; O, 20.7. Found: C, 58.0; H, 7.1; N, 14.1; O, 20.4.

(Positions 59–65) Tryptophylprolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected heptapeptide hydrazide (6.85 g) was hydrogenated in MeOH (150 ml) and 10% acetic acid (20 ml) for 24 hr. The catalyst was removed by filtration and the solution was evaporated to dryness. The residue was dissolved in MeOH, ether was added, and the precipitate was collected and dried: 5.77 g (91%); $[\alpha]^{28}D$ −54.9° (*c* 2.54, MeOH); R_f^{I} 0.6; R_f^{III} 0.8. A sample (50 mg) was dissolved in TFA (0.4 ml) and the solution was kept at room temperature for 30 min. Ether was then added and the precipitate was collected, washed with ether, and dried: 45 mg; R_f^{I} 0.3; amino acid ratios in AP-M digest, $Trp_{1.4}Pro_{1.1}Ile_{0.9}Leu_{1.0}Ser_{2.0}Gly_{0.7}$ (76%).

(Positions 58–65) Benzyloxycarbonyl-γ-*tert*-butylglutamyltryptophylprolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide. α-*N*-Hydroxysuccinimido γ-*tert*-butyl benzyloxycarbonylglutamate[25] (1.74 g) was added to a solution of tryptophylprolylisoleucylleucylserylserylglycine *tert*-butoxycarbonylhydrazide acetate (3.73 g) in DMF (16 ml), THF (16 ml), and TEA (1.12 ml). The mixture was stirred for 5 hr at room temperature and overnight at 4° and the THF was removed by evaporation. Ethyl acetate (400 ml) was added to the residue and the solution was washed in the usual manner and dried. The ethyl acetate layers were pooled and concentrated to a small volume, and ether was added to the residue. The precipitate was collected and dried; 4.5 g. Since tlc indicated the presence of unreacted amino component, the material was dissolved in DMF (30 ml) and ethyl acetate (600 ml) and the solution was rewashed and the material reprecipitated as described above: 4.0 g (84%); mp 160°; $[\alpha]^{28}D$ −58.9° (*c* 2.55, MeOH); R_f^{I} 0.8; R_f^{IV} 0.9.

Anal. Calcd for $C_{58}H_{85}O_{16}N_{11}$: C, 58.4; H, 7.2; N, 12.9; O, 21.5. Found: C, 58.2; H, 7.1; N 12.7; O 21.3.

(Positions 58–65) γ-*tert*-Butylglutamyltryptophylprolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected octapeptide hydrazide (5.96 g) was hydrogenated for 20 hr in MeOH (100 ml) and 10% acetic acid (20 ml). The catalyst was removed and the solvents were evaporated. The residue was precipitated from EtOH with ether: 5.30 g (95%); $[\alpha]^{25}D$ −60.1° (*c* 2.63, MeOH); hygroscopic, after storing for several weeks in the air; $[\alpha]^{25}D$ −57.7° (*c* 2.65, MeOH); R_f^{I} 0.8; R_f^{IV} 0.9; R_f^{VI} 0.2.

A sample (30 mg) was dissolved in anhydrous TFA (0.3 ml) and the solution kept at room temperature for 1 hr. Ether was added to the reddish-pink solution and the pink solid was collected and washed with ether. The material was dissolved in a small volume of water and the solution lyophilized: 25 mg; R_f^{I} 0.4; amino acid ratios in 48 hr AP-M digest, $Glu_{1.0}Trp_{1.1}Pro_{0.9}Ile_{1.0}Leu_{1.0}Ser_{2.1}Gly_{0.8}$ (63%).

(Positions 57–65) *N,O*-Dibenzyloxycarbonyltyrosyl-γ-*tert*-butylglutamyltryptophylprolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide. 2,4,5-Trichlorophenyl *N,O*-dibenzyloxycarbonyltyrosinate[17] (2.52 g) was added to a solution of γ-*tert*-butylglutamyltryptophylprolylisoleucylleucylserylserylglycine *tert*-butoxycarbonylhydrazide acetate (4.47 g) in DMF (25 ml), THF (50 ml), and TEA (1.12 ml). The mixture was stirred at room temperature for 15 hr, the THF removed, and the residue diluted with ethyl acetate (500 ml). The solution was washed in the usual manner and dried. The solvent was evaporated and the residue precipitated from warm ethyl acetate with ether: 5.86 g (98%); R_f^{I} 0.8 (slight tailing); R_f^{III} 0.9; R_f^{IV} 0.9; R_f^{VI} 0.3 with trace impurity at 0.12.

The product was twice reprecipitated from ethyl acetate with ether: 5.72 g (96%); $[\alpha]^{25}D$ −50.2° (*c* 2.60, MeOH); tlc showed a single spot without tailing in systems I, III, and IV; with system VI slight tailing was observed.

Anal. Calcd for $C_{75}H_{100}O_{20}N_{12}$: C, 60.5; H, 6.8; N, 11.3; O, 21.5. Found: C, 60.2; H, 6.8; N, 11.6; O, 21.6.

(Positions 57–65) Tyrosyl-γ-*tert*-butylglutamyltryptophylprolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected nonapeptide hydrazide (5.0 g) was hydrogenated for 12 hr in MeOH (150 ml) and 10% acetic acid (25 ml). The catalyst was removed and the solvents were evaporated. The residue was reprecipitated from EtOH with ether and dried: hygroscopic powder; 4.06 g (94%); $[\alpha]^{25}D$ −66.3° (*c* 2.34, MeOH); R_f^{I} 0.7; R_f^{III} 0.8; R_f^{IV} 0.8; single ninhydrin, chlorine, and hydrazide positive spot.

A sample (30 mg) was dissolved in anhydrous TFA (0.3 ml) and the solution kept at room temperature for 1 hr. Ether was added and the pink precipitate was collected, washed with ether, and dissolved in a small volume of water. The solution was lyophilized to give a pink solid: 24 mg; amino acid ratios in 48 hr AP-M digest $Tyr_{1.0}Glu_{1.0}Trp_{1.0}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.2}Gly_{0.9}$ (69%).

(Positions 56–65) *N,O*-Dibenzyloxycarbonyltyrosyltyrosyl-γ-*tert*-butylglutamyltryptophylprolylisoleucylleucylserylserylglycine *tert*-Butoxycarbonylhydrazide (Subfragment E_2). 2,4,5-Trichlorophenyl *N,O*-dibenzyloxycarbonyltyrosinate[17] (2.1 g) was added to a solution of tyrosyl-γ-*tert*-butylglutamyltryptophylprolylisoleucylleucylserylserylglycine *tert*-butoxycarbonylhydrazide acetate (4.07 g) in DMF (20 ml) and THF (40 ml). The mixture was stirred for 40 hr at room temperature when the THF was removed and the residue was diluted with ethyl acetate (300 ml). The solution was washed in the usual manner and dried. The residue obtained after evaporation was twice reprecipitated from EtOH with ether: 5.0 g (95%); $[\alpha]^{27}D$ −43.1° (*c* 2.13, MeOH); R_f^{I} 0.7; R_f^{VII} 0.5 with faint traces at 0.4; R_f^{VI} 0.4 with faint traces at 0.3.

Anal. Calcd for $C_{84}H_{109}O_{22}N_{13}$: C, 61.0; H, 6.6; N, 11.0. Found: C, 61.1; H, 6.8; N, 10.7.

Subfragment E_2 *tert*-Butoxycarbonylhydrazide. Subfragment E_2 (600 mg) was hydrogenated in MeOH (30 ml) for 12 hr. The catalyst was removed by filtration and the solvents were evaporated. The residue was dissolved in a small volume of EtOH and ether was added. The precipitate was collected and dried: 473 mg (97%); $[\alpha]^{27}D$ −54.0° (*c* 1.17, MeOH); R_f^{I} 0.9; R_f^{I} 0.6; R_f^{III} 0.8; R_f^{IV} 0.4 with trace at 0.1; R_f^{VI} 0.9; R_f^{VII} 0.4. This material (15 mg) was dissolved in TFA (0.2 ml) and the solution was kept at room temperature for 1 hr. Ether was added and the precipitate was collected, washed with ether, and dried: 14 mg; R_f^{I} 0.6; R_f^{III} 0.8; R_f^{IV} 0.9; amino acid ratios in 48 hr AP-M digest, $Tyr_{2.0}Glu_{1.0}Trp_{0.8}Pro_{1.0}Ile_{1.0}Leu_{1.0}Ser_{2.8}Gly_{1.0}$ (82%).

Preparation of Fragment E (Positions 48–65). A solution of subfragment E_1 hydrazide trifluoroacetate (642 mg) in DMF (3.5 ml) was cooled at −25° and 6.9 *N* HCl in dioxane (0.4 ml) was added with stirring followed by *tert*-butyl nitrite (0.7 ml of a 10% solution in DMF). The solution was stirred for 15 min at −25° and then cooled to −60° and TEA (2.63 ml of a 20% solution in DMF) was added dropwise. To the stirred suspension (pH 7.5–8.0) was added dropwise a solution of subfragment E_2 *tert*-butoxycarbonylhydrazide (380 mg) in DMF (3 ml). The reaction mixture was stirred for 3 days at 4°, the solvent evaporated, and water (~70 ml) was added to the residue. The precipitate was collected, washed with water, and dried: 898 mg; R_f^{I} 0.7 containing ninhydrin positive material with R_f^{I} 0.6. This material was dissolved in DMF–MeOH–H_2O (1:7:2) (150 ml) and the solution cooled to −10°. Chilled Dowex 50W-X2 (H^+ cycle) (10 ml settled in water) was added and the mixture stirred for 30 min at −5 to −10°. A further addition of resin (10 ml) was made after 15 min. The mixture was rapidly filtered and the resin washed with ice-cold solvent. The filtrate was refiltered through a bed of Celite; a few drops of TEA were added to the filtrate which was evaporated. The residue was dissolved in DMF–MeOH–H_2O and the resin treatment repeated. The residue was dissolved in a small volume of DMF and precipitated by addition of ethyl acetate: 703 mg; R_f^{I} 0.7 ninhydrin negative, chlorine and hydrazide positive spot. This material, in 350-mg batches, was dissolved in DMF (4 ml) and the solution added to a Sephadex LH-20 column (2 × 150 cm) which was eluted with DMF. Absorbancy measurements at 280 nm served to locate the desired peptide in the various fractions. Tubes which contained homogeneous material by tlc were pooled and evaporated. The residue was precipitated from DMF with ethyl acetate and rechromatographed on LH-20 in the manner described: 310 mg (48%); $[\alpha]^{27}D$ −35.8° (*c* 1.44, DMF); R_f^{I} 0.7; R_f^{III} 0.8, ninhydrin negative, chlorine, hydrazide, and Ehrlich positive spot; amino acid ratios in 48-hr acid hydrolysates of two samples

Phe	Asp	Ser	Val	Pro	Tyr	Glu	Ile	Leu	Gly	
1.9	1.0	4.3	1.0	2.0	1.7	1.1	1.0	1.0	1.0	(92%)
2.0	1.0	4.2	1.0	2.0	1.7	1.1	1.0	1.0	1.0	(95%)

ratio of Val/Ile = 1.0

Anal. Calcd for $C_{121}H_{163}O_{33}N_{21}$: C, 59.5; H, 6.8; N, 12.0; O, 21.6. Found: C, 59.3; H, 7.0; N, 11.9; O, 21.8.

Fragment E *tert*-Butoxycarbonylhydrazide Acetate. A solution of fragment E (50 mg) in MeOH–5% acetic acid (9:1) (50 ml) was

(25) R. Zabel and H. Zahn, *Z. Naturforsch. B*, 20, 650 (1965).

hydrogenated for 12 hr. The catalyst was removed by filtration and the solvent was evaporated. The residue was reprecipitated from EtOH with ether and the solid collected and dried: 32.7 mg (68%); R_f^1 0.6; R_f^{111} 0.8; ninhydrin, chlorine, hydrazide, and Ehrlich positive spot; amino acid ratios in 72-hr acid hydrolysate, $Phe_{1.7}Asp_{1.0}Ser_{4.7}Val_{1.0}Pro_{2.1}Tyr_{1.5}Glu_{1.0}Ile_{1.0}Leu_{1.0}Gly_{1.1}$ (Trp destroyed).

Fragment E Hydrazide Trifluoroacetate. Fragment E (80 mg) was dissolved in ice-cold 90% TFA containing 1% mercaptoethanol and 1% anisole and the mixture was kept at this temperature for 10 min. The solution was then kept at room temperature for 40 min and was concentrated to a small volume at 20°. Ether (approximately 50 ml) was added to the residue and the precipitate was collected by centrifugation and washed with three portions of ether: 74 mg (92%); R_f^1 0.6; R_f^{111} 0.8; amino acid ratios in acid hydrolysate, $Phe_{1.8}Asp_{1.1}Ser_{4.4}Val_{1.1}Pro_{2.1}Tyr_{2.0}Glu_{1.1}Ile_{1.1}Leu_{1.1}$-$Gly_{1.2}$.

Preparation of Subfragment F_1 (Positions 66–74). (Positions 73–74) Benzyloxycarbonylserylglycine *tert*-Butoxycarbonylhydrazide. DCC (10.2 g) was added to a solution of benzyloxycarbonylserylglycine[26] (14.8 g) and *tert*-butoxycarbonylhydrazine[13] (6.6 g) in dioxane (25 ml) and DMF (25 ml). The mixture was stirred at 0° for 1 hr and at room temperature for 15 hr and was filtered. The solvents were removed, the residue was dissolved in ethyl acetate, and the solution was washed in the usual manner and dried. The solvent was evaporated to give a crystalline residue, which was recrystallized from ethyl acetate: needles; 16.6 g (77%); mp 112–114°; $[\alpha]^{25}D$ −6.3° (*c* 3.7, MeOH); ninhydrin negative.

Anal. Calcd for $C_{18}H_{26}O_7N_4$: C, 52.7; H, 6.4; N, 13.7. Found: C, 52.9; H, 6.6; N, 13.8.

(Positions 73–74) Serylglycine *tert*-Butoxycarbonylhydrazide. Benzyloxycarbonylserylglycine *tert*-butoxycarbonylhydrazide (12.3 g) in MeOH (100 ml) was hydrogenated. The product was isolated in the usual manner and was lyophilized from dioxane–water: 8.3 g (100%); R_f^1 0.5; R_f^2 0.8; R_f^1 0.4. The product was employed for the next step without further purification.

(Positions 71–72) *N*-Hydroxysuccinimido Benzyloxycarbonylprolylglycinate. DCC (8.2 g) was added to a solution of benzyloxycarbonylprolylglycine[27] (12.2 g) and *N*-hydroxysuccinimide (4.4 g) in dioxane (150 ml). Following stirring at 4° for 20 hr, the mixture was filtered and the filtrate evaporated to dryness. Ether was added to the residue to initiate crystallization. The product was recrystallized from ethyl acetate: needles; 12.7 g (79%); mp 149–150°; $[\alpha]^{24}D$ −59.1° (*c* 5.35, MeOH).

Anal. Calcd for $C_{19}H_{21}O_7N_3$: C, 56.6; H, 5.2; N, 10.4. Found: C, 56.3; H, 5.4; N, 10.7.

(Positions 71–74) Benzyloxycarbonylprolylglycylserylglycine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylprolylglycinate (12.1 g) in dioxane (50 ml) was added to a solution of serylglycine *tert*-butoxycarbonylhydrazide (8.24 g) in dioxane (50 ml) containing TEA (2.1 ml). The mixture was stirred at room temperature for 5 hr and water (50 ml) was added. The solution was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed in the usual manner and dried. Evaporation of the solvent gave a viscous oil which was dissolved in a small volume of EtOH and ether was added. The precipitate was collected and crystallized from acetonitrile: needles; 15.0 g (87%); mp 112–115°; $[\alpha]^{25}D$ −29.3° (*c* 1.00, MeOH); R_f^1 0.5.

Anal. Calcd for $C_{25}H_{36}O_9N_6$: C, 53.2; H, 6.4; N, 14.9. Found: C, 52.8; H, 6.6; N, 14.4.

(Positions 71–74) Prolylglycylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected tetrapeptide hydrazide (8.47 g) was hydrogenated in MeOH (100 ml) and 10% acetic acid (30 ml). The product was isolated in the usual manner and was lyophilized from water–dioxane: 7.35 g (100%); $[\alpha]^{27}D$ −29.6° (*c* 1.50, MeOH); R_f^1 0.5; R_f^2 0.5; R_f^1 0.1; amino acid ratios in AP-M digest, $Pro_{1.0}Gly_{2.0}Ser_{1.0}$ (59%).

(Positions 69–74) Benzyloxycarbonylserylglycylprolylglycylserylglycine *tert*-Butoxycarbonylhydrazide. Benzyloxycarbonylserylglycine *tert*-butoxycarbonylhydrazide (6.98 g) was dissolved in 5.8 *N* hydrogen chloride in dioxane (15 ml) and the solution was kept at room temperature for 30 min; then THF (20 ml) was added. The solution was cooled at −30°, *tert*-butyl nitrite (2.0 ml) was added, and the mixture was stirred at −20 to −25° for 15 min. The mixture was then cooled at −60° and TEA (9.50 ml) was added. To this solution containing ZSerGly azide was added a chilled solution of prolylglycylserylglycine *tert*-butoxycarbonylhydrazide acetate (7.35 g) and TEA (2.1 ml) in DMF (20 ml) and THF (20 ml). The mixture was stirred at 4° for 24 hr and the bulk of the solvents was evaporated. The residue was diluted with water (150 ml) and extracted twice with ethyl acetate. The organic layers were in turn washed twice with water. The combined aqueous solutions were extracted six times with 1-butanol and the butanol layers were washed six times with 5% acetic acid and evaporated. To the residue was added EtOH and the precipitate was collected, washed with ether, and dried. For further purification the compound was dissolved in hot ethanol, the solution was left to cool, and the gelatinous material was collected and dried: 8.51 g (80%); mp 168–169° dec; $[\alpha]^{25}D$ −21.9° (*c* 3.99, DMF); R_f^1 0.4.

Anal. Calcd for $C_{30}H_{44}O_{12}N_8$: C, 50.8; H, 6.3; N, 15.8. Found: C, 50.7; H, 6.5; N, 15.5.

(Positions 69–74) Serylglycylprolylglycylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected hexapeptide hydrazide (7.09 g) was hydrogenated in MeOH (100 ml) and 5% acetic acid (30 ml). The catalyst was removed by filtration and the solvents were evaporated. The residue was dissolved in a small volume of EtOH and ether was added. The precipitate was collected and dried: 6.25 g (98%); $[\alpha]^{25}D$ −10.6° (*c* 2.5 MeOH); R_f^1 0.4; R_f^2 0.5; R_f^1 0.1; amino acid ratios in AP-M digest, $Ser_{2.0}Pro_{1.1}Gly_{2.9}$ (88%).

(Positions 67–74) Benzyloxycarbonylvalyltyrosylserylglycylprolylglycylserylglycine *tert*-Butoxycarbonylhydrazide. *tert*-Butyl nitrite (1.3 ml) was added to a solution cooled at −30° of benzyloxycarbonylvalyltyrosine hydrazide[28] (4.72 g) containing DMF (20 ml) and THF (20 ml) in 5.8 *N* hydrogen chloride in dioxane (9.5 ml). The mixture was stirred at −20 to −25° for 15 min and then cooled to −60°, and TEA (6.2 ml) was added. This mixture containing ZValTyr azide was added to a chilled solution of serylglycylprolylglycylserylglycine *tert*-butoxycarbonylhydrazide acetate (6.35 g) in DMF (20 ml) and THF (20 ml) containing TEA (1.4 ml). The mixture was stirred at 4° for 20 hr when a second portion of azide (prepared from 4.72 g of hydrazide) was added. Stirring was continued for 20 hr at 4°, then the bulk of the solvents was evaporated. The residual solution was diluted with water (200 ml) and was extracted with three portions of ethyl acetate. The aqueous layers were extracted six times with 1-butanol and the butanol phases were washed six times with 5% acetic acid and evaporated. To the residue was added EtOH (30 ml) and ether (60 ml) and the mixture was stored at 4° for 20 hr. The resulting gelatinous material was collected and dried. This material was suspended in hot MeOH and water was added to obtain a clear solution which was left to cool slowly. The crystalline product was collected and dried: needles; 7.12 g (73%); mp 215–217° dec; $[\alpha]^{25}D$ −23.0° (*c* 1.17, DMF); R_f^1 0.4.

Anal. Calcd for $C_{44}H_{62}O_{15}N_{10}$: C, 54.4; H, 6.4; N, 14.4. Found: C, 54.1; H, 6.3; N, 14.1.

(Positions 67–74) Valyltyrosylserylglycylprolylglycylserylglycine *tert*-Butoxycarbonylhydrazide Acetate. The protected octapeptide hydrazide (5.8 g) was hydrogenated in MeOH (120 ml), 5% acetic acid (25 ml), and water (15 ml). The catalyst was removed by filtration and the solution was evaporated. The residue was dissolved in EtOH and ether was added. The ensuing precipitate was collected and dried: 5.3 g (100%); $[\alpha]^{24}D$ −13.5° (*c* 2.12, MeOH); R_f^1 0.6; R_f^2 0.7; R_f^1 0.2; amino acid ratios in AP-M digest $Val_{1.0}$-$Tyr_{0.9}Ser_{2.0}Gly_{2.9}Pro_{1.2}$ (86%).

(Positions 66–74) Benzyloxycarbonyl-β-*tert*-butylaspartylvalyltyrosylserylglycylprolylglycylserylglycine *tert*-Butoxycarbonylhydrazide (Subfragment F_1). α-*N*-Hydroxysuccinimido β-*tert*-butyl benzyloxycarbonylaspartate[15] (2.1 g) was added to a solution of the above octapeptide *tert*-butoxycarbonylhydrazide acetate (4.5 g) in DMF (20 ml), THF (10 ml), and TEA (0.7 ml). The mixture was stirred at room temperature for 4 hr, then water (approximately 200 ml) was added. The solution was extracted six times with 1-butanol and the butanol phases were washed six times with 5% acetic acid and evaporated. To the residue was added EtOH (50 ml) and ether (200 ml) and the solution was stored at 4° overnight. The resulting gelatinous material was collected and dried. This compound was dissolved in hot MeOH and the solution was left to cool. The resulting needles were collected and dried: 4.7 g (82%); mp 190–191° dec; $[\alpha]^{24}D$ −22.5° (*c* 1.19, DMF); R_f^1 0.6 with faint trace at 0.4; R_f^{1V} 0.8; ninhydrin negative,

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(27) M. A. Ondetti, *J. Med. Chem.*, **6**, 10 (1963).
(28) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Amer. Chem. Soc.*, **79**, 5697 (1957).

chlorine and hydrazide positive spots; amino acid ratios in acid hydrolysate, $Asp_{1.0}Val_{1.0}Tyr_{0.7}Ser_{1.8}Pro_{1.1}Gly_{3.0}$ (89%).

Anal. Calcd for $C_{52}H_{75}O_{18}N_{11}$: C, 54.7; H, 6.6; N, 13.5. Found: C, 54.6; H, 6.9; N, 13.7.

Subfragment F_1 *tert*-Butoxycarbonylhydrazide. The benzyloxycarbonylnonapeptide *tert*-butoxycarbonylhydrazide (570 mg) was hydrogenated in MeOH (70 ml) and 10% acetic acid (1 ml). The catalyst was removed and the solution was evaporated. Ether was added to the residue and the resulting solid was collected and dried: 510 mg (95%); $[\alpha]^{24}D$ −39.5° (*c* 1.93, 50% acetic acid); R_f^1 0.7; R_f^2 0.9; R_f^1 0.3 with traces at 0.2 and 0.4; ninhydrin, chlorine, and hydrazide positive spots; amino acid ratios in AP-M digest, $(O\text{-}t\text{-BuAsp} + Asp)_{1.0}Val_{1.0}Tyr_{1.0}Ser_{2.0}Gly_{2.9}Pro_{1.1}$ (83%).[24]

Subfragment F_1 Hydrazide Trifluoroacetate. Subfragment F_1 (114 mg) was dissolved in chilled 90% TFA (2 ml) and the solution kept at room temperature for 30 min. The solvent was evaporated, the residue was triturated with ether, and the white solid precipitate was collected and washed thoroughly with ether. The product was precipitated from a small volume of DMF with ether. The solid was collected, washed with ether, and dried: 101 mg (88%); R_f^1 0.4; R_f^{III} 0.6; amino acid ratios in acid hydrolysate, $Asp_{1.0}Val_{1.1}Tyr_{0.6}Ser_{1.8}Gly_{3.1}Pro_{1.0}$ (84%).

Preparation of Subfragment F_2 (Positions 75–80). (Positions 79–80) Benzyloxycarbonylvalylphenylalanine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylvalinate[16] (34.8 g) in THF (150 ml) was added to a solution of phenylalanine *tert*-butoxycarbonylhydrazide (27.9 g) in 90% (v/v) aqueous THF (200 ml). The mixture was stirred for 5 hr at room temperature, maintaining the pH at 7.5–8.0 by addition of TEA. The solution was concentrated and water was added. The ensuing crystalline material was washed by trituration and decantation with three 100-ml portions each of 1 *N* sodium bicarbonate, water, 1 *N* citric acid, and water, and was dried. The peptide was crystallized from chloroform–petroleum ether: 41.0 g (84%); mp 162–163°; $[\alpha]^{25}D$ −45.0° (*c* 3.21, MeOH); R_f^1 0.9; R_f^{IV} 0.9; amino acid ratio in acid hydrolysate $Val_{1.0}Phe_{1.0}$.

Anal. Calcd for $C_{27}H_{36}O_6N_4$: C, 63.3; H, 7.1; N, 10.9. Found: C, 63.5; H, 7.2; N, 11.0.

(Positions 79–80) Valylphenylalanine *tert*-Butoxycarbonylhydrazide Acetate. The protected dipeptide hydrazide (15.4 g) was hydrogenated in MeOH (200 ml) in the presence of 10% (v/v) aqueous acetic acid (18 ml). After 5 hr the catalyst was removed by filtration and the solvent evaporated. The residue was dissolved in water and the solution was filtered, concentrated to a small volume, and lyophilized: 12.5 g (95%); $[\alpha]^{24}D$ +16.7° (*c* 2.80, H_2O); $[\alpha]^{27}D$ +23.7° (*c* 1.59, 1 *N* HCl); R_f^1 0.6; R_f^{IV} 0.6.

(Positions 78–80) Benzyloxycarbonylvalylvalylphenylalanine *tert*-Butoxycarbonylhydrazide. *N*-Hydroxysuccinimido benzyloxycarbonylvalinate[16] (10.5 g) was added to a solution of valylphenylalanine *tert*-butoxycarbonylhydrazide acetate (13.0 g) in 90% (v/v) aqueous THF (150 ml). The solution was stirred for 10 hr at room temperature, maintaining the pH at 7.5–8.0 by addition of TEA. The solution was concentrated and water was added. The crystalline residue was washed by trituration and decantation with four 100-ml portions each of 1 *N* sodium bicarbonate, water 1 *N* citric acid, and water, and was dried. The peptide was recrystallized from THF–water: laminar crystals; 15.0 g (83%); mp 215–216°; $[\alpha]^{25}D$ −5.5° (*c* 4.22, DMF); R_f^I 0.8; R_f^{III} 0.9; R_f^{IV} 0.9.

Anal. Calcd for $C_{32}H_{45}O_7N_5$: C, 62.8; H, 7.4; N, 11.4. Found: C, 63.0; H, 7.4; N, 11.4.

(Positions 78–80) Valylvalylphenylalanine *tert*-Butoxycarbonylhydrazide Acetate. The protected tripeptide hydrazide (18.3 g) was hydrogenated in MeOH (300 ml) containing 10% (v/v) aqueous acetic acid (18.0 ml). The catalyst was removed by filtration and the solvent was evaporated. The residue was dissolved in a small volume of EtOH and the material precipitated by addition of ether: 16.0 g (99%); mp 133–134°; $[\alpha]^{27}D$ −39.8° (*c* 1.23, MeOH); R_f^1 0.5; R_f^{III} 0.7; amino acid ratios in AP-M digest, $Val_{2.1}Phe_{0.9}$ (95%).

(Positions 77–80) N^{α}-Benzyloxycarbonyl-N^{ω}-nitroarginylvalylvalylphenylalanine *tert*-Butoxycarbonylhydrazide. To a solution cooled at −15°, containing N^{α}-benzyloxycarbonyl-N^{ω}-nitroarginine[11] (5.4 g) and NMM (1.68 ml) in THF (50 ml), was added dropwise isobutyl chloroformate (1.77 ml). After 1 min an ice-cold solution of valylvalylphenylalanine *tert*-butoxycarbonylhydrazide acetate (5.4 g) in DMF (30 ml), THF (50 ml), and TEA (1.38 ml) was added. The mixture was stirred for 10 min at 0° and for 2 min at 40° and was concentrated to a small volume. The solution was poured into ether (1 l.) and the mixture was kept at 0° for 12 hr when the precipitate was collected by filtration. The solid was washed by trituration and decantation with ten 200-ml portions each of 2 *N* ammonium hydroxide and water and four 200-ml portions each of 1 *N* citric acid and water. The material was suspended in ethyl acetate (800 ml) and the suspension was washed successively with five 200-ml portions each of 1 *N* sodium bicarbonate and water. The organic layer was evaporated, the residue was dissolved in DMF, and the solution was filtered and concentrated to a small volume. Addition of water afforded a precipitate which was collected and dried: 7.9 g (96%); mp 238° dec; $[\alpha]^{27}D$ −8.4° (*c* 2.42, DMF); R_f^1 0.8; R_f^{III} 0.9.

Anal. Calcd for $C_{38}H_{56}O_{10}N_{10}$: C, 56.1; H, 6.9; N, 17.2. Found: C, 56.2; H, 7.0; N, 17.0.

(Positions 77–80) Arginylvalylvalylphenylalanine *tert*-Butoxycarbonylhydrazide Diacetate. The protected tetrapeptide hydrazide (7.3 g) was hydrogenated in MeOH (160 ml) containing 10% (v/v) aqueous acetic acid (160 ml) and 1-butanol (1 ml). After 24 hr the catalyst was removed by filtration and the solvents were evaporated. The residue was dissolved in water, the solution was filtered, and the material lyophilized to constant weight from water: 6.4 g (94%); $[\alpha]^{28}D$ −33.1° (*c* 3.78, H_2O); R_f^1 0.5; R_f^{III} 0.7; amino acid ratios in AP-M digest, $Arg_{1.0}Val_{2.0}Phe_{1.0}$ (84%).

(Positions 76–80) Benzyloxycarbonyl-β-*tert*-butylaspartylarginylvalylvalylphenylalanine *tert*-Butoxycarbonylhydrazide Acetate Monohydrate. *N*-Hydroxysuccinimido β-*tert*-butyl benzyloxycarbonylaspartate[15] (1.26 g) was added to a solution of arginylvalylvalylphenylalanine *tert*-butoxycarbonylhydrazide diacetate (2.26 g) in DMF (12 ml). The pH was adjusted to 7.0 by addition of a 10% solution of TEA in DMF (2.1 ml) and the mixture was stirred at room temperature for 24 hr. The bulk of the solvents was evaporated at a bath temperature not exceeding 36° and the residue was distributed between 1-butanol and 5% acetic acid (60-ml upper and 60-ml lower phase) in three separatory funnels. The butanol layers were washed with 15 60-ml portions of 5% acetic acid and 15 60-ml portions of water. The butanol layer from the first funnel, containing the desired product, was evaporated to a small volume and the product precipitated by addition of ether: 2.48 g (82%); mp 173° dec; $[\alpha]^{28}D$ −39.4° (*c* 3.05, MeOH); R_f^1 0.7; R_f^{III} 0.8; amino acid ratios in 48-hr acid hydrolysate, $Asp_{1.1}Arg_{1.0}Val_{1.8}Phe_{1.1}$ (97%).

Anal. Calcd for $C_{48}H_{74}O_{13}N_{10}\cdot H_2O$: C, 56.7; H, 7.5; N, 13.8; O, 22.0. Found: C, 56.5; H, 7.5; N, 13.7; O, 22.8.

(Positions 76–80) β-*tert*-Butylaspartylarginylvalylvalylphenylalanine *tert*-Butoxycarbonylhydrazide Diacetate. The protected pentapeptide hydrazide acetate monohydrate (2.0 g) was hydrogenated in MeOH (100 ml) containing 10% acetic acid (1.2 ml). After 18 hr the catalyst was removed by filtration and the solvent was evaporated. Ether was added and the precipitate was collected and dried: 1.7 g (94%); $[\alpha]^{28}D$ −11.0° (*c* 2.30, DMF); R_f^1 0.4; R_f^{III} 0.7; amino acid ratios in 48-hr AP-M digest, $(O\text{-}t\text{-BuAsp} + Asp)_{1.2}Arg_{0.9}Val_{1.9}Phe_{1.0}$ (85%).[24]

(Positions 75–80) Benzyloxycarbonylalanyl-β-*tert*-butylaspartylarginylvalylvalylphenylalanine *tert*-Butoxycarbonylhydrazide Acetate (Subfragment F_2). *N*-Hydroxysuccinimido benzyloxycarbonylalaninate[16] (320 mg) was added to a solution of β-*tert*-butylaspartylarginylvalylvalylphenylalanine *tert*-butoxycarbonylhydrazide (930 mg) in DMF (12 ml) and the pH of the solution was adjusted to 7.5 by addition of 10% TEA in DMF (1.4 ml). The mixture was stirred at room temperature for 40 hr, then 3-dimethylaminopropylamine (0.05 ml) was added, and stirring was continued for 1 hr. The solvents were evaporated and the product was distributed between 1-butanol and 5% acetic acid (50 ml each) in three separatory funnels. The butanol layers were washed with 15 50-ml portions of 5% acetic acid and 15 50-ml portions of water. The butanol layer of the first funnel was evaporated and the product precipitated by addition of ether: 830 mg (77%); mp 189° dec; $[\alpha]^{28}D$ −19.1° (*c* 2.23, DMF); R_f^1 0.7; R_f^{III} 0.8; amino acid ratios in 48-hr acid hydrolysate, $Ala_{1.1}Asp_{1.2}Arg_{0.9}Val_{1.7}Phe_{1.0}$ (95%).

Anal. Calcd for $C_{61}H_{79}O_{14}N_{11}$: C, 57.2; H, 7.4; N, 14.4; O, 20.9. Found: C, 57.5; H, 7.3; N, 14.3; O, 21.6.

Subfragment F_2 *tert*-Butoxycarbonylhydrazide Diacetate. Subfragment F_2 acetate (326 mg) was hydrogenated in MeOH (50 ml) containing 10% (v/v) aqueous acetic acid (0.4 ml). After 18 hr the catalyst was removed by filtration, the solvents were evaporated, and the product was precipitated by addition of ether; 290 mg (97%). This material was distributed between 1-butanol and 5% acetic acid in 15 separatory funnels (25-ml upper and 25-ml lower phase). Usually 40 transfers were sufficient to remove impurities. The butanol phases were then washed with ten 25-ml portions of water and the homogeneous material was located by tlc in funnels 3–13. Butanol layers containing homogeneous material

were pooled, the solvent was evaporated, and the residue was lyophilized from water: 220 mg (73%); $[\alpha]^{26}D$ −13.5° (*c* 1.02, DMF); R_f^I 0.5; R_f^{III} 0.7; amino acid ratios in 48-hr AP-M digest, (Ala + *O*-*t*-BuAsp)$_{1.7}$Asp$_{0.2}$Arg$_{1.1}$Val$_{2.0}$Phe$_{1.1}$ (77%).[24]

Preparation of Fragment F (Positions 66–80). To a solution cooled at −15° of fragment F_1 hydrazide trifluoroacetate (375 ml) in DMF (3.5 ml) was added 7.73 *N* HCl in dioxane (0.22 ml) followed by a 10% (v/v) solution of *tert*-butyl nitrite in DMF (0.43 ml). The mixture was stirred for 20 min at −15°, then cooled to −50°, and a 10% (v/v) solution of TEA in DMF (approximately 3.3 ml) was added to adjust the pH to 7.5–8.0. The temperature of the reaction mixture was allowed to reach −20° and a solution cooled at −10° of fragment F_2 *tert*-butoxycarbonylhydrazide diacetate (213 mg) in DMF (1.5 ml) was added dropwise. The reaction mixture was stirred for 24 hr at 4°, the pH being maintained at 7.5–8.0 by addition of 10% TEA in DMF. The mixture was evaporated to a small volume, the residue was dissolved in 150 ml of isopropyl alcohol–methanol–water (1:1:1), the pH was adjusted to 7.5–8.0 with TEA, and the solution was applied to an acetate cycle AG 1-X2 column (2.5 × 20 cm) which was eluted with the same solvent mixture until the eluates became chlorine negative. The chlorine positive eluates were evaporated, the residue was suspended in 20% acetic acid, and the solution was lyophilized. The residue was extracted with two 10-ml portions of boiling ethanol and dried. The material was then dissolved in 1-butanol–methanol–water (1:1:1) (80 ml) and the cloudy solution filtered through a layer of Filter Cel. The clear filtrate was concentrated to a small volume, the residue was dissolved in 20% acetic acid and the solution was lyophilized: 305 mg (75%); $[\alpha]^{27}D$ −28.3° (*c* 0.95, DMSO); R_f^I 0.6; R_f^{III} 0.7; chlorine and hydrazide positive single spot with slight tailing; amino acid ratios in 48-hr acid hydrolysate Asp$_{2.2}$Val$_{3.2}$Tyr$_{0.9}$Ser$_{1.8}$Gly$_{3.1}$Pro$_{1.2}$Ala$_{1.1}$Arg$_{0.9}$Phe$_{1.1}$ (88%); ratio of Gly/3:Ala = 0.94.

Anal. Calcd for $C_{86}H_{128}O_{28}N_{20}$: C, 54.6; H, 6.8; N, 14.8; O, 23.7. Found: C, 54.4; H, 6.9; N, 14.5; O, 22.9.

Fragment F *tert*-Butoxycarbonylhydrazide Diacetate. Fragment F (200 mg) was hydrogenated for 20 hr in 1-butanol–methanol–20% acetic acid (1:1:1) (40 ml). The catalyst was removed by filtration, the filtrate was evaporated at a temperature of 36°, the residue was dissolved in 20% acetic acid, and the solution was lyophilized: 180 mg (97%); $[\alpha]^{26}D$ −28.6° (*c* 1.03, DMSO); R_f^I 0.4; R_f^{III} 0.7; amino acid ratios in 48-hr acid hydrolysate, Asp$_{2.1}$Val$_{3.0}$Tyr$_{0.9}$Ser$_{1.8}$Gly$_{3.0}$Pro$_{1.1}$Ala$_{1.1}$Arg$_{1.1}$Phe$_{1.1}$ (83%). Amino acid ratios in 48-hr *p*-toluenesulfonic acid hydrolysate:[20] Asp$_{2.2}$Val$_{2.2}$Tyr$_{1.0}$Ser$_{2.0}$Gly$_{3.3}$Pro$_{1.1}$Ala$_{1.1}$Arg$_{1.1}$Phe$_{1.1}$ (81%); ratio of Gly/3:Ala = 1.0.

Fragment F Hydrazide Ditrifluoroacetate. Fragment F (100 mg) was dissolved in 90% (v/v) aqueous TFA and the solution was kept at room temperature for 45 min. Excess TFA was evaporated and the residue was lyophilized from water: 100 mg; mp 210°; $[\alpha]^{27}D$ −27.1° (*c* 1.67, DMF); R_f^I 0.4; amino acid ratios in 48-hr acid hydrolysate; Asp$_{2.1}$Val$_{2.8}$Tyr$_{0.8}$Ser$_{1.8}$Gly$_{3.0}$Pro$_{1.1}$Ala$_{1.1}$-Arg$_{1.0}$Phe$_{1.0}$ (62%).

Preparation of Fragment EF (Positions 48–80). Fragment E hydrazide trifluoroacetate (73 mg) was dissolved in DMF (1.5 ml) and the solution was cooled at −10°. To this solution was added 6.91 *N* hydrogen chloride in dioxane (10% in DMF) (0.22 ml) followed by *tert*-butyl nitrite (1% in DMF) (0.36 ml). The mixture was stirred at −10° for 20 min and was then cooled at −20°. After 10 min at this temperature, 10% TEA in DMF (0.33 ml) was added followed by a solution of fragment F *tert*-butoxycarbonylhydrazide (52 mg) dissolved in DMSO (0.5 ml), DMF (0.5 ml), and TEA (0.06 ml). The mixture, which was green to "pHydrion paper," was stirred at 4° for 3 days. Ethyl acetate (approximately 100 ml) was added, the precipitate was collected by centrifugation, washed with the same solvent, and dried. The material was then washed with water, redried, and dissolved in 50% acetic acid (3.5 ml). This solution was added to a Sephadex G-100 column (2 × 140 cm) which was eluted at 4° with the same solvent. Fractions (approximately 3-ml each) were collected at a flow rate of 6 ml/hr. Absorbancy measurements at 280 nm and tlc in solvent system I (chlorine test) served to locate the desired compound R_f^I 0.5 in fractions 93–100. These fractions were pooled and lyophilized, and the residue was precipitated from DMF with ethyl acetate and dried: 48 mg (39%); $[\alpha]^{29}D$ −24.5° (*c* 0.92, DMF); R_f^I 0.5; R_f^{III} 0.8, single chlorine and Ehrlich positive spot: amino acid ratios in 48-hr *p*-toluenesulfonic acid hydrolysates[20]

Phe	Asp	Ser	Pro	Val	Tyr	Glu	
3.2	3.6	6.7	3.0	3.2	3.0	1.1	
3.3	3.2	7.2	2.7	3.0	2.9	1.0	
Trp	Ile	Leu	Gly	Ala	Arg		
0.8	1.0	1.1	4.1	1.0	0.9	(102%)	
1.1	1.0	1.0	4.2	1.0	0.9	(80%)	

ratio of Ile/Ala = 1.0.

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