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Toward a Synthesis of *Clostridium butyricum* Apoferredoxin: Two Tetradecapeptides Comprising Half the Sequence (Residues 7–20 and 21–34)[1]

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The two protected tetradecapeptides Z·Ser·Cys[Bzl(OMe)]·Val·Ser·Cys[Bzl(OMe]·Gly·Ala·Cys[Bzl(OMe)]·Ala·Gly·Glu(OBu^t)·Cys[Bzl(OMe)]·Pro·Val·NH·NH·Boc and Z·Ser·Ala·Ile·Thr·Gln·Gly·Asp(OBu^t)·Thr(Bu^t)·Gln·Phe·Val·Ile·Asp(OBu^t)·Ala·NH·NH·Boc, corresponding to residues 7–20 and 21–34 in the amino acid sequence of *Clostridium butyricum* apoferredoxin have been synthesized as a first stage in a total synthesis of the apoferredoxin. The former peptide has been deprotected to the tetra-thiol peptide H·Ser·Cys·Val·Ser·Cys·Gly·Ala·Cys·Ala·Gly·Glu·Cys·Pro·Val·NH·NH_2, and two tri-thiol and three di-thiol peptide components of this have also been synthesized for iron–sulfur complexing studies.

INTRODUCTION

This paper is an account of the first half of a projected synthesis of *Clostridium butyricum* apoferredoxin (*1*). This particular apoferredoxin was chosen for synthesis since its amino acid sequence appeared to present marginally fewer synthetic difficulties than those of other bacterial apoferredoxins known at the commencement of our work (*1*).

The amino acid sequence (*2*) of *Clostridium butyricum* apoferredoxin is as follows:[3]

H·Ala·Phe·Val·Ile·Asn·Asp | Ser·Cys·Val·Ser·Cys·Gly·Ala·Cys·Ala·Gly.Glu·Cys·Pro·
5 10 15
Val | Ser·Ala·Ile·Thr·Gln·Gly·Asp·Thr·Gln·Phe·Val·Ile·Asp·Ala | Asp·Thr·Cys·Ile·Asp·
20 25 30 35
Cys·Gly·Asn·Cys·Ala·Asn·Val·Cys·Pro·Val | Gly·Ala·Pro·Asn·Gln·Glu·OH.
40 45 50 55

(1)

By analogy with *Micrococcus aerogenes* ferredoxin (*3*, *4*) there is no reasonable doubt that the two Fe_4S_4 cubic clusters in the ferredoxin involve cysteine residues 8, 11, 14, 47, and 18, 37, 40, 43, respectively.

[1] Dedicated to the memory of the late Professor George Kenner.

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[3] All chiral amino acids have the L-configuration. Abbreviations for amino acid residues are those recommended by IUPAC-IUB [*Biochem. J.* **126**, 773 (1972)]. Other abbreviations: Boc = Bu^{t}O·CO–; Bpoc = *p*-C_6H_5·C_6H_4·CMe_2O·CO–; Bzl(OMe) = *p*-MeO·C_6H_4·CH_2–; Dmpoc = CH≡C·CMe_2O·CO–; Np = *p*-O_2N·C_6H_4–; NSu = CO·CH_2·CH_2·CO·N–; Tcp = 2,4,5-$C_6H_2Cl_3$–; Z = Ph·CH_2O·CO–.

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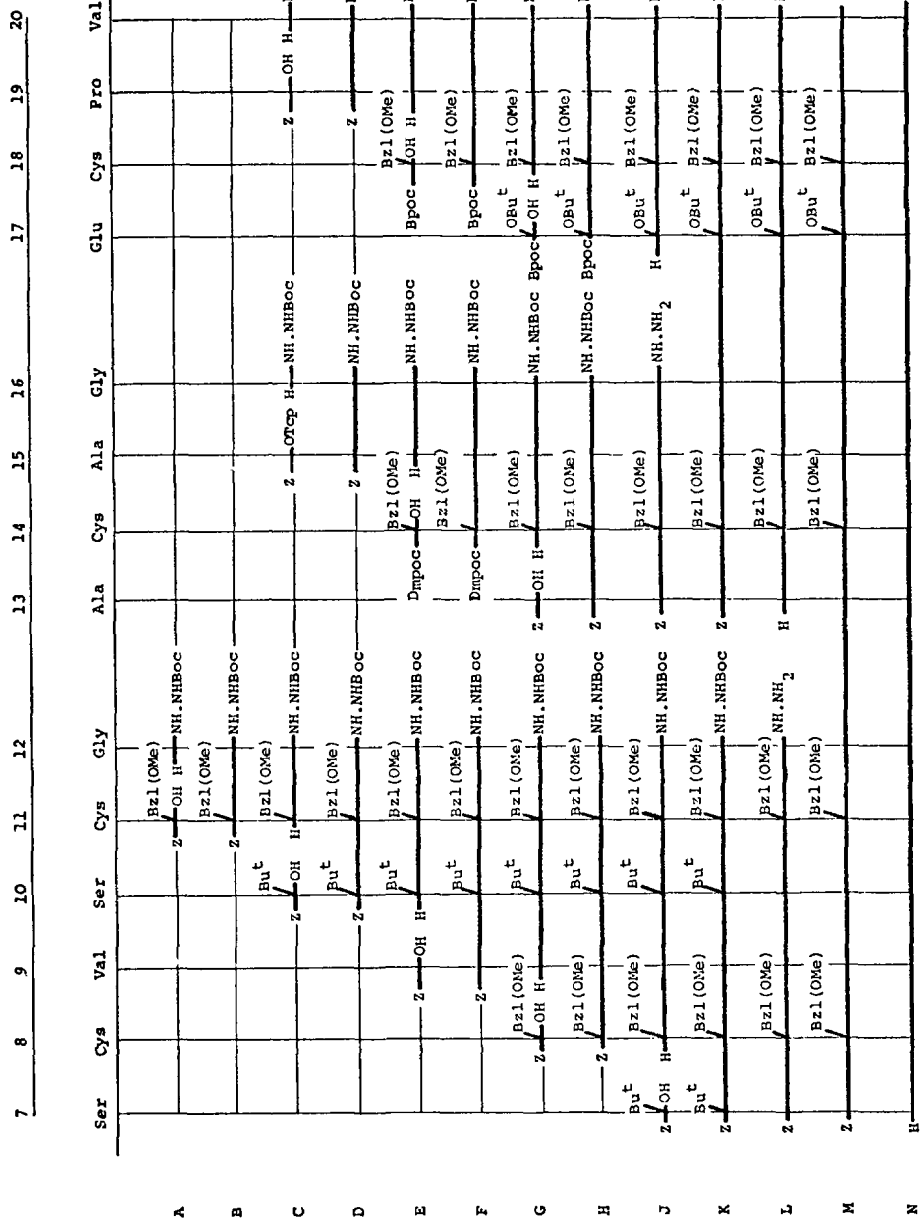
Our work had as its objective not only the synthesis of (1) but also the study of the influence of the different side chains in the amino acid sequence of the apoferredoxin on its ability to form biochemically active iron–sulfur complexes. For this reason the sequence was broken down for synthetic purposes into the five fragments indicated in structure (1), thus: (a) Two end pieces, residues 1–6 and 50–55, each ending in a residue bearing a side-chain carboxyl preceded by one with a side-chain carboxamido group. (b) Two side pieces, residues 7–20 and 35–49, each containing four cysteine residues with the spacing $Cys \cdot X_2 \cdot Cys \cdot X_2 \cdot Cys \cdot X_3 \cdot Cys$ and ending in the sequence $Pro \cdot Val$. (c) A midpiece, residues 21–34, joining the side pieces together. Such a breakdown allows, *inter alia*, for the synthesis from only three fragments of the analog (1–6)·(7–20)·(21–34)·(7–20)·(1–6) which contains all the obvious structural features of a typical bacterial apoferredoxin and of other analogs in which the cysteine-free portions (1–6), (21–34) and (50–55) are omitted or replaced by shorter or simpler amino acid sequences. Special interest attaches to the first-mentioned analog in view of the suggestion (*5*) that the eight-iron bacterial ferredoxins may have arisen by gene duplication. We now report syntheses of the side piece (7–20) and the mid piece (21–34).

Synthesis of $H \cdot Ser \cdot Cys \cdot Val \cdot Ser \cdot Cys \cdot Gly \cdot Ala \cdot Cys \cdot Ala \cdot Gly \cdot Glu \cdot Cys \cdot Pro \cdot Val \cdot NH \cdot NH_2$ *(I N 7–20)*[4]

This synthesis is outlined in Scheme I. The subfragment peptides (I J 17–20), (I J 13–16), and (I L 7–12) were prepared by stepwise syntheses and then coupled together, as indicated, to give the fully protected tetradecapeptide (I M 7–20), which was finally deprotected to give the desired product (I N 7–20). These particular subfragments were chosen since we were thereby enabled to prepare the three dicysteine and two tricysteine peptides contained within the tetradecapeptide, which we required for iron–sulfur complexing experiments (*6*). The coupling method used for the stepwise synthesis of the subfragments was the mixed isobutyl carbonic anhydride procedure, using a 50% excess of the anhydride [except for the synthesis of the protected dipeptide (I C 15–16) where the trichlorophenyl ester method was used]. The subfragments were coupled using Rudinger's modification (*7*) of the azide method. For this reason, the *t*-butoxycarbonylhydrazides (*8*) were used throughout for carboxyl protection; the presence of this group in the penultimate product (I M 7–20) allows for continuation of the synthesis and the synthesis of the analogs mentioned above.

An overriding consideration in planning the synthesis was the need for an S-protecting group which could be removed cleanly at the end of the synthesis to yield the free thiol peptide directly, without the need for a lengthy purification procedure and the accompanying risk of autooxidation. We chose the *S-p*-methoxybenzyl group, which is removed by treatment with hydrogen fluoride/anisole under mild conditions (*9*). For protecting the side-chain hydroxyl of serine and the side-chain carboxyl of glutamic acid we used the *t*-butyl ether and ester, respectively. These groups were removed at the same time as the *N-t*-butoxycarbonyl group by treatment with trifluoroacetic acid, in preparing the protected subfragment hexapeptide (I K 7–12) for azide coupling, and

[4] Individual peptides are numbered with reference to the schemes; (I N 7–20) indicates Scheme I, line N, residues 7–20.


7 8 9 10 11 12 13 14 15 16 17 18 19 20
Ser Cys Val Ser Cys Gly Ala Cys Ala Gly Glu Cys Pro Val
A
B
C
D
E
F
G
H
J
K
L
M
N

SCHEME I

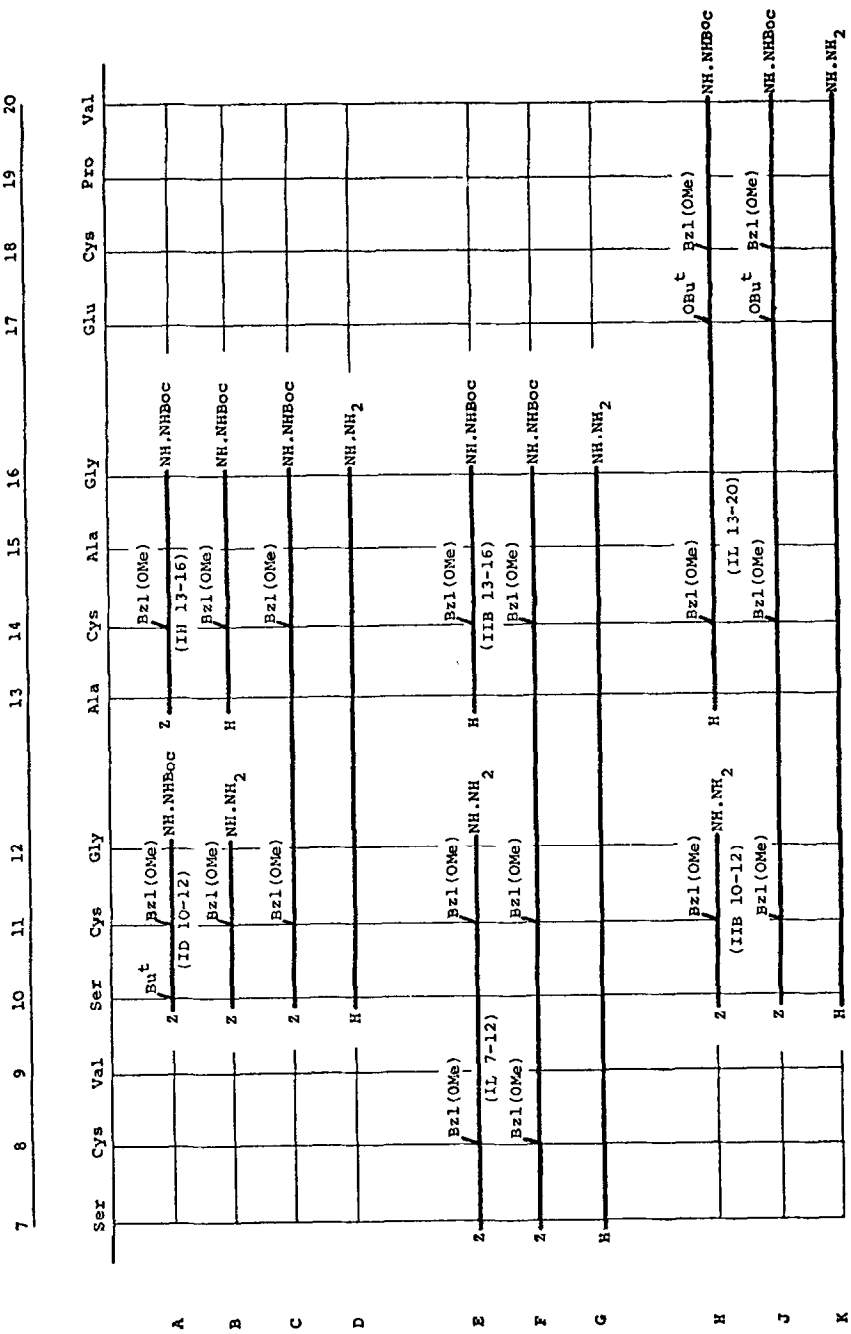
together with all the other protecting groups in the final stage of the synthesis by the action of hydrogen fluoride/anisole at 0°C.

For N-protection of the cysteine-free peptides, the benzyloxycarbonyl group was chosen, since it is cleanly and selectively removed by catalytic hydrogenolysis in the presence of the other, t-butyl based, protecting groups. Initially, however, we could not use this method with cysteine-containing peptides (e.g., I F 13–16 and I F 18–20), owing to poisoning of the hydrogenation catalyst. We therefore used the biphenylylisopropoxycarbonyl (Bpoc) group of Sieber and Iselin (*10*) in the latter case and the 1,1-dimethyl-2-propynyloxycarbonyl (Dmpoc) group of Southard *et al.* (*11*) in the former. Neither was entirely satisfactory; and later in the synthesis we were able to revert to the benzyloxycarbonyl group, which was removed, following Meienhofer and Kuromizu (*12*), by hydrogenolysis over palladium black in liquid ammonia. This procedure we found entirely satisfactory, except in the cases of the two peptides (I B 11–12 and I H 8–12) with N-terminal *S-p*-methoxybenzylcysteinyl residues; in both, hydrogenolysis was incomplete, coming to a standstill after about 70% reaction, the reason for this behaviour not being clear.

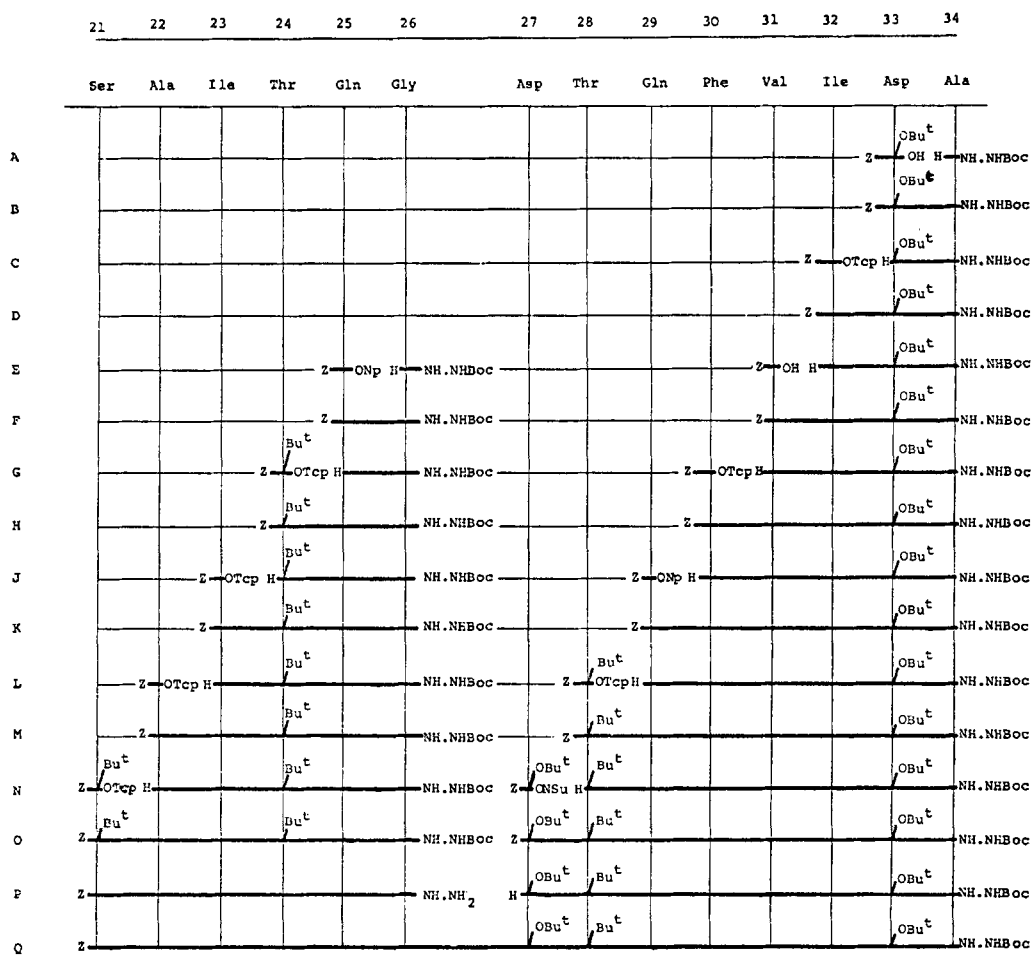
Of the di- and tri-cysteine peptides required for complexing studies, two, *viz*, H · Ala · Cys · Ala · Gly · Glu · Cys · Pro · Val · NH · NH_2 and H · Ser · Cys · Val · Ser · Cys · Gly · NH · NH_2, were readily available by deprotection with hydrogen fluoride/anisole of the intermediates (I K 13–20) and (I K 7–12). The other three (II D 10–16, II G 7–16, and II K 10–20), were prepared from other intermediates in the manner shown in Scheme II. All the couplings were carried out by the azide method, and the final deprotections brought about with hydrogen fluoride/anisole.

Synthesis of Z · Ser · Ala · Ile · Thr · Gln · Gly · Asp(OBu^t) · Thr(Bu^t) · Gln · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc(III Q 21–34)

The synthesis of this protected tetradecapeptide is outlined in Scheme III. Deprotection of the final product was not carried out, since this peptide was required only for continuation of the synthesis of the apoferredoxin and its analogs. The strategy of the synthesis involved the stepwise synthesis of the two subfragments (III O 21–26 and III O 27–34) and azide coupling of their partial deprotection products. N-Protection throughout was by the benzyloxycarbonyl group, removed by catalytic hydrogenolysis in methanol or, for the larger, less soluble, peptides, dimethylformamide. C-Protection was by the t-butoxycarbonylhydrazides to enable fragment couplings to be carried out by the azide method. Side-chain protection was as t-butyl ethers (Ser and Thr residues) or esters (Asp residues). In the stepwise syntheses of the subfragments we used mixed carbonic anhydrides and various active esters as indicated in Scheme III. In two instances (III H 24–26 and III M 28–34), owing to the non-crystalline nature of Z · Thr(Bu^t) · OTcp, the azide-coupling method was also carried out with the crystalline hydrazide; the yields by the azide method were inferior, but the products obtained by the two methods were identical. The overall yields of the subfragments (III O 21–26) and (III O 27–34) from the initial t-butoxycarbonylhydrazides were 26 and 24%, respectively, and that for the combination of the two 40%. The chiral homogeneity of the final protected tetradecapeptide was confirmed by the virtually identical amino acid analyses of its complete acid hydrolysate before and after treatment with D-amino acid oxidase.

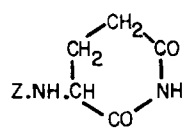

7 8 9 10 11 12 13 14 15 16 17 18 19 20
Ser Cys Val Ser Cys Gly Ala Cys Ala Gly Glu Cys Pro Val
A
B
C
D
E
F
G
H
J
K
Bu^t
Bzl(OMe)
NH.NHBoc
NH.NH$_2$
OBu^t
(ID 10-12)
(IH 13-16)
(IL 7-12)
(IIB 13-16)
(IIB 10-12)
(IL 13-20)
Z
H

SCHEME II

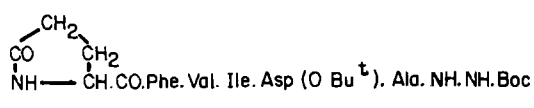


SCHEME III

We encountered only two minor difficulties in working with glutamine derivatives. Attempted recrystallization of Z · Gln · ONp from hot methanol gave the cyclic imide (2) and *p*-nitrophenol, the structure (2) being supported by the ir, ^1H nmr and mass spectra. Schnabel *et al.* (*13*) observed a similar cyclization of the mixed isobutyl carbonic anhydride from Boc · Glu · NH_2. Cyclization in the opposite sense to give the pyroglutamyl peptide (3) was encountered in the hydrogenolysis of the protected hexapeptide (III K 29–34); working up in the usual way gave (3) as the sole product, but its formation was entirely avoided by removing the hydrogenolysis solvent (dimethylformamide) at 20°C or below. The pyroglutamyl peptide (3) was formed from the



(2)



(3)

hydrogenolysis product (III L 29–34) by storage in acetic acid at room temperature or in dimethylformamide at 40°C.

Some difficulty was experienced in the selective deprotection of (III O 21–26). Schnabel *et al.* (*14*) recommended 70% trifluoroacetic acid or boron trifluoride etherate for removal of t-butoxycarbonyl groups in the presence of benzyloxycarbonyl, but neither reagent was satisfactory in our case. The latter reagent gave no useful selectivity; whereas with the former, although the Boc group was removed almost instantaneously, one of the t-butyl groups resisted the reagent for more than 12 h. Model experiments with Z · Ser(Bu^t) · OH and Z · Thr(Bu^t) · OH showed that the serine side-chain t-butyl was removed much more slowly than that attached to threonine. We therefore had recourse to anhydrous trifluoroacetic acid at room temperature, following the reaction by ^{1}H nmr spectroscopy; this reagent removed all three t-butyl groups rapidly, a reaction time of 30 min being optimal and giving a good yield of the selective deprotection product (III P 21–26). The compound was ninhydrin negative, showing that no N → O acyl shift had occurred, and showed no absorption at 1780 cm^{-1}, indicating the absence of *O*-trifluoroacetylation.

EXPERIMENTAL

Organic solutions were dried over $MgSO_4$ and evaporated or concentrated at 10–20 mm Hg on a rotary evaporator. The purity of most compounds was confirmed by thin-layer chromatography on Kieselgel GF254 in two or three solvent systems;[5] compounds with free amino groups were located by spraying with 0.3% ninhydrin in butan-1-ol and heating at 100°C for 10 min, N-acyl compounds by the chlorine–starch–iodide method (*15*), and hydrazides by the method of Hofmann *et al.* (*16*). All new compounds had ^{1}H nmr spectra in accordance with the structures assigned to them.

The ir spectra were recorded on a Perkin–Elmer grating spectrophotometer, Model 357, ^{1}H nmr spectra at 33·5°C on a Jeol JMH-100 100 MHz instrument with $SiMe_4$ as internal reference and mass spectra with a Perkin–Elmer/Hitachi RMU6 instrument. Optical rotations were measured with a Bendix-NPL polarimeter, Model 143C. C, H, N analyses were carried out with a Carlo Erba elemental analyzer, Model 1102, and amino acid analyses with a Jeol JLC-5AH amino acid analyzer on samples hydrolyzed with redistilled 6 *M* hydrochloric acid at 110°C for 24 hr in evacuated tubes.

Starting materials were prepared by literature methods and had the properties described in the literature (*17*). Z · Thr(Bu^t) · OTcp was prepared by the general method of Pless and Boissonnas (*18*) but could not be induced to crystallize. Use of the crude product (ca 90% pure) in coupling reactions gave rise to side products difficult to remove. The ester was therefore purified by adsorption on Kieselgel GF254, washing with chloroform–light petroleum (1 : 1), and elution with tetrachloromethane; owing to the instability of the ester, the purification should be completed and the ester used within 1 day.

[5] The following solvent systems were used: *A* $CHCl_3$/MeOH (9 : 1); *B* Bu^{n}OH/AcOH/H_2O (3 : 1 : 1); *D* Bu^{n}OH/AcOH/C_5H_5N/H_2O (15:3:10:6); *F* Bu^{s}OH/3% aq. NH_4OH (100:44); *G* $CHCl_3$/MeOH/AcOH (9:1:1); *AN* C_5H_5N/AcOH/H_2O (10:6:3); *AO* Aq·NH_4OH, *d* 0.880/AcOH/H_2O (10:10:80).

A. Synthesis of H·Ser·Cys·Val·Ser·Cys·Gly·Ala·Cys·Ala·Gly·Glu·Cys·Pro·Val·NH·NH_2, (I N 7–20).

Mixed anhydride couplings. To a stirred solution of the carboxyl component (1.5 mmol) in anhydrous dimethylformamide (15 ml) at −15°C, *N*-methylmorpholine (1.5 mmol) was added, followed by isobutyl chloroformate (1.4 mmol). After stirring for 2 min at −15°C, a solution of the amino component (1.0 mmol) in dimethylformamide (15 ml) was added over about 5 min, with external cooling, at such a rate that the temperature did not rise by more than 2°C. The mixture was then stirred at −15°C until ninhydrin negative (1.5–3 hr). The temperature was then raised to 0°C and 2 *M* potassium hydrogen carbonate (10 ml) added. The mixture was stirred at 0°C for 30 min and poured into 75% saturated brine (200 ml). If the product separated as a crystalline solid it was collected, washed with water, dried, and recrystallized. If the precipitate was oily, it was extracted into ethyl acetate (3 × 20 ml), washed successively with saturated brine (10 ml), 1 *M* citric acid (10 ml), saturated sodium hydrogen carbonate (10 ml), and brine (10 ml), dried and evaporated to dryness, and the solid residue was recrystallized. The compounds listed in Table 1 were prepared in this way.

Active ester coupling. Z·Ala·OTcp (8.44 g, 21 mmol) and H·Gly·NH·NH·Boc (3.96 g, 21 mmol) were kept overnight at room temperature in dimethylformamide (40 ml). Saturated brine (150 ml) was then added and the solution extracted with ethyl acetate (150 ml). The organic phase was washed with 1 *M* citric acid (75 ml), brine (50 ml), saturated sodium hydrogen carbonate (50 ml), and brine (2 × 50 ml), dried, and evaporated. The residue was dissolved in ether and washed with 3% aqueous ammonia (25 ml). The product, which precipitated from the ether layer, was collected by filtration. Recrystallization from aqueous methanol gave 5.5 g of Z·Ala·Gly·NH·NH·Boc (I D 15–16) (70% yield), mp 83–84.5°C, $[\alpha]_D^{22}$ −9.0° ($c = 1$ in MeOH).

Anal. Calcd for $C_{18}H_{26}N_4O_6$: C, 54.8; H, 6.6; N, 14.2. Found: C, 54.6; H, 6.7; N, 14.1.

Azide couplings. To a stirred solution of the hydrazide component (0.2 mmol) in anhydrous dimethylformamide (3 ml) at −20°C, a solution of HCl in dioxan (3–4 *M*) (0.8 mmol) was added, followed by t-butyl nitrite (0.22 mmol). The mixture was stirred at −20°C until a small portion gave no blue coloration with the hydrazide reagent (*16*) (1–2 hr). The mixture was then cooled to −55°C and triethylamine (0.8 mmol) was added, followed by the amino component (0.2 mmol) in dimethylformamide (4 ml). The reaction mixture was allowed to warm up to −10°C and stirred at this temperature for 2–4 hr and then at +4°C until tlc indicated no further reaction (2–4 days). The solution was poured into water, and the precipitate dried and recrystallized. The compounds listed in Table 2 were prepared in this way.

Removal of benzyloxycarbonyl groups by hydrogenolysis. (a) In liquid ammonia. Anhydrous ammonia was condensed directly into a three-necked round-bottomed flask fitted with a solid CO_2/acetone reflux condenser, a magnetic stirrer, and a gas inlet tube reaching to the bottom of the flask, cooled in a solid CO_2/acetone bath. The *N*-benzyloxycarbonyl-peptide (0.5 mmol) was dissolved in the liquid ammonia (400 ml) and freshly prepared palladium black (from $PdCl_2$, 500 mg, hydrogenated in ethanol, 150 ml, at room temperature and atmospheric pressure, and washed with methanol) added under nitrogen. Dry hydrogen was bubbled through the stirred suspension at the

Compound	Yield (%)	Properties	Analysis
Z · Cys[Bzl(OMe)] · Gly · NH · NH · Boc (I B 11–12)	88	mp 72–73°C from MeOH; $[\alpha]_D^{22}$ −19.5° (c = 1 in MeOH)	Calcd for $C_{26}H_{34}N_4O_7S$: C, 57.1; H, 6.25; N, 10.25. Found: C, 56.75; H, 6.05; N, 10.1.
Z · Ser(Bu^t) · Cys[Bzl(OMe)] · Gly · NH · NH · Boc (I D 10–12)	64	mp 114–116°C from EtOAc/light petroleum; $[\alpha]_D^{26}$ −22.0° (c = 1 in MeOH)	Calcd for $C_{33}H_{47}N_5O_9S$: C, 57.45; H, 6.85; N, 10.15. Found: C, 57.1; H, 6.65; N, 10.25.
Z · Val · Ser(Bu^t) · Cys[Bzl(OMe)] · Gly · NH · NH · Boc (I F 9–12)	92	mp 194–195°C from EtOAc; $[\alpha]_D^{30}$ −20.0° (c = 1 in MeOH)	Calcd for $C_{38}H_{56}N_6O_{10}S$: C, 57.85; H, 7.15; N, 10.65. Found: C, 57.6; H, 6.7; N, 10.8.
Z · Cys[Bzl(OMe)] · Val · Ser(Bu^t) · Cys[Bzl(OMe)] · Gly · NH · NH · Boc (I H 8–12)	71	mp 181–182°C from MeOH; $[\alpha]_D^{27}$ −17.0° (c = 1 in DMF)	Calcd for $C_{49}H_{69}N_7O_{12}S_2$: C, 58.15; H, 6.85; N, 9.7. Found: C, 58.1; H, 6.8; N, 9.8.
Z · Ser(Bu^t) · Cys[Bzl(OMe)] · Val · Ser(Bu^t) · Cys[Bzl(OMe)] · Gly · NH · NH · Boc (I K 7–12)	76	mp 249–251°C dec. from MeOH; $[\alpha]_D^{27}$ −15.0° (c = 1 in DMF)	Calcd for $C_{56}H_{82}N_8O_{14}S_2$: C, 58.2; H, 7.15; N, 9.7. Found: C, 57.9; H, 7.2; N, 9.85.
Dmpoc · Cys[Bzl(OMe)] · Ala · Gly · NH · NH · Boc (I F 14–16)	83[a]	mp 120°C dec. from EtOAc/Et_2O; $[\alpha]_D^{25}$ −5.0° (c = 1 in MeOH)	Calcd for $C_{27}H_{39}N_5O_8S$, $\frac{1}{2}H_2O$: C, 53.8; H, 6.7; N, 11.6. Found: C, 53.8; H, 6.6; N, 11.3.
Z · Ala · Cys[Bzl(OMe)] · Ala · Gly · NH · NH · Boc (I H 13–16)	77[b,c]	mp 179.5°C from MeOH/Et_2O; $[\alpha]_D^{27}$ −26.0° (c = 1 in MeOH)	Calcd for $C_{32}H_{44}N_6O_9S$: C, 55.8; H, 6.45; N, 12.2. Found: C, 56.0; H, 6.4; N, 12.3.
Z · Pro · Val · NH · NH · Boc (I D 19–20)	80[a,b,d]	mp 159–161°C from EtOAc/light petroleum; $[\alpha]_D^{25}$ − 98.0° (c = 1 in MeOH)	Calcd for $C_{23}H_{34}N_4O_6$, $\frac{1}{2}H_2O$: C, 58.6; H, 7.5; N, 11.9. Found: C, 58.7; H, 7.5; N, 12.1.
Bpoc · Cys[Bzl(OMe)] · Pro · Val · NH · NH · Boc (I F 18–20)	90[b]	mp 90–110°C dec. from EtOAc/light petroleum; $[\alpha]_D^{25}$ −89.0° (c = 1 in MeOH)	Calcd for $C_{42}H_{55}N_5O_8S$, $\frac{1}{2}H_2O$: C, 63.15; H, 7.05; N, 8.75. Found: C, 63.0; H, 7.1; N, 8.4.
Bpoc · Glu(OBu^t) · Cys[Bzl(OMe)] · Pro · Val · NH · NH · Boc (I H 17–20)	72[b,e]	mp 98–100°C dec. from EtOAc/light petroleum; $[\alpha]_D^{25}$ −81.0° (c = 1 in MeOH)	Calcd for $C_{51}H_{70}N_6O_{11}S$: C, 62.8; H, 7.25; N, 8.6. Found: C, 62.8; H, 7.3; N, 8.2

[a] In this case equimolar quantities of carboxyl and amino components were used.

[b] Coupling reaction performed in tetrahydrofuran.

[c] Amino component liberated from *H*-oxalate by partitioning between EtOAc and saturated $NaHCO_3$.

[d] Triethylamine used in place of *N*-methylmorpholine.

[e] Amino component liberated from *H*-oxalate *in situ* with NEt_3.

TABLE 2

AZIDE COUPLING PRODUCTS

Compound	Yield (%)	Properties	Analysis
Z · Ala · Cys[Bzl(OMe)] · Ala · Gly · Glu(OBu^t) · Cys[Bzl(OMe)] · Pro · Val · NH · NH · Boc (I K 13–20)	68[a]	mp 208–209°C from MeOH; $[\alpha]_D^{31}$ −51.5° (c = 1 in DMF)	Calcd for $C_{62}H_{88}N_{10}O_{16}S_2$: C, 57.55; H, 6.85; N, 10.8. Found: C, 57.4; H, 6.8; N, 10.7. Amino acid anal.: Glu, 1.02; Pro, 1.00; Gly, 0.95; Ala, 1.96; Val, 0.95.
Z · Ser · Cys[Bzl(OMe)] · Val · Ser · Cys[Bzl(OMe)] · Gly · Ala · Cys[Bzl(OMe)] · Ala · Gly · Glu(OBu^t) · Cys[Bzl(OMe)] · Pro · Val · NH · NH · Boc (I M 7–20)	33	mp 252–253°C; $[\alpha]_D^{25}$ − 49.0° (c = 2 in DMSO)	Calcd for $C_{97}H_{136}N_{16}O_{26}S_4$: C, 56.2; H, 6.6; N, 10.8. Found:[b] C, 56.7; H, 6.8; N, 10.8. Amino acid anal.: Ser, 2.03; Glu, 0.91; Pro, 0.83; Gly, 2.00; Ala, 2.04; Val, 1.9.
Z · Ser · Cys[Bzl(OMe)] · Gly · Ala · Cys[Bzl(OMe)] · Ala · Gly · NH · NH · Boc (II C 10–16)	52	mp 201–203°C from aq. AcOH; $[\alpha]_D^{27}$ −32.0° (c = 1 in DMF)	Calcd for $C_{48}H_{65}N_9O_{14}S_2$: C, 54.4; H, 6.2; N, 11.9; S, 6.05. Found:[b] C, 54.5; H, 6.1; N, 11.8; S, 6.0. Amino acid anal.: Ser, 1.05; Gly, 2.00; Ala, 1.94.
Z · Ser · Cys[Bzl(OMe)] · Val · Ser · Cys[Bzl(OMe)] · Gly · Ala · Cys[Bzl(OMe)] · Ala · Gly · NH · NH · Boc (II F 7–16)	50	mp 243–245°C; $[\alpha]_D^{24}$ −68.0° (c = 1 in DMSO)	Calcd for $C_{67}H_{92}N_{12}O_{19}S_3$: C, 54.9; H, 6.35; N, 11.45. Found:[b] C, 55.0; H, 6.45; N, 11.4. Amino acid anal.; Ser, 1.91; Gly, 1.72; Ala, 2.00; Val, 0.98.
Z · Ser · Cys[Bzl(OMe)] · Gly · Ala · Cys[Bzl(OMe)] · Ala · Gly · Glu(OBu^t) · Cys[Bzl(OMe)] · Pro · Val · NH · NH · Boc (II J 10–20)	45	mp 237–238°C from aq. AcOH; $[\alpha]_D^{31}$ − 51.5° (c = 1 in DMF)	Calcd for $C_{78}H_{109}N_{13}O_{21}S_3$: C, 56.4; H, 6.6; N, 11.0; S, 5.8. Found:[b] C, 56.5; H, 6.6; N, 10.9; S, 6.0. Amino acid anal.: Ser, 1.0; Glu, 0.98; Pro, 1.00; Gly, 1.87; Ala, 2.00; Val, 1.00.

[a] Amino component liberated from *H*-oxalate *in situ* with NEt_3.

[b] Analyses by Butterworth Microanalytical Consultancy Ltd., Teddington.

temperature (ca −30°C) of refluxing ammonia until tlc indicated that the reaction was complete or proceeding no further. The ammonia was then allowed to evaporate overnight in a slow stream of nitrogen. The residue was taken up in methanol (300 ml), the catalyst was filtered off, the filtrate was evaporated to dryness, and the residue was triturated with ether. In most cases tlc showed the residual solid to be homogeneous and it was used directly for the subsequent coupling.

The hydrogenolysis product from (I B 11–12) was dissolved in ethyl acetate (30 ml) and extracted with 1 *M* citric acid (3 × 5 ml). The aqueous layers were combined, washed with ethyl acetate (10 ml), and neutralized with solid sodium hydrogen carbonate. Extraction with ethyl acetate (3 × 10 ml), drying, and evaporation gave homogeneous (I C 11–12). Unreacted starting material (24%) was recovered from the ethyl acetate solutions by evaporation.

TABLE 3

PRODUCTS OF HYDROGENOLYSIS IN LIQUID AMMONIA

Compound	Reaction time (hr)	Yield (%)	R_f values
H·Cys[Bzl(OMe)]·Gly·NH·NH·Boc (I C 11–12)	7	70	*A*, 0.30; *B*, 0.63; *G*, 0.11.
H·Ser(Bu^t)·Cys[Bzl(OMe)]·Gly·NH·NH·Boc (I E 10–12)	2	95	*A*, 0.42; *B*, 0.60.
H·Val·Ser(Bu^t)·Cys[Bzl(OMe)]·Gly·NH·NH·Boc (I G 9–12)	2	100	*A*, 0.23; *B*, 0.58; *G*, 0.18.
H·Cys[Bzl(OMe)]·Val·Ser(Bu^t)·Cys[Bzl(OMe)]·Gly·NH·NH·Boc (I J 8–12)	6	70	*A*, 0.36; *B*, 0.65.
H·Ala·Cys[Bzl(OMe)]·Ala·Gly·NH·NH·Boc (II B 13–16)	5	98	*A*, 0.10; *B*, 0.50.
H·Ala·Cys[Bzl(OMe)]·Ala·Gly·Glu(OBu^t)·Cys[Bzl(OMe)]·Pro·Val·NH·NH·Boc (I L 13–20)	4.5	100	*A*, 0.05; *B*, 0.54; *G*, 0.12.

In the hydrogenolysis of (I H 8–12) the crude product was dissolved in methanol (10 ml) and purified by preparative tlc on seven 20 × 40-cm plates coated with 1 mm of Kieselgel GF254. The plates were developed with $CHCl_3$–MeOH (9 : 1). Elution of the main band with methanol gave (I J 8–12); unchanged starting material (14%) was recovered from the other principal band.

Compounds prepared in this way are listed in Table 3.

(*b*) In methanol. Z·Ala·Gly·NH·NH·Boc (I D 15–16) (12.0 g) was hydrogenated over 5% palladized barium sulfate (4.8 g) in methanol (120 ml) for 3 hr. Filtration, evaporation, and recrystallization from ethyl acetate gave 5.95 g of H·Ala·Gly·NH·NHBoc (I E 15–16) (77% yield) as needles, mp 140–141°C, $[\alpha]_D^{22}$ + 6.0° (c = 1 in MeOH).

Anal. Calcd for $C_{10}H_{20}N_4O_4$: C, 46.1; H, 7.7; N, 21.5. Found: C, 46.2; H, 7.8; N, 21.5.

Similar hydrogenolysis of Z·Pro·Val·NH·NH·Boc (I D 19–20) and recrystallization of the crude product from ethyl acetate–diisopropyl ether gave H·Pro·Val·NH·NH·Boc (I E 19–20) in 86% yield; mp 138–139°C, $[\alpha]_D^{25}$ −89.5° (c = 1 in MeOH).

Anal. Calcd for $C_{15}H_{28}N_4O_4$: C, 54.85; H, 8.6; N, 17.05. Found: C, 54.65; H, 8.7; N, 17.2.

Removal of dimethylpropynyloxycarbonyl group. The protected tripeptide (I F 14–16) (5.95 g, 10 mmol) was hydrogenated for 2.5 hr over 5% palladized charcoal (5.0 g) in anhydrous methanol (400 ml) containing triethylamine (1.13 g, 11 mmol). The solution was filtered and the spent catalyst was washed well with methanol. The combined filtrate and washings were evaporated to dryness and the residue dissolved in ethyl acetate (50 ml). The solution was washed with water (20 ml) and then extracted with 1 *M* citric acid (3 × 25 ml). The combined extracts were washed with ethyl acetate (10 ml) and then neutralized with solid sodium hydrogen carbonate. The oily solid which separated was extracted with ethyl acetate (3 × 25 ml) and the extract was dried and evaporated. The noncrystalline product, H·Cys[Bzl(OMe)]·Ala·Gly·NH·NH·Boc (I G 14–16) (4.08 g, 82% yield) was homogeneous by tlc in three systems[6] and used for the next coupling step without further purification.

Removal of biphenylisopropoxycarbonyl group. The protected tripeptide (I F 18–20) (3.9 g, 4.9 mmol) was kept for 10 min at room temperature in 1.5% trifluoroacetic acid in dichloromethane (100 ml). The solution was washed with saturated sodium hydrogen carbonate (25 ml) and water (25 ml), dried, and evaporated. The residue was dissolved in methanol (25 ml) and oxalic acid (0.47 g 5.3 mmol) was added. After 45 min the solution was evaporated to dryness and the residue was triturated with ether to give the hydrogen oxalate of H·Cys[Bzl(OMe)]·Pro·Val·NH·NH·Boc (I G 18–20), mp 135°C (dec.), (2.74 g; 86% yield).

Anal. Calcd for $C_{28}H_{43}N_5O_{10}S$: C, 52.4; H, 6.8; N, 10.9. Found: C, 51.8; H, 6.7; N, 10.5.

The hydrogen oxalate of H·Glu(OBu^t)·Cys[Bzl(OMe)]·Pro·Val·NH·NH·Boc (I J 17–20), was prepared similarly from (I H 17–20) in 73% yield.

Removal of t-butoxycarbonyl and t-butyl groups. (a) The protected tetrapeptide (I H 13–16) (160 mg, 0.23 mmol) was kept at room temperature for 30 min in a 1:1 mixture of trifluoroacetic acid and dichloromethane (0.2 ml). The solution was poured into 2 *M* potassium hydrogen carbonate (10 ml) and the precipitate was collected by filtration, washed with water, dried, and recrystallized from ethanol to give Z·Ala·Cys[Bzl(OMe)]·Ala·Gly·NH·NH_2 (I J 13–16) (103 mg, 73% yield), mp 197–200°C (dec.), $[\alpha]_D^{26}$ −8.0° ($c = 1$ in $HCONMe_2$).

Anal. Calcd for $C_{27}H_{36}N_6O_7S$: C, 55.1; H, 6.15; N, 14.25. Found: C, 54.8; H, 6.1; N, 14.0.

(b) The protected hexapeptide (I K 7–12) (100 mg, 86.5 μmol) was kept at room temperature for 80 min in anhydrous trifluoroacetic acid (0.4 ml). The mixture was poured into water and the product was collected by filtration, washed with water, and triturated with hot methanol to give 65 mg (80% yield) of Z·Ser·Cys[Bzl(OMe)]·Val·Ser·Cys[Bzl(OMe)]·Gly·NH·NH_2 (I L 7–12), homogeneous by tlc in two solvent systems,[7] which was used directly in the next coupling step.

(c) The protected tripeptide (I D 10–12) (200 mg, 0.29 mmol) was kept at room temperature for 30 min in anhydrous trifluoroacetic acid (1.0 ml). The solution was poured into ice-cold 2 *M* potassium hydrogen carbonate (6 ml) and the precipitate was

[6] R_f values: *A*, 0.16; *D*, 0.67; *F* 0.61.

[7] R_f values: *A*, 0.27; *B*, 0.73.

collected by filtration and recrystallized from methanol to give 100 mg (65% yield) of Z·Ser·Cys[Bzl(OMe)]·Gly·NH·NH_2 (II B 10–12),[8] $[\alpha]_D^{31}$ −24.0° (*c* = 1 in $HCONMe_2$), mp 174–175°C.

Final deprotection. The protected peptide (50 μmol) was stirred at 0°C for 40–60 min in anhydrous hydrogen fluoride (5–10 ml) containing anisole (4 equivalents for each protecting group to be removed). The solution was then evaporated to dryness and the residue was dried over potassium hydroxide pellets overnight at 0.01 mm Hg. The residue was dissolved in water (10 ml) containing 2-mercaptoethanol (0.1 ml) and extracted with ether (4 × 5 ml). The ether extracts were washed with water (3 ml). This washing and the original aqueous solution were combined and evaporated to dryness, and the residual oil was triturated with ethyl acetate containing 5% mercaptoethanol. The resulting solid was collected by filtration, washed with ethyl acetate, and finally lyophilized from water containing 2% of mercaptoethanol. Details of the compounds prepared in this way are given in Table 4. None of these compounds had definite mp's; ^1H nmr spectra showed no residual peaks in the aromatic region.

B. Synthesis of Z·Ser·Ala·Ile·Thr·Gln·Gly·Asp(OBu^t)·Thr(Bu^t)·Gln·Phe·Val·Ile·Asp(OBu^t)·Ala·NH·NH·Boc (III Q 21–34)

Active ester couplings. A solution of the amino component in tetrahydrofuran or dimethylformamide (for concentrations see Table5) was treated with the active ester of the carboxyl component (1 eq plus sufficient excess to make the residual solution 0.05 *M* in active ester after total consumption of the amino component) (*19*), and the mixture was stirred at room temperature until reaction was complete (tlc, ninhydrin). The solvent was removed by evaporation and the residue triturated with a suitable solvent (Et_2O, MeOH, or EtOAc). The solid product was collected, dried, and recrystallized. The compounds listed in Table 5 were prepared in this way.

Mixed anhydride couplings. (a) Triethylamine (10.26 ml, 74 mmol) was added to a solution of Z·Asp(OBu^t)·OH [from the dicyclohexylammonium salt (39.7 g, 77 mmol) and *M*-H_3PO_4] in anhydrous tetrahydrofuran (100 ml) at −20°C. Ethyl chloroformate (7.05 ml, 74 mmol) was added and the mixture was stirred at −20°C for 8 min, when a precooled solution of H·Ala·NH·NH·Boc (14.98 g, 74 mmol) in tetrahydrofuran (90 ml) was added. The mixture was kept for 1 hr at −10°C and then overnight at room temperature. It was then partitioned between ether and water and the organic phase was washed successively with 1 *M* hydrochloric acid, saturated sodium hydrogen carbonate, and brine. The solution was dried and evaporated, and the solid residue was twice recrystallized from ethyl acetate/light petroleum, giving 26.24 g (70%) of Z·Asp(OBu^t)·Ala·NH·NH·Boc (III B 33–34), mp 90–93°C, $[\alpha]_D^{25}$ −38.0° (*c* = 1 in MeOH).

Anal. Calcd for $C_{24}H_{36}N_4O_8$: C, 56.7; H, 7.1; N, 11.0. Found: C, 56.6; H, 7.25; N, 11.1.

(b) A similar coupling, using *N*-methylmorpholine in place of triethylamine, isobutyl in place of ethyl chloroformate, and ethyl acetate in place of ether, gave an 87% yield of Z·Val·Ile·Asp(OBu^t)·Ala·NH·NH·Boc (III F 31–34) mp 231°C (dec.) from aqueous ethanol, $[\alpha]_D^{27}$ −66.0° (*c* = 1 in MeOH).

[8] R_f values: *A*, 0.35; *B*, 0.85.

TABLE 4

FINAL DEPROTECTION PRODUCTS

Compound	Yield (%)	$[\alpha]_D^{25}$ ($c = 1$)	R_f values[a]					Amino-acid analysis					
			AO	*AN*	*B*	*D*	*G*	Ser	Glu	Pro	Gly	Ala	Val
H·Ser·Cys·Val·Ser·Cys·Gly·Ala·Cys·Ala·Gly·Glu·Cys·Pro·Val·NH·NH_2 (I N 7–20)	93	−112.0° (Me_2SO)	—	—	0.27	0.40	0.0	1.35	1.01	0.90	2.00	1.99	1.86
H·Ala·Cys·Ala·Gly·Glu·Cys·Pro·Val·NH·NH_2	99	−102.0° (H_2O)	0.61	0.95	—	—	0.0	—	0.97	1.08	1.00	1.97	1.06
H·Ser·Cys·Val·Ser·Cys·Gly·NH·NH_2	95	−54.0° (H_2O)	—	—	0.31	0.50	0.0	1.83	—	—	1.00	—	0.94
H·Ser·Cys·Gly·Ala·Cys·Ala·Gly·NH·NH_2 (II D 10–16)	96	−37.0° (Me_2SO)	—	—	0.42	—	0.0	1.05	—	—	2.00	2.10	—
H·Ser·Cys·Val·Ser·Cys·Gly·Ala·Cys·Ala·Gly·NH·NH_2 (II G 7–16)	96	−87.0° (Me_2SO)	—	—	0.31	0.50	0.0	1.77	—	—	2.00	2.08	0.97
H·Ser·Cys·Gly·Ala·Cys·Ala·Gly·Glu·Cys·Pro·Val·NH·NH_2 (II K 10–20)	97	−56.0° (Me_2SO)	—	—	0.40	0.56	0.0	1.00	0.93	0.91	2.00	1.98	1.03

[a] Mercaptoethanol (4%) was added to all systems.

Compound	Solvent	Yield (%)	Properties	Analysis
Z · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III D 32–34)	DMF (0.5 *M*)	93	mp 203°C dec. from aq. EtOH; $[\alpha]_D^{25}$ −53.0° (c = 1 in MeOH)	Calcd for $C_{30}H_{47}N_5O_9$: C, 57.95; H, 7.6; N, 11.3. Found: C, 58.1; H, 7.9; N, 11.2.
Z · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III H 30–34)	DMF (0.1 *M*)	84	mp 243°C dec. from MeOH; $[\alpha]_D^{25}$ − 22.0° (c = 1 in Me_2SO)	Calcd for $C_{44}H_{65}N_7O_{11}$ · MeOH: C, 60.0; H, 7.7; N, 10.9. Found: C, 60.2; H, 7.4; N, 11.2.
Z · Gln · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III K 29–34)	DMF (0.1 *M*)	86	mp 255–260°C dec. from aq. AcOH; $[\alpha]_D^{25}$ −26.9° (c = 0.5 in Me_2SO)	Calcd for $C_{49}H_{73}N_9O_{13}$: C, 59.1; H, 7.4; N, 12.65. Found: C, 58.7; H, 7.4; N, 12.4
Z · Thr(Bu^t) · Gln · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III M 28–34)	DMF (0.1 *M*)	90	mp 230°C dec. from aq. Me_2SO; $[\alpha]_D^{24}$ −25.4° (c = 0.5 in Me_2SO)	Calcd for $C_{57}H_{88}N_{10}O_{15}$. H_2O: C, 58.45; H, 7.75; N, 11.95. Found: C, 58.6; H, 8.0; N, 12.0.
Z · Asp(OBu^t) · Thr(Bu^t) · Gln · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III O 27–34)	DMF (0.15 *M*)	88	mp 235°C dec. from MeOH; $[\alpha]_D^{24}$ −21.4° (c = 0.5 in Me_2SO)	Calcd for $C_{65}H_{101}N_{11}O_{18}$. H_2O: C, 58.15; H, 7.75; N, 11.5. Found: C, 58.25; H, 7.55; N, 11.25. Amino acid anal.: Asp, 1.88; Thr[9], 1.01; Glu, 1.10; Phe, 1.05; Val, 0.86; Ile, 0.90; Ala, 1.06.
Z · Gln · Gly · NH · NH · Boc (III F 25–26)	THF (0.23 *M*)	85[a]	mp 175–176°C from aq. EtOH; $[\alpha]_D^{25}$ −14.5° (c = 0.4 in MeOH)	Calcd for $C_{20}H_{29}N_5O_7$: C, 53.2; H, 6.5; N, 15.5. Found: C, 53.55; H, 6.65; N, 15.65.
Z · Thr(Bu^t) · Gln · Gly · NH · NH · Boc (III H 24–26)	DMF (1 *M*)	71	mp 115–125°C dec. from $CHCl_3$/Pr^i_2O; $[\alpha]_D^{25}$ −3.0° (c = 4.5 in MeOH)	Calcd for $C_{28}H_{44}N_6O_9$. H_2O: C, 53.7; H, 7.4; N, 13.4. Found: C, 53.7; H, 7.25; N, 13.3.
Z · Ile · Thr(Bu^t) · Gln · Gly · NH · NH · Boc (III K 23–26)	DMF (0.2 *M*)	92	mp 213–215°C from aq. Pr^{i}OH; $[\alpha]_D^{25}$ −11.0° (c = 1 in MeOH)	Calcd for $C_{34}H_{55}N_7O_{10}$: C, 56.6; H, 7.7; N, 13.6. Found: C, 56.8; H, 7.9; N, 13.8.
Z · Ala · Ile · Thr(Bu^t) · Gln · Gly · NH · NH · Boc (III M 22–26)	DMF (0.05 *M*)	85	mp 218°C dec. from aq. EtOH; $[\alpha]_D^{25}$ −28.9° (c = 0.6 in MeOH)	Calcd for $C_{37}H_{60}N_8O_{11}$ · EtOH: C, 55.8; H, 7.9; N, 13.35. Found: C, 55.6; H, 7.7; N, 13.35.
Z · Ser(Bu^t) · Ala · Ile · Thr(Bu^t) · Gln · Gly · NH · NH · Boc (III O 21–26)	DMF (0.1 *M*)	85	mp 232–240°C dec. from MeCN/MeOH; $[\alpha]_D^{25}$ +5.0° (c = 1 in DMF)	Calcd for $C_{44}H_{71}N_9O_{13}$: C, 56.45; H, 7.85; N, 13.45. Found: C, 56.2; H, 8.1; N, 13.3. Amino acid anal.: Ser[9], 0.97; Ala, 1.03; Ile, 1.09; Thr[9], 0.97; Glu, 1.08; Gly, 1.00.

[a] In this case the amino component rather than the active ester was used in excess.

Anal. Calcd for $C_{35}H_{56}N_6O_{10}$: C, 58.3; H, 7.8; N, 11.65. Found: C, 58.0; H, 8.2; N, 11.5.

Removal of benzyloxycarbonyl groups by hydrogenolysis. A solution or suspension of the *N*-benzyloxycarbonyl peptide in methanol (10–40 ml g^{-1}) or dimethylformamide

TABLE 6

PRODUCTS OF HYDROGENOLYSIS IN MeOH OR DMF

Compound	Solvent	Yield (%)	
H · Asp(OBu^t) · Ala · NH · NH · Boc (III C 33–34)	MeOH	100	Characterized as the hydrogen oxalate, mp 146°C dec. from EtOH/Et_2O. *Anal.* Calcd for $C_{18}H_{32}N_4O_{10}$: C, 46.5; H, 6.9; N, 12.1. Found: C, 46.2; H, 7.2; N, 11.85.
H · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III E 32–34)	MeOH	92	Recrystallized from EtOAc/light petroleum; mp 161–162°C. *Anal.* Calcd for $C_{22}H_{41}N_5O_7$: C, 54.2; H, 8.5; N, 14.4. Found: C, 54.1; H, 8.8; N, 14.2.
H · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III G 31–34)	MeOH	87	Recrystallized from ethyl acetate/light petroleum; mp 235°C dec. *Anal.* Calcd for $C_{27}H_{50}N_6O_8$: C, 55.3; H, 8.6; N, 14.3. Found: C, 55.5; H, 8.95; N, 14.2.
H · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III J 30–34)	DMF	96	
H · Gln · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III L 29–34)	DMF	100	Hydrogenated in suspension; solution evaporated at 20°C.
H · Thr(Bu^t) · Gln · Phe · Val · Ile Asp(OBu^t) · Ala · NH · NH · Boc (III N 28–34)	DMF	97	Hydrogenated in suspension.
H · Asp(OBu^t) · Thr(Bu^t) · Gln · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III P 27–34)	DMF	98	Hydrogenated in suspension. Purified by absorption on Kieselgel GF254 from DMF and elution with $CHCl_3$/MeOH (98:2).
H · Gln · Gly · NH · NH · Boc (III G 25–26)	MeOH	97	
H · Thr(Bu^t) · Gln · Gly · NH · NH · Boc (III J 24–26)	MeOH	85	Recrystallized from $CHCl_3/Et_2O$; mp 90°C. *Anal.* Calcd for $C_{20}H_{38}N_6O_7 \cdot H_2O$: C, 48.8; H, 8.2; N, 17.1. Found: C, 48.5; H, 8.2; N, 16.7.
H · Ile · Thr(Bu^t) · Gln · Gly · NH · NH · Boc (III L 23–26)	MeOH	90	mp 164–166.5°C from $CHCl_3/Et_2O$. *Anal.* Calcd for $C_{26}H_{49}N_7O_8 \cdot H_2O$; C, 51.55; H, 8.5; N, 16.2. Found: C, 51.3; H, 8.35; N, 15.5.
H · Ala · Ile · Thr(Bu^t) · Gln · Gly · NH · NH · Boc (III N 22–26)	MeOH	100	

(40 ml g^{-1}) was hydrogenated at room temperature over 5% palladized barium sulfate (0.2 g per gram of peptide). When deprotection was complete (tlc) the catalyst was filtered off, the filtrate was evaporated to dryness, and the residue was triturated with ether or ethanol. In most cases the product (homogeneous by tlc) was used in the subsequent coupling step without further purification. The compounds listed in Table 6 were prepared in this way.

Final azide coupling. The fully protected N-terminal hexapeptide (III O 21–26) (200 mg, 0.214 mmol) was kept at room temperature in anhydrous trifluoroacetic acid (2 ml) for 30 min. The solution was then diluted with toluene and evaporated to dryness at 25°C (bath temperature). After two further evaporations with added toluene, the residue was triturated with ether. The product was dissolved in the minimum of dimethyl sulfoxide; two drops of pyridine were added, followed by methanol to incipient crystallization, and the solution was set aside to crystallize. Filtration and washing with methanol and ether gave 136 mg (88% yield) of Z · Ser · Ala · Ile · Thr · Gln · Gly · NH · NH_2 (III P 21–26), mp 240°C (dec.), $[\alpha]_D^{25}$ −22.8° ($c = 0.6$ in Me_2SO).

Anal. Calcd for $C_{31}H_{49}N_9O_{11}$ · MeOH: C, 50.8; H, 7.1; N, 16.6. Found: C, 50.4; H, 6.8; N, 16.4. Amino acid analysis: Ser, 0.97;[9] Ala, 1.01; Ile, 1.06; Thr, 0.97;[9] Glu, 1.06; Gly, 1.00.

This hydrazide was converted into the azide and coupled with the partially protected C-terminal octapeptide (III P 27–34) by the general procedure (see above), but at half the concentration. The dimethylformamide solution was evaporated to dryness at 20°C and the solid residue triturated with methanol to constant weight. The partially protected tetradecapeptide, Z · Ser · Ala · Ile · Thr · Gln · Gly · Asp(OBu^t) · Thr(Bu^t) · Gln · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (III Q 21–34) so obtained in 40% yield had no definite mp; concentration of the methanol washings and chromatography on Sephadex LH20 gave recovered C-terminal octapeptide (50%).

Anal. Calcd for $C_{88}H_{140}N_{18}O_{27} \cdot 4H_2O$: C, 54.1; H, 7.6; N, 12.9. Found: C, 53.8; H, 7.3; N, 12.9. Amino acid analysis: Ser, 1.02;[9] Ala, 2.02; Ile, 1.89; Thr, 1.99;[9] Glu, 2.11; Gly, 1.00; Asp, 1.98; Phe, 0.93; Val, 0.91.

Optical purity of product. A mixture of amino acids and hydrazine (molar proportions: H · Asp · OH, 2.00; H · Thr · OH, 2.00; H · Ser · OH, 1.00; H · Gln · OH, 2.00; H · Gly · OH, 1.00; H · Ala · OH, 2.00; H · Val · OH, 1.00; H · Ile · OH, 2.00; H · Phe · OH, 1.00; N_2H_4, 1.00) was subjected to the standard procedure for complete acid hydrolysis (see above) and the product was subjected to amino acid analysis, with and without treatment with D-amino acid oxidase.[10] The following results were obtained:

	Asp	Thr	Ser	Glu	Gly	Ala	Val	Ile	Phe
1. Acid hydrolysis only	2.00	1.84	0.85	2.04	1.00	1.94	1.02	1.70	0.93
2. Acid hydrolysis and DAO treatment	1.85	1.74	0.81	1.87	1.00	1.81	0.94	1.61	0.78

The degree of hydrolytic destruction of Thr (8%) and Ser (15%) residues found in Expt 1 was used to correct the values found for these residues in amino acid analyses (see footnote 9). The degree of racemization of the various residues caused by the acid hydrolysis, as estimated from Expt 2, was used to correct the results of Expt 4.

The protected tetradecapeptide (III Q 21–34) was hydrolyzed as usual and the

[9] Corrected for destruction during hydrolysis; see above.

[10] The solution was evaporated to dryness and the residue was dissolved in sodium phosphate buffer, pH 8.1, at a concentration of 10^{-3} *M*. To 0.5 ml of this solution D-amino acid oxidase (DAO) (0.7 mg; 0.013 enzyme units) in 0.5 ml buffer was added. The reaction vessel was flushed out with oxygen, stoppered, and kept at 37°C for 20 hr. The solution was evaporated to dryness at room temperature and the residue was dissolved in 0.01 *M* hydrochloric acid and subjected to amino acid analysis.

hydrolysate was subjected to amino acid analysis, with and without treatment with DAO, with the following results:

	Asp	Thr	Ser	Glu	Gly	Ala	Val	Ile	Phe
3. Acid hydrolysis only	1.98	1.86	0.87	2.11	1.00	2.02	0.91	1.89	0.93
4. Acid hydrolysis and DAO treatment	1.98	1.86	0.86	2.09	1.00	2.02	0.98	1.91	0.95

Cyclization of glutamine derivatives. (a) Z · Gln · ONp (500 mg) was refluxed for 48 hr in methanol (10 ml). Water was added to the cooled solution and the crystalline precipitate was recrystallized from aqueous methanol to give 270 mg (84% yield) of *α-benzyloxycarbonylaminoglutarimide* (2), mp 120–121°C. δ ($CDCl_3$): 8.85 (1H, s, D_2O exchangeable, imide NH); 7.37 (5H, s, C_6H_5); 5.78 (1H, d, urethane NH); 5.14 (2H, s, benzyl CH_2); 4.34 (1H, m, α-CH); 2.68 (2H, m, γ-CH_2); 2.38 (1H, br m, β-CH); 1.84 (1H, br m, β-CH). ν_{max} ($CHCl_3$): 3425 (NH str); 3370 (NH str); 2930 (CH str); 2860 (CH str); 1715 (br, imide and urethane CO str).

Anal. Calcd for $C_{13}H_{14}N_2O_4$: C, 59.5; H, 5.4; N, 10.7; *M*, 262. Found: C, 59.75; H, 5.4; N, 10.6; *m/e*, 262 (263, 264).

(b) A solution of the partially protected hexapeptide (III L 29–34) in acetic acid was kept at room temperature for 3 days. Evaporation and trituration of the residue with methanol gave Glp · Phe · Val · Ile · Asp(OBu^t) · Ala · NH · NH · Boc (3), ninhydrin negative, mp 230°C (dec.). δ[$(CD_3)_2SO$]: 8.84 (1H, s) and 7.85–8.40 (7H, complex) (D_2O exchangeable, NH's); 7.33 (5H, s, C_6H_5); 4.64 (2H, m), 4.30 (3H, m) and 4.00 (1H, m) (α-CH's); 2.5–3.0 (4H, complex, Asp β-CH_2 and Gln γ-CH_2); 1.6–2.2 (6H, complex, Gln and Phe β-CH_2's, Val and Ile β-CH's); 1.39 (18H, s, Bu^t's); 1.20 (5H, d, Ala β-CH_3 and Ile γ-CH_2); 0.84 (12H, d, Val and Ile CH_3's). The same compound (tlc, ir, nmr) was obtained by heating a solution of (III L 29–34) in dimethylformamide at 40°C.

Anal. Calcd for $C_{41}H_{64}N_8O_{11}$: C, 58.2; H, 7.6; N, 13.3. Found: C, 57.5; H, 7.8; N, 13.2.

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