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STUDIES ON CYTOCHROME *C*

XII. Synthesis of the Protected Undecapeptide (Sequence 66–76) and Pentadecapeptide (Sequence 66–80) of Horse Heart Cytochrome *c**

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This paper is part of a series on synthesis of suitably protected peptides covering the 66–104 sequence of horse heart cytochrome c. It describes the preparation, by conventional procedures, of a partially protected N^{α}-benzyloxycarbonyl-undecapeptide hydrazide corresponding to the sequence from 66 to 76 (Fragment F), which represents a building block for the synthesis of the entire 66–104 sequence.

Moreover, the preparation is described of a partially protected pentadecapeptide corresponding to the sequence region 66 to 80, which represents the key peptide for the semisynthesis of the same COOH-terminal sequence utilizing the natural 81–104 N^{ϵ}-trifluoroacetylated CNBr fragment.

A cyanogen bromide-mediated fragmentation of horse heart cytochrome *c* yields primarily the heme-peptide corresponding to the sequence 1–65 and the COOH-terminal segment 66–104 (Corradin & Harbury, 1970). The two fragments recombine to an almost fully active 1:1 complex in well-defined experimental conditions (Corradin & Harbury, 1971). The subsequent observation that Hse^{65}-cytochrome *c* is regenerated in the recombination process, by means of homoserine lactone-mediated peptide bond formation (Corradin & Harbury, 1974; Wilgus &

* The amino acid residues, except glycine, are of the L-configuration. For a simpler description the customary L-designation for individual amino acid residues is omitted. Standard abbreviations for amino acid derivatives and peptides are used according to the suggestion of the IUPAC-IUB Commission on Biochemical Nomenclature (1972) *Biochemistry* **11**, 1726–1732.

Additional abbreviations used: BuOH, butanol; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicycloexylurea; DCHA, dicyclohexylamine; DMF, dimethylformamide; EtOAc, ethyl acetate; EtOH, ethanol; HOBt, 1-hydroxybenzotriazole; HOAc, acetic acid; Me Morph, *N*-methylmorpholine; MeOH, methanol; OSu, *N*-hydroxysuccinimide ester; TEA, triethylamine; Tfa, trifluoroacetyl; THF, tetrahydrofuran; t.l.c., thin-layer chromatography.

Preliminary accounts of this work have been presented at *13th European Peptide Symposium* [Moroder, L., Borin, G., Filippi, B. & Marchiori, F. (1975) in *Peptides 1974* (Wolman, Y., ed.) pp. 1–8, J. Wiley and Sons, Toronto – Israel Universities press, Jerusalem], and *14th European Peptide Symposium* [Borin, G. Filippi, B., Moroder, L. & Marchiori, F. in *Peptides 1976*, (Loffet, A., ed.), pp. 233–238, Editions de l'Université de Bruxelles, Bruxelles].

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Stellwagen, 1974) at position 65, constitutes an attractive finding for a semisynthetic approach to the preparation of Hse[65]-horse heart cytochrome *c* and related analogues in view of structure-activity studies on this interesting family of evolutionarily differentiated haemoproteins. It may be worth noting that, except for tryptophan 59, all amino acids which have been involved in the proposed electron transfer mechanisms (Takano *et al.*, 1972; Salemme *et al.*, 1973) are localized on the COOH-terminal part of the molecule. Thus with synthetic 66–104 COOH-terminal peptides and analogues which contain amino acid substitutions at different positions of the sequence, selectively modified semisynthetic cytochrome *c* should become available, providing a means for a critical evaluation of the proposed electron transfer mechanisms. Moreover, detailed investigations on the nature of interactions with both partners in the respiratory chain, i.e. cytochrome oxidase and reductase, would be possible.

Tentatively, this promising approach has been applied to baker's yeast iso-l-cytochrome *c* – the object of our previous synthetic studies (Moroder *et al.*, 1973*a*) – , but so far it has failed because of the difficult CNBr selective cleavage at Met 69 of latter haemoprotein in the same experimental conditions elaborated for the fragmentation of horse cytochrome *c*. These negative preliminary results prompted us to conduct simultaneously some semisynthetic studies on horse heart cytochrome *c*. Therefore two routes were designed for the synthesis of the COOH-terminal fragment corresponding to sequence 66–104. The route for total synthesis was based on the preparation of three fragments F, G and H (Fig. 1) corresponding to the sequence 66–76, 77–87 and 88–104, respectively. The semisynthetic route was based on the preparation of a suitably protected subunit corresponding to the sequence 66–80 and its condensation via the azide procedure with the partially protected fragment 81–104 obtained by CNBr cleavage of N^{ϵ}-trifluoroacetylated horse cytochrome *c* (Fontana, A., unpublished results). The last strategy may be particularly useful for studying the functional and structural importance of the invariant sequence 70 to 80 in eukariotic cytochromes *c*.

The synthetic routes for both approaches were elaborated in view of the optimal utilization of intermediate products identical with those used in our previous studies on baker's yeast iso-l-cytochrome *c* (Moroder *et al.*, 1973 *b–d*, 1974). Thus the strategy of minimum side chain protection using a proven combination of protecting groups (Moroder *et al.*, 1975) was applied.

Synthesis of the partially protected fragments 66–76 and 66–80

Fig. 2 shows a scheme of the synthesis elaborated for the preparation of the partially protected N^{α}-benzyloxycarbonyl-N^{ϵ}-trifluoroacetyl-undecapeptide hydrazide (Sequence 66–76), fragment F for the synthesis of the horse heart cytochrome *c* sequence 66–104. Stepwise acylation of methyl γ-*tert*-butylglutamate (obtained from methyl *N*-benzyloxycarbonyl-γ-*tert*-butylglutamate (Klieger & Gibian, 1962)) via mixed anhydride procedure (Anderson *et al.*, 1967) with benzyloxycarbonylleucine (Losse & Demuth, 1961), *N*, *O*-dibenzyloxycarbonyl-tyrosine (Katchalski & Sela, 1953; Panneman

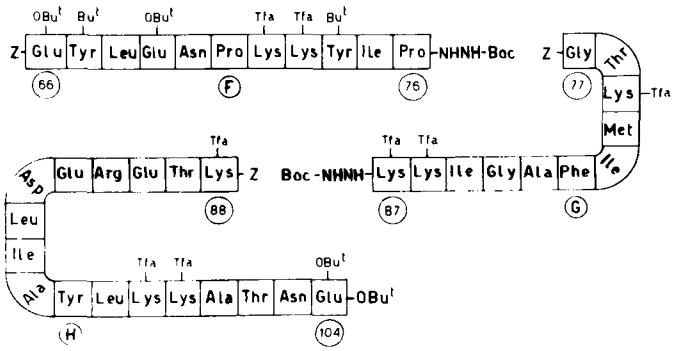

FIGURE 1
Scheme of synthesis of the COOH-terminal portion of horse heart cytochrome *c*.

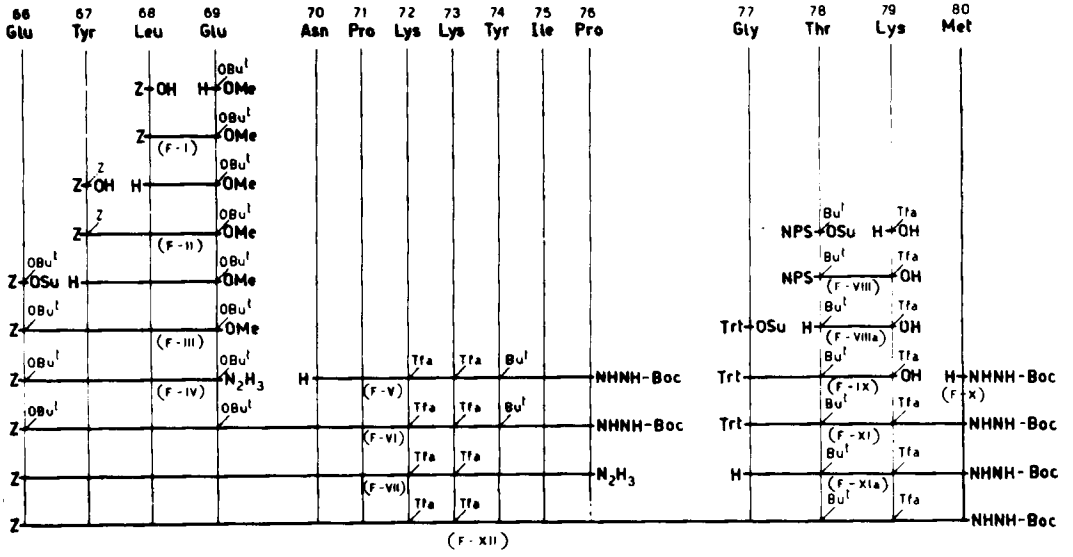

FIGURE 2

Scheme for the synthesis of the protected peptide derivative corresponding to sequence 66 to 76 (fragment F) and 66 to 80 of horse heart cytochrome *c*.

et al., 1959), and finally via the *N*-hydroxysuccinimido *N*-benzyloxycarbonyl-γ-*tert*-butylglutamate (Beacham *et al.*, 1971; Zabel & Zahn, 1965) led to the homogeneous tetrapeptide F-III in a good overall yield. Treatment of this compound with hydrazine hydrate and successive conversion of the resulting tetrapeptide hydrazide (F-IV) to the corresponding azide by the Rudinger method (Honzl & Rudinger, 1961) yielded the activated acylation reagent for the heptapeptide 70–76 free amine (Zabel & Zahn, 1965). The resulting homogeneous undecapeptide *tert*-butoxycarbonylhydrazide (F-VI) was partially deprotected by treatment with trifluoroacetic acid to produce fragment F (F-VII).

Difficulties arising in the synthesis of the tetrapeptide glycyl-*O*-*tert*-butylthreonyl-N^{ϵ}-trifluoroacetyllsyl-methionine *tert*-butoxycarbonylhydrazide (F-XIa) were overcome following the synthetic route shown in the scheme of Fig. 2. The tripeptide tritylglycyl-*O*-*tert*-butylthreonyl-N^{ϵ}-trifluoroacetyllysine [F-IX, prepared by stepwise acylation of N^{ϵ}-trifluoroacetyllysine (Anfinsen *et al.*, 1967) with *N*-hydroxysuccinimido N^{α}-*o*-nitrophenylsulfenyl-*O*-*tert*-butylthreoninate (Wünsch & Fontana, 1968) and, after removal of the *o*-nitrophenylsulfenyl group by thioacetamide treatment (Kessler & Iselin, 1966) with *N*-hydroxysuccinimido tritylglycinate (Anderson *et al.*, 1964)] was coupled with methionine *tert*-butoxycarbonylhydrazide (F-X) via DCC/*N*-hydroxybenzotriazole (König & Geiger, 1970, 1971) to yield the fully protected tetrapeptide F-XI. The use of the trityl (Helferich *et al.*, 1925) group at this stage of the synthesis allowed the selective removal, by 50% aqueous acetic acid treatment, of the N^{α}-protecting group in the presence of both the methionine residue and the acid labile *tert*-butoxycarbonyl at protection of the hydrazide. A final azide coupling step of fragment F (F-VII) with the tetrapeptide glycyl-*O*-*tert*-butylthreonyl - N^{ϵ} - trifluoroacetyllysyl - methionine *tert*-butoxycarbonylhydrazide (F-XII), as necessary intermediate for the semisynthetic approach, in satisfactory yield and homogeneous form, within the limits of the analytical methods.

EXPERIMENTAL PROCEDURES

In this series of papers concerning the synthesis of peptide derivatives, related to the amino acid sequence of the COOH-terminal portion of horse heart cytochrome *c*, the following materials, preparative and analytical methods are used.

Melting points were determined by the Tottoli's apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. Samples for elemental analysis were dried *in vacuo* over P_2O_5 at 60°. Acid hydrolyses were performed in constant boiling HCl for 22 h at 110° in evacuated vials. Thr and Tyr values are corrected for amino acid decomposition. AP-M digests were obtained as described by Hofmann *et al.* (1966). The amino acid analyses of the acid and enzymic hydrolysates were performed on a Carlo Erba 3A 27 or on a Jeol JLC-6AH amino acid analyser. Solvent systems for ascending t.l.c. on silica gel plates F-254 and DC cellulose F plates (from Merck A. G., Darmstadt, West Germany) were numbered as: I) 1-BuOH-HOAc-H_2O (3:1:1); II) 1-BuOH-pyridine-HOAc-H_2O (60:40:12:48); III) $CHCl_3$-HOAc-benzene (85–10–5); IV) H_2O-MeOH-pyridine (80:20:4); V) MeOH-benzene (2:8); VII) MeOH-$CHCl_3$ (15:85).

Amino acid derivatives and peptides were located by spraying the chromatograms with ninhydrin (Connel *et al.*, 1955) for free amino groups, with Sakaguchi reagent (Hamilton, 1960) for the guanido groups, with hydrazide reagent (Ertel & Horner, 1962) for the *C*-terminal hydrazide or protected hydrazide group and with a modified chlorine reagent (Barrolier, 1961) for all peptide derivatives.

Unless stated otherwise solvents were evaporated at a bath temperature of 30–40° under reduced pressure. The hydrogenations were performed at room temperature and atmospheric pressure in presence of 10% palladized charcoal. Routinely, the catalyst was removed by filtering the hydrogenation mixture through a bed of Filter Cel. In some cases spongy palladium was used as the catalyst.

Except when noted otherwise, after each condensation step the bulk of the solvent was evaporated and the residue distributed between EtOAc and H_2O. The organic layer was washed with 0.5 M citric acid, 0.5 M Na_2CO_3 and H_2O and dried (Na_2SO_4).

Yields are based on weight of vacuum dried (over P_2O_5) substance.

For column-chromatography, silica gel 0.05–0.2 mm (70–325 mesh ASTM, Merck A. G., Darmstadt, West Germany) was used. The ion exchange resin AG 1-X2 was purchased from Bio-Rad Laboratories, Richmond, California; U.S.A. The silica gel as well as ion exchange resins were preequilibrated with the appropriate solvents or solvent systems before use.

The analytical and physical data for new compounds which were characterized are listed in Table 1.

Synthesis of Fragment F (Position 67–69) Z-Tyr(Z)-Leu-Glu(OBu^t)-OMe (F-II). The dipeptide Z-Leu-Glu(OBu^t)-OMe (König & Geiger, 1970) (F-I) was synthesized by the condensation of the mixed anhydride prepared from Z-Leu-OH (Losse & Demuth; 1961) (4.11 g, 15.49 mmol) and isobutyl chloroformate (1.96 ml, 15.01 mmol) in THF (70 ml) in presence of Me Morph (1.68 ml, 15.49 mmol) with H-Glu (OBu^t)-OMe [obtained by hydrogenolysis of Z-Glu (OBu^t)-OMe (Klieger & Gibian, 1962); 5.17 g, 14.71 mmol]. The reaction mixture was worked up in the usual fashion and the oily product [5.50 g (80.5%), 11.83 mmol; R_f^I 0.95, R_f^{II} 0.9, R_f^{IV} 0.6] was hydrogenated in MeOH (100 ml) over spongy palladium. The catalyst was removed by filtration and the solution was evaporated to an oil at room temperature. To the ice-cold solution of the residue (R_f^I 0.8) in THF (50 ml) was added under stirring, a mixed anhydride prepared from Z-Tyr(Z)-OH (Katchalski & Sela 1953; Panneman *et al.*, 1959) (5.85 g, 13.01 mmol) in THF (70 ml) with Me Morph (1.43 ml 13.01 mmol) and isobutyl chloroformate (1.57 ml, 12.00 mmol) at −15° in 2 min. The reaction mixture was stirred for 2 h at ice bath temp. Standard work-up produced an oil which crystallized from ether; yield 5.30 g (58.8%).

(Positions 66–69) Z-Glu (OBu^t)-Tyr-Leu-Glu (OBu^t)-OMe (F-III). Compound F-II (5.00 g, 6.56 mmol) was hydrogenated in MeOH (100 ml) and HOAc (50 ml). The catalyst was filtered off and the filtrate evaporated to dryness. To the solution of the N^{α}-deprotected tripeptide (R_f^I 0.8) in THF (100 ml) Z-Glu (OBu^t)-OSu (Zabel & Zahn, 1965; Beacham *et al.*, 1971) (2.85 g, 6.56 mmol) and TEA (0.92 ml, 6.56 mmol) were added. The reaction mixture was kept overnight at room temp. The solvent was then evaporated and the residue dissolved in EtOAc. The solution was washed in the usual

TABLE 1

Analytical and other data on new compound[a]

	Compound (mol. formula; mol. wt.)		Microanalysis C	H	N	S	Melting point °C	Optical rotation (25°C) (conc., solvent)	T.l.c.[b]
F–II	Z[67–69] OMe	Calc.	64.6	6.75	5.5		120–122	–21.95°	0.9 (III); 0.8 (IV)
	($C_{41}H_{51}N_3O_{11}$; 761.87)	Found	64.3	6.6	5.5			(*c* = 1.08, MeOH)	
F–III	Z[66–69] OMe	Calc.	62.05	7.45	6.9		191–193	– 35.6°	0.55 (III); 0.5 (IV)
	($C_{42}H_{60}N_4O_{12}$; 812.96)	Found	62.0	7.45	6.85			(*c* = 1.05, MeOH)	
F–IV	Z[66–69] $NHNH_2$	Calc.	60.6	7.4	10.4		207–208	– 21.8°	0.8 (I); 0.85 (II)
	($C_{41}H_{60}N_6O_{11}$; 812.96)	Found	60.2	7.4	10.4			(*c* = 1.13, DMF)	0.4 (IV); 0.8 (V)
F–VI	Z[66–76] NHNH-Boc	Calc.	57.0	6.9	11.2		202–203	– 48.4°	0.9 (I); 0.65 (IV)
	($C_{95}H_{138}N_{16}O_{24}F_6$; 2002.24)	Found	56.8	6.9	10.9			(*c* = 0.975, DMF)	
F–VII	Z[66–76] $NHNH_2$ acetate							– 42.0°	0.7 (I); 0.55 (II)
								(*c* = 1.01, DMF)	
F–VIII	Nps[78–79] OH	Calc.	47.8	5.65	10.15	5.8		– 39.1°	0.95 (I); 0.9 (II)
	($C_{22}H_{31}N_4O_7SF_3$; 552.57)	Found	48.0	5.7	10.0	6.0		(*c* = 1.07, MeOH)	0.3 (IV); 0.5 (V)
	Nps[78–79] OH.DCHA	Calc.	55.6	7.4	9.5	4.4		– 37.5°	id.
	($C_{22}H_{31}N_4O_7SF_3.C_{12}H_{23}N$; 733.89)	Found	55.8	7.5	9.4	4.45		(*c* = 0.97, MeOH)	
F–VIII a	H[78–79] OH monohydrate	Calc.	46.0	7.2	10.0			+ 10.7°	0.75 (I); 0.8 (II)
	($C_{16}H_{28}N_3O_5F_3 \cdot H_2O$; 417.43)	Found	46.2	7.2	9.8			(*c* = 1.05, MeOH)	0.1 (IV)
F–IX	Trt[77–79] OH	Calc.	63.6	6.5	8.0			+ 39.6°	0.85 (II); 0.45 (IV)
	($C_{37}H_{45}N_4O_6F_3$; 698.78)	Found	64.0	6.6	7.9			(*c* = 1.015, MeOH)	0.5 (V)
F–X	H-Met-NHNH-Boc	Calc.	45.6	8.05	15.95	12.2	73	+ 15.5°	0.75 (I); 0.8 (II)
	($C_{10}H_{21}N_3O_3S$; 263.36)	Found	45.3	8.1	15.6	12.4		(*c* = 1.015, MeOH)	0.4 (IV); 0.6 (V)
F–XI	Trt[77–80] NHNH-Boc	Calc.	59.8	6.8	10.4		185–186	– 3.4°	0.4 (III); 0.6 (IV)
	($C_{47}H_{64}N_7O_8SF_3$; 944.13)	Found	59.7	7.0	10.1			(*c* = 0.995, MeOH)	
F–XI a	H[77–80] NHNH-Boc acetate	Calc.	47.3	7.15	12.9		148–150	– 20.5°	0.7 (I); 0.8 (II)
	($C_{28}H_{50}N_7O_8SF_3.C_2H_4O_2$; 761.86)	Found	47.2	7.2	12.7			(*c* = 1.04, MeOH)	0.2 (IV); 0.35 (V)
F–XII	Z[66–80] NHNH-Boc							– 37.9°	0.9 (I)
								(*c* = 0.475, DMF)	

[a] See text for amino acid composition.

[b] Solvents (in parentheses) are listed under Experimental Procedures.

fashion, dried and then concentrated to a small volume. The product precipitated on standing in the cold; yield 4.40 g (82.6%).

(Positions 66–69) Z-Glu (OBu^t)-Tyr-Leu-Glu (OBu^t)-NHNH$_2$ (F-IV). To a solution of F-III (4.11 g, 5.06 mmol) in MeOH (80 ml) $N_2H_4 . H_2O$ (5.06 ml) was added. The reaction mixture was kept for 3 days at room temp. The tetrapeptide hydrazide, which precipitated in the cold, was filtered off, washed with ice-cold MeOH and ether, and dried (H_2SO_4); yield 3.68 g (89.5%); amino acid ratios in acid hydrolysate: $Glu_{1.99}$, $Leu_{1.09}$, $Tyr_{0.93}$.

(Positions 66–76) Z-Glu (OBu^t)-Tyr-Leu-Glu (OBu^t)-Asn-Pro-Lys (Tfa)-Lys(Tfa)-Tyr(Bu^t)-Ile-Pro-NHNH-Boc (F-VI). 10% *tert*-Butyl nitrite (1.26 ml, 1.06 mmol) was added to a stirred solution, at $-15°$, of F-IV (0.78 g, 0.96 mmol) in DMF (25 ml) containing 4.27 N HCl in dioxane (0.90 ml, 3.84 mmol). The solution was stirred for 10 min at $-15°$ and then cooled to $-60°$ and neutralized with TEA (0.54 ml, 3.84 mmol). To this solution of the azide the ice-cold solution of H-Asn-Pro-Lys (Tfa)-Lys(Tfa)-Tyr(Bu^t)-Ile-Pro-NHNH-Boc acetate (Moroder *et al.*, 1973*b*) (0.99 g, 0.77 mmol) and TEA (0.11 ml, 0.77 mmol) in DMF (10 ml) was added. The reaction mixture was kept under stirring at 5° for 2 days and then evaporated to a small volume. The solution was diluted with MeOH and the crude product obtained on addition of H_2O was reprecipitated from MeOH with H_2O and from MeOH/EtOAc (1:1) with ether; yield 1.09 g (71.2%); amino acid ratios in acid hydrolysate: $Asp_{0.96}$, $Glu_{1.95}$, $Pro_{1.96}$, $Ile_{0.96}$, $Leu_{1.03}$, $Tyr_{2.08}$, $Lys_{2.02}$.

(Positions 66–76) Z-Glu-Tyr-Leu-Glu-Asn-Pro-Lys (Tfa)-Lys (Tfa)-Tyr-Ile-Pro-NHNH$_2$ acetate (F-VII). A solution of F-VI (0.68 g, 0.34 mmol) in trifluoroacetic acid (2 ml) was kept for 1 h at room temp. The bulk of the solvent was then evaporated, the residue dissolved in MeOH (20 ml) and precipitated with ether. The crude product was dissolved in t.l.c. solvent system I and chromatographed on a silica gel column (2.5 × 50) using the same solvent as an eluent. Fractions of 10 ml each were collected and analysed by t.l.c. Tubes containing single-spot material were pooled, concentrated and precipitated with ether; yield 0.31 g (50.8%); amino acid ratios in acid hydrolysate: $Asp_{1.00}$, $Glu_{2.03}$ $Pro_{2.11}$, $Ile_{0.98}$, $Leu_{1.01}$, $Tyr_{1.96}$, $Lys_{2.04}$.

Synthesis of the Sequence 66–80

(Positions 78–79) Nps-Thr (Bu^t)-Lys (Tfa)-OH (F-VIII). To a solution of H-Lys (Tfa)-OH (Anfinsen *et al.*, 1967) (2.56 g, 10.57 mmol) and TEA (1.49 ml, 10.57 mmol) in DMF (50 ml) Nps-Thr (Bu^t)-OSu (Wünsch & Fontana, 1968) (3.00 g, 7.05 mmol) was added. The reaction mixture was kept overnight at room temp. and then evaporated to dryness. The residue was distributed between ice-cold H_2O, acidified with precooled 0.5 M citric acid, and ice-cold EtOAc. The aqueous layer was extracted two more times with EtOAc, then the combined organic layers were washed with 0.5 M citric acid and H_2O, and dried (Na_2SO_4). The solution was evaporated to dryness and the oil was dissolved in ether. The pure product was isolated by addition of petroleum ether; 3.85 g (98.8%). The dipeptide was also characterized as dicyclohexylamine salt.

(Positions 78–79) H-Thr (Bu^t)-Lys (Tfa)-OH monohydrate (F-VIIIa). A solution of F-VIII (3.64 g, 6.58 mmol) and tioacetamide (1.48 g, 19.74 mmol) in MeOH-HOAc (60 ml, 5:1 v/v) was stirred at room temp. for 45 min. The yellow precipitate was removed by filtration and the filtrate was evaporated. The solution of the oily residue in 5% acetic acid was extracted with EtOAc (2 × 100 ml) and the organic layers reextracted with 5% acetic acid. The combined aqueous layers were evaporated to dryness at room temp. The crude product obtained from EtOH on addition of ether was dissolved in 1-BuOH-H_2O-HOAc (3:1:1) (12 ml) and applied to a silica gel column (5.5 × 60 cm). The column was eluted with the same solvent at a flow rate of 40 ml/h collecting fractions of 10 ml each. The fractions were analysed by t.l.c. in solvent system I. The fractions containing single-spot material were pooled and concentrated to a small volume. The product was obtained on addition of ether; yield 1.82 g (66.3%).

(Positions 77–79) Trt-Gly-Thr (Bu^t)-Lys (Tfa)-OH (F-IX). To a solution of F-VIIIa (1.53 g, 3.67 mmol) and TEA (0.52 ml, 3.67 mmol) in DMF (50 ml) Trt-Gly-OSu (Anderson *et al.*, 1964) (1.82 g, 4.40 mmol) was added. After 12 h at room temp. the bulk of the solvent was evaporated and the residue distributed between EtOAc and ice-cold 0.5 M citric acid. The organic layer was washed with H_2O and dried (Na_2SO_4). The solution was concentrated and the product was obtained on addition of petroleum ether; yield 1.98 g (77.2%).

Methionine tert-*butoxycarbonylhydrazide (F-X).* A solution of Tfa-Met-NHNH-Boc (Wünsch *et al.*, 1965) (6.61 g, 18.40 mmol) in EtOH (60 ml) and 2 N NaOH (18.40 ml, 36.80 mmol) was kept at 50° for 45 min, then the solution was diluted with H_2O (200 ml) and extracted with EtOAc (4 x 150 ml). The combined organic layers were extracted with ice-cold 0.5 M citric acid and H_2O. The aqueous extracts were neutralized with 2 N NaOH and the product extracted with EtOAc (4 x 150 ml). The combined organic layers were washed with H_2O, dried (Na_2SO_4) and concentrated to a small volume. The pure product was precipitated on addition of petroleum ether; yield 2.93 g (60.5%).

(Positions 77–80) Trt-Gly-Thr (Bu^t)-Lys (Tfa)-Met-NHNH-Boc (F-XI). DCC (0.317 g, 1.536 mmol) and HOBt (0.226 g, 1.676 mmol) were added to a stirred solution of F-IX (0.98 g, 1.397 mmol) and F-X (0.441 g, 1.676 mmol) in THF (50 ml) at −10°. The reaction mixture was stirred for 2 h at −10° and overnight at 5°. The precipitate (DCU) was removed by filtration and the solution evaporated to dryness. The residue was dissolved in EtOAc and the solution, washed in the usual fashion, was concentrated. The product, obtained on addition of ether, was reprecipitated from EtOAc with ether; yield 0.79 g (54.6%); amino acid ratios in acid hydrolysate: $Thr_{1.03}$, $Gly_{1.00}$, $Met_{0.97}$, $Lys_{0.98}$.

(Positions 77–80) H-Gly-Thr (Bu^t)-Lys (Tfa)-Met-NHNH-Boc acetate (F-XIa). Compound F-XI (1.22 g, 1.29 mmol) was treated for 3 min with boiling 50% HOAc; then the resulting solution was evaporated at room temp. to dryness and the residue was triturated with ether. The crude product was dissolved in solvent system I (10 ml) and applied to a silica gel column (5.5 x 60 cm), which was eluted with the same solvent at a flow rate of 48 ml/h. Fractions of 12 ml were collected and analysed by t.l.c. Fractions from 75 to 91 were pooled and evaporated at room temp. to a small volume. The pure product was isolated on addition of ether; 0.72 g (73.3%).

(Positions 66–80) Z-Glu–Tyr-Leu-Glu-Asn-Pro-Lys (Tfa)-Lys (Tfa)-Tyr-Ile-Pro-Gly-Thr (Bu^t)-Lys (Tfa)-Met-NHNH-Boc (F-XII). To a solution of F-VII (0.343 g, 0.191 mmol) in DMF (10 ml) at −15° containing 4.27 N HCl in dioxane (1.79 ml of a 10% solution in DMF; 0.764 mmol) 0.25 ml of a 10% *tert*-butylnitrite solution in DMF (0.210 mmol) were added with stirring. The mixture was stirred for 10 min, cooled to −60° and neutralized with TEA (1.34 ml of a 10% solution in DMF; 0.955 mmol). To this azide solution an ice-cold solution of F-XIa (0.291 g, 0.382 mmol) in DMF (3 ml) and TEA (0.54 ml of a 10% solution in DMF; 0.382 mmol) was added. The reaction mixture was kept under stirring at 5° for 2 days and then evaporated to a small volume. The solid obtained on additon of H_2O was collected, dissolved in l-BuOH equilibrated with 5% HOAc and distributed repeatedly with 5% acetic acid equilibrated with l-BuOH. The organic layers were combined and evaporated to dryness. The residue was dissolved in MeOH and the product precipitated with EtOAc. The final purification was achieved by chromatography on a silica gel column (3.5 x 50 cm) eluted with solvent system I (flow rate 40 ml/h; fraction 10 ml) and the fractions containing pure product were collected and concentrated. The product was finally precipitated with ether; yield 0.314 (68.4%); amino acid ratios in acid hydrolysate: $Asp_{0.97}$, $Thr_{0.98}$, $Glu_{1.94}$, $Pro_{2.08}$, $Gly_{0.94}$, $Met_{0.93}$, $Ile_{0.99}$ $Leu_{1.03}$, $Tyr_{2.09}$, $Lys_{2.96}$.

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