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SYNTHESIS OF HUMAN β-ENDORPHIN IN SOLUTION USING BENZYL-TYPE SIDE CHAIN PROTECTIVE GROUPS

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A solution synthesis of human β-endorphin (β-EP) was carried out by condensation of protected peptide segments bearing N^{α}*-tert.-butyloxycarbonyl groups and benzyl-derived groups for the protection of functionalities in amino acid side chains. Five intermediate segments were assembled in a stepwise manner starting at the carboxyl terminus. Thus, the segment of sequence region (27–31) was coupled to segment (22–26) by the azide method. Segment (19–21) was incorporated into the growing chain by azide coupling, and segment (10–18) by dicyclohexylcarbodiimide coupling in the presence of 1-hydroxybenzotriazole (DDC–HOBt). Solubility problems in condensing the ensuing 22-peptide with segment (1–9) by DDC–HOBt were overcome by using a dimethylformamide-phenol mixture as a solvent. Protecting group cleavage by Na in liquid* NH_3 *was much superior to liquid HF which gave rise to many decomposition products. Homogeneous* β_h*-EP indistinguishable from authentic material in physiochemical and biological properties, was obtained in a single preparative reversed phase liquid chromatographic step after protecting group cleavage.*

Key words: N^{α}-Boc and benzyl-derived side chain protection; deprotection by Na-liq. NH_3; purification by reversed phase HPLC; segment condensation in solution.

The discoveries of endogenous opioid peptides, i.e. the enkephalins (Hughes *et al.*, 1975) and the endorphins from pituitary glands of several mammalian species (see reviews: Li, 1977, 1978), and observations of central nervous system effects and behavioral changes in laboratory animals (e.g. Bloom *et al.*, 1976; Jacquet & Marks, 1976) were soon followed by the isolation, structure determination and solid phase synthesis of human β-endorphin (β_h-EP) by Li *et al.* (1976, 1977). Several other syntheses by solid phase techniques (Atherton

Abbreviations are mostly those of the IUPAC-IUB Commission. AcOH, acetic acid; β_h-EP, human β-endorphin; Boc, *tert.*-butyloxycarbonyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DCU, *N,N'*-dicyclohexylurea; DMF, dimethylformamide; DMSO, dimethylsulfoxide; Et_3N, triethylamine; Et_2O, diethylether; EtOAc, ethyl acetate; EtOH, ethanol; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; HPLC, high performance liquid chromatography; MeOH, methanol; OBzl, benzyl ester; OPfp, pentafluorophenyl ester; OSu, *N*-hydroxysuccinimide ester, Pyr, pyridine, THF, tetrahydrofuran; Z, benzyloxcarbonyl.

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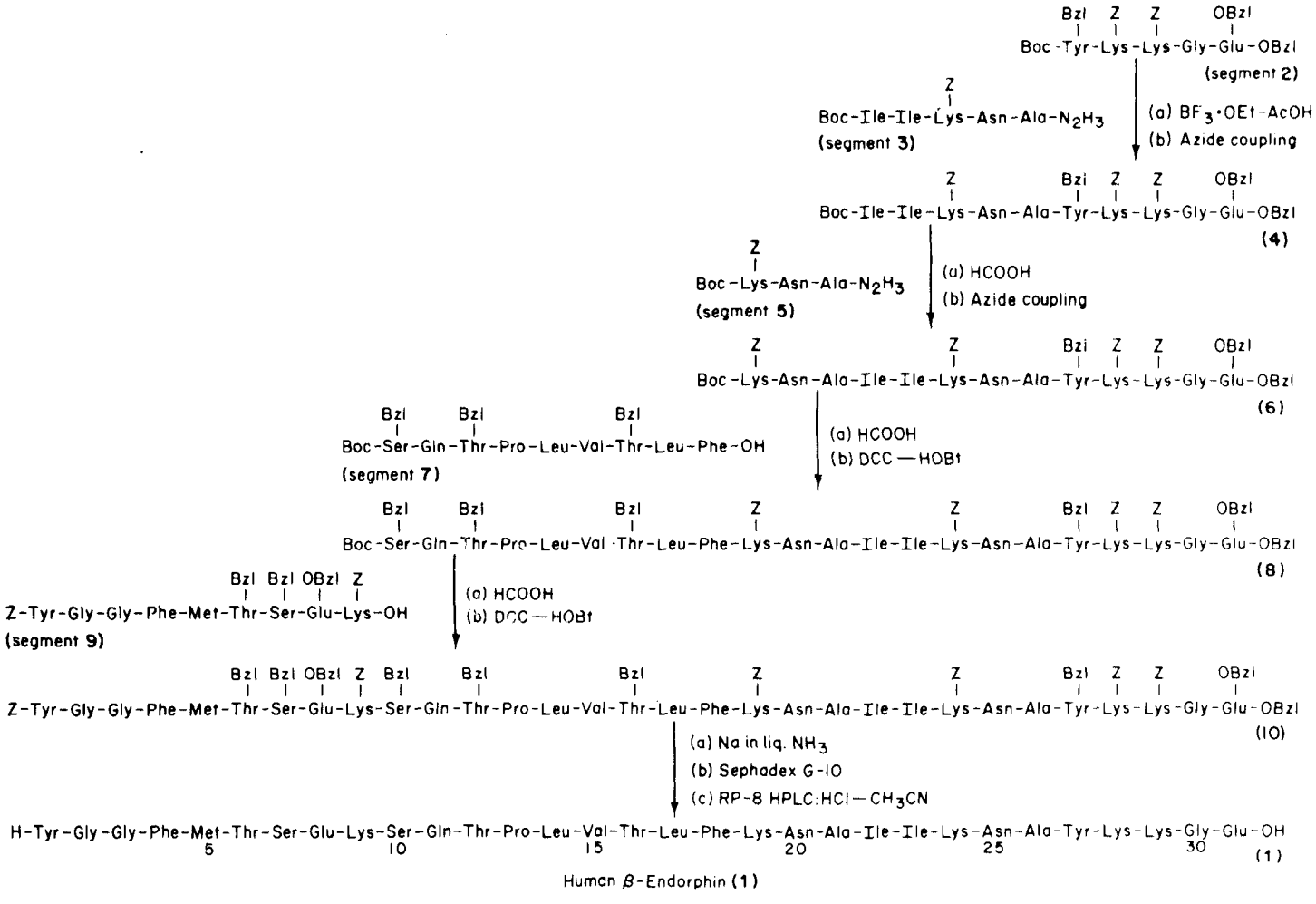
Scheme 1


Boc-Tyr-Lys-Lys-Gly-Glu-OBzl
(segment 2)
Boc-Ile-Ile-Lys-Asn-Ala-N2H3
(segment 3)
(a) BF3·OEt-AcOH
(b) Azide coupling
Boc-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OBzl
(4)
Boc-Lys-Asn-Ala-N2H3
(segment 5)
(a) HCOOH
(b) Azide coupling
Boc-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OBzl
(6)
Boc-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-OH
(segment 7)
(a) HCOOH
(b) DCC—HOBt
Boc-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OBzl
(8)
Z-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-OH
(segment 9)
(a) HCOOH
(b) DCC—HOBt
Z-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OBzl
(10)
(a) Na in liq. NH3
(b) Sephadex G-10
(c) RP-8 HPLC:HCl—CH3CN
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
(1)
Human β-Endorphin (1)

et al., 1977, 1978; Coy *et al.*, 1977; Segal *et al.*, 1977) and in solution (Kubota *et al.*, 1978; Nishimura *et al.*, 1978) have since been reported.

This paper described details of a solution synthesis of human β-EP (**1**) which was disclosed previously in a short communication (Tzougraki *et al.*, 1978). Stepwise condensation of five intermediate protected peptide segments, as shown in Scheme 1, provided a 31-peptide (**10**) with benzyl-type side chain protective groups. The synthetic approach differed substantially from those of Kubota *et al.* (1978) and Nishimura *et al.* (1978) in the nature of protective groups and their cleavage, the selection of intermediate protected segments and the purification procedures used for the final product.

In this synthesis, the *tert.*-butyloxycarbonyl (Boc) group (Anderson & McGregor, 1957; McKay & Albertson, 1957) was used for the temporary protection of the α-amine groups. Maximum protection of all side chain functionalities was affected by N^{ω}-benzyloxycarbonyl (Bergmann & Zervas, 1932), benzyl ester (Miller & Waelsch, 1952) and benzyl ether groups (Wünsch & Jentsch, 1964). The stepwise assembly of the 31-peptide chain (see Scheme 1) was carried out by utilizing intermediate protected segments of the following sequence regions: 1–9, 10–18, 19–21, 22–26 and 27–31. Following protective group cleavage from the final 31-peptide by Na in liquid ammonia the ensuing crude β_h-EP was purified by reversed phase liquid chromatography in up to 400-mg column loads (Gabriel *et al.*, 1979). Homogeneous human β-endorphin, indistinguishable from authentic material (Li *et al.*, 1976, 1977), was obtained.

For the assembly of the 31-peptide chain, the COOH-terminal protected pentapeptide segment-(27–31), Boc-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl (**2**), was prepared by dicyclohexylcarbodiimide and *N*-hydroxysuccinimide-mediated coupling (Weygand *et al.*, 1966; Wünsch & Drees, 1966) of Boc-Tyr(Bzl)-Lys(Z)-Lys(Z)-OH (**2b**) with HCl × H-Gly-Glu(OBzl)-OBzl, obtained from Boc-Gly-Glu(OBzl)-OBzl (**2c**) by treatment with 4 N HCl in tetrahydrofuran. Compound **2b** was synthesized by reaction of Boc-Tyr(Bzl)-OPfp (Kisfaludy & Nyeki, 1975) with HCOOH × H-Lys(Z)-Lys(Z)-OH (**2a**), which was obtained from Boc-Lys(Z)-Lys(Z)-OH (Wang *et al.*, 1979) by treatment with formic acid (Halpern & Nitecki, 1967). Compound **2c** was synthesized from H-Glu(OBzl)-OBzl (Shields *et al.*, 1961) and Boc-Gly-OSu (Anderson *et al.*, 1964).

Intermediate protected segment-(22–26), Boc-Ile-Ile-Lys(Z)-Asn-Ala-N_2H_3 (**3**), was obtained by dicyclohexylcarbodiimide and 1-hydroxybenzotriazol-mediated coupling (König & Geiger, 1972, 1973) of Boc-Ile-Ile-OH (**3a**) with H-Lys(Z)-Asn-Ala-OBzl [produced from Boc-Lys(Z)-Asn-Ala-OBzl (**3c**) by treatment with boron trifluoride etherate (Hiskey & Adams, 1966)] and hydrazinolysis of the ensuing Boc-pentapeptide benzyl ester (**3d**). Compound **3a** was prepared by treatment of H-Ile-OH with Boc-Ile-OSu (Anderson *et al.*, 1964; Ondetti *et al.*, 1970). For the synthesis of **3c**, Boc-Asn-OH (Schröder & Klieger, 1964) was coupled with H-Ala-OBzl (Erlanger & Brand, 1951) with the use of dicyclohexylcarbodiimide, and the ensuing Boc-Asn-Ala-OBzl (**3b**) treated with HCl tetrahydrofuran for cleavage of the Boc group and subsequently with Boc-Lys(Z)-OPfp (Kisfaludy *et al.*, 1973).

The protected tripeptide segment-(19–21), Boc-Lys(Z)-Asn-Ala-N_2H_3 (**5**) was obtained by hydrazinolysis of the corresponding benzyl ester **3c**.

Intermediate nonapeptide segment-(10–18), Boc-Ser(Bzl)-Gln-Thr(Bzl)-Pro-Leu-Val-Thr(Bzl)-Leu-Phe-OH (**7**) was produced by azide coupling (Honzl & Rudinger, 1961; see also Meienhofer, 1979) from Boc-Ser(Bzl)-Gln-Thr(Bzl)-Pro-Leu-Val-Thr(Bzl)-N_2H_3 (**7h**) and Boc-Leu-Phe-OH (**7i**) after Boc group cleavage with HCl in tetrahydrofuran. Compound **7i** was synthesized by treatment of H-Phe-OH with Boc-Leu-OPfp (Kisfaludy *et al.*, 1973). The benzyl ester precursor (**7g**) of the hepapeptide hydrazide (**7h**) was obtained by a dicyclohexylcarbodiimide and hydroxybenzotriazole mediated coupling of Boc-Ser(Bzl)-Gln-OH from (**7f**) and H-Thr(Bzl)-Pro-Leu-Val-Thr(Bzl)-OBzl (produced by Boc group cleavage with HCl in tetrahydrofuran from the Boc-pentapeptide benzyl ester **7e**). Compound **7f** resulted from a reaction of Boc-Ser(Bzl)-OSu (Laufer & Blout, 1967) with H-Gln-OH. Compound **7e** was synthesized by a dicyclohexylcarbodiimide and *N*-hydroxysuc-

cinimide mediated coupling of Boc-Thr(Bzl)-Pro-OH (**7d**) and the tripeptide benzyl ester obtained from Boc-Leu-Val-Thr(Bzl)-OBzl (**7b**) by Boc group cleavage with HCl in tetrahydrofuran. Compound **7d** was prepared by treatment of Pro-OH with Boc-Thr(Bzl)-OSu (**7c**). For the synthesis of **7b**, Boc-Leu-OH (Anderson & McGregor, 1957) and H-Val-Thr(Bzl)-OBzl, obtained from the Boc protected precursor **7a**, were coupled by dicyclohexylcarbodiimide. Compound **7a** was produced by treatment of H-Thr(Bzl)-OBzl (Mizoguchi *et al.*, 1968) with Boc-Val-OSu (Anderson *et al.*, 1964).

The NH_2-terminal protected nonapeptide segment-(1–9), Z-Tyr-Gly-Gly-Phe-Met-Thr(Bzl)-Ser(Bzl)-Glu(OBzl)-Lys(Z)-OH (**9**) was synthesized from benzyloxycarbonyl enkephalin hydrazide, Z-Tyr-Gly-Gly-Phe-Met-N_2H_3 (**9e**) (produced by hydrazinolysis of the corresponding benzyl ester, **9d**) via azide coupling with H-Thr(Bzl)-Ser(Bzl)-Glu(OBzl)-Lys(Z)-OH [which was obtained from the corresponding Boc protected precursor (**9c**) by treatment with boron trifluoride etherate (Hiskey & Adams, 1966)]. Compound **9c** was prepared by treatment of H-Glu(OBzl)-Lys(Z)-OH (derived from Boc-Glu(OBzl)-Lys(Z)-OH (**9a**) by Boc group cleavage with BF_3 • etherate) with Boc-Thr(Bzl)-Ser(Bzl)-OSu (**9b**). Compound **9a** was produced by reaction of Boc-Glu(OBzl)-OSu (Nakajima & Okawa, 1973) with H-Lys(Z)-OH (Ledger & Stewart, 1965; Costopanagiotis *et al.*, 1968) and compound **9b** by treatment of H-Ser(Bzl)-OH (Hayakawa *et al.*, 1966) with Boc-Thr(Bzl)-OSu and conversion of the ensuing dipeptide derivative to its *N*-hydroxysuccinimide ester. A sample of segment **9** was treated with hydrazine in the presence of dicyclohexylcarbodiimide and 1-hydroxybenzotriazole (Wang *et al.*, 1978) to produce the protected nonapeptide hydrazide (**9f**).

The assembly of the 31-residue protected peptide chain (**10**) from the five intermediates, **2**, **3**, **5**, **7**, and **9**, starting from the COOH-terminus is shown in Scheme 1. For the synthesis of decapeptide **4**, Boc-Ile-Ile-Lys(Z)-Asn-Ala-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl, the pentapeptide hydrazide **3**, in dimethylformamide, was converted (Honzl & Rudinger, 1961; see also Meienhofer, 1979) to the azide and coupled with the pentapeptide amine obtained from **2** by Boc group cleavage with borontrifluoride etherate in glacial acetic acid (Hiskey & Adams, 1966). Decapeptide **4** was obtained as a colorless powder in 69% yield, with a ratio of diagnostic amino acids (Beacham *et al.*, 1971) of $Asp_{1.0}$, $Glu_{1.0}$. To prepare the 13-peptide **6**, Boc-Lys(Z)-Asn-Ala-Ile-Ile-Lys(Z)-Asn-Ala-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl, the protected tripeptide azide prepared from intermediate segment **5** was coupled in a dimethylformamide–dimethylsulfoxide solution with decapeptide amine resulting from treatment of compound **4** with formic acid (Halpern & Nitecki, 1967). The 13-peptide was obtained in 82.5% yield. Synthesis of the 22-peptide, **8**, Boc-Ser(Bzl)-Gln-Thr(Bzl)-Pro-Leu-Val-Thr(Bzl)-Leu-Phe-Lys(Z)-Asn-Ala-Ile-Ile-Lys(Z)-Asn-Ala-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl, was carried out by dicyclohexylcarbodiimide and hydroxybenzotriazole mediated preactivation coupling (König & Geiger, 1973) of the protected intermediate nonapeptide acid, **7**, with the 13-peptide amine, prepared from **6** by Boc group cleavage with formic acid. Compound **8** was isolated in 83% yield, with diagnostic amino acid values of $Val_{0.98}$, $Gly_{1.00}$. To prepare the final protected 31-peptide, **10**, Z-Tyr-Gly-Gly-Phe-Met-Thr(Bzl)-Ser(Bzl)-Glu(OBzl)-Lys(Z)-Ser(Bzl)-Gln-Thr(Bzl)-Pro-Leu-Val-Thr(Bzl)-Leu-Phe-Lys(Z)-Asn-Ala-Ile-Ile-Lys(Z)-Asn-Ala-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl, the dicyclohexylcarbodiimide and hydroxybenzotriazole preactivation coupling of the protected nonapeptide acid, **9**, and the 22-peptide-amine, produced from compound **8** by formic acid treatment, had to be carried out in a 1:1 mixture of dimethylformamide and phenol* because of the limited solubility of the 22-peptide amine in other solvents including DMSO. Compound **10** was obtained as a colorless powder in 82% yield, with diagnostic amino acid values of $Met_{1.12}$, $Val_{0.93}$.

The cleavage of all 15 benzyl-type protective groups from **10** was initially carried out by

* The 1:1 phenol–DMF mixture can be cooled and remains liquid even below −5° which is an advantage over the use of pure phenol as a solvent (Geiger *et al.*, 1969) which requires working at temperatures above 35°.

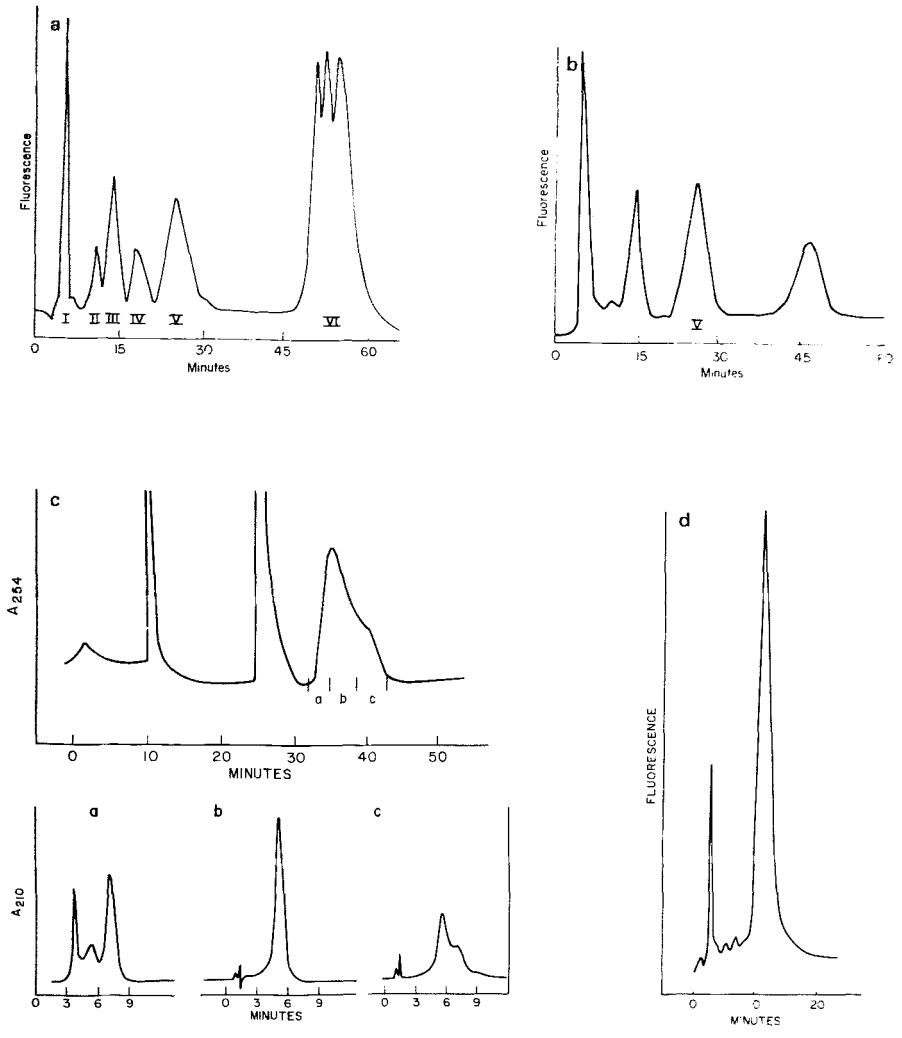


FIGURE 1

(a) HPLC purification of synthetic human β-endorphin (1) after liquid hydrogen fluoride deblocking, 76 mg, RP-18 0.9 X 50 cm Partisil ODS-2 column, Pyr–AcOH–CH_3CN-iC_3H_7OH–H_2O (5.9:1.0:13:13:66.2, v/v), isocratic 4 ml/min, *o*-phthalaldehyde–fluorescence monitoring, 4-ml fractions. I: void volume; II, III: probably decomposed material; IV: [Met(O)^5]-EP; V: β_h-EP; VI: probably incompletely deprotected. (b) HPLC purification of synthetic human β-endorphin (1) after deblocking with Na in liquid NH_3, chromatographic conditions as in (A). (c) *Top:* HPLC purification of synthetic human β-endorphin (1) (Na in liquid NH_3 deblocking), 150 mg, using a "u.v.-clear" solvent system: 0.01 N HCl modified with acetonitrile gradient (for details, see Experimental part), RP-8 3.7 X 44 cm LiChroprep Lobar column, 10 ml/min, u.v. monitoring (254 nm), 20 ml fractions. *Bottom:* Analytical HPLC of individual fractions (a, b, c, in top chromatogram) to determine pooling of pure center fractions (b) and contaminated shoulder fractions (a, c). System was RP-18 0.4 X 30 cm Waters Bondapak C18 column, 28% CH_3CN in 0.1 N HCl, isocratic, 2 ml/min, u.v. monitoring (210 nm). (d) Analytical HPLC of purified human β-endorphin (1), 80 μg, RP-8 0.46 X 15 cm ES Industries column, 1 ml/min solvent and monitoring system as in (A), showing a single symmetrical peak at K′ = 2.8.

treatment with anhydrous hydrogen fluoride (Sakakibara, 1971 [review]; Sakakibara & Shimonishi, 1965; Sakakibara *et al.*, 1967) in the presence of anisole and diethyl sulfide. Typical workup (see e.g. Watanabe *et al.*, 1975): i.e. evaporation of HF, dissolution of residue in 0.5 M AcOH, extraction of ether, lyophilization, Sephadex G-15 gel filtration using 0.5 M AcOH, yielded 60–84% of a white lyophilizate in several experiments. This crude material was purified to homogeneity by single preparative high performance liquid chromatography (Fig. 1a) on a reversed phase 0.9 × 500 cm Partisil ODS-2 column using a volatile buffer system. However, recoveries of human β-endorphin (**1**) did not exceed 13%. The HPLC elution diagram Fig. 1A indicated the presence of both degradation products eluting before β_h-EP and more hydrophobic material after β_h-EP that might possibly originate from incomplete protective group cleavage. Literature evidence for destructive effects of liquid HF on peptides and proteins†, side effects due to carbonium ion formation during protective group cleavage**, and incomplete deprotection†† is abundant and calls for a critical reassessment of the usefulness of this reagent for protective group cleavage from larger synthetic peptides.

Protective group cleavage by sodium in liquid ammonia (Sifferd & du Vigneaud, 1935; Roberts, 1954) was then examined***. Treatment of a solution of the protected 31-residue peptide, **10**, in refluxing anhydrous liquid NH_3 with 5- to 10-fold excess sodium for 30–60 min followed by evaporation and desalting on Sephadex G-10 (or G-15) provided crude material, **1b**, in 65–85% yield, which exhibited a considerably less complex HPLC elution profile, Fig. 1b, than that observed after liquid HF cleavage (Fig. 1a). Purified β_h-EP was obtained in 20–30% yield. Because of the superior results obtained by reductive protecting group cleavage, the sodium in liquid ammonia procedure was subsequently used for the deprotection of the 31-peptide **10** in up to 1-g batches.

Purification of crude products, **1a** or **1b**, to homogeneity in single chromatographic runs was initially carried out by C-18 reversed-phase liquid chromatography on a 0.9 × 50 cm column with 10 μ ODS-2 packing. A salt-free solvent system was developed, consisting of aq. pyridine–acetic acid with acetonitrile–isopropanol mixtures as a modifier (Meienhofer *et al.*, 1979), which can be removed by lyophilization of pooled fractions to provide directly the desired product, thus eliminating the need for desalting after HPLC. Loads of up to 100 mg of crude material were processed. Although the columns were heavily overloaded under these conditions and baseline separations from impurities close to the main peak were not always obtained, careful cutting of the center fractions of the product peak provided homogeneous β_h-endorphin. Product remaining in the ascending and descending parts of the peak were pooled from several chromatographic runs and rechromatographed to yield additional homogeneous β_h-endorphin. The resolving power and speed of these operations and of similar HPLC systems (Rubinstein *et al.*, 1979) compared with those of conventional chroma-

† Loss of biological activity upon exposure to liquid HF has been reported, amongst others, for: α-MSH (Lenard & Hess, 1964), native Staphylococcal nuclease (Anfinsen *et al.*, 1967), reduced and reoxidized somatotropin (Li & Yamashiro, 1970), reduced and reoxidized ribonuclease A (Gutte & Merrifield, 1971), reduced and reoxidized lyozyme (Sharp *et al.*, 1973), native acyl carrier protein (Marshall *et al.*, 1973).

** Observed side reactions during protective group cleavage by liquid HF include: N → O peptidyl shift [Ser, Thr] (Sakakibara *et al.*, 1962; Iwai & Ando, 1967), Met peptide bond cleavage (Lenard & Hess, 1964), Gln and Asn deamination (Robinson *et al.*, 1970), Tyr C-alkylation (Erickson & Merrifield, 1973; Engelhard & Merrifield, 1978), γ-Glu acylcarbonium ion formation (Feinberg & Merrifield, 1975; Sano & Kawanishi, 1975), Asp(OR) cyclic imide formation (Wang *et al.*, 1974; Yang & Merrifield, 1976; Tam *et al.*, 1979), product aggregation with Cys(MeOBzl) (Sheppard, 1976), methionine *tert.*-butylation (Noble *et al.*, 1976), Trp *tert.*-butylation (Wünsch *et al.*, 1977), see also Merrifield *et al.* (1979).

†† Incomplete protective group cleavage by liquid HF was reported for: insulin chains containing Cys(MeOBzl) (Berndt, 1976), urogastrone (Camble & Petter, 1976), gastric inhibitory polypeptide, GIP (Ogawa *et al.*, 1976).

*** This alternative procedure was not originally planned or considered because of the presence of 3 ammonolysable benzyl ester group in the protected 31-peptide (**10**).

TABLE 1

Amino acid analyses of synthetic human β-endorphin

Acid hydrolysate[a]			Enzyme hydrolysate[b]		
Amino acid	Expected	Found	Amino acid	Expected	Found[c]
Lys	5	5.00	Lys	5	5.33
Asp	2	2.08	Asp	0	0
Thr	3	2.97	Thr + Ser + Asn + Gln	8	7.51
Ser	2	1.90	Ser	0	
Glu	3	3.13	Glu	2	2.26
Pro	1	0.95	Pro	1	0.82
Gly	3	3.20	Gly	3	2.67
Ala	2	2.08	Ala	2	2.07
Val	1	0.95	Val	1	1.13
Met	1	1.03	Met	1	0.98
Ile	2	1.70[d]	Ile	2	2.02
Leu	2	2.05	Leu	2	2.16
Tyr	2	1.92	Tyr	2	1.99
Phe	2	1.93	Phe	2	1.96

[a] 6 N HCl–phenol, 110°, 24 h.
[b] With trypsin and chymotrypsin followed by leucine aminopeptidase.
[c] Degree of hydrolysis was 92% relative to acid hydrolysis.
[d] 72-hour hydrolysis.

tographic procedures for peptides and proteins provide considerable advantages.

Recently, another solvent system for reversed phase HPLC of peptides was developed (Gabriel *et al.*, 1979) which is transparent to u.v. light, even below 210 nm, and permits the use of the much simpler on-stream u.v. monitoring. It consists of 0.01 N HCl modified with acetonitrile in isocratic or gradient modes and has the additional advantage of providing hydrochloride salts of peptides upon lyophilization. This system was used for the purification of human β-EP by step gradient elution on LiChroprep RP-8 Lobar 3.7 x 44 cm columns at up to 150 mg loads (see Fig. 1c for a typical run) and on a Prep Pak C-18 cartridge with a 400-mg load using a Waters Prep 500 instrument. For analytical purposes to demonstrate the homogeneity of individual eluant fractions, isocratic elution with 0.01 N HCl modified by 29% acetonitrile was used on a 0.39 x 30 cm Bondapak C-18 column (Fig. 1c, bottom).

Overall, the pyridine–acetate system proved to be more versatile for a larger range of different peptides than the HCl-based system because its buffering properties permit better pH adjustment for optimizing resolution. The fluorescence detection system measures amine and is not responsive to artifactual contamination by non-peptidic material.

The synthetic product was homogeneous and identical with authentic human β-endorphin* (Li, 1977, 1978) on paper electrophoresis and on thin-layer chromatography. Analytical HPLC on an RP-8 column showed a single symmetrical peak, $K' = 2.8$ (Fig. 1d). Amino acid analysis after acid hydrolysis was in agreement with the expected values and data obtained after enzymatic hydrolysis were in close agreement with those exhibited by authentic β_h-endorphin (see Table 1).

The specific rotation, $[\alpha]$, of the synthetic peptide (1) and authentic β_h-EP (Li *et al.*, 1976) at several wavelengths in water is shown in

* Synthetic human β-EP prepared in this work was compared with material synthesized by Li *et al.* (1976, 1977) which in turn had been shown to be indistinguishable from isolated natural material (Li, 1977, 1978).

TABLE 2

Specific optical rotation of synthetic peptide and authentic β_h-EP in H_2O

	$[\alpha]_\lambda^{27°}$, deg.	
λ, nm	Peptide (1)	β_h-EP
589	− 57[a]	− 58
400	− 206	− 189
300	− 538	− 553
240	−2190	−2280

[a] In acidic solution a higher value, $[\alpha]_D^{25} - 83.5°$ (*c* 0.1, 0.01 N HCl) was found. Lit. (Kubota *et al.*, 1978) $[\alpha]_{25}^{D} - 76.6°$ (0.5 N AcOH).

Table 2. The specific rotations of the two peptides at each wavelength shown differ only slightly, and these differences are probably not significant. The $[\alpha]_D^{25} - 83.5°$ (*c* 0.1, 0.1 N HCl) in acidic solution exhibited by β_h-EP prepared in this work was somewhat higher than that in water and that reported by Kubota *et al.* (1978), i.e. $[\alpha]_D^{25} - 76.6°$ (*c* 0.3, 0.5 N AcOH).

The CD spectra in the far u.v. region are shown in Fig. 2. In both water and 90% aqueous methanol, the spectra for the synthetic product (1) and authentic β_h-EP are nearly identical. It is known that β_h-EP in H_2O has no secondary structure but exhibits helical conformation in aqueous methanol (Yang *et al.*, 1977).

The opioid activity of synthetic human β-EP (1) was identical with that of authentic material in the guinea pig ileum assay (Kosterlitz *et al.*, 1970) (50% inhibition at 1.8×10^{-9} M (see Fig. 3) and was blocked by the specific opiate antagonist naloxone (complete reversal at 3.0×10^{-8} M). In the stereospecific receptor binding assay (Ferrara *et al.*, 1979), the synthetic peptide (1) and β_h-EP are indistinguishable (Fig. 4). As shown in Fig. 5, the synthetic peptide has the same immunoreactivity of β_h-EP in the β_h-EP radioimmunoassay system (Chang *et al.*, 1979).

From the above data, it may be concluded that the human β-endorphin, synthesized by conventional methods in solution, is a homogeneous product.

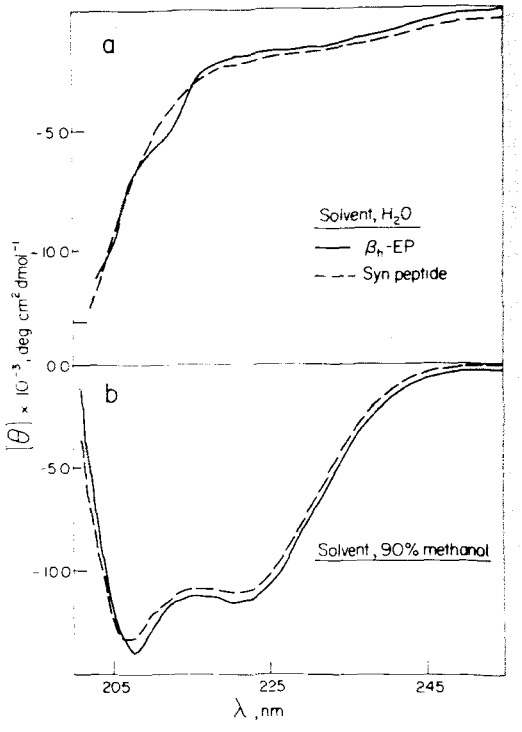


FIGURE 2

Circular dichroism spectra of synthetic peptide (1) and authentic β_h-EP in (a) water and (b) 90% aqueous methanol in the ultraviolet region.

EXPERIMENTAL PROCEDURES

Material

All asymmetric amino acid derivatives were of the L-configuration and were synthesized by literature procedures or purchased from Bachem, Inc., Torrence, CA, or Chemical Dynamics Corp., South Plainfield, NJ. Dimethylformamide (reagent grade, Matheson Coleman and Bell) was distilled from ninhydrin at reduced pressure and stored over molecular sieve. Tetrahydrofuran (reagent grade, Matheson Coleman and Bell) was distilled from $LiAlH_4$. Hydrogen fluoride (Matheson Gas Products) was dried over cobalt trifluoride (Alpha Inorganics) and distilled into the reaction vessel through an apparatus supplied by Toho Kasei Company Ltd., Osaka, Japan. Triethylamine (Pierce Chemical Co.) was of sequenal grade purity. All other solvents were of reagent grade and used without further

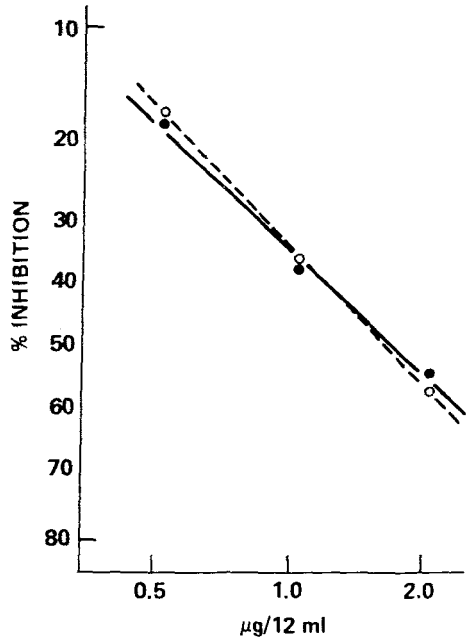


FIGURE 3

Guinea pig ileum bioassay of synthetic peptide (1), - - o - -, and authentic β_h-EP, —●—.

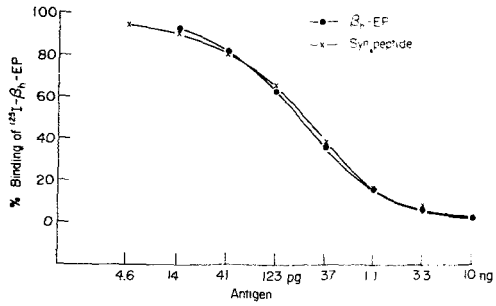


FIGURE 4

Radioimmunoassay of synthetic peptide (1), —X—, and authentic β_h-EP, —●—.

purification. Hydrogen chloride (Matheson Gas Products) was thoroughly dried (H_2SO_4) prior to use.

Methods

Elemental and amino acid analyses, and physiochemical measurements (i.r., n.m.r., $[\alpha]_D$) for

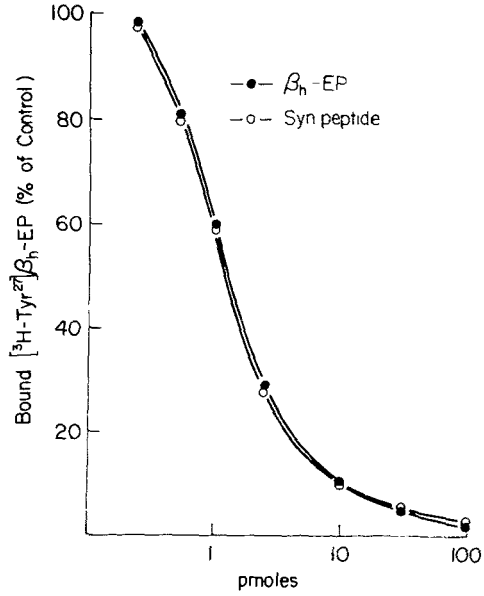


FIGURE 5

Competition of binding of [^{3}H-Tyr^{27}]-β_h-EP to rat brain membrane preparation by synthetic peptide (1), — o —, and authentic β_h-EP, — ● —.

all peptide intermediates were carried out by the Physicochemical Department of Hoffmann-La Roche Inc. Amino acid analyses were determined on the Beckman 121M amino acid analyzer. Solvent systems for thin-layer chromatography on silica gel G (Analtech, Inc.) or F-254 plates (Merck) were (A) 1-butanol–acetic acid–water (4:1:1), (B) 1-butanol–pyridine–acetic acid–water (15:10:3:12), (C) 1-butanol–ethyl acetate–acetic acid–water (1:1:1:1), (D) chloroform–methanol–acetic acid (85:10:5), and (E) chloroform–methanol (50:10) or (90:10). Solvent fronts were run for 12–14 cm and spots (30–50 μg loads) were visualized by ninhydrin or the fluorescamine spray procedure (Felix & Jimenez, 1974) or by the chlorine-tolidine procedure (Zahn & Rexroth, 1955; Nitecki & Goodman, 1966). Melting points were determined on a Büchi apparatus and are uncorrected. I.r. and n.m.r. spectra were compatible for all new products synthesized. Optical rotations were measured in a jacketed 1-dm cell on a Perkin-Elmer Model 141 Polarimeter.

High performance liquid chromatography
Several systems were suitable for the purification of β_h-EP.

A. Reversed phase C-18 0.9 x 500 cm Partisil 10 ODS-2 columns (Whatman Inc.) were used for loads of up to 100 mg. The pumping system consisted of Constametric I and II G pumps and gradient master (Laboratory Data Control). The flow rate was 4 ml per min. The solvent system was basically composed of a mixture of 8% pyridine with 2.5% AcOH in deionized H_2O, modified with a 1:1 mixture of acetonitrile and isopropanol. In this work Pyr–AcOH–CH_3CN–iC_3H_7OH–H_2O (5.9:1.9:13:13:66.2, v/v) was used in isocratic mode. It was removed from pooled fractions by lyophilization after the addition of deionized water (2–3 vol.). It was essential to use solvents leaving no residues, e.g. Burdick and Jackson "Distilled in Glass". Peptides were detected by automatic periodic sampling of aliquots of the effluent into a stream of *o*-phthalaldehyde (Fluoropa®, Pierce Chemical Co.) and fluorescence monitoring (Benson & Hare, 1975) on a Varian Fluorochrome using #9780 and #3387 emission and #9863 excitation filters. A full scale recorder response was obtained by 100 pmol of tyrosine at a sensitivity in which the baseline noise was 0.5% of scale. Less than 1% of peptide was consumed by sampling and 70–80% were recovered.

B. HCl based system. A solvent system, transparent at 210 nm, was used for analytical separations. To monitor the homogeneity of peaks produced on preparative separation, loads of 10 μg run on a Waters 0.39 x 30 cm Bondapak C-18 column with 0.01 N HCl containing 29% acetonitrile, at a flow rate of 2 ml/min, produced peak heights of 30% of scale on the recorder at 0.08 AUFS on the spectromonitor measuring the 210 nm absorption (Spectro-Monitor II, Laboratory Data Control). The system was also used for preparative separations with loads of up to 150 mg on 3.7 x 44 cm LiChroprep RP-8 Lobar columns (E.M. Laboratories) with HCl–acetonitrile gradient elution at 10 ml/min. An FMI model RP2SYSS pump (Fluid Metering Inc.) was used. Column effluents were monitored at 254 nm by a model 1205 detector (Laboratory Data Control). Care must be taken to avoid corrosion damage by the 0.01 N HCl to the pumps and other components by flushing the system with water to pH > 4 at the end of each day. A larger scale purification (400-mg load) was carried out on a Waters Prep 500 instrument using a single Prep Pak C-18 cartridge with an HCl–acetonitrile step gradient program.

Optical rotatory dispersion and circular dichroism spectra of synthetic β_h-EP were measured in the Hormone Research Laboratory, San Francisco, CA, in a Cary 60 recording spectropolarimeter, equipped with a model 6002 circular dichroism attachment, using procedures described before (Bewley & Li, 1967; Bewley *et al.*, 1969).

For the CD spectra, β_h-EP samples were dissolved in deionized water, followed by addition of methanol (MCB, spectroquality) to 90% (v/v). Concentrations were determined by measuring absorbance at 276 nm, correcting for light scattering according to the method of Beaven & Holiday (1952). Absorptivity ($D^{0.1\%}_{1\,cm,\,276\,nm}$) was calculated to be 0.77, according to the method of Wetlaufer (1962), assuming the same amino acid composition as β_h-endorphin.

CD spectra were taken from 255 nm to 200 nm, using a 2.0 cm pathlength. In all cases, peptide concentrations were approximately 0.5 mg/ml. All data were collected at dynode voltages less than 500 V. The mean residue weight of 112, calculated for the sequence of β_h-endorphin, was used for both peptides. Peptide spectra were scanned three or four times each, and baselines were scanned two or three times each. Content of α-helix was calculated according to the method of Bewley *et al.* (1969).

For the ORD spectra, the peptides were dissolved in deionized water. Peptide concentrations were determined as described above. ORD spectra were taken from 595 nm to 585 nm, 405 nm to 395 nm, and 300 nm to 240 nm, using a 1.0 cm pathlength. Peptide concentrations were varied from 0.38 mg/ml to 2.5 mg/ml. All data were collected at dynode voltages less than 480 V. Peptide spectra were scanned three to four times each, and baselines were scanned two to three times each.

Bioassays. Opioid activity was measured by the guinea pig ileum assay as described by Kosterlitz *et al.* (1970). IC_{50} was determined by plotting log molar concentration against mean percent inhibition. The peptides were

assayed twice at five different concentrations (2–10 μg/ml).

For receptor binding assay, the procedure recently described by Ferrara *et al.* (1979) was employed using tritiated β_h-EP as the primary ligand. Radioimmunoassay was carried out as previously described (Chang *et al.*, 1979), using a specific antiserum which showed slight cross-reaction with human β-lipotropin but none with human ACTH or with human β-melanotropin.

SYNTHESIS OF PROTECTED PEPTIDE SEGMENTS

Coupling reactions were generally carried out at pH 7.5–8.0 (moist pH paper) with initial addition of an equivalent of Et_3N or *N*-methylmorpholine followed by periodic adjustment of more base during reactions.

Unless otherwise indicated N^{α}*-Boc group cleavage* was affected by treatment of protected peptides with freshly prepared dry 4 N HCl in peroxide-free THF for 30–60 min, evaporation to dryness and precipitation or trituration of residues with dry ether to obtain partially protected peptide hydrochloride salts.

Work-up generally involved concentration of reaction mixture or evaporation to dryness *in vacuo* followed by repeated washing of the product in EtOAc, $CHCl_3$ or Et_2O with H_2O, 5% AcOH or 10% citric acid, H_2O, 5% $NaHCO_3$, H_2O, drying over Na_2SO_4 or $MgSO_4$ and evaporation of the organic solvent to provide crude products. (The $NaHCO_3$ wash is eliminated for peptides with free carboxyl groups.)

Segment 2

N^{ϵ}*-Benzyloxycarbonyl-*L*-lysyl-*N^{ϵ}*-benzyloxycarbonyl-*L*-lysine formiate (2a).* Boc-Lys(Z)-Lys(Z)-OH · DCHA (Wang *et al.*, 1979)* (0.8 g, 1 mmol) was partitioned between EtOAc and 0.1 N H_2SO_4. The aqueous layer was extracted once more with EtOAc and the combined extracts were washed (3 × H_2O), dried (Na_2SO_4) and evaporated to dryness. The oily residue was treated with 93% HCOOH (2.63 ml) at 25° for 5 h and evaporated to dryness. It was re-evaporated from H_2O and then from DMF. Addition of ether produced **2a** as a white crystalline solid which after filtration was washed with EtOH. Yield, 0.42 g (71%); m.p. 210–213°; $[\alpha]_D^{25}$ + 3.8° (*c* 1.53, DMF).

Anal. calc. for $C_{29}H_{40}N_4O_9$ (588.67): C, 59.17; H, 6.85; N, 9.52. Found: C, 59.41; H, 6.70; N, 9.76.

N^{α}*-tert-Butyloxycarbonyl-*O*-benzyl-*L*-tyrosyl-*N^{ϵ}*-benzyloxycarbonyl-*L*-lysyl-*N^{ϵ}*-benzyloxycarbonyl-*L*-lysine (2b).* A solution of **2a** (0.23 g, 4 mmol) in DMF (25 ml) was cooled to 0° and the pH adjusted to 8.5 by dropwise addition of Et_3N. Boc-Tyr(Bzl)-OPfp (Kisfaludy & Nyeki, 1975) was added. After stirring the mixture for 1 h at 0° and 1 h at 25° the solvent was evaporated and the residue treated with 10% aqueous citric acid. The product was extracted with EtOAc and the organic phase was washed with 10% citric acid and H_2O and dried (Na_2SO_4). During evaporation to a smaller volume **2b** precipitated as a white solid which was filtered off to yield 0.13 g; m.p. 171–174°. A second fraction was obtained by addition of petroleum ether to the filtrate until cloudiness, cooling, and thorough trituration of the ensuing precipitate with refluxing EtOAc; 0.16 g; m.p. 170–174°. The combined fractions were again triturated with hot EtOAc to yield 0.27 g (75%); m.p. 174–175°; $[\alpha]_D^{25}$ + 1.4° (*c* 1, MeOH).

Anal. calc. for $C_{49}H_{61}N_5O_{11} \cdot H_2O$ (914.08): C, 64.38; H, 6.95; N, 7.66. Found: C, 64.60; H, 6.76; N, 7.61.

An identical product was obtained by coupling of Boc-Tyr(Bzl)-OPfp with the salt formed by treatment of Boc-Lys(Z)-Lys(Z)-OH with 0.4 M $BF_3 \cdot OEt_2$ in AcOH.

Tert.*-Butyloxycarbonylglycyl-*L*-glutamic acid-α, γ-dibenzyl ester (2c).* To a solution of H-Glu(OBzl)-OBzl · HCl (1.82 g, 5 mmol) in DMF (35 ml) at 0° was added Et_3N (0.7 ml, 5 mmol) followed by Boc-Gly-OSu (1.5 g, 5.5 mmol). The mixture was stirred for 2 h at 0° and 17 h at 25° and the pH maintained at 8. The reaction mixture was then evaporated to a small volume and H_2O was added. The precipitated

* Prepared by treatment of H-Lys(Z)-OH (Ledger & Stewart, 1965; Costopanagiotis *et al.*, 1968) with Boc-Lys(Z)-OSu in DMF for 48 h. Crystallization of DCHA salt from EtOAc: m.p. 160–162°, $[\alpha]_D^{25}$ −3.2° (*c* 1.16, DMF).

oil was extracted with EtOAc and the organic phase washed in standard fashion and dried (Na_2SO_4). Evaporation provided an oil which was purified on a Silica gel 60 column (Gabriel *et al.*, 1976, 1977) using $CHCl_3$: MeOH (5:1) as eluant. The pooled fractions containing (**2c**) were evaporated yielding a colorless oil which failed to crystallize, 1.62 g (67%) homogeneous on t.l.c. (D, E), $[\alpha]_D^{25} -15.1°$ (*c* 2, MeOH).
Anal. calc. for $C_{26}H_{32}N_2O_7$ (484.56): C, 64.45; H, 6.66; N, 5.78. Found: C, 64.23; H, 6.72; N, 5.66.

Compound **2c** was also prepared from Boc-Gly-Glu(OBzl)-OH via the cesium salt procedure (Wang *et al.*, 1977).

N^{α}-tert.-*Butyloxycarbonyl*-O-*benzyl*-L-*tyrosyl*-N^{ϵ}-*benzyloxycarbonyl*-L-*lysyl*-N^{ϵ}-*benzyloxycarbonyl*-L-*lysyl*-*glycyl*-L-*glutamic acid*-α,γ-*dibenzyl ester (segment 2).* Compound **2c** (0.28 g, 0.57 mmol) was treated with freshly prepared 4 N HCl in THF (6 ml) for 30 min at 25°. The excess acid and solvent were evaporated and the remaining syrup re-evaporated twice from fresh THF. The residue was solidified by treatment with petroleum ether. The ensuing dipeptide ester hydrochloride was dissolved in DMF (1.5 ml), the solution was cooled to 0°, and neutralized with *N*-methylmorpholine (0.04 ml, 0.57 mmol). To this mixture, HOSu (0.12 g, 1.06 mmol) was added followed by a solution of Boc-Tyr(Bzl)-Lys(Z)-Lys(Z)-OH (**2b**) (0.48 g, 0.53 mmol) in DMF (3 ml) and by DCC (0.12 g, 0.58 mmol). The reaction was adjusted to pH 7.5 (wet pH paper) with a few drops of *N*-methylmorpholine and was stirred for 1 h at 0° and overnight at 25°. Insoluble by-products were filtered off and the filtrate was evaporated to a small volume. The residue was treated with H_2O and the precipitated white solid was taken up in $CHCl_3$. The organic phase was washed with 10% aq. citric acid, H_2O, 5% aq. $NaHCO_3$ and H_2O, dried (Na_2SO_4), and evaporated to dryness. It was redissolved in $CHCl_3$ and precipitated as a solid powder with petroleum ether. The crude product (0.56 g) was purified on a Silica gel 60 column (Gabriel *et al.*, 1976, 1977) using 5% MeOH in $CHCl_3$ as eluant. The pooled fractions containing 2 were concentrated to a small volume. Precipitation with petroleum ether provided (0.38 g, 57.4%) of crystalline material; m.p. 154–157°. Recrystallized from isopropanol or DMF-H_2O; m.p. 157–159°; $[\alpha]_D^{25} -7.2°$ (*c* 1.65, DMF).
Anal. calc. for $C_{70}H_{83}N_7O_{15}$ (1262.49): C, 66.60; H, 6.63; N, 7.77. Found C, 66.76; H, 6.66; N, 7.84.

Segment 3

Tert.-*Butyloxycarbonyl*-L-*isoleucyl*-L-*isoleucine dicyclohexylammonium salt (3a).* To a suspension of finely powdered isoleucine (1.97 g, 15 mmol) in DMF (100 ml) at 0° was added Boc-Ile-OSu (Anderson *et al.*, 1964; Ondetti *et al.*, 1970) (4.92 g, 15 mmol) and the pH adjusted to 8 by addition of Et_3N (1.12 ml, 8 mmol). The mixture was stirred for 2 h at 0° and 5 days at 25° and pH 8 maintained by periodic addition of Et_3N (0.98 ml, 7 mmol). Insoluble material was filtered off, the filtrate evaporated to dryness, the residue treated with 0.5 N HCl and the precipitated solid extracted with EtOAc. The organic layer was washed as usual, dried, concentrated to a small volume and titrated with DCHA to pH 8. The product, **3a**, precipitated as a crystalline dicyclohexylammonium salt which was collected and washed with EtOAc. Yield, 4.6 g (58.3%); m.p. 160–162°; $[\alpha]_D^{25} -18.5°$ (*c* 2, MeOH).
Anal. calc. for $C_{29}H_{55}N_3O_5$ (525.78): C, 66.25; H, 10.54; N, 7.99. Found: C, 66.03; H, 10.57; N, 7.94.

Tert.-*Butyloxycarbonyl*-L-*asparaginyl*-L-*alanine benzyl ester (3b).* To a stirred solution of H-Ala-OBzl · HCl (Erlanger & Brand, 1951) (43 g, 200 mmol) in DMF (500 ml) at 0° was added *N*-methylmorpholine (27 ml, 241 mmol), followed by Boc-Asn-OH (Schröder & Klieger, 1964) (46.5 g, 200 mmol), HOBt (54 g, 353 mmol) and DCC (45 g, 218 mmol). After reaction for 1.5 h at 0° and 17 h at 25° the mixture was worked up in general fashion and the crude product crystallized from THF-hexane to yield **3b**, 62.5 g (79.4%); m.p. 140–141°; $[\alpha]_D^{25} -31.1°$ (*c* 1, MeOH).
Anal. calc. for $C_{19}H_{27}N_3O_6$ (393.44): C, 58.00; H, 6.92; N, 10.68. Found: C, 57.77; H, 7.09; N, 10.52.

N^{α} - tert. - *Butyloxycarbonyl* - N^{ϵ} - *benzyloxycarbonyl*-L-*lysyl*-L-*asparaginyl*-L-*alanine benzyl*

ester (3c). Compound **3b** (27.5 g, 70 mmol) was treated with 4 N HCl in THF (875 ml) for 30 min at 25°. Evaporation and trituration of the residue with dry ether provided H-Asn-Ala-OBzl · HCl (23.1 g, 100%) which was dissolved in DMF (250 ml). The stirred solution was cooled to 0° and *N*-methylmorpholine (4.9 ml, 70 mmol) was added followed by Boc-Lys(Z)-OPfp (Kisfaludy *et al.*, 1973) (39.5 g, 72.3 mmol). After reaction for 1 h at 0° and 3 h at 25°, concentration to a small volume, precipitation of the product with 0.5 N HCl, filtration and washing with H_2O, 5% $NaHCO_3$ and H_2O, the dried **3c** was triturated with EtOAc and hexane to yield 41.5 g (90.4%) of colorless solid; m.p. 148–150°; $[\alpha]_D^{25} -15.3°$ (*c* 2, DMF).

Anal. calc. for $C_{33}H_{45}N_5O_9$ (655.76): C, 60.44; H, 6.92; N, 10.68. Found: C, 60.50; H, 6.97; N, 10.70.

Tert.-*Butyloxycarbonyl-L-isoleucyl-L-isoleucyl-*N^{ϵ}*-benzyloxycarbonyl-L-lysyl-L-asparaginyl-L-alanine benzyl ester (3d).* Compound **3c** (3.94 g, 6 mmol) was treated with 0.4 M $BF_3 \cdot OEt_2$ in glacial AcOH (60 ml) for 2.5 h at 25°. Evaporation and trituration of the residue with dry ether afforded H-Lys(Z)-Asn-Ala-OBzl · HCl as a white powder. A solution of **3a** (3.16 g, 6 mmol) in EtOAc was treated with 0.1 N H_2SO_4 and the organic phase was washed with H_2O, dried (Na_2SO_4) and evaporated. The residue was triturated with hexane to produce Boc-Ile-Ile-OH as a white solid which was dissolved in DMF (45 ml). The stirred solution was cooled to 0°, the above tripeptide ester hydrochloride added and the pH adjusted to 8 by addition of *N*-methylmorpholine. HOBt (1.95 g, 12 mmol) and DCC (1.36 g, 6.6 mmol) were added and the mixture was stirred for 2 h at 0° and for 48 h at 25°. Precipitated by-products were filtered off and washed thoroughly with DMF. After evaporation of the combined filtrates to a small volume the product was precipitated with 0.1 N H_2SO_4, filtered off and washed with H_2O, 5% $NaHCO_3$, and H_2O and dried to provide a white solid (5 g) which was repeatedly precipitated from DMF–isopropanol to yield **3d**, 3.24 g (61.2%); m.p. 222–224°; $[\alpha]_D^{25} -20.1°$ (*c* 1.5, DMF).

Anal. calc. for $C_{45}H_{67}N_7O_{11}$ (882.09): C, 61.27; H, 7.66; N, 11.12. Found: C, 61.59; H, 7.80; N, 11.09.

Tert.-*Butyloxycarbonyl-L-isoleucyl-L-isoleucyl-*N^{ϵ}*-benzyloxycarbonyl-L-lysyl-L-asparaginyl-L-alanine hydrazide (segment 3).* A solution of **3d** (0.88 g, 1 mmol) in DMF (20 ml) was treated with anhydrous hydrazine (0.5 ml, 15.6 mmol) for 48 h at 25°. MeOH was added to the cloudy mixture followed by refrigeration for 2 h. The ensuing white precipitate was filtered off and washed with MeOH and ether to yield **3** as a colorless solid, 0.61 g (75.7%); m.p. 252–253°; $[\alpha]_D^{25} -33.7°$ (*c* 1, DMSO).

Anal. calc. for $C_{38}H_{63}N_9O_{10}$ (805.99): C, 56.63; H, 7.88; N, 15.64. Found: C, 56.79; H, 7.91; N, 15.64.

Segment 5

N^{α} - tert. - *Butyloxycarbonyl* - N^{ϵ} - *benzyloxy - carbonyl - L - lysyl - L - asparaginyl - L - alanine hydrazide (segment 5).* Compound 3c (2.3 g, 3.5 mmol) was dissolved in MeOH (20 ml) with warming. After cooling to 4°, anhydrous hydrazine (1.1 ml, 34 mmol) was added, and the mixture was kept for 72 h at 4°. The precipitated product was filtered off, washed with MeOH and ether and crystallized from DMF–isopropanol. Yield, 1.0 g (49.3%); m.p. 177–180°; $[\alpha]_D^{25} -14.9°$ (*c* 1.5, DMF).

Anal. calc. for $C_{26}H_{41}N_7O_8$ (579.67): C, 53.87; H, 7.13; N, 16.91. Found: C, 53.75; H, 7.19; N, 17.00.

Segment 7

Tert.-*Butyloxycarbonyl-L-valyl-O-benzyl-L-threonine benzyl ester (7a).* H-Thr(Bzl)-OBzl hemioxalate (Mizoguchi *et al.*, 1968) (58 g, 168.4 mmol) was suspended in DMF (700 ml) and stirred with Boc-Val-OSu (Anderson *et al.*, 1964) (48 g, 153.1 mmol) and Et_3N (16 ml) at 0° for 1 h, 4° for 72 h, and 25° for 24 h and the pH maintained at 7.5–8. The reaction mixture was worked up as described for 2 to produce an oil which crystallized from EtOAc–hexane, yield 40.8 g (53.5%); m.p. 99–100.5°; $[\alpha]_D^{25} -26.0°$ (*c* 1, MeOH).

Anal. calc. for $C_{28}H_{38}N_2O_6$ (498.62): C, 67.45; H, 7.68; N, 5.62. Found: C, 67.57; H, 7.55; N, 5.70.

Tert.-*Butyloxycarbonyl-L-valyl-O-benzyl-L-threonine (7aa).* A suspension of H-Thr(Bzl)-OH (Mizoguchi *et al.*, 1968) (3.0 g, 14.3 mmol) in DMF (60 ml) was stirred with Et_3N (2 ml, 14.3 mmol) and Boc-Val-OSu (4.96 g, 15.8 mmol) at 0° for 2 h and 25° for 24 h, AcOH was added to about pH 3 and the solvents removed under reduced pressure. The product was extracted into EtOAc, washed with 5% AcOH and H_2O, dried ($MgSO_4$) and evaporated to produce a clear oil which crystallized from EtOAc–hexane to yield 4.9 g (83.1%); m.p. 132–135°; $[\alpha]_D^{25}$ + 21.5° (*c* 1, $CHCl_3$).
Anal. calc. for $C_{21}H_{32}N_2O_6$ (408.49): C, 61.74; H, 7.89; N, 6.85. Found: C, 61.61; H, 7.76; N, 6.80.

Tert.-*Butyloxycarbonyl-L-leucyl-L-valyl-O-benzyl-L-threonine benzylester (7b).* Compound **7a** (40.0 g, 80.2 mmol) was treated with 4 N HCl in THF (1003 ml) for 20 min. Evaporation and treatment of the residue with dry ether afforded an amorphous white solid, 28.0 g (80.1%) which was dissolved in DMF (150 ml) and stirred with Et_3N (9 ml, 64.3 mmol), Boc-Leu-OH · H_2O (Anderson & McGregor, 1957) (16.0 g, 64.3 mmol) and DCC (14.6 g, 71 mmol) at 0° for 1 h and 25° for 24 h. The reaction mixture was worked up as described for 2 to produce an oil, yield 33.0 g (83.9%). The oil was subjected to silica gel chromatography (Gabriel *et al.*, 1976, 1977) with a stepwise gradient elution in the solvent system, chlorobutane and acetonitrile. A clear oil was obtained which failed to crystallize; $[\alpha]_D^{25}$ − 31.0° (*c* 1, $CHCl_3$).
Anal. calc. for $C_{34}H_{49}N_3O_7$ (611.79): C, 66.75; H, 8.07; N, 6.86. Found: C, 66.71; H, 8.08; N, 6.61.

N-tert.-*Butyloxycarbonyl*-O-*benzyl-L-threonine* N-*hydroxysuccinimide ester (7c).* Boc-Thr(Bzl)-OH (Mizoguchi *et al.*, 1968) (47 g, 151.6 mmol), dissolved in THF (450 ml), was stirred with *N*-hydroxysuccinimide (1.2 equiv.) and DCC (1.1 equiv.) at 0° for 1.5 h and 25° for 1 h. Insoluble DCU was filtered off and the solvent evaporated to one-third volume when more DCU was filtered off after cooling. The solvent was evaporated to a clear oil which crystallized from isopropanol–hexane to provide very fine white crystals, yield 58.8 g (95.5%); m.p. 101–102°; $[\alpha]_D^{25}$ + 6.04° (*c* 1, $CHCl_3$).
Anal. calc. for $C_{20}H_{26}N_2O_7$ (406.44): C, 59.10; H, 6.45; N, 6.89. Found: C, 58.89; H, 6.29; N, 6.87.

N-tert.-*Butyloxycarbonyl*-O-*benzyl-L-threonyl-L-proline (7d).* A suspension of Pro-OH (1.0 g, 8.69 mmol) in DMF (15 ml) was stirred with Et_3N (2.44 ml) and Boc-Thr(Bzl)-OSu (**7c**) (3.88 g, 9.56 mmol) at 0° for 1 h and 25° for 72 h. The reaction mixture was acidified to pH 3 with AcOH and worked up as described for **2b** to produce **7d** as a crystalline product from EtOAc–hexane, 3.0 g (85.0%); m.p. 84–88°; $[\alpha]_D^{25}$ − 52.2° (*c* 1, $CHCl_3$).
Anal. calc. for $C_{21}H_{30}N_2O_6$ (406.48): C, 62.05; H, 7.43; N, 6.89. Found: C, 62.29; H, 7.52; N, 6.86.

N-tert.-*Butyloxycarbonyl*-O-*benzyl-L-threonyl-L-prolyl-L-leucyl-L-valyl*-O-*benzyl-L-threonine benzyl ester (7e).* Compound **7b** (0.88 g, 1.43 mmol) was treated with 4 N HCl in THF (17.9 ml) for 45 min. Evaporation and treatment of the residue with dry ether gave an amorphous white solid, 0.55 g (70.5%) which was dissolved in DMF (20 ml) and stirred with *N*-methylmorpholine (0.11 ml, 1.0 mmol), compound **7d** (0.4 g, 1.0 mmol), HOSu (0.23 g, 2.0 mmol) and DCC (0.227 g, 1.1 mmol) at 0° for 1.5 h and 25° for 24 h. The reaction was worked up as described for 2. After silica gel 60 chromatography (Gabriel *et al.*, 1976, 1977) using the isocratic system of $CHCl_3$–MeOH (18:1) the product (**7e**) was obtained as an amorphous solid: 0.6 g (66.7%); $[\alpha]_D^{25}$ − 44.0° (*c* 1, $CHCl_3$).
Anal. calc. for $C_{50}H_{69}N_5O_{10}$ (900.14): C, 66.72; H, 7.73; N, 7.78. Found: C, 66.73; H, 7.52; N, 7.71.

N-tert.-*Butyloxycarbonyl*-O-*benzyl-L-seryl-L-glutamine dicyclohexylammonium salt (7f).* Glutamine (15.0 g, 104.2 mmol) was dissolved in 51.5 ml of 40% Triton B and evaporated to an oil. It was re-evaporated twice with DMF and the salt obtained was stirred with Boc-Ser(Bzl)-OSu (Laufer & Blout, 1967) (45.0 g, 114.6 mmol) in DMF (400 ml) at 0° for 2 h and

25° for 24 h. The solvent was evaporated to a syrup which was dissolved in $CHCl_3$, washed with 5% AcOH and H_2O, dried (Na_2SO_4) and evaporated to yield an oil which was dissolved in EtOAc (500 ml) and titrated with DCHA to pH 8–9. The resulting crystals were filtered off, washed with EtOAc and Et_2O and dried, yield 39.4 g (62.5%); m.p. 124–127°; $[\alpha]_D^{25}$ + 8.3° (*c* 1, MeOH).

Anal. calc. for $C_{32}H_{52}N_4O_7$ (604.79): C, 63.55; H, 8.67; N, 9.26. Found: C, 63.28; H, 8.82; N, 9.07.

N-tert.-*Butyloxycarbonyl-O-benzyl-L-seryl-L-glutaminyl-O-benzyl-L-threonyl-L-prolyl-L-leucyl-L-valyl-O-benzyl-L-threonine benzyl ester (7g).* Compound **7e** (1.0 g, 1.11 mmol) was treated with 4 N HCl in THF (13.9 ml) for 45 min. Evaporation and treatment of the residue with dry ether produced an amorphous white solid (0.84 g, 92.9%), which was dissolved (0.76 g 0.91 mmol) in DMF (20 ml). The solution was cooled to 0° and *N*-methylmorpholine (0.1 ml, 0.90 mmol) was added, followed by Boc-Ser(Bzl)-Gln-OH (0.39 g, 0.91 mmol) obtained by partitioning **7f** (0.6 g, 0.99 mmol) between EtOAc and 0.1 N H_2SO_4 as described for **2a**), HOBt (0.25 g, 1.6 mmol) and DCC (0.21 g, 1.0 mmol). After stirring the mixture for 2 h at 0° and 24 h at 25° it was worked up as described for **2**. The crude product was crystallized from isopropanol to yield **7g** as colorless crystals (0.61 g, 55.4%); m.p. 157–159°; $[\alpha]_D^{25}$ −44.7° (*c* 1, $CHCl_3$).

Anal. calc. for $C_{65}H_{88}N_8O_{14}$ (1205.47): C, 64.77; H, 7.36; N, 9.29. Found: C, 64.84; H, 7.50; N, 9.13.

Amino acid analysis (6 N HCl–phenol, 110°, 24 h): $Thr_{1.90}$, $Ser_{0.78}$, $Glu_{0.99}$, $Pro_{0.99}$, $Val_{1.04}$, $Leu_{1.04}$.

N-tert.-*Butyloxycarbonyl-O-benzyl-L-seryl-L-glutaminyl-O-benzyl-L-threonyl-L-prolyl-L-leucyl-L-valyl-O-benzyl-L-threonine hydrazide (7h).* Compound **7g** (1.6 g, 1.32 mmol) was dissolved in DMF–MeOH (5:3; 16 ml) and treated with H_2NNH_2 (0.48 ml, 24 mmol) for 72 h at 25°. The precipitated white solid was filtered and washed with MeOH and H_2O, yield 1.05 g (70.5%); m.p. 210–210.5°; $[\alpha]_D^{25}$ −22.5° (*c* 1, DMF).

Anal. calc. for $C_{58}H_{84}N_{10}O_{13}$ (1129.37): C, 61.68; H, 7.50; N, 12.40. Found: C, 61.72; H, 7.26; N, 12.30.

Tert.-*Butyloxycarbonyl-L-leucyl-L-phenylalanine (7i).* To a suspension of finely powdered phenylalanine (1.49 g, 9 mmol) in DMF (20 ml) at 0° was added Et_3N (1.26 ml, 9 mmol) followed by Boc-Leu-OPfp (Kisfaludy *et al.*, 1973) (3.93 g, 9.9 mmol). The mixture was stirred for 1 h at 0° and 2.5 h at 25°, filtered, the solvent evaporated and the residue treated with H_2O. The separated oil was extracted into EtOA and washed as described in **2b**. Crystallization from EtOAc–hexane provided **7i** as colorless crystals (2.3 g, 67.6%); m.p. 104–106°; $[\alpha]_D^{25}$ −3.4° (*c* 1, EtOH).

Anal. calc. for $C_{20}H_{30}N_2O_5$ (378.47): C, 63.47; H, 7.99; N, 7.40. Found: C, 63.46; H, 8.18; N, 7.49.

N-tert.-*Butyloxycarbonyl-O-benzyl-L-seryl-L-glutaminyl-O-benzyl-L-threonyl-L-prolyl-L-leucyl-L-valyl-O-benzyl-L-threonyl-L-leucyl-L-phenylalanine (segment 7).* Compound **7i** (0.53 g, 1.4 mmol) was treated with 4 N HCl in THF for 45 min. Evaporation and treatment of the residue with dry ether produced crystalline Leu-Phe hydrochloride which was dissolved in DMF (5 ml) in the presence of Et_3N (0.28 ml, 2 mmol). Compound **7h** (0.79 g, 0.7 mmol) in DMF (10 ml) was converted into the azide by treatment with 2.8 N HCl in THF (1.25 ml) and isoamylnitrite (0.14 ml) at −20° for 25 min. The temperature was lowered to −30° and Et_3N (0.49 ml) was added followed by the above pre-cooled solution of Leu-Phe. The mixture was stirred for 1.5 h at −15° and for 4 days at 4°. It was acidified with glacial AcOH, evaporated to a smaller volume, and treated with 1 M AcOH. The precipitated solid was filtered and washed with H_2O and triturated with EtOAc. The ensuing crude product (0.92 g); m.p. 194–196.5° was crystallized from hot MeOH to provide segment **7** in fine crystals, 0.51 g (53%); m.p. 205–207°; $[\alpha]_D^{25}$ −20.1° (*c* 1, DMF).

Anal. calc. for $C_{73}H_{102}N_{10}O_{16}$ (1375.70): C, 63.73; H, 7.47; N, 10.18. Found: C, 63.26; H, 7.43; N, 10.23.

Amino acid analysis (6 N HCl–phenol, 110°,

24 h): $Thr_{2.00}$, $Ser_{0.91}$, $Glu_{1.03}$, $Pro_{1.05}$, $Val_{0.99}$, $Leu_{2.08}$, $Phe_{1.00}$.

Segment 9

N-tert.-*Butyloxycarbonyl-L-glutamyl-γ-benzyl ester-*N^{ϵ}*-benzyloxycarbonyl-L-lysine (9a)* A stirred suspension of H-Lys(Z)-OH (Ledger & Stewart, 1965; Costopanagiotis *et al.*, 1968) (0.89 g, 3.18 mmol) in DMF (25 ml) at 0° was treated with Boc-Glu(OBzl)-OSu (Nakajima & Okawa, 1973) (1.58 g, 3.5 mmol) in the presence of Et_3N (0.5 ml, 3.6 mmol). After stirring for 1 h at 0° and 24 h at 25° the mixture was worked up, as described for **2b**, to produce a clear oil which was crystallized from EtOAc–hexane to yield 1.47 g (77.4%) of **9a**; m.p. 110–112°; $[\alpha]_D^{25}$ + 6.0° (*c* 1, $CHCl_3$).
Anal. calc. for $C_{31}H_{41}N_3O_9$ (599.69): C, 62.08; H, 6.89; N, 7.00. Found: C, 62.04, H, 6.96; N, 7.10.

N-tert.-*Butyloxycarbonyl-*O*-benzyl-L-threonyl-*O*-benzyl-L-serine* N*-hydroxysuccinimide ester (9b).* A stirred suspension of H-Ser(Bzl)-OH (Hayakawa *et al.*, 1966) (2.59 g, 13.3 mmol) in DMF (70 ml) was treated with Boc-Thr(Bzl)-OSu (**7c**; 6.0 g, 14.8 mmol) in the presence of Et_3N (2.0 ml, 14.3 mmol) at 0° for 2 h and 25° for 72 h. The reaction mixture was acidified to about pH 3 with AcOH and the solvents removed under reduced pressure. The resulting oil was extracted into EtOAc, washed with 5% AcOH and H_2O, dried ($MgSO_4$) and treated with HOSu (1.8 g, 15.7 mmol) and DCC (3.0 g, 14.5 mmol) for 1.5 h at 0° and 3 h at 25°. DCU was removed by filtration and the filtrate evaporated to a clear oil which was crystallized from EtOH, yield 5.3 g (70.4%); m.p. 112–113°; $[\alpha]_D^{25}$ + 18.7° (*c* 1, $CHCl_3$).
Anal. calc. for $C_{30}H_{37}N_3O_9$ (583.65): C, 61.72; H, 6.39; N, 7.20. Found: C, 61.75: H. 6.49; N, 7.17.

N-tert.-*Butyloxycarbonyl-*O*-benzyl-L-threonyl-*O*-benzyl-L-seryl-L-glutamyl-γ-benzyl ester-*N^{ϵ}*-benzyloxycarbonyl-L-lysine (9c).* Compound **9a** (2.0 g, 3.33 mmol) was dissolved in 0.4 M $BF_3 \cdot Et_2O$ in AcOH (16.7 ml) and stirred at 25° for 4 h. The solvent was evaporated to a clear oil which was solidified by treatment with dry ether and drying over KOH. This material was dissolved in DMF (25 ml) and treated with Boc-Thr(Bzl)-Ser(Bzl)-OSu (**9b**; 1.89 g, 3.33 mmol) in the presence of Et_3N (0.47 ml, 3.33 mmol) at 0° for 1.5 h and 25° for 24 h while pH 8 was maintained. The mixture was worked up as described for **2b** to afford a crystalline product from EtOAc–hexane (2.5 g, 78.1%); m.p. 111–115°; $[\alpha]_D^{25}$ −4.8° (*c* 1, $CHCl_3$).
Anal. calc. for $C_{52}H_{65}N_5O_{13}$ (968.12): C, 64.51; H, 6.76; N, 7.23. Found: C, 64.40; H, 6.60; N, 7.11.

N-*Benzyloxycarbonyl-L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-methionine benzyl ester (9d).* Prepared by coupling of Z-Tyr-Gly-Gly-N_3 with H-Phe-Met-OBzl as described by Wang *et al.* (1977), **9d** had m.p. 182–185°; $[\alpha]_D^{25}$ −26.8° (*c* 1, DMF).

N-*Benzyloxycarbonyl-L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-methionine hydrazide (9e).* Compound **9d** (0.85 g, 1.07 mmol) was dissolved in DMF (10 ml, purged with argon for 15 min) and treated with H_2NNH_2 (0.34 ml, 10.7 mmol) for 72 h at 25°. The solvent was removed under reduced pressure to yield a crystalline residue which was triturated with H_2O and recrystallized from DMF–EtOH to form colorless needles (0.65 g, 84%); m.p. 209–213°; $[\alpha]_D^{25}$ −27.6° (*c* 1, DMF).
Anal. calc. for $C_{35}H_{43}N_7O_8S_1$ (721.84): C, 58.24; H, 6.00; N, 13.58; S, 4.44. Found: C, 57.95; H, 6.04; N, 13.56; S, 4.67.

N-*Benzyloxycarbonyl-L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-methionyl-*O*-benzyl-L-threonyl-*O*-benzyl-L-seryl-L-glutamyl-γ-benzyl ester-*N^{ϵ}*-benzyloxycarbonyl-L-lysine hydrazide (9f).* Compound **9** (0.1 g, 0.064 mmol) was dissolved in DMF (2 ml) and stirred with H_2NNH_2 (0.05 ml, 1.5 mmol), HOBt (19.6 mg, 0.13 mmol) and DCC (14.5 mg, 0.07 mmol) at 0° for 1 h and 25° for 24 h (Wang *et al.*, 1978). During this time a few drops of *N*-methylmorpholine were added to the reaction to maintain a pH between 7 and 8. For work-up, the reaction mixture was added dropwise into rapidly stirred ice water to form a white precipitate which was filtered and washed with H_2O and Et_2O; it was recrystallized from DMF and EtOH, yield 67 mg (67%); m.p. 245–249° dec; $[\alpha]_D^{25}$ −4.3° (*c* 1, DMF).

Anal. calc. for $C_{82}H_{98}N_{12}O_{18}S$ (1571.82): C, 62.66; H, 6.28; N, 10.69; S, 2.04. Found: C, 62.78; H, 6.35; N, 10.61; S, 2.03.

N-Benzyloxycarbonyl-L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-methionyl-O-benzyl-L-threonyl-O-benzyl-L-seryl-L-glutamyl-γ-benzyl ester-N$^{\epsilon}$-benzyloxycarbonyl-L-lysine (segment 9). Compound **9c** (4.2 g, 4.33 mmol) was treated with 0.4 M $BF_3 \cdot Et_2O$ in AcOH (27 ml, 10.8 mmol) for 4 h at 25°. Evaporation, treatment of the residue with dry Et_2O and drying over KOH gave the tetrapeptide derivative as an amorphous solid which was dissolved in DMF (20 ml). To prepare the pentapeptide azide, a solution of **9e** (3.13 g, 4.33 mmol) in DMF (50 ml, purged with argon for 15 min) at −20° was treated with isoamyl nitrite (0.88 ml, 6.5 mmol) in the presence of 4 N HCl (5.93 ml) in THF. After 30 min the mixture was cooled to −30° and the above tetrapeptide solution in the presence of Et_3N (3.64 ml, 26 mmol) was added. The mixture was stirred for 72 h at 4° and the pH maintained at 8. Work-up by acidification to pH 3 with AcOH, evaporation, trituration of the residue with H_2O, filtration, washing with 5% AcOH and H_2O and repeated trituration with EtOH provided **9** as an amorphous solid, 5.5 g (81.5%); m.p. 227–231° dec; $[\alpha]_D^{25}$ − 3.2° (*c* 1, DMF).

Anal. calc. for $C_{82}H_{96}N_{10}O_{19}S$ (1157.79): C, 63.22; H, 6.21; N, 8.99; S, 2.06. Found: C, 63.03; H, 6.24; N, 9.12; S, 2.12.

Amino acid analysis (6 N HCl–phenol, 110°, 24 h): $Lys_{1.11}$, $Thr_{1.05}$, $Ser_{0.86}$, $Glu_{1.07}$, $Gly_{1.86}$, $Met_{0.95}$, $Tyr_{0.81}$, $Phe_{1.02}$.

SEGMENT CONDENSATION

Boc-Ile-Ile-Lys(Z)-Asn-Ala-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl (4; sequence region 22–31). Boc-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl (segment **2**; 6.58 g, 5.21 mmol) was treated for 4 h at 25° with 0.4 M $BF_3 \cdot OEt_2$ in AcOH (26 ml). The amorphous solid material obtained after evaporation and trituration with ether was directly used for coupling. Boc-Ile-Ile-Lys(Z)-Asn-Ala-$NHNH_2$ (segment **3**; 4.2 g, 5.21 mmol) was suspended in DMF (50 ml) and treated with 4.04 N HCl (6.45 ml) and isoamyl nitrite (1.05 ml, 7.8 mmol) at −20° for 30 min. After lowering the temperature of the bath to −30°, Et_3N (3.65 ml, 26.1 mmol) was added followed by a precooled mixture of the salt of the pentapeptide, described above, in DMF (20 ml) in the presence of Et_3N (0.73 ml, 5.21 mmol). The reaction mixture was stirred at −20° to −15° for 30 min and 0° for 3 days and the pH maintained at 8. After 5 h the product started to precipitate in gelatinous form. For work-up, the mixture was acidified to pH 5 with glacial AcOH and the solvent evaporated. The ensuing residue was triturated with 0.05 N HCl, filtered, and washed with H_2O to yield 8.6 g of crude **4**. Reprecipitation from DMSO–EtOH provided 6.3 g (63%); m.p. 247–250° dec; $[\alpha]_D^{25}$ − 18.5° (*c* 1, DMSO).

Anal. calc. for $C_{103}H_{134}N_{14}O_{23}$ (1936.32): C, 63.89; H, 6.98; N, 10.13. Found: C, 63.72; H, 7.00; N, 9.98.

Amino acid analysis (6 N HCl–phenol, 110°, 24 h): $Lys_{3.15}$, $Asp_{1.00}$, $Glu_{1.03}$, $Gly_{1.00}$, $Ala_{0.97}$, $Ile_{1.93}$, $Tyr_{0.95}$.

Boc-Lys(Z)-Asn-Ala-Ile-Ile-Lys(Z)-Asn-Ala-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl (6; sequence region 19–31).

A) Cleavage of the N^{α}-Boc group from protected decapeptide **4**. Compound **4** (3.87 g, 2 mmol) was treated with 98% HCOOH (35 ml) for 3.5 h at 25°. Excess HCOOH was then evaporated (at a bath temperature below 30°). The residue was re-evaporated from DMF and treated with dry Et_2O. The resulting white solid was dissolved with warming in DMF–DMSO (1:1, 50 ml). The decapeptide amine (**4a**) was precipitated by adjusting the pH to 8 with 0.5 N NH_4OH, and isolated as a white amorphous powder by repeated centrifugation and washing, and drying in a desiccator (KOH, H_2SO_4); yield 3.22 g (87.7%).

B) Azide coupling. Boc-Lys(Z)-Asn-Ala-$NHNH_2$ (segment **5**; 2.32 g, 4 mmol) was dissolved in DMF (15 ml) cooled to −20° and treated with isoamyl nitrite (0.8 ml, 6 mmol) in the presence of 3.8 N HCl in THF (5.2 ml). After stirring at −20° for 30 min the temperature was lowered to −30°. Et_3N (2.8 ml, 20 mmol) was added followed by the precooled solution of the decapeptide amine (**4a**) in DMF–DMSO (10:3, 13 ml). The pH was adjusted to 8 by the addition of Et_3N and the mixture stirred for 1 h at −15° and 4 days at

4°. For work-up, it was acidified with glacial AcOH and concentrated to a smaller volume. The product was precipitated with 1 M AcOH, filtered, and washed thoroughly with H_2O. After drying it was triturated with EtOH and with boiling MeOH to yield a white powder which was reprecipitated from DMF–DMSO (1:1, 11 ml) by addition of 90% MeOH to provide 3.45 g (32.5%, based on **4a**) m.p. 257–259° dec; $[\alpha]_D^{25}$ −24.8° (*c* 1, DMSO).
Anal. calc. for $C_{124}H_{163}N_{19}O_{29}$ (2383.82): C, 62.48; H, 6.89; N, 11.16. Found: C, 62.21; H, 6.76; N, 11.25.

Amino acid analysis (6 N HCl–phenol, 110°, 25 h): $Lys_{4.07}$, $Asp_{2.04}$, $Glu_{1.00}$, $Gly_{1.00}$, $Ala_{2.07}$, $Ile_{1.91}$, $Tyr_{0.91}$.

Boc-Ser(Bzl)-Gln-Thr(Bzl)-Pro-Leu-Val-Thr(Bzl)-Leu-Phe-Lys(Z)-Asn-Ala-Ile-Ile-Lys(Z)-Asn-Ala-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl (8; sequence region 10–31).

A) Cleavage of the N^{α}-Boc group from protected 13-peptide **6**. Compound **6** (2.86 g, 1.2 mmol) was treated with 98% HCOOH (20 ml), as described above for **4**, to yield a white solid (2.79 g); m.p. 272–275° dec. Titration with 0.5 N NH_4OH in DMF–DMSO (4:3; 21 ml) yielded a white powder (**6a**; 2.65 g, 96.7%), m.p. 263–267° dec.

B) Segment condensation by DCC–HOBt (König & Geiger, 1972, 1973). To a cold (0°) solution of Boc-Ser(Bzl)-Gln-Thr(Bzl)-Pro-Leu-Val-Thr(Bzl)-Leu-Phe-OH (segment **7**; 1.98 g, 1.44 mmol) in DMF (23 ml) HOBt (0.49 g, 3.2 mmol) was added, followed by DCC (0.33 g, 1.6 mmol). The mixture was stirred for 1 h at 0° while pH 7 was maintained by addition of *N*-methylmorpholine. A precooled solution of the 13-peptide amine (**6a**) was added and the pH adjusted to 8 with *N*-methylmorpholine. Stirring was continued for 2 h at 0° and for 3 days at 25°. For work-up, the mixture was concentrated to a small volume and treated with 5% aq. $NaHCO_3$. The crude precipitate was filtered off and washed to neutral with H_2O. After drying it was triturated repeatedly with boiling MeOH to obtain a white powder, 3.5 g (83% based on the 13-peptide amine). For elemental analysis a sample was freeze-dried from DMSO; m.p. (sintered) 283–290° dec; $[\alpha]_D^{25}$ −23.8° (*c* 0.5, DMSO).

Anal. calc. for $C_{192}H_{255}N_{29}O_{42}$ (3641.38): C, 63.33; H, 7.06; N, 11.16. Found: C, 63.16; H, 7.07; N, 10.96.

Amino acid analysis (6 N HCl–phenol, 110°, 24 h): $Lys_{4.18}$, $Asp_{2.14}$, $Thr_{1.97}$, $Ser_{0.98}$, $Glu_{2.06}$, $Pro_{0.98}$, $Gly_{1.00}$, $Ala_{2.02}$, $Val_{0.98}$, $Ile_{2.04}$, $Leu_{2.02}$, $Tyr_{1.02}$, $Phe_{1.00}$.

Z-Tyr-Gly-Gly-Phe-Met-Thr(Bzl)-Ser(Bzl)-Glu(OBzl)-Lys(Z)-Ser(Bzl)-Gln-Thr(Bzl)-Pro-Leu-Val-Thr(Bzl)-Leu-Phe-Lyz(Z)-Asn-Ala-Ile-Ile-Lys(Z)-Asn-Ala-Tyr(Bzl)-Lys(Z)-Lys(Z)-Gly-Glu(OBzl)-OBzl (10; sequence 1–31).

A) Cleavage of the N^{α}-Boc group from protected 22-peptide **8**. Compound **8** (1.5 g, 0.41 mmol) was treated with 98% HCOOH (11 ml) for 3.5 h at 25°. After filtration and concentration to a small volume, the residue was dissolved in DMSO–DMF (1:1, 20 ml) and a few drops of Et_3N were added. The pH was then titrated to 8 with 0.5 N NH_4OH. The precipitated 22-peptide amine was washed thoroughly to neutral with H_2O (by repeated centrifugation and decantation), filtered off and dried (KOH, H_2SO_4) to yield a white powder (**8a**; 1.33 g, 91.7%).

B) Segment condensation by DCC–HOBt. Z-Tyr-Gly-Gly-Phe-Met-Thr(Bzl)-Ser(Bzl)-Glu(OBzl)-Lys(Z)-OH (segment **9**; 1.6 g, 1.03 mmol) was dissolved in DMF (10 ml) and the solution was cooled to 0°. HOBt (0.35 g, 2.3 mmol) was added, followed by DCC (0.24 g, 1.15 mmol). The mixture was stirred for 1 h each at 0° and 25°. After cooling to 0° it was combined with a cold solution of the 22-peptide amine (**8a**) in DMF–phenol which was prepared by dissolving **8a** (1.33 g, 0.38 mmol) in melted phenol (12.5 ml) at 40–50°, followed by the addition of DMF (12.5 ml) and cooling to 0°. This was stirred for 1 h at 0° and for 3 days at 25°. For work-up, the mixture was slowly added to cold (0°) stirred 0.5% aq. AcOH (1200 ml). The ensuing white precipitation from warm DMF–MeOH and from hexafluoroisopropanol–MeOH provided **10** as a white amorphous powder (1.58 g, 82% based on **8a**); m.p. 273–276° dec; $[\alpha]_D^{25}$ −36.4° (*c* 0.5, hexafluoroisopropanol).
Anal. calc. for $C_{269}H_{341}N_{39}O_{58}S$ (5081.07): C, 63.59; H, 6.77; N, 10.75; S, 0.63. Found: C, 63.15; H, 6.77; N, 10.64; S, 0.86.

Amino acid analysis (6 N HCl–phenol, 110°,

24 h): $Lys_{5.07}$, $Asp_{1.94}$, $Thr_{2.75}$, $Ser_{1.63}$, $Glu_{3.15}$, $Pro_{0.91}$, $Gly_{3.00}$, $Ala_{1.97}$, $Val_{0.93}$, $Met_{1.12}$, $Ile_{1.74}$, $Leu_{1.94}$, $Tyr_{1.78}$, $Phe_{2.06}$.

HUMAN β-ENDORPHIN (1)

Protective group cleavage by liquid hydrogen fluoride

Protected 31-peptide, **10** (153 mg, 0.03 mmol) was stirred in anhydrous HF (6 ml; in a Kel-F reactor vessel) in the presence of anisole (1 ml) and diethylsulfide (2 ml) at 0° for 30 min. HF was then evaporated, using a water aspirator followed by a high vacuum pump, to leave a gelatinous residue which was dissolved in 0.5 M AcOH (20 ml). The solution was washed three times with peroxide-free Et_2O and lyophilized. The ensuing solid material was dissolved in 0.5 M AcOH and passed through a 2.5 x 100 cm column of Sephadex G-15. The fractions of the main peak (280 nm detection) were pooled and lyophilized to yield a crude product, **1a** (100 mg, 84%), which was further purified by reversed phase liquid chromatography, see below.

Protective group cleavage by sodium in liquid ammonia

Protected 31-peptide, **10** (0.95 g, 0.187 mmol) was dissolved in refluxing anhydrous liquid NH_3 (1000 ml) which was agitated by a magnetic stirrer. Sodium (650 mg, 28 mmol, *ca.* 10-fold excess) was added in portions over a 45-min period. After 15 more min NH_4Cl (1.6 g, 30 mmol) was added and the mixture allowed to concentrate to 50 ml by spontaneous evaporation of NH_3. The mixture was then frozen in a liquid N_2 bath and freeze-dried by connection to a water aspirator via a drying tube filled with KOH pellets. The residual white fluffy residue was further dried in a vacuum desiccator (P_2O_5–KOH). Desalting of this material by passage through a 2.5 x 100 cm column of Sephadex G-10 in 0.01 N HCl provided a crude product, **1b** (610 mg, 87%, based on a mol. wt. of 3755 for β_h-EP)* which was further purified by reversed-phase liquid chromatography, see below.

* Molecular weight of lyophilized βh-EP was estimated at 3755 daltons, based on a peptide of ~92%.

Purification by liquid chromatography

HCl-acetonitrile system (UV-clear System: Gabriel *et al.*, 1979). In a typical run, crude β_h-EP (150 mg) was dissolved in 0.01 N HCl (5 ml) and injected or pumped on a 3.5 x 44 cm LiChroprep RP-8 Lobar column. The mobile phase program consisted of successive 500-ml portions of 0.01 N HCl, 10%, 12% and 15% acetonitrile in 0.01 N HCl, followed by a linear gradient from 15 to 25% acetonitrile in 0.01 N HCl during which the major peak emerged from the column (monitored at λ 254 nm). The chromatogram was completed by a step gradient of 500 ml each of 25%, 40%, and 90% acetonitrile in 0.01 N HCl (Fig. 1c, top). Each 20-ml fraction was analyzed by injecting 100 μl onto the analytical column (Fig. 1c, bottom). Pooling limits for clean and contaminated fractions were set based on these analytical chromatograms.

The bulk of the β_h-EP was found in the 15–25% acetronitrile eluted peak. A typical run produced 27 mg of β_h-EP, 68 mg of β_h-EP contaminated with other species, and 50 mg of non-EP material. The appropriate fractions were pooled and concentrated each to a small volume (*ca.* 1/3) to remove most of the organic modifier. The residual aqueous solution was then diluted two-fold and lyophilized to a light yellow residue. This was passed through a Sephadex G-10 column (2.5 x 100 cm) in 0.01 N HCl. The fractions from a single major peak were pooled and lyophilized to yield β_h-EP as a white product (27 mg, 18%).

The combined pooled side fractions (400 mg) from eight Lobar separations, described above, were chromatographed in a single run on a Waters Prep 500 instrument, using a Prep Pak C-18 cartridge with a similar step gradient, to produce 132 mg homogeneous β_h-EP and 33 mg contaminated β_h-EP. Overall yield of purified human β-EP from 1.2 g crude product was about 348 mg (29%).

The somewhat cumbersome gradient elution program of the HCl–acetonitrile system was found empirically to give larger amounts of "clean" β_h-EP than either continuous gradient or isocratic elutions. Typical chromatograms of a preparative run and analytical runs on the ascending limb, center, and descending limb of the β_h-EP peak are shown in Fig. 1c.

Pyridine–acetate system (Meienhofer *et al.*, 1979). In a typical run, crude β_h-endorphin (**1a**, after HF treatment: 76 mg) was dissolved in 0.5 ml of 0.01 N HCl and loaded onto an 0.9 × 50 cm ODS-2 column which had been equilibrated with 10% of a 1:1 (v/v) mixture of isopropanol and acetonitrile in aqueous 8% pyridine – 2.6% AcOH. Elution was carried out at 4 ml/min for one column volume, followed by 24% of 1:1 isopropanol–acetonitrile until the β_h-EP (peak V) eluted. Finally a gradient to 75% of 1:1 isopropanol–acetonitrile was run to remove the less polar materials (peak VI). The elution is shown in Fig. 1a. Appropriate fraction pools were made, diluted with 2–3 vol. H_2O and lyophilized. Combined pools, I–IV weighed 26 mg, pool V, 12 mg (β_h-EP) and VI 21.5 mg. Fig. 1d shows an analytical chromatogram of the pool V. Total recovery of material was 59.5 mg, 78% of the load.

Physicochemical characterization

Paper electrophoresis of synthetic material (100-μg samples) on Whatman 3 MM at pH 3.5 (pyridine acetate buffer) and 6.5 (collidine acetate buffer) for 3 h at 800 V each showed a single spot (fluorescamine detection) with R_f Lys values of 0.60 and 0.44, respectively, identical with authentic material. Thin-layer chromatography of synthetic material (50 μg) on silica gel in *n*-butanol–pyridine–acetic acid–water (5:5:1:4) showed a single spot with R_f 0.5 (ninhydrin and Cl_2-tolidine). Amino acid analysis (Spackman *et al.*, 1958) of synthetic material (0.6 mg) after 24 h hydrolysis in constant boiling HCl–phenol gave values shown in Table 1. For total enzyme digestion synthetic material (1 mg) was treated in 0.28 ml of 0.05 M Tris buffer (pH 8; 0.01 M Mg^{2+}) with 14 μg each of trypsin (Worthington) and chymotrypsin (Worthington) for 24 h at 37°. The solution was heated at 100° for 15 min, cooled, and treated with 28 μg leucine aminopeptidase (Worthington) at 37° for 48 h. After treatment with 1 N NaOH (35 μl) and lyophilization, amino acid analysis gave the results shown in Table 1.

Anal. calc. for $C_{158}H_{251}N_{31}O_{46}S \cdot 5HCl \cdot 6H_2O$ (3755.46): C, 50.53; H, 7.19; N, 14.55; Cl, 4.72; S, 0.85. Found: C, 50.10; H, 7.12; N, 14.14; Cl, 4.93; S, 0.97; H_2O (Karl Fisher), 2.74.

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