Reaction of sulphonium salt **8** *with benzenethiolate giving* **10a** *and* **10b**

A solution of **8** (0.1 g, 0.28 mmol) in methanol (5 ml) was added to a stirred solution of benzenethiol (0.034 g, 0.31 mmol) and KOH (0.025 g, 0.45 mmol). After 2 h, water was added and work-up with ether followed by TLC afforded 95 mg (88%) of **10**. ^{1}H NMR: δ 3.10 (AB quartet, CH_2S); 2.9–3.6 (m, CHS).

Methyl 1,4-dioxaspiro[4,5]decane-8-carboxylate (**14**)

A solution of ketone **13** (10.5 g, 0.067 mol), prepared according to *Jung*[10], glycol and *p*-toluenesulphonic acid (0.1 g) was refluxed in 300 ml of benzene with continuous removal of water. When no more water separated the cooled solution was washed with bicarbonate and water. After drying and concentration, distillation afforded 12.5 g (93%) of **14**. B.p. (17 mm) 138–140°C. IR: 1740 cm^{-1}. ^{1}H NMR: δ 3.63 (s, CH_3O); 3.90 (s, dioxolanylidene).

8-(Hydroxymethyl)-1,4-dioxaspiro[4.5]decane (**15**)

A solution of ester **14** (10.0 g, 0.05 mol) in 50 ml of ether was added drop by drop to a stirred suspension of $LiAlH_4$ (1.75 g, 0.046 mol) in 200 ml of ether (under nitrogen). After the addition was complete, the mixture was refluxed for 1 h. The excess $LiAlH_4$ was destroyed by the subsequent addition of 1.75 ml of water, 1.75 ml of 15% NaOH and 1.75 ml of water. After filtration the resulting solution was washed with water and brine. Drying and concentration followed by distillation afforded 7.6 g (90%) of **15**. B.p. (8 mm) 138–142°C. IR: 3450 cm^{-1}. ^{1}H NMR: δ 3.90 (s, dioxolanylidene); 3.40 (d, CH_2OH).

8-(Bromomethyl)-1,4-dioxaspiro[4,5]decane (**16**)

To a solution of CBr_4 (20.3 g, 0.061 mol) (dried before use) and alcohol **15** (7.0 g, 0.04 mol) in 100 ml of benzene was added $P(C_6H_5)_3$ (7 g, 0.44 mol) in benzene. The mixture was stirred for 2 h and allowed to stand overnight. Pentane was added and the precipitated $O{=}P(C_6H_5)_3$ was removed by filtration. Concentration followed by chromatography on silica gel using hexane/ethyl acetate (3/1) as eluent gave 6.8 g (69%) of **16**. ^{1}H NMR: δ 3.90 (s, dioxolanylidene); 3.28 (d, CH_2Br).

8-(Phenylthiomethyl)-1,4-dioxaspiro[4,5]decane (**17**)

A solution of bromide **16** (6.5 g, 0.027 mol) was added to a sodium benzenethiolate solution (prepared *in situ* by the subsequent addition of Na (0.7 g, 0.030 mol) and benzenethiol (3.04 g, 0.033 mol)) in ethanol (50 ml). The mixture was left overnight, water was added and normal ethereal work-up afforded 8.5 g of **17** which was used without purification. ^{1}H NMR: δ 2.85 (d, CH_2S); 3.90 (s, dioxolanylidene); 7.1–7.5 (m, Ar).

4-(Phenylthiomethyl)cyclohexanone (**18**)

Sulphide **17** (8.5 g, crude product) was dissolved in a mixture of 200 ml of acetone and 75 ml of 10% HCl. The solution was refluxed for 1 h to afford after work-up with ether and chromatography on silica gel ($CHCl_3$ as eluent) 5.1 g of **18** (84% on the basis of bromide **16**). IR: 1710 cm^{-1}). ^{1}H NMR: δ 2.92 (d, CH_2S); 7.1–7.5 (m, Ar).

2-(4,8-Dimethylnona-3,6-dienyl)-4-(phenylthiomethyl)cyclohexanone (**19**)

Compound **19** was prepared in 35% yield by the method of *Harding* et al.[9]. The *cis* and *trans* isomers were formed in about equal amounts. Isomerization of 1 g of ketone in 50 ml of *t*-BuOH containing 0.1 g of *t*-BuOK for 2.5 h changed the ratio to 1/5 in favour of the *cis* isomer. Pure products were obtained by chromatography on silica gel using ethyl acetate/hexane (1/4) as eluent. ^{1}H NMR: *trans:* δ 1.58, 1.67, 5.02, 2.93 (d, CH_2S); 7.1–7.5 (m, Ar); *cis:* as for *trans* with 2.85 (d) instead of 2.93.

2-t-(4,8-Dimethylnona-3,6-dienyl)-4-c-bis(phenylthiomethyl)-1-r-cyclohexanol (**20**)

Prepared as for **2a** and **2b** from *cis* **19** in 43% yield. ^{1}H NMR: δ 1.58, 1.67, 5.02, 7.1–7.5 (as for **19**); 2.83 (d, CH_2S); 3.12 (AB quartet, CH_2S).

1-n-Bromo-2-t-(dimethylnona-3,8-dienyl)-4-c-bis(phenylthiomethyl)cyclohexane (**21**)

Prepared as for **7a** and **7b** (quantitatively). ^{1}H NMR: δ 1.58, 1.67, 5.02, 7.1–7.5 (as for **20**), 2.83 (d, CH_2S); 3.68 (AB quartet, CH_2S).

Formation of a bridged sulphonium ion (**12**) *from an open carbenium ion*

To a stirred solution of CH_3MgI, prepared from Mg (0.5 g, 0.021 mol) and CH_3I (2.5 g, 0.018 mol), in 20 ml of ether was added a solution of **18** (1 g, 0.0045 mol) in ether (5 ml). After 1 h at room temperature the mixture was poured over ice. Work-up of the ethereal extract afforded 1.1 g (quantitatively) of a mixture of (*cis* and *trans*) alcohols. ^{1}H NMR: δ 1.20 and 1.22 (s, CH_3); 2.83 (broad, CH_2S); 7.1–7.5 (m, Ar). The formation of a bridged sulphonium ion (**12**) from an open carbenium ion could be demonstrated by the reaction of a mixture of alcohols prepared from ketone **18**, with perchloric acid in CD_3NO_2. ^{1}H NMR: δ 7.77 (m, phenyl H); 3.87 (d, *J* 3 Hz CH_2 next to S); 1.21 (CH_3).

[9] *K. E. Harding, R. C. Ligon, C. Tsench* and *L. Wu*, J. Org. Chem. **38**, 3478 (1973).

[10] *M. E. Jung* and *C. A. McCombs*, Tetrahedron Lett. 2935 (1976).

Synthesis of fragments of human β-lipotropin, β_h-LPH. Part II†. The synthesis of β_h-LPH-(61–91), β_h-endorphin

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Abstract. The classical fragment condensation approach was used to synthesize the hentriacontapeptide human β-endorphin [β_h-LPH-(61–91)].

Introduction

Approximately at the same time but independently, several groups of investigators[1-6] were involved in isolating peptides, which showed opiate-like activity, from brain and pituitary tissue.

† Fort part I see: Recl. Trav. Chim. Pays-Bas **98**, 571 (1979).

[1] *A. F. Bradbury, D. G. Smyth, C. R. Snell, N. J. M. Birdsall* and *E. C. Hulme*, Nature **260**, 793 (1976).

[2] *R. Guillemin*, C.R. Acad. Sci., Ser. D, **282**, 783 (1976).

[3] *C. H. Li* and *D. Chung*, Proc. Natl. Acad. Sci. U.S.A. **73**, 1145 (1976).

[4] *A. Goldstein*, Science **193**, 1081 (1976).

[5] *M. Chrétien, S. Benjannet, N. Dragon, N. G. Seidah* and *M. Lis*, Biochem. Biophys. Res. Commun. **72**, 472 (1976).

[6] *L. Gráf, E. Barat* and *A. Patthy*, Acta Biochim. Biophys. Acad. Sci. Hung. **11**, 121 (1976).

The term endorphin, coined by Eric Simon (mentioned in references 2 and 4), was readily accepted as a generic descriptor for this family of endogenous peptides which interact with receptors for which morphine has a strong affinity.

β-Endorphin, a peptide with 31 amino acid residues corresponding to the C-terminal fragment of β-lipotropin, was isolated by *Bradbury* et al.[7] and *Gráf* et al.[6] from porcine pituitary glands, by *Li* and *Chung* first from camel[3] and then from human[8] and bovine[9] pituitaries, and by *Seidah* et al.[10] from sheep pituitaries. With the help of pulse-chase labelling experiments, *Chrétien* et al. demonstrated that β-lipotropin may function as a prohormone for β-endorphin[11]. In addition to the opiate-like activities, *De Wied* et al. found that endorphins induce behavioural effects, which, as extra-morphine-like activities, are not antagonized by naloxone[12,13].

In this paper, the second of a series, the synthesis of human β-endorphin is described, making use of the classical fragment condensation approach. A preliminary communication appears in the Proceedings of the XVth European Peptide Symposium[14].

Strategy of the synthesis

Solid phase syntheses of human β-endorphin have been described by *Li* et al.[15] and *Coy* et al.[16], making use of a 1% cross-linked *Merrifield* resin[17] and by *Atherton* et al.[18] who use an amorphous copolymer of *N,N'*-dimethylacrylamide, *N,N'*-diacryloylethylenediamine and *N*-acryloyl-*N'*-(*tert*-butoxycarbonyl)-β-alanylhexamethylenediamine[19].

We preferred the classical fragment condensation approach for reasons described in part I, and were able to obtain the 31-peptide 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 and several of its fragments in satisfactory yield and of good quality*.

For general remarks concerning the strategy, see part I†. Our route to β-endorphin has been planned in such a way that the three main, protected fragments 61–69, 70–77 and 78–91 could be used in the synthesis of γ-endorphin and β-endorphin. Since the synthesis of the protected fragments 61–69 and 70–77 has already been described (part I), we will only give details of the synthesis of protected 78–91 and the final assembly of the fragments leading to β_h-LPH-(61–91) in this paper.

After our work was completed, the solid-phase synthesis of β_h-endorphin making use of base-labile Fmoc-amino acids was reported[20,21] as well as two syntheses of β_h-endorphin via classical solution techniques[22,23]; these approaches, however, were different from our own.

Description and discussion of the synthesis

Figure 1 shows the sequence of fragment 78–91. It should be noted that the sequence -Lys-Asn-Ala- is present twice, and we used this in the planning of the synthesis. Four protected fragments, i.e. 78–81, 82–83, 84–86 and 87–91 were prepared.

Our initially chosen route is shown in the upper part of the figure: a coupling of fragments 82–86 and 87–91. We had to depart from this approach for reasons mentioned below. Saponification of the rather insoluble pentapeptide Z-Ile-Ile-Lys(Boc)-Asn-Ala-OMe gave rise to the rapid formation of by-products. Conversion into the corresponding hydrazide was also not satisfactory. Saponification of the tripeptide Z-Lys(Boc)-Asn-Ala-OMe with aqueous NaOH was not successful; addition of a large excess of a Na_2CO_3 solution to a solution of the tripeptide in DMF, however, gave the protected tripeptide acid in about 50–60% yield. The reaction of Z-Ile-Ile-OH with H-Lys(Boc)-Asn-Ala-OH, after pre-activation with DCC and HOOBt, was not satisfactory, and we decided to change our strategy in the following way: Z-Lys(Boc)-Asn-Ala-OH was built up starting with free alanine using active esters, and was then coupled with the C-terminal pentapeptide H-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu (also obtained via a stepwise procedure) using DCC and HOBt: nearly 80% of pure octapeptide, sequence 84–91, was obtained (see Fig . 2). Hydrogenation, followed by acylation of the resulting product with Z-Ile-Ile-OH gave, again in good yield, the protected decapeptide 82–91.

† For part I see: Recl. Trav. Chim. Pays-Bas **98**, 571 (1979).

* Standard abbreviations are used for amino acids and protecting groups [IUPAC-IUB Commission on Biochemical Nomenclature, Biochem. J. **126**, 773 (1972)]. Other abbreviations are: DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; DMF, *N,N'*-dimethylformamide; EtOAc, ethyl acetate; Fmoc, 9-fluorenylmethyloxycarbonyl; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HOOBt, 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine; LAO, L-amino acid oxidase; NEM, N-ethylmorpholine; MeOH, methanol; Pd/C, 10% palladium on charcoal; pet. ether, petroleum ether 40–60; TLC, thin-layer chromatography; TFA, trifluoroacetic acid; TMAH, tetramethylammonium hydroxide.

[7] *A. F. Bradbury, D. G. Smyth* and *C. R. Snell*, Proceedings of the IVth Amer. Peptide Symposium, eds. *R. Walter* and *J. Meienhofer*, Ann Arbor Sci. Publ. Inc., Ann Arbor, 1975, p. 609.

[8] *C. H. Li, D. Chung* and *B. A. Doneen*, Biochem. Biophys. Res. Commun. **72**, 1542 (1976).

[9] *C. H. Li, L. Tan* and *D. Chung*, Biochem. Biophys. Res. Commun. **77**, 1088 (1977).

[10] *N. G. Seidah, N. Dragon, S. Benjannet, R. Routhier* and *M. Chrétien*, Biochem. Biophys. Res. Commun. **74**, 1528 (1977).

[11] *M. Chrétien, P. Crine, M. Lis, S. Benjannet* and *N. G. Seidah* in Central Nervous System Effects of Hypothalamic Hormones and other Peptides (ed. *R. Collu*), Raven Press, New York, 1979, p. 237.

[12] *D. de Wied, B. Bohus, J. M. van Ree* and *I. Urban*, J. Pharmacol. Exp. Ther. **204**, 570 (1978).

[13] *D. de Wied, G. L. Kovács, B. Bohus, J. M. van Ree* and *H. M. Greven*, Eur. J. Pharmacol. **49**, 427 (1978).

[14] *W. A. A. J. Bijl, J. W. van Nispen* and *H. M. Greven* in Peptides 1978, Proceedings of the XVth European Peptide Symposium, Gdánsk, Poland (eds. *I. Z. Siemion* and *G. Kupryszewski*), Wroclaw Univ. Press, Wroclaw, 1979, p. 569.

[15] *C. H. Li, D. Yamashiro, L. F. Tseng* and *H. H. Loh*, J. Med. Chem. **20**, 325 (1977).

[16] *D. H. Coy, P. Gill, A. J. Kastin, A. Dupont, L. Cusan, F. Labrie, D. Britton* and *R. Fertel*, Proceedings of the Vth Amer. Peptide Symposium, eds. *M. Goodman* and *J. Meienhofer*, J. Wiley & Sons, New York, 1977, p. 107.

[17] *R. B. Merrifield*, J. Am. Chem. Soc. **85**, 2149 (1963).

[18] *E. Atherton, M. Caviezel, H. Over* and *R. C. Sheppard*, J. Chem. Soc. Chem. Commun. 819 (1977).

[19] *E. Atherton, D. L. J. Clive, D. A. East* and *R. C. Sheppard* in Peptides 1976, Proceedings of the XIVth European Peptide Symposium, ed. *A. Loffet*, Editions de l'Université de Bruxelles, 1976, p. 291; J. Am. Chem. Soc. **97**, 6584 (1975).

[20] *E. Atherton, H. Fox, D. Harkiss* and *R. C. Sheppard*, J. Chem. Soc. Chem. Commun. 539 (1978).

[21] *J. Meienhofer* and *C. D. Chang* in Peptides 1978, Proceedings of the XVth European Peptide Symposium, Gdánsk, Poland (eds. *I. Z. Siemion* and *G. Kupryszewski*), Wroclaw Univ. Press, Wroclaw, 1979, p. 573.

[22] *M. Kubota, T. Hirayama, O. Nagase* and *H. Yajima*, Chem. Pharm. Bull. **26**, 2139 (1978).

[23] *C. Tzougraki, R. C. Makofske, T. F. Gabriel, S. S. Wang, R. Kutny, J. Meienhofer* and *C. H. Li*, J. Am. Chem. Soc. **100**, 6248 (1978).

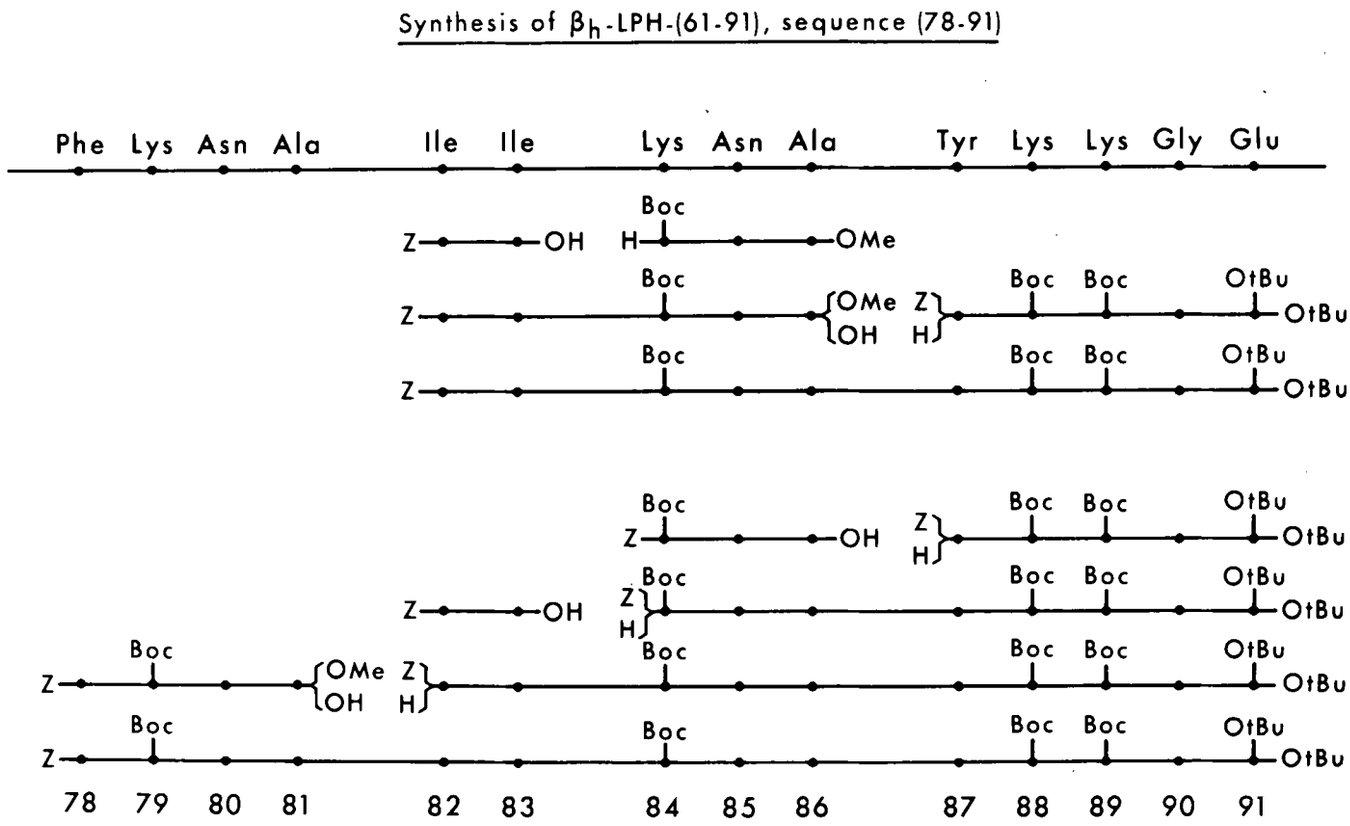

Fig. 1. Schematic representation of the initially chosen route to synthesize protected fragment 78–91 (upper part), and the finally adapted one (lower part).

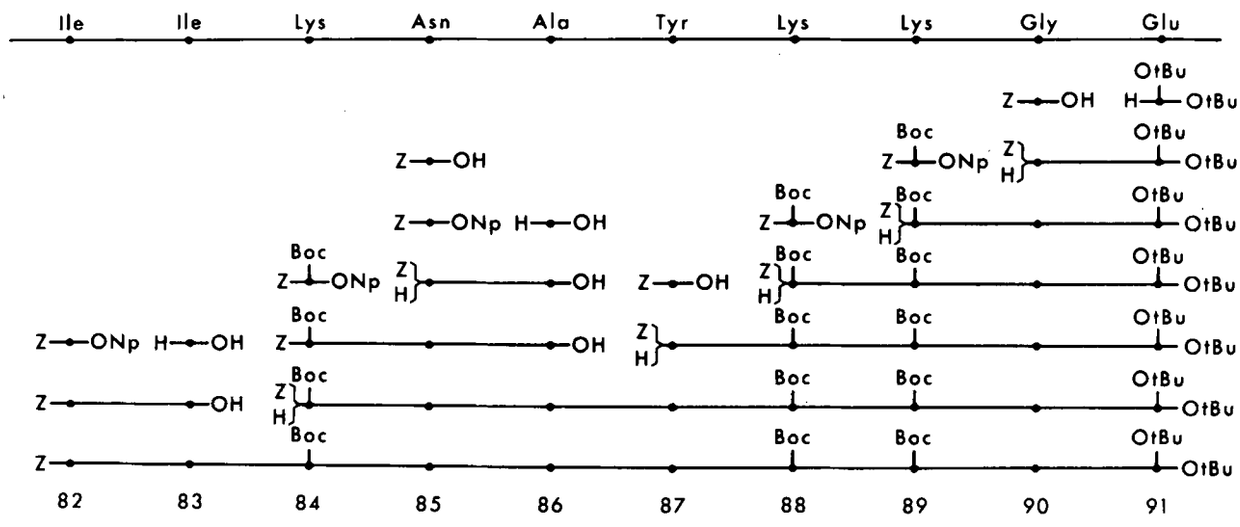

Fig. 2. Detailed representation of the synthesis of protected fragment 82–91.

Z-Phe-Lys(Boc)-Asn-Ala-OMe, sequence 78–81, was built up stepwise. The saponification of the methyl ester with aqueous K_2CO_3 in DMF gave the corresponding acid in good yield and quality. The fragments Z-(78–81)-OH and H-(82–91)-OtBu (the latter was obtained after hydrogenation of Z-(82–91)-OtBu in DMF) were condensed using DCC and HOBt. The final assembly of the three main, protected fragments 61–69, 70–77 and 78–91 is given in Figure 3. The C-terminal 14-peptide was hydrogenated in 1-methyl-2-pyrrolidone instead of DMF since the solubility in the latter solvent was too low. The resulting product was acylated with Z-(70–77)-OH, which was obtained from its -OtBu ester after treatment with trifluoroacetic acid; DCC and HOBt served as the condensing agents, to give 78% of the 22-peptide.

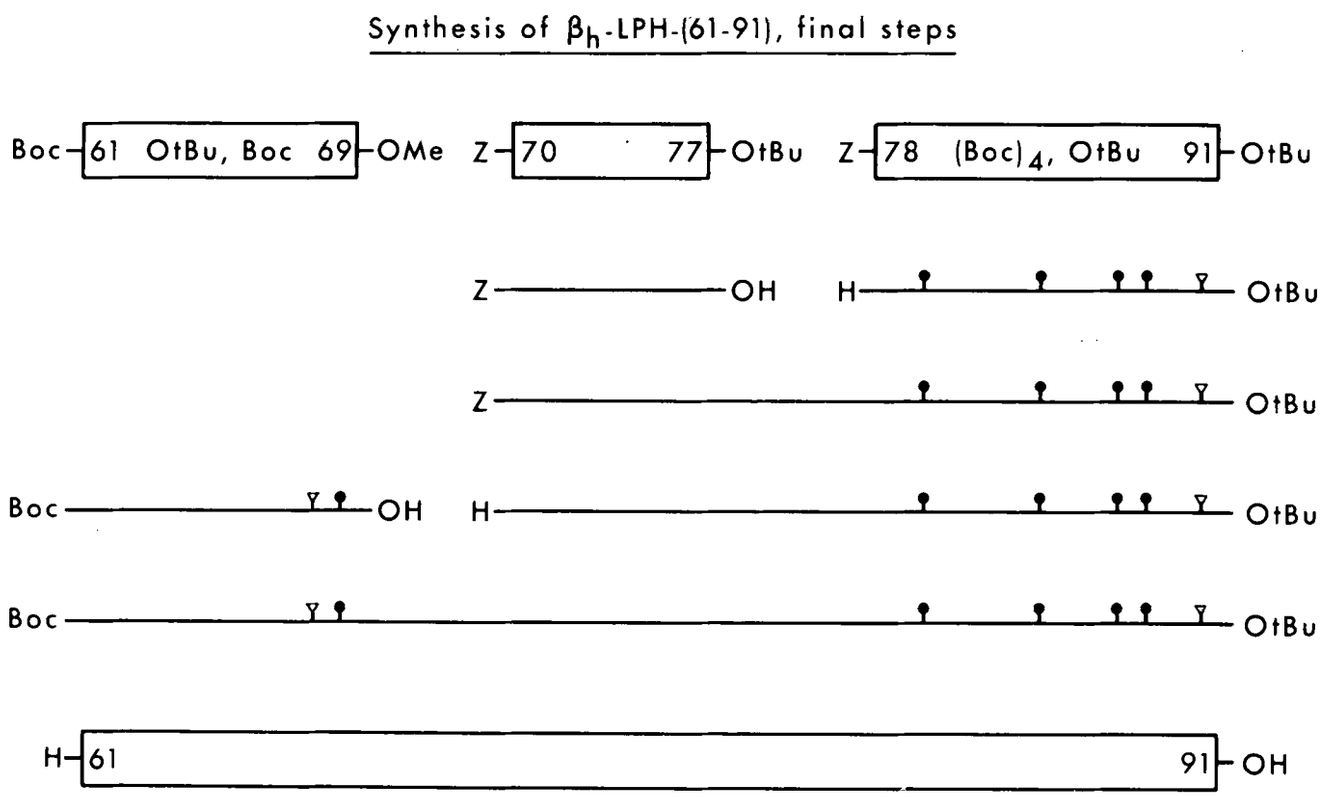

Fig. 3. The final steps in the synthesis of human β-endorphin (♀ represents a N^{ε}-tert-butyloxycarbonyl group of a lysine residue, ▽ a γ-tert-butyl ester function of a glutamic acid residue).

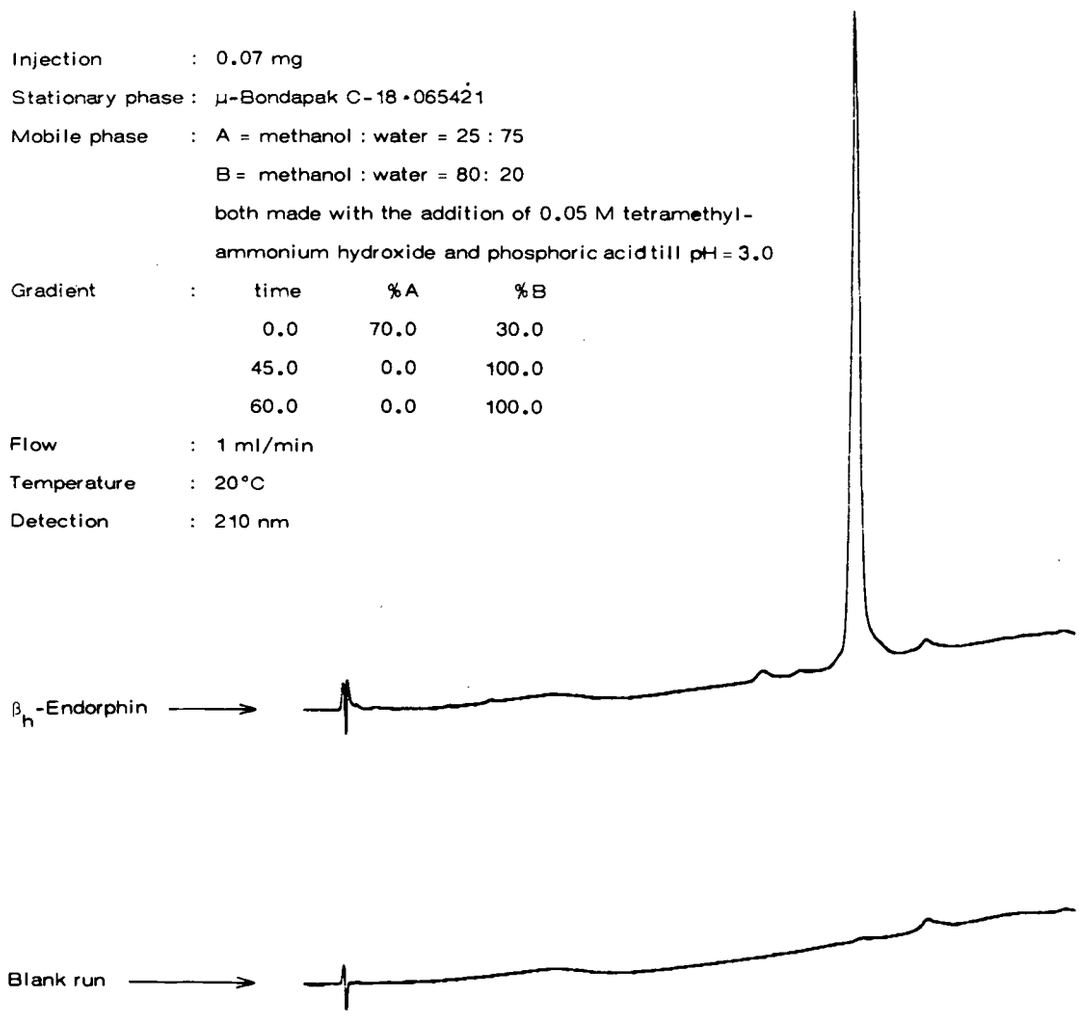

Fig. 4. HPLC elution profile of β_h-endorphin.

The Z group in protected 70–91 was again removed by hydrogenolysis in 1-methyl-2-pyrrolidone. The final coupling reaction between Boc-(61–69)-OH (see part I) and H-(70–91)-OtBu was mediated by DCC and HOBt in a mixture of 1-methyl-2-pyrrolidone and DMF. After the reaction, the 31-peptide was isolated by precipitation with water and purified by the addition of ethyl acetate to a solution of the peptide in DMF/ethanol (15/10, v/v); 65% of protected β_h-endorphin with the correct amino acid composition was obtained. Deprotection with trifluoroacetic acid in the presence of *tert*-butyl sulfide under N_2 was followed by the exchange of the trifluoroacetate salt into the acetate salt. Several purification methods were attempted (e.g. counter current distribution, Sephadex G-25) but the best results were obtained with column chromatography on a Merck Fertigsäule, Kieselgel 60, with the eluent 1-butanol/pyridine/acetic acid/water = 8/3/1/4. The purity of β_h-endorphin was assessed using TLC, HPLC (a typical run is shown in Fig. 4), circular paper chromatography, electrophoresis, amino acid analysis and L-amino acid oxidase (the L-amino acids Tyr, Phe, Met, Lys, Leu, Val, Ala, Ile are converted into their corresponding α-keto acids by this enzyme; the conversions of Thr, Ser, Glu, Pro and Asp are not reproducible).

Experimental section

For details of the methods, solvent systems etc., see part I*.

Synthesis of Z-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu, sequence 87–91, Fig. 2

Z-Lys(Boc)-Gly-Glu(OtBu)-OtBu (89–91). To a solution containing 37.2 g (118 mmol) of H-Gly-Glu(OtBu)-OtBu[24] in 400 ml of DMF, were added 56.0 g (112 mmol) of Z-Lys(Boc)-ONp. The solution was stirred at room temperature for 24 h, and then evaporated to dryness. The residue was dissolved in EtOAc (400 ml) and the solution was extracted successively with 5% $KHSO_4$, 5% $NaHCO_3$ and saturated NaCl solutions. After drying (Na_2SO_4) the solution was concentrated to about 150 ml and 100 ml of pet. ether were added. The precipitate was filtered off, and triturated with ether/pet. ether (2/1, v/v).
Yield 48.7 g (64.2%); m.p. 76–78°C; $[\alpha]_D^{21}$ −15.0° (*c* 1, DMF). TLC: R_f 0.44 (b).

Z-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu (88–91). 34.1 g (68.0 mmol) of Z-Lys(Boc)-ONp and 39.0 g (71.7 mmol) of H-Lys(Boc)-Gly-Glu(OtBu)-OtBu [obtained after hydrogenation of 48.7 g (71.7 mmol) of Z-Lys(Boc)-Gly-Glu(OtBu)-OtBu in DMF with Pd/C as the catalyst] were coupled in DMF (250 ml) and the reaction mixture was worked up as described for 89–91.
Yield 54.6 g (88.0%); m.p. 101–102°C; $[\alpha]_D^{21}$ −7.9° (*c* 1, DMF). TLC: R_f 0.45 (b).

Z-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu (87–91). To a cooled (−20°C), stirred solution containing 18.4 g (58.5 mmol) of Z-Tyr-OH, 45.4 g (one equivalent) of H-Lys(Boc)-Lys(Boc)-Gly-Glu-(OtBu)-OtBu (prepared by hydrogenation of the corresponding Z-peptide in MeOH) and 7.90 g (58.5 mmol) of HOBt in 400 ml of DMF, DCC was added (58.5 mmol, 12.1 g). After 30 min at −15°C and 4 days at +4°C, the filtered solution was evaporated to dryness. The oily residue was dissolved in EtOAc and extracted as described for 89–91. After drying and concentrating of the solution, crystallization was achieved by the addition of pet. ether.
Yield 43.1 g (68.7%); m.p. 168–169°C; $[\alpha]_D^{22}$ −12.1° (*c* 1, DMF). TLC: R_f 0.34 (b).

Synthesis of Z-Ile-Ile-Lys(Boc)-Asn-Ala-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu, sequence 82–91, Fig. 2

Z-Asn-Ala-OH (85–86). 25.5 g (286 mmol) of H-Ala-OH were dissolved in a solution containing 48.1 g (573 mmol) of $NaHCO_3$ in 400 ml of H_2O. A solution of 122 g (315 mmol) containing Z-Asn-ONp in 500 ml of DMF was added and the mixture was stirred for two days at room temperature. The filtered solution was then evaporated to dryness and the residue was crystallized from EtOAc. The precipitate was filtered off and triturated with water.
Yield 30.0 g (31.1%); m.p. 217–219°C (dec.); $[\alpha]_D^{21}$ +1.1° (*c* 1, DMF). TLC: R_f 0.17 (a).

Z-Lys(Boc)-Asn-Ala-OH (84–86). Z-Asn-Ala-OH (30.0 g, 88.9 mmol) was dissolved in 300 ml of DMF and 50 ml of H_2O. After the addition of Pd/C, hydrogen was bubbled through the solution overnight. The next morning 90 ml of acetic acid were added to dissolve the partially precipitated H-Asn-Ala-OH. Then the catalyst was removed by filtration, the filtrate evaporated and the residue was triturated with EtOAc. After drying, 16.3 g (80.0 mmol) of H-Asn-Ala-OH were dissolved in 500 ml of DMF/H_2O (1/1, v/v) and to this solution were added NEM (1.03 equiv. giving a pH of 7.2) and 42.2 g (84.1 mmol) of Z-Lys(Boc)-ONp. After 24 h of stirring at room temperature, DMF was added to the solution until clear (500 ml). After standing for another night, the solution was evaporated to dryness and the concentrate was dissolved in DMF (150 ml); water was added and the precipitate was filtered and dried. Recrystallization was performed from DMF/EtOAc.
Yield 36.6 g (80.9%); m.p. 158°C (dec.); $[\alpha]_D^{21}$ −11.0° (*c* 1, DMF). TLC: R_f 0.36 (a).

Z-Lys(Boc)-Asn-Ala-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu (84–91). 2.83 g (5.00 mmol) of Z-Lys(Boc)-Asn-Ala-OH and H-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu [prepared by hydrogenation of Z-87–91-OtBu (5.35 g, 5.00 mmol) in 100 ml of MeOH with Pd/C as the catalyst] in DMF (50 ml) were coupled by the DCC/HOBt method. The filtered solution was evaporated, the residue dissolved in *sec*-butyl alcohol/chloroform (2/3, v/v) and the solution was extracted and worked up as described for fragment 89–91. The peptide was crystallized from isopropanol.
Yield 4.43 g (59.7%); m.p. 209–210°C (dec); $[\alpha]_D^{21}$ −26.2° (*c* 1, DMF). TLC: R_f 0.28 (b).

Z-Ile-Ile-Lys(Boc)-Asn-Ala-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu (82–91). 510 mg (1.35 mmol) of Z-Ile-Ile-OH[25] and 1.82 g (one equiv.) of H-84–91-OtBu (obtained after hydrogenation of the corresponding Z-compound in DMF) were coupled in DMF by the DCC/HOBt method. After removal of the precipitated DCU, the filtrate was added dropwise to 150 ml of MeOH, the precipitate was filtered, washed with MeOH and dried.
Yield 1.68 g (72.9%); m.p. >250°C; $[\alpha]_D^{21}$ −27.5° (*c* 1, DMF). TLC: R_f 0.24 (b).
Amino acid analysis (hydrolysis time 96 h): Asp 1.00, Glu 1.01, Gly 1.03, Ala 1.04, Ile 1.90, Tyr 1.02, Lys 2.98. Peptide content 99.6%.

Synthesis of Z-Phe-Lys(Boc)-Asn-Ala-Ile-Ile-Lys(Boc)-Asn-Ala-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu, sequence 78–91, Fig. 1

Z-Lys(Boc)-Asn-Ala-OMe (79–81). 24.8 g (49.5 mmol) of Z-Lys-(Boc)-ONp and 6.64 g (30.6 mmol) of H-Asn-Ala-OMe (obtained after hydrogenation of Z-Asn-Ala-OMe[26] in DMF) were coupled by the p-nitrophenyl ester method. After evaporation of the solvent, the residue was triturated with EtOAc. The precipitate was then dissolved in DMF and the solution was added to EtOAc; recrystallization from DMF/EtOAc gave 13.5 g (76.2%) of pure tripeptide with m.p. 181.5–182.5°C and $[\alpha]_D^{20}$ −16.7° (*c* 1, DMF). TLC: R_f 0.77 (a).

Z-Phe-Lys(Boc)-Asn-Ala-OMe (78–81). 10.1 g (24.0 mmol) of Z-Phe-ONp and 10.2 g (22.9 mmol) of H-Lys(Boc)-Asn-Ala-OMe (obtained after hydrogenation of Z-79–81-OMe in DMF) were coupled in the same way as described for Z-79–81-OMe. The crude product was triturated with EtOAc to give a yield of 94.6% (15.7 g). M.p. 221°C; $[\alpha]_D^{20}$ −14.9° (*c* 1, DMF). TLC: R_f 0.75 (e).

Z-Phe-Lys(Boc)-Asn-Ala-OH (78–81). 10.5 g (14.4 mmol) of Z-78–81-OMe were dissolved in DMF (500 ml). To this solution 500 ml of 10% aqueous K_2CO_3 were added with stirring. After 1 h at room temperature, the pH was carefully adjusted to 2 with 4N HCl, resulting in the precipitation of Z-Phe-Lys(Boc)-Asn-Ala-OH. The precipitate was filtered, washed thoroughly with water, and dried.

* See preceeding paper in this issue.

[24] *B. Kamber*, Helv. Chim. Acta **54**, 398 (1971).
[25] *Y. Okumura* and *A. Sakurai*, Bull. Chem. Soc. Japan **46**, 2190 (1973).
[26] *M. Zaoral*, Coll. Czech. Chem. Commun. **27**, 1273 (1962).

Yield 9.76 g (94.8%); m.p. 201–203°C; $[\alpha]_D^{21.5}$ −10.9° (*c* 1, DMF). TLC: R_f 0.31 (a).
The correct amino acid composition was found and no racemization (LAO digestion) was observed.

Z-Phe-Lys(Boc)-Asn-Ala-Ile-Ile-Lys(Boc)-Asn-Ala-Tyr-Lys(Boc)-Lys(Boc)-Gly-Glu(OtBu)-OtBu (78–91). 5.79 g (8.12 mmol) of Z-Phe-Lys(Boc)-Asn-Ala-OH and H-82–91-OtBu [obtained after hydrogenation of 14.62 g (8.55 mmol) of Z-82–91-OtBu in DMF] were coupled using the DCC/HOBt method as described before. After filtration of the DCU precipitate, the filtrate was poured into 2500 ml of MeOH and the resulting precipitate was filtered, washed with MeOH and dried.
Yield 12.27 g (66.5%); m.p. >250°C; $[\alpha]_D^{22}$ −12.6° (*c* 0.5, 1-methyl-2-pyrrolidone). TLC: R_f 0.99 (d).
Amino acid analysis (hydrolysis time 24 h; value for Ile after 96 h): Asp 1.98, Glu 1.01, Gly 1.01, Ala 2.02, Ile 1.84, Tyr 1.07, Phe 1.10, Lys 3.96. Peptide content 94.5%.

Synthesis of 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, β_h-endorphin, Fig. 3

Z-70–77-OH. 1.60 g (1.52 mmol) of Z-70–77-OtBu were dissolved in 20 ml of 90% aqueous TFA. After 30 min, the solution was poured into ether (210 ml) and the precipitate was filtered, washed with ether and dried.
Yield 1.25 g (82.8%); m.p. 117–119°C (dec.); $[\alpha]_D^{21}$ −38.8° (*c* 1, DMF). TLC: R_f 0.26 (a).

H-78–91-OtBu. 2.27 g (1.00 mmol) of Z-78–91-OtBu were dissolved in 200 ml of 1-methyl-2-pyrrolidone. Pd/C was added and H_2 was bubbled through the solution. The reduction was complete within 1½ h as judged by TLC. The filtered solution was concentrated in vacuo at 50°C to about 20 ml and used directly in the next coupling reaction.

Z-70–91-OtBu. 1.09 g (1.10 mmol) of Z-70–77-OH in DMF (20 ml) and the above-mentioned concentrated solution of H-78–91-OtBu were coupled using the DCC/HOBt method as described for Boc-61–77-OtBu (see part I). The filtered solution was poured into 800 ml of water, the precipitate isolated by filtration, washed with water, and dried. 2.57 g of crude Z-70–91-OtBu were then dissolved in 1-methyl-2-pyrrolidone/DMF (1/2, 30 ml total volume) and the solution was again added to water (750 ml). The precipitate was filtered, washed with water and dried.
Yield 2.42 g (77.8%); m.p. >250°C; $[\alpha]_D^{21.5}$ +1.5° (*c* 1, 1-methyl-2-pyrrolidone). TLC: R_f 0.30 (c).

Amino acid analysis (hydrolysis time 24 h; value for Ile after 96 h): Asp 2.03, Thr 1.83, Ser 0.90, Glu 1.96, Pro 0.99, Gly 1.07, Ala 2.02, Val 0.98, Ile 1.80, Leu 1.91, Tyr 0.95, Phe 1.00 and Lys 4.26. Peptide content 95.2%. LAO digestion: no racemization could be detected for Ala, Val, Ile, Leu, Tyr, Phe and Lys.

Boc-61–91-OtBu. 574 mg (0.45 mmol) of Boc-61–69-OH (see part I) and H-70–91-OtBu [prepared by hydrogenation of 0.45 mmol (1.40 g) of Z-70–91-OtBu in 1-methyl-2-pyrrolidone as described for Z-78–91-OtBu] were coupled in DMF/1-methyl-2-pyrrolidone using the DCC/HOBt method as described for Boc-61–77-OtBu (see part I of this series). After removal of the precipitated DCU the filtrate was poured into 300 ml of H_2O and the peptide was filtered, washed and dried.
1.70 g of crude, protected 61–91 were dissolved in 15 ml of DMF and 10 ml of ethanol (100%); 175 ml of EtOAc were then added and the resulting precipitate was filtered, washed with EtOAc and dried.
Yield 1.24 g (65.2%), $[\alpha]_D^{22}$ −0.98° (*c* 0.5 in DMF). TLC: R_f 0.71 (a).
Amino acid analysis (hydrolysis time 24 h; value for Ile after 96 h): Asp 1.98, Thr 2.88, Ser 1.87, Glu 3.05, Pro 0.98, Gly 3.16, Ala 2.10, Val 0.91, Met 0.97, Ile 1.90, Leu 1.86, Tyr 1.99, Phe 2.12, Lys 5.28, NH_3 3.30. Peptide content 96.4%.
Results of the LAO digestion: no racemization of any importance was detected (Tyr, Phe, Met, Lys, Leu, Val, Ala, Ile).

β_h-LPH-(61–91), β_h-endorphin. 1.18 g (0.28 mmol) of Boc-61–91-OtBu were treated with 20 ml of TFA/H_2O (9/1, v/v) under N_2 for 2 h, in the presence of a few drops of *tert*-butyl sulfide. The solution was poured into ether, the precipitate was filtered and dried, and then dissolved in *tert*-butanol/H_2O 1/1, v/v. After the exchange of TFA ions for acetate ions, the solution was lyophilized to yield 800 mg of crude H-61–91-OH. Purification of 136 mg of this material using column chromatography (Merck Fertigsäule, Kieselgel 60) with the solvent system 1-butanol/pyridine/acetic acid/water = 8/3/1/4, by volume, as the eluent, gave 53.8 mg (39.6%) of a product that was nearly homogeneous upon TLC (R_f 0.20 (e), 0.38 (solvent system 1-butanol/pyridine/acetic acid/water 38/24/8/30, by volume), circular paper chromatography on Whatman no 20 (solvent system 1-butanol/pyridine/acetic acid/water 39/20/6/24, by volume) and electrophoresis on cellogel sheets (Chemetron, Italy) at pH 1.9 and 6.4. $[\alpha]_D^{20}$ −74° (*c* 0.3 in 0.5 N HOAc).

Amino acid analysis (hydrolysis time 24 h; value for Ile after 96 h): Asp 1.98, Thr 2.92, Ser 1.84, Glu 3.08, Pro 1.00, Gly 2.98, Ala 2.08, Val 1.04, Met 0.90, Ile 1.84, Leu 1.89, Tyr 1.95, Phe 1.95, Lys 5.10. Peptide content 67%. No racemization of the amino acids Tyr, Phe, Met, Lys, Leu, Val, Ala and Ile could be detected after LAO digestion. HPLC: a linear gradient of 30%–100% B was run in 45 min followed by an isocratic elution (0% A–100% B) for another 15 min; main component 91.0% (see also Fig. 4).

H-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH, β_h-LPH-(78–91). Treatment of protected 78–91 with 90% aqueous TFA in the presence of anisole and isolation of H-78–91-OH as described in the previous paragraph, followed by counter current distribution in the solvent system 1-butanol/acetic acid/water (4/1/5, by volume).
Yield 232 mg (27.3%); $[\alpha]_D^{21}$ −63.8° (*c* 0.5, 10% aqueous HOAc). TLC: R_f 0.33 (1-butanol/pyridine/acetic acid/water 38/24/8/30, by volume; Nano plate SIL-20 from Machery-Nagel & Co).

Amino acid analysis (hydrolysis time 24 h; value for Ile after 96 h): Asp 1.98, Glu 1.02, Gly 1.04, Ala 2.03, Ile 1.99, Tyr 1.02, Phe 1.05, Lys 3.88. Peptide content 82.0%. No racemization was detected after a LAO digestion. HPLC: a linear gradient from 0–100% B was run in 15 min. (A = MeOH/H_2O 10/90, B = MeOH/H_2O 50/50, v/v, both with the addition of 0.05 M TMAH and phosphoric acid to adjust the pH to 3.0.) Main component: 99%.

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