SOLID PHASE SYNTHESIS OF PORCINE α-ENDORPHIN AND γ-ENDORPHIN, TWO HYPOTHALAMIC-PITUITARY PEPTIDES WITH OPIATE ACTIVITY

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SUMMARY

The synthesis by solid phase methodology of α-endorphin (Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-OH)* and γ-endorphin (Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-OH), two morphinomimetic peptides isolated from pig hypothalamus-pituitary extracts, is described. The sequences of these two peptides correspond to residues 61-76 and 61-77, respectively, of porcine β-lipotropin. The two synthetic compounds were shown to have the same physical, chemical and opiate activity as the respective native substances.

α-Endorphin and γ-endorphin were isolated from porcine hypothalamus-pituitary extracts (1) on the basis of their activity to inhibit the amplitude of the muscle contraction of the electrically stimulated myenteric plexus-longitudinal muscle of the guinea pig ileum (2); this inhibition is reversed or prevented by the opiate antagonist naloxone and is thus recognized to involve "opiate receptors" (3). The primary structure of α-endorphin and γ-endorphin were determined by mass spectrometry and dansyl-Edman methods as H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-OH and H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-OH (4); these correspond to sequences 61-76 and 61-77 of the various β-lipotropins (5). This paper describes the synthesis of these two peptides by solid phase method (6) and comparison of their physical chemical and biological properties with the respective natural substances.

*Symbols for amino acid derivatives and substituents are according to IUPAC-IUB recommendations published in J. Biol. Chem., 247, 977-983 (1972). Other abbreviations are as follows: TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; DMF, dimethylformamide; TFA, trifluoroacetic acid.

MATERIALS AND METHODS

Mass spectra of the derivatized peptides were obtained from a Varian Mat CH-5 single-focusing mass spectrometer with the direct inlet system. The electron current was set at 300 μA with an ionizing energy of 70 eV. Amino acid analyses were determined on peptide hydrolysates using a Beckman/Spinco Model 119 amino acid analyzer. Hydrolyses were performed in 6 N HCl containing 0.5% thioglycollic acid at 110° in evacuated sealed tubes for 20 h or enzymatic digestion with papain and leucineaminopeptidase (7). Optical rotations were measured in 1% HOAc solution in a Perkin-Elmer Model 141 polarimeter. Ascending thin layer chromatography (TLC) on silica gel was performed with Eastman Chromatogram Sheet No. 13191. About 20 μg samples were spotted in 5 μl of 2 N HOAc and the solvent front was allowed to travel 10-12 cm. The spots were detected by ninhydrin and Pauly reagents. High pressure liquid chromatography (HPLC) was performed with a 4 mm x 30 cm μ-Bondapak/C_{18} column in the Waters Associate Model 204 liquid chromatography system. The column flow rate was 2.5 ml per min and a Schoeffel Model 770 monochromator was used to detect the peptide components. Trypsin or α-chymotrypsin digestion of the endorphins were each performed with 20 μg of peptide and 1 μg of enzyme in 20 μl of 0.1 M NH_4OAc-0.001 M $CaCl_2$ pH 8.1or 0.1 M NH_4OAc pH 8.1, respectively, at 37°C for 4 h. Amino acid derivatives used for the synthesis were of the L configuration and were purchased from Bachem, Inc. The α-amino function was protected exclusively with the Boc group. Other side-chain protecting groups consisted of 2,6-dichlorobenzyl for Tyr; benzyl for Thr, Ser, and Glu; 2-chlorobenzyloxycarbonyl for Lys.

α-Endorphin. Coupling of Boc-(OBzl)Thr to the chloromethyl-resin (0.9 mEq Cl/g, Lab Systems, Inc.) was performed by a modification of the Monahan and Gilon procedure (8). A ratio of 0.4 mEq of Boc-(OBzl)Thr and $KOBu^t$ per gram resin was used, which resulted in a substitution of 0.064 mmol Thr per gram resin (17).

Coupling of the other amino acids to form the rest of the peptide chain on 4 g of the Boc-(OBzl)Thr-resin was accomplished according to the following schedule, except for Gln which was coupled as its p-nitrophenyl ester in DMF for over-night: (I) 50% TFA/CH_2Cl_2, 1 min*; 50% TFA/CH_2Cl_2, 15 min*; (II) wash with 3 X CH_2Cl_2, 1 X MeOH; (III) 12.5% Et_3N/CH_2Cl_2 1 min*; 12.5% Et_3N/H_2Cl_2, 5 min*; (IV) wash with 2 X (CH_2Cl_2, MeOH), 2 X CH_2Cl_2; (V)Boc-amino acid (1 mmol/g resin in CH_2Cl_2) plus equivalent amount 2 M dicyclohexylcarbodiimide in CH_2Cl_2, 2 h*; (VI) wash with 3 X CH_2Cl_2, 1 X 12.5% Et_3N/CH_2Cl_2, 2 X (CH_2Cl_2, MeOH), 2 X CH_2Cl_2; (VII) go back to step I. The coupling reaction was monitored by the ninhydrin test of Kaiser et al (9). After incorporation of the Met the TFA deblocking step was modified with the inclusion of 5% $HSCH_2CH_2SH$ (10). After the last amino acid had been incorporated, the Boc group was removed (11) before the protected peptide-resin was treated with 10% anisole in HF (12,13) for 1 h at -5°. The HF was evacuated in vacuo at 0° and the resulting cleavage product washed with 2 X Et_2O and alternately 2 X($CHCl_3$ and Et_2O). The peptide was extracted with 5 X 2 M HOAc. Lyophilization of the extract left 447 mg of crude peptide. The crude material was first applied to a Whatman microgranular CM-32 carboxymethyl cellulose column (3.7 X 28 cm, V_t = 250 ml) and eluted with 0.01 M NH_4OAC at pH 7 (14). Fractions of 6.5 ml were collected at a flow rate of 100 ml/h. The peptides in the effluent fractions were monitored by TLC with the upper phase of 1-BuOH:HOAc:H_2O(4:1:5). Fractions between 168 and 200 ml contained the major compound and they were pooled and lyophilized repetitively to 158 mg peptide. Gel filtration chromatography (15) in a Sephadex G-25F column (2 X 101 cm, V_t = 256 ml) was further used to purify the product. Elution with 0.5 M HOAc were performed at a flow rate of 3.3 ml per 2 min. The fractions were again evaluated by TLC as above and those that emerged between 141 and 179 ml were pooled and lyophilized to 137 mg

*1 min, 15 min, etc. denote prewashing and reaction times, respectively.

Table 1. Amino Acid Analyses of Native and Synthetic Porcine Endorphins

Sample	porcine α-endorphin	synthetic α-endorphin	synthetic α-endorphin	porcine γ-endorphin	synthetic γ-endorphin	synthetic γ-endorphin
Conditions[a]	1	1	2	1	1	2
Thr	2.7	2.9	2.3	2.7	2.8	2.3
Gln	-	-	-	-	-	-
Ser	1.6	1.7	2.3[b]	1.6	1.8	2.4[b]
Glu	2.0	2.0	1.0	1.9	2.0	1.0
Pro	1.0	1.0	-	1.0	0.9	-
Gly	1.9	2.1	2.0	2.0	2.0	2.0
Val	1.0	1.0	1.1[c]	1.0	1.0	1.1[c]
Met	1.0	1.0	1.0	0.9	1.0	1.0
Leu	1.1	1.0	1.2[c]	2.0	2.0	2.3[c]
Tyr	1.0	1.0	1.0	0.9	1.0	1.1
Phe	1.0	1.0	1.0	1.0	1.0	1.0
Lys	1.0	1.0	1.0	1.0	1.0	1.0
NH_3	2.3	1.2	-	2.3	1.3	-

a) 1, 6 N HCl (0.5% thioglycollic acid); 2, enzymatic hydrolysis with papain and leucineaminopeptidase.
b) Gln does not resolve from Ser under these conditions.
c) This peak includes a shoulder, probably due to incompletely hydrolyzed peptides.

peptide. Final purification was carried out on a partition chromatography (16) column of Sephadex G-25F (2 X 109 cm, V_t = 257 ml, V_o = 78 ml) with the solvent system 1-BuOH:HOAc:H_2O(4:1:5). Fractions of 5.5 ml per 20 min were collected and monitored by TLC likewise. The pure peptide emerged between 234 and 274 ml. The fractions were pooled and lyophilized repetitively to 81 mg product (18% yield), $[\alpha]_D^{23}$ - 76.5° (c = 1).

γ-Endorphin. Similarly 6 g of chloromethyl-resin was coupled with 2.4 mEq of Boc-Leu and $KOBu^t$ to give the Boc-Leu-resin. A substitution of 0.104 mmol Leu per gram resin was found by the Gisin method (17). The remaining part of the peptide-chain was synthesized in the same manner and the protected peptide-

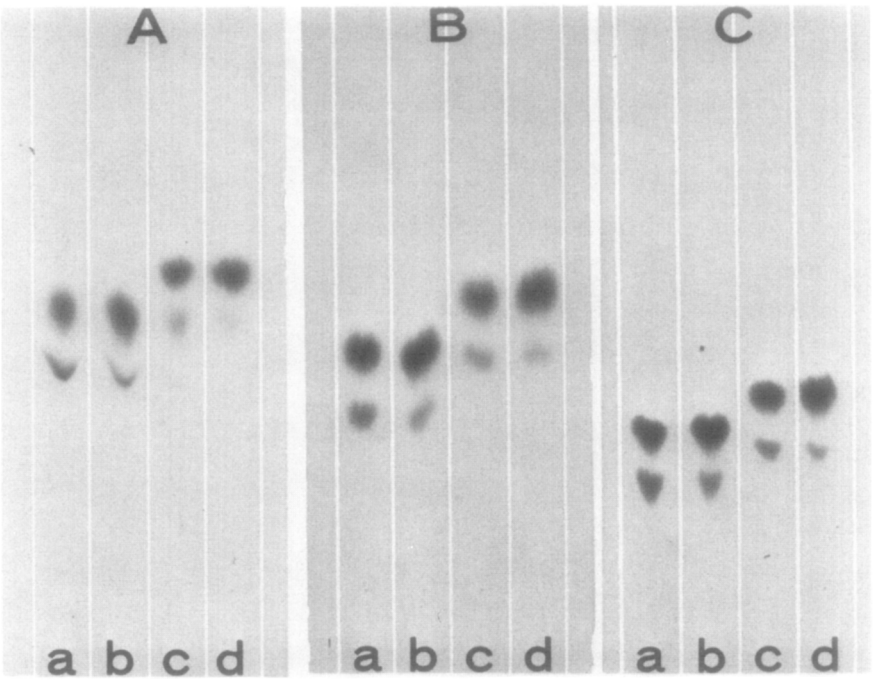

Figure 1. TLC of native (a) and synthetic (b) α-endorphin; native (c) and synthetic (d) γ-endorphin. Solvent systems are: (A) 1-BuOH: acetone:conc. NH_4OH:H_2O (10:10:5:2); (B) 1-BuOH:pyridine:HOAc:H_2O (6: 4:1:9, upper phase); (C) 1-BuOH:HOAc:H_2O (4:1:5, upper phase).

resin treated with 10% anisole in HF. After the usual work-up the peptide was extracted with 2 M HOAc. Lyophilization of the extract left 1161 mg white powder. The crude peptide was purified through carboxymethyl cellulose chromatography, gel filtration and partition chromatography as above to yield 347 mg product (30% yield), $[\alpha]_D^{23}$ - 80.5° (c = 1).

RESULTS

Amino acid analyses of the synthetic α-endorphin and γ-endorphin are in agreement with those of the respective native substances (Table 1). On TLC the synthetic materials have identical patterns as the natural products in three solvent systems (Fig. 1)*. The peptide fragments from trypsin or α-chymotrypsin digestion of synthetic and native α-endorphin as well as γ-endorphin yielded the same TLC pattern, respectively (Fig. 2). Co-injection of synthetic and porcine α-endorphin into a Waters Associate Model 204 liquid chromatography system gave

*The lower spot on the TLC of each compound probably results from the formation of the methionine sulfoxide by-product during the application of the respective peptide solutions on the TLC plates.

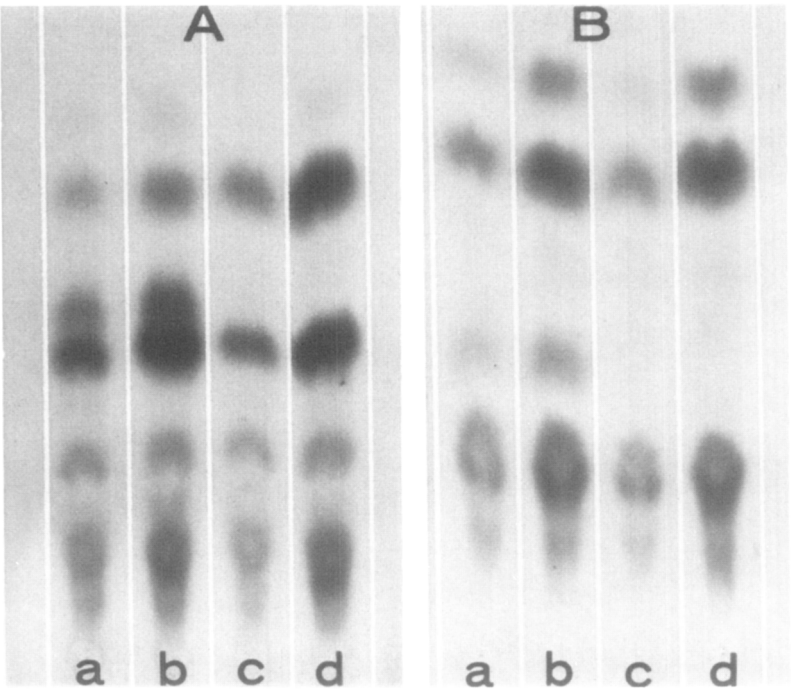

Figure 2. TLC of the trypsin (A) and α-chymotrypsin (B) digestion products from native (a) and synthetic (b) α-endorphin; native (c) and synthetic (d) γ-endorphin. Solvent system is 1-BuOH:pyridine: HOAc:H_2O (6:4:1:9, upper phase).

only one peak (Fig. 3A). The same result was obtained with a mixture of synthetic and porcine γ-endorphin (Fig. 3B). Both synthetic endorphins were subjected to the same derivatization procedure for direct mass spectrometric sequence determination as the porcine materials. The mass spectra obtained were identical with those of the corresponding native substances (4). The opiate activity of synthetic and native α- and γ-endorphins was compared in the myenteric plexus bioassay (2). Results showed that the potency ratios of the synthetic versus the respective native substances are statistically not different from unity (Table 2).

DISCUSSION

While our work on the characterization of the endorphins was in progress, Hughes et al. (18) reported the isolation from pig brain of two pentapeptides, Tyr-Gly-Gly-Phe-Met-OH and Tyr-Gly-Gly-Phe-Leu-OH, with morphinomimetic activ-

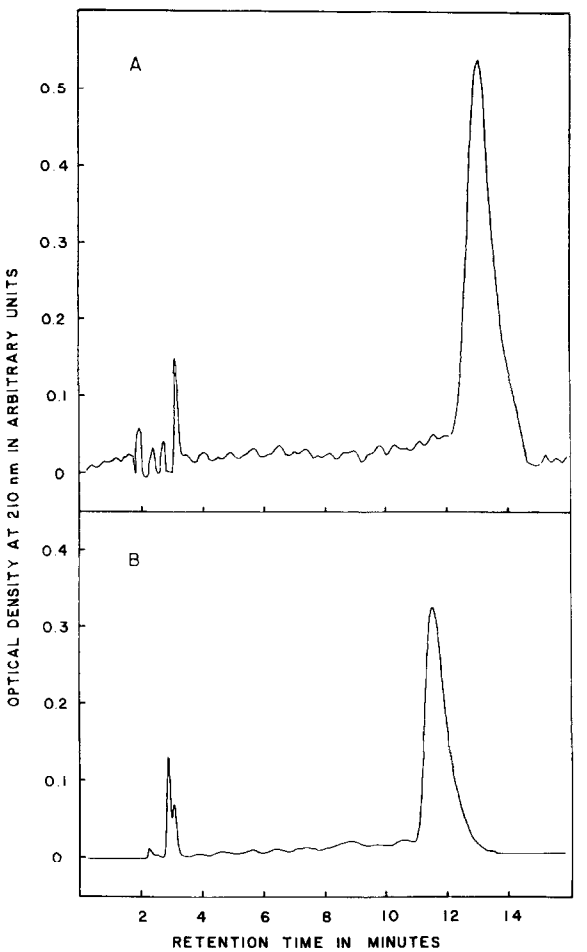

Figure 3. (A) HPLC pattern from a co-injection of 26 µg porcine and 12 µg synthetic α-endorphin. Elution buffer is 20% CH_3CN in 0.01 M NH_4OAc pH 4. (B) HPLC pattern from a co-injection of 20 µg porcine and 25 µg synthetic γ-endorphin. Elution buffer is 24.5% CH_3CN in 0.01 M NH_4OAc pH 4.

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ity. The former compound was named Met-enkephalin and the latter Leu-enkephalin. In the same report they also noted that Met-enkephalin corresponds to the β-LPH sequence from Tyr^{61} to Met^{65} of the various β-lipotropins (β-LPH) (5), first isolated by C.H. Li et al. (19) in 1965 from ovine pituitary glands. While no β-LPH with leucine at the 65th position has ever been reported, Leu-enkephalin would share with β-LPH at least the Tyr^{61} to Phe^{64} sequence. α-Endorphin and

Table 2. Potency Ratios of Synthetic vs. Native Endorphins with 95% Fiducial Limits as Tested in the Myenteric Plexus Bioassay.

Compounds	Potency Ratio
Synthetic α-endorphin/native α-endorphin	0.97 (0.72-1.29)
Synthetic γ-endorphin/native γ-endorphin	1.08 (0.94-1.24)

γ-endorphin have respectively, for primary structure, the same amino acid sequence as β-LPH(61-76) and β-LPH-(61-77). The C-terminal fragment of β-LPH, β-LPH-(61-91), identified earlier on basis of its chemical properties by Bradbury et al. (20) and Li and Chung (21) from whole pituitary extracts was also shown to have morphinomimetic activity. This compound later synthesized by Li et al. (22) was named β-endorphin by C.H. Li (21) in the interim between the characterization of the two endorphins reported here; hence our terminology from α- to γ-endorphin. Several other fragments of the (61-91)-COOH-terminal of β-LPH have recently been characterized; all have opiate-like activity (23, 28). Hence, a number of papers appear in the literature lately (24-28), speculating on the possible role of β-LPH as a prohormone for the endorphins and enkephalins.

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REFERENCES

1. Guillemin, R., Ling, N., and Burgus, R. (1976) C.R. Acad. Sci. Paris, Ser. D, 283, 783-785.
2. Paton, D.M. and Zars, M.A. (1968) J. Physiol., 194, 13-33.
3. Pert, C.B., and Snyder, S.H. (1973) Science, 179, 1011-1014.
4. Ling, N., Burgus, R., and Guillemin, R. (1976) Proc. Nat. Acad. Sci. USA, 70, (in press).
5. Li, C.H., and Chung, D. (1976) Nature, 260, 622-624.
6. Merrifield, R.B. (1963) J. Am. Chem. Soc., 85, 2149-2154.
7. Stewart, J., and Young, J. (1969) in Solid Phase Peptide Synthesis, pp. 54-55, W.H. Freeman, San Francisco.

8. Monahan, M.W., and Gilon, C. (1973) Biopolymers, 12, 2513-2519.
9. Kaiser, E., Colescott, R., Bossinger, C., and Cook, P. (1970) Anal. Biochem., 34, 595-598.
10. Westall, F.C., and Robinson, A.B. (1970) J. Org. Chem., 35, 2842-2844.
11. Noble, R., Yamashiro, D., and Li, C.H. (1976) J. Am. Chem. Soc., 98, 2324-2328.
12. Sakakibara, S., Shimonishi, Y., Okada, M., and Kishida, Y. (1967) in Peptides, pp. 44-49, North-Holland Publishing Co., Amsterdam.
13. Lenard, J. and Robinson, A.B. (1967) J. Am. Chem. Soc., 89, 181-182.
14. Peterson, E.A., and Sober, H.A. (1956) J. Am. Chem. Soc., 78, 751-755.
15. Porath, J., and Flodin, P. (1959) Nature, 183, 1657-1659.
16. Yamashiro, D. (1964) Nature, 201, 76-77.
17. Gisin, B.F. (1972) Anal. Chimica. Acta., 58, 248-249.
18. Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, C.A., Morgan, B.A., and Morris, H.R. (1975) Nature, 258, 577-579.
19. Li, C.H., Barnafi, C., Chretien, M., Chung, D. (1965) Nature, 208, 1093-1094.
20. Bradbury, A.F., Smyth, D.G., and Snell, C.R. (1975) in Peptides - Chemistry Structure Biology, pp. 609-715, Ann Arbor Science Publishers, Ann Arbor.
21. Li, C.H., and Chung, D. (1976) Proc. Nat. Acad. Sci. USA, 73, 1145-1148.
22. Li, C.H., Lemaire, S., Yamashiro, D. and Doneen, B.A. (1976) Biochem. Biophys. Res. Commun., 71, 19-25.
23. Bradbury, A.F., Smyth, D.G., Snell, C.R., Birdsall, N.J.M., and Hulme, E.C. (1976) Nature, 260, 793-795.
24. Bradbury, A.F., Smyth, D.G. and Snell, C.R. (1976) Biochem. Biophys. Res. Commun., 69, 950-956.
25. Cox, B.M., Goldstein, A., and Li, C.H. (1976) Proc. Nat. Acad. Sci. USA, 73, 1821-1823.
26. Lazarus, L., Ling, N., and Guillemin, R. (1976) Proc. Nat. Acad. Sci. USA, 73, 2156-2159.
27. Goldstein, A. (1976) Science, 193, 1081-1086.
28. Graf, L., Ronai, A.Z., Bajusz, S., Czeh, G., and Szekely, J.I. (1976) FEBS Lett. 64, 181-184.