Journal of Medicinal Chemistry

Volume 20, Number 3 March 1977

Synthesis and Analgesic Activity of Human β-Endorphin

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The solid-phase synthesis of human β-endorphin is described. A yield of 32% is achieved based on starting resin. The synthetic product behaves as a homogeneous peptide in partition chromatography, paper electrophoresis, thin-layer chromatography, disc electrophoresis, amino acid composition, and a tryptic map. The synthetic β_h-endorphin possesses antinociceptive properties as estimated by the tail-flick, hot-plate, and writhing tests in mice. When applied centrally, β_h-endorphin is 17–48 times more potent than morphine. It is 3.4 times more potent than morphine when injected intravenously. The analgesic responses are blocked by the specific opiate antagonist, naloxone.

The discovery that β-lipotropin[2a] may serve as prohormone for the opiate active pentapeptide enkaphalin[2b] has drawn attention to the possibility that other fragments may act as neurohormones. Isolation and characterization of the untriakontapeptide β-endorphin[3a] from camel pituitary glands have shown it to be identical in sequence with positions 61–91 of ovine[2a,4] β-LPH[5] (Figure 1) and to possess high opiate activity.[3b] Synthesis of β-endorphin[6] by the solid-phase method[7] in good yield has been reported. Since the structure of human β-LPH has been elucidated[4] and no report[8] has appeared of the isolation of β_h-endorphin, it was of interest to synthesize that fragment which would correspond to a β-endorphin molecule (Figure 1). We report herein the synthesis and analgesic activity of β_h-endorphin.

Synthesis of the protected peptide corresponding to β_h-endorphin was performed by the solid-phase method on brominated styrene–1% divinylbenzene polymer.[7] The standard ester attachment has been reported to be cleaved 1.4% per cycle.[9] The same ester linkage to bromo polymer is sufficiently stable to acidolytic cleavage in TFA that only 0.03% of peptide chains are lost per cycle as measured with a model peptide (see Experimental Section). On the other hand, the linkage is sufficiently labile in liquid HF[10,11] to be cleaved 90% in 75 min at 0 °C. Esterification of Boc(Bzl)Glu-OH to chloromethylated bromo polymer was effected as described previously.[12,13] Synthesis of β_h-endorphin was carried out essentially by procedures discussed elsewhere[6,14,15] with the exception that *N*-methylmorpholine replaced most of the diisopropylethylamine used for the neutralization process. The Boc group of the final protected peptide resin was removed before the HF treatment to reduce tertiary butylation of the methionine residue.[16] Purification was effected as described for β_c-endorphin.[6] The synthetic β_h-endorphin was characterized by partition chromatography in Sephadex G-50 (Figure 2), paper electrophoresis at pH 3.7 and 6.7, thin-layer chromatography, disc gel electrophoresis, and amino acid analyses of acid and total enzymic digests. Peptide mapping of a tryptic digest gave the five expected peptides (Figure 3). Amino acid analyses confirmed the identities of these peptides and accounted for 94% of the peptides detected on the map.

Table I. Median Antinociceptive Doses (AD_{50}) of β_h-Endorphin in Mice

	AD_{50}[a]	Potency ratio[a] (morphine = 1) human	camel
(a) Intracerebroventricular Injection, nmol/Mouse			
Tail-flick test	0.026 (0.017–0.043)	48.4	33.0
Hot-plate test	0.031 (0.023–0.01)	32.8	17.5
Writhing test	0.026 (0.017–0.40)	17.3	19.5
(b) Intravenous Injection, μmol/kg			
Tail-flick test	3.32 (1.86–5.69)	3.4	3.5

[a] The values of AD_{50} of morphine and potency ratio for camel β-endorphin were obtained from our previous studies.[17,18] Values in parentheses are the 95% confidence limits of AD_{50}.

β_h-Endorphin, at doses ranging from 0.09 to 0.38 μg per mouse injected icv, produced a dose-related inhibition of the tail-flick response of mice to this nociceptive heat stimulus (Figure 4). This effect lasted 30–60 min depending on the dose used. Subcutaneous injection of naloxone (1 mg/kg) 10 min after injection of β_h-endorphin completely reversed the analgesic response to β_h-endorphin. Similar results were obtained by the hot-plate test. Additionally, in the writhing assay, β_h-endorphin (0.38 μg per mouse injected 5 min before the injection of acetic acid) completely inhibited the writhing response to the stimulus of acetic acid. Pretreatment of mice with naloxone hydrochloride (1 mg/kg sc) 5 min before the injection of β_h-endorphin completely abolished the inhibitory effect of β_h-endorphin on writhing.

In another experiment, β_h-endorphin was injected iv and the analgesic response was again determined by the tail-flick method. β_h-Endorphin, at doses of 9.4, 18.8, and 28.2 mg/kg, produced a dose-related inhibition of the tail-flick response after peripheral administration (Figure 5). The effects lasted 20–30 min depending on the dose

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                    5                                   10
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-

                              15                                  20
Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-

                                        25                                  31
Ala- Ile - Ile -Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
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Figure 1. Amino acid sequence of β_h-endorphin [β_h-LPH-(61–91)].

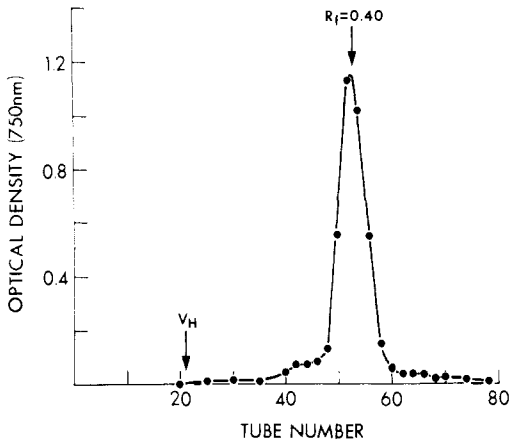

Figure 2. Partition chromatography of synthetic β_h-endorphin (12.4 mg) on Sephadex G-50: column size, 1.18 × 60 cm; solvent system, 1-butanol–pyridine–0.6 M NH_4OAc (5:3:10); hold-up volume (V_h), 19 ml; 0.89 ml per tube; detection, Folin–Lowry method.

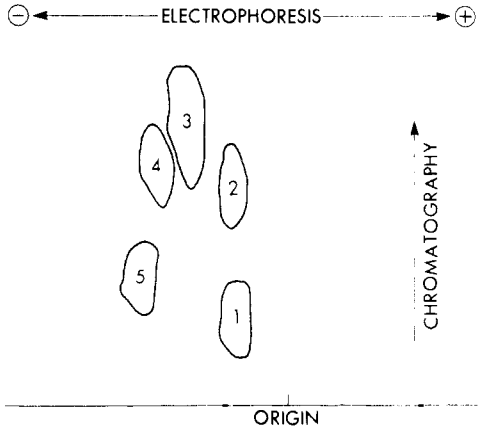

Figure 3. Peptide map of tryptic digest of synthetic β_h-endorphin.

used. Pretreatment of mice with naloxone hydrochloride (1 mg/kg sc) 5 min before the injection of 20.1 mg/kg of β_h-endorphin completely abolished the inhibitory effect of β_h-endorphin. Thus, β_h-endorphin, injected either iv or icv, was effective in producing analgesic effects.

A quantitative determination of analgesic potency of β_h-endorphin after icv and iv injection is shown in Table I. Morphine is used as a reference compound for the comparison of potency with β_h-endorphin. When potency was compared on a molar basis, β_h-endorphin, when applied centrally, was 17–48 times more potent than morphine as estimated by the three bioassays. β_h-Endorphin was 3.4 times more potent than morphine when injected iv. Thus, the analgesic potency of β_h-endorphin is comparable to that of β_c-endorphin (see Table I). It may be noted that there are only two residues which are different in the human and camel β-endorphins: the former has tyrosine in position 87 instead of histidine, and glutamic acid in position 91 instead of glutamine (4). Apparently,

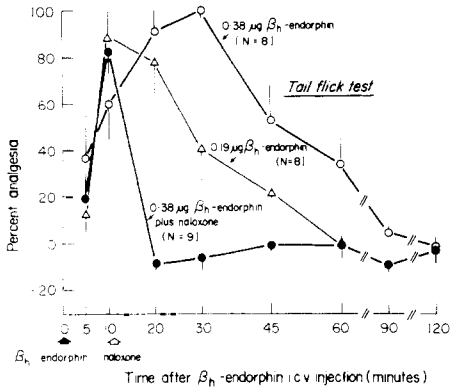

Figure 4. Inhibitory effect on tail-flick response following intracerebroventricular (icv) injection of β_h-endorphin and its reversal by naloxone. β_h-Endorphin was injected icv at 0 time. Naloxone (1 mg/kg ic) was injected at 10 min after β_h-endorphin. N = number of mice studied. The vertical bars indicate the SEM.

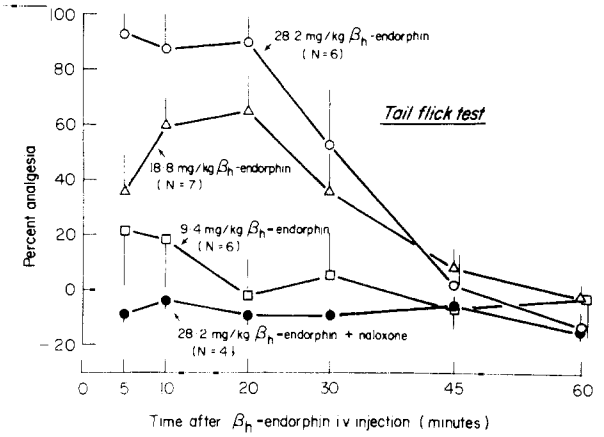

Figure 5. Inhibitory effect on tail-flick response following intravenous (iv) injection of β_h-endorphin and its blockade by naloxone. β_h-Endorphin was injected iv via the tail vein at 0 time. Naloxone hydrochloride (1 mg/kg sc) was injected 5 min before the injection of β_h-endorphin. N = number of mice studied. The vertical bars indicate the SEM.

substitution of these two residues in positions 87 and 91 of β_c-endorphin does not change its analgesic potency.

Since the molecular weight of β_h-endorphin is about ten times higher than morphine, it is likely that the lower potency ratio of β_h-endorphin to morphine when applied peripherally is due to the relatively poorer penetration of this peptide into the brain when compared to morphine. Also, a different rate of degradation after peripheral administration is a possible factor.

Experimental Section

Tests of Brominated Polymer. Chloromethylated styrene–1% divinylbenzene (5.013 g, Lab Systems LS-601, 0.9 mmol of Cl/g) was stirred in CCl_4 (40 ml) for 10 min. Iodine (165 mg) and a solution of bromine (8 ml) in CCl_4 (17 ml) were added, and the mixture was stirred for 20 h at 24 °C in the dark.[7] The resin was filtered off and washed with 150 ml each of dioxane, H_2O, 1 M $NaHCO_3$, H_2O, DMF, H_2O, and methanol: yield, 8.423 g (weight gain, 66%, theoretical weight gain for monobromination of every aromatic ring, 71%).

The tetramethylammonium salt of Boc-Phe-OH (3.7 mmol) was allowed to react with brominated resin (3.365 g) in DMF (16 ml) for 21 h at 25 °C and worked up as previously described:[13] yield, 3.880 g. Removal of the Boc group and amine determination[19] gave 0.51 mmol/g. The model peptide H-Lys^5-Glu^3-Leu^2-Trp(NPS)-Phe-OH was synthesized by procedures detailed elsewhere including use of TFE to enhance coupling efficiency.[15] Chain cleavage during removal of the Boc group was measured by detection of the NPS chromophore[20] at 365 nm in the eluate. Cleavage was measured after deprotection of the

second Leu residue and the last Lys residue; 0.03% of the peptide load was cleaved in each case. Final deblocking and cleavage in liquid HF[10,11] for 75 min at 0 °C gave 90% cleavage. The overall yield of H-Lys[5]-Glu[3]-Leu[2]-Trp(NPS)-Phe-OH was 77% and the error peptide H-Lys[4]-Glu[3]-Leu[2]-Trp(NPS)-Phe-OH was 2.6% of that.

Protected β_h-Endorphin Bromo Polymer. Starting with chloromethylated styrene–1% divinylbenzene (Bio-Beads S.X-1, 200–400 mesh, 0.69 mmol/g) bromination and esterification with Boc(Bzl)Glu-OH was carried out as described above to give a load of 0.28 mmol/g. Boc(Bzl)Glu bromo polymer was carried through the same schedule for synthesis described previously[15] with the following exceptions: (1) in steps 7 and 9, 5% *N*-methylmorpholine in CH_2Cl_2 (prepared fresh each day) was used; (2) steps 12 and 13 were deleted; (3) in step 16, 0.5 equiv of *N*-methylmorpholine in TFE was used in place of the diisopropylethylamine. The last change has been found to be helpful in reducing nucleophilic attack on the *o*-BrZ protecting group of tyrosine when TFE is present while at the same time effective in neutralizing the acid contamination in the Beckman 990 peptide synthesizer.[21] The following side-chain protecting groups were employed: *o*-BrZ for Lys[13] and Tyr[22]; Bzl for Glu, Thr, and Ser. All amino acids except glutamine and asparagine were coupled by preforming the symmetrical anhydride of the Boc derivative in CH_2Cl_2 as described previously.[14,23] The low solubility of Boc-Leu-OH in CH_2Cl_2 necessitated running this reaction with minimal cooling. Boc-Gln-OH was coupled by the symmetrical anhydride procedure in a DMF–CH_2Cl_2 mixture[24] while Boc-Asn-OH was coupled by the DCC–1-hydroxybenzotriazole method.[25,26] Since the beneficial effects of TFE in coupling efficiency is nullified by the presence of DMF,[21] it was not used for coupling Boc-Gln-OH and Boc-Asn-OH. The Boc group of the last amino acid residue was removed with TFA. The yield of final protected peptide resin from 0.51 g of Boc(Bzl)Glu bromo polymer (141 μmol) was 1.367 g.

β_h-Endorphin. Protected β_h-endorphin bromo polymer (680 mg) was treated in HF (10 ml) for 75 min at 0 °C in the presence of anisole (1.5 ml). After removal of HF, the oily residue was washed with ethyl acetate (25 ml). The product was extracted with 0.5 N acetic acid (5 ml) and subjected to gel filtration on a 2.16 × 25 cm column of Sephadex G-10 in 0.5 N acetic acid. A single peak was detected (280 nm) and isolation by lyophilization gave 272 mg. A portion (260 mg) upon gel filtration on a 2.5 × 137 cm column of Sephadex G-25 in 0.5 N acetic acid gave essentially one major peak which on lyophilization gave 212 mg. A portion (206 mg) on CMC chromatography[6] gave a major peak followed by a substantial second peak; isolation by lyophilization gave 119 and 48 mg, respectively. An aliquot of the major product (49 mg) was subjected to partition chromatography on Sephadex G-50 in a 1.76 × 47 cm column[6] to give essentially one sharp symmetrical peak with R_f 0.37, and isolation by lyophilization gave 33 mg (90% peptide content by amino acid analysis) of highly purified β_h-endorphin (32% yield based on starting resin).

Paper electrophoresis (110-μg samples) at pH 3.7 (pyridine acetate buffer) and pH 6.7 (collidine acetate buffer) for 4.5 h at 400 V each gave a single spot (ninhydrin detection) with R_f values of 0.56 and 0.23 relative to lysine. Thin-layer chromatography (BPAW) (65 μg) gave one spot (ninhydrin) with R_f 0.52. A sample (12.4 mg) on partition chromatography in a 1.18 × 60 cm column of Sephadex G-50 gave the results in Figure 2. Disc electrophoresis (0.1 mg) on polyacrylamide gel[27] at pH 4.5 gave one intense band. Amino acid analysis[28] of a 24-h HCl hydrolysis gave $Lys_{5.3}Asp_{2.0}Thr_{2.8}Ser_{1.7}Glu_{3.1}Pro_{1.0}Gly_{3.1}Ala_{1.8}Val_{1.0}Met_{0.9}Ile_{1.4}Leu_{2.1}Tyr_{1.9}Phe_{1.9}$ (the Ile-Ile moiety is resistant). Amino acid analysis after complete enzymic digestion (first with trypsin and chymotrypsin and then leucine aminopeptidase) gave $Lys_{5.1}$(Thr + Ser + Asn + Gln)$_{7.7}Glu_{2.0}Pro_{1.1}Gly_{3.0}Ala_{1.8}Val_{1.1}Met_{1.0}Ile_{1.9}Leu_{2.1}Tyr_{2.0}Phe_{2.1}$.

For peptide mapping a sample (1.30 mg) was treated in 0.23 ml of 0.2 M NH_4OAc, pH 8, with 26 μg of tryptin at 37 °C for 6 h. After repeated lyophilization an aliquot (0.80 mg) was spotted in Whatman 3 MM and mapped by chromatography (BAW) followed by electrophoresis (collidine acetate), pH 6.7, 400 V, 6 h). Revelation by ninhydrin (0.01% in ethanol) gave five major spots (Figure 3) which were cut out and extracted with 0.1 N NH_4OH for 24 h. All minor spots were cut out and pooled for extraction. Amino acid analyses of HCl hydrolysates gave for spot 1, $Lys_{1.0}Glu_{1.1}Gly_{0.9}$; for spot 2, $Lys_{0.9}Thr_{1.1}Ser_{0.9}Glu_{1.2}Gly_{2.2}Met_{0.8}Tyr_{0.8}Phe_{1.1}$; for spot 3, $Lys_{0.8}Thr_{1.9}Ser_{0.9}Glu_{1.2}Pro_{0.9}Val_{1.0}Leu_{1.9}Phe_{1.1}$; for spot 4, $Lys_{0.9}Asp_{1.0}Ala_{1.1}Ile_{1.7}$; and for spot 5, $Lys_{0.9}Asp_{1.1}Ala_{1.1}Tyr_{0.9}$. The five major spots accounted for 94% of the peptides detected by this procedure.

For analgesic assays, male ICR mice weighing 25–30 g (Simonsen Laboratories, Gilroy, Calif.) were used. Naloxone hydrochloride was a gift from Endo Laboratories (Garden City, N.Y.). The antinociceptive or analgesic properties of β_h-endorphin were assessed in mice by the tail-flick method,[29] the hot-plate method,[30] and the acetic acid induced writhing method.[31] The peptide was injected either icv in a volume of 5 μl according to the method described by Haley and McCormick[32] or iv via the tail vein.

To evaluate the tail-flick and hot-plate responses, a control latency (T_0) was obtained from the mean of two latencies determined prior to drug injection; the test latencies (T_1) were determined at various times after injection for each animal. "Percent analgesia" was calculated as $[(T_1 - T_0)/(T_2 - T_0)] \times 100$, where the cutoff times (T_2) for the tail-flick and hot-plate tests were 10 and 60 s,[33] respectively. With a twofold increase in latency of reaction time of tail-flick response and hot-plate response as a quantal index of inhibition, the median antinociceptive dose (AD_{50}) and 95% confidence limits were calculated according to the method of Litchfield and Wilcoxon.[34] When "analgesia" was measured by the writhing method,[31] acetic acid (0.1 ml/10 g of body weight of 0.6% acetic acid) was administered intraperitoneally 5 min after the icv injection of β_h-endorphin. The number of writhes was counted for 15 min. To determine the AD_{50} for writhing, a quantal index of inhibited response was individually defined as any mouse which writhed less than twice after drug administration.

Acknowledgment. We thank K. Hoey, J. Knorr, and S. Petluck for assistance. This was supported in part by an NIH grant (GM 2907) and NIDA grants (DA-01314 and DA-00091).

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(5) Symbols and abbreviations are in accordance with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature, Collected Tentative Rules and Recommendations (1973). Other abbreviations used are LPH, lipotropin; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; TFE, 2,2,2-trifluoroethanol; NPS, *o*-nitrophenylsulfenyl; DCC, *N,N*^{1}-dicyclohexylcarbodiimide; CMC, carboxymethylcellulose; BPAW, 1-butanol–pyridine–acetic acid–water (5:5:1:4); BAW, 1-butanol–acetic acid–water (4:1:5); β_h-endorphin, β-endorphin from human pituitary glands; β_c-endorphin, β-endorphin from camel glands; icv, intracerebroventricularly; iv, intravenously.
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Synthesis of Cephalotaxine Esters and Correlation of Their Structures with Antitumor Activity

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Received August 2, 1976

Twenty-two new esters of natural (–)-cephalotaxine with synthetic acids possessing widely divergent structural features have been synthesized. Murine antitumor (P388 system) test data reveal that the methyl itaconate (**7a**) and trichloroethyl carbonate (**27**) esters of cephalotaxine are the most active of this group; this activity is less than that of harringtonine and other naturally occurring cephalotaxine esters. Other synthetic esters exhibiting activity are methyl cephalotaxylfumarate (**4**) and the trichloroethyl carbonate of cephalotaxyl-L-mandelate (**21**). The specificity of this experimental tumor system apparently requires esters of (–)-cephalotaxine for tumor inhibition because methyl cephalotaxylitaconate (**7b**) prepared from the synthetic (+) enantiomer of cephalotaxine is inactive.

Investigation of alkaloids isolated from *Cephalotaxus harringtonia* extracts demonstrated that several natural esters of the parent alkaloid, cephalotaxine (**1**),[1-4] exhibit significant activity against experimental leukemia systems.[3] However, plant material from which to extract these esters is in critically short supply. This shortage, coupled with the fact that cephalotaxine has now been synthesized,[5] has stimulated efforts to convert cephalotaxine (which possesses no activity in the unesterified form) to some of the active, naturally occurring esters. However, very unfavorable steric (and perhaps electronic) interactions at the reaction sites of both the cephalotaxine and the acyl moiety preclude direct esterification of these acids with cephalotaxine as a route to the active natural esters.[4,6a] Conversion of cephalotaxine (**1**) to its active esters has been achieved in the case of deoxyharringtonine[7a,b] (**10a**) and harringtonine;[7c] all three syntheses were carried out by indirect routes. Although significant, our partial synthesis of **10a** did not appear to be sufficiently high yielding to provide a practical source of active esters. Yield data for the Chinese worker's syntheses are not available at this time.

Chemical aspects of these active alkaloid esters have recently been supplemented by results of biological investigations concerned with their mode of action.[8] Huang[8c] states that harringtonine is the only small molecule thus far reported that penetrates animal cell membranes and selectively inhibits initiation of protein synthesis without affecting chain elongation.

Our current research was designed to further delineate structure–antitumor activity relationships[6] (beyond those provided by the natural esters) by preparing, as shown in Table I, various types of cephalotaxine esters (Chart I) not subject to the severe steric requirements mentioned earlier. In view of the plant material shortage, we also hoped to discover new, easily prepared esters with tumor inhibitory properties comparable to those of the natural esters. The esters to be synthesized (shown in Chart I) were selected to include acyl groupings with widely diverse structural features and also to include series of α,β-unsaturated, dicarboxylic, and aromatic esters.

Chemistry. In general, acylation reactions between cephalotaxine and the acylating agents produced highest yields if allowed to proceed at room temperature for an extended time period. Refluxing the reactants gave a greater mixture of products and greatly enhanced the tarry nature of the mixture. An interesting feature of the acylations that has not been explored concerns the four syntheses involving inverse addition of only a slight excess of acid chloride to alcohol (compounds **2**, **25**, **26**, and **27** in Table I). Two of these reactions yielded products (**2** and **27**) that were pure as isolated but this result could also be due in part to the fact that these particular two esters were extracted from pH 7.0 phosphate buffer instead of Na_2CO_3 solution. Buffer was used to provide conditions as mild as possible for these structures whose stabilities were unknown at the time. The other two products of inverse addition, while not pure as isolated, were easier to purify than those produced by the normal addition procedure. Our earlier work[6] indicated that an excess of at least 50% of acid chloride was desirable, if not mandatory, for good acylation yields, but results with the chloro-