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AMINO-TERMINAL EXTENSION ANALOGS OF METHIONINE-ENKEPHALIN

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Summary: Five NH_2-terminal extension analogs of methionine-enkephalin have been synthesized by solid phase methodology and their morphinomimetic activity determined in the guinea pig ileum-myenteric plexus preparation. Relative to methionine-enkephalin which corresponds to β-LPH-(61-65) and arbitrarily assigned a potency value of 100, β-LPH-(60-65), β-LPH-(59-65), β-LPH-(58-65), β-LPH-(57-65) and β-LPH-(56-65) have potencies of 90, 7, 4.4, 0.3 and 0.11 respectively.

The existence of endogenous substances with morphinomimetic activity in mammalian brain and pituitary has been reported by several groups of workers (1-4). Hughes et al. (5) were the first to isolate and characterize two pentapeptides, named methionine-enkephalin (Tyr-Gly-Gly-Phe-Met-OH*) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu-OH), from pig brain which behave like opiates in various in vitro assay systems. Later Simantov and Snyder (6) also reported the isolation of the same two pentapeptides from bovine brain. Meanwhile, three additional endogenous opioid peptides were identified and characterized from mammalian pituitary and hypothalamus extracts as α-, β-, and γ-endorphins (7-12). What is remarkable is that, with the exception of leucine-enkephalin, all the other four opioid peptides are various COOH-terminal fragments starting from the Tyr^{61} residue of β-lipotropin (13). Thus methionine-enkephalin is the fragment corresponding to β-LPH-(61-65), α-endorphin to β-LPH-(61-76), β-endorphin to β-LPH-(61-91) and γ-endorphin to β-LPH-(61-77). Other COOH-terminal fragments of β-LPH that were produced either by chemical synthesis, enzymatic digestion or isolated from tissue extracts and all of which

*Symbols for amino acid derivatives and substituents are according to IUPAC-IUB recommendations in J. Biol. Chem. (1972) 247, 977-983. Other abbreviations are as follows: TLC, thin layer chromatography; β-LPH, β-lipotropin.

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begin with the Tyr^{61} residue were also reported to have opiate activity (14-16). In view of the invariable presence of the Tyr^{61} residue at the NH_2-terminus of these opioid peptides, it is important to determine whether biological activity is preserved when the NH_2-terminus is extended beyond the Tyr^{61} position towards the NH_2-terminal of β-LPH. For this reason we have synthesized by solid phase methodology (17) β-LPH-(60-65), β-LPH-(59-65), β-LPH-(58-65), β-LPH-(57-65) and β-LPH-(56-65) and assayed their morphinomimetic activity in the guinea pig ileum-myenteric plexus bioassay preparation (18).

MATERIALS AND METHODS

Amino acid derivatives used for the synthesis of the peptides 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 were 2,6-dichlorobenzyl for Tyr, Tosyl for Arg, 2-chlorobenzyloxycarbonyl for Lys, benzyl for Asp. Amino acid analyses were determined on peptide hydrolyzates using a Beckman/Spinco Model 119 Amino Acid Analyzer. Hydrolyses were performed in 6 N HCl containing 2.5% thioglycolic acid at 110° in evacuated sealed tubes for 20 hours. Optical rotations were measured at c = 1 in 1% HOAc solution at 23°C in a Perkin-Elmer Model 141 Polarimeter. Ascending thin layer chromatography on silica gel was performed with Eastman chromatogram sheet No. 13191. About 20 µg samples were spotted in 2 µl H_2O and the solvent front was allowed to travel 10-12 cm. The spots were detected by ninhydrin and Pauly reagents. Chloromethylated polystyrene resin cross-linked with 1% divinylbenzene was obtained from Lab Systems, Inc. The substitution according to the manufacturer was 0.90 mmol Cl/g resin.

Synthesis. The chloromethyl resin was transformed to the hydroxymethyl resin by the method of Bodansky and Sheehan (19). Briefly, 20.00 g chloromethyl resin (18 mmol Cl) was incubated with 7.84 g KOAc (80 mmol) in 240 ml benzyl alcohol at 80°C for 6 hours. The resulting acetoxymethyl resin was washed with 3 X CH_3OH, 3 X (CH_2Cl_2, CH_3OH) and dried *in vacuo* to yield 20.09 g. The substitution of the acetoxy group as estimated by the intensity of the ester carbonyl band in infra-red absorption was 0.18 mmol/g resin. The acetyl grouping was removed by incubating the acetoxymethyl resin in 240 ml 1 N NaOH in benzyl alcohol at 80°C for 1 hour. After the usual washing and drying 19.97 g resin was recovered. Infra-red analysis showed that the carbonyl band had completely disappeared.

Coupling of Boc-Met to the hydroxymethyl resin was accomplished with carbonyldiimidazole. Ten grams hydroxylmethyl resin (1.8 mmol $HOCH_2$ group) was stirred with 2.49 g Boc-Met (10 mmol) and 1.62 g carbonyldiimidazole (10 mmol) in 30 ml CH_2Cl_2 at room temperature overnight. After the usual washing and drying 10.25 g Boc-Met resin was obtained with a substitution of 0.15 mmol Boc-Met/g resin as determined by the Gisin method (20). If the substitution is not high enough, the coupling reaction can be repeated to give a high incorporation of Boc-Met. The remaining hydroxyl group was blocked by 0.5 hour acetylation with Ac_2O in CH_2Cl_2.

Incorporation of the peptide chain was accomplished on 10.00 g of the acetylated Boc-Met resin with the appropriate Boc-amino acid and dicyclohexylcarbodiimide as previously described (21). After coupling and deblocking of the Arg^{60} residue, 2.0 g of the protected β-LPH-(60-65) resin was removed and the remaining peptide resin coupled with Lys^{59} followed by deblocking and removal of another 2.0 g resin and so on until the β-LPH-(56-65) fragment.

The protected peptide resin was treated with HF containing 1.5 ml anisole and 0.2 ml methylethyl sulfide per gram resin for 1 hour at 0°C as described before (21). After rapid removal of the HF, the resin was washed with Et_2O, CH_2Cl_2 and the peptide extracted with 2 N HOAc and the extract lyophilized. The resulting lyophilized powder was purified through carboxymethyl cellulose cation-exchange chromatography and partition chromatography on Sephadex G-25F as previously described (21). Fractions were monitored by TLC and pooled to favor purity rather than quantity. Generally, a yield of 20-40% of the final product based on the substituting of the Boc-Met on the resin was obtained. The purified peptides were characterized by TLC in three solvent systems, optical rotation and amino acid analysis.

Bioassays. The peptide fragments were assayed by the guinea pig ileum-myenteric plexus muscle preparation as described by Paton and Zar (18).

RESULTS AND DISCUSSION

Five NH_2-terminal extension peptides of methionine-enkephalin corresponding to β-LPH-(60-65), β-LPH-(59-65), β-LPH-(58-65), β-LPH-(57-65) and β-LPH-(56-65) have been synthesized by solid phase methodology. The purity of these peptides has been verified by TLC (Table 1) and amino acid analysis (Table 2). Morphinomimetic activity of these peptides has been determined by the *in vitro* guinea pig ileum-myenteric plexus bioassay (Table 3).

It is interesting to note that addition of the Arg^{60} residue to the NH_2-terminal of methionine-enkephalin does not change the *in vitro* activity with respect to the parent compound. However, incorporation of the next Lys^{59} residue drastically lowers the activity of this analog. Further addition of the Asp^{58}, Lys^{57} and Pro^{56} residues decreases the activity even more with the activity of β-LPH-(56-65) practically equal to zero.

The β-LPH-(60-65) fragment has been prepared by many workers (14,22-24). Using the rat brain opiate receptor binding assay, Chang *et al.* (14) reported that this compound has a potency of 40% with respect to methionine-enkephalin while Law and co-workers (24) found that the two peptides were equipotent. This discrepancy may be due to the variation of the binding

TABLE 1. PHYSICAL CONSTANTS OF β-LPH FRAGMENTS

	β-LPH	TLC*			$[\alpha]_D^{23}$
		BPyA	BAW	BPyAW	
Arg-methionine-enkephalin	(60-65)	0.45	0.45	0.56	+20.2°
Lys-Arg-methionine-enkephalin	(59-65)	0.15	0.26	0.43	+3.6°
Asp-Lys-Arg-methionine-enkephalin	(58-65)	0.27	0.25	0.39	-15.1°
Lys-Asp-Lys-Arg-methionine-enkephalin	(57-65)	0.08	0.13	0.26	-19.0°
Pro-Lys-Asp-Lys-Arg-methionine-enkephalin	(56-65)	0.01	0.10	0.24	-40.7°

*BPyA, 1-butanol:pyridine:0.1 M acetic acid (5:3:11, upper phase); BAW, 1-butanol:acetic acid:water (4:1:5, upper phase); BPyAW, 1-butanol: pyridine: acetic acid:water (6:4:1:9, upper phase). All these methionine containing peptides gave a small spot which ran slower than the major spot. The slower spot probably resulted from the formation of the corresponding methionine sulfoxide analog during the application of the respective peptide solutions on the TLC plate.

TABLE 2. AMINO ACID ANALYSES OF β-LPH FRAGMENTS

	β-LPH-(60-65)	β-LPH-(59-65)	β-LPH-(58-65)	β-LPH-(57-65)	β-LPH-(56-65)
Met	1.05	0.99	0.95	0.96	0.96
Phe	1.00	1.00	1.00	1.00	1.00
Gly	1.98	1.92	1.94	1.90	1.98
Tyr	0.98	0.97	0.97	0.99	0.99
Arg	0.97	0.99	1.00	1.01	1.00
Lys	-	0.97	0.99	2.04	2.05
Asp	-	-	0.99	0.96	1.01
Pro	-	-	-	-	1.03

assay in each laboratory. Law et al. (24) also reported that β-LPH-(60-65) was equipotent to methionine-enkephalin in the guinea pig ileum-myenteric plexus preparation which is in agreement with our finding. However, Day and co-workers (23) found that in the guinea pig ileum bioassay β-LPH-(60-65) was only about 40% as potent as methionine-enkephalin.

TABLE 3. RELATIVE POTENCIES OF β-LPH FRAGMENTS IN GUINEA PIG ILEUM-MYENTERIC PLEXUS PREPARATION*

β-LPH-(60-65)	90	(84-98)
β-LPH-(59-65)	7	(4-47)
β-LPH-(58-65)	4.4	(3.3-6.1)
β-LPH-(57-65)	0.3	(0.2-0.4)
β-LPH-(56-65)	0.11	(0.07-0.15)

*methionine-enkephalin (β-LPH-(61-65)) = 100

Ungar et al. (22) had reported that β-LPH-(60-65) was an antagonist of morphine analgesia in vivo. This finding has not been confirmed in other laboratories including our own, either in vitro or by in vivo administration. The β-LPH-(59-65) analog was found to be much less potent than β-LPH-(60-65) in the binding assay (14) which is in agreement with our results.

Graf and co-workers (25) have demonstrated that pars distalis and pars intermedia contain endopeptidase which cleave specifically the Arg^{60}-Tyr^{61} bond of β-LPH to yield the various endogenous opioid peptides. However, no such enzymic activity has been reported in the guinea pig ileum-myenteric plexus. As a matter of fact, Law et al. (24) have shown that the activity of β-LPH-(60-65) on the guinea pig ileum-longitudinal muscle preparation appeared to be due to the intact peptide rather than its conversion to β-LPH-(61-65). As a result, the question of how biological activities of the longer NH_2-terminal extension analogs of methionine-enkephalin are generated remains open.

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