SYNTHESIS OF AN INTRINSICALLY RADIOLABELED ENKEPHALIN ANALOG: [p-TRITIO-PHENYLALANYL]^4-NORLEUCYL^5-ENKEPHALIN

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SUMMARY

In a previous study methionine-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and four analogs and homologs were synthesized and compared to morphine with respect to their ability to inhibit electrically evoked contractions of the isolated guinea pig ileum. The compounds were: Tyr-Gly-Gly-Phe-Leu (Leu^5-enkephalin), Tyr-Gly-Gly-Phe (des-Met^5-enkephalin), Tyr-Gly-Gly-Phe-Nle (Nle^5-enkephalin) and Tyr-Gly-Gly-[p-chloro-Phe]-Nle ([p-chloro-Phe]^4-Nle^5-enkephalin). The IC_{50} concentrations on the guinea pig ileum assay were 3.0×10^{-7} M, 4.0×10^{-5} M, 1.1×10^{-7} M and 1.6×10^{-7} M respectively. The IC_{50} for Met^5-enkephalin was 5.0×10^{-8} M and for morphine 2.0×10^{-7} M. These data indicate that the methionine residue is important for biological activity and that norleucine is a more acceptable substitution at this position than is leucine. Therefore, the [p-chloro-Phe]^4-[Nle]^5-enkephalin was synthesized, dehalogenated by catalytic hydrogenation in the presence of $^3H_2$ to yield the intrinsically labeled [p-tritio-Phe]^4-Nle^5-enkephalin. The labeled peptide (9 Ci/mmole) was completely stable for at least 9 months at -20°C in 50% aqueous acetic acid.

Key Words: Enkephalin, Opiate, Radiolabeled peptide

INTRODUCTION

Enkephalin, a mixture of two naturally occurring pentapeptides with primary sequences H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH, has been shown to have a potent opiate like activity (1). This important observation may provide an explanation for the unique pharmacological and physiological effects of narcotic

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alkaloids. Already data has been published demonstrating that methionine enkephalin (Met^5-enkephalin) displaces specifically bound radiolabeled narcotics from rat brain homogenates (2,3,4), possesses short term antinociceptive activity when injected intraventricularly into the brains of mice and rats (5,6) and alters the levels of cyclic nucleotides in neuroblastoma glioma hybrid cells (7). Enkephalin a part sequence of β-lipotropin (61-65)(1) appears to be one of a family of related peptides derived from this "prohormone". Most notable of these are β-lipotropin 61-76 (α-endorphin), 61-77 (γ-endorphin) and 61-91 (β-endorphin). a common feature of all of these "hormones" is the N-terminal pentapeptide. Indeed structure activity studies within the enkephalin series have shown that the pentapeptide unit (61-65) is essential for opiate activity in the guinea pig ileum (8) and brain homogenate binding assay (2).

In order to study the possible biosynthesis, storage, release and degradation of Met^5-enkephalin an intrinsically radiolabeled peptide would be of value. The di-iodinated tyrosine analog of Leu^5-enkephalin has been synthesized and shown to retain less than 1% of the activity of the parent peptide in the opiate receptor binding assay (9). This indicates a di-^{125}I peptide would be unsuitable although it does not rule out the possibility that monoiodinated material could be of use. In Met^5-enkephalin, however, oxidation of the sulfur containing side chain by chloramine T or peroxide during iodination would also be of concern.

High-specific activity peptides can be prepared by synthesis of a halogen containing analog which can then be subjected to catalytic hydrogenation in the presence of $^3H_2$ gas. However, as with the iodinated analogs, the presence of methionine in this peptide, could present difficulties. It is well known, for example, that catalytic hydrogenation of methionine containing peptides may

result in serious poisoning of the catalyst and therefore incomplete reaction (10). This might account for the relatively low incorporation of $^3H_2$ into synthetic (3,5-dibromo-tyrosyl)^2-α-MSH (11). In addition, actual desulfuration has been reported (12). Therefore, in a previous study (8) a series of analogs were synthesized in an attempt to identify a precursor peptide which both retained high biological activity but did not contain methionine. The data indicated that Nle^5-enkephalin fulfilled these requirements. Therefore, [p-chloro-Phe]^4-Nle^5-enkephalin was prepared and subjected to catalytic hydrogenation in the presence of $^3H_2$ to yield the intrinsically labeled product.

CHEMISTRY

Peptide synthesis was carried out by standard solid-phase methodology essentially as described by Stewart and Young (13). L-Norleucine (Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo.) was converted to the t-butyloxycarbonyl derivative by the method of Anderson and McGregor (14) and isolated as its dicyclohelylammonium salt. A portion of the t-Boc-Norleucine was converted to its cesium salt and reacted with 1% cross-linked chloromethylated Bio-Beads (200-400 mesh) S-X-1 according to the procedure outlined by Gisin (15). Peptides were cleaved from the resin by treatment with anhydrous HF in the presence of anisole (50 equiv). The crude peptides were purified by countercurrent distribution (100 transfers) using n-butanol/acetic acid/water (4:1:5). Racemic p-chlorophenylalanine (Sigma Chemical Company) was trifluoroacetylated (16) and treated with carboxypeptidase A (17). The L-isomer was obtained in good yield and protected by reaction with t-butyloxycarbonyl azide.

Dehalotritiation was performed as a customer service by New England Nuclear. The label was incorporated to an extent of 9.0 curies per mmole. Chemical homogeneity was confirmed by thin

layer chromatography and electrophoresis against standard Nle^5-enkephalin. Localization of labeled peptide was carried out on a Packard Model 7201 Radiochromatogram Scanner.

RESULTS AND DISCUSSION

Our previously reported (8) concentrations (IC_{50}) of morphine, Met^5-enkephalin and its analogs and homologs required to inhibit electrically evoked contractions of the isolated guinea pig ileum are listed in Table 1. That study showed that Met^5-enkephalin was clearly the most potent of the synthetic peptides tested. It was also found to be 4 times more active than morphine itself in the stimulated gut preparation. Because of the potential importance of this group of compounds the availability of a labeled derivative of Met^5-enkephalin would be most desirable for subsequent biochemical studies. However, as mentioned previously, the presence of the sulfur containing methionine residue precludes the use of dehalotritiation and therefore alternative precursors were sought. The other naturally occurring peptide Leu^5-enkephalin, would not present a problem with respect to synthesis and has been reported (1) to have good biological activity (20%) in the guinea pig ileum assay. We have confirmed this in our previous studies (8). However, methionine, not leucine, appears as the residue in position 65 in human, as well as ovine and porcine β-lipotropin (18). In light of this observation and its lesser biological activity, this analog was not considered a prime candidate for radiolabeling. It was clear from our earlier studies (8) that simple removal of the methionine residue to give the des-Met^5-enkephalin would not give a suitable precursor since it severely depressed biological activity (<1% of Met^5-enkephalin in stimulated ileum). Other lower homologs (Tyr-Gly-Gly and Gly-Gly-Phe) tested showed no activity whatsoever. However, substitution of an isosteric norleucyl residue for the methionine gave an analog

Table 1. Stimulated Guinea Pig Ileum*

Compound	IC_{50}(M)**	Rel.Potency***
Morphine	2.0×10^{-7}	1.0
Tyr-Gly-Gly-Phe-Met	5.0×10^{-8}	4.0
Tyr-Gly-Gly-Phe-Leu	3.0×10^{-7}	0.7
Tyr-Gly-Gly-Phe-Nle	1.1×10^{-7}	1.8
Tyr-Gly-Gly-Phe	5±2% @ 1.0×10^{-6}	---
Tyr-Gly-Gly	inactive @ 10^{-6}	---
Gly-Gly-Phe	inactive @ 10^{-6}	---
Tyr-Gly-Gly-[p-chloro-Phe]-Nle	1.6×10^{-7}	1.2

* Table compiled from data reported in Day, A.R., et al., Res. Comm. Chem. Path. and Pharm. 14:597, 1976.
** IC_{50}= dose of compound required to inhibit contraction of stimulated ileum to 50% control.
*** Relative potency calculated versus morphine.

which showed good biological activity (45%) when compared to the parent Met^5-enkephalin. Furthermore, it was more than 2.5 times more active than Leu^5-enkephalin.

These data indicated that the Nle^5-enkephalin would be the most suitable precursor for radiolabeling because of its relatively high potency and non-sulfur containing side chain. Therefore, the [p-chloro-Phe]^4-Nle^5-enkephalin analog was synthesized, purified, and tritiated as a customer service by New England Nuclear. The resulting product was labeled to the extent of 9.0 Ci $^3H_2$/mmole and co-chromatographed with unlabeled Nle^5-enkephalin in the analytical systems described in the experimental section (Figure 1). In addition, the labeled peptide showed no deterioration whatsoever after 9 months storage at -20°C in 50% aqueous acetic acid.

EXPERIMENTAL

t-Butyloxycarbonyl amino acids were purchased from Bachem Incorporated (Marina Del Ray, California). The hydroxyl group

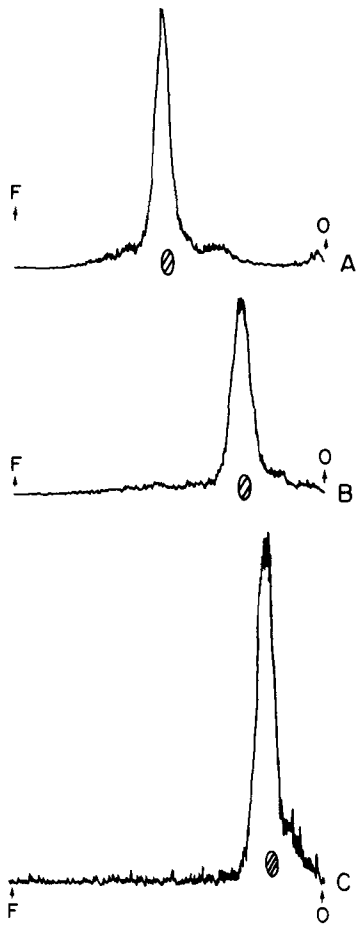

Figure 1. Tracings of radiochromatogram scans of [p-tritiophenylalanyl]^4-Norleucyl^5-enkephalin following thin layer chromatography in systems A, B and C (see Experimental section). Plates were scanned on a Packard Model 7201 Radiochromatogram Scanner at a rate of 1 cm/min. The time constant was 30 and the linear range was 1-3 x 10^4. Identical traces were found after 9 months storage at -20°C in 50% aqueous acetic acid. ⌀ = position of cold Nle^5-enkephalin visualized by chlorine/o-tolidine spray.

of tyrosine was protected by etherification to the 2,6-dichlorobenzyl derivative. Triethylamine was redistilled from NaOH pellets, collected and stored in brown bottles prior to use. Analytical

reagent grade solvents were used without further purification.

Thin layer chromatography was performed on pre-coated Merck silica gel 60 F254 glass plates using the solvent systems: A. n-Butanol-water-acetic acid (4:1:1); B. Chloroform-methanol-water-acetic acid (60:30:1:4); C. Benzene-acetic acid-water (9:1:9); D. Chloroform-acetic acid (95:5). Peptides were visualized by spraying the plates with ninhydrin, Pauly and chlorine/o-tolidine reagents. Electrophoreses at pH 2 and 5 were carried out routinely on Whatman 3MM paper at 1000v for 60 minutes (ISCO 490 power pack). Rotary evaporations were done in vacuo at a bath temperature of 40°. Peptides were hydrolysed in constant boiling hydrochloric acid containing 1% w/v phenol and mercaptoethanol for 24 hours at 110°. Amino acid analyses were performed using a Beckman 119C amino acid analyser.

TFA-DL-Phe(Cl)-OH

DL-p-Chlorophenylalanine (4.0 g, 20 mmole) was dissolved in trifluoroacetic acid and the solution cooled to 10°. Trifluoroacetic anhydride (6 ml, 40 mmole) was added slowly (10 minutes) and the reaction mixture stirred for 60 minutes at 10°. Evaporation in vacuo gave a crystalline residue which was dissolved in ethyl acetate (30 ml) and washed with saturated sodium chloride solution (2x20 ml). The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residual oil crystallized on standing and the crystals were isolated by filtration from hexane. A single spot was found by thin layer chromatography in systems A. R_f=0.8 and B. R_f=0.95. Yield 4.8 g (78%); m.p. 154°. Anal. ($C_{11}H_9NO_3ClF_3$)C,H,N.

L-p-Chlorophenylalanine

TFA-DL-P-chlorophenylalanine (4.29 g, 14.5 mmoles) was suspended in distilled water (140 ml) and the pH of the solution adjusted carefully to 7.0 with 1M KOH. The solution was incubated

with carboxypeptidase A (16 mg, 1000 units mg^{-1}) for 3 hours at 37°. A few drops of conc. HCl was added to lower the pH to 5.5 and the digest heated at 70° for 30 minutes. The mixture was filtered, cooled and acidified to pH 3.0. Crystalline TFA-D-p-chlorophenylalanine was isolated by filtration. The aqueous phase was extracted twice with ethyl acetate (30 ml), its pH was adjusted to 7.0 and evaporated to dryness. The residue was suspended in 20 ml of cold distilled water and filtered. Crystallization of the solid from water/methanol gave L-p-chlorophenylalanine. Yield 1.03 g (71%); d.p. 248-250° [Lit. 241-243°] (19). A single spot was found by thin layer chromatography in systems A. R_f=0.41; B. R_f=0.28 and C. R_f=0.38. $[\alpha]_D^{25}$ = -23° (C 0.4, H_2O) [Lit. -27.8° (C 0.4, H_2O) and -23° (C 0.5, H_2O)] (19,20). Anal. ($C_9H_{10}NO_2Cl$)C,H,N. The amino acid eluted at 133 min. under standard 3 hour hydrolysate conditions on a Beckman 119C amino acid analyser.

Boc-Phe(Cl)-OH

The N-protected derivative was prepared by stirring a suspension of p-chlorophenylalanine (710 mg, 3.55 mmoles) in dimethylsulfoxide (9 ml) together with triethylamine (0.42 ml, 3 mmoles) and t-butoxycarbonyl azide (1 ml). The reaction was complete when all the solid had dissolved. The reaction mixture was diluted with water (50 ml) and extracted with ethyl acetate (2x10 ml). The aqueous phase was cooled in ice to 4°, acidified to pH 3.0 with 2N HCl and extracted with ethyl acetate (3x10 ml). The organic phases were combined, washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The product was crystallized from ethyl acetate/hexane. Yield 787 mg (74%); m.p. 111-111.5°. A single spot was found on thin layer chromatography in system C. (R_f=0.73). No free amino acid could be detected.

t-Butyloxycarbonyl L-Norleucine (I)

This compound was prepared on a 30 mmolar scale according to the method of Anderson and McGregor (14). The free acid was isolated as a colorless oil. Half of the oil was converted to the dicyclohexylammonium salt. Yield 4.2 g (70%); m.p. 134.5-135.5°. The material was homogeneous in solvent systems A. R_f=0.81, B. R_f=0.90 and C. R_f=0.85. Anal. ($C_{22}H_{42}N_2O_4$)C,H,N. The remaining material was converted to the cesium salt and reacted with 1% crosslinked chloromethylated Bio-Beads S-X-1 resin according to the method of Gisin (15). A resin substitution of 0.62 mequiv g^{-1} was obtained.

Boc-O-2,6-dichlorobenzyl-L-Tyrosyl-Glycyl-Glycyl-L-Phenylalayn-L-Norleucyl-resin (II)

Boc-Nle-resin (645 mg, 0.4 mmole norleucine) was placed in a solid phase reaction vessel and washed six times with methylene chloride. The subsequent steps followed the pattern outlined in scheme I.

L-Tyrosyl-Glycyl-Glycyl-L-Phenylalanyl-L-Norleucine (III)

Compound II was dried and placed in Kel-F reaction vessel of an HF apparatus (Protein Research Foundation, Osaka, Japan). Anisole (50 equiv) and HF (15 ml) were added and allowed to react for 45 min at 0°. The HF was removed by vacuum distillation. The dried resin was extracted with ethyl acetate (3x20 ml) and then with 0.1M acetic acid (5x20 ml). Lyophilization of the pooled aqueous phase gave 162 mg (67% yield based on resin substitution) of crude peptide. The crude peptide was purified by countercurrent distribution (100 transfers) in butanol-acetic acid-water (4:1:5). The contents of tubes 50-67 were pooled, evaporated, redissolved in distilled water and lyophilized to give 54 mg (22% yield) of white powder. One ninhydrin, Pauly and chlorine/o-tolidine positive spot was shown in solvent systems A. R_f=0.3; B. R_f=0.54;

Scheme I

Step Number	Reagent, Solvent	Total time (min)
1	Methylene chloride wash (6x)	6
2	Trifluoroacetic acid[a]	1.5
3	Trifluoroacetic acid[a]	30
4	Methylene chloride wash (6x)	6
5	Triethylamine[b]	10
6	Methylene chloride wash (6x)	6
7	Boc-amino acid (2.5 equivalents)	5
8	Dicyclohexylcarbodiimide (2.5 equiv.)	120
9	Chloroform wash (3x)	3
10	Ethanol wash (3x)	3
11	Return to step number 1	

[a]25% v/v solution in methylene chloride containing mercaptoethanol (0.4 ml per 100 ml of methylene chloride).

[b]10% v/v solution in methylene chloride. Neutralization and subsequent washing of the dipeptide trifluoroacetate salt were performed using ice cold triethylamine, methylene chloride and methylene chloride solution of the Boc-amino acid to prevent diketopiperazine formation.

C. R_f=0.13. The material migrated as a single component on electrophoresis at pH 2 and 5. Amino acid analysis: Glycine 2.2; Tyrosine 1.10; Phenylalanine 1.00; Norleucine 1.00.

L-Tyrosyl-Glycyl-Glycyl-L-Chlorophenylalanyl-L-Norleucine (IV)

This peptide, obtained in crude form (80 mg; 64.6%), was purified by countercurrent distribution (100 transfers) in the solvent system butanol-acetic acid-water (4:1:5). Tubes 65-75 were pooled and the peptide (48 mg; 37%) isolated as described above. One ninhydrin, Pauly and chlorine/o-tolidine positive spot was shown on TLC in solvent systems A. R_f=0.61; B. R_f=0.35; C. R_f=0.45. The material also migrated as a single component on electrophoresis at both pH 2 and pH 5. Amino acid analysis: Glycine 2.04; Tyrosine 1.0; Norleucine 0.91; Chlorophenylalanine 1.0.

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