TOTAL SYNTHESIS OF FULLY TRITIATED LEU-ENKEPHALIN BY ENZYMATIC COUPLING

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SUMMARY

This paper describes the total enzymatic synthesis of Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) in which all residues were labelled with tritium. Carboxypeptidase Y from *Saccharomyces cerevisiae* was the coupling enzyme. [^{3}H]-Tyr-NH_2, [^{3}H]-Gly-Oet, [^{3}H]-Phe-NH_2 and [^{3}H]-Leu-NH_2 were prepared with specific radioactivities ranging between 20 and 60 Ci/mmol (740 to 2220 GBq/mmol). Using a microscale procedure, we obtained a fully tritiated hormone having a specific radioactivity equal to 139 Ci/mmol (5143 GBq/mmol), in agreement with the summation of the specific radioactivities of constituting residue. The radioactive hormone had antigenic properties identical to those of native Leu-enkephalin. It also bound to rat brain opiate receptors like the parental hormone.

Key words:

Peptide radiosynthesis, enzymatic synthesis, carboxypeptidase Y, enkephalin.

INTRODUCTION

Binding properties of a ligand with its physiological target are currently investigated using a radioactive form of the ligand [1]. Tritium is a well-suited isotope since its presence does not affect the properties of biological molecules [2]. Several methods have described the incorporation of one or two tritium atom(s) in a peptide or a protein [3,4,5]. However, the final specific radioactivity of such tritiated molecules is not greater than 60 Ci/mmol (2220 GBq/mmol), a value that may be too low for sensitive binding studies. We therefore investigated the possibility of designing a convenient and generalizable method for synthesizing a radioactive peptide in which each amino acid would possess at least one tritium atom. Chemical synthesis of radioactive polypeptide chains has sometimes been explored, using micro methods, however, such approaches require the use of large amounts of radioactive aminoacids.

Enzymatic procedures may present a number of advantages over chemical methods. In particular, they should not require protecting and deprotecting steps [6-7] Also, no racemization

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is expected. Furthermore, enzymatic coupling reactions are known to take place in mild conditions [8,9,10,11,12]. In the present paper, we describe a microscale procedure of enzymatic coupling which was applied to the synthesis of fully tritiated Leu-enkephalin. The enzyme used for synthesis was carboxypeptidase Y (CP-Y) from *Saccharomyces cerevisiae*. The labelled hormone had a high specific radioactivity and its biological properties were identical to those of the parental molecule. The approach described in this paper may have general implications for labelling of peptides with tritium.

MATERIALS & METHODS

1 - MATERIALS

CP-Y from backer's yeast was purchased from Merck. Trypsin pretreated by N-tosyl-L-Phenylalanyl chloromethyl keton, and α-chymotrypsin were obtained from Mann Research Laboratories. Aminoacids and peptides were bought form SIGMA or BACHEM. The precursor Boc-L-Phe$(Br)_3$-NH_2 was synthesized by B. Charpentier (laboratory of B. Roques, Faculté de Pharmacie, Paris). The precursor L-Δ 3,4-Leu was provided by the Service des Molécules Marquées (Commissariat à l'Energie Atomique, Saclay, France). Pure tritium gas was made by the Commissariat à l Energie Atomique, Saclay, (France) and distributed by Lumina. The tritium concentration was determined using an Intertechnique SL3000 or SL30, liquid scintillation counter. The Tacussel pHmeter was equipped with a MI410 microelectrode. U.V. absorbance was measured with a VARIAN CARRY 210. Analytical and preparative thin layer chromatography was carried out on Merck Silicagel 60 F 254 precoated plates. [^{3}H] scannings of TLC plates were done with a Berthold scanner. The final[^{3}H] aminoacids and peptides were analyzed and purified by either TLC (autoradiography of the plates was done using a Kodak film). The compounds were purified on a Gilson chromatographic system (High performance liquid chromatography) using reverse phase columns form Waters (μBondapack, C_{18}) or from the Société Française Chromato Colonne : S.F.C.C. (nucleosil 10μm, C_{18}). Acetonitrile, used for HPLC was bought from BDH, all others solvents were purchased from Merck or Prolabo. Aminoacid analyses were carried out on a LKB 4400 autoanalyzer.

Abbreviations are used :

CP-Y: carboxypeptidase Y (E.C. 3.4.2.3.); Bz-Arg-Oet: Benzoyl Arginine ethyl ester; BOC: Tertiobutyloxycarbonyl; TLC: Thin Layer Chromatography; HPLC: High Performance Liquid Chromatography; BAW: Butanol/Acetic Acid/Water; DFP: Diisopropyl-fluorophosphate; DMF: N,N Dimethylformamide; DMSO: Dimethylsulfoxide; TEA: Triethylamine; PMSF: Phenylmethanesulfonyl fluoride; CT: α chrymotrypsin (E.C. 3.4.2.1.1.); T: Trypsin [E.C. 3.4.2.1.4.].

2 - METHODS

2.1 - Preparation of labelled aminoacids

2.1.1 - Tritiation of the precursor DIT-NH_2

The precursor diiodo-tyrosinamide (DIT-NH_2) was synthesized from Tyr-NH_2 [13]. Then DIT-NH_2 (1.23 mg), 1 μmole was dissolved in 1 ml of methanol in the reaction vial. 2 μl of

TEA was added before freezing the solution, to neutralise hydriodic acid formed during the reduction.

The catalyst (10 mg of PdO) was then dispersed on the surface and the reaction vial was connected to the automatic tritium gas transfer unit [14].

After a pressure of 1 mPa was reached, pure tritium gas (1110 GBq i.e. 30 Ci) was compressed to 100 kPa, and the catalyst was satured for 15 minutes. The solution was frozen again before the reaction vial was brought to 20°C and magnetically stirred for 2.5 hours. Finally, the solution was refrozen before the reaction vial was removed from the tritium supply unit. The catalyst was filtered through a millipore FG filter, and labile tritium atoms were eliminated by successive evaporations in a rotavapor with methanol (4x20 ml). The aminoacid derivative was purified by HPLC (Nucleosil 10µm, C_{18}, detection : wavelength 275 nm).

2.1.2 Tritiation of the precursors ethyl-cyanoformiate, Boc-Phe(Br)$_3$-NH_2 and L-Δ 3,4 Leu

The catalytic hydrogenation procedure is identical to that described in 2.1.1. The tritiation parameters which change for each precursor, are summarized in the **Table 1.** [^{3}H]-Gly-Oet, [^{3}H]-Boc-Phe-NH_2 and [^{3}H]-Leu were analyzed and purified by TLC on Silicagel (BAW 40/10/10/ v/v/v) or by HPLC (nucleosil 10µm C_{18}), respectively.

2.1.3. - Chemical modifications of [^{3}H] aminoacids

The aminoacid derivative [^{3}H]-Boc-Phe-NH_2 (6.7 µmoles) was desacylated in the presence of TFA. for 30 mn at 0°C [16], to obtain [^{3}H] Phe-NH_2. 38.2 µmoles of [^{3}H] Leu were first acylated with a tertio-butyloxycarbonyl (Boc) group [15]. The protected aminoacid derivative was purified by HPLC (see conditions in paragraph 2.1.2) column Nucleosil 10 µm C18, 30 µmoles of [^{3}H]-Boc-Leu were obtained and amidated with anhydrous ammonia gas [16,17]. [^{3}H]-Boc-Leu-NH_2 was then purified by HPLC (same conditions as described above). The Boc group was removed in the presence of TFA and [^{3}H]-Leu-NH_2 was purified by HPLC.

2.2 Enzymatic and chemical reactions

In the interactions between the peptidic substrate and the protease to form an intermediate complex, the acylenzyme gives a hydrolysis or the synthesis product, depending on the thermodynamic and kinetic conditions [18,19].

In the first step, starting with N-blocked aminoacid carboxyethylester (Bz-Arg-Oet) and an amidated aminoacid nucleophiles (Tyr-NH_2), CP-Y catalyzed the formation of peptide bonds and gave the dipeptide Bz-Arg-Tyr-NH_2 [20,21].

In the following step, we proceed to desamidation of the above substrate with α-chymotrypsin or CP-Y, and we activated the α-carboxyl group with thionyl chloride in anhydrous ethanol to form Bz-Arg-Tyr-Oet.

We used the same procedure to incorporate the second and other aminoacids. After incorporation to the first three aminoacids of Leu-Enkephalin, Bz-Arg was removed by trypsin.

PRECURSORS	Dit - NH_2	Ethylcyanoformiate	Boc-L-Phe (Br3)	L - 3,4 Leu OH
Amount of precursor	23.1 μ moles	200 μ moles	8 μ moles	40.3 μ moles
Solvent	MeOH 1 ml T.E.A 2 μl	T.F.A. 1ml	D.M.F. 1 ml T.E.A 2 μl	MeOH/Water 50 % 1 ml
Catalyst	Pd O 10 mg	Pd/Charcoal 50 mg	Pd/Charcoal 28 mg	Pd O 35 mg
Tritiation Time	2.30 hrs	1 hr	1 hr	1.30 hrs
Catalyst filtrations	FG Filter (Millipore)	FG Filter (Millipore)	FG Filter (Millipore)	GS Filter (Millipore)
Elimination of labile Tritium	MeOH 80 ml	MeOH 80 ml	MeOH 80 ml	MeOH/Water 50 % 80 ml
Total Activity	*Tyr-NH_2 5.32 μmoles 282 mCi	*Gly-Oet 37.3 μmoles 2089 mCi	*Phe-NH_2 4 μmoles 200 mCi	*Leu-NH_2 11.4 μmoles 627 mCi
S.A. GBq/mmol Ci/ mmol	1961 53	2072 56	1850 50	2035 55

Table 1 : *Tritiation Conditions.*

The first three aminoacids were then adequate for the interactions between enzyme and substrate in the next steps of the synthesis. All the optimal conditions, for each enzymatic reaction are specified and summarized in **Table 2**. After each step, the reaction mixture was analyzed and purified by HPLC. under the following conditions : reverse phase column nucleosil 10 µm C_{18}, solvent TEAF pH3/acetonitrile 20/80, flow rate 1ml/min, wavelength : 250 nm. The aminoacid composition of the isolated products was determined using LKB autoanalyzer.

2.3 Antigenicity of the fully tritiated hormone.

The antigenic properties of the radioactive neuropeptide were assessed by a radioimmunoassay procedure described by G.Patey et al. [22].

2.4 Binding of fully tritiated [Leu]-enkephalin to opiate receptor from rat brain.

Membranes rich in brain rat opiate receptor were prepared according to Zajac & Roques [23]. O.6-O.7 mg of proteins were incubated 45 mn at 35° with varying amounts of tritiated hormone, in the presence of 50 µM puromycine in a final volume of 1ml Tris-HCl buffer pH 7.4. In control experiments 10 µM levorphanol was added. The suspensions were filtrated at 4° on GF/B Whatman filters which were rinsed twice with 5 ml Tris buffer. Filters were counted for radioactivity using a Kontron Betamatic liquid scintillator.

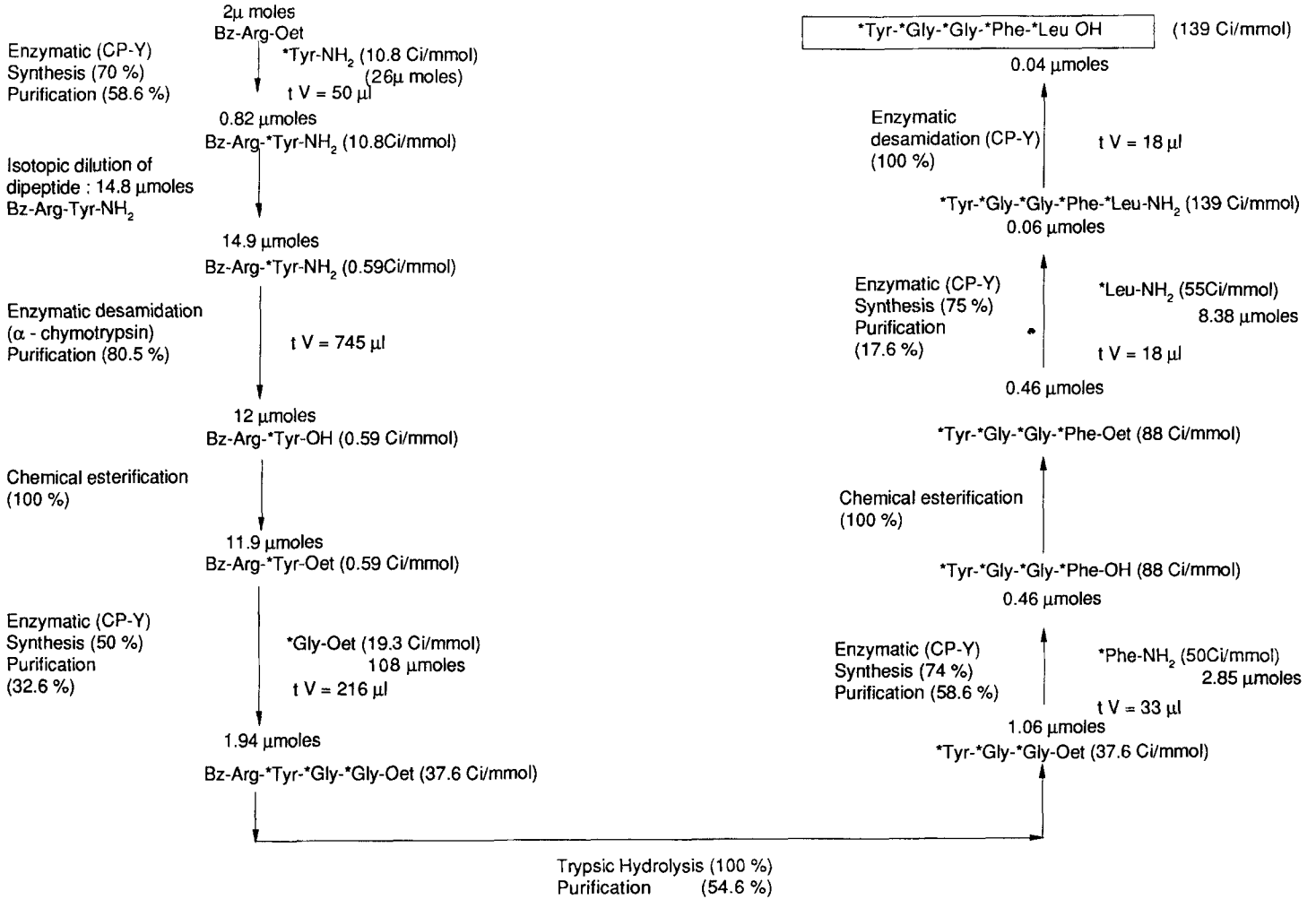
RESULTS & DISCUSSION

The first part of our study reports on the preparation of [^{3}H]-Tyr-NH_2, [^{3}H]-Gly-Oet, [^{3}H]-Phe-NH_2 and [^{3}H]-Leu-NH_2 using classical procedures. The specific radioactivities of the labelled amino acids were in agreement with theoritical expectations, except in the case of [^{3}H]-Gly-Oet, which, for unclear reasons, was lower. The second part of the paper described the coupling of these labelled residues using CP-Y, according to a microscale procedure [12]. To initiate the synthesis, we blocked the free N-terminal group of the first residue (Tyr-NH2) by coupling it to Bz-Arg-Oet. The presence of this additional residue increased the solubility of the peptide during synthesis. The peptide bond formed between Bz-Arg and [^{3}H]-Tyr was readily hydrolysed at the second step of the synthesis by trypsin.

In general, coupling yields ranged between 60 and 80%. The highest values were obtained in the following order: Phe-NH_2> Tyr-NH_2> Leu-NH_2> Gly-Oet as shown on **scheme 1** and **Table 2**. These yields, however, are not totally satisfactory and need to be improved. Previous works [12] have tentatively optimized a number of parameters, including the choice of pH, the enzyme concentration and the ratio of the substrate to nucleophile.These parameters have been taken into account in the present study. Further improvements can nevertheless be still envisionned since we observed that the overall coupling yield of [^{3}H] Gly-Oet substantially increased by adding glycerol (40% final contration) to the aqueous solvent.

Substrate	Nucleophile	Ratio N/S	Enzyme	Reaction medium P H	Incubation temperature °C	Incubation time Hours	Inhibitor	Yield %
Bz-Arg-Oet 40 m M	*Tyr-NH_2 0.52 M	13	Y 82 μ M	KCl 0.1M EDTA 1m M 9.6	37	1	D.F.P	70
Bz-Arg-*Tyr-NH_2 20 m M	-	-	C T desamidation 145 μ M	Tris/Hcl 50 m M $CaCl_2$ 100 m M 8	25	19	D.F.P	100
Bz-Arg-*Tyr-OH esterification $SOCl_2$/EtOH	-	-	-	-	-	-	-	100
Bz-Arg-*Tyr-Oet 55 m M	*Gly-Oet 0.5 M	9.1	Y 16 μ M	KCl 0.1 M EDTA 1 m M Glycerol 40 % 9	37	0.25	D.F.P	50
Bz-Arg-*Tyr-*Gly-*Gly-Oet			T 100 μ M (Bz-Arg Hydrolysis)	Tris/HCl 10^{-2}M $CaCl_2$ 2.10^{-2}M 8	25	5	P.M.S.F	100
*Tyr-*Gly-*Gly-Oet 31.9 m M	*Phe-NH_2 86 m M	2.69	Y 226 μ M	KCl 0.1 M EDTA 1 m M 8	37	1	D.F.P	70
*Tyr-*Gly-*Gly-*Phe-NH_2	-	-	Y simultaneous desamidation	-	-	-	-	70
*Tyr-*Gly-*Gly-*Phe-OH esterification $SOCl_2$/EtOH	-	-	-	-	-	-	-	100
*Tyr-*Gly-*Gly-*Phe-Oet 25 m M	*Leu-NH_2 0.46 M	18.4	Y 16 μ M	KCl 0.1 M EDTA 1 m M 9.5	37	1	D.F.P	80
*Tyr-*Gly-*Gly-*Phe-*Leu-NH_2			Y désamidation 0.5 μ M	KCl 0.1 M EDTA 10 m M carbonate buffer 0.1 M 10	37	0.25	D.F.P	100
*Tyr-*Gly-*Gly-*Phe-*Leu-OH								

Table 2 : Enzymatic and Chemical reactions
Y = CP - Y ; CT = α-Chymotrypsin ; T = Trypsin
N/S = Nucleophile concentration / Substrate concentration


2μ moles
Bz-Arg-Oet
Enzymatic (CP-Y)
Synthesis (70 %)
Purification (58.6 %)
*Tyr-NH2 (10.8 Ci/mmol)
(26μ moles)
t V = 50 μl
0.82 μmoles
Bz-Arg-*Tyr-NH2 (10.8Ci/mmol)
Isotopic dilution of
dipeptide : 14.8 μmoles
Bz-Arg-Tyr-NH2
14.9 μmoles
Bz-Arg-*Tyr-NH2 (0.59Ci/mmol)
Enzymatic desamidation
(α - chymotrypsin)
Purification (80.5 %)
t V = 745 μl
12 μmoles
Bz-Arg-*Tyr-OH (0.59 Ci/mmol)
Chemical esterification
(100 %)
11.9 μmoles
Bz-Arg-*Tyr-Oet (0.59 Ci/mmol)
Enzymatic (CP-Y)
Synthesis (50 %)
Purification
(32.6 %)
*Gly-Oet (19.3 Ci/mmol)
108 μmoles
t V = 216 μl
1.94 μmoles
Bz-Arg-*Tyr-*Gly-*Gly-Oet (37.6 Ci/mmol)
Trypsic Hydrolysis (100 %)
Purification (54.6 %)
1.06 μmoles
*Tyr-*Gly-*Gly-Oet (37.6 Ci/mmol)
Enzymatic (CP-Y)
Synthesis (74 %)
Purification (58.6 %)
*Phe-NH2 (50Ci/mmol)
2.85 μmoles
t V = 33 μl
*Tyr-*Gly-*Gly-*Phe-OH (88 Ci/mmol)
0.46 μmoles
Chemical esterification
(100 %)
*Tyr-*Gly-*Gly-*Phe-Oet (88 Ci/mmol)
0.46 μmoles
Enzymatic (CP-Y)
Synthesis (75 %)
Purification
(17.6 %)
*Leu-NH2 (55Ci/mmol)
8.38 μmoles
t V = 18 μl
*Tyr-*Gly-*Gly-*Phe-*Leu-NH2 (139 Ci/mmol)
0.06 μmoles
Enzymatic
desamidation (CP-Y)
(100 %)
t V = 18 μl
0.04 μmoles
*Tyr-*Gly-*Gly-*Phe-*Leu OH
(139 Ci/mmol)

SCHEME 1

We deliberately reduced the initial specific radioactivities of [^{3}H] Tyr-NH_2 and [^{3}H] Gly-NH_2 by isotopic dilution to reach respectively 10.8 and 19.3 Ci/mmol (399.6 and 714.1 GBq./mmol). As a result, the final synthetic radioactive neuropeptide had a specific radioactivity equal to 139 Ci/mmol. (5143 GBq./mmol), a value close to that, 154 Ci/mmol (5698 GBq./mmol), theoritically expected from the summation of the specific radioactivities of the individual tritiated residues. The aminoacid composition of the fully tritiated hormone was identical to that of the parental molecule (not shown). We then investigated the biological properties of the radioactive peptide. First, we studied its antigenic properties, on the basis of a radioimmunoassay competition using anti-[Leu]-Enkephalin antiserum and iodinated [Leu]-Enkephalin as a tracer, under non equilibrium conditions as previously described [22]. The standard competition curve was obtained by adding increasing amounts of the native neuropeptide.

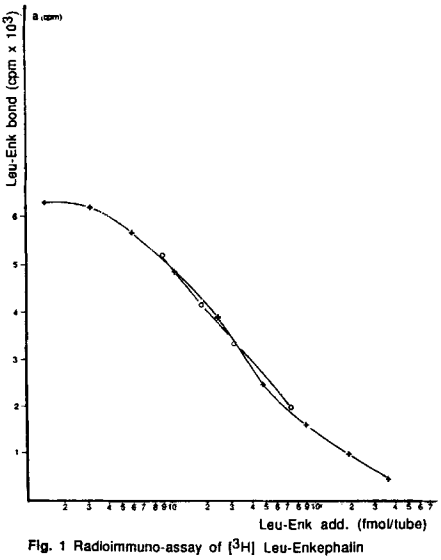


Fig. 1 Radioimmuno-assay of [^{3}H] Leu-Enkephalin
+------+ Standard curve
o------o Tritiated Leu-Enkephalin

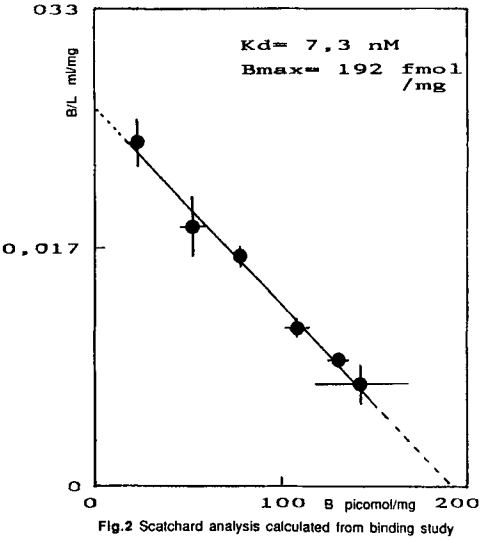


Fig.2 Scatchard analysis calculated from binding study of [^{3}H] Leu-Enkephalin on rat brain receptors

As shown in **Fig. 1** the radioactive [Leu]-enkephalin had retained full antigenic integrity since it behaved like the parental molecule.

Secondly, we investigated the direct binding of [^{3}H] Leu-Enkephalin on purified opiate receptors, according to Zajac et al [23]. **Fig. 2** shows a typical binding experiment represented according to Scathard. Fully tritiated [Leu]-enkephalin was characterized by an equilibrium dissociation constant equal to approximately 7 nM, a value which is similar to that expected for the binding of enkephalins to opiate receptor [24]. All these findings indicate that the fully tritiated Leu-enkephalin behave like the parental neuropeptide.

In conclusion, we have shown that total synthesis of a fully tritiated peptide can be readily achieved by enzymatic coupling. This approach is now currently under investigation in our laboratory with the view to generalize it. At present, our major goal will focus on the necessity to improve coupling yields.

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