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SYNTHESIS OF [^{3}H] TYR-D-THR-GLY-PHE(pN_3)-LEU-THR AN IRREVERSIBLE PHOTOAFFINITY PROBE FOR THE OPIOID δ RECEPTORS.

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SUMMARY

In a previous work, the synthesis of [^{3}H] Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET), a highly selective probe for δ opioid receptors has been described. More recently, Az-DTLET (Tyr-D-Thr-Gly-Phe (pN_3)-Leu-Thr) has been shown to be a specific and irreversible photoaffinity ligand for δ opioid sites. In the present paper, we reported the synthesis of the 3,5 dibromotyrosyl 4'-amino-3',5' diiodophenylalanyl precursor of [^{3}H] Az-DTLET. Reductive tritiation by exchange with $^3H_2$ followed by the introduction of the azido group leads to the labelled azido analog of enkephalin with specific activity of 50 Ci/mmole. This compound is now used to characterize the subunits of opioid δ receptors and to visualize these targets in the brain by electron microscopy.

Key words : Peptide synthesis, tritiated azido enkephalin, photo-affinity labelling, opiate receptor, specific δ agonists.

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INTRODUCTION

It is now well established that brain opioid peptides interact with at least three types of binding sites (μ, δ and κ) at the level of the central nervous system (1,2). The existence of such a plurality of putative receptors could explain the multiple pharmacological responses elicited by morphine and opioid peptides. Therefore the biochemical and pharmacological characterization of these different types of binding sites remains a very interesting challenge. In this field, the use of specific probes , able to bind irreversibly one kind of subsites represents a very useful approach. Numerous alkylating agents or photoaffinity labeling probes of opioid receptors already synthetized (3,6) were affected by a poor selectivity or induced a large amount of irreversible nonspecifc binding. In order to circumvent these inconvenients, an azido derivative of DTLET, a highly specific δ ligand (7,9) was developed in our laboratory (10). This probe was shown to display an even better selectivity for δ binding site that its parent compound (10). According to these results, it appeared suitable to design a labeled derivative of Az-DTLET for biochemical investigations.

To preserve the structure of Az-DTLET, tritium was prefered to ^{125}iodide for the labeling of the probe and synthesis of [^{3}H] Az-DTLET was performed from a precursor containing two dihalogenated aminoacids, to ensure a high specific radioactivity. Indeed, in addition to the usual dibromo-3,5-tyrosine, a L-4'-amino-3',5'-diiodophenylalanine (Adip) was introduced in place of the Phe residue. In these conditions, tritium exchange led to the incorporation of four tritium atoms.

A good yield was obtained for the synthesis of the precursor, greatly facilitated, by the occurence of the two bulky iodide atoms, able to protect the amino group of Adip during the coupling steps (11).

After tritiation of the precursor, the azido group was successfully introduced in the labelled 4'-amino-phenylalanyl derivative of DTLET. A specific activity of 50 Ci/mmole was obtained for [^{3}H] Az-DTLET. Preliminary biological

results show that [^{3}H] Az-DTLET is a suitable photoaffinity probe for the characterization and the purification of the δ opioid receptor and for the visualization of these sites in the central nervous system by electron microscopy.

EXPERIMENTAL PART

Materials

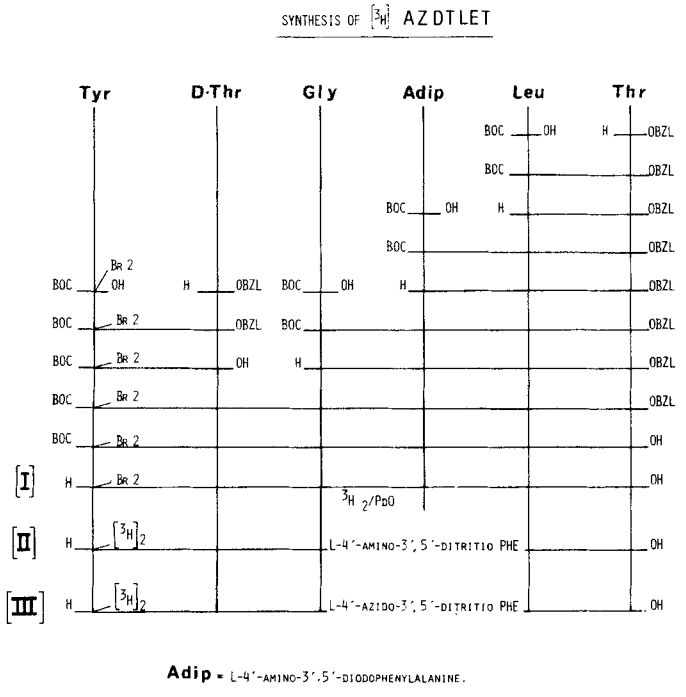
Protected aminoacids are from Bachem (Switzerland). The peptides were synthetized by liquid phase method using tert butyloxycarbonyl (Boc) and benzylesters as protecting groups and dicyclohexylcarbodiimide (DCC) with hydroxybenzotriazole (HOBt) as coupling reagents. the structure of all the compounds and of all the intermediates were confirmed by ^{1}H NMR spectroscopy (Bruker WH 270 MHz and WH 400 MHz). The purity was checked by thin layer chromatography on silicagel plates (Merck) in the following systems (v/v) : A, chloroform-methanol (9:1) ; B, BuOH-AcOH-H_2O (4:1:1) and by HPLC at 210 m on a Waters apparatus (μ-Bondapak C_{18} column) with different NH_4OAc buffer 10^{-2}M, pH 4.3/CH_3CN systems as solvents. Melting points of crystallized products are reported uncorrected.

The catalyst PdO was supplied by Engelhardt (France). Tritium gas was made by the Commissariat à l'Energie Atomique (France). The automatic gas transfert device for catalytic tritiation was previously described (12). The catalyst was separated from the reacting solution by filtration over Millipore GS. The tritium determinations were made with an Intertechnique liquid scintillation counter (SL 3000). The final ^{3}H-derivatives were purified by HPLC (Waters). After HCl hydrolysis, peptide weight determinations were carried out with a LKB 4400 amino acid analyser (U.K.).

The following abbreviations are used : THF, tetrahydrofuran ; MeOH, methanol ; $CHCl_3$, Chloroform ; EtOAc, ethylacetate ; DCC, dicyclohexylcarbodiimide ; HOBt, 1-hydroxybenzotriazole ; TFA, trifluoroacetic acid and Boc, tert-butyloxycarbonyl.

METHODS

Synthesis of [^{3}H] Az-DTLET is reported in the following scheme :



I - Peptide synthesis : Synthesis of 3,5 - dibromo - L - tyrosyl - D - threonyl - glycyl - L - 4' - amino - 3',5' - diiodophenylalanyl - L - Leucyl - L - threonine (I).

N-(tert-butyloxycarbonyl)-3,5-dibromo-L-tyrosyl-D-threonine benzyl ester

To a solution of N-(tert-butyloxycarbonyl)-3,5-dibromo-L-tyrosine (1.32 g, 3 mmol) in THF (25 ml) cooled at 0°C, were added successively a solution of D-threonine benzyl ester hemioxalate (0.90 g, 3 mmol) and triethylamine (0.42 ml) in $CHCl_3$ (25 ml), a solution of HOBt (0.46 g, 3 mmol) in THF (5 ml) and a solution of DCC (0.62 g, 3 mmol) in $CHCl_3$ (5 ml). After 1 hour at 0°C, the mixture was allowed to come to room temperature and then stirred overnight. After removal of dicyclohexylurea and evaporation of solvents in vacuo, the residue was dissolved in EtOAc (40 ml) and washed successively with a saturated solution of NaCl (20 ml), a 10 % solution of citric acid (3 x 20 ml), water (20 ml), a 10 % solution of $NaHCO_3$ (4 x 20 ml) and finally, with a saturated

solution of NaCl (20 ml). The solvent was dried on Na_2SO_4 and evaporated in vacuo. This procedure is designated as standard treatment. The protected dipeptide was obtained as a white solid : yield 1.8 g (95 %) ; m.p. > 260°C ; TLC Rf (A) = 0.58.

N-(tert-butyloxycarbonyl)-3,5-dibromo-L-tyrosyl-D-threonine

To a solution of the preceding compound (0.63 g, 1 mmol) in MeOH (10 ml) cooled at 0°C , 2 ml of 1 N NaOH were added. The mixture was stirred at 0°C for 1 hour and at room temperature for 2 hours. The solution was concentrated in vacuo, diluted with 10 ml of water, filtered and acidified to pH 2 with 1N HCl. After extraction of the aqueous solution by EtOAc, the organic layer was dried and evaporated in vacuo. This treatment is designated as "standard procedure for alkaline hydrolysis". The white solid was recrystallized from EtOAc, yielding 0.50 g (95 %) of the pure protected dipeptide, m.p. > 260°C ; TLC Rf (B) = 0.90.

N-(tert-butyloxycarbonyl)-L-leucyl-L-threonine benzyl ester

To a solution of N-(tert-butyloxycarbonyl)-L-leucine mono hydrate (2.49 g, 10 mmol) in THF (15 ml) cooled in an ice-water bath, were added successively a solution of L-threonine benzyl ester hemioxalate (2.1 g, 10 mmol) and triethylamine (1.4 ml) in $CHCl_3$ (50 ml), a solution of HOBt (1.53 g, 10 mmol) in THF (25 ml) and a solution of DCC (2.06 g, 10 mmol) in $CHCl_3$ (10 ml). After 1 hour at 0°C, the mixture was allowed to come to room temperature and then stirred overnight. The reaction was treated following the standard procedure and a white solid (3.2 g, 75 %) was obtained which showed a single spot on TLC Rf(A) = 0.73 ; m.p. 114-115°C.

L-leucyl-L-threonine benzyl ester trifluoroacetate

The preceding compound (1.69 g, 4 mmol) was dissolved in TFA (6 ml) at 0°C. After 30 min, the mixture was allowed to come to room temperature and stirred for 30 mn. Evaporation in vacuo, several addition of ether and evaporation in vacuo led to an oily residue of the pure compound (1.71 g, 98 %) ; TLC Rf (C) = 0.57.

N-(tert-butyloxycarbonyl)-L-4'amino-3',5'- diiodophenylalanyl-L-leucyl-L-threonine benzyl ester

To a solution of the preceding compound (0.44 g, 1 mmol) and triethylamine (0.14 ml) in $CHCl_3$ (30 ml) cooled at 0°C, were added successively a solution of N-(tert-butyloxycarbonyl)-L-4'amino-3',5'-diiodophenyl alanine (0.53 g, 1 mmol) in $CHCl_3$ (20 ml), a solution of HOBt (0.15 g, 1 mmol) in THF (5 ml) and a solution of DCC (0.21 g, 1 mmol) in $CHCl_3$ (5 ml). After 1 hour at 0°C, the mixture was allowed to come to room temperature and then stirred overnight. The reaction was treated as usual leading 0.60 g (72 %) of a white solid ; m.p. 174-175°C ; TLC Rf(A) = 0.77.

L-4'-amino-3',5'-diiodophenylalanyl-L-leucyl-L-threonine benzyl ester

The preceding compound (0.50 g, 0.6 mmol) was dissolved in TFA (0.9 ml) at 0°C. After 30 min, the mixture was allowed to come to room temperature and stirred for 30 min. The addition of ether (100 ml) led to the precipitation of the crude product which was washed with ether (4 x 100 ml) and dried in vacuo ; yield 0.42 g (83 %) ; TLC Rf (C) = 0.78.

N-(tert-butyloxycarbonyl)-glycyl-L-4'amino-3',5'-diiodophenylalanyl-L-leucyl-L-threonine benzyl ester

To a solution of the preceding compound (0.38 g, 0.45 mmole) and triethylamine (0.06 ml) in $CHCl_3$ (8 ml) cooled at 0°C, were added successively a solution of N-(tert-butyloxycarbonyl)-glycine (0.08 g, 0.45 mmol) in THF (1 ml), a solution of HOBt (0.07 g, 0.45 mmol) in THF (1 ml) and a solution of DCC (0.1 g, 0.5 mmol) in $CHCl_3$ (1 ml).

After 1 hour at 0°C, the mixture was allowed to come to room temperature and then stirred overnight. The reaction was treated as usual leading 0.36 g (89%) of the pure diprotected tetrapeptide ; m.p. 150-152°C ; TLC Rf(A) 0.42.

Glycyl-L-4' amino-3',5'-diiodophenylalanyl-L-leucyl-L-threonine benzyl ester

The preceding compound (0.35 g, 0.40 mmol) was dissolved in TFA (0.6 ml) at 0°C. After 30 min, the mixture was allowed to come to room temperature and

stirred for 30 min. The addition of ether (80 ml) led to the precipitation of a part of the peptide which was washed with ether (3 x 80 ml). A second crop of the compound was obtained by evaporation and treatment of the first ether phase. The two crops of pure product were mixed yielding 0.35 g (96 %) of a white solid ; TLC Rf (B) = 0.85.

N-(tert-butyloxycarbonyl)-3,5-dibromo-L-tyrosyl-D-threonylglycyl-L-4'-amino-3',5'-diiodophenylalanyl-L-leucyl-L-threonine benzyl ester

To a solution of the preceding compound (0.23 g, 0.25 mmol) and triethylamine (0.035 ml) in $CHCl_3$ (15 ml) cooled at 0°C, were added successively a solution of N-(tert-butyloxycarbonyl)-3,5-dibromo-L-tyrosyl-D-threonine (0.135 g, 0.25 mmol) in THF (5 ml), a solution of HOBt (0.038 g, 0,25 mmol) in THF (3 ml) and a solution of DCC (0.052 g, 0.25 mmol) in $CHCl_3$ (3 ml). After 1 hour at 0°C, the mixture was stirred at room temperature for 24 hours. The reaction was treated as usual and the crude product was obtained and purified by "flash" chromatography on Kieselgel 60 with $CHCl_3$/MeOH, 20/1 as eluent. Fractions containing pure diprotected hexapeptide ; yield 0.23 g (70 %) ; m.p. > 260°C ; TLC, Rf(A) : 0.85.

N-(tert-butyloxycarbonyl)-3,5-dibromo-L-tyrosyl-D-threonylglycyl-L-4'-amino-3',5'-diiodophenylalanyl-L-leucyl-L-threonine

To a solution of the preceding compound (0.17 g, 0.13 mmol) in MeOH (4 ml) cooled at 0°C, 0.30 ml of 1N NaOH was added. The mixture was stirred at 0°C for 1 hour and at room temperature for 3 hours. The reaction was treated following the standard procedure for alkaline hydrolysis and produced a white solid ; yield 0.13 g (82 %) ; TLC Rf(B) = 0.83.

3,5-dibromo-L-tyrosyl-D-threonylglycyl-L-4'-amino-3',5'-diiodophenylalanyl-L-leucyl-L-threonine (I)

The preceding compound (0.1 g, 0.08 mmol) was dissolved in TFA (0.2 ml) at 0°C. After 30 min, the mixture was allowed to come to room temperature and stirred for 30 min. The addition of ether (40 ml) led to the precipitation of

the crude product which was washed with ether (3 x 40 ml) and dried in vacuo ; yield 80 mg (80 %). The purity of the compound was checked both by TLC : single spot Rf(B) = 0.65 and by HPLC : reversed phase μ Bondapak C_{18}, diam. 7.8 mm ; solvent : NH_4OAc buffer 10^{-2} M, pH = 4.3 / CH_3CN ; elution conditions : 16 % CH_3CN for 12 mm, linear gradient 16 % to 40 % in 10 min and isocratic conditions with 40 % CH_3CN ; flow rate 2.5 ml/mn : retention time of the single peak : 1792s. NMR (Me_2SO-d_6), : 4.24 ($TyrH_\alpha$), 7.42 (ArH), 2.50 and 2.80 (H_β), 4.05 (D-Thr H_α), 3.90 (H_β), 0.90 (CH_3), 3.68 (Gly H_α), 4.38 (Phe H_α), 7.55 (ArH), 2.72 and 2.95 (H_β), 4.20 (Leu H_α), 1.50 (H_β), 1.50 (H_γ), 0.85 (CH_3), 4.05 (L-Thr-H_α), 4.05 (H_β), 0.97 (CH_3).

II - Tritiation

Preparation of [3,5-^{3}H]L-tyrosyl-D-threonyl-glycyl-4'-amino-[3,5-^{3}H]-L-phenylalanyl-L-leucyl-L-threonine (II).

1.92 μmoles (2.35 mg) of compound I (3,5-dibromo-L-tyrosyl-D-threonyl-glycyl-L-4'-amino-3',5'-diiodophenylalanyl-L-leucyl-L-threonine) was dissolved in 1 ml of pure methanol and then frozen by liquid nitrogen. 14.5 mg (6 times) of the catalyst (PdO) was dispersed on the surface and the reacting vial was connected to the tritiation manifold. The vial was evacuated (10^{-3} Torr) and 70-80 curies (2580-2960 GBq) of pure tritium gas (99.9 %) were introduced and compressed until 1.12 Bars (Pressure red at low temperature) and the catalyst was flushed 15 minutes whereas the solution was kept cooled. After thawing, the reaction mixture was vigorously stirred at room temperature for 3.4 hours. The absorption of tritium gas produced a reduction in pressure of about 0.17 Bar (measured at low temperature). The catalyst was removed by Millipore filtration (GS) and washed with 50 ml of diluted methanol (methanol/water, 50/50, v/v). The labile tritium atoms were eliminated by successive flash rotative evaporations whith 50 ml of diluted methanol ($MeOH/H_2O$, 50/50, v/v). Total radioactivities recovered were : 82 mCi (3.034 GBq). Semi preparative purifications by HPLC were carried out on μ Bondapak C-18 (7.8 mm X 30 cm) column. The crude labelled material was chromatographed eluting with NH_4OAc (10^{-2}M, pH : 4.3/CH_3CN (75/25), flow rate

2.5 ml/min. The main peak commigrating with the reference was collected and rechromatographed giving one single peak by both uv and radioactivity detection (15 mCi, 555 MBq, yield : 15 %). Quantitative and comparative estimations indicated that the specific radioactivity was found to be : 50 Ci/mmole (1.850 TBq/mmol). The solution was evaporated to dryness by lyophilisation and the remaining solid dissolved in methanol. After several months of storage in liquid nitrogen, (para amino - [3',5'-^{3}H] phenylalanyl) DTLET retained its chemical property.

III - Introduction of the azido group

To a solution of compound II (15 mCi, 0.2 mg) in H_2O (80 µl) were added successively at 0°C 5 µl of 1 N HCl and 5 µl of 0.08 M solution of $NaNO_2$. After stirring for 10 min at 0°C, 5 µl of 0.08 M solution of NaN_3 were added and the mixture was stirred at 0°C, in the darkness for 1 h. Then the reaction mixture was neutralized with 1 N NaOH and evaporated. Then the residue was dissolved in MeOH (1 ml) and purified by HPLC (µ Bondapak C_{18}, diam. 7.8 mm ; NH_4OAc/CH_3CN , 75/25 ; flow rate : 2.5 ml/min). 35 % (6.8 mCi) of total radioactivity was found commigrating with Az-DTLET (retention time : 847 s). After acid hydrolysis of an aliquot (80 µCi) of the labeled compound (6 N HCl, 16-17 h) quantitative and comparative estimations indicated that the specific radioactivity was found to be 50 Ci/mmol (1.850 TBq/mmol).

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