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TRITIUM LABELLING OF HIGHLY SELECTIVE PROBES FOR δ-OPIOID RECEPTORS : [^{3}H] Tyr-D-Ser(O-t-Bu)-Gly-Phe-Leu-Thr(DSTBULET) and [^{3}H]Tyr-D-Ser(O-t-Bu)-Gly-Phe-Leu-Thr(O-t-Bu)(BUBU).

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SUMMARY

The introduction of bulky residue (s) in linear enkephalin-related hexapeptides represents a new approach in the design of selective probes for δ-opioid receptors, displaying the appropriate criteria to investigate biological and pharmacological properties of the assumed binding site (δ) of endogenous enkephalins. The selectivities and high affinities of Tyr-D-Ser(O-t-Bu)-Gly-Phe-Leu-Thr(DSTBULET) and especially Tyr-D-Ser(O-t-Bu)-Gly-Phe-Leu-Thr(O-t-Bu) (BUBU) associated with a satisfactory resistance to peptidases, make them the most suitable δ–probes reported to date. In the present paper, we report the synthesis of DSTBULET and BUBU under tritiated forms with high specific radioactivities. These radio-labelled probes will enable extensive in vitro and in vivo investigations of δ–opioid receptors properties to be carried out.

Keywords : Peptide synthesis, tritiated enkephalins, specific δ agonists.

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INTRODUCTION and RESULTS.

Endogenous opioid peptides are involved in the modulation of a variety of physiologic processes (1-5) which appear to be mediated by several types of opioid receptors (μ, δ, κ...) (6-8) requiring highly selective agonists and antagonists as biochemical as well as pharmacological probes.

Among the several series of enkephalin-related peptides so far synthesized, two have been found to display a high selectivity for the δ binding site. The first includes linear peptides obtained by modifications of the sequence of the endogenous peptides after extensive conformational studies of the native peptides. Among these compounds were the first reported relatively selective δ agonists DSLET and DTLET (9-11). The second type of δ selective probes belongs to the group of cyclic peptides. Although most of the cyclic enkephalins exhibit μ-selectivity, the penicillamino-containing-peptides : Tyr-D-Pen-Gly-Phe-Pen (DPLPE) and Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE) (12) were reported to be even more δ–selective than the linear hexapeptides DSLET and DTLET, although they were subsequently shown to have weaker affinities and consequent higher non-specific binding (13-15).

New δ-selective ligands have now been designed endowed with the affinity of DTLET and the selectivity of DPLPE, taking into account the comparative conformational properties of DTLET and DPLPE studied by ^{1}H NMR (16) and by theoritical calculations (17). The results of these studies suggested that an increase in the size of the residue in position 2 and/or 6 of DSLET might induce a partial inhibition of μ receptor recognition (16). Therefore, hexapeptides bearing bulky residues in these crucial positions were synthesized. Amongst these compounds, Tyr-D-Ser-(O-t-Bu)-Gly-Phe-Leu-Thr (DSTBULET) and Tyr-D-Ser-(O-t-Bu)-Gly-Phe-Leu-Thr (O-t-Bu) (BUBU) were shown to be among the most potent and selective δ probes reported to date (18-19), and these have now been tritiated.

Moreover, the usefullness of such probes being related to their specific radioactivity, two halogenated aminoacids were introduced into their precursors. In addition to the dibromo-3,5-L-tyrosine, a iodo-4-L-phenylalanine was included in place of the phenylalanine residue, allowing the incorporation ot three tritium atoms by reductive deshalogenation. The two precursors were obtained with a good field via a synthetic pathway which included the preparation of a common N-protected pentapeptide Z-Tyr-D-Ser (O-t-Bu)-Gly-Phe-Leu.

After tritiation of these two precursors, specific activities of 72 and 75 Ci/mmol (2664 GBq and 2275 GBq/mmol) were obtained for [^{3}H] DSTBULET and [^{3}H] BUBU respectively and the biological results have shown that [^{3}H] DSTBULET and [^{3}H] BUBU are suitable labelled probes for the exploration of the biochemical properties of the δ opioid receptor (19-20). In particular, the use of [^{3}H] BUBU has demonstrated the ability of this peptide analog to cross the blood-brain barrier (20) making it ideal choice to investigate the pharmacological responses associated to δ receptor activation, after systemic administration..

EXPERIMENTAL PART

MATERIALS.

Protected aminoacids are from Bachem (Switzerland). The peptides were synthesized by liquid phase method using tert-butyloxycarbonyl (Boc), benzyloxycarbonyl (Z) and methylesters as protecting groups and dicyclohexylcarbodiimide (DCC) with hydroxybenzotriazole (HOBt) or hydroxysuccinimide (HONSu) as coupling reagents.

The structure and the lack of racemisation of the compounds and all of the intermediates were established by ^{1}H NMR spectroscopy (Bruker WH 270 and 400 MHz). The purity was checked by thin layer chromatography on silicagel plates (Merck) in the following solvent systems (v/v) : A, chloroform-methanol (9:1) ; B, BuOH-AcOH-H_2O (4 : 1 : 1) ; C, EtOAc-pyridine-AcOH-H_2O (260 : 20 : 6 : 11) and by HPLC at 210 nm on a Waters apparatus (μ Bondapak C18 column) with Et_3N-H_3PO_4 buffer (TEAP, 0.025M, pH 6.5)/ CH_3CN as eluents (flow rate, 1.5 -2 ml/mn).

Amino acid analysis was carried out on a LKB biochrom 4400 analyzer after hydrolysis by 6 N HCL at 110°C for 24h. Mass spectra were recorded on a double-focusing VG 70-250 instrument. The FAB ionization was obtained with a FAB saddle field source (Ion Tech Ltd, Teddington, UK)operated with Xenon at 8 kV and 1 mA. Glycerol or cesium iodide was used for calibration. Accelerating voltage was set at 6 k V, and resolution was 1200. Mass spectra were obtained in different matrices and processed by means of the VG-250 software package.

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 (21). The catalyst was separated from the

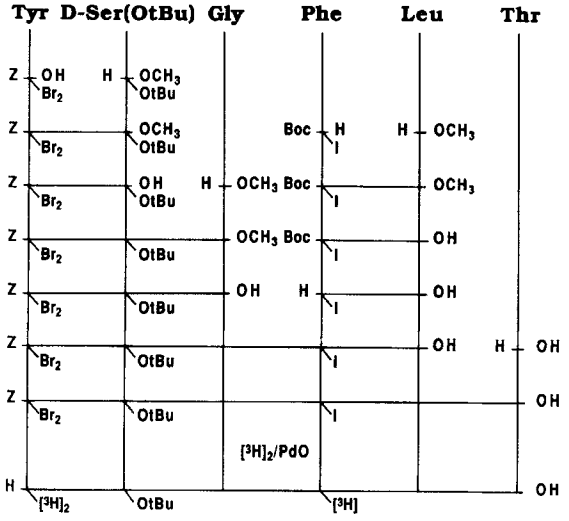
reacting solution by fliltration over Millipore GS. The tritium determinations were made with an Intertechnique liquid scintillation counter (SL 3000). The final ^{3}H-derivatives were purified by HPLC (Waters). After HCL hydrolysis, peptide weight determinations were carried out with a LKB 4400 amino acid analyser (U.K).

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

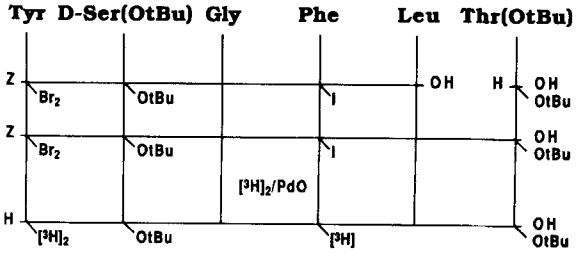
METHODS

Syntheses of [^{3}H] DSTBULET and [^{3}H] BUBU are reported schemes 1 and 2:

Scheme 1



Scheme 2



I - Peptide synthesis

A) Synthesis of 3,5-dibromo-L-tyrosyl-O-tert-butyl-D-serylglycyl-4-iodo-L-phenylalanyl-L-leucyl-L-threonine

N-(benzyloxycarbonyl)-3,5-dibromo-L-tyrosyl-O-tert-butyl-D-serine.

To a solution of Z-dibromo-L-tyrosine (11) (1.890 g, 4 mmol) in THF (30ml), cooled at 0°C, were added successively a solution of (O-t-Bu)-D-serine methylester hydrochloride (0.846 g, 4mmol) and triethylamine (0.56 ml) in $CHCl_3$ (20 ml), a solution HOBt (0.612 g, 4 mmol) in THF (10 ml) and a solution of DCC (0.824 g, 4 mmol) in $CHCl_3$ (10 ml). After 1 h at 0°C, the mixture was allowed to come to room temperature and 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 (20ml). The solution was dried (Na_2SO_4) and evaporated in vacuo. This procedure is designated as "the standard treatment". The protected dipeptide was obtained as a solid : yield 1,840 g (74%) ; mp > 260° C ; TLC Rf (A) 0.80, Rf (B) 0.90.

To the preceding compound (0.945 g, 1.5 mmol) in MeOH (5ml) cooled at O°C was added 5 ml of 1N NaOH. The mixture was stirred at 0°C for 1 h and overnight at room temperature. The solution was concentrated in vacuo, diluted with 30 ml of water, filtered and acidified to pH 2 with 1 N HCl. After extraction of the aqueous solution by EtOAc, the organic layer was dried and evaporated in vacuo. This treatment is designated as "the standard procedure for alkaline hydrolysis". The white solid was recrystallized from EtOAc, yielding 0.770 g (83%) of the pure protected dipeptide : mp > 260°C ; TLC Rf (B) 0.97, Rf(C) 0.65.

N-(benzyloxycarbonyl)-3,5-dibromo-L-tyrosyl-O-tert-butyl-serylglycine.

To a solution of the preceding compound (0.616 g, 1 mmol) in THF (20 ml) cooled at 0°C were added successively a solution of Gly-OMe, HCl (O,125 g, 1 mmol), and triethylamine (0.14 ml) in $CHCl_3$ (20 ml), a solution of HOBt (0.153 g, 1 mmol) in THF (10 ml) and a solution of DCC (0.206 g, 1 mmol) in $CHCl_3$ (10 ml).

After 1 at 0°C, the mixture was allowed to come to room temperature and then stirred overnight. The reaction was worked up via the standard treatment and yielded a white solid, recrystallized

from EtOAC, yielding O.645 g (94%) : mp > 260°C ; TLC Rf (A) 0.64.

To the preceding compound (0.623 g, 0.90 mmol) in MeOH (2 ml) cooled at 0°C, was added 1.8 ml of 1 N NaOH. The mixture was stirred at 0°C for 1 h and overnight at room temperature. The reaction was treated via the standard procedure for alcaline hydrolysis and yielded the N-protected tripeptide : 0.5 g (80%) ; mp > 260°C ; TLC Rf (B) 0.98 ; Rf(C) 0.70 FAB-MS (MH^+) calcd 674, found 674.

N-(tert-butyloxycarbonyl)-4'-iodo-L-phenylanyl-L-leucine methylester

To a solution of Boc-4'-iodo-L-Phe (0.587 g, 1.5 mmol) in THF (25 ml) cooled in an ice-water bath, were added successively a solution of L-Leu-OMe, HCl (0.273 g, 1.5 mmol) and triethylamine (0.21 ml) in $CHCl_3$ (25 ml), a solution of HOBt (0.23 g, 1.5 mmol) in THF (10 ml) and a solution of DCC (0.31 g, 1.5 mmol) in $CHCl_3$ (10 ml). After 1 h at 0°C, the mixture was allowed to come room temperature and then stirred overnight. The reaction was then worked up via the standard treatment, yielding 0.721 g (93%) of the pure protected dipeptide as a white solid : TLC Rf (A) 0.95, Rf(D) 0.80.

4'-iodo-L-phenylalanyl-L-leucine trifluoroacetate

To the preceding compound (0.720 g, 1.4 mmol) in MeOH (10 ml) cooled at 0°C, was added 1.4 ml of 1 N NaOH. The mixture was stirred at 0°C for 1 h and at room temperature for 6 h. The reaction was treated via the standard procedure for alcaline hydrolysis and yielded the N-protected dipeptide : 0,52 g (75%) ; TLC Rf (B) 0.98, Rf(C) 0.60. The latter compound (0.50 g, 1 mmol) was then dissolved in TFA (1.5 ml) at 0°C. After 30 min, the mixture was allowed to come to room temperature and stirred for 30 min. After evaporation of TFA in vacuo, the addition of ether (30 ml) led to the precipitation of the compound. The white solid was washed with ether (4 x 30 ml) and dried in vacuo : yield 0.50 (96%); TLC Rf (B) : 0.62.

N-(benzyloxycarbonyl)-3,5-dibromo-L-tyrosyl-O-tert-butyl-D-serylglycyl-4'-iodo-L-phenylalanyl -L-leucine (1)

To a sample of Z-3,5 dibromo-Tyr-D-Ser-(OtBu)-Gly (236 mg, 0.35 mmol) dissolved in DMF (2 ml) and cooled at - 20°C were added HONSu (50 mg, 0.42 mmol) and DCC (80 mg, 0.38 mmol). The mixture was then stirred for 0.5 h at -20°C, 1 h at 0°C and 15 h at room temperature and DCU was removed.

To the preceding solution, cooled at 0°C was added a solution of 4'-iodo-L-Phe-Leu, TFA (182 mg, 0.35 mmol) and triethylamine (0.05 ml) inDMF (1.5 ml). The reaction mixture was stirred 1 h at 0°C and at room temperature overnight. After filtration and evaporation of DMF in vacuo, the residue was dissolved in EtOAc (10 ml) and washed successively with a 10% solution of citric acid (3 x 5 ml) and a saturated solution of NaCl (5 ml). The solvent was dried on Na_2SO_4 and evaporated in vacuo, leading to the pure compound : 300 mg (81%) ; TLC Rf (C) 0.61 ; FAB-MS (MH^+) calcd 1060, found 1060.

N-(benzyloxycarbonyl)-3,5-dibromo-L-tyrosyl-O-tert-butyl-D-serylglycyl-4'-iodo-L-phenylalanyl-L-leucyl-L-threonine (2)

To a sample of Z-3,5-dibromo-Tyr-D-Ser(OtBu)-Gly-4'-iodo-L-Phe-Leu (1) (150 mg, 0.14 mmol) dissolved in DMF (1ml) and cooled at - 20°C, were added HONSu (20 mg), O.17 mmol) and DCC (32 mg, 0.15 mmol). The mixture was stirred for 0.5 h at - 20°C, 1 h at 0°C and at room temperature overnight. DCU was removed and L-Thr (17 mg, 0.14 mmol) was added at 0°C. Then the reaction mixture was stirred for 1 h at 0°C and at room temperature overnight. The treatment previously described for compound 1 led to 114 mg (70%) of the crude compound, which was purified by "flash chromatography" an Kieselgel with 3 parts of AcOEt and 1 part of $CHCl_3$-MeOH-H_2O-AcOH (5 : 5 : 1 : 0.5) as eluent. Fractions containing pure compound (Rf : 0.45) were collected : yield 80 mg (50%) ; TLC Rf (B) 0.96, Rf (C) 0.65. The amino acid analysis, analytical HPLC, and FAB-MS data are given in Table I.

N-(benzyloxycarbonyl)-3,5-dibromo-L-tyrosyl-O-tert-butyl-D-serylglycyl-4'-iodo-L-phenylalanyl-L-leucyl-O-tert-butyl-L-threonine (3).

To a sample of Z-3,5-dibromo-Tyr-D-Ser-(OtBu)-Gly-4'-iodo-L-Phe-Leu (1) (150 mg, 0.14 mmol) dissolved in DMF (1 ml) and cooled at - 20°C, were added HONSu (20 mg, 0.17 mmol) and DCC (32 mg, 0.15 mmol). The mixture was stirred for 0.5 h at - 20°C, 1 h at 0°C and at room temperature overnight. DCU was removed and L-Thr(OtBu) (25 mg, 0.14 mmol) was added at 0°C. Then the reaction mixture was stirred for 1 h at 0°C and at room temperature overnight. The treatment previously described for compound 1 led to 120 mg (70%) of the pure compound ; TLC Rf (B) 0.98, Rf (C) O.70. The amino acid analysis, analytical HPLC and FAB-MS data are given in Table I.

Table I Analytical data of DSTBULET, BUBU and their halogenated precursors

N°	Compound	amino. acid anal. Tyr-	D-X-	Gly-	Phe	-Leu	-Thr	HPLC TEAP/CH_3CN %	elut. time	FAB-MS MH^+ calcd.	found
	DSTBULET	0.96	1.01	0.98	1.02	1.00	0.96	75/25	14.4	743	743
	BUBU	0.89	0.86	1.02	1.04	1.00	0.85	68/32	9.4	799	799
2	Z diBr Tyr-D-Ser (OtBu)-Gly-I-Phe-Leu-Thr		0.85	1.01		1.00	0.91	50/50	4.9	1161	1161
3	Z diBr Tyr-D-Ser(OtBu)-Gly-I-Phe-Leu-Thr(OtBu)		0.87	1.03		1.00	0.86	50/50	12.4	1217	1217

II - Tritiations

Preparations of [^{3}H] DSTBULET and [^{3}H] BUBU. 1.75 µmoles (2mg) of Z-3,5-dibromo-Tyr-D-Ser(OtBu)-Gly-4'-iodo-L-Phe-Leu-Thr (DSTBULET) and 1.93 µmoles (2.4 mg) of Z-3,5-dibromo-Tyr-D-Ser (OtBu)-Gly-4'-iodo-Phe-Leu-Thr (OtBu) (BUBU) were dissolved in 1 ml of pure methanol with 2 µl of triethylamine and then frozen by liquid nitrogen. 10 mg or 12 mg (for DSTBULET and BUBU, 5 times) of catalyst (PdO) were dispersed on the surface and the reacting vial was connected to the automatic gas transfer unit (21). When the vial and all tubings had been evacuated (vacuum : 0.13 Pa), 1.295-1.480 TBq (35-40 curies) of pure gas tritium were introduced and compressed until 1.1 - 1.2 .10^3hPa and the catalyst was then flushed 15 minutes (pressure red at low temperature : - 196°C) into the still frozen solution.

After thawing, the reaction mixtures were vigorously stirred at room temperature for 2 hours. The absorption of tritium gas produced an identical pressure reduction of about 0.2 .10^3 hPa for the two peptides (pressure red at low temperature). PdO was easily removed by filtration over Millipore filter (FG) and washed with 2 x 20 ml of pure methanol and labile tritium atoms eliminated by successive flash rotative evaporations with 4 x 20 ml of diluted methanol (MeOH/H_2O, 50:50 v/v).Total radioactivities recovered was : 1909.2 TBq: 51.6 mCi (DSTBULET) and 2053.5 TBq : 55.5 mCi (BUBU). The first attempt to analyse the crude labelled products were performed using TLC on silicagel with solvent system B. The analytical autoradio-chromatograms and ^{3}H scannings performed on each peptide revealed a major peak (DSTBULET, Rf : 0.58, BUBU, Rf: 0.87) commigrating with the reference and corresponding to the

spot detectable with 4,4'-tetramethyldiamino-diphenyl methane (TDM).

Purifications of crude peptides were then performed by preparative thin layer chromatography and after visualization by autoradiochromatography, labelled peptides were extracted from silicagel powder with 5 x 10 ml of pure methanol. After centrifugation and concentration, peptides were also checked by high performance liquid chromatography (see conditions in Table I) and were chromatographically identical with authentic references. Ultra-violet spectra of tritiated peptides were found to be exactly the same as that of references. After acid hydrolysis of an aliquot (5 nmoles) of the two labelled agonists (6N HCl, 16-17 hours), the following amino acid analyses were found: Tyr, 0.86; Gly, 1.00; Phe, 1.00; Leu, 0.98; Thr, O.92 (DSTBULET and Tyr, 0.84; Gly, 1.00; Phe, 0.99; Leu, 1.00 (BUBU). Quantitative and comparative estimation (by u.v. spectrometric titration and amino acid analysis) indicated and confirmed that the specific activities were: 2590-2664 GBq/mmol (70-72 Ci/mmol) for [^{3}H]DSTBULET and 2775 GBq/mmol (75 Ci/mmol) for [^{3}H]BUBU. The overall radiochemical yields of DSTBULET and BUBU are 29.9% and 30.5% respectively; an absorption into the catalyst and a low recovery from the TLC purification could explain these percentages seeing that the hydrogenolysis of the two halogenated peptides was complete, as well for dibromo-Tyr than for iodo-Phe. Radiochemical purity, greater than 97%, was obtained and no significant difference was found in binding studies between tritiated and non tritiated products (19).

After several months of storage in liquid nitrogen, the tritiated compounds retained both their chemical and biological potencies.

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