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# Synthesis of 2',6'-Dimethyltyrosine Containing Tritiated Delta Opioid-Receptor Selective Antagonist Dipeptide Ligands with Extraordinary Affinity

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**Summary:**

A new class of δ opioid antagonists was recently discovered in which the sequence Tyr-Tic was used as a message domain. The substitution of $Tyr^1$ by Dmt enhanced the δ selectivity and antagonist activity. The excellent properties of these ligands stimulated us to prepare the corresponding tritiated derivatives. Peptides containing Tic at position 2 undergo spontaneous diketopiperazine formation in some solvents, with a reduction in opioid activity. To avoid this side-reaction we synthesised the N,N-dimethylated analogue ($N,N(Me)_2$-Dmt-Tic-OH), which was found to be stable. On the basis of this result, we prepared diiodinated analogues of H-Dmt-Tic-OH and $N,N(Me)_2$-Dmt-Tic-OH to undergo catalytic dehalotritiation. Products of high specific radioactivity were obtained: 44.67 Ci/mmol for [$^3$H]Dmt-Tic-OH and 59.88 Ci/mmol for [$^3$H]$N,N(Me)_2$-Dmt-Tic-OH.

Keywords: δ opioid antagonist, dipeptide, tritium

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## Introduction:

The heterogeneity of opioid receptors is well established and it is accepted that there are at least three types of opioid receptor (μ, δ and κ). As no opioid ligand has been found to exhibit absolute specificity for any particular receptor type, several laboratories have made an effort to develop ligands of high specificity. Simple δ opioid dipeptide antagonists containing the sequence Tyr-Tic (Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) (1) have recently been described. Replacement of Tyr by the more hydrophobic residue 2',6'-dimethyltyrosine (Dmt) resulted in new analogues with high *in vitro* δ opioid antagonism (2) and exceptional δ receptor affinity ($K_i^{\delta}$ = 0.022 nM) and selectivity ($K_i^{\mu}/K_i^{\delta}$ = 150000). Peptides containing imino acids such as Tic in position 2 are prone to cyclization during synthesis and storage, with diketopiperazine formation (3,4,5). This side-reaction decreases the opioid activity of these ligands. This phenomenon occurs not only for peptides containing Tic or other imino acids in the proper sequence, but also for other residues during peptide synthesis (6). On the basis of these results, we have synthesised an N-alkylated analogue of Dmt-Tic with the purpose of avoiding diketopiperazine formation. N,N-Dimethylation affected the δ receptor binding properties, and the affinity of N,N(Me)$_2$-Dmt-Tic-OH was slightly lower (5-fold) in comparison with that of H-Dmt-Tic-OH, but the antagonist activity of the new analogue was better (7).

## Experimental section:

Materials:

H-L-Dmt-OH was a generous gift from and synthesised by Dygos et al. (8). H-Tic-OH was obtained from Bachem Feinchemikalien AG. Melting points were determined on a Kofler apparatus and were uncorrected. Optical rotations were determined at 10 mg/ml in methanol with a Perkin-Elmer 241 polarimeter with a 10 cm water-jacketed cell. Analytical HPLC analyses were carried out with a Bruker liquid chromatography LC 21-C instrument, using a

Vydac 218 TP 5415 C18 column (250×4.6 mm, 5 μm particle size) and a Bruker LC 313 W variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled with an Epson computer system (QX10). TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany). Ninhydrin (1%, Merck) and UV light were employed to detect the peptides. NMR samples were run at 400 MHz on a Bruker AM-400 instrument equipped with an Aspect 3000 computer. For the determination of mass ions, a triple-stage quadrupole mass spectrometer (TSQ 700; Finnigan MAT, San Jose, CA, U.S.A.) was used. $PdO/BaSO_4$ (10 % Pd) catalyst was from Merck. $^3H_2$ gas was purchased from Technobexport, Russia, and contained at least 98 % tritium. The amount of tritiated material was measured by UV detection on a Shimadzu-160 spectrophotometer. For radiochemical purification, we used a Jasco PU 980 HPLC equipped with a Merck 50943 LiChroCart 125-4 LiChrospher 100 RP-18 (5 μm particle size) column. Detection was performed with a Jasco UV-975 detector at 214 nm and a Packard Flow-one/β A-500 radiodetector. Radioactivity was counted with a Searle-Delta-300 liquid scintillation counter in a toluene-Triton X-100 scintillation cocktail. Radiochemical purity was checked with a Berthold Radichromatogram Tracemaster.

**Methods:**

The peptides were prepared by standard solution methods. N-Dimethylated peptide was obtained by exhaustive methylation of the corresponding deprotected linear dipeptide with aqueous formaldehyde and $NaBH_3CN$ in acetonitrile (9). The peptides containing L-Dmt were prepared from the optically pure L-Dmt amino acid. All analogues showed less than 1 % impurities when monitored at 220 nm.

*Synthesis of H-Dmt(3',5'-$I_2$)-Tic-OH:*— The diiodinated precursor H-Dmt(3',5'-$I_2$)-Tic-OH was prepared by treating H-Dmt-Tic-OH with chloramine T and sodium iodide according to reported methods (10). H-Dmt-Tic-OH (0.1 g; 0.21 mM) was dissolved in

water/acetonitrile 1:1 (10 ml) at room temperature and treated by the simultaneous addition of chloramine T (0.3 g; 1.05 mM) and sodium iodide (0.16 g; 1.05 mM). After 5 min., the reaction mixture containing mono- and diiodinated peptides were cooled in ice and lyophilized. The residue was purified on a preparative HPLC [Waters Delta Prep 3000 L 30×3 cm; 15 μm] column, with a linear gradient of 0-60 % B in 25 min. at a flow rate of 50 ml/min. (solvent A = 10 v/v % acetonitrile in 0.1 % trifluoroacetic acid; solvent B = 60 v/v % acetonitrile in 0.1 % trifluoroacetic acid) and identified by NMR, MALDI-TOF mass spectrometry and elemental analysis. Analytical characterization of H-Dmt(3',5'-$I_2$)-Tic-OH: yield 0.12 g (81 %); TLC $R_f$ = 0.51 (mobile phase: nBuOH/AcOH/$H_2O$ 4:1:1); m.p. 166-168 °C; $[\alpha]D^{20}$ +21.4 (c = 1, MeOH); $MH^+$ 621; $^1$H-NMR (DMSO) δ = 2.18 (s,6H); 3.16-3.46 (m,4H); 4.16-4.67 (m,4H); 7.08 (s,4H); 9.12 (s,1H). HPLC analysis was monitored at 220 nm. The peptide was eluted with a linear gradient of 0-100 % B in 25 min. at a flow rate of 1 ml/min. k' = 5.61 $t_R$ = 16.16 min. During HPLC analysis, the formation of a small amount of cyclic derivative may occur, which under the same conditions as reported above has $t_R$ = 21.03 min.. Analytical characterization of H-Dmt(3'-I)-Tic-OH: yield 0.02 g (19 %); TLC $R_f$ = 0.45 (mobile phase: nBuOH/AcOH/$H_2O$ 4:1:1); m.p. 150-152 °C; $[\alpha]D^{20}$ +22.2 (c = 1, MeOH); $MH^+$ 495; $^1$H-NMR (DMSO) δ = 2.17 (s,6H); 2.79-3.25 (m,4H); 4.13-4.58 (m,4H); 6.56 (s,1H); 7.13 (s,4H); 9.9 (s,1H). HPLC analysis was monitored at 220 nm. The peptide was eluted with a linear gradient of 0-100 % B in 25 min. at a flow rate of 1 ml/min. k' = 5.19.

*Synthesis of N,N(Me)$_2$-Dmt(3',5'-$I_2$)-Tic-OH:*—The diiodinated precursor N,N(Me)$_2$-Dmt(3',5'-$I_2$)-Tic-OH was prepared by treating N,N(Me)$_2$-Dmt-Tic-OH (7) with chloramine T and sodium iodide according to reported methods (10). Briefly, N,N(Me)$_2$-Dmt-Tic-OH (0.11 g; 0.21 mM) was dissolved in water/acetonitrile 1:1 (10 ml) at room temperature and treated by the simultaneous addition of chloramine T (0.3 g; 1.05 mM) and sodium iodide (0.16 g; 1.05 mM). After 5 min., the reaction mixture containing mono- and diiodinated peptides were cooled in ice and lyophilized. The residue was purified on a preparative HPLC

[Waters Delta Prep 3000 L 30×3 cm; 15 μm particle size] column, with a linear gradient of 0-60 % B in 25 min. at a flow rate of 50 ml/min. (solvent A = 10 v/v % acetonitrile in 0.1 % trifluoroacetic acid; solvent B = 60 v/v % acetonitrile in 0.1 % trifluoroacetic acid) and identified by NMR, MALDI-TOF mass spectrometry and elemental analysis. Analytical characterization of N,N(Me)$_2$-Dmt(3',5'-I$_2$)-Tic-OH: yield 0.13 g (80 %); TLC $R_f$ = 0.53 (mobile phase: nBuOH/AcOH/$H_2O$ 4:1:1); m.p. 153-155 °C; $[\alpha]D^{20}$ +25 (c = 1, MeOH); $MH^+$ 649; $^1$H-NMR (DMSO) δ = 2.29 (s,6H); 2.65 (s,6H); 2.77-3.24 (m,4H); 4.16-4.67 (m,4H); 7.17 (s,4H); 9.41 (s,1H). HPLC analysis was monitored at 220 nm. The peptide was eluted with a linear gradient of 0-100 % B in 25 min. at a flow rate of 1 ml/min. k' = 5.84. Analytical characterization of N,N(Me)$_2$-Dmt(3'-I)-Tic-OH: yield 0.03 g (20 %); TLC $R_f$ = 0.47 (mobile phase: nBuOH/AcOH/$H_2O$ 4:1:1); m.p. 140-142 °C; $[\alpha]D^{20}$ +126.7 (c = 1, MeOH); $MH^+$ 523; $^1$H-NMR (DMSO) δ = 2.19 (s,6H); 2.44 (s,6H); 2.74-3.34 (m,4H); 4.16-4.62 (m,4H); 6.54 (s,1H); 7.18 (s,4H); 10.2 (s,1H). HPLC analysis was monitored at 220 nm. The peptide was eluted with a linear gradient of 0-100 % B in 25 min. at a flow rate of 1 ml/min. k' = 4.84.

*Preparation of H-[3',5'-$^3$H]Dmt-Tic-OH:*— H-[$^3$H]Dmt-Tic-OH was prepared by catalytic dehalogenation of the precursor using $^3H_2$ gas and Pd as catalyst (Figure 1). To a solution of 1.6 mg (~2.5 μmol) H-[3',5'-I$_2$]-Dmt-Tic-OH in 1 ml DMF, 12 mg PdO/$BaSO_4$ (10 % Pd) and 0.8 μl TEA were added and the reaction vessel was connected to the tritium manifold (11). After freezing of the reaction mixture with liquid nitrogen and evacuation, tritium gas (555 GBq [15 Ci]) was introduced. The mixture was stirred with a magnetic stirrer and the reaction was followed by a manometer measuring the tritium pressure. The reaction time was 80 min. The reaction was terminated by adsorption of the unreacted tritium gas on pyrophoric uranium.

The vessel was removed from the tritium manifold to work up the crude mixture. The catalyst was removed by filtration through a Whatman GF/C filter and washed several times with ethanol. The traces of labile tritium were removed from the ethanol/water (1:1) solution of the

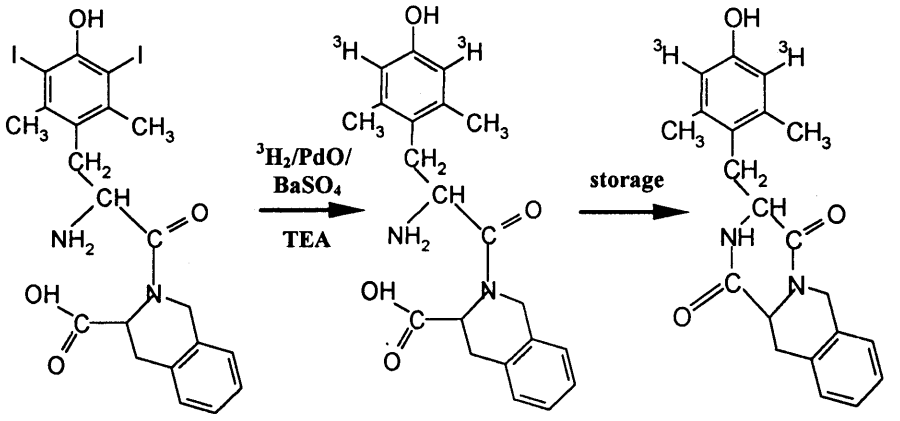
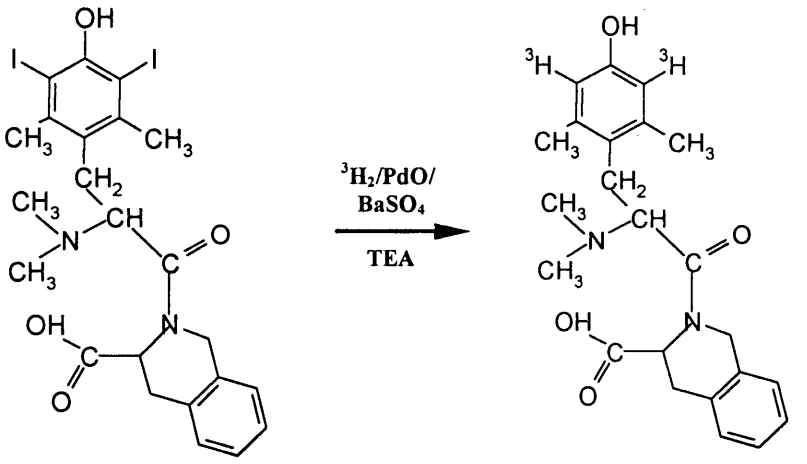



Figure 1. The tritiation of 3',5'-$I_2$-Dmt -Tic-OH, and the side-reaction under storage

radiolabelled product by repeated evaporation. The total radioactivity of the radiolabelled peptide was measured by liquid scintillation counting, and it proved to be 3.96 GBq (107 mCi). The crude tritiated peptide was purified by RP-HPLC. Gradient elution was used and the solvent system was A: acetonitrile/0.1 % TFA, B: water/0.1 % TFA. The purified peptide was stored in 18.5 MBq/ml (0.5 mCi/ml) concentration in methanol/water 1:4, with the pH adjusted to 9 with $NH_4OH$, and with the solution under liquid nitrogen.

*Preparation of N,N(Me)$_2$-[3',5'-$^3$H]Dmt-Tic-OH:*— N,N(Me)$_2$-[$^3$H]Dmt-Tic-OH was prepared by catalytic dehalogenation of the precursor using $^3H_2$ gas and Pd as catalyst (Figure 2). To a solution of 2 mg (~2.5 μmol) N,N(Me)$_2$[3',5'-$I_2$]-Dmt-Tic-OH in 1 ml DMF, 12 mg PdO/$BaSO_4$ (10 % Pd) and 0.9 μl TEA were added and the reaction vessel was connected to the tritium manifold. After freezing of the reaction mixture with liquid nitrogen and evacuation, tritium gas (555 GBq [15 Ci]) was introduced. The mixture was stirred with a magnetic stirrer and the reaction was controlled by following the tritium pressure. The reaction was terminated by adsorption of the unreacted tritium gas on pyrophoric uranium. The reaction




**3',5'-$I_2$-N,N-dimethyl-Dmt-Tic** **3',5'-$^3H_2$-N,N-dimethyl-Dmt-Tic**

Figure 2. The tritiation of 3',5'-$I_2$-N,N(Me)$_2$-Dmt-Tic-OH

was completed in 80 min. The catalyst was removed by filtration through a Whatman GF/C filter and washed several times with ethanol. The traces of labile tritium were removed from the ethanol/water (1:1) solution of the radiolabelled product by repeated evaporation. The total radioactivity of the tritiated peptide was measured by liquid scintillation counting, and it proved to be 2.96 GBq (80 mCi). The crude tritiated peptide was purified by RP-HPLC using the same instrumentation as described above. Gradient elution was used and the solvent system was A: acetonitrile/0.1 % TFA, B: water/0.1 % TFA. The purified peptide was stored in 18.5 MBq/ml (0.5 mCi/ml) concentration in acetonitrile/water 1:4 under liquid nitrogen.

**Results and Discussion:**

Iodination of the dipeptides H-Dmt-Tic-OH and N,N(Me)$_2$-Dmt-Tic-OH gave both mono- and diiodinated compounds (20 % and 80 %, respectively), which were separated by HPLC. In the case of H-Dmt-Tic-OH, diketopiperazine formation was also observed, depending on the solvents and pH conditions. H-[$^3$H]Dmt-Tic-OH was characterized by analytical HPLC under

the following gradient conditions: at 0 min. 15 % A/85 % B, at 10 min. 20 % A/ 80 % B, at 15 min. 32 % A/68 % B and at 25 min. 50 % A/50 % B. The flow rate was 1 ml/min. $t_R$ = 15.97 min. and k' = 10.2. During tritiation, we also observed the formation of diketopiperazine, with a retention time of 19.3 min. k' = 12.7. The purity of the radiolabelled peptide was assessed by RP-HPLC under the same conditions, and was found to be at least 95 %. After purification, we found 1.6 % of diketopiperazine. During storage in methanol this increased to 18.9% at 24 hours. TLC was found to increase diketopiperazine formation. The amount of peptide was determined by UV spectrometry using unlabelled TFA×H-Dmt-Tic-OH as a standard ($\varepsilon$ = 29.74 $dm^3$/mmol×cm at 214 nm) [29740 $cm^2$/mmol]. The specific activity of the purified H-[$^3$H]Dmt-Tic-OH was 1.65 TBq/mmol (44.67 Ci/mmol).

The purity of the N,N(Me)$_2$-[$^3$H]Dmt-Tic-OH was assessed by RP-HPLC under the following gradient conditions: at 0 min. 20 % A/80 % B, at 10 min. 30 % A/70 % B, and at 15min. 40 % A/60 % B, and also by TLC, and was found to be higher than 95 %. The flow rate was 1 ml/min. $t_R$ = 10.2 min., k' = 8.1. The amount of peptide was determined by UV spectrometry using unlabelled TFA×N,N(Me)$_2$-Dmt-Tic-OH as a standard ($\varepsilon$ = 30.92 $dm^3$/mmol×cm at 214 nm) [30920 $cm^2$/mmol]. The specific activity of the purified N,N(Me)$_2$-[$^3$H]Dmt-Tic-OH was 2.22 TBq/mmol (59.88 Ci/mmol). We investigated the radiochemical purity of the N,N(Me)$_2$-[$^3$H]Dmt-Tic-OH repeatedly and found that this ligand was very stable under our storage conditions. In the binding assay, the affinity of N,N(Me)$_2$-Dmt-Tic-OH was somewhat lower (5-fold) in comparison with that of H-Dmt-Tic-OH; in an *in vitro* assay, the dimethylated analogue was more potent than the parent compound. This affords a good opportunity to use it as a $\delta$ ligand, and the tritiated form will be a useful tool for the characterization of opioid receptors. The binding data (published elsewhere) are promising for this new potent and selective $\delta$ opioid receptor ligand with very low non-specific binding and with high specific radioactivity.

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