Journal of Labelled Compounds and Radiopharmaceuticals
J Label Compd Radiopharm 2007; **50**: 1148–1152.
Published online 31 August 2007 in Wiley InterScience
(www.interscience.wiley.com). DOI: 10.1002/jlcr.1402

JLCR

Research Article

Tritium labelling and degradation studies of Dmt^1-endomorphin 2

ERZSÉBET SZEMENYEI and GÉZA TÓTH*

Isotope Laboratory, Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary

Received 17 April 2007; Revised 14 May 2007; Accepted 14 May 2007

Abstract: Two tritiated derivatives of Dmt^1-endomorphin 2 (Dmt^1-EM2) were prepared by the catalytic tritiodehalogenation of 3′,5′-diiodo-Dmt^1-EM2 and the saturation of $^{3,4}\Delta$Pro^2-Dmt^1-EM2, resulting in [3′,5′-$^3H_2$]Dmt^1-EM2 and [$^3H_2$]Pro^2-Dmt^1-EM2 with specific activities of 2.88 TBq/mmol (77.8 Ci/mmol) and 1.95 TBq/mmol (52.8 Ci/mmol), respectively. 3′,5′-Diiodo-Dmt^1-EM2 was synthesized by the chloramine T method from Dmt^1-EM2. $^{3,4}\Delta$Pro^2-Dmt^1-EM2 was synthesized by using the Merrifield solid-phase method. The distributions of the tritium in the labelled peptides were investigated by reversed-phase high-performance liquid chromatography after acidic hydrolysis. The stability of Dmt^1-EM2 in a rat brain membrane homogenate was also determined. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: opioid peptides; catalytic tritiation; Dmt^1-endomorphin 2; degradation

Introduction

Since the discovery 10 years ago of the endomorphins[1] (endomorphin 1, H-Tyr-Pro-Trp-Phe-NH_2, EM1, and endomorphin 2, H-Tyr-Pro-Phe-Phe-NH_2, EM2), which are endogenous neuropeptides with high affinity for the μ-opioid receptor, several new analogues have been developed, mainly with the aim of increasing the opioid receptor affinity, the bioactivity and the enzyme resistance. The substitution of Dmt (2′,6′-dimethyltyrosine) for Tyr in EM2 (Dmt^1-EM2) resulted in a 5-fold increase in the μ-opioid receptor affinity.[2,3] Simultaneously, the δ-opioid receptor affinity was increased 2–3-fold, Dmt^1-EM2 thereby being a bivalent EM2 derivative.[2,4] Through this substitution, the action was shifted more spinally than supraspinally.[5] The pharmacological potency of most Dmt analogues can presumably be attributed to the enzyme resistance at the N-terminal.[6] The increase in hydrophobicity of Dmt opioid peptides resulting from dimethylation of the phenol ring may lead to an improved ligand–receptor interaction. The appropriately oriented phenol ring arising from the dimethylation can probably play a role in the formation of hydrophobic forces with the aliphatic or aromatic residues of the receptors.[7]

The present article relates to the synthesis of two novel, labelled opioid ligands: [3′,5′-$^3H_2$]Dmt^1-EM2 (H-[3′,5′-$^3H_2$]Dmt-Pro-Phe-Phe-NH_2) and [$^3H_2$]Pro^2-Dmt^1-EM2 (H-Dmt-[$^3H_2$]Pro-Phe-Phe-NH_2). The aim of the work was to acquire suitable tools for use in *in vitro* and *in vivo* biological assays.

Results and Discussion

Dmt^1-EM2 and Dmt^1-$^{3,4}\Delta$Pro^2-EM2 were synthesized by the SPPS method on 4-methylbenzhydrylamine resin by means of Boc chemistry. 3′,5′-Diiodo-Dmt^1-EM2 was synthesized by the chloramine T method.[8,9] The radioligands were prepared by tritiation of the precursor peptides containing 3′,5′-diiodo-Dmt and 3,4-dehydroproline. The crude tritiated products were purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The quantitative analyses of the chemical amounts and radioactivities of the labelled peptides were performed by RP-HPLC via UV and radioactivity detection using a calibration curve produced with unlabelled Dmt^1-EM2. Analytical data

*Correspondence to: Géza Tóth, Isotope Laboratory, Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary. E-mail: geza@brc.hu
Contract/grant sponsor: Hungarian National Bureau of Research and Technology; contract/grant numbers: NKTH and DNT 08/2004
Contract/grant sponsor: Hungarian Research Foundation; contract/grant numbers: OTKA 046514 and OTKA TS 09817

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on the unlabelled and radioactive peptides were obtained by TLC, HPLC and MS (see Tables 1 and 2).

The distributions of the tritium labels in [$^3H_2$]Dmt^1-EM2 and [$^3H_2$]Pro^2-Dmt^1-EM2 were determined after acidic hydrolysis and Fmoc derivatization by HPLC. The Dmt and Pro contained tritium in >90% of the theoretical level. The Phe residues in the peptides were also partially labelled (Table 3). This phenomenon presumably caused the overall higher specific activity of [$^3H_2$]Dmt^1-EM2 than the theoretical level. The specific activity of [$^3H_2$]Pro^2-Dmt^1-EM2 was >90% of theoretical level, and the specificity of the label was satisfactory, resulting in an appropriate radioligand for radioligand-binding experiments and metabolic studies.

The stability of a radioligand in radioligand-binding studies is essential, and it is therefore necessary to determine the biological half-life of the ligand in the biological matrix used. In our earlier investigations, EM1 and EM2 demonstrated long half-lives in the presence of a rat brain membrane preparation (0.3 mg/ml protein): 295 and 230 min, respectively, accordingly they do not degrade during binding assays.[10] In the present study, the half-life of [$^3H_2$]Pro^2-Dmt^1-EM2 under the above conditions was 515 min.

The kinetics of degradation of Dmt^1-EM2 was studied in a rat brain homogenate, as compared with the earlier published degradation kinetics of EM2.[11] The protein content of the homogenate was 5.92 mg/ml and Dmt^1-EM2 concentration was 100 μM. Figure 1 shows the kinetics of degradation of EM2 and Dmt^1-EM2 in the rat brain homogenate. After 12 min of incubation, only 24% of the parent EM2 remained in the samples, whereas 72% of the initial Dmt^1-EM2 concentration remained after 15 min of incubation. The logarithmic forms of these curves were analysed by linear regres-

Table 1 Analytical data on Dmt-EM2 analogues

Peptides	TLC[a]			HPLC[b]	MS[c]	
	R_f (A)	R_f (B)	R_f(C)	k'	$[M+H]^+$	M_r
Dmt-Pro-Phe-Phe-NH_2	0.36	0.53	0.33	3.04	600.37	599
DiiodoDmt-Pro-Phe-Phe-NH_2	0.50	0.57	0.43	5.91	852.24	851
Dmt-ΔPro-Phe-Phe-NH_2	0.39	0.53	0.33	3.01	598.33	597

[a] R_f values on silica gel 60 F_{254}-precoated glass plates (Merck) solvent systems: (A) acetonitrile:methanol:water (4:1:1); (B) 1-butanol:acetic acid:water (4:1:1); (C) ethyl acetate:pyridine:acetic acid:water (60:20:6:11).
[b] Capacity factor for a Vydac 218TP54 C_{18} reversed-phase column (25 × 0.46 cm) with a gradient of 20–40% of organic modifier in 20 min. Flow rate 1 ml/min. All peptides were monitored at 216 nm.
[c] Measured and calculated molecular weights.

Table 2 Radioanalytical data on Dmt-EMs

Peptides	a (TBq/mmol)	TLC[a]			HPLC[b]
		R_f (A)	R_f (B)	R_f(C)	k'
[3′,5′-$^3H_2$]Dmt-Pro-Phe-Phe-NH_2	2.88	0.36	0.53	0.33	3.26
Dmt-[3,4-$^3H_2$]Pro-Phe-Phe-NH_2	1.95	0.36	0.53	0.33	3.26

[a] For conditions, see Table 1.
[b] For conditions, see Table 1.

Table 3 Tritium distributions in Dmt-EMs

Tritiated peptides	a/a_{max}[a]	HPLC[b]		
		Fmoc-[^{3}H]Dmt	Fmoc-[^{3}H]Pro	Fmoc-[^{3}H]Phe
[^{3}H]Dmt^1-EM2 (%)	133	92	—	8
Dmt^1-[^{3}H]Pro-EM2 (%)	92	1	95	4

[a] a/a_{max} is the ratio of the specific to the theoretically maximum specific activity.
[b] Analysis on a Vydac 218TP54 C18 column (25 × 0.46 cm) with a gradient of 30–80% of organic modifier in 20 min. Flow rate 1 ml/min; $\lambda = 216$ nm.

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DOI: 10.1002.jlcr

sion, which allowed calculation of the half-lives of the EMs. As shown in Table 4, Dmt^1-EM2 broke down relatively slowly in the brain homogenate. Dmt^1-EM2 had a half-life of 33.64 min, while EM2 was almost six times less resistant than Dmt^1-EM2 to the peptidases: its half-life was 5.88 min.

Experimental

Materials

Protected and unprotected amino acids and 4-methylbenzhydrylamine resin were purchased from Calbiochem-Novabiochem or Bachem.

The reagents were from Merck or Fluka. All reagents and solvents were of analytical or reagent grade and were used without further purification. The mobile phases for linear gradient elutions in RP-HPLC were mixed from 0.1% (v/v) trifluoroacetic acid (TFA) in water and 0.08% (v/v) TFA in acetonitrile. A Bruker Reflex III MALDI-TOF mass spectrometer was used to identify the peptides.

Radio-thin layer chromatography (TLC) was performed on silica gel 60 F_{254}-precoated glass plates (Merck), and the radioactive spots were detected with a Berthold LD 2821 flow-through (9.9 methane/Ar) Geiger–Müller counter. Radio-HPLC was performed on a Jasco instrument with a Vydac 218TP54 C_{18} (25×0.46 cm, 5 μm) column and liquid scintillation detection on a Canberra Packard Radiomatic 505TR Flow Radiochromatography Detector with the Ultima-Flo M scintillation cocktail. Tritiation was carried out in a self-designed vacuum manifold.[12] $^3H_2$ gas was purchased from Technobexport, Russia, and contained ⩾98% tritium. The radioactivities of the tritiated compounds were measured with a TRI-CARB 2100TR liquid scintillation counter in a toluene–Triton X-100 cocktail.

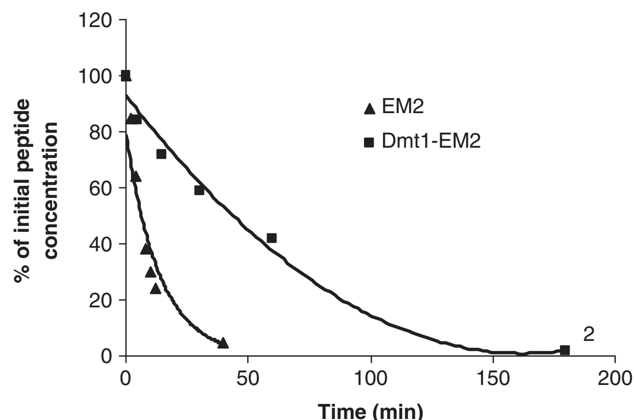

Figure 1 Degradation of EM2 and Dmt^1-EM2 in rat brain homogenate. EM2 and Dmt^1-EM2 were added to the brain homogenate at a final concentration of 100 μM. HPLC analysis was performed on supernatants of the centrifuged homogenate, and 20 μl of aliquots was analysed after digestion for 0–40 and 0–180 min. Each point represents the mean ± SEM of the EMs concentration, expressed as a percentage of the initial EM2 and Dmt^1-EM2 ($n = 4$–6). EM2, endomorphin-2; Dmt1-EM2, Dmt^1-endomorphin-2.

Table 4 Half-lives of endomorphins

EM2		Dmt^1-EM2	
$100 \times k$ (min^{-1})	$t_{1/2}$ (min)	$100 \times k$ (min^{-1})	$t_{1/2}$ (min)
11.79 ± 0.75	5.88 ± 0.39	2.09 ± 0.39	33.64 ± 6.81

Data are arithmetic means of three to six measurements ± SEM. Protein content in the brain homogenate: 5.92 mg/ml. The EMs concentrations were 100 μM; k, rate constant; $t_{1/2}$, half-life.

Methods

Solid-phase syntheses of peptides. Peptides were synthesized manually on 4-methylbenzhydrylamine resin by using the Merrifield solid-phase method. N^{α}-*t*-Boc chemistry with *N*-hydroxybenzotriazole and *N*,*N*′-dicyclohexylcarbodiimide as coupling agents were employed for peptide elongation. The peptides were cleaved from the resin with anhydrous HF (10 ml/g resin) in the presence of anisole (1 ml/g resin) at 0°C for 60 min. The peptide–resin mixtures were washed with diethyl ether to remove the scavengers, the peptides were then dissolved in 30% acetic acid, and the filtrate was lyophilized. The crude peptides were purified by RP-HPLC on a Vydac 218TP1010 C_{18} (25×1 cm, 12 μm) column, using a linear gradient of 20–50% of the organic modifier within 25 min at a flow rate of 4 ml/min, with UV detection at 220 nm. Peptide purity was assessed by TLC and HPLC, and the molecular weights of the peptides were established by MALDI-TOF-MS (Table 1).

Syntheses of 3′,5′-diiodo-Dmt^1-EM2. 3′,5′-Diiodo-Dmt^1-EM2 was synthesized by the chloramine T method. Testing was performed to establish the optimum reaction conditions. Accordingly, 1.4 μmol of Dmt^1-EM2 was dissolved in acetonitrile:water (1:1) and 5 equivalents of the reagents (0.067 M NaI + 0.022 M chloramine T in phosphate buffer, pH = 7.4) was added amount to the solution. The reaction was stopped after 30 s by adding 0.053 M $Na_2S_2O_5$. The reaction mixture was analysed and purified by RP-HPLC (a Vydac

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DOI: 10.1002.jlcr

218TP54 C_{18} RP column with a gradient of 20–50% of organic modifier in 25 min, flow rate 1 ml/min).

Tritium labelling. An amount of 2.4 mg (2.88 μmol) of 3′,5′-diiodo-Dmt[1]-EM2 and 2.8 mg (3.92 μmol) of $^{3,4}\Delta$Pro[2]-Dmt[1]-EM2 were dissolved separately in 1 ml of dimethylformamide and labelled with tritium gas. The reaction mixture contained 1.5 μl of triethylamine and 12 or 14 mg of $PdO/BaSO_4$ catalyst, respectively. Tritium gas was liberated from uranium tritide by heating, and 555 GBq (15 Ci) of this gas was introduced into the reaction vessel.[10,12] The reaction mixture was stirred at room temperature for 1 or 2 h and the unreacted tritium gas was then adsorbed onto pyrophoric uranium. The catalyst was filtered off on a Whatman GF/C filter and the labile tritium was removed by repeated vacuum evaporation of an aqueous ethanolic solution of the radiolabelled product. The crude products were purified by HPLC to give a radioactive purity of >95%, checked by both TLC and HPLC. The quantitative analysis of the pure, labelled peptides was performed by HPLC with a UV detector, using a calibration curve prepared with unlabelled Dmt[1]-EM2, and the total activities of the products were measured by liquid scintillation counting. The specific activity of [^{3}H]-Dmt[1]-EM2 was 2.88 TBq/mmol (77.8 Ci/mmol), and that of [$^3H_2$]Pro[2]-Dmt[1]-EM2 was 1.95 TBq/mmol (52.8 Ci/mmol) (Table 2). The pure, tritiated peptides were dissolved in ethanol and were stored at a concentration of 37 MBq/ml under liquid nitrogen. The stabilities of both tritiated endomorphin analogues under these storage conditions were really good. After 6 months, the purities were checked and proved to be >95%.

Tritium distributions in labelled peptides. An amount of 0.74 MBq of [$^3H_2$]Dmt[1]-EM2 or [$^3H_2$]Pro[2]-Dmt[1]-EM2 and 0.05 mg of unlabelled Dmt[1]-EM2 were hydrolysed separately for 24 h in 1 ml of 6 M HCl at 110°C under argon pressure in sealed ampoule. The solvent was then removed by evaporation, and the sample was next dissolved in 1 ml of 0.2 M borate buffer, pH = 7.7. The remaining radioactivity was 0.39 MBq for [$^3H_2$] Pro[2]Dmt[1]-EM2 or 0.1 MBq for [$^3H_2$]Dmt[1]-EM2. To 0.2 ml of each aqueous sample, 0.2 cm^3 of 9-fluorenylmethyl chloroformate in acetone (15 mM) was added. After about 45 s, the mixtures were extracted with *n*-pentane and the aqueous phase was analysed by HPLC (Table 3).

Digestion of [$^3H_2$]Pro[2]-Dmt[1]-EM 2. After preincubation of the rat brain homogenate (protein content 0.3 mg/ml), 945 nM [$^3H_2$]Pro[2]-Dmt[1]-EM2 (370 kBq) was incubated with it at 37°C in a final volume of 200 μl. Twenty microlitre aliquots were withdrawn after incubation for 5, 15, 30 or 60 min, and immediately acidified with 20 μl of 0.1 M HCl solution. Following centrifugation of the samples (11 340 × *g*, 5 min, 25°C), 20 μl of supernatant was analysed by radio-HPLC.

Determination of the rates of degradation of the peptides. About 20 μl aliquots of the 1 mM peptide stock solutions in 50 mM Tris–HCl buffer (pH = 7.4) were added to 180 μl of rat brain homogenate, and the mixtures were incubated at 37°C. Aliquots of 20 μl were taken from these incubation mixtures and immediately acidified with 25 μl of 0.1 M aqueous HCl solution. About 10 μl of each supernatant obtained after centrifugation (11 340 × *g*, 5 min, 25°C) of the samples was analysed by HPLC. The degradation rate constants (k) were obtained by least-square linear regression analysis of the plots of logarithmic EM peak areas ($\ln(A/A_0)$) versus time, using a minimum of four points. Degradation half-lives ($t_{1/2}$) were calculated from the rate constants as $\ln(2/k)$.

Conclusions

Two Dmt[1]-EM2 isotopomers were labelled with tritium in position 1 or position 2. Both radioligands exhibited high specific radioactivity. Tritiated Dmt[1]-EM2 may become a useful research tool for direct radioreceptor binding[13] (to be published elsewhere) and the isotopomers may serve as important tools for degradation studies in rat brain homogenates. Different rates of degradation of EM2 and of Dmt[1]-EM2 were observed in the rat brain homogenate. Dmt[1]-EM2 was six times more resistant than EM2 to peptidases, the half-lives being 33.64 and 5.88 min, respectively. The half-life of [$^3H_2$]Pro[2]-Dmt[1]-EM2 proved almost 2.5 times longer than that of EM2.

Acknowledgements

This work was supported by grant NKTH, DNT 08/2004 from the Hungarian National Bureau of Research and Technology, and grants OTKA 046514 and OTKA TS 09817 from the Hungarian Research Foundation.

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DOI: 10.1002.jlcr