Journal of Peptide Science
J. Pept. Sci. 2006; **12**: 505–508
Published online 2 May 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.757

JOURNAL OF PEPTIDE SCIENCE

Fluorescein-labeled stable neurotensin derivatives

VERONIQUE MAES,[a] CHRISTINA HULTSCH,[b] SUZANN KOHL,[b] RALF BERGMANN,[b] THOMAS HANKE[c] and DIRK TOURWÉ[a]*

[a] Organic Chemistry Department, Vrije Universiteit Brussel, B-1050 Brussels, Belgium
[b] Institut für Radiopharmazie, Forschungszentrum Rossendorf, Germany
[c] Max-Bergmann-Zentrum für Biomaterialen, Institut für Werkstoffforschung, TU-Dresden, Germany

Received 25 January 2006; Revised 3 February 2006; Accepted 9 February 2006

Abstract: Neurotensin(8-13) analogs containing a glycine or 5-aminovaleroyl spacer were labeled with fluorescein through formation of an *N*-terminal thiourea function. The receptor binding was measured in HT-29 cell cultures and showed a substantial decrease in affinity, especially for the metabolically stabilized [MeArg^9, Tle^{11}] analog. Using fluorescence microscopy, the internalization of the fluorescent neurotensin analogs into HT-29 cells was observed. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: neurotensin; fluorescein; receptor binding; fluorescence microscopy

INTRODUCTION

In recent years, it has become evident that neuropeptide receptors allow the successful *in vivo* targeting of many tumors. Labeling of neuropeptides with a γ-tracer such as ^{99m}Tc, or a positron emitter such as ^{18}F, allows scintigraphic detection of malignancies that overexpress the peptide receptor on the tumor cells. Similarly, labeling with β-emitters can be used for therapeutic applications [1–5]. A major limitation of the use of neuropeptides as targeting molecules is their rapid degradation by plasma and tissue proteases. Therefore, the peptide sequence has to be modified to increase the metabolic stability, while preserving the receptor affinity and the biodistribution properties. An example is NT, and particularly its *C*-terminal hexapeptide fragment NT(8–13) Arg-Arg-Pro-Tyr-Ile-Leu-OH. The NT receptor subtype NTR-1 is overexpressed in human meningiomas, Ewing sarcoma and ductal pancreatic carcinoma [5,6]. We have demonstrated that the plasma half-life of ^{18}F- or ^{99m}Tc-labeled NT(8–13) can be prolonged by using a strategy based on the reduction or *N*-methylation of the Arg^8–Arg^9 amide bond and the use of the bulky Tle^{12} residue [7–11]. A preliminary clinical study with the analog [^{99m}Tc(CO)$_3$](N$^{\alpha}$His)Ac-LysΨ(CH$_2$NH)Arg-Pro-Tyr-Tle-Leu-OH (Figure 1) allowed the visualization of the tumor in a patient with a ductal pancreatic carcinoma [12].

Very often, a surgical intervention is required to remove the tumor. In such a case, the distinction between tumor and normal tissues is often difficult. For large tumors, the challenge is the delineation from normal tissue, whereas for early carcinoma, the recognition in itself may be a problem. In such cases fluorescence-based diagnostics may be very helpful [13]. Moreover, the availability of confocal microscopy has provided a powerful method to study receptor-mediated mechanisms in cells and tissues [14,15]. Fluorophore-tagged peptides are also of interest as a safer and more versatile alternative to radioligands in pharmacology and drug discovery [16].

As an extension of our studies on radiolabeled NTs for tumor diagnosis and therapy, we present here our results on the synthesis and characterization of fluorescein-labeled NT analogs, and their uptake in HT-29 tumor cells.

MATERIALS AND METHODS

Boc-protected amino acids Tyr[Bzl(Cl$_2$)]-OH, Pro-OH, Arg(Tos)-OH, Gly and the preloaded Leu-Merrifield resin used in solid-phase peptide synthesis (SPPS) were obtained from NovaBiochem (Läufelfingen, Switzerland). Boc-MeArg(Tos)-OH was purchased from Bachem (Bubendorf, Switzerland); DIC, HOBt, TFA, dichloromethane, Boc-Ava-OH and Boc-Tle-OH were obtained from Fluka (Bornem, Belgium); anisole, DIPEA, DMF and FITC were from Acros (Geel, Belgium). HATU was purchased from Applied Biosystems. SPPS was performed on a semiautomated peptide synthesizer SP640B (Labortec, Bubendorf, Switzerland). Purification of the peptides was performed on a semipreparative high-performance liquid chromatograph (Gilson) on an RP C_{18} column (Discovery BIO SUPELCO Wide Pore, 25 × 2.21 cm, 5 μm) with a linear gradient (3% to 80% CH_3CN in H_2O, containing 0.1% TFA in 30 min), a flow rate of 20 ml/min and UV detection at

Abbreviations: As recommended in *J. Pept. Sci.* 2006: **12**, 1–12. Other abbreviations used are: Ava, 5-aminovaleric acid; FITC, fluorescein isothiocyanate; NT, neurotensin; (*N*$^{\alpha}$His)Ac, "retro-*N*$^{\alpha}$-carboxymethyl-histidine" (see Figure 1); Tle, *t*-butylglycine.

*Correspondence to: D. Tourwé, Vrije Universiteit Brussel, Organic Chemistry Department, Pleinlaan 2, B-1050 Brussels, Belgium;
e-mail: datourwe@vub.ac.be

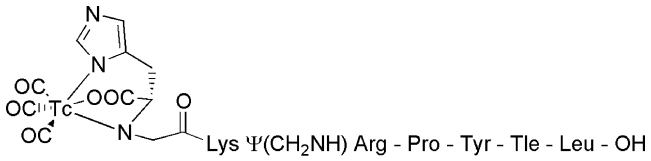

Figure 1 Structure of [^{99m}Tc(CO)$_3$](N$^{\alpha}$His)Ac-LysΨ(CH$_2$NH) Arg-Pro-Tyr-Tle-Leu-OH.

215 nm. The peptides were analyzed by HPLC on a Discovery BIO SUPELCO Wide Pore column (25 × 0.46 cm, 5 μm) using a flow rate of 1 ml/min and the same gradient as indicated above. Mass spectrometry detection was performed with a VG Quattro II spectrometer (ESI) using MassLynx 2.22 software for data analysis.

Solid-phase Peptide Synthesis of Fluorescein-labeled Neurotensins

The synthesis was carried out using a stepwise solid-phase procedure using Boc-protected amino acids and Boc-Leu-Merrifield resin (0.6 mmol/g). Typically, the Boc-deprotections were performed in a mixture of TFA/CH_2Cl_2/anisole (49/49/2) (5 min + 20 min), followed by filtration of the TFA mixture and neutralization by washing with 20% DIPEA/CH_2Cl_2. The couplings were performed by adding protected amino acid (3 equiv.) and DIC (3 equiv.) in the presence of HOBt (3 equiv.) in DMF to the resin. Boc-MeArg(Tos)-OH and Boc-Arg(Tos)-OH (4 equiv.) were coupled to Pro and MeArg, respectively, using HATU in four-fold excess and 8 equiv. DIPEA in a CH_2Cl_2/DMF (1/1) mixture. The completion of the couplings was checked with the ninhydrin or NF 31 color test [17]. After Boc deprotection of Gly or Ava, the amine was reacted with fluorescein isothiocyanate (6 equiv.) in a mixture of DMF/DIPEA (95/5) for 2 h. The peptides were cleaved from the resin by treatment with $HF_{(l)}$ for 1 h at 0 °C, followed by HPLC purification.

Determination of Binding Affinity

Cell cultures. The cells HT-29 were obtained from ECACC-Oxford, England (ECACC Ref. No: 91 072 201). They were routinely cultured in T-25/75 tissue culture flasks (Cellstar, Greiner bio-one) with McCoy's 5a medium containing Glutamax-I (Gibco BRL), 10% fetal bovine serum (Sigma) and penicillin/streptomycin (10 000 U/ml, Gibco). Cells were grown in the culture media in humidified 5% CO_2/95% air at 37 °C. In the T-25/75 flasks the cells reached confluence after 3–5 days. The experiments were conducted at confluence with cell densities between 4–7 × 10^6 cells per ml (equivalent to about 300–700 μg protein) of passage 5–20. For the determination of binding affinity, the cells were detached by trypsin/EDTA (0.02–0.05%).

Receptor binding. The experiments were initiated by the preparation of a dilution series of the peptides. A 1 mM stock solution of the peptides dissolved in DMSO was prepared. Final concentrations of 10 μM, 6 μM, 1 μM, 600 nM, 200 nM, 100 nM, 60 nM, 10 nM, 6 nM, 2 nM and 1 nM were obtained by dilution with Tris buffer (50 mM Tris HCl, 1 mM EDTA, 0.1% BSA, 0.5 mM *o*-phenanthroline, pH 7.4) containing 10% DMSO. The cells in the flasks were washed thrice with Tris buffer. They were subsequently scraped out from the flask and resuspended in 8 ml Tris buffer. The cell suspension was then homogenized in a glass homogenizer. Two hundred microliters of the cell homogenate was mixed with 100 μl of the peptide solution and 500 μl of Tris buffer. Two hundred microliters [^{3}H]NT was added and the mixture was allowed to incubate for 30 min at room temperature. Nonspecific binding was assessed in the presence of 100 mM NT(8–13).

Separation of bound and free ligands was performed by rapid filtration through Whatman GF/B glass fiber filters using a Brandel (Gaithersburg, MD) cell harvester. The filters were washed with three volumes of Tris buffer and were placed into vials with 4 ml scintillation cocktail (ULTIMA Gold, Packard Instruments, USA). The [^{3}H]NT remaining on the filters was quantified by liquid scintillation spectrometry (TriCarb, Packard Instruments).

Fluorescence Microscopy and Confocal LSM

Cells were incubated with 200 nmol–10 μmol of NT analogs for 60 to 120 min, after which the incubation liquid was removed.

Fluorescence images are two-channel images observed on a wide-field fluorescence microscope, Zeiss Axioscope 2 FS mot, equipped with a Zeiss AxioCam color camera. Excitation was done by an HBO 50 mercury lamp. For the blue channel the filter set Zeiss 01 (DAPI) was used, and for the green channel, the filter set Zeiss 09 (FITC). The images were framed and processed by AxioVis 3.1 software.

In order to identify the NT-FITC fluorescence in the presence of large broadband intrinsic cell fluorescence, a confocal laser scanning microscope, LSM 510 META, was used. For each pixel, a fluorescence spectrum after excitation at 488 nm (Ar^+-ion laser) was recorded. FITC was identified by its typical emission maximum around 525 nm.

RESULTS AND DISCUSSION

Design and Synthesis of Peptides

It has been shown that the introduction of a bulky [^{99m}Tc(CO)$_3$]–chelator complex at the *N*-terminus of NT(8–13) analogs does not significantly influence receptor recognition [8–11]. However, such an influence of the fluorescein group, which is linked through a thiourea function to the peptide, cannot be excluded. Therefore, a Gly spacer and the longer 5-aminovaleroyl (Ava) spacer were inserted, leading to FITC-Gly-NT(8–13) **1** and FITC-Ava-NT(8–13) **2**. Subsequently, FITC-Ava-Arg-MeArg-Pro-Tyr-Tle-Leu-OH **3** was prepared to study the influence of the metabolic stabilizations. All peptides were synthesized on a Merrifield resin using Boc-protected amino acids using diisopropylcarbodiimide couplings in the presence of HOBt. For the coupling of Boc-MeArg(Tos)-OH to Pro and Boc-Arg(Tos)-OH to the MeArg residue, the coupling reagent HATU was used. The completion of this coupling was checked with NF 31 color tests [15]. The FITC group was introduced by reaction with fluorescein isothiocyanate. After cleavage from the resin using liquid HF, the peptides were purified by reversed-phase HPLC. The final

products were identified by HPLC–ESI-MS: analog **1**, t_R, 17.5 min, $[M + H]^+ = 1263.6$, $[M - FITC]^+ = 874.7$; Analog **2**, t_R, 17.5 min, $[M + H]^+ = 1304.5$, $[M - FITC]^+ = 916.6$; Analog **3**, t_R, 17.5 min, $[M + H]^+ = 1318.5$, $[M - FITC]^+ = 930.5$.

Receptor Affinity

The affinity of the peptides for NTR-1 was determined in HT-29 cells. The IC_{50} values were determined from competition binding curves using ^3H–NT as a radioligand.

The results are shown in Figure 2, and the data are reported in Table 1.

In contrast to the corresponding [^{99m}Tc(CO)$_3$](N^{α}His)Ac-NT(8–13) analog, for which low nanomolar affinities for the NTR-1 receptor in HT-29 cells have been observed [8], the introduction of an *N*-terminal FITC group strongly reduces the receptor affinity. The effect is clearly smaller when the longer aminovaleroyl spacer is inserted than when the glycine spacer is inserted. Modifications of the peptide to reduce metabolic breakdown further reduce the affinity in **3**. This is again in contrast to what is observed for the corresponding [^{99m}Tc(CO)$_3$](N^{α}His)Ac-NT analogs, where these modifications had only a minor effect (log K_d 9.3 *vs* 9.5) [11], and to FITC-labeled NTs containing various Arg mimetics [18].

Fluorescence Microscopy

HT-29 cells were incubated with the NT analogs, and after removal of the incubation liquid, the cells were examined using a wide-field fluorescence microscope. After binding to the NT receptor, the receptor–ligand complex is internalized [14]. Figure 3 shows the recordings for compound **2**, the analog with the highest receptor affinity, after incubation with 10 μmol of compound **2** for 60 min. The figure clearly shows the internalization of the NT analog into the HT-29 cells. Furthermore, the displacement of compound **2** from the NT-receptors on the HT-29 cells by NT(8–13) could be clearly observed.

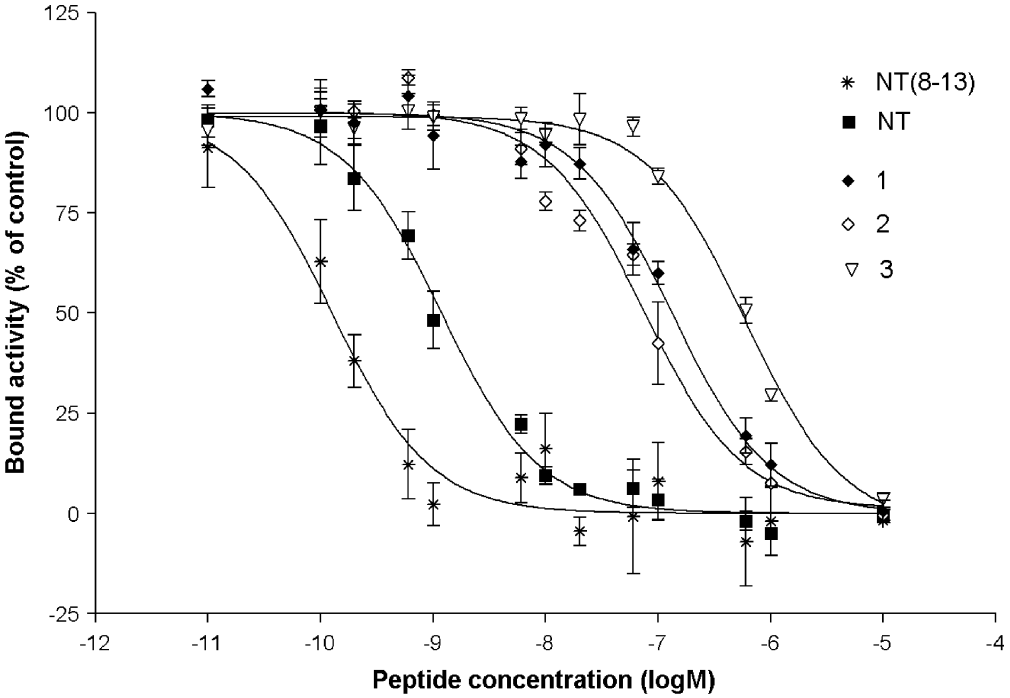

Figure 2 Inhibition of ^3H-neurotensin binding to HT-29 cell membranes.

Table 1 Peptide structure and receptor affinity of FITC-neurotensin analogs. Data are from two independent experiments in duplicate

Analog	Structure	IC_{50} (nM)
NT	PGlu-Leu-Tyr-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH	1.0 ± 0.3
NT(8–13)	Arg-Arg-Pro-Tyr-Ile-Leu-OH	0.4 ± 0.2
1	FITC-Gly-Arg-Arg-Pro-Tyr-Ile-Leu-OH	157 ± 47
2	FITC-Ava-Arg-Arg-Pro-Tyr-Ile-Leu-OH	75 ± 23
3	FITC-Ava-Arg-MeArg-Pro-Tyr-Tle-Leu-OH	479 ± 207

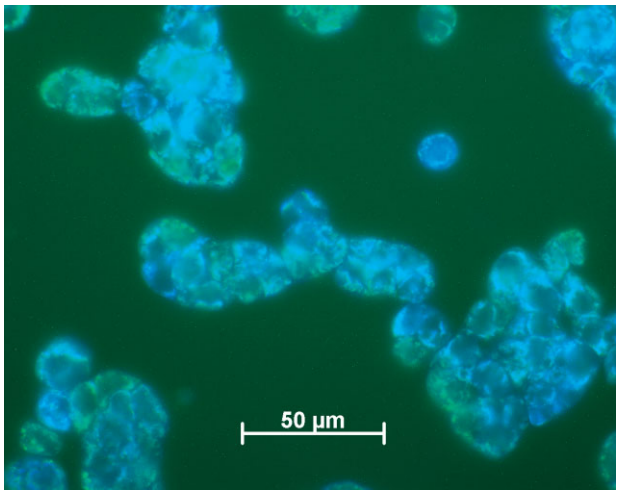

Figure 3 The image representing a superposition of the blue and green channel images. In this figure, the FITC-labeled ligand **2** appears in a light greenish color. The remainder is cell autofluorescence. In a different experiment, FITC-fluorescence was identified by recording the fluorescence spectra of each pixel using a confocal LSM 510 META.

CONCLUSIONS

The introduction of a fluorescein group at the *N*-terminus of NT(8–13) reduces the receptor affinity. This reduction is limited by an aminovaleroyl spacer. The modifications of the peptide sequence in order to limit the metabolic breakdown further reduce the receptor affinity, in contrast to what has been observed for the corresponding ^{99m}Tc-labeled analogs. Nevertheless, for the most potent analog **1** the internalization into HT-29 cells can be visualized using confocal microscopy. The application of a fluorescent-labeled NT analog will require the development of more potent analogs. Since previous studies on ^{99m}Tc- or ^{18}F-benzoyl-labeled analogs indicated that the *N*-terminal modifications of the peptide sequence are well tolerated by the NTR-1, improvements are likely to be linked to the structure of the spacer and/or of the fluorophore. One option might be to use less sterically hindering fluorophores such as 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), 7-methoxycoumarin-4-acetyl acid (MCA) and, especially, the small *o*-anthaniloyl (ATN) group [19–21].

Acknowledgements

V. Maes is a Research Assistant of the Fund for Scientific Research-Flanders (Belgium), which we also acknowledge for financial support (grant G.0036.04). This work was also partially supported by the BIOMED grant BMCH4-CT98-3198 of the EC.

REFERENCES

1. Heppeler A, Froideveaux S, Eberle AN, Maecke HR. Receptor targetting for tumor localisation and therapy with radiopeptides. *Curr. Med. Chem.* 2000; **7**: 971–994.
2. Kwekkeboom DJ, Krenning EP, De Jong M. Peptide receptor imaging and therapy. *J. Nucl. Med.* 2000; **41**: 1704–1713.
3. Reubi JC, Mäcke HR, Krenning EP. Candidates for peptide receptor radiotherapy today and in the future. *J. Nucl. Med.* 2005; **46**: 67S–75S.
4. Fishman AJ, Babich JW, Strauss HW. A ticket to ride: peptide radiopharmaceuticals. *J. Nucl. Med.* 1993; **43**: 2253–2263.
5. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr. Rev.* 2003; **24**: 389–427.
6. Reubi JC, Waser B, Friess H, Büchler M, Laissue J. Neurotensin receptors: a new marker for human ductal pancreatic adenocarcinoma. *Gut* 1998; **42**: 546–550.
7. Bergmann R, Scheunemann M, Heichert C, Mäding P, Wittrisch H, Kretzmar M, Rodig H, Tourwé D, Iterbeke K, Chavatte K, Zips D, Reubi JC, Johannsen B. Biodistribution and catabolism of ^{18}F-labeled neurotensin(8–13) analogs. *Nucl. Med. Biol.* 2002; **29**: 61–72.
8. Garcia-Garayoa E, Allemann-Tannahill L, Bläuenstein P, Willmann M, Carrel-Rémy N, Tourwé D, Iterbeke K, Conrath P, Schubiger PA. In vitro and in vivo evaluation of new radiolabeled neurotensin(8–13) analogues with high affinity for NT1 receptors. *Nucl. Med. Biol.* 2001; **28**: 75–84.
9. Garcia-Garayoa E, Bläuenstein P, Bruehlmeier M, Blanc A, Iterbeke K, Conrath P, Tourwé D, Schubiger PA. Preclinical Evaluation of a new, stabilized neurotensin(8–13) pseudopeptide radiolabeled with ^{99m}Tc. *J. Nucl. Med.* 2002; **43**: 374–383.
10. Bruehlmeier M, Garcia-Garayoa E, Blanc A, Holzer B, Gergely S, Tourwé D, Schubiger PA, Bläuenstein P. Stabilization of neurotensin analogues: effect on peptide catabolism, biodistribution and tumor binding. *Nucl. Med. Biol.* 2002; **29**: 321–327.
11. Bläuenstein P, Garcia-Garayoa E, Rüegg D, Blanc A, Tourwé D, Beck-Sickinger A, Schubiger PA. Improving the tumor uptake of ^{99m}Tc-labeled neuropeptides using stabilized peptide analogues. *Cancer. Biother. Radiopharm.* 2004; **19**: 181–188.
12. Buchegger F, Bonvin F, Kosinski M, Schaffland AO, Prior J, Reubi JC, Bläuenstein P, Tourwé D, Garcia-Garayoa E, Bischof Delaloye A. Radiolabeled neurotensin analog, ^{99m}Tc-NT-XI, evaluated in ductal pancreatic adenocarcinoma patients. *J. Nucl. Med.* 2003; **44**: 1649–1654.
13. Gutowski M, Carcenac M, Pourquier D, Larroque C, Saint-Aubert B, Rouanet P, Pèlegrin A. Intraoperative immunophotodetection for radical resection of cancers: evaluation in an experimental model. *Clin. Cancer Res.* 2001; **7**: 1142–1148.
14. Beaudet A, Nouel D, Stroh T, Vandenbulcke F, Dal-Farra C, Vincent J-P. Fluorescent ligands for studying neuropeptide receptors by confocal microscopy. *Braz. J. Med. Biol. Res.* 1998; **31**: 1479–1489.
15. Daly CJ, McGrath JC. Fluorescent ligands, antibodies, and proteins for the study of receptors. *Pharmacol. Ther.* 2003; **100**: 101–118.
16. Middleton RJ, Kellam B. Fluorophore-tagged GPCR ligands. *Curr. Opin. Chem. Biol.* 2005; **9**: 517–525.
17. Madder A, Farcy N, Hosten NGC, De Muynck H, De Clerq PJ, Barry J, Davis AP. A novel sensitive assay for visual detection of solid-phase bound amines. *Eur. J. Org. Chem.* 1999; 2787–2791.
18. Achilefu S, Srinivasan A, Schmidt MA, Jimenez HN, Bugaj JE, Erion JL. Novel bioactive and stable neurotensin peptide analogues capable of delivering radiopharmaceuticals and molecular beacons to tumors. *J. Med. Chem.* 2003; **46**: 3403–3411.
19. Kele P, Illyés E, Mezö G, Doda M, Mak M, Kaposi AD, Sebestyén F. Fluorescent derivatives of bovine neurotensin 8–13 fragment. *Lett. Pept. Sci.* 1999; **6**: 235–238.
20. Ciencialova A, Zakova L, Jiracek J, Barthova J, Barth T. Preparation and characterization of two LysB29 specifically labelled fluorescent derivatives of human insulin. *J. Pept. Sci.* 2004; **10**: 470–478.
21. Schiller PW, Berezowska I, Weltrowska G, Chen H, Lemieux C, Chung NN. Type and location of fluorescent probes incorporated into the potent μ-opioid peptide [Dmt^1]DALDA affect potency, receptor selectivity and intrinsic efficacy. *J. Pept. Res.* 2005; **65**: 556–563.