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Role of 2′,6′-Dimethyl-L-Tyrosine (Dmt) in Some Opioid Lead Compounds

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Abstract

Here we evaluated how the interchange of the amino acids 2′,6′-dimethyl-L-tyrosine (Dmt), 2′,6′-difluoro-L-tyrosine (Dft), and tyrosine in position 1 can affect the pharmacological characterization of some reference opioid peptides and pseudopeptides. Generally, Dft and Tyr provide analogues with a similar pharmacological profile, despite different pK_a values. Dmt/Tyr(Dft) replacement gives activity changes depending on the reference opioid in which the modification was made. Whereas, H-Dmt-Tic-Asp*-Bid is a potent and selective δ agonist (MVD, IC_{50} = 0.12 nM); H-Dft-Tic-Asp*-Bid and H-Tyr-Tic-Asp*-Bid are potent and selective δ antagonists (pA_2 = 8.95 and 8.85, respectively). When these amino acids are employed in the synthesis of deltorphin B and its Dmt^1 and Dft^1 analogues, the three compounds maintain a very similar δ agonism (MVD, IC_{50} 0.32–0.53 nM) with a decrease in selectivity relative to the Dmt^1 analogue. In the less selective H-Dmt-Tic-Gly*-Bid the replacement of Dmt with Dft and Tyr retains the δ agonism but with a decrease in potency. Antagonists containing the Dmt-Tic pharmacophore do not support the exchange of Dmt with Dft or Tyr.

Keywords

Dmt-Tic pharmacophore; opioid peptides; opioid receptors; δ opioid agonists; UFP-512; δ opioid antagonists

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Supplementary Data

Supplementary data (synthetic schemes 1–4) associated with this article can be found in the online version at doi:

1. Introduction

2′,6′-Dimethyl-L-tyrosine (Dmt)a has become one of the most widely used amino acids in the synthesis of opioid peptides and pseudopeptides.[1, 2] To the best of our knowledge, Dmt was first introduced in opioid peptides by Hansen Jr. et al. in 1985.[3] After this seminal contribution nearly all research groups working in the opioid peptide field used this amino acid in the synthesis of new and interesting opioids. The importance of Dmt is further documented by different synthetic methods published or patented until now in order to obtain the chiral amino acid in high yield in order to avoid the use of very expensive chiral catalysts.[4–7] Generally, structure-activity studies on a variety of opioid ligands containing Dmt revealed important alterations in their activities through the elevation of affinity (especially for the μ receptor), modification of receptor selectivity, and change in the spectrum of their bioactivity profile.[1, 2] Once the importance of the Dmt substitution in lieu of Tyr[1] was established, other Tyr/Dmt surrogates were also considered in further structure-activity studies.[8, 9] In some cases the new analogues yielded interesting results,[9] but the use of Dmt in opioids still remains unsurpassed.

Starting from this point it is quite difficult to suggest new Tyr/Dmt analogues able to maintain or improve the activity when inserted in opioid peptides or pseudopeptides. Nonetheless, we focused our attention on a new Tyr/Dmt analogue in which the 2′ and 6′ methyl groups are substituted by fluorine atoms (2′,6′-difluoro-L-tyrosine; Dft). This amino acid was selected because is commercially available at the same costs of Dmt (RSP Amino Acids), fluorine atoms maintain the same positions in the aromatic ring, and finally as generally asserted by Thayer A. M.; “Fluorine can be highly advantageous in pharmaceutical and agrochemical compounds. One or just a few atoms in an organic molecule can dramatically alter its chemical and biological nature, including its stability, lipophilicity, and bioavailability”.[10, 11] Furthermore, in the light of the relatively small dimensions of fluorine atoms, we also revaluated the possibility of using Tyr in place of Dmt. The biological effects induced by Dft and Tyr were assessed by their insertion in lieu of Dmt[1] in some of our opioid lead compounds containing the Dmt-Tic pharmacophore: H-Dmt-Tic-Asp*-Bid[12] and H-Dmt-Tic-Gly*-Bid[13] (δ agonists); H-Dmt-Tic-Lys-NH-Bzl (μ antagonist/weak δ antagonist);[14] *N*,*N*(Me)$_2$-Dmt-Tic-OH[15] (δ antagonist) and *N*,*N*(Me)$_2$-Dmt-Tic- NH$_2$[15, 16] (δ inverse agonist). Deltorphin B was also modified in light of the fact that its Dmt[1] analogue was previously synthesized and pharmacologically evaluated.[17]

2. Chemistry

All new peptides and pseudopeptides **(2, 3, 5, 6, 11, 12, 14, 15, 17, 18)** (except deltorphin B and its analogues) were prepared in solution by the same methods we previously used for the corresponding lead compounds **(1, 4, 9, 10, 13, 16)**. Briefly, mixed carbonic anhydride coupling of Boc-Asp(OMe)-OH or Boc-Gly-OH with *o*-phenylendiamine gave the corresponding crude intermediate monoamides, which were converted without purification

[a]**Abbreviations**. In addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem*. **1985**, *260*, 14–42), this paper uses the following additional symbols and abbreviations: AcOEt, ethyl acetate; AcOH, acetic acid; Asp*, -NH-CH(CH$_2$-COOH)-; Bid, 1*H*- benzimidazol-2-yl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin; Deltorphin B or Deltorphin I, H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH$_2$; deltorphin C or Deltorphin-II, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH$_2$; Dft, 2′,6′-difluoro-L-tyrosine; DMF, *N*,*N*-dimethylformamide; DMSO-d_6, hexadeuteriodimethyl sulfoxide; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, (D-Pen^2,D-Pen^5)-enkephalin; Endomorphin-2, H-Tyr-Pro-Phe-Phe-NH$_2$; Et$_2$O, diethyl ether; Gly*, -NH-CH$_2$-; GPI, guinea-pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IBCF, isobutyl chloroformate; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; MVD, mouse vas deferens; NMM, 4-methylmorpholine; OBzl, benzyl ester; pA$_2$, negative log of the molar concentration required to double the agonist concentration to achieve the original response; PE, petroleum ether; PL017, H-Tyr-Pro-(*N*-Me)Phe-D-Pro-NH$_2$; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TLC, thin-layer chromatography; WSC, 1-ethyl-3-[3′-dimethyl)aminopropyl]-carbodiimide hydrochloride; Z, benzyloxycarbonyl

to the desired 1*H*-benzimidazol-2-yl (Bid) derivatives by cyclization and dehydration in acetic acid (AcOH) as outlined in Scheme 1 (Supporting Information) for analogues **2** and **3**. Each Boc-protected intermediate was treated with trifluoroacetic acid (TFA) and condensed with Boc-Tic-OH via WSC/HOBt. After Tic deprotection by TFA, the pseudodipeptides were subsequently condensed with Boc-Dft-OH (Dft = 2′,6′-difluoro-L-tyrosine) or Boc-Tyr-OH. In final compounds **(2, 3, 5, 6)**, methyl esters and Boc protecting groups were removed by basic hydrolysis (1N NaOH) and TFA treatment, respectively.[12, 13]

Compounds **(11, 12)** were prepared essentially (Scheme 2, Supporting Information) as reported previously[14] starting from the condensation of Z-Lys(Boc)-OH with benzylamine via WSC/HOBt. The Z-protected intermediate was treated with catalytic hydrogenation (10%, Pd/C) and condensed with Z-Tic-OH via WSC/HOBt. After Tic deprotection by catalytic hydrogenation (10%, Pd/C), the dipeptide was subsequently condensed with Boc-Dft-OH or Boc-Tyr-OH. In final compounds **(11, 12)**, Boc protecting groups were simultaneously removed by TFA treatment.

N-terminal dimethylated dipeptides were obtained as reported in Scheme 3 and 4 (Supporting Information) by condensation via WSC/HOBt of the commercially available H-Tic-O*t*Bu with Boc-Dft-OH **(14)** or Boc-Tyr-OH **(15);** or H-Tic-NH_2 with Boc-Dft-OH **(17)** or Boc-Tyr-OH **(18)**, respectively. After N-terminal deprotection, dipeptides were *N*-dimethylated by aqueous formaldehyde and $NaBH_3CN$ treatment in acetonitrile solution at room temperature.[15] Deltorphin B (**9**) and its analogues (**7**, **8**) were prepared by solid phase peptide synthesis using a Fmoc strategy as detailed in the Experimental Section.

3. Results and Discussion

3.1. Receptor Affinity Analysis

Receptor binding data for δ- and μ-opioid receptors and δ-selectivity ($K_i{}^{\mu}/K_i{}^{\delta}$) are reported in Table 1. Interestingly, analogues of the more highly selective δ agonist reference compounds **1** and **7** maintained the same affinity for δ receptors despite the replacement of Dmt by Dft or Tyr. In the less selective reference δ agonist (**4**), the same substitutions caused a ca. 30-fold drop in δ affinity. On the other hand, Dft and Tyr when introduced in place of Dmt in non-selective (weak δ antagonist/μ antagonist; **10**), selective δ antagonist (**13**) or selective δ inverse agonist (**16**), decreased δ affinity at least of 2 orders of magnitude. The substitution of Dmt in lieu of Tyr is generally thought to be linked with an increase in μ affinity and consequently to a loss of δ selectivity. While this observation holds for the references δ agonists (**1**, **4** and **7**) in comparison with their corresponding analogues (**2**, **3; 5**, **6; 8**, **9**), antagonists and the inverse agonist revealed a different behaviour. In fact, whereas reference (**10**) and its analogues exhibited only a minimal variation in δ selectivity, references (**13**, **16**) and their analogues (**14**, **15; 17**, **18**) yielded the opposite receptor binding behaviour; namely, the compounds containing Dmt were far more δ selective than the analogues containing either Dft or Tyr. This remarkable differential profile was essentially due to a loss in δ affinity by more than 2 orders of magnitude.

3.2. Functional Bioactivity

All newly synthesized compounds were evaluated in the electrical stimulated MVD and GPI pharmacological assays for intrinsic functional bioactivity (Table 1). Interestingly and totally unexpectedly, in the δ opioid agonist H-Dmt-Tic-Asp*-Bid (**1**; coded UFP-512) the substitution of Dmt[1] with Dft or Tyr reverted its activity from a potent and selective δ agonism (IC_{50} = 0.12 nM) into potent and selective δ antagonism (pA_2 = 8.95 and 8.85, respectively). The same substitution in the less selective δ agonist H-Dmt-Tic-Gly*-Bid (MVD IC_{50} = 0.13 nM; GPI IC_{50} = 26.92 nM) yielded less active δ agonists [MVD (**5**) IC_{50}

= 67.3 nM; (**6**) IC_{50} = 50.1 nM]. Furthermore, analogues (**7**, **8**) of the δ selective agonist deltorphin B (**9**) maintained the same degree of δ agonism [MVD (**7**) IC_{50} = 0.32 nM; (**8**) IC_{50} = 0.40 nM; (**9**) IC_{50} = 0.53 nM], but with a loss of δ selectivity relative to the Dmt^1 analogue (**7**). Derivatives (**11**, **12**), (**14**, **15**) and (**17**, **18**) of the corresponding reference antagonists (**10**), (**13**) and (**16**) respectively, on the basis of their poor affinities for δ and μ receptors were not assessed for functional bioactivities.

4. Conclusion

To shed more light in the structure activity relationship of opioid peptides and pseudopeptides, we synthesized analogues in which 2′,6′-dimethyl-L-tyrosine in position 1 was substituted by 2′,6′-difluoro-L-tyrosine (Dft) or L-tyrosine. From this study some interesting observations can be drawn: Tyr and Dft seem to show a very similar behaviour when inserted in position 1 of opioid peptides and pseudopeptides despite their very different pK_a values (Dft has a pK_a of 8.1, about 2 units lower than Tyr).[18] To give more complexity, if possible, to the SAR related to the δ agonist containing the Dmt-Tic pharmacophore (**1**, **4**), here we complete the demonstration that all parts of the compounds can be altered resulting in different activities. For example: alkylation of 1*H*-benzimidazol-2-yl (Bid) at N^1 yields only δ antagonists;[19–21] substitution of Bid with other aromatic nuclei (benzoxazol-2-y; benzothiazol-2-yl; 1*H*-indol-2-yl; 4-phenyl-1*H*-imidazol-2-yl) generally furnish δ antagonists, but occasionally δ agonism can be maintained (1*H*-imidazol-2-yl).[22] The portion -Gly*-Bid can be replaced by – Gly-NH-Ph or – Gly-NH-Bzl resulting in a dual μ agonist/δ agonist or a μ agonist/δ antagonist, respectively.[13] Again, if we consider the replacement of the benzyl group with surrogates or the introduction of substituents in the *para* position, its pharmacological profile further changes.[23] Furthermore, the portion -Asp*-Bid can be replaced by – Asp-NH-Ph or – Asp-NH-Bzl giving δ antagonists in both cases.[20] The side chain next to Bid can be altered to obtain a variety of different activities: e. g. Gly, Ala and Asp side-chains result in δ agonism; Phe side chain results in δ antagonism, and the side chains of the other more representing amino acids yield μ agonism.[24] Substitution of Tic^2 by 4-amino-1,2,4,5-tetrahydrobenzo[*c*]-azepin-3-one[25] or D-Tic yields compounds endowed with μ agonism.[23] Finally, in the highly selective δ agonist (**1**) (MVD, IC_{50} = 0.12 nM), replacement of Dmt^1 by Dft or Tyr provides potent and selective δ antagonists (**2**, MVD, pA_2 = 8.95; **3**, MVD, pA_2 = 8.85, respectively); the same modification made with the less selective δ agonist (**4**) carries to less potent δ agonists. Deltorphin B (**9**) supports the same degree of δ agonism in spite of the amino acid introduced in position 1 (Dmt, Dft, or Tyr) the only variation concerns the increase of μ affinity and functional bioactivity induced by Dmt in comparison to Dft or Tyr.

Antagonists (**11**, **12**, **14**, **15**, **17**, **18**) listed in Table 1 do not tolerate such a change, but in other compounds it is well tolerated; such as the pair formed by the δ antagonist H-Tyr-Tic-Phe-Phe-NH_2 and the μ agonist/δ antagonist H-Dmt-Tic-Phe-Phe-NH_2.[26] Furthermore, H-Tyr-Tic-Phe-Phe-OH, H-Dmt-Tic-Phe-Phe-OH, H-Dbcp-Tic-Phe-Phe-OH; H-Cdp-Tic-Phe-Phe-OH have δ antagonist activities with K_e values ranging from 0.196 to 4.80 nM, whereas H-Bcp-Tic-Phe-Phe-OH is a δ agonist (IC_{50} = 3.42 nM).[9] In conclusion, we should recall that the history of Tyr/Dmt substitution started from the couple of δ selective antagonist dipeptides H-Tyr-Tic-OH/NH_2[27] and H-Dmt-Tic-OH/NH_2[28] initially reported by us and later by Schiller et al.,[9] in which the difference in activity induced by Dmt aroused great interest.

In summary, especially working in the field of δ selective opioid peptide/pseudopeptide ligands, besides to consider the insertion of expensive or difficult to prepare Tyr/Phe analogues in position 1, it could also be helpful to prepare the corresponding analogue containing the inexpensive Tyr in position 1, it could raise interesting surprises.

5. Experimental Section

5.1. Chemistry

5.1.1. General Methods—Crude peptides and pseudopeptides were purified by preparative reversed-phase HPLC [Waters Delta Prep 4000 system with Waters Prep LC 40 mm Assembly column C18 (30 cm x 4 cm, 15 μm particle)] and eluted at a flow rate of 20 mL/min with mobile phase solvent A (10% acetonitrile + 0.1% TFA in H_2O, v/v), and a linear gradient from 10 to 60% B (60%, acetonitrile + 0.1% TFA in H_2O, v/v) in 25 min. Analytical HPLC analyses were performed with a Beckman System Gold (Beckman ultrasphere ODS column, 250 mm x 4.6 mm, 5 μm particle). Analytical determinations and capacity factor (*K′*) of the products used HPLC in solvents A and B programmed at flow rate of 1 mL/min with linear gradients from 0 to 100% B in 25 min. Analogues had less than 5% impurities at 220 and 254 nm.

TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany): (A) 1- butanol/AcOH/H_2O (3:1:1, v/v/v); (B) CH_2Cl_2/toluene/methanol (17:1:2). Ninhydrin (1% ethanol, Merck), fluorescamine (Hoffman-La Roche) and chlorine spray reagents. Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were assessed at 10 mg/mL in methanol with a Perkin-Elmer 241 polarimeter in a 10 cm water-jacketed cell. Molecular weights of the compounds were determined by a MALDI-TOF analysis (Hewlett Packard G2025A LD-TOF system mass spectrometer) and α-cyano-4-hydroxycinnamic acid as a matrix. ^{1}H NMR (δ) spectra were measured, when not specified, in DMSO-d_6 solution using a Bruker AC-200 spectrometer, and peak positions are given in parts per million downfield from tetramethylsilane as internal standard. The purity of tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Chemistry Department, University of Ferrara, with a Yanagimoto MT-5 CHN recorder elemental analyzer. All tested compounds possess a purity of at least 95% of the theoretical values.

5.2. Synthesis

5.2.1. *tert*-Butyl (*S*)-2-(methoxycarbonyl)-1-(1*H*-benzo[*d*]imidazol-2-yl)ethylcarbamate [Boc-Asp(OMe)*-Bid]—(Asterisk represents an abbreviation as suggested by Maekawa et al.[29]) A solution of Boc-Asp(OMe)-OH (1 g, 4.05 mmol) and NMM (0.4 mL, 4.05 mmol) in DMF (10 mL) was treated at −20°C with IBCF (0.5 mL, 4.05 mmol). After 10 min. at −20°C, *o*-phenylendiamine (0.44 g, 4.05 mmol) was added. The reaction mixture was allowed to stir while slowly warming to room temperature (1h) and was then stirred for 3 h. The solvent was evaporated, and the residue was partitioned between AcOEt and H_2O. The AcOEt layer was washed with $NaHCO_3$ (5% in H_2O) and brine and dried over Na_2SO_4. The solution was filtered, the solvent was evaporated, and the residual solid was dissolved in glacial acetic acid (10 mL). The solution was heated at 60°C for 1 h. After the solvent was evaporated and the residue was precipitated from Et_2O/PE (1:9, v/v): yield 1.07 g (83%); *Rf*(B) 0.45; HPLC *K′* 6.52; mp 129–131 °C; $[\alpha]^{20}_D$ +16.4; *m/*z 320 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) *δ* 1.40 (s, 9H), 2.67–2.92 (m, 2H), 3.67 (s, 3H), 5.48–5.53 (m, 1H), 7.26–7.70 (m, 4H).

5.2.2. 2TFA.H-Asp(OMe)*-Bid—Boc-Asp(OMe)*-Bid (1.02 g, 3.2 mmol) was treated with TFA (2 mL) for 0.5 h at room temperature. Et_2O/PE (1:1, v/v) were added to the solution until the product precipitated: yield 1.33 g (93%); *Rf*(A) 0.46; HPLC *K′* 5.20; mp 137–139 °C; $[\alpha]^{20}_D$ +18.1; *m/*z 220 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) *δ* 2.67–2.92 (m, 2H), 3.67 (s, 3H), 4.51–4.56 (m, 1H), 7.26–7.70 (m, 4H).

5.2.3. Boc-Tic-Asp(OMe)*-Bid—To a solution of Boc-Tic-OH (0.79 g, 2.86 mmol) and 2TFA.H-Asp(OMe)*-Bid (1.28 g, 2.86 mmol) in DMF (10 mL) at 0 °C, NMM (0.62 mL, 5.72 mmol), HOBt (0.48 g, 3.15 mmol), and WSC (0.60 g, 3.15 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in AcOEt and washed with $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 1.18 g (86%); *Rf*(B) 0.88; HPLC *K′* 8.14; mp 149–151 °C; $[\alpha]^{20}_D$ +12.2; *m/*z 480 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38–1.42 (d, 9H), 2.67–3.17 (m, 4H), 3.67 (s, 3H), 4.17–4.27 (m, 2H), 4.92–5.51 (m, 2H), 6.96–7.70 (m, 8H).

5.2.4. 2TFA.H-Tic-Asp(OMe)*-Bid—Boc-Tic-Asp(OMe)*-Bid (1.13 g, 2.36 mmol) was treated with TFA (1.5 mL) for 0.5 h at room temperature. Et_2O/PE (1:1, v/v) were added to the solution until the product precipitated: yield 1.29 g (90%); *Rf*(A) 0.57; HPLC *K′* 6.48; mp 145–147 °C; $[\alpha]^{20}_D$ +13.1; *m/*z 379 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.67–3.17 (m, 4H), 3.67–3.95 (m, 6H), 5.47–5.52 (m, 1H), 6.96–7.70 (m, 8H).

5.2.5. Boc-Dft-OH—To a solution of H-Dft-OH (0.5 g, 2.3 mmol) in *t*BuOH/H_2O (2:1, 15 mL), 1N NaOH (2.3 mL, 2.3 mmol) and di-*tert*-butyl dicarbonate (0.55 g, 2.53 mmol) at 0 °C, were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. H_2O (20 mL) and solid citric acid (5 g) were added. The product was extracted with AcOEt (100 mL), dried (Na_2SO_4), and evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 0.66 g (91%); *Rf*(B) 0.27; HPLC *K′* 6.76; mp 148–150 °C; $[\alpha]^{20}_D$ −5.02; *m/*z 317 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.91–3.16 (dd, 2H), 4.72–4.93 (m, 1H), 6.16 (s, 2H).

5.2.6. Boc-Dft-Tic-Asp(OMe)*-Bid—To a solution of Boc-Dft-OH (0.09 g, 0.27 mmol) and 2TFA.H-Tic-Asp(OMe)*-Bid (0.16 g, 0.27 mmol) in DMF (10 mL) at 0 °C, NMM (0.06 mL, 0.54 mmol), HOBt (0.05 g, 0.30 mmol), and WSC (0.06 g, 0.30 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in AcOEt and washed with $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 0.16 g (88%); *Rf*(B) 0.91; HPLC *K′* 8.94; mp 162–164 °C; $[\alpha]^{20}_D$ +6.3; *m/*z 679 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38–1.42 (d, 9H), 2.67–3.17 (m, 6H), 3.67 (s, 3H), 4.41–4.51 (m, 2H), 4.92–5.51 (m, 3H), 6.16 (s, 2H), 6.96–7.70 (m, 8H).

5.2.7. Boc-Dft-Tic-Asp*-Bid—To a solution of Boc-Dft-Tic-Asp(OMe)*-Bid (0.13 g, 0.19 mmol) in methanol (10 mL) at room temperature, 1 N NaOH (0.38 mL, 0.38 mmol) was added. The reaction mixture was stirred for 3 h at room temperature. After methanol was evaporated, the residue was dissolved in solvent B (60% acetonitrile + 0.1% TFA in H_2O, v/v) and directly lyophilized. A small amount were purified by preparative HPLC for characterization: yield 0.12 g (93%); *Rf*(B) 0.84; HPLC *K′* 8.54; mp 168–170 °C; $[\alpha]^{20}_D$ +7.4; *m/*z 665 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38–1.42 (d, 9H), 2.67–3.17 (m, 6H), 4.41–4.51 (m, 2H), 4.92–5.20 (m, 3H), 6.16 (s, 2H), 6.96–7.70 (m, 8H).

5.2.8. 2TFA.H-Dft-Tic-Asp*-Bid (2)—Boc-Dft-Tic-Asp*-Bid (0.09 g, 0.14 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/PE (1:1, v/v) were added to the solution until the product precipitated: yield 0.10 g (94%); *Rf*(A) 0.63; HPLC *K′* 7.12; mp 172–154 °C; $[\alpha]^{20}_D$ +8.6; *m/*z 565 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.65–3.17 (m, 6H), 3.91–3.99 (m, 1H), 4.41–4.51 (m, 2H), 4.92–5.20 (m, 2H), 6.16 (s, 2H), 6.96–7.70 (m, 8H). Anal. $C_{33}H_{29}F_8N_5O_9$: C; H; N.

5.2.9. Boc-Tyr-Tic-Asp(OMe)*-Bid—This intermediate was obtained by condensation of Boc-Tyr-OH with 2TFA.H-Tic-Asp(OMe)*-Bid via WSC/HOBt as reported for Boc-Dft-Tic- Asp(OMe)*-Bid: yield 0.21 g (86%); *Rf*(B) 0.64; HPLC *K′* 6.28; mp 143–145 °C; $[\alpha]^{20}_{D}$ +5.8; *m/*z 643 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38–1.42 (d, 9H), 2.67–3.17 (m, 6H), 3.67 (s, 3H), 4.41–4.51 (m, 2H), 4.92–5.51 (m, 3H), 6.68–7.70 (m, 12H).

5.2.10. Boc-Tyr-Tic-Asp*-Bid—Boc-Tyr-Tic-Asp(OMe)*-Bid was treated with 1 N NaOH as reported for Boc-Dft-Tic-Asp*-Bid: yield 0.13 g (94%); *Rf*(B) 0.58; HPLC *K′* 5.85; mp 150–152 °C; $[\alpha]^{20}_{D}$ +6.2; *m/*z 629 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38–1.42 (d, 9H), 2.67–3.17 (m, 6H), 4.41–4.51 (m, 2H), 4.92–5.20 (m, 3H), 6.68–7.70 (m, 12H).

5.2.11. 2TFA.H-Tyr-Tic-Asp*-Bid (3)—Boc-Tyr-Tic-Asp*-Bid was treated with TFA as reported for 2TFA.H-Dft-Tic-Asp*-Bid: yield 0.09 g (94%); *Rf*(A) 0.35; HPLC *K′* 4.01; mp 165–167 °C; $[\alpha]^{20}_{D}$ +7.5; *m/*z 529 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.65–3.17 (m, 6H), 3.91–3.99 (m, 1H), 4.41–4.51 (m, 2H), 4.92–5.20 (m, 2H), 6.68–7.70 (m, 12H). Anal. $C_{33}H_{31}F_6N_5O_9$: C; H; N.

5.2.12. Boc-Dft-Tic-Gly*-Bid—This intermediate was obtained by condensation of Boc-Dft-OH with 2TFA.H-Tic-Gly*-Bid[13] via WSC/HOBt as reported for Boc-Dft-Tic-Asp(OMe)*-Bid: yield 0.15 g (85%); *Rf*(B) 0.65; HPLC *K′* 6.34; mp 149–151 °C; $[\alpha]^{20}_{D}$ +5.1; *m/*z 607 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38–1.42 (d, 9H), 2.92–3.17 (m, 4H), 4.41–4.51 (m, 4H), 4.85–4.96 (m, 2H), 6.16 (s, 2H), 6.96–7.70 (m, 8H).

5.2.13. 2TFA.H-Dft-Tic-Gly*-Bid (5)—Boc-Dft-Tic-Gly*-Bid was treated with TFA as reported for 2TFA.H-Dft-Tic-Asp*-Bid: yield 0.11 g (94%); *Rf*(A) 0.37; HPLC *K′* 4.19; mp 158–160 °C; $[\alpha]^{20}_{D}$ −4.3; *m/*z 507 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.92–3.17 (m, 4H), 3.91–3.99 (m, 1H), 4.41–4.92 (m, 5H), 6.16 (s, 2H), 6.96–7.70 (m, 8H). Anal. $C_{31}H_{27}F_8N_5O_7$: C; H; N.

5.2.14. Boc-Tyr-Tic-Gly*-Bid—This intermediate was obtained by condensation of Boc-Tyr-OH with 2TFA.H-Tic-Gly*-Bid[13] via WSC/HOBt as reported for Boc-Dft-Tic-Asp(OMe)*-Bid: yield 0.28 g (87%); *Rf*(B) 0.61; HPLC *K′* 5.98; mp 142–144 °C; $[\alpha]^{20}_{D}$ +6.2; *m/*z 571 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38–1.42 (d, 9H), 2.92–3.17 (m, 4H), 4.41–4.51 (m, 4H), 4.85–4.96 (m, 2H), 6.68–7.70 (m, 12H).

5.2.15. 2TFA.H-Tyr-Tic-Gly*-Bid (6)—Boc-Tyr-Tic-Gly*-Bid was treated with TFA as reported for 2TFA.H-Dft-Tic-Asp*-Bid: yield 0.18 g (95%); *Rf*(A) 0.32; HPLC *K′* 3.57; mp 151–153 °C; $[\alpha]^{20}_{D}$ −6.1; *m/*z 471 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.92–3.17 (m, 4H), 3.91–3.99 (m, 1H), 4.41–4.92 (m, 5H), 6.68–7.70 (m, 12H). Anal. $C_{31}H_{29}F_6N_5O_7$: C; H; N.

5.2.16. Z-Lys(Boc)-NH-Bzl—To a solution of Z-Lys(Boc)-OH (1 g, 2.63 mmol) and benzylamine (0.29 mL, 2.63 mmol) in DMF (10 mL) at 0 °C, HOBt (0.44 g, 2.89 mmol), and WSC (0.56 g, 2.89 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in AcOEt and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 1.09 g (88%); *Rf*(B) 0.89; HPLC *K′* 7.99; mp 104–106 °C; $[\alpha]^{20}_{D}$ −11.8; *m/*z 471 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29–1.79 (m, 15H), 2.92–2.99 (t, 2H), 4.46–4.53 (m, 3H), 5.34 (s, 2H), 7.06–7.19 (m, 10H).

5.2.17. H-Lys(Boc)-NH-Bzl—To a solution of Z-Lys(Boc)-NH-Bzl (1.04 g, 2.21 mmol) in methanol (30 mL) was added Pd/C (10%, 0.1 g), and H_2 was bubbled for 1 h at room

temperature. After filtration, the solution was evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 0.62 g (84%); *Rf*(A) 0.64; HPLC *K*′ 5.56; mp 104–106 °C; $[\alpha]^{20}_D$ −13.2; *m/*z 336 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29–1.79 (m, 15H), 2.92–2.99 (t, 2H), 3.56–4.46 (m, 3H), 7.06–7.14 (m, 5H).

5.2.18. Z-Tic-Lys(Boc)-NH-Bzl—To a solution of Z-Tic-OH (0.53 g, 1.70 mmol) and H-Lys(Boc)-NH-Bzl (0.57 g, 1.70 mmol) in DMF (10 mL) at 0 °C, HOBt (0.29 g, 1.87 mmol), and WSC (0.36 g, 1.87 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in AcOEt and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 0.93 g (87%); *Rf*(B) 0.92; HPLC *K*′ 8.56; mp 110–112 °C; $[\alpha]^{20}_D$ −17.9; *m/*z 630 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29–1.79 (m, 15H), 2.92–3.17 (m, 4H), 4.17–4.53 (m, 5H), 4.92–5.34 (m, 3H), 7.06–7.19 (m, 14H).

5.2.19. H-Tic-Lys(Boc)-NH-Bzl—To a solution of Z-Tic-Lys(Boc)-NH-Bzl (0.88 g, 1.40 mmol) in methanol (30 mL) was added Pd/C (10%, 0.09 g), and H_2 was bubbled for 1 h at room temperature. After filtration, the solution was evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 0.59 g (85%); *Rf*(A) 0.68; HPLC *K*′ 6.72; mp 123–125 °C; $[\alpha]^{20}_D$ −18.5; *m/*z 496 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29–1.79 (m, 15H), 2.92–3.17 (m, 4H), 3.76–3.95 (m, 3H), 4.46–4.53 (m, 3H), 6.96–7.14 (m, 9H).

5.2.20. Boc-Dft-Tic-Lys(Boc)-NH-Bzl—To a solution of Boc-Dft-OH (0.1 g, 0.32 mmol) and H-Tic-Lys(Boc)-NH-Bzl (0.16 g, 0.32 mmol) in DMF (10 mL) at 0 °C, HOBt (0.05 g, 0.35 mmol), and WSC (0.07 g, 0.35 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in AcOEt and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 0.23 g (90%); *Rf*(B) 0.93; HPLC *K*′ 8.76; mp 138–140 °C; $[\alpha]^{20}_D$ −19.1; *m/*z 795 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29–1.79 (m, 24H), 2.92–3.17 (m, 6H), 4.41–4.53 (m, 5H), 4.88–4.95 (m, 2H), 6.16 (s, 2H), 6.96–7.14 (m, 9H).

5.2.21. 2TFA.H-Dft-Tic-Lys-NH-Bzl (11)—Boc-Dft-Tic-Lys(Boc)-NH-Bzl (0.18 g, 0.23 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/PE (1:1, v/v) were added to the solution until the product precipitated: yield 0.17 g (92%); *Rf*(A) 0.41; HPLC *K*′ 5.19; mp 152–154 °C; $[\alpha]^{20}_D$ −17.1; *m/*z 595 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29–1.79 (m, 6H), 2.65–3.17 (m, 6H), 3.95–4.53 (m, 6H), 4.90–4.94 (m, 1H), 6.16 (s, 2H), 6.96–7.14 (m, 9H). Anal. $C_{36}H_{39}F_8N_5O_8$: C; H; N.

5.2.22. Boc-Tyr-Tic-Lys(Boc)-NH-Bzl—This intermediate was obtained by condensation of Boc-Tyr-OH with H-Tic-Lys(Boc)-NH-Bzl via WSC/HOBt as reported for Boc-Dft-Tic-Lys(Boc)- NH-Bzl: yield 0.25 g (88%); *Rf*(B) 0.87; HPLC *K*′ 8.32; mp 132–134 °C; $[\alpha]^{20}_D$ −20.3; *m/*z 759 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29–1.79 (m, 24H), 2.92–3.17 (m, 6H), 4.41–4.53 (m, 5H), 4.88– 4.95 (m, 2H), 6.68–7.14 (m, 13H).

5.2.23. 2TFA.H-Tyr-Tic-Lys-NH-Bzl (12)—Boc-Tyr-Tic-Lys(Boc)-NH-Bzl was treated with TFA as reported for 2TFA.H-Dft-Tic-Lys-NH-Bzl: yield 0.19 g (92%); *Rf*(A) 0.35; HPLC *K*′ 4.36; mp 147–149 °C; $[\alpha]^{20}_D$ −18.9; *m/*z 559 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29–1.79 (m, 6H), 2.65–3.17 (m, 6H), 3.95–4.53 (m, 6H), 4.90–4.94 (m, 1H), 6.68–7.14 (m, 13H). Anal. $C_{36}H_{41}F_6N_5O_8$: C; H; N.

5.2.24. Boc-Dft-Tic-O*t*Bu—To a solution of Boc-Dft-OH (0.11 g, 0.34 mmol) and H-Tic-O*t*Bu (0.08 g, 0.34 mmol) in DMF (10 mL) at 0 °C, HOBt (0.06 g, 0.37 mmol), and WSC (0.07 g, 0.37 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in AcOEt and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 0.15 g (84%); *Rf*(B) 0.76; HPLC *K′* 8.12; mp 112–115 °C; $[\alpha]^{20}_D$ +11.3; *m/*z 534 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.29 (s, 9H), 1.38 (s, 9H), 2.92–3.29 (m, 4H), 4.41–4.51 (m, 2H), 4.81–4.92 (m, 2H), 6.16 (s, 2H), 6.96–7.02 (m, 4H).

5.2.25. TFA.H-Dft-Tic-OH—Boc-Dft-Tic-O*t*Bu (0.12 g, 0.23 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/PE (1:1, v/v) were added to the solution until the product precipitated: yield 0.1 g (93%); *Rf*(A) 0.57; HPLC *K′* 3.13; mp 141–143 °C; $[\alpha]^{20}_D$ +16.3; *m/*z 377 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.91–3.17 (m, 4H), 3.92–3.98 (m, 1H), 4.41–4.85 (m, 3H), 6.16 (s, 2H), 6.96–7.02 (m, 4H).

5.2.26. TFA.*N*,*N*(Me)$_2$-Dft-Tic-OH (14)—To a stirred solution of TFA.H-Dft-Tic-OH (0.07 g, 0.14 mmol) in acetonitrile/H_2O (1:1, v/v; 5 mL) at room temperature, NMM (0.03 mL, 0.28 mmol), 37% aqueous formaldehyde (0.1 mL, 1.4 mmol), and $NaBH_3CN$ (0.03 g, 0.42 mmol) were added. Acetic acid (0.04 mL) was added over 10 min. and the reaction was stirred for 15 min. The reaction mixture was acidified with TFA (0.1 mL) and directly purified by reverse phase preparative HPLC: yield 0.07 g (94%); *Rf*(A) 0.59; HPLC *K′* 3.23; mp 149–151 °C; $[\alpha]^{20}_D$ +24.3; *m/*z 405 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.27 (s, 6H), 2.77–3.16 (m, 4H), 3.95–4.51 (m, 3H), 4.82–4.88 (m, 1H), 6.16 (s, 2H), 6.96–7.02 (m, 4H). Anal. $C_{23}H_{23}F_5N_2O_6$: C; H; N.

5.2.27. Boc-Dft-Tic-NH$_2$—To a solution of Boc-Dft-OH (0.1 g, 0.32 mmol) and TFA.H-Tic-NH_2 (0.09 g, 0.32 mmol) in DMF (10 mL) at 0 °C, NMM (0.03 mL, 0.32 mmol), HOBt (0.05 g, 0.35 mmol), and WSC (0.07 g, 0.35 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in AcOEt and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/PE (1:9, v/v): yield 0.14 g (89%); *Rf*(B) 0.63; HPLC *K′* 7.54; mp 121–123 °C; $[\alpha]^{20}_D$ +15.4; *m/*z 476 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38 (s, 9H), 2.92–3.17 (m, 4H), 4.41–4.51 (m, 2H), 4.85–4.94 (m, 2H), 6.16 (s, 2H), 6.96–7.02 (m, 4H).

5.2.28. TFA.H-Dft-Tic-NH$_2$—Boc-Dft-Tic-NH_2 (0.11 g, 0.23 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.08 g (94%); *Rf*(A) 0.65; HPLC *K′* 3.42; mp 137–139 °C; $[\alpha]^{20}_D$ +15.8; *m/*z 376 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.92–3.17 (m, 4H), 3.92–3.98 (m, 1H), 4.41–4.92 (m, 3H), 6.16 (s, 2H), 6.96–7.02 (m, 4H).

5.2.29. TFA.*N*,*N*(Me)$_2$-Dft-Tic-NH$_2$ (17)—To a stirred solution of TFA.H-Dft-Tic-NH_2 (0.06 g, 0.12 mmol) in acetonitrile/H_2O (1:1, v/v; 5 mL) at room temperature, NMM (0.01 mL, 0.12 mmol), 37% aqueous formaldehyde (0.09 mL, 1.2 mmol), and $NaBH_3CN$ (0.02 g, 0.42 mmol) were added. Acetic acid (0.04 mL) was added over 10 min. and the reaction was stirred for 15 min. The reaction mixture was acidified with TFA (0.1 mL) and directly purified by reverse phase preparative HPLC: yield 0.06 g (95%); *Rf*(A) 0.69; HPLC *K′* 3.58; mp 144–146 °C; $[\alpha]^{20}_D$ +18.6; *m/*z 404 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.27 (s, 6H), 2.77–3.17 (m, 4H), 3.95–4.51 (m, 3H), 4.89–4.95 (m, 1H), 6.16 (s, 2H), 6.96–7.02 (m, 4H); Anal. $C_{23}H_{23}F_5N_3O_5$: C; H; N.

5.2.30. Boc-Tyr-Tic-NH_2—This intermediate was obtained by condensation of Boc-Tyr-OH with TFA.H-Tic-NH_2 via WSC/HOBt as reported for Boc-Dft-Tic-NH_2: yield 0.23 g (87%); *Rf*(B) 0.58; HPLC *K*′ 6.84; mp 129–131 °C; $[\alpha]^{20}_D$ +16.3; *m/*z 441 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.38 (s, 9H), 2.92–3.17 (m, 4H), 4.41–4.51 (m, 2H), 4.85–4.94 (m, 2H), 6.68–7.02 (m, 8H).

5.2.31. TFA.H-Tyr-Tic-NH_2—Boc-Tyr-Tic-NH_2 was treated with TFA as reported for TFA.H-Dft-Tic-NH_2: yield 0.15 g (94%); *Rf*(A) 0.62; HPLC *K*′ 3.31; mp 132–134 °C; $[\alpha]^{20}_D$ +19.5; *m/*z 340 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.92–3.17 (m, 4H), 3.92–3.98 (m, 1H), 4.41–4.92 (m, 3H), 6.68–7.02 (m, 8H).

5.2.32. TFA.*N*,*N*$(Me)_2$-Tyr-Tic-NH_2 (18)—This compound was obtained by *N*, *N*-dimethylation of TFA.H-Tyr-Tic-NH_2 as reported for TFA.*N*,*N*$(Me)_2$-Dft-Tic-NH_2: yield 0.09 g (93%); *Rf*(A) 0.61; HPLC *K*′ 3.39; mp 138–140 °C; $[\alpha]^{20}_D$ +20.2; *m/*z 368 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 2.27 (s, 6H), 2.77–3.17 (m, 4H), 3.95–4.51 (m, 3H), 4.89–4.95 (m, 1H), 6.68–7.02 (m, 8H); Anal. $C_{23}H_{26}F_3N_3O_5$: C; H; N.

Deltorphin B (**9**) and its analogues (**7**, **8**) were prepared by solid phase peptide synthesis. Fmoc Rink Amide resin (0.69 mmol/g, 0.2 g) was treated with 40% piperidine in DMF and linked with Fmoc-Gly-OH using DIPCI/HOBt as coupling reagent. The following protected amino acids were sequentially coupled to the growing peptide chain: Fmoc-Val-OH, Fmoc-Val-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-D-Ala-OH and Boc-Dmt-OH or Boc-Dft-OH or Boc-Tyr-OH. Protected aminoacids were reacted in a 4-fold excess using DIPCI/HOBt (4-fold excess) for 1 h of coupling. To improve the purity of the final crude peptides, capping with acetic anhydride (0.5 M/DMF) in the presence of NMM (0.25M/DMF) (3:1 v/v; 2 mL/0.2 g resin) was performed at any step. Fmoc protecting group was removed at every step by 40% piperidine/DMF treatment for 25 min. Each protected peptide linked to the resin, after washing with methanol and drying in vacuo, was fully deprotected and removed from the resin by treatment with a mixture containing TFA/triethylsilane/H_2O (10 mL; 9/0.5/0.5) for 1.5h at room temperature. The resin was filtered and the solution was evaporated in vacuo to yield an oil that was triturated with diethyl ether and collected by centrifugation. Each crude peptide was purified by reverse phase preparative HPLC.

5.2.33. TFA.[Dft^1]Deltorphin B (8)—Estimated yield 0.05 g (39%); *Rf*(A) 0.67; HPLC *K*′ 4.39; mp 176–178 °C; $[\alpha]^{20}_D$ −22.5; *m/*z 820 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 1.01–1.48 (m, 15H), 2.06–2.23 (m, 4H), 2.68–3.17 (m, 6H), 3.95–4.09 (m, 3H), 4.52–4.92 (m, 5H), 6.16 (s, 2H), 7.08–7.21 (m, 5H); Anal. $C_{40}H_{53}F_5N_8O_{12}$: C; H; N.

5.3. Pharmacology

5.3.1. Competitive Binding Assays—Opioid receptor affinities were determined under equilibrium conditions [2.5 h room temperature (23 °C)] in competition assays using brain P_2 synaptosomal membranes prepared from Sprague-Dawley rats.[30, 31] Synaptosomes were preincubated to remove endogenous opioid peptides and stored at − 80 °C in buffered 20% glycerol.[30, 32] Each analogue was analyzed in duplicate assays using five to eight dosages and conducting three to five independent repetitions with different synaptosomal preparations (*n* values are listed in Table 1 in parenthesis and results are mean ± SE). Unlabeled peptide (2 μM) was used to determine non-specific binding in the presence of 1.9 nM [^{3}H]deltorphin II (45.0 Ci/mmol, Perkin Elmer, Boston, MA; K_D = 1.4 nM) for δ-opioid receptors and 3.5 nM [^{3}H]DAMGO (50.0 Ci/mmol), Amersham Bioscience, Buckinghamshire, U. K.; K_D = 1.5 nM) for μ-opioid receptors. Glass fibre filters (Whatman GFC) were soaked in 0.1% polyethylenimine in order to enhance the signal-to-noise ratio of the bound radiolabeled-synaptosome complex, and the filters were washed thrice in ice-cold

buffered 0.1% BSA,[30] and the affinity constants (K_i) were calculated according to Cheng and Prusoff.[33]

5.3.2. Biological Activity in Isolate Tissue Preparations—The myenteric plexus longitudinal muscle preparations (2–3 cm segments) from the small intestine of male Hartley strain of guinea pigs (GPI) measured μ-opioid receptor agonism, and a single mouse vas deferens (MVD) was used to determine δ-opioid receptor agonism as described previously.[34] The isolated tissues were suspended in organ baths containing balanced salt solutions in a physiological buffer, pH 7.5. Agonists were tested for the inhibition of electrically evoked contraction and expressed as IC_{50} (nM) obtained from the dose-response curves. The IC_{50} values represent the mean ± SE of five or six separate assays, and the δ-antagonist potencies in the MVD assay were determined against the δ agonist deltorphin-II, while μ antagonism (GPI assay) used the μ agonist endomorphin-2. Antagonism is expressed as pA_2 determined using the Schild Plot.[35]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Receptor Binding Affinities and Functional Bioactivities of Compounds **1–14**.

	Structure	receptor affinity[b] (nM)		selectivity	functional bioactivity			
		K_i^{δ}	K_i^{μ}	K_i^{μ}/K_i^{δ}	MVD pA_2[d]	MVD IC_{50}[c] (nM)	GPI pA_2[d]	GPI IC_{50}[c] (nM)
1	***H-Dmt-Tic-Asp*-Bid***[e]	***0.44***	***53.9***	***122***		***0.12***		***1724***
2	H-Dft-Tic-Asp*-Bid	0.162±0.02 (5)	225.1±36 (4)	1390	8.95			
3	H-Tyr-Tic-Asp*-Bid	0.177±0.009 (4)	683.9±91 (6)	3864	8.85			
4	***H-Dmt-Tic-Gly*-Bid***[f]	***0.035***	***0.50***	***14***		***0.13***		***26.92***
5	H-Dft-Tic-Gly*-Bid	1.01±0.05 (3)	54.5±4.4 (5)	54		67.3±6.8		>10000
6	H-Tyr-Tic-Gly*-Bid	0.913±0.03 (3)	66.9±8.8 (6)	73		50.1±1.67		4216±544
7	***[Dmt[1]]Deltorphin B***[g]	***0.16±0.03 (4)***	***1.68±0.14 (3)***	***10***		***0.32±0.02***		***48.2±1.76***
8	[Dft[1]]Deltorphin B	0.241±0.03 (3)	154.3±19 (3)	640		0.40±0.08		3559±566
9	Deltorphin B[g]	0.12	638	5317		0.53±0.02		>10000
10	***H-Dmt-Tic-Lys-NH-Bzl***[h]	***0.50***	***4.05***	***8***	***6.01***		***7.96***	
11	H-Dft-Tic-Lys-NH-Bzl	153.4±21 (6)	1029±83 (4)	7				
12	H-Tyr-Tic-Lys-NH-Bzl	63.9±7.6 (3)	1649±231 (6)	26				
13	***N,N(Me)$_2$-Dmt-Tic-OH***[i]	***0.118***	***2435***	***20636***	***9.4***		***5.8***	
14	*N*,*N*(Me)$_2$-Dft-Tic-OH	29.2±3.8 (5)	573.3±69 (4)	20				
15	*N*,*N*(Me)$_2$-Tyr-Tic-OH[i]	34.8±1.7 (4)	1234±48.5 (3)	35				
16	***N,N(Me)$_2$-Dmt-Tic-NH$_2$***[i]	***0.309***	***511.4***	***1655***	***9.9***		***6.3***	
17	*N*,*N*(Me)$_2$-Dft-Tic-NH$_2$	176.4±7.5 (5)	596.4±58 (4)	3				
18	*N*,*N*(Me)$_2$-Tyr-Tic-NH$_2$	201.3±14 (4)	1451±25 (5)	7				

[b] The K_i values (nM) were determined according to Cheng and Prusoff.[33] The mean ± SEM with *n* repetitions in parenthesis is based on independent duplicate binding assays conducted with five to eight peptide doses using several different synaptosomal preparations.

[c] Agonist activity is expressed as IC_{50} obtained from dose-response curves. These values represent the mean ± SE for at least four tissue samples. DPDPE and PL017 were the internal standards for MVD (δ-opioid receptor bioactivity) and GPI (μ-opioid receptor bioactivity) tissue preparation, respectively.

[d] The pA_2 values of opioid antagonists against the δ and μ agonists (deltorphin II and endomorphin-2, respectively) were determined by the method of Kosterlitz and Watt.[36]

[e] Data taken from Balboni et al.[12]

[f] Data taken from Balboni et al.[13]

[g] Affinity data taken from Guerrini et al.[17]

[h] Data taken from Balboni et al.[14]

[i] Data taken from Salvadori et al.[15]