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Further studies on the effect of lysine at the C-terminus of the Dmt-Tic opioid pharmacophore

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Abstract—A wide range of activities are induced by Lys when introduced at C-terminus of the δ-opioid Dmt-Tic pharmacophore through the α-amine group, including: improved δ-antagonism, μ-agonism and μ-antagonism. Here we report the synthesis of a new series of compounds with the general formula H-Dmt-Tic-NH-$(CH_2)_4$-CH(R)-R′ (R = -NH_2, -NH-Ac, -NH-Z; R′ = CO-NH-Ph, -CO-NH-CH_2-Ph, -Bid) in which Lys is linked to Dmt-Tic through its side-chain amine group. All new compounds (**1**–**9**) displayed potent and selective δ-antagonism (MVD, pA_2 = 7.81–8.27), which was independent of the functionalized α-amine and carboxylic groups of C-terminal Lys. This behaviour suggests a direct application as a prototype intermediate, such as Boc-Dmt-Tic-ε-Lys(Z)-OMe, which could be successfully applied in the synthesis (after Z or methyl ester removal) of unique designed multiple ligands containing the pharmacophore of the quintessential δ-antagonist Dmt-Tic and another opioid or biologically active non-opioid ligand.

1. Introduction

Extensive structure–activity studies on the prototype δ-opioid receptor antagonist, H-Dmt-Tic-OH,[†,1] revealed that even minor chemical modifications changed its pharmacological profile,[2] including enhanced δ-antagonism,[3] the reversal to δ-agonism,[4] the appearance of mixed μ-agonism/δ-agonism,[5] as well as formation of mixed μ-agonism/δ-antagonism,[5] μ-agonism[6] and -antagonism.[6] Each pharmacological profile indicated interesting potential for therapeutic applications, such as analgesia with low tolerance and dependence,[5] antidepressant activity,[7,8] neuroprotection and neurogenesis,[9] regulation of food intake[10] and in the treatment of alcoholism.[11]

Recently, we demonstrated that the substitution of C-terminal amino acids in tri- and tetrapeptides containing the Dmt-Tic pharmacophore with side-chain protected Lys improved δ-antagonist potency.[12,13] On the basis of these results, we extended the substitution of the side-chain protected or unprotected Lys to other biologically active compounds previously developed by us [H-Dmt-Tic-NH-CH(CH_2-COOH)-Bid (Bid = 1*H*-benzimidazole-2-yl) a δ-agonist; H-Dmt-Tic-Gly-NH-Ph a μ-agonist/

Keywords: δ-Opioid receptors; Dmt-Tic pharmacophore; Designed multiple ligands; Opioid peptides.
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† *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: Ac, acetyl; Bid, 1*H*-benzimidazole-2-yl; Boc, *tert*-butyloxycarbonyl; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin; Deltorphin II, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2; DMF, *N*,*N*-dimethylformamide; DMSO-d_6, hexadeuteriodimethyl sulfoxide; Dmt, 2′,6′-dimethyl-L-tyrosine; EtOAc, ethyl acetate; 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; MeOH, methanol; MVD, mouse vas deferens; NMM, 4-methylmorpholine; pA_2, negative log of the molar concentration required to double the agonist concentration to achieve the original response; Pe, petroleum ether; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TLC, thin-layer chromatography; WSC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; Z, benzyloxycarbonyl.

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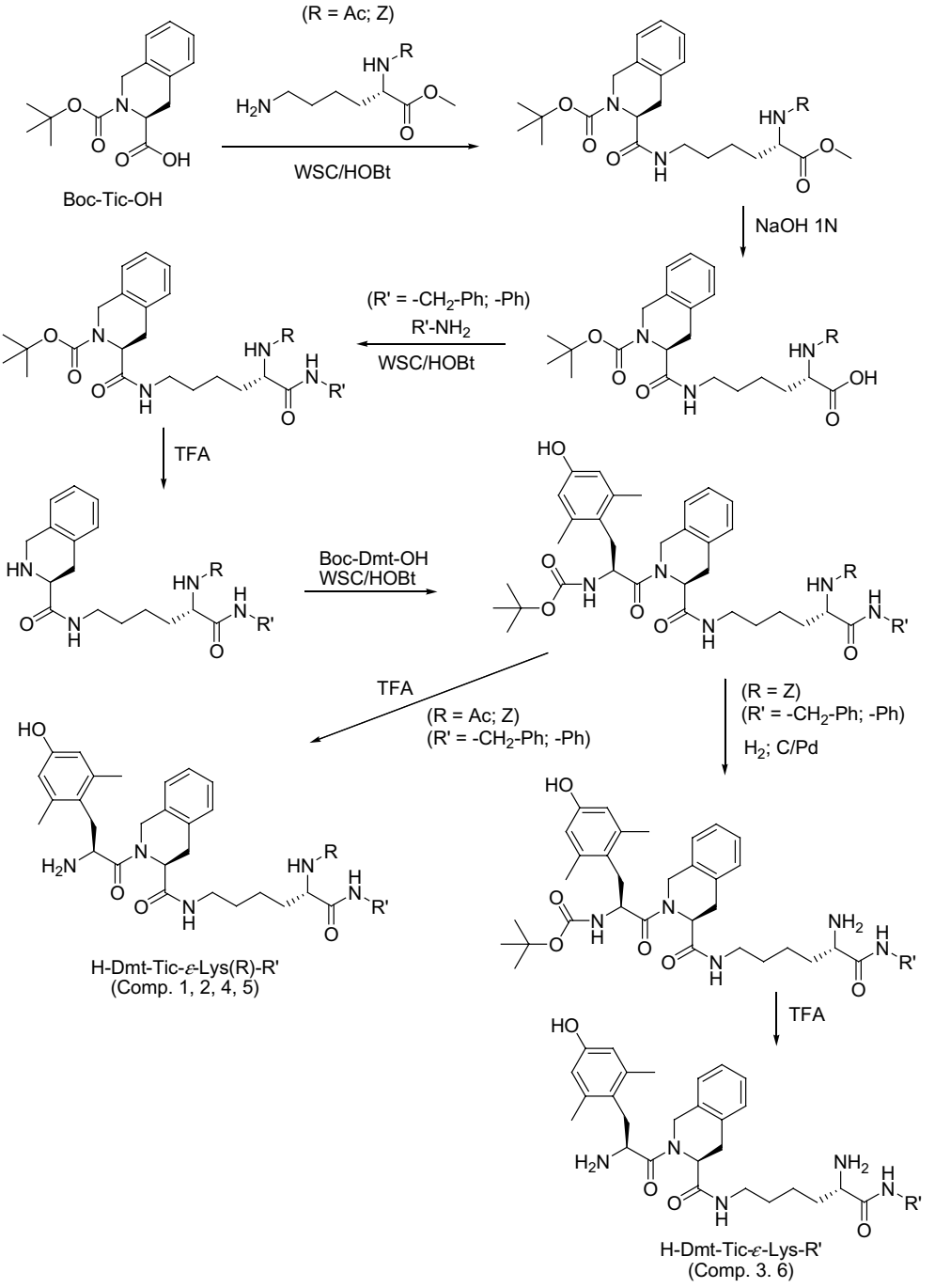
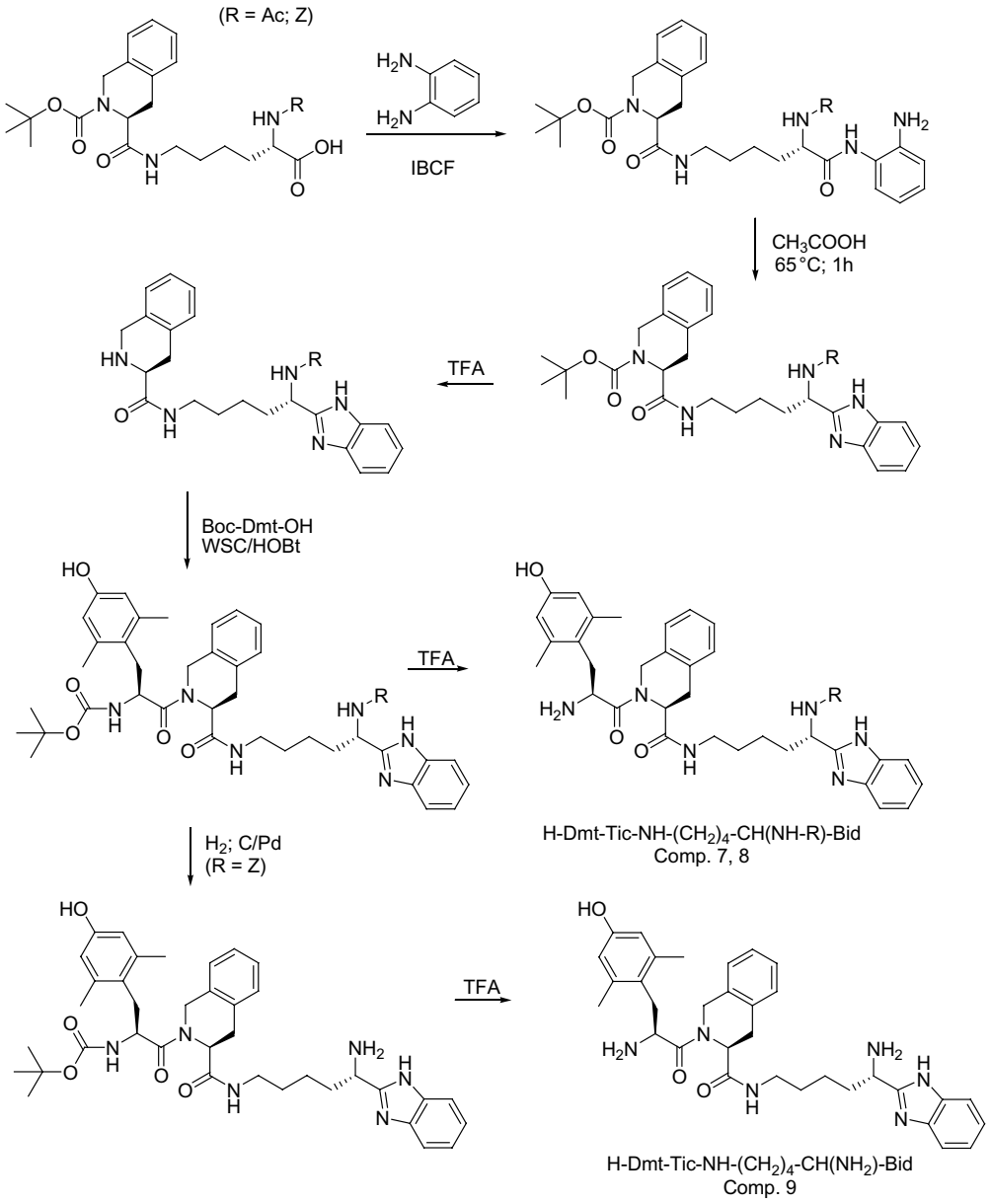
δ-agonist and H-Dmt-Tic-Gly-NH-CH_2-Ph a μ-agonist/δ-antagonist] with a quite surprising array of interesting results. Lysine, when introduced in place of the C-terminal amino acid in the above reference compounds, did not produce a simple improvement in the original pharmacological activities but provided opioid ligands which exhibited mixed properties ranging from δ-antagonism, μ-agonism and interestingly, μ-antagonism.[6] Considering the variety of biological effects induced by Lys in tripeptides and pseudotripeptides of the general formula H-Dmt-Tic-Lys(R)-R′, the studies described herein extend our initial investigations on the synthesis and biological evaluation of a new series of constitutional isomers developed on the framework of H-Dmt-Tic-ε-Lys(R)-R′, where Lys is linked to the Dmt-Tic dipeptide through the ε-amine group, in order to further evaluate the important influence of Lys on opioid receptor interactions and functional bioactivities to produce opioid ligands for potential translation into human health initiatives.

2. Chemistry

Peptides (**1**–**6**) and pseudopeptides (**7**–**9**) were prepared stepwise by solution peptide synthetic methods, as outlined in Schemes 1 and 2, respectively. Boc-Tic-OH was



Scheme 1. Synthesis of compounds **1**–**6**.



Scheme 2. Synthesis of compounds **7**–**9**.

condensed with commercially available Z-Lys-OMe or Ac-Lys-OMe via WSC/HOBt obtaining the corresponding Boc-Tic-ε-Lys(Z)-OMe or Boc-Tic-ε-Lys(Ac)-OMe. C-Terminal methyl ester protecting groups were removed by hydrolysis with 1 N NaOH and then each pseudodipeptide was condensed with benzylamine or aniline via WSC/HOBt. N-terminal Boc-protected pseudodipeptide amides were treated with TFA and condensed with Boc-Dmt-OH via WSC/HOBt. Final N-terminal Boc deprotection with TFA gave compounds **1**, **2**, **4** and **5** (Scheme 1). Catalytic hydrogenation (10% Pd/C) and TFA treatment of Boc-Dmt-Tic-ε-Lys(Z)-amides gave the final products **3** and **6** (Scheme 1). Pseudopeptides (**7**–**9**), containing C-terminal 1*H*-benzimidazol-2-yl (Bid), were synthesized in a similar manner (Scheme 2). Mixed carbonic anhydride coupling of Boc-Tic-ε-Lys(Z)-OH or Boc-Tic-ε-Lys(Ac)-OH with *o*-phenylendiamine gave the corresponding crude intermediate monoamides, which were converted without purification to the desired heteroaromatic derivatives by cyclization and dehydration in acetic acid. As detailed in Scheme 1, after N^{α} deprotection with TFA, each derivative was condensed with Boc-Dmt-OH via WSC/HOBt. Final N-terminal Boc deprotection with TFA gave compounds **7**, **8** (Scheme 2). Catalytic hydrogenation (10% Pd/C) and TFA treatment of Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid provided the product **9** (Scheme 2). Final compounds (**1**–**9**) were purified by preparative HPLC as described in Experimental Section.

3. Results and discussion

3.1. Receptor affinity analysis

Receptor binding and functional bioactivities are reported in Table 1. All the compounds (**1**–**9**) exhibited

Table 1. Receptor binding affinities and functional bioactivities of compounds **1–9**

Compound	Structure	Receptor affinity[a] (nM)		Selectivity	Functional bioactivity	
		K_i^δ	K_i^μ		MVD pA_2[c]	GPI IC_{50}[b] (nM)
	H-Dmt-Tic-NH_2[d]	*1.22*	*277*	*227*	*7.2*	*>10,000*
1	H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph	0.53 ± 0.08 (4)	3.15 ± 0.39 (4)	5.9	8.27	1451 ± 200
2	H-Dmt-Tic-ε-Lys(Ac)-NH-CH_2-Ph	0.21 ± 0.01 (3)	3.43 ± 0.54 (4)	16.3	8.07	1990 ± 674
3	H-Dmt-Tic-ε-Lys-NH-CH_2-Ph	1.00 ± 0.02 (4)	0.60 ± 0.11 (4)	1.7*	7.81	553 ± 173
4	H-Dmt-Tic-ε-Lys(Z)-NH-Ph	0.47 ± 0.04 (4)	2.67 ± 0.41 (4)	5.7	8.23	711 ± 194
5	H-Dmt-Tic-ε-Lys(Ac)-NH-Ph	0.22 ± 0.005 (3)	2.57 ± 0.34 (4)	11.7	8.18	486 ± 63
6	H-Dmt-Tic-ε-Lys-NH-Ph	2.02 ± 0.20 (4)	0.89 ± 0.12 (4)	2.3*	7.92	515 ± 73
7	H-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid	0.86 ± 0.06 (3)	2.78 ± 0.27 (4)	3.2	7.82	618 ± 140
8	H-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Ac)-Bid	0.21 ± 0.01 (3)	0.99 ± 0.06 (4)	4.7	8.12	610 ± 185
9	H-Dmt-Tic-NH-$(CH_2)_4$-CH(NH_2)-Bid	2.64 ± 0.18 (3)	1.02 ± 0.08 (3)	2.6*	8.09	434 ± 26

[a] The K_i values (nM) were determined according to Cheng and Prusoff.[24] The means ± SE with *n* repetitions in parentheses is based on independent duplicate binding assays with 5–8 peptide doses using several different synaptosomal preparations.

[b] Agonist activity was expressed as IC_{50} obtained from dose–response curves. These values represent means ± SE for at least 5–6 fresh tissue samples. Deltorphin II and endomorphin-2 were the internal standards for MVD (δ-opioid receptor bioactivity) and GPI (μ-opioid receptor bioactivity) tissue preparation, respectively.

[c] The pA_2 values of opioid antagonists against the agonist deltorphin II were determined by the method of Kosterlitz and Watt.[25]

[d] Data taken from Salvadori et al.[1]

* μ-Opioid receptor selectivity K_i^δ/K_i^μ.

nanomolar affinity for δ-opioid receptors (K_i^δ = 0.21–2.64 nM). As expected, the lack of a free carboxylic function in molecules containing the Dmt-Tic pharmacophore induces a substantial increase in μ-opioid receptor affinity (K_i^μ = 0.60–3.43 nM).[12,4] Compounds (**1**, **2**, **4**, **5**, **7**, **8**) containing a Lys residue protected at the α-amine function (Z, Ac) had weak δ-opioid receptor selectivity (K_i^μ/K_i^δ = 16.3–3.2); the acetyl protecting group confers marginally better selectivity than the Z group. Removal of the α-amine protecting group of Lys (**3**, **6**, **9**) shifted the δ-opioid selectivity to a very weak μ-selective ligands essentially attributable to the presence of the additional positive charge.[14] The same general behaviour was observed previously in the series of Dmt-Tic containing peptides with Lys in the third position.[6]

3.2. Functional bioactivity

Compounds **1–9** were tested in the electrically stimulated MVD and GPI assays for intrinsic functional bioactivity (Table 1). We and other investigators have previously discussed the discrepancy in the correlation between receptor binding affinities and functional bioactivity. Unfortunately, we have neither definitive nor comprehensive explanations for these observations.[12,6] In comparison to the Dmt-Tic peptides containing a protected or unprotected Lys residue at the C-terminus[6] all the analogues are inactive as δ-opioid agonists in the MVD assay (Table 1). Furthermore, they exhibited a weak or very weak μ-agonism in the GPI assay (GPI, IC_{50} 434–1990 nM), which is in quite good agreement with the previous studies, except analogues containing Bid at C-terminus.[6] When Lys was linked to Dmt-Tic through the α-amine group and its carboxylic function transformed into Bid, the pseudopeptides predominately had selective μ-agonism. On the other hand, when Lys was linked to Dmt-Tic through the ε-amine group and its carboxylic function once again transformed into Bid, no interesting μ-agonism activity was observed. Interestingly, all these compounds (**1–9**) had about the same order of magnitude of δ-antagonism (MVD, pA_2 7.82–8.27), which was independent of the substitutions and modifications made on the α amine and carboxylic functions of Lys, but in quite good agreement with the reference dipeptide H-Dmt-Tic-NH_2.[1]

4. Conclusions

Considering the new derivatives (**1–9**) as analogues of the published reference compounds [H-Dmt-Tic-NH-CH_2-Bid δ-agonist, H-Dmt-Tic-NH-CH(CH_2-COOH)-Bid δ-agonist, H-Dmt-Tic-Gly-NH-Ph μ-agonist/δ-agonist and H-Dmt-Tic-Gly-NH-CH_2-Ph μ-agonist/δ-antagonist] the introduction of Lys (linked through its ε amine group to the Dmt-Tic pharmacophore) in place of the C-terminal amino acid failed to maintain the original pharmacological activity, as previously reported for the corresponding isomers containing Lys linked through its α-amine group.[6] While isomers containing the C-terminal α-Lys revealed a variety of opioid effects (δ-antagonism, μ-agonism and μ-antagonism),[6] the isomers containing C-terminal ε-Lys demonstrated a unique δ-opioid antagonism of about the same order of magnitude, and independent of the substituents linked on the α position. Without taking into considerations the different behaviour of Lys when coupled to the Dmt-Tic pharmacophore through its α- or ε-amine group, it is of notable significance to utilize these new isomers as potential precursors in the synthesis of 'designed multiple ligands', where one of the two pharmacophores is represented by the δ-selective antagonist dipeptide Dmt-Tic.[15,16] The four methylene groups of Lys side chain can be considered the spacer linked to the first pharmacophore (Dmt-Tic) and, as reported by Neumeyer et al., its length generally does not influence the biological activity of either pharmacophores.[17,18]

The second pharmacophore, required to complete the potential designed multiple ligands, preferentially should be selected among the compounds in the opioid field, but other pharmacophores endowed with activity for other receptors would be readily inserted as well.[16,19] More importantly, the second pharmacophore can be conveniently inserted using the deprotected α-amino or carboxylic function depending on the required final bioactive product. Furthermore, very similar compounds were obtained when using fluorescent chromophores in place of the second pharmacophore.[20,21] Recently, Okada et al. reported the synthesis of similar compounds with the general formula H-Dmt-Tic-NH-$(CH_2)_6$-NH-R (where R = Dmt, Phe, Tic and Tic-Dmt) in which all the opioids displayed increased δ antagonism attributable to the additional aromatic amino acids.[22] However, the same enhancement in δ-antagonist activity could be derived, at least in part, by the presence of the spacer as reported here and in preceding studies.[20,21]

In summary, we suggest the possibility to use a unique intermediate [for example Boc-Dmt-Tic-ε-Lys(Z)-OMe] for the synthesis of designed multiple ligands containing: (a) the δ-antagonist pharmacophore Dmt-Tic, (b) a spacer of defined length and (c) two different protected functionalities (amine and carboxylic functions) for the linkage to a variety of second pharmacophores. As further explorations of this proposal, the synthesis of multiple ligands derived from the coupling of the selectively deprotected δ-antagonist intermediate Boc-Dmt-Tic-ε-Lys(Z)-OMe with salvinorin A[23a] (κ-agonist); and the synthesis of H-Dmt-Tic-ε-Lys(4-Fluorobenzoyl)-OH as a potential pharmacological tool for PET imaging of δ receptors,[23b,c] are currently in progress in our laboratory.

5. Experimental

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 × 4 cm, 15 μm particle)] and eluted at a flow rate of 25 mL/min with mobile phase solvent A (10% acetonitrile + 0.1% TFA in H_2O, v/v), and a linear gradient from 25% to 75% 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 × 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 1% 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.

5.2. Peptide synthesis

5.2.1. Boc-Tic-ε-Lys(Z)-OMe. To a solution of Boc-Tic-OH (0.9 g, 3.24 mmol) and HCl·Z-Lys-OMe (0.95 g, 3.24 mmol) in DMF (10 mL) at 0 °C, NMM (0.35 mL, 3.24 mmol), HOBt (0.54 g, 3.56 mmol) and WSC (0.68 g, 3.56 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 EtOAc 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 1.4 g (78%); R_f (B) 0.89; HPLC K' 5.43; mp 101–103 °C; $[\alpha]_D^{20}$ −20.1; m/z 554 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.90 (m, 15H), 3.05–3.67 (m, 7H), 4.22–4.42 (m, 3H), 4.92–5.34 (m, 3H), 6.96–7.19 (m, 9H).

5.2.2. Boc-Tic-ε-Lys(Z)-OH. To a solution of Boc-Tic-ε-Lys(Z)-OMe (1.4 g, 2.53 mmol) in MeOH (10 mL) was added 1 N NaOH (2.8 mL). The reaction mixture was stirred for 24 h at room temperature. After solvent evaporation, the residue was dissolved in EtOAc and washed with citric acid (10% 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 (1:1, v/v): yield 1.1 g (81%); R_f (B) 0.42; HPLC K' 3.71; mp 120–122 °C; $[\alpha]_D^{20}$ −21.2; m/z 540 $(M+H)^+$.

5.2.3. Boc-Tic-ε-Lys(Z)-NH-CH_2-Ph. To a solution of Boc-Tic-ε-Lys(Z)-OH (0.52 g, 0.96 mmol) and benzylamine (0.1 mL, 0.96 mmol) in DMF (10 mL) at 0 °C, HOBt (0.16 g, 1.06 mmol) and WSC (0.2 g, 1.06 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 EtOAc 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 0.53 g (88%); R_f (B) 0.85; HPLC K' 5.37; mp 102–104 °C; $[\alpha]_D^{20}$ −17.7; m/z 629 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.79 (m, 15H), 3.05–3.20 (m, 4H), 4.22–4.53 (m, 5H), 4.92–5.34 (m, 3H), 6.96–7.14 (m, 14H).

5.2.4. TFA·H-Tic-ε-Lys(Z)-NH-CH_2-Ph. Boc-Tic-ε-Lys(Z)-NH-CH_2-Ph (0.47 g, 0.75 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 0.34 g (87%); R_f (A) 0.51; HPLC K' 4.3; mp 115–117 °C; $[\alpha]_D^{20}$ −19.8; *m/z* 529 $(M+H)^+$.

5.2.5. Boc-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph. To a solution of Boc-Dmt-OH (0.075 g, 0.24 mmol) and TFA·H-Tic-ε-Lys(Z)-NH-CH_2-Ph (0.15 g, 0.24 mmol) in DMF (10 mL) at 0 °C, NMM (0.03 mL, 0.24 mmol), HOBt (0.04 g, 0.26 mmol) and WSC (0.05 g, 0.26 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 EtOAc 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 0.17 g (87%); R_f (B) 0.81; HPLC K' 5.23; mp 129–131 °C; $[\alpha]_D^{20}$ −16.6; *m/z* 820 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.79 (m, 15H), 2.35 (s, 6H), 3.05–3.20 (m, 6H), 4.46–4.53 (m, 5H), 4.92–5.34 (m, 4H), 6.29 (s, 2H), 6.96–7.19 (m, 14H).

5.2.6. TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph (1). Boc-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph (0.11 g, 0.13 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 0.09 g (96%); R_f (A) 0.47; HPLC K' 3.63; mp 125–127 °C; $[\alpha]_D^{20}$ −14.6; *m/z* 721 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.79 (m, 6H), 2.35 (s, 6H), 3.05–3.20 (m, 6H), 3.95–4.53 (m, 6H), 4.92–5.34 (m, 3H), 6.29 (s, 2H), 6.96–7.19 (m, 14H). Anal. Calcd for $C_{44}H_{50}F_3N_5O_8$: C, 63.37; H, 6.04; N, 8.40. Found: C, 63.22; H, 5.98; N, 8.21.

5.2.7. Boc-Tic-ε-Lys(Ac)-OMe. This intermediate was obtained by condensation of Boc-Tic-OH with HCl·Ac-Lys-OMe via WSC/HOBt, as reported for Boc-Tic-ε-Lys(Z)-OMe: yield 1.6 g (82%); R_f (B) 0.77; HPLC K' 5.32; mp 127–129 °C; $[\alpha]_D^{20}$ −20.5; *m/z* 463 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.90 (m, 15H), 2.02 (s, 3H), 2.92–3.67 (m, 7H), 4.17–4.92 (m, 4H), 6.96–7.02 (m, 4H).

5.2.8. Boc-Tic-ε-Lys(Ac)-OH. This intermediate was obtained by hydrolysis of Boc-Tic-ε-Lys(Ac)-OMe as reported for Boc-Tic-ε-Lys(Z)-OH: yield 1.26 g (82%); R_f (B) 0.45; HPLC K' 5.18; mp 135–137 °C; $[\alpha]_D^{20}$ −22.3; *m/z* 449 $(M+H)^+$.

5.2.9. Boc-Tic-ε-Lys(Ac)-NH-CH_2-Ph. This intermediate was obtained by condensation of Boc-Tic-ε-Lys(Ac)-OH with benzylamine via WSC/HOBt as reported for Boc-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.33 g (81%); R_f (B) 0.79; HPLC K' 5.32; mp 108–110 °C; $[\alpha]_D^{20}$ −18.6; *m/z* 537 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.79 (m, 15H), 2.02 (s, 3H), 2.92–3.20 (m, 4H), 4.17–4.92 (m, 6H), 6.96–7.14 (m, 9H).

5.2.10. TFA·H-Tic-ε-Lys(Ac)-NH-CH_2-Ph. Boc-Tic-ε-Lys(Ac)-NH-CH_2-Ph was treated with TFA as reported for TFA·H-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.21 g (97%); R_f (A) 0.48; HPLC K' 3.92; mp 121–123 °C; $[\alpha]_D^{20}$ −20.7; *m/z* 437 $(M+H)^+$.

5.2.11. Boc-Dmt-Tic-ε-Lys(Ac)-NH-CH_2-Ph. This intermediate was obtained by condensation of Boc-Dmt-OH with TFA·H-Tic-ε-Lys(Ac)-NH-CH_2-Ph via WSC/HOBt as reported for Boc-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.14 g (83%); R_f (B) 0.75; HPLC K' 4.92; mp 135–137 °C; $[\alpha]_D^{20}$ −17.5; *m/z* 729 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.79 (m, 15H), 2.02 (s, 3H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 4.41–4.92 (m, 7H), 6.29 (s, 2H), 6.96–7.14 (m, 9H).

5.2.12. TFA·H-Dmt-Tic-ε-Lys(Ac)-NH-CH_2-Ph (2). Boc-Dmt-Tic-ε-Lys(Ac)-NH-CH_2-Ph was treated with TFA as reported for TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.08 g (96%); R_f (A) 0.45; HPLC K' 3.21; mp 131-133 °C; $[\alpha]_D^{20}$ −15.5; *m/z* 629 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.79 (m, 6H), 2.02 (s, 3H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 3.95–4.92 (m, 7H), 6.29 (s, 2H), 6.96–7.14 (m, 9H). Anal Calcd for $C_{38}H_{46}F_3N_5O_7$: C, 61.53; H, 6.25; N, 9.44. Found: C, 61.77; H, 6.39; N, 9.15.

5.2.13. Boc-Dmt-Tic-ε-Lys-NH-CH_2-Ph. To a solution of Boc-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph (0.1 g, 0.12 mmol) in methanol (30 mL) was added Pd/C (10%, 0.07 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 0.07 g (85%); R_f (B) 0.58; HPLC K' 4.98; mp 144–145 °C; $[\alpha]_D^{20}$ −18.7; *m/z* 687 $(M+H)^+$.

5.2.14. 2TFA·H-Dmt-Tic-ε-Lys-NH-CH_2-Ph (3). Boc-Dmt-Tic-ε-Lys-NH-CH_2-Ph was treated with TFA as reported for TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.07 g (95%); R_f (A) 0.39; HPLC K' 3.32; mp 148–150 °C; $[\alpha]_D^{20}$ −16.2; *m/z* 587 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.79 (m, 6H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 3.56–4.92 (m, 7H), 6.29 (s, 2H), 6.96–7.14 (m, 9H). Anal Calcd for $C_{38}H_{45}F_6N_5O_8$: C, 56.08; H, 5.57; N, 8.61. Found: C, 56.30; H, 5.68; N, 8.70.

5.2.15. Boc-Tic-ε-Lys(Z)-NH-Ph. This intermediate was obtained by condensation of Boc-Tic-ε-Lys(Z)-OH with aniline via WSC/HOBt as reported for Boc-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.52 g (88%); R_f (B) 0.81; HPLC K' 5.61; mp 94–96 °C; $[\alpha]_D^{20}$ −19.4; *m/z* 615 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.89 (m, 15H), 2.92–3.20 (m, 4H), 4.17–5.34 (m, 6H), 6.96–7.64 (m, 14H).

5.2.16. TFA·H-Tic-ε-Lys(Z)-NH-Ph. Boc-Tic-ε-Lys(Z)-NH-Ph was treated with TFA as reported for TFA·H-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.37 g (95%); R_f (A) 0.46; HPLC K' 4.32; mp 111–113 °C; $[\alpha]_D^{20}$ −19.9; *m/z* 515 $(M+H)^+$.

5.2.17. Boc-Dmt-Tic-ε-Lys(Z)-NH-Ph. This intermediate was obtained by condensation of Boc-Dmt-OH with TFA·H-Tic-ε-Lys(Z)-NH-Ph via WSC/HOBt as reported for Boc-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.17 g (87%); R_f (B) 0.76; HPLC K' 5.73; mp 124–126 °C; $[\alpha]_D^{20}$ −15.9; *m/z* 807 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.89 (m, 15H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 4.41–5.34 (m, 7H), 6.29 (s, 2H), 6.96–7.64 (m, 14H).

5.2.18. TFA·H-Dmt-Tic-ε-Lys(Z)-NH-Ph (4). Boc-Dmt-Tic-ε-Lys(Z)-NH-Ph was treated with TFA as reported for TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.09 g (90%); R_f (A) 0.40; HPLC K' 3.70; mp 133–135 °C; $[\alpha]_D^{20}$ −13.9; *m/z* 707 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.89 (m, 6H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 3.95–5.34 (m, 7H), 6.29 (s, 2H), 6.96–7.64 (m, 14H). Anal Calcd for $C_{43}H_{48}F_3N_5O_8$: C, 62.99; H, 5.90; N, 8.54. Found: C, 62.95; H, 5.76; N, 8.41.

5.2.19. Boc-Tic-ε-Lys(Ac)-NH-Ph. This intermediate was obtained by condensation of Boc-Tic-ε-Lys(Ac)-OH with aniline via WSC/HOBt as reported for Boc-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.32 g (81%); R_f (B) 0.74; HPLC K' 4.21; mp 100–102 °C; $[\alpha]_D^{20}$ −19.9; *m/z* 524 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.89 (m, 15H), 2.02 (s, 3H), 2.92–3.20 (m, 4H), 4.17–4.92 (m, 4H), 6.96–7.64 (m, 9H).

5.2.20. TFA·H-Tic-ε-Lys(Ac)-NH-Ph. Boc-Tic-ε-Lys(Ac)-NH-Ph was treated with TFA as reported for TFA·H-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.27 g (96%); R_f (A) 0.43; HPLC K' 3.47; mp 117–119 °C; $[\alpha]_D^{20}$ −20.8; *m/z* 424 $(M+H)^+$.

5.2.21. Boc-Dmt-Tic-ε-Lys(Ac)-NH-Ph. This intermediate was obtained by condensation of Boc-Dmt-OH with TFA·H-Tic-ε-Lys(Ac)-NH-Ph via WSC/HOBt as reported for Boc-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.15 g (88%); R_f (B) 0.71; HPLC K' 5.21; mp 130–132 °C; $[\alpha]_D^{20}$ −16.8; *m/z* 715 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.89 (m, 15H), 2.02 (s, 3H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 4.41–4.92 (m, 5H), 6.29 (s, 2H), 6.96–7.64 (m, 9H).

5.2.22. TFA·H-Dmt-Tic-ε-Lys(Ac)-NH-Ph (5). Boc-Dmt-Tic-ε-Lys(Ac)-NH-Ph was treated with TFA as reported for TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.08 g (98%); R_f (A) 0.37; HPLC K' 2.89; mp 127–129 °C; $[\alpha]_D^{20}$ −14.8; *m/z* 615 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.89 (m, 6H), 2.02 (s, 3H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 3.95–4.92 (m, 5H), 6.29 (s, 2H), 6.96–7.64 (m, 9H). Anal Calcd for $C_{37}H_{44}F_3N_5O_7$: C, 61.06; H, 6.09; N, 9.62. Found: C, 60.96; H, 6.03; N, 9.48.

5.2.23. Boc-Dmt-Tic-ε-Lys-NH-Ph. Boc-Dmt-Tic-ε-Lys(Z)-NH-Ph was dissolved in methanol and treated with Pd/C (10%) and H_2 as reported for Boc-Dmt-Tic-ε-Lys-NH-CH_2-Ph: yield 0.08 g (87%); R_f (B) 0.55; HPLC K' 5.12; mp 146–148 °C; $[\alpha]_D^{20}$ −19.1; *m/z* 673 $(M+H)^+$.

5.2.24. 2TFA·H-Dmt-Tic-ε-Lys-NH-Ph (6). Boc-Dmt-Tic-ε-Lys-NH-Ph was treated with TFA as reported for TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.04 g (95%); R_f (A) 0.37; HPLC K' 2.58; mp 147–149 °C; $[\alpha]_D^{20}$ −14.8; *m/z* 573 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.89 (m, 6H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 3.56–4.92 (m, 5H), 6.29 (s, 2H), 6.96–7.64 (m, 9H). Anal Calcd for $C_{37}H_{43}F_6N_5O_8$: C, 55.57; H, 5.42; N, 8.76. Found: C, 55.82; H, 5.53; N, 8.47.

5.2.25. Boc-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid. A solution of Boc-Tic-ε-Lys(Z)-OH (0.5 g, 0.93 mmol) and NMM (0.1 mL, 0.93 mmol) in DMF(10 mL) was treated at −20 °C with IBCF (0.12 mL, 0.93 mmol). After 10 min at −20 °C, *o*-phenylendiamine (0.1 g, 0.93 mmol) was added. The reaction mixture was allowed to stir while slowly warming to room temperature (1 h) and was then stirred for an additional 3 h. The solvent was evaporated and the residue was partitioned between EtOAc and H_2O. The EtOAc layer was washed with $NaHCO_3$ (5% in H_2O) and brine and dried over Na_2SO_4. The solution was filtered, the solvent evaporated, and the residual solid was dissolved in glacial acetic acid (10 mL). The solution was heated at 65 °C for 1 h. After the solvent was evaporated, the residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.47 g (82%); R_f (B) 0.66; HPLC K' 4.92; mp 134–136 °C; $[\alpha]_D^{20}$ −12.8; *m/z* 613 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.84 (m, 15H), 2.92–3.20 (m, 4H), 4.17–5.34 (m, 6H), 6.96–7.70 (m, 13H).

5.2.26. 2TFA·H-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid. Boc-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid was treated with TFA as reported for TFA·H-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.33 g (96%); R_f (A) 0.41; HPLC K' 3.57; mp 137–139 °C; $[\alpha]_D^{20}$ −14.1; *m/z* 513 $(M+H)^+$.

5.2.27. Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid. To a solution of Boc-Dmt-OH (0.075 g, 0.24 mmol) and 2TFA·H-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid (0.18 g, 0.24 mmol) in DMF (10 mL) at 0 °C, NMM (0.05 mL, 0.48 mmol), HOBt (0.04 g, 0.26 mmol) and WSC (0.05 g, 0.26 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 EtOAc 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 (85%); R_f (B) 0.66; HPLC K' 4.93; mp 137–139 °C; $[\alpha]_D^{20}$ −14.3; *m/z* 804 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.84 (m, 15 H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 4.41–5.34 (m, 7H), 6.29 (s, 2H), 6.96–7.70 (m, 13H).

5.2.28. 2TFA·H-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid (7). Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid was treated with TFA as reported for TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.05 g (94%); R_f (A) 0.33; HPLC K' 2.95; mp 143–145 °C; $[\alpha]_D^{20}$ −17.8; *m/z* 704 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.84 (m, 6H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 3.95–5.34 (m, 7H), 6.29 (s, 2H), 6.96–7.70 (m, 13H). Anal Calcd for $C_{45}H_{48}F_6N_6O_9$: C, 58.06; H, 5.20; N, 9.06. Found: C, 57.97; H, 5.16; N, 8.89.

5.2.29. Boc-Tic-NH-$(CH_2)_4$-CH(NH-Ac)-Bid. This intermediate was obtained by condensation of Boc-Tic-ε-Lys(Ac)-OH with *o*-phenylendiamine via mixed anhydrides (IBCF) as reported for Boc-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid: yield 0.34 g (88%); R_f (B) 0.60; HPLC K' 4.31; mp 140–142 °C; $[\alpha]_D^{20}$ −13.7; *m/z* 521 $(M+H)^+$; ^1H NMR (DMSO-d_6) δ 1.29–1.84 (m, 15H), 2.02 (s, 3H), 2.92–3.20 (m, 4H), 4.17–4.92 (m, 4H), 6.96–7.70 (m, 8H).

5.2.30. 2TFA·H-Tic-NH-$(CH_2)_4$-CH(NH-Ac)-Bid. Boc-Tic-NH-$(CH_2)_4$-CH(NH-Ac)-Bid was treated with TFA as reported for TFA·H-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.20 g (90%); R_f (A) 0.44; HPLC K' 3.21; mp 143–145 °C; $[\alpha]_D^{20}$ −15.0; *m/z* 421 $(M+H)^+$.

5.2.31. Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Ac)-Bid. This intermediate was obtained by condensation of Boc-Dmt-OH with 2TFA·H-Tic-NH-$(CH_2)_4$-CH(NH-Ac)-Bid via WSC/HOBt as reported for Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid: yield 0.14 g (85%); R_f (B) 0.60; HPLC K' 4.21; mp 132–134 °C; $[\alpha]_D^{20}$ −15.2; *m/z* 712 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.84 (m, 15H), 2.02 (s, 3H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 4.41–4.92 (m, 5H), 6.29 (s, 2H), 6.96–7.70 (m, 8H).

5.2.32. 2TFA·H-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Ac)-Bid (8). Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Ac)-Bid was treated with TFA as reported for TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.08 g (98%); R_f (A) 0.30; HPLC K' 2.63; mp 149–151 °C; $[\alpha]_D^{20}$ −18.7; *m/z* 612 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.84 (m, 6H), 2.02 (s, 3H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 3.95–4.92 (m, 5H), 6.29 (s, 2H), 6.96–7.70 (m, 8H). Anal Calcd for $C_{39}H_{44}F_6N_6O_8$: C, 55.84; H, 5.29; N, 10.02. Found: C, 56.01; H, 5.38; N, 10.12.

5.2.33. Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH_2)-Bid. Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH-Z)-Bid was dissolved in methanol and treated with Pd/C (10%) and H_2 as reported for Boc-Dmt-Tic-ε-Lys-NH-CH_2-Ph: yield 0.08 g (86%); R_f (B) 0.51; HPLC K' 3.56; mp 140–142 °C; $[\alpha]_D^{20}$ −19.3; *m/z* 670 $(M+H)^+$.

5.2.34. 3TFA·H-Dmt-Tic-NH-$(CH_2)_4$-CH(NH_2)-Bid (9). Boc-Dmt-Tic-NH-$(CH_2)_4$-CH(NH_2)-Bid was treated with TFA as reported for TFA·H-Dmt-Tic-ε-Lys(Z)-NH-CH_2-Ph: yield 0.06 g (95%); R_f (A) 0.29; HPLC K' 2.86; mp 154–156 °C; $[\alpha]_D^{20}$ −19.9; *m/z* 570 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.29–1.84 (m, 6H), 2.35 (s, 6H), 2.92–3.20 (m, 6H), 3.90–4.92 (m, 5H), 6.29 (s, 2H), 6.96–7.70 (m, 8H). Anal Calcd for $C_{39}H_{43}F_9N_6O_9$: C, 51.43; H, 4.76; N, 9.23. Found: C, 51.32; H, 4.62; N, 9.12.

5.3. Pharmacology

5.3.1. Radioreceptor binding assays. Opioid receptor affinity was determined under equilibrium conditions [2.5 h at room temperature (23 °C)] in a competition assay using brain P_2 synaptosomal membranes prepared from Sprague–Dawley rats.[26,27] Synaptosomes were pre-incubated to remove endogenous opioid peptides and stored at −80 °C in buffered 20% glycerol.[26,28] Each analogue was analyzed in duplicate assays using 5–8 dosages and 3–5 independent repetitions with different synaptosomal preparations (*n* values are listed in Table 1 in parentheses and results are means ± SE). Unlabelled 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, UK; 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 radiolabelled-synaptosome complex, and the filters were washed thrice in ice-cold buffered BSA.[26] The affinity constants (K_i) were calculated according to Cheng and Prusoff.[24]

5.3.2. Biological activity in isolated tissue preparations. The myenteric plexus longitudinal muscle preparations (2–3 cm segments) from the small intestine of male Hartley strain 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.[6,29] 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 means ± SE of five or six separate assays. δ-antagonist potencies in the MVD assay were determined against the δ-agonist deltorphin II and are expressed as pA_2 determined using the Schild Plot.[30]

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