The *cis*-4-Amino-L-proline Residue as a Scaffold for the Synthesis of Cyclic and Linear Endomorphin-2 Analogues: Part 2

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SUPPORTING INFORMATION

GENERAL PROCEDURES

General synthetic procedure for C-terminal amidation: to the mixture of tetrapeptide COOH terminal **12a-c** (1mmol) in THF at -10° C was added NMM (2.2 mmol) and isobutyl chloroformate (IBCF) (1.1 mmol) under stirring. After 15 min. NH_4OH s.s. was added in excess and the mixture was kept for 15 min at -10° C, then at room temperature for 3 h. The solvent was evaporated under reduced pressure and the oily residue was dissolved in EtOAc. The organic layer was washed with 5% citric acid, $NaHCO_3$ s.s. and brine. The organic layers were combined and dried under Na_2SO_4, filtered and evaporated under reduced pressure to give the crude products **15a-c**, that were purified with silica gel column chromatography.

General synthetic procedure of cyclization: a mixture of the tetrapeptides **5a-c** N^{α}-Boc protected, deprotected on N^{ε} of cAmp residue and on the C-terminal, in DMF (10^{-3} mol) and DIPEA (0.5 ml) was added dropwise to a solution of PyBop (1.5 equiv) in DMF and DIPEA (3 equiv) under stirring. The mixture was kept 12 h at room temperature, then DMF was evaporated under reduced pressure and the oily residue was dissolved in EtOAc. The organic layer was washed with 5% citric acid, $NaHCO_3$ s.s. and brine. The organic layers were combined and dried under Na_2SO_4, filtered and evaporated under reduced pressure to give the crude products **6a-b**, that were purified by silica gel column chromatography.

Catalitic hydrogenolysis: the products **4a-c** were dissolved in MeOH, Pd/C 10% was added and the mixture was kept under H_2 atmosphere for 2-6 h at room temperature stirring vigorously. Then catalyzer was removed by paper filtration and the solvent was evaporated under reduced pressure, the obtained crude products **5a-c** were used in the next step without further purification.

General coupling procedure: the *N*-protected amino acid (1mmol) or peptide (1 mmol) was dissolved in DMF at 0° C, and EDC (1.1 equiv), $HOBt{\cdot}H_2O$ (1.1.equiv) and NMM (2.2 equiv) were added. After 10 min TFA salt or HCl salt and a second portion of NMM (1.1 equiv) were added. The mixture was kept 12 h at room temperature, DMF was evaporated under reduced pressure, the oily residue was dissolved in EtOAc and the organic layer was washed with 5% citric acid, $NaHCO_3$ s.s. and brine. The organic layers were combined and dried under Na_2SO_4, filtered and evaporated under reduced pressure to give the crude product that was purified with silica gel column chromatography.

Saponification of Methyl esters: NaOH (1 N, 3 equiv) was added slowly to the mixture of methyl ester in MeOH at 0° C, then 3 h at room temperature under stirring. The organic solvent was evaporate, distilled water was added to the residue, and extracted with one portion of diethyl ether. Then the aqueous layer was acidified by 1 N HCl to pH = 3, the precipitated product was extracted by three portions of EtOAc. The organic layers were combined, dried under Na_2SO_4, filtered and evaporated under reduced pressure to give the free C-terminal acid, that was used into the next step without further purification.

Deprotection of Boc group: The Boc protected product was dissolved in a 1:1 TFA/DCM mixture for 3 h at r.t. under N_2 atmosphere. Then the mixture was evaporated under reduced pressure until complete elimination of TFA, to give the crude intermediate product that was used in the next step without further purification.

Deprotection of the Cbz group: The *N*-protected product was dissolved in HBr/acetic acid 33% for 3 h at room temperature, then the mixture was evaporated under reduced pressure. All HBr salts obtained were purified by HPLC C_{18} (H_2O/CH_3CN).

Synthesis and characterization of products 2-15

TFA·NH$_2$-Phe-Phe-OMe (2a-d). To a solution of *N*-Boc-L-Phe-OH or *N*-Boc-D-Phe-OH (1.1 equiv) in DMF, EDC (1.1 equiv), HOBt·H_2O (1.1 equiv) and one portion of NMM (2.2 equiv) were added at 0° C under stirring. After 10 min HCl·L-Phe-OMe or HCl·D-Phe-OMe (1 equiv) and a second portion of NMM (1.1 equiv) were added, the mixture was warmed to room temperature overnight, according to the coupling general procedure, to obtain the crude products **1a-c** (90-95%). **1a**: ^{1}H NMR ($CDCl_3$) δ: 1.39 (s, 9H, $C(CH_3)_3$), 2.99-3.07 (m, 4H, βCH_2 Phe), 3.67 (s, 3H, OCH_3), 4.28-4.38 (m, 1H, αCH Phe^1), 4-74-4.81 (m, 1H, αCH Phe^2), 4.93 (d, 1H, Boc-NH), 6.25 (d, 1H, NH Phe^2), 6.95-7.32 (m, 10H, Ar). ESI-HRMS for $C_{24}H_{31}N_2O_5$ [MH^+], calcd 427.2254; found, 427.2253. LRMS (ESI) *m/z* = 427.2. Anal. calcd for $C_{24}H_{30}N_2O_5$: C, 67.59; H, 7.09; N, 6.57; O, 18.76. Found: C, 67.57; H, 7.13; N, 6.55; O, 18.72. **1b**: ^{1}H NMR ($CDCl_3$) δ: 1.38 (s, 9H, $C(CH_3)_3$), 2.89-3.10 (m, 4H, βCH_2 Phe), 3.67 (s, 3H, OCH_3), 4.30-4.40 (m, 1H, αCH Phe^1), 4-80-4.86 (m, 1H, αCH Phe^2), 4.93 (d, 1H, Boc-NH), 6.35 (d, 1H, NH Phe^2), 6.91-7.33 (m, 10H, Ar). ESI-HRMS for $C_{24}H_{31}N_2O_5$ [MH^+], calcd 427.2254; found, 427.2255. LRMS (ESI) *m/z* = 427.2. Anal. calcd for $C_{24}H_{30}N_2O_5$: C, 67.59; H, 7.09; N, 6.57; O, 18.76. Found: C, 67.61; H, 7.06; N, 6.60; O, 18.79. **1c**: ^{1}H NMR ($CDCl_3$) δ: 1.38 (s, 9H, $C(CH_3)_3$), 2.89-3.10 (m, 4H, βCH_2 Phe), 3.67 (s, 3H, OCH_3), 4.30-4.40 (m, 1H, αCH Phe^1), 4-80-4.86 (m, 1H, αCH Phe^2), 4.91 (d, 1H, Boc-NH), 6.35 (d, 1H, NH Phe^2), 7.15-7.33 (m, 10H, Ar). ESI-HRMS for $C_{24}H_{31}N_2O_5$ [MH^+], calcd 427.2254; found, 427.2256. LRMS (ESI) *m/z* = 427.2. Anal. calcd for $C_{24}H_{30}N_2O_5$: C, 67.59; H, 7.09; N, 6.57; O, 18.76. Found: C, 67.63; H, 7.07; N, 6.62; O, 18.78. Compounds **1a-c** were deprotected in standard condition to give the pure products **2a** (84%), **2b** (81%), **2c** (79%). **2a**: ESI-HRMS for $C_{19}H_{23}N_2O_3$ (**2c**) [MH^+], calcd 327.1735; found, 327.1734. LRMS (ESI) for compound **2a** *m/z* = 327.2. Anal. calcd for $C_{19}H_{22}N_2O_3$: C, 69.92; H, 6.79; N, 8.58; O, 14.71. Found: C, 69.88; H, 6.84; N, 8.62; O, 14.67. **2b**: ESI-HRMS for $C_{19}H_{23}N_2O_3$ [MH^+], calcd 327.1735; found, 327.1737. LRMS (ESI) *m/z* = 327.2. Anal. calcd for $C_{19}H_{22}N_2O_3$: C, 69.92; H, 6.79; N, 8.58; O, 14.71. Found: C, 69.90; H, 6.75; N, 8.60; O, 14.66. **2c**: ESI-HRMS for $C_{19}H_{23}N_2O_3$ [MH^+], calcd 327.1735; found, 327.1736. LRMS (ESI) *m/z* = 327.2. Anal. calcd for $C_{19}H_{22}N_2O_3$: C, 69.92; H, 6.79; N, 8.58; O, 14.71. Found: C, 69.94; H, 6.76; N, 8.56; O, 14.75.

***N*-Boc-cAmp(Cbz)-Phe-Phe-OMe (3a-c).** *N*-Boc-cAmp(Cbz)-OH (1.2 equiv) was dissolved in DMF, and EDC (1.35 equiv), HOBt·H_2O (1.35 equiv) were added to the mixture. Then TFA·Phe-Phe-OMe (**2a-c**) (1.30 equiv) and NMM (0.56 mL) were added according to the coupling general procedure to give the crude products **3a** (86%), **3b** (88%), **3c** (77%). **3a**: ^{1}H NMR ($CDCl_3$) δ: 1.40 (s, 9H, $C(CH_3)_3$), 2.02-2.24 (m, 2H, Pro C^3H_2), 2.94-3.14 (m, 4H, βCH_2Phe), 3.42-3.50 (m, 2H, Pro C^5H_2), 3.63 (s, 3H, OCH_3), 4.15 (m, 1H, αCHPro), 4.24-4.34 (m, 1H, Pro C^4H), 4.57-4.65 (m, 1H, αCHPhe^2), 4.68-4.75 (m, 1H, αCH Phe^3), 5.00-5.14 (m, 2H, CH_2 Cbz), 6.74-7.36 (m, 18H, Ar and NH Cbz and NH Phe^{2,3}). ESI-HRMS for $C_{37}H_{45}N_4O_8$ [MH^+], calcd 673.3225; found, 673.3228. LRMS (ESI) *m/z* = 673.3. Anal. calcd for $C_{37}H_{44}N_4O_8$: C, 66.05; H, 6.59; N, 8.33; O, 19.03. Found: C, 66.08; H, 6.57; N, 8.28; O, 19.05. **3b**: ^{1}H NMR ($CDCl_3$) δ: 1.39 (s, 9H, $C(CH_3)_3$), 1.92-2.19 (m, 2H, Pro C^3H_2), 2.89-3.10 (m, 4H, βCH_2Phe), 3.42-3.50 (m, 2H, Pro C^5H_2), 3.58 (s, 3H, OCH_3), 4.18 (m, 1H, αCHPro), 4.28 (m, 1H, Pro C^4H), 4.61 (m, 1H, αCHPhe^2), 4.73 (m, 1H, αCH Phe^3), 5.00-5.14 (m, 2H, CH_2 Cbz), 6.68-7.36 (m, 18H, Ar and NH Cbz and NH Phe^{2,3}). ESI-HRMS for $C_{37}H_{45}N_4O_8$ [MH^+], calcd 673.3225; found, 673.3226. LRMS (ESI) *m/z* = 673.3. Anal. calcd for $C_{37}H_{44}N_4O_8$: C, 66.05; H, 6.59; N, 8.33; O, 19.03. Found: C, 66.10; H, 6.55; N, 8.30; O, 19.08. **3c**: ^{1}H NMR ($CDCl_3$) δ: 1.44 (s, 9H, $C(CH_3)_3$), 2.08-2.24 (m, 2H, Pro C^3H_2), 2.88-3.15 (m, 4H, βCH_2Phe), 3.27-3.50 (m, 2H, Pro C^5H_2), 3.66 (s, 3H, OCH_3), 4.22-4.32 (d, 1H, αCHPro), 4.58-4.66 (m, 1H, αCH Phe^3), 4.74-4.81 (m, 1H, αCHPhe^2), 5.04-5.15 (m, 2H, CH_2 Cbz), 6.28 (d, 1H, NH Cbz), 6.37 (d, 1H, NH Phe^2) 6.91-7.36 (m, 15H, Ar), 7.65 (d, 1H, NH Phe^3). ESI-HRMS for $C_{37}H_{45}N_4O_8$ [MH^+], calcd 673.3225; found, 673.3227. LRMS (ESI) *m/z* = 673.3. Anal. calcd for $C_{37}H_{44}N_4O_8$: C, 66.05; H, 6.59; N, 8.33; O, 19.03. Found: C, 66.03; H, 6.61; N, 8.35; O, 18.98.

TFA·H-cAmp(Cbz)-Phe-Phe-OMe (10a-c). 3a-c (0.27 equiv) were dissolved in 1:1 CH_2Cl_2/TFA mixture according to general procedure. **10a**: ESI-HRMS for $C_{32}H_{37}N_4O_6$ [MH^+], calcd 573.2783; found, 573.2781. LRMS (ESI) *m/z* = 573.3. Anal. calcd for $C_{32}H_{36}N_4O_6$: C, 67.12; H, 6.34; N, 9.78; O, 16.76. Found: C, 67.17; H, 6.36; N, 9.80; O, 16.81. **10b**: ESI-HRMS for $C_{32}H_{37}N_4O_6$ [MH^+], calcd 573.2783; found, 573.2785. LRMS (ESI) *m/z* = 573.3. Anal. calcd for $C_{32}H_{36}N_4O_6$: C, 67.12; H, 6.34; N, 9.78; O, 16.76. Found: C, 67.08; H, 6.30; N, 9.74; O, 16.80. **10c**: ESI-HRMS for $C_{32}H_{37}N_4O_6$ [MH^+], calcd 573.2786; found, 573.2781. LRMS (ESI) *m/z* = 573.3. Anal. calcd for $C_{32}H_{36}N_4O_6$: C, 67.12; H, 6.34; N, 9.78; O, 16.76. Found: C, 67.07; H, 6.39; N, 9.73; O, 16.71.

Cbz-Tyr-cAmp(Cbz)-Phe-Phe-OMe (11a-c). To a solution of Cbz-Tyr-OH (0.32 equiv) in DMF, EDC (0.32 equiv), HOBt·H_2O (0.32 equiv) and NMM (0.075 mL) were added at 0° C. Then TFA·H-cAmp(Cbz)-Phe-Phe in DMF and NMM were added to the reaction mixture according to general procedure to give **11a** (81 %), **11b** (80%), **11c** (89%). **11a**: NMR ($(CD_3)_2SO$) δ: 1.33-2.15 (m, 2H, Pro C^3H_2), 2.60-2.74 (m, 2H, βCH_2 Tyr^1), 2.66-3.09 (m, 2H, βCH_2 Phe^3), 3.17-3.94 (m, 2H, Pro C^5H_2), 3.08 (m, 2H, βCH_2 Phe^4), 3.53 (s, 3H, OCH_3), 4.06 (m, 1H, Pro C^4H), 4.26 (m, 1H, αCH Tyr^1), 4.29 (m, 1H, αCH Pro), 4.47 (m, 1H, αCH Phe^4), 4.54 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.64 (d, 2H, $C^{3,5}H$ Tyr^1), 7.04 (d, 2H, $C^{2,6}H$ Tyr^1), 7.39 (d, 1H, NH Pro), 7.55 (d, 1H, NH Tyr^1), 8.38 (d, 1H, NH Phe^4), 8.45 (d, 1H, NH Phe^3). ESI-HRMS for $C_{49}H_{52}N_5O_{10}$ [MH^+], calcd 870.3715; found, 870.3717. LRMS (ESI) *m/z* = 870.4. Anal. calcd for $C_{49}H_{51}N_5O_{10}$: C, 67.65; H, 5.91; N, 8.05; O, 18.39. Found: C, 67.70; H, 5.94; N, 8.03; O, 18.44. **11b**: ^1H NMR ($(CD_3)_2SO$) δ: 1.30-2.16 (m, 2H, Pro C^3H_2), 2.53-2.88 (m, 2H, βCH_2 Phe^3), 2.59-2.73 (m, 2H, βCH_2 Tyr^1), 2.89-3.04 (m, 2H, βCH_2 Phe^4), 3.22-3.94 (m, 2H, Pro C^5H_2), 3.60 (s, 3H, OCH_3), 4.06 (m, 1H, Pro C^4H), 4.27 (m, 1H, αCH Tyr^1), 4.30 (m, 1H, αCH Pro), 4.50 (m, 1H, αCH Phe^4), 4.53 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.62 (d, 2H, $C^{3,5}H$ Tyr^1), 7.00 (d, 2H, $C^{2,6}H$ Tyr^1), 7.37 (d, 1H, NH Pro), 7.42 (d, 1H, NH Tyr^1), 8.42 (d, 1H, NH Phe^4), 8.43 (d, 1H, NH Phe^3). ESI-HRMS for $C_{49}H_{52}N_5O_{10}$ [MH^+], calcd 870.3715; found, 870.3712. LRMS (ESI) *m/z* = 870.4. Anal. calcd for $C_{49}H_{51}N_5O_{10}$ (**11b**): C, 67.65; H, 5.91; N, 8.05; O, 18.39. Found: C, 67.62; H, 5.87; N, 8.09; O, 18.35. **11c**: ^1H NMR ($(CD_3)_2SO$) δ: 1.68-2.35 (m, 2H, Pro C^3H_2), 2.62-2.78 (m, 2H, βCH_2 Tyr^1), 2.69-2.81 (m, 2H, βCH_2 Phe^3), 2.84-3.02 (m, 2H, βCH_2 Phe^4), 3.30-4.00 (m, 2H, Pro C^5H_2), 3.58 (s, 3H, OCH_3), 4.09 (m, 1H, Pro C^4H), 4.31 (m, 1H, αCH Tyr^1), 4.37 (m, 1H, αCH Pro), 4.42 (m, 1H, αCH Phe^4), 4.50 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.65 (d, 2H, $C^{3,5}H$ Tyr^1), 7.06 (d, 2H, $C^{2,6}H$ Tyr^1), 7.51 (d, 1H, NH Pro), 7.57 (d, 1H, NH Tyr^1), 7.98 (d, 1H, NH Phe^3), 8.43 (d, 1H, NH Phe^4). ESI-HRMS for $C_{49}H_{52}N_5O_{10}$ [MH^+], calcd 870.3715; found, 870.3713. LRMS (ESI) *m/z* = 870.4. Anal. calcd for $C_{49}H_{51}N_5O_{10}$: C, 67.65; H, 5.91; N, 8.05; O, 18.39. Found: C, 67.60; H, 5.88; N, 8.08; O, 18.36.

Cbz-Tyr-cAmp(Cbz)-Phe-Phe-OH (12a-c). To a solution of Cbz-Tyr-cAmp(Cbz)-Phe-Phe-OMe (**11a-c**) (0.54 equiv) in MeOH, 1 N NaOH (3 equiv) was added according to the general procedure to give **12a-c**. **12a**: ^1H NMR ($(CD_3)_2SO$) δ: 1.30-2.15 (m, 2H, Pro C^3H_2), 2.59-2.74 (m, 2H, βCH_2 Tyr^1), 2.66-3.10 (m, 2H, βCH_2 Phe^3), 3.18-3.92 (m, 2H, Pro C^5H_2), 3.08 (m, 2H, βCH_2 Phe^4), 4.05 (m, 1H, Pro C^4H), 4.26 (m, 1H, αCH Tyr^1), 4.30 (m, 1H, αCH Pro), 4.42 (m, 1H, αCH Phe^4), 4.55 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.64 (d, 2H, $C^{3,5}H$ Tyr^1), 7.05 (d, 2H, $C^{2,6}H$ Tyr^1), 7.37 (d, 1H, NH Pro), 7.53 (d, 1H, NH Tyr^1), 8.26 (d, 1H, NH Phe^4), 8.40 (d, 1H, NH Phe^3). ESI-HRMS for $C_{48}H_{50}N_5O_{10}$ [MH^+], calcd 856.3624; found, 856.3625. LRMS (ESI) *m/z* = 856.4. Anal. calcd for $C_{48}H_{49}N_5O_{10}$: C, 67.36; H, 5.77; N, 8.18; O, 18.69. Found: C, 67.41; H, 5.72; N, 8.14; O, 18.66. **12b**: ^1H NMR ($(CD_3)_2SO$) δ: 1.27-2.15 (m, 2H, Pro C^3H_2), 2.49-2.86 (m, 2H, βCH_2 Phe^3), 2.58-2.72 (m, 2H, βCH_2 Tyr^1), 2.88-3.08 (m, 2H, βCH_2 Phe^4), 3.22-3.93 (m, 2H, Pro C^5H_2), 4.05 (m, 1H, Pro C^4H), 4.26 (m, 1H, αCH Tyr^1), 4.30 (m, 1H, αCH Pro), 4.47 (m, 1H, αCH Phe^4), 4.54 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.62 (d, 2H, $C^{3,5}H$ Tyr^1), 7.01 (d, 2H, $C^{2,6}H$ Tyr^1), 7.36 (d, 1H, NH Pro), 7.48 (d, 1H, NH Tyr^1), 8.31 (d, 1H, NH Phe^4), 8.35 (d, 1H, NH Phe^3). ESI-HRMS for $C_{48}H_{50}N_5O_{10}$ [MH^+], calcd 856.3624; found, 856.3622. LRMS (ESI) *m/z* = 856.4. Anal. calcd for $C_{48}H_{49}N_5O_{10}$: C, 67.36; H, 5.77; N, 8.18; O, 18.69. Found: C, 67.32; H, 5.73; N, 8.16; O, 18.73.

12c: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.67-2.34 (m, 2H, Pro C^3H_2), 2.62-2.78 (m, 2H, βCH_2 Tyr^1), 2.66-2.80 (m, 2H, βCH_2 Phe^3), 2.82-3.05 (m, 2H, βCH_2 Phe^4), 3.30-4.01 (m, 2H, Pro C^5H_2), 4.08 (m, 1H, Pro C^4H), 4.30 (m, 1H, αCH Tyr^1), 4.37 (m, 1H, αCH Pro), 4.39 (m, 1H, αCH Phe^4), 4.51 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.64 (d, 2H, $C^{3,5}$H Tyr^1), 7.06 (d, 2H, $C^{2,6}$H Tyr^1), 7.51 (d, 1H, NH Pro), 7.56 (d, 1H, NH Tyr^1), 7.94 (d, 1H, NH Phe^3), 8.31 (d, 1H, NH Phe^4)**.** ESI-HRMS for $C_{48}H_{50}N_5O_{10}$ [MH$^+$], calcd 856.3624; found, 856.3621. LRMS (ESI) *m/z* = 856.4. Anal. calcd for $C_{48}H_{49}N_5O_{10}$: C, 67.36; H, 5.77; N, 8.18; O, 18.69. Found: C, 67.39; H, 5.82; N, 8.22; O, 18.71.

Cbz-Tyr-cAmp(Cbz)-Phe-Phe-NH$_2$ (14a-c). Products **12a-c** were dissolved in THF at -10° C. IBCF (0.20 mL) and NMM (0.21 mL) were added. After 15 min at -10° C a solution of NH_4OH s.s. (0.27 mL) was added according to procedure to give products **14a-c**. **14a**: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.47-2.17 (m, 2H, Pro C^3H_2), 2.62-2.73 (m, 2H, βCH_2 Tyr^1), 2.66-2.99 (m, 2H, βCH_2 Phe^3), 3.04-3.12 (m, 2H, βCH_2 Phe^4), 3.24-4.01 (m, 2H, Pro C^5H_2), 4.12 (m, 1H, Pro C^4H), 4.28 (m, 1H, αCH Tyr^1), 4.32 (m, 1H, αCH Phe^3), 4.33 (m, 1H, αCH Pro), 4.37 (m, 1H, αCH Phe^4), 5.03 (m, 2H, CH_2 Cbz), 6.65 (d, 2H, $C^{3,5}$H Tyr^1), 7.08 (d, 2H, $C^{2,6}$H Tyr^1), 7.45 (d, 1H, NH Pro), 7.61 (d, 1H, NH Tyr^1), 8.00 (d, 1H, NH Phe^4), 8.68 (d, 1H, NH Phe^3). ESI-HRMS for $C_{48}H_{51}N_6O_9$ [MH$^+$], calcd 855.3744; found, 855.3742. LRMS (ESI) *m/z* = 855.4. Anal. calcd for $C_{48}H_{50}N_6O_9$: C, 67.43; H, 5.89; N, 9.83; O, 16.84. Found: C C, 67.46; H, 5.94; N, 9.85; O, 16.80. **14b**: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.30-2.17 (m, 2H, Pro C^3H_2), 2.50-2.82 (m, 2H, βCH_2 Phe^3), 2.56-2.72 (m, 2H, βCH_2 Tyr^1), 2.82-3.07 (m, 2H, βCH_2 Phe^4), 3.23-3.94 (m, 2H, Pro C^5H_2), 4.07 (m, 1H, Pro C^4H), 4.26 (m, 1H, αCH Tyr^1), 4.26 (m, 1H, αCH Pro), 4.45 (m, 1H, αCH Phe^4), 4.50 (m, 1H, αCH Phe^3), 5.03 (m, 2H, CH_2 Cbz), 6.63 (d, 2H, $C^{3,5}$H Tyr^1), 7.02 (d, 2H, $C^{2,6}$H Tyr^1), 7.39 (d, 1H, NH Pro), 7.54 (d, 1H, NH Tyr^1), 8.20 (d, 1H, NH Phe^4), 8.33 (d, 1H, NH Phe^3)**.** ESI-HRMS for $C_{48}H_{51}N_6O_9$ [MH$^+$], calcd 855.3744; found, 855.3746. LRMS (ESI) *m/z* = 855.4. Anal. calcd for $C_{48}H_{50}N_6O_9$: C, 67.43; H, 5.89; N, 9.83; O, 16.84. Found: C, 67.39; H, 5.85; N, 9.79; O, 16.89. **14c**: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.69-2.28 (m, 2H, Pro C^3H_2), 2.63-2.77 (m, 2H, βCH_2 Tyr^1), 2.76-2.90 (m, 2H, βCH_2 Phe^3), 2.86-3.05 (m, 2H, βCH_2 Phe^4), 3.29-4.04 (m, 2H, Pro C^5H_2), 4.11 (m, 1H, Pro C^4H), 4.30 (m, 1H, αCH Phe^3), 4.30 (m, 1H, αCH Pro), 4.30 (m, 1H, αCH Tyr^1), 4.34 (m, 1H, αCH Phe^4), 5.03 (m, 2H, CH_2 Cbz), 6.65 (d, 2H, $C^{3,5}$H Tyr^1), 7.07 (d, 2H, $C^{2,6}$H Tyr^1), 7.53 (d, 1H, NH Pro), 7.62 (d, 1H, NH Tyr^1), 8.11 (d, 1H, NH Phe^4), 8.22 (d, 1H, NH Phe^3)**.** ESI-HRMS for $C_{48}H_{51}N_6O_9$ [MH$^+$], calcd 855.3744; found, 855.3747. LRMS (ESI) *m/z* = 855.4. Anal. calcd for $C_{48}H_{50}N_6O_9$: C, 67.43; H, 5.89; N, 9.83; O, 16.84. Found: C, 67.40; H, 5.87; N, 9.78; O, 16.86.

***N*$^{\alpha}$-Boc-cAmp(Cbz)-Phe-Phe-OH (4a-c).** To a solution of *N*$^{\alpha}$-Boc-cAmp(Cbz)-Phe-Phe-OMe (**3a-c**) (0.54 eq.) in MeOH, 1N NaOH (3 eq.) was added according to the general procedure to give **4a-c**. **4a**: ESI-HRMS for $C_{36}H_{43}N_4O_8$ [MH$^+$], calcd 659.3127; found, 659.3129. LRMS (ESI) *m/z* = 659.3. Anal. calcd for $C_{36}H_{42}N_4O_8$: C, 65.64; H, 6.43; N, 8.51; O, 19.43. Found: C, 65.63; H, 6.40; N, 8.55; O, 19.40. **4b**: ESI-HRMS for $C_{36}H_{43}N_4O_8$ [MH$^+$], calcd 659.3127; found, 659.3125. LRMS (ESI) *m/z* = 659.3. Anal. calcd for $C_{36}H_{42}N_4O_8$: C, 65.64; H, 6.43; N, 8.51; O, 19.43. Found: C, 65.69; H, 6.39; N, 8.48; O, 19.45. **4c**: ESI-HRMS for $C_{36}H_{43}N_4O_8$ [MH$^+$], calcd 659.3127; found, 659.3129. LRMS (ESI) *m/z* = 659.3. Anal. calcd for $C_{36}H_{42}N_4O_8$: C, 65.64; H, 6.43; N, 8.51; O, 19.43. Found: C, 65.60; H, 6.47; N, 8.46; O, 19.48.

***N*$^{\alpha}$-Boc-cAmp-Phe-Phe-OH (5a-c).** To a solution of *N*$^{\alpha}$-Boc-cAmp(Cbz)-Phe-Phe-OH (**4a-c**) (0.54 equiv) in CH_3OH, Pd/C 10% (36 mg) was added, according to general procedure to give crude **5a** (73%), **5b** (79%), **5c** (81 %). **5a**: ESI-HRMS for $C_{28}H_{37}N_4O_6$ [MH$^+$], calcd 525.2789; found, 525.2792. LRMS (ESI) *m/z* = 535.3. Anal. calcd for $C_{28}H_{36}N_4O_6$: C, 64.10; H, 6.92; N, 10.68; O, 18.30. Found: C, 64.06; H, 6.97; N, 10.71; O, 18.33. **5b**: ESI-HRMS for $C_{28}H_{37}N_4O_6$ [MH$^+$], calcd 525.2789; found, 525.2787. LRMS (ESI) *m/z* = 535.3. Anal. calcd for $C_{28}H_{36}N_4O_6$: C, 64.10; H, 6.92; N, 10.68; O, 18.30. Found: C, 64.13; H, 6.87; N, 10.73; O, 18.27. **5c**: ESI-HRMS for $C_{28}H_{37}N_4O_6$ [MH$^+$], calcd 525.2786; found, 525.2792. LRMS (ESI) *m/z* = 535.3. Anal. calcd for $C_{28}H_{36}N_4O_6$: C, 64.10; H, 6.92; N, 10.68; O, 18.30. Found: C, 64.12; H, 6.89; N, 10.63; O, 18.25.

N^{α}-Boc-*c*[4-NH-Pro-Phe-Phe] (6a-b). To a solution of N^{α}-Boc-cAmp-Phe-Phe-OH (**5a-b**) (0.47 equiv) in DMF (1000 mL) and DIEA (0.46 mL), a mixture of PyBop (1.50 equiv) and DIPEA (0.45 mL) in DMF was added slowly, according to general cyclization procedure to give **6a** (60%), **6b** (58%). **6c** was not isolated from the mixture. **6a**: ^{1}H NMR ($CDCl_3$) δ: 1.28 (s, 9H, $C(CH_3)_3$), 2.06-2.16 (m, 1H, Pro C^3 H_a), 2.82 (m, 1H, βCHPhe), 2.91-3.00 (m, 2H, βCHPhe and Pro C^3H_b), 3.20-3.29 (m, 3H, Pro C^5H_a and βCH_2Phe), 3.56 (dd, 1H, Pro C^5H_b), 4.13-4.19 (m, 1H, Pro C^4H), 4.30-4.38 (m, 1H, αCHPro), 4.68-4.85 (m, 2H, αCHPhe), 5.45 (d, 1H, γNHPro), 6.16 (d, 1H, NH Phe^2), 6.32 (d, 1H, NH Phe^3), 7.16-7.29 (m, 10H, Ar). ESI-HRMS for $C_{28}H_{35}N_4O_5$ [MH$^+$], calcd 507.2637; found, 507.2640. LRMS (ESI) *m/z* = 507.3. Anal. calcd for $C_{28}H_{34}N_4O_5$: C, 66.38; H, 6.76; N, 11.06; O, 15.79. Found: C, 66.40; H, 6.70; N, 11.03; O, 15.77. **6b**: ^{1}H NMR ($CDCl_3$) δ: 1.32 (s, 9H, $C(CH_3)_3$), 2.09-2.17 (m, 1H, Pro C^3 H_a), 2.77-2.97 (m, 3H, Pro C^3H_b and βCH_2Phe), 3.16-3.26 (m, 3H, Pro C^5Ha and βCH_2Phe), 3.48 (dd, 1H, Pro C^5H_b), 3.56 (dd, 1H, Pro C^5H_b), 4.08-4.15 (m, 1H, Pro C^4H), 4.22-4.26 and 4.33-4.36 (2m, 1H, αCHPro), 4.64-4.82 (m, 2H, αCHPhe), 5.76 (br, 1H, γNHPro), 6.51 (d, 2H, NH Phe), 7.13-7.26 (m, 10H, Ar). ESI-HRMS for $C_{28}H_{35}N_4O_5$ [MH$^+$], calcd 507.2637; found, 507.2634. LRMS (ESI) *m/z* = 507.3. Anal. calcd for $C_{28}H_{34}N_4O_5$: C, 66.38; H, 6.76; N, 11.06; O, 15.79. Found: C, 66.35; H, 6.72; N, 11.10; O, 15.81.

TFA·*c*[4-NH-Pro-Phe-Phe] (7a-b). 6a-b (0.27 equiv) was dissolved in 1:1 CH_2Cl_2/TFA mixture according to general procedure. **7a**: ESI-HRMS for $C_{23}H_{27}N_4O_3$ [MH$^+$], calcd 407.2158; found, 407.2161. LRMS (ESI) *m/z* = 407.2. Anal. calcd for $C_{23}H_{26}N_4O_3$: C, 67.96; H, 6.45; N, 13.78; O, 11.81. Found: C, 67.91; H, 6.50; N, 13.73; O, 11.77. **7b**: ESI-HRMS for $C_{23}H_{27}N_4O_3$ [MH$^+$], calcd 407.2158; found, 407.2156. LRMS (ESI) *m/z* = 407.2. Anal. calcd for $C_{23}H_{26}N_4O_3$ (**7b**): C, 67.96; H, 6.45; N, 13.78; O, 11.81. Found: C, 67.93; H, 6.40; N, 13.82; O, 11.85.

Boc-Tyr-*c*[4-NH-Pro-Phe-Phe] (8a-b). To a solution of Boc-Tyr-OH (0.32 equiv) in DMF, EDC (0.32 equiv), HOBt·H_2O (0.32 equiv) and NMM (0.075 mL) were added at 0° C. Then TFA·[4-NH-Pro-Phe-Phe] in DMF and NMM were added to the reaction mixture according to general procedure A to give **8a** (81 %), **8b** (80%). **8a**: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.29 (s, 9H, $C(CH_3)_3$), 1.53-2.55 (m, 2H, Pro C^3H_2), 2.70-2.91 (m, 2H, βCH_2 Phe^4), 2.74 (m, 2H, βCH_2 Tyr^1), 2.82-3.20 (m, 2H, βCH_2 Phe^3), 3.36-3.47 (m, 2H, Pro C^5H_2), 3.65 (m, 1H, αCH Pro), 3.80 (m, 1H, Pro C^4H), 4.02 (m, 1H, αCH Tyr^1), 4.39 (m, 1H, αCH Phe^4), 4.62 (m, 1H, αCH Phe^3), 6.50 (d, 1H, NH Pro), 6.68 (d, 2H, $C^{3,5}$H Tyr^1), 6.98 (d, 2H, $C^{2,6}$H Tyr^1), 7.36 (d, 1H, NH Tyr^1), 7.47 (d, 1H, NH Phe^4), 7.98 (d, 1H, NH Phe^3). ESI-HRMS for $C_{37}H_{44}N_5O_7$ [MH$^+$], calcd 670.3228; found, 670.3230. LRMS (ESI) *m/z* = 670.3. Anal. calcd for $C_{37}H_{43}N_5O_7$: C, 66.35; H, 6.47; N, 10.46; O, 16.72. Found: C, 66.30; H, 6.42; N, 10.44; O, 16.77. **8b**: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.29 (s, 9H, $C(CH_3)_3$), 1.54-2.59 (m, 2H, Pro C^3H_2), 2.71-2.93 (m, 2H, βCH_2 Phe^4), 2.75 (m, 2H, βCH_2 Tyr^1), 2.84-3.23 (m, 2H, βCH_2 Phe^3), 3.36-3.51 (m, 2H, Pro C^5H_2), 3.70 (m, 1H, αCH Pro), 3.80 (m, 1H, Pro C^4H), 4.04 (m, 1H, αCH Tyr^1), 4.39 (m, 1H, αCH Phe^4), 4.64 (m, 1H, αCH Phe^3), 6.53 (d, 1H, NH Pro), 6.69 (d, 2H, $C^{3,5}$H Tyr^1), 6.99 (d, 2H, $C^{2,6}$H Tyr^1), 7.40 (d, 1H, NH Tyr^1), 7.47 (d, 1H, NH Phe^4), 7.97 (d, 1H, NH Phe^3). ESI-HRMS for $C_{37}H_{44}N_5O_7$ [MH$^+$], calcd 670.3228; found, 670.3227. LRMS (ESI) *m/z* = 670.3. Anal. calcd for $C_{37}H_{43}N_5O_7$: C, 66.35; H, 6.47; N, 10.46; O, 16.72. Found: C, 66.32; H, 6.45; N, 10.49; O, 16.68.

TFA·Tyr-*c*[4-NH-Pro-Phe-Phe] (9a-b). 8a-b (0.22 equiv), was dissolved in 1:1 CH_2Cl_2/TFA mixture according to general procedure to give **9a** (76%), **9b** (93%). **9a**: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.39-2.38 (m, 2H, Pro C^3H_2), 2.76-2.93 (m, 2H, βCH_2 Phe^4), 2.84-3.09 (m, 2H, βCH_2 Phe^3), 2.89-2.97 (m, 2H, βCH_2 Tyr^1), 3.42-3.50 (m, 2H, Pro C^5H_2), 3.50 (m, 1H, αCH Pro), 3.86 (m, 1H, αCH Tyr^1), 3.89 (m, 1H, Pro C^4H), 4.41 (m, 1H, αCH Phe^4), 4.62 (m, 1H, αCH Phe^3), 6.15 (d, 1H, NH Pro), 6.74 (d, 2H, $C^{3,5}$H Tyr^1), 6.98 (d, 2H, $C^{2,6}$H Tyr^1), 7.65 (d, 1H, NH Phe^3), 7.94 (d, 1H, NH Phe^4), 8.36 (br, 3H, NH_3^+ Tyr^1). ESI-HRMS for $C_{32}H_{36}N_5O_5$ [MH$^+$], calcd 570.2712; found, 570.2714. LRMS (ESI) *m/z* = 570.3. Anal. calcd for $C_{32}H_{35}N_5O_5$: C, 67.47; H, 6.19; N, 12.29; O, 14.04. Found: C, 67.43; H, 6.24; N, 12.34; O, 14.07. **9b**: ^{1}H NMR ($(CD_3)_2SO$) δ: 1.39-2.38 (m, 2H, Pro C^3H_2), 2.76-2.93 (m, 2H, βCH_2 Phe^4), 2.84-3.09 (m, 2H, βCH_2 Phe^3), 2.89-2.97 (m, 2H, βCH_2

Tyr^1), 3.42-3.50 (m, 2H, Pro C^5H_2), 3.50 (m, 1H, αCH Pro), 3.86 (m, 1H, αCH Tyr^1), 3.89 (m, 1H, Pro C^4H), 4.41 (m, 1H, αCH Phe^4), 4.62 (m, 1H, αCH Phe^3), 6.15 (d, 1H, NH Pro), 6.74 (d, 2H, $C^{3,5}H$ Tyr^1), 6.98 (d, 2H, $C^{2,6}H$ Tyr^1), 7.65 (d, 1H, NH Phe^3), 7.94 (d, 1H, NH Phe^4), 8.36 (br, 3H, NH_3^+ Tyr^1). ESI-HRMS for $C_{32}H_{36}N_5O_5$ [MH^+], calcd 570.2712; found, 570.2715. LRMS (ESI) *m/z* = 570.3. Anal. calcd for $C_{32}H_{35}N_5O_5$: C, 67.47; H, 6.19; N, 12.29; O, 14.04. Found: C, 67.45; H, 6.15; N, 12.32; O, 14.09.

2HBr·Tyr-cAmp-Phe-Phe-OH (13a-c). 12a-c was dissolved in HBr/AcOH 33% at r.t. for 3 h according to procedure to give crude products **13a-c**. **13a**: ^1H NMR ($(CD_3)_2SO$) δ: 1.39-2.33 (m, 2H, Pro C^3H_2), 2.74-3.11 (m, 2H, βCH_2 Phe^3), 2.90-3.01 (m, 2H, βCH_2 Tyr^1), 3.01-3.10 (m, 2H, βCH_2 Phe^4), 3.26-3.89 (m, 2H, Pro C^5H_2), 3.77 (m, 1H, Pro C^4H), 4.36 (m, 1H, αCH Tyr^1), 4.46 (m, 1H, αCH Phe^4), 4.52 (m, 1H, αCH Pro), 4.62 (m, 1H, αCH Phe^3), 6.72 (d, 2H, $C^{3,5}H$ Tyr^1), 7.06 (d, 2H, $C^{2,6}H$ Tyr^1), 7.92 (d, 1H, NH Pro), 8.15 (br, 3H, NH_3^+ Tyr^1), 8.41 (d, 1H, NH Phe^4), 8.79 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{38}N_5O_6$ [MH^+], calcd 588.2875; found, 588.2874. LRMS (ESI) *m/z* = 588.3. Anal. calcd for $C_{32}H_{37}N_5O_6$: C, 65.40; H, 6.35; N, 11.92; O, 16.34. Found: C, 65.45; H, 6.31; N, 11.95; O, 16.39. **13b**: ^1H NMR ($(CD_3)_2SO$) δ: 1.42-2.34 (m, 2H, Pro C^3H_2), 2.49-2.75 (m, 2H, βCH_2 Phe^3), 2.86-3.13 (m, 2H, βCH_2 Phe^4), 2.89-3.03 (m, 2H, βCH_2 Tyr^1), 3.32-3.92 (m, 2H, Pro C^5H_2), 3.78 (m, 1H, Pro C^4H), 4.38 (m, 1H, αCH Tyr^1), 4.53 (m, 1H, αCH Pro), 4.53 (m, 1H, αCH Phe^4), 4.62 (m, 1H, αCH Phe^3), 6.72 (d, 2H, $C^{3,5}H$ Tyr^1), 7.08 (d, 2H, $C^{2,6}H$ Tyr^1), 7.95 (d, 1H, NH Pro), 8.15 (br, 3H, NH_3^+ Tyr^1), 8.54 (d, 1H, NH Phe^4), 8.64 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{38}N_5O_6$ [MH^+], calcd 588.2875; found, 588.2877. LRMS (ESI) *m/z* = 588.3. Anal. calcd for $C_{32}H_{37}N_5O_6$: C, 65.40; H, 6.35; N, 11.92; O, 16.34. Found: C, 65.43; H, 6.38; N, 11.88; O, 16.29. **13c**: ^1H NMR ($(CD_3)_2SO$) δ: 1.85-2.49 (m, 2H, Pro C^3H_2), 2.63-2.73 (m, 2H, βCH_2 Phe^3), 2.82-3.09 (m, 2H, βCH_2 Phe^4), 2.96-3.02 (m, 2H, βCH_2 Tyr^1), 3.35-3.96 (m, 2H, Pro C^5H_2), 3.85 (m, 1H, Pro C^4H), 4.39 (m, 1H, αCH Tyr^1), 4.44 (m, 1H, αCH Phe^4), 4.60 (m, 1H, αCH Phe^3), 4.64 (m, 1H, αCH Pro), 6.69 (d, 2H, $C^{3,5}H$ Tyr^1), 7.06 (d, 2H, $C^{2,6}H$ Tyr^1), 7.96 (d, 1H, NH Pro), 8.17 (br, 3H, NH_3^+ Tyr^1), 8.56 (d, 1H, NH Phe^4), 8.59 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{38}N_5O_6$ [MH^+], calcd 588.2875; found, 588.2878. LRMS (ESI) *m/z* = 588.3. Anal. calcd for $C_{32}H_{37}N_5O_6$: C, 65.40; H, 6.35; N, 11.92; O, 16.34. Found: C, 66.36; H, 6.39; N, 11.87; O, 16.37.

2HBr·Tyr-cAmp-Phe-Phe-NH$_2$ (15a-c). 14a-c was dissolved in HBr/AcOH 33% at r.t. for 3 h according to the general procedure to give crude products **15a-c**. **15a**: ^1H NMR ($(CD_3)_2SO$) δ: 1.46-2.37 (m, 2H, Pro C^3H_2), 2.71-2.97 (m, 2H, βCH_2 Tyr^1), 2.75-3.08 (m, 2H, βCH_2 Phe^3), 2.90-3.05 (m, 2H, βCH_2 Phe^4), 3.40-3.94 (m, 2H, Pro C^5H_2), 3.79 (m, 1H, Pro C^4H), 4.27 (m, 1H, αCH Tyr^1), 4.47 (m, 1H, αCH Phe^4), 4.52 (m, 1H, αCH Pro), 4.55 (m, 1H, αCH Phe^3), 6.73 (d, 2H, $C^{3,5}H$ Tyr^1), 7.09 (d, 2H, $C^{2,6}H$ Tyr^1), 8.02 (br, 3H, NH_3^+ Tyr^1), 8.02 (d, 1H, NH Pro), 8.19 (d, 1H, NH Phe^4), 8.75 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{39}N_6O_5$ [MH^+], calcd 587.3047; found, 587.3049. LRMS (ESI) *m/z* = 587.3. Anal. calcd for $C_{32}H_{38}N_6O_5$: C, 65.51; H, 6.53; N, 14.32; O, 13.64. Found: C, 65.50; H, 6.55; N, 14.30; O, 13.62. **15b**: ^1H NMR ($(CD_3)_2SO$) δ: 1.42-2.34 (m, 2H, Pro C^3H_2), 2.45-2.71 (m, 2H, βCH_2 Phe^3), 2.78-3.05 (m, 2H, βCH_2 Phe^4), 2.87-3.04 (m, 2H, βCH_2 Tyr^1), 3.36-3.91 (m, 2H, Pro C^5H_2), 3.78 (m, 1H, Pro C^4H), 4.39 (m, 1H, αCH Tyr^1), 4.51 (m, 1H, αCH Pro), 4.54 (m, 1H, αCH Phe^4), 4.61 (m, 1H, αCH Phe^3), 6.72 (d, 2H, $C^{3,5}H$ Tyr^1), 7.07 (d, 2H, $C^{2,6}H$ Tyr^1), 7.95 (d, 1H, NH Pro), 8.13 (br, 3H, NH_3^+ Tyr^1), 8.44 (d, 1H, NH Phe^4), 8.61 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{39}N_6O_5$ [MH^+], calcd 587.3047; found, 587.3050. LRMS (ESI) *m/z* = 587.3. Anal. calcd for $C_{32}H_{38}N_6O_5$: C, 65.51; H, 6.53; N, 14.32; O, 13.64. Found: C, 65.47; H, 6.50; N, 14.35; O, 13.60. **15c**: ^1H NMR ($(CD_3)_2SO$) δ: 1.87-2.50 (m, 2H, Pro C^3H_2), 2.62-2.72 (m, 2H, βCH_2 Phe^3), 2.75-3.03 (m, 2H, βCH_2 Phe^4), 2.80-2.97 (m, 2H, βCH_2 Tyr^1), 3.47-3.97 (m, 2H, Pro C^5H_2), 3.85 (m, 1H, Pro C^4H), 4.28 (m, 1H, αCH Tyr^1), 4.44 (m, 1H, αCH Phe^4), 4.59 (m, 1H, αCH Phe^3), 4.62 (m, 1H, αCH Pro), 6.70 (d, 2H, $C^{3,5}H$ Tyr^1), 7.06 (d, 2H, $C^{2,6}H$ Tyr^1), 8.04 (d, 1H, NH Pro), 8.05 (br, 3H, NH_3^+ Tyr^1), 8.41 (d, 1H, NH Phe^4), 8.51 (d, 1H, NH Phe^3). ESI-HRMS for $C_{32}H_{39}N_6O_5$ [MH^+], calcd 587.3047; found, 587.3047. LRMS (ESI) *m/z* = 587.3. Anal. calcd for $C_{32}H_{38}N_6O_5$: C, 65.51; H, 6.53; N, 14.32; O, 13.64. Found: C, 65.53; H, 6.48; N, 14.37; O, 13.59.

BIOLOGICAL ASSAYS

Functional Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays. In vitro biological assays were performed on **9a-b** as TFA salts and **13a-c**, **15a-c** as hydrobromide salts. Electrically induced smooth muscle contractions of mouse vas deferens and guinea pig ileum longitudinal muscle-myenteric plexus were used as bioassays. Tissue came from male ICR mice weighing 25-30 g and from male Hartley guinea pigs weighing 300-500 g. The tissues were tied to gold chains with suture silk, suspended in 20 mL baths containing 37°C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium-free for MVD), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length previously determined to be 1 g tension (0.5 g for MVD) and allowed to equilibrate for 15 min, then stimulated transmurally between platinum plate electrodes at 0.1 Hz for 0.4 ms pulses (2.0 ms pulses for MVD) and supramaximal voltage. Drugs were added to the baths in 20-60 μL volumes. The agonists remained in contact with the tissue for 3 min before addition of the next split log dose to construct cumulative dose-response curves. IC_{50} values represent the mean of not less than four tissues. IC_{50} estimates and relative potency estimates were determined by fitting the mean data to the Hill equation by using a computerized nonlinear least-squares method. All biological data are summarized in Table 1.

Radioligand Labeled Binding Assays

μ and δ Opioid Receptors. Crude membranes were prepared from transfected cells that express the MOR or the DOR. The protein concentration of the membrane preparations was determined by the Lowry method and the membranes were stored at -80 °C until use. Membranes were resuspended in assay buffer [50 mM Tris, pH 7.4, containing 50 μg/mL bacitracin, 30 μM bestatin, 10 μM captopril, 100 μM PMSF, 1 mg/mL BSA]. For saturation analysis, six concentrations of [^{3}H]DAMGO (0.02-6 nM, 47.2 Ci/mmol) or six concentrations of [^{3}H]DPDPE (0.1-10 nM, 44 Ci/mmol) were each mixed with 200 μg of membranes from MOR or DOR expressing cells, respectively, in a final volume of 1 mL. For competition analysis, 10 concentrations of a test compound were each incubated with 50 μg of membranes from MOR- or DOR-expressing cells and the K_d concentration of [^{3}H]DAMGO (1.0 nM, 50 Ci/mmol) or of [^{3}H]DPDPE (1.0 nM, 44 Ci/mmol), respectively. Naloxone at 10 μM was used to define the nonspecific binding of the radioligands in all assays. All samples were carried out in duplicate. The samples were incubated in a shaking water bath at 25 °C for 3 h, followed by a rapid filtration through Whatman grade GF/B filter paper (Gaithersburg, MD) presoaked in 1% polyethyleneimine and washing four times each with 2 mL of cold saline, and the radioactivity was determined by liquid scintillation counting (Beckman LS5000 TD).

κ opioid receptors. κ opioid receptor (KOR) binding affinities were carried out by CEREP - Rue du Bois l'Eveque - BP 30001 - 86600 Celle l'Evescault (FRANCE) following a slightly modified procedure previously reported by Meng et al. by using Chinese hamster ovary (CHO) cell lines that stably express human KOP (see ref. 27). The K_d values of [^{3}H]U69593 binding to KOP were 2.0 ± 0.5 nM ($n = 3$). Expressing cells were harvested after 65 h in culture, homogenized in 50 mM Tris buffer (pH 7.4) that contained 10 mM $MgCl_2$ and 1 mM EDTA, pelleted by centrifugation for 20 min at 30000 × g, and resuspended in the same buffer. For the saturation binding assays, cell membrane suspensions were incubated for 60 min at 25°C with various concentrations [^{3}H]U69593. Nonspecific binding was determined in the presence of 10 μM of naloxone. For the competitive binding assays, the cell membrane suspensions were incubated for 60 min at RT with 3 nM [^{3}H]U69593 in the presence of various concentrations of ligands. After incubation for 60 min, the membrane suspensions were rapidly filtrated and the radioactivity of each filter was then measured by liquid scintillation counting. The binding assay results are the mean ± SEM of four independent experiments, each performed in duplicate.