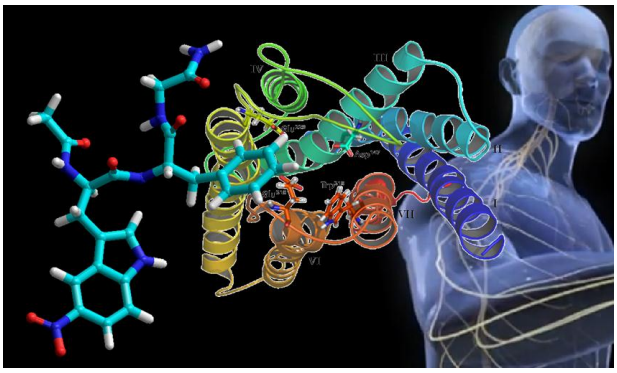
SUPPORTING INFORMATION

Synthesis of tripeptides containing D-Trp substituted at the indole ring, assessment of opioid receptor binding and *in vivo* central antinociception.

Rossella De Marco,[a,§] Andrea Bedini,[b,§] Santi Spampinato,[b]* and Luca Gentilucci.[a,]*

[a] *Dept of Chemistry "G. Ciamician", University of Bologna, via Selmi 2, 40126 Bologna Italy.* [b] *Dept. of Pharmacy and BioTechnology, University of Bologna, via Irnerio 48, 40126 Bologna, Italy.* [§] *Contributed equally to this work*

KEYWORDS. Endomorphin; opioid peptide; μ-opioid receptor; peptidomimetic; substituted D-Trp; displacement binding assay; antinociception; tail flick test



Contents

*Preparation of Ac-D/L-Tryptophans (**4**).*

L-Serine (1.06 g, 10.3 mmol) and the substituted indole (5.13 mmol) were dissolved in AcOH (12.0 mL) and Ac_2O (4.0 mL). The mixture was stirred for 30 min under MW irradiation using a MicroSYNTH microwave labstation equipped with a built-in ATC-FO advanced fiber optic automatic temperature control, with a initial power of 150W and monitoring the internal reaction temperature at 80°C. Then pH was adjusted to 11 with 30% NaOH (40 mL) and the mixture was extracted with Et_2O (100 mL). The water layer was adjusted to pH 3 with 1N HCl and extracted with AcOEt (3x20 mL). The collected organic layers were dried over Na_2SO_4 and concentrated at reduced pressure. The Ac-tryptophans D/L-**4** were dissolved in MeOH (5 mL) and precipitated from ice-cold Et_2O (35 mL) and collected by filtration (80-90%, 70-80% pure by analytical RP-HPLC). The crude D/L-**4** were identified by ESI MS, and were utilized without further purifications.

*Preparation of D-**4**.*

Porcine Kidney Acylase, Grade II, salt-free, lyophilized powder, 500-1500 units/mg protein (40 mg), and $CoCl_2(6H_2O)$ (0.20 mg), suspended in phosphate buffer pH 7.4 (5 mL), were added to a suspension of the crude D/L-**4** (0.41 mmol) in phosphate buffer pH 7.4 (15 mL), and the mixture was shaken for 24-72 h at 37°C. After 24 h, the reaction was monitored by RP-HPLC-ESI. To this purpose, an aliquot of 10 μL was withdrawn from the incubation mixture and enzyme activity was terminated by precipitating proteins with 0.1 mL of glacial acetonitrile. The sample was then diluted with 0.1 mL of 0.5% AcOH to prevent further enzymatic breakdown, and centrifuged at 13,000×g for 15 min. The supernatant was collected and the peak area of Ac-tryptophan **4** and *N*-deprotected tryptophan was measured by RP-HPLC (for a representative example, see Figure S1). The reaction was allowed to continue until the peak areas of **4** and *N*-deprotected tryptophan became nearly equivalent. Eventually, the reaction was monitored again by RP-HPLC-ESI after 24 h. In any case, the reaction was stopped after 72 h. Then pH was adjusted to 3 with 10% HCl and the mixture was filtered. The aqueous solution was extracted with AcOEt (3x20 mL), the collected organic layers were dried over Na_2SO_4, and solvent was evaporated at reduced pressure. The de-acetylated *L*-tryptophan was not isolated, nor characterized. The resulting crude D-**4** (55-62%, 85-90% pure by analytical RP-HPLC, e.e. not determined) was analyzed by RP-HPLC to confirm the absence of de-acetylated *L*-tryptophan, and was identified by ^{1}H NMR and ESI MS. The crude D-**4** was utilized for the coupling reaction with H-Phe-GlyNH_2 without further purifications.

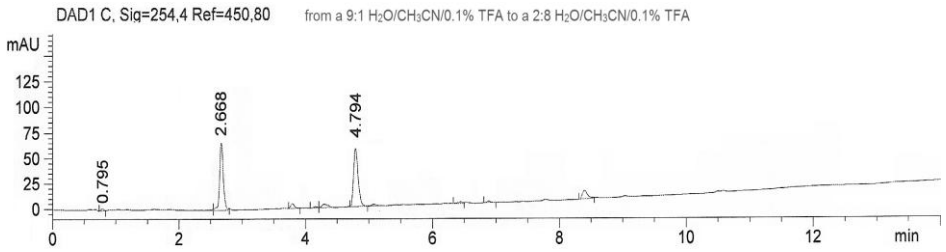


Figure S1. RP-HPLC analysis (General Methods) of the enzymatic resolution of Ac-5-F-D/L-TrpOH after 48 h, showing a *circa* 1:1 ratio between the peak of Ac-5-F-TrpOH (4.794 min) enriched in D-enantiomer, and the peak of 5-F-L-TrpOH (2.688 min).

Ac-5'-Me-D-TrpOH (**4c**)[a,1] ES-MS *(m/z)* 260.1 [M+1], calcd 260.1. Ac-5'-F-D-TrpOH (**4e**)[a,2] ES-MS *(m/z)* 264.2 [M+1], calcd 264.1. Ac-6'-F-D-TrpOH (**4f**)[a,2] ES-MS *(m/z)* 264.1 [M+1], calcd 264.1. Ac-5'-Cl-D-TrpOH (**4g**)[a,2] ES-MS *(m/z)* 280.1 [M+1], calcd 280.1. Ac-6'-Cl-D-TrpOH (**4h**)[a,2] ES-MS *(m/z)* 280.2 [M+1], calcd 280.1. Ac-5'-Br-D-TrpOH (**4i**).[a,2] ES-MS *(m/z)* 324.1/326.2 [M+1], calcd 324.0/326.0. Ac-7'-Br-D-TrpOH (**4l**)[a,3] ES-MS *(m/z)* 324.1/326.1 [M+1], calcd 324.0/326. Ac-5'-NO_2-D-TrpOH (**4m**)[a,2] ES-MS *(m/z)* 291.2 [M+1], calcd 291.1.

[a] ^{1}H NMR analysis was found to match with the literature.

Ac-2'-Me-D-TrpOH (**4b**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.76 (s, 3H, Ac), 2.30 (s, 3H, Me), 2.93 (dd, J=7.4, 14.2 Hz, 1H, TrpHβ), 3.07 (dd, J=6.2, 14.2 Hz, 1H, TrpHβ), 4.41 (ddd, J=6.2, 7.4, 7.6 Hz, 1H, TrpHα), 6.91 (t, J=7.4 Hz, 1H, $TrpH_5$), 6.96 (t, J=7.6 Hz, 1H, $TrpH_6$), 7.21 (d, J=8.0, 1H, $TrpH_7$), 7.43 (d, J=7.6, 1H, $TrpH_4$), 8.10 (d, J=7.6, 1H, TrpNH), 10.71 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 260.2 [M+1], calcd 260.1.

Ac-7'-Me-D-TrpOH (**4d**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.98 (s, 3H, Ac), 2.60 (s, 3H, TrpMe), 3.04 (dd, J=9.0, 14.6 Hz, 1H,TrpHβ), 3.22 (dd, J=5.0, 14.6 Hz, 1H,TrpHβ), 4.52 (ddd, J=5.0, 8.0, 9.0 Hz, 1H, TrpHα), 6.88-7.00 (m, 2H, $TrpH_{5,6}$), 7.20 (s, 1H, $TrpH_2$), 7.42 (d, J=7.2 Hz, 1H, $TrpH_4$), 8.20 (d, J=8.0 Hz, 1H, TrpNH), 10.89 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 260.3 [M+1], calcd 260.1.

Ac-5'-Cl-2'Me-D-TrpOH (**4n**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.81 (s, 3H, Ac), 2.02 (s, 3H, TrpMe), 2.93 (dd, J=7.2, 14.4 Hz, 1H,TrpHβ), 3.05 (dd, J=6.4, 14.4 Hz, 1H,TrpHβ), 4.41 (ddd, J=6.4, 7.2, 8.0 Hz, 1H, TrpHα), 6.95 (d, J=8.4 Hz, 1H, $TrpH_6$), 7.21 (d, J=8.8 Hz, 1H, $TrpH_7$), 8.14 (d, J=8.0 Hz, 1H, TrpNH), 10.90 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 295.0 [M+1], calcd 295.1

Ac-7'-Br-2'-Me-D-TrpOH (**4o**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 2.01 (s, 3H, Ac), 2.33 (s, 3H, TrpMe), 2.94 (dd, J=8.0, 14.8 Hz, 1H,TrpHβ), 3.07 (dd, J=6.2, 14.8 Hz, 1H,TrpHβ), 4.40 (ddd, J=6.2, 8.0, 8.6 Hz, 1H, TrpHα), 6.87-6.96 (m, 2H, $TrpH_{4,5}$), 7.37 (br.d, 1H, H_6), 8.15 (d, J=8.6 Hz, 1H, TrpNH), 9.89 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 339.2/341.2 [M+1], calcd 339.0/341.0.

---

[1] Porter, J.; Dykert, J.; Rivier, J. Synthesis, resolution and characterization of ring substituted phenylalanines and tryptophans. *Int. J. Pept. Prot. Res*. **1987**, *30*, 13-21.

[2] Blaser, G.; Sanderson, J. M.; Batsanov, A. S.; Howard, J. A. K. The facile synthesis of a series of tryptophan derivatives. *Tetrahedron Lett.* **2008**, *49*, 2795–2798.

[3] Konda-Yamada, Y.; Okada, C.; Yoshida, K.; Umeda, Y.; Arima, S.; Sato, N.; Kai, T.; Takayanagi, H.; Harigaya, Y. Convenient synthesis of 7' and 6'-bromo-D-tryptophan and their derivatives by enzymatic optical resolution using D-aminoacylase. *Tetrahedron* **2002**, *58*, 7851-7861.

*Preparation of Ac-tripeptides (**2**).*

HOBt (1.1 mmol) and HBTU (1.1 mmol) were added to a stirred solution of the crude **4** (1.0 mmol) in 4:1 DCM/DMF (5 mL) at r.t. under inert atmosphere. After 10 min, the H-Phe-GlyNH_2 (1.1 mmol) and DIPEA (2.2 mmol) were added at r.t., and the mixture was stirred under MW irradiation with a initial power of 150W, and monitoring the internal reaction temperature at 80°C. After 10 min the mixture was diluted with DCM, and the solution was washed with 0.5 M HCl (5 mL) and saturated $NaHCO_3$ (5 mL). The organic layer was dried over Na_2SO_4, and solvent was removed at reduced pressure. The crude peptides were precipitated from ice-cold MeOH/Et_2O and collected by centrifuge. The analyses of the resulting crude **2** revealed the presence of traces of the epimer containing L-Trp (5-12%). The peptides **2** (75-85%) were isolated as single stereoisomers (Figure S2) by semi-preparative RP-HPLC (95-98% pure by analytical RP-HPLC, General Methods).

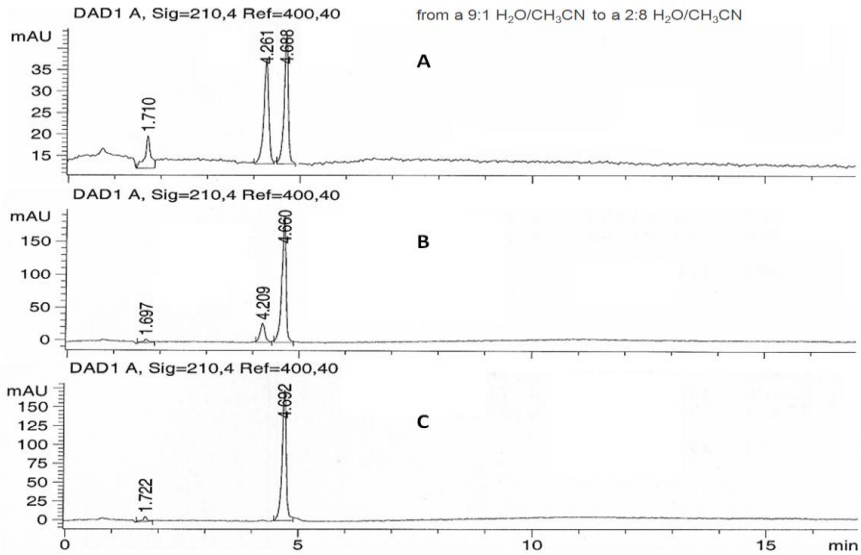


Figure S2. (**A**) RP-HPLC analysis (General Methods) of Ac-5-F-D/L-Trp-Phe-GlyNH_2 obtained from racemic Ac-5-F-TrpOH; the separation of this 1:1 mixture by semi-preparative RP-HPLC did not afford peptides >95% pure. (**B**) Analysis of Ac-5-F-D-Trp-Phe-GlyNH_2 (4.660 min) and residual Ac-5-F-L-Trp-Phe-GlyNH_2 (4.209 min) prepared from enriched Ac-5-F-D-TrpOH, obtained in turn by enzymatic resolution of the racemate; the separation of this mixture gave purity >95% (see **C**). (**C**) Analysis of Ac-5-F-D-Trp-Phe-GlyNH_2 after semi-preparative RP-HPLC as described in General Methods showing complete removal of the L-stereoisomer.

*Analytical characterization of peptides **2b**-**o***

Ac-2-methyl-D-Trp-Phe-GlyNH_2 (**2b**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.79 (s, 3H, Ac), 2.20 (s, 3H, Me), 2.68-2.77 (m, 2H, PheHβ+TrpHβ), 2.82 (dd, J=6.4, 14.4 Hz, 1H, PheHβ), 2.96 (dd, J=4.4, 14.0 Hz, 1H, TrpHβ), 3.65 (dd, J=6.4, 17.6 Hz, 1H, GlyHα), 3.71 (dd, J=5.6, 17,6 Hz, 1H, GlyHα), 4.45-4.51 (m, 2H, TrpHα+PheHα), 6.95 (t, J=8.0 Hz, 1H, $TrpH_5$), 7.00 (t, J=8.0 Hz, 1H, $TrpH_6$), 7.17-7.30 (m, 8H, PheArH+$CONH_2$+$TrpH_7$), 7.46 (d, J=7.6 Hz, 1H, $TrpH_4$), 8.02 (d, J=8.0 Hz, 1H, TrpNH), 8.23 (t, J=6.0 Hz, 1H, GlyNH), 8.29 (d, J=8.0 Hz, PheNH), 10.70 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 464.1/486.2 [M+1/M+23], calcd 464.2.

Ac-5-methyl-D-Trp-Phe-GlyNH_2 (**2c**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.75 (s, 3H, Ac), 2.40 (s, 3H, Me), 2.62 (dd, J=9.4, 14.6 Hz, 1H, PheHβ), 2.72 (dd, J=4.6, 14.6 Hz, 1H, PheHβ), 2.79 (dd, J=10.4, 13.6 Hz, 1H, TrpHβ), 3.06 (dd, J=4.0, 13.6 Hz, 1H, TrpHβ), 3.62 (dd, J=6.0, 16.8 Hz, 1H, GlyHα), 3.68 (dd, J=6.0, 16.8 Hz, 1H, GlyHα), 4.42 (ddd, J=4.6, 7.6, 9.4 Hz, 1H, PheHα), 4.50 (ddd, J=4.0, 8.4, 10.4 Hz, 1H, TrpHα), 6.88 (d, J=8.8 Hz, 1H, $TrpH_6$), 6.90 (s, 1H, $TrpH_2$), 7.10 (s, 1H, $CONH_2$), 7.12 (s, 1H, $CONH_2$), 7.19-7.26 (m, 6H, PheArH+$TrpH_7$), 7.33 (s, 1H, $TrpH_4$), 8.00 (d, J=7.6 Hz, 1H, PheNH), 8.18 (t, J=6.0 Hz, 1H, GlyNH), 8.39 (d, J=8.4 Hz, 1H, TrpNH), 10.60 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 464.2/486.2 [M+1/M+23], calcd 464.2.

Ac-7-methyl-D-Trp-Phe-GlyNH_2 (**2d**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.74 (s, 3H, Ac), 2.38 (s, 3H, Me), 2.74-2-82 (m, 3H, PheHβ+TrpHβ), 3.02 (dd, J=3.8, 14.0 Hz, 1H, TrpHβ), 3.55 (dd, J=5.6, 16.8 Hz, 1H, GlyHα), 3.65 (dd, J=6.2, 16.8 Hz, 1H, GlyHα), 4.33-4.41 (m, 2H, TrpHα+PheHα), 6.81 (br.t, 1H, $TrpH_5$), 6.92-6.98 (m, 2H, $TrpH_2$+$CONH_2$), 7.05-7.12 (m, 7H, PheArH+$CONH_2$+$TrpH_6$), 7.27 (br.d, 1H, $TrpH_4$), 7.92 (br.d, 1H, PheNH), 8.04 (br.t, 1H, GlyNH), 8.27 (br.d, 1H, TrpNH), 10.53 (br.s, 1H, $TrpH_1$); ES-MS *(m/z)* 464.1/486.1 [M+1/M+23], calcd 464.2.

Ac-5-fluoro-D-Trp-Phe-GlyNH_2 (**2e**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 2.15 (s, 3H, Ac), 2.64 (dd, J=9.2, 14.4 Hz, 1H, PheHβ), 2.73 (dd, J=4.4, 14.4 Hz, 1H, PheHβ), 2.83 (dd, J=10.6, 13.5 Hz, 1H, TrpHβ), 3.12 (dd, J=4.0, 13.5 Hz, 1H, TrpHβ), 3.68 (dd, J=6.0, 17.2 Hz, 1H, GlyHα), 3.75 (dd, J=6.0, 17.2 Hz, 1H, GlyHα), 4.50 (ddd, J=4.4, 7.6, 9.2 Hz, 1H, PheHα), 4.57 (ddd, J=4.0, 8.4, 10.6 Hz, 1H, TrpHα), 6.88 (t, J=9.0 Hz, 1H, $TrpH_6$), 7.03 (s, 1H, $TrpH_2$), 7.07 (s, 1H, $CONH_2$), 7.12-7.30 (m, 7H, PheArH+$TrpH_4$+$CONH_2$), 7.34 (d, J=9.0 Hz, 1H, $TrpH_7$), 8.00 (d, J=7.6 Hz, 1H, PheNH), 8.19 (t, J=6.0 Hz, 1H, GlyNH), 8.47 (d, J=8.4 Hz, 1H, TrpNH), 10.84 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 468.2/490.1 [M+1/M+23], calcd 468.2.

Ac-6-fluoro-D-Trp-Phe-GlyNH_2 (**2f**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.80 (s, 3H, Ac), 2.68 (dd, J=9.2, 14.8 Hz, 1H, PheHβ), 2.78 (dd, J=4.4, 14.8 Hz, 1H, PheHβ), 2.83 (dd, J=10.8, 14.0 Hz, 1H, TrpHβ), 3.11 (dd, J=4.4, 14.0 Hz, 1H, TrpHβ), 3.68 (dd, J=5.6, 16.8 Hz, 1H, GlyHα), 3.74 (dd, J=5.6, 16.8 Hz, 1H, GlyHα), 4.48-4.59 (m, 2H, TrpHα+PheHα), 6.89 (t, J=8.8 Hz, 1H, $TrpH_5$), 7.00 (s, 1H, $TrpH_2$), 7.10-7.31 (m, 8H, PheArH+$CONH_2$+$TrpH_7$), 7.55 (dd, J=5.6, 8.8 Hz, 1H, $TrpH_4$), 8.06 (d, J=7.6 Hz, 1H, PheNH), 8.27 (t, J=5.6 Hz, 1H, GlyNH), 8.51 (d, J=8.0 Hz, 1H, TrpNH), 10.87 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 468.3/490.3 [M+1/M+23], calcd 468.2.

Ac-5-chloro-D-Trp-Phe-GlyNH_2 (**2g**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.69 (s, 3H, Ac), 2.54 (dd, J=9.4, 14.4 Hz, 1H, PheHβ), 2.64 (dd, J=4.4, 14.4 Hz, 1H, PheHβ), 2.72 (dd, J=4.0, 14.8 Hz, 1H, TrpHβ), 3.02 (dd, J=4.4, 14.8 Hz, 1H, TrpHβ), 3.60 (dd, J=6.0, 16.8 Hz, 1H, GlyHα), 3.64 (dd, J=6.0, 16.8 Hz,1H, GlyHα), 4.28 (ddd, J=4.4, 8.0, 9.4 Hz, 1H, PheHα), 4.50 (ddd, J=4.0, 4.4, 8.4 Hz, 1H, TrpHα), 6.97-7.03 (m, 2H, $TrpH_2$+$TrpH_6$), 7.04 (s, 1H, $CONH_2$), 7.10 (s, 1H, $CONH_2$), 7.16-7.25 (m, 5H, PheArH), 7.28 (d, J=8.4 Hz, 1H, $TrpH_7$), 7.60 (s, 1H, $TrpH_4$), 7.97 (d, J=8.0 Hz, 1H, PheNH), 8.16 (t, J=6.0 Hz, 1H, GlyNH), 8.46 (d, J=8.4 Hz, 1H, TrpNH), 10.91 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 484.2/506.2 [M+1/M+23], 484.2.

Ac-6-chloro-D-Trp-Phe-GlyNH_2 (**2h**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.73 (s, 3H, Ac), 2.61 (dd, J=9.4, 14.4 Hz, 1H, PheHβ), 2.71 (dd, J=4.6, 14.4 Hz, 1H, PheHβ), 2.75 (dd, J=10.4, 13.6 Hz, 1H, TrpHβ), 3.04 (dd, J=4.4, 13.6 Hz, 1H, TrpHβ), 3.62 (dd, J=5.8, 16.8 Hz, 1H, GlyHα), 3.67 (dd, J=5.8, 16.8 Hz, 1H, GlyHα), 4.41-4.51 (m, 2H, PheHα+TrpHα), 6.94-7.00 (m, 2H, $TrpH_2$+$TrpH_5$), 7.10 (s, 1H, $CONH_2$), 7.13 (s, 1H, $CONH_2$), 7.18-7.26 (m, 5H, PheArH), 7.34 (s, 1H, $TrpH_7$), 7.50 (d, J=8.8 Hz, 1H, $TrpH_4$), 7.98 (d, J=8.0 Hz, 1H, PheNH), 8.20 (t, J=5.8 Hz, 1H, GlyNH), 8.43 (d, J=8.0 Hz, 1H, TrpNH), 10.88 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 484.2/506.1 [M+1/M+23], 484.2.

Ac-5-bromo-D-Trp-Phe-GlyNH_2 (**2i**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.78 (s, 3H, Ac), 2.58 (dd, J=9.4, 14.5 Hz, 1H, PheHβ), 2.67 (dd, J=4.4, 14.5 Hz, 1H, PheHβ), 2.74 (dd, J=4.0, 14.8 Hz, 1H, TrpHβ), 3.05 (dd, J=4.4, 14.8 Hz, 1H, TrpHβ), 3.65 (dd, J=6.0, 16.8 Hz, 1H, GlyHα), 3.70 (dd, J=6.0, 16.8 Hz, 1H, GlyHα), 4.40 (ddd, J=4.4, 8.0, 9.4 Hz, 1H PheHα), 4.51 (ddd, 4.0, 4.4, 8.4, 1H, TrpHα), 6.99 (s, 1H, $TrpH_2$), 7.08 (s, 1H, $CONH_2$), 7.10-7.18 (m, 2H, $TrpH_6$+$CONH_2$), 7.20-7.28 (m, 6H, PheArH+$TrpH_7$), 7.76 (s, 1H, $TrpH_4$), 8.00 (d, J=8.0 Hz, 1H, PheNH), 8.20 (t, J=6.0 Hz, 1H, GlyNH), 8.50 (d, J=8.4 Hz, 1H, TrpNH), 10.97 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 528.1/530.2 [M+1], calcd 528.1/530.1.

Ac-7-Br-D-Trp-Phe-GlyNH_2 (**2l**). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.80 (s, 3H, Ac), 2.86-2.95 (m, 4H, TrpHβ+PheHβ), 3.69 (dd, J=6.0, 17.0 Hz, 1H, GlyHα), 3.82 (dd, J=6.0, 17.0 Hz, 1H, GlyHα), 4.35-4.48 (m, 2H, TrpHα+PheHα), 6.24 (s, 1H, $CONH_2$), 6.78 (s, 1H, $CONH_2$), 6.83-6.97 (m, 2H, $TrpH_2$+$TrpH_5$), 7.10-7.17 (m, 5H,

PheArH), 7.25 (d, J=8.0 Hz, 1H, $TrpH_6$), 7.34-7.40 (m, 2H, PheNH+$TrpH_4$), 7.52 (d, J=7.0 Hz, 1H, TrpNH), 7.77 (br.t, 1H, GlyNH), 9.17 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 528.0/530.2 [M+1], calcd 528.1/530.1.

Ac-5-NO_2-D-Trp-Phe-GlyNH_2 (**2m**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.80 (s, 3H, Ac), 2.82-2.95 (m, 2H, PheHβ), 2.92-3.12 (m, 2H, TrpHβ), 3.80 (d, J=6.0 Hz, 2H, GlyHα), 4.45-4.57 (m, 2H, TrpHα+PheHα), 5.90 (br.s, 1H, $CONH_2$), 6.61 (br.s, 1H, $CONH_2$), 6.90-6.95 (m, 3H, PheArH+$TrpH_2$), 6.96-7.02 (m, 3H, PheArH), 7.22 (d, J=8.2 Hz, 1H, $TrpH_7$), 7.40 (d, J=5.2 Hz, 1H, TrpNH), 7.70 (br.t, 1H, GlyNH), 7.80 (d, J=7.2 Hz, 1H, PheNH), 7.90 (d, J=8.2 Hz, $TrpH_6$), 8.40 (s, 1H, $TrpH_4$), 10.61 (s, 1H, $TrpH_1$); ^{13}C NMR (100 MHz, [D6]DMSO) δ: 22.9, 27.9, 37.4, 42.3, 58.0, 59.1, 109.7, 112.0, 114.0, 123.0, 125.9, 126.7, 127.7, 128.3, 128.6, 132.2, 136.6, 142.6, 169.8, 171.7, 172.0. Elem. Anal. for $C_{24}H_{26}N_6O_6$ calcd: C 58.29, H 5.30, N 16.99 found: C 59.16, H 5.35, N 16.81 ES-MS (m/z) 495.2/517.2 [M+1/M+23], calcd 495.2.

Ac-5-chloro-2-methyl-D-Trp-Phe-GlyNH_2 (**2n**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.80 (s, 3H, Ac), 2.18 (s, 3H, Me), 2.67-2.74 (m, 2H, PheHβ+TrpHβ), 2.77-2.89 (m, 2H, TrpHβ+PheHβ), 3.58 (dd, J=5.6, 16.8 Hz, 1H, GlyHα), 3.66 (dd, J=5.6, 16.8 Hz, 1H, GlyHα), 4.33-4.40 (m, 2H, TrpHα+PheHα), 6.90 (d, J=8.6 Hz, 1H, $TrpH_6$), 6.84-7.01 (m, 3H, PheArH+$CONH_2$), 7.13-7.23 (m, 4H, PheArH+$CONH_2$), 7.13 (d, J=8.6 Hz, 1H, $TrpH_7$), 7.41 (d, J=5.6 Hz, 1H, $TrpH_4$), 7.99 (br.d, 1H, PheNH), 8.07 (br.t, 1H, GlyNH), 8.20 (br.d, 1H, TrpNH), 10.70 (s, 1H, $TrpH_1$); ES-MS *(m/z)* 498.3/520.1 [M+1/M+23], calcd 498.2.

Ac-7-Br-2-Me-D-Trp-Phe-GlyNH_2 (**2o**). ^{1}H NMR (400 MHz, [D6]DMSO) δ: 1.76 (s, 3H, Ac), 2.22 (s, 3H, CH_3), 2.68 (dd, J=8.4, 14.4 Hz, 1H, PheHβ), 2.93-3.05 (m, 3H, TrpHβ+PheHβ), 3.37 (dd, J=6.0, 13.8 Hz, 1H, GlyHα), 3.79 (dd, J=6.0, 16.8 Hz, 1H, GlyHα), 4.23 (m, 1H, PheHα), 4.47 (m, 1H, TrpHα), 6.00 (s, 1H, $CONH_2$), 6.64 (s, 1H, $CONH_2$), 6.75 (t, J=7.4 Hz, 1H, $TrpH_5$), 6.83 (br.t, 1H, GlyNH), 6.98-7.20 (m, 6H, PheArH+TrpNH), 7.27 (d, J=7.4 Hz, 1H, $TrpH_6$), 7.42 (d, J=7.6 Hz, 1H, PheNH), 7.68 (d, J=8.4 Hz, 1H, $TrpH_4$), 9.62 (s, 1H, $TrpH_1$); ^{13}C NMR (100 MHz, [D6]DMSO) δ: 12.1, 22.9, 28.6, 37.4, 42.3, 58.0, 59.4, 100.0, 108.2, 117.8, 121.1, 124.5, 125.9, 127.7, 128.6, 129.5, 131.2, 135.2, 136.6, 169.8, 171.7, 172.0. Elem. Anal. for $C_{25}H_{28}BrN_5O_4$ calcd: C 55.36, H 5.20, N 12.91; found: C 56.46, H 5.09, N 12.78 ES-MS (m/z) 542.1/544.2 [M+1], calcd 542.1/544.1.

Preparation of HEK-293 cells stably expressing human MOR

HEK-293 cells (ATCC, U.S.A.) were transfected with the human MOR encoding plasmid pcDNA3.1+OPRM1 (UMR cDNA Resource Center, Rolla, MO, USA) by using EXGEN 500 (Fermentas, Hanover, MD, USA). Stable transfectants were selected by exposure to G418 (500 mg mL^{-1}) for four weeks and then seeded in normal minimum essential medium (MEM; Sigma, Steinheim, Germany) to perform binding assays.

Determination of surface MOR

Cell surface human MOR receptors were measured on intact cells using [^{3}H]DAMGO (0.1-5.0 nM) as radioligand, and naloxone (30 μM) to determine nonspecific binding. [^{3}H]DAMGO binding to HEK-293 cells expressing human MOR was saturable, with a K_d (apparent dissociation constant of the radioligand) of 1.45±0.14 nM and a B_{max} (maximal number of binding sites) of 3189±18 dpm $(5x10^5 \text{ cells})^{-1}$ (n=4). No any specific radioligand binding was detectable in HEK-293 cells transfected with an empty pcDNA3.1 plasmid (data not shown). In a separate set of experiments, following the protocol adopted to carry out receptor binding assays, HEK-293 cells transfected with the empty vector were incubated at r.t. for 120 min with 5 nM

[^{3}H]DAMGO to measure any non-specific, MOR independent, cellular uptake of the radioligand. At the end of the incubation, the cells were washed two times with PBS (pH 7.4), scraped of and lysed with 0.1 N NaOH and then buffered with an equal amount of 0.1N HCl and left in scintillation fluid for 8 h before counting by liquid scintillation spectrophotometry. In the experimental conditions adopted to carry out receptor binding assays, cellular uptake of [^{3}H]DAMGO was low and did not reach 10% (n=5)

Receptor binding assays to cloned human MOR.

HEK-293 cells stably expressing human MOR were incubated at r.t. for 2 h with [^{3}H]DAMGO (5.0 nM), in the presence or absence of compounds in the range of concentrations (10^{-12}-10^{-4}M); nonspecific binding was determined in the presence of naloxone (30 μM). Compounds were prepared as stock solutions (10^{-2} M) in ethanol and protected from light; compound dilutions were made in assay buffer. After incubation with the listed ligands, cells were washed in PBS (pH 7.4) and lysed with 0.1 N NaOH. Lysed samples were buffered with an equal amount of 0.1 N HCl and left in scintillation fluid for 8h before counting. Data from at least three independent experiments, each performed in triplicate, were fitted by nonlinear regression analysis using GraphPad Prism and K_i values were calculated using the Cheng-Prusoff equation. Data to the Hill equation were fitted by a computerized nonlinear least-squares method.

Receptor binding assays to cloned human DOR and KOR

HEK-293 cells stably expressing human DOR (2700±100 fmol mg^{-1} protein; n=6) or KOR (2600±400 fmol mg^{-1} protein; n=6) were generated by EXGEN500 (Fermentas) by transfection with cDNAs cloned into the pcDNA3.1(+) vector (Invitrogen). The cDNAs were obtained from UMR cDNA Resource Center (Rolla, MO, USA). HEK-293 cells expressing DOR or KOR were grown as a monolayer culture in tissue culture flasks that were incubated at 37°C in a humidified atmosphere (5% CO_2) in MEM (Lonza) containing 2 mM L-glutamine, 1 x nonessential amino acids (Invitrogen) and supplemented with 10% fetal calf serum (Lonza), and maintained in the presence of 500 mg mL^{-1} geneticin (Invitrogen). Cells were washed with ice-cold PBS, pH 7.4; scraped into an ice-cold buffer containing 10 mm HEPES/NaOH, pH 7.4 and 1 mm EDTA; and lysed with a Dounce tissue grinder. The cell lysate was centrifuged at 1000 g for 2 min at 4°C. The supernatant was collected and centrifuged at 32000 g for 20 min at 4°C. The pellet was re-suspended in homogenization buffer at a protein concentration (determined by BCA assay) of 1.0 to 1.5 mg mL^{-1} and stored in aliquots at -80°C. Receptor binding assays were carried out by using [^{3}H]diprenorphine to label DOR and [^{3}H]U69,593 to label KOR and by incubating the membrane preparations at 25°C for 90 min in buffer containing 100 mM Tris-HCl and 0.3% BSA. For saturation binding assays, the concentrations of [^{3}H]diprenorphine and [3H]U69,593 ranged from 40 pM to 3 nM and from 20 pM to 5 nM, respectively ([^{3}H]diprenorphine K_d = 0.22±0.03 nM; n=3) ([^{3}H]U69,593 K_d=1.1±0.1 nM; n=3). For competition binding assays, the concentration of [^{3}H]diprenorphine or [^{3}H]U69,593 was 1 nM and 2 nM, respectively. Nonspecific binding was determined in the presence of either 10 μM DPDPE (DOR) or 10 μM U50,488 (KOR) and corresponded to 8-12% and 12-15% of total [3H]diprenorphine and [^{3}H]U69,593 binding, respectively. Triplicate determinations were made for each experiment. Reactions were terminated by filtration through Whatman GF/C filters presoaked with 0.3% polyethylenimine, which were washed three times with 5 mL of ice-cold buffer containing 50 mM Tris-HCl, pH 7.4. The radioactivity trapped was determined by liquid scintillation spectrometry. Data from at least three independent experiments were

fitted by nonlinear regression analysis using GraphPad Prism. K_i values were calculated from IC_{50} values by the Cheng-Prusoff equation. IC_{50} values represent mean values from no fewer than four experiments. IC_{50} values, relative potency estimates, and their associated standard errors were determined by fitting the data to the Hill equation by a computerized nonlinear least-squares method.

Animals and treatments

Adult male CD-1 mice (Charles River, Calco, Como, Italy) weighing 25-30 g were used. They were housed in a light- and temperature-controlled room (light on 08.00–20.00 h, temperature 24°C) and had free access to food and water. This research was conducted in compliance with the guidelines of the Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purpose. The procedures employed in this study were approved by the Animal Care and Use Committee of the University of Bologna (Prot. n. 29-IX/9, 25th July 2012) and conformed to the International Association for the Study of Pain (IASP) guidelines on ethical standards for investigations of experimental pain in animals.

For intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) injections, the compounds were dissolved in a 1:1 v/v solution of propylene glycol and saline (vehicle) and injected in a volume of 0.1 ml/10 g body weight for i.p. administration, and in a volume of 2 μL/mouse for i.c.v. administration. For i.c.v. injections, 2 μL of sterile vehicle were administered under ether anesthesia as previously described [Haley, T.J.; McCormick, W. G. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br. J. Pharmacol.* **1957**, *12*, 12-15]. The anesthetic or the vehicle alone (2 μL i.c.v. injected) did not affect antinociceptive measurements up to 60 min later (data not shown).

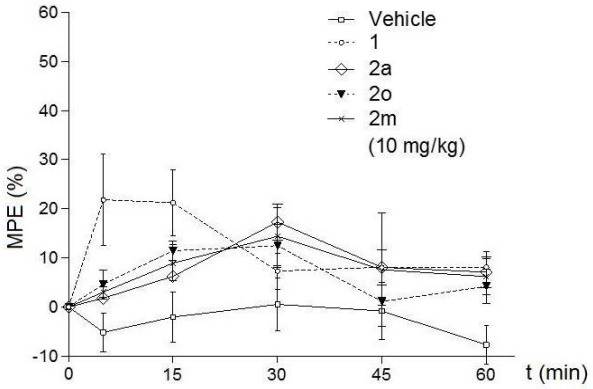


Figure S3. Antinociception produced by the compounds in the mouse tail-flick assay: effects elicited by i.p. administered **1**, **2a**, **2o**, **2m** or vehicle (10 mg/kg).

Peptide stability in mouse serum.

Enzymatic degradation studies of the peptides and EM1 were carried out in triplicate and repeated three times using mouse serum purchased from Sigma-Aldrich. Peptides were dissolved in Tris buffer pH 7.4 and 10 μL aliquots of a 10 μM peptide stock solution were added to 190 μL of serum. Incubations were carried out at 37°C. Sampling intervals were chosen so that a kinetic curve could be constructed. Aliquots of 20 μL were withdrawn from the incubation mixtures and enzyme activity was terminated by precipitating proteins with 90μL of glacial acetonitrile. Samples were then diluted with 90 μL of 0.5% acetic acid to prevent further enzymatic breakdown and centrifuged at 13,000×g for 15 min. The supernatants were collected and the stability of peptides was determined by RP-HPLC analysis.

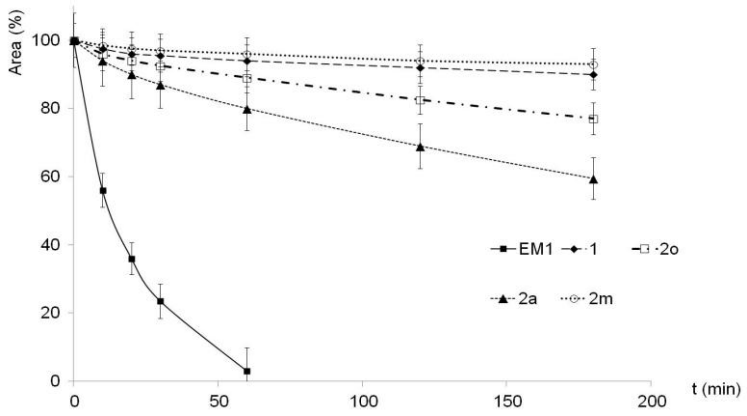


Figure S4. Stability curves of EM1 and compounds **1**, **2a**, **2m**, **2o**, and EM1, in mouse serum. Sampling intervals were chosen so that a kinetic curve could be constructed. Peak areas were normalized to 100 at *t*=0. Each hydrolysis experiment was repeated 3 times, and the reported data are mean values. Error ranges were estimated on the basis of the standard deviation.

References

Selection of references on EM mimetics showing central antinociceptive activity.[4,5,6,7,8,9]

[4] Cardillo, G.; Gentilucci, L.; Qasem, A. R.; Sgarzi, F.; Spampinato, S. Endomorphin-1 analogues containing β-proline are μ-opioid receptor agonists and display enhanced enzymatic hydrolysis resistance. *J. Med. Chem.* **2002**, *45*, 2571-2578.

[5] Spampinato, S.; Qasem, A. R.; Calienni, M.; Murari, G.; Gentilucci, L.; Tolomelli, A.; Cardillo, G. Antinociception by a peripherally administered novel endomorphin-1 analogue containing β-proline. *Eur. J. Pharmacol.* **2003**, *469*, 89-95.

[6] Liu, X.; Wang, Y.; Xing, Y.; Yu, J.; Ji, H.; Kai, M.; Wang, Z.; Wang, D.; Zhang, Y.; Zhao, D.; Wang, R. Design, synthesis, and pharmacological characterization of novel endomorphin-1 analogues as extremely potent μ-opioid agonists. *J. Med. Chem.* **2013**, *56*, 3102-3114.

[7] Janecka, A.; Fichna, J.; Kruszynski, R.; Sasaki, Y.; Ambo, A.; Costentin, J.; do-Rego, J. C. Synthesis and antinociceptive activity of cyclic endomorphin-2 and morphiceptin analogs. *Biochem. Pharmacol.* **2005**, *71*, 188-195.

[8] Perlikowska, R.; do-Rego, J. C.; Cravezic, A.; Fichna, J.; Wyrebska, A.; Toth, G.; Janecka, A. Synthesis and biological evaluation of cyclic endomorphin-2 analogs. *Peptides* **2010**, *31*, 339-345.

[9] Fichna, J.; Perlikowska, R.; Wyrebska, A.; Gach, K.; Piekielna, J.; do-Rego, J. C.; Toth, G.; Kluczyk, A.; Janecki, T.; Janecka, A. Effect of 2',6'-dimethyl-L-tyrosine(Dmt) on pharmacological activity of cyclic endomorphin-2 and morphiceptin analogs. *Bioorg. Med. Chem*. **2011**, *19*, 6977-6981.

Selection of references for the preparation of EMs by "green chemistry" protocols.[10,11,12]

[10] Xu, J.; Sun, H.; He, X.; Bai, Z.; He, B. Highly efficient synthesis of endomorphin-2 under thermodynamic control catalyzed by organic solvent stable proteases with in situ product removal. *Bioresource Tech.* **2013**, *129*, 663-666.

[11] Torres-García, C.; Pulido, D.; Carceller, M.; Ramos, I.; Royo, M.; Nicolas, E. Solid-Phase Synthesis of phenylalanine containing peptides using a traceless triazene linker. *J. Org. Chem.* **2012**, *77*, 9852-9858.

[12] Sun, H.; He, B.; Xu, J.; Wu, B.; Ouyang, P. Efficient chemo-enzymatic synthesis of endomorphin-1 using organic solvent stable proteases to green the synthesis of the peptide. *Green Chem*. **2011**, *13*, 1680-1685.

Selection of references of EM mimetics showing improved CNS bioavailability,[13] *including cationization,*[14] *lipidation,*[15,16] *glycosylation,*[17,18,19] *vector-mediated transport,*[20] *halogenation*[21,22,23]

[13] Liu, H. M.; Liu, X. F.; Yao, J. L.; Wang, C. L.; Yu, Y.; Wang, R. Utilization of combined chemical modifications to enhance the blood-brain barrier permeability and pharmacological activity of endomorphin-1. *J. Pharm. Exp. Ther*. **2006**, *319*, 308-316.

[14] Hau, V. S.; Huber, J. D.; Campos, C. R.; Lipkowski, A. W.; Misicka, A.; Davis, T. Effect of guanidino modification and proline substitution on the in vitro stability and blood-brain barrier permeabilità of endomorphin-2. *J. Pharm. Sci.* **2002**, *91*, 2140-2149.

[15] Varamini, P.; Mansfeld, F. M.; Blanchfield, J. T.; Wyse, B. D.; Smith, M. T.; Toth, I. Lipo-endomorphin-1 derivatives with systemic activity against neuropathic pain without producing constipation. *PLoS One* **2012**, *7*, e41909.

[16] Varamini, P.; Goh, W. H.; Mansfeld, F. M.; Blanchfield, J. T.; Wyse, B. D.; Smith, M. T.; Toth, I. Peripherally acting novel lipo-endomorphin-1 peptides in neuropathic pain without producing constipation. *Biorg. Med. Chem.* **2013**, *21*, 1898-1904.

[17] Varamini, P.; Mansfeld, F. M.; Blanchfield, J. T.; Wyse, B. D.; Smith, M. T.; Toth, I. Synthesis and biological evaluation of an orally active glycosylated endomorphin-1. *J. Med. Chem.* **2012**, *55*, 5859-5867.

[18] Biondi, B.; Giannini, E.; Negri, L.; Melchiorri, P.; Lattanzi, R.; Rosso, F.; Ciocca, L.; Rocchi, R. Opioid peptides: synthesis and biological activity of new endomorphin analogues. *Int. J. Peptide Res. Ther.* **2006**, *12*, 145-151.

[19] Koda, Y.; Del Borgo, M.; Wessling, S. T.; Lazarus, L. H.; Okada, Y.; Toth, I.; Blanchfield, J. T. Synthesis and in vitro evaluation of a library of modified endomorphin 1 peptides. *Bioorg. Med. Chem.* **2008**, *16*, 6286-6296.

[20] Wang, C. L.; Guo, C.; Wang, Y. Q.; Zhou, Y.; Li, Q.; Ni, J. M.; Wang, R. Synthesis and antinociceptive effects of endomorphin-1 analogs with C-terminal linked by oligo-arginine. *Peptides* **2011**, *32*, 293-299.

[21] Liu, H.; Zhang, B.; Liu, X.; Wang, C.; Ni, J.; Wang, R. Endomorphin-1 analogs with enhanced metabolic stability and systemic analgesic activity: design, synthesis, and pharmacological characterization. *Bioorg*. *Med*. *Chem*. **2007**, *15*, 1694-1702.

[22] Mallareddy, J. R.; Borics, A.; Keresztes, A.; Kover, K. E.; Tourwe, D.; Töth, G. Design, synthesis, pharmacological evaluation, and structure-activity study of novel endomorphin analogues with multiple structural modifications. *J. Med. Chem.* **2011**, *54*, 1462-1472.

[23] Nishio, K.; Nishimura, H.; Suyama, K.; Matsushima, A.; Nose, T.; Shimohigashi, Y. Halogenated Phe-containing endomorphin-2 analogs with mixed agonist and antagonist activities. *Pep. Science* **2013**, *49*, 25-26.

Selection of references reporting the K_i values DAMGO,[24] DPDPE,[25] and U50,488[26] from displacement binding experiments

[24] Harrison, C.; McNulty, S.; Smart, D.; Rowbotham, D. J.; Grandy, D. K.; Devi, L. A.; Lambert, D. G. The effects of endomorphin-1 and endomorphin-2 in CHO cells expressing recombinant μ-opioid receptors and SH-SY5Y cells. *Br. J. Pharmacol.* **1999**, *128*, 472-478.

[25] Schmidt, H.; Vormfelde, S.; Klinder, K.; Gundert-Remy, U.; Gleiter, C. H.; Skopp, G.; Aderjan, R.; Fuhr, U. Affinities of dihydrocodeine and its metabolites to opioid receptors. *Pharmacol*. *Toxicol*. **2002**, *91*, 57-63.

[26] Clark, J. A.; Pasternak, G. W. U50,488: a kappa-selective agent with poor affinity for μ1 opiate binding sites. *Neuropharm.* **1988**, *27*, 331−332.

Selection of references on physicochemical drug properties and CNS exposure[27,28,29,30]

[27] Clark, D. E. Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena. 2. Prediction of blood-brain barrier penetration. *J. Pharm. Sci*. **1999**, *88*, 815-821.

[28] Keserű, G. M.; Makara, G. M. The influence of lead discovery strategies on the properties of drug candidates. *Nat. Rev. Drug Discov.* **2009**, *8*, 203-212.

[29] Perola, E. An analysis of the binding efficiencies of drugs and their leads in successful drug discovery programs. *J. Med. Chem*. **2010**, *53*, 2986-2997.

[30] Wager, T. T.; Chandrasekaran, R. Y.; Hou, X.; Troutman, M. D.; Verhoest, P. R.; Villalobos, A.; Will, Y. Defining desirable central nervous system drug space through the alignment of molecular properties, in vitro ADME, and safety attributes. *ACS Chem. Neurosci*. **2010**, *1*, 420-434.