Modulation of the interaction between a peptide ligand and a G protein-coupled receptor by halogen atoms

Supporting Information

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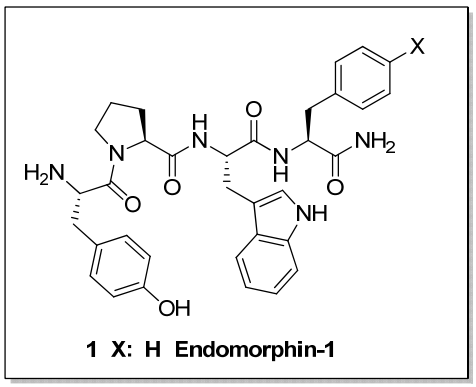
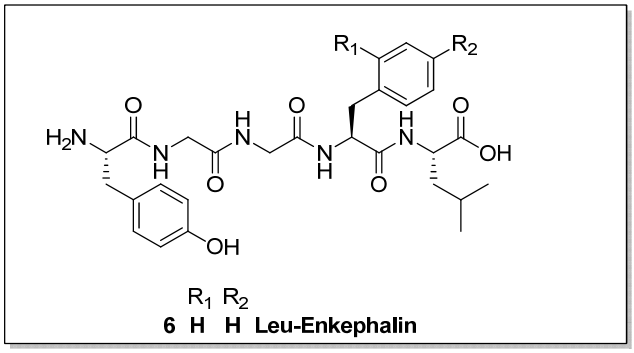


1 X: H Endomorphin-1

2 X: F [4-F-Phe^4]-Endo-1
3 X: Cl [4-Cl-Phe^4]-Endo-1
4 X: Br [4-Br-Phe$_4$]-Endo-1
5 X: I [4-I-Phe$_4$]-Endo-1

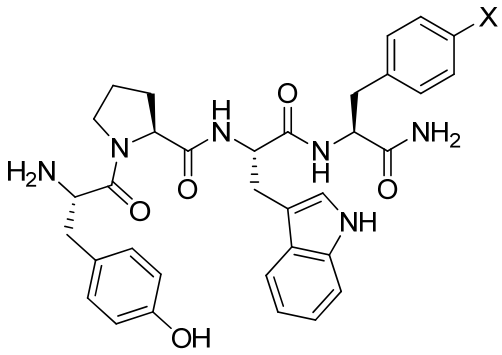


6 H H Leu-Enkephalin

[2-X-Phe^4]-Leu-ENK				**[4-X-Phe^4]-Leu-ENK**			
	R_1	R_2			R_1	R_2	
7	F	H	[2-F-Phe^4]-Leu-ENK	11	H	F	[4-F-Phe^4]-Leu-ENK
8	Cl	H	[2-Cl-Phe^4]-Leu-ENK	12	H	Cl	[4-Cl-Phe^4]-Leu-ENK
9	Br	H	[2-Br-Phe^4]-Leu-ENK	13	H	Br	[4-Br-Phe^4]-Leu-ENK
10	I	H	[2-I-Phe^4]-Leu-ENK	14	H	I	[4-I-Phe^4]-Leu-ENK

Scheme S1: Endomorphin and Leu-enkephalin analogues

Nomenclature

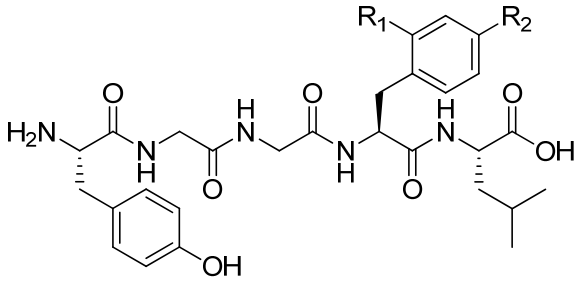


Scheme S2. Family of halogenated Endomorphin-1 analogues synthesized.

Peptide	X	Name	PEPTIDE
1	H	Endomorphin-1	H-Tyr-Pro-Trp-Phe-NH_2
2	F	[4-F-Phe^4]-Endo-1	H-Tyr-Pro-Trp-**(4-F)**Phe-NH_2
3	Cl	[4-Cl-Phe^4]-Endo-1	H-Tyr-Pro-Trp-**(4-Cl)**Phe-NH_2
4	Br	[4-Br-Phe^4]-Endo-1	H-Tyr-Pro-Trp-**(4-Br)**Phe-NH_2
5	I	[4-I-Phe^4]-Endo-1	H-Tyr-Pro-Trp-**(4-I)**Phe-NH_2

Table S1. Family of halogenated Endomorphin-1 analogues synthesized.

Nomenclature



Scheme S3. Family of halogenated Leu-Enkephalin analogues synthesized.

Peptide	R_1	R_2	Name	PEPTIDES
6	H	H	Leu-Enkephalin	H-Tyr-Gly-Gly-Phe-Leu-OH
7	F	H	[2-F-Phe^4]-Leu-ENK	H-Tyr-Gly-Gly-**(2-F)**Phe-Leu-OH
8	Cl	H	[2-Cl-Phe^4]-Leu-ENK	H-Tyr-Gly-Gly-**(2-Cl)**Phe-Leu-OH
9	Br	H	[2-Br-Phe^4]-Leu-ENK	H-Tyr-Gly-Gly-**(2-Br)**Phe-Leu-OH
10	I	H	[2-I-Phe^4]-Leu-ENK	H-Tyr-Gly-Gly-**(2-I)**Phe-Leu-OH
11	H	F	[4-F-Phe^4]-Leu-ENK	H-Tyr-Gly-Gly-**(4-F)**Phe-Leu-OH
12	H	Cl	[4-Cl-Phe^4]-Leu-ENK	H-Tyr-Gly-Gly-**(4-Cl)**Phe-Leu-OH
13	H	Br	[4-Br-Phe^4]-Leu-ENK	H-Tyr-Gly-Gly-**(4-Br)**Phe-Leu-OH
14	H	I	[4-I-Phe^4]-Leu-ENK	H-Tyr-Gly-Gly-**(4-I)**Phe-Leu-OH

Table S2. Family of halogenated Leu-Enkephalin analogues synthesized.

Computational studies

Molecular models of peptide-receptor complexes. Modeller 9v8[1] was used to model zebrafish μ- opioid receptors using the crystal structure of mouse μ-opioid receptor (PDB 4DKL)[2] as a template. The alignment of both sequences (85% of identity and 89% of similarity) is shown in Figure S2. Similarly, zebrafish δ1b-opioid receptor was modeled from human δ-opioid receptor (PDB 4N6H)[3]. These receptors share 87% of sequence identity and 89% of sequence similarity (Figure S2). No significant structural differences among the models obtained with Modeller were observed, given the high level of sequence identity and similarity between target and template. Thus, the model with the lowest energy was selected. The extended conformation of Endo-1 and LeuENK, together with the conformational restraint determined by NMR, were docked into the homology models using the Autodock Vina tool.[4] All docking solutions were visually inspected and the poses in which the *N*-terminus amine of the peptide forms an ionic interaction with Asp3.32 and Tyr[1] interacts with a conserved water molecule, as observed in crystal structures of opioid receptors in complex with non-peptidic ligands, were selected for energy minimization and MD simulations. In a second step, these complexes were embedded in a lipid bilayer (358 molecules of POPC) with explicit solvent (30,858 water molecules) and counterions (155 Na+ and 169 Cl-). Model systems were energy minimized and subsequently subjected to a 1 ns MD equilibration, with positional restraints on the Cα atoms of the receptor, to remove possible voids present in protein/lipids or protein/water interfaces. These restraints were released and 100 ns MD trajectories were produced at constant pressure and temperature, using the particle mesh Ewald method to evaluate electrostatic interactions with the GROMACS software[5] v4.53, the AMBER99SB force field for the amino acids, and Berger parameters for POPC lipids, using the protocol previously described.[6] Cl, Br, and I were modeled following the procedure proposed by Ibrahim[7], which consists in adding a positively charged particle (extra-point) on the opposite side of the C—X axis in order to reproduce the σ-hole. The C—X parameters and charges were those proposed by Ibrahim[7] but the mass and Van der Waal's radius of the extra-point was set to 1 and 0, respectively. The stability of the peptide-receptor complexes was monitored by root-mean-square deviations (rmsd). The molecular electrostatic potential depicted in Figure S3 were calculated with GAUSSIAN 09[8] using the B3LYP level of theory and the 6-31g** basis for F, Cl and Br and the lanl2dz8 basis set for I.

References:

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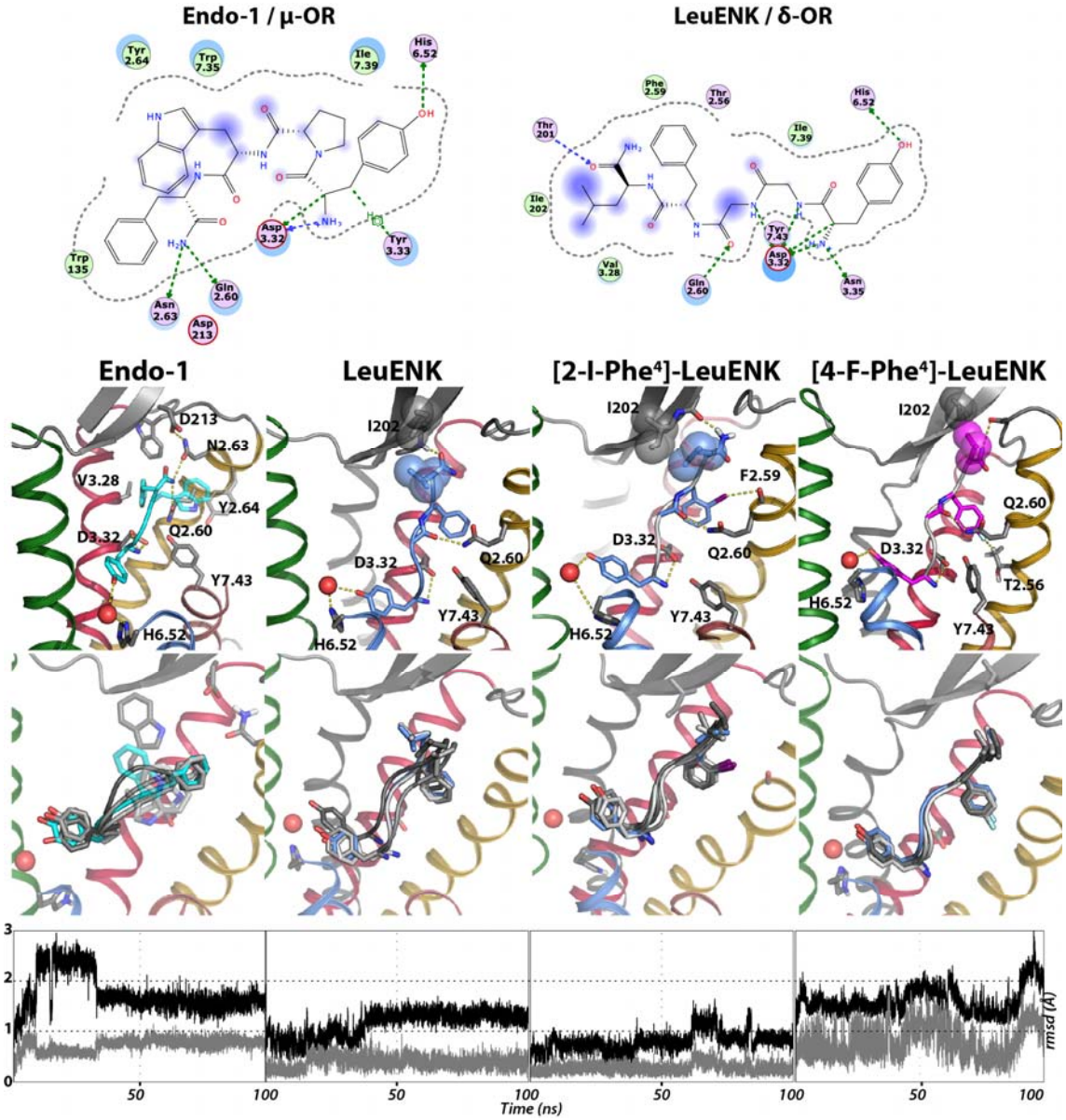

Figure S1. Computational models of the complexes between Endo-1, LeuENK, [2-I-Phe^4]-LeuENK and [4-F-Phe^4]-LeuENK with μ- and δ1b-opioid receptors. The *message* part of all peptides interacts in a similar manner in both receptors. The protonated N-terminus amine of the peptide forms an ionic interaction with Asp3.32, whereas Tyr^1 interacts with His6.52 via a conserved water molecule, present in all released crystal structures of opioid receptors (red spheres). The selective *address* part interacts differently in the two receptors. **Endo-1:** Trp^3 of Endo-1 (cyan) in complex with μ-OR makes an aromatic-aromatic stacking interaction with Tyr2.64 (in transparent) and Phe^4 in placed in a hydrophobic pocket created by Thr2.56, Phe2.59, Val3.38, and Trp127 in ECL 1. The –$CONH_2$ C-terminus forms a μ-specific hydrogen bond with Gln2.60 and Asn2.63. In the complex between LeuENK and its halogenated forms (light blue and magenta) and δ-ORs the Phe^4 in positioned in the same hydrophobic pocket as before, but with different conformation (see text and Figure 2D) while Leu5 forms hydrophobic interactions with δ-OR-specific Ile202 in ECL 2, and the –CONH2 C-terminus of the peptide hydrogen bonds with the exposed backbones of ECL2/ECL1. Schematic representations showing these interactions are shown (top). Representative snapshots (3 structures collected every 33 ns. plus the representative structures shown in first row) extracted from the molecular dynamics simulations of the peptide-receptor complexes are shown in middle panels (33ns in light gray, 66 ns in grey and 100ns in dark grey. Colored ligands are those shown in first row). The key proposed interactions remain stable

during the simulation. Root-mean-square deviations (rmsd) on peptides α-carbons (in grey) and all atoms (in black) throughout the molecular dynamics simulations are shown in bottom panels. The colour code of the helices is TMs 1 in white, 2 in yellow, 3 in red, 4 in gray, 5 in green, 6 in blue, and 7 in brown.

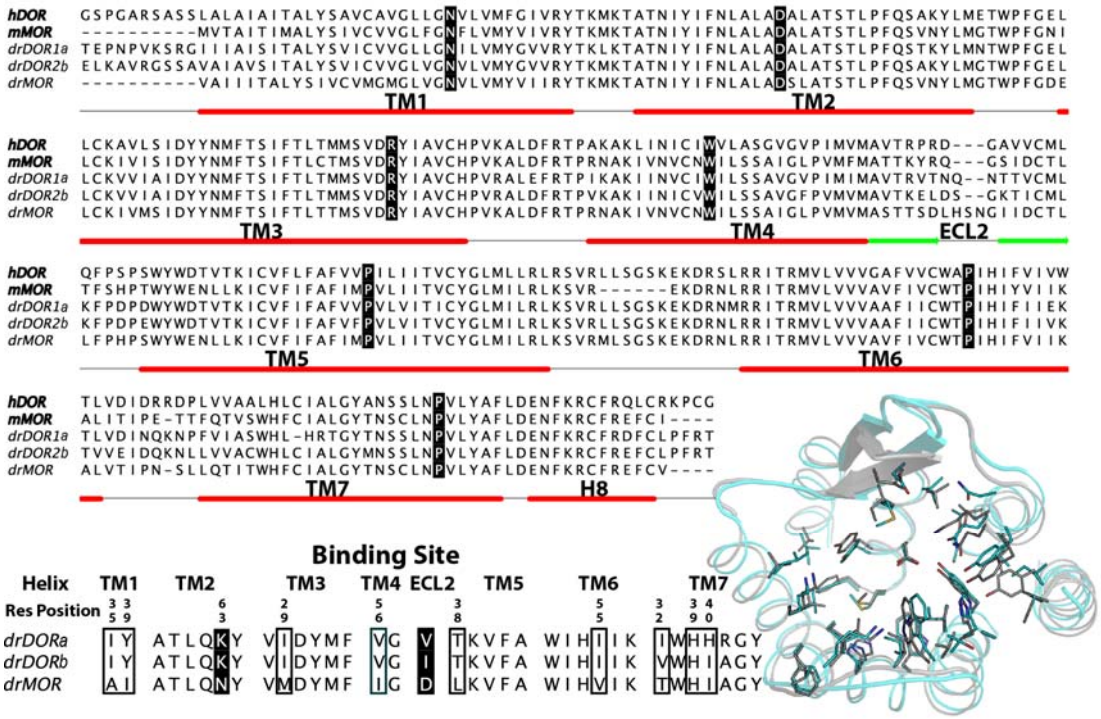

Figure S2. Sequence alignment of crystallized δ-OR and μ-OR (in bold) and danio rerio (dr) δa-OR, δb-OR and μ-OR (upper panel). The highly conserved residues TM.50 (Ballesteros numbering scheme) are shown in black. Sequence alignment of the residues forming the binding site (lower panel). Non-conserved but similar residues are boxed while chemically different residues are shown in black. These residues are displayed in sticks in the right lower panel, where the superposition of dr-δ-OR and dr-μ-OR homology models (grey and cyan, respectively) is shown.

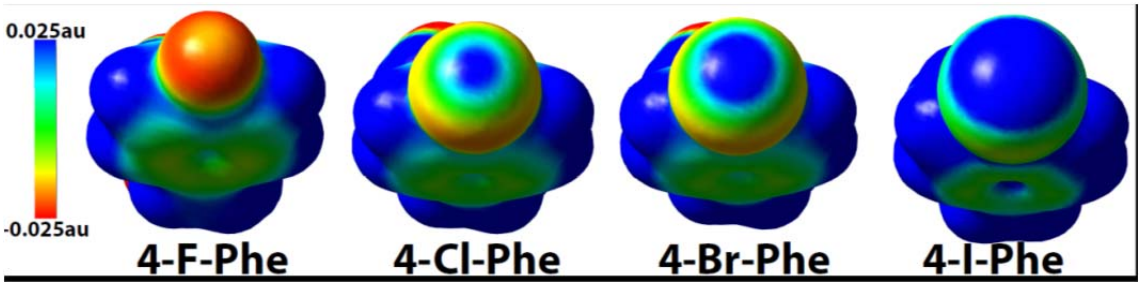

Figure S3. Electrostatic potential surfaces mapped on the surface of molecular electron density (0.02e au^{-3}) of *para* halogenated phenylalanine. The electrostatic potential shown varies between -0.025 (red) and 0.025 (blue) au. These maps show the type and extension of the charge of the σ-hole: a hollow negative charge for F and a positive charge, which increase in size and intensity with the mass of the halogen, for Cl, Br and I. The molecular electrostatic potential were calculated with GAUSSIAN 09 using the B3LYP level of theory and the 6-31g** basis for F, Cl and Br and the lanl2dz8 basis set for I.

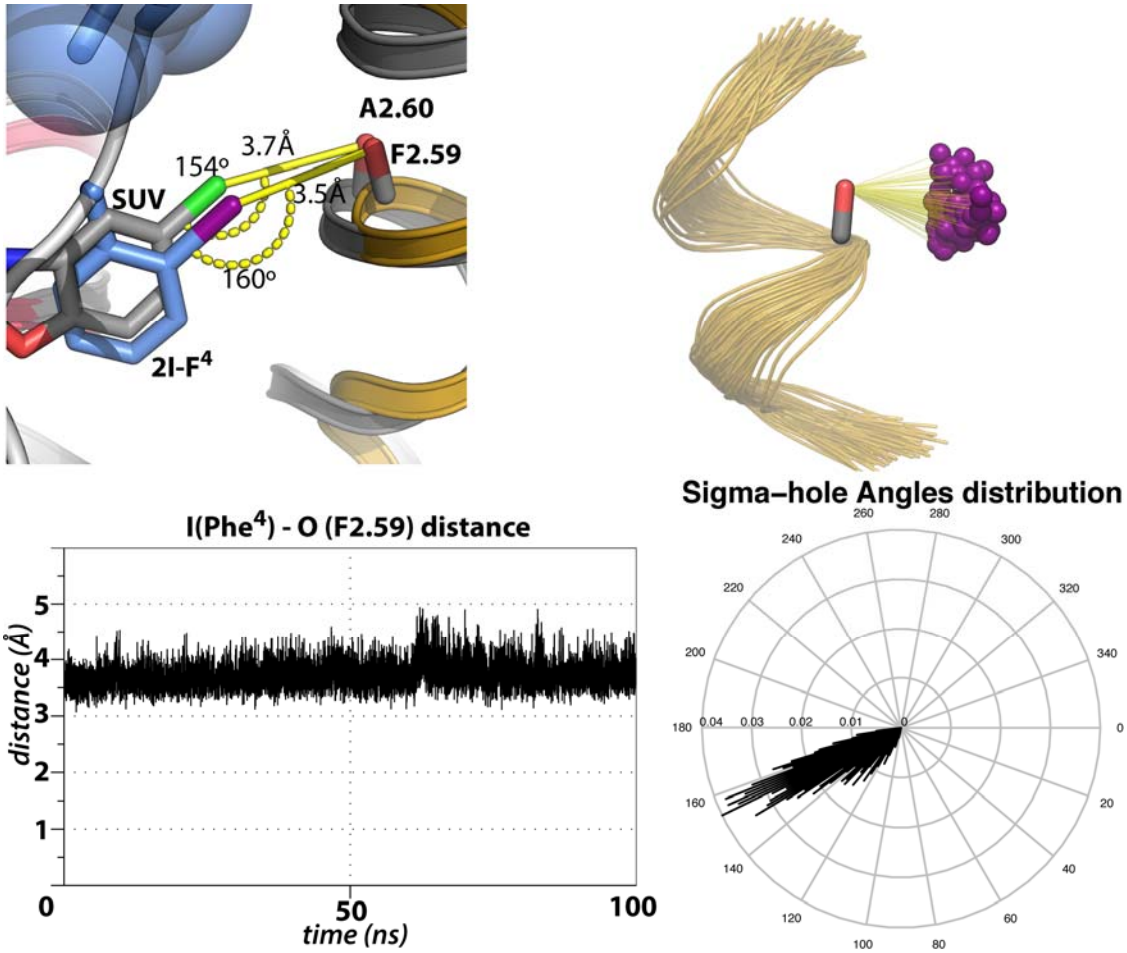

Figure S4. Detailed view of the crystal structure of OX_2 orexin receptor (grey cartoon) in complex with the chlorinated compound Suvorexant (SUV, in grey sticks) superposed to the homology model of δ1b-opioid receptor in complex with [2-I-Phe[4]]-LeuENK (upper left). Clearly, the observed halogen bond in the OX_2 crystal structure resembles the proposed halogen bond between [2-I-Phe[4]]-LeuENK and δ1b. Evolution of i) the interaction between the Iodine atom (spheres) and the carbonyl oxygen of residue Phe2.59 (upper right) (the C=O bond superposed the different snapshots), ii) the distance (Angströms) between the Iodine atom and the carbonyl oxygen of residue Phe2.59 (bottom left) and iii) distribution of the "sigma-hole" angle (C-X···O=C) (bottom right) along the 100ns MD trajectory. These values are in agreement with bibliographic data (9).

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NMR studies

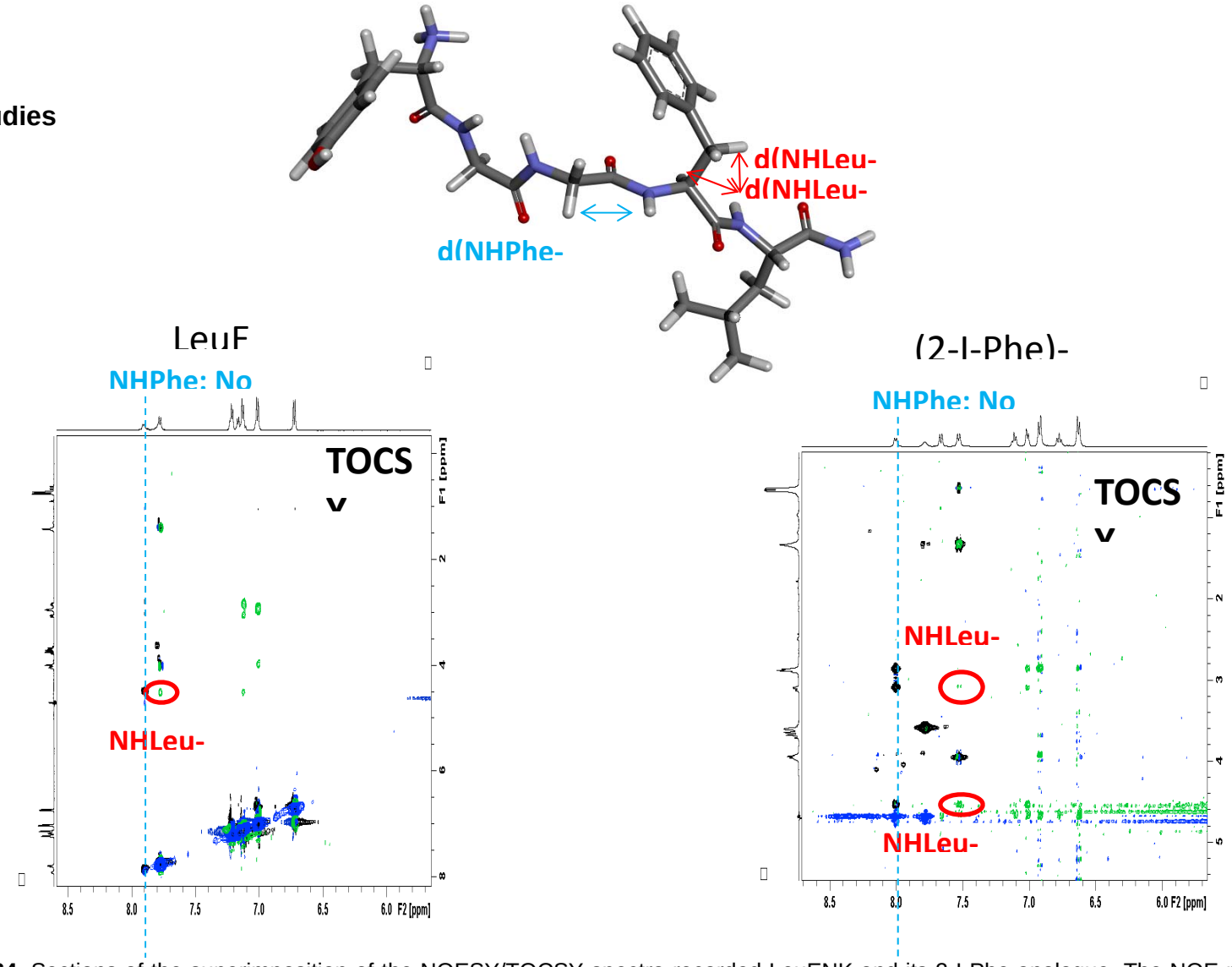

Figure S4. Sections of the superimposition of the NOESY/TOCSY spectra recorded LeuENK and its 2-I-Phe analogue. The NOE patterns are very similar and do not show any long range connectivity. This evidence strongly suggests the presence of major extended and flexible conformations in solution. One possible solution is shown on the top part of the figure.

Biological studies

EXPERIMENTAL SECTION

Drugs and radioligands. Naloxone (Nx) was purchased from Sigma-Aldrich (Madrid, Spain) and [^{3}H]-diprenorphine (DPN) (50 Ci/mmol) from Perkin-Elmer (Boston MA). All other reagents used were from analytical grade.

Cell culture and membrane preparation. Stably transfected HEK293 cells expressing the μ receptor (dre-*oprm1*, ENSEMBL gene ID ENSDARG00000039434), the δ1a receptor (dre-*oprd1a*, ENSEMBL gene ID ENSDARG00000041660) or the δ1b receptor (dre- *oprd1b*, ENSEMBL gene ID ENSDARG00000037159) from zebrafish were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 U mL-1 penicillin, 0.1 mg mL-1 streptomycin and 250 μg mL-1 Geneticin (G-418) (all from Gibco-BRL Life Technology Inc., Grand Island, NY, USA), at 37 ºC in humidified atmosphere containing 5% (v/v) CO_2 in a Forma incubator.

Cells were grown to 80% confluence, harvested in phosphate buffered saline (PBS) pH 7.4 containing 2 mM EDTA and collected by centrifugation at 500 g. The cell pellets were frozen at -80 ºC and resuspended in 50 mM Tris HCl buffer pH 7.4 (assay buffer) with protease inhibitors (0.1 mg mL^{-1} bacitracin, 3.3 μM captopril and protease inhibitor cocktail, from Sigma-Aldrich). Cell suspensions were homogenized with a Potter-Elvehjem tissue grinder in assay buffer and the homogenates were centrifuged at 500 g for 10 min at 4 ºC. The nuclear pellet was homogenized again, centrifuged and discarded. The two supernatants were combined, homogenized again with the tissue grinder and the membrane pellet was collected upon centrifugation at 18000 g for 30 min at 4 ºC. The crude membrane fraction was resuspended in ice-cold assay buffer with protease inhibitors and protein concentration was determined by Bradford (BioRad Laboratories, Alcobendas, Madrid, Spain).

Competition binding assays and data analysis. Radioligand binding was performed as previously described.[1] 10 μg protein were incubated with different concentrations of unlabelled ligand ranging from 0.3 nM to 10 μM, and using [^{3}H]-DPN as radioligand (the working concentration was similar to the affinity constant, KD = 1 nM for dre-μ and 3.4 nM for both δ receptors). Reactions were incubated for 1 h (for dre-μ and dre-δ1a) or for 4 h (for dre-δ1b) at 25 ºC in a final volume of 250 μL assay buffer. 10 μM Nx was used to determine nonspecific binding. After incubation, the reaction was stopped by adding 4 mL of ice-cold 50 mM Tris HCl buffer pH 7.4, the mixture was rapidly filtrated using a Brandel Cell Harvester and washed two times onto GF/B glass-fiber filters that were presoaked with 0.2% (v/v) polyethylenimine for at least 1 h. The filters were placed in scintillation vials and incubated overnight at room temperature in EcoScint A scintillation liquid (London, England). Radioactivity was counted using a Beckman Coulter 6500 scintillation counter (Pasadena, CA). All experiments were performed in triplicate and repeated three times.

Specific Binding was defined as the difference between total binding and non-specific binding, as measured in presence of 10 μM Nx. Radioligand binding data

were analyzed by computer-assisted non linear regression analysis using GraphPad Prism software (San Diego, CA, USA), and inhibition constants (Ki) were obtained for each ligand using Cheng and Prusoff's equation, which corrects for the concentration of radioligand used in each experiment as well as for the affinity of the radioligand for its binding site (K_D).[2]

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Figure S5: Diprenorphine displacement curves.

Competition binding assays of the different series of halogenated peptides on μ and δ opioid receptor membrane homogenates. Data were fit to the one-site competition model and each point represents the mean ± S.E.M. (capped bars) of three independent experiments performed in triplicate. Legends: parent compound (not halogenated): black; F: red; Cl: blue; Br: green; I: purple.

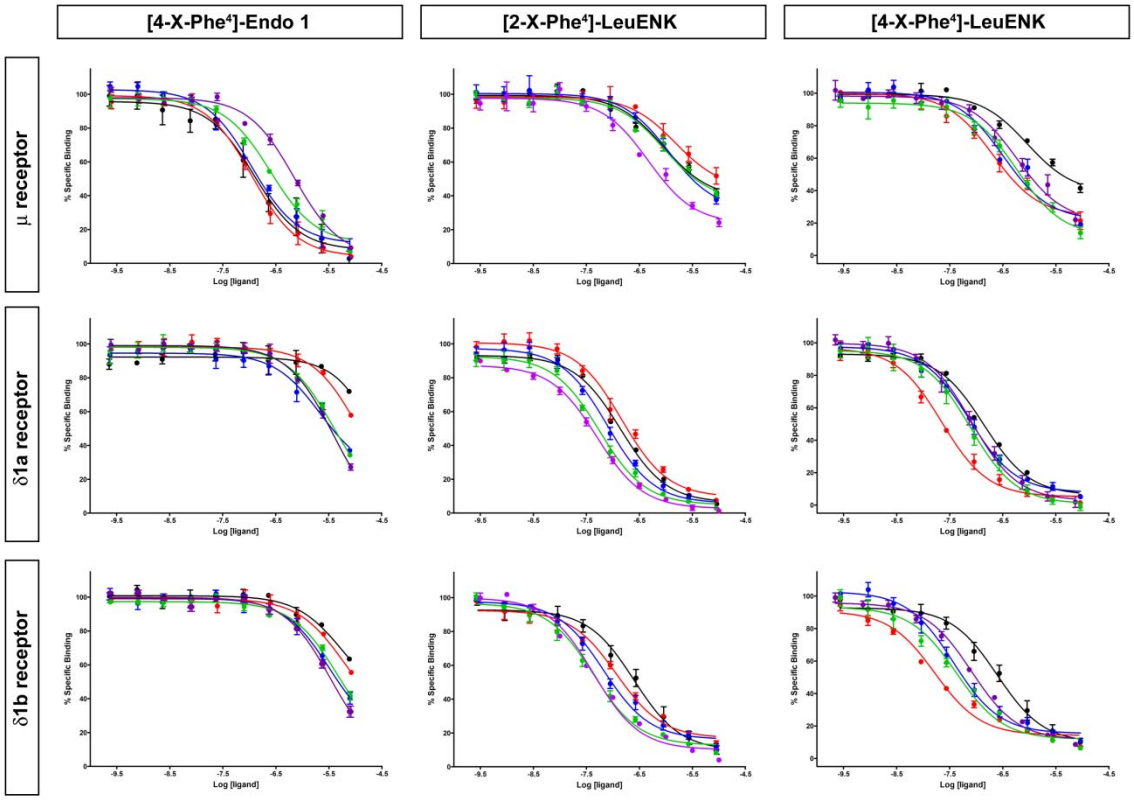
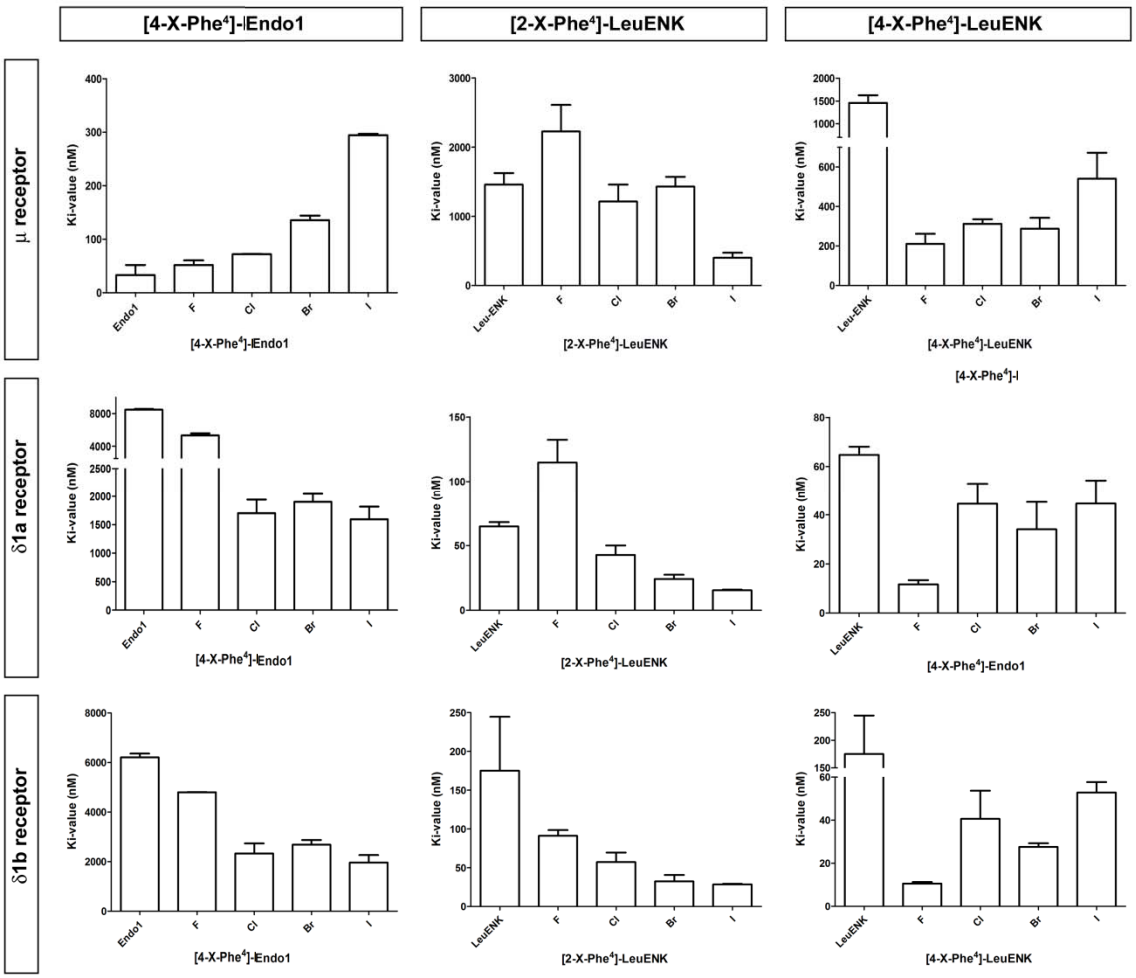


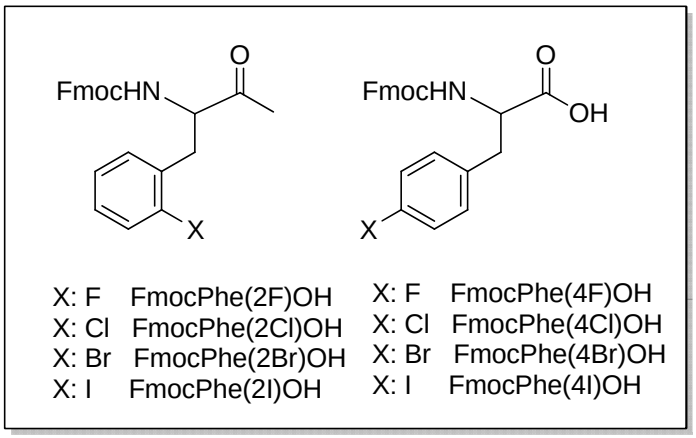
Figure S6: Ki-values obtained for each halogenated series of Endo1 and Leu-ENK when tested on the μ and δ receptors.



General procedure for Solid Phase Peptide Synthesis (SPPS).

Amino acids, building blocks (Scheme 4), coupling reagents and Wang resins were purchased from Novabiochem AG. All reagents used for synthesis were from Anal. grade. Peptides were synthesized manually following standard solid-phase methods and Fmoc protocols on Wang resin using amino acids with orthogonal protections on lateral chains. Amide couplings were performed manually in a peptide synthesis column using DIC/HOBt in DMF under reciprocal oscillating agitation. Coupling efficiencies were monitored by Kaiser ninhydrin test. Fmoc groups were removed with a 20% piperidine in DMF solution. Peptides were cleaved from the resin by shaking with a cleavage cocktail consisting of TFA:H_2O:TIS (95:2.5:2.5) for 2 h. The filtrate was evaporated, washed several times with cold ^{t}butyl methyl ether and concentrated under reduced pressure. Crude peptides were purified by C-18 RP-LC (VersaFlash^{TM} Flash Chromatography System) using a water-acetonitrile gradient. Analytical RP-HPLC were performed using the following solvents A (0.1% TFA in H_2O) and B (0.1% TFA in acetonitrile) and the Nucleosil 100 RP-18 (5µm) C18 column (4x 250 mm).

FmocPhe BUILDING BLOCKS

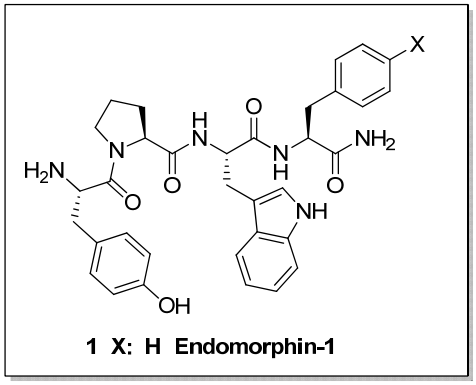


X: F FmocPhe(2F)OH
X: Cl FmocPhe(2Cl)OH
X: Br FmocPhe(2Br)OH
X: I FmocPhe(2I)OH

X: F FmocPhe(4F)OH
X: Cl FmocPhe(4Cl)OH
X: Br FmocPhe(4Br)OH
X: I FmocPhe(4I)OH

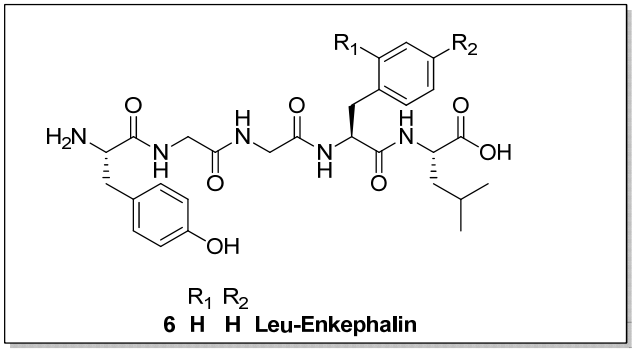
Scheme 4: Commercially available halogenated Phe building blocks used in SPPS (X: F, Cl, Br, and I).

Synthesis and characterization of peptides



1 X: H Endomorphin-1

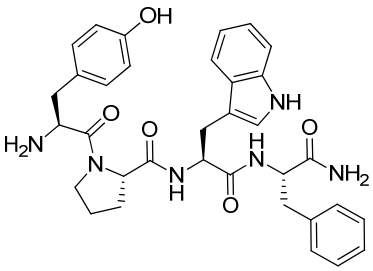
2 X: F [4-F-Phe^4]-Endo-1
3 X: Cl [4-Cl-Phe^4]-Endo-1
4 X: Br [4-Br-Phe$_4$]-Endo-1
5 X: I [4-I-Phe$_4$]-Endo-1



6 H H Leu-Enkephalin

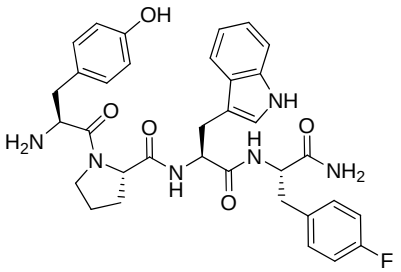
[2-X-Phe^4]-Leu-ENK				**[4-X-Phe^4]-Leu-ENK**			
	R_1	R_2			R_1	R_2	
7	F	H	[2-F-Phe^4]-Leu-ENK	11	H	F	[4-F-Phe^4]-Leu-ENK
8	Cl	H	[2-Cl-Phe^4]-Leu-ENK	12	H	Cl	[4-Cl-Phe^4]-Leu-ENK
9	Br	H	[2-Br-Phe^4]-Leu-ENK	13	H	Br	[4-Br-Phe^4]-Leu-ENK
10	I	H	[2-I-Phe^4]-Leu-ENK	14	H	I	[4-I-Phe^4]-Leu-ENK

Synthesis and characterization of endomorphin-1 (1)



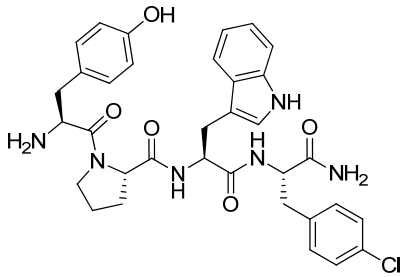
Peptide 1 (H-Tyr-Pro-Trp-Phe-NH_2) was synthesized from 100 mg (0,067 mmol) of Fmoc-Rink Amide resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-Phe-OH, Fmoc-Trp(Boc)-OH, Fmoc-Pro-OH and Fmoc-Tyr(OtBu)-OH (4 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (20:80) (ACN:H_2O) to (100:0) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 36 mg of endomorphin-1. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 12,86 min). UPLC-HRMS (ESI/TOF): 611,2974 ([M+H]$^+$, $C_{34}H_{39}N_6O_5^+$; calc. 611,2982). Peptidic content 76,5% (Elemental analysis 10,5% N; $C_{34}H_{38}N_6O_5$ calc. 11,6% N).

[4-F-Phe^4]-endomorphin-1 (2)



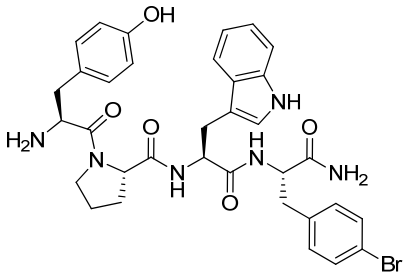
Peptide **2** (H-Tyr-Pro-Trp-(4-F)Phe-NH_2) was synthesized from 200 mg (0,122 mmol) of Fmoc-Rink Amide resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(4-F)Phe-OH (2 eq., 20 h), Fmoc-Trp(N-Boc)-OH, Fmoc-Pro-OH and Fmoc-Tyr(tBu)-OH (4 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (20:80) (ACN:H_2O) to (100:0) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 56 mg of [4-F-Phe^4]-endomorphin-1, as an orange solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 12,61 min). UPLC-HRMS(ESI/TOF): 629,2869 ([M+H]$^+$, $C_{34}H_{38}N_6O_5F^+$; calc. 629,2888). Peptidic content 83.0% (Elemental analysis 11,1% N; $C_{34}H_{37}N_6O_5F$ calc. 13,3% N).

[4-Cl-Phe^4]endomorphin-1 (3)



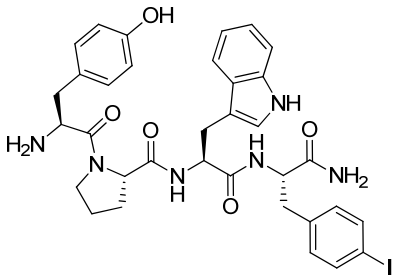
Peptide **3** (H-Tyr-Pro-Trp-(4-Cl)Phe-NH_2) was synthesized from 300 mg (0,183 mmol) of Fmoc-Rink Amide resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(4-Cl)Phe-OH (2 eq., 20 h), Fmoc-Trp(N-Boc)-OH, Fmoc-Pro-OH and Fmoc-Tyr(tBu)-OH (4 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (20:80) (ACN:H_2O) to (100:0) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 39 mg of [4-Cl-Phe^4]-endomorphin-1, as a yellow solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 14,51 min). UPLC-HRMS(ESI/TOF): 645,2601 ([M+H]$^+$, $C_{34}H_{38}N_6O_5Cl^+$; calc. 645,2592). Peptidic content 92.4% (Elemental analysis 11,0% N; $C_{34}H_{37}N_6O_5Cl$ calc. 11,9% N).

[4-Br-Phe^4]endomorphin-1 (4)



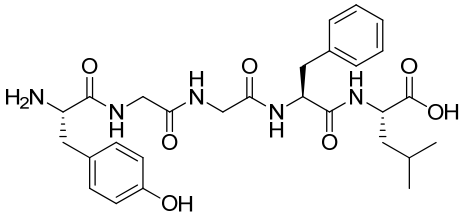
Peptide **4** (H-Tyr-Pro-Trp-(4-Br)Phe-NH_2) was synthesized from 200 mg (0,122 mmol) of Fmoc-Rink Amide resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(4-Br)Phe-OH (2 eq., 20 h), Fmoc-Trp(N-Boc)-OH, Fmoc-Pro-OH and Fmoc-Tyr(tBu)-OH (20 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (20:80) (ACN:H_2O) to (100:0) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 84 mg of [4-Br-Phe^4]-endomorphin-1 as an orange solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 13,97 min). UPLC-HRMS(ESI/TOF): 689,2084 ([M+H]$^+$, $C_{34}H_{38}N_6O_5{}^{79}Br^+$; calc. 689,2087), 691,2067 ([M+H]$^+$, $C_{34}H_{38}N_6O_5{}^{81}Br^+$; calc. 691,2067). Peptidic content 79,6% (Elemental analysis 9,7% N; $C_{34}H_{37}N_6O_5Br$ calc. 12,2% N).

[4-I-Phe^4]endomorphin-1 (5)



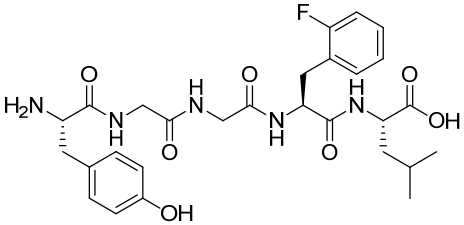
Peptide **5** (H-Tyr-Pro-Trp-(4-I)Phe-NH_2) was synthesized from 200 mg (0,122 mmol) of Fmoc-Rink Amide resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(4-I)Phe-OH (2 eq., 20 h), Fmoc-Trp(Boc)-OH, Fmoc-Pro-OH and Fmoc-Tyr(tBu)-OH (20 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (20:80) (ACN:H_2O) to (100:0) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 47 mg of [4-I-Phe^4]-endomorphin-1 as a yellow solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 14,62 min). UPLC-HRMS(ESI/TOF): 737,1962 ([M+H]$^+$, $C_{34}H_{38}N_6O_5I^+$; calc. 737,1948). Peptidic content 82,4% (Elemental analysis 9,4% N; $C_{34}H_{37}N_6O_5I$ calc. 11,4% N).

Leu-enkephalin (Leu-ENK) (6)



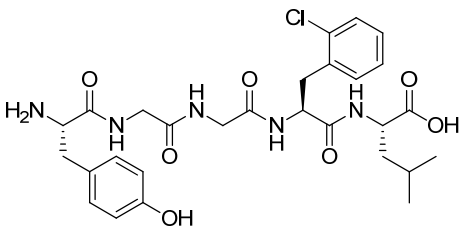
Leu-ENK (**6**) (H-Tyr-Gly-Gly-Phe-Leu-OH) was synthesized from 120 mg (0,067 mmol) of Fmoc-Leu-Wang resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH. Crude was purified by Prep. RP-HPLC with *Versaflash*™ (From (5:95) (ACN:H_2O) to (50:50) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 30 mg of Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 11,3 min). UPLC-HRMS (ESI/TOF): 590,2385 ([M+H]$^+$, $C_{28}H_{38}N_5O_7^+$; calc. 555,.2693). Peptidic content 76,1% (Elemental analysis 9,3% N; $C_{28}H_{37}N_5O_7$ calc. 12,2% N).

[2-F-Phe^4]-Leu-ENK (7)



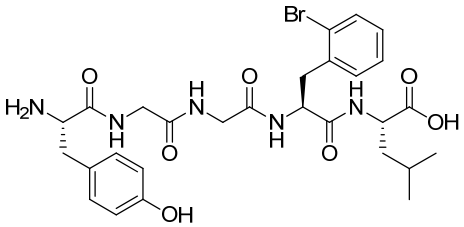
Peptide **7** (H-Tyr-Gly-Gly-(2-F)Phe-Leu-OH) was synthesized from 120 mg (0.087 mmol) of Fmoc-Leu-Wang resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(2-F)Phe-OH (2eq., 20 h), Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH (5 h). Crude was purified by Prep. RP-HPLC with *Versaflash*™ (From (5:95) (ACN:H_2O) to (50:50) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 34 mg of [2-F-Phe^4]-Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 9,7 min). UPLC-HRMS(ESI/TOF): 574,2670 ([M+H]$^+$, $C_{28}H_{37}N_5O_7F^+$; calc. 574,2677). Peptidic content 89,0% (Elemental analysis 11,2% N; $C_{28}H_{36}N_5O_7F$ calc. 12,6% N).

[2-Cl-Phe^4]-Leu-ENK (8)



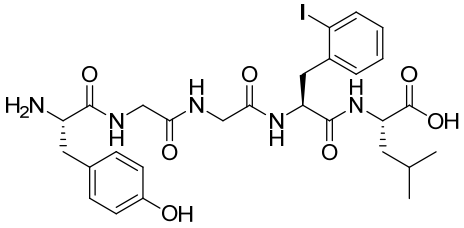
Peptide **8** (H-Tyr-Gly-Gly-(2-Cl)Phe-Leu-OH) was synthesized from 120 mg (0.087 mmol) of Fmoc-Leu-Wang resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(2-Cl)Phe-OH (2eq., 20 h), Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH (5 h). Crude was purified by Prep. RP-HPLC with *Versaflash*™ (From (5:95) (ACN:H_2O) to (50:50) (ACN:H_2O) in 50 with a RP-C_{18} cartridge) to obtain 30 mg of [2-Cl-Phe^4]-Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 12,6 min). UPLC-HRMS (ESI/TOF): 590,2385 ([M+H]$^+$, $C_{28}H_{37}N_5O_7Cl^+$; calc. 590,2382). Peptidic content 91,6% (Elemental analysis 10,9% N; $C_{28}H_{36}N_5O_7Cl$ calc. 11,9% N).

[2-Br-Phe^4]-Leu-ENK (9)



Peptide **9** (H-Tyr-Gly-Gly-(2-Br)Phe-Leu-OH) was synthesized from 120 mg (0.087 mmol) of Fmoc-Leu-Wang resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(2-Br)Phe-OH (2 eq., 20 h), Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH (5 h). Crude was purified by Prep. RP-HPLC with *Versaflash*™ (From (5:95) (ACN:H_2O) to (50:50) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 10 mg of [2-Br-Phe^4]-Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 11,9 min). UPLC-HRMS(ESI/TOF): 634,1891 ([M+H]$^+$, $C_{28}H_{37}N_5O_7Br^+$; calc. 634,1849). Peptidic content 88,4% (Elemental analysis 9,8% N; $C_{28}H_{36}N_5O_7Br$ calc. 11,0% N).

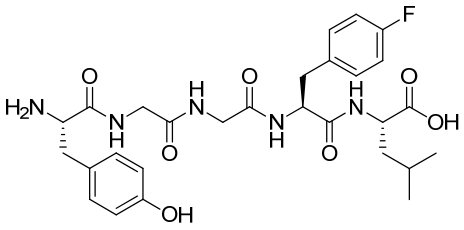
[2-I-Phe^4]-Leu-ENK (10)



Peptide **10** (H-Tyr-Gly-Gly-(2-I)Phe-Leu-OH) was synthesized from 150 mg (0.096 mmol) of Fmoc-Leu-Wang resin (substitution: 0,64 mmol/g resin) using following protected peptides: Fmoc-(2-I)Phe-OH (2 eq., 20 h), Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH. Crude was purified by Prep. RP-HPLC with *Versaflash*™ (From (5:95) (ACN:H_2O) a (50:50) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 42

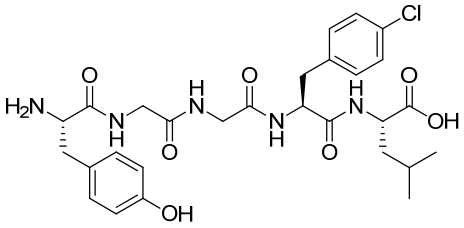
mg of [2-I-Phe^4]-Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 13,4 min). UPLC-HRMS (ESI/TOF): 682.1768 ([M+H]$^+$, $C_{28}H_{37}N_5O_7I^+$; calc. 682.1762). Peptidic content 89.3% (Elemental analysis 9,18% N; $C_{28}H_{36}N_5O_7I$ calc. 10,3% N).

[4-F-Phe^4]-Leu-ENK (11)



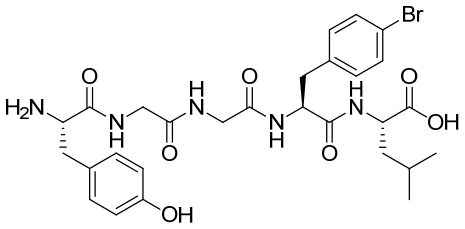
Peptide **11** [H-Tyr-Gly-Gly-(4-F)Phe-Leu-OH] was synthesized from 120 mg (0.087 mmol) of Fmoc-Leu-Wang resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(4-F)Phe-OH (2 eq., 20 h), Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH (5 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (5:95) (ACN:H_2O) to (50:50) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 29 mg of [4-F-Phe^4]-Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 11,8 min). UPLC-HRMS (ESI/TOF): 574,2670 ([M+H]$^+$, $C_{28}H_{37}N_5O_7F^+$; calc. 574,2677). Peptidic content 91,3% (Elemental analysis 11,5% N; $C_{28}H_{36}N_5O_7F$ calc. 12,6% N).

[4-Cl-Phe^4]-Leu-ENK (12)



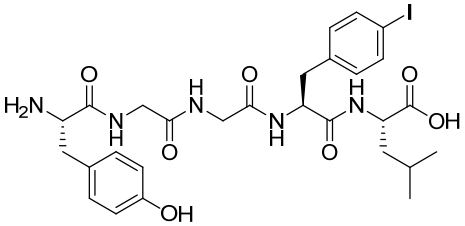
Peptide **12** [H-Tyr-Gly-Gly-(4-Cl)Phe-Leu-OH] was synthesized from 120 mg (0.087 mmol) of Fmoc-Leu-Wang resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(4-Cl)Phe-OH (2 eq., 20 h), Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH (5 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (5:95) (ACN:H_2O) to (50:50) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 37 mg of [4-Cl-Phe^4]-Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% Bin 25 min, r.t.: 12,6 min). UPLC-HRMS (ESI/TOF): 590,2376 ([M+H]$^+$, $C_{28}H_{37}N_5O_7Cl^+$; calc. 590,2382). Peptidic content 92,4% (Elemental analysis 11,0% N; $C_{28}H_{36}N_5O_7Cl$ calc. 11,9% N).

[4-Br-Phe^4]-Leu-ENK (13)



Peptide **13** [H-Tyr-Gly-Gly-(4-Br)Phe-Leu-OH] was synthesized from 120 mg (0.087 mmol) of Fmoc-Leu-Wang resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(4-Br)Phe-OH (2 eq., 20 h), Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH (5 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (5:95) (ACN:H_2O) to (50:50) (ACN:H_2O) in 50 min with a RP-C_{18} cartridge) to obtain 40 mg of [4-Br-Phe^4]-Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% Bin 25 min, r.t.: 13,2 min). UPLC-HRMS (ESI/TOF): 634,1849 ([M+H]$^+$, $C_{28}H_{37}N_5O_7Br^+$; calc. 634,1849). Peptidic content 91,8% (Elemental analysis 10,1% N; $C_{28}H_{36}N_5O_7Br$ calc. 11,0% N).

[4-I-Phe^4]-Leu-ENK (14)



Peptide **14** [H-Tyr-Gly-Gly-(4-I)Phe-Leu-OH] was synthesized from 120 mg (0.087 mmol) of Fmoc-Leu-Wang resin (substitution: 0,61 mmol/g resin) using following protected peptides: Fmoc-(4-I)Phe-OH (2 eq., 20 h), Fmoc-Gly-OH, Fmoc-Gly-OH and Fmoc-Tyr(tBu)-OH (5 h). Crude was purified by Prep. RP-HPLC with *Versaflash*^{TM} (From (5:95) (ACN:H_2O) to (50:50) (ACN:H_2O) in 50 min with RP-C_{18} cartridge) to obtain 36 mg of [4-I-Phe^4]-Leu-ENK as a white solid. Characterization: Anal. RP-HPLC (20-80% B in 25 min, r.t.: 14,4 min). UPLC-HRMS (ESI/TOF): 682.1762 ([M+H]$^+$, $C_{28}H_{37}N_5O_7I^+$; calc. 682.1762). Peptidic content 82,4% (Elemental analysis 9,4% N; $C_{28}H_{36}N_5O_7I$ calc. 11,4% N).

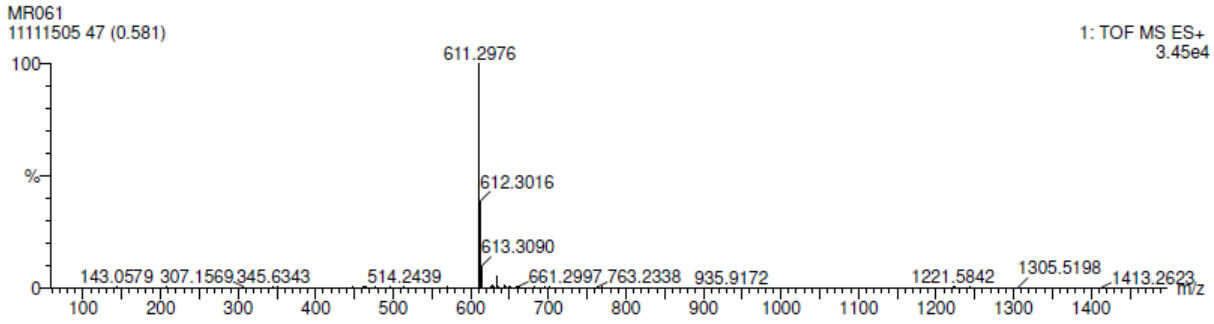
HRMS (UPLC-TOF/MS) OF PEPTIDES

HRMS spectra (UPLC-TOF/MS) were recorded on a Waters ACQUITY UPLC System with Waters LCT Premier™ XE Mass Spectrometer operating either in the positive ion electrospray mode or in negative electrospray mode. Water and acetonitrile were used as carrier solvents.

Table

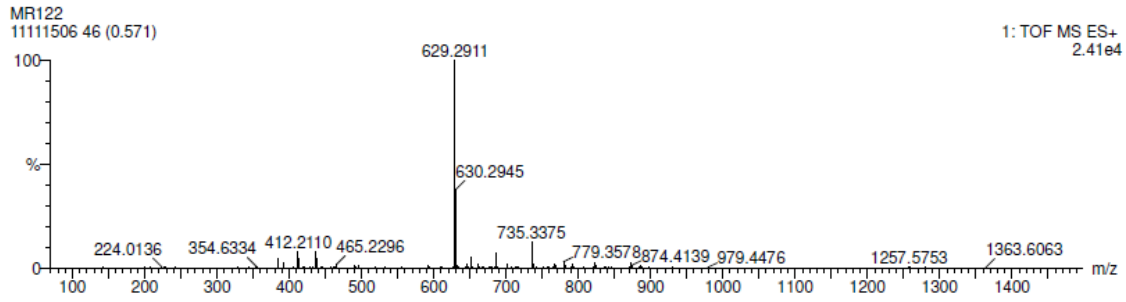
Peptide	Name	FORMULA $([M+H]^+$	*m/z Calc. $([M+H]^+$*	m/z FOUND $([M+H]^+$
Endomorphin-1 and halogenated analogues				
1	Endomorphin-1	$C_{34}H_{39}N_6O_5^+$	*611,2982*	611,2974
2	[4-F-Phe^4]-Endo-1	$C_{34}H_{38}N_6O_5F^+$	*629,2911*	629,2888
3	[4-Cl-Phe^4]-Endo-1	$C_{34}H_{38}N_6O_5Cl^+$	*645,2592*	645,2579
4	[4-Br-Phe^4]-Endo-1	$C_{34}H_{38}N_6O_5{}^{79}Br^+$ $C_{34}H_{38}N_6O_5{}^{81}Br^+$	*689,2087* *691,2067*	689,2025 691,2043
5	[4-I-Phe^4]-Endo-1	$C_{34}H_{38}N_6O_5I^+$	*737,1960*	737,1948
Leu-ENK and halogenated analogues				
6	Leu-ENK	$C_{28}H_{38}N_5O_7^+$	*556,2771*	556,2781
7	[2-F-Phe^4]-Leu-ENK	$C_{28}H_{37}N_5O_7F^+$	*574,2677*	574,2646
8	[2-Cl-Phe^4]-Leu-ENK	$C_{28}H_{37}N_5O_7Cl^+$	*590,2350*	590,2382
9	[2-Br-Phe^4]-Leu-ENK	$C_{28}H_{37}N_5O_7{}^{79}Br^+$ $C_{28}H_{37}N_5O_7{}^{81}Br^+$	*634,1876* *636,1856*	634,1882 636,1902
10	[2-I-Phe^4]-Leu-ENK	$C_{28}H_{37}N_5O_7I^+$	*682.1738*	682.1768
11	[4-F-Phe^4]-Leu-ENK	$C_{28}H_{37}N_5O_7F^+$	*574,2677*	574,2687
12	[4-Cl-Phe^4]-Leu-ENK	$C_{28}H_{37}N_5O_7Cl^+$	*590,2382*	590,2376
13	[4-Br-Phe^4]-Leu-ENK	$C_{28}H_{37}N_5O_7{}^{79}Br^+$ $C_{28}H_{37}N_5O_7{}^{81}Br^+$	*634,1876* *636,1856*	634,1868 636,1847
14	[4-I-Phe^4]-Leu-ENK	$C_{28}H_{37}N_5O_7I^+$	*682.1738*	682.1736

HRMS of endomorphin-1 (1)



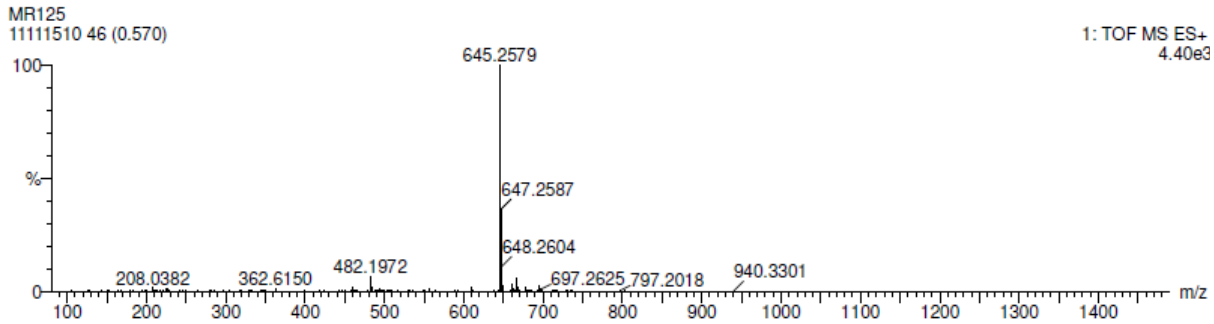
Minimum:				-1.5		
Maximum:		10.0	10.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
611.2976	611.2982	-0.6	-1.0	18.5	20.2	C34 H39 N6 O5

HRMS of [4-F-Phe^4]-ENDOMORPHIN-1 (2)



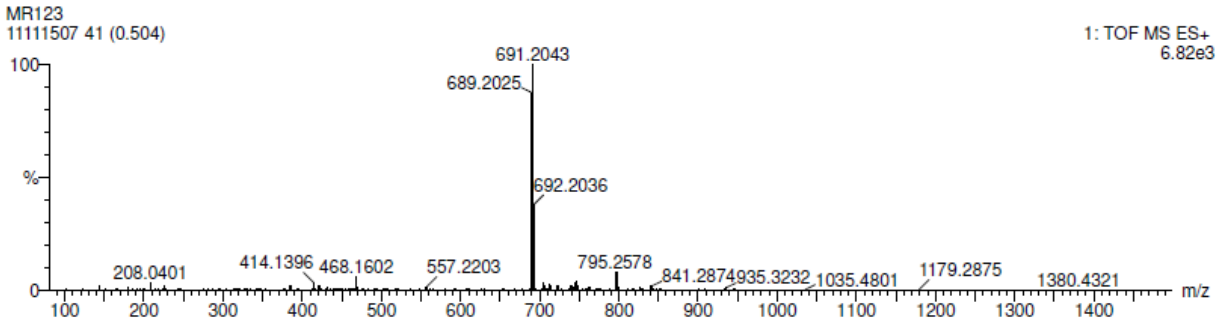
Minimum:				-1.5		
Maximum:		10.0	10.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
629.2911	629.2888	2.3	3.7	18.5	32.1	C34 H38 N6 O5 F

HRMS of [4-Cl-Phe^4]-endomorphin-1 (3)



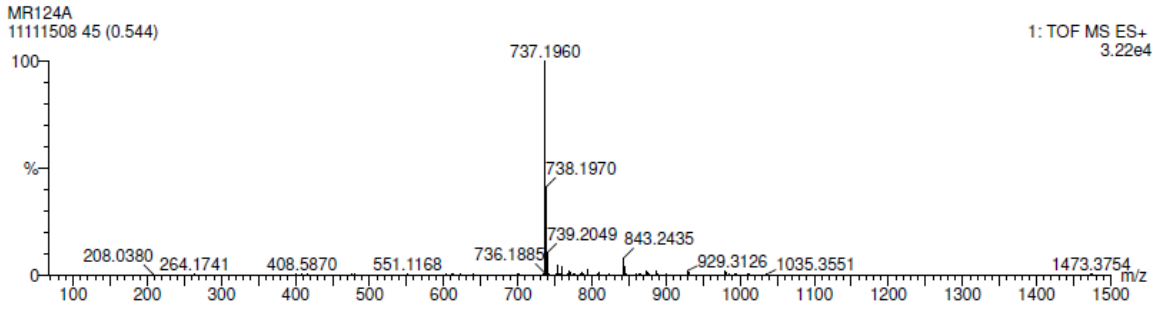
Minimum:				-1.5		
Maximum:		10.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
645.2579	645.2592	-1.3	-2.0	18.5	20.5	C34 H38 N6 O5 Cl

HRMS of [4-Br-Phe^4]-endomorphin-1 (4)



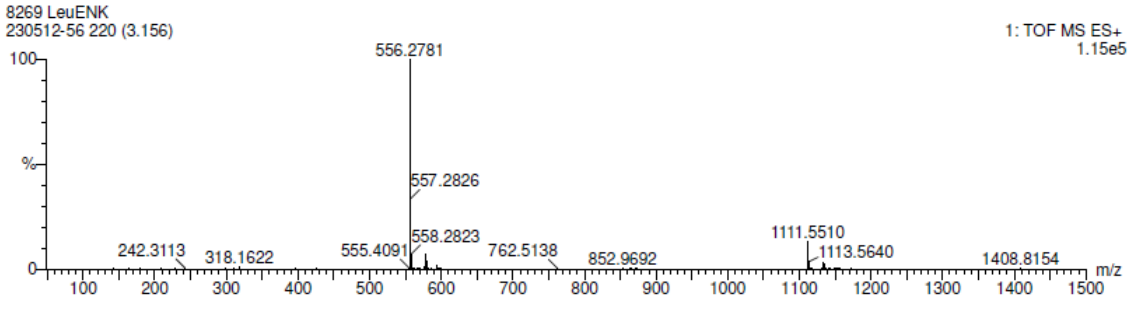
Minimum:	80.00				-1.5		
Maximum:	100.00		10.0	10.0	50.0		
Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
689.2025	87.54	689.2087	-6.2	-9.0	18.5	16.2	C34 H38 N6 O5 79Br
691.2043	100.00	691.2067	-2.4	-3.5	18.5	4.8	C34 H38 N6 O5 81Br

HRMS of [4-I-Phe^4]-endomorphin-1 (5)



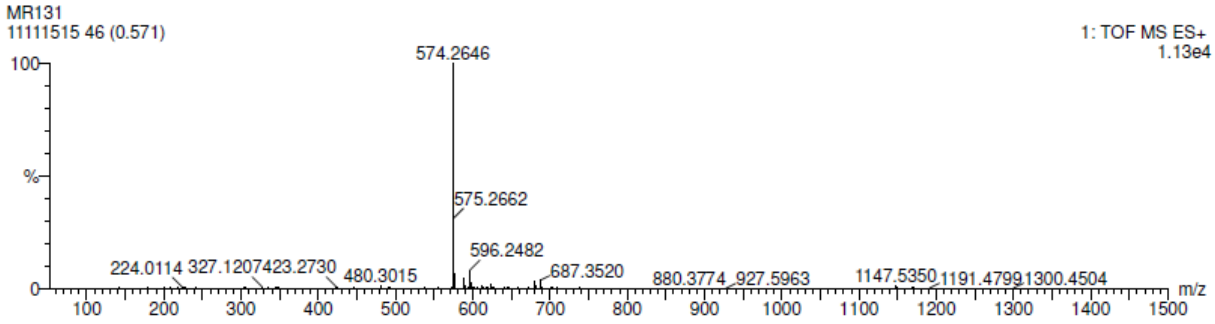
Minimum:				-1.5		
Maximum:		10.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
737.1960	737.1948	1.2	1.6	18.5	19.4	C34 H38 N6 O5 I

HRMS of Leu-enkephalin (6)



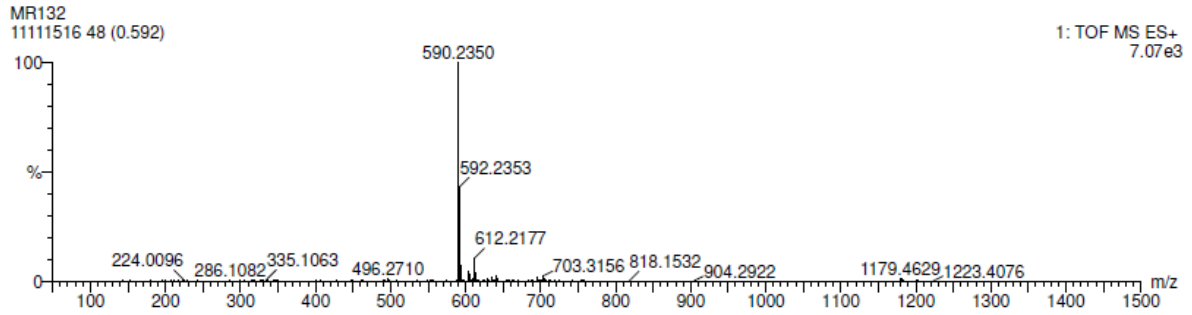
Minimum:				-1.5		
Maximum:		10.0	30.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
556.2781	556.2771	1.0	1.8	12.5	8.4	C28 H38 N5 O7

HRMS of [2-F-Phe^4]-Leu-ENK (7)



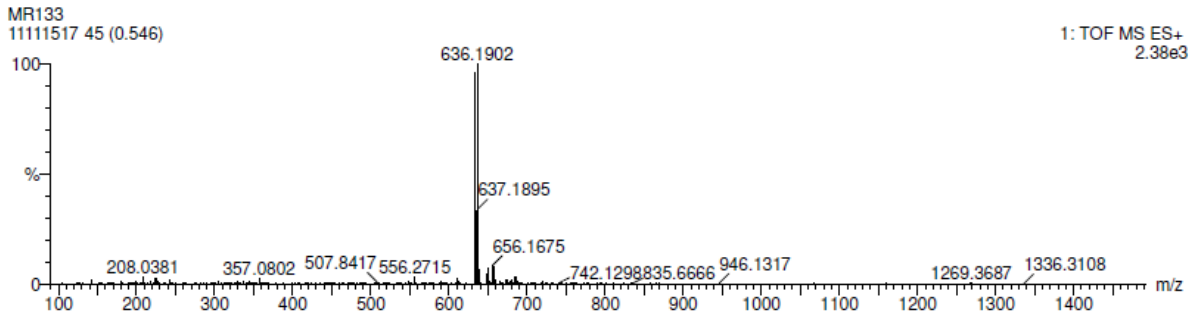
Minimum:				-1.5		
Maximum:		10.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
574.2646	574.2677	-3.1	-5.4	12.5	11.2	C28 H37 N5 O7 F

HRMS of [2-Cl-Phe^4]-Leu-ENK (8)



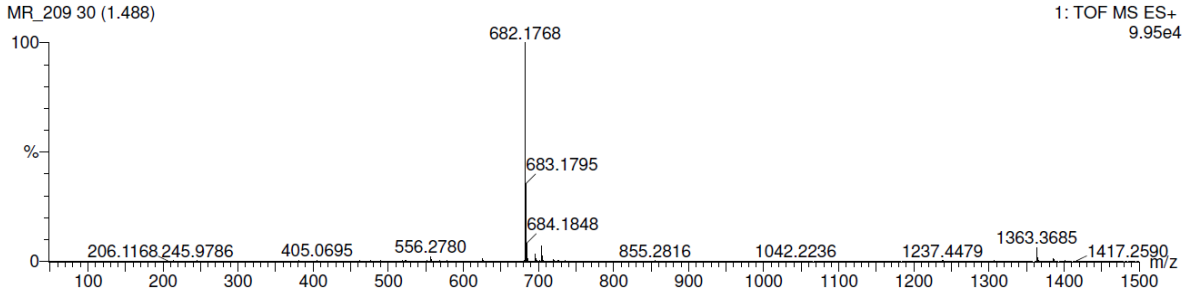
Minimum:				-1.5		
Maximum:		10.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
590.2350	590.2382	-3.2	-5.4	12.5	23.4	C28 H37 N5 O7 Cl

HRMS of [2-Br-Phe^4]-Leu-ENK (9)



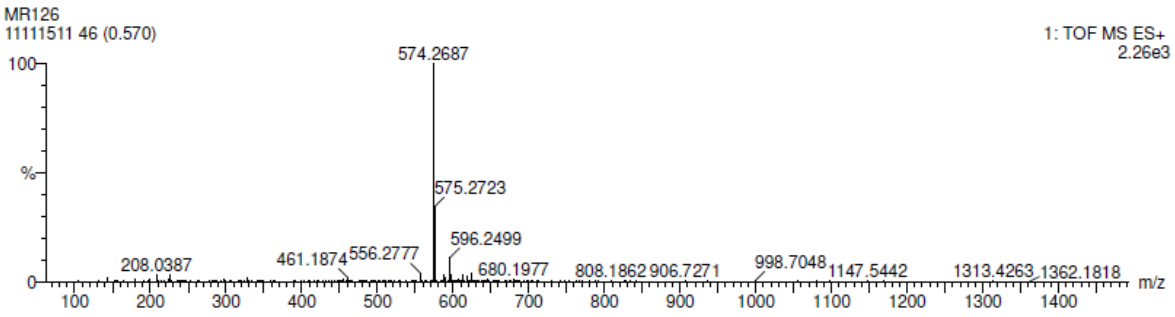
Minimum:	80.00				-1.5			
Maximum:	100.00		10.0	20.0	50.0			
Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula	
634.1882	95.64	634.1876	0.6	0.9	12.5	0.4	C28 H37 N5 O7	79Br
636.1902	100.00	636.1856	4.6	7.2	12.5	1.0	C28 H37 N5 O7	81Br

HRMS of [2-I-Phe^4]-Leu-ENK (10)



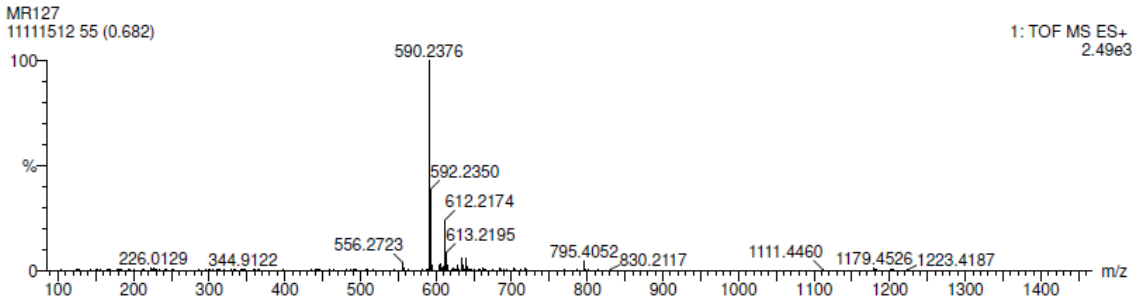
Minimum:				-1.5		
Maximum:		10.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
682.1768	682.1738	3.0	4.4	12.5	100.7	C28 H37 N5 O7 I

HRMS of [4-F-Phe^4]-Leu-ENK (11)



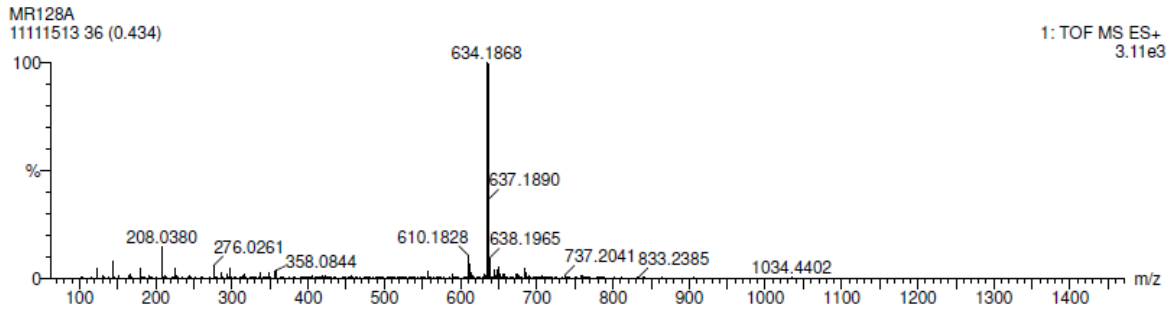
Minimum:				-1.5		
Maximum:		10.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
574.2687	574.2677	1.0	1.7	12.5	0.1	C28 H37 N5 O7 F

HRMS of [4-Cl-Phe^4]-Leu-ENK (12)



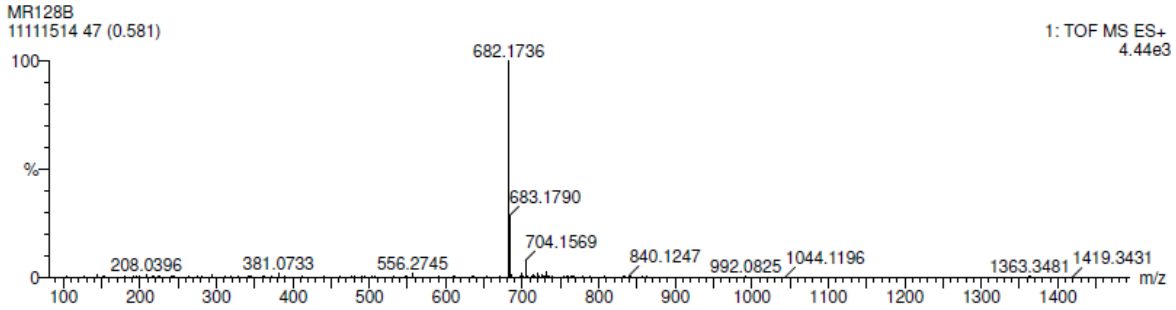
Minimum:				-1.5		
Maximum:		10.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
590.2376	590.2382	-0.6	-1.0	12.5	0.7	C28 H37 N5 O7 Cl

HRMS of [4-Br-Phe^4]-Leu-ENK (13)



Minimum:	80.00				-1.5		
Maximum:	100.00		10.0	20.0	50.0		
Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
634.1868	100.00	634.1876	-0.8	-1.3	12.5	8.8	C28 H37 N5 O7 79Br
636.1847	99.13	636.1856	-0.9	-1.4	12.5	16.9	C28 H37 N5 O7 81Br

HRMS of [4-I-Phe^4]-Leu-ENK (14)



Minimum:				-1.5		
Maximum:		10.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
682.1736	682.1738	-0.2	-0.3	12.5	122.6	C28 H37 N5 O7 I

HPLC DATA FOR PEPTIDES SYNTHESIZED

HPLC solvents:

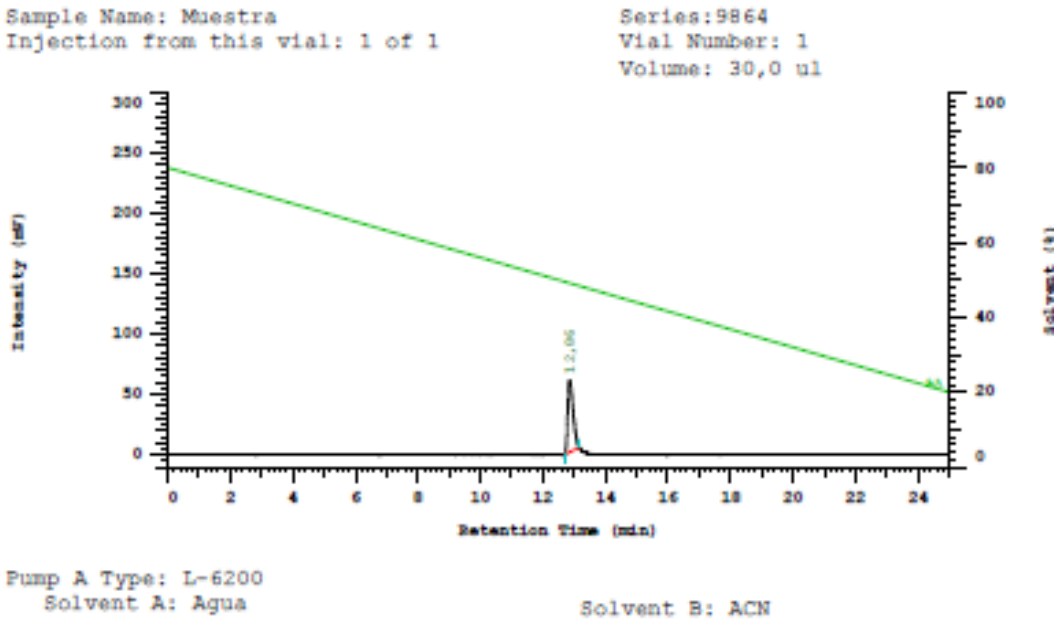
A: 0.1% TFA in H_2O
B: 0.1% TFA in ACN

HPLC gradient:

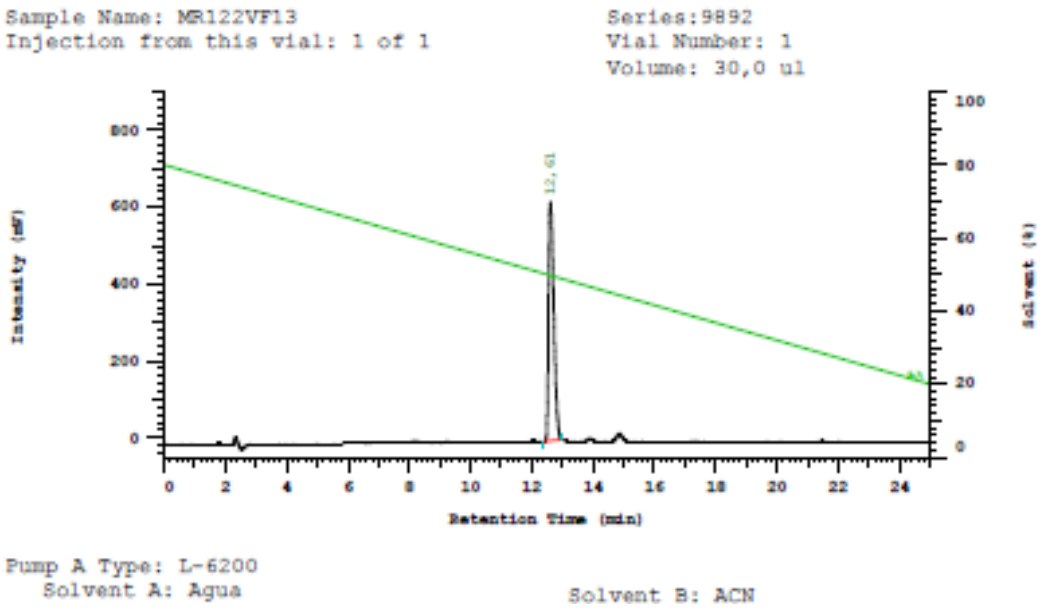
From (80:20) (A:B) to (20:80) (A:B) in 25 min

Peptide	Name	RETENTION TIME (min)
ENDO-1 and ENDO-1 analogues		
1	Endomorphin-1	12,86
2	[4-F-Phe^4]-Endo-1	12,61
3	[4-Cl-Phe^4]-Endo-1	14,51
4	[4-Br-Phe^4]-Endo-1	13,97
5	[4-I-Phe^4]-Endo-1	14,62
Leu-ENK and Leu-ENK analogues		
6	Leu-ENK	11,3
7	[2-F-Phe^4]-Leu-ENK	9,7
8	[2-Cl-Phe^4]-Leu-ENK	12,6
9	[2-Br-Phe^4]-Leu-ENK	11,9
10	[2-I-Phe^4]-Leu-ENK	13,4
11	[4-F-Phe^4]-Leu-ENK	11,8
12	[4-Cl-Phe^4]-Leu-ENK	12,6
13	[4-Br-Phe^4]-Leu-ENK	13,2
14	[4-I-Phe^4]-Leu-ENK	14,4

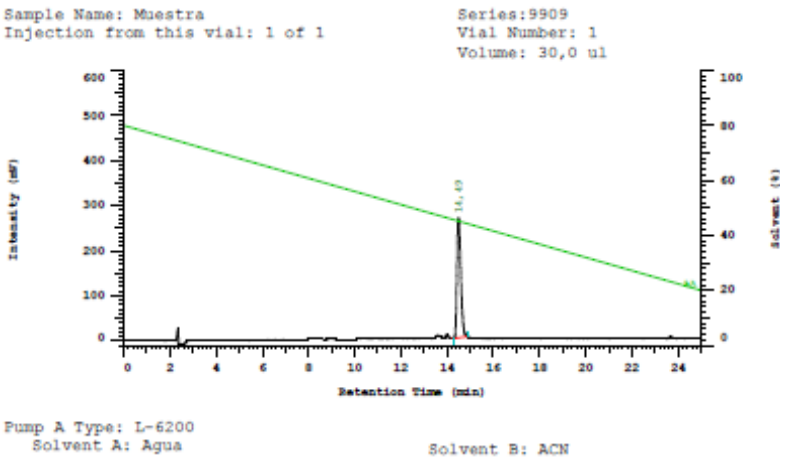
HPLC of endomorphin-1 (1)



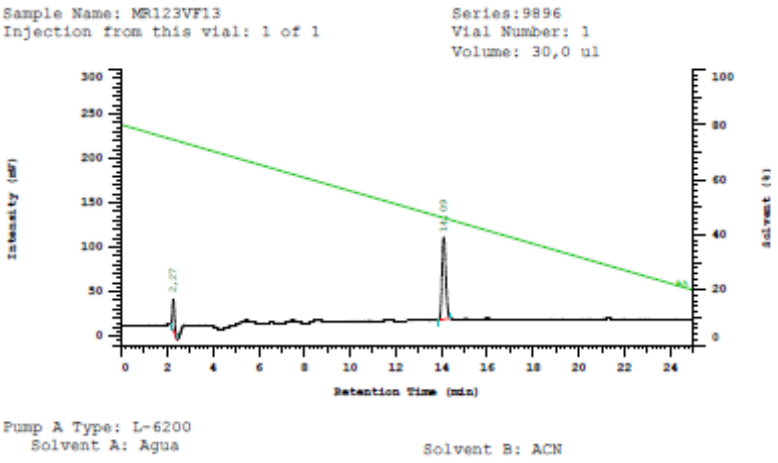
HPLC of [4-F-Phe^4]-endomorphin-1 (2)



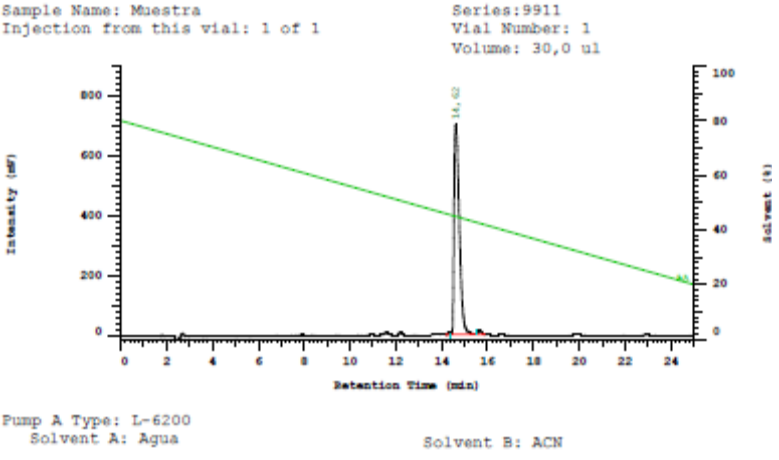
HPLC of [4-Cl-Phe^4]-endomorphin-1 (3)



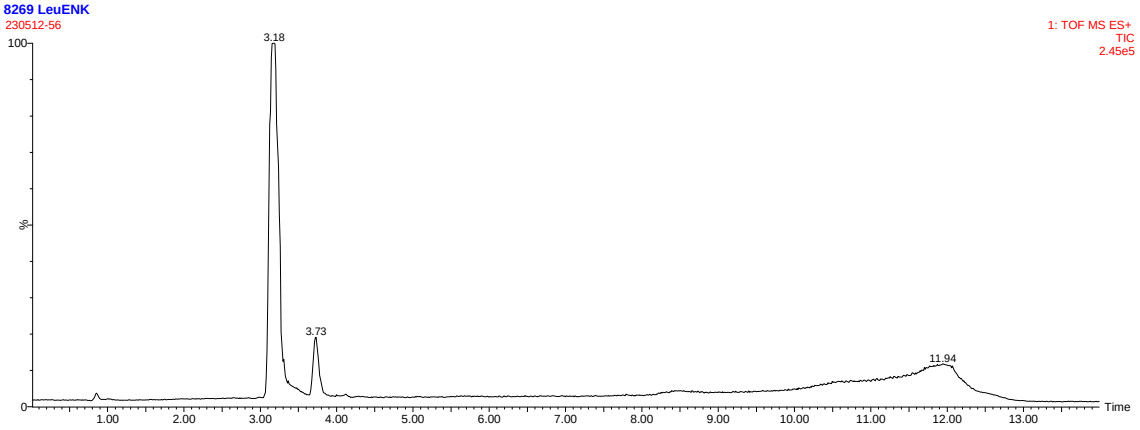
HPLC of [4-Br-Phe^4]-endomorphin-1 (4)



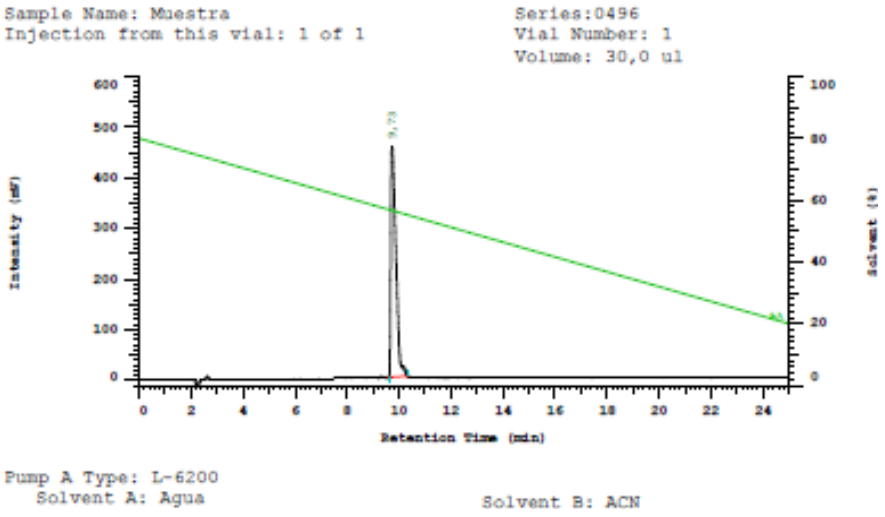
HPLC of [4-I-Phe^4]-endomorphin-1 (5)



UPLC of Leu-enkephalin (6)



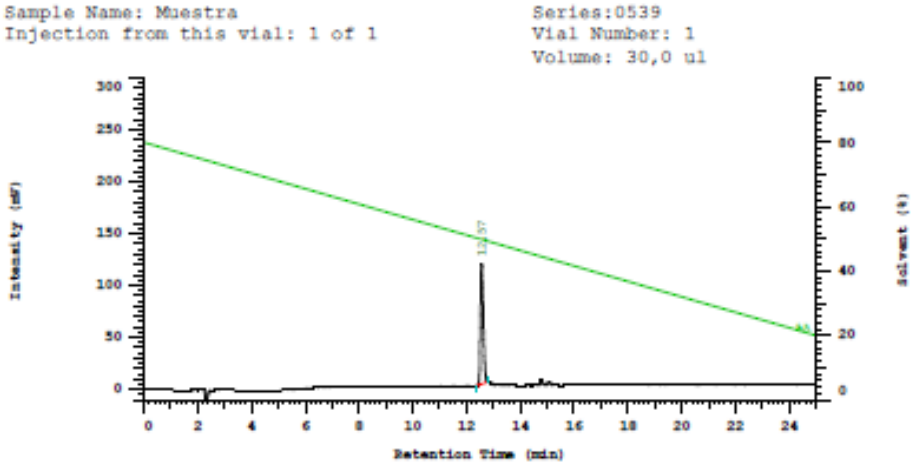
HPLC of [2-F-Phe^4]-Leu-ENK (7)



Pump A Type: L-6200
Solvent A: Agua

Solvent B: ACN

HPLC of [2-Cl-Phe^4]-Leu-ENK (8)



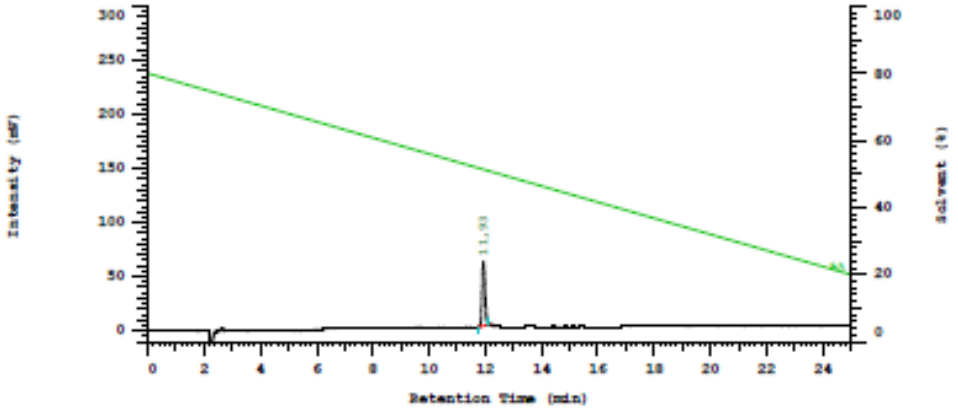
Pump A Type: L-6200
Solvent A: Agua

Solvent B: ACN

HPLC of [2-Br-Phe^4]-Leu-ENK (9)

Sample Name: Muestra
Injection from this vial: 1 of 1

Series:0542
Vial Number: 1
Volume: 30,0 ul



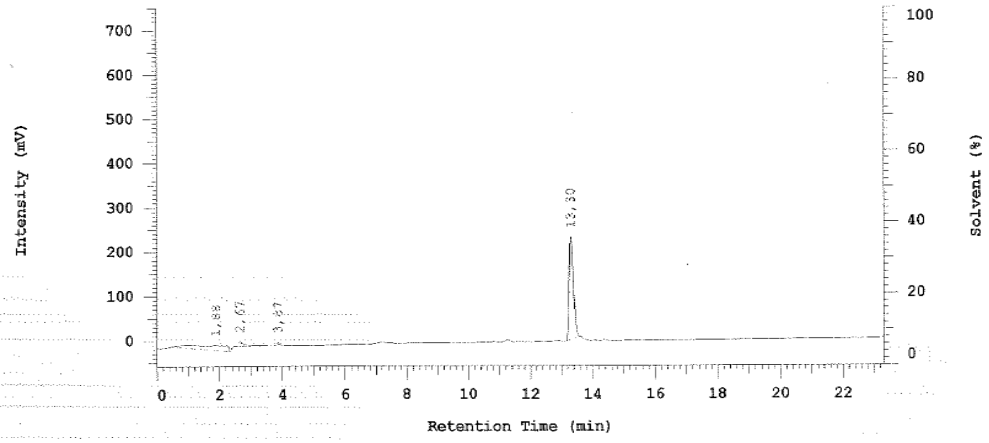
Pump A Type: L-6200
Solvent A: Agua

Solvent B: ACN

HPLC of [2-I-Phe^4]-Leu-ENK (10)

Sample Name:
Injection from this vial: 1 of 1

Series:5120
Vial Number: 1
Volume: 10,0 ul



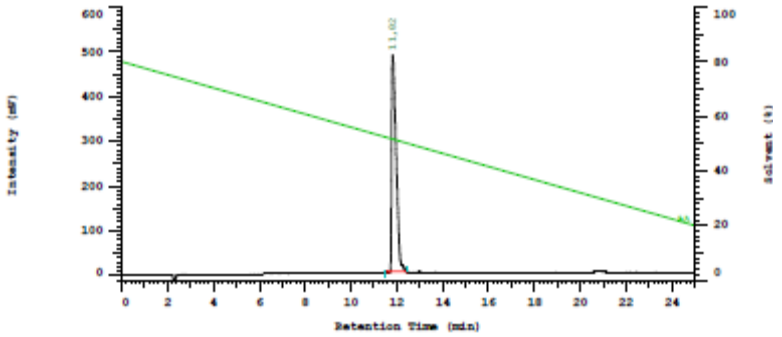
Pump A Type: L-6200
Solvent A: Agua

Solvent B: Acetonitrilo

HPLC of [4-F-Phe^4]-Leu-ENK (11)

Sample Name: Muestra
Injection from this vial: 1 of 1

Series:0024
Vial Number: 1
Volume: 30,0 ul



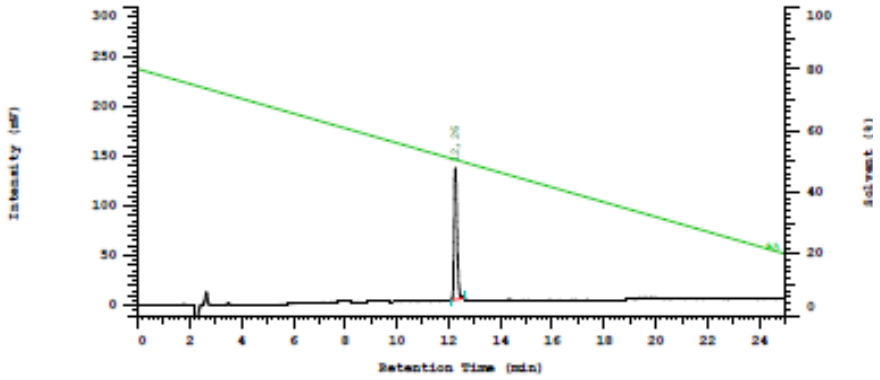
Pump A Type: L-6200
Solvent A: Agua

Solvent B: ACN

HPLC of [4-Cl-Phe^4]-Leu-ENK (12)

Sample Name: Muestra
Injection from this vial: 1 of 1

Series:0019
Vial Number: 1
Volume: 30,0 ul



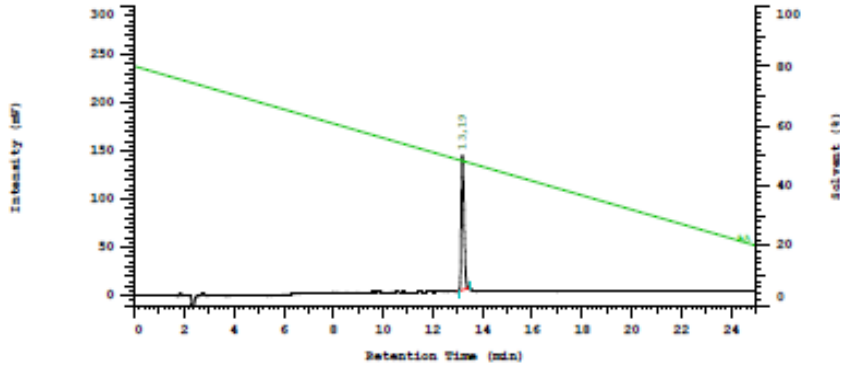
Pump A Type: L-6200
Solvent A: Agua

Solvent B: ACN

HPLC of [4-Br-Phe^4]-Leu-ENK (13)

Sample Name: Muestra
Injection from this vial: 1 of 1

Series:0039
Vial Number: 1
Volume: 30,0 ul



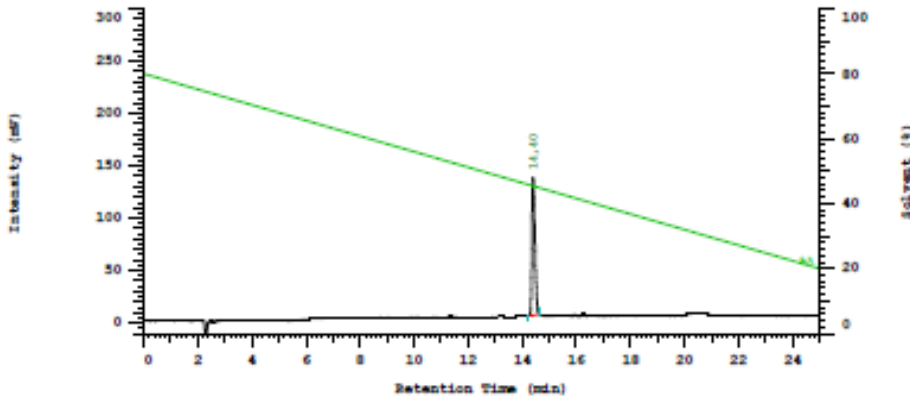
Pump A Type: L-6200
Solvent A: Agua

Solvent B: ACN

HPLC of [4-I-Phe^4]-Leu-ENK (14)

Sample Name: Muestra
Injection from this vial: 1 of 1

Series:9974
Vial Number: 1
Volume: 30,0 ul



Pump A Type: L-6200
Solvent A: Agua

Solvent B: ACN