Discovery of KOR selective D-tetrapeptides with improved *in vivo* antinociceptive effect after peripheral administration

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Experimental procedures

Chemistry

Materials and Methods

All reagents, Fmoc-protected amino acids and solvents were purchased from VWR (MI, Italy), Merck (MI, Italy) and GLS Shanghai (China). All the *C*-terminal amides obtained as TFA salts were triturated in diethyl ether and purified on C18 prep RP-HPLC recorded at 213, 254, and 275 nm (Waters XBridgeTM Prep BEH C18, 130 Å, 5.0 μm, i.d. 19 mm × 250 mm length, 19 mm × 10 mm column) at a flow rate of 7 mL/min; eluent: H_2O/ACN-0.1% TFA from 5% ACN to 90% ACN in 32 min. The purity of each final product was assessed by C18 analytical RP-HPLC recorded at 213, 254, and 275 nm (Waters C18 4.6 mm × 150 mm) at a flow rate of 1 mL/min; eluent: H_2O/ACN-0.1% TFA from 5% ACN to 90% ACN in 30 minutes. ^{1}H-NMR spectra were recorded at 25°C on a 300 MHz Varian Oxford spectrometer, DMSO-d_6 as solvent (chemical shifts in parts per million (δ) downfield from the internal standard TMS). LRMS was performed on a LCQ Finnigan-Mat mass spectrometer (San Jose, CA) by ESI-spray source and ion trap analyzer, capillary temperature at 200°C, the spray voltage at 4.00 kV. Nitrogen (N_2) and helium were used as sheath gas and auxiliary gas. All the final products show a purity ≥ 90% as detected by analytical RP-HPLC (see SI).

Solid phase peptide synthesis procedure

The novel *C*-terminal amides were prepared using Fmoc protection strategy via solid phase peptide synthesis, on Rink amide resin (loading coefficient 1.2 mMol/g). The following protected amino acids were used: *tert*-butyloxy-carbonyl (Boc) for D-tryptophan, *O-tert*-butyl (O-*tert*-Bu) for D-tyrosine, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for D-arginine side chain. Repeated steps of coupling reactions and Fmoc-removal were done following the procedure previously described by us. The novel tetrapeptides have been obtained as TFA salts following a strong cleavage treatment of the resin with TFA/DCM= 9:1 for 1h at r.t.

H-(D)Phe-(D)Phe-(D)Nle-(D)Arg-NH$_2$: 64% overall yield; rt (RP-HPLC anal.): 15.20 min. ^{1}H-NMR (DMSO-d_6), δ: 8.04 (d, 1H, NH (D)Phe^2), 7.35-7.13 (m, 16H, NH$_2$ *C*-terminal amide + NH (D)Arg + NH (D)Nle + 2*NH (D)Arg + 10H aromatics), 4.56 (q, 1H, CH^α (D)Phe^1), 4.17 (m, 2H, CH^α (D)Nle, (D)Arg), 3.88 (q, 1H, CH^α (D)Phe^2), 3.06 (m, 4H, 2*CH$_2$^{β} (D)Phe^{1,2}), 2.85 (m, 2H, CH$_2$^{β} (D)Nle), 1.61-1.26 (m, 10H, 2*CH$_2$^{γ,δ} (D)Nle and

$3*CH_2^{\beta,\gamma,\delta}$ (D)Arg), 0.84 (d, 3H, CH_3 (D)Nle). LRMS $C_{30}H_{44}N_8O_4$ without TFA, m/z: 580.3, found: 603.2 [M+Na]$^+$

H-(D)-*p*F-Phe-(D)Phe-(D)Nle-(D)Arg-NH$_2$ (**1**): 82% overall yield; rt (RP-HPLC anal.): 15.37 min. ^{1}H-NMR (DMSO-d$_6$), δ: 8.13 (t, 1H, NH (D)Phe), 7.26-7.01 (m, 15H, NH$_2$ *C*-terminal amide + NH (D)Arg + NH (D)Nle + 2*NH guanidinium + 9H aromatics), 4.49 (q, 1H, CH$^\alpha$ (D)-pF-Phe), 4.11 (m, 2H, CH$^\alpha$ (D)Nle, (D)Arg), 3.78 (q, 1H, CH$^\alpha$ (D)Phe), 3.10-2.74 (m, 8H, 4*CH$_2^\beta$ (D)Phe, (D)-pF-Phe, (D)Nle, (D)Arg), 1.64-1.25 (m, 10H, 2*CH$_2^{\gamma,\delta}$ (D)Nle and 3*CH$_2^{\beta,\gamma,\delta}$ (D)Arg), 0.84 (d, 3H, CH$_3$ (D)Nle). LRMS $C_{30}H_{43}FN_8O_4$ without TFA, m/z: 598.3, found: 599.3 [M+H]$^+$

H-(D)-*m*F-Phe-(D)Phe-(D)Nle-(D)Arg-NH$_2$ (**2**): 98% overall yield; rt (RP-HPLC anal.): 15.53 min. ^{1}H-NMR (DMSO-d$_6$), δ: 8.74 (d, 1H, NH (D)Phe), 8.39 (d, 1H, NH (D)Nle), 8.01 (bs, 3H, NH$_3^+$ (D)-mF-Phe), 7.91 (d, 1H, NH (D)Arg), 7.48 (t, 1H, NH guanidinium), 7.38-7.05 (m, 11H, NH$_2$ *C*-terminal amide + 9H aromatics), 4.65 (q, 1H, CH$^\alpha$ (D)-mF-Phe), 4.34 - 4.16 (m, 2H, CH$^\alpha$ (D)Nle + (D)Arg), 3.98 (q, 1H, CH$^\alpha$ (D)Phe), 3.11-2.70 (m, 8H, 4*CH$_2^\beta$ (D)Phe, (D)-mF-Phe, (D)Nle, (D)Arg), 1.76-1.20 (m, 10H, 2*CH$_2^{\gamma,\delta}$ (D)Nle and 3*CH$_2^{\beta,\gamma,\delta}$ (D)Arg), 0.84 (d, 3H, CH$_3$ (D)Nle). LRMS $C_{30}H_{43}FN_8O_4$ without TFA, m/z: 598.3, found: 599.4 [M+H]$^+$

H-(D)-*o*F-Phe-(D)Phe-(D)Nle-(D)Arg-NH$_2$ (**3**): 35% overall yield; rt (RP-HPLC anal.): 15.16 min. ^{1}H-NMR (DMSO-d$_6$), δ: 8.74 (d, 1H, NH (D)Phe), 8.33 (d, 1H, NH (D)Nle), 8.11 (bs, 3H, NH$_3^+$ (D)-oF-Phe), 7.89 (d, 1H, NH (D)Arg), 7.50 (t, 1H, NH guanidinium), 7.36-7.06 (m, 11H, NH$_2$ *C*-terminal amide + 9H aromatics), 4.63 (q, 1H, CH$^\alpha$ (D)-oF-Phe), 4.26-4.13 (m, 2H, CH$^\alpha$ (D)Nle, (D)Arg), 4.02 (q, 1H, CH$^\alpha$ (D)Phe), 3.10-2.70 (m, 8H, 4*CH$_2^\beta$ (D)Phe, (D)-oF-Phe, (D)Nle, (D)Arg), 1.63-1.25 (m, 10H, 2*CH$_2^{\gamma,\delta}$ (D)Nle and 3*CH$_2^{\beta,\gamma,\delta}$ (D)Arg), 0.84 (d, 3H, CH$_3$ (D)Nle). LRMS $C_{30}H_{43}FN_8O_4$ without TFA, m/z: 598.3, found: 599.4 [M+H]$^+$

H-(D)Tic-(D)Phe-(D)Nle-(D)Arg-NH$_2$ (**4**): 48% overall yield; rt (RP-HPLC anal.): 15.46 min. ^{1}H-NMR (DMSO-d$_6$), δ: 8.76 (d, 1H, NH (D)Phe), 8.51 (d, 1H, NH (D)Nle), 8.02 (d, 1H, NH (D)Arg), 7.75 (t, 1H, NH guanidinium), 7.32-7.11 (m, 13H, NH$_2$ *C*-terminal amide + 9H aromatics + NH$_2$ guanidinium), 4.62 (q, 1H, CH$^\alpha$ (D)Tic), 4.20-02 (m, 2H, CH$^\alpha$ (D)Nle + (D)Arg), 3.85 (q, 1H, CH$^\alpha$ (D)Phe), 3.10-2.73 (m, 8H, CH$_2^\beta$ (D)Phe and 2*CH$_2$ (D)Tic, CH$_2^\beta$

(D)Nle), 1.72-1.25 (m, 10H, $2*CH_2^{\gamma,\delta}$ (D)Nle and $3*CH_2^{\beta,\gamma,\delta}$ (D)Arg), 0.85 (d, 3H, CH_3 (D)Nle). LRMS $C_{31}H_{44}N_8O_4$ without TFA, m/z: 592.3, found: 592.8 [M]

H-(D)Trp-(D)Phe-(D)Nle-(D)Arg-NH_2 (**5**): 97% overall yield; rt (RP-HPLC anal.): 15.80 min. ^{1}H-NMR (DMSO-d$_6$), δ: 11.01 (s, 1H, NH indole), 8.87 (d, 1H, NH (D)Phe), 8.34 (d, 1H, NH (D)Nle), 7.93-7.91 (m, 4H, NH_3^+ Trp and NH (D)Arg), 7.69 (d, 1H, H-indole), 7.49 (t, 1H, NH guanidinium), 7.35-6.95 (m, 13H, NH_2 *C*-terminal amide + 9H aromatics + NH_2 guanidinium), 4.67 (q, 1H, CH^α (D)Trp), 4.30-4.14 (m, 2H, CH^α (D)Nle, (D)Arg), 3.93 (q, 1H, CH^α (D)Phe), 3.24-2.69 (m, 6H, CH_2^β (D)Phe, CH_2^β (D)Trp, CH_2^β (D)Nle), 1.63-1.26 (m, 10H, $2*CH_2^{\gamma,\delta}$ (D)Nle and $3*CH_2^{\beta,\gamma,\delta}$ (D)Arg), 0.83 (d, 3H, CH_3 (D)Nle). LRMS $C_{32}H_{45}N_9O_4$ without TFA, m/z: 619.3, found: 620.3 [M+H]$^+$

H-(D)Tyr-(D)Phe-(D)Nle-(D)Arg-NH_2 (**6**): 31% overall yield; rt (RP-HPLC anal.): 14.90 min. ^{1}H-NMR (DMSO-d$_6$), δ: 9.35 (s, 1H, OH Tyr), 8.72 (d, 1H, NH (D)Phe), 8.36 (d, 1H, NH (D)Nle), 7.92-7.89 (m, 4H, NH_3^+ and NH (D)Arg), 7.54 (t, 1H, NH guanidinium), 7.36-7.11 (m, 9H, NH_2 *C*-terminal amide + 5H aromatics + NH_2 guanidinium), 6.99 and 6.65 (dd, 4H, aromatics Tyr), 4.64 (q, 1H, CH^α (D)Tyr), 4.25-4.16 (m, 2H, CH^α (D)Nle, (D)Arg), 3.82 (q, 1H, CH^α (D)Phe), 3.08-2.69 (m, 6H, CH_2^β (D)Phe, CH_2^β (D)Tyr, CH_2^β (D)Nle), 1.63-1.25 (m, 10H, $2*CH_2^{\gamma,\delta}$ (D)Nle and $3*CH_2^{\beta,\gamma,\delta}$ (D)Arg), 0.84 (d, 3H, CH_3 (D)Nle). LRMS $C_{30}H_{44}N_8O_5$ without TFA, m/z: 596.3, found: 597.4 [M+H]$^+$

H-(D)Phe-(D)-*p*F-Phe-(D)Nle-(D)Arg-NH_2 (**7**): 37% overall yield; rt (RP-HPLC anal.): 15.39 min. ^{1}H-NMR (DMSO-d$_6$), δ: 8.73 (d, 1H, NH (D)-pF-Phe), 8.34 (d, 1H, NH (D)Phe), 7.92 (d, 2H, NH (D)Nle, (D)Arg), 7.49 (t, 1H, NH guanydinium (D)Arg), 7.37-7.04 (m, 13H, NH_2 *C*-terminal amide + NH_2 (D)Arg + 9H aromatics), 4.63 (q, 1H, CH^α (D)-pF-Phe), 4.25-4.09 (m, 2H, CH^α (D)Nle, (D)Arg), 3.93 (q, 1H, CH^α (D)Phe), 3.14-2.70 (m, 8H, $4*CH_2^\beta$ (D)Phe, (D)-pF-Phe, (D)Nle, (D)Arg), 1.63-1.24 (m, 10H, $2*CH_2^{\gamma,\delta}$ (D)Nle and $3*CH_2^{\beta,\gamma,\delta}$ (D)Arg), 0.83 (d, 3H, CH_3 (D)Nle). LRMS $C_{30}H_{43}FN_8O_4$ without TFA, m/z: 598.3, found: 599.4 [M+H]$^+$

H-(D)Phe-(D)-*m*F-Phe-(D)Nle-(D)Arg-NH_2 (**8**): 59% overall yield; rt (RP-HPLC anal.): 15.24 min. ^{1}H-NMR (DMSO-d$_6$), δ: 8.75 (d, 1H, NH (D)Phe), 8.36 (d, 1H, NH (D)Nle), 7.95 (m, 4H, NH_3^+ (D)-mF-Phe and NH (D)Arg), 7.51 (t, 1H, NH guanidinium), 7.36-6.99 (m, 13H, NH_2 *C*-terminal amide + 9H aromatics + NH_2 guanydinium), 4.66 (q, 1H, CH^α (D)-mF-Phe), 4.27-4.16 (m, 2H, CH^α (D)Nle, (D)Arg), 3.96 (q, 1H, CH^α (D)Phe), 3.06-2.70 (m, 8H,

4*CH$_2^\beta$ (D)Phe, (D)-mF-Phe, (D)Nle, (D)Arg), 1.63-1.20 (m, 10H, 2*CH$_2^{\gamma,\delta}$ (D)Nle and 3*CH$_2^{\beta,\gamma,\delta}$ (D)Arg), 0.84 (d, 3H, CH$_3$ (D)Nle). LRMS C$_{30}$H$_{43}$FN$_8$O$_4$ without TFA, m/z: 598.3, found: 300.3 [M/2]$^{++}$

H-(D)Phe-(D)-oF-Phe-(D)Nle-(D)Arg-NH$_2$ (**9**): 96% overall yield; rt (RP-HPLC anal.): 15.40 min. ^{1}H-NMR (DMSO-d$_6$), δ: 8.74 (d, 1H, NH (D)Phe), 8.33 (d, 1H, NH (D)Nle), 8.11 (bs, 3H, NH$_3^+$ (D)-oF-Phe), 7.89 (d, 1H, NH (D)Arg), 7.50 (t, 1H, NH guanidinium), 7.32-7.05 (m, 11H, NH$_2$ *C*-terminal amide + 9H aromatics), 4.66 (q, 1H, CH$^\alpha$ (D)-oF-Phe), 4.20-4.12 (m, 2H, CH$^\alpha$ (D)Nle, (D)Arg), 3.91 (q, 1H, CH$^\alpha$ (D)Phe), 3.06-2.79 (m, 8H, 4*CH$_2^\beta$ (D)Phe, (D)-oF-Phe, (D)Nle, (D)Arg), 1.60-1.23 (m, 10H, 2*CH$_2^{\gamma,\delta}$ (D)Nle and 3*CH$_2^{\beta,\gamma,\delta}$ (D)Arg), 0.81 (d, 3H, CH$_3$ (D)Nle). LRMS C$_{30}$H$_{43}$FN$_8$O$_4$ without TFA, m/z: 598.3, found: 599.5 [M+H]$^+$

H-(D)Phe-(D)Tyr-(D)Nle-(D)Arg-NH$_2$ (**10**): 17% overall yield; rt (RP-HPLC anal.): 14.92 min. ^{1}H-NMR (DMSO-d$_6$), δ: 9.22 (s, 1H, OH Tyr), 8.72 (d, 1H, NH (D)Phe), 8.32 (d, 1H, NH (D)Nle), 7.89 (d, 1H, NH (D)Arg), 7.49 (t, 1H, NH guanidinium), 7.36-7.11 (m, 9H, NH$_2$ *C*-terminal amide + 5H aromatics + NH$_2$ guanidinium), 7.05 and 6.62 (dd, 4H, aromatics Tyr), 4.55 (q, 1H, CH$^\alpha$ (D)Tyr), 4.27-4.16 (m, 2H, CH$^\alpha$ (D)Nle, (D)Arg), 3.90 (q, 1H, CH$^\alpha$ (D)Phe), 3.08-2.89 (m, 6H, CH$_2^\beta$ (D)Phe, CH$_2^\beta$ (D)Tyr, CH$_2^\beta$ (D)Nle), 1.63-1.24 (m, 10H, 2*CH$_2^{\gamma,\delta}$ (D)Nle and 3*CH$_2^{\beta,\gamma,\delta}$ (D)Arg), 0.83 (d, 3H, CH$_3$ (D)Nle). LRMS C$_{30}$H$_{44}$N$_8$O$_5$ without TFA, m/z: 596.3, found: 597.4 [M+H]$^+$

H-(D)Phe-(D)Trp-(D)Nle-(D)Arg-NH$_2$ (**11**): 40% overall yield; rt (RP-HPLC anal.): 15.65 min. ^{1}H-NMR (DMSO-d$_6$), δ: 10.82 (s, 1H, NH indole), 8.74 (d, 1H, NH (D)Phe), 8.38 (d, 1H, NH (D)Nle), 7.98-7.86 (m, 4H, NH$_3^+$ and NH (D)Arg), 7.68 (d, 1H, H-indole), 7.49 (t, 1H, NH guanidinium), 7.35-6.93 (m, 13H, NH$_2$ *C*-terminal amide + 9H aromatics + NH$_2$ guanidinium), 4.68 (q, 1H, CH$^\alpha$ (D)Trp), 4.30-4.14 (m, 2H, CH$^\alpha$ (D)Nle, (D)Arg), 3.94 (q, 1H, CH$^\alpha$ (D)Phe), 3.16-2.84 (m, 6H, CH$_2^\beta$ (D)Phe, (D)Trp, (D)Nle), 1.64-1.20 (m, 10H, 2*CH$_2^{\gamma,\delta}$ (D)Nle and 3*CH$_2^{\beta,\gamma,\delta}$ (D)Arg), 0.83 (d, 3H, CH$_3$ (D)Nle). LRMS C$_{32}$H$_{45}$N$_9$O$_4$ without TFA, m/z: 619.3, found: 620.3 [M+H]$^+$

H-(D)Phe-(D)Tic-(D)Nle-(D)Arg-NH$_2$ (**12**): 61% overall yield; rt (RP-HPLC anal.): 16.37 min. ^{1}H-NMR (DMSO-d$_6$), δ: 8.33 (d, 1H, NH (D)Phe), 8.10-8.05 (m, 3H, NH (D)Nle + NH$_2^+$ (D)Tic), 7.86 (d, 1H, NH (D)Arg), 7.56 (t, 1H, NH guanidinium), 7.39-7.11 (m, 13H, NH$_2$ *C*-terminal amide + 9H aromatics + NH$_2$ guanidinium), 4.84-4.76 (m, 2H, CH$^\alpha$ (D)Tic,

(D)Nle), 4.20-4.06 (m, 2H, CH^α (D)Arg, (D)Phe), 3.16-2.91 (m, 8H, CH_2^β (D)Phe and 2*CH_2 (D)Tic, CH_2^β (D)Nle), 1.59-1.20 (m, 10H, 2*$CH_2^{\gamma,\delta}$ (D)Nle and 3*$CH_2^{\beta,\gamma,\delta}$ (D)Arg), 0.81 (d, 3H, CH_3 (D)Nle). LRMS $C_{31}H_{44}N_8O_4$ without TFA, m/z: 592.3, found: 593.4 $[M+H]^+$

Molecular Modelling

Molecular Docking

The docking of the novel molecule was done on the crystallized receptor-ligand complex KOR (6B73) obtained from the RCSB protein databank and submitted to a preparation by the Protein Preparation Wizard module present in Maestro 10.2. Several errors in the raw crystal structures have been amended such as the addition of the missing side chains, all the molecules belonging to the crystallization buffer were eliminated from the files, with the only exception for the crystallographic ligand; the protonation state was calculated at pH 7.4 and the hydrogens minimized by OPLS3 methods.

Following previously well-established protocol on these targets reported by us, the software Glide implemented in the Maestro 10.2 package was employed to perform the docking study. As a first step, the self-docking experiment was carried out to validate the docking procedure. Glide XP was used to perform the self-docking validation process. The docking cavity was defined as a cubic space of 20 Å side, centered at the crystallographic ligand, then Glide XP was employed in the *in silico* experiments for **7** and the parent compound FE200041.

In vitro assays

Materials and Methods

Chemicals

The radiolabelled GTP analogue [^{35}S]GTPγS (specific activity: 1250 Ci/mmol) and the Ultima GoldTM MV harmless scintillation cocktail were acquired from PerkinElmer (Boston, USA).

*Opioid receptor binding and G-protein stimulation assays*

Opioid receptor radiolabelled competition assay and G-protein stimulation assay were executed on MOR, DOR and KOR, following the procedures previously described.[1]

Data analysis

Data analysis of GTPγS binding was performed with GraphPad Prism 5.0 software (GraphPad Prism Software Inc., San Diego, CA, USA).

1 Szűcs, E.; Büki, A.; Kékesi, G.; Horváth, G.; Benyhe, S. Mu-Opioid (MOP) receptor mediated G-protein signaling is impaired in specific brain regions in a rat model of schizophrenia. *Neurosci Lett.* **2016**, *21*, 29-33. doi: 10.1016/j.neulet.2016.02.060.

Figure 1S. MOR (A), DOR (B) and KOR (C) binding of *lead compound*, **1-12** ligands and reference compounds.
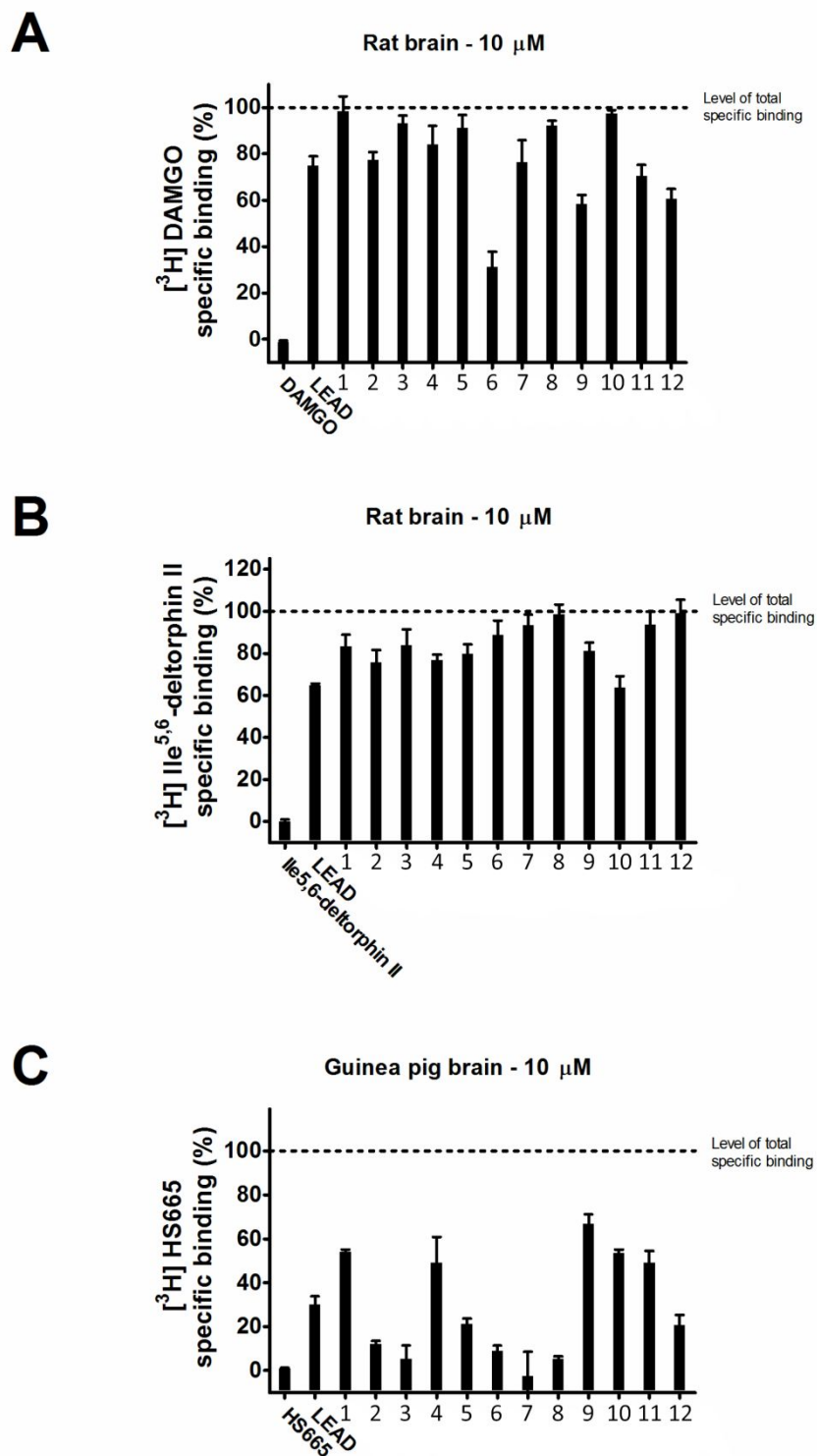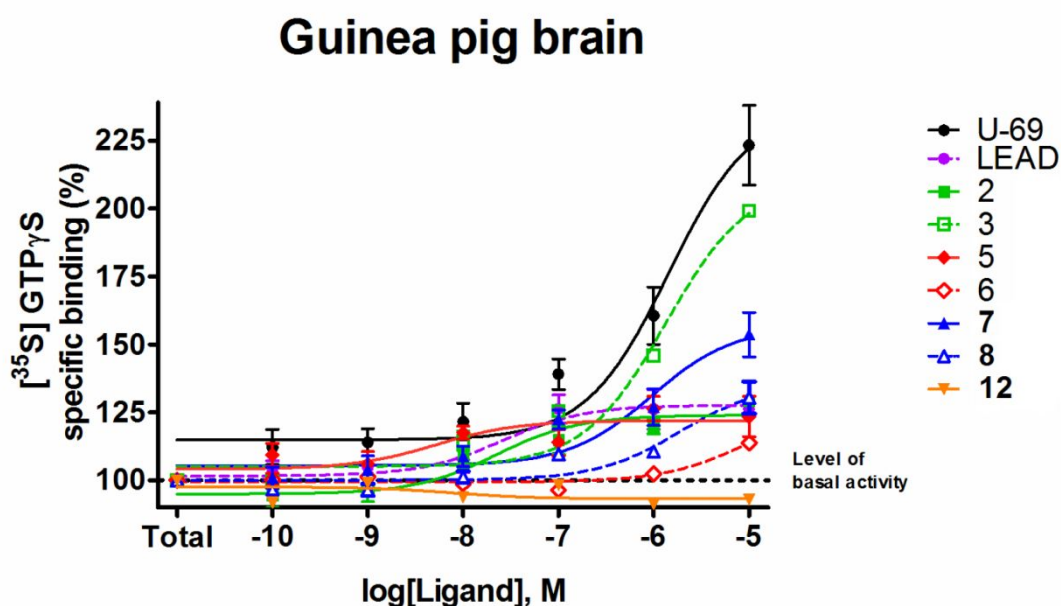
Figure 2S. Binding affinity of the best ligands **2,3,5,6** and **7**,**8**,**12** in KOR-opioid system against HS665 and the *lead compound*.
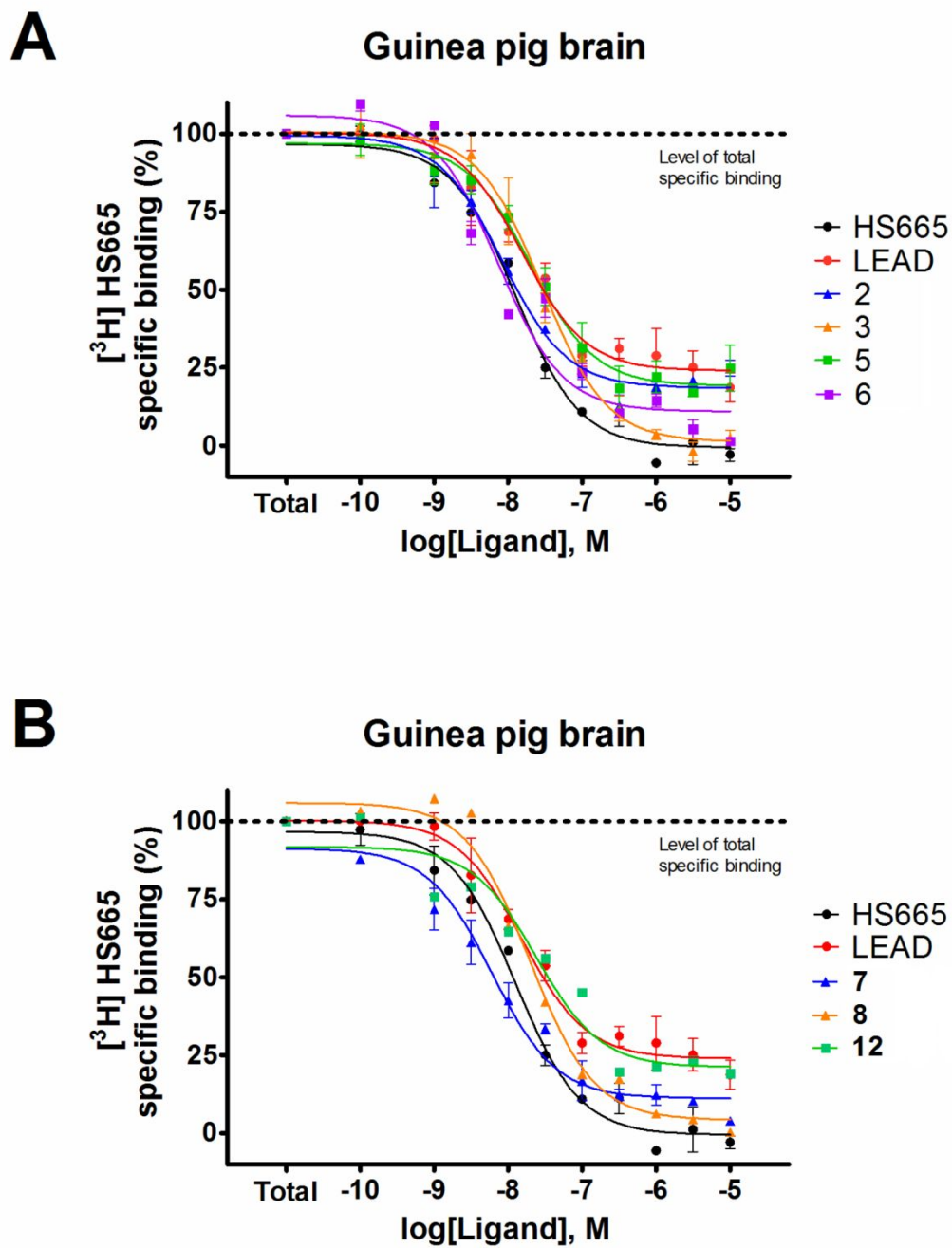
Figure 3S. G-protein activation of U-69, *lead compound,* **2,3,5,6** and **7,8**,**12** analogues.

In vivo assays

Animals

CD-1 male mice (Harlan, Italy) weighing 25-30 g were used in all experiments. Before the experimental sessions, the mice were maintained in colony, housed in cages (7 mice per cage) under standard light/dark cycle (from 7:00 AM to 7:00 PM), temperature (21±1°C) and relative humidity (60±10%) for at least 1 week. Food and water were available *ad libitum*. The research protocol was approved by the Service for Biotechnology and Animal Welfare of the Istituto Superiore di Sanità and authorized by the Italian Ministry of Health, according to Legislative Decree 26/14, which implemented the European Directive 2010/63/UE on the protection of laboratory animals in Italy (authorization number, 756/2018-PR). Animal welfare was routinely checked by veterinarians from the Service for Biotechnology and Animal Welfare.

Treatment Procedure

DMSO was purchased from Merck (Rome, Italy). Peptide solutions were freshly prepared using saline containing 0.9% NaCl and DMSO 0.1% every experimental day. These solutions were injected at a volume of 10 μL/mouse for intracerebroventricular (i.c.v.) administrations,

at a volume of 20 μL/mouse for subcutaneous (s.c.) administrations or at a volume of 10 ml/kg for intravenous (i.v.) administration.

Surgery for Intracerebroventricular Injection

For i.c.v. injections, mice were implanted with a 22-gauge stainless steel guide cannula aimed at the lateral ventricle. Implantation was done under ketamine-xylazine (80 mg/kg ketamine-10 mg/kg xylazine mixture, intraperitoneally (i.p.)) anesthesia, and was performed at least 1 week prior to the behavioral tests. Stereotaxic coordinates for the left lateral ventricle were as follows: anteroposterior (AP) $=-0.5$ mm from the bregma; mediolateral $=-1.0$ mm from the sagittal suture; and dorsoventral $=-1$ mm from the skull surface. The cannula was subsequently fixed to the skull by one screw and dental acrylic. A stylet was inserted within the cannula to preserve its patent before infusions. Drug infusions were done by a 27-gauge stainless steel needle (1 mm longer than the guide cannula) attached to a Hamilton micro-syringe via polyethylene tubing. The mice were allowed to move freely in the test cage during injection performed at 2 μl/min. After injection, the injection probe was kept in place for at least 5 min to prevent backflow. Before the experiments, the mice had at least 5–7 days recovery period.

Tail flick test

The tail flick latency was obtained using a commercial unit (Ugo Basile, Italy), consisting of an infrared radiant light source (100 W, 20 V bulb) focused onto a photocell utilizing an aluminum parabolic mirror. During the trials the mice were gently hand-restrained with a glove. Radiant heat was focused 3-4 cm from the tip of the tail, and the latency (s) of the tail withdrawal recorded. The measurement was interrupted if the latency exceeded the cut off time (30 s). The baseline was calculated as mean of three readings recorded before testing at intervals of 15-30 min and the time course of latency determined at 15, 30, 45, 60, 90 and 120 min after treatment. In the tail flick test, data were expressed as time course of the percentage of maximum effect (%MPE)=(post drug latency – baseline latency)/(cut-off time – baseline latency) x 100. Then, the area under the curve was calculated with the aid of a computer program (GraphPad Prism 9.3.1).

Formalin Test

In the formalin test, the injection of a dilute solution of formalin (1%, 20 μL/paw) into the dorsal surface of the mouse hind paw evoked biphasic nociceptive behavioral responses, such as licking, biting the injected paw, or both, occurring from 0 to 10 min after formalin

injection (the early phase) and a prolonged phase, occurring from 10 to 40 min (the late phase). Before the test, mice were individually placed in a Plexiglas observation cage (30 × 14 × 12 cm) for one hour, to acclimatize to the testing environment. The total time the animal spent licking or biting its paw during the early and late phase of formalin-induced nociception was recorded.

Data Analysis and Statistics

Experimental in vivo data were expressed as mean ± s.e.m. Significant differences among the groups were evaluated with one-way ANOVA followed by Dunnett's multiple comparisons test. GraphPad Prism 9.3.1 software was used for all the analyses. Statistical significance was set at $p < 0.05$. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology.

RP-HPLC traces

1: *p*(F)-D-Phe-D-Phe-D-NLeu-D-Arg-NH$_2$
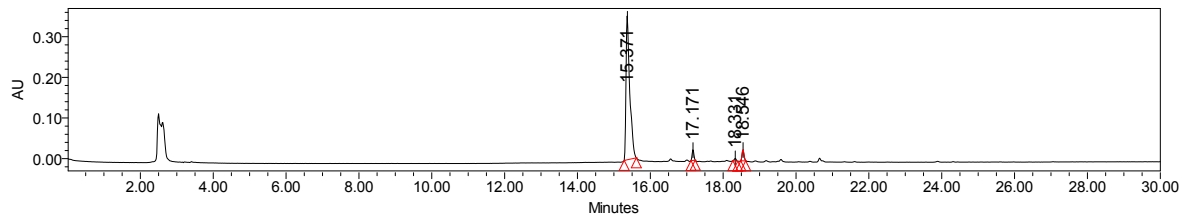
	Retention Time	% Area	Height
1	15.371	92.08	353107
2	17.171	3.30	25371
3	18.331	0.86	6176
4	18.546	3.76	26434

2: *m*(F)-D-Phe-D-Phe-D-NLeu-D-Arg-NH$_2$
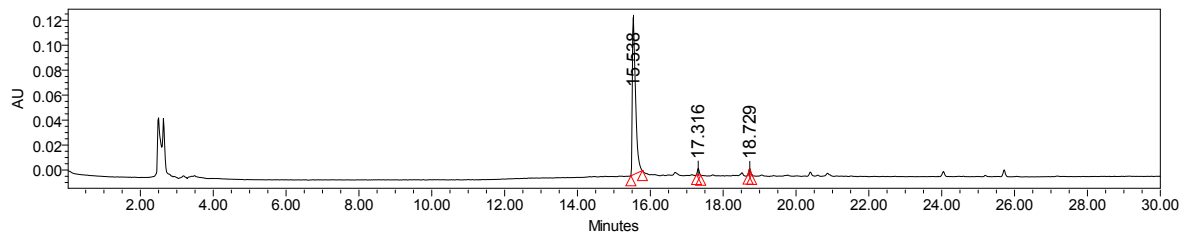
	Retention Time	% Area	Height
1	15.538	96.01	126563
2	17.316	2.16	4952
3	18.729	1.83	4380

3: *o*(F)-D-Phe-D-Phe-D-NLeu-D-Arg-NH$_2$
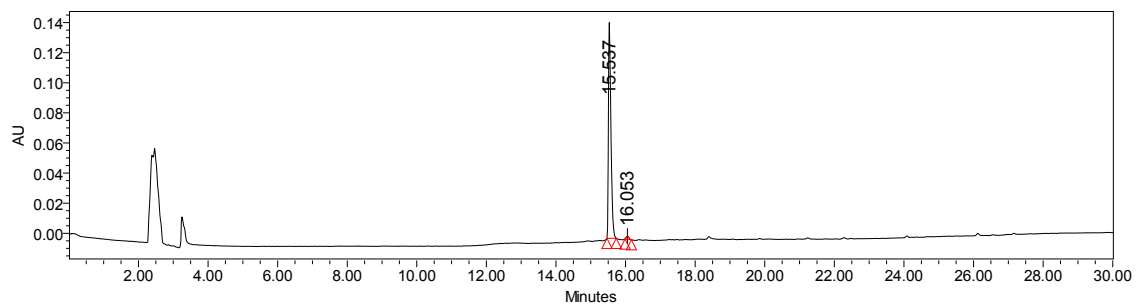
4: D-Tic-D-Phe-D-NLeu-D-Arg-NH$_2$
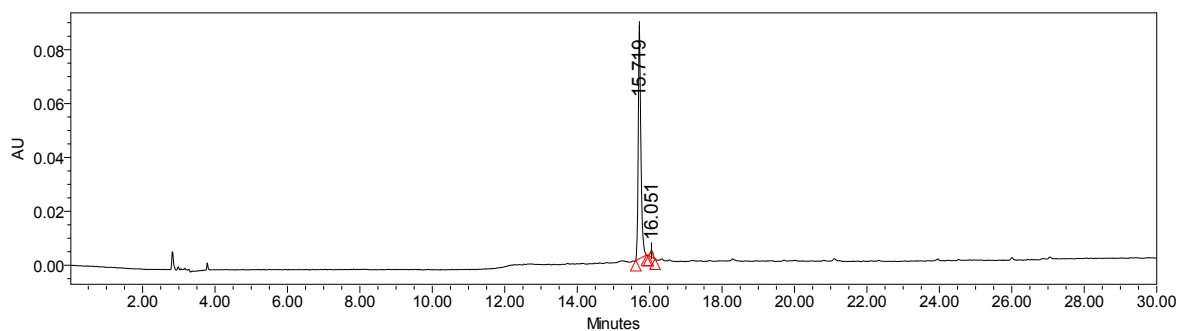
	Retention Time	% Area	Height
1	15.719	97.23	85012
2	16.051	2.77	2392

5: D-Trp-D-Phe-D-NLeu-D-Arg-NH$_2$
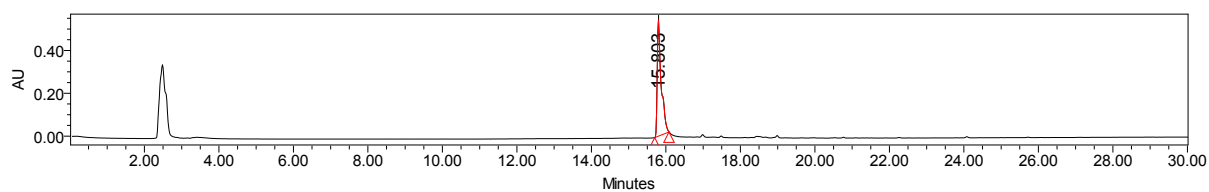
6: D-Tyr-D-Phe-D-NLeu-D-Arg-NH$_2$
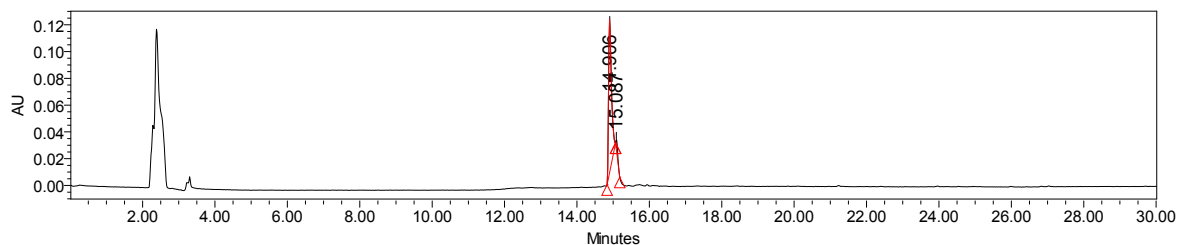
	Retention Time	% Area	Height
1	14.906	95.77	113917
2	15.087	4.23	7072

S14

7: D-Phe-*p*(F)-D-Phe-D-NLeu-D-Arg-NH$_2$
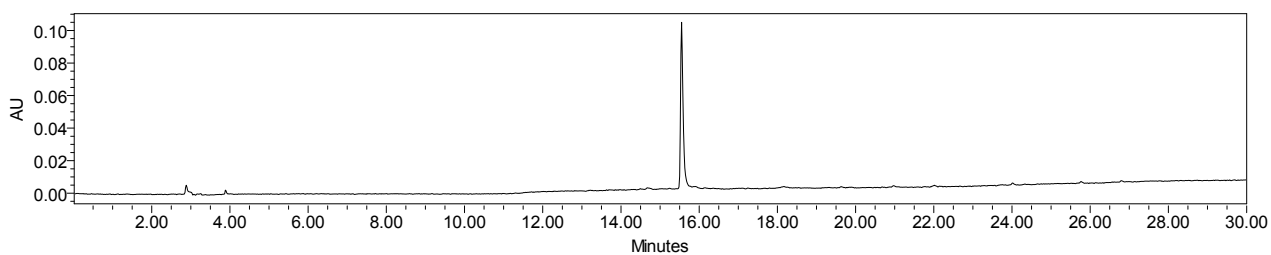
	Retention Time	Height
1	15.553	99177

8: D-Phe-*m*(F)-D-Phe-D-NLeu-D-Arg-NH$_2$
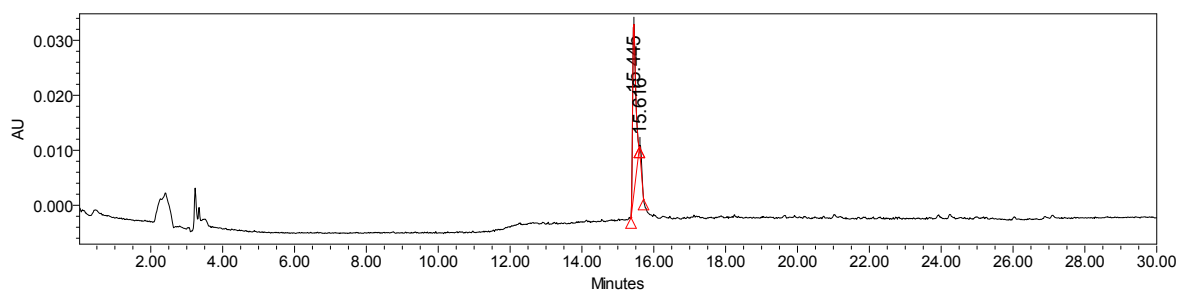
	Retention Time	% Area	Height
1	15.445	95.06	31438
2	15.616	4.94	1814

9: D-Phe-*o*(F)-D-Phe-D-NLeu-D-Arg-NH$_2$
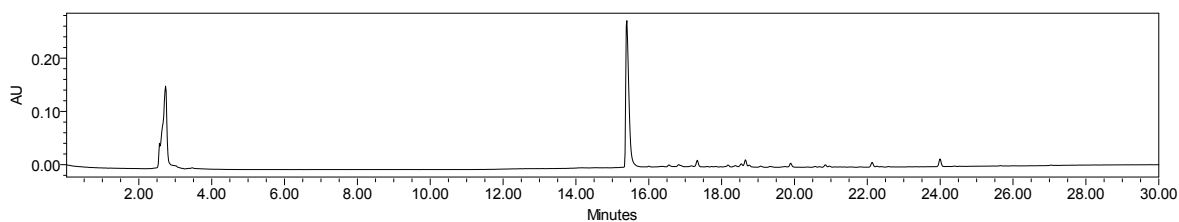
	Retention Time	% Area	Height
1	15.402	100.00	273212

S15

10: D-Phe-D-Tyr-D-NLeu-D-Arg-NH$_2$
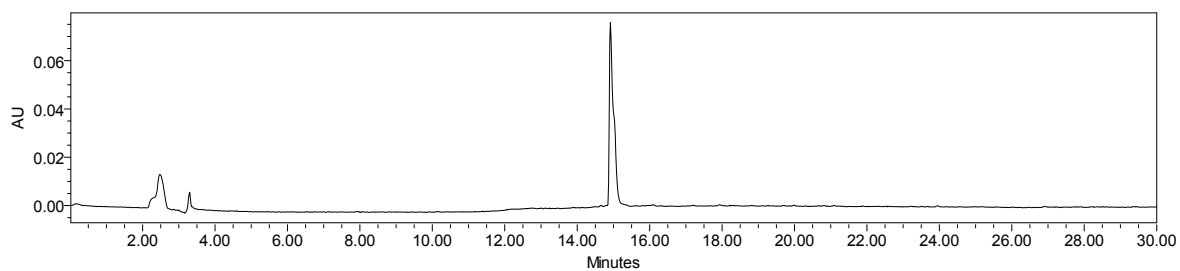
	Retention Time	Height
1	14.922	75458

11: D-Phe-D-Trp-D-NLeu-D-Arg-NH$_2$
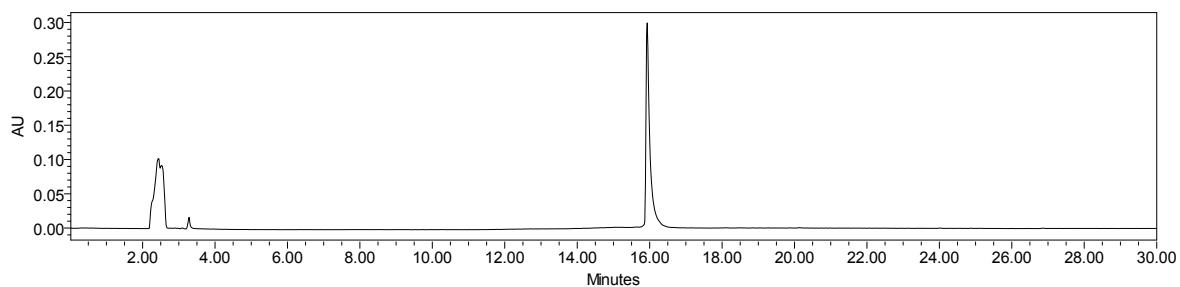
	Retention Time	Height
1	15.934	232392

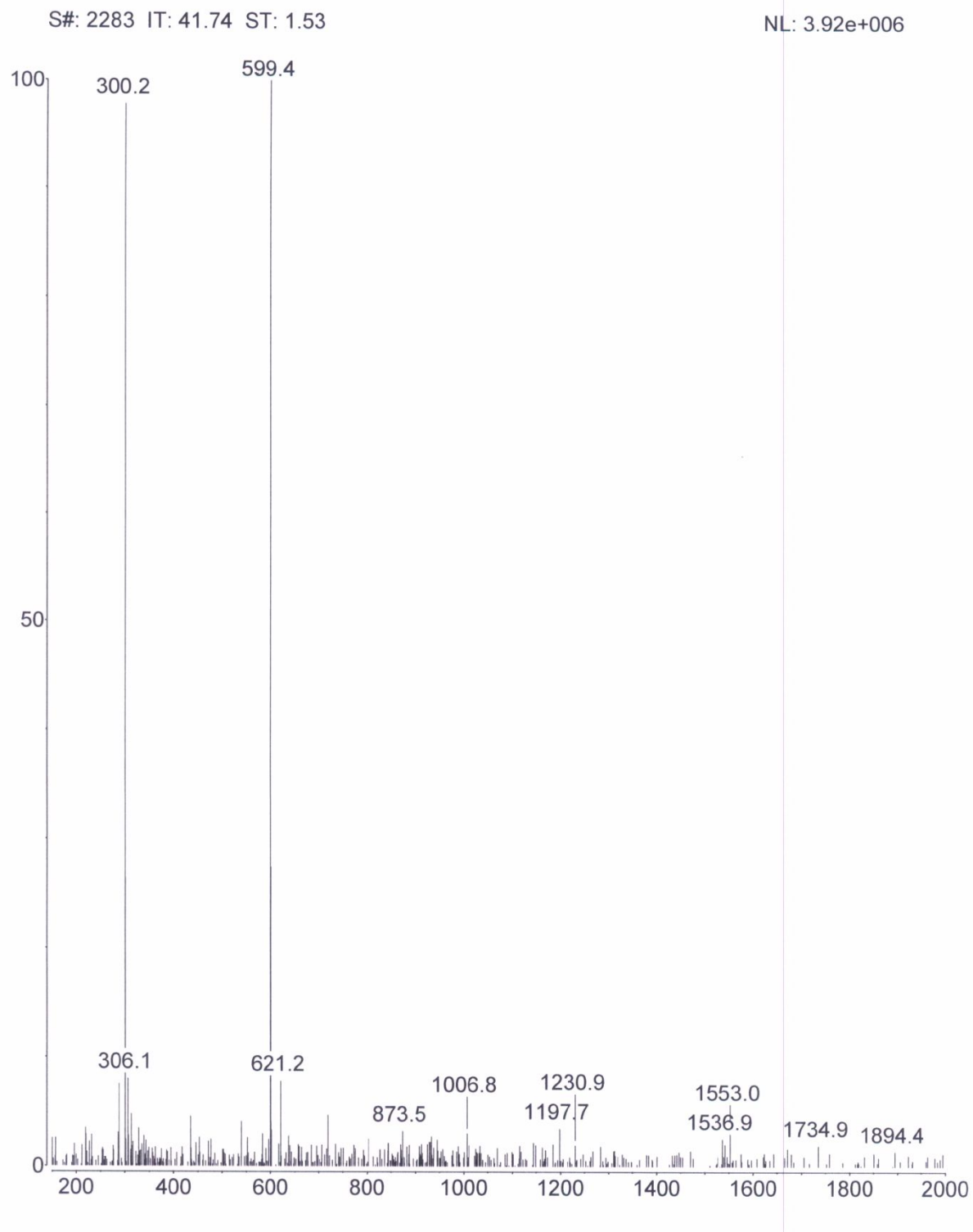**12**: D-Phe-D-Tic-D-NLeu-D-Arg-NH$_2$
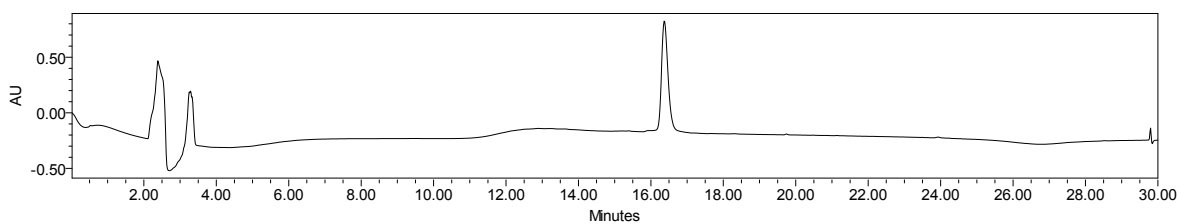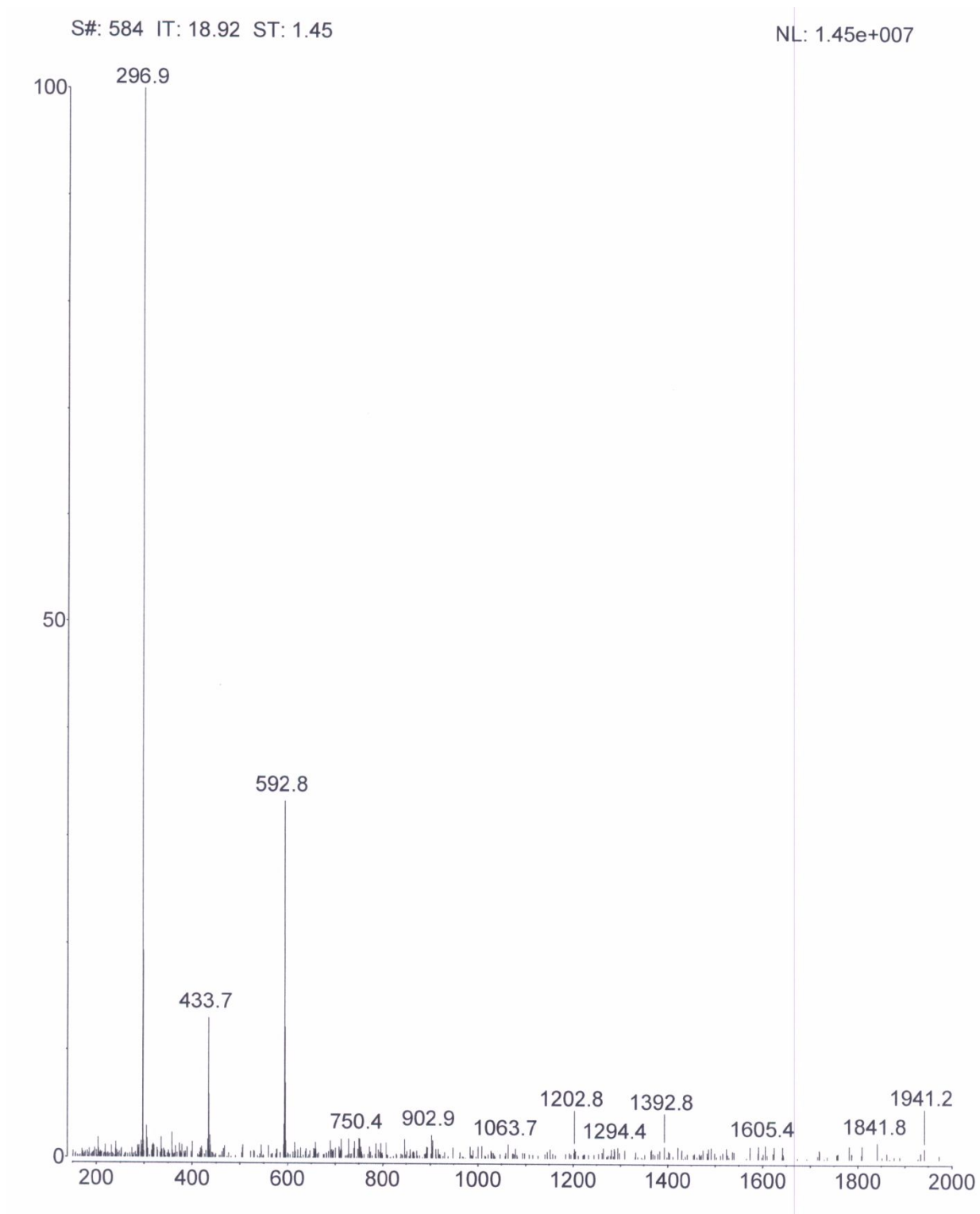

	Retention Time	Height
1	16.372	10880

LRMS of tetrapeptides 1-12

1

S#: 3616 IT: 27.43 ST: 1.46

NL: 5.71e+006

300.3

599.3

735.3

434.3

879.9

1097.5

1048.8

1280.3

1402.5

1584.0

1725.0

1970.8

200 400 600 800 1000 1200 1400 1600 1800 2000

2

S#: 4099 IT: 40.87 ST: 1.55

NL: 4.65e+006

3

S#: 2283 IT: 41.74 ST: 1.53

NL: 3.92e+006

100 ┐

300.2

599.4

50

306.1   621.2

1006.8   1230.9

873.5   1197.7

1553.0

1536.9

1734.9 1894.4

0

200   400   600   800   1000 1200 1400 1600 1800 2000

4

5

6

7

8

NL: 9.11e+005

9

S#: 711 IT: 46.81 ST: 1.52   NL: 1.85e+006

S#: 2631 IT: 14.54 ST: 1.48

NL: 6.95e+006

11

NL: 1.86e+008

Pharmacokinetic study

Materials
C57/BL6 Mouse Plasma
5.00 mM DMSO stock solutions of FP200041 and **7**
Microcentrifuge tubes
Microcentrifuge
MilliQ water
Methanol
Formic acid
Samples of plasma and brain collected during in vivo study
Pipettes and Pipette tips
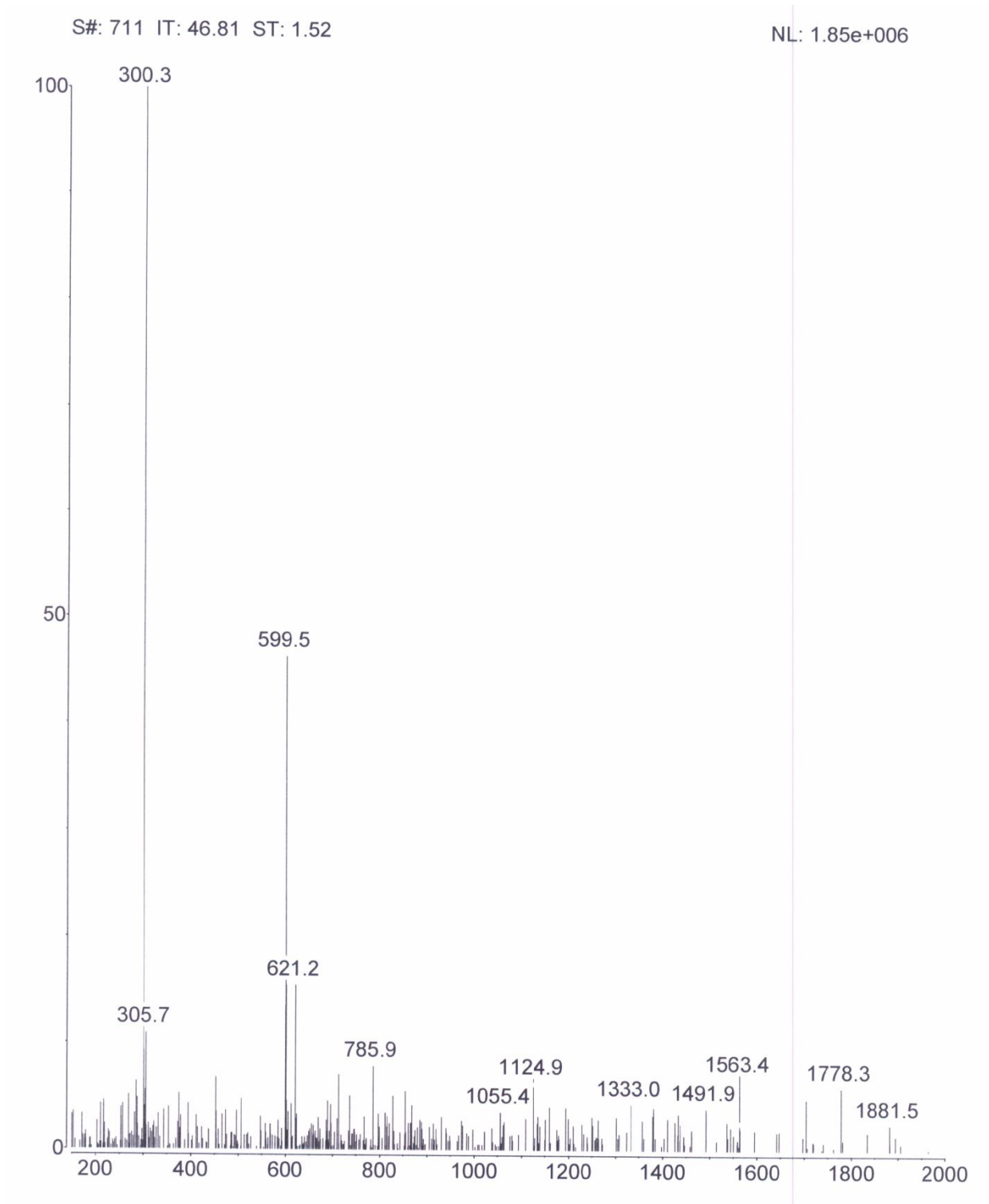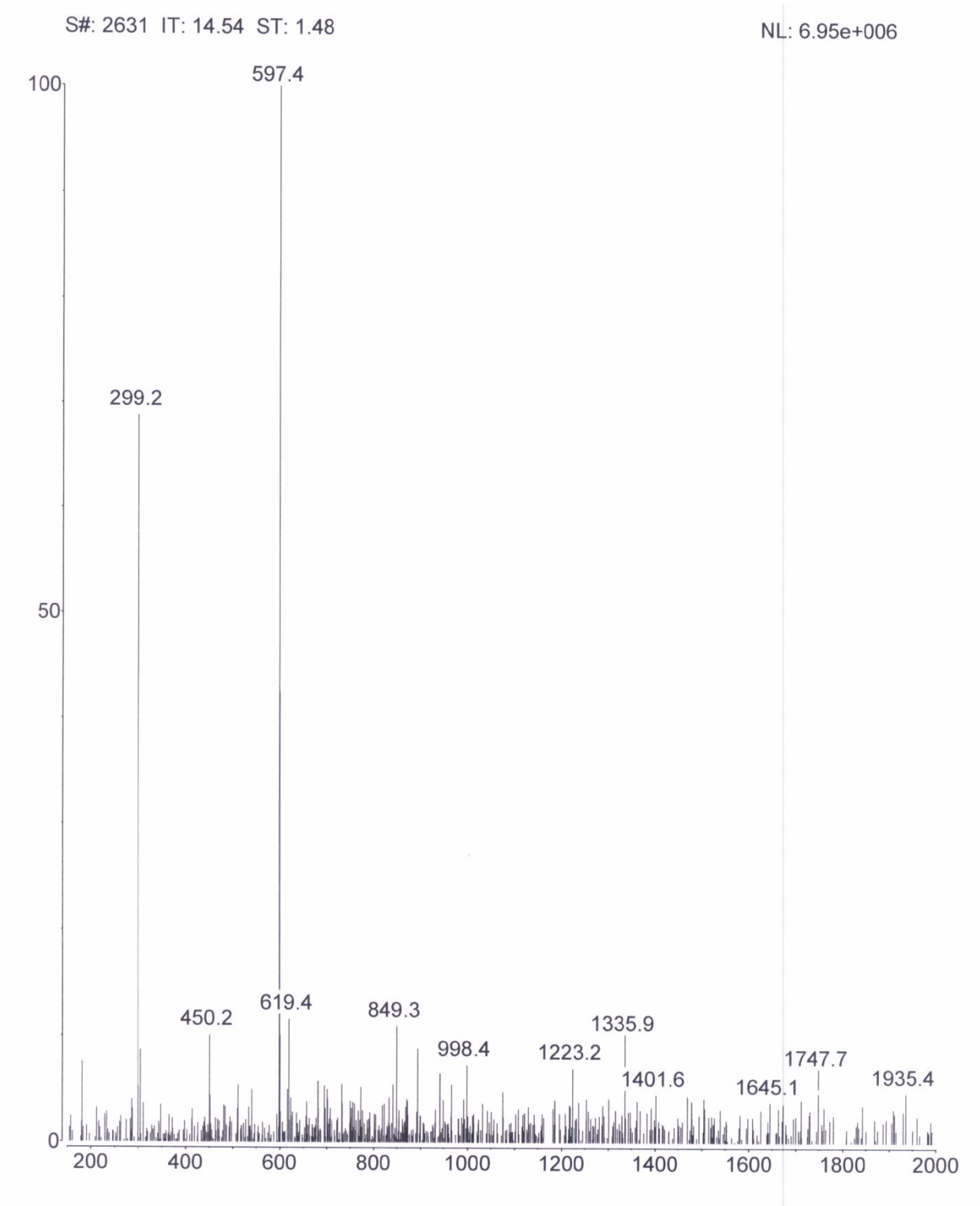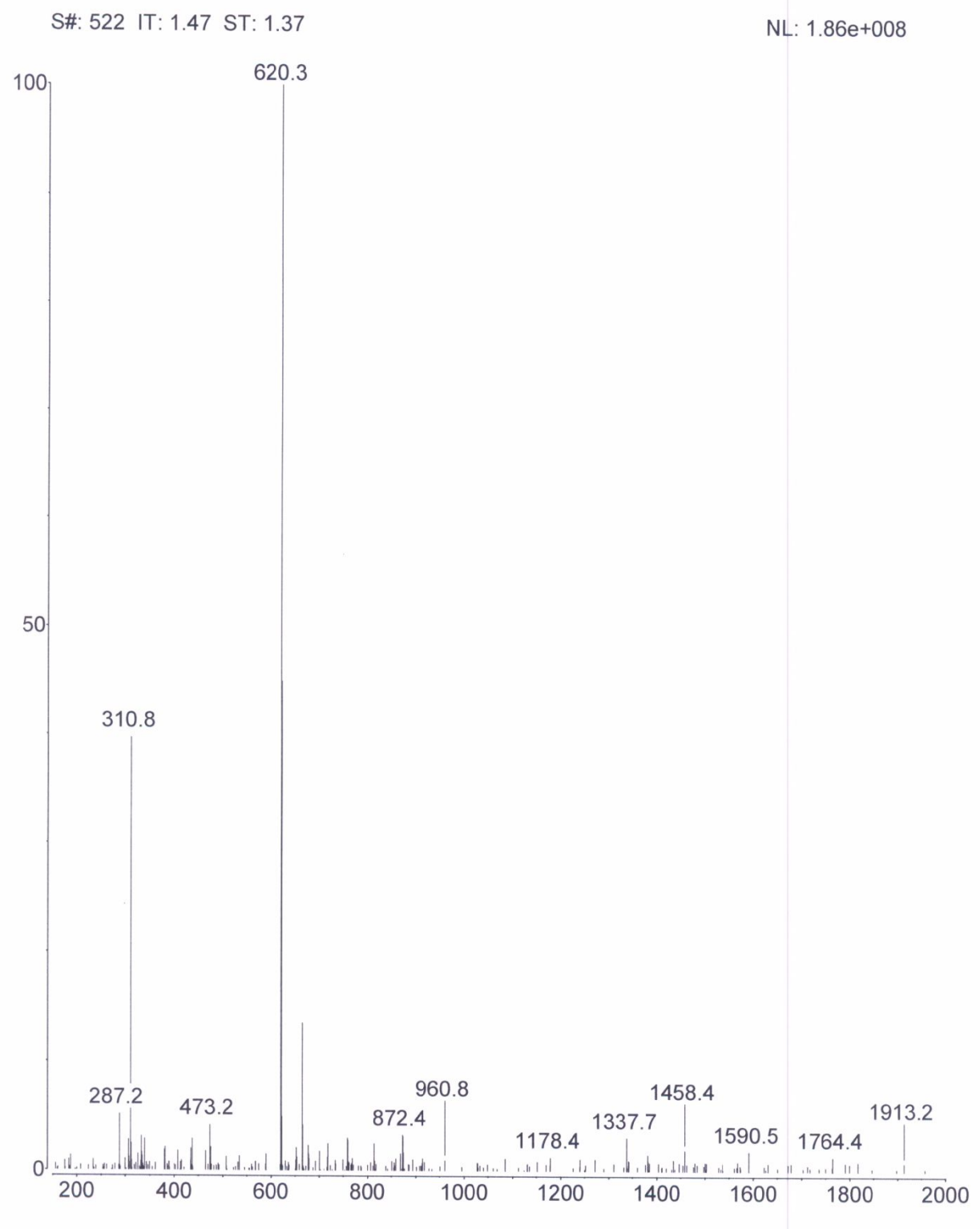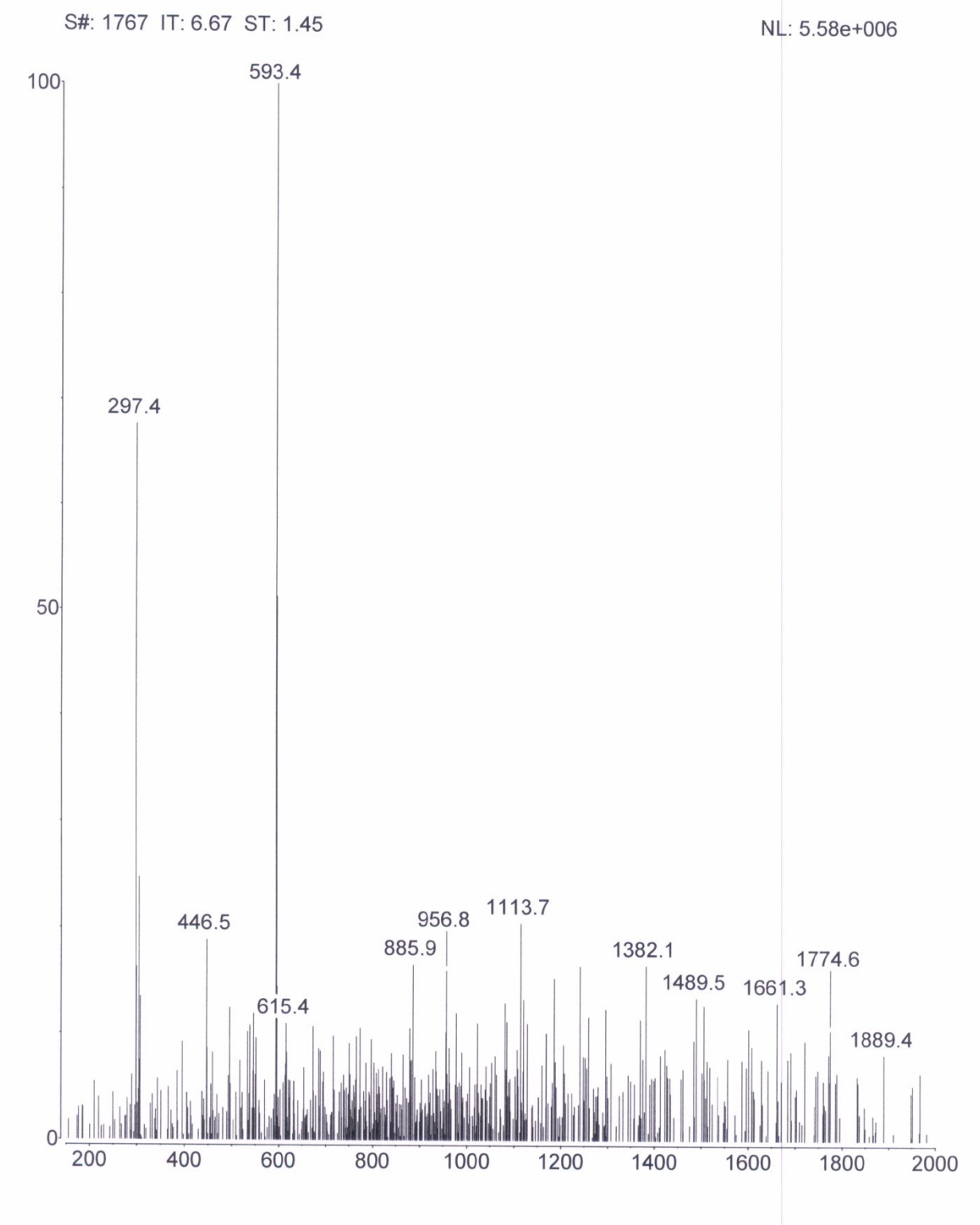150 μL conical bottom 96 well plates
Homogenizer

Plasma Extraction
1. Calibration standards in plasma were prepared in mouse plasma via serial dilution from a concentration of 5000 nM to a concentration of 1.00 nM using a dilution pattern of 1:2.5:2:2. The actual range of the calibration curve was to be tailored to the concentration observed in the samples at the time of analysis.
2. Study samples were thawed on ice.
3. A volume of 50 μL sample, blank plasma, or calibration standard was transferred to a microcentrifuge tube.
4. Add 200 μL methanol to each microcentrifuge tube.
5. Vortex for 2 minutes.
6. Centrifuge tubes for 10 minutes at 14000 rpm in a microcentrifuge.
7. Transfer 100 μL of the supernatant to a 96 well plate for analysis.
8. Inject 10 μL for LC/MS/MS analysis.

Brain Extraction
1. Brains were individually weighed on a microbalance, and weights were recorded.
2. A volume of MilliQ water was added to each tube such that the resulting tissue concentration was 0.5 mg/mL.
3. Brain tissue and water was homogenized with a Fisher Scientific PowerGen 700 at a speed of 4000 rpm until the resulting solution was uniform in appearance.
4. Homogenizer probe was rinsed at 4000 rpm in fresh HPLC grade water, and then disassembled and washed in MilliQ water subsequent to the processing of each sample.
5. Homogenized brain samples were stored at -80ºC until analysis.
6. Samples were thawed at room temperature, and thoroughly mixed.
7. Calibration standards in blank mouse plasma were prepared via serial dilution from a concentration of 5000 nM to a concentration of 1.00 nM using a dilution pattern of 1:2.5:2:2. The actual range of the calibration curve would be tailored to the concentration observed in the samples at the time of analysis.
8. A volume of 50 μL of freshly thawed and mixed brain homogenate blank, calibration standard, or sample was transferred to a microcentrifuge tube.
9. Add 200 μL methanol to each microcentrifuge tube.
10. Vortex for 2 minutes.
11. Centrifuge tubes for 2 minutes at 14000 rpm in a microcentrifuge.

12. Transfer 100 μL of the supernatant to a 96 well plate for analysis.
13. Inject 10 μL for LC/MS/MS analysis.

LC/MS/MS Conditions
Agilent 6460 mass spectrometer
Source
Gas temperature: 350°C
Gas flow: 11 L/min
Nebulizer: 45 psi
Sheath gas temperature: 400°C
Sheath gas flow: 11 L/min
Capillary: 4000V
Nozzle voltage: 500V

MS/MS Detection
Dwell time: 50 ms

Compound	Transition	Fragmentor	Collision Energy	Retention Time	Ion Mode
FP200041	693.3→113.0	125	19	3.90	negative
7	711.3→113.0	130	27	4.43	negative

LC Conditions
Agilent 1200
Agilent XDB C18 2.0 x 150 mm 5 μ
Column temperature: 30°C
A: 10 mM ammonium formate pH 4
B: methanol
Flow rate: 0.50 mL/min

Time	%A	%B
0.00	95	5
2.00	95	5
3.00	5	95
4.50	5	95
4.60	95	5
5.50	95	5

Table 1S. Individual concentration results in mouse plasma subsequent to IV dosing at 13.9 mg/kg

Time (min)	Dose Route	Concentration of FP200041 (nM)	Concentration of 7 (nM)
5	IV	BLOQ (< 10.0 nM)	BLOQ (< 10.0 nM)
5	IV	109	28.2
5	IV	80.6	265
5	IV	182	131
15	IV	64.8	106
15	IV	53.6	231
15	IV	BLOQ (< 10.0 nM)	BLOQ (< 10.0 nM)
15	IV	85.6	100
30	IV	BLOQ (< 10.0 nM)	59.1
30	IV	94.5	104
30	IV	BLOQ (< 10.0 nM)	34.3
30	IV	BLOQ (< 10.0 nM)	31.4
60	IV	BLOQ (< 10.0 nM)	30.5
60	IV	BLOQ (< 10.0 nM)	24.5
60	IV	BLOQ (< 10.0 nM)	42.0
60	IV	BLOQ (< 10.0 nM)	36.6
90	IV	BLOQ (< 10.0 nM)	BLOQ (< 10.0 nM)
90	IV	BLOQ (< 10.0 nM)	13.5
90	IV	BLOQ (< 10.0 nM)	16.8
90	IV	BLOQ (< 10.0 nM)	BLOQ (< 10.0 nM)
120	IV	BLOQ (< 10.0 nM)	BLOQ (< 10.0 nM)
120	IV	BLOQ (< 10.0 nM)	BLOQ (< 10.0 nM)
120	IV	BLOQ (< 10.0 nM)	BLOQ (< 10.0 nM)
120	IV	BLOQ (< 10.0 nM)	BLOQ (< 10.0 nM)

Figure 4S. Concentrations of FP200041 (left side) and peptide **7** (right side) in mouse plasma subsequent to IV dosing at 13.9 mg/kg (conc ± SEM).
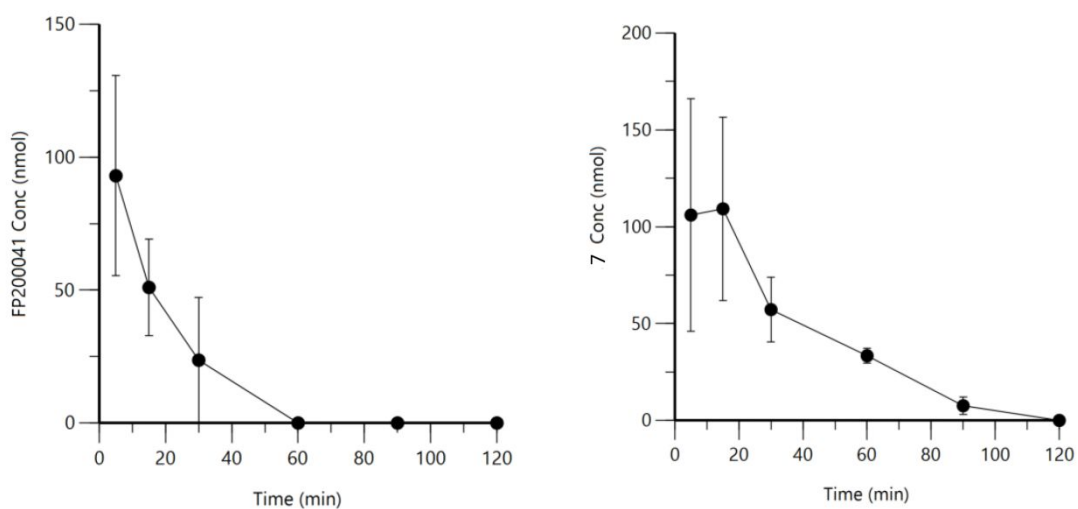
There was a large variability in the concentrations for each plasma time point, as demonstrated by SEM. This variability can be due to several reasons, most likely to variation in dosing (dose delivery) and or sample collection across animals. Additionally, variability can be introduced if the compounds are relatively insoluble in the dosing formulation.