Opioid receptor activity and analgesic potency of DPDPE peptide analogues containing a xylene bridge

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General Procedure for the Synthesis of 7a-c. D-Pen-OH was functionalized as previously reported.[1,2] All reactions were built under nitrogen atmosphere unless otherwise noted. All coupling reactions of the linear intermediates were performed using the standard coupling method of carbodiimmide (EDC/HOBt/DIPEA) in DMF,[3] as described below. The synthesis of the cyclic peptides **5a-c** started with the coupling between L-Boc-Phe-OH and D-PenOMe (Scheme 1) to obtain compound **2** in 56% yield after column chromatography. The dipeptide **2** was *N*-terminal deprotected in TFA 1:1 DCM. The resulting TFA salt was coupled with N^{α}-Boc-Gly-OH to yield the tripeptide **3** in 67% yield. Then Boc-D-PenOH was prepared following the literature's procedure,[2] and was coupled to the tripeptide **3**, to give product **4** in 64% yield after silica gel chromatography. Cyclic peptides **5a-c** were prepared from **4** following Scheme 1, then they were deprotected as TFA salts and coupled to Boc-TyrOH to afford cyclic pentapeptides **6a-c**, which were isolated on silica gel chromatography in 60%, 58%, 60% yields respectively. The three cyclic products **7a-c** were obtained deprotecting the pentapeptides **6a-c** at the COOMe terminal by hydrolysis with NaOH 1M in THF, followed by removal of *N*-Boc protecting group.

Tyr-*c*[D-Pen-Gly-Phe-D-Pen]*o*-xylene (**7a**). 41% overall yields. ^{1}H NMR ($(CD_3)_2SO$) δ 1.08 (s, 3H, CH3 Pen^5), 1.36 (s, 3H, CH3 Pen^5), 1.41 (s, 3H, CH3 Pen^2), 1.45 (s, 3H, CH3 Pen^2), 2.77-2.63 (m, 2H, $^{\beta}$CH2 Tyr and 1H, $^{\beta}$CH Phe), 3.03 (m, 1H, $^{\beta}$CH Phe), 3.22 (s, 2H, CH2 xylene), 3.71 (s, 2H, CH2 xylene), 4.12 (m, 1H, $^{\alpha}$CH Tyr), 4.23 (m, 2H, $^{\alpha}$CH2 Gly), 4.37-4.41 (m, 1H, $^{\alpha}$CH Phe and 1H, $^{\alpha}$CH Pen^5), 4.63 (d, 1H, $^{\alpha}$CH Pen^2), 6.68 (d, 2H, C3,5H Tyr), 7.09 (d, 2H, C2,4H Tyr), 7.31-7.11 (m, 9H, H Phe and H xylene aromatics), 7.58 (d, 1H, NH Pen^2), 8.01 (br, 3H, NH_3^+ Tyr), 8.43 (d, 1H, NH Pen^5), 8.54 (d, 1H, NH Phe), 8.68 (t, 1H, NH Gly), 9.34 (s, 1H, OH Tyr). ESI-LRMS for $C_{38}H_{47}N_5O_7S_2$, calcd. m/z = 749.9; found 750.7 [M+H].

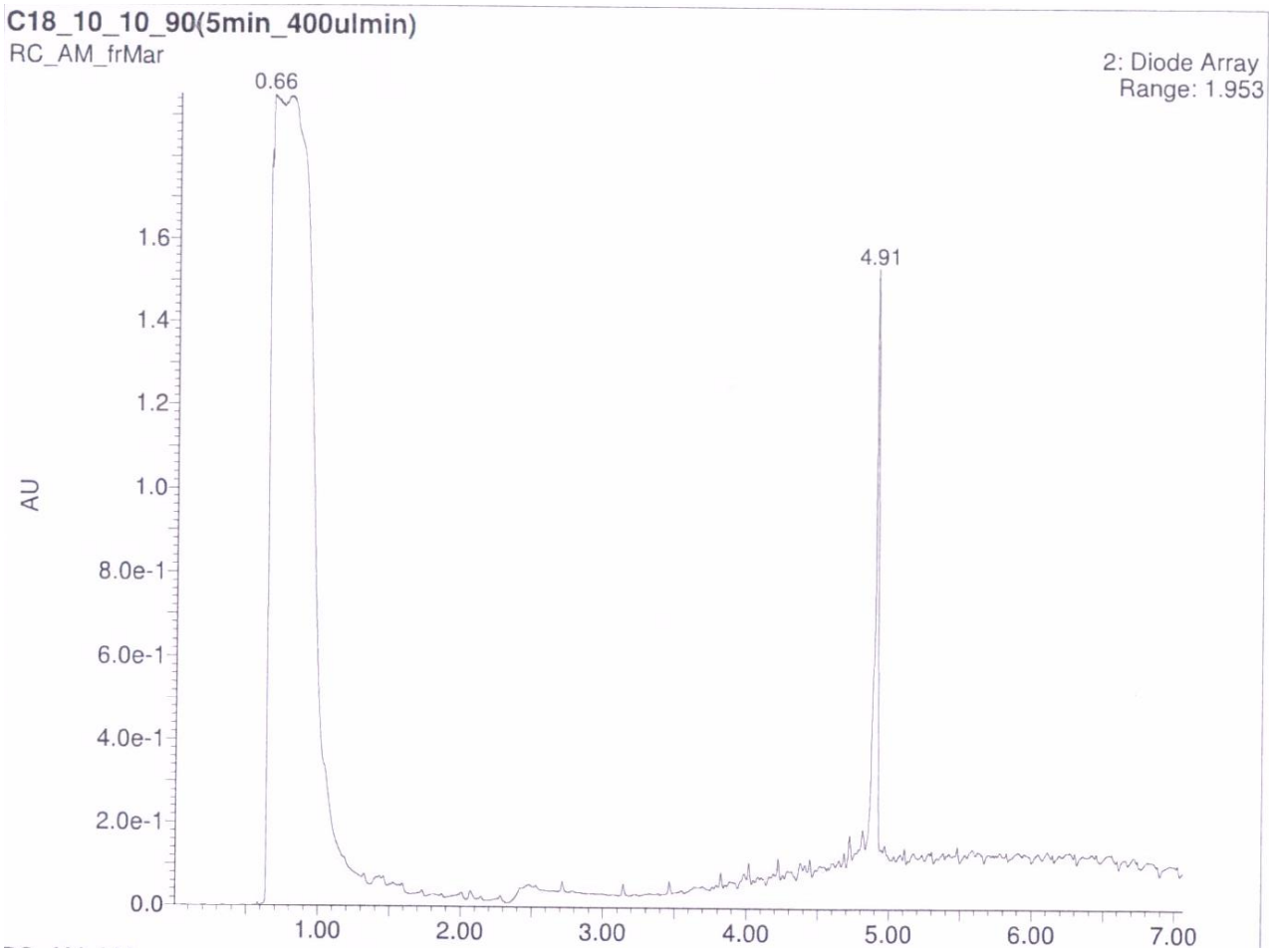
Tyr-*c*[D-Pen-Gly-Phe-D-Pen]*m*-xylene (**7b**). 55% overall yield. ^{1}H NMR ($(CD_3)_2SO$) δ 1.16 (s, 9H, CH3 Pen^5 and 2CH3 Pen^2), 1.53 (s, 3H, CH3 Pen^5), 2.52 (m, 2H, $^{\beta}$CH2 Phe), 2.76 (m, 1H, $^{\beta}$CH Tyr), 2.94 (m, 1H, $^{\beta}$CH Tyr), 3.19 (s, 2H, CH2 xylene), 3.71 (s, 2H, CH2 xylene), 3.91 (m, 2H, $^{\alpha}$CH2 Gly), 4.12 (m, 1H, $^{\alpha}$CH Tyr), 4.42 (d, 1H, $^{\alpha}$CH Pen^5), 4.73 (d, 1H, $^{\alpha}$CH Phe), 4.81 (d, 1H, Pen2), 6.71 (d, 2H, C3,5H Tyr), 7.06-7.34 (m, 2H, C2,4H Tyr; 9H, H Phe and H xylene aromatics), 7.67 (d, 1H, NH Pen^2), 8.05 (br, 3H, NH_3^+ Tyr), 8.86 (t, 2H, NH Phe plus NH Pen^5), 9.23 (t, 1H, NH Gly), 9.38 (s, 1H, OH Tyr). ESI-LRMS for $C_{38}H_{47}N_5O_7S_2$, calcd. m/z = 749.9; found 750.5 [M+H].

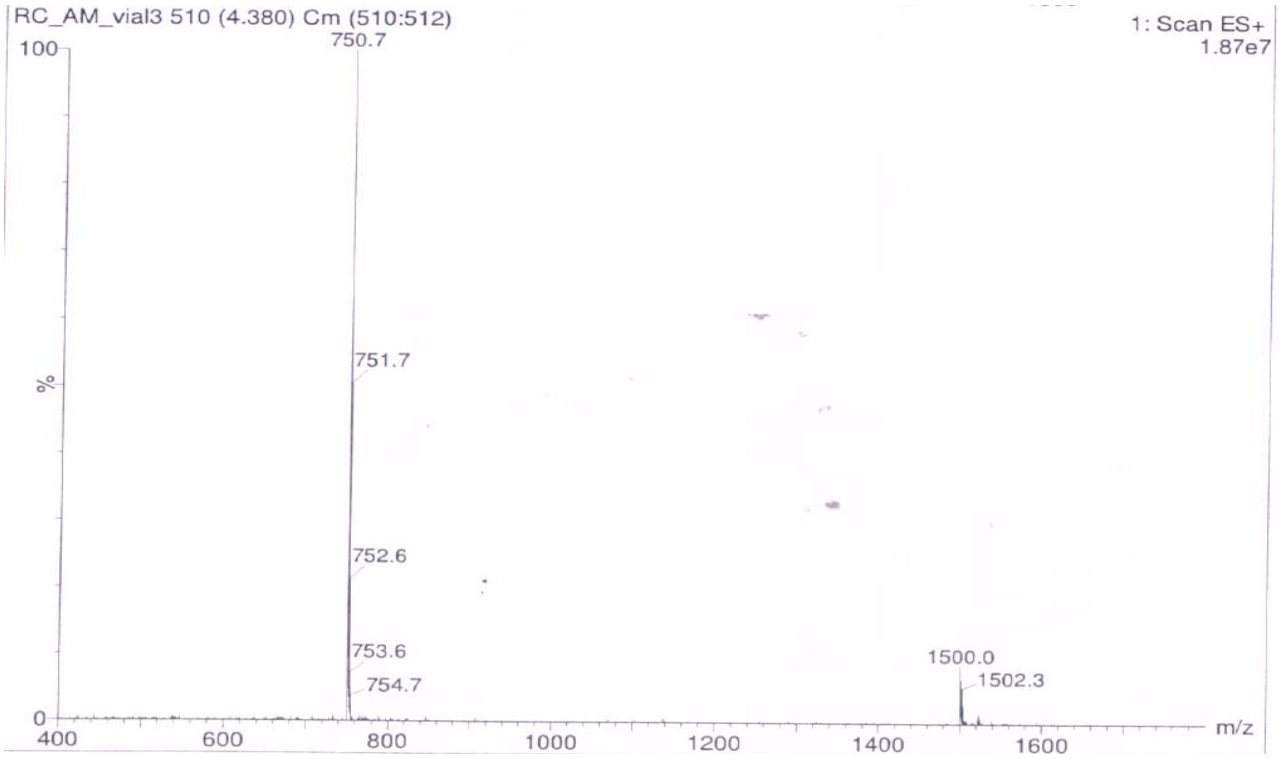
Tyr-*c*[D-Pen-Gly-Phe-D-Pen]*p*-xylene (**7c**). 40% overall yield. ^{1}H NMR ($(CD_3)_2SO$) δ 1.11 (s, 3H, CH3 Pen^5) 1.23 (s, 3H, CH3 Pen^5), 1.33 (s, 3H, CH3 Pen^2), 1.57 (s, 3H, CH3 Pen^2), 2.01 (m, 1H, $^{\beta}$CH Phe), 2.78 (m, 1H, $^{\beta}$CH Phe), 3.01 (m, 2H, $^{\beta}$CH2 Tyr), 3.19 (s, 2H, CH2 xylene), 3.68 (s, 2H, CH2 xylene), 3.76 (m, 1H, $^{\alpha}$CH Gly), 3.93 (m, 1H, $^{\alpha}$CH Gly), 4.03 (d, 1H, $^{\alpha}$CH Pen^5), 4.15 (m, 1H,

$^{\alpha}$CH Tyr), 4.52 (m, 1H, $^{\alpha}$CH Phe), 4.68 (d, 1H, Pen^{2}), 6.71 (d, 2H, C3,5H Tyr), 7.06-7.23 (m, 12H, C2,4H Tyr, H Phe, NH Phe and H xylene aromatics), 7.73 (d, 1H, NH Pen^{2}), 8.03 (br, 3H, NH_3^+ Tyr), 8.71 (t, 1H, NH Gly), 8.85 (d, 1H, NH Pen^{5}), 9.35 (s, 1H, OH Tyr). ESI-LRMS for $C_{38}H_{47}N_5O_7S_2$, calcd. m/z = 749.9; found 750.4 [M+H].

Figure S1. MS-UPLC of compound **7a.** ESI-LRMS for $C_{38}H_{47}N_5O_7S_2$, calcd. m/z = 749.9; found 750.7 [M+H].

UPLC RP: C18 linear gradient of H_2O/acetonitrile 0.1% TFA starting from 10% acetonitrile to 90% acetonitrile in 20 min.

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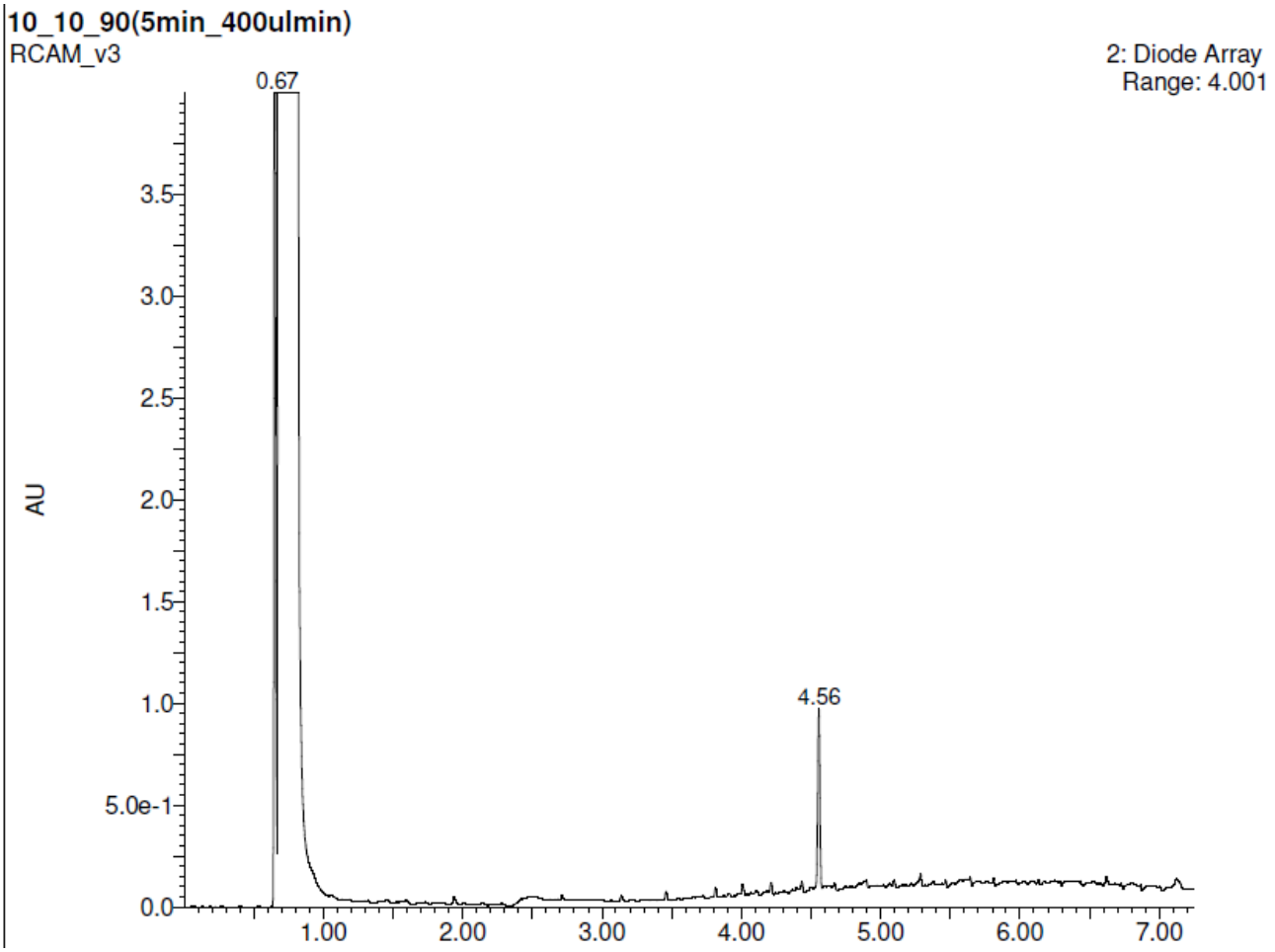
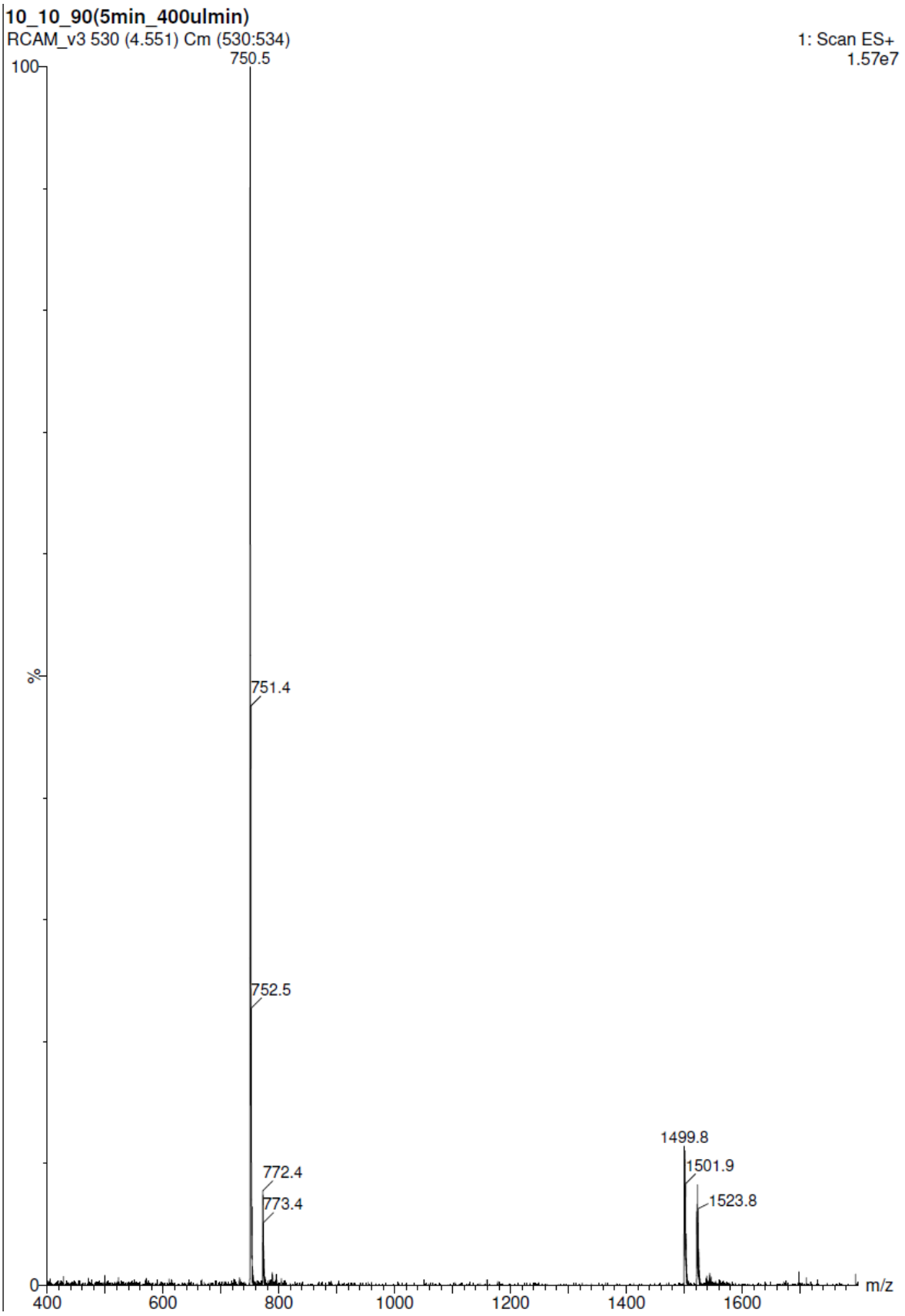
%
0
750.7
751.7
752.6
753.6
754.7
1500.0
1502.3
400
600
800
1000
1200
1400
1600
m/z

Figure S2. MS-UPLC of compound **7b.** ESI-LRMS for $C_{38}H_{47}N_5O_7S_2$, calcd. m/z = 749.9; found 750.5 [M+H].

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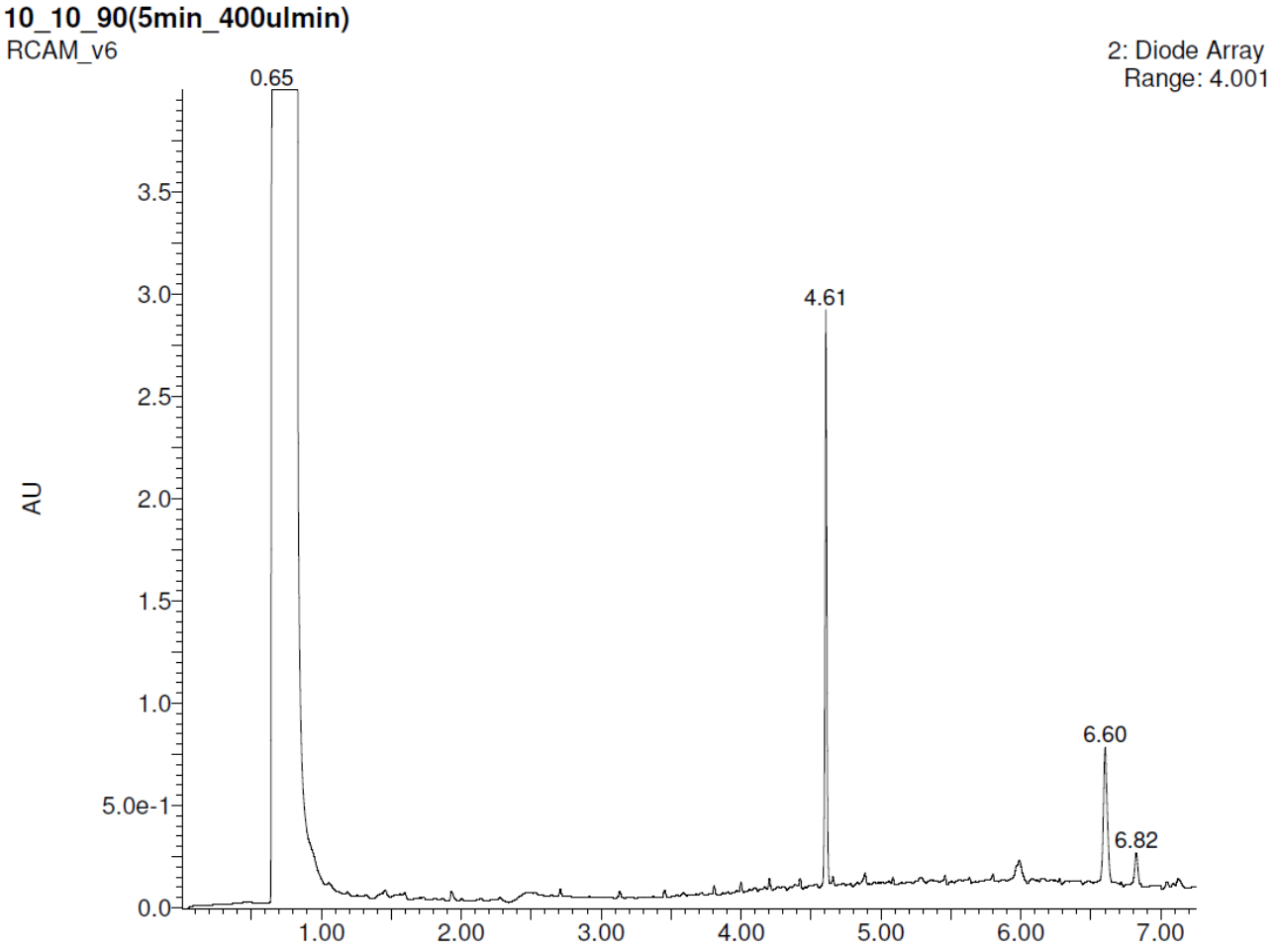
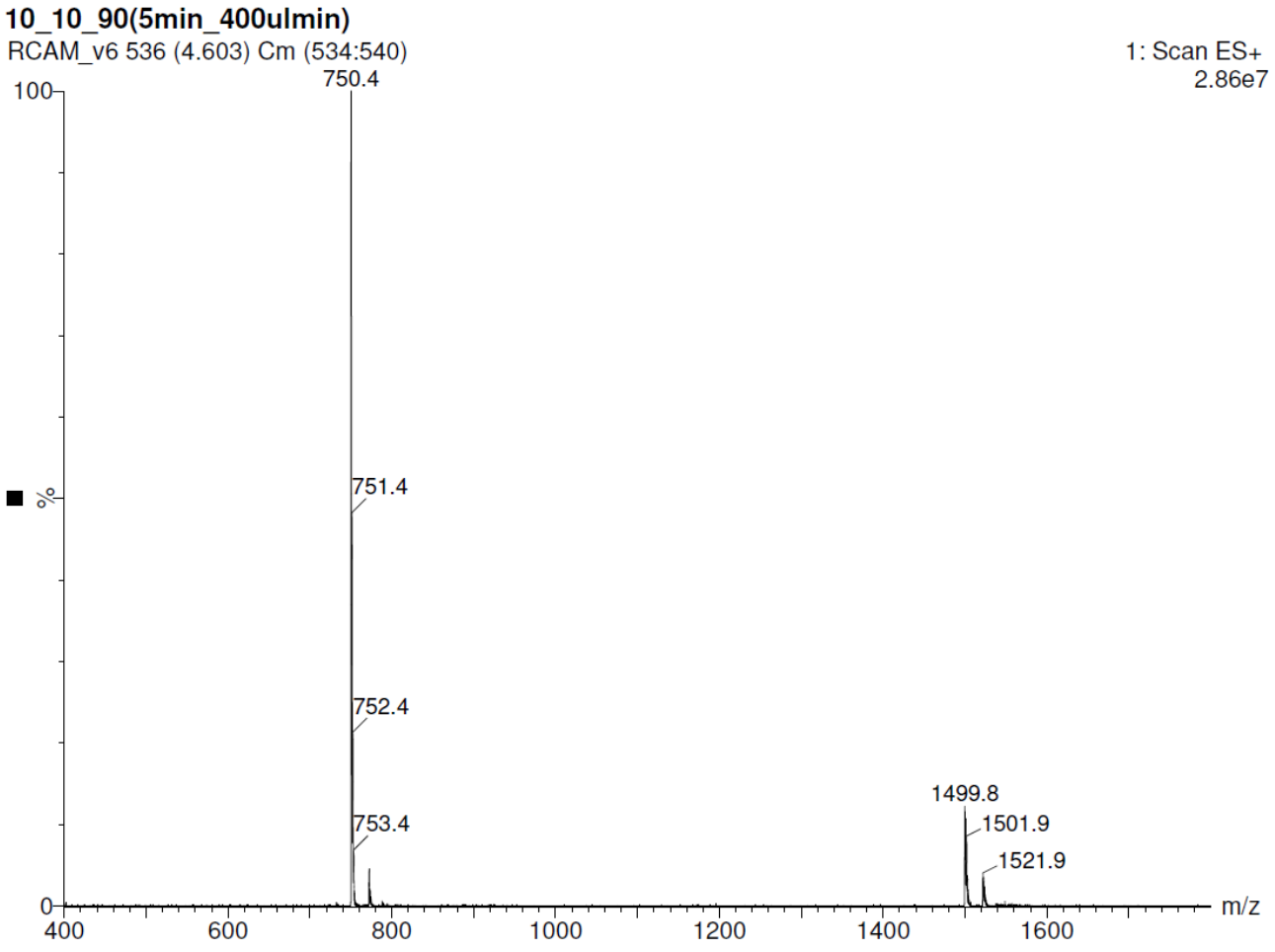
%
0
750.5
751.4
752.5
772.4
773.4
1499.8
1501.9
1523.8
400
600
800
1000
1200
1400
1600
m/z

Figure S3. MS-UPLC of compound **7c.** ESI-LRMS for $C_{38}H_{47}N_5O_7S_2$, calcd. m/z = 749.9; found 750.4 [M+H].

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100
%
0
750.4
751.4
752.4
753.4
1499.8
1501.9
1521.9
400
600
800
1000
1200
1400
1600
m/z

***In vitro* competition binding assays**

Chemicals

Tris-HCl and DPDPE were purchased from Sigma-Aldrich (Budapest, Hungary). Naloxone was kindly provided by the company Endo Laboratories DuPont de Nemours (Wilmington, DE, USA). The DPDPE analogues **7a-c** were provided by Mollica's group. Ligands were dissolved in water and stored in 1 mM stock solution at -20˚C. Ligands were stored in 1 mM stock solution. [^{3}H]IleDelt II (specific activity: 19,6 Ci/mmol),[4] and [^{3}H]DAMGO (specific activity: 38.8 Ci/mmol) were radiolabeled by the Laboratory of Chemical Biology group in BRC (Szeged, Hungary). The Ultima Gold^{TM} MV aqueous scintillation cocktail was purchased from PerkinElmer (Budapest, Hungary).

Animals

Male and female Wistar rats (250-300g body weight) were used for binding assays. Rats were housed in the local animal house of BRC (Szeged, Hungary) and were kept in a temperature controlled room (21-24^{o}C) under a 12:12 light and dark cycle, and were provided with water and food *ad libitum*. All housing and experiments were conducted in accordance with the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§). The total number of animals as well as their suffering was minimized.

Rat whole brain membrane homogenate preparation for binding assays

Rats were decapitated and their brains were quickly removed. Samples were prepared for membrane homogenate preparation as previously described;[5] Briefly the brains were homogenized, centrifuged and incubated, afterwards the centrifugation was repeated and the final pellet was suspended in 50 mM Tris-HCl pH 7.4 buffer containing 0.32 M sucrose, and stored at -80^{o}C.

Competition radioligand binding assay

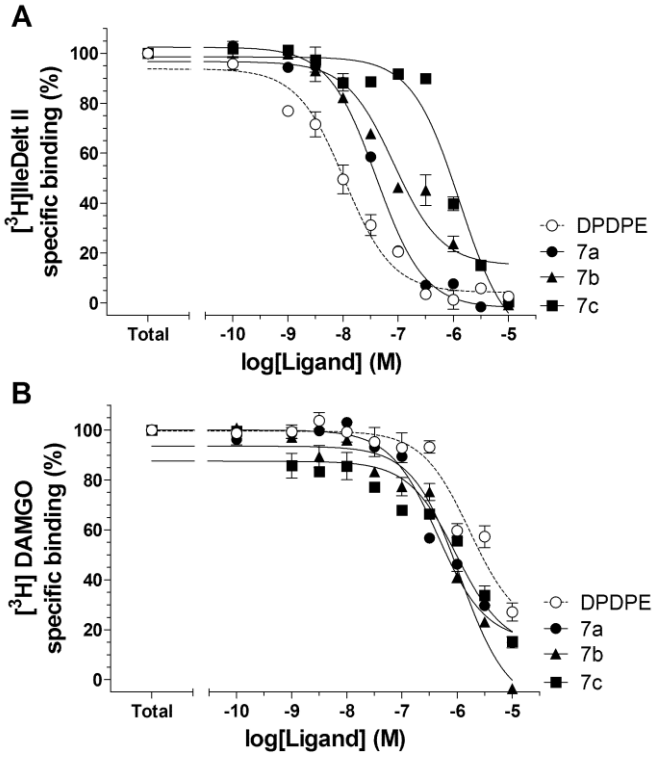
Aliquots of frozen rat whole brain membrane homogenates were centrifuged (18 000 rpm, 20 min, 4˚C) to remove sucrose, afterwards the pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) to reach the appropriate protein content. Rat whole brain membrane homogenates containing 0.3-0.6 mg/mL of protein were incubated at 35^{o}C for 45 minutes together with 2 nM of [^{3}H]lleDelt II or [^{3}H]DAMGO in the presence of unlabeled DPDPE and **7a-c** at increasing concentrations (10^{-11}-10^{-5} M) in a final volume of 1 mL. Incubation was followed by rapid filtration under vacuum, washing three times with 5 mL ice-cold Tris-HCl (50 mM, pH 7.4) buffer through Whatman GF/B glass fiber filters, using Brandel M24R cell harvester. Non-specific binding was defined in the presence of 10 µM unlabelled naloxone, while total binding was measured in the absence of the unlabelled ligands. The radioactivity of the filters was detected in Ultima Gold^{TM} MV aqueous scintillation

cocktail with Packard TriCarb 2300TR liquid scintillation counter. Competition binding experiments were performed in duplicates and repeated at least three times.

Data analysis

The specific binding of [^{3}H]lleDelt II and [^{3}H]DAMGO was calculated by subtracting the data of non-specific binding from total binding and was given in percentage. The data was normalized to total specific binding, which was settled 100%. Experimental data were presented as means ± S.E.M in the function of the applied ligand concentration range in logarithm form. Points were fitted using non-linear regression with the professional curve fitting program, GraphPad Prism 5.0 (GraphPad Prism Software Inc., San Diego, CA). The 'One-site competition' equation was applied for the data obtained from the radioligand competition binding assays to determine the logIC_{50} (unlabeled ligand affinity) value. The K_i value was also calculated according to the Cheng-Prusoff equation,[6] and based on the values selectivity ratios were calculated to examine DOR selectivity of the compounds. For IC_{50} value standard error is only given in their logarithm form by the curve fitting program due to the data representation.

Figure S4. The δ and μ opioid receptors binding properties of 7a-c compared to DPDPE in radioligand competition binding experiments. Figures represents the specific binding of [^{3}H]IleDelt II (**A**) and [^{3}H]DAMGO (**B**) in percentage in the presence of increasing concentrations (10^{-10}-10^{-5} M) of the indicated unlabeled ligands in rat brain membrane homogenates. "Total" on the x-axis indicates the total specific binding of the radioligand, which is measured in the absence of the unlabeled compounds. The level of total specific binding was defined as 100%. Points represent means ± S.E.M. for at least three experiments performed in duplicate.



***In vivo* assays**

Animals

Male CD-1 mice (Harlan, Italy) weighing 25-30g were used for all experiments. Mice were housed for at least 1 week before the experimental sessions in colony cages (7 mice in each cage) under standard light (from 7.00 a.m. to 7.00 p.m.), temperature (21±1°C), relative humidity (60±10%) with food and water 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/EEC on laboratory animal protection. Animal welfare was routinely checked by veterinarians from the Service for Biotechnology and Animal Welfare.

Assessment of nociception

Thermal-induced nociception: hot plate and tail flick test

Hot plate test was performed using a commercially available apparatus consisting of a metal plate 25x25 cm (Ugo Basile, Italy) heated to a constant temperature of 55.0 ± 0.1°C, on which a plastic cylinder (20 cm diameter, 18 cm high) was placed. The time of latency (s) was recorded from the moment the animal was placed in the cylinder on the hot plate until it licked its paws or jumped; the cut-off time was 60 s. The baseline was calculated as mean of three readings recorded before testing at intervals of 15 min, and was in the same order of magnitude in all experimental groups (mean 9.8±1.2 s, N=8-10). The time course of latency was then determined at 15, 30, 45, 60, 90 and 120 min after compound treatment.

The tail flick latency was obtained using a commercial unit (Ugo Basile, Italy), consisting of an infrared radiant light source (100 W, 15 V bulb) focused onto a photocell utilizing an aluminum parabolic mirror.[7] 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 (15 s at 15 V). Also in this case, the baseline was calculated as mean of three readings recorded before testing at intervals of 15 min (mean 3.4±0.8 s, N=8-10) and the time course of latency determined at 15, 30, 45, 60, 90 and 120 min after treatment. In both 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. In both test, drugs were injected in the cerebral ventricle (i.c.v.) at the dose of 23 nmol/mouse. For i.c.v. injections, mice were lightly anesthetized with isoflurane, and an incision was made in the scalp. Injections were performed using a 10 μL Hamilton microsyringe at a point 2-mm caudal and 2-mm lateral from the bregma.[8]

Chemical-induced nociception: formalin test

Subcutaneous injection of a dilute solution of formalin (1%, 20 μL/paw) into the mice hind paw evokes nociceptive behavioral responses, such as licking, biting the injected paw or both, which are considered indices of nociception.[7] The nociceptive response shows a biphasic trend, consisting of an early phase occurring from 0 to 10 min after the formalin injection, due to the direct stimulation of peripheral nociceptors, followed by a late prolonged phase occurring from 15 to 40 min, that reflects the response to inflammatory pain. During the test, the mouse was placed in a Plexiglas observation cage (30×14×12 cm), 1h before the formalin administration to allow it to acclimatize to its surroundings. The total time (s) that the animal spent licking or biting its paw during the formalin-induced early and late phase of nociception was recorded. In the formalin test, drugs were administered subcutaneously (s.c.) at the dose of 150 nmol/mouse, 15 minutes before formalin.

Data analysis and statistics

Experimental data were expressed as mean ± S.E.M. The significance among groups was evaluated with the analysis of variance (two-way ANOVA) followed by Bonferroni's post-hoc comparisons using the statistical software Prism 6.0 (GraphPad Software Inc.). Statistical significance was assumed at $P<0.05$.

Drugs and treatment procedure

DMSO was purchased from Merck (Italy). DPDPE was purchased from Sigma Aldrich (Italy). On each test day, peptides solutions were freshly prepared using saline containing 0.9% NaCl and DMSO in the ratio DMSO:saline = 1:5v/v.

These solutions were injected at a volume of 5 μL/mouse for i.c.v. administrations or in a volume of 20 mL/mouse for s.c. administration.

Molecular Modeling

Protein preparation

The X-Ray structure of δ opioid receptor co-crystallized with pseudopeptide DIPP-NH_2 was downloaded from the Swiss Protein Databank, pdb code: 4RWD.[9] The receptor was prepared for molecular docking by removing the crystallographic ligand and all the small molecules present in the pdb file, with exception of the Na^+ atom and the water coordinated to it. Then the protein was processed with the Protein Wizard suite,[10] embedded in Maestro 10.2.[11] which fixed automatically all the errors in the crystal and a minimization of the hydrogens was also carried out by OPLS3 force field. The protein was used for molecular modeling without any further modification.

Ligands preparation

Fully deprotected DPDPE and DPDPE analogues **7a-c** were drawn by Chemdraw14.0 and converted into 3D models by Maestro 10.2. Then the structures were prepared for docking by LigPrep embedded in Maestro 10.2 suite, minimized by OPLS2005 force field and submitted to molecular docking.

Figure S5. Representation of the interactions between the best docking pose of DPDPE (A), compound **7a** (B), compound **7b** (C) and compound **7c** (D) at the DOR.

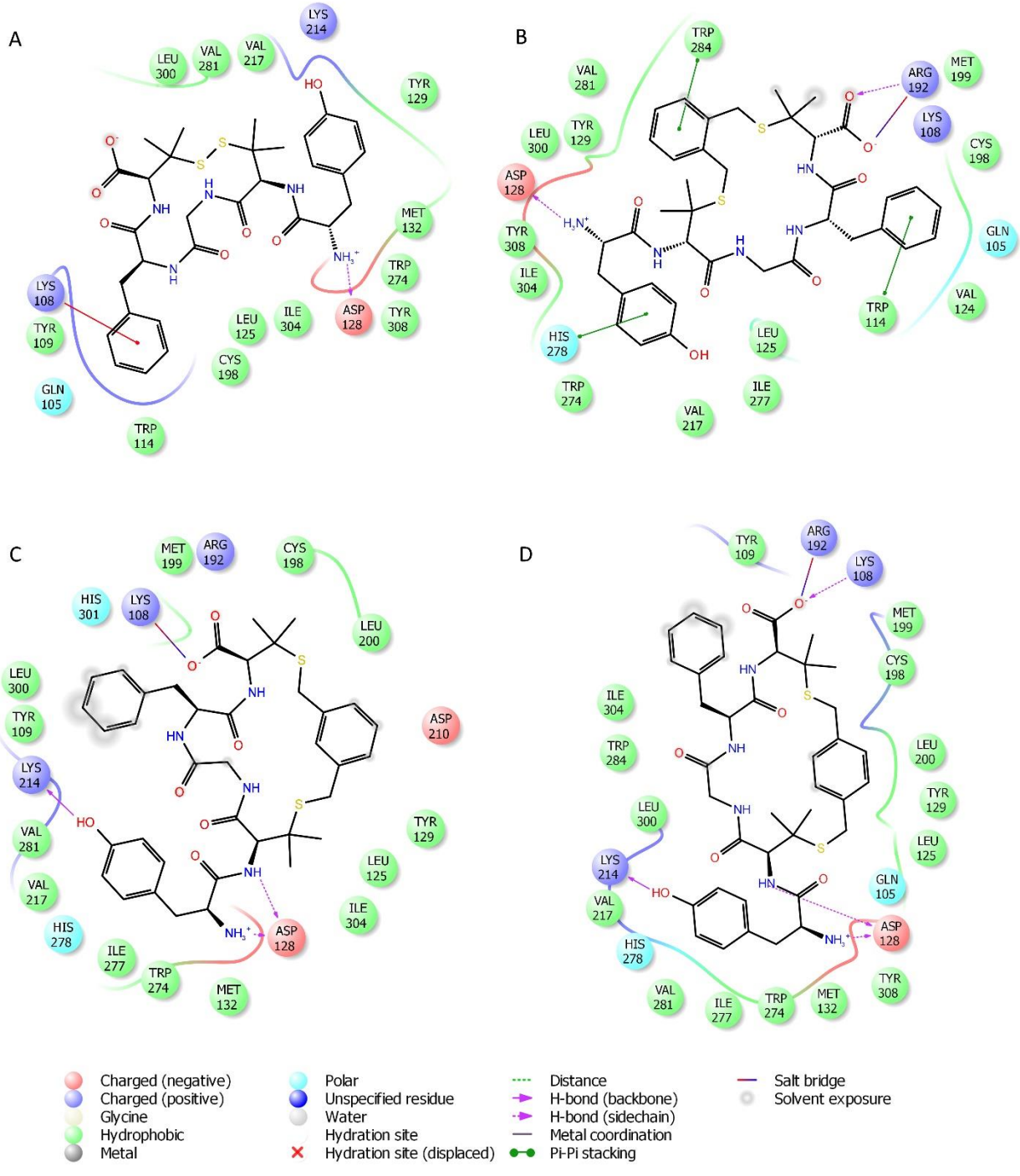
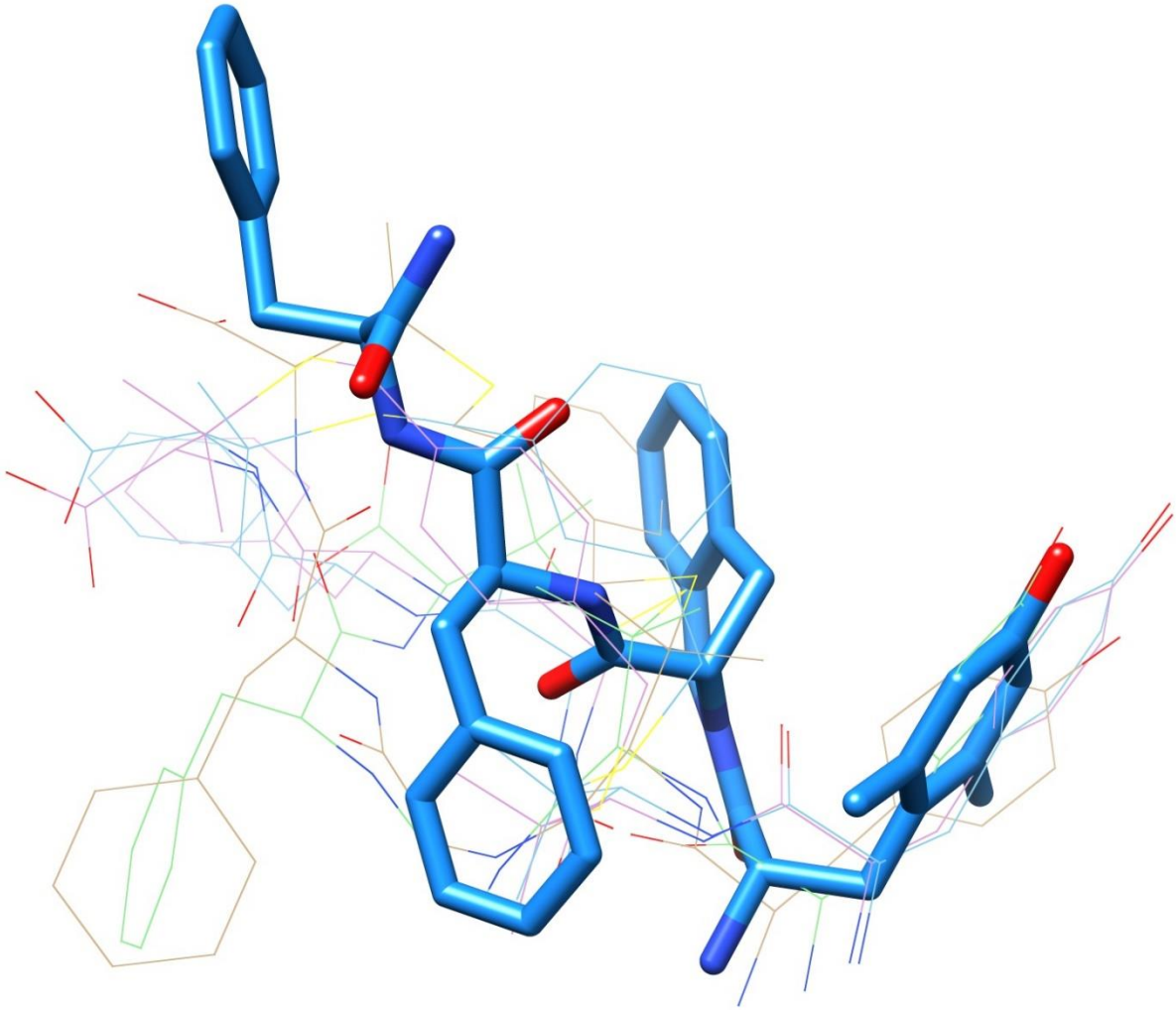


Figure S6. Best docking pose of DPDPE (green), compound **7a** (brown), compound **7b** (cyan), compound **7c** (blue) in comparison with the crystallographic ligand DIPP-NH_2 (bold blue).



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