

# Synthesis, Pharmacological Evaluation, and Computational Studies of Cyclic Opioid Peptidomimetics Containing $\beta^3$ -Lysine

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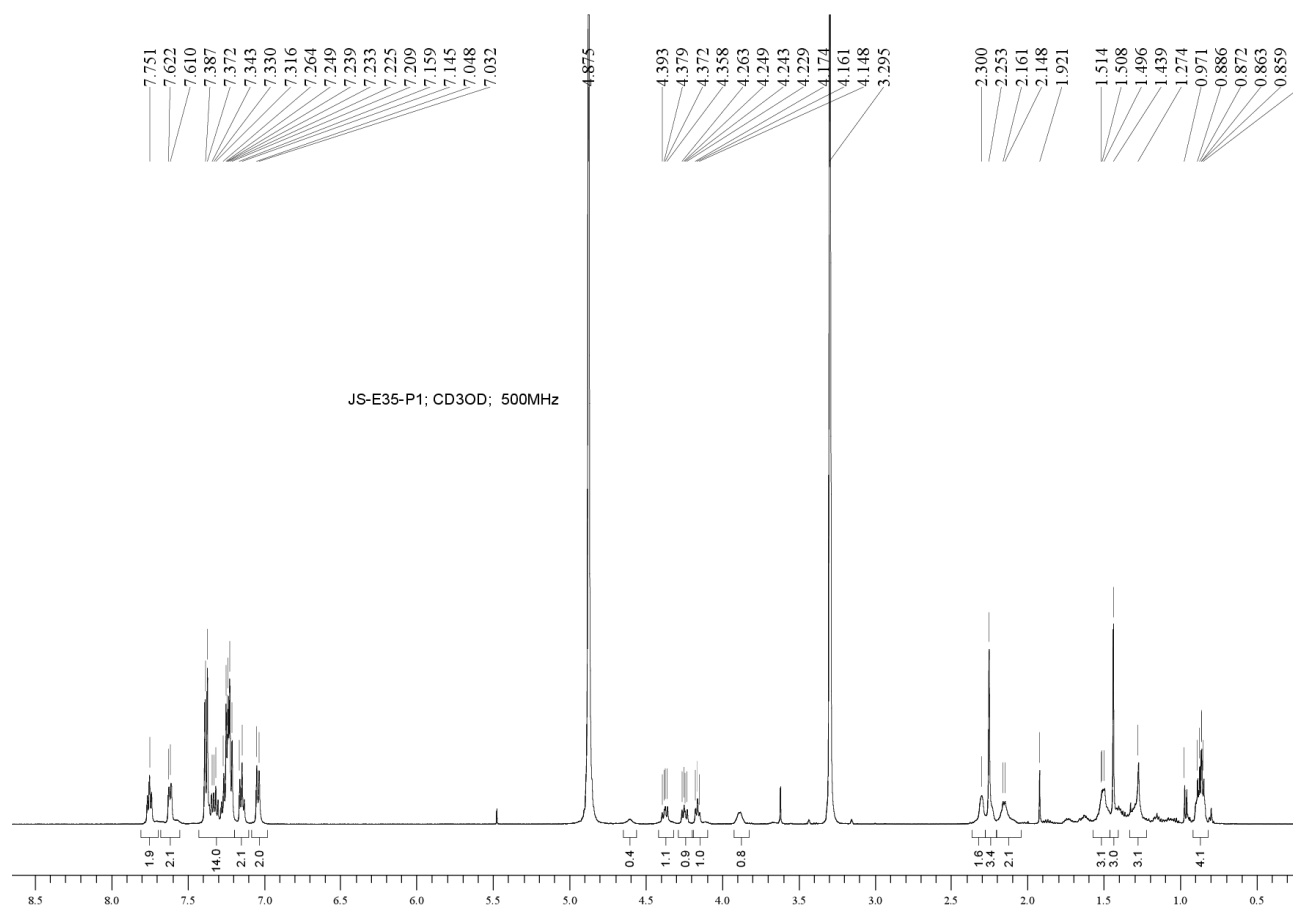
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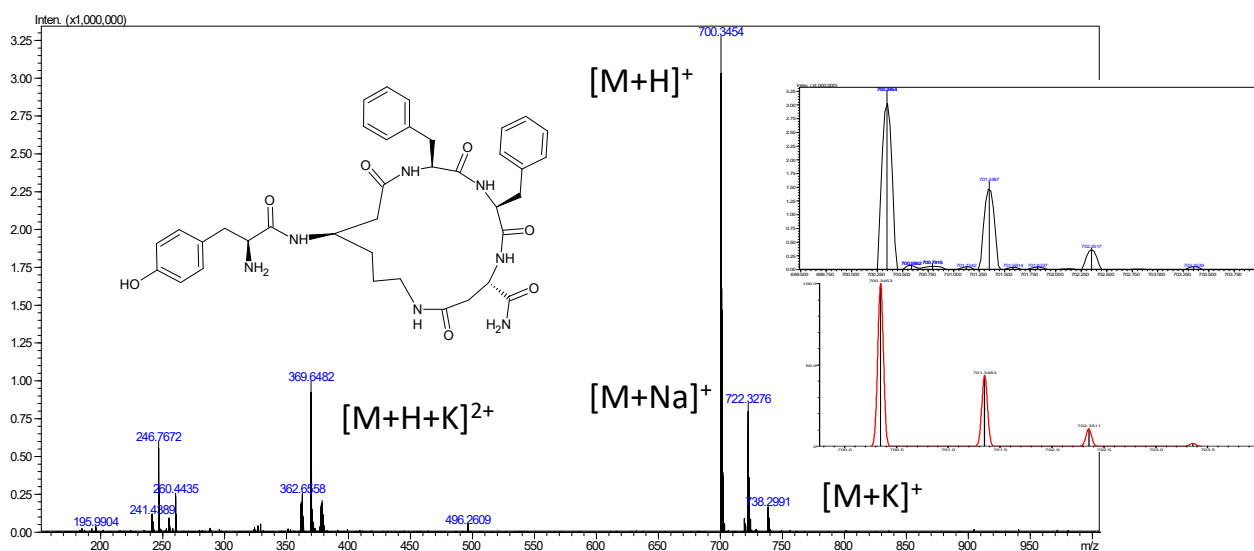
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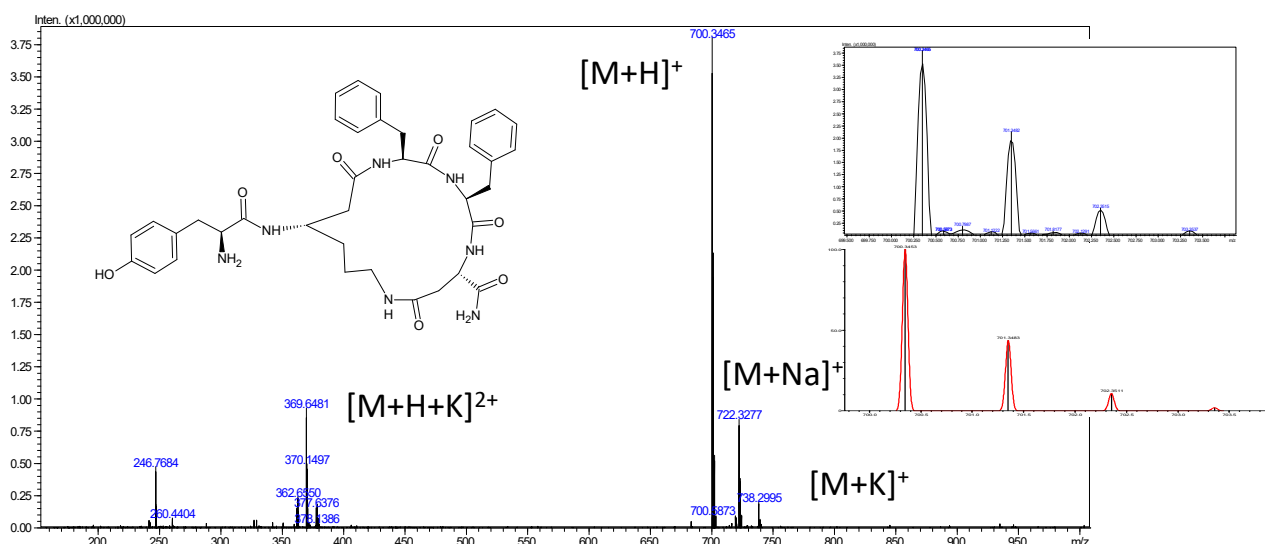
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**Figure S1.**  $^1\text{H}$  NMR spectrum of Fmoc-(*R*)- $\beta^3$ -Lys(Mtt).

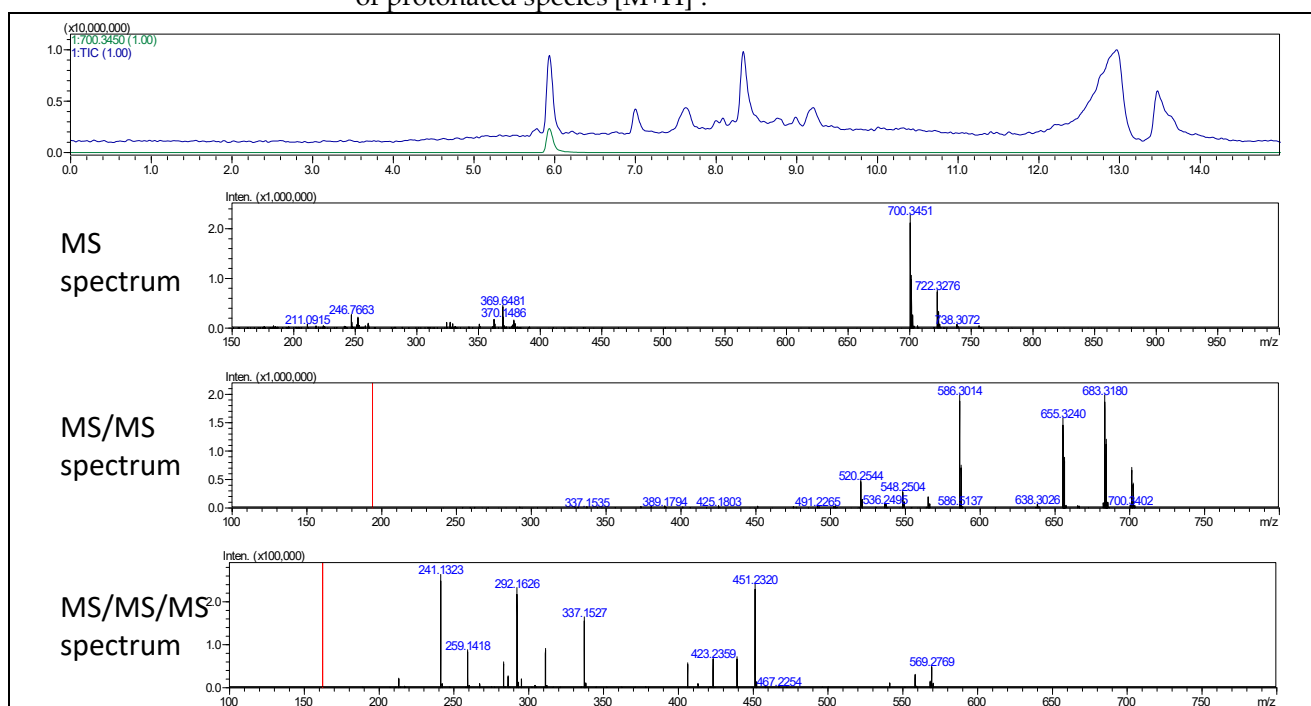


**Figure S2.** Analysis of peptide Tyr-c[(*R*)- $\beta^3$ Lys-Phe-Phe-Asp]NH<sub>2</sub> (RP-171) by mass spectrometry. High resolution MS spectrum. In inset, fragment of the experimental spectrum is compared with the simulated isotopic profile calculated for the expected molecular formula of protonated species  $[\text{M}+\text{H}]^+$ .

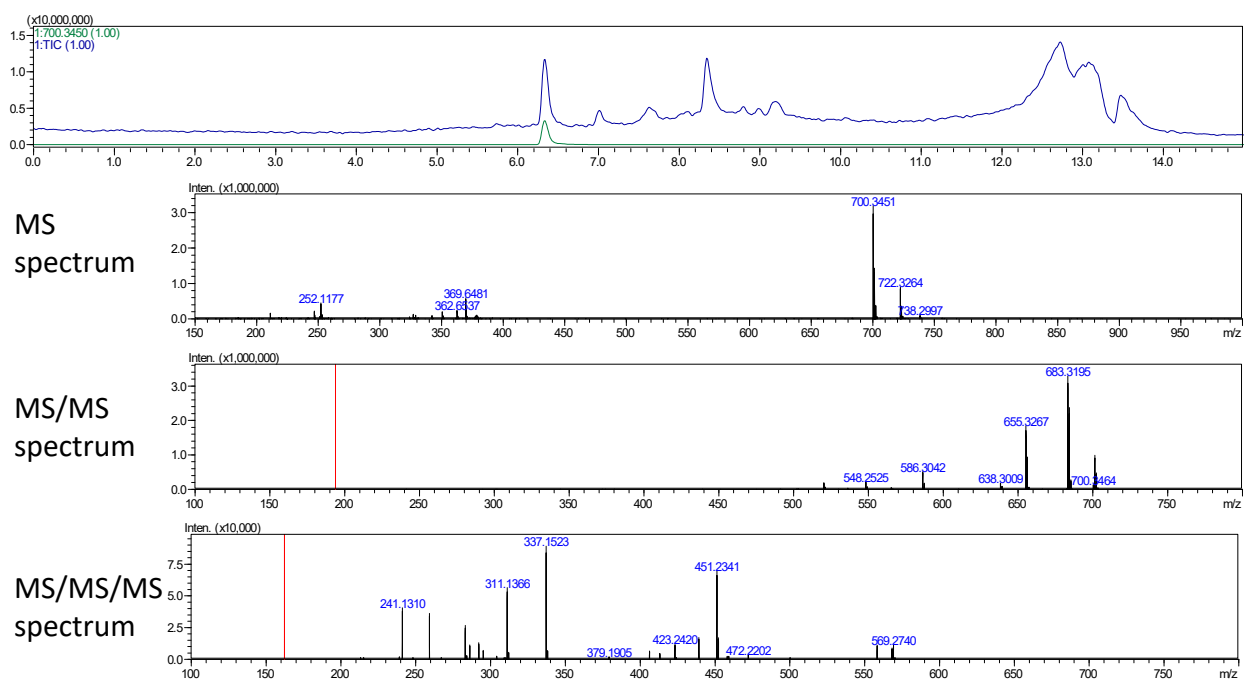


**Figure S3.** Analysis of peptide of peptide Tyr-c[(S)-β³Lys-Phe-Phe-Asp]NH₂ (**RP-172**).

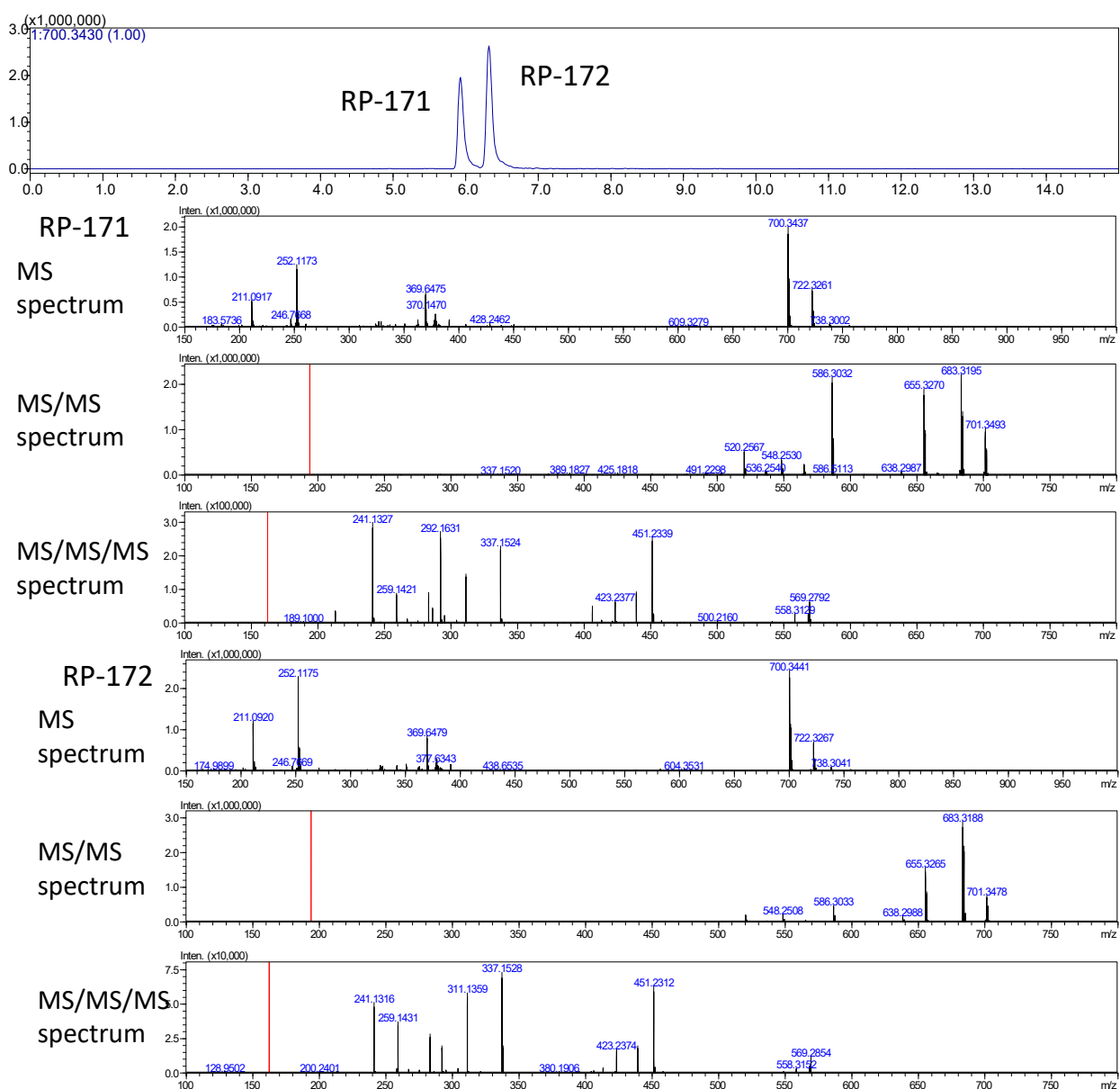
High resolution MS analysis. In inset, fragment of the experimental spectrum is compared with the simulated isotopic profile calculated for the expected molecular formula of protonated species  $[M+H]^+$ .



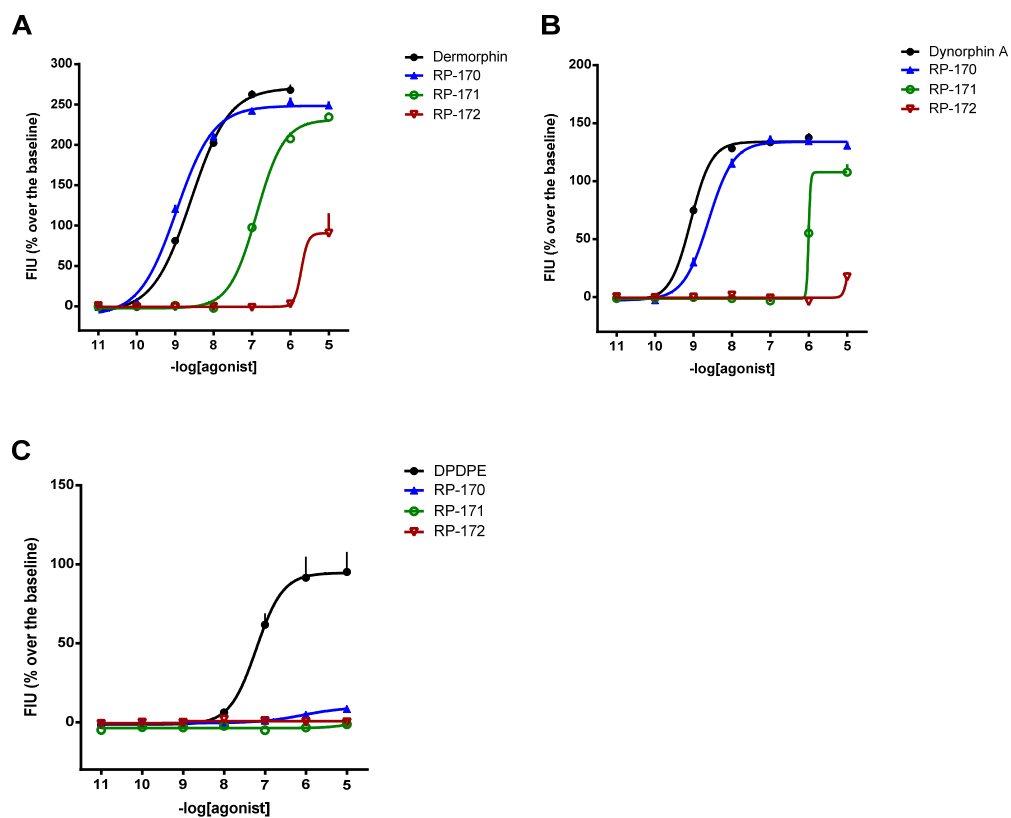
**Figure S4.** LC-MS analysis of peptide Tyr-c[(R)-β³Lys-Phe-Phe-Asp]NH₂ (**RP-171**). Top panel: total ion chromatogram (TIC, blue line) and extracted ion chromatogram for  $m/z$  700.345 (XIC, green line). MS<sup>n</sup> spectra recorded for the retention time in XIC. Kinetex Biphenyl column was used for chromatographic separation.



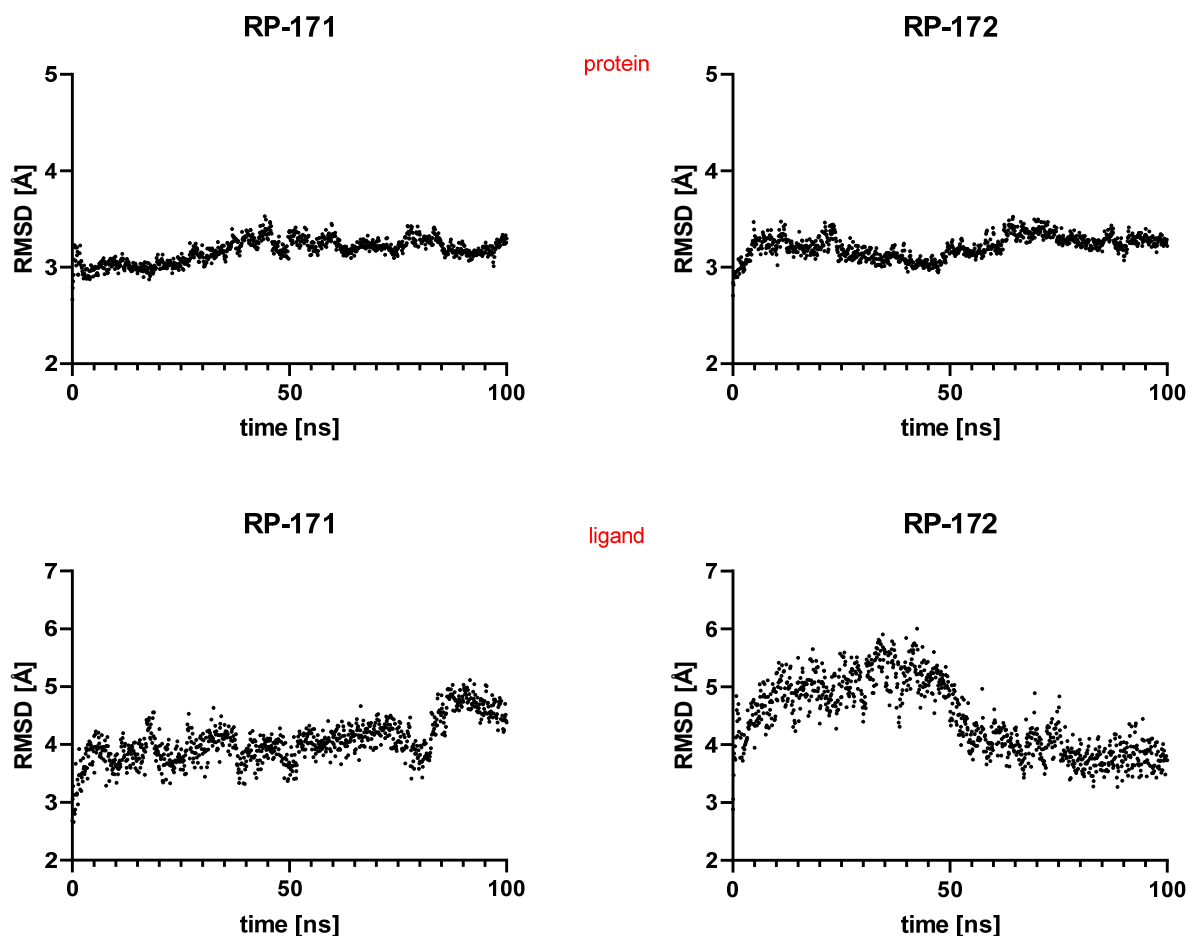
**Figure S5.** LC-MS analysis of peptide Tyr-c(S)-β<sup>3</sup>Lys-Phe-Phe-Asp]NH<sub>2</sub> (**RP-172**). Top panel: total ion chromatogram (TIC, blue line) and extracted ion chromatogram for *m/z* 700.345 (XIC, green line). MS<sup>n</sup> spectra recorded for the retention time in XIC. Kinetex Biphenyl column was used for chromatographic separation.



**Figure S6.** LC-MS analysis of peptide mixture, containing Tyr-c(R)- $\beta^3$ -Lys-Phe-Phe-Asp]NH<sub>2</sub> (**RP-171**) and Tyr-c(S)- $\beta^3$ -Lys-Phe-Phe-Asp]NH<sub>2</sub> (**RP-172**). Top panel: total ion chromatogram (TIC, blue line) and extracted ion chromatogram for  $m/z$  700.345 (XIC, green line). MS<sup>n</sup> spectra recorded for the retention times in XIC. Kinetex Biphenyl column was used for chromatographic separation.



**Figure S7.** Concentration-response curves to reference agonists and tested peptides in calcium mobilization experiments performed in CHO cells stably co-expressing the human MOR (Panel A) or KOR (Panel B) and the C-terminally modified  $G\alpha_{q15}$  and CHO cells co-expressing DOR (Panel C) and the  $G\alpha_{qG66D15}$  protein. Data are the mean $\pm$ SEM of at least 4 separate experiments performed in duplicate.



**Figure S8.** Root mean square deviations (RMSD) of protein (top) and ligand (bottom) positions for MD simulations of **RP-171** and **RP-172** with MOR.

**Table S1.** Physicochemical characterization of analogs.

No.	Sequence	Formula	m/z [ $M + H$ ] <sup>†a</sup>		HPLC $t_R$ <sup>b</sup> [min]
			Calcd	Obsd	
<b>RP-171</b>	Tyr-c[( <i>R</i> )-β <sup>3</sup> Lys-Phe-Phe-Asp]NH <sub>2</sub>	C <sub>37</sub> H <sub>44</sub> N <sub>7</sub> O <sub>6</sub>	700.3453	700.3454	14.05
<b>RP-172</b>	Tyr-c[( <i>S</i> )-β <sup>3</sup> Lys-Phe-Phe-Asp]NH <sub>2</sub>	C <sub>37</sub> H <sub>44</sub> N <sub>7</sub> O <sub>6</sub>	700.3453	700.3465	14.36

<sup>†a</sup> Observed by ESI MS<sup>+</sup> ionization. Mass spectra of peptides were recorded using Shimadzu IT-TOF mass spectrometer equipped with ESI ion source and operated in positive ion mode.

<sup>b</sup> RP-HPLC was performed on a Vydac C<sub>18</sub> column (5 μm, 4.6 × 250 mm) using the solvent system of 0.1% TFA in water (A) and 80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 0–100% solvent B over 50 min, with a flow rate of 1 mL/min.

In the MS<sup>3</sup> experiment, the 292 *m/z* ion corresponds to Tyr-β<sup>3</sup>-Lys fragment, 451 *m/z* and 337 *m/z* ion are probably formed by removal of Tyr from the 586 *m/z* ion and internal cyclization, whereas 311 *m/z* ion represents fragment containing Phe and Tyr residues, formed during internal rearrangement, a frequent occurrence in MS analysis of cyclic peptides [1,2,3,4]. There are at least two pathways in 586 *m/z* ion fragmentation, with the internal cyclization as well as rearrangement leading to Tyr-Phe fragment formation predominant in **RP-172**, probably resulting from a more stable structure due to orientation of Tyr residue, although a final assignment requires additional MS<sup>n</sup> studies.

**Table S2.** Total energies of top 15 conformers for **RP-171** and **RP-172**, obtained at the B3LYP/6-31G(d,p) level.

	Conformer code	Total energy (Hartrees)
	<b>RP-171</b>	
<b>1</b>	RP-171 31	-2347.500135
<b>2</b>	RP-171 43	-2347.500042
<b>3</b>	RP-171 25	-2347.494393
<b>4</b>	RP-171 39	-2347.493544
<b>5</b>	RP-171 12	-2347.489088
<b>6</b>	RP-171 92	-2347.487881
<b>7</b>	RP-171 56	-2347.485198
<b>8</b>	RP-171 60	-2347.484992
<b>9</b>	RP-171 96	-2347.483326
<b>10</b>	RP-171 30	-2347.479364
<b>11</b>	RP-171 27	-2347.474592
<b>12</b>	RP-171 3	-2347.474055
<b>13</b>	RP-171 35	-2347.473866
<b>14</b>	RP-171 0	-2347.473614
<b>15</b>	RP-171 72	-2347.472941
	<b>RP-172</b>	
<b>1</b>	RP-172 20	-2347.502704
<b>2</b>	RP-172 41	-2347.498904
<b>3</b>	RP-172 84	-2347.498412
<b>4</b>	RP-172 62	-2347.498134
<b>5</b>	RP-172 11	-2347.496409
<b>6</b>	RP-172 8	-2347.495252
<b>7</b>	RP-172 31	-2347.493671
<b>8</b>	RP-172 37	-2347.493462
<b>9</b>	RP-172 56	-2347.493301
<b>10</b>	RP-172 49	-2347.493077
<b>11</b>	RP-172 6	-2347.492747
<b>12</b>	RP-172 51	-2347.492158
<b>13</b>	RP-172 71	-2347.491597
<b>14</b>	RP-172 70	-2347.49103
<b>15</b>	RP-172 64	-2347.490503

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