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RE: REVISED

Potent m-Opioid Receptor Agonists from Cyclic Peptides Tyr-c[D-Lys-Xxx-Tyr-Gly]: Synthesis, Biological, and Structural Evaluation

By: Nicholas Stein and Amber Gerheart

April 10, 2018

Dr. Glaser,

Thank you for your expedient communication with the peer reviews for our original manuscript. We are grateful for all of the suggestions and have put much thought and consideration into all of them during the revising process of our manuscript. We hope that our manuscript is satisfactory after making changes in accordance with the recommendations given to us by our peer reviewers. The changes that have been made are described as follows:

**Review 1**

Major Revisions:

[M1.1] An abstract has been added between the cover letter and introduction detailing the important points of the paper.

[M1.2] We did not move the first few sentences/paragraph of our Results and Discussion section to the Materials and Methods, but the methods for the stability testing was added to the Materials and Methods and the Results and Discussion section was mentioned as the area to find the results of the testing. Additional information on the stability testing was also added into the Results and Discussion section.
[M1.3] More information on R-, S-, and Racemic Methadone was added, which should at some distinction to them. The data explanation was also briefly added onto

[M1.4] The LC-MS was used for detection of $\alpha$-thioesters during synthesis and due to the number of spectra provided, it was decided that highlighting a few key points taken from the spectra would be best over giving long explanations about the spectra themselves. More information about the key aspects taken from NMR conformation analysis has been added to the Results and Discussions section.

Minor Revisions:

[1.1] Many minor changes were made based on the photocopy provided. This was used to help fix minor formatting, grammar, and wording mistakes and was greatly appreciated.

[1.2] Access dates were added to the online sources.

**Review 2**

(Please Note: As no major or minor revisions were explicitly stated, we took the liberty of determining what was deemed as major and minor based on the change recommendations provided.)

Major Revisions:

[M2.1] Abstract added. Please see [M1.1].

[M2.2] Scheme 1 reproduced and is hopefully of a higher quality than before. Scheme 3 was also reproduced along with S3.

[M2.3] A detailed method of cyclization has been added to the supporting information section in hopes to make it more easily reproducible by the readers.

[M2.4] Higher Definition LC-MS and NMR was added that will hopefully help in being able to read the spectra. See [M1.4] concerning other detailed about LC-MS and NMR discussions.

Minor Revisions:

[2.1] Mentions of Table 1, Figure 1, and Table 2 have been removed from Materials and Methods section.

[2.2] Attempted to provide higher resolution Figure 1.

[2.3] Table 2 was separated so that the format would not be so wide.

[2.4] IC$_{50}$ was discussed a bit more in depth and references to the comparable nine cyclic peptides (“STAR” compounds) was made.

[2.5] Answers to questions provided by reviewer deemed pertinent was added.

**Review 3**
Major Revisions:

No major revisions were mentioned.

Minor Revisions:

[3.1] As this study focuses on the identification on optimization of μ-opioid receptor ligands to provide high binding affinity, selectivity, and functional activity toward the μ-opioid receptor, it is hard to determine potential cost effectiveness of production of analogues as these analogues are used to simply explore binding characteristics of the binding ligand and not be analgesics peptides themselves for the time being.

[3.2] Introduction was expanded to further elaborate on impact and significance of this study.

Sincerely,

Nicholas Stein and Amber Gerheart
Potent \( \mu \)-Opioid Receptor Agonists from Cyclic Peptides Tyr-c[D-Lys-Xxx-Tyr-Gly]: Synthesis, Biological, and Structural Evaluation

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Tyr-c[D-Lys-Xxx\(^3\)-Tyr-Gly]

Xxx\(^3\): Ph, K, 0.99nM; 3Pal, K, 1.95nM; Trp, K, 1.99nM
Abstract

Nine cyclic analogues of endomorphin-1 and endomorphine-2 were synthesized in order to study their binding affinities towards μ-opioid receptors, κ-opioid receptors, and δ-opioid receptors as well as their agonist capabilities toward μ-opioid receptors. The nine cyclic peptide analogues H-Tyr-c[D-Lys-Xxx-Tyr-Gly] have varying residues of Xxx³. These varying residues consist of aromatic, linear, basic, and constrained aromatic structures, which will show to have an important role in binding affinities towards μ-opioid receptors, κ-opioid receptors, and δ-opioid receptors and even effect the agonist capabilities of the cyclic peptide analogue. This study hopes to find important structural information that might help in the development of new analgesics as opioid peptides.
Introduction

Analgesics are a class of drugs designed specifically to relieve pain.\textsuperscript{1} Opioids are analgesics that act on opioid receptors to produce a feeling of euphoria and pain relief.\textsuperscript{1} In 2016 more than 2.1 million people in the United States used opioids for pain relief.\textsuperscript{2} One drawback to this is the addictive nature of opioids, which causes tens of thousands of deaths in the United States every year.\textsuperscript{2} The purpose of this paper is to propose possible opioid peptides that can be used for pain relief with minimal tolerance and addiction side effects.\textsuperscript{3}

Opioids offer a variety of means for synthesis, which can include opioids developed from peptides. Endogenous opioid peptides are naturally produced in the central nervous system of mammalian brains and spinal cords and are used to relieve pain.\textsuperscript{4} The primary endogenous opioid peptides that will be looked at are endomorphin-1 and endomorphine-2. These endogenous opioid peptides showed promise in providing pain relief in rats with minimal dose-dependent activity but failed in metabolic stability.\textsuperscript{5} Cyclic analogues of the opioid peptides can be developed, which helps maintain potency and greatly increases the metabolic stability.\textsuperscript{6}

A variety of cyclic analogues of endomorpine-1 and endomorpine-2 will be synthesized and their binding affinities towards $\mu$-opioid receptors (MOR), $\kappa$-opioid receptors (KOR), and $\delta$-opioid receptors (DOR) will be examined. We will show that cyclic peptides Tyr-c[D-Lys-Xxx-Tyr-Gly] with various residues for Xxx show promise as potent cyclic peptides opioids with acceptable affinities and tolerance side effects. This
will help provide identification on optimization of \( \mu \)-opioid receptor ligands to provide high binding affinity, selectivity, and functional activity toward the \( \mu \)-opioid receptor. These cyclic peptide opioids will be compared to other similar peptide opioids for reference on potency and receptor binding capabilities.

**Materials and Methods**

The endogenous opioid peptides endomorphine-1 and endomorphine-2 can be purchased commercially or can be obtained as a natural product produced in mammalian brains and spinal cords. Scheme 1 shows the structures of both endomorphine-1 and endomorphine-2. For this experiment the endogenous opioid peptides will be obtained commercially.

**Scheme 1. Structures of Endomorphine-1 and Endomorphine-2**

Cyclization of these endomorphins to form head-to-tail cyclic analogs, along with the replacing various targeted amino acids can increase the probability of an optimum response with bio-receptors.\(^7\) The cyclization method employed imidazole-promoted cyclization of thioesters.\(^8\) This cyclization produced a number of cyclic peptides and the
structures of cyclic peptides as well as the scaffold of the mixture-based cyclic pentapeptide can be found in the supporting information sections. Out of the numerous cyclic peptides produced during the cyclization process, nine analogues with likely dramatically higher biological activity with the attachment of peptide thioesters H-Tyr-D-Lys-Xxx-Tyr-Gly-SCH₂Ph were synthesized. The analogues were synthesized using the cyclization method mentioned earlier and the peptide thioesters were synthesized using a solid-phase approach, which used mercaptomethylphenyl-functionalized silica gel as a volatilizable support. A cleavage with anhydrous HF was used to release the unprotected peptide thioesters from the silica gel and the HF was removed with a nitrogen stream. The peptide thioesters were then dissolved in a 1:7 v/v ratio of 1.5 M aqueous imidazole and 1 mM acetonitrile and stirred for 72 hours to form the cyclic peptides. The amino acids used and the sequences of the cyclic peptides along with the synthetic approach to the cyclic peptides can be found in Scheme 2. H NMR was employed to probe the conformations of the cyclic peptides and this information can be found in the supporting information section. The stability against trypsin of the cyclic peptides were tested using compound 8 as the model since compound 8 has Arg, a trypsin cleavable site. Compound 8 was tested against a commercially purchased nonapeptide by incubation of 1 mg of peptide with a 20 μg trypsin solution and a pH 8.2 buffer. The results of this stability testing can be found in the results and discussion section. The nine analogs were tested for their binding affinities toward the μ-opioid receptor (MOR). This was done with a radioactive receptor binding assay which used ³H-DAMGO as a competing radioligand. It is important to note that analogs having the same
Xxx³ residues showed comparable binding affinities to one another. The nine analogs were also tested for their binding affinities toward the δ-opioid receptor (DOR) and κ-opioid receptor using a radioactive receptor binding assay. These binding affinities to the opioid receptors can be found in Table 1.

Scheme 2. (a) Amino Acids Employed and Sequences of the Resulting Cyclic Peptides and (b) Synthetic Approach to the Cyclic Peptides

The nine analogs were also tested for their binding affinities toward the δ-opioid receptor (DOR) and κ-opioid receptor using a radioactive receptor binding assay. The function activity toward MOR was also looked at by using fluorescent membrane potential assay to evaluate agonist and antagonist activity toward G-protein-coupled receptors.¹¹
Results and Discussion

To test the stability of the cyclic peptides against trypsin, compound 8 seen in Scheme 2 was used as a model and tested against a linear nonapeptide by incubation of 1 mg of peptide with a 20 μg trypsin solution and a pH 8.2 buffer. Compound 8 was chosen due to the possession of Arg\(^3\), which is a trypsin cleavable site. The linear nonapeptide was used to monitor the activity of trypsin. No significant change was found for compound 8 after 24 hours of incubation due to an increased stability against proteolysis as a result of cyclization increased the stability of the cyclic peptide.\(^{12}\) The control linear peptide was completely degraded after a one hour incubation. The nine analogs found in Scheme 2 were then tested for their binding affinities toward MOR, KOR, and DOR in a radioactive receptor binding assay, which was first mentioned in the materials and methods section. An in-depth explanation on the process of the radioactive receptor binding assay can be found in the supporting information section.

For MOR it was found that compounds with the same type of Xxx\(^3\) residues showed comparable binding affinities to one another. Detailed values of this result can be found in Table 1. Compared to the original compound of endomorphine-1 and the cyclic peptide with a Dap(Ant) residue, the binding affinities increased by 14-fold and 7-fold when the peptide was treated by cyclization and the residues were replaced with Phe\(^3\) and Trp\(^3\), respectively. This data shows that generally compounds with aromatic Xxx\(^3\) residues exhibited much higher binding affinities to the MOR. This suggests that an aromatic residue at Xxx\(^3\) favored ligand binding to the MOR due to the aromatic structure, which can be compared to residues that contained linear or constrained
aromatic residues at Xxx. Having a linear peptide Xxx residue showed binding affinities that were comparable to the original molecule, such as with compounds 8 and 9. The compounds with constrained aromatic residues, such as compounds 4 and 5, showed

Table 1. Binding Affinities of the Tyr-c[D-Lys-Xxx-Tyr-Gly] to the Opioid Receptors

<table>
<thead>
<tr>
<th>Compd</th>
<th>Xxx³</th>
<th>Kᵢ ± SD (nM)</th>
<th>Kᵢ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MOR</td>
<td>KOR</td>
</tr>
<tr>
<td>EM-1</td>
<td></td>
<td>0.7 ± 0.1</td>
<td>5069 ± 3003</td>
</tr>
<tr>
<td>0</td>
<td>Dap(Ant)</td>
<td>14 ± 0.54</td>
<td>3231 ± 863</td>
</tr>
<tr>
<td>1</td>
<td>Phe</td>
<td>0.99 ± 0.14</td>
<td>4450 ± 1245</td>
</tr>
<tr>
<td>2</td>
<td>Tyr</td>
<td>114 ± 27</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>3</td>
<td>Trp</td>
<td>1.99 ± 1.6</td>
<td>8160 ± 3271</td>
</tr>
<tr>
<td>4</td>
<td>Tic</td>
<td>991 ± 326</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>5</td>
<td>7-OH-Tic</td>
<td>1129 ± 65.8</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>6</td>
<td>3 Pal</td>
<td>1.95 ± 0.6</td>
<td>5330 ± 3896</td>
</tr>
<tr>
<td>7</td>
<td>4 Pal</td>
<td>9.21 ± 1.2</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>8</td>
<td>Arg</td>
<td>17.4 ± 0.6</td>
<td>8530 ± 2070</td>
</tr>
<tr>
<td>9</td>
<td>Cit</td>
<td>9.04 ± 2.0</td>
<td>8430 ± 2220</td>
</tr>
</tbody>
</table>

a decrease in binding affinity to the MOR. This decrease in binding affinity was almost 71-fold compared to the original compound. Since these constrained aromatic residues
may take trans or cis conformation, the decrease in binding affinities may be due to conformational alterations affecting the binding. Using NMR to probe the conformational preferences of cyclic peptides showed that compounds 4 and 5 contained a cross peak between H(d-Lys\textsuperscript{2})-H(Tic\textsuperscript{3})/H(7-OH-Tic\textsuperscript{3}), which indicated that both peptides were in cis conformation at the constrained amino acid residue, which decreased binding affinity to the MOR as supported by our data. Compounds 1 and 3 showed trans conformation preferences and temperature dependence of backbone H\textsuperscript{N} chemical shifts by \textsuperscript{1}H NMR experiments showed similar backbone conformation, which leads to the highest MOR binding affinities as noted by the data in Table 1.

The nine analogs were also tested for their binding affinities towards KOR and DOR. None of the nine analogs showed any binding affinity higher than the original compound toward the KOR, which indicated that the single-residue replacement of Xxx\textsuperscript{3} did not improve biological activity toward the KOR. Analogs 1 and 3 did show higher binding affinities toward DOR with a 20-fold and 8-fold increase in binding activity when compared to the original compound, respectively. These analogs contained an aromatic residue replacement, which was also characteristic with an increase in binding affinity to the MOR as well. The selectivity for MOR over KOR and over DOR was also calculated using the ratio of binding affinity to each receptor. The residues that have a decrease in binding affinity to the MOR, such as the constrained aromatic residue replacements, will show a very high calculated binding affinity to the DOR since the binding affinities did not change. Other structures, such as the aromatic residue replacements, with increased selectivity to the MOR and DOR, will still have higher
calculated binding affinities to the MOR due to the much higher increase in binding affinities shown by the assay toward the MOR. These relationships will be useful for future designs of opioid ligands that are selective toward MOR.\(^\text{13}\)

Fluorescent membrane potential assay was used to evaluate agonist and antagonist activity toward G-protein-coupled receptors. The nine analogs were tested for their function activity at the MOR using morphine as a positive control. Figure 1 shows that all nine analogs activated the MOR to increase membrane potential, indicating that they are all MOR agonists.

**Figure 1.** Agonist Potency of the Compounds from a Fluorescent Membrane Potential Assay.

The calculated agonist potency (EC\(_{50}\)) for each analog can be found in Table 2. Out of the nine analogs, compound 1 possessed the highest agonist potency, which was nearly 3-fold more potent than morphine. This suggests that compound 1 will have more agonist potency than morphine in vitro.\(^\text{14}\)
Table 2. Agonist Potency of the Compounds to MOR

<table>
<thead>
<tr>
<th>Compound</th>
<th>Morphine</th>
<th>DAMGO</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$ ± SD (nM)</td>
<td>6.3 ± 3.0</td>
<td>2.5 ± 0.6</td>
<td>2.3 ± 1.0</td>
<td>70.4 ± 52.0</td>
<td>24 ± 9.2</td>
<td>677.8 ± 313.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$ ± SD (nM)</td>
<td>1250.0 ± 316.1</td>
<td>11.1 ± 5.2</td>
<td>32.0 ± 3.9</td>
<td>130.7 ± 95.6</td>
<td>66.9 ± 19.8</td>
</tr>
</tbody>
</table>

These binding profiles can be compared to those of other similar compounds to understand the MOR agonist capabilities of the nine analogue cyclic peptide opioids. One such compound that can be compared is the binding profiled of methadone stereoisomers, studied at the Department of Pharmaceutics and Department of Pharmacology at the Royal Danish School of Pharmacy and the University of Copenhagen, respectively.\textsuperscript{15} Methadone is a synthetic narcotic analgesic, and is commonly used clinically as a racemic mixture of levorotatory methadone (R-Methadone) and dextrorotatory methadone (S-Methadone).\textsuperscript{15} The structures for R- and S-methadone are given in Scheme 3. Although bout R- and S-methadone possess analgesic activity, it has been found that the analgesic activity of the racemic mixture is almost entirely due to its content of R-methadone.\textsuperscript{15} Studies have shown that R-methadone is around 50 times more potent than the S-isomer and time times as effective in competition with naloxone for binding sites in rat brain homogenate. Although there is an obvious high potency ratio in favor of R-
methadone, comparing the binding profiles of the nine analogue cyclic peptide opioids against R- and S-methadone, as well as the racemic mixture will provide a variable potency ratio to compare against.

**Scheme 3. Structures of R- and S-methadone**

The binding affinities to Mu$_1$ (MOR1), Mu$_2$ (MOR2), δ (DOR), and κ opioid receptors (KOR) for R-methadone, S-methadone, and racemic methadone were studied in the described paper by various assays.$^{15}$ The results contained can be found in Table 3, which will be compared to the results listed in table 1 for the cyclic peptide opioid analogues.$^{15}$ R-methadone, S-methadone, and racemic methadone all had higher binding affinities to MOR1, MOR2, DOR, and KOR. S-methadone had the highest binding affinity ratio for all four receptors. These binding affinities in Table 3, where IC$_{50}$ is the concentration of compound which inhibits 50% of the specific binding of the ligand, are generally higher than the binding affinities for the nine analogues but considering the binding ratio shows
similar high binding affinities for the MOR for aromatic residues such as compound 1 and 3, which can be comparable to R-methadone in affinity.\textsuperscript{15}

Table 3. Affinities of R-, S-, Racemic Methadone and Morphine for Mu\textsubscript{1}, Mu\textsubscript{2}, Delta and Kappa Opioid Receptors in Bovine Caudate Nucleus

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Mu\textsubscript{1}</th>
<th>Mu\textsubscript{2}</th>
<th>Delta</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-Methadone</td>
<td>3.01 ± 0.18</td>
<td>6.94 ± 1.3</td>
<td>371 ± 75</td>
<td>1332 ± 280</td>
</tr>
<tr>
<td>S-Methadone</td>
<td>26.4 ± 3.7</td>
<td>87.5 ± 9.0</td>
<td>9532 ± 854</td>
<td>2137 ± 881</td>
</tr>
<tr>
<td>Racemic Methadone</td>
<td>5.73 ± 1.5</td>
<td>10.0 ± 3.1</td>
<td>752 ± 686</td>
<td>1817 ± 573</td>
</tr>
<tr>
<td>Morphine</td>
<td>2.55 ± 0.37</td>
<td>6.59 ± 1.73</td>
<td>365 ± 97</td>
<td>213 ± 102</td>
</tr>
</tbody>
</table>

The comparable binding affinities to the cyclic peptide analogues 1 and 3 shows a structural importance of aromaticity related to the ligand for high affinity binding.

A quick note can be made about the aromatic nature of the R- and S-methadone in comparison to their binding profiles. Although the binding profiles seem to be in part due to the stereochemistry of the attached methyl group near the nitrogen, the aromatic residues of the nine analogues presented played a role in the binding profile for cyclic peptide opioids, which presents the suggestion that the aromatic rings in the methadone structures play a role in specific binding to the MOR\textsubscript{1}, MOR\textsubscript{2}, DOR, and KOR.\textsuperscript{15} The other cyclic peptide analogues showed poor binding affinities that is comparable to S-
methadone, although compound 9 shows similar binding affinities to the racemic methadone, which is in part due to the restrained aromatic Xxx\(^3\) residue.

**Conclusion**

Cyclic peptide analogs were synthesized to act as a MOR targeting peptide opioids. Nine residues, which generated analogs of H-Tyr-c[D-Lys-Xxx-Tyr-Gly] with varying Xxx\(^3\) residues were examined for binding affinities and agonist capabilities. These residues showed great importance in effecting the binding affinities of the cyclic peptide analogs. Half of the analogs showed binding affinity no lower than the original compound. Those analogs with aromatic residues showed the highest binding affinity toward MOR, and showed binding affinities that were 7- to 14-fold higher than that of the original compound. The agonist potency of the analogs was tested in which all analogs activated the MOR. Their agonist potencies were in close agreement with their binding affinities. The most active analog exhibited an agonist potency 3-fold more potent than morphine.

The NMR analysis showed that a trans conformation near the Xxx\(^3\) residue is crucial for the cyclic peptide opioids to maintain high affinity and functional activity toward the MOR. When compared to three other MOR binding compounds, such as R-,S-, and racemic methadone, the cyclic analogs showed comparable binding affinities most notably those cyclic analogs with aromatic residues.
References


Supporting Information

Potent μ-Opioid Receptor Agonists from Cyclic Peptides Tyr-c[D-Lys-Xxx-Tyr-Gly]: Synthesis, Biological, and Structural Evaluation

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Scheme S1. Structure of Cyclic Peptides: (a) Structure of Original Scaffold (b) Structure of Mixture-based Cyclic Scaffold

Structure of Mixture-based Cyclic Scaffold

P1: fixed Gly
P2: mixture of 36 amino acids
P3: fixed Dap(Ant)
P4: mixture of 19 amino acids
P5: mixture of 36 amino acids
Synthesis of Cyclic Peptides H-Tyr-c[D-Lys-Xxx-Tyr-Gly]

All of the cyclic peptides were prepared through the cyclization of peptide thioesters. These peptide α-thioesters were prepared by solid phase peptide synthesis using mercaptomethylphenyl-functional silica gel. The solid phase peptide synthesis was carried out using a “teabag” approach. The teabag approach is essentially breaking up the reaction into single peptide sequences in order to speed up the process and be more effective. For the teabag approach synthesis, a PyBOP/DIEA coupling protocol was used. After peptide elongation, the peptides were treated with anhydrous HF at zero degrees Celsius. In order to get the final peptide α-thioester products, the HF was evaporated off using gaseous nitrogen.

In order to make them cyclic, the linear peptide α-thioesters were dissolved in 7:1 acetonitrile: 1.5M aqueous imidazole solution. This reaction was carried out at room temperature for 72 hours. A 0.1mL sample was taken out and quenched with 15% TFA in water. The sample was quenched again after no linear peptide α-thioesters were detected by liquid chromatography – mass spectroscopy. Solvents were removed through lyophilization and the cyclic peptide product was isolated using preparative HPLC.
Information on Cyclization Process

All of the cyclic peptides were prepared through the cyclization of peptide thioesters. Cyclization is promoted by imidazole and proceeds in three basic steps to form cyclic peptides. A radical forms a cyclic radical which then converts to the cyclic product. Although cyclization has no regioselectivity preference, it is used for metabolic stability. These peptide α-thioesters were prepared by solid phase peptide synthesis using mercaptomethylphenyl-functional silica gel. The solid phase peptide synthesis was carried out using a “teabag” approach. The teabag approach is essentially breaking up the reaction into single peptide sequences in order to speed up the process and be more effective. For the teabag approach synthesis, a PyBOP/DIEA coupling protocol was used. After peptide elongation, the peptides were treated with anhydrous HF at zero degrees Celsius. In order to get the final peptide α-thioester products, the HF was evaporated off using gaseous nitrogen.
**Radioactive Receptor Binding Assay**

The nine analogues were tested for their binding affinities toward MOR using a radioactive receptor binding assay. This assay used $^3$H-DAMGO as a competing radioligand. For testing binding affinities toward KOR and DOR $^3$H-U69593 and $^3$H-DPDPE were used as competing radioligands, respectively. In this method the binding of the listed radiolabeled ligands to the receptors of interest is measures. These competing radioligands were chosen due to the $^3$H release of beta energy, which can be measured with ease on a scintillation counter after the addition of scintillant in the form of a scintillation cocktail. These radioligands also presented a high specific activity and radiochemical purity suitable for such testing.
LC-MS Results of Peptides

**Tyr-c[D-Lys-Phe-Tyr-Gly] (1)**

Molecular Weight: 669.74

**Tyr-c[D-Lys-Tyr-Tyr-Gly] (2)**

Molecular Weight: 874.74

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S7
Tyr-c[D-Lys-Trp-Tyr-Gly] (3)

Molecular Weight: 687.76

Tyr-c[D-Lys-Tic-Tyr-Gly] (4)

Molecular Weight: 673.70
Tyr-c[α-Lys-4PAl-Tyr-Gly] (7)

Molecular Weight: 659.73

Tyr-c[α-Lys-Arg-Tyr-Gly] (8)

Molecular Weight: 657.70
Tyr-c[ε-Lys-Cit-Tyr-Gly] (9)

Mass: 660.34

Peak 2: R. Time: 2.863 min (Scan #: 265)
Mass peaks: 53
Spectrum Mode: Averaged 2.849-2.871 (264-266)
BG Mode: Calc. Segment 1 - Event 1
NMR Profiles of Peptides
Tyr-c[Lys-Phe-Tyr-Gly] (1)
Tyr-c[α-Lys-Tic-Tyr-Gly] (4)
Tyr-c[β-Lys-Cit-Tyr-Gly] [9]
Bibliography


