Linear Scaffolds for Multivalent Targeting of Melanocortin Receptors

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$^{13}$C NMR Spectrum of Compound 12
$^1\text{H NMR Spectrum of Compound 14}$
$^{13}$C NMR Spectrum of Compound 14
$^{13}$C NMR Spectrum of Compound 15
$^1$H NMR Spectrum of Compound 18
**Sample Information**

- **Sample Name:** Elshan CH-1-226
- **Sample Type:** Unknown
- **Vial:** 80
- **Injection #:** 1
- **Injection Volume:** 10.00 µL
- **Run Time:** 45.0 Minutes
- **Sample Set Name:** Prime_Run_07_19_07

**Acquired By:** System
**Date Acquired:** 6/5/2014 4:21:08 PM MDT
**Acq. Method Set:** 10_90B_in
**Date Processed:** 6/11/2014 1:41:38 PM MDT
**Processing Method:** Peptide_general
**Channel Name:** 2487Channel 1, 2487Channel 2
**Proc. Chnl. Descr.:** 220nm, 280nm

### Channel 220 nm
**Channel:** 2487Channel 1

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<th>RT</th>
<th>Area (µV·sec)</th>
<th>% Area</th>
<th>Channel</th>
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<tbody>
<tr>
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<td>2.74e+007</td>
<td>97.69</td>
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### Channel 280 nm
**Channel:** 2487Channel 2

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<th>Channel</th>
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<tr>
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**Notes:**
Sample Information

Sample Name: Elshan CH-1-54
Sample Type: Unknown
Vial: 77
Injection #: 1
Injection Volume: 10.00 ul
Run Time: 45.0 Minutes
Sample Set Name: Prime_Run_07_19_07

Acquired By: System
Date Acquired: 6/5/2014 2:02:47 PM MDT
Acq. Method Set: 10_90B_in
Date Processed: 6/11/2014 1:37:21 PM MDT
Processing Method: Peptide_general
Channel Name: 2487Channel 1, 2487Channel 2
Proc. Chnl. Descr.: 220nm, 280nm

NOTES:

Report Method: Dual Channel Summary
SAMPLE INFORMATION

Sample Name: Elshan CH-1-267
Sample Type: Unknown
Vial: 84
Injection #: 1
Injection Volume: 10.00 ul
Run Time: 45.0 Minutes
Sample Set Name: Prime_Run_07_19_07

Acquired By: System
Date Acquired: 6/5/2014 7:25:54 PM MDT
Acq. Method Set: 10_90B_in
Date Processed: 6/11/2014 1:46:00 PM MDT,
Processing Method: Peptide General
Channel Name: 2487Channel 1, 2487Channel 2
Proc. Chnl. Descr.: 220nm, 280nm

Channel 220 nm
Channel: 2487Channel 1

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Channel 280 nm
Channel: 2487Channel 2

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NOTES:

Sample Information

Sample Name: Elshon CH-1-259
Sample Type: Unknown
Vial: 82
Injection #: 1
Injection Volume: 10.00 ul
Run Time: 45.0 Minutes
Sample Set Name: Prime_Run_07_19_07

Acquired By: System
Date Acquired: 6/5/2014 5:53:42 PM MDT
Acq. Method Set: 10_9CB_in
Date Processed: 6/11/2014 1:43:55 PM MDT,
Processing Method: Peptide_general
Channel Name: 2487Channel 1, 2487Channel 2
Proc. Chnl. Descr.: 220nm, 280nm

Channel 220 nm
Channel: 2487Channel 1

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<th>Channel</th>
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Channel 280 nm
Channel: 2487Channel 2

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<th>Channel</th>
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<td>1.53e+006</td>
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NOTES:

- Sample Name: Elshon CH-1-259; Proc. Chnl. Descr. 220nm
- Sample Name: Elshon CH-1-259; Proc. Chnl. Descr. 280nm

**Sample Information**

Sample Name: Elshan CH-1-261  
Sample Type: Unknown  
Vial: 83  
Injection #: 1  
Injection Volume: 10.00 ul  
Run Time: 45.0 Minutes  
Sample Set Name: Prime_Run_07_19_07  
Acquired By: System  
Date Acquired: 6/5/2014 6:39:47 PM MDT  
Acq. Method Set: 10_90B_in  
Date Processed: 6/11/2014 1:44:56 PM MDT  
Processing Method: Peptide_general  
Channel Name: 2487Channel 1, 2487Channel 2  
Proc. Chnl. Descr.: 220nm, 280nm

---

**Graphs**

Graphs showing UV absorption at 220 nm and 280 nm with peaks at 12.950 and 12.958 minutes.

**Table**

<table>
<thead>
<tr>
<th>RT</th>
<th>Area (µV*sec)</th>
<th>% Area</th>
<th>Channel</th>
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</thead>
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<td>100.00</td>
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</table>

**Channel 220 nm**

Channel: 2487Channel 1

<table>
<thead>
<tr>
<th>RT</th>
<th>Area (µV*sec)</th>
<th>% Area</th>
<th>Channel</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>3.80e+006</td>
<td>100.00</td>
<td>2487Channel 2</td>
</tr>
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</table>

**Channel 280 nm**

Channel: 2487Channel 2

**NOTES**

- 23
- R = (CH₃)₄

**Report Method:** Dual Channel Summary  
**Printed:** 1:45:29 PM 6/11/2014  
**Page:** 1 of 1
Data Analysis for the Binding Assays

Biological data analysis was performed using GraphPad Prism software (version 5.04) using the following analysis methods.

Saturation Binding Data
The Total Binding and Non-specific Binding curves in Figure 1 were generated from the binding assay data using nonlinear regression analysis and fitted to the “One site - Total and nonspecific binding” equations (Equations S1 and S2).

\[
\text{Specific} = \frac{B_{\text{max}} \cdot X}{(X + K_d)} \quad \text{(Equation S1)}
\]
\[
\text{Nonspecific} = \text{NS} \cdot X + \text{Background} \quad \text{(Equation S2)}
\]
For Total Binding: \( Y = \text{specific} + \text{nonspecific} \)
For Nonspecific binding: \( Y = \text{nonspecific} \)

Where
- \( B_{\text{max}} \) is the maximum specific binding in the same units as \( Y \).
- \( K_d \) is the equilibrium binding constant, in the same units as \( X \). It is the labeled ligand concentration needed to achieve a half-maximum binding at equilibrium.
- \( \text{NS} \) is the slope of nonspecific binding in \( Y \) units divided by \( X \) units.
- Background is the amount of nonspecific binding with no added labeled ligand. This represents counter background. If the counter automatically subtracts off the background signal, Background can be constrained to a constant value of zero.

The Specific Binding curve in Figure 1 was generated from data derived by taking the difference between the Total and Nonspecific binding data, using nonlinear regression analysis, and fitting to the “One site - Specific binding” equation (Equation S3).

\[
\text{Specific} = \frac{B_{\text{max}} \cdot X}{(X + K_d)} \quad \text{(Equation S3)}
\]

Competitive Binding Data
Competitive binding data were analyzed using nonlinear regression analysis and fitted to the “One site - Fit \( K_i \)” equation.

\[
\log \text{EC}_{50} = \log (10^\cdot \log K_i \cdot (1 + [L]/[\text{Hot } K_d])) \quad \text{(Equation S4)}
\]
\[
Y = \text{Bottom} + \frac{(\text{Top-Bottom})}{(1+10^\cdot (X - \text{Log } \text{EC}_{50}))} \quad \text{(Equation S5)}
\]

Where
- Top and Bottom are plateaus in the units of the \( Y \) axis.
- \( K_i \) is the molar equilibrium dissociation constant of the unlabeled ligand.
- \([L]\) is the concentration of labeled ligand in nM. Here \([L] = 20 \text{ nM} \)
- \([\text{Hot } K_d]\) is the equilibrium dissociation constant of the labeled ligand in nM. Here \([\text{Hot } K_d] = 21 \text{ nM} \).
Molecular Dynamics Studies

Molecular dynamics (MD) calculation trials were performed in triplicate on compounds 21-23 using the AMBER99 force field and the MD program contained in MOE® to obtain representative conformations at 310K. The initial structure of each molecule was minimized, then solvated in a water sphere (compound 21- 4487 H₂O, compound 22- 5490 H₂O, compound 23- 5308 H₂O), and again minimized. The results (depicted) were used as the starting structures for each calculation trial. One carbon of the core scaffold was fixed within the solvation sphere (21, ethylene glycol C1; 22, glycerol C2; 23, mannitol C3) to avoid migration of the molecule toward the edge of the water sphere during MD. Initial calculations performed using compound 22 showed convergence of the structure within 1 ns of a 5 ns production run, therefore subsequent production calculation durations were set at 1 ns. MD consisted of heating the molecule from 300 to 310 K over 250 ps, equilibration for 250 ps, and production for 1 ns. Trajectories were recorded every 0.5 ps.

The lowest energy structure of the production run for each trial was compared to the starting structure by graphical overlayment (see Figure 4 in the article). Inter-ligand distance measurements (from the N-terminal nitrogen atoms of the histidine residues) are shown for the starting structures. The graphs represent the inter-ligand distances for each individual trial with the exception of the graph for compound 21, which contains all three trials on one graph.

<table>
<thead>
<tr>
<th>scaffold origin</th>
<th>ligand labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethylene glycol</td>
<td>( L^1 \leftrightarrow L^2 )</td>
</tr>
<tr>
<td>glycerol</td>
<td>( L^1 \leftrightarrow L^2 \leftrightarrow L^3 )</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>( CH_3O \leftrightarrow L^3 \leftrightarrow L^4 \leftrightarrow L^2 \leftrightarrow OCH_3 )</td>
</tr>
</tbody>
</table>
Average Inter-Ligand Distances$^a$

<table>
<thead>
<tr>
<th>Compound (inter-ligand distance measured)</th>
<th>Average Inter-Ligand Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 (L₁-L₂)</td>
<td>21.06±0.62</td>
</tr>
<tr>
<td>22 (L₁-L₂)</td>
<td>22.99±0.28</td>
</tr>
<tr>
<td>23 (L₂-L₄)</td>
<td>27.84±0.64</td>
</tr>
</tbody>
</table>

$^a$The values reported for compounds 22 and 23 are the averages for the two ligands giving the greatest inter-ligand distances for that compound.
Figure S1: Starting minimized structure with inter-ligand distance measurement.
Figure S2: Average Inter-Ligand Distance for Each Compound 21 Trial

<table>
<thead>
<tr>
<th>Compound 21</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L_1-L_2$ Distance (Å)</td>
<td>21.45±0.26</td>
<td>20.23±0.16</td>
<td>21.49±0.18</td>
</tr>
</tbody>
</table>

Inter-Ligand Distance (Å)
Compound 21

Figure S3: Lowest energy structure from each trial with starting minimized structure in green.
Figure S4: Starting minimized structure with inter-ligand distance measurements.
## Compound 22

<table>
<thead>
<tr>
<th>Distance (Å)</th>
<th>$L_1$-$L_2$</th>
<th>$L_2$-$L_3$</th>
<th>$L_1$-$L_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>20.13±0.17</td>
<td>18.69±0.14</td>
<td>22.59±0.15</td>
</tr>
<tr>
<td>Trial 2</td>
<td>19.92±0.23</td>
<td>19.71±0.16</td>
<td>23.21±0.16</td>
</tr>
<tr>
<td>Trial 3</td>
<td>21.53±0.18</td>
<td>18.64±0.19</td>
<td>22.86±0.21</td>
</tr>
</tbody>
</table>

**Figure S5**: Average Inter-Ligand Distances for Each Compound 22 Trial
Figure S6: Lowest energy structure from each trial with starting minimized structure in green.
Figure S7: Starting minimized structure with inter-ligand distance measurements.
Compound 23

Figure S8: Average Inter-Ligand Distances for Each Compound 23 Trial
Figure S9: Lowest energy structure from each trial with starting minimized structure in green.