**Supplement**

# A hybrid structural approach to analyze ligand binding by the 5-HT4 receptor

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** contributed equally

Supplementary Table S1. Ligand docking results analyzed with Autodock[a].

| Ligand | Binding energy | Inhibitory constant (Ki) | Ki (nM)[b], [$^3$H] GR113808 |
|---|---|---|---|
| Serotonin | -6.89 | 204.09 μM | 500.73 |
| Cisapride | -5.56 | 13.05 μM | 69.90 |
| GR125487 | -10.68 | 14.8 nM | 0.28 |

[a]Predictions from Autodock are in good agreement with experimentally determined binding ranges for these ligands. Values shown are from ligand docking studies done with various ligands for 5-HT4R along with antagonist GR125487 binding experiment detailed in this study. [b]Average experimentally determined values of Ki are taken from the PDSP database (http://pdsp.med.unc.edu/pdsp.php).

Supplementary Table S2. List of oxidized peptides from 5-HT4R samples with no first order oxidation rates calculated[a]

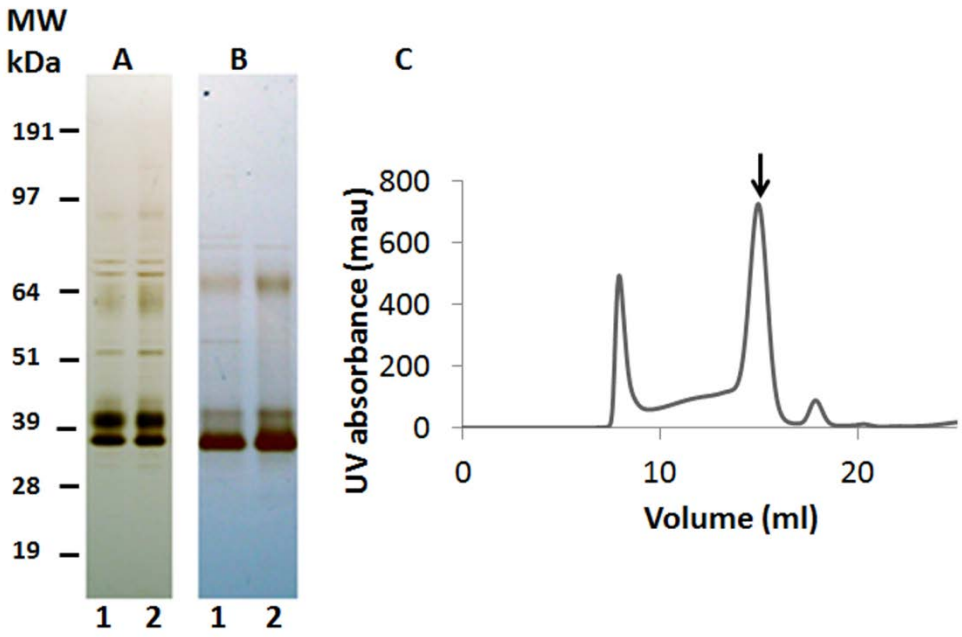
| *Peptide* | Range | Domain | Oxidized AA | Mass shift (Da) |
|---|---|---|---|---|
| VSSEEGF | 8-14 | N terminus | E11 | -30 |
| ELVQDIWIYGEVF | 80-92 | ECL1 to TM3 | W86 and/or Y88, F92$^{3.24}$ | 32 |
| IYGEV | 87-91 | ECL1 to TM3 | V91$^{3.23}$ | 16 |
| CLVRTSLDVLLTT | 93-105 | TM3 | L102$^{3.31}$ | 16 |
| RTSLDVLL | 96-103 | TM3 | R96$^{3.28}$ | -43 |
| ICCQPLV | 122-128 | ICL2 | I122$^{3.54}$ | 16 |
| TPLRIAL | 134-140 | TM4 | P135$^{4.39}$ and I138$^{4.42}$ | 32 |
| VIPTF | 147-151 | TM4 | V147$^{4.51}$ and I148$^{4.52}$/P149$^{4.53}$ | 32 |
| | 147-151 | TM4 | P 149$^{4.53}$ | 14 |
| WVIPTF | 146-151 | TM4 | W146$^{4.50}$ | 16 |
| | 146-151 | TM4 | W146$^{4.50}$ | 32 |
| FISFL | 151-155 | TM4 | F151$^{4.55}$/I152$^{4.56}$ | 16 |
| PIMQGWNNIG | 156-165 | TM4 to ECL2 | M158$^{4.62}$ | 16 |
| MQGWNNIGIIDLIEK | 158-172 | ECL2 | M158$^{4.62}$/I164/W161 | 16 |

| | | | | |
|---|---|---|---|---|
| MQGWNNIGIIDLIEK | 158-172 | ECL2 | M158 and W161 | 48 |
| WNNIG | 161-165 | ECL2 | W161 | 32 |
| IEKRKFNQNSNSTY | 170-183 | ECL2 | F175 | 16 |
| VNKPYAIT | 188-195 | ECL2 to TM5 | V188/Y192$^{5.38}$ | 16 |
| YRIYVT | 213-218 | TM5 | Y213$^{5.58}$/Y216$^{5.62}$ | 16 |
| YVTAKEHAHQIQML | 216-229 | ICL3 | M228 | 16 |
| AKEHAHQIQML | 219-229 | ICL3 | H222/H224 | -22 |
| AKEHAHQIQML | 219-229 | ICL3 | M228 | 16 |
| AKEHAHQIQMLQ | 219-230 | ICL3 | M228 | 16 |
| KEHAHQIQML | 220-229 | ICL3 | M228/I226 | 16 |
| HAHQIQML | 222-229 | ICL3 | M228 | 16 |
| IQMLQRAGAS | 226-235 | ICL3 | M228 | 16 |
| IVDPF | 280-284 | TM6 | I280$^{6.56}$ | 16 |
| NIVDPFID | 279-286 | TM6 to ECL3 | D282$^{6.58}$ | -30 |
| CWAPFF | 271-276 | TM6 | W272$^{6.48}$ | 32 |
| VTNIVDPFI | 277-285 | TM6 | V277$^{6.53}$ | 16 |
| YTVPGQVW | 287-294 | TM7 | W294$^{7.35}$ | 32 |
| YTVPGQVWTA | 287-296 | TM7 | W294$^{7.35}$ and P290$^{7.31}$ | 48 (32 and 16) |
| FLWLG | 297-301 | TM7 | W 299$^{7.40}$ | 32 |
| INSGLNPFL | 303-311 | TM7 | I303$^{7.44}$ | 16 |

| | | | | |
|---|---|---|---|---|
| GLNPFL | 306-311 | TM7 | P309$^{7.50}$ | 16 |
| FLII | 323-326 | H8 | F323$^{8.54}$ | 16 |
| AVECGGQW | 361-368 | C terminus | W368 | 32 |
| ASMTGGQQMGSATAS KTETSQVAPA | 394-417 | Tags | M 395 and M 401 | 32 |

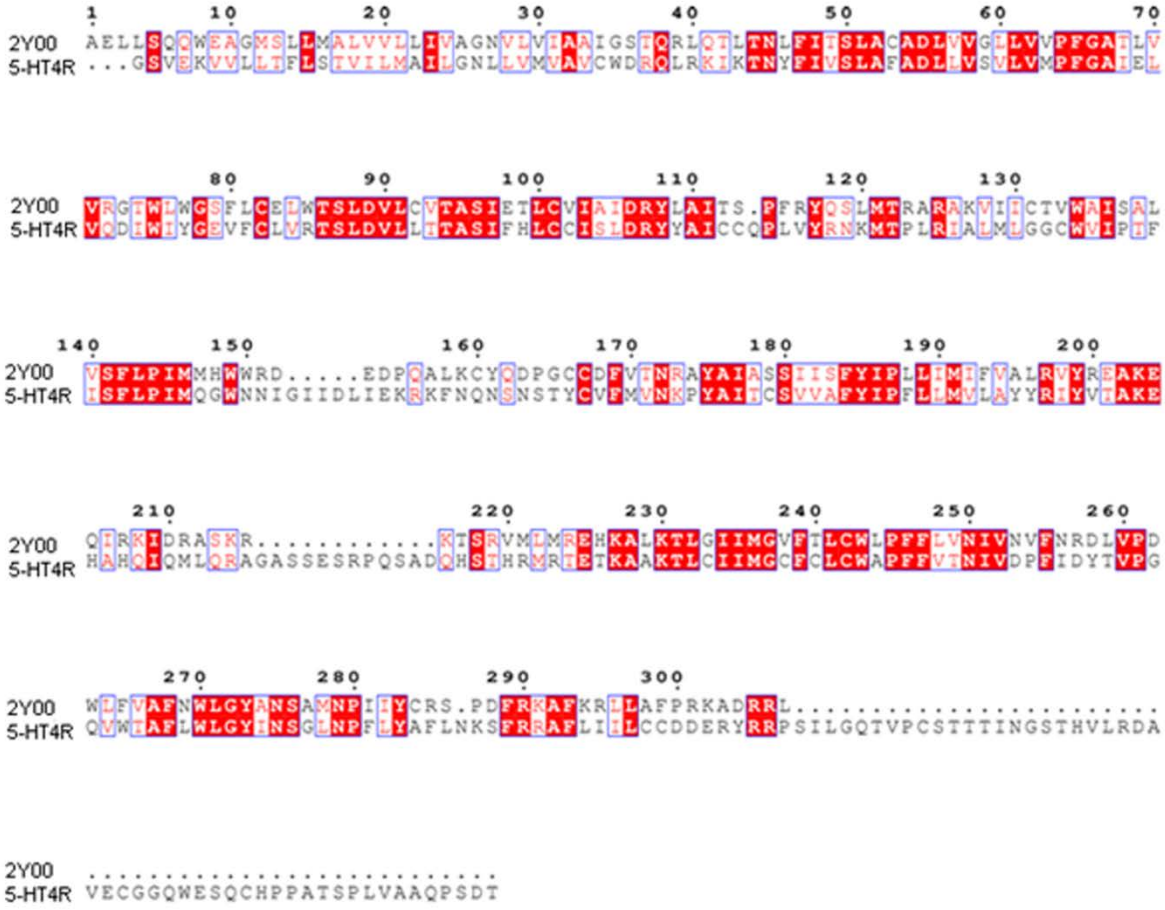
[a] Thirty four unique peptides, apart from the seven peptides shown in Table 1, were detected in ligand-bound and ligand-free 5-HT4R with modifications, but the rates were not reproducible.

Supplementary Figure S1. Purification and analysis of 5-HT4R. SDS-PAGE in panels A and B. Panel (A) Immunopurified receptor without bound ligand (free) (lane 1) and bound with antagonist GR125487 (lane 2). The final purified ligand-free (lane 1) and bound (lane 2) 5-HT4R preparation used for these experiments is shown in panel (B). The ligand-free samples were prepared by extensive washing during 1D4 affinity chromatography. Soon after elution, both ligand-free and ligand-bound receptors were subjected to PNGase F digestion and then further purified by size exclusion chromatography as shown in (C). The major peak of interest is indicated by an arrow. PNGase F eluted later (smaller peak).



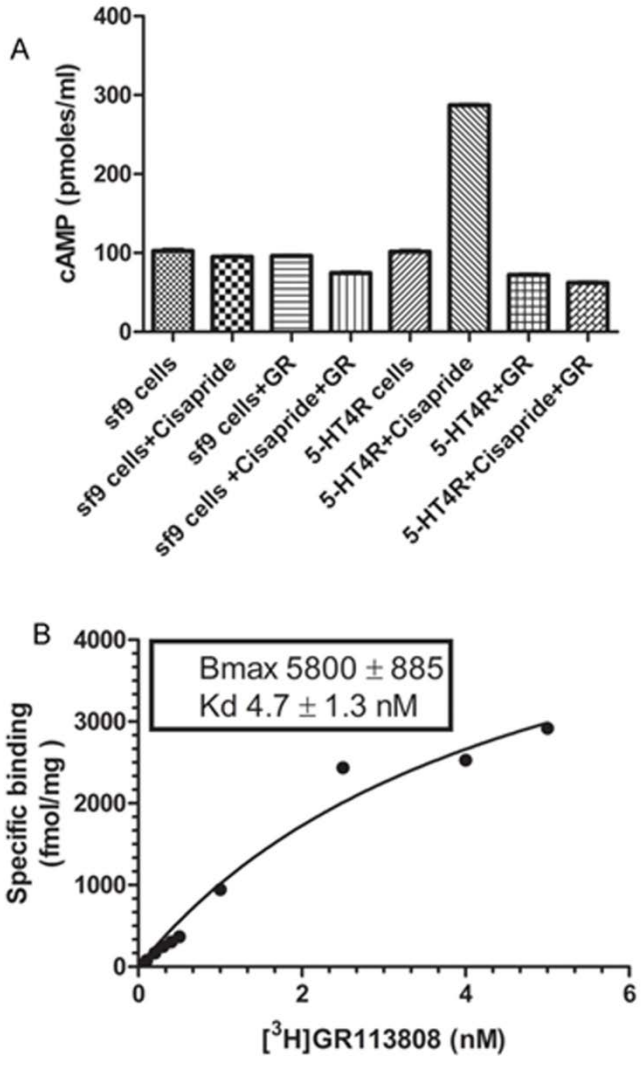
Supplementary Figure S2. Sequence coverage comparison of antagonist-bound and ligand-free samples of 5-HT4R. A) Sequence coverage (95.4%) of GR125487-bound 5-HT4R by LC-MS analysis: Gray lines indicate pepsin digests and purple lines, elastase digests. B) Sequence coverage (94.5%) of ligand-free 5-HT4R achieved by LC-MS analysis.

Supplementary Figure S3. Sequence alignment of model template PDB ID 2Y00 with the 5-HT4R target sequence (388 residues). The alignment was performed with ClustalW. Residue number one for 2Y00 in this figure corresponds to residue number 33 in the PDB and the first 14 residues of 5-HT4R N-terminus are not included in this alignment. Red filled boxes correspond to 100% identity, empty blue outlined boxes with red letters are partial identity and black residues indicate no conservation.



Supplementary Figure S4. Biochemical and functional characterization of 5-HT4R

(A) Concentrations of cAMP formed *in vivo* by cultured insect cells (Sf9). An ELISA-based method was used to quantify cAMP in insect cells cultured on plates. The potent agonist cisapride elevated cAMP levels significantly, whereas incubation of cells with GR125487 (GR) prior to cisapride exposure inhibited cAMP production. (B) Purified 5-HT4R (free) solubilized in DDM was analyzed for ligand binding with the tritiated 5-HT4R antagonist GR113808. Estimated affinity (Kd) for the ligand was about 4.7 nM. The binding analysis was performed with GraphPad software Prism 5.

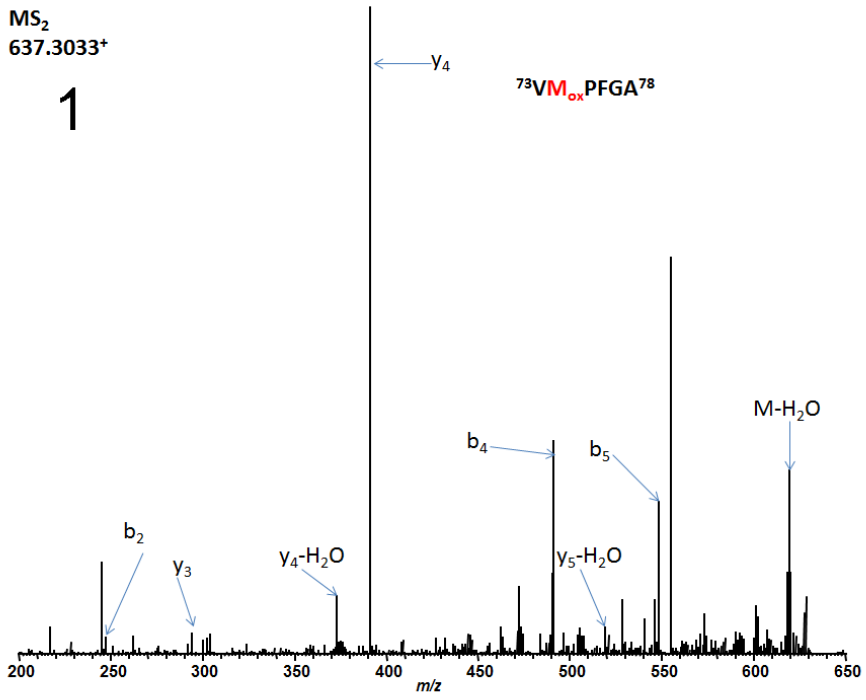


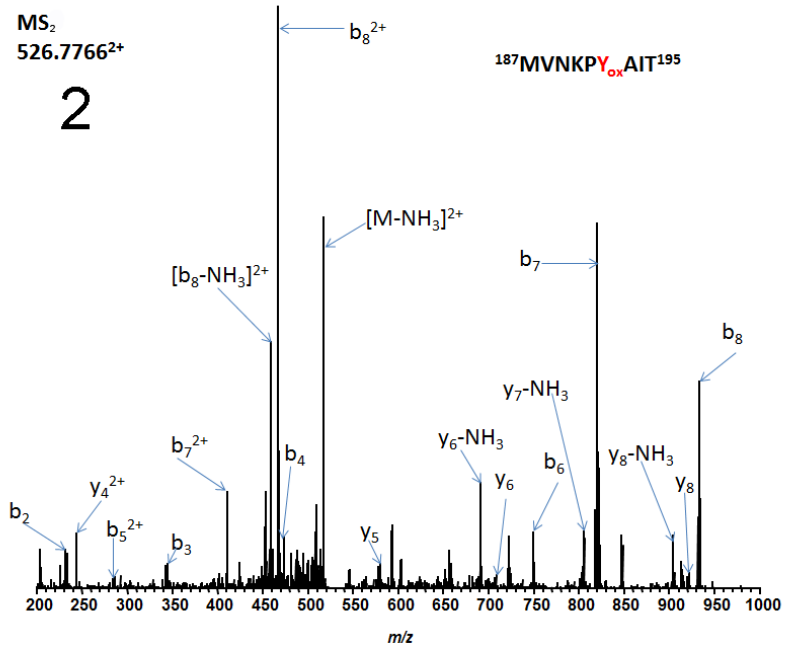
Supplementary Figure S5. Partial proteolysis of purified 5-HT4R in (A) a ligand-free state (B) an antagonist-bound (GR125487) state. Detergent-solubilized 5-HT4R in DDM/CHS micelles was digested by trypsin followed by digestion with CNBr. Red letters represent 5-HT4R sequence form tryptic peptides identified by MS. Blue lines drawn under the amino acid sequences represent multiple peptides detected that correspond to trypsin sensitive sites. Trypsin digestion sites at R or K residues are indicated by cyan letters. The pattern of digestion in free *vs*. bound samples is almost identical. Protection against trypsin digestion of one or more R or K residues within TM5 and TM6 helices is indicated by green boxes. Tentative positions of helices TM1 through H8 (brick red), ICL1 through ICL3 (green) and extracellular domains ECL1 through ECL3 (yellow) are indicated by solid lines above the protein sequences. Trypsin cleaved all R and K residues at their C-termini, and CNBr cleaved Met residues at their C-termini.

Supplementary Figure S6. HPLC chromatogram and mass spectrometric identification of oxidized residues from seven peptides for which rates of oxidation were calculated.

Mass spectrometry was used to identify the oxidized residues in the following peptides. (1) Peptide 73-78 with oxidized M74, (2) Peptide 187-195 with oxidized Y192, (3) Peptide 187-195 with oxidized M187, (4) Peptide 187-195 with oxidized Y192, (5) Peptide 187-195 with oxidized M187 and Y192, (6) Peptide 130-140 with oxidized M133, (7) Peptide 227-243 with oxidized M228, (8) Peptide 236-257 with oxidized M251, (9) Peptide 302-308 with oxidized Y302, and (10) Peptide 383-393 with oxidized M392.

Supplementary Figure S6. (contd.)



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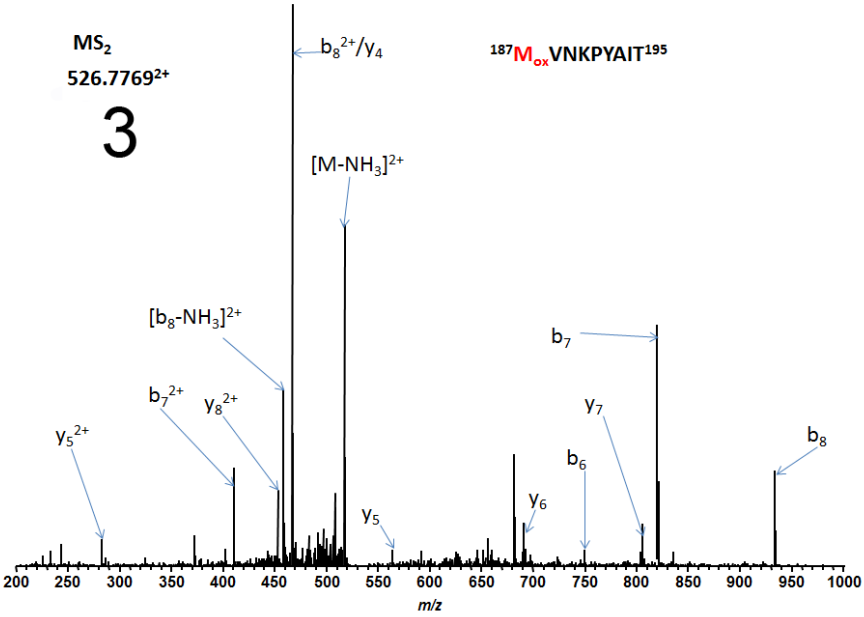


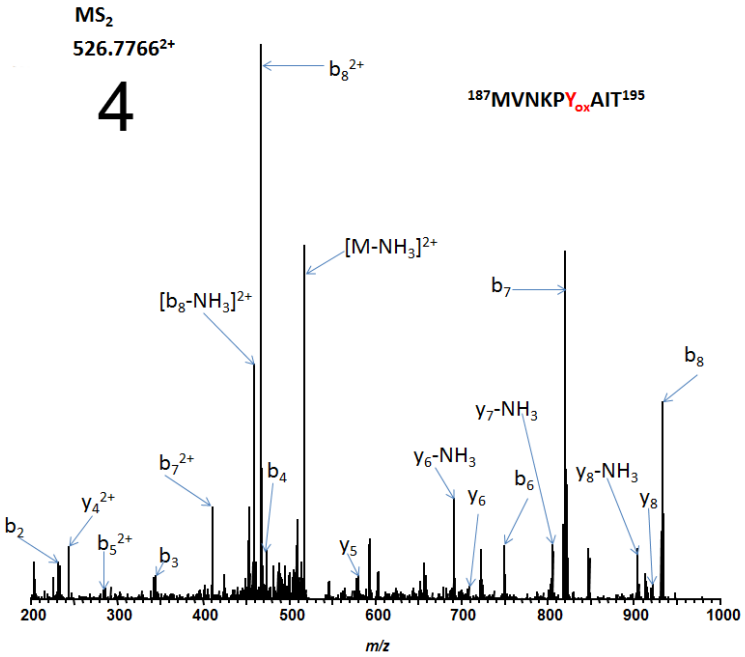

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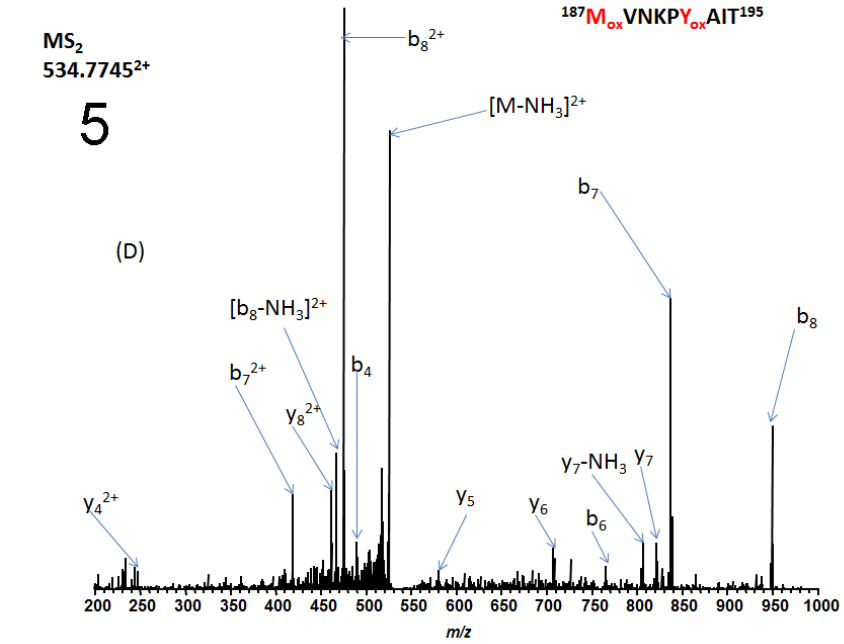


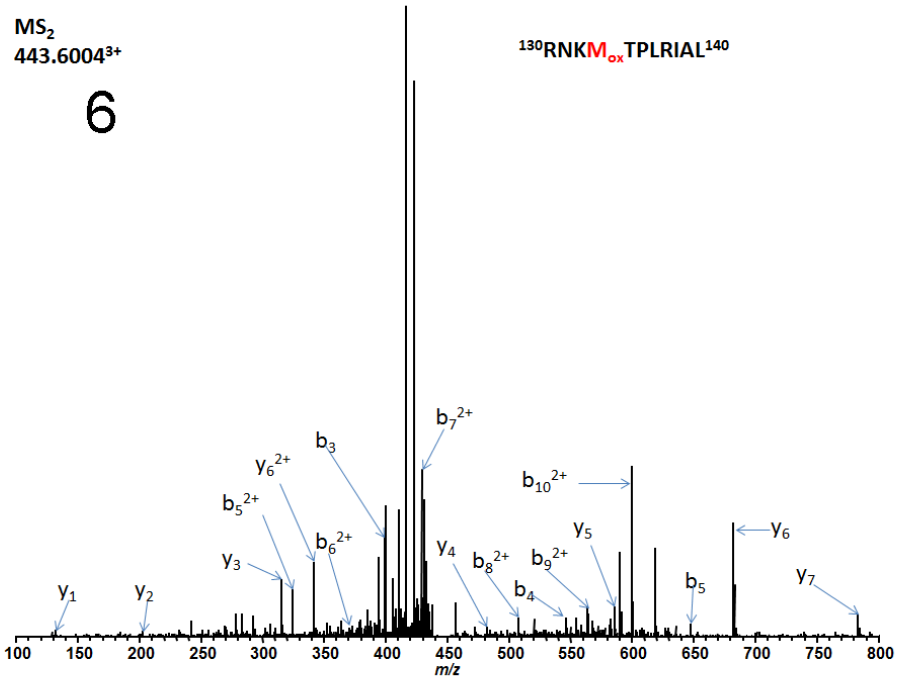

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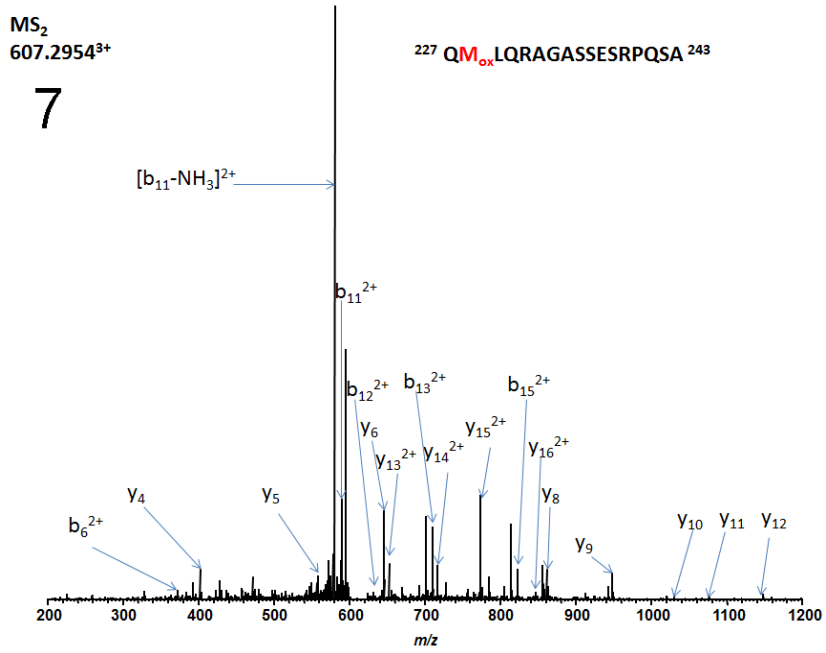


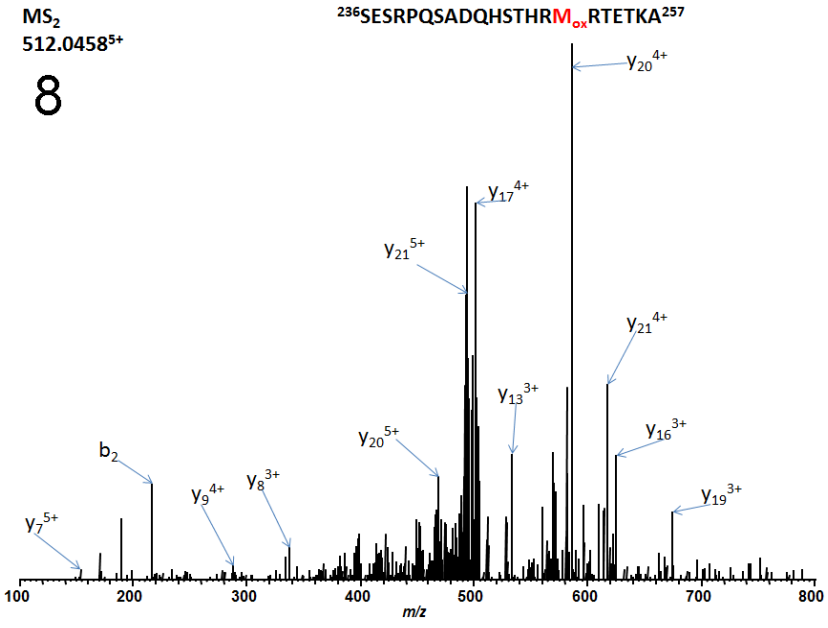

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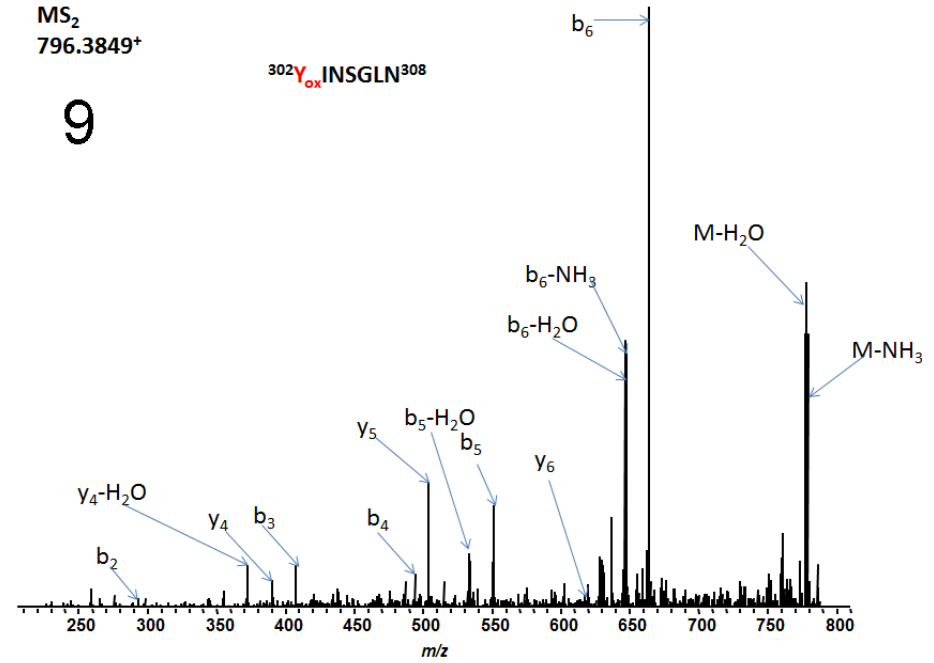
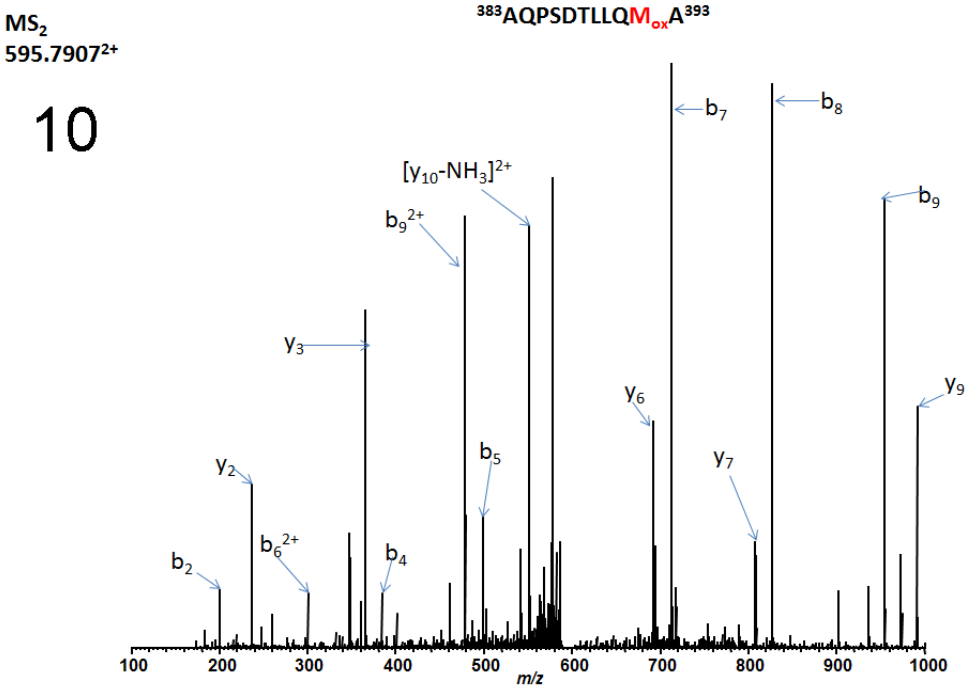
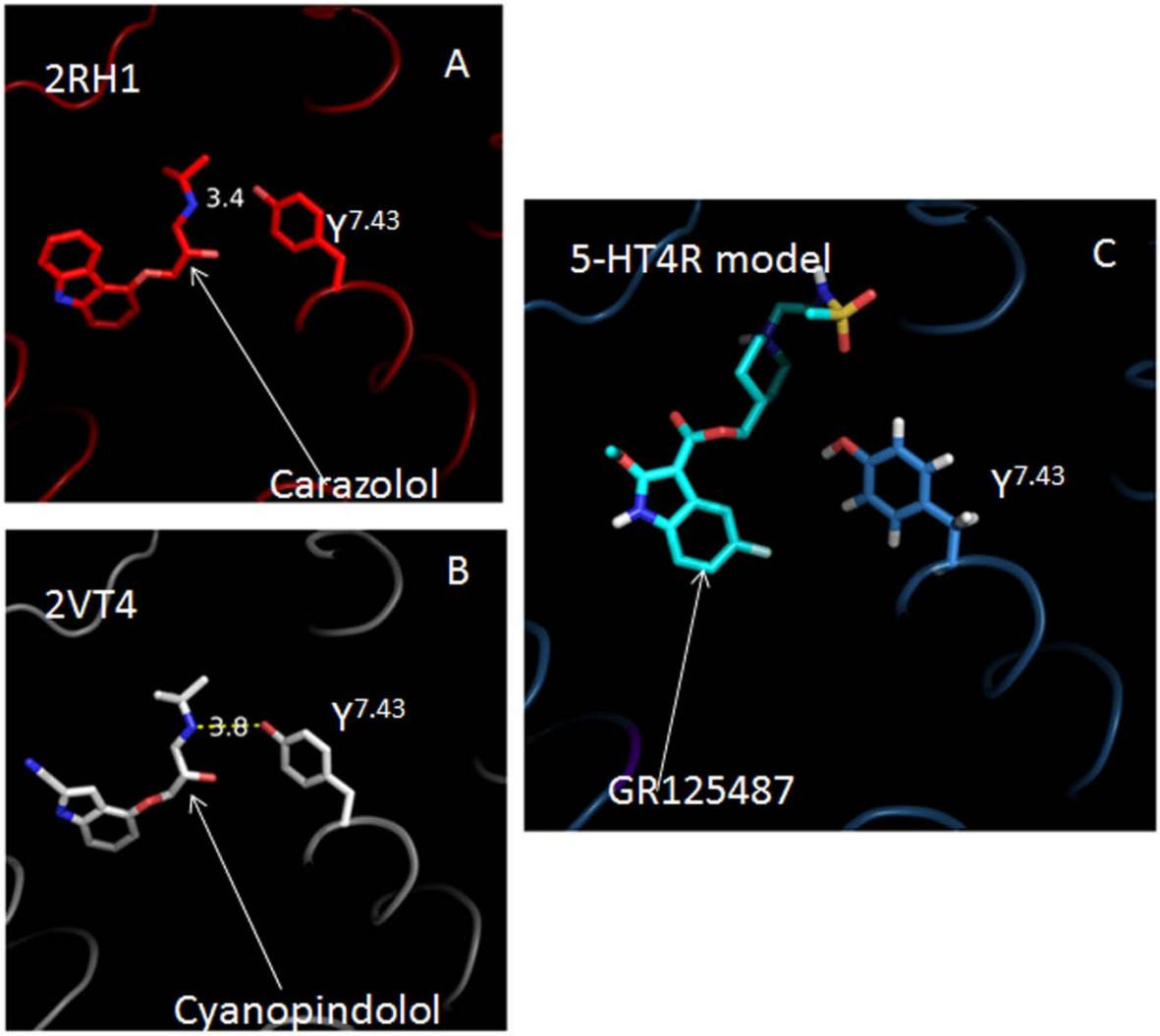
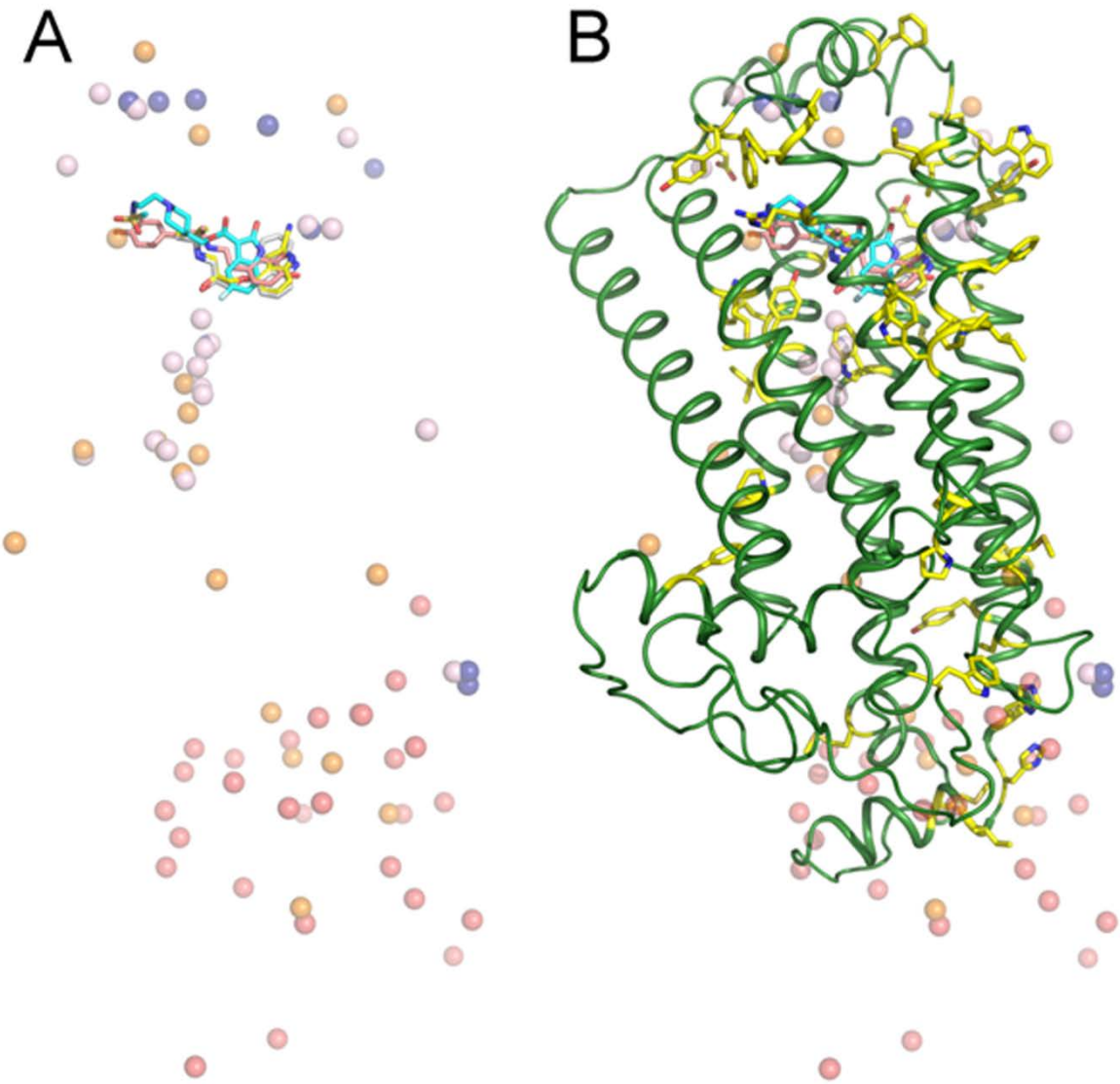


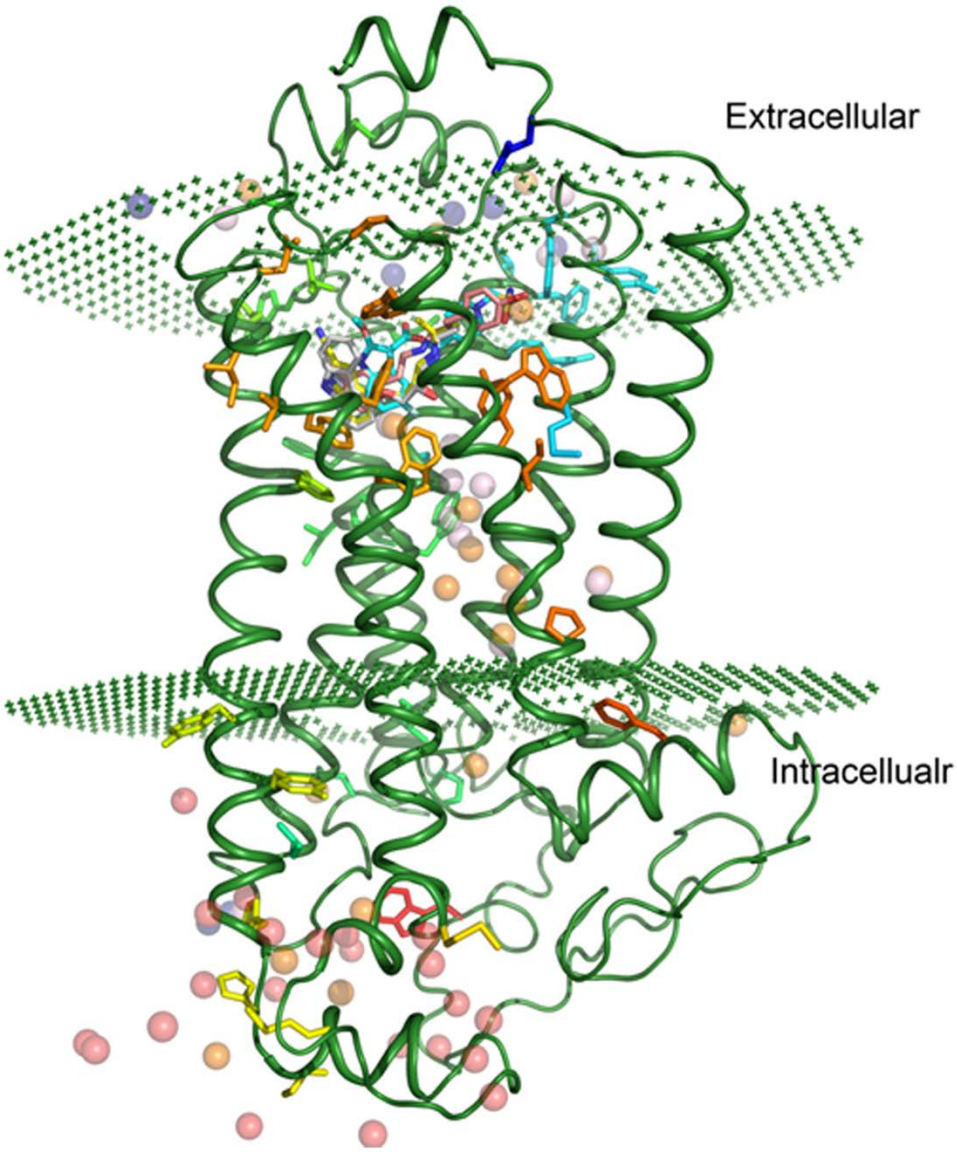
Supplementary Figure S7. Conserved residue $Y^{7.43}$ in the binding pocket of 2RH1, 2VT4 and 5-HT4R (model) bound antagonist. A) Carazolol bound in β2-AR in close proximity to the antagonist to make a favorable hydrogen bond with $Y^{7.43}$. B) Cyanopindolol bound to β1-AR is positioned similar to β2-AR. C) The same as in the case of β1-AR, 5-HT4R model with docked GR125487also positioned favorably at a position to $Y^{7.43}$.



Supplementary Figure S8. Adding waters from known homologous crystal structures to 5-HT4R model. (A) Overall spatial distribution of waters from crystal structures PDB IDs 2Y00 (pink balls), 2VT4 (dark blue balls) and 2RH1 (golden brown balls) structurally aligned to the 5-HT4R model. Binding of antagonist ligands GR125487 (cyan, docking), carazolol (yellow) and cyanopindolol (silver grey) with respect to the agonist dobutamine (flesh pink) demonstrates highly conserved binding pockets for class A rhodopsin family GPCRs. The distribution of waters is asymmetric within these GPCRs. (B) Overlay of the 5-HT4R model cartoon showing the positions of mass spectrometrically-identified oxidized residues with respect to waters.



Supplementary Figure S9. Lipid bilayer interactions of the 5-HT4R calculated with the OPM server (http://opm.phar.umich.edu/). The 5-HT4R model with positions of oxidized residues observed by mass spectrometry is shown with respect to the lipid bilayer. Crystallographic waters were placed into the model as described under Supplementary Figure S5. Residues distributed on the extracellular side most likely are oxidized by the surrounding bulk water because residues within TM helices are oxidized by nearby buried waters located inside the helix bundle. Oxidized residues are colored in rainbow colors starting from the N-terminus to the C-terminus.



Supplementary Figure S10. A compartive view of the binding site interactions showing free against antagonist bound 5-HT4R. (A) In free receptors the Y302$^{7.43}$ and D100$^{3.32}$ are both free to interact with each other. (B) Upon binding of antagonist the Y302$^{7.43}$ is kept in an alternate conformation bound to the ligand so that D100 no longer capable of making  a favorable interaction.