Chapter 3: Probing side chain conformations and rearrangements in receptor binding sites

3.1 Abstract

The large-scale gating motions of ligand-activated transmembrane receptors are likely correlated with motions at the ligand binding site, such as side chain rearrangements upon ligand binding. In GPCRs and in nAChRs, the nature of these conformational changes, and the precise orientation of side chains in the ligand-bound, active state, have been widely debated. Activation models have been proposed, such as the rotamer toggle switch model for GPCR activation, though there is little experimental evidence to support them. Crystallography and molecular dynamics of these systems have offered some insight, though have largely complicated, rather than clarified, models of gating at the binding site. Here we present several mutagenesis approaches to study side chain conformations and rearrangements. Rotamer-biased β -methyl analogs of Trp and Phe, and other unnatural analogs, were employed at several sites in the muscle-type nAChR and in the D2 dopamine and M2 acetylcholine GPCRs. These data provide some suggestive evidence for specific side chain geometries, corroborated by modeling of the conformational bias of these unnatural side chains. However, much of the data cannot be reconciled with proposed steric clashes or activation models, underscoring the difficulty of rationalizing conformation by mutagenesis.

3.2 Introduction

Gating of GPCRs and nAChRs is initiated by ligand binding, and ultimately results in substantial conformational changes of the receptor that enable association with a G protein (in the case of GPCRs) or opening of an ion-conducting pore (in the case of nAChRs). Presumably these large-scale gating motions are coupled to smaller scale rearrangements of the binding site, which also assumes active and inactive states. The structural details of these active and inactive states of the binding site are debated in both of these systems.

3.2.1 GPCR activation

In GPCRs, receptor activation is characterized by an outward displacement of the intracellular end of transmembrane helix 6 (TM6), which creates a binding site for the G protein.¹ This is thought to be a conserved mode of activation among GPCRs and was originally proposed on the basis of site-directed spin labeling,² disulfide crosslinking,³ and fluorescence experiments.⁴ TM6 displacement upon activation has also been borne out by crystallography of Rhodopsin and of the β 2 adrenergic receptor, both of which have been captured in both inactive and active states, the latter in complex with its cognate G protein.⁵⁻⁸ These structures also indicate more subtle movements of the intracellular ends of TM5 and TM7.

Presumably, a local rearrangement in TM6 at or near the ligand-binding site gets propagated into the larger conformational changes observed. Much attention has been focused on the highly conserved CWxPx(Y/F) motif in the middle of TM6, centered around the helical kink associated with P6.50 (residue numbering follows the Ballesteros-Weinstein scheme, in which the most conserved residue of helix X is denoted X.50).⁹

37

Based on biased Monte Carlo simulations and mutagenesis of the β 2 adrenergic receptor, Javitch and co-workers (expanding upon a proposal by Weinstein and co-workers¹⁰) proposed that the binding site residues C6.47, W6.48, and F6.52 form a rotamer toggle switch, in which receptor activation involves a correlated "switch" of these residues' sidechain χ^1 rotamers (Figures 3.1 and 3.2).¹¹ The authors further propose that the rotamer switching, including a *gauche*(-) to *trans* transition of the W6.48 χ^1 angle, increases the TM6 kink angle, inducing the key displacement of the intracellular end of TM6. These residues line the binding site and ligand binding would presumably induce the rotamer switching.



Figure 3.1. (A) Angles describing a residue's conformation, shown for Trp. (B) χ^1 rotamer definitions



Figure 3.2. The TM6 rotamer toggle switch model for GPCR activation, involving changes in χ^1 for residues C6.47, W6.48, and F6.52.

NMR and fluorescence experiments on rhodopsin suggest rotamer switching or some other local rearrangement of the W6.48 side chain upon receptor activation,^{12,13} and molecular dynamics simulations of rhodopsin show rotamer switching of W6.48.¹⁴ In detailed molecular dynamics experiments on the A2A receptor that draw upon multiple ligand-bound crystal structures, Voth and co-workers have proposed a model in which receptor activation is intimately connected to rotamer transitions of W6.48. The proposed rotamer transitions are the same as those suggested by Javitch: a *gauche*(-) to *trans* switch in χ^1 and a 180° flip in χ^2 .¹⁵

However, crystal structures that are thought to represent active GPCR conformations consistently show binding sites essentially unchanged from the putative inactive structures (Figure 3.3).^{8,16,17} A slight lateral displacement of W6.48 toward TM5 has been observed, especially in rhodopsin structures (Figure 3.3B), but no rotamer changes are seen. A different orientation of the W6.48 side chain is observed in a structure of the M2 acetylcholine receptor (Figure 3.3D), but this is an antagonist-bound, inactive structure.¹⁸ Thus the role of TM6 side chain rearrangements in GPCR activation remains unresolved. Interestingly, a different "switch" was observed one helical turn below W6.48 in crystal structures of the β 2 adrenergic receptor. F6.44 moves laterally past the I3.40 side chain "gate" (I3.40 undergoes a rotamer transition). The experiments described in this chapter interrogate the rotamer toggle switch and the F6.44/I3.40 switch in the D2 dopamine receptor and also interrogate W6.48 in the M2 muscarinic acetylcholine receptor.



Figure 3.3. W6.48 conformations seen in GPCR crystal structures. Binding sites are shown with W6.48 and co-crystallized ligand.

3.2.2 nAChR activation

Activation of nAChRs and other pentameric receptors opens the ion-conducting transmembrane pore approximately 60 Å away from the ligand binding site. This is thought to occur through a twisting of the extracellular domain, which induces a tilting of TM2 and TM3 to open the pore. This general gating mechanism has been borne out both computationally, through normal mode analysis of an α 7 receptor model,¹⁹ and by crystallography, through structures of pentameric bacterial channels in open and closed pore conformations.²⁰⁻²²

40

Other structural data have guided our understanding of gating motions at the ligand binding site. Mollusc-derived acetylcholine binding proteins (AChBPs) with homology to the extracellular domain of nAChRs have been crystallized at high resolution, offering a structural template for the ligand binding region of these receptors. Structures of several variants of this protein are available, in complex with antagonists, partial agonists, and full agonists.²³ A consensus model has emerged in which agonist binding induces closure of the C loop around the ligand binding site. Very subtle changes have been observed for ligand binding residues, for example, in apo versus agonist-bound structures of an AChBP/ α 7 receptor chimera: subtle side chain displacements are seen for TyrC1, TyrA, and TrpD.²⁴ However, as AChBPs simply bind acetylcholine and do not open a transmembrane pore, it is unclear how well, if at all, these binding site "gating" motions are applicable to nAChRs.

Other insights into nAChR gating have come from cryo-EM structures of the *Torpedo* nAChR in native membrane. One recent structure obtained from rapid freezing after exposure to acetylcholine corroborates some gating motions, including C loop closure and pore opening (though these changes were not observed in all subunits).²⁵ The resolution of this structure (~6 Å) is too low for insight into side chain geometry. A higher resolution (4 Å) structure is available for the closed, ligand-free receptor.²⁶ This structure reveals an extended C loop and a more open binding site, relative to AChBP structures, suggesting side chain rearrangements that must occur upon ligand binding (Figure 3.4). In particular, TrpD is seen in a different side chain rotamer, and the critical cation- π binding residue TrpB shows a displacement as well. The nAChR experiments in

41

this chapter interrogate the mouse muscle-type nAChR ($\alpha_2\beta\gamma\delta$), which has very high homology to the *Torpedo* nAChR.



Figure 3.4. The nAChR binding site in a cryo-EM structure of the inactive receptor shows different side chain conformations for TrpB and TrpD, compared to an agonist-bound AChBP structure.

3.2.3 Probing side chain conformation by mutagenesis

Here we use mutagenesis to probe side chain conformation in these transmembrane receptor systems. In one approach, we assess the effect of appending a charged amine to the Trp side chain using 5-aminomethyltryptophan, a novel amino acid that was synthesized for these experiments. The tolerance of this significant structural and electrostatic perturbation helps inform the positioning of the W6.48 side chain within GPCR binding sites. In another approach, we employ conformationally-biased unnatural side chains to probe for rotameric transitions upon receptor activation. For Phe and Trp, we use conformationally constrained β -methyl analogs – a method introduced by Victor Hruby to study the orientation of aromatic side chains.²⁷ The β -methyl group (in either (R) or (S) stereochemistry, Figure 3.5) alters the conformational landscape of these amino acids, changing their side chain rotamer preferences. This reshaping of the χ^1 , χ^2 conformational landscape was modeled by Hruby using molecular mechanics calculations on a model peptide,²⁷ and we performed similar calculations here to produce energy maps for these unnatural side chains.



Figure 3.5. β -methyl amino acids employed in this chapter

We probed the W6.48 site of the M2 muscarinic GPCR and the W6.48, F6.51, and F6.52 sites of the D2 dopamine GPCR to test the rotamer toggle switch hypothesis. We also used β -methyltryptophan to probe the TrpB and TrpD sites of the muscle-type nAChR, to test for rotamer transitions of these residues. To explore possible steric effects of the introduced methyl groups with adjacent residues, we performed double mutants of the β -methyl analog and potentially clashing side chains. Finally, a similar approach was applied using the *allo* isomers of β -branched amino acids Thr and Ile – unnatural analogs with inverted stereochemistry at the β carbon. These were used as probes of Thr and Ile rotamer transitions.

3.3 Results and Discussion

3.3.1 Mutagenesis probing conformational rearrangements of C6.47 upon D2 dopamine receptor activation

In their study proposing the rotamer toggle switch model for GPCR activation, Javitch and co-workers supported their model though mutagenesis of the β 2 adrenergic receptor.¹¹ In their model (Figure 3.2), C6.47 adopts the *trans* rotamer in the inactive state, contacting W6.48, and switches to the *gauche*(-) rotamer in the active state. Mutation of C6.47 to Ser, which has a similar rotamer distribution to Cys in an α -helix, largely preserved wild type function in the β 2 receptor. They rationalized that since the "inactive" *trans* rotamer is highly disfavored for Thr, a C6.47T mutation would promote receptor activation. Indeed, the C6.47T mutant of the β 2 receptor showed enhanced constitutive activity (basal signaling in the absence of agonist) and a reduced EC₅₀. We sought to test these and other mutations in the D2 dopamine receptor to probe the proposed rotamer toggle switch in that system.

In the D2 dopamine receptor we also observe a small gain of function for the C6.47T mutant and a borderline loss of function for C6.47S (Table 3.1) – consistent with the Javitch results for the β 2 receptor. To test the hypothesis that the C6.47T mutation causes a gain of function because it favors the "active" *gauche*(-) rotamer, we evaluated *allo*-threonine (*a*Thr) at this position, which has opposite stereochemistry at the β carbon. On the basis of simple conformational analysis (Figure 3.6), the "inactive" *trans* rotamer of *a*Thr should be favored and the active *gauche*(-) rotamer disfavored, relative to Thr. Surprisingly, the *a*Thr mutation gives an even larger gain of function than C6.47T, inconsistent with this model. C6.47A, which removes all side chain functionality, also gives a gain of function. This latter observation could be consistent with the Javitch

model, in that no interaction with W6.48 is possible to stabilize the inactive state. Overall, this data set does not lend strong support for the Javitch model of C6.47's role in activation. The nature of an interaction between C6.47 and the W6.48 side chain is unclear. While the native Cys would be anticipated to undergo a sulfur- π -type interaction, a comparable interaction is not very favorable for the –OH of Ser and Thr. As such, it is difficult to anticipate how these mutants would adapt to the toggle switch model.

Table 3.1. C6.47 mutations in the D2 dopamine receptor. Dopamine EC_{50} and Hill coefficient (n_H) are \pm SEM for goodness of fit to the Hill equation.

	EC ₅₀ (µM Dopamine)	Fold Shift	n _H	n
wt	0.043 ± 0.001		1.13 ± 0.02	57
C6.47S	0.072 ± 0.002	1.7	1.17 ± 0.04	20
C6.47A	0.010 ± 0.002	1 / 4.3	1.1 ± 0.1	5
C6.47T	0.0152 ± 0.0009	1 / 2.8	1.07 ± 0.06	28
C6.47Thr ^a	0.014 ± 0.001	1/3.1	1.12 ± 0.08	11
C6.47aThr	0.0082 ± 0.0004	1 / 5.2	1.21 ± 0.06	14

^aExpression of the wild type receptor with the natural amino acid incorporated by nonsense suppression



Figure 3.6. Conformational analysis indicates that the putative "inactive" *trans* χ^1 rotamer will be more accessible to *a*Thr than to Thr.

Also casting doubt on this model, C6.47 is found in the "inactive" *trans* rotamer in the crystal structure of rhodopsin in the inactive state (the structural template for the Javitch study), but is actually found in the putative "active" *gauche*(-) rotamer (pointing away from W6.48) in many other "inactive" GPCR crystal structures. These include antagonist-bound structures of the β 2 adrenergic receptor and the D3 dopamine receptor.^{5,7,28} As such, the role of C6.47 in activation of the D2 receptor and other GPCRs remains unclear.

3.3.2 Investigating the role of a F6.44/I3.40 switch in activation of the D2 dopamine receptor

GPCR crystal structures thought to represent active conformations have not shown any of the side chain transitions predicted by the rotamer toggle switch model. However, for the β 2 adrenergic receptor, the closest homolog to the D2 receptor for which we have an active state crystal structure, another switch was found. One turn below W6.48, F6.44 was observed to slide laterally, with the lower half of TM6 following it. This rigid body rotation contributes to the large displacement of TM6 to accommodate the G protein. The F6.44 side chain does not undergo a conformational change, but rather moves past I3.40, which serves as a "swinging gate," adopting a new side chain rotamer to follow the Phe side chain in the active state (Figure 3.7). These residues, especially F6.44, are highly conserved. Previous mutagenesis studies have pointed to the importance of F6.44 for receptor activation in the β 2 adrenergic receptor, among other systems, and computational work has supported the idea of a F6.44/I3.40 activation "switch."²⁹⁻³¹ In particular, previous studies in the M3 muscarinic receptor, the β 2 adrenergic receptor, and the α IB-adrenergic receptor have shown that mutating F6.44 to smaller side chains such as Leu, Val, or Ala results in a gain of receptor function and/or an increase in constitutive activity.^{29,31,32}



Figure 3.7. F6.44/I3.40 activation switch revealed by structures of the β 2-adrenergic receptor. Inactive (blue) and active (green) structures are overlaid.

To investigate the role of these residues in the D2 dopamine receptor, we also characterized the F6.44L mutant, for which a gain of function was observed, consistent with the results described above from other systems (Table 3.2). This could reflect a weakening of the inactive state "lock" imposed by the interaction of F6.44 with I3.40. We saw no EC_{50} shift for the 3,4,5-trifluorophenylalanine mutant of F6.44, indicating that side chain electrostatics are unimportant and suggesting no involvement in significant aromatic-aromatic interactions with F5.47, W6.48, or other nearby residues.

At the I3.40 site, we observed a significant loss of function for the I3.40V mutant – simply a methyl group deletion from the native Ile side chain. Such sensitivity confirms the importance of this side chain, but as this methyl contacts F6.44 in both active and

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	EC50 (µM Dopamine)	Fold Shift	Hill	n	
wt	0.043 ± 0.001		1.13 ± 0.02	57	
F6.44L	0.013 ± 0.001	1 / 3.3	1.3 ± 0.1	5	
F6.44Phe ^a	0.047 ± 0.005		1.0 ± 0.1	5	
F6.44F ₃ -Phe	0.046 ± 0.003	1.0	1.3 ± 0.1	4	
I3.40V	0.280 ± 0.020	6.5	1.10 ± 0.08	5	
I3.40A	ND^{b}				
I3.40Ile ^a	0.034 ± 0.002		1.13 ± 0.09	9	
I3.40 <i>a</i> Ile	0.059 ± 0.007	1.7	0.95 ± 0.10	10	

Table 3.2. Mutations to F6.44 and I3.40 in the D2 dopamine receptor. Dopamine EC_{50} and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

^{*a}*Expression of the wild type receptor with the natural amino acid incorporated by nonsense suppression ^{*b*}No dopamine-induced current detected</sup>

inactive states of the putative "switch," it is difficult to ascribe these data a mechanistic significance. No response was detected from the I3.40A mutant (Table 3.2). Finally, to probe the potential role of a rotamer switch, Ile was mutated to *allo*-isoleucine (*alle*), the Ile analog with inverted stereochemistry at the β carbon. I3.40 undergoes a *trans* to *gauche(-)* rotamer transition upon activation in the β 2 receptor crystal structures. *a*Ile is expected to favor the *trans* rotamer and disfavor *gauche(-)*, by the same logic applied to *a*Thr in section 3.3.1 above, and thus should be deleterious to receptor function. A very small loss of function was measured, below the 2-fold threshold we typically consider to be meaningful for this assay, so it is difficult to rule on the importance of the rotamer transition based on this data point. Of course, the stereochemistry inversion also alters the packing of this side chain with its neighbors.

Overall, these various mutagenesis results could be consistent with a F6.44/I3.40 rotamer switch, and indicate some functional importance of these residues. However, our observations are certainly insufficient to confirm the presence of this "switch" in the D2 dopamine receptor.

3.3.3 Synthesis and use of 5-aminomethyltryptophan to probe the W6.48 site in GPCRs

It remains unclear what conformation W6.48 assumes in the active state of various GPCRs, and whether rotamer switching has a role in activation. We reasoned that we could learn about the Trp's orientation by measuring the tolerance for a significant structural perturbation added to the W6.48 side chain. We chose to introduce an aminomethyl group at the 5-position of the indole, a group that will be positively charged at physiological pH. We further envisioned that by also introducing a negative charge in the receptor (i.e., Asp or Glu) near the Trp side chain, we might create a salt bridge involving W6.48 (Figure 3.8). If such a salt bridge could be engineered and was functionally tolerated, this would help inform the preferred orientation of the W6.48 side chain.



Figure 3.8. Scheme for an engineered salt bridge involving 5-aminomethyltryptophan to test for W6.48 rotamer switching

In particular, we were interested in probing W6.48 in the M2 muscarinic acetylcholine receptor and in the D2 dopamine receptor. Inactive, antagonist-bound crystal structures are available for the M2 receptor and for the D3 dopamine receptor, a very close homolog to the D2 receptor.^{18,28} Interestingly, these structures show different orientations of W6.48: the usual "vertical" Trp orientation in the D3 structure, and a "tilted" orientation with a different χ^2 rotamer in the M2 structure (Figure 3.3, C and D).

We wondered whether these different orientations seen by crystallography would be reflected in different functional tolerance for 5-aminomethyltryptophan (5-NH₂CH₂Trp).

5-NH₂CH₂Trp was synthesized employing a Friedel-Crafts-type indole coupling (Scheme 3.1).³³ 5-Aminomethyl-indole was Boc protected, and this species (1), was coupled to ethyl 3-bromo-2-hydroxyiminopropanoate.³³ The hydroximine (2) was reduced by an aluminum/mercury amalgam, and the Boc group removed by trifluoroacetic acid. This racemic diamine (4) was protected with nitroveratryloxycarbonyl (NVOC) groups and the ester was hydrolyzed. Reaction with chloroacetonitrile gave the cyanomethyl ester 7, which was coupled to dCA and ligated to tRNA by standard methods.





 $5-NH_2CH_2Trp$ was surprisingly well tolerated at the W6.48 site in the M2 receptor, giving an EC₅₀ less than 2-fold shifted from wild type (Table 3.3). In contrast, no response was detected from efforts to incorporate W6.48[$5-NH_2CH_2Trp$] in the D2 receptor under otherwise identical conditions. It is unclear whether the mutation rendered the receptor nonfunctional or reduced receptor expression levels. The salt bridge conceived for W6.48 in the "active" conformation (Figure 3.8) suggested by the rotamer toggle switch pairs $5-NH_2CH_2Trp$ with Y5.48E. No response was detected for this double mutant either, and again, it is unclear whether low expression or lack of receptor function is the reason, though the Y5.48E single mutant gave a very large loss of function.

Table 3.3. 5-Aminomethyltryptophan mutations to W6.48 in the M2 acetylcholine and D2 dopamine receptors. EC_{50} and Hill coefficient (n_H) are \pm SEM for goodness of fit to the Hill equation.

M2 Acetylcholine Receptor	EC ₅₀ (µM ACh)	Fold Shift	n _H	n	
W6.48Trp ^a	0.27 ± 0.01		1.32 ± 0.06	7	
W6.48[5-NH ₂ CH ₂ Trp]	0.47 ± 0.09	1.7	1.5 ± 0.4	4	
D2 Dopamine Receptor	EC ₅₀ (µM Dopamine)	Fold Shift	n _H	n	
W6.48Trp ^a	0.025 ± 0.002		1.00 ± 0.06	8	
W6.48[5-NH ₂ CH ₂ Trp]	ND^b				
Y5.48E	6 ± 2	240	0.56 ± 0.05	4	
Y5.48E W6.48[5-NH ₂ CH ₂ Trp]	ND^{b}				

^{*a}*Expression of the wild type receptor with the natural amino acid incorporated by nonsense suppression ^{*b*}No dopamine-induced current detected</sup>

In the speculative case that the D2 W6.48[5-NH₂CH₂Trp] mutant expresses but is nonfunctional, an interesting contrast emerges with the M2 receptor. If W6.48 does not move upon activation, as suggested by crystallography, 5-NH₂CH₂Trp is expected to be especially deleterious in the D2 receptor, for which the D3 structure suggests a "vertical" orientation. The cationic amine of dopamine is expected to meet steric and electrostatic repulsion from the introduced aminomethyl substitutent (Figure 3.9).



Figure 3.9. Structures of GPCR binding sites with the 5-aminomethyl substituent modeled onto the W6.48 side chain. The approximate predicted location of agonist binding is noted.

Also supporting this "vertical" tryptophan conformation in the active D2 receptor, a functionally important sulfur- π interaction between W6.48 and C3.36 has been identified.³⁴ Weakening this interaction by indole fluorination is detrimental to receptor function, suggesting that the interaction is present in the active receptor, which would require the Trp to maintain its "vertical" conformation. In contrast, the "tilted" orientation of W6.48 in the M2 structure would place the aminomethyl group clear of the agonist in a pocket between helices 3, 4, and 5 (Figure 3.9). This could account for the surprisingly good tolerance for this side chain in the M2 receptor. These data are consistent with a lack of Trp movement upon activation, and with different Trp conformations in the D2 and M2 receptors, as suggested by crystallography.

3.3.4 Synthesis, mutagenesis, and modeling of β -methyltryptophan and β -methylphenylalanine

 β -methyl analogs of Phe and Trp were employed to probe the conformation of aromatic side chains within nAChR and GPCR binding sites. β -methyl substituents introduce conformational constraints to the side chain and have been employed to study the conformation of aromatic amino acids in various peptide contexts by Hruby and coworkers.²⁷

3.3.4.1 Synthesis

In these studies, we employed (2S, 3S) and (2S, 3R) stereoisomers of both βmethyltryptophan and β-methylphenylalanine. (2S, 3S)-β-methylphenylalanine [hereafter (S)-β-MePhe] was obtained from a commercial supplier (Chem-Impex). (2S, 3R)-β-methylphenylalanine [(R)-β-MePhe] and (2S, 3S)-β-methyltryptophan [(S)-β-MeTrp] were generous gifts from Victor Hruby. These three compounds were NVOCprotected, activated as cyanomethyl esters, and coupled to dCA by standard methods (compounds **9-14**). (2S, 3R)-β-methyltryptophan [(R)-β-MeTrp] was synthesized by a route drawing upon two previously reported syntheses by Hruby (Scheme 3.2).^{35,36}

Indoleacrylic acid was Boc-protected and the resulting compound (15) was coupled to the (S)-4-phenyl-2-oxazolidinone chiral auxiliary via a pivalate ester to yield compound 16. The chiral auxiliary directs installation of the methyl and amine groups with good stereoselectivity. Conjugate cuprate addition of methyl Grignard installed the β -methyl group (17). The azide precursor to the amine was installed via deprotonation and electrophilic azide addition with trisyl azide to yield compound 18. The chiral auxiliary was removed via hydrogen peroxide-mediated hydrolysis, and the azide of this compound (19) was reduced to the amine (compound 20) via a Staudinger reduction. Removal of the Boc group yielded the amino acid (21), which was NVOC protected (compound 22), activated as a cyanomethyl ester (compound 23), and coupled to dCA (24) by standard methods.

Scheme 3.2. Synthesis of (2S,3R)-β-methyltryptophan



3.3.4.2 Mutagenesis

(R) and (S) β -methyl analogs of both Phe and Trp proved viable for incorporation by nonsense suppression; robust currents were measured from receptors expressing these unnatural amino acid analogs in both GPCRs and in the muscle-type nAChR. While very large losses of receptor function were recorded for the β -MeTrp analogs at the W6.48 site of the D2 dopamine and M2 acetylcholine GPCRs, these side chains caused little, or no, perturbation to nAChR function at the TrpB (α W149) and TrpD (γ W55/ δ W57) sites (Table 3.4). The β -MePhe analogs were well-tolerated at the F6.52 site in the D2 dopamine receptor. Additionally, both β -MePhe analogs were incorporated at the TrpD site, which interestingly yielded equivalent losses of function, in contrast to the β -MeTrp derivatives. Both conformational and steric effects could contribute to the EC₅₀ shifts observed in Table 3.4. Conformational (rotamer-bias) effects are considered in section 3.3.4.3 and steric effects are considered in section 3.3.4.4.

Table 3.4. β -methyl mutations to W6.48 of the M2 acetycholine receptor, W6.48 and F6.52 of the D2 dopamine receptor, and TrpB (α W149) and TrpD (γ W55/ δ W57) of the muscle-type nAChR. EC₅₀ and Hill coefficient ($n_{\rm H}$) are \pm SEM for goodness of fit to the Hill equation.

M2 Acetylcholine Receptor	EC ₅₀ (µM ACh)	Fold Shift	n _H	n
W6.48Trp ^a	0.27 ± 0.01		1.32 ± 0.06	7
W6.48(R)βMeTrp	54 ± 1	200	0.95 ± 0.02	7
W6.48(S)βMeTrp	73 ± 2	270	0.91 ± 0.02	7
D2 Dopamine Receptor	EC50 (µM Dopamine)	Fold Shift	n _H	n
W6.48Trp ^a	0.025 ± 0.002		1.00 ± 0.06	8
W6.48(R)-β-MeTrp	4.3 ± 0.4	170	0.70 ± 0.03	5
W6.48(S)-β-MeTrp	0.69 ± 0.10	28	1.18 ± 0.16	7
F6.52Phe ^a	0.052 ± 0.003		1.44 ± 0.08	5
F6.52(R)-β-MePhe	0.067 ± 0.007	1.3	1.19 ± 0.05	10
F6.52(S)-β-MePhe	0.127 ± 0.008	2.4	1.12 ± 0.07	7
Muscle-Type nAChR	EC ₅₀ (µM ACh)	Fold Shift	n _H	n
$\alpha W149 Trp^{a}$	22 ± 2		1.5 ± 0.1	6
αW149(R)-β-MeTrp	22 ± 1	1.0	1.6 ± 0.1	4
αW149(S)-β-MeTrp	17.1 ± 0.4	1 / 1.3	1.39 ± 0.04	7
$\gamma W55/\delta W57Trp^{a}$	23.4 ± 0.7		1.35 ± 0.05	3
γW55/δW57(R)-β-MeTrp	95 ± 3	4.0	1.44 ± 0.05	4
γW55/δW57(S)-β-MeTrp	23.6 ± 0.8	1.0	1.30 ± 0.05	5
γW55/δW57Phe	271 ± 7	12	1.51 ± 0.05	4
γW55/δW57(R)-β-MePhe	330 ± 20	14	1.35 ± 0.09	4
γW55/δW57(S)-β-MePhe	290 ± 30	12	1.2 ± 0.1	6

^aExpression of the wild type receptor with the natural amino acid incorporated by nonsense suppression

3.3.4.3 Conformational effects

To gain insight into the conformational bias of these β -methyl analogs relative to the native Phe and Trp side chains, we performed calculations to map the energy landscape of these amino acids as a function of χ^1 and χ^2 . Using a protocol similar to that previously employed by Hruby,²⁷ molecular mechanics calculations (MMFF) were performed for each amino acid, capped in the form: Ac-Xaa-NH-Me (where Xaa is the β methyl amino acid). Energies were determined at all angles of χ^1 and χ^2 , in 10° increments. The angles φ and ψ were constrained to either the β -sheet values observed for TrpD in the nicotine-bound *Ls*-AChBP crystal structure³⁷ ($\varphi = -86^\circ$, $\psi = 120^\circ$), or to idealized α -helix values ($\varphi = -48^\circ$, $\psi = -57^\circ$) which are closer to the appropriate angles for TrpB and for W6.48. The plots (Figures 3.10 through 3.12) show the same general shape as those previously constructed by Hruby,²⁷ and the location of wells in these plots generally (though not perfectly) corresponds to the predominant rotamers represented in the PDB for Phe and Trp.³⁸



Figure 3.10. Energy maps for Trp, (R)- β -MeTrp, and (S)- β -MeTrp as a function of the side chain dihedrals χ^1 and χ^2 , with the backbone constrained to idealized α -helix dihedral values ($\phi = -48^\circ$, $\psi = -57^\circ$).



Figure 3.11. Energy maps for Trp, (R)- β -MeTrp, and (S)- β -MeTrp as a function of the side chain dihedrals χ^1 and χ^2 , with the backbone constrained to the β -sheet dihedral values observed for TrpD in the nicotine-bound *Ls*-AChBP crystal structure³⁷ (ϕ = -86°, ψ = 120°).



Figure 3.12. Energy maps for Phe, (R)- β -MePhe, and (S)- β -MePhe as a function of the side chain dihedrals χ^1 and χ^2 , with the backbone constrained to the β -sheet dihedral values observed for TrpD in the nicotine-bound *Ls*-AChBP crystal structure³⁷ ($\varphi = -86^\circ$, $\psi = 120^\circ$).

To evaluate the effect of a β -methyl mutation on the conformational landscape of a Trp or Phe side chain, we created " β -methyl effect" plots, in which the plot for the parent side chain (Trp or Phe) is subtracted from the plot for one of its β -methyl analogs (Figures 3.13 through 3.16). Note that the two plots from which the difference is taken are for different compounds and have different global minima, so the value $\Delta\Delta G = 0$ should not be ascribed special significance.

We can use these plots to make predictions about how β -methyl mutations should affect receptor activation on the basis of specific activation models. We first consider the "rotamer toggle switch" activation model for W6.48 in GPCR binding sites (Figure 3.13). As W6.48 in the inactive M2 acetylcholine receptor structure was observed in a noncanonical conformation unaccounted for in models of GPCR activation, the M2 receptor is not included in this analysis. Instead, we consider the D3 dopamine receptor structure as a model for the inactive D2 dopamine receptor. The Javitch/Voth "rotamer toggle switch" model for GPCR activation posits a W6.48 χ^1 rotamer change from *gauche*(-) to *trans* and a 180° flip in $\chi^{2,11,15}$ On Figure 3.13A, this is a transition from the black \times (χ^1 , χ^2 for the canonical inactive W6.48 rotamer seen in the D3 structure) to the purple X. The difference in energy between these two (χ^1, χ^2) points is 0.3 kcal/mol greater for (R)- β -MeTrp than for Trp and 0.4 kcal/mol less for (S)- β -MeTrp than for Trp (Figure 3.13B, blue values). Thus, β -methyl substitutions are expected to have modest but opposite effects for the two stereoisomers. This stands in stark contrast to the large losses of function recorded for both stereoisomers in the D2 dopamine receptor. Steric effects, discussed in section 3.3.4.4, most likely dominate.



Figure 3.13. (A) " β -methyl effect" difference plots for (R) and (S) β -methyls of tryptophan with α -helix backbone dihedral constraints. χ^1, χ^2 values for W6.48 observed in crystal structures and predicted for the active state are denoted with an X. (B) Conformational energy analysis. Boxed values are energies (in kcal/mol) calculated for the indicated χ^1, χ^2 , taken from the corresponding plot in Figure 3.10. Values associated with arrows are energy differences. Note that the green "methyl effect" values correspond to $\Delta\Delta G$ values at the appropriate X in part A. Blue values are the differential effect of the methyl on activation.

In an alternative model, W6.48 does not move upon activation, as suggested by crystal structures of active GPCRs, mutagenesis suggesting a sulfur- π interaction between W6.48 and C3.36,³⁴ and perhaps consistent with the 5-NH₂CH₂Trp results in section 3.3.3. In this scenario, β -methyl groups could conceivably have a functional effect if they

destabilize the W6.48 conformation observed in inactive GPCR crystal structures (which is also the active conformation). Note that the different W6.48 conformations (specifically, different χ^2 values) seen in the D3 and M2 structures are indeed differentially affected by the β -methyl analogs (Figure 3.13A, black × and green ×). While (R)- β -MeTrp should have a similar effect on both receptors, (S)- β -MeTrp will significantly destabilize the M2 tryptophan conformation. This effect could in part be responsible for the larger loss of function observed for (S)- β -MeTrp in the M2 acetylcholine receptor, compared to the D2 dopamine receptor (Table 3.4).

In the nAChR, a speculative gating model emerges by comparing agonist-bound AChBP structures (presumably the active conformation) to Unwin's cryo-EM structure of the inactive nAChR (Figure 3.4). An analysis of predicted β -methyl effects for this Unwin cryo-EM-to-AChBP activation model is presented in Figures 3.14 through 3.16 using the same approach applied to GPCR W6.48 activation in Figure 3.13. For TrpB, the inactive to active energy gap is predicted to be 3.4 kcal/mol larger for (R)- β -MeTrp than for Trp and 3.4 kcal/mol smaller for (S)-β-MeTrp than for Trp (blue values, Figure 3.14B). However, both of these analogs gave wild type function at TrpB (Table 3.4), calling into question the validity of this activation model and/or energy analysis. For TrpD, smaller but still significant β -methyl perturbations are predicted (+1.5 kcal/mol for (R)-β-MeTrp and -2.2 kcal/mol for (S)-β-MeTrp) (Figure 3.15). (R)-β-MeTrp did indeed give a loss of function for TrpD, as predicted, while the (S) analog was wild type (Table 3.4). Interestingly, the loss of function for the (R) analog was not observed for the series Phe, (S)- β -MePhe, and (R)- β -MePhe. However, the energy analysis for the β -methyl Phe analogs suggests that (R)- β -MePhe should destabilize activation by 2.6 kcal/mol, even

more than (R)- β -MeTrp (Figure 3.16). Clearly, the predictions made by computational work on model systems are inconsistent with our mutagenesis results. This could reflect incorrect assignment of inactive and active Trp conformations, certainly possible for the inactive state given the low resolution of the Unwin structure. Alternatively, steric effects in the actual protein are unaccounted for in the model system used for the calculations. We consider the latter possibility in the following section.



Figure 3.14. (A) " β -methyl effect" difference plots for (R) and (S) β -methyls of tryptophan with α -helix backbone dihedral constraints (note that these are the same plots as in Figure 3.13A). χ^1 , χ^2 values for TrpB observed in cryo-EM and crystal structures are denoted with an X. (B) Conformational energy analysis. Boxed values are energies (in kcal/mol) calculated for the indicated χ^1 , χ^2 , taken from the corresponding plot in Figure 3.10. Values associated with arrows are energy differences. Note that the green "methyl effect" values correspond to $\Delta\Delta G$ values at the appropriate X in part A. Blue values are the differential effect of the methyl on activation.



Figure 3.15. (A) " β -methyl effect" difference plots for (R) and (S) β -methyls of tryptophan with β -sheet backbone dihedral constraints. χ^1, χ^2 values for TrpD observed in cryo-EM and crystal structures are denoted with an X. (B) Conformational energy analysis. Boxed values are energies (in kcal/mol) calculated for the indicated χ^1, χ^2 , taken from the corresponding plot in Figure 3.11. Values associated with arrows are energy differences. Note that the green "methyl effect" values correspond to $\Delta\Delta G$ values at the appropriate X in part A. Blue values are the differential effect of the methyl on activation.



Figure 3.16. (A) " β -methyl effect" difference plots for (R) and (S) β -methyls of phenylalanine with β sheet backbone dihedral constraints. χ^1, χ^2 values for TrpD observed in cryo-EM and crystal structures are denoted with an X. (B) Conformational energy analysis. Boxed values are energies (in kcal/mol) calculated for the indicated χ^1, χ^2 , taken from the corresponding plot in Figure 3.12. Values associated with arrows are energy differences. Note that the green "methyl effect" values correspond to $\Delta\Delta G$ values at the appropriate X in part A. Blue values are the differential effect of the methyl on activation.

3.3.4.4 Double mutant cycle analysis to probe steric effects of β -methyl substituents

To probe for steric effects of the introduced β -methyl substituents, we made β methyl mutations in combination with mutations to adjacent side chains. Double mutant cycle analyses could then be used to test for interactions, and perhaps clarify why energy analyses in the preceding section failed. Mutant cycle analysis can determine whether two single mutants' perturbations are functionally coupled or act independently. This is done by calculating a coupling coefficient { $\Omega = [EC_{50} (mut_{1,2}) \times EC_{50} (WT)]/[EC_{50} (mut_1) \times EC_{50} (mut_2)]$ }, which can in turn be converted to a coupling energy [$\Delta\Delta G =$ -RTln(Ω)]. If the two mutations are independent of each other, $\Omega \sim 1$. A

"compensatory" coupling, in which the double mutant is less deleterious than the effect of the two single mutants multiplied, will yield $\Omega < 1$ and a positive $\Delta\Delta G$. By defining specific steric clashes, we can place constraints on the side chain χ^1 angle (i.e., in which direction the β -methyl group is pointing).

In the D3 and M2 GPCR crystal structures, the F5.47 side chain is expected to clash with both (R) and (S) β -methyl substituents on W6.48, and a clash between the (S)- β -methyl and the F6.52 side chain is anticipated in the D3 structure (Figure 3.17). No clash with the ligand is predicted for either receptor (neither for the antagonist co-crystallized in each structure nor for the agonist used to probe each receptor).

The F5.47L mutation in the M2 receptor, which reduces the size of the side chain, caused a large loss of function (Table 3.5). The EC₅₀ estimates for the double mutants of F5.47L with the β -MeTrp analogs predictably indicate large losses of function, though a strong "compensatory" coupling is seen: +1.8 kcal/mol with the (S)- β -methyl and +2.4 kcal/mol with the (R)- β -methyl. The compensatory nature of the coupling ($\Omega < 1$,

Figure 3.17. Structures of GPCR binding sites with β -methyl substituents modeled onto the W6.48 side chain

Table 3.5. W6.48/F5.47 double mutants of the M2 acetylcholine receptor. EC_{50} and Hill coefficient (n_H) are \pm SEM for goodness of fit to the Hill equation.

	EC ₅₀ (µM ACh)	Fold Shift	n _H	n	Ω	ΔΔG (kcal/mol)
W6.48Trp ^{a,b}	0.27 ± 0.01		1.32 ± 0.06	7		
W6.48(R)-β-MeTrp ^a	54 ± 1	200	0.95 ± 0.02	7		
W6.48(S)-β-MeTrp ^a	73 ± 2	270	0.91 ± 0.02	7		
F5.47L	25 ± 2	93	1.2 ± 0.1	4		
F5.47L W6.48Trp	Low Currents					
F5.47L W6.48(R)-β-MeTrp	$80 \pm 10^{\circ}$	300	1.1 ± 0.1	6	1 / 62.5	+2.4
F5.47L W6.48(S)-β-MeTrp	$310 \pm 70^{\circ}$	1100	0.67 ± 0.05	4	1 / 21.8	+1.8

^aData reproduced from Table 3.4

^bExpression of the wild type receptor with the natural amino acid incorporated by nonsense suppression $^{c}EC_{50}$ estimate from truncated dose-response data

positive $\Delta\Delta G$) is consistent with the envisioned "bump-hole" pair created in the double mutant. The fact that both methyls couple strongly with the 5.47 side chain suggests that the *gauche*(-) χ^1 angle seen for W6.48 in the crystal structure may indeed be the approximate χ^1 angle of the active receptor, as both proposed steric clashes are evidently detrimental to receptor activation. Further, W6.48 and F5.47 move slightly closer to one

67

another in crystal structures of the active $\beta 2$ adrenergic receptor and of rhodopsin: the C α -C α distance decreases by 1.2 Å for the $\beta 2$ receptor and by 2.5 Å for rhodopsin. Thus, the β -methyl perturbation might be telling us more about subtle repacking upon activation rather than about side chain rotamer changes.

A similar approach to study steric interactions with F5.47 in the D2 dopamine receptor was attempted, but the F5.47L mutant gave too large of a loss of function to accurately measure an EC₅₀. However, previously published results for single mutants in this region are consistent with a similar steric clash as seen in the M2 receptor. A 14-fold loss of function was recorded for mutation of F5.47 to the bulkier 3,5dimethylphenylalanine analog, and an extremely large 1300-fold loss of function was recorded for the same mutation to F6.52.^{34,39} These results are consistent with tight packing in this region where the β -methyls were introduced.

In the muscle-type nAChR, we have identified several potential steric clashes between adjacent side chains and the β -methyl groups introduced, using a homology model of this receptor based on AChBP that should represent the active state (Figure 3.18). Steric clashes in this active conformation of the receptor are predicted to give a loss of function. Little, if any, steric clash is predicted for either β -methyl introduced to TrpB, in keeping with the wild type EC₅₀ values measured for both (R) and (S) analogs at this site (Figure 3.18A).

However, it is predicted that extra bulk at the α V91 site will result in a steric clash with the (R) β -methyl. In an effort to verify the χ^1 rotamer of TrpB in the active receptor, double mutants of α V91 with (R)- β -MeTrp were made, with (S)- β -MeTrp mutations serving as controls (Table 3.6). Lengthening the α 91 side chain from Val to Leu had no

Figure 3.18. Two views of the α/γ binding site of the muscle-type nAChR in the presumed active conformation from a homology model based on AChBP by Kristin Rule Gleitsman,⁴⁰ with β -methyl substituents modeled onto the side chains of TrpB and TrpD. Similar conformations are seen at the α/δ binding site. Corresponding residues in the δ subunit are T38, W57, and L121.

	EC ₅₀ (µM ACh)	Fold Shift	n _H	n	Ω	ΔΔG (kcal/mol)
$\alpha W149Trp^{a,b}$	22 ± 2		1.5 ± 0.1	6		
αW149(R)-β-MeTrp ^a	22 ± 1	1.0	1.6 ± 0.1	4		
αW149(S)-β-MeTrp ^a	17.1 ± 0.4	1 / 1.3	1.39 ± 0.04	7		
aV91L aW149Trp	23 ± 1	1.0	1.4 ± 0.1	7		
αV91L αW149(R)-β-MeTrp	63 ± 2	2.9	1.52 ± 0.06	4	2.74	-0.60
αV91L αW149(S)-β-MeTrp	57 ± 3	2.6	1.46 ± 0.08	4	3.19	-0.69
αV91I αW149Trp	33 ± 1	1.5	1.39 ± 0.07	7		
αV91I αW149(R)-β-MeTrp	38 ± 2	1.7	1.20 ± 0.05	5	1.15	-0.084
αV91I αW149(S)-β-MeTrp	108 ± 2	4.9	1.43 ± 0.04	4	4.21	-0.85
αV91Val αW149Trp ^b	22.5 ± 0.3		1.57 ± 0.03	8		
αV91Tle αW149Trp	93 ± 2	4.1	1.55 ± 0.05	7		
αV91Tle αW149(S)-β-MeTrp	260 ± 10	12	1.6 ± 0.1	7	3.59	-0.76
αV91Tle αW149(R)-β-MeTrp	ND ^c					

Table 3.6. TrpB (α W149) double mutants of the muscle-type nAChR. EC₅₀ and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

^{*a}</sup>Data reproduced from Table 3.4*</sup>

^bExpression of the wild type receptor with the natural amino acid(s) incorporated by nonsense suppression ^cNo current detected from identical expression conditions as $\alpha V91Tle \alpha W149(S)$ - β -MeTrp effect on the wild type receptor, and unexpectedly gave a similar modest coupling energy for both (R) and (S) β -methyl analogs. Also unexpectedly, the larger IIe side chain had a meaningful coupling with the (S) β -methyl, but not the (R). Finally, α V91 was mutated to *tert*-

leucine (Tle), which adds an additional methyl group to the β carbon (Figure 3.19) that is expected to clash significantly with an (R) β -methyl on TrpB. The α V91Tle single mutant itself gave a modest loss of function and a measurable +0.76 kcal/mol coupling energy was calculated for the Tle/(S)- β -MeTrp double mutant cycle. The (R)- β -MeTrp/Tle double mutant gave very little, if any, detectable currents (*versus* average currents of 1.3 μ A for the Tle/(S)- β -MeTrp double mutant also measured at 24 hrs postinjection). This observation could be consistent with a major perturbation to this key residue of the ligand binding site, which would confirm the proposed TrpB χ^1 rotamer, though it is unknown whether the deficit is in ligand binding, activation, or expression.

At the TrpD site, steric clashes are predicted for the (R) β -methyl with the γ T36/ δ T38 side chain and for the (S) β -methyl with the γ L119/ δ L121 side chain (Figure 3.18B). We wondered whether the loss of function recorded for the (R) β -methyl analog was due to a clash with the adjacent Thr. In support of this hypothesis, double mutant cycles with (R)- β -MeTrp in which the Thr side chain was made progressively smaller – to Ser, to Ala, and to Gly – gave progressively larger "compensatory" coupling energies of +0.069, +0.50, and +0.72 kcal/mol (Table 3.7). However, moderate coupling energies (also positive in sign) were also observed for the same Thr mutations in conjunction with (S)- β -MeTrp. As this β -methyl should not interact with the Thr side chain, the trend

70

Figure 3.19. *Tert*-leucine (Tle)

described above cannot reasonably be attributed to a specific interaction between the (R)

 β -methyl and the Thr.

	EC ₅₀ (µM ACh)	Fold Shift	n _H	n	Ω	ΔΔG (kcal/mol)
$\gamma W55/\delta W57Trp^{a,b}$	23.4 ± 0.7		1.35 ± 0.05	3		
$\gamma W55/\delta W57(R)$ - β -MeTrp ^a	95 ± 3	4.0	1.44 ± 0.05	4		
$\gamma W55/\delta W57(S)$ - β -MeTrp ^a	23.6 ± 0.8	1.0	1.30 ± 0.05	5		
γT36/δT38S γW55/δW57Trp	44 ± 1	1.9	1.24 ± 0.04	5		
γT36/δT38S γW55/δW57(R)β-MeTrp	160 ± 3	6.8	1.40 ± 0.03	6	1 / 1.13	+0.069
γT36/δT38S γW55/δW57(S)β-MeTrp	22.0 ± 0.6	1/1.1	1.32 ± 0.04	4	1 / 2.07	+0.43
γT36/δT38A γW55/δW57Trp	63 ± 1	2.7	1.35 ± 0.03	5		
γT36/δT38A γW55/δW57(R)β-MeTrp	112 ± 3	4.8	1.45 ± 0.05	4	1 / 2.34	+0.5
γT36/δT38A γW55/δW57(S)β-MeTrp	40.1 ± 0.8	1.7	1.28 ± 0.03	5	1 / 1.66	+0.29
γT36/δT38G γW55/δW57Trp	192 ± 3	8.2	1.43 ± 0.03	5		
γT36/δT38G γW55/δW57(R)β-MeTrp	230 ± 10	9.8	1.34 ± 0.07	5	1/3.41	+0.72
γT36/δT38G γW55/δW57(S)β-MeTrp	117 ± 3	5.0	1.27 ± 0.03	5	1 / 1.70	+0.31
γL119/δL121F γW55/δW57Trp	86 ± 3	3.7	1.73 ± 0.09	3		
γL119/δL121F γW55/δW57(R)β-MeTrp	390 ± 10	17	1.36 ± 0.05	4	1.12	-0.066
γL119/δL121F γW55/δW57(S)β-MeTrp	142 ± 5	6.1	1.17 ± 0.04	4	1.63	-0.29

Table 3.7. TrpD (γ W55/ δ W57) double mutants of the muscle-type nAChR. EC₅₀ and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

^aData reproduced from Table 3.4

^bExpression of the wild type receptor with the natural amino acid incorporated by nonsense suppression

While the TrpD mutation to (S)- β -MeTrp gave a wild type EC₅₀, a modest steric clash is predicted with the γ L119/ δ L121 side chain. We wondered if mutation to a larger Phe side chain at this site might induce a functionally significant clash. The (S)- β -MeTrp/Phe double mutant cycle indeed yields a coupling (-0.29 kcal/mol), though modest, and the sign (negative $\Delta\Delta G$, $\Omega > 1$) is consistent with an engineered clash. Reassuringly, the (R)- β -MeTrp/Phe double mutant cycle has a negligible coupling energy. While a sensible result emerges from this series of mutations of TrpD with γ L119/ δ L121, the majority of these nAChR double mutants probing sterics (Tables 3.6 and 3.7) give unexpected results. Overall, the lack of predictability suggests that either our model of the active receptor's binding site is incorrect, or that the mutations designed to simply alter sterics have unforeseen consequences.

3.4 Conclusions

The experiments described in this chapter use mutagenesis to probe side chain conformation in receptor binding sites, and changes to side chain conformation upon receptor activation. The D2 and M2 GPCR results are most consistent with a model in which the W6.48 side chain does not change its conformation upon activation, and suggest different χ^2 rotamers for W6.48 in the D2 and M2 receptors, consistent with crystallography. However, no definitive conclusions emerge. The nAChR results are largely inconclusive. Thus, even very subtle mutations like those considered here can produce ambiguous results when used to probe the complex motions associated with integral membrane receptors.

To be able to interpret the effect of a mutation on a conformational change, there are two requirements. First, a fairly precise description of the protein's inactive and active conformations is necessary (if indeed a two-state model adequately describes the system). From the current limited body of structural data and of proposed activation mechanisms for GPCRs and nAChRs, this requirement is a significant challenge.

Second, the energetic consequence of the mutation on each state must be accurately predicted. On this point, it is hard to know the true energetic consequence of a mutation in the actual receptor. Steric and electrostatic impacts of the local protein environment certainly complicate the simple conformational analysis applied to Thr/*a*Thr or Ile/*a*Ile or the more sophisticated χ^1 , χ^2 energy analysis applied to the β -methyl analogs in this chapter. Recently developed molecular dynamics approaches termed "metadynamics" allow the energy of an entire system to be determined as a function of a set of collective variables, such as χ^1 , χ^2 of a specific residue.⁴¹ This effectively allows for the construction of plots such as those in Figures 3.10 through 3.12 for a given side chain in its actual protein context. Such an approach has been used by Voth and coworkers to suggest χ^1 , χ^2 rearrangements of W6.48 in the A2A adenosine GPCR.¹⁵ If extended to unnatural amino acid analogs at W6.48 or other protein sites, this approach could aid the interpretation of experimental data to inform activation mechanisms.

3.5 Experimental

3.5.1 Molecular biology

cDNA for the mouse muscle nAChR $\alpha 1$, $\beta 1$, γ , and δ subunits was in the pAMV plasmid, the human D2 dopamine receptor (long isoform) was in the pGEMhe plasmid, the human M2 muscarinic acetylcholine receptor was in the pGEM3 plasmid, GIRK1 and GIRK4 were in pBSMXT plasmids, and RGS4 was in the pcDNA3.1 plasmid. Sitedirected mutagenesis was performed using the QuikChange protocol (Agilent Technologies). For nonsense suppression experiments, the site of interest was mutated to the amber stop codon, with the exception of muscle-type nAChR α V91, which was mutated to the opal stop codon. Circular DNA was linearized with the appropriate restriction enzyme (NotI for nAChR subunits, NheI or SbfI for the D2 receptor, HindIII for the M2 receptor, SalI for GIRK1 and GIRK4, and StuI for RGS4). After purification (Qiagen), linearized DNA was used as a template for runoff *in vitro* transcription using the T7 mMessage mMachine kit (Life Technologies). The amber suppressor tRNA THG73⁴² was used for nonsense suppression at all sites except α V91 in the muscle-type nAChR, for which the opal suppressor TQOpS^{,43,44} was used.

Amino acids were appended to the dinucleotide dCA and enzymatically ligated to the appropriate truncated 74mer suppressor tRNA as previously described.⁴⁵ Crude tRNA-amino acid product was used without desalting, and the product was confirmed by MALDI-TOF MS on a 3-hydropicolinic acid matrix. tRNA-amino acids bearing a 6nitroveratryloxycarbonyl protecting group were deprotected prior to injection *via* irradiation with a 500 W Hg/Xe arc lamp, filtered with WG-334 and UG-11 filters prior to injection.

3.5.2 Microinjection

Stage V–VI *Xenopus laevis* oocytes were harvested and injected with RNAs as described previously.⁴⁵ For the muscle-type nAChR, oocytes were injected with 10 - 20 ng mRNA in a 10:1:1:1 ratio of α 1: β 1: γ : δ for nonsense suppression in the α 1 subunit or in a 1:1:5:5 ratio for nonsense suppression in the γ and δ subunits, together with ~25 ng of the appropriate tRNA. Oocytes were incubated 18 - 24 hrs before recording.

For the M2 acetylcholine receptor, approximately 10 ng M2 receptor mRNA (2 ng for conventional mutagenesis experiments), 10 ng each of GIRK1 and GIRK4 mRNA,

and ~25 ng of the appropriate tRNA (for nonsense suppression) were injected 48 hrs prior to recording. An additional ~25 ng tRNA (for nonsense suppression) and 10 ng of RGS4 mRNA were injected 24 hrs prior to recording.

For the D2 dopamine receptor, receptor mRNA (4 - 25 ng for suppression experiments, 0.16 ng for wild type, and 1 ng for conventional mutagenesis), 10 ng each of GIRK1 and GIRK4, and ~25 ng tRNA (for nonsense suppression) were injected 48 hrs prior to recording. For low-expressing mutants generated by nonsense suppression, an additional ~25 ng receptor mRNA and ~25 ng tRNA were injected 24 hrs prior to recording.

As a negative control for all suppression experiments, unacylated full length tRNA was co-injected with mRNA in the same manner as charged tRNA. These control experiments yielded negligible responses for all sites studied.

3.5.3 Electrophysiology

Receptor function was assayed using the OpusXpress 6000A (Molecular Devices, Sunnyvale, CA) in two-electrode voltage clamp mode. The oocytes were clamped at a holding potential of -60 mV. For the muscle-type nAChR, acetylcholine doses in Ca²⁺free ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5) were applied for 15 s followed by a 116 s wash with Ca²⁺-free ND96. For the D2 dopamine receptor and M2 muscarinic acetylcholine receptor, cells were subjected to a ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.5) pre–wash for 10 s, a high K⁺ buffer (96 mM NaCl, 24 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 1.8 mM CaCl₂, pH 7.5) application for 50 s to establish basal currents, and agonist application in high K⁺ ringer for 25 s, followed by high K^+ and ND96 washings for 45 s and 90 s in duration, respectively. Agonist–induced currents were measured over the basal K^+ current as described previously.³⁹ Dopamine solutions in high K^+ buffer were prepared immediately before recording by dilutions from a 1 M stock in water. The EC₅₀ and Hill coefficient (n_H) values for each condition were obtained by fitting the averaged, normalized dose-response data to the Hill equation.

3.5.4 Energy calculations

Using the Spartan '08 software package, molecular mechanics (MMFF) energies were calculated for the amino acids (R)-β-MeTrp, (S)-β-MeTrp, (R)-β-MePhe, and (S)-β-MePhe capped in the form: Ac-Xaa-NH-Me (where Xaa is the β -methyl amino acid). Energies were determined at all angles of χ^1 and χ^2 , in 10° increments. For Phe and β -MePhe, the backbone dihedrals were constrained to $\varphi = -86^\circ$, $\psi = 120^\circ$ (β -sheet values observed for TrpD in the nicotine-bound *Ls*-AChBP crystal structure).³⁷ For Trp and β-MeTrp, the backbone dihedrals were constrained to either $\varphi = -86^\circ$, $\psi = 120^\circ$ (β -sheet) or to $\varphi = -48^\circ$, $\psi = -57^\circ$ (idealized α -helix). For all calculations, the backbone amide dihedrals were constrained to 180°. The resulting energies, relative to the global minimum for each condition, are plotted *versus* χ^1 and χ^2 in Figures 3.10 through 3.12. A cubic interpolation surface fit of each data set was constructed using MATLAB, from which energy values for a specific χ^1 , χ^2 were extracted. These values are shown in the tables in Figures 3.13 through 3.16,. The "methyl effect" plots (Figures 3.13 through 3.16) are generated by subtracting the relative energies for the parent amino acid (Trp or Phe) from the relative energies of the β -methyl analog.

3.5.5 Synthesis

Synthesis of Boc-5-aminomethyl indole (1). 5-aminomethyl indole (Aldrich, 500 mg, 3.42 mmol, 1 eq) was dissolved in a solution of H₂O (20 mL) and THF (20 mL). Boc₂O (Fluka, 0.864 mL, 3.76 mmol, 1.1 eq) was added with stirring, followed by NaOH (3.76 mL of a 1 M aqueous solution, 3.76 mmol, 1.1 eq), and stirred for 12 hrs. The THF was removed *in vacuo* and the aqueous layer extracted with dichloromethane (3x), washed with brine, dried over MgSO₄, and concentrated. The resulting crude product was purified by flash chromatography (2.5:1 hexanes/ethyl acetate). Compound **1** (786 mg, 93% yield) was recovered as a clear, colorless tar. ¹H NMR (300 MHz, CDCl₃) δ 8.51 (s, 1H), 7.55 (s, 1H), 7.33 (d, *J* = 8.3 Hz, 1H), 7.16 (m, 1H), 7.12 (d, *J* = 8.3 Hz, 1H), 6.51 (ddd, *J* = 3.0, 2.0, 0.9 Hz, 1H), 4.90 (s, 1H), 4.42 (d, *J* = 5.5 Hz, 2H), 1.50 (s, 9H).

Synthesis of hydroxyimine (2). Ethyl 3-bromo-2-hydroxyiminopropanoate³³ (298 mg, 1.42 mmol, 1 eq) was dissolved in 20 mL dichloromethane. Boc-5-aminomethyl indole **1** (700 mg, 2.84 mmol, 2 eq) followed by anhydrous Na₂CO₃ (226 mg, 2.13 mmol, 1.5 eq) were added, and the reaction suspension was stirred for 20 hrs under argon. Dichloromethane (25 mL) and H₂O (25 mL) were added, and the layers were partitioned. The aqueous layer was extracted (3x) with dichloromethane, the combined organics were washed with brine, dried over MgSO₄, and concentrated. The crude product was purified by flash chromatography (1.25:1 to 1:1.1 hexanes/ethyl acetate) to yield compound **2** (339 mg, 64% yield). ¹H NMR (300 MHz, CDCl₃) δ 10.34 (s, 1H), 8.32 (s, 1H), 7.64 (s, 1H), 7.22 (d, *J* = 8.2 Hz, 1H), 7.09 (s, 1H), 7.06 (s, 1H), 4.91 (s, 1H), 4.37 (d, *J* = 4.8 Hz, 2H), 4.20 (q, *J* = 7.2 Hz, 2H), 4.05 (s, 2H), 1.47 (s, 9H), 1.23 (t, *J* = 7 Hz, 3H).

Synthesis of Boc-5-aminomethyl-Trp ethyl ester (3). Hydroximine 2 (300 mg, 0.80 mmol) was dissolved in a solution of THF (27 mL) and H₂O (3 mL). Aluminum pellets (2 g) were treated with 10% NaOH (50 mL) for 3 min, washed (3x) with water, treated with 2% Hg₂Cl₂ (50 mL) for 5 min, washed (3x) with water, and dropped into the solution of hydroximine 2. After 16 hrs stirring, the reaction was filtered through a short plug of silica, washing with ethyl acetate, then 5:2 ethyl acetate/methanol, and the combined organics were concentrated. The crude product (327 mg recovered) was carried on to the next step without further purification.

Synthesis of 5-aminomethyl-Trp ethyl ester (4). Crude amine 3 (100 mg, ~0.28 mmol) was dissolved in dichloromethane (1 mL). Trifluoroacetic acid was added (1 mL) and the reaction was stirred for 1 hr under argon, after which the reaction solution was concentrated *in vacuo*. The crude product (75 mg recovered) was carried on to the next step without further purification.

Synthesis of (NVOC)₂-5-aminomethyl-Trp ethyl ester (5). Crude diamine 4 (75 mg, \sim 0.29 mmol, 1 eq) was dissolved in H₂O (6 mL). 6-Nitroveratryloxycarbonyl (NVOC) chloride (158 mg, 0.574 mmol, 3 eq) dissolved in dioxane (6 mL) was added, and the reaction solution was stirred for 4 hrs. An additional portion of 6-nitroveratryloxycarbonyl (NVOC) chloride (39 mg, 0.191 mmol, 0.5 eq) dissolved in dioxane (1 mL) was added and the reaction solution was stirred for an additional 4 hrs. The reaction solution was poured into 75 mL H₂O, acidified to pH = 3 with 0.2 M aqueous HCl, and extracted (3x) with ethyl acetate. The combined organics were washed with brine, dried over Mg₂SO₄, and concentrated. The crude product was purified by flash chromatography (2.5:1 ethyl acetate/hexanes, followed by 100% ethyl acetate) to

yield compound **5** (48 mg, 20% yield over three steps from hydroximine **2**). ¹H NMR (500 MHz, DMSO-d₆) δ 10.84 (s, 1H), 8.01 (d, *J* = 7.8 Hz, 1H), 7.97 (t, *J* = 6.0 Hz, 1H), 7.69 (s, 2H), 7.42 (s, 1H), 7.29 (d, *J* = 8.3 Hz, 1H), 7.17 (s, 2H), 7.13 (s, 1H), 7.03 (dd, *J* = 8.3, 1.1 Hz, 1H), 5.37-5.26 (m, 4H), 4.29 (d, *J* = 5.1 Hz, 2H), 4.05 (dd, *J* = 7.0, 2.2 Hz, 1H), 3.86-3.74 (m, 14H), 3.14 (dd, *J* = 14.3, 5.2 Hz, 1H), 3.03 (dd, *J* = 14.5, 9.2 Hz, 1H), 1.09 (t, *J* = 7.1 Hz, 2H).

Synthesis of (NVOC)₂-5-aminomethyl-Trp (6). (NVOC)₂-5-aminomethyl-Trp ethyl ester **5** (43 mg, 0.058 mmol, 1 eq) was dissolved in a solution of H₂O (1 mL) and dioxane (5 mL). Aqueous 1M NaOH was added (0.5 mL) and the reaction was stirred for 20 hrs at room temperature. Additional 1M NaOH (1 mL) was added and the reaction was heated at 40°C for 1.5 hrs, after which the reaction was complete by TLC. The reaction solution was cooled to room temperature, diluted with H₂O, and aqueous KHSO₄ was added until the pH reached 2.5, causing the solution to turn cloudy. This suspension was extracted with dichloromethane (3x) and the combined organics were washed with brine, dried over Mg₂SO₄, and concentrated, to yield the crude product (42 mg recovered), which was carried on to the next step without further purification. LRMS (ESI-) calculated for [C₃₂H₃₂N₅O₁₄]⁻ ([M-H]⁻) 710.2, found 710.0.

Synthesis of (NVOC)₂-5-aminomethyl-Trp cyanomethyl ester (7). Crude (NVOC)₂-5aminomethyl-Trp 6 (42 mg, ~0.058 mmol, 1 eq) was dissolved in a solution of DMF (1 mL) and chloroacetonitrile (1 mL, 16 mmol, 270 eq). Triethylamine (24 μ L, 0.17 mmol, 3 eq) was added and the reaction was stirred at room temperature for 6 hrs. The reaction solution was concentrated *in vacuo* and the crude product was dissolved in dichloromethane. This solution was washed with 0.2 M aqueous HCl, 1 M aqueous NaHCO₃, and brine, dried over MgSO₄, and concentrated. The crude product was recrystallized by dissolving in a minimal volume (~1 mL) dichloromethane, diluting with hexanes to precipitate the product, and cooling this suspension to 0°C. The precipitate was recovered by filtration, washing with cold hexanes, to yield compound **7** (21 mg, 47% yield over two steps). ¹H NMR (300 MHz, DMSO-d₆) δ 10.90 (s, 1H), 8.21 (d, *J* = 7.5 Hz, 1H), 7.97 (t, *J* = 5.8 Hz, 1H), 7.69 (s, 2H), 7.43 (s, 1H), 7.30 (d, *J* = 8.3 Hz, 1H), 7.19 (d, *J* = 1.9 Hz, 1H), 7.17 (s, 1H), 7.11 (s, 1H), 7.04 (d, *J* = 8.3 Hz, 1H), 5.40-5.27 (m, 4H), 4.99 (s, 2H), 4.42 (dd, *J* = 14.1, 7.9 Hz, 1H), 4.29 (d, *J* = 5.7 Hz, 2H), 3.86-3.79 (m, 12H), 3.23-3.05 (m, 2H). ¹³C NMR (126 MHz, DMSO-d₆) δ 171.26, 155.78, 155.56, 153.40, 153.33, 147.67, 147.61, 139.15, 139.04, 135.36, 129.67, 128.07, 127.69, 126.75, 124.40, 121.13, 116.70, 115.66, 112.07, 111.42, 110.33, 110.13, 109.53, 108.81, 108.12, 62.67, 62.35, 56.13, 56.06, 54.60, 49.46, 44.62, 26.64.

Synthesis of (NVOC)₂-5-aminomethyl-Trp-dCA (8). dCA•2.2 TBA (10 mg, 8.6 μ mol, 1 eq) and (NVOC)₂-5-aminomethyl-Trp cyanomethyl ester 7 (10 mg, 13 μ mol, 1.5 eq) were dissolved in DMF (0.2 mL) and stirred at room temperature for 22 hrs under argon, then stirred at 50°C for 1 hr. The reaction was purified by HPLC to yield compound **8** (347 μ g, 3% yield). MALDI-MS calculated for [C₅₁H₅₈N₁₃O₂₆P₂]⁺ ([M+H]⁺) 1330.31, found 1330.54.

NVOC-(2S,3S)-β-methylPhe cyanomethyl ester (9). Prepared from (2S,3S)-βmethylPhe (Chem-Impex) by standard methods as for compounds **22** and **23**. ¹H NMR (300 MHz, CDCl₃) δ 7.70 (s, 1H), 7.37-7.17 (m, 5H), 6.88 (s, 1H), 5.50 (dd, *J* = 34.8, 15.0 Hz, 2H), 5.12 (d, *J* = 8.8 Hz, 1H), 4.85-4.62 (m, 3H), 3.94 (s, 3H), 3.92 (s, 3H), 3.42 (m, 1H), 1.43 (d, *J* = 7.1 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 170.42, 155.76, 153.72, 148.22, 139.70, 129.19, 127.97, 127.83, 127.52, 113.81, 109.79, 108.23, 64.23, 59.17, 56.52, 48.94, 41.75, 17.88.

NVOC-(2S,3S)-\beta-methylPhe-dCA (10) Prepared from NVOC-(2S,3S)- β -methylPhe cyanomethyl ester (9) by standard methods as for compound **24**. MALDI-MS calculated for $[C_{39}H_{47}N_{10}O_{20}P_2]^+$ ($[M+H]^+$) 1037.24, found 1037.45.

NVOC-(2S,3R)-β-methylPhe cyanomethyl ester (11). Prepared from (2S,3R)-βmethylPhe (generous gift from Victor Hruby, University of Arizona) by standard methods as for compounds **22** and **23**. ¹H NMR (300 MHz, CDCl₃) δ 7.70 (s, 1H), 7.35-7.19 (m, 5H), 6.93 (s, 1H), 5.50 (dd, J = 21, 15 Hz, 2H), 5.33 (d, J = 8.7 Hz, 1H), 4.64-4.56 (m, 3H), 3.95 (s, 6H), 3.32 (m, 1H), 1.43 (d, J = 7.2 Hz, 3H).

NVOC-(2S,3R)-\beta-methylPhe-dCA (12). Prepared from NVOC-(2S,3R)- β -methylPhe cyanomethyl ester (11) by standard methods as for compound 24. MALDI-MS calculated for $[C_{39}H_{47}N_{10}O_{20}P_2]^+$ ($[M+H]^+$) 1037.24, found 1037.39.

NVOC-(2S,3S)-β-methylTrp cyanomethyl ester (13). Prepared from (2S,3S)-βmethylTrp (generous gift from Victor Hruby, University of Arizona) by standard methods as for compounds **22** and **23**. ¹H NMR (300 MHz, CDCl₃) δ 8.17 (s, 1H), 7.70 (s, 1H), 7.62 (d, J = 7.9 Hz, 1H), 7.38 (d, J = 8.1 Hz, 1H), 7.22 (t, J = 7.6 Hz, 1H), 7.14 (t, J = 7.5Hz, 1H), 7.09 (d, J = 2.5 Hz, 1H), 6.91 (s, 1H), 5.52 (dd, J = 15, 15 Hz, 2H), 5.35 (d, J =8.7 Hz, 1H), 4.79-4.49 (m, 3H), 3.94 (s, 3H), 3.89 (s, 3H), 3.82 (m, 1H), 1.54 (d, J = 7.2Hz, 4H). ¹³C NMR (CDCl₃) δ 170.58, 155.90, 153.77, 148.31, 139.82, 136.56, 127.85, 126.41, 122.82, 122.13, 120.19, 118.94, 114.84, 113.93, 111.78, 110.08, 108.34, 64.30, 58.96, 56.60, 56.56, 48.95, 34.18, 18.10. **NVOC-(2S,3S)-\beta-methylTrp-dCA (14).** Prepared from NVOC-(2S,3S)- β -methylTrp cyanomethyl ester (11) by standard methods as for compound 24. MALDI-MS calculated for $[C_{41}H_{48}N_{11}O_{20}P_2]^+$ ($[M+H]^+$) 1076.25, found 1076.29.

Boc-indoleacrylic acid (15). Trans-3-indoleacrylic acid (2 g, 10.7 mmol, 1 eq) was added to a solution of acetonitrile (20 mL) and water (1.5 mL) in a 100 mL round bottom flask. Triethylamine (1.57 mL, 11.2 mmol, 1.05 eq) was added and all solids dissolved after several minutes of stirring. 4-Dimethylaminopyridine (131 mg, 1.07 mmol, 0.1 eq) was added, followed by Boc_2O (2.58 mL, 11.2 mmol, 1.05 eq), added dropwise over ~2 min, and the reaction solution was stirred at room temperature overnight. Additional Boc₂O (2.46 mL, 10.7 mmol, 1 eq) was added and the reaction was stirred at 45°C for 2 hrs, after which the reaction was complete by TLC. The reaction solution was diluted with 60 mL Et₂O and washed with 1M aqueous KHSO₄ (5x). The organic layer was extracted with 5% aqueous NaHCO₃ (5x) and the combined aqueous NaHCO₃ layers were acidified with aqueous HCl to pH 1.5, then extracted with $Et_2O(5x)$ and these combined ether layers were washed with brine, dried over Na₂SO₄, and concentrated. The resulting solid was purified by flash chromatography (5:1 to 1:1 dichloromethane/ethyl acetate), to give 2.15 g (\sim 70% yield) of crude product, which was carried on to the next step without further purification. ¹H NMR (300 MHz, CDCl₃) δ 8.21 (d, J = 7.9 Hz, 1H), 7.97-7.85 (m, 3H), 7.38 (m, 2H), 6.56 (d, J = 16.1 Hz, 1H), 1.70 (s, 9H).

Boc-indoleacrylic acid-oxazolidinone (16). Crude Boc-indoleacrylic acid **15** (1.5 g, ~5.2 mmol, 1.3 eq) was dissolved in dry THF (20.2 mL) in a 250 mL oven-dried Schlenk flask under argon. Triethylamine (1.41 mL, 10 mmol, 2.5 eq) was added, and the

reaction solution was cooled to ~-10°C. Trimethylacetyl chloride (0.595 mL, 4.8 mmol, 1.2 eq) was added dropwise, upon which a yellow precipitate formed, and the reaction suspension was stirred at -10°C for 1 hr. Dry LiBr (0.384 g, 4.4 mmol, 1.1 eq) was added, followed by (S)-4-phenyl-2-oxazolidinone (0.656 g, 4.0 mmol, 1.0 eq), the reaction was warmed to room temperature, and stirred at room temperature for 4 hrs. The reaction was quenched by addition of 0.2 M aqueous HCl (40 mL, 2 eq), and the THF was removed *in vacuo*, ethyl acetate was added, and this organic layer was washed with 0.2 M aqueous HCl, then 1M aqueous NaHCO₃ (2x), then brine, dried over Na₂SO₄, and concentrated. The crude product was purified by flash chromatography (4:1 hexanes/ethyl acetate) to yield compound **16** (1.51 g, 47% yield over 2 steps from *trans*-3-indoleacrylic acid). ¹H NMR (300 MHz, CDCl₃) δ 8.19 (dd, *J* = 5.9, 3.3 Hz, 1H), 8.08 (d, *J* = 15.8 Hz, 1H), 7.99-7.89 (m, 3H), 7.43-7.33 (m, 7H), 5.59 (dd, *J* = 8.7, 3.8 Hz, 1H), 4.33 (dd, *J* = 8.8, 3.8 Hz, 1H), 1.68 (s, 9H).

(3R)-Boc-β-methylindolepropanoic acid-oxazolidinone (17). A solution of methylmagnesium bromide (3M in Et₂O, 1.75 mL, 5.24 mmol, 1.5 eq) in dry THF (18 mL) and dimethyl sulfide (5.4 mL) in an oven-dried Schlenk flask was subjected to three freeze-pump-thaw cycles, placed under an atmosphere of argon, and cooled to 0°C. CuBr•DMS (1.08g, 5.24 mmol, 1.5 eq) was added all at once and the solution was stirred for 30 min at 0°C. A solution of Boc-indoleacrylic acid-oxazolidinone (16) in THF (10 mL), which had also been subjected to three freeze-pump-thaw cycles, was added via cannula over 5 min. The reaction solution was stirred at 0°C for 2 hrs, then at room temperature for 45 min, quenched by the addition of 30 mL saturated aqueous NH₄Cl, and stirred at room temperature for 30 min. The layers were partitioned and the organic

layer was washed with saturated aqueous NH₄Cl (4x), then brine (2x), then dried over Mg₂SO₄ and concentrated. The major diastereomer was isolated from the crude product by flash chromatography (5:1 hexanes/ethyl acetate) to yield compound **17** (1.12 g, 72% yield). ¹H NMR (300 MHz, CDCl₃) δ 8.12 (d, *J* = 7.3 Hz, 1H), 7.60 (d, *J* = 7.3 Hz, 1H), 7.36-7.21 (m, 8H), 5.36 (dd, *J* = 8.7, 3.7 Hz, 1H), 4.58 (t, *J* = 8.8 Hz, 1H), 4.22 (dd, *J* = 8.9, 3.8 Hz, 1H), 3.61 (dd, *J* = 13.9, 7.0 Hz, 1H), 3.47 (dd, *J* = 16.5, 7.1 Hz, 1H), 3.25 (dd, *J* = 16.5, 7.2 Hz, 1H), 1.66 (s, 9H), 1.33 (d, *J* = 6.9 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 171.51, 153.84, 139.05, 129.68, 129.23, 128.78, 125.94, 125.04, 124.42, 122.47, 121.80, 119.39, 115.44, 83.57, 70.05, 57.77, 42.24, 28.36, 26.97, 21.11.

(2S, 3R)-Boc- β -methyl- α -azido-indolepropanoic acid-oxazolidinone (18). (3R)-Boc- β -methylindolepropanoic acid-oxazolidinone 17 (600 mg, 1.34 mmol, 1 eq) was dissolved in THF (7 mL) under argon in a 50 mL oven-dried Schlenk flask. A solution of 0.5 M KHMDS in toluene (2.94 mL, 1.47 mmol, 1.1 eq) was added to THF (5 mL) in a 100 mL oven-dried Schlenk flask under argon. Both flasks were cooled to -78°C and the solution of compound 17 was transferred to the KHMDS solution by cannula. This solution was stirred at -78°C for 30 min. Trisyl azide^{46,47} was dissolved in THF (5 mL) in a 100 mL oven-dried Schlenk flask under argon and cooled to -78°C. The compound 17/KHMDS solution was transferred to this flask via cannula transfer and the solution was stirred at -78°C for 5 min. The reaction was quenched by addition of glacial acetic acid (0.368 mL, 6.44 mmol, 4.8 eq), and the solution was diluted with Et₂O and 1M aqueous sodium chloride and mixed. The aqueous phase was extracted with 1:1 Et₂O/THF (3x) and the combined organics washed with 1M NaHCO₃ (2x) then brine

(2x), dried over MgSO₄, and concentrated. The crude product was purified by flash chromatography (5:1 hexanes/ethyl acetate) to yield compound **18** (455 mg, 69% yield). ¹H NMR (300 MHz, CDCl₃) δ 8.18 (d, *J* = 8.1 Hz, 1H), 7.65 (d, *J* = 7.4 Hz, 1H), 7.53 (s, 1H), 7.36-7.26 (m, 5H), 7.18 (dd, *J* = 7.6, 1.8 Hz, 2H), 5.36 (d, *J* = 9.4 Hz, 1H), 4.79 (dd, *J* = 8.3, 2.9 Hz, 1H), 4.02 (dd, *J* = 8.8, 3.0 Hz, 1H), 3.85 (t, *J* = 8.6 Hz, 1H), 3.64 (dq, *J* = 14.1, 7.0 Hz, 1H), 1.69 (s, 9H), 1.57 (d, *J* = 7.0 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 169.63, 153.02, 149.58, 138.19, 135.25, 129.34, 129.26, 128.97, 125.77, 124.89, 124.11, 122.76, 120.30, 119.40, 115.34, 84.13, 70.22, 63.64, 58.02, 32.84, 28.31, 17.98.

(2S,3R)-Boc-β-methyl-α-azido-indolepropanoic acid (19). (2S,3R)-Boc-β-methyl-αazido-indolepropanoic acid-oxazolidinone 18 (400 mg, 0.817 mmol, 1 eq) was dissolved in THF (10 mL) and H₂O (10 mL). This solution was cooled to 0°C and hydrogen peroxide (30% aqueous solution, 0.508 mL) was added dropwise over 1 min with stirring, then LiOH \bullet H₂O (68 mg, 1.63 mmol, 2 eq) was added all at once, and the reaction solution was stirred at 0°C for 30 min, after which the reaction was complete by TLC. NaHSO₃ (512 mg in 0.6 mL H₂O, 4.92 mmol, 3 eq) was added at 0°C, followed by saturated aqueous NaHCO₃ (10 mL), and the mixture was stirred at room temperature for 30 min. THF was removed in vacuo, Et₂O (15 mL) was added and the layers were partitioned after mixing. The organic layer was extracted with saturated aqueous NaHCO₃ (3x) and the combined aqueous layers were acidified to pH = 1.5 with 1M HCl. The resulting suspension was extracted with ethyl acetate (4x) and these combined ethyl acetate layers were washed with $H_2O(2x)$ and brine (2x), dried over MgSO₄, and concentrated to yield crude product 1. The original Et₂O organic layer was extracted again with saturated aqueous NaHCO₃ (3x), acidified to pH = 1, and this suspension was

extracted with ethyl acetate (4x). The combined organic layers were washed with brine (2x), dried over MgSO₄, and concentrated to yield crude product 2. Crude product 1 was partially purified by flash chromatography (1:1 ethyl acetate/hexanes, then 100% ethyl acetate, then 5% methanol and 1% acetic acid in ethyl acetate) to yield compound **19** (92 mg, 33% yield). ¹H NMR (300 MHz, CDCl₃) δ 9.62 (s, 1H), 8.16 (d, *J* = 8.0 Hz, 1H), 7.60 (d, *J* = 7.6 Hz, 1H), 7.54 (s, 1H), 7.31 (dt, *J* = 23.6, 7.0 Hz, 2H), 4.41 (d, *J* = 4.3 Hz, 1H), 3.79-3.71 (m, 1H), 1.69 (s, 9H), 1.44 (d, *J* = 7.0 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 175.38, 149.84, 135.58, 129.21, 124.85, 123.65, 122.81, 121.07, 118.70, 115.70, 84.12, 66.28, 33.07, 28.34, 14.87. IR (NaCl) 2978 (m), 2928 (m), 2109 (s), 1733 (s), 1370 (s), 1157 (s) cm⁻¹.

(2S,3R)-Boc-β-methyltryptophan (20). (2S,3R)-Boc-β-methyl-α-azido-indolepropanoic acid 19 (81 mg, 0.235 mmol, 1 eq) was dissolved in THF (2 mL), trimethylphosphine (1M in THF, 0.47 mL, 0.47 mmol, 2 eq) was added, and the reaction solution was stirred at room temperature under argon for 1 hr. H₂O was added (0.1 mL, 5.6 mmol, 24 eq) and this solution was stirred at room temperature for 14 hrs, after which it was concentrated *in vacuo*. The resulting solid was partially purified by flash chromatography (1% acetic acid in ethyl acetate to 5% acetic acid in 1:1 ethyl acetate/methanol) to give 18 mg (~24% yield) of crude product, which was carried on to the next step without further purification. ¹H NMR (300 MHz, CD₃OD) δ 8.14 (d, *J* = 8.1 Hz, 1H), 7.81 (d, *J* = 7.1 Hz, 3H). ¹³C NMR (126 MHz, CD₃OD) δ 178.44, 150.99, 130.48, 125.89, 124.69, 123.89, 121.97, 120.16, 116.39, 85.05, 32.49, 28.40, 22.81, 13.03. HRMS (APCI/ESI+) calculated for $[C_{17}H_{23}N_2O_4]^+$ ([M+H]⁺) 319.1658, found 319.1663. (2S,3R)-β-methyltryptophan (21). Crude (2S,3R)-Boc-β-methyltryptophan 20 (18 mg, ~0.058 mmol) was suspended in H₂O and heated to 170°C for 3 min by microwave irradiation. The resulting solution was lyophilized to give 15.2 mg crude compound 21, which was carried on to the next step without further purification. ¹H NMR (300 MHz, CD₃OD) δ 7.74 (d, J = 7.8 Hz, 1H), 7.37 (d, J = 7.9 Hz, 1H), 7.18 (s, 1H), 7.13 (t, J = 7.5 Hz, 1H), 7.05 (t, J = 7.5 Hz, 1H), 4.01-3.95 (m, 2H), 1.39 (d, J = 6.6 Hz, 3H). HRMS (APCI/ESI+) calculated for [C₁₂H₁₅N₂O₂]⁺ ([M+H]⁺) 219.1128, found 219.1148.

NVOC-(2S,3R)-β-methyltryptophan (22). Crude (2S,3R)-β-methyltryptophan **21** (15.2 mg, ~0.07 mmol, 1 eq) and Na₂CO₃ (36.9 mg, 0.348 mmol, 5 eq) were dissolved in a solution of water (1 mL) and dioxane (1 mL). This solution was cooled to 0°C, 6-nitroveratryloxycarbonyl (NVOC) chloride (19.3 mg, 0.07 mmol, 1 eq) was added, and the reaction solution was stirred at room temperature for 3 hrs. The reaction solution was poured into H₂O (15 mL) and washed with Et₂O (3x). The aqueous layer was acidified to pH = 1.5 with 6M HCl, then extracted (4x) with Et₂O. These combined Et₂O layers were dried over MgSO₄ and concentrated. The resulting solid was purified by flash chromatography (1% acetic acid in 1.5:1 dichloromethane/ethyl acetate) to give 12.7 mg crude compound **22**, which was carried on to the next step without further purification. HRMS (APCI/ESI-) calculated for [C₂₂H₂₄N₃O₈]⁻ ([M-H]⁻) 458.1558, found 458.1616.

NVOC-(2S,3R)-\beta-methyltryptophan cyanomethyl ester (23). Crude NVOC-(2S,3R)- β -methyltryptophan **22** (12.7 mg, ~ 0.028 mmol, 1 eq) was dissolved in DMF (0.2 mL) and chloroacetonitrile (0.2 mL, 3.16 mmol, 113 eq) under argon. Triethylamine (11.6 μ L, 0.083 mmol, 3 eq) was added and the reaction was stirred at room temperature for 2 hrs, then concentrated *in vacuo*. The resulting solid was partially purified by flash

chromatography (15:1 to 2.5:1 dichloromethane/ethyl acetate), and further purified by additional flash chromatography (1:10 to 1:2 ethyl acetate/hexanes), to yield compound **23** (6.3 mg, 5% yield over 4 steps from compound **19**). ¹H NMR (300 MHz, CDCl₃) δ 8.17 (s, 1H), 7.70 (s, 1H), 7.60 (d, J = 7.8 Hz, 1H), 7.38 (d, J = 8.0 Hz, 1H), 7.22 (t, J = 7.2 Hz, 1H), 7.13 (t, J = 7.4 Hz, 1H), 7.08 (d, J = 2.4 Hz, 1H), 6.91 (s, 1H), 5.56-5.38 (m, 3H), 4.75 (dd, J = 8.7, 6.1 Hz, 1H), 4.56 (q, J = 15.6 Hz, 2H), 3.95 (s, 3H), 3.90 (s, 3H), 3.72-3.63 (m, 1H), 1.52 (d, J = 7.2 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 170.61, 155.58, 153.76, 148.31, 139.83, 136.37, 127.83, 126.55, 122.71, 122.03, 120.05, 118.66, 115.34, 113.88, 111.70, 110.13, 108.32, 64.23, 59.24, 56.60, 56.56, 48.75, 34.04, 17.18. HRMS (APCI/ESI+) calculated for [C₂₄H₂₅N₄O₈]⁺ ([M+H]⁺) 497.1667, found 497.1677.

NVOC-(2S,3R)-\beta-methylTrp-dCA (24). dCA•2.2 TBA (10 mg, 8.6 µmol, 1 eq) and NVOC-(2S,3R)- β -methyltryptophan cyanomethyl ester **23** were dissolved in DMF (0.135 mL) and stirred at room temperature for 40 hrs under argon. The reaction was purified by HPLC to yield compound **24** (283 µg, 3% yield). MALDI-MS calculated for $[C_{41}H_{48}N_{11}O_{20}P_2]^+$ ([M+H]⁺) 1076.25, found 1076.65.

NVOC-*tert*-leucine cyanomethyl ester. *Tert*-leucine (Aldrich) was NVOC-protected and activated as a cyanomethyl ester by standard procedures. ¹H NMR (300 MHz, CDCl₃) δ 7.71 (s, 1H), 6.96 (s, 1H), 5.52 (d, *J* = 3.6 Hz, 2H), 5.41 (d, *J* = 9.4 Hz, 1H), 4.77 (dd, *J* = 53.3, 15.6 Hz, 2H), 4.25 (d, *J* = 9.4 Hz, 1H), 3.99 (s, 3H), 3.95 (s, 3H), 1.04 (s, 9H). ¹³C NMR (126 MHz, CDCl₃) δ 170.63, 155.74, 153.71, 148.33, 139.89, 127.74, 113.89, 110.12, 108.31, 64.27, 62.18, 56.61, 56.55, 48.61, 34.94, 26.60. NVOC-tert-leucine-dCA. Prepared from NVOC-tert-leucine cyanomethyl ester by

standard methods as for compound 24. MALDI-MS calculated for $[C_{35}H_{47}N_{10}O_{20}P_2]^+$

 $([M+H]^+)$ 989.24, found 989.68.

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