Supplemental Materials

for "**Mapping general anesthetic binding site(s) in human α1β3 γ-aminobutyric acid type A ($GABA_A$) receptors with [^{3}H]TDBzl-etomidate, a photoreactive etomidate analog**" by Chiara, DC, Dostalova, Z, Jayakar, SS, Zhou, X, Miller, KW, and Cohen, JB.

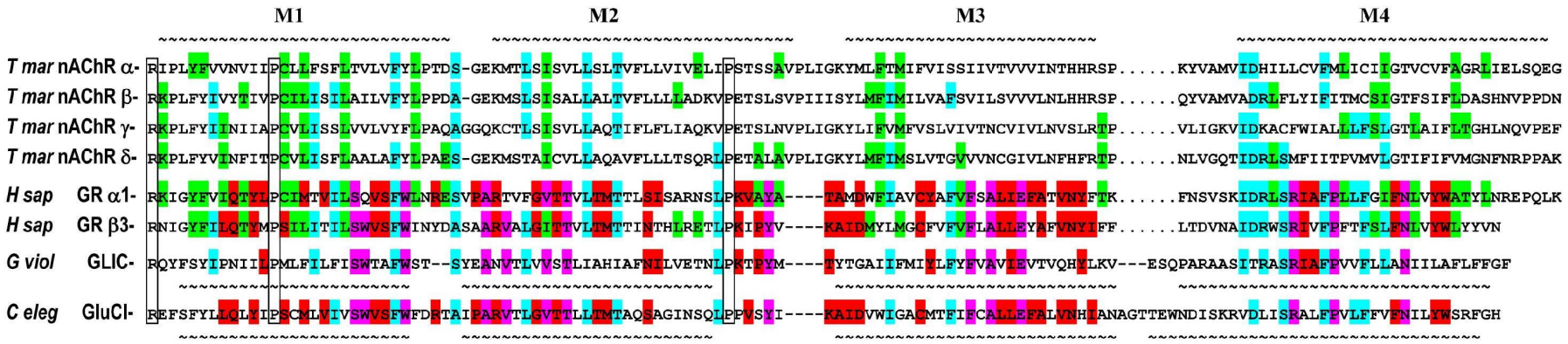
Methods

Docking anesthetics in $GABA_AR$ homology models. CDocker (Wu et al., (2003) *J. Comp. Chem. 24,* 1549-1562), a CHARMm-based molecular dynamics simulated annealing program, was used to dock drugs into potential binding pockets in the αβ $GABA_AR$ transmembrane domain (β3-α1 or β3-β3 interfaces, the α1 and β3 subunit helix bundles, and the ion channel) in the homology models based upon the GLIC and the GluCl structures. Because the side chains of $GABA_AR$ amino acids that are not conserved in the homology model templates (GLIC or GluCl) have random rotamers, the rotamers of those nonconserved amino acids are not identical at each of the β3-α1 interfaces despite the fact that the orientations of the helices are the same. The homology models were initially subjected to twenty cycles of energy minimization to ensure that no side chains were positioned in energetically unfavorable positions. Prior to minimization, *S*-TDBzl-etomidate was positioned in the GLIC-based model in each interface site at the level of β3Phe-289 in β3M3, while no drug was positioned in that site in the GluCl-based model, which was derived from a structure with ivermectin bound at that interface between subunits.

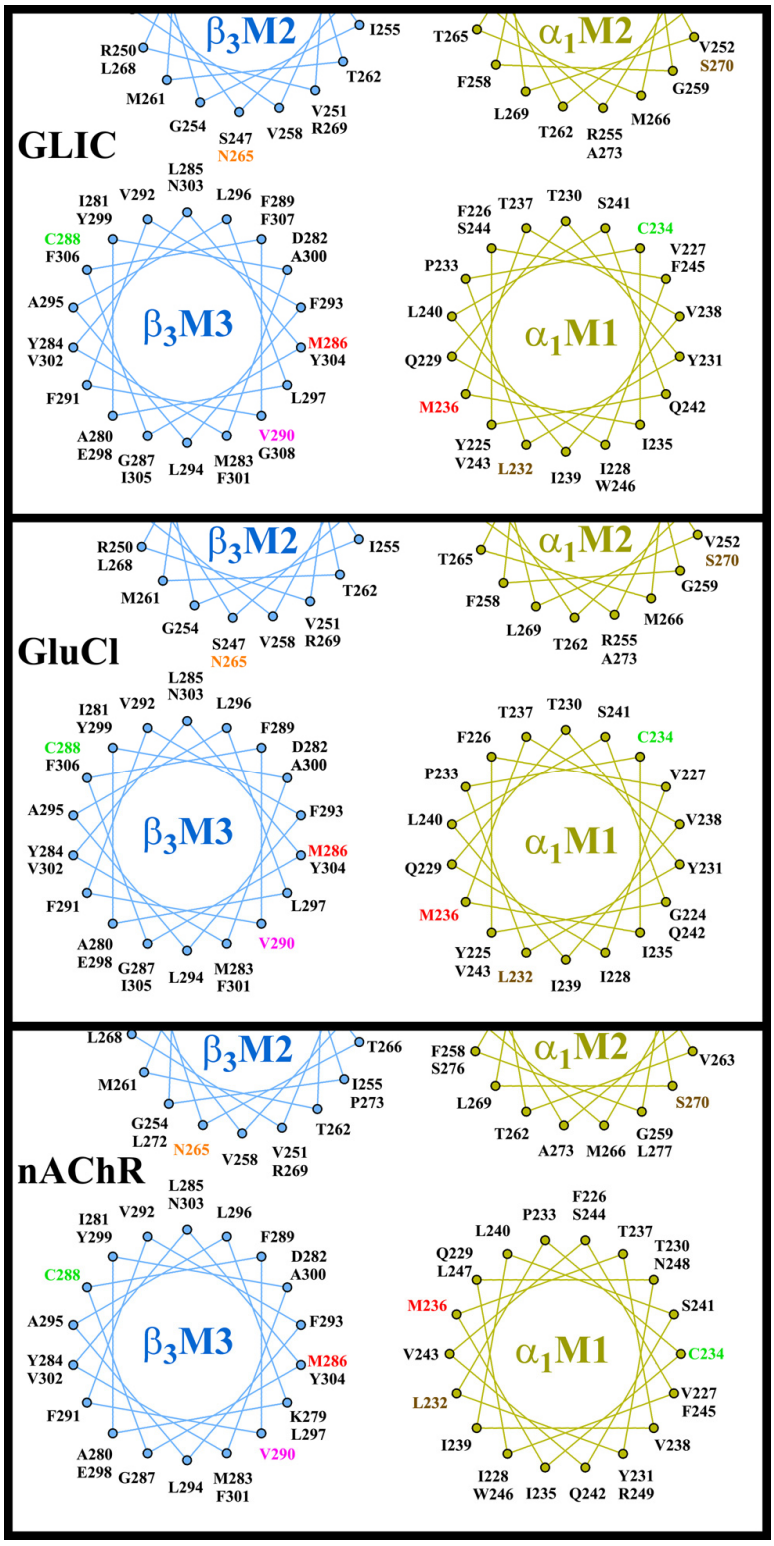
For the β3-α1 interface site in the GLIC-derived model, a 16 Å diameter binding site sphere was centered at the level of β3Phe-289. CDocker was configured to generate, using high temperature molecular dynamics, 50 random ligand conformations which were each docked in 30 random orientations and refined, using simulated annealing and a full potential final minimization step, to obtain energy minimized solutions for each drug. Solutions were ranked according to the calculated CDocker interaction energies, and the orientations of bound drugs were compared for the 100 lowest energy solutions. The

Connolly surfaces (Connolly (1983) *Science 221,* 709-713) defined by a 1.4 Å probe, of *R*-TDBzl-etomidate (volume, 325 $Å^3$) docked at the β3-α1 sites in the 100 lowest energy solutions define volumes of 717 and 810 $Å^3$ in the ββ-α and αβ-α sites, respectively, and for the 14 lowest energy solutions a volume of 511 $Å^3$ (Figures 8B-E). The amino acids contributing to the predicted TDBzl-etomidate binding site are shown in stereo representation in Supplemental Figures S4 and S5. A similar binding site for TDBzl-etomidate was obtained when the diameter of the binding site sphere was increased to 18 or 20 Å (not shown), or when TDBzl-etomidate was docked in the GluCl-derived model (Supplemental Figure S5).

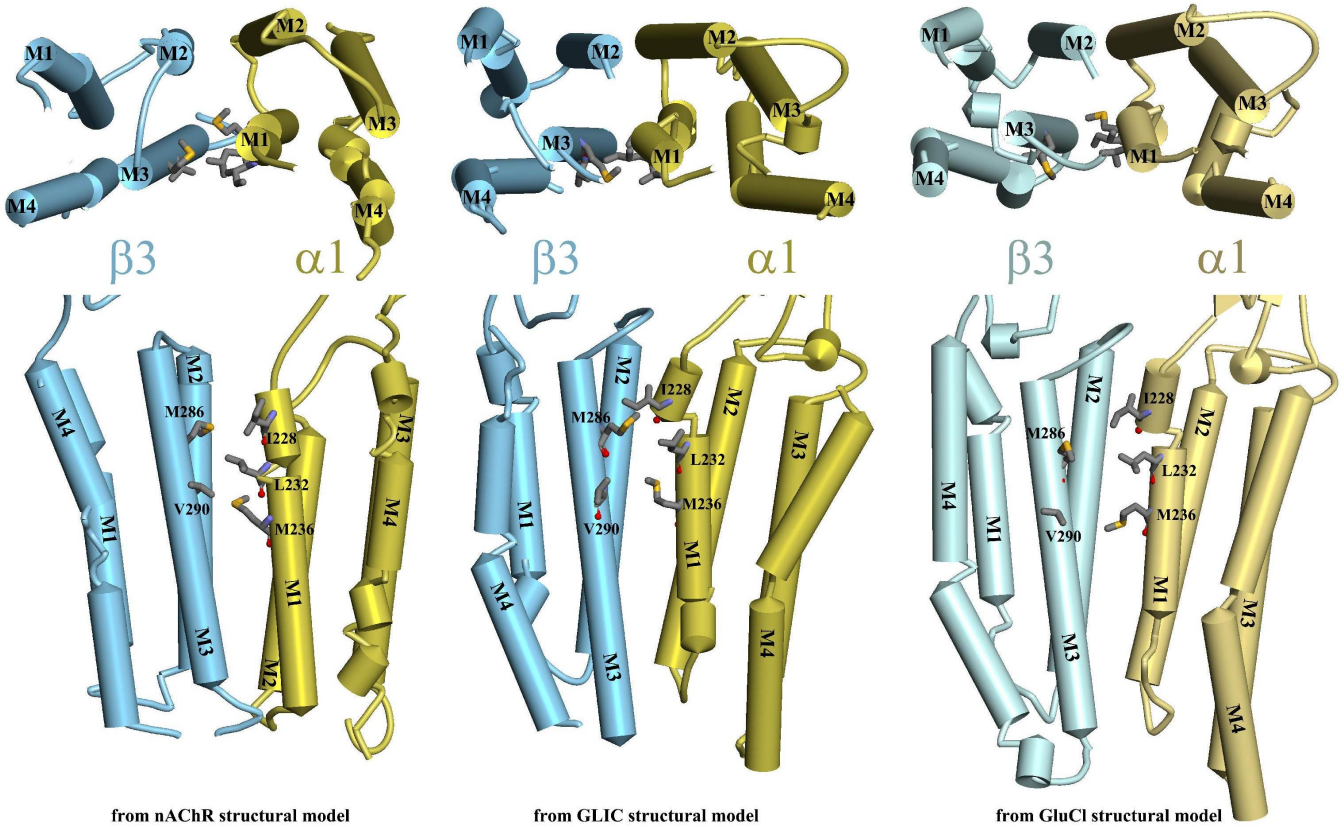
A similar protocol, using an 18 Å diameter sphere, was used to dock drugs at the β3-β3 interface (Supplemental Figure S6). TDBzl-etomidate was docked in the β3 and α1 subunit helix bundles, using binding site spheres (diameter, 20 Å) centered at the level of M2-13 (80 initial ligand conformations, each in 30 random orientations) (Supplemental Figure S7).



Supplemental Figure S1: The amino acid sequence alignments in receptor transmembrane domains used to create the $GABA_AR$ homology models from the structures of: 1) the *Torpedo marmorata* nAChR (PDB 2BG9) (homology model of Li et al (2006); 2) a *Gloebacter violaceus* ligand gated ion channel (GLIC) structure (PDB 3P50); and 3) a *Caenorhabditis elegans* glutamate-gated chloride channel (GluCl) structure (PDB 3RIF). Boxed residues are conserved in all sequences. Color coding: Magenta, identity between one or both $GABA_AR$ subunits and both GLIC & GluCl; Red, identity between one or both $GABA_AR$ subunits and GLIC or GluCl; Green, identity between one or more $GABA_AR$ subunits and one or more *Torpedo marmorata* nAChR subunits; Cyan, identity between one or more $GABA_AR$ subunits, GLIC and/or GluCl, and one or more nAChR subunits. The lines (~~) above (nAChR) or below (GLIC and GluCl) the alignments denote the extent of each transmembrane helix in the nAChR, GLIC, or GluCl molecular models. Comparisons between the GLIC and GluCl models in the transmembrane region show that the sequence homology translates into structure homology for M1, M2, and M3. Despite a strong sequence homology between GLIC and GluCl M4, the alignment does not translate structurally in the M4 helices as the conserved residues are displaced by about 1 helical turn.

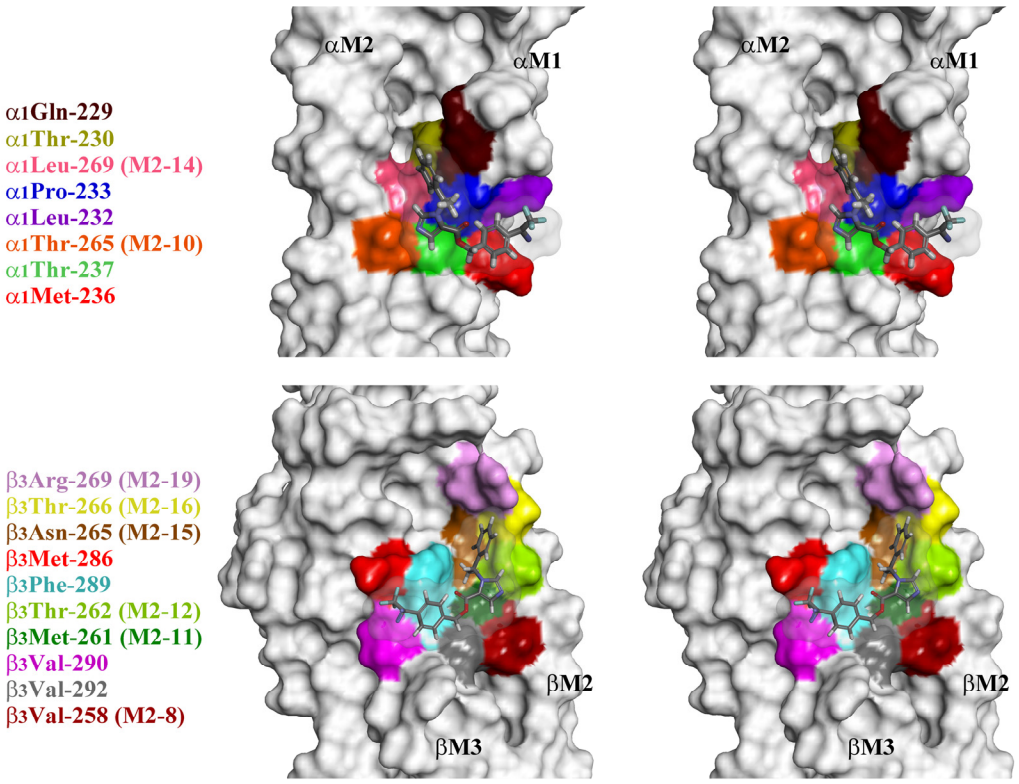


Supplemental Figure S2: Helical wheel representations of the transmembrane domain β3-α1 interface in the 3 $GABA_AR$ homology models, viewed from the base of the extracellular domain. Residues photolabeled by azietomidate (α1Met-236, β3Met-286) or TDBzl-etomidate (β3Val-290, α1Met-236, β3Met-286, βCys-288, & αCys-234) and sensitivity determinants for etomidate (βAsn-265) and volatile anesthetics (α1Ser-270 & α1Leu-232) are color coded.



Supplemental Figure S3: Comparison of the $GABA_AR$ transmembrane domain β3-α1 interface in the 3 $GABA_AR$ homology models, viewed from the base of the extracellular domain (upper figures) or from the lipid (lower figures). Helices are denoted as cylinders. To illustrate the degree of similarity or difference between the models, the Table below tabulates the α-carbon to α-carbon distances (in Å) between β3M3 residues (Val-290 and Met-286) and α1M1 residues (Met-236, Leu-232, and Ile-228). The two values for each measurement are from the two β3-α1 interfaces in each model. These residues are shown in stick format color-coded by atom type: gray, carbon; red, oxygen; blue, nitrogen; and gold, sulfur.

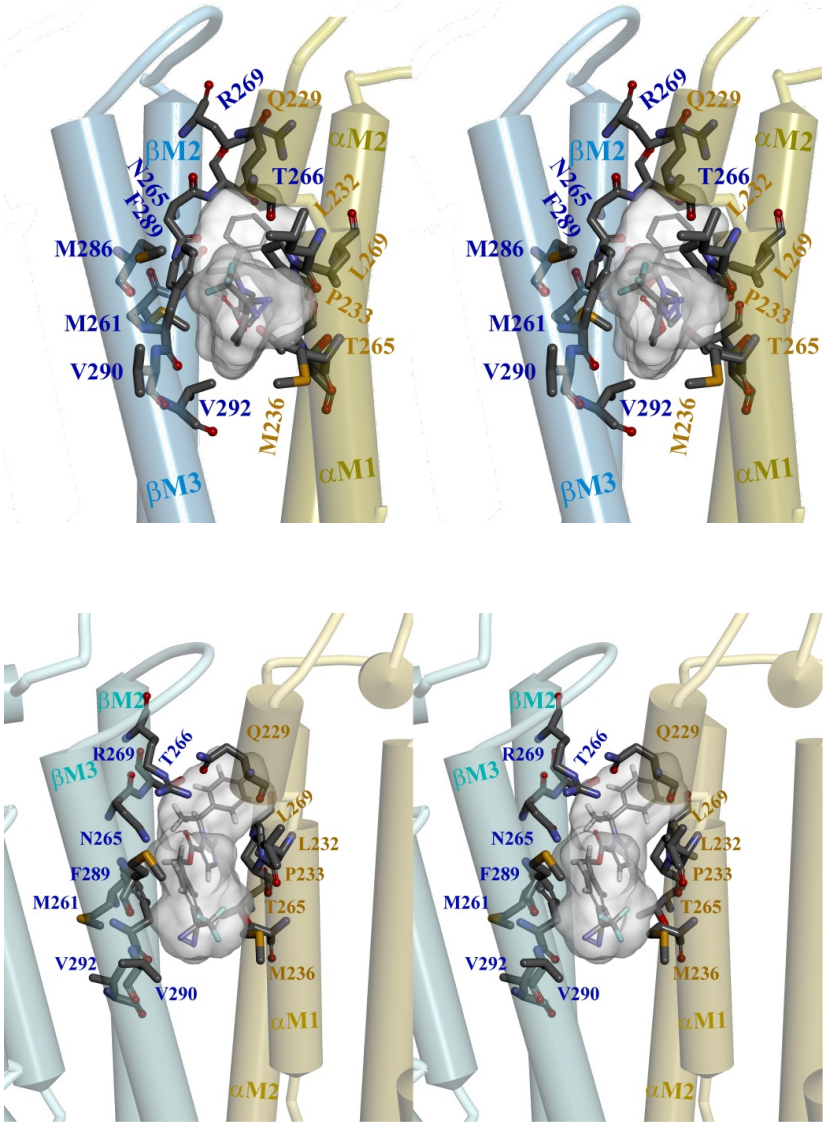
		nAChR		GLIC		GluCl
β3Met-286	α1Met-236	13.3, 13.0		11.4, 10.8		10.7, 10.9
β3Met-286	α1Leu-232	11.5, 9.0		8.8, 9.1		10.0, 10.0
β3Met-286	α1Ile-228	11.9, 9.3		6.6, 7.8		9.5, 9.5
β3Val-290	α1Met-236	9.7, 8.8		8.7, 8.4		9.6, 9.7
β3Val-290	α1Leu-232	10.5, 7.4		10.1, 9.9		12.3, 12.2
β3Val-290	α1Ile-228	13.5, 11.1		11.7, 11.6		14.2, 14.2



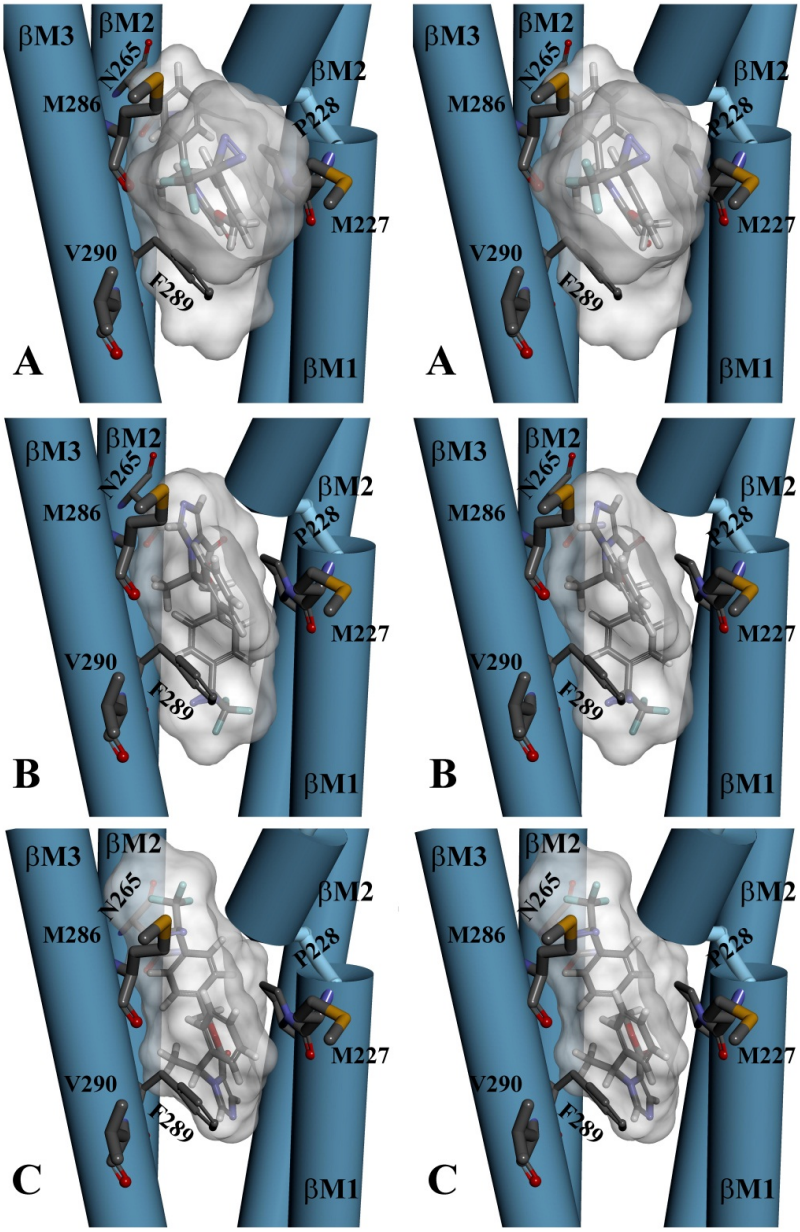
Supplemental Figure S4: $GABA_AR$ amino acids contributing to the TDBzl-etomidate pocket at the β3-α1 interface. Connolly surfaces are shown in stereo representation of the opposing surfaces from the α1 (upper panels) and β3 (lower panels) subunits, with color-coding for the amino acids contributing to the pocket. TDBzl-etomidate, in its predicted lowest energy orientation, is included in stick structure, color-coded by atom type: gray, carbon; blue, nitrogen; red, oxygen, white, hydrogen; and cyan, fluorine).

GLIC

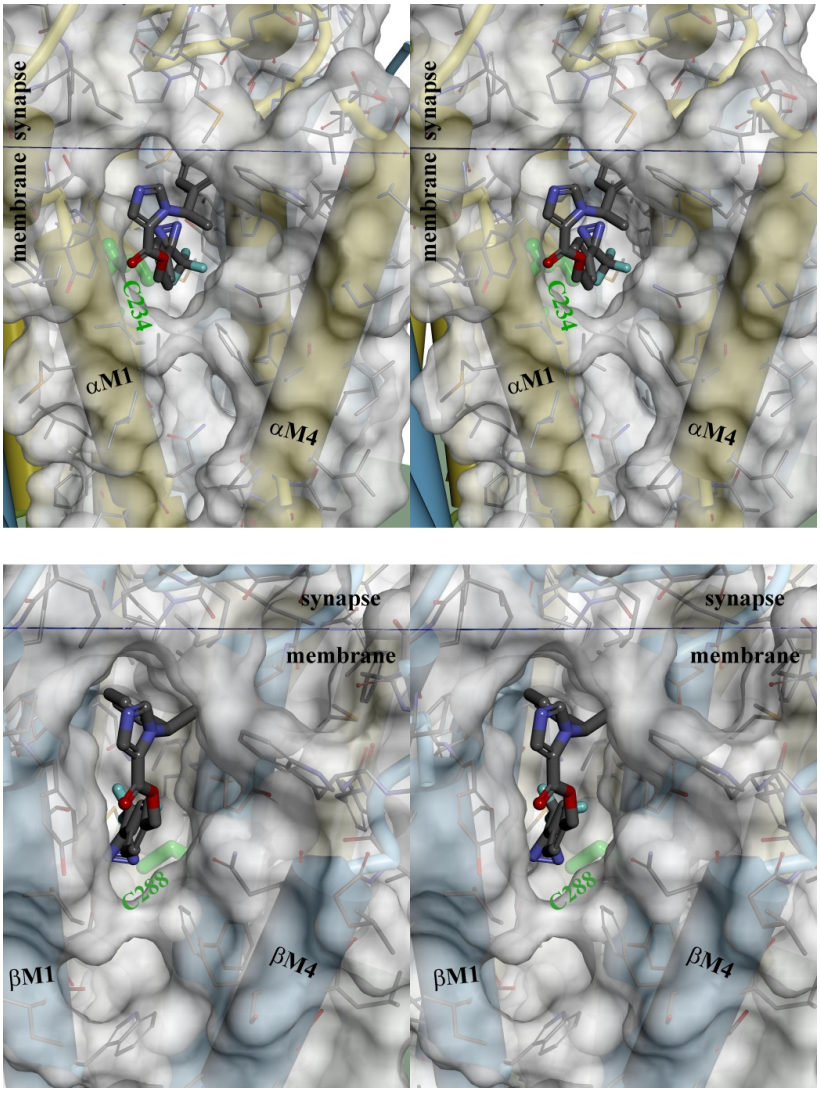
GluCl



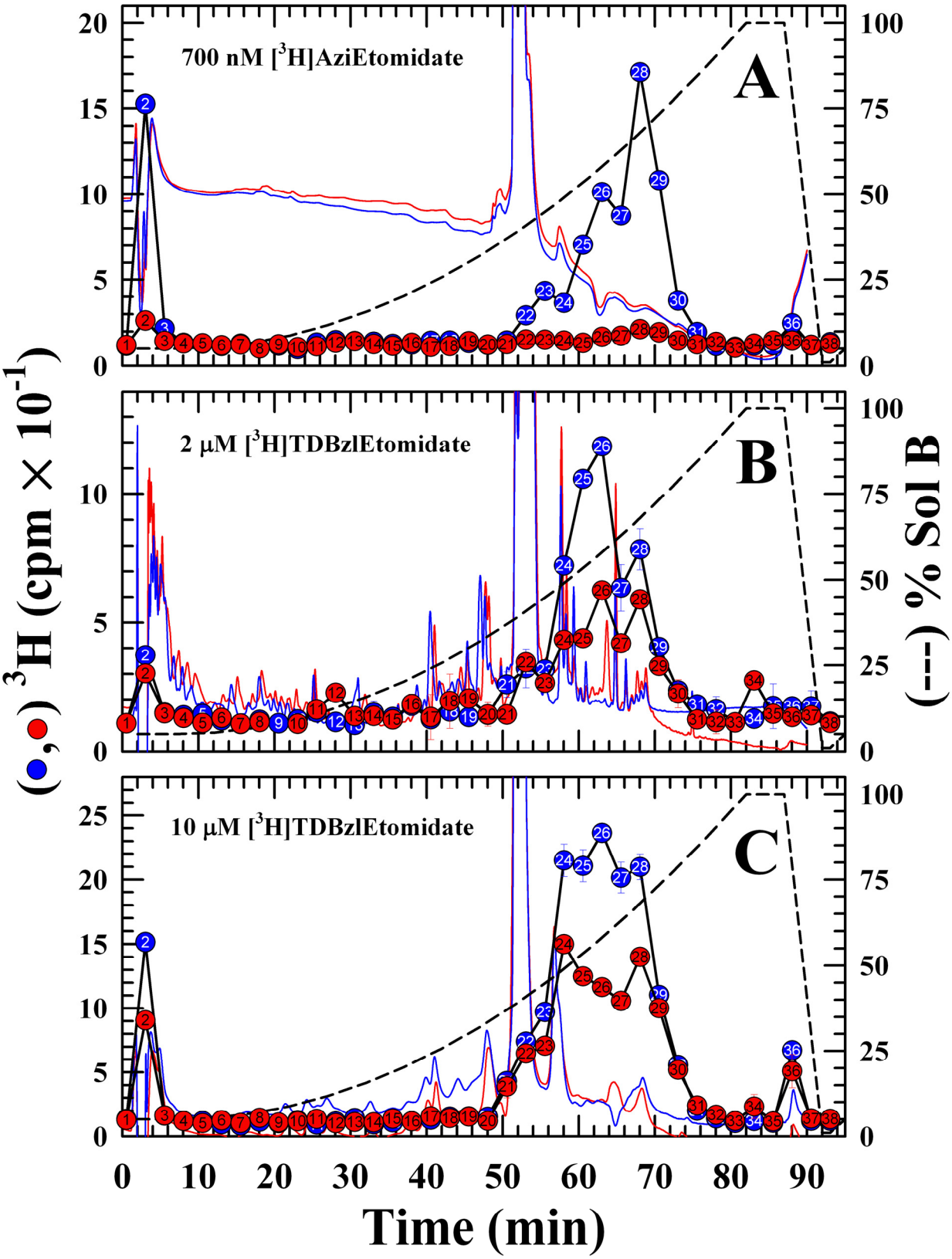
Supplemental Figure S5: Comparison of *R*-TDBzl-etomidate docked into a β3-α1 interface site in $GABA_AR$ homology models derived from the structure of GLIC and of GluCl. In the stereo representations, helices are shown as cylinders whereas *R*-TDBzl-etomidate, in its lowest energy orientation, and $GABA_AR$ amino acids in proximity are shown in stick format, color-coded by atom type: gray, carbon; blue, nitrogen; red, oxygen, white, hydrogen (excluded for residues); gold, sulfur; and cyan, fluorine. Connolly surfaces (transparent white) are included in the upper panel for the 14 (of 100) lowest energy orientations, or, in the lower panel, for the 126 (of 400) orientations with the diazirine facing β3Val-290/α1Met-236.



Supplemental Figure S6: *R*-TDBzl-etomidate docks in a pocket at the $GABA_AR$ β3-β3 interface in 3 orientations. In a β3β3αβ3α $GABA_AR$, the interface between adjacent β3 subunits (shown in stereo) juxtaposes βM3/βM2 from one subunit and βM1/βM2 from the other. *R*-TDBzl-etomidate and selected nearby $GABA_AR$ amino acids are shown in stick format, color-coded by atom type: gray, carbon; blue, nitrogen; red, oxygen, white, hydrogen (excluded for residues); gold, sulfur; and cyan, fluorine. Connolly surfaces (transparent white) are included for the composite volumes defined in the 100 lowest energy docking solutions, for *R*-TDBzl-etomidate docked: **A**, horizontally with the diazirine in proximity to photolabeled β3Met-227 in βM1 (14/100); B, vertically, with the diazirine in proximity to β3Thr-260 (βM2-10, not highlighted, 66/100), or C, vertically with the diazirinenear β3Asn-265 (βM2-15, 19/100).



Supplemental Figure S7: In the $GABA_AR$ homology model derived from the structure of GLIC, TDBzl-etomidate has access from the lipid to the pockets within the α and β subunit helix bundles. Stereo views from the lipid are shown of an α1 subunit (upper) and a β3 subunit (lower), each covered by transparent Connolly surfaces with their amino acids in thin stick format. Included in thick stick format is TDBzl-etomidate, docked in its lowest energy orientation (out of 300) in the pockets in each subunit formed by the helix bundles. TDBzl-etomidate is colored by atom type (black, carbon; red, oxygen; blue, nitrogen; cyan, fluorine). In this orientation its diazirine is predicted to be in proximity to the photolabeled α1Cys-234 (green, upper figure) and β3Cys-288 (green, lower figure).

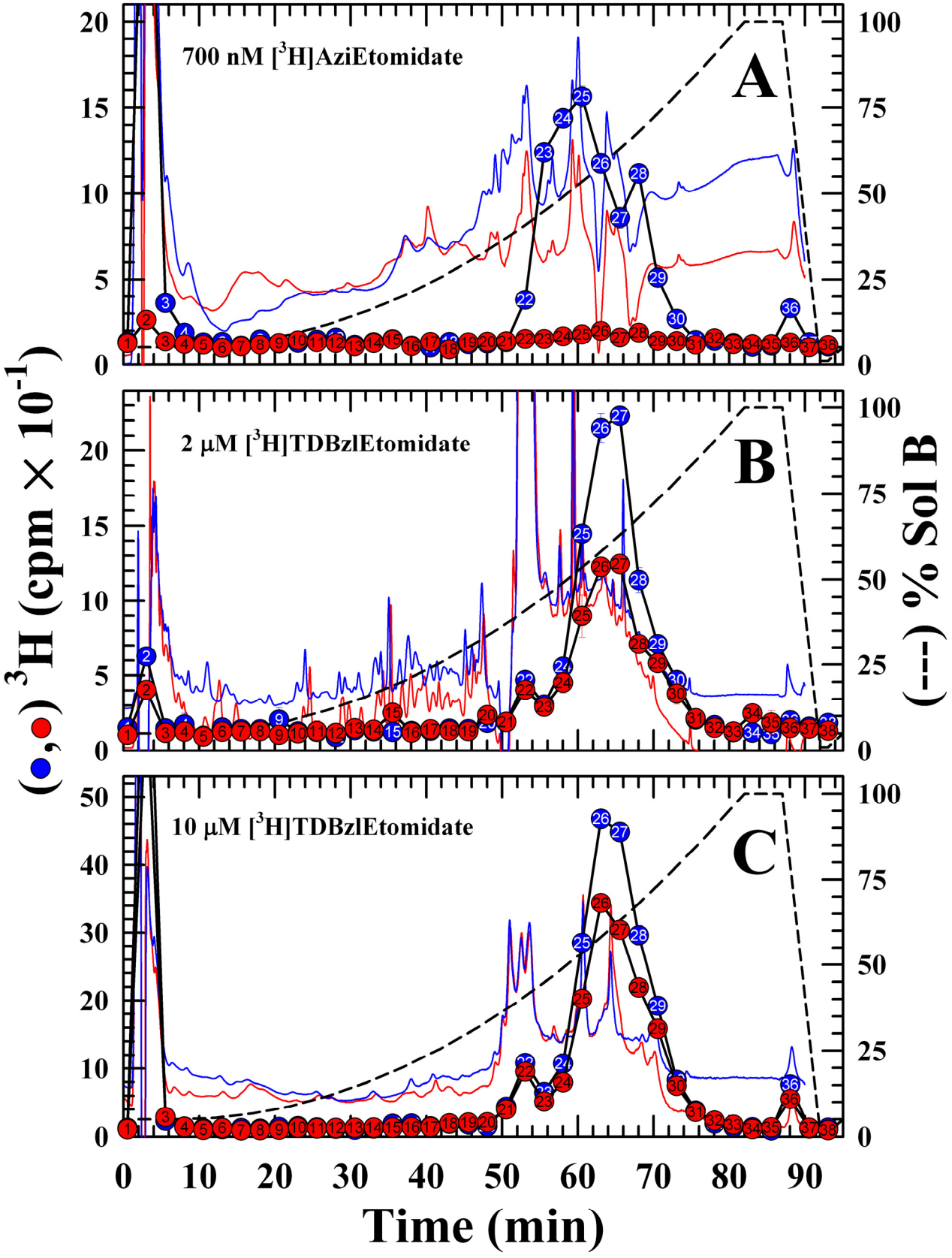


Supplemental Figure S8

Supplemental Figure S8: Reversed-phase HPLC fractionations of EndoGlu-C digests of [^{3}H]azietomidate (A) and [^{3}H]TDBzl-etomidate (B &C) photolabeled β3 subunits. Aliquots of β3 subunit, eluted from SDS-PAGE separations of α1β3 $GABA_AR$ photolabeled with 700 nM [^{3}H]AziEtomidate (**A**), 2 μM [^{3}H]TDBzl-etomidate (**B**), or 10 μM [^{3}H]TDBzlEtomidate (**C**) in the presence of 1 mM GABA and in the absence (**blue**) or presence (**red**) of 100 μM etomidate were digested with EndoGlu-C (Princeton Separations, 5 μg, 2-3 days, 20 °C) and separated by reversed-phase HPLC. Ten percent of each fraction was assayed for ^{3}H (●,●). Also shown are the absorbance at 215 nm (**red** / **blue** line) and the HPLC gradient (in % organic phase, ---). Total ^{3}H cpm injected and recovered (**-Et** /**+Et**): **A,** **10,640** and **8,020** cpm/ **810** and **1,000** cpm; **B,** **5,320** and **5,820** cpm/ **3,330** and **3,660** cpm; **C**, **23,080** and **15,760** cpm/ **14,990** cpm and **10,410** cpm .

Previous HPLC fractionations of EndoGlu-C digests of the β3 subunit (Li, Chiara et al., J. Neurosci.26:11599-11605 (2006)) established that a fragment beginning at β3Gly-386 and containing M4 elutes in fraction 24 (50 % organic), a fragment beginning at β3Thr-271 and including β3M3 elutes between fractions 25 and 27 (55-65% organic), and a fragment beginning at β3His-191 that contains β3M1 and M2 elutes around fraction 28 (70 % organic). For each HPLC run, fractions 25-27 were pooled and sequenced as shown in Figs. **6A, C, & E**.

Sequence analysis of fractions 28-29 from **A** is shown in Fig **7A**. For unknown reasons, this peptide, when sequenced, began at β3Gly-203 rather than the expected V8 protease cleavage site (β3His-191). While this unusual cleavage occurred in both samples from the labeling in **A**, the fragment beginning at β3His-191 was found in those fractions in three other preparative labelings, including Fig. 7B.

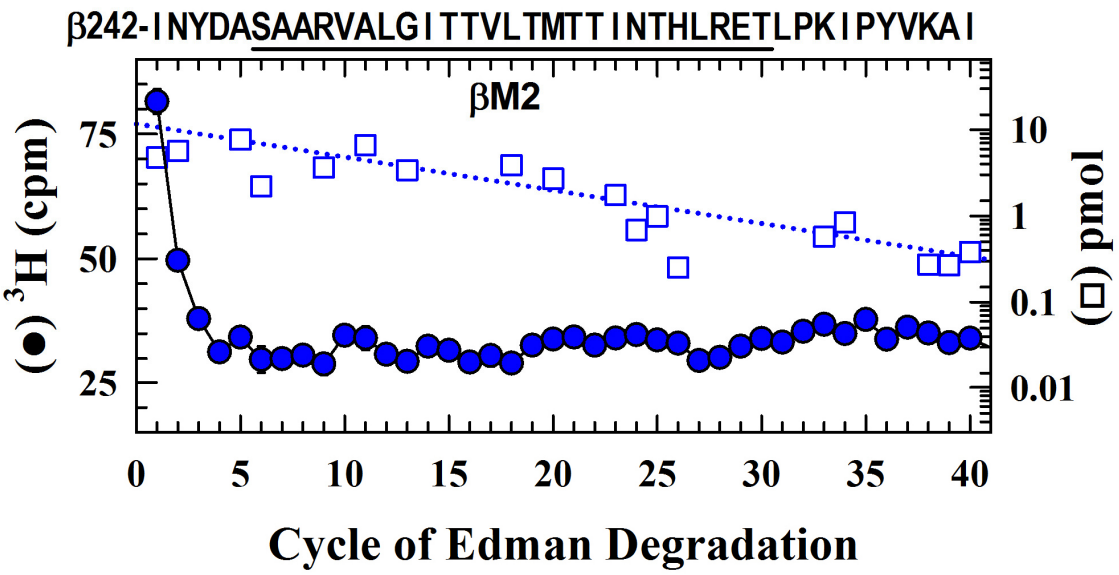


Supplemental Figure S9

Supplemental Figure S9: Reversed-phase HPLC fractionations of Trypsin or EndoLys-C digests of [^{3}H]azietomidate and [^{3}H]TDBzl-etomidate photolabeled α1 subunits. From the same photolabeling experiment of Figure S8, aliquots of α1 subunit from α1β3 $GABA_AR$ photolabeled with [^{3}H]AziEtomidate (**A**) or [^{3}H]TDBzlEtomidate (**B,** 2 μM; **C**, 10 μM) in the absence (**blue**) or presence (**red**) of 100 μM etomidate were digested with either trypsin (**A**) or EndoLys-C (**B**, Princeton Separations, 2.5 μg, 4 days, 20 °C; **C**, Roche, 0.5 U, 2 weeks, 20 °C) and separated by rpHPLC. Ten percent of each fraction was assayed for ^{3}H (●,●). Also plotted are the absorbance at 215 nm (**red** / **blue** line) and the HPLC gradient (in % organic phase, ---). Total ^{3}H cpm injected and recovered (**-Et** /**+Et**): **A,** **17,270** and **12,310** cpm/ **770** and **950** cpm; **B,** **9,930** and **10,040** cpm/ **5,450** and **6,530** cpm; **C**, **13,370** and **9,830** cpm/ **10,340** cpm and **8,550** cpm .

Previous HPLC fractionations of EndoLys-C digests of the α1 subunit (Li, Chiara et al., J. Neurosci.26:11599-11605 (2006)) established that a fragment beginning at α1Ile-392 and containing M4 elutes in fraction 24 or 25 (50-55 % organic) and that fragments beginning at α1Arg-221/Ile-223 containing α1M1 & M2 and at α1Val-280 containing α1M3 eluted in fractions 25-28 (55-70% organic). For the HPLC runs in **A**, fractions 25-26 were pooled and sequenced as shown in Figs. **6B**. For the HPLC runs in **B** & **C**, fractions 26-27 were pooled and sequenced as shown in Figs. **6D** & **6E**.

Sequence analysis of fraction 25 from **C** confirmed the presence of the fragment beginning at α1Ile-392, with no peaks of ^{3}H release seen while sequencing through α1M4.



Supplemental Figure S10: Sequence analysis of β3M2 from [^{3}H]TDBzl-etomidate labeled α1β3 GABA$_A$R found no photoincorporation. An aliquot of β3 subunit, eluted from an SDS-PAGE separation of α1β3 GABA$_A$R photolabeled with 3.5 μM [^{3}H]azietomidate in the absence (**blue**) of etomidate, was cleaved with BNPS-skatole and sequenced (7,550 cpm loaded, 1990 remaining on the fiter after 50 cycles of Edman degradation). (The sample labeled in the presence of 100 μM etomidate was not sequenced). The peptide beginning at β3Ile-242 was detected (□, I_0 = 12 pmol; R = 92 %) along with the other 7 expected peptides produced from cleaving after the 7 Trp residues in β3 (range 4 – 11 pmol). There was no photolabeling of the etomidate affinity determinant, β3Asn-265 (cycle 24, <1 cpm/pmol), and the background variations of ^{3}H release while sequencing through β3M2 indicated that photoincorporation, if it occurred, was at < 5 cpm/pmol. A small peak of ^{3}H release (6 cpm) was detected in cycle 49, consistent with photolabeling of β3Val-290 at 80 cpm/pmol, the level of β3Val-290 photolabeling determined by sequence analysis of the appropriate rpHPLC fraction from an EndoGlu-C digest of β3 subunit isolated in this photolabeling experiment.