

1 **Engineering transcription factor BmoR mutants for constructing multifunctional**

2 **alcohol biosensors**

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4

5 **Abstract**

6 Native transcription factor-based biosensors (TFBs) have the potential for in situ detection
7 of value-added chemicals or byproducts. However, their industrial application is limited by
8 their ligand promiscuity, low sensitivity, and narrow detection range. Alcohols exhibit
9 similar structures, and no reported TFB can distinguish a specific alcohol from its analogs.
10 Here, we engineered an alcohol-regulated transcription factor, BmoR, and obtained various
11 mutants with remarkable properties. For example, the generated signal-molecule-specific
12 BmoRs could distinguish the constitutional isomers n-butanol and isobutanol, with
13 insensitivity up to an ethanol concentration of 800 mM (36.9 g/L). Linear detection of 0–
14 60 mM of a specific higher alcohol could be achieved in the presence of up to 500 mM
15 (23.0 g/L) ethanol as background noise. Notably, two mutants with raised outputs and over
16 1.0×10^7 -fold higher sensitivity, and one mutant with an increased upper detection limit
17 (14.8 g/L n-butanol or isobutanol) were screened out. Using BmoR as an example, this
18 study systematically explored the ultimate detection limit of a TFB towards its small-
19 molecule ligands, paving the way for in situ detection in the biofuel and wine industries.

20 **Keywords:** transcription factor, BmoR, biosensor, specificity, high sensitivity, wider
21 detection

22

23 **Introduction**

24 Upon sensing specific signal molecules, transcription factors (TFs) can bind or unbind to
25 the DNA-regulatory sequences of target genes to induce or repress gene transcription. TFs
26 can be utilized as biosensors by coupling the transcriptional alteration with the expression
27 of a reporter protein¹. Transcription factor-based biosensors (TFB) have been designed and
28 constructed to detect toxic metals², regulate metabolic flux^{3,4}, and screen highly active
29 enzymes⁵ or chemical overproducers^{6,7}. However, several drawbacks limit the industrial
30 applications of TFBs. First, TFB signaling can be saturated by the medium concentration
31 of intracellular signal products and cannot distinguish chemical overproducers from
32 producers⁸. Second, TFBs can be interfered by the byproducts, which are structurally
33 similar to the products⁹. Third, in many cases, TFBs are not sensitive enough to detect low
34 concentrations of value-added chemicals^{10,11}. Therefore, it is necessary to develop TFBs
35 with low ligand promiscuity, high sensitivity, and a wide detection range.

36

37 n-Butanol and isobutanol are considered as promising substitutes for gasoline because of
38 their special properties¹². Butanols are also crucial precursors for the production of plastics
39 and polymers¹³. Metabolic engineering has been used to produce n-butanol and isobutanol
40 in various hosts¹⁴⁻²². However, an efficient TFB with low ligand promiscuity, high
41 sensitivity, and a wide detection range has yet to be achieved for in situ detection and high-
42 throughput screening. *Pseudomonas butanovora* BmoR is an activated transcription factor
43 that belongs to the bacterial enhancer-binding proteins (bEBPs) and can induce the

44 transcription of σ^{54} -dependent P_{bmo} promoter by combining with C2-C5 n-alcohols^{23, 24}.

45 The activation mechanism of BmoR is shown in Fig. 1A. The N-terminal, central domain,

46 and C-terminal of BmoR are responsible for sensing and combining the signal molecules,

47 contacting the σ^{54} factor of holoenzyme and hydrolyzing ATP, and binding to the upstream

48 sequence of the σ^{54} -dependent promoter, respectively²⁴. Native BmoR has been shown to

49 respond to C3-C5 alcohols, and a BmoR-based biosensor has been utilized to screen n-

50 butanol or isobutanol producers¹⁰.

51

52 In this study, we aimed to obtain efficient BmoR mutants with specificity, high sensitivity,

53 and a wide detection range towards special higher alcohols for the purpose of in situ

54 detection and high-throughput screening. The development of a biosensor that was

55 sensitive to butanols but completely insensitive to 0–36 g/L ethanol was another significant

56 purpose of this study. This type of biosensor is urgently needed in the wine industry for the

57 in situ detection of byproducts (n-butanol or isobutanol) production without interfering

58 with ethanol production. Here, we utilized error-prone PCR to construct a BmoR random

59 mutagenesis library based on the understanding of the BmoR N-terminal and a variety of

60 BmoR mutants with desired properties, such as specificity, sensitivity or a wide detection

61 range were obtained and are shown in Table 1. These mutants could be widely utilized in

62 various fields, including metabolic regulation, enzyme or production strain screening, and

63 byproduct detection.

64

65 **Results and discussion**

66 **Analysis of the wild-type BmoR structure and establishment of the mutagenesis** 67 **library**

68 We first analyzed the binding regions of different molecules and attempted to utilize
69 rational design to endow BmoR with signal-molecule specificity. In our previous study, the
70 biofilm regulator FleQ (PDB code: 5EXP) and the transcription factor PobR (PDB code:
71 5W1E) were used as templates to simulate wild-type BmoR (Fig. 1B). In this study,
72 molecular docking of BmoR with different alcohols (n-butanol, isobutanol, ethanol, and
73 glycol) was conducted. The results in Fig. 1C show that the binding pockets of different
74 signal molecules were similar, and the signal molecules bound to BmoR mainly via the
75 formation of hydrogen bonds with Arg211, Gln212, and Asn259. We first performed site-
76 saturation mutagenesis at these sites. The generated mutants had decreased response values
77 when compared to the wild-type (Fig. S1A, B, and C) and did not display signal-molecule
78 specificity. These results suggest the complexity of the response mechanisms of BmoR and
79 indicate that obtaining BmoR with desirable properties cannot be easily achieved through
80 rational design. Therefore, we performed random mutagenesis of BmoR. The N-terminus
81 of BmoR is responsible for recognizing and binding signal molecules and modulating the
82 activity of bEBP^{25,26}. Modifying the region could directly influence the response of BmoR
83 to the signal molecule and further change its properties. Therefore, error-prone PCR was
84 conducted at the N-terminal of BmoR to endow BmoR with desirable properties. After
85 screening the mutagenesis library, we acquired 400 colonies that had different GFP values

86 when compared with wild-type BmoR. The *bmoR* genes in these colonies were then
87 extracted and sequenced.

88

89 Besides, we assumed the initiation mechanism of BmoR. BmoR and NtrC could both
90 activate the σ^{54} -dependent transcription. The regulatory mechanism of NtrC was positive
91 regulation²⁷. In our previous study we have proved that the regulatory mechanism of BmoR
92 was also positive regulation²⁴. Therefore, we speculated the initiation mechanism of BmoR
93 might be similar to NtrC because of their structural and functional similarity. To induce the
94 transcription, BmoR might be firstly expressed and formed as dimers. The dimers of BmoR
95 would then capture the signal molecules and the σ^{54} of σ^{54} -RNAP holoenzyme ($E\sigma^{54}$)
96 would bind to the -24 (GG) and -12 (TGC) regions of σ^{54} -dependent promoter (P_{bmo}). After
97 that, the dimers with signal molecules would form as hexamer to initiate the transcription
98 of P_{bmo} with the energy which would be released by ATP hydrolysis. The whole activation
99 process was shown in Fig. S2. The true regulation mechanism of BmoR needed to be
100 exploited by crystallization of BmoR with signal molecules and in-depth analysis of the
101 generated structures.

102

103 **Specific response of BmoR mutants towards n-butanol**

104 We isolated two special mutants (W21R/E54V and I183T/D273N) that show a significant
105 response towards 10 mM of n-butanol and a weak response towards 10 mM of isobutanol
106 (Fig. 2A). The GFP/OD₆₀₀ values of W21R/E54V and I183T/D273N towards n-butanol

107 were 92.4 ± 14 and 15.6 ± 0.8 , respectively. A series of gradient addition experiments with
108 different concentrations of n-butanol or isobutanol were carried out to measure the apparent
109 K_m value and to verify the detection potential of these two mutants. Significantly, the
110 GFP/OD₆₀₀ values of W21R/E54V and I183T/D273N increased with the increase of n-
111 butanol concentration (Fig. 2B), and the response values of W21R/E54V and
112 I183T/D273N reached 325 ± 17 and 147 ± 12 when 100 mM n-butanol was added to the
113 culture, respectively, while the response values of these two mutants have no obvious
114 change with the increase of isobutanol concentration (Fig. 2C). These results suggest that
115 W21R/E54V and I183T/D273N could perceive the change of n-butanol concentration and
116 further give the corresponding response, and exhibited insensitivity towards isobutanol. In
117 addition, the apparent K_m value of W21R/E54V was 42.6 mM towards n-butanol. The
118 response value of I183T/D273N was linear to the concentration of n-butanol. Based on the
119 above results, we verified the mutants W21R/E54V and I183T/D273N as n-butanol-
120 specific BmoR mutants.

121

122 In addition, simulation and molecular docking of W21R/E54V and I183T/D273N with
123 isobutanol or n-butanol were conducted. The model in Fig. 2D shows that a hydrogen bond
124 was formed between n-butanol and the 261st glutamate of W21R/E54V, and there was no
125 force between isobutanol and W21R/E54V, suggesting that the interaction between n-
126 butanol and W21R/E54V was stronger than that between isobutanol and W21R/E54V. In
127 addition, we mutated the 261st glutamate to the other 19 amino acids. The generated 19

128 mutants have significantly lower response values when compared with W21R/E54V, which
129 demonstrates the significance of Glu261 in the response to n-butanol (Fig. S3). Similar to
130 W21R/E54V, two hydrogen bonds between n-butanol and I183T/D273N were observed in
131 the complex, and no force was formed between isobutanol and I183T/D273N,
132 demonstrating that n-butanol could bind I183T/D273N tightly (Fig. 2E).

133

134 **Specific response of BmoR mutants towards isobutanol**

135 In addition to the n-butanol-specific BmoR mutants, two mutants (M94V/F272L and
136 S240P) that significantly responded to 10 mM isobutanol and hardly responded to 10 mM
137 n-butanol, were screened from the mutagenesis library (Fig. 3A). The results of the gradient
138 addition experiment in Fig. 3B and C show that the GFP/OD₆₀₀ values of M94V/F272L
139 and S240P exhibited an increasing trend with the increase of isobutanol concentration and
140 had no significant change with the increase of n-butanol concentration. These distinct
141 responses illustrated that M94V/F272L and S240P have the ability to perceive the change
142 of isobutanol concentration and then output the corresponding response signal; on the
143 contrary, they could not sense n-butanol. Hence, the mutants M94V/F272L and S240P
144 could be considered as isobutanol-specific BmoR mutants.

145

146 Furthermore, complexes containing any of the above mutants and signal molecules (n-
147 butanol or isobutanol) were modeled, and the interactions between mutated BmoR and the
148 signal molecule were analyzed to explain the isobutanol specificity of M94V/F272L and

149 S240P. As shown in Fig. 3D, hydrophobic interactions existed between the signal molecule
150 isobutanol and Phe276 of M94V/F272L; meanwhile, no interaction was observed in the
151 complex of M94V/F272L and n-butanol. For the complexes of mutant S240P and signal
152 molecules, isobutanol bound to S240P via the formation of a hydrogen bond at Glu261 and
153 n-butanol did not interact with S240P (Fig. 3E). The interaction between the isobutanol-
154 specific mutants and signal molecules proved the isobutanol specificity of the mutants.

155

156 **Further verification of the specificity via the addition of mixed signal molecules**

157 To further confirm the specificity and test whether the two kinds of signal molecules
158 competed to combine the mutants, the response of n-butanol-specific and isobutanol-
159 specific BmoR mutants was measured by adding mixed signal molecules to the culture. We
160 used an n-butanol-specific mutant (W21R/E54V) and an isobutanol-specific mutant
161 (M94V/F272L) as examples to conduct the experiments. As shown in Fig. 4A, the
162 GFP/OD₆₀₀ value of the n-butanol-specific mutant W21R/E54V increased with an increase
163 in the n-butanol proportion, reaching 342 ± 7 when the ratio of n-butanol to isobutanol in
164 the culture was 10:1 (100 mM:10 mM). As a comparison, the GFP/OD₆₀₀ value of
165 W21R/E54V did not change with the increase of isobutanol proportion. The response of
166 W21R/E54V only varied with the change of n-butanol proportion, proving that there is no
167 competition between n-butanol and isobutanol in the n-butanol-specific mutant and that
168 W21R/E54V could maintain its specificity in the case of mixed signal molecules. Similar
169 to W21R/E54V, the GFP/OD₆₀₀ value of M94V/F272L only varied with the change of the

170 isobutanol proportion. In summary, we screened n-butanol-specific BmoR mutants
171 (W21R/E54V and I183T/D273N) and isobutanol-specific BmoR mutants (M94V/F272L
172 and S240P) from the mutagenesis library, and these mutants showed satisfactory specificity.

173

174 **BmoR mutants with higher output and sensitivity**

175 In addition to the above signal molecule-specific mutants, two mutants with higher outputs
176 were isolated from the random mutagenesis library. The sequencing results show that these
177 two mutants have mutations of E54G and V311A, respectively. Notably, mutant E54G have
178 a 27.5-fold higher GFP/OD₆₀₀ value (1941 ± 17) towards 1 mM n-butanol and a 12.1-fold
179 higher GFP/OD₆₀₀ value (1020 ± 17) towards 1 mM isobutanol when compared with wild-
180 type BmoR. In comparison, the GFP/OD₆₀₀ values of V311A towards 1 mM n-butanol and
181 1 mM isobutanol were 34.2- and 19.5-fold higher than wild-type BmoR, respectively (Fig.
182 4B). Based on this, we assumed that these two mutants with higher output might respond
183 to the low concentration of butanols, signifying that the mutants have higher sensitivity. To
184 validate this assumption, we added 0–1 mM n-butanol or isobutanol to the culture to test
185 the response of mutants E54G and V311A. The lower detection limit of BmoR was defined
186 as the concentration of butanols that could achieve a maximum GFP/OD₆₀₀ value of 75%.
187 Based on this, we estimated the lower detection limits of mutants E54G and V311A based
188 on the Michaelis-Menten equation of Origin8.5. As shown in Fig. 4C and D, the lower
189 detection limits of mutants E54G and V311A towards n-butanol were 2.64×10^{-6} mM and
190 2.13×10^{-6} mM, respectively, which demonstrated that the sensitivity of these two mutants

191 towards n-butanol was over 1.0×10^7 -fold higher than that of wild-type BmoR (28 mM).
192 In comparison, mutants E54G and V311A have lower detection limits of 2.16×10^{-6} mM
193 and 1.94×10^{-6} mM towards isobutanol, respectively, which suggests that these two
194 mutants possess over 1.0×10^7 -fold higher sensitivity to isobutanol when compared with
195 wild-type BmoR (26 mM). In addition, we attempted to introduce the mutations in the
196 mutant with high output into the signal molecule-specific mutants in order to enhance the
197 response values of the signal molecule-specific mutants. However, the mutations in the
198 mutant with high output did not significantly enhance the output of signal molecule-
199 specific mutants (data not shown). Future work might focus on enhancing the sensitivity
200 and output of signal molecule-specific mutants in order to sense the trace accumulation of
201 butanols.

202

203 **BmoR mutant with wider detection range**

204 To utilize the BmoR-based biosensor to screen industrial high-level n-butanol or isobutanol
205 production strains, we needed to broaden the detection range of BmoR. In this study, we
206 screened out a mutant (T12N) with a wider detection range (0–200 mM) when compared
207 with wild-type BmoR. Fig. 5A and B showed mutant T12N outputted significantly distinct
208 GFP/OD₆₀₀ values when feeding the 150 mM and 200 mM butanol solutions and the
209 apparent K_m values of T12N were 37.3 mM towards n-butanol and 89.9 mM towards
210 isobutanol. These results suggest that the upper detection limit of mutant T12N could reach
211 200 mM.

212 We then introduced mutant T12N into an isobutanol-producing strain to confirm its wider
213 detection range for *in vivo* host-producing isobutanol. First, plasmids pYH10 and pYH10-
214 T12N were individually introduced into the isobutanol-producing strain TW, resulting in
215 strains TW1 and TW2, respectively. TW1 and TW2 were used for subsequent fermentation
216 and GFP/OD₆₀₀ value detection. As shown in Fig. 5C and D, the GFP/OD₆₀₀ value of strain
217 TW2 with T12N BmoR expression displayed an upward trend as the isobutanol titer during
218 the 84-hour fermentation. The GFP/OD₆₀₀ values at 72 h and 84 h were significantly
219 different, and the value at 84 h reached 835 ± 25 . Meanwhile, 215 mM isobutanol was
220 accumulated in the culture. In comparison, the GFP/OD₆₀₀ value of strain TW1 with wild-
221 type BmoR expression increased with the production of isobutanol in the first 36 h, and the
222 isobutanol titer was 67 mM at 36 h. In the next 48 h, isobutanol was continuously produced.
223 However, the GFP/OD₆₀₀ value of TW1 did not increase significantly. These results
224 illustrate that mutant T12N demonstrates a wider detection range (0–200 mM) towards *in*
225 *vivo* host-producing isobutanol, and this mutant could be used to construct a sensor to
226 screen overproducers.

227

228 **BmoR mutants with ethanol insensitivity**

229 In the ethanol fermentation industry, n-butanol and isobutanol are usually produced as
230 byproducts. We assumed that the BmoR-based biosensor could serve as a powerful tool to
231 detect the accumulation of higher alcohols in ethanol-producing strains. However, native
232 BmoR showed a significant response to ethanol, which could induce signal interference

233 and lead to inaccurate measurement of higher alcohol concentrations. Therefore, a
234 biosensor that is insensitive to ethanol and sensitive to higher alcohols needs to be urgently
235 explored. Fortunately, the abovementioned two n-butanol-specific mutants and two
236 isobutanol-specific mutants did not respond to 0–200 mM ethanol (Fig. S4). The apparent
237 K_m values of mutants W21R/E54V and I183T/D273N towards ethanol were 228 and 174
238 mM, respectively, which were 23.5- and 17.9-fold higher than that of the wild-type.
239 Similarly, mutants M94V/F272L and S240P possess 13.6- and 12.1-fold higher apparent
240 K_m towards ethanol when compared with the wild type, respectively. The insensitivity of
241 these mutants to ethanol was maintained at a concentration of 800 mM (Fig. 5E). The
242 complexes in Fig. S5 show that there was no interaction between any of the
243 abovementioned mutants and ethanol. Notably, W21R/E54V and M94V/F272L maintained
244 their respective specificities even with background interference of 500 mM ethanol (Fig.
245 5F). In addition, we tested the response of special BmoR mutants to other alcohols
246 including methanol, glycol, isopropanol, isopentanol, and 2-methyl-1-pentanol. Fig. S6
247 shows that mutant T12N could respond to the abovementioned alcohols as well as wild-
248 type BmoR, and mutant W21R/E54V has a narrower signal molecule range when
249 compared with wild-type BmoR, suggesting that mutations in the mutants influenced the
250 range of signal molecules.

251

252 The promiscuity of TFs is a double-edged sword. On the one hand, promiscuity could
253 provide some benefits. For instance, TFs with promiscuity can expand the signal

254 molecule spectrum. On the other hand, the signal molecule diversity of TFBs could result
255 in an inaccurate response. Many studies have engineered biosensors to realize specific
256 responses ^{28, 29}. Endowing a BmoR-based biosensor with n-butanol specificity or
257 isobutanol specificity is required to expand its application. In addition to the native sensing
258 elements, some *de novo* designed biosensors that assemble specific protein functional
259 domains were also developed ³⁰. Based on the analysis of BmoR in this work, the specific
260 domains of BmoR might be served as crucial units for establishing novel biosensors to
261 detect other vital compounds.
262

263 **Conclusions**

264 The non-ideal properties of wild-type BmoR limit its applications for the screening of
265 single-higher-alcohol industrial-producing strains or the detection of byproducts in the
266 fermentation process. In this study, we engineered a significant region of BmoR and
267 acquired two n-butanol-specific mutants, two isobutanol-specific mutants, and two
268 ultrasensitive mutants. In addition, a mutant with a wider detection range (0–200 mM) was
269 screened out, which could also be reflected in the isobutanol-producing strain. Notably, we
270 observed that the signal-molecule-specific mutants display insensitivity towards ethanol,
271 indicating that these specific mutants could be used to detect the production of byproducts
272 (butanols) in the ethanol fermentation industry. In summary, this work supplied various
273 desirable BmoR mutants that could be employed for constructing biosensors to screen ideal
274 strains or establish dynamic control systems in the field of metabolic engineering.

275

276 **Methods**

277 **Strains, media, and materials**

278 *E. coli* XL10-Gold was used for plasmid construction, screening, and construction of the
279 BmoR N-terminal-based random mutagenesis library. LB medium (10 g/L tryptone, 5 g/L
280 yeast extract, and 10 g/L NaCl) was used for strain incubation and library screening. M9
281 medium (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, and 0.5 g/L NaCl, 1 mM MgSO₄,
282 0.1 mM CaCl₂, 10 mg/L VB1, 4 g/L yeast extract, and 40 g/L glucose) was used for the
283 fermentation experiment. Plasmids pSA65, pSA69, pYH1, and pYH10 were obtained from
284 our previous study^{16,24}. The details of the strains and plasmids used in this study are listed
285 in Table S1.

286

287 **Homology modeling and molecular docking of BmoR mutants with signal molecules**

288 AUTODOCK and Chimera 1.14 were used for homology modeling and molecular docking
289 of BmoR with signal molecules (n-butanol, isobutanol, ethanol, or glycol). The tertiary
290 structure of wild-type BmoR, which was modeled in our previous study²⁴, was used as a
291 template to simulate and dock BmoR mutants with signal molecules. All BmoR mutant
292 models were evaluated by PROCHECK, and all models have satisfactory quality, with over
293 80% of the residues in the most favored region of the calculated z-scores. A grid box (10 ×
294 17 × 8) encompassing the binding pocket of BmoR was set as the search space to explore
295 suitable substrate-binding regions. The interactions between the mutants and signal
296 molecules were analyzed and are shown in the corresponding figures.

297 **Establishment of the BmoR N-terminal-based mutagenesis library**

298 Error-prone PCR was carried out on the N-terminus of BmoR to generate an N-terminal-
299 based mutagenesis library. The details of the error-prone PCR method are provided in the
300 Supplementary Material. To carry out site-directed mutagenesis of *bmoR*, a pair of primers,
301 including the desired mutations, was synthesized
302 and used to amplify a linearized fragment from pYH1. The PCR products were purified
303 and digested with DpnI and then transferred into *E. coli* XL10-Gold. The primers used in
304 this study are listed in Table S2.

305

306 **High-throughput screening of the mutagenesis library**

307 Single colonies harboring plasmid pYH1 containing wild-type or mutated BmoR were pre-
308 inoculated into 5 mL LB with 100 µg/mL ampicillin and then cultured at 37 °C overnight.
309 Next, 50 µL of the seed culture was transferred into 950 µL LB, which was supplemented
310 with appropriate antibiotics and 10 mM n-butanol or isobutanol in 96-deep-well plates. The
311 cultures were then left at 30 °C for 16 h. To measure the apparent K_m values of the special
312 BmoR mutants towards the butanols or ethanol, the butanol concentrations in 96-deep-well
313 plates ranged between 0–100 mM, and the ethanol concentrations in 96-deep-well plates
314 ranged between 0–800 mM. A microplate reader (BioTek Cytation 3) was used to detect
315 the OD₆₀₀ values and GFP fluorescence. The excitation and emission wavelengths were set
316 at 470 nm and 510 nm, respectively. The *bmoR* in the colonies that have distinct GFP/OD₆₀₀
317 values compared to the wild type were sequenced. Apparent K_m values were estimated

318 using Origin8.5 through non-linear regression of the Michaelis-Menten equation. The
319 entire screening process is illustrated in Fig. S7. The ratio of n-butanol to isobutanol in a
320 mixture of butanol in the culture is described in the Supplementary Material.

321

322 **Fermentation verification of BmoR with a wider detection range**

323 Plasmids pYH10 containing wild-type *bmoR* and pYH10-T12N containing *T12N* were
324 individually introduced into strain TW (JCL260 with pSA65, which contained genes *kivd*
325 and *adhA*, and pSA69, which contained genes *alsS*, *ilvC*, and *ilvD*), resulting in strains
326 TW1 and TW2, respectively. For the fermentation experiment, the single colonies were
327 pre-inoculated into 5 mL LB medium with associated antibiotics at 37 °C for 12–14 h. Then,
328 200 µL of culture was inoculated into 20 mL M9 with 0.1 mM IPTG in a 250 mL screw
329 cap conical flask and left at 30 °C in a shaker at 220 rpm. After 36 h, 40 g/L glucose was
330 added to the culture. Samples were taken every 12 h for GFP/OD₆₀₀ measurements and
331 isobutanol detection. GC analysis of the samples was performed as described in our
332 previous study³¹.

333

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349

350 **Author Contributions**

351 Y.-X.H. generated the idea. Y.-X.H., Z.C. and T.W. designed the project. T.W. and Z.C.
352 carried out the experiments. T.W., Z.C., C.Z. and Y.-X.H. analyzed the data. T.W., Z.C. and
353 Y.-X.H. wrote the manuscript.

354

355 **Author Contributions**

356 #T.W. and Z.C. contributed equally to this work.

357

358 **Notes**

359 The authors declare no competing financial interest.

360

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444

445

446 **Figure legends**

447 **Fig. 1. The activation mechanism and simulated structure of wild-type BmoR.** (A) The
448 simulation mechanism of wild-type BmoR. (B) The simulated structure and the binding
449 pocket of wild-type BmoR. (C) The binding pocket and binding sites of wild-type BmoR
450 with different signal molecules (isobutanol, n-butanol, ethanol or glycol).

451

452 **Fig. 2. Specific response of BmoR mutants towards n-butanol.** (A) The response values
453 of BmoR mutants W21R/E54V and I183T/D273N towards 10 mM n-butanol or isobutanol.
454 (B) The response curves of BmoR mutants towards n-butanol. (C) The response curves of
455 BmoR mutants towards isobutanol. (D) Simulation and molecule docking of mutant
456 W21R/E54V with n-butanol or isobutanol, and confirmation of the binding sites with signal
457 molecules. (E) Simulation and molecule docking of mutant I183T/D273N with n-butanol
458 or isobutanol, and confirmation of the binding sites with signal molecules. Values and error
459 bars represent mean and s.d. (n = 3), respectively.

460

461 **Fig. 3. Specific response of BmoR mutants towards isobutanol.** (A) The response values
462 of BmoR mutants M94V/F272L and S240P towards 10 mM n-butanol or isobutanol. (B)
463 The response curves of BmoR mutants towards n-butanol. (C) The response curves of
464 BmoR mutants towards isobutanol. (D) Simulation and molecule docking of mutant
465 M94V/F272L with n-butanol or isobutanol, and confirmation of the binding sites with
466 signal molecules. (E) Simulation molecule docking of mutant S240P with n-butanol or

467 isobutanol, and confirmation of the binding sites with signal molecules. Values and error
468 bars represent mean and s.d. (n = 3), respectively.

469

470 **Fig. 4. Verification of the specificity via adding mixed signal molecules and BmoR**

471 **mutants with higher outputs and sensitivity. (A)** Maintaining the concentration (10 mM)

472 of one kind of butanols and increasing the concentration of the other kind of butanols with

473 a gradient (0-100 mM) to confirm the specificity of BmoR mutants W21R/E54V and

474 M94V/F272L. **(B)** The response values of BmoR mutants E54G and V311A towards 1 mM

475 n-butanol or isobutanol. **(C)** The response curves of BmoR mutants E54G and V311A

476 towards n-butanol. **(D)** The response curves of BmoR mutants E54G and V311A towards

477 isobutanol. Values and error bars represent mean and s.d. (n = 3), respectively.

478

479 **Fig. 5. BmoR mutant with wider detection range or ethanol insensitivity. (A)** The

480 response curves of wild-type BmoR and mutant T12N towards n-butanol. **(B)** The response

481 curves of wild-type BmoR and mutant T12N towards isobutanol. **(C)** The response curves

482 of wild-type BmoR and mutant T12N towards isobutanol which was produced by the

483 isobutanol-producing strain. **(D)** The response values of wild-type BmoR and mutant T12N

484 towards 67 mM, 114 mM and 215 mM isobutanol. **(E)** The response values of specific

485 mutants via adding 0-800 mM ethanol in culture. **(F)** The response values of W21R/E54V

486 and M94V/F272L via adding 0-60 mM n-butanol or isobutanol and 500 mM ethanol as

487 noise. Values and error bars represent mean and s.d. (n = 3), respectively. *P < 0.1, **P <

488 0.01 as determined by two-tailed t-test.

489

490 **Table 1. Representative BmoR mutants with remarkable properties in this study**
491

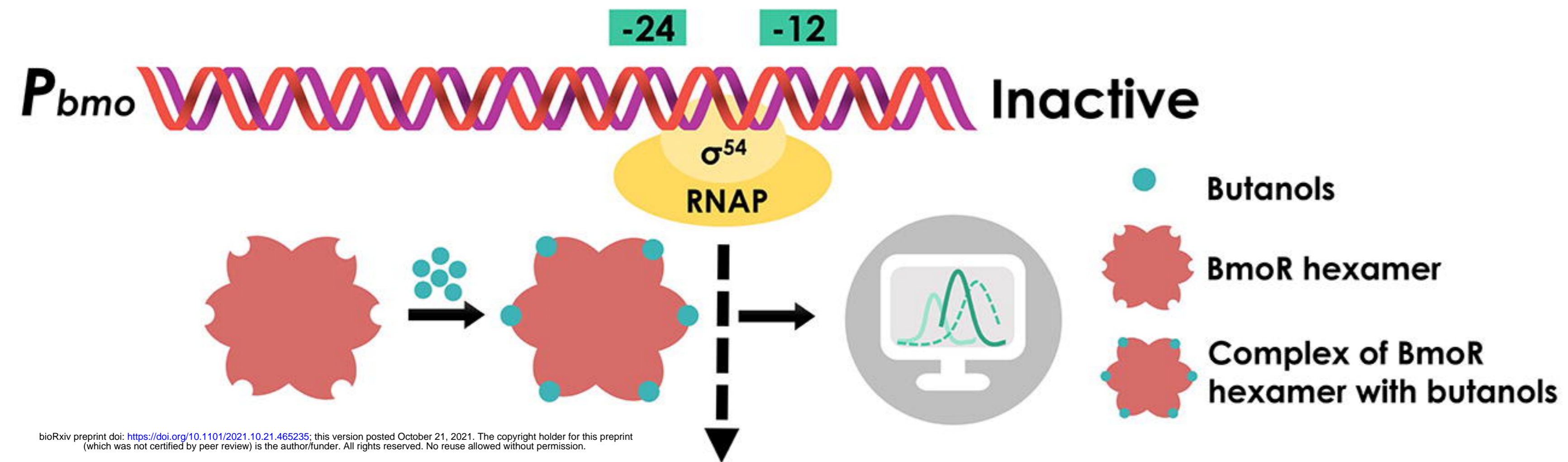
BmoR Mutant	Property					
	n-Butanol specificity	Isobutanol specificity	Insensitivity to ethanol	Higher sensitivity	Wider detection range	Higher output
W21R/E54V	√		√			
I183T/D273N	√		√			
M94V/F272L		√	√			
S240P		√	√			
E54G				√		√
V311A				√		√
T12N					√	

492

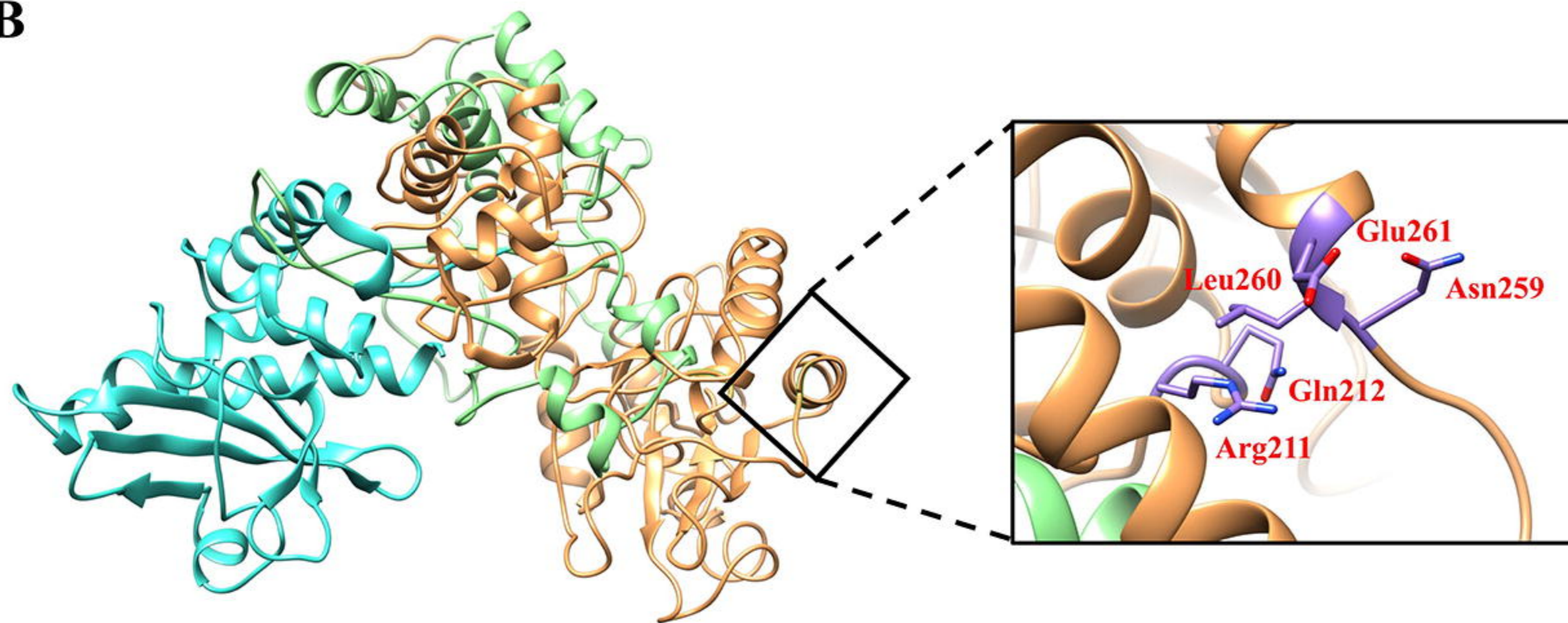
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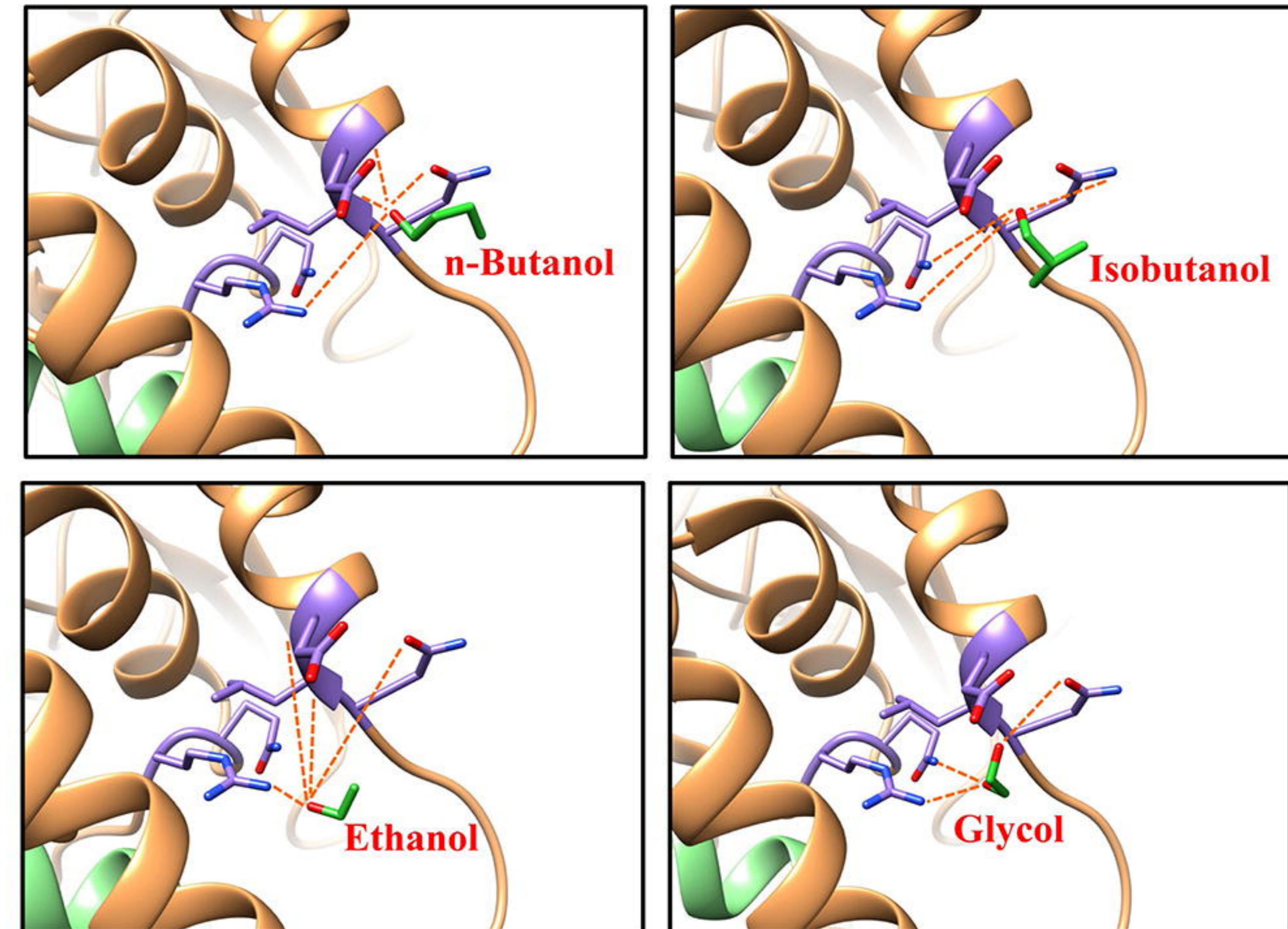
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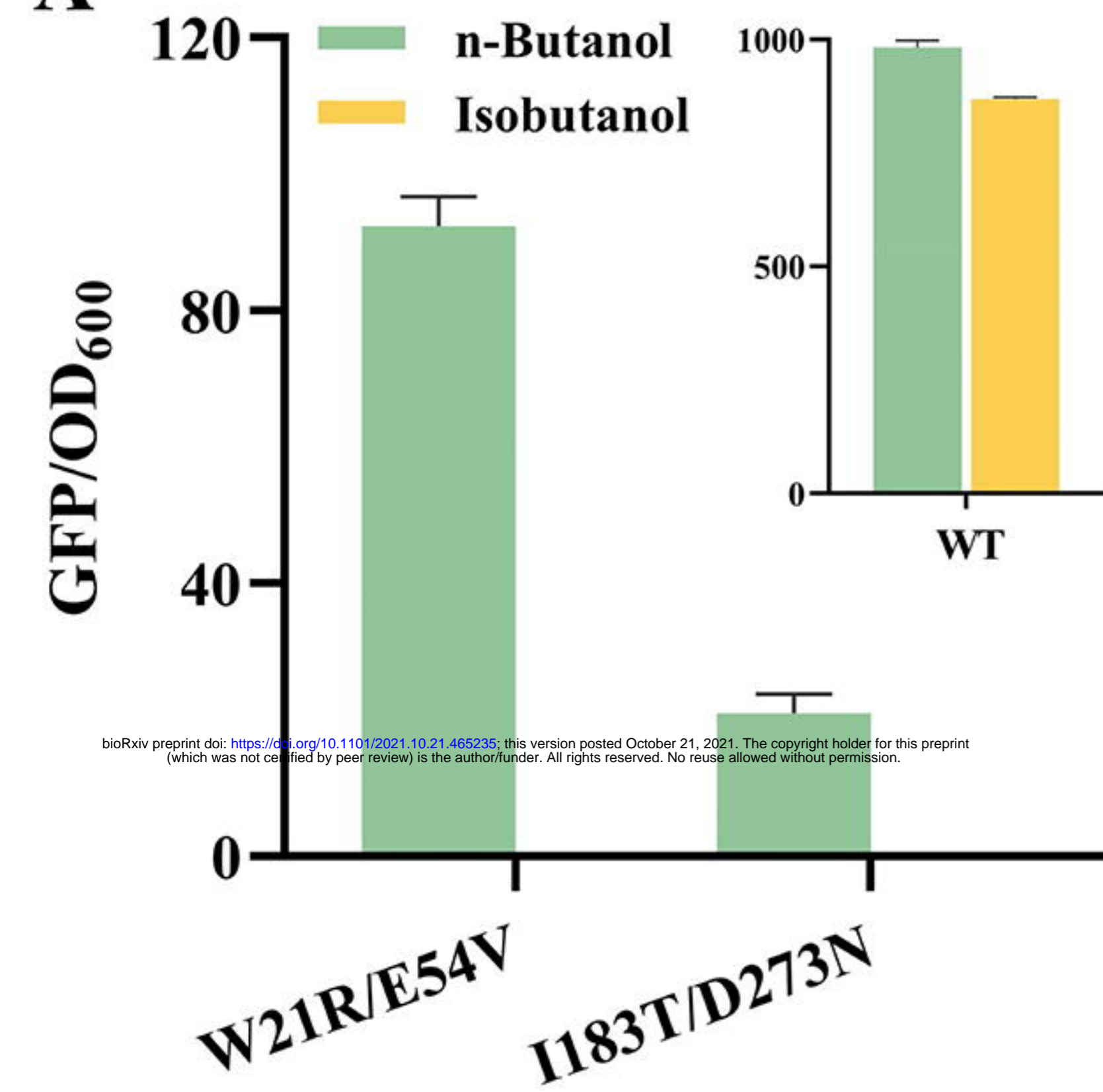
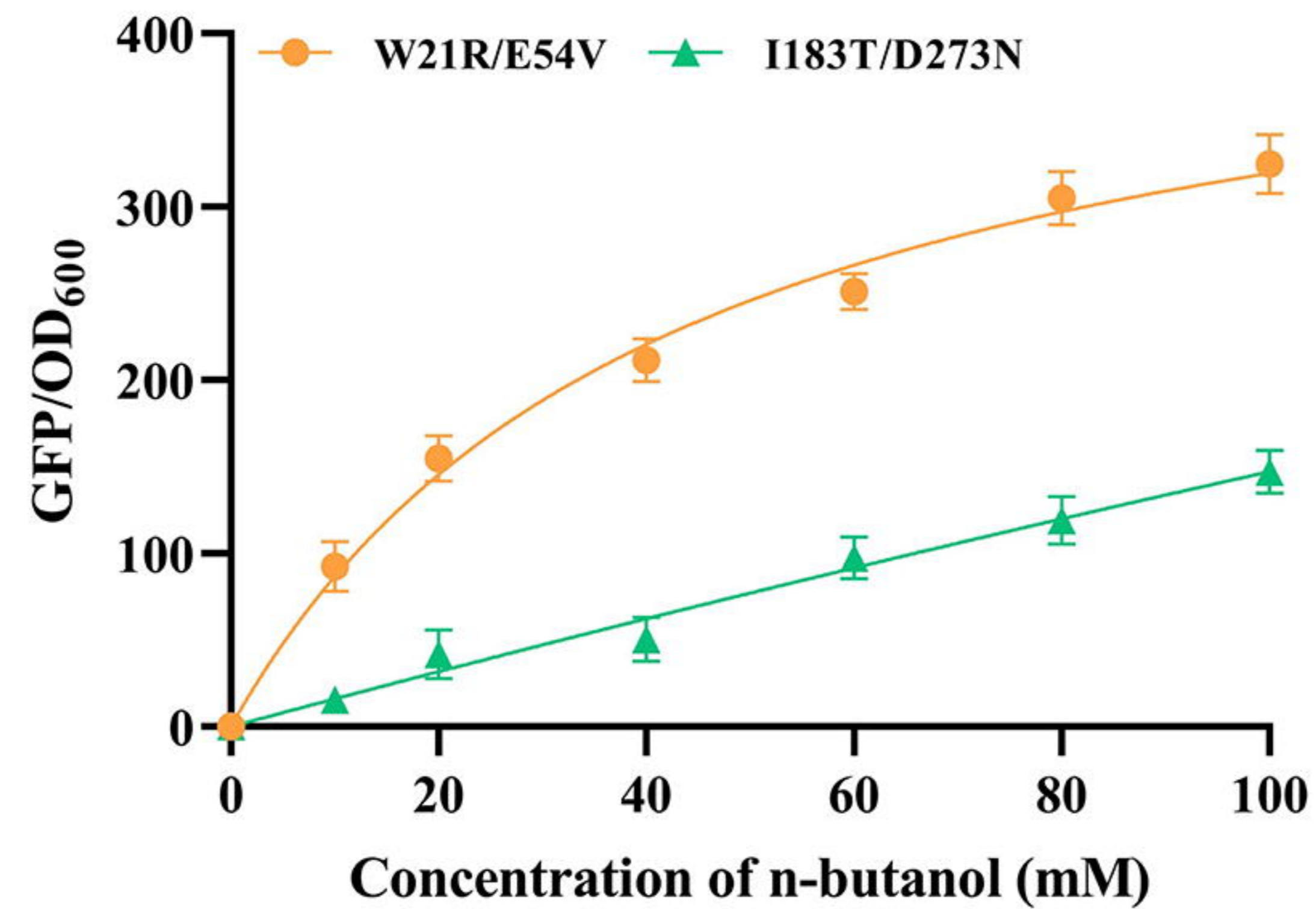
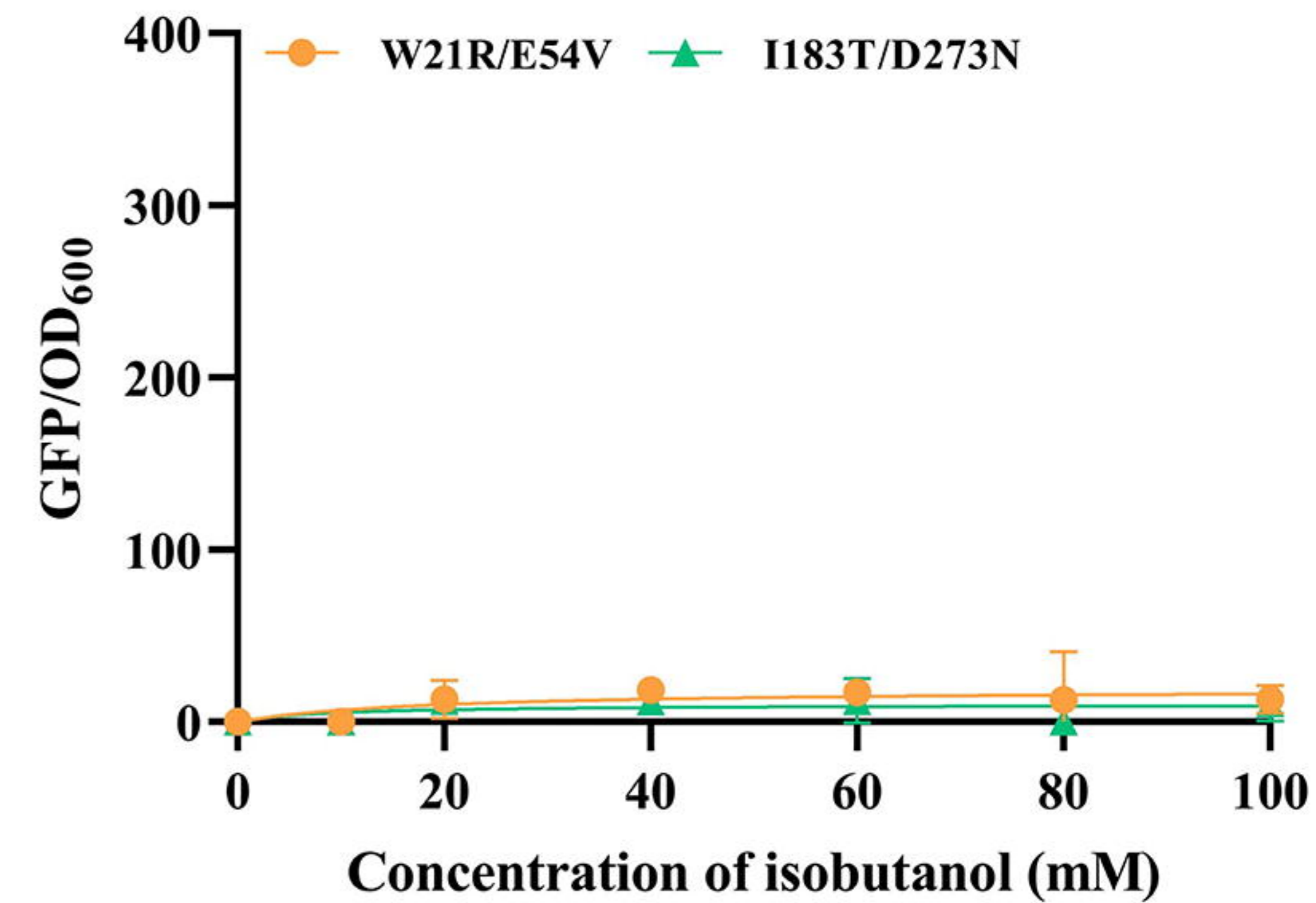
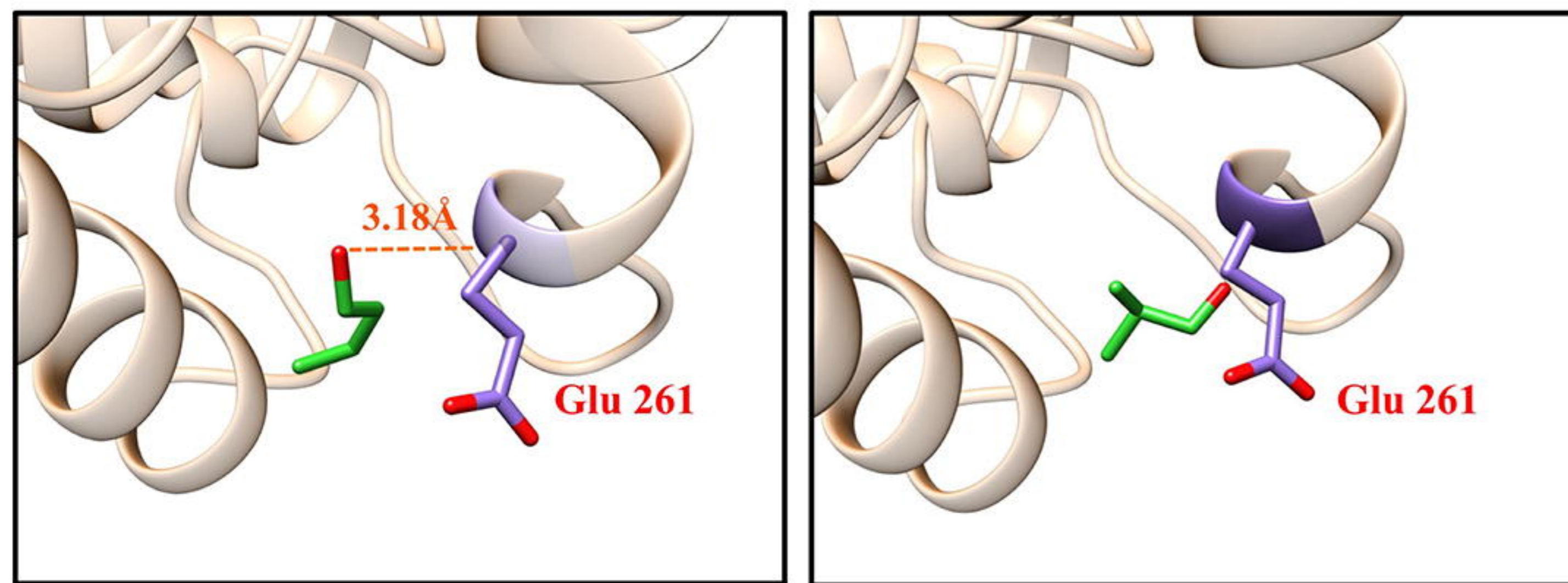
B



C



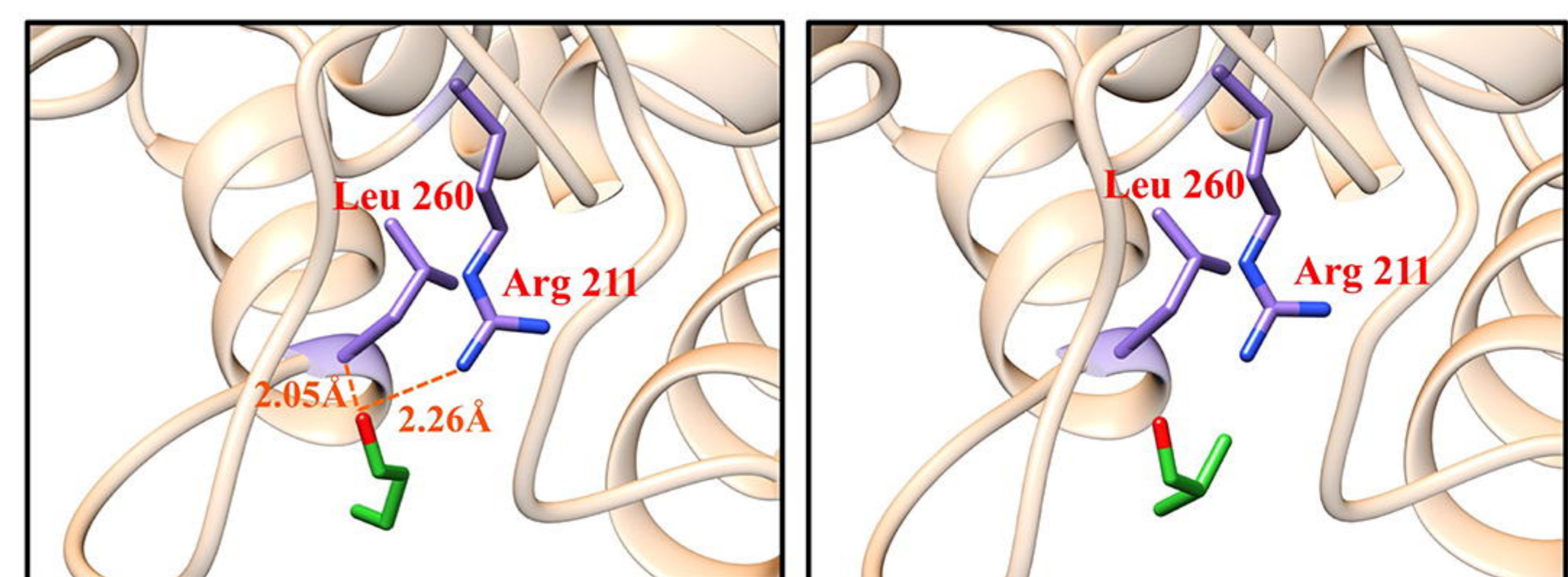
	n-Butanol	Isobutanol	Ethanol	Glycol
Binding sites	Arg 211 Gln 212 Asn 259 Leu 260 Glu 261	Arg 211 Gln 212 Asn 259	Arg 211 Gln 212 Asn 259 Leu 260 Glu 261	Arg 211 Gln 212 Leu 260

A**B****C****D**

W21R/E54V with n-butanol

W21R/E54V with isobutanol

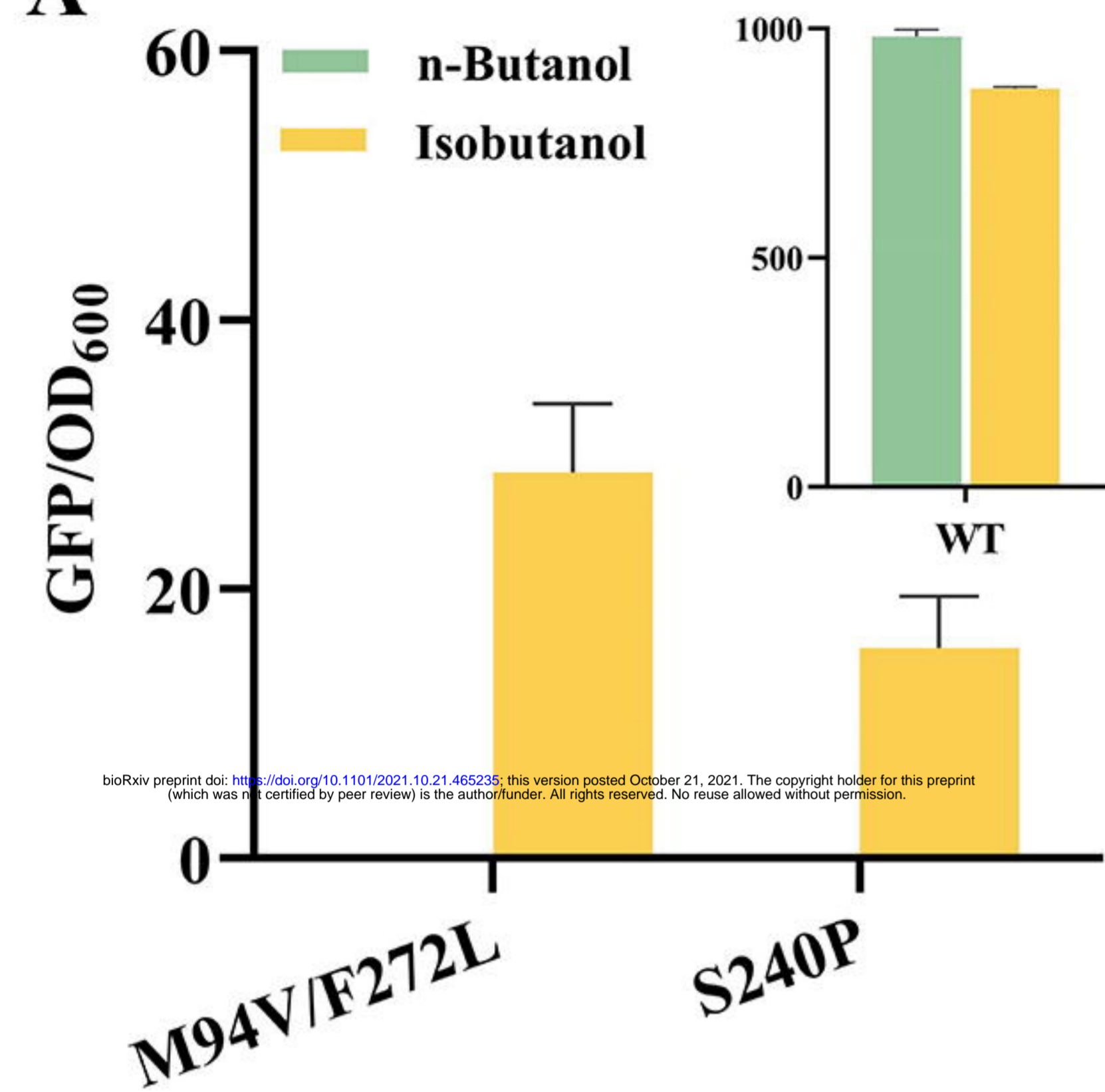
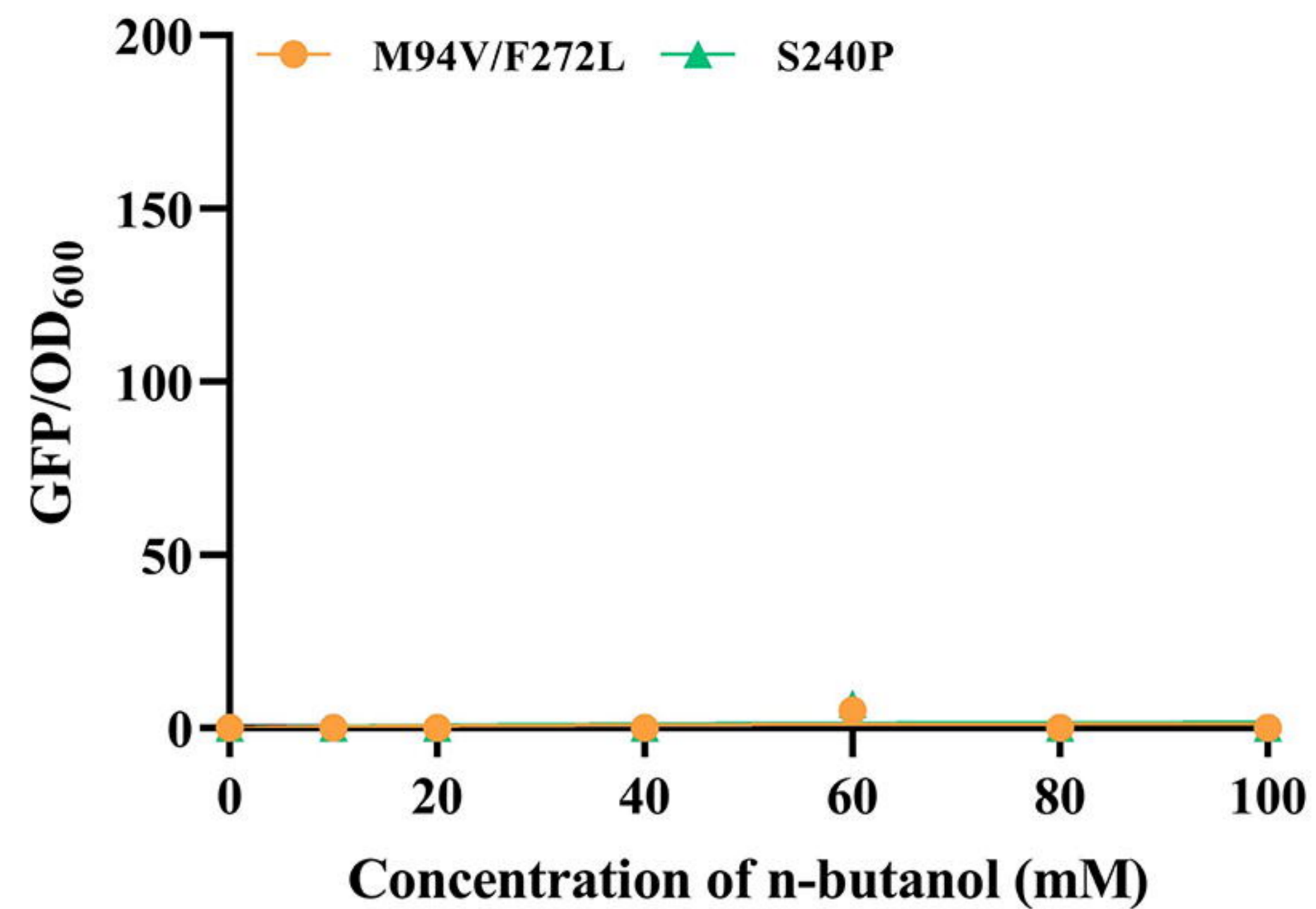
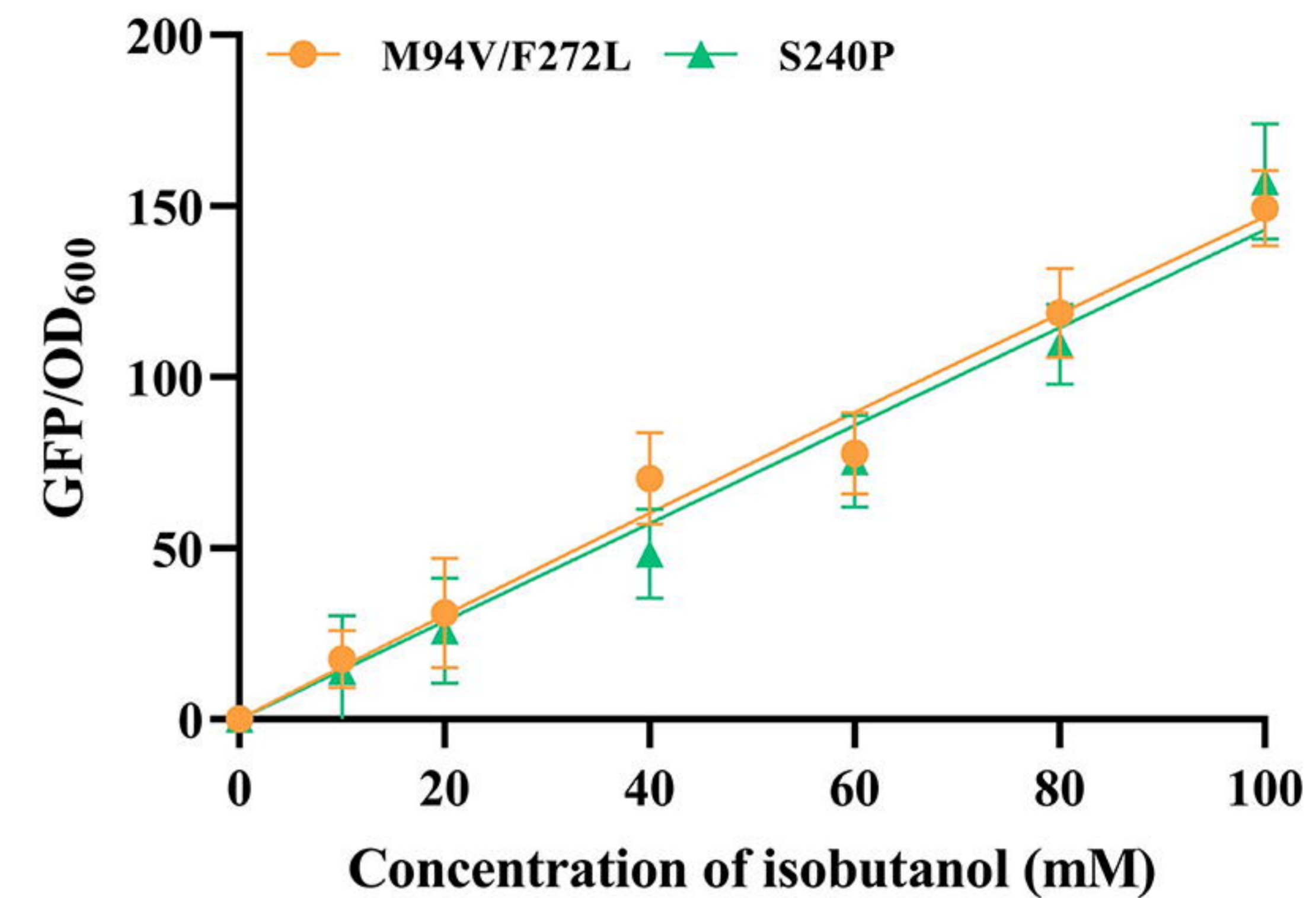
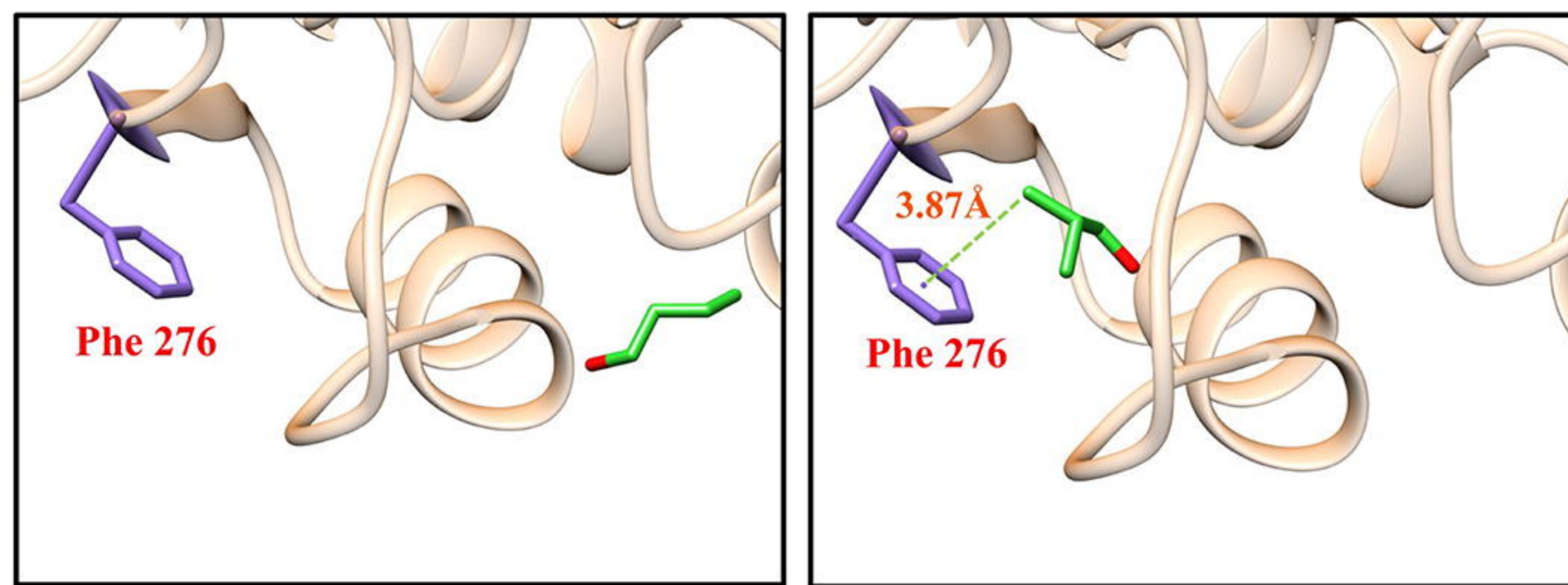
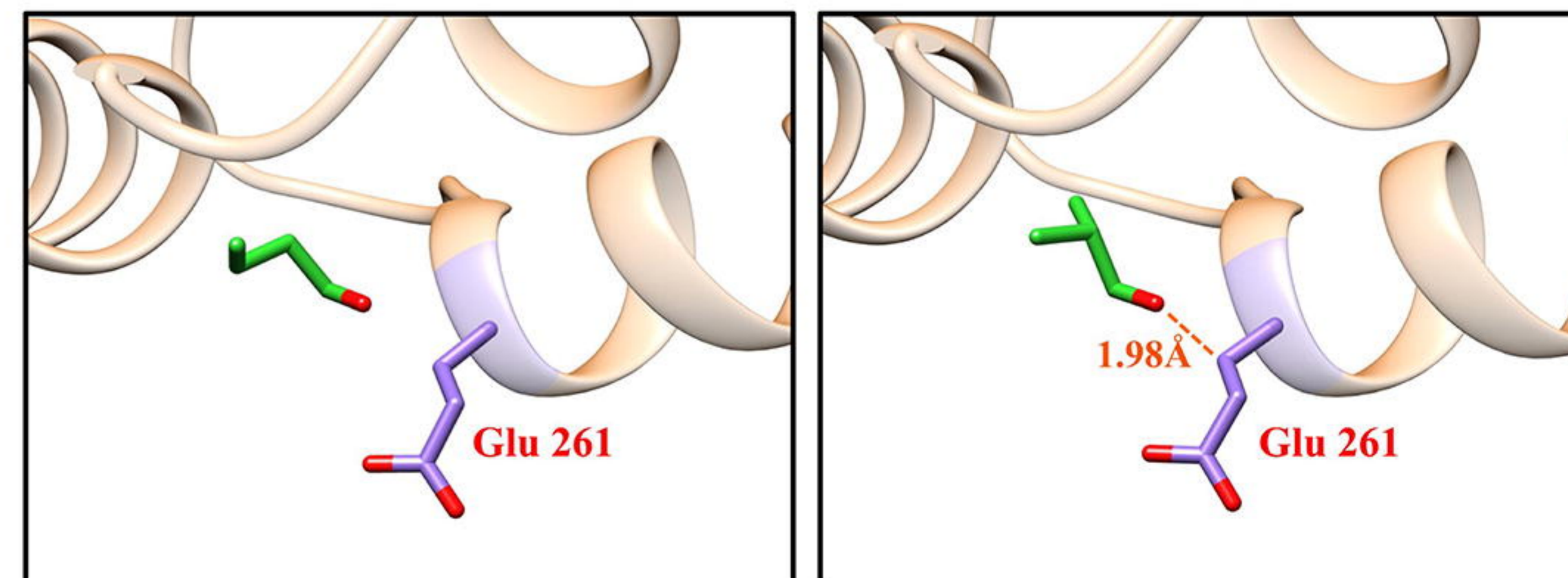
No interaction

E

I183T/D273N with n-butanol

I183T/D273N with isobutanol

No interaction

A**B****C****D****No interaction****M94V/F272L with n-butanol****M94V/F272L with isobutanol****E****No interaction****S240P with n-butanol****S240P with isobutanol**

