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# Interaction of picolinamide fungicide primary metabolites UK-2A and CAS -649 with the cytochrome bc 1 complex Qi site: mutation effects and modeling in *Saccharomyces cerevisiae*

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1 **Title: Interaction of picolinamide fungicide primary metabolites UK-2A and CAS-649 with the**  
2 **cytochrome *bc*<sub>1</sub> complex Qi site: mutation effects and modeling in *Saccharomyces cerevisiae***

3

4 **Running title: Effects of Qi site mutations on UK-2A and CAS-649**

5

6 **David H. Young,<sup>a,\*</sup> Brigitte Meunier<sup>b</sup> and Nick X. Wang<sup>a</sup>**

7

8 **Abstract**

9

10 **BACKGROUND:** Fenpicoxamid and florylpicoxamid are picolinamide fungicides targeting the Qi site of  
11 the cytochrome *bc*<sub>1</sub> complex, *via* their primary metabolites UK-2A and CAS-649, respectively. We  
12 explore binding interactions and resistance mechanisms for picolinamides, antimycin A and ilicicolin H in  
13 yeast by testing effects of cytochrome *b* amino acid changes on fungicide sensitivity and interpreting  
14 results using molecular docking.

15

16 **RESULTS:** Effects of amino acid changes on sensitivity to UK-2A and CAS-649 were similar, with highest  
17 resistance associated with exchanges involving G37 and substitutions N31K and L198F. These changes,  
18 as well as K228M, also affected antimycin A, while ilicicolin H was affected by changes at G37 and L198,  
19 as well as Q22E. N31 substitution patterns suggest that a lysine at position 31 introduces an electrostatic  
20 interaction with neighboring D229, causing disruption of a key salt-bridge interaction with  
21 picolinamides. Changes involving G37 and L198 imply resistance primarily through steric interference.  
22 G37 changes also showed differences between CAS-649 and UK-2A or antimycin A with respect to  
23 branched versus unbranched amino acids. N31K and substitution of G37 by large amino acids reduced  
24 growth rate substantially while L198 substitutions showed little effect on growth.

25

26 **CONCLUSION:** Binding of UK-2A and CAS-649 at the Qi site involves similar interactions such that general  
27 cross-resistance between fenpicoxamid and florylpicoxamid is anticipated in target pathogens. Some  
28 resistance mutations reduced growth rate and could carry a fitness penalty in pathogens. However,  
29 certain changes involving G37 and L198 carry little or no growth penalty and may pose the greatest risk  
30 for resistance development in the field.

31

32

33 **Keywords:** UK-2A; CAS-649; picolinamide fungicide; antimycin A; ilicicolin H; resistance mutation

34

35

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41

## 42 1 INTRODUCTION

43 Fenpicoxamid (Fig. 1) was recently introduced as a new fungicide which acts at the Qi site of the  
44 cytochrome  $bc_1$  complex (complex III) in the mitochondrial electron transport chain.<sup>1</sup> As the first Qi  
45 fungicide with efficacy against Ascomycete pathogens, fenpicoxamid provides a new mode of action for  
46 control of Septoria leaf blotch (*Zymoseptoria tritici*) and other diseases in cereals, as well as black  
47 Sigatoka (*Mycosphaerella fijiensis*) in bananas. Chemically, fenpicoxamid belongs to a new  
48 “picolinamide” class of fungicides and is derived from the natural product UK-2A,<sup>2</sup> a highly potent Qi site  
49 inhibitor.<sup>3,4</sup> UK-2A is structurally related to the Qi site inhibitor antimycin A and is produced by  
50 fermentation of *Streptomyces* s. 517-02.<sup>1</sup> Conversion of UK-2A to fenpicoxamid is achieved by addition  
51 of an isobutyryloxymethyl ether substituent. This modification to the hydroxyl substituent on the  
52 picolinamide head group increases stability on the plant surface, and results in greatly improved disease  
53 control.<sup>1</sup> Within the plant or pathogen, fenpicoxamid is then converted back to UK-2A, as the  
54 fungicidally active species.

55  
56 Exploration of structure-activity relationships for UK-2A analogs enabled the design of a fully synthetic  
57 new Qi fungicide florylpicoxamid (Fig. 1), which is currently in commercial development and lacks the  
58 complex 9-member macrocycle of UK-2A.<sup>5</sup> The primary metabolite CAS-649 is responsible for activity of  
59 florylpicoxamid, and is generated *in vivo* by removal of the acetyl substituent on the picolinamide head.<sup>5</sup>  
60 An important feature of florylpicoxamid is its strong efficacy against a broad range of Ascomycetes,  
61 including *Botrytis*, *Colletotrichum* and *Alternaria* species, as well as powdery mildew pathogens.<sup>6</sup> This  
62 will expand the use of picolinamide fungicides to many additional markets and provide a new mode of  
63 action for controlling important diseases in row crops, fruits and vegetables.

64

65 Fungicides which target the  $bc_1$  complex act at either the Qo or Qi sites that are formed by the central  
66 subunit of the multisubunit complex, namely cytochrome *b* (cyt *b*). They are referred to as QoI and QiI  
67 fungicides, respectively. Resistance to QoIs and QiIs is most commonly caused by cyt *b* changes at their  
68 respective target sites, such that resistance mutations at the Qo site do not reduce efficacy of QiI  
69 fungicides, and vice-versa.<sup>7,8</sup> In the specific case of picolinamide fungicides, the G143A change  
70 responsible for strobilurin-resistance does not reduce efficacy of fenpicoxamid and florylpicoxamid in *Z.*  
71 *tritici*,<sup>1,6,7</sup> and there is some evidence for negatively correlated cross-resistance.<sup>8</sup> Strobilurins represent  
72 the largest class of  $bc_1$  complex inhibitors, which when first introduced were believed to be at low risk  
73 for resistance development because the target protein, cyt *b*, is encoded by a mitochondrial gene.  
74 Multiple applications per season and use in solo applications rather than mixtures likely contributed to a  
75 rapid development of resistance.<sup>9</sup> The mutation G143A is responsible for efficacy loss in most cases of  
76 strobilurin resistance, while the F129L mutation confers lower levels of field resistance in *Phakopsora*  
77 *pachyrhizi* (soybean rust), *Pyrenophora teres* (net blotch in barley), *Alternaria solani* (potato/tomato  
78 early blight) and *Pythium aphanidermatum* (Pythium blight of turfgrass).<sup>10,11</sup> Effects of these mutations  
79 have been explained by their impacts on binding interactions at the Qo site in cyt *b* molecular docking  
80 experiments.<sup>12</sup> The QiI fungicides cyazofamid and amisulbrom were launched in 2001 and 2003,  
81 respectively, and are used to control Oomycete diseases. Cyt *b* modifications which cause resistance  
82 have been reported in *Plasmopara viticola* (grape downy mildew), involving the insertions E203-DE-  
83 V204 and E203-VE-V204, as well as substitution L201S.<sup>13</sup> Another Oomycete fungicide, ametoctradin, is  
84 believed to bind to both Qo and Qi sites<sup>14</sup> and the Qi site resistance mutation S34L has been reported.<sup>15</sup>  
85  
86 Baseline sensitivity monitoring for fenpicoxamid in *Z. tritici* populations has shown a relatively narrow  
87 range of sensitivities and no detectable resistance.<sup>9</sup> In the laboratory, however, mutants resistant to  
88 fenpicoxamid and UK-2A have been isolated in yeast and *Z. tritici*: amino acid substitutions N31K, G37C

89 and L198F at the Qi site conferred resistance in *Saccharomyces cerevisiae*,<sup>7</sup> and the mutation G37V was  
90 identified in lab mutants of *Zymoseptoria tritici* by selection in the presence of fenpicoxamid using an  
91 experimental evolution approach.<sup>8</sup> Target site changes conferring resistance to fenpicoxamid have not  
92 yet been reported in field populations. However, the aforementioned examples of resistance to  
93 commercialized  $bc_1$  complex inhibitor fungicides, as well as lab studies with fenpicoxamid, suggest that  
94 target site resistance to both fenpicoxamid and florypicoxamid must be anticipated and appropriate  
95 resistance management practices implemented. To preserve its effectiveness, fenpicoxamid should be  
96 applied only once per season and in mixture with another effective mode of action. Suitable resistance  
97 management strategies will also be employed for florypicoxamid when it is commercialized, as  
98 appropriate for the targeted disease and crop.

99

100 The isolation of resistant mutants in yeast, or modification of binding sites through introduction of  
101 specific amino acid changes, combined with crystallographic and modeling studies has been a successful  
102 strategy in understanding binding site interactions for a wide variety of  $bc_1$  complex inhibitors.<sup>16-19</sup>  
103 Moreover, *S. cerevisiae* is the only fungal species currently amenable to introduction of specific amino  
104 acid substitutions in the mitochondrially encoded *cyt b*. This allows proposed inhibitor docking poses to  
105 be evaluated experimentally, resulting in more robust binding site models. In addition, mechanisms  
106 responsible for target site resistance can be explored by analyzing effects of amino acid exchange. In our  
107 current study, we use yeast as a model system to compare binding site interactions and resistance  
108 mechanisms for UK-2A and CAS-649, as well as two other natural product Qi site inhibitors antimycin A  
109 and ilicicolin H, providing insights into similarities and differences between these structurally different  
110 ligands.

111

112 The ability of amino acid changes to disrupt target site interactions without significant fitness penalty  
113 will likely determine the identity of resistance mutations which might emerge in pathogen populations  
114 exposed to picolinamide fungicides. Consequently, similarities and differences between UK-2A and CAS-  
115 649 with respect to effects of mutations on sensitivity are of practical importance in assessing the  
116 likelihood of cross-resistance in pathogens on certain crops where both fenpicoxamid and  
117 florylpicoxamid might be registered, and in providing recommendations for resistance management.  
118

## 119 **2 MATERIALS AND METHODS**

### 120 **2.1 Cytochrome *b* mutants**

121 A wild-type diploid strain of *S. cerevisiae* containing an intron-free version of the cytochrome *b* gene was  
122 used as the parental strain. Mutants containing the G37C, L198F and N31K changes were isolated by  
123 selection on fenpicoxamid or antimycin A.<sup>7</sup> All other mutants were generated by site-directed  
124 mutagenesis and mitochondrial transformation by methods described previously.<sup>20,21</sup>

125

### 126 **2.2 Fungitoxicity assays**

127 Compounds were dissolved in DMSO and 3-fold dilutions in DMSO were prepared by serial dilution.  
128 Aliquots (1  $\mu$ L) of each dilution were transferred to wells of flat-bottomed 96-well microtiter plates, with  
129 three replicate wells for each treatment, and DMSO alone in control wells. Wells then received 100  $\mu$ L of  
130 YPEth broth (10 g of yeast extract, 20 g of Bacto peptone and 2% (v/v) of ethanol per liter). Cells taken  
131 from overnight cultures grown on YPD agar (Becton, Dickinson and Co., Sparks, MD, USA) at 30 °C were  
132 suspended in YPEth broth at a cell density of  $2 \times 10^5$  cells mL<sup>-1</sup>. Wells were inoculated with 100  $\mu$ L of cell  
133 suspension and the plates were incubated at 30 °C for 45 h. After mixing well contents to provide  
134 homogeneous cell suspension, growth was measured using a NEPHELOstar Galaxy plate reader (BMG  
135 Labtech, Cary, NC, USA) and 50% effective concentration (EC50) values for growth inhibition were  
136 determined using the CALCUSYN program (Biosoft, Cambridge, UK). Mutants were tested in two or more  
137 independent experiments and average EC50 values  $\pm$  SEM determined across all replicates.

138

### 139 **2.3 Molecular docking of ligands at the *S. cerevisiae* Qi site**

140 The crystal structure of the *S. cerevisiae* cytochrome *bc*<sub>1</sub> complex (PDB: 1EZV)<sup>22</sup> was used as the protein  
141 target. The structure was first parameterized and then refined in the QuickPrep of MOE (CCG Inc.,  
142 Montreal, Canada) using Amber14:EHT force field. After the protein system had been well prepared, UK-



143 2A, CAS-649, antimycin A and ilicicolin H were docked into the Qi site using the Induce Fit docking  
144 method with MMFF94x force field in MOE. Each docking run generated 30 docking poses. The most  
145 likely binding pose for each molecule was selected based on measured target site mutation effects and  
146 docking score.

147

#### 148 **2.4 Evaluation of kinetic growth rate**

149 Cells from overnight cultures grown on YPD medium at 30 °C were suspended in YPEth broth and  
150 adjusted to  $A_{600} = 0.3$  in a 1 cm cuvette. Cell suspensions (100  $\mu$ L) were added to wells of a 96-well flat-  
151 bottom tissue culture plate (Cat. No. 655180, Greiner Bio-One, Monroe, NC, USA) with 8 replicate wells  
152 per strain. The plate was sealed with Breathe-Easy™ sealing film (Cat. No. BEM-1, Diversified Biotech,  
153 Dedham, MA, USA). Growth curves were measured in a Cytation 3 instrument (BioTek Instruments,  
154 Winooski, VT, USA) at 30 °C using continuous double orbital shaking at 425 cpm with 3 mm amplitude  
155 and measurements taken at 1 h intervals. Specific growth rates were determined during exponential  
156 growth over the 6 - 11 h period after start of the experiment and used to calculate doubling time. All  
157 experiments were repeated twice with 8 replicates per experiment and growth rate  $\pm$  SD determined  
158 across all replicates.

159

## 160 **3 RESULTS AND DISCUSSION**

### 161 **3.1 Qi site mutants**

162 The Qi site mutant strains used in the study were selected based on various criteria (Table 1). Different  
163 amino acid substitutions at N31, G37 and L198 were designed to explore mechanisms responsible for  
164 reduced sensitivity of N31K, G37C/V and L198F, which confer resistance to UK-2A,<sup>7,8</sup> to characterize  
165 effects of these changes on CAS-649, antimycin A and illicicolin H, and evaluate natural variants found in  
166 fungal pathogens. Mutations Y16A and M221F were studied to investigate proposed roles in ligand  
167 binding of Y16<sup>23</sup> and F221,<sup>33</sup> a natural variant in *Z. tritici*. Several additional mutations known to confer  
168 reduced sensitivity to other Qi site ligands were also included to evaluate potential cross-resistance to  
169 UK-2A and CAS-649.

170

### 171 **3.2 Effects of mutations on inhibitor sensitivity**

172 Despite considerable structural differences between UK-2A and CAS-649, the effects of mutations at the  
173 Qi site on sensitivity were quite similar overall for the two molecules (Figs. 2 and 3). Among the various  
174 amino acid changes examined, those associated with high resistance factors for the two molecules  
175 involved various G37 changes, and substitutions N31K and L198F. The same changes affected sensitivity  
176 to antimycin A, although resistance factors were lower (Fig. 4). Sensitivity to antimycin A was also  
177 reduced by mutation K228M, which did not affect UK-2A or CAS-649. The similarity in mutation effects  
178 for UK-2A and CAS-649 in yeast suggests that cross-resistance between fenpicoxamid and  
179 florylpicoxamid is most likely to be observed in plant pathogens should target site resistance develop in  
180 the field.

181

182 Illicicolin H is structurally different from UK-2A, antimycin A and CAS-649, and is known to be affected by  
183 a number of Qi site mutations including S20I/L/T, Q22E/T, G37C/D/S, L198F and M221E/Q.<sup>19,25,26,30</sup> The

184 strong resistance observed in our study for Q22E, G37C/S and L198F (Fig. 5) is in agreement with  
185 previous work. M221Q conferred only a low level of resistance, consistent with the weak effect reported  
186 for this mutation at the enzyme level.<sup>26</sup> Whereas cross-resistance between illicicolin H, antimycin A and  
187 picolinamides was seen for L198F and G37 substitutions, differences between illicicolin H and the other  
188 ligands were apparent for N31K, which did not confer resistance to illicicolin H, and for Q22E which  
189 strongly reduced sensitivity to illicicolin H but did not affect the other inhibitors.

190

### 191 **3.3 Binding poses for UK-2A, CAS-649 and antimycin A**

192 The preferred docking poses for UK-2A and CAS-649 show the flat, polar picolinamide head located deep  
193 within the binding pocket and the bulky hydrophobic tail portions extending towards the outside of  
194 pocket (Figs. 6 and 7). For the head moiety, both molecules use the protonated pyridine N to form a  
195 strong salt bridge with the deprotonated carboxylic side chain of D229. As reported previously, this pose  
196 for UK-2A is strongly supported by analysis of yeast mutation effects on activity of analogs with different  
197 head groups and the importance of the pyridyl N for activity.<sup>7</sup> Different binding interactions are  
198 employed by antimycin A (Fig. 8), which instead forms two hydrogen bonds with D229 using the  
199 hydroxyl and formamide substituents of the phenyl head. The carbonyl of the formylamino group also  
200 forms a H-bond network via a water molecule with K228 and N31. UK-2A, CAS-649 and antimycin A  
201 share the same amide linker which forms an intramolecular hydrogen bond between the amide N and  
202 the OH substituent on the aryl ring to stabilize the co-planar conformation of the head. The tail portions  
203 of UK-2A and antimycin A share the same bislactone core but differ with respect to side chain  
204 substituents. UK-2A has 2-methyl propanoate and benzyl at the C-7 and C-8 positions, respectively,  
205 whereas antimycin A has 3-methyl butanoate and a hexyl moiety. Binding interactions for the tail  
206 portions of UK-2A, CAS-649 and antimycin A are primarily hydrophobic and the ligands are near residues  
207 G37 and L198, which can explain altered sensitivity in response to substitution of these residues.

208 In a *Z. tritici* binding model, an alternative binding orientation with the picolinamide head of UK-2A  
209 flipped by 180° had been postulated primarily to explain high potency towards *Z. tritici* of certain  
210 analogs with alternative head groups which lack the ability to form a salt bridge, yet are highly active  
211 against *Z. tritici*.<sup>33</sup> In this pose, the OH substituent on the picolinamide head binds to D229, rather than  
212 the pyridyl N, and this pose is enabled by pi-pi stacking with F221 as a key interaction. Since in yeast the  
213 pi-pi stacking interaction is not feasible due to a methionine instead of a phenylalanine at position 221,  
214 we used the yeast system to compare these two amino acids in terms of their impact on sensitivity to  
215 UK-2A and CAS-649, based on the assumption that an increase in sensitivity should be observed if pi-pi  
216 stacking with F221 was enabled to mimic the *Z. tritici* binding pocket. The fact that the M221F  
217 substitution did not alter sensitivity to UK-2A and CAS-649 (Figs. 2 and 3) argues against the suggested  
218 pi-pi interaction with F221 in *Z. tritici* as a key interaction for analogs with the picolinamide head. A  
219 critical role for F221 in binding is also not supported by sequence analysis of other pathogens since  
220 some pathogens which are highly sensitive to picolinamides, such as *Erysiphe necator*, *Blumeria graminis*  
221 and *Puccinia triticina*, have M221 like yeast, or I221 in the case of *Phakopsora pachyrhizi* (Table 2). While  
222 the potency against *Z. tritici* of picolinamide analogs with alternative head groups that lack the ability to  
223 form a salt bridge may indeed involve an alternative pose enabled by pi-pi stacking with F221, for  
224 picolinamides like UK-2A and CAS-649 the pose involving the salt bridge interaction between the pyridyl  
225 N and D229 is the most likely.

226 Another amino acid which has been proposed to play a possible role in binding of UK-2A is Y16,<sup>23</sup> a  
227 residue which is highly conserved across fungi. In the binding pocket this residue lies in close proximity  
228 to the benzyl substituent at C8 of the bislactone ring with the possibility of participation in a pi-pi  
229 stacking interaction. Although CAS-649 and UK-2A adopt a similar binding pose, pi-pi stacking with Y16  
230 would be less likely for CAS-649 because the branched *p*-fluoro phenyl substituent of CAS-649 is farther  
231 from Y16 than the benzyl of UK-2A. To test the possible role for Y16 in binding, we evaluated the Y16A

232 mutation since introduction of an alanine would negate any pi-pi stacking interaction. In fact, the Y16A  
233 change did not reduce sensitivity to UK-2A and CAS-649, but instead increased sensitivity, particularly  
234 for CAS-649 (Figs. 2 and 3). These results suggest that interaction with Y16 is not required for binding of  
235 picolinamides.

236

### 237 **3.4 Binding pose for ilicicolin H**

238 The most likely binding pose of ilicicolin H (Fig. 9) overlaps those of UK-2A, CAS-649 and antimycin A but  
239 involves different ligand-protein interactions. A key interaction involves H-bonding of the phenol head  
240 to Q22. The high level of resistance conferred by the Q22E mutation can be explained by loss of this  
241 interaction. In contrast to Q22E, the Q22M change reduced sensitivity to ilicicolin H only slightly  
242 suggesting that a methionine can substitute for glutamine in H-bonding. The hydroxyl group in the  
243 middle ring of ilicicolin H forms an intramolecular hydrogen bond with the nearby ketone group to  
244 position the bulky fused ring portion between the G37 and L198 residues. As for picolinamides and  
245 antimycin A, proximity to these residues explains the mutation effects resulting from amino acid  
246 substitution. Unlike the other ligands, no involvement of N31 or D229 is apparent in this binding pose.  
247 Our binding pose differs from an earlier model in *Candida albicans* in which a key interaction of the  
248 phenol moiety with N31 was proposed.<sup>30</sup> However, such an interaction is not consistent with the lack of  
249 effect of N31K on sensitivity (Fig. 5). Ilicicolin H is positioned very close to both G37 and L198, with  
250 closest distances of 3.6 and 3.5 Å, respectively. This close proximity suggests that replacement of these  
251 amino acids by larger residues would interfere with binding due to steric effects and likely explains the  
252 high levels of resistance associated with G37 changes and L198F (Fig. 5).

253

### 254 **3.5 N31 Mutations**

255 Since changes involving N31, G37 and L198 are the primary ones associated with reduced sensitivity to  
256 UK-2A and CAS-649, we explored the mechanisms responsible for resistance by comparing the effects of  
257 various substitutions at these positions. We also examined effects of these changes on growth rate in  
258 the hope of gaining insight into potential fitness penalties, which could determine mutations most likely  
259 to appear in pathogens in nature in response to fungicide selection pressure.

260

261 In the crystal structure of the  $bc_1$  complex from *Bos taurus* with bound antimycin A<sup>35</sup> and in our yeast  
262 models for binding of UK-2A and CAS-649, there is no direct interaction between the ligands and N31  
263 (N32 in *B. taurus*). According to the bovine structure, N31 participates in an extensive H-bonding chain  
264 involving other amino acids and water molecules: one water molecule forms a link between N31, K228  
265 and I26, while a second water links N31, S34 and D229.<sup>35</sup> The latter residue has been proposed to bind  
266 to the benzoquinone moiety of coenzyme Q6 and act as a proton donor during reduction of the  
267 substrate.<sup>22</sup> Exchange of N31 by other amino acids or substitution of S34 should disrupt this H-bonding  
268 network and would likely impact D229, potentially interfering with its key role in binding of picolinamide  
269 fungicides and antimycin A (Figs. 6 - 8). However, in the yeast crystal structure with the bound natural  
270 substrate coenzyme Q6 (ubiquinone 6),<sup>22</sup> N31 does not interact with D229 via water. It is not clear  
271 whether the apparent differences in binding of N31 to D229 via water between the bovine structure  
272 with bound antimycin A and the yeast structure with bound coenzyme Q6 are a consequence of  
273 different induced fit conformational changes during binding of antimycin A versus coenzyme Q6, the  
274 different protein sources, or methods for preparation of crystals. It is significant that resistance to UK-  
275 2A, CAS-649 and antimycin A was associated with introduction of the basic lysine side chain and not with  
276 acidic (aspartate), polar neutral (serine) or glycine residues, or with the S34L exchange (Figs. 2-4). If  
277 resistance from substitution of N31 was due to disruption of a H-bonding network, these other  
278 mutations would also be expected to confer resistance. A more likely explanation is that introduction of

279 a lysine at position 31 affects D229 by electrostatic lysine-aspartate attraction and prevents or weakens  
280 the critical ligand binding interactions involving D229. In Ascomycete and Basidiomycete pathogens, N31  
281 is conserved (Table 2), and in yeast its replacement by other amino acids resulted in markedly lower  
282 growth rates (Table 3). It is possible that this reflects a loss in efficiency of the  $bc_1$  complex resulting  
283 from an effect, via the H-bonding network mentioned above, on the nearby D229 through interference  
284 with its proposed catalytic function in substrate reduction. These considerations suggests that the N31K  
285 mutation, although conferring a high level of resistance in yeast, might carry a considerable fitness  
286 penalty if it were to occur in field populations of plant pathogens.

287

### 288 **3.6 G37 Mutations**

289 Among the G37 modifications explored, a high level of resistance to both UK-2A and CAS-649 was found  
290 for G37V/C/I/L, while G37M conferred a moderate level of resistance (Figs. 2 and 3). G37V/C/I/L also  
291 conferred resistance to antimycin A (Fig. 4) although resistance factors (RFs) were lower than for  
292 picolinamides. The data suggest a rough correlation between RFs and amino acid size, consistent with  
293 steric effects on binding since the highest RFs were found for G37V/C/I/L and the smallest for G37A/S.  
294 However, branching at the  $\beta$  carbon atom of amino acids also impacted the level of resistance in the  
295 case of UK-2A and antimycin A, since the branched amino acids valine and isoleucine produced  
296 significantly higher RFs than unbranched residues of comparable size, cysteine and leucine. Interestingly,  
297 the differential effect of branched and unbranched amino acids observed for these natural products,  
298 which have the same 9-membered macrocyclic scaffold, was not apparent for CAS-649. These data  
299 suggest that subtle differences in binding interactions may exist between UK-2A and CAS-649 in the  
300 region near G37. In the case of ilicicolin H, the exchange of G37 by other amino acids resulted in even  
301 higher RFs than observed for the other ligands, suggesting even more stringent steric limitations in this  
302 region of the binding site. The G37M exchange presented a curious exception to the general correlation

303 between level of resistance and residue size. Although methionine was the largest amino acid among  
304 those examined, the G37M change resulted in lower RFs against all ligands than was observed for  
305 substitution of G37 by various smaller amino acids. The reason for this is unknown but these data  
306 suggest that the G37M change may alter the binding pocket in such a way as to modulate the effect of  
307 size alone on ligand binding.

308

309 Pathogens which are most sensitive to fenpicoxamid and florylpicoxamid have a glycine or an alanine in  
310 position 37 (Table 2). This appears consistent with the relatively small effect of the yeast G37A mutation  
311 on sensitivity. *Fusarium* spp. are relatively insensitive to fenpicoxamid and florylpicoxamid (unpublished  
312 results, Corteva Agriscience) and contain L37 and M198. In yeast, the change L198M showed little effect  
313 on sensitivity to all ligands and no reduction in growth rate. However, G37L caused very high resistance  
314 to the picolinamides, suggesting that the L37 variant may explain insensitivity of *Fusarium* spp. Yeast  
315 mutant G37L+L198M was designed to mimic the *Fusarium* Qi site more closely, and showed interesting  
316 differences from the G37L mutant in terms of inhibitor sensitivity and growth rate. For G37L+L198M,  
317 resistance to UK-2A and antimycin A was lower than for the G37L mutant. However, this was not the  
318 case for CAS-649 and ilicicolin H which had equivalent RFs for both the single and double mutant.  
319 Moreover, in the double mutant, the L198M change compensated partially for a large reduction in  
320 growth rate associated with the G37L change. One can speculate that occurrence of these two variations  
321 together in *Fusarium* spp. may overcome effects of an otherwise deleterious G37L change, if it occurred  
322 alone.

323

324 Mutations involving G37 impacted growth rate to varying degrees (Table 3). Substitution of glycine by  
325 relatively small amino acids, alanine and serine, did not affect growth rate. Among mutants which  
326 showed strong resistance to picolinamides, G37S showed no reduction in growth rate, G37C showed a



327 slight reduction in growth rate, while growth of G37V/I/M was substantially reduced. It is interesting to  
328 note that G37C was the mutation identified in yeast through selection in the presence of fenpicoxamid<sup>7</sup>  
329 although G37V/I mutants have higher resistance factors. Similarly, for ilicicolin H G37V/I mutants had  
330 higher resistance factors than G37S. Yet G37S/D were the mutants isolated by selection on ilicicolin.<sup>25</sup> It  
331 is likely that the smaller fitness penalties in terms of growth rate for G37C/S favored their isolation in  
332 these cases. By contrast, in *Z. tritici* the only mutants obtained by selection on fenpicoxamid using  
333 experimental evolution contained the G37V mutation.<sup>8</sup> This mutation, which produced a high level of  
334 resistance to UK-2A, caused a lower efficiency of *bc*<sub>1</sub> complex in a cell-free *Z. tritici* mitochondrial assay  
335 but, unlike in yeast, did not affect the *in vitro* growth rate. These examples highlight the fact that the  
336 particular mutant(s) selected in the laboratory or field may differ depending on the organism, and is  
337 dependent on both the associated level of resistance and effect on growth or fitness.

338

### 339 3.7 L198 Mutations

340 The L198F mutation was found previously by selection in the presence of fenpicoxamid<sup>7</sup> and ilicicolin  
341 H,<sup>25</sup> and showed cross-resistance to antimycin A. This mutation also conferred resistance to CAS-649  
342 (Fig. 3). Binding poses indicate a similar distance of 3.8 or 3.9 Å between L198 and these various ligands  
343 (Figs. 6 – 9). L198F introduces the largest amino acid among the variants tested and showed the greatest  
344 mutation effect against all compounds. Other changes, involving smaller amino acids, had little or no  
345 effect on sensitivity to the picolinamides and antimycin A and relatively small effects on ilicicolin H.  
346 These data suggest that L198F confers resistance due to a steric clash with the ligands

347

348 For the L198F mutant, RFs for UK-2A and CAS-649 were lower than for some G37 mutants: G37V/I/L in  
349 the case of UK-2A, and G37I/L in the case of CAS-649. However, in contrast to those changes involving  
350 G37, substitution of L198F did not reduce growth rate (Table 3). Occurrence of F198 as a natural variant

351 in *Rhizoctonia solani* has been suggested as a possible explanation for the low sensitivity of this  
352 pathogens to fenpicoxamid.<sup>36</sup> The combination of a fairly high level of resistance and little impact on  
353 growth in the yeast model highlights L198F as a mutation of interest which potentially could lead to field  
354 resistance to picolinamide fungicides in plant pathogens.

355

## 356 **5 CONCLUSIONS**

357 Comparison of picolinamides with antimycin A and ilicicolin H showed both similarities and differences  
358 in mutation effects which could be explained by overlapping binding poses. For UK-2A and CAS-649,  
359 mutation effects were similar and molecular docking at the Qi site indicated common binding  
360 interactions. This suggests that cross-resistance between fenpicoxamid and florypicoxamid is likely in  
361 plant pathogens should target site resistance develop in the field. Florypicoxamid is intended for use to  
362 control diseases on many crops not targeted by fenpicoxamid, such that cross-resistance would have no  
363 practical implications. However, the likelihood of cross-resistance is an important consideration for  
364 resistance management in the case of crops like wheat and bananas where both compounds might be  
365 used in future years. Certain changes involving G37 and substitution L198F were associated with high  
366 levels of resistance and little or no effect on growth rate, as well as insensitivity in some species due to  
367 natural variants. Notwithstanding obvious limitations in use of a yeast model to predict resistance and  
368 disease-associated fitness in a different organism, our results highlight these residues as ones most likely  
369 to be involved in target site resistance to picolinamide fungicides in pathogens under selection pressure  
370 in the field.

371

## 372 **ACKNOWLEDGMENTS**

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374 and development of florylpicoxamid. We would also like to acknowledge Meiji Seika Pharma Co. Ltd. for  
375 their collaboration on the development of fenpicoxamid.

376

#### 377 **SUPPORTING INFORMATION**

378 Supporting information may be found in the online version of this article.

379

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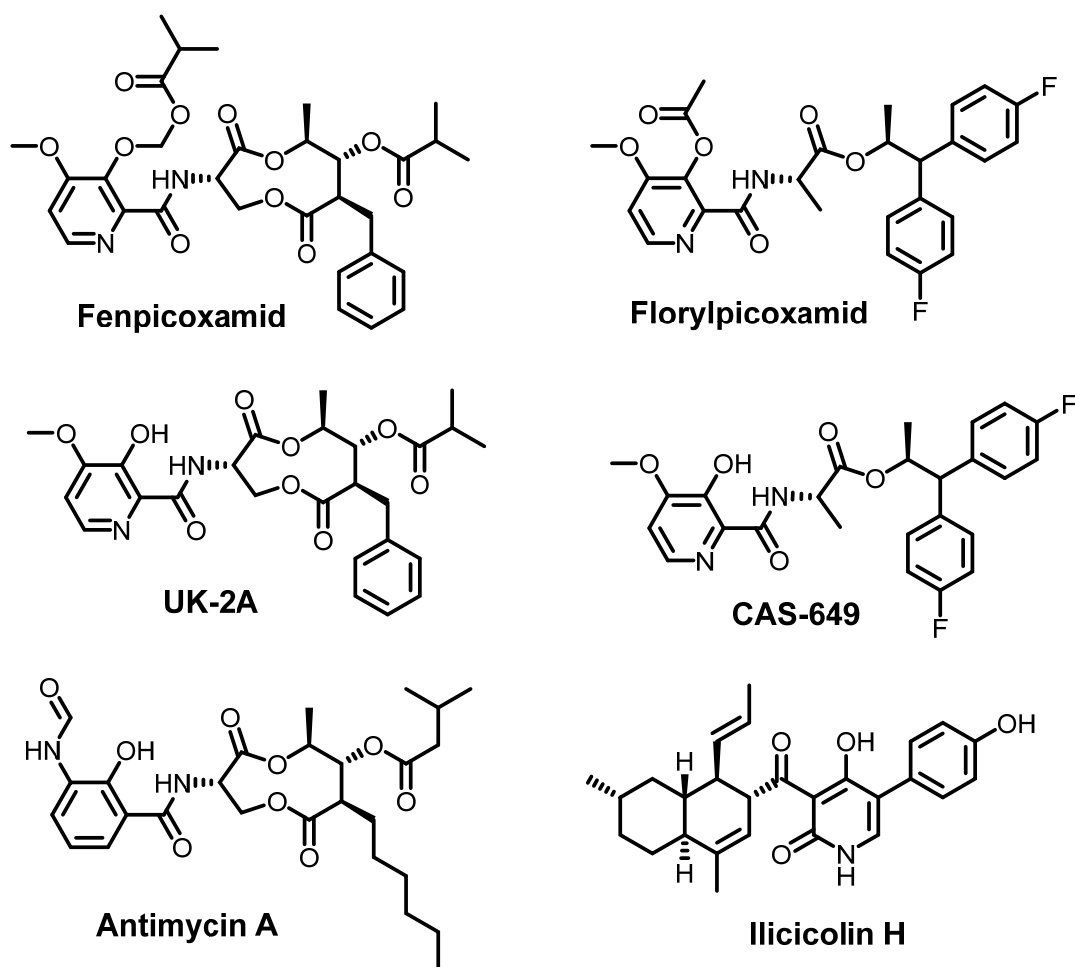
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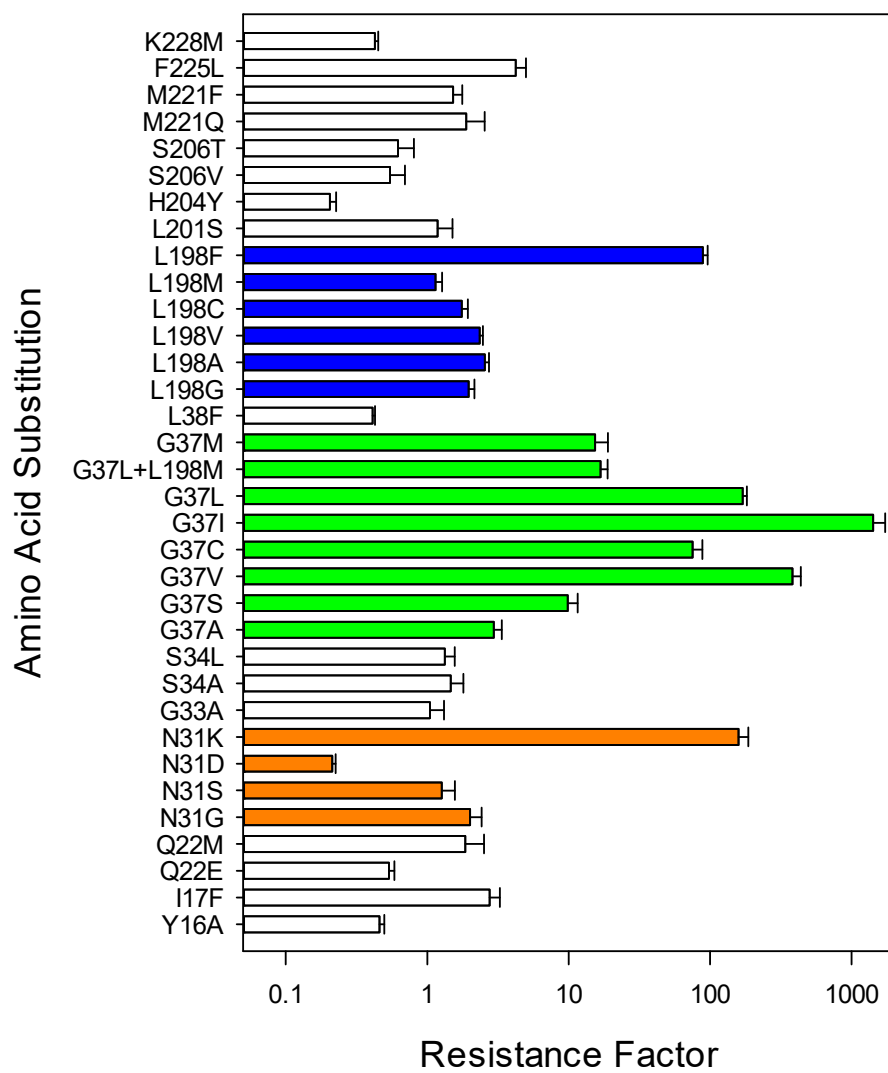
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**Figure 1.** Structures of picolinamide fungicides fenpicoxamid and florypicoxamid, their primary metabolites UK-2A and CAS-649, antimycin A and illicolin H.

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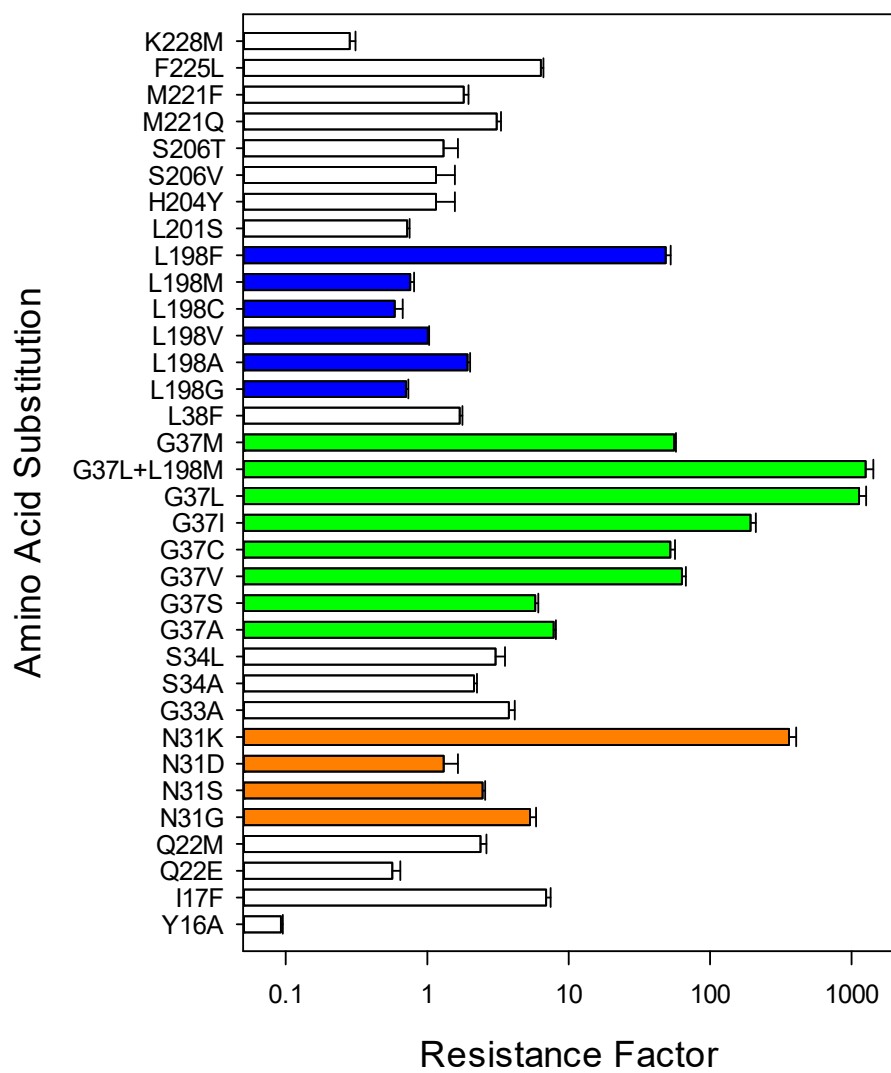
498 **Figure 2.** Effect of Qi site amino acid substitutions on sensitivity to UK-2A. Changes involving N31, G37

499 and L198 are highlighted in orange, green and blue, respectively. Resistance factor values represent

500 mean values, with SEM indicated by error bars.

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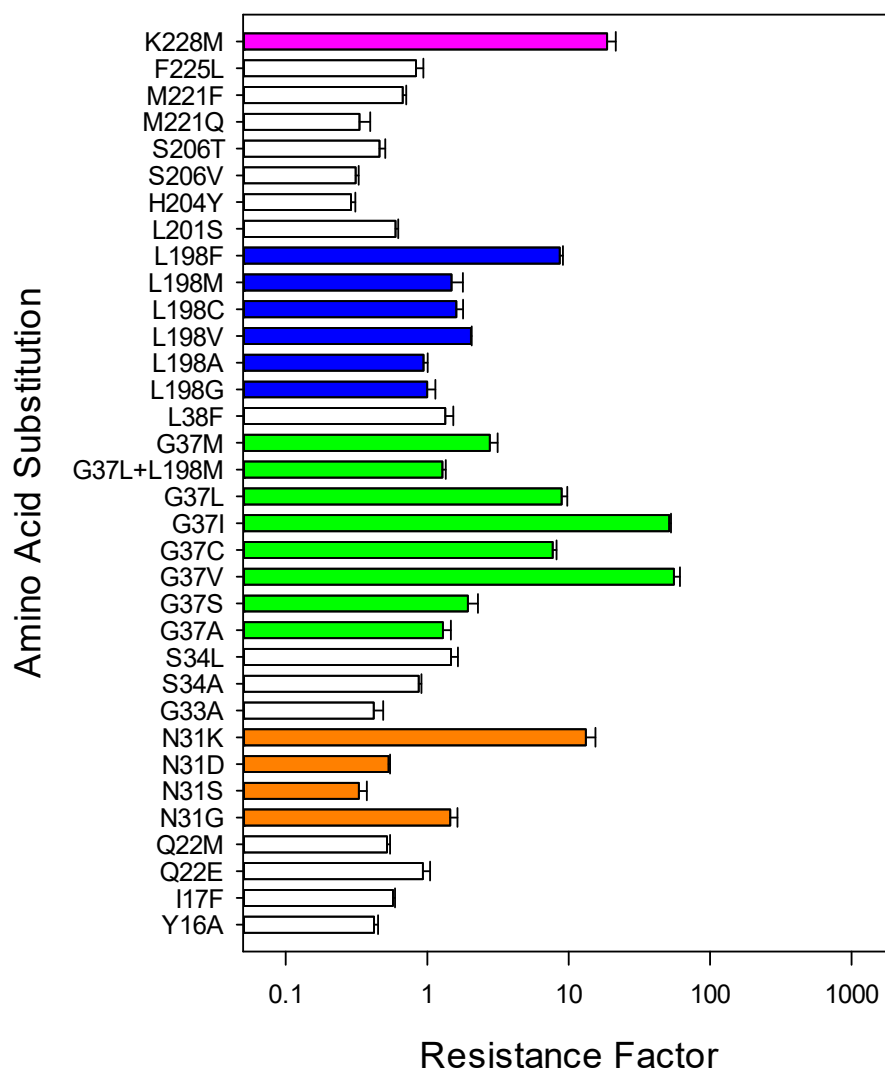
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504 **Figure 3.** Effect of Qi site amino acid substitutions on sensitivity to CAS-649. Changes involving N31, G37

505 and L198 are highlighted in orange, green and blue, respectively. Resistance factor values represent

506 mean values, with SEM indicated by error bars.

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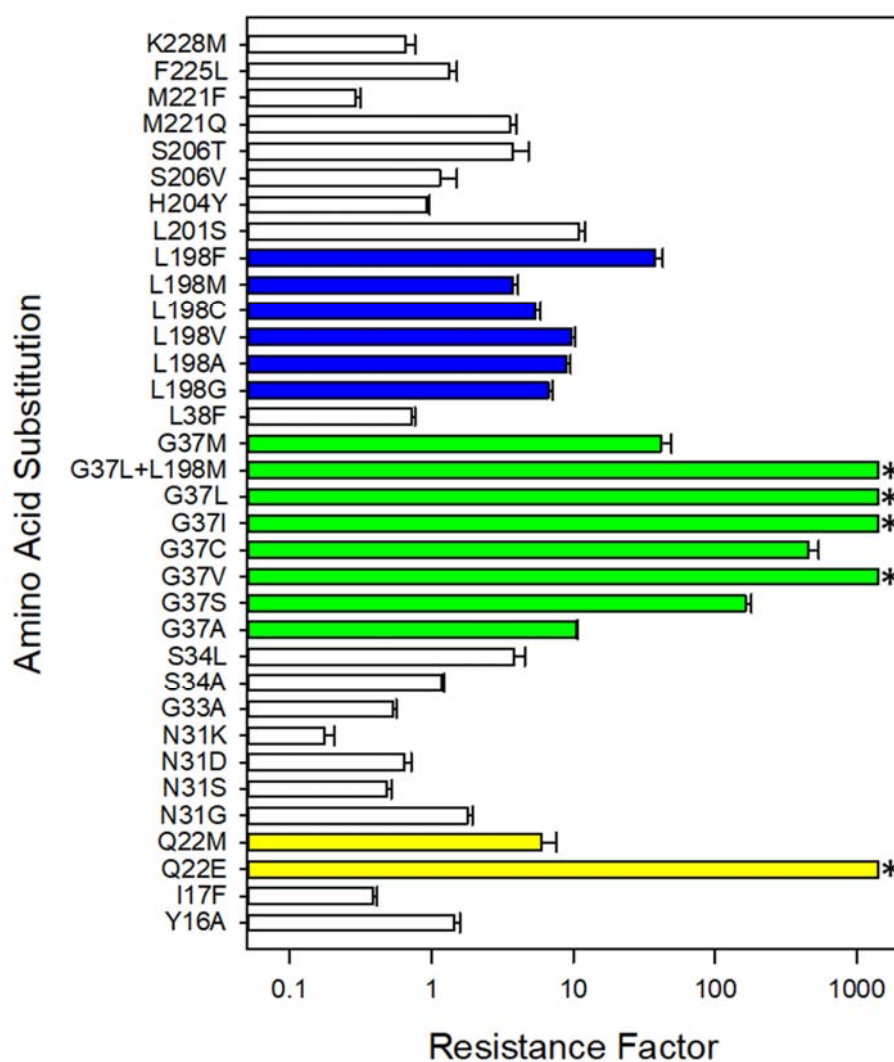
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509 **Figure 4.** Effect of Qi site amino acid substitutions on sensitivity to antimycin A. Changes involving N31,

510 G37, L198 and K228 are highlighted in orange, green, blue and pink, respectively. Resistance factor

511 values represent mean values, with SEM indicated by error bars.

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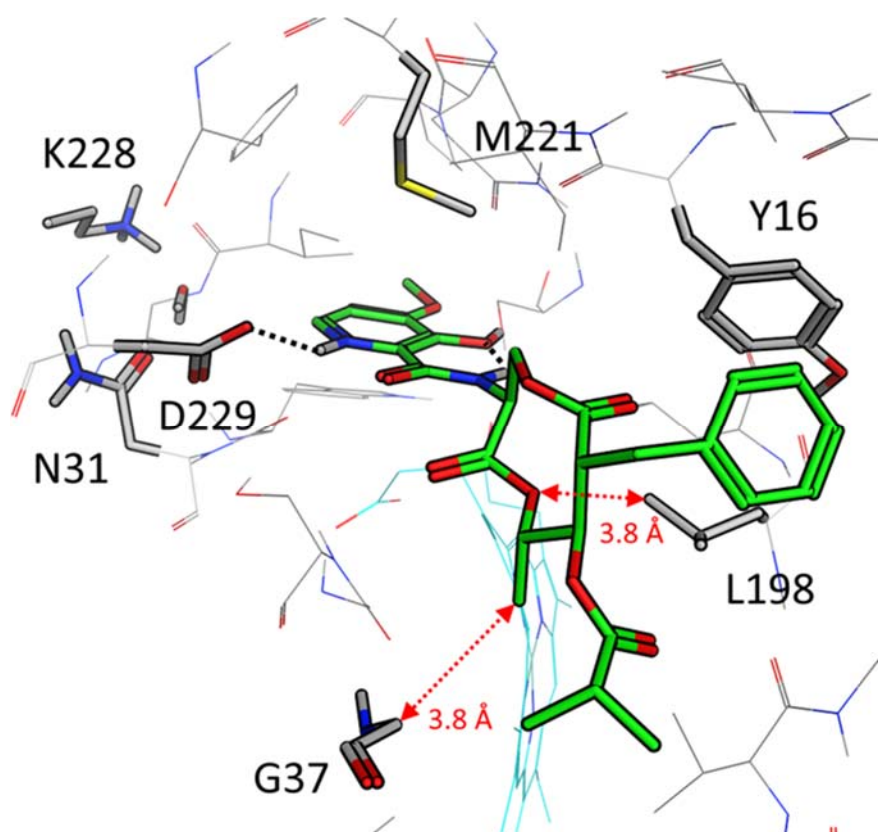
514 **Figure 5.** Effect of Qi site amino acid substitutions on sensitivity to ilicicolin H. Changes involving Q22,

515 G37 and L198 are highlighted in yellow, green and blue, respectively. Resistance factor values represent

516 mean values, with SEM indicated by error bars. Asterisks indicate RF &gt; indicated value due to &lt;50%

517 inhibition at highest test concentration of 10  $\mu\text{g mL}^{-1}$ .

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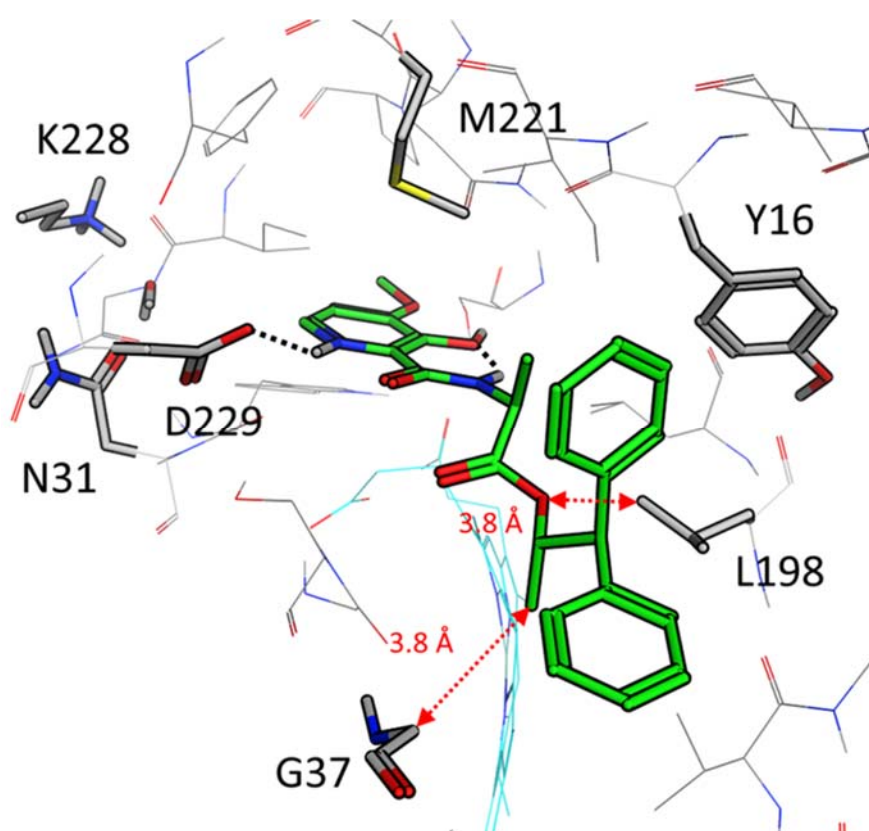
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521 **Figure 6.** Docking pose of UK-2A bound to the Qi site of cytochrome *b*, showing the salt bridge

522 interaction with D229. Red arrows indicate the distances (Å) between the ligand and residues G37 and

523 L198.

524



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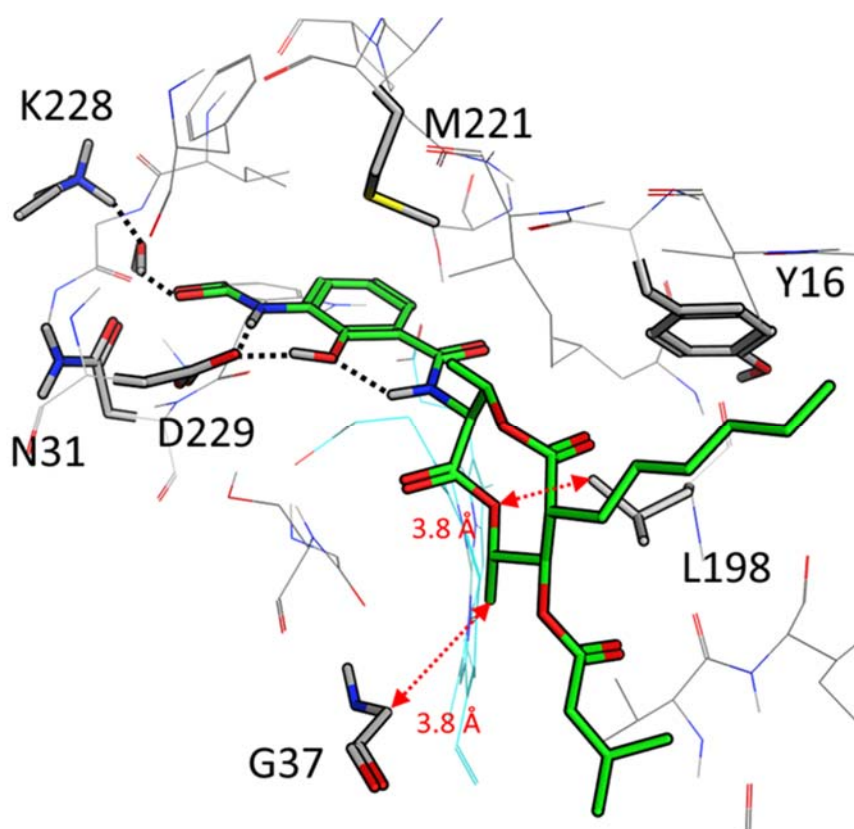
526

527 **Figure 7.** Docking pose of CAS-649 bound to the Qi site of cytochrome *b*, showing the salt bridge

528 interaction with D229. Red arrows indicate the distances (Å) between the ligand and residues G37 and

529 L198.

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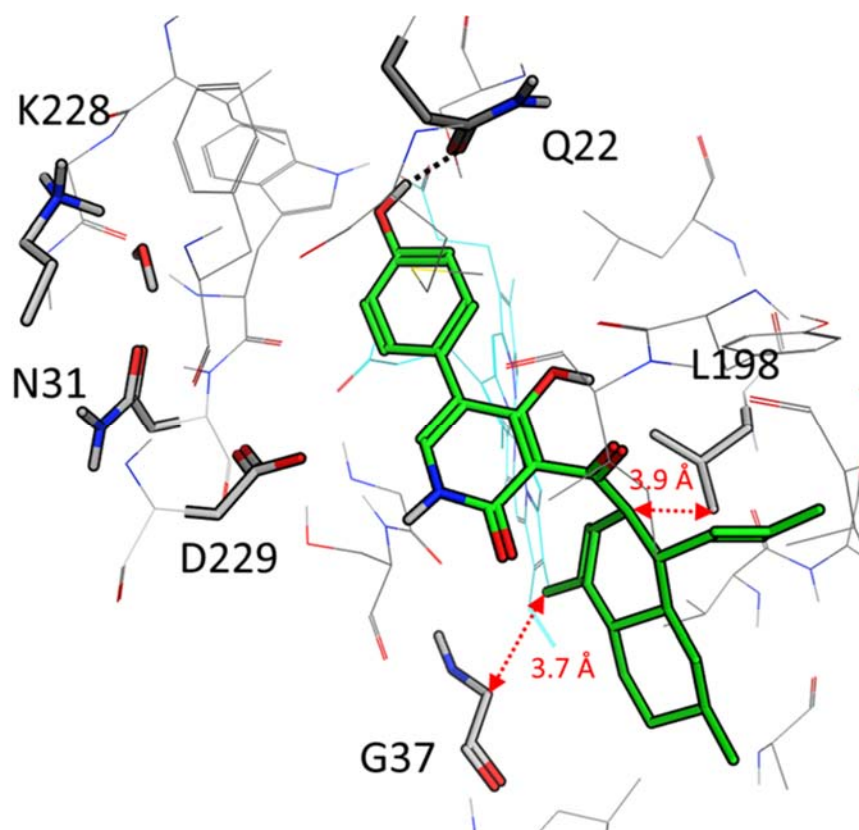
533 **Figure 8.** Docking pose of antimycin A bound to the Qi site of cytochrome *b*, showing the H-bond

534 interactions with D229 and K228. Red arrows indicate the distances (Å) between the ligand and residues

535 G37 and L198.

536





537

538

539 **Figure 9.** Docking pose of ilicicolin H bound to the Qi site of cytochrome *b*, showing the H-bond

540 interaction with Q22. Red arrows indicate the distances (Å) between the ligand and residues G37 and

541 L198.

542

543 **Table 1.** Yeast mutants used in the current study containing amino acid substitutions in cytochrome *b*  
 544 and relevance of mutations

545

Mutation	Relevance
Y16A	Y16 was hypothesized to participate in UK-2A binding <sup>23</sup>
I17F	Resistance to diuron and HQNO in <i>S. cerevisiae</i> <sup>24</sup>
Q22E/M	Q22E confers resistance to ilicicolin H in <i>S. cerevisiae</i> <sup>25,26</sup>
N31K	N31K confers resistance in <i>S. cerevisiae</i> to UK-2A, <sup>7</sup> antimycin A, diuron, and HQNO <sup>24,27</sup>
N31G/S/D	Exploration of substitution at N31
G33A	Resistance to funiculosin and HQNO in <i>S. cerevisiae</i> <sup>28</sup>
S34A/L	S34L confers resistance to ametoctradin in <i>Plasmopara viticola</i> <sup>15</sup>
G37A	Resistance to ML238, BRD6923 in <i>Plasmodium falciparum</i> <sup>29</sup>
G37S	Resistance to ilicicolin H in <i>S. cerevisiae</i> <sup>25</sup>
G37V	Resistance to UK-2A in <i>Z. tritici</i> , <sup>8</sup> antimycin A in <i>S. cerevisiae</i> , <sup>27</sup> ML238, BRD6923 and antimycin A in <i>Plasmodium falciparum</i> <sup>29</sup>
G37C	Resistance to UK-2A in <i>S. cerevisiae</i> <sup>7</sup> and ilicicolin H in <i>Candida albicans</i> <sup>30</sup>
G37I/M	Exploration of substitution at G37
G37L	Exploration of substitution at G37 and natural variant in <i>Fusarium</i> spp.
G37L+L198M	Natural variant in <i>Fusarium</i> spp.
L38F	Proximity to G37 and natural variant in <i>Z. tritici</i>
L198F	Resistance to UK-2A, antimycin A, <sup>7</sup> funiculosin <sup>31</sup> and ilicicolin H <sup>25</sup> in <i>S. cerevisiae</i>
L198G/A/V/C/M	Exploration of substitution at L198
L201S	Resistance to cyazofamid in <i>Plasmopara viticola</i> <sup>13</sup>
H204Y, S206T/V	Resistance to HQNO in <i>S. cerevisiae</i> <sup>32</sup>
M221F	F221 is a natural variant in <i>Z. tritici</i> and was hypothesized to participate in UK-2A binding <sup>33</sup>
M221Q	Resistance to HQNO, <sup>28</sup> ELQ 271 <sup>34</sup> and ilicicolin H <sup>26</sup> in <i>S. cerevisiae</i>
F225L	Resistance to diuron in <i>S. cerevisiae</i> <sup>27</sup>
K228M	Resistance to antimycin A in <i>S. cerevisiae</i> <sup>27</sup>

546

547 **Table 2.** Amino acids at positions 16, 31, 37, 198 and 221 in various plant pathogens based on yeast  
 548 numbering

Organism	NCBI Accession #	Amino Acid				
		16	31	37	198	221
<i>Saccharomyces cerevisiae</i>	YP_009144709	Y	N	G	L	M
<i>Zyoseptoria tritici</i> <sup>a</sup>	AAP81933	Y	N	G	L	F
<i>Pseudocercospora fijiensis</i> <sup>a</sup>	YP_009681541	Y	N	G	L	F
<i>Erysiphe necator</i> <sup>a</sup>	QQY98156	Y	N	G	L	M
<i>Cercospora beticola</i> <sup>a</sup>	AFQ23210	Y	N	G	L	F
<i>Puccinia triticina</i> <sup>a</sup>	YP_009680272	Y	N	G	L	M
<i>Phaeosphaeria nodorum</i> <sup>a</sup>	YP_001427390	Y	N	G	L	F
<i>Phakopsora pachyrhizi</i> <sup>a</sup>	ACT15476	Y	N	G	L	I
<i>Botrytis cinerea</i> <sup>a</sup>	ACL50595	Y	N	A	L	F
<i>Magnaporthe grisea</i> <sup>a</sup>	AAO91631	Y	N	A	L	F
<i>Blumeria graminis f. sp. tritici</i> <sup>a</sup>	QQY98353	Y	N	A	L	M
<i>Alternaria alternata</i>	QGA30432	Y	N	A	L	F
<i>Venturia inaequalis</i> <sup>a</sup>	AAB95255	Y	N	A	L	F
<i>Colletotrichum acutatum</i>	YP_009145212	Y	N	A	L	F
<i>Colletotrichum gloeosporioides</i>	AQT39566	Y	N	A	L	F
<i>Colletotrichum graminicola</i>	EFQ24843	Y	N	A	L	F
<i>Monilinia laxa</i>	ADF43785	Y	N	A	L	F
<i>Monilinia fructigena</i>	ADK35189	Y	N	A	L	F
<i>Monilinia fructicola</i>	ACT75643	Y	N	A	L	F
<i>Rhynchosporium commune</i> <sup>a</sup>	YP_008965385	Y	N	A	L	F
<i>Fusarium solani</i>	YP_005088108	Y	N	L	M	M
<i>Fusarium graminearum</i>	DAC73963	Y	N	L	M	M
<i>Fusarium oxysporum</i>	QJQ35616	Y	N	L	M	M
<i>Rhizoctonia solani</i>	YP_008082056	Y	N	G	F	F

549 <sup>a</sup>Highly sensitive to fenpicoxamid and/or florypicoxamid.<sup>6</sup>

550 **Table 3.** Effects of amino acid change at N31, G37 and L198 on doubling time

Change	Doubling time $\pm$ SD (h) <sup>a</sup>
None	4.33 $\pm$ 0.67 (n = 88)
N31G	6.21 $\pm$ 0.75 (n = 16)**
N31S	7.14 $\pm$ 1.27 (n = 16)**
N31D	5.84 $\pm$ 1.04 (n = 16)**
N31K	7.10 $\pm$ 0.66 (n = 16)**
G37A	4.22 $\pm$ 0.38 (n = 16)
G37S	4.14 $\pm$ 0.39 (n = 16)
G37V	7.36 $\pm$ 1.49 (n = 16)**
G37C	4.52 $\pm$ 0.60 (n = 16)
G37L	13.44 $\pm$ 3.50 (n = 16)**
G37L+L198M	8.26 $\pm$ 2.78 (n = 16)**
G37I	11.60 $\pm$ 2.47 (n = 16)**
G37M	7.78 $\pm$ 1.37 (n = 16)**
L198G	4.76 $\pm$ 0.69 (n = 16)*
L198A	4.53 $\pm$ 0.38 (n = 16)
L198V	4.25 $\pm$ 0.37 (n = 16)
L198C	3.74 $\pm$ 0.19 (n = 16)**
L198M	3.70 $\pm$ 0.20 (n = 16)**
L198F	3.91 $\pm$ 0.33 (n = 16)*

551 <sup>a</sup>One and two asterisks indicate a statistically significant difference in doubling time from the parent  
552 strain at P < 0.05 and P < 0.001, respectively (t-test).

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