Thiocillin and Micrococcin Exploit the Ferrioxamine Receptor of *Pseudomonas aeruginosa* for Uptake

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KEYWORDS: drug discovery, mechanism of uptake, thiopeptide, antimicrobial activity, siderophore, trojan horse,

ABSTRACT: Thiopeptides are a class of Gram-positive antibiotics that inhibit protein synthesis. They have been underutilized as therapeutics due to solubility issues, poor bioavailability, and lack of activity against Gram-negative pathogens. We discovered recently that a member of this family, thiostrepton, has activity against *Pseudomonas aeruginosa and Acinetobacter baumannii* under iron-limiting conditions. Thiostrepton uses pyoverdine siderophore receptors to cross the outer membrane, and combining thiostrepton with an iron chelator yielded remarkable synergy, significantly reducing the minimal inhibitory concentration. These results led to the hypothesis that other thiopeptides could also inhibit growth by using siderophore receptors to gain access to the cell. Here, we screened six thiopeptides for synergy with the iron chelator deferasirox against *P. aeruginosa* and a mutant lacking the pyoverdine receptors FpvA and FpvB. Our findings suggest that thiopeptides such as thiocillin cross the outer membrane using FoxA, the ferrioxamine siderophore receptor. Other structurally related thiopeptides did not inhibit growth of *P. aeruginosa*, but had greater potency against methicillin-resistant *Staphylococcus aureus* than thiostrepton and related thiopeptides. These results suggest that thiopeptide structures have evolved with considerations for target affinity and entry into cells.

Thiopeptides are a class of natural products with potent antibacterial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA)[1]. They are characterized chemically by the oxidation state of a central pyridine ring and by the size of a core macrocyclic ring comprised of thiazole moieties[2]. Thiopeptides act on the ribosome to inhibit protein translation but the exact mechanism depends on the number of members in the core macrocyclic ring: 26-membered macrocycles inhibit elongation factor G whereas 29-membered macrocycles block elongation factor Tu binding[3–5]. The exact mechanism of action for 35-membered macrocycles remains unknown, although they are reported to target the 50S ribosome, similar to thiostrepton (TS)[6]. TS is among the best-studied 26-membered thiopeptides and has multiple biological properties, including anticancer and antimalarial activities[7,8].

32 In 1948, Su isolated a strain of *Micrococcus* from sewage samples that produced a compound with
33 activity against Gram positive but not Gram negative bacteria[9]. The compound, micrococcin, was the first
34 thiopeptide to be discovered. Since then, the thiopeptide family has expanded to over 100 members of
35 diverse structure and origin[10]. Thiopeptides belong to a larger class of natural products called ribosomally
36 synthesized and post-translationally modified peptides (RiPPs)[11]. These natural products are produced
37 from a peptide precursor whose sequence varies depending on the thiopeptide, and undergo many post-
38 translational modifications to reach their final structure[12]. Most thiopeptides are produced by Gram-
39 positive soil and marine organisms including *Bacillus cereus, Streptomyces* spp., and *Nocardopsis* spp.,
40 although some Gram negatives such as *Serratia marcesens* have biosynthetic gene clusters encoding
41 thiopeptide synthesis[13–17].

42 Due to their poor solubility, challenging synthesis, and limited potency against Gram-negative
43 bacteria, pharmaceutical companies ruled out thiopeptides for development as clinically useful
44 antibiotics. Instead, researchers focused on addressing these issues through total synthesis, discovery of
45 new thiopeptides and structure elucidation, identification of thiopeptide biosynthetic gene clusters, and
46 modification of existing thiopeptides to improve solubility and activity [12,17–25]. However, there is a gap in
47 knowledge regarding their utility against Gram-negative bacteria which likely stems from the assumption
48 that thiopeptides cannot cross the outer membrane.

49 We showed recently that TS hijacks pyoverdine receptors to cross the outer membranes of
50 *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* among the World Health Organization's priority
51 pathogens for new antibiotic development[26 27] *P. aeruginosa* and *A. baumannii* are opportunistic
52 pathogens with intrinsic resistance to many antibiotics and are causative agents of healthcare-acquired
53 infections[28]. Both express receptors for pyoverdine, a siderophore with high affinity for iron. TS uptake
54 through pyoverdine receptors occurred under iron-limited conditions, and deferasirox (DSX), an FDA-
55 approved oral iron chelator, strongly synergized with TS to inhibit the growth of clinical isolates of both
56 species. Combinations of TS with other iron chelators were also effective[29]. These findings showed that
57 thiopeptides can cross the outer membranes of specific Gram-negative bacteria. TS lacks activity against
58 *Escherichia coli* because it does not express pyoverdine receptors. The thiopeptide family has over 100
59 members and recent bioinformatic analyses suggest that there could be hundreds more thiopeptides[30,31].
60 We hypothesized that other thiopeptides may also have activity against Gram-negative pathogens.

61 Here, we screened six thiopeptides (Figure 1, Supplementary Figure 1) for activity against *P. aeruginosa*
62 and potential synergy with DSX. Combinatorial assays showed that two of these used the ferrioxamine
63 siderophore receptor, FoxA, to enter the cell, confirming our hypothesis that other thiopeptides could
64 cross the outer membrane. The 26-membered macrocycles are of particular interest because

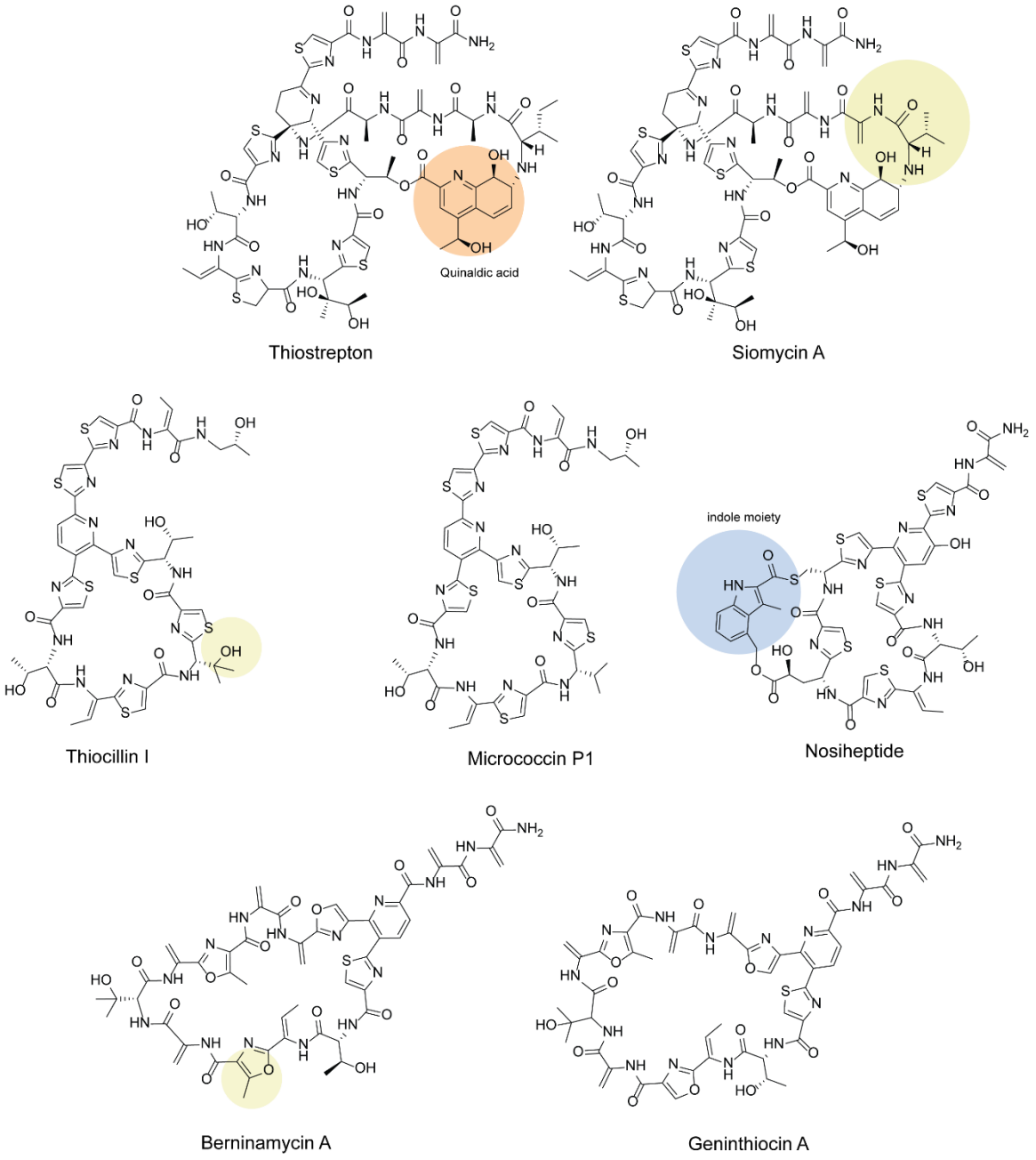

Figure 1. Structures of Thiopeptides Tested for Synergy with DSX. Differences between structural analogues are highlighted in yellow. The following are structural analogues: TS and SM, TC and MC, (26-membered macrocycles) and BER & GEN (35-membered macrocycles). NH is structurally similar to TC and MC, but has a unique indole moiety (blue). SM and TS have a unique quinaldic acid moiety (orange), but SM differs by two amino acids.

65 modifications to the core macrocyclic ring altered siderophore receptor specificity. Other modifications
66 abolished Gram-negative activity but improved Gram-positive activity. Thiopeptides are an underutilitzed
67 class of antibiotics with potential for development as clinically useful drugs, especially as iron limitation
68 at sites of infection could promote thiopeptide uptake.

69

70 RESULTS

71 *Siomycin A Uses the Pyoverdine Receptors for Uptake*

72 We screened the activity of six thiopeptides in combination with DSX against *P. aeruginosa* PA14
73 using checkerboard assays. We looked for individual thiopeptide activity, synergy, and activity against a
74 mutant lacking pyoverdine receptors FpvA and FpvB, which results in TS resistance. If the combination
75 was active against the wild type but not the *fpvAB* mutant, the thiopeptide likely uses those receptors to
76 cross the membrane. The six thiopeptides in the screen included siomycin A (SM), thiocillin I (TC),
77 micrococcin P1 (MC), nosiheptide (NH), berninamycin A (BER), and geninthiocin A (GEN) (Figure 1).

78 SM differs from TS in that the two amino acids adjacent to the quinaldic acid moiety are dehydroalanine
79 and isoleucine instead of alanine and leucine. TC and MC are nearly identical except that TC has a
80 hydroxyvaline and MC has a valine in its macrocyclic ring. BER and GEN differ by a single methyl group on

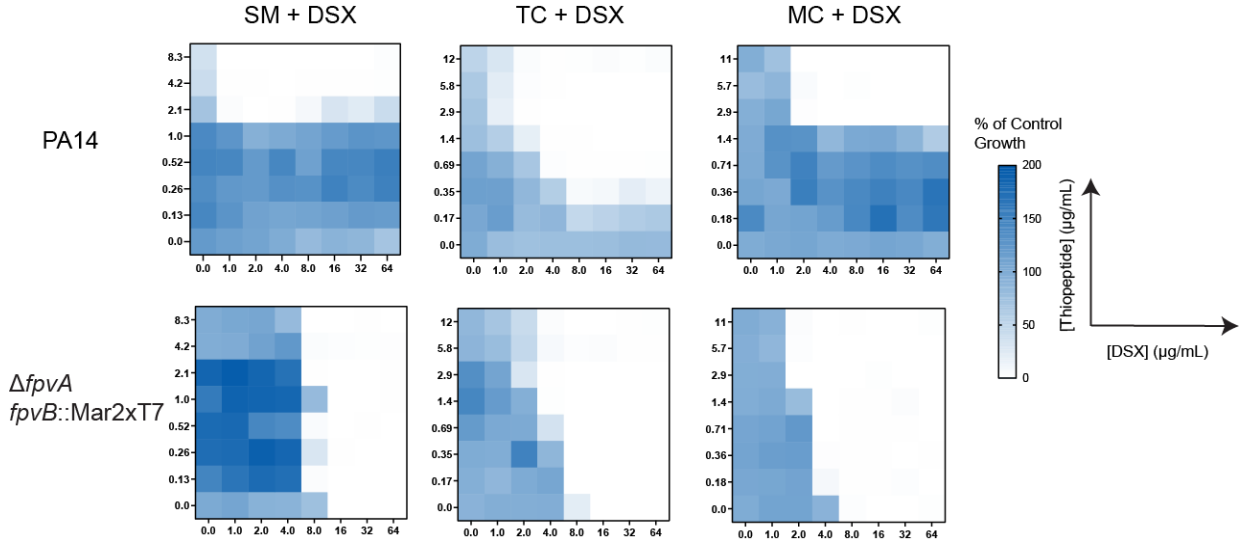

Figure 2. Thiopeptides synergize with DSX against PA14 and PA14 *ΔfpvA fpvB*::Mar2xT7. Checkerboard assays were conducted in 10:90 medium. Top row are checkerboards conducted with each thiopeptide against wild type PA14 whereas bottom row are checkerboards conducted against its pyoverdine receptor-null mutant. The concentration of the thiopeptides are indicated along the column (maximum concentration of 10 µM in µg/mL except for SM which was tested at 5 µM) and the concentration of DSX is indicated along the row (maximum concentration of 64 µg/mL). Growth is proportional to the intensity of blue whereas white indicates no growth. Results are averaged from three independent
81 experiments.

one of the oxazole rings. NH resembles TC and MC but is structurally unique from the other thiopeptides, with an indole moiety linked to the core macrocyclic ring to form a secondary ring. TS, SM, TC, MP, and NH belong to the 26-membered macrocycles whereas BER and GEN have 35-membered macrocycles.

Based on checkerboard assays, BER, GEN, and NH did not synergize with DSX (Supplementary Figure 1). SM synergized with DSX against wild type PA14 but the combination had no effect against the *fpvAB* mutant (Figure 2). Unlike the wild type, growth of the mutant was inhibited by 16 µg/ml DSX alone. These data are similar to those obtained previously with TS + DSX[26] and indicates that SM likely crosses the outer membrane using the pyoverdine receptors. However, the synergistic profile of SM + DSX was less pronounced compared to TS + DSX. There are two potential explanations for its weaker synergistic profile: 1) siomycin has less affinity for the target or 2) siomycin has less affinity for the receptors. To distinguish between these possibilities, we tested each thiopeptdide against the Gram-positive pathogen MRSA USA 300 in a broth dilution assay (Supplementary Figure 2). MRSA lacks the outer membrane; therefore, the main consideration would be differences in affinity for the highly-conserved ribosomal target. No differences between the minimal inhibitory concentration (MIC) of cells treated with TS or SM were observed, suggesting that the differences in susceptibility in *P. aeruginosa* were related to receptor affinity. Further, this result provides some structure-activity relationship (SAR) information regarding interactions between TS and the pyoverdine receptors, specifically that the alanine and leucine adjacent to the quinaldic acid do not affect target activity but improve uptake; however, they are not essential because SM can still use the pyoverdine receptors to cross the outer membrane.

Thiocillin I and Micrococcin P1 Exploit the Ferrioxamine Receptor

In checkerboard assays, TC and MC synergized with DSX against *P. aeruginosa* PA14 (Figure 2). The concentration of DSX was kept constant at 64 µg/mL, while the highest concentration of TC and MC tested due to solubility limitations was 12 µg/mL (10 µM). Remarkably, this combination inhibited growth of the *fpvAB* mutant (Figure 2). This result indicated that that TC and MC likely use a different siderophore receptor to enter the cell. To identify that receptor, we screened a panel of 16 siderophore receptor-deficient transposon mutants of PA14 (Supplementary Table 2). Only a *foxA* transposon mutant, lacking the ferrioxamine receptor, displayed resistance to TC + DSX (Figure 3AB). When the mutant was complemented with *foxA* in trans*,* susceptibility to TC was restored, confirming that *foxA* is necessary for TC uptake (Figure 3C). The susceptibility of the complemented strain was slightly reduced compared to the wild type, likely due to the low copy number of the vector in *P. aeruginosa*.

113

114 *TC and MC inhibit the growth of TS-resistant clinical*
115 *isolates*

116 In our previous studies, we identified *P.*
117 *aeruginosa* clinical isolates resistant to the TS + DSX
118 combination. One isolate, C0379, was resistant due
119 to a 5' truncation of the gene encoding the FpvB
120 receptor[26,29]. We hypothesized that TC or MC might
121 inhibit the growth of this isolate because the only
122 differences in its *foxA* gene compared to that of
123 PAO1 were five silent mutations and a single base
124 pair mutation (D760G) in one beta-strand
125 belonging to a structural component of the
126 receptor (Supplementary Table 3). Consistent with
127 this idea, C0379 was susceptible to combinations
128 of TC + DSX or MC + DSX (Supplementary Figure
129 3A). Bioinformatic analysis showed that *A.*
130 *baumannii* also encodes the FoxA receptor. We
131 tested one multidrug-resistant clinical isolate,
132 C0286, for susceptibility to TC + DSX and found
133 that it was susceptible even at the lowest
134 concentration of TC tested (0.17 µg/mL)
135 (Supplementary Figure 3B). However, the lab
136 strain ATCC 17978 was not susceptible to the
137 combination, suggesting inter-strain differences.
138 This is not unusual as it is well established that lab
139 adapted strains may not behave similarly to
140 clinical isolates[32]. Overall, we conclude that TC +
141 DSX can inhibit the growth of *P. aeruginosa* and *A.*
142 *baumannii* clinical isolates including those
143 resistant to TS.

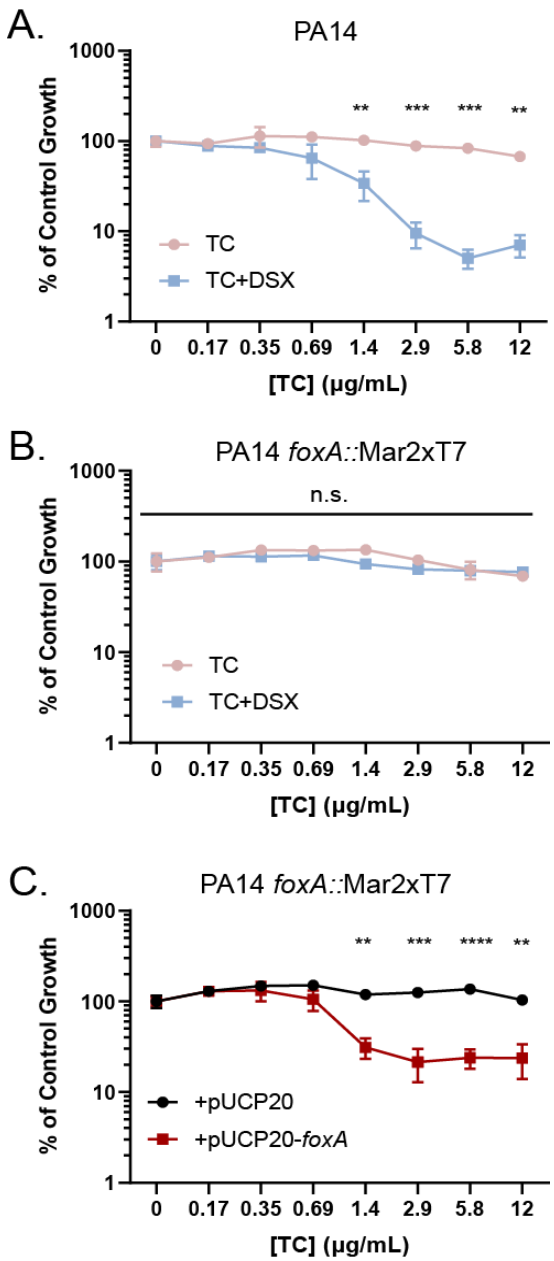

Figure 3. TC crosses the outer membrane through the FoxA siderophore receptor. Wild type PA14 (A) and PA14 foxA::Mar2xT7 (B) were challenged with TC (pink) and TC + DSX (blue). Dose response assays were conducted in 10:90. The transposon mutant was complemented with wild type PA14 foxA (C) and challenged with TC in VBMM. The vector control is in black whereas the complemented mutant is in red. Results were averaged from three independent replicates. n.s, not significant; **, p<0.01; ***, p<0.0005; ****, p<0.0001. Significance was determined using two-way ANOVA followed by Sidak's multiple-comparison test.

144

145 *A cocktail of TS + TC + DSX has an additive effect*

146 Our observation that thiopeptides
147 synergize with DSX against *P. aeruginosa*
148 demonstrated the potential to target gram-
149 negatives expressing either pyoverdine or
150 ferrioxamine receptors using thiopeptide-chelator
151 cocktails. Sub-MIC combinations of TS (0.1 µg/mL)
152 + 64 µg/mL DSX and TC (0.2 µg/mL) + 64 µg/mL
153 DSX, as well as TS + TC + DSX were tested against
154 PA14 in VBMM (Figure 4). Each double
155 combination inhibited growth to ~30% of control;
156 however, the triple combination decreased the
157 growth to only 10% of control. A multiplicative rule
158 approach was used to determine the effect of
159 combining both thiopeptides where the expected
160 growth is the product of the growth inhibition for
161 the individual combinations (i.e. TS + DSX or TC +
162 DSX). For TS + DSX, growth was reduced to 31% of
163 control and for TC + DSX, growth was reduced to
164 29% of control. Therefore, the expected growth for the combination of TC + TS + DSX would be 9% of
165 control. DSX alone had no effect on growth. The experimental value yielded 8.8% of control, suggesting
166 an additive effect of the thiopeptides. These results indicate that thiopeptide cocktails could offer an
167 advantage compared over single thiopeptide treatment by using different siderophore receptors to enter
168 the cell.

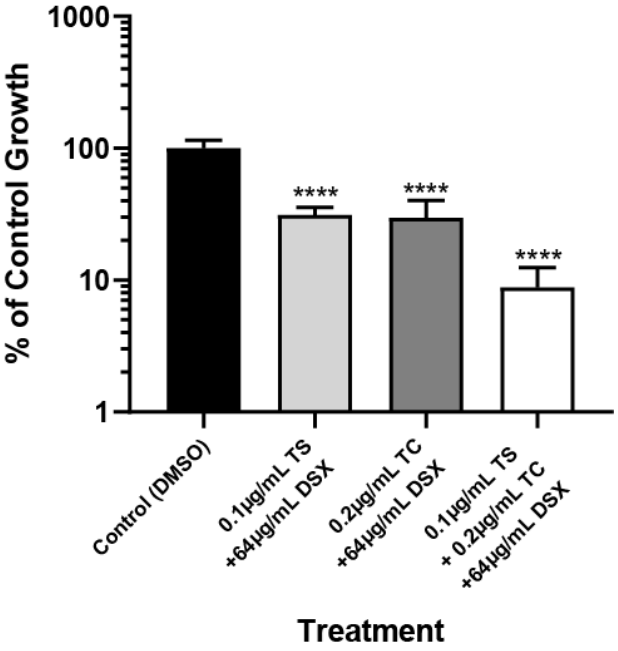

Figure 4. A thiopeptide cocktail significantly reduces PA14 growth. Control growth less than 20% was considered as the MIC cut-off. Assays were conducted in VBMM. Results are averaged from three independent experiments. ****,p<0.0001. Significance was determined using one-way ANOVA followed by Tukey honest significant difference post test.

169

170 *Thiopeptides target specific bacterial genera and species*

171 Unlike the pyoverdine receptors, the ferrioxamine receptor is not unique to Pseudomonads.
172 Therefore, we tested whether other Gram-negative bacteria were susceptible to TC. Bioinformatic
173 analyses of other priority Gram-negative pathogens such as *Escherichia coli, Klebsiella pneumoniae,* and
174 *Salmonella* Typhimurium showed that they carry *foxA* in their genome. However, they were not

175 susceptible to TC or the combination of TC + DSX
176 at the highest concentrations tested, suggesting
177 that the receptors are sufficiently different in
178 sequence to prevent recognition of TC as a
179 ligand. FoxA proteins from *S.* Typhimurium
180 SL1344 and *P. aeruginosa* PAO1 share 40% amino
181 acid sequence identity (Supplementary Figure 4).
182 Residues Y218, H374, and Q441 that interact
183 with the native ferroxamine ligand are conserved
184 in both species, indicating that TC and MC must
185 rely on interactions with other amino acids for
186 uptake into Pseudomonads[33].

187 To determine if there were differences
188 in susceptibilities between different species of
189 *Pseudomonas*, we conducted dose-response
190 assays with TC and TC + DSX against *P. putida*
191 KT2440, *P. marginalis* CVCOB 1152, *P. protegens*
192 Pf-5, and *P. fluorescens* PV5(Supplementary
193 Figure 5. Similar experiments were done with TS
194 and TS + DSX. We defined resistance if the
195 percent of control growth exceeded 80%,
196 susceptible if below 20%, and intermediate if
197 between 20-80%. Interestingly, there was
198 variability in the susceptibility to the
199 combinations between species. *P. putida* was resistant to TS but susceptible to TC (Figure 5,
200 Supplementary Figure 5). *P. protegens* was resistant to all combinations (Figure 5, Supplementary Figure
201 5). Alignments of the FoxA sequences for each species showed 65%, 65%, and 60% similarity for *P.*
202 *fluorescens, P. protegens,* and *P. putida* compared to *P. aeruginosa* PAO1. Despite the differences in
203 susceptibility to TC + DSX, the key residues that interact with the native ligand are conserved, similar to *S.*
204 Typhimurium, suggesting that TC interacts with other amino acids. To focus on possible sites of
205 interaction, we looked for residues shared between *P. aeruginosa*, *P. putida*, and *P. fluorescens*, but not

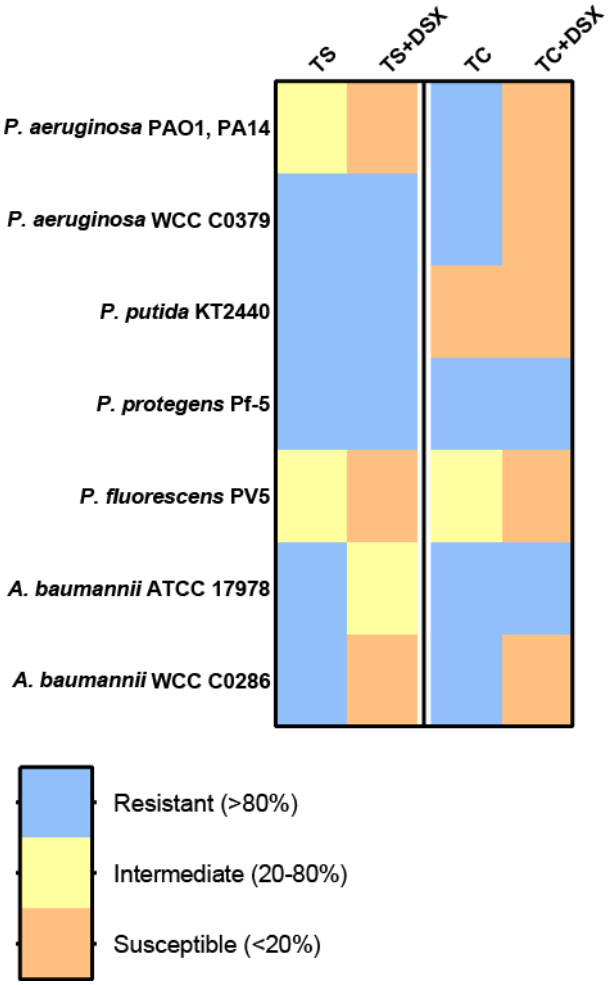

Figure 5. Susceptibility to TS and TC are different between species and strains. Susceptibility of *Pseudomonas* and *Acinetobacter* to TS and TC in combination with DSX. Blue boxes indicate resistance (>80% of control growth). Yellow boxes indicate intermedicate susceptibility (20-80% of control growth). Orange boxes indicate susceptible (<20% of control growth).

206 *P. protegens*, which was resistant to TC and TC + DSX (Figure 5, Supplementary Figure 5). A total of 17
207 residues were identified following this criterion and mapped onto a co-crystal structure of FoxA bound to
208 ferrioxamine (PDB ID: 6I96)[33]. Only three were found in proximity to the native ligand – A620(P), S612(A),
209 and D380(N) (Supplementary Table 4). A629(P) indicates that in the FoxA receptor of *P. aeruginosa*, *P.*
210 *putida,* and *P. fluorescens* at position 620, the amino acid is alanine. However, in *P. protegens* the amino
211 acid at the same position is proline. A620(P) and S612(A) are located in loop 7 of FoxA, which closes upon
212 interaction of the ligand with the active site[33]. Closure of loop 7 prevents the native ligand from
213 dissociation. D380(N) is close to conserved residue H374, which interacts with the native ligand.

214 A BLAST search using the PAO1 FoxA amino acid sequence against *P. aeruginosa, P. putida, P.*
215 *fluorescens* (all TC susceptible under low iron), and *P. protegens* (TC resistant) was conducted to see if
216 A620(P), S612(A), and D380(N) are conserved (Supplementary Figure 6). The top 100 hits were aligned
217 (MUSCLE), which revealed that alanine at position 620 and aspartic acid at position 380 are well conserved
218 in *P. aeruginosa, P. fluorescens,* and *P. putida*. Alanine was the most common amino acid at position 612
219 in *P. fluorescens* rather than serine, suggesting that this residue may not be important for TC interaction
220 with FoxA since this species was susceptible to TC + DSX. In *P. protegens,* which is resistant to TC + DSX,
221 lysine was the most abundant at residue 620. At residue 380, glutamine occurred more frequently. These
222 differences suggest that alanine and aspartic acid at residues 620 and 380 respectively may be involved
223 in TC uptake. These data suggest that TC uptake requires specific amino acid interactions that differ
224 between species.

225

226 *Nosiheptide is a potent inhibitor of MRSA USA 300*

227 The core structure of NH is nearly identical to TC and MC; however, a single indole is linked to the
228 core 26-membered macrocyclic ring (Figure 1). This small change in structure results in loss of synergy
229 with DSX (Supplementary Figure 1). Although synergy was not observed, there was a reduction in growth
230 when NH was combined with DSX. When tested against the *fpvAB* mutant, potentiation was lost
231 (Supplementary Figure 1).

232 There are examples – such as psychotrimine and daptomycin – of natural product antibiotics
233 containing tryptophan or its side chain, indole [34]. Daptomycin is a Gram-positive antibiotic that disrupts
234 the cytoplasmic membrane[35]. Like NH, daptomycin is a bulky molecule whose size prevents it from
235 crossing the outer membrane, making it ineffective against Gram-negative bacteria. Based on its unique
236 indole content, we hypothesized that NH might have improved efficacy against Gram-positive bacteria

237 compared to the other thiopeptides tested. All seven thiopeptides were tested in a dose-response assay
238 against MRSA USA 300, with 260 ng/mL as the highest concentration (Supplementary Figure 2). Consistent
239 with our hypothesis, NH was the most potent against MRSA, with a MIC of <4 ng/mL, followed by TS and
240 SM (32 ng/mL), TC and MC (130 ng/mL), and BER and GEN (>260 ng/mL). These results show that the
241 indole moiety of NH may confer an advantage against Gram-positives compared to other thiopeptides.

242

243 **DISCUSSION**

244 We previously showed that TS hijacked the pyoverdine receptors of *P. aeruginosa* and *A.*
245 *baumannii* to cross the outer membrane. These data informed the hypothesis that other thiopeptides
246 might similarly act as Trojan Horse antibiotics. In this work, we explored the ability of six other
247 thiopeptides to exploit siderophore receptors under iron-limited conditions. We showed that SM – a
248 thiopeptide structurally similar to TS – also uses pyoverdine receptors to enter cells. The differences in
249 structure of the two compounds provided SAR information. The IC_{50} of TS and SM are similar (0.1 µM vs
250 0.25 µM) based on *in vitro* studies of *E. coli* ribosomal activity[36]. This suggests that SM has weaker synergy
251 with DSX because of reduced affinity for the receptor compared to TS. Combinatorial analyses by
252 checkerboard assays suggested that TC and MC used a different siderophore receptor. We screened
253 mutants lacking various receptors in low iron growth media, leading to the discovery that those
254 thiopeptides used FoxA, a xenosiderophore receptor in *P. aeruginosa*. TC was also effective against a TS-
255 resistant *P. aeruginosa* clinical isolate. However, despite widespread expression of FoxA homologues,
256 susceptibility to TC was not universal among Gram-negative bacteria. Even within the genus
257 *Pseudomonas*, there was variability in susceptibility between species. Differences in the levels of
258 siderophore receptor expression could be a factor in the susceptibility profiles as a higher abundance
259 would allow more thiopeptide uptake.

260 This work suggests links between thiopeptide structure and activity against specific Gram-
261 negatives. Thiopeptides were thought to have little or no activity against Gram-negative pathogens due
262 to their inability to cross the outer membrane. However, thiopeptide producers are mainly marine and
263 soil bacteria, which inhabit environments shared by Pseudomonads. We propose that thiopeptide
264 structures evolved to target the siderophore receptors of cohabiting Gram-negatives. This is not
265 unprecedented as many other natural products can use such receptors to cross the outer membrane. For
266 example, *P. aeruginosa* produces pyocins that target the pyoverdine receptors of competing strains[37]..

267 NH failed to synergize with DSX, although the combination reduced growth. Potentiation was lost against
268 the *fpvAB* mutant. This result suggests that NH could have poor affinity for pyoverdine receptors, but
269 further work needs to be done to demonstrate this. We also showed that of the thiopeptides tested, NH
270 had the greatest activity against MRSA USA 300. The exact mechanism remains unknown, but while the
271 core of NH is nearly identical to TC and MC, the indole moiety makes it unique compared to the other
272 thiopeptides. NH does not synergize with DSX which suggests that the indole moiety could interfere with
273 its recognition by the FoxA receptor. Tryptophan and indole increase interactions of peptides with
274 membranes and can act as signaling molecules[38–41]. We speculate that the indole moiety may provide
275 enhanced peptide-membrane interactions, facilitating the ability of NH to cross the membrane and target
276 Gram-positive pathogens like MRSA. We observed a lower MIC with NH (<4 ng/mL) compared to TS (32
277 ng/mL) against MRSA in 10:90 medium, consistent with results from other groups using Mueller-Hinton
278 Broth[1,42]. In contrast, assays using purified ribosomes showed that TS had 40% greater inhibitory activity
279 compared to NH[43]. While NH has weaker inhibitory activity against purified ribosomes, it may compensate
280 with improved membrane permeability. NH has been tested against MRSA in an intraperitoneal mouse
281 infection model[1]. Mice treated with 20 mg/kg NH showed 90% survival over 5 days compared to 40% in
282 the vehicle control group.

283 Comparing TC and TS, it is apparent that the core macrocycle is nearly identical. However, TS has
284 a second macrocyclic ring with a structurally-distinct quinaldic acid moiety. It is likely that this second ring
285 allows TS to interact with the pyoverdine receptors rather than FoxA. In contrast, BER and GEN had no
286 activity against *P. aeruginosa* under iron-limitation. Even against MRSA, these 35-membered macrocycle
287 thiopeptides had no activity at the highest concentration tested (260 ng/mL). This is unsurprising as it was
288 previously reported that BER has weaker inhibitory activity on purified ribosomes compared to TS[6]. Our
289 results do not rule out the potential of 35-membered thiopeptides having other advantages. Furthermore,
290 in this study we did not test 29-membered thiopeptides such as GE2270A, thiomuracin A, and GE37468[44–
291 46]. These molecules could also have potential activity against *P. aeruginosa* and *A. baumannii* under iron-
292 limited conditions.

293 Schwalen et al. recently used a bioinformatics approach to search for enzymes that catalyze non-
294 spontaneous [4+2]-cycloadditions characteristic of thiopeptide biosynthesis. They identified 508 [4+2]-
295 cycloaddition enzymes, 372 of which were unique at the amino acid level[31]. These results suggest that
296 there are many more structurally unique thiopeptides than previously estimated. Diverse thiopeptides
297 may reveal interesting new activities with antimicrobial applications. One example is cyclothioazomycin,

298 a structurally distinct compound with activity against *Bacillus* spp., although weaker relative to other
299 thiopeptides[47]. Interestingly, this thiopeptide has potent antifungal activity via its interactions with
300 chitin[48]. It also was reported to be a human plasma renin inhibitor[49].

301 Trojan horse antibiotics are an interesting avenue for drug development. They have advantages
302 over antibiotics that must simply diffuse through the outer membrane because of their greater specificity
303 and uptake. Moreover, sites of infection are frequently iron-limited, which naturally potentiates such
304 molecules. Hosts produce iron sequestration proteins such as transferrin and lactoferrin that restrict iron,
305 a process called nutritional immunity[50–52].

306 While thiopeptides been overlooked for use in the clinic, we show the potential for these
307 antibiotics to be developed as selective Gram-negative antimicrobials that can target high priority
308 pathogens including *P. aeruginosa* and *A. baumannii*. Our work also supports the future development of
309 NH as an anti-MRSA therapeutic.

310

311 **METHODS**

312 Chemicals

313 Thiopeptides were purchased from Cayman Chemicals and stored at -20°C. Thiopeptide purity was at least
314 ≥95%. All thiopeptides were dissolved in dimethylsulfoxide (DMSO) with no solubility issues ranging from
315 1mg/mL to 20mg/mL. Deferasirox was purchased from AK Scientific (98% purity). A stock solution of 20
316 mg/mL in DMSO was made. Stock solutions were stored at -20°C until use and all serial dilutions were
317 prepared using fresh DMSO at 75x the final concentration.

318

319 Bacterial Strains and Culture Conditions

320 All bacterial strains used in this study are listed in Supplementary Table 1. Bacteria were inoculated from
321 glycerol stocks stored at -80°C and grown overnight in LB at 37°C with shaking (200 rpm). Bacteria were
322 subcultured (1/500 dilution) into fresh 10:90 media or Vogel-Bonner Minimal Media (VBMM) and grown
323 for 16 hours. Optical densities were standardized to OD_{600} 0.1 and diluted 1/500 into fresh 10:90 or
324 VBMM, which was used in microbroth dilution MIC assays or checkerboard assays.

325

326

327 Generation of PA14 f*oxA*::Mar2xT7 pUCP20-*foxA*

328 *foxA* was PCR amplified from wild type PA14 genomic DNA prepared using Qiagen genomic DNA isolation
329 kit. The following forward and reverse primers were used to obtain wild type *foxA* with SacI and XbaI
330 restriction sites respectively (underlined): forward 5'GAAGGAGCTCGTGGACGCTTGCTTTCGT and reverse
331 5'GCGTTCTAGAGACGCCGGCGAATGCCC. The PCR product and pUCP20 were digested with SacI and XbaI
332 (Thermo Scientific) using Thermo Scientific Fast Digest. The products were run on a 1% agarose gel for
333 30mins at 120V, cut out, and purified using GeneJET Extraction Kit (Thermo Scientific). The purified
334 linearized vector and digested *foxA* gene were ligated (1:3 molar ratio) overnight at 4°C using T4 DNA
335 ligase (Thermo Scientific). The construct was transformed into competent *E. coli* DH5α by heat shock at
336 42°C and plated on LB plates with 100 µg/mL ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-
337 galactopyranoside (IPTG) for blue-white screening (BioShop). Plates were incubated at 37°C overnight.
338 White single colonies were streaked on fresh LB plates with ampicillin to confirm that colonies contained
339 the construct. Minipreps were done using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and
340 quantified by NanoDrop. The *foxA* transposon mutant was grown overnight in LB. 1 mL was centrifuged
341 and washed twice with nuclease-free water (Qiagen). The cell pellet was resuspended in 500 µL of
342 nuclease-free water. 100 ng of the construct was added to 100 µL of resuspended cells and electroporated
343 (2.50 V). After a recovery period of 2 hours, cells were plated onto LB agar with 200 µg/mL carbenicillin
344 (Bioshop). Plates were incubated at 37°C overnight. Single colonies were streaked on fresh plates to
345 confirm successful electroporation. A single colony was picked and grown in liquid for MIC assays. The
346 susceptibility of the *foxA* transposon mutant harbouring pUCP20-*foxA* to TC indicated successful
347 complementation. Similarly, the same *foxA* transposon mutant was electroporated with empty pUCP20
348 as vector control.

349

350 Microbroth Dilution and Checkerboard Assays

351 Stock solutions were diluted in DMSO and 2µL of the resulting solutions plus 148µL of a bacterial
352 suspension standardized to an OD600 of ~ 0.1 and diluted 1:500 in 10:90 was added to a 96 well plate
353 (Nunc) in triplicate. Control wells contained 148µL of 10:90 + 2µL DMSO (sterility control) or standardized
354 bacterial suspension + 2µL DMSO (growth control). After incubating at 37°C for 16h, with shaking at 200
355 rpm, the OD600 was read using a plate reader (Thermo Scientific). Assays were performed in triplicate,
356 calculations were done in Microsoft Excel, and results were graphed using Prism (GraphPad) as a

357 percentage of the DMSO control. For wells with 2 compounds, such as increasing TS concentrations with
358 64µg/mL of DSX, 4µL of DMSO was used in the sterility and growth controls.
359
360 Checkerboard assays were set-up using Nunc 96-well plates in an 8-well by 8-well format. Two columns
361 were allocated for vehicle controls and two columns for sterility controls. Vehicle controls contained 4 µL
362 DMSO, in 146 µL of 10:90 inoculated with PA14 as described in Growth Curves. Sterile controls contained
363 the same components in 10:90, without cells. Serial dilutions of each thiopeptide – with 10 µM being the
364 highest final concentration – was added along the ordinate of the checkerboard (increasing concentration
365 from bottom to top) whereas serial dilutions of DSX – with 64 µg/mL being the highest final concentration
366 – was added along the abscissa (increasing concentration from left to right). The final volume of each well
367 was 150 µL and each checkerboard was repeated at least three times. Plates were incubated and the final
368 OD600 determined as detailed above. Checkerboards were graphed as heatmaps in Prism (GraphPad).
369
370 Bioinformatic Analysis
371 MUSCLE alignments were conducted using Unipro UGENE and Geneious Prime. Accession numbers of *P.*
372 *aeruginosa, P. fluorescens, P. protegens, P. putida,* and *S.* Typimurium FoxA are as follows: AAG05854.1,
373 WP_122305244.1, WP_003218248.1, AAY95537.2, NP_742329.1, and CBW16459.1. Genomic analyses for
374 mutations in WCC C0379 were done with breseq by Kara Tsang using *P. aeruginosa* PAO1 as the reference
375 genome.
376
377 ASSOCIATED CONTENT
378 **Supporting Information**
379 Table S1, list of strains; Table S2, mutants tested to show that TC uses the FoxA receptor to cross the outer
380 membrane; Table S3, breseq genomic annotations of the FoxA receptor for WCC C0379; Table S4, FoxA
381 amino acid residues that are different in *P. protegens* compared to other *Pseudomonas* spp; Figure S1,
382 checkerboard assays for thiopeptides that did not synergize with DSX; Figure S2, thiopeptide activity
383 against MRSA USA300; Figure S3, thiopeptide activity against *P. aeruginosa* and *A. baumannii* TS-resistant
384 clinical isolates; Figure S4, MUSCLE alignment of *S.* Typhimurium and *P. aeruginosa* FoxA receptors; Figure
385 S5, dose-response assays of thiopeptides against different *Pseudomonas* spp. Figure S6, Sequence logos
386 of FoxA amino acid sequences of *Pseudomonas* spp.

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392 **Author Contributions**

393 DCKC performed all experiments. DCKC and LLB wrote the paper.

394 **Funding Sources**

395 This work was funded by grants to LLB from the Natural Sciences and Engineering Research Council
396 (NSERC) RGPIN-2016-06521 and the Ontario Research Fund RE07-048. DCKC holds an NSERC Canadian
397 Graduate Scholarship – Master's Award.

398 **ACKNOWLEDGMENTS**

399 We thank Gerry Wright for strains from the Wright Clinical Collection, as well as Eric Brown and Brian
400 Coombes for the following strains: MRSA USA 300 and *K. pneumoniae*, and S. Typhimurium SL1344. We
401 thank John Whitney for the following strains: *P. protegens* Pf-5, and *P. putida* KT2440 and David Guttman
402 for *P. fluorescens*. We thank Kara Tsang for her help with breseq analyses. We thank Christy Groves for
403 assistance with the abstract graphic.

404 **ABBREVIATIONS**

405 TS, thiostrepton; TC, thiocillin; MC, micrococcin; NH, nosiheptide; MIC, minimal inhibitory concentration;
406 SM, siomycin; BER, berninamycin; GEN, geninthiocin; DSX, deferasirox; FpvA, ferripyoverdine receptor A;
407 FpvB, ferripyoverdine receptor B; FoxA, ferrioxamine receptor; WCC, Wright Clinical Collection; VBMM,
408 Vogel-Bonner Minimal Media; MRSA, methicillin-resistant *Staphylococcus aureus*; *E. coli*, *Escherichia coli*;
409 *K. pneumoniae, Klebsiella pneumoniae*; *S.* Typhimurium, *Salmonella* Typhimurium; *P. aeruginosa,*
410 *Pseudomonas aeruginosa*; *P. fluorescens*, *Pseudomonas fluorescens*; *P. putida*, *Pseudomonas putida*; *P.*
411 *protegens, Pseudomonas protegens; A. baumannii*, *Acinetobacter baumannii*.

412

413 **REFERENCES**

414 (1) Haste, N. M.; Thienphrapa, W.; Tran, D. N.; Loesgen, S.; Sun, P.; Nam, S. J.; Jensen, P. R.; Fenical,
415 W.; Sakoulas, G.; Nizet, V.; Hensler, M. E. Activity of the Thiopeptide Antibiotic Nosiheptide against

416 Contemporary Strains of Methicillin-Resistant Staphylococcus Aureus. *J. Antibiot. (Tokyo).* **2012**, *65* (12),
417 593–598. https://doi.org/10.1038/ja.2012.77.

418 (2) Bagley, M. C.; Dale, J. W.; Merritt, E. A.; Xiong, X. Thiopeptide Antibiotics. *Chemical Reviews*. 2005,
419 pp 685–714. https://doi.org/10.1021/cr0300441.

420 (3) Walter, J. D.; Hunter, M.; Cobb, M.; Traeger, G.; Spiegel, P. C. Thiostrepton Inhibits Stable 70S
421 Ribosome Binding and Ribosome-Dependent GTPase Activation of Elongation Factor G and Elongation
422 Factor 4. *Nucleic Acids Res.* **2012**, *40* (1), 360–370. https://doi.org/10.1093/nar/gkr623.

423 (4) Bowen, W. S.; Van Dyke, N.; Murgola, E. J.; Lodmell, J. S.; Hill, W. E. Interaction of Thiostrepton
424 and Elongation Factor-G with the Ribosomal Protein L11-Binding Domain. *J. Biol. Chem.* **2005**, *280* (4),
425 2934–2943. https://doi.org/10.1074/jbc.M407008200.

426 (5) Heffron, S. E.; Jurnak, F. Structure of an EF-Tu Complex with a Thiazolyl Peptide Antibiotic
427 Determined at 2.35 Å Resolution: Atomic Basis for GE2270A Inhibition of EF- Tu. *Biochemistry* **2000**, *39*
428 (1), 37–45. https://doi.org/10.1021/bi9913597.

429 (6) Thompson, J.; Cundliffe, E.; Stark, M. J. R. The Mode of Action of Berninamycin and the Mechanism
430 of Resistance in the Producing Organism, Streptomyces Bernensis. *J. Gen. Microbiol.* **1982**, *128* (4), 875–
431 884. https://doi.org/10.1099/00221287-128-4-875.

432 (7) Hegde, N. S.; Sanders, D. A.; Rodriguez, R.; Balasubramanian, S. The Transcription Factor FOXM1
433 Is a Cellular Target of the Natural Product Thiostrepton. *Nat. Chem.* **2011**, *3* (9), 725–731.
434 https://doi.org/10.1038/nchem.1114.

435 (8) Aminake, M. N.; Schoof, S.; Sologub, L.; Leubner, M.; Kirschner, M.; Arndt, H. D.; Pradel, G.
436 Thiostrepton and Derivatives Exhibit Antimalarial and Gametocytocidal Activity by Dually Targeting
437 Parasite Proteasome and Apicoplast. *Antimicrob. Agents Chemother.* **2011**, *55* (4), 1338–1348.
438 https://doi.org/10.1128/AAC.01096-10.

439 (9) SU, T. L. Micrococcin, an Antibacterial Substance Formed by a Strain Of. *Br. J. Exp. Pathol.* **1948**,
440 *29* (5), 473–481.

441 (10) Li, J.; Qu, X.; He, X.; Duan, L.; Wu, G.; Bi, D.; Deng, Z.; Liu, W.; Ou, H. Y. ThioFinder: A Web-Based
442 Tool for the Identification of Thiopeptide Gene Clusters in DNA Sequences. *PLoS One* **2012**, *7* (9).
443 https://doi.org/10.1371/journal.pone.0045878.

444 (11) Arnison, P. G.; Bibb, M. J.; Bierbaum, G.; Bowers, A. A.; Bugni, T. S.; Bulaj, G.; Camarero, J. A.;
445 Campopiano, D. J.; Challis, G. L.; Clardy, J.; Cotter, P. D.; Craik, D. J.; Dawson, M.; Dittmann, E.; Donadio,

446 S.; Dorrestein, P. C.; Entian, K. D.; Fischbach, M. A.; Garavelli, J. S.; Göransson, U.; Gruber, C. W.; Haft, D.
447 H.; Hemscheidt, T. K.; Hertweck, C.; Hill, C.; Horswill, A. R.; Jaspars, M.; Kelly, W. L.; Klinman, J. P.; Kuipers,
448 O. P.; Link, A. J.; Liu, W.; Marahiel, M. A.; Mitchell, D. A.; Moll, G. N.; Moore, B. S.; Müller, R.; Nair, S. K.;
449 Nes, I. F.; Norris, G. E.; Olivera, B. M.; Onaka, H.; Patchett, M. L.; Piel, J.; Reaney, M. J. T.; Rebuffat, S.; Ross,
450 R. P.; Sahl, H. G.; Schmidt, E. W.; Selsted, M. E.; Severinov, K.; Shen, B.; Sivonen, K.; Smith, L.; Stein, T.;
451 Süssmuth, R. D.; Tagg, J. R.; Tang, G. L.; Truman, A. W.; Vederas, J. C.; Walsh, C. T.; Walton, J. D.; Wenzel,
452 S. C.; Willey, J. M.; Van Der Donk, W. A. Ribosomally Synthesized and Post-Translationally Modified
453 Peptide Natural Products: Overview and Recommendations for a Universal Nomenclature. *Natural*
454 *Product Reports*. Royal Society of Chemistry 2013, pp 108–160. https://doi.org/10.1039/c2np20085f.

455 (12) Brown, L. C. W.; Acker, M. G.; Clardy, J.; Walsh, C. T.; Fischbach, M. A. Thirteen Posttranslational
456 Modifications Convert a 14-Residue Peptide into the Antibiotic Thiocillin. *Proc. Natl. Acad. Sci. U. S. A.*
457 **2009**, *106* (8), 2549–2553. https://doi.org/10.1073/pnas.0900008106.

458 (13) Khilyas, I. V.; Tursunov, K. A.; Shirshikova, T. V.; Kamaletdinova, L. K.; Matrosova, L. E.; Desai, P.
459 T.; McClelland, M.; Bogomolnaya, L. M. Genome Sequence of Pigmented Siderophore-Producing Strain
460 Serratia Marcescens SM6. *Microbiol. Resour. Announc.* **2019**, *8* (18). https://doi.org/10.1128/mra.00247-
461 19.

462 (14) Cheng, T. H.; Saidin, J.; Danish-Daniel, M.; Gan, H. M.; Isa, M. N. M.; Bakar, M. F. A.; Ismail, N.
463 Genome Sequence of Serratia Marcescens Subsp. Sakuensis Strain K27, a Marine Bacterium Isolated from
464 Sponge (Haliclona Amboinensis). *Genome Announc.* **2018**, *6* (6).
465 https://doi.org/10.1128/genomeA.00022-18.

466 (15) Engelhardt, K.; Degnes, K. F.; Kemmler, M.; Bredholt, H.; Fjaervik, E.; Klinkenberg, G.; Sletta, H.;
467 Ellingsen, T. E.; Zotchev, S. B. Production of a New Thiopeptide Antibiotic, TP-1161, by a Marine
468 Nocardiopsis Species. *Appl. Environ. Microbiol.* **2010**, *76* (15), 4969–4976.
469 https://doi.org/10.1128/AEM.00741-10.

470 (16) Trejo, W. H.; Dean, L. D.; Pluscec, J.; Meyers, E.; Brown, W. E. Streptomyces Laurentii, a New
471 Species Producing Thiostrepton. *J. Antibiot. (Tokyo).* **1977**, *30* (8), 639–643.
472 https://doi.org/10.7164/antibiotics.30.639.

473 (17) Acker, M. G.; Bowers, A. A.; Walsh, C. T. Generation of Thiocillin Variants by Prepeptide Gene
474 Replacement and in Vivo Processing by Bacillus Cereus. *J. Am. Chem. Soc.* **2009**, *131* (48), 17563–17565.
475 https://doi.org/10.1021/ja908777t.

476 (18) Akasapu, S.; Hinds, A. B.; Powell, W. C.; Walczak, M. A. Total Synthesis of Micrococcin P1 and
477 Thiocillin I Enabled by Mo(vi) Catalyst. *Chem. Sci.* **2019**, *10* (7), 1971–1975.
478 https://doi.org/10.1039/C8SC04885A.

479 (19) Luo, X.; Zambaldo, C.; Liu, T.; Zhang, Y.; Xuan, W.; Wang, C.; Reed, S. A.; Yang, P. Y.; Wang, R. E.;
480 Javahishvili, T.; Schultz, P. G.; Young, T. S. Recombinant Thiopeptides Containing Noncanonical Amino
481 Acids. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (13), 3615–3620.
482 https://doi.org/10.1073/pnas.1602733113.

483 (20) Wever, W. J.; Bogart, J. W.; Baccile, J. A.; Chan, A. N.; Schroeder, F. C.; Bowers, A. A.
484 Chemoenzymatic Synthesis of Thiazolyl Peptide Natural Products Featuring an Enzyme-Catalyzed Formal
485 [4 + 2] Cycloaddition. *J. Am. Chem. Soc.* **2015**, *137* (10), 3494–3497. https://doi.org/10.1021/jacs.5b00940.

486 (21) Bogart, J. W.; Bowers, A. A. Thiopeptide Pyridine Synthase TbtD Catalyzes an Intermolecular
487 Formal Aza-Diels-Alder Reaction. *J. Am. Chem. Soc.* **2019**, *141* (5), 1842–1846.
488 https://doi.org/10.1021/jacs.8b11852.

489 (22) Liao, R.; Duan, L.; Lei, C.; Pan, H.; Ding, Y.; Zhang, Q.; Chen, D.; Shen, B.; Yu, Y.; Liu, W. Thiopeptide
490 Biosynthesis Featuring Ribosomally Synthesized Precursor Peptides and Conserved Posttranslational
491 Modifications. *Chem. Biol.* **2009**, *16* (2), 141–147. https://doi.org/10.1016/j.chembiol.2009.01.007.

492 (23) Tran, H. L.; Lexa, K. W.; Julien, O.; Young, T. S.; Walsh, C. T.; Jacobson, M. P.; Wells, J. A. Structure-
493 Activity Relationship and Molecular Mechanics Reveal the Importance of Ring Entropy in the Biosynthesis
494 and Activity of a Natural Product. *J. Am. Chem. Soc.* **2017**, *139* (7), 2541–2544.
495 https://doi.org/10.1021/jacs.6b10792.

496 (24) Wang, J.; Lin, Z.; Bai, X.; Tao, J.; Liu, W. Optimal Design of Thiostrepton-Derived Thiopeptide
497 Antibiotics and Their Potential Application against Oral Pathogens. *Org. Chem. Front.* **2019**, *6* (8), 1194–
498 1199. https://doi.org/10.1039/c9qo00219g.

499 (25) Kelly, W. L.; Pan, L.; Li, C. Thiostrepton Biosynthesis: Prototype for a New Family of Bacteriocins.
500 *J. Am. Chem. Soc.* **2009**, *131* (12), 4327–4334. https://doi.org/10.1021/ja807890a.

501 (26) Ranieri, M. R. M.; Chan, D. C. K.; Yaeger, L. N.; Rudolph, M.; Karabelas-Pittman, S.; Abdo, H.; Chee,
502 J.; Harvey, H.; Nguyen, U.; Burrows, L. L. Thiostrepton Hijacks Pyoverdine Receptors to Inhibit Growth of
503 Pseudomonas Aeruginosa. *Antimicrob. Agents Chemother.* **2019**, *63* (9).
504 https://doi.org/10.1128/AAC.00472-19.

(27) Tacconelli, E., Magrini, N., Kahlmeter, G. & Singh, N. Global Priority List of Antibiotic-Resistant Batceria to Guide Research, Discovery, and Development of New Antibiotics. *WHO Publ.* **2017**, 7.

(28) National Nosocomial Infection Surveillance System. National Nosocomial Infections Surveillance (NNIS) System Report, Data Summary from January 1992 through June 2004, Issued October 2004. *American Journal of Infection Control*. Mosby Inc. December 1, 2004, pp 470–485. https://doi.org/10.1016/j.ajic.2004.10.001.

(29) Chan, D. C. K.; Guo, I.; Burrows, L. L. Forging New Antibiotic Combinations under Iron-Limiting Conditions. *Antimicrob. Agents Chemother.* **2020**, *64* (3). https://doi.org/10.1128/AAC.01909-19.

(30) Merwin, N. J.; Mousa, W. K.; Dejong, C. A.; Skinnider, M. A.; Cannon, M. J.; Li, H.; Dial, K.; Gunabalasingam, M.; Johnston, C.; Magarvey, N. A. DeepRiPP Integrates Multiomics Data to Automate Discovery of Novel Ribosomally Synthesized Natural Products. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117* (1), 371–380. https://doi.org/10.1073/pnas.1901493116.

(31) Schwalen, C. J.; Hudson, G. A.; Kille, B.; Mitchell, D. A. Bioinformatic Expansion and Discovery of Thiopeptide Antibiotics. *J. Am. Chem. Soc.* **2018**, *140* (30), 9494–9501. https://doi.org/10.1021/jacs.8b03896.

(32) Di Venanzio, G.; Flores-Mireles, A. L.; Calix, J. J.; Haurat, M. F.; Scott, N. E.; Palmer, L. D.; Potter, R. F.; Hibbing, M. E.; Friedman, L.; Wang, B.; Dantas, G.; Skaar, E. P.; Hultgren, S. J.; Feldman, M. F. Urinary Tract Colonization Is Enhanced by a Plasmid That Regulates Uropathogenic Acinetobacter Baumannii Chromosomal Genes. *Nat. Commun.* **2019**, *10* (1). https://doi.org/10.1038/s41467-019-10706-y.

(33) Josts, I.; Veith, K.; Tidow, H. Ternary Structure of the Outer Membrane Transporter FoxA with Resolved Signaling Domain Provides Insights into TonB-Mediated Siderophore Uptake. *Elife* **2019**, *8*. https://doi.org/10.7554/eLife.48528.

(34) Schallenberger, M. A.; Newhouse, T.; Baran, P. S.; Romesberg, F. E. The Psychotrimine Natural Products Have Antibacterial Activity against Gram-Positive Bacteria and Act via Membrane Disruption. *J. Antibiot. (Tokyo).* **2010**, *63* (11), 685–687. https://doi.org/10.1038/ja.2010.107.

(35) Randall, C. P.; Mariner, K. R.; Chopra, I.; O'Neill, A. J. The Target of Daptomycin Is Absent from Escherichia Coli and Other Gram-Negative Pathogens. *Antimicrob. Agents Chemother.* **2013**, *57* (1), 637–639. https://doi.org/10.1128/AAC.02005-12.

533 (36) Lentzen, G.; Klinck, R.; Matassova, N.; Aboul-Ela, F.; Murchie, A. I. H. Structural Basis for
534 Contrasting Activities of Ribosome Binding Thiazole Antibiotics. *Chem. Biol.* **2003**, *10* (8), 769–778.
535 https://doi.org/10.1016/S1074-5521(03)00173-X.

536 (37) White, P.; Joshi, A.; Rassam, P.; Housden, N. G.; Kaminska, R.; Goult, J. D.; Redfield, C.; McCaughey,
537 L. C.; Walker, D.; Mohammed, S.; Kleanthous, C. Exploitation of an Iron Transporter for Bacterial Protein
538 Antibiotic Import. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114* (45), 12051–12056.
539 https://doi.org/10.1073/pnas.1713741114.

540 (38) Chan, D. I.; Prenner, E. J.; Vogel, H. J. Tryptophan- and Arginine-Rich Antimicrobial Peptides:
541 Structures and Mechanisms of Action. *Biochimica et Biophysica Acta - Biomembranes*. Elsevier September
542 1, 2006, pp 1184–1202. https://doi.org/10.1016/j.bbamem.2006.04.006.

543 (39) Gaede, H. C.; Yau, W. M.; Gawrisch, K. Electrostatic Contributions to Indole-Lipid Interactions. *J.*
544 *Phys. Chem. B* **2005**, *109* (26), 13014–13023. https://doi.org/10.1021/jp0511000.

545 (40) Lee, J. H.; Lee, J. Indole as an Intercellular Signal in Microbial Communities. *FEMS Microbiology*
546 *Reviews*. Blackwell Publishing Ltd July 1, 2010, pp 426–444. https://doi.org/10.1111/j.1574-
547 6976.2009.00204.x.

548 (41) Zhu, X.; Ma, Z.; Wang, J.; Chou, S.; Shan, A. Importance of Tryptophan in Transforming an
549 Amphipathic Peptide into a Pseudomonas Aeruginosa-Targeted Antimicrobial Peptide. *PLoS One* **2014**, *9*
550 (12), e114605. https://doi.org/10.1371/journal.pone.0114605.

551 (42) Niu, H.; Yee, R.; Cui, P.; Tian, L.; Zhang, S.; Shi, W.; Sullivan, D.; Zhu, B.; Zhang, W.; Zhang, Y.
552 Identification of Agents Active against Methicillin- Resistant Staphylococcus Aureus Usa300 from a Clinical
553 Compound Library. *Pathogens* **2017**, *6* (3). https://doi.org/10.3390/pathogens6030044.

554 (43) Cundliffe, E.; Thompson, J. The Mode of Action of Nosiheptide (Multhiomycin) and the Mechanism
555 of Resistance in the Producing Organism. *J. Gen. Microbiol.* **1981**, *126* (1), 185–192.
556 https://doi.org/10.1099/00221287-126-1-185.

557 (44) Stella, S.; Montanini, N.; Le Monnier, F.; Ferrari, P.; Colombo, L.; Landini, P.; Ciciliato, I.; Goldstein,
558 B. P.; Selva, E.; Denaro, M. Antibiotic GE37468 A: A New Inhibitor of Bacterial Protein Synthesis: I. Isolation
559 and Characterization. *J. Antibiot. (Tokyo).* **1995**, *48* (8), 780–786.
560 https://doi.org/10.7164/antibiotics.48.780.

561 (45) Fabbretti, A.; He, C. G.; Gaspari, E.; Maffioli, S.; Brandi, L.; Spurio, R.; Sosio, M.; Jabes, D.; Donadio,
562 S. A Derivative of the Thiopeptide GE2270A Highly Selective against Propionibacterium Acnes. *Antimicrob.*
563 *Agents Chemother.* **2015**, *59* (8), 4560–4568. https://doi.org/10.1128/AAC.05155-14.

564 (46) LaMarche, M. J.; Leeds, J. A.; Brewer, J.; Dean, K.; Ding, J.; Dzink-Fox, J.; Gamber, G.; Jain, A.;
565 Kerrigan, R.; Krastel, P.; Lee, K.; Lombardo, F.; McKenney, D.; Neckermann, G.; Osborne, C.; Palestrant, D.;
566 Patane, M. A.; Rann, E. M.; Robinson, Z.; Schmitt, E.; Stams, T.; Tiamfook, S.; Yu, D.; Whitehead, L.
567 Antibacterial and Solubility Optimization of Thiomuracin A. *J. Med. Chem.* **2016**, *59* (14), 6920–6928.
568 https://doi.org/10.1021/acs.jmedchem.6b00726.

569 (47) Cox, C. L.; Tietz, J. I.; Sokolowski, K.; Melby, J. O.; Doroghazi, J. R.; Mitchell, D. A. Nucleophilic 1,4-
570 Additions for Natural Product Discovery. *ACS Chem. Biol.* **2014**, *9* (9), 2014–2022.
571 https://doi.org/10.1021/cb500324n.

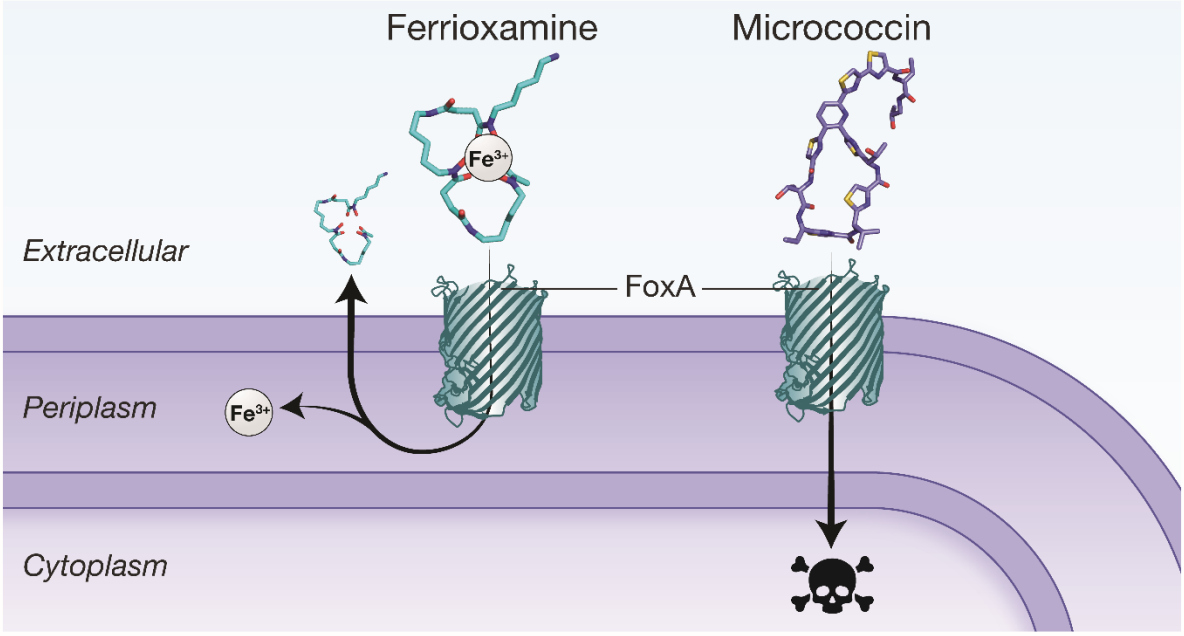
572 (48) Mizuhara, N.; Kuroda, M.; Ogita, A.; Tanaka, T.; Usuki, Y.; Fujita, K. I. Antifungal Thiopeptide
573 Cyclothiazomycin B1 Exhibits Growth Inhibition Accompanying Morphological Changes via Binding to
574 Fungal Cell Wall Chitin. *Bioorganic Med. Chem.* **2011**, *19* (18), 5300–5310.
575 https://doi.org/10.1016/j.bmc.2011.08.010.

576 (49) Aoki, M.; Ohtsuka, T.; Yamada, M.; Ohba, Y.; Yoshizaki, H.; Yasuno, H.; Sano, T.; Watanabe, J.;
577 Yokose, K.; Seto, H. Cyclothiazomycin, a Novel Polythiazole-Containing Peptide with Renin Inhibitory
578 Activity. Taxonomy, Fermentation, Isolation and Physico-Chemical Characterization. *J. Antibiot. (Tokyo).*
579 **1991**, *44* (6), 582–588. https://doi.org/10.7164/antibiotics.44.582.

580 (50) Hood, M. I.; Skaar, E. P. Nutritional Immunity: Transition Metals at the Pathogen-Host Interface.
581 *Nature Reviews Microbiology*. NIH Public Access August 2012, pp 525–537.
582 https://doi.org/10.1038/nrmicro2836.

583 (51) Iatsenko, I.; Marra, A.; Boquete, J.-P.; Peña, J.; Lemaitre, B. Iron Sequestration by Transferrin 1
584 Mediates Nutritional Immunity in Drosophila Melanogaster. *Proc. Natl. Acad. Sci.* **2020**, *117* (13),
585 201914830. https://doi.org/10.1073/pnas.1914830117.

586 (52) Kortman, G. A. M.; Raffatellu, M.; Swinkels, D. W.; Tjalsma, H. Nutritional Iron Turned inside out:
587 Intestinal Stress from a Gut Microbial Perspective. *FEMS Microbiology Reviews*. 2014, pp 1202–1234.
588 https://doi.org/10.1111/1574-6976.12086.

589

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