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Phenolic Substitution in Fidaxomicin: A Semisynthetic Approach to Antibiotic Activity Across Species**

Erik Jung,[a] Anastassia Kraimps,[a] Silvia Dittmann,[b] Tizian Griesser,[c] Jordan Costafrolaz,[d] Yves Mattenberger,[d] Simon Jurt,[a] Patrick H. Viollier,[d] Peter Sander,[c] Susanne Sievers,[b] and Karl Gademann*[a]

Fidaxomicin (Fdx) is a natural product antibiotic with potent activity against *Clostridioides difficile* and other Gram-positive bacteria such as *Mycobacterium tuberculosis*. Only a few Fdx derivatives have been synthesized and examined for their biological activity in the 50 years since its discovery. Fdx has a well-studied mechanism of action, namely inhibition of the bacterial RNA polymerase. Yet, the targeted organisms harbor different target protein sequences, which poses a challenge for the rational development of new semisynthetic Fdx derivatives. We introduced substituents on the two phenolic hydroxy groups of Fdx and evaluated the resulting trends in antibiotic activity against *M. tuberculosis*, *C. difficile*, and the Gram-negative model organism *Caulobacter crescentus*. As suggested by the target protein structures, we identified the preferable derivatisation site for each organism. The derivative *ortho*-methyl Fdx also exhibited activity against the Gram-negative *C. crescentus* wild type, a first for fidaxomicin antibiotics. These insights will guide the synthesis of next-generation fidaxomicin antibiotics.

Introduction

Only a few new antibiotics were commercially registered in the last two decades.[1] Among them, the natural product fidaxomicin (Fdx, **1**, tiacumicin B, lipiarmycin A3) was approved for the treatment of *Clostridioides difficile* infections in 2011.[2–7] Fdx also exhibits antibiotic activity against a broad range of Gram-positive bacteria, most notably against *Mycobacterium tuberculosis* (MIC 0.25 μg/mL).[8–11] Recently, the first occurrence of Fdx resistance was observed in clinical isolates of *C. difficile*, urging the development of 2nd generation fidaxomicin antibiotics.[12] Other antibiotic classes such as the cephalosporins have been under active development for decades, resulting in greatly expanded potency and spectrum of activity.[13,14] The potential to develop a class of fidaxomicin-based antibiotics has been largely untapped, despite more than 50 years passing since its discovery in 1972.[15–17] Progress has been hampered by difficulties to selectively functionalize its complex structure that contains multiple reactive sites. Our group achieved the first total synthesis of fidaxomicin in 2015[18,19] and developed protocols for the semisynthesis of new derivatives.[20–23] In addition, several other groups achieved the synthesis of Fdx fragments or intermediates,[24–27] and also achieved total synthesis.[28]

A remaining challenge consists in the identification of the best approach to develop promising new derivatives for the different target organisms. Consequently, species differences with regard to envelope permeability, efflux, or target structure (RNA polymerase, RNAP)[29–34] must be considered. Not all residues in RNAP that interact with Fdx are conserved between different bacterial species and thus, the binding site needs to be evaluated for each species separately. These structural requirements therefore result in species-specific design considerations for the derivatisation of Fdx and offer opportunities for increasing the potency of Fdx. The present study aimed to test this hypothesis *via* functionalization of the two most reactive sites of fidaxomicin: the two phenolic hydroxy groups of the dichlorohomoorsellinic acid moiety. We planned to synthesize new derivatives that contain substituents attached in the *para*- and *ortho*-positions of the arene fragment.

The instalment of substituents such as ethyl acetate and benzyl sulfonyl fluoride on the phenolic hydroxy groups (herein referred to as *p*-, bis-, *o*-) led to a puzzling observation in our previous study: while *p*-substitution was generally detrimental

[a] E. Jung, A. Kraimps, S. Jurt, Prof. Dr. K. Gademann
Department of Chemistry, University of Zurich
8057 Zürich (Switzerland)
E-mail: karl.gademann@uzh.ch

[b] S. Dittmann, Dr. S. Sievers
Department of Microbial Physiology and Molecular Biology
Institute of Microbiology
Center for Functional Genomics of Microbes
University of Greifswald
Greifswald (Germany)

[c] T. Griesser, Prof. Dr. P. Sander
Institute of Medical Microbiology
University of Zurich
Zurich (Switzerland)

[d] Dr. J. Costafrolaz, Dr. Y. Mattenberger, Prof. Dr. P. H. Viollier
Department of Microbiology and Molecular Medicine
Faculty of Medicine, University of Geneva
Geneva (Switzerland)

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to antibiotic activity against *M. tuberculosis*, addition of a second substituent (resulting in bis-substituted derivatives) partially rescued the activity in some cases (Figure 1B).[21] We hypothesized that in fact *o*-substitution could be beneficial to antibiotic activity and responsible for this observation (Figure 1C). To mechanistically support this hypothesis, we turned to a cryo-EM structure of Fdx in complex with *M. tuberculosis* RNAP and the transcription factor RbpA, elucidated by Campbell and co-workers (*M. tuberculosis* RNAP, PDB: 6BZO).[34] A schematic representation of the binding site around the dichlorohomoorsellinic acid moiety of Fdx is shown in Figure 1A. The *o*-hydroxy group engages in hydrogen bonding with Lys86 (of the β′-subunit, RpoC). The *p*-hydroxy group interacts with Lys1101 (of the β-subunit, RpoB) and RbpA. Ebright and co-workers conducted an alanine-scan of RNAP residues in contact with Fdx and observed that RNAP^{K1101A} (RpoB) was >400-fold more resistant to Fdx.[33] RbpA is essential for *M. tuberculosis*, but Campbell and co-workers were able to truncate the *N*-terminal tail region (NTT) interacting with Fdx resulting in a 35-fold decrease of Fdx susceptibility in an *in vitro* abortive initiation assay.[34] Stallings and co-workers found that Fdx susceptibility in whole cell assays is associated with RNAP-promoter open complex (RP_O) stability. The sigma interaction domain of RbpA increases RP_O stability and this is attenuated by its NTT.

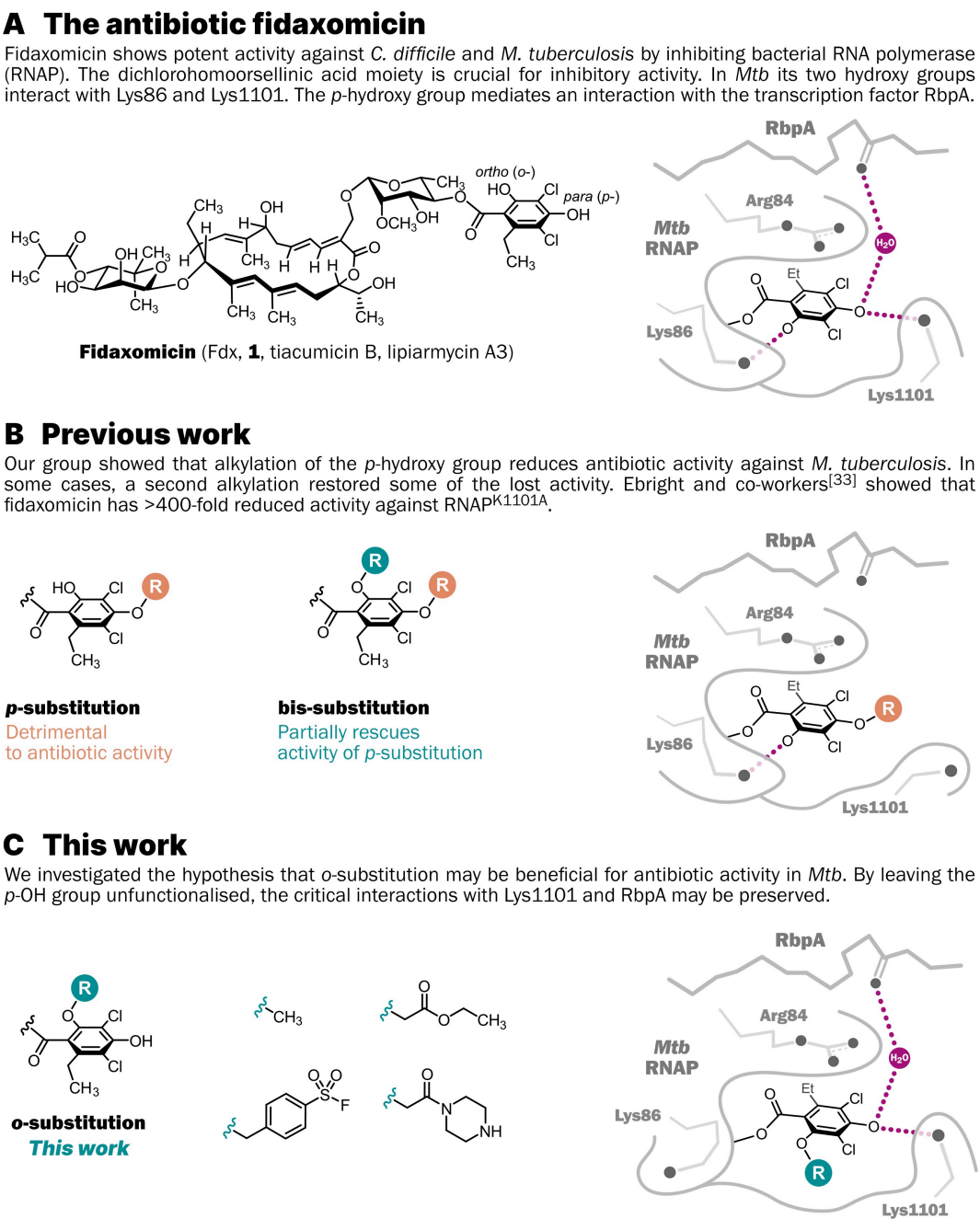


Figure 1. Design of *ortho*-substituted fidaxomicin derivatives.

Therefore, interaction of Fdx or its derivatives with RbpA^{NTT} should increase antibacterial activity.[35] These observations suggest that *p*-substitution is likely unfavourable in *M. tuberculosis*. Important differences between *C. difficile* RNAP and *M. tuberculosis* RNAP include Arg84→Lys84 in RpoC and Lys1101→Arg1121 in RpoB. We adjusted our hypothesis for *C. difficile* based on this sequence difference. Campbell and co-workers determined a cryo-EM structure of Fdx in complex with *C. difficile* RNAP to reveal that the *o*-hydroxy group of the dichlorohomoorsellinic acid moiety interacts with Lys84 and Lys86. Through single-point mutations they uncovered the crucial role of Lys84 for RNAP inhibition by Fdx and termed it the "Fdx sensitizer position".[36] Based on this we expected *o*-substitution to perform poorly in *C. difficile*, where *p*-substitution should be preferred.

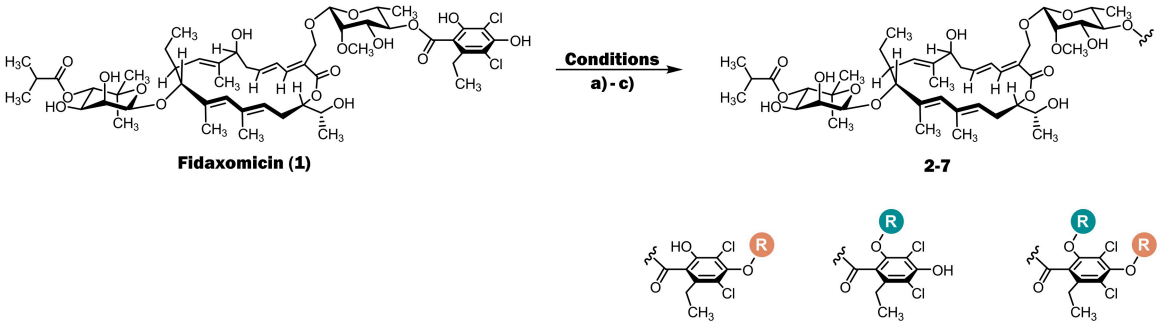
With our hypotheses backed by structural data and *in vitro* experiments, we selected four substituents for the preparation of novel *o*-substituted derivatives. We based this selection on the most active *p*- and bis-substituted derivatives from our previous study, which contained benzyl sulfonyl fluoride, ethyl acetate, and acetyl piperazine substituents.[21] Bis-methyl fidaxomicin (**4**) has been described previously in a patent with reported antibiotic activity equal to or exceeding that of Fdx in *Mycobacterium smegmatis*, *Micrococcus luteus*, and *Nocardia asteroides*.[37] Therefore, the synthesis of **4** and the to-date undescribed mono-methylation of fidaxomicin was targeted as well.

Results and Discussion

We began our investigation with the synthesis of *p*-methyl fidaxomicin (**2**) and *o*-methyl fidaxomicin (**3**) which have not been prepared to date. Our lab has previously developed conditions for the alkylation of fidaxomicin (K_2CO_3, DMF, 45 °C) to synthesize several new fidaxomicin analogues.[21] These conditions delivered *p*-substituted derivatives accompanied by double alkylated bis-substituted derivatives. In combination with MeI, these conditions do result in a mixture of mono- and dimethylation (Fdx:*o*-Me:*p*-Me:bis-Me 51:not detected:17:32). We found that the use of TMS-diazomethane (Scheme 1, Conditions A) gives the *para*-derivative **2** preferentially (Fdx:*o*-Me:*p*-Me:bis-Me 27:7:43:23), yielding *p*-methylated **2** in 33% and the dimethylated **4** in 17%. To date, monoalkylation was exclusively observed for the *p*-position. Consequently, for reliable access to *o*-substituted derivatives, a *p*-protection, *o*-alkylation, *p*-deprotection strategy was established. The allyl-protecting group has been used successfully for the total synthesis of fidaxomicin and some of its derivatives by our group.[19,20,22] Applying this knowledge, *o*-methyl fidaxomicin (**3**) was obtained in 13% over three steps (Scheme 1, Conditions B). Since scalable access to *p*-allyl fidaxomicin is crucial for the synthesis of further derivatives, we optimized the alkylation by varying solvent and temperature. Tsuji-Trost type allylation and Mitsunobu alkylation were investigated as well (Supporting Information, Table S1). Using our optimal conditions, allylation of fidaxomicin was carried out on gram-scale in DMSO at room temperature (Scheme 1, Conditions C) to yield *p*-allyl (**5**, 21%), bis-allyl (**7**, 36%), and *o*-allyl (**6**, 4%) fidaxomicin. We have thus established an operationally simple protocol for the methylation and *p*-allylation of fidaxomicin.

The structure of the obtained regioisomers was investigated next by spectroscopic techniques. The hexasubstituted arene of fidaxomicin presents a challenging structure determination problem. The alkylation site was previously tentatively assigned based on estimated relative reactivities of the two phenolic hydroxy groups.[21] A conventional HMBC experiment is not sufficient for this assignment since the C(2‴)- and C(6‴)-carbon atoms have similar chemical shifts (119/121 ppm) (Figure 2). For the analysis of carbon-rich structures, such as polyphenol natural products, 2-dimensional INADEQUATE (Incredible Natural Abundance DoublE QUAntum Transfer Experiment) represents a powerful technique since it reveals ^{13}C—^{13}C connectivity.[38,39] We acquired a 2D-INADEQUATE spectrum

Synthetic strategy for fidaxomicin alkylation



a)	R = CH_3	$TMSCHN_2$ (2.2 eq.), $CHCl_3$/MeOH 3:1	33% **(2)**	n.d. **(3)**	17% **(4)**
b)	R = CH_3	(i) Allyl bromide (1.2 eq.), K_2CO_3 (3.0 eq.), DMF *then* MeI (3.0 eq.) (ii) morpholine (1.4 eq.), $Pd(PPh_3)_4$ (5 mol%), THF. *Yields over 3 steps.*	n.d. **(2)**	13% **(3)**	16% **(4)**
c)	R = Allyl	Allyl bromide (1.2 eq.), K_2CO_3 (3.0 eq.), DMSO	21% **(5)**	4% **(6)**	36% **(7)**

Scheme 1. Methylation and allylation of fidaxomicin. n.d. = not detected.

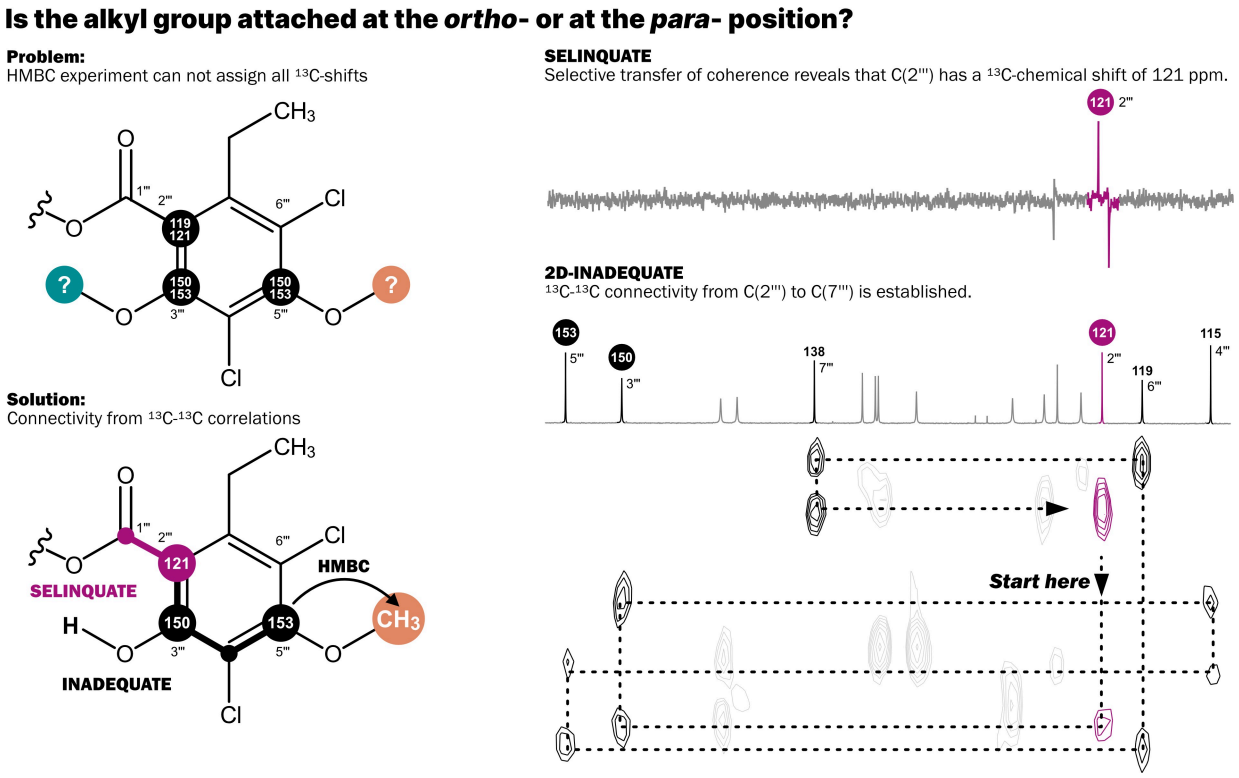
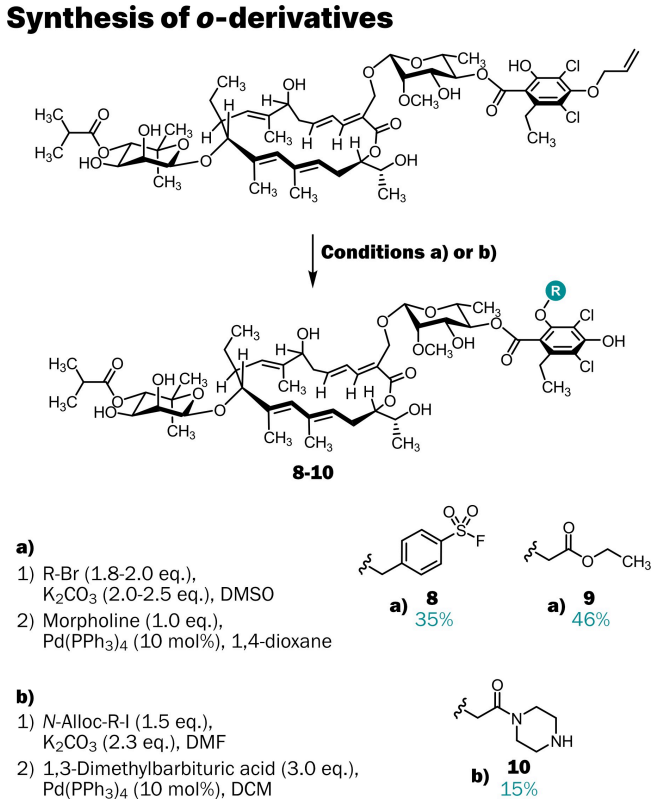


Figure 2. Assignment of alkylation regioselectivity, Numbers in circles denote ^{13}C-chemical shifts in ppm.

using 134 mg of **2** in DMSO-d_6 on a 500 MHz spectrometer. Double quantum correlations were observed for all dichlorohomoorsellinic acid carbon atoms, with the exception of the carbonyl C(1'''). A SELINQUATE (selective INADEQUATE) was carried out to obtain selective coherence transfer from C(1''') to C(2'''), assigning the chemical shift of C(2''') (121 ppm).[40] From there, tracing the carbon skeleton of the ring by INADEQUATE to C(5''') allowed assignment of the methylation site *via* HMBC correlation (CH_3→C(5''')) (Figure 2). Thus, the structure of **2** was unambiguously assigned as *p*-methyl fidaxomicin.

We next identified suitable targets for Fdx modification. Based on the most potent *p*- and bis-substituted derivatives of our previous study, we selected benzyl sulfonyl fluoride **8**, the ethyl acetate **9**, and acyl piperazine **10**. After alkylation and palladium-catalysed deprotection, compounds **8** and **9** were obtained in 35% and 46% yield respectively over two steps (Scheme 2). To synthesize acyl piperazine **10**, an Alloc-protected alkyl iodide was chosen for the alkylation, which was concomitantly deprotected in the palladium-catalysed second step. Here, 1,3-dimethylbarbituric acid was chosen as the allyl scavenger since the resulting by-products are not basic, facilitating separation from the piperazine-containing product (**10**, 15% over two steps).

We evaluated the antibacterial properties of our new derivatives by determining their Minimal Inhibitory Concentration (MIC) values against *M. tuberculosis* and *C. difficile*. Bismethyl Fdx (**4**) was reported in a patent to be more potent than Fdx (MIC 1.56 *vs* 12.5 μg/mL) against *Mycobacterium smegmatis*, a non-pathogenic model organism for *M. tuberculosis*.[37] In



Scheme 2. Derivatisation of fidaxomicin. Yields given are calculated over two steps.

contrast to this, we observed nearly a complete loss of activity against *M. tuberculosis* after dimethylation (Figure 3). In line with our hypothesis that *o*-substitution is favourable against *M. tuberculosis*, *p*-methyl fidaxomicin is also nearly inactive (8–16 μg/mL). To our delight, methylation in the *o*-position reduces activity only slightly compared to Fdx (1 *vs* 0.25 μg/mL). The same trend is observed for the benzyl sulfonyl fluorides (*p*→bis-→*o*-, MIC 8–16→2–4→2 μg/mL). Ethyl acetate substitution is tolerated equally well in the *o*- and *p*-positions, while the bis-substituted derivative is the most potent. Unlike the other derivatives, acetyl piperazine is more potent in the *p*-position, possibly resulting from unique binding interactions of this moiety.

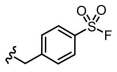
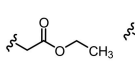
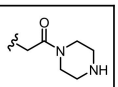
We need to account for the differences in RNAP sequence for every evaluated species of bacteria in the design of new antibiotics. Based on structural and biochemical evidence that concluded that the interactions with the *o*-hydroxy group are essential for activity against *C. difficile*, we hypothesized that *p*-substitution should be favoured over *o*-substitution in *C. difficile*. After observing that *p*-methyl Fdx (**2**, MIC 0.125 μg/mL) and *o*-methyl Fdx (**3**, MIC ≤ 0.0625 μg/mL) are nearly equipotent, we evaluated derivatives containing larger substituents. *p*-Ethyl acetate Fdx (MIC ≤ 0.0625 μg/mL) and *p*-acetyl piperazine Fdx (MIC 0.5 μg/mL), exhibit 16- and 64-fold lower MIC values than their *o*-substituted counterparts. The sulfonyl fluoride **8** opposes this trend, which could be explained by its potential reactivity with Lys84 or Lys86 which may be more facile from the *ortho*-position. The reduced activity of the sulfonyl fluorides compared to activity against *M. tuberculosis* may also be caused by the high cysteine content (0.4%) of the *C. difficile* medium. We therefore conclude that derivatives targeting *M. tuberculosis* should be *ortho*-substituted. Molecular modelling suggests that derivatives targeting *C. difficile* should be *para*-substituted. Our results indicate that depending on the nature of the substituent, *ortho*- or *para*-substitution results in higher activity against *C. difficile*.

Treating infections caused by Gram-negative bacteria can be highly challenging since they naturally exhibit high levels of antibiotic resistance owing to the outer membrane (OM)[41] which presents an additional barrier that antibiotics need to cross to reach their cytosolic target. Antibiotic resistance can be exacerbated by multi-drug efflux pumps such as the widespread AcrAB-NodT pump, a tripartite trans-envelope assembly spanning the cytoplasmic membrane, cell wall (peptidoglycan) layer, and the OM. Since Fdx is generally not active against Gram-negative bacteria,[11] we wondered if this lack of activity is caused by insufficient RNAP inhibition, impaired cell permeation, or efflux.[42,43] In addition, Fdx-dependent inhibition of Gram-negative RNAP is generally weaker than that of *C. difficile* RNAP. The "Fdx sensitizer position" Lys84 of *C. difficile* is exchanged for neutral or negatively charged residues in Gram-negative bacteria, greatly reducing activity.[36] We used three mutant strains of the Gram-negative model organism *Caulobacter crescentus* to dissect the molecular reasons for limited Fdx susceptibility of this organism (Figure 4). The first harbours an aqueous (ungated) protein pore in the OM through which

Activity against *M. tuberculosis*

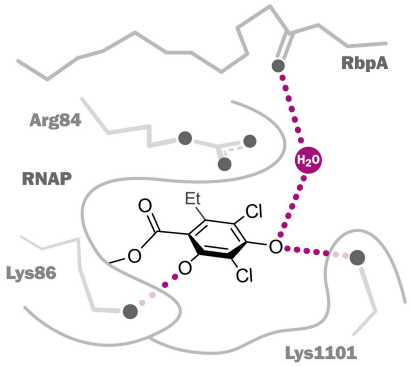
p-Methylation dramatically reduces activity, while *o*-methylation is well tolerated. This also extends to the sulfonyl fluorides. Ethyl acetate is equally potent in the *o*- and *p*-position. The trend is reversed for the basic piperazine.

MIC_{90} values against *M. tuberculosis* (μg/mL)

	H (1)				
p-	-	8-16	8-16[a]	2[a]	1-2[a]
bis-	0.25	8-16	2-4[a]	0.5-1[a]	-
o-	-	1	2	1-2	4-8

[a]Previously synthesized and previously reported MIC values.[21]

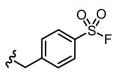
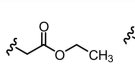
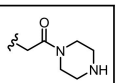
Crucial interaction in *para*-position



Activity against *C. difficile*

p-substituted derivatives are up to 64-fold more potent than their *ortho*-counterparts by preserving the interactions with the "Fdx sensitizer position". The sulfonyl fluorides are deactivated by high cysteine levels in the medium.

MIC values against *C. difficile* 630 (μg/mL)

	H (1)				
p-	-	0.125	32[a]	≤0.0625[a]	0.5[a]
bis-	≤0.004	2	>32[a]	4[a]	-
o-	-	≤0.0625	4	1	32

[a]Previously synthesized[21], MIC values determined in this work.

Crucial interaction in *ortho*-position

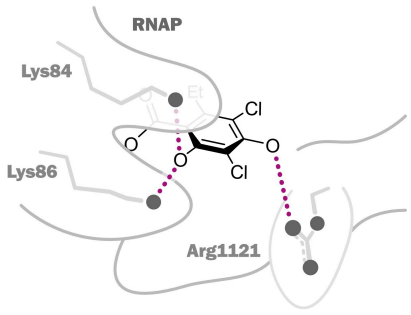


Figure 3. Effect of target protein structure on antibiotic activity against *M. tuberculosis* and *C. difficile*.

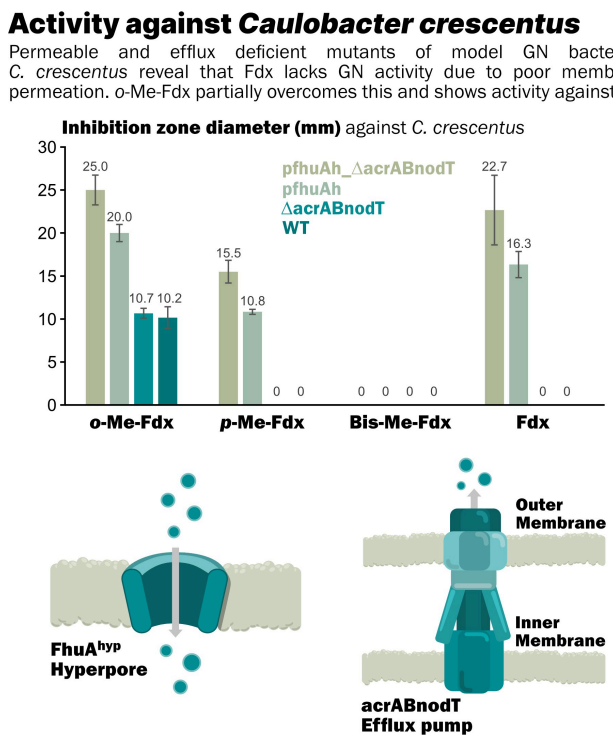


Figure 4. Influence of membrane permeability and efflux in Gram-negative bacteria on Fdx activity. Inhibition diameter is the average of three experiments. Growth up to the disk is reported as 0 mm.

soluble antibiotics can pass (hyperpore). This permeable state is achieved by expression of the hyperpore mutant of *Escherichia coli* ferrichrome outer membrane transporter ($FhuA^{hyp}$) in *C. crescentus* (from plasmid p$fhuA^{hyp}$).[44,45] The second strain contains a deletion in the *acrAB-nodT* genes (Δ*acrAB-nodT*) to impair antibiotic efflux.[46] The third strain is deleted for *acrAB-nodT* and expresses $FhuA^{hyp}$ to maximize entry in the absence of efflux (Δ*acrAB-nodT* + p$fhuA^{hyp}$). We evaluated the methylated derivatives against the three mutant strains and *C. crescentus* wild type (WT). As expected, Fdx activity is highest against Δ*acrAB-nodT* + p$fhuA^{hyp}$ cells. Reinstating efflux reduces the activity of Fdx while elimination of $FhuA^{hyp}$ completely abolishes activity. Thus, we can conclude that the major factor preventing antibiotic activity of Fdx in Gram-negative bacteria is poor cell permeation, but efflux also contributes to Fdx resistance. By contrast, *p*-methyl Fdx exhibits an inhibition zone smaller than Fdx in Δ*acrAB-nodT* + p$fhuA^{hyp}$ and WT + p$fhuA^{hyp}$ cells. *C. crescentus* has a hydrophobic leucine residue at the corresponding position 84 of RpoC which may make alkylation in the *o*-position favourable.[47] *o*-Methyl Fdx exhibited higher activity than Fdx in the Δ*acrAB-nodT* + p$fhuA^{hyp}$ cells while also inhibiting growth of the single deletion mutant Δ*acrAB-nodT* and even *WT C. crescentus* cells. These results suggest that *o*-methyl fidaxomicin penetrates better into *C. crescentus* or that it inhibits RNAP of *C. crescentus* better. Nonetheless, at least one hydroxy group of the dichlorohomoorsellinic acid moiety seems to be needed to inhibit *C. crescentus* RNAP. We hope that the spectrum of activity of Fdx can be fully expanded into Gram-negative bacteria with novel *o*-substituted derivatives.

Phenolic functionalization of fidaxomicin not only alters RNAP inhibitory activities, but also changes the lipophilicity of the derivatives. The observed MIC values obtained in whole-cell assays are dependent on RNAP inhibition, cell permeation, metabolism, and efflux.[48] Retention times from reverse-phase liquid chromatography can serve as an indicator of a compound's lipophilicity.[49] *o*-Methyl Fdx (t_R = 3.53 min, Figure S1) is significantly more polar than *p*-methyl Fdx (t_R = 4.25 min, Figure S1) and its retention time closely matches that of Fdx (t_R = 3.40 min, Figure S1). The proton of the *ortho*-hydroxy group of **1** can engage in an intramolecular hydrogen bond with the orsellinic carbonyl oxygen atom, resulting in a 6-membered cyclic geometry.[50] This is commonly exploited in medicinal chemistry to mask hydrogen bond donors and increase membrane permeability.[51,52] Therefore, exchanging the *o*-proton for a methyl group has a smaller impact on lipophilicity than an exchange of the exposed *p*-proton.

Conclusions

We established a protocol for the *ortho*-functionalization of fidaxomicin. This enabled us to synthesize new *o*-substituted derivatives, filling the gaps of previous work. In addition, *p*-, bis-, and *o*-methyl Fdx were successfully obtained. We then unambiguously assigned their structure using ^{13}C–^{13}C correlation experiments. The obtained derivatives were evaluated against *M. tuberculosis*, *C. difficile*, and *C. crescentus*. From the resulting activities and models of the molecular targets, we propose design guidelines for the derivatisation of fidaxomicin by functionalization of the phenolic hydroxy groups: (i) derivatives targeting *M. tuberculosis* should be *ortho*-substituted, (ii) derivatives targeting *C. crescentus* should be *ortho*-substituted. Against *C. difficile* either *para*- or *ortho*-substitution leads to higher activity than bis-substitution, suggesting that mono-substitution should be prioritized. We hope that these structure activity relationship insights will guide the design of future 2nd-generation fidaxomicin antibiotics. In general, species-dependent differences in the molecular target should be considered in the design of new antibiotics.

CRediT Statement

Erik Jung: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft, Visualization, Project administration

Anastassia Kraimps: Methodology, Investigation, Formal analysis, Writing – Review & Editing

Karl Gademann: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition

Silvia Dittmann: Investigation, Formal analysis

Susanne Sievers: Formal analysis, Resources, Supervision, Project administration, Funding acquisition

Tizian Griesser: Investigation, Formal analysis

Peter Sander: Resources, Supervision, Project administration, Funding acquisition

Jordan Costafrolaz: Methodology, Investigation

Yves Mattenberger: Methodology, Investigation

Patrick H. Viollier: Resources, Supervision, Project administration, Funding acquisition

Simon Jurt: Investigation

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Conflict of Interests

The authors declare no competing financial interest.

Data Availability Statement

Supporting Information contains supplementary figures and tables, experimental procedures, compound characterization, and NMR spectra. Additional data were deposited at zenodo, https://doi.org/10.5281/zenodo.8344957.

Keywords: antibiotics · fidaxomicin · natural products · semisynthesis · structure-activity relationships

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