Semisynthetic Analogs of the Antibiotic Fidaxomicin – Design, Synthesis, and Biological Evaluation

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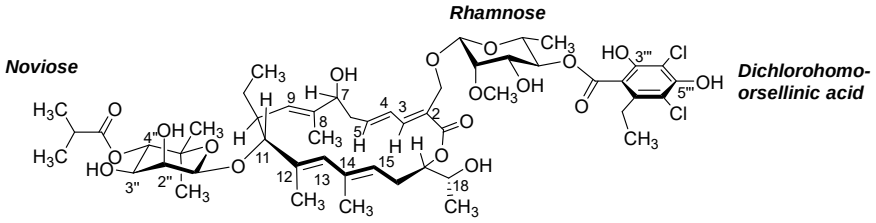
ABSTRACT: The glycoslated macrocyclic antibiotic fidaxomicin (**1**, tiacumicin B, lipiarmycin A3) displays good to excellent activity against Gram-positive bacteria and was approved for the treatment of *Clostridium difficile* infections (CDI). Among the main limitations for this compound, its low water solubility impacts further clinical uses. We report on the synthesis of new fidaxomicin derivatives based on structural design and utilizing an operationally simple one-step protecting group-free preparative approach from the natural product. An increase in solubility of up to 25-fold with largely retained activity was observed. Furthermore, hybrid antibiotics were prepared that show improved antibiotic activities.

KEYWORDS fidaxomicin, antibiotics, semisynthesis, homology modeling, water solubility

Fidaxomicin (**1**, tiacumicin B, lipiarmycin A3) is a glycosylated macrocyclic lactone produced by actinomycetes and has been isolated from four different soil bacteria.[1–7] Fidaxomicin shows good antibiotic activity in vitro against many Gram-positive bacteria, with minimum inhibitory concentration (MIC) values between 0.012 µg/mL and 0.25 µg/mL for *Clostridium difficile*,[8,9] a pathogen causing nosocomial diarrhea. Since 2011, fidaxomicin has been approved for the treatment of inflammations of the intestine caused by *C. difficile*.[10–12] Furthermore, excellent activity against multi-resistant *M. tuberculosis* (MIC values <0.008 – 0.045 µg/mL) and *S. aureus* (MIC values 2 - 16 µg/mL) has been reported.[7,13] Due to the low water solubility of fidaxomicin (**1**) and in consequence its poor systemic absorption, its application for the treatment of systemic infections has not yet been achieved. The development of semisynthetic derivatives is therefore a promising approach to render this class of antibiotics available for such treatments of systemic infections, in particular by improving water solubility.



Fidaxomicin (**1**, tiacumicin B, lipiarmycin A3)

Figure 1. Structure of fidaxomicin (tiacumicin B, lipiarmycin A3).

With regard to its aglycon ring system, fidaxomicin (**1**) structurally belongs to the macrocyclic lactone antibiotics (Figure 1), but features a larger 18-membered ring.[14,15] The aglycon is connected to a modified noviose (isobutyl ester instead of a methoxy group in 4''-position) as well as to a rhamnose substituent, which is esterified to a dichlorohomoorsellinic acid subunit. The complex structure and the remarkable biological properties of fidaxomicin (**1**) aroused the interest of many research groups and led to several synthetic studies towards the preparation of the aglycon[16–20] and finally the first total synthesis of this natural product reported by some of us,[21] and subsequently by others.[22] Furthermore, investigations of the mechanism of action revealed that fidaxomicin, in contrast to other macrolide antibiotics, does not affect ribosomal protein synthesis, but rather inhibits the transcription process catalyzed by bacterial RNA-Polymerase (RNAP).[8,23–30] In order to identify promising semisynthetic target molecules based on structural considerations, docking calculations of fidaxomicin on a homology model of *M. tuberculosis* RNAP, as well as stability assays[31] under various conditions were carried out first.

We started our work with the prediction of the binding mode of fidaxomicin in a structural model of *M. tuberculosis* RNAP. We applied multi-template homology modeling and a multi-model docking approach.[32,33] In the predicted complex, fidaxomicin forms one direct and three indirect interactions (mediated by water) with an RNAP residue whose mutation triggered resistance (Figure 2).[7,26,34–36] The dichlorohomoorsellinic acid is located outside the binding pocket and its phenolic hydroxy groups do not take part in any of these interactions. This led to the hypothesis that modifications at these positions represent a promising strategy when aiming to improve water solubility. Furthermore, these hydroxy groups are in proximity to the binding pocket of the antibiotic rifampicin (Figure S1) and we used our binding mode prediction to calculate the optimal linker length (24 atoms of a polyethylene glycol chain) to covalently connect the two antibiotics in order to target both binding sites at the same time. The actual binding mode of fidaxomicin in *M. tuberculosis* RNAP was recently elucidated using cryo-electron microscopy (cryo-EM).[29,30] A comparison with our binding mode prediction revealed that the macrocycles are overlapping, but the resorcinol moieties point in opposite directions (Figure S2). However, the cryo-EM structure also revealed a solvent pocket around the dichlorohomoorsellinic acid subunit, thus further corroborating the hypothesis that this part is a suitable site for modifications.

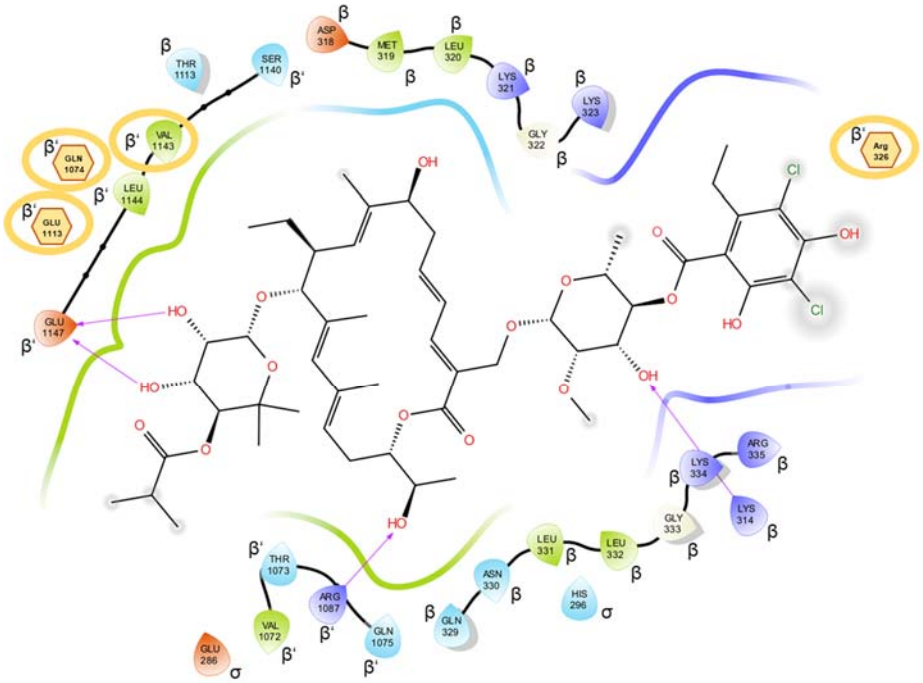
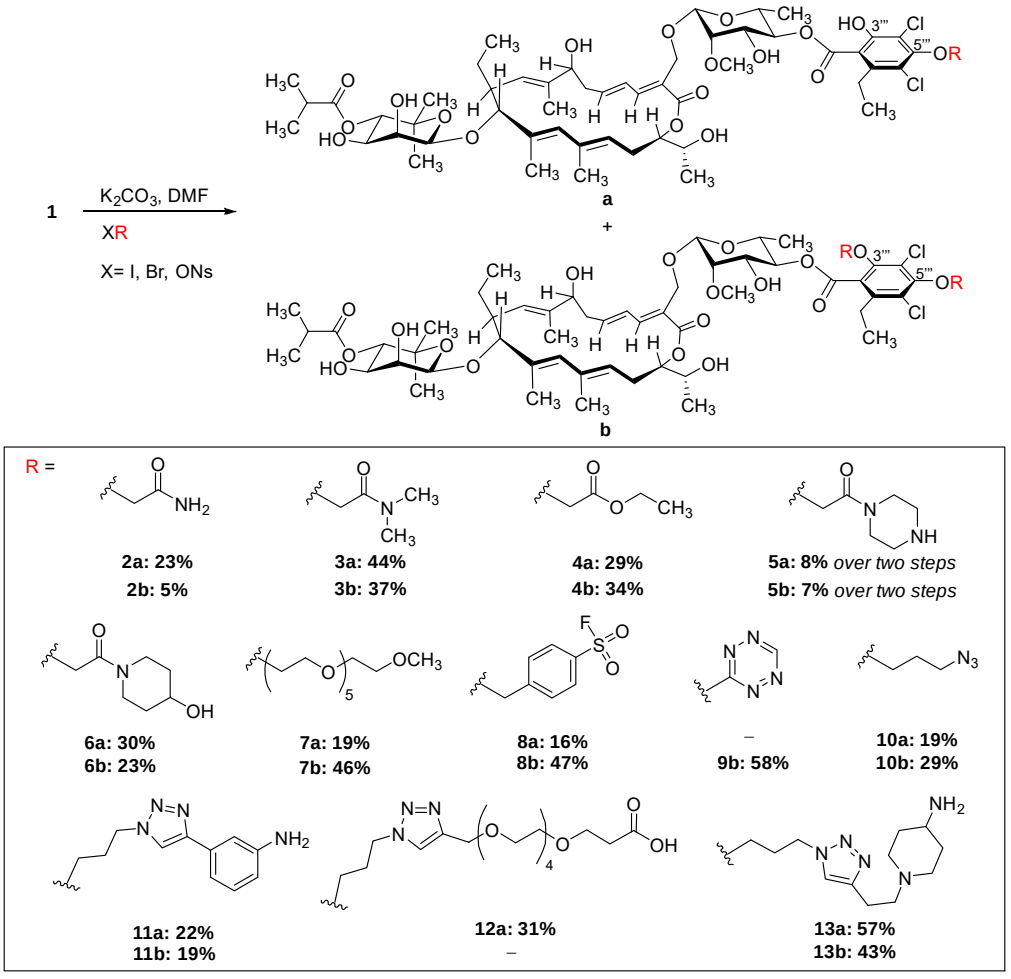


Figure 2. Interaction diagram of the predicted fidaxomicin binding mode in *M. tuberculosis* RNAP. The protein subunits are given in Greek letters. Grey circles around atoms show exposition to solvent. Orange circles show residues that lead to resistance when mutated, indirectly affected residues are displayed as hexagons.

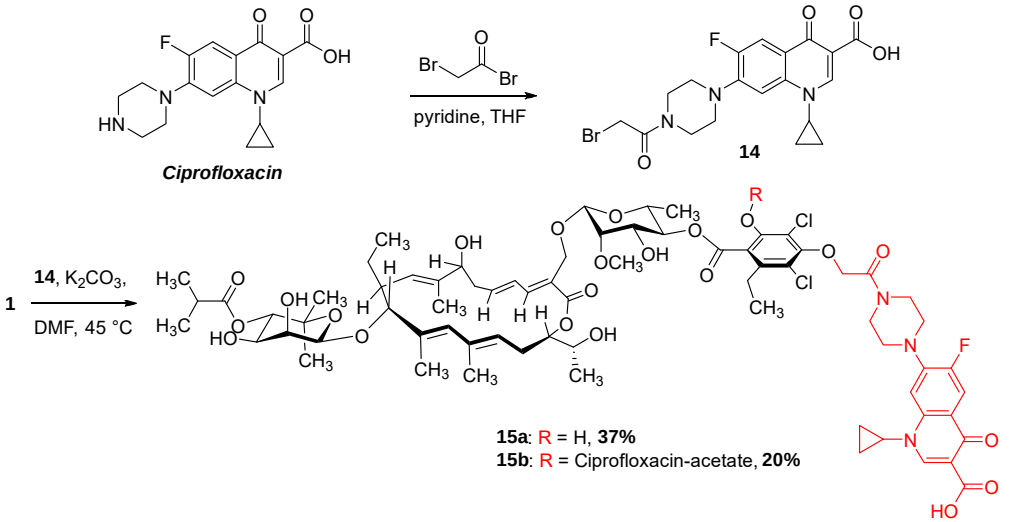
According to our binding mode prediction, 3'''- and 5'''-positions displayed promising sites for modifications as they are exposed to solvent. Furthermore, functionalizations in these positions turned out to be synthetically feasible without the use of any protecting group, thus analogs were accessible with only one preparative step.[37] Therefore, fidaxomicin (**1**) was treated with different electrophiles under slightly basic conditions in DMF at elevated temperatures (Scheme 1). The products **2**−**10** were obtained as separable mixtures of mono- and disubstituted compounds (except **9b**[38]). Noteworthy, the monosubstitutions were exclusively observed at 5'''-hydroxy group since electronic effects might render this hydroxy group more nucleophilic. Under basic conditions, formation of trace amounts of isomers, which proved to be the transacylated isobutyric ester, could not be prevented.[31] After purification by preparative HPLC, the desired compounds were obtained in moderate to good yields (28−81%).



Scheme 1. Functionalization at positions 3''' and 5''' of the resorcinol unit. (Ns = Nosyl).

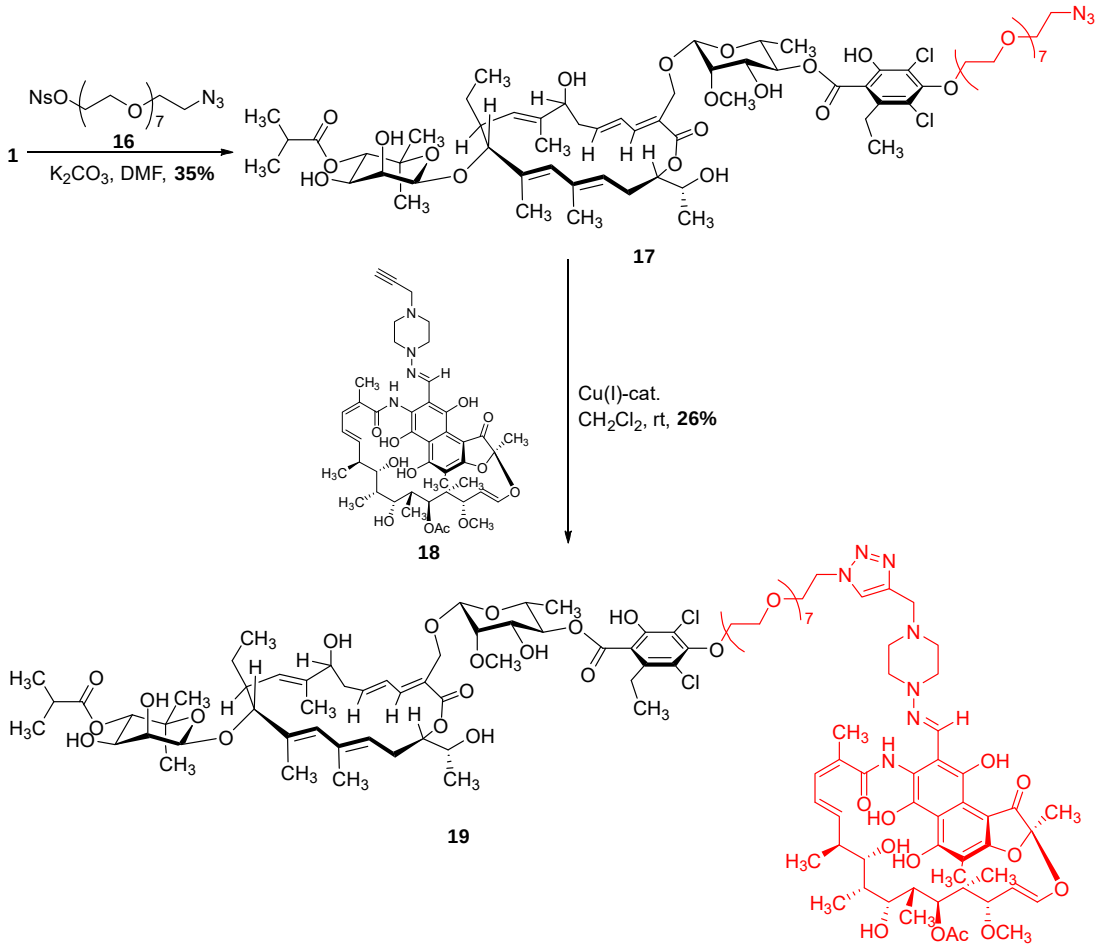
The substituents in compounds **2**–**7** were installed for their high polarity and their common use in medicinal chemistry. The sulfonyl fluorides **8a**/**8b** were synthesized for their ability to form covalent bonds with the enzyme of interest (SuFEx-Click chemistry),[39] which might also be useful for MS-based studies on the binding site. In the context of a study on labeling of macromolecules with tetrazine moieties, we synthesized tetrazine-fidaxomicin **9b**.[38] Furthermore, we reacted the nosylated azidopropanol to obtain azide derivatives **10a**/**b**, which were used as a platform to connect several structurally diverse alkynes to the resorcinol moiety using Click chemistry (CuAAC) with a dicopper-catalyst developed by Straub and coworkers[40] or commercially available Cu(I)OAc. Using azide **10a** as starting material, anilines **11**, PEG5-acid **12a** and piperidinamine **13a**/**b** were obtained, which showed improved water solubility in comparison with fidaxomicin (see below).

In addition to the derivatives discussed so far, hybrids of fidaxomicin with other antibiotics were synthesized. These either have different targets or interact with different binding sites within a common target. Fluoroquinolones are common components of hybrid antibiotics due to their stability in a great variety of reaction conditions and their broad spectrum of activity.[41,42] Modifications on the amine of the piperazine moiety were shown to barely influence the activity.[43] Therefore, this position was chosen for covalently connecting ciprofloxacin to fidaxomicin, thus expecting only minor influences on both molecules' biological properties. For the synthesis of the fidaxomicin-ciprofloxacin hybrid **15**, ciprofloxacin was first transformed into the corresponding bromoacetyl-ciprofloxacin **14** using bromoacetyl bromide (Scheme 2). Subsequent exposure to basic conditions (K_2CO_3 in DMF) together with fidaxomicin resulted in the desired hybrid **15**.



Scheme 2. Synthesis of a fidaxomicin-ciprofloxacin hybrid.

As fidaxomicin and rifampicin share the same target enzyme (RNAP), but interact with different binding sites (Figure S1),[8] we synthesized a fidaxomicin-rifampicin hybrid. Based on our calculations on the predicted binding mode, an octaethylene glycol linker was deemed suitable in order to covalently connect the two antibiotics while retaining their ability to interact with their respective binding sites at the same time. Starting from fidaxomicin (**1**) and nosylated octaethylene glycol azide **16,** the octaethlyene glycol linker was introduced and azide **17** was then connected with literature known alkynylated rifampicin **18**[44] using a Cu(I)-catalyst[40] to give the desired fidaxomicin-rifampicin hybrid **19** (Scheme 3).



Scheme 3. Synthesis of the fidaxomicin-rifampicin hybrid **19.**

We investigated the antibiotic activity of the synthesized derivatives on different bacterial strains such as *B. subtilis* (DSM3256), *S. aureus* (ATCC29213) and *M. tuberculosis* by evaluation of the minimum inhibitory concentration (MIC) (see SI). Derivatives that showed promising antibiotic activity in these tests where further assessed for their antibiotic activity against several isolates of *C. difficile* (Table 1). Although some of the derivatives did not retain their antibiotic activity, it was demonstrated that large substituents on the resorcinol do not necessarily impair the biological activity, which is shown by the good biological activities of derivatives **4a/b**, **5a, 9b, 13a** and hybrids **15a** and **19**. Although derivatives **4a/b** and **13a/b** display decreased activity against *C. difficile*, some promising activity was observed against *M. tuberculosis, S. aureus* and *B. subtilis*. Moreover, compounds **5a** and **9b** retained excellent activity against *C. difficile*.

Table 1. Summary of the minimum inhibitory concentrations (MIC) of selected derivatives.

Compound	MIC in µg/mL												
	C. difficile ATCC 43255	*C. difficile* ATCC 700057	*C. difficile* BAA-1805	*C. difficile* BAA-1875	*C. difficile* ATCC 9689 (rt 001)	*C. difficile* 8260 (rt 017)	*C. difficile* 8282 (rt 017)	*C. difficile* 5680 (rt 027)	*C. difficile* 8264 (rt 027)	*C. difficile* 8290 (rt 078)	*M. tuberculosis*	*S.aureus* ATCC 29213	*B. subtilis* DSM3256
1	0.03	0.03	0.12	0.03	≤0.015	0.06	0.03	0.06	0.12	0.03	0.25	8−16	8−16
4a	>16	8	>16	8	2	16	8	>16	>16	16	2	16	32
4b	>16	16	>16	16	4	16	16	>16	>16	16	0.5−1	32	8
5a	0.5	0.25	0.5	0.5	0.03	0.03	0.06	0.5	0.5	0.25	1-2	>64	>64
9b	0.12	0.06	0.25	0.25	0.03	0.12	0.06	0.5	0.25	0.12	n.d.	n.d.	n.d.
13a	4	2	8	2	0.5	4	1	8	8	4	4-8	>64	>64
13b	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	16−32	>64	8
15a	0.03	0.06	0.12	0.06	≤0.015	0.03	0.03	0.12	0.12	0.12	1-2	>64	32 − 64
19	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015	4	≤0.015	0.03	0.03	0.5	4	4−16
Rifampicin	≤0.015	≤0.015	≤0.015	≤0.015	≤0.015	>16	>16	≤0.015	≤0.015	≤0.015	0.004	0.008	0.25
Ciprofloxacin	>16	>16	>16	>16	16	>16	>16	>16	>16	>16	1	n.d.	n.d.

The fidaxomicin-rifampicin hybrid **19** shows an improved activity compared to fidaxomicin against all investigated strains and similar activity against *C. difficile* when compared to rifampicin. Interestingly, hybrid **19** retains its biological activity even against strains which are not susceptible to rifampicin (*C. difficile* 8260 and 8282). Though no antibiotic activity against *S. aureus* and *B. subtilis* has been observed, the fidaxomicin-ciprofloxacin hybrid **15a** retains its excellent activity against all *C. difficile* strains even though ciprofloxacin itself is inactive against the latter.

Additionally, we investigated the water solubility of the obtained derivatives at pH = 7 by HPLC. For this purpose, saturated solutions of the derivatives in phosphate buffer were prepared and the concentration was determined after filtration of the resulting suspensions. The results (Figure 3) provide evidence for higher water solubility of PEG-derivatives **7b** and **12a**, piperidinol **6a**/**b** and piperidinamine **13b**. Thus, PEG5-acid **12a** acid displays 25-fold and amine **13b** 5-fold increase in water solubility, though its antibiotic activity is reduced.

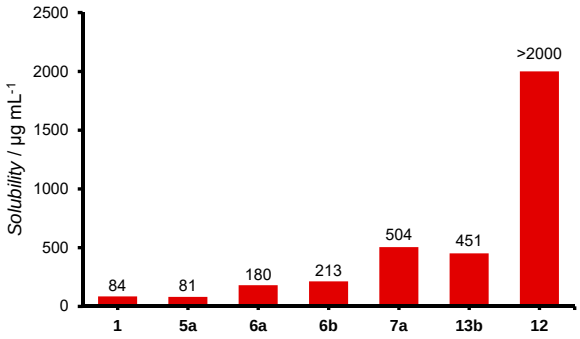


Figure 3. Solubility of selected derivatives in phosphate buffer (pH = 7).

In conclusion, apart from methylations[45] and benzylations[30] at the phenolic hydroxy groups, the new analogs presented here are, to our knowledge, the first examples of complex semisynthetic derivatives of fidaxomicin obtained via phenolic modifications. Other derivatives recently reported are obtained by semisynthesis,[46–48] fermentation[49,50] and fermentation of gene-knockout mutants.[51–54] Based on our predictions of a binding mode of fidaxomicin in a homology model of *M. tuberculosis* RNAP, which suggested that modifications on the dichlorohomoorsellinic acid would be promising, several analogs have been synthesized and tested for their biological activity and water solubility. Some of these compounds showed improved water solubility with maintained antibiotic activity. The synthesized hybrid antibiotics **15** and **19** show improved activity compared to fidaxomicin,

while also retaining activity against strains, that show resistance against the attached antibiotic itself. Access to a great variety of derivatives of this complex natural product was achieved by using easy and reliable synthetic methods. These derivatives could provide an important contribution in ongoing efforts to reduce the rate of antibiotic resistance development in bacteria and broaden the scope of application of fidaxomicin.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supplementary Figures S1 and S2, experimental procedures and methods, characterization data, ^{1}H and ^{13}C NMR spectra, supplementary MIC tables, computational details. (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. A.D., R.B., and K.G. designed the study. A.D., R.B., and S.D.S. carried out the synthesis and characterization of the derivatives. C.G., W.G. and H.G. performed the homology modelling and computational studies. D.S. and P.S. developed the MIC tests for *M. tuberculosis* and performed the biological evaluation of the derivatives. K.Z. and M.G. performed the MIC tests against *S. aureus* and *B. subtilis*. A.D., R.B., and K.G. analyzed and discussed the results. A.D. and K.G. wrote the manuscript. All authors have given approval to the final version of the manuscript.
‡These authors contributed equally.

Notes

A patent application (WO2019135010A1, EP18150671.8A) was filed Jan 8th, 2018 that includes antibiotics presented in this work.

ACKNOWLEDGMENT

We thank the Swiss National Science Foundation for financial support (200021_182043 (K.G.) and 31003A_153349/1 (P.S.)). Work in the laboratory of P.S. is supported by Swiss Lung Association (2018-02). We acknowledge partial financial support by the DFG (GRK 2158, P4 for H.G.). We gratefully acknowledge the NMR- und MS services of the University of Zurich and F. Fazzi und G. Uzunidis for skillful laboratory assistance. We further thank Dr. A. Siegle and Prof. Dr. O. Trapp (LMU München) for using the HPLC in their research groups and Dr. R. Wehlauch and J. Michel for proofreading the manuscript.

ABBREVIATIONS

CDI *C. difficile* infection, MIC minimum inhibitory concentration, RNAP RNA polymerase, cryo-EM cryo-electron microscopy, HPLC high performance liquid chromatography, PEG polyethylene glycol.

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