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

Andrea Dorst, Regina Berg, Christoph G. W. Gertzen, Daniel Schäfe, Katja Zerbe, Myriam Gwerder, Simon D. Schnell, Peter Sander, Holger Gohlke, and Karl Gademann*

ABSTRACT: The glycoslated macrocyclic antibiotic fidaxomicin (1, tiacumin B, lipiarycin A3) displays good to excellent antibiotic 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, tiacumin B, lipiarycin A3) is a glycosylated macrocyclic antibiotic 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 multiresistant Mycobacterium tuberculosis (MIC values <0.008−0.045 μg/mL) and Staphylococcus 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.

With regard to its aglycon ring system, fidaxomicin (1) structurally belongs to the macrolide 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 toward the preparation of the aglycon16−20 and finally the first total synthesis of this natural product reported by some of us21 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 assays31 under various conditions were carried out first.

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We started our work with the prediction of the binding mode of fidaxomicin in a structural model of *M. tuberculosis* RNAP. We applied multitemplate homology modeling and a multimodel docking approach. 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). 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). 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.

According to our binding mode prediction, 3′′′- and 5′′′-positions displayed promising sites for modifications as they are exposed to the 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. 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). It is noteworthy that the monosubstitutions were exclusively observed at 5′′′-hydroxy...
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After purification by preparative HPLC, the desired compounds were obtained in moderate to good yields (28−81%).

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The sulfonyl fluorides 8a/8b were synthesized for their ability to form covalent bonds with the enzyme of interest (SuFEx-Click chemistry), 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. 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 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. Modifications on the amine of the piperazine moiety were shown to barely influence their biological activity. Therefore, this position was chosen for covalently connecting ciprofloxacin to fidaxomicin, thus expecting only minor effects on both molecules’ biological properties. For the synthesis of the fidaxomicin-ciprofloxacin hybrid 15, ciprofloxacin was first transformed into the corresponding bromoacetil-ciprofloxacin 14 using bromoacetyl bromide (Scheme 2). Subsequent exposure to basic conditions (K₂CO₃ in DMF) together with fidaxomicin resulted in the desired hybrid 15.

As fidaxomicin and rifampicin share the same target enzyme (RNAP), but interact with different binding sites (Figure S1), 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 octaethylene glycol linker was introduced and azide 17 was then connected with literature-known alkynylated rifampicin 18 using a Cu(I)-catalyst to give the desired fidaxomicin-rifampicin hybrid 19 (Scheme 3).

We investigated the antibiotic activity of the synthesized derivatives on different bacterial strains such as Bacillus subtilis (DSM3256), S. aureus (ATCC29213), and M. tuberculosis by evaluation of the minimum inhibitory concentration (MIC) (see SI). Derivatives featuring promising antibiotic activity in these tests were 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 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*.

The fidaxomycin−rifampicin hybrid 19 shows an improved activity compared to fidaxomycin 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 fidaxomycin-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 displays 25-fold and amine 13b 5-fold increase in water solubility, though its antibiotic activity is reduced.

In conclusion, apart from methylations and benzylations at the phenolic hydroxy groups, the new analogs presented here are, to our knowledge, the first examples of complex semi-synthetic derivatives of fidaxomycin obtained via phenolic modifications. Other derivatives recently reported are obtained by semisynthesis, fermentation, and fermentation of knockout mutants. Based on our predictions of a binding mode of fidaxomycin 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 fidaxomycin, 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 fidaxomycin.

### Table 1. Summary of the Minimum Inhibitory Concentrations (MIC) of Selected Derivatives

| Compound  | C. difficile ATCC 29213 | C. difficile ATCC 43506 | C. difficile ATCC 70067 | C. difficile BAA-1805 | C. difficile BAA-1875 | C. difficile ATCC 9089 (R 001) | C. difficile 8260 (R 017) | C. difficile 8282 (R 017) | C. difficile 5860 (R 027) | C. difficile 8264 (R 027) | C. difficile 8290 (R 078) | M. tuberculosis | S. aureus ATCC 29213 | S. subtilis DSM256 |
|-----------|-------------------------|-------------------------|-------------------------|-----------------------|-----------------------|-----------------------------|--------------------------|--------------------------|------------------------|------------------------|------------------------|-----------------|---------------------|------------------|
| 1         | 0.03 0.03               | 0.12 0.03               | 0.03 0.03               | ≤0.015                | 0.06 0.03             | 0.06 0.03                   | 0.12 0.03               | 0.03 0.03               | 0.03 0.03               | 0.03 0.03               | 0.12 0.03               | ≥0.25            | 8–16                | 8–16              |
| 4a        | >0.16 0.16              | >0.16 0.16              | >0.16 0.16              | >0.16 0.16            | >0.16 0.16            | >0.16 0.16                  | >0.16 0.16              | >0.16 0.16              | >0.16 0.16              | 0.5–1 0.15             | 0.25 0.15             | 4–8              | >0.64               | >0.64             |
| 5a        | 0.5 0.25                | 0.5 0.25                | 0.5 0.25                | 0.03 0.03             | 0.03 0.03             | 0.03 0.03                   | 0.5 0.25                | 0.25 0.12                | 0.12 0.12               | n.d. n.d.              | n.d. n.d.              | n.d.             | 8–16                | 8–16              |
| 9b        | 0.12 0.06               | 0.25 0.25               | 0.25 0.25               | 0.12 0.06             | 0.12 0.06             | 0.25 0.12                   | 0.12 0.06               | 0.12 0.06               | 0.12 0.06               | 0.12 0.06               | 0.12 0.06               | 1.2–3            | 8–16                | 8–16              |
| 13a       | 4 2                     | 8 2                     | 2 0.5                   | 4 1                   | 8 8                   | 4 8                         | 4 8                      | 4–8                     | >0.64 0.64             | >0.64 0.64             | >0.64 0.64             | 8–16            | >0.64               | >0.64             |
| 13b       | >0.16 >0.16             | >0.16 >0.16             | >0.16 >0.16             | >0.16 >0.16           | >0.16 >0.16           | >0.16 >0.16                 | >0.16 >0.16             | >0.16 >0.16             | >0.16 >0.16           | 16–32 16–32           | >0.64 0.64           | 8–16            | >0.64               | >0.64             |
| 15a       | 0.03 0.06               | 0.12 0.06               | 0.06 ≤0.015             | 0.03 0.03             | 0.12 0.12             | 0.12 0.12                   | 0.12 0.12               | 0.12 0.12               | 0.12 0.12             | >0.64 >0.64           | 32–64 32–64         | 8–16            | >0.64               | >0.64             |
| 19        | ≤0.015 ≤0.015           | ≤0.015 ≤0.015           | ≤0.015 ≤0.015           | ≤0.015 ≤0.015         | ≤0.015 ≤0.015         | ≤0.015 ≤0.015               | ≤0.015 ≤0.015           | ≤0.015 ≤0.015           | ≤0.015 ≤0.015         | ≤0.015 ≤0.015         | 0.004 0.008         | 0.25            | n.d. n.d.           | n.d.              |
| Rifampicin| ≤0.015 ≤0.015           | ≤0.015 ≤0.015           | ≤0.015 ≤0.015           | ≤0.015 ≤0.015         | ≤0.015 ≤0.015         | ≤0.015 ≤0.015               | ≤0.015 ≤0.015           | ≤0.015 ≤0.015           | ≤0.015 ≤0.015         | ≤0.015 ≤0.015         | ≤0.015 ≤0.015       | 4–8             | 4–16                | 4–16              |
| Ciprofloxacin| >0.16 >0.16          | >0.16 >0.16             | >0.16 >0.16             | >0.16 >0.16           | >0.16 >0.16           | >0.16 >0.16                 | >0.16 >0.16             | >0.16 >0.16             | >0.16 >0.16           | >0.16 >0.16           | >0.16 >0.16           | 1.2–3           | n.d. n.d.           | n.d.              |

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

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00381.

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

AUTHOR INFORMATION

Corresponding Author
Karl Gademann — Department of Chemistry, University of Zurich, 8057 Zürich, Switzerland; orcid.org/0000-0003-3053-0689; Email: karl.gademann@chem.uzh.ch

Authors
Andrea Dorst — Department of Chemistry, University of Zurich, 8057 Zürich, Switzerland
Regina Berg — Department of Chemistry, University of Zurich, 8057 Zürich, Switzerland
Christoph G. W. Gertzen — Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany
Daniel Schäfer — Institute of Medical Microbiology, University of Zurich, 8006 Zurich, Switzerland
Katja Zerbe — Department of Chemistry, University of Zurich, 8057 Zürich, Switzerland
Myriam Gwerder — Department of Chemistry, University of Zurich, 8057 Zürich, Switzerland
Simon D. Schnell — Department of Chemistry, University of Zurich, 8057 Zürich, Switzerland
Peter Sanders — Institute of Medical Microbiology and National Center for Mycobiology, University of Zurich, 8006 Zurich, Switzerland
Holger Gohlke — Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany; orcid.org/0000-0001-8613-1447

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00381

Author Contributions
A.D. and R.B. contributed equally. 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 modeling and computational studies. D.S. and P.S. developed the MIC tests for S. aureus and B. subtilis. A.D., R.B., and K.G. analyzed and discussed the results. A.D., R.B., and K.G. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes
The authors declare the following competing financial interest(s): A patent application (WO2019135010A1, EP18150671.8A) was filed Jan 8th, 2018 that includes antibiotics presented in this work.

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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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