

Development of Novel Bacterial Topoisomerase Inhibitors Assisted by Computational Screening

Joshua W. Powell, Chelsea A. Mann, Paul D. Toth, Sheri Nolan, Anja Steinert, Clarissa Ove, Justin T. Seffernick, Daniel J. Wozniak, Razieh Kebraiee, Steffen Lindert, Neil Osherooff, Jack C. Yalowich, and Mark J. Mitton-Fry*



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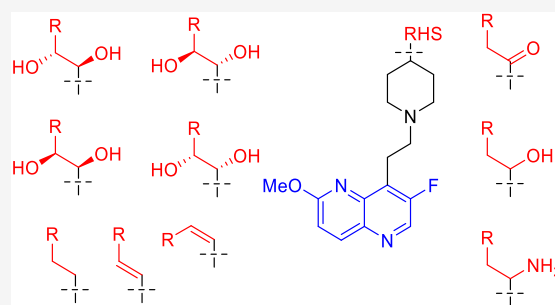
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ABSTRACT: Multidrug-resistant bacterial infections pose an ever-evolving threat to public health. Since the outset of the antibacterial age, bacteria have developed a multitude of diverse resistance mechanisms that suppress the effectiveness of current therapies. New drug entities, such as Novel Bacterial Topoisomerase Inhibitors (NBTIs), can circumvent this major issue. A computational docking model was employed to predict the binding to DNA gyrase of atypical NBTIs with novel pharmacophores. Synthesis of NBTIs based on computational docking and subsequent antibacterial evaluation against both Gram-positive and Gram-negative bacteria yielded congeners with outstanding anti-staphylococcal activity and varying activity against select Gram-negative pathogens.

KEYWORDS: MRSA, gyrase, antimicrobial resistance, cleavage, efflux



Antibacterial resistance is an ever-growing threat to humanity. The essential bacterial topoisomerase enzymes, DNA gyrase¹ and topoisomerase IV (TopoIV),² are attractive targets, as exemplified by the fluoroquinolone class of antibacterials. However, a rapid increase in resistance in recent decades, driven primarily by mutations to the genes encoding the topoisomerase targets, has reduced their effectiveness.^{3,4} One strategy to attack such resistant bacteria is to target these essential enzymes at novel binding sites. Novel Bacterial Topoisomerase Inhibitors (NBTIs), such as gepotidacin (now known as a triazaacenaphthylene), elude fluoroquinolone resistance using this strategy and display a differentiated pharmacological profile.⁵

The pharmacophore of gepotidacin is typical for most NBTIs (see Figure 1).^{5,6} The left-hand side (LHS, blue) utilizes either a bi- or tricyclic ring system to bind bacterial DNA. The linker (black) connects the LHS and the right-hand side (RHS, red), a moiety binding to a hydrophobic pocket in the topoisomerase. The RHS secondary amine is prototypical and interacts with a highly conserved aspartate (D83, *Staphylococcus aureus* numbering) in the GyrA subunit of DNA gyrase, at the entrance to the binding pocket.^{7,8} A mono- or bicyclic ring system binds nonspecifically to this pocket. The binding mode identified by X-ray crystallographic studies has been further validated by selection for NBTI-resistant mutants, of which GyrA D83N is common.⁹ Extensive optimization efforts have varied each portion of the pharmacophore, with the aims of improving antibacterial activity, enhancing pharmacokinetic properties, and reducing key safety liabilities including hERG inhibition.^{5,6}

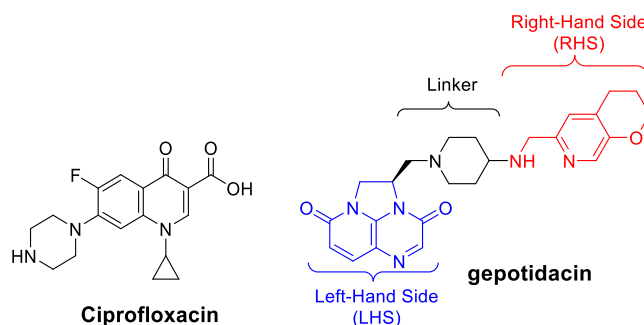


Figure 1. Examples of a fluoroquinolone (ciprofloxacin) and NBTI (gepotidacin) are shown, with the key elements of the NBTI pharmacophore highlighted.

In this study, we have combined experimental results from previously synthesized molecules with a computational model to predict binding affinities of novel structures, enabling evaluation and prioritization of unique RHS pharmacophores prior to synthesis. In doing so, we identified atypical NBTIs with potent Gram-positive antibacterial activity. Gram-negative activity has

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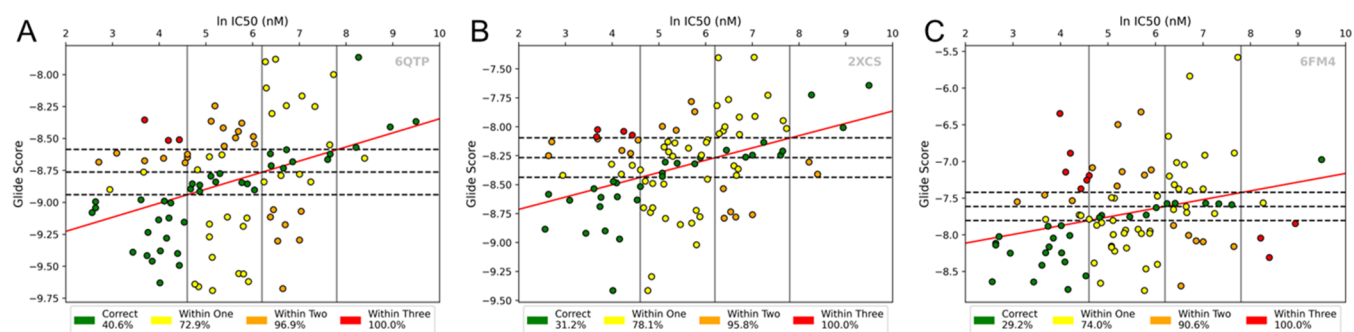


Figure 2. Assessing the predictive accuracy of docking results using binned classification: binning assessment of systems selected from benchmarking: (A) 6QTP, (B) 2XCS, and (C) 6FM4. For a series of known NBTIs, the predicted binding score was plotted versus the natural logarithm of the measured IC_{50} value from a *S. aureus* DNA gyrase supercoiling assay. Agreement between experimental values and average Glide docking score was measured to determine the predictive accuracy of each DNA gyrase system (green: correct; yellow: within one bin; orange: within two; red: within three).

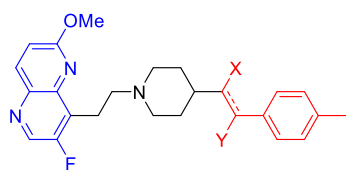


Figure 3. General structure of the targeted compounds.

Table 1. Predicted Binding Scores of Atypical NBTIs

RHS	Avg Z-Score	RHS	Avg Z-Score
	-1.510		-1.907
	-1.012		-1.210
	-1.012		-1.210

proven more challenging, with efflux posing a particular challenge, as has been observed previously.^{5,10}

Our efforts began with a set of 96 compounds, for which we had experimentally determined IC_{50} values for inhibition of gyrase-mediated supercoiling, which were used as a rough surrogate for binding affinity. Using Schrödinger Glide,¹¹ each compound was docked into seven receptors constructed from high-resolution DNA gyrase crystals with bound NBTIs: PDB IDs 2XCS, 4PLB, 5BS3, 5NPP, 6FM4, 6QTK, and 6QTP. An initial self-docking screening was conducted to evaluate the utility of each crystal as a docking receptor (Figure S1, Supporting Information [SI]). For each of these systems, we created four experimental binding affinity bins based on the $\ln IC_{50}$ (nM) values of the benchmark compounds with bounds at 4.6 (100 nM), 6.2 (500 nM), and 7.8 (2500 nM). We then calculated least-squares linear regressions from the Glide docking data and natural log IC_{50} values of each receptor. A second set of cutoffs to represent docking scores was then defined, where the regressions intersected the IC_{50} bounds. This gave us a total of 16 bins, into which the benchmark compounds fell (Figure 2). We could subsequently evaluate the performance of each system based on the number of benchmark compounds that had a matching docking score and IC_{50} bins. Those along the positive diagonal had consistent experimental and computational classifications, i.e., both values were ranked excellent or both poor. These compounds were categorized as “correctly predicted.” The inhibitors that fell outside of these diagonal bins,

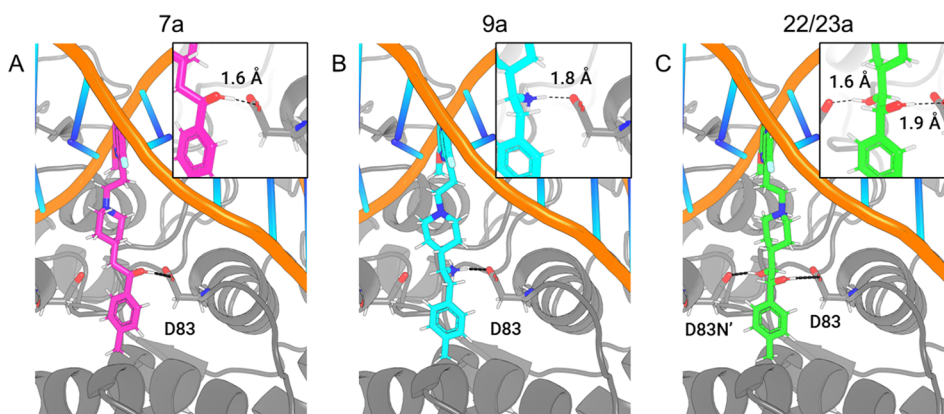


Figure 4. Predicted docking poses and interactions with GyrA D83 in 2XCS of (A) monohydroxyl (7a) (1.6 Å to D83), (B) primary amine (9a) (1.8 Å to D83), and (C) anti-diol (22/23a) (1.6 and 1.9 Å to D83).

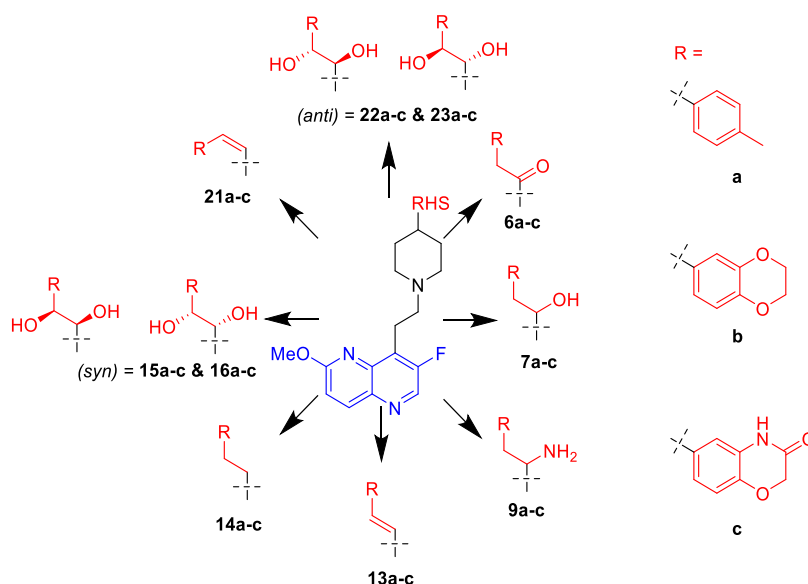
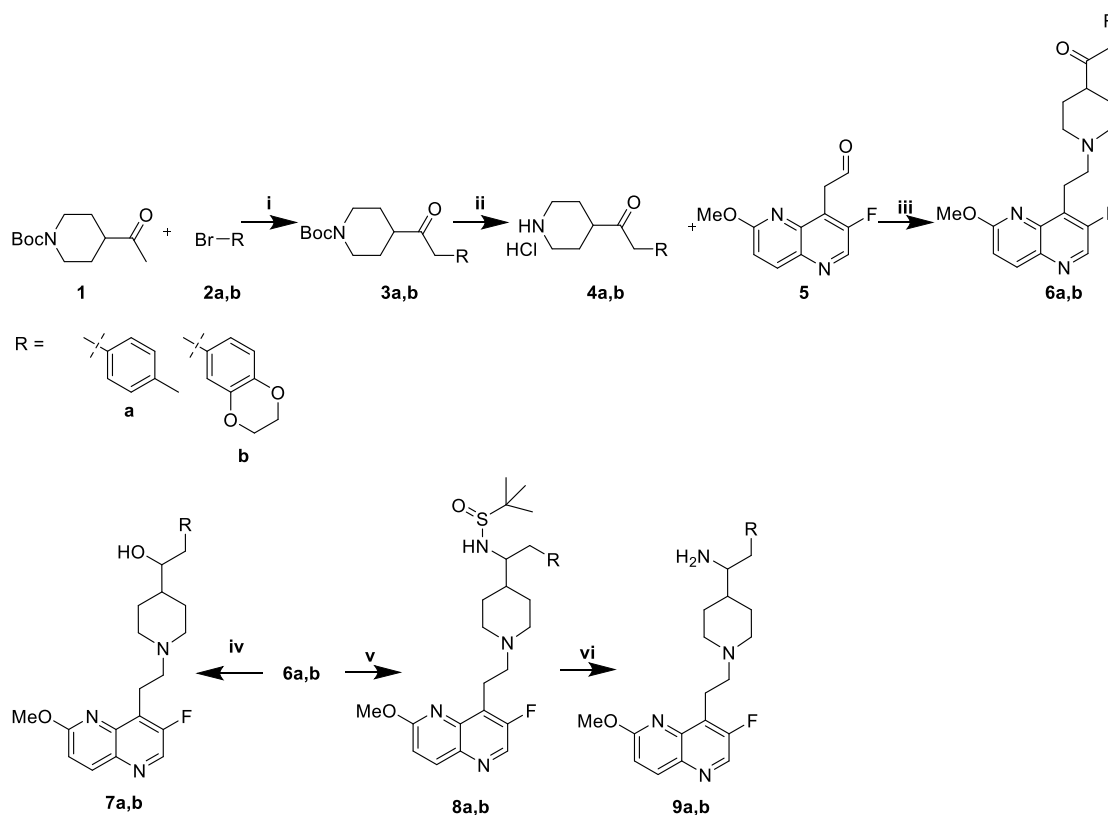


Figure 5. Overview of synthesized compounds.

Scheme 1. Synthesis of Ketones and Monohydroxyls, and Primary Amines^{4a}

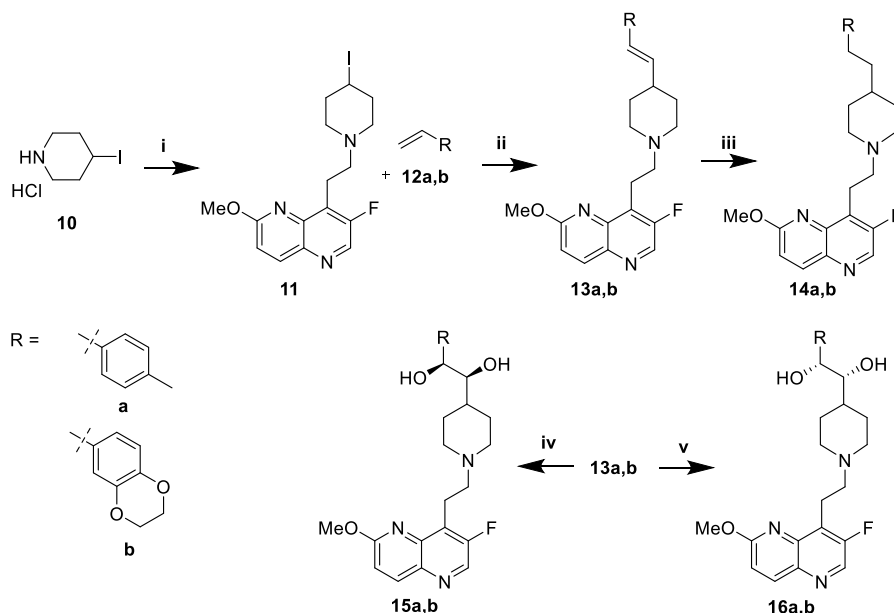


^{4a}Reaction conditions: (i) NaO^tBu, Pd₂dba₃, (±)-BINAP, THF, 70 °C; (ii) HCl, MeOH; (iii) 1. AcOH, THF/MeOH, 4 Å MS. 2. NaCNBH₃; (iv) NaBH₄, MeOH; (v) 1. H₂NSOC(CH₃)₃, Ti(OEt)₄, THF, 70 °C. 2. NaBH₄, MeOH; (vi) HCl, MeOH.

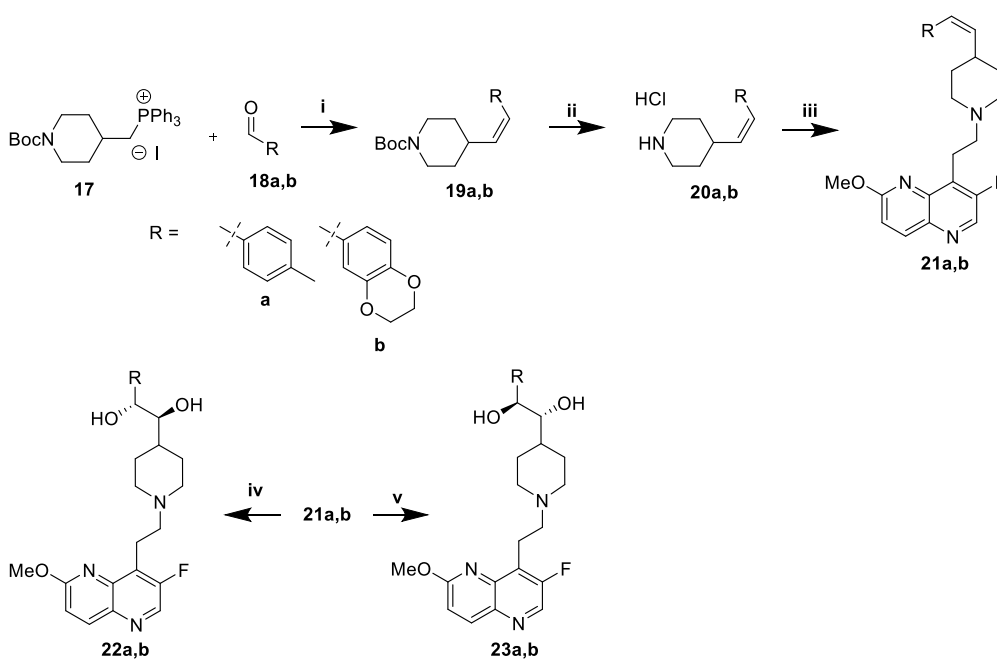
where the experimental and computational classifications did not agree, were “incorrectly predicted”. Depending on the number of bins by which the docking classification deviated from the ln IC₅₀ classification, they were labeled as “within one”, “within two”, or “within three” of their correct bin.

Three systems were identified for downstream analysis based on a combination of their ability to predict compounds as “within one” or better and the R² of their regression line: 6QTP

(R²: 0.14, correct: 40.6%, within one bin: 72.9%), 2XCS (R²: 0.16, correct: 31.2%, within one bin: 78.1%), and 6FM4 (R²: 0.08, correct: 30%, within one bin: 74.0%). These were then used to screen all potential synthesis candidates as an initial evaluation. Docking score distributions of the 443 compounds tested are shown in Figure S2, SI. As docking scores cannot be directly compared across different structures, the results from all systems were normalized to Z-scores (see Equation S1, SI) as

Scheme 2. Synthesis of (*E*)-Olefins, Alkanes, and *Syn*-Diols^a

^aReaction conditions: (i) 1. 5, AcOH, THF/MeOH, 4 Å MS. 2. NaCNBH₃; (ii) 4-CZIPN, Co(dmgH)(dmgH₂)Cl₂, HK₂PO₄, Et₃N, DMF, 440 nm light; (iii) H₂, 10% Pd/C, EtOH; (iv) AD-mix- α , ^tBuOH/water; (v) AD-mix- β , ^tBuOH/water.

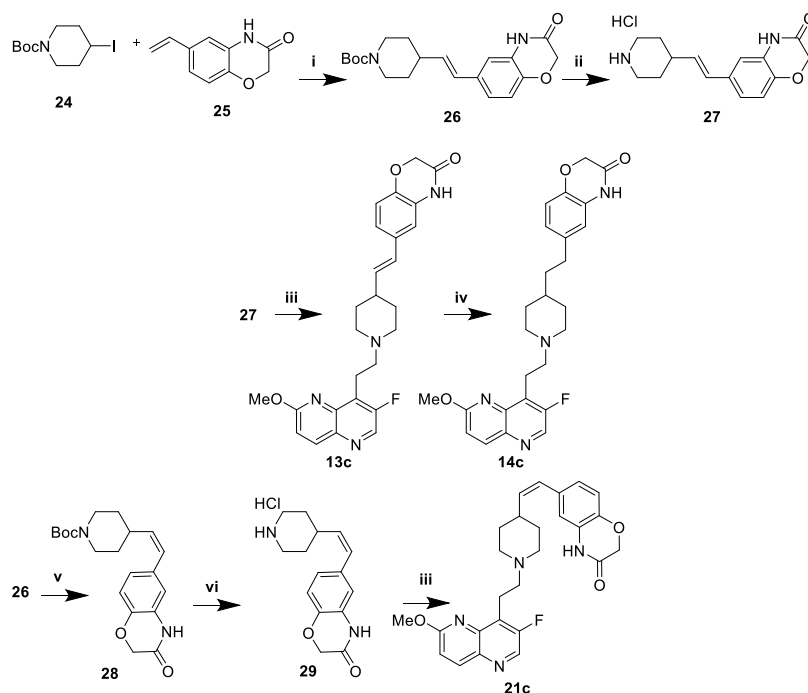
Scheme 3. Synthesis of (*Z*)-Olefins and *Anti*-Diols^a

^aReaction conditions: (i) KO^tBu, THF, 0 °C; (ii) HCl, MeOH; (iii) 1. 5, AcOH, THF/MeOH, 4 Å MS. 2. NaCNBH₃; (iv) AD-mix- α , ^tBuOH/water; (v) AD-mix- β , ^tBuOH/water.

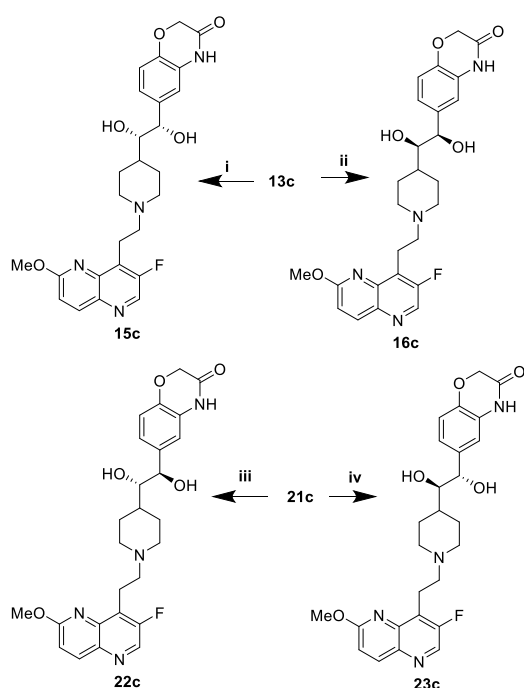
seen in previous publications.^{12–14} Compounds were selected for synthesis and experimental analysis based on the average of their Z-scores across the three systems mentioned above, with more negative values reflecting better predicted binding.

To simplify the workflow, docking experiments were conducted with targeted variations in the RHS moiety while holding other components consistent (Figure 3 and Table 1). Docking of the “traditional” NBTI with a secondary amine (40) afforded an average Z-score of −0.949, and novel structures with superior (more negative) scores were prioritized for synthesis

and experimental evaluation. In this research, our molecular design strategy focused on replacement of the secondary amine with other functional groups capable of engaging GyrA D83; four classes of analogues were of particular interest (alcohol 7a–c, *syn*- and *anti*-diols (15a–c and 16a–c; 22a–c and 23a–c), and primary amine (9a–c) (Figure 5). Primary amines have been associated with increased intracellular accumulation and reduced efflux in Gram-negative bacteria,^{10,15} and our chosen compound set enables a matched-pair comparison of primary amines with analogous alcohols and the secondary amine.

Scheme 4. Synthesis of Alkane and Alkenes with Oxazinone RHS^a

^aReaction conditions: (i) 4-CZIPN, Co(dmgH)(dmgH₂)Cl₂, HK₂PO₄, Et₃N, DMF, 440 nm light; (ii) HCl, MeOH; (iii) 1. 5, AcOH, THF/MeOH, 4 Å MS. 2. NaCNBH₃; (iv) H₂, 10% Pd/C, MeOH; (v) [Ir{dF(CF₃)ppy}₂(dtbpy)]PF₆, ACN, 456 nm light; (vi) HCl, MeOH.

Scheme 5. Synthesis of *Syn*- and *Anti*-Diols with Oxazinone RHS^a

^aReaction conditions: (i) AD-mix- α , ^tBuOH/water; (ii) AD-mix- β , ^tBuOH/water; (iii) AD-mix- α , ^tBuOH/water; (iv) AD-mix- β , ^tBuOH/water.

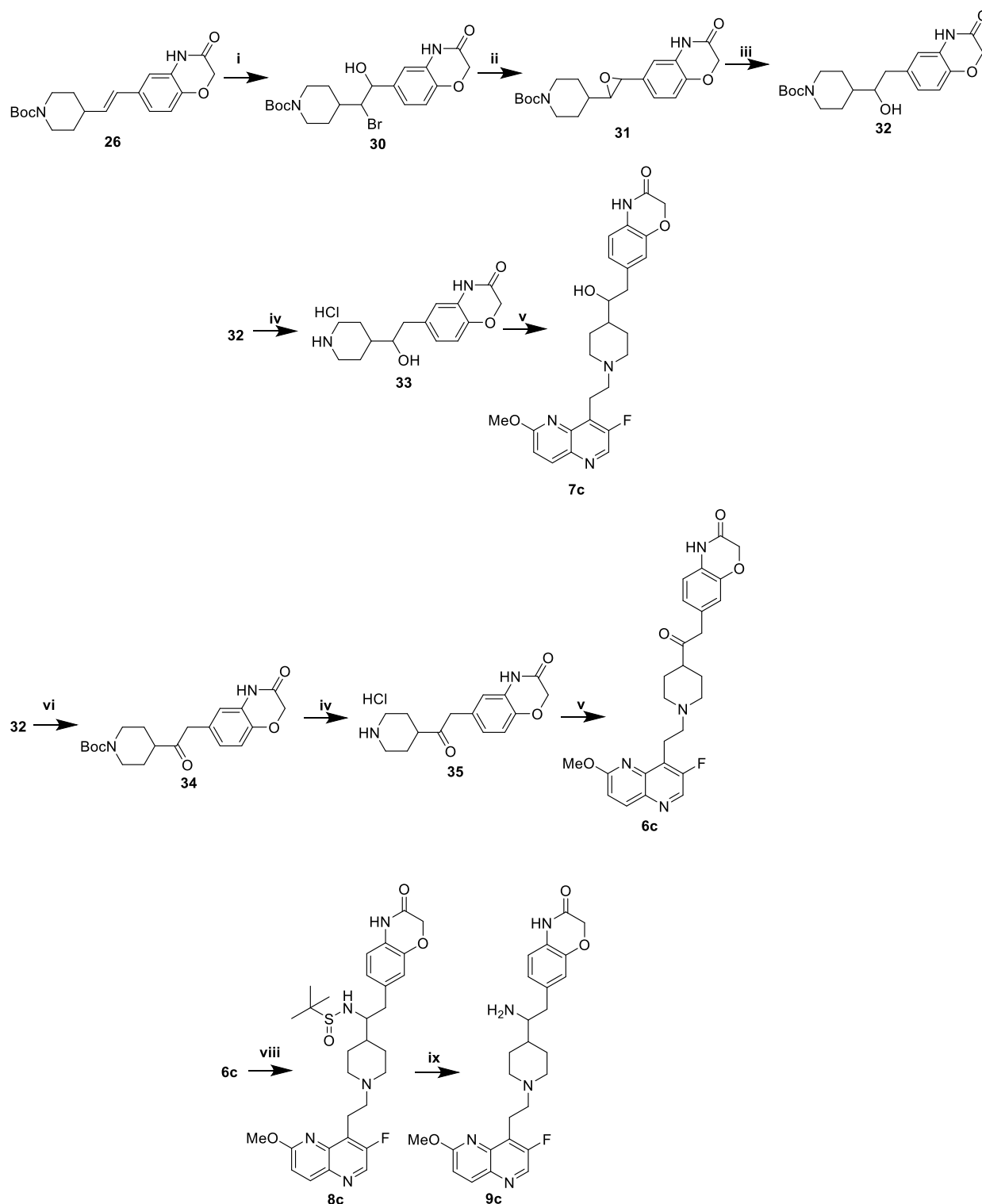
Predicted docking poses (Figure 4) display interactions with the D83 residue at the entrance to the binding pocket of DNA gyrase. This prediction is in line with the known binding mode of traditional NBTIs.^{5–8} The diol (22/23a) and monohydroxyl

(7a) form a hydrogen bonding interaction, while the amine (9a) forms an ionic interaction. Within each compound class, analogues with three diverse RHS moieties were synthesized, the hydrophobic *para*-toluyl group (a), the more polar benzodioxane possessing two potential hydrogen bond acceptors (b), and benzoxazinone (c) containing both hydrogen bond donor and acceptor moieties. The compounds shared similar synthetic routes. Moreover, antibacterial testing of various intermediates, namely, ketones (6a–c), (*E*)-olefins (13a–c), and (*Z*)-olefins (21a–c) as well as the readily accessible alkanes (14a–c) further expanded our understanding of structure–activity relationships.

The tolyl and benzodioxane compounds were prepared by analogous routes (Schemes 1–3), while the oxazinones generally necessitated modified approaches (Schemes 4–7).

Ketone (6a,b), monohydroxyl (7a,b), and primary amine (9a,b) analogues were prepared from ketone 1 (Scheme 1). Preliminary studies (not shown) indicated little dependence of the antibacterial activity on the absolute stereochemistry of the monohydroxyl or primary amine compounds. Consequently, racemic mixtures were synthesized. Pd-catalyzed arylation with the requisite aryl bromides 2a,b afforded intermediates 3a,b. Boc deprotection with methanolic HCl yielded amines 4a,b as hydrochloride salts. Reductive amination of aldehyde 5 afforded ketones 6a,b. Reduction with NaBH₄ provided racemic monohydroxyls 7a,b, whereas reductive amination with racemic *tert*-butylsulfonamide provided sulfonamide intermediates 8a,b. Acidic deprotection afforded racemic primary amines 9a,b.

The synthesis of (*E*)-alkene 13a,b, alkane 14a,b, and *syn*-diol compounds (15a,b and 16a,b) began with reductive amination of aldehyde 5 with 4-iodopiperidine 10 to afford intermediate 11 (Scheme 2). A Heck-type coupling under photocatalytic conditions with styrenes 12a,b produced the (*E*)-olefins 13a,b stereoselectively (dr >10:1).¹⁶ Hydrogenation afforded alkanes

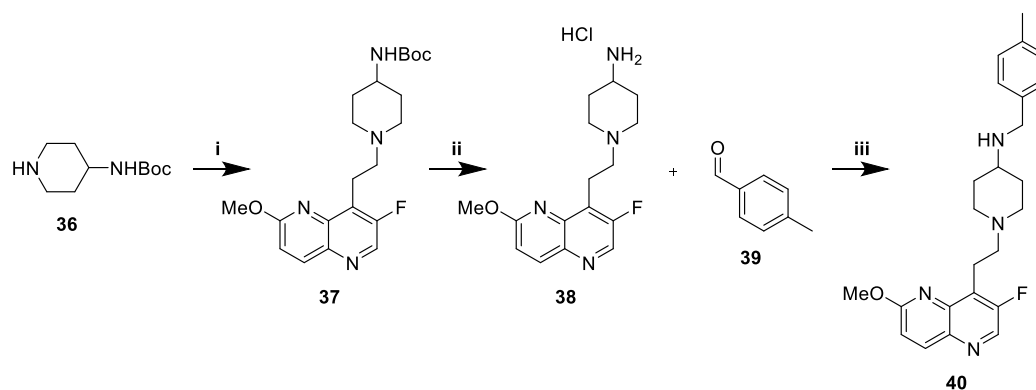
Scheme 6. Synthesis of Ketone, Monohydroxyl, and Monoamine with Oxazinone RHS^{4a}

^{4a}Reaction conditions: (i) NBS, ACN/water; (ii) K₂CO₃, MeOH; (iii) H₂, 10% Pd/C, EtOH; (iv) HCl, MeOH; (v) 1. AcOH, THF/MeOH, 4 Å MS. 2. NaCNBH₃; (vi) DMP, DCM; (vii) HCl, MeOH; (viii) 1. H₂NSOC(CH₃)₃, Ti(OEt)₄, THF, 2. NaBH₄, MeOH; (ix) HCl, MeOH.

14a,b. Alternatively, Sharpless asymmetric dihydroxylation of the (*E*)-olefins using commercially available AD-mix- α or - β provided *syn*-diols **15a,b** and **16a,b**, respectively.

The synthesis of (*Z*)-alkene **21a,b** and *anti*-diol compounds (**22a,b** and **23a,b**) began with Wittig reaction of **17** with commercially available aldehydes **18a,b** to afford (*Z*)-alkene

intermediates **19a,b** in high stereoselectivity (dr ~25:1, [Scheme 3](#)). Boc deprotection to afford **20a,b** and subsequent reductive amination of aldehyde **5** yielded (*Z*)-alkenes **21a,b**. Sharpless asymmetric dihydroxylation using AD-mix- α or - β delivered *anti*-diols **22a,b** and **23a,b**, respectively.

Scheme 7. Synthesis of Secondary Amine^a

^aReaction conditions: (i) 1. 5, AcOH, THF/MeOH, 4 Å MS. 2. NaCNBH₃; (ii) HCl, MeOH; (iii) AcOH, THF/MeOH, 4 Å MS. 2. NaCNBH₃.

The synthesis of benzoxazinone-type analogues was generally more difficult owing to the presence of an abstractable proton, requiring modified routes. Heck-type coupling of iodide **24** and styrene **25** under photocatalytic conditions afforded (*E*)-alkene **26** (Scheme 4).¹⁶ Boc deprotection to afford **27**, followed by reductive amination with **5**, yielded (*E*)-olefin **13c**, which was hydrogenated to provide **14c**. Alternatively, photocatalytic isomerization of (*E*)-olefin **26** at 456 nm with iridium catalysis afforded (*Z*)-olefin **28**. Boc deprotection to provide **29** and subsequent reductive amination of aldehyde **5** yielded (*Z*)-olefin **21c**.

The *syn*- (**15c**, **16c**) and *anti*-diols (**22c**, **23c**) were prepared via Sharpless asymmetric dihydroxylation of the (*E*)- and (*Z*)-alkenes, respectively (Scheme 5).

Ketone **6c**, monohydroxyl **7c**, and primary amine **9c** were synthesized beginning with (*E*)-alkene **26** (Scheme 6). Reaction with NBS afforded bromohydrin **30**, which was converted to epoxide **31** under basic conditions. Hydrogenolysis using 10% Pd/C provided alcohol **32**. Deprotection with methanolic HCl afforded piperidine **33**, and reductive amination of aldehyde **5** yielded monohydroxylated analogue **7c**. Alternatively, the oxidation of **32** with Dess-Martin periodinane yielded ketone **34**. Acidic Boc deprotection to provide piperidine **35** and reductive amination with aldehyde **5** afforded ketone analogue **6c**. Reductive amination of the ketone with racemic *tert*-butylsulfonamide afforded **8c**, and a final deprotection with methanolic HCl provided racemic primary amine **9c**.

A single secondary amine **40** was synthesized as a comparator representing the “traditional” NBTI scaffold (Scheme 7). Aldehyde **5** and piperidine **36** were reacted via reductive amination to afford Boc protected **37**. Deprotection under acidic conditions and reductive amination with aldehyde **39** produced secondary amine **40**.

Minimal inhibitory concentrations (MICs) were determined using three strains of *S. aureus* and two Gram-negative pathogens (*Escherichia coli* and *Acinetobacter baumannii*). The strains of *S. aureus* include one wildtype (ATCC 29213), one NBTI-resistant (GyrA D83N amino acid substitution¹⁷), and one fluoroquinolone-resistant MRSA (USA300).

Surprisingly, (*E*)-olefins **13a–c** and alkanes **14a–c** displayed anti-staphylococcal activity, suggesting that NBTIs do not absolutely require the prototypical interaction with D83 at the entrance of the DNA gyrase binding pocket if the appropriate binding vector is achieved. Meanwhile, (*Z*)-olefins **21a–c** did not retain the same activity, perhaps due to the misorientation of the RHS by the bond angle produced by the (*Z*)-olefin.

Monohydroxylated compounds **7a–c** were more potent than the alkanes and alkenes and were comparable to other synthesized compounds when comparing Gram-positive MICs. This finding was also intriguing, as the monohydroxyls are incapable of forming an ionic interaction with D83 but can hydrogen bond, as predicted in Figure 4. Ketones (**6a** and **6c**) were similarly active vs *S. aureus*, whereas compound **6b** showed elevated MICs. Primary amines **9a–c** were less potent than matched monohydroxyls **7a–c**. The computational model (Table 1) predicted equivalent GyrA binding between diol enantiomers. Table 2 results confirmed this prediction: only minimal differences were observed for *S. aureus* [ATCC 29213, USA300 (MRSA)] MICs in head-to-head comparisons of **15a,b** with **16a,b** or **22a–c** with **23a–c**. Results for **15c** and **16c** were within the standard variability in the MIC assays. In contrast, docking results (Table 1) predicted a greater GyrA binding affinity for the *anti*- (**22a–c** and **23a–c**) versus the *syn*-diols (**15a–c** and **16a–c**). Results in Table 2 were consistent with this prediction, with *S. aureus* [ATCC 29213, USA300 (MRSA)] MICs values indicating improved activity for the *anti*- compared to the *syn*-diol analogues.

All compounds lost activity in the NBTI-resistant strain (GyrA D83N) suggesting that the atypical NBTIs described here derive their antibacterial activity primarily from targeting DNA gyrase. The loss of activity for (*E*)-alkenes, alkanes, and (*Z*)-alkenes is particularly striking, given that they are incapable of direct interaction with D83. The precise origins of this effect are not fully understood. The analogous GyrA D82N mutation is in *E. coli* leads to diminished target inhibition by fluoroquinolones and imidazopyrazinones,¹⁹ neither of which directly contacts the aspartate. This observation has been attributed to reductions in the ability of gyrase to cleave DNA.

Compared to *S. aureus*, the MICs for these atypical NBTIs against Gram-negative pathogens were elevated. However, several structure–activity relationships were observed. Oxazinone-containing compounds often outperformed other RHS moieties (e.g., **6c**, **7c**, **13c**, and **14c**). Incorporating primary amines has previously been associated with a higher intracellular accumulation in *E. coli*.^{10,15} In contrast with their diminished anti-staphylococcal activity, primary amines (**9a–c**) displayed comparable *E. coli* MICs to the matched monohydroxyls (**7a–c**), potentially reflecting improved accumulation. Primary amine **9a** was 4-fold less potent than the structure-matched secondary amine control (**40**) for both Gram-negative bacteria. As with *S. aureus*, MICs for (*Z*)-olefins (**21a–c**) were higher, whereas those of (*E*)-olefins (**13b,c**) were similar to other chemotypes.

Table 2. Minimal Inhibitory Concentrations ($\mu\text{g/mL}$) of Atypical NBTIs^a

Cmpd	<i>S. aureus</i> ATCC 29213	<i>S. aureus</i> D83N ^b	<i>S. aureus</i> USA300 (MRSA)	<i>E. coli</i> ATCC 25922	<i>A. baumannii</i> ATCC 19606
Ketones					
6a	0.13	4	0.13	16	32
6b	4	32	4	64	64
6c	≤ 0.13	16	≤ 0.13	2	4
Monohydroxyls					
7a	0.06	8	0.25	16	32
7b	0.25	4	0.25	8	8
7c	≤ 0.13	16	≤ 0.13	4	4
Primary amines					
9a	1	32	4	16	16
9b	2	≥ 16	4	8	16
9c	16	64	8	8	32
(E)-Alkenes					
13a	0.25–0.5 ^c	≥ 32	0.5	≥ 64	≥ 64
13b	0.25	2	0.5	8	8
13c	0.5	16	≤ 0.25	4	8
Alkanes					
14a	0.5	4	0.5	64	64
14b	0.5	8	1	16	32
14c	1	32	0.5	4	8
(syn)-Diols					
15a	1–2 ^c	≥ 32	2–4 ^c	64	32–64 ^c
15b	1	≥ 32	2	32	16
15c	1	≥ 32	1	32	16
16a	1	≥ 32	2	64	64
16b	1–4 ^c	16	1–4 ^c	16	8–16 ^c
16c	8	≥ 32	8	16	32
(Z)-Alkenes					
21a	4–8 ^c	32	4–8 ^c	≥ 64	≥ 64
21b	8	≥ 16	8	≥ 64	64
21c	≥ 64	≥ 64	64	64	32
(anti)-Diols					
22a	0.13	16	0.13	16	16
22b	≤ 0.13	16	0.25	16	8
22c	2	64	4	8	32
23a	0.13	≥ 32	0.25	32	16
23b	0.03	8	0.03	8	4
23c	2	64	2	8	32
Controls					
40	≤ 0.25	16	≤ 0.25	4	4
Gep. ^d	0.125–1 ^c	8–32 ^c	0.125–0.5 ^c	1–4 ^c	8–32 ^c
Cipro. ^d	0.25–0.5 ^c	0.25–1 ^c	16–64 ^c	0.0075–0.25 ^c	1–2 ^c

^aMinimal inhibitory concentrations (MICs) determined in triplicate (at a minimum) according to CLSI guidelines.¹⁸ ^bFirst-step mutant of *S. aureus* 29213.¹⁷ ^cRanges of observed values given where appropriate. ^dGepotidacin and ciprofloxacin employed as controls.

The *syn*- (15a–c and 16a–c) and *anti*-diols (22a–c and 23a–c) had comparable activity against Gram-negative pathogens. Overall, the compounds displayed more modest antibacterial activity against the studied Gram-negative pathogens, as is commonly seen with NBTIs,^{5,17} albeit with some exceptions.²⁰ We suspect that the reduced activity may be due to lower intracellular accumulation as a result of efflux (see below).

To address the question of efflux, compounds were tested against wildtype and efflux-deficient strains of *Salmonella enterica* serovar Typhimurium (Table 3), a clinically important Gram-negative pathogen causing salmonellosis.²¹ The tolC-knockout lacks a prominent efflux channel present in the wildtype.²² By comparing the MICs for both strains, we can estimate the impact of the efflux.

Whereas compounds displayed poor activity against the wildtype strain, MICs were significantly improved against the efflux-deficient strain. Primary amines 9a–c displayed reduced susceptibility to efflux, with 8- to 16-fold changes observed between strains, whereas changes for other compounds ranged as high as 512-fold. Thus, the primary amines are not effluxed as efficiently as other chemotypes,¹⁵ as has been previously observed in a structurally distinct series of NBTIs.¹⁰

Biochemical evaluations using isolated bacterial topoisomerases were conducted to validate the mechanism of action and further support the MIC results and the computational model. The inhibition of supercoiling by *S. aureus* DNA gyrase was measured as previously described.²⁵ The secondary amine control (40), monohydroxyl (7a), monoamine (9a), and *anti*-diol (22a) were chosen as representative compounds, holding

Table 3. Antibacterial Activity vs *Salmonella* Strains^a

Cmpd	<i>S. enterica</i> Typhimurium ATCC 14028 MIC ($\mu\text{g/mL}$)	<i>S. enterica</i> Typhimurium 14028 $\Delta\text{tolC1}::\text{kan}^b$ MIC ($\mu\text{g/mL}$)	Fold Change
6a	64	0.5	128
6b	≥ 64	1 to $>1^c$	≥ 64
6c	16	≤ 0.125	≥ 128
7a	64	1	64
7b	32	0.25	128
7c	16	≤ 0.125	≥ 128
9a	64	4	16
9b	32	2	16
9c	32	4	8
13a	≥ 64	0.5–1 ^c	≥ 64
13b	64	0.125	512
13c	16	0.25	64
14a	≥ 64	1	≥ 64
14b	64	0.25	256
14c	16	0.5	32
15a	≥ 64	1–2 ^b	≥ 64
15b	64	1	64
15c	32	0.25	128
16a	≥ 64	0.5–1 ^c	≥ 128
16b	64	0.5	128
16c	64	1	64
21a	≥ 64	8	≥ 8
21b	≥ 64	4	≥ 16
21c	64	16	4
22a	64	1	64
22b	32	0.25	128
22c	≥ 64	8	≥ 8
23a	64	1	64
23b	32	0.125	256
23c	32	0.5	64
40	32	0.5	64
Gep. ^d	2–8 ^c	0.125–0.5 ^c	4–16
Cipro. ^d	≤ 0.13	≤ 0.13	N/A

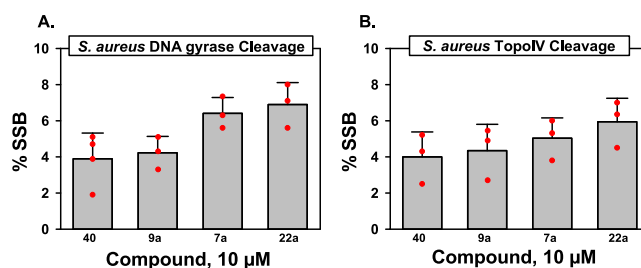
^aMinimal inhibitory concentrations (MICs) determined in triplicate (at a minimum) according to CLSI guidelines.¹⁸ ^bEfflux-deficient strain EFB044. This strain was constructed by moving mutation $\Delta\text{tolC1}::\text{kan}$ from the McClelland mutant collection²³ into wildtype strain 14028 using P22 transduction.²⁴ ^cRanges of observed values given where appropriate. ^dGepotidacin and ciprofloxacin employed as controls.

Table 4. Supercoiling IC₅₀ against DNA Gyrase and Decatenation IC₅₀ Against TopoIV^a

Cmpd	<i>S. aureus</i> DNA gyrase IC ₅₀ (μM) ^a	<i>S. aureus</i> TopoIV IC ₅₀ (μM) ^b	<i>S. aureus</i> TopoIV/gyrase ratio	<i>E. coli</i> DNA gyrase IC ₅₀ (μM) ^a
7a	0.24	2.29	9.5	12.37
9a	5.05	82.6	16.4	>400
22a	0.065	3.31	50.9	17.09
40	0.004	0.69	176.9	NT ^c

^aInhibition of supercoiling activity ($n = 3$). ^bInhibition of decatenation activity ($n = 3$). ^cNot tested.

the LHS and RHS constant to assess the impact of the novel functionality. Secondary amine **40** afforded the most potent inhibition despite its predicted weaker binding (Table 4). For the novel compounds, the relative binding affinities predicted by the computational model (Table 1) were not always consistent with the inhibition of *S. aureus* DNA gyrase, with **9a** being the

Figure 6. DNA cleavage assays with *S. aureus* DNA gyrase (left, A) and TopoIV (right, B).

least potent inhibitor, despite having the best predicted binding affinity. However, the relative whole cell anti-staphylococcal activity followed the same trend as gyrase inhibition (Table 2), supporting DNA gyrase as the principal target in *S. aureus*. Compounds were also evaluated by using *E. coli* DNA gyrase, where inhibition was considerably weaker. The correlation between binding affinities and biochemical inhibition was also less robust, perhaps attributable to the derivation of the docking model from *S. aureus* gyrase structures.

Decatenation experiments with *S. aureus* TopoIV were conducted with the same select compounds to assess the potential for dual targeting of both bacterial topoisomerases. All compounds displayed elevated IC₅₀ values for TopoIV over DNA gyrase, and compound **9a** displayed the highest IC₅₀ value (Table 4). Compounds **7a** and **22a** had comparable activity in the TopoIV assay, in contrast to their ~4-fold differences in gyrase IC₅₀ values, and **7a** afforded the best TopoIV/gyrase ratio at 9.5. Despite the much higher TopoIV/gyrase ratio for **22a** (51), its potent targeting of DNA gyrase nevertheless afforded excellent anti-staphylococcal activity (Table 2).

NBTIs typically lead to the accumulation of single-strand breaks (SSBs) with both DNA gyrase and TopoIV.^{5,7} Given the atypical structures of the NBTIs described herein, an assessment of cleavage activity was warranted. All compounds tested displayed typical NBTI behavior and resulted in an accumulation of SSBs, with minimal to no DSBs observed (<1%, data not shown). For *S. aureus* gyrase at 10 μM, lower levels of SSBs were observed for primary amine **9a** and secondary amine **40** as compared to those of monohydroxyl **7a** and anti-diol **22a** (Figure 6A). Similar results were obtained for the TopoIV cleavage assay (Figure 6B).

In this study, we employed a computational docking model to prioritize compounds for the synthesis and testing. Diverse novel chemotypes delivered potent MICs with the Gram-positive pathogen *S. aureus*. Elevated MICs in the NBTI-resistant GyrA D83N mutant *S. aureus* and biochemical testing with isolated enzymes were consistent with gyrase as the primary antibacterial target. Using a test set of three novel chemotypes, we found predicted docking scores were consistent with relative inhibition of *S. aureus* DNA gyrase and with whole cell anti-staphylococcal activity. Antibacterial activity was significantly reduced in Gram-negative bacteria, including *E. coli*, *A. baumannii*, and *S. enterica* Typhimurium. Results from wildtype and efflux-deficient *Salmonella* strains demonstrated that efflux significantly compromised activity in the wildtype strain, indicating a need for continued optimization. In total, these results open the door for further chemical innovation of the NBTI class of antibacterials.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmmedchemlett.4c00162>.

Assay methods, computational methods, synthesis and characterization of tested compounds. ^1H NMR spectra of tested compounds, ^{13}C NMR spectra, UPLC, and HRMS of selected compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Mark J. Mitton-Fry – Division of Medicinal Chemistry and Pharmacognosy, The Ohio State University, Columbus, Ohio 43210, United States; orcid.org/0000-0002-6715-8836; Email: mitton-fry.1@osu.edu

Authors

Joshua W. Powell – Division of Medicinal Chemistry and Pharmacognosy, The Ohio State University, Columbus, Ohio 43210, United States

Chelsea A. Mann – Division of Medicinal Chemistry and Pharmacognosy, The Ohio State University, Columbus, Ohio 43210, United States

Paul D. Toth – Department of Chemistry and Biochemistry, The Ohio State University, Columbus, Ohio 43210, United States

Sheri Nolan – Microbial Infection and Immunity, The Ohio State University, Columbus, Ohio 43210, United States

Anja Steinert – Division of Outcomes and Translational Sciences, The Ohio State University, Columbus, Ohio 43210, United States

Clarissa Ove – Division of Medicinal Chemistry and Pharmacognosy, The Ohio State University, Columbus, Ohio 43210, United States

Justin T. Seffernick – Department of Chemistry and Biochemistry, The Ohio State University, Columbus, Ohio 43210, United States

Daniel J. Wozniak – Microbial Infection and Immunity and Department of Microbiology, The Ohio State University, Columbus, Ohio 43210, United States

Razieh Kebraei – Division of Outcomes and Translational Sciences, The Ohio State University, Columbus, Ohio 43210, United States

Steffen Lindert – Department of Chemistry and Biochemistry, The Ohio State University, Columbus, Ohio 43210, United States; orcid.org/0000-0002-3976-3473

Neil Osheroﬀ – Department of Biochemistry and Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, United States; orcid.org/0000-0002-2550-4884

Jack C. Yalowich – Division of Pharmaceutics and Pharmacology, The Ohio State University, Columbus, Ohio 43210, United States

Complete contact information is available at <https://pubs.acs.org/doi/10.1021/acsmmedchemlett.4c00162>

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Notes

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■ ABBREVIATIONS

NBTI, novel bacterial topoisomerase inhibitor; TopoIV, topoisomerase IV; CLSI, Clinical and Laboratory Standards Institute; SSB, single-strand break; DSB, double-strand break

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