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Triaryl Benzimidazoles as a New Class of Antibacterial Agents against Resistant Pathogenic Microorganisms

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Supporting Information

ABSTRACT: A new class of nontoxic triaryl benzimidazole compounds, derived from existing classes of DNA minor groove binders, were designed, synthesized, and evaluated for their antibacterial activity against multidrug resistant (MDR) Gram-positive and Gram-negative species. Molecular modeling experiments suggest that the newly synthesized class cannot be accommodated within the minor groove of DNA due to a change in the shape of the molecules. Compounds 8, 13, and 14 were found to be the most active of the series, with MICs in the range of 0.5–4 μg/mL against the MDR Staphylococci and Enterococci species. Compound 13 showed moderate activity against the MDR Gram-negative strains, with MICs in the range of 16–32 μg/mL. Active compounds showed a bactericidal mode of action, and a mechanistic study suggested the inhibition of bacterial gyrase as the mechanism of action (MOA) of this chemical class. The MOA was further supported by the molecular modeling study.

INTRODUCTION

Antibiotic resistance is a major global issue. The rapid spread of resistance, along with the so-called “discovery void” of new classes of antibiotics, brought the prospect of a “post-antibiotic era”, where limited therapeutic alternatives are available for the treatment of infections caused by multidrug resistant (MDR) pathogens.1,2 Increasing occurrence of infection caused by MDR strains belonging to the ESKAPE group of pathogens is a matter of great concern across the world.3,4 According to WHO and ECDC reports, diseases related to MDR pathogens are responsible for 25000 and 23000 deaths a year in Europe and the U.S., respectively. Those values are predicted to increase to 10 million deaths a year in 2050 if the surge in antimicrobial resistance (AMR) is left unchecked.5 This phenomenon is caused primarily by the lack of discovery of new classes of antibiotics in the last 30 years and the rapid increase in resistance to existing classes of antibiotic.6 The last classes of antibacterial drugs with activity against Gram-positive bacteria (mutilins, lipopeptides, and oxazolidinones) were introduced in the U.S., respectively. Those values are predicted to increase to 10 million deaths a year in 2050 if the surge in antimicrobial resistance (AMR) is left unchecked.5 This phenomenon is caused primarily by the lack of discovery of new classes of antibiotics in the last 30 years and the rapid increase in resistance to existing classes of antibiotic.6 The last classes of antibacterial drugs with activity against Gram-positive bacteria (mutilins, lipopeptides, and oxazolidinones) were introduced in the 2000s, but their discovery goes as far back as the 1980s while the last broad spectrum antibiotic class, quinolones, was introduced almost 50 years ago in 1964.6,7 This extended period, often described as a “discovery void”, reflects the failure of the antibacterial drug discovery processes.6,8,9 The target-based, genomic-driven approach, while presenting enormous advantages in terms of rationalization of the research and reduced costs, failed in finding scaffolds belonging to new chemical classes with antibacterial activity.6,10 This is due to the fact that in most cases, the translation of compounds identified through cell-free HTS assay into candidates with in vitro and ultimately in vivo efficacy was unsuccessful. This failure could be partly due to either the target not being sufficiently validated or the inability of the compounds to penetrate bacteria and partly due to their inability to overcome the resistance mechanisms that are commonly associated with MDR bacteria. A solution proposed to overcome this impasse in the antibacterial drug discovery process can be found in the expansion of the chemical space of antibacterial drugs.10 The discovery of new scaffolds derived from natural sources or chemical synthesis could open the way to alternative classes of antibiotics with new mechanisms of action. Several results indicated benzofused rings, especially benzimidazole, as promising moieties to design novel scaffolds with antibacterial activity.11–14 According to the literature, several pharmacological activities (such as antibacterial, anticancer, and anti-inflammatory) are attributed to benzimidazole derivatives.15,16 This wide range of activity is attributed to the peculiar chemical features ofazole rings, which are able to interact in a noncovalent way with a range of targets due to the presence of an electron-rich aromatic system and heteroatoms.17,18 Different derivatives of the bis-benzimidazole fluorescent dye 4-((6-(4-methylpiperazin-1-yl)-1H,3′H-2,5′-bibenzo[d]imidazo)-2′-yl)phenol (Hoechst 33258, Figure 1) have shown notable antibacterial activity against MRSA and VRE.19 The Hoechst series of dyes is a group of molecules widely used to stain DNA sequences in biology due to their ability to bind to AT-rich sequences in the minor groove of DNA. By simply changing the
configuration of the binding of the two sequential benzimidazo- 

cles units from head to tail to symmetric head to head, a new 

series of derivatives with increased anticancer and antibacterial 

activity against the MRSA and VRE strains has been developed. 

Recently, compounds belonging to this class have been 

reported to be active against MRSA and VRE pathogenic 

strains, with MICs in the range between 0.06 and 8 μg/mL and 

an example is compound 1 (compound A, Figure 1).20 This 

process also lead to the discovery of 2,2′-di(pyridin-4-yl)- 

1H,3′H-5,5′-bibenzimidazole (SMT-19969, Figure 1), a 

nonabsorbable agent for the treatment of Gram-positive 

Clostridium difficile infections that recently entered phase II 

clinical trials.21 Other research groups focused their attention 

on the Hoechst derivatives by designing a series of diamidine 

dication molecules, in which the two benzimidazole units are 

separated by a symmetric diaryl spacer. The most interesting 

molecul in this class was represented by compound 2 (DB- 

325, Figure 1); along with its derivatives, it presented good 

antibacterial activity against MRSA and VRE pathogenic 

agents with MICs between 0.06 and 1 μg/mL.22−24 Although different 

mechanisms of action that are able to explain the activity of 

these molecules have been proposed,25,26 a possible major issue 

for this series of derivatives is related to their ability to bind to 

the minor groove of DNA. This could cause unwanted 

eukaryotic toxicity which would be undesirable, especially for 

compounds that are only active against Gram-positive strains 

for which limited, but safer, therapeutic alternatives are still 

available. For this reason, we designed a new triaryl 

benzimidazole scaffold with the aim of developing a new, 

safer antibacterial agent against multidrug resistant MRSA and 

VRE strains. In this article, we report the design, molecular 

modeling study, synthesis, and microbiological profile evalu- 

ation of this new class of compounds.

■ RESULTS AND DISCUSSION

Design of Compounds and Molecular Modeling. We 
designed a new triaryl benzimidazole scaffold characterized 
by its reduced affinity toward the minor groove of DNA with 
the aim of improving the cytotoxicity profile against eukaryotic 
cell lines. The new scaffold (Figure 2) presents a central 
biaryl-pyridino-phenyl spacer linked to two benzimidazole 
units. The central linker in the bis-amidine and bis- 
benzimidazole compounds has been studied by different 

research groups, and the biphenyl spacers have been shown to 
reduce the interaction of molecules with the minor groove of 
DNA compared to alkyl and alkenyl spacers.27 To change the 
shape of the molecule, one benzimidazole moiety is directly 
linked through a C−N bond to the pyridine ring, while the 
other one is connected through an amide linker to the phenyl 
ring. Moreover, to disrupt the linearity of the molecule and 
conferring a different three-dimensional shape compared to 
previously reported molecules, the benzimidazole moieties are 
linked in a nonsymmetric way to the central spacer. The 
scaffold is then completed by a protonable tertiary amionic 
lateral chain that has been reported to be important in 
conferring antibacterial activity against MRSA and VRE 
strains.

To prove the hypothesis that the new scaffold has reduced 
affinity to the minor groove of DNA, in comparison with 
previously reported bis-benzimidazole antibacterial molecules, 
we performed molecular modeling of the selected compounds 
against different sequences of DNA. Compounds 1, 2, and 
the newly designed scaffold completed by a diethylamino tail 
(compound 8) underwent molecular docking against three 
different sequences of DNA (Figure 3A,B). The selected 
sequences represent a mixed sequence, a GC-rich sequence and 
an AT-rich sequence and are reported in Table 1 along with 
the result of the docking experiment expressed in predicted 
affinity (kcal/mol) for the DNA sequences. The experiment indicates 
that the literature compounds bind preferentially to the minor 
groove of DNA through a network of conventional hydrogen 
bonds and carbon−hydrogen bonds between the hydrogen rich 
molecules and the DNA sequences, with some additionally 
hydrophobic interaction reported for compound 8. The 
behavior is consistent with the one reported for Hoechst 
derivatives, with a higher predicted affinity for the AT-rich 
DNA sequences.28 The newly designed scaffold presents a 
lower predicted affinity for the minor groove of DNA compared 
to the examples from the literature, indicating that the scaffold 
could not be accommodated favorably within the minor groove. 
According to the best docking pose reported in Figure 3 for the 
random DNA sequence, the second benzimidazole moiety of 
the newly designed scaffold stays outside the minor groove of 
DNA. This behavior is not consistent to the one shown by 
examples from the literature that bind well with the minor 
groove of DNA. The modeling study indicated that the 
introduction of the amide linkage disrupted the isohelicity of 
the newly designed molecules. We hypothesized that this 
ability to bind to the minor groove of DNA would result in 
loss of eukaryotic cell toxicity which has previously prevented 
the introduction of bisbenzimidazoles and Hoechst 
derivatives as antibacterial agents.

Synthesis and Preliminary SAR. A new synthetic route 
was designed for the synthesis of the novel series of compounds 
by taking into account the commercial availability of building 

Figure 1. Structure of previously synthesized bis-benzimidazole 
compounds with antibacterial activity.

Figure 2. General structure of newly designed triaryl benzimidazole 
class of compounds.
blocks (Scheme 1). A retrosynthetic disconnection approach allowed us to identify the N-pyridyl-benzimidazole moiety as a key intermediate for the synthesis of the desired compound. The process started with an aromatic nucleophilic substitution of 2-amino-5-bromopyridine on methyl-(3-nitro-4-fluoro)phenylcarboxylate. The reaction was carried out in THF, using NaH as a base. In the next step, we used Raney nickel as a catalyst in a H₂ atmosphere (4 atm.) to reduce the nitro group of 3, thereby obtaining a rapid and complete conversion to the aniline derivative 5. The formation of the N-pyridyl-benzimidazole intermediate 5 was achieved by simply refluxing 4 in THF in the presence of methyl orthoacetate and p-toluensulfonic acid. Compound 6 was obtained via a Suzuki coupling reaction with 3-aminophenylboronic acid using tetrakis(triphenyl)palladium (0) in the presence of K₂CO₃ as a catalytic system. The aniline derivative 6 was subsequently coupled to 2-methyl-1H-benzimidazole-5-carboxylic acid to give compound 7, using HBTU/DIPEA as coupling reagents. For the synthesis of 8, compound 7 was first hydrolyzed in an aqueous basic condition to the corresponding carboxylic acid that was coupled to dimethylaminoethylamine, giving the final product that was synthesized as a proof of principle of our idea.

Figure 3. (A,B) Molecular model showing literature compounds 1 and 2 fits well with the DNA minor groove of a random sequence, while the designed ligand 8 cannot be accommodated within the DNA minor groove (A). Details of the interaction of the compounds within the minor groove of DNA (B).

Table 1. Molecular Docking Experiment Showing Affinity of Literature Compounds and Newly Designed Compound 8 against DNA Sequences

| Ligand | random seq | AT-rich seq | GC-rich seq |
|--------|------------|-------------|-------------|
| 1      | −11.0      | −12.3       | −10.5       |
| 2      | −11.4      | −11.9       | −12.0       |
| 8      | −10.2      | −10.4       | −10.0       |

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Also, in this case, the HBTU/DIPEA coupling system was used for the formation of the amide bond.

Compound 8 was tested for its microbiological activity against VRE-12201 and EMRSA-15 (HO 5096 0412) strains, resulting in activity with an MIC of 1 and 2 μg/mL, respectively (Table 2). These results showed that the newly designed triaryl benzimidazole scaffold possesses antibacterial activity and could be considered as an ideal starting point for medicinal chemistry optimization of the series. To proceed with a systematic exploration of the different components of the molecule, we initially focused on possible modifications which did not change the central triaryl benzimidazole scaffold. Initially, we substituted the dimethylaminoethyl tail with other tertiary amine tails that differ in their shape or carbon chain length (Scheme 1, compound 8–14). The importance of a protonable chemical moiety in antibacterial drugs has been investigated in different studies.29,30 Particularly, previous research focused their attention on the pK_a of the selected moieties, with weak bases found to be more effective in conferring antibacterial activity to the molecules by providing better penetration through the bacterial cell wall.31

These modifications caused a loss of antibacterial activity with MIC values between 64 and >128 μg/mL for the strains VRE-12201 and EMRSA-15 (Table 2). Compounds 13 and 14 with the same dimethylamino substitution with longer carbon chains (3-carbon and 4-carbon, respectively), showed improved antibacterial activities with MICs of 0.25 and 0.5 μg/mL for compound 13 and 0.25 and 1 mg/mL for compound 14, respectively. These two synthesized compounds showed notably superior activity compared to the 2-carbon tail analogue, with antibacterial activity comparable to the bis-benzimidazole compounds reported by Moreira and co-workers.20 This result demonstrated the importance of the dimethyl amino substitution pattern on the tertiary amine tail for antibacterial activity. A confirmation of the importance of the protonable moiety was shown by the lack of antibacterial activity shown by intermediate 7, a direct analogue of compound 13 without the tertiary amine tail.

With the aim of improving the activity of 13, we synthesized compound 17 (Scheme 2) in which an additional dimethylaminopropyl tail is added in position 2 of the benzimidazole ring attached to the central triaryl scaffold. Position 2 of the benzimidazole ring can be modified through simple synthetic steps involving the condensation of the diaminobenzoate derivative with carbonyl compounds. The synthesis of the required building block 15 was achieved by an MW-assisted condensation reaction between methyl 3,4-diaminobenzoate and 4-(dimethylamino)butanoic acid, using propylphosphonic anhydride (T3P) as condensing agent. The reaction allowed the synthesis of 15 with a good yield without the formation of byproducts. The building block obtained was then hydrolyzed in a basic environment to the corresponding carboxylic acid and coupled to 6, giving 16. The final step involved the hydrolysis of the methyl ester and the sequential

Table 2. Antibacterial Activity of the Synthesized Compounds against MDR VRE and MRSA Strains

| compd | VRE NCTC 12201 | MRSA EMRSA-15 |
|-------|---------------|---------------|
| 7     | >32           | >32           |
| 8     | 1             | 2             |
| 9     | >128          | >128          |
| 10    | 64            | >128          |
| 11    | >128          | >128          |
| 12    | >128          | >128          |
| 13    | 0.25          | 0.5           |
| 14    | 0.25          | 1             |
| 16    | >128          | >128          |
| 17    | 64            | >128          |
| 18    | >128          | >128          |
| 19    | >128          | >128          |
| 21    | >128          | >128          |
coupling to the dimethylaminopropyl tail to give 17. The compound was tested for its antibacterial activity; it showed partial activity (MIC of 64 μg/mL) against the VRE-12201 strain (Table 2). The addition of a second tertiary aminic tail caused no improvement in the antibacterial activity, contrary to what was observed in the series of compounds related to 2, where the deletion of one of the two amidine protonable moieties diminished the antibacterial activity against Gram-positive strains.

To verify the importance of the structural constituents of 13, we synthesized compounds 18 and 19 (Scheme 3). Together with compound 7, they represent all the possible fragments of the most active molecule 13. The antibacterial screening showed no antibacterial activity for the tested molecules against VRE-12201 and EMRSA-15, revealing the importance of all the moieties constituting compounds 13 (Table 2).

The importance of the second benzimidazole moiety linked through amide bond to the triaryl scaffold was evaluated with the synthesis of compound 21 (Scheme 4), in which the azole fused ring was substituted by a simple benzene ring. Compound 21 did not show activity against VRE-12201 and EMRSA-15 strains, confirming the role of benzimidazole ring in conferring antibacterial activity for this class of molecules.

Compounds having an MIC ≤ 64 μg/mL were screened against the extended panel of Gram-positive bacteria in order to verify the antibacterial activity of the selected molecules (Table 3). Compounds 13 and 14 were found to be most active molecule in this chemical series, with MIC values between 0.25 and 2 μg/mL for both MRSA and VRE strains with activity comparable or better compared to vancomycin, amoxicillin, and levofloxacin (Table 3). Good activity was also shown by compound 8, however, this activity was 4-fold less than 13. Compound 10 was inactive against all the strains tested, apart from VRE-12201. Compound 17 showed partial activity against VRE strains, with an MIC between 32 and 64 μg/mL, but showed no activity against S. aureus strains. Interestingly, the most active compounds (8, 13, and 14) showed no eukaryotic toxicity in the MTT test against HeLa cell lines at a concentration of 25 μM. The concentration tested is between 30 and 60 times the MIC for compounds 13 and 14, suggesting an adequate selectivity index between the eukaryotic and prokaryotic cells’ toxicity (Table 3).

To further test our hypothesis that DNA binding is associated with increased eukaryotic toxicity, we designed and then synthesized compound 24. This new molecule is
Table 3. Antibacterial Activity of Compounds 8, 10, 13, 14, 17, and 24 against the Extended Panel of Gram-Positive Bacteria and Their Toxicity against the Eukaryotic HeLa Cell Line

| Compd | NCTC 12201 | NCTC 12204 | NCTC 775 | EMRSA 15 | EMRSA 16 (MRSA 252) | ATCC 9144 | % viability in HeLa cells (MTT test 25 μM) |
|-------|------------|------------|----------|----------|----------------------|-----------|------------------------------------------|
| 8     | 1          | 2          | 4        | 2        | 2                    | 2         | >98                                      |
| 10    | 64         | >128       | >128     | >128     | >32                  | 16        | >98                                      |
| 13    | 0.25       | 0.5        | 0.5      | 0.5      | 2                    | 0.25      | >98                                      |
| 14    | 0.25       | <0.125     | 0.5      | 1        | 2                    | 0.5       | >98                                      |
| 17    | 32         | 64         | >128     | >128     | >128                 | >128      | 39                                       |
| 24    | 64         | 64         | 64       | >128     | >128                 | >128      | 62                                       |
| Vancomycin | >128 | >128     | 1        | 2        | 2                    | 0.5       |                                          |
| Amoxicillin | 0.5     | 16         | 0.5      | 128      | >128                 | 1         |                                          |
| Levofloxacin | 1        | 0.5       | 1        | 8        | 8                    | 0.12      |                                          |

Scheme 5. Synthetic Procedures and Reagents for Compound 24

Reagents and conditions: (a) HBr aq solution, 0 °C, 10 min then NaNO₂, CuBr, 50 °C; (b) 5-benzimidazole boronic acid, tetrakis(triphenyl)palladium(0), 1 MW irradiation, 150 °C, 15 min; (c) NaOH aq solution, MeOH, 25 °C, 15 h then dimethylaminopropylamine, HBTU, DIPEA, DMF, 25 °C, 2 h.
characterized by the presence of a direct C–C bond between the triaryl benzimidazole scaffold and the second benzimidazole moiety. The deletion of the amide bond led to a new tetra-aryl scaffold, which resulted in less flexibility and shape similarities to previous classes of bis-benzimidazole molecules. This was confirmed by molecular docking experiments of the new compound that were realized on different DNA sequences. The best resulting docking poses (Supporting Information, Figure S1) showed that the compound binds well to the minor groove of DNA, with a binding mode comparable to those predicted for previously reported examples; this was confirmed by comparing the estimated affinity values with DNA sequences (Supporting Information, Table S2). Synthetically, the constrained tetra-aryl compound 24 was obtained by subjecting the intermediate 6 to a Sandmeyer-type reaction to obtain the aryl halide 22. The low yield of the reaction was due to the simultaneous hydrolysis of the ester moiety caused by strong acidic conditions, as verified by the recovery of the corresponding acid derivative in the reaction mixture. A Suzuki coupling reaction gave the tetra-aryl scaffold 23, which was then hydrolyzed and coupled with the dimethylaminopropyl tail in order to give the final compound 24 (Scheme 5). The new tetra-aryl derivative showed reduced antibacterial activity, with an MIC value of 64 μg/mL against the strain VRE-12201 (Table 3). In addition, the compound was observed to be more toxic in comparison with its analogue 13, suggesting a good relationship between the binding mode of tetra-aryl compounds in the minor groove DNA and their eukaryotic toxicity.

**Evaluation of DNA Binding Ability of the Synthesized Compounds.** The ability of compounds 8, 13, and 14 to bind and stabilize the DNA was evaluated using a FRET-based DNA melting assay. The compounds were tested using an AT-rich DNA sequence. Netropsin, a known DNA minor groove binder, was used as a positive control. The result of the assay is reported in Table 4. Compounds 8, 13, and 14 did not stabilize the DNA sequence, with ΔTm values <1 °C at 1 μM concentration (drug:DNA ratio 5:1), while netropsin showed a significant 16.5 °C stabilization at 1 μM. Compound 13 and 8 showed weak DNA stabilization at 5 μM concentration (drug:DNA ratio 25:1), with 2.1 and 0.9 °C stabilization, respectively, while Netropsin stabilized the DNA by 22.2 °C. Compound 14 with the 4-carbon tail did not stabilize the DNA even at 5 μM concentration with only 0.6 °C stabilization at 25:1 drug:DNA ratio. The result supports the molecular docking experiment result, as the compounds did not interact favorably with the DNA sequence and failed to stabilize it compared to a control DNA minor groove binder.

**Mode of Killing of EMRSA-15 and VRE-12201 by Compounds 8, 13, and 14.** The mode of killing of the three most active compounds (8, 13, and 14) was determined at a supra-inhibitory concentration (4 × MIC) against VRE-12201 and EMRSA-15; the results are reported in Figure 4. All three compounds showed rapid bactericidal activity against the two strains tested, with the number of colonies below the limit of detection of the assay by 2 h after exposure to compounds 13 and 14 and 4 h with compound 8. A small population of cells was detected after 24 h of treatment of strain VRE 12201 with compound 8. This population was subsequently tested for increased resistance to compound 8; no change in MIC level was observed, indicating that this population is not comprised of true resistant mutants but rather is potentially a “persister population” of cells with reduced metabolic activity. No bacterial survival was detected for compound 13 in either species or for compound 8 in EMRSA-15. Interestingly, a small population of bacterial recovery was observed in both species for compound 14 after 10 h.

**Evaluation of Microbiological Activity against the Gram-Negative Panel.** The most active compounds (8, 10, 13, and 14) were also evaluated against Gram-negative bacteria. Two strains each (one drug-sensitive and one multidrug resistant) from Acinetobacter baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae were tested in the presence or absence of the efflux pump inhibitor phenylalanine-arginine β-naphthylamide (PA/N) in the presence of Mg2+ ions (Table 5). All strains tested showed no susceptibility to compounds 8 and 10 (MIC values of >32 μg/mL). Some activity was shown for compound 13 against A. baumannii (MIC 16 μg/mL) and the drug sensitive K. pneumoniae strain M6 (MIC 32 μg/mL), but no activity was observed against P. aeruginosa. To assess the effect of the Gram-negative membrane permeability barrier on the activity of the compounds, the selective membrane permeabilizer, polymixin B nonapeptide (PMBN) was used. Little effect was observed with either compound 8 or 10, with only strain M6 showing any significant reduction in MIC. In the case of compounds 13 and 14, MICs were significantly reduced by addition of PMBN by at least 4-fold for the two K. pneumoniae isolates and by greater than 16-fold for the two P. aeruginosa isolates, with a 2-fold reduction also seen in A. baumannii (final MICs being 8–16, 4, and 8 μg/mL, respectively). The addition of PA/N in the presence of Mg2+ to stabilize the membrane reduced the MIC value for all strains, except PA01 by 2–4-fold. As only one strain (K. pneumoniae M6) showed a ≥4-fold reduction in MIC, the data does not conclusively show that RND-family efflux pumps play a role in efflux of the compounds from the cell; further studies would be needed to understand the role of RND pumps and their specificity for these compounds. The poor activity of compounds 13 and 14 and more generally of the series against Gram-negative strains could be explained by the intrinsic chemical–physical properties of this class of molecules. Triaryl benzimidazole molecules have relatively high lipophilicity, which is not usually associated with molecules that are active against Gram-negative bacteria as they typically fit in a more hydrophilic chemical space. In addition the membrane structure of Gram-negative bacteria (having an inner and outer membrane) means that activity of compounds is often decreased relative to Gram-positive bacteria due to poor penetration of the compound through the cell membrane. The

| ligand | ΔTm (°C) |
|-------|----------|
| 8 (1 μM) | 0.2 |
| 8 (5 μM) | 0.9 |
| 13 (1 μM) | 0.4 |
| 13 (5 μM) | 2.1 |
| 14 (1 μM) | 0.3 |
| 14 (5 μM) | 0.6 |
| Netropsin (1 μM) | 16.5 |
| Netropsin (5 μM) | 22.2 |

“ΔTm values are reported in the table in °C in comparison to the control. Assay conducted in triplicate.
potentiation observed with PMBN in the *K. pneumoniae* and *P. aeruginosa* strains reflects the poor relative permeability of these membranes compared to *A. baumannii* isolates where only limited potentiation is observed. This is in agreement with observations suggesting that the membrane of *P. aeruginosa*, in particular, is less permeable than other Gram-negative bacteria.

**Preliminary Mechanistic Evaluation.** DNA-interactive molecules are known to inhibit DNA-processing enzymes,\(^{32,33}\) and bis-benzimidazoles have been shown to inhibit gyrases in *S. aureus*.\(^{34}\) Therefore, we investigated the possibility that these compounds might also exert their antimicrobial activity by interacting with DNA processing enzymes. A molecular docking experiment with the bacterial gyrase from *Staphylococcus aureus* (PDB 2XCT) showed compound 13 favorably interacted with the binding pocket of both the subunits of the enzyme, and the best pose gave a CHEM Score of 28.65 and affinity of \(-34.27\) (kcal/mol) for subunit 1 of gyrase A and a CHEM Score of 26.52 and affinity of \(-32.50\) (kcal/mol) for subunit 2 of gyrase A (Figure 5 and Supporting Information, Table S3). The 2D models shown in Figure 5C suggests compound 13 forms three conventional hydrogen bonds with lysine 43, arginine 92, and phenylalanine 97 of the subunit 1 of DNA gyrase A. Similarly, compound 13 also forms three conventional hydrogen bonds with serine 85, arginine 92, and serine 98 of the subunit 2 of gyrase A. A number of hydrophobic interactions with different amino acids of subunit 1 of gyrase A and electrostatic interactions with the subunit 2 of gyrase A enzyme were also observed. The molecular modeling study suggested that the newly designed scaffold inhibits DNA gyrase by directly interacting with the enzyme. The in silico observation was validated by carrying out a DNA gyrase inhibition assay using a commercial *S. aureus* supercoiling high throughput plate assay (no. SATRG01) kit obtained from Inspiralis (Norwich, UK), and the result is shown in Figure 6. At concentrations around the MIC for *S. aureus* (0.5 \(\mu\)g/mL), compound 13 showed 65.2 ± 9.8% inhibition of the gyrase and this was comparable to levofloxacin, a known DNA gyrase inhibitor, which showed inhibition of 57.9 ± 3.5% at the same concentration. Although only assessed over a small concentration range and under standard conditions as defined for the gyrase assay kit, these results suggest that compound 13 is an efficient inhibitor of *S. aureus* gyrase and this is likely to be the principle mechanism by which this compound kills the bacterial cells. Interestingly, compound 13 showed rapid bactericidal activity in time kill studies with both EMRSA-15 and VRE12201. When levofloxacin was used, also at 4 × MIC, against the same strains it achieved less than a 3-log bacterial kill with VRE12201 at 24 h (and would be defined as being bacteriostatic on this basis) and showed a 3-log kill against EMRSA15 at 6 h with a further log-reduction to below the limits of detection of viable bacteria (>6-log reduction) only at 24 h (Figure 4). This suggests that the mechanism of inhibition of *S. aureus* gyrase by compound 13 is probably

### Table S5. Antibacterial Activity of Compounds 8, 10, 13, and 14 against Gram-Negative Bacteria in the Presence and Absence of Efflux Pump Inhibitor PaβN and Membrane Permeabilizer PMBN

| compd | A. baumannii (µg/mL) | K. pneumoniae (µg/mL) | P. aeruginosa (µg/mL) |
|-------|----------------------|-----------------------|-----------------------|
|       | AYE      | ATCC 17978  | M6        | NCTC 13368 | PA01     | NCTC 13437 |
| 8     | >32      | >32        | >32      | >32      | >32      | >32        |
| 8+PaβN+Mg²⁺ | >32  | 32        | >32      | >32      | >32      | >32        |
| 8+PMBN | 32      | 32        | 16       | >32      | >32      | >32        |
| 10    | >32      | >32        | >32      | >32      | >32      | >32        |
| 10+PaβN+Mg²⁺ | >32  | >32      | >32      | >32      | >32      | >32        |
| 10+PMBN | >32    | >32      | >32      | >32      | >32      | >32        |
| 13    | 16       | 16        | 16       | 16       | 4        | 4          |
| 13+PaβN+Mg²⁺ | 8     | 8        | 8        | 16       | 4        | 4          |
| 13+PMBN | 8      | 8        | 8        | 16       | 4        | 4          |
| 14    | >32      | >32        | >32      | >32      | >32      | >32        |
| 14+PaβN+Mg²⁺ | 16    | 16        | 16       | 16       | 16       | 16          |
| 14+PMBN | 8      | 8        | 16       | 16       | 16       | 16          |

Figure 4. Response of EMRSA-15 and VRE-12201 strains to treatment with suprainhibitory concentrations (4 × MIC) of compounds 8, 13, and 14.
different from levofloxacin and that this results in a much more potent bactericidal kill in the two Gram-positive strains tested.

■ CONCLUSION

A new triaryl benzimidazole scaffold with a lower predicted affinity for DNA binding has been designed, synthesized, and evaluated for its antibacterial activity. The compounds showed a negligible DNA binding at high concentrations and were found to be nontoxic against eukaryotic cell lines. The most active compounds 8, 13, and 14 showed rapid bactericidal activity against multidrug-resistant Gram-positive MRSA and VRE bacteria, and, for the first time in the case of this class of compound, activity against specific strains of Gram-negative bacteria. The chemical scaffold appears to have a very restricted SAR, with limited modifications allowed in order to retain activity. The lack of DNA stabilization suggests that the antibacterial activity of these compounds is not related to their DNA binding ability. The compounds appeared to work by inhibiting DNA gyrase, and the most active compound showed notable inhibition of the gyrase enzyme from S. aureus. Compounds with antibacterial activity are in urgent need to combat the growing antimicrobial resistance problem, and this new scaffold provides an opportunity to develop a more potent antibacterial agent by carrying out further medicinal chemistry modifications. Further studies on the mechanism of gyrase inhibition, to understand the fundamental differences with fluoroquinolones, are also merited.

■ EXPERIMENTAL SECTION

Synthesis: General Material and Methods. All solvents and reagents for the synthesis were obtained from commercially available sources including, among others, Sigma-Aldrich, Fisher Scientific, Fluorochem, and Alfa Aesar. Thin-layer-chromatography (TLC) analysis was performed on silica gel plates (E. Merck silica gel 60 F254 plates) and visualized by ultraviolet (UV) radiation at 254 nm. Flash chromatography for the purification of the compound was performed with silica gel as a stationary phase (Merck 60, 230–400 mesh). 1H and 13C magnetic resonance (NMR) analyses were performed on a Bruker Avance 400 MHz spectrometer. LC-MS analyses were performed on a Waters Alliance 2695 system, eluting in gradient. The analyses were performed on a Monolithic C18 50 mm ×
Synthesis of Triaryl Benzimidazole Series Analogues. Synthesis of 4-(5-Bromo-pyridin-2-ylamino)-3-nitro-benzene Acid Methy Ether (3). NaH 60% dispersion in mineral oil (1.340 g, 1.5 equiv) was added to a solution of 5-bromo-pyridin-2-ylamine (4.275 g, 1 equiv) in THF (60 mL), with the suspension kept under a magnetic stirrer at 0 °C. After 20 min, 4-fluoro-3-nitro-benzene acid methyl ester (5.0 g, 1 equiv) was added to the suspension that was left under a magnetic stirrer, at 25 °C, for 15 h and monitored by TLC until completion. After 15 h, EtOH (40 mL) and H2O (150 mL) were added to the reaction mixture and a yellow solid precipitated. The suspension was filtered under vacuum, collected, and dissolved in DMF (50 mL), and sequentially washed with a saturated solution of NaHCO3 (60 mL) and brine (60 mL). The organic phase was dried over anhydrous MgSO4 and evaporated using a rotary evaporator. The residue was then evaporated in vacuum, with the residue dissolved in AcOEt (50 mL) and sequentially washed with a saturated solution of NaHCO3 (60 mL) and brine (60 mL). The organic phase was then evaporated using a rotary evaporator to give pure 3 (5.9 g, 68%), as a dark-yellow solid, mp 172.2 − 172.8 °C.

1H NMR (400 MHz, chloroform-d): δ 10.52 (1 H, s), 8.94 (1 H, d, J = 2.01 Hz), 8.86 (1 H, d, J = 9.06 Hz), 8.44 (1 H, d, J = 2.52 Hz), 8.19 (1 H, dd, J = 9.14, 2.14 Hz), 7.79 (1 H, dd, J = 8.56, 2.52 Hz), 6.93 (1 H, d, J = 8.81 Hz), 3.95 (3 H, s). 13C NMR (101 MHz, chloroform-d): δ 164.8, 151.1, 148.4, 141.2, 140.5, 135.8, 133.5, 128.1, 121.3, 118.7, 115.4, 113.6, 52.1. m/z (+EI) calcd for C13H12BrN3O2 (M)+ 321.0, found 351.8 (M+H)+.

Synthesis of 3-Amino-4-(5-bromo-pyridin-2-ylamino)-benzoic Acid Methylester (4). A solution was prepared by dissolving 3 (5.9 g, 1 equiv) in a mixture of MeOH and DCM (60 mL, 50:50, v/v). A catalytic amount of Raney nickel slurry in H2O (400 mg) was added to the solution, and the resulting solution was transferred to a vial and hydrogenated at 40 psi for 1 h in a Parr hydrogenation system until TLC showed completion of the reaction. Subsequently, the reaction mixture was filtered on a Celite path, washing with DCM. The organic phase was then evaporated by flushing with nitrogen to give 4 (5.2 g, > 95%) as a light-brown solid, mp 194.5–195.8 °C. 1H NMR (400 MHz, DMSO-d6): δ 8.31 (1 H, s), 8.19 (1 H, d, J = 2.52 Hz), 7.73 (1 H, dd, J = 8.94, 2.64 Hz), 7.67 (1 H, d, J = 8.31 Hz), 7.39 (1 H, d, J = 2.01 Hz), 7.19 (1 H, dd, J = 8.31, 2.01 Hz), 6.81 (1 H, d, J = 9.06 Hz), 5.17 (2 H, s), 3.79 (3 H, s). 13C NMR (101 MHz, DMSO-d6): δ 166.4, 154.8, 147.6, 140.1, 139.7, 130.6, 124.1, 117.8, 115.9, 112.4, 108.2, 54.9, 51.6. m/z (+EI) calcd for C13H16BrN2O (M)+ 320.1, found 321.9 ([M+H]+).

Synthesis of 1-(5-Bromo-pyridin-2-yl)-2-methyl-1H-benzoimidazole-5-carboxylic Acid Methylester (5). A solution was prepared by dissolving 4 (5 g, 1 equiv) in THF (130 mL). Trimethyl orthoacetate (4.07 mL, 2 equiv) and p-TsOH (1.577 g, 0.5 equiv) were sequentially added to this solution, and the reaction mixture was kept at reflux for 15 h until TLC showed completion of the reaction. The reaction mixture was then evaporated in vacuum, with the residue dissolved in AcOEt (50 mL) and sequentially washed with a saturated solution of NaHCO3 (60 mL) and brine (60 mL). The organic phase was dried over anhydrous MgSO4 and evaporated using a rotary evaporator. The obtained crude was purified by flash column chromatography (mobile phase: DCM/MeOH, 80/20, v/v), obtaining pure 5 (3.7 g, 69%) as a white solid, mp 141.5–141.9 °C. 1H NMR (400 MHz, chloroform-d): δ 8.77 (1 H, d, J = 2.52 Hz), 8.45 (1 H, d, J = 1.76 Hz), 7.85 (1 H, d, J = 8.31 Hz), 7.80 (1 H, d, J = 8.18, 1.64 Hz), 7.41 (1 H, d, J = 8.31 Hz), 7.38 (1 H, d, J = 8.56 Hz), 3.96 (3 H, s), 2.70 (3 H, s). 13C NMR (101 MHz, chloroform-d): δ 153.1, 151.2, 147.9, 142.5, 141.6, 137.9, 136.6, 125.3, 124.9, 121.6, 121.0, 120.0, 109.8, 52.2, 15.5. m/z (+EI) calcd for C13H16BrN2O (M)+ 345.0, found 345.8 ([M+H]+).
1.78 (6 H, m). 13C NMR (101 MHz, MeOH-CD3) δ 615.3190, found 615.3189. C37H39N9O2 ([M] + H)+ 642.3299, found 642.3302. 7.53–7.59 (4 H, m), 3.5 (2 H, m), 2.66 (3 H, s), 2.5 (H, m), 2.32 (6 H, m).

1H NMR (100 MHz, DMSO-d6) δ 666.3, 166.2, 166.5, 159.1, 152.7, 150.3, 149.0, 147.9, 145.5, 142.1, 140.9, 137.5, 136.6, 135.5, 133.7, 130.3, 129.5, 129.1 125.5, 125.1, 122.3, 120.4, 120.3, 118.7, 117.6, 113.7, 110.4, 56.4, 43.5, 37.5, 26.4, 15.0. 14H. HRMS (EI, m/z): calcld for C37H39N9O2 ([M] + H)+ 642.3299, found 642.3302.

**N-(4-Dimethylaminobutyl)-2-methyl-5-[3-(2-Methyl-1H-benzo[d]imidazol-6-carboxamido)phenyl]pyridin-2-yl)-1H-benzo[d]imidazol-6-carboxamide (14).** Yield 0.035 g (71%) as a brown oil. 1H NMR (400 MHz, DMSO-d6) δ: 10.40 (1 H, s), 9.01 (t, J = 2.04 Hz, 1 H), 8.39 (1 H, dt, J = 8.32 Hz, 8.32 (1 H, s), 8.19–8.23 (2 H, m), 7.91–7.94 (1 H, m), 7.87–7.90 (1 H, m), 8.82–8.72 (2 H, m), 7.52–7.58 (4 H, m), 3.23–3.26 (2 H, m), 3.06–3.07 (6 H, m), 2.65 (3 H, s), 2.54 (3 H, s), 1.82–1.85 (2 H, m). 1.51–1.63 (4 H, m). C NMR (101 MHz, DMSO-d6) δ: 166.2, 163.8, 152.6, 147.8, 147.4, 141.9, 140.4, 137.4, 136.5, 135.4, 129.5, 129.3, 129.1, 127.7, 127.5, 124.6, 123.3, 121.8, 120.4, 120.2, 119.2, 118.8, 117.7, 110.3, 69.7, 45.1, 41.9, 31.8, 30.3, 15.0, 14.7. HRMS (EI, m/z): calcld for C44H35N12O4 ([M] + H)+ 690.3043, found 690.3043.

## Synthesis of Double Aminic Tails Derivative 17. **Synthesis of Methyl 2-(3-[Dimethylamino)propyl]-1H-benzo[d]imidazol-6-carboxylate (15).** Methyl 3,4-diaminobenzoate (0.050 g, 1 equiv) and 4-[3-(Dimethylamino)butanoyl]benzoyl chloride (0.65 g, 2 equiv) were put into a MW vial in the presence of DPEPA (2 equiv) and propylphosphonic anhydride (T,P) 50% solution in ethyl acetate (1 equiv). The reaction mixture was heated at 160 °C for 40 min. The obtained crude of the reaction was dissolved in ethyl acetate (10 mL) and subsequently extracted with NaHCO3 saturated aqueous solution (2 × 15 mL). The collected organic phase was dried over anhydrous MgSO4 and evaporated using a rotary evaporator. The obtained crude was purified by flash column chromatography (mobile phase: initial DCM/MeOH, 90/10, v/v then DCM/NH3 solution in MeOH, 95/5, v/v), obtaining pure 15 (0.054 g, 69%) as a transparent oil. 1H NMR (400 MHz, chloroform-d) δ: 8.24 (s, 1H), 7.84–7.99 (m, 1H), 7.55 (d, J = 8.56 Hz, 1H), 3.94 (3H, 3H), 3.08–3.20 (m, 2H), 2.51–2.63 (m, 2H), 2.49 (s, 6H), 1.95–2.02 (m, 2H). C NMR (101 MHz, chloroform-d) δ: 167.9, 158.2, 143.1, 138.3, 132.6, 132.4, 116.6, 114.5, 59.6, 52.0, 45.0, 28.8, 24.4. m/z (+EI) calcld for C19H16N2O2 ([M] + H)+ 261.1, found 262.0 ([M] + H)+.

## Synthesis of Methyl 1-[5-(2-[3-(3-[Dimethylamino)propyl]amino)phenyl]-pyridin-2-yl]-1H-benzo[d]imidazol-6-carboxylate (16). Compound 15 (0.126 g, 2 equiv) was dissolved in MeOH (12 mL) and added to 0.5 M NaOH aqueous solution (6 equiv). The solution was left under a magnetic stirrer for 15 h until TLC showed total hydrolysis of the ester moiety. The pH of the solution was adjusted to 3 through the addition of HCl 1 M aqueous solution, with the solvent then evaporated under reduced pressure. The obtained white solid was dissolved in DCM (4 mL) and the same procedure used for the synthesis of benzofused amide derivative of 6 was applied. The purification of the crude of reaction was performed using a SCX cartridge as described in the section “general chemistry”. After that, a purification by column chromatography (mobile phase: from DCM/MeOH, 90/10, v/v to DCM/MeOH/NH3 in MeOH, 90/10/1, v/v/v) was required to obtain pure 16 (0.050 g, 35%) as a brown oil. 1H NMR (400 MHz, chloroform-d) δ: 8.94 (d, J = 3.02 Hz, 1H), 8.50 (s, 1H), 8.46 (d, J = 1.26 Hz, 1H), 8.28 (t, J = 1.89 Hz, 1H), 8.20 (dd, J = 2.52, 8.31 Hz, 1H), 8.17 (s, 1H), 8.00 (dd, J = 1.51, 8.56 Hz, 1H), 7.77 (dd, J = 1.64, 8.44 Hz, 1H), 7.67 (dd, J = 7.30 Hz, 1H), 7.58 (d, J = 8.31 Hz, 1H), 7.50–7.56 (m, 2H), 7.41–7.47 (m, 2H, 3H), 3.96 (s, 3H), 3.11–3.13 (m, 2H), 2.74 (s, 3H), 2.60–2.66 (m, 2H), 2.45 (s, 6H), 2.01–2.05 (m, 2H). C NMR (101 MHz, chloroform-d) δ: 166.7, 166.7, 153.4, 148.4, 148.2, 142.4, 139.5, 134.1, 137.6, 137.4, 136.7, 136.2, 135.5, 129.5, 128.8,123.2, 121.9, 120.4, 120.3, 118.8, 117.7, 112.2, 110.4, 48.8, 46.0, 35.8, 29.0, 15.0, 14.7. HRMS (EI, m/z): calcld for C24H20N2O2 ([M] + H)+ 315.1900, found 315.1899.

## Synthesis of 1-(5-[(2-Methyl-3H-benzoimidazole-5-carboxylamido)-phenyl]-pyridin-2-yl)-1H-benzo[d]imidazol-6-carboxamide (17). Compound 16 (0.020 g, 1.3 equiv) was dissolved in MeOH (5 mL) and added to NaOH 0.5 M aqueous solution (5.2 equiv). The
solution was left under a magnetic stirrer for 15 h until TLC showed total hydrosynthesis of the ester moiety. The pH of the solution was adjusted to 3 through the addition of HCl 1 M aqueous solution, with the solvent then evaporated under reduced pressure. The obtained white solid was dissolved in DMP (4 mL), and the same procedure used for the synthesis of tertiary aminic tail derivatives was employed. Purification was performed using a SCX cartridge as described in the section “general chemistry”. After that, a purification by column chromatography (mobile phase: from DCM/MeOH/MeOH 90/10, v/v/v) was required to obtain pure 17 (0.014 g, 82%) as a brown oil. 1H NMR (400 MHz, MeOH-d4): δ = 9.02 (d, J = 2.52 Hz, 1H), 8.44 (dd, J = 2.52, 8.31 Hz, 1H), 8.24–8.26 (m, 2H), 8.19 (d, J = 1.51 Hz, 1H), 7.92 (dd, J = 1.76, 8.56 Hz, 1H). 13C NMR (100 MHz, MeOH-d4): δ = 170.7, 169.4, 163.1, 158.4, 155.3, 149.3, 148.1, 142.8, 139.2, 138.3, 130.9, 130.6, 130.3, 124.2, 124.3, 122.5, 122.0, 120.9, 118.8, 111.9, 159.3, 57.8, 45.2, 44.5, 38.3, 27.8, 27.4, 25.5, 14.98. HRMS (EI, m/z): calc for C24H23N3O3 [M+H]+ 459.7965, found 459.7867.

Synthesis of Compound 18–19. Synthesis of 1-(5-Bromopyridin-2-yl)-2-methyl-1H-benzoimidazole-5-carboxylic Acid (3-Dimethylamino-propyl)-amine (19). Starting from compound 18 (0.031 mg, 1 equiv) was dissolved in DCM (10 mL) and brine (2 mL), obtaining pure 19 (0.49, 1H, s), 7.80 (1H, d, J = 8.00 Hz), 7.66 (1H, d, J = 6.40 Hz), 7.51 (1H, d, J = 7.2 Hz), 3.46 (2H, t, J = 5.93 Hz), 2.67 (3H, s), 2.47 (2H, d, J = 6.40 Hz), 2.30 (6H, s), 1.82–1.89 (2H, m). 13C NMR (100 MHz, MeOH-d4): δ = 170.1, 151.5, 152.2, 148.9, 143.6, 142.7, 138.0, 131.1, 124.0, 123.2, 121.6, 118.8, 111.8, 58.7, 45.4, 39.5, 37.0, 28.2, 15.0. HRMS (EI, m/z): calc for C16H15N3O3 [M+H]+ 299.1080, found 299.1078.

Synthesis of 1-(3-Amino-phenyl)pyridin-2-yl)-2-methyl-1H-benzoimidazole-5-carboxylic Acid (3-Dimethylamino-propyl)-amine (20). Starting from compound 6 (0.1 g), the same procedure used for the synthesis of compound 15 was applied, affording pure 19 (0.095 g, 79%) as a yellow oil. 1H NMR (400 MHz, DMSO-d6): δ = 8.89 (1H, s), 8.17 (1H, d, J = 2.04 Hz), 8.20 (1H, d, J = 2.80, 8.00 Hz), 8.20 (1H, s), 7.81 (2H, m), 7.01 (1H, d, J = 8.00 Hz), 7.19 (1H, t, J = 8.00 Hz), 6.97 (2H, m), 6.87 (1H, d, J = 8.00 Hz), 5.32 (2H, br s), 3.5 (2H, m), 2.70 (2H, t, J = 2.80 Hz), 2.67 (3H, s), 2.45 (6H, s), 1.79–1.87 (2H, m). 13C NMR (100 MHz, DMSO-d6): δ = 166.3, 152.6, 149.4, 147.5, 147.2, 141.9, 137.1, 136.6, 135.6, 136.2, 129.8, 129.0, 122.3, 120.3, 113.7, 117.4, 114.1, 112.0, 110.3, 55.7, 48.5, 43.5, 25.6, 14.98. HRMS (EI, m/z): calc for C21H20N3O3 [M+H]+ 343.1450, found 343.1440.
The oligonucleotides were annealed through heating the samples to 85 °C for 6 min followed by cooling to 25 °C and storing at this temperature for 5 h. Annealed DNA (25 μL) and sample solution (25 μL) were added to each well of a 96-well plate (MJ Research, Waltham, MA) and processed in a DNA Engine Opticon (MJ Research). Fluorescence readings were taken at intervals of 0.5 °C over the range 30–100 °C, with a constant temperature maintained for 30 s prior to each reading. The raw data was imported into the program Origin (Version 7.0, OriginLab Corp.), and the graphs were smoothed using a 10-point running average and then normalized. The determination of melting temperatures was based on values at the maxima of the first derivative of the smoothed melting curves using a script. The difference between the melting temperature of each sample and that of the blank (ΔTm) was used for comparative purposes.

Microbiological Evaluation of the Compounds. Determination of Minimum Inhibitory Concentration. MICs were determined using the broth microdilution method as described previously. Cell growth in Tryptic Soy Broth (TSB) was determined by measuring optical density (600 nm) using a FLUOstar Omega microplate reader (BMG Labtech). The MIC was determined as the lowest concentration of drug at which growth was below an optical density 600 nm of 0.1 after 20 h growth. Tests were conducted in triplicate. Where necessary, MIC determinations were carried out in the presence of the efflux pump inhibitor phenylalanine-arginine β-naphthylamide (PAMβNA) at 25 μg/mL. Studies with permeabilised membranes used polymyxin B nonapeptide (PMBXβ) at a final concentration of 30 μg/mL.

Time Kill Assays. Flasks of TSB were inoculated with test organisms at a concentration of ~105 cfu/mL in a total volume of 10 mL. The antimicrobial agents were then added at a concentration of 4 × MIC and incubated at 37 °C in a shaking incubator at 200 rpm. Samples (0.1 mL) were taken from each sample 1, 2, 4, 6, and 24 h following inoculation. The effect on bacterial growth was determined using the Miles-Misra dilution method to estimate total colony forming units (CFU) per mL. A compound was defined as bactericidal if it resulted in a loss of >3log CFU/mL. The data is representative of three independent repeats and error bars are the standard deviation from the mean.

DNA Gyrase Inhibition Assay. The inhibition of gyrase by the compounds was evaluated using the S. aureus gyrase supercoiling high throughput plate assay (no. SATRGG01), obtained from Inspiralis (Norwich, UK). Methods were conducted as per the manufacturer’s instructions. Briefly, compounds were incubated with gyrase enzyme and the plasmid pNO1, which contains sequences with triplex forming potential. Triplex forming oligonucleotides captured the negatively supercoiled plasmids and are stained with fluorescent dye (Promega Diamond dye), which was read using a fluorescent plate reader (Ex, 485 nm; Em, 520 nm) (FLUOstar Omega, BMG Labtech, Germany).

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b00108.

LCMs method, NMR spectra, and HRMS (PDF)
Docking poses of selected compounds with DNA sequences (PDBs) ZIP

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ABBREVIATIONS USED

MDR, multidrug resistant; HTS, high-throughput screening; MRSA, methicillin resistant S. aureus; VRE, vancomycin resistant Enterococci; HBTU, 2-(1H-benzo triazol-1-yl)-1,1,3,3-tetramethyllumonium hexafluorophosphate; DPEA, N,N-dissopropylethylamine; FRET, fluorescence resonance energy transfer; FAM, 6-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine.

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