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DNA binding ligands with in vivo efficacy in murine models of bacterial infection: optimization of internal aromatic amino acids

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Abstract—DNA binding ligands with potent antimicrobial activity against Gram-positive bacteria were further optimized by variation of the internal aromatic amino acids. This modification led to compounds with improved in vivo efficacy in lethal murine models of peritonitis (methicillin-resistant *S. aureus,* MRSA) and lung infection (*S. pneumoniae*).

We have identified a novel class of antimicrobial compounds with high activity against drug resistant, Gram-positive bacteria. These molecules have been shown to exercise bactericidal activity by interacting with functionally important A/T-rich sites of bacterial DNA and thereby inhibiting DNA replication and RNA transcription.[1] Previous lead optimization to improve antibacterial spectrum, potency, tolerability, and in vivo efficacy was focused on C-terminal amines and N-terminal hetero-aromatic groups[2,3] and resulted in well-tolerated compounds with broad and potent activity against Gram-positive bacteria. One of these prototypic antibiotics, **1**, was used to demonstrate the in vivo proof of concept in a murine lethal peritonitis model using methicillin-sensitive *S. aureus* (MSSA).[3] However, the same compound failed in this model using MRSA. The increasing threat by multi-drug-resistant bacteria, MRSA in particular,[4] indicates the importance of this milestone. This article focuses on the optimization of the internal, aromatic amino acids using **1** as the starting point with the goal to achieve in vivo efficacy in murine models of MRSA infection while retaining antibacterial spectrum and potency and in vivo tolerability. We chose **1**, not the most potent compound, for our initial investigations based on the large available dataset. In addition, we anticipated that the influence of subtle structural modifications on the biological parameters would be easily detected with a moderately potent lead structure.

Previous optimization studies have identified 4-(2-aminoethyl)morpholine and 2-carboxy-3-chlorothiophene as valuable end caps for this class of DNA-binding antibiotics.[3] In order to better understand the importance of internal N-methylpyrrole-2-carboxamide units (Py), we synthesized a small library of **1** analogs, in which a single Py unit is replaced by various aromatic amino acids in all three positions (Table 1, Scheme 1). In the context of larger DNA minor-groove binding ligands, referred to as polyamides, substitution of a Py by desmethyl pyrrole (Ds) has led to compounds with similar binding affinity, but reduced sequence specificity.[5] Hence, demethylation of a Py unit was expected to only minimally affect DNA interaction, but alter other physicochemical parameters such as lipophilicity and molecular weight. Each replacement (**2**–**4**) resulted in loss of potency, as evidenced most dramatically for Ds in position 3 (**4**). The novel building block *N*-isopropylpyrrole[6] (^{i}PrPy) was investigated in order to study the effect of increased steric bulk on solubility and/or aggregation; we anticipated a positive influence on these parameters due to steric interference on the stacking properties of these molecules (**5**–**7**). However, the aqueous solubility remained low. Nevertheless, **6** and **7** retained good antibacterial potency and **7** was further evaluated in vivo. The compound passed the acute tolerability screen but failed to protect infected mice in the

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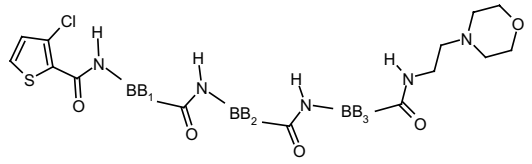
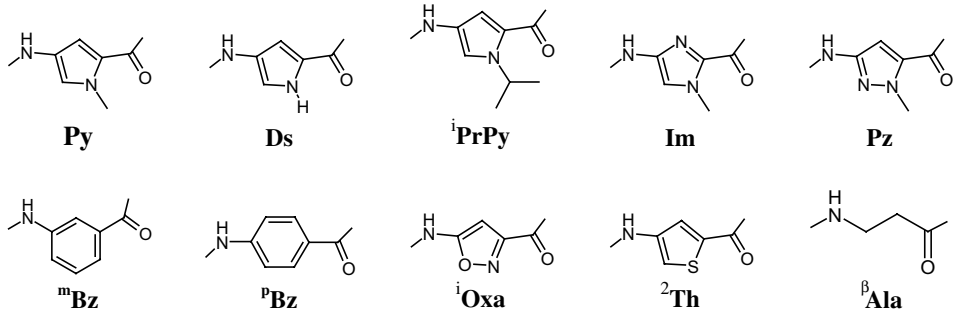
Table 1. Antimicrobial activity of single aromatic substitutions in analogs of **1**



Entry	BB_1	BB_2	BB_3	MRSA[a] 27660	VSEF[a] 29212	PISP[a] 49619
1	Py	Py	Py	2–4	0.5–1	0.13–1
2	Ds	Py	Py	8	2	2
3	Py	Ds	Py	8	2–8	0.5–2
4	Py	Py	Ds	16	32	8
5	^{i}PrPy	Py	Py	4	4	2
6	Py	^{i}PrPy	Py	1	1	0.5
7	Py	Py	^{i}PrPy	1	1	0.25
8	Im	Py	Py	2–4	0.13–1	0.03–0.13
9	Py	Im	Py	>32[b]	>32	16
10	Py	Py	Im	>32	>32	>32
11	Pz	Py	Py	8	8	0.25
12	Py	Pz	Py	32	>32	1–32
13	Py	Py	Pz	>32	>32	>32
14	^{m}Bz	Py	Py	>32	>32	>32
15	Py	^{m}Bz	Py	>32	>32	>32
16	Py	Py	^{m}Bz	16	16	8
17	^{p}Bz	Py	Py	32	16	32
18	Py	^{p}Bz	Py	8	2	1
19	Py	Py	^{p}Bz	>32	>32	>32

[a] MIC values against ATCC strains are given in μg/mL and were measured according to standard guidelines.[25] MRSA, methicillin-resistant *S. aureus* VREF, vancomycin-resistant *E. faecalis* VSEF, vancomycin-susceptible *E. faecalis* PISP, penicillin-intermediate *S. pneumoniae*. Ranges of values are given for multiple determinations.

[b] Determined with MSSA (ATCC 29213).



Scheme 1. Structures and abbreviations of internal aromatic amino acids. The compounds were prepared either in solution[3] or on solid support[23] using a thiol resin.[24] The building blocks were incorporated as N-Boc protected derivatives. Py,[23] Im,[23] Ds,[5] Pz,[11] and ^{2}Th[14] have been previously described; ^{m}Bz,[14] ^{p}Bz,[14] and $^{\beta}$Ala are commercially available and ^{i}Oxa was prepared as described.[16] All compounds were characterized by ^{1}H NMR and mass spectrometry and showed purity of at least 90% by analytical reversed phase HPLC. The isolated yields after HPLC purification ranged from 10–30%.[3]

peritonitis model (MSSA, 50 mg/kg). Similar results were obtained with the *N*-isobutyl pyrrole derivative, an analog of the previously described isoamylpyrrole[7] (data not shown). *N*-Methyl-imidazole-2-carboxamide (Im) has been studied extensively in polyamides; when paired with Py in a side-by-side mode, it specifically recognizes a G/C base pair.[8] In a one to one binding mode, however, Im has been shown to interact with A/T-rich sequences albeit lacking the specificity of Py.[9,10] Interestingly, the in vitro potency of Im-containing compounds is highly dependent on the position: as compared to **1**, an Im in positions 2 or 3 led to complete loss of activity (**9**, **10**), while the analogous modification in position 1 improved potency (**8**). Unfortunately, **8**

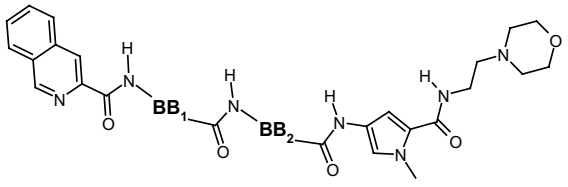
was not tolerated well in the acute murine screen as demonstrated by 11% weight loss of animals after three days (single dose at 50 mg/kg). A similar effect was observed for the pyrazole derivatives (Pz; **11**–**13**).[11] In this case, even the most potent derivative **11** revealed a decrease of antibacterial activity as compared to the parent compound **1**.

Little investigation has been reported for the replacement of internal five-membered ring systems by their higher homologues in Py-containing polyamides.[12–14] Six-membered aromatic amino acids have a different geometry and consequently are expected to affect DNA interaction by influencing the curvature of the crescent-shaped ligands. However, derivatization and lead optimization of six-membered rings would be straightforward and was considered potentially beneficial. Replacement of Py by *meta*-aminobenzoic acid (^{m}Bz; **14**–**16**) resulted in almost complete loss of activity, irrespective of the position of the newly introduced ring. The isomeric series with *para*-aminobenzoic acid (^{p}Bz) revealed an unexpected result; while substitution in positions 1 and 3 (**17**, **19**) yielded compounds with limited or no potency, replacement in position 2 (**18**) retained most activity (2–4-fold less than **1**). In summary, five-membered ring analogs of pyrrole appear to be better tolerated in positions 1 and to a lesser extent 2, whereas *para*-substituted six-membered rings are best placed in position 2.

Next, we intended to identify the optimal five-membered hetero-aromatic groups in positions 1 and 2 (Table 2). Rather than repeating part of the work done with 3-chlorothiophene, we used the isoquinoline N-terminus. This group proved very useful in earlier studies with respect to antibacterial potency, tolerability, molecular weight, and solubility.[3] The parent compound containing three internal Py units (**20**) demonstrated improved in vitro potency as compared to the 3-chlorothiophene derivative **1**, but failed to protect mice in the MSSA peritonitis model at 20 mg/kg. The Ds substitution in position 2 (**22**) yielded a derivative with equal potency against MRSA and improved in vivo efficacy in the murine peritonitis model (MSSA: 60% protection at 10 mg/kg, MRSA: 100% at 50 mg/kg, no protection at 20 mg/kg).[15] The compound was safe in mice (single dose at 50 mg/kg), but indicated poorer tolerability in the multiple dose evaluation (four daily doses of 30 mg/kg) with loss of body weight (5%), elevated BUN (blood urea nitrogen), AST (aspartate amino transferase), ALT (alanine amino transferase), and triglycerides (all roughly 2-fold increased) and 2–3 fold decreased glucose levels.

In contrast to the findings with the 3-chlorothiophene derivatives, Im-substitution in position 1 (**23**) did not improve antibacterial potency in the isoquinoline series. This indicates that optimized groups in various positions are not simply additive, but have to be evaluated in the context of the entire molecule. Replacement of Py by Pz supported this statement: in contrast to the 3-chlorothiophene series, substitution in position 2 (**25**) resulted in a compound with good in vitro activity. Unfortunately, **25** did not protect mice in the peritonitis model (MSSA, 20 mg/kg) and displayed mild lethargy in animals when tested for acute tolerability (50 mg/kg). Based on antibacterial potency, the isooxazole[16] (^{i}Oxa) in position 1 (**26**) looked promising. However, the poor solubility of this compound did not allow formulation for an in vivo experiment. The last heterocycle evaluated was 4-amino-2-carboxy-thiophene (^{2}Th).[14] When introduced in position 1 (**27**), it resulted in moderately good

Table 2. Antimicrobial activity of various five-membered heteroaromatic groups in positions 1 and 2



Entry	BB_1	BB_2	MRSA[a] 27660	VSEF[a] 29212	PISP[a] 49619
20	Py	Py	0.5	0.25	0.06
21	Ds	Py	1–2	0.5	0.5–1
22	Py	Ds	0.25–1	0.5–2	1–2
23	Im	Py	1	1	0.25
24	Pz	Py	1	8	0.5
25	Py	Pz	0.5	1	0.25
26	^{i}Oxa	Py	0.06	0.5	0.13
27	^{2}Th	Py	1–2[b]	0.25–0.5	0.13–0.25
28	Py	^{2}Th	8	>32	0.5
29	$^{\beta}$Ala	Py	32	16	8

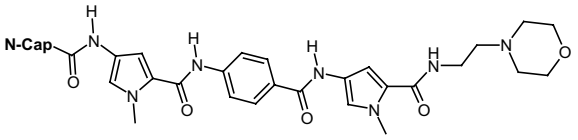
See legend to Table 1 for details.

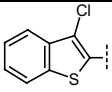
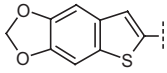
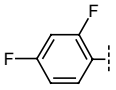
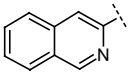
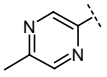
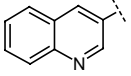
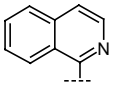
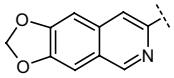
potency, even though the *S. aureus* activity did not meet our requirements. Among multiple non-aromatic substituents, β-alanine ($^{\beta}$Ala) has previously been proven a valuable module for DNA-binding polyamides[9] and indeed performed best compared to a variety of non-aromatic replacements (data not shown). However, **29** still revealed a dramatic loss of antibacterial potency indicating that the use of non-aromatic internal building blocks is not a promising avenue for advanced lead optimization.

Finally, we studied compounds containing ^{p}Bz at position 2 and various N-terminal caps that have led to highly potent molecules in the Py_3 series (Table 3).[3] Interestingly, neither 3-chloro-benzothiophene (**30**), its des-chloro-dioxolane derivative (**31**),[17] nor 2,4-difluoro-benzene (**32**) yielded compounds with good activity against MRSA, whereas the isoquinoline **33** showed excellent antibacterial potency. Attempts to better understand this unique combination of the N-terminal isoquinoline and ^{p}Bz at position 2 revealed that pyridines, pyrimidines, or pyrazines (**34**–**36**) were not equal substitutes for the isoquinoline. The position of the ring nitrogen and connection point appeared to be crucial for activity (**37**, **38**). Even the dioxolane analog, a derivatization that previously has led to compounds with improved properties in the context of benzothiophenes,[3] did not enhance potency (**39**).

Even though compound **33** appears to kill bacteria by interacting with A/T-rich DNA target sites (K_d = 7.5 nM[15,22]) as demonstrated with earlier prototype molecules,[1–3] its unique properties cannot be explained in a rational way. We observed a general correlation with exceptions between DNA-interaction and antibacterial activity, however, this correlation has expectedly

Table 3. Antimicrobial activity of ^{p}Bz containing compounds (position 2) with various end-caps



Entry	N-Cap	MRSA[a] 27660	VSEF[a] 29212	PISP[a] 49619
30		>32	16	0.25
31		32	>32	0.5
32		32	8	0.5
33		0.06–0.13	0.25–0.5	0.03–0.06
34		>32	>32	0.5
35		>32	32	0.5
36		0.5	2	0.13
37		>32	>32	2
38		1	2	0.25[c]
39		0.06	0.5	0.5
	Linezolid	2	2	1
	Vancomycin	1	1	0.25

See legend to Table 1 for details.

[c] Determined with *S. pneumoniae* ATCC 51422.

limitations due to the comparison between a molecular interaction and a whole cell assay. Whereas we never characterized compounds in this series that had antibacterial activity but did not bind A/T-rich DNA, we found a few compounds with opposite properties (strong DNA interaction with little or no antibacterial activity). These observed differences originate presumably in varying properties of membrane transport; a hypothesis that still needs to be validated experimentally. We also assumed a beneficial influence of isoquinoline on the physico-chemical properties of corresponding compounds. Even though isoquinoline derivatives appeared generally to have slightly higher solubility as compared to other bicyclic moieties, aqueous solubility of **33** at pH 7.4 was still poor (<1 ng/mL, *data not shown*) and protein binding remained high at 94% within the same range as measured for other representative compounds of this class.[15]

Based on its promising profile, **33** was examined in a recently isolated strain of vancomycin-resistant *S. aureus* (VRSA) and, not surprisingly, found to be active (MIC = 0.25 μg/mL).[21]

In the murine lethal peritonitis model (MRSA infection, Fig. 1), **33** showed remarkable efficacy with an ED_{50} of roughly 11 mg/kg (IV administration) even though it is still inferior to vancomycin (ED_{50} varies between 1.0 and 4.4 mg/kg).[15] The compound was also efficacious in a lethal murine model of lung infection by *S. pneumoniae* (Fig. 2). Compound **33** was the first one to demonstrate in vivo efficacy in these tough murine models at dose levels that are well tolerated.[15]

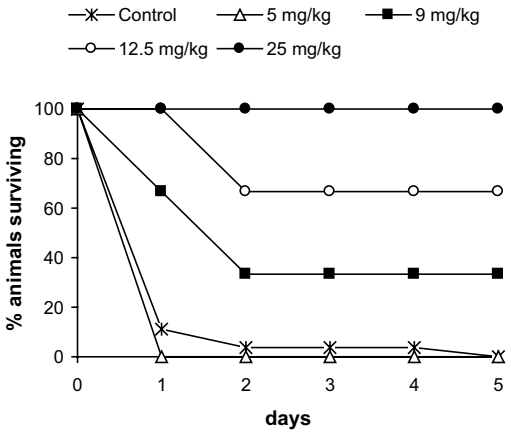


Figure 1. Dose dependence of **33** in the mouse peritonitis model (MRSA). The mouse peritionitis model[26] was performed as previously described.[15] Fresh colonies of ATCC 27660 (MRSA, β-lactamase$^+$) were grown in Brain Heart Infusion broth (BHI, International Bio-Products, Bothell, WA) at 37 °C with gentle agitation and adjusted to a concentration of 2×10^7 cfu/mL. A solution of 10% gastric mucin porcine (ICN Biomedicals Inc., Aurora, OH) was added at an equal volume to achieve the final inoculum of 1.0×10^7 cfu/mL in 5% mucin, representing a 5–10× LD_{50} inoculum with 100% mortality rate in vehicle treated animals by 48 h. Groups of 5 ICR female mice (~25 g from Taconic Farms) were inoculated intraperitoneally with 0.5 mL of the inoculum described above. Groups were treated intravenously with vehicle, positive control (Vancomycin, Linezolid) or test articles with a single dose 1 h post infection. Clinical observations were monitored over 5 days with a primary endpoint of survival. For all in vivo studies described herein, test antibacterials were formulated in 40% hydroxypropyl-β-cyclodextrin/10% D-mannitol/50 mM NaOAc buffer pH 4.5 ± 0.1 to achieve concentrations necessary for in vivo profiling. Number of animals represented in the graph above: control (vehicle alone; 27), 5 mg/kg (5), 9 mg/kg (6), 12.5 mg/kg (9), 25 mg/kg (24).

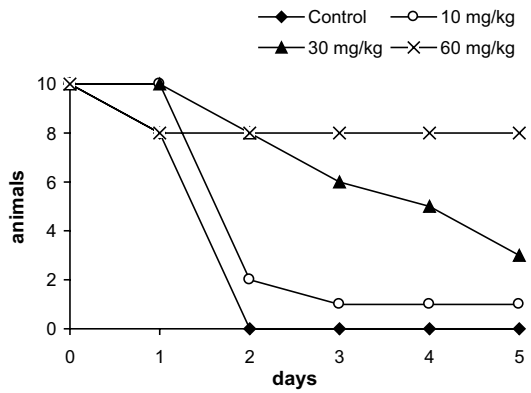


Figure 2. Dose dependence of **33** in the mouse pneumococcal pneumonia model. Murine lung infection model with *S. pneumoniae* (ATCC 6303, penicillin sensitive):[27] The organism was grown overnight in Müller–Hinton broth (BD Biosciences, Sparks, MD) containing 5% lysed horse blood to a concentration of $\sim5\times10^6$ cfu/mL. Mice (ICR) were anesthetized with isoflourane (Baxter, Deerfield, IL) and 50 μL/mouse of the inoculum above was instilled via the intranasal route. Compound or vehicle was administered IV at 1 and 5 h post-inoculation and survival was monitored over 5 days.

Safety profiling was undertaken with **33** to assess its potential as candidate for therapeutic development. Acute and short-term multi-day toxicity studies in mice were encouraging in that no adverse effects were observed at dose levels (30 mg/kg) above efficacious doses.[15] Importantly, the extensive evaluation of the potential for genotoxic properties was negative, indicating that reversible DNA binding does not necessarily result in undesirable genetic effects on mammalian cells.

In conclusion, optimization of internal building blocks combined with optimized terminal groups yielded **33**, the first DNA-binding ligand with potent Gram-positive antibacterial potency and in vivo efficacy against MRSA at safe doses in relevant murine models of infection. Further optimization of this promising scaffold has been initiated and will be reported shortly.

Acknowledgements

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References and notes

1. Ge, Y.; Difuntorum, S.; Touami, S.; Critchley, I.; Bürli, R.; Jiang, V.; Drazan, K.; Moser, H. *Antimicrob. Agents Chemother.* **2002**, *46*, 3168.
2. Bürli, R. W.; Ge, Y.; White, S.; Baird, E. E.; Touami, S. M.; Taylor, M.; Kaizerman, J. A.; Moser, H. E. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2591.
3. Kaizerman, J. A.; Gross, M. I.; Ge, Y.; White, S.; Hu, W.; Duan, J.-X.; Baird, E. E.; Johnson, K. W.; Tanaka, R. D.; Moser, H. E.; Bürli, R. W. *J. Med. Chem.* **2003**, *46*, 3914.
4. Eady, E. A.; Cove, J. H. *Curr. Opin. Infect. Dis.* **2003**, *16*, 103.
5. Bremer, R. E.; Szewczyk, J. W.; Baird, E. E.; Dervan, P. B. *Bioorg. Med. Chem.* **2000**, *8*, 1947.
6. ^{i}PrPy was incorporated as the N-Boc protected amino acid. This compound was prepared starting from ethyl 4-nitropyrrole-2-carboxylate[5] by N-alkylation with 2-bromopropane (2.1 equiv, 1 equiv KI, 2 equiv K_2CO_3 in DMF; 75 °C) followed by hydrogenation (H_2, 5% Pd–C, THF; rt), protection (Boc_2O, DMF; rt), and hydrolysis (4 equiv KOH in EtOH/H_2O; 60 °C, 72 h) in 62% overall yield.
7. Dyatkina, N. B.; Roberts, C. D.; Keicher, J. D.; Dai, Y.; Nadherny, J. P.; Zhang, W.; Schmitz, U.; Kongpachith, A.; Fung, K.; Novikov, A. A.; Lou, L.; Velligan, M.; Khorlin, A. A.; Chen, M. S. *J. Med. Chem.* **2002**, *45*, 805.
8. White, S.; Baird, E. E.; Dervan, P. B. *Chem. Biol.* **1997**, *4*, 569.
9. Urbach, A. R.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4343.
10. Maeshima, K.; Janssen, S.; Laemmli, U. K. *The EMBO J.* **2001**, *20*, 3218.
11. Nguyen, D. H.; Szewczyk, J. W.; Baird, E. E.; Dervan, P. B. *Bioorg. Med. Chem.* **2001**, *9*, 7.
12. Reddy, B. S. P.; Sharma, S. K.; Lown, J. W. *Curr. Med. Chem.* **2001**, *8*, 475.
13. Neidle, S. *Nat. Prod. Rep.* **2001**, *18*, 291.
14. Boger, D. L.; Fink, B. E.; Hedrick, M. P. *J. Am. Chem. Soc.* **2000**, *122*, 6382.
15. Gross, M.; Bürli, R.; Jones, P.; Garcia, M.; Batiste, B.; Kaizerman, J.; Moser, H.; Jiang, V.; Hoch, U.; Duan, J.-X.; Tanaka, R.; Johnson, K. *Antimicrob. Agents Chemother.* **2003**, *47*, 3448.
16. Adolphe, P. S.; Hublot, B.; Lepage, F. FR Patent 2,750,425, 1998; (CAN 128:192643 AN 1998:195243).
17. The synthesis of [1,3]dioxolo[4,5-g]isoquinoline-7-carboxylic acid was performed as follows: Commercially available benzo[1,3]dioxole-5-carbaldehyde was brominated in AcOH (2.5 equiv Br_2 in AcOH; rt, 24 h, then 50 °C, 2 h; 85%)[18] followed by acetal formation ($HC(OEt)_3$, 5 mol% *p*-TsOH; reflux, 24 h; 94%), formylation (1.07 equiv BuLi, Et_2O; 0 ° C, 30 min; then, −78 ° C, 1.3 equiv DMF; then rt, 2 h; 69%)[19] and deprotection (dioxane/H_20/85% H_3PO_4 3:3:2; rt, 12 h; 84%)[20] to give benzo[1,3]dioxole-5,6-dicarbaldehyde. Treatment of the dialdehyde with 1 equiv glycine methylester hydrochloride (dioxane/DBU 5:1; reflux, 2 h; 10%) followed by hydrolysis (MeOH/2 M aq NaOH 1:5; 70 °C, 12 h; 43%) gave the title compound.
18. Harrowven, D. C.; Newman, N. A.; Knight, C. A. *Tetrahedron Lett.* **1998**, *39*, 6757.
19. Boger, D. L.; Wolkenberg, S. E. *J. Org. Chem.* **2000**, *65*, 9120.
20. Dallacker, F.; Schleuter, H.-J.; Schneider, P. Z. *Naturforsch. B Anorg. Chem. Org. Chem.* **1986**, *41*, 1273.
21. Bozdogan, B.; Esel, D.; Whitener, C.; Browne, F. A.; Appelbaum, P. C. *J. Antimicrob. Chemother.* **2003**, *52*, 864.
22. Compound **33** was shown to interact with A/T-rich DNA: DNase I footprinting[2] on the σ70 target sequence 5′-ACAATTAA-3′ revealed a K_d (app.) of ~7.5 nM.
23. Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1996**, *118*, 6141.
24. To avoid the incorporation of a C-terminal β-alanine as described,[23] a thiol containing resin (thiomethyl-polystyrene used as scavenger for alkylating agents, NovaBiochem #01-64-0319) was used. After washing the resin with *N*-methyl pyrrolidine (NMP), the first Boc-protected amino acid (1.3 equiv) was coupled in NMP/^{i}PrEt$_2$N (4:1) for 24 h at 37 °C. The resin was treated with Ac_2O (1 h, rt) and thoroughly washed (2× each with NMP, MeOH, and CH_2Cl_2) prior to the next step. Deprotection was achieved by incubating the resin twice with CF_3COOH (10 min, rt) followed by washing. The following coupling steps were accomplished as described.[23] Cleavage of the final product from the resin was achieved by aminolysis (RNH_2/NMP 2:1, 60 °C, 48–96 h). This procedure was mainly used for compounds carrying Py, ^{i}PrPy, or Pz in position 3.
25. National Committee for Clinical Laboratory Standards 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard (M7-A5)-Fifth Edition. National Committee for Clinical Laboratory Standards, Wayne, PA.
26. Frimodt-Møller, N.; Knudsen, J. D.; Espersen, F. In *Handbook of Animal Models of Infection*; Zak, O., Sande, M. A., Eds.; Academic: London, San Diego, 1999; pp 127–136.
27. Azoulay-Dupuis, E.; Moine, P. In *Handbook of Animal Models of Infection*; Zak, O., Sande, M. A., Eds.; Academic: London, San Diego, 1999; pp 481–493.