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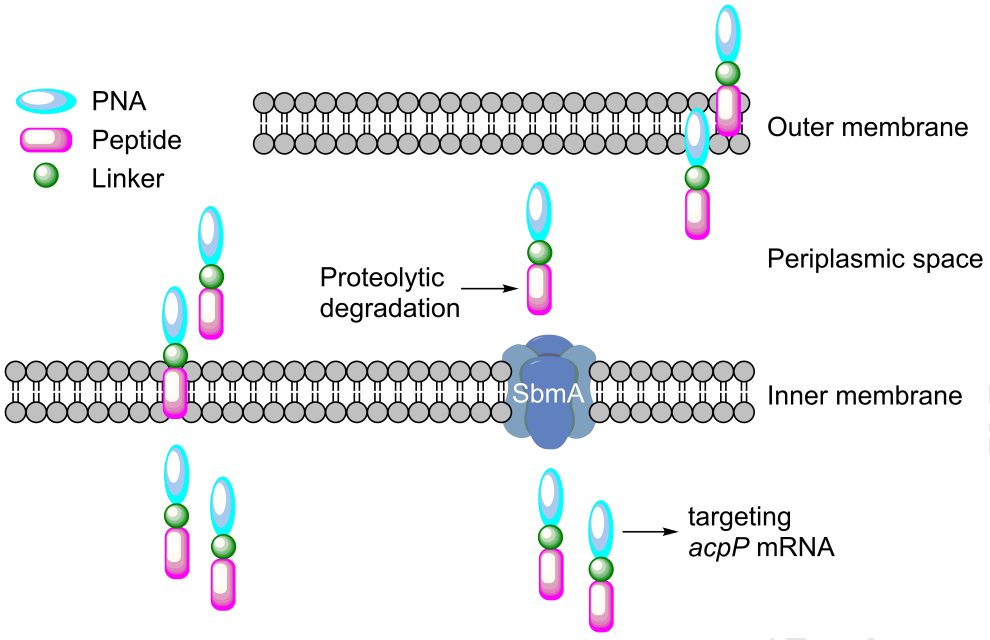

Microwave-assisted solid-phase synthesis of antisense *acpP* peptide nucleic acid-peptide conjugates active against colistin- and tigecycline-resistant *E. coli* and *K. pneumoniae*

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Abstract

Recent discovery of potent antibacterial antisense PNA-peptide conjugates encouraged development of a fast and efficient synthesis protocol that facilitates structure-activity studies. The use of an Fmoc/Boc protection scheme for both PNA monomers and amino acid building blocks in combination with microwave-assisted solid-phase synthesis proved to be a convenient procedure for continuous assembly of antisense PNA-peptide conjugates.

A validated antisense PNA oligomer (CTC-ATA-CTC-T; targeting mRNA of the *acpP* gene) was linked to N-terminally modified drosocin (i.e., RXR-PRPYSPRPTSHPRPIRV; X = aminohexanoic acid) or to a truncated Pip1 peptide (i.e., RXRRXR-IKILFQNRRMKWKK; X = aminohexanoic acid), and determination of the antibacterial effects of the resulting conjugates allowed assessment of the influence of different linkers as well as differences between the L- and D-forms of the peptides.

The drosocin-derived compound without a linker moiety exhibited highest antibacterial activity against both wild-type *Escherichia coli* and *Klebsiella pneumoniae* (MICs in the range 2-4 μg/mL ~ 0.3-0.7 μM), while analogues displaying an ethylene glycol (eg1) moiety or a polar maleimide linker also possessed activity toward wild-type *K. pneumoniae* (MICs of 4-8 μg/mL ~ 0.6-1.3 μM).

Against two colistin-resistant *E. coli* strains the linker-deficient compound proved most potent (with MICs in the range 2-4 μg/mL ~ 0.3-0.7 μM).

The truncated all-L Pip1 peptide had moderate inherent activity against *E. coli*, and this was unaltered or reduced upon conjugation to the antisense PNA oligomer. By contrast, this peptide was 8-fold less potent against *K. pneumoniae*, but in this case some PNA-peptide conjugates exhibited potent antisense activity (MICs of 2-8 μg/mL ~ 0.3-1.2 μM). Most interestingly, the antibacterial activity of the D-form peptide itself was 2- to 16-fold higher than that of the L-form, even for the colistin- and tigecycline-resistant *E. coli* strains (MIC of 1-2 μg/mL ~ 0.25-0.5 μM). Low activity was found for conjugates with a two-mismatch PNA sequence corroborating an antisense mode of action. Conjugates containing a D-form peptide were also significantly less active. In conclusion, we have designed and synthesized antisense PNA-drosocin conjugates with potent antibacterial activity against colistin- and tigecycline-resistant *E. coli* and *K. pneumonia* without concomitant hemolytic properties. In addition, a truncated D-form of Pip1 was identified as a peptide exhibiting potent activity against both wild- type and multidrug-resistant *E. coli*, *P. aeruginosa*, and *A. baumannii* (MICs within the range 1-4 μg/mL ~ 0.25-1 μM) as well as toward wild-type *Staphylococcus aureus* (MIC of 2-4 μg/mL ~ 0.5-1.0 μM).

Keywords: antisense PNA; delivery peptide; conjugates; antibacterial; *Escherichia coli*; *Klebsiella pneumoniae*.

Abbreviations:

A: adenine; Alloc: allyloxycarbonyl; AMP: antimicrobial peptide; AMR: antimicrobial resistance; B: β-alanine; Bhoc: benzhydryloxycarbonyl; Boc: *tert*-butyloxycarbonyl; BSA: bovine serum albumin; Bts: 1,3-benzothiazol-2-yl; Cl-Bhoc: chloro-benzhydryloxycarbonyl; C: cytosine; CFU: colony-forming unit; CST: colistin; CST-R: colistin-resistant; CST-S: colistin-susceptible; DIPEA: *N*,*N*-Diisopropylethylamine; DMT: 4,4'-dimethoxytrityl; Dde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; Dts: dithiasuccinoyl; ESBL: extended-spectrum β-lactamase; eg1: O-linker = AEEA linker = [2-(2-aminoethoxy)ethoxy]acetic acid; Fmoc: fluoren-9-ylmethoxycarbonyl; G: guanine; I: intermediate; KPC: *Klebsiella pneumoniae* carbapenemase; MCR: mobile colistin resistance; MDR: multidrug-resistant; MIC: minimal inhibitory concentration; Mmt: monomethoxytrityl; Mtt: monomethyltrityl; MW: microwave; NDM: New Delhi metallo-β-lactamase; NHS: N-hydroxysuccinimidyl; NMI: National Medicine Institute, Warzaw, Poland; NVOC: nitroveratryloxycarbonyl; 4-OMe-Z: *p*-methoxybenzyloxycarbonyl; PMB: *p*-methoxybenzyl; PNA:

peptide nucleic acid; pNZ: *p*-nitrobenzyloxycarbonyl; R: resistant; S: susceptible; SMCC: succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SPPS: solid-phase peptide synthesis; SPS: solid-phase synthesis; Teoc: 2-(trimethylsilyl)ethyloxycarbonyl; T: thymine; TGC: tigecycline; TGC-I: tigecycline-intermediate; TGC-R: tigecycline-resistant; TGC-S: tigecycline-susceptible; X: 6-aminohexanoic acid; Z: benzyloxycarbonyl; TSTU: *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

1. Introduction

Antimicrobial resistance (AMR) is a continually growing global threat to human health, and already in 2011 more than 2 million infections caused approx. 23.000 deaths in the US [1,2]. In particular, AMR in the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae* constitutes a challenge due to high prevalence of multidrug-resistant (MDR) clinical isolates [3]. The situation is further aggravated by the fact even though 30 antibiotics have been launched since 2000, only five represent novel classes of antibiotics, and these are all limited to targeting Gram-positive bacteria. This emphasizes the importance of identifying new classes of antibiotics with efficacy against Gram-negative pathogens [4]. As of 2015 the pipeline of compounds in clinical development for treatment of Gram-negative infections comprised 24 potential antibiotics. However, these comprise primarily improved analogues belonging to the classes of β-lactams (including combinations with β-lactamase inhibitors), tetracyclines, aminoglycosides and topoisomerase inhibitors that all interact with targets well-known for their propensity to undergo rapid mutation with ensuing short expected clinical lifetime of the antibiotics [5]. Consequently, novel antibacterial agents with alternative targets and/or novel mode of action as compared to current antibiotics are needed.

An antisense approach relying on peptide nucleic acid (PNA) oligomers for silencing of essential bacterial gene expression was introduced two decades ago [6-9]. In particular, the *acpP* gene, essential for fatty acid biosynthesis, has been validated as a sensitive target for antisense PNA-peptide conjugates [6,7,10]. Originally, conjugation to the peptide $(KFF)_3K$, and subsequently to arginine-rich peptides [7-13], was employed as a means for enhancing delivery of antisense PNA oligomers that inherently display poor bacterial uptake. Moreover, site-specific activity of the anti-acpP PNA sequence was confirmed by the real-time quantitative PCR [14]. Recently, antimicrobial peptides (AMPs) with an intracellular target were shown to constitute alternative carriers enabling efficient bacterial delivery of PNA oligomers [13].

Bacterial resistance to certain PNA-peptide conjugates has been correlated to the presence of a functional *sbmA* gene encoding an ABC transporter involved in translocation of Pro-rich AMPs, PNA oligomers, and PNA-peptide conjugates across the inner membrane of Gram-negative bacteria [10,13,15-18]. However, delivery moieties (e.g., repetitive arginine/6-aminohexanoic acid motifs as well as the AMPs Pep-1-K, KLW-9,13-a, and the C-terminally modified drosocin-RXR with X = 6-aminohexanoic acid) that confer PNA antisense activity in a Δ*sbmA E. coli* strain have recently been identified [10,13]. In the present work, we investigated the synthesis and antibacterial activity of a 10-mer PNA oligomer conjugated to N-terminally RXR-modified drosocin or a shortened version of PNA-internalizing peptide 1 (Pip1) [19], the latter being a hybrid between the cell-penetrating RXR repeats and R_6-penetratin [19,20].

Originally, PNA oligomers were obtained via assembly of Boc/Z-protected monomers [21-24], and subsequently by a number of synthetic protocols involving Fmoc-protected building blocks displaying a variety of nucleobase protecting groups: Z [25], Bhoc (commercially available) [26,27], Cl-Bhoc [27], F-Bhoc [27], 4-OMe-Z [27], Teoc [27], Boc [27,28], and bis(Boc) [29-31]. In addition, alternatives to Fmoc protection as temporary backbone amine protecting group have been proposed (with the nucleobase protecting groups stated in the brackets): Mmt (anisoyl/4-*t*Bu-benzoyl/isobutanoyl) [32], Dts (Z) [33], DMT (PMB) [34], azido (Bhoc) [35], Alloc (Bhoc) [36], NVOC (anisoyl/isobutyryl) [37], Dde (Mmt) [38], Bts (Boc) [39], Mtt (Boc) [40], and pNZ (bis-Boc) [41].

Methodologies for obtaining PNA-peptide conjugates involve either continuous Boc-, Fmoc- or combined Boc(PNA)/Fmoc(peptide)-based solid-phase synthesis (SPS), or alternatively a two-step assembly of purified PNA oligomer and peptide by using a bisfunctional linker [20,42]. However, the latter approach is time-consuming as it involves three separate syntheses each requiring purification by HPLC. In previous protocols for manual PNA synthesis [43] and continuous automated synthesis of PNA-peptide conjugates the commercially available Fmoc/Bhoc monomers have been applied since this enables a convenient simultaneous removal of all protecting groups and cleavage from resin under acidic conditions [44-46]. To our knowledge only building blocks displaying a traditional Bhoc protection of the nucleobases in combination with a temporary Fmoc protection are commercially available. Nevertheless, these Bhoc-protected monomers are both expensive and possess reduced solubility in the solvents usually employed for SPS (i.e., DMF, NMP or CH_2Cl_2). Addition of DMSO may promote dissolution, but then different coupling conditions with other MW settings may need to be used for the C monomer due to the large difference in the degree of MW energy absorption between DMF/NMP and DMSO, thus complicating the

methodology. Another advantage of applying this protection scheme for the nucleobases is that Boc constitutes a "traceless protecting group" when employed in combination with a TFA-H_2O-TIS cleavage mixture (in many cases TIS may be omitted). Thus, when using Boc protection of the nucleobases it is possible to avoid any sample clean-up prior to purification by HPLC. Hence, alternative strategy using Fmoc/Boc monomers [27] was chosen for exploration of a novel protocol for automated microwave (MW)-assisted continuous synthesis of PNA-peptide conjugates.

Existing protocols for synthesis of PNA-peptide conjugates by MW-assisted SPS comprise: (i) separate peptide synthesis and PNA oligomer synthesis by using Fmoc/Bhoc-protected PNA monomers [19] followed by conjugation, or (ii) continuous synthesis of the entire PNA-peptide conjugate [47]; an alternative method for the latter involves an Fmoc/Dde protection scheme [48]. By contrast, there appears to be no reports on the use of Fmoc/Boc PNA monomers [27] for MW-assisted assembly of PNA alone or in combination with Fmoc-protected amino acids with acid-labile side-chain protecting groups. Nevertheless, since Fmoc-based solid-phase peptide synthesis (SPPS) is the most commonly used methodology in automated assembly of peptides, we have investigated the use of such PNA monomers in a versatile strategy for automated continuous synthesis of PNA-peptide constructs.

2. Results and Discussion

2.1. Chemistry

2.1.1. Monomer synthesis

Fmoc/Bhoc-protected PNA monomers as well as Fmoc-protected thymine monomer were obtained from a commercial supplier, while Fmoc/Boc-protected cytosine, adenine and guanine monomers were prepared via modifications of conversions reported in earlier synthesis studies [27], combined with alternative starting materials and strategies, thereby enabling overall shorter synthetic sequences (Fig. 1).

The starting Fmoc-protected aminoethylglycine PNA backbone (**1**) was prepared in large scale according to previously reported procedures [46,49]. Next, the Fmoc/Boc-protected cytosine PNA monomer (**3**) was obtained from acylation of **1** with the intermediate acetic acid **2** in high yield (93% cf. 72% in [27]) by using TSTU as the coupling reagent, albeit extended activation and prolonged reaction times were needed. Synthesis of the Fmoc/Boc-protected adenine PNA monomer (**7**) was achieved in three steps starting from the commercially available ethyl ester **4**. Initial Boc protection of the aminopurine moiety followed by saponification of the ethyl ester **5**

afforded Boc-protected acetic acid **6** in 40% overall yield, which was comparable to the original more time-consuming sequence starting from adenine with conversion into the benzyl ester corresponding to **4**. Finally, coupling of **6** to the PNA backbone **1** resulted in an acceptable yield (66% cf. 64% in [26]) of the Fmoc/Boc-protected adenine PNA monomer (**7**). Importantly, this modified shorter sequence constitutes a cheap, fast and readily scalable protocol.

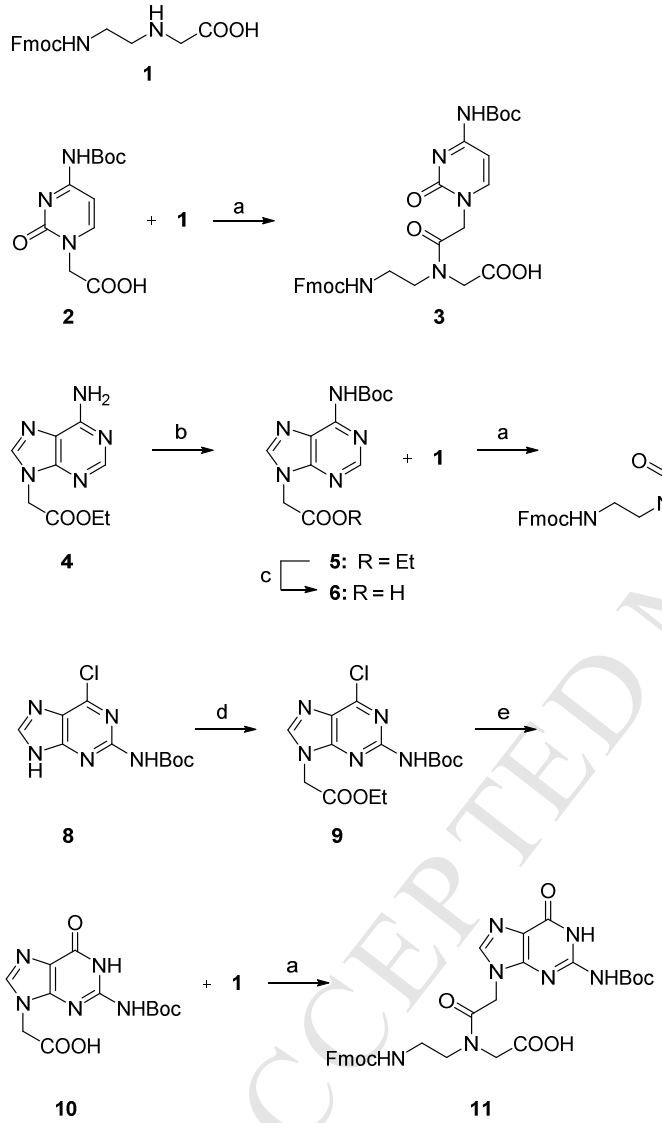

Figure 1. Reaction scheme for synthesis of Fmoc/Boc-protected PNA monomers. (a) TSTU and DIPEA, rt, 2 h; then **1** and DIPEA, 3 h, rt (**3**: 93%; **7**: 66%; **11**: 82%); (b) carbonyldiimidazole (CDI), 105 °C, 1 h; *tert*-butanol, 95°C → rt, overnight (45%); (c) LiOH, 48 h, rt (89%); (d) K_2CO_3, ethyl 2-bromoacetate (dropwise during 30 min), rt, overnight (87%); (e) NaH and 2-cyanoethanol, THF, -78 °C → 0 °C; then **9**, 0 °C → rt, 48 h, followed by H_2O, 0 °C, pH 2-3 (67%).

The Fmoc/Boc-protected guanine PNA monomer (**11**) was obtained in four steps starting from **8** [50]. First, alkylation of 2-amino-6-chloropurine building block **8** with ethyl bromoacetate afforded **9** in good yield (87%), while subsequent one-pot saponification and transformation into guanine resulted in Boc-guanine acetic acid **10** in a moderate yield (67%). Similarly, the Fmoc/Boc-protected guanine PNA monomer (**11**) was assembled in good yield (82%) by TSTU coupling of Boc-guanine acetic acid **10** with backbone **1**, which gave an 48% overall yield of guanine PNA building block **11**, while a 36% overall yield was obtained in four steps starting from 6-chloro-2-aminopurine via the corresponding benzyl acetic ester [27]. Despite the present improvements, purification of the desired protected monomers remained a challenge due to low solubility in solvent mixtures suitable for extraction and/or column chromatography, however, by combining previous and modified methods gram-scale syntheses of all three monomers were accomplished with purities appropriate for use in SPS.

2.1.2. Microwave-assisted solid-phase synthesis

Initially, the efficiency of Fmoc/Boc-protected PNA monomers in assembly of PNA oligomers was assessed by performing SPS of a PNA pentamer, containing all four nucleobases (i.e., Fmoc-Gly-TGCAT-NH_2 retaining the Fmoc at the N-terminus to confer sufficient retention on HPLC), on an Fmoc-Rink amide polystyrene resin as well as on an H-Rink amide ChemMatrix® resin. Comparison of the HPLC chromatograms of the crude products (see Fig. S1 in Supplementary Material) indicated that the ChemMatrix® resin was superior in terms of purity of the crude product as reported earlier for similarly complex compounds [51].

Standard MW-assisted SPPS is conducted at 75 °C for most couplings (when using a CEM Liberty synthesizer [52,53]). However, the potential thermolability of Boc protecting groups [54,55] present on the nucleobases constitutes a risk for spontaneous Boc deprotection during the repeated cycles of MW heating, potentially leading to branched byproducts. To investigate at which temperature the Boc group might undergo spontaneous degradation Fmoc-Gly-TGCAT-NH_2 was synthesized manually (with conventional heating), while Fmoc-Gly-GCAT-NH_2 was prepared by automated synthesis (with MW heating) at 45 °C, 55 °C, 65 °C and 75 °C. HPLC chromatograms of the crude oligomers (see Fig. S2 in Supplementary Material) showed that high-purity crude products was formed at 45 °C, while several byproducts were produced at temperatures above 45 °C. Additional experiments, in which each Fmoc/Boc-protected PNA monomer was heated to 45 °C, 55 °C, 65 °C and 75 °C, confirmed that Boc groups on nucleobases indeed are labile at temperatures higher than 45 °C as judged by analytical HPLC and MALDI-TOF analysis. Thus,

assembly of both PNA oligomer and peptide should proceed cleanly when performed at 45 °C, thereby enabling continuous synthesis of the entire PNA-peptide conjugate. When a (reversed) peptide-PNA conjugate is desired the peptide may be synthesized at a higher coupling temperature followed by PNA assembly at a lower temperature.

The excess of PNA monomer required to achieve a satisfactory purity of the resulting oligomer and ultimately of the conjugate is another variable. For MW-assisted SPPS, the recommended default excess of amino acid building block is 5 equivalents. However, due to the considerably higher cost of PNA monomers it was attractive to investigate whether use of a lower excess might be feasible. Experiments using 2, 3, or 4 equivalents of each PNA monomer for preparation of Fmoc-Gly-GCAT-NH_2 showed that purity did not improve significantly beyond the use of 3 equivalents. Finally, in order to achieve a fast and efficient methodology, the coupling time was likewise varied (5, 10 and 15 min), and similar purity was obtained with both the standard period of 10 min and with a prolonged coupling of 15 min, while a coupling time of 5 min proved less efficient.

These optimized conditions were employed for the continuous synthesis of PNA-peptide compounds lacking a linker or having an eg1 moiety as spacer, as the latter previously has been incorporated in conjugates obtained by manual continuous Boc-based synthesis.

2.1.3. Approaches for obtaining PNA-peptide conjugates

Encouraged by our recent finding that SbmA-independent antibacterial conjugates could be obtained by combining a validated 10-mer acpP antisense PNA oligomer (CTC-ATA-CTC-T) with AMPs possessing a propensity for bacterial internalization [13], we investigated similar conjugates based on an RXR-modified drosocin (i.e., RXR-PRPYSPRPTSHPRPIRV-NH_2; **L-12**) and a truncated analogue of Pip1 ($[RXR]_3$IKILFQNRRMKWKKM) with only two RXR repeats. Conjugates having different linkers between the two parts as well as the conjugate devoid of a linker were synthesized, since previous studies had inferred that this structural feature might influence the antibacterial activity [7] and/or delivery to human cells [20,56,57].

Previously, several linker moieties were utilized for connecting antisense PNA oligomers with suitable delivery peptides [20], e.g., one or more repeats of the ethylene glycol linker (eg1; also denoted as the O-linker) or the succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). These as well as a more hydrophilic maleimide ethylene glycol linker (i.e., Mal-eg1; not previously explored for this purpose) were studied in the present work (Fig. 2A). The initial step involved formation of an amide linkage between the N-terminus of the PNA oligomer

and the appropriate bisfunctional reagent containing an N-hydroxysuccimide (NHS) ester moiety as well as a maleimide group (i.e., SMCC NHS ester [7,58] or Mal-eg1 NHS ester [59,60]; Fig. 2B). After full conversion into an intermediate modified PNA the final conjugate was obtained upon addition of a Cys-containing delivery peptide to the maleimide moiety (a one-pot protocol may be utilized for Mal-eg1 while a precipitation step is included for SMCC).

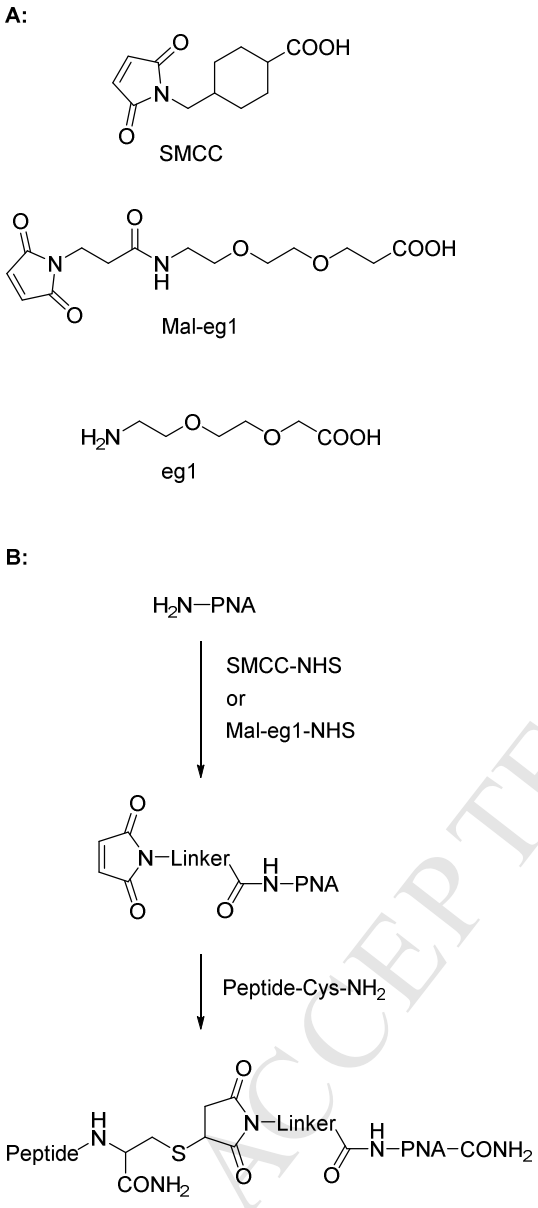

Figure 2. Synthesis of conjugates via bisfunctional linkers. (A) Different linkers used in the present work; (B) Two-step one-pot assembly protocol (see Experimental Section for details).

2.1.3. Peptides and PNA-peptide conjugates

For all-L RXR-drosocin (**L-12**) either the hydrophobic SMCC linker or the more hydrophilic Mal-eg1 linker was inserted between the anti-*acpP* PNA oligomer (targeting *acpP*

expression) and the delivery peptide to give conjugates **L-13** and **L-14**, respectively (see Table 1). Also, the corresponding all-D peptide (i.e., **D-12**) and the corresponding antisense PNA conjugate (i.e., **D-14**) were included for comparison. In addition, PNA-peptide conjugate **L-15** (having a single eg1 residue as linker) and **L-16** (lacking a linker between the PNA and peptide moieties) as well as its mismatch control **L-16m** (displaying a PNA oligomer with a shuffled base sequence) and the conjugate containing the all-D peptide (i.e., **D-16**) were synthesized.

Likewise, the truncated Pip1-derivative (i.e., $[RXR]_2$IKILFQNRRMKWKK-NH_2; **L-17**) was conjugated to anti-*acpP* PNA via the SMCC, Mal-eg1 or eg1 moieties to give **L-18**, **L-19**, and **L-20**, respectively. Also, the all-D peptide **D-17** was conjugated to anti-*acpP* PNA to give conjugate **D-19**. A compound without a linker moiety (i.e., **L-21**), as well as mismatch controls (**L-19m** and **L-21m**) were included in this series of compounds.

2.2. Microbiological evaluation

2.2.1. Panel of bacteria and activity trends

Minimal inhibitory concentrations (MICs) for the peptides and PNA-peptide conjugates were determined against a panel of pathogenic bacteria (capable of causing human diseases) comprising wild-type (WT) ATCC strains of *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus* as well as an *E. coli* Δ*sbmA* strain lacking a functional SbmA inner-membrane peptide transporter (Table 1). In addition, the conjugates were tested against four selected MDR Enterobacteriaceae strains comprising *K. pneumoniae* NMI 2697/14 and NMI 3081/09, and *E. coli* NMI3898/15 and NMI 3548/14, all being clinical isolates obtained from Polish hospitals during the period of 2009-2015. These MDR strains represent various resistance patterns, levels and mechanisms (see Table 2, and Table S2 in Supplementary Material), including plasmidic (*mcr-1*) and chromosomally encoded resistance to colistin, tigecycline, and/or β-lactams (associated with expression of various β-lactamases e.g., CTX-M-like ESBLs, carbapenemase KPC, or CMY-2 cephalosporinase).

Table 1. MIC values (in μg/mL) against the screening panel of bacteria.

		E. coli ATCC 25922	*E. coli* Δ*sbmA*	*K. pneumoniae* ATCC 13883	*P. aeruginosa* ATCC 27853	*P. aeruginosa* PAO1	*A. baumannii* ATCC 19606	*S. aureus* ATCC 29213
L-12	(RXR)PRPYSPRPTSHPRPIRV-NH_2	>64	>64	>64	-	>64	64	>64
D-12	(rXr)prpysprptshprpirv-NH_2	>64	>64	>64	-	>64	>64	>64
L-13	(RXR)PRPYSPRPTSHPRPIRVC-SMCC-(PNA2301)	16	>64	8	-	>64	>64	>64
L-14	(RXR)PRPYSPRPTSHPRPIRVC-(Mal-eg1)-(PNA2301)	16	>64	4-8	-	>64	>64	>64
D-14	(rXr)prpysprptshprpirvc-(Mal-eg1)-(PNA2301)	32	64	8-16	-	>64	64	>64
L-15	(RXR)PRPYSPRPTSHPRPIRV-eg1-(PNA2301)	32	>64	4-8	-	>64	>64	>64
L-16	(RXR)PRPYSPRPTSHPRPIRV-(PNA2301)	4	>64	2-4	>64	>64	64	>64
L-16m	(RXR)PRPYSPRPTSHPRPIRV-(PNA3961)	>64	>64	64	-	>64	>64	>64
D-16	(rXr)prpysprptshprpirv-(PNA2301)	>64	>64	8-16	-	>64	>64	>64
L-17	$(RXR)_2$IKILFQNRRMKWKK-NH_2	8	8	64	-	8	8	32-64
D-17	$(rXr)_2$ikilfqnrrmkwkk-NH_2	2-4	2	4	2	1	2	2-4
L-18	$(RXR)_2$IKILFQNRRMKWKKC-SMCC-(PNA2301)	16	16	4-8	-	32	16	64
L-19	$(RXR)_2$IKILFQNRRMKWKKC-(Mal-eg1)-(PNA2301)	8	8-16	2-4	-	32-64	16	>64
L-19m	$(RXR)_2$IKILFQNRRMKWKKC-(Mal-eg1)-(PNA3961)	32	32	64	-	32	16	>64
D-19	$(rXr)_2$ikilfqnrrmkwkkc-(Mal-eg1)-(PNA2301)	8	8	16	-	8	4	32
L-20	$(RXR)_2$IKILFQNRRMKWKK-eg1-(PNA2301)	16	16	8	-	32	32	>64
L-21	$(RXR)_2$IKILFQNRRMKWKK-(PNA2301)	16	16	8	-	32	16	>64
L-21m	$(RXR)_2$IKILFQNRRMKWKK-(PNA3961)	64	64	>64	-	32	16-32	>64
CST		0.125-0.5	0.5	2-4	0.25	0.5	0.25	>16

Compounds with prefices L and D indicates that peptide is the all-L and all-D form, respectively, while suffix m indicates that the conjugate contains a control PNA with two mismatched (interchanged) nucleobases. X = 6-aminohexanoic acid = H_2N-$(CH_2)_5$COOH residue. Anti-*acpP*: PNA2301 = H-CTCATACTCT-NH_2; Mismatch: PNA3961 = H-CTCTTACACT-NH_2. CST = colistin.

The minimal inhibitory concentrations (MICs) revealed that only PNA-peptide **L-16** exhibited efficient antisense activity in *E. coli* ATCC 25922 (MIC = 4 μg/mL ~ 0.7 μM), while the activity of conjugates **L-19** and **D-19** appears to arise mainly from the delivery peptide (i.e., **L-17**). Moreover, in *E. coli* antibacterial activity was abolished in the mutant lacking the SbmA peptide transporter, which recently was reported to be the case for several similar PNA-AMP conjugates [13]. The peptide **D-17** was significantly more potent (MIC values of 1-4 μg/mL ~ 0.25-1 μM) than **L-17** (MICs in the range 8-64 μg/mL). *K. pneumoniae,* displaying an identical *acpP* antisense target sequence, proved to be susceptible to several PNA-peptide conjugates (MICs in the range 2-8 μg/mL ~ 0.3-1.3 μM). By contrast, in *P. aeruginosa*, *A. baumannii*, and *S. aureus,* having a different *acpP* target sequence, the antibacterial effect of the conjugates containing the modified pip1 appears to arise from the peptide. Noticeably, peptide **D-17** exhibited broad-spectrum activity (with MICs in the range 1-4 μg/mL ~ 0.25-1 μM) against all Gram-negative pathogens included in the panel as well as toward the Gram-positive *S. aureus*.

The two most potent compounds **L-16** and **D-17** were also tested against additional strains, including wild-type *P. aeruginosa* ATCC 27853, two colistin-resistant *P. aeruginosa* and *A. baumannii* strains (CST-R; MIC >16 μg/mL), and three *E. coli* clinical isolates obtained from Polish hospitals during the period of 2011-1016: susceptible to colistin but resistant to carbapenems due to expression of NDM-1 or NDM-5 carbapenemases (Table 3). Both compounds **L-16** and **D-17** exhibited potent activity against all NDM producers (MIC range 0.5-2 μg/mL ~ 0.1-0.5 μM), but only **D-17** was efficient against colistin-resistant *P. aeruginosa* and *A. baumannii* strains (MIC 2-4 μg/mL ~ 0.5-1 μM).

2.2.2. PNA-drosocin series

Compound **L-16**, lacking a linker, exhibited potent activity against WT *E. coli* (MIC of 4 μg/mL ~ 0.7 μM) that may be ascribed to an SbmA-dependent antisense mechanism, based on the finding of an at least 16-fold lower activity of the delivery peptide (i.e., **L-12**) and of the mismatched PNA conjugate **L-16m** in combination with a similarly low activity of **L-16** against the *E. coli* Δ*sbmA* strain.

Noticeably, the linker-containing antisense conjugates had only moderate activity against WT *E. coli* (i.e., **L-13**, **L-14** and **L-15** had MIC values in the range 16-32 μg/mL). This observation infers that the presence of a linker moiety may indeed influence the bacterial delivery. That linkers appear to influence PNA uptake by cells was earlier found also by Hatamoto et al. [61],

and in more recent work with a vitamin B12-mediated transport of PNA to *E. coli* cells [62,63]. Similarly, differences between linker types were observed in an earlier study where a PNA-peptide conjugate with an SMCC linker gave rise to a lower antibacterial activity in *E. coli* than the corresponding conjugate with an eg1 spacer [7]. Moreover, the potency of **L-16** is comparable to that of the analogue with the RXR motif inserted at the C-terminus of drosocin (i.e., GKPRPYSPRPTSHPRPI-RXR-NH_2) [13]. Interestingly, for the latter conjugate activity was retained when tested against an *E. coli* Δ*sbmA* strain [13]. In addition **D-16**, exhibited a more than 16-fold lower activity in WT *E. coli*. Surprisingly, the tigecyline- and colistin-resistant *E. coli* isolate NMI3898/15 was more susceptible than the WT strain to conjugates based on drosocin (Table 2). Thus, compounds **L-13**, **L-14** and **L-16** had 2- to 8-fold lower MIC values (as compared to those against the WT strain), again with **L-16** being the most potent (MIC of 1-2 μg/mL ~ 0.15-0.3 μM) and retaining high activity against *E. coli* mcr-1 (NMI 3898/15; MIC of 4 μg/mL ~ 0.6 μM).

Table 2. MIC values (in μg/mL) against drug-resistant strains.

	E. coli NMI3548/14	*E. coli* NMI 3898/15	*K. pneumoniae* NMI 2697/14	*K. pneumoniae* NMI 3081/09	Hemolysis (%) at 100 μM
L-12	32->64	32- >64	>64	>64	<1%
D-12	>64	>64	>64	>64	<1%
L-13	4-8	16-64	32 - >64	32	<1%
L-14	2-8	8-32	32 - >64	64	<1%
D-14	32	64/32	>64/64	64	<1%
L-15	16-32	32- >64	>64	32-64	<1%
L-16	1-2	4	32	8	<1%
L-16m	>64	>64	>64	8-16	<1%
D-16	64	>64	>64	>64	<1%
L-17	4-8	16	>64	>64	<1%
D-17	1	2	16	16	<1%
L-18	8-16	16	32	16	<1%
L-19	4-8	16	16-32	8-16	<1%
L-19	16-32	32-64	>64	>64	<1%
D-19	8	8	64	16	1.6%
L-20	8	32	>64	16	<1%
L-21	8-16	32	64 - >64	16	<1%
L-21	32	64- >64	>64	>64	<1%
CST	-	2-4	-	-	-

MDR Enterobacteriaceae strains used for the evaluation: *E. coli* NMI3548/14: tigecycline-resistant (TGC-R with MIC = 16) and colistin (CST-R with MIC = 4; *mcr*-negative), ESBL (CTX-M-positive); *E. coli* NMI 3898/15: tigecycline-susceptible (TGC-S, MIC = 0.5), colistin-resistant (CST-R, MIC =4; *mcr1*-positive), CMY-2-positive [64]; *K. pneumoniae* NMI 2697/14: tigecycline-susceptible (TGC-S, MIC = 0.5), colistin-resistant (CST-R, MIC >16; *mcr*-negative), ESBL (CTX-M)-positive; *K. pneumoniae* NMI 3081/09: tigecycline-intermediate (TGC-I, MIC =2), colistin-susceptible (CST-S, MIC = 1), KPC-positive.

See also Table S2 in Supplementary Material for further details on resistance patterns. CST = colistin. NMI = National Medicine Institute, Warzaw, Poland.

Likewise, conjugate **L-16** was the most active (MIC of 2-4 μg/mL ~ 0.3-0.7 μM) against WT *K. pneumoniae*, albeit also conjugates **L-13**, **L-14**, and **L-15** displayed 2- to 4-fold lower MIC values (4-8 μg/mL ~ 0.6-1.2 μM) as compared to those found against *E. coli*. Notably, the compound based on the all-D peptide (i.e., **D-16** lacking a spacer/linker) was 2- to 8-fold less active than the **L-16**. With respect to the resistant *K. pneumoniae* isolates, only **L-16** retained activity against the MDR (but colistin-susceptible) strain NMI 3081/09 with a MIC value of 8 μg/mL, whereas all analogues lacked activity in the highly colistin-resistant *K. pneumoniae* NMI 2697/14 isolate. Finally, none of the conjugates showed significant activity against *P. aeruginosa*, *A. baumannii*, or *S. aureus* (Table 1).

Table 3. MIC values (in μg/mL) of compounds **L-16** and **D-17** against MDR clinical.

	E. coli NMI 3371/16 ST101 NDM-1	*E. coli* NMI 636/15 ST405 NDM-5	*E. coli* NMI 5428/11 ST410 NDM-1	*P. aeruginosa* ATCC 27853	*P. aeruginosa* NMI 506/17	*A. baumannii* NMI 3658/17
L-16	0.5	1	1	>64	>64	32
D-17	2	2	1-2	2	4	2

MDR Enterobacteriaceae strains: *E. coli* NMI 3371/16: carbapenem-resistant, colistin-susceptible (CST-S), and NDM-1-positive; *E. coli* NMI 636/15: carbapenem-resistant, colistin-susceptible (CST-S), and NDM-5-positive; *E. coli* NMI 5428/11: carbapenem-resistant, colistin-susceptible (CST-S), and NDM-1-positive [65]; *P. aeruginosa* NMI 506/17: colistin-resistant (CST-R, MIC >16; *mcr*-negative); *A. baumannii* NMI 3658/17: colistin-resistant (CST-R, MIC >16; *mcr*-negative).

The corresponding $(KFF)_3K$-eg1-PNA2301 was previously found to exhibit low micromolar potency against the *E. coli* strains K12 [7] and DH10B [9], while another $(KFF)_3K$-based conjugate with a 12-mer PNA (targeting rpoD gene expression) was somewhat less active toward *E. coli* MG1655 and an ESBL-producing strain (ATCC 35218) [66]. Also, the anti-acpP PNA with a C-terminally added lysine, i.e., $(KFF)_3K$-eg1-PNA2301-K (MIC of 16 μg/mL) was also found to be active against the pathogenic E. coli O157:H7 ST2-8624 strain [67].

Importantly, all peptides and conjugates in this series were found to be essentially non-hemolytic, as less than 1% hemolysis typically was observed at 100 μM, which corresponds to a cell selectivity (based on the concentration giving 10% hemolysis relative to the MIC) well above 100 for the most potent compounds.

2.2.3. PNA-Pip1 series

Inspired by previous reports on targeting gene expression in the Gram-positive *Streptomyces pyogenes* [68] and *S. aureus* [69] by using a common eukaryotic CPP, such as Tat (GRKKKRRQRRRYK), for bacterial delivery, it was explored whether a shortened analogue of one of the so far most efficient CPPs for delivery of PNA to human cells, namely the Pip1 peptide [19], likewise might facilitate efficient uptake in bacteria. Since modification of AMPs with a single repeat of the RXR motif may be sufficient to confer SbmA-independent enhanced uptake in *E. coli* [13], an analogue of Pip1 ($[RXR]_2$IKILFQNRRMKWKK-NH_2; **L-17**), having only two instead of three RXR repeats at the N-terminus, was examined as delivery peptide in anti-*acpP* PNA-peptide conjugates. In this case the peptide itself (**L-17**) exhibited activity in the Gram-negative species (albeit weakly in *K. pneumoniae*). Interestingly, all antisense-based conjugates (**L-18**, **L-19**, **L-20**, and **L-21**) were in general equipotent or less active than peptide **L-17** itself against all bacteria, except in the case of *K. pneumoniae* where all conjugates containing the anti-*acpP* PNA showed higher activity than that of peptide **L-17** and PNA mismatch control conjugates (**L-19m** and **L-21m**), thereby inferring an antisense mode of action. Finally, **L-19** retained significant activity (MIC of 8-16 μg/mL) against the KPC-producing, colistin-susceptible *K. pneumoniae* strain NMI 3081/09 (Table 2). In this series, in which the activity is due to the CPP, rather than to a PNA antisense mechanism, the conjugate lacking a linker or with a short eg1 moiety inserted (i.e., **L-21** and **L-20**, respectively) were slightly less potent against WT *K. pneumoniae* than the conjugates containing either an SMCC or a Mal-eg1 linker (i.e., **L-18** or **L-19**, respectively) with the latter conjugate being most potent (MIC of 2-4 μg/mL ~ 0.3-0.6 μM).

The antibacterial activity of anti-*acpP* PNA conjugated to peptide **L-17** may relate to its resemblance to $(RXR)_4$-XB (B = β-alanine) used as an efficient vehicle for delivery of antisense PMOs and PNAs in *E. coli* [70,10], *K. pneumoniae* [66] and *P. aeruginosa* [12,71]. However, previous findings suggest that the antibacterial activity of such conjugates may to some extent be exerted by the $(RXR)_4$-XB peptide moiety [12]. This also seems to be the case for conjugates based on **L-17**, albeit less pronounced for *K. pneumoniae*.

Quite surprisingly, **D-17** was significantly more potent (MICs in the range 1-4 μg/mL) than **L-17** (MICs of 8 μg/mL) against all WT strains (Table 1), while **D-17** retained activity against the two colistin-resistant *E. coli* strains NMI 3548/14 and NMI 3898/15 (MICs of 1 and 2 μg/mL, respectively, corresponding to 0.25 and 0.5 μM). This could simply be a result of the increased stability to enzymatic degradation conferred by the all-D stereochemistry or alternatively be due to another mechanism of action for the D-form.

Most analogues in this series, including the all-D peptide **D-17**, were found to be essentially non-hemolytic, as less than 1% hemolysis typically was observed at 100 μM, which further infers that both peptide **D-17** and conjugate **L-19** constitute promising hit compounds.

2.3. Conclusion

The present work shows that assembly of PNA-peptide conjugates may be performed conveniently by an Fmoc/Boc-based continuous MW-assisted SPS strategy. This methodology has the advantage of utilizing monomer building blocks with suitable solubility properties as well as a decreased workload in synthesis (avoiding a solution-phase conjugation) and purification.

How introduction of a linker between the antisense PNA and the delivery peptide affects activity remains to be elucidated in detail as it appears to depend on both the delivery peptide and the targeted bacteria. However, it was demonstrated that absence or proper selection of a linker or spacer moiety indeed constitutes an optimization feature for PNA-peptide conjugates. This is exemplified by the finding that the most active conjugate against *E. coli* (i.e., **L-16**) was devoid of a linker residue while for *K. pneumoniae* also the conjugate containing the Mal-eg1 linker (i.e., **L-14**) was almost equipotent, while the SMCC linker was slightly less favorable.

Interestingly, the drosocin-based conjugate **D-16** having an all-D peptide moiety was inactive in *E. coli* (while **D-14** also containing an all-D peptide displayed weak activity), indicating that either translocation across the outer membrane is compromised or the SbmA transporter discriminates between enantiomers or is preferentially transporting shorter (proteolytically degraded) fragments of the all-L drosocin conjugates. For the corresponding pip1-derived compounds any such effects were totally dominated by the inherent potent antibacterial activity of the all-D pip1 peptide **D-17**.

Together with earlier findings on the effect of introducing RXR motifs at the termini of delivery peptides [13], it appears that this approach is not a general solution to circumvent SbmA-dependent uptake that constitutes a potential Achilles´ heel with respect to resistance development. However, further optimization concerning size and sequence of RXR-based motifs may address this. Provided that SbmA-dependent uptake can be abolished, the present bacterial delivery approach may be useful for exploring other antisense-based therapies targeting bacterial ribosomes via domain II of 23S rRNA [72] or 16S rRNA [73], or as a means of overcoming resistance to β-lactam antibiotics as recently explored in *E. coli* [74]. In relation to *K. pneumoniae*, earlier work on antisense-mediated growth inhibition comprise: (i) $(KFF)_3K$ conjugated to 16-mer anti-*gyrA* or anti-*ompA* PNA oligomers [74], (ii) $(KFF)_3K$- and $(RXR)_4XB$-conjugated 10-mer PNAs targeting

rpoD expression [66], and (iii) $(KFF)_3K$-conjugated anti-*acpP* and anti-*murA* 10-mer PNAs [9]. It is noteworthy that out of these conjugates, only the latter displayed low micromolar activity. Therefore, several of the novel compounds described here constitute interesting starting points for further optimization of PNA delivery in *K. pneumoniae*. In conclusion, the most potent conjugates exhibit promising potency even in certain MDR clinical isolates of *E. coli* and *K. pneumoniae*, which warrant further optimization studies of these.

3. Experimental section

3.1. General procedures

Starting materials and solvents were purchased from commercial suppliers (ABCR GmbH, Alfa Aesar, AppliChem, CHEMsolute, Fluka, Iris Biotech, LabScan, Merck, Sigma Aldrich and VWR and used without further purification. Deionized water was filtered (0.22 μm) in-house by use of a Milli-Q plus system (Millipore, Billerica, MA). Column chromatography was performed on Screening Devices silica gel 60 (0.040-0.063 mm). TLC analysis was conducted on HPTLC aluminium sheets (Merck, silica gel 60, F245). Compounds were visualized by UV absorption (245 nm), by spraying with 20% H_2SO_4 in ethanol or with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$ 25 g/L and $(NH_4)_4Ce(SO_4)_4{\cdot}2\ H_2O$ 10 g/L, in 10% aqueous H_2SO_4 followed by heating. ^1H, and ^{13}C NMR spectra were recorded on a Bruker Avance III HD equipped with a cryogenically cooled 5 mm dual probe optimized for ^1H and ^{13}C (600 MHz and 150 MHz respectively). NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 with chemical shift (δ) relative to tetramethylsilane. All intermediates were more than 95% pure as determined by HPLC, and identity established by MALDI-TOF (on a Bruker SolariX XR in MALDI mode or on a Bruker MicroTOF-Q II equipped with an electrospray ionization interface operated in positive ionization mode. For peptides preparative reversed-phase high pressure liquid chromatography (RP-HPLC) was performed by using a Phenomenex Luna C18(2) column (250 × 21.2 mm, particle size: 5 μm) on a Shimadzu system consisting of a CBM-20A Prominence communication bus module, two LC-20AP Prominence pumps, an SPD-M20A Prominence diode array detector, and an SIL-20A HT Prominence autosampler. Elution was performed by using an aqueous MeCN gradient with 0.1% TFA (eluent A: 5:95 MeCN–H_2O + 0.1% TFA, eluent B: 95:5 MeCN–H_2O + 0.1% TFA). Analytical UHPLC was performed on a Shimadzu Prominence UHPLC system using a Phenomenex Luna C18(2) HTS column (100 × 3.0 mm; particle size: 2.5 μm) eluted at a rate of 0.5 mL/min (using either 0 → 40B% or 0 → 60B% gradient) by using same eluents as for preparative RP-HPLC. High-resolution mass spectra were obtained on an

Agilent (Santa Clara, CA, USA) 1260 series chromatographic HPLC system consisting of a G1311B quaternary pump with built-in degasser, a G1316A thermostated column compartment, a G1315D photodiode-array detector and a G1329B autosampler. Chromatographic separation of was performed on a Phenomenex Luna C18(2) column (150 mm × 4.6 mm; particle size: 3 µm, pore size: 100 Å) at 40 °C using an elution system consisting of H_2O–MeCN (95:5) (eluent A) and MeCN–H_2O (95:5) (eluent B), both acidified with formic acid 0.1%, in a flow rate of 0.8 mL/min with linear gradient from 0% → 100% B over 15 min. HRMS spectra were acquired using a Bruker micrOTOF-Q II mass spectrometer equipped with an electrospray ionization (ESI) interface (Bruker Daltonik, Bremen, Germany). Mass spectra were acquired in positive ionization mode, at 200 °C drying temperature, capillary voltage of 4100 V, nebulizer pressure of 2.0 bar, and drying gas flow of 7 L/min. A solution of sodium formate clusters was automatically injected at the beginning of each run to enable internal mass calibration. By means of a syringe pump (KD Scientific), selected peptides were continually infused into the mass spectrometer at 3 µL/min with the settings described above. Prior to the analyses, the spectrometer was calibrated using a solution of sodium formate clusters.

3.2. Cytosine PNA monomer (3)

To a solution of Boc-protected cytosine acetic acid **2** (8.08 g, 30.0 mmol) in DMF (100 mL) were added TSTU (9.48 g, 31.5 mmol) and DIPEA (6.80 mL, 5.04 g, 39.0 mmol). The resulting mixture was stirred for 2 h, and then finely ground PNA backbone **1** (11.24 g, 33.0 mmol) was added in portions. Alternately, the suspension was stirred, heated to 45 ^{o}C, and sonicated until a clear solution was obtained. Additional DIPEA (6.80 mL, 5.04 g, 39.0 mmol) was added, and then the mixture was stirred for 3 h, after which it was cooled to 0 ^{o}C. Then 1M $KHSO_4$ (100 mL) was slowly added, followed by more water to a total volume of ~500 ml, at which point the product precipitated. The resulting suspension was filtered, and the solid washed with water (50 mL). The dried crude product was purified by flash column chromatography (MeOH:acetone:$CHCl_3$, 0:1:1 → 0:1:0 → 1:1:0) to afford a slightly yellow solid, which was further purified by dissolution in chloroform (~100 mL) followed by precipitation upon addition of petrol ether (~500 mL) and cooling to 0 ^{o}C. Monomer **3** (16.5 g, 93%) was obtained as an off-white solid. M.p. >165 ^{o}C (decomposition). ^{1}H NMR (600 MHz, DMSO-d_6): δ ppm 1.45 (s, 9H, 3 × CH_3 in Boc), 3.10–3.45 (m, 4H, 2 × CH_2, appears under H_2O peak, signals assigned by COSY), 3.93–4.05 (m, 2H, CH_2), 4.18–4.35 (m, 3H, $CHCH_2$ in Fmoc), 4.60 (s, 1H, CH*H*), 4.79 (s, 1H, CH*H*), 6.90–7.00 (m, 1H, H-5), 7.26–7.92 (m, 10H, H-6, N*H* Fmoc, 8 × Ar-H), 10.22–10.32 (m, 1H, N*H* Boc); ^{13}C NMR (150 MHz, DMSO-d_6,): δ ppm

27.8, 38.0, 38.7, 46.7, 47.0, 47.3, 49.4, 49.5, 65.5*, 80.9*, 93.9, 120.1*, 120.1*, 125.1, 125.2, 127.1*, 127.6*, 140.7*, 143.9*, 150.5, 152.1, 155.1, 156.1, 156.3, 163.3, 167.0, 167.6; * two close peaks; several rotamers were present. HRMS: calcd for $[M+H]^+$ 592.2407, found 592.2401; ΔM = 1.0 ppm.

3.3. Boc-protected adenine acetic acid ethyl ester (5)

To a suspension of adenine-AcOEt (**4**; 50.0 g, 226 mmol, co-evaporated with 2 × 100 mL of dry DMF) in DMF (450 mL, dried over 3Å MS) was added 1,1'-carbonyldiimidazole (CDI, 73.3 g, 452 mmol). The mixture was heated to 105 ^{o}C and stirred for 1 h, and then additional CDI (27.5 g, 170 mmol) was added. After 1 h, the mixture was cooled to 90 ^{o}C and *tert*-BuOH (64.8 mL, 50.3 g, 678 mmol) was added, after which the mixture was stirred at rt overnight. Most of the solvent was removed *in vacuo* leaving a residue (~300 g) to which EtOAc (500 mL) was added. The resulting organic solution was washed with water (500 mL). The aqueous layer was extracted back with EtOAc (2 × 500 mL), and the combined organic layers were washed with brine (500 mL). The brine layer was extracted back with EtOAc (250 mL). All EtOAc phases were combined and dried (Na_2SO_4) and concentrated *in vacuo*. The residue (~300 g) was diluted with petrol ether (500 mL), and then the mixture was cooled to 0 ^{o}C and stirred for 1 h during which a precipitate formed. The white solid was filtered and washed further with petrol ether. The mother liquor was evaporated and treated similarly with EtOAc and petrol ether to yield a second crop of solid. Drying overnight under vacuum of the combined portions afforded **5** (33.0 g, 103 mmol, 45%) as an amorphous white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 1.21 (t, 3H, *J* = 7.1 Hz, CH_2CH_3), 1.48 (s, 9H, 3 × CH_3 in Boc), 4.18 (q, 2H, *J* = 7.1 Hz, CH_2CH_3), 5.18 (s, 2H, CH_2), 8.40, 8.58 (each s, 1H, H-2/H-8), 10.09 (s, 1H, N*H*Boc); ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 14.0, 27.8, 44.2, 61.5, 80.1, 123.0, 144.4, 149.9, 151.1, 151.6, 152.0, 167.7; HRMS: calcd for $[M+H]^+$ 322.15153, found 322.15221; ΔM = 2.1 ppm.

3.4. Boc-protected adenine acetic acid (6)

To ethyl ester **5** (30.85 g, 96.0 mmol) in a mixture of 1,4-dioxane (432 mL) and water (48 mL) was added LiOH (4.60 g, 192 mmol). After stirring for 48 h, the mixture was diluted with CH_2Cl_2 (1200 mL) and freshly solvent-rinsed Dowex H^+ was added until pH 5-6. Then Na_2SO_4 was added, and the mixture filtered on a glassfilter with a bed (~5 cm) of Na_2SO_4. The filtrate was evaporated to give **6** (25.1 g, 85.6 mmol, 89%) as an amorphous white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 1.48 (s, 9H, 3 × CH_3 in Boc), 5.07 (s, 2H, CH_2), 8.40, 8.57 (each s, 1H, H-2/H-8), 10.09 (s, 1H, N*H*Boc); ^{13}C

NMR (150 MHz, $CDCl_3$): δ ppm 27.9, 44.2, 80.1, 123.0, 144.5, 149.8, 151.2, 151.5, 152.1, 169.1; HRMS: calcd for $[M+H]^+$ 294.12023, found 294.12060; ΔM = 1.2 ppm.

3.5. Adenine PNA monomer (7)

To a solution of Boc-Adenine-AcOH **6** (9.27 g, 31.6 mmol) in DMF (105 ml) were added TSTU (9.99 g, 33.2 mmol) and DIPEA (7.16 mL, 5.31 g, 41.1 mmol). The resulting mixture was stirred for 2 h, and then finely ground PNA backbone **1** (11.84 g, 34.8 mmol) was added in portions. Alternately, the mixture was stirred, heated to 45 ^{o}C, and sonicated until it a clear solution was obtained. More DIPEA (7.16 mL, 5.31 g, 41.1 mmol) was added, and the mixture stirred for 2 h. The resulting reaction mixture was cooled to 0 ^{o}C, 1M $KHSO_4$ (105 mL) was slowly added, after which water was added (to give a total volume of ~500 mL) to precipitate the product. The solid was filtered and washed with water (50 mL). After drying, the crude product was purified by flash column chromatography (MeOH:acetone:$CHCl_3$, 0:1:1 → 0:1:0 → 1:1:0), giving a slightly yellow solid which was purified further by dissolution in chloroform (~100 mL) followed by precipitation upon addition of petrol ether (~500 mL) and cooling to 0 ^{o}C. The monomer **7** (12.8 g, 20.8 mmol, 66%) was obtained as an off-white solid. M.p. >153 ^{o}C (decomposition). ^{1}H NMR (600 MHz, DMSO-d_6): δ ppm 1.48 (s, 9H, 3 × CH_3 in Boc), 3.11–3.17 (m, 1H, CH*H*), 3.30–3.39 (m, 2H, 2 × CH*H*), 3.52–3.57 (m, 1H, CH*H*), 3.95–4.11 (m, 2H, CH_2), 4.17–4.37 (m, 3H, $CHCH_2$ in Fmoc), 5.13–5.31 (m, 2H, CH_2), 7.28–7.33 (m, 2H, Ar-H), 7.37–7.42 (m, 2H, Ar-H), 7.48–7.56 (m, 1H, N*H*Fmoc), 7.64–7.69 (m, 2H, Ar-H), 7.85–7.89 (m, 2H, Ar-H), 8.24–8.52 (m, 2H, H-2, H-8), 10.02–10.04 (m, 1H, N*H*Boc); ^{13}C NMR (150 MHz, DMSO-d_6): δ ppm 27.9, 37.9, 38.7, 43.8, 44.0, 46.7*, 46.9, 47.4, 48.1, 51.0, 65.5*, 79.2, 79.9, 80.0*, 120.1*, 122.9*, 125.1, 125.2, 127.0, 127.1, 127.6*, 140.7*, 143.8, 143.9, 144.9, 145.0, 149.6, 149.7, 151.1, 151.3, 151.4, 152.2, 152.3, 156.1, 156.4, 166.4, 167.1, 170.6, 171.1; * two close peaks; several rotamers were present. HRMS: calcd for $[M+H]^+$ 616.2419, found 616.2514; ΔM = 0.8 ppm.

3.6. 2-N-Boc-6-Cl-purine acetic acid ethyl ester (9)

Ethyl bromoacetate (3.88 mL, 5.85 g, 35.0 mmol) was added dropwise (over 30 min) to a mixture of 2-N-Boc-6-Cl-purine **8** (9.45 g, 35.0 mmol) and K_2CO_3 (9.69 g, 70.1 mmol) in dry DMF (470 mL). After stirring for 16 h, the mixture was filtered over celite and the filtrate concentrated *in vacuo*. The residue was dissolved in EtOAc (100 mL) and washed with a 1:1-mixture of water and brine (100 mL) and then with brine (100 mL). The combined aqeous layers were extracted with EtOAc (50 mL), and the resulting organic layer was washed with brine (50 mL). The combined organic

layers were dried (Na_2SO_4), filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (petrol ether–EtOAc, 1:1 → 1:2) to give **9** (10.9 g, 30.6 mmol, 87%) as an off-white amorphous solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 1.22 (t, 3H, *J* = 7.1 Hz, CH_2CH_3), 1.46 (s, 9H, 3 × CH_3 in Boc), 4.19 (q, 2H, *J* = 7.1 Hz, CH_2CH_3), 5.11 (s, 2H, CH_2), 8.45 (s, 1H, H-8), 10.30 (s, 1H, N*H*Boc); ^{13}C NMR (150 MHz): δ ppm 14.0, 27.9, 44.4, 61.7, 79.8, 126.4, 146.5, 149.1, 150.9, 152.7, 153.1, 167.4; HRMS: calcd for $[M+H]^+$ 356.11256, found 356.11356; ΔM = 2.8 ppm.

3.7. Boc-protected guanine acetic acid (10)

To a cooled (–78 °C) suspension of sodium hydride (7.69 g, 192 mmol, 60% dispersion in mineral oil) in THF (225 mL) was added 2-cyanoethanol (13.1 g, 13.7 g, 192 mmol). The mixture was allowed to warm to 0 °C, after which a solution of **9** (11.4 g, 32.0 mmol) in THF (100 mL) was added dropwise during 5 min. Then the mixture was allowed to warm up to rt, and then stirring was continued for 48 h. The solvent was removed under reduced pressure after which water (130 mL) was added, and then the mixture was extracted with hexane (150 mL) and EtOAc (150 mL). Next, the aqeous layer was cooled to 0 °C and carefully acidified to pH 2-3 with hydrochloric acid (37% aq.) Then EtOAc (120 mL) was added, and the mixture was stirred vigorously until the product precipitated. The crude product was filtered and washed on the filter with water, EtOAc and petrol ether. The solid was recrystallized from EtOAc–petrol ether (1:1, 200 mL), giving **10** (6.65 g, 21.5 mmol, 67%) as a pale amber solid. ^{1}H NMR (600 MHz; $CDCl_3$): δ ppm 1.48 (s, 9H, 3 × CH_3 in Boc), 4.85 (s, 2H, CH_2), 7.91 (s, 1H, H-8), 11.11, 11.39 (each s, 1H, NH guanine/N*H*Boc), 13.20– 3.42 (s, 1H, COOH); ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 27.8, 44.2, 82.5, 119.1, 147.7, 149.4, 153.8, 155.1, 169.0; HRMS: calcd for $[M+H]^+$ 310.11514, found 310.11557; ΔM = 1.3 ppm.

3.8. Guanine PNA monomer (11)

To a solution of Boc-Guanine-AcOH (**10**; 1.86 g, 6.00 mmol) in DMF (20 mL), TSTU (1.99 g, 6.60 mmol) and DIPEA (1.36 mL, 1.01 g, 7.80 mmol) were added. The resulting mixture was stirred for 1.5 h, and then finely ground PNA backbone **1** (2.25 g, 6.60 mmol) was added in portions. Alternately, the mixture was stirred, heated to 45 °C, and sonicated until a clear solution was obtained. More DIPEA (1.36 ml, 1.01 g, 7.80 mmol) was added, and then the mixture was stirred for 2 h. Then the mixture was cooled to 0 °C, 1M $KHSO_4$ (20 mL) was slowly added, after which additional water was added (to a total volume of ~100 mL), at which point the product precipitated. The solid was filtered and washed with water (50 mL). After drying, the crude product was purified by flash column chromatography (MeOH:acetone:$CHCl_3$, 0:1:1 → 0:1:0 →

1:1:0), giving a pale amber solid, which was further purified by dissolution in chloroform (~20 mL) followed by precipitation upon addition of petrol ether (~80 mL) and cooling to 0 ^{o}C. The G PNA monomer **11** (3.10 g, 4.91 mmol, 82%) was obtained as an off-white solid. M.p. >175 ^{o}C (decomposition). ^{1}H NMR (600 MHz, DMSO-d_6): δ ppm 1.44–1.46 (m, 9H, 3 × CH_3 in Boc), 3.13–3.49 (m, 4H, 2 × CH_2, appears under H_2O peak, signals assigned by 2D NMR), 3.87–3.91 (m, 2H, CH_2), 4.19–4.35 (m, 3H, $CHCH_2$ in Fmoc), 4.89, 5.04 (each s, 1H, CH_2), 7.27–7.31 (m, 2H, 2 × Ar-H), 7.37–7.41 (m, 2H, 2 × Ar-H), 7.57–7.88 (m, 6H, H-8, N*H*Fmoc, 4 × Ar-H), 10.98–11.42 (m, 2H, NH guanine, N*H*Boc); ^{13}C NMR (150 MHz, DMSO-d_6): δ ppm 27.7, 38.0, 38.9, 43.9, 46.6, 46.7, 47.8, 52.4, 65.5, 82.3, 82.4, 118.8, 120.0, 120.1, 125.0, 125.3, 127.0, 127.1, 127.5, 127.6, 140.4, 140.6, 140.7, 143.8, 143.9, 147.5, 149.5, 149.7, 153.7, 153.9, 155.1, 156.0, 156.3, 166.1, 167.1, 170.6; several rotamers were present. HRMS: calcd for $[M+H]^+$ 632.2469, found 632.2468; ΔM = 0.1 ppm.

3.9. **Peptide synthesis.**

All peptides listed in Table 1 (characterization data are listed in Table S1) were synthesized on a CEM Liberty™ MW peptide synthesizer by using MW-assisted Fmoc-based SPPS on an H-Rink-Amide ChemMatrix® resin (loading 0.52 mmol/g, 0.1 mmol; 100-200 mesh) or on an Fmoc-Rink-Amide AM resin (loading 0.7 mmol/g, 0.1 mmol; 100-200 mesh). Chain elongation was performed by using 0.2 M N^{α}-Fmoc-protected amino acid building blocks (5 equiv; with acid-labile tBu/Trt/Boc/Pbf as side chain protecting groups) in DMF in combination with 0.5 M HBTU (5.0 equiv) as coupling reagent and 2 M DIPEA in NMP as activator base. Fmoc deprotection was performed with 20% piperidine in DMF at 75 °C (37 W for 30 sec followed by 40 W for 180 sec), while amino acid couplings were conducted at 75 °C (25 W for 300 sec). Couplings involving Arg or hindered amino acid building blocks (Val, Pro, Ile, Thr) as well as the subsequent elongations were performed as double couplings (each perfomed as above). After assembly of the delivery peptide sequence, the final tagging with a cysteine residue was carried out by manual coupling (for 2 h) of Boc-Cys(Trt)-OH (5 equiv) to the deprotected N-terminus in the presence of HBTU (5 equiv), 1-hydroxybenzotriazole (HOBt) (5 equiv) and DIPEA (10 equiv). All peptides were cleaved by using TFA–TIS–H_2O (95:2.5:2.5; 4 mL) for 2 × 1 h, eluting the resin with additional CH_2Cl_2. The combined eluates were kept at room temp for at least 8 h, and then co-evaporated with toluene under vacuum. The residue was then triturated twice with Et_2O giving crude peptides with C-terminal amides. Peptides were purified by preparative RP-HPLC, and purity (>95%; typically with 2-3% dimer present in Cys-containing peptides) was confirmed by analytical RP-HPLC (see Table S1 in supporting information), while the molecular masses of the peptides were confirmed by HRMS.

3.10. **PNA synthesis and solution-phase conjugation of PNA and peptides.**

The PNAs for solution-phase conjugation were synthesized on a CEM Liberty^{TM} MW peptide synthesizer by using MW-assisted Fmoc/Boc-based SPS (0.1 mmol scale) on an H-Rink-Amide ChemMatrix® resin (loading 0.52 mmol/g). Chain elongation was performed by using 0.2 M Fmoc-protected monomer building blocks (with Boc-protected nucleobases; 5 equiv) in DMF in combination with 0.5 M HBTU (5 equiv) as coupling reagent and 2 M DIPEA in NMP as activator base. Fmoc deprotection was performed with 20% piperidine in DMF at 45 °C (15 W for 60 sec followed by 15 W for 300 sec), while monomer building block couplings were conducted at 45 °C (15 W for 600 sec).

PNA-peptide conjugates via an SMCC NHS ester were obtained as previously described [7,57]. Conjugates with the polar Mal-eg1 linker were obtained as follows (adapted method from [58,59]): PNA oligomer (1.0 equiv) and the Mal-eg1 NHS ester (0.95 eq) were dissolved in 1.5 mL phosphate buffer (pH 7.4) and left stirring for two hours. The reaction mixture was checked by UHPLC to confirm conversion of the starting PNA into the adduct. The linker-modified PNA was transferred to a vial containing the Cys-modified peptide, and then the mixture was stirred for 16 h. The resulting conjugate was purified by preparative HPLC, and identity was verified by MALDI-TOF and HRMS.

3.11. **Continuous synthesis**.

The PNA-peptides without a linker or with an eg1 spacer were synthesized on a CEM Liberty^{TM} MW peptide synthesizer by using MW-assisted Fmoc-based SPPS on an H-Rink-Amide ChemMatrix® resin (loading 0.52 mmol/g, 0.1 mmol). Assembly of the PNA part was performed as described in section 3.10, followed by chain elongation with 0.2 M N^{α}-Fmoc-protected amino acid building blocks (with acid-labile tBu/Trt/Boc/Pbf as side-chain protecting groups; 5 equiv) in DMF in combination with 0.5 M HBTU (5 equiv) as coupling reagent and 2 M DIPEA in NMP as activator base. Fmoc deprotection was performed with 20% piperidine in DMF at 45°C (15 W for 60 sec followed by 15 W for 300 sec), while amino acid couplings were conducted at 45 °C (25 W for 600 sec). Couplings involving Arg or hindered amino acid building blocks (Val, Pro, Ile, Thr) as well as the subsequent elongations were performed as double couplings.

3.12. **MIC determinations**

MICs were evaluated by the modified Hancock lab protocol [76] in non-binding polystyrene microtitre plates, using bacterial suspensions of ~2 × 10^5 CFU/mL in Mueller-Hinton broth (Difco, Sparks MD, USA), with Mg^{2+} and Ca^{2+} concentrations of 4 mg/L each. The compounds were dissolved in H_2O and diluted in 0.01% acetic acid, 0.2% BSA (final concentration), and aliquots of 11 µL of 10× test compounds were transferred to the appropriate wells with bacterial suspensions. To prepare the compounds, low-binding sterile tubes and tips (Axygen, Union City CA, USA) were used. The MICs were read after 20 h incubation of covered plates at 35 °C (± 2 °C) with circular shaking 220 rpm. MDR strains and clinical isolates used in the MIC assays were obtained from the National Medicine Institute (NMI), Warzaw, Poland.

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Appendix A. Supplementary Material.

Supplementary data related to this article can be found at…

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Highlights

- Improved synthesis of Fmoc-protected PNA monomers with Boc-protected nucleobases
- Microwave-assisted assembly of Fmoc/Boc monomers into PNA-peptide conjugates
- Identification of delivery peptides enabling bacterial delivery of PNA oligomers
- Submicromolar minimal inhibitory concentrations in *E. coli* and *K. pneumoniae*
- Presence of linker/spacer and peptide stereochemistry affect antibacterial activity