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Activity and Predicted Nephrotoxicity of Synthetic Antibiotics Based on Polymyxin B

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ABSTRACT

The polymyxin lipodecapeptides colistin and polymyxin B have become last resort therapies for infections caused by highly drug-resistant Gram-negative bacteria. Unfortunately their utility is compromised by significant nephrotoxicity and polymyxin-resistant bacterial strains. We have conducted a systematic activity-toxicity investigation by varying eight of the nine polymyxin amino acid free side chains, preparing over thirty analogs using a novel solid-phase synthetic route. Compounds were tested against a panel of Gram-negative bacteria and counter-screened for *in vitro* cell toxicity. Promising compounds underwent additional testing against primary kidney cells isolated from human kidneys to better predict their nephrotoxic potential. Many of

the new compounds possessed equal or better antimicrobial potency compared to polymyxin B, and some were less toxic than polymyxin B and colistin against mammalian HepG2 cells and human primary kidney cells. These initial structure-activity and structure-toxicity studies set the stage for further improvements to the polymyxin class of antibiotics.

KEY WORDS: polymyxin, colistin, antimicrobial resistance, multidrug-resistant bacteria, antibiotics, nephrotoxicity

INTRODUCTION

Bacterial sepsis kills millions of people every year. In the United States, patients hospitalized for septicemia or sepsis are more than eight times as likely to die during their hospitalization compared to those hospitalized for other diagnoses,[1] with over 200,000 deaths in 2008.[2] Over one third of patients with sepsis treated in an intensive care unit die in the hospital.[3] Sepsis is the most expensive condition treated in U.S. hospitals, costing more than $20 billion in 2011.[4] Sepsis is most commonly caused by Gram-positive (G+ve) *Staphylococcus aureus* and *Streptococcus pyogenes* and Gram-negative (G-ve) Klebsiella spp., *Escherichia coli*, and *Pseudomonas aeruginosa*.[5] Unfortunately there has been an ominous rise in highly drug-resistant G-ve bacteria that are able to overcome almost every known antibiotic, threatening a return to a 'pre-antibiotic' era if new therapies are not discovered. The current clinical pipeline is mainly populated by G+ve drug candidates.[6] One possible approach to antibiotic development is to revisit antibiotics discovered in the 'golden age' of antibiotic discovery (the 1950-70s) to see if they can be optimized or improved.

In 1947 two American groups independently discovered antibiotic substances produced by *Bacillus polymyxa*.[7-9] The same year a group in England described a new antibiotic they named aerosporin isolated from a strain at first thought to be *B. aerosporus*, but later shown to be identical to the strain *B. polymyxa*.[10] Many examples of polymyxin derivatives have since been isolated from nature, all of them with various degrees of activity against bacteria.[11-15] A review by Stansly published in 1949 on early human clinical use of four distinct polymyxin antibiotic complexes A, B, C and D concluded that there is "little doubt that polymyxins have exhibited effective therapeutic activity in certain infections of man produced by Gram-negative bacteria. It is apparent, however that their application in this field is likely to be limited because of the occurrence of untoward reactions elicited by material used in the clinical trials."[16] Nonetheless, two of the polymyxins, Polymyxin B **1** and Polymyxin E (colistin) **2** entered clinical use, and in recent years have become last-resort antibiotics for multi-drug resistant G-ve infections, fueling a surge of interest in their use.[17-22] The main limitation of the polymyxins is nephrotoxicity, with adverse renal toxicity observed in over 50% of cases in some studies.[23,24] This has led to a systematic effort to improve dosing regimens.[25,26]

Polymyxins **1** and **2** are both positively charged macrocyclic peptide antibiotics composed of a heptapeptide ring and three amino acid exocyclic tail capped by a mixture of two fatty acids (see Figure 1), differing only in a D-Phe/D-Leu alteration at position-6. They are highly basic, containing six 2,4-diaminobutyric acid (Dab) residues, including one residue whose side chain is used for lactam formation with the C-terminal Thr residue. While Polymyxin B is administered as the parent compound sulfate salt, colistin is almost always used as a poly-methanesulfonylated prodrug, colistin methanesulfonate sodium, which spontaneously hydrolyses to release

Polymyxin E **2**. Given that the polymyxins are natural products produced by fermentation, their composition does vary considerably between manufacturers.[27]

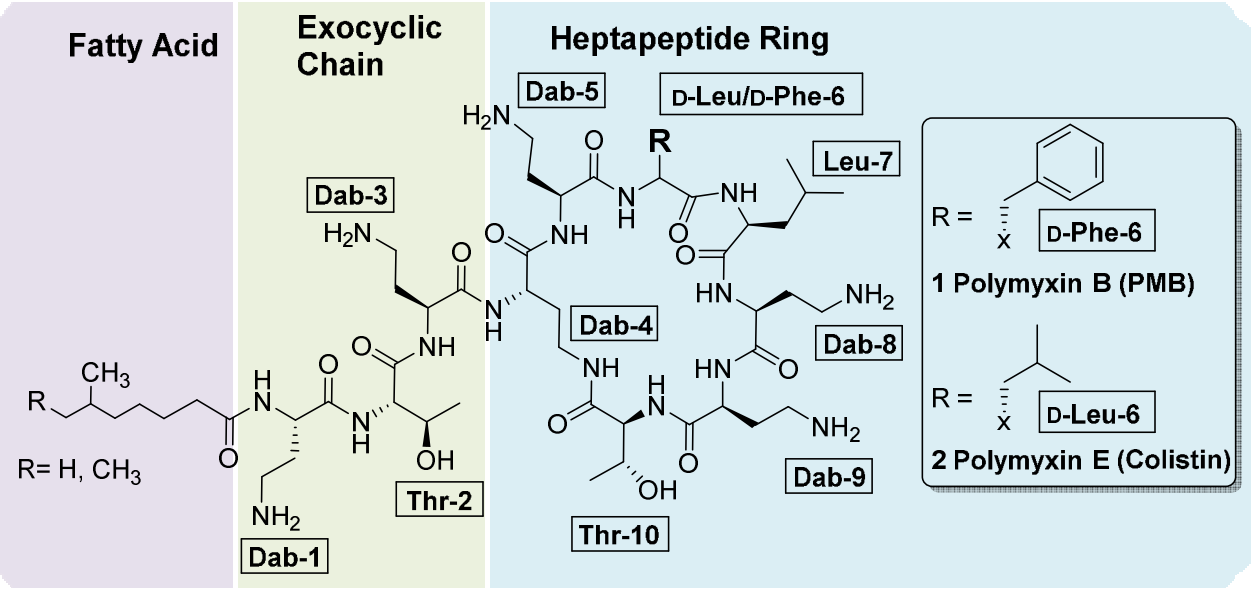


Figure 1. Structures of Polymyxin B 1 and Polymyxin E (Colistin) 2

The antimicrobial activity of the polymyxins is initiated by binding to Lipid A, the membrane-anchoring component of lipopolysaccharide (LPS) found in the outer membrane of G–ve bacteria.[17,28,29]. Displacement of membrane-stabilizing divalent cations and insertion of the fatty acyl chain expands and weakens the bacterial outer membrane, causing cell leakage and allowing polymyxin entry.[17,30,31] Once inside the cell, further modes of action have been postulated, including the generation of hydroxyl radical production[32] and inhibition of the respiratory enzyme NADH-quinone oxidoreductase.[33,34]

Unfortunately, resistance to the polymyxins is appearing and has been identified in most major pathogens, including *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*.[17] The main mechanisms of resistance involve alterations to LPS, in particular via addition of phosphoethanolamine and/or L-4-amino-arabinose to Lipid A, which lowers the binding affinity with the Dab residues of the polymyxins due to reduction of negative charge.[17,35,36] This increase in resistance, coupled with the growing importance of polymyxins in treating highly drug-resistant G-ve infections, has led to a number of efforts in recent years to prepare polymyxin analogs to understand structure-activity relationships, improve potency, reduce nephrotoxicity and overcome resistance. Despite their length of time in use, there have been relatively few structure-activity relationship (SAR) studies conducted to date compared to other classes of antibiotics, with most publications focusing on the readily modified exocyclic tail (Pfizer,[37-39] Novacta Biosystems Ltd/Cab-Tab Anti-Infectives Ltd,[40-42] Northern Antibiotics,[43-45] Sakura *et al.*[46]) or selective/nonselective modifications of the multiple Dab side chains (Northern Antibiotics,[43-45] Pfizer,[37-39,47] and Schering-Plough[48]). Biosource Pharm Inc/Cubist developed a method for fatty acid deacylation and linear tail amino acid hydrolysis, followed by reconstruction of the linear chain.[49-54] From this study, CB182,804[55] entered Phase I clinical trials, but development was discontinued in 2009. In one of the few studies to investigate changes within the polymyxin ring system, Li *et al.* at Monash University patented a set of polymyxin derivatives that retain activity against polymyxin-resistant strains; this group centered their efforts on the variation of hydrophobicity at positions 6 and 7 (numbering as in Figure 1).[56-58] Early efforts at polymyxin SAR were summarized by Storm *et al.* in 1977,[59] while more recent developments were reviewed by Velkov *et al.* in 2010[30] and Vaara in 2013.[60]

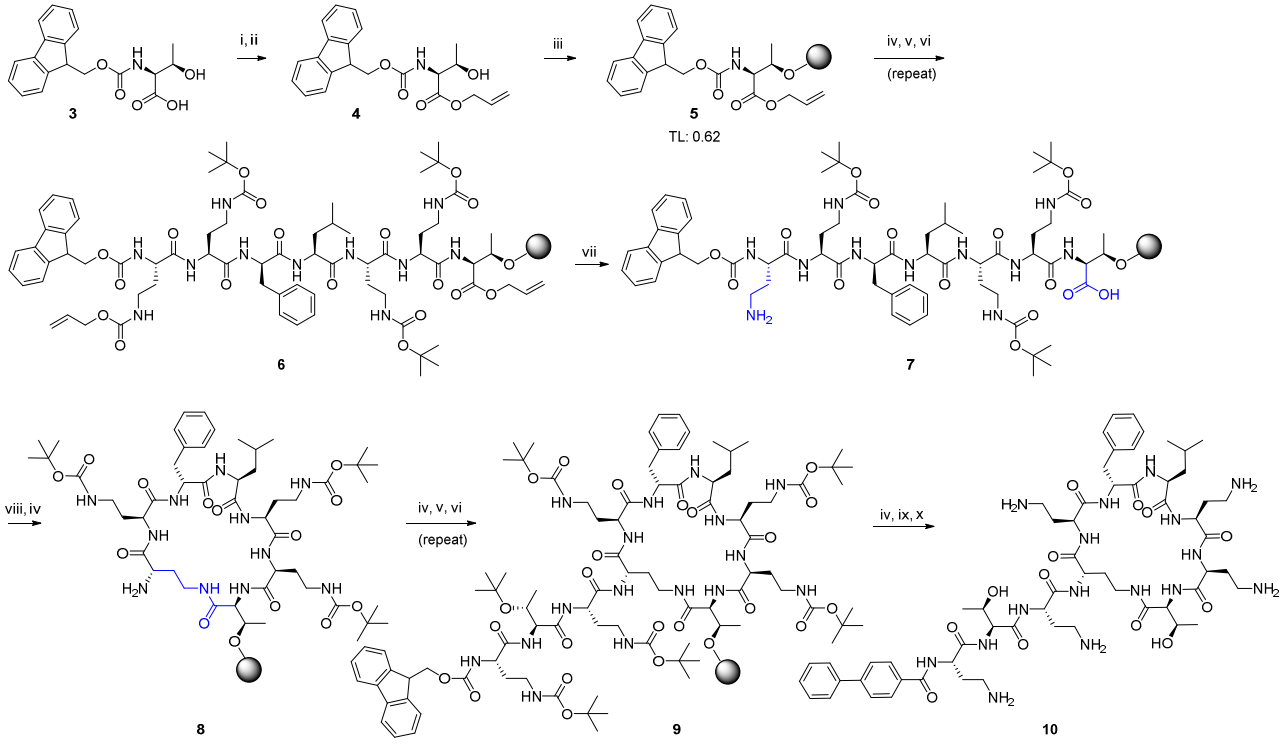
A set of novel polymyxin derivatives developed by Northern Antibiotics, with the exocyclic Dab-1 residue removed/substituted and the fatty acid tail altered, were found to be less toxic than polymyxin B[61] based on cytotoxicity testing in the porcine renal proximal tubular cell line LLC-PK1. It was proposed that this was due to a reduction in the number of positive charges coupled with alteration of hydrophobicity of the fatty acid. Based on these results, and the improved activity of the Monash compounds, we decided to investigate the effects of alterations at other positions within the polymyxins, with a particular focus on modifying the basic Dab residues contained within the ring to alter their basicity. Given that the Dab residues are proposed to be integral to binding to Lipid A, and that mutation of Lipid A to introduce phosphoethanolamine is a key polymyxin resistance mechanism, we also wanted to examine whether introducing zwitterionic residues or substituents capable of hydrogen-bonding at these positions might help overcome resistance.

Twenty-six new analogs were prepared and assayed. In addition, six key compounds from the literature were also prepared to provide a systematic SAR comparison assessed under common conditions. Some of the compounds retained better antimicrobial activity against polymyxin-resistant strains compared to polymyxin B and colistin . Most importantly, some of the most promising compounds showed less cytotoxicity than polymyxin B and colistin when tested in mammalian cell lines, and less toxicity than polymyxin B, colistin and gentamicin in a cytotoxicity assay potentially predictive of nephrotoxicity as measured by LDH and GGT release from human primary kidney cells.

RESULTS AND DISCUSSION

Chemistry. A total of 32 compounds were synthesized (six of these have been reported previously[46-48,53,54,57,58]) with the structures presented in Tables 1 and S1 (Supporting Information). All compounds possessed > 95% purity, as determined by LCMS analysis using both ELSD and UV (210 nm) detection. The synthetic routes towards the final compounds in this library are summarized in Schemes 1, 2 and S2 (Supporting Information). The first solid-phase total synthesis of polymyxin B1 was developed in 1999 by Sharma[62] using Fmoc-Thr(tBu)-SASRIN resin, with the linear peptide cleaved from the resin and cyclized in solution. In the current study we present a new synthetic route to the polymyxins employing an on-resin cyclization that allows for alteration of any substituent position other than Thr-10 (see Scheme 1 for a representative synthesis of **10**). The first step towards this library of compounds was the *O*-allyl ester protection of the C-terminal amino acid Fmoc-L-Thr-OH **3** (Thr10 in the final product). The side-chain alcohol of the protected amino acid **4** was then loaded onto a dihydropyran DHP HM resin (3,4-dihydro-2H-pyran-2-yl-methoxymethyl polystyrene). The peptide sequence was constructed by standard solid-phase peptide synthesis (SPPS) using sequential Fmoc deprotection followed by amino acid attachment using a systematic double coupling protocol, including a capping step with acetic anhydride and pyridine (50:50 v/v) to avoid the formation of deletion products due to unreacted free amino groups. On-resin cyclization was conducted following incorporation of the Fmoc-Dab(Alloc) residue by *in situ* deprotection of both Thr10 allyl ester and the Dab-4 side chain amine N^{γ}-Alloc protecting groups (Scheme 1, step vii), followed by overnight cyclization using DPPA and DIPEA in DMF at room temperature. Fmoc deprotection of the cyclized product then allowed completion of the exocyclic linear tail by further amino acid couplings. Following each step, an analytical sample of resin

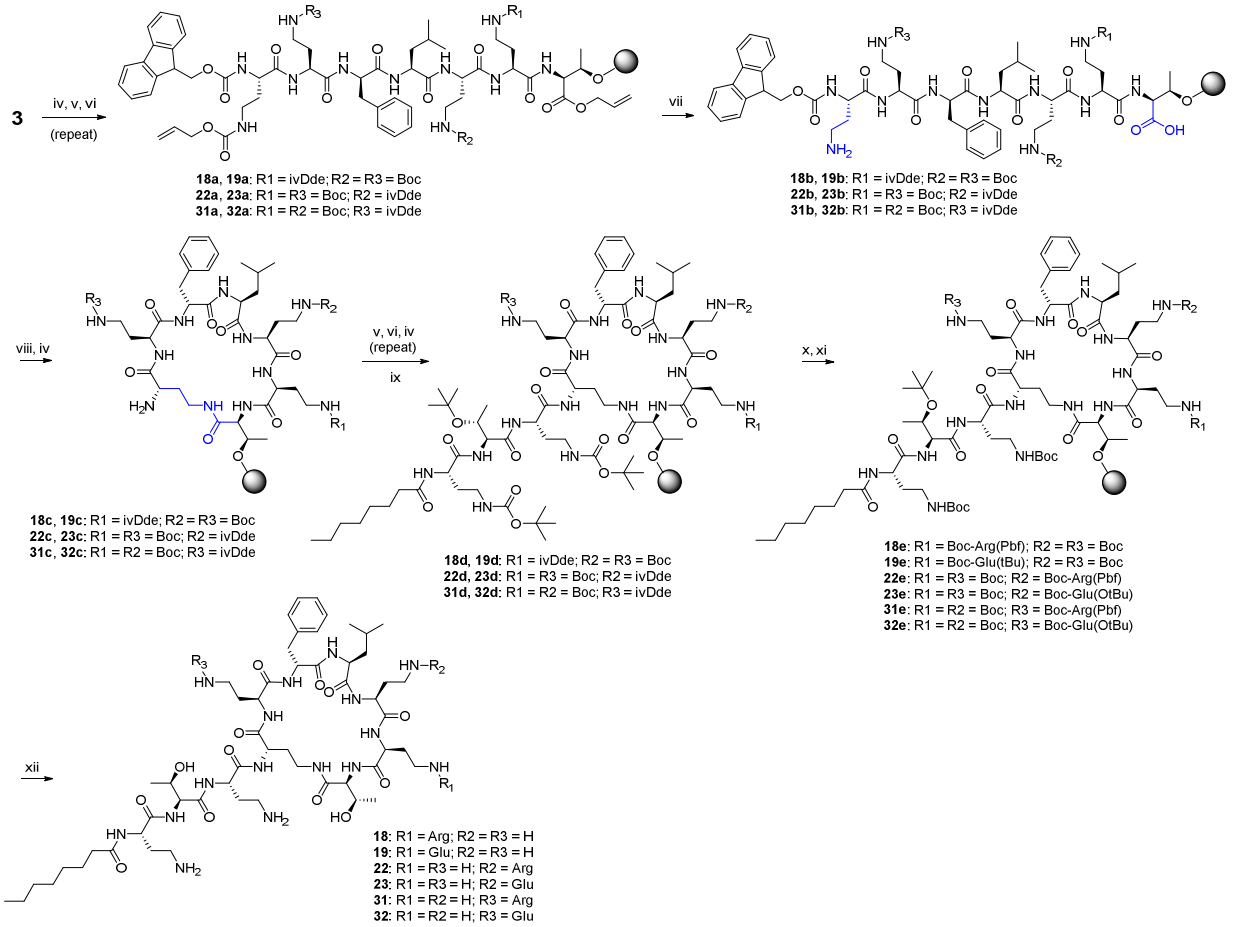
was cleaved with TFA/Et_3SiH/H_2O (95:1:4) followed by LCMS analysis to confirm the coupling with the corresponding amino acid. The final coupling capped the peptides with the appropriate fatty acid. Cleavage of the cyclic decapeptides from the resin with concomitant side chain deprotection was achieved with the addition of a solution of TFA/Et_3SiH/H_2O (95:1:4) for 30 min at room temperature, providing crude compounds that were then HPLC-purified to afford the final polymyxin analog TFA salts as white powders; total yields were generally between 5-30% based on the initial resin loading.



Scheme 1. General on-resin cyclization synthetic route exemplified by synthesis of 10. Reagent and Conditions: (i) Cs_2CO_3, MeOH, H_2O, pH 7-8; (ii) Allyl bromide, dry DMF; (iii) DHP polystyrene resin, PPTS, dry DCE; (iv) 30% piperidine, DMF; (v) Solid-phase peptide synthesis (SPPS) with corresponding amino acid (Table S3), HCTU, DIPEA (vi) Acetic

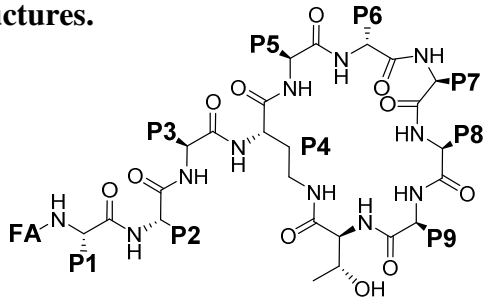
anhydride, pyridine; (vii) $Pd(PPh_3)$, $PhSiH_3$; (viii) DPPA, DIPEA, DMF; (ix) 4-Ph-$PhCO_2H$, HCTU, DIPEA; (x) TFA/Et_3SiH/H_2O (95:1:4).

For some syntheses, an orthogonal protecting group was employed for one of the multiple Dab residues, allowing for selective modification of individual Dab side chains. Thus, side-chain ivDde-protected Dab was used at positions P5, P8 or P9 (see Scheme 2 for generic route; Scheme S1 for explicit synthesis of **18** and **19**). The P4 Alloc-protected Dab was deprotected and used for cyclization as before, with the fully protected resin-bound peptide treated with 2% hydrazine to selectively unmask the desired Dab residue. This was then derivatized by coupling with Boc-Arg(Pbf)-OH or Boc-Glu(OtBu)-OH, with global deprotection and purification as previously described.



Scheme 2. General on-resin cyclization synthetic route with selective Dab modification used for synthesis of compounds 18, 19, 22, 23, 31 and 32. Reagent and Conditions: (iv) 30% piperidine, DMF; (v) Solid-phase peptide synthesis (SPPS) with corresponding amino acid (Table S3), HCTU, DIPEA (vi) Acetic anhydride, pyridine; (vii) $Pd(PPh_3)$, $PhSiH_3$; (viii) DPPA, DIPEA, DMF; (ix) *n*-C_7CO, HCTU, DIPEA; (x) 2% H_2NNH_2 in DMF; (xi) peptide coupling with corresponding amino acid: Boc-L-Arg(Pbf)-OH or Boc-L-Glu(OtBu)-OH, HCTU, DIPEA; (xii) TFA/Et_3SiH/H_2O (95:1:4).

Table 1. Compound Structures.



Cmpd	FA	P1	P2	P3	P4	P5	P6	P7	P8	P9
1 Pmx-B	6Me-C7CO/ 6Me-C6CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab
2 Colistin	6Me-C7CO/ 6Me-C6CO	Dab	Thr	Dab		Dab	**D-Leu**	Leu	Dab	Dab
11[a]	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab
12[b]	nC7CO	-	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab
13[c]	2-ClPhNHCO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab
10	Ph-4-PhCO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab
14	PhO-4-PhCO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab
15[d]	4-Ph-PhNHCO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab
16	2-ClPhNHCO	Dab	Thr	**D-Dab**		Dab	**D-Phe**	Leu	Dab	Dab
17	2-ClPhNHCO	Dab	Thr	**D-Ser**		Dab	**D-Phe**	Leu	Dab	Dab
18[e]	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab(Arg)
19	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Dab(Glu)
20	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	$4NH_2$Phe
21	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab	Arg
22	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab(Arg)	Dab
23	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Dab(Glu)	Dab
24	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	Hser	Dab
25	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Leu	$4NH_2$Phe	Dab
26	nC7CO	Dab	Thr	Dab	Dab	Dab	**D-Phe**	Leu	Arg	Dab
27	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	Bip	Dab	Dab
28[f]	nC7CO	Dab	Thr	Dab		Dab	**D-Phe**	DL-OctGly	Dab	Dab
29	nC7CO	Dab	Thr	Dab		Dab	**D-Bip**	Leu	Dab	Dab
30[g]	nC7CO	Dab	Thr	Dab		Dab	DL-OctGly	Leu	Dab	Dab
31	nC7CO	Dab	Thr	Dab		Dab(Arg)	**D-Phe**	Leu	Dab	Dab
32	nC7CO	Dab	Thr	Dab		Dab(Glu)	**D-Phe**	Leu	Dab	Dab
33	nC7CO	Dab	Thr	Dab		Hser	**D-Phe**	Leu	Dab	Dab
34	nC7CO	Dab	Thr	Dab		$4NH_2$Phe	**D-Phe**	Leu	Dab	Dab
35	nC7CO	Dab	Thr	Dab		Arg	**D-Phe**	Leu	Dab	Dab
36	nC7CO	Dab	Thr	**D-Dab**		Dab	**D-Phe**	Leu	Dab	Dab
37	nC7CO	Dab	Thr	**D-Ser**		Dab	**D-Phe**	Leu	Dab	Dab
38	nC7CO	Dab	Thr	Gly		Dab	**D-Phe**	Leu	Dab	Dab
39	nC7CO	-	Thr	Gly		Dab	**D-Phe**	Leu	Dab	Dab
40	nC7CO	**D-Dab**	Thr	Gly		Dab	**D-Phe**	Leu	Dab	Dab
41	nC7CO	Gly	Dab	Thr		Dab	**D-Phe**	Leu	Dab	Dab

All amino acids are L-isomers unless otherwise indicated. [a]**11** = Polymyxin B3. [b]**12** = compound 5 in O'Dowd *et al.*[47] [c] **13** = compound 5 in Leese *et al.*[54] [d] **15** = compound 18 in Leese *et al.*[53] [e] **18** is similar to compound 24 (retains PmxB mixture of fatty acids) in Weinstein *et al.*[48] [f] **28** =

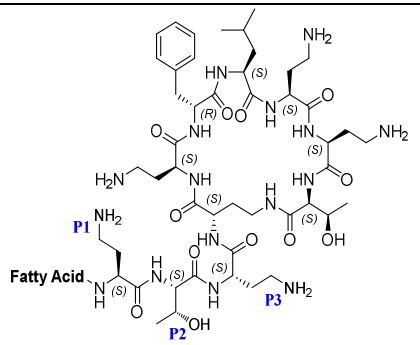
DL- OctGly version of L-Ade compound 2 in Li *et al.*[57] [g] **30** = DL-OctGly version of D-Ade compound 1 in Li *et al.*[58]

Biological activity. The 32 compounds, along with vancomycin, gentamicin, colistin and polymyxin B, were assessed against a panel of predominantly G-ve bacteria, with *S. aureus* used as a representative G+ve strain. Five antibiotic-sensitive and resistant ATCC reference strains covering the G-ve ESKAPE pathogens (*E. coli, K. pneumoniae, A. baumannii, P. aeruginosa)* were used, along with polymyxin-resistant clinical isolates of *K. pneumoniae, A. baumannii,* and *P. aeruginosa* (Table S2). Notably, compounds that were resyntheses of those previously reported demonstrated similar activity to the earlier literature values (footnotes in Tables 2,3). Counter-screening for general cytotoxicity was conducted using human liver hepatocellular carcinoma cells (HepG2). Our previous studies have demonstrated that cytotoxicity measurements against these immortalized cells were not predictive of nephrotoxicity,[63,64] but they do provide an indication of potential general cell toxicity.

Alterations to the fatty acid tail (Table 2) had minimal effect on activity against the reference strains, but biphenyl or biphenyl ether substituents (**10**, **14** and **15**) did promote improved activity against polymyxin-resistant strains, albeit accompanied by greater cytotoxicity. In the linear exocyclic peptide tail (Table 2), replacement of Dab-3 with D-Dab (**36**, **16**) or Gly (**38**) had minimal effect. Analog **38** is particularly interesting since it retained MIC activity among the reference strains (0.5-2 μg/mL for most strains) with low cytotoxicity seen in HepG2 cells (CC_{50} > 300 μM). This is the only polymyxin B example where Dab-3 has been replaced by a nonbasic amino acid, where the amino acid replacement also potentially impacts on the final structural geometry. The effects of substitution of Dab-3 with D-Ser depended on the fatty acid; with the heptanoic acid **37** activity was slightly decreased (vs **11**), but with *o*-chlorophenylurea **17** most activity was lost. The inversion of L-Dab-1 to D-Dab-1 in **40** abrogated most activity, a much larger effect than the inversion use of L-Dab-3 to D-Dab-3 in **36/16**. Finally, replacing the linear

chain Dab-Thr-Dab sequence with Gly-Dab-Thr in **41** was also detrimental to antibacterial activity.

Table 2. Summary of MIC against a panel of sensitive and resistant bacteria and cytotoxicity (CC_{50}) against HepG2 cells for a set of compounds with diversity in the exocyclic chain and fatty acid tail.



			E. coli ATCC 25922	*K. pneumoniae* ATCC 13883	*K. pneumoniae* ATCC 700603, MDR	*K. pneumoniae* ATTC BAA-2146, NDM-1 pos	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 27853	*P. aeruginosa* FADDI-PA070, PmxR	*P. aeruginosa* PA9704, PmxR	*A. baumannii* CI Ptyela 100734512:2, PmxR	*K. pneumoniae* CI Koprana 100650661:1, PmxR	*K. pneumoniae* CI 138-16357-20362, PmxR	*S. aureus* ATCC 25923, MSSA	HepG2 - CC_{50} (µM)
Controls	Vancomycin		>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	2	>300
	Gentamicin		0.25	0.25	8	>32	32	1	32	>32	>32	>32	1	0.5	>300
	Colistin **2**		0.25	0.25	0.25	0.25	0.25	0.5	>32	128	>64	>128	64	>128	>300
	Polymyxin B **1**		1	1	0.5	0.25	0.25	0.5	32	128	64	64	64	>64	>300
Sample	**Fatty Acid**	**Position**													
11[a]	nC7CO		0.5	2	1	0.5	0.5	1	>32	>128	>64	>128	>128	>128	>300
12[b]	nC7CO	P1=None	1	1	2	2	2	0.5	>32	>32	>32	>32	>32	>32	>300
13[c]	2-ClPhNHCO		1	2	2	4	1	0.5	>32	>32	>32	>32	>32	>32	>300
10	Ph-4-PhCO		0.5	0.5	1	0.5	0.5	1	8	>32	8	>32	>32	>32	298
14	PhO-4-PhCO		0.5	0.5	0.5	0.5	0.5	1	8	32	16	>32	>32	>32	287
15[d]	Ph-4-PhNHCO		2	2	2	1	1	1	16	8	16	16	>32	32	316
16	2-ClPhNHCO	P3=D-Dab	1	0.5	0.5	1	2	0.5	16	>32	>32	>32	>32	>32	>300
17	2-ClPhNHCO	P3=D-Ser	32	32	16	>32	>32	8	>32	>32	>32	>32	>32	>32	>300
41	nC7CO	P1=Gly, P2=Dab; P3=Thr	16	32	8	16	>32	2	>32	>32	>32	>32	>32	>32	>300
40	nC7CO	P1=D-Dab, P3=Gly	>32	>32	8	32	>64	16	>32	>32	>32	>32	>32	>64	>300
38	nC7CO	P3=Gly	0.5	0.5	1	0.5	1	2	>32	>32	>32	>32	>32	>64	>300
39	nC7CO	P1=None, P3=Gly	4	>32	>32	>32	>32	16	>32	>32	>32	>32	>32	>32	>300
36	nC7CO	P3=D-Dab	4	0.5	1	4	1	2	>32	32	>32	>32	>32	>32	>300
37	nC7CO	P3=D-Ser	4	0.5	2	16	1	2	>32	>32	>32	>32	>32	>32	>300

All amino acids are L-isomers unless otherwise indicated.

ND: Not determined. PmxR: Polymyxin resistant. CI: Clinical isolate. MSSA: Methicillin resistant *S. aureus*. MICs were determined visually after 18 hours of incubation at 37°C, with the MIC defined as the lowest compound concentration at which no bacterial growth was visible. MIC in μg/mL. 2-Chlorophenyl carbamoyl (2-ClPhNHCO), 4-biphenyl-benzoyl (Ph-4-PhCO), 4-phenoxybenzoyl (PhO4-PhCO), 4-biphenyl carbamoyl (Ph-4-PhNHCO), octanoyl (nC7CO)**.**

[a]**11** is Polymyxin B3. Literature MIC *E. coli* = 0.5 and *P. aeruginosa* = 1.0 nmol/mL. *E. coli* IFO12734, and *P. aeruginosa* IFO3080 from the Institute for Fermentation, Osaka, Japan.[46]

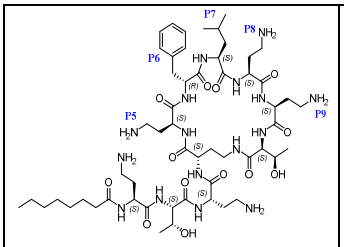
[b]**12** is compound 5 in O'Dowd *et al.*; MIC *E. coli* = 0.03, *P. aeruginosa* = 4, *S. aureus* = >16 ug/mL.[47]

[c]**13** is compound 5 in Leese *et al.*; MIC90 *P. aeruginosa* (100 strains) = 2, *A. baumannii* (81 strains) = 4, *E.coli* (80 strains) = 2, *K. pneumoniae* (81 strains) = 4 μg/mL.[54]

[d]**15** is compound 18 in Leese *et al.*; MIC *E. coli* = 2.5, *P. aeruginosa ATCC 27853* = 2.5 μg/mL; MIC values were determined by serial twofold broth dilution method.[53]

For alterations within the heptapeptide ring system (Table 3), replacement of D-Phe/D-Leu-6 or Leu-7 with the more lipophilic residues DL-Ade or Bip/D-Bip (**27-30**) replicated the results of Velkov *et al.*,[56] where these substitutions restored activity against polymyxin-resistant strains. However, these changes also caused increased cytotoxicity against HepG2 cell lines. The effects of altering the ring Dab residues varied significantly depending on which Dab was altered. Replacement of Dab-5 with homoserine (Hse), Arg, or 4′-amino-Phe (**33-35**), or acylation with Arg or Glu (**31, 32**), almost completely abolished activity (16 - > 32 μg/mL against all tested strains). The same modifications at Dab-9 (**18-21**) gave more variable results, retaining moderate levels of activity (4-16 μg/mL for most strains). The Dab-8 position was most tolerant of alterations, with even neutral Hse **24** and zwitterionic Dab(Glu) **23** retaining good activity (1-8 μg/mL for most strains). Unfortunately, the hypothesis that a zwitterionic substituent might overcome Lipid A resistance modifications was not correct, with all of the Dab(Glu) substitutions inactive against the polymyxin-resistant strains. The Dab-8 replacement with Arg-8 **26** (Table 3) had the best activity of all the analogs made, and showed some activity against the polymyxin-resistant strains. While not as potent as the Arg-8 **26** analog, the 4′-amino-Phe-8 analog **25** also showed good activity, this is an interesting result due to the aromatic nature of the replacement for the otherwise aliphatic amino acid Dab. Further investigation to elaborate on the SAR trends observed with these type of compounds is in progress in our laboratory.

Table 3. Summary of MIC against a panel of sensitive and resistant bacteria and cytotoxicity (CC_{50}) against HepG2 cells for a set of compounds with diversity in the heptapeptide ring.

		*E. coli* ATCC 25922	*K. pneumoniae* ATCC 13883	*K. pneumoniae* ATCC 700603, MDR	*K. pneumoniae* ATTC BAA-2146, NDM-1 pos	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 27853	*P. aeruginosa* FADDI-PA070, PmxR	*P. aeruginosa* PA9704, PmxR	*A. baumannii* CI Ptyela 100734512-2, PmxR	*K. pneumoniae* CI Koprana 100650661-1, PmxR	*K. pneumoniae* CI 138-16357-20362, PmxR	*S. aureus* ATCC 25923, MSSA	HepG2 - CC_{50} (µM)
Controls	Vancomycin	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	2	>300
	Gentamicin	0.5	0.25	8	>32	32	1	32	>32	>32	>32	1	0.5	>300
	Colistin **2**	0.25	0.25	0.25	0.25	0.25	0.5	>32	128	>64	>128	64	>128	>300
	Polymyxin B **1**	0.25	1	0.5	0.25	0.25	0.5	32	128	64	64	64	>64	>300
Sample	**Position**													
31	P5=Dab(Arg)	32	32	16	16	32	32	>32	>32	>32	>32	>32	>32	>300
32	P5=Dab(Glu)	>32	>32	>32	32	>32	>32	>32	>32	>32	>32	>32	>32	>300
33	P5=Hser	16	>32	>32	32	>32	>32	>32	>32	>32	>32	>32	>32	>300
34	P5=4-NH_2-Phe	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>300
35	P5=Arg	16	>32	32	32	>32	32	>32	>32	>32	>32	>32	>32	>300
29	P6=D-Bip	1	4	2	1	0.5	1	16	16	8	32	32	8	119
30[a]	P6=DL-OctGly	2	2(4)	1	1	1(2)	2(1)	4	8	16	16	16	16	254
27	P7=Bip	1	4	2	1	1	2	16	16	32	32	>32	8	117
28[b]	P7= DL-OctGly	1	2(1)	2	1	1(1)	2(4)	16	8	8	32	32	8	116
22	P8=Dab(Arg)	0.5	4	0.5	1	4	2	>32	>32	>32	>32	>32	>32	ND
23	P8=Dab(Glu)	1	8	4	4	4	8	>32	>32	>32	>32	>32	>32	>300
24	P8=Hser	1	>32	2	2	8	4	>32	>32	>32	>32	>32	>32	>300
25	P8=4-NH_2-Phe	0.5	8	1	2	4	8	32	>32	>32	>32	>32	>32	ND
26	P8=Arg	0.25	4	0.5	0.5	0.5	1	32	>32	32	>32	>32	>32	ND
18[c]	P9=Dab(Arg)	32	32	8	8	16	32	>32	>32	>32	>32	>32	>32	>300
19	P9=Dab(Glu)	4	16	4	4	16	8	>32	>32	>32	>32	>32	>32	>300
20	P9=4-NH_2-Phe	16	>32	16	16	>32	32	>32	>32	>32	>32	>32	>32	>300
21	P9=Arg	16	>32	8	4	32	1	>32	>32	>32	>32	>32	>32	>300

All amino acids are L-isomers unless otherwise indicated. ND: Not determined. PmxR: Polymyxin resistant. CI: Clinical isolate. MSSA: Methicillin resistant *S. aureus*. MICs were determined visually after 18 hours of incubation at 37°C, with the MIC defined as the lowest compound concentration at which no bacterial growth was visible. MIC in µg/mL. 2-Chlorophenyl carbamoyl (2-ClPhNHCO), 4-biphenyl-benzoyl (Ph-4-PhCO), 4-phenoxybenzoyl (PhO4-PhCO), 4-biphenyl carbamoyl (Ph-4-PhNHCO), octanoyl (nC7CO)**.**

[a] values are reported MIC for compound 2 = L-Ade version of **28** in Li *et al.* WO2010/130007[57]

[b] values are reported MIC for compound 1 = D-Ade version of **30** in Li *et al.* WO2012/051663[58]

[c] **18** similar to compound 24 (retains polymyxin B fatty acid) in Weinstein *et al.*; MIC *E. coli* = 0.16, *P. aeruginosa* = 0.03, *S. aureus* = 2 µg/mL[48]

Nephrotoxicity. Traditional cytotoxicity assays have been confirmed as improper indicators for nephrotoxicity screening.[63,64] Therefore, in addition to general cytotoxicity screening assessed using HepG2 cells, we selected four compounds with promising antimicrobial potency and reasonable cytotoxicity against the HepG2 cell lines for further assessment for toxicity using primary kidney cells freshly isolated from human kidneys (see Table 4). This assay, measuring LDH and GGT release, has been reported to be a better *in vitro* model for predicating *in vivo* nephrotoxicity.[63-65] We also assessed cytotoxicity against immortalized HEK293 cells. Colistin, polymyxin B and gentamicin were employed as control nephrotoxic compounds, and clearly showed the greatest toxicity in both primary renal cell assay readouts (Figures 2A and 2B), in contrast to their apparent lack of cytotoxicity in the HepG2/HEK293 assays. Compounds **10** and **14**, with non-alkyl fatty acids, had shown greater cytotoxicity than polymyxin B and colistin, but induced almost no release of LDH or GGT at 100 μg/mL in the primary kidney cells. Compound **38** was the least cytotoxic (HepG2/HEK293 assays) of the four new analogs tested, but possessed intermediate levels of "nephrotoxicity" against primary kidney cells, as did **15** which had similar cytotoxicity against HepG2 cells as **10** and **14**. The results support our previous assessment of *in vitro* predictors of nephrotoxicity, in that there is little correlation between general cytotoxicity measurements and the human kidney results.[63,64]

Table 4. Summary of MIC, cytotoxicity CC_{50} data (HepG2 and HEK293 cell lines) and nephrotoxicity CC_{50} data measuring LDH **(A)** and GGT **(B)** release from primary human kidney cells using lead compounds (**10**, 14, **15** and **38**) compared to control compounds (colistin, polymyxin B and gentamicin).

Sample	*K. pneumoniae ATCC 13883*	*K. pneumoniae ATCC 700603, MDR*	*K. pneumoniae ATTC BAA-2146, NDM-1 pos*	HepG2 - CC_{50} (μM)	HEK293 - CC_{50} (μM)	LDH release CC_{50} (□g/mL)	GGT release CC_{50} (□g/mL)
Gentamicin	0.25	8	>32	>300	>300	33.5	32.6
1 Polymyxin B	1	0.5	0.25	>300	>300	23.2	176.6
2 Colistin	0.25	0.25	0.25	>300	>300	25.4	48.5
10	0.5	1	0.5	298	297	>128	>128
14	0.5	0.5	0.5	287	296	>128	>128
15	2	2	1	316	>300	>128	>128
38	0.5	1	0.5	>300	>300	122.0	>128

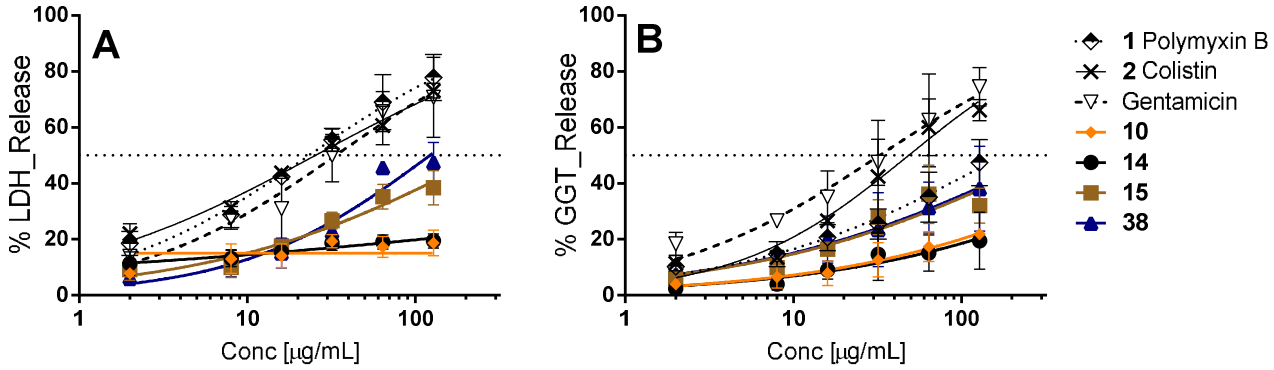


Figure 2. *In vitro* nephrotoxicity studies measuring LDH **(A)** and GGT **(B)** release from primary human kidney cells using lead compounds (**10**, **14**, **15** and **38**) compared to control compounds (colistin, polymyxin B and gentamicin).

Conclusions. A new solid-phase synthesis of the polymyxin class of antibiotics has been developed based on attachment to the resin through the side chain of Thr-10, allowing for on-resin cyclization, variation at any other position, and selective derivatization of the side chains of individual Dab residues. Thirty-two analogs were prepared and tested against a panel of reference and clinical isolates, including polymyxin-resistant MDR strains. Cytotoxicity screening was performed against the HepG2 cell line. The results confirmed that more lipophilic groups at P6/P7 improved activity against polymyxin-resistant strains but increased cytotoxicity against mammalian cells. Similar effects were observed by replacement of the alkyl fatty acid with biaryl substituents. Further testing of these compounds by assessing cytotoxicity in primary human renal cells as a potential surrogate for nephrotoxicity conversely found reduced toxicity compared to the increased general cytotoxicity seen in an immortalized cell line. Large differences were found when varying the five free side-chain Dab residues: Dab-5 could not be replaced or altered without loss of activity, while alterations at Dab-9 also severely affected potency, though to a slightly less extent. In contrast Dab-8 tolerated a range of basic, neutral or zwitterionic replacements. Similarly, Dab-3 could be replaced by non-basic Gly or Ser with minimal effects, while inversion of the configuration of Dab-1 abolished activity. Further studies of this class of compounds will investigate combinations of promising substituent alterations, particularly those that reduce the overall basic character, improve activity against polymyxin-resistant bacteria, and demonstrate reduced potential for nephrotoxicity in human kidney cells.

EXPERIMENTAL SECTION

Synthetic Material and Method. Experimental procedures are described in the Supporting Information. All chemicals were obtained from commercial suppliers and used without further purification. LC-MS analysis were conducted using Agilent Technologies 1200 Series Instrument with a G1316A variable wavelength detector set at λ= 210 nm, 1200 Series ELSD, 6110 quadrupole ESI-MS, using an Agilent Eclipse XDB-Phenyl (3 × 100mm, 3.5 μm particle size, flow rate 1 mL/min, the mobile phases 0.05% formic acid in water and 0.05% formic acid in acetonitrile). Compound purification was done using a Agilent 1260 Infinity Preparative HPLC with a G1365D multiple wavelength detector set at λ= 210 nm and an Agilent Eclipse XDB-Phenyl column 21.2 x 100mm, 5 μm particle size. Ultraviolet/visible spectra were recorded with a Varian Cary 50 Bio spectrophotometer. Identities of final products were confirmed by MS/MS spectra, obtained using an API QSTAR™ Pulsar Hybrid LC-MS/MS System, high resolution mass spectrometry (HRMS), performed on a Bruker Micro TOF mass spectrometer using (+)-ESI calibrated to sodium formate, and by ^{1}H (600 MHz) and 2D NMR spectra, obtained using a Bruker Avance-600 spectrometer equipped with a TXI cryoprobe in D_2O, referenced externally with NaOAc (δ_H 1.90 and 8.44; 10 mg in 500 μL D_2O) and then internally with the HDO resonance at δ 4.77.[66] Final purity of more than 95% for all compounds was confirmed by LCMS analysis using both ELSD and UV (210 nm) detection.

Minimum Inhibitory Concentration (MIC) determination. Bacteria were either obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) or independent academic clinical isolate collections, as listed in Table S2. Bacteria were cultured in Nutrient broth (NB; Bacto Laboratories, catalog No. 234000) or Muller Hinton broth (MHB; Bacto Laboratories, catalog No. 211443) at 37 °C overnight with shaking (~180 rpm). A sample of each culture was then diluted 50-

fold in fresh MHB and incubated at 37 °C for 1.5-3 h with shaking (~180 rpm). Compound stock solutions were prepared as 0.64 mg/mL or 2.56 mg/mL in water. The compounds, at twice the final desired concentration, were serially diluted two-fold across the wells of 96-well plates (Polystyrene, Corning, catalogue No. 3370). Mid-log phase bacterial cultures (after 1.5-3 h incubation) were diluted to a final concentration of 5×10^5 colony forming units (CFU)/mL, and 50 μL was added to each well giving a final compound concentration range of 32 μg/mL to 0.015 μg/mL (128-0.06 μg/mL for colistin and polymyxin B against the polymyxin resistant clinical isolates). MICs were determined visually after 18 h of incubation at 37 °C, with the MIC defined as the lowest compound concentration at which no bacterial growth was visible.

Cytotoxicity Assay (Resazurin assay). Cytotoxicity to HEK293 and HepG2 cells was determined using the resazurin assay.[67,68] In brief, HEK293 and HepG2 cells were seeded as 4000 cells/well in black-wall, clear-bottom 384-well plates (Corning, Australia) and incubated for 24 h at 37°C, 5% CO_2. Compounds were then added into each well. After 24 h incubation, 5 μM resazurin were added per well and incubated at 37 °C for 2 h. Fluorescence intensity was then read using a Polarstar Omega with excitation/emission 560/590. The data were then analyzed using GraphPad Prism 6 software. CC_{50} values were calculated using the following equation: Percentage Viability = $(FI_{TEST} - FI_{Negative}/FI_{UNTREATED} - FI_{Negative})*100$.

Nephrotoxicity Assay (Lactate Dehydrogenase (LDH) and Gamma Glutamyl Transferase (GGT) release in primary cells freshly isolated from human kidneys). Primary human renal proximal tubule epithelial (hPT) cells were isolated and used for *in vitro* prediction of drug-induced nephrotoxicity[63,64,69] by measuring the release of metabolism enzymes, LDH and GGT as previously described.[70]

ANCILLARY INFORMATION

Supporting Information: Tables of structures, characterization data and bacterial strain descriptions, detailed synthetic procedures and detailed characterization of four representative compounds.

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Abbreviations Used: ATCC, American Type Culture Collection; Dab 2,3-diaminobutyric acid; DIPEA, diisopropylethylamine; DMF, dimethylformamide; *E. coli*, *Escherichia coli*; GGT, γ-glutamyltransferase; G-ve, Gram-negative; G+ve, Gram-positive; HCTU, (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate); HepG2, human liver hepatocellular carcinoma cells; HEK293, human embryonic kidney 293; HK2, human kidney proximal tubule epithelial cell line; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl; LDH, lactate dehydrogenase; *P. aeruginosa*, *Pseudomonas aeruginosa*; *S. aureus*, *Staphylococcus aureus*; *S. pyogenes*, *Streptococcus pyogenes*; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid.

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