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Structure–activity relationships of bivalent aminoglycosides and evaluation of their microbiological activities

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Abstract—A library of 4,5- and 4,6-linked bivalent aminoglycoside (AMG) antibiotics consisting of neamine and nebramine pharmacophores have been synthesized. We probed the effect of the linker on antibiotic activity with a series of selected synthetic analogues with varied length and substituents. A number of compounds demonstrated in vitro activity against several bacterial strains and showed activity against drug resistant strains of *Pseudomonas aeruginosa*. Among the compounds prepared, analogues **12a–d** were novel 4,6-linked AMGs containing the nebramine pharmacophore. In addition the lead compound OPT-11 possessed an ED_{50} of $\leqslant 5$ mg/kg in a *Staphylococcus aureus* ATCC 29213 mouse protection model.
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1. Introduction

Nosocomial infections of *P. aeruginosa* are associated with high mortality and morbidity; in addition, *P. aeruginosa* is the primary pathogen associated with the genetic disease cystic fibrosis (CF).[1,2] Treatment options for infections caused by *P. aeruginosa* are limited in comparison to many Gram-positive infections due to the organism's intrinsic resistance to antibiotics. Aminoglycosides (AMGs) are particularly active against several Gram-negative pathogens and are commonly used to treat severe *P. aeruginosa* infections. However, resistance to this important class of antibiotics as well as other antipseudomonals is growing. Recently, a new class of linked AMGs was reported to have good antimicrobial activity against AMG sensitive strains of *S. aureus* and *P. aeruginosa*.[3] The linked AMGs consist of the 2-deoxystreptamine (2-DOS) pharmacophore connected through a diamine linker and are believed to interact with the target 16S rRNA in a bivalent fashion, as shown in Figure 1A. Furthermore, these novel AMGs were found to be nanomolar inhibitors of the APH(2″) activity of the AMG modifying-enzyme AAC(6′)–APH(2″) making the linked AMGs 'bifunctional' as well.

OPT-11, shown in Figure 1B, is a bivalent AMG with potent antibacterial activity.[3] The neamine motif present in OPT-11 has been shown to complex the 16S rRNA model AS–wt with a unique 2:1 binding stoichiometry in contrast to other naturally occurring AMGs, which bind AS–wt in a 1:1 fashion. Tethering neamine with an appropriate linker resulted in OPT-11, which reverted to 1:1 binding and showed a 250-fold improvement in K_d over monomeric neamine and showed a significant increase in antimicrobial activity compared to neamine. Figure 2 illustrates a theoretical model of OPT-11 bound to the AS–wt. The model was constructed using the reported structure of AS–wt bound to paromomycin.[4] In our model one molecule of neamine is docked to the A^{1408}–A^{1493} base pair in the bulge region of the RNA while the tether lies in the major

Keywords: Aminoglycosides; RNA; Antibiotics; Ribosome; 2-Deoxystreptamine.
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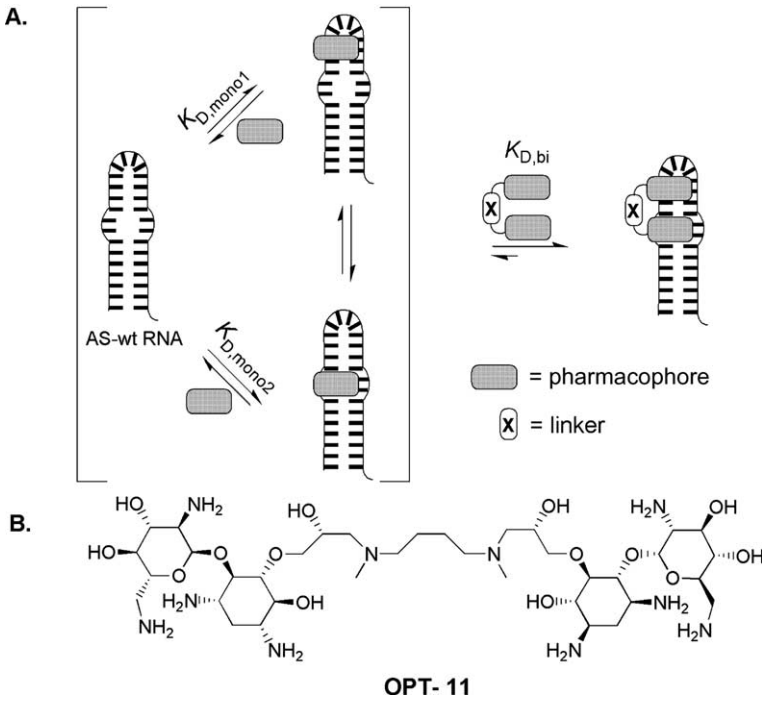


Figure 1. (A) Model of a bivalent AMG binding an AS–wt rRNA model sequence. The pharmacophore is neamine or nebramine (shaded). (B) The structure of OPT-11, a bivalent AMG.

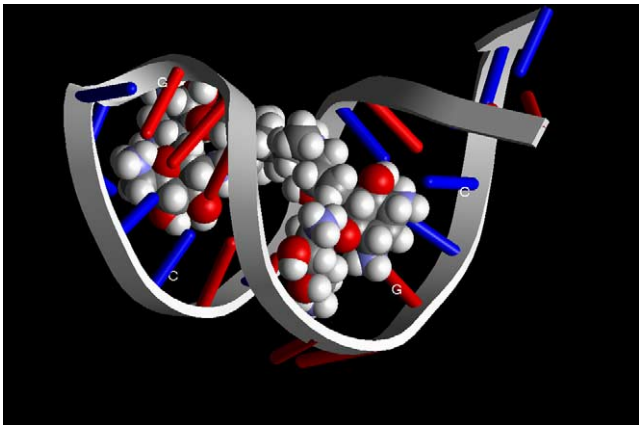


Figure 2. A model of OPT-11 bound to the AS–wt rRNA, a model for the 16S rRNA domain.

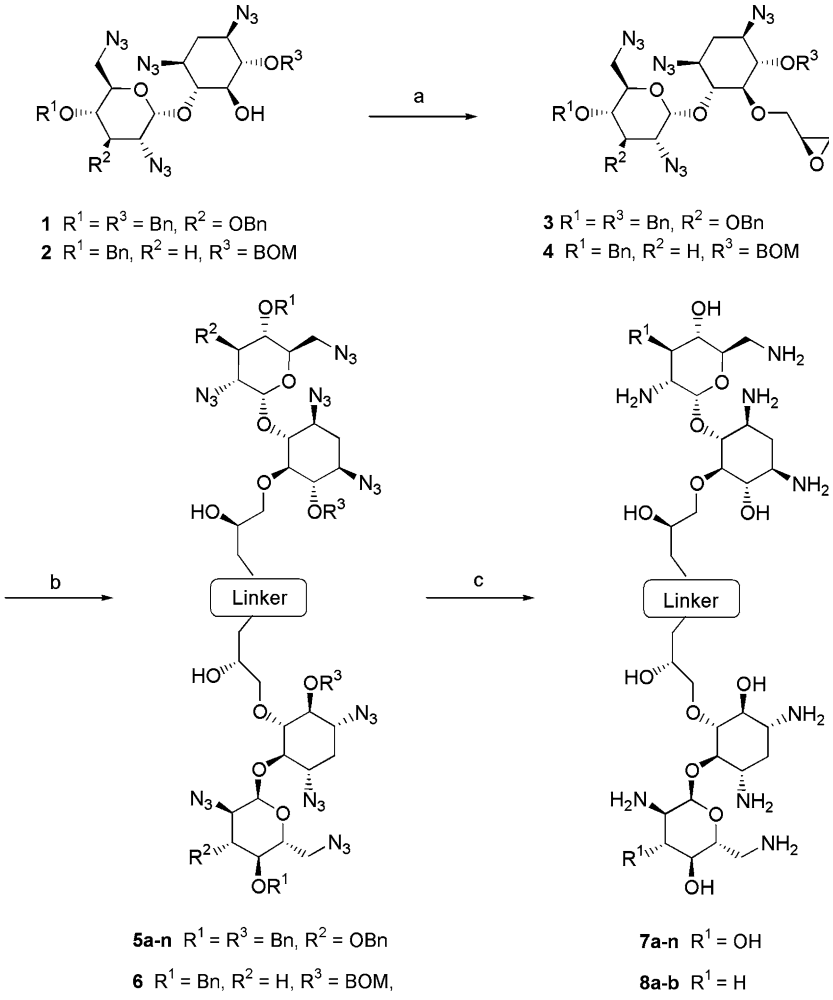
groove of the RNA and the distal neamine motif interacts near the loop domain.

Based on this model we aimed to determine whether modification of the linker domain and the linkage position could further enhance the antimicrobial activity of bivalent AMGs because optimized binding or selectivity for 16S rRNA would result in the improvement in antimicrobial activity (vide infra).

To accomplish this aim we selected a series of substituted and unsubstituted linkers with lengths between 4 and 10 atoms that were either flexible or rigid. In addition we also wanted to assess linking the 2-DOS pharmacophore through the 6-position because of the abundance of naturally occurring AMGs linked through that position. And finally, since our ultimate goal was to achieve high levels of activity against *P. aeruginosa* we wanted to evaluate the effect of replacing the neamine motif with the nebramine motif which is found in the AMG tobramycin, the AMG with the highest potency against *P. aeruginosa*.

2. Chemistry

The synthesis of 4,5-linked neamine, 4,5-linked nebramine and 4,6-linked nebramine analogues is outlined in Schemes 1 and 2, respectively. 5-free OH neamine **1**[3] and 5-free OH nebramine **2**[5] were obtained following previously reported procedures. The alcohols **1** and **2** were reacted with (*R*)-glycidol tosylate to afford the epoxides **3** and **4** which were subsequently linked with a variety of amines using 2 equiv of epoxide heated at 70 °C in ethanol. After 24–48 h of heating, the resulting linked AMGs were purified by silica gel and de-protected in a single step by hydrogenation over 20% $Pd(OH)_2/C$ in 1:1 acetic acid–water. The resulting AMGs were efficiently purified by mass-directed preparative RP-HPLC. Alternatively, the compounds could be purified by Amberlite^{TM} CG50 cation exchange resin using the ammonium form of the resin. Elution of the resin with a linear gradient 0–3% NH_4OH was generally sufficient to purify the compounds. The latter method was more convenient than RP-HPLC when multigram quantities of linked AMG were needed for in vivo studies. Table 1 shows compounds **7a**–**n**, which contain a representative selection of flexible and rigid linkers in the 4,5-linked neamine series, while Table 2 shows **8a**,**b** the nebramine homologues of OPT-11. In order to determine if the 4,5-linking pattern was critical, protected nebramine **9** was prepared as recently



1 $R^1 = R^3 = Bn$, $R^2 = OBn$
2 $R^1 = Bn$, $R^2 = H$, $R^3 = BOM$

3 $R^1 = R^3 = Bn$, $R^2 = OBn$
4 $R^1 = Bn$, $R^2 = H$, $R^3 = BOM$

5a-n $R^1 = R^3 = Bn$, $R^2 = OBn$
6 $R^1 = Bn$, $R^2 = H$, $R^3 = BOM$,

7a-n $R^1 = OH$
8a-b $R^1 = H$

Scheme 1. Synthesis of 4,5-linked neamine and nebramine congeners. Reagents and conditions: (a) (*R*)-glycidyl tosylate, NaH, DMF; (b) 0.5 equiv diamine, EtOH, 70 °C, 48 h; (c) 20% $Pd(OH)_2/C$, H_2, H_2O, AcOH.

described.[5] The linking chemistry was repeated to afford the 4,6-linked compounds **12a**–**d**, Scheme 2 and Table 3, respectively.

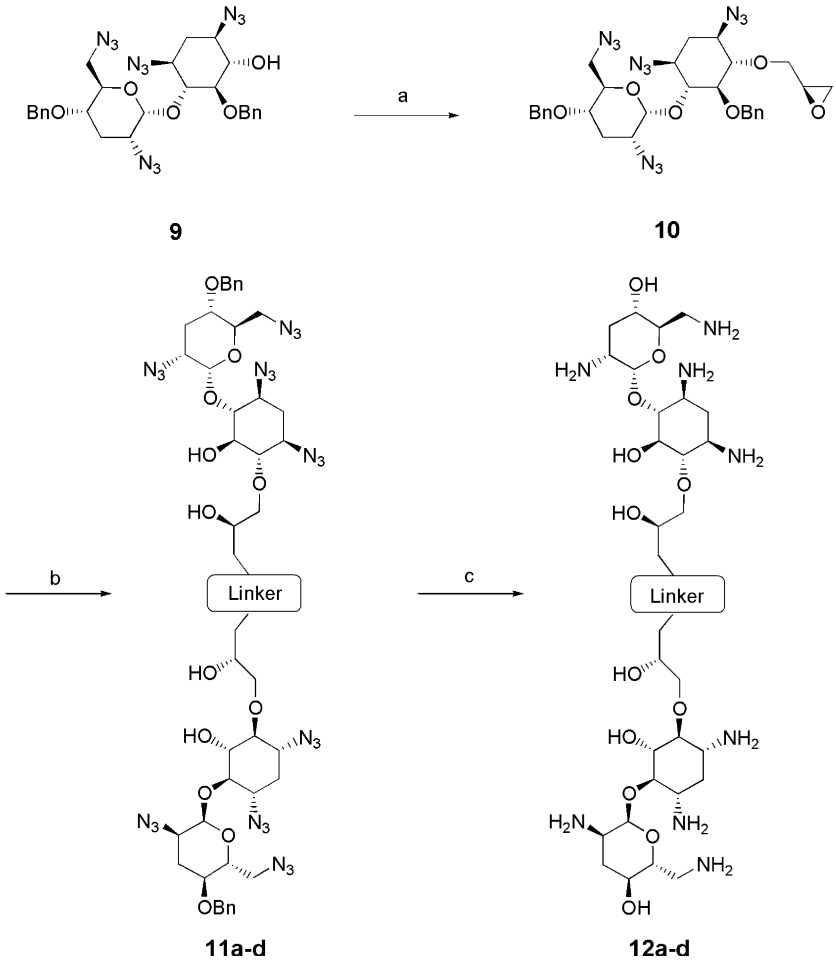
3. Biology

As a screen for broad spectrum activity, minimum inhibitory concentrations (MICs) in microgram per milliliter values were determined after 16–20 h incubation[6] for compounds **7a**–**n**, **8a**,**b** and **12a**–**d** in comparison with tobramycin against reference strains *S. aureus* ATCC 29213; *Enterococcus faecalis* ATCC 29212; *Escherichia coli* ATCC 25922; *P. aeruginosa* ATCC 27853, and PAO-1 and *S. aureus* MRSA 33591, Table 4. As a secondary panel, 16 clinical isolates of *P. aeruginosa* (PAE_NUH01-7, 17-25) were obtained which include both sensitive and resistant strains (National University Hospital, National University of Singapore, Department of Laboratory Medicine, Clinical Microbiology Lab, Singapore). The disk diffusion method was used to obtain the antibiotic susceptibility profiles against a variety of empirically used antipseudomonal antibiotics (Table 5).[7] Also shown are the MICs for tobramycin, OPT-11 and the des-methyl analogue **7h**.

The lead compound OPT-11 was further evaluated for efficacy in a *S. aureus* mouse protection model (Table 6). A fresh inoculum of *S. aureus* ATCC 29213 was adjusted to $100 \times LD_{50}$ dose and 500 mL was injected intraperitoneally to five female Balb/c mice. The test compound was dissolved in a sterile normal saline (0.85% NaCl) solution. The pH was between 7.0 and 7.5 and required no adjustment. The drug (60 mg) was dissolved in 3 mL of sterile saline, the drug was rapidly solubilized and this solution was then used as the stock solution for all lower concentrations used in this study. OPT-11 was administered over a five-dose range by intravenous injection into the tail vein 1 h and 5 h post-infection and observed for the following five days. An exact ED_{50} was not calculated because there were not sufficient mortalities in the lower dosing groups, but was less than 5 mg/kg. The results show protection of 1/2 of the mice at concentrations below 5 mg/kg, a result that compares favourably with currently used AMGs.

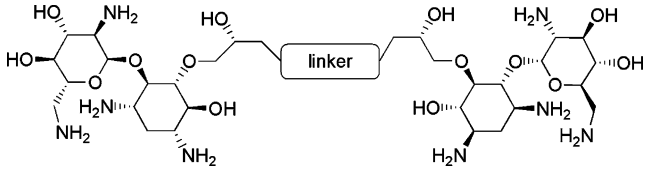
4. Discussion

Derivatives **7a**–**n**, **8a**,**b** and **12a**–**d** were synthesized utilizing two pharmacophore domains, neamine and



Scheme 2. Synthesis of 4,6-linked nebramine congeners. Reagents and conditions: (a) (*R*)-glycidyl tosylate, NaH, DMF; (b) 0.5 equiv diamine, EtOH, 70 °C, 48 h; (c) 20% $Pd(OH)_2/C$, H_2, H_2O, AcOH.

Table 1. 4,5-Linked neamine structures



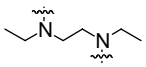
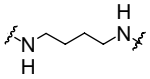
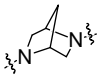
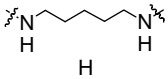
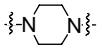
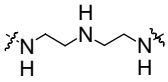
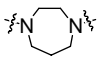
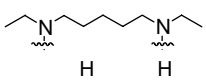
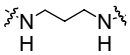
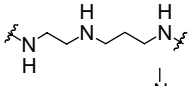
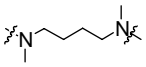
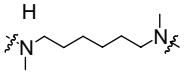
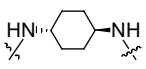
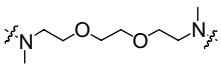
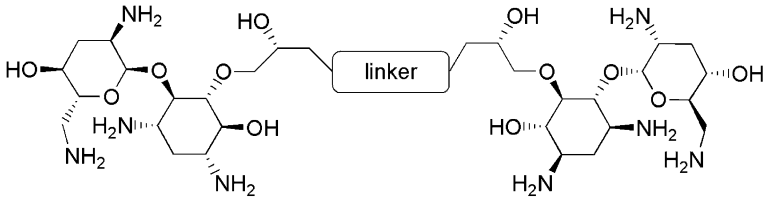
No.	Linker	No.	Linker
7a		**7h**	
7b		**7i**	
7c		**7j**	
7d		**7k**	
7e		**7l**	
7f (OPT-11)		**7m**	
7g		**7n**	

Table 2. 4,5-Linked nebramine structures



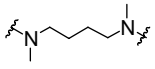
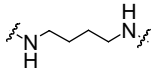
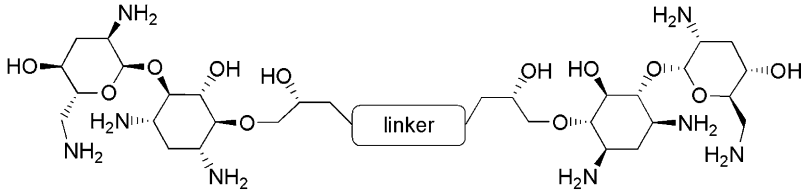
No.	Linker	No.	Linker
8a		**8b**	

Table 3. 4,6-Linked nebramine structures



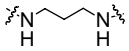
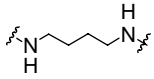
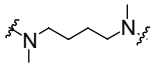
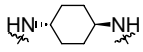
No.	Linker	No.	Linker
12a		**12c**	
12b		**12d**	

Table 4. In vitro antimicrobial activities of (**7a**–**n**), (**8a**,**b**) and (**12a**–**d**)

No.	MIC (μg/mL)					
	Sa	*Ef*	*Ec*	*Pa*	*Pa*[a]	*Sa*[b]
7a	>50	>50	>50	>50	>50	>50
7b	12.5	>50	>50	12.5	50	>50
7c	12.5	>50	25	25	25	>50
7d	12.5	>50	50	12.5	50	>50
7e	3.1	>50	12.5	6.3	6.3	50
7f (OPT-11)	1	>50	4	8	8	16
7g	50	>50	25	1.6	—	50
7h	1	>50	4	4	2	16
7i	1.6	>50	12.5	6.3	12.5	50
7j	6.3	>50	12.5	3.1	—	12.5
7k	12.5	>50	25	25	50	>50
7l	>50	>50	>50	>50	>50	>50
7m	6.3	>50	25	12.5	50	>50
7n	>50	>50	>50	>50	>50	>50
8a	2	>50	16	16	16	32
8b	2	32	16	4	4	16
12a	1.3	>20.9	20.9	2.6	1.3	10.4
12b	0.6	20.9	5.2	10.4	2.6	20.9
12c	0.5	32	4	2	2	16
12d	0.5	32	4	2	2	16
Tobramycin range	0.12–1	8–32	0.25–1	0.25–1	0.25–1	>64

Sa, *Staphylococcus aureus* ATCC 29213; *Ef*, *Enterococcus faecalis* 29212; *Ec*, *Escherichia coli* ATCC 25922; *Pa*, *Pseudomonas aeruginosa* ATCC 27853.

[a] *Pa*, *Pseudomonas aeruginosa* PAO-1.

[b] *Sa*, *Staphylococcus aureus* MRSA 3359.

nebramine. A number of compounds demonstrated MICs below 6 μg/mL against several bacterial strains. In the **7a**–**n** series the conformationally restrained linkers **7b**–**d** and **g** all showed reduced activities as did linkers shorter or longer than 4Cs, **7a**,**e** and **7i**–**n**, respectively (Table 4). Thus, the C4 linker remained the optimal linker length. The des-methyl analogue **7h** was slightly more potent against *P. aeruginosa* whilst linkers of any length with substitution larger in size than a methyl group lost activity (data not shown). These data are

Table 5. Susceptibility testing of *P. aeruginosa* clinical isolates and in vitro antimicrobial activities of OPT-11 and **7h**

Strain	Antibiotic											MIC (μg/mL)		
	AMK	GM	TOB	IMP	CEF	CIP	AZT	PTZ	CO-T	CHL	TET	TOB	OPT-11	**7h**
ATCC 27853	S	S	S	S	S	S	S	S	R	R	S	1	8	1
PAE_NUH01	R	R	R	S	R	R	R	R	R	R	R	>200	12.5	6.3
PAE_NUH02	R	R	R	S	R	R	R	R	R	R	R	50	6.3	6.3
PAE_NUH03	R	R	R	S	R	R	R	S	R	R	R	50	6.3	6.3
PAE_NUH04	R	R	R	S	R	R	R	S	R	R	R	100	6.3	3.1
PAE_NUH05	R	R	R	S	R	R	R	S	R	R	R	100	6.3	3.1
PAE_NUH06	R	R	R	S	R	R	R	S	R	R	R	100	12.5	6.3
PAE_NUH07	S	R	S	S	I	R	R	R	R	R	R	6.3	6.3	3.1
PAE_NUH17	S	S	S	S	S	S	S	S	S	S	R	1	>32	32
PAE_NUH18	S	I	S	S	S	S	R	S	R	R	R	0.5	16	8
PAE_NUH19	S	S	S	S	S	S	R	S	R	R	R	0.5	16	8
PAE_NUH20	S	S	S	S	S	S	R	S	S	S	R	2	16	16
PAE_NUH21	S	S	S	S	S	S	R	S	R	I	I	1	16	16
PAE_NUH22	S	S	S	S	S	S	R	S	R	I	R	0.25	8	16
PAE_NUH23	S	I	R	S	S	S	R	S	R	I	R	>32	>32	16
PAE_NUH24	S	I	S	S	S	S	R	S	R	I	R	1	32	16
PAE_NUH25	S	S	S	S	S	S	R	S	S	R	R	0.5	8	8

AMK = amikacin; GM = gentamicin, TOB = tobramycin; IMP = imipenem; CEF = ceftazidime; CIP = ciprofloxacin; AZT = aztreonam; PTZ = piperacillin/tazobactam, CO-T = co-trimoxazole; CHL = chloramphenicol; TET = tetracycline.

Table 6. In vivo efficacy of OPT-11 in a *S. aureus* mouse protection model

Dose	Survivors	Mortalities	% Survivors
Control	1 of 5	4 of 5	20
5 mg/kg	4 of 5	1 of 5	66.6
10 mg/kg	5 of 5	0 of 5	90
25 mg/kg	4 of 5	1 of 5	92.9

consistent with a model where the linker lies in the groove of the RNA. The 4,5-linked nebramine compounds **8a**,**b** were less active than their neamine homologues while the 4,6-linked nebramine dimers were consistently more active than their 4,5-linked homologues **12a**–**d**. Also intriguing were the MIC results obtained with the extended panel of *P. aeruginosa* (PAE_NUH01-7, 17-25), Table 5. The bivalent AMGs were demonstrated to retain activity against *P. aeruginosa* including strains resistant to a variety of antipseudomonal agents. In conclusion, these studies have established that the length of the linker and its substituents were shown to be important determinants of antibacterial activity in bivalent AMGs and the results appear to be consistent with the model proposed in Figure 2. Furthermore, bivalent AMGs showed potent activity against drug resistant strains of *P. aeruginosa* and the lead compound OPT-11 demonstrated the ability to protect mice in an infection model. Taken together, these data suggest that bivalent AMGs may lead to the development of AMGs active against bacteria that are resistant to a wide variety of antipseudomonal drugs.

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6. Individual compounds are tested for growth inhibition and minimum inhibitory concentrations (MIC) values calculated according to the protocols outlined by the NCCLS in their publication M7-A5, 'Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically,' 5th ed., 2000; with reference to the standards in their publication M100-S12, 'Performance Standards for Antimicrobial Susceptibility Testing, Twelfth Informational Supplement,' 2002. For most (nonfastidious) organisms, the protocol is the same: compounds are dissolved to 10 mg/mL in water and diluted to 128 μg/mL in Mueller–Hinton Broth, then successively diluted (2-fold dilutions) in microtiter plates. Bacteria are suspended in Mueller–Hinton Broth (cation adjusted) and mixed 1:1 with the diluted antibiotic solution. Plates are incubated at 35 °C for 16–20 h. The MIC is read as the lowest antibiotic concentration at which no growth is observed. All MICs are performed in duplicate, with a no-antibiotic and a no-inoculum (sterility) control.
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