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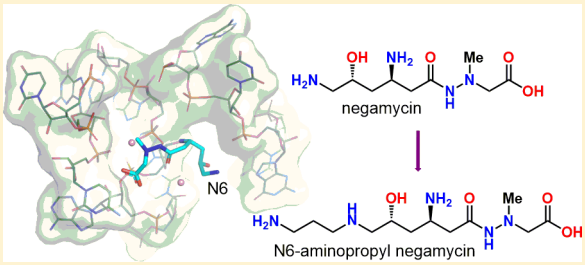
Structural Insights Lead to a Negamycin Analogue with Improved Antimicrobial Activity against Gram-Negative Pathogens

David C. McKinney,[†] Gregory S. Basarab,[†,⊥] Alexis I. Cocozaki,[§] Melinda A. Foulk,[†] Matthew D. Miller,[†,#] Anatoly M. Ruvinsky,[†,○] Clay W Scott,[∥] Kumar Thakur,[†] Liang Zhao,[†,▽] Ed T. Buurman,[‡,□] and Sridhar Narayan*[,†,◇]

Departments of [†]Chemistry and [‡]Bioscience, Infection Innovative Medicines; [§]Structure and Biophysics, Discovery Sciences; [∥]Discovery Safety, Drug Safety and Metabolism, AstraZeneca R&D Boston, 35 Gatehouse Drive, Waltham, Massachusetts 02451, United States

Supporting Information

ABSTRACT: Negamycin is a natural product with antibacterial activity against a broad range of Gram-negative pathogens. Recent revelation of its ribosomal binding site and mode of inhibition has reinvigorated efforts to identify improved analogues with clinical potential. Translation-inhibitory potency and antimicrobial activity upon modification of different moieties of negamycin were in line with its observed ribosomal binding conformation, reaffirming stringent structural requirements for activity. However, substitutions on the N6 amine were tolerated and led to N6-(3-aminopropyl)-negamycin (**31f**), an analogue showing 4-fold improvement in antibacterial activity against key bacterial pathogens. This represents the most potent negamycin derivative to date and may be a stepping stone toward clinical development of this novel antibacterial class.



KEYWORDS: *Antibacterial, ribosome, natural product, Gram-negative, negamycin*

The threat of bacterial resistance to clinically used antibiotics has reached alarming proportions, with an estimated 23,000 deaths and over 2 million infections each year in the United States alone. The biggest threats come from six species collectively termed the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) that have become challenging in the hospital setting.[1] This rise of resistance, coupled with a decrease in antibacterial approvals, has recently led to several public policy changes and private initiatives to spur research and development in this important area.[2] These developments notwithstanding, the need for novel antimicrobial agents—against Gram-negative pathogens in particular—remain acute.[3]

Natural products have served as excellent leads since the early days of antibiotics research. Almost all known classes of antibiotics have originated from natural substances, with oxazolidinones[4] and fluoroquinolones[5] being notable exceptions. Natural product β-lactams, macrolides, tetracyclines, aminoglycosides, and glycopeptides have all been the subject of derivatization leading to newer generations of antibiotics. Steady progress in the clinical development of the aminoglycoside plazomicin,[6] the tetracycline eravacycline,[7] and the cephalosporin ceftolozane[8] for the treatment of Gram-negative infections is a recent reminder that chemical modification of established classes of natural products is still a viable route for drug discovery.

Negamycin (**1**, Figure 1) was originally isolated from a culture of *Streptomyces purpeofuscus* in 1970.[9] Efficacy of this natural product was observed in immunocompetent mouse models of sepsis caused by important Gram-negative pathogens including *P. aeruginosa* and *K. pneumoniae*, thus affording the compound its name. Early studies revealed that negamycin inhibits bacterial protein synthesis, affecting translation initiation, elongation, and termination.[10−14] The inability for target-based mutations to be isolated, as is typical for translation inhibitors due to redundancy of rRNA operons, combined with the absence of ribosome crystallographic systems precluded definition of its ribosomal binding site(s).

Following its isolation and structure elucidation, a number of synthetic routes to negamycin and analogues thereof were designed in attempts to improve biochemical and antibacterial activity of the scaffold (Figure 1).[15,16] In the absence of guidance by structural information, biochemical progress was monitored by activity in a coupled transcription−translation (TT) assay using S30 extracts of *E. coli*.[17] Umezawa et al. found that deoxynegamycin (**2**) and *O*-methylnegamycin (**3**) maintained antimicrobial activity albeit with 3- to 4-fold loss of potency compared to negamycin. Inversion of the C3 stereocenter (3-*epi*-negamycin **4** and *ent*-negamycin **5**) resulted

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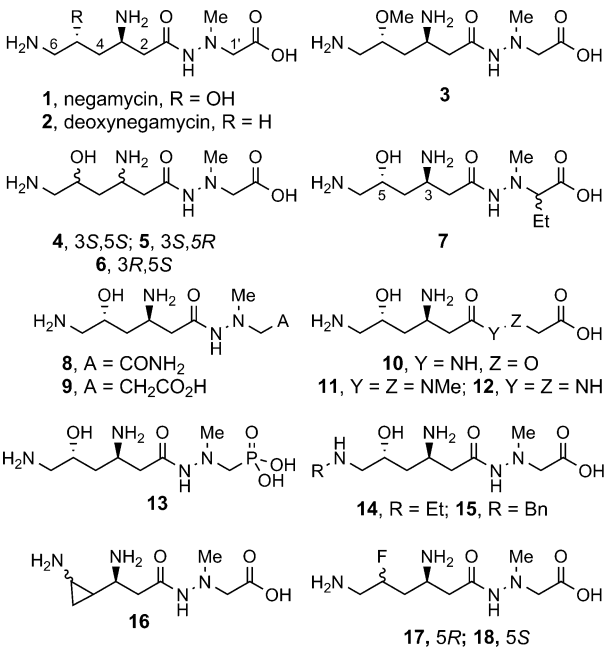

Figure 1. Negamycin and some of its reported analogues.

in roughly a 10-fold loss in activity. Inversion of the C5 stereocenter to 5-*epi*-negamycin **6** resulted in a 25-fold loss in biochemical potency, despite the fact that concomitant inversion of both stereocenters (**5**) only resulted in an 8-fold loss in activity.[13] Alterations in the methylhydrazine acetic acid (MHA) portion (**7**–**9**) of negamycin led to compounds lacking discernible biochemical or antimicrobial activities at the concentrations tested.

A more thorough evaluation of structure–activity relationships (SARs) resulted from the synthesis of dozens of analogues as part of an extensive drug discovery program at Vicuron in the early 2000s.[18,19] Modifications to the C-terminus of negamycin (**10**–**13**) resulted in compounds with greatly diminished biochemical potency, whereas N-terminal derivatives **14** and **15** were reported to maintain biochemical potency. A conformationally constrained analogue of deoxynegamycin **16** showed 8-fold improvement in biochemical potency; however, this compound did not inhibit bacterial growth within the range of concentrations tested, suggesting detrimental cellular permeation. Fluorinated analogue **17** reportedly showed biochemical and antibacterial activities comparable to negamycin.

Two recent concomitant crystallographic efforts have revealed the negamycin binding site to overlap that of tetracycline and tigecycline.[20,21] Mechanistic studies using smFRET also showed negamycin, unlike tetracycline, to enable accommodation of a ternary complex using near cognate tRNA leading to mistranslation and cellular death.[21] This breakthrough in the understanding of the binding mode and the mechanism of action spurred us to initiate an antibiotic discovery program based on negamycin. Negamycin's route of bacterial entry at the site of infection is similar to that of aminoglycosides in that it is driven by the inner membrane potential seemingly without involvement of a membrane carrier under physiological conditions.[22,23] The aim therefore was to incorporate structural guidance to engineer novel interactions with the ribosome, while maintaining the physicochemical parameters important for membrane penetration, and thus improve upon its antibacterial activity. A negamycin class of antibiotics would be a significant addition to the antibiotic arsenal because it would likely avoid the molecular modification mechanisms that lead to aminoglycoside clinical resistance.[24] Furthermore, the alternative ribosomal binding site of negamycin could potentially exclude the occurrence of ototoxicity with aminoglycosides observed in a subset of patients.[25]

In addition to confirming established SARs, modifications to the negamycin structure were explored to identify new areas for design. Accordingly, multiple synthetic routes were adapted from the literature for the synthesis of diverse analogues (see Supporting Information). To support biochemical inhibition data, antibacterial activities (presented as minimum inhibitory concentrations, MICs) were determined against four strains representing each of the Gram-negative ESKAPE pathogens (Table 1). In our studies, deoxynegamycin (**2**) was biochemically only 2-fold less potent, although its antibacterial activity was 4- to 8-fold lower than negamycin. We previously reported that the three stereoisomers **4**–**6** of negamycin showed diminished activity relative to negamycin indicating that the 3*R*,5*R* configuration is optimal.[22] As previously noted, both N6-alkyl analogues **14** and **15** showed biochemical potency comparable to negamycin; however, benzyl derivate **15** was significantly worse in terms of antibacterial activity. 5-Fluoro analogue **17** also showed moderately weaker biochemical and antimicrobial activities compared to negamycin, whereas the 5*S* fluoro derivative **18**, similar to the 5*S* negamycin **6**, was considerably less active. Our observations with compounds substituted on the C-terminal end of negamycin (**12**, **19**, **20**) were similar to previous studies; all changes led to abolishment

Table 1. Biochemical Potency (TT IC_{50}) and MIC Values of Previously-Known and Novel Negamycin Analogues Synthesized in This Study

Cmpd	TT *Eco*[a]	*Aba*[b]	*Kpn*[c]	*Eco*[d]	*Pae*[e]
gentamicin	0.4	>64	8	0.5	1
tetracycline	1.4	>64	16	1	32
1	1.7	16	32	16	32
2	3.3	128	128	128	128
4	12	128	256	512	256
5	13	256	256	>256	>256
6	51	512	1024	1024	>1024
12	34	>64	>64	>64	>64
14	0.5	64	64	64	512
15	1.0	>256	128	128	>256
17	2.9	128	64	64	128
18	26	>512	>512	>512	>512
20	7.4	256	128	256	128
21	12	128	256	128	256
22	3.2	64	>64	>64	>64
23	43	>256	>256	>256	>256
24	7.3	128	>128	>128	>128
25	78	>512	>512	>512	>512

[a]IC_{50} (μM) in *E. coli* TT assay. [b]MIC (μg/mL) against *A. baumannii* (ARC3495). [c]MIC (μg/mL) against *K. pneumoniae* (ATCC700603). [d]MIC (μg/mL) against *E. coli* (ATCC25922). [e]MIC (μg/mL) against *P. aeruginosa* (PAO1). Analogues were tested up to their highest soluble concentrations. Compounds **10**, **11**, and **19** had TT IC_{50} >100 μM and MICs >256 μg/mL. Compounds **3**, **7**, **8**, **9**, **13**, and **16** were not synthesized in this study.

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of TT and antibacterial activities at the concentrations tested. Substitutions at C2, C4, or C6 of negamycin (**21**−**25**), which were designed to improve potency by establishing additional interactions with the ribosome, also showed lower biochemical potencies and antibacterial activities (Figure 2).

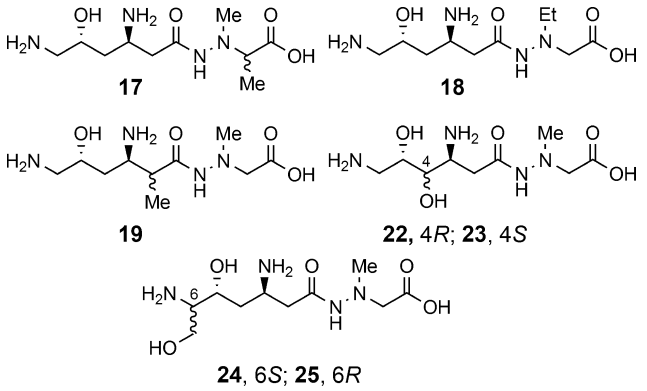


Figure 2. Some novel analogues of negamycin synthesized in this study.

This initial SAR exploration confirmed that the C-terminal MHA region of negamycin was intolerant to a range of substitutions or modifications. A potential concern is that hydrolysis of the carboxamide linkage in negamycin derivatives would release MHA. Pharmacokinetic studies on negamycin have revealed very low levels of MHA in the urine of rats and dogs upon administration of negamycin.[26] Since the presence of MHA has been linked to hepatoxicity, albeit without supporting data,[27] its metabolic formation and toxicological profile would warrant monitoring. Nonetheless, we focused our efforts on generating novel N6-substituted analogues of negamycin where there appeared to be more opportunity for maintaining and improving potency.

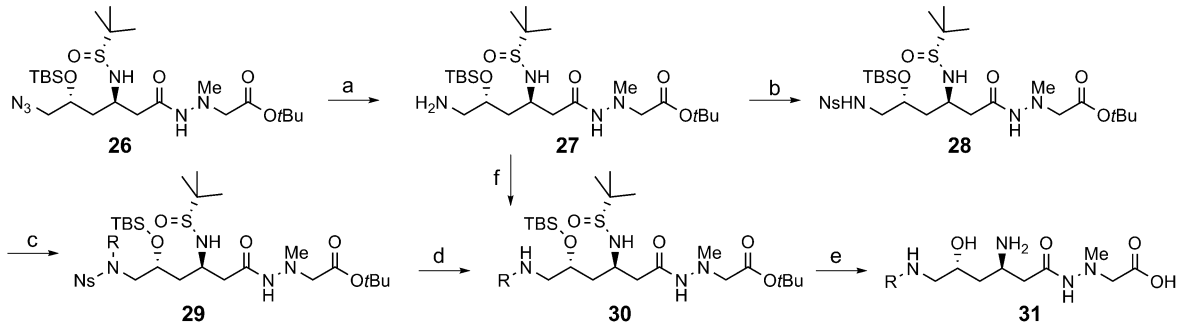
The general protocol used for generation of such analogues is depicted in Scheme 1. Briefly, catalytic hydrogenation of azide **26**, previously utilized in a total synthesis of negamycin,[21] was followed by treatment with *p*-nitrosulfonyl chloride to afford the key intermediate **28**. A variety of substituents was introduced onto **28** under Mitsunobu conditions. Alternately, a few analogues were generated from **27** by reductive amination or by alkylation with activated alkyl bromides. Subsequent global deprotection produced the desired final compounds (**31**).

The activity of various N6-substituted negamycin analogues is shown in Table 2. In addition to the four strains of Gram-negative bacteria described above, compounds were also tested in isogenic efflux pump debilitated strains of *E. coli* and *P. aeruginosa* to help understand the contribution of efflux toward antibacterial activity.[28]

Hydroxyethyl analogue **31a** showed a 3-fold improvement in biochemical potency compared to negamycin. Other compounds bearing substituted ethyl side-chains (**31b**−**d**) showed a 10-fold range in TT inhibitory potency depending on the terminal functional group. However, these modifications resulted in diminishment of antimicrobial activity. A 30-fold decrease in biochemical potency was observed with hydroxypropyl analogue **31e** along with a commensurate decrease in antibacterial activity. In marked contrast, aminopropyl compound **31f** showed significantly improved biochemical and antimicrobial potencies compared to negamycin. *To our knowledge, **31f** shows the highest antibacterial activity of any negamycin analogue reported to date*. Moreover, it appears that the terminal amine of **31f** contributes an additional binding interaction not previously recognized.

We next explored substitutions on the aminopropyl chain of **31f**. Methyl substitution on the carbon adjacent to N6 (**31g**) had only a minimal impact, whereas methylation at the other carbons had progressively detrimental effects (**31h** and **31i**) on TT and antibacterial activities. The presence of amino (**31j**) and fluoro (**31k**) substituents on the propyl chain also resulted in significant diminishment of biochemical potency and corresponding decrease in antimicrobial activity compared to **31f**. The effect of addition of a hydroxyl group at the middle carbon was dependent upon its configuration (**31l** vs **31m**) suggesting that the conformation of the propyl chain was important to gaining the added interaction from the terminal amine. Substitution on the terminal nitrogen atom of **31f** was detrimental to potency (**31n, 31o**, and **31p**) providing further evidence that the improved activity of **31f** is likely due to an added interaction with the ribosome. Other modifications such as guanidine **31q** and carboxamide **31r** had weaker biochemical and antimicrobial potencies compared to **31f**, pointing to the unique requirement for a primary amine group. Furthermore, aminobutyl analogue **31s** with a 4- rather than a 3-atom linkage showed a 2-fold improvement in inhibitory potency relative to negamycin and commensurate level of antibacterial activity. Compounds in which the 3-atom linkage was constrained within a ring (**31t** and **31u**) were generally 10-fold less active than **31f** indicating that the constraint did not well-approximate

Scheme 1. General Synthetic Route for N6 Analogues[a]



[a]Reagents and conditions: (a) H_2, Pd/C, MeOH, 25 °C, 98%; (b) *p*-NO_2PhSO_2Cl, CH_2Cl_2, 25 °C, 75%; (c) ROH, Ph_3P, DEAD, THF; 0 °C; (d) PhSNa, THF, 25 °C; (e) HCl, dioxane; 25 °C; (f) R′CHO, $NaBH_3CN$, 0 °C or RBr, K_2CO_3, DMF, 0 °C.

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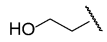
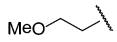
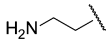
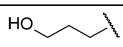
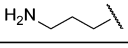
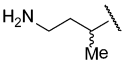
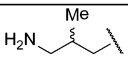
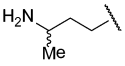
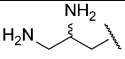
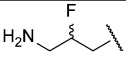
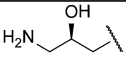
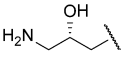
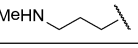
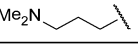
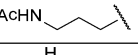
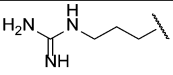
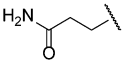
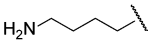
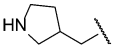
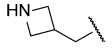
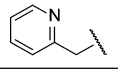
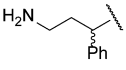
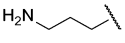
Table 2. Biochemical and Antibacterial Activity of Novel N6 Analogues

Cmpd	R-group	TT *Eco*[a]	*Aba*[b]	*Kpn*[c]	*Eco*[d]	*EcoΔ*[e]	*Pae*[f]	*PaeΔ*[g]
	gentamicin	0.41	>64	8	0.5	0.5	1	0.13
	tetracycline	1.5	>64	16	1	0.5	32	0.25
1	H (negamycin)	1.7	16	32	16	16	32	16
31a		0.63	64	64	64	128	512	128
31b		5.3	256	128	>256	128	>256	>256
31c		2.3	128	64	64	128	>256	256
31d		2.1	64	32	64	64	128	64
31e		11	256	512	512	128	>1024	1024
31f		0.34	4	4	8	16	16	8
31g		0.44	8	8	16	16	32	32
31h		1.1	16	16	32	16	32	16
31i		3.4	64	64	64	64	128	64
31j		10	32	64	128	128	128	128
31k		3.6	256	128	256	128	256	128
31l		0.73	32	64	64	32	128	64
31m		2.5	128	64	256	64	256	128
31n		5.8	64	64	256	256	256	256
31o		15	256	256	>256	>256	>256	>256
31p		19	>512	>512	>512	>512	>512	>512
31q		1.7	64	128	512	256	512	256
31r		4.0	>256	>256	>256	256	>256	>256
31s		0.74	16	16	32	32	128	64
31t		3.3	64	128	256	256	256	128
31u		3.9	128	64	256	128	256	128
31v		11	>256	>256	>256	256	>256	>256
31w		2.3	>256	64	256	128	>256	32
32	 (*3S*) epimer of negamycin	2.8	32	64	128	128	128	128

[a]IC_{50} (μM) in *E. coli* TT assay. [b]MIC (μg/mL) against *A. baumannii* (ARC3495). [c]MIC (μg/mL) against *K. pneumoniae* (ATCC700603). [d]MIC (μg/mL) against *E. coli* (ATCC25922). [e]MIC (μg/mL) against *E. coli* (ATCC25922 tolC$^-$). [f]MIC (μg/mL) against *P. aeruginosa* (PAO1). [g]MIC (μg/mL) against *P. aeruginosa* (PAO1 *mexABCDXY*$^-$). Analogues were tested up to their highest soluble concentrations in water.

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the aminopropyl binding conformation. Although the *N*-benzyl derivative of negamycin (**15**) had comparable biochemical potency, the N6 region of negamycin was generally intolerant of aromatic groups. The 2-pyridylmethyl analogue **31v** had 11-fold weaker biochemical potency compared to **15**, while **31w**, a hybrid of **15** and **31f**, was closer in activity to **15**, rather than **31f**. Finally, installation of the aminopropyl side-chain on **5** (3-*epi*-negamycin) provided **32**, which resulted in a 5-fold increase in biochemical potency and corresponding improvement in antibacterial activity, indicating the possibility that the amino-propyl modification could also serve to enhance the potency of other newer negamycin analogues.

As seen from Table 2, potency in the TT assay was generally indicative of antimicrobial activity. However, compounds containing amino groups in the N6 side-chain showed MICs lower than expected (cf. **31c** vs **31d**, **31j** vs **31k**, **31l**, or **31m**) indicating a beneficial effect of basicity upon cellular penetration. Significantly, negamycin and most of its analogues did not appear to be subject to efflux pumps that shuttle xenobiotics out of the bacterium through the outer membrane.[29] MIC values determined for such efflux pump knockout strains typically varied little from the isogenic parent. This is in marked contrast to tetracycline for example, which showed a 128-fold decrease in MIC for the pump knockout *P. aeruginosa* strain versus the wild-type (Table 2).

The recently described 3.1 Å X-ray crystal structure of negamycin bound to the *E. coli* ribosome suggests that modification of the N6 moiety would be tolerated, allowing for establishment of new interactions (Figure 3).[21] To understand the contribution of the aminopropyl group of **31f** to binding, multiple attempts were made to soak this compound into the ribosome crystal analogous to negamycin. However, no density for **31f** was observed, implying that there is limited physiological relevance of negamycin binding to the ribosome only. This is consistent with single molecule fluorescence resonance energy transfer (smFRET) studies, which suggest that negamycin acts while enabling the binding of a ternary complex with a near-cognate tRNA and a differently ratcheted state of the 30S and 50S ribosomal subunits.[21]

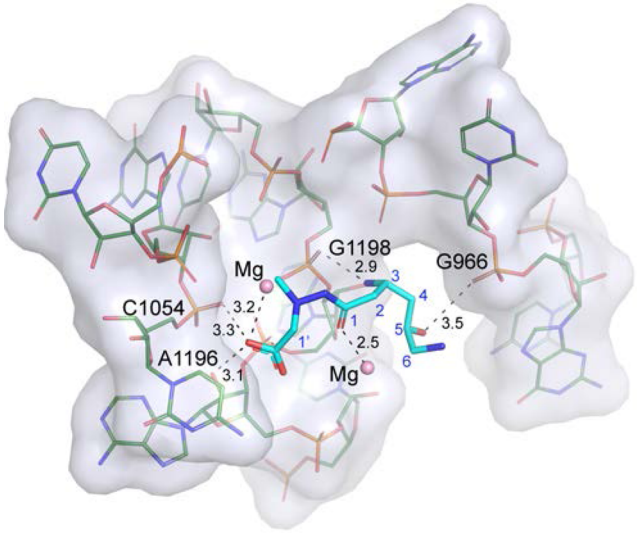

Figure 3. Schematic representation of negamycin (cyan) bound to the *E. coli* ribosome. Dotted lines indicate polar interactions; distances are in Å.

The MIC_{90} of **31f** against a panel of 100 clinical isolates of *P. aeruginosa* maintained in the AstraZeneca collection improved 4-fold over that of negamycin (16 vs 64 μg/mL). Another 4-fold improvement in the MIC_{90} would approximate the range of the recommended clinical breakpoints for aminoglycosides.[30] The in vitro clearance and physicochemical properties of **31f** are similar to that of negamycin. In addition, no discernible differences were observed in the activities of negamycin and **31f** in secondary pharmacology assays; both compounds showed only minimal effects when tested at concentrations as high as 1 mM in a range of biochemical and cell-based assays encompassing common enzyme, ion channel, and G-coupled protein receptor (GPCR) targets (full details are provided in the Supporting Information). These data suggest that the aminopropyl modification does not introduce any unexpected toxicities into this chemical scaffold.

In summary, we have carried out detailed SAR studies on the natural product negamycin confirming that the structural requirements for antimicrobial activity are rigorous. Using a combination of empiric design and structural insights, we identified a novel analogue with superior activity, bringing this chemical class closer to clinical evaluation. We believe that building upon the crystallographic work carried out to date could allow the delineation of the precise interactions of the aminopropyl side-chain leading to further improvements. Starting with a natural product lead with demonstrated pharmacological activity continues to be a viable path to identifying novel antibacterial agents with clinical utility, and we are hopeful that additional work could allow the addition of a negamycin derivative to our antibiotic armamentarium.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic schemes for compounds **2**, **21**−**25**, and **32**, full experimental details for synthesis of **31f**, **31j**, and **31v**, and secondary pharmacology assay results for negamycin and **31f**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchem-lett.5b00205.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: snarayan@navitorpharma.com.

Present Addresses

[⊥]University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa.
[#]Semichem, Inc., Shawnee, Kansas 66216, United States.
[○]Schrödinger Inc., Cambridge, Massachusetts 02142, United States.
[▽]Cerulean Pharma Inc., Cambridge, Massachusetts 02139, United States.
[□]Pfizer Vaccine Research and Development, Pearl River, New York 10965, United States.
[◊]Navitor Pharmaceuticals, Cambridge, Massachusetts 02138, United States.

Notes

The authors declare the following competing financial interest(s): All authors are current or former employees of AstraZeneca.

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■ ABBREVIATIONS

DEAD, diethylazodicarboxylate; DMF, dimethylformamide; GPCR, G-coupled protein receptor; MHA, methylhydrazine-acetic acid; MIC, minimum inhibitory concentration; SAR, structure−activity relationship; smFRET, single molecule fluorescence resonance energy transfer; THF, tetrahydrofuran; TT, coupled transcription-translation

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