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Bioorganic & Medicinal Chemistry Letters 13 (2003) 2413–2418

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

N- and C-Terminal Modifications of Negamycin†

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Received 21 November 2002; accepted 7 February 2003

Abstract—Negamycin **1** is a bactericidal antibiotic with activity against Gram-negative bacteria, and served as a template in an antibiotic discovery program. An orthogonally protected β-amino acid derivative **3a** was synthesized and used in parallel synthesis of negamycin derivatives on solid support. This advanced intermediate was also used for N- and C-terminal modifications using solution-phase methodologies. The N-terminal modifications have resulted in the identification of active analogues, whereas the C-terminal modifications resulted in complete loss of antibacterial activity. The *N*-methyl negamycin analogue, **19a**, inhibits protein synthesis ($IC_{50} = 2.3\,\mu M$), has antibacterial activity (*Escherichia coli*, MIC = 16 μg/mL), and is efficacious in an *E. coli* murine septicemia model ($ED_{50} = 16.3$ mg/kg).

Introduction

The emergence and spread of bacterial resistance[1] to clinically useful antibiotics is a major threat to the health care system worldwide. This observation has rekindled interest in the discovery of new antibacterial agents, preferably with new mode of action.[2] Pursuing novel targets based on bacterial genomics[3] is an elegant approach towards this goal. An alternative approach is to re-investigate previously discovered molecules with antibacterial activity that remained under-explored for various reasons.[4] Negamycin, a natural product isolated[5] in 1970 from the culture filtrate of *Streptomyces purpeofuscus*, is one such antibiotic. It is active mainly against Gram-negative organisms with weak activity against *Staphylococcus aureus*. The mechanism of action is through the inhibition of protein synthesis and miscoding activity.[7] Negamycin is bactericidal and shown to be efficacious with low acute toxicity[5] in animal models. However, there are limited structure–activity relationship (SAR) studies reported in the literature.[6] This has prompted us to undertake a SAR investigation around this template. To this end, modifications on the N- and C-terminal regions of negamycin were undertaken to assess the influence of such modifications on antibacterial activity.

Chemistry

We have identified the orthogonally protected β-amino ester **3a** (Fig. 1) as a key building block for the solution- or solid-phase parallel synthesis of negamycin (**1**) and various other C- and N-terminal derivatives. The β-amino acid derivative **3a** can be obtained from homoallylglycine **4** using classical organic transformations.

Thus, homoallylglycine[8] **4** (Scheme 1) was subjected to iodolactonization conditions[9] followed by treatment with sodium azide to give azidolactone **5** as a mixture of diastereomers (1:1). After base hydrolysis of azidolactone **5**, the resulting hydroxy acid was reacted with *t*-butyldimethylsilyl chloride (TBS-Cl). Hydrolysis of the silyl ester gave acid **6** as a mixture of diastereomers. The activated ester of acids **6** was formed with pentafluorophenyl trifluoroacetate in pyridine and the diastereomers were separated by column chromatography to give **3a** and **3b**. Azide **7a**, obtained by condensing the β-amino ester **3a** with hydrazine **2b**,[10] was reduced to the corresponding amine using catalytic hydrogenation. Cleavage of silyl ether by treatment with tetrabutylammonium fluoride followed by acidic treatment

†Part of this work was presented at 42nd ICAAC. Annual meeting of the American Society for Microbiology. September 27–30, 2002, San Diego, CA, USA.

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doi:10.1016/S0960-894X(03)00393-7

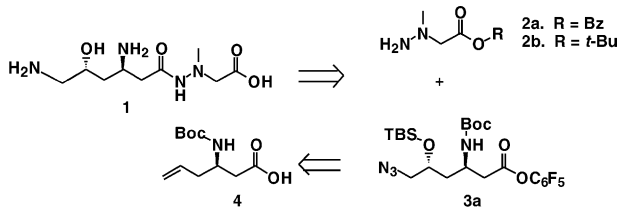
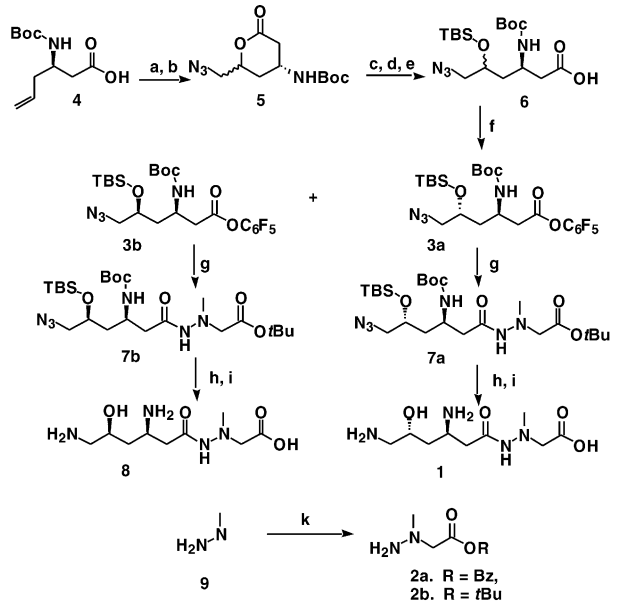


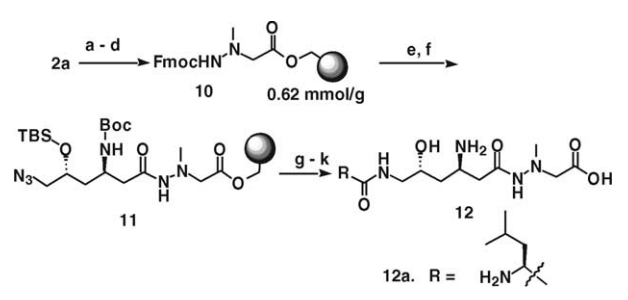
Figure 1. Retrosynthetic analysis.



Scheme 1. Negamycin and epinegamycin. Reagents and conditions: (a) I_2, KI, $NaHCO_3$, H_2O, DCM, rt, 2.5 h; (b) NaN_3, DMF, 55 °C, 4 h (62% for two steps); (c) LiOH, H_2O, MeOH, rt, 2 h (89%); (d) TBS-Cl, imidazole, DCM, rt, 18 h; (e) K_2CO_3, MeOH, H_2O, rt, 2 h (80%); (f) $CF_3CO_2C_6F_5$, pyridine, DCM, rt, 24 h (79%, for three steps); (g) **2b**, DMF, rt, 3 h (96%); (h) TBAF, THF, rt, 1.25 h (70%); (i) H_2, Pd/C, EtOAc, rt, 3 h (90%); (j) 4 M HCl, dioxane, H_2O, rt, 9 h (78%); (k) benzyl 2-bromoacetate or *t*-butyl 2-bromoacetate, Et_3N, DCM, 0 °C to rt, 2 h (99%).

furnished negamycin (**1**). Similar transformations on β-amino ester **3b** gave 5-epinegamycin (**8**).

Protection of the amine group in **2a** (Scheme 2) with a fluorenylmethoxycarbonyl group (Fmoc) and removal of the benzyl protecting group via catalytic hydrogenation gave the acid, which was then immobilized on Sasrin resin under Mitsunobu reaction conditions to give resin bound hydrazine derivative **10**. The unreacted hydroxyl groups on the resin were capped with acetyl groups. The Fmoc group was removed and resin bound hydrazine **10** was reacted with activated β-amino acid **3a** to give resin bound azide **11**. The residual amino groups on the resin were capped with acetyl groups. The azide was reduced to an amine using triphenylphosphine, then acylated with various acids under standard coupling conditions to give the resin bound amides. Cleavage of the amides from the resin with 10% TFA followed by treatment with 4 M hydrochloric acid in dioxane resulted in global deprotection to furnish amides **12** in a library format.

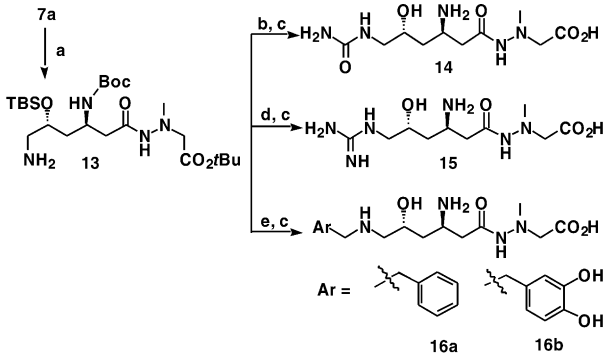


Scheme 2. Solid-phase parallel synthesis of N-terminal amides. Reagents and conditions: (a) FmocOSu, Na_2CO_3, dioxane, 0 °C to rt, 18 h (80%); (b) H_2, Pd/C, EtOAc, rt, 18 h, (100%); (c) sasrin resin, PPh_3, DIAD, THF, 0 °C to rt, 16 h; (d) Ac_2O, pyridine, rt, 3 h; (e) 20% piperidine in DMF, 30 min; (f) **3a**, Et_3N, DMF, rt, 24 h; (g) Ac_2O, pyridine, rt, 3 h; (h) PPh_3, THF, H_2O, 28 h; (i) RCO_2H, HATU, DIEA, DMF, rt, 16 h; (j) 10% TFA in DCM, rt, 1 h; (k) 4 M HCl in dioxane, rt, 1 h.

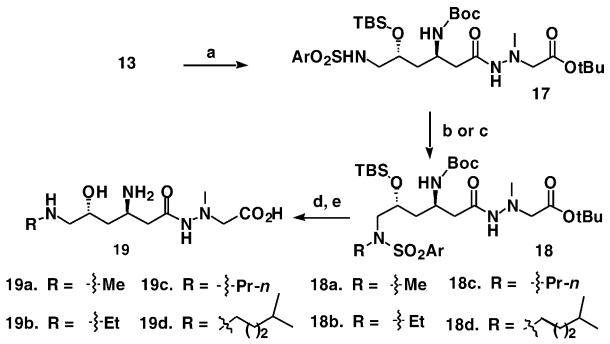
Reduction of azide **7a** (Scheme 3) with triphenylphosphine gave amine **13**. Reaction of amine **13** with trimethylsilylisocyanate and removal of the protecting groups furnished urea analogue **14**. Reaction of amine **13** with 2-methyl-2-thiopseudourea sulfate followed by removal of the protecting groups under acidic conditions gave guanidine analogue **15**. Further, amine **13** was also reacted with benzyl bromide and 3,4-diacetoxybenzyl bromide[11] and treated with acid to generate monoalkylated analogues **16a** and **16b**, respectively.

An alternative approach to the synthesis of *N*-alkyl negamycin derivatives based on Fukuyama chemistry[12] is briefly summarized in Scheme 4. Sulfonamide **17**, obtained by treating amine **13** with 2-nitrobenzenesulfonyl chloride, was reacted with a set of alkyl halides using cesium carbonate as a base to produce alkylated sulfonamides **18a–c**. The alkylated analogue **18d** was obtained by treatment of sulfonamide **17** with 4-methyl-1-pentanol under Mitsunobu reaction conditions. Removal of the 2-nitrobenzenesulfonamide protecting group in **18a–d** by treatment with thiophenol in the presence of potassium carbonate followed by global deprotection under acidic conditions gave the desired *N*-alkylated negamycins **19a–d**.

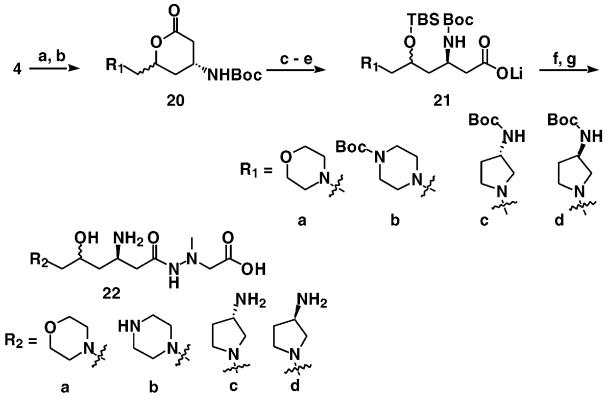
To access *N*,*N*-dialkylated analogues, the iodolactone (Scheme 5) obtained from homoallylglycine **4** as descri-



Scheme 3. N-terminal modifications of negamycin. Reagents and conditions: (a) PPh_3, THF, H_2O, 14 h (80%); (b) trimethylsilyl isocyanate, DCM, rt, 18 h (60%); (c) 4 M HCl in dioxane, H_2O, rt, 2 h (96%); (d) 2-methyl-2-thiopseudourea sulfate, NaOAc, *i*-PrOH, reflux, 8 h (40%); (e) $ArCH_2Br$, DIEA, DMF, rt, 16 h (67–77%, for two steps).



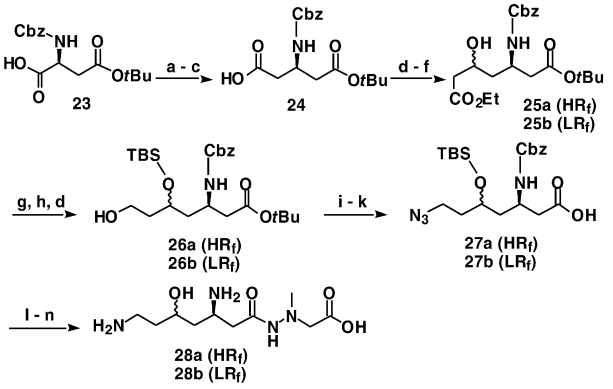
Scheme 4. Mono *N*-alkylated negamycin analogues. Reagents and conditions: (a) 2-nitrobenzenesulfonyl chloride, collidine, DCM, rt, 8 h (90–97%); (b) ROH, PPh_3, DIAD, DCM, 4 °C to rt, 3 h (80%); (c) $CsCO_3$, RI, DMF, rt, 2 h (94%); (d) PhSH, K_2CO_3, DMF, rt, 12 h (76%); (e) 4 M HCl in dioxane, H_2O, rt, 2 h (96%).



Scheme 5. *N,N*-dialkylated negamycin analogues. Reagents and conditions: (a) I_2, KI, $NaHCO_3$, H_2O, DCM, rt, 2.5 h (88%); (b) R_1NHR_2, DMF, 70 °C, 17 h (38–65%); (c) LiOH, H_2O, MeOH, 0 °C to rt, 24 h; (d) TBS-Cl, imidazole, DCM, 0 °C to rt, 24 h; (e) K_2CO_3, MeOH, H_2O, rt, 3 h; (f) HATU, DIEA, DMF, 0 °C to rt, 17 h (35–47%, last four steps); (g) 4 M HCl in dioxane, H_2O, rt, 7 h (quantitative).

bed above (Scheme 1), was alkylated with various cyclic amines to obtain lactones **20a–d**. Opening of lactones **20a–d** under basic conditions followed by protection of the secondary hydroxyl group as a TBS ether then base hydrolysis of the silyl ester furnished amino acids **21a–d** as lithium salts on lyophilization. Coupling of lithium salts **21a–d** with hydrazine **2b** using HATU as a coupling agent followed by acidic treatment gave the desired analogues **22a–d** as a mixture of diastereomers.

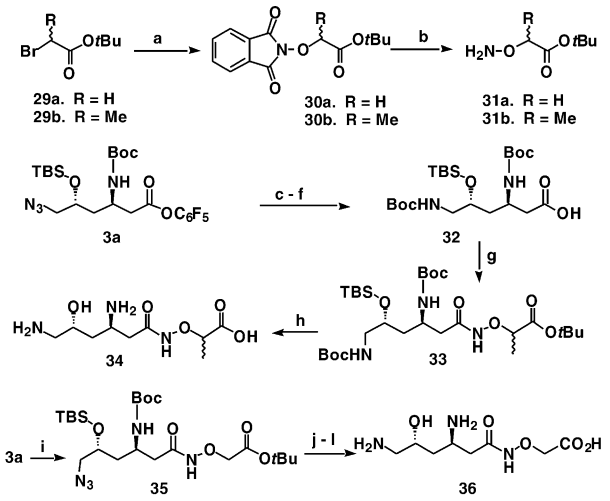
An analogue in which the terminal amine was extended by one methylene unit was desired to investigate the role of chain length in the β-amino acid portion of the molecule. Homologation of aspartic acid **23** (Scheme 6) under Arndt–Eistert[13] reaction conditions, followed by ester hydrolysis gave desired acid **24**. The alcohol obtained by reduction of acid **24** under mixed anhydride[14] reaction conditions was oxidized to the corresponding aldehyde then reacted with ethyl bromoacetate under modified Reformatsky[15] reaction conditions to furnish a mixture of diastereomeric alcohols **25a** and **25b**, separated by column chromatography. After protecting the secondary hydroxyl group in **25a** as its TBS ether, the ethyl ester was selectively hydrolyzed and reduced to alcohol **26a** by the mixed



Scheme 6. Synthesis of homonegamycin. Reagents and conditions: (a) *i*-butyl chloroformate, NMM, THF, −15 °C, 45 min; then ethereal solution of CH_2N_2; (b) $C_6H_5CO_2Ag$, Et_3N, MeOH, (79%, for two steps); (c) LiOH, H_2O, THF, 0 °C to rt, 1.5 h (89%); (d) *i*-butyl chloroformate, NMM, THF, −15 °C, 45 min; then $NaBH_4$ followed by H_2O (68%); (e) DMSO, oxalyl chloride, TEA, −60 to −40 °C (93%); (f) $BrCH_2CO_2Et$, Et_2Zn, $RhCl(PPh_3)_3$, THF (57%); (g)TBS-Cl, imidazole, THF, rt, 18 h (81%); (h) LiOH, H_2O, MeOH, rt, 20 h (93%); (i) CH_3SO_2Cl, Et_3N, DCM, 0 °C to rt, 2 h; (j) NaN_3, DMF, 70 °C, 2 h (69%); (k) 2 M KOH, MeOH, rt, 16 h, (89%); (l) **2b**, HATU, DMF, 0 °C to rt, 14 h (62%); (m) H_2, Pd/C, EtOAc, rt, 2.5 h (92%); (n) 4 M HCl, dioxane, H_2O (100%).

anhydride[14] procedure. Hydroxy derivative **26a** was further transformed to the corresponding azido acid **27a** in three classical steps: mesylation, nucleophilic displacement using sodium azide then hydrolysis of the *t*-butyl ester under basic conditions. β-Amino acid **27a** was then coupled with hydrazine **2b**, using HATU as a coupling reagent. Reduction of the azide to the corresponding amine by catalytic hydrogenation and removal of the protecting groups under acidic conditions gave homonegamycin **28a**. Similar transformations on **25b** gave homonegamycin analogue **28b**, a diasteromer of **28a**.

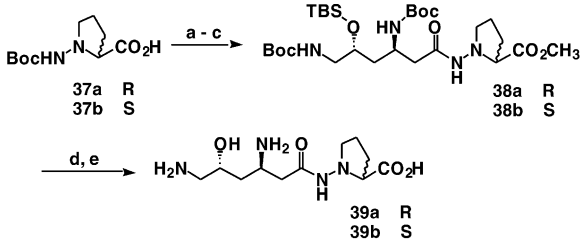
C-Terminal analogues were obtained by coupling of β-amino acid intermediate with various derivatives. Alkylation of *N*-hydroxyphthalimide with *t*-butyl 2-bromoacetate **29a** or with *t*-butyl 2-bromopropionate **29b** (Scheme 7) followed by removal of the phthalimide protecting group using hydrazine gave the desired *O*-alkyl hydroxylamines **31a** and **31b**, respectively. *Trans* esterification of activated ester **3a** with methanol followed by reduction of the azide group using catalytic hydrogenation gave the primary amine. The amine was then protected with a Boc group and the ester group was hydrolyzed to give desired β-amino acid **32**. Coupling of β-amino acid **32** with **31b** using HATU as a coupling reagent and global deprotection gave hydroxamate **34**. Condensation of β-amino ester **3a** with *O*-alkyl hydroxylamine **31a** gave the fully protected hydroxamate **35**. Reduction of azide **35** to the corresponding amine using triphenylphosphine followed by treatment with 30% trifluoroacetic acid in dichloromethane resulted in removal of the Boc and *t*-butyl protecting groups, with the TBS group intact. Removal of the TBS group was accomplished using HF in pyridine which was buffered with excess pyridine to generate desired hydroxamate **36**.



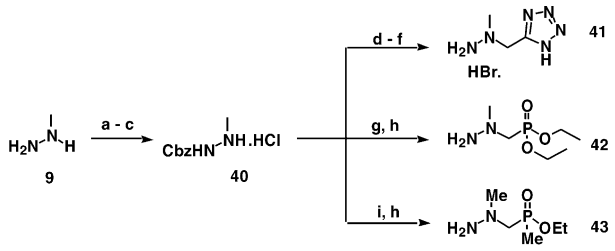
Scheme 7. Hydroxamate ethers. Reagents and conditions: (a) NaH, DMF, 0 °C to rt (85%); (b) NH_2NH_2, EtOH, rt (50%); (c) MeOH, Et_3N, rt, 8 h (quantitative); (d) H_2, 5% Pd/C, MeOH, 4 h; (e) $(Boc)_2O$, THF, Et_3N, rt; (f) KOH, MeOH, rt, 14 h (75%, three steps); (g) **31b**, HATU, DIEA, DMF; (h) 4 M HCl in dioxane, H_2O, rt (30%, for two steps); (i) **31a**, DMF, rt, 24 h (68%); (j) PPh_3, THF, H_2O, rt, 16 h (93%); (k) 30% TFA in DCM, rt, 4 h; (l) HF, pyridine, rt, 2.5 h (67%, for two steps).

Conformationally restricted C-terminal analogues were generated from D- and L-aminoproline derivatives. Aminoprolines **37a** and **37b** (Scheme 8) were treated with diazomethane to produce the corresponding methyl esters, which were treated with trifluoroacetic acid in DCM to remove the Boc group. The resultant amines were coupled with β-amino acid **32** using HATU as coupling reagent. Removal of the protecting groups in **38a** and **38b** gave analogues **39a** and **39b**, respectively.

Obtaining analogues with C-terminal acid surrogates required the synthesis of several building blocks. Selective protection of the more reactive secondary amine[16] in *N*-methylhydrazine **9** (Scheme 9) with a Boc group followed by treatment with benzyl chloroformate and removal of the Boc group under acidic conditions gave hydrazine **40**. *N*-Alkylation of hydrazine **40** using chloroacetonitrile followed by cycloaddition with azidotributyltin[17] and then treatment with hydrobromic acid furnished hydrazine **41**. For the preparation of phosphonate analogue **42**, the triflate derived from di-



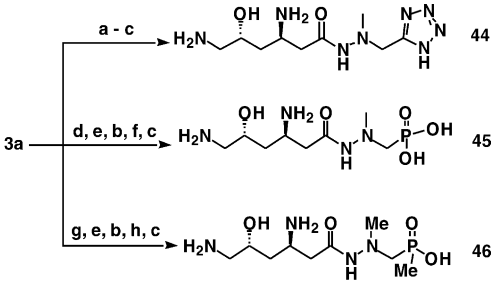
Scheme 8. Conformationally restricted C-terminal analogues. Reagents and conditions: (a) CH_2N_2, Et_2O, THF, 0 °C; (b) 20% 4 N HCl in dioxane, 30 min, rt (98%) ; (c) **32**, HATU, DIEA, DMF, 0 °C to rt, 8 h (44%); (d) 1 N NaOH, MeOH, rt, 3 h; (e) 4 M HCl in dioxane, H_2O, rt, 4 h (90%, two steps).



Scheme 9. Acid surrogate building blocks. Reagents and conditions: (a) $(Boc)_2O$, $NaHCO_3$, EtOAc, 0 °C to rt, 3 h (100%); (b) Cbz-Cl, $NaHCO_3$, EtOAc, 0 °C to rt, 1 h (60%); (c) 4 M HCl in dioxane, rt, 4 h (100%); (d) $ClCH_2CN$, DMF, rt, 14 h (87%); (e) Bu_3SnN_3, 110 °C, 72 h (83%); (f) HBr, AcOH, rt, 1 h (64%); (g) diethyl (trifluoromethylsulfonylmethyl)phosphonate, DIEA, toluene, rt, 4 h (47%); (h) Pd/C, EtOAc, rt, 1 h (92%); (i) diethyl methylphosphonate, HCHO, THF, rt, 1 h, reflux, 1 h (51%).

ethyl (1-hydroxymethyl)phosphonate[18] was used to *N*-alkylate hydrazine **40** and the benzyloxycarbonyl group was removed under catalytic hydrogenation conditions. A three component condensation[19] between hydrazine **40**, formaldehyde and diethyl methylphosphonate followed by removal of the benzyloxycarbonyl group by catalytic hydrogenation resulted in phosphinate **43**.

Condensation of hydrazine **41** (Scheme 10) with activated ester **3a** followed by reduction of the azide to the corresponding amine and removal of the protecting groups under acidic conditions gave tetrazole analogue **44**. Hydrazine **42** was condensed with activated ester **3a** and the TBS protecting group was removed using acetic acid and water containing a catalytic amount of hydrofluoric acid to give the hydroxy azide intermediate. Reduction of the azide via hydrogenation followed by treatment with trimethylsilyl iodide, then with hydrochloric acid in dioxane, furnished phosphonic acid **45**. Similarly, condensation of activated ester **3a** with hydrazine **43** and removal of the silyl-protecting group gave a hydroxy azide intermediate. The azide was reduced to the amine by catalytic hydrogenation, the phosphinate ester was hydrolyzed to the corresponding phoshinic acid under basic conditions and the Boc group was cleaved in acidic conditions to generate phosphinic acid **46**.



Scheme 10. Negamycin analogues with C-terminal acid surrogates. Reagents and conditions: (a) **41**, Et_3N, DMF, rt, 24 h (31%); (b) PPh_3, THF, H_2O, rt 16 h or H_2, Pd/C, EtOAc, rt, 4 h (60–87%); (c) 4 M HCl in dioxane, H_2O; (d) **42**, Et_3N, DMF, rt, 4 h (78%); (e) AcOH, HF, THF, H_2O, rt, 11 h (72%); (f) TMSI, CH_3CN, 0 °C to rt, 18 h (9%); (g) **43**, Et_3N, DMF; (h) LiOH, H_2O, EtOH, 18 h (12% for two steps).

Table 1. MICs[21] and protein synthesis inhibition data (TC/TL)[23]

Organisims/Compd	MIC (μg/mL)										
	1	**8**	**12a**	**14**	**15**	**16a**	**16b**	**19a**	**19b**	**19c**	**19d**
E. coli ATCC 25922	4	128	16	>256	>256	64	128	16	16	>256	>256
E. coli MG1655	4	128	16	>256	>256	32	32	8	8	>256	>256
E. coli MG1655 *tolC*	2	32	8	>256	128	16	8	4	4	>256	>256
Klebsiella pneumoniae ATCC 113882	2	64	32	>256	>256	32	32	4	8	>256	>256
Enterobacter cloacae ATCC35030	8	128	16	>256	>256	64	128	16	16	>256	>256
Pseudomonas aeruginosa PA013	32	>512	64	>256	>256	>128	>128	128	128	>256	>256
S. aureus ATCC 25293	32	512	256	>256	>256	>128	128	128	>256	>256	>256
Streptococcus pneumoniae VSPN1005	32	ND	ND	ND	ND	128	>128	256	ND	>256	>256
TC/TL IC_{50} (μM)	1.0	23.4	3.5	>80	>80	0.8	0.6	2.3	4.5	20.3	>80

ND, not determined. Analogues **22a–d**, **28a–b**, **34**, **36**, **39a–b**, **44**, **45** and **46** are inactive (MIC >256 μg/mL and IC_{50} >80 μM).

Results and Discussion

A flexible method for the synthesis of negamycin and its analogues was developed starting from commercially available 3-*R*-*t*-butoxycarbonylaminohex-5-enoic acid[8] (**4**). The orthogonally protected advanced intermediate **3a** was used in the synthesis of *N*-acylated analogues on a solid support. This method enabled us to generate a library of 180 discrete compounds.[20] In vitro evaluation of the antimicrobial properties[21] of amide analogues against Gram-positive and Gram-negative organisms indicated that all analogues were inactive except leucylnegamycin **12a**, which is 2–16 times less active than negamycin (Table 1). Interestingly, leucylnegamycin has been proposed as a biosynthetic precursor of negamycin.[7a] The transformation of the N-terminal amine in negamycin to the corresponding urea and guanidine derivatives resulted in analogues **14** and **15**, respectively, which are devoid of antibacterial activity. On the other hand, the antibacterial activity of *N*-methyl and *N*-ethyl derivatives **19a** and **19b** are comparable to that of negamycin (**1**). The *N*-methyl analogue is also efficacious in an *E. coli* murine septicemia model[22] (ED_{50} = 16.3 mg/kg). The analogues bearing slightly longer alkyl groups (**19c** and **19d**) and the cyclic-alkyl groups (**22a–d**) exhibited a drastic loss in antibacterial activity. These results dictate a stringent requirement for a basic amino group that is either unsubstituted or has small linear alkyl groups (methyl or ethyl) on it. An exception to this observation are the *N*-benzyl derivatives **16a** and **16b**, which are significantly less active than negamycin (**1**) but retain some antibacterial activity. In contrast, homonegamycin analogues **28a** or **28b** lack antibacterial activity. These observations indicate that not only is the basic N-terminal primary amine essential for antibacterial activity, but its distance from the hydroxyl group or from the internal amine is also critical for antibacterial activity. Replacement of the C-terminal acid with the hydroxamate ether moiety, as in analogue **34** and **36**, resulted in inactive analogues. Replacement of the acid with surrogates such as tetrazole, phosphonic acid, and phosphinic acid (**44**, **45** and **46**, respectively) also resulted in complete loss of antibacterial activity. In addition to a whole cell assay,[21] all the analogues were evaluated in a cell-free protein synthesis assay.[23] The *N*-methyl and *N*-ethyl analogues, **19a** and **19b** respectively, are slightly less active than negamycin in both assays. The *N*-benzylated analogues **16a** and **16b** are equipotent to negamycin in cell-free protein synthesis assay but these analogues are significantly less active than negamycin in the whole cell assay. These analogues are not significantly effluxed in *E. coli* (Table 1) suggesting they may not be getting transported as efficiently into the bacterial cell. All other analogues are inactive in either the whole cell or the protein synthesis assays (MIC >256 μg/mL; IC_{50} >80 μM).

In conclusion, the small molecule antibacterial agent negamycin appears to have stringent requirements with regard to the chemical nature of the basic N-terminal and acidic C-terminal groups and also with regard to the spacing of the basic amino group relative to the remainder of the functional groups on the scaffold. These results suggest an active-transport mediated event may account for the permeability of such a small but highly charged molecule across the cell wall membrane of Gram-positive and across Gram-positive organisms. Our efforts are now focused on probing the importance of the internal functional groups of negamycin for antibacterial activity and will be communicated in due course.

References and Notes

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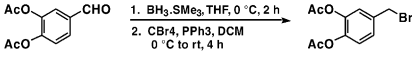
11. 

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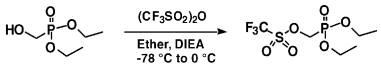
18. 

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20. Final compounds were cleaved from resign into 96-well plate and screened against *S. aureus* ATCC25293, *E. coli* ATCC25922 and *E. coli* MG1655. Hits identified from this screen were re-synthesized and their MIC's were determined against an expanded panel of organisms. *E. coli* MG1655 *tolC* is an efflux pump mutant.

21. Micro dilution MICs were determined in Mueller–Hinton broth supplemented with 50% human serum and an inoculum size of 5×10^5 cfu/mL.

22. In vivo efficacy: Mice were infected ip with approximately 1×10^5 cfu of *E. coli* ATCC25922. The compound was administered iv at 1 and 5 h after infection. Survival was monitored for 7 days and the ED_{50} calculated by non-linear regression. Ampicillin was used as a standard in this study ($ED_{50} = 2.3$ mg/kg). ED_{50} for negamycin 5.1 mg/kg.

23. Inhibition of protein synthesis was measured with a cell-free transcription/translation coupled assay (*E. coli* S30 Extract System, Promega, Madison, WI, USA) using pGEMbgal as a DNA template. Compounds were pre-incubated with the reaction mixture for 10 min prior to the addition of template. After 1 h of incubation, ONPG was added and β-galactosidase activity was monitored at 420 nm.