New Inhibitors of Bacterial Protein Synthesis from a Combinatorial Library of Macrocycles

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A mixture-based combinatorial library of 14-membered macrocycles was synthesized to target ribosomal RNA and uncover a new class of antibacterial agents. High-throughput screening identified a macrocyclic mixture that inhibited cell-free-coupled transcription/translation in *Escherichia coli*-derived extracts, with an IC$_{50}$ value in the 25−50 μM range. In a follow-up library of 64 single macrocycles, 8 gave IC$_{50}$ values ranging from 12 to 50 μM in the cell-free protein synthesis inhibition assay. Some of the macrocycles were screened in a translation inhibition assay, and IC$_{50}$ values generally paralleled those obtained in the uncoupled transcription/translation assay. Additional analogues were prepared in a preliminary structure−activity relationship study, and more potent macrocycles were identified with low micromolar activity (IC$_{50}$ values = 2−3 μM). Some of these macrocycles displayed antibacterial activity against lipopolysaccharide mutant *E. coli* bacterial cells (IC$_{50}$ values = 12−50 μM).

Introduction

Due to the rapid growth of drug resistant strains of pathogenic bacteria, the search for new antibacterial agents is of utmost importance.[1] Bacterial ribosomal RNA (rRNA) is an attractive target for developing new antibacterial drugs: the targets are universally conserved in pathogens, are critical to their life cycle, represent core pathogen vulnerability, and have appealing biochemical properties for drug targeting.[2−4]

There are many clinically useful antibiotic classes of drugs that interact with rRNA and inhibit protein synthesis. Examples include aminoglycosides,[5,6] macrolides,[7] tetracyclines,[8] glycylcyclines,[9] ketolides,[10] oxazolidinones,[11] and streptogramins.[12] The structurally diverse nature of these inhibitors suggests that a multiplicity of unique rRNA binding sites are available for effective small molecule drug interactions. Selectivity in RNA drug binding can be achieved by targeting specific three-dimensional structural elements formed by bulges, hairpins, junctions, or asymmetries in the deep and shallow grooves around noncanonical base pairs.[4]

Our initial goal was to prepare compounds targeted at rRNA that may serve as new antibacterial agents. It has been reported that aminoglycosides induce a conformational change on the target RNA sequence, supporting an induced-fit model for ligand−RNA binding.[13,14] With this in mind, we decided to pursue a cyclic scaffold to constrain the potential drug−RNA complex and strengthen the inherent binding interactions. From a synthesis perspective, we decided to prepare a large number of macrocycles on solid support using combinatorial chemistry. This led us to pursue a 14-membered aryl ether-linked scaffold incorporating a backbone of amino acid-type building blocks (Figure 1).[15] The template appealed to us because a variety of RNA recogni-
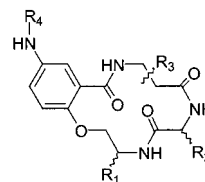

Figure 1. Macrocyclic scaffold.

tion elements could be incorporated as building block side chains, many antibiotics are known to have cyclic amino acid sequences,[16,17] and several macrolide antibiotics have 14-membered ring backbones.[7]

Our synthetic strategy for the 14-membered macrocyclic template was described in a preliminary publication.[19] We reported that the macrocycle can be varied in a combinatorial fashion using readily available building block α-amino alcohols, α-amino acids, β-amino acids, carboxylic acids, and isocyanates. Therefore, functional groups important for RNA recognition can be conveniently incorporated into the macrocyclic ligand to optimize potential hydrogen-bonding, electrostatic, and π-stacking interactions.

In this paper, we report the discovery of a new class of in vitro bacterial (prepared from *Escherichia coli* extracts[20]) protein synthesis inhibitors derived from a mixture-based macrocyclic combinatorial library. Analogues were prepared to optimize potency in the coupled transcription/translation assay and gain further knowledge about structure−activity relationships. Because bacterial translation inhibition IC$_{50}$ values roughly parallel uncoupled transcription/translation inhibition IC$_{50}$ values, it is suggested that the protein synthesis inhibition arises primarily from a translation inhibition mechanism.

Chemistry

General Macrocycle Synthesis. The solid-phase synthesis of the macrocycles is outlined in Scheme 1.

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Scheme 1[a]

[Scheme showing reaction sequence with steps i,ii; iii; iv,v; vi; vii,viii; ix; x,xi]
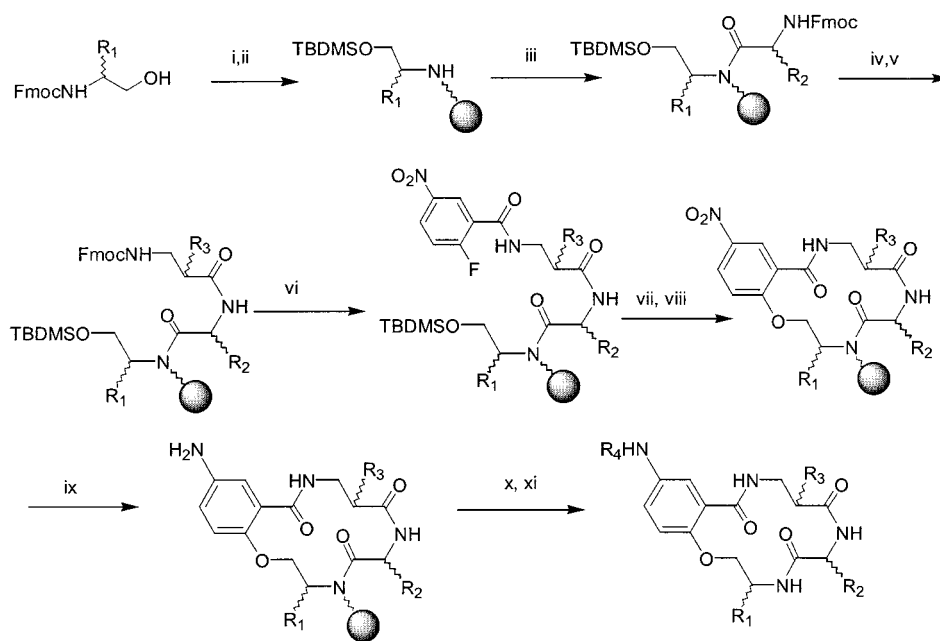
[a] Reagents and conditions: (i) 1.3 equiv of Fmoc-amino alcohol (Fmoc-NHR$_1$CO$_2$H), 2.0 equiv of NaOH, MeOH, 4 h; 2.2 equiv of AcOH, 30 min; 1.0 equiv of ArgoGel-MB-CHO, CH(OMe)$_3$, RT, 24 h; 2 equiv of BH$_3$-pyridine, 2 equiv of AcOH, RT, 24 h; (ii) 3.0 equiv of TBDMS-Cl, 3.0 equiv of TEA, 0.1 equiv of DMAP, DCM, RT, 24 h; (iii) 0.2 M Fmoc-α-amino acid, 0.2 M DIC, DMF, RT, 24 h; (iv) 20% piperidine, DMF; (v) 0.12 M Fmoc-β-amino acid (Fmoc-NHCH$_2$CHR$_3$CO$_2$H), 0.12 M HATU, 0.25 M collidine, DMF, RT, 24 h; (vi) 0.12 M 2-fluoro-5-nitrobenzoic acid, 0.12 M HATU, 0.25 M collidine, DMF/DCM (1/1; v/v), RT, 24 h; (vii) 0.2 M TREAT-HF, THF, RT, 24 h; (viii) 0.2 M DBU, DMF; (ix) 1.5 M SnCl$_2$, DMF/EtOH (10:1); (x) 0.12 M carboxylic acid, 0.12 M HATU, 0.25 M collidine, DMF, RT, 24 h or 0.2 M isocyanate, DMF, RT, 24 h; (xi) TFA/TIS (95:5).

For Fmoc-protected amino alcohols, a one-pot Fmoc deprotection/reductive amination protocol was followed, while for amino alcohols without side chain protection, the reductive amination step was carried out directly. In the reductive amination step, the amino alcohol was reacted with ArgoGel-MB-CHO resin using BH$_3$-pyridine.[21] The resin-bound amino alcohol was then *tert*-butyldimethylsilyl (TBDMS) protected, and then an Fmoc-protected α-amino acid was reacted with the secondary amine. After Fmoc deprotection, an Fmoc-protected β-amino acid was coupled to the N-terminus. After another Fmoc deprotection step, the cyclization linker 2-fluoro-5-nitrobenzoic acid was incorporated into the backbone. TBDMS deprotection was then removed with triethylamine trihydrofluoride (TREAT-HF), and the structure was cyclized by a base-induced S$_N$Ar reaction.[22,23] The aryl nitro group was reduced with tin-(II) chloride,[22] and then functionalized. The macrocycles were cleaved from solid support with TFA to provide library members in >85% purity, according to LC−MS analysis. All macrocycles were obtained as TFA salts.

Mixture-Based Combinatorial Library. A combinatorial library of approximately 12 000 macrocycles was prepared using IRORI technology[24] for directed-sorting split-and-mix synthesis. The side chain diversity incorporated in the library is outlined in Figure 2. Each IRORI MicroKan was loaded with a mixture of the TBDMS-protected amino alcohol resins. Each canister contained an equimolar mixture of the amino alcohol-loaded resins. A total of 4 different resin mixtures derived from 8 different amino alcohols (Fmoc-D,L-Tyr-(*t*Bu)-ol/D,L-Phe-ol, Fmoc-D,L-Gln(Trt)-ol/Fmoc-D,L-Ser-(*t*Bu)-ol, Fmoc-D,L-Arg(*t*Bu)-ol/Fmoc-D,L-Lys(*t*Bu)-ol, Gly-ol/Fmoc-Glu(*t*Bu)-ol) were used in the library. In the first synthesis step with the derivatized resins, a total

of 10 Fmoc-amino acids were reacted as single D- or L-isomers (step iii, Scheme 1). After Fmoc deprotection, 4 β-amino acid racemic mixtures and Fmoc-β-alanine were coupled (step v, Scheme 1). In the final diversity step, 7 carboxylic acids and 1 isocyanate (4-methoxy-benzylisocyanate) were used to functionalize the arylamine (step x, Scheme 1). For one set of macrocycles, the arylamine was left unreacted. Resin cleavage into individual wells gave 3−8 product macrocycles depending on the starting resin mixture. It was estimated that 0.10 mmol of a macrocycle mixture was obtained from the resin cleavage from the contents of a single canister. After resin cleavage and sample drying, the samples were dissolved to ca. 20 mM in DMSO. The library was designed so that each well contained a mixture of macrocycles with only two different molecular weights to simplify LC−MS analysis. All wells were analyzed by LC−MS, and the sum of the integrated peaks of the expected molecular ions was generally >85%.[25]

Synthesis of the Follow-Up Library. We identified one active well (mixture of 8 macrocycles, Figure 3) in the cell-free-coupled transcription/translation inhibition assay from high-throughput screening. None of the wells gave MIC (minimum inhibitory concentration) activity against either *E. coli* or *S. aureus* pathogenic bacterial strains at 100 μM. To follow up on the activity in the transcription/translation assay, a 64-member library of single compounds was prepared (4 R$_1$ × 8 R$_2$ × 2 R$_3$, Figure 4). The library included the 8 macrocycles from the mixture along with additional analogues incorporating other aromatic diversity at R$_2$. The library was designed to explore all stereoisomers. The synthesis scale was doubled (IRORI MiniKans with 60 mg of amino alcohol-loaded resin) so sufficient material (ca.
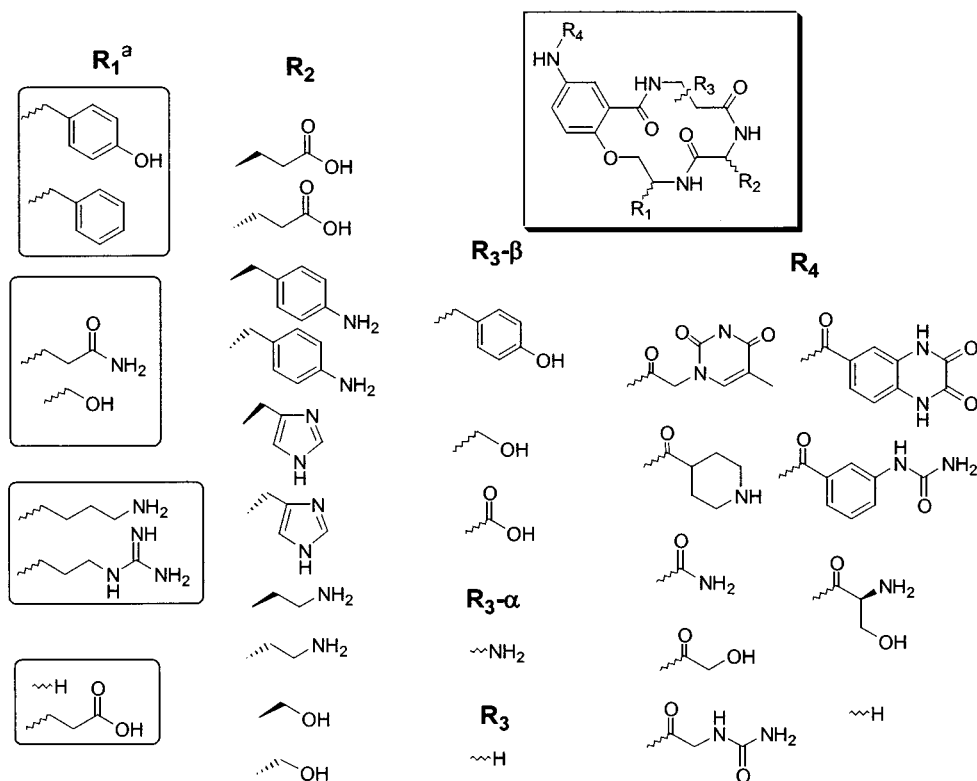
Figure 2. Mixture-based combinatorial library (15 $R_1 \times 10 R_2 \times 9 R_3 \times 9 R_4$). R_1 denotes R_1-encapsulated product functionalities which came from amino alcohol-derivatized resins that were premixed in a single canister at the outset of the combinatorial synthesis.
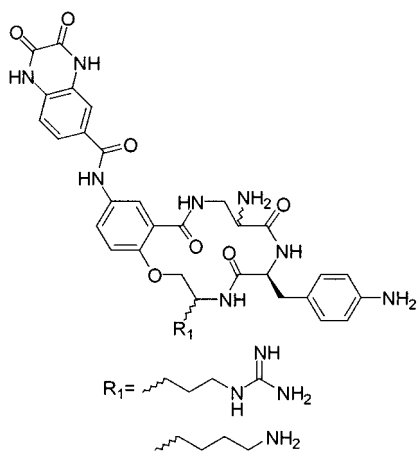

Figure 3. Active mixture (in vitro protein synthesis inhibition, $IC_{50} = 25-50 \ \mu M$).

0.2 mmol) could be obtained for future testing and characterization.

The 64-member library was prepared, using 4 amino alcohol-derived resins (R_1), 8 α-amino acids (R_2), and 2 β-amino acids (R_3) according to Scheme 1. After cleavage and drying of the resin, product macrocycles were dissolved to 20 mM in DMSO (>85% purity) and screened in the transcription/translation inhibition assay. In an initial screening, 8 macrocycles (Table 1) inhibited the assay >70% at 100 μM. Only macrocycle **1** was a member of the macrocyclic mixture of Figure 3. This suggests that the inhibitory activity of the macrocyclic mixture comes primarily from macrocycle **1**. None of the 64 macrocycles gave MIC activity against either *E. coli* or *S. aureus* pathogenic bacterial strains at 100 μM. All compounds in Table 1 were HPLC-

purified and rescreened in the transcription/translation assay to obtain IC_{50} values. Some representative compounds (**1**–**3**, **6**, and **7**) were screened in a translation inhibition assay, and those IC_{50} values are also reported in Table 1.

Analogues from a Resin-Bound Macrocyclic Precursor. Additional macrocycles were prepared from a common resin-bound macrocycle. The macrocyclic resin (150 mg) was loaded into canisters, and the remaining synthesis steps were carried out according to Scheme 2. For the synthesis of macrocycles **9**–**16** (Table 2), the starting resin was reacted with a variety of carboxylic acids and 2 sulfonyl chlorides (step i, Scheme 2). For the synthesis of macrocycles **17**–**21** (Table 3), the starting resin was reacted with an Fmoc-N-protected carboxylic acid, deprotected, and then coupled to 1,4-dihydroquinoxaline-2,3-dione-6-carboxylic acid (steps iii–v, Scheme 2). Macrocycles **11**–**14** and **17**–**21** were prepared using commercially available carboxylic acids. Macrocycles **9**, **10**, **15**, and **16** were synthesized using building blocks prepared by standard literature procedures. After the initial screening with unpurified samples, macrocycles **15**–**17**, **19**, and **20** were HPLC-purified and rescreened in the transcription/translation assay.

Results and Discussion

In Vitro Transcription/Translation. A cell-free-coupled transcription/translation assay derived from *E. coli* extracts is often used to find leads for the development of ribosomally active antibacterial agents.[26] When our large mixture-based macrocyclic library was screened in this assay, we identified one well with an IC_{50} value of 25–50 μM (total macrocycle concentration). All other
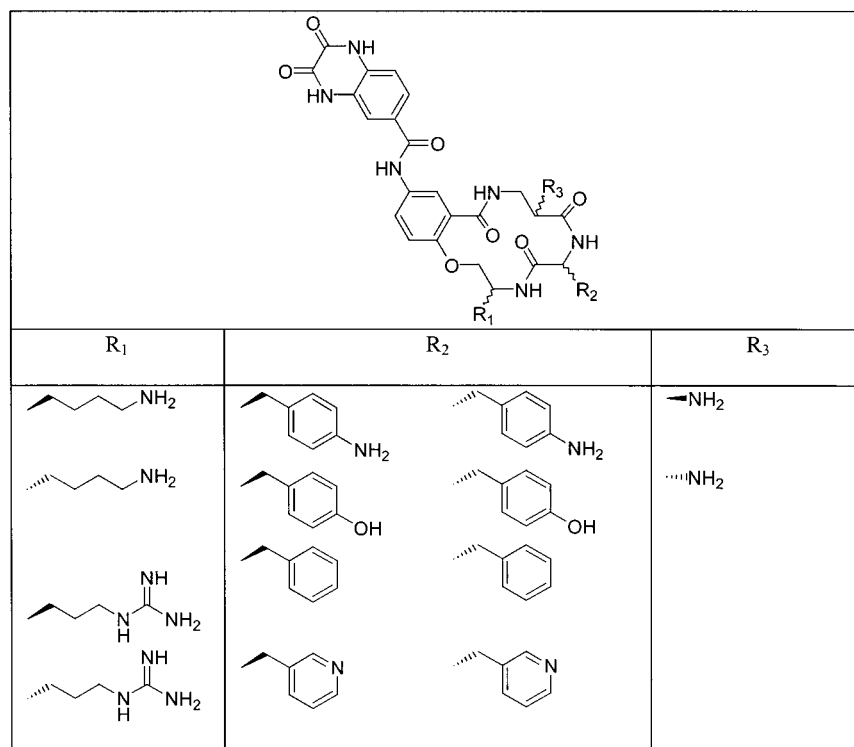
Figure 4. Combinatorial library of 64 macrocycles.

wells were considered inactive as they did not inhibit the assay at a minimum level of 70% at 100 μM. For the large combinatorial library, the well "hit rate" in the transcription/translation assay is approximately 0.06% (1 active well/1800 wells). The activity is very sensitive to the character of the R_1–R_4 substituents as well as the stereochemistries of the R_1–R_3 side chains. We are able to gain some valuable structure–activity relationship (SAR) information from the large mixture-based combinatorial library and the 64-member library of single macrocycles.

First, only amino alcohol imputs with positively charged side chains at R_1 (Arg-ol and Lys-ol) gave active macrocycles (Table 1). Second, because only the *p*-aminobenzyl substituent at R_2 gave active compounds, an aromatic functionality is presumably required at this position. Third, for the β-amino acid-derived portion of the macrocycle, only the α-amino side chain functionality provided active compounds. For the last diversity position, only some 1,4-dihydroquinoxaline-2,3-dione-functionalized macrocycles were effective at inhibiting the assay.

In the follow-up library of 64 macrocycles, a variety of macrocycles with aromatic R_2 substituents were also found to be active, indicating that the nature of the aromatic residue does not have a strong influence on transcription/translation activity. However, a wider range of aromatic substitutions would have to be incorporated in a future SAR study to test this generality.

Seven out of eight macrocycles that were potent in the coupled transcription/translation assay had an Arg side chain incorporated at R_1. The importance of the guanidino functionality for activity is in line with other reports where the group has been identified as a type of RNA recognition element.[27] The guanidino group is unique in its ability to remain protonated over a large pH range, thus, enhancing its affinity for phosphate group interaction.
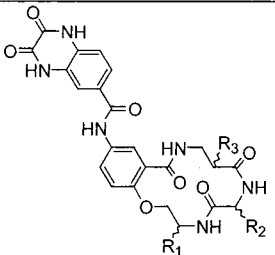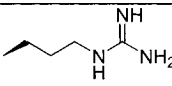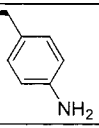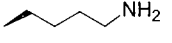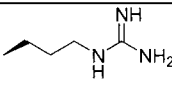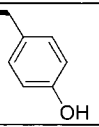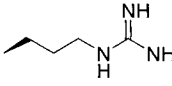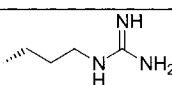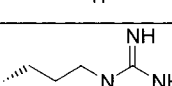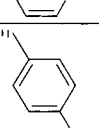
The relative stereochemical orientation of side chain functionality (R_1–R_3) is important for activity. The R_1 and R_2 groups are required to be on the same face of the macrocyclic structure, with R_3 on the opposite face. However, because the enantiomeric pairs [(**1**,**6**), (**3**,**7**), and (**4**,**5**)] have similar activity, the absolute stereochemistry does not appear to be important.

In our macrocyclic series, only the 1,2,3,4-tetrahydro-2,3-dioxo-6-quinoxalinecarboxyl functionality at R_4 led to active macrocycles in addition to a specific R_1–R_3 substitution pattern. Some preliminary SARs were explored at R_4, keeping the R_1–R_3 groups constant (Table 2). The parent macrocycle **1** had an IC_{50} of 16 μM in the transcription/translation assay. The introduction of a 7-nitro substituent (macrocycle **9**) and 1,4-dimethyl substituents (macrocycle **10**) to the 1,2,3,4-tetrahydro-2,3-dioxo-6-quinoxalinecarboxyl group led to inactive compounds. Other related R_4 substitutions also gave inactive macrocycles (**11**–**14**). The 1,2,3,4-tetrahydro-2,3-dioxo-6-quinoxalinesulfonyl-derivatized macrocycle (**15**) gave an IC_{50} value of 62 μM. Interestingly, the introduction of a 7-methyl substituent (**16**) to this functionality improved the activity 30-fold.
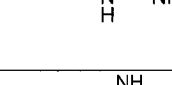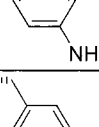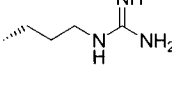
In this study, the quinoxalinedione functionality was identified as a critical structural element for activity in the in vitro protein synthesis assay. In another one of our antibacterial programs, it was also found to be an important motif for binding to a 23S rRNA fragment, according to our mass spectrometry binding assay.[28−30] Quite interestingly, in another unrelated study, a quinoxalinedione derivative was found to bind to RNA, inhibiting an HIV-1 Tat−TAR interaction.[31]

Because the quinoxalinedione functionality was an essential structural element for activity, we decided to investigate its spacing from the macrocycle moiety in a

Table 1. Inhibition of Protein Synthesis in Vitro

| No.	R$_1$	R$_2$	R$_3$	transcription /translation, IC$_{50}$ (μM)^a	translation, IC$_{50}$ (μM)^c
1	propyl-guanidine	4-aminobenzyl	NH$_2$	16 (30)^b	13
2	butyl-amine	benzyl	NH$_2$	40 (37)	50
3	propyl-guanidine	4-hydroxybenzyl	NH$_2$	25 (39)	50
4	propyl-guanidine	pyridin-3-ylmethyl	NH$_2$	50 (81)	-
5	propyl-guanidine	pyridin-3-ylmethyl	NH$_2$	20 (41)	-
6	propyl-guanidine	4-aminobenzyl	NH$_2$	17 (37)	25
7	propyl-guanidine	4-hydroxybenzyl	NH$_2$	15 (30)	35
8	propyl-guanidine	naphthylmethyl	NH$_2$	12 (31)	-

^{a} Kanamycin gave IC$_{50}$ values of 0.04 and 0.08 μM in the transcription/translation and translation assays, respectively. ^{b} Numbers in parentheses were obtained from unpurified samples. ^{c} Five representative macrocycles were screened in this assay.

homologous series (Table 3, macrocycles **17–21**). The addition of a glycine spacer (**17**) decreased the activity 3-fold, while the addition of a sarcosine spacer gave inactive macrocycle **18**. The addition of a β-alanine spacer in **19** improved the activity 5-fold compared to that of parent macrocycle **1**. Macrocycles incorporating longer spacers (**20** and **21**) were less active then parent macrocycle **1**. This series shows that the transcription/translation activity is very sensitive to the distance between the quinoxalinedione moiety and the positively charged macrocyclic ring.[32,33]

In Vitro Translation. Some of the macrocycles were screened in a translation inhibition assay, and IC$_{50}$ values roughly paralleled the uncoupled transcription/translation inhibition IC$_{50}$ values (Table 1). It is likely that the macrocycles target rRNA and block protein synthesis by some mechanism involving the rRNA. The

14-membered macrocycles, therefore, represent a new class of RNA binders.

Bacterial Minimal Inhibitory Concentrations. Although the macrocycles clearly inhibit protein synthesis in a cell-free assay, they did not show MIC activity against either our *E. coli* or *S. aureus* pathogenic bacterial strains at 100 μM.[34,35] This can usually be attributed to low intracellular concentrations because of either poor membrane permeability or active efflux pumps.[36,37] Some of the macrocycles (Table 4) were tested in MIC against an outer membrane lipopolysaccharide (LPS) mutant[38] as well as two efflux pump mutant bacterial strains[39,40] obtained from another laboratory (a wild-type *E. coli* strain was used as the control[41]). Some of the macrocycles were found to be sensitive to the LPS mutant and exhibited weak MIC activity (25–50 μM for **1**, **3**, and **8** and 12–25 μM for **2**,

Scheme 2[a]

1, 10-16

17-21
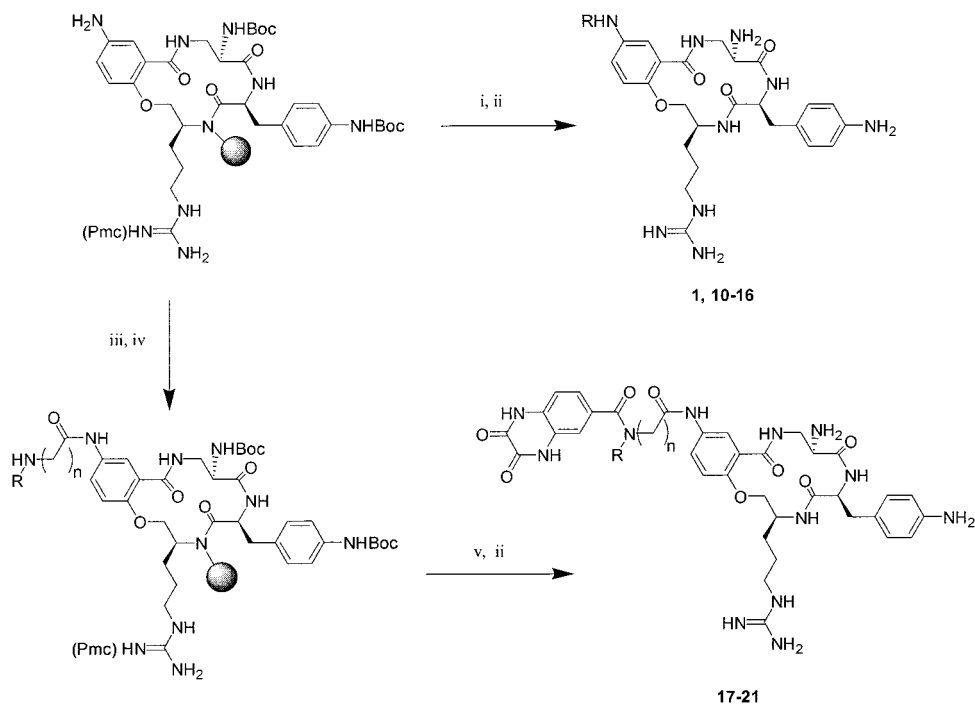
[a] Reagents and conditions: (i) 0.12 M carboxylic acid, 0.12 M HATU, 0.25 M collidine, DMF, RT, 24 h or 0.2 M sulfonyl chloride, DCM/NMP (10:1), RT, 24 h; (ii) TFA/TIS (95:5); (iii) 0.12 M Fmoc-amino acid or mimetic, 0.12 M HATU, 0.25 M collidine, DMF, RT, 24 h; (iv) 20% piperidine, DMF; (v) 1,4-dihydroquinoxaline-2,3-dione-6-carboxylic acid, 0.12 M HATU, DMF (Pmc = 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl).

6, and **7**). Macrocycle **8** exhibited weak MIC activity against the wild-type *E. coli* strain as well as the *E. coli* LPS and pump mutant strains.

Conclusions

In conclusion, we have identified a novel class of in vitro bacterial synthesis inhibitors with IC_{50} values as low as $2-3$ μM from a combinatorial library of 14-membered macrocycles. The SAR appears very narrow, with the required quinoxalinedione moiety and strict positioning of positive charges around a macrocyclic core. Some macrocycles had weak antibacterial activity against *E. coli* LPS mutant bacterial cells. Presumably, insufficient penetration of the outer membrane against intact cells is responsible for the general lack of antibacterial activity with this series of macrocycles.

Experimental Section

General Methods. All reagents were purchased from Aldrich, Sigma, Bachem, Novabiochem, and ChemImpex and used without further purification. Resins were purchased from Argonaut Technologies. NMR spectra were obtained on either a Varian Gemini 200 MHz instrument or a Varian Unity 200 MHz instrument. High-resolution mass spectra (HRMS) were obtained by Mass Consortium, San Diego, CA. Combinatorial library synthesis was carried out using the Irori AccuTag-100 Combinatorial Chemistry System and the AccuCleave-96 Cleavage Station. HPLC analyses were performed on a Waters LC system equipped with a Phenomenex Luna 3 μm C18 (100 × 4.60 mm) column and a SEDERE (France) Sedex 55 Evaporative Light Scattering detector. The instrument was also equipped with a Waters 717 Autosampler. The HPLC eluent was acetonitrile (0.1% TFA)/H$_2$O (0.1% TFA) in a gradient mode. Electrospray mass spectra were obtained on a Hewlett-Packard Electrospray 59987A instrument equipped with a Hewlett-Packard Series II 1090 Liquid Chromatograph. Chemical ionization mass spectra were obtained on a Hewlett-
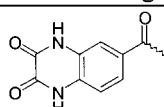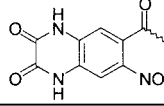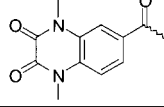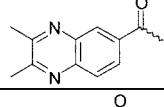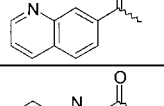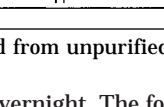
Packard Series 1100 MSD equipped with a Sedex 75 LC and a Gilson 215 Liquid Handler. Purified macrocycles were obtained using a Gilson 215 system with a Waters PrepPak (25 × 100 mm) C18 column, eluting with a linear gradient of 5−45% mobile phase B for 30 min with a flow rate of 5 mL/min (A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile).

Biology. All library members were screened in a luciferase-based protein synthesis inhibition assay,[20] and the results are expressed as IC_{50} values where the IC_{50} value is defined as the concentration of compound required to reduce the expression of luciferase protein activity to 50% of control values. A "hit" is defined as a well that inhibited the assay >75% at 100 μM. Compounds were also screened in MIC assays[27,28] against *E. coli* and *S. aureus* bacterial strains at 100 μM. A hit in MIC inhibited the assay >75% at a ligand concentration of 100 μM.

General Procedure for Attachment of Fmoc-Amino Alcohol to ArgoGel-MB-CHO Resin. To a flask containing the Fmoc-amino alcohol (15 mmol) was added MeOH (90 mL) followed by NaOMe (6.5 mL, 30 mmol, 25 wt% in MeOH). The mixture was swirled for 4−5 h on an orbital shaker. Acetic acid (1.89 mL, 33 mmol) was then added, and the mixture was left swirling for 30 min. Trimethylorthoformate (60 mL) was added followed by ArgoGel-MB-CHO resin (30 g, 0.40 mmol/g, 12 mmol). Additional MeOH was added to keep the resin wet (80−100 mL), and the mixture was kept on the orbital shaker overnight. The next day, pyridine-borane (8 M, 3.8 mL, 30 mmol) was added to the mixture followed by acetic acid (1.8 mL, 30 mmol), and it was kept on the orbital shaker overnight. The resin was filtered and washed with MeOH, DMF, DCM, and MeOH (3 times each). The resin was then dried under high vacuum over P$_2$O$_5$.
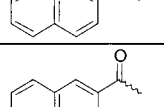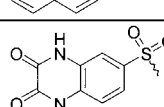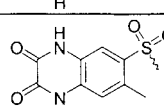
General Procedure for Attachment of Unprotected Amino Alcohol to ArgoGel-MB-CHO Resin. To the amino alcohol (15 mmol) were added MeOH (ca. 150 mL) and trimethylorthoformate (39 mL) followed by ArgoGel-MB-CHO resin (30 g). The mixture was left on the orbital shaker overnight. The next day, pyridine-borane (8 M, 11.6 mL, 93 mmol) and acetic acid (5.3 mL, 93 mmol) were added to the flask. Additional MeOH was added to keep the resin wet. The

Table 2. Inhibition of Protein Synthesis in Vitro

No.	R group	IC$_{50}$ (μM)
1		16 (30) [a]
9		(>100)
10		(>100)
11		(>100)
12		(>100)
13		(>100)
14		(>100)
15		62
16		2

[a] Numbers in parentheses were obtained from unpurified samples.

mixture was kept on the orbital shaker overnight. The following day, the resin was filtered and washed with MeOH, DMF, DCM, and MeOH (3 times each). The resin was then dried under high vacuum over P$_2$O$_5$.
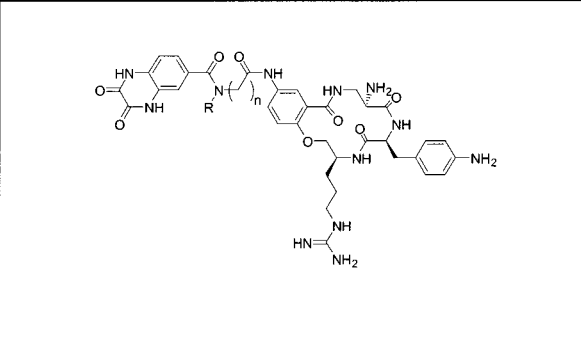
General Procedure for TBDMS Protection of Resin-Bound Amino Alcohol. The amino alcohol-derivatized resin (ca. 30 g, ca. 10 mmol) was suspended in dichloromethane (ca. 250 mL), and TBDMS-Cl (8.7 g, 58 mmol), triethylamine (8 mL, 58 mmol), and DMAP (2.3 g, 19 mmol) were added. The mixture was left swirling on the orbital shaker overnight. The next day, the resin was filtered and washed with DCM, DMF, and MeOH (3 times each). The resin was then dried under high vacuum over P$_2$O$_5$.

General Procedures for Library Synthesis Using Irori Canisters. Preparation of Irori Canisters for Library Synthesis. The derivatized amino alcohol resins were suspended in DCM/DMF (1:1) and dispensed into IroriKans (30 mg of resin/canister for MicroKans and 60 mg of resin/canister for MiniKans).

Reaction of Fmoc-α-Amino Acid (Combinatorial Step). The canisters were divided equally based on the number of

Fmoc-amino acids chosen for the combinatorial step. For the initial library, acylations were carried out with the following acids: Fmoc-L-Ser(tBu)-OH, Fmoc-D-Ser(tBu)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-D-Gln(Trt)-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-His(Trt)-OH, Fmoc-p-amino(Boc)-L-Phe-OH, Fmoc-p-amino-(Boc)-L-Phe-OH, Fmoc-N-Boc-L-aminobutyric acid, and Fmoc-N-Boc-D-aminobutyric acid. For the second generation library, acylations were also carried out with Fmoc-L-Phe-OH, Fmoc-D-Phe-OH, Fmoc-β-(3-pyridyl)-L-Ala-OH, Fmoc-β-(3-pyridyl)-D-Ala-OH, Fmoc-L-Tyr(tBu)-OH, and Fmoc-D-Tyr(tBu)-OH. To each flask were added DMF, 0.2 M Fmoc-amino acid, and 0.2 M DIC. The contents from the appropriate canisters were then added to the flasks. After the flask mixtures were kept on an orbital shaker for 48 h at RT under Ar, the contents of the canisters were combined and washed with DMF, DCM, and DMF (3 times each). The contents of the canisters were treated with 20% piperidine/DMF for 1 h, washed with DMF, DCM, and MeOH, and dried overnight under high vacuum over P$_2$O$_5$.

Reactions of Fmoc-β-Amino Acid (Combinatorial Step). The canisters were divided equally based on the number of Fmoc-β-amino acids chosen for the combinatorial step. The

Table 3. Inhibition of Protein Synthesis in Vitro

No	R	N	IC$_{50}$ (μM)
1^a	-	-	16
17	H	1	50
18	CH$_3$	1	(>100)^b
19	H	2	3
20	H	3	75
21	H	5	(33)^c

^{a} Structure in Table 1. ^{b} Numbers in parentheses are IC$_{50}$ values obtained from unpurified samples. ^{c} Estimated value with unsatisfactory dose curve.

Table 4. Minimum Inhibitory Concentration (MIC) Activity with Mutant *E. coli* Bacterial Strains

no.^{a}	*E. coli* K-12 wild-type MIC (μM)	D21f2, LPS mutant MIC (μM)^b	KZM120, pump mutant MIC (μM)^c	Ram195, pump mutant MIC (μM)^d
1	>100	25−50	>100	>100
2	>100	12−25	>100	>100
3	>100	25−50	>100	>100
6	>100	12−25	>100	>100
7	>100	12−25	>100	>100
8	50−100	25−50	50−100	50−100

^{a} Macrocycles **4**, **5**, and **15−19** were tested in the same MIC assays and did not show activity at 100 μM. ^{b} From ref 38. ^{c} From ref 39. ^{d} From ref 40.

following β-amino acids were reacted: Fmoc-β-alanine, *N*-α-Boc-*N*-β-Fmoc-D,L-diaminopropionic acid, *N*-α-Boc-*N*-β-Fmoc-L-diaminopropionic acid, *N*-α-Boc-*N*-β-Fmoc-D-diaminopropionic acid, Fmoc-D,L-β-Ser(O*t*Bu), Fmoc-D,L-β-Tyr(O*t*Bu)-OH, and Fmoc-D,L-Asp(O*t*Bu).

To each flask were added HATU (0.12 M), Fmoc-β-amino acid (0.12 M), DMF, and collidine (0.25 M). The contents of the canisters were added, and the mixtures were left on the orbital shaker overnight at RT. The next day, the solvent was removed, and the contents of the canisters were pooled and washed with DMF, DCM, and DMF. The canister mixtures were treated with 20% piperidine/DMF and kept on the orbital shaker for 1 h. The contents of the canisters were then filtered, washed with DMF, DCM, and MeOH (3 times each), and dried under high vacuum over P$_2$O$_5$.

Reaction of 2-Fluoro-5-nitrobenzoic Acid. To the flask were added 2-fluoro-5-nitrobenzoic acid (0.12 M) and HATU (0.12 M) in DMF/DCM (1:1). Collidine (0.25 M) was then added followed by the contents of the canisters. The mixture was kept on the orbital shaker overnight at RT. The following day, the contents of the canisters were filtered, washed with DMF, DCM, and MeOH, and dried under high vacuum over P$_2$O$_5$.

TBDMS Deprotection. To a flask was added THF followed by the contents of the canisters. TREAT-HF (0.2 M) was then added, and the mixture was left on the orbital shaker overnight at RT. The next day, the contents of the canisters

were filtered and washed with DMF, DCM, DMF, DCM, and MeOH (3 times each). The contents of the canisters were dried under high vacuum over P$_2$O$_5$.

Macrocyclization with DBU. The contents of the canisters were added to a solution of DBU (0.2 M) in DMF. The flask was kept on the orbital shaker at RT over argon for 3 d. The contents of the canisters were then filtered and washed with DMF, DCM, DMF, DCM, and MeOH (3 times each). The contents of the canisters were dried under high vacuum over P$_2$O$_5$.

Aryl Nitro Group Reduction. To a solution of SnCl$_2$ (1.5 M) in DMF/EtOH (10:1) were added the contents of the canisters. The flask was kept on the orbital shaker at RT over Ar. The contents of the canisters were filtered and washed with isopropyl alcohol (IPA), DCM, IPA, CHCl$_3$, DMF, and MeOH. The contents of the canisters were dried under high vacuum over P$_2$O$_5$.

Functionalization of Arylamine. Reaction with Carboxylic Acids (Combinatorial Step). The canisters were divided equally based on the number of carboxylic acids chosen for the combinatorial step. To each flask were added the carboxylic acid (0.12 M), HATU (0.12 M), DMF, and collidine (0.25 M). The contents of the canisters were added to the solution, and the mixture was kept on the orbital shaker overnight at RT. The following day, the contents of the canisters were filtered and pooled together. They were washed with DMF, DCM, and MeOH and dried under high vacuum over P$_2$O$_5$. The following commercially available carboxylic acids were used: Boc-Ser(*t*Bu)-OH, 3-({[4-(methoxyphenyl)-methyl]amino}carbonylamino)benzoic acid, hydantoic acid, *tert*-butoxyacetic acid, Boc-isonipecotic acid, thymine-L-acetic acid, and 1,4-dihydroquinoxaline-2,3-dione-6-carboxylic acid.

Aryl Modifications. 2-Quinolinecarboxylic acid and 2-naphthalenecarboxylic acid were commercially available. 2,3-Dimethylquinoxaline-6-carboxylic acid was obtained from the hydrolysis of commercially available methyl 2,3-dimethylquinoxaline-6-carboxylate.[42] 1,4-Dihydro-7-nitro-quinoxaline-2,3-dione-6-carboxylic acid was prepared from the nitration of 1,4-dihydroquinoxaline-2,3-dione-6-carboxylic acid using standard procedures.[43] 6-(Chlorosulfonyl)-1,4-dihydroquinoxaline-2,3-dione and 7-(chlorosulfonyl)-6-methyl-1,4-dihydroxyquinoxaline-2,3-dione were prepared from a modification of a literature procedure using commercially available 1,4-dihydroquinoxaline-2,3-dione and 6-methyl-1,4-dihydroquinoxaline-2,3-dione, respectively.[44]

Preparation of 1,4-Dimethyl-2,3-dioxo-1,2,3,4-tetrahydroquinoxaline-6-carboxylic Acid (for the Preparation of 10). To a mixture of sodium hydride (5.8 g, 175 mmol, 3.6 equiv, 60% dispersion in mineral oil) and DMSO (500 mL) was added 1,4-dihydroquinoxaline-2,3-dione-6-carboxylic acid (10 g, 49 mmol). Methyl iodide (9 mL, 145 mmol) was then added under Ar. The mixture was stirred for 2 h under Ar. The product was precipitated with H$_2$O, filtered, and dried. To a mixture of NaOH (1.44 g, 36 mmol) in MeOH (250 mL) was added 1,4-dimethyl-2,3-dioxo-1,2,3,4-tetrahydroquinoxaline-6-carboxylic acid methyl ester (8 g, 32 mmol). The solution was refluxed overnight under Ar. The solvent was then removed by rotary evaporation, and H$_2$O was added; the solution was cooled on an ice bath. Concentrated HCl was slowly added to precipitate the desired carboxylic acid (6 g, 53% yield for 2 steps): ^{1}H NMR (200 MHz, DMSO) δ 13.01 (s, 1 H), 7.49−7.54 (m, 1 H, Ar), 7.81−7.89 (m, 2 H, Ar), 2.50 (s, 6 H); HRMS-MALDI (m/z) [M + Na]$^+$ calcd for C$_{11}$H$_{10}$N$_2$O$_4$ + Na, 257.0544; found, 257.0533.

Reaction with 4-Methoxybenzylisocyanate. To a solution of 4-methoxybenzylisocyanate (0.2 M) in DMF were added the contents of the canisters. The mixture was kept on the orbital shaker overnight. The contents of the canisters were filtered and pooled together with the other canister contents for washing.

Cleavage of Product from Solid Support. The contents of each canister were archived into a 96-well plate format and treated with a solution of TFA/TIS (95:5). The volatiles were removed under high vacuum, and 500 μL of dioxane/H$_2$O (1:

1) was then added to the residue of each well. The dioxane/water mixture was removed under high vacuum, and residues were dried under high vacuum over P_2O_5.

General Reaction of the Arylamine Precursor of 1 with Sulfonyl Chloride (for the Synthesis of 15 and 16). To a solution of sulfonyl chloride (0.2 M) and DIEA (0.2 M) in DCM/NMP (10:1) was added the resin-bound macrocyclic arylamine. The resin was reacted overnight, filtered, and then washed with DMF, DCM, and MeOH (3 times each).

General Synthesis of Macrocycles 17–21. To a solution of amino acid or analogue (0.2 M, Fmoc-Gly-OH, Fmoc-β-Ala-OH, Fmoc-γ-Abu-OH, or Fmoc-ϵ-aminocaproic acid), HATU (0.2 M), and collidine (0.5 M) in DMF was added the resin-bound arylamine precursor of **1**. The mixture was left swirling on the orbital shaker overnight. The following day, the resin was filtered and washed with DMF, DCM, and MeOH (3 times). The Fmoc group was then removed with 20% piperidine/DMF, and 1,4-dihyroquinoxaline-2,3-dione-6-carboxylic acid was coupled to the N-terminus as described above.

Coupled Bacterial Transcription/Translation Inhibition Assay. The DNA template, pBest Luc (Promega), is a plasmid containing a reporter gene for firefly luciferase fused to a strong *tac* promoter and ribosome binding site. Messenger RNA from the DNA template was transcribed and translated in *E. coli* S30 bacterial extract in the presence or absence of the test compound. Compounds were tested in a black 96-well microtiter plate with an assay volume of 35 μL. Each test well contained 5 μL of test compound, 13 μL of S30 premix (Promega), 4 μL of 10× complete amino acid mix (1 mM each), 5 μL of an *E. coli* S30 extract, and 8 μL of 0.125 μg/μL pBest Luc. The transcription/translation reaction mixture was incubated for 35 min at 37 °C followed by the detection of functional luciferase with the addition of 30 μL of LucLite (Packard). Light output was quantitated on a Packard Top-Count.

Bacterial Translation Inhibition Assay. Luciferase SP6 Control Plasmid (Promega L474B) is a plasmid containing a reporter gene for firefly luciferase under the transcriptional control of a phage RNA polymerase promoter. This DNA produces an RNA transcript approximately 1800 bases in length.

RNA from this luciferase SP6 plasmid was transcribed using SP6 RNA polymerase in the RiboMAX RNA Production System (Promega P1280). This RNA was subsequently translated in an *E. coli* S30 extract for linear templates (Promega L1030). The extract was prepared according to a published procedure.[20] The translation reactions were performed in a black 96-well microtiter plate in an assay volume of 60 μL. Each test well contained 12 μL of test compound, 20 μL of S30 premix (Promega), 6 μL of 10× complete amino acid mix (1 mM each), 15 μL of an *E. coli* S30 extract, and 7 μL of 1 μg/μL luciferase mRNA. The translation mixture was incubated for 90 min at 37 °C followed by the detection of functional luciferase with the addition of 7 μL of Steady-Glo luciferase substrate (Promega E2510). Light output was quantitated on a Packard TopCount.

Minimum Inhibitory Concentrations (MICs). The assays were carried out in a 150 μL volume in duplicate in 96-well clear flat-bottom plates. The bacterial suspension from an overnight culture growth in the appropriate medium was added to a solution of test compound in 2.5% DMSO in water. Final bacterial inoculum was approximately $10^2–10^3$ CFU/well. The percentage growth of the bacteria in the test wells relative to that observed for a control well containing no compound was determined by measuring absorbance at 595 nm (A_{595}) after 20–24 h at 37 °C. The MIC was determined as a range of concentrations where complete inhibition of growth was observed at the higher concentration and bacterial cells are viable at the lower concentration. Both ampicillin and tetracycline were used as antibiotic positive controls in each screening assay for *E. coli* (ATCC 25922) and *S. aureus* (ATCC 13709). The bacterial strains in Table 4, including the *E. coli* K-12 wild-type, D21f2 (LPS mutant), KZM120 (pump mutant), and Ram195 (pump mutant), were obtained from Hiroshi Nikaido at the Department of Microbiology and Immunology, University of California, Berkeley, CA.

HRMS of Macrocycles. 1: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{34}H_{40}N_{11}O_7$, 714.3107; found, 714.3087. **2**: HRMS-MALDI (*m/z*) [M + Na]$^+$ calcd for $C_{37}H_{38}N_8O_7$ + Na, 693.2756; found, 693.2756. **3**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{34}H_{39}N_{10}O_8$, 715.2947; found, 715.2949. **4**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{33}H_{38}N_{11}O_7$, 700.2950; found, 700.2969. **5**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{33}H_{38}N_{11}O_7$, 700.2950; found, 700.2958. **6**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{34}H_{40}N_{11}O_7$, 714.3107; found, 714.3083. **7**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{34}H_{39}N_{10}O_8$, 715.2947; found, 715.2953. **8**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{34}H_{39}N_{10}O_7$, 699.2998; found, 699.2993. **15**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{33}H_{40}N_{11}O_8S$, 750.2776; found, 170.2775. **16**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{34}H_{42}N_{11}O_8S$, 764.2933; found, 764.2935. **17**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{36}H_{43}N_{12}O_8$, 771.3321; found, 771.3349. **19**: HRMS-MALDI (*m/z*) [M + H]$^+$ calcd for $C_{37}H_{45}N_{12}O_8$, 785.3478; found, 785.3458. **20**: HRMS-MALDI (*m/z*) [M + Na]$^+$ calcd for $C_{38}H_{46}N_{12}O_8$ + Na, 821.3454; found, 821.3436.

Supporting Information Available: LC–MS of macrocyclic mixture (Figure 3); LC–MS of HPLC-purified macrocycles **1–8**, **15–17**, **19**, and **20**; LC–MS of **9–14** (crude samples); and ^{1}H NMR spectra of HPLC-purified macrocycles **1–8**, **15–17**, **19**, and **20**. This material is available free of charge via the Internet at http://pubs.acs.org.

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