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The Synthesis and Screening of 1,4,5,8-Naphthalenetetracarboxylic Diimide–Peptide Conjugates with Antibacterial Activity

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Abstract—We have employed an initial combinatorial approach followed by systematic lead optimization to investigate a series of novel molecules that exhibit antimicrobial activity against Gram-negative and Gram-positive bacteria. The new molecules contain various sequences of amino acids, generally L-lysine and glycine, attached to the 1,4,5,8-naphthalenetetracarboxylic diimide aromatic unit. Systematic structure–activity studies found that increasing positive charge enhanced activity and molecules containing one naphthalenetetracarboxylic diimide unit as well as at least seven lysine residues were optimum for antimicrobial activity. The naphthalenetetracarboxylic diimide derivatives were found to be inactive against mammalian cell lines, making them excellent antimicrobial candidates. Our results indicate that combining positive charge with aromatic and/or hydrophobic elements may be an interesting new approach to antimicrobial agents and adds an important new dimension to the field of cationic peptides.

Introduction

The emerging crisis in antibiotic resistance has renewed interest in the investigation of novel chemical structures with antimicrobial activity.[1,2] Two major approaches for combating resistant microbial pathogens include (1) targeting the 'resistance factors' and (2) the generation of novel antimicrobial agents, ideally with new modes of action.[2]

Among the different classes of antimicrobial agents currently being investigated are the cationic peptides that exhibit strong activity against both Gram-negative and Gram-positive bacteria. This class of antibiotics currently consists of a rapidly expanding list of naturally occurring cationic peptides that have been isolated from bacteria, mammals, amphibians, plants, fungi, insects, crustaceans and humans.[3,4] The cationic peptides as a class possess two elements in common, the presence of both basic (i.e., arginine and lysine) and hydrophobic amino acids. These cationic peptides are thus amphipathic molecules.[1,3–5] The antibacterial mode of action has been generally attributed to the formation of ion-channels or other types of membrane disruption/disorganization, although research remains active in this area.[2–8]

The homopolymer poly-L-lysine also displays antibacterial activity against both Gram-positive and Gram-negative bacteria.[9] The naturally occurring homopolymer contains anywhere between 25 and 30 lysine residues and has been isolated from *Streptomycin albulus*.[9,10] A minimum of 10 lysine residues have been found necessary to inhibit bacterial growth using synthetic peptides, and increasing the number of lysine residues enhanced bacterial growth inhibition.[11]

The biological activity of *N*-substituted naphthalimides has been studied since the early 1940's.[12] Biological potencies of simple analogues include anti-tumor, anti-neoplastic and anesthetic activity. To the best of our knowledge, however, there has never been a systematic study examining the antimicrobial properties of these species. More recently, naphthalimide and 1,4,5,8-naphthalenetetracarboxylic diimides (NDI) derivatives have also attracted attention because of their ability to intercalate into double-stranded DNA.[13,14]

Herein is reported the synthesis and preliminary evaluation of the antimicrobial properties of a series of novel cationic NDI–peptide hybrid molecules. Initial combinatorial studies identified lead structures that were optimized using systematic structure–activity approaches. Several of the more positively-charged molecules exhibited activity against Gram-negative and

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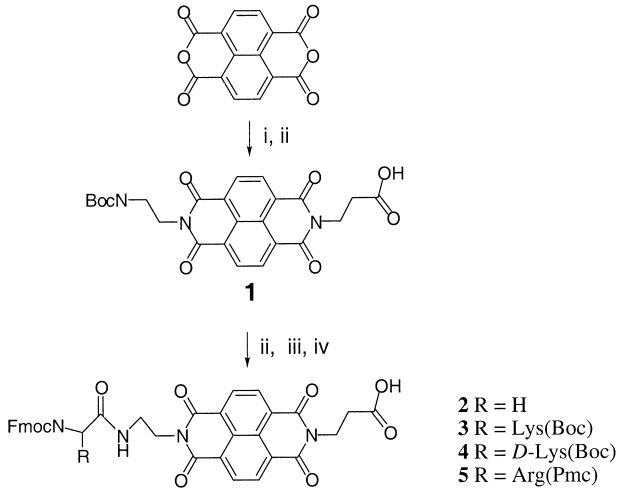
especially Gram-positive bacteria, with no detectable activity against mammalian cell lines in preliminary studies. Taken together, our results indicate the cationic peptide motif of antimicrobial activity is not restricted to molecules composed of amino acids only, perhaps paving the way for new generations of novel cationic molecules with potent antimicrobial activity.

Results

To investigate the antimicrobial activity of compounds based on peptide hybrids of the NDI moiety, a series of protected NDI derivatives (**2**–**5**) were synthesized in preparation for solid-phase synthesis (Schemes 1–3).

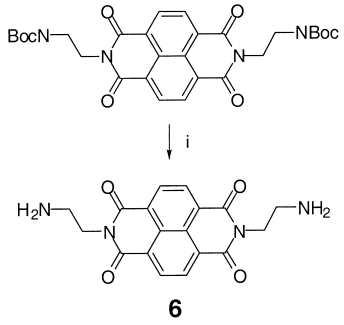
Split synthesis was employed to create a library of compounds varying in hydrophobic and electrostatic properties. The library was based on a so-called dimer, in which two NDI units are separated by four amino acids (Fig. 1), and glycine residues on each terminus. Nineteen amino acids were used as building blocks for the polypeptides in the library. Cysteine was not included in the study due to potential complications arising from disulfide bond formation. Fmoc-NovaSyn TGA resin was evenly divided into 19 reaction vessels, wherein each vessel represented an amino acid in the tetrapeptide. The NDI was attached to glycine prior to solid-phase synthesis. Consequently, only three amino acid positions were varied in the initial library. The compounds were not mixed after the addition of the third amino acid. In this way, a total library of approximately 6900 dimers were divided among nineteen vessels with 361 compounds per vessel. The material from each vessel was cleaved from the resin then placed on filter paper at known concentrations in preparation for testing.



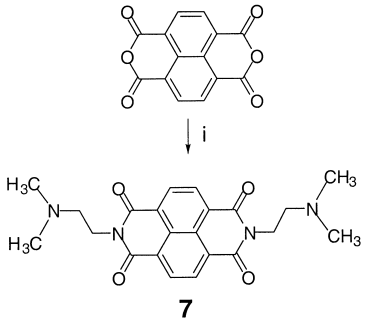
Scheme 1. Synthesis of diimide amino acid precursor. Reagents: (i) β-alanine benzyl ester *p*-toluene sulfonate salt, $BocN(CH_2)_2NH_2$, *i*-Pr_2-NEt; (ii) H_2, Pd/C; (iii) TFA/CH_2Cl_2; (iv) Fmoc-Gly-OC_6F_6,[a] HOBT, 2,6-lutidine. [a]For compounds with lysine and arginine residues, Fmoc-Lys(Boc)-OC_6F_6 and Fmoc-Arg(PMC)-OC_6F_6 were substituted.

The Kirby–Bauer disk assay was used to identify active mixtures.[15–17] In these assays, *E. coli* and *Bacillus subtilis* were used as representative Gram-negative and Gram-positive bacterial strains, respectively. The antibiotic streptomycin was used as a calibration in all tests. Screening of the 19 different libraries indicated that at 300 nmol on the filter, only the mixture containing a lysine residue in the X_3 position of the tetrapeptide exhibited slight antibacterial activity in *B. subtilis*. No activity was observed in *E. coli*. The 19 libraries were not deconvoluted further, but rather, a series of studies were initiated to identify parameters important for antimicrobial activity.

A number of molecular parameters were investigated, including the influence of (1) linker amino acid sequence; (2) increasing positive charge; (3) arginine versus lysine; (4) L versus D amino acids; (5) altering the number of NDI units; (6) *N*-alkylamino substituents versus peptide containing substituents; (7) variation of the aromatic moiety. The qualitative and quantitative



Scheme 2. Synthesis of *N,N*-bis(2,2′-ethylamine) 1,4,5,8-naphthalenetetracarboxylic diimide. (i) TFA/CH_2Cl_2.



Scheme 3. Synthesis of *N,N*-bis(2,2′-dimethylmino)-1,4,5,8-naphthalenetetracarboxylic diimide. (i) 2-(dimethylamino)ethylamine, THF.

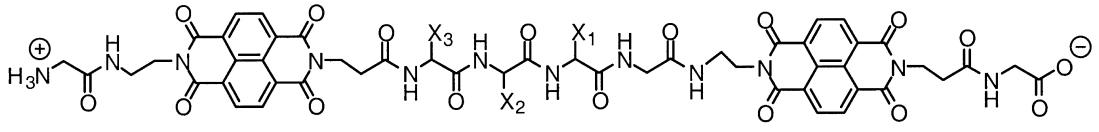


Figure 1. Variation of three of the four amino acids in the polypeptide linker where X_n represents one of the 19 amino acids.

structure–activity relationship (SAR) of each derivative was explored first using the Kirby–Bauer assay. The amounts used for each test ranged from 33–300 nmol and were used to assay the biological activity against two bacterial reference strains, *E. coli* and *B. subtilis*. Each study was repeated five times to assess reproducibility. On average, the studies resulted in zones of inhibition with a difference of ±2 mm.

Fifty NDI–peptide conjugates, **8**–**39** and **44**–**61**, ranging in selected properties were prepared using solid-phase chemistry and analyzed in an effort to probe potential relationships between chemical structure and antimicrobial activity. RP HPLC was used to purify each compound and molecular weight was characterized by mass spectrometry.

Six compounds were initially synthesized (**8**–**13**), varying in the location of a single L-lysine residue in the context of five glycine residues and two NDI aromatic units. The resulting compounds were inactive in both *E. coli* and *B. subtilis* at 300 nmol per disk (Table 1). Fifteen dimers (**14**–**28**) were then synthesized containing the various possible sequences of two L-lysine residues in the context of four glycine residues and two NDI aromatic units. At 300 nmol, all but one, compound **28**, displayed similar activities against *B. subtilis*, but were inactive against *E. coli*. Compound **28** contained lysine residues on both the N- and C-termini and was completely inactive. It is unclear why having the two lysine residues in the terminal positions precluded activity with **28**, while the activities of the all other compounds in the series, **14**–**27**, were not noticeably dependent on the location of the lysine residues.

The results using compounds **14**–**27** indicated that greater positive charge (+2 vs +1) produced generally greater activity, so the influence of positive charge was investigated in more detail using compounds **29**–**34**. Compounds **29**–**34** have total charges ranging from −2 to +6. *A clear trend toward greater activity with increasing positive charge was observed in the series*. Particularly noteworthy are compounds **33** and **34**, with total charges of +5 and +6, respectively, which showed substantial activity against *B. subtilis* even with the 33 nmol disks, and activity against *E. coli* on the 100 nmol disks. The bottom line is that within this series of molecules, a minimum of two positive charges are needed to show activity against *B. subtilis* and at least five positive charges are needed to show activity against *E. coli* in this assay system.

Table 1. Observed and calculated HR-MS (FAB) data for selected compounds

Compound	Molecular weight		Molecular formula
	Observed	Calculated	
14	1230.4741	1230.4730	$C_{58}H_{66}N_{14}O_{17}$
27	1229.4623	1229.4652	$C_{58}H_{65}N_{14}O_{17}$
28	1229.4674	1229.4652	$C_{58}H_{65}N_{14}O_{17}$
33	1442.6892	1442.6857	$C_{70}H_{92}N_{17}O_{17}$
34	1514.7654	1514.7670	$C_{74}H_{102}N_{18}O_{17}$
46	638.29337	638.29384	$C_{31}H_{40}N_7O_8$
47	766.38857	766.38880	$C_{37}H_{52}N_9O_9$
49	1022.5802	1022.5787	$C_{49}H_{76}N_{13}O_{11}$
53	1335.7904	1335.7901	$C_{63}H_{103}N_{18}O_{14}$
54	1663.0634	1663.0642	$C_{79}H_{145}N_{23}O_{16}$
61	1963.7926	1963.7869	$C_{93}H_{109}N_{23}O_{26}$
62	944.6322	944.6296	$C_{47}H_{82}N_{11}O_9$
63	1357.8600	1357.86115	$C_{70}H_{113}N_{14}O_{13}$

Compounds **35** and **36** contain only D-lysine, but are otherwise identical to the L-lysine containing compounds **27** and **33**, respectively. In both cases, the D-lysine containing analogues displayed antimicrobial activities that were indistinguishable from the molecules containing L-lysine, indicating that the stereochemistry of the lysine residue is not relevant to overall activity. In addition, L-arginine was used in place of L-lysine in compounds **37** and **38**. Here, the arginine-containing compounds displayed similar but measurably lower activity than the reference compounds **27** and **33**. Finally, as important controls, an NDI unit alone (**39**) and lysine containing peptides of differing lengths were analyzed (**40**–**43**), but failed to show any measurable antimicrobial activity. These latter results are consistent with literature precedent in which at least 10 lysine residues are required for significant antimicrobial activity in peptides.[9]

A series of derivatives with a single NDI unit attached to various numbers of glycine, L-lysine, D-lysine and L-arginine residues were prepared (**44**–**60**) and analyzed for antimicrobial activity using the same Kirby–Bauer disk diffusion assay (Table 2). The results with these so-called 'NDI monomers' were entirely consistent with the results obtained with the NDI dimers listed in Table 1. In particular, with the NDI monomers, increasing antimicrobial activity was correlated with increasing positive charge (**44**–**54**). Again, a minimum of two positive charges was needed for activity against *B. subtilis* and at least five positive charges were needed for activity against *E. coli*. Increasing the number of L-lysine residues to greater than six had only a minimal effect on activity in this assay. Substitution of L-lysine residues with D-lysine (**55**) or L-arginine (**56**) also had little effect. In addition, the NDI unit position within the peptide conjugate did not matter (**57**–**60**) and having three NDI units in the context of a longer peptide showed no advantage (**61**) in terms of antimicrobial activity.

As a first attempt to see if other naphthalene derivatives would function in a manner similar to the NDI unit, another aromatic moiety, 1,8-dialkoxynaphthalene, was used in place of the NDI unit in compounds **62** and **63**. No activity was observed with either compound. Finally, two NDI derivatives were investigated in which aminoethyl (**6**) or (dimethylamino)ethyl (**7**) groups were attached in place of the peptide. These molecules displayed the highest activity of any of the derivatives in the Kirby–Bauer disk diffusion assay, against both *E. coli* and *B. subtilis*.

In order to probe further the antimicrobial activities of the NDI–peptide conjugates, minimum inhibitory concentrations (MIC's)[15–17] were determined for selected compounds varying in their number of NDI units and

cationic properties (Table 3). The same general trends were seen in the MIC's as with the Kirby–Bauer studies. In general, activity increased with increasing positive charge, and was rather insensitive to the number of NDI units (as long as at least one was present), or the stereochemistry of the lysine residues. However, as opposed to the Kirby–Bauer assays, there was a dramatic difference between derivatives with six versus seven L-lysine residues. The molecules with seven L-lysine residues, **52** and **53**, showed significantly higher activity than the corresponding molecule with only six L-lysine residues (**51**). Increasing to 10 L-lysine residues, **54**, did not increase activity further. Interestingly, in *B. subtilis*, compounds **52**, **53** and **54** containing 7 and 10 lysine residues, respectively, displayed the same MIC, 3.1 μM, as the antibiotic streptomycin.

In contrast to the Kirby–Bauer assay results, the MICs in Table 4 indicate that the amino alkyl NDI derivatives **6** and **7** are significantly less active in culture than the NDI–peptide conjugates **52**, **53**, and **54**. The discrepancy in results between measured MICs and Kirby–Bauer assays can be attributed to the differences in the molecular weights of the compounds. The Kirby–Bauer method is highly dependent on diffusion and thus the molecular weight and solubility of a compound, whereas molecular weight is less important when determining the MIC by broth dilution since diffusion is no longer a significant consideration. This rationale may also explain why the zone of inhibition of the most active NDI–peptide conjugates, **52**, **53**, and **54,** were less than streptomycin **66** (data not shown) whereas the MIC's were identical in *B. subtilis*.

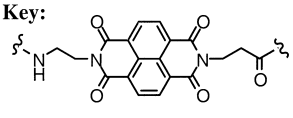
Poly-L-lysines ranging in molecular weights (2900, 111,000, and 134,000 g/mol) were also tested to determine the correlation between enhanced antimicrobial activity and increasing molecular weight/positive charge. Poly-L-lysine (2900 g/mol) **43** displayed an MIC of 50 μM whereas the larger poly-L-lysine (111,000 and

Table 2. Peptide and Dimer results; variation of charge, location of chromophore, increasing positive charge, D versus L amino acids and arginine versus lysine[a]

	E. coli			*B. subtilis*			
	300	100	33	300	100	33	Charge
8 GAGGGGAK	NI	NI	NI	NI	NI	NI	+1
9 GAGGGKAG	NI	NI	NI	NI	NI	NI	+1
10 GAGGKGAG	NI	NI	NI	NI	NI	NI	+1
11GAGKGGAG	NI	NI	NI	NI	NI	NI	+1
12GAKGGGAG	NI	NI	NI	NI	NI	NI	+1
13 KAGGGGAG	NI	NI	NI	NI	NI	NI	+1
14 GAGKGKAG	NI	NI	NI	10	NI	NI	+2
15 GAKKGGAG	NI	NI	NI	8	NI	NI	+2
16 GAGKKGAG	NI	NI	NI	10	NI	NI	+2
17 KAKGGGAG	NI	NI	NI	8	NI	NI	+2
18 KAGKGGAG	NI	NI	NI	7	NI	NI	+2
19 GAKGGKAG	NI	NI	NI	9	NI	NI	+2
20 GAGGKKAG	NI	NI	NI	10	NI	NI	+2
21 KAGGGKAG	NI	NI	NI	7	NI	NI	+2
22 KAGGKGAG	NI	NI	NI	10	NI	NI	+2
23 GAGGGKAK	NI	NI	NI	10	NI	NI	+2
24 GAGGKGAK	NI	NI	NI	10	NI	NI	+2
25 GAGKGGAK	NI	NI	NI	11	NI	NI	+2
26 GAKGGGAK	NI	NI	NI	11	NI	NI	+2
27 GAKGKGAG	NI	NI	NI	10	NI	NI	+2
28 KAGGGGAK	NI	NI	NI	NI	NI	NI	+2
29 GAEGEGAG	NI	NI	NI	NI	NI	NI	−2
30 GAGGGGAG	NI	NI	NI	NI	NI	NI	0
31 GAKKKGAG	NI	NI	NI	11	8	NI	+3
32 GAKKKKAG	NI	NI	NI	12	10	NI	+4
33 KAKKKKAG	11	8	NI	13	11	9	+5
34 KAKKKKAK	12	8	NI	14	11	9	+6
35 GAKGKGAG (*D*)	NI	NI	NI	10	NI	NI	+2
36 KAKKKKAG (D)	12	9	NI	12	10	8	+5
37 GARGRGAG	NI	NI	NI	8	NI	NI	+2
38 RARRRRAG	10	NI	NI	11	8	NI	+5
39 H-A-OH	NI	NI	NI	NI	NI	NI	0
40 Lys–Lys–Lys	NI	NI	NI	NI	NI	NI	+3
41 Lys–Lys–Lys–Lys	NI	NI	NI	NI	NI	NI	+4
42 Lys–Lys–Lys–Lys–Lys	NI	NI	NI	NI	NI	NI	+5
43 Poly-L-lysine (2900 g/mol)	NI	NI	NI	NI	NI	NI	+*n*

[a]Diameters of zones of inhibition (DZI), mm 300, 100 and 33 nmol.

Key:



A

NI = no inhibition
K or Lys = Lysine
R = Arginine
G = Glycine

134,000 g/mol) derivatives (**64** and **65**) exhibited an MIC of 0.78 μM. Since the shorter poly-L-lysine (2900 g/mol) **43**, containing 15 L-lysine residues, displayed relatively poor MIC activity, these data confirm the importance of the NDI moiety for enhancing activity in small oligo-L-lysine peptide conjugates.

To probe the bactericidal activity of the most active compounds, minimal lethal concentrations (MLC's) were measured for the most active NDI–peptide conjugates (**52**–**54**), the aminoalkyl NDIs (**6**, **7**) and streptomycin (**66**). Briefly, in these experiments, broth from each *B. subtilis* culture of the MIC determination experiments was plated to find the minimum concentration of compound in which all bacteria had been killed, as opposed to just growth arrested, following incubation with compound in solution. Interestingly, the NDI–peptide conjugates displayed bactericidal activity similar to that of streptomycin, but the aminoalkyl NDI derivatives **6** and **7** were not bactericidal even at the highest concentrations examined, 100 μM. Thus, these latter two compounds appear to be bacteriostatic, as opposed to bactericidal.

The activities of selected NDI derivatives in mammalian cell lines were investigated using normal (NCM-460) and cancer (HT-29) cell lines (Table 5). Two-fold serial dilutions starting at 100 μM were used with the MTT assay.[18] None of the NDI–peptide conjugates showed activity even at 100 μM. On the other hand, the aminoalkyl derivatives **6** and **7** displayed IC_{50} values of 1 μM and 390 nM in both cell lines. The mammalian cell activity observed with **6** is consistent with literature reports of this compound.[19,20]

Discussion

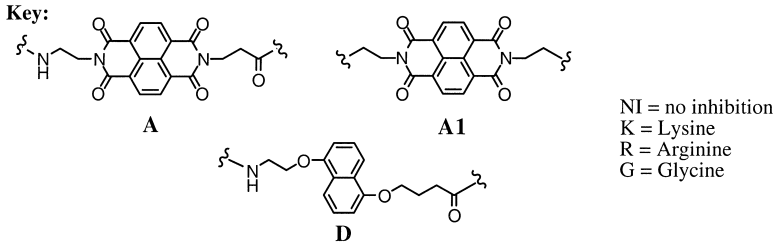
Positively-charged NDI–peptide conjugates display antimicrobial activity against the representative Gram-negative *E. coli* and especially the Gram-positive *B. subtilis*, with increasing activity being correlated to increasing numbers of positively-charged lysine residues in the conjugate. Maximal activity is seen with seven or greater lysines. The activity is dependent on the presence of at least one NDI residue, but the position of this unit within the molecule does not appear to be critical and additional NDI units provide no discernable benefit. Importantly, the NDI–peptide conjugates display bactericidal activity and have no activity against the mammalian cell lines tested.

Taken together, the data for all of the NDI–peptide conjugates investigated provide circumstantial support

Table 3. Monomer results; variation of charge, location of chromophore, increasing positive charge, D versus L amino acids and arginine versus lysine[a]

	E. coli			*B. subtilis*			
	300	100	33	300	100	33	Charge
44 GAG	NI	NI	NI	NI	NI	NI	0
45 GAK	NI	NI	NI	NI	NI	NI	+1
46 KAK	NI	NI	NI	10	7	NI	+2
47 KKAK	NI	NI	NI	12	10	NI	+3
48 KKKAK	NI	NI	NI	12	10	NI	+4
49 KKKKAK	8	NI	NI	14	10	NI	+5
50 KKKKKAG	12	NI	NI	13	10	NI	+5
51 KKKKKAK	11	8	NI	12	10	9	+6
52 KKKKKKAK	10	NI	NI	12	10	7	+7
53 KKKKKKKAG	11	7	NI	12	10	7	+7
54 KKKKKKKKKAK	11	10	NI	13	10	8	+10
55 KKKKKAG (D)	12	11	NI	12	11	9	+5
56 RRRRRAG	11	6	NI	12	10	6	+5
57 KKKKAKK	11	9	NI	14	12	8	+6
58 KKKAKKK	11	8	NI	13	12	12	+6
59 KKAKKKK	11	9	NI	14	12	10	+6
60 KAKKKKK	12	9	NI	13	11	10	+6
61 GAKGKGAKGKGAG	NI	NI	NI	11	9	NI	+4
62 KKKKDK	NI	NI	NI	NI	NI	NI	+5
63 KDKKKKDK	NI	NI	NI	NI	NI	NI	+6
6 $[H_3N\mathbf{A1}NH_3]^{2+}$	20	16	15	20	19	18	+2
7 $[(CH_3)_2N\mathbf{A1}N(CH_3)_2]^{2+}$	16	10	NI	25	20	19	+2

[a]Diameters of zones of inhibition (DZI), mm 1300, 100 and 33 nmol.



for a mode of action involving the bacterial membranes or components thereof, as it is unlikely that highly positively-charged molecules can penetrate into the interior of bacterial cells. In addition, the observed lack of amino acid sequence dependence and equivalent activity of D-lysine containing derivatives argue against a specific receptor mediated process. Rather, the activity seems more correlated with the overall physical properties of the molecules, namely the positive charges of the lysine residues coupled to the aromatic surfaces of the NDI unit. Since no activity with the NDI–peptide conjugates was seen in mammalian cells, similar membrane-based interactions presumably do not occur.

The activity of the NDI–peptide conjugates can be contrasted with the activities of the aminoalkyl NDI derivatives **6** and **7**. Only **6** and **7** displayed relatively strong activity in mammalian cells. Sami et al. have shown that the mammalian cell activities of naphthalimide analogues are related to DNA intercalation and enhanced DNA interactions parallel cytotoxicity.[21–23] Assuming a DNA intercalation mechanism of action, the 3-fold difference in IC_{50} values observed in our study is likely due to greater membrane permeability of the tertiary amino groups of **6** compared to the primary amino groups of **7**.

To the best of our knowledge, the antimicrobial properties of the NDI species **6** and **7** have not been reported. Although **6** and **7** both show strong activity in the Kirby–Bauer assay, they appeared to be bacteriostatic, not bactericidal. Such distinct behavior underscores the

Table 4. Minimum inhibitory and minimum lethal concentrations of selected compounds reported in μM and μg/mL

	E. coli		*B. subtilis*			
	MIC (μM)	MIC (μg/mL)	MIC (μM)	MIC (μg/mL)	MLC (μM)	MLC (μg/mL)
34 KAKKKKAK	50	76	25	38		
47 KKAK	>100	>77	>100	>77		
48 KKKAK	>100	>90	>100	>90		
49 KKKKAK		>100	>103	50	51	
50 KKKKKAG	50	51	25	26		
55 KKKKKAG (D)	50	51	25	26		
51 KKKKKAK	50	58	25	29		
52 KKKKKKAK	25	64	3.1	4.2	12.5	19
53 KKKKKKKAG	50	67	3.1	4.2	12.5	20
33 KAKKKKAG	50	72	50	72		
36 KAKKKKAG (D)	50	72	50	72		
54 KKKKKKKKKAK	25	42	3.1	5.2	12.5	25
6 $[H_3N\mathbf{A1}NH_3]^{2+}$	50	18	25	8.8	>100	>35
7 $[(CH_3)_2N\mathbf{A1}N(CH_3)_2]^{2+}$	50	20	25	10	>100	>41
40 Lys–Lys–Lys	>100	>41	>100	>41		
41 Lys–Lys–Lys–Lys	>100	>54	>100	>54		
42 Lys–Lys–Lys–Lys–Lys	>100	>66	>100	>66		
43 Poly-L-lysine (2900 g/mol)	50	145	50	145		
64 Poly-L-lysine (111,000 g/mol)	0.78	87	0.78	87		
65 Poly-L-lysine (134,000 g/mol)	0.78	104	0.78	104		
66 Streptomycin	0.39	1.1	3.1	4.6	25	36

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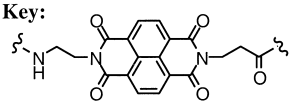

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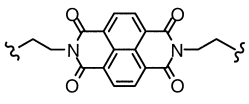


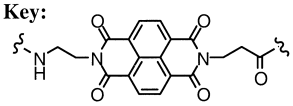
A1

K = Lysine
G = Glycine

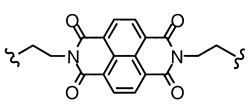
Table 5. Mammalian cell results obtained from the MTT assay of various compounds tested against human normal and colon carcinoma cell lines

	NCM-460 IC_{50}	HT-29 IC_{50}
6 KAK	>100 μM	>100 μM
50 KKKKKAG (L)	>100 μM	>100 μM
55 KKKKKAG (D)	>100 μM	>100 μM
54 KKKKKKKKKAK	>100 μM	>100 μM
27 GAKGKGAG	>100 μM	>100 μM
6 $[H_3N\mathbf{A1}NH_3]^{2+}$	1 μM	1 μM
7 $([(CH_3)_2N\mathbf{A1}N(CH_3)_2]^{2+}$	390 nM	390 nM

Key:



A



A1

K = Lysine
G = Glycine

likely different mode of action of **6** and **7** compared to the NDI–peptide conjugates such as **52**–**54**.

Conclusion

The NDI–peptide conjugates such as **52**–**54** should be considered as starting points for further antibacterial drug development due to relatively strong antimicrobial activity coupled to the complete lack of observed activity in mammalian cell lines. At this point, it is tempting to ascribe the antimicrobial activity to membrane interactions/disruption in analogy to previous studies with poly-L-lysine and cationic peptides.[3,4] Nevertheless, detailed mechanistic studies with the most active NDI–peptide conjugates are currently being planned that will address in detail their mode of action.

The studies described herein have demonstrated that the relatively hydrophobic and electron deficient aromatic NDI unit can be used to enhance dramatically the antimicrobial activities of positively-charged peptides, and this activity enhancement is likely independent of any preformed secondary structure. Such findings add a new dimension to the field of antimicrobial cationic peptides. Taken one step further, our results indicate that a systematic investigation of novel hydrophobic and positively-charged units, including non-peptide systems, might uncover a fertile new source of potent antimicrobial agents.

Experimental

General considerations

Proton and carbon NMR were obtained on a Bruker AC 250 spectrometer or a Bruker AMX-500 spectrometer. Chemical shifts are reported in ppm relative to tetramethylsilane (δ units). Chemical ionization mass spectra and fast atom bombardment (FAB) mass spectra were recorded on a Finnigan TSQ-700 and Micromass ZAB spectrometer at the Analytical Facility of The University of Texas at Austin. Combinatorial chemistry and parallel synthesis were performed manually on an apparatus designed in our laboratory. To insure proper mixing of the reactants the apparatus was attached to a teflon block, argon outlet, vacuum manifold and connected to a shaker. HPLC data were obtained on a Hewlett-Packard 1090 UV–Vis Spectrophotometer. Unless indicated, all solvents and materials were used without further purification. Amino acids and coupling reagents were purchased from Novabiochem, Advanced Chemtech and BACHEM. Anhydrous (99.8%) DMF was purchased from Aldrich and stored over molecular sieves. All reactions were performed under argon atmosphere and glassware used for SPPS were pre-treated with SIGMACOTE to prevent the resin from sticking to the glass.

Mono-*tert*-butoxycarbonylaminoethylamine

Ethylenediamine (57 mL, 852 mmol) was dissolved in CH_2Cl_2 (100 mL) and added to a 500-mL three-neck round-bottom flask equipped with gas inlet stopper and dropping funnel. Di-*tert*-butyl dicarbonate (34 mL, 147 mmol) was suspended in 100 mL of dichloromethane and slowly added to the flask via dropping funnel. The reaction was stirred 18 h and concentrated in vacuo. H_2O (100 mL) was added to the flask and the filtrate was partitioned between CH_2Cl_2 and 10% Na_2CO_3. The organic layer was washed with Na_2CO_3 (4×100 mL), water (4×100 mL), brine (150 mL), dried over anhydrous sodium Na_2SO_4, filtered and concentrated to produce a viscous yellow oil (20.3 g, 86%). ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.65 (δ br, 1 H), 3.18 (s, 1 H), 2.82 (q, 2 H, $J=6$ Hz), 2.78 (t, 2 H, $J=6$ Hz), 1.46 (s, 9 H);^{13}C NMR (63 MHz, $CDCl_3$) δ 156.1, 42.9, 41.1, 30.8, 28.0; HR-MS (FAB) m/z 161.1284 (161.1290 calcd for $C_7H_{17}N_2O_2$, $M^+ + H$).

***N*-(2-*tert*-Butoxycarbonylaminoethyl)-*N′*-(2-carboxyethyl)-1,4,5,8-naphthalenetetracarboxylic diimide (1).** 1,4,5,8-naphthalenetetracarboxylic dianhydride (34.06 g, 127 mmol), mono-*tert*-butoxycarbonylaminoethylamine (20.31 g, 127 mmol), β-alanine benzyl ester *p*-toluenesulfonate salt (44.63 g, 127 mmol) and *N,N*-diisopropylyethylamine (24 mL, 140 mmol) were suspended in 1 L of *i*-PrOH. The mixture was heated at reflux for 24 h then concentrated in vacuo to give a pink solid. The solid was dissolved in CH_2Cl_2 (550 mL) and the organic layer was washed with 0.2 M sodium citrate buffer (4×250 mL, pH 4.5), saturated $NaHCO_3$ (5×250 mL), brine (250 mL), and then dried over anhydrous Na_2SO_4. The combined organic layers were concentrated to afford a statistical mixture of products, including **1**.

The resulting tan solid was suspended in absolute EtOH (2 L) and purged with argon for 3 h. To the mixture was added 10% Pd/C (15 g) and then the solution was charged with H_2 at atmospheric pressure for 72 h. The solvents were evaporated and the resulting solid was dissolved in 200 mL of 20% TEA/CH_2Cl_2. The solution was filtered through Celite and the filtrate was concentrated under vacuum onto 100 g of silica. The resulting solid was purified by column chromatography using a gradient from 0 to 10% MeOH in 90:10 CH_2Cl_2 /TEA. The product was taken from the second band, concentrated and dissolved in 10% MeOH/CH_2Cl_2 (200 mL) and HOAc (30 mL). Pentane (300 mL) was added to the solution to facilitate precipitation of the product. The mixture was sonicated, filtered, washed with more pentane (30 mL) and dried in vacuo to yield **1** as a tan powder (18.4 g, 30%). ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.61 (s, 4H), 6.91 (t, 1H, $J=6$ Hz), 4.25 (t, 2H, $J=7.4$ Hz), 4.12 (t, 2H, $J=6$ Hz), 3.27 (br, 2H), 2.61 (t, 2H, $J=7.5$ Hz), 1.21 (s, 9H); ^{13}C NMR (63 MHz, DMSO-d_6) δ 172.5, 162.2, 162.0, 155.8, 130.2, 130.1, 125.8, 125.6, 77.6, 37.6, 36.1, 32.1, 28.0; HR-MS (FAB) m/z 481.1467 (481.1486 calcd for $C_{24}H_{23}N_3O_8$, $M^+ + H$).

***N*-2-(*N*-α-9-Fluorenylmethoxycarbonylglycyl)aminoethyl-*N′*-(2–carboethyl)-1,4,5,8-naphthalenetetracarboxylic diimide (2).** Compound **1** (2 g, 4.15 mmol) was suspended in CH_2Cl_2 (12 mL) and was added slowly TFA (12 mL). The solution stirred for 30 min, concentrated and residual TFA was removed by azeotropic evaporation

from heptane (5×30 mL). The mixture was triturated with ether (5×30 mL), filtered and dried in vacuo. The resulting solid was dissolved in DMF (15 mL) and Fmoc-Gly-OC_6F_5 (1.92 g, 4.15 mmol) was added followed by the addition of 1-hydroxybenzotriazole (HOBT, 0.561 mg, 4.15 mmol) and 2,6-lutidine (1.32 g, 12.5 mmol). The mixture was stirred for 18 h then poured into citric acid buffer (100 mL, 1 M, pH 4.5) and filtered. The yellow solid was thoroughly rinsed with H_2O (3×30 mL), ether (3×30 mL) and dried in the presence of P_2O_5 in a vacuum dessicator for 7 h. The solid was triturated with heptane (4×50 mL) (to remove the excess pentafluorophenol and HOBT), filtered and dried overnight to give **2** (2.5 g, 92%) as an orange powder. ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.61 (s, 4H), 8.02 (t, 1H, J=6 Hz), 7.86 (d, 2H, J=7.3 Hz), 7.62 (d, 2H, J=7.3 Hz), 7.43 (m, 1H, J=6.4 Hz), 7.39 (t, 2H, J=7.3 Hz), 7.27 (t, 2H, J=7.6 Hz), 4.24 (t, 2H, J=6.9 Hz), 4.15 (br, 2H), 4.06 (d, 2H, J=6.4 Hz), 3.60 (br, 1H), 3.50 (d, 2H, J=6.4 Hz), 3.47 (m, 2H), 2.61 (t, 2H, J=7.6 Hz); ^{13}C NMR (63 MHz, DMSO-d_6) δ 172.4, 169.57, 162.5, 162.1, 156.1, 143.7, 143.3, 140.5, 130.3, 127.6, 127.0, 126.0, 125.7, 125.2, 120.0, 63.1, 46.4, 43.5, 36.5, 36.4, 32.0; HR-MS (FAB) m/z 661.1941 (661.1934 calcd for $C_{36}H_{29}N_4O_9$, $M^+ + H$).

Preparation of L and D *N*-2-(*N*-αα-Fluorenylmethoxycarbonyl-*N*-ε-*tert*-butoxycarbonyl-lysyl)aminoethyl-*N*′-(2-carboethyl)-1,4,5,8-naphthalenetetracarboxylic diimide (3) and (4). Compounds **3** and **4** were prepared according to the procedure for the preparation of **2**. However, Fmoc-L-Lys(Boc)-OC6F5 and Fmoc-D-Lys(Boc)-OC6F5 (2.63 g, 4.15 mmol) was used instead of Fmoc-Gly-OC_6F_5. ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.55 (s, 4H), 8.07 (br, 1H), 7.83 (d, 2H,O J=7.3 Hz), 7.61 (t, 2H, J=7.3 Hz), 7.30 (m, 1H), 7.38 (d, 2H, J=6 Hz), 7.28 (br, 2H), 7.07 (br t, 1H) 4.24 (t, 2H, J=6.4 Hz), 4.11 (t, 2H, J=7.3 Hz), 4.04 (d, 2H, J=6.8 Hz), 3.78 (br, 1H), 3.57 (br, 2H), 3.39 (m, 2H), 2.85 (br, 2H), 2.63 (t, 2H, J=7.3 Hz), 1.49 (m, 2H), 1.38 (s, 9H) 1.29 (br m, 4H); ^{13}C NMR (63 MHz, DMSO-d_6) δ 172.5, 63.8, 162.4, 162.1, 155.8, 155.6, 143.7, 140.5, 131.6, 128.9, 128.3, 126.5, 125.7, 123.9, 121.8, 77.3, 55.9, 53.7, 47.5, 45.4, 34.7, 29.9, 29.7, 28.2, 22.1; HR-MS (FAB) m/z 832.3218 (832.3194 calcd for $C_{45}H_{46}N_5O_{11}$, $M^+ + H$).

***N*-2-(*N*-αα-9-Fluorenylmethoxycarbonyl-*N*^{G}-2,2,5,7,8-pentamethylchroman-6-sulfonyl-L-argyl)aminoethyl)-*N*′-(2-carboethyl)-1,4,5,8-naphthalenetetracarboxylic diimide (5).** Compound **5** was prepared according to the procedure for the preparation of **2**. However, Fmoc-Arg(PMC)-OC_6F_5 (3.43 g, 4.15 mmol) was used instead of Fmoc-Gly-OC_6F_5. ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.71(s, 4H), 8.02 (br, 1H), 7.98 (d, 2H, J=7.3 Hz), 7.61 (t, 2H, J=7.5 Hz), 7.37 (d, 2H, J=7.4 Hz), 7.30 (m, 1H), 7.27 (br, 2H), 7.07 (br t, 1H), 6.89 (br, 1H) 6.56 (br, 1H), 4.18 (br m, 7H), 3.38 (br, 1H), 3.39 (m, 2H), 2.98 (br, 2H), 2.00 (s, 6H), 1.90 (br t, 1H), 1.72 (br t, 4H), 1.53 (m, 1H), 1.35 (br, 4H), 1.21 (s, 9H); ^{13}C NMR (63 MHz, DMSO-d_6) δ 170.7, 172.2, 162.5, 162.1, 156.1, 155.8, 152.3, 143.6, 140.5, 134.5, 134.0, 131.6, 128.8, 128.2, 126.5, 126.0, 125.8, 123.9, 122.7, 121.3, 121.1, 118.7, 117.8, 73.4, 65.7, 55.6, 53.3, 47.6, 45.4, 36.2, 34.1, 32.2, 27.4, 25.4, 20.8, 19.2, 18.1, 17.1, 16.1; δ HR-MS (FAB) m/z 1026.3705 (1026.3707 calcd for $C_{54}H_{56}N_7O_{12}S_1$, $M^+ + H$).

***N*′*N*-bis(2,2′-*tert*-Butoxycarbonylaminoethyl)-1,4,5,8-naphthalenetetracarboxylic diimide.** The bis-Boc compound was formed as a by-product during the synthesis of **1**. Chromatographic separation of the statistical mixture using a gradient from 0 to 10% MeOH in 90:10 CH_2Cl_2/TEA and isolation of the first band afforded a tan solid in 15% yield. ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.75 (s, 4H), 4.87 (br, 2H), 4.38 (t, 4H, J=5.7 Hz), 3.57 (m, 4H), 1.22 (s, 18H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 163.14, 156.18, 130.94, 128.43, 128.25, 40.62, 39.19, 28.14, 21.67; HR-MS (FAB) 552.2208 (552.2220 calcd for $C_{28}H_{32}N_4O_8$, $M^+ + H$).

***N*,*N*′-bis(2,2′-Ethylamine)-1,4,5,8-naphthalenetetracarboxylic diimide (6).** The above compound (1 g, 1.81 mmol) was suspended in CH_2Cl_2 (12 mL) and was added dropwise TFA (12 mL). The solution stirred for 30 min, was concentrated in vacuo and the remaining TFA was removed by azeotropic evaporation (5×30 mL) from heptane. The solid was triturated with ether (4×25 mL), filtered and dried in vacuo to give rise to **6** as a yellow powder (630 mg, 97%). ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.72 (s, 4H), 7.93 (br, 4H), 4.35 (t, 4H, J=5.9 Hz), 3.19 (t, 4H, J=5.6 Hz); ^{13}C NMR (63 MHz, DMSO-d_6) δ 162.7, 129.8, 126.0, 125.7, 37.5, 36.9; HR-MS (FAB) m/z 353.1245 (353.1249 calcd for $C_{18}H_{17}N_4O_4$, $M^+ + H$).

***N*,*N*′-bis-(2,2′-Dimethylamino)ethylamine-1,4,5,8-naphthalenetetracarboxylic diimide (7).** The synthesis of **7** is a slight modification of the synthesis reported by Tanious.[24] 1,4,5,8-naphthalenetetracarboxylic dianhydride (1 g, 3.72 mmol) and *N*, *N*-Dimethylethylenediamine (8.1 g, 91.8 mmol) were suspended in THF (15 mL) and heated at reflux for 8 h. The excess volatiles were removed in vacuo, then the mixture was taken up in CH_2Cl_2 (100 mL), washed with a saturated sodium bicarbonate solution (2×20 mL) followed by a water (20 mL) wash and concentrated. The resulting orange solid was suspended in water (5 mL) and acidified to pH 6 with cold HCl. Trituration of the mixture resulted in the formation of a precipitate that was filtered and dried. Recrystallization of the solid from water afforded pure **7** (1.4 g, 92%). ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.74 (s, 4H), 4.35 (t, 4H, J=6.7 Hz), 2.68 (t, J=6.8 Hz, 4H), 2.35 (s, 12H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 162.8, 130.9, 126.05, 126.4, 56.9, 45.7, 380.6; HR-MS (FAB) m/z 409.1874 (409.1875 calcd for $C_{22}H_{25}N_4O_4$, $M^+ + H$).

Combinatorial library

1.5 g of Fmoc-Gly-functionalized NovaSyn TGA resin (0.17 mmol/g loading) was deprotected with 2×20 mL of 50% piperidine/DMF (2×15 min), washed with DMF (5×10 mL), *i*-PrOH (5×10 mL), DMF (5×10 mL), *i*-PrOH (5×10 mL) and DMF (5×10 mL). 674 mg of **2** (1.2 mmol, 4 equiv), 1.95 mL of PyBOP/DMF (0.4 M, 4 equiv) and 1.95 mL of NMM/DMF (0.8 M, 8

equiv) were added to the reaction and shaken for 45 min. The resin was washed with *i*-PrOH (3×10 mL), DMSO (3×10 mL) followed by the wash procedure described above. The resin was suspended in 50 mL DCM with stirring and the mixture was divided into 20 reaction vessels using disposable pipettes. Each vessel represented one of the standard amino acids, whereas one corresponded to the control. The DCM was filtered, the resin was washed with DMF and deprotected as previously described. The appropriate Fmoc-amino acids (4 equiv); Ala, Arg(Pmc), Asn(Trt), Asp(OBu), Glu(OBu), Gln(Trt), Gly, His(Trt), Ile, Leu, Lys(Boc), Met, Phe, Pro, Ser(*t*-Bu), Thr(*t*-Bu), Trp(Boc), Tyr(*t*-Bu), Val, were coupled to the resin using PyBOP/NMM (see above). The resin was washed with DMF, *i*-PrOH, DMF, *i*-PrOH, DMF and the resin from the vessels was suspended in DCM (50 mL) with stirring and was evenly divided into the 19 reaction vessels. This process was repeated until all of the units had been coupled, however, the resin was not mixed and pooled after the addition of the last amino acid. Instead, 674 mg of **2** was dissolved in 1.95 mL of DMF, 260 μL of the solution was added to each vessel and 260 μμL of PyBOP/DMF (0.4 M, 4 equiv) and 260 μL μ of NMM/DMF (0.8 M, 8 equiv) were added and shaken for 45 min. The resin was washed with *i*-PrOH (3×10 mL), DMSO (3×10 mL), followed by the wash procedure described above. The resin in each vessel was then suspended in DCM (5 mL) and transferred to twenty 10 mL glass vials with a screw cap. The resin was dried in vacuo and the acid labile side chains and mixtures were cleaved from the resin with 5 mL TFA/H_2O/TIS (95:2.5:2.5, 18 h). The resin was filtered off and washed several times with TFA (0.5 mL) and DCM (0.5 mL). Each solution was transferred to a 50 mL plastic tube and triturated with cold ether (20 mL). The products precipitated and the solutions were centrifuged for 15 min, the ether was decanted and cold ether (20 mL) was added. The process was repeated five times to remove residual TFA and the resulting solids were dried in vacuo for 1 h, dissolved in 10 mL of H_2O and lyophilized. All mixtures were analyzed on RP HPLC (C_{18}) monitored by UV (λ = 386 nm) over a 20 min gradient. The gradient consisted of 90% TFA/H_2O (0.07:99.93) to 100% TFA/CH_3CN (0.07:99.93).

Parallel synthesis

Compounds **8–39** and **44–63** were synthesized using the procedure described above. However, the compounds were not mixed after couplings and in some cases, Fmoc-Lys(Boc) or Fmoc-Arg(Pmc) resin was used instead of Fmoc-Gly resin.

Biological screening: Kirby–Bauer assay

The following studies were conducted in the absence of light due to the sensitivity of the NDI units. The compounds synthesized via combinatorial and parallel synthesis were tested for biological activity against *B. subtilis* and *E. coli* using the Kirby–Bauer disk diffusion method.[15–17] The tests were performed using the reference strains *E. coli* JM109 and *B. subtilis* 1012M15. Monomer concentrations were determined by UV measurements at 386 nm (ε_{386} = 23,300, 1 cm) and dimer concentrations were determined at 386 nm (ε_{386} = 27,400, 1 cm). Stock concentrations (15, 5, and 1.66 mM) of the compounds to be tested were prepared using sterile dd H_2O. The desired compound (20 μL) was added to a sterile 4 inch concentration disk (Difco) and allowed to dry overnight to afford compounds of 300, 100, and 33 nmol, respectively.

Minimum inhibitory concentrations (MICS)

In addition to the Kirby–Bauer test, the MIC of various compounds were determined in microtiter plates by making 2-fold serial dilutions of each compound in Mueller–Hinton broth, adding the test organism and incubating the plate for 16–20 h.[16,17] *E. coli* JM109 and *B. subtilis* 1012M15 were grown in Muller–Hinton broth for 4 h and diluted to an OD_{600} of 0.1. Microtiter plates were covered with a sterile lid and incubated at 37 °C for 18 to 20 h and the MICs were determined. The wells were examined for bacterial growth at OD_{600} and the MIC was defined as the lowest concentration of the agent that prevented growth of the test organism. The activity in mammalian cells of selected compounds were determined through 2-fold serial dilutions starting at 100 μM concentrations using the MTT assay.[18]

References and Notes

1. Oren, Z.; Hong, J.; Shai, Y. *J. Biol. Chem.* **1997**, *272*, 1463.
2. Setti, E. L.; Micetich, R. G. *Curr. Med. Chem.* **1998**, *5*, 101.
3. Hancock, R. E.; Falla, J. T. In *Biotechnology of Antibiotics*, 2nd ed.; Strohl, W. R., Ed.; Marcel Dekker: New York, 1978; pp 471–494.
4. Tossi, A.; Sandri, L.; Giangasporo, A. *Biopolymers* **2000**, *55*, 4. See also the other comprehensive reviews in this issue.
5. Hancock, L.; Lehrer, R. *Trends in Biotech.* **1998**, *16*, 82.
6. Vaara, M.; Vaara, T. *Anti. Agents Chemother.* **1983**, *24*, 107.
7. He, K.; Ludtke, J. S.; Huang, H. W. *Biochemistry* **1995**, *34*, 15614.
8. Nikaido, H.; Vaara, M. *Micro. Rev.* **1985**, *49*, 1.
9. Shima, S.; Matsuoka, H.; Iwamoto, T.; Sakai, H. *J. Antibiot.* **1984**, *37*, 1149.
10. Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205.
11. Lee, S.; Mihara, H.; Aoyagi, H.; Kato, T.; Izumiya, N.; Yamasaki, N. *Biochim. Biophys. Acta* **1986**, *862*, 211.
12. Cheng, C. C. In *Cancer Chemotherapuetic Agents*; Foye, W. O., Ed.; American Chemical Society: Washington, DC, 1995; pp 239–260.
13. Yeng, S.; Gabbay, E. J.; Wilson, D. W. *Biochemistry* **1982**, *21*, 2070.
14. Lokey, R. S.; Kwok, Y.; Guelev, V.; Pursell, C. J.; Hurley, L. H.; Iverson, B. L. *J. Am. Chem. Soc.* **1997**, *119*, 7202.
15. Alper, P. B.; Hendrix, M.; Sears, P.; Wong, C. *J. Am. Chem. Soc.* **1998**, *120*, 1965.
16. Phillips, I.; Williams, J. D.; Wise, R. In *Laboratory Methods in Antimicrobial Chemotherapy*; Garrod, L., Ed.; Churchill Livingston: Edinburgh, 1978; pp 3–30.
17. Waterworth, P. M. In *Laboratory Methods in Antimicrobial Chemotherapy*; Garrod, L., Ed.; Churchill Livingston: Endinburgh, 1978; pp 31–40.
18. Sami, S. M.; Dorr, R. T.; Aniko, M. S.; Alberts, D. S.; Bhashyam, S. I.; Remers, W. A. *J. Med. Chem.* **1995**, *39*, 1609.

19. Schutz, V. S.; Kurz, J.; Plumpe, H.; Bock, M. O.; Otten, H. *Arzn-Forsch*. **1971**, *21*, 739.
20. Paull, K. D.; Nasr, M.; Narayanan, V. L. *Arzn-Forsch* **1984**, *34*, 1243.
21. Sami, S. M.; Dorr, R. T.; Alberts, D. S.; Remers, W. A. *J. Med. Chem*. **1993**, *36*, 765.
22. Sami, S. M.; Dorr, R. T.; Aniko, M. S.; Alberts, D. S.; Remers, W. A. *J. Med. Chem*. **1995**, *38*, 983.
23. Sami, S. M.; Dorr, R. T.; Aniko, M. S.; Alberts, D. S.; Remers, W. A. *J. Med. Chem*. **1996**, *39*, 4978.
24. Tanious, F. A.; Yen, S.; Wilson, W. D. *Biochemistry* **1991**, *30*, 1813.