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Chemoenzymatic Synthesis and High-Throughput Screening of an Aminoglycoside−Polyamine Library: Identification of High-Affinity Displacers and DNA-Binding Ligands

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Abstract: Chemoenzymatic parallel synthesis and high-throughput screening were employed to develop a multivalent aminoglycoside−polyamine library for use as high-affinity cation-exchange displacers and DNA-binding ligands. Regioselective lipase-catalyzed acylation, followed by chemical aminolysis, was used to generate vinyl carbonate and vinyl carbamate linkers, respectively, of the aminoglycosidic cores. These were further derivatized with polyamines, leading to library generation. A parallel batch-displacement assay was employed to identify the efficacy of the library candidates as potential displacers for protein purification. Using this approach, low-molecular-mass displacers with affinities higher than those previously observed have been identified. The aminoglycoside−polyamine library was also screened for DNA binding efficacy using an ethidium bromide displacement assay. These highly cationic molecules exhibited strong DNA-binding properties and may have potential for enhanced gene delivery.

Introduction

Displacement chromatography has attracted significant attention as a powerful technique for the purification of biotherapeutic proteins and oligonucleotides.[1−9] In particular, low-molecular-weight (MW < 2000) displacers have been shown to have significant advantages for high-resolution protein purifications. Displacement chromatography enables simultaneous concentration and purification in a single step, which is significant in the purification of biopharmaceuticals. However, the major obstacle in implementing this technique is the lack of a sufficient diversity of appropriate displacer candidates. Low-molecular-weight displacers employed to date possess moderate to high affinities, yet are unable to displace highly retained proteins on a variety of hydrophilic and hydrophobic resins. Thus, there is an urgent need to develop a wide range of appropriate high-affinity and selective displacers that are applicable across a wide spectrum of bioseparation demands.

The identification of appropriate displacer candidates has traditionally relied on estimation of the steric mass action (SMA) parameters[10,11] of potential candidates. Shukla et al.[12,13] used a dynamic affinity analysis to assess the effectiveness of displacers as a function of key structural characteristics, including spacing of the charged moieties, number of charges, types of charged functional groups, presence of aromatic groups, and overall geometry. While these approaches yielded important information on actual column performance of the displacer candidates, they were time-consuming. They also involved a number of linear gradient experiments. Furthermore, this approach does not allow for the a priori prediction of displacer efficacy for new displacer lead compounds.

Empirical screening of chemical compound libraries is of growing importance for the identification of chemical leads in the pharmaceutical industry.[14−16] Combinatorial techniques and high-throughput screening (HTS) have also been widely employed for the identification of ligands for affinity chromatog-

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raphy from combinatorial libraries.[17] Although the earliest reports on the use of combinatorial synthesis of affinity ligands involved screening of epitope peptide libraries,[18−22] small-molecule ligands have been identified for a variety of targets, including kallikrien,[23] IgG,[24] and elastases[25] from focused libraries.

We have recently developed a batch-displacement assay as a high-throughput screening technique for the rapid identification of potential displacer molecules.[26] The screening results indicated that a variety of aminoglycosides and linear polyamines show moderate to high affinities as displacers in cation exchange systems. The high-throughput screening technique is a powerful route for the rapid identification of efficient displacer molecules in ion-exchange systems, and it has been recently extended to identify selective and high-affinity displacers for protein mixtures.[27] These HTS results have also been used to develop quantitative structure−efficacy relationship (QSER) models,[26] and they have been employed in concert with the multicomponent steric mass action (SMA) formalism for the prediction of chromatographic behavior of selected displacer leads.[28]

The complexation of DNA by polyamines has been investigated in great detail.[29−34] The addition of multivalent cations with a charge greater than three in each molecule induces the condensation of DNA in aqueous solutions. Multivalent cations effectively reduce the electrostatic repulsion between DNA molecules by charge neutralization, ultimately leading to DNA collapse or condensation, which is an important first step for gene therapy applications. Measurement of the ability of a drug to displace ethidium bromide from DNA is established as a valid measurement of its DNA binding ability. It is hypothesized that DNA binding induced by polyamine binding above a critical concentration causes conformational changes within the double helix leading, to the release of bound ethidium bromide.[35,36]

In this paper, we describe the development of a small-molecule library for potential use as both high-affinity displacers in ion-exchange chromatography and as DNA-binding ligands. Previous work on parallel screening of commercially available displacer candidates in our laboratory resulted in the identification of aminoglycosides and linear polyamines as moderate- to high-affinity displacers in cation-exchange systems.[26,27] It was therefore hypothesized that the derivatization of aminoglycosidic core molecules with multiple copies of linear polyamines at different positions on the core would result in multivalent derivatives. Consequently, chemoenzymatic synthesis was used to generate libraries based on polyamine derivatives of aminoglycosides. Enzymes were used to regioselectively acylate aminoglycoside-linker derivatives, which were further modified by linear polyamines at the selected positions. This enabled a multivalent approach whereby linear polyamines were grafted onto an aminoglycosidic core to generate multivalent cationic molecules. The components of the library were screened for their displacement efficacy using a batch displacement assay[26] and for their DNA-binding efficacy using an ethidium bromide displacement assay.[35,36] The results presented in this paper demonstrate that this approach can be successfully employed to identify several powerful multivalent ligands for both protein purification and DNA condensation.

Experimental Section

Materials. *Candida antarctica* lipase B (CAL-B, Novozyme 435) was obtained from Novozymes North America, (Franklinton, NC) as a gift. *Pseudomonas fluorescence* lipase (PFL) was purchased from Amano (Nagoya, Japan). Fast Flow Sepharose SP (FF Sepharose SP), High Performance SP Sepharose (HP Sepharose SP), and Source 15S stationary phase materials were donated by Amersham Pharmacia (Uppsala, Sweden). (Note that while the Sepharose materials are agarose-based, the Source resins consist of hydrophilized poly(styrene-divinylbenzene.) The HTS experiments were carried out using 96-well Multiscreen, 0.45-μm Durapore membrane-bottomed plates donated by Millipore. The 2′-deoxyadenosine, glucosamine, mannosamine, kanamycin A & B, neomycin sulfate, spermine, calf-thymus DNA, chicken egg lysozyme, horse heart cytochrome-C, ammonium bicarbonate, sodium phosphate (dibasic), and sodium phosphate (monobasic) were purchased from Sigma (Saint Louis, MO). Ethylenediamine, diethylenetriamine, vinyl chloroformate, and dry THF were purchased from Aldrich (Milwaukee WI). Acetone-*O*-(vinyloxy)carbonyl)oxime was prepared according to a literature protocol.[37] Glucosamine analogues (Scheme 1, molecules **1b**−**1e**) neamine (Figure 2, molecule **15**) and methyl neobiosamine (Figure 2, molecule **18**) were prepared according to literature procedures.[38−40]

The ^{1}H and ^{13}C NMR spectra were recorded on a Varian spectrometer with TMS as the internal standard. Chemical shifts are reported in ppm and the coupling constants (*J*) are given in Hertz (Hz). The ESI-MS and MALDI-TOF were measured on a Varian mass spectrometer. Flash chromatography was performed on 60−200-mesh silica gel (Sigma MO). Product yields, purities, and spectroscopic data are provided in the Supporting Information section. Cation-exchange chromatography was performed on Fast Flow Sepharose SP (FF Sepharose SP) using ammonium bicarbonate (NH_4HCO_3) as a mobile phase. Fluorescence and absorbance analyses were carried out using a Perkin-Elmer plate reader and the results were analyzed using the software HTSoft 2.0.

Procedures. I. Generation of Glucosamine/Mannosamine Derivatives (Scheme 1). Synthesis of Vinyl Carbamate Linkers, 2a−2e (Procedure 1). Vinyl chloroformate (426 μL, 5.0 mmol) was added

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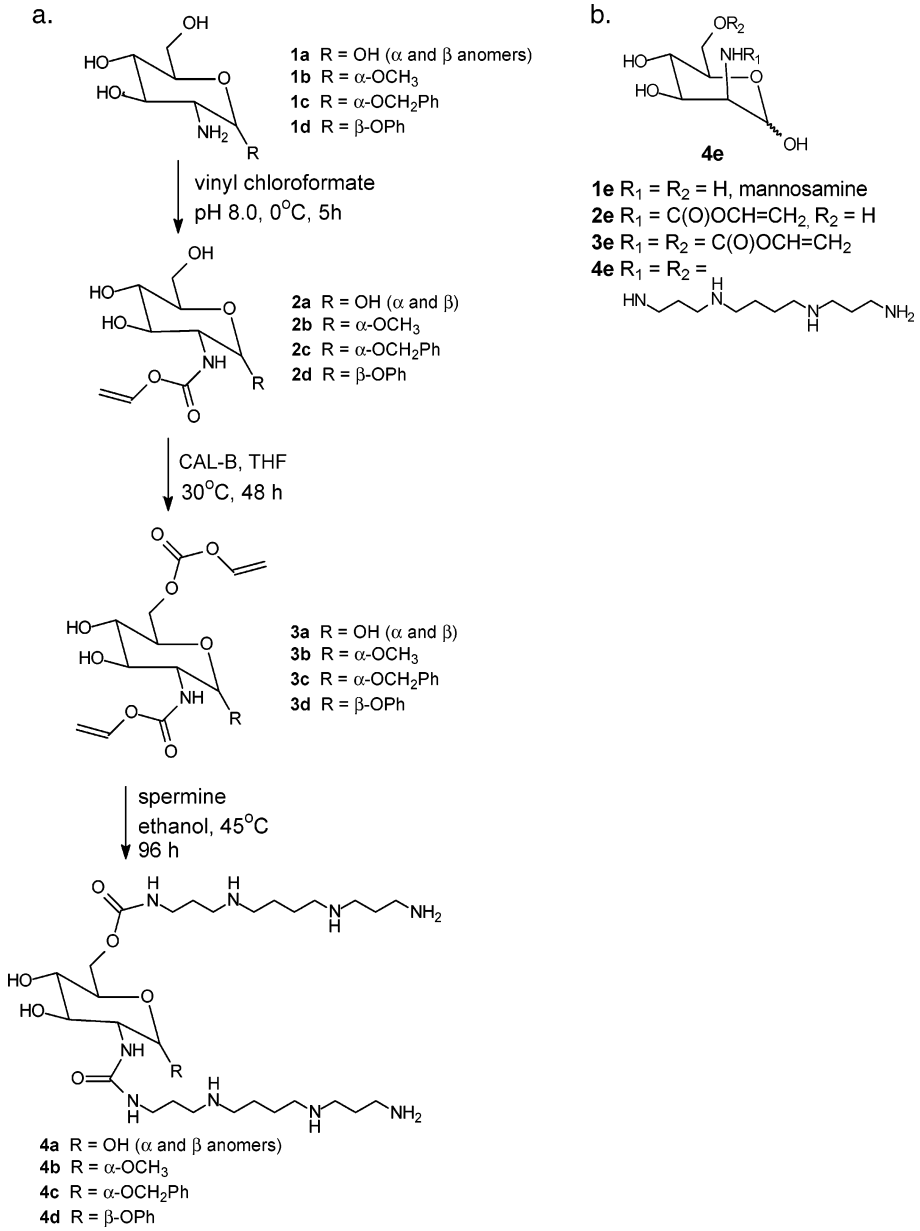
Scheme 1. (a) Chemoenzymatic Synthesis of Glucosamine-Based Derivatives and (b) Mannosamine Derivatives



dropwise to a phosphate buffer solution (40 mL, pH 8.0, 50 mM) of compounds (**1a**−**1e**) (2.0 mmol) for 1 h at 0 °C. The reaction mixture was vigorously stirred and the solution was maintained at pH 8.0 by continuously adding 1.0 N NaOH. After 5 h, compounds **2c** and **2d** were directly precipitated from the buffer solution, then they were washed by distilled H_2O and dried under vacuum with >95% purity. For the other compounds, the reaction mixture was lyophilized for 24 h to afford crude product, which was purified by silica gel flash chromatography using EtOAc/MeOH (5:1 and 2:1). The above procedure resulted in moderate to good yields: **2a**, 88%; **2b**, 33%; **2c**, 42%; **2d**, 67%, **2e**, 80%.

Lipase-Catalyzed Regioselective Synthesis of 6-Vinyl Carbonate Linkers, 3a−3e (Procedure 2). A mixture of **2a**−**e** (0.6 mmol), acetone *O*-(vinyloxy) carbonyl)oxime (1.8 mmol), and *Candida antarctica* lipase B (CAL) in 15 mL of dry THF were shaken at 200 rpm at 45 °C for 24−96 h. The reaction was monitored by TLC. After evaporation of the solvent, the residue was purified by flash chromatography using hexane/EtOAc (2:1, 1:2) to afford **3a**−**3e** (**Scheme 1**). The yields were generally good (**3a**, 91%; **3b**, 73%; **3c**, 83%; **3d**, 48%; **3e**, 82%) and the 6-vinyloxy derivatives were the sole products in most cases.

Generation of Spermine Derivatives of Glucosamine and Mannosamine, **4a**−**4e (Procedure 3).** A solution of vinyloxy linkers (**3a**−**3e**, **Scheme 1**) (0.2−0.4 mmol) and spermine (3 equiv relative to the free amine group) in ethanol (10 mL) was shaken at 45 °C and 250 rpm for 96 h. After removal of ethanol under pressure, the residue was neutralized with 1.0 N HCl at 0 °C. After lyophilization, the powder was purified using cation-exchange chromatography (resin: FF Sepharose SP, eluent: 0.1−0.5 M NH_4HCO_3 solution) as described subsequently. The fraction collection was monitored by TLC and detected by ninhydrin reagent solution after heating at 100 °C. The NH_4HCO_3 solution was removed by lyophilization to yield desired products (**4a**−**4e**) with purities in the range 85 ∼ 95% based on NMR and MS analyses (Scheme 1) and moderate isolated yields in the range 10−25% after cation-exchange chromatography (described below). It was observed that the formation of isomers was usually lower than 20−40%, presumably due to stronger steric effects of the secondary amino than the primary amino groups in spermine. The mannosamine-spermine derivative (**4e**) was synthesized in a similar manner with a 23% overall yield.

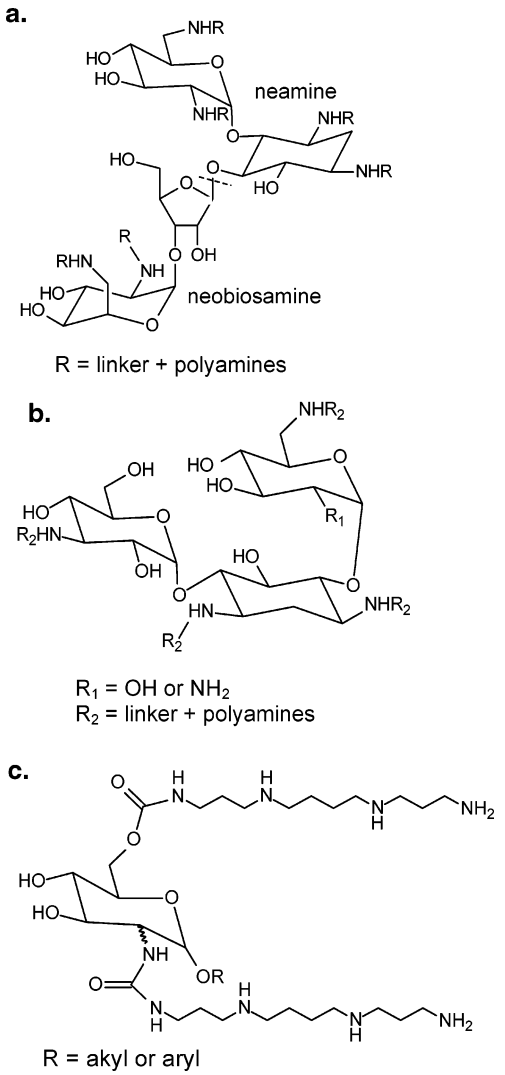


Figure 1. Structures of the aminoglycosidic leads used for derivatization with polyamines. (a) Neomycin, neamine, neobiosamine, and potential polyamine library using CO linkers. (b) Kanamycin A and B and potential polyamine library using CO linkers. (c) Glucosamine- and mannosamine-based dispermine derivatives.

II. Generation of 2′-Deoxyadenosine Derivatives (Schemes 2 and 3). Monospermine Derivatives of 2′-Deoxyadenosine (Scheme 2a,b). The vinyl linker **6** was readily formed using CAL-B catalyzed acylation with high yields (87%).[40] Aminolysis with excess spermine gave the desired product, **7**. A solution of vinyloxy derivative **6** (0.3 mmol) and spermine (0.9 mmol) in THF (20 mL) was shaken at 30 °C and 250 rpm for 24 h. After removal of THF, the residue was purified by silica gel flash chromatography using MeOH/1.0 M NaCl (10:1) to yield the pure product **7** (69% yield after flash chromatography, Scheme 2a).

Pseudomonas fluorescence lipase (PFL) was used to catalyze the acylation of 2′-deoxyadenosine; the vinyl carbonate linker was introduced at 3′-hydroxyl as described above, with high regioselectivity (>10:1, 3′-OH versus 6′-OH group) to yield the intermediate product, **8** in reasonable yield (28%).[40] The desired monospermine derivative, **9**, was obtained upon aminolysis with spermine (50% yield, 95% purity).

Trispermine Derivatives of 2′-Deoxyadenosine. Vinyl chloroformate (307μL, 5.4 mmol) was added dropwise to **5** (Scheme 2a) (377 mg, 1.5 mmol) in pyridine (5 mL) and DMAP (122 mg, 1.0 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 24 h, after which the reaction mixture was diluted with EtOAc (20 mL) and the mixture was washed with 1.0 N HCl, saturated $NaHCO_3$, and then saturated NaCl. The organic phase was dried over $MgSO_4$. After evaporation of solvent the residue was purified by flash chromatography (hexane/EtOAc, 1:1 and 1:5) to afford the desired product **10** (yield, 28%). The trispermine derivative, **11**, was synthesized in a manner similar to that described in Procedure 3 (yield 32%, purity, 85%).

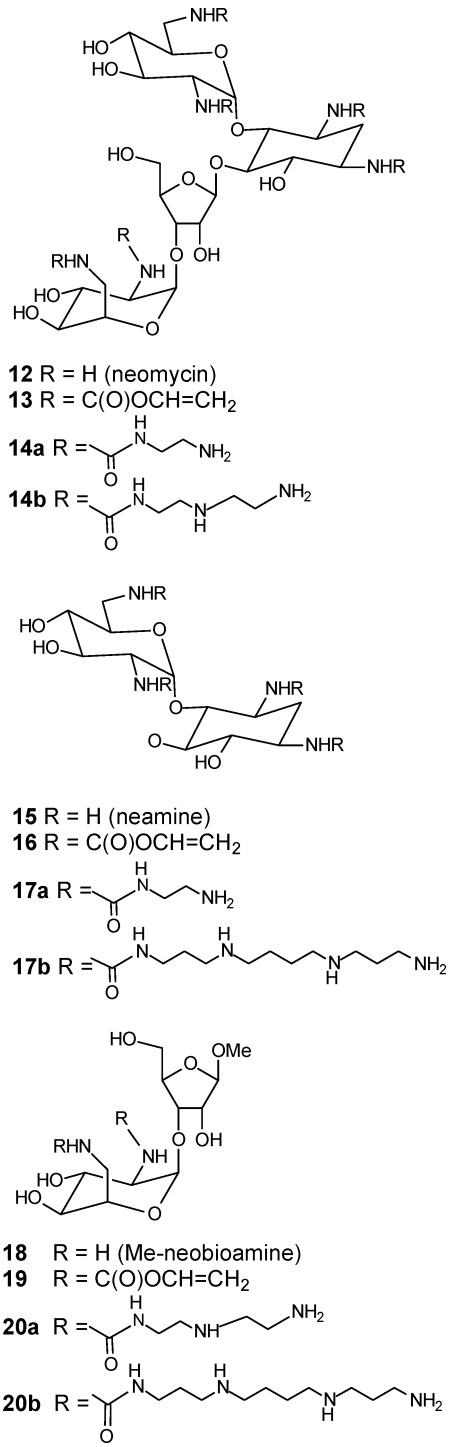
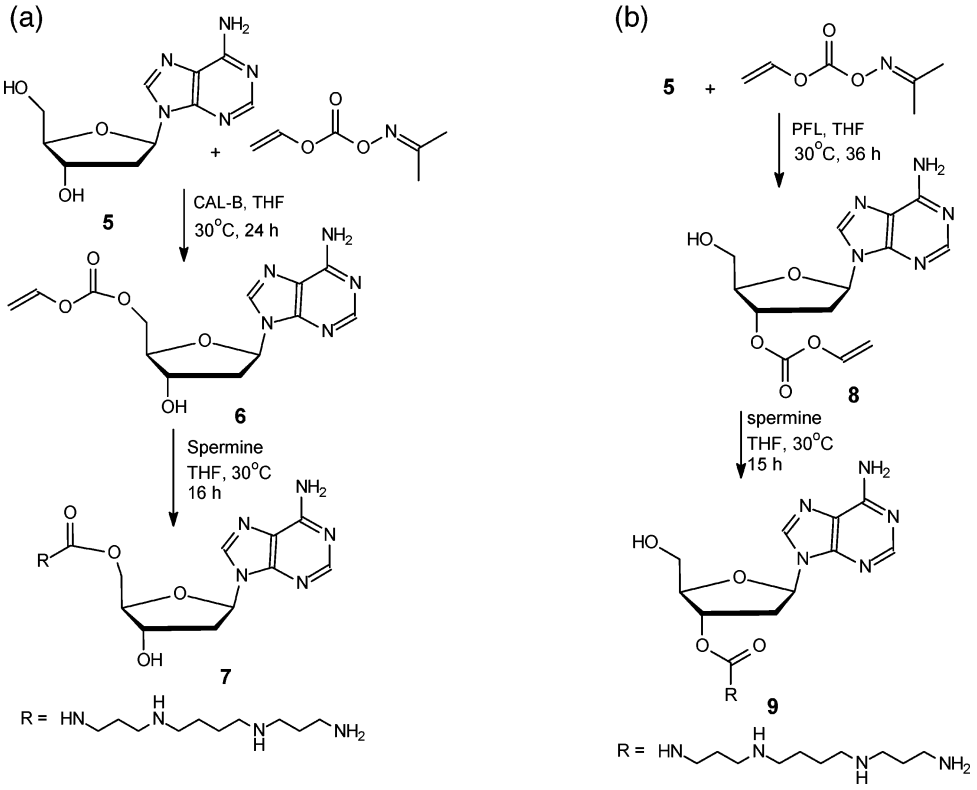


Figure 2. Neomycin-, neamine-, and neobiosamine-based polyamine library.

III. Generation of Polyamine Derivatives of Neomycin (12), Neamine (15), and Methyl-Neobiosamine (18) (Figure 2). Synthesis of Vinyl Carbamate Linkers (13, 16, 19). The vinyl carbamate linkers of neomycin, neamine, and methyl-neobiosamine were synthesized as described in Procedure 1 in moderate to good yields (13, 71%; 16, 66%; 19, 48%, respectively).

Ethylenediamine-Based Derivatives (Procedure 4). A solution of vinyloxy linkers **13** and **16**, (0.2−0.4 mmol) and ethylenediamine (2

Scheme 2. (a) Chemoenzymatic Synthesis of 2′-Deoxyadenosine Derivative Containing a Spermine Chain at the 6′-Hydroxyl Group and (b) Chemoenzymatic Synthesis of 2′-Deoxyadenosine Derivative Containing a Spermine Chain at the 3′-Hydroxyl Group



mL) were shaken at 35 °C and 250 rpm for 24−48 h. The excess ethylenediamine was evaporated under pressure and the residue was precipitated by MeOH/EtOAC; it was then washed with EtOAc to yield pure products **14a** and **17a**, respectively.

Diethylenetriamine-Based Derivatives (Procedure 5). A solution of vinyloxy linkers **13** and **19** (0.3 mmol) and diethylenetriamine (4 equiv relative to the free amine group) in ethanol (10 mL) was shaken at 45 °C and 250 rpm for 96 h. After removal of ethanol under vacuum, the residue was precipitated and washed as described in procedure 4 to afford **14b** and **20a**, respectively.

Spermine-Based Derivatives. The spermine derivatives of neamine and methyl-neobiosamine, **17b** and **20b**, respectively, were synthesized according to Procedure 3 above and were obtained in moderate to good yields.

IV. Generation of Polyamine Derivatives of Kanamycin (Figure 3). Synthesis of Vinyl Carbamate Linkers (22a and 22b). The carbamate linkers for kanamycin A and B were synthesized according to Procedure 1 and were obtained in moderate to good yields: **22a**, yield 37%, **22b**, yield 84%.

Ethylenediamine-Based Derivatives. A solution of **22a** or **22b** (0.2−0.4 mmol) and ethylenediamine (2 mL) was shaken at 35 °C and 250 rpm for 24−48 h. The excess ethylenediamine was evaporated under pressure, and the residue was precipitated by MeOH/EtOAC and then washed with EtOAc to yield pure products **23a** and **24**, respectively.

Diethylenetriamine-Based Derivatives. A solution of **22a** (0.3 mmol) and diethylenetriamine (4 equiv relative to free amine group) in ethanol (10 mL) was shaken at 45 °C and 250 rpm for 96 h. After removal of ethanol in vacuo the residue was precipitated and washed as procedure A to afford **23b** (76% yield).

Spermine-Based Derivatives. The spermine derivatives **23c** and **25** (structure deduction based on ESI-MS, ^{1}H and ^{13}C NMR data) were synthesized according to Procedure 3 above and were obtained in moderate to good yields (7% and 27%, respectively) with purities >95% after cation exchange chromatography.

Purification of Aminoglycoside−Polyamine Library. The purification of the components of the aminoglycoside−polyamine library was carried out as follows. In the case of ethylenediamine, the desired aminoglycoside polyamines were produced in a high yield and good purity after the evaporation of excess ethylenediamine in vacuo and simple precipitation with a mixture of ethyl acetate and methanol (for example, **14a** and **17a**, >99% yield, >95% purity).

For the purification of diethylenetriamine and spermine derivatives, a parallel batch screen using resin packed in a membrane-bottomed 96-well microtiter plate was used to identify the correct resin-mobile phase combination for the subsequent chromatography steps. Cation-exchange chromatography using Fast Flow Sulfopropyl Sepharose (FF Sepharose SP) with a multistep gradient of ammonium bicarbonate were identified as the best candidates for the stationary phase and the mobile phase conditions, respectively (screening data not shown). The fact that ammonium bicarbonate could be removed from the final product by freezedrying was also a significant factor in its selection as the mobile phase.

Parallel Screening of Displacers.[26] Protein Adsorption. The bulk stationary phase (HP Sepharose SP or Source 15S) was first washed once with deionized water and then three times with the buffer, (50 mM phosphate, pH 6.0) and was allowed to equilibrate for 2 h. After gravity settling of the stationary phase, the supernatant was removed and 3.0 mL of the remaining stationary phase slurry was equilibrated with 36 mL containing 3 mg/mL of the protein (horse-heart cytochrome-C or chicken egg lysozyme) in 50 mM phosphate buffer, pH 6.0, at 20 °C. The protein was equilibrated with the resin for 5 h to attain complete equilibration, during which the stationary phase was allowed to gravity settle. Upon settling, the supernatant was removed and the protein content in the supernatant was determined using absorbance detection at 280 or 405 nm, using a plate reader. The mass of the protein adsorbed on the stationary phase was determined by mass balance.

Determination of DC_{50}. For the screening experiments, 300 μL of different initial concentrations (ranging from 0.3 to 5 mM) of a displacer

solution was added to 25-μL aliquots of the stationary phase slurry with bound protein. (Note that a different displacer molecule and concentration were employed for each vial to enable parallel screening.) The system was equilibrated for 5 h. After equilibrium was achieved, the supernatant was removed and the protein content was determined by absorbance detection at 280 or 405 nm, using a plate reader. This entire procedure was carried out in parallel in 96-well membrane-bottomed plates to enable rapid screening of potential displacer candidates. The *percent protein displaced* was calculated for each aliquot based on protein mass balance and the data were plotted as a function of the initial displacer concentration. The resulting plots were then employed to determine the initial displacer concentration required to displace 50% of the adsorbed protein (i.e., the DC_{50}).

Identification of DNA-Binding Ligands. The components of the aminoglycoside−polyamine library were screened for their DNA-binding efficacy using an ethidium bromide displacement assay.[35,36] Parallel screening of DNA-binding activity was carried out in the following manner: 3 mL of 6 μg/mL double-stranded calf-thymus DNA were equilibrated with 15 μL of 0.5 mg/mL ethidium bromide. After equilibration, 25 μL of each potential DNA-binding compound (0.3 mM) were added to the DNA−ethidium bromide mixture and equilibrated for 20 min. A portion (75 μL) of the resulting solution was transferred into a 96-well microtiter plate and the percent fluorescence decrease was monitored using excitation at 260 nm and emission at 595 nm. All solutions were prepared in 20 mM Tris buffer, pH 8.0.

Results and Discussion

In this paper, we describe the parallel synthesis and high-throughput evaluation of a multivalent cationic library designed as high efficacy displacers for protein purification and high-affinity DNA-binding ligands. Previous work on parallel screening of commercially available displacer candidates in our laboratory resulted in the identification of aminoglycosides and linear polyamines as moderate- to high-affinity displacers in cation-exchange systems.[26,27] Furthermore, structure-efficacy relationship modeling results in concert with the screening data indicated that charge content and spacing, inclusion of aromatic moieties in the molecule, and molecular flexibility resulted in increased affinity of displacer candidate molecules.[26,27] Based on these results, it was determined that the generation of a multivalent library based on the derivatization of aminoglycosides by linear polyamines would result in candidates with increased displacement affinities. Further, the availability of a large number of reactive sites for functionalization makes aminoglycosides (Figure 1) promising lead candidates for chemoenzymatic syntheses of these libraries. As will be shown later in this paper, it turns out that the high cationic content of these library candidates also make them promising candidates for DNA binding/complexation. Accordingly, the library was screened for both displacer efficacy and DNA-binding affinity. The yields, purities, and spectroscopic data of the library candidates are described in the Supporting Information.

Chemoenzymatic Synthesis of the Aminoglycoside−Polyamine Library. The structural and functional diversity of aminoglycosides was expanded using chemoenzymatic syntheses. Selective incorporation of a vinyl carbamate linker on amino groups in a molecule containing multiple hydroxyl groups was readily achieved through chemical means in yields in the range 35−90%. A vinyl carbamate linker was initially introduced on the 2α-amino group of glucosamine (**1a**) and its analogues, **1b**−**1d** (Scheme 1) using vinyl chloroformate as the acyl donor. However, regioselective introduction of a vinyl carbonate linker is very challenging and led us to consider enzymatic acylation as an alternative synthetic approach.[41−43] We used *Candida antarctica* lipase B (CAL-B) to incorporate the carbonate linker at the 6-OH using acetone O−(vinyloxy)carbonyl)oxime as the acyl donor in THF.[37] Based on this strategy, we envisioned that a series of different oligosaccharide-based branched polyamines with different orientations could be prepared, each with different efficacies as displacers in ion-exchange chromatography. To that end, the acylated derivatives prepared above were further derivatized with spermine, as shown in Scheme 1. The purification of the spermine-conjugated molecules was performed using cation-exchange chromatography with FF Sepharose SP as the stationary phase and a linear gradient of NH_4HCO_3 as the mobile phase. The combination of the stationary and mobile phases was based on a microtiter-plate screen for a variety of resins and eluents. Although other eluents also showed comparable performance, NH_4HCO_3 was chosen as the mobile phase, particularly because it could be lyophilized from the final product.

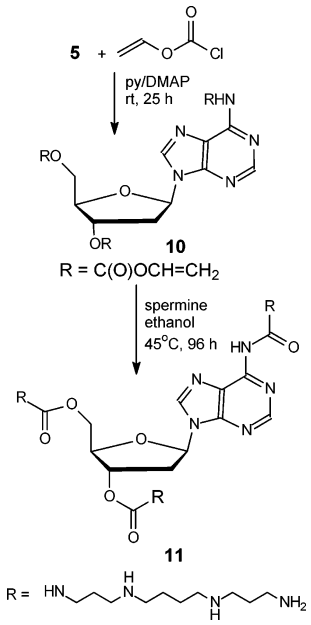
The synthesis of monospermine compounds based on 2′-deoxyadenosine is described in Scheme 2. The monospermine derivative of 2′-deoxyadenosine, **7** (spermine incorporated on the primary hydroxyl group of 2′-deoxyadenosine), was synthesized chemoenzymatically; the vinyl linker, **6**, was readily formed using CAL-B-catalyzed acylation.[40] Aminolysis with excess spermine resulted in desired product, **7**. For the synthesis of the regioisomer **9**, the regioselective generation of the vinyl carbonate linker at the 3′-hydroxyl group is challenging. Nevertheless, *P fluorescence* lipase (PFL) did catalyze the 3′-acylation with high regioselectivity (>10:1, 3′-OH versus **5**′-OH group) to afford **8** in reasonable yield (28%).[40] After aminolysis of **8** with spermine similar to that described above, the desired product, **9,** was obtained in 50% yield and ∼95% purity resulting in a 14% overall yield of **9**. Finally, the trispermine derivative of 2′-deoxy-adenosine, **11**, was synthesized via chemical acylation of 2′-deoxyadenosine with vinyl chloroformate, followed by the aminolysis with spermine in a 9% overall yield for the two steps (Scheme 3).

Neomycin-, neamine-, methyl-neobiosamine- (Figure 2) and kanamycin-based polyamine derivatives (Figure 3) were synthesized using a procedure similar to that used in generating the glucosamine/mannosamine derivatives. Incorporation of the vinyl carbamate linker on the amino groups of these molecules was performed chemically in yields ranging from ∼50 to 70%, using vinyl chloroformate as the acyl donor. The acylated derivatives were further derivatized with ethylenediamine, diethylenetriamine, and spermine to yield **14a**, **14b**, **17a**, **17b**, **20a**, **20b**, **23a**, **23b**, **23c**, **24**, and **25** in yields ranging from ∼40 to 85%.

Evaluation of the Aminoglycoside−Polyamine Library. Identification of High-Affinity Cation-Exchange Displacers. As described in the Introduction, we have recently developed an efficient technique for the high-throughput screening of displacer molecules.[26] In this approach, small-scale parallel batch displacement experiments are used for rapidly screening a large number of potential displacer candidates. The technique can be

(41) Riva, S.; Chopineau, J.; Kieboom, A. P. G.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 584−589.
(42) Hennen, W. J.; Sweers, H. M.; Wang, Y.-F.; Wong, C.-H. *J. Org. Chem.* **1988**, *53*, 4939−4945.
(43) Ferreira, L.; Ramos, M. A.; Gil, M. H.; Dordick, J. S. *Biotechnol. Prog.* **2002**, *18*, 986−993.

Scheme 3. Synthesis of a 2′-Deoxyadenosine Derivative Having Three Spermine Chains



applied to screen a diverse library of molecules and can be used for different modes of interactions, such as ion exchange, hydrophobic interaction, and reversed phase, and presents a significant advantage over previous column based techniques.[12,13]

In the present work, we used a modified batch-displacement assay to investigate displacer efficacy over a wide range of concentrations. Different initial concentrations of the displacer candidates were employed to determine the DC_{50} value that is the initial displacer concentration required to displace 50% of the initially bound protein; clearly, the lower the DC_{50} value, the more efficacious the displacer. Figure 4 presents representative data for the displacement of cytochrome-C by various initial concentrations of neamine and its derivatives on HP Sepharose SP. The resulting plots were then employed to determine the DC_{50} values of neamine (DC_{50}, 5.6 mM), **17a** (DC_{50}, 2.1 mM), and **17b** (DC_{50}, 0.7 mM).

The above approach was employed to determine the DC_{50} values of the library candidates (Table 1) for the displacement of horse cytochrome-C on HP Sepharose SP and Source 15S cation-exchange resins at pH 6.0. As seen in the table, while commercially available displacer candidates such as spermine and neomycin show moderate to high affinities as displacers with DC_{50} values of 4.8 and 2.8 mM, respectively, compounds **17b** (neamine tetraspermine, Figure 2) and **23c** (kanamycin A tetraspermine, Figure 3) showed submillimolar DC_{50} values. Furthermore, 11 displacers from the library exhibited higher affinities than neomycin, while the others had comparable affinities. In previous work, spermine and neomycin were among the highest-affinity displacers identified by our laboratory.[12,13,26] These results are important in that they demonstrate that individual displacers with moderate affinities can be conjugated to generate a new class of high-affinity displacers. This represents a novel approach for the design of high-affinity displacers for biomolecule purification by displacement chromatography. The DC_{50} values of neomycin and its derivatives **14a** (neomycin derivatized with ethylenediamine, Figure 2) and **14b** (neomycin derivatized with diethylenetriamine, Figure 2)

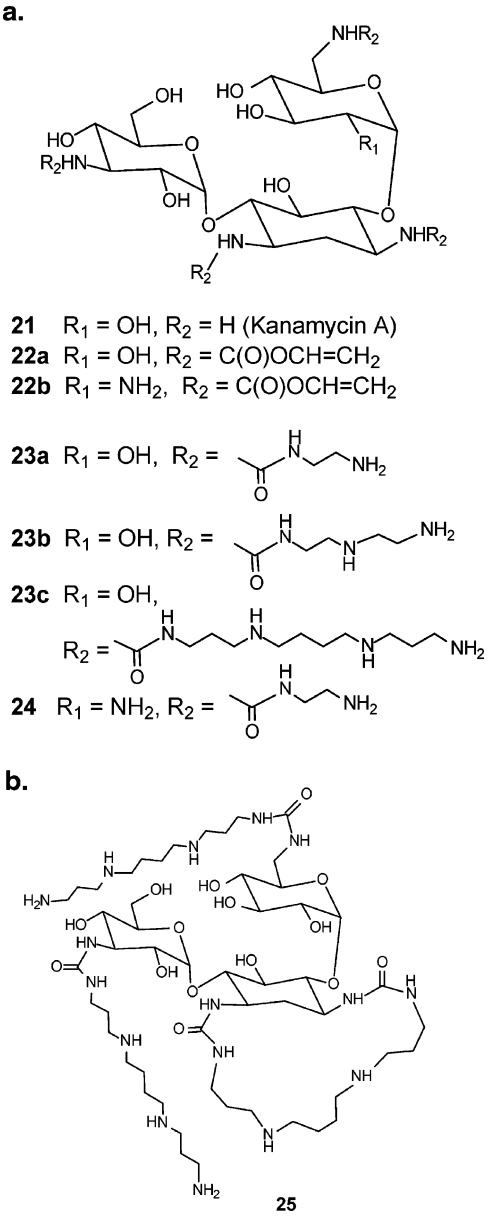


Figure 3. (a) Kanamycin−polyamine library. (b) Proposed structure of one of the major isomers of kanamycin−trispermine derivatives.

were 2.8, 1.6, and 1.1 mM, respectively, for the displacement on HP Sepharose SP. This indicates that displacer affinity increases as larger polyamine homologues are conjugated to neomycin. We hypothesize that the derivatization of neomycin with ethylenediamine (**14b)** serves to increase the flexibility of the primary amines on the resulting molecule as compared to the parent neomycin. The greater flexibility of the cationic charge results in an increased affinity of the molecule, a result consistent with previous observations.[26,27] Neomycin derivatized with diethylenetriamine (**14a)** was found to have the highest affinity among the neomycin derivatives, the increased affinity due to an increase in charge on the molecules as well as added flexibility of the charged groups. It turns out that the displacer affinity for the molecules, as well as for their derivatives, followed the trend neomycin > neamine > neobiosamine. Glucosamine and mannosamine derivatives **4a**−**e** (Scheme 1) show interesting trends in their DC_{50} values on HP Sepharose SP (Table 1). The DC_{50} values of **4a**, **4d**, and **4e** are similar

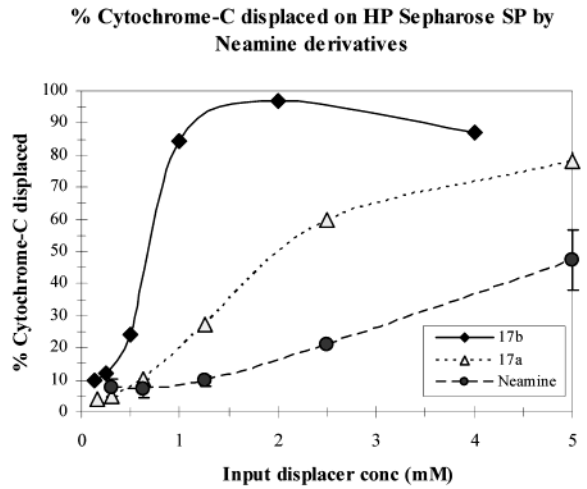


Figure 4. Determination of DC_{50} values from percentage protein displaced data for cytochrome-C displaced on HP Sepharose SP by neamine and its derivatives. The lines in the graph are for visualization only. DC_{50} values: neamine, 5.6 mM; **17a**, 2.1 mM; **17b**, 0.7 mM.

Table 1. Performance of the Novel Aminoglycoside Library: DC_{50} (mM) Values of Horse Cytochrome C Displaced on HP Sepharose SP and Source 15 S (Data Arranged in Increasing Order of DC_{50} on HP Sepharose SP)

displacer	DC_{50}-cytochrome C on HP Sepharose SP (mM)	DC_{50}-cytochrome C on source 15S (mM)
17b	0.7 ± na	0.6 ± 0.03
23c	1.0 ± na	0.9 ± 0.08
25	1.0 ± 0.02	2.4 ± 0.13
14b	1.1 ± 0.06	0.9 ± 0.12
20b	1.1 ± na	0.8 ± 0.04
4c	1.3 ± 0.08	0.8 ± 0.02
14a	1.6 ± 0.01	1.3 ± 0.09
24	1.9 ± 0.01	1.9 ± 0.06
23a	2.0 ± 0.15	1.7 ± 0.02
17a	2.1 ± 0.06	2.3 ± 0.04
11	2.3 ± na	1.1 ± 0.14
4e	2.7 ± 0.68	1.1 ± 0.00
4a	2.8 ± na	0.9 ± 0.07
4d	2.8 ± 0.20	0.8 ± 0.00
neomycin	2.8 ± 0.87	1.7 ± 0.02
4b	3.6 ± 0.25	0.8 ± 0.04
23b	4.1 ± 0.29	2.4 ± 0.13
spermine	4.8 ± 1.04	4.3 ± 0.64
neamine	5.6 ± 1.99	4.9 ± 1.67
bekanamycin	6.2 ± 0.37	3.1 ± 2.29
spermidine	7.7 ± 1.44	5.8 ± 0.44
20a	9.4 ± 2.65	4.8 ± 0.03
diethylenetriamine	10.8 ± 0.19	14.4 ± 3.67
7	11.7 ± 0.10	6.2 ± 0.46
9	12.3 ± 0.25	12.9 ± 1.46
ethylenediamine	20.2 ± 2.67	17.6 ± 2.15

(∼2.7−2.8 mM). However, **4c** and **4b** show very different affinities. While **4c** (DC_{50} = 1.3 mM) has the highest affinity among this family of molecules, **4b** (DC_{50} = 3.6 mM) has the lowest affinity. These results indicate that the introduction of the benzyl glycoside moiety in molecule **4c** increases displacer efficacy, while the introduction of a methyl group (**4b**) decreases the affinity. This may be due to greater van der Waals interactions of the benzyl moiety of **4c** with the stationary phase resin, a phenomenon known to enhance displacer affinity in ion-exchange systems.[12,13,26] In addition, the increased flexibility of the benzyl group as compared to the phenyl group could contribute to the increased affinity of **4c** over **4d**. These results, along with the general trends observed in the preceding paragraphs, indicate that the affinity of the resulting multivalent displacers can be significantly improved by the conjugation of appropriate moieties.

The influence of the stationary phase on displacer affinity was investigated using a second strong cation-exchange resin, Source 15S, and the resulting displacement data are also shown in Table 1. As seen in the table, the performance of the displacers on Source 15S (backbone matrix: crosslinked hydrophilized poly(styrene-divinyl benzene)) is significantly different from that observed on HP Sepharose SP (backbone matrix: crosslinked agarose), with as many as eight displacers exhibiting submillimolar affinities on the Source resin. Although neamine-tetraspermine (**17b**, Figure 2) and kanamycin A-tetraspermine (**23c**, Figure 3) were still among the highest-affinity displacers, with DC_{50} values of 0.6 and 0.9 mM, respectively, other displacers (including **4b**, **4c**, **4d**, **4a**, **14b**, and **20b**) had comparable submillimolar affinities. Furthermore, the affinities of the glucosamine/mannosamine derivatives (**4a**−**e**, Scheme 1) on the Source 15S resin showed significant differences when compared to their affinities on HP Sepharose SP. The DC_{50} values of these molecules ranged between 0.8 and 1.1 mM for the Source resin. This indicated that these were among the highest-affinity molecules on that resin. The glucosamine/mannosamine derivatives (**4a**−**e**) follow the affinity trend **4b** ∼ **4c** ∼ **4d** > **4a** > **4e** (DC_{50} values: 0.8, 0.8, 0.8, 0.9, and 1.1 mM, respectively). It is also interesting to note that **4b** (DC_{50}, 3.6 mM), the least-effective displacer among this family of molecules on the HP Sepharose SP resin, was the most effective on the Source resin (DC_{50}, 0.8 mM). The trend described above indicates that displacer efficacy on the Source resin increased with the introduction of hydrophobic/aromatic moieties in the molecule. This is probably due to the fact that the backbone of the Source resin is based on poly(styrene-divinyl benzene) and that displacers with hydrophobic/aromatic moieties may be able to exploit secondary interactions with the resin backbone.[12,13] These results indicate that although this library was designed for generic high-affinity displacers, unique selectivities can be observed on different resins.

To evaluate this displacer library with another protein, experiments were carried out with chicken-egg lysozyme adsorbed on HP Sepharose SP. Figure 5 shows a comparison of the percent protein displaced for both lysozyme and cytochrome-C at displacer concentrations of 2.5 mM; the data are arranged in increasing order of percent lysozyme displaced. As seen in the figure, a large number of library compounds had significantly higher affinities than those of the previously identified commercial compounds neomycin and spermine. In addition, molecules such as kanamycin A tetraspermine (**23b**, Figure 3) and neamine tetraspermine (**17b**, Figure 2) were found to be particularly effective at displacing lysozyme with values of 67.8 and 52.8% protein displaced, respectively. It may be noted further that while these molecules demonstrated differences in affinities for displacing lysozyme, they resulted in almost 100% displacement of cytochrome-C from the resin. This is indicative of the fact that the relative efficacies of these high-affinity displacers were strongly influenced by both the displacer chemistry and the protein being displaced.

As seen in Figure 5, the displacer candidates exhibited a wide range of efficacies for displacing lysozyme on HP Sepharose SP. Consequently, interesting structure-efficacy information may

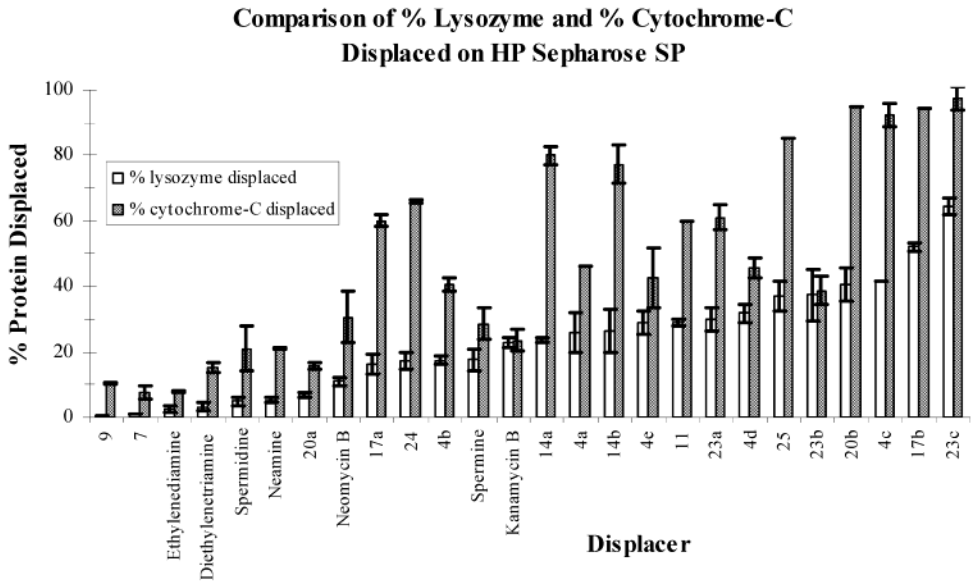


Figure 5. Comparison of percent lysozyme and percent cytochrome-C displaced on HP Sepharose SP at input displacer concentration = 2.5 mM. Data increased in increasing order of percent cytochrome-C displaced.

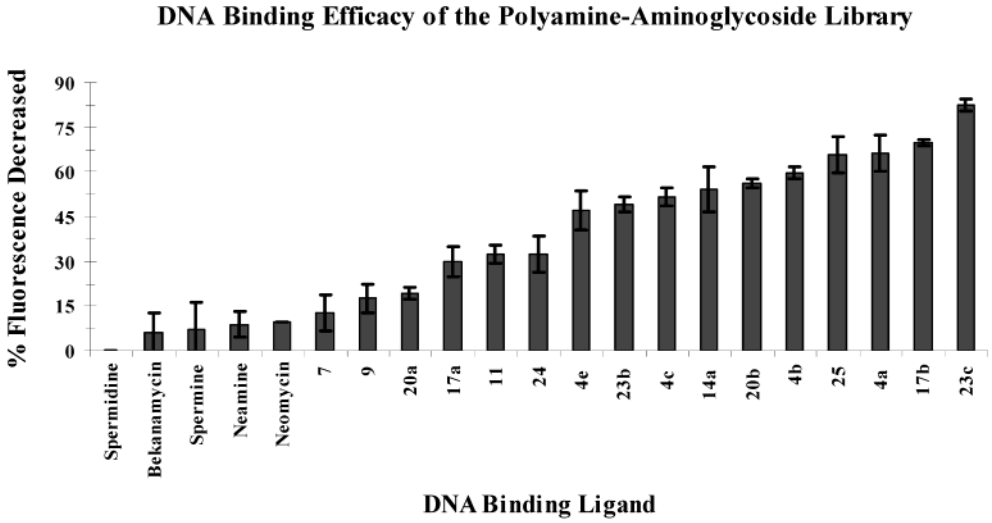


Figure 6. DNA-binding performance of the aminoglycoside−polyamine library: percent fluorescence decreased using ethidium bromide displacement assay. Ligand concentration: 0.3 mM.

be observed in this case. For example, although **20b** (Figure 2) has the same number of amines as **4a** (Scheme 1), the latter showed much lower affinity for displacing lysozyme (**4a**: 23%, **20b**: ~41%). This suggests that the presence of an additional 1-methoxyribose unit may be responsible for the enhanced affinity of **20b**. Furthermore, an increase in the aromatic character of the substituents on glucosamine−dispermine (Scheme 1) derivatives resulted in increased displacement affinities for lysozyme. As seen in Figure 5, while **4c** and **4d** have the same cationic content as **4a**, **4b**, and **4e** due to the presence of the two spermine chains on the molecules (Scheme 1), the presence of the benzyl group on **4c** and the phenyl group of **4d** resulted in high affinities of these displacers. These results are in agreement with previous reports from our group that the presence of terminal aromatic groups serves to enhance the efficacies of displacer candidates in ion exchange systems.[12,13,26]

As seen from these screening results, the rational design of a displacer library has resulted in the identification of several novel high-affinity displacers for cation exchange systems. These molecules have the potential to significantly improve the efficiency of ion-exchange processes ranging from large-scale preparative chromatography to microscale proteomic applications.

Evaluation of the Aminoglycoside−Polyamine Library. Identification of High-Affinity DNA-Binding Ligands. We hypothesized that the constituents of the aminoglycoside−polyamine library could also be employed as high-affinity DNA-binding ligands due to the high cationic nature of this library. Accordingly, the ethidium bromide displacement assay[35,36] was employed to evaluate the DNA-binding affinity of this library in a 96-well format. Using this screen, the percent fluorescence decreased[35,36] was used as a parameter to rank the DNA-binding efficacy of the various compounds (Figure 6).

As seen in the figure, commercially available polyamines such as spermidine, spermine, bekanamycin, and neomycin showed low efficacies (the percent fluorescence decreased values ranging from 0 to 15%). On the other hand, a large number of the library constituents acted as effective DNA binding agents. Particularly

significant are the results with **23c** (Figure 3), **17b** (Figure 2), **4a** (Scheme 1), **25** (Figure 3), and **4b** (Scheme 1), which resulted in values of 82, 70, 66, 66, and 60% fluorescence decreased, respectively. While the trend in Figure 6 clearly reveals that an increase in cationic charge leads to the generation of higher DNA-binding affinity ligands, some interesting trends can also be observed. For example, an increase in the hydrophobic character of the substituents on glucosamine−dispermine (Scheme 1) derivatives resulted in an overall decrease in DNA-binding efficacy of the molecules; the results followed the trend **4a** (66%) > **4b** (60%) > **4c** (52%) > **4e** (47%). In particular, differences in the position of spermine (2-α versus 2-*β*) led to significant differences in efficacy, as seen in case of **4a** (glucosamine-dispermine, 66% fluorescence decreased) and **4e** (mannosamine-dispermine, 47% fluorescence decreased). Clearly, these data demonstrates that several candidates from the aminoglycoside−polyamine library exhibited high DNA-binding efficacies. Furthermore, differences in the chemistry and location of the substituted moieties resulted in different DNA-binding efficacies.

Conclusions

In this paper, chemoenzymatic syntheses have been employed with rapid, parallel screening techniques for the identification of both high-affinity displacers and DNA-binding ligands. Regioselective lipase-catalyzed acylation, followed by chemical aminolysis, was used to generate vinyl carbonate and vinyl carbamate linkers, respectively, of the aminoglycosidic lead molecules. The resulting derivatives were further derivatized, using polyamines to generate the library.

Screening results indicate that many components of the library indeed show high affinities as cation-exchange displacers and DNA-binding ligands. Particularly, neamine and kanamycin conjugated with four spermine molecules show enhanced displacer and DNA-binding activities, due primarily to an overall increased cationic content of the molecules. It was found that increased charge, size, and charge spacing contributed to overall efficacy of the molecules. These results justify the rational, multivalent design of high-affinity displacers and DNA-binding ligands from low- to moderate-affinity leads. Future work will involve the use of the displacer efficacy DC_{50} data and the DNA-binding data for the generation of predictive structure−property models, which will then be employed to direct the a priori design of a second generation of high-affinity displacers and DNA-binding ligands. The identification of high-affinity displacers will enable relatively low displacer concentrations to be employed in protein separation processes, resulting in dramatic improvements in yield and purity. This will have implications for protein separation processes ranging from large-scale manufacturing to microscale proteomic applications. The identification of high-affinity DNA-binding ligands will set the stage for further evaluation of these compounds for their efficacies as transfection agents.

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Supporting Information Available: Yields and spectroscopic data of the aminoglycoside−polyamine library. This material is available free of charge via the Internet at http://pubs.acs.org.

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