Designing the Polyamine Pharmacophore: Influence of *N*-Substituents on the Transport Behavior of Polyamine Conjugates

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N-Ethylated *N*-arylmethyl polyamine conjugates were synthesized and evaluated for their ability to target the polyamine transporter (PAT). To understand the effect of *N*-ethylation upon PAT selectivity, ethyl groups were appended onto a PAT-selective N^1-anthracenenylmethyl homospermidine derivative, **1b**. Bioevaluation in L1210 murine leukemia cells and in two Chinese hamster ovary cell lines (PAT-active CHO and PAT-deficient CHO-MG) revealed a dramatic decrease in PAT targeting ability upon N^1 or N^5 ethylation of the pharmacophore **1b**. Experiments using the amine oxidase inhibitor, aminoguanidine (AG, 2 mM), revealed that the N^9-ethyl and N^9-methyl analogues were able to retain their PAT selectivity and cytotoxicity properties in the presence or absence of AG. In contrast, the lead compound **1b** (containing a terminal NH_2 group) revealed a dramatic reduction in both its PAT-targeting ability and cytotoxicity in the absence of AG. An improved balance between these three properties of PAT-targeting, cytotoxicity and metabolic stability can be attained via *N*-methylation at the N^9-position.

Introduction

Polyamines are essential cellular growth factors.[1] Tumor cells have been shown to contain elevated polyamine levels and have active polyamine transport systems to import exogenous polyamines.[1] Due to the enhanced cellular need for these amine growth factors and an activated transport system for their import, one can selectively deliver polyamine-drug conjugates to particular cell types.[1–8] The structural tolerances accommodated by the polyamine transporter (PAT[a]) allow for the import of non-native polyamine conjugates.[2–6]

Prior work in our laboratories and others have revealed the structural requirements associated with the delivery of polyamine conjugates via the PAT in murine leukemia (L1210) and Chinese hamster ovary (CHO) cells.[1–11] In short, the methylene "spacer" units (Figure 1, **1**: x, y, and z), the size of the N^1-substituent, and the degree of N^1-substitution all influence PAT-mediated delivery.[4,6–8] Earlier studies in our laboratory revealed that the homospermidine conjugate **1b** (e.g., a 4,4-triamine) had 150-fold higher cytotoxicity in CHO cells than in a mutant PAT-deficient CHO cell line (CHO-MG).[4,5,12,13]

Recently, we demonstrated that the presence of a secondary nitrogen at the N^1 position (e.g., **1b**) is a critical structural element for utilizing PAT for cellular entry.[7,14] The presence of a tertiary amine at the N^1-position blocked the ability of both the dihydromotuporamines **3** and **4** and the N^1-ethyl-N^1-(anthracenylmethyl)-polyamine **5** to utilize the PAT for cellular entry. Molecular modeling studies revealed that molecular shape may explain this sensitivity. While both **1b** and N^1 tertiary amines (**3**–**5**) prefer a hoe shape, **1b** has ready access to a shovel shape.[15] Furthermore, these shape preferences were observed in solution by ^{1}H NMR studies.[15] Because N^1-ethylation had such a dramatic impact on PAT targeting, we were interested if other positions along the polyamine chain had the same sensitivity to further *N*-alkylation.

N-Alkylation has many effects on the biological properties of polyamines. For example, *N*-alkylated polyamines have been shown to have enhanced metabolic stability due to their ability to avoid *N*-acetylation by spermidine (SPD)-spermine acetyl transferase, SSAT, and subsequent degradation by polyamine oxidase (PAO).[10a,16] *N*-Alkylated polyamines also have enhanced stability in the culture medium, wherein the additional alkyl group prevents their oxidation by serum amine oxidases.[16e] In all of our previous experiments, aminoguanidine (AG, 2 mM) was routinely added in order to inhibit the serum amine oxidase activity.[16e,f] This was important because the polyamine drug conjugates, which contain a primary amine, for example, **1b**, could act as substrates for these enzymes. Indeed, secondary amine motifs at the terminal position have been shown to impart enhanced metabolic stability and improve bioactivity.[10a]

Compounds **6** and **7** were synthesized to better understand how additional *N*-alkylation of the **1b** motif altered the ability of these derivatives to enter cells via the polyamine transport system. Specifically, these new derivatives of **1b** provided an opportunity to probe how *N*-ethylation at the N^5 and N^9 positions perturbed PAT-mediated delivery. In previous work,[3,10a] both dimethyl and diethyl SPD (**12a** and **12b**) were shown to be competitive antagonists for the PAT via competition experiments with SPD. In particular, SPD rescue experiments revealed that the N^1,N^8-dimethyl SPD (**12a**) was more sensitive to SPD rescue than the related N^1,N^8-diethyl compound (**12b**).[2,3] Because *N*-methyl derivatives may have higher affinity for PAT,[10a] compound **8** was also synthesized for comparison to **7**.

Tetraamines were previously shown to have high affinity for the PAT (e.g., the L1210 K_i value of **9**: 51 nM) but lower PAT selectivity than **1b**.[5] Tetraamine **10** was synthesized to explore

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[a] Abbreviations: PAT, polyamine transporter; CHO, Chinese hamster ovary; CHO-MG, Chinese hamster ovary cells polyamine transport deficient mutant; L1210, mouse leukemia cells; HBBS, Hanks balanced salt solution; PBS, Phosphate buffered saline.

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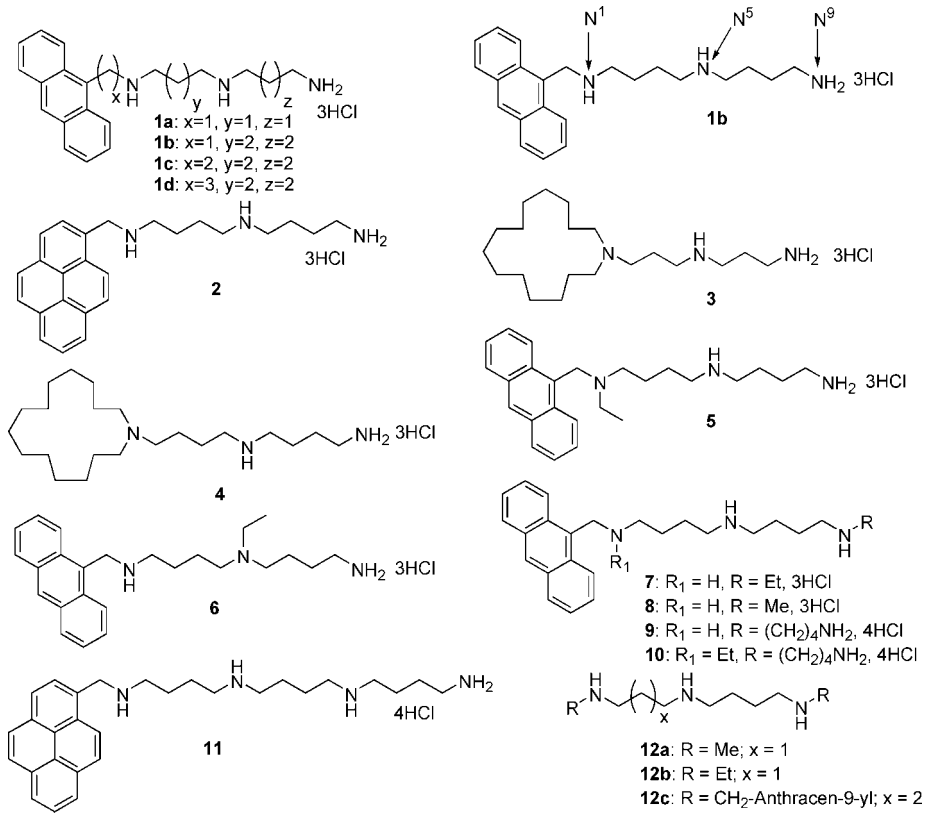


Figure 1. Polyamine Architectures **1**–**12**.

how N^1-ethylation of a tetraamine influenced PAT selectivity. Previous results showed that N^1-ethyl,N^1-anthracen-9-ylmethyltriamine **5** had a complete loss of PAT selectivity when compared to **1b**. However, while tetraamine **10** contains this same N^1 tertiary center, it also retains the homospermidine motif (e.g., the two secondary and one primary nitrogen motifs) needed for PAT recognition. Indeed, large N^1 "cargoes" (e.g., **2**) have been shown to be delivered to cells via the PAT. Tetraamine **11** was synthesized to test whether a large pyrenyl group could also be delivered via a tetraamine motif and for comparisons with **10**.

With the above compounds in mind, we had certain expectations. For example, the bis-alkylated polyamine analogues have been found to mimic the functions of the natural polyamines and are more stable to intracellular polyamine metabolism and degradation pathways (i.e., SSAT and PAO).[10a,16] Related studies with N^1-(methylaryl)polyamines (e.g., **1b** and **2**) revealed that large N^1-substituents are not cleaved (e.g., no homospermidine is observed).[6] Therefore, in terms of design, one should be able to use either *N*-alkyl or larger alkylaryl substituents to avoid degradation. As shown in this study, the *N*-methyl substituents were superior, as they retained good PAT selectivity and avoided degradation. Using these insights compounds **6**, **7**, **8**, **10**, and **11** were designed to probe the effect of additional *N*-alkylation and terminal aminobutylation on compound **1b** (the most PAT-selective ligand). These studies are important as they further define the structural tolerances of PAT and provide insight into the design constraints for the polyamine vector and placement of the cytotoxic "cargo".

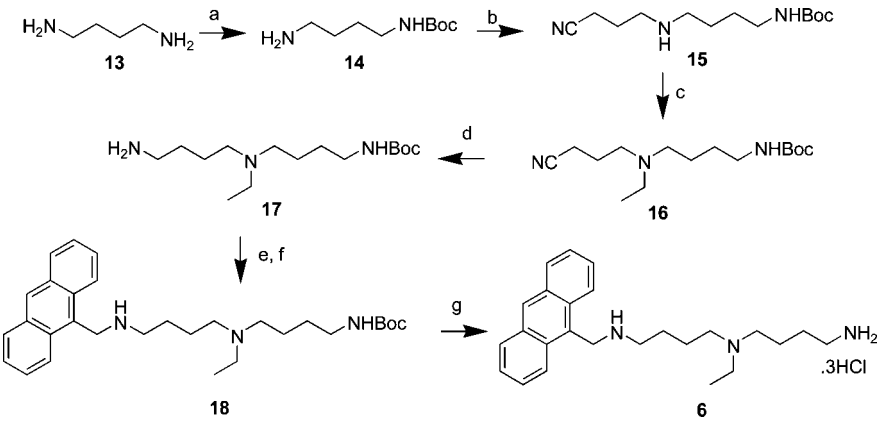
Results and Discussion

Synthesis. New conjugates **6**−**8**, **10**, and **11** were synthesized to probe the role of *N*-ethylation at the N^5 and N^9 positions and whether tetraamines were as sensitive as the triamines to N^1 substitution and to the size of the N^1-substituent.

The N^5-ethyl derivative **6** was synthesized from commercially available 1,4-diaminobutane **13** (shown in Scheme 1), which was selectively converted to mono Boc-protected putrescine **14** in 90% yield.[8] Amine **14** was then alkylated using 4-bromobutyronitrile to give secondary amine **15** in 65% yield.[8] *N*-Alkylation of **15** using EtBr and K_2CO_3 generated nitrile **16** in 85% yield. Reduction of **16** by hydrogenation over Raney Ni gave the primary amine **17**, which upon reductive amination with 9-anthraldehyde resulted in the secondary amine **18** in 72% yield. Lastly, the Boc group was removed using 4 N HCl in ethanol to provide the N^5-ethyl derivative **6** in 95% yield.

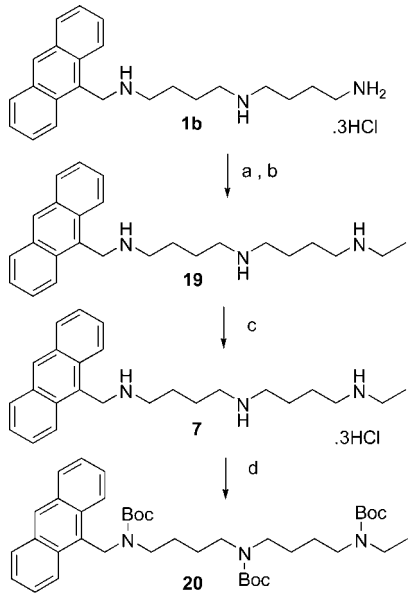
Previously synthesized **1b**[4] was used in the synthesis of the N^9-ethyl derivative **7**. The selective *N*-alkylation of the primary amine in **1b** was accomplished with the cesium method reported by Jung.[17] When $CsOH \cdot H_2O$ (1 equiv) in DMF, 4 Å molecular sieves, and ethyl bromide were used, the secondary amine **19** was synthesized in 21% yield (Scheme 2). Some of the *N*,*N*-diethylated compound was also separated by column chromatography. Further treatment of **19** with 4 N HCl resulted in compound **7**. To confirm the structure of compounds **7** and **19**, di-*tert*-butyldicarbonate was used in excess to Boc-protect all the available amines of **7** to synthesize the tricarbamate **20**. The ^{1}H NMR spectrum of **20** showed the presence of three Boc groups and the absence of a doublet of triplets at 3.03 ppm, which indicated that a RCH_2CH_2NHBOC group was not present. This observation confirmed that there is no NH carbamate available in compound **20**. Both observations confirmed the regiochemistry of the *N*-ethyl group in compounds **19** and **7**. Indeed, a comparison of the ^{1}H NMR spectra of **5**, **6**, and **7** (see Supporting Information) showed distinct differences and ruled out conclusively any misassignment of the N-Et regiochemistry.

Scheme 1[a]



[a] Reagents and conditions: (a) di-*t*-butyl dicarbonate/10% TEA/MeOH, 90%; (b) K_2CO_3/CH_3CN/4-bromobutyronitrile, 65%; (c) $K_2CO_3/CH_3CN/C_2H_5Br$, 85%; (d) Raney Ni/NH_4OH/EtOH/NH_3, 94%; (e) 9-anthraldehyde/25% MeOH/CH_2Cl_2; (f) 50% MeOH/CH_2Cl_2/$NaBH_4$, 72%; (g) EtOH/4 N HCl, 95%.

Scheme 2[a]



[a] Reagents and conditions: (a) Na_2CO_3/CH_2Cl_2; (b) CsOH·H_2O/molecular sieves/DMF/C_2H_5Br, 21%; (c) 4 N HCl/EtOH, 93%; (d) di-*t*-butyl dicarbonate/10% TEA/MeOH, 94%.

As shown in Scheme 3, the synthesis of compound **8** utilized the previously synthesized alcohol **21**.[7] The HCl salt of amine **22** was obtained using 4 N HCl in ethanol. Regioselective reductive amination of amine **22** with 9-anthraldehyde in the presence of TEA resulted in the desired secondary amine **23** in 95% yield. Reaction of **23** with di-*tert*-butyl dicarbonate provided the alcohol **24** in 96% yield.[5] Mesylation of alcohol **24** resulted in mesylate **25**,[5] which was reacted with excess methylamine to obtain amine **26** in 73% yield. Lastly, removal of the Boc groups with 4 N HCl provided the N^9-methyl derivative **8** in 95% yield.

For the synthesis of tetraamines **10** and **11**, triBoc-amine **31**[18] was synthesized as depicted in Scheme 4. Secondary amine **15** (Scheme 4) was Boc-protected using di-*tert*-butyl dicarbonate to provide nitrile **27** in 95% yield, which was then reduced to amine **28** in 90% yield by hydrogenation over Raney Ni.[8] Amine **28** was then alkylated using 4-bromo-butyronitrile to give secondary amine **29** in 62% yield, which was further Boc-protected to give nitrile **30** in 94% yield. Reduction of nitrile **30** by hydrogenation over Raney Ni gave the triBoc-amine **31** in 90% yield.

As shown in Scheme 5, reductive amination of amine **31** with 9-anthraldehyde resulted in the secondary amine **32** in 72% yield, which upon *N*-alkylation with EtBr and K_2CO_3 in acetonitrile resulted in tertiary amine **33** in 81% yield. Lastly, the Boc groups were removed using 4 N HCl in ethanol to provide the N^1-ethyl-N^1-anthracenylmethyl tetraamine **10** in 96% yield.

Finally, as shown in Scheme 6, reductive amination of amine **31** with 1-pyrenyl aldehyde resulted in the secondary amine **34** in 70% yield. Removal of the Boc groups with 4 N HCl gave the N^1-pyrenyl tetraamine **11** in 90% yield.

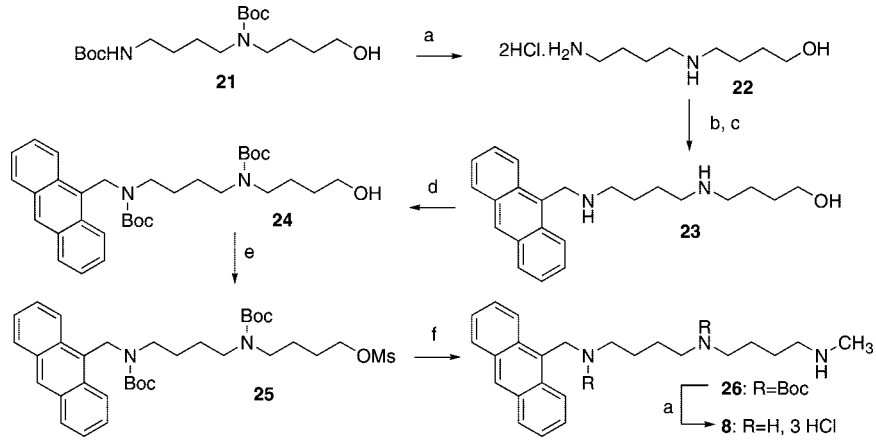
Biological Evaluation. Once synthesized, the conjugates were screened for cytotoxicity in L1210, CHO, and CHO-MG cells. L1210 (mouse leukemia) cells were selected to enable comparisons with the published IC_{50} and K_i values for a variety of polyamine substrates.[2–6] CHO cells were chosen along with a mutant PAT deficient cell line (CHO-MG) to comment on selective transport via the PAT.[4–6,9] The results are shown in Table 1.

L1210 IC_{50} and K_i Studies. The IC_{50} values listed in Table 1 represent the concentration of the polyamine conjugate required to reduce the relative cell growth by 50%. The K_i values in Table 1 were determined for [^{14}C]SPD uptake and reflect the affinity of the polyamine derivative for the PAT on the cell surface. With both parameters, one can determine whether high affinity for the transporter (e.g., low K_i value) translated into high cytotoxicity (e.g., low IC_{50} value) and so on.

Previous results[8] showed that although the K_i values provide relative affinity measures of polyamine derivatives toward the PAT system, they were of minimal value in predicting their cytotoxicity. This is likely due to the fact that polyamine transport is a multistep process, which involves cell surface interactions followed by uptake across the cell membrane.[1,5] In addition, some polyamine derivatives have been shown to have a high affinity for the PAT but are not transported into the cells.[11]

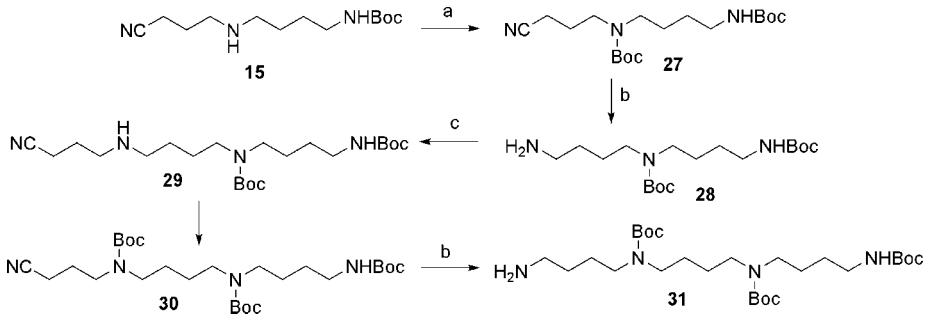
Nevertheless, sometimes correlations between K_i values and cytotoxicity become apparent. For example, upon N^1-alkylation of **1b** to provide the N^1-Et derivative **5**, the K_i value increased (**1b**: 1.8 μM; **5**: 24.4 μM). Indeed, this simple N^1-alkylation resulted in a nearly 14-fold increase in K_i value (1.8 vs 24.4 μM) and a 74-fold increase in L1210 IC_{50} value (0.3 vs 22.2 μM, i.e., a dramatic shift to lower cytotoxicity). Molecular modeling studies suggested that this was related to changes in molecular shape induced by the tertiary amine (N^1-ethylation in compound **1b**).[15]

Scheme 3[a]



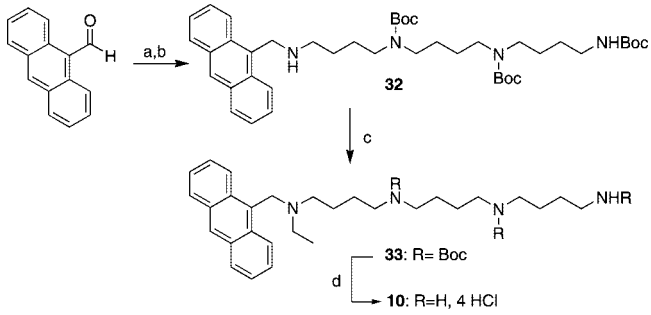
[a] Reagents and conditions: (a) EtOH/4 N HCl, 97%; (b) 9-anthraldehyde/TEA/25% MeOH/CH_2Cl_2; (c) 50% MeOH/CH_2Cl_2/$NaBH_4$ 95%; (d) di-*t*-butyl dicarbonate/10% TEA/MeOH, 96%; (e) methane sulfonyl chloride/TEA, 94%; (f) methylamine/CH_3CN, 73%.

Scheme 4[a]



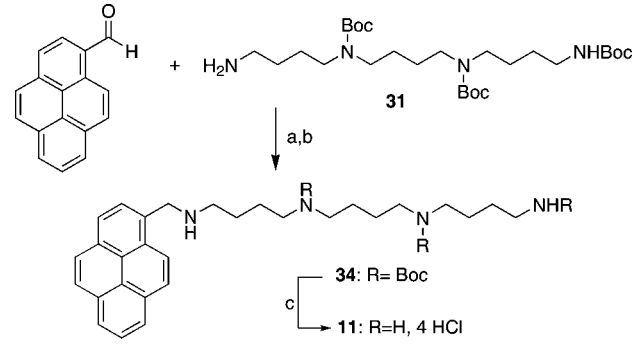
[a] Reagents and conditions: (a) di-*t*-butyl dicarbonate/10% TEA/MeOH, 95%; (b) Raney Ni/NH_4OH/EtOH/NEt_3, 90%; (c) K_2CO_3/CH_3CN/4-bromobutyronitrile, 62%; (d) Raney Ni/NH_4OH/EtOH/NEt_3, 90%.

Scheme 5[a]



[a] Reagents and conditions: (a) **31**/25% MeOH/CH_2Cl_2; (b) 50% MeOH/CH_2Cl_2/$NaBH_4$ 72%; (c) K_2CO_3/CH_3CN/C_2H_5Br, 81%; (d) EtOH/4 N HCl, 96%.

Scheme 6[a]



[a] Reagents conditions: (a) 25% MeOH/CH_2Cl_2; (b) 50% MeOH/CH_2Cl_2/$NaBH_4$ 70%; (c) EtOH/4 N HCl, 90%.

In the present investigation, when the position of the N^1-ethyl group was changed to N^5- and N^9-ethyl (i.e., compounds **6** and **7**), the K_i values approached that of compound **1b**. As the position of the tertiary nitrogen center moves away from the anthracene unit, the K_i value decreases (**5**: 24.4; **6:** 5.5; **7**: 3.5 μM), implying an increased affinity for PAT in this series. To explore how N^9-substituents affected PAT-binding affinity, the K_i values of compounds **7** (N^9-Et) and **8** (N^9-Me) were also determined. As listed in Table 1, the K_i values for the N^9-derivatives (**7**: 3.5 μM and **8**: 8.2 μM) were similar and yet significantly lower than the N^1-derivative **5** (24.4 μM). These data suggest that in terms of additional *N*-alkylation of **1b** polyamine conjugates containing N^5- and N^9-alkyl substituents have higher PAT affinity than those with additional N^1-substituents (e.g., **5**).

In the case of tetraamines **9**[5] and **10**, the K_i values were significantly lower, which was consistent with previous results with other tetraamines,[5] and reflected their higher binding affinity. N^1-Ethylation of tetraamine **9** resulted in nearly a 10-fold increase in K_i value for compound **10** (**9**: 0.05 vs **10**: 0.46 μM), which is consistent with the above trend observed with triamines **1b** and **5** (**1b**: 1.8 vs **5**: 24.4 μM). This observation indicated that the presence of the tertiary amine at the N^1 position also lowers the PAT binding affinity of tetraamines. The presence of the larger pyrenyl cargo in compound **11** also increased the K_i value to 0.14 μM (vs **9**: 0.051 μM). This suggested that the size of the N^1-substituent can also alter the affinity of the polyamine conjugate for the PAT, a finding that is consistent with previous results with N^1-substituted polyamines.[8]

Table 1. Biological Evaluation of Polyamine Derivatives in L1210, CHO, and CHO-MG Cells in the Presence of AG[a]

cmpd (tether)	L1210 IC_{50} in μM	L1210 K_i values (μM)	ref	CHO-MG IC_{50} in μM	CHO IC_{50} in μM	IC_{50} ratio[b]
1b: Antmethyl (4,4)	0.30 (±0.04)	1.8 (±0.1)	*4*	66.7 (±4.1)	0.45 (±0.10)	148
5: N^1-Ethyl-N^1-antmethyl (4,4)	22.2 (±1.2)	24.4 (±1.5)	*7*	21.9 (±0.9)	22.2 (±0.7)	1
6: N^5-Ethyl-N^1-antmethyl (4,4)	0.88 (±0.02)	5.5 (±1.5)		7.0 (±0.2)	3.9 (±0.5)	1.8
7: N^9-Ethyl-N^1-antmethyl (4,4)	1.01 (±0.04)	3.5 (±0.2)		57.9 (±2.3)	9.8 (±0.3)	5.9
8: N^9-Methyl-N^1-antmethyl (4,4)	0.40 (±0.05)	8.2 (±0.6)		60.0 (±2.6)	4.9 (±0.2)	12.2
9: Antmethyl (4,4,4)	7.5 (±0.3)	0.05 (±0.06)	*5*	33.2 (±1.7)	10.6 (±3.1)	3.1
10: N^1-Ethyl-N^1-antmethyl (4,4,4)	3.4 (±0.3)	0.46 (±0.10)		55.7 (±5.1)	17.1 (±1.9)	3.3
11: Pyr-methyl (4,4,4)	1.2 (±0.1)	0.14 (±0.02)		25.3 (±2.9)	2.4 (±0.1)	10.5
12c: Bis-N^1,N^9-antmethyl (4,4)	1.5 (±0.1)	4.3 (±0.2)	*8*	1.2 (±0.1)	1.1 (±0.1)	1.1

[a] Definitions used in Table 1, column 1: Ant = Anthracen-9-yl, column 4: ref denotes the reference number in which the data was originally reported. A blank in the ref column denotes new data. Cells were incubated for 48 h at 37 °C with the respective conjugate. [b] The ratio denotes the (CHO-MG/CHO) IC_{50} ratio, a measure of PAT selectivity.

In the L1210 experiments, the higher K_i value of **5** correlated with its lower cytotoxicity (IC_{50} of **5**: 22.2 μM). Indeed, the IC_{50} value increased significantly from the parent system, **1b** (0.3 μM). This revealed that at least for these compounds both the PAT binding affinity and the conjugate's cytotoxicity were sensitive to the degree of alkylation at the N^1 position. As the position of the tertiary amine was moved from N^1 (**5**) to N^5 (**6**) and N^9 (**7**), the cytotoxicity increased (IC_{50} value: **5**: 22.2 μM; **6**: 0.88 μM; **7**: 1.01 μM). In terms of N^9-substituent effects, the small N^9-alkyl groups of **7** and **8** did not significantly alter the cytotoxicity profile seen with the parent **1b** (e.g., IC_{50} **1b**: 0.30 μM; **7**: 1.01 μM; IC_{50} **8**: 0.40 μM). Indeed, a SPD rescue experiment, wherein the competitive antagonist SPD is added, showed significant rescue of cells from compound **8**. Specifically, a significant increase in the IC_{50} value was observed in the presence of added SPD (IC_{50} **8**: 0.4 μM; IC_{50} **8**+SPD: 1.38 μM). Similar SPD protection was previously observed with **12a** and **12b**.[2,3] This result suggests that **8** is able to access cells via the same PAT in L1210 cells, as utilized by the native SPD.

In general, the SPD protection assays were performed to determine whether uptake of the selected polyamine analogues is mediated, in part or in whole, by the polyamine transport apparatus. To answer this question, competition assays were performed in the absence and presence of SPD. To maximize the protective effects of SPD, an excess of SPD (200 μM) was used during these experiments. Use of excess SPD provided a high level of competition with the selected polyamine derivatives in terms of using the polyamine transport system for cellular entry. Note: a similar SPD protection effect was observed in CHO cells for compounds **1b**, **7**, and **8**.

In the case of the tetraamines **10** and **11**, the opposite trend was observed. A modest increase in cytotoxicity was observed upon *N*-ethylation (IC_{50} value decreased from 7.5 μM (**9**) to 3.4 μM (**10**)). This suggests that a motif with two secondary nitrogens and one primary nitrogen is sufficient for cellular entry. Interestingly, this premise could also explain the increase in cytotoxicity of the N^1-Et tetraamine **10** (3.4 μM) over its N^1-Et triamine **5** (IC_{50}: 22.2 μM) counterpart. As will be demonstrated in the following CHO studies, this is due to the enhanced PAT targeting abilities of **10**.

In summary, the L1210 K_i and IC_{50} values allowed comparisons to published polyamine systems and revealed that, at least in the series **5**−**7**, the higher affinity for PAT translated into a more cytotoxic compound.[8] The CHO and CHO-MG IC_{50} comparisons were even more informative.

CHO and CHO-MG Studies. CHO cells were chosen along with a mutant cell line (CHO-MG) to comment on how the synthetic conjugates gain access to cells.[4–6,9] The CHO-MG cell line is polyamine-transport deficient and was isolated after selection for growth resistance to methylglyoxalbis(guanylhydrazone), MGBG, (CH_3C[=N−NHC(=NH)NH_2]CH[=N−NHC(=NH)NH_2]) using a single-step selection after mutagenesis with ethylmethanesulfonate.[12,13]

For the purposes of this study, the CHO-MG cell line represents cells with no PAT activity and provided a model for alternative modes of entry or action, which are independent of PAT. These alternative modes of entry include passive diffusion or utilization of another transporter. The alternative modes of action may also include interactions on the outer surface of the plasma membrane or other membrane receptor interactions.

In contrast, the parent CHO cell line represents a cell type with high PAT activity.[12,13] Comparison of conjugate cytotoxicity in these two CHO cell lines provided an important screen to detect selective conjugate delivery via the PAT. For example, a conjugate with high utilization of the PAT would be very toxic to CHO cells, but less so to CHO-MG cells.[4–6,9] In short, highly selective PAT ligands should give high (CHO-MG/CHO) IC_{50} ratios.

As reported earlier,[8] dramatic differences in cytotoxicity were observed with **1b** (CHO-MG/CHO IC_{50} ratio: 148), a highly PAT-selective substrate. The CHO-MG/CHO IC_{50} ratios listed in Table 1 suggested that PAT targeting is influenced by the degree of substitution of nitrogen at the N^1 position of the polyamine vector. A direct correlation was observed between cytotoxicity and polyamine conjugate uptake.[8] Therefore, the relative toxicities observed in CHO and CHO-MG cells represent a measure of differential uptake via PAT and provide a measure of PAT selectivity.[8]

When this CHO screen was used, N^1-alkylation (**5**) gave a complete loss of PAT selectivity (IC_{50} ratio CHO-MG/CHO = 1). As expected, cytotoxicity was also effected, wherein compound **5** (CHO IC_{50} of **5**: 22.2 μM) was 49 times less cytotoxic then compound **1b** (0.45 μM). Thus, the presence of the tertiary amine at the N^1 position of the homospermidine vector resulted in a loss of ability to use PAT and reduced cytotoxicity. When the position of the tertiary nitrogen was moved away from the N^1 position (**5**) to the N^5 (**6**) and N^9 (**7**) positions, it also resulted in a decrease in cytotoxicity when compared to the parent compound **1b**.

The CHO-MG/CHO IC_{50} ratios revealed that PAT selectivity was very sensitive to alkylation at N^1 and N^5, with CHO-MG/CHO ratios of 1 and 1.8, respectively. Interestingly, N^9-ethylation of **1b** provided compound **7**, which was 5.9 times more toxic to CHO than CHO-MG cells. Although compound **1b** was more selective in using PAT, the tertiary N^9-amine, **7**, could also be accommodated by PAT, albeit to a lesser degree of selectivity. However, previous results showed that very large N^9-substituents (e.g., compound **12c**) resulted in the complete loss of PAT selectivity (Table 1). Pursuing this insight, compound **8** with a methyl group at the N^9 position was evaluated in the CHO cell lines. The smaller N^9-methyl substituent resulted in increased PAT selectivity (ratio IC_{50}

Table 2. Biological Evaluation of Polyamine Derivatives in CHO and CHO-MG Cells in the Absence of AG (IC_{50} Values in μM)[a]

cmpd (tether)	CHO-MG IC_{50} w/o AG	CHO IC_{50} w/o AG	IC_{50} ratio[b] w/o AG	IC_{50} ratio[b] with AG
1b: Antmethyl(4,4)	7.1 (±0.3)	1.7 (±0.1)	4.2	148
7: N^9-Ethyl-N^1-antmethyl (4,4)	51.2 (±1.8)	13.2 (±0.5)	3.9	5.9
8: N^9-Methyl-N^1-antmethyl (4,4)	56.2 (±2.0)	4.9 (±0.1)	11.5	12.3

[a] Definitions used in Table 1, column 1: ant = anthracen-9-yl; cells were incubated for 48 h with the respective conjugate. [b] The ratio denotes the (CHO-MG/CHO) IC_{50} ratio, a measure of PAT selectivity.

CHO-MG/CHO for **8**: 12.2 vs **7**: 5.9). The increased PAT selectivity of *N*-methyl analogue **8** over its *N*-ethyl counterpart **7** is likely due to steric effects, wherein the smaller substituent at the N^9 position is better accommodated by PAT. Indeed, the ability to target PAT increased as one reduced the size of the N^9-substituent within the series **12c**, **7**, **8**, and **1b**.

Conclusively, the presence of the tertiary center at N^1 and N^5 positions either obliterated or significantly diminished the ability of conjugates to utilize the PAT for cellular entry, whereas N^9-methylation retained significant PAT selectivity.

Interestingly, N^1-alkylation in the case of tetraamines **9** and **10** did not affect the PAT selectivity. Although the PAT selectivity was reduced relative to triamine **1b**, the CHO-MG/CHO IC_{50} ratios for tetraamines **9** and **10** were 3.1 and 3.3, respectively. This suggested that the presence of two secondary amines and one primary amine is a sufficient motif for cellular entry via PAT. Indeed, the truncated motif **5** had no PAT selectivity, whereas tetraamine **10** (with the intact motif) showed 3-fold selectivity. The presence of the larger cargo (pyrenyl) in tetraamine **11** resulted in a 10.5-fold increase in PAT selectivity, which suggests that very large N^1 cargoes could also be accommodated by the PAT as long as the proper polyamine motif was present.

Prior work with extended tethers in the N^1 position (**1c** and **1d**) revealed decreases in PAT selectivity as the N^1-tether was extended from methylene (**1b**) to ethylene (**1c**) and propylene (**1d**). The current finding with **11** is significant because it implies that larger cargoes can be accommodated if the correct motif is present.[18] The nature of this amine message will be explored in further work. The present findings have direct applications in the future design of PAT selective drug conjugates.

N-Alkylated polyamines have been shown to have enhanced metabolic stability due to their ability to avoid degradation by serum amine oxidases (present in the culture medium) and by the intracellular polyamine oxidase (PAO).[10a,16] Aminoguanidine[16e,f] (AG) is a known inhibitor of the serum amine oxidases and is routinely added (at 2 mM) during our cell culture experiments to avoid polyamine drug degradation by the serum oxidase. We speculated that, in the absence of AG, the polyamine conjugates could be degraded by the serum oxidases and converted to other metabolites, which could affect the measured cytotoxicity and PAT selectivity of the conjugates. Prior experiments revealed that polyamine metabolic stability could be modulated via steric effects.[10a] Therefore, even though the N^9-alkylated polyamine conjugates **7** and **8** showed lower PAT selectivity than the lead compound **1b**, it was possible that they were more metabolically stable. To test this hypothesis, we determined the cytotoxicity of **1b**, **7**, and **8** in the presence and absence of AG (Table 2).

As expected, the PAT selectivity of lead compound **1b** was lowered from 148 to 4 (Table 2) in the absence of AG, which clearly suggests that **1b** is a substrate for serum amine oxidases. In contrast, the PAT selectivities of N^9-alkylated compounds **7** and **8** were retained in the absence of AG, suggesting that these compounds are not the substrates for serum amine oxidases and are still able to utilize the PAT. In the absence of AG, compounds **7** and **1b** have the same PAT selectivity (ratio IC_{50} CHO-MG/CHO: 4). However, in the absence of AG, the N^9-methyl analogue **8** has a higher selectivity (ratio IC_{50} ratio: 11.5) than either its *N*-ethyl derivative **7** or the parent system, **1b**.

Conclusions

A series of polyamine conjugates were synthesized to probe the sensitivity of the PAT to small structural changes in its substrate and to provide insight into potential "points of attachment" onto the polyamine pharmacophore. A direct correlation was found between PAT selectivity and the degree of alkylation at the N^1, N^5, and N^9 positions. *N*-Alkylation with small substituents at the terminal N^9 position still retained modest PAT selectivity. This PAT selectivity was maintained even in the absence of AG, whereas the lead compound, **1b**, showed a dramatic reduction in PAT selectivity in the absence of AG. Large N^1-cargoes could be accommodated (e.g., **11**) by PAT as long as the proper polyamine motif was in place. These insights will be applied to the future design of polyamine-drug conjugates, which are metabolically stable and still retain PAT selectivity (e.g., **8**).

Experimental Section

Materials. Silica gel (32–63 μm) and chemical reagents were purchased from commercial sources and used without further purification. All solvents were distilled prior to use. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively. TLC solvent systems are based on volume % and NH_4OH refers to concentrated aqueous NH_4OH. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA).

Biological Studies. Murine leukemia (L1210), CHO, and CHO-MG cells were grown in RPMI medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (50 μg/mL). L-Proline (2 μg/mL) was added to the culture medium for CHO-MG cells. Cells were grown at 37 °C under a humidified 5% CO_2 atmosphere. Aminoguanidine (AG, 2 mM) was added to the culture medium to prevent oxidation of the drugs by the enzyme (bovine serum amine oxidase) present in calf serum. Trypan blue staining was used to determine cell viability before the initiation of a cytotoxicity experiment. Cells in early to mid log-phase were used.

IC_{50} Determinations. Cell growth was assayed in sterile 96-well microtiter plates (Becton-Dickinson, Oxnard, CA, U.S.A.). L1210 cells were seeded at 5e^4 cells/mL of medium (100 μL/well). CHO and CHO-MG cells were plated at 2e^3 cells/mL. Drug solutions (10 μL per well) of appropriate concentration were added at the time of seeding for L1210 cells and after an overnight incubation for the CHO cell lines. After exposure to the drug for 48 h, cell growth was determined by measuring formazan formation from 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium using a Titertek Multiskan MCC/340 microplate reader for absorbance (540 nm) measurements.[19]

***K*$_i$ Procedure.** The ability of the conjugates to interact with the polyamine transport system was determined by measuring competition by the conjugates against radiolabeled SPD uptake in L1210 cells. This procedure was used to obtain the data listed in Table 1. Initially, the K_m value of SPD transport was determined as previously described.[20]

The ability of conjugates to compete for [^{14}C]SPD uptake were determined in L1210 cells by a 10-min uptake assay in the presence of increasing concentrations of competitor using 1 μM [^{14}C]SPD as substrate. K_i values for inhibition of SPD uptake were determined using the Cheng−Prusoff equation[21] from the IC_{50} value derived by iterative curve fitting of the sigmoidal equation describing the

velocity of SPD uptake in the presence of the respective competitor.[11,22] L1210 cells were grown and maintained according to established procedures[10d] and were washed twice in HBSS prior to the transport assay.

***N*^{1}-{4-[(Anthracen-9-ylmethyl)-amino]-butyl}-*N*^{1}-ethyl-butane-1,4-diamine, Hydrochloride Salt, 6.** A solution of Boc-protected **18** (300 mg, 0.63 mmole) was dissolved in absolute ethanol (13 mL) and stirred at 0 °C for 10 min. A 4 N HCl solution (22 mL) was added to the reaction mixture dropwise and stirred at 0 °C for 20 min and then at room temperature overnight. The solution was concentrated in vacuo to give **6** as a yellow solid in 95% yield. ^{1}H NMR (D_2O) δ 8.69 (s, 1H), 8.26 (d, 2H), 8.16 (d, 2H), 7.73 (m, 2H), 7.63 (m, 2H), 5.25 (s, 2H), 3.30 (t, 2H), 3.20 (q, 2H), 3.16 (m, 4H), 3.03 (t, 2H), 1.75 (m, 8H), 1.26 (t, 3H); ^{13}C NMR (D_2O) δ 131.3, 130.9, 130.7, 129.9, 128.2, 126.0, 122.9, 121.2, 52.0, 51.8, 48.3, 47.4, 46.9, 43.3, 39.2, 24.4, 23.2, 21.1, 18.2, 8.5. HRMS (FAB) calcd for $C_{25}H_{35}N_3 \cdot 3HCl$ $(M + H - 3HCl)^+$, 378.2909; found, 378.2916.

***N*-Anthracen-9-ylmethyl-*N*′-(4-ethylamino-butyl)-butane-1,4-diamine, Hydrochloride Salt, 7.** A solution of **19** (49 mg, 0.13 mmol) was dissolved in absolute ethanol (6 mL) and stirred at 0 °C for 10 min. A 4 N HCl solution (11 mL) was added to the reaction mixture dropwise and stirred at 0 °C for 20 min and then at room temperature overnight. The solution was concentrated *in vacuo* to give **7** as a yellow solid in 93% yield. ^{1}H NMR (D_2O) δ 8.64 (s, 1H), 8.22 (d, 2H), 8.13 (d, 2H), 7.71 (m, 2H), 7.61 (m, 2H), 5.19 (s, 2H), 3.28 (t, 2H), 3.09 (m, 8H), 1.77 (m, 8H), 1.30 (t, 3H); ^{13}C NMR (D_2O) δ 133.7, 133.3, 133.1, 132.2, 130.5, 128.3, 125.3, 123.5, 49.9, 49.6, 49.1, 45.7, 25.7, 13.4. HRMS (FAB) calcd for $C_{25}H_{35}N_3 \cdot 3HCl$ $(M + H - 3HCl)^+$, 378.2909; found, 378.2906.

***N*-Anthracen-9-ylmethyl-*N*′-(4-methylamino-butyl)-butane-1,4-diamine, Hydrochloride Salt, 8.** A solution of Boc-protected **26** (180 mg, 0.32 mmole) was dissolved in absolute ethanol (13 mL) and stirred at 0 °C for 10 min. A 4 N HCl solution (22 mL) was added to the reaction mixture dropwise and stirred at 0 °C for 20 min and then at room temperature overnight. The solution was concentrated in vacuo to give **8** as a yellow solid in 95% yield. ^{1}H NMR (300 MHz, D_2O) δ 8.32 (s, 1H), 7.99 (d, 2H), 7.93 (d, 2H), 7.60 (m, 2H), 7.50 (m, 2H), 4.87 (s, 2H), 3.16 (t, 2H), 3.02 (m, 6H), 2.70 (s, 3H), 1.72 (m, 8H); ^{13}C NMR (D_2O) δ 130.7, 130.4, 130.1, 129.5, 127.7, 125.5, 122.5, 120.4, 48.4, 47.2, 47.1, 47.0, 42.8, 32.9, 23.1, 23.0, 22.9. HRMS (FAB) calcd for $C_{24}H_{33}N_3 \cdot 3HCl$ $(M + H - 3HCl)$, 364.2747; found, 364.2715.

***N*-(4-Amino-butyl)-*N*′-[4-(anthracen-9-ylmethyl-ethyl-amino)-butyl]-butane-1,4-diamine, Hydrochloride Salt, 10.** A solution of **33** (140 mg, 0.18 mmol) was dissolved in absolute ethanol (10 mL) and stirred at 0 °C for 10 min. A 4 N HCl solution (20 mL) was added to the reaction mixture dropwise and stirred at 0 °C for 20 min and then at room temperature overnight. The solution was concentrated in vacuo to give **10** as a yellow solid in 96% yield. ^{1}H NMR (D_2O) δ 8.68 (s, 1H), 8.17 (m, 4H), 7.75 (m, 2H), 7.64 (m, 2H), 5.25 (s, 2H), 3.09–3.00 (m, 12H), 2.82 (m, 2H), 1.78 (m, 10H), 1.44 (m, 5H); ^{13}C NMR (D_2O) δ 131.3, 131.0, 129.8, 128.2, 125.7, 122.7, 119.6, 107.1, 49.6, 49.5, 47.2, 47.1, 47.0, 46.7, 39.0, 24.2, 23.1, 23.0, 22.9, 20.9, 8.8. HRMS (FAB) calcd for $C_{29}H_{44}N_4 \cdot 4HCl$ $(M + H - 4HCl)^+$, 449.3639; found, 449.3629.

***N*-[4-(4-Amino-butylamino)-butyl]-*N*′-(4,6-dihydro-pyren-1-ylmethyl)-butane-1,4-diamine, Hydrochloride Salt, 11.** A solution of **34** (200 mg, 0.27 mmol) was dissolved in absolute ethanol (10 mL) and stirred at 0 °C for 10 min. A 4 N HCl solution (20 mL) was added to the reaction mixture dropwise and stirred at 0 °C for 20 min and then at room temperature overnight. The solution was concentrated in vacuo to give **11** as a yellow solid in 90% yield. ^{1}H NMR (D_2O) δ 8.25 (m, 2H), 8.15 (m, 2H), 7.95 (m, 3H), 7.90 (m, 2H), 4.64 (s, 2H), 3.1 - 2.90 (m, 12H), 1.85–1.60 (m, 12H); ^{13}C NMR (D_2O) δ 131.8, 130.7, 130.0, 128.8, 128.6, 128.3, 127.2, 126.7, 126.0, 125.9, 124.9, 123.6, 123.3, 123.0, 121.4, 64.5, 57.7, 49.1, 48.2, 47.2, 47.1, 46.7, 39.1, 24.2, 23.1, 17.1. HRMS (FAB) calcd for $C_{29}H_{40}N_4 \cdot 4HCl$ $(M + H - 4HCl)^+$, 445.3326; found, 445.3321.

(4-Amino-butyl)-carbamic Acid *tert*-Butyl Ester, 14.[8] 1,4-Diaminobutane (**13**; 30 g, 341 mmol) was dissolved in a solution of triethylamine and methanol (10% TEA in MeOH, 250 mL). A solution of di-*tert*-butyl dicarbonate (25 g, 115 mmol) in methanol (50 mL) was added dropwise to this mixture with vigorous stirring at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, then warmed slowly to rt and stirred overnight. The methanol and TEA were removed in vacuo to yield an oily residue that was dissolved in CH_2Cl_2 (200 mL) and washed with a solution 10% aq sodium carbonate (3 × 80 mL). The CH_2Cl_2 layer was dried over anhydrous sodium sulfate, filtered, the solvent was removed in vacuo, and the oily residue was purified by flash column chromatography to give the product **14**[8] as a clear oil (90%), R_f = 0.38 (NH_4OH/MeOH/$CHCl_3$, 1:10:89); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.68 (br s, 1H, NHCO), 3.13 (m, 2H), 2.70 (t, 2H), 1.62–1.33 (m, 13H, 2 × CH_2, 3 × CH_3).

[4-(3-Cyano-propylamino)-butyl]-carbamic Acid *tert*-Butyl Ester, 15.[8] 4-Bromo-butyronitrile (11.8 g, 80 mmol) was dissolved in anhydrous acetonitrile and was added to the stirring mixture of compound **14** (17 g, 90 mmol) and anhydrous K_2CO_3 (22 g, 159 mmol). The mixture was then stirred overnight at 75 °C under a N_2 atmosphere. After confirmation of the disappearance of the nitrile by TLC, the solution was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (100 mL) and washed three times with 10% aqueous sodium carbonate. The organic layer was separated, dried with anhydrous Na_2SO_4, filtered, and concentrated under vacuum. Flash column chromatography of the residue gave **15** as a light yellow oil. Yield 65%; R_f = 0.35 (MeOH/NH_4OH/$CHCl_3$, 5:0.5:94.5); ^{1}H NMR ($CDCl_3$) δ 4.92 (bt, 1H), 3.12 (q, 2H), 2.73 (t, 2H), 2.61 (t, 2H), 2.45 (t, 2H), 1.81 (q, 2H), 1.39–1.6 (m, 13H).

{4-[(3-Cyano-propyl)-ethyl-amino]-butyl}-carbamic Acid *tert*-Butyl Ester, 16. Bromoethane (385 mg, 3.53 mmol) was dissolved in anhydrous acetonitrile and was added to a stirred mixture of compound **15** (300 mg, 1.18 mmol) and anhydrous K_2CO_3 (487 mg, 3.53 mmol). The mixture was then stirred overnight at 75 °C under a N_2 atmosphere. After confirmation of the disappearance of **15** by TLC, the solution was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (100 mL) and washed three times with aqueous Na_2CO_3. The organic layer was separated, dried with anhydrous Na_2SO_4, filtered, and concentrated under vacuum. Flash column chromatography of the residue gave **16** as a light yellow oil. Yield 85%; R_f = 0.35 (MeOH/CH_2Cl_2, 3:97); ^{1}H NMR ($CDCl_3$) δ 4.94 (b, 1H), 3.10 (q, 2H), 2.37–2.52 (m, 8H), 1.76 (q, 2H), 1.44 (m, 13H), 1.00 (t, 3H); ^{13}C NMR ($CDCl_3$) δ 155.9, 119.9, 78.9, 53.3, 51.6, 47.3, 40.6, 28.6, 28.2, 24.7, 23.7, 14.9, 11.8.

{4-[(4-Amino-butyl)-ethyl-amino]-butyl}-carbamic Acid *tert*-Butyl Ester, 17. The nitrile **16** (290 mg, 1.02 mmol) was dissolved in ethanol (30 mL). NH_4OH (3 mL) and Raney nickel (1.5 g) were added and ammonia gas was bubbled through the solution for 10 min at 0 °C. The suspension was hydrogenated at 50 psi for 24 h. Air was bubbled through the solution, and the Raney nickel was removed by filtering through a sintered glass funnel keeping the Raney nickel residue moist at all times. The ethanol and NH_4OH were removed in vacuo, and the oily residue dissolved in CH_2Cl_2 and was washed with a 10% aqueous sodium carbonate solution (2 × 50 mL). The organic layer was dried over anhydrous sodium sulfate and filtered, and the solvent was removed in vacuo to give the product **17** as a light yellow oil (94% yield) that was used in the next step without further purification; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.20 (br s, 1H), 3.35 (m, 2H), 2.15–2.80 (m, 8H), 0.90–1.70 (m, 21H); ^{13}C NMR ($CDCl_3$) δ 156.1, 78.9, 53.4, 47.5, 42.4, 40.8, 32.1, 28.7, 28.4, 24.9, 24.5, 11.8.

[4-({4-[(Anthracen-9-ylmethyl)-amino]-butyl}-ethyl-amino)-butyl]-carbamic Acid *tert*-Butyl Ester, 18. To a stirred solution of **17** (275 mg, 0.96 mmol) in 25% MeOH/CH_2Cl_2 (20 mL) was added a solution of 9-anthraldehyde (164 mg, 0.80 mmol) in 25% MeOH/CH_2Cl_2 (15 mL) under N_2. The mixture was stirred at room temperature overnight until the imine formation was complete (monitored by disappearance of the ^{1}H NMR ($CDCl_3$) signal at 11.40 ppm). The solvent was removed in vacuo, the solid residue

was dissolved in 50% MeOH/CH_2Cl_2 (40 mL), and the solution was cooled to 0 °C. $NaBH_4$ (2.40 mmol) was added in small portions to the solution, and the mixture was stirred at rt overnight. The solvent was removed in vacuo, the solid residue was dissolved in CH_2Cl_2 (50 mL) and washed with 10% aq Na_2CO_3 solution (3 × 30 mL). The CH_2Cl_2 layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give an oily residue. The oil was purified by flash column chromatography to yield the product **18** as a pale yellow viscous oil (72%), $R_f = 0.3$ (MeOH/NH_4OH/CH_2Cl_2, 6:0.5:93.5); ^1H NMR ($CDCl_3$) δ 8.30 (d, 2H), 8.27 (s, 1H), 7.93 (d, 2H), 7.48 (m, 2H), 7.40 (m, 2H), 4.66 (s, 2H), 3.03 (q, 2H), 2.85 (t, 2H), 2.44 (q, 2H), 2.35 (m, 4H), 1.40–1.54 (m, 17H), 0.96 (t, 3H); ^{13}C NMR ($CDCl_3$) δ 156.0, 131.7, 131.5, 130.2, 129.1, 127.1, 126.0, 124.9, 124.1, 78.8, 53.3, 53.2, 50.5, 47.3, 45.9, 40.6, 28.6, 28.3, 24.7, 24.7, 11.6. HRMS (FAB) *m/z* calcd for $C_{30}H_{44}N_3O_2$ $(M + H)^+$, 478.3434; found, 478.3442.

***N*-Anthracen-9-ylmethyl-*N′*-(4-ethylamino-butyl)-butane-1,4-diamine, 19.** A saturated sodium carbonate solution (20 mL) was added to a vigorously stirred solution of **1b** (500 mg, 1.09 mmol) in CH_2Cl_2 (50 mL). The organic layer was separated and was washed twice with saturated sodium carbonate. The combined organic layers were dried with anhydrous Na_2SO_4, filtered, and concentrated to give the free amine of **1b** as a pale yellow thick oil in 98% yield.

To the stirred solution of the free amine of **1b** (368 mg, 1.05 mmol) in DMF (6 mL), flame dried 4 Å powdered molecular sieves (500 mg) and $CsOH \cdot H_2O$ (177 mg, 1.05 mmol) were added. The solution was stirred for 30 min, bromoethane (77 mg, 0.96 mmol) dissolved in DMF (3 mL) was added, and the reaction was stirred overnight at rt. The molecular sieves were filtered off, DMF was removed in vacuo, and the residue was dissolved in CH_2Cl_2 (50 mL) and washed with 10% aq Na_2CO_3 solution (3 × 30 mL). The CH_2Cl_2 layer was separated, dried over anhydrous Na_2SO_4, filtered, and removed in vacuo to give an oily residue. The oil was purified by flash column chromatography (MeOH/NH_4OH/CH_2Cl_2, 12:1:87) to yield the product **19** as a pale yellow oil (21%). ^1H NMR ($CDCl_3$) δ 8.37 (s, 1H), 8.30 (d, 2H), 7.98 (d, 2H), 7.52 (m, 2H), 7.44 (m, 2H), 4.70 (s, 2H), 2.87 (m, 2H), 2.57 (m, 8H), 1.45–1.57 (m, 8H), 1.09 (t, 3H); ^{13}C NMR ($CDCl_3$) δ 131.9, 131.6, 130.3, 129.2, 127.2, 126.2, 125.0, 124.2, 50.7, 50.0, 49.9, 46.0, 44.3, 28.2, 28.2, 28.1, 15.5.

Anthracen-9-ylmethyl-(4-{*tert*-butoxycarbonyl-[4-(*tert*-butoxycarbonyl-ethyl-amino)-butyl]-amino}-butyl)-carbamic Acid *tert*-Butyl Ester, 20. Compound **7** (5 mg, 0.01 mmol) was dissolved in a solution of triethylamine and methanol (10% TEA in MeOH, 10 mL). A solution of di-*tert*-butyl dicarbonate (11.20 mg, 0.05 mmol) in methanol (3 mL) was added dropwise to this solution with vigorous stirring at 0 °C. The reaction was stirred at 0 °C for 30 min and then warmed slowly to rt. The solution was stirred overnight. The methanol and TEA were removed in vacuo to yield an oily residue that was dissolved in CH_2Cl_2 (30 mL) and washed with a solution of 10% aq sodium carbonate (3 × 30 mL). The CH_2Cl_2 layer was dried over anhydrous sodium sulfate, separated, filtered, and concentrated in vacuo to provide a thick oil **20** (94%). ^1H NMR (300 MHz, $CDCl_3$) δ 8.45 (s, 1H), 8.38 (d, 2H), 8.02 (d, 2H), 7.51 (m, 4H), 5.52 (s, 2H), 3.15 (m, 4H), 2.60–2.97 (m, 6H), 1.05–1.59 (m, 38H).

4-(4-Amino-butylamino)-butan-1-ol, Hydrochloride Salt, 22. A solution of Boc-derivative **21** (600 mg, 1.67 mmol) was dissolved in absolute ethanol (20 mL) and stirred at 0 °C for 10 min. A 4 N HCl solution (35 mL) was added to the reaction mixture dropwise and stirred at 0 °C for 20 min and then at room temperature overnight. The solution was concentrated in vacuo to give **22** as a white solid in 97% yield. ^1H NMR (300 MHz, D_2O) δ 3.61 (t, 2H, OCH_2), 3.05 (m, 6H, NCH_2), 1.75 (m, 6H, CH_2), 1.61 (m, 2H, CH_2); ^{13}C NMR (300 MHz, D_2O): δ 60.9, 47.5, 46.9, 38.9, 28.4, 24.1, 22.9, 22.5.

4-{4-[(Anthracen-9-ylmethyl)-amino]-butylamino}-butan-1-ol, 23. To a stirred solution of **22** (386 mg, 1.66 mmol) and TEA (418 mg, 4.14 mmol) in 25% MeOH/CH_2Cl_2 (20 mL) was added a solution of 9-anthraldehyde (310 mg, 1.50 mmol) in 25% MeOH/CH_2Cl_2 (15 mL) under N_2. The mixture was stirred at room temperature overnight until the imine formation was complete (monitored by NMR). The solvent was removed in vacuo, the solid residue was dissolved in 50% MeOH/CH_2Cl_2 (40 mL), and the solution was cooled to 0 °C. $NaBH_4$ (4.50 mmol) was added in small portions to the solution, and the mixture was stirred at rt overnight. The solvent was removed in vacuo, and the solid residue was dissolved in CH_2Cl_2 (50 mL) and washed with 10% aq Na_2CO_3 solution (3 × 30 mL). The CH_2Cl_2 layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give an oily residue, **23** (95%), which was used in the next step without further purification. ^1H NMR (300 MHz, $CDCl_3$) δ 8.31 (s, 1H), 8.26 (d, 2H), 7.92 (d, 2H), 7.48 (m, 2H), 7.40 (m, 2H), 4.63 (s, 2H), 3.48 (t, 2H), 2.80 (t, 2H), 2.51 (t, 4H), 1.46–1.58 (m, 8H); ^{13}C NMR ($CDCl_3$) δ 131.7, 131.4, 130.1, 129.1, 127.0, 126.0, 124.8, 124.0, 62.4, 50.4, 49.6, 49.4, 45.8, 32.5, 28.7, 28.0, 27.7.

[4-(Anthracen-9-ylmethyl-tert-butoxycarbonyl-amino)-butyl]-(4-hydroxy-butyl)-carbamic Acid *tert*-Butyl Ester, 24. The synthesis of compound **24** has been reported previously.[5]

Methanesulfonic Acid 4-{[4-(Anthracen-9-ylmethyl-*tert*-butoxycarbonyl-amino)-butyl]-*tert*-butoxycarbonyl-amino}-butyl Ester, 25. The synthesis of compound **25** has been reported previously.[5]

[4-(Anthracen-9-ylmethyl-*tert*-butoxycarbonyl-amino)-butyl]-(4-methylamino-butyl)-carbamic Acid *tert*-Butyl Ester, 26. Compound **25** (425 mg, 0.68 mmol) was dissolved in acetonitrile (10 mL) and added to a stirring solution of methylamine (4.77 mL of 2.0 M in MeOH, 9.55 mmol) in acetonitrile (20 mL). The mixture was then stirred at 75 °C overnight under a N_2 atmosphere. TLC showed the absence of starting material **25** after 24 h and the solution was then concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (30 mL) and washed three times with aqueous sodium carbonate. The organic layer was separated, dried with anhydrous Na_2SO_4, filtered, and concentrated under vacuum. Flash column chromatography of the residue gave **26** as a yellow oil (73%). **26**: $R_f = 0.3$ (MeOH/NH_4OH/CH_2Cl_2, 8:1:91); ^1H NMR (300 MHz, $CDCl_3$) δ 8.40 (s, 1H), 8.36 (d, 2H), 7.98 (d, 2H), 7.48 (m, 4H), 5.50 (s, 2H), 2.93–2.79 (m, 6H), 2.53 (t, 2H), 2.40 (s, 3H), 1.76–1.43 (m, 8H), 1.41–1.28 (m, 14H), 1.11 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 155.3, 131.3, 131.3, 129.2, 128.2, 126.3, 125.0, 124.1, 79.8, 79.0, 53.6, 51.9, 46.7, 44.5, 41.2, 36.6, 28.7, 28.6, 27.2, 26.1, 25.7. HRMS (FAB) *m/z* calcd for $C_{34}H_{49}N_3O_4$ $(M + Na)^+$, 586.3615; found, 586.3608.

(4-*tert*-Butoxycarbonylamino-butyl)-[4-(3-cyano-propylamino)-butyl]-carbamic Acid *tert*-Butyl Ester, 29. To a solution of the amine **28** (1.32 g, 3.68 mmol) in anhydrous acetonitrile (20 mL) was added potassium carbonate (1.7 g), and the suspension was stirred at rt for 10 min. A solution of 4-bromobutyronitrile (0.54 g, 3.68 mmol) in acetonitrile (10 mL) was added, and the resulting mixture was stirred at 50 °C under nitrogen for 24 h. TLC (1:10:89 NH_4OH/MeOH/$CHCl_3$) showed the reaction was 95% complete. The mixture was filtered and the volatiles were removed in vacuo to give a semisolid residue. The residue was redissolved in CH_2Cl_2 (70 mL) and washed with saturated aq Na_2CO_3 (50 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The oily residue was purified by flash column chromatography (MeOH/NH_4OH/CH_2Cl_2, 7:1:92) to give the product **29** as an oil in 62% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 4.60 (br s, 1H, NHCO), 3.15 (m, 6H, 3 × CH_2), 2.74 (t, 2H, CH_2), 2.62 (t, 2H, CH_2), 2.46 (t, 2H, CH_2), 1.83 (quin, 2H, CH_2), 1.55–1.39 (m, 26H, 4 × CH_2, 6 × CH_3); ^{13}C NMR ($CDCl_3$) δ 156.0, 155.6, 119.8, 79.3, 79.2, 53.6, 49.5, 48.1, 46.9, 40.4, 28.7, 28.6, 27.6, 27.3, 26.1, 25.9, 15.2.

(4-*tert*-Butoxycarbonylamino-butyl)-{4-[*tert*-butoxycarbonyl-(3-cyano-propyl)-amino]-butyl}-carbamic Acid *tert*-Butyl Ester, 30. A solution of di-*tert*-butyl dicarbonate (0.92 g, 0.42 mmol) in methanol (10 mL) was added dropwise to a vigorously stirred solution of **29** (0.12 g, 0.28 mmol) in 10% TEA/MeOH at 0 °C. The mixture was stirred at rt overnight. TLC (NH_4OH/MeOH/$CHCl_3$, 1:10:89) showed the *tert*-butoxycarbonylation was complete. The solvent was removed in vacuo to yield an oily residue that

was then dissolved in dichloromethane (100 mL) and washed with a solution of saturated aqueous Na_2CO_3 (3 × 30 mL). The organic layer was dried over anhydrous Na_2SO_4, separated, filtered, and concentrated in vacuo to give an oily residue, which was purified by flash column chromatography ($CHCl_3$/MeOH 99:1) to give **30** as an oil (94% yield), $R_f = 0.5$ ($CHCl_3$/MeOH, 98:2); ^{1}H NMR ($CDCl_3$) δ 4.63 (br s, 1H, NHCO), 3.28 (t, 2H, CH_2), 3.14 (m, 8H, 4 × CH_2), 2.36 (t, 2H, CH_2), 1.88 (quin, 2H, CH_2), 1.58–1.35 (m, 35H, 4 × CH_2, 9 × CH_3); ^{13}C NMR δ 155.9, 155.5, 79.9, 79.3, 79.0, 46.7, 45.8, 40.3, 28.6, 28.5, 27.5, 26.0, 25.7, 24.6, 14.9.

(4-Amino-butyl)-{4-[*tert*-butoxycarbonyl-(4-*tert*-butoxycarbonylamino-butyl)-amino]-butyl}-carbamic Acid *tert*-Butyl Ester, 31. The nitrile **30** (500 mg, 0.95 mmol) was dissolved in ethanol (30 mL). NH_4OH (3 mL) and Raney nickel (1.5 g) were added and ammonia gas was bubbled through the solution for 10 min at 0 °C. The suspension was hydrogenated at 50 psi for 24 h. Air was bubbled through the solution and the Raney nickel was removed by filtering through a sintered glass funnel, keeping the Raney nickel residue moist at all times. The ethanol and NH_4OH were removed in vacuo, and the oily residue was dissolved in CH_2Cl_2 and washed with 10% aq sodium carbonate (2 × 50 mL). The organic layer was separated, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to give the product **31** as a light yellow oil in 90% yield. The oil was used in the next step without further purification; $R_f = 0.5$ (NH_4OH/MeOH/$CHCl_3$, 1:10:89). ^{1}H NMR ($CDCl_3$) δ 4.65 (s, 1H, NHCO), 3.15 (m, 10H, 5 × CH_2), 2.70 (t, 2H, CH_2NH_2), 1.58–1.39 (m, 39H, 6 × CH_2, 9 × CH_3); ^{13}C NMR ($CDCl_3$) δ 155.9, 155.4, 79.2, 79.1, 47.0, 46.7, 40.3, 28.6, 28.5, 27.5, 26.1, 25.7. HRMS (FAB) calcd for $C_{27}H_{55}N_4O_6$ (M + 1), 531.4122; found (M + 1), 531.4111.

[4-({4-[(Anthracen-9-ylmethyl)-amino]-butyl}-*tert*-butoxycarbonyl-amino)-butyl]-(4-*tert*-butoxycarbonylamino-butyl)-carbamic Acid *tert*-Butyl Ester, 32. To a stirred solution of **31** (204 mg, 0.38 mmol) in 25% MeOH/CH_2Cl_2 (20 mL) was added a solution of 9-anthraldehyde (87.2 mg, 0.42 mmol) in 25% MeOH/CH_2Cl_2 (15 mL) under N_2. The mixture was stirred at room temperature overnight until the imine formation was complete (as monitored by ^{1}H NMR). The solvent was removed in vacuo. The solid residue was dissolved in 50% MeOH/CH_2Cl_2 (40 mL) and the solution was cooled to 0 °C. $NaBH_4$ (2.40 mmol) was added in small portions to the solution, and the mixture was stirred at rt overnight. The solvent was removed in vacuo, and the solid residue was dissolved in CH_2Cl_2 (50 mL) and washed with 10% aq Na_2CO_3 solution (3 × 30 mL). The CH_2Cl_2 layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give an oily residue. The oil was purified by flash column chromatography (MeOH/NH_4OH/CH_2Cl_2, 6:0.5:93.5) to yield **32** as a pale yellow viscous oil (72%), $R_f = 0.3$ (MeOH/NH_4OH/CH_2Cl_2, 6:0.5:93.5; ^{1}H NMR ($CDCl_3$) δ 8.35 (s, 1H), 8.29 (d, 2H), 7.96 (d, 2H), 7.44 (m, 4H), 5.24 (s, 2H), 3.12 (m, 10H), 2.87 (m, 2H), 1.24–1.55 (m, 39H); ^{13}C NMR ($CDCl_3$) δ 155.9, 155.5, 131.7, 131.5, 130.2, 129.1, 127.1, 126.1, 124.9, 124.1, 79.2, 79.2, 50.4, 47.0, 46.7, 45.9, 40.4, 28.7, 28.6, 27.6, 26.2, 25.8. HRMS (FAB) *m/z* calcd for $C_{42}H_{64}N_4O_6$ $(M + H)^+$, 721.4893; found, 721.4904.

(4-{[4-(Anthracen-9-ylmethyl-ethyl-amino)-butyl]-*tert*-butoxycarbonyl-amino}-butyl)-(4-*tert*-butoxycarbonylamino-butyl)-carbamic Acid *tert*-Butyl Ester, 33. Ethylbromide (78 mg, 0.71 mmol) was dissolved in anhydrous acetonitrile and added to a stirred mixture of compound **32** (172 mg, 0.24 mmol) and anhydrous K_2CO_3 (98.9 mg, 0.71 mmol). The mixture was then stirred overnight at 75 °C under a N_2 atmosphere. After confirmation of the disappearance of **32** by TLC, the solution was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (20 mL) and washed three times with 10% aqueous sodium carbonate. The organic layer was separated, dried with anhydrous Na_2SO_4, filtered, and concentrated under vacuum. Flash column chromatography of the residue gave **33** as a light yellow oil. Yield 81%; $R_f = 0.35$ (MeOH/NH_4OH/CH_2Cl_2, 5:0.5:94.5); ^{1}H NMR ($CDCl_3$) δ 8.45 (d, 2H), 8.36 (s, 1H), 7.95 (d, 2H), 7.45 (m, 4H), 4.47 (s, 2H), 3.07 (m, 6H), 2.97 (m, 4H), 2.62 (q, 2H), 2.50 (m, 2H), 1.20–1.60 (m, 39H), 1.15 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 156.0, 155.6, 131.4, 131.4, 131.0, 129.0, 127.2, 125.4, 125.3, 124.8, 79.3, 79.1, 52.7, 50.9, 47.5, 47.0, 46.8, 40.5, 28.7, 28.7, 27.6, 26.2, 25.8, 24.6, 12.2. HRMS (FAB) *m/z* calcd for $C_{44}H_{68}N_4O_6$ $(M + H)^+$, 749.5212; found, 749.5210.

{4-[*tert*-Butoxycarbonyl-(4-*tert*-butoxycarbonylamino-butyl)-amino]-butyl}-{4-[(4,6-dihydro-pyren-1-ylmethyl)-amino]-butyl}-carbamic Acid *tert*-Butyl Ester, 34. To a stirred solution of **31** (150 mg, 0.28 mmol) in 25% MeOH/CH_2Cl_2 (20 mL) was added a solution of 1-pyrenecarboxaldehyde (71 mg, 0.31 mmol) in 25% MeOH/CH_2Cl_2 (15 mL) under N_2. The mixture was stirred at room temperature overnight until the imine formation was complete (as monitored by ^{1}H NMR). The solvent was removed in vacuo, the solid residue was dissolved in 50% MeOH/CH_2Cl_2 (40 mL), and the solution was cooled to 0 °C. $NaBH_4$ (2.40 mmol) was added in small portions to the solution, and the mixture was stirred at rt overnight. The volatiles were removed in vacuo, and the solid residue was dissolved in CH_2Cl_2 (50 mL) and washed with 10% aq Na_2CO_3 solution (3 × 30 mL). The CH_2Cl_2 layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give an oily residue. The oil was purified by flash column chromatography (MeOH/NH_4OH/CH_2Cl_2, 5:0.5:94.5) to yield the product **34** as a pale yellow oil (70%), $R_f = 0.3$ (5% MeOH/0.5% NH_4OH/CH_2Cl_2; ^{1}H NMR ($CDCl_3$) δ 8.32 (d, 1H), 8.07 (m, 4H), 7.96 (m, 4H), 4.43 (s, 2H), 3.06 (m, 10H), 2.78 (m, 2H), 1.20–1.63 (m, 39H); ^{13}C NMR ($CDCl_3$) δ 156.0, 155.5, 133.9, 131.3, 130.8, 130.6, 129.1, 127.6, 127.5, 127.1, 125.9, 125.1, 125.1, 125.0, 125.0, 124.9, 124.7, 123.2, 79.3, 79.2, 52.0, 50.0, 47.1, 46.8, 40.4, 28.7, 28.6, 27.7, 26.3, 25.8. HRMS (FAB) *m/z* calcd for $C_{44}H_{64}N_4O_6$ $(M + H)^+$, 745.4899; found, 745.4872.

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Supporting Information Available: Elemental analyses for compounds **5**−**8**, **10**, **11**, **18**, **26**, and **32**−**34** and the ^{1}H and ^{13}C spectra of compounds **6**−**8**, **10**, **11**, **16**−**20**, **22**−**24**, **26**, and **29**−**34** are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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