Expedited Articles

The Role of Charge in Polyamine Analogue Recognition

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A series of analogues and homologues of N^1,N^{12}-diethylspermine (DESPM) was synthesized, and their biological properties were evaluated. These tetraamines include a simple linear analogue of DESPM, N^1,N^{12}-bis(2,2,2-trifluoroethyl)spermine (FDESPM), the cyclic analogues of DESPM, N,N'-bis(4-piperidinylmethyl)-1,4-diaminobutane [PIP(4,4,4)] and N,N'-bis[2-(4-piperidinyl)ethyl]-1,4-diaminobutane [PIP(5,4,5)], and their aromatic counterparts, N,N'-bis-(4-pyridylmethyl)-1,4-diaminobutane [PYR(4,4,4)] and N,N'-bis[2-(4-pyridyl)ethyl]-1,4-diaminobutane [PYR(5,4,5)]. The analogues FDESPM, PIP(4,4,4), and PYR(4,4,4) have distances between their nitrogen atoms almost identical to those of DESPM. The longer analogues PIP-(5,4,5) and PYR(5,4,5) are very similar in the spacing of their amino groups. However, the pK_a of the nitrogens in the groups differ; thus, the extent of protonation and the charge characteristics among the members of the groups differ. A comparison of the biological properties of these compounds clearly demonstrates that the tetraamines must be charged to be "recognized" by the cell. Analogues with low nitrogen pK_a's such that the nitrogens are poorly protonated at physiological pH do not compete well with spermidine for uptake and, as expected, have high 96 h IC_{50} values and have little effect on *S*-adenosylmethionine decarboxylase, ornithine decarboxylase, and spermidine/spermine N^1-acetyltransferase activities and on intracellular polyamine pools.

Introduction

In recent years a great deal of attention has been given to the polyamine biosynthetic network as a target in antineoplastic strategies. The concept is predicated on the observation that the level of polyamine biosynthesis is very high in transformed cells as reflected by both the level of polyamine enzymes, e.g., ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC), and cellular polyamine concentrations. Initial work focused on the design and synthesis of compounds which would inhibit ODC and AdoMetDC. Some success was achieved through this approach in that difluoromethylornithine (DFMO), an ODC inhibitor, and methylglyoxylbis(guanylhydrazone) (MGBG), an AdoMetDC inhibitor, were effective against both *in vivo* and *in vitro* tumors.[1] However, clinical trials did not mirror the success realized in the model systems; the drug either was too toxic as with MGBG or was unable to show significant impact on tumors in humans as with DFMO. The design concept was somewhat flawed in that both ODC and AdoMetDC are regulatory enzymes with very short half-lives: 20 min; consequently the tumor cells and thus the patients must be exposed to inhibitors for extended periods of time to be effective. Nonetheless, the drastic reduction of ODC and AdoMetDC activities observed in tumor cells grown in exogenous spermine encouraged further pursuit of the polyamine biosynthetic network as an antineoplastic target.

The idea was to design polyamine analogues which would appear enough like the natural polyamines to shut down polyamine enzymes just as when the cells were exposed to exogenous spermine. It was thus anticipated that such analogues would be incorporated via the polyamine transport apparatus, and once in the cell would find their way to the same subcellular distribution sites as the normal polyamines do. It was important to design these analogues in such a way that they would be unable to be further metabolized once in these sites.

In fact, a series of *N*-alkylated polyamines, terminally dialkylated analogues and homologues of spermine, which exhibit antineoplastic activity against a number of murine and human tumor lines both *in vitro* and *in vivo*, was designed and synthesized in these laboratories.[1-3] These compounds have been shown to utilize the polyamine transport apparatus for incorporation,[4,5] deplete polyamine pools,[6] drastically reduce the level of ODC[7,8] and AdoMetDC activities,[9] and in some cases to upregulate spermidine/spermine N^1-acetyltransferase (SSAT).[10-16]

Very small structural alterations in these polyamine analogues result in substantial differences in their biological activity.[6] While the tetraamines N^1,N^{12}-diethylspermine (DESPM), N^1,N^{11}-diethylnorspermine (DENSPM), and N^1,N^{14}-diethylhomospermine (DEHSPM) suppress ODC and AdoMetDC to about the same level at equimolar concentrations, the effect of both DESPM and DEHSPM on cell growth is much faster than that observed for DENSPM. The K_i value of DENSPM is over 10 times as great[6] as those of DESPM and DEHSPM for the polyamine transport system.

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However, the most notable difference between the three analogues is related to their ability to stimulate SSAT.[11-13] The tetraamine DENSPM upregulates SSAT by 1200-fold in MALME-3 cells, while DESPM and DEHSPM stimulate SSAT by 250- and 30-fold, respectively.[14] The question is: What structural parameters of the polyamine analogues do the cell "recognize" in order to respond so differently to such subtle structural differences? It is clear that there are a number of levels at which this "recognition" needs to occur, the first of which is at the polyamine transport apparatus.

On the basis of pK_a values measured for DENSPM and MINSQ calculations using published pK_a values for homospermine and spermine,[17] DENSPM, DEHSPM, and DESPM are highly protonated at physiological pH. At pH 7.2, spermine and homospermine are 85% and 97% tetracation, respectively, while DENSPM is 74% in this form, the remainder being the trication. The ability of the analogues to interact with biological counterions, e.g., a group of anions fixed to a platform, as in nucleic acids, and thus to be "read" by the cell as "normal" polyamines could well be related to these cationic properties. The distance between the charged centers is critical in such interactions. In the simplest terms, the force vector between charged centers falls off as the inverse square of the distance between them. This implies that in a set of homologous polyamine analogues with different nitrogen pK_a's, the uncharged, unprotonated homologue will not interact with the same biological counteranion as its charged counterparts. Thus, if the activity of the analogues is dependent on their ability to electrostatically interact with a biological counteranion, weakly protonated analogues would not be expected to be active. The current study focuses on how charge impacts on transport apparatus recognition of the polyamine analogues.

Design Concept

Because terminally diethylated spermine (DESPM) is structurally the most similar of all of the polyamine analogues to the natural cellular polyamine spermine, it was elected as the positive control in these studies. The first compound chosen to investigate the significance of charge in cellular polyamine recognition was N^1,N^{12}-bis(2,2,2-trifluoroethyl)spermine (FDESPM) (**1**) (Figure 1). The trifluoroethyl groups reduce the pK_a of the terminal nitrogens such that they are not significantly protonated at physiological pH.[18] Thus from a charge perspective, FDESPM should appear to the cell as an N^1,N^4-dialkylated putrescine.

In order to further verify the significance of charge in analogue recognition, a set of piperidine analogues and their pyridine counterparts was also explored: N,N'-bis(4-piperidinylmethyl)-1,4-diaminobutane [(PIP(4,4,4)] (**2**), N,N'-bis[2-(4-piperidinyl)ethyl]-1,4-diaminobutane [PIP(5,4,5)] (**3**), N,N'-bis(4-pyridylmethyl)-1,4-diaminobutane [PYR(4,4,4)] (**4**), and N,N'-bis[2-(4-pyridyl)ethyl]-1,4,-diaminobutane [PYR(5,4,5)] (**5**) (Figure 1). In designing these molecules a BIOSYM molecular modeling program was employed. The synthetic candidates were set in their potential minimum, and the through-space distances between the respective $N^{\alpha}-N^{\beta}$ nitrogens, the external nitrogen and closest neighboring nitrogn, were measured along with the $N^{\alpha}-N^{\alpha'}$ value, the distance between the two external nitrogens. These

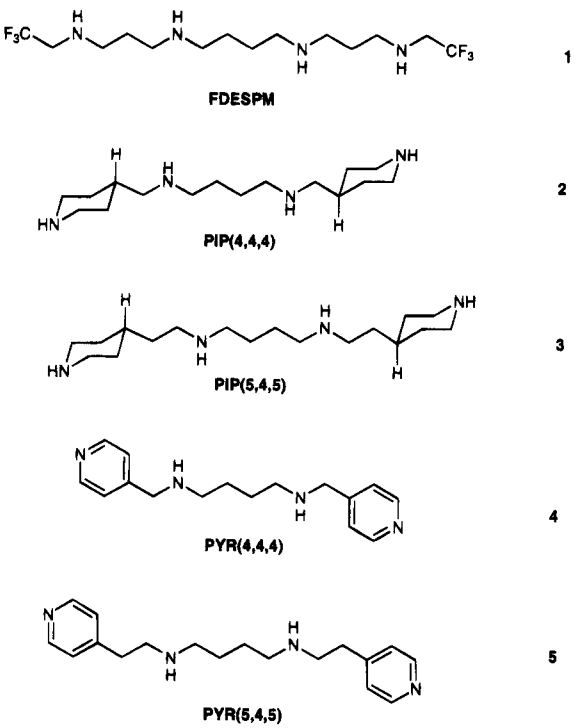


Figure 1. Hexafluoro, dipiperidyl, and dipyridyl analogues of N^1,N^{12}-diethylspermine (DESPM).

Table 1. BIOSYM-Generated Distances between Nitrogens on Polyamine Analogues[a]

polyamine	$N^{\alpha}-N^{\beta}$ (Å)	$N^{\alpha}-N^{\alpha'}$ (Å)
DESPM (3,4,3)	5.04	16.43
FDESPM (3,4,3)	5.04	16.43
PIP(4,4,4)	5.31	16.84
PYR(4,4,4)	5.21	16.47
PIP(5,4,5)	6.78	19.66
PYR(5,4,5)	6.51	18.86
PIP(3,4,3)	4.24	14.56
PYR(3,4,3)	4.21	14.40

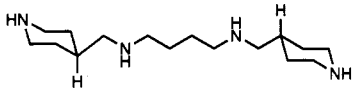
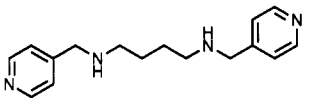
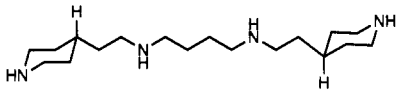
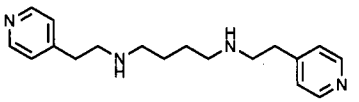
[a] The distances are through-space distances and taken from the tetracation in the conformationally most stable state.

calculations revealed some rather interesting findings (Table 1). The $N^{\alpha}-N^{\beta}$ and $N^{\alpha}-N^{\alpha'}$ distances of PIP(4,4,4) and PYR(4,4,4) are very close to those of DESPM. Interestingly the $N^{\alpha}-N^{\beta}$ and $N^{\alpha}-N^{\alpha'}$ distances in cyclic DESPM analogues N,N'-bis(4-piperidinyl)-1,4-diaminobutane [PIP(3,4,3)] and N,N'-bis(4-pyridyl)-1,4-diaminobutane [PYR(3,4,3)] are much shorter than the corresponding distances in DESPM. The longer analogues PIP(5,4,5) and PYR(5,4,5), whose $N^{\alpha}-N^{\beta}$ and $N^{\alpha}-N^{\alpha'}$ distances were, as anticipated, substantially greater than those in DESPM, were also explored.

The terminal bis-piperidines **2** and **3**, can be regarded as ethylated linear polyamines in which the ethyl groups are tied back into the chains, forming a cyclic compound. All four of the secondary amino groups in these aliphatic cyclic compounds are sufficiently basic[19,20] to be highly protonated at physiological pH, as is DESPM. The aromatic nitrogens of dipyridines **4** and **5**, which correspond to bis-piperidines, should have pK_a values approximately five units below their aliphatic counterparts.[20] Since the pyridine rings will be largely unprotonated at physiological pH, compounds **4** and **5** should be "seen" by biological systems as terminally dialkylated derivatives of putrescine.

Finally, in order to verify our assumptions about the protonation states of the model systems, we carried out

Table 2. Titrimetrically Measured pK_a Values and Calculated Relative Abundances of Ionic Species at pH 7.4 for FDESPM, PIP(4,4,4), PIP(5,4,5), PYR(4,4,4), and PYR(5,4,5)

structure	compound abbreviation	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}	relative abundance of species vs charge (pH = 7.4) 0	1+	2+	3+	4+
$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	SPM(3,4,3)	10.86	10.05	8.82	7.95	0.0%	0.0%	1.0%	23.4%	75.6%
$CF_3CH_2NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2CF_3$	FDESPM(3,4,3)	10.48	9.41	4.81	4.09	0.0%	1.0%	98.6%	0.3%	0.0%
	PIP(4,4,4)	10.93	10.69	9.43	8.45	0.0%	0.0%	0.1%	8.2%	91.7%
	PYR(4,4,4)	9.23	7.64	4.43	3.77	0.7%	36.3%	63.0%	0.0%	0.0%
	PIP(5,4,5)	10.93	10.82	10.01	9.10	0.0%	0.0%	0.0%	2.0%	98.0%
	PYR(5,4,5)	9.81	8.85	5.69	4.93	0.0%	3.3%	94.7%	2.0%	0.0%

a series of pK_a studies, the results of which are included in Table 2. On the basis of pK_a values measured for SPM (3,4,3), FDESPM (3,4,3), PIP(4,4,4), PYR(4,4,4), PIP(5,4,5), and PYR(5,4,5), the nonaromatic, nontrifluoroethylated tetraamines are indeed largely tetracationic at pH 7.4. FDESPM and PYR(5,4,5) are almost exclusively in the dicationic state, and while PYR(4,4,4) is predominately in the dicationic state, there is also a significant fraction of monocation.

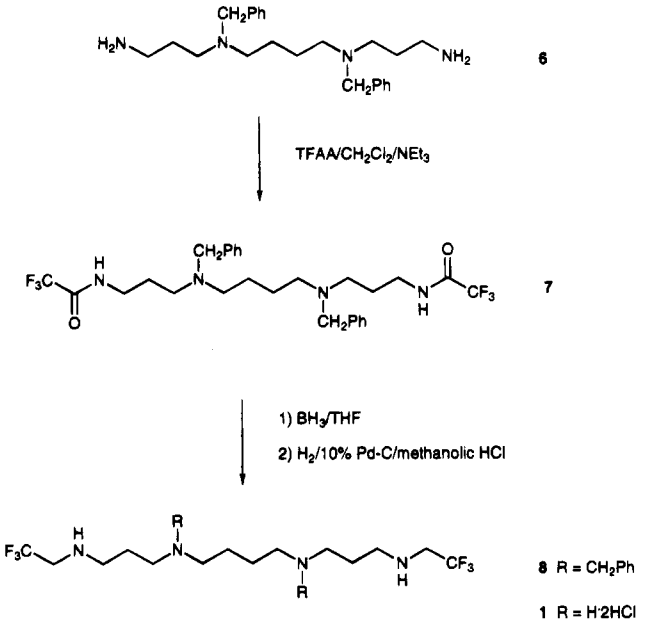
Synthesis

Three different types of tetraamines were synthesized (Figure 1). These included a simple linear analogue of DESPM, FDESPM (**1**), the alicyclic analogues of DESPM, PIP(4,4,4) (**2**), PIP(5,4,5) (**3**), and their aromatic counterparts, PYR(4,4,4) (**4**) and PYR(5,4,5) (**5**). The sets FDESPM, DESPM, PIP(4,4,4) and PYR(4,4,4), and PIP(5,4,5) and PYR(5,4,5) have almost identical distances between their nitrogen atoms. However, as described above, the pK_a of the nitrogens in the pairs differ; thus, the extent of protonation and the charge characteristics of the pairs FDESPM vs DESPM, PIP(4,4,4) vs PYR(4,4,4), and PIP(5,4,5) vs PYR(5,4,5) differ.

FDESPM (**1**) was synthesized by first reacting N^4,N^9-dibenzylspermine[21] with trifluoroacetic anhydride (CH_2Cl_2/NEt_3), to produce N^1,N^{12}-bis(2,2,2-trifluoroacetyl)-N^4,N^9-dibenzylspermine (**7**) (Scheme 1). The amides were reduced using borane–dimethyl sulfide complex in THF to afford dibenzylspermine derivative **8**. The internal amino groups of **8** were unmasked employing mild catalytic reduction conditions (Pd–C/methanolic HCl), giving the hexafluorinated DESPM **1**.

The aliphatic cyclic analogues of DESPM, PIP(4,4,4) (**2**) and PIP(5,4,5) (**3**), were prepared according to Schemes 2 and 3, respectively, utilizing the mesitylsulfonyl protecting group.[2] 4-(Aminomethyl)piperidine was reacted with mesitylenesulfonyl chloride under Schotten–Baumann conditions to give bis-sulfonamide **9**, which was alkylated with 1,4-dibromobutane (0.5 equiv)/NaH/DMF (Scheme 2) to provide the tetrasulfonamide **10**. Removal of the sulfonyl protecting groups occurred cleanly with 30% HBr in HOAc/(PhOH/CH_2Cl_2), furnishing cyclic DESPM analogue **2**. The longer dipiperidyl tetraamine **3** was synthesized from 4-piperidineethanol, which was converted to its *N*-protected *O*-activated derivative **12** with mesitylenesulfonyl chloride in pyridine (Scheme 3). *N*,*N*′-Bis(mesitylsulfonyl)-putrescine **11**[2] was alkylated at each terminus with **12** (NaH/DMF). Deprotection of the resulting tetrasulfonamide **13** with HBr provided spermine homologue **3**.

Scheme 1. Synthesis of N^1,N^{12}-Bis(2,2,2-trifluoroethyl)spermine (FDESPM) (**1**)

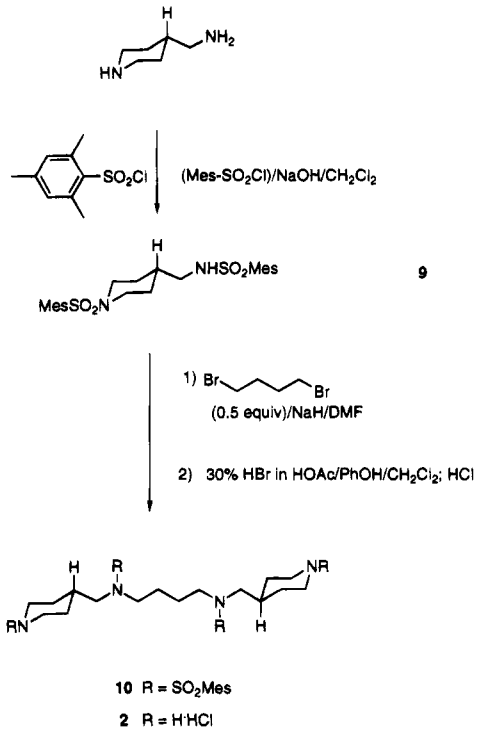


PYR(4,4,4) (**4**) and PYR(5,4,5) (**5**), aromatic analogues of DESPM, were each prepared in one step (Scheme 4). Putrescine and 4-pyridinecarboxaldehyde (2 equiv) were condensed to produce a bis-imine, which was reduced *in situ* using sodium borohydride to provide analogue **4**. 1,6-Addition of putrescine to 4-vinylpyridine (>2 equiv)[22] furnished dipyridinyl tetraamine **5**.

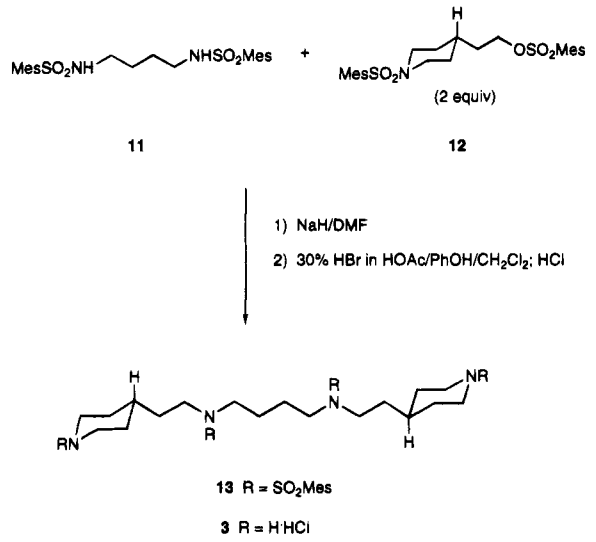
Biological Evaluations

In summarizing the biological properties of the polyamine analogues, the results will be separated into two sets of measurements: (1) the 96 h IC_{50} values

Scheme 2. Synthesis of *N,N'*-Bis(4-piperidinylmethyl)-1,4-diaminobutane [PIP(4,4,4)] (**2**)



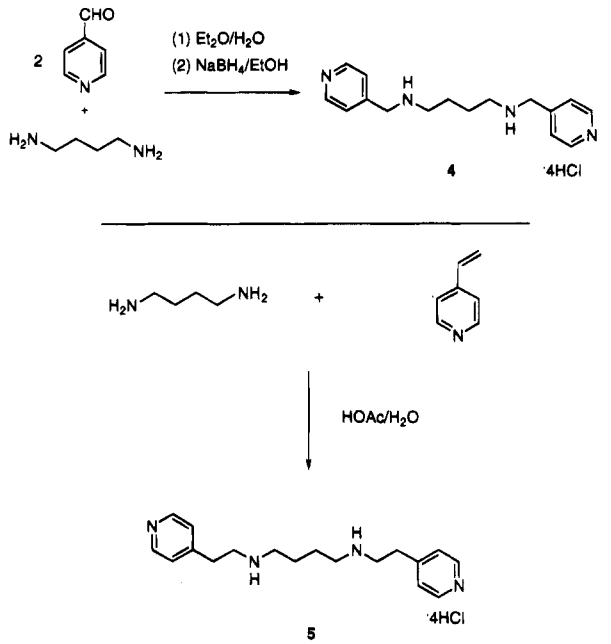
Scheme 3. Synthesis of *N,N'*-Bis[2-(4-piperidinyl)ethyl]-1,4-diaminobutane [PIP(5,4,5)] (**3**)



against L1210 cells and the corresponding K_i values for the polyamine transport apparatus (Table 3) and (2) the effect on polyamine pools, the impact on ODC, AdoMetDC, and SSAT activities (Table 4). While we have reported the effect of DESPM[2] on these same biological parameters, the results of these studies are included for comparative purposes where relevant.

IC_{50} Studies. The most notable feature of the IC_{50} measurements is the fact that all of the tetraamines which are not significantly in the tetraprotonated state at physiological pH are not active (Table 3). For example, DESPM has a 96 h IC_{50} of 0.2 μM, while the corresponding terminal hexafluoro derivative has an IC_{50} in excess of 100 μM. The fluorinated compound is only protonated at the two central nitrogens at physiological pH. The cyclic analogues of DESPM follow the same trends in that diprotonated species are not active against L1210 cells in cell culture. The DESPM piperidine analogue PIP(4,4,4) with the distances between the nitrogens almost identical to those of DESPM itself has a 96 h IC_{50} of 0.1 μM, while its pyridine analogue has an IC_{50} of 80 μM. Again the pyridine analogue is largely in the diprotonated state under cell culture conditions. Extending the concept to a higher homologue PIP(5,4,5) and its pyridine counterpart PYR(5,4,5) produced similar results. The piperidine compound had an IC_{50} of 0.3 μM and the pyridine analogue an IC_{50} in excess of 100 μM.

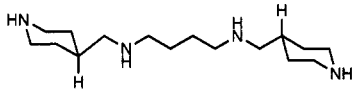
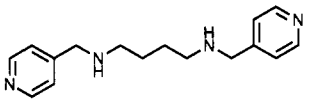
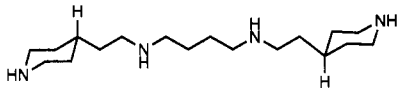
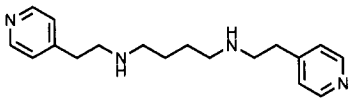
Scheme 4. Synthesis of *N,N'*-Bis(4-pyridylmethyl)-1,4-diaminobutane [PYR(4,4,4)] (**4**) and *N,N'*-Bis[2-(4-pyridyl)ethyl]-1,4-diaminobutane [PYR(5,4,5)] (**5**)



K_i Studies. The ability of the analogues to compete with radiolabeled spermidine (SPD) for uptake followed trends similar to those of the IC_{50} determinations (Table 3). DESPM has a K_i value of 1.6 μM, while FDESPM has a K_i value of 285 μM. The DESPM cyclic analogues follow the same trends, i.e., compounds which are largely in the dicationic state at physiological pH do not compete well for uptake. Thus the DESPM piperidine analogue PIP(4,4,4) has a K_i value of 4.9 μM, while its pyridine analogue has a K_i > 500 μM. The same trend applies to the homologue PIP(5,4,5) and its pyridine counterpart PYR(5,4,5). The piperidine compound has a K_i value of 18.1 μM and the pyridine analogue, >500 μM.

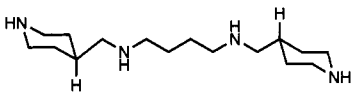
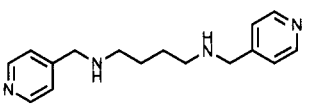
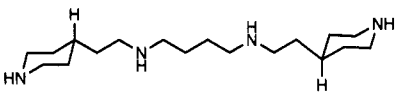
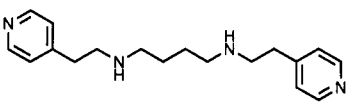
Polyamine Pools. The impact of DESPM and its cyclic analogue PIP(4,4,4) on polyamine pools was very similar, although the latter was not quite as effective as DESPM at depleting spermine (Table 4). The higher homologue PIP(5,4,5) was less effective than PIP(4,4,4) at polyamine pool depletion; nevertheless, it still significantly reduced the levels of putrescine and spermidine. Again, the fluorinated and pyridine analogues had little effect on the polyamine pools. While it was not possible to use the same analytical methods to follow the pyridine analogues, concentrations of the piperidine

Table 3. IC_{50} and K_i Values As Determined for Their Analogues in an L1210 Cell Culture Model[a]

analogue structure	compound abbreviation	96 h IC_{50}	K_i[a] (μM)
$CH_3CH_2NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2CH_3$	DESPM (3,4,3)	0.2	1.6
$CF_3CH_2NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2CF_3$	FDESPM (3,4,3)	>100	285
	PIP(4,4,4)	0.1	4.9
	PYR(4,4,4)	80	>500
	PIP(5,4,5)	0.3	18.1
	PYR(5,4,5)	>100	>500

[a] The K_i values were measured against spermidine uptake, simple substrate-competitive inhibition of radiolabeled spermidine transport in L1210 cells.

Table 4. Impact of the Analogues on the Polyamine Biosynthetic Enzymes ODC, AdoMetDC, and SSAT and Polyamine Pools in an L1210 Cell Model

analogue structure	compound abbreviation	ODC[a] % control	AdoMetDC[b] % control	SSAT[c] % control	concn[d] (μM)	% polyamine pools[e] Put	Spd	Spm	analogue level (pmol/10^6 cells)
$CH_3CH_2NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2CH_3$	DESPM (3,4,3)	3	28	460	30	0	0	22	1780 ± 20
$CF_3CH_2NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2CF_3$	FDESPM (3,4,3)	100	100	97	100	103	103	108	<20
	PIP(4,4,4)	6	39	325	2	0	5	50	2518 ± 91
	PYR(4,4,4)	90	100	87	100	87	95	99	
	PIP(5,4,5)	75	50	145	100	0	27	89	1465 ± 143
	PYR(5,4,5)	100	100	140	100	124	85	96	

[a] Ornithine decarboxylase (ODC) activity in L1210 cells treated with analogue for 4 h. Values are expressed relative to untreated controls (control = 100%). [b] *S*-Adenosylmethionine decarboxylase (AdoMetDC) activity in L1210 cells treated with analogue for 6 h. Values are expressed relative to untreated controls (control = 100%). [c] Spermine/spermidine *N*-acetyl transferase (SSAT) activity in L1210 cells treated with analogue for 48 h. Values are expressed relative to untreated controls (control = 100%). [d] L1210 cells were grown 48 h in the presence of this concentration of analogue (equivalent to the 48 h IC_{50}) to assess the effect on polyamine pools. [e] Native polyamine levels in analogue-treated L1210 cells are expressed relative to untreated controls (control = 100%). Typical control values in pmol/10^6 L1210 cells are Put = 192 ± 38; Spd = 2751 ± 163; Spm = 726 ± 34.

and fluorinated analogues in the cells could be monitored. In the case of DESPM, PIP(4,4,4), and PIP(5,4,5), the analogues achieved total amine equivalents in the cell substantially higher than the total remaining natural polyamines. Finally, the concentration of FDESPM achieved in the cells was exceptionally low relative to DESPM, PIP(4,4,4), and PIP(5,4,5).

Impact of Analogues on ODC, AdoMetDC, and SSAT. Not surprisingly, the effect of the analogues on the polyamine enzymes ODC, AdoMetDC, and SSAT followed the same trends as observed for the other biological parameters (Table 4). At 10 μM, the parent compound DESPM reduced the ODC to 3% of control level, reduced the AdoMetDC to 28% of control, and upregulated the SSAT to 460%. Even at 100 μM FDESPM had no significant impact on any of these systems. At 10 μM PIP(4,4,4) reduced the ODC to 6% of control level, reduced the AdoMetDC to 39% of control, and upregulated the SSAT to 325%. PYR(4,4,4) at 100 μM had a marginal impact on these systems with a 10% reduction in ODC activity and a 13% reduction in SSAT activity. Interestingly, even at 100 μM PIP(5,4,5), well above its 96 h IC_{50}, there is relatively little impact on ODC, a 25% reduction, with a 50% reduction in the AdoMetDC level. Also the SSAT was only increased to 145%. The corresponding PYR(5,4,5) had no impact on ODC and AdoMetDC levels but stimulated SSAT to 140% at 10 μM.

Discussion

All of the above data are consistent with the idea that charge is critical to cellular recognition of the polyamine

analogues. The original polyamine analogue antineoplastic design strategy was aimed at the synthesis of compounds which would "appear" to the cell as if they were natural polyamines. This strategy was predicated on the observation that when cells were grown in exogenous spermine, ODC and AdoMetDC were down regulated. Thus in principle polyamine analogues could be expected to also shut down these two key enzymes. Indeed this was the case. The analogues assembled were structurally similar enough to spermine to not only be incorporated via the polyamine transport apparatus but to shut down both AdoMetDC and ODC as well to as to upregulate SSAT.

Studies in these laboratories indicated that the ability of these analogues to impact on cell growth was dependent on (1) the number of nitrogens in the molecule, (2) the distance between the nitrogens, and (3) the nature of the terminal alkyl substituents. Tetraamines, e.g., DESPM, were typically more active than the corresponding triamine analogues, e.g., N^1,N^8-diethylspermidine.[1] In a family of tetraamines with the same terminal alkyl groups, those with fewer methylenes between nitrogens were often less active and less toxic in whole animals, e.g., DEHSPM is more active than DENSPM.[2] The impact of altering the terminal alkyl group on the compound's activity is most obvious when looking at the K_i values. On moving from smaller substituents, e.g., methyl to *tert*-butyl groups, the K_i rises sharply. Two alternative explanations for the polyamine transport apparatus recognition of these analogues are possible: either the *tert*-butyl substituents were so large the compound could not "fit" into the transport apparatus or the *tert*-butyl group inhibited interaction of the protonated terminal nitrogen cation with a biological counteranion in the transport apparatus. A large group such as *tert*-butyl would certainly be expected to increase the distance between the two ions, thus weakening their interaction and diminishing "polyamine recognition".

The significance of polyamine cationic charge is clear from this study. For example, in the set DESPM, PIP(4,4,4), FDESPM, and PYR(4,4,4), the first two of these tetraamines, both of which form tetracations at physiological pH, compete well with SPD for uptake and are effective at controlling cell growth. The latter two compounds, FDESPM and PYR(4,4,4), are dicationic at pH 7.4, compete poorly with spermidine for uptake, and are inactive. The same relationship holds between PIP(5,4,5) and PYR(5,4,5). PIP(5,4,5), highly protonated at physiological pH, is active, and its internally dicationic counterpart PYR(5,4,5) is not.

While PIP(3,4,3) and PYR(3,4,3) seemed a better choice initially as cyclic analogues to DESPM (3,4,3), two problems emerge. First the literature suggests[23] that 4-aminopyridines like PYR(3,4,3) would be protonated at the pyridine nitrogens at physiological pH because of resonance, forming the terminal dication. Second, a BIOSYM modeling of PIP(3,4,3) and PYR(3,4,3) (Table 1) revealed that in their lowest energy conformations the N^{α}–N^{β} (4.24; 4.21 Å) and N^{α}–$N^{\alpha'}$ (14.56; 14.40 Å) distances are not similar to those of the BIOSYM-generated DESPM, N^{α}–N^{β} (5.04 Å) and N^{α}–$N^{\alpha'}$ (16.43 Å). However PIP(4,4,4) and PYR(4,4,4) have N^{α}–N^{β} (5.31, 5.21 Å) and N^{α}–$N^{\alpha'}$ (16.84, 16.47 Å) distances much closer to the parent DESPM. While it is certainly true that the BIOSYM models say nothing about what is occurring in a cellular envelope, they nevertheless provide a comparative starting point. In the final analysis, the data are consistent with the idea that charge is critical to cellular recognition of the polyamine analogues at the level of transport.

Experimental Section

Reagents were purchased from Aldrich Chemical Co. Reactions using hydride reagents were run in distilled DMF under a nitrogen atmosphere. Fisher Optima grade solvents were routinely used, and organic extracts were dried with sodium sulfate. Silica gel 60 (70–230 mesh) obtained from EM Science (Darmstadt, Germany) or silica gel (32–63 (40 μM "flash") from Selecto, Inc. (Kennesaw, GA) was used for column chromatography. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Proton NMR spectra were run at 90, 300, or 600 MHz in $CDCl_3$ (not indicated) or D_2O with chemical shifts given in parts per million downfield from tetramethylsilane or 3-(trimethylsilyl)-propionic-*2,2,3,3-d*$_4$ acid, sodium salt, respectively. Coupling constants (J) are in hertz. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA.

Computer Modeling. The distance between nitrogens on polyamine analogues was taken from their most stable conformations. The search for low-energy conformations of polyamine analogues was done using BIOSYM program 2.3.0 running on a SiliconGraphics Indigo workstation, and the results are similar to those using the conformational search program supported by Tripos Assoc. Inc. in Sybyl 6.02.

Potentiometric Measurements. The amine tetrahydrochloride (0.300 mmol) was dissolved in carbon dioxide-free 0.1 M KCl (20.0 mL) and converted to the free base by addition of a slight excess of 1 N NaOH (1.25 mequiv). This solution was then *immediately* titrated with 1 N HCl using a Radiometer-Copenhagen DTS833 digital titration system. The pK_a's were estimated from a computer fit of the titration data using MINSQ v. 2.0 nonlinear parameter estimation software (Micromath Scientific Software, Salt Lake City, UT). The relative abundance of each charged species present was calculated by MINSQ in the simulation mode given the four pK_a values.

Cell Culture. Murine L1210 leukemia cells were maintained in logarithmic growth as a suspension culture in RPMI-1640 medium containing 10% NuSerum (Collaborative Research, Bedford, MA), 2% HEPES-MOPS buffer, and 1 mM aminoguanidine (Sigma) at 37 °C in a water-jacketed 5% CO_2 incubator.

IC_{50} Determinations. Cells were grown in 25 cm^2 tissue culture flasks in a total volume of 10 mL. Cultures were treated while in logarithmic growth [$(0.5–1.0) \times 10^5$ cells/mL] with the polyamine derivatives diluted in sterile water and filtered through a 0.2 μm filter. Following a 48 h period, cells were reseeded and incubated for an additional 48 h.

After the indicated time periods, cells were removed from flasks for counting. Cell number was determined by electronic particle counting (Model Z_F coulter counter, Coulter Electronics, Hialeah, FL) and confirmed periodically with hemocytometer measurements.

The percentage of control growth was determined as follows:

$$\% \text{ of control growth} = \left(\frac{\text{final treated cell no.} - \text{initial inoculum}}{\text{final untreated cell no.} - \text{initial inoculum}}\right) \times 100$$

The IC_{50} is defined as the concentration of compound necessary to reduce cell growth to 50% of control growth after defined intervals of exposure.

Polyamine Pool Analysis. While in logarithmic growth, cells were treated with the polyamine derivatives. At the end of the treatment period, cell suspensions were sampled, washed twice in cold medium RPMI-1640, and pelleted for extraction using 0.6 N perchloric acid.[6] Each supernatant was frozen at −20 °C until analysis by HPLC.

Uptake Determinations. The polyamine derivatives were studied for their ability to compete with [^{3}H]SPD or [^{14}C]SPD for uptake into L1210 leukemia cells *in vitro*.[6] Cell suspensions were incubated in 1 mL of RPMI-1640 containing 1, 2, 4, 6, 8, and 10 μM radiolabeled SPD alone or with the additional presence of 10, 25, and 50 μM polyamine analogue for 20 min at 37 °C. At the end of the incubation period, tubes were centrifuged at 900*g* for 5 min at 0–4 °C. The pellet was washed twice with 5 mL of cold RPMI-1640 containing 1 μM SPD, dissolved in 200 mL of 1 N NaOH at 60 °C for 1 h, and neutralized with 1 N HCl. The material was transferred to a vial for scintillation counting. Lineweaver–Burk plots indicated a simple competitive inhibition with respect to SPD.

Enzyme Assays. ODC and AdoMetDC activities were determined according to the procedures of Seely and Pegg[24] and Pegg and Pösö,[25] respectively, on the basis of quantitation of $^{14}CO_2$ released from [^{14}C]carboxyl-labeled L-ornithine or *S*-adenosyl-L-methionine. Spermidine/spermine N^1-acetyl transferase (SSAT) activity was assayed by quantitation of [^{14}C]-N^1-acetylspermidine formed by acetylation of SPD with [^{14}C]-acetyl coenzyme A according to the method of Libby *et al.*[13] Every experiment was performed *in triplicate* and included both untreated control cells in addition to positive controls where cells were treated with a polyamine drug having a known reproducible effect on the particular enzyme assayed: ODC and AdoMetDC (10 μM DEHSPM) and SSAT (10 μM DENSPM).

N^1,N^{12}-Bis(trifluoroacetyl)-N^4,N^9-dibenzylspermine (7). Trifluoroacetic anhydride (2.0 mL, 14 mmol) in CH_2Cl_2 (50 mL) was added to a solution of **6**[21] (2.63 g, 6.87 mmol) and NEt_3 (2.0 mL, 14 mmol) in CH_2Cl_2 (50 mL) at 0 °C. The reactants were stirred (0 °C to room temperature) for 21 h, followed by addition of 5% $NaHCO_3$ (50 mL). The layers were separated, and the aqueous portion was extracted further with CH_2Cl_2 (2 × 50 mL). The extracts were washed with saturated $NaHCO_3$ (50 mL) and H_2O (50 mL). Solvent removal and column chromatography on silica gel, eluting with 5% EtOH/$CHCl_3$, gave 3.11 g (79%) of **7** as an amorphous solid: NMR δ 1.40–1.85 (m, 8 H), 2.32–2.66 (m, 8 H), 3.31 (q, 4 H, J = 5), 3.53 (s, 4 H), 7.33 (s, 10 H), 8.56 (br s, 2 H). Anal. ($C_{28}H_{36}F_6N_4O_2$) C, H, N.

N^1,N^{12}-Bis(2,2,2-trifluoroethyl)-N^4,N^9-dibenzylspermine (8). Borane–methyl sulfide complex (2 M in THF, 20 mL, 40 mmol) was added to **7** (2.53 g, 4.40 mmol) in THF (20 mL). The reactants were heated at 75 °C for 1 day and then cooled to 0 °C. HCl (6 N, 20 mL) was cautiously added, and the mixture was heated at 84 °C. Ethanol was added, and solvents were evaporated. Ice water (200 mL) and KOH (13.97 g) were added to the residue, followed by extraction with Et_2O (4 × 50 mL). Organic extracts were washed with brine, concentrated, and purified by silica gel column chromatography, eluting with 10% MeOH/$CHCl_3$, to produce 1.80 g (75%) of **8** as an oil: NMR δ 1.32–1.75 (m, 8 H), 2.26–2.52 (m, 8 H), 2.65 (t, 4 H, J = 6), 3.01 (q, 4 H, J = 9), 3.46 (s, 4 H), 7.23 (s, 10 H). Anal. ($C_{28}H_{40}F_6N_4$) C, H, N.

N^1,N^{12}-Bis(2,2,2-trifluoroethyl)spermine (1). Pd–C (10%, 0.50 g) was added to a solution of **8** (1.50 g, 2.74 mmol) in 0.05 M methanolic HCl (210 mL). The mixture was hydrogenated for 2.5 h (room temperature, 1 atm). Solids were filtered and washed with 1 N HCl, and solvents were removed. The residue was taken up in 1 N HCl, which was extracted with CH_2Cl_2 (5 × 80 mL). The aqueous portion was concentrated *in vacuo*. Recrystallization from aqueous EtOH afforded 1.08 g (77%) of **1** as plates: NMR (D_2O) δ 1.67–1.90 (m, 4 H), 2.08–2.35 (m, 4 H), 3.0–3.4 (m, 12 H), 4.00 (q, 4 H, J = 9). Anal. ($C_{14}H_{32}Cl_4F_6N_4$) C, H, N.

***N*,*N*′-Bis(mesitylsulfonyl)-4-(aminomethyl)piperidine (9).** A solution of mesitylenesulfonyl chloride (19.49 g, 89.1 mmol) in CH_2Cl_2 (100 mL) was added to 4-(aminomethyl)-piperidine (5.15 g, 45.1 mmol) in 1 N NaOH (100 mL) at 0 °C. After addition was complete, the biphasic mixture was stirred for 1 day (0 °C to room temperature). The layers were separated, and the aqueous portion was extracted with $CHCl_3$ (2×). The combined organic phase was washed with 0.5 N HCl (200 mL) and H_2O (100 mL) and evaporated *in vacuo*. Recrystallization from aqueous ethanol produced 18.72 g (88%) of **9** as plates: mp 158.5–160 °C; NMR δ 0.8–2.0 (m, 5 H), 2.25 (s, 6 H), 2.46–2.93 (m + 2 s, 16 H), 3.37–3.65 (m, 2 H), 4.67 (t, 1 H, J = 6), 6.90 (s, 4 H). Anal. ($C_{24}H_{34}N_2O_4S_2$) C, H, N.

Tetrakis(mesitylsulfonyl)-*N*,*N*′-bis(4-piperidinylmethyl)-1,4-diaminobutane (10). Sodium hydride (80% in oil, 1.411 g, 47.0 mmol) was added to **9** (18.43 g, 38.5 mmol) and NaI (0.146 g, 0.97 mmol) in DMF (165 mL) at 0 °C. The suspension was stirred for 1 h 45 min at room temperature under nitrogen. 1,4-Dibromobutane (2.2 mL, 18.4 mmol) was added by syringe, and the reaction mixture was heated at 84 °C for 19 h. After cooling to 0 °C, H_2O (200 mL) was cautiously added to quench residual NaH, followed by extraction with $CHCl_3$ (300 mL, 2 × 100 mL). The combined organic phase was washed with 1% Na_2SO_3 (100 mL) and H_2O (2 × 100 mL) and evaporated under high vacuum. Recrystallization from EtOAc/$CHCl_3$ gave 13.00 g (70%) of **10** as a solid: mp 202–203.5 °C; NMR δ 0.75–1.90 (m, 14 H), 2.25 (s, 12 H), 2.40–3.18 (m + 2 s, 36 H), 3.3–3.6 (m, 4 H), 6.87 (s, 8 H). Anal. ($C_{52}H_{74}N_4O_8S_4$) C, H, N.

***N*,*N*′-Bis(4-piperidinylmethyl)-1,4-diaminobutane Tetrahydrochloride (2).** HBr in acetic acid (30%, 100 mL) was added over 10 min to a solution of **10** (5.34 g, 5.28 mmol) and phenol (18.97 g, 0.202 mol) in CH_2Cl_2 (75 mL) at 0 °C. The reaction mixture was stirred for 1 day (0 °C to room temperature) and cooled to 0 °C. Distilled H_2O (120 mL) was added, followed by extraction with CH_2Cl_2 (3 × 100 mL). The aqueous layer was evaporated under high vacuum. The residue was basified with 1 N NaOH (12 mL) and 50% (w/w) NaOH (20 mL) with ice cooling, followed by extraction with $CHCl_3$ (10 × 50 mL), while adding NaCl to salt out the aqueous layer. After $CHCl_3$ extracts were evaporated, the residue was taken up in ethanol (200 mL) and acidified with concentrated HCl (3.5 mL) and solvents were removed under vacuum. Recrystallization from 7% aqueous EtOH furnished 1.318 g (58%) of **2** as a white solid: 300 MHz NMR (D_2O) δ 1.45–1.63 (m, 4 H), 1.75–1.86 (m, 4 H), 2.01–2.22 (m, 6 H), 2.99–3.18 (m, 12 H), 3.44–3.55 (m, 4 H). Anal. ($C_{16}H_{38}Cl_4N_4$) C, H, N.

***N*,*O*-Bis(mesitylsulfonyl)-4-piperidineethanol (12).** Mesitylenesulfonyl chloride (24.78 g, 0.113 mol) in pyridine (60 mL) was added all at once to 4-piperidineethanol (5.58 g, 43.2 mmol) in pyridine (25 mL) at −16 °C; the temperature rose to −11 °C. The flask was stored in the refrigerator at 5.5 °C for 44 h under argon. The reaction mixture was poured into ice (1 kg), and after 3 h, 16.00 g (75%) of **12** as a yellow solid was filtered off: mp 93.5–94 °C; NMR δ 1.4–2.1 (m, 7 H), 2.27 (s, 6 H), 2.44–2.96 (m + s, 14 H), 3.37–3.69 (m, 2 H), 3.97 (t, 2 H, J = 5), 6.90 and 6.93 (2 s, 4 H). Anal. ($C_{25}H_{35}NO_5S_2$) C, H, N.

Tetrakis(mesitylsulfonyl)-*N*,*N*′-bis[2-(4-piperidinyl)-ethyl]-1,4-diaminobutane (13). Sodium hydride (80% in oil, 0.783 g, 26.1 mmol) was added to **11**[2] (5.15 g, 11.4 mmol) and NaI (0.376 g, 2.5 mmol) in DMF (140 mL) at 0 °C. The suspension was stirred for 23 min at room temperature, followed by introduction of **12** (15.84 g, 32.1 mmol). The reaction mixture was heated at 58–67 °C for 18 h and then poured into H_2O (300 mL), followed by extraction with $CHCl_3$ (4 × 100 mL). The combined extracts were washed with saturated $NaHCO_3$ (100 mL), 1% $NaHSO_3$ (100 mL), and H_2O (100 mL) and evaporated under high vacuum. Column chromatography on silica gel eluting with 1 to 2% CH_3OH/$CHCl_3$ furnished 10.03 g (85%) of **13** as a solid: mp 152–153.5 °C (EtOAc); NMR δ 0.8–2.0 (m, 18 H), 2.08–2.71 (m + 3 s, 40 H), 2.8–3.5 (m, 12 H), 6.87 (s, 8 H). Anal. ($C_{54}H_{78}N_4O_8S_4 \cdot H_2O$) C, H, N.

***N*,*N*′-Bis[2-(4-piperidinyl)ethyl]-1,4-diaminobutane Tetrahydrochloride (3).** HBr in acetic acid (30%, 180 mL) was added over 30 min to a solution of **13** (9.83 g, 9.45 mmol) and phenol (33.38 g, 0.355 mol) in CH_2Cl_2 (135 mL) at 0 °C. The reaction mixture was stirred for 1 day (0 °C to room temperature) and cooled to 0 °C. Distilled H_2O (200 mL) was added, followed by extraction with CH_2Cl_2 (2 × 100 mL). The aqueous portion was evaporated under high vacuum. The residue was basified with 1 N NaOH (50 mL) and 50% (w/w) NaOH (10 mL) (ice cooling), followed by extraction with $CHCl_3$ (10×), while adding NaCl to salt out the aqueous layer. The latter

organic extracts were evaporated, concentrated HCl (5 mL) in ethanol (300 mL) was added to the residue, and solvents were removed under vacuum. Recrystallization from 3% aqueous EtOH gave 2.91 g (68%) of **3** as a white solid: 300 MHz NMR (D_2O) δ 1.37–2.04 (m, 18 H), 2.94–3.18 (m, 12 H), 3.4–3.5 (m, 4 H). Anal. ($C_{18}H_{42}Cl_4N_4$) C, H, N.

***N,N'*-Bis(4-pyridylmethyl)-1,4-diaminobutane Tetrahydrochloride (4).** 4-Pyridinecarboxaldehyde (2.55 g 23.8 mmol) in diethyl ether (20 mL) was added to 1,4-diaminobutane (1.02 g, 11.6 mmol) in water (10 mL). The biphasic reaction was stirred for 3 h and separated. The ether layer was evaporated, dissolved in EtOH (15 mL), and treated with $NaBH_4$ (0.25 g, 6.6 mmol). EtOH (20 mL) was added to the aqueous portion and then $NaBH_4$ (0.88 g, 23 mmol) was introduced with brief ice cooling. Both reactions stood for 15 h at room temperature and were acidified with 1 N HCl at 0 °C and stood overnight. The reactions were basified to pH 14 with solid NaOH with ice cooling and extracted with $CHCl_3$ (4×). Solvent was removed, and silica gel column chromatography, eluting with 2% concentrated NH_4OH/CH_3OH, gave the free tetraamine, which was dissolved in EtOH (70 mL) and acidified with concentrated HCl (4 mL). After solvent removal, the residue was recrystallized with 10% aqueous EtOH, providing 2.402 g (76%) of **4**: NMR (D_2O) δ 1.8–2.0 (m, 4 H), 3.14–3.88 (m, 4 H), 4.58 (s, 4 H), 8.10 (d, 4 H, J = 7), 8.82 (d, 4 H, J = 7). Anal. ($C_{16}H_{26}Cl_4N_4$) C, H, N.

***N,N'*-Bis[2-(4-pyridyl)ethyl]-1,4-diaminobutane Tetrahydrochloride (5).** A mixture of 4-vinylpyridine (5 mL, 46 mmol), 1,4-diaminobutane (1.95 g, 22.1 mmol), and HOAc (2.5 mL, 44 mmol) in H_2O (6 mL) was heated for 1 day at 108 °C under N_2. NaOH (1 N, 100 mL) was added to the cooled reaction, and extraction with $CHCl_3$ (9 × 50 mL) was carried out. After the solvent was removed, the residue was purified by flash chromatography, eluting with 4% concentrated NH_4OH/CH_3OH. Free amine was dissolved in EtOH, and concentrated HCl (3 mL) was added. After solvent removal, recrystallization from 5% aqueous EtOH afforded 1.45 g (15%) of **5** as a solid: 600 MHz NMR (D_2O) δ 1.79–1.84 (m, 4 H), 3.14–3.19 (m, 4 H), 3.36 (t, 4 H, J = 9), 3.49 (t, 4 H, J = 9), 7.96 (d, 4 H, J = 7), 8.71–8.74 (m, 4 H). Anal. ($C_{18}H_{30}Cl_4N_4$) C, H, N.

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