Design, Synthesis, and Testing of Polyamine Vectored Iron Chelators

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Abstract: Iron chelators have been shown to control the growth of cancer cells in culture by sequestering exogenous iron in the media. Thus, the ligands prevent cellular access to the metal. However, because transferrin provides iron to tumor cells in animals, chelators have not been effective antitumor agents. Polyamine chelator conjugates in which the polyamine vectored ligands into cells were far more active than the free chelators themselves. However, the free ligands were not released from the vector once in the cell. The current study focuses on the synthesis and preliminary evaluation of a polyamine chelator conjugate capable of releasing the free ligand intracellularly via a nonspecific esterase.

Key words: iron, chelator, conjugate, polyamine vector, intracellular esterase

It is well established that iron chelators can significantly reduce the growth of tumor cells in vitro.[1] It has been demonstrated that this reduction could be accounted for by the inhibition of ribonucleotide reductase, as evidenced by thymidine uptake studies and arrest of cell division at the G1-S border.[2] One major shortcoming with iron chelators as antineoplastics is that most of these ligands [e.g., desferrioxamine (DFO)] do not cross the cell membrane particularly well.[3] Thus, while chelators are effective as growth inhibitors of tumor cells in culture, where the iron sources are limited, this has not been the case in tumor xenografts in whole animals.[4] In the former instance, extracellular iron in the culture medium is ligated, preventing uptake of the metal and transformation of apo-ribonucleotide reductase to its active form. In the latter instance, there is a continual and ample supply of protein-bound iron, for example, as transferrin.

For intracellular chelation to be a therapeutically significant antineoplastic strategy, higher levels of chelator must be transported into the cell. We elected to exploit polyamines as vectors to achieve this goal. However, in each instance, the polyamine vector was linked to the chelator such that the free ligand could not be liberated intracellularly. The sustained increases in polyamine biosynthesis in preneoplastic and neoplastic tissues led to a great deal of attention focused on the polyamine biosynthetic network as a target in antineoplastics therapy.[5] In the course of developing N-alkylated analogues of the natural polyamines as anticancer drugs,[6,7] the structural boundary conditions set by the polyamine transporter on these substrates were defined. All of the findings[8,9] are consistent with the idea that charge is critical to transporter recognition of the polyamine analogues. We have in fact been able to successfully exploit the polyamine transport apparatus in delivering iron chelators intracellularly.

Initial experiments focused on the delivery of a simple bidentate ligand, 1,2-dimethyl-3-hydroxypyridin-4-one (L1, **2**, Figure 1, Table 1) using polyamines as vectors. The chelator itself does not achieve a high concentration (~1 μM) in cultured cells[10] (L1, **2**). L1 was linked to spermidine via a propyl tether to generate 1-(12-amino-4,9-diazadodecyl)-2-methyl-3-hydroxy-4(1*H*)-pyridinone (**3**, Figure 1). Job's plots revealed the expected 3:1 ligand/Fe(III) complex. In conjugate **3**, the hydroxypyridone, the chelating cargo fragment covalently bonded to the polyamine, is neutral at physiological pH. All parameters measured in murine leukemia L1210 cells treated with conjugate **3**, including (1) the effect on cell proliferation (IC_{50} values), (2) the ability to compete with radiolabeled spermidine for the polyamine transport apparatus (K_i), (3) impact on polyamine pools, and (4) effects on the polyamine enzymes L-ornithine decarboxylase (ODC), *S*-adenosyl-L-methionine decarboxylase (AdoMetDC), and spermidine/spermine/N^1-acetyltransferase (SSAT), suggested that this L1 analogue both competed well for the polyamine transport apparatus and indeed gained intracellular access (390 μM) in contrast to L1 (**2**) itself. This finding encouraged us to explore additional iron chelator polyamine adducts. The norspermidine adducts, (*S*)-4,5-dihydro-2-[2-hydroxy-4-(12-amino-5,9-diazadodecyloxy)phenyl]-4-methyl-4-thiazolecarboxylic acid (**4**) and its ester **5** (Table 1) were thus assembled and evaluated.[9]

Again, the IC_{50} of **4** and **5** were measured in L1210 cells and compared with the parent drug, (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-4-methyl-4-thiazolecarboxylic acid (**6**, Figure 1). The parent acid **6** presented with IC_{50} values of 20 μM at 96 hours, the polyamine adduct ethyl ester **5** had a 96-hour IC_{50} of 1.5 μM, and the corresponding polyamine free acid conjugate **4** had a 40 μM IC_{50} at this time point (Table 1). The parent acid **6** did not effectively accumulate in L1210 cells (<50 μM). The acid polyamine adduct **4** achieved levels of <95 μM. However, the ester adduct **5** reached intracellular levels of ~1000 μM. Furthermore, concentrations of ~3000 μM of the free acid adduct **4** were seen in these same cells, obviously deriving from nonspecific serum esterase hydrolysis of the parent ester **5**.[9] Interestingly, the polyamine ester adduct **5** competed well for the polyamine transporter, K_i 5.7 μM, while the acid conjugate **4** did not, K_i 73 μM. In keeping with the idea, negatively charged cargo fragments fixed to

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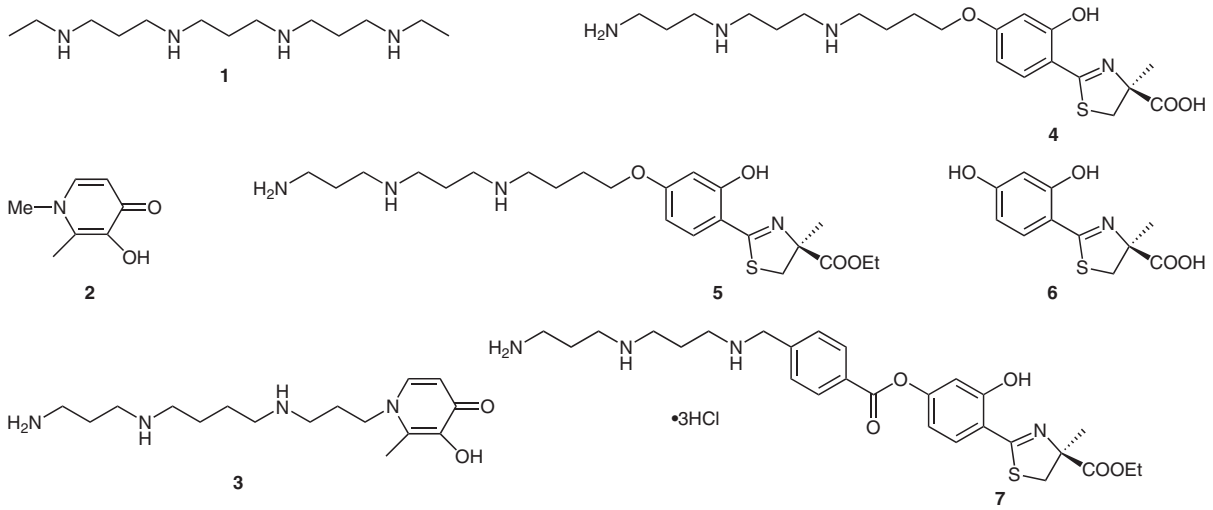


Figure 1 Polyamine analogue DENSPM (**1**), chelators **2** and **6**, and polyamine analogue chelator conjugates **3**, **4**, **5**, **7**

Table 1 L1210 Cell Growth Inhibition and Transport for Selected Polyamines, Iron Chelators, and Polyamine Conjugate Iron Chelators

Compound structure/abbreviation	IC_{50} (μM)		K_i (μM)
	48 h	96 h	
DENSPM (**1**)	20	2	17
L1 (**2**)	46	55	>500
L1-SPM-conjugate (**3**)	0.2	0.2	3.7
NSPD-(*S*)-4′-(HO)-DADFT (**4**)	40	40	73
NSPD-(*S*)-4′-(HO)-DADFT-EE (**5**)	1.5	1.5	5.7
(*S*)-4′-(HO)-DADFT (**6**)	19	20	>500
NSPDBn-(*S*)-4′-(HO)-DADFT-EE (**7**)	1.8	2.1	4.0

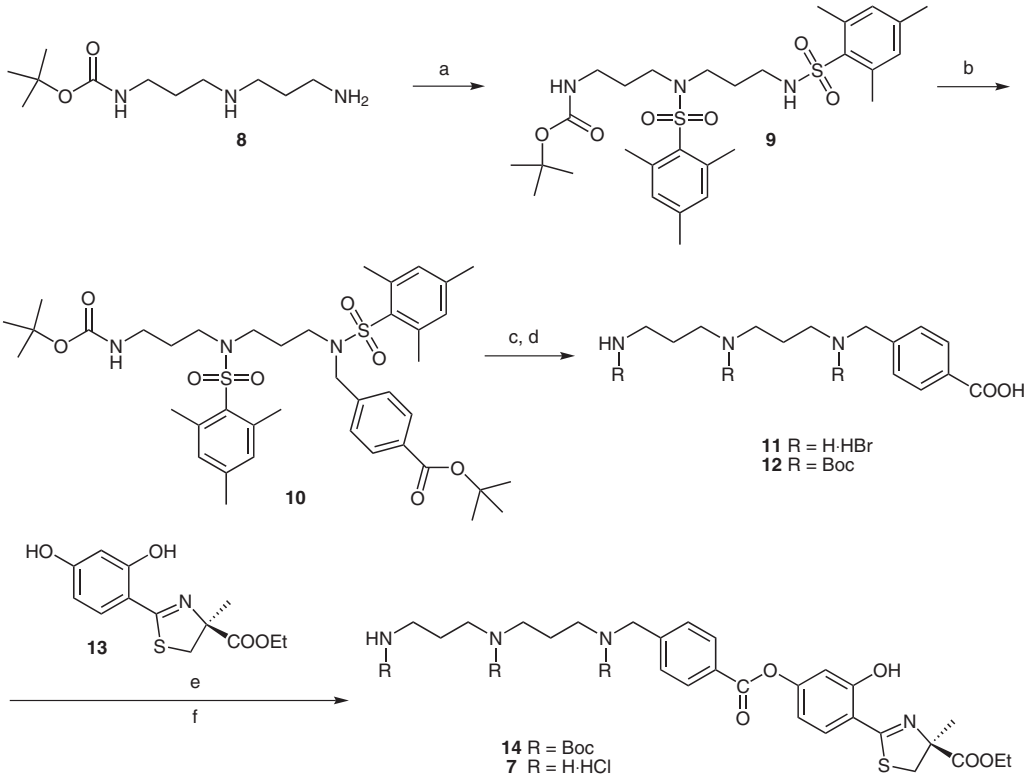
polyamine vectors, for example, the carboxylate of **4** at physiologic pH, are not well transported. It was clear that cargo molecules needed to be neutral or positively charged. In the same set of experiments, we were also able to demonstrate that the norspermidine backbones of the chelator conjugates were metabolized. Specifically, the 3-aminopropyl segments were successively removed, resulting in a diamine and a monoamine. Also the primary amines of the parent conjugates and the diamine metabolite were oxidized, resulting in dicarboxylic acids. However we were unable to demonstrate any transformation of the final 4-aminobutyl metabolite.[9] These findings forced the question of whether a polyamine chelator conjugate could be assembled such that the chelating unit could be released from the cargo intracellularly. The current study focuses on the synthesis of such a conjugate with a brief description of the ligand's biological properties. The vectored molecule is designed for nonspecific esterase release of the iron ligand from the cargo.

Labile Polyamine Vectors

The above findings support the idea that polyamines can be utilized to vector various molecules into cells. However, the conjugates studied do not allow for the release of the cargo molecule. They remain attached to the polyamine vector and/or one of the vector's metabolites.[9] This encouraged the design of a system in which the polyamine cargo linkage would allow for intracellular release of an active fragment. The simplest concept revolves around the idea that intracellular esterase can be exploited to cleave cargo molecules fixed to polyamine vectors as esters. The first element of the program was to explore the accessibility and stability of such molecules. It is not the purpose of the study to develop a 'more active' chelator conjugate, but rather, to assess whether or not a labile polyamine conjugate ester can be assembled that utilizes the polyamine transporter. The ultimate goal is to articulate a polyamine iron chelator conjugate that will release the free chelator once in the cell.

One of the concerns was, of course, that a polyamine carrier with a labile ester linkage could, by its very nature, cyclize to amides. In order to overcome this, the molecule was designed to render cyclization difficult because of steric hindrance. Thus the target molecule, ethyl (*S*)-4,5-dihydro-2-{2-hydroxy-4-[4-(9-amino-2,6-diazanonyl)benzoyloxy]}-4-methyl-4-thiazolecarboxylate (**7**, Table 1, Scheme 1), consists of iron chelator **6**, masked as its ethyl ester, and norspermidine covalently linked by a spacer. Moreover, the cargo molecule is neutral.

The para-disubstituted aromatic ring fixed to the polyamine vector of 'the presumed esterase labile' polyamine chelator conjugate sets significant restrictions on potential intramolecular cyclization. The benzylic nitrogen cannot close onto the benzoate ester for steric reasons. Similar constraints exist for cyclocondensation of the internal norspermidine nitrogen with the benzoate ester. While it is true in principle that the primary amine of **7** could form a 15-membered ring by displacing a molecule of **13** (Scheme 1), the ethyl ester of chelator **6** (Figure 1), the ensuing decrease in entropy would be unfavorable. Similar arguments can be made against nitrogen closures onto the thiazoline ester.



Scheme 1 *Reagents and conditions*: (a) mesitylenesulfonyl chloride, aq 1 N NaOH, CH_2Cl_2, 67%; (b) 4-(*tert*-butoxycarbonyl)benzyl bromide, 60% NaH, DMF, 70 °C, 70%; (c) 30% HBr in AcOH, PhOH, CH_2Cl_2, 84%; (d) Boc_2O, aq THF, Et_3N, 85%; (e) EDAC, DMAP, CH_2Cl_2, 55%; (f) TFA, CH_2Cl_2, ion exchange, aq 1 N HCl, quant.

N^1-(*tert*-Butoxycarbonyl)norspermidine (**8**)[11] was reacted with mesitylenesulfonyl chloride (2 equiv) under biphasic conditions to produce N^1,N^4-bis(mesitylenesulfonyl)-N^7-(*tert*-butoxycarbonyl)norspermidine (**9**) in 67% yield (Scheme 1). The difference in acidic strength[12] between the sulfonamide and carbamate hydrogens on **9** allowed monoalkylation of this norspermidine system. Specific deprotonation of **9** with NaH (1.1 equiv) and treatment with 4-(*tert*-butyloxycarbonyl)benzyl bromide[13] in DMF at 70 °C provided *tert*-butyl ester **10** in 70% yield. The four protecting groups of **10** were removed with 30% HBr in acetic acid and phenol in CH_2Cl_2, giving N^1-(4-carboxybenzyl)norspermidine trihydrobromide (**11**) in 84% yield. The amino groups were masked as their *tert*-butyl carbamates utilizing di-*tert*-butyl dicarbonate and Et_3N in aqueous THF, affording N^1,N^4,N^7-tris(*tert*-butoxycarbonyl)-N^1-(4-carboxybenzyl)norspermidine (**12**) in 85% yield. Ethyl (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-4-methyl-4-thiazolecarboxylate (**13**),[9] in which the carboxylate group of (*S*)-4′-(HO)-DADFT (**6**) is protected, has been alkylated at the less hindered 4′-hydroxy in the presence of the 2′-hydroxy, an iron chelating site, providing numerous DFT analogues.[14] A corresponding selective acylation of **13** with substituted benzoic acid **12** using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) with 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2 furnished diester **14** in 55% yield. The Boc protecting groups of **14** were removed with trifluoroacetic acid (TFA) in CH_2Cl_2, followed by ion exchange to provide NSPDBn-(*S*)-4′-(HO)-DADFT-EE (**7**) as its trihydrochloride salt in quantitative yield (Scheme 1).

Preliminary Bioassays

Two pieces of data are consistent with the idea that the polyamine vector is indeed working as planned; the 48-hour and 96-hour IC_{50} values are 1.8 and 2.1 μM, respectively, for conjugate **7**. Recall that the parent ligand **6** had 19 and 20 μM IC_{50}s at 48 hours and 96 hours. There is a notable and consistent observation regarding IC_{50} values of polyamine analogues, for example, DENSPM (**1**), versus chelators **2** and **6**, and polyamine analogue chelator conjugates **3**, **4**, **5**, **7** (Table 1). Comparing the 48- versus 96-hour IC_{50} values for the polyamine analogues, for example, **1**, the 96-hour values are always much lower. We have seen this consistently over the years with nearly all polyamine analogues.[7f] However, L1210 cells exposed to chelators or polyamine chelator conjugates always present with very similar 48- and 96-hour IC_{50} values.[9] This is consistent with the idea that the ethyl ester chelator conjugate **7** is gaining intracellular access and the chelator is being released. Finally, further underscoring this idea, while the parent ligand **6** did not compete well for the polyamine transporter K_i >500 μM, the conjugate **7** did, K_i = 4.0 μM.

Studies now underway include: 1) assessment of the intracellular concentration of free ligand **6** as a function of external adduct **7** concentration, 2) evaluation the intracellular metabolites, 3) an assessment of the impact of the conjugate **7** on polyamine pools, and 4) a study the

impact of adduct **7** on key polyamine biosynthetic enzymes. This protracted biological screen is the subject of another paper.

Reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Fisher Optima grade solvents were routinely used, and DMF was distilled. Reactions were run under a N_2 atmosphere, and organic extracts were dried with Na_2SO_4. Silica gel 40–63 from SiliCycle, Inc. (Quebec City, Quebec, Canada) was used for column chromatography. Glassware that was presoaked in aq 3 N HCl for 15 min, washed with distilled H_2O and distilled EtOH, and oven-dried was used during the isolation of **7**. Sephadex LH-20 was obtained from Amersham Biosciences (Piscataway, NJ, USA) and AG1-X8 (hydroxide form) anion exchange resin from Bio-Rad Laboratoties, Inc. (Hercules, CA, USA) was used for ion exchange chromatography. Optical rotations were run at 589 nm (sodium D line) and 20 °C on a Perkin-Elmer 341 polarimeter, with *c* being concentration in grams of compound per 100 mL of solution. NMR spectra were obtained at 400 MHz (^{1}H) or 100 MHz (^{13}C) on a Varian Mercury 400BB. Chemical shifts (δ) for ^{1}H spectra are given in parts per million downfield from TMS for $CDCl_3$ (not indicated) or sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 for D_2O. Chemical shifts (δ) for ^{13}C spectra are given in parts per million referenced to the residual solvent resonance (δ = 77.16) in $CDCl_3$ (not indicated) or to 1,4-dioxane (δ = 67.19) in D_2O. The base peaks are reported for the ESI-FTICR mass spectra.

N^1,N^4-Bis(mesitylenesulfonyl)-N^7-(*tert*-butoxycarbonyl)norspermidine (9)

A solution of mesitylenesulfonyl chloride (2.19 g, 10.0 mmol) in CH_2Cl_2 (30 mL) was added dropwise to a mixture of **8**[11] (1.16 g, 5.0 mmol), aq 1 N NaOH (43 mL), and CH_2Cl_2 (20 mL) with ice bath cooling. The mixture was stirred at r.t. for 16 h. The layers were separated, and the aqueous phase was further extracted with CH_2Cl_2 (2 × 50 mL). The combined organic extracts were washed with aq 1 N NaOH (50 mL), H_2O (50 mL), and brine (50 mL). After solvent removal in vacuo, flash chromatography, eluting with 33% EtOAc in $CHCl_3$ furnished 2.0 g (67%) of **9** as a colorless oil.

^{1}H NMR: δ = 1.41 [s, 9 H, $(CH_3)_3C$], 1.54–1.60 (m, 2 H, $CH_2CH_2CH_2$), 1.64–1.71 (m, 2 H, $CH_2CH_2CH_2$), 2.30 (s, 6 H, 2 CH_3Ar), 2.56 (s, 6 H, 2 CH_3Ar), 2.61 (s, 6 H, 2 CH_3Ar), 2.88 (q, *J* = 6.4 Hz, 2 H, CH_2NH), 2.94 (q, *J* = 6.4 Hz, 2 H, CH_2N), 3.10 (t, *J* = 6.8 Hz, 2 H, CH_2N), 3.28 (q, *J* = 6.8 Hz, 2 H, CH_2NH), 4.33 (br s, 1 H), 4.92 (t, *J* = 6.8 Hz, 1 H), 6.95 (s, 4 H, Ar).

^{13}C NMR: δ = 21.01, 21.05, 22.96, 22.98, 23.04, 27.57, 27.75, 28.38, 28.74, 37.56, 39.48, 42.97, 43.08, 79.30, 80.52, 125.39, 128.31, 129.12, 132.06, 132.23, 132.26, 133.10, 134.00, 139.00, 140.07, 142.16, 142.90, 155.97.

HRMS: *m*/*z* calcd for $C_{29}H_{46}N_3O_6S_2$: 596.2783 $(M + H)^+$; found: 596.2744.

N^1,N^4-Bis(mesitylenesulfonyl)-N^7-(*tert*-butoxycarbonyl)-N^1-[4-(*tert*-butoxycarbonyl)benzyl]norspermidine (10)

NaH (60%, 0.44 g, 11.0 mmol) was added in portions to a solution of **9** (5.9 g, 9.9 mmol) in DMF (100 mL) with ice bath cooling, and the mixture was stirred at 0 °C for 20 min. A solution of 4-(*tert*-butyloxycarbonyl)benzyl bromide[13] (2.82 g, 10.4 mmol) in DMF (50 mL) was added to the reaction mixture dropwise over 30 min, and the mixture was stirred for 12 h at 70 °C. After solvent removal by rotary evaporation, H_2O (100 mL) was added to the residue and extracted with $CHCl_3$ (4 × 75 mL). The organic phase was concentrated in vacuo, and column chromatography, eluting with 3:3:1 hexanes–$CHCl_3$–EtOAc, gave 5.44 g (70%) of **10** as a white solid; mp 65–66 °C.

^{1}H NMR: δ = 1.42 [s, 9 H, $(CH_3)_3C$], 1.51–1.56 (m, 2 H, $CH_2CH_2CH_2$), 1.59 [s, 9 H, $(CH_3)_3C$], 1.61–1.65 (m, 2 H, $CH_2CH_2CH_2$), 2.29 (s, 3 H, CH_3Ar), 2.32 (s, 3 H, CH_3Ar), 2.51 (s, 6 H, 2 CH_3Ar), 2.59 (s, 6 H, 2 CH_3Ar), 2.87 (t, *J* = 7.2 Hz, 2 H, CH_2NH), 2.91–2.99 (m, 4 H, 2 CH_2N), 3.07 (t, 6.8 Hz, 2 H, CH_2N), 4.26 (s, 2 H, NCH_2Ar), 4.48 (br s, 1 H, NHCO), 6.91 (s, 2 H, Ar), 6.97 (s, 2 H, Ar), 7.09 (d, *J* = 8.4 Hz, 2 H, Ar), 7.88 (d, *J* = 8.4 Hz, 2 H, Ar).

^{13}C NMR: δ = 21.04, 21.07, 22.88, 22.99, 24.68, 27.48, 28.27, 28.50, 37.43, 42.89, 43.00, 43.04, 49.38, 79.79, 81.31, 128.42, 129.85, 131.75, 132.13, 132.23, 132.93, 133.12, 140.10, 140.24, 140.32, 142.69, 142.93, 155.97, 165.39.

HRMS: *m*/*z* calcd for $C_{41}H_{60}N_3O_8S_2$: 786.3777 $(M + H)^+$; found: 786.3795.

N^1,N^4,N^7-Tris(*tert*-butoxycarbonyl)-N^1-(4-carboxybenzyl)norspermidine (12)

*Formation of **11***: HBr (30% in HOAc, 100 mL) was added dropwise to a solution of **10** (5.0 g, 6.4 mmol) and phenol (24.1 g, 0.256 mol) in CH_2Cl_2 (200 mL) with ice bath cooling. The reaction mixture was stirred for 96 h at r.t. and was quenched with dropwise addition of H_2O (100 mL) under ice bath cooling. The layers were separated and the aqueous layer was further extracted with CH_2Cl_2 (3 × 100 mL). The aqueous portion was evaporated under high vacuum to give 2.70 g (84%) of **11** as a light colored solid; mp 280–282 °C.

^{1}H NMR (D_2O): δ = 2.05–2.18 (s, 4 H, 2 $CH_2CH_2CH_2$), 3.11 (t, *J* = 8.0 Hz, 2 H, CH_2ND), 3.16–3.25 (m, 6 H, 2 CH_2N + CH_2ND), 4.35 (s, 2 H, $NDCH_2Ar$), 7.59 (d, *J* = 7.6 Hz, 2 H, Ar), 8.06 (m, *J* = 7.6 Hz, 2 H, Ar).

HRMS: *m*/*z* calcd for $C_{14}H_{23}N_3O_2$: 266.1863 $(M + H)^+$ (free amine); found: 266.1876.

*Conversion of **11** to the Boc Derivative **12***: Et_3N (1.1 mL, 7.9 mmol) and di-*tert*-butyl dicarbonate (0.78 g, 3.6 mmol) in THF (60 mL) were added successively to **11** (0.54 g, 1.06 mmol) in 5% aq THF (15 mL) with ice bath cooling, and the reaction mixture was stirred for 16 h at r.t. Following solvent removal by rotary evaporation, the residue was dissolved in EtOAc (75 mL) and washed with aq 0.25 M citric acid (50 mL), H_2O (50 mL) ,and brine (50 mL). After solvent removal in vacuo, flash chromatography eluting with 5% MeOH–$CHCl_3$ gave 0.51 g (85%) of **12** as a colorless oil.

^{1}H NMR: δ = 1.43 [s, 27 H, 3 $(CH_3)_3C$], 1.58–1.78 (m, 4 H, $CH_2CH_2CH_2$), 3.08–3.20 (m, 8 H, CH_2NH + 3 CH_2N), 4.47 (s, 2 H, NCH_2Ar), 7.29 (br s, 2 H, Ar), 8.04 (d, *J* = 7.2 Hz, 2 H, Ar).

^{13}C NMR: δ = 27.55, 28.41, 37.42, 43.82, 44.45, 44.91, 49.99, 50.58, 79.06, 79.85, 80.26, 125.49, 127.03, 128.42, 129.22, 130.57, 144.50, 155.75, 156.18, 166.61.

HRMS: *m*/*z* calcd for $C_{29}H_{48}N_3O_8$: 566.3436 $(M + H)^+$; found: 566.3444.

Ethyl (*S*)-4,5-Dihydro-2-(2-hydroxy-4-{4-[(9-*tert*-butoxycarbonylamino)-2,6-bis(*tert*-butoxycarbonyl)-2,6-diazanonyl]benzoyloxy})-4-methyl-4-thiazolecarboxylate (14)

Compounds **12** (0.27 g, 0.48 mmol), **13** (0.141 g, 0.50 mmol), and DMAP (0.0122 g, 0.11 mmol) were dissolved in anhyd CH_2Cl_2 (50 mL). EDAC (0.10 g, 0.52 mmol) was added and the mixture was stirred at r.t. for 20 h. After solvent was removed by rotary evaporation, the residue was combined with aq 0.25 M citric acid–brine (1:1, 50 mL) and extracted with EtOAc (3 × 50 mL). The organic extracts were washed with H_2O (50 mL) and brine (50 mL). Solvent removal and column chromatography on silica gel eluting with 10% EtOAc in CH_2Cl_2 gave 220 mg (55%) of **14** as a viscous pale oil; $[\alpha]_D^{20}$ +15.2 (*c* 0.25, $CHCl_3$).

^{1}H NMR: δ = 1.31 (t, *J* = 7.2 Hz, 3 H, CH_3CH_2), 1.44 [s, 27 H, 3 $(CH_3)_3C$], 1.61–1.82 (s + m, 7 H, 4-CH_3 + 2 $CH_2CH_2CH_2$), 3.05–

3.21 (m, 8 H, CH_2NH + 3 CH_2N), 3.24 (d, J = 11.2 Hz, 1 H, H-5), 3.89 (d, J = 11.2 Hz, 1 H, H-5), 4.26 (dq, J = 4.0, 7.2 Hz, 2 H, CH_2CH$_3$), 4.51 (s, 2 H, NCH_2Ar), 6.77 (dd, J = 2.4, 8.4 Hz, 1 H, Ar), 6.88 (d, J = 2.0 Hz, 1 H, Ar), 7.36 (br s, 2 H, Ar), 7.45 (d, J = 8.8 Hz, 1 H, Ar), 8.14 (d, J = 8.0 Hz, 2 H, Ar), 12.72 (s, 1 H, ArO*H*).

^{13}C NMR: δ = 14.29, 21.24, 24.61, 28.63, 37.62, 40.12, 44.01, 44.50, 45.19, 50.79, 62.22, 79.09, 79.91, 80.38, 83.58, 110.63, 112.96, 114.34, 127.22, 127.85, 128.34, 130.76, 131.67, 145.17, 154.88, 155.61, 156.05, 160.79, 164.49, 171.21, 172.72.

HRMS: *m*/*z* calcd for $C_{42}H_{61}N_4O_{11}S$: 829.4052 (M + H)$^+$; found: 829.4037.

Ethyl (*S*)-4,5-Dihydro-2-{2-hydroxy-4-[4-(9-amino-2,6-diazanonyl)benzoyloxy]}-4-methyl-4-thiazolecarboxylate Trihydrochloride (7)

Freshly distilled TFA (3 mL) in CH_2Cl_2 (5 mL) was added dropwise to **14** (0.157 g, 0.189 mmol) in CH_2Cl_2 (10 mL) with ice bath cooling. The mixture was stirred at 0 °C for 1 h and at r.t. for 1 h, and volatiles were removed by rotary evaporation. The residue was passed through a short Sephadex LH-20 column, eluting with 2% aq EtOH. The iron active band was lyophilized and passed through an ion exchange column (AG1-X8, hydroxide form) eluting with aq 1 N HCl to yield 125 mg (quant) of **7** as a white solid; mp 267–269 °C; $[\alpha]_D^{20}$ +57.5 (*c* 0.12, H_2O).

^{1}H NMR (D_2O): δ = 1.33 (t, J = 6.8 Hz, 3 H, CH_3CH$_2$), 1.74 (m, 3 H, 4 CH_3), 2.08–2.22 (m, 4 H, 2 CH$_2$CH_2CH$_2$), 3.12–3.27 (m, 8 H, 4 CH_2N), 3.49 (d, J = 11.6 Hz, 1 H, H-5), 3.92 (d, J = 12 Hz, 1 H, H-5), 4.32 (q, J = 6.8 Hz, 2 H, CH_2CH$_3$), 4.40 (s, 2 H, NCH_2Ar), 6.91–6.93 (d + s, J = 9.2 Hz, 2 H, Ar), 7.66 (d, J = 8.4 Hz, 2 H, Ar), 7.70 (d, J = 8.4 Hz, 1 H, Ar), 8.28 (d, J = 7.6 Hz, 2 H, Ar).

^{13}C NMR (D_2O): δ = 13.90, 23.33, 24.03, 24.38, 37.13, 40.13, 44.96, 45.25, 45.36, 51.35, 63.74, 83.35, 110.71, 113.85, 114.81, 130.14, 130.79, 131.39, 132.57, 137.23, 154.82, 159.90, 166.43, 172.85, 175.12.

HRMS: *m*/*z* calcd for $C_{27}H_{37}N_4O_5S$: 529.2479 (M + H)$^+$ (free amine); found: 529.2489.

Preparation of Cell Culture

Murine L1210 leukemia cells were maintained in logarithmic growth as a suspension culture in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco), 2% HEPES-MOPS buffer, 1 mM L-glutamine (Gibco), and 1 mM aminoguanidine at 37 °C in a water-jacketed 5% CO_2 incubator.

IC_{50} Determination

Cells were grown in 25 cm^2 tissue culture flasks in a total volume of 10 mL. Culture were treated during logarithmic growth (0.5–1.0 × 10^5 cells/mL) with the compounds of interest, reseeded, and incubated as described previously.[7g] Cell counting and calculation of percent of control growth were also carried out as given in an earlier publication.[7g] 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.

Uptake Determination; General Procedure

The molecules of interest were studied for their ability to compete with [^{3}H]SPD for uptake into L1210 leukemia cell suspension in vitro as given in details in previous publications.[6e,7d,g] Briefly, cell suspension were incubated in 1 mL of culture medium containing radiolabeled SPD alone or radiolabeled SPD in the presence of graduated concentration of a molecule. At the end of the incubation period the tubes were centrifuged; the pellets was washed, digested, and neutralized prior to scintillation counting. Lineweaver–Burk plots indicated simple competitive inhibition with respect to SPD.

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