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Synthesis and Biological Characterization of Novel Charge-Deficient Spermine Analogues

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Biogenic polyamines, spermidine and spermine, are positively charged at physiological pH. They are present in all cells and essential for their growth and viability. Here we synthesized three novel derivatives of the isosteric charge-deficient spermine analogue 1,12-diamino-3,6,9-triazadodecane (SpmTrien, **5a**) that are N^1-Ac-SpmTrien (**5c**), N^{12}-Ac-SpmTrien (**5b**), and N^1,N^{12}-diethyl-1,12-diamino-3,6,9-triazadodecane (N^1,N^{12}-Et_2-SpmTrien, **5d**). **5a** and **5d** readily accumulated in DU145 cells at the same concentration range as natural polyamines and moderately competed for the uptake with putrescine (**1**) but not with spermine (**4a**) or spermidine (**2**). **5a** efficiently down-regulated ornithine decarboxylase and decreased polyamine levels, while **5d** proved to be inefficient, compared with N^1,N^{11}-diethylnorspermine (**6**). None of the tested analogues were substrates for human recombinant spermine oxidase, but those having free aminoterminus, including 1,8-diamino-3,6-diazaoctane (Trien, **3a**), were acetylated by mouse recombinant spermidine/spermine N^1-acetyltransferase. **5a** was acetylated to **5c** and **5b**, and the latter was further metabolized by acetylpolyamine oxidase to **3a**, a drug used to treat Wilson's disease. Thus, **5a** is a bioactive precursor of **3a** with enhanced bioavailability.

Introduction

The polyamines spermidine (Spd,[a] **2**) and spermine (Spm, **4a**), and their precursor putrescine (Put, **1**), are organic cations existing at millimolar concentrations in eukaryotic cells.[1] At physiological pH their amino groups are protonated,[2] enabling them to interact with negatively charged cellular components such as DNA, RNA, and anionic phospholipids. Polyamines participate in the regulation of important cellular functions, such as proliferation, differentiation, and functioning of *N*-methyl-D-aspartate receptor and inward rectifier potassium channel.[1] Disturbances in the polyamine homeostasis have been linked to various pathological conditions, such as cancer, acute pancreatitis, diabetes, and decreased immune response.

The main regulatory enzymes of polyamine biosynthesis are ornithine decarboxylase (ODC) and *S*-adenosyl-L-methionine decarboxylase (AdoMetDC), while spermidine/spermine N^1-acetyltransferase (SSAT) is the key enzyme of acetylpolyamine oxidase (APAO)-mediated catabolism/interconversion.[1] **4a** can also be catabolized to **2** by spermine oxidase (SMO) without prior acetylation step. The expression of the rate-limiting enzymes of polyamine metabolism is tightly regulated by multiple steps, including complex transcriptional, posttranscriptional, translational, and posttranslational mechanisms. Spd and Spm synthases are constitutively expressed and regulated by the abundance of their substrates, especially by the level of decarboxylated *S*-adenosylmethionine.

Different polyamine structural analogues have been designed for research purposes and as potential therapeutic compounds.[3] Among the most studied potential polyamine-based cancer chemotherapeutics are the terminally bis-N-alkylated analogues, such as N^1,N^{11}-diethylnorspermine (DENSpm, **6**). Many of them superinduce SSAT, induce polyamine efflux, down-regulate biosynthetic enzymes, and compete for polyamine uptake, resulting in dramatic polyamine depletion and cessation of cell growth or apoptosis. In addition, some of these analogues seem to have cytostatic/cytotoxic effects that are not directly related to polyamine depletion.[4] By contrast, C-methylated polyamine analogues, such as α-methylspermidine, generally support cell growth and mimic the natural polyamines in many of their cellular functions.[5−7] Apart from these analogue types, charge-deficient analogues have been synthesized in order to elucidate the importance of proper charge distribution. Among these compounds are difluorospermidines[8] and aminooxy[9] and oxa derivatives[10] of **2** and **4a**.

Triethylenetetraamine (1,8-diamino-3,6-diazaoctane, Trien, **3a**) is a charge-deficient analogue of **2**, having two positive charges at physiological pH (Table 1). Unlike **2**, **3a** is a selective and effective Cu^{2+} chelator (pK_{uns} = 20.4 at pH 14 and pK_{uns} = 14.2 at pH 7),[11] and it is used as an alternative treatment of Wilson's disease (hepatolenticular degeneration,

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[a]Abbreviations: AdoMetDC, *S*-adenosyl-L-methionine decarboxylase; APAO, acetylpolyamine oxidase; N^1,N^{12}-Et_2-SpmTrien, N^1,N^{12}-diethyl-1,12-diamino-3,6,9-triazadodecane; DFMO, α-difluoromethylornithine (2,5-diamino-2-(difluoromethyl)pentanoic acid); DENSpm, N^1,N^{11}-diethylnorspermine (N^1,N^{11}-Et_2-norSpm); N^1-Ac-Spd, N^1-acetylspermidine; N^1-Ac-Spm, N^1-acetylspermine; NMD, nonsense-mediated mRNA decay; ODC, ornithine decarboxylase; Put, putrescine (1,4-diaminobutane); Spd, spermidine (1,8-diamino-4-azaoctane); Spm, spermine (1,12-diamino-4,9-diazadodecane); SMO, spermine oxidase; SpmTrien, 1,12-diamino-3,6,9-triazadodecane; SSAT, spermidine/spermine N^1-acetyltransferase; Trien, triethylenetetraamine (1,8-diamino-3,6-diazaoctane).

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Table 1. pK_a Values of **2**, **4a**, and Their Charge-Deficient Isosteric Analogues **3a** and **5a**[a]

	pK_a				
	1	**2**	**3**	**4**	**5**
2	8.24	9.81	10.89		
3a	3.27	6.56	9.07	9.74	
4a	7.96	8.85	10.02	10.80	
5a	3.3	6.3	8.5	9.5	10.3

[a] Table adapted from Weisell et al. (2010).[17]

a genetic disorder in which copper accumulates in tissues) if the patient is intolerant to the primary medication with penicillamine.[12] Recently, **3a** has gained attention in ameliorating left ventricular hypertrophy in diabetic patients.[13] It was also successfully used to regenerate the heart by selective Cu^{2+} chelation in a rat model of streptotoxin-induced diabetes.[14] Very little is known about its mechanism of action, excluding its role as a selective Cu^{2+} chelator, and only a few of these studies have focused on polyamine metabolism, although **3a** resembles **2** in structure. A recent publication speculated that SSAT could metabolize **3a**, but no data have been published to support that hypothesis.[15]

Replacement of the **2** moiety from **4a** with **3a** residue gives rise to **5a**,[16−18] which is an isoster of **4a**. **5a** exists as nonsymmetrical trivalent cation at physiological pH (Table 1), and it can induce DNA condensation more efficiently than **2**.[16] The condensation ability of **5a** is more pronounced at slightly acidic pH where the protonation status of the analogue is close to that of **4a**. Therefore, the presence of the **3a** moiety gives good chelating properties for **5a**, and the presence of the aminopropyl group seems to evoke additional effects on cell growth and polyamine metabolism compared to **3a**.[17] At the moment, the only known charge-deficient terminally bis-N-alkylated polyamine derivative is N^1,N^{12}-bis-(2,2,2-trifluoroethyl)-1,12-diamino-4,9-diazadodecane, but the electronegative effect of the trifluoroethyl substituent turns the terminal amino groups nonprotonated at physiological pH.[2] However, terminally diethylated spermine derivatives with partly protonated central fragment have still remained unknown.

Here we describe synthesis of N^1,N^{12}-Et_2-SpmTrien (**5d**) and terminally monoacetylated SpmTrien's, i.e., N^1-Ac-SpmTrien (**5c**) and N^{12}-Ac-SpmTrien (**5b**) (Figure 1). We have investigated the uptake of **5c** and the earlier synthesized **5a** and **3a** into cells, including the impact of AZ in this process, their effects on cell growth, and their ability to mimic the cellular functions of the natural polyamines in DU145 prostate carcinoma cells. In addition, the substrate properties of these analogues for polyamine catabolic enzymes SSAT, SMO, and APAO were studied. The present data demonstrate that not only the proper geometry but also the proper charge distribution is important for the analogue to be recognized as a polyamine and to completely fulfill the cellular functions of polyamines and that the alteration of the degree of protonation may provide an analogue with unusual biological activities.

Results and Discussion

Chemistry. A rational approach to prepare monoacetylated derivatives of **5a** and terminally bis-ethylated **5a** consisted of the use of orthogonally protected precursors. Synthesis of **5b** (Scheme 1) was started from commercially available N-(2-aminoethyl)aminoethanol **7**, which was converted into bis-Cbz derivative, mesylated, and then the chain

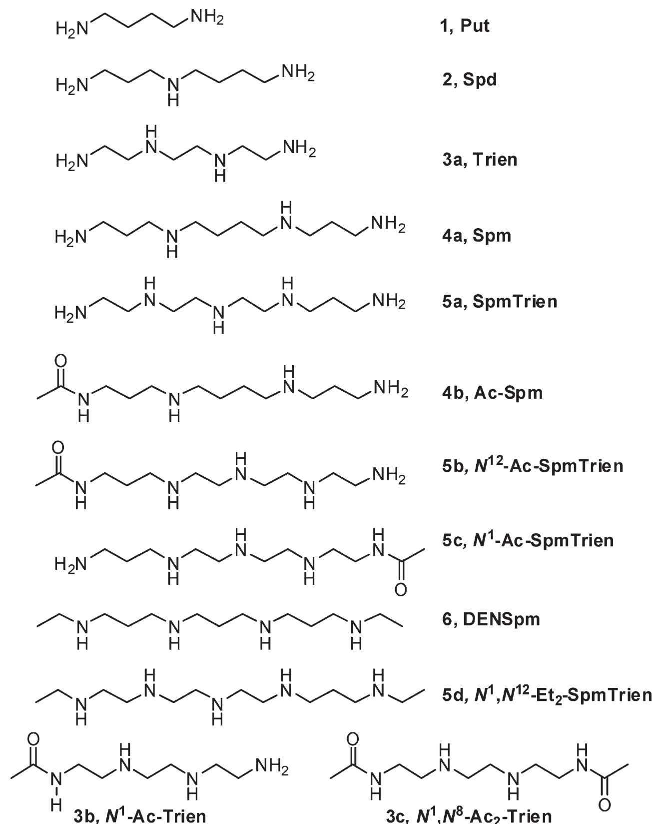

Figure 1. Structures of the studied compounds.

was elongated with aminoethanol followed by the addition of the third benzyloxycarbonyl protection group to give the intermediate **8a**, whose synthesis was described earlier in detail.[18] Following the same strategy alcohol **8a** was converted via the corresponding mesylate to **9a** using N-acetyl-1,3-diaminopropane[19] to give the target compound after the removal of Cbz groups by catalytic hydrogenation.

Synthesis of **5d** (Scheme 1) was started from N-(benzyloxycarbonyl)-3-azapentanol-1[20] using the same sequence of the reactions as above, but in this case the chain elongation reagent was N-(2-aminoethyl)aminoethanol, to give the intermediate **8b**, which was first mesylated and then aminated with an excess of N-ethyl-1,3-diaminopropane to give the protected intermediate **9b**. In this case, the reaction mixture contained, in addition to the target linear compound **9b**, a branched byproduct of the alkylation of the secondary amino group of N-ethyl-1,3-diaminopropane, which was hardly separated by flash chromatography on silica gel under the used conditions. However, the addition of 20 mol % of salicylic aldehyde to crude **9b** resulted in the formation of a stable Shiff base with the primary amino group of the byproduct. Thereafter, the formed Schiff base adduct was separated from target **9b** by flash chromatography. Finally, N-protection groups were removed by catalytic hydrogenation to give target **5d** as a pentahydrochloride with 24% overall yield, calculating from **10**.

Synthesis of **5c** was started from N-(benzyloxycarbonyl)-3-amino-1-propyl methanesulfonate **12**,[21] used for the chain elongation, the procedure being close to the above-described that resulted in the key intermediate **5b** with a good yield. To prepare orthogonally protected **14** (Scheme 2), it was necessary to discriminate the primary and secondary amino groups of the intermediate **13**. Required selective N-Cbz protection

Scheme 1. Synthesis of **5b** and **5d**[a]

[a] (a) CbzCl, $NaHCO_3$; (b) MsCl, Et_3N; (c) $H_2N(CH_2)_2OH$; (d) $H_2N(CH_2)_2NH(CH_2)_2OH$, THF; (e) $H_2N(CH_2)_3NHAc$; (f) $EtNH(CH_2)_3NH_2$, THF; (g) H_2/Pd, AcOH, MeOH.

Scheme 2. Synthesis of **5c**[a]

[a] (a) $H_2N(CH_2)_2NH(CH_2)_2OH$; (b) CbzCl; (c) MsCl, Et_3N, $NaHCO_3$; (d) $H_2N(CH_2)_2NH_2$; (e) *o*-$C_6H_4(OH)CHO$/THF; (f) $MeONH_2$; (g) AcCl, Et_3N; (h) H_2/Pd, AcOH, MeOH.

Table 2. Effect of 50 μM Polyamines and Charge-Deficient Analogues on Polyamine Metabolizing Enzyme Activities in DU145 Cells[a]

	metabolic enzymes ((pmol/h)/μg DNA)				
	ODC	AdoMetDC	SSAT	SMO	APAO
			At 24 h		
control	56 ± 9	236 ± 29	138 ± 21	537 ± 102	334 ± 8
2	10 ± 1***	179 ± 3**	117 ± 2	374 ± 33	277 ± 15
4a	13 ± 3***	108 ± 9***	132 ± 14	552 ± 34	284 ± 14
3a	77 ± 15*	241 ± 21	140 ± 22	509 ± 86	316 ± 49
5a	3 ± 0***	52 ± 3***	236 ± 1	478 ± 32	274 ± 17
5d	53 ± 0	166 ± 16***	178 ± 8	495 ± 7	309 ± 10
6	0.5 ± 0.1***	17 ± 3***	22019 ± 3659***	3205 ± 329***	315 ± 48
			At 48 h		
control	29 ± 3	223 ± 10	122 ± 4	330 ± 63	274 ± 19
2	4 ± 0***	179 ± 7***	104 ± 6	310 ± 40	233 ± 20
4a	6 ± 1***	112 ± 4***	125 ± 10	388 ± 66	254 ± 37
3a	31 ± 2	171 ± 6***	104 ± 5	298 ± 15	231 ± 14
5a	1 ± 0***	48 ± 1***	177 ± 17	379 ± 26	249 ± 18
5d	13 ± 0***	143 ± 15***	129 ± 12	395 ± 86	253 ± 35
6	0.1 ± 0.0***	14 ± 1***	12644 ± 758***	2233 ± 15***	278 ± 34
			At 72 h		
control	9 ± 1	174 ± 11	98 ± 13	312 ± 7	230 ± 18
DFMO	0.3 ± 0.0***	652 ± 25***	93 ± 2	105 ± 11**	237 ± 6
DFMO + **4a**	0.2 ± 0.0***	105 ± 14*	128 ± 22	450 ± 62	259 ± 26
DFMO + **3a**	0.3 ± 0.1***	441 ± 41***	85 ± 5	68 ± 27**	198 ± 16
DFMO + **5a**	0.2 ± 0.1***	38 ± 1***	178 ± 14	284 ± 39	206 ± 25
DFMO + **5d**	0.3 ± 0.2***	69 ± 14***	163 ± 33	292 ± 62	304 ± 42
DFMO + **6**	0.2 ± 0.0***	20 ± 2***	9486 ± 1181***	1456 ± 108***	197 ± 20

[a] Cells were cultured with 1 mM AG and 50 μM polyamines/analogues with or without 5 mM DFMO. Data (in (pmol/h)/μg DNA) are the mean ± SD, $n = 3$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to AG-treated control.

was achieved in one-pot with 75% yield by using salicylic aldehyde[22] as protection agent for terminal primary amino group followed by the addition of a Cbz group to the remaining NH group, and in the last step the salicylidene group was removed with $MeONH_2$ base to give **14**, which was acetylated to **15** and deprotected to target **5c** with 51% overall yield, calculating from compound **13**.

Effects on Cell Growth and Polyamine Metabolism. To investigate the metabolism and effect of the analogues on cell growth and polyamine metabolism, DU145 prostate carcinoma cells were incubated with 50 μM natural polyamines, charge-deficient analogues, or the reference compound **6** for 24 or 48 h. There were dramatic differences between the natural polyamines and their charge-deficient isosters in their effects on polyamine metabolism. **5a** was a very efficient down-regulator of ODC and AdoMetDC and moderate inductor of SSAT, while **3a** had very little, if any, effect toward polyamine metabolizing enzymes (Table 2). **5a** was

almost as potent an ODC down-regulator as **6**, which encouraged us to synthesize **5d**, which is a charge-deficient analogue of N^1,N^{12}-diethylspermine (DESpm). Surprisingly, **5d** exerted only modest effects on polyamine metabolism. All charge-deficient analogues showed slight (up to 25%) cytostatic effect after 24−48 h of culture, in the order of **3a** < **5a** < **5d** (Figure 2A). When incubated in the presence of the ODC inhibitor α-difluoromethylornithine (DFMO) for 72 h, **5a** was able to support cell growth for about 75% (Figure 2B), while in the absence of DFMO after 48 h of incubation with **5a** the growth was 80% (Figure 2A), compared with nontreated control. When **3a** was incubated in the presence of DFMO for 72 h, it was able to restore cell growth for about 75% (Figure 2B), while in the absence of DFMO after 48 h of incubation with **3a** the growth was 90% (Figure 2A), compared to growth of control cells. Altogether, the data show that charge-deficient **5a** can fulfill some but not all cellular functions of polyamines, whereas diethylation of **5a** (resulting in the molecule **5d**) abolished biological activity toward regulatory enzymes.

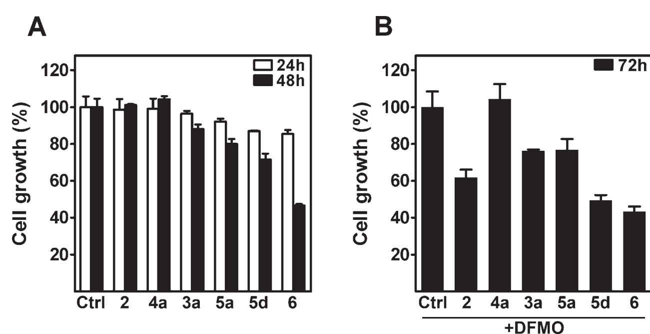

Figure 2. Growth of DU145 cells incubated with 50 μM polyamines/analogues (A) without or (B) with 5 mM DFMO. All plates contained 1 mM AG. Results are the mean ± SD, $n = 3$, compared to (A) untreated control, (B) DFMO-treated group: Ctrl, control.

Cellular Uptake and Its Regulation by Antizyme. **5a** and **5d** were accumulated intracellularly in the same concentration range as the natural polyamines, whereas the intracellular concentration of **3a** was 8−10 times lower (Table 3). Compared to the natural polyamines and **6**, compounds **3a**, **5a**, and **5d** were moderately competing for uptake with [^{14}C]-labeled **1** (10 μM) but very poorly with **2** or **4a**, as measured by 10 min experiments in DU145 cells (Figure 3). A previous report using rat intestinal brush-border vesicles determined K_m of 1.13 mM for the uptake of **3a**, which is much higher than K_m of 1.44 μM for **4a**.[23,24] Accordingly, the tested analogues competed efficiently only with **1** for uptake, suggesting that **3a**, **5a**, and **5d** are taken up by polyamine transport but their affinity is low.

It is known that polyamine uptake is negatively regulated by antizyme (AZ), a small protein also binding to ODC and targeting it for degradation.[25,26] To further investigate whether the charge-deficient analogues are taken up via AZ-regulated polyamine transporter, we measured the ability of AZ to decrease the uptake of the analogues. For that purpose, we constructed doxycycline (Dox) inducible AZ-overexpressing DU145 cell line. The AZ frameshift site was removed in order to get high expression of AZ regardless of the intracellular polyamine level. As shown in Figure 4A, in the presence of Dox (250 ng/mL, 24 h), DU145 cells synthesized a high amount of AZ protein. Forced overexpression of AZ significantly decreased the level of **5d** and the reference compound **6** (Figure 4B), which is in accordance with

Table 3. Effect of 50 μM Polyamines and Charge-Deficient Analogues on Polyamine Pools in DU145 Cells[a]

	polyamines (pmol/μg DNA)					
	1	**2**	**4a**	**4b**	analog	metabolites
			At 24 h			
control	18 ± 5	277 ± 37	245 ± 37	nd		
2	nd***	294 ± 11***	174 ± 8**	11 ± 2		
4a	nd***	142 ± 10**	273 ± 26	nd		
3a	27 ± 6*	206 ± 20***	263 ± 14	nd	66 ± 14	
5a	6 ± 1**	88 ± 4***	38 ± 2***	40 ± 3***	475 ± 38	44 ± 7,[b] 19 ± 0[c]
5d	19 ± 1	166 ± 20***	197 ± 6	15 ± 2	642 ± 77	
6	nd***	25 ± 5***	36 ± 8***	29 ± 17**	921 ± 100	
			At 48 h			
control	12 ± 2	206 ± 4	232 ± 6	nd		
2	3 ± 0***	250 ± 13***	160 ± 13**	6 ± 0***		
4a	2 ± 1***	127 ± 9***	272 ± 24	nd		
3a	13 ± 2	132 ± 6***	207 ± 22	nd	38 ± 7	
5a	2 ± 1***	58 ± 2***	24 ± 1***	21 ± 2***	351 ± 39	19 ± 2,[b] 10 ± 1[c]
5d	12 ± 2	135 ± 7***	184 ± 28	10 ± 1***	617 ± 33	
6	nd***	11 ± 2***	18 ± 2***	nd	578 ± 44	
			At 72 h			
control	7 ± 1	161 ± 7	196 ± 13	nd		
DFMO	nd***	11 ± 1***	148 ± 14	nd		
DFMO + **4a**	nd***	132 ± 21*	251 ± 45	nd		
DFMO + **3a**	nd***	10 ± 2***	126 ± 6**	nd	115 ± 8	
DFMO + **5a**	nd***	24 ± 5***	14 ± 1***	11 ± 0***	304 ± 22	16 ± 3[c]
DFMO + **5d**	nd***	40 ± 12***	67 ± 17***	nd	1365 ± 271	
DFMO + **6**	nd***	nd***	13 ± 1***	nd	413 ± 48	

[a] Cells were cultured with 1 mM AG and 50 μM polyamines/analogues with or without 5 mM DFMO. Data (in (pmol/μg DNA)) are the mean ± SD, $n = 3$. nd, not detectable. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to AG-treated control. [b] **5a** is metabolized to **3a**. [c] **5a** is metabolized to **5c**.

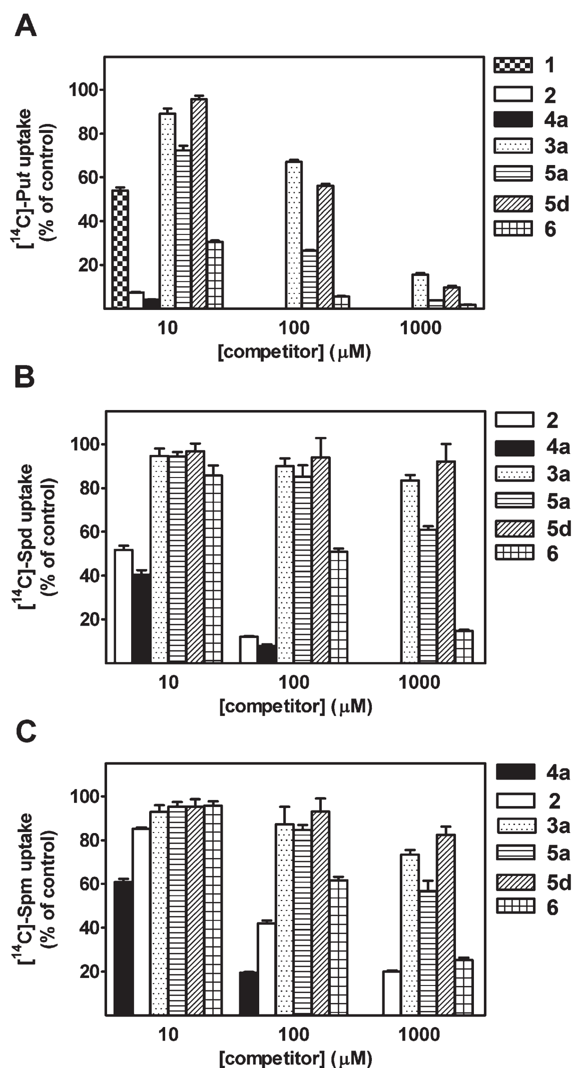

Figure 3. Uptake competition of the analogues with [^{14}C]-labeled (A) **1**, (B) **2**, or (C) **4a** in DU145 cells. Compounds were tested for 10 min at 10, 100, and 1000 μM against 10 μM labeled **1**, **2**, or **4a**. Results are the mean ± SD, n = 3.

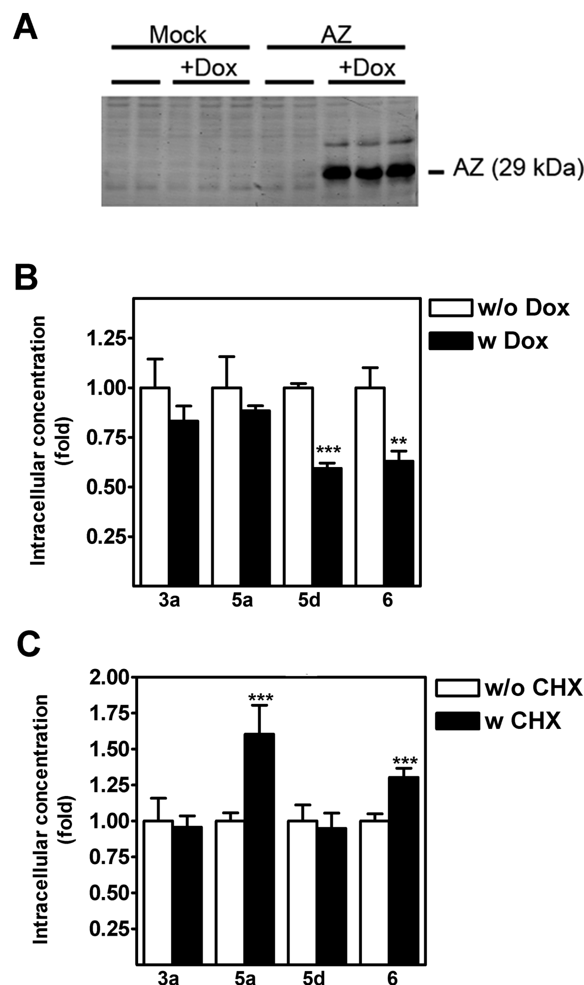

Figure 4. (A) AZ expression in doxycycline-inducible AZ-overexpesing cells, showing Western blot of AZ in mock- or AZ-transfected DU145 cells, with or without 24 h of preincubation with Dox. (B) Effect of forced AZ overexpression on accumulation of the analogues in DU145 cells. Doxycycline-inducible AZ-overexpressing cells were incubated with 50 μM analogues in the presence or absence of doxycycline (Dox, 250 ng/mL, preincubation 24 h) for 6 h. (C) Effect of CHX on the intracellular accumulation of the analogues in DU145 cells. Cells were incubated in the presence or absence of CHX (10 μg/mL) for 1 h after which the incubation was continued with 50 μM analogue for 4 h, in the presence or absence of CHX. Results show the intracellular analogue concentrations, mean ± SD, n = 3. No intracellular metabolites of the analogues were detected by HPLC. *, **, and *** refer to p values of < 0.05, < 0.01, and < 0.001, respectively, compared to corresponding without-CHX or without-Dox group.

the well-known competition of **6** with **4a**/**2** for transportation and its ability not only to induce SSAT but also to down-regulate ODC AZ-dependently.[27,28] In contrast to **6** and **5d**, the intracellular levels of **3a** and **5a** were slightly but not significantly decreased by AZ overexpression and the observed difference between **5a** and **5d** is of special interest.

We also investigated whether the analogues are able to induce AZ expression. Previous reports have indicated that compounds bearing even some resemblance to polyamines can induce AZ.[29] Since AZ is an inducible protein with a very short half-life and regulates polyamine uptake, we utilized an indirect method where we measured the intracellular accumulation of the analogues in the presence and absence of protein synthesis inhibitor cycloheximide (CHX) (Figure 4C). As expected, CHX treatment (prevention of AZ induction) increased the intracellular accumulation of **6**. However, it did not affect the intracellular level of **3a** or **5d**. Since the level of **5d** was not increased by CHX treatment but was decreased by forced AZ expression, it is possible that **5d** is not able to induce AZ. This view is supported by the finding that **5d** did not down-regulate ODC (Table 2). In contrast, **5a** did down-regulate ODC, and its accumulation was significantly increased by CHX treatment, suggesting that it induces AZ. As AZ production is regulated by a unique mechanism involving polyamine-induced +1 ribosomal frameshift on AZ mRNA, the ability of **5a** and **5d** to induce AZ frameshifting should be studied in more detail. Altogether, the results from the uptake experiments suggest that the charge-deficient analogues **5a**, **5d**, and **3a** have low affinity for the polyamine transporter, but they readily accumulate in DU145 cells at the same concentration range as natural polyamines with the exception of **3a**.

Catabolism of 5a. The analysis of the polyamine pool in **5a**-treated DU145 cells demonstrated that the drug was metabolized to **3a** and independently to **5c** (based on HPLC retention time, i.e., coelution with authentic reference compound),

Table 4. Kinetic Values for Mouse Recombinant SSAT[a]

compd	K_m (μM)	V_{max} ((μmol/min)/mg SSAT)
5a	106 ± 6	1.17 ± 0.02
5c	250 ± 18	0.44 ± 0.01
5b	435 ± 30	1.18 ± 0.03
3a	144 ± 6	1.36 ± 0.02
3b	79 ± 6	0.57 ± 0.02
2	151 ± 15	8.85 ± 0.5[b]
4a	nd	6.39 ± 0.6[b]

[a] Mouse recombinant SSAT (20 or 2 ng) was incubated with supplemented analogue at 50, 100, 250, 500, and 2500 μM substrate concentration range in triplicate. Blank reaction mixtures were done in triplicate and treated identically as earlier samples but without SSAT and by using 2500 μM concentration of each analogue. Values are the mean ± SD, n = 3. nd, not determined. Kinetic values for **3b** were determined by using 50, 100, and 250 μM because of substrate inhibition. [b] Reference V_{max} of **2** and **4a** has been determined by using 2500 μM and 2 ng of mouse recombinant SSAT.

while no intracellular metabolites were detectable for **3a** or **5d** (Table 3). Obviously, **3a** can be formed from **5a** only either by the result of direct oxidation of **5a** by SMO or APAO or via the SSAT/APAO pathway. The latter pathway requires an intermediate **5b** to be formed first. To study whether the enzymes of polyamine metabolism are involved in biotransformation of **5a** into **3a**, we investigated the interaction of **5a** with recombinant SMO, SSAT, and APAO.

5a exhibited very minor substrate properties with SMO or APAO; HPLC analysis of the 200 μM **5a** substrate mixtures showed less than 5% metabolism in the presence of 1 μg of SMO or APAO after 30 min of incubation at 37 °C (data not shown). Therefore, a direct intracellular transformation of **5a** into **3a** by APAO or SMO is unlikely. However, **5a** proved to be a substrate of SSAT (Table 4), but in contrast to **4a**, **5a** is an asymmetric molecule. To determine the site of its acetylation, two earlier unknown monoacetylated derivatives, i.e., **5c** and **5b** (Figure 1), were synthesized and used as reference compounds in HPLC coelution analysis as described above. **5b** was a good substrate of APAO, thus producing **3a** (K_m = 382 ± 23 μM, k_{cat} = 6.17 ± 0.15 s^{-1}; reference value for **4b** was k_{cat} = 3.15 ± 0.1 s^{-1}, determined under similar conditions at 0.5 mM; K_m of **4b** is 14 μM[30]). Taken together, these data indicate that the biotransformation of **5a** into **5c** and into **3a** via **5b** can take place with the consequent action of SSAT/APAO enzymes. Furthermore, we studied the metabolism of 50 μM **5a** for 24 h in SSAT-deficient mouse fetal fibroblasts and did not detect any conversion of **5a** to **3a** or **5b** or **5c**, while syngenic fibroblast did metabolize **5a** to **3a** and **5c** (data not shown). It is noteworthy that the amount of **3a** produced from supplementation of DU145 cells with 50 μM **5a** is about the same as after the treatment of the cells with 50 μM **3a** itself (Table 3), demonstrating that efficiently transported **5a** is a bioactive precursor of **3a**.

Interaction of Charge-Deficient Analogues with SSAT, SMO, and APAO. Typically SSAT favors charged amino groups separated by three carbons,[31] but under certain conditions SSAT can also acetylate compounds having charged amino groups separated by two-carbon-atom backbone and even N^8-aminoterminus of spermidine.[32,33] All tested compounds having a free aminoterminal moiety (**3a**, **5a**, **5b**, and **5c**) proved to be substrates of SSAT under the standard assay conditions (Table 4). Therefore, we ruled out the nonenzymatic acetylation by using a reaction mixture without recombinant SSAT and did not detect significant

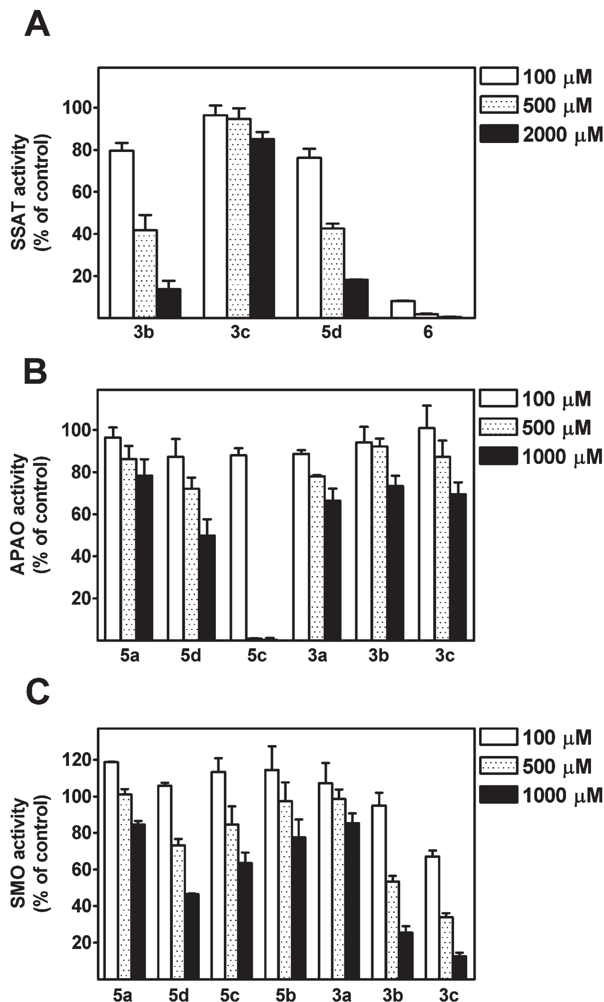

Figure 5. Competition of the analogues with (A) **2** for mouse recombinant SSAT, (B) **4b** for human recombinant APAO, and (C) **4a** for human recombinant SMO. Analogues were tested at 100, 500, and 1000 μM (or 2000 μM) against (A) 150 μM **2**, (B) 10 μM **4b**, or (C) 10 μM **4a**. Results are the mean ± SD, n = 3.

incorporation of [^{14}C]-label from Ac-CoA to the compounds tested (data not shown). We found that **5a**, **3a**, and **5b** showed similar maximal reaction velocity of about 1.2 (μmol/min)/mg enzyme, but their affinities for SSAT were different (Table 4). Kinetic values for N^1-Ac-Trien (**3b**) were determined by using 50, 100, and 250 μM substrate ranges. With higher concentration we detected increasing substrate inhibition retarding observed maximal reaction, since we excluded product (N^1,N^8-Ac$_2$-Trien, **3c**) feedback inhibition (Figure 5A). **5c** was acetylated with reasonable velocity but less efficiently than **5a** itself. However, **5d** proved to be a relatively inefficient inhibitor of SSAT in comparison with **6** (Figure 5A).

We did not detect **3b** in DU145 cells supplemented with 50 μM **3a** (Table 3). However, prior induction of SSAT by a 24 h treatment with compound **6** in mouse fetal fibroblasts induced a detectable accumulation of **3b** (data not shown), suggesting that SSAT may be a functional acetylator of **3a**. **3b** elutes just after **2** in the used HPLC method, and small amounts of **3b** remain undetectable in the presence of **2**. However, treatment with compound **6** induced SSAT, and furthermore, it depleted the **2** pool that facilitated detecting **3b**. Charge-deficiency of the central part of this type of

Table 5. SSATX/Total SSAT mRNA Ratio and Polyamine Levels in Mouse Primary Fetal Fibroblasts Treated with CHX and Polyamine Analogues[a]

		level (pmol/μg DNA)				
treatment	SSATX/total SSAT ratio (fold)	**1**	**2**	**4a**	analogue	total PA
control	1.00 ± 0.08***	72 ± 6***	411 ± 4	88 ± 11		499 ± 15
CHX	9.51 ± 2.58	10 ± 8	390 ± 33	89 ± 15		479 ± 47
CHX + **3a**	7.92 ± 1.15	< 10	389 ± 25	92 ± 2	176 ± 31***	658 ± 9**
CHX + **5a**	4.46 ± 0.33***	< 10	307 ± 23	95 ± 13	264 ± 31***	666 ± 53**
CHX + **5d**	5.87 ± 0.27**	< 10	380 ± 15	106 ± 20	354 ± 41***	841 ± 63***
CHX + **6**	4.92 ± 0.74***	< 10	203 ± 21	90 ± 15	352 ± 17***	647 ± 41 **

[a] Cells were cultured with CHX (10 μg/mL) for 8 h, of which the last 7 h were with polyamine analogues (50 μM). The ratio of SSATX to total SSAT mRNA (SSATX + SSAT) was analyzed by quantitative RT-PCR. Data are the mean ± SD, $n = 3$. *, **, and *** refer to p values of < 0.05, < 0.01, and < 0.001, respectively, compared to CHX-treated group. "Total PA" indicates the sum of **2**, **4**, and analogue. No metabolism of the analogues was detectable by HPLC at 8 h.

Spm/Spd analogues has little affect in productive binding at the SSAT active center.

SMO is known to prefer **4a** and then N^1-Ac-Spm (**4b**) as its substrates, while APAO has been shown to strongly prefer acetylated polyamines.[34,35] Such a substrate specificity of SMO in conjunction with our recent finding that both oxidases metabolize some N-alkylated polyamine analogues[36] validated the testing of synthesized analogues for their substrate properties. However, none of the charge-deficient analogues exhibited substrate properties in a SMO-catalyzed reaction (less than 5% product formation in 30 min in the presence of 1 μg of SMO; data not shown). Hence, in contrast with SSAT, the adequate protonation of the central part of pseudosubstrate molecules is essential for SMO-catalyzed transformations of these analogues because in competition studies all the analogues and their metabolites showed some degree of inhibition (Figure 5C). Both APAO and SMO are FAD-dependent oxidases, but unlike SMO, APAO does not have such precise requirements for the degree of protonation of the inner part of the substrate molecule(s). Thus, **5b** was a substrate of APAO (see above), while **5c** turned out to be an effective inhibitor of the enzyme (Figure 5B).

Taken together, these data clearly indicate that the addition of the 3-aminopropyl group to **3a** provides novel properties to the analogue and converts it into a rather good mimetic of **4a**, i.e., **5a**, which provides additional information about the differences between SMO and APAO, especially in the recognition of **4a** derivatives with substrate properties.

Ability To Regulate the Alternative Splicing of SSAT. To further study whether the charge-deficient analogues differ from the natural polyamines in their cellular functions, their ability to modulate the alternative splicing of SSAT was investigated. Our previous work demonstrated that the intracellular polyamine level regulates the alternative splicing of SSAT; high polyamine level promotes the production of normal SSAT mRNA, and low polyamine level promotes the generation of an unproductive splice variant (SSATX), which is subsequently targeted to protein-synthesis-dependent degradation pathway called nonsense-mediated mRNA decay (NMD).[37] Primary fetal fibroblasts were treated with CHX to shut down the rapid degradation of SSATX via NMD. Then the effect of the analogues on the abundance of SSATX mRNA relative to total SSAT was measured by quantitative RT-PCR. As indicated in Table 5, **5a**, **5d**, and **6** decreased the SSATX/total SSAT ratio, whereas **3a** did not show statistically significant effect. In the case of the charge-deficient analogues, SSATX/total SSAT mRNA ratio did not correlate with polyamine concentration (Table 5). Although the total polyamine level in **5d**-treated cells was much higher than that of **6**-treated cells, the latter showed more efficient reduction in SSATX/total SSAT ratio. Since both compounds reduced SSATX mRNA level but only **6** caused SSAT superinduction (Table 2), it is likely that **5d** is not able to prolong the SSAT protein half-life, like **6**,[38] because of its low affinity toward SSAT (Figure 5A). The fact that **5a** and **5d**, but not **3a**, were able to regulate the alternative splicing of SSAT implies that the charge of the polyamine is important in the alternative splicing process. These charge-deficient analogues may therefore help to dissect the mechanism of polyamines' action in alternative splicing.

Conclusion

The obtained data clearly demonstrate that charge deficiency of the central moiety of the **4a** isoster, i.e., **5a**, has in some cases practically no effect on the biochemical properties of the analogue: (i) **5a** was a relatively good substrate of mouse recombinant SSAT; (ii) **5b** was a substrate of human recombinant APAO; (iii) **5a** very effectively down-regulated ODC; (iv) both **5a** and **5d** positively controlled the productive splicing of SSAT mRNA.

In other cases, charge deficiency of the central moiety restricted or diminished the recognition of the analogues as polyamines: (i) **5d** did not induce SSAT or SMO in DU145 cells; (ii) **5a** and **5d** accumulated efficiently inside DU145 cells, although their affinity for polyamine transporter was low; (iii) none of the compounds tested, except for **5b**, was a substrate of human recombinant SMO or APAO.

Finally, an effective intracellular conversion of **5a** into **3a** makes it possible to consider **5a** as a bioactive precursor of **3a** with enhanced bioavailability. Hence, the synthesized isosteric analogues of **4a** having charge-deficient central moieties are promising tools for investigating the cellular functions of the natural polyamines and also the peculiarities of their recognition by intracellular binding sites and their metabolic enzymes. Our data also imply that SSAT could be a physiological acetylator of **3a**.

Experimental Section

Materials. The human prostate carcinoma cell line DU145 was obtained from American Type Culture Collection. Nontransgenic mouse primary fetal fibroblasts were prepared as previously published.[39] DFMO was obtained from ILEX Oncology Inc., and putrescine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride were from Sigma Aldrich. [^{14}C]-Labeled putrescine dihydrochloride (specific activity 107 mCi/mmol), spermine tetrahydrochloride (specific activity 112 mCi/mmol), spermidine trihydrochloride (specific activity 113 mCi/mmol), L-ornithine (specific activity 57 mCi/mmol), acetyl-CoA (specific activity 60 mCi/mmol), and *S*-adenosyl-L-methionine (specific activity 54 mCi/mmol) were obtained from GE Healthcare.

Syntheses. *N*-(2-Aminoethyl)aminoethanol, ethylenediamine, methanesulfonyl chloride (MsCl), and acetyl chloride (AcCl) were obtained from Fluka (Switzerland); triethylene tetraamine (**3a**), benzyl chloroformate (CbzCl), salicylic aldehyde, *O*-methylhydroxylamine hydrochloride were from Aldrich. *N*-(Benzyloxycarbonyl)-3-azapentanol-1 was synthesized as described earlier.[20] *N*-(Benzyloxycarbonyl)-3-amino-1-propyl methanesulfonate was prepared following a published procedure.[21] *N*-Ethyl-1,3-diaminopropane was synthesized by $LiAlH_4$ reduction of corresponding nitrile as described earlier,[40] and physicochemical properties of the obtained product were identical with the data published earlier.[41] *N*-Acetyl-1,3-diaminopropane hydrochloride was synthesized as described previously.[19] **6** was synthesized essentially as described previously.[42] **5a** was synthesized as described previously.[17,18] **8a** and **13** were synthesized as described previously.[18] **3b** and **3c** were synthesized as described earlier.[43]

Flash chromatography was performed on Kieselgel (40−63 μm, Merck, Germany), and systems for elution are indicated in the text. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 500 DRX (Germany) using tetramethylsilane (TMS) in $CDCl_3$ or sodium 3-(trimethylsilyl)-1-propanesulfonate (TSP) in D_2O as internal standards. Chemical shifts are given in ppm. The letter "*J*" indicates normal $^3J_{HH}$ couplings, and all *J* values are given in Hz. High-resolution electrospray mass spectra were obtained on Applied Biosystems/MDS Sciex QSTAR XL. All final compounds were ≥98% pure, which was confirmed with analytical HPLC used according to the published method.[44]

***N*^{12}-Acetyl-*N*^{1},*N*^{3},*N*^{6}-tris(benzyloxycarbonyl)-1,12-diamino-3,6,9-triazadodecane (9a).** Methanesulfonyl chloride (0.163 mL, 2.1 mmol) in dry DCM (3 mL) was added dropwise with stirring to a cooled (0 °C) solution of **8a**[18] (1.1 g, 2.0 mmol) and Et_3N (0.39 mL, 2.8 mmol) in dry DCM (10 mL). Stirring was continued for 1 h at 0 °C and 1 h at 20 °C, and the reaction mixture was poured into a 1 M $NaHCO_3$ (5 mL) solution. The organic layer was separated, washed with H_2O (3 mL), 0.5 M H_2SO_4 (3 × 4 mL), H_2O (3 mL), 1 M $NaHCO_3$ (3 mL), H_2O (3 mL), brine (5 mL) and dried ($MgSO_4$). The solvent was evaporated in vacuo to give intermediate *N*^{3},*N*^{6},*N*^{8}-tris(benzyloxycarbonyl)-8-amino-3,6-diaza-1-octyl methanesulfonate, which was used (without purification) to alkylate *N*-(3-aminopropyl)acetamide (2.3 g, 20 mmol) in dry THF (10 mL) first for 6 h at 20 °C and then for 48 h at 37 °C. The reaction mixture was evaporated to dryness in vacuo, NaOH (1M, 5 mL) was added, and the resulting mixture was extracted with DCM (2 × 3 mL). Solvent was evaporated in vacuo and the residue was purified on a silica gel column (120 g) using a mixture of dioxane−25% NH_4OH (100−0.7) as an eluent to give **9a** (0.97 g, 75%) as a viscous oil. TLC, NMR, and HRMS were conducted.

***N*^{12}-Acetyl-1,12-diamino-3,6,9-triazadodecane Tetrahydrochloride, *N*^{12}-Ac-SpmTrien (5b).** Pd black in MeOH (~0.5 mL) was added to a solution of **9a** (0.9 g, 1.39 mmol) in a mixture of AcOH−MeOH (1:1, 20 mL), and hydrogenation was carried out at atmospheric pressure. Solids were filtered off, washed with MeOH (5 mL), and combined filtrates were evaporated to dryness in vacuo. The residue was dissolved in MeOH (5 mL), diluted with 5 M HCl (2 mL), evaporated to dryness in vacuo and the residue was recrystallized from a mixture H_2O−MeOH−EtOH to give **7** (0.42 g, 77%) as colorless crystals, which did not melt below 250 °C. R_f = 0.27 (E); ^{1}H NMR (D_2O) δ 3.44−3.26 (12H, m, NHC$\underline{H_2}$C$\underline{H_2}$NH), 3.21−3.14 (2H, m, C$\underline{H_2}$NHAc), 3.07−2.99 (2H, m, AcNH(CH$_2$)$_2$C$\underline{H_2}$), 1.88 (3H, s, C$\underline{H_3}$), 1.86−1.78 (2 H, m, CC$\underline{H_2}$C); ^{13}C NMR (D_2O) δ 174.99, 45.80, 44.76, 43.74 (2 C), 43.70, 43.32, 36.01, 35.56, 25.80, 21.95. HRMS (ESI+) calcd for $C_{11}H_{28}N_5O$ $[M + H]^+$ 246.2294, found 246.2298. Anal. C, H, N.

***N*^{9}-(Benzyloxycarbonyl)-3,6,9-triazaundecanol-1 (11).** An intermediate *N*-(benzyloxycarbonyl)-3-aza-1-pentyl methanesulfonate was prepared as *N*^{3},*N*^{6},*N*^{8}-tris(benzyloxycarbonyl)-8-amino-3,6-diaza-1-octyl methanesulfonate (synthesis of **9a**) from **10** (4.5 g, 20.2 mmol) and MsCl (1.72 mL, 22.2 mmol) in dry DCM (100 mL) containing Et_3N (4.23 mL, 30.3 mmol) and was used (without purification) to alkylate *N*-(2-aminoethyl)-aminoethanol (31.7 mL, 0.318 mol) in dry THF (40 mL) for 20 h at 20 °C. THF and unreacted amine were removed in vacuo (0.1 mbar). To the residue 2 M NaOH (20 mL) was added, and the resulting mixture was extracted with DCM (2 × 20 mL). Extracts were washed with H_2O (5 mL), brine (10 mL), dried (K_2CO_3), and DCM was evaporated in vacuo. The residue was purified on a silica gel column (125 g) using a mixture of MeOH−Et_3N (95−5) as an eluent to give **11** (3.1 g, 50%) as a viscous oil. TLC, NMR, and ESI-MS were conducted.

***N*^{3},*N*^{6},*N*^{9}-Tris(benzyloxycarbonyl)-3,6,9-diazaundecanol-1 (8b).** Benzyl chloroformate (1.7 mL, 11.46 mmol) was added in three portions within 15 min intervals to a cooled (0 °C) and vigorously stirred mixture of **11** (1.69 g, 5.5 mmol), Na_2CO_3 (2M, 6 mL), $NaHCO_3$ (1.0 g, 11.9 mmol), THF (15 mL), and H_2O (5 mL). Stirring was continued for 1 h at 0 °C and 4 h at room temperature. The organic layer was separated, and the water layer was extracted with DCM (2 × 10 mL). The combined organic extracts were evaporated to dryness in vacuo. The residue was dissolved in EtOAc (20 mL), washed with 1 M HCl (3 × 5 mL), H_2O (5 mL), 1 M $NaHCO_3$ (5 mL), H_2O (5 mL), brine (10 mL) and dried ($MgSO_4$). Solvent was distilled off in vacuo and the residue was purified on a silica gel column (60 g) using first $CHCl_3$ followed by a mixture of $CHCl_3$−MeOH (95−5) as eluents to give **8b** (2.8 g, 88%) as a viscous oil. TLC, NMR, and ESI-MS were conducted.

***N*^{10},*N*^{13},*N*^{16}-Tris(benzyloxycarbonyl)-3,7,10,13,16-pentaazaoctadecane (9b).** An intermediate *N*^{3},*N*^{6},*N*^{9}-tris(benzyloxycarbonyl)-3,6,9-diaza-1-undecanoyl methanesulfonate was prepared as *N*^{3},*N*^{6},*N*^{8}-tris(benzyloxycarbonyl)-8-amino-3,6-diaza-1-octyl methanesulfonate (synthesis of **9a**) from **8b** (2.74 g, 4.8 mmol) and MsCl (0.4 mL, 5.2 mmol) in dry DCM (25 mL) containing Et_3N (1.0 mL, 7.1 mmol) and was used (without purification) to alkylate *N*-ethyl-1,3-diaminopropane (5.1 g, 50 mmol) in THF (10 mL) first for 6 h at 0 °C and then for 24 h at 20 °C. THF and an excess of *N*-ethyl-1,3-diaminopropane were distilled off in vacuo and the residue was purified on a silica gel column (60 g) using a mixture of MeOH−Et_3N (9−1) as an eluent to give crude **9b** (2.0 g). To remove the product of the alkylation of the secondary amino group of *N*-ethyl-1,3-diaminopropane, crude **9b** was treated with salicylic aldehyde (0.074 g, 0.61 mmol) in MeOH (12 mL) for 3 h at 20 °C. The reaction mixture was evaporated to dryness in vacuo and the residue was purified on a silica gel column (110 g) using a mixture of MeOH−Et_3N (9−1) as an eluent to give **9b** (1.2 g, 32%) as a viscous oil. TLC, NMR, and ESI-MS were conducted.

3,7,10,13,16-Pentaazaoctadecane Pentahydrochloride, *N*^{1},*N*^{11}-Et$_2$-SpmTrien (5d). The compound was prepared as **5b** from **9b** (1.15 g, 1.7 mmol) to give **5d** (0.54 g, 71%) as colorless crystals, which did not melt below 250 °C. R_f = 0.26; (*E*) ^{1}H NMR (D_2O) δ 3.42−3.34 (10H, m, C$\underline{H_2}$NH), 3.34−3.28 (2H, m, C$\underline{H_2}$NH), 3.10 (2H, t, *J* = 8.0 Hz, C$\underline{H_2}$NH), 3.07−2.93 (6H, m, C$\underline{H_2}$NH), 2.04−1.94 (2H, m, CC$\underline{H_2}$C), 1.16 (3H, t, *J* = 7.2 Hz, C$\underline{H_3}$), 1.14 (3H, t, *J* = 7.3 Hz, C$\underline{H_3}$); ^{13}C NMR (D_2O) δ 48.05, 46.79, 46.74, 46.70, 46.55 (2 C), 46.49, 46.38, 46.07, 45.55, 25.63, 13.45 (2 C). HRMS (ESI+) calcd for $C_{13}H_{34}N_5$ $[M + H]^+$ 260.2814, found 260.2812. Anal. C, H, N.

***N*^{3},*N*^{6},*N*^{9},*N*^{12}-Tetrakis(benzyloxycarbonyl)-1,12-diamino-3,6,9-triazadodecane (14).** A solution of **13**[18] (1.30 g, 2.15 mmol) and salicylic aldehyde (0.275 g, 2.26 mmol) in THF (4 mL) was kept for 1 h at 20 °C followed by adding H_2O (1.5 mL), $NaHCO_3$ (0.200 g, 2.4 mmol), and Na_2CO_3 (2 M, 1.2 mL). The reaction mixture was cooled to 0 °C, and benzyl chloroformate (0.35 mL, 2.38 mmol) was added with vigorous stirring. After the mixture was stirred at 0 °C for 1 h and at 20 °C for 3 h, the organic phase was separated and a mixture of CH_3ONH_2 (0.425 g, 9 mmol) and AcOH (30 μL, 0.5 mmol) was added. After being stirred for 2 h at 20 °C, the reaction mixture was evaporated to dryness in vacuo. The residue was dissolved in DCM (20 mL), washed with

1 M $NaHCO_3$ (5 mL), H_2O (5 mL), brine (10 mL), and dried (K_2CO_3). The residue after evaporation was purified on a silica gel column (65 g) using a mixture $CHCl_3$−MeOH (85−15) as an eluent to give **14** (1.25 g, 75%) as a viscous oil. TLC, NMR, and HRMS were conducted.

N^1-Acetyl-N^3,N^6,N^9,N^{12}-Tetrakis(benzyloxycarbonyl)-1,12-diamino-3,6,9-triazadodecane (15). To a cooled (0 °C) and stirred mixture of **14** (1.2 g, 1.62 mmol) and Et_3N (0.28 mL, 2 mmol) in dry THF (8 mL) was added dropwise within 5 min a solution of AcCl (0.13 mL, 1.86 mmol) in dry THF (2 mL). Stirring was continued for 30 min at 0 °C, MeOH (10 mL) was added, and after 10 min of stirring the mixture was evaporated to dryness in vacuo. The residue was dissolved in $CHCl_3$ (20 mL), washed with $NaHCO_3$ (1 M, 3 × 5 mL), H_2O (5 mL), H_2SO_4 (0.5 M, 3 × 5 mL), H_2O (5 mL), brine (10 mL), and dried ($MgSO_4$). The solvent was evaporated in vacuo to give **15** (1.22 g, 95%) as a viscous oil. TLC, NMR, and HRMS were conducted.

N^1-Acetyl-1,12-diamino-3,6,9-triazadodecane Tetrahydrochloride, N^1-Ac-SpmTrien (5c). The compound was prepared as **5b** from **15** (1.17 g, 1.5 mmol) to give **5c** (0.424 g, 72%) as colorless crystals, which did not melt below 250 °C. R_f = 0.27; (*E*) ^{1}H NMR (D_2O) δ 3.55−3.40 (10H, m, $C\underline{H_2}NH$), 3.26−3.16 (4H, m, $C\underline{H_2}NHAc$ + $CCC\underline{H_2}$), 3.09−3.01 (2H, m, $C\underline{H_2}NH_2$), 2.11−2.01 (2H, m, $CC\underline{H_2}C$), 1.95 (3H, s, $C\underline{H_3}$); ^{13}C NMR (D_2O) δ 177.24, 51.11, 48.14, 46.55, 46.43, 46.15 (2 C), 39.45, 38.78, 26.69, 24.90. HRMS (ESI+) calcd for $C_{11}H_{28}N_5O$ $[M + H]^+$ 246.2294, found 246.2297. Anal. C, H, N.

Cell Culture. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich) and 50 μg/mL gentamycin (Sigma Aldrich). The cells were incubated in a humidified atmosphere at +37 °C, 10% CO_2. The cells were detached using a solution containing 0.25% trypsin and 1 mM EDTA. The pelleted cells were washed with phosphate-buffered saline (PBS), pelleted, and stored at −70 °C. The cell number was measured with the Coulter Counter model Z1. Cell pellets were lysed in buffer (50 mM potassium phosphate buffer, pH 7.2, 1 mM EDTA, 0.1% Triton X-100, 0.1 mM dithiothreitol, complete EDTA-free protease inhibitor cocktail (Roche Diagnostics)) and incubated for 20 min on ice. Samples for polyamine measurement were taken and mixed 9:1 with 50% sulfosalicylic acid containing 100 μM diaminoheptane. The rest of the lysate was centrifuged at 13 000 rpm for 20 min at +4 °C. The supernatant fraction was used for the assay of SSAT, ODC, AdoMetDC, SMO, and APAO activities.

Enzyme Activities, Polyamines, and Analogues. Intracellular polyamines and polyamine analogues were measured with HPLC according to published methods.[44] The amount of DNA was measured from the pellets of polyamine samples using PicoGreen (Invitrogen) according to the manufacturer's instructions. Dilutions of calf thymus DNA (Sigma Aldrich) were used as standards. ODC, AdoMetDC, and SSAT activities were measured as described earlier.[7,45,46] SMO and APAO activities were measured as follows. Cell samples (20 μL) were preincubated in black 96-well plates for 15 min in an assay buffer containing 50 mM potassium phosphate buffer, pH 7.8, 1 mM pargyline, 0.1 mM semicarbazide, and 0.02 U/mL horseradish peroxidase (Roche Diagnostics). Then a mixture of 50 μM Ampliflu Red (Sigma Aldrich) and 1 mM **4a** (SMO) or **4b** (APAO) was added to a total volume of 200 μL. Fluorescence was measured for 30−60 min at excitation 540 nm and emission 590 nm with a Victor^2 counter (PerkinElmer) at 2 min intervals. A standard curve (0−2500 pmol) was prepared immediately before measurement using dilutions of H_2O_2 from fresh 30% stock solution.

Uptake Experiments. For competition experiments, DU145 cells were plated onto six-well plates at a density of 1 × 10^6 cells/well. After overnight incubation, the medium was changed to prewarmed serum-free medium supplemented with 10 μM [^{14}C]-labeled **1**, **2**, or **4a** (specific activity 25 mCi/mmol) and competing polyamine/analogues (10, 100, 1000 μM). After a 10 min incubation, the plates were washed twice with ice-cold PBS and the cells lysed in 500 μL of 0.1 M NaOH. Then an amount of 200 μL of the lysate was mixed with 3 mL of Optiphase "HiSafe" scintillation cocktail (PerkinElmer) and counted with liquid scintillation counter (1450 Microbeta PLUS, Wallac). For antizyme-depletion experiments, the cells were incubated in the presence or absence of CHX (10 μg/mL) for 1 h after which the incubation was continued with 50 μM analogue for 4 h in the presence or absence of CHX before the determination of the intracellular concentrations of analogues.

Experiments with Antizyme-Overexpressing DU145 Cells. A mutant mouse AZ cDNA construct lacking T at site 205 was used to get efficient AZ expression without the need for frameshifting.[47] For doxycycline (Dox) inducible expression, a novel system containing a reverse transactivator, the gene construct of choice under tetracycline responsive element, and a selection marker in one lentiviral vector was used.[48] The lentiviral particles were produced as described earlier.[48] DU145 cells were infected at MOI 1 and placed under neomycin selection (0.5 mg/mL Geneticin (Sigma Aldrich)) for 2 weeks. For uptake experiments, the cells were plated onto six-well plates at a density of 1 × 10^6 cells/well and incubated for 24 h in the presence or absence of Dox (250 ng/mL). Medium containing analogues (50 μM) with or without doxycycline was then changed, and the cells were harvested after 6 h of incubation. The intracellular concentrations of analogues were measured with HPLC.

Experiments with Recombinant Proteins. Plasmids encoding human recombinant SMO or APAO were a kind gift from Dr. Carl Porter, Roswell Park Cancer Institute, Buffalo, NY. The enzymes were produced as described previously.[49] Recombinant SSAT was purified, and kinetic measurements were done as described previously.[6] Initial studies with APAO and SMO were performed using fixed analogue concentration (200 μM) with 0.1 or 1.0 μg of recombinant protein and incubated for 30 min in a 37 °C water bath. Samples were analyzed with HPLC with postcolumn *o*-phthaldialdehyde derivatization to determine the concentrations of the reaction products. **5b** proved to be a substrate of APAO in initial screening; therefore, a more detailed kinetic was carried out in triplicate using a 50, 100, 250, 500, and 2500 μM substrate concentration range with 0.2 μg of APAO incubated for 5 or 10 min to verify the linearity of the observed reaction velocity. Kinetic values were determined by Lineweaver−Burk plotting using Graph Pad Prism 4.03 software with nonlinear fitting. K_{cat} values were determined using an M_w of 55 382 for recombinant human APAO as monomer consisting of one catalytically active center.

APAO and SMO inhibition experiments were measured as follows. Competitor (50 μL) was pipetted in black 96-well plates containing assay buffer (50 mM potassium phosphate buffer, pH 7.8, 1 mM EDTA, 0.02 U/mL horseradish peroxidase, and 10 μM **4a** (SMO) or 10 μM **4b** (APAO)). The reaction was initiated by the addition of 50 μL of enzyme mixture (50 μM Ampliflu Red, 0.1% Triton X-100, and 0.05 μg of recombinant SMO/APAO) to a total reaction volume of 200 μL. Fluorescence was measured for 15−30 min at excitation 540 nm and emission 590 nm with a Victor^2 counter (PerkinElmer) at 1 min intervals. A standard curve (0−3175 pmol) was prepared immediately before measurement using dilutions of H_2O_2 from fresh 30% stock solution.

Alternative Splicing of SSAT. Mouse primary fetal fibroblasts were seeded in six-well plates and incubated overnight. The cells were incubated in the presence of CHX (10 μg/mL) for 1 h after which 50 μM analogue was added, and incubation continued for a further 7 h. SSAT-X/total SSAT ratio was analyzed by quantitative RT-PCR as described earlier.[37]

Western Blotting. Total protein concentrations were measured using Coomassie Brilliant Blue (Bio-Rad) with dilutions of bovine serum albumin (Bio-Rad) as standards. Equal amounts

of proteins (10 μg) were run on 12% SDS−polyacrylamide gel, and samples were then transferred onto a PVDF membrane (Immobilon, Millipore). The AZ protein was detected by using 1 μg/mL rabbit anti-mouse AZ antibody (a kind gift from Prof. Olli Jänne, Finland) and anti-rabbit Dylight 800 secondary antibody (Thermo Fischer Scientific). The antibody-bound protein was visualized by an infrared scanner (Odyssey Imager, Li-Cor Biosciences).

Statistical Analysis. Values are the mean ± SD. One-way analysis of variance (ANOVA) with Tuckey's or Dunnett's post hoc test was used for multiple comparisons with the aid of a software package, GraphPad Prism 4.03 (GraphPad Software Inc.).

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Supporting Information Available: Synthesis and the spectroscopic details for compounds **8a** and **13**; physicochemical data for compounds **9a**, **11**, **8b**, **9b**, and **13**−**15**; and elementary analysis results of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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