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SYNTHESIS OF SPERMIDINE AND NORSPERMIDINE DIMERS AS HIGH AFFINITY POLYAMINE TRANSPORT INHIBITORS

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Abstract: A series of novel spermidine and *sym*-norspermidine dimers was synthesized by crosslinking the polyamine backbones via alkylation of their secondary amino groups to butyl, *trans*-2-butenyl, 2-butynyl or *p*-xylyl bridges. The resulting hexamines behaved as high-affinity antagonists of polyamine uptake, with a relative potency that was dependent on the geometry of the linker structure.

Putrescine and the naturally occurring polyamines, spermidine and spermine, are ubiquitous compounds that are essential for eukaryotic cell proliferation.[1] These compounds can be synthesized by most cell types, but can also be accumulated from exogenous sources via at least one membrane transport system.[1] The enhanced polyamine transport capacity demonstrated in tumor cells[1] represents a major obstacle for chemopreventive and chemotherapeutic strategies aimed at depleting polyamines through the use of specific biosynthesis inhibitors such as α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase.[1-3] Moreover, the in vivo supply of exogenous polyamines is an important promoter of preneoplastic events in the ontogeny of gastrointestinal tumors in rats.[4] Therefore, developing specific inhibitors of polyamine transport with high potency and low toxicity is a major goal toward the improvement of in vivo polyamine depletion and tumor growth arrest in the context of cancer therapy.

Only a few attempts have been recently made to design specific inhibitors of polyamine transport. A series of polypyridinium salts has been synthesized, some of them exhibiting a low K_i against putrescine uptake and low acute toxicity for mammalian cells.[5] However, the synthesis of these compounds was quite involved, and it is unclear whether such compounds efficiently blocked the uptake of spermidine and spermine. More recently, a high molecular mass ($M_r \approx 25$ kDa) spermine polymer has been described as a competitive inhibitor of polyamine transport with a K_i in the 10^{-6} M range, but its usefulness at specifically blocking polyamine accumulation is uncertain because of its marked cytotoxicity toward mammalian cells.[6]

We have previously shown that spermine analogs crosslinked into a dimeric structure exhibit a higher affinity for the mammalian polyamine carrier than the corresponding monomers.[7] 2,2'-Dithiobis(*N*-ethyl-spermine-5-carboxamide) (DESC) proved to be a purely competitive inhibitor of putrescine, spermidine and spermine transport without being used as a substrate. However, DESC was initially designed as a precursor of the corresponding thiol for the synthesis of fluorescent and radioactive probes for polyamine-binding proteins. Therefore, DESC was not suitable for its prolonged use in biological media containing thiols and disulfides, and was found to react

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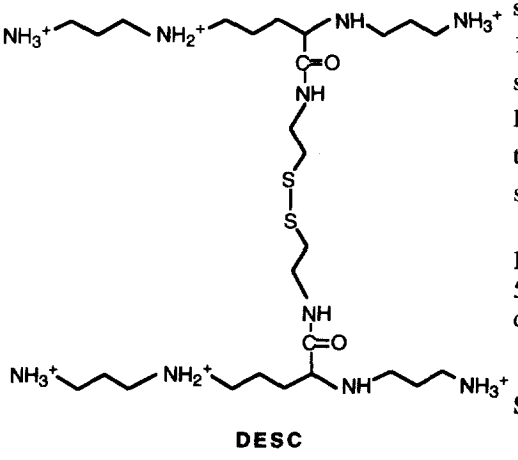
readily with L-cystine.[8] Moreover, the disulfide form of N^4-mercaptoethylspermidine, synthesized in the presence of 2,2'-dithiobis(benzothiazole)[9] along a route first described by Cohen et al.,[10] proved a much more potent inhibitor of polyamine uptake than DESC,[11] suggesting that crosslinking two polyamine chains via direct alkylation of the secondary amines led to an optimal interaction with the polyamine transporter. Thus, the stability of DESC and its affinity toward the polyamine carrier was expected to be improved by eliminating the disulfide and amide bonds, respectively. In this report, we describe the synthesis of symmetrical hexamines prepared from spermidine and *sym*-norspermidine (*N*-(3-aminopropyl)-1,3-diaminopropane) that are dimerized through their central secondary nitrogen using four different tethers of similar length. These new compounds have been tested in vitro for their inhibitory properties against the polyamine transport system.



DESC

Figure 1. Structure of 2,2'-dithiobis(*N*-ethyl-spermine-5-carboxamide) (DESC), a prototype for the design of dimeric polyamine transport inhibitors.

Synthesis

To determine the structure/activity relationships of different linkers of nearly equivalent length, we synthesized spermidine and *sym*-norspermidine dimers crosslinked with C_4 aliphatic chains with increasing degrees of bond unsaturation at carbon 2, or with a *p*-xylyl group (Figure 2). Primary amino groups of spermidine **1** and *sym*-norspermidine **2** were selectively protected by trityl groups and then reacted with the appropriate dihalide.[12] *p*-Xylyl derivatives (**9d** and **10d**) were synthesized using 1,4-dibromo *p*-xylene; *trans*-2-butenyl (**9b** and **10b**) and 2-butynyl (**9c** and **10c**) derivatives were obtained from the corresponding dichloride. The saturated *n*-butyl derivatives (**9a** and **10a**) were prepared via amidation from succinyl chloride followed by reduction with $LiAlH_4$. The latter route of synthesis was preferred over direct alkylation with a dihalogenated saturated alkane due to the formation of heterocyclic side products using the latter method.[12] Following acid hydrolysis of the trityl groups[12] and purification by cation exchange chromatography on Dowex 50X8-100 using a HCl gradient (from 0 to 6 N), the final hexamine derivatives **9a/d** and **10a/d** were obtained as their hexahydrochloride salts. Compounds **11** and **12** with saturated R linkers were similarly obtained (as their tetrahydrochloride salt), except that the reduction step was omitted. All compounds gave analytical and spectral data in agreement with the proposed structure.[13] DESC was prepared from L-ornithine and cystamine as described.[7] Purity of the final compounds was assessed by ion pairing HPLC using post-column derivatization with *o*-phthaldialdehyde for the on-line detection of amines.[7]

Polyamine uptake inhibition

The velocity of polyamine uptake was determined in T-47D and ZR-75-1 human breast cancer cells in mid-exponential growth phase and incubated in serum-free RPMI 1640 medium with 20 μM [^{3}H]putrescine, 3 μM

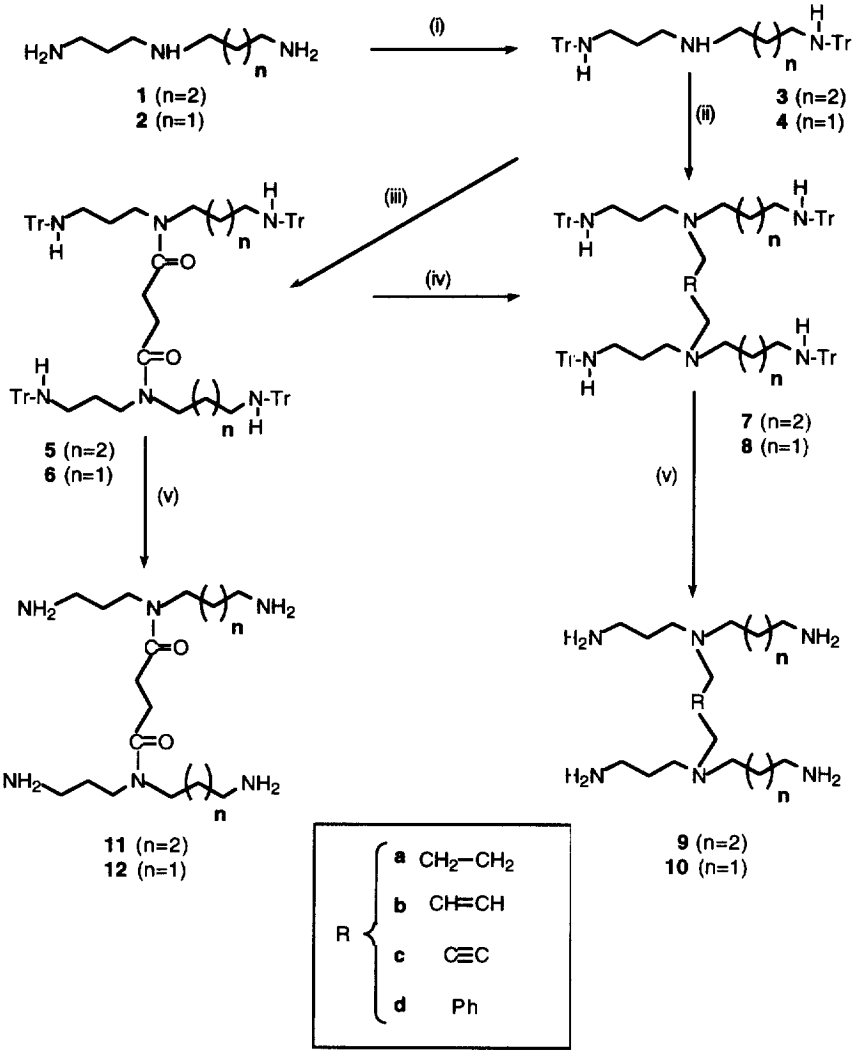


Figure 2. General route of synthesis for spermidine and *sym*-norspermidine dimers. (i) TrCl, NEt_3, chloroform; (ii) XCH_2-R-CH_2X, K_2CO_3, DMF (cat), acetonitrile, reflux; (iii) $(ClCOCH_2)_2$, NEt_3, chloroform, reflux; (iv) $LiAlH_4$, THF, reflux; (v) 3 N HCl , reflux.

[^{3}H]spermidine, or 3 μM [^{14}C]spermine as substrates for a 20-min assay period, as described.[7] Uptake activity was expressed per amount of total cellular DNA as fluorometrically determined using 3,5-diaminobenzoic acid.[14]

K_i values of competitive inhibitors were calculated from the half-maximal inhibitory concentration (IC_{50}) estimated by iterative curve fitting for sigmoidal equations describing polyamine uptake velocity in the presence of increasing concentrations of antagonist according to the equation

$$K_i = \frac{IC_{50}}{1+\left(\frac{S}{K_m}\right)}$$

where S is the substrate concentration.[15] The K_m of putrescine, spermidine and spermine uptake were independently determined by Lineweaver-Burke analysis of transport velocity data within a range of increasing substrate concentrations (0.01-300 μM).

Results and discussion

Table 1. K_i values for the inhibition of putrescine, spermidine and spermine transport by DESC and dimers of spermidine and *sym*-norspermidine in T-47D human breast cancer cells.

	K_m or K_i (μM)		
Inhibitor	[^{3}H]Putrescine	[^{3}H]Spermidine	[^{14}C]Spermine
Putrescine	7.4 ± 1.1	ND[a]	ND
Spermidine	ND	4.4 ± 0.9	ND
Spermine	0.65 ± 0.12	0.92 ± 0.08	2.7 ± 0.4
DESC	3.1 ± 0.4	12.6 ± 1.4	15.9 ± 4.0
9a, R → CH_2CH_2	0.72 ± 0.20	4.9 ± 0.1	4.3 ± 0.7
10a, R → CH_2CH_2	0.52 ± 0.06	2.4 ± 0.4	6.2 ± 0.6
9b, R → CH=CH	0.10 ± 0.01	1.8 ± 0.1	1.5 ± 0.1
10b, R → CH=CH	0.27 ± 0.04	2.0 ± 0.5	1.6 ± 0.2
9c, R → C≡C	1.9 ± 0.4	16.9 ± 2.0	27.7 ± 3.2
10c, R → C≡C	2.6 ± 0.4	25.6 ± 4.0	12.8 ± 2.2
9d, R → Ph	0.28 ± 0.02	2.0 ± 0.2	2.9 ± 0.6
10d, R → Ph	0.16 ± 0.03	1.5 ± 0.1	1.5 ± 0.1

[a] ND, not determined.

Data are presented as the mean ± SD of at least two independent determinations of IC_{50} values, each based on triplicate determinations of uptake velocity at increasing inhibitor concentrations.

As shown in Table 1, the spermidine and *sym*-norspermidine dimers synthesized in the present study displayed a 4- to 30-fold greater inhibitory potency than DESC toward putrescine, spermidine and spermine transport in T-47D cells, except for those crosslinked with a 2-butynyl chain (**9c** and **10c**). The latter type of crosslinker led to compounds with K_i values against diamine and polyamine uptake similar to those noted for DESC. Poorer competition for polyamine transport by compounds **9c** and **10c** was not solely due to conformational constraints conferred by the triple bond, as evidenced by the strong inhibitory potency displayed by the triamine dimers bridged with the similarly planar *p*-xylyl crosslinker (**9d** and **10d**). In fact, the *p*-xylyl and *trans*-butenyl derivatives displayed lower K_i values than the n-butyl derivatives, suggesting that the more rigid conformation imposed by an unsaturated tether interacts preferentially with the polyamine transporter. When compared pairwise, spermidine and *sym*-norspermidine derivatives dimerized with the same crosslinker behaved very closely as

inhibitors of diamine or polyamine transport. Thus, aminopropyl and aminobutyl moieties interact with nearly equivalent efficiency with the polyamine carrier in the context of these dimeric structures, although spermidine usually behaves as a slightly better substrate than *sym*-norspermidine for the mammalian polyamine uptake system.[12,13]

The relative potency of transport inhibition by the hexamines was greater for putrescine than either spermidine or spermine uptake, as expected from the observation that the K_i values of spermidine and spermine toward putrescine transport are lower than the K_m of putrescine in mammalian cells.[12,14,15] It is also noteworthy that the *p*-xylyl and *trans*-2-butenyl derivatives were more potent inhibitors of putrescine uptake than either spermidine or spermine. Likewise, these compounds had a K_i value lower than the K_m of spermidine toward spermidine uptake, with a potency nearly equal to that of spermine in that respect. Finally, the *p*-xylyl and *trans*-2-butenyl derivatives inhibited spermine uptake with a potency similar or even greater than the substrate affinity in T-47D cells. Thus, for the examples provided here, dimerization of spermidine or *sym*-norspermidine through their central amino groups does not impair the ability of the resulting molecule to interact with the polyamine transport system, and can in fact substantially increase their affinity for the carrier as compared with the parent polyamine species, as previously demonstrated for DESC.[7]

Table 2. K_i values for the inhibition of spermine transport by DESC and dimers of spermidine and *sym*-norspermidine in ZR-75-1 human breast cancer cells.

Inhibitor	Linker	K_m or K_i (μM)
Spermine		0.20 ± 0.02
DESC	-CONH(CH_2)$_2$S-S(CH_2)$_2$NHCO-	4.0 ± 1.2
9d	-CH_2-Ph-CH_2-	0.24 ± 0.08
10d	-CH_2-Ph-CH_2-	0.70 ± 0.09
12	-CO(CH_2)$_2$CO-	1.7 ± 0.5
10a	-(CH_2)$_4$-	0.56 ± 0.03

[a] The linker of DESC is tethered to the terminal carbon of the central butyl chain of a spermine backbone (cf. Fig. 1), whereas the linkers of compounds **9d**, **10d**, **12**, and **10a** are tethered to the central nitrogen atom of spermidine or *sym*-norspermidine.

The potency of the novel hexamines as polyamine transport inhibitors was also examined in other cell types, including the ZR-75-1 cell line in which the polyamine transport system has been extensively characterized.[7,16,17] An example of a limited subset of comparisons for the spermine uptake system present in the latter cell line is presented in Table 2. As in T-47D cells, the *p*-xylyl and *n*-butyl derivatives were substantially better inhibitors of spermine uptake than DESC. The ability of the spermidine dimer crosslinked with a saturated butyl chain (**9a**) to inhibit spermine uptake was also compared with that of the corresponding secondary diamide **12**. The weaker potency of **12** as a spermine transport inhibitor likely reflects the absence of charged central nitrogen atoms in its spermidine-derived moieties, but could also be accounted in part on the steric and rotational constraints imposed by the bulky amido groups, as previously suggested for N^4-acylated vs. N^4-alkylated spermidine derivatives with short side chains.[18,19] Likewise, crosslinking of spermine via an amide bond at the C5 position in the DESC structure, which virtually eliminates the charge of the neighboring amino group at physiological pH by decreasing its pK_a,[20] may also contribute to the lower potency of DESC as a transport antagonist, as compared with the present series of triamine dimers.

Most of the novel spermidine and *sym*-norspermidine dimers described in the present study thus present a substantial improvement over DESC[7] as polyamine transport antagonists, in terms of both relative potency and simplicity of synthesis. Among possible factors underlying the lower K_i values of these hexamines against diamine and polyamine uptake are the structure of the linker, its tethering to a secondary amino group of the polyamine backbone rather to the proximal methylene group, and the greater flexibility of the alkyl linkage as compared to the amide bond previously used.[7] A detailed account of the synthesis and biological properties of an extensive series of spermidine and *sym*-norspermidine dimers as cell-impermeant and efficient inhibitors of polyamine uptake in mammalian cells will be presented elsewhere.

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