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PUTRESCINE OR SPERMIDINE BINDING SITE OF AMINOPROPYLTRANSFERASES AND COMPETITIVE INHIBITORS

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Abstract—A model of the active site of aminopropyltransferases was proposed based on the study of a number of monoamino and diamino compounds as potential inhibitors and substrates, respectively, of spermidine synthase purified from pig liver. The active site seems to have a relatively large hydrophobic cavity adjacent to a negatively charged site, to which a protonated amino group of putrescine binds, with another amino group of putrescine being situated in the hydrophobic cavity as a free form to be aminopropylated by decarboxylated *S*-adenosylmethionine. On the basis of the above-mentioned model, another modified one was proposed for spermine synthase, and several compounds designed according to the modified model were found to potently inhibit spermine synthase, purified from rat brain, in competition with spermidine. The newly developed inhibitors were about two orders of magnitude more potent *in vitro* than a known inhibitor of spermine synthase, dimethyl(5′-adenosyl)sulfonium perchlorate.

Aminopropyltransferases catalyze the transfer of the aminopropyl moiety of *S*-adenosyl-5′-deoxy-(5′)-3-methylthiopropylamine (decarboxylated AdoMet†) to putrescine to form spermidine (spermidine synthase) and to spermidine to form spermine (spermine synthase). These reactions are believed to be sequential SN_2 reactions [1] like many enzymatic methyl transfer reactions with *S*-adenosylmethionine. Some physical and catalytic properties of the two mammalian enzymes have been described, using homogeneous enzymes [2, 3]. Recent work on spermidine synthase protein has revealed an immunochemical similarity among the mammalian native enzymes [4] and a primary amino acid sequence of the human enzyme [5]. However, few attempts have been made to clarify the structure of the active site of aminopropyltransferases. Elucidation of the active site should offer valuable information on the detailed mechanism of the enzymatic transfer reaction, the structure of the polyamine binding site of proteins, and possible designs for new inhibitors.

Known inhibitors of aminopropyltransferases have been reviewed by Pegg and Williams-Ashman [6]. Most of them are competitive inhibitors directed at the binding site of decarboxylated AdoMet, probably owing to the low K_m value of the common nucleoside substrate to the two enzymes compared with the K_m value of the putrescine or spermidine substrate. Therefore, it is difficult to specifically inhibit the two enzymes using these inhibitors, except for *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDato) [7] and *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDatad) [1], transition state analogues for spermidine and spermine synthase, respectively, and dimethyl(5′-adenosyl)sulfonium perchlorate [8] for spermine synthase. Also, the potency of these inhibitors could be weakened by a significant increase of decarboxylated AdoMet which is usually observed in biological systems depleted of cellular polyamines [9]. In view of these facts, inhibitors directed at the binding site of putrescine or spermidine should be of practical use *in vivo* for the specific inhibition of spermidine synthase or spermine synthase respectively. Cyclohexylamine (often incorrectly designated as dicyclohexylamine [10]), an inhibitor of spermidine synthase [11], is known to compete with putrescine at the active site. Although there is an apparent lack of structural similarity between putrescine and cyclohexylamine, the idea that cyclohexylamine may partially reflect a fixed conformation of putrescine at the active site led us to examine the inhibitor or substrate capability of various cycloalkane compounds as tools for collecting spacial information on the active site of spermidine synthase.

The present paper proposes a model for the active site of aminopropyltransferase and discusses the development of new potent inhibitors directed at the polyamine binding site.

MATERIALS AND METHODS

Putrescine (**53**) dihydrochloride and spermidine trihydrochloride were purchased from Sigma (St. Louis, MO). *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (81 Ci/mmol) was purchased from NEN (Boston, MA)

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† Abbreviations: decarboxylated AdoMet, *S*-adenosyl-5′-deoxy-(5′)-3-methylthiopropylamine; and MTA, 5′-methylthioadenosine.

and converted to decarboxylated *S*-adenosyl-[*methyl*-^{3}H]methionine by treatment with bacterial *S*-adenosylmethionine decarboxylase as described in Ref. 12. Unlabeled decarboxylated AdoMet was synthesized as previously described [13] and used for the dilution of the labeled compound. Since the synthetic decarboxylated AdoMet was a 1:1 mixture of *S* and *R* stereoisomers, the concentration is expressed as that of the *S* isomer. Dimethyl(5'-adenosyl)sulfonium perchlorate was synthesized according to a reported method [14].

Tested compounds. Cyclohexylamine (**1**), *N*-methylcyclohexylamine (**2**), aniline (**9**) hydrochloride, toluidine (**10**) hydrochloride, 4-ethylaniline (**11**), *n*-propylamine (**26**), *n*-butylamine (**27**) hydrochloride, *n*-pentylamine (**28**), *n*-hexylamine (**29**), *n*-heptylamine (**30**), *iso*-propylamine (**31**) hydrochloride, 2-aminobutane (**32**), 1-ethylpropylamine (**35**), *iso*-butylamine (**36**), 2-methylbutylamine (**39**), *iso*-amylamine (**38**), and 1,3-diaminopropane (**52**) dihydrochloride were obtained from Wako Pure Chemical Industries (Osaka). *N,N*-Dimethylcyclohexylamine (**3**), 1-methylbutylamine (**33**), 1,3-bis(aminomethyl)cyclohexane (**43**), 3-butoxypropylamine (**70**), and dibutylamine (**71**) were obtained from Tokyo Kasei (Tokyo). *N*-(3-Aminopropyl)-cyclohexylamine (**60**), 2-methylcyclohexylamine (**4**), 3-methylcyclohexylamine (**5**), 4-propylaniline (**12**), 4-butylaniline (**13**), cyclohexanemethylamine (**14**), cyclopentylamine (**18**), cycloheptylamine (**20**), 3-aminonoradamantane (**21**) hydrochloride, 1-aminoadamantane (**22**) hydrochloride, 2-aminoadamantane (**23**) hydrochloride, *exo*-2-aminonorbornane (**24**), *endo*-2-aminonorbornane (**25**) hydrochloride, 1,2-diaminocyclohexane (**39**), 1,3-diaminocyclohexane (**40**), 1-aminopiperidine (**45**), 2-iminopiperidine (**46**) hydrochloride, 3-aminopiperidine (**47**) dihydrochloride, 4-aminomethylpiperidine (**49**), 1,4-diaminopiperidine (**50**), and 4,4'-bispiperidine (**51**) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). 1,4-Bis(aminomethyl)cyclohexane (**44**) was obtained from the Eastman Kodak Co. (Rochester, NY). In the case of free base, the compounds were crystallized as the hydrochloride forms.

The structure and purity of the hydrochloride form of all of the above compounds and the compounds synthesized in this laboratory as described below were confirmed by elemental analysis (within 0.3% of calculated values for C, H, and N) and/or NMR spectrometry with a JOEL-GX270 FT spectrometer. NMR data showed that the ratio of the *cis/trans* isomer was 3/7 for the sample of compound **4**, 7/3 for compound **5**, 1/1 for compound **39**, and 3/7 for compound **40**, respectively, and that the sample of compound **25** contained 2% of compound **24**.

The following compounds were prepared from each corresponding alcohol as a starting material by the Mitsunobu reaction [15]: *cis*-4-methylcyclohexylamine (**6**) hydrochloride from *trans*-4-methylcyclohexanol, m.p. 229–230° (lit. [16] m.p. 233–234°), Anal. ($C_7H_{15}N \cdot HCl$); *trans*-4-methylcyclohexylamine (**7**) hydrochloride from *cis*-4-methylcyclohexanol, m.p. 259–260° (lit. [16] m.p. 260–261.5°), Anal. ($C_7H_{15}N \cdot HCl$); 4-ethylcyclohexylamine (**8**) hydrochloride from 4-ethylcyclohexanol, m.p. 228–240° (lit. [17] m.p. 234–246°), Anal. ($C_8H_{17}N \cdot HCl$), ^{1}H-NMR data showing that the ratio of *cis/trans* isomer was 2:1, 2-aminoethylcyclohexane (**15**) hydrochloride from 2-cyclohexaneethanol, m.p. 246–253° (lit. [18] m.p. 253–254°), Anal. ($C_8H_{17}N \cdot HCl$); 3-aminopropylcyclohexane (**16**) hydrochloride from 3-cyclohexylpropanol, m.p. 224–234° (lit. [18] m.p. 224–228°), Anal. ($C_9H_{19}N \cdot HCl$); 4-aminobutylcyclohexane (**17**) hydrochloride from 4-cyclohexylbutanol, m.p. 246° (lit. [18] m.p. 246°), Anal. ($C_{10}H_{21}N \cdot HCl$); 3-methylcyclopentylamine (**19**) hydrochloride from 3-methylcyclopentanol, m.p. 135–140°, Anal. ($C_6H_{13}N \cdot HCl$); ^{1}H-NMR data showing that the ratio of *cis/trans* isomer was 2/3; 2-aminohexane (**34**) hydrochloride from 2-hexanol, m.p. 105–109°, Anal. ($C_6H_{13}N \cdot HCl$).

Cis-1,4-Diaminocyclohexane (**41**) and *trans*-1,4-diaminocyclohexane (**42**) were prepared according to the method of Suess and Hesse [19]: **41** dihydrochloride, Anal. ($C_6H_{14}N_2 \cdot 2HCl$), ^{1}H-NMR data showing that this preparation contained 2% of the *trans* isomer **42**; **42** dihydrochloride, m.p. > 300°, Anal. ($C_6H_{14}N_2 \cdot 2HCl$). 4-Aminopiperidine (**48**) dihydrochloride was prepared by hydrogenolysis of 1-benzyl-4-aminopiperidine, Anal. ($C_5H_{12}N_2 \cdot 2HCl$). *N*-Benzylputrescine (**55**) dihydrochloride was prepared by hydrolysis of *N*-(4-phthalimidobutyl)benzylamine [20], m.p. 152°, Anal. ($C_{11}H_{18}N_2 \cdot 2HCl$).

The following aminopropylated alkylamines were prepared from corresponding alkylamine using *N*-(3-bromopropyl)phthalimide according to our method [20] for the synthesis of spermine from putrescine: *N*-(3-aminopropyl)-*trans*-4-methylcyclohexylamine (**56**) dihydrochloride from compound **7**, m.p. 227°, Anal. ($C_{10}H_{22}N_2 \cdot 2HCl$); *N*-(3-aminopropyl)-*exo*-2-aminonorbornane (**57**) dihydrochloride from compound **24**, m.p. 235°, Anal. ($C_{10}H_{20}N_2 \cdot 2HCl$); *N*-(3-aminopropyl)-*endo*-2-aminonorbornane (**58**) dihydrochloride from compound **25**, m.p. 165°, Anal. ($C_{10}H_{20}N_2 \cdot 2HCl$); *N*-(3-aminopropyl)cyclopentylamine (**59**) dihydrochloride from compound **18**, m.p. 184°, Anal. ($C_8H_{18}N_2 \cdot 2HCl$); *N*-(3-aminopropyl)cycloheptylamine (**61**) dihydrochloride from compound **20**, m.p. 170°, Anal. ($C_{10}H_{22}N_2 \cdot 2HCl$); N-(3-aminopropyl)hexylamine (**62**) dihydrochloride from compound **29**, m.p. 294°, Anal. ($C_9H_{22}N_2 \cdot 2HCl$); *N*-(3-aminopropyl)pentylamine (**63**) dihydrochloride from compound **28**, m.p. 295°, Anal. ($C_8H_{20}N_2 \cdot 2HCl$); *N*-(3-aminopropyl)butylamine (**64**) dihydrochloride from compound **27**, m.p. 288° (lit. [21] m.p. 296°), Anal. ($C_7H_{18}N_2 \cdot 2HCl$); *N*-(3-aminopropyl)propylamine (**65**) dihydrochloride from compound **26**, m.p. 277° (lit. [21] m.p. 282°), Anal. ($C_6H_{16}N_2 \cdot 2HCl$); *N*-(3-aminopropyl)ethylamine (**66**) dihydrochloride from *N*-benzylethylamine, m.p. 221° (lit. [21] m.p. 222°), Anal. ($C_5H_{14}N_2 \cdot 2HCl$).

N-(2-Aminoethyl)cyclohexyl amine (**67**) dihydrochloride was prepared from compound **1** in the same manner as described for compound **56** except for using *N*-(2-bromoethyl)phthalimide instead of *N*-(3-bromopropyl)phthalimide, m.p. 204–206° (lit. [22] m.p. 212–214°), Anal. ($C_8H_{18}N_2 \cdot 2HCl$). *N*-(4-Aminobutyl)cyclohexylamine (**68**) dihydrochloride was prepared from compound **1** in the same manner

as described for compound **56** except for using *N*-(4-bromobutyl)phthalimide instead of *N*-(3-bromopropyl)phthalimide, m.p. 248–249° (lit. [22] m.p. 249–250°), Anal. ($C_{10}H_{22}N_2 \cdot 2HCl$). *N*-(5-Aminopentyl)cyclohexylamine (**69**) dihydrochloride was prepared from compound **1** in the same manner as described for compound **56** except for using *N*-(5-bromopentyl)phthalimide instead of *N*-(3-bromopropyl)phthalimide, m.p. 239–240° (lit. [22] m.p. 239–240°), Anal. ($C_{11}H_{24}N_2 \cdot 2HCl$). *N*-Methylputrescine (**54**) dihydrochloride was prepared from *N*-methylbenzylamine as a starting material in the same manner as described for compound **68**, m.p. 177–179° (lit. [23] m.p. 176°), Anal. ($C_5H_{14}N \cdot 2HCl$).

Enzyme. Spermidine synthase (515 nmol/min/mg) was purified to homogeneity from pig liver according to a reported method [24] and spermine synthase (440 nmol/min/mg) from rat brain according to the method described in Ref. 25 for the bovine enzyme with some modifications.

Assay of aminopropyltransferases. The assay was carried out by measuring the production of labeled 5′-methylthioadenosine (MTA) from labeled decarboxylated AdoMet in the presence of an appropriate amine acceptor. Labeled MTA was separated with a small column of phosphocellulose as described by Raina *et al.* [26]. Standard assay medium contained 10 μM decarboxylated AdoMet, 1 mM putrescine for spermidine synthase or 1 mM spermidine for spermine synthase, 5 mM dithiothreitol, 0.75 mg/mL bovine serum albumin and 0.1 M potassium phosphate, pH 7.4. The inhibition studies were performed by measuring the enzyme activities in the presence of a 0 to 1.0 mM concentration of the tested compound. The capability of the compounds to inhibit the enzyme activity was expressed as IC_{50} (the concentration of the compound which inhibited enzyme activity by 50%). The acceptor capability of compounds for spermidine synthase was examined by measuring the production of labeled MTA in the presence of a 1 mM concentration of each tested compound instead of putrescine under the standard assay conditions and was expressed as the relative rate of MTA production to that in the case of putrescine.

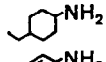
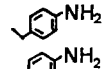
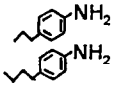
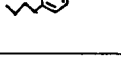
RESULTS

Inhibition of spermidine synthase activity by various cyclic or chain monoamino compounds. To study the structural requirements of cyclohexylamine for inhibiting mammalian spermidine synthase, a series of cyclic monoamino compounds were tested for their capabilities, expressed as IC_{50} values, to inhibit the enzyme activity using purified spermidine synthase from pig liver (Table 1). Among the methylated derivatives of cyclohexylamine (**1**), compounds **2–7**, *trans*-4-methylcyclohexylamine (**7**) is the most potent inhibitor [27], with an IC_{50} value of one-fifth that of cyclohexylamine [11]. When the 4-methyl group was replaced by a 4-ethyl group to form compound **8**, the IC_{50} value increased markedly. The results with aniline derivatives, compounds **9–13**, suggested that further elongation of the alkyl chain at the 4-position of cyclohexylamine would result in a loss of inhibition. The effect of the distance between the cyclohexyl ring and the amino group on the IC_{50} value was examined using compounds **14–17**, and cyclohexylamine was found to be the most potent. Some other cycloalkanes, compounds **18–25**, were also tested, and *exo*-2-aminonorbornane (**24**) showed a lower IC_{50} value than cyclohexylamine. These results suggested that there might be a fairly large hydrophobic cavity adjacent to a negative charge at the putrescine binding site, and led us to test simple alkylamines with three to seven carbons, as shown in Table 2. Although compounds **31–35** have an α-branched primary amino group similar to cyclohexylamine, their IC_{50} values were high compared with those of simple *n*-alkylamines. Among them, *n*-butylamine (**27**) and *n*-pentylamine (**28**) showed lower IC_{50} values than cyclohexylamine. To ascertain that all the compounds actually acted at the putrescine binding site, some typical potent ones with low IC_{50} values, i.e. *trans*-4-methylcyclohexylamine, *n*-pentylamine, *exo*-2-aminonorbornane, and cyclohexylamine, were examined kinetically in the presence of 0.08 to 1.0 mM putrescine and 10 μM decarboxylated AdoMet and found to be competitive with putrescine with K_i values of 0.04, 0.10, 0.15, and 0.2 μM respectively.

Substrate properties of various diamino compounds for spermidine synthase. To collect additional information on the putrescine binding site of spermidine synthase, various diamino compounds possessing a cyclohexyl or piperidine ring, including some putrescine analogues, were tested for their substrate properties as aminopropyl acceptors (Table 3). Values were expressed as the relative rate of tritium MTA formation in the presence of each compound to that in the presence of putrescine, as described under Materials and Methods. Among the compounds tested, *N*-monomethylputrescine (**54**), *trans*-1,4-diaminocyclohexane (**42**), and 4-aminomethylpiperidine (**49**), could more or less act as aminopropyl acceptors. The value for *trans*-1,4-diaminocyclohexane was half that reported for bovine spermidine synthase by Sarhan *et al.* [28], but the difference could be explained by the different experimental conditions. The apparent MTA formation of the *cis* isomer evidently depended on 2% contamination with the *trans* isomer. Their data also showed that the *cis* isomer could not serve as a substrate. The kinetics data of putrescine and the three compounds in the aminopropyl transfer reaction were as follows for K_m (mM), V_{max} (nmol/min/mg) and V_{max}/K_m in the presence of 10 μM decarboxylated AdoMet: 0.09, 515 and 5700 for putrescine; 0.10, 68 and 680 for *trans*-1,4-diaminocyclohexane; 2.2, 280 and 130 for *N*-monomethylputrescine; and 2.2, 37 and 17 for 4-aminomethylpiperidine. There may be some significance in the similar K_m values of the first two compounds and those of the last two.

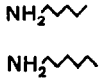
Putrescine binding site of spermidine synthase. As *trans*-1,4-diaminocyclohexane was found to act as an aminopropyl acceptor and *trans*-4-methylcyclohexylamine was considered to fit better to the putrescine binding site than cyclohexylamine, we

Table 1. Inhibition of spermidine synthase activity by cyclohexylamine-related compounds*

No.	Structure	IC_{50} (μM)	% Inhibition at 1 mM	No.	Structure	IC_{50} (μM)	% Inhibition at 1 mM
1		8.1		**14**		68	
2		103		**15**		>1000	33.2
3		>1000	40.4	**16**		>1000	8.2
4†		>1000	37.0	**17**		>1000	<1.0
5†		300		**18**		19	
6	 (*cis*)	430		**19**†		15	
7	 (*trans*)	1.7		**20**		30	
8†		330		**21**		>1000	18.5
9		>1000	29.1	**22**		>1000	<1.0
10		108		**23**		>1000	<1.0
11		>1000	<1.0	**24**		5.5	
12		>1000	<1.0	**25**		107	
13		>1000	<1.0				

* [Putrescine] = 1.0 mM, and [decarboxylated AdoMet] = 10 μM.
† A mixture of *cis* and *trans* isomers.

Table 2. Inhibition of spermidine synthase activity by alkylamines*

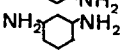
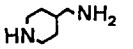
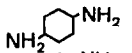
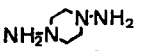
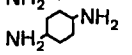
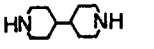
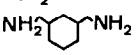
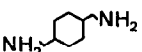
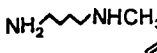
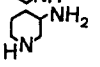
No.	Structure	IC_{50} (μM)	No.	Structure	IC_{50} (μM)
26		35	**33**		450
27		3.8	**34**		150
28		3.6	**35**		>1000
29		104	**36**		45
30		>1000	**37**		20
31		1000	**38**		7.8
32		250			

* [Putrescine] = 1.0 mM, and [decarboxylated AdoMet] = 10 μM.

speculated that the probable conformation of putrescine at the active site was one stretching in the 1,4-direction of cyclohexylamine. In such a conformation, the amino group of putrescine to be alkylated by the aminopropyl donor should be situated near the methyl group of *trans*-4-methylcyclohexylamine, perhaps as a non-protonated form in a hydrophobic environment. This led us to depict model A, an active site of spermidine synthase with decarboxylated AdoMet, on the basis of a sequential SN_2 reaction [29] (Fig. 1A).

Design of new inhibitors for spermine synthase and their effectiveness. Model A for spermidine synthase suggested model B for spermine synthase (Fig. 1), assuming that the aminopropyl transfer reaction would be similar in both enzymes. Model B has an additional cavity for the binding of the 1,3-diaminopropane moiety to the putrescine binding site of spermidine synthase. The importance of the aminopropyl moiety of spermidine for efficient binding to spermine synthase has already been suggested [1, 30]. Hence, N-aminopropylation of the above-mentioned compounds exhibiting potent inhibition for spermidine synthase was thought to be a reasonable way of obtaining new inhibitors of spermine synthase. A series of compounds was prepared to test their inhibitory effects on spermine synthase activity and also the validity of the two models. The assay conditions are given in Materials and Methods. Purified spermine synthase from rat brain was used, and the results are summarized in Table 4. As expected, all the aminopropyl derivatives of the compounds which showed low IC_{50} values in the inhibition of spermidine synthase, i.e. compounds **56**, **57**, **59**, **60**, **61**, **63** and **64**, potently inhibited spermine synthase activity, but had no inhibitory effect on spermidine synthase at 100 μM. Among them, *N*-(3-aminopropyl)cyclohexylamine, com-

Table 3. Screening of potential diamine substrates for spermidine synthase

No.	Structure	% MTA production*	No.	Structure	% MTA production
39†		<1.0	48		<1.0
40†		<1.0	49		2.7
41‡	 (*cis*)	3.0	50		<1.0
42	 (*trans*)	11.6	51†		<1.0
43†		<1.0	52		<1.0
44†		<1.0	53		100
45		<1.0	54		18
46		<1.0	55		<1.0
47		<1.0			

* MTA production at a 1 mM concentration of diamines in the presence of 10 μM decarboxylated AdoMet.

† A mixture of *cis* and *trans* isomers.

‡ This preparation contained 2% of *trans* isomer **42**.

Fig. 1. Active site models of aminopropyltransferases. (A) spermidine synthase, and (B) spermine synthase. The shaded region represents a hydrophobic area.

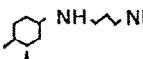
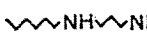
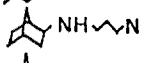
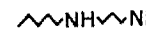
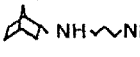
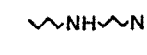
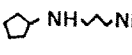
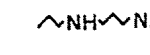
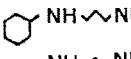
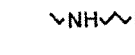
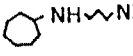
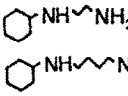
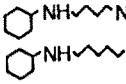
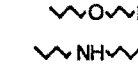
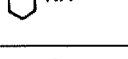
pound **60**, showed the lowest IC_{50} value. Through inspection of a series of related aminoalkyl derivatives of cyclohexylamine, compounds **67–69**, the presence of the aminopropyl moiety was further confirmed to be important. In addition, the importance of the terminal nitrogen of the aminopropyl group and of the secondary amino nitrogen for effective inhibition was respectively demonstrated by comparison of IC_{50} values of compounds **64** and **71**, and those of compounds **60** and **17**, or compounds **64** and **70**. Kinetics studies of *N*-(3-aminopropyl)cyclohexylamine showed that it inhibited spermine synthase activity competitively with respect to spermidine. In addition, the inhibition was not affected by increased concentrations of decarboxylated AdoMet in the assay (Fig. 2), further suggesting that the compound displays tight affinity at the spermidine binding site of spermidine synthase. All the other potent inhibitors described above were also shown to be competitive with respect to spermidine. These findings show the validity of model B as a representation of the active site of spermine synthase.

Comparison of the new spermine synthase inhibitors with dimethyl(5'-adenosyl)sulfonium perchlorate. The present inhibitors were compared with a known inhibitor, dimethyl(5'-adenosyl)sulfonium perchlorate, which is quite specific toward spermine synthase and capable of blocking the synthesis of spermine in SV-3T3 cells [8]. The relative activities to the control measured at different concentrations of each inhibitor in the presence of a 10–50 μM concentration of decarboxylated AdoMet are shown in Fig. 2, where the vertical line is expressed by a logit scale and the horizontal line by a log scale. The parallel lines for the dimethyl sulfonium compound depend on increased concentrations of decarboxylated AdoMet in the assays. On the other hand, the present inhibitors were not influenced by the concentrations of the nucleoside substrate, again strongly supporting the idea that they compete for the spermidine binding site of the enzyme. The IC_{50} value for the dimethyl sulfonium compound was about 30 μM under the present standard assay conditions containing 10 μM decarboxylated AdoMet. This value was about two orders of magnitude higher than those of the inhibitors presented here.

DISCUSSION

The present findings on the putrescine binding site of spermidine synthase led to the proposal of model A (Fig. 1) for the active site, in which a relatively large hydrophobic cavity lies adjacent to a negatively charged site, as schematically shown. Interestingly, the hydrophobic region is not a narrow groove to

Table 4. Inhibition of spermine synthase by *N*-(3-aminopropyl)alkylamine and related compounds*

No.	Structure	IC_{50} (μM)	No.	Structure	IC_{50} (μM)
56	 (*trans*)	0.50	**62**		>100
57		0.27	**63**		4.2
58		10	**64**		0.42
59		7.5	**65**		11
60		0.17	**66**		>100
61		1.1			
67		37	**17**		>100
68		7.0	**70**		>100
69		>100	**71**		>100

* [Spermidine] = 1.0 mM, and [decarboxylated AdoMet] = 10 μM.

accommodate the alkyl chain of putrescine, but a wide space capable of including a 4-methylcyclohexane or norbornane ring. A relatively large cavity may be a common structure for effectively binding a linear molecule with a high degree of freedom in its conformation, like putrescine. With this in mind, the polyamine binding sites of other proteins should also be examined. Another important proposal of this model is that the amino group of putrescine, which accepts the aminopropyl group, is located in the hydrophobic cavity. This comes from the interesting substrate and inhibitor properties of *trans*-1,4-diaminocyclohexane and *trans*-4-methylcyclohexylamine respectively. As described above, it is reasonable from the SN_2 mechanism for aminopropyl transfer reaction that the amino group of putrescine be present as a non-protonated form in a hydrophobic environment. This is consistent with the observation (unpublished data) that the K_m value for putrescine at pH 8.0 was a quarter of that at pH 7.2 in assays using a homogeneous rat enzyme. Another amino group of putrescine, after protonation, should bind electrostatically to the negatively charged site. Comparison of IC_{50} values of cyclohexylamine and aniline, or those of aminomethylcyclohexane and benzylamine should be helpful for understanding the situation. Namely, the IC_{50} value of cyclohexylamine was about a hundred-fold lower than that of aniline (Table 1), whereas the value of aminomethylcyclohexane, compound **14**, was half of the value (150 μM) of benzylamine. These results suggest that the hydrophobic cavity can accept not only an aliphatic cyclohexane ring, but also aromatic benzene, and that the aliphatic primary amino group with higher basicity than the aromatic one plays a role in the elevated affinity of the interacting compound. Model B (Fig. 1) for spermine synthase, which includes an additional cavity for the binding of the aminopropyl moiety of spermidine substrate, was formulated based on our findings. The aminopropylated derivatives of the inhibitors for spermidine synthase, prepared on the basis of model B, were all found to be potent inhibitors for spermine synthase. These results suggest that both models are valid, and the active sites of the two enzymes are closely related, thus arousing evolutionary interest.

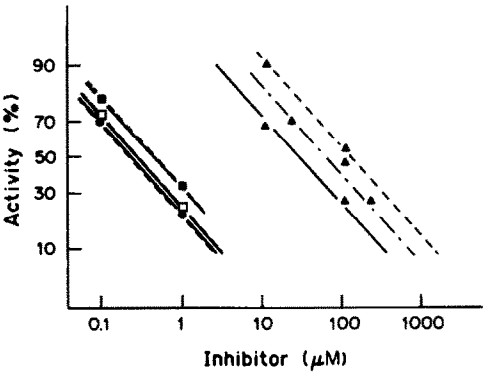


Fig. 2. Comparison of the present inhibitors for spermine synthase with dimethyl(5′-adenosyl)sulfonium. The inhibitory effects of compound **57** (□), **60** (●), **64** (■) and dimethyl (5′-adenosyl)sulfonium (▲) on purified spermine synthase (3 pmol/min) were compared in the presence of 10 μM (——), 20 μM (– – –) or 50 μM (------) decarboxylated AdoMet and 1.0 mM spermidine. The vertical line is expressed by logit scale and the values are percentages of the remaining activities in the presence of various concentrations of the tested compound.

Several new potent inhibitors for spermine synthase (Table 4) were developed based on the present findings. One of them was *N*-(3-aminopropyl)butylamine, compond **64**, which has

been reported recently by Baillon *et al.* [31]. Their approach to the inhibitor was as follows. Using various compounds with a fluorine atom in place of a hydrogen atom of the methylene chain of spermidine, they found [32] that protonation of the terminal nitrogen of the propylamino group and of the central amine function of spermidine was essential for efficient binding to the enzyme active site, and that a decrease of the pK_a value of the terminal butylamino group was correlated with an increase of binding affinity, which agrees with the presence of a non-protonated amino group of spermidine in model B. These findings led them to synthesize a series of N-alkylated derivatives of 1,3-diaminopropane. Their work proceeded from the need for a 1,3-diaminopropane binding site to the existence of a hydrophobic region, while ours proceeded in the opposite direction, with characterization of the hydrophobic cavity, as described above, in an effort to clarify the active site of spermidine synthase.

Our work offers a basic knowledge of the active site of aminopropyltransferase as well as the new inhibitors. Our model should be useful for designing compounds which bind irreversibly to the active site and can also be used to study the fine structures of the site. The new competitive inhibitors directed to the polyamine binding site of aminopropyltransferases were potent and not influenced by the high concentration of decarboxylated AdoMet *in vitro* as was expected. *In vivo* studies of these inhibitors are now underway.

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