Effects of Ethyl and Benzyl Analogues of Spermine on Escherichia coli Peptidyltransferase Activity, Polyamine Transport, and Cellular Growth

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Various ethyl and benzyl spermine analogues, including the anticancer agent N\textsubscript{4},N\textsubscript{12}-bis(ethyl)spermine, were studied for their ability to affect the growth of cultured Escherichia coli cells, to inhibit \[^{3}H\]\putrescine and \[^{3}H\]\spermine uptake into cells, and to modulate the peptidyltransferase activity (EC 2.3.2.12). Relative to other cell lines, growth of E. coli was uniquely insensitive to these analogues. Nevertheless, these analogues conferred similar modulation of in vitro protein synthesis and inhibition of \[^{3}H\]\putrescine and \[^{3}H\]\spermine uptake, as is seen in other cell types. Thus, both ethyl and benzyl analogues of spermine not only promote the formation and stabilization of the initiator ribosomal ternary complex, but they also have a sparing effect on the Mg\textsuperscript{2+} requirements. Also, in a complete cell-free protein-synthesizing system, these analogues at low concentrations stimulated peptide bond formation, whereas at higher concentrations, they inhibited the reaction. The ranking order for stimulation of peptide-bond formation by the analogues was N\textsubscript{4},N\textsubscript{12}-dibenzylspermine > N\textsubscript{4},N\textsubscript{2}-bis(ethyl)spermine \equiv N\textsuperscript{1}-ethylyspermine > N\textsubscript{4},N\textsubscript{12}-bis(ethyl)spermine, whereas the order of analogue potency regarding the inhibitory effect was inverted, with inhibition constant values of 10, 3.1, 1.5, and 0.98 \(\mu\)M, respectively. Although the above analogues failed to interact with the putrescine-specific uptake system, they exhibited high affinity for the polyamine uptake system encoded by the potABCD operon. Despite this fact, none of the analogues could be internalized by the polyamine transport system, and therefore they could not influence the intracellular polyamine pools and growth of E. coli cells.

The polycationic polyamines putrescine, spermidine, and spermine are present in all living organisms and engage in noncovalent interactions with a wide variety of cellular targets, including nucleic acids, proteins, and phospholipids (26, 29, 39). These interactions affect various processes of cell growth. For instance, preferential stimulation or inhibition of the in vivo synthesis of specific proteins is one of the important functions of polyamines in cell growth and regulation of differentiation (see reference 14 and references therein). Due to its four positive charges at physiological pH, spermine is the most effective of the naturally occurring polyamines both in regulating the in vitro translation process at several levels and in decreasing (but not abolishing) the Mg\textsuperscript{2+} requirements for protein synthesis (8, 11, 25–27, 39). We have previously demonstrated (3) that in an Escherichia coli cell-free system, spermine at 6 mM Mg\textsuperscript{2+} displays a concentration-dependent allosteric biphasic activity on ribosomal peptidyltransferase. In agreement with this, accumulation of excess polyamines causes inhibition of cell growth or a decrease in cell viability, primarily through inhibition of protein synthesis (9). On the other hand, blockage of polyamine synthesis by mutations or by inhibitors leads to a virtual cessation of growth, unless exogenous polyamines are provided (29). Accumulating evidence suggests that these inhibitors may be useful therapeutic agents for treatment of a variety of diseases, including cancer (23, 29).

N\textsuperscript{3},N\textsuperscript{12}-Bis(ethyl)spermine, the most active derivative in depleting intracellular polyamine pools, not only negatively reg-
flexibility, as determinants of these bacterial functions. Since acetyl polyanimes per se have no pharmacological significance, it was of interest to extend our knowledge by using a series of spermine analogues which are known to have an antiproliferative effect on eukaryotic cells. An investigation of their effects on prokaryotic cells could have an impact not only on basic research, but also on interpretation of potential symbiotic relationships between prokaryotic and eukaryotic cells.

MATERIALS AND METHODS

Materials. GTP (disodium salt), poly(U), ATP (disodium salt), phenylalanine, puromycin dihydrochloride, heterogeneous tRNA from E. coli W, spermine tetrahydrochloride, N^1-acetyl spermine, and vitamin B1 were obtained from Sigma Chemical Co. (St. Louis, Mo.). 1-Phenyl-[2-3H]alanine, [1,4-14C]puresine dihydrochloride were purchased from Amersham (Arlington Heights, Ill.). Cellulose acetate filters (type Sepaphore III) were obtained from Gelman Sciences (Ann Arbor, Mich.), and cellulose nitrate filters (type HA, 24-mm diameter, 0.45-μm pore size) were obtained from Millipore (Bedford, Mass.). Casamino Acids were obtained from Difco Laboratories (Detroit, Mich.).

Drugs. N^2-Ethyl spermine, N^2,N^6-bis(ethyl)spermine, and N^2,N^6-bis(ethyl) spermine were synthesized by reduction of the corresponding acetyl analogues of spermine with lithium aluminium hydride for 12 h in refluxing tetrahydrofuran (THF). N^2-Acetyl spermine, N^2,N^4-diacetyspermine, N^2,N^4-diacetyl spermine, and N^2,N^4-dibenzyloxy spermine were synthesized by the application of a general methodology (22), with N-trifluoroacetyl amino acids used as building blocks.

Biochemical preparations. Salt-washed (0.5 M NaCl) and polyamine-depleted ribosomes were obtained from E. coli B cells, as described previously (15). Partially purified translation factors (FWR fraction) and crude acetyl-[Ac]^1[^H]Phe-tRNA charged with 16.3 pmol of [3H]Phe (86 kcpm total) per A_{260} unit were prepared as reported previously (15). Complex C, i.e., the Ac[^H]Phe-tRNA-poly(U)-ribosome complex, was prepared and purified through adsorption on cellulose nitrate filters, as described elsewhere (18). The radioactivity trapped on the filters was counted in a liquid scintillation spectrometer. Controls without poly(U) were included in each experiment, and the values obtained were subtracted.

Cell culture. E. coli B cells were grown aerobically in M9 medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl), supplemented with 0.03 mM FeCl_3, 0.1 mM CaCl_2, 1 mM MgSO_4, 0.01 mM vitamin B1, 0.6% glucose, and 0.1% Casamino Acids, at 37°C in shaking Erlenmeyer flasks. Polamine analogues (100 μM, total concentration) were added at the time of culture initiation, and growth was followed by measuring the A_{600}.

Peptide bond formation assay and first-order analysis.

Cell-free system. The effects of spermine analogues and spermine on the functions of complex C were examined by using a cell-free system by using the puromycin reaction as a model reaction (38).

Effect of spermine analogues on ribosomal functions. (i) Effect on the stability of complex C. The stability of complex C, i.e., the AcPhe-tRNA-poly(U)-ribosome complex, was examined by incubation of complex C at 25°C with or without spermine analogues and monitoring the percentage of surviving complex C. In accordance with previous results (16, 18), we observed that 58% of complex C formed in the absence of translation factors (FWR fraction) was inactivated after 20 min of incubation in the absence of spermine or spermine analogues. However, the stability of complex C was greatly enhanced by the presence of spermine analogues, in a dose-dependent manner. Complex C formed in the presence of the FWR fraction exhibited high stability, and therefore the presence of spermine analogues caused only a slight improvement.

(ii) Effect on poly(U)-directed Ac[^H]Phe-tRNA binding to ribosomes. In the absence of spermine or spermine analogues, the optimum concentration of Mg^{2+} for AcPhe-tRNA binding to poly(U)-programmed ribosomes has been found in our laboratory to equal 10 mM (see reference 3 and references therein). Addition of spermine or spermine analogues at each optimal concentration resulted in a lowering of the Mg^{2+} optimum to about 6 mM (data not shown). When translation factors were omitted from the standard reaction mixture (Fig. 2A), addition of spermine or spermine analogues at 6 mM Mg^{2+} caused substantial stimulation of AcPhe-tRNA binding. The stimulatory effect was not moderated when the reaction mixture included the FWR fraction (Fig. 2B).

(iii) Effect on peptide bond formation. The effects of spermine and spermine analogues on peptide bond formation were compared in an E. coli cell-free system by using the puromycin reaction as a model reaction (38).

The effects of spermine analogues and spermine on the extent of puromycin reaction resembled each other and depended on the experimental conditions under which complex C was formed. When complex C was formed in a complete reaction mixture (containing FWR), all analogues examined appeared to have diminished activity, whereas, when complex C was formed in the absence of the FWR fraction, the extent of peptide bond formation was elevated by spermine analogues and spermine to a comparable degree. For instance, the extent of peptide bond formation was raised from 21% to 60% by increasing the concentration of N^2-ethyl spermine from zero to 0.2 mM. Based on the concept that the extent of puromycin reaction is a reliable measurement of the AcPhe-tRNA distribution between the ribosomal A site and P site (33), the data support the notion that during complex C formation, the analogues enhance the binding of AcPhe-tRNA at both P and A sites, the former being more stimulated.

In accordance with previous results obtained with spermine (3), polamine analogues inhibited AcPhe-puromycin synthesis.

\[ k_{\text{on}} = \frac{k_{\text{cat}}[S]}{K_s + [S]} \]
carried out with complex C formed in the absence of translation factors. Detailed kinetic analysis revealed that again the inhibition is of partial noncompetitive type (34), with one molecule of ligand involved in the mechanism of inhibition. Therefore, the CSI complex is catalytically active, but with a lower rate constant \(k_{93}\) than CS. The dissociation constant \(K_i\) and the \(b(k_{93}/k_3)\) values obtained from the corresponding 1/\(D\) intercept replots (not shown) are summarized in Table 1.

In experiments carried out with complex C formed in the presence of translation factors, the kinetic pattern of spermine analogue impact on peptidyltransferase activity was more complicated, but resembled that of spermine (3). As shown in Fig. 3, there was a biphasic dose response: concentrations up to a certain limit for each analogue stimulated peptidyltransferase activity, with higher concentrations being inhibitory. Among the analogues, \(N^4,N^9\)-dibenzyl spermine exhibited the strongest stimulatory effect in a broad concentration range. As shown in Fig. 3, the \(k_{\text{max}}\) value increased and peaked at 100 \(\mu\)M with 67% enhancement of its value, approaching an inhibitory phase at around 500 \(\mu\)M. Based on detailed kinetic analysis, similar to that applied for the interpretation of the spermine-bimodal action (3), we concluded that the kinetic scheme of Fig. 1 can adequately explain the present results. It is noteworthy that the resulting fit of theoretical curves to the experimentally measured curves was greatly satisfying. The values of the kinetic parameters, obtained from the best fits, are presented in Table 2.

**Polyamine uptake.** According to recent observations in our laboratory (17), spermine transport in *E. coli* cells obeys simple Michaelis-Menten kinetics (Fig. 4, lower line) with \(V_{\text{max}}\) and \(K_i\) values of 83.3 \pm 2.4 nmol · min \(^{-1}\) per g of protein and 25.16 \pm 1.58 \(\mu\)M, respectively. Competitive inhibition of spermine uptake was observed when *E. coli* cells were treated with ethyl or benzyl analogues of spermine (Fig. 4). From the \(K_i\) values presented in Table 3, it is obvious that the carrier affinity is highest for \(N^4,N^9\)-dibenzyl spermine, then lower for \(N^1\)-ethyl spermine and \(N^3,N^9\)-bis(ethyl)-spermine, and lowest for \(N^1,N^{12}\)-bis(ethyl)spermine.

Consistent with previous results (17, 19), the Lineweaver-Burk plot of the initial putrescine uptake was biphasic (Fig. 5, lower line). This suggests that two independent transporters with different affinities for putrescine are involved. The \(K_i\) and \(V_{\text{max}}\) values for the first transport system (carrier 1) as well as the \(K_i\) and \(V_{\text{max}}\) values for the other system (carrier 2) are shown in Table 3. All of the analogues behave as competitive inhibitors. However, as indicated by the \(K_i\) values shown in

FIG. 1. Kinetic model for AcPhe–puromycin synthesis carried out in the presence of spermine analogues, with complex C formed in the presence of the FWR fraction. C, complex C; S, puromycin; I and I (A), spermine analogue bound to the inhibition site and activation site of complex C, respectively; C', CI', CI(A), and CI(A)', ribosomal complexes after their reaction with puromycin.
Table 3, they failed to inhibit the high-affinity putrescine uptake system (carrier 2). In contrast, they exhibited significant effectiveness in competing with putrescine for carrier 1, the ranking order once again being \(N^4,N^9\)-dibenzylspermine, followed by \(N^1\)-ethylspermine and \(N^4,N^9\)-bis(ethyl)spermine, and then \(N^1,N^{12}\)-bis(ethyl)spermine (Table 3).

Effect of spermine analogues on *E. coli* cell growth and polyamine pools. The effects of spermine analogues on the growth of cultured *E. coli* cells were screened at 100 mM. Surprisingly, none of the analogues examined showed any measurable effect, either stimulatory or inhibitory, on the growth of this strain. At first sight, these results seem to be in contradiction to the effects of analogues on the polyamine uptake system and peptidyltransferase activity. It is known from several reports (23, 29, 30, 35) that some of the analogues examined exert their action after internalization into the cells. In addition, inhibited uptake of \([14C]\)putrescine or \([14C]\)spermidine is usually taken as a criterion for the ability of the inhibitor to use the polyamine transporters. However, it could not be discounted that one or the other of the spermine analogues men-

<table>
<thead>
<tr>
<th>Effector (\delta)</th>
<th>(k_+^\prime) (min(^{-1}))</th>
<th>(K_s^\prime) ((\mu)M)</th>
<th>(K_i^\prime) ((\mu)M)</th>
<th>(\beta^\prime)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1.11</td>
<td>660</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPM</td>
<td>118</td>
<td>0.165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N^1)-MESPM</td>
<td>138</td>
<td>0.138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N^4,N^{12})-BESPM</td>
<td>225</td>
<td>0.360</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N^4,N^9)-BESPM</td>
<td>222</td>
<td>0.345</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N^4,N^9)-DBzSPM</td>
<td>130</td>
<td>0.522</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\delta\) SPM, spermine; \(N^3\)-MESPM, \(N^3\)-ethylspermine; \(N^4,N^{12}\)-BESPM, \(N^4,N^{12}\)-bis(ethyl)spermine; \(N^5\)-BESPM, \(N^5\)-bis(ethyl)spermine; \(N^4,N^9\)-DBzSPM, \(N^4,N^9\)-dibenzylspermine.

\(\beta \) The \(k_+^\prime\) and \(K_s^\prime\) values were calculated by fitting the experimental data to equation 2 by a least-square procedure provided by Microcal Software, Inc.

\(\beta \) The \(K_i^\prime\) and \(\beta\) values were obtained from the corresponding 1/\(D\) intercept replots.

FIG. 2. Effect of spermine and spermine analogues on poly(U)-directed Ac\([3H]\)Phe-tRNA binding to ribosomes. The binding mixture (25 \(\mu\)l) was prepared in the absence (A) or presence (B) of the FWR fraction (10 \(\mu\)g of protein) and contained 100 mM Tris-HCl (pH 7.2), 100 mM NH\(_4\)\(_2\)SO\(_4\), 25.6 \(A_{260}\) units of washed ribosomes per ml, 320 \(\mu\)g of poly(U) per ml, and, finally, spermine (\(\oplus\)), \(N^4,N^9\)-dibenzylspermine (\(\bigotimes\)), \(N^1\)-ethylspermine (\(\bigotimes\)), \(N^4,N^9\)-bis(ethyl)spermine (\(\triangle\)), or \(N^1,N^{12}\)-bis(ethyl)spermine (\(\Delta\)) at the indicated concentrations. The time course of the reaction was monitored up to 30 min at 25°C. The values of bound Ac\([3H]\)Phe-tRNA were estimated from the maximum level of binding curves.

FIG. 3. Variation of \(k_{\text{max}}/k_3\) as a function of spermine-analogue concentration. Complex C, formed in the presence of the FWR fraction at spermine or spermine analogue concentrations such as those used during the puromycin reaction, was adsorbed on a cellulose nitrate filter, washed, and then reacted with the appropriate concentration of puromycin in reaction buffer containing 6 mM Mg\(^{2+}\) and spermine (\(\oplus\)), \(N^4,N^9\)-dibenzylspermine (\(\bigotimes\)), \(N^1\)-ethylspermine (\(\bigotimes\)), or \(N^1,N^{12}\)-bis(ethyl)spermine (\(\triangle\)) as indicated. The \(k_{\text{max}}\) value was calculated by fitting the experimental data to equation 3 by nonlinear regression. Similarly, the \(K_i\) and \(k_3\) values for the control experiment (no amine added) were estimated by using equation 2 and were found to be 660 \(\pm\) 58 \(\mu\)M and 2.10 \(\pm\) 0.22 min\(^{-1}\), respectively.
tion may block the transporters without being efficiently internalized. The ability of E. coli cells to accumulate spermine analogues was thus determined. The results revealed that none of the analogues is able to penetrate the plasma membrane. Only trace amounts of \(N^4,N^9\)-dibenzylspermine could be recovered in E. coli cells after a 24-h incubation with 100 \(\mu\)M, despite the high potency of this analogue as an uptake antagonist. Also, none of the analogues altered the intracellular concentrations of polyamines or the putrescine/spermidine molar ratio. The parent compound, spermine, exhibited the same behavior, although it was accumulated in cells at a concentration of 22 \(\mu\)mol/g of protein.

**DISCUSSION**

The use of alkyl and aryllic derivatives of spermine for the chemotherapy of cancer and several parasitic diseases, including trypanosomiasis and leishmaniasis, has been greatly increased in the past few years (23). \(N^4,N^9\)-Bis(ethyl) analogues of spermine have been of particular interest, and some members of this family are now in clinical trials. Given that the fate of certain pathogenic bacteria in animal infection is important in both fundamental and applied microbiology, we decided to explore the ability of a series of spermine analogues to affect the growth of cultured E. coli cells, to inhibit putrescine and spermine transport into cells, and to modulate several ribosomal functions.

We have previously demonstrated that the effects of spermine on protein synthesis are exerted at both the stages of initiation and elongation and that the charge distribution, as well as the chain flexibility of the ligand, plays a crucial role in its mode of action (3, 16, 18). From the present results, it is evident that the spermine analogues examined not only promote the formation and stabilization of the initiator ribosomal ternary complex, but also have a sparing effect on the \(Mg^{2+}\) requirements; although weaker, these effects are reminiscent of the behavior of spermine (3, 16). We have previously observed (18) that spermine selectively acylated at each amino group shows a much higher \(Mg^{2+}\) optimum, with a plateau at values above 9 mM. It is noteworthy that the analogues’ potential is inversely related to their \(K_i\) values, estimated by kinetic analysis (Table 1). This suggests that the ability of analogues to stimulate the binding may be closely related to their affinity for one or more constituents of complex C. The consequence of amino group ethylation to the analogue’s inhibitory activity is quite evident by the \(K_i\) and \(\beta\) values shown in Table 1; in going from bis-ethylated compounds to spermine, the ability of each analogue to interact with complex C increases (decrease in \(K_i\) values), whereas the reactivity of the CSI complex is concomitantly reduced (decrease in \(\beta\) values). It may be possible that the ethyl substituents at the nitrogens of spermine compromise some electrostatic interactions by increasing the distance between the charged sites of this compound and group of anions fixed to complex C. Thus, one might expect \(N^4,N^9\)-dibenzylspermine to bind more poorly than \(N^4,N^9\)-bis(ethyl)spermine. However, the \(K_i\) value of \(N^4,N^9\)-dibenzylyspermine was about twofold smaller than that obtained with the second analogue. A reason for such behavior may be the existence of a hydrophobic pocket at the binding site on complex C which helps ligand anchoring. Alternatively, a restricted freedom of rotation around the neighboring carbon and nitrogen atoms, due to the introduction of benzyl groups, may stabilize the positive charges on secondary nitrogens and allow the development of strong hydrogen bond links. The second explanation is consistent with the finding of Frydman et al. (5) that the secondary amines of spermine bind more strongly to tRNA than the more electropositive primary amino groups. Despite the comparable \(K_i\) values between \(N^4,N^9\)-dibenzylyspermine and spermine, the analogue behaved as a weaker inhibitor, since it has its high \(\beta\) value that increases the reactivity of the corresponding CSI complex.

**TABLE 2.** Kinetic parameters of AcPhe-puromycin synthesis carried out in the presence of spermine analogues, with complex C formed in the presence of the FWR fraction

<table>
<thead>
<tr>
<th>Effector*</th>
<th>([I]_{opt}) (mM)</th>
<th>(\beta(I)^2/\Delta [E]_tK_i)</th>
<th>(\beta)</th>
<th>(\beta(K_i)) (mM (^{-1}))</th>
<th>(K_i(d)) (mM)</th>
<th>(\frac{[I]_{opt}}{[E]}_t)</th>
<th>% Relative activity of peptidyltransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.050</td>
<td>0.443</td>
<td>0.448</td>
<td>1.100</td>
<td>3.20</td>
<td>2.340</td>
<td>100</td>
</tr>
<tr>
<td>SPM</td>
<td>0.050</td>
<td>0.443</td>
<td>0.448</td>
<td>1.100</td>
<td>3.20</td>
<td>2.340</td>
<td>100</td>
</tr>
<tr>
<td>(N^4)-MESPM</td>
<td>0.050</td>
<td>0.093</td>
<td>0.200</td>
<td>0.844</td>
<td>3.60</td>
<td>1.107</td>
<td>1.50</td>
</tr>
<tr>
<td>(N^4,N^9)-BESPM</td>
<td>0.025</td>
<td>0.082</td>
<td>0.450</td>
<td>1.510</td>
<td>3.47</td>
<td>0.277</td>
<td>0.98</td>
</tr>
<tr>
<td>(N^4,N^9)-9-DBzSPM</td>
<td>0.060</td>
<td>0.130</td>
<td>0.345</td>
<td>1.478</td>
<td>3.08</td>
<td>0.931</td>
<td>3.10</td>
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<tr>
<td>(N^4,N^9)-DBzSPM</td>
<td>0.100</td>
<td>0.168</td>
<td>0.300</td>
<td>1.90</td>
<td>2.000</td>
<td>10.00</td>
<td>167</td>
</tr>
</tbody>
</table>

*Abbreviations of effectors are defined in Table 1.
In experiments carried out in the presence of the FWR fraction, addition of spermine analogue had a dose-dependent biphasic effect on peptide bond formation (Fig. 3). These results suggest that there are two separate binding sites for analogues on complex C, an activation site and an inhibition site. Once again, the polyamine analogues mimic the functions of spermine (3). The potency of each ligand depends on several features. Thus, N°,N°-substitution affords a high affinity to the ligand for the inhibition site (low K value), but dramatically lowers the stimulatory effect of ligand on peptidyltransferase activity (low α value). This is in accordance with observations in rabbit reticulocyte translation systems (10, 36). In contrast, derivatization of internal amines by ethyl or benzyl groups has only a little influence on the ability of ligand to stimulate translation. Thus, the degree of peptidyltransferase activation by N°,N°-dibenzylspermine is similar to that obtained by spermine (Table 2), although the optimal concentration of analogue ([I]opt) is twofold greater than that of the parent compound. Taking into account that polyamines with terminal benzyl groups show diminished activity in protein-synthesizing eukaryotic systems (36), our results can be interpreted on the basis of restricted rotation effects described above.

Since all analogues tested behave as competitive inhibitors of polyamine uptake, full characterization of their potency can be made exclusively on the basis of the K value, independently of whether putrescine or spermine is used as a substrate. As shown in Table 3, the analogues exhibit similar K values for both the carrier of spermine and carrier 1 of putrescine, suggesting the existence, in fact, of a common transporter. Moreover, the K value of carrier 1 is in the vicinity of that reported for the putrescine transport system encoded by the potABCD operon (19). These findings, taken together, suggest that all spermine analogues examined compete for the PotD protein. Obviously, ethylation of the terminal amino groups decreases the affinity of ligand for the uptake system, whereas ethyl or benzyl substitution of N°,N°-imino groups can be greatly tolerated. All of the data presented above are consistent with the idea that in addition to the key role of cationic centers, specific hydrophobic interactions must also largely contribute to the affinity of analogues for polyamine transporter. Furthermore, none of the analogues tested behaved as competitive inhibitors of spermine in reference to the parent compound. Finally, it is important to point out that E. coli cells, in contrast to erythrocytes and other mammalian cells (2), do not appear to have a specific uptake system for bis(benzyl)polyamine analogues; both spermine and N°,N°-dibenzylyspermine compete for the PotD protein (Table 3).

Surprisingly, none of the analogues added to the medium affected E. coli cell growth. Even prolonged culture in the presence or absence of various ethyl and benzyl analogues of spermine (40), according to which PotF recruits a bulky side chain that protrudes deeply into the binding cavity. In conclusion, the polyamine uptake results in E. coli further support the mimetic character of ethyl and benzyl derivatives of spermine in reference to the parent compound. Finally, it is important to point out that E. coli cells, in contrast to erythrocytes and other mammalian cells (2), do not appear to have a specific uptake system for bis(benzyl)polyamine analogues; both spermine and N°,N°-dibenzylyspermine compete for the PotD protein (Table 3).

![Graph](http://jb.asm.org/Downloadedfromhttp://jp.assm.org/)

**FIG. 5.** Double-reciprocal plots of [14C]putrescine uptake by E. coli B cells, in the presence or absence of various ethyl and benzyl analogues of spermine. The incubation mixture of cells was prepared as described in the legend to Fig. 4. The [14C]putrescine uptake was determined in the absence of analogues (○) or in the presence of 100 nM N°-N°-bis(ethyl)spermine (□), N°-N°-bis(benzyl) spermine (△), N°-ethylspermine (○), and N°,N°-dibenzylyspermine (●). Inset, detail of the kinetic plots at high concentrations of [14C]putrescine.

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**TABLE 3.** Kinetic parameters of putrescine and spermine transport under the influence of various spermine analoguesa

<table>
<thead>
<tr>
<th>Effectorb</th>
<th>Spermine transport</th>
<th>Putrescine transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>None</td>
<td>25.16 ± 1.58</td>
<td>3.63 ± 0.43</td>
</tr>
<tr>
<td>N°-MESPM</td>
<td>59.66 ± 7.43</td>
<td>61.32 ± 2.90</td>
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<tr>
<td>N°,N°-BESPM</td>
<td>109.92 ± 13.48</td>
<td>92.60 ± 6.85</td>
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<tr>
<td>N°,N°-BESPM</td>
<td>70.86 ± 8.50</td>
<td>65.15 ± 3.51</td>
</tr>
<tr>
<td>N°,N°-DBzESPM</td>
<td>23.72 ± 2.30</td>
<td>27.21 ± 5.12</td>
</tr>
</tbody>
</table>

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a The values are expressed as means ± standard deviations. In spermine transport, the V<sub>max</sub> value was 83.3 ± 2.4 nmol min<sup>-1</sup> g of protein<sup>-1</sup>. In putrescine transport, the V<sub>max</sub> and V<sub>max</sub> values were 220.5 ± 12.1 nmol min<sup>-1</sup> g of protein<sup>-1</sup> and 63.8 ± 5.1 nmol min<sup>-1</sup> g of protein<sup>-1</sup>, respectively.

b Abbreviations of effectors are defined in Table 1.
presence of 100 μM spermine analogue did not show any deviation from the behavior of untreated cells. Also, we failed to detect any effect of the spermine analogues on intracellular polyamine pools. Moreover, no spermine analogue was detected by HPLC analysis in exposed cells, nor did any intermediate product result from stepwise desubstitution and subsequent oxidative degradation (data not shown). Relatively, it has been demonstrated that N1,N2,N7-trialkyl analogues of spermine cannot be used as substrates in spermidine/spermine-N-acetyltransferase (SSAT)/polyamine oxidase (PAO)-catalyzed oxidation, even though they act as precursors (30, 31). In addition, SSAT and PAO are not as active in E. coli cells as they are in eukaryotic cells (7). Traces of N1,N2-dibenzylspermine found in E. coli cells after a 24-h incubation with this analogue can be attributed to secondary diffusion effects. In combination, these observations suggest that the spermine analogues examined, although endowed with high affinity for the E. coli polyamine transport system encoded by \textit{potABCD}, are not efficiently internalized into the cells. Consequently, they should be characterized as pure competitive antagonists of polyamine uptake. Thereby, the failure of SSAT induction in \textit{E. coli} cells by exogenous N1,N2,N7-tris(ethyl) spermine (7) seems to be reasonable: it is rather the lack of permeation of analogue through the \textit{E. coli} membrane which explains the above effect, not the inability per se to induce the enzyme.

Distinct structure-activity relationships relevant to the spermin molecule have become apparent during this study. Our findings also reinforce the notion that high heterogeneity of polyamine transport-deficient mutants of \textit{Escherichia coli} and cloning of the genes for polyamine transport proteins. J. Biol. Chem. 265:20893–20897.


