Biochem. J. (1991) 274, 167–171 (Printed in Great Britain)

Role of unsaturated derivatives of spermidine as substrates for spermine synthase and in supporting growth of SV-3T3 cells

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Synthetic unsaturated analogues of the natural polyamines were examined as possible substrates for spermine synthase and as replacements for spermidine in supporting the growth of SV-3T3 cells. It was found that *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene [the *cis* isomer of the alkene analogue of spermidine] was a good substrate for spermine synthase, but that the *trans* isomer [*N*-(3-aminopropyl)-1,4-diamino-*trans*-but-2-ene] and the alkyne analogue [*N*-(3-aminopropyl)-1,4-diaminobut-2-yne] were not substrates. These results provide the first demonstration of stereospecificity in the spermine synthase reaction. All three of the unsaturated spermidine analogues described above and the *cis*-alkene analogue of spermine [N^1N^4-bis-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene] were able to support the growth of SV-3T3 cells that were prevented from the endogenous synthesis of spermidine by treatment with α-difluoromethylornithine. Since *N*-(3-aminopropyl)-1,4-diamino-*trans*-but-2-ene] and *N*-(3-aminopropyl)-1,4-diaminobut-2-yne were not converted into a spermine derivative, it is apparent that this conversion is not needed for the stimulation of growth. However, since N^1N^4-bis-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene was also able to support growth and was not degraded to the spermidine derivative, it appears that either polyamine can be effective in this respect. All of the unsaturated analogues tested accumulated in the SV-3T3 cells to a much greater extent than spermidine itself. This indicates that these compounds are substrates for the polyamine transport system, but that they are less effective than the natural polyamines in the feedback regulation of this system.

INTRODUCTION

The polyamines spermidine and spermine are essential for the growth of mammalian cells and may have vital roles in the maintenance of a number of cellular functions, although these have not yet been fully defined (Pegg & McCann, 1982; Tabor & Tabor, 1984; Pegg, 1986, 1988; Marton & Morris, 1987; Morgan, 1987). Many papers have described effects of polyamines on biochemical reactions *in vitro*, but it is difficult to establish the physiological significance of these observations, because the effects may result from non-specific interaction of the positively charged polyamines with acidic macromolecules such as nucleic acids, proteins and membranes. One possible approach to this problem is to attempt to define more closely the structural characteristics of the polyamines needed to support growth and to enter into the polyamine metabolic pathway. Our initial studies of a series of non-metabolizable polyamines showed that five *gem*-dimethylspermidines were able to support the growth of polyamine-depleted SV-3T3 cells (Nagarajan *et al.*, 1988). Other groups have reported on the properties of halogenated, hydroxylated or alkylated polyamine derivatives (Samejima & Nakazawa, 1980; Porter *et al.*, 1982, 1985, 1987; Sarhan *et al.*, 1987*a*,*b*; Baillon *et al.*, 1988; Dezeure *et al.*, 1988; Bergeron *et al.*, 1989; Mamont *et al.*, 1989). In general, while the *N*-substituted derivatives do not substitute for natural polyamines in growth, they nevertheless have significant pharmacological potential (Porter & Sufrin, 1986; Bergeron *et al.*, 1989; Casero *et al.*, 1989).

In the present work, we have examined the ability of unsaturated derivatives of spermidine to serve as substrates in aminopropyltransferase reactions and to act to reverse the effects of α-difluoromethylornithine (DFMO), an inhibitor of polyamine production.

MATERIALS AND METHODS

Materials

The polyamine derivatives tested are shown in Fig. 1 and were prepared as described by Nagarajan & Ganem (1987). Compound **3**, the *trans* isomer of the alkene analogue of spermidine contained a small amount of the *cis* isomer. DFMO was generously provided by Merrell Dow Research Institute, Cincinnati, OH, U.S.A. Radioactive materials were obtained from du Pont–New England Nuclear Products, Boston, MA, U.S.A. Biochemical reagents and polyamines were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Enzyme assays

Rat brain spermine synthase was isolated as previously described (Raina *et al.*, 1983; Pegg *et al.*, 1986) and its activity determined by measuring the production of 5′-methyl[^{35}S]thioadenosine from decarboxylated *S*-adenosyl[*methyl*-^{35}S]methionine (AdoMet) (Hibasami & Pegg, 1978). Activity was measured using spermidine as a control and in the presence of compounds **1**–**4**. The labelled product was separated on small columns of cellulose phosphate as described by Raina *et al.* (1983). The standard assay medium contained 5 μM labelled decarboxylated AdoMet (0.1–0.25 μCi), 0.5 mM-spermidine (or compounds **1**–**4**), 50 mM-sodium phosphate buffer, pH 7.2, and 0.1 mM-dithiothreitol in a total volume of 0.2 ml. Sufficient enzyme was added to ensure that 5–15000 c.p.m. was incorporated into product during a 30 min incubation at 37 °C with spermidine as acceptor.

Cell culture and polyamine analysis

SV-3T3 cells were maintained and transferred as described by

Abbreviations used: DFMO, α-difluoromethylornithine; AdoMet, *S*-adenosylmethionine.

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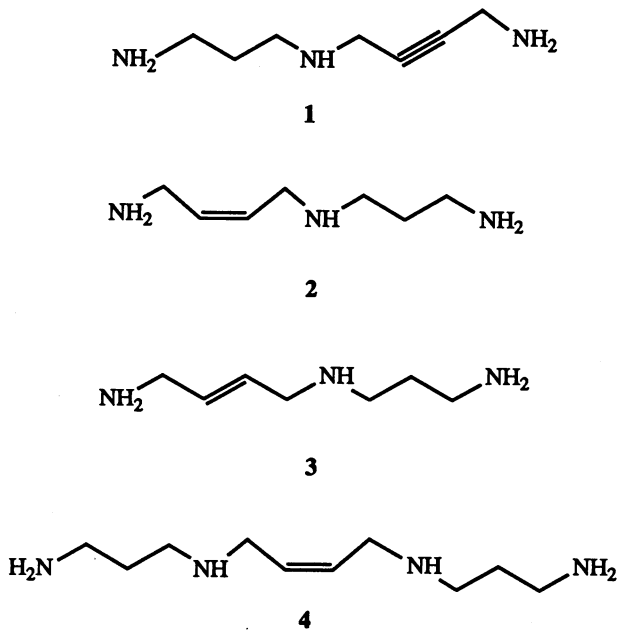

Fig. 1. Structures of polyamine derivatives 1–4

The compounds are: **1**, *N*-(3-aminopropyl)-1,4-diaminobut-2-yne; **2**, *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene; **3**, *N*-(3-aminopropyl)-1,4-diamino-*trans*-but-2-ene; **4**, N^1N^4-bis-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene.

Pegg (1984). They were plated at a density of 0.2×10^6 per dish in Dulbecco's modified Eagle's medium in the presence of 3% (v/v) horse serum, 2% (v/v) fetal-calf serum and 1 mM-aminoguanidine to prevent oxidation of the added polyamines. In some experiments, 5 mM-DFMO was added at the time of plating. Compounds **1–4** were added at the time and concentration shown in the legends to the Figures and Table. Intracellular polyamine concentrations were determined as previously described (Nagarajan *et al.*, 1988) using ion-paired reversed-phase h.p.l.c. and detection and quantification after post-column derivatization with *o*-phthalaldehyde (Seiler & Knödgen, 1985). The retention times for the polyamines were: **1**, 39.5 min; spermidine, 40.0 min; **3**, 40.5 min; **2**, 40.6 min; spermine, 48.7 min; and **4**, 49.3 min.

RESULTS

In order to examine the ability of unsaturated polyamine derivatives to substitute for their natural polyamine counterparts in cell growth, compounds **1–4** were tested for their ability to restore a normal growth rate in SV-3T3 cells in which polyamine synthesis was blocked by exposure to DFMO (Figs. 2*a*–2*d*). None of the compounds was quite as effective as spermidine itself, but all stimulated growth significantly when added at concentrations of 10 or 25 μM. Fig. 2 also shows that 10–25 μM concentrations of spermidine or of the analogues were needed to support growth in these cells, whereas addition of 1 μM was not sufficient.

The polyamine content in these cells is shown in Table 1. The alkyne analogue of spermidine, **1**, was not converted into any metabolites detectable by the h.p.l.c. analysis system used and, at concentrations of 10–25 μM, it was accumulated to much higher levels than spermidine itself. The fall in the spermine content of cells treated with **1** is consistent with the growth of these cells in the absence of spermine synthesis. Addition of **1** at 1 μM concentration, which did not support growth (Fig. 2*a*), was sufficient to provide an intracellular content of **1** equivalent to

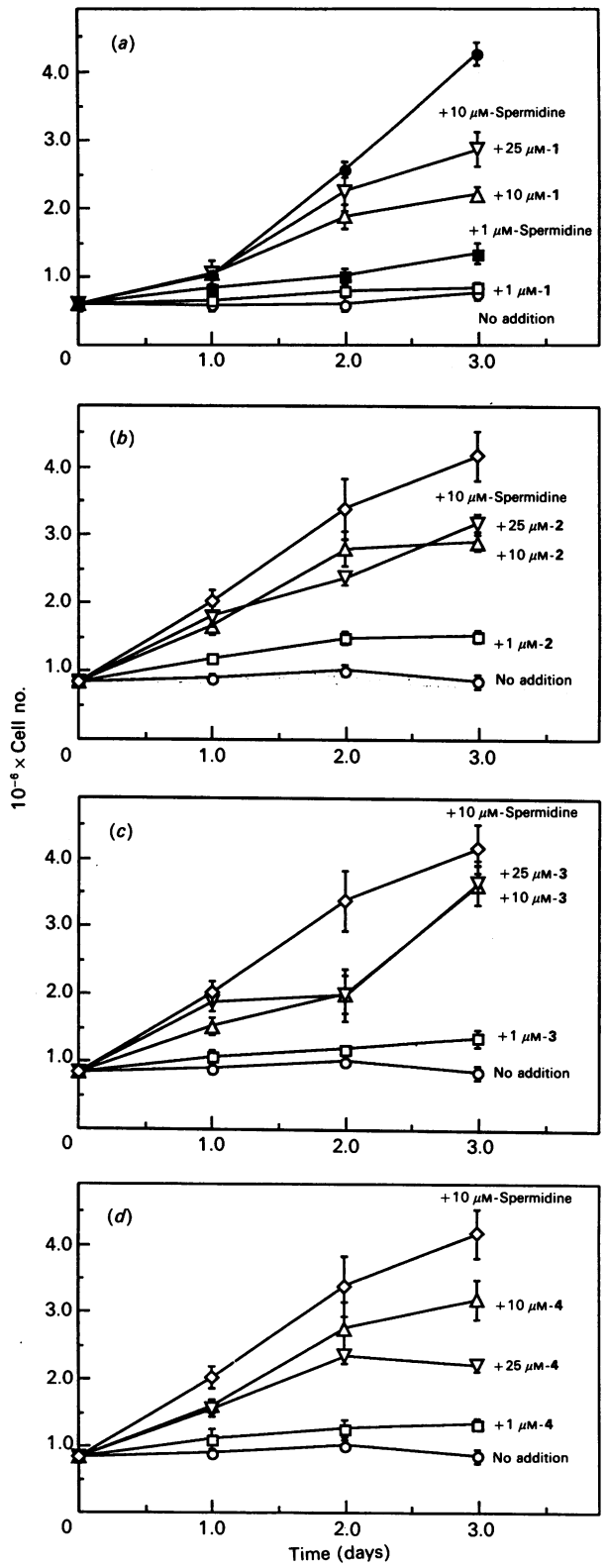

Fig. 2. Effect of spermidine and unsaturated polyamine derivatives on the growth of SV-3T3 cells in the presence of DFMO

Cells were grown for 72 h in the presence of DFMO and the medium was then changed to fresh medium containing 5 mM-DFMO and the other additions indicated. Cells were then counted at the time shown. Results are given as means ± S.D. Where no S.D. is shown, the variation was less than the size of the symbol. Results are shown in (*a*) for compound **1**, in (*b*) for compound **2**, in (*c*) for compound **3** and in panel (*d*) for compound **4**. Results with 10 μM-spermidine are shown in all panels for comparison, and results with 1 μM-spermidine are shown in (*a*).

Table 1. Levels of polyamines in DFMO-treated SV 3T3 cells after addition of unsaturated polyamines

Cells were grown for 72 h in the presence of DFMO, and the medium was then changed to fresh medium containing 5 mM-DFMO and the other additions indicated. The polyamine content was determined as described in the Materials and methods section in cells harvested at 48 and 72 h as indicated. The results are given as means ± S.D. for at least four estimations.

Addition* and concn. (μM)	Time (h)	Polyamine content (nmol/mg)		
		Spermidine or derivative	Spermine	**4**
None	48	< 0.05	11.1 ± 0.8	–
	72	< 0.05	10.9 ± 2.0	–
Spermidine				
1	48	< 0.05	18.4 ± 1.6	–
1	72	< 0.05	14.2 ± 2.8	–
10	48	16.8 ± 3.0	17.1 ± 3.1	–
10	72	14.4 ± 2.1	20.4 ± 1.9	–
Compound **1**				
1	48	13.8 ± 0.5 (**1**)	11.1 ± 0.4†	–
1	72	9.6 ± 0.5 (**1**)	9.2 ± 0.6†	–
10	48	47.2 ± 2.3 (**1**)	4.7 ± 0.4†	–
10	72	47.8 ± 1.9 (**1**)	5.3 ± 0.2†	–
25	48	93.1 ± 5.5 (**1**)	4.7 ± 0.7†	–
25	72	62.2 ± 3.5 (**1**)	4.7 ± 0.6†	–
Compound **2**				
1	48	< 0.05	8.1 ± 0.4	13.4 ± 0.7
1	72	< 0.05	7.0 ± 0.5	11.9 ± 0.4
10	48	14.2 ± 1.9 (**2**)	4.5 ± 0.5	18.6 ± 3.2
10	72	13.1 ± 2.1 (**2**)	4.1 ± 0.5	29.8 ± 1.3
25	48	45.6 ± 5.3 (**2**)	3.4 ± 0.4	9.4 ± 1.0
25	72	34.8 ± 5.1 (**2**)	3.7 ± 0.4	12.3 ± 0.9
Compound **3**				
1	48	8.4 ± 0.8 (**3**)	9.7 ± 0.3†	–
1	72	6.6 ± 0.6 (**3**)	8.9 ± 0.5†	–
10	48	33.9 ± 4.6 (**3**)	5.6 ± 0.6†	–
10	72	34.8 ± 0.9 (**3**)	6.7 ± 0.5†	–
25	48	48.7 ± 9.5 (**3**)	4.6 ± 0.8†	–
25	72	45.1 ± 1.0 (**3**)	5.7 ± 0.4†	–
Compound **4**				
1	48	< 0.05	9.1 ± 1.1	12.3 ± 1.7
1	72	< 0.05	7.6 ± 0.6	10.4 ± 0.7
10	48	0.9 ± 0.2	3.0 ± 0.5	54.9 ± 11.1
10	72	0.7 ± 0.2	2.9 ± 0.4	41.4 ± 1.9
25	48	0.3 ± 0.1	3.1 ± 0.7	83.4 ± 9.8
25	72	0.3 ± 0.1	1.2 ± 0.1	54.1 ± 4.5

* In all groups cells were grown in DFMO for 72 h to deplete internal polyamines before the start of the experiment.
† These values may include a spermine analogue which is not well resolved from spermine.

that of spermidine found in cells growing at a maximal rate after exposure to 10 μM-spermidine. This suggests that **1** is considerably less potent than spermidine in stimulating growth. Essentially similar results were found with the *trans* isomer of the alkene analogue of spermidine, **3**. However, the *cis* isomer, **2**, was efficiently converted into the *cis* alkene analogue of spermine, **4** (Table 1). This conversion was sufficiently active that, after exposure to 1 μM concentrations of **2**, there was no accumulation of **2** in the cells, but large amounts of **4** were present. The total of **2** and **4** in the cells treated with 10–25 μM-**2** was greater than the content of spermidine and spermine in cells treated with spermidine (Table 1). Cells treated with **4** accumulated large amounts of the *cis* alkene analogue of spermine and were able to grow with very low levels of spermidine and spermine.

Direct proof that **2** is able to serve as a substrate for spermine synthase was obtained by testing the ability of compounds **1**–**4** to serve as substrates in the reaction catalysed by the enzyme from rat brain. Measurement of the production of 5′-methylthioadenosine from decarboxylated AdoMet in this assay in the presence of 0.5 mM-**2** indicated that **2** had activity equivalent to about 35% of that of spermidine, that **1** and **4** were totally inactive and that **3** had about 3% of the activity of spermidine (results not shown). A more detailed investigation of the reaction as a function of the acceptor polyamine concentration is shown in Fig. 3. Activity with **3** is only manifest at high concentrations and is likely to be due to the minor contamination of **3** with **2**. The *cis* alkene analogue of spermidine, **2**, is clearly a substrate for the spermidine synthase, and the calculated $V_{max.}$ is about 80% of that for spermidine. The apparent K_m value for **2** is 0.7 mM, which is significantly higher than the value of 0.09 mM for spermidine in these experiments. However, it should be noted that these values are only crude approximations, and a full analysis over a wider range of conditions and concentrations of both substrates was not carried out because the kinetics of spermine synthase, which is very strongly inhibited by the 5′-methylthioadenosine product, are complex (Pegg *et al.*, 1981; Pajula, 1983). When tested in the presence of 0.025 mM-spermidine or 0.5 mM-spermidine as substrate, compounds **1**, **3** and **4** were not inhibitory to the spermidine synthase reaction (results not shown).

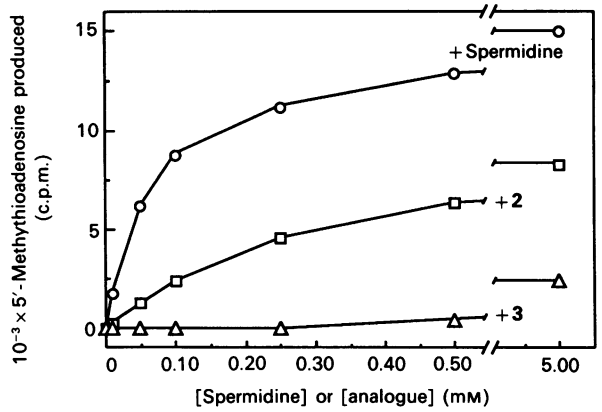

Fig. 3. Ability of spermidine and of compounds 2 and 3 to serve as substrates for spermine synthase

The standard assay which measures the production of 5'-methylthioadenosine from decarboxylated AdoMet was carried out using either spermidine (○), **2** (□) or **3** (△) as the amine acceptor.

DISCUSSION

These results indicate that the unsaturated analogues of spermidine are able to support the growth of cells prevented from synthesizing the normal amount of spermidine by DFMO. Since both **1** and **3** support growth without giving rise to a spermine analogue, it is clear that this conversion is not essential for continuing growth. Conversely, since **4**, the *cis*-alkene analogue of spermine, also supported growth without being converted into a spermidine derivative, it appears that either spermidine or spermine can fulfil this requirement. Comparing compounds **2** and **3**, the *cis*- and *trans*-alkene isomers of spermidine, is particularly interesting in this respect. These compounds are equally effective in supporting growth, and **2** is converted efficiently into the spermine analogue, whereas **3** is not.

The results are consistent with previous studies in which either spermidine or spermine and various analogues of these polyamines have been shown to stimulate the growth of cells treated with ornithine decarboxylase inhibitors (Gerner & Mamont, 1986; McGovern *et al.*, 1986; Pegg, 1986; Porter & Sufrin, 1986; Baillon *et al.*, 1989; Mamont *et al.*, 1989; Pegg *et al.*, 1989). It is clear that the spatial separation of the positively charged atoms is critical for the function of polyamines. It is therefore of considerable interest that the introduction of either an alkyne or alkene function into spermidine does not interfere with their function. Further molecular-modelling studies of the interactions of polyamines with cellular macromolecules (Basu *et al.*, 1989; Feuerstein *et al.*, 1989, 1990) incorporating this information should help to define the critical interaction sites.

It is known that both 1,4-diaminobut-2-ene (isomer not specified) and 1,4-diaminobut-2-yne are substrates for spermidine synthase, although the rate of reaction was reduced and the K_m was increased compared with the normal putrescine substrate (Sarhan *et al.*, 1987*b*; Samejima & Nakazawa, 1980). Our results indicate that spermidine synthase is more specific, in that **1**, the alkyne derivative of spermidine, is not converted into the spermine equivalent. This is consistent with previous work in which it has been shown that the substrate specificity of spermine synthase is quite strict (Pegg *et al.*, 1981; Dezeure *et al.*, 1988; Baillon *et al.*, 1989; Mamont *et al.*, 1989). We also demonstrate, for the first time, stereospecificity in the spermine synthase reaction. The *cis*-alkene analogue of spermidine, **2**, is a good substrate for this enzyme, but the *trans*-alkene, **3**, and the alkyne **1**, are not converted into their spermine analogues. Since mechanism-based inhibitors of spermine synthase have been reported (Baillon *et al.*, 1989; Pegg *et al.*, 1989; Woster *et al.*, 1989), this information may allow modifications of increased potency.

The stimulatory effect of **1** on cell growth contrasts with the effects of 1,4-diaminobut-2-yne, which produced cytoplasmic vacuolation in L 1210 cells (Porter *et al.*, 1990), but the doses used in that study were very high (1.2 mM), and it is likely that this dose leads to the accumulation of substantial levels of the compound in the lysosomes. The finding by Porter *et al.* (1988) that the alkyne spermine derivative N^1N^4-bis-(3-aminopropyl)-1,4-diaminobut-2-yne was able to support the growth of L 1210 cells is consistent with our results with the alkyne spermidine derivative.

The unsaturated spermidine and spermidine derivatives do not appear to be substrates for the acetylase/oxidase pathway for interconverting and degrading polyamines. No evidence for the formation of **2** from **4** or for the formation of unsaturated derivatives of putrescine from **1**, **2** or **3** was seen in cells treated with these compounds, despite the fact that they accumulated to high levels. Such accumulation indicates that these compounds are taken up by the polyamine-transport system. This transport system is highly regulated in response to the internal polyamine concentration, and the reduction in its activity in response to a rise in intracellular polyamines limits the uptake of spermidine or spermine (Alhonen-Hongisto *et al.*, 1980; Porter & Jänne, 1987; Byers & Pegg, 1990; Seiler & Dezeure, 1990). However, all of the unsaturated derivatives were accumulated to levels considerably higher than spermidine, suggesting that they are less effective in bringing about this regulation. It has been shown that certain drugs which are substrates for the polyamine-transport system, such as methylgloxal bis(guanylhydrazone), accumulate to very high levels in cells, because they do not down-regulate transport (Seppänen *et al.*, 1981; Byers & Pegg, 1990). Polyamine analogues such as N^1N^{12}-bis(ethyl)spermine and related compounds show significant promise as anti-tumour agents (Porter & Sufrin, 1986; Bergeron *et al.*, 1989; Casero *et al.*, 1989). Their activities may be improved by modifications which increase their uptake and, therefore, synthesis of unsaturated derivatives of these agents should be considered.

This research was supported in part by grants AM-26754, GM-26290 and CA-37606 from the National Cancer Institute and by a grant from the National Foundation for Cancer Research. We thank Ms. R. Wechter for expert technical assistance.

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Received 14 August 1990; accepted 13 September 1990

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