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Novel Metabolically Stable and Functionally Active Mimetic of Spermidine[1]

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Abstract—Earlier unknown 1,8-diamino-3-methyl-4-azanonane (γ-MeSpd) was synthesized. The analogue was a substrate of neither spermine/spermidine N^1-acetyltransferase nor spermine synthase, but was capable to support the growth of DU145 cells having depleted polyamine pools. Such a combination of γ-MeSpd properties discloses novel opportunities to study cellular functions of catabolically unstable and easily interconvertible spermine and spermidine.

Keywords: *polyamines, spermidine analogues, γ-MeSpd, spermine synthase, spermine/spermidine N^1-acetyltransferase, DU145 cells*

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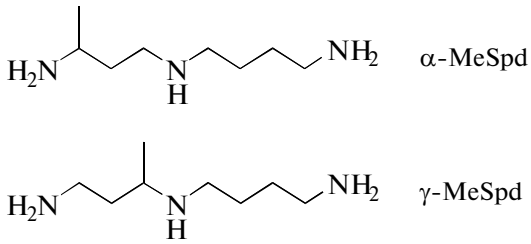
INTRODUCTION

High intracellular concentrations of biogenic polyamines spermine (Spm) and spermidine (Spd) determine their participation in the regulation of many vitally important processes (for reviews, see [1–3]). Investigation of the cellular functions of Spm and Spd at the molecular level is rather complicated due to their partial interchangeability and also due to their feasible interconversion (Fig. 1). Moreover, the activities and biosyntheses of the key enzymes of polyamine metabolism are precisely regulated at different levels of gene expression including the down-regulatory effects exerted by Spm and Spd [1, 2]. To study the cellular functions of Spm and Spd, cells and microorganisms deficient of the key enzymes of polyamine metabolism are being used. One of the recent examples of such an approach is the construction of *E. coli* strains deficient of all the enzymes of polyamine biosynthesis [4]. Different lines of transgenic animals have been generated, which has given an opportunity either to activate or to "switch off the targeted steps of polyamine metabolism even in vivo (for review, see [5]). Application of specific inhibitors of the enzymes of polyamine metabolism is an efficient approach in vitro, but the necessity to inhibit several enzymes leads to cumbrous combinations of the inhibitors (for reviews, see [6, 7]). A relatively simple method to discriminate the cellular effects of Spm and Spd is the depletion of the polyamine pool and subsequent application of functionally active metabolically stable mimetics of Spm and Spd.[3] However, the analogues having such a set of properties are still unknown amongst *mono-C*-methylated polyamine derivatives.



In the present paper, we describe synthesis of earlier unknown γ-MeSpd and demonstrate the analogue not to be a substrate of SSAT which makes γ-MeSpd catabolically stable. Under cell culture conditions there was no detectable conversion of γ-MeSpd to the

Abbreviations: AG, aminoguanidine; Ms, methanesulfonyl-; Spd, spermidine(1,8-diamino-4-azaoctane); γ-MeSpd (1,8-diamino-5-azanonane); Spm, spermine(1,12-diamino-4,8-diazadodecane); γ-MeSpd (1,8-diamino-2-methyl-4-azaoctane); γ-eSpd (1,8-diamino-3- methyl-4-azanoctane).

[1] The article was translated by the authors.

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[3] Earlier synthesized optical isomers of α-MeSpd [8] were catabolically stable [9], but in cell culture they were converted into corresponding analogues of Spm [9]. Furthermore, α-MeSpm is readily metabolized to α-MeSpd and Spd by SMO [10].

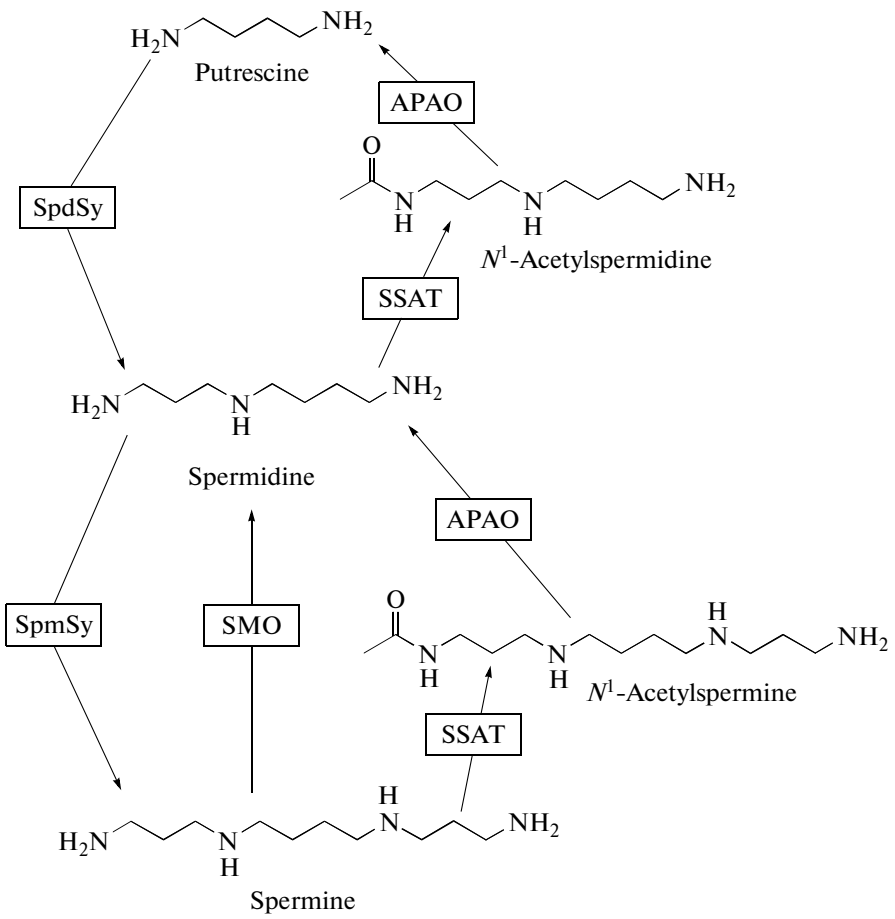
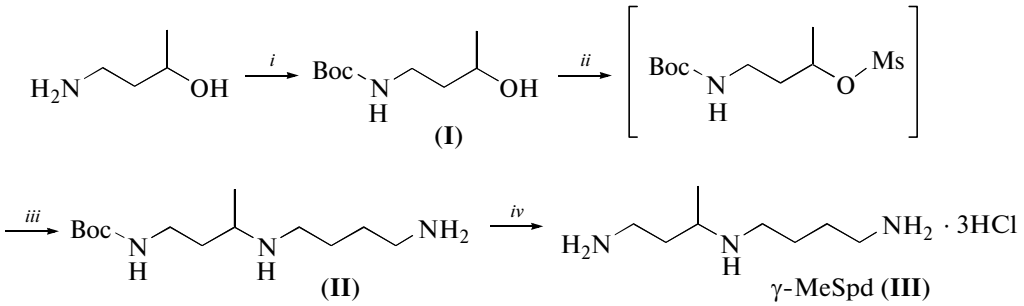


Fig. 1. Interconversions of spermine and spermidine. APAO—acetylpolyamine oxidase; SMO—spermine oxidase; SpdSy—spermidine synthase; SpmSy—spermine synthase; SSAT—spermine/spermidine N^1-acetyltransferase.

corresponding Spm derivative. These characteristics, together with the ability of the analogue to restore the growth of DU15 cells with depleted polyamine pool renders γ-MeSpd the first *mono-C*-methylated metabolically stable functionally active mimetic of Spd that is suitable to study the cellular effects of easily interconvertible Spm and Spd.

RESULTS AND DISCUSSION

Synthesis of γ-MeSpd (struct.) may be performed according to different schemes, using the methods being applied in the chemistry of polyamines to build up the C–N-bond [11, 12]. The most simple approach seems to be the addition of putrescine (Put, 1,4-diaminobutane) to crotononitrile with a subsequent reduction of the nitrile group. However, here we used feasible strategy employed earlier for the synthesis of α-methylated polyamine analogues, i.e. alkylation of the excess of Put with the methane sulfonate of 4-(*N-tert*-butyloxycarbonyl)amino-2-butanole to build up the polyamine backbone (Scheme). [13,14]. *N*-Boc-aminoalcohol (**I**) was prepared from commercially available 4-amino-2-butanol under standard conditions, using Boc_2O for the protection of the amino group. Conversion of the secondary alcohol (**I**) into the corresponding methane sulfonate was performed with a practically quantitative yield that gave a possibility to use it at the next stage without isolation. However, the alkylation of the excess of Put with the methane sulfonate of the secondary alcohol was not as successful as in the case of the preparation of α-MeSpd from 3-(*N*-benzyloxycarbonyl)amino-1-propyl methane sulfonate and Put. Intermediate 1-(*N-tert*-butyloxycarbonyl)amino-3-methyl-4-aza-8-aminooctane (**II**) was isolated by flash chromatography on silicagel with only 31% yield. The relatively low yield was partly due to the necessity of complete separation of *N*-Boc-triamine (**II**) from minor ninhydrin-positive by-products. Removal of *N*-Boc-group was performed with HCl in aq. alcohol that resulted in target γ-MeSpd (**III**) with an overall yield of 19%, as calculated for 4-amino-2-butanol.



Reaction conditions: *i*—Boc_2O/THF; *ii*—MsCl/C_6H_6/Et_3N; *iii*—$H_2N(CH_2)_4NH_2$/TNF; *iv*—H_2/Pd.

Scheme.

Investigation of biochemical properties of γ-MeSpd was initiated from the study of its substrate properties in SSAT reaction. It turned out that γ-MeSpd, like α-MeSpd [15], was a very poor substrate of mouse recombinant SSAT. Practically, the incorporation of [^{14}C]-Ac-group into γ-MeSpd was about three-fold as compared to α-MeSpd (Fig. 2). Thus, γ-MeSpd was a competitive inhibitor of the SSAT with of K_i 50 μM (γ-MeSpd has K_i 140 μM [10]).

Catabolism of Spd is initiated by N^1-acetylation by SSAT and the use of α-/γ-methyl-substituent renders the analogue stable against SSAT/APAO-mediated degradation. However, chemically synthesized N^1-acetyl-α-MeSpd, like N^1-Ac-Spd, can be splitted by APAO [16, 17]. Finally, γ-MeSpd proved to be nontoxic to cultured cells without aminoguanidine (AG) supplementation under conditions were Spd was toxic, thus showing stability against serum aminooxidases (data not shown). Hence, γ-MeSpd is a new catabolically stable analogue of Spd.

At the next step, we investigated the effects of γ-MeSpd on the level of polyamines in DU145 cells and its ability to restore the growth of these cells with depleted Spd and Put pools as the result of DFMO (suicide inhibitor of ornithine decarboxylase) treatment. To prevent nonspecific oxidation of Spd by serum aminooxidases, the experiments were carried out in the presence of AG.

γ-MeSpd efficiently penetrated into DU145 cells, competing for the transport with Spd (data not shown), and its intracellular content was about the same as that of α-MeSpd and Spd (table). Both γ-MeSpd and γ-MeSpd decreased the levels of Put and Spd in DU145 cells (table) suggesting that they down-regulated polyamine synthesis. The effect of γ-MeSpd on the level of Spm was comparable with the effect of Spd, but was much less than the effect of α-MeSpd (table), which may be due to the fact that in DU145 cells about 20% of γ-MeSpd is converted to α-MeSpm, which is a metabolically competent analogue of Spm. Thus, γ-MeSpd, like α-MeSpd and Spd itself, can participate in maintaining of polyamine homeostasis in DU145 cells.

γ-MeSpd, like Spd and α-MeSpd (this analogue fulfills the cellular functions of Spd both in vitro and in vivo [18]), effectively restored the growth of DU145 cells with decreased levels of Put and Spd as the result of 72 h incubation with DFMO (Fig. 4). The ability of γ-MeSpd to neutralize efficiently the acute deficiency of polyamines and to regulate polyamine homeostasis (see above) makes γ-MeSpd a functionally active mimetic of Spd.

Finally, we investigated the intracellular conversion of γ-MeSpd to γ-MeSpm. As depicted at table, the formation of γ-MeSpm in DU145 cells was not detected

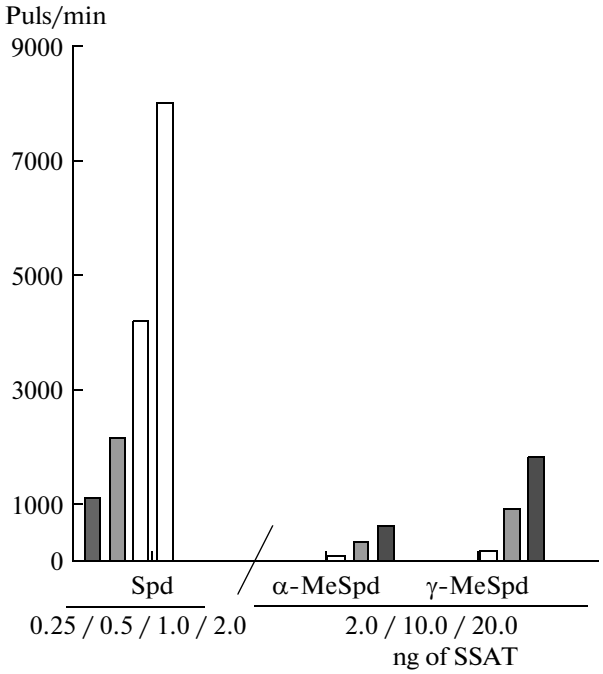


Fig. 2. Incorporation of [^{14}C]-acetyl group in Spd, γ-MeSpd, or γ-MeSpd as a function of the amount of SSAT.

Effect of the analogs on growth and polyamine levels in DU145 cells after 72 h of culture

Cells DU145	Growth, %	Polyamines				
		Put	*Spd*	*Spm*	*MeSpd*	*MeSpm*
		(pmol/10^6 cells)				
AG	100 ± 4	93 ± 25	1065 ± 58	1513 ± 78		
AG + *Spd*	94 ± 3	48 ± 21	2416 ± 103***	976 ± 36***		
AG + α-*MeSpd*	97 ± 3	34 ± 14*	115 ± 12***	549 ± 20***	3053 ± 47	858 ± 95
AG + γ-*MeSpd*	101 ± 4	20 ± 4**	136 ± 14***	1026 ± 59***	3030 ± 160	n.d.
AG + *DFMO*	49 ± 2***	59 ± 25	55 ± 6***	1197 ± 57***		
AG + *DFMO* + *Spd*	89 ± 4	35 ± 3***	2567 ± 196***	831 ± 21***		
AG + *DFMO* + α-*MeSpd*	86 ± 4	21 ± 2***	47 ± 5***	246 ± 26***	4223 ± 509	959 ± 119
AG + *DFMO* + γ-*MeSpd*	91 ± 5	18 ± 1***	n.d.	486 ± 36***	5131 ± 159	n.d.

Note: Cells were cultured for 72 h in the presence of 100 μM *Spd*, or its analogues and 1 mM *AG* in the presence or absence of 5 mM *DFMO*. Results are means *SD*, $n = 3$. n.d., not detectable; *, ** and *** refer to statistical significance of <0.05, 0.01 and 0.001, respectively, as compared to *AG*-treated control.

by HPLC. Moreover, HPLC analysis did not show the presence of any acetylated γ-MeSpm intermediate even when MDL72,527 (20 μM) was used to prevent APAO/SMO-mediated backconversion (data not shown). Hence, γ-MeSpd, in contrast to γ-MeSpd, was not a substrate of spermine synthase.

The obtained data proves γ-MeSpd to be the first metabolically stable and functionally active mimetic of Spd among *mono-C*-methylated polyamine analogues. This analogue is a promising tool for the investigation of individual cellular functions of readily interconvertible Spd.

EXPERIMENTAL

4-Amino-2-butanol was obtained from Acros (Belgium); 1,4-diaminobutane from Aldrich (United States); Boc_2O and MsCl from Fluka (Switzerland), α-MeSpd was synthesized following the earlier published method [13].

Flash chromatography was carried out on Kieselgel (40–63 μm, Merck), systems for elution are specified in the text. TLC was carried out on precoated Kieselgel 60 F_{254} plates (Merck) in systems: $CHCl_3$–MeOH, 100 : 2 (A); dioxane–25% NH_4OH, 8 : 2 (B); *n*-BuOH–AcOH–pyridine–H_2O, 4 : 2 : 1 : 2 (C). Substances on chromatograms were detected by bromophenol blue solution (Boc-derivatives) and the compounds with free amino group were developed using color reaction with ninhydrin.

NMR spectra were registered on a Bruker Avance 500 DRX (Germany) instrument with working frequency of 500.1 MHz for ^1H nuclei and 125.8 MHz for ^{13}C nuclei. Tetramethylsilane was used as an internal standard ($CDCl_3$) and sodium 3-trimethyl-1-propanesulfonate (D_2O). Chemical shifts are given in ppm, and spin–spin coupling constants in Hz. Melting points were determined in open capillary tubes using Electrotermals Mel-Temp 1202D apparatus and are uncorrected. Elemental analysis was performed in Nesmeyanov Institute of Organoelement Compounds, RAS, Moscow, using CHN-analyser Carlo Erba 1106.

DU145 prostate carcinoma cells were grown as described earlier [9]. The effects of Spd analogues on the intracellular content of polyamines, as well as the investigation of the ability of γ-MeSpd to restore the growth of DU145 cells with depleted polyamine pool were performed as described earlier [19]. Intracellular polyamines were measured with HPLC according to the published method [20]. Substrate properties of α-MeSpd and γ-MeSpd in SSAT reaction were determined following the incorporation of [^{14}C]-group of Ac-CoA in α-MeSpd or γ-MeSpd in comparison with that for Spd, in accordance with the published protocol for polyamines [21], but the incubation time was 10 min. Concentrations of Spd and its *C*-methylated derivatives in the substrate mixture were 2.5 μM, while the amounts of mouse recombinant SSAT were 0.25, 0.50, 1.0 and 2.0 ng of the protein/per probe in the case of Spd; and 2.0,10.0 and 20.0 ng of the protein/per probe in the case of α-MeSpd and γ-MeSpd. The experiments were performed twice in triplicates.

Statistical analysis: values are means *SD*. One-way analysis of variance (ANOVA) with Tuckey's post-hoc test was used for multiple comparisons with the aid of a software package, GraphPad Prism 4.03 (GraphPad Software Inc.). *, ** and *** refer to *p* values of <0.05, <0.01 and <0.001, respectively.

4-(*N-tert*-Butyloxycarbonyl)amino-2-butanol (I). Boc_2O (2.4 g, 11 mmol) in THF (10 ml) was added slowly with stirring to a cooled (+4°C) solution of

4-amino-2-butanol (1.04 g, 11.6 mmol) in THF (10 ml). Stirring was continued for 1 h at +4°C and 16 h at 20°C and the reaction mixture was evaporated to dryness in vacuo. The residue was taken into $CHCl_3$ (30 ml), washed with 10% citric acid (4 × 7 ml), H_2O (5 ml), 1 M $NaHCO_3$ (5 ml), H_2O (5 ml), brine (10 ml) and dried ($MgSO_4$). Solvent was distilled off in vacuo and the residue was dried in vacuo over P_2O_5 to give (**I**) (1.82 g, 87.5%) as a viscous oil; R_f 0.29 (A). ^{1}H NMR ($CDCl_3$): 4.91–4.76 (1H, bs, NHBoc), 3.88–3.80 (1H, m, CHOH), 3.50–3.37 (1H, m, CH_2NHBoc), 3.14–3.04 (1H, m, CH_2NHBoc), 3.00–2.90 (1H, bs, OH), 1.64–1.55 (1H, m, CH_2CHOH), 1.54–1.46 (1H, m, CH_2CHOH), 1.43 (9H, s, $(CH_3)_3$), 1.22 (3H, d, *J* 1.2, CHCH_3). ^{13}C NMR ($CDCl_3$): 157.04, 79.48, 65.20, 39.50, 37.51, 28.48, 23.36. Found, %: C 56.95; H 10.27; N 7.14. $C_9H_{19}NO_3$. Calculated, %: C 57.12; H 10.12; N 7.40.

***N*^{1}-(*tert*-Butyloxycarbonyl)-1,8-diamino-3-methyl-4-azaoctane (II).** Methanesulfonyl chloride (1.03 g, 9.0 mmol) in dry benzene (10 ml) was added dropwise with stirring to a cooled (+4°C) solution of (**I**) (1.75 g, 9.26 mmol) and Et_3N (2.08 ml, 15 mmol) in dry benzene (20 ml). Stirring was continued for 1 h at +4°C and 4 h at 20°C, then the precipitated triethylamine hydrochloride was filtered off. Combined filtrates were washed with H_2O (5 ml), 1 M $NaHCO_3$ (3 × 5 ml), H_2O (5 ml), 10% citric acid (4 × 5 ml), H_2O (5 ml), brine (10 ml) and dried ($MgSO_4$/$NaHCO_3$). Solvent was evaporated in vacuo, to give intermediate 4-(*N*-*tert*-butyloxycarbonyl)amino-2-butyl methanesulfonate {R_f 0.59 (A)}, which was used (without purification) to alkylate 1,4-diaminobutane (8.8 g, 100 mmol) in dry THF (50 ml) first for 16 h at +4°C and then for 48 h at 20°C. The reaction mixture was evaporated to dryness at 1 mm Hg, the residue was taken into 2 M NaOH (10 ml) and extracted with DCM (2 × 10 ml). Combined organic extracts were washed with H_2O (3 ml), brine (5 ml) and evaporated to dryness in vacuo. The residue was purified on a silica gel column (120 g) using a mixture of dioxane—25% NH_4OH (95 : 5) as an eluent. Fractions, containing (**II**) were combined and evaporated to dryness in vacuo that after drying of the residue in vacuo over P_2O_5 gave (**II**) (0.72 g, 31%) as a viscous oil; R_f 0.43 (B). ^{1}H NMR ($CDCl_3$): 5.53–5.42 (1H, bs, NHBoc), 3.31–3.21 (1H, m, CH_2NHB0C), 3.19–3.10 (1H, m, CH_2NHBoc), 2.77–2.65 (4H, m, NHCH_2CH_2 + CH_2NH_2), 2.57–2.51 (1H, m, CHCH_3), 1.62–1.31 (18H, m, $(CH_3)_3$ + NHCH_2CH_2CH2CH_2NH_2), 1.09 (3H, d, *J* 7.2, CHCH_3). ^{13}C NMR ($CDCl_3$): 156.11, 78.85, 51.96, 46.88, 42.10, 38.27, 36.15, 31.60, 29.71, 28.48, 27.77, 20.33. Found, %: C 60.42; H 11.22; N 16.11. $C_{13}H_{29}N_3O_2$. Calculated, %; C 60.20; H 11.27; N 16.20.

1,8-Diamino-3-methyl-4-azaoctane trihydrochloride, γ-MeSpd (III). To a solution of (**II**) (0.65 g, 2.5 mmol) in EtOH (15 ml) 37% HCl (2.5 ml) was added and the reaction mixture as kept for 30 min at 20°C. The reaction mixture was evaporated to dryness in vacuo, the residue was co-evaporated with abs. EtOH (4 × 15 ml), the resulted semi-solid residue was recrystallized from abs. EtOH that after drying in vacuo over P_2O_5/KOH gave γ-MeSpd (**III**) (0.48 g, 71.5%), mp 231–232°C, R_f 0.24 (C). ^{1}H NMR (D_2O): 3.48–3.41 (1H, m, CH(CH_3)); 3.20–3.03 (6H, m, H_2NCH_2(CH_2)$_2$CH_2NH + NH_2CH_2); 2.24–2.17 (1H, m, CHCH_2); 2.00–1.91 (1H, m, CHCH_2); 1.83–1.74 (4H, m, C–CH_2CH_2–C); 1.37 (3H, d, *J* 6.7, CH(CH_3)). ^{13}C NMR (D_2O): 54.88, 47.10, 41.68, 38.75, 33.07, 26.84, 25.83, 17.96. Found, %: C 35.61; H 9.07; N 15.59. $C_8H_{24}Cl_3N_3$. Calculated, %: C 35.77; H 9.00; N 15.64.

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