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AKI JÄRVINEN

α-Methylated Polyamine Analogues

Tools to Study Polyamine Metabolism and Polyamide Oxidase

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L22, Snellmania building, University of Kuopio, on Saturday 19th November 2005, at 12 noon

Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Kuopio

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A.I. Virtanen Institute for Molecular Sciences

Author's address:
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
P.O. Box 1627
University of Kuopio
FIN-70211 KUOPIO
Tel. +358 17 163 672
Fax +358 17 163 025
E-mail: aki.jarvinen@uku.fi

Supervisors:
Professor Juhani Jänne, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

Professor Leena Alhonen, Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

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ABSTRACT

The polyamines, spermidine and spermine as well as their precursor putrescine, are found in all mammalian cells. They are essential for cell growth, the maintenance of cell physiology and they participate in the regulation of cellular metabolism. Excess amounts of polyamines are associated with malignant growth whereas insufficient amounts evoke programmed cell death i.e. apoptosis. The cellular levels of polyamines are delicately controlled via a balance between synthesis, degradation, uptake and excretion. Furthermore, polyamines are effectively interconverted in concerted manner by spermidine/spermine N^1–acetyltransferase, the enzyme that acetylates polyamines, and polyamine oxidase, which readily oxidizes acetylated polyamines. Spermidine/spermine N^1–acetyltransferase is a very inducible enzyme when compared to polyamine oxidase which appears rather to be constitutively expressed throughout the mammalian system. All of the above metabolic events allow for fine–tuning of the amounts of each polyamine to match their use in essential cellular processes. However, the efficient interconversion of polyamines makes any assessment of the roles of each polyamine very challenging. Therefore, we utilized metabolically stable α–methylated polyamines that are able to mimic the crucial cellular functions of their natural counterparts.

Metallothionein promoter driven spermidine/spermine N^1–acetyltransferase transgenic rodents have proven invaluable for studies aimed at elucidating the mechanisms and consequences of the interconversion of the polyamines. Recombinant proteins (spermidine/spermine N^1–acetyltransferase, polyamine oxidase and recently discovered spermine oxidase) used in this study are excellent tools to evaluate the biological stabilities of different polyamines and their analogues. Furthermore, immortalized rat fibroblast cell lines derived from syngenic and the metallothionein promoter driven spermidine/spermine N^1–acetyltransferase transgenic rat complete the tools available for a detailed exploration of polyamine metabolism.

Both α–methylspermidine and bis–α–methylspermine are resistant to spermidine/spermine N^1–acetyltransferase–mediated acetylation. Neither polyamine nor spermine oxidase can degrade α–methylspermidine but both oxidases use bis–α–methylspermine poorly as a substrate. *In vivo*, both compounds are able to prevent acute pancreatitis and initiate early liver regeneration in the partially hepatectomized spermidine/spermine N^1–acetyltransferase transgenic rat. Both of these above conditions are characterized by severe depletions of the natural polyamines.

Recombinant protein studies with enantiomers of acetyl–α–methylspermidine showed that polyamine oxidase expresses stereoselectivity for the (*R*)–isomer of α–methylspermidine though the natural substrates for this enzyme are achiral. Aldehyde supplementation in the reaction buffer has been shown to enhance polyamine oxidase–mediated oxidation. Surprisingly, the stereoselectivity of the enzyme becomes also changed in the presence of different aldehydes. Benzaldehyde enhances selectively the degradation of (*R*)– and pyridoxal (*S*)–isomer of α–methylspermidine from a racemic substrate mixture in glycine–NaOH buffer at pH 9.5. The same stereoselectivity is retained in other studied buffer systems at high pH and also at pH 7.4 though at a reduced rate. There are indications that the Schiff base between aldehyde and polyamine is the target for enzymatic catalysis by polyamine oxidase. For the first time, the stereospecificity of a FAD–dependent oxidase has been shown to be guided by simple aldehyde supplementation in aqueous media. Understanding these phenomena may help clarify FAD–dependent oxidative reactions and may eventually lead to some applications in biocatalysis.

National Library of Medicine Classification: QU 61, QU 55, QU 450, WI 702, WI 805
Medical Subject Headings: polyamines/metabolism; spermidine/analogs & derivatives;
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liver regeneration; fibroblasts; recombinant proteins; acetyltransferases; oxidoreductases;
molecular conformation; aldehydes; benzaldehydes; Schiff bases; transgenes; animals, genetically modified;
rats; humans

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Kuopio, November 2005

Aki Järvinen

Mother Earth, the guardian and provider of us all, I look forward to our refreshing, inspiring and encouraging moments in the woods.

ABBREVIATIONS

AA	amino acid
AbeAdo	5′–{[(*Z*)–4–amino–2–butenyl]methylamino}–5′–deoxyadenosine
AcSPD	N^1–acetylspermidine
AcSPM	N^1–acetylspermine
AdoMet	(*S*)–adenosyl–(*L*)–methionine
AdoMetDC	(*S*)–adenosyl–(*L*)–methionine decarboxylase
AG	aminoguanidine
AOE–PUT	aminooxy–ethyl–putrescine
AZ	antizyme
BA	benzaldehyde
DFMO	α–difluoromethylornithine
DENSPM	diethylnorspermine
DESPM	diethylspermine
dcAdoMet	decarboxylated (*S*)–adenosyl–(*L*)–methionine
diAcSPM	N^1,N^{12}–diacetylspermine
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiotreitol
eIF5A	eukaryotic initiation factor 5A
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
MeSPD	α–methylspermidine
MeSPM	α–methylspermine
Me_2SPM	bis–α–methylspermine
MDL 72527	N^1,N^2–bis(2,3–butadienyl)–1,4–butanediamine
MGBG	methylglyoxal–bis(guanylhydrazone)
mRNA	messenger ribonucleic acid
MT	metallothionein I promoter
Nrf–2	Nf–E2 related transcription factor
ODC	ornithine decarboxylase
OPA	phthaldialdehyde
ORF	open reading frame
PA	polyamine
PAO	polyamine oxidase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEST	proline (P), glutamic acid (E), serine (S) and threonine (T)
PL	pyridoxal
PL=SPM	adduct of spermine with pyridoxal
PMF–1	polyamine–modulated factor–1
PRE	polyamine response element
PUT	putrescine
P4CA	pyridine 4–carboxaldehyde
RNA	ribonucleic acid
SPD	spermidine
SPDSy	spermidine synthase
SPM	spermine
SPMSy	spermine synthase
SSAT	spermidine/spermine N^1–acetyltransferase
SMO	spermine oxidase

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by their corresponding Roman numerals:

I Järvinen A, Grigorenko N, Khomutov AR, Hyvönen MT, Uimari A, Vepsäläinen J, Sinervirta R, Keinänen TA, Vujcic S, Alhonen L, Porter CW, and Jänne J.

Metabolic stability of α–methylated polyamine derivatives and their use as substitutes for the natural polyamines.

Journal of Biological Chemistry (2005) **280**; 6595-6601

II Järvinen A, Cerrada–Gimenez M, Grigorenko N, Khomutov AR, Vepsäläinen J, Sinervirta R, Keinänen TA, Alhonen L, and Jänne J.

α–Methyl polyamines: efficient synthesis and tolerance studies in vivo and in vitro. The first evidence for dormant stereospecificity of polyamine oxidase

Journal of Medicinal Chemistry (2005) **submitted**

III Järvinen A, Keinänen TA, Grigorenko N, Khomutov AR, Uimari A, Vepsäläinen J, Närvänen A, Alhonen L, and Jänne J.

Guide–molecule driven stereospecific degradation of α–methylpolyamines by polyamine oxidase

Journal of Biological Chemistry (2005) **submitted**

Some unpublished data is also presented.

CONTENTS

1 INTRODUCTION

Polyamines (PAs) are found in all living organisms except for a few bacteria. In mammalian cells, these small molecules are characterized by a straight carbon backbone which separates two or more amino groups. Putrescine, (PUT) found in large quantities in decomposing flesh, is the simplest of the PAs and acts as a precursor for the two longer molecules: spermidine (SPD) and spermine (SPM, Fig. 1). SPM was originally discovered in the human semen and both SPD and SPM are named accordingly. Contrary to the situation in eukaryotes, in prokaryotes a far wider variety of PAs has been found. For example, in the thermophilic bacteria, branched PAs are common (Hamana et al., 1992).

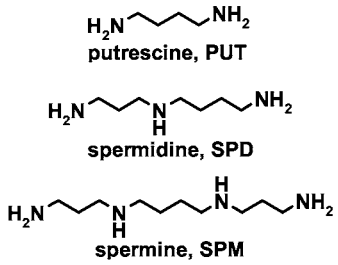


Figure 1 The three polyamines found in mammalian cells

Under normal physiological conditions, all amino groups in PUT, SPD and SPM are protonated. Due to their polycationic nature, PAs interact with negatively charged macromolecules. However, unlike the common cellular ions (Ca^{2+}, Mg^{2+} etc.), these compounds have their positive charges divided by few carbon atoms along the entire length of the molecule. This unique feature allows PAs to link to different regions of anionic macromolecules and even entirely separate molecules can be bridged by the flexible carbon chain. Both SPD and SPM fit in between the major and minor grooves of DNA, stabilizing the nucleic acid structure. Thus, PA depletion partially unwinds DNA in nucleosomes and may reveal potential sequences for transcription regulating factors (Morgan et al., 1987). However, in cells, most of the PAs appear to form complexes with RNA (Watanabe et al., 1991). The above interactions indicate that PAs have important roles in the regulation of both transcription and translation. For example, PA depletion stabilizes both p53 mRNA and protein in rat intestinal epithelial cells, inhibiting normal proliferation (Li et al., 2001). Furthermore, PAs form complexes with proteins and phospholipids, strengthening cellular membranes. PAs also appear to regulate many membrane–bound enzymes and ion channels (Fakler et al., 1995). All these properties emphasize the importance of controlled PA levels in order to maintain the cellular functionality.

In prokaryotes, PAs appear to stabilize nucleic acids under extreme conditions (Terui et al., 2005) and are also used as carbon and nitrogen sources. In eukaryotes, detoxification processes and regulation of fundamental cellular processes appear to be the main roles for PAs. Recently numerous terminally *N*–alkylated PA analogues have been studied in PA metabolism associated effects but these PA mimetics have limited stabilities. Therefore, the development of metabolically stable PA derivatives would be extremely useful in elucidating the exact roles of each individual PA. Several apparently stable and differently methylated PA analogues were introduced in 1980s (Nagarajan and Ganem, 1986) but due to their limited supply these compounds have so far been studied only *in vitro*. The methylation apparently protects these PA derivatives against acetylation as well as degradation by mono– and diamino oxidases, making them metabolically far more stable than

their natural counterparts (Nagarajan et al., 1988). Spermidine/spermine N^1–acetyltransferase (SSAT, EC 2.3.1.57) is considered as the key enzyme in interconversion of PAs and transgenic rodents over–expressing SSAT are characterized by enhanced PA catabolism (Alhonen et al., 2000; Pietilä et al., 1997; Suppola et al., 1999). We previously demonstrated that α–methylated SPD (MeSPD, Fig. 2) can mimic the cellular functions of SPD and can prevent acute pancreatitis in the mouse metallothionein I (MT) promoter driven SSAT transgenic rats (Räsänen et al., 2002).

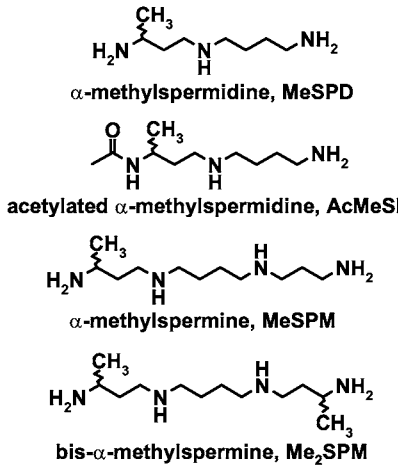


Figure 2 Four studied α–methylated polyamine analogues.

In vitro studies with recombinant human SSAT showed that a single methyl group is indeed enough to prevent acetylation of MeSPD. Neither recombinant human polyamine nor human spermine oxidase (PAO, EC 1.5.3.11 and SMO, respectively) could utilize MeSPD as a substrate. However, the SPM derivative with a single α–methyl group (MeSPM, Fig. 2) was readily degraded by hSMO but bis–α–methylated SPM (Me_2SPM, Fig. 2) was more resistant to hSMO–mediated oxidation. On the other hand, hPAO used Me_2SPM more readily than MeSPM, though both were poor substrates for this enzyme. SPD has been considered as the essential PA to sustain cellular functionality. Hepatic PAs are severely depleted in the MT–SSAT transgenic rat after hepatectomy. However, Me_2SPM treatment prior to the operation prevented the delay in liver regeneration in these animals. In fact, Me_2SPM appeared to be a more effective drug than MeSPD on a molar basis. Extensive *in vivo* studies showed that the MT–SSAT transgenic mice tolerated all of the α–methylated PA analogues.

Many enzymes preferentially use one isomer as their substrate(s). Natural substrates for PAO are achiral but since the studied α–methylated PA analogues were chiral we suspected that PAO might express stereospecificity. The enantiomers of acetylated MeSPD (AcMeSPD, Fig. 2) were indeed degraded differently by hPAO with (*R*)–AcMeSPD being a far better substrate than the (*S*)–isomer.

Aldehyde supplementation increases the reaction rates of PAO (Hölttä, 1977). However, the ability to predetermine the stereoselectivity of hPAO–mediated oxidation of the chiral substrate with appropriate aldehyde supplementation was totally unexpected. Benzaldehyde (BA) supplementation resulted mostly in degradation of (*R*)–MeSPD by hPAO whereas (*S*)–MeSPD was exclusively used by hPAO when pyridoxal (PL) was included into the reaction mixture. In the presence of the above aldehydes, the same stereoselective degradations of MeSPM isomers and Me_2SPM diastereomers were also observed. The current study suggests that the Schiff base formed between the aldehyde and the PA may be the target for recognition by PAO.

The stereospecific nature of enzymatic oxidation with supplementary guide molecules is a unique finding. The present results may be used to develop PAO specific inhibitors. Also the reaction mechanisms of FAD–dependent oxidases might be understood better in the light of this

study. Furthermore, as the demand for tools to produce enantiomerically pure compounds as part of drug synthesis increases, PAO might represent a model enzyme for further exploitation.

2 REVIEW OF LITERATURE

2.1 TOOLS TO STUDY POLYAMINE METABOLISM

The PAs have been extensively studied since the beginning of the last century but the first report about SPM dates back to the 17th century. However, the exact roles of PAs in different cellular functions have proved hard to clarify. This is most likely due to the abundance of these molecules and due to their polycationic nature, their strong association with multiple cellular organelles (Igarashi and Kashiwagi, 2000). It is not possible to accurately measure the amounts of free PAs in cells with the current methods and their compartmentalization within intact cells remains a mystery. The regulation of PA homeostasis has proven to be complicated and highly variable from cell type to cell type let alone from species to species. Some approaches used in the PA related studies are presented with a critique.

2.1.1 Cell culture

The number of published research papers on PAs easily passes five digits and out of all these papers a significant proportion deals with cell culture studies. Innumerable cell lines have been derived from both human and animal tissues; moreover, several embryonic cell lines have been established from both intact and genetically manipulated animals. During the past years, these techniques have given rise to specifically engineered cells where a selected gene can either be over–expressed or knocked out.

In general, studies with a single cell type can give very clear answers to well defined questions. However, cultured cells are devoid of all tissue–tissue interactions and with time tend to adapt to their environment, losing some essential features required *in vivo* or the cells may acquire new properties. It is essential to measure intracellular and occasionally even extracellular levels of each drug as they may compete for the same cellular transport system(s). Furthermore, the compounds studied *in vitro* are quite often administered at high doses and at concentrations not necessarily relevant to conditions prevailing *in vivo*. Accordingly, *in vitro* results have rather limited value if one wishes to extrapolate the results to the situation in intact animals and humans.

2.1.2 Experimental animals

Different rodent models have proven very valuable. However, one always has to bear in mind the species–specific differences and the fact that no single animal model can perfectly reflect the metabolism of humans.

The past two decades have witnessed a large number of genetically modified animal studies (Jänne et al., 2004) but they have their own limitations. When a transgene is introduced into the host genome it can integrate in the middle of an active gene possibly knocking it out. It is extremely common that the transgene expression results in some compensatory effects in the organism but such metabolic adjustments may not always be sufficient and an abnormal phenotype may well be observed. The heredity of transgene(s) is also sometimes problematic and does not necessarily follow Mendelian law in live offspring.

2.1.3 Recombinant proteins

Recombinant protein production in bacteria, yeasts and different cell cultures has advanced significantly during the last few years. The desired protein(s) can be purified on a large scale with a few steps by using commercially available kits (Terpe, 2003). Such pure enzymes are excellent tools to study enzyme kinetics under precisely controlled conditions. However, one should be

aware that the enzymes in their natural cellular environment will be exposed to multiple effectors that are absent in test tubes.

Lately, the traditional practice of tissue extract studies has more or less been discarded in favor of more modern approaches. However, such extracts reflect the cellular metabolism better than any cell line in an artificial buffer system.

2.2 POLYAMINES AND MAMMALIAN CELLS

The PA homeostasis is maintained via an intricate metabolic system in mammals. The

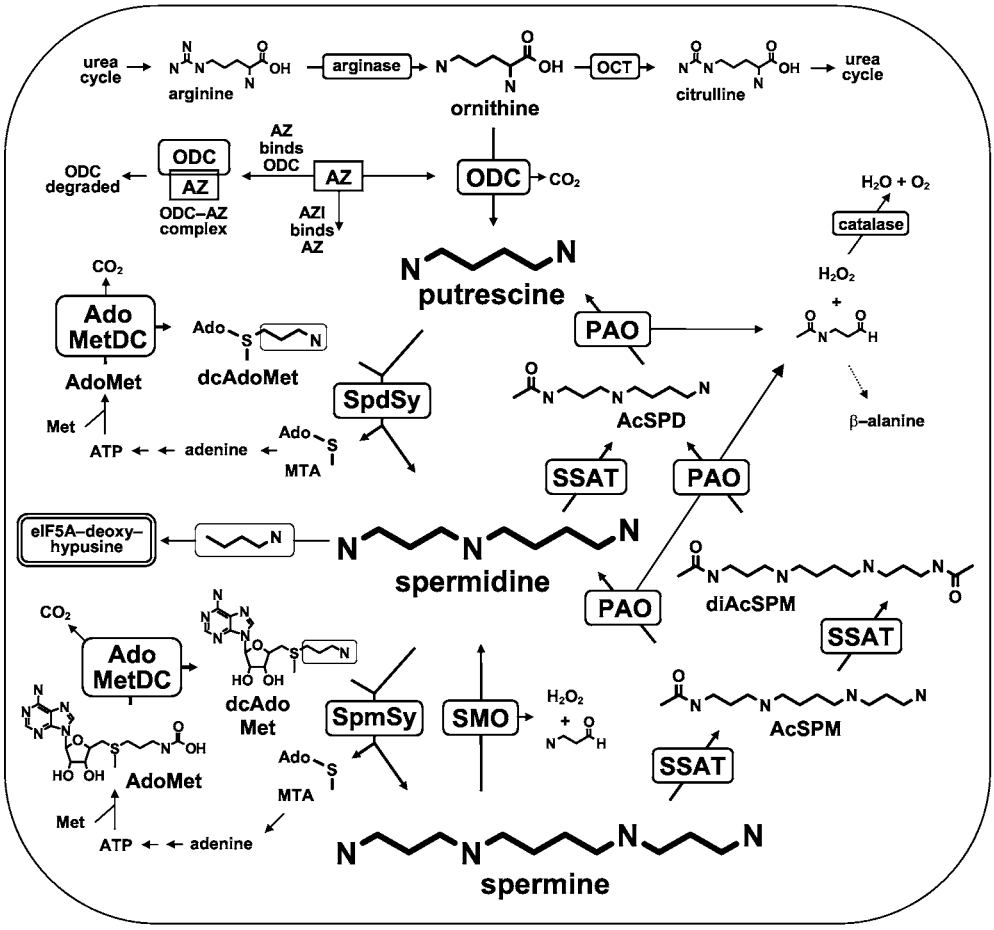


Figure 3 Polyamine biosynthesis and interconversion in mammalian cells

AcSPD, N^1–acetylspermidine; AcSPM, N^1–acetylspermine; AdoMet, (*S*)–adenosyl–(*L*)–methionine; AdoMetDC, (*S*)–adenosyl–(*L*)–methionine decarboxylase; ATP, adenosinetriphosphate; AZ, antizyme; AZI, antizyme inhibitor; dcAdoMet, decarboxylated (*S*)–adenosyl–(*L*)–methionine; diAcSPM, N^1,N^{12}–diacetylspermine; eIF5A, eukaryotic initiation factor 5A; Met, (*L*)–methionine; MTA, methylthioadenosine; OCT, ornithine carbamoyltransferase; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SMO, spermine oxidase; SPDSy, spermidine synthase; SPMSy, spermine synthase; SSAT, spermidine/spermine N^1-acetyltransferase.

biosynthesis of PAs starts from ornithine to form PUT, SPD and SPM sequentially (Fig. 3). SPM and SPD can either be interconverted (Fig. 3) or terminally degraded with different enzymes (Fig. 7, page NN). All the enzymes involved in the synthesis and degradation of PAs appear to have multiple interlocking feedback mechanisms. Furthermore, there have been recent reports of splice variants of enzymes involved in the PA metabolism (Casero et al., 2003; Cervelli et al., 2004; Pyronnet et al., 2005) giving rise to several isoenzymes with different properties and this makes the elucidation of the mechanisms controlling PA homeostasis even more challenging.

One can discern how such a sophisticated maintenance system allows fine–tuning of PA levels throughout the diversity of tissues, each with entirely different requirements for PAs. The demand for PAs is intimately linked with the cell–cycle (Oredsson, 2003) and the proliferation rate of the tissue. Dramatic consequences can occur when excess or insufficient amounts of PAs are present. These changes can lead to malignancy (reviewed in detail by Gerner and Meyskens, 2004) or apoptosis i.e. programmed cell death (Nitta et al., 2002). Furthermore, the species–specific requirements for PAs can also have huge variability; in this review PA metabolism in plants or prokaryotes will not be covered in any detail.

2.2.1 Polyamine biosynthesis

The primary precursor of PAs, (*L*)–ornithine, can either be derived from the diet or produced from (*L*)–arginine by mitochondrial arginase II (EC 3.5.3.1). Previously it was thought that arginase II, instead of cytosolic arginase I, was more important. However, now it appears that isoform I is the enzyme predominantly responsible (especially during development). Knock–out mice for arginase I survive only 12 days whereas knockout mice for arginase II have no disturbed phenotype (Cederbaum et al., 2004). These results raise questions about the exact roles of these two isoenzymes.

Ornithine can be converted to citrulline by ornithine carbamoyltransferase (EC 2.1.3.3) within the urea cycle. However, in the case of the PA biosynthesis, ornithine decarboxylase (ODC, EC 4.1.1.17) is the sole key enzyme producing PUT. ODC is a very fascinating enzyme (extensive reviews by Schipper and Verhofstad, 2002; Shantz and Pegg, 1999). It has a very short half–life (a short as 10 to 20 minutes up to 2 hours) and it has been localized mostly in cytoplasm (inducible form) and occasionally in the nucleus (uninducible form) depending on the cell type. Active ODC uses PL phosphate as its cofactor and is a homodimer where the two active sites are located between the monomers. ODC is inactivated when it forms a heterodimer with antizyme (AZ).

AZ is a unique protein as complete AZ protein expression requires a +1 translational frameshift which is triggered by increasing PA concentrations (Matsufuji et al., 1995). Functional AZ binds ODC protein (Murakami et al., 1985) and alters its conformation, revealing the C–terminal PEST sequence (rich in proline (P), glutamic acid (E), serine (S) and threonine (T)) a sequence which is common in proteins with rapid turnover rates (Rogers et al., 1986). ODC is degraded by 26 S proteosome in an ATP–dependent, but ubiquitin–independent, manner (Murakami et al., 1992). At least three different AZs have been reported; AZ1 is strongly associated with ODC degradation, AZ2 down–regulates PA transport (Zhu et al., 1999) and AZ3 is expressed only in the testis (Tosaka et al., 2000). Growth stimuli can cause the release of ODC from AZ by the so–called antizyme inhibitor, a compound which has higher affinity for AZ than the affinity of AZ for ODC. Interestingly AZ inhibits the degradation of AZI (Bercovich and Kahana, 2004).

The next step in the synthesis of higher PAs which includes decarboxylation is the conversion of (*S*)–adenosyl–(*L*)–methionine (AdoMet) to its

decarboxylated form (dcAdoMet) by adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50). AdoMet, derived from (*L*)–methionine, is a common methyl group donor in biological methylations (Chiang et al., 1996). Like ODC, AdoMetDC has a short half–life of about 1 hour in most mammalian cells (reviewed by (Jänne et al., 1991).

Spermidine synthase (SPDSy, EC 2.5.1.16) uses PUT as an acceptor for the aminopropyl group from dcAdoMet to form SPD. Another similar aminopropyl transfer reaction from dcAdoMet to SPD is catalyzed by spermine synthase (SPMSy, EC 2.5.1.22) to produce SPM. Even though these two enzymes are functionally similar, they represent two distinctly different enzymes and contain only one homologous domain, which most likely is related to the binding of dcAdoMet (Korhonen et al., 1995). SPDSy can be found in all organisms whereas SPMSy exists only in mammalian cells. Both synthases are rather stable (half–lives of about 12 hours) and their turnover appears to be regulated by the availability of their substrates (reviewed by Jänne et al., 1991).

Both of the above synthases also produce 5'–methylthioadenosine (MTA), the significance of which is often neglected. MTA acts as a feedback regulator of SPMSy (strongest), SPDSy and ODC. The crucial salvage of adenine from MTA is catalyzed by an MTA–specific phosphorylase. Interestingly, many malignant cells lack this phosphorylase activity and simply secrete MTA (Nobori et al., 1996). Furthermore, MTA appears to have cell signaling characteristics and can evoke inhibition of protein methylation and/or phosphorylation (reviewed by Avila et al., 2004).

SPD appears to be the key compound in the studies of PA–related tissue regeneration and SPM is considered more or less to be a storage pool for SPD (Alhonen et al., 2002). However, SPM can act as a free radical scavenger (Ha et al., 1998) and appears to be a regulator of mitochondrial Ca^{2+} transport (Salvi and Toninello, 2004). On the other hand, both SPM and SPD can stabilize soluble DNA as effectively and probably have an impact on the DNA packaging (Saminathan et al., 2002). Surprisingly, SPMSy deficient Gy–mice (virtually without any SPM but with compensatory high SPD contents in all organs) grow to full adulthood in spite of some neuronal defects and compromised male fertility. The growth rate of fibroblasts derived from the Gy–mice is normal (Mackintosh and Pegg, 2000). Therefore, SPD and SPM seem to have multiple and different roles in maintaining cellular function, but may be interchangeable to some extent.

During deoxyhypusine synthesis, the aminobutyl moiety of SPD is transferred to the intermediate deoxyhypusal–residue (Park et al., 1981). Deoxyhypusine is further hydrolyzed to hypusine which is an integral part of eukaryotic initiation factor 5A (eIF5A) and essential for eukaryotic cell proliferation (Park et al., 1993). Furthermore, the accumulation of unmodified, hypusine–lacking eIF5A leads to apoptosis (Jin et al., 2003). Since eIF5A has quite a long biological half–life, it is sometimes difficult to differentiate between a low amount of SPD and loss of eIF5A as the cause of cytostatic effects.

Under physiological conditions, all the above reactions are practically irreversible and the two decarboxylases are considered as the rate–controlling enzymes in PA biosynthesis (reviewed by Shantz and Pegg, 1999). The regulation of ODC is complex and occurs at the levels of transcription, translation and protein degradation. PAs also have a strong feedback effect in the regulation of both decarboxylases: the ODC activity is down–regulated by all PAs whereas AdoMetDC is positively controlled by the amount of PUT. The encoding regions of both ODC and AdoMetDC genes contain multiple regulatory elements that recognize different anabolic and

catabolic inducers such as growth factors, tumor promoters and hormones.

2.2.2 Polyamine interconversion

In mammals the interconversion of SPM and SPD to SPD and PUT, respectively, involves successive acetylation by SSAT and oxidation by PAO. In addition, SPM can also be converted directly to SPD by the recently discovered SMO which was initially mistaken for PAO (Wang et al., 2001a).

Acetylation is considered as the rate–limiting step in the interconversion of higher PAs due to the fact that SSAT is inducible by a huge number of different stimuli and is localized to cytosol (reviewed by Casero and Pegg, 1993; Seiler, 1987). A 250–fold SSAT induction was first detected in the rat liver after CCl_4 treatment (Matsui and Pegg, 1980). The basal level of SSAT is very low and it has one of the shortest half–lives of all enzymes (Matsui et al., 1981). However, regulation of SSAT seems to include many mechanisms from transcription through to protein degradation. The SSAT gene is preceded by a TATA–less promoter which contains several elements such as the PA responsive element (PRE) (Tomitori et al., 2002) and cis–elements for regulation. SSAT has an MATEE sequence (Coleman et al., 1995) which may partly be responsible for its rapid turnover, acting similarly to PEST sequence in the case of ODC. The natural substrates for SSAT are SPD and SPM producing N^1–acetyl–SPD (AcSPD, Fig. 3) and N^1–acetyl–SPM (AcSPM, Fig. 3), respectively (Ragione and Pegg, 1983). SSAT also acetylates AcSPM further to N^1,N^{12}–diacetyl–SPM (diAcSPM, Fig. 3) (Vujcic et al., 2000).

PAO has been studied from many sources (Suzuki et al., 1984; Tsukada et al., 1988) and the enzyme was first purified from the rat liver (Hölttä, 1977). It seems that purified rat liver PAO has a M_w of 60'000, is sensitive to sulfhydryl and carbonyl group reagents, has an optimum pH of 10 for oxidation of PAs in the presence of molecular oxygen and may require Fe^{2+} ions (Hölttä, 1977). PAO has been localized to both cytosol and peroxisomes in the rat liver (Van den Munckhof et al., 1995) and PAO activity can be found virtually in all vertebrate tissues (Seiler, 1995). Mammalian PAO has high affinity towards acetylated PAs; AcSPD, AcSPM and diAcSPM being excellent substrates; however, N^8–acetyl–SPD is not a substrate (Seiler et al., 1980; Suzuki et al., 1981). Interestingly, two PAO isozymes with slightly different enzymatic properties were found in the L1210 cell line (Libby and Porter, 1987). The mammalian PAO attacks secondary N^4 of the substrate from the *exo*–side whereas maize PAO attacks the same atom from the *endo*–side; moreover, the maize PAO does not act on acetylated PAs and actually may be part of a distinct oxidase sub–family. Both mammalian and plant PAO require O_2 and utilize tightly but not covalently bound flavin adenine dinucleotide (FAD) as the cofactor (Fig. 5).

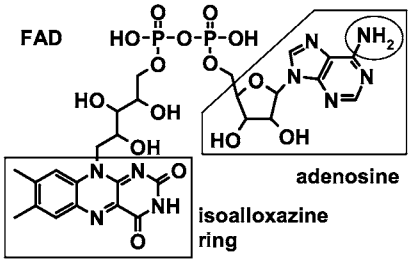


Figure 4 Flavin adenine dinucleotide (FAD), the cofactor of polyamine oxidase.

The FAD cofactor includes an adenosine–residue (with one free amine, circled in Fig. 4) a common feature of other cofactors. A flexible carbon chain (containing two phosphate groups) forms a bridge between the adenosine–residue and the isoalloxazine ring. The oxidized isoalloxazine ring is a good electron sink and readily accepts electrons from the substrate (i.e. it is reduced). The reduced ring structure reduces

molecular O_2 to form one molecule of H_2O_2 per carbon–nitrogen bond cleaved and the aromatic ring structure is oxidized. The free oxidized isoalloxazine ring is planar while the reduced form is bent at the N^5–N^{10} axis (Fig. 5) and this feature may be important in the control of the redox state of the enzyme (Silverman, 2000).

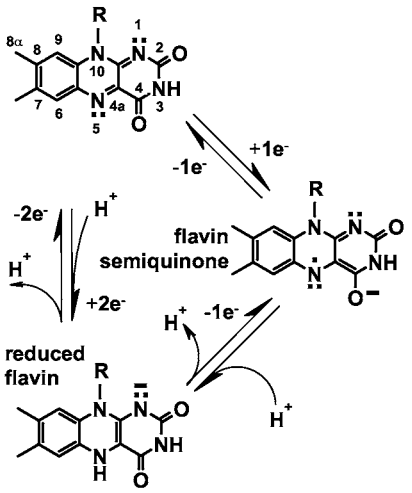


Figure 5 Three redox states of flavin according to Silverman (2000)

Crystallized maize PAO contains 470 amino acids (AAs) and has a 30 Å long U–shaped catalytic tunnel which appears to be optimal for catalysis of linear PAs and the innermost section of this tunnel is located in front of flavin (Binda et al., 1999). The active site is embedded with aromatic side chains and many carbonyl groups that can form hydrogen bonds with the substrate. The larger end of the catalytic tunnel is lined with several acidic AA–residues which may help to steer protonated PAs into the active site and may permit the passage of bulky substrates. No charged groups are detected in the catalytic tunnel probably allowing for a varied nitrogen–carbon organization in the substrate (Binda et al., 2001). General features of the maize PAO (Cona et al., 2004) appear similar to both the barley PAO (Cervelli et al., 2001) and the mouse SMO (Cervelli et al., 2003).

Recently different mammalian PAOs have been cloned by two groups (Wu et al., 2003; Vujcic et al., 2003) but currently no crystallized mammalian PAOs are available nor has their regulation been described. The human PAO codes for a protein with 511 AAs with M_w of 55'500 and the mouse PAO contains 504 AAs with M_w of 55'000.

When we reported the degradation of SPM to SPD in SSAT–deficient cells (Niiranen et al., 2002), it was evident that a new player controlling the PA homeostasis had been discovered. Shortly thereafter the properties of recombinant SMO from multiple sources were described (Cervelli et al., 2003; Vujcic et al., 2002). Four different splice variants of human SMO with slightly different properties have been reported (Murray-Stewart et al., 2002). However, from nine mouse SMO splice variants, the one with the highest activity was cytoplasmic while another, less active, SMO variant was found in both nucleus and cytoplasm after transfection into a murine neuroblastoma cell line (Cervelli et al., 2004). There is a strong indication that SMO utilizes FAD as a cofactor since its sequence contains flavin–binding domains (Cervelli et al., 2004). SMO strongly favors SPM over its acetylated derivatives and does not use SPD as a substrate at all. SMO attacks the same carbon–nitrogen bond as the mammalian PAO and similarly produces H_2O_2. It seems that the H_2O_2 specifically produced by SMO leads to DNA damage and mitochondrial membrane depolarization as signs of apoptosis (Amendola et al., 2005; Chaturvedi et al., 2004; Xu et al., 2004). Presently, the only crystal structure of an SMO–like protein has been that obtained from yeast (Huang et al., 2005). The

mammalian SMO appears to be slightly larger than PAO with 555 AAs and M_w of 62'000.

2.2.3 Terminal degradation of polyamines

Amine oxidase studies have a long history and one of the first extensive reviews dates back to 1950s (Blaschko, 1952). In mammals, there are several other amino oxidases in addition to PAO and SMO. These oxidases can be divided into two subgroups according to their cofactors (flavin or copper–containing oxidases).

Monoamine oxidase (MAO, EC 1.4.3.4) has two variants (A and B) which have over 70 % sequence similarity. MAOs are located in the mitochondria and use covalently bound (via 8α–carbon) flavin mononucleotide (FMN, Fig. 6) as their cofactor (Binda et al., 2002a). However, the significant difference to PAO is that MAOs exclusively attack primary amines.

The overall structure of crystallized human MAO B resembles only slightly the maize PAO; however, the active sites in both oxidases

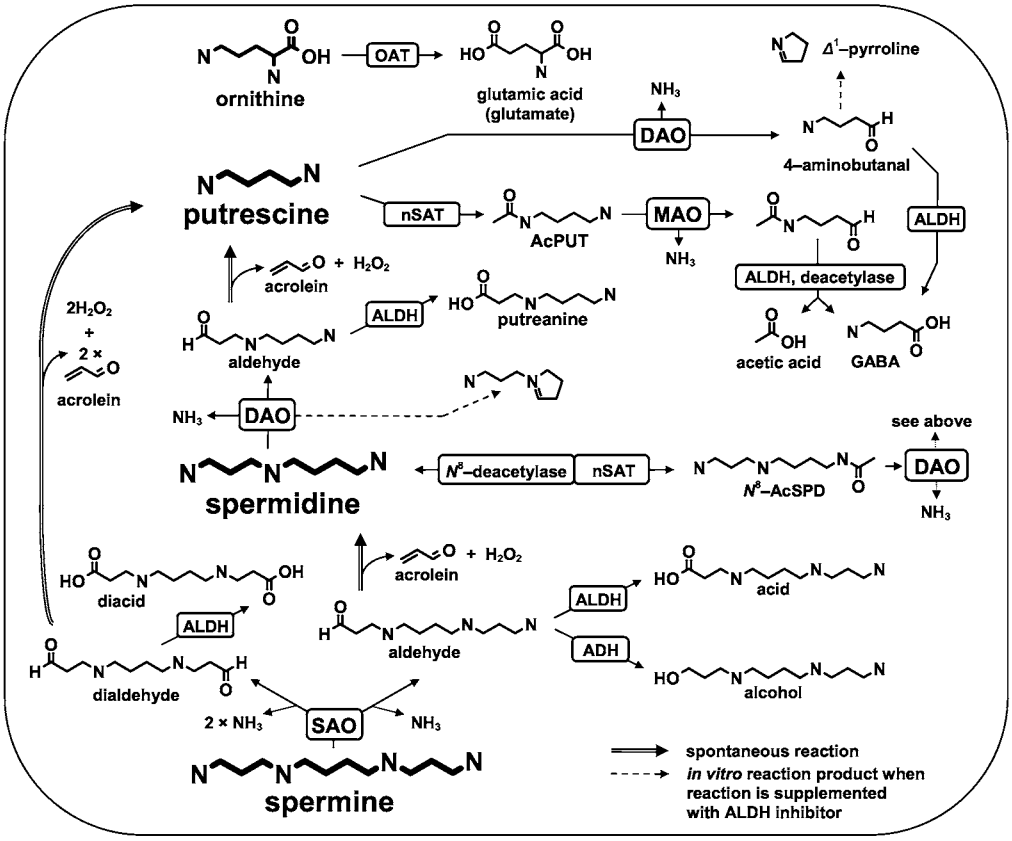


Figure 7 Terminal degradation of polyamines in mammalian cells

AcPUT, acetylputrescine; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; SAO, serum amineoxidase; DAO, diamine oxidase; GABA, γ–aminobutyric acid; MAO, monoamine oxidase; N^8–AcSPD, N^8–acetylspermidine; nSAT, nuclear spermidine N^8–acetyltransferase; OAT, ornithine aminotransferase.

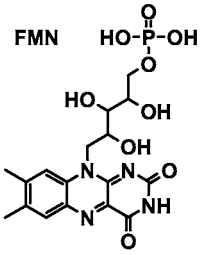


Figure 6 Flavin mono nucleotide (FMN), the cofactor of monoamine oxidases.

are lined by many aromatic and aliphatic residues forming a highly apolar environment for substrates (Binda et al., 2002b). The only apparent structural difference between MAO A and B is a 50 AA–long C–terminal segment in MAO B that binds it to the outer mitochondrial membrane. Both MAO A and B take part in the biogenic monoamine neurotransmitter metabolism but have a lesser role in the catabolism of acetylated amines. MAO inhibitors are of clinical importance in the treatment of depression (MAO A inhibitors e.g. moclobemide) and Parkinson's disease (MAO–B inhibitors e.g. selegiline, reviewed by Agostinelli et al., 2004).

Diamine oxidase (DAO, EC 1.4.3.6) was originally known as histaminase. It requires copper and utilizes a post–translationally altered tyrosine residue, 2,4,5–trihydroxyphenylalanine quinone (Dooley, 1999), as its cofactor (TPQ, Fig. 8) which is an absolute requirement for catalytic activity (Brazeau et al., 2004). DAO can be

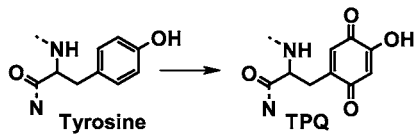


Figure 8 Post–translationally modified tyrosine–residue in the active site of diamine oxidase. TPQ, 2,4,5–trihydroxyphenylalanine quinone.

detected in many mammalian organs but shows high activity in rapidly proliferating tissues like intestinal mucosa. However, in brain, virtually no DAO activity is detectable. The best substrates for DAO are histamine, PUT and SPD. Cyclic amines can also be formed as the DAO–reaction products *in vitro* or in the presence of aldehyde metabolizing enzyme inhibitors (Sessa and Perin, 1994). Increased DAO activities have been associated with tumor promotion (Kusche et al., 1988).

Other amine oxidases similar to DAO have also been detected from different animal sources (like bovine serum, pig and equine plasmas). They are here collectively named as serum amine oxidases (SAOs) and have the same cofactor requirements as DAO. These SAOs use both SPM and SPD as substrates and attack the primary amino groups. The reaction products are ammonia and aldehydes that degrade spontaneously giving rise to another very reactive aldehyde, acrolein (Lee and Sayre, 1998). Numerous other copper–containing amine oxidases have also been identified from a wide variety of species (including micro–organisms and plants); they are all dimers and exhibit very similar properties (extensively reviewed by Agostinelli et al., 2004). To make the tale of amine degradation even more complicated, there is evidence that polyphenolic compounds from plants also have oxidative deamination activities (possibly via their quinone forming properties) in the presence of copper (Akagawa and Suyama, 2001).

There is a nuclear SPD acetyltransferase that can use both PUT and SPD (only from the aminobutyl moiety) as substrates but, in contrast to SSAT, is not inducible. This nuclear acetyltransferase also appears to be linked with histone acetylation and it is suggested that transporting SPD out of the nucleus could be involved in the cell growth regulation via histone

acetylation (Desiderio et al., 1992; Desiderio, 1992). N^8–acetyl–SPD is a poor substrate for DAO but can be returned into circulation by N^8–acetylspermidine deacetylase (EC 3.5.1.48).

Ornithine can also be converted to glutamate by ornithine specific aminotransferase, OAT (EC 2.6.1.13). PUT is a precursor for γ–aminobutyric acid, GABA. Both glutamate and GABA are important neurotransmitters.

2.3 POLYAMINE ANALOGUES

The PAs have central roles in many cellular processes (Wallace et al., 2003). As the population ages, the incidence of many diseases will increase and many of these diseases, e.g. cancer, are strongly associated with distorted PA homeostasis (Thomas and Thomas, 2003). Therefore, understanding PAs is important if we wish to understand the exact mechanisms of cellular functions under extreme conditions.

There are two alternative ways to alter PA levels i.e. the inhibition of their biosynthesis or enhancement of their degradation. Both options are possible with compounds that either mimic the natural substrates or affect the activities of the enzymes involved in the regulation of PA homeostasis. It is not rare that such artificial compounds have more than one effect.

There are at least three possibilities to alter natural PAs: 1) substituents at one or both ends of the PA, 2) substituents along the backbone of the parent PA or 3) altering the original PA backbone length or replacing atom(s) within it. Figure 9 shows schemes for SPD and SPM analogues. In prokaryotes, the PA backbone may

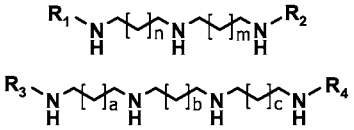


Figure 9 Schemes for SPD and SPM analogues.

contain up to 6 nitrogen atoms (Terui et al., 2005). If n=m=a=b=c=1 or 2, the derivatives are called nor–PAs or homo–PAs, respectively. R_1, R_2, R_3 and R_4 can be almost any moiety (–CH_3, –CH_2CH_3 etc.) and often the above approaches are combined. It appears that the charge distribution is decisive in the recognition and subsequent fate of each analogue (Bergeron et al., 1995a). Here, no PA derivatives particularly related to MAOs, DAO or SAOs will be reviewed.

2.3.1 Analogues affecting polyamine synthesis

The initial promise of blocking the PA biosynthesis as cancer treatment has not been realized – it seems that the inhibition of PA synthesis results in multiple compensatory effects. Nevertheless, a few such inhibitory drugs are shortly described here for their scientific value.

ODC is a logical target for drugs designed to interfere with PA biosynthesis as PUT is the

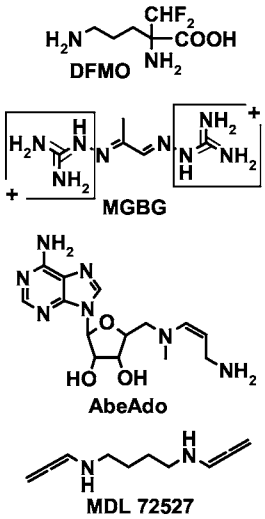


Figure 10 Some PA metabolism inhibitors. The resonance structures of MGBG are marked with boxes. DFMO, ODC inhibitor; MGBG; AdoMetDC inhibitor; AbeAdo; AdoMetDC inhibitor; MDL 72527, PAO inhibitor.

precursor for all higher PAs. Irreversible ODC inhibitors, such as α–difluoromethylornithine, (DFMO, Fig. 10) were originally developed as anticancer drugs. DFMO decreases ODC activity and depletes PUT time–dependently. It is usually cytostatic and its effects are reversed with PA replenishment (extensively reviewed by Seiler, 2003a). Even though native ODC uses exclusively (*L*)–ornithine, the different enantiomers of DFMO appear to have minimal differences when used to inhibit recombinant ODC (Qu et al., 2003). An earlier study with differently methylated PUT analogues showed only slight inhibition of ODC, comparable to that of PUT (Ruiz et al., 1986). DFMO is quite well tolerated by humans but causes some troublesome complications (such as loss of hearing). Fortunately these side effects are reversible. Presently DFMO is used as an anti–parasitic drug in developing countries and has been tried as a cancer chemopreventive agent when combined with other drugs.

The selective AdoMetDC inhibitors such as methylglyoxal–bis(guanylhydrazone), (MGBG, Fig. 10) deplete higher PAs and result in the induction of ODC and the accumulation of PUT. Unfortunately, in humans, MGBG proved to be too toxic to permit clinical use. In rats, MGBG

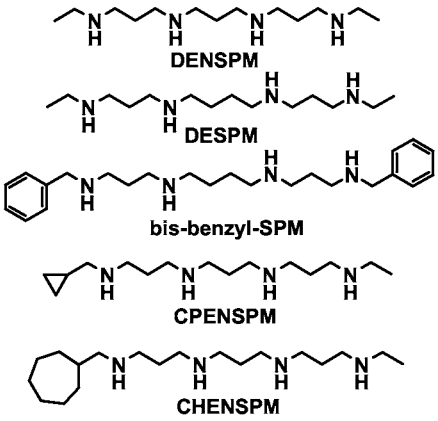


Figure 11 Some differently *N*–alkylated SPM analogues.

treatment increases PAO activity in liver and spleen but decreases it in thymus (Ferioli and Armanni, 2003; Ferioli et al., 2004). Enzyme activated AdoMetDC inhibitor 5'–{[(*Z*)–4–amino–2–butenyl] methylamino}–5'–deoxyadenosine, (AbeAdo, Fig. 10) has been successfully used in the treatment of African trypanosomas (Bitonti et al., 1990). It contains two resonance structures (guanyls). It is important to note that the use of AdoMetDC inhibitors results in the accumulation of unmodified eIF5A. Inhibition of either SPDSy or SPMSy appears to have only a limited effect *in vitro* as usually new PA homeostasis is reached rather rapidly and the treated cells divide only at a somewhat reduced rate (reviewed by Seiler, 2003a).

2.3.2 Analogues affecting polyamine catabolism

Different SPM derivatives (Fig. 11) have proven to be valuable tools in PA catabolism–related studies (extensively reviewed by Seiler, 2003b). Bis–ethylated SPM analogues (DESPM and DENSPM, Fig. 11) are very powerful SSAT inducers (Casero et al., 1989) and deplete natural PAs effectively (Libby et al., 1989). It seems that SPM derivatives stabilize the SSAT protein preventing its ubiquitination (necessary for many short–lived enzymes targeted to proteosomal degradation) by conformational changes (Coleman and Pegg, 2001). Furthermore, these SPM analogues also down–regulate both ODC and AdoMetDC (Chang et al., 1992). Due to these properties, DENSPM is currently in clinical trials for treatment of several cancer types (Hahm et al., 2002).

Only in DENSPM–responsive cells, are there significant expressions of polyamine–modulated factor–1 (PMF–1) and Nf–E2 related transcription factor (Nrf–2, Casero et al., 2003). Nrf–2 binds to PRE of SSAT and transcription cofactor, PMF–1 (with no DNA–binding domain) binds to Nrf–2 via

a unique leuzine–zipper–coil (Wang et al., 2001b) to modulate SSAT transcription. DENSPM treatment significantly increases the expression of PMF–1 and the extremely rapid induction of SSAT suggests that some PMF–1 binds to Nrf–2 even without external stimuli.

DENSPM is proposed to be degraded in several steps *in vivo* (Bergeron et al., 2000; Bergeron et al., 1995b) and it seems that PAO is the enzyme responsible for its degradation (Vujcic et al., 2003). Mechanistic studies with DESPM shows that reduction of flavin is the rate–controlling step in the substrate degradation (Royo and Fitzpatrick, 2005).

Only berenil and pentamidine have been described being as effective SSAT inhibitors (Libby and Porter, 1992). However, new methods are being used more frequently and SSAT suppression by small interfering RNA directly revealed a link between SSAT induction by DENSPM and apoptosis (Chen et al., 2003).

Both CHENSPM and CPENSPM deplete PAs but only CPENSPM can induce SSAT in 24 h while CHENSPM increases DNA fragmentation, alters cell morphology and induces G_2/M cell cycle arrest (Nairn et al., 2000). Furthermore, both analogues have very different effects on tubulin polymerization; CHENSPM enhances this process while CPENSPM slows the polymerization as compared to SPM (Webb et al., 1999). *In vitro* exposure to CPENSPM causes increased transcription of hSMO, mRNA stabilization, *de novo* synthesis of protein and massive SMO activity (Devereux et al., 2003; Wang et al., 2005). Furthermore, the SMO promoter appears to be sensitive to some PA analogues, pointing to a complicated transcriptional regulation.

It seems that differences in terminal alkyl groups are important for the observed effects of SPM mimetics. Bis–methylated SPM analogues are only cytostatic whereas bis–ethyl– and bis–propyl–SPM derivatives are cytotoxic (Kramer et al., 1997).

In mice a single injection of MDL 72527 (N^1,N^2 –bis(2,3–butadienyl) –1,4– butanediamine, 20 mg/kg, Fig. 10, p. 24) rapidly inhibits PAO and results in an accumulation of AcSPD (Bolkenius et al., 1985). However, within two days, the PAO activity reappears slowly in liver and kidney. Long–term treatment with the drug does not appear to cause any toxic effects. It seems that MDL 72527 reacts with mammalian PAO differently than other substrates and binds covalently to N^5 of isoalloxazine ring, thus inactivating the enzyme (Wu et al., 2005). SMO, on the other hand, is less susceptible than PAO to inhibition by MDL 72527 exposure (Vujcic et al., 2002).

2.3.3 α–Methylated polyamine analogues

From the late 1970s onwards, differently alkylated PAs were synthesized in order to inhibit PA synthesis, and in the mid–1980s, several structural PA derivatives with dimethylated carbons were introduced (Nagarajan and Ganem, 1986). SPD containing dimethylated C^1, C^2, C^3 or C^5 carbons (Fig. 12) is resistant to acetylation. Similarly, bis–α– and bis–γ–dimethylated SPM analogues are not acetylated. However, C^8–dimethyl–SPD is acetylated by SSAT as efficiently as SPD. None of the SPD mimetics are substrates for SPMSy. All the mentioned SPD and SPM analogues can support cell growth when natural PAs are depleted by DFMO (Nagarajan et al., 1988).

The first study with MeSPD, MeSPM and Me_2SPM showed that a single methyl group in α–position is enough to protect against acetylation.

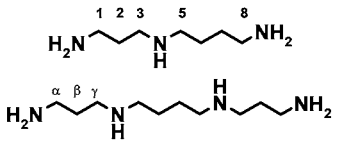


Figure 12 Possible numbers or names of carbons in SPD and SPM.

Furthermore, the analogues restore cell growth in the presence of DFMO similarly to dimethylated PA derivatives. All of the studied analogues deplete natural PAs effectively; MeSPD is not degraded *in vitro* but slow turnover into MeSPM is detected and Me_2SPM is not entirely stable (Lakanen et al., 1992). Natural PAs; SPD and SPM (via interconversion to SPD), and MeSPD rescue the L1210 cells from AdoMetDC inhibitor AbeAdo–induced hypusine depletion whereas Me_2SPM does not (Byers et al., 1994). Interestingly, in CHO cells, AbeAdo treatment results in high PUT contents whereas the DFMO treatment depletes PUT neither effect being modified by extensive uptake of MeSPD or Me_2SPM. The amount of PUT does not affect the uptake of higher PAs, indicating that there are two alternate PA transport systems in these cells. Furthermore, a study in CHO–MG cells lacking the PA uptake system suggests that PA–uptake is essential to maintain the cellular PA levels in this CHO cell line (Byers et al., 1994).

Many PA analogues cause extensive SSAT induction with simultaneous ODC suppression and cell growth arrest. Nevertheless, Me_2SPM can maintain the cell growth even though the SSAT activity increases significantly (and the ODC activity remains unaltered) in a human lung carcinoma cell line NCI H157 (Yang et al., 1995). It seems that cells can survive very high SSAT activities when supplemented with a growth–supporting PA mimetic. Furthermore, MeSPD, MeSPM and Me_2SPM are as effective in inducing the conversion of right–handed *B*–DNA to left–handed *Z*–DNA (Varnado et al., 2000) as their unmethylated parent molecules.

2.3.4 Polyamine transport system and polyamine analogues

Acetylation may be a recognition signal for "unnecessary" PAs as acetylated PAs are usually exported out of the cells (Seiler, 1987). However, there are significant species–specific differences in excretion. In human urine, most of the PAs are in the acetylated form (10–fold difference) but in the rat urine mostly PUT is detected, indicating that acetylation is not required for excretion in the rat. On the other hand, PA–depleted cells (i.e. after DFMO treatment) import extracellular PAs very effectively. However, the PA uptake in cells is not dependent on the export and generally the intracellular PA contents appear to have the opposite effect on uptake (reviewed in detail by Seiler and Moulinoux, 1996).

In spite of extensive efforts, no PA specific transporter has been cloned. Different PA transporter systems seem to exist depending on the cell type. In some cells there is one system for PUT whereas other PAs use a separate transport system. In some other cases, all PAs appear to use the same system for transmembrane movement. Since the stringency of these transporter systems seems to be rather low, PAs have been evaluated as possible vehicles for selective drug delivery into malignant cells that often require substantial PA uptake to maintain growth.

PAs appear to be good carriers for drug delivery, as even anthracene (an intercalator that prevents topoisomerases from resealing DNA breaks during DNA synthesis and transcription;

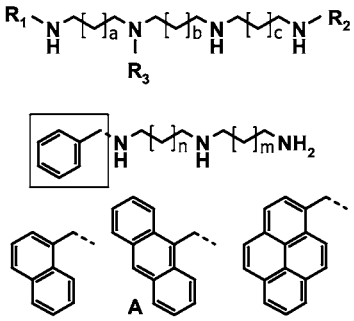


Figure 13 Schemes for PA analogues with ring structure containing conjugates. A, anthracene.

compound A in Fig. 13) conjugated homo–SPD (n=m=2) is readily imported into cells (Wang et al., 2003a). Anthracene–conjugated SPM analogues (R_1=A, R_2=H or aminopropyl, R_3=H) appear to be even better vehicles. Interestingly, a SPM analogue with anthracene in the side–chain (R_3=–$CH_2CH_2CH_2$–A) is also taken up effectively (Phanstiel et al., 2000). However, homo–SPD containing four interlocked 6–carbon ring structure conjugate is too bulky (compound on right in Fig. 13). As expected, the SPD derivatives compete with SPD but in the L1210 cell–line anthracene–SPM analogues seem to use some other transport system than SPD. Also longer SPM derivatives (a, b, c, >=1, R_2=aminopropyl etc.) appear to be potential tools for studying transmembrane trafficking (Phanstiel et al., 2000). Studies with CHO and CHO–MG cell lines confirmed that PA transport system is used for the uptake of these analogues (Wang et al., 2003b).

2.4 TRANSGENIC RODENTS WITH ALTERED POLYAMINE METABOLISM PRODUCED IN THE UNIVERSITY OF KUOPIO

Animals over–expressing human ODC, rat AdoMetDC, human SPDSy and mouse SSAT have been generated. Transgenic animals carrying mouse MT promoter–driven human ODC and mouse SSAT are also available. In the ODC transgenic rodents, ODC is expressed in all tissues with the greatest activities (20 to 80–fold increases) being detected in testis, brain, muscle and heart (Halmekytö et al., 1991). Interestingly, the accumulated PUT is not converted to higher PAs (Halmekytö et al., 1993) and no activation of PA catabolism or urinary excretion is evident in the ODC over–expressing rodents, indicating that some other mechanisms can prevent excessive accumulation of higher PAs. Both the ODC transgenic male mice and rats suffer from greatly decreased fertility but show no signs of generally increased tumorigenesis (Alhonen et al., 1995) even though elevated ODC activity has been linked with transformation (Auvinen et al., 1992). However, the ODC transgenic mice are more sensitive than their syngenic littermates to chemically induced skin papillomas and there is evidence that ODC is linked with skin tumorigenesis though the actual mechanisms are unknown.

The AdoMetDC and SPDSy transgenic animals express the transgenes only moderately (up to 5–fold) and do not show any apparent phenotypic alterations. Furthermore, both the AdoMetDC/ODC (Heljasvaara et al., 1997) and the SPDSy/ODC (Kauppinen et al., 1993) hybrid mice appear normal and PA pools in these animals remain practically unchanged.

Activated PA catabolism is clearly evident in both the SSAT over–expressing mice and the MT–SSAT transgenic rodents. The SSAT over–expression greatly affects the transgenic mice; PUT and AcSPD become greatly accumulated while higher PAs are depleted. Interestingly, PUT accumulation (be it from ODC or SSAT over–expression) is tolerated in brain and may even be neuroprotective (Kaasinen et al., 2000; Lukkarinen et al., 1999). Surprisingly, the low degree of basal SSAT elevation results in female infertility, skin wrinkling and hair loss (Pietilä et al., 1997). Unexpectedly, the MT–ODC/MT–SSAT hybrid mice show signs of even more severe PA catabolism as the accumulating PUT is not converted to SPD (Suppola et al., 2001). Furthermore, MT–SSAT transgenic mice are extremely sensitive to continuous DENSPM treatment as indicated by the high mortality rate in five days associated with ultrastructural abnormalities (i.e. mitochondrial swelling) in the liver (Alhonen et al., 1999; Suppola et al., 1999). Here, some characteristics of different tissues of the MT–SSAT transgenic mice and rat are briefly described (for an extensive review see Jänne et al., 2004).

LIVER: Liver is the organ processing most of the toxic substances and as such is responsible for maintaining balanced homeostasis of many vital functions. The regeneration potential of liver is essential as any damage may be life–threatening. The initiation of the liver regeneration after partial hepatectomy is directly dependent on hepatic PA contents in rat (Alhonen et al., 2002). Partial hepatectomy is characterized with severe SPD depletion in the MT–SSAT rats and SPD level appears to be critical for early liver regeneration. The hepatic recovery processes are initiated as effectively in MeSPD pretreated MT–SSAT rats as in their syngenic littermates without MeSPD treatment but not in transgenic rats without SPD analogue supplementation (Räsänen et al., 2002).

PANCREAS: Pancreas with its endocrine and exocrine portions is another tissue with multiple functions. The endocrine portion is involved in sugar metabolism while the exocrine portion excretes digestive enzymes into the duodenum. Under normal conditions, autodigestion is prevented as the digestive enzymes are mostly produced in inactive forms and stored in the zymogen granules. However, in acute pancreatitis (common causes are alcohol and drug abuse) premature activation of the digestive enzymes (most notably trypsinogen) seems to cause inflammation and pancreatic tissue injury. Zinc administration in MT–SSAT transgenic rat induces acute pancreatitis (Alhonen et al., 2000) whereas DENSPM treatment alone is not sufficient to cause the inflammation. However, combined treatment with DENSPM/MDL 72527 also induces acute pancreatitis indicating that the loss of SPD, not the H_2O_2 production, is the key factor in the development of the disease in these animals. The pretreatment with MeSPD prevents the SSAT induction mediated development of pancreatitis and confirms the crucial importance of SPD in these animals.

SKIN: The skin of the MT–SSAT rat wrinkles with age and the animals start losing hair at the age of 6 to 8 weeks, similar to the MT–SSAT transgenic mice. The keratinocyte differentiation in the MT–SSAT mice is clearly abnormal (Pietilä et al., 2005) and suggests that the availability of PUT is linked to correct keratinocyte maturation. As a proof of the significance of PUT, DFMO treatment of transgenic mice restores hair growth to some extent. However, no studies on the MT–SSAT transgenic rat skin have been carried out.

2.5 OPTIMIZING ENZYME ACTION

By definition, enzymes are biological catalysts that accelerate chemical reactions by many orders of magnitude (acceleration ranges for example from 10^6 for carbonic anhydrase to 10^{17} for alkaline phosphatase compared to the non–enzymatic reaction). The rate of acceleration is expressed as k_{cat} and it stands for the maximum number of substrate molecules converted to product molecules per active site per second (for example, the k_{cat} value for papain is 10 while for catalase it is 10^7). The size of enzymes varies from fairly small (M_w of few thousands g/mol) to extremely large molecules (M_w of millions g/mol and composed of multiple sub–units). Enzymes may have high substrate specificities that depend on environmental factors. Interestingly RNA can also catalyze reactions (reviewed by Lorsch and Szostak, 1996). The enzymatic reaction can be presented as free substrate (S) forming a complex with enzyme (E–S), conversion of substrate to product in the active centre (E–P) from which the product is released (Scheme 1).

The enzymes are composed of hundreds or thousands of covalently bound AAs which fold in according to the nature of AAs. Also other

$$E + S \rightleftharpoons E\text{-}S \rightleftharpoons E\text{-}P \rightleftharpoons E + P$$

Scheme 1 Basic scheme for enzymatic reaction from free substrate to released product.

non–covalent bonds maintain the three dimensional structure of the proteins intact; hydrogen bonds are essential for α–helix and β–sheet integrity but electrostatic interactions between the side–chains of AAs are also important. Ionic, ion–dipole and dipole–dipole interactions as well as charge transfer complexes are present in proteins. The essential phenomena in many enzymatic reactions are hydrophobic interactions i.e. organized water molecules are pushed away from the hydrophobic surfaces of the enzyme and the substrate as they approach each other (entropy increases). Only an accumulation of these rather weak non–covalent interactions permits the substrate binding. The mechanisms of the enzymatic catalysis include approximation (two substrates are close to each other), nucleophilic (covalent) catalysis (enzyme binds substrate covalently forming a more reactive intermediate), acid–base catalysis (proton transfer), electrostatic catalysis (the ionic charge stabilizes transition state), desolvation (H_2O removal destabilizes the charged group of the substrate in a lower dielectric constant environment) and strain which is characterized by conformational distortion(s) in the enzyme and/or the substrate (reviewed in an excellent book by Silverman, 2000).

2.5.1 Stereospecificity of enzymes

Proteins are extremely chiral compounds, thus it is no surprise that enzymes express a great degree of selectivity towards the different isomers of the substrates. The stereoselectivity of the enzymatic reaction arises from the conformation of the active site which depends on the specific functional groups of AAs. One isomer of the substrate simply may fit poorly or not at all into the active site whereas a product of the enzymatic reaction with an unfavorable conformation may be released from the enzyme very slowly or not at all (enzyme activated inhibitors). The production of enantiomerically pure compounds is imperative in pharmaceutical industry as many drugs are more active in one conformation and the "wrong" enantiomer may be inactive, inhibitory or even toxic.

2.5.2 Forced evolution of enzymes

Enzymes have tremendous potential for use as practical catalysts in the pharmaceutical industry. Currently there are not many industrially used enzymes and these are mostly limited to some rather simple enzymes like lipases, esterases, catalases, peroxidases and hydroxylases (reviewed by (Zaks and Dodds, 1997). During the last few decades, several approaches have been introduced to increase the utility of the enzymes in the chemical industry. Improving the stability of the enzymes and altering the substrate specificities are of obvious interest.

Directed evolution is a method with which new enzymes are developed and screened for greatly improved performance (Chirumamilla et al., 2001). The procedure involves mutation of the gene of the target enzyme, expression of the new gene(s) and screening of the freshly–synthesized enzymes for the desired properties. When small regions of the enzymes are targeted, site–directed mutagenesis with degenerate oligonucleotides is a simple method (Hermes et al., 1990). Another effective method is error prone PCR where the target gene is amplified in varying concentrations of Mn^{2+}. However, the most successful method is DNA shuffling where the gene of interest is cleaved into many random fragments, purified and recombined in a PCR like process without exogenous primers. After terminal primer addition in the final step of extension, full–length sequences are amplified and cloned (Crameri et al., 1998). The greatest advantage is the fact that wild type genes can be pooled from the same family of enzymes (possibly from different organisms) and amplified under mutagenic conditions (Stemmer, 1994). The described

methods have produced some very useful industrial applications such as enzymes that can utilize benzene, toluene, PCB and related biphenyl compounds as their substrate (Kumamaru et al., 1998). The are many successful applications i.e. a peroxidase (used in oxidation of dyes) that is stable in alkaline (pH 8.5–10) and highly oxidative conditions, proteases and phospholipases with greatly improved thermal stabilities and a subtilisin (protease) mutant that does not require calcium and is 100–fold more stable in strongly chelating conditions than the wild–type enzyme (reviewed by Chirumamilla et al., 2001).

2.5.3 Allosteric regulation

Enzymatic reactions are equilibrium reactions and the products often have feedback effects. A product may compete for the active site with the substrate (negative feedback) or interact with the enzyme protein at some other site (allosteric site). Binding of the regulator to the allosteric site may induce positive or negative conformational changes in the enzyme (Kern and Zuiderweg, 2003) i.e. the substrates may access the active site more or less easily, respectively. The affinity of G418 to ODC is rather low (K_I=8 mM at pH 7.0 with (*L*)–ornithine) but clearly causes conformational alterations to ODC protein (Jackson et al., 2003). It appears that allosteric ligands are often small hydrophobic molecules (mostly inhibitors) that may bind on the surface but also deep into the core of the enzyme or between the dimerization interfaces of the subunits of the enzyme stabilizing one conformation (reviewed by Hardy and Wells, 2004).

2.5.4 Other factors affecting enzymatic reactions

The pharmaceutical industry aims for cost–effective methods. However, protein–protein interactions become more likely when the protein concentration is increased. This phenomenon exhibits similar characteristics to allosteric regulation (Goh et al., 2004). Protein immobilization is a way to counter many negative effects and the method also enhances the use of enzymes in continuous processes. The stabilities, substrate specificities and activities of the enzymes used may improve after immobilization. Also the separation of the products as well as recycling and disposal of the enzymes is straightforward (reviewed by Cao et al., 2003).

2.5.5 Enzymes in organic solvents

As long as the use of enzymes is limited to aqueous media, the bioconversion of many organic chemicals is limited. The solubility of many potential compounds is very low in water and the recovery of the products may be difficult. Furthermore, common organic reagents degrade in water, unwanted side reactions are frequent and thermodynamic equilibria of many processes are unfavorable. Thus, the use of an organic solvent would circumvent many of the problems associated with water–based reaction media and the concept of "medium engineering" has surfaced after the appearance of some interesting findings of enzyme behavior in non–aqueous solvents (reviewed by Klibanov, 1997).

The major parameter in aqueous media is pH but this has no significance in organic solvents. However, enzymes have "pH memory" i.e. the catalytic activity reflects the pH of the aqueous solvent from which the enzymes were collected. Proteins are also very rigid in non–aqueous media because intra–protein interactions are far stronger when there are no H_2O–molecules present. Structural inflexibility also means that enzymes have "ligand memory" and the active site retains the conformation of the ligand with which it was lyophilized. This structural stability is especially valuable when enantiomeric selectivity of the enzyme is sought (reviewed by Klibanov, 2001).

Enzymes often express totally unpredictable properties in organic media. Such qualities include extreme thermostability and dramatic substrate specificity alterations: enantiomer selectivity may change between different organic solvents; different functional groups are targeted etc. The absence of hydrogen bonds may even permit for reverse reactions to occur e.g. ester formation from alcohols and acids (reviewed by Carrea and Riva, 2000).

Unfortunately, enzymes tend to be far less active in organic media. However, some solutions are available: hydrogen bond forming solvents (glycerol, ethylene glycol) may be used to "relax" proteins and even small amounts of water (< 3 %) can be added into the reaction media. Lyophilization appears to be major reason for the low activities of enzymes in organic media and thus lyoprotectants like poly(ethyleneglycol), crown ethers, sugars and even salts have been used during the enzyme preparation (reviewed by Klibanov, 2001).

3 AIMS OF THE STUDY

The PA derivatives with a single methyl group in α–position (MeSPD, MeSPM and Me_2SPM) were first introduced in the early 1990s. However, the synthetic protocols proposed at that time were inefficient and appropriate only for milligram–scale production. Accordingly, these compounds have previously been studied only *in vitro*.

This study was initiated when it was shown that MeSPD can fulfill extremely well the cellular role of natural SPD during severe PA depletion (Räsänen et al., 2002). However, a good supply of these substances was necessary for extensive *in vitro* and *in vivo* studies. Furthermore, to obtain a thorough understanding of the fates of these PA analogues in the cellular environment and their actual relevance to the PA homeostasis, multiple simultaneous approaches were required.

The major goals in this study were:

► Establish an immortalized fibroblast cell line from the MT–SSAT transgenic rat to be used as a tool to test hypotheses before *in vivo* studies.

► Examine the stabilities of all three α–methylated PA derivatives in rat liver extracts and as substrates for recombinant oxidases (SMO and PAO) *in vitro*.

► Study the fates and significances of the PA derivatives in the MT–SSAT transgenic animals *in vivo*.

► Study the significance of substrate chirality in PAO–mediated oxidation.

4 MATERIALS AND METHODS

4.1 POLYAMINE ANALOGUES AND OTHER CHEMICALS (I–III)

The synthesis of the α–methylated PA derivatives is described in detail in **II**. The enantiomers of MeSpd and AcMeSpd (1–amino–8–acetamido–5–azanonane) were prepared as presented earlier (Grigorenko et al., 2005) and in **II**, respectively. The two isomers of MeSPM and the diastereomers of Me_2SPM were also synthesized according to the principles presented in **II** and described in greater detail in **III**. The purities of all α–methylated PA analogues and their different enantiomers were checked with ^{1}H– and ^{13}C–NMRs (Avance DRX Spectrometer at 500.13 MHz) and all proved to be >99.5 % pure. DENSPM, DESPM and bis–benzyl–SPM (Fig. 10, page 24) were synthesized principally as described earlier (Rehse et al., 1990) and proved to be >98 % pure according to ^{1}H– and ^{13}C–NMR. All compounds were further studied with the HPLC and again showed excellent purities. MDL 72527 was a generous gift from Hoechst–Roussel.

Aminooxy–ethyl–putrescine (AOE–PUT) and its acetyl and oxime derivatives were synthesized as referred to in **III**, lyophilized and dissolved in 100 mM stock solutions in water. All proved to be >95 % pure according to both NMR and HPLC studies. Preformed Schiff bases of SPM with PL (PL=SPM) was prepared as described in **III**.

The short PA analogues (2–2–SPD and 2–3–2–SPM, Fig. 14) and their bis–substituted derivatives were prepared (R_1=R_2=R_3=R_4= –ethyl or –acetyl groups) as described earlier (Saramäki, 2004) .

All other chemicals were purchased from Sigma–Aldrich and Fluka. Freshly distilled carbonyl compounds were checked by ^{1}H–NMR, dissolved as 100 mM stock solutions in ethanol and stored in the refrigerator prior to studies. Hydrochloride salt of PL was dissolved in H_2O and stored in –20 °C. [6–^{3}H]Thymidine (specific radioactivity 18 μCi/mmol) for DNA–synthesis studies was obtained from Perkin Elmer Life Sciences.

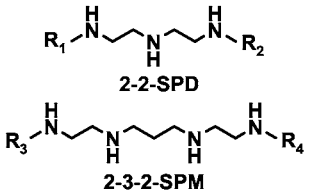


Figure 14 Schemes for short SPD and SPM derivatives.

4.2 TRANSGENIC ANIMALS (I, II)

The generation of the MT–SSAT transgenic rats and mice has been described earlier in detail (Alhonen et al., 2000; Suppola et al., 1999). Both animal lines were produced by the standard pronuclear injection technique using the same transgene construct; the mouse MT–promoter driven mouse SSAT. The animals were housed in the National Laboratory Animal Centre of Kuopio University under controlled temperature (20 ± 2 °C), humidity (50–70 %) and lighting (12 h/12 h) and fed *ad libitum*.

Different α–methylated PA analogues and MDL 72527 were dissolved in saline and administered i.p. according to Table I. After sacrifice, the tissue pieces were frozen in liquid nitrogen and homogenized in standard buffer (25 mM Tris–HCl pH 7.4 with 0.1 mM EDTA and 1 mM dithiotreitol, DTT). The 20 μl aliquots of the homogenates, supplemented with 180 μl of 5 % w/v sulphosalicylic acid in 10 μM 1,7–diaminoheptane, were used for the PA assays. The homogenates were centrifuged (at +4 °C and

Table I Different polyamine analogue doses and treatment timetables for animal studies

ANIMAL	TREATMENT	DOSAGE	TIME BEFORE SACRIFICE	PUBL.
RAT	*Liver regeneration studies*			
MT–SSAT	MeSPD	5 mg/kg	20 h + 4 h	I
transgenic	MeSPD	25 mg/kg	20 h + 4 h	I
	Me_2SPM	25 mg/kg	20 h + 4 h	I
RAT	*Analogue stability / toxicity studies*			
MT–SSAT	MeSPD	25 mg/kg	22 h + 16 h	I + II
transgenic	MeSPM	25 mg/kg	22 h + 16 h	I + II
	Me_2SPM	25 mg/kg	22 h + 16 h	I + II
	MDL 72527 with or without analogue treatment	50 mg/kg	40 h + 24 h	I
MOUSE	*Analogue stability / toxicity studies*			
MT–SSAT	MeSPD	50–500 mg/kg	24 h	II
transgenic and	MeSPM	12.5–50 mg/kg	24 h	II
syngenic mice	Me_2SPM	12.5–50 mg/kg	24 h	II

13'000 × g for 30 min) and the supernatant fractions were used for the enzyme assays.

In the liver regeneration experiments, the rats were sedated with a 0.1 ml dose of fluanisone (Hypnorm, Janssen) and fentanyl citrate mixture (10 mg/ml and 0.315 mg/ml; respectively). The anesthetic was a mixture of midazolam (Dormicum, Roche), fluanisone and fentanyl citrate (1.25 mg/ml, 2.5 mg/ml and 0.07875 mg/ml; respectively). The anesthetic was administered at 0.2 ml/100 g dose. The Institutional Animal Care and Use Committee of the University of Kuopio and the Provincial Government approved the animal experiments.

4.3 IMMORTALIZED MT–SSAT TRANSGENIC RAT FIBROBLASTS (I, III)

The 13 days old MT–SSAT transgenic rat fetuses were used as the sources for the fibroblasts. The fibroblast processing was performed as described earlier (Mackintosh and Pegg, 2000). Transfection was performed with the same commercial kit LIPOFECTAMINE™ plus; the same plasmid expressing SV40 small *t* and large *T* antigens (a kind gift from Dr. M. J. Tevethia, the Department of Microbiology and Immunology, Pennsylvania State University College of Medicine, Hershey, PA, USA) was also used. Thirty one different cell populations out of 240 were identified with the aid of the quantitative PCR and appropriate cell populations were selected for further studies according to their growth rates, external appearance and SSAT expression.

In different experiments, the immortalized fibroblasts were grown in DMEM (Invitrogen) supplemented with heat–inactivated 10 % FBS, gentamycin (50 μg/ml; Gibco) and with or without geneticin (G418) supplementation (500 μg/ml;

Sigma). The freshly plated cells were allowed to adhere for 24 h before fresh medium and the drugs were supplemented. After the incubation, the cells were washed once with PBS, detached with trypsin and counted. The PA concentrations were measured from the supernatant fractions after sulphosalicylic acid precipitation.

4.4 RECOMBINANT PROTEINS (I, III)

The production of the plasmids containing cDNAs coding the human SMO and human PAO ORFs with 6×His at the N–terminus is explained in detail in **I**. These plasmids were provided by Dr. Vujcic, Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, New York 14263, USA. Bacteria containing plasmids were cultured and collected according to Qiagen Qiaexpressionist™ manual and the proteins were purified under native conditions using Ni–NTA His Bind Resin (Novagen) according to the manufacturer's instructions. PAO was further purified with affinity chromatography and the procedure is explained in detail in **I**. As the last step proved to be ineffective, in **II** and **III** it was not performed. The production of the plasmid containing the recombinant human SSAT cDNA was performed by Dr. Anne Uimari and the exact procedure is described in **I**. Similarly to the oxidases, hSSAT was also purified with Ni–NTA Resin under native conditions.

Both hSMO and hSSAT were very pure according to SDS–PAGE while hPAO was estimated to be 80 % pure. However, the contaminating bacterial protein in hPAO preparation did not have any PAO–like activity. The protein concentrations were measured by using the Bio–Rad Protein Assay (Bio–Rad, CA, USA). The kinetic studies of the oxidizing enzymes were performed mostly in duplicate with three to six different 10 up to 1000 μM substrate concentrations (**I–III**) and a few in triplicate (**III**). The hSMO and hPAO reactions were carried out in a total volume of 180 μl 100 mM glycine–NaOH at pH 9.5 (**I–III**), 100 mM alanine–NaOH at pH 9.5 (**III**), 50 mM borate at pH 9.3 (**III**), 50 mM phosphate at pH 7.4 or pH 9.5 buffers (**III**); all buffers contained 5 mM DTT as well. Different freshly distilled 5 mM aldehydes (**I**, **III**) were used to decrease the K_m values and to increase the reaction velocities of hPAO (Hölttä, 1977). The effects of different structurally related 5 mM ketones (**III**) on the metabolism of the different isomers of MeSPD were similarly tested with hPAO. The reactions were allowed to proceed from 10 to 60 min at +37 °C before stopping the reactions with sulphosalicylic acid precipitation. The reaction mixtures without hPAO were routinely checked to exclude any enzyme independent degradation of substrates. The kinetic studies of hSSAT were performed with four different 100 up to 1000 μM SPD or SPM substrate concentrations. Similarly, four different 100 up to 1000 μM MeSPD or Me_2SPM concentrations were used as competitive inhibitors.

4.5 LIVER EXTRACTS (I, III)

Wild–type Wistar rats were sacrificed and livers were processed as described in **4.2**. All supernatants were combined and eluted at +4 ºC in the standard buffer (**4.2**, **I**) or the borate buffer (**4.4**, **III**) with high–salt supplementation (500 mM NaCl) to remove the natural PAs. The elutions were performed with Amicon Ultra–15 centrifugal filter devices (Millipore) with nominal M_w limit of 30,000. The resulting liver eluates were desalted thrice in the same columns with non–salt buffer to remove excess salt. The final eluates were pooled and the protein concentrations measured with the aid of the Bio–Rad protein assay.

The reaction buffers, 100 mM glycine–NaOH at pH 9.5 (**I**) or 50 mM borate at pH 9.3 (**III**), were used to study the metabolism of the natural PAs and the α–methylated derivatives. An appropriate amount of the liver extract was used

in the total reaction volume of 180 μl. The reactions were initiated with the addition of the PAs or their analogues; in the case of MDL 72527 (250 μM), extracts were pre–incubated for 10 min to inactivate PAO before substrate addition. Different 5 mM aldehydes were used similarly as in **4.4** to enhance the activity of PAO. The reaction tubes were incubated for 60 (**I**) or 120 (**III**) min in +37 ºC water–bath and stopped as in **4.2**.

4.6 ANALYTICAL METHODS

The treatment of the transgenic rats before partial hepatectomy and the determination of DNA synthesis rate were performed as described earlier (Räsänen et al., 2002). The ODC activities (**I**) were measured as described by Jänne and Williams-Ashman (1971). The SSAT activities (**I**–**III**) were measured essentially as described by Bernacki et al. (1995). Furthermore, the tissue PAO activities (**I**) were measured principally as described by Kumazawa et al. (1990) except that radioactive N^1,N^{11}–diacetylnorspermine was used as the substrate. Alanine aminotransferase and α–amylase were determined from heparinized blood plasma of the mice using an analyzer system Microlab 200 from Merck.

The concentrations of the PAs and analogues were determined with the aid of HPLC (**I**–**III**) essentially as described by Hyvönen et al. (1992). Separation of (*R*)– and (*S*)–isomers of MeSPD (**III**) was performed with Chiral–HPLC (Whelk–O 1 (*R*,*R*) 25 cm * 4.6 mm column and isocratic run 0–75 min with a flow–rate of 0.55 ml/min 70 % EtOH) after dansylation and treatment as described earlier (Porter et al., 1985).

4.7 STATISTICAL METHODS

Where applicable, the data is expressed as means ± SD and analyzed by two–tailed Student's test. For multiple comparisons, one–way analysis of variance with the Dunnett's *post hoc* test was used with the aid of a software package, GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA).

5 RESULTS

5.1 METABOLIC STABILITY OF α–METHYLATED POLYAMINE ANALOGUES (I)

The recombinant protein studies showed that MeSPD similarly to SPD was not a substrate for either hPAO or hSMO. However, both MeSPM and Me_2SPM were degraded by both oxidases. Surprisingly, the activity of hPAO seemed to increase with the number of methyl substituents, whereas the activity of hSMO clearly decreased with the same substrates when compared to SPM as a substrate. As expected, the activity of hPAO was enhanced by aldehyde supplementation. Furthermore, MeSPD and Me_2SPM were not substrates but competitive inhibitors of hSSAT when natural PAs were used as substrates, confirming that the α–methylation protects the compound from acetylation but not from oxidation. The fates of the analogues in the liver extract studies (corresponding to the cellular environment) supported the results achieved with the recombinant proteins.

The studies with the immortalized MT–SSAT fibroblasts verified that both SPM derivatives were not metabolically totally stable. However, Me_2SPM was clearly more resistant to degradation than MeSPM. Furthermore, MeSPD appeared to be metabolically stable and was not converted to MeSPM. The PA analogues accumulated efficiently in the cells, induced the SSAT activity to some extent and effectively substituted for the natural PAs. As expected, MeSPM was the least tolerated analogue due to its more extensive degradation.

MeSPD did not appear to undergo any metabolism *in vivo*. In line with the *in vitro* studies, both SPM analogues were degraded in the liver of the MT–SSAT transgenic rat. Pretreatment of the transgenic rats with a low MeSPD dose (2 × 5 mg/kg) did not restore the early liver regeneration after partial hepatectomy. However, transgenic rats pretreated with Me_2SPM (2 × 25 mg/kg) and higher MeSPD (2 × 25 mg/kg) dose did not suffer from delayed liver regeneration after surgical removal of two thirds of the liver. In fact, Me_2SPM appeared to be even more effective than MeSPD on a molar basis. MeSPD detected in the liver of Me_2SPM treated animals was only half of MeSPD detected in the liver of rats given the lower MeSPD dose. The salvaging effect of Me_2SPM was verified *in vitro* revealing that Me_2SPM reversed the growth inhibition by DFMO in the MT–SSAT fibroblasts.

5.2 EFFICIENT SYNTHESIS PROTOCOLS FOR α–METHYLATED POLYAMINE ANALOGUES AND THEIR *IN VIVO* AND *IN VITRO* STUDIES (II)

Straightforward synthetic protocols for each α–methylated PA derivative were developed. Commercially available ethyl 3–aminobutyrate was used as the starting material for MeSPD, MeSPM and Me_2SPM syntheses. The mesylated derivative of benzyl chloroformate protected 3–aminobutanol was the key intermediate for the preparation of all PA mimetics. MeSPD, MeSPM and Me_2SPM were prepared in five, seven and seven steps, respectively. The synthesis yields were satisfactory: 40–60 % of the starting material was converted to the target compound. Furthermore, (*R*)– and (*S*)–alaninols were used as the starting material for the production of the two isomers of AcMeSPD. The preparations of both AcMeSPD enantiomers in seven steps were successful and about 50 % yields for both (*R*)– and (*S*)–AcMeSPD were achieved.

The toxicity of the three α–methylated PA derivatives was extensively tested with syngenic and the MT–SSAT transgenic mice and rats. It can be concluded that all the analogues were well tolerated by mice and SPD was depleted in a

dose–dependent manner. α–Amylase and alanine aminotransferase (ALAT) activities (as determined from a heparinized blood plasma) remained virtually unaltered and the insignificantly increased SSAT activities also did not appear to have any deleterious effects. The transgenic rats tolerated only 2 × 25 mg/kg doses of the drugs.

The fibroblast studies showed effective depletion of natural PAs in both control and the SSAT over–expressing cell lines. In both cell lines, 1 mM MeSPD resulted in total loss of PUT and 90–95 % reduction of SPD in 48 h whereas SPM levels remained at about two thirds when compared to untreated cells. One millimolar Me_2SPM supplementation in both cell lines was even more effective resulting in a total loss of PUT and 90–95 % depletions of both SPD and SPM. At concentrations higher than 10 μM, MeSPM was toxic to both cell lines. Supplementation of 1 mM aminoguanidine (AG) prevented the degradation of MeSPM effectively indicating that the unmethylated end of the SPM derivative is readily targeted by AG–sensitive oxidases. The SSAT activities remained unaffected in the control cell line and increased only slightly in transgenic cells as a result of exposure to the PA analogues. When comparing the uptake of the three PA derivatives, it seemed that the maximal uptake of these compounds was achievable with rather low concentrations in the studied cell lines. Ten micromolar MeSPM supplementation resulted in accumulation of MeSPD (as a degradation product of MeSPM) to about two thirds of the level achieved with PA derivatives in 1 mM MeSPD or Me_2SPM treated cells. In the presence of AG, the accumulation of MeSPM (combined with the minimal amount of MeSPD) was only marginally lower (about 50–60 % of detected analogues in 1 mM MeSPD/Me_2SPM treated cells). However, *in vivo*, the drug accumulation was dose–dependent.

Racemic AcMeSPD was a substrate (K_m = 100 μM, k_{cat} = 1.3 s^{-1}) of hPAO suggesting that acetylation is essential for the degradation of MeSPD and acetylation should greatly improve the degradation of both MeSPM and Me_2SPM by hPAO. The important finding was that (*R*)–AcMeSpd (K_m = 95 μM, k_{cat} = 9.0 s^{-1}) was almost as good a substrate as AcSpd (K_m = 14 μM, k_{cat} = 8.5 s^{-1}) for hPAO. (*S*)–AcMeSpd (K_m = 170 μM, k_{cat} = 1.2 s^{-1}), however, appeared to be rather poor a substrate suggesting that hPAO has hidden potency for stereospecificity and may readily use (*R*)–enantiomer of AcMeSPD.

5.3 STEREOSPECIFICALLY FLEXIBLE POLYAMINE OXIDASE (III)

Seventeen aldehydes were tested for their ability to enhance hPAO–mediated degradation of racemic, (*R*)– and (*S*)–MeSPD (Fig. 16). The structures of the aldehydes greatly influenced the degradation rates of the substrates. For example, aliphatic aldehydes (Fig. 16, line E) appeared to be less effective than aromatic aldehydes (Fig. 16, lines A–D). The aldehyde sensitive site of hPAO appears to be restricted in its crucial dimensions as the position of carboxaldehyde group in structures with two aromatic rings is critical (Fig. 16, line A). Furthermore, nitrogen in the *p*–position to the carboxaldehyde group appears to be essential for effective degradation of (*S*)–MeSPD (Fig. 16, line B). PL with two additional hydroxyl groups (Fig. 16, line B on right) greatly increased the oxidation of (*S*)–isomer which was generally an unfavored substrate with the tested aldehydes. Furthermore, the position of the hydroxyl group in the three different hydroxyl–BAs was critical to the substrate degradation and only the *m*–position allowed any marked degradation of (*R*)–MeSPD (Fig. 16, line C). Cyclohexane carboxaldehyde also allowed efficient degradation of both racemic and the (*R*)– enantiomer of MeSPD. The position of the nitro group did not appear to have any major effect on the oxidation

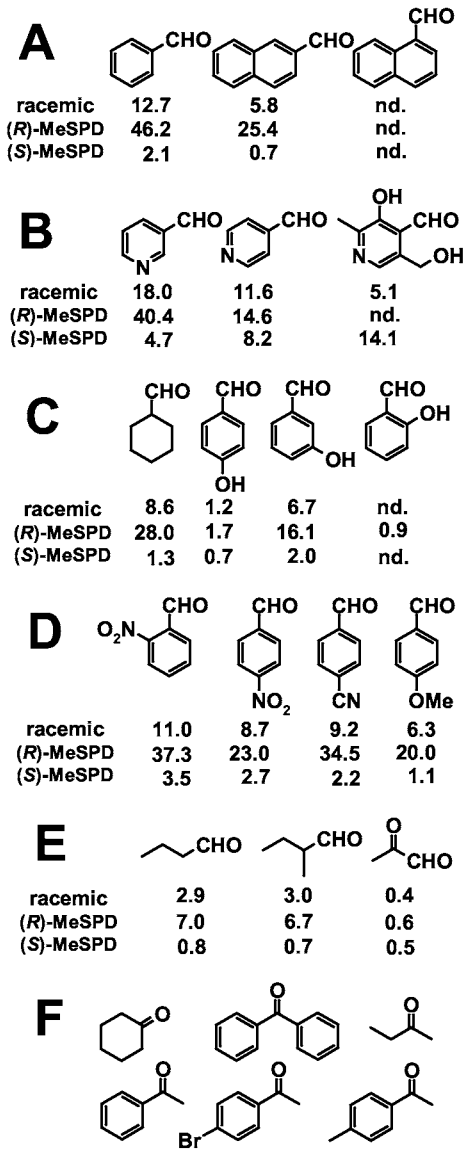


Figure 16, lines A–E The percentages of hPAO–mediated oxidations of 200 μM racemic, (*R*)– and (*S*)–MeSPD in the presence of 5 mM aldehydes in 1 hour at +37 ºC in 100 mM glycine–NaOH at pH 9.5. **Line F** Structurally related ketones that proved ineffective. nd., not detected.

rates (Fig. 16, line D). Moreover, electron–repulsing (–OCH_3) or electron–withdrawing (–CN) substituents opposite to the carboxaldehyde group had no marked differences on the oxidation rates of the two MeSPD isomers (Fig.16, line D). The structurally related and tested ketones proved ineffective as hPAO enhancers (Fig. 16, line F).

Based on the screening studies with different aldehydes it seemed possible to enrich different isomers of MeSPD from racemic MeSPD by hPAO in the presence of guide–molecules (Fig. 17). BA supplementation in the reaction mixture was expected to result in the predominant degradation of (*R*)–MeSPD whereas PL should have evoked an opposite affect i.e. the degradation of (*S*)–MeSPD. Moreover, pyridine 4–carboxaldehyde (P4CA) appeared to be an unselective enhancer for the degradations of both MeSPD enantiomers in the presence of hPAO (Fig. 16, aldehyde in the middle of line B). Chiral–HPLC analysis after 5 mM BA and PL supplemented reactions revealed that this indeed had occurred (Fig. 18). No reduction of reaction products was performed. Therefore, it is not clear whether 3–aminobutanals retain their conformation or if they are racemized during the reaction. Nonetheless, the 3–aminobutanal formed is labile in the aqueous environment and degrades rapidly. The rat liver extract studies showed that native PAO is also prone to stereospecific steering by BA and PL.

The key aldehydes were also studied with the three diastereomers of Me_2SPM and the two enantiomers of MeSPM. BA increased the reaction rates of hPAO with all chiral substrates whereas PL supplementation clearly enhanced the hPAO–mediated degradation of only (*S*,*S*)–Me_2SPM and did not enhance the use of the methylated terminus of (*R*)–MeSPM. The kinetic studies revealed that successful 5 mM BA or PL enhanced

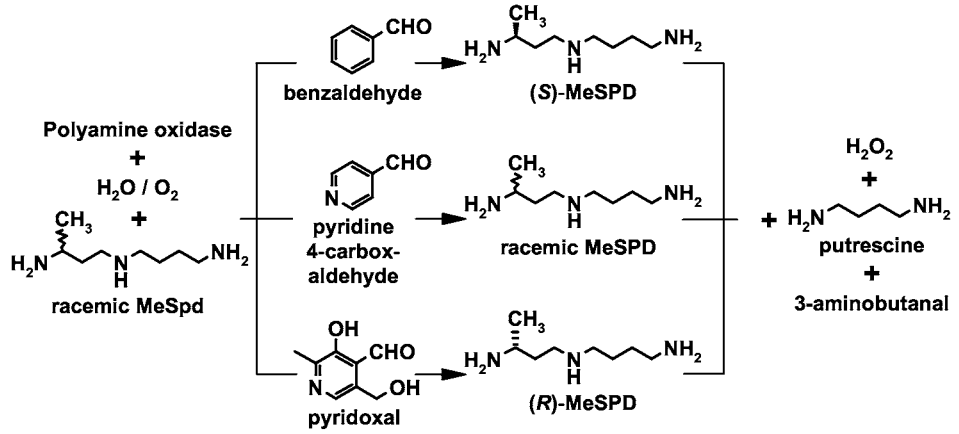


Figure 17 Scheme for stereospecific degradation of MeSPD by PAO in the presence of aromatic aldehydes

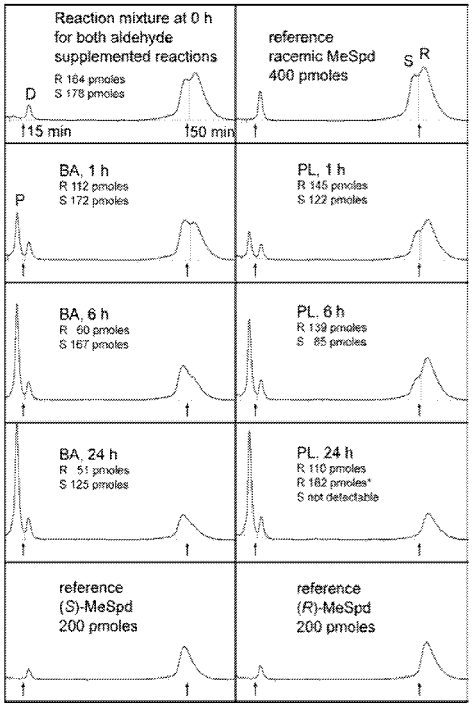


Figure 18 Chiral–HPLC analysis of 5 mM BA or PL supplemented hPAO with 200 μM MeSPD as the substrate in 100 mM glycine–NaOH at pH 9.5 during 24 h. D, 1,7–diaminoheptane; P, PUT; R, (*R*)–MeSPD; S, (*S*)–MeSPD.

hPAO reactions exhibited increased k_{cat} values and almost invariably reduced K_m values (Table II). In the presence of 5 mM P4CA, the affinities of the substrates to hPAO were poorer (especially with SPM mimetics) than with 5 mM BA and PL.

Increasing the aldehyde concentration clearly increased the hPAO–mediated oxidation rates when both SPM and Me_2SPM were used as substrates in both glycine and borate buffers at high pH. However, the reaction rates were not dependent on the order of PA, aldehyde and hPAO addition. Aldehyde enhanced PAO–mediated reaction is clearly characterized by a complex equilibrium between aldehyde, PA and the enzyme. Kinetic studies showed that, in the presence of 5 mM BA or PL, the affinities of PAs to hPAO were slightly increased. However, when the enzyme was saturated (>90 %) with high substrate concentration (K_m for SPM = 47 μM), it was possible to estimate the affinities of aldehydes to the hPAO–PA adduct. A 1 mM SPM concentration was used with BA, P4CA or PL concentrations increasing from 0.1 to 5 mM but very low affinities were observed. Both BA and P4CA increased the reaction rates up to 5 mM concentration in the glycine buffer but PL

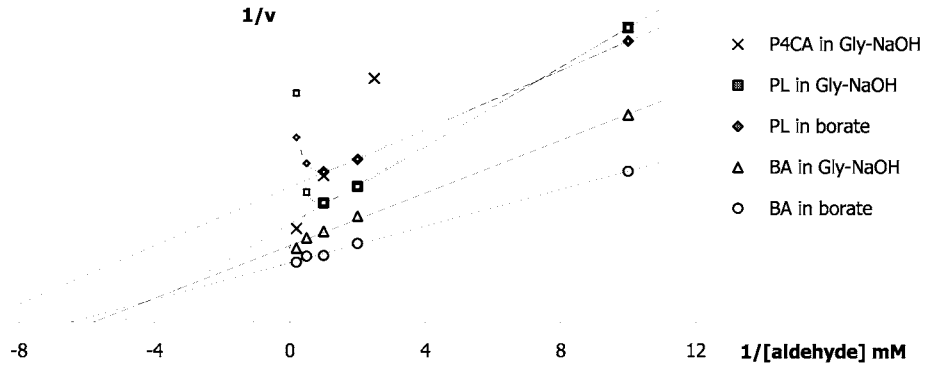


Figure 19 Lineweaver–Burk plotting with increasing aldehydes and 1 mM SPM as a substrate for hPAO. The reactions were carried out in 100 mM glycine–NaOH at pH 9.5 or 50 mM borate at pH 9.3 for 25 min with 0.1 μg of the protein.

appeared to behave inconsistently (Fig. 19) and the reaction rate enhancement was greatest at 1 mM PL. The phenomenon with PL was observed when SPM (but not SPD) was used as a substrate for hPAO in both 50 mM borate and 100 mM glycine–NaOH buffers at high pH (Fig. 19). Therefore, the kinetic values for SPM and the diastereomers of Me_2SPM were determined in the presence of both 1 and 5 mM PL in the glycine buffer at pH 9.5 (Table II). The kinetic values of hPAO at constant 1 mM Spm and in the presence of increasing aldehyde supplementation were as follows. In the glycine buffer K_m = 200 μM and k_{cat} = 5.1 s^{-1} for PL, K_m = 170 μM and k_{cat} = 6.6 s^{-1} for BA and K_m = 930 μM and k_{cat} = 6.6 s^{-1} values for P4CA were determined. In the borate buffer K_m = 110 μM and k_{cat} = 3.7 s^{-1} for PL and K_m = 150 μM and k_{cat} = 8.4 s^{-1} for BA were similarly determined. It may be that at the higher concentrations PL interacted with the enzyme. The degradation efficacy of 200 μM SPM by 0.1 μg hPAO (nmoles of SPD produced per μg of enzyme per min) in the presence of 5 mM PL was about 70 % compared to the SPM degraded by 0.5 μg hPAO supplemented with 5 mM PL (results not shown). It is also possible that the aldehyde

Table II Some kinetic values of hPAO in 100 mM glycine–NaOH at pH 9.5. (*R*), (*R*)–MeSPD; (*S*), (*S*)–MeSPD; (*R,R*), (*R,R*)–Me_2SPM; (*S,S*), (*S,S*)–Me_2SPM.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
AcSPD	**14**	**8.5**	**610 x 10^3**
SPD + 5 mM BA	**9.4**	**0.85**	**79 x 10^3**
SPD + 5 mM PL	**25**	**0.49**	**20 x 10^3**
(*R*) + 5 mM BA	**20**	**0.68**	**34 x 10^3**
(*R*) + 5 mM PL	**5.9**	**0.01**	**1.7 x 10^3**
(*S*) + 5 mM BA	**14**	**0.06**	**4.3 x 10^3**
(*S*) + 5 mM PL	**5.1**	**0.24**	**47 x 10^3**
AcSPM	**1.1**	**17**	**15 x 10^6**
SPM	**47**	**0.4**	**8.5 x 10^3**
SPM + 5 mM BA	**4.5**	**12**	**2.6 x 10^6**
SPM + 5 mM PL	**4.1**	**2.9**	**700 x 10^6**
SPM + 1 mM PL	**3.9**	**2.7**	**710 x 10^6**
(*R,R*)-Me_2SPM	**55**	**0.12**	**2.2 x 10^3**
(*R,R*) + 5 mM BA	**13**	**2.7**	**210 x 10^3**
(*R,R*) + 5 mM PL	**12**	**0.08**	**6.7 x 10^3**
(*R,R*) + 1 mM PL	**21**	**0.13**	**6.2 x 10^3**
(*S,S*)-Me_2SPM	**16**	**0.97**	**61 x 10^3**
(*S,S*) + 5 mM BA	**21**	**6.1**	**290 x 10^3**
(*S,S*) + 5 mM PL	**5.7**	**2.4**	**420 x 10^3**
(*S,S*) + 1 mM PL	**27**	**6.0**	**220 x 10^3**

reacted with the reaction intermediate(s)and/or product(s) and or perhaps the resulting adduct(s) eluted similarly with SPM during the HPLC analysis. The two hydroxyl groups in PL probably play some role in the observed phenomenon. Whatever the reason, it appears that the aldehydes interact poorly with the targets for hPAO but the hPAO–PA–aldehyde–complex is efficiently processed as the k_{cat} values are rather high for hPAO (for AcSPD the k_{cat} value = 8.5 s^{-1}). Furthermore, preformed PL=SPM adducts were studied as substrates for hPAO to circumvent the low affinities of aldehydes for hPAO. As expected, 1 mM PL=SPM was more effectively degraded by hPAO than 1 mM SPM in the presence of 2 mM PL. The kinetic values for PL=SPM were estimated as K_m > 1 mM and k_{cat} ~20 s^{-1}.

It has been suggested that the Schiff base of aldehyde and PA would be the target for PAO–mediated oxidation (Hölttä, 1977). Initial studies were performed in 100 mM glycine–NaOH and since glycine possesses the potential to form Schiff bases, other buffers were required to rule out possible buffer related artefacts. The general stereoselective degradation of MeSPD by the key aldehyde supplemented hPAO was retained in all three buffer systems studied at high pH (Table III). However, the stringency and efficacies of MeSPD oxidations varied greatly. BA supplemented hPAO–reaction appeared the least stereospecific in borate buffer though the degradation of (*R*)–MeSPD was as effective in both borate and NaH_2PO_4 buffers. PL addition to the substrate mixture exhibited the highest reaction rates with racemic, (*R*)– and (*S*)–MeSPD in borate buffer but, similarly to BA, the stereoselectivity of the reaction was best in glycine buffer. In all buffers, P4CA seemed to be an equally unselective guide molecule for enhanced oxidation by hPAO but it was also very ineffective in borate buffer. Brief studies with 100 mM (*D*)– and (*L*)–alanine–NaOH buffers at pH 9.5 detected no differences whatsoever with 100 mM glycine–NaOH on the stereoselectivity of hPAO in 5 mM BA or PL supplemented reactions. It seems that glycine or alanine do not participate in the isomer selective oxidation by hPAO in the presence of aldehydes.

Table III PUT (nmoles) detected in three buffers at pH 9.3–9.5 after 1 h reaction with 1 μg of hPAO and indicated amounts of MeSPD in the presence of the key aldehydes.

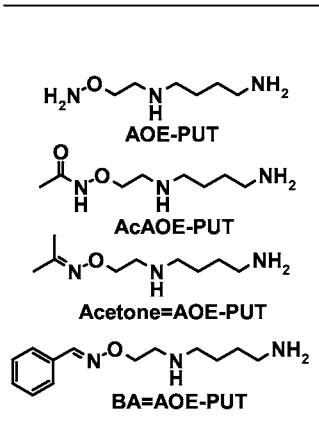
	racemic MeSPD	(*R*) MeSPD	(*S*) MeSPD	
5 mM BA	72 nmoles	36 nmoles	36 nmoles	(*R* / *S*)
NaH_2PO_4	3.8	8.4	0.7	12
Borate	6.7	8.5	1.1	8
Gly-NaOH	6.5	14.6	1.1	14
5 mM PL				(*S* / *R*)
NaH_2PO_4	1.2	ND	2.3	---
Borate	5.8	0.4	11.5	31
Gly-NaOH	3.5	0.2	9.0	50
5 mM P4CA				(*R* / *S*)
NaH_2PO_4	2.0	1.5	0.9	1.6
Borate	0.4	0.3	0.1	1.9
Gly-NaOH	2.5	2.3	1.5	1.5

Aldehydes can form Schiff bases only with the primary amino groups of PAs. As terminally *N*–alkylated PA derivatives are substrates for hPAO, the enhanced degradations of such compounds would indicate that aldehydes bind to an allosteric site in hPAO. DENSPM, DESPM and bis–benzyl–SPM were efficiently degraded by hPAO and the reactions evidenced the formations of nor–SPD, SPD, ethyl–nor–SPD, ethyl–SPD and probably also monoethyl–1,3–diaminopropane. Both reactions were inhibited by 20–50 % by either 5 mM BA or PL supplementation. Similarly, the oxidation of bis–benzyl–SPM was inhibited when 5 mM BA was added to the reaction

mixture. Furthermore, the oxidations of AcSPD and AcMeSPD were markedly inhibited by the supplementation with either 5 mM BA or PL. It seems that the primary amino group(s) in the substrate is (are) required for the observed aldehyde stimulus.

Reduced Schiff bases might be targets for hPAO mediated oxidation. However, reduction changes the properties of the nitrogen next to the C=N double bond. The Schiff base nitrogen becomes similar to other nitrogens in the SPD backbone and is protonated at physiological pH. We used AOE–PUT and its stable oxime analogues with acetone and BA (Fig. 20). Acetylated AOE–PUT (AcAOE–PUT) was also studied (Fig. 20). The presence of oxygen next to the Schiff base decreased the reaction rates of all AOE–PUT derivatives clearly when compared to AcSPD and BA–SPD adducts. However, the introduction of a hydrophobic phenyl group into AOE–PUT resulted in about a 100–fold difference in the affinities of AcAOE–PUT and acetone oxime to hPAO (Fig. 20). BA=AOE–PUT appears to be a potent competitive inhibitor for PAO.

Further studies aimed at clarifying the mechanism of aldehyde stimulus on hPAO proved



K_m (μM)	k_{cat} (s^{-1})
very poor substrate	
290	0.54
380	0.17
3.4	0.04

Figure 20 The kinetic values of hPAO with different AOE–PUT derivatives as substrates in 100 mM glycine–NaOH at pH 9.5.

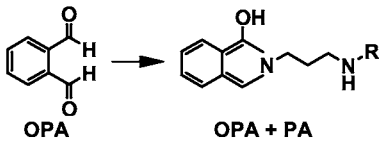


Figure 21 Phthaldialdehyde (OPA) attacks primary amino groups.

unsuccessful. No substrate degradation was observed at 50 % concentration of DMSO in the reaction mixture. Phthaldialdehyde (OPA, Fig. 21) was used to reduce primary amines of hPAO but it inactivated hPAO during overnight incubation. In fact, already 15 min pre–incubation with the enzyme resulted in hardly detectable product formation (results not shown). However, 15 min pre–incubation of OPA with either SPM or Me_2SPM resulted in marked degradations of both compounds by hPAO (results not shown). The possible stereoselective degradation of OPA–derivatized α–methyl–PAs by hPAO remains to be elucidated.

5.4 OTHER RESULTS

PA homeostasis is highly dependent on the tissue and organism. PAO activity in the rat liver was approximately 10–fold higher than in the mouse liver as evidenced by the degradation rates of AcSPD.

The plasmid used for the immortalization of the rat fibroblasts contained a neomycin selection marker. In the presence of G418, cells recovered faster after plating and DENSPM–induced SSAT activity was about 2–fold higher than without G418 supplementation. The PA transport system in these cells greatly favored Me_2SPM over DENSPM and after a 48 hour simultaneous incubation with both drugs at 20 μM concentrations, a 50–fold difference in their intracellular amounts was observed.

AcSPD and AcMeSPD supplementation (up to 1 mM) had no effect on the growth rates of either syngenic or transgenic immortalized fibroblasts. It

is likely that the uptakes of these compounds are limited. Nevertheless, they might be degraded by SAOs but the presence of these SPD analogues in the growth media did not seem to affect the cells.

Recombinant hPAO did not use the 2–2–SPD or its derivatives as substrates at all. In addition, hPAO supplemented with 5 mM BA did not result in degradation of any of the SPD analogues. Only N^1,N^9–diacetyl–2–3–2–SPM from the three studied SPM analogues, was slightly degraded by hPAO but no kinetic values were measurable. The results are in line with the literature, suggesting that mono–acetylated nor–SPD is the shortest possible substrate for PAO. These compounds were also tested as substrates of hSMO but proved to be inert.

Figure 22 shows the structures of some aldehydes that were also tested as enhancers for hPAO–mediated oxidation of MeSPD. Cinnamaldehyde (aldehyde 1) apparently polymerized when added to the substrate mixture and no substrate degradation was detected. Aldehyde 2, on the other hand, appeared in some way to mediate un–enzymatic degradation of MeSPD. Pyridine 2–carboxaldehyde (aldehyde 3) resulted only in the degradation of (*R*)– MeSPD similarly to most of the other studied aldehydes (Fig. 16).

In addition to PL=Spm adduct studies, kinetic studies with 2:1 (aldehyde [BA and PL] to SPM) substrate mixture as substrates for hPAO were attempted. This approach results in more dynamic reaction conditions than with constant aldehyde and increasing substrate concentrations or vice versa. However, similar results were achieved to those obtained with increasing

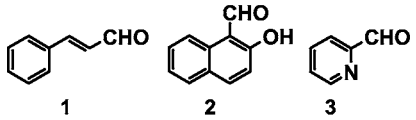


Figure 22 Other tested aldehydes on hPAO–mediated degradation of MeSPD.

aldehyde supplemented reactions in the presence of a constant SPM concentration. Both BA and PL expressed very poor affinities (the K_m values of ~700 μM and >1 mM, respectively, were measured) but the reaction rates were high (the k_{cat} values of 10–20 s^{-1} with both aldehydes were estimated).

SPM was the best substrate of hSMO but its analogues were degraded quite differently (Table IV). Five millimolar BA or PL addition to the hSMO reaction mixture with SPM as a substrate appeared to have only a limited effect and only slightly decreased reaction rates were observed (results not shown). Similarly, the oxidation of both racemic MeSPM and Me_2SPM by hSMO appeared unresponsive to the key aldehyde supplementation (Table V). However, different enantiomers of MeSPM and diastereomers of

Table IV The kinetic values of hSMO for SPM and its different analogues as substrates in 100 mM glycine–NaOH at pH 9.5. MeSPM is an asymmetric compound and two sets of values were measured (for methylated and unmethylated terminus).

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
SPM	20	14	700×10^3
racemic Me_2SPM	110	1.6	15×10^3
(*R*,*R*)-Me_2SPM	25	0.06	2.9×10^3
(*R*,*S*)-Me_2SPM	28	0.34	12×10^3
(*S*,*S*)-Me_2SPM	53	9.7	180×10^3
racemic MeSPM			
(methyl)	31	1.0	32×10^3
(no methyl)	76	2.1	28×10^3
(*R*)-MeSPM			
(methyl)	2.2	0.19	86×10^3
(no methyl)	3.5	0.20	57×10^3
(*S*)-MeSPM			
(methyl)	19	2.2	120×10^3
(no methyl)	29	5.1	180×10^3

Table V Nmoles of 500 μM SPM analogues (corresponding to 90 nmoles at the beginning of the reaction) degraded by hSMO. Five millimolar aldehydes and 30 min reaction times in 100 mM glycine–NaOH at pH 9.5 were used with the indicated protein amounts.

Substrate	hSMO 0.5 μg	+5 mM BA	+5 mM PL
racemic Me_2SPM	12	11	12
(*R,R*)-Me_2SPM	0.6	2.9	1.9
(*R,S*)-Me_2SPM	2.3	7.5	3.7
(*S,S*)-Me_2SPM	36	24	9.4
Substrate	**hSMO 0.3 μg**	**+5 mM BA**	**+5 mM PL**
racemic MeSPM			
(methyl)	8.9	10	7.8
(no methyl)	9.9	9.0	7.1
(*R*)-MeSPM			
(methyl)	1.3	4.1	1.3
(no methyl)	0.9	1.2	0.8
(*S*)-MeSPM			
(methyl)	20	8.2	7.1
(no methyl)	24	9.7	7.0

Me_2SPM were degraded quite differently by hSMO in reaction mixtures containing 5 mM aldehyde (Table V). It seems that hSMO expresses stereoselectivity and the hSMO–mediated oxidations of different enantiomers of α–methylated SPM mimetics are susceptible to aldehydes.

6 DISCUSSION

PAs are essential to sustain normal cellular metabolism both in prokaryotes and eukaryotes. PA homeostasis is a balance between the biosynthesis of these compounds, their degradation, export and uptake. Proliferative stimuli lead to both increased import and enhanced biosynthesis of PAs. On the other hand, the accumulation of PAs not only stimulates PA export and degradation but also promotes inhibitions of both the PA uptake and the PA biosynthesis. As the maintenance of optimal PA concentrations appears to include many interlocking feedback routes, comprehending the mechanisms by which PAs regulate cellular functions has proven elusive.

The PA–mediated proliferation is considered to occur via nucleic acid and protein synthesis. As no direct measurement of cellular localization of PAs is available, the association of PAs can only be measured after disintegrating the cells. In such cell–free studies, most of the cellular PAs appear to be associated with RNA and only a fraction of PAs are free. PAs seem to alter the conformations of different RNAs as both RNA stabilization and destabilization has been observed. As a result, protein synthesis at various stages may be affected. In cell–free DNA, GC–rich regions are targeted by PAs, a phenomenon which may correspond to the situation *in vivo*. PAs also participate in the condensation of histones and the PA export from the nucleus may be one way to enhance transcription. There are also indications that PAs can affect protein phosphorylation (reviewed in detail by Igarashi and Kashiwagi, 2000).

In *E. coli* there is a fairly large group of genes (about 10 % of all studied genes and referred to as "PA modulon") whose initiation codon is preceded by the unusual Shine–Dalgarno sequence. Small alterations to these sequences result in loss of PA–dependent transcription. In *E. coli* there is also a group of genes that are transcriptionally repressed by PAs. PAs are proposed to "modulate" the levels of many proteins and indirectly maintain optimal conditions of cell functions. It seems that PA–dependent cellular phenomena are slowly being revealed as there is evidence of similar PA–mediated transcriptional control in mammalian cells, too (Yoshida et al., 2004).

The mRNA splicing has lately been investigated in more detail. The phenomenon seems to be associated with extraordinary situations like the response to external stimuli and unregulated cell division. Full SSAT mRNA codes for 171 AAs but a shorter splice variant (responding for 71 AAs) was detected in the presence of DENSPM *in vitro* (unpublished observations) and SSAT splicing appears to be sensitive to environmental stress factors (Kim et al., 2005; Mita et al., 2004). The observed enzyme activity differences between organs may well be due to alternate splicing of mRNA.

PA homeostasis is highly dependent on tissue and organism. In normal rat pancreas the total pool of PAs is 3–fold higher than in liver and the high PA content in pancreas appears to be necessary to maintain the integrity of cellular membranes. On the other hand, the roles of PAs in central nervous system appear to be many–fold and opposite. For example, PUT is thought to be a weak antagonist of *N*–methyl–(*D*)–aspartate receptor whereas both SPD and SPM are agonists of the same receptor and appear to be able to substitute for each other to some extent (Williams, 1997).

Numerous PA derivatives are extensively used as tools to elucidate the cellular functions of PAs. Different PA analogues can be divided into two main classes: terminally *N*–alkylated (R_6

and/or R_7= $–CH_3$, $–CH_2CH_3$ etc., Fig. 23) and others with alterations along the backbone of the parent PA. The latter group includes compounds with different lengths of the carbon chains between the nitrogen atoms and alkyl side–chains at carbons or internal nitrogens. The difference between these compounds is that the drugs in the former group do not have free terminal amino groups but the compounds in the latter group do and it may be that this property is the reason for different effects of the PA derivatives in cells.

Even though under physiological conditions all the nitrogens are positively charged in both Me_2SPM and DENSPM, the compounds are treated quite differently. Both are competitive inhibitors of SSAT but DENSPM induces SSAT very effectively whereas Me_2SPM results in only moderate SSAT induction. One explanation of the different properties may be that the α–methyl groups do not reach out far enough to induce conformational changes in the SPM analogue sensitive sites whereas the terminal *N*–ethyl groups of DENSPM may distort the positions of their neighboring groups more effectively. This conformational change in the ligand binding site(s) of the macromolecules, due to the interaction(s) with PA(s) or PA analogue(s), may stabilize the ligand–protein complexes differently and/or exhibit allosteric effects, either of which may explain the observed differences.

Here, the reported biological stabilities of both MeSPD and Me_2SPM were confirmed both *in vivo* and *in vitro*. These PA derivatives appear to be excellent tools with which to elucidate the different cellular roles of SPD and SPM. Furthermore, both MeSPD and Me_2SPM mimic and substitute for natural PAs extremely well: both prevent acute pancreatitis and rescue the early liver regeneration in the MT–SSAT transgenic rat. These compounds do not cause any significant SSAT induction or inflammation *in vivo*. However,

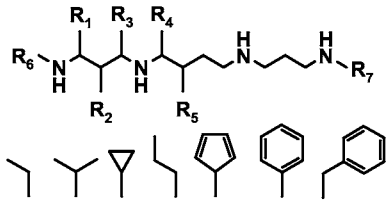


Figure 23 Some possible substituents and their positions at SPM.

only racemic MeSPD and Me_2SPM have been studied in cell cultures and *in vivo*. Enantiomerically pure drugs may very well evoke different effects. The dormant stereoselectivity of PAO has also been revealed due to the chiral nature of these compounds.

A single methyl group at α–position is sufficient to prevent acetylation by SSAT. It would be of interest to study methyl substituents in other positions i.e. R_2 to R_5 (Fig. 23) and the stabilities of such PA derivatives. A single methyl substituent at R_2–position may be close enough to both N^1 and N^4 to prevent both acetylation by SSAT and degradation by PAO and other amine oxidases. R_3–substitution might result in a stable compound against PAO–mediated degradation. Furthermore, examination of other singly α–substituted PAs (R_1= $–CH_2CH_3$, $–CH_2C_5H6$ etc., Fig. 23) is necessary to determine the properties of potential substrates for PAO in the presence of aldehydes. Substituents containing functional groups (–SH, $–NH_2$, –COOH etc.) or atoms other than carbons would also be interesting to investigate. Such PA mimetics might also provide some insight into the mechanisms of hSMO–mediated catalysis.

The aldehyde stimulated degradations of the different enantiomers of α–methylated PAs by hPAO are known to be dependent on the properties of the supplemented aldehyde. However, the studies aimed at understanding the mechanism of aldehyde stimulus on PAO proved

problematic. Aldehydes form Schiff bases with primary amines. However, the possibility that buffer is a mediator in the stereoselective reaction was excluded as the same general stereoselective degradation of MeSPD isomers by the key aldehydes supplemented hPAO was observed in the two AA–free buffers.

The free amino group of FAD (Fig. 4, page 20) and lysine(s) (Fig. 24) in PAO are also possible targets for Schiff base formation. In the active site of maize PAO, a lysine–residue is bridged to N^5 of the isoalloxazine ring by a water molecule (Binda et al., 1999) and that particular lysine is a promising target for the Schiff base. On the other hand, many cofactors (NAD, NADP and ATP) contain free amino groups similarly to FAD. If covalent modification of cofactors were to result in permanent substrate specificity changes, this might have some industrial applications. To rule out allosteric interaction of aldehyde with the enzyme, reduction of OPA to hPAO was attempted but this resulted in inactivated enzyme. However, binding to the aldehyde–responsive allosteric site in hPAO should enhance the oxidation of terminally *N*–alkylated PAs (compounds like DESPM, DENSPM and bis–benzyl–SPM). Supplementation of aldehydes in the hPAO reaction mixture inhibited the catalysis of these three substrates indicating that there is no positive allosteric site for these aldehydes.

It is plausible that Schiff base with the primary amino groups of the substrate enhances the reaction rates. This is because Schiff base formation results in an uncharged nitrogen and the organization of the molecule may resemble that of AcSPD. However, whether the Schiff base is required for the substrate recognition by PAO or is essential for the catalysis in the active site is harder to elucidate.

As aldehydes are unstable under alkaline conditions increasing aldehyde concentrations

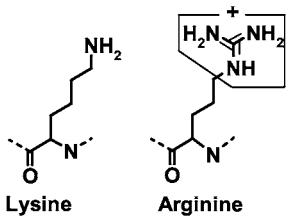


Figure 24 Lysine has a free amino group whereas arginine contains a resonance structure that does not allow Schiff base formation.

were used to allow effective Schiff base formation. The aldehyde concentration appeared to increase the reaction rates of hPAO with both BA and P4CA up to 5 mM. However, the PL–mediated hPAO enhancement appeared greatest at 1 mM PL concentration. It may very well be that PL interacts with the enzyme at higher concentrations and inhibits the reaction. There are also other possibilities e.g. PL may attack the degradation products of the reaction or the resulting compounds may not be detectable with the HPLC analysis system used.

PL forms a hydrogen bond stabilized Schiff base (boxes in Fig. 25) and the preformed PL=SPM adduct was studied as a substrate of hPAO to confirm the degradation of Schiff bases. It seems that the Schiff base enhances the catalysis as the PL=SPM adduct was readily degraded by hPAO. However, the estimated kinetic values of PL=SPM were K_m > 1 mM and k_{cat} ~20 s^{-1}. The reaction rate is exceptionally good for hPAO (the k_{cat} values of AcSPM and AcSPD are 17 s^{-1} and 8.5 s^{-1}, respectively) but the

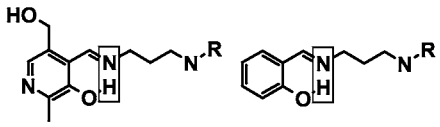


Figure 25 Hydrogen bond stabilized Schiff bases of PL and salicylaldehyde with PAs.

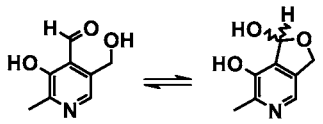


Figure 26 Cyclization of PL results in a chiral carbon.

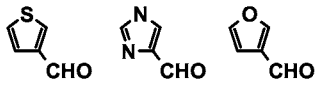


Figure 27 Structures for five atoms containing cyclic aldehydes.

affinity of the PL=SPM adduct to PAO appears to be very weak. These results might be interpreted to mean that aldehyde reacts with PA in solution and the aldehyde–PA adduct serves as a substrate for hPAO. Furthermore, it could not be confirmed whether chirality of the cyclic form of PL plays any role (Fig. 26). A fraction of PL certainly is in the cyclic conformation in aqueous media.

The observed very weak MeSPD degradation by hPAO in the presence of salicylaldehyde is confusing as this molecule also (similarly to PL) can form a hydrogen bond stabilized Schiff base (Fig. 25, on right). It may be that the Schiff base of salicylaldehyde is ineffective without an additional hydroxyl group to stabilize the intermediate during the catalysis in the active site of hPAO. Another possibility is that salicylaldehyde in some way reacts with the produced PUT and the resulting adducts are not detected by the HPLC analysis (similarly to apparently lowered reaction rates of hPAO in high PL concentration).

Another approach to deduce the significance of the Schiff base was AOE–PUT and its derivatives. Oximes are exact Schiff base mimetics and the carbon=nitrogen bond in oximes is stable. AOE–PUT was a very poor substrate of PAO (similar to SPD) and from the studied molecules the acetyl derivative exhibited the best k_{cat} value (although only $^1/_{15}$th of the k_{cat} value for AcSPD). Preformed Schiff bases of AOE–PUT with BA and acetone exhibited discrepancies: it seems that BA=AOE–PUT has far better affinity (two orders of magnitude) to PAO but a very poor k_{cat} value. The k_{cat} value of acetone=AOE–PUT was only marginally better. However, the k_{cat}/K_m value of BA=AOE–PUT was the highest of the studied AOE–PUT derivatives. The results with these SPD derivatives indicate that the aromatic aldehyde–PA adduct is the target for recognition by hPAO. Further investigations to elucidate the mechanism of the observed phenomenon might include studies with the crystallized mammalian PAO and BA=AOE–PUT (k_{cat}~$^1/_{10}$th of SPM) appears to be interesting ligand to study in that system. At present, mechanistic studies with mammalian PAOs have been only performed with the UV spectroscopy (Royo and Fitzpatrick, 2005).

The underlying mechanism of the aldehyde stimulus for PAO has proved to be elusive and difficult to comprehend. It certainly seems that aldehydes (BA and PL) do not have a positive allosteric site(s) outside the catalytic site of PAO. A six carbon ring structure (aromatic or not) containing Schiff base adduct appears to be essential for the effective MeSPD degradation as the supplementation of hPAO with aliphatic aldehydes resulted in lower reaction rates. In the active site of maize PAO, there are two aromatic AAs (tyrosine and phenylalanine, Binda et al., 1999) that form a sandwich–like structure and the phenyl group of Schiff base adduct might fit between them in hPAO (if a similar structure is present in mammalian PAO). In the future five atom ring structure (Fig. 27) containing aldehydes might prove interesting to investigate.

Aromatic aldehydes might form a cyclic aminal with the aminopropyl moiety of PAs (Ledbetter, 1982; McQuate and Leussing, 1975; Metzler et al., 1980) and this kind of cyclic structure would be energetically favorable (Fig. 28A). The cyclization would fix the α–methyl

group closer to the cleavage site and result in another chiral center that might in part explain the stereoselective degradation of MeSPD by PAO. On the other hand, the active site of maize PAO is rather narrow and a compound with two cyclic rings may have difficulty fitting into the catalytic tunnel (Fig. 28B). However, no mammalian PAOs have been crystallized so the conformation of the active site of hPAO can only be speculated. It might even be possible that acetylated PAs are converted to a cyclic form (Fig. 28C) at the active site before undergoing catalysis.

The aromatic aldehydes might react differently from each other in the active site of PAO. The nitrogen opposite to the carboxaldehyde group might bridge to some AA in the active site "locking" aminal in place for the duration of catalysis allowing the use of (*S*)–MeSPD. In the case of PL, additional hydroxyl groups may contribute to the reaction. The degradation patterns of MeSPD in the presence of P4CA, pyridine–3–carboxyaldehyde and pyridine–2–carboxyaldehyde (nitrogens at positions 3, 2 and 1, respectively, Fig. 23B) by hPAO could be explained by this hypothesis. As the distance between the nitrogen and its counterpart in the active site increases, the less (*S*)–MeSPD degradation that occurs. This "locking" might not happen with the other studied aldehydes and the phenyl ring would rotate into another orientation. That conformation might be energetically more favorable as the enhancement of (*R*)–MeSPD degradation is the dominant observation. As the aldehydes have not been tested extensively with the Me_2SPM diastereomers, one can only speculate about the significance of other chiral carbon(s) of the substrate. Furthermore, the interaction studies with aldehydes and hSMO may be also informative as different enantiomers of α–methylated SPM analogues appear to be degraded with alternating efficacies in the presence of BA and PL.

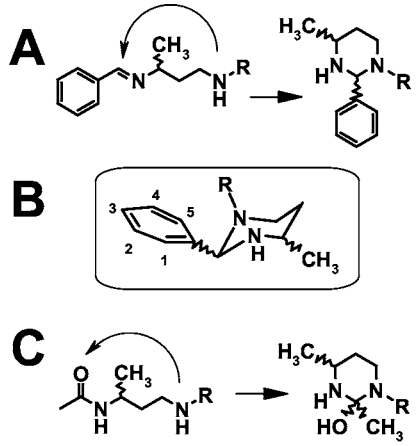


Figure 28 A Scheme for cyclic aminal conversion from Schiff base adduct of BA and MeSPD. **B** Cross section of hypothetic cyclic aminal in the active site of PAO. **C** Scheme for cyclic conversion of AcMeSPD at the active site of PAO.

The pharmaceutical industry is now searching for usable enzymes and their modifications suitable for large–scale applications. Versatile chemical tools are continually being sought and easy–to–prepare, stereoselective, stable enzymes are of interest (Zaks and Dodds, 1997). Another vigorously studied research area is nonaqueous enzymology as water–based reaction buffers can pose problems for many chemical syntheses (Krishna, 2002). Enzymatic polymerization has been confirmed in supercritical fluids, gases, eutectic mixtures, liquid crystals, melts and low–vapor–pressure ionic liquids. Ultimately whole–cell catalysis in a nonaqueous environment may be achieved (extensively reviewed by Klibanov, 2001). Whether PAO (or SMO) has potential as a synthetic chemistry tool remains to be determined.

7 SUMMARY

The synthetic routes for preparation of the drugs enabled extensive research with MeSPD, MeSPM and Me_2SPM. The immortalized rat fibroblasts proved to be useful tools to evaluate the fates of the studied PA analogues *in vitro*. The rat liver extract and recombinant protein investigations complemented the cell culture results. *In vivo* studies with the MT–SSAT transgenic rodents were in line with *in vitro* studies.

The presence of a single α–methyl group greatly increases the biological stabilities of the studied PA mimetics. SSAT–mediated acetylation is prevented and hSMO–mediated degradations of both MeSPM and Me_2SPM are clearly diminished. However, hPAO–mediated degradations of both α–methylated SPM derivatives are insignificantly enhanced when compared to SPM. Neither hSMO nor hPAO uses MeSPD as a substrate.

Me_2SPM, similarly to MeSPD, restores the early liver regeneration in the partially hepatectomized MT–SSAT transgenic rat and on a molar basis Me_2SPM appears to be more effective than MeSPD. (*R*,*R*)–Me_2SPM is the most stable diastereomer against both hSMO– and hPAO–mediated degradations. However, further studies are required to understand the relevance of chirality to the cellular effects of α–methylated PA derivatives.

The dormant stereospecificity of PAO was revealed. The possibility to predetermine the stereoselectivity of PAO–mediated oxidation with guide molecule supplementation was demonstrated. PAO is highly sensitive to aromatic aldehydes and addition of BA to the reaction mixture greatly enhanced the oxidation of (*R*)–MeSPD. PL in turn modified the dominant stereospecificity of PAO and allowed efficient degradation of (*S*)–MeSPD. Furthermore, the present study indicated that the Schiff base between PA and aldehyde is the target of recognition for PAO. However, the exact mechanism for the observed phenomenon remains to be elucidated.

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Metabolic Stability of α–Methylated Polyamine Derivatives and Their Use as Substitutes for the Natural Polyamines.

Järvinen A, Grigorenko N, Khomutov AR, Hyvönen MT, Uimari A, Vepsäläinen J, Sinervirta R, Keinänen TA, Vujcic S, Alhonen L, Porter CW, and Jänne J.

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Metabolic Stability of α-Methylated Polyamine Derivatives and Their Use as Substitutes for the Natural Polyamines*

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Aki Järvinen‡, Nikolay Grigorenko§, Alex R. Khomutov§, Mervi T. Hyvönen‡, Anne Uimari‡, Jouko Vepsäläinen¶, Riitta Sinervirta‡, Tuomo A. Keinänen‡, Slavoljub Vujcic‖, Leena Alhonen‡, Carl W. Porter‖, and Juhani Jänne‡**

From the ‡A. I. Virtanen Institute for Molecular Sciences and the ¶Department of Chemistry, University of Kuopio, P. O. Box 1627, FIN-70211 Kuopio, Finland, the §Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov Street 32, Moscow 117984, Russia, and the ‖Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, New York 14263

Metabolically stable polyamine derivatives may serve as useful surrogates for the natural polyamines in studies aimed to elucidate the functions of individual polyamines. Here we studied the metabolic stability of α-methylspermidine, α-methylspermine, and bis-α-methylspermine, which all have been reported to fulfill many of the putative physiological functions of the natural polyamines. *In vivo* studies were performed with the transgenic rats overexpressing spermidine/spermine N^1-acetyltransferase. α-Methylspermidine effectively accumulated in the liver and did not appear to undergo any further metabolism. On the other hand, α-methylspermine was readily converted to α-methylspermidine and spermidine; similarly, bis-α-methylspermine was converted to α-methylspermidine to some extent, both conversions being inhibited by the polyamine oxidase inhibitor N^1,N^2-bis(2,3-butadienyl)-1,4-butanediamine. Furthermore, we used recombinant polyamine oxidase, spermidine/spermine N^1-acetyltransferase, and the recently discovered spermine oxidase in the kinetic studies. *In vitro* studies confirmed that methylation did not protect spermine analogs from degradation, whereas the spermidine analog was stable. Both α-methylspermidine and bis-α-methylspermine overcame the proliferative block of early liver regeneration in transgenic rats and reversed the cytostasis induced by an inhibition of ornithine decarboxylase in cultured fetal fibroblasts.

Although the requirement of the natural polyamines spermidine, spermine, and their precursor putrescine for the growth of mammalian cells is extremely well documented, their specific functions in proliferative processes are largely unknown (1). Some of the published data appear to assign a central role to spermidine, whereas putrescine is supposed to serve as its precursor and spermine as a storage pool convertible back to spermidine. For the elucidation of the physiological roles of individual polyamines, metabolically stable derivatives of polyamines fulfilling their specific cellular functions would be extremely valuable. Methyl derivatives of spermidine and spermine have been used as substitutes for the natural polyamines both *in vitro* and *in vivo*. α-Methylspermidine (MeSpd),[1] α-methylspermine (MeSpm), and bis-α-methylspermine (1,12-dimethylspermine, Me_2Spm) are equally effective as the natural polyamines in inducing the conversion of right-handed B-DNA to left-handed Z-DNA (2). In addition to spermidine and spermine, cytostasis that resulted from the inhibition of the *S*-adenosylmethionine decarboxylase can be reversed by MeSpd but not by Me_2Spm (3). Spermidine, spermine (because of its conversion to spermidine), and MeSpd serve as substrates for the synthesis of deoxyhypusine (an integral part of eukaryotic initiation factor 5A), whereas Me_2Spm does not (3). Interestingly, all of the mentioned methylated derivatives of spermidine and spermine have been reported to reverse the cytostasis induced by difluoromethylornithine, a specific inhibitor of mammalian ornithine decarboxylase (4). MeSpd appears to undergo slow conversion to MeSpm. Me_2Spm has not been reported to be metabolized. MeSpd is not a substrate for spermidine/spermine N^1-acetyltransferase (SSAT) (4). In line with the above studies, we found that MeSpd prevents zinc-induced pancreatitis and restores liver regeneration in transgenic rats overexpressing SSAT under the control of the metallothionein promoter (5). Under these conditions where polyamine catabolism was intensely activated, the natural polyamines could not be used as they were rapidly acetylated and degraded with no net tissue accumulation (5).

Here we studied the metabolic stability of the three methylated polyamine derivatives, namely MeSpd, MeSpm, and Me_2Spm. Transgenic rats with activated polyamine catabolism because of overexpression of SSAT were used for the experiments *in vivo*, immortalized fibroblasts derived from the same animals and liver extracts from normal rats were used for experiments *in vitro*. The metabolisms of these polyamine analogs were also studied by using purified recombinant human polyamine oxidase, human spermine oxidase, and mouse SSAT. We found no evidence indicating that MeSpd would be further metabolized *in vivo* and the compound was also a poor substrate for the studied enzymes *in vitro*. Surprisingly, both MeSpm and Me_2Spm were catabolized at the methylated ends both *in vivo* and *in vitro* although Me_2Spm was far more stable. Furthermore, MeSpd and Me_2Spm were competitive inhibitors of SSAT.

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** To whom correspondence should be addressed: Dept. of Biotechnology and Molecular Medicine, A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, P. O. Box 1627, FIN-70211 Kuopio, Finland. Tel.: 358-17-163049; Fax: 358-17-163025; E-mail: Juhani.Janne@uku.fi.

[1] The abbreviations used are: MeSpd, α-methylspermidine; SSAT, spermidine/spermine N^1-acetyltransferase; PAO, polyamine oxidase; SMO, spermine oxidase; MeSpm, α-methylspermine; Me_2Spm, bis-α-methylspermine.

Both MeSpd and Me_2Spm restored early liver regeneration in transgenic rats with activated polyamine catabolism, in which a profound spermidine and spermine depletion developed after partial hepatectomy. Although Me_2Spm was metabolized to MeSpd to some extent, the restoration of the proliferative activity was attributable to Me_2Spm and not to the low contents of MeSpd.

MATERIALS AND METHODS

Chemicals—α-Methylated polyamine analogs were synthesized as described in Refs. 6 and 7 and administered in saline. The PAO inhibitor MDL72527 (N^1,N^2-bis(2,3-butadienyl)-1,4-butanediamine) was a generous gift from Hoechst-Roussel. All other chemicals were purchased from Sigma and Fluka. [6-^{3}H]Thymidine (specific radioactivity 18 Ci/mmol) was obtained from PerkinElmer Life Sciences.

Transgenic Rats and the Studies of α-Methylated Spermine Analogs—The production of transgenic rats has been described in detail earlier (8). Partial hepatectomy of the transgenic rats was carried out essentially as described in Ref. 9. Treatments before partial hepatectomy and the determination of DNA synthesis were carried out as described in the legend to Ref. 5. Transgenic 10-week-old male rats were injected twice with MDL72527 (50 mg/kg intraperitoneal) at 16-h intervals to inactivate PAO according to Bolkenius *et al.* (10) and further with MeSpd, MeSpm, or Me_2Spm twice (25 mg/kg intraperitoneal) 2 and 8 h after the second MDL72527 treatment. Animals not treated with the PAO inhibitor were injected with α-methylated spermine analogs or MeSpd at the same time points as MDL72527-pretreated animals. The animals were sacrificed 24 h after the second MDL72527 injection; liver pieces were frozen in liquid nitrogen and homogenized in the standard buffer (25 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol). An aliquot of the homogenates was used for the polyamine assays. The homogenates were centrifuged (at 13,000 × *g*, for 30 min, at 4 °C) and the supernatant fractions were used for the enzyme activity assays. The Institutional Animal Care and Use Committee of the University of Kuopio and the Provincial Government approved the animal experiments.

Immortalized Fibroblasts Overexpressing SSAT—Rat fibroblasts from MT-SSAT transgenic rats were derived from 13-day-old fetuses. The fibroblast processing was basically the same as described by Mackintosh *et al.* (11), and transfection was performed with the same plasmid and the commercial kit Lipofectamine™ Plus. Plasmid expressing SV40 large and small T antigens was a kind gift from Dr. M. J. Tevethia of the Department of Microbiology (Pennsylvania State University College of Medicine, Hershey, PA). The transgenic cell populations were identified with the aid of the quantitative PCR. The immortalized fibroblasts were plated in 6-well culture plates in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum, gentamycin (50 μg/ml; Invitrogen), and geneticin G418 (500 μg/ml; Sigma). The cells were left to adhere for 24 h before the growth medium was replaced with fresh medium and drugs. After the incubation, cells were washed with phosphate-buffered saline, detached with trypsin, counted, and the polyamines were determined after sulfosalisylic acid precipitation from the supernatant fractions with the aid of high performance liquid chromatography.

The Back-conversion Studies of the α-Methylated Polyamine Analogs with the Liver Extracts in Vitro—Wild-type Wistar male rats were sacrificed and livers were processed as above. All supernatants were combined and eluted at 4 °C through Amicon Ultra-15 centrifugal filter devices (Millipore) with a nominal molecular weight limit of 30,000 with high-salt (500 mM NaCl) buffer to remove the natural polyamines. The resulting eluates were desalted in the same columns with non-salt buffer three times to remove excess salt. The final eluates were pooled and the protein concentrations were determined. Experiments were carried out in triplicates where 40 μl of liver extract was used per total reaction volume of 180 μl. Reaction buffer containing 0.1 M glycine-NaOH, pH 9.5, and 5 mM dithiothreitol was used to study the metabolism of the polyamines and their α-methylated analogs in 1 mM concentrations. The reactions were initiated with the studied polyamine or analog addition and the reaction tubes were incubated at 37 °C water bath for 60 min. Five millimolar freshly distilled benzaldehyde was used to decrease the K_m values and to increase the reaction velocity of PAO according to Hölttä (12). Where indicated, the reaction mixtures were preincubated for 10 min with 250 μM MDL72527 to inactivate PAO before polyamine or analog addition (Table I). The reactions were stopped with the addition of 20 μl of 100 μM diaminohexane in 50% (w/v) sulfosalisylic acid.

Production of Recombinant PAO and SMO, Expression Systems—The cDNA coding for the hSMO open reading frame and containing His_6 at the N terminus was synthesized by PCR using the following primers: 5′-GAAGGAGATATACATATGCACCATCATCACCATCACATTGAGGGTCGCATGCAAAGTTGTGAATCCAGTGG-3′ and 5′-GAGAAGGTCGTCCCCTGGACTGAGCTCTTCGAACATACG-3′. The cDNA coding for the hPAO open reading frame and containing His_6 at the N terminus was synthesized by PCR using the following primers: 5′-GAAGGAGATATACATATGCACCATCATCACCATCACATTGAGGGTCGCATGGAGTCGACCGGCAGCGTC-3′ and 5′-CGGGTCCGGCTCCGAGATCGAGCTCTTCGAACATACG-3′. The PCR fragments were cloned into NdeI/XhoI of the pET 30a vector (Novagen, Inc., Madison, WI). Bacteria containing plasmids were cultured and collected according to the Qiagen Qiaexpressionist™ manual and the proteins were purified under native conditions using nickel-nitrilotriacetic acid His Bind resin (Novagen) according to the manufacturer's instructions.

Both PAO and SMO proteins were further purified with affinity chromatography. For the affinity matrix NHS-activated Sepharose™ 4 Fast Flow was used according to the manufacturer's instructions (Amersham Biosciences). N^8-Acetylspermidine and spermidine were used as ligands for PAO, whereas for SMO, spermidine and Me_2Spm were used. The SMO elutes were concentrated and washed with the standard buffer (25 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol) several times using Amicon Ultra-15 centrifugal filter devices (Millipore). PAO, however, could not be concentrated nor washed in a similar way as it bound to the filter membrane very tightly. Desalting of the PAO elutes after affinity chromatography were performed with NAP-10™ gel filtration columns according to the manufacturer's instructions (Amersham Biosciences).

Production of Recombinant SSAT—To obtain the SSAT cDNA (exons one to six) the pool of the first strand cDNA was PCR amplified using primers 5′-TACGTCGACACGAATGAGGAACCACC-3′ and 5′-CTAGCGGCCGCAGGTTGTCATTGTCTAC-3′. The resultant PCR product was gel-purified, restriction enzyme digested, and cloned. For protein expression the coding sequence of SSAT was amplified by PCR using the cloned SSAT cDNA as a template. The primers used are: 5′-TTAGCCATATGCATCATCATCATCATCATGATGACGACGACAAGATGGCTAAATTTAAGATCCG-3′ and 5′-CTACTCGAGCTCACTCCTCTGCTGCC-3′. The upstream primer contained His_6 and an enterokinase cleavage site. The NdeI/XhoI-digested PCR products were cloned into the pET 30a vector (Novagen, Inc., Madison, WI) and sequenced. For the protein production *Escherichia coli* strain BL21(DE3) was used as the host and the recombinant protein was purified under native conditions using nickel-nitrilotriacetic acid His Bind resin (Novagen) according to the manufacturer's instructions.

All purified proteins were analyzed by SDS-PAGE, and the protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad). The kinetic studies of the oxidizing enzymes were performed in duplicates with 4 to 6 different 10 to 1000 μM substrate concentrations. The SMO and PAO reactions were carried out in a total volume of 180 μl in the same buffer as the liver extract reactions and were allowed to proceed for 10 to 30 min at 37 °C before addition of 20 μl of 100 μM diaminohexane in 50% (w/v) sulfosalisylic acid. The kinetic study of SSAT was performed with 100, 400, 700, and 1000 μM spermidine or spermine as a substrate and 100, 400, 700, and 1000 μM MeSpd or Me_2Spm as a competitive inhibitor.

Determination of Ornithine Decarboxylase, SSAT, PAO Activities, and Polyamines—The activity of ornithine decarboxylase was assayed as described previously (13), and SSAT activity was assayed as described in Ref. 14. The PAO activity was assayed essentially as described by Kumazawa *et al.* (15) using radioactive N^1,N^{11}-diacetylnorspermine, instead of N^1-acetylspermine, as the substrate. High performance liquid chromatography was used to determine the concentrations of the polyamines and their methylated analogs essentially as described by Hyvönen *et al.* (16).

Statistical Analyses—The data were expressed as mean ± S.D. One-way analysis of variance with Dunnett's post-hoc test for multiple comparisons was used for statistical analyses with the aid of a software package, GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Purity of α-Methylated Polyamine Analogs: NMR and High Performance Liquid Chromatography Studies—^{1}H and ^{13}C spectra were recorded on an Avance DRX spectrometer operating at 500.13 MHz. NMR showed >99.5% purity for all studied polyamine analogs. MeSpd, MeSpm, and Me_2Spm

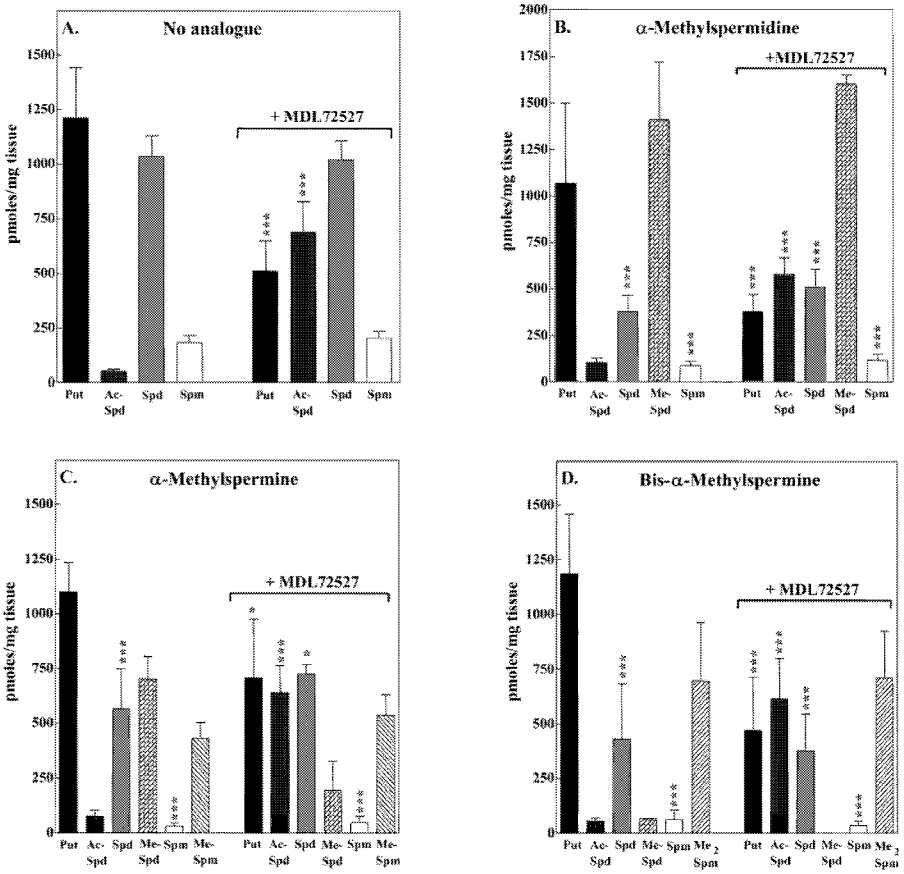

FIG. 1. **Hepatic polyamine pools in SSAT transgenic rats.** *A*, control animals ± MDL72527; *B*, animals treated with α-methylspermidine ± MDL72527; *C*, animals treated with α-methylspermine ± MDL72527; *D*, animals treated with bis-α-methylspermine ± MDL72527. The rats were injected twice with MDL72527 (50 mg/kg intraperitoneal) at 16-h intervals. The polyamine analogs (25 mg/kg intraperitoneal) were injected 2 and 8 h later. The animals were sacrificed 24 h after the last MDL72527 injection. Three to four animals were in each group. *Put*, putrescine; *AcSpd*, N^1-acetylspermidine; *Spd*, spermidine. *, $p < 0.05$ as compared with untreated animals; ***, $p < 0.001$ as compared with untreated animals.

were further tested with high performance liquid chromatography for typical orthophtalaldehyde reactive impurities. In 10,000 pmol of each sample, the amount of orthophtalaldehyde reactive impurities were less than 50 pmol (results not shown).

Stability of the Analogs in the Liver of SSAT Transgenic Rats—Fig. 1*A* displays the typical polyamine pattern in the liver of SSAT overexpressing rats. Putrescine pool was greatly increased, whereas the spermine level was decreased when compared with wild-type rat liver where putrescine remains almost undetectable (8). An exposure of the rats to the polyamine oxidase inhibitor MDL72527 expectedly greatly reduced putrescine content and expanded the pool of N^1-acetylspermidine (Fig. 1*A*). All the analogs accumulated in the liver of the transgenic rats, MeSpd showing the highest tissue concentrations (Fig. 1, *A–D*). The latter analog likewise appeared to be metabolically stable and, in contrast to an earlier report (4), we found no evidence that MeSpd was converted to MeSpm (Fig. 1*B*). As indicated in Fig. 1*C*, MeSpm was effectively converted to MeSpd. The latter conversion was markedly inhibited by the MDL72527 compound (Fig. 1*C*). Fig. 1*D* shows that also Me_2Spm was converted to MeSpd, but to a much lesser extent than MeSpm. The conversion of Me_2Spm to MeSpd was totally prevented by MDL72527 (Fig. 1*D*).

Effect of the Analogs on SSAT and PAO Activities in the Liver of SSAT Transgenic Rats—The methylated polyamine analogs did not appear to be very effective inducers of SSAT activity. In fact, only Me_2Spm significantly increased the hepatic SSAT activity, which was further enhanced by combining the latter analog with MDL72527 (Fig. 2*A*). However, the modest induction of the SSAT activity in response to the methylated analogs may be tissue-specific as the analogs much more effectively induced SSAT activity in the pancreas of the transgenic rats (results not shown). The analogs had little effect on PAO activity, whereas the used doses of MDL72527 alone or in combination with the analogs virtually completely inhibited PAO activity (Fig. 2*B*). All the analogs stimulated ornithine decarboxylase activity to some extent (results not shown).

Studies with Liver Extracts Obtained from Wild-type Rats—We used crude liver extracts to study the stability of

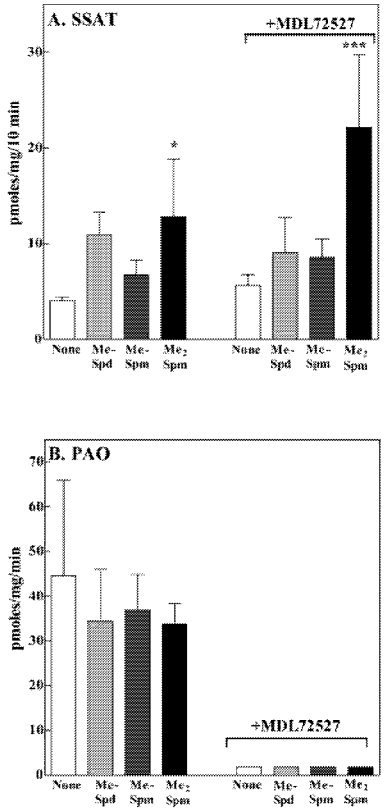

FIG. 2. **Effect of polyamine analogs without or with MDL72527 on hepatic SSAT (*A*) and PAO (*B*) activities in SSAT transgenic rats.** The rats were treated with the drugs as described in the legend to Fig. 1. Three to four animals were in each group. *, $p < 0.05$ as compared with untreated animals; ***, $p < 0.001$ as compared with untreated animals.

analogs *in vitro* under conditions corresponding to the situation *in vivo*. High-salt extraction was performed (see "Materials and Methods") to remove or decrease the content of small molecular weight compounds. The extraction procedure certainly removed all acetyl-CoA (and probably also inactivated the labile SSAT protein) thus preventing acetylation of the polyamine substrates during the experiments. We used benzaldehyde supplementation as it is known to greatly enhance PAO activity when nonacetylated spermidine or spermine is used as a substrate (but not when N^1-acetylspermidine is used) (12). Benzaldehyde in all likelihood forms a Schiff base with the polyamines mimicking the structure and charge distribution of the acetylated polyamines, which are much more preferred substrates for PAO than the unmodified polyamines (12).

As shown in Table I, substantial amounts of spermidine and spermine (but no putrescine) remained in the extracts even after the high-salt procedure. During the 60-min incubation, some putrescine was formed and the production was greatly enhanced upon an inclusion of 5 mM benzaldehyde in the incubation mixture. Interestingly, the formation of putrescine was associated with the disappearance of the endogenous spermine, but not spermidine. However, the view that spermine was directly converted to putrescine was ruled out by tracer studies indicating that labeled spermine was first converted to spermidine followed by the appearance of the label in putrescine (results not shown). Putrescine formation without benzaldehyde addition was probably because of mono- and/or diaminooxidases as we did not use any inhibitors. Inclusion of 1 mM N^1-acetylspermidine yielded large amounts of putrescine, the formation of which was not enhanced by benzaldehyde. In fact, the observed slight inhibition in the presence of benzaldehyde could be attributable to the formation N^1-acetyl/N^8-benzyl-Schiff base-spermidine and N^8-acetylspermidine is an inhibitor for PAO (17). MDL72527 prevented any formation of putrescine from N^1-acetylspermidine (Table I). In comparison with N^1-acetylspermidine, 1 mM spermidine produced relatively little putrescine even in the presence of benzaldehyde, probably because Schiff base can be formed in both primary amino groups of spermidine (N^1/N^8). Inclusion of 1 mM MeSpd appeared to yield some putrescine in the presence of benzaldehyde, but this putrescine was in all likelihood derived from endogenous spermine (Table I). Exogenous spermine was converted to spermidine even in the absence of benzaldehyde but the conversion was greatly enhanced by the latter compound. Inclusion of 1 mM MeSpm yielded substantial amounts of spermidine and MeSpd already without benzaldehyde, which further enhanced the conversions by a factor of about 3 (Table I). The only product derived from 1 mM Me_2Spm was MeSpd, the formation of which was stimulated nearly 5-fold by benzaldehyde. It is interesting to note that Me_2Spm was even more effectively converted to MeSpd in the presence of benzaldehyde than was MeSpm. The metabolism of spermine and its methylated derivatives was fully or partially blocked by MDL72527 (Table I).

Polyamines and Their Analogs as Substrates for Recombinant SSAT, PAO, and SMO—We also produced purified recombinant mouse SSAT, human PAO, and human SMO to study the substrate specificities of these enzymes. SSAT and SMO purifications yielded very pure enzymes according to SDS-PAGE, whereas purification of PAO resulted in about 80% pure enzyme. However, the contaminating bacterial protein did not have any PAO-like activity (data not shown).

K_m and V_{max} values of spermidine and spermine as substrates for SSAT were 52 μM and 2.4 nmol/min/μg; 33 μM and 0.43 nmol/min/μg, respectively. The K_i value of MeSpd with spermidine as substrate was 144 μM, K_i values of Me_2Spm with spermidine and spermine as substrates were 30 μM and 34 μM, respectively. MeSpm was not tested as it could serve as a substrate for SSAT at the unmethylated end. The kinetic values for different analogs are listed in Table II in respect to both recombinant oxidases. PAO strongly preferred N^1-acetylspermidine; furthermore, PAO was able to oxidize methylated spermine derivatives, but not MeSpd, to spermidine and MeSpd. In the presence of benzaldehyde, the K_m values for PAO decreased and the enzyme readily used spermine and its analogs but not very effectively spermidine or MeSpd, probably because of the inhibitory N^8 Schiff base. Spermidine, N^1-acetylspermidine, and MeSpd did not serve as substrates for SMO, whereas spermine and its mono- and dimethyl derivatives did. Expectedly, neither oxidase produced spermidine from Me_2Spm. Interestingly, the effect of methylation on the kinetic values seems to be opposite for the oxidases; the activity of PAO without benzaldehyde increased with the number of the methyl groups, whereas that of SMO decreased. As a conclusion it seems that α-methylation prevents polyamine derivatives from acetylation but not their oxidation.

Restoration of Early Liver Regeneration in Transgenic Rats Overexpressing SSAT—We have earlier found that partial hep-

TABLE I

Metabolism of polyamines and their analogs in rat liver extracts

The liver extracts were prepared as described under "Materials and Methods" and incubated for 60 min at +37 °C with the indicated additions. Reactions with MDL72527 were preincubated for 10 min before other drug addition. The concentration of added polyamine was 1 mM, benzaldehyde 5 mM, and MDL 72527 250 μM. The abbreviations used are: Put, putrescine; N^1-AcSpd, N^1-acetylspermidine; Spd, spermidine; Spm, spermine; BA, benzaldehyde.

Treatment	Polyamine or analog			
	Put	Spd	MeSpd	Spm
	pmol/mg protein			
Liver extracts				
without 1 h + 37 °C incubation	ND	796 ± 27		1,490 ± 190
after 1 h + 37 °C incubation	70 ± 45	824 ± 68		1,270 ± 88
+ 5 mM BA	635 ± 22	825 ± 110		20 ± 34
+ 250 μM MDL72527	51 ± 24	739 ± 110		1,430 ± 120
+ 5 mM BA + 250 μM MDL72527	4 ± 5	835 ± 49		1,270 ± 110
1 mM N^1-AcSpd	18,900 ± 940	1,120 ± 100		1,520 ± 310
+ 5 mM BA	15,100 ± 1100	1,080 ± 210		1,190 ± 67
+ 250 μM MDL72527	ND[a]	1,210 ± 140		1,300 ± 74
+ 5 mM BA + 250 μM MDL72527	ND	899 ± 150		1,600 ± 93
1 mM Spd	186 ± 24	—[b]		905 ± 78
+ 5 mM BA	3330 ± 290	—[b]		310 ± 83
+ 250 μM MDL 72527	44 ± 30	—[b]		876 ± 78
+ 5 mM BA + 250 μM MDL72527	9 ± 10	—[b]		784 ± 51
1 mM MeSpd	ND	394 ± 10	—[b]	839 ± 130
+ 5 mM BA	1030 ± 55	620 ± 74	—[b]	210 ± 72
+ 250 μM MDL72527	ND	387 ± 9	—[b]	733 ± 54
+ 5 mM BA + 250 μM MDL72527	ND	344 ± 12	—[b]	789 ± 120
1 mM Spm	ND	3,140 ± 390		—[b]
+ 5 mM BA	ND	21,000 ± 1700		—[b]
+ 250 μM MDL72527	ND	397 ± 64		—[b]
+ 5 mM BA + 250 μM MDL72527	ND	659 ± 39		—[b]
1 mM MeSpm	ND	1,830 ± 170	3,100 ± 390	441 ± 36
+ 5 mM BA	ND	6,240 ± 110	9,220 ± 270	343 ± 25
+ 250 μM MDL72527	ND	735 ± 99	ND	375 ± 18
+ 5 mM BA + 250 μM MDL72527	ND	568 ± 140	ND	389 ± 33
1 mM Me_2Spm	ND	744 ± 89	4,390 ± 680	1,110 ± 140
+ 5 mM BA	ND	999 ± 66	20,400 ± 1,300	640 ± 85
+ 250 μM MDL72527	ND	697 ± 81	617 ± 32	968 ± 120
+ 5 mM BA + 250 μM MDL72527	ND	746 ± 42	602 ± 26	1,050 ± 61

[a] ND, not detected.
[b] The beginning of the reaction polyamine equaled 84,700 pmol/mg protein.

TABLE II

Kinetic values of recombinant polyamine and spermine oxidases

The enzyme activities were measured as described under "Materials and Methods." Methylspermine can be catabolized from either end of the spermine backbone. The abbreviations used are: SMO, spermineoxidase; BA, benzaldehyde; N^1-AcSpd, N^1-acetylspermidine; Spd, spermidine; Spm, spermine.

Substrate	K_m			V_{max}		
	PAO	PAO + 5 mM BA	SMO	PAO	PAO + 5 mM BA	SMO
	μM			*pmol/μg protein/min*		
N^1-AcSpd	14	28		5900	4000	
Spd		15			710	
MeSpd		16			140	
Spm	47	9.3	20	340	4600	12000
MeSpm	19[a]	12[a]	67[a]	530[a]	4000[a]	2300[a]
	17[b]	11[b]	34[b]	340[b]	1500[b]	1100[b]
Me_2Spm	47	29	100	900	4100	1400

[a] Kinetic values for the unmethylated end.
[b] Kinetic values for the methylated end.

atectomy of the transgenic rats overexpressing SSAT results in a profound spermidine and spermine depletion because of SSAT induction at 24 h postoperatively and failure to initiate liver regeneration (5, 18). Liver regeneration could be restored by a prior administration of MeSpd (5). Table III shows thymidine incorporation in livers of transgenic rats before and 24 h after partial hepatectomy. As shown, thymidine incorporation remained at the preoperative level at 24 h in untreated rats, whereas small doses (5 mg/kg) of MeSpd only insignificantly increased DNA synthesis. Higher doses (25 mg/kg) of both MeSpd and Me_2Spm resulted in about a 10-fold stimulation of DNA synthesis. Table III also lists the hepatic pools of poly-

TABLE III

Accumulation of polyamines, their analogs, and thymidine incorporation in regenerating liver

Transgenic rats were partially hepatectomized and sacrificed at 24 h. The polyamine analogs were given 20 and 4 h before operation (5 mg or 25 mg/kg). Ten μCi of tritiated thymidine was given 30 min before sacrifice. There were 3 to 4 animals in each group. The abbreviations used are: Put, putrescine; Spd, spermidine; Spm, spermine.

Time of regeneration/treatment	Polyamines					Thymidine
	Put	Spd	MeSpd	Spm	Me_2Spm	
	pmol/mg tissue					*cpm/g liver*
0 h	1030 ± 95	1170 ± 140		158 ± 45		5640 ± 800
24 h	4600 ± 710[a]	405 ± 91[a]		45 ± 31[a]		6210 ± 600
24 h + MeSpd (2 × 5 mg)	4540 ± 690[a]	183 ± 47[a]	462 ± 41	18 ± 5[a]		14600 ± 4800
24 h + MeSpd (2 × 25 mg)	3280 ± 600[a]	278 ± 130[a]	1070 ± 230	38 ± 29[a]		60100 ± 22000[b]
24 h + Me_2Spm (2 × 25 mg)	4300 ± 250[a]	241 ± 28[a]	213 ± 98	2 ± 2[a]	422 ± 110	49800 ± 11000[b]

[a] $p < 0.001$.
[b] $p < 0.05$ as compared with unoperated animals.

amines and their analogs before and after partial hepatectomy. As found earlier, partial hepatectomy brought about profound depletions of the natural polyamines spermidine and spermine at 24 h after the operation in the transgenic rats, whereas the analogs readily accumulated in the liver. N^1-Acetylspermidine levels remained unaffected in all animal groups at about 100 pmol/mg of tissue (results not shown). Even though Me_2Spm was converted to MeSpd, the hepatic concentration of the latter compound remained clearly below (about half) the level achieved with the smaller dose of MeSpd that failed to stimulate thymidine incorporation (Table III). It thus appears that the observed stimulation of DNA synthesis after Me_2Spm was attributable to the spermine derivative and not to MeSpd. In fact, Me_2Spm appeared to be even more effective than MeSpd on the molar basis.

Reversal of Difluoromethylornithine-induced Cytostasis by Bis-α-methylspermine in Immortalized Fibroblasts—We next tested whether difluoromethylornithine (DFMO)-induced cytostasis in fibroblasts derived from transgenic fetuses overexpressing SSAT could be reversed with Me_2Spm. As shown in Fig. 3, 5 mM DFMO distinctly inhibited cell growth. An addition of 20 μM Me_2Spm fully reversed growth inhibition. Polyamine analyses showed that under these conditions, Me_2Spm was not converted to MeSpd to any appreciable extent. As also shown in Fig. 3, a combination of Me_2Spm with MDL72527 (inhibiting PAO and SMO) equally effectively reversed DFMO-induced growth inhibition (Me_2Spm, MDL72527, and their combination did not have any effect on cell growth in the absence of DFMO; results not shown). These results can be understood in terms that Me_2Spm fulfills the requirements for the natural polyamines without being converted to MeSpd.

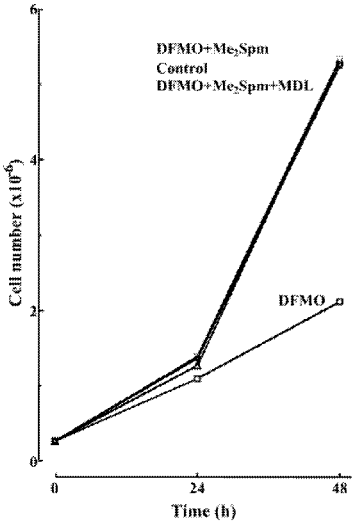

FIG. 3. **Growth curves of the immortalized fibroblasts derived from the SSAT transgenic rats treated without or with bis-α-methylspermine ± DFMO ± MDL72527.** The cells were plated in triplicates on 6-well plates (75,000 cells/well), after 24 h fresh medium was changed and the cell cultures were treated with different combinations of 20 μM bis-α-methylspermine, 5 mM DFMO, and 20 μM MDL72527. MDL, MDL72527.

DISCUSSION

The natural polyamines spermidine and spermine are ultimately converted to putrescine via the concerted action of SSAT and PAO. As PAO strongly prefers acetylated polyamines to the unmodified spermidine and spermine, SSAT is generally considered as the rate-controlling enzyme of the back-conversion pathway. However, when working with SSAT-deficient mouse embryonic stem cells we found that SSAT is absolutely necessary for the conversion of spermidine to putrescine, but not for the degradation of spermine to spermidine (19). In fact, the targeted cells appeared to convert spermine to spermidine much more efficiently than did their wild-type counterparts (19). The conversion of spermine to spermidine in the absence of SSAT activity is obviously attributable to a recently discovered oxidase, which, when first cloned, was thought to be PAO (20), but was later identified as a novel flavin-containing spermine oxidase (21). Unlike PAO, SMO strongly prefers spermine to its acetylated derivatives. Spermidine is not degraded at all, but monoethylspermine is as a good substrate for SMO as is spermine (21, 22). SMO, although inhibited to some extent, is much more resistant to the PAO inhibitor MDL72527 (21).

The present results have revealed that both MeSpm and Me_2Spm serve as substrates for both PAO and SMO *in vivo* and *in vitro*. MeSpm yielded both spermidine and MeSpd, whereas Me_2Spm was converted only to MeSpd. Like in the case of MeSpd (4), it is highly unlikely that doubly methylated spermine would serve as a substrate for SSAT. This view is supported by the findings that in the presence of benzaldehyde *in vitro* (Table I) Me_2Spm was converted to MeSpd much more effectively than MeSpm, whereas *in vivo* (Fig. 1,*C* and *D*), the latter compound yielded many times more MeSpd than its bis-methylated counterpart.

The results obtained with crude liver extracts and transgenic animals were largely confirmed with the use of purified recombinant PAO, SMO, and SSAT. Whereas PAO strongly preferred N^1-acetylspermidine as a substrate, SMO most effectively degraded spermine, but did not use spermidine or any of its tested analogs as substrates. The methyl derivatives of spermine were readily metabolized by SMO and PAO (in the presence of benzaldehyde) (Table II). PAO did not use MeSpd as a substrate and

it was only poorly metabolized when supplemented with benzaldehyde (Table II). The recombinant SSAT studies confirmed that MeSpd and Me_2Spm were not acetylated. However, they were competitive inhibitors of SSAT.

Our earlier studies with transgenic rats overexpressing SSAT have indicated that partial hepatectomy of these animals resulted in an induction of SSAT and a profound depletion of the hepatic spermidine pool that was associated with failure to initiate liver regeneration (5, 18). Liver regeneration could be fully restored with prior administration of MeSpd (5). Based on these studies and earlier work indicating that partial hepatectomy rapidly elevates the hepatic spermidine, but not spermine, pool, we assigned a critical role to spermidine in liver regeneration (5, 18). However, the present results indicated that liver regeneration, as judged by thymidine incorporation, was equally well restored by Me_2Spm, even at lower concentrations than required for MeSpd (Table III), indicating that spermidine and spermine may be fully exchangeable in this system. Straightforward interpretation of the experimental results with the transgenic animals are somewhat complicated by the fact that Me_2Spm was converted to MeSpd to some extent. However, according to the present results this conversion appeared to be slow yielding insufficient levels of MeSpd for the correction of the polyamine depletion and initiation of liver regeneration (Table III). To minimize Me_2Spm conversion to MeSpd, we used cell cultures with low Me_2Spm concentration. In cultured cells, DFMO-induced growth inhibition was fully reversed by Me_2Spm in the absence of any conversion of the latter compound to MeSpd. The combination of Me_2Spm with MDL72527 equally effectively reversed the antiproliferative effect of DFMO thus totally excluding the contribution of MeSpd. These results confirmed the observed restoration of liver regeneration by Me_2Spm as directly attributable to a polyamine (spermine) function, as Me_2Spm cannot be converted to deoxyhypusine (3). Our present study shows the necessity of combining *in vivo* and *in vitro* methods to indisputably confirm the conclusions drawn in modern biochemical experiments and clearly shows the value of the analogs in the polyamine metabolism studies.

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II

α–Methylated Polyamines: Efficient Synthesis and Tolerance Studies *in vivo* and *in vitro*. The First Evidence for Dormant Stereospecificity of Polyamine Oxidase

Järvinen A, Cerrada–Gimenez M, Grigorenko N, Khomutov AR, Vepsäläinen J, Sinervirta R, Keinänen TA, Alhonen L, and Jänne J.

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α-Methyl Polyamines: Efficient Synthesis and Tolerance Studies *in vivo* and *in vitro*. The First Evidence for Dormant Stereospecificity of Polyamine Oxidase

Aki Järvinen[§#], *Marc Cerrada-Gimenez*[§#], *Nikolay A. Grigorenko*[‡], *Alex R. Khomutov*[‡], *Jouko Vepsäläinen*[†*], *Riitta Sinervirta*[#], *Tuomo A. Keinänen*[#], *Leena Alhonen*[#] *and Juhani Jänne*[#]

[#]Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, [†]Department of Chemistry, University of Kuopio, Kuopio, Finland and [‡]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

[§] These authors share equal contribution
[*] To whom the correspondence should be addressed. Tel: +358–17–163256; Fax: +358–17–163259

TITLE RUNNING HEAD α-methylated polyamine analogs

[*] CORRESPONDING AUTHOR FOOTNOTE Jouko Vepsäläinen, Department of Chemistry, University of Kuopio, P. O. Box 1627, FI-70211 Kuopio, Finland. Street address (for courier): Savilahdentie 9E, Snellmania, FI-70210 Kuopio, Finland. Tel.: 358-17-163256; Fax: 358-17-163259; Email: Jouko.Vepsalainen@uku.fi.

Abstract

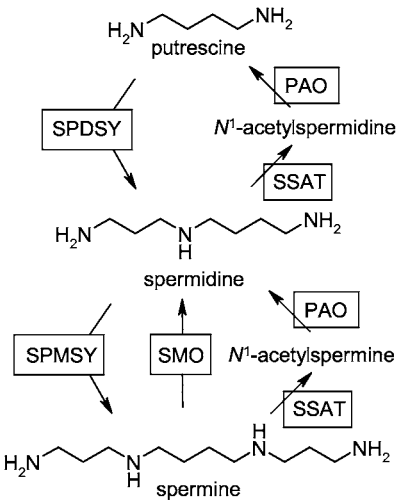
Efficient syntheses of metabolically stable α–methylspermidine **1**, α–methylspermine **2** and bis–α,α'–methylated spermine **3** starting from ethyl 3–aminobutyrate are described. The biological tolerance for these compounds was tested in wild–type mice and transgenic mice carrying the metallothionein promoter–driven spermidine/spermine N^1–acetyltransferase gene (MT–SSAT). The efficient substitution of natural polyamines by their derivatives was confirmed *in vivo* with the rats harboring the same MT–SSAT transgene and *in vitro* with the immortalized fibroblasts derived from these animals. Enantiomers of previously unknown 1–amino–8–acetamido–5–azanonane dihydrochloride **4** were synthesized starting from enantiomerically pure (*R*)– and (*S*)–alaninols. The studies with recombinant human polyamine oxidase (PAO) showed that PAO (usually splits achiral

substrates) strongly favors *R*–isomer of **4** that demonstrates for the first time that the enzyme has hidden potency for stereospecificity.

KEYWORDS. Polyamines, metabolically stable, polyamine oxidase, stereospecificity.

Introduction

Under natural physiological conditions biogenic polyamines are polycations that interact with many cellular macromolecules and participate in several distinct functions in the mammalian physiology[1]. Precisely maintained polyamine homeostasis is imperative for cell proliferation as both the accumulation and depletion of polyamines can lead to disorders of cellular metabolism[2]. In many tumour cell lines the polyamine levels are high compared to normal cells supporting accelerated cell proliferation. On the other hand, the depletion of the polyamine pools with inhibitors of their biosynthesis or compounds activating the catabolism of the polyamines lead to cell growth inhibition and even to apoptosis[3].



Scheme 1. Polyamine metabolism in mammals. PAO, polyamine oxidase; SMO, spermine oxidase; SPDSY, spermidine synthase; SPMSY, spermine synthase; SSAT, spermidine/spermine N^1–acetyltransferase.

In mammalian cells (Scheme 1) spermine can be oxidized directly to spermidine by spermine oxidase[4, 5]. Other possibility for the back–conversion of spermine to spermidine (and similarly

spermidine to putrescine) is the concatenated actions of spermidine/spermine N^1–acetyltransferase (SSAT) and polyamine oxidase (PAO). SMO uses only spermine, whereas PAO strongly favours N^1–acetylated substrates; both singly or doubly acetylated spermine and N^1–acetylspermidine are excellent substrates for PAO[6]. This back–conversion of the polyamines produces one H_2O_2 molecule per each splitted carbon–nitrogen bond thus causing oxidative stress. Drugs affecting polyamine catabolism may induce SSAT activity up to 1000 fold and cause the depletion of the higher polyamines, spermidine and spermine. Such compounds are of great interest and are being studied as anti–cancer drugs[7].

Alkylated polyamine derivatives are widely used for the investigation of biochemistry of spermine and spermidine. Most of these studies have concentrated on the analogues of spermine and its homologues which are symmetrically (e.g. diethylnorspermine, DENSpm) or non–symmetrically bis–alkylated at terminal amino groups. These compounds similar to spermine are actively transported into the cells but are incapable of fulfilling crucial cellular functions of polyamines[8]. The accumulation of DENSpm, or other compounds of similar nature, results in the down–regulation of the polyamine synthesis and/or the activation of the polyamine catabolism. Such terminally *N*–alkylated analogues are substrates for PAO[9] and after terminal dealkylation are prone to SSAT–PAO pathway.

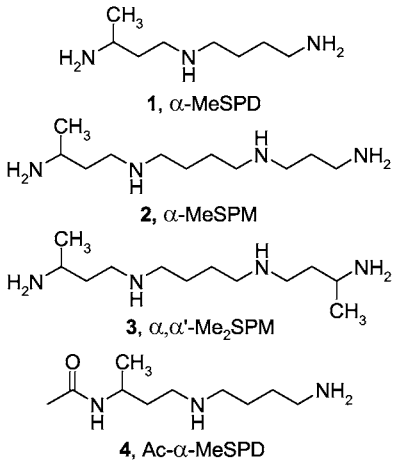


Figure 1. The structures of the α–methylated polyamine analogues used in this study.

Less investigated group of polyamine derivatives comprises the analogues with an alkyl substituent(s) attached to carbon atom(s) of polyamine backbone. Contrary to terminally *N*–alkylated derivatives of spermine, some of these compounds are able to sustain cell viability with depleted polyamine pools[10, 11]. The single methyl group in α–position (Figure 1) prevents the acetylation of the

adjacent amine of α–methylspermidine (**1**, α–MeSPD), α–methylspermine (**2**, α–MeSPM) and bis–α,α'–methylated spermine (**3**, α,α'–Me_2SPM) by SSAT which is the rate limiting step in the interconversion of the natural polyamines. However, **1–3** turned capable of inducing biosynthesis of SSAT similarly to spermine and spermidine[12].

Analogues **1-3** are as effective as the natural polyamines in inducing the conversion of the right–handed B–DNA to left–handed Z–DNA[13]. Furthermore, **1** (similarly to spermidine) and **2** (similarly to spermine due to its conversion to spermidine) serve as substrates for the deoxyhypusine synthase which posttranslationally modifies the eukaryotic initiation factor 5A[14]. Moreover, **1** and **3** proved to be metabolically quite stable as they are not substrates for diamine or monoamine oxidases[15, 16] and as such are auspicious tools to evaluate the roles of the polyamines in different cellular functions both *in vivo* and *in vitro*. Compound **2**, on the other hand, is more complicated as one end of the spermine backbone is unmodified and this analogue can serve as a substrate for the catabolic enzymes.

We have recently published results covering the effects of these analogues in the transgenic rats[17, 18]. The SSAT transgene under the control of the mouse metallothionein I promoter (MT) is greatly induced in the MT–SSAT transgenic rats by zinc administration. This leads to drastically reduced levels of the higher polyamines in the pancreas and results in a severe necrotizing pancreatitis[18]. *In vitro* studies have shown that in the case of SSAT over–induction exogenous supplementation of the natural polyamines does not restore cell viability due to their rapid intracellular catabolism[19]. However, the treatment with **1** prevented the acute pancreatitis altogether *in vivo*[18] and both **1** and **3** restored the early liver regeneration in the partially hepatectomized transgenic rats[17, 18]. Moreover, we showed that **1** and **3** are competitive SSAT inhibitors and rather poor substrates for the oxidases (especially **1**) involved in the catabolism of the polyamines *in vitro*[17].

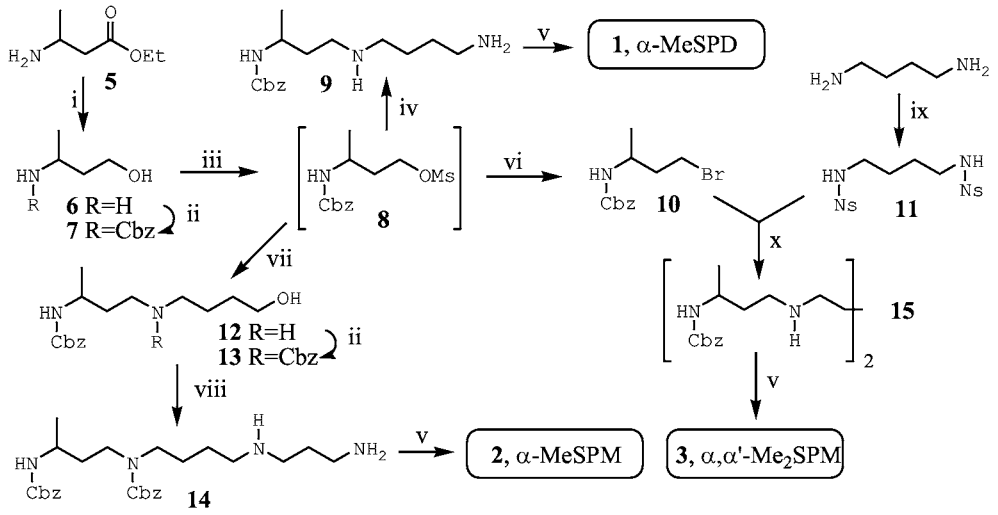
Here we present efficient synthetic protocols to prepare **1–3** in quantities sufficient for animal studies. Synthesis of previously unknown 1–amino–8–acetamido–5–azanonane (**4**, Ac–α–MeSPD) mimicing N^1–acetylspermidine (an excellent substrate for PAO) is also described. This compound and its different enantiomers are essential to bypass the acetylation block of α–methylated polyamine analogues by SSAT and to study the substrate requirements of PAO. The biological tolerances of **1–3** were for the first time measured in MT-SSAT transgenic and syngenic mice *in vivo*. Furthermore, these analogues were also studied with MT-SSAT transgenic rats *in vivo* and with the immortalized MT-SSAT transgenic rat fibroblasts *in vitro*.

Chemical synthesis

Scheme 2 outlines the synthesis of the studied α-methylated polyamine analogues **1**–**3**. Synthesis was started from commercially available ethyl 3-aminobutyrate (**5**), which was reduced by $LiAlH_4$ to aminoalcohol **6**, protected with benzylchloroformate (CbzCl) to **7** and mesylated to our key intermediate **8**. This mesyl derivative, which was not further purified, was easily converted either to *N*-protected methylspermidine **9**, bromide derivative **10** or to aminoalcohol **12**.

To prepare compound **1** the intermediate **8** was first aminated with an excess of putrescine to **9** at 0 to 20°C, that decreased the number of minor by-products that were difficult to separate and enabled good yield (72%). The target trihydrochloride of **1** was obtained after catalytic hydrogenation and crystallisation from aqueous acidic alcohol in 46 % overall yield as calculated for starting **5**.

Scheme 2. Synthesis of racemic α–methylated polyamines **1**–**3**[a]



[a] Reagents and conditions: (i) $LiAlH_4$, THF, reflux; (ii) Cbz–Cl, H_2O, $NaHCO_3$; (iii) MsCl, Et_3N, 0°C; (iv) $H_2N(CH_2)_4NH_2$, THF, 0 to 20°C; (v) (1) H_2/Pd, AcOH–MeOH 1:1; (2) HCl, MeOH; (vi) LiBr, THF; (vii) $H_2N(CH_2)_4OH$, THF, 0 to 37°C; (viii) (1) MsCl, Et_3N, (2) $H_2N(CH_2)_3NH_2$, THF, 0 to 20°C; (ix) NsCl, Et_3N, CH_2Cl_2, 0°C; (x) (1) K_2CO_3, DMF; (2) PhSH, K_2CO_3, DMF.

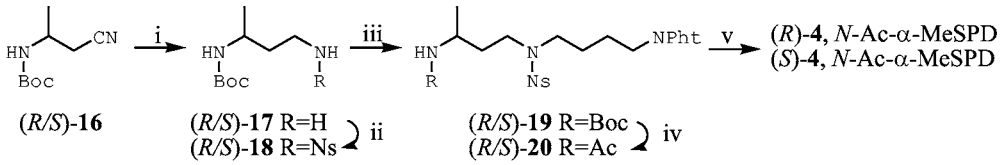
The synthesis of corresponding spermine analogue **2** was started from mesyl intermediate **8** by elongation of the protected polyamine backbone first with an excess of 4-aminobutanol at 0° to 37°C to give **12**, which was then protected to **13**, mesylated and treated with an excess of 1,3–diaminopropane

in THF at 0° to 37°C to give **14** in 70% yield. We also tried amination at 20°C, but this only increased the number of minor by–products that made purification of the compound **14** more laborious. The target tetrahydrochloride of **2** was obtained after deprotection similar to **1** in 41% overall yield calculated for *N*–Cbz–3–aminobutanol **7**.

The above linear strategy was ineffective for the synthesis of α,α'–Me_2SPM **3**. However, due to symmetry of the target molecule a straightforward convergent strategy was developed starting from bis-nosyl protected putrescine **11**. This derivative was not readily soluble in most of organic solvents except DMF and DMSO. The attempts to alkylate **11** with mesyl or tosyl derivatives of **7** gave low yields. However, the bromide derivative **10** reacted smoothly with **11** in the presence of K_2CO_3 and after selective removal of Ns–groups with small excess of thiophenol symmetrical **15** was obtained in a high yield. As previously, Cbz–groups were removed by catalytic hydrogenation and tetrahydrochloride of **3** was isolated in 57% overall yield as calculated for **7**.

To prepare isomers (*R*)–**4** and (*S*)–**4**, the corresponding enatiomerically pure nitriles **16**, were synthesized as described earlier[20], and were reduced by $LiAlH_4$ without racemisation to give the key amines (*R*)–**17** and (*S*)–**17** (Scheme 3). These amines were converted into nosylates **18** and then smoothly alkylated with 4–iodophthalimidobutane to give enantiomers (*R*)–**19** and (*S*)–**19** in 89% and 91% yields, respectively. After removal of Boc–group with HCl/EtOH followed by acetylation and subsequent one–pot removal of nosyl– and phthaloyl–groups with PhSH and $H_2NNH_2 \cdot H_2O$, respectively, resulted in (*R*)–**4** and (*S*)–**4**, which were converted to hydrochlorides and crystallized. In conclusion, the earlier unknown (*R*)–**4** and (*S*)–**4** were prepared in 7 steps with good overall yields 51% and 52%, respectively, as calculated for starting material **16**.

Scheme 3. Synthesis of enantiomers of acetyl–α–methylspermidine, **4**[a].



[a] Reagents and conditions: (i) $LiAlH_4$, Et_2O, -5°C; (ii) Ns–Cl, Et_3N, CH_2Cl_2, 0°C; (iii) $PhtN(CH_2)_4I$, K_2CO_3, DMF; (iv) (1) HCl, EtOH, EtAc; (2) AcCl, Et_3N, CH_2Cl_2, 0°C; (v) (1) PhSH, K_2CO_3, DMF; (2) $H_2NNH_2*H_2O$, EtOH, refl.; (3) HCl, MeOH.

Biological results and discussion

The methylated polyamines accumulated in a dose–dependent manner in the three tissues studied: liver (Figure 2), pancreas (supplemental Tables S4, S9 and S14) and kidney (supplemental Tables S5, S10 and S15) of the MT–SSAT transgenic and syngenic mice. Target tissues were selected based on our focus in current research; liver for toxicity and regeneration, pancreas due to pancreatitis study and kidney because higher polyamines are associated with the nephrotoxicity. The dosages used in the studies 50–500 mg/kg of **1** (equalling 1–10 fold of the dosage used for the liver regeneration or pancreatitis studies in rat), 12.5–50 mg/kg of **2** and 12.5–50 mg/kg of **3** (equalling 0.25–1 fold of the dosage used in the pancreatitis studies in rat) were well tolerated in mice. The total polyamine levels were not much affected in either mouse line by the treatments, with the exception of liver where spermidine was markedly depleted (Figure 2, supplemental Tables S3-S5, S8-10 and S13-15). In both mouse lines the SSAT induction was greatest with **1** (2–20 fold) whereas **3** resulted practically in no SSAT induction at all (supplemental Tables S2, S7 and S12). As expected the results from mice treated with **2** were more complicated. The induction of SSAT by the compounds pronounced in the transgenic mice but only in the transgenic liver **1** was detected as the degradation product of **2**. On the other hand, in the syngenic pancreas **2** appeared to be completely degraded as shown by the large accumulation of spermidine. However, whether **2** degraded to **1** or spermidine was impossible to distinguish due to the large spermidine peak in the HPLC graphs that obscured possible appearance of **1**. Interestingly, **2** appeared to be stable in the pancreas of the transgenic mice (supplemental Table S14). The spermine levels remained mostly unaffected or decreased slightly in response to the treatments; only in the livers of the syngenic animals and in the kidneys of both mouse lines treated with **3** spermine levels were significantly depleted. The analogues did not affect plasma α–amylase or ALAT activities (supplemental Tables S1, S6 and S11). All analogues were well tolerated and the general tolerability was comparable to that of the natural polyamines in mice (LD_{50} values (i.p.; as hydrochloride) of 870 mg/kg for spermidine and 370 mg/kg for spermine).

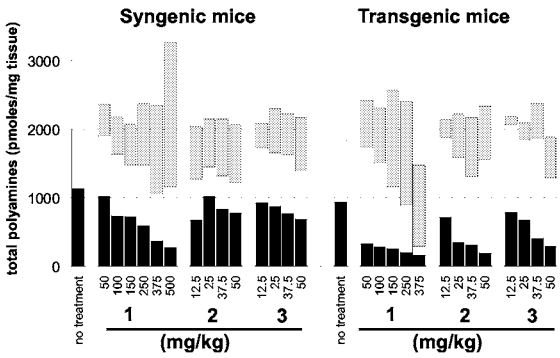


Figure 2. The distribution of polyamines and their α–methylated analogues in the liver of MT–SSAT transgenic mice and their syngenic littermates after 24 h treatment with the indicated dosages.

The MT–SSAT transgenic rats were treated twice with **1**–**3** at 25 mg/kg dose and none of the animals were lost during the study. However, at higher doses mortality rate quickly rose (results not shown). While tissue SSAT activities were only marginally affected, the concentrations of spermidine and spermine were reduced especially in the liver (supplemental Table S17). As in the mouse, **1** appeared to be metabolically stable in the transgenic rat whereas **2** was readily degraded in both the liver and the kidney. Furthermore, **3** degraded to **1** to some extent only in the liver (supplemental Table S17). No degradation of spermine analogues was detected in the rat pancreas but again low amounts of **1** could have remained undetected with the used HPLC system due to the spermidine peak. As a conclusion, it appears that there are some species–specific and/or the transgenecity–related differences in the maintenance of the polyamine homeostasis in the studied animals.

The *in vitro* studies with immortalized rat fibroblasts derived from the MT–SSAT transgenic and syngenic rats showed that the treatment with **1**–**3** effectively reduced the pools of natural polyamines (Figure 3). Both **1** and **3** were well tolerated at one millimolar concentration whereas higher than 10 μM concentrations of **2** were toxic. The supplementation of the cells with one millimolar aminoguanidine (AG) had insignificant effects on the polyamine contents of the fibroblasts. However, **2** was readily degraded in the absence of AG in both cell lines while the degradation of **3** was detected only in the transgenic cells independently of the AG treatment (supplemental Tables S19A-B). Moreover, in the transgenic cells **2** clearly inhibited the proliferation of the fibroblasts in the absence of AG while in rodents both spermine analogues were equally well tolerated (supplemental Table S17).

Interestingly, the SSAT activity increased slightly (2–10 fold) in the transgenic cell lines upon exposure to **1–3** but remained unaffected in the control cells, whereas DENSpm induced SSAT 200–300 fold in the transgenic cells but only about 10 fold in the control cells (supplemental Table S18). The maximal uptake of the analogues appeared to be reachable at low concentrations *in vitro* whereas *in vivo* the analogue accumulation depended on the dose (supplemental Tables S3-S5, S8-S10, S13-S15, S17 and S19A-B).

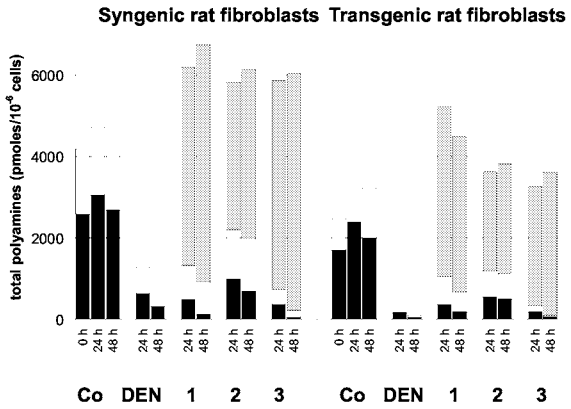


Figure 3. The distribution of polyamines and their α–methylated analogues in the immortalized rat fibroblasts during 48 h culture.

To study the fate of **4**, the use of recombinant human PAO was essential, as α–methylated polyamine analogues are not acetylated *in vivo* or *in vitro*. Furthermore, **4** is not effectively transported into the immortalized fibroblasts (unpublished observations) and, in fact, acetylation is considered to facilitate the export of excess polyamines. In addition, **4** is an excellent compound to assess stereospecific substrate requirements for PAO. We found that PAO strongly preferred (Figure 4) the *R*–isomer of **4** (K_m=95 μM, k_{cat}=9.0 s^{-1}) to *S*–isomer (K_m=170 μM, k_{cat}=1.2 s^{-1}). Expectedly the degradation of racemic **4** (K_m=100 μM, k_{cat}=1.3 s^{-1}) was strongly affected by the presence of (*S*)–**4**. Interestingly, k_{cat} value for (*R*)–**4** was slightly higher than that for N^1–acetylspermidine (K_m=14 μM, k_{cat}=8.5 s^{-1}) suggesting that PAO could readily use acetylated *R*–isomers of α–substituted polyamine analogues. Thus, the polyamine analogues with other alkyl and/or aromatic substituents in the α–position are interesting compounds for further studies to determine the substrate requirements of PAO.

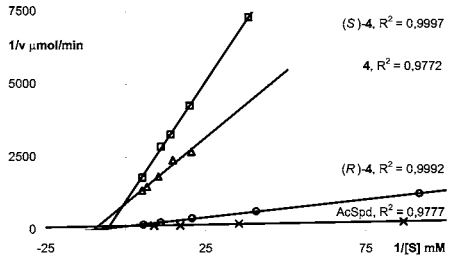


Figure 4. Determination of kinetic values for degradation of racemic and different isomers of acetyl–α–methylspermidine using Lineweaver–Burk plot.

In summary, the synthetic schemes described in the present paper allow allow the preparation of **1**–**4** in gram quantities with more than 99 % purity. Suggested synthetic schemes are also suitable for the preparation of (*R*)– and (*S*)–isomers of **1**–**3**, which may exhibit different biological activities. The strong stereoselectivity of PAO, which was demonstrated for the first time with the different isomers of **4**, may have biological implications. The presented animal experiments show that these polyamine analogues are well tolerated in mice. Furthermore, the studies *in vitro* confirm that these analogues are effectively transported into fibroblasts and can support cell proliferation when the natural polyamines are severely depleted. These compounds have proven to be useful tools in studies aimed to elucidate the functions of single polyamines both *in vivo* and *in vitro*.

Experimental section

Chemistry: Ethyl 3-aminobutyrate, (*R*)-alaninol, (*S*)-alaninol, 1,4-diaminobutane, 4-(*N*-iodobutyl)phthalimide, thiophenol, methanesulfonyl chloride (MsCl), $LiAlH_4$, and *o*-nitrophenylsulfonyl chloride (NsCl) were purchased from Aldrich (United States); and the rest of chemicals from Fluka (Switzerland). (*R*)- and (*S*)-*N*-(*tert*-Butyloxycarbonyl)-3-amino-butyronitrile (**16**) were prepared following published procedures[20].TLC was carried out on precoated Kieselgel 60 F254 plates and column chromatography with Kieselgel (40-63 µm, Merck, Germany) using the following elution systems: (A) 97:3 $CHCl_3$–MeOH, (B) 9:1 dioxane–25% ammonia, and (C) 4:2:1:2 *n*-butanol–AcOH–pyridine–H_2O, (D) $CHCl_3$, (E) 200:1 $CHCl_3$–MeOH, (F) 97:3 dioxane–25% ammonia, (G) 95:5 $CHCl_3$–MeOH, (H) 96:4 dioxane–25% ammonia, (I) 98:2 dioxane–25% ammonia, (J) 95:5 dioxane–25% ammonia, (K) 1:2 EtOAc–hexane, and (L) 75:25 dioxane–25% ammonia. Melting points were determined in open capillary tubes and are uncorrected. Optical rotation angles were measured with Perkin-Elmer 241 digital polarimeter. Chiral–HPLC analysis was performed using Whelk–O 1 (*R*,*R*) 25 cm * 4.6 mm column; isocratic run 0–75 min with flow rate of 0.5 mL/min, 60 % ethanol to separate

(*R*)– and (*S*)–isomers of Ac-α-MeSPD after dansylation and treatment as described previously[21]. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 500 DRX (Germany) using tetramethylsilane (TMS) in $CDCl_3$ or sodium 3-(trimethylsilyl)-propanesulfonate (TSP) in D_2O as internal standards. Chemical shifts are given in ppm, and the letter "*J*" indicates normal $^3J_{HH}$ couplings and all *J* values are given in Hz.

3-Aminobutan-1-ol (6). A solution of freshly distilled ethyl 3-aminobutyrate (**5**, 13.5 g, 0.103 mol) in dry THF (50 mL) was added dropwise to the stirred suspension of $LiAlH_4$ (8 g, 0.21 mol) in dry THF (150 mL). The reaction mixture was refluxed with stirring for 3 h and kept at 20°C overnight. The residue was quenched first with water (11.4 mL), 20% NaOH (10.6 mL), water (29.0 mL), and finally with 40% NaOH (34.2 mL). The organic phase was separated and the solids were extracted with hot chloroform (4 × 80 mL). The combined organic layers were dried ($MgSO_4$), evaporated *in vacuo*, and the residue was distilled (bp 108–9°C/42 mmHg, lit.[22] bp 73°C/7 mmHg) to give **6** (7.3 g, 80%) as a colorless liquid: ^{1}H NMR ($CDCl_3$) δ 3.79–3.68 (2H, m, CH_2O), 3.12–3.03 (1H, m, MeCH), 2.58 (3H, bs, OH, NH_2), 1.62–1.54 (1H, m, CHC<u>H</u>$_2$), 1.50–1.40 (1H, m, CHC<u>H</u>$_2$ [*see footnote]), 1.09 (3H, d, *J*=6.5, CH_3).

***N*-(Benzyloxycarbonyl)-3-aminobutan-1-ol (7).** Prepared from benzyl chloroformate (4.65 mL, 33 mmol), **6** (2.7 g, 30 mmol), THF (30 mL), water (5 mL), and $NaHCO_3$ (3.81 g, 45 mmol) using the known method[23]. The residue was triturated with a 1:2 ether–hexane mixture (80 mL), and the precipitate was filtered and dried in vacuum over P_2O_5, to give **7** (6.03 g, 90%): mp 60°C; *Rf* 0.38 (A); ^{1}H NMR ($CDCl_3$) δ 7.37–7.28 (5H, m, Ph), 5.09 (2H, s, CH_2Ph), 4.75 (1H, bs, NHCbz), 4.01–3.93 (1H, m, MeCH), 3.67–3.59 (2H, m, CH_2O), 2.98 (1H, bs, OH), 1.83–1.72 (1H, m), 1.45–1.35 (1H, m, CHC<u>H</u>$_2$ [*see footnote on page 13]), 1.20 (3H, d, *J*=6.5, CH_3).

***N*^{8}-(Benzyloxycarbonyl)-1,8-diamino-5-azanonane (9).** To a stirred and cooled (0°C) solution of **7** (5.51 g, 24.7 mmol) and Et_3N (5.22 mL, 37.5 mmol) in dry DCM (60 mL) MsCl (2.14 mL, 27.5 mmol) in dry DCM (10 mL) was added within 10 min. Stirring was continued for 1h at 0°C, 30 min at room temperature and the reaction mixture was poured into 1 M $NaHCO_3$ (40 mL) solution. The organic layer was separated, washed with water (2x10 mL), 0.5 M H_2SO_4 (3x35 mL), water (2x10 mL) and 1 M $NaHCO_3$ (10 mL). The dried ($MgSO_4$) and evaporated residue of **8** was dissolved in anhydrous THF (40 mL), cooled to 0°C and cold (0°C) solution of 1,4-diaminobutane (35.1 g, 400 mmol) in dry THF (40 mL) was added in one portion and the reaction mixture was kept for 6 h at 0°C and 16 h at room temperature. THF and excess of 1,4-diaminobutane were evaporated *in vacuo* and the residue was purified on a silica gel column using eluent (B) to give **9** (5.3 g, 72%) as a viscous oil: *Rf* 0.15 (B); ^{1}H NMR ($CDCl_3$) δ 7.37–7.27 (5H, m, Ph), 5.57 (1H, bs, NHCbz), 5.08 (2H, s, CH_2Ph), 3.85-3.76 (1H, m, MeCH), 2.74-2.52 (6H, m, CH_2NH), 1.71-1.41 (6H, m, CCH_2C), 1.40 (3H, bs, NH, NH_2), 1.17 (3H, d, *J*=6.5, CH_3); ^{13}C NMR δ 156.04 s, 136.85 s, 128.49 s, 128.01 s (2C), 66.39 t, 49.77 t, 46.43 t, 45.96 d, 42.11 t, 36.64 t, 31.57 t, 27.43 t, 21.25 q.

1,8-Diamino-5-azanonane trihydrochloride (1, α-MeSPD). Pd black in methanol (~1 mL) was added to a solution of **9** (5.2 g, 17.7 mmol) in a mixture of AcOH–MeOH (1:1, 40 mL) and hydrogenation was carried out at atmospheric pressure. Solids were filtered off, washed with MeOH, and the combined filtrates were evaporated *in vacuo*. The residue was dissolved in ethanol, diluted with 7 M HCl (6.6 mL), evaporated to dryness *in vacuo* and the residue was recrystallized from a H_2O–MeOH–EtOH mixture to give **1** (4.23 g, 89%) as colourless crystals: mp 191–2°C (lit.: mp 283°C[16] for lyophilized powder); *Rf* 0.45 (C); ^{1}H NMR (D_2O) δ 3.53 (1H, m, MeCH), 3.21 (2H, m, NCH_2), 3.15 (2 H, m, NCH_2), 3.07 (2H, m, NCH_2), 2.16 (1H, m, CCH_2C [*see footnote on page 13]), 2.02 (1H, m, CCH_2C [*see footnote on page 13]), 1.86–1.74 (4H, m, CCH_2C), 1.36 (3H, d, *J*=6.5, CH_3); ^{13}C NMR δ 50.03 t, 48.36 d, 46.89 t, 41.85 t, 33.34 t, 26.85 t, 25.69 t, 20.38 q. Anal. ($C_8H_{24}N_3Cl_3$, C, H, N).

***N*-(Benzyloxycarbonyl)-3-amino-1-bromobutane (10).** The mesyl intermediate **8** was prepared as in **9** from **7** (3.01 g, 13.5 mmol), Et_3N (2.52 mL, 18.1 mmol) and MsCl (1.16 mL, 15 mmol). The evaporated and dried residue was dissolved in THF (5 mL), and LiBr (3.5 g, 40 mmol) in THF (20 mL) was added in one portion. The reaction mixture was stirred overnight at 20°C, chloroform (30 mL) was added, solids were filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in chloroform (50 mL), washed with water (2x30 mL), 1 M $NaHCO_3$ (2x15 mL), brine (20 mL) and dried ($MgSO_4$). The evaporated residue was dried *in vacuo* over P_2O_5/KOH to give **10** (3.78 g, 95%) as a viscous oil: R_f 0.61 (D); ^{1}H NMR ($CDCl_3$) δ 7.37-7.26 (5H, m, Ph), 7.11 (1H, br s, NHCbz), 5.03 (2H, s, CH_2Ph), 3.78-3.72 (1H, m, MeCH), 3.46-3.41 (2H, t, *J*=7.4, CH_2Br), 2.10-1.99 (1H, m [*see footnote on page 13]), 1.95-1.85 (1H, m, $CHCH_2$ [*see footnote on page 13]), 1.12 (3H, d, *J*=6.6, CH_3).

***bis*-N^1,N^4-*o*-Nitrophenylsulfonyl-1,4-diaminobutane (11).** To a cooled (0°C) solution of freshly distilled 1,4-diaminobutane (1.1 g, 12.5 mmol) and Et_3N (5.2 mL, 37.5 mmol) in dry DCM (50 mL) NsCl (6.05 g, 27.3 mmol) in dry DCM (30 mL) was added within 40 min with vigorous stirring. After addition stirring was continued for 1 h at 0°C and 3 h at room temperature. The precipitate was filtered off, washed with MeOH (3x15 mL), chloroform (3x10 mL) and dried *in vacuo* over P_2O_5 to give **11** (5.3 g, 91%) as a pale-yellow crystals: R_f 0.55 (E); m.p. 185-6°C; ^{1}H NMR (DMSO-d_6) δ 7.98-7.91 (4 H, m, Ph-), 7.86-7.81 (4H, m, Ph); 2.87-2.82 (4H, m, CH_2N), 1.44-1.37 (4H, m, CCH_2C).

N^8-(Benzyloxycarbonyl)-8-amino-5-azanonan-1-ol (12). Mesyl intermediate **8** was prepared as in **9** from **7** (3.34 g, 15 mmol), Et_3N (2.61 mL, 19 mmol) and MsCl (1.24 mL, 16 mmol). The evaporated residue was dissolved in THF (10 mL), cooled to 0°C and to the resulting solution the cold (0°C) mixture of 4-aminobutanol (12.3 g, 138 mmol) and THF (20 mL) was added in one portion and reaction mixture was stirred at each three temperatures (0°C, 20°C and 37°C) for 12 h, totally 36h. The solvent and an excess of 4-aminobutanol were evaporated *in vacuo*, the residue was dissolved in 2 M NaOH (25 mL) and extracted with DCM (2x45 mL). The organic layer was separated, washed with brine (15 mL), dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified on a silica gel with eluent (F) which resulted in **12** (3.75 g, 85%) as a viscous oil: R_f 0.45 (B); ^{1}H NMR ($CDCl_3$) δ 7.40-7.29 (5H, m, Ph), 5.09 (2H, s, CH_2Ph), 5.03 (0.5H, s, NHCbz), 5.01 (0.5H, s, NHCbz), 3.85-3.77 (1H, m, MeCH), 3.57 (2H, t, *J* 6.3, CH_2OH), 2.70-2.55 (4H, m, CH_2N), 1.76-1.51 (8H, m, NH, OH, CCH_2C), 1.18 (3H, d, *J*=6.5, CH_3).

***bis*-N^5,N^8-(Benzyloxycarbonyl)-8-amino-5-azanonan-1-ol (13).** Benzyl chloroformate (1.55 mL, 11 mmol) was added in three portions with 15-min intervals to a cooled (0°C) and vigorously stirred mixture of **12** (2.94 g, 10 mmol), THF (20 mL), $NaHCO_3$ (0.84 g, 10 mmol), and 2 M Na_2CO_3 (6 mL). Stirring was continued for 1 h at 0°C, 3 h at room temperature, organic layer was separated and the residue was extracted with chloroform (15 mL). Organic layers were combined and evaporated to dryness *in vacuo*, the residue was dissolved in chloroform (30 mL) and washed with 0.5 M H_2SO_4 (2x10 mL), water (15 mL), and 1 M $NaHCO_3$ (2x10 mL) dried ($MgSO_4$) and evaporated *in vacuo*. The residue was purified on a silica gel using first eluent E and then G to give **13** (3.94 g, 92%) a viscous oil; R_f 0.27 (A); ^{1}H NMR ($CDCl_3$) δ 7.34-7.27 (10H, m, Ph), 5.11 (2H, s, CH_2Ph), 5.07 (2H, s, CH_2Ph), 4.96 (0.5H, s, NHCbz), 4.57 (0.5H, s, NHCbz), 3.73-3.55 (3H, m, MeCH, CH_2O), 3.30-3.18 (4H, m, CH_2N), 1.75-1.45 (7H, m, OH, CCH_2C), 1.17-1.10 (3H, m, CH_3); ^{13}C NMR ($CDCl_3$) δ 156.16, 155.90, 136.82, 128.52, 128.07, 128.00, 127.85, 67.07, 66.59, 62.33, 47.44, 45.41, 44.78, 44.34, 36.22, 35.13, 29.66, 25.02, 24.70, 21.32.

***bis*-N^9,N^{12}-(Benzyloxycarbonyl)-1,12-diamino-4,9-diazatridecane (14).** The mesyl intermediate was prepared as in **9** from **13** (3.85 g, 9 mmol), Et_3N (1.53 mL, 11 mmol) and MsCl (0.77 mL, 10 mmol). The evaporated and dried residue was dissolved in THF (15 mL), cooled to 0°C, and

cold (0°C) solution of 1,3-diaminopropane (6.66 g, 90 mmol) in THF (5 mL) was added in one portion. After 6 h at 0°C and overnight at room temperature the reaction mixture was concentrated *in vacuo*. The residue was poured into 1 M NaOH (15 mL), extracted with DCM (2x15 mL), evaporated *in vacuo* and the residue was purified on a silica gel using eluent H to give **14** (3.05 g, 70%) as a viscous oil; R_f 0.25 (F); ^{1}H NMR ($CDCl_3$) δ 7.34-7.29 (10H, m, Ph), 5.10 (2H, s, C$\underline{H}_2$Ph), 5.07 (2H, s, C$\underline{H}_2$Ph), 4.79 (1H, s, N$\underline{H}$Cbz), 3.35-3.20 (5H, m, MeCH, CH_2N), 2.73 (2H, t, *J*=6.8, C$\underline{H}_2$N), 2.66-2.53 (4H, m, C$\underline{H}_2$N), 1.70-1.35 (11H, m, NH, NH_2, CC$\underline{H}_2$C), 1.16-1.10 (3H, m, CH_3); ^{13}C NMR ($CDCl_3$) δ 155.85, 136.88, 136.70, 128.48, 128.02, 127.92, 127.79, 67.07, 66.96, 66.48, 49.63, 47.85, 47.62, 47.17, 45.37, 44.77, 44.32, 40.54, 36.20, 35.06, 33.77, 27.25, 26.48, 25.95, 21.33.

1,12-Diamino-4,9-diazatridecane tetrahydrochloride (2, α-MeSPM). Prepared as **1** from **14** (3.0 g, 6.2 mmol), recrystallized from a H_2O-MeOH-EtOH mixture to give **2** (1.97 g, 88%) as colourless crystals: mp 250-1°C, dec., (lit. mp 247°C[16] for liophylized powder); R_f 0.13 (C); ^{1}H NMR (D_2O) δ 3.56-3.48 (1H, m, MeCH); 3.24-3.10 (10H, m, C$\underline{H}_2$N); 2.18-2.08 (3H, m, NHCHC$\underline{H}_2$, C$\underline{H}_2$CH_2NH_2); 2.04-1.95 (1H, m, NHCHC$\underline{H}_2$); 1.85-1.77 (4H, m, CCH_2C); 1.36 (3H, d, *J*=6.6, CH_3); ^{13}C NMR (D_2O) δ 47.07, 45.41, 44.62, 44.00, 36.67, 30.47, 23.79, 22.83, 17.58, 17.34; Anal. ($C_{11}H_{32}N_4Cl_4$·0.25 H_2O, C, H, N).

***bis*-N^2,N^{13}-(Benzyloxycarbonyl)-2,13-diamino-5,10-diazatetradecane (15).** Bromide **10** (3.78 g, 13.2 mmol), *bis*-sulfamide **11** (2.16 g, 4.7 mmol), and K_2CO_3 (4.3 g, 31 mmol) were stirred in DMF (30 mL) for 48 h at room temperature followed by addition of a mixture of K_2CO_3 (3.7 g, 27 mmol) and PhSH (1.4 mL, 13.5 mmol) in DMF (10 mL). After stirring for additional 12 h at 20°C salts were filtered off and the filtrate was evaporated to dryness *in vacuo*, the residue was suspended in chloroform (50 mL), solids were filtered off and washed with chloroform (3x10 mL). Combined filtrates were washed with water (2x30 mL), dried ($MgSO_4$), evaporated to dryness *in vacuo* and the residue was purified on a silica gel using eluent (F) that resulted in **15** (2.3 g, 70%) as a colourless solid: R_f 0.46 (F); m.p. 107-8°C; ^{1}H NMR ($CDCl_3$) δ 7.36-7.26 (10H, m, Ph), 5.51 (2H, bs, N$\underline{H}$Cbz), 5.08 (4H, s, C$\underline{H}_2$Ph), 3.85-3.74 (2H, m, MeCH), 2.73-2.65 (2H, m, CH_2N), 2.64-2.54 (2H, m, CH_2N), 2.54-2.45 (4H, m, CH_2N), 1.72-1.40 (8H, m, CC$\underline{H}_2$C), 1.16 (6 H, d, *J*=6.5, CH_3); ^{13}C NMR ($CDCl_3$) δ 156.03, 136.86, 128.53, 128.05, 67.14, 66.41, 49.82, 46.45, 46.01, 36.66, 27.87, 21.28.

2,13-Diamino-5,10-diazatetradecane tetrahydrochloride (3, α,α'-Me_2SPM). Prepared as **2** from **15** (2.3 g, 4.6 mmol) to give **3** (1.47 g, 85%) as colourless crystals: mp 224-5°C, dec., (lit. mp 180°C[16] for liophylized powder); R_f 0.13 (C). ^{1}H NMR (D_2O) δ 3.55-3.46 (2H, m, MeCH); 3.21 (4H, t, *J*=6.8, CH_2N); 3.17-3.11 (4H, m, CH_2N), 2.18-2.09 (2H, m, CCH_2C), 2.04-1.93 (2H, m, CCH_2C), 1.84-1.75 (4H, m, CCH_2C); 1.35 (6H, d, *J*=6.6, CH_3); ^{13}C NMR (D_2O) δ 49.86, 48.14, 46.78, 33.27, 25.64, 20.09. Anal.($C_{12}H_{34}N_4Cl_4$, C, H, N).

***(R)*-N^3-(*tert*-Butyloxycarbonyl)-1,3-diaminobutane ((*R*)-17).** To the cooled (-5°C) suspension of $LiAlH_4$ (0.96 g, 25 mmol) in Et_2O (20 mL) the solution of (*R*)-**16**[20] (1.7 g, 9.2 mmol) in Et_2O (15 mL) was added with stirring within 20 min and stirring was continued for 45 min at -5°C followed by quenching of the reaction mixture with 20 % (w/w) aq. NaOH. Organic phase was separated, the residue was extracted with Et_2O (3x20 mL) and the combined organic phases were washed with brine (20 mL). Ether was evaporated *in vacuo* and the residue was purified on silica gel using eluent (I) to give (*R*)-**17** (1.56 g, 90 %) as a colorless oil: R_f 0.33 (J); $[\alpha]_D^{20}$ - 12.0° (*c* 2.0, $CHCl_3$, lit.[20] $[\alpha]_D^{20}$ - 12.0°); ^{1}H NMR ($CDCl_3$) δ 4.61 (1H, bs, NH); 3.77 (1H, bs, MeCH), 2.79-2.69 (2H, m, CH_2N), 1.60-1.44 (m, 2H), 1.43 (s, 9H), 1.08 (3H, d, *J*=6.6 Hz); ^{13}C NMR ($CDCl_3$) δ 155.51, 78.89, 44.26, 40.99, 38.81, 28.34, 21.43.

(*S*)-N^3-(*tert*-Butyloxycarbonyl)-1,3-diaminobutane ((*S*)-17). Prepared as (*R*)-**17** starting from (*S*)-**16**[20] to give (*S*)-**17** as a colorless oil (91 %): $[\alpha]_D^{20}$ +12.0° (*c* 2.0, $CHCl_3$, lit.[20] $[\alpha]_D^{20}$ +12.0°). ^1H NMR shifts identical to (*R*)-17.

(*R*)-N^1-(*o*-Nitrophenylsulfonyl)-N^3-(*tert*-butyloxycarbonyl)-1,3-diaminobutane ((*R*)-18). To the cooled (0°C) solution of (*R*)-**17** (1.43 g, 7.6 mmol) and Et_3N (1.21 mL, 8.8 mmol) in dry DCM (15 mL) the solution of NsCl (1.77 g, 8.0 mmol) in dry DCM (7 mL) was added with stirring within 30 min. Strirring was continued for 1 h at 0°C followed by washing the reaction mixture with H_2O (10 mL), 10 % citric acid (15 mL), 1 M $NaHCO_3$ (10 mL), brine (10 mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* to afford (*R*)-**18** (2.78 g, 98 %) as colorless solid: m.p. 115-6 °C (H_2O–MeOH); R_f 0.21 (K); $[\alpha]_D^{20}$ -13.0° (*c* 5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 8.14-8.10 (1H, m, Ar), 7.85-7.81 (1H, m, Ar), 7.74-7.69 (2H, m, Ar), 6.23 (1H, bs, NsNH), 4.31 (1H, d *J*=6.5, NH), 3.80-3.69 (1H, m, MeCH), 3.33-3.22 (1H, m, CH_2N), 3.01 (1H, m, CH_2N), 1.78-1.69 (1H, m, $CHC\underline{H}_2$ [*see footnote on page 13]), 1.51-1.43 (1H, m, $CHC\underline{H}_2$ [*see footnote on page 13]), 1.37 (9H, s, CMe_3), 1.11 (3H, d, *J*=6.7); ^{13}C NMR δ 155.91, 148.07, 134.24, 133.28, 132.49, 130.70, 125.06, 79.59, 43.81, 40.81, 38.10, 28.26, 21.37. Anal. ($C_{15}H_{23}N_3O_6S$, C, H, N).

(*S*)-N^1-(*o*-Nitrophenylsulfonyl)-N^3-(*tert*-butyloxycarbonyl)-1,3-diaminobutane ((*S*)-18). Prepared as (*R*)-**18** starting from (*S*)-**17** to give (*S*)-**18** as a colorless solid (99 %); m.p. 115-6 °C (H_2O–MeOH); $[\alpha]_D^{20}$ +13.0° (*c* 5, $CHCl_3$). ^1H NMR shifts identical to (*R*)-**18**. Anal. ($C_{15}H_{23}N_3O_6S$, C, H, N).

(*R*)-N^1-(Phthaloyl)-N^5-(*o*-nitrophenylsulfonyl)-N^8-(*tert*-butyloxycarbonyl)-1,8-diamino-5-azanonane ((*R*)-19). The mixture of (*R*)-**18** (0.75 g, 2.0 mmol), *N*-(4-iodobutyl)phthalimide (0.725 g, 2.2 mmol) and K_2CO_3 (0.85 g, 6.2 mmol) in dry DMF (4 mL) was stirred for 24 h at 20°C. Solvent was evaporated *in vacuo*, the residue was treated with EtOAc-H_2O (2:1, 30 mL) and the organic layer was separated, washed with H_2O (10 mL), brine (20 mL), dried ($MgSO_4$) and evaporated *in vacuo*. The residue was crystallized from EtOAc-*n*-hexane to give (*R*)-**19** as pale-yellow crystals (1.03 g, 89%): mp 109-110°C (EtOAc-*n*-hexane); $[\alpha]_D^{20}$ -7.0° (*c* 2, EtOAc); ^1H NMR ($CDCl_3$) δ 8.00-7.96 (1H, m, Ar), 7.86-7.80 (2H, m, Ar), 7.73-7.68 (2H, m, Ar), 7.66-7.61 (2H, m, Ar), 7.58-7.53 (1H, m, Ar), 4.48 (1H, bs, NH), 3.66 (2H, t, *J*=6.5, CH_2NPht), 3.62-3.55 (1H, m, MeCH), 3.40-3.21 (4H, m, CH_2N); 1.76-1.52 (6H, m, CCH_2C), 1.42 (9H, s), 1.11 (3H, d, *J*=6.5 Hz); ^{13}C NMR δ 168.35, 133.99, 133.49, 133.33, 132.09, 131.54, 130.68, 124.10, 123.28, 78.80, 47.40, 45.14, 37.11; 36.18, 28.41, 25.70, 25.53, 21.47.

(*S*)-N^1-(Phthaloyl)-N^5-(*o*-nitrophenylsulfonyl)-N^8-(*tert*-butyloxycarbonyl)-1,8-diamino-5-azanonane((*S*)-19). Prepared as (*R*)-**19** starting from (*S*)-**18** to give (*S*)-**19** as a pale-yellow crystals (91 %); mp 109-110°C (EtOAc-*n*-hexane); $[\alpha]_D^{20}$ +7.0° (*c* 2, EtOAc). ^1H NMR shifts identical to (*R*)-**19**.

(*R*)-N^1-(Phthaloyl)-N^5-(*o*-nitrophenylsulfonyl)-N^8-(acetyl)-1,8-diamino-5-azanonane ((*R*)-20). To the solution of (*R*)-**19** (0.97 g, 1.69 mmol) in EtOAc (8 mL) the solution of dry HCl in EtOH (10 M, 4 mL) was added and the mixture was stirred for 30 min at 20 °C. Solvents were evaporated *in vacuo*, the residue was co-evaporated with DMF (2x15 mL) *in vacuo* and the residual oil was dissolved in dry DCM (8 mL) and Et_3N (1.41 mL, 10.2 mmol). After cooling (0°C), AcCl (1.17 mL, 2.38 mmol) in dry DCM (3 mL) was added with stirring over 10 min and stirring was continued for additional 15 min at 0°C followed by adding MeOH (3 mL). The solvents were evaporated *in vacuo*, the residue was dissolved in EtOAc (40 mL), washed with H_2O (20 mL), 10% citric acid (10 mL), brine (20 mL), dried ($MgSO_4$) and evaporated *in vacuo*. The residue was crystallized from (EtOAc–*n*-hexane, 1:2) to give (*R*)-**20** as a pale-yellow crystals (0.8 g, 92%): R_f 0.54 (J); m.p. 125-6°C (EtOAc–*n*-hexane, 1:2); $[\alpha]_D^{20}$ -6.7° (*c* 2, EtOAc). ^1H NMR ($CDCl_3$) δ 7.96-7.92 (1H, m, Ar), 7.86-7.80 (2H, m, Ar), 7.74-7.69 (2H, m, Ar), 7.66-7.61 (2H, m, Ar), 7.57-7.52 (1H, m, Ar), 5.58 (1H, d, *J*=8.1, NH); 4.00-3.89 (1H, m, MeCH); 3.67 (2H, t, *J*=6.9, CH_2NPht), 3.40-3.17 (4H, m, CH_2N), 1.97 (s, 3 H), 1.85-1.54 (6H, m, CCH_2C), 1.14

(3H, d, *J*=6.5 Hz); ^{13}C NMR δ 169.94, 168.55, 148.37, 134.21, 133.60, 133.36, 132.22, 131.77, 130.63, 124.21, 123.43, 48.02, 45.47, 43.60, 37.28, 36.29, 25.85, 25.81, 23.60, 21.32.

(*S*)-N^1-(Phthaloyl)-N^5-(*o*-nitrophenylsulfonyl)-N^8-(acetyl)-1,8-diamino-5-azanonane ((*S*)-20). Prepared as (*R*)-**20** starting from (*S*)-**19** to give (*S*)-**20** as a pale-yellow crystals (91 %); m.p. 125-6°C (EtOAc–*n*-hexane, 1:2); $[\alpha]_D^{20}$ +6.7° (*c* 2, EtOAc). ^{1}H NMR shifts identical to (*R*)-20.

(*R*)-N^8-(Acetyl)-1,8-diamino-5-azanonane ((*R*)-4). A mixture of (*R*)-**20** (0.75 g, 1.45 mmol), PhSH (0.23 mL, 2.25 mmol) and K_2CO_3 (0.62 g, 4.5 mmol) in DMF (3 mL) was stirred for 16 h at 20°C, the solvent was removed *in vacuo* and the residue was dissolved in the mixture of DCM-H_2O (3:1, 50 mL). Water layer was separated, extracted with DCM and combined organic phases were washed with brine (15 mL), dried ($MgSO_4$) and solvent was evaporated *in vacuo*. The residue was dissolved in EtOH (6 mL) containing $N_2H_4 \cdot H_2O$ (0.06 g, 1.83 mmol), refluxed for 3 h and solvent was evaporated *in vacuo*. The residue was treated with EtOH-water (1:1) mixture (5 mL), insoluble oil was separated and the solution was applied on Dowex 1x8 (200-400 mesh, HO^-form, 10 mL) and eluted with EtOH-H_2O (1:1) to get rid of phthalyl hydrazide. Fractions containing crude (*R*)-**4** were evaporated to dryness *in vacuo* and the residue was purified on silica gel column using eluent (L) that resulted in pure (*R*)-4 as a base. After treatment with HCl in EtOH and recrystallization from MeOH-EtOH mixture (*R*)-**4** (0.21 g, 72%) was obtained as colorless crystals: R_f 0.39 (C); m.p. 200-1°C dec. (MeOH-EtOH); $[\alpha]_D^{20}$ +4.0° (*c* 2, H_2O). ^{1}H NMR (D_2O) δ 3.98-3.89 (1H, m, MeC<u>H</u>), 3.14 (6H, m, C<u>H</u>$_2$N), 2.00 (3H, s, $COCH_3$) 1.97-1.87 (1H, m, CHC<u>H</u>$_2$ [*see footnote on page 13]), 1.83-1.72 (5H, m, 5H, CC<u>H</u>$_2$C), 1.19 (3H, d, *J*=6.6, CH_3); ^{13}C NMR δ 174.00, 47.04, 44.91, 43.05, 38.96, 32.56, 24.02, 22.85, 22.05, 19.58. Anal.($C_{10}H_{25}N_3Cl_2O$, C, H, N). ee 95%.

(*S*)-N^8-(Acetyl)-1,8-diamino-5-azanonane ((*S*)-4). Prepared as (*R*)-**4** starting from (*S*)-**20** to give (*S*)-**4** (70 %): m.p. 200-1°C dec. (MeOH-EtOH); $[\alpha]_D^{20}$ -4.0° (*c* 2, H_2O). ^{1}H NMR shifts identical to (*R*)-4. Anal.($C_{10}H_{25}N_3Cl_2O$, C, H, N). ee 95%.

Transgenic animal studies: The production of the transgenic mice and rats has been described earlier in detail[17]. Both animal lines were produced by the standard pronuclear injection technique using the same transgene construct. The methylated polyamine analogues were administered in saline. Three to four months old male mice were injected once with 0, 50, 100, 150, 250, 375 or 500 mg/kg (**1**) or with 0, 12.5, 25, 37.5 or 50 mg/kg (**2** and **3**) intraperitoneally 24 h before sacrifice. Ten weeks old transgenic male rats were treated twice with 25 mg/kg (**1**–**3**) intraperitoneally 22 and 16 hours before sacrifice. Tissue samples were frozen in liquid nitrogen and homogenized in the standard buffer (25 mM Tris–HCl pH 7.4, 0.1 mM EDTA, 1 mM DTT). An aliquot of the homogenates was used for the polyamine assays. The homogenates were centrifuged (at 13 000×g, for 30 min, at +4 °C) and the supernatant fractions were used for the enzyme activity assays. The Institutional Animal Care and Use Committee of the University of Kuopio and the Provincial Government approved the animal experiments.

Immortalized rat fibroblast studies: The production of the immortalized fibroblasts is explained earlier in detail[17]. The cells were plated in triplicates in six–well culture plates in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with heat–inactivated 10% fetal bovine serum and gentamycin (50 μg/mL; Gibco). The cells were let to adhere for 24 h before the growth medium was replaced with fresh medium and the drugs. After the incubation, the cells were washed with PBS, detached with trypsin, counted and the polyamines were determined after sulphosalisylic acid precipitation from the supernatant fractions with the aid of the HPLC.

Recombinant protein studies: The human recombinant PAO was produced according to Qiagen Qiaexpressionist™ manual and the protein was purified under native conditions using Ni–NTA His Bind Resin (Novagen) as described in[17] except without affinity purification. PAO was estimated to

be 80% pure according to SDS–PAGE. However, the contaminating bacterial protein did not have any PAO like activity (data not shown). The kinetic studies of PAO were performed in duplicates with 4 to 5 different 10 to 200 μM substrate concentrations. The total volumes of reactions were 180 μl containing 100 mM glycine–NaOH pH 9.5, 5 mM DTT and appropriate amount of PAO. The reactions were allowed to proceed for 10 to 60 min at +37°C before addition of 20 μl of 100 μM diaminohexane as internal standard in 50 % w/v sulphosalisylic acid prior to HPLC analysis.

Assays related to biological studies: The SSAT activity was assayed as described earlier[24]. HPLC was used to determine the concentrations of the polyamines and their α–methylated analogues essentially as described by Hyvönen *et al.*[25] α–Amylase and alanine amino transferase (ALAT) were determined from heparinized plasma of the mice using an analyzer system Microlab 200 from Merck.

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FOOTNOTE

* Due to chiral center on the next carbon these CH_2-protons have different chemical shifts

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Figure legends

Figure 1. The structures of the α–methylated polyamine analogues used in this study. **1**, α–MeSPD, α–methylspermidine; **2**, α–MeSPM, α–methylspermine; **3**, α,α'–Me_2SPM, bis–α,α'–methylspermine; **4**, Ac–α–MeSPD, N^8–acetyl–α–methylspermidine.

Figure 2 The distribution of polyamines and their α–methylated analogues in the liver of MT–SSAT transgenic mice and their syngenic littermates after 24 h treatment with the indicated dosages. N = 3–5; **1**, α–methylspermidine; **2**, α–methylspermine; **3**, bis–α,α'–methylspermine;■, spermidine;□, combined amounts of putrescine, N^1–acetylspermidine, and spermine; ▨, α–methylated polyamine analogues.

Figure 3 The distribution of polyamines and their α–methylated analogues in the immortalized rat fibroblasts during 48 h culture. Cells were plated in triplicates in six–well culture plates in Dulbecco's modified Eagle's medium supplemented with heat–inactivated 10% fetal bovine serum and gentamycin 50 μg/mL 24 h before the growth medium was replaced with fresh medium and the drugs. Co, untreated cells; DENSpm, 10 μM diethylnorspermine; **1**, 1 mM α–methylspermidine; **2**, 10 μM α–methylspermine; **3**, 1 mM bis–α,α'–methylspermine;■, spermidine;□, combined amounts of putrescine and spermine; ▨, α–methylated polyamine analogues. No N^1–acetylspermidine was detected in either fibroblast cell lines.

Figure 4 Lineweaver–Burk plot to calculate kinetic values of racemic and different isomers of N^8–acetyl–α–methylspermidine. The kinetic studies of human recombinant polyamine oxidase were performed in duplicates with 4 to 5 different 10 to 200 μM substrate concentrations. Reactions were carried out in total volumes of 180 μl containing 100 mM glycine–NaOH pH 9.5 and 5 mM DTT and were allowed to proceed for 10 to 60 min at +37°C before HPLC analysis. No degradation of the substrates was detected in the absence of the enzyme. (*S*)-**4**, (*S*)-N^8-acetyl–α–methylspermidine; **4**, racemic N^8-acetyl–α–methylspermidine; (*R*)-**4**, (*R*)-N^8-acetyl–α–methylspermidine; AcSPD, N^1–acetylspermidine.

α-Methyl Polyamines: Efficient Synthesis and Tolerance Studies *in vivo* and *in vitro*. The First Evidence for Dormant Stereospecificity of Polyamine Oxidase

Aki Järvinen, Marc Cerrada-Gimenez, Nikolay Grigorenko, Alex R. Khomutov, Jouko Vepsäläinen, Riitta Sinervirta, Tuomo A. Keinänen, Leena Alhonen and Juhani Jänne

SUPPORTING INFORMATION

Material and Methods

Chemicals

The polyamine analogs, namely α–methylspermidine, α–methylspermine and bis–α,α'–methylspermine, were administered in saline.

Animals and immortalized fibroblasts

The drugs were injected intraperitoneally to syngenic and the mouse methallothionein promoter I driven mouse spermidine/spermine N^1–acetyltransferase (MT–SSAT) transgenic animals. The cells were plated in dublicates in culturing bottles in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with heat–inactivated 10% fetal bovine serum and gentamycin (50 μg/mL; Gibco). The cells were seeded 24 h before the growth medium was replaced with fresh medium and the drugs. After the incubation, the cells were washed with PBS, detached with trypsin and counted.

Analytical Methods

Polyamines and their derivatives were determined with the aid of high–performance liquid chromatography as described by Hyvönen *et al.*(1). SSAT activity was assayed according to Bernacki *et al.* (2). α–amylase and alanine amino transferase (ALAT) were determined from heparinized plasma using an analyzer system Microlab 200 from Merck.

Statistical Analysis

The data are expressed as means ± S.D. For statistical analysis, the two-tailed Student's *t* test was used.

References

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α–Methylspermidine studies with mice

Table S1. Plasma α–amylase and ALAT in syngenic and transgenic mice after α–methylspermidine treatment.
The mice were injected once with α–methylspermidine, as presented in the table, 24 h before sacrifice. There were four to five animals per group.

Group	α–amylase (U/l)	ALAT (U/l)
Sg 50 mg/kg	267 ± 23	18 ± 3
Sg 100 mg/kg	282 ± 24	19 ± 6
Sg 150 mg/kg	285 ± 27	18 ± 2
Sg 250 mg/kg	287 ± 75	66 ± 42
Sg 375 mg/kg	654 ± 400	111 ± 89
Sg 500 mg/kg	370 ± 100	33 ± 10
Tg 50 mg/kg	342 ± 45	35 ± 4
Tg 100 mg/kg	303 ± 30	45 ± 7
Tg 150 mg/kg	276 ± 34	42 ± 10
Tg 250 mg/kg	389 ± 210	34 ± 15
Tg 375 mg/kg	315 ± 21	52 ± 10

Table S2. Spermidine/spermine N^1–acetyltransferase activities in the liver, pancreas and kidney of the syngenic and MT–SSAT transgenic mice after α–methylspermidine treatment.
The mice were injected once with α–methylspermidine, as presented in the table, 24 h before sacrifice. There were four to five animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic.

	pmoles / mg tissue / 10 min		
Group	Liver	Pancreas	Kidney
Sg Control	0.7 ± 0.2	1.3 ± 0.7	0.7 ± 0.3
Sg 50 mg/kg	3.7 ± 0.5 ***	6.6 ± 1 ***	5.0 ± 0.4 ***
Sg 100 mg/kg	3.8 ± 0.2 ***	7.5 ± 2 ***	4.6 ± 0.5 ***
Sg 150 mg/kg	4.2 ± 0.7 ***	6.9 ± 2 ***	5.1 ± 0.8 ***
Sg 250 mg/kg	3.8 ± 0.3 ***	12 ± 4 ***	6.5 ± 0.7 ***
Sg 375 mg/kg	10 ± 9 ***	18 ± 6 ***	9.4 ± 5 ***
Sg 500 mg/kg	16 ± 13 ***	27 ± 11 ***	15 ± 5 ***
Tg Control	13 ± 6	72 ± 30	1.8 ± 0.5
Tg 50 mg/kg	51 ± 6 ***	179 ± 21 ***	3.2 ± 0.4 ***
Tg 100 mg/kg	75 ± 22 ***	226 ± 41 ***	3.1 ± 0.4 ***
Tg 150 mg/kg	82 ± 29 ***	183 ± 79 ***	3.4 ± 2 ***
Tg 250 mg/kg	95 ± 28 ***	188 ± 62 ***	2.9 ± 0.4 ***
Tg 375 mg/kg	136 ± 47 ***	134 ± 38 ***	3.4 ± 0.5 ***

Table S3. The hepatic polyamine concentrations in the syngenic and MT–SSAT transgenic mice after α–methylspermidine treatment.

The mice were injected once with α–methylspermidine, as presented in the table, 24 h before sacrifice. There were four to five animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic; NA, not applicable; ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; αMeSPD, α–methylspermidine; SPM, spermine.

	POLYAMINES pmoles / mg tissue				
Group	PUT	N^1–AcSPD	SPD	αMeSPD	SPM
Sg Control	ND	ND	1130 ± 36		863 ± 58
Sg 50 mg/kg	ND	21 ± 11	1020 ± 260	470 ± 74	864 ± 91
Sg 100 mg/kg	15 ± NA	13 ± 4	730 ± 100 *	549 ± 42	893 ± 79
Sg 150 mg/kg	ND	25 ± 9	723 ± 120	599 ± 130	740 ± 170
Sg 250 mg/kg	22 ± 2	19 ± 10	593 ± 30 ***	902 ± 52	855 ± 110
Sg 375 mg/kg	29 ± 1	32 ± 35	370 ± 170 *	1290 ± 260	645 ± 180
Sg 500 mg/kg	119 ± 160	133 ± 180	273 ± 110 *	2110 ± 870	666 ± 190
Tg Control	645 ± 130	159 ± 52	936 ± 110		108 ± 27
Tg 50 mg/kg	1060 ± 180	245 ± 85	330 ± 110 ***	691 ± 230	103 ± 16
Tg 100 mg/kg	835 ± 170	*294 ± 19*	286 ± 220 ***	800 ± 120	98 ± 52
Tg 150 mg/kg	603 ± 140	215 ± 34	260 ± 160 ***	1420 ± 240	79 ± 25
Tg 250 mg/kg	445 ± 160	212 ± 73	200 ± 100 ***	1520 ± 100	36 ± 10 *
Tg 375 mg/kg	861 ± 510	252 ± 110	168 ± 11 ***	1190 ± 340	42 ± 8

Table S4. The pancreatic polyamine concentrations in the syngenic and MT–SSAT transgenic mice after α–methylspermidine treatment.

The mice were injected once with α–methylspermidine, as presented in the table, 24 h before sacrifice. There were four to five animals per group. * correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic; NA, not applicable; ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; αMeSPD, α–methylspermidine; SPM, spermine.

	POLYAMINES pmoles / mg tissue				
Group	PUT	N^1–AcSPD	SPD	αMeSPD	SPM
Sg Control	28 ± 8	21 ± NA	3580 ± 260		648 ± 81
Sg 50 mg/kg	3 ± NA	ND	6600 ± 1000 *	ND	911 ± 140
Sg 100 mg/kg	ND	66 ± NA	6090 ± 1000 *	ND	815 ± 180
Sg 150 mg/kg	2 ± NA	ND	6350 ± 1000 *	ND	834 ± 53
Sg 250 mg/kg	159 ± 210	ND	4770 ± 840	1900 ± 240	763 ± 110
Sg 375 mg/kg	727 ± 460 *	14 ± NA	4070 ± 670	1840 ± 320	741 ± 110
Sg 500 mg/kg	813 ± 190 *	11 ± 0.3	3670 ± 1240	1730 ± 730	652 ± 160
Tg Control	4730 ± 1100	89 ± 27	2000 ± 760		221 ± 70
Tg 50 mg/kg	4970 ± 380	204 ± 65	1320 ± 250	699 ± 65	224 ± 47
Tg 100 mg/kg	4470 ± 410	657 ± 310 *	1540 ± 350	813 ± 140	212 ± 21
Tg 150 mg/kg	4460 ± 370	167 ± 100	1240 ± 570	1050 ± 260	214 ± 77
Tg 250 mg/kg	4640 ± 210	137 ± 95	968 ± 400	1290 ± 190	157 ± 80
Tg 375 mg/kg	4830 ± 190	95 ± 21	1000 ± 370	1520 ± 170	122 ± 28

Table S5. The renal polyamine concentrations in the syngenic and MT–SSAT transgenic mice after α–methylspermidine treatment.

The mice were injected once with α–methylspermidine, as presented in the table, 24 h before sacrifice. There were four to five animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic; ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; αMeSPD, α–methylspermidine; SPM, spermine.

	POLYAMINES pmoles / mg tissue				
Group	PUT	N^1–AcSPD	SPD	αMeSPD	SPM
Sg Control	75 ± 16	31 ± NA	231 ± 13		606 ± 59
Sg 50 mg/kg	46 ± 11	37 ± 4	315 ± 46	153 ± 17	856 ± 68 ***
Sg 100 mg/kg	69 ± 12	39 ± 8	232 ± 25	164 ± 9	686 ± 76
Sg 150 mg/kg	65 ± 13	25 ± 9	241 ± 29	153 ± 22	635 ± 42
Sg 250 mg/kg	42 ± 8	31 ± 12	226 ± 29	312 ± 63	519 ± 38
Sg 375 mg/kg	59 ± 53	27 ± 10	189 ± 3	559 ± 290	454 ± 57
Sg 500 mg/kg	790 ± 1500	35 ± 30	181 ± 29	1380 ± 800	458 ± 110
Tg Control	160 ± 21	23 ± 3	191 ± 16		600 ± 57
Tg 50 mg/kg	179 ± 84	49 ± 15	231 ± 16 ***	94 ± 20	897 ± 26
Tg 100 mg/kg	134 ± 47	65 ± 27	212 ± 11 ***	100 ± 25	841 ± 67
Tg 150 mg/kg	119 ± 20	43 ± 9	201 ± 19 ***	108 ± 52	721 ± 65
Tg 250 mg/kg	104 ± 25	48 ± 5	186 ± 19 ***	127 ± 14	597 ± 91
Tg 375 mg/kg	127 ± 31	45 ± 10	174 ± 14 ***	167 ± 19	580 ± 52

Bis–α–methylspermine studies with mice

Table S6. Plasma α–amylase and ALAT in syngenic and transgenic mice after bis–α,α'–methylspermine treatment.

The mice were injected once with bis–α–methylspermine, as presented in the table, 24 h before sacrifice. There were three to five animals per group. * corresponds to $p < 0.05$ as compared to the correspondent control. Sg, syngenic; Tg, transgenic.

Group	α–amylase (U/l)	ALAT (U/l)
Sg Control	3470 ± 250	52 ± 9
Sg 12.5 mg/kg	3170 ± 86	49 ± 9
Sg 25 mg/kg	2930 ± 240	58 ± 16
Sg 37.5 mg/kg	2830 ± 120	36 ± 3
Sg 50 mg/kg	2720 ± 530	62 ± 25
Tg Control	2640 ± 250	57 ± 9
Tg 12.5 mg/kg	2820 ± 430	51 ± 10
Tg 25 mg/kg	3240 ± 210	45 ± 21
Tg 37.5 mg/kg	2540 ± 420 *	27 ± 9
Tg 50 mg/kg	2890 ± 520	60 ± 29

Table S7. Spermidine/spermine N^1–acetyltransferase activities in the liver, pancreas and kidney of the syngenic and MT–SSAT transgenic mice after bis–α–methylspermine treatment.

The mice were injected once with bis–α–methylspermine, as presented in the table, 24 h before sacrifice. There were three to five animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic; ND, not detected.

	pmoles / mg tissue / 10 min		
Group	Liver	Group	Liver
Sg Control	0.7 ± 0.2	1.3 ± 0.7	0.7 ± 0.3
Sg 12.5 mg/kg	ND	3.4 ± 1 ***	0.3 ± 0.1 *
Sg 25 mg/kg	ND	4.2 ± 1 ***	0.6 ± 0.4
Sg 37.5 mg/kg	ND	3.2 ± 1 ***	0.3 ± 0.1 *
Sg 50 mg/kg	ND	8.4 ± 5 ***	0.6 ± 0.2
Tg Control	13 ± 6	72 ± 30	1.8 ± 0.5
Tg 12.5 mg/kg	7 ± 3	34 ± 11 *	0.7 ± 0.2 ***
Tg 25 mg/kg	9 ± 2	65 ± 13	1.5 ± 0.9
Tg 37.5 mg/kg	56 ± 55 ***	125 ± 59 *	8 ± 14 *
Tg 50 mg/kg	13 ± 3	62 ± 25	1.2 ± 0.4

Table S8. The hepatic polyamine concentrations in the syngenic and MT–SSAT transgenic mice after bis–α–methylspermine treatment.

The mice were injected once with bis–α–methylspermine, as presented in the table, 24 h before sacrifice. There were three to five animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic; NA, not applicable; ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; SPM, spermine; α,α'–Me_2SPM, bis–α–methylspermine.

	POLYAMINES pmoles / mg tissue				
Group	PUT	N^1–AcSPD	SPD	SPM	α,α'–Me_2SPM
Sg Control	ND	ND	1130 ± 36	863 ± 58	
Sg 12.5 mg/kg	92 ± 26	ND	927 ± 61	708 ± 46	359 ± 49
Sg 25 mg/kg	74 ± 41	ND	879 ± 130 *	700 ± 56	646 ± 96
Sg 37.5 mg/kg	96 ± 32	15 ± NA	773 ± 93 *	755 ± 150	603 ± 77
Sg 50 mg/kg	52 ± NA	ND	688 ± 75 ***	688 ± 47	771 ± 78
Tg Control	645 ± 130	159 ± 52	936 ± 110	108 ± 27	
Tg 12.5 mg/kg	944 ± 220	227 ± 47	793 ± 310	104 ± 18	125 ± 81
Tg 25 mg/kg	895 ± 190	220 ± 20	673 ± 280	67 ± 16	246 ± 28
Tg 37.5 mg/kg	1030 ± 450	355 ± 63	404 ± 290 *	74 ± 18	515 ± 160
Tg 50 mg/kg	576 ± 360	196 ± 98	266 ± 97 *	208 ± 220	603 ± 200

Table S9. The pancreatic polyamine concentrations in the syngenic and MT–SSAT transgenic mice after bis–α–methylspermine treatment.

The mice were injected once with bis–α,α'–methylspermine, as presented in the table, 24 h before sacrifice. There were three to five animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic; NA, not applicable; ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; SPM, spermine; α,α'–Me_2SPM, bis–α–methylspermine.

	POLYAMINES pmoles / mg tissue				
Group	PUT	N^1–AcSPD	SPD	SPM	α,α'–Me_2SPM
Sg Control	28 ± 8	21 ± NA	3580 ± 260	648 ± 81	
Sg 12.5 mg/kg	299 ± 75	ND	6580 ± 610 ***	981 ± 200	ND
Sg 25 mg/kg	467 ± 420	ND	5730 ± 300 ***	529 ± 240	478 ± 15
Sg 37.5 mg/kg	233 ± 46	124 ± NA	6000 ± 24 ***	709 ± 180	515 ± 64
Sg 50 mg/kg	297 ± 120	ND	5610 ± 610 ***	562 ± 78	457 ± 34
Tg Control	4730 ± 1100	89 ± 27	2000 ± 760	221 ± 70	
Tg 12.5 mg/kg	6170 ± 1300	267 ± 20	3650 ± 370	187 ± 65	ND
Tg 25 mg/kg	7570 ± 1100	474 ± 140 *	3390 ± 450	73 ± 57	247 ± 29
Tg 37.5 mg/kg	8160 ± 2900 *	340 ± 170	2270 ± 1800	99 ± 83	327 ± 160
Tg 50 mg/kg	6140 ± 1100	323 ± 130	1640 ± 680	119 ± 80	327 ± 150

Table S10. The renal polyamine concentrations in the syngenic and MT–SSAT transgenic mice after bis–α–methylspermine treatment.

The mice were injected once with bis–α,α'–methylspermine, as presented in the table, 24 h before sacrifice. There were three to five animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic; NA, not applicable; ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; SPM, spermine; α,α'–Me_2SPM, bis–α–methylspermine.

	POLYAMINES pmoles / mg tissue				
Group	PUT	N^1–AcSPD	SPD	SPM	α,α^1–Me_2SPM
Sg Control	75 ± 16	31 ± NA	231 ± 13	606 ± 59	
Sg 12.5 mg/kg	142 ± 100	33 ± NA	246 ± 56	457 ± 8 *	560 ± 27
Sg 25 mg/kg	88 ± 9	28 ± 3	257 ± 42	460 ± 49 *	712 ± 71
Sg 37.5 mg/kg	94 ± 22	29 ± 8	290 ± 26	514 ± 21	847 ± 170
Sg 50 mg/kg	98 ± 43	ND	249 ± 72	424 ± 74 *	927 ± 38
Tg Control	160 ± 21	23 ± 3	191 ± 16	600 ± 57	
Tg 12.5 mg/kg	184 ± 71	20 ± NA	236 ± 2	739 ± 44	ND
Tg 25 mg/kg	137 ± 20	26 ± NA	217 ± 31	565 ± 44	659 ± 150
Tg 37.5 mg/kg	226 ± 94	27 ± 10	186 ± 8	427 ± 50 *	854 ± 280
Tg 50 mg/kg	393 ± 410	ND	195 ± 37	325 ± 220	547 ± 180

α–Methylspermine studies with mice

Table S11. Plasma α–amylase and ALAT in syngenic and transgenic mice after α–methylspermine treatment.

The mice were injected once with α–methylspermine, as presented in the table, 24 h before sacrifice. There were three animals per group. * corresponds to $p < 0.05$ as compared to the correspondent control. Sg, syngenic; Tg, transgenic.

Group	α–amylase (U/l)	ALAT (U/l)
Sg Control	3470 ± 250	52 ± 9
Sg 12.5 mg/kg	2350 ± 210 ***	54 ± 4
Sg 25 mg/kg	2650 ± 160 *	44 ± 4
Sg 37.5 mg/kg	2610 ± 140 *	48 ± 13
Sg 50 mg/kg	2510 ± 280 *	52 ± 6
Tg Control	2640 ± 250	57 ± 9
Tg 12.5 mg/kg	2680 ± 310	59 ± 8
Tg 25 mg/kg	2040 ± 110	45 ± 6
Tg 37.5 mg/kg	2410 ± 300	42 ± 8
Tg 50 mg/kg	2670 ± 300	41 ± 4

Table S12. Spermidine/spermine N^1–acetyltransferase activities in the liver, pancreas and kidney of the syngenic and MT–SSAT transgenic mice after α–methylspermine treatment.

The mice were injected once with α–methylspermine, as presented in the table, 24 h before sacrifice. There were three animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic.

	pmoles / mg tissue / 10 min		
Group	Liver	Group	Liver
Sg Control	0.7 ± 0.2	1.3 ± 0.7	0.7 ± 0.3
Sg 12.5 mg/kg	0.5 ± 0.2	2.0 ± 1	3.7 ± 2 ***
Sg 25 mg/kg	0.3 ± 0.2 *	1.7 ± 0.5	2.8 ± 3 ***
Sg 37.5 mg/kg	0.8 ± 0.7	1.5 ± 0.9	4.3 ± 2 ***
Sg 50 mg/kg	0.5 ± 0.3	2.2 ± 1	3.2 ± 2
Tg Control	13 ± 6	72 ± 30	1.8 ± 0.5
Tg 12.5 mg/kg	21 ± 11 *	609 ± 400 ***	5.9 ± 2 ***
Tg 25 mg/kg	31 ± 18 *	561 ± 240 ***	3.1 ± 2
Tg 37.5 mg/kg	25 ± 2 ***	544 ± 180 ***	3.5 ± 3
Tg 50 mg/kg	39 ± 11 ***	707 ± 120 ***	3.0 ± 2

Table S13. The hepatic polyamine concentrations in the syngenic and MT–SSAT transgenic mice after α–methylspermine treatment.

The mice were injected once with α–methylspermine, as presented in the table, 24 h before sacrifice. There were three animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. Sg, syngenic; Tg, transgenic; NA, not applicable; ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; αMeSPD, α–methylspermidine; SPM, spermine; αMeSPM, α–methylspermine.

	POLYAMINES pmoles / mg tissue					
Group	PUT	N^1–AcSPD	SPD	αMeSPD	SPM	αMeSPM
Sg Control	ND	ND	1130 ± 36		863 ± 58	
Sg 12.5 mg/kg	40 ± NA	37 ± 1	670 ± 540	ND	568 ± 57 ***	766 ± 100
Sg 25 mg/kg	44 ± 2	ND	1020 ± 200	ND	411 ± 66 ***	692 ± 95
Sg 37.5 mg/kg	ND	ND	831 ± 53 *	ND	486 ± 24 ***	831 ± 53
Sg 50 mg/kg	23 ± NA	46 ± NA	780 ± 86	ND	420 ± 60 ***	849 ± 130
Tg Control	645 ± 130	159 ± 52	936 ± 110		108 ± 27	
Tg 12.5 mg/kg	857 ± 150	243 ± 82	717 ± 420	ND	63 ± 14	259 ± 37
Tg 25 mg/kg	872 ± 150	318 ± 140	350 ± 150	366 ± 88	50 ± 9 *	271 ± 21
Tg 37.5 mg/kg	774 ± 170	180 ± 17	306 ± 88 *	506 ± 34	47 ± 11 *	357 ± 42
Tg 50 mg/kg	1060 ± 130 *	266 ± 43	197 ± 53 *	471 ± 110	38 ± 17 *	308 ± 17

Table S14. The pancreatic polyamine concentrations in the syngenic and MT–SSAT transgenic mice after α–methylspermine treatment.

The mice were injected once with α–methylspermine, as presented in the table, 24 h before sacrifice. There were three animals per group. * corresponds to $p < 0.05$ as compared to the correspondent control. α–Methylspermidine was not detected in the pancreas of α–methylspermine treated mice. Sg, syngenic; Tg, transgenic; NA, not applicable; ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; SPM, spermine; αMeSPM, α–methylspermine.

	POLYAMINES pmoles / mg tissue				
Group	PUT	N^1–AcSPD	SPD	SPM	αMeSPM
Sg Control	28 ± 8	21 ± NA	3580 ± 260	648 ± 81	
Sg 12.5 mg/kg	81 ± 66	56 ± NA	7950 ± 3000	1050 ± 400	ND
Sg 25 mg/kg	51 ± 21	20 ± NA	5420 ± 730	729 ± 100	ND
Sg 37.5 mg/kg	36 ± 13	ND	5810 ± 1100	805 ± 170	ND
Sg 50 mg/kg	108 ± 33	ND	6840 ± 1600	811 ± 360	ND
Tg Control	4730 ± 1100	89 ± 27	2000 ± 760	221 ± 70	
Tg 12.5 mg/kg	5690 ± 940	155 ± 150	2300 ± 620	42 ± 25	249 ± 37
Tg 25 mg/kg	5800 ± 1400	328 ± 310	2450 ± 380	38 ± 8	241 ± 51
Tg 37.5 mg/kg	7430 ± 850	176 ± 68	2110 ± 560	52 ± 45	322 ± 51
Tg 50 mg/kg	9030 ± 4200	285 ± 37	3570 ± 1300	71 ± 20	334 ± 41

Table S15. The renal polyamine concentrations in the syngenic and MT–SSAT transgenic mice after α–methylspermine treatment.

The mice were injected once with α–methylspermine, as presented in the table, 24 h before sacrifice. There were three animals per group. * and ***, correspond to $p < 0.05$ and $p < 0.001$ respectively, as compared to the correspondent control. α–Methylspermidine was not detected in the kidney of α–methylspermine treated mice. Sg, syngenic; Tg, transgenic; NA, not applicable; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; SPM, spermine; αMeSPM, α–methylspermine.

	POLYAMINES pmoles / mg tissue				
Group	PUT	N^1–AcSPD	SPD	SPM	αMeSPM
Sg Control	75 ± 16	31 ± NA	231 ± 13	606 ± 59	
Sg 12.5 mg/kg	48 ± 2	46 ± 18	313 ± 55	247 ± 18 ***	349 ± 17
Sg 25 mg/kg	123 ± 76	59 ± 8	215 ± 25	272 ± 41	426 ± 16
Sg 37.5 mg/kg	59 ± 33	31 ± 2	245 ± 58	208 ± 22 ***	468 ± 96
Sg 50 mg/kg	160 ± 31	48 ± 8	193 ± 33	258 ± 16 ***	512 ± 120
Tg Control	160 ± 21	23 ± 3	191 ± 16	600 ± 57	
Tg 12.5 mg/kg	58 ± 24	39 ± 6	248 ± 57	208 ± 36 ***	351 ± 25
Tg 25 mg/kg	68 ± 12	43 ± NA	157 ± 16	210 ± 20 ***	424 ± 29
Tg 37.5 mg/kg	96 ± 73	32 ± 13	234 ± 49	159 ± 42 ***	414 ± 67
Tg 50 mg/kg	92 ± 11	44 ± 7	156 ± 28	205 ± 11 ***	649 ± 48

The MT-SSAT transgenic rat studies

Table S16. Spermidine/spermine N^1–acetyltransferase activities in the liver, pancreas and kidney of the MT–SSAT transgenic rat after α–methylated polyamine treatments.

The rats were injected twice with analog (25 mg/kg), as presented in the table, 22 and 16 h before sacrifice. There were three to four animals per group. * correspond to $p < 0.05$ as compared to the control. NA, not applicable; αMeSPD, α–methylspermidine; αMeSPM, α–methylspermine; α,α'–Me_2SPM, bis–α–methylspermine.

	pmoles / mg tissue / 10 min		
Group	Liver	Pancreas	Kidney
no treatment	4.5 ± 0.4	44 ± 34	2.2 ± 0.8
2×αMeSPD	11.0 ± 2.3	NA	NA
2×αMeSPM	7.5 ± 1.7	61 ± 20	1.5 ± 0.5
2×α,α'–Me_2SPM	14.2 ± 6.7 *	48 ± 12	1.2 ± 0.3

Table S17. The polyamine concentrations in the liver, pancreas and kidney of the MT–SSAT transgenic rat after α–methylated polyamine analog treatments.

The rats were injected twice with analog (25 mg/kg), as presented in the table, 22 and 16 h before sacrifice. There were three to four animals per group. *, ** and ***, correspond to $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively, as compared to the control. ND, not detected; PUT, putrescine; N^1–AcSPD, N^1–acetylspermidine; SPD, spermidine; αMeSPD, α–methylspermidine; SPM, spermine; αMeSPM, α–methylspermine; α,α'–Me_2SPM, bis–α–methylspermine.

	POLYAMINES pmoles / mg tissue					αMeSPM/
Liver	PUT	N^1–AcSPD	SPD	αMeSPD	SPM	α,α'–Me_2SPM
no treatment	1210 ± 230	53 ± 9	1040 ± 93		183 ± 33	
2×αMeSPD	1070 ± 430	104 ± 24 *	378 ± 87 ***	1410 ± 308	87 ± 27 ***	
2×αMeSPM	1100 ± 130	76 ± 26	567 ± 180 **	701 ± 100	31 ± 13 ***	429 ± 76
2×α,α'–Me_2SPM	1190 ± 270	57 ± 14	430 ± 250 **	69 ± 1	64 ± 41 ***	694 ± 270
Pancreas						
no treatment	3300 ± 1600	21 ± 38	3400 ± 420		482 ± 94	
2×αMeSPM	3660 ± 1400	43 ± 18	3600 ± 470	ND	215 ± 40 **	943 ± 180
2×α,α'–Me_2SPM	3240 ± 760	49 ±15	3250 ± 760	ND	388 ±79	555 ± 220
Kidney						
no treatment	23 ± 11	27 ± 5	255 ± 19		684 ± 100	
2×αMeSPM	41 ± 10	49 ± 5 ***	123 ± 12 ***	91 ± 2	222 ± 45 ***	982 ± 93
2×α,α'–Me_2SPM	56 ± 25	46 ± 4 **	173 ± 18 ***	ND	441 ± 65 **	1070 ± 290

The immortalized rat fibroblast studies

Tables S18A–B. The spermidine/spermine N^1–acetyltransferase activities in the immortalized syngenic and MT–SSAT transgenic fibroblasts after α–methylated polyamine analog treatments. The cells were cultured in triplicates and seeded 24 h before the growth medium was replaced with fresh medium and drugs. Time indicates the time after medium replacement. Sg, syngenic; Tg, transgenic; αMeSPD, α–methylspermidine; αMeSPM, α–methylspermine; α,α'–Me_2SPM, bis–α–methylspermine; DENSPM, N,N'–diethylnorspermine.

A, Sg cell line	SSAT pmoles / 10 min / 10^6 cells			number of cells (10^3)		
	0 h	24 h	48 h	0 h	24 h	48 h
no treatment	5 ± 2	9 ± 4	12 ± 2	480 ± 10	1400 ± 60	3300 ± 300
+ 1 mM AG		9 ± 2	12 ± 1		1400 ± 50	3600 ± 200
10 μM DENSPM		90 ± 10	190 ± 10		1300 ± 80	2400 ± 100
+ 1 mM AG		80 ± 9	180 ±9		1300 ± 40	2200 ± 100
1 mM αMeSPD		4 ± 4	13 ± 3		1600 ± 60	3400 ± 100
+ 1 mM AG		7 ± 7	15 ± 3		1600 ± 90	3100 ± 200
10 μM αMeSPM		8 ± 5	15 ± 1		1500 ± 100	2700 ± 300
+ 1 mM AG		8 ± 8	16 ±2		1700 ± 50	2800 ± 200
1 mM α,α'–Me_2SPM		7 ± 1	17 ±7		1500 ± 50	3300 ± 100
+ 1 mM AG		5 ± 2	21 ± 1		1500 ± 60	3000 ± 200

B, Tg cell line	SSAT pmoles / 10 min / 10^6 cells			number of cells (10^3)		
	0 h	24 h	48 h	0 h	24 h	48 h
no treatment	25 ± 2	39 ± 10	32 ± 2	230 ± 5	770 ± 20	3700 ± 200
+ 1 mM AG		35 ± 10	30 ± 1		780 ± 60	3500 ± 100
10 μM DENSPM		3600 ± 300	7000 ± 200		640 ± 6	1700 ± 60
+ 1 mM AG		3300 ± 200	8700 ± 400		630 ± 50	1600 ± 30
1 mM αMeSPD		64 ± 10	110 ± 7		730 ± 20	3300 ± 100
+ 1 mM AG		60 ± 8	120 ± 4		760 ± 30	3300 ± 200
10 μM αMeSPM		32 ± 10	44 ± 2		590 ± 70	1900 ± 500
+ 1 mM AG		50 ± 10	81 ± 5		750 ± 40	3000 ± 100
1 mM α,α'–Me_2SPM		120 ± 10	470 ± 40		710 ± 10	2700 ± 100
+ 1 mM AG		130 ± 7	440 ± 50		710 ± 20	2800 ± 100

Table S19A. The polyamine concentrations in the immortalized syngenic and MT–SSAT transgenic fibroblasts after α–methylated polyamine analog treatments.
The cells were cultured in triplicates and seeded 24 h before the growth medium was replaced with fresh medium and drugs. Time indicates the time after medium replacement. No N^1–acetylspermidine was detected. ND, not detected; SG, syngenic; PUT, putrescine; SPD, spermidine; αMeSPD, α–methylspermidine; SPM, spermine; αMeSPM, α–methylspermine; α,α'–Me_2SPM, bis–α–methylspermine; DENSPM, N,N'–diethylnorspermine.

A, SG cell line		POLYAMINES pmoles / 10^6 cells				
		PUT	SPD	αMeSPD	SPM	αMeSPM/ α,α'–Me_2SPM
0 h	no treatment	630 ± 50	2600 ± 30		970 ± 20	
24 h	no treatment	560 ± 70	3100 ± 200		1100 ± 60	
	+ 1 mM AG	440 ± 70	3000 ± 300		1100 ± 100	
	10 μM DENSPM	220 ± 20	640 ± 100		410 ± 50	
	+ 1 mM AG	120 ± 100	790 ± 5		410 ± 60	
	1 mM αMeSPD	61 ± 100	480 ± 40	4900 ± 200	790 ± 60	ND
	+ 1 mM AG	ND.	360 ± 20	4800 ± 300	820 ± 40	ND
	10 μM αMeSPM	210 ± 20	1000 ± 40	3600 ± 200	980 ± 90	ND
	+ 1 mM AG	220 ± 4	780 ± 70	160 ± 70	320 ± 20	2400 ± 200
	1 mM α,α'–Me_2SPM	ND	360 ± 30	ND	380 ± 20	5100 ± 400
	+ 1 mM AG	ND	290 ± 50	ND	380 ± 30	4700 ± 100
48 h	no treatment	260 ± 50	2700 ± 200		1200 ± 40	
	+ 1 mM AG	66 ± 60	2000 ± 200		1100 ± 80	
	10 μM DENSPM	ND	320 ± 60		240 ± 10	
	+ 1 mM AG	ND	540 ± 30		340 ± 10	
	1 mM αMeSPD	ND	120 ± 20	5800 ± 80	790 ± 30	ND
	+ 1 mM AG	ND	50 ± 20	5800 ± 300	670 ± 80	ND
	10 μM αMeSPM	85 ± 10	680 ± 90	4100 ± 900	1200 ± 200	ND
	+ 1 mM AG	ND	410 ± 70	280 ± 60	240 ± 30	3000 ± 60
	1 mM α,α'–Me_2SPM	ND	48 ± 7	ND	180 ± 6	5800 ± 200
	+ 1 mM AG	ND	42 ± 10	ND	170 ± 7	5700 ± 400

Table S19B. The polyamine concentrations in the immortalized syngenic and MT–SSAT transgenic fibroblasts after α–methylated polyamine analog treatments.

The cells were cultured in triplicates and seeded 24 h before the growth medium was replaced with fresh medium and drugs. Time indicates the time after medium replacement. No N^1–acetylspermidine was detected. ND, not detected; TG, transgenic; PUT, putrescine; SPD, spermidine; αMeSPD, α–methylspermidine; SPM, spermine; αMeSPM, α–methylspermine; α,α'–Me_2SPM, bis–α–methylspermine; DENSPM, N,N'–diethylnorspermine.

		POLYAMINES pmoles / 10^6 cells				
B, TG cell line		PUT	SPD	αMeSPD	SPM	αMeSPM/ α,α'–Me_2SPM
0 h	no treatment	220 ± 30	1700 ± 200		520 ± 90	
24 h	no treatment	350 ± 20	2400 ± 200		750 ± 90	
	+ 1 mM AG	250 ± 20	2500 ± 50		820 ± 50	
	10 μM DENSPM	83 ± 20	160 ± 30		96 ± 10	
	+ 1 mM AG	100 ± 20	200 ± 10		120 ± 4	
	1 mM αMeSPD	24 ± 2	360 ± 50	4200 ± 500	660 ± 100	ND
	+ 1 mM AG	ND	210 ± 10	3800 ± 100	550 ± 30	ND
	10 μM αMeSPM	50 ± 30	550 ± 200	2500 ± 500	580 ± 100	ND
	+ 1 mM AG	120 ± 20	510 ± 80	89 ± 7	140 ± 30	2000 ± 400
	1 mM α,α'–Me_2SPM	8 ± 10	190 ± 20	ND	140 ± 10	2900 ± 300
	+ 1 mM AG	ND	230 ± 10	ND	170 ± 3	3200 ± 100
48 h	no treatment	520 ± 20	2000 ± 90		700 ± 50	
	+ 1 mM AG	350 ± 5	2100 ± 30		790 ± 20	
	10 μM DENSPM	15 ± 6	49 ± 20		27 ± 1	
	+ 1 mM AG	22 ± 5	65 ± 10		41 ± 3	
	1 mM αMeSPD	33 ± 10	180 ± 10	3800 ± 200	470 ± 30	ND
	+ 1 mM AG	ND	86 ± 7	4100 ± 200	410 ± 20	ND
	10 μM αMeSPM	ND	500 ± 40	2700 ± 50	620 ± 20	ND
	+ 1 mM AG	ND	220 ± 8	380 ± 5	49 ± 7	2200 ± 100
	1 mM α,α'–Me_2SPM	ND	64 ± 3	58 ± 10	26 ± 4	3800 ± 200
	+ 1 mM AG	ND	46 ± 6	48 ± 10	21 ± 2	3600 ± 200

Table S20. Microanalysis data for target compounds.

		Calcd.			Found		
Compound	Formula	C	H	N	C	H	N
1	$C_8H_{24}N_3Cl_3$	35.76	9.00	15.64	35.88	9.10	15.63
2	$C_{11}H_{32}N_4Cl_4$*0.25 H_2O	36.03	8.93	15.28	36.03	9.00	15.04
3	$C_{12}H_{34}N_4Cl_4$	38.31	9.11	14.89	38.08	9.24	14.67
(R)-4	$C_{10}H_{25}N_3Cl_2O$	43.80	9.19	15.32	43.87	9.15	15.41
(S)-4	$C_{10}H_{25}N_3Cl_2O$	43.80	9.19	15.32	43.83	9.28	15.36

III

Guide–molecule Driven Stereospecific Degradation of α–Methylated Polyamines by Polyamine Oxidase

Järvinen A, Keinänen TA, Grigorenko N, Khomutov AR, Uimari A, Vepsäläinen J, Närvänen A, Alhonen L, and Jänne J.

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GUIDE MOLECULE–DRIVEN STEREOSPECIFIC DEGRADATION OF α–METHYLPOLYAMINES BY POLYAMINE OXIDASE

Aki Järvinen•*, Tuomo A. Keinänen•*, Nikolay A. Grigorenko‡, Alex R. Khomutov‡, Anne Uimari*, Jouko Vepsäläinen§, Ale Närvänen§, Leena Alhonen* and Juhani Jänne*¶

***A. I. Virtanen Institute for Molecular Sciences and the §Department of Chemistry, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland, the ‡Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov Street 32, Moscow 119991, Russia**

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¶Address correspondence to: Juhani Jänne, Department of Biotechnology and Molecular Medicine, A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, P.O. Box 1627 FI-70211 Kuopio, Finland. Street address (for courier): Neulaniementie 2, 70210 Kuopio, Finland, Tel. +358-17-163049; Fax: +358-17-163025; E-mail: Juhani.Janne@uku.fi

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•*These authors contributed equally to this work*

Flavin adenine dinucleotide (FAD)–dependent polyamine oxidase (PAO; EC 1.5.3.11) is one of the key enzymes in the catabolism of polyamines spermidine and spermine. The natural substrates for the enzyme are N^1–acetylspermidine (AcSpd), N^1–acetylspermine and N^1,N^{12}–diacetylspermine. Here we report that PAO, which normally metabolizes achiral substrates, oxidized (*R*)–isomer of 1–amino–8–acetamido–5–azanonane (AcMeSpd) and AcSpd as efficiently while (*S*)–AcMeSpd was much less preferred substrate. It has been shown that in the presence of certain aldehydes, the substrate specificity of PAO and the kinetics of the reaction are changed to favor spermine and spermidine as substrates. Therefore, we examined the effect of several aldehydes on the ability of PAO to oxidize different enantiomers of α–methylated polyamines. PAO supplemented with benzaldehyde predominantly catalyzed the cleavage of (*R*)–isomer of α–methylspermidine (MeSpd) while in the presence of pyridoxal the (*S*)–MeSpd was preferred. PAO displayed the same stereospecificity with both singly and doubly α–methylated spermine derivatives when supplemented with the same aldehydes. Structurally related ketones proved to be ineffective. This is the first time that the stereospecificity of FAD–dependent oxidase has been successfully regulated by changing the supplementary aldehyde. These findings might facilitate the chemical regulation of stereospecificity of the enzymes.

Polyamines, spermidine (Spd) and spermine (Spm), and their precursor putrescine, are present at millimolar concentrations in mammalian cells (1). They are essential for cell growth, participate in the regulation of cellular metabolism and physiology (2,3). Their cellular levels are strictly controlled at various levels including the synthesis, degradation, uptake and excretion (4,5). Furthermore, the polyamine homeostasis is also regulated by rapid interconversion (6-8) that in part offers means to supply proper polyamines for each individual processes. Spd and Spm appear to be able to substitute for each other to some extent (9,10). The exact cellular roles of each individual polyamine, however, are not well known (11). One of the approaches to elucidate the impact of each individual polyamine in biological processes is the use of metabolically stable Spd and Spm analogues. Examples of such compounds are MeSpd and bis–α,α'–methylspermine (Me_2Spm) that are able to fulfill many of the cellular functions of their native counterparts.

Earlier, we have successfully used racemic α–methyl analogs of polyamines to study the role of each individual polyamine *in vivo*. Both MeSpd and Me_2Spm are capable of preventing acute pancreatitis and restoring the early liver regeneration after partial hepatectomy under

condition of severe depletion of the natural polyamines in transgenic rat (12,13).

The interaction of racemic MeSpd with recombinant human PAO (hPAO) in the presence of benzaldehyde (BA), which allows the enzyme to oxidize non–acetylated Spd, led to the formation of putrescine (13). This prompted us to study the effect of aldehydes on this reaction using recently synthesized isomers of MeSpd and α–methylspermine (MeSpm) as well as the three diastereomers of Me_2Spm (Fig. 1). In the present paper the interactions of recombinant hPAO with different enantiomers of AcMeSpd have been studied.

We found that for hPAO, using achiral molecules as native substrates, (*S*)–AcMeSpd was clearly an inferior substrate in comparison with (*R*)–AcMeSpd, which itself was as effectively catabolized as AcSpd. Moreover, the supplementation of hPAO with different aldehydes regulated the stereospecificity of this enzyme. Thus, PAO in the presence of BA predominantly oxidized (*R*)–MeSpd, while in the presence of pyridoxal (PL) the (*S*)–isomer was preferred. In the case of MeSpm and Me_2Spm, the stereospecificity of oxidation could also be controlled by different aldehydes.

MATERIALS AND METHODS

Chemicals– Racemic α–methylated polyamine analogs were synthesized as described in (14,15). The two enantiomers of MeSpd were prepared as described in (16). Syntheses of (*R*)–AcMeSpd, (*S*)–AcMeSpd, both isomers of MeSpm and all diastereomers of Me_2Spm were performed using the same (*R*)– and (*S*)–N^1–(*o*–nitrophenylsulfonyl)–N^3–(*tert*–butyloxycarbonyl)–1,3–diaminobutane as the key chiral intermediates, which after alkylation with appropriate halides and removal of protecting groups afforded the target isomers of α–methylated analogs. Both racemic α–methylated polyamine analogs and their isomers showed purity >99.5 % according to ^{1}H/^{13}C–NMR data and HPLC–analysis. Preformed adduct of spermine with PL (PL=Spm) was prepared as described in (17). Aminooxyethyl putrescine (AOE–PU) and its acetylated derivative (AcAOE–PU) were synthesized as described in (18). The oximes of AOE–PU with BA and acetone were prepared as described (19), and showed >95 % purity according to ^{1}H–NMR– and HPLC–analyses. Diethylnorspermine (DENSpm) and diethylspermine (DESpm) were prepared principally as described earlier (20) and all proved to be >97 % pure according to ^{1}H–NMR and HPLC analysis. The PAO inhibitor MDL 72527 [N^1,N^4–*bis*(2,3–butadienyl)–1,4–diaminobutane] was a generous gift from Hoechst–Roussel. N^1,N^{11}–Diacetylnorspermine was synthesized essentially as described earlier (21). N^1–Acetylspermine was purchased from Sigma–Aldrich, dissolved in H_2O and stored at –20 °C. All other chemicals were purchased from Sigma–Aldrich and Fluka. The carbonyl compounds used for the study were freshly distilled and checked with ^{1}H–NMR. Stock solutions of 100 mM carbonyl compounds in ethanol were prepared prior to studies and stored in refrigerator. PL as a hydrochloride salt was dissolved in H_2O and stored at –20 °C.

Rat liver extract– Rat liver extracts were prepared as described earlier (13) except of using 50 mM borate buffer pH 9.3 with appropriate salt supplementation to remove natural polyamines.

HPLC analysis and kinetic studies– The investigation of the kinetics of hPAO–reaction was performed in duplicates at 3 to 6 different (10 to 1000 μM) substrate concentrations in the presence of 5 mM BA, pyridine 4–carboxaldehyde (P4CA) or PL. All the kinetic values with different substrates were determined with Lineweaver–Burk plotting. Some kinetic determinations were also carried out in duplicates at a fixed 1 mM Spm concentration supplemented with 3 to 4 different concentrations of 0.1 to 1 mM aldehydes (BA, P4CA or PL). The PAO reactions were carried out in a total volume of 180 μl in different buffers as described for each experiment separately (see legends for Tables and Figures) and were allowed to proceed for indicated time at +37 °C before addition of 20 μl of 50 % w/v sulfosalisylic acid containing 100 μM 1,7–diaminoheptane as internal standard. HPLC with post–column phthaldialdehyde derivatization was used to determine the concentrations of the polyamines and their methylated analogs essentially as described earlier (22). Chiral–HPLC analysis was performed using Whelk–O 1 (*R*,*R*) 25 cm * 4.6 mm column; isocratic run 0–75 min with flow rate of 0.55 ml/min, 70 % ethanol to separate (*R*)– and (*S*)–isomers of MeSpd after dansylation and treatment as described in (23).

Recombinant hPAO– The enzyme was produced as described earlier (13) omitting the affinity purification step.

RESULTS

Interaction of (R)–AcMeSpd and (S)–AcMeSpd with recombinant hPAO– An incubation of (*R*)– and (*S*)–isomers of AcMeSpd with hPAO in 100 mM glycine–NaOH buffer at pH 9.5 resulted in predominant oxidation of the (*R*)–AcMeSpd while (*S*)–enantiomer was much less efficiently catabolized. The (*R*)–AcMeSpd had K_m = 95 μM and k_{cat} = 9.0 s^{-1}, while for (*S*)–AcMeSpd the K_m and k_{cat} were 170 μM and 1.2 s^{-1}, respectively. It should be noted that AcSpd, the native substrate of PAO, had K_m = 14 μM and k_{cat} = 8.5 s^{-1} under the same conditions.

Initial screening studies with hPAO supplemented with various carbonyl compounds– The effect of seven aromatic aldehydes on the stereoselectivity of hPAO is depicted in Table 1 (for results with 17 tested aldehydes see supplemental Table S1). All reaction mixtures containing racemic, (*R*)– or (*S*)–MeSpd were supplemented with 5 mM of the tested aldehyde and the same mixtures without the enzyme were used as controls. Aromatic aldehydes were superior to aliphatic ones in enhancing the reaction rate of hPAO (supplemental Table S1). The enzyme showed strong preference towards (*R*)–MeSpd with most of the tested aldehydes, of which BA was most effective. The structure of the aldehyde was one of the key factors determining the extent of the enhancement. For example, the effect of *o*–, *m*– or *p*–hydroxy benzaldehydes totally depended on the position of hydroxy group (Table 1). Among the tested aldehydes, PL was the only (*S*)–guiding aldehyde changing the preference of hPAO to (*S*)–MeSpd. Thus, in the initial screening we were able to identify (*S*)–guiding PL, (*R*)–guiding BA and P4CA that enhanced the degradation of both isomers of MeSpd. All of the structurally related and tested ketones proved to be inactive at 5 mM concentrations (data not shown, structures of the used ketones are given in supplemental Table S2).

Studies with liver extracts obtained from wild–type Wistar rats– To exclude the possibility that hPAO, as a recombinant protein containing 6×HIS–tag fragment, would behave differently in comparison with the native enzyme, crude liver extracts were used as a source of PAO to study the effects of guide molecules *in vitro*. The data in Table 2 clearly show that the reaction rate was increased by the key aldehydes similarly for both recombinant hPAO and rat liver enzyme. Moreover, steering of the isomer specificity was also possible in the crude liver extract.

Guide molecule–controlled degradation of selected isomer from racemic MeSpd– Based on the above data, BA and PL were chosen to investigate the oxidation of racemic MeSpd. The reaction mixtures were subjected to chiral–HPLC analysis after dansylation of the polyamines. The used conditions (see Materials and Methods) did not yield complete separation of distinct isomers from the reaction mixture (elution times for (*S*)– and (*R*)–MeSpd were 49.2 min and 52.7 min, respectively). However, the preference for selected isomer with hPAO was clear and in the presence of BA, only traces of (*R*)–MeSpd were detected after 24 hours. In the case of PL, after 24 hour incubation (*S*)–MeSpd was completely oxidized (supplemental Fig. S1).

The kinetics of hPAO–dependent oxidation of different enantiomers of MeSpd supplemented with BA, PL or P4CA– The above described experiment showed that it was possible to enrich racemic MeSpd with the preferred enantiomer by the addition of the proper aldehyde into the substrate mixture of PAO. Therefore, the kinetic parameters of the hPAO–dependent oxidations of Spd, racemic and the two enantiomers of MeSpd were determined in the presence of fixed 5 mM aldehyde concentration (Table 3). Among the tested aldehydes, BA was the most effective with Spd (k_{cat} = 0.85 s^{-1}) though clearly inferior to AcSpd as a substrate. This may partly be due to the reaction of aldehyde with N^8–nitrogen, thus reducing the reaction rate. PL treatment provided practically the same affinity for both MeSpd isomers for hPAO, but the k_{cat} of 0.24 s^{-1} for (*S*)–MeSpd was much higher than the k_{cat} of 0.01 s^{-1} for (*R*)–MeSpd. P4CA enhanced the degradation both isomers of MeSpd to about the same extent (Table 3).

Stereospecificity and kinetics of hPAO–catalyzed oxidation of two enantiomers of MeSpm and three diastereomers of Me_2Spm supplemented with the key aldehydes– Spm was a rather poor substrate for hPAO and, expectedly, aldehyde supplementation greatly increased its degradation (Table 4). In the case of MeSpm both Spd and MeSpd were produced by hPAO whereas only MeSpd was obtained from Me_2Spm (Fig. 1). The addition of BA enhanced the reaction rates of all diastereomers of Me_2Spm but PL clearly enhanced only the degradation of (*S*,*S*')–Me_2Spm (Table 4). Similarly, addition of BA enhanced the degradation rates of both isomers of MeSpm. The presence of methyl

group did not influence the efficiency of the degradation, i.e. the cleavage of MeSpm took place at both methylated and non–methylated ends of the molecule. However, PL–supplementation did not enhance the hPAO–mediated oxidation of (*R*)–MeSpm from the methylated terminus (supplemental Table S3).

The effect of increasing aldehyde concentration on hPAO–dependent oxidation– The reaction rates of hPAO with Spm as substrate were enhanced upon increasing BA concentration up to 5 mM in both 100 mM glycine–NaOH and 50 mM borate buffer at high pH (supplemental Table S4). However, 10 mM BA was inhibitory for the enzyme reaction (data not shown). The reaction rate acceleration of hPAO was retarded in the presence of 2 mM or higher PL concentration when Spm or its methylated derivatives were used as a substrate (Table 5, data not shown). Therefore, we determined kinetic values for Spm derivatives with both 1 and 5 mM PL supplementation (Table 4). However, with Spd as a substrate the reaction rate increased up to 5 mM PL (data not shown). The kinetic values of hPAO–catalyzed oxidation were measured at constant 1 mM Spm concentration and increasing amount of supplemented aldehydes. In 100 mM glycine–NaOH K_m = 200 μM and k_{cat} = 5.1 s^{-1} for PL, K_m = 170 μM and k_{cat} = 6.6 s^{-1} for BA and K_m = 930 μM and k_{cat} = 6.6 s^{-1} values for P4CA were determined. In 50 mM borate buffer K_m = 110 μM and k_{cat} = 3.7 s^{-1} for PL and K_m = 150 μM and k_{cat} = 8.4 s^{-1} for BA were obtained. We also tested DMSO in increasing concentrations as the substitute for water (24) but already 50 % DMSO proved to inhibit the enzyme reaction (data not shown).

The effect of incubation order with substrate, aldehyde and hPAO– Both 1 mM Spm and racemic Me_2Spm were used as substrates for hPAO (0.3 and 0.6 μg per reaction, respectively) in the presence of 5 mM BA in 100 mM glycine–NaOH at pH 9.5. Two of the three components (substrate, aldehyde and enzyme) were incubated on ice for 15 min before the addition of the third. The reactions were carried out at +37 °C for 20 min. In all combinations with both substrates no differences in the reaction rates were observed (results not shown).

Terminally N–substituted polyamines as substrates for hPAO– It is known that DENSpm is a substrate of PAO yielding N^1–ethyl–Spd as the product (25). Inclusion of either 5 mM BA or PL to DENSpm or DESpm containing substrate mixtures decreased the rate of PAO–dependent oxidation (data not shown). Furthermore, oxidation of AcSpd was inhibited by the addition of BA or PL (data not shown).

Preformed adducts of Spm with PL as substrates for hPAO– The stock solution of the adduct was prepared by the incubation of 100 mM solution of Spm–base with 200 mM solution of PL–base in methanol in the dark at 20 °C overnight. The obtained adduct was oxidized by hPAO far more effectively than Spm supplemented with the same amount of PL (Table 5).

Buffer effect– The stereoselectivity of hPAO towards MeSpd isomers was very strict in 100 mM glycine–NaOH (Table 6) and 100 mM alanine–NaOH buffers at pH 9.5 (results not shown). Glycine and alanine as key constituents of these buffers have the potency to react with aldehydes. Therefore, two other buffer systems were used to exclude any buffer–related effects. Stereoselectivity of hPAO was sustained in all tested buffer systems (Table 6). However, the stringency for enantiomer selection and efficacy of aldehyde–enhanced degradations differed in the studied buffers (Table 6). Moreover, aldehyde–dependent oxidation carried out at pH 7.4 (NaH_2PO_4–buffer) showed reduced reaction rates but the stereoselectivity of degradation was retained (data not shown).

Oximes of AOE–PU– We have prepared stable oximes of AOE–PU with BA and acetone as well as AcAOE–PU (Fig. 2). These oximes may be considered as mimetics of Schiff base, which was suggested to be a substrate of the oxidation in the case of incubation of Spm or Spd with PAO in the presence of BA (26). It was found that AOE–PU, like Spd, was not a substrate in hPAO reaction. However, AcAOE–PU, which is an isoster of AcSpd, was a substrate. The k_{cat} value of AcAOE–PU was $^1/_{15}$th of the value for AcSpd (k_{cat} = 0.54 s^{-1} and 8.5 s^{-1}, respectively). Benzaldoxime of AOE–PU showed a good affinity for the enzyme, but the reaction rate was very low (Fig. 2) and k_{cat} was about 5 % of that obtained with Spd supplemented with 5 mM BA (Table 3). Acetone oxime of AOE–PU may be considered as a mimetic of AcSpd and showed a the k_{cat} value of one third of the value obtained for AcAOE–PU (Fig. 2).

DISCUSSION

PAO is one of the key enzymes in the catabolism of Spm and Spd. The natural substrates for PAO are N^1,N^{12}–diacetylspermine, N^1–acetylspermine and AcSpd (27) (Fig. 3), which are formed in cells as the result of acetyl–CoA–dependent acetylation of Spm and Spd by spermidine/spermine N^1–acetyltransferase (28). Hence, the substrates and the products of PAO–catalyzed reactions are achiral molecules (29). Recently we demonstrated that racemic MeSpd and Me_2Spm are effective surrogates of native polyamines (12,13). MeSpd proved to be metabolically stable as it is not a substrate for the spermidine/spermine N^1–acetyltransferase–mediated acetylation (13). Therefore, we prepared the corresponding (*R*)– and (*S*)–isomers of AcMeSpd (Fig. 1) that are the simplest chiral analogues of AcSpd and tested these compounds as substrates for hPAO. It turned out that the enzyme preferred (*R*)–AcMeSpd to the corresponding (*S*)–isomer as (*R*)–isomer had a k_{cat}/K_m value of less than 10 % of the (*S*)–isomer. This indicates for the first time that PAO has a hidden potency of stereospecificity.

In the presence of certain aldehydes the substrate specificity of rat liver PAO and the kinetics of the reaction are changed in a way that Spm and Spd are efficiently catabolized (26). This observation was later verified with purified PAO from porcine liver (30), but the exact mechanism of the aldehyde action remained unclear. Initially, the formation of the Schiff base between the aldehyde and amino group of Spm or Spd, which might mimic the charge distribution of AcSpd, was suggested (26). Moreover, a dependance between the aldehyde structure and its effect on PAO reaction was observed (26). Having the complete set of optical isomers of α–methylated Spd and Spm analogs, we checked the stereoselectivity of hPAO in the presence of some aldehydes. *A priori,* it was difficult to predict whether PAO would retain its potency to oxidize (*R*)–isomers, because it is known that the alteration in substrate structure may control the stereospecificity of the reaction. For example, in the case of well–studied ornithine decarboxylase only *L*–Orn is a substrate while *D*–isomer is a competitive inhibitor (31). However, in the case of α–difluoromethylornithine, which is an enzyme–activated inhibitor of ornithine decarboxylase, both *L*– and *D*–isomers are substrates (32), as the inhibitor must be first decarboxylated in order to produce reactive intermediate, which is responsible for irreversible inhibition of the enzyme (33). We tested several aldehydes in PAO–dependent oxidation of (*R*)– and (*S*)–MeSpd (Table 1 and supplemental Table S1). Most of the tested aromatic aldehydes enhanced the oxidation of (*R*)–isomer, the rate of which was dependent on the structure of the aldehyde (Table 1 and supplemental Table S1). However, supplementation of the substrate mixture with PL enhanced the oxidation of (*S*)–MeSpd, but not that of the (*R*)–isomer. P4CA stimulated degradation of both (*R*)– and (*S*)–MeSpd (Table 3). In spite of more complicated substrate properties of Spm derivatives, as compared with isomers of MeSpd, the enhancement and stereospecificity of reaction in the presence of key aldehydes were similar to those for MeSpd. The presence of the aldehyde in the substrate mixture resulted in increased k_{cat} and slightly lowered K_m values for reaction with Spm and the different diastereomers of Me_2Spm as substrates for hPAO (Table 4). For the first time, according to our knowledge, it was possible to regulate the stereospecificity of the FAD–dependent oxidase simply by adding appropriate aldehyde into the substrate mixture.

It is known that the introduction of a guide molecule in the substrate mixture may effect the rate of enzymatic reaction. One of the first examples is the acceleration of the solvolysis of acetyl chymotrypsin in the presence of indole due to its binding at a specific site of the enzyme (34). Hölttä's finding of the stimulation of PAO activity in the presence of BA (Spm and Spd were used as substrates) is another example, which served as the background of the present work. Another example is the effect of Mg^{2+}–ions on the activity of S–adenosylmethionine decarboxylase from *E.coli* via Mg^{2+}–induced conformational changes of of the enzyme, which are necessary for catalytic activity (35). In the absence of Mg^{2+}–ions the pyruvate residue, responsible for the decarboxylation, does not react with powerful and irreversible substrate–like hydroxylamine–containing inhibitor of the enzyme (35).

The mechanism of the substrate specificity change of PAO as a result of aldehyde treatment is unknown. Most likely it includes the interaction of aldehyde with the primary amino group(s) of the substrate. PAO has been shown to have potency to effectively oxidize many terminally *N*–alkylated polyamines and the main product in the case of DESpm is N^1–ethyl–Spd (25). The supplementation of the substrate mixture (containing DENSpm or DESpm) with

either BA or PL did not increase the reaction rate but resulted in the inhibition of the enzyme activity (20 to 50 %) at 5 mM BA or PL (data not shown). This might be due to the interaction of the aldehyde(s) with the reduced FAD. Similar effect was observed earlier when the interaction of N^1–cycloheptylmethyl–N^{11}–ethyl–norspermine with the maize PAO was studied. In that case the formed aldehyde, as the result of oxidative de–ethylation of the substrate, is covalently attached to the reduced FAD (36).

The interaction of aromatic aldehydes in water solution with amines, including amino acids, ethylenediamine and trimethylenediamine is well known and results in the formation of a complex equilibrium mixture of products, which in the case of diamines includes even cyclic aminals (37-39). The latter might be of importance in the case of aldehyde–driven oxidation of α–methylpolyamines by PAO because the breaking bond is in γ–position to the chiral center and the formation of cyclic aminal fixes chiral center closer to the cleavage site. The enhanced rate of the oxidase reaction by the increased concentration of PL at constant 1 mM Spm as the substrates in two different buffer systems (Table 5) indicate that glycine does not interfere with the formation of the "substrate adduct" for hPAO. However, with BA borate buffer was somewhat better reaction media compared to glycine–NaOH (supplemental Table S4). Furthermore, the order of substrate, aldehyde and enzyme addition to the reaction mixtures did not have any effect on the reaction rates.

The reaction between an aldehyde and the polyamines may take place either directly in solution or the aldehyde may attack the polyamine–PAO complex. The reaction rate with 1 mM preformed PL=Spm adduct was higher than the rate with 1 mM Spm supplemented with 2 mM PL (Table 5) and no further enhancement was detected at higher PL concentration (data not shown). Accordingly, it is likely that the aldehyde reacts with a polyamine in solution and the resultant product(s) serves as a substrate for PAO.

In the next set of experiments we first saturated hPAO with 1 mM Spm (over 90 %, as the K_m of Spm is 47 μM) and then added increasing concentrations (from 0.1 to 1 mM) of either BA or PL. At this range of aldehyde concentration the reaction rate enhanced upon increasing of the aldehyde concentration. This could be considered as an evidence indicating that polyamine binds first to PAO and only then reacts with an aldehyde. However, the K_m value of BA=Spm for hPAO is 10 times lower than with Spm (Table 4), that may result in the dissociation of the initial Spm–PAO comples.

Schiff bases were initially suggested by Hölttä (26) as the natural substrates for PAO and we decided to mimic them with isosteric oximes in order to work with individual compounds and not with the equilibrium mixtures. X–ray study of the complexes of the pyridoxal–5'–phosphate–dependent aspartate aminotransferase with the aminooxy analogues of the substrates showed that the pyridoxal–5'–phosphate–oxime binds at the active center and is a good mimetic of the external aldimine (40). Therefore, we used AOE–PU, AcAOE–PU as well as stable BA– and acetoneoximes (Fig. 2), instead of Spd, AcSpd and BA=Spd adducts. Introduction of oxygen in the β–position into the splitting bond decreased the affinity of AcAOE–PU towards hPAO and reduced the k_{cat} value to $^1/_{15}$th of the k_{cat} value of AcSpd. Resulting from the introduction of hydrophobic phenyl group, for both BA=AOE–PU and BA=Spd adduct, only slightly increased affinities for hPAO but dramatically decreased k_{cat} values were observed, as compared with AcSpd. Similarity in the behavior of BA=AOE–PU and BA=Spd adduct may indicate that Schiff base is a substrate for PAO.

The studied buffer systems (at pH 9.5) showed that the general isomer selectivity by hPAO with the key aldehydes was not dependent on the nature of the buffer (Table 6). Furthermore, at pH 7.4 the same isomer selectivity by hPAO in the presence of BA and PL was retained even though the reaction rates were reduced. However, the oxidations of both Spm and DESpm by PAO are similarly reduced at physiological pH (30,41) indicating that these compounds behave similarly as the substrates of PAO.

The present results distinctly indicate that the stereospecificity of a FAD–dependent oxidase can be regulated with small molecules. Using MeSpd as a substrate, the addition of either (*R*)–guiding BA or (*S*)–guiding PL was sufficient to change the stereoselectivity of the enzyme. Such a dormant stereoselectivity of PAO may contribute to the metabolism of the biologically active chiral polyamine derivatives. The observed phenomenon could be a basis to develop novel inhibitors for PAO and may serve

as an approach for some practical applications of biocatalysis.

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FOOTNOTES

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[1] The abbreviations used are: AcAOE–PU, acetylated aminooxyethyl putrescine; AcSpd, N^1–acetylspermidine; AcMeSpd, acetylated α–methylspermidine (1–amino–8–acetamido–5–azanonane); AOE–PU, aminooxyethyl putrescine; BA, benzaldehyde; DENSpm, diethylnorspermine; DESpm, diethylspermine; FAD, flavin adenine dinucleotide; MeSpd, α–methylspermidine; MeSpm, α–methylspermine; Me_2Spm, bis–α,α'–methylspermine; PAO, polyamine oxidase; PL, pyridoxal; PL=Spm, adduct of pyridoxal with spermine; P4CA, pyridine 4–carboxaldehyde; Spd, spermidine; Spm, spermine.

FIGURE LEGENDS

Fig. 1. Degradation of α–methylated polyamine analogues by polyamine oxidase (PAO). Methylated polyamines need to be supplemented with aldehydes in order to be degraded by PAO. All reactions should yield corresponding 3–aminobutanal or 3–acetamidobutanal which are spontaneously degraded. AcMeSpd, acetylated α–methylspermidine; MeSpd, α–methylspermidine; MeSpm, α–methylspermine; Me_2Spm, bis–α,α'–methylspermine.

Fig. 2. Structures of *N*–acetylated and oxime derivatives of aminooxyethylputrescine. Kinetic values were determined when possible in 100 mM glycine–NaOH at pH 9.5 containing 5 mM DTT. The reactions were stopped and analyzed as described in Materials and Methods. AcAOE–PU, acetylated aminooxyethyl putrescine; AOE–PU, aminooxyethyl putrescine; NA, not applicable (30 min reaction with 2 μg of hPAO and 1 mM aminooxyethyl putrescine evidenced less than 0.5 % degradation of the substrate).

Fig. 3. Natural substrates of polyamine oxidase (PAO). PAO catalyzes the degradation of spermidine and spermine derivatives, being acetylated by spermidine/spermine N^1–acetyltransferase, into 3–acetamidopropanal and putrescine and spermidine, respectively.

Figure 1

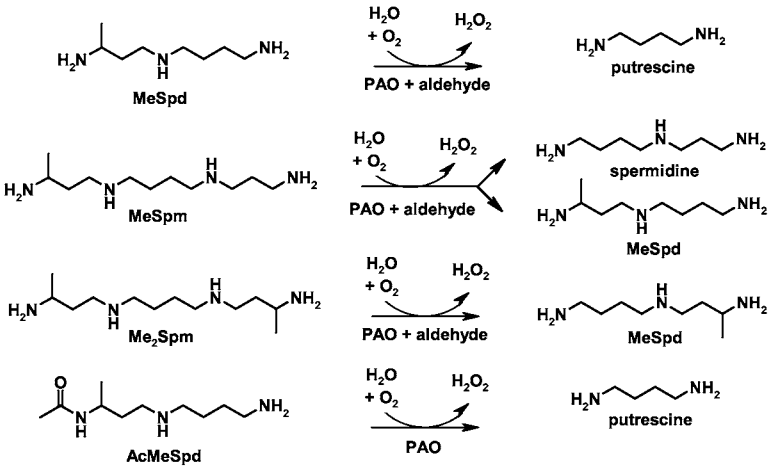

MeSpd
MeSpm
Me2Spm
AcMeSpd
H2O + O2
H2O2
PAO + aldehyde
PAO
putrescine
spermidine
MeSpd
MeSpd
putrescine

Figure 2

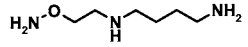
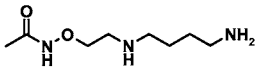
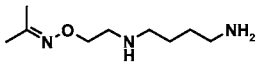
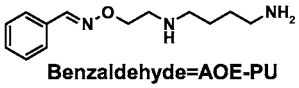
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
 AOE-PU	NA	NA	NA
 AcAOE-PU	290	0.54	1.9×10^3
 Acetone=AOE-PU	380	0.17	0.45×10^3
 Benzaldehyde=AOE-PU	3.4	0.04	12×10^3

Figure 3

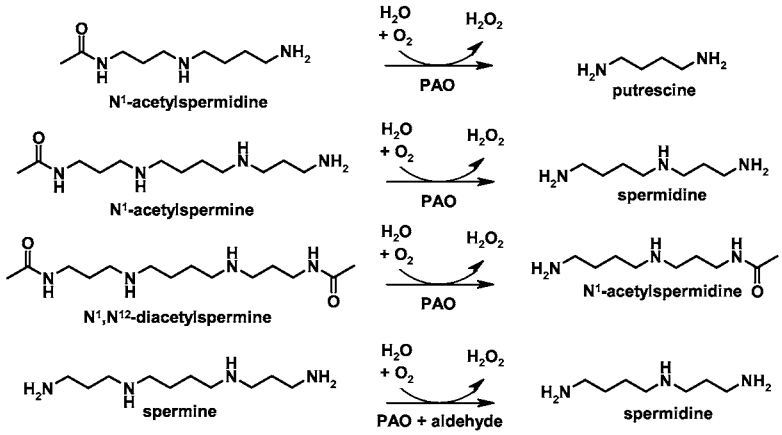
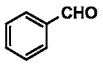
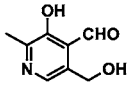
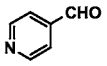
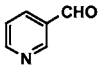
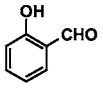
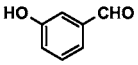
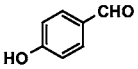

N1-acetylspermidine
H2O + O2
H2O2
PAO
putrescine
N1-acetylspermine
spermidine
N1,N12-diacetylspermine
N1-acetylspermidine
spermine
PAO + aldehyde

Table 1 The effects of selected aromatic aldehydes to isomer specificity of human polyamine oxidase

	Racemic MeSpd	(*R*)–MeSpd	(*S*)–MeSpd
Aldehyde		% used	
 **BA**	12.7	46.2	2.1
 **PL**	5.1	ND	14.1
 **P4CA**	11.6	14.6	8.2
	18.0	40.4	4.7
	ND	0.9	ND
	6.7	16.1	2.0
	1.2	1.7	0.7

The percentages of the substrate used by hPAO in the reaction containing 200 μM racemic, (*R*)– or (*S*)–α–methylspermidine and different 5 mM aldehydes. Reactions were carried out in duplicates at +37 ºC for 1 h in 100 mM glycine–NaOH at pH 9.5 supplemented with 5 mM DTT, 5 % EtOH and 1 μg recombinant protein. Reactions were stopped and measured with HPLC as described in Materials and Methods. No substrate degradation was detected in the reaction mixture in the absence of the enzyme. The duplicates differed less than 5 % from average. BA, benzaldehyde; PL, pyridoxal; P4CA, pyridine 4–carboxaldehyde; MeSpd, α–methylspermidine; ND, not detected.

Table 2 Degradation of racemic, (*R*)– and (*S*)–α–methylspermidine in the rat liver extract in the presence of pyridoxal or benzaldehyde

	Polyamine (pmoles/mg protein)		
	putrescine	spermidine	spermine
2 h, 400 µM racemic MeSpd	301 ± 23	206 ± 27	212 ± 41
+ 5 mM benzaldehyde	2320 ± 70	166 ± 12	37 ± 3
+ 5 mM pyridoxal	1170 ± 100	247 ± 14	63 ± 3
+ 250 µM MDL72527	182	191	191
2 h, 200 µM (*R*)–MeSpd	261 ± 6	217 ± 6	169 ± 8
+ 5 mM benzaldehyde	2550 ± 30	139 ± 24	21 ± 18
+ 5 mM pyridoxal	350 ± 0	233 ± 12	55 ± 3
+ 250 µM MDL72527	72	192	179
2 h, 200 µM (*S*)–MeSpd	221 ± 0	181 ± 35	172 ± 29
+ 5 mM benzaldehyde	451 ± 69	158 ± 12	27 ± 6
+ 5 mM pyridoxal	1450 ± 69	232 ± 23	49 ± 2
+ 250 µM MDL72527	85	188	185

200 µM substrate equalled to 9060 pmoles of analogue/mg of protein in the beginning of the reaction. Reactions were carried out in triplicates (except MDL72527 reactions in duplicates) at +37 °C for 2 h in 50 mM borate buffer at pH 9.3 containing 5 mM DTT, 100 µM pargyline and 1 mM semicarbazide. Reactions were stopped and measured with HPLC as described in Materials and Methods. The duplicates differed less than 5 % from average. MDL72527, polyamine and spermine oxidase inhibitor; MeSpd, α–methylspermidine.

Table 3 Kinetic characteristics of the degradation of spermidine, racemic and different isomers of α–methylspermidine with human polyamine oxidase in the presence of different aldehydes

	K_m (μM)	k_{cat}(s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
AcSpd	14	8.5	610×10^3
Spd	NA	NA	NA
+ 5 mM benzaldehyde	9.4	0.85	79×10^3
+ 5 mM P4CA	37	0.31	8.4×10^3
+ 5 mM pyridoxal	25	0.49	20×10^3
Racemic AcMeSpd	100	1.3	13×10^3
Racemic MeSpd	NA	NA	NA
+ 5 mM benzaldehyde	14	0.19	13×10^3
+ 5 mM P4CA	55	0.11	2.0×10^3
+ 5 mM pyridoxal	31	0.18	5.8×10^3
(*R*)–AcMeSpd	95	9.0	95×10^3
(*R*)–MeSpd	NA	NA	NA
+ 5 mM benzaldehyde	20	0.68	34×10^3
+ 5 mM P4CA	110	0.18	1.6×10^3
+ 5 mM pyridoxal	5.9	0.01	1.7×10^3

(*S*)–AcMeSpd	170	1.2	7.1×10^3
(*S*)–MeSpd	NA	NA	NA
+ 5 mM benzaldehyde	14	0.06	4.3×10^3
+ 5 mM P4CA	44	0.09	2.0×10^3
+ 5 mM pyridoxal	5.1	0.24	47×10^3

Reactions were carried out in 3 to 6 different substrate concentrations ranging from 10 to 500 µM in duplicates at +37 °C for 5 to 30 min in 100 mM glycine–NaOH at pH 9.5 containing 5 mM DTT and 0.05 to 2 µg recombinant protein. Reactions were stopped and measured with HPLC as described in Materials and Methods. NA, not applicable (30 min reaction with 2 µg of recombinant protein and 500 µM substrate evidenced less than 0.5 % degradation of the substrate); AcSpd, N^1–acetylspermidine; Spd, spermidine; AcMeSpd, acetylated α–methylspermidine; MeSpd, α–methylspermidine; P4CA, pyridine 4–carboxaldehyde.

Table 4 Kinetic values of spermine and different diastereomers of bis–α,α'–methylspermine for human polyamine oxidase in the presence of different aldehydes

	K_m (μM)	k_{cat}(s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
AcSpm	1.1	17	15×10^6
DiAcNSpm	2.7	19	7.0×10^6
Spm	47	0.4	8.5×10^3
+ 5 mM benzaldehyde	4.5	12	2.6×10^6
+ 5 mM P4CA	15	9.6	640×10^3
+ 5 mM pyridoxal	4.1	2.9	700×10^3
+ 1 mM pyridoxal	3.9	2.7	710×10^3
Racemic Me_2Spm	47	1.0	21×10^3
+ 5 mM benzaldehyde	28	5.8	210×10^3
+ 5 mM P4CA	110	4.5	42×10^3
+ 5 mM pyridoxal	23	2.0	87×10^3
+ 1 mM pyridoxal	14	1.64	120×10^3
(*R*,*R*')–Me_2Spm	55	0.12	2.2×10^3
+ 5 mM benzaldehyde	13	2.7	210×10^3
+ 5 mM P4CA	170	0.53	3.0×10^3
+ 5 mM pyridoxal	12	0.08	6.7×10^3

+ 1 mM pyridoxal	21	0.13	6.2×10^3
(*R*,*S*')–Me_2Spm	27	1.1	41×10^3
+ 5 mM benzaldehyde	21	4.5	210×10^3
+ 5 mM P4CA	110	4.1	39×10^3
+ 5 mM pyridoxal	6.6	1.2	180×10^3
+ 1 mM pyridoxal	8.1	1.9	230×10^3
(*S*,*S*')–Me_2Spm	16	0.97	61×10^3
+ 5 mM benzaldehyde	21	6.1	290×10^3
+ 5 mM P4CA	330	7.8	24×10^3
+ 5 mM pyridoxal	5.7	2.4	420×10^3
+ 1 mM pyridoxal	27	6.0	220×10^3

Reactions were carried out with 3 to 6 different 10 to 500 µM substrate concentrations in duplicates at +37 °C for 5 to 30 min in 100 mM glycine–NaOH at pH 9.5 containing 5 mM DTT and 0.025 to 2 µg recombinant protein. Reactions were stopped and measured with HPLC as described in Materials and Methods. AcSpm, N^1–acetylspermine; DiAcNSpm, N^1,N^{11}–diacetylnorspermine; Spm, spermine; Me_2Spm, bis–α,α'–methylspermine; P4CA, pyridine 4–carboxaldehyde.

Table 5 Human polyamine oxidase–catalyzed production of spermidine from spermine–pyridoxal adduct and from spermine supplemented with different amounts of pyridoxal

	100 mM glycine–NaOH pH 9.5	50 mM borate pH 9.3
	Spd nmoles	Spd nmoles
1000 μM PL=Spm	11.95 ± 0.37	12.49 ± 0.36
1000 μM Spm	1.09 ± 0.03	1.07 ± 0.05
+ 100 μM pyridoxal	3.64 ± 0.08	3.81 ± 0.04
+ 500 μM pyridoxal	7.92 ± 0.33	6.58 ± 0.12
+ 1 mM pyridoxal	9.01 ± 0.27 ***	7.13 ± 0.12 ***
+ 2 mM pyridoxal	8.25 ± 0.18 ***	6.75 ± 0.29 ***

1000 μM substrate equalled to 180 nmoles in the beginning of the reaction. Reactions were carried out in triplicates at 37°C for 25 min in the indicated buffers containing 5 mM DTT and 0.1 μg recombinant protein. Reactions were stopped and measured with HPLC as described in Materials and Methods. No substrate degradation was detected in the reaction mixture in the absence of the enzyme. Two tailed Student's test was used for statistical analysis between PL=Spm adduct and 1 or 2 mM pyridoxal supplemented 1000 μM Spm reactions. PL=Spm, adduct of spermine with pyridoxal; Spd, spermidine; Spm, spermine, ***, $p<0.001$.

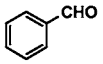
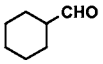
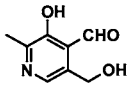
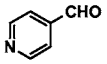
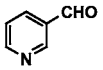
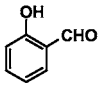
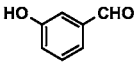
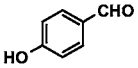
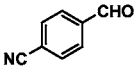
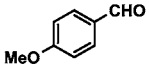
Table 6 Putrescine production by human polyamine oxidase from racemic, (*R*)– and (*S*)–α–methylspermidine in the presence of pyridoxal, benzaldehyde or pyridine 4–carboxaldehyde

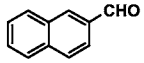
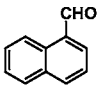
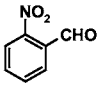
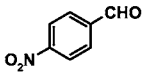
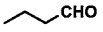
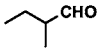
	racemic MeSpd 72 nmoles	(*R*)–MeSpd 36 nmoles	(*S*)–MeSpd 36 nmoles	Ratio
Buffer system	Supplemented with 5 mM benzaldehyde			(*R*) / (*S*)
50 mM Na_2HPO_4 pH 9.5	3.8	8.4	0.7	12
50 mM borate pH 9.3	6.7	8.5	1.1	8
100 mM glycine–NaOH pH 9.5	6.5	14.6	1.1	14
Buffer system	Supplemented with 5 mM pyridoxal			(*S*) / (*R*)
50 mM Na_2HPO_4 pH 9.5	1.2	ND	2.3	–
50 mM borate pH 9.3	5.8	0.4	11.5	31
100 mM glycine–NaOH pH 9.5	3.5	0.2	9.0	50
Buffer system	Supplemented with 5 mM pyridine 4–carboxaldehyde			(*R*) / (*S*)
50 mM Na_2HPO_4 pH 9.5	2.0	1.5	0.9	1.6
50 mM borate pH 9.3	0.4	0.3	0.1	1.9
100 mM glycine–NaOH pH 9.5	2.5	2.3	1.5	1.5

Reactions were carried out in duplicates at +37 °C for 1 h in the indicated buffers containing 5 mM DTT and 1 µg recombinant polyamine oxidase. Reactions were stopped and measured with HPLC as described in Materials and Methods. No substrate degradation was detected in the reaction mixtures in the absence of the enzyme. The duplicates differed less than 5 % from average. MeSpd, α-methylspermidine; ND, not detected; Put, putrescine.

Supplemental Tables and Figure

Table S1 The effects of 17 aldehydes on the stereospecificity of human polyamine oxidase

	Racemic MeSpd	*R*–MeSpd	*S*–MeSpd
Aldehyde		% used	
 **BA**	12.7	46.2	2.1
	8.6	28.0	1.3
 **PL**	5.1	ND	14.1
 **P4CA**	11.6	14.6	8.2
	18.0	40.4	4.7
	ND	0.9	ND
	6.7	16.1	2.0
	1.2	1.7	0.7
	9.2	34.5	2.2
	6.3	20.0	1.1

	5.8	25.4	0.7
	ND	ND	ND
	11.0	37.3	3.5
	8.7	23.0	2.7
	2.9	7.0	0.8
	3.0	6.7	0.7
	0.4	0.6	0.5

The percentages of the substrate used by hPAO in the reaction containing 200 µM racemic, (*R*)– or (*S*)–α–methylspermidine and different 5 mM aldehydes. Reactions were carried out in duplicates at +37 ºC for 1 h in 100 mM glycine–NaOH at pH 9.5 supplemented with 5 mM DTT, 5 % EtOH and 1 µg recombinant protein. Reactions were stopped and measured with HPLC as described in Materials and Methods. No substrate degradation was detected in the reaction mixture in the absence of the enzyme. The duplicates differed less than 5 % from average. BA, benzaldehyde; PL, pyridoxal; P4CA, pyridine 4–carboxaldehyde; MeSpd, α–methylspermidine; ND, not detected.

Table S2 The structures of tested ketones from which none had effect on the reaction rate of human polyamine oxidase

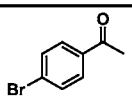
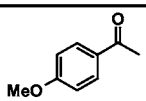
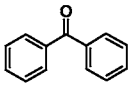
		
		

Table S3 Degradation of racemic, (*R*)– and (*S*)–α–methylspermine by human polyamine oxidase in response to different aldehydes

	MeSpd	Spd
racemic MeSpm	1.2	1.1
+ 5 mM benzaldehyde	21.7	12.0
+ 5 mM P4CA	13.6	6.5
+ 5 mM pyridoxal	8.9	5.7
(*R*)–MeSpm	0.8	1.9
+ 5 mM benzaldehyde[a]	13.6	22.3
+ 5 mM P4CA	3.2	9.6
+ 5 mM pyridoxal	3.1	2.0
(*S*)–MeSpm	2.0	0.7
+ 5 mM benzaldehyde	26.9	5.7
+ 5 mM P4CA	21.5	5.5
+ 5 mM pyridoxal	13.5	8.7

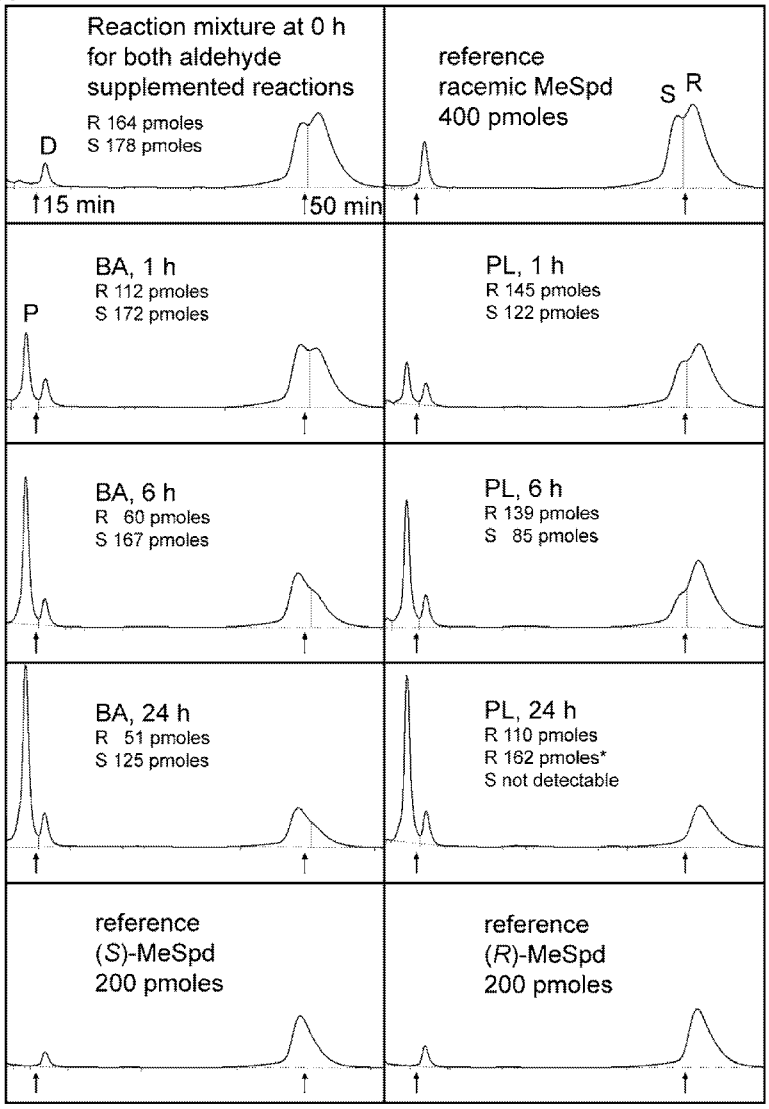
500 μM substrate equalled to 90 nmoles in the beginning of the reaction. Reactions were carried out in duplicates at +37 °C for 30 min in 100 mM glycine–NaOH at pH 9.5 containing 5 mM DTT and 0.2 μg recombinant protein. Reactions were stopped and measured with HPLC as described in Materials and Methods. No substrate degradation was detected in the reaction mixture in the absence of the enzyme. The duplicates differed less than 5 % from average. *a*, 0.2 nmoles of putrescine was also detected; Spd, spermidine; MeSpd, α–methylspermidine; MeSpm, α–methylspermine; P4CA, pyridine 4–carboxaldehyde. Please, note that MeSpm may be degraded from either end (Fig. 1).

Table S4 The effect of increasing benzaldehyde concentration to the degradation of spermine by human polyamine oxidase

	100 mM glycine–NaOH pH 9.5	50 mM borate pH 9.3
	Spd nmoles	Spd nmoles
1000 μM Spm	1.09 ± 0.03	1.07 ± 0.05
+ 100 μM benzaldehyde	5.17 ± 0.11	7.11 ± 0.04
+ 500 μM benzaldehyde	10.13 ± 0.32	13.65 ± 0.80
+ 1 mM benzaldehyde	11.83 ± 0.56	16.10 ± 0.59
+ 2 mM benzaldehyde	12.74 ± 0.39	16.29 ± 0.24
+ 5 mM benzaldehyde	14.48 ± 0.41	17.92 ± 0.46

1000 μM substrate equalled to 180 nmoles in the beginning of the reaction. Reactions were carried out in triplicates at +37 °C for 25 min in the indicated buffers containing 5 mM DTT and 0.1 μg recombinant protein. Reactions were stopped and measured with HPLC as described in Materials and Methods. No substrate degradation was detected in the reaction mixture in the absence of the enzyme. Spd, spermidine; Spm, spermine.

Figure S1



Polyamine oxidase–mediated stereospecific degradation of racemic α–methylspermidine in the presence of benzaldehyde or pyridoxal. Reactions were carried out in duplicates at +37 °C for up to 24 h in 100 mM glycine–NaOH at pH 9.5 containing 5 mM aldehyde, 5 mM DTT, 5 % EtOH and 1 µg recombinant protein. Reactions were stopped and measured with chiral–HPLC analysis as described in Materials and Methods. BA, benzaldehyde; D, 1,7–diaminoheptane; P, putrescine; PL, pyridoxal; MeSpd, α–methylspermidine; R; (*R*)–α–methylspermidine; S, (*S*)–α–methylspermidine; Standard curve for racemic MeSpd [(100, 200, 400 pmoles equalling to 50, 100 and 200 pmoles of single enantiomer; (*R*)–MeSpd (r^2 = 0.998; y= 526374x - 12048362); (*S*)–MeSpd (r^2 = 0.999; y= 280813x -

6911740)] was used to determine (R/S)–enantiomer content in each sample. *, pmoles for (*R*)–MeSpd calculated with pure (*R*)–MeSpd enantiomer standard curve (r^2 = 0.999; y= 336860x - 8595502).

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