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Substrate specificity of the bovine serum amine oxidase and in situ characterisation of aminoaldehydes by NMR spectroscopy

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Abstract—The oxidation of spermidine or homospermidine with bovine serum amine oxidase (BSAO) was monitored in situ, using proton nuclear magnetic resonance spectroscopy in water with 10% D_2O. NMR assignments were performed by spin decoupling and COSY spectra or by comparison with data from synthetic aminoaldehydes. The results represent the first in situ characterisation of the highly reactive aminoaldehydes and showed oxidation at the N^1 amino group of spermidine and homospermidine.

Comparison of homospermidine with a variety of substrates revealed that among straight chain di- and polyamines both an aminopropyl group and two primary amino groups separated by seven (norspermidine) or eight (spermidine) carbon atoms were required for optimal substrate ability. However, highest activity was seen with the substrate *N*-(4-aminobutyl)hexahydropyrimidine, showing that the substrate channel of BSAO has a dual substrate preference, with moderately bulky substituents at the distal end of a diamine contributing equally well as an alkyl amino group. Cytotoxic investigations of a variety of substrates for BSAO, confirmed previous results, that cytotoxicity is primarily linked to polyamines encompassing the aminopropyl moiety. No acrolein was observed at any time during the oxidation showing that it reacts very fast with available amino groups forming a variety of derivatives.

1. Introduction

The intracellular polyamine level is intimately linked to cell proliferation and apoptosis[1] and is regulated by multiple pathways including acetylation and oxidative degradation by polyamine oxidase (PAO).[2,3] Extracellularly, the oxidative deamination of polyamines and other amines is catalysed by copper-containing amine oxidases (CAOs).[4–8] The bovine serum amine oxidase (BSAO) is a particularly interesting enzyme, which is easily purified and gives rise to highly cytotoxic products by the oxidation of the natural polyamines spermidine and spermine.[9–24] BSAO is present in foetal calf serum (FCS), and cellular studies carried out in the presence of FCS must take into account the possible oxidation of polyamines by BSAO. The importance of being able to identify the oxidation products has given rise to several studies of substrate selectivity and regiospecificity. Previous investigations were based on studies of secondary transformations followed by product analysis, using TLC, HPLC, MS and NMR spectroscopy.[25–27] Tabor et al., using

Abbreviations: CAO, copper-containing amine oxidase; BSAO, bovine serum amine oxidase; HRP, horseradish peroxidase; *p*-NPP, *p*-nitrophenyl phosphate substrate tablets; bFGF, recombinant human basic fibroblast growth factor; VEGF, recombinant human vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cells; PET, polyethylene terephthalate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EBM-2, endothelial cell basal medium-2; FBS, foetal bovine serum; hbFGF, human basic fibroblast growth factor; hVEGF, human vascular endothelial growth factor; hEGF, human epidermal growth factor; R^3-IGF-1, long R insulin-like growth factor-1; GA-1000, gentamicin sulfate; PBS, phosphate-buffered saline; VLC, vacuum liquid chromatography; ECAO, *Escherichia coli* amine oxidase; PSAO, pea seedling amine oxidase; AGAO, *Arthrobacter globiformis* amine oxidase; HPAO, *Hansenula polymorpha* amine oxidase; PAO, polyamine oxidase; DQF-COSY, double-quantum filtered correlation spectroscopy.

Keywords: Bovine serum amine oxidase; Spermidine; Homospermidine; *N*-(4-Aminobutyl)hexahydropyrimidine; NMR spectroscopy; Cytotoxicity; Acrolein.

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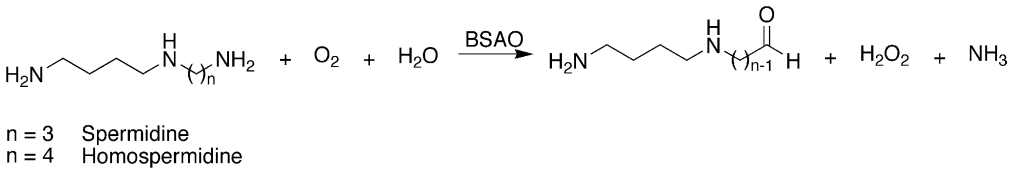


Scheme 1. The BSAO oxidation of spermidine and homospermidine.

TLC in combination with $NaBH_4$ reduction of aldehydes, concluded that spermidine and spermine were oxidised at primary amino groups[25] (Scheme 1). Houen et al., using NMR spectroscopy, HPLC and MS, concluded that oxidation of these two polyamines occurred at secondary amino groups.[26] In both of these studies, β-elimination of acrolein was a major complicating problem. Consequently, Lee and Sayre[27] used homospermidine as a substrate incapable of β-elimination (Scheme 1) in combination with $NaBH_4$ reduction and acetylation followed by NMR spectroscopy of the reduced and acetylated aminoaldehydes and concluded that oxidation had occurred at the primary amino group. The stereochemistry of the oxidation of dopamine was elucidated by deuterium labelling and reduction prior to ^1H NMR analysis[28] and the structure of reaction intermediates, for example, from benzylamine has been probed using UV/vis and Resonance Raman spectroscopy.[29] We have carried out a comparative analysis of homospermidine and other amines with respect to substrate specificity and cytotoxicity, and report an improved method for monitoring and identifying the oxidation products of polyamines in situ using ^1H NMR spectroscopy in water containing 10% D_2O.

2. Results

2.1. The substrate specificity of BSAO

In order to examine the substrate specificity of BSAO, and in particular to compare homospermidine with spermidine and related polyamines, a number of amines were selected for comparison. Compounds were either commercially available or synthesised, and the relative substrate activities were determined using a peroxidase-coupled chemiluminescence assay. The results appear in Table 1. The data first of all demonstrate that the presence of long chains and hydrophobic character of the amines favour an accelerated rate of enzymatic oxidation. The diamines ($H_2N(CH_2)_nNH_2$) in particular demonstrate this trend as illustrated graphically in Figure 1, that is, a long chain is required for maximum activity ($n = 8$ to 10) while the shorter diamines ($n = 3$ to 7) show diminished substrate activity. In the case of decane-2,9-diamine a lower activity is observed compared to those of both decane-1,10-diamine and octane-1,8-diamine. Secondly, substrates containing the aminopropyl moiety are always superior to those lacking this group but with comparable chain length. Thus, norspermidine containing two aminopropyl groups reacts faster than both homospermidine (two aminobutyl groups) and *N*-(2-aminoethyl)-putrescine (carrying one aminobutyl and one aminoethyl group). The presence of moderately bulky substituents in the non-oxidised end of the amines does not appear to have a major influence on the substrate activities, provided that the substituents are removed from the amino group. In particular the high activity of *N*-(4-aminobutyl)-hexahydropyrimidine renders it among the best substrates for BSAO.

Table 1. Substrate activities towards BSAO

Substrate	Relative activity[a]
Polyamines	
Norspermidine	100
Spermidine	86
Spermine	53
Homospermidine	49
1,1,9,9-Tetradeuterio-homospermidine	8
N-(2-Aminoethyl)-putrescine	7
Straight chain mono- and diamines	
Octylamine	46
Octane-1,8-diamine	38
Decane-1,10-diamine	30
Dodecane-1,12-diamine	23
Putrescine	13
Propane-1,3-diamine	13
Heptane-1,7-diamine	9
Hexane-1,6-diamine	8
Cadaverine	7
Moderately bulky amines	
N-(4-Aminobutyl)hexahydropyrimidine	110
N-(3-Aminopropyl)hexahydropyrimidine	61
Benzylamine	30
N-Aminobutylpyrrolidine	19
Decane-2,9-diamine	11
Histamine	7

[a] Relative substrate activities of selected amines (0.1 mM) with BSAO (0.01 mg/mL), in 20 mM bis–tris pH 7.0.

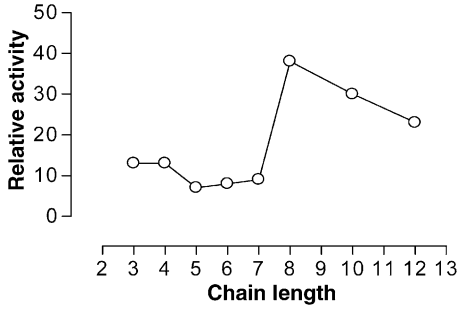


Figure 1. Relative substrate activities of straight-chain diamines at a concentration of 0.1 mM.

2.2. Enzymatic reaction with spermidine

The oxidation of spermidine by purified BSAO in H_2O with 10% D_2O was monitored by NMR spectroscopy using a jump-return sequence or water presaturation. Using 100% D_2O as a solvent, it was previously reported that spermidine is converted to putrescine.[26] However, the intermediate aldehyde could not be identified with certainty from the NMR pattern due to deuteration of the CH_2 group α to the aldehyde group. Based upon other evidence, an oxidation sequence involving the secondary amino group seemed probable, and the NMR spectra could, in fact, be interpreted to support this contention. Lee and Sayre[27] later obtained results for homospermidine supporting a regiospecific oxidation at the primary amino group. Therefore, we decided to reinvestigate the enzymatic oxidation of spermidine and homospermidine by NMR spectroscopy. The concentration of the aqueous solution was chosen to be 1 mM in spermidine. This is small enough to ascertain the natural function of the enzyme but simultaneously allows a satisfactory signal-to-noise ratio. The enzymatic conversion was followed either at 400 or 800 MHz, and the signals assigned from 1D ^1H and 2D DQF-COSY spectra at 800 MHz.

Before the enzyme is added to the NMR tube, spermidine, $NH_3^+CH_2^ACH_2^BCH_2^CCH_2^DNH_2^+CH_2^ECH_2^F$-$CH_2^GNH_3^+$, displayed the following NMR spectrum: δ_H 3.00 and 2.98 (2H, t, $J(H,H)$ = 7.8 Hz, CH_2^E) and CH_2^G, 2.96 (2H, t, $J(H,H)$ = 7.5 Hz, CH_2^D), 2.93 (2H, t, $J(H,H)$ = 7.2 Hz, CH_2^A), 1.99 (2H, quintet, $J(H,H)$ = 7.8 Hz, CH_2^F), 1.68 (2H, quintet, CH_2^C), 1.65 (2H, quintet, CH_2^B).

Hydrated 3-(ω-aminobutylamino)-propionaldehyde, or 3-(ω-aminobutylamino)-propane-1,1-dioldihydrochloride, $NH_3^+CH_2^ACH_2^BCH_2^CCH_2^DNH_2^+CH_2^ECH_2^FCH^G(OH)_2$ as present in almost pure state after addition of three portions of enzyme exhibited the following NMR signals: 3.05 (2H, t, $J(H,H)$ = 7.3 Hz, CH_2^E), 2.98 (2H, t, $J(H,H)$ = 7.5 Hz, CH_2^D), 2.93 (2H, t, $J(H,H)$ = 7.2 Hz, CH_2^A), 1.88 (2H, dt, $J(H,H)$ = 4.8 Hz and 6.6 Hz, CH_2^F), 1.67 (2H, quintet, CH_2^C), 1.65 (2H, quintet, CH_2^B).

The signal arising from $-CH^G(OH)_2$ was not observed because it is assumed to be almost coincident with the water signal. However, the presence of the –CHO group could be excluded from the absence of signals in the relevant region and the observation of a coupling constant, $J(H,H)$, for the CH_2^F group of 4.8 Hz, which is well beyond the upper limit (3 Hz) for couplings to the –CHO group. It was confirmed by spin decoupling that the signal at 1.88 ppm collapsed to a doublet with $J(H,H)$ = 4.8 Hz on irradiation at the triplet at 3.05 ppm.

The spectra are shown in Figure 2A. The upper spectrum was recorded of the spermidine solution prior to addition of enzyme and is characterised by three multiplet patterns. DQF-COSY spectra (Fig. 2B) demonstrated the presence of four partly overlapping triplets around 3 ppm arising from the CH_2 groups attached to the protonated amino groups, a quintet at 2 ppm due to the central CH_2 group of the trimethylene moiety and two superimposed quintets around 1.65 ppm attributed to the corresponding CH_2CH_2 groups of the tetramethylene grouping. Addition of three portions of enzyme resulted in almost complete conversion to the aminoaldehyde after 24 h (30% after 2 h, 40% after 5 h) as apparent from the replacement of the quintet at 2 ppm with a new signal at 1.86 ppm in the lower spectrum (Fig. 2A). The spectrum is identical with the spectrum previously obtained in D_2O[26] with the sole exception that the signal at 1.86 ppm was absent in D_2O, corresponding to a CH_2 group, which is easily deuterated. Irradiation at 3 ppm verified that this signal is due to a CH_2 group attached to a protonated amino group, and since the remaining doublet showed a coupling constant of $J(H,H)$ = 4.8 Hz it must be situated α to a hydrated aldehyde group. The DQF-COSY spectra (Fig. 2C) further served to establish the presence of the sequence N^+–$CH_2CH_2CH(OH)_2$. The deuteration with formation of N^+–$CH_2CD_2CH(OH)_2$ must be due to an equilibrium with free aldehyde, however, in amounts too small to be observed in the NMR spectra. Nevertheless, the NMR spectra confirm that oxidation does in fact occur at the primary amino group. When taking the signal-to-noise ratio of the ^1H NMR spectra into account, it can be seen, that over 90% of the oxidation takes place at the aminopropyl moiety of spermidine.

2.3. The pH equilibrium of 1-(4′-aminobutyl)-pyrrolinium dihydrochloride investigated by ^1H NMR

A total of 19 proton NMR spectra (400 MHz) were recorded at the following pH: 1.14, **1.43**, 1.35, 2.75, 3.78, 6.56, **7.22**, 7.75, **7.95**, **8.40**, 8.86, **9.05**, **9.50**, 10.16, **10.52**, 11.49, **11.51**, **12.25** and 12.70 (bold-faced values indicate spectra recorded using 3.2 mg instead of 6.8 mg substrate per sample). The spectra proved independent of concentration. Scheme 2 shows the structure of aminoaldehyde *1* in different ranges of pH based on assignment of the spectra. The entropically disfavoured cyclisation to a 10-membered ring, and the possibility of imines arising from bimolecular reactions have been omitted in Scheme 2.

At acidic pH (1.14–6.56) the NMR spectra displayed all the characteristic signals of *1a*, which had previously been assigned (^1H, ^{13}C NMR, COSY, HETCOR). At pH 7.22–7.95 the eight individual peaks arising from *1a* broaden and loses the coupling information although they still retain the overall appearance of spectra recorded at more acidic pH's.

In the region from pH 7.95 to 10.5 it proved difficult to obtain a clear-cut assignment of the spectrum due to lack of characteristic signals and broad overlapping signals. Deprotonation of *1a* yields very little *1b* evidenced by the lack of an imine signal around 7.7 ppm, although in the range pH 7.22–7.95 a broad singlet at 6.9 ppm can be observed. This suggests that upon deprotonation *1b* is immediately transformed to one or more of the species

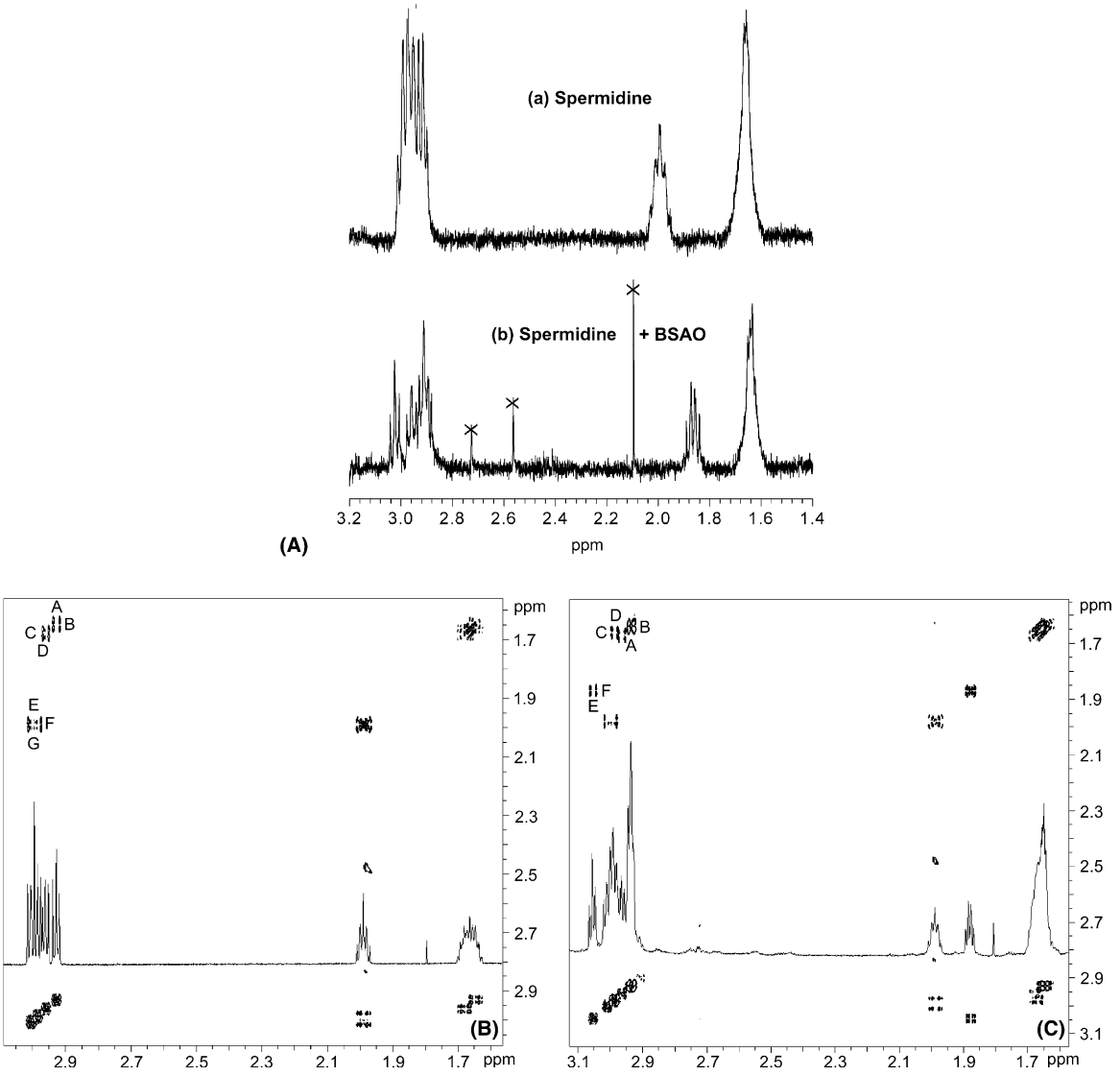
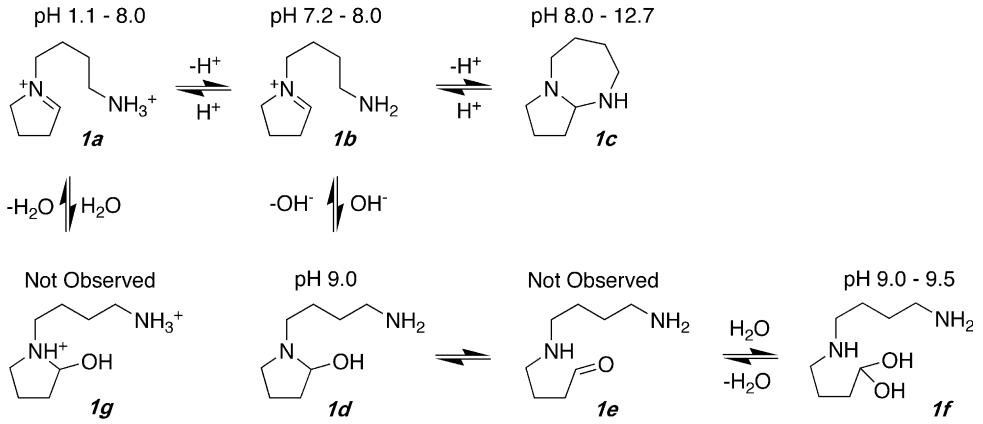


Figure 2. Oxidation of spermidine. A: Proton NMR spectra (400 MHz) of: Spermidine, pH 7.2 (top) and enzymatically oxidised spermidine, pH 7.2 (bottom). B: Proton NMR spectrum and DQF-COSY (800 MHz) of spermidine. C: Proton NMR spectrum and DQF-COSY (800 MHz) of partly oxidised spermidine.

1c, *1d*, *1e* and *1f*. The aldehyde *1e* is not observed at any pH, as the aldehyde proton around δ 9.7 ppm is never observed.

Spectra recorded in the region of pH 9.05–9.50 reveal a proton appearing at δ 5.08 ppm from the hydrated aldehyde *1f*. A mixture of hemiaminal *1d* and hydrated



Scheme 2. The structures of oxidised homospermidine at different pH.

aldehyde *1f* is found at pH 9.05 indicated by a broad signal at 5.24 ppm. When pH is changed from 9.05 to 9.50 the signal at 5.24 ppm sharpens and moves to 5.08 ppm (characteristic of the hydrated aldehyde), which indicates that the equilibrium between *1d* and *1f* is shifted towards *1f*. The remaining peaks around pH 7.95–10.5 may be due to protonated *1c*, provided that the characteristic triplet from the aminal proton is disguised by the broad HDO signal at 4.8 ppm.

In the basic region from pH 11.49 to 12.70, the characteristic triplet at 3.99 ppm due to the N–CH–N proton clearly demonstrates the presence of bicyclic *1c*. Although the rest of the signals are broad, due to the different conformations for the two fused five- and seven-membered rings of *1c*, they correlate with a previous assignment of 1,6-diazabicyclo[5.3.0]decane *1c* based on ^{1}H, ^{13}C NMR, DEPT, COSY and HETCOR spectra.

2.4. Enzymatic reaction with homospermidine

Before addition of BSAO, the only observable peaks in the ^{1}H NMR spectrum (400 MHz) arose from homospermidine $NH_2^+(CH_2^ACH_2^BCH_2^BCH_2^ANH_3^+)_2$. δ_H 2.94–3.02 (8H, m, CH_2^A), 1.66–1.76 (8H, m, CH_2^B). After addition of 25 μL BSAO solution, the reaction subsided after 7–24 h yielding 10% turnover and no further oxidation could be detected over the course of 48 h. If the oxidation was carried out at 37 °C, the signal-to-noise ratio of the resulting spectrum was much lower suggesting either inactivation of the enzyme or decomposition of the products. Using 50 μL BSAO resulted in 15% turnover after 24 h. Adding 50 μL BSAO every 7 h (four times in total), and leaving the sample for a total of 48 h, resulted in a maximum of 40% turnover. Since this is sufficient to characterise the oxidation product adequately, no further attempts were made to oxidise the remaining homospermidine. The aminoaldehyde could be unambiguously identified by comparison with the spectra of synthetic 1-(4′-aminobutyl)-pyrrolinium dihydrochloride at pH 7.2 and at pH 1. An expansion of the relevant spectra is shown in Figure 3. The upper spectra were recorded of the synthetically prepared aminoaldehyde (1-(4′-aminobutyl)-pyrrolinium dihydrochloride) at neutral pH and acidic pH, while the corresponding lower spectra were obtained from enzymatic oxidation of homospermidine. A primary kinetic isotope effect, $k_H/k_D \approx 6.2$, was found for homospermidine deuterated α to the two primary amino groups using the luminescence assay (Table 1). These results demonstrate that the oxidation of homospermidine is regioselective and occurs principally at the primary amino groups (Scheme 1).

2.5. Cytotoxic investigations

The cytotoxic effect (IC_{50} values) for some of the amines used in Table 1 was determined using human umbilical vein endothelial cells (HUVEC) (Table 2). It may be seen from the table that an aminopropyl moiety confers cytotoxicity. Thus, even though homospermidine is a relatively good substrate, it does not exert any cytotoxic effect. It may thus be concluded that H_2O_2 and NH_3 do not contribute appreciably to cytotoxicity, and hence that the aminoaldehyde is the major cytotoxic agent. Similar results were obtained with HeLa and EaHY 926 cells (results not shown). Decane-1,10-diamine, dodecane-1,12-diamine and octylamine also exhibited cytotoxic effects. These, however, were not influenced by aminoguanidine, showing that cytotoxicity was not due to enzymatic oxidation, but rather due to an inherent cytotoxicity of these compounds.

The prerequisite of an aminopropyl group for cytotoxicity suggested that it was mediated by acrolein. However, acrolein was not observed in the NMR spectra of the reaction mixture from oxidation of spermidine, not even when oxidised spermidine was spontaneously converted

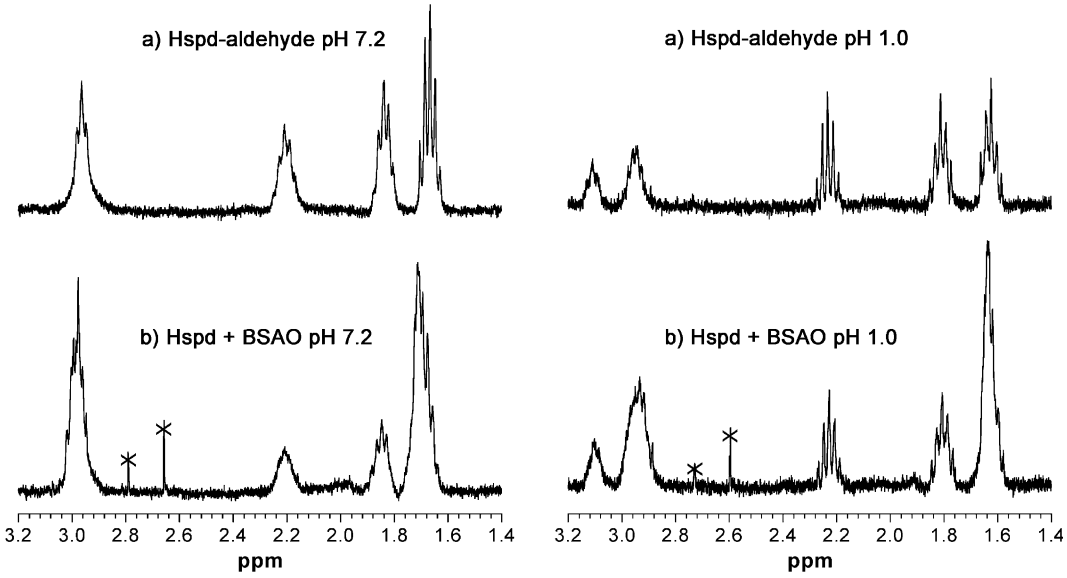


Figure 3. Oxidation of homospermidine. Proton NMR spectra (400 MHz) of: (a) synthetically prepared 'homospermidine aldehyde' (1-(4′-aminobutyl)-pyrrolinium dihydrochloride), pH 7.2 and 1.0 and (b) enzymatically oxidised homospermidine, pH 7.2 and 1.0.

Table 2. Cytotoxic effect towards human umbilical vein endothelial cells

Substrate	IC_{50} (μM)[a]	AG effect[b]
Spermine	3	>1000
Spermidine	14	>1000
Norspermidine	26	>1000
Homospermidine	>1000	ND
N-(2-Aminoethyl)-putrescine	>1000	ND
Histamine	>1000	ND
Propane-1,3-diamine	>1000	ND
Butane-1,4-diamine (Putrescine)	>1000	ND
Pentane-1,5-diamine (Cadaverine)	>1000	ND
Hexane-1,6-diamine	>1000	ND
Heptane-1,7-diamine	>1000	ND
Octane-1,8-diamine	>1000	ND
Decane-1,10-diamine	380	NE
Dodecane-1,12-diamine	50	NE
Benzylamine	>1000	ND
Octylamine	250	NE

[a] Cytotoxic effect (IC_{50}) of selected amines added to human umbilical vein endothelial cells (HUVEC) in the presence of bovine serum amine oxidase.

[b] AG, aminoguanidine; ND, not determined; NE, no effect.

to putrescine. This result presumably reflects that acrolein reacts promptly with amino groups present in the reaction mixture, that is, NH_3 and protein lysine side chains. To test this hypothesis we mixed acrolein (1 mM) with the same amount of enzyme as used for the enzymatic oxidation reactions. Control spectra recorded for 1 mM acrolein showed the signals characteristic for acrolein, but no signals were observed when acrolein had been mixed with enzyme (result not shown). This showed that all acrolein was transformed to products of a concentration too low to be observed in the NMR spectra.

3. Discussion and conclusion

3.1. The substrate specificity of BSAO

Copper-containing amine oxidases (CAOs) have been the subject of extensive biochemical, spectroscopic and kinetic studies for many years.[8,30–32] Crystal structure determinations of bacterial, yeast and plant CAOs have revealed these as mushroom-shaped homodimers with a close similarity between the active site structures of the enzymes.[33–41] The active site is buried inside the enzymes and the substrate access is provided by a channel leading from the outer surface. Negatively charged groups primarily surround the entrance to the channel, providing excellent conditions for trapping protonated amines. The channel becomes increasingly lipophilic as the active site is approached, favouring the unprotonated amines necessary for oxidation.

CAOs generally oxidise a wide variety of amines, but the relative rates and stereochemistries vary significantly from enzyme to enzyme.[8,30–32] These differences in substrate selectivity are not consistent with the close resemblance of the active sites, and Wilce et al.[38] suggest that the differences in catalytic activities to be caused by the channel leading from the outer surface to the active site. Comparisons of crystal structures[33–42] show a partial hindrance of the channel in *Escherichia coli* amine oxidase (ECAO) and a narrower channel in pea seedling amine oxidase (PSAO) compared to the more accessible channels of *Arthrobacter globiformis* amine oxidase (AGAO), *Hansenula polymorpha* amine oxidase (HPAO) and BSAO, implying that the internal structure of the channel is different in each protein. Furthermore, steric and electrostatic differences are observed near the channel entrance and along the channel enabling substrate differentiation.

Our results on the substrate specificity for BSAO (Table 1) are consistent with previous results[26,43–49] and confirm the existence of a substrate channel with a lipophilic interior and an attachment point for a secondary amino group with a spacing of 3–4 CH_2 groups to the primary amino group. Due to the lipophilic interior, an increased chain length confers increased substrate activity. Both straight-chain diamines and polyamines lacking the aminopropyl moiety are poor substrates when the spacing between the primary amino groups is less than eight atoms. This could indicate an attachment point fitting the aminooctyl moiety, but the fact that octylamine is a better substrate than octane-1,8-diamine shows that the lipophilic properties of the amine play a key role in the differentiation between good and poor substrates. The presence of moderately bulky substituents does not seem to have a major influence on the substrate activities, suggesting a flexible substrate channel able to accommodate a wide substrate variety. In particular, the high activity of BSAO towards *N*-(4-aminobutyl)hexahydropyrimidine renders this substrate the best in this investigation. The presence of a secondary amino group containing an aminopropyl moiety also enhances substrate activity due to the attachment point for the positive charge. For example, when comparing the substrate activities of norspermidine and (*N*-(2-aminoethyl)-putrescine), the former is by far the better substrate. Although both have a spacing of seven atoms between the two primary amino groups, only norspermidine contains the aminopropyl group. These results indicate the presence of an attachment point fitting the aminopropyl moiety near the active site. The fact that the hydrophilic propane-1,3-diamine is a poor substrate, even though it contains the aminopropyl moiety, can be explained from the general tendency in lipophilicity. The lack of a secondary amino group in the δ position also leads to reduced substrate activity (e.g., heptane-1,7-diamine vs norspermidine and octane-1,8-diamine vs spermidine). In conclusion, among straight chain amines both an aminopropyl group and two primary amino groups separated by seven or eight carbon or nitrogen atoms were required for optimal substrate ability, while for the lipophilic bulky hexahydropyrimidines a tetramethylene spacing between the primary and tertiary amino groups was superior to a trimethylene spacing. Di Paolo et al.[43] studied the kinetics of BSAO oxidation of spermine, spermidine, N^8-Ac-spermidine, 1,8-diaminooctane, nonylamine, butylamine, 1,3-diaminopropane and benzylamine, and concluded that the enzyme had a dual specificity reflecting a substrate channel with a

lipophilic interior and attachment points for positive charges corresponding to the geometry of the natural polyamines and this conclusion is supported by the X-ray crystal structure of the enzyme.[42] Apparently, the geometry of the substrate channel does allow bulky amines to reach the active site (e.g., benzyl amine) and also allows some secondary amines to react with the active site TPQ.[50,51]

3.2. Enzymatic reaction with spermidine and homospermidine monitored by NMR spectroscopy

The NMR method for identifying the major products of enzymatic oxidation is a time consuming but rewarding method. It is currently the only method of following the oxidation process with detection of unstable intermediates. The results show that NMR spectroscopy in 10% D_2O allows the monitoring of the major products hour by hour, at physiological concentrations, using the jump-return sequence or water presaturation. Analysing the spectra using COSY and spin decoupling identified the products arising from enzymatic oxidation of spermidine. Moreover, indication may appear in the spectra for the formation and structure of minor products not apparent after secondary transformations. The problems encountered using the above method, however, are considerable. In order to characterise the spectra of the aminoaldehydes adequately it may be necessary to prepare authentic samples. Since the spectra are recorded of extremely low concentrations in aqueous solution, they often lack information, for example, coupling constants. Signals close to the water signal are not observable, due to the inherent nature of the experiment.[52] It has proved important to use H_2O containing 10% D_2O in order not to deuterate exchangeable protons. For identification purposes it has furthermore proved useful to compare the product from enzymatic oxidation at a different pH even though the oxidation with the enzyme occurs at physiological pH. Thus, for homospermidine a much more informative spectrum is obtained by adjusting the pH of the solution to the acidic range and comparison with a synthetically prepared reference compound proved necessary.

3.3. Cytotoxicity of substrates for BSAO

An aminopropyl group was a prerequisite for cytotoxicity. Thus, although homospermidine was almost as good a substrate as spermine only the latter exhibited cytotoxicity. These results are in agreement with previous results obtained by others[9–27,53] and show that acrolein, and not the aminoaldehydes, is the main cytotoxic product. The lack of cytotoxicity of putrescine and homospermidine could in principle be due to cyclisation of the corresponding aminoaldehydes. The oxidation products of putrescine and homospermidine can both cyclise to five-membered rings at physiological pH,[54] whereas spermidine oxidised at N^1 does not cyclise due to the fact that the resulting four-membered ring would be too strained. The relative contribution of the various reaction products (NH_3, H_2O_2, aminoaldehydes and acrolein) has been the subject of several studies.[55–65] Acrolein, which has been isolated from reaction mixtures from BSAO oxidation of spermidine and spermine, has an extreme cytotoxicity[55,56,64–67] and has been shown to induce gene products promoting cell survival.[68] The fast reaction of acrolein with NH_3 and other amines explains the appearance of 3-aminopropanal as previously reported[26] and explains why no acrolein is observed since reaction with protein side chains leads to a heterogeneous mixture. The extreme cytotoxicity of acrolein also indicates why intracellular oxidation of polyamines takes place by acetylation and oxidation by polyamine oxidase (PAO). Apparently, it is imperative to prevent β-elimination and generation of acrolein. As an illustration of this, it has been shown that microinjection of CAOs causes cell death by a process resembling apoptosis.[69,70] Extracellularly, polyamines are present only at low concentrations. Therefore, CAOs presumably do not oxidise polyamines to any appreciable extent except in cases of cell death by apoptosis or necrosis, where the intracellular polyamines are released.

4. Experimental

4.1. General

All reagents and solvents were obtained from commercial suppliers and used without further purification. Hydroxylammonium chloride (99%) was from Riedel-de Haën. 4-Chlorobutyraldehyde diethylacetal is 98% pure from ABCR, Germany. *n*-Octylamine, 1 M Borane–tetrahydrofurane complex solution in THF, ~10 M Borane–methylsulfide complex solution and $LiAlD_4$ (98% Atom D) were obtained from Aldrich. Putrescine, spermidine, spermine, norspermidine, benzylamine, histamine, pentane-1,5-diamine, hexane-1,6-diamine, heptane-1,7-diamine, octane-1,8-diamine, dodecane-1,12-diamine, bis–tris, horseradish peroxidase (HRP), alkaline phosphatase-conjugated goat anti-mouse IgG, diethanolamine, *p*-nitrophenyl phosphate substrate tablets (*p*-NPP), α-Me-mannopyranoside, amino guanidine and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Decane-1,10-diamine was from Fluka (Buchs, Germany). Propane-1,3-diamine, $MgCl_2$, 37% HCl, NaCl, Na_2HPO_4, NaH_2PO_4 and Tween 20 were from Merck (Darmstadt, Germany). EAH Sepharose, Con A Sepharose, Q Sepharose Fast Flow and ECL reagents were from Amersham Pharmacia (Buckinghamshire, UK). Mouse anti-human ds DNA, TTN buffer (0.05 M tris buffer, pH 7.5, 0.3 M NaCl, 1% v/v Tween 20) and purified BSAO were from Statens Serum Institute (Copenhagen, Denmark). Recombinant human basic fibroblast growth factor (bFGF) and recombinant human vascular endothelial growth factor (VEGF) were from R & D Systems (Minneapolis, MN, USA). Human umbilical vein endothelial cells (HUVEC) and the HUVEC media-kit EGM-2 Bullet kit were obtained from Clonetics, BioWhittaker (Walkersville, MD, USA). Polyethylene terephthalate (PET) plates were from Wallac (Turku, Finland).

The ^1H NMR spectra were recorded at 25 °C on a Varian XL-400 FT NMR spectrometer operating at

400 MHz for ^{1}H and 100 MHz for ^{13}C , respectively, or on a Varian UNITY INOVA 800 spectrometer operating at 799.6 MHz for ^{1}H. The DQF-COSY spectra were obtained using the standard Varian pulse program tndqcosy. 400 MHz NMR experiments and synthesis of novel compounds were conducted at the University of Copenhagen. 800 MHz NMR spectra were obtained at the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules. The spectra are reported in δ units (ppm) relative to tetramethylsilane while the coupling constants, $J(H,H)$, are given in Hz. The largest solvent peak is used as reference, unless otherwise stated. Mass spectra were recorded on a JEOL JMS-HX/HX 110A Tandem Mass spectrometer. Samples for GC–MS were injected into a HP 5890 Gas chromatograph connected to a HP 5972 mass spectrometer. IR spectra were obtained on a Perkin–Elmer 1760X FT-IR spectrophotometer from KBr pellets or from a film. Elemental analyses were obtained on a Flash EA 1112 series elemental analyser. Purification of BSAO, luminescence and cytotoxic assays were performed at Statens Serum Institut. A Wallac 1450 Microbeta Trilux (Turku, Finland) was used as luminescence counter in the chemiluminescence assays.

4.2. The pH equilibrium of 1-(4′-aminobutyl)-pyrrolinium dihydrochloride investigated by ^{1}H NMR spectroscopy (Scheme 2)

The samples (0.6 mL) were prepared in NMR tubes (4 mm) using the following procedure, which ensures uniformity. A stock solution was prepared by dissolving 1-(4′-aminobutyl)-pyrrolinium dihydrochloride (113.5 mg, 0.532 mmol) in D_2O (9.7 mL) and adding DSS (2,2-dimethyl-2-silapentane-5-sulfonate, 0.5 mM) in D_2O (50 mM, 0.1 mL). One-half of the sample (4.9 mL) is transferred to a beaker fitted with the pH electrode and DCl is added (20%, 0.1 mL) to give a total volume of 5.0 mL. Next, the pH (close to 1) is measured accurately, 0.6 mL is placed in a NMR tube, and the ^{1}H NMR spectrum was recorded (400 MHz). D_2O (0.1 mL) is added to the remaining stock solution to a total volume of 5.0 mL. Transferring 0.6 mL of the stock solution to the beaker leaves the concentration unhanged and restores the volume to 5.0 mL. The pH can now be adjusted to a higher value with 12 M NaOD using capillary tubes without significantly changing the volume, and thus the concentration. Again, 0.6 mL is placed in a NMR tube, and the ^{1}H NMR spectrum was recorded. By repeating this procedure, ^{1}H NMR spectra covering the pH range 1–12 can be recorded. ^{1}H NMR spectra of the samples were recorded within 3 h after preparation, using 40 scans per spectrum. DSS is used as internal standard and the largest peak arising from DSS is referenced at 0.00 ppm. DSS is preferred to the readily available solvent peak HDO because of the narrow line width and chemical shift insensitivity to pH in the range 2–11.[71] pH values are calculated by adding 0.40 units[72] to the pH meter reading, obtained from a standard pH-meter calibrated to read pH in aqueous solutions. One of the alkaline samples was titrated to an acidic pH. The spectrum proved identical to that recorded for the acidic range demonstrating that the equilibrium is reversible.

4.3. Enzymatic reaction with spermidine investigated by ^{1}H NMR spectroscopy

The oxidation of a terminal $–CH_2NH_2$ group in spermidine with formation of the corresponding aminoaldehyde was followed by ^{1}H NMR spectroscopy (400 MHz). In order to mimic the physiological conditions as closely as possible very dilute solutions were used. In a NMR tube, an aqueous solution (54 μL) of spermidine trihydrochloride (10 mM, 0.078 mg) was mixed with a 10 mM sodium phosphate buffer (486 μL, pH 7.2), and D_2O was added (60 μL) to give a total of 600 μL solution containing 10% D_2O. After recording the spectrum BSAO (75 μL, 1.65 mg/mL, dialysed against a 5 mM phosphate buffer at pH 8.0 to remove bis–tris) was added. The oxidation proceeded rapidly initially but then subsided (30% conversion after 2 h, 40% after 5 h). Addition of two additional portions of enzyme with an interval of 2 h resulted in virtually complete oxidation of spermidine as evidenced by the lack of spermidine signals.

The oxidation was also monitored on a Varian Unity Inova 800 instrument using identical conditions except that spectra were recorded at 799.6 MHz at 25 °C in 5 mm tubes.

The ^{1}H NMR spectra indicated almost complete hydration of the initially formed aminoaldehyde in the dilute aqueous solution allowing for further NMR spectroscopic investigations necessary to analyse the spectra (spin decoupling, COSY). However, the small amount of free aminoaldehyde present in the equilibrium after 24 h gives rise to almost complete conversion to putrescine by elimination of acrolein. Signals which could be attributed to acrolein or its dimerisation or hydration products were not observed at any time although the oxidation was followed closely by recording an NMR spectrum for each hour. From the observation of a large number of small peaks scattered around in the final spectrum we conclude that acrolein is converted to a large number of products each with intensity too small to allow identification. Since addition of 1 equiv of acrolein to a solution of enzyme did not change the activity, reaction of acrolein with the active site can be excluded. Therefore acrolein probably is removed by reaction with, for example, free amino groups of the enzyme.

^{1}H NMR spectra were recorded in H_2O containing 10% D_2O with chemical shifts being referenced to the solvent peak ($\delta = 4.7$). The very strong water signal was suppressed using the jump-return procedure. In the jump-return sequence two 90° pulses are applied directly on the water signal with a delay time of 200 μs between the pulses. Alternatively a water presaturation for 1.5 s was used for the data at 800 MHz. The assignments of all spectra were confirmed by COSY spectra performed in the phase-sensitive mode combined with spin decoupling in order to resolve overlapping signals.

4.4. Enzymatic reaction with homospermidine investigated by ^{1}H NMR spectroscopy

In contrast to spermidine, the reaction of BSAO with homospermidine gives rise to an aminoaldehyde unable to undergo β-elimination (retro-Michael addition). The reaction was followed by ^{1}H NMR using the same jump-return sequence described previously. In a 4 mm NMR tube, an aqueous solution of 10 mM homospermidine trihydrochloride (75 μL) was mixed with 10 mM sodium phosphate buffer (pH 7.2, 415 μL) and D_2O (60 μL). Finally, purified BSAO (50 μL, 1.65 mg/mL) was added to yield a total volume of 600 μL. Furthermore, BSAO was added in portions of 50 μL at intervals of 7 h, amounting to a final volume of 750 μL. After 48 h, the reaction subsided (as monitored via ^{1}H NMR) resulting in approximately 40% conversion. Since substantial amounts of homospermidine were still present, direct assignment of the resulting spectrum proved difficult. However, the aminoaldehyde could be unambiguously identified by comparison with the spectra of synthetic 1-(4′-aminobutyl)-pyrrolinium dihydrochloride both at pH 7.2 and by acidification to pH = 1.

4.5. Enzyme activity assays

The amine was dissolved in 20 mM bis–tris (pH 7.0) to a concentration of 10 mM. HRP was dissolved in 20 mM bis–tris (10 mg/mL) and BSAO was diluted to 0.1 mg/mL in 20 mM bis–tris. HRP was diluted in ECL-2 solution to furnish a final concentration of 10 μg/mL and amines were added until the desired concentration was reached (0.1 mM). Reactions were then started by the addition of 10 μL BSAO (final concentration 0.01 mg/mL) to the substrate- and HRP-containing ECL-2 solution (90 μL). All measurements were carried out in 96-well PET plates, which were read immediately in the luminescence reader.

4.6. Purification of bovine serum amine oxidase

BSAO was purified from bovine serum essentially as described previously.[73] Bovine serum (915 mL) was stirred overnight at 5 °C with ammonium sulfate (209 g/L). The precipitate was collected by centrifugation (20 min, 16.300 g) and the supernatant stirred overnight at 5 °C with ammonium sulphate (129 g/L). The precipitate was collected by centrifugation as above, dissolved in 10 mM Na_2HPO_4 (170 mL, pH 8.0) and dialysed three times against 5 mM Na_2HPO_4 (pH 8.0). The dialysed solution was clarified by centrifugation and chromatographed on an EAH column (100 mL) eluted with stepwise increasing concentrations of Na_2HPO_4 (pH 8.0, 0.1 M, 0.2 M, 0.3 M, 0.4 M). The enzyme-containing fraction was located by activity assays as described above using spermine as substrate. Pooled fractions were chromatographed on a 100 mL con A Sepharose column, which was equilibrated and washed with 5 mM Na_2HPO_4 (pH 8.0). Enzyme was eluted by 0.3 M α-Me-mannopyranoside. Enzyme-containing fractions were located as above, pooled and dialysed against 5 mM Na_2HPO_4 (pH 8.0). The dialysed fractions were chromatographed on a Q Sepharose column, which was eluted with a stepwise gradient of NaCl (0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, 1 M) in 5 mM Na_2HPO_4 (pH 8.0). Enzyme activity was determined in fractions as above and fractions containing enzyme were pooled and dialysed against 5 mM Na_2HPO_4 (pH 8.0). A_{280} was measured to be 1.65 and the final specific activity using 1 mM benzylamine as substrate was found to be 0.96 μmol min mg^{-1} (20 °C, 20 mM bis–tris, pH 7.0).

4.7. Cell culture

4.7.1. Human umbilical vein endothelial cell propagation. Human umbilical vein endothelial cells (HUVEC) were maintained in culture at 37 °C, 5% CO_2, in an EGM-2 Bullet kit composed of endothelial cell basal medium-2 (EBM-2 medium) supplemented with ascorbic acid, foetal bovine serum (FBS), hydrocortisone, human basic fibroblast growth factor (hbFGF), human vascular endothelial growth factor (hVEGF), human epidermal growth factor (hEGF), long R insulin-like growth factor-1 (R^3-IGF-1), gentamicin sulfate (GA-1000) and heparin as described by the manufacturer (BioWhittaker).

When HUVEC were used for an experiment, the cells were seeded in 96-microwell polystyrene plates from Nunc (Kamstrup, Denmark) in a reduced medium called TFSM + 10%. This is composed of 500 mL EBM-2 medium supplemented with 0.55 mL ascorbic acid, 2.2% FBS, 0.55 mL gentamicin sulfate, 0.55 mL heparin (all from the HUVEC medium kit), 1.1 ng/mL hbFGF, and 11 ng/mL hVEGF.

4.7.2. Cytotoxicity assays. In each well of a 96-microwell plate, 1×10^3 HUVEC were seeded in 90 μL TFSM + 10% with or without the presence of 0.11 mM amino guanidine (TFSM + 10% and amino guanidine had been incubated over night at 4 °C). Immediately after seeding the cells each well was added 10 μL vehicle or diamine/polyamine resulting in a final concentration ranging from 1 to 1000 μM. After three days of culture at 37 °C and 5% CO_2 the medium was removed from the 96-microwell plate, and the cells were washed with phosphate-buffered saline (PBS) (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, $2H_2O$, 0.15 M NaCl, pH 7.3) for 1 min. All washings in this assay were carried out with 200 μL/well at room temperature on an orbital shaker. Incubations were performed with 100 μL/well. The cells were fixed with ice-cold 96% ethanol for 2 h at room temperature. This was followed by three washings with TTN buffer (50 mM tris, pH 7.5, 1% Tween 20, 0.3 M NaCl) and permeabilisation for 2 h at room temperature with 1% Triton X-100. The wells were washed three times with TTN buffer and blocked for 1 h with 200 μL TTN buffer. Cells were then incubated either overnight at 4 °C or for 1 h at room temperature with 100 μL mouse anti-human ds DNA diluted 1:2000 in TTN buffer. Subsequently, the cells were washed three times with TTN buffer, followed by 1 h of incubation with alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:1000 in TTN buffer. After being washed three times with TTN buffer the amount of detected

antigen was quantitated using 1 mg/mL *p*-nitro phenyl phosphate in alkaline substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8). The absorbance was read on a Versamax plate reader at 405 nm with background subtraction at 690 nm. After being washed three times with 200 μL Milli-Q water the cells were inspected in an Olympus CK2 microscope.

4.8. Synthesis

4.8.1. *N*-Benzyl-5-aza-nonanedinitrile. The dinitrile was prepared essentially by the method previously described.[74] However, substantial amounts (20–30%, GC–MS) of *N*-benzyl-2-pyrrolidone were formed by cyclisation and hydrolysis of the initially formed *N*-benzyl-4-aminobutanenitrile. This by-product was removed by taking the filtered reaction mixture to dryness, dissolving it in hydrochloric acid, and washing repeatedly with ether. The acidic mixture was then made basic with the addition of sodium carbonate and extracted with ether (3×100 mL). Evaporation of the solvent left the crude dinitrile still containing minor impurities (TLC). The crude product was purified by vacuum liquid chromatography (VLC) using heptane–ethylacetate as the eluent to give the pure dinitrile (50%) as a colourless oil (Found: C, 74.51; H, 8.00; N, 17.35. $C_{15}H_{19}N_3$ requires C, 74.65; H, 7.94; N, 17.41%); ν_{max} (film)/cm^{-1} 2245s (CN), 1602m, 1564w and 1494s; δ_H ($CDCl_3$) 1.76 (8H, q, $J(H,H) = 6.8$ Hz, CH_2), 2.32 (4H, t, $J(H,H) = 7.0$ Hz, CH_2), 2.52 (4H, t, $J(H,H) = 6.8$ Hz, CH_2), 3.51 (2H, s, CH_2Ph), 7.21–7.34 (5H, m, Ph); δ_C ($CDCl_3$) 14.3, 22.8, 51.8, 58.0 (CH_2Ph), 119.3 (CN), 126.8, 128.0, 128.3, 138.2; *m/z* (EI) 241 (M^+, 1%), 187 (33), 91 (100). Catalytic hydrogenation at atmospheric pressure and room temperature with PtO_2 in glacial acetic acid has been reported[72] to furnish homospermidine triacetate. However, complete conversion of both the benzyl and nitrile groups proved impossible without simultaneous formation of a considerable amount of polymeric material. This method was thus abandoned for the synthesis of larger quantities of homospermidine in favour of the following approach. However, the purified *N*-benzyl-5-aza-nonanedinitrile was still a convenient precursor for preparation of the deuterium labelled homospermidine.

4.8.2. 1-Acetamido-4-aminobutane. Following the reported procedure,[75] a mixture of 1,4-butanediamine (23.1 g, 0.262 mol) and ethylacetate (7.4 g, 0.084 mol) was sealed in a tube and heated to 100 °C for 24 h. Kugelrohr distillation afforded the pure compound (7.2 g, 65%), δ_H ($CDCl_3$) 1.25–1.40 (6H, m, CH_2CH_2 and NH_2), 1.79 (3H, s, CH_3CO), 2.54 (2H, t, $J(H,H) = 6.6$ Hz, CH_2NH_2), 3.03 (2H, q, $J(H,H) = 6.7$ Hz, CH_2NH), 6.96 (1H, s, NH); δ_C ($CDCl_3$) 22.6, 26.5, 30.4, 38.9, 41.3, 169.9 (CO); *m/z* (EI) 130 (M^+, 3%), 101 (90), 73 (88), 43 (100).

4.8.3. 1,4-Diacetamidobutane. The remanent from the distillation of 1-acetamido-4-aminobutane crystallised to give almost pure diacetylated product (2.0 g, 28%). Mp 137–138.5 °C (EtOAc); (Found: C, 55.49; H, 9.76; N, 16.27. $C_8H_{16}N_2O_2$ requires C, 55.79; H, 9.36; N, 16.27%); δ_H (DMSO-d_6) 1.35 (4H, m, CH_2), 1.77 (6H, s, CH_3), 3.0 (4H, m, CH_2NH), 7.81 (2H, s, NH); δ_C (DMSO-d_6) 22.6, 26.7, 38.2, 168.9 (CO); *m/z* (FAB) 173 ($[M+H]^+$).

4.8.4. 1,11-Diacetylhomospermidine. Essentially as previously reported,[75] 1-acetamido-4-aminobutane (5.5 g, 0.042 mol) was refluxed in toluene with dehydrated Raney-Ni (3 g) for 6.5 h to give the pure product in quantitative yield. Mp 74–75 °C (Et_2O–MeCN 95:5); (Found: C, 58.95; H, 10.39; N, 17.02. $C_{12}H_{25}N_3O_2$ requires C, 59.23; H, 10.36; N, 17.27%); ν_{max} (KBr)/cm^{-1} 3424vs br (NH), 1635vs (CO), 1567s (NH bend); δ_H (D_2O) 1.47 (8H, s), 1.92 (6H, s, CH_3CO), 2.60 (4H, s), 3.12 (4H, s); δ_C (D_2O) 22.5, 26.6, 38.8, 38.9, 48.7, 170.6 (CO); *m/z* (FAB, glycerol) 244 ($[M+H]^+$).

4.8.5. *N*-(4-Aminobutyl)-pyrrolidine. This compound was prepared using the two-step procedure previously reported.[27] The crude slightly yellow oil was purified by distillation under reduced pressure (120–140 °C/ 5–10 mbar) to yield a colourless oil. Spectroscopic data (^1H, ^{13}C NMR, MS) proved identical to the previously reported.[27]

4.8.6. *N*-(4-Aminobutyl)hexahydropyrimidine. This compound was prepared from spermidine essentially as described[76] except that formaldehyde was used in 10% excess to ensure complete conversion of spermidine. Distillation of the crude product (66–68 °C, 0.1 mbar) left a colourless oil (47%). However, after allowing it to stand for some time, colourless crystals (mp 42–44 °C) were secured. The product was very hygroscopic and analysed as $C_8H_{19}N_3$ with varying amounts of water. ν_{max} (KBr)/cm^{-1} 3316s, 3268m, 3215s and 3200s (νNH), 1614s (δNH_2); Other spectroscopic data (^1H, ^{13}C NMR, MS) proved identical to the previously reported.[76]

4.8.7. *N*-(3-Aminopropyl)hexahydropyrimidine. This compound was prepared from norspermidine using the same procedure as described for *N*-(4-aminobutyl)hexahydropyrimidine. The crude product was distilled in vacuo (59 °C/0.2 mbar) to yield a colourless oil (38%), which crystallised near room temperature, mp 33–34 °C. The product is extremely hygroscopic and analysed as $C_7H_{17}N_3$ with varying amounts of water. It rapidly liquefies when exposed to air but recrystallises when dried in vacuo over NaOH. ν_{max} (KBr)/cm^{-1} 3200–3400s (νNH), 1596s (δNH_2); δ_H ($CDCl_3$) 1.61 (2H, quintet, $J(H,H) = 5.5$ Hz, CH_2), 1.63 (2H, quintet, $J(H,H) = 7.1$ Hz, CH_2), 2.27 (3H, s, NH and NH_2), 2.31 (2H, t, $J(H,H) = 7.2$ Hz, NCH_2), 2.57 (2H, br s, NCH_2), 2.77 (2H, t, $J(H,H) = 6.8$ Hz, NCH_2), 2.81 (2H, t, $J(H,H) = 5.1$ Hz, NCH_2), 3.38 (2H, s, NCH_2N); δ_C ($CDCl_3$), 26.6, 29.9, 40.2, 44.6, 52.6, 52.8, 69.5; *m/z* (FAB) 144 (100%, $[M+H]^+$), 142 (64, $[M+H-H_2]^+$).

4.8.8. *N,N′*-Di-(methyl-d_3)-1,8-octanediamine dihydrochloride. This compound proved readily available from 1,8-octane-diamine using $LiAlD_4$ for reduction using the two-step procedure described by Skinner et al.[77] for the parent compound. Recrystallisation from EtOH

furnished white crystals, mp 240–241 °C; (Found: C, 47.64; H, 10.57; N, 11.12. $C_{10}H_{20}D_6Cl_2N_2$ requires C, 47.80; 'H', 10.43; N, 11.15%); ν_{max} (KBr)/cm^{-1} 2582m, 2363m (νCD_3), 1601m (δNH_2); δ_H (DMSO-d_6) 1.28 (8 H, m, CH_2), 1.59 (4H, quintet, $J(H,H)$ = 7.0 Hz, CH_2), 2.81 (4H, t, $J(H,H)$ = 8.0 Hz, NCH_2), 8.92 (4H, s, NH_2); δ_C (DMSO-d_6) 25.0, 25.2, 27.6, 31.5 (N-CD_3), 48.8 (N-CH_2); *m/z* (FAB) 179 (100%, $[M+H]^+$), 215 (52, $[M+H+HCl]^+$).

4.8.9. Homospermidine. In contrast to previous suggestions,[75] the diacetyl compound had to be heated under reflux with 6 M HCl for 9 h to ensure complete hydrolysis. After evaporation of the solvent the crude hydrochloride (4.1 g) was thoroughly mixed with finely powdered KOH (3 g) and distilled in a Kugelrohr apparatus to give pure homospermidine as a colourless oil. δ_H (DMSO-d_6) 1.3–1.4 (8H, m, CH_2CH_2), 2.42 (4H, t, $J(H,H)$ = 6.9 Hz, CH_2N), 2.48 (4 H, t, $J(H,H)$ = 6.6 Hz, CH_2N); δ_C (DMSO-d_6) 27.3, 31.5, 41.9, 49.7; *m/z* (FAB, glycerol) 160 ($[M+H]^+$).

4.8.10. Homospermidine dihydrochloride. An ethereal solution of homospermidine was treated with gaseous HCl to give the product as white crystals. Mp 230–233 °C (Et_2O); (Found: C, 41.16; H, 10.08; N, 17.95; Cl, 30.09. $C_8H_{23}Cl_2N_3$ requires C, 41.38; H, 9.98; N, 18.10; Cl, 30.54%); ν_{max} (KBr)/cm^{-1} 2800–3000vs, br (NH), 1631s, 1597s and 1557s (NH_3 and NH_2); δ_H (D_2O) 1.52 (8H, m, CH_2CH_2), 2.74 (4H, t, $J(H,H)$ = 7.3 Hz, CH_2N), 2.77 (4H, t, $J(H,H)$ = 7.1 Hz, CH_2N); δ_C (D_2O) 23.9, 25.6, 39.2, 47.2; *m/z* (FAB, glycerol) 160 ($[M+H]^+$).

4.8.11. Homospermidine trihydrochloride. When an ethanolic solution of homospermidine is treated with gaseous HCl, a white precipitate of homospermidine trihydrochloride is formed. Mp 278–280 °C (EtOH, Lit.[78] 283–285 °C, dec. on slow melting); (Found: C, 35.74; H, 9.13; N, 15.48; Cl, 39.60. $C_8H_{24}Cl_3N_3$ requires C, 35.77; H, 9.00; N, 15.64; Cl, 39.59%); ν_{max} (KBr)/cm^{-1} 2700–3100vs, br (NH), 1610s, 1598s, and 1516s (NH_3^+ and NH_2^+); δ_H (D_2O) 1.73 (8H, m, CH_2CH_2), 3.01 (4H, t, $J(H,H)$ = 7.0 Hz, CH_2N), 3.07 (4H, t, $J(H,H)$ = 7.6 Hz, CH_2N); δ_C (D_2O) 22.7, 23.9, 38.8, 46.8; *m/z* (FAB, glycerol) 160 ($[M+H]^+$).

4.8.12. *N*,*N*-Bis(4-amino-4,4-dideuteriobutyl)benzylamine. Following the reported procedure,[74] a solution of $AlCl_3$ (3.2 g, 24.0 mmol) in dry ether (75 mL) was added to a suspension of $LiAlD_4$ (1.03 g, 24.6 mmol) in dry ether (225 mL). The mixture was stirred under a nitrogen atmosphere for 15 min, followed by dropwise addition of *N*-Benzyl-5-aza-nonanedinitrile (2.69 g, 11.2 mmol) dissolved in dry ether (50 mL). Stirring was continued for 24 h at room temperature. After cooling to 0 °C the reaction mixture was quenched by dropwise addition of H_2O (10 mL) followed by 1 M HCl (150 mL). Stirring was continued until a clear slightly yellow solution was obtained. Slow addition of solid NaOH (6 g) gave a precipitate, which was separated from the mother liquor by filtration and washed repeatedly with ether. The combined ether extracts were dried over Na_2SO_4 and the solvent was evaporated to yield the product as a colourless oil (0.78 g, 28%), which was used without further purification in the subsequent step. *m/z* (FAB) 254.27 ($[M+H]^+$); *m/z* (EI) 193 (3%), 179 (6), 162 (16), 91 (100).

4.8.13. *N*,*N*-Bis(4-amino-4,4-dideuteriobutyl)amine trihydrochloride. *N*,*N*-Bis(4-amino-4,4-dideuteriobutyl)benzylamine (0.60 g, 2.37 mmol) dissolved in ethanol (30 mL) was hydrogenated for 22 h at atmospheric pressure using palladium hydroxide on carbon (0.36 g, 20% pH) as the catalyst. The catalyst was removed by filtration, and the clear ethanolic solution was treated with gaseous HCl to give the trihydrochloride as white colourless crystals. The trihydrochloride was collected on a sintered glass funnel, washed repeatedly with dry ether, and dried in an exsiccator over KOH. The pure trihydrochloride of the deuterated triamine (0.32 g, 50%) was obtained as fine white crystals, mp 279–281 °C (dec. on slow melting); (Found: C, 35.15; 'H', 9.05; N, 15.28; Cl, 38.73. $C_8H_{20}D_4Cl_3N_3$ requires C, 35.24; 'H', 8.87; N, 15.41; Cl, 39.00%); ν_{max} (KBr)/cm^{-1} 3426, 2948 (CH_2), 2020 (CD_2), 1763, 1688 and 1489 (CH_2); δ_H (D_2O) 1.73 (8 H, m, CH_2CH_2), 3.05 (4 H, t, CH_2N); δ_C (D_2O) 22.6, 23.6, 38.2(t), 46.8; *m/z* (FAB, glycerol + 5% TFA) 164.17 (100%, $[M(d_4)+H]^+$), 163 (7, $[M(d_3H)+H]^+$), 162 (3, $[M(d_2H_2)+H]^+$), 161 (<1, $[M(d_1H_3)+H]^+$), 160 (<1, $[M(H_4)+H]^+$). Approx. 96% deuterium incorporation is found (FAB).

4.8.14. 5,10-Diazadecanal diethyl acetal. A solution of 4-chlorobutyraldehyde diethyl acetal (5.0 g, 0.028 mol) was added to 1,4-diaminobutane (25 mL, 0.25 mol), which was stirred at 85 °C overnight. After cooling to rt, the mixture was slowly added to a stirred cold solution of 2 M NaOH (50 mL, 0.1 mol) and extracted with ether (3 × 25 mL). From the combined organic extracts the solvent is evaporated. The remanent is redissolved in cold 2 M NaOH (50 mL) and extracted with ether (3 × 25 mL). The combined organic extracts are dried over anhydrous K_2CO_3 followed by evaporation of the solvent. The crude oil is distilled in vacuo (115 °C/0.5 mbar) to yield the aminoacetal (1.36 g, 21%) as a hygroscopic colourless oil δ_H ($CDCl_3$) 1.02 (3H, br s, NH_2+NH), 1.05 (6H, t, $J(H,H)$ = 7.0 Hz, CH_3), 1.28–1.53 (8H, m), 2.46 (2H, t, $J(H,H)$ = 7.0 Hz), 2.47 (2H, t, $J(H,H)$ = 7.1 Hz), 2.55 (2H, t, $J(H,H)$ = 7.0 Hz), 3.34 and 3.49 (4H, dq, $J(H,H)$ = 9.5 Hz and 7.1 Hz, CH_2), 4.34 (1H, t, $J(H,H)$ = 5.5 Hz, H-1); δ_C ($CDCl_3$) 15.0 (CH_3), 25.0, 27.2, 31.1, 31.3, 41.8 (C-9), 49.4 and 49.5 (C-6 and C-4), 60.6 (CH_2), 102.4 (C-1); *m/z* (FAB, 3-nitrobenzyl alcohol) 233.12 ($[M+H]^+$); *m/z* (EI) 203 (22%, $M-C_2H_5$), 141 (11), 84 (100), 72 (70), 70 (64), 43 (47), 30 (77).

4.8.15. 1-(4′-Aminobutyl)-pyrrolinium dihydrochloride. 5,10-Diazadecanal diethyl acetal (0.625 g, 2.93 mmol) was hydrolysed in water (20 mL) with 2 M HCl added (3.25 mL, 6.5 mmol) for 25 min at rt. Lyophilisation for two days furnished hygroscopic white amorphous crystals, which were used without further purification. δ_H (D_2O) 1.74 (2H, m, H-3′), 1.93 (2H, m, H-2′), 2.35 (2H, quintet, $J(H,H)$ = 8.1 Hz, H-4), 3.06 (2H, br t, $J(H,H)$ = 7.7 Hz, H-4′), 3.22 (2H, br t, $J(H,H)$ =

7.0 Hz, H-3), 3.98 (2H, br t, $J(H,H)$ = 7.5 Hz, H-1′), 4.21 (2H, tq, $J(H,H)$ = 8.1 Hz and 1.8 Hz, H-5), 8.73 (1H, m, $J(H,H)$ = 1.5 Hz, H-2); δ_C (D_2O) 19.1 (C-4), 23.2 and 23.5 (C-2′ and C-3′), 35.6 (C-3), 38.6 (C-4′), 53.0 (C-1′), 58.7 (C-5), 181.3 (C-2); *m/z* (FAB, 3-nitrobenzyl alcohol) 141.11 ($[C_8H_{16}N_2+H]^+$). Analyses as 1,6-diazabicyclo[5.3.0]decane in gas phase.

4.8.16. 1,6-Diazabicyclo[5.3.0]decane. 2 M HCl (5.92 mL, 11.84 mmol) was added to a solution of 5,10-diazadecanal diethyl acetal (1.25 g, 5.38 mmol) in water (25 mL). The solution was stirred for 25 min at rt, followed by addition of 1 M K_2CO_3 (25 mL). Extraction with CH_2Cl_2 (3 × 25 mL), followed by drying over K_2CO_3, and evaporation of the solvent afforded the bicyclic aminal (0.47 g, 62%) as a colourless oil. δ_H ($CDCl_3$, 0 °C) 1.40–1.50 (1H, m), 1.60–1.87 (6H, m), 1.94–2.30 (1H, vbr s, NH), 2.12–2.22 (1H, m), 2.44–2.54 (2H, m), 2.73–2.81 (1H, m), 2.88–3.02 (3H, m), 3.61 (1H, t, $J(H,H)$ = 7.3 Hz, H-1); δ_C (75 MHz, $CDCl_3$) 22.0, 26.7, 30.4, 33.9, 47.3, 53.7, 54.6, 78.9; δ_H (D_2O, 2 °C) 1.10 and 1.83 (2H, br s, H-2), 1.28 and 1.40 (6H, br s, H-3, H-7, H-8), 2.25 and 2.42 (4H, br s, H-4, H-6), 2.25 and 2.59 (2H, br s, H-9), 3.37 (1H, br t, $J(H,H)$ = 6.4 Hz, H-1); δ_C (D_2O, 2 °C) 21.5 and 25.4 and 28.8 (C-3, C-7, C-8), 32.4 (C-2), 46.5 (C-9), 51.9 and 53.4 (C-4, C-6) 77.4 (C-1); δ_C (75 MHz, CD_3OD) 23.4, 27.7, 31.0, 34.5, 48.7, 54.3, 55.7, 80.1; *m/z* (EI) 140 (M^+, 65%), 139 (59, M–H), 83 (100).

4.8.17. Boc-glycine. Boc-glycine was prepared from glycine and di-*tert*-butyldicarbonate essentially by the procedure described,[79] scaled to 1:10. Recrystallisation yielded pure white crystals (89%), mp 87–88 °C. The presence of both the *E*- and *Z*-isomers of the amide is observed (^1H and ^{13}C NMR). (Found: C, 47.91; H, 7.55; N, 8.08. $C_7H_{13}NO_4$ requires C, 47.99; H, 7.48; N, 8.00%); ν_{max} (KBr)/cm^{-1} 3500–2400s (*ν*COOH), 3407m, 3343s (*δ*NH), 1749vs (*ν*COOH), 1668vs (*ν*CONH), 1541vs (*δ*NH), 1411s, 1369m, 1215vs, 1198vs, 1058m, 959m, 860m; δ_H ($CDCl_3$) 1.43 (9H, s, CH_3), 3.86 and 3.93 (2H, s, CH_2), 5.23 and 6.65 (1H, s, NH), 10.05 (1H, s, COOH); δ_C ($CDCl_3$) 28.1, 42.1 and 43.2, 80.3 and 81.7, 155.9 and 157.1, 173.8 and 174.4; *m/z* (EI) 160 (2%, M−CH_3), 130 (11, M−COOH), 120 (60), 116 (4, M−CH_2COOH), 102 (5, M−OC$(CH_3)_3$), 76 (21), 59 (90, M−$(CH_3)_3$COCONH), 57 (100, M−O_2CNCH_2COOH), 41 (40), 29 (24).

4.8.18. Boc-putrescine. Using the previously described procedure[80] Boc-putrescine was prepared from putrescine and di-*tert*-butyldicarbonate (83%). The colourless oil was shown to be pure by GC–MS but too hygroscopic for elemental analysis. ν_{max} (KBr)/cm^{-1} 3363vs, 2934vs, 2175w, 1688vs, 1525vs, 1365s, 1173vs, 1042m, 993m, 868m; δ_H ($CDCl_3$) 1.3–1.5 (13H, m, CH_3+2 × CH_2), 2.07 (2H, s, NH_2), 2.64 (2H, t, $J(H,H)$ = 6.6 Hz, N–CH_2), 3.03 (2H, t, $J(H,H)$ = 6.6 Hz, N–CH_2), 4.88 (1H, s, NH); δ_C ($CDCl_3$) 27.3, 28.2, 30.1, 40.2, 41.3, 78.7, 155.9; *m/z* (EI) 188 (0.1%, M^+), 143 (2), 132 (28), 115 (22, M−OC$(CH_3)_3$), 114 (19), 103 (19), 98 (17), 74 (33), 70 (64), 57 (79, $(CH_3)_3C$), 59 (58), 41 (45), 30 (100, CH_2=NH_2).

4.8.19. *N*-(Boc-glycyl)-*N*′-Boc-putrescine. Boc-putrescine (5.65 g, 30 mmol) dissolved in dichloro-methane (300 mL) was added slowly to a stirred mixture of Boc-glycine (5.35 g, 30 mmol) and dicyclohexylcarbodiimide (6.80 g, 33 mmol) cooled to 0 °C. The stirred mixture was kept at 0 °C for 1 h and then at ambient temperature for 24 h, whereupon the precipitated dicyclohexylurea was filtered off and washed with dichloromethane. Washing with saturated $NaHCO_3$ (30 mL), cold 2 M $NaHSO_4$ (30 mL) and water (2 × 30 mL) ensured removal of unreacted Boc-glycine and Boc-putrescine. The organic phase was dried over $MgSO_4$ and the solvent evaporated. Minor impurities of dicyclohexylurea were removed by dissolving the residue in ether followed by filtration and evaporation. Recrystallisation from ether afforded the desired compound as colourless crystals (8.16 g, 79%), mp 79–80 °C. (Found: C, 55.67; H, 9.21; N, 12.08. $C_{16}H_{31}N_3O_5$ requires C, 55.63; H, 9.05; N, 12.16%); ν_{max} (KBr)/cm^{-1} 3349vs and 3267vs (*ν*NH), 3053m, 2978s, 2940m, 2871w, 1718vs (*ν*CO), 1695vs (*ν*CO), 1657vs (*ν*CO), 1555 (*δ*NH), 1381s, 1364m, 1294vs, 1251vs, 1181vs, 1134s; δ_H ($CDCl_3$) 1.37 (18H, s, CH_3), 1.44 (4H, m, 2 × CH_2), 3.04 (2H, t, $J(H,H)$ = 6.1 Hz, CH_2–N), 3.25 (2H, q, $J(H,H)$ = 5.3 Hz, CH_2–N), 3.71 (2H, s, N–CH_2–CON), 4.72 (1H, s, NH), 5.40 (1H, s, NH), 6.57 (1H, s, NH); δ_C ($CDCl_3$) 26.4, 27.3, 28.2, 39.0, 40.0, 44.2, 79.1, 80.0, 156.0, 169.4; *m/z* (FAB) 346 ($[M+H]^+$), 691 ($[(2 \times M)+H]^+$).

4.8.20. *N*-(2-Aminoacetyl)-putrescine dihydrochloride. *N*-(Boc-glycyl)-*N*′-Boc-putrescine (4.14 g, 12 mmol) was dissolved in dichloromethane (40 mL), cooled to 0 °C and treated with TFA (9.18 mL, 0.12 mol). The reaction mixture was stirred at room temperature for 30 min and then evaporated to dryness leaving a slightly pink oil. In order to remove excess TFA, toluene (50 mL) was added and subsequently evaporated at reduced pressure. This procedure was repeated three times. The resulting oil was dissolved in HCl-saturated methanol followed by slow addition of HCl-saturated diethyl ether causing precipitation of the hydrochloride to occur. The white precipitate was filtered off, washed with ether and dried at reduced pressure over NaOH pellets. Recrystallisation from methanol gave white needle-like crystals (1.91 g, 73%), mp 183–184 °C. (Found C, 33.08; H, 7.89; N, 19.06. $C_6H_{17}Cl_2N_3O$ requires C, 33.04; H, 7.86; N, 19.26%); ν_{max} (KBr)/cm^{-1} 3500–2200vs (νNH_3^+), 3252vs (*ν*NH), 1969m, 1679vs (*ν*CO); δ_H (DMSO-d_6) 1.46 (2H, m, CH_2), 1.59 (2H, m, CH_2), 2.74 (2H, m, CH_2), 3.10 (2H, m, CH_2), 3.51 (2H, s, N–CH_2–CO), 8.21 (3H, s, NH_3^+), 8.29 (3H, s, NH_3^+), 8.70 (1H, t, $J(H,H)$ = 5.7 Hz, NH); δ_C (DMSO-d_6) 24.3, 25.8, 38.0, 38.3, 40.1, 165.8; *m/z* (FAB) 146 ($[M+H]^+$).

4.8.21. *N*-(2-Aminoethyl)-putrescine trihydrochloride. A suspension of *N*-(2-aminoacetyl)-putrescine dihydrochloride (1.45 g, 6.6 mmol) in dry THF (135 mL) was added slowly to a stirred solution of borane–THF complex (1.0 M, 75 mL, 75 mmol) under nitrogen. The reaction mixture was refluxed for 48 h and cooled to room temperature, whereupon it was quenched by slow addition of methanol (180 mL) followed by reflux overnight.

After filtration and evaporation of the solvents the crude products were dissolved in methanol (100 mL), cooled to 0 °C and HCl-saturated methanol (150 mL) was added. Evaporation of methanol and excess hydrogen chloride left a mixture of white crystals and yellowish oil. It was found that the starting materials had only partially reacted (^{1}H NMR), and the crude product required recrystallisation from methanol in three turns to provide pure *N*-(2-aminoethyl)-putrescine trihydrochloride as white crystals (0.51 g, 32%), mp 217–218 °C. (Found C, 30.02; H, 8.34; N, 17.14. $C_6H_{20}Cl_3N_3$ requires C, 29.95; H, 8.38; N, 17.46%); ν_{max} (KBr)/cm^{-1} 3500–2400vs (νNH_3^+ and νNH_2^+), 2049m, 1865w, 1600s, 1521s, 1466s, 1396m, 1345m, 1028s, 879m, 815m; δ_H (D_2O) 1.75 (4 H, m, $2\times CH_2$), 3.01 (2H, t, $J(H,H) = 7.0$ Hz, CH_2–N), 3.15 (2H, t, $J(H,H) = 7.0$ Hz, CH_2–N), 3.39 (4H, m, $2\times CH_2$–N); δ_C (D_2O) 22.5, 23.6, 35.2, 38.6, 43.9, 47.2; *m/z* (FAB) 132 ([M+H]$^+$).

4.8.22. Decane-2,9-dione dioxime. Pure decane-2,9-dione was prepared in two steps using the procedure previously described[81] and used as starting material. Decane-2,9-dione (5.45 g, 32 mmol) and hydroxylammonium-chloride (7.02 g, 0.1 mol) were dissolved in 60% ethanol (50 mL) and cooled to 0 °C. Potassium carbonate (13.8 g, 0.1 mol) was added to the stirred mixture that was allowed to return to rt, followed by reflux for 3 h. Addition of water (100 mL) followed by cooling to 0 °C led to precipitation of the crude product, which was filtered off and washed with water. The remaining product was extracted with ether (3×50 mL). The ether phase was dried over $MgSO_4$, followed by evaporation of the solvent. The extracted and the precipitated crude products were combined and recrystallised from water–ethanol (60:40) to yield pure white crystals (3.95 g), mp 129–133 °C. Additional pure product (1.35 g, mp 124–131 °C) could be secured by evaporation of the mother liquor and recrystallisation of the remanent. The difference in melting points of the first and second crop was ascribed to the presence of more than one isomer of the dioxime. (total yield, 5.30 g, 83%). (Found: C, 59.78; H, 10.10; N, 13.96. $C_{10}H_{20}N_2O_2$ requires C, 59.97; H, 10.07; N, 13.99%); ν_{max} (KBr)/cm^{-1} 3234s br (νOH), 2926vs, 2883s, 2852s, 2824m (νCH), 1679m (νC=N), 1462s (δCH_2), 1411s (δOH), 1368s (δCH_3), 951vs (νN–O), 729s (ρCH_2); δ_H (DMSO-d_6) 1.24 (4H, m, CH_2), 1.42 (4H, m, CH_2), 2.08 (4H, t, $J(H,H) = 7.2$ Hz, CH_2–CNOH), 10.18 (2H, s, OH); δ_C (DMSO-d_6) 13.0, 25.8, 28.4, 35.0, 155.1; *m/z* (EI) 200 (1%, M$^+$), 183 (14, M−OH), 168 (39), 167 (46), 150 (9), 128 (100, M−C_3H_6NO), 100 (10), 86 (42), 73 (95, M−$C_7H_{13}NO$), 55 (36), 42 (50), 41 (51).

4.8.23. Decane-2,9-diamine. Decane-2,9-dione dioxime (2.00 g, 10 mmol) dissolved in THF (80 mL) was slowly added to an ice-cold solution of ~10 M boran–dimethylsulfide complex (12 mL, 120 mmol) under nitrogen. The mixture was heated to rt and refluxed for 24 h, whereupon it was cooled to 0 °C and quenched with ethanol (120 mL). The THF–ethanol solution was then refluxed for 6 h, followed by slow addition of an aqueous solution of 2.3 M KOH (60 mL, 140 mmol) and refluxed for an additional 12 h. The organic solvents were removed by evaporation and the remaining water phase was extracted with ether (4×40 mL). The combined extracts were dried over $MgSO_4$ followed by evaporation of the solvent. The crude product was mixed with finely powdered KOH and distilled using Kugelrohr to obtain the pure product as a colourless oil (1.07 g, 58%). The compound was too hygroscopic for elemental analysis. ν_{max} (KBr)/cm^{-1} 3325s and 3260s (νNH_2), 2955s, 2925s, 2854s, 1592s (δNH_2), 1461s, 1373s, 1152m, 1075m (νCN), 819m br, 724m; δ_H (DMSO-d_6) 2.71 (2H, sextet, $J(H,H) = 6.3$ Hz, CH), 1.59 (4H, br s, NH_2), 1.17–1.27 (12H, m, CH_2), 0.93 (6H, d, $J(H,H) = 6.3$ Hz, CH_3); δ_C (DMSO-d_6) 46.3, 39.9, 29.4, 25.9, 24,1; *m/z* (EI) 171 (<1%, M−H), 157 (3, M−CH_3), 145 (2, M−HCN), 129 (6, $C_8H_{19}N$), 112 (4), 70 (9), 56 (12), 44 (100, C_2H_6N), 30 (6, CH_2=NH_2).

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