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Polyamine modification by acrolein exclusively produces 1,5-diazacyclooctanes: a previously unrecognized mechanism for acrolein-mediated oxidative stress†

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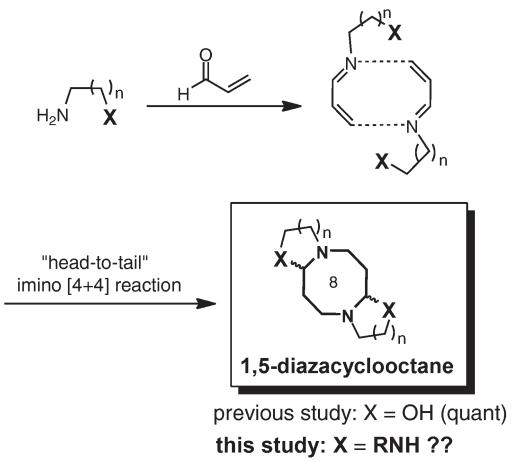
Acrolein, a toxic unsaturated aldehyde generated as a result of oxidative stress, readily reacts with a variety of nucleophilic biomolecules. Polyamines, which produced acrolein in the presence of amine oxidase, were then found to react with acrolein to produce 1,5-diazacyclooctane, a previously unrecognized but significant downstream product of oxidative stress. Although diazacyclooctane formation effectively neutralized acrolein toxicity, the diazacyclooctane hydrogel produced through a sequential diazacyclooctane polymerization reaction was highly cytotoxic. This study suggests that diazacyclooctane formation is involved in the mechanism underlying acrolein-mediated oxidative stress.

Introduction

Acrolein is a highly toxic unsaturated aldehyde[1] that can be produced during the burning of oils, charcoal, wood, or plastic. It can also be generated by cells under oxidative stress conditions (through the enzymatic oxidation of threonine or polyamines[2–4]) or during reactive oxygen species (ROS)-mediated oxidation of highly unsaturated lipids.[5] The unsubstituted and most reactive 2-alkenal produced through the latter pathway can react with nearby thiol, hydroxyl, or amino functional groups on DNA,[6] proteins,[7] or phosphatidyl ethanolamines to accelerate the oxidative stress processes associated with various disease states.[8,9] Studies of acrolein conjugates could, therefore, contribute to an understanding of the relationship between acrolein and oxidative stress and, hence, disease at a molecular level.

Acrolein conjugates are currently used as biomarkers of oxidative stress[7] in the context of a variety of diseases. Acrolein–amino conjugates involving, for example, lysine δ-amino groups, 3-formyl-3,4-dehydropiperidine (FDP),[7] or 3-methylpyridinium (MP) derivatives[10] have been described. Antibodies[11] to these conjugates are widely used for the immunochemical detection of various disease states, such as arteriosclerosis,[11–14] Alzheimer's disease,[15,16] tumors,[17–21] diabetes,[22–26] autoimmune



Scheme 1 1,5-Diazacyclooctanes are previously unrecognized products of the reaction of polyamines with acrolein. Proposed reaction mechanism governing the OH- and amine-mediated "head-to-tail" imino [4 + 4] reaction of unsaturated imines.

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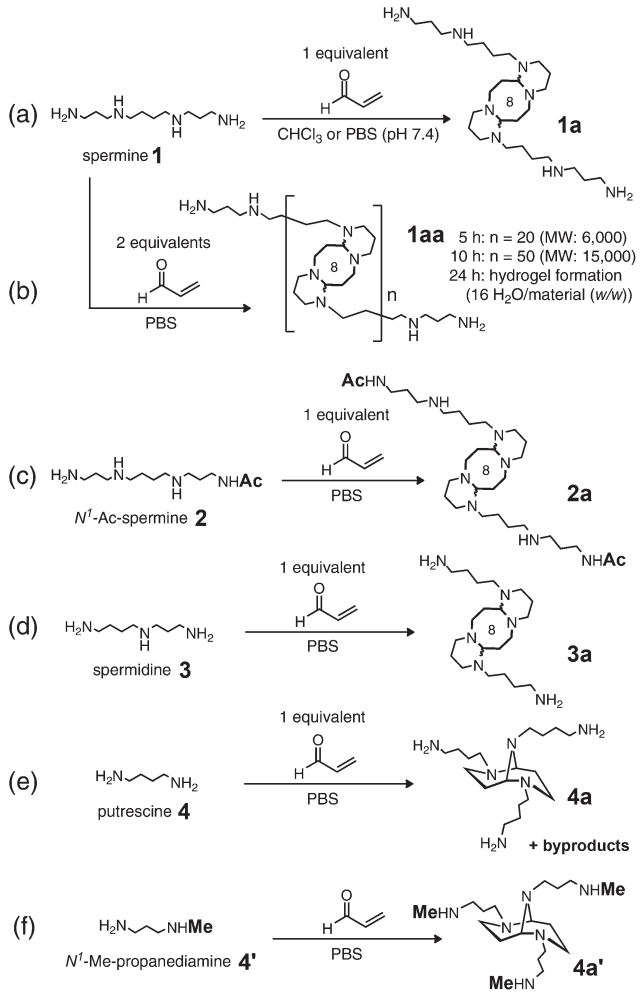
disease,[27,28] high blood pressure,[29] and so on.[30–34] Alternatively, acrolein can react with polyamines to produce FDP conjugates that modulate the cytotoxicity of acrolein.[9]

In our research program, which explores the novel reactivities of imines,[35–38] we recently found by chance that the unsaturated *N*-alkylimines participated in the hitherto unknown "head-to-tail" [4 + 4] dimerization in the presence of hydroxylated alkyl groups on the imino nitrogen (Scheme 1).[39] The reaction readily provided eight-membered heterocycles, the 1,5-diazacyclooctanes, at micromolar concentrations. The nucleophilic hydroxyl groups on the imino nitrogen (X = OH in Scheme 1) accelerated and stabilized 1,5-diazacyclooctane formation. Based on the reactivity profiles, we hypothesized that amino groups (X = RNH in Scheme 1) could similarly form diazacyclooctanes. For example, these results suggested that polyamines could react with acrolein to provide diazacyclooctanes through the [4 + 4] dimerization of the imines, as illustrated in Scheme 2. Considering that acrolein is produced as a polyamine metabolite by amine oxidase[2–4] during oxidative stress processes, the diazacyclooctanes (acrolein-modified polyamines), if formed, may be involved in the mechanisms underlying acrolein-mediated oxidative stress.

In this study, we investigated the reactions of the polyamines with acrolein. We revealed for the first time that 1,5-diazacyclooctanes are the exclusive and previously unrecognized products of polyamine modification by acrolein. The cytotoxicity, effects on oxidative stress, and oxidative metabolism of the acrolein-modified polyamines were evaluated in an effort to elucidate the mechanism underlying acrolein-mediated oxidative stress.



Scheme 2 Reaction of various polyamines, at concentrations ranging from 10 μM to 100 mM, with acrolein in either chloroform ($CHCl_3$) or PBS buffer at room temperature. (a) Reaction of spermine **1** with 1 equivalent acrolein to provide the 1,5-diazacyclooctane **1a**. (b) The reaction of spermine **1** with 2 equivalents acrolein gradually produced the 1,5-diazacyclooctane polymers **1aa**, *i.e.*, the polymers with molecular weights of *ca.* 6000 after 5 h, 15 000 after 10 h, and an insoluble hydrogel that adsorbed 16 g water g^{-1} material (w/w) after 24 h. The molecular weight was estimated using diffusion ordered NMR spectroscopy (DOSY) techniques. (c) The reaction of N^1-acetyl spermine **2** with 1 equivalent acrolein yielded 1,5-diazacyclooctane **2a**. (d) The reaction of spermidine **3** with 1 equivalent acrolein provided 1,5-diazacyclooctane **3a**. (e) The reaction of putrescine **4** with 1 equivalent acrolein provided a mixture of products that contained the 2,6,9-triazabicyclo[3.3.1]nonane **4a** as the main product (in approximately 50% yield, based on the ^{1}H NMR results). (f) Quantitative reaction of N^1-Me-propanediamine with 0.7 equivalents acrolein to provide the caged compound **4a**′.

Results and discussion

The reaction of spermine **1**, N^1-Ac-spermine **2**, and spermidine **3** with 1 equivalent acrolein at room temperature smoothly produced the corresponding eight-membered diazacyclooctane derivatives **1a–3a** within 15 min (Scheme 2(a)–(d)). These products could exist as a 1 : 1 mixture of the diastereomers at the acetal stereogenic centers, which are interconvertible with each other under equilibrium conditions. It should be noted that the reaction proceeded in both chloroform and PBS buffer with similar efficiencies and at a concentration of 100 μM; that is, the reactivity was tested under biologically relevant concentrations of polyamines present in and on cells.[40–42] Although an MS analysis of the crude mixture detected small quantities of the FDP derivatives among the starting polyamines, the 1,5-diazacyclooctane derivatives were generated exclusively, *i.e.*, in more than 80% yield, and very rapidly under mild conditions, as shown in Scheme 2. The structures of 1,5-diazacyclooctane were unambiguously determined for the spermine derivative **1a** using MS, ^{1}H, ^{13}C, and various 2D NMR techniques. The spectra of the derivatives **2a** and **3a** were compared with those of **1a**. The structurally more simple diamine, putrescine **4**, on the other hand, gave a mixture of products, approximately 50% of which comprised the bridged irreversible 2,6,9-triazabicyclo[3.3.1]nonane structure **4a** (Scheme 2(e)). The reactivity of acrolein toward the simple diamine was similar to that of N^1-Me-propanediamine **4**′ (Scheme 2(f)), which smoothly produced the caged product **4a**′ in quantitative yield, as reported previously.[43]

The reaction of spermine **1** with 2 equivalents acrolein in PBS (Scheme 2(b)) resulted in a [4 + 4] polymerization reaction between the two imines generated at the two amino termini of

(a)

compounds	IC_{50} (μM)	IC_{50} (μM) (+ aminoguanidine: 1 mM)
acrolein	17.2	14.9
spermine **1**	2.5	> 50
acrolein-spermine **1a**	0.14	> 50
N^1-Ac-spermine **2**	11.6	> 50
acrolein-N^1-Ac-spermine **2a**	15.5	> 50
spermidine **3**	11.8	> 50
acrolein-spermidine **3a**	4.6	> 50
N^1-Me-propanediamine **4'**	> 50	> 50
acrolein-N^1-Me-propanediamine **4a'**	14.5	22.0

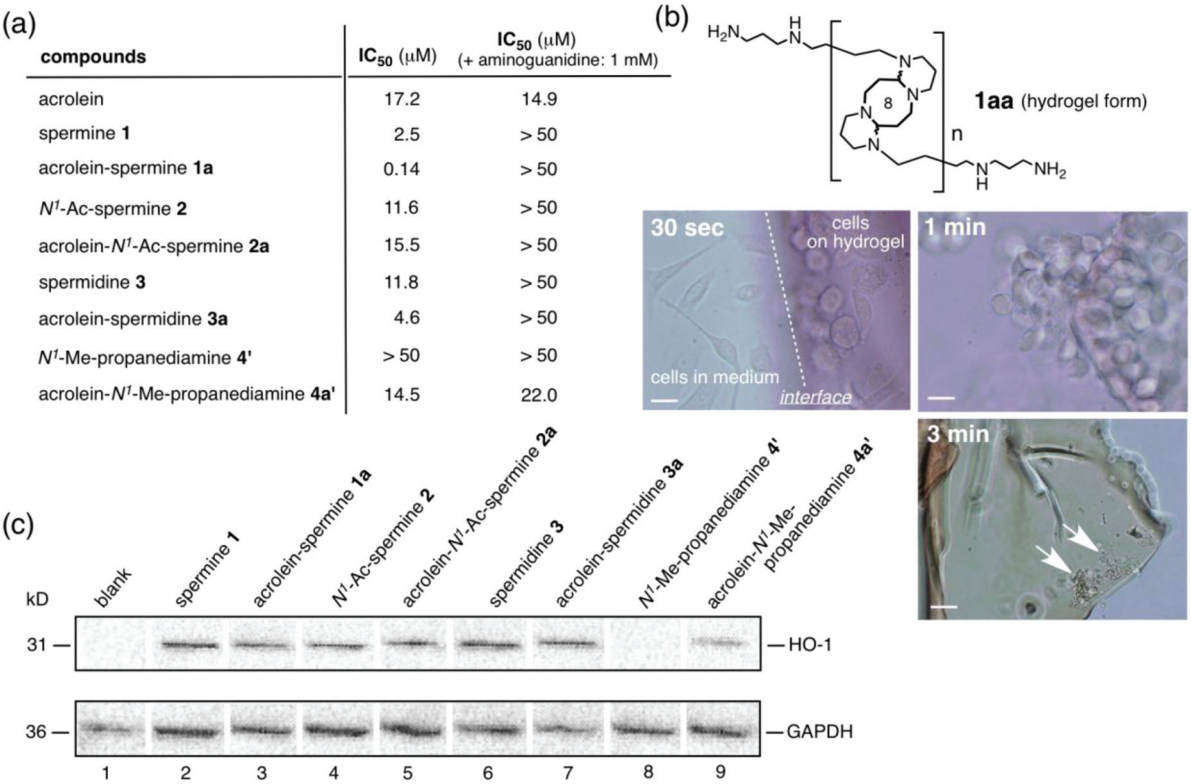


Fig. 1 Cytotoxic activities and effects on oxidative stress of polyamines and their acrolein-modified products. (a) Cytotoxic activities of the polyamines, the 1,5-diazacyclooctanes, and the 2,6,9-triazabicyclo[3.3.1]nonane derivatives **1–4a'**. HeLa cells were treated with compounds **1–4a'** for 72 h at 37 °C, and the cytotoxicities were evaluated using the MTS method. For the amine oxidase inhibition experiments, 1 mM of the aminoguanidine was applied. The acrolein–putrescine conjugate, *i.e.*, the caged compound **4a**, was obtained as a mixture with other byproducts, and the pure **4a** was not isolated; therefore, the cytotoxicity and the oxidative stress activities were evaluated using a similar caged compound, **4a'**, which was obtained as a single product from the corresponding N^1-Me-propanediamine. Reference IC_{50} of putrescine **4**: >50 μM both in the presence and absence of aminoguanidine; IC_{50} of **4a** (mixtures of the products): 17.6 and 28.2 μM, respectively, in the absence and presence of aminoguanidine. (b) HeLa cells treated with the diazacyclooctane hydrogel **1aa** in the medium at 37 °C. The cells immediately adhered to the hydrogel and were lysed within a few minutes. The dashed line shows the interface between the cells in the medium and those that adhered to the hydrogel. The scale bars indicate 10 μm for the images collected at 30 s or 1 min and 100 μm for the image collected at 3 min. (c) Western blots of the cell lysates using *anti*-HO-1 (upper) and *anti*-GAPDH (lower) antibodies after treatment with **1–4a'** in the absence of aminoguanidine. HO-1: heme oxygenase-1. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

spermine. This reaction produced the diazacyclooctane polymers **1aa** with an average molecular weight of 6000 after 5 h and of 15 000 after 10 h (estimated based on diffusion ordered NMR spectroscopy (DOSY) methods,[44] ESI Fig. S2†). Interestingly, the polymerization reaction ultimately produced an insoluble hydrogel that contained 16 water molecules in the material (w/w) (see the pictures in Fig. 1(b)).

Acrolein may be generated along the polyamine metabolic pathway by amine oxidase.[2–4] Polyamines are also degraded to acrolein when they are released from the cells and exposed to serum amine oxidase. The treatment of spermine **1** with serum amine oxidase[4] did not produce detectable levels of the [4 + 4] dimerization products, *i.e.*, diazacyclooctanes, because these products were further oxidized by the enzyme (*vide infra*). Nevertheless, the enzymatic reaction ultimately provided a suspension of the polymers over 24 h that displayed physiological properties that were very similar to those of the hydrogel **1aa**. Although conclusive solid-phase NMR spectroscopic data could not be obtained, the enzymatic oxidation reaction with polyamine mediated the polymerization reaction.

The cytotoxicities of the 1,5-diazacyclooctanes products **1a–4a'** and their effects on oxidative stress were evaluated in cell-based assays using HeLa and A549 (human lung adenocarcinoma epithelial) cells (Fig. 1). The putrescine-derived **4a** was obtained in a mixture with other byproducts and could not be further purified; therefore a similar caged product **4a'** was used in place of **4a**. The compounds exhibited similar effects on both cell types. The data from the HeLa cell assay are provided in Fig. 1 (cytotoxic activities on A549 cells are provided in ESI Table S1†). All of the diazacyclooctanes exhibited cytotoxicity profiles with IC_{50} values in the micromolar range based on the MTS method (Fig. 1(a)). These IC_{50} values were lower than those of acrolein (17.2 μM) and, in most cases, than the IC_{50} values of the starting polyamines. All products **1a–4a'** notably increased the cellular levels of heme oxygenase-1 (HO-1), which is a marker for oxidative stress (Fig. 1(c)).[45]

On the other hand, the cytotoxic activities of the eight-membered diazacyclooctanes **1a–3a**, except for the caged compound **4a′**, could be markedly reduced in the presence of aminoguanidine, an amine oxidase inhibitor (Fig. 1(a)).[8] The data indicated that the cytotoxicities of the diazaoctanes **1a–3a** were most likely derived from their oxidation by the serum amine oxidase, which was included in the assay media.

The oxidation products generated by amine oxidase were examined by treating one of the diazacyclooctanes, the acrolein-modified spermidine **3a**, with serum amine oxidase (Fig. 2).[4] The oxidation reaction was directly monitored by ^{1}H NMR (Fig. 2(a)). We clearly detected proton signals corresponding to putrescine **4** over 24 h, together with other insoluble polymeric products, presumably the mixed diazacyclooctane and triazabicyclo[3.3.1]nonane polymers, suggesting that 4 molecules of the cytotoxic acrolein were generated during the oxidation process of the diazacyclooctane **3a** (Fig. 2(b)).

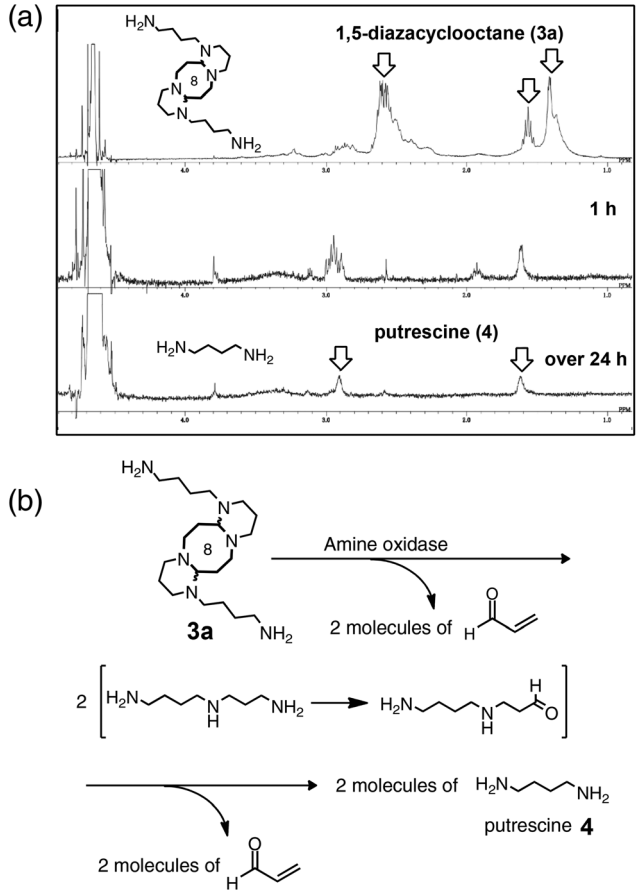
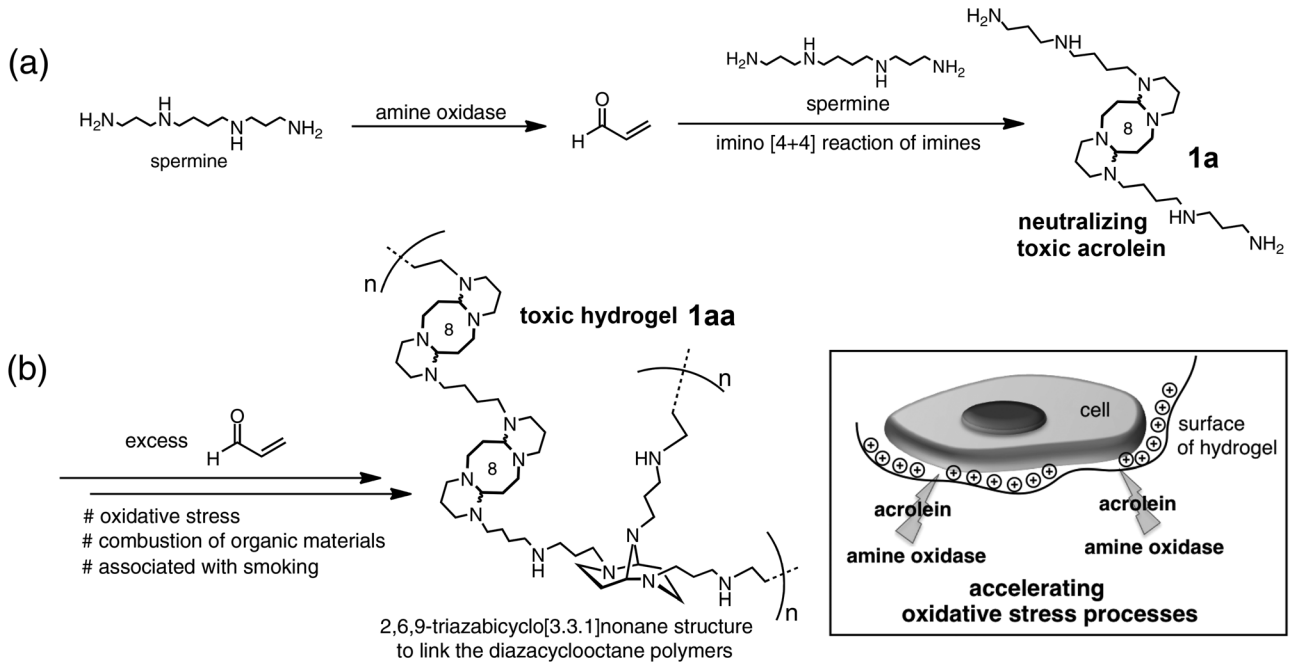


Fig. 2 Oxidation of the 1,5-diazacyclooctane **3a** with the serum amine oxidase. (a) The reaction was performed in PBS-*d* at 37 °C, pH 7.2, using 10 μM **3a** and 3.8 units bovine serum amine oxidase and was directly monitored using ^{1}H NMR techniques. Diazacyclooctane **3a** was gradually transformed into the putrescine **4** over 24 h. Lower signal-to-noise ratios of the spectra in the process of the reaction are due to the production of other insoluble polymeric products, *e.g.*, eight-membered polymers. (b) Possible reaction mechanism for the enzyme-catalyzed oxidation of the diazacyclooctane **3a** and the production of 4 molecules of acrolein.

The diazacyclooctane hydrogel **1aa**, produced through the [4 + 4] polymerization of the bis-imine derivative of spermine **1**, on the other hand, exhibited notable toxicity toward the cells, regardless of the presence or absence of the aminoguanidine (Fig. 1(b)). Thus, upon introduction of the hydrogel into a suspension of HeLa cells in DMED medium, the cells immediately adhered to the cationic hydrogel and were lysed within a few minutes.

Acrolein is the most cytotoxic unsaturated aldehyde yet characterized. It readily reacts with a variety of functional groups common to certain biomolecules (*i.e.*, amines, thiols, or hydroxyls), the modification of which is correlated with a variety of disease states.[1] Piperidine and pyridine-type derivatives, *i.e.*, 3-formyl-3,4-dehydropiperidine (FDP) and 3-methylpyridinium (MP) derivatives,[7,9,10] were previously known to be the major products resulting from reactions with the amino groups of, *e.g.*, lysine or polyamines. Some FDP and MP derivatives are used as biomarkers. The present study clearly showed that the main acrolein-modified products of the polyamines were the diazacyclooctanes. The facile formation of eight-membered heterocycles as the exclusive acrolein–polyamine conjugates, *i.e.*, the formation at μM concentrations under physiological conditions, has been overlooked until now. This could be because (i) the 1,5-diazacyclooctanes existed in equilibrium with the starting imines[39] or (ii) these heterocycles are unstable under the biological conditions, *i.e.*, in the presence of amine oxidase, which facilely oxidized and metabolized these products, as demonstrated experimentally in Fig. 2. Furthermore, (iii) these products were transformed into a variety of unidentified compounds upon exposure to acidic, basic, and heated conditions;[38,39,43] hence, they would have been undetectable using standard analytical methods (chromatographic separation conditions, including exposure to silica gel or LC-MS techniques). The FDP and MP derivatives are, therefore, the only derivatives that have previously been examined in depth, as these compounds are stable under the standard analytical conditions used previously to examine both chemical and biological samples.[7,9,10] Even if the byproducts had been identified previously, the simple ^{1}H NMR spectra of the symmetrical structures, such as **1a–3a** (see ESI Fig. S2†), which displayed the marked absence of vinyl protons, may have been assumed to be a polymerized byproduct due to the presumed instabilities of the alkylamine-derived unsaturated imines. We succeeded in identifying a novel polyamine modification by acrolein during a basic research study in which the reactivity profiles of the unsaturated imines were explored.[35–39]

Polyamines, *i.e.*, spermine and spermidine, are present in cells at concentrations of a few mM and mostly bind to RNA.[42,46–48] Polyamines are essential for cell growth, and their expression levels inside mammalian cells are precisely regulated by biosynthesis, degradation, and transport;[49] however, once cells are damaged, *e.g.*, under oxidative stress conditions, polyamines are released from RNA and then from the cells,[40] and they are oxidized by the serum amine oxidase to generate acrolein. The reactive and, therefore, toxic acrolein in turn further accelerate cell damage. Comprehensive toxicity investigations by Igarashi and co-workers revealed that acrolein



Scheme 3 A new proposed mechanism for the generation of acrolein-mediated oxidative stress, based on the reactivity profiles of the polyamines with acrolein and the cytotoxicity of their products. (a) 1,5-Diazacyclooctanes, *e.g.*, **1a**, were obtained smoothly by the reaction of polyamines with acrolein, which neutralized the toxic acrolein. (b) Large quantities of acrolein, generated by amine oxidase during oxidative stress and/or being present in cigarette and other environmental sources, produced a highly toxic diazacyclooctane hydrogel. The 2,6,9-triazabicyclo[3.3.1]nonane structure efficiently linked the diazacyclooctane polymers to form a polymer lattice structure with the characteristic structural features of a hydrogel. The resulting cationic hydrogel adhered to the cell surfaces, leading to immediate cell death and further accelerating oxidative stress processes. Both the oxidase-mediated production of acrolein and the oxidase-independent cytotoxicity of 2,6,9-triazabicyclo[3.3.1]nonane compounds were responsible for the high toxicity.

is more toxic to cells[9] than reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) or hydroxy radical (·OH), the major oxidative stress factors that led to a variety of disorders.[5]

Considering that acrolein is produced from polyamines by amine oxidase and that its production is involved in the progression of disorders, our finding of the facile production of 1,5-diazacyclooctanes, a previously unrecognized acrolein-modified polyamine, suggests a new mechanism for acrolein-mediated oxidative stress (Scheme 3).

Once acrolein was produced through the oxidation of polyamine or by the other sources, the diazacyclooctanes were immediately produced through a reaction with nearby polyamines (Scheme 3(a)). The cytotoxicity test revealed that the diazacyclooctanes themselves were not toxic; hence the formation of the eight-membered product *via* the newly identified chemical reaction effectively neutralized the toxic acrolein. The acrolein-neutralizing effects of FDP derivatives have previously been suggested.[9]

When large quantities of polyamines are released from disordered cells, polyamines as well as cyclic products are readily oxidized by serum amine oxidase to continuously produce acrolein (Scheme 3(b)). The excess acrolein in turn mediates a sequential [4 + 4] imino-polymerization reaction with polyamines, yielding a diazacyclooctane hydrogel. Polymer formation under physiological conditions was strongly supported by chemical and enzymatic reactions with spermine **1**. Although a detailed spectroscopic analysis using solid-phase NMR techniques could not precisely elucidate the structural constituents of the hydrogel due to the similarities between the proton and carbon signals of the polyamine-derivatized products, the reactivity profiles presented in Scheme 1 strongly suggest that the 2,6,9-triazabicyclo[3.3.1]nonane structure **4a** constitutes a ridged and irreversible product and could play an important role in hydrogel formation (see the structure **1aa** in Scheme 3(b)). Thus, (i) the spermine amino terminus of the developing diazacyclooctane polymers and/or (ii) the putrescine produced during the enzyme-catalyzed oxidation of the polyamines and diazacyclooctanes could spatially link the diazacyclooctane polymers by forming 2,6,9-triazabicyclo[3.3.1]nonane lattice polymer structures characteristic of a hydrogel. This enzymatic process efficiently produced the hitherto unrecognized hydrogel.

Finally, the resulting diazacyclooctane hydrogel, which is a highly cationic and conformationally flexible material, strongly adhered to the negatively charged cell surfaces (Scheme 3(b)) and led to immediate cell death. The presumed oxidase-mediated production of large quantities of acrolein near cell adhesion regions on the diazacyclooctane polymeric materials, together with the oxidase-independent cytotoxic properties of the 2,6,9-triazabicyclo[3.3.1]nonane structures present in the partial hydrogel constituents, may account for the high cellular toxicity of acrolein.

It should be noted that the acrolein is not only generated extracellularly by the serum amine oxidase as discussed above, but also found in cigarette and other environmental sources.[1] The excess acrolein being produced during the combustion of organic materials or associated with smoking could produce the toxic diazacyclooctane hydrogel, leading to cell damage. Alternatively, it is known that the amine oxidase levels are also elevated inside diseased cells under oxidative stress. In fact, increased intracellular levels of polyamine oxidase and acrolein are consistently evaluated as good markers of chronic failure and brain stroke.[29,50,51] Although we have not examined the intracellular effects of the diazacyclooctane or the hydrogel, these previously unrecognized products could pivotally damage the cells, if formed inside the cells, based on the high reactivity and toxicity profiles of the cyclic products reported in this paper.

Conclusions

In conclusion, we discovered that the exclusive products of the acrolein modification of polyamines were 1,5-diazacyclooctanes, rather than the previously reported piperidine and pyridine-type derivatives. The previously unrecognized eight-membered heterocycles were rapidly produced under mild aqueous physiological conditions. Although diazacyclooctane formation effectively neutralized acrolein toxicity, the diazacyclooctane polymers produced in the presence of excess acrolein, such as is present under oxidative stress conditions, were highly toxic to cells. This study strongly suggests that diazacyclooctane formation is involved in the mechanism underlying acrolein-mediated oxidative stress.

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