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CONCISE ARTICLE

Exclusive Formation of Imino[4+4]cycloaddition Products with Biologically Relevant Amines: Plausible Candidates of Acrolein Biomarkers and Biofunctional Modulators

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We synthetically demonstrated that eight-membered heterocycles, namely, 2,6,9-triazabicyclo[3.3.1]nonanes and 1,5-diazacyclooctanes, are the exclusive products of the reaction of acrolein with biologically relevant amines via an imino[4+4]cycloaddition. These compounds are produced in much higher amounts and efficiencies than the acrolein biomarker in current use, 3-formyl-3,4-dehydropiperidine (FDP). Our results not only indicate that eight-membered heterocycles may potentially be used as new biomarkers, but also strongly suggest the involvement of these heterocycles in various important biological phenomena, e.g., an acrolein-mediated mechanism underlying oxidative stress.

Introduction

Acrolein is a highly toxic unsaturated aldehyde[1] that is 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 (Fig. 1). Antibodies[11] to these conjugates are widely used for the immunochemical detection of various disease states, including arteriosclerosis,[11,12-14] Alzheimer's disease,[15,16] tumors,[17-21] diabetes,[22-26] autoimmune disease,[27,28] high blood pressure,[29] and others.[30-34] Alternatively, acrolein can react with polyamines to produce FDP conjugates that modulate the cytotoxicity of acrolein.[9]

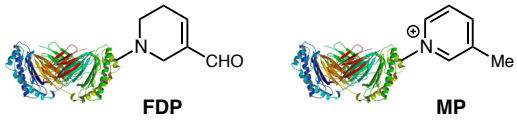
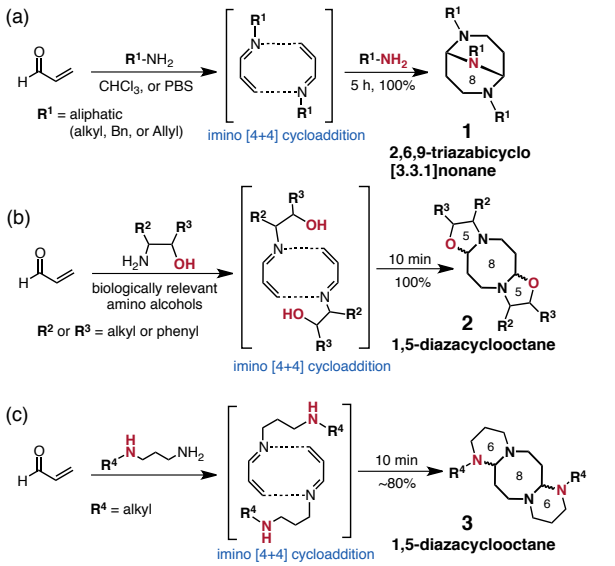


Fig. 1 Chemical structures of 3-formyl-3,4-dehydropiperidine (FDP) and 3-methylpyridinium (MP) as oxidative stress markers.



Scheme 1 Formation of eight-membered azaheterocycles from acrolein and amines through a [4+4] cycloaddition of intermediary imines. (a) 2,6,9-Triazabicyclo[3.3.1]nonanes from aliphatic amines. (b) 1,5-Diazacyclooctanes from the biologically relevant 1,2-aminoalcohols through a hydroxyl-mediated cycloaddition. (c) 1,5-Diazacyclooctanes from polyamines through an amine-mediated cycloaddition.

In our research program, which explores the novel reactivities of *N*-alkyl unsaturated imines,[35-37] we recently found by chance that the imines derived from the *N*-alkyl amines and acrolein participate in the hitherto unknown "head-to-tail" [4+4] dimerization in organic solvents or, more importantly, in aqueous media (Scheme 1).[38-42] Depending on the structure of the alkyl substituents on the nitrogen atom, the reactions provide 2,6,9-triazabicyclononanes (Scheme 1a)[38,39] and 1,5-diazaoctanes

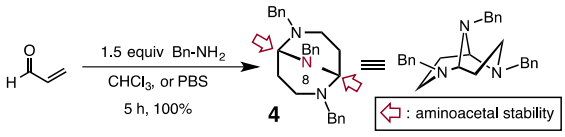
(Schemes 1b and 1c)[40-42] as eight-membered heterocyclic products, within 30 min and in nearly quantitative yields. The extremely high reactivities of the unsaturated imines at concentrations of a few μM under physiological conditions are noteworthy and strongly suggest that these reactions occur in biological systems, i.e., in and on cells. Interestingly, diazaheterocycles are the exclusive acrolein-modified products of these reactions, and no FDP derivatives, which were previously believed to be the preferred acrolein aminoadducts and widely used as oxidative stress biomarkers,[11] could be observed. Our independent study of the acrolein reactivity profiles revealed that FDP production is, in fact, observed to a lesser degree than the production of diazaheterocycles. These results suggest that the eight-membered heterocycles may potentially be used as new biomarkers. These previously unrecognized diazaheterocycles might be responsible for a variety of biological functions.

This paper summarizes, in detail and provides new data in support of the reactivity profiles of the newly discovered *N*-alkyl unsaturated imines. The possible utility of these imines as a new biomarker of oxidative stress is also discussed. A new mechanism for acrolein-mediated oxidative stress is proposed based on the cytotoxicity and the effects on oxidative stress on the cells. This mechanism is discussed as a potentially unrecognized function of these eight-membered diazaheterocycles.

Results and discussion

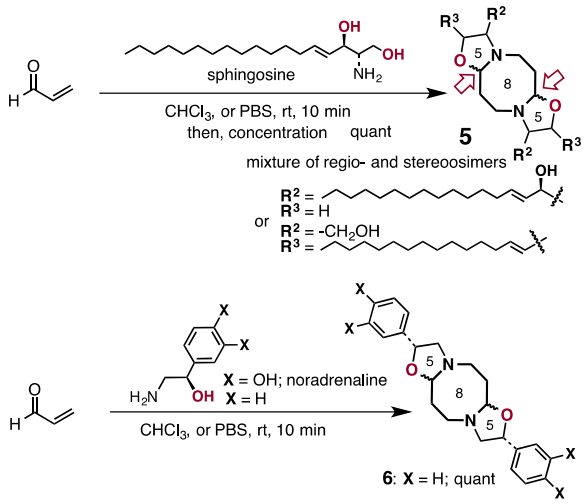
Reaction of acrolein with various biologically relevant amines

Acrolein readily reacts with various amines via a [4+4] cycloaddition to produce intermediary imines. The resulting eight-membered diazaheterocycle structures depend on the structure of the initial amine. A reaction with excess aliphatic amines, e.g., alkyl, benzyl, or allyl amines, which are abundant among biomolecules such as the lysine ε-amino group, smoothly provided the 2,6,9-triazabicyclo[3.3.1]nonane derivatives **4** in quantitative yield.[38,39] (The reaction with benzyl amine is illustrated in Scheme 2.) Although the unsaturated imines obtained from acrolein and the aliphatic amines were thought to be in equilibrium with the [4+4] dimerization products, they have not been observed previously. This is presumably due to the unfavorable strain in the eight-membered ring, i.e., eight-membered dienamine, which shifts the equilibrium toward the starting unsaturated imines. The use of an excess amine could potentially efficiently trap the eight-membered enamines as aminoacetals to produce the thermodynamically stable triazabicyclo[3.3.1]nonanes (Scheme 2).



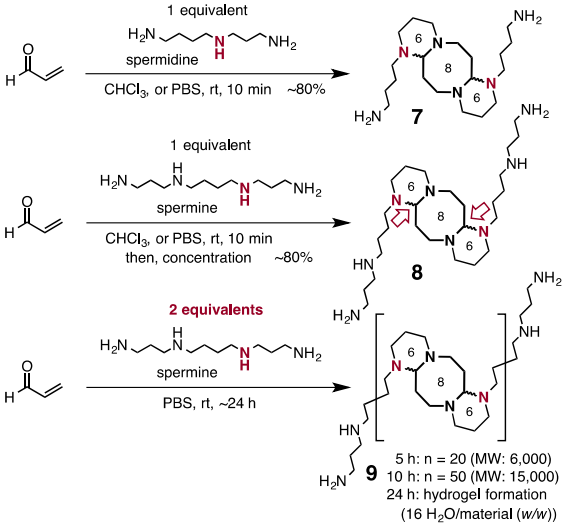
Scheme 2 2,6,9-Triazabicyclo[3.3.1]nonane from the reaction of acrolein with benzylamine, a model of the lysine ε-amino group.

The reaction of acrolein with one equivalent of the biologically relevant 1,2-aminoalcohols, i.e., sphingosine or a noradrenaline derivative, on the other hand, readily provided the eight-membered azaheterocycles, 1,5-diazacyclooctanes **5** and **6** in quantitative yield (Scheme 3).[42] The nucleophilic hydroxyl groups in the 1,5-diazacyclooctane molecules could intramolecularly stabilize the 1,5-diazacyclooctane formation more efficiently than it could stabilize the 2,6,9-triazabicyclo[3.3.1]nonane formation.



Scheme 3 1,5-diazacyclooctanes from the reaction of acrolein with the biologically relevant 1,2-aminoalcohols.

Polyamines, such as spermidine and spermine, which contain consecutive 1,3- and 1,4-diamine moieties (Scheme 4), could also participate in a smooth imino [4+4] cycloaddition reaction with one equivalent of acrolein, providing the corresponding 1,5-diazacyclooctane derivatives **7** and **8**.[40] These diazaheterocycles could be stabilized through a bis-aminoacetal formation via the amino groups substituted on the imino nitrogen. The reaction of spermine with 2 equivalents acrolein in PBS (Scheme 4), on the other hand, resulted in a [4+4] polymerization reaction between the two imines generated at the two amino termini of spermine.[40,41] This reaction produced the diazacyclooctane polymers **9**, which ultimately produced an insoluble hydrogel that contained 16 water molecules in the material (*w/w*).

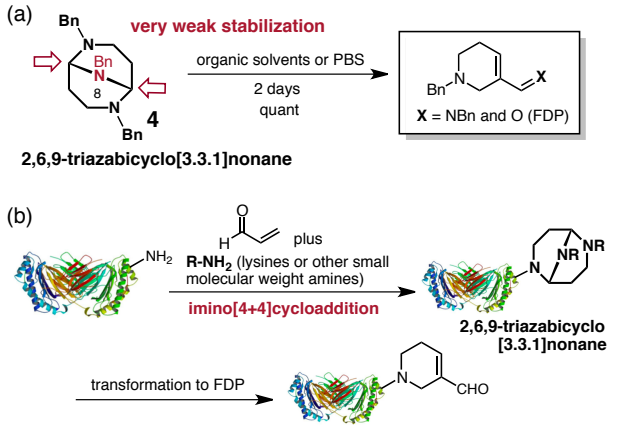


Scheme 4 1,5-diazacyclooctanes from the reaction of acrolein with the polyamines.

Chemical Properties and possible application of the eight-membered diazaheterocycles as biomarkers

Our studies of the reactions illustrated in Schemes 2–4 suggested that the eight-membered diazaheterocycles, i.e., the 2,6,9-triazabicyclononanes and the 1,5-diazacyclooctanes, were the exclusive acrolein–amine conjugates. Surprisingly, the production of 3-formyl-3,4-dehydropiperidine (FDP) conjugates,[7] which are now widely used as biomarkers of oxidative stress, was almost undetectable in these reactions. In reactivity and stability studies of 2,6,9-triazabicyclononane **4**, the aminoalcohol-derived diazacyclooctane **6**, and the spermine-derived **8**, led to a very interesting observation. Whereas the 1,5-diazacyclooctanes **6** and **8** remained almost intact over a day, 2,6,9-triazabicyclononane **4** was gradually transformed to the corresponding FDP and its imine derivatives at room temperature simply through dissolution in chloroform, acetonitrile, or in a buffer solution (Scheme 5a).

Our studies suggested that 2,6,9-triazabicyclononanes, such as **4**, which do not contain an internal enamine-stabilizing hydroxy or amino group (see the arrows in **5** and **8**, Schemes 3–4), could be readily transformed to the FDP derivatives. In other words, acrolein reacted with the various biologically relevant amines to produce, exclusively, the eight-membered heterocycles as the initial acrolein-modification products. These products gradually converted, under physiological conditions and depending on the bis-aminoacetal stability of the eight-membered ring system, into the corresponding stable six-membered FDP, which could only be detected under standard analytical conditions and are recognized as oxidative stress markers.



Scheme 5 Transformation of 2,6,9-triazabicyclo[3.3.1]nonane to 3-formyl-3,4-dehydropiperidine (FDP). (a) Chemical stability in organic solvents or PBS. (b) New mechanism proposed for the production of FDP from acrolein and lysines.

The FDP-lysine conjugate is thought to be produced by the initial double conjugate addition of a lysine amino group with two equivalents of acrolein, followed by an intramolecular aldol condensation.[7] In addition to this mechanism, our results presented in Scheme 5a suggest an alternative mechanism (Scheme 5b). The 2,6,9-triazabicyclononanes, which were initially produced from the lysines and the other small molecule amines, could eventually be converted to the FDP derivatives. Triazabicyclononane formation may, thus, mediate and facilitate FDP production.

FDP detection using antibodies has been widely applied to a variety of disease states.[11] Our independent model reactions using primary amines indicated, however, that the reaction was very sluggish. Only a tiny amount of the FDP derivatives could be produced. The eight-membered heterocycles, including the 2,6,9-triazabicyclononanes and 1,5-diazacyclooctanes, on the other hand, reacted smoothly to produce much larger amounts of FDP. These products potentially provide new candidate biomarkers of oxidative stress.

The previously unrecognized effects of the polyamine-derived 1,5-diazacyclooctanes on acrolein-mediated oxidative stress

Unlike the FDP derivatives, which are the final amino-modification products of the acrolein reactions and which do not display significant biological activity,[7] we found that the eight-membered diazaheterocycles may play an important and previously overlooked role in biological activity. The reactivity profiles presented in Scheme 4 indicated that the biologically relevant polyamines, e.g., spermine and spermidine, smoothly reacted with acrolein to provide diazacyclooctanes through a [4+4] dimerization of the imines. 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. 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.[40]

The cytotoxicities of the 1,5-diazacyclooctanes products **7**–**9** and their effects on oxidative stress were evaluated in cell-based assays using HeLa and A549 (human lung adenocarcinoma epithelial) cells (Fig. 2). The 1,5-diazacyclooctanes **7** and **8** exhibited cytotoxicity profiles with IC_{50} values in the micromolar range based on the MTS method (Fig. 2a). These IC_{50} values were lower than those of acrolein (17.2 μM) and were comparable or even lower than the IC_{50} values of the starting polyamines. The products **7** and **8** notably increased the cellular levels of heme oxygenase-1 (HO-1), which is a marker of oxidative stress (Fig. 2b).[43] On the other hand, the cytotoxic activities of the eight-membered diazacyclooctanes could be markedly reduced in the presence of aminoguanidine, an amine oxidase inhibitor (Fig. 2a).[8] We found that the spermidine-derived diazacyclooctane **7** was oxidized by the serum amine oxidase to produce putrecine, similar to the metabolic pathway followed by natural polyamines.[40] The data presented in Figs. 2a and 2b indicated that the cytotoxicities of the diazaoctanes **7** and **8** were most likely derived from acrolein, which is generated during oxidation processes by serum amine oxidase in the assay media.

The diazacyclooctane hydrogel **9**, produced through the [4+4] polymerization of the bis-imine derivative of spermine (Scheme 4), on the other hand, exhibited notable toxicity toward the cells, regardless of the presence or absence of the aminoguanidine (Fig. 2c). 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.

(a)

compounds	HeLa cell: IC_{50} (μM) (- AG)	HeLa cell: IC_{50} (μM) (+ AG)	A549 cell: IC_{50} (μM) (- AG)	A549 cell: IC_{50} (μM) (+ AG)
acrolein	17.2	14.9	NA	NA
spermidine	11.8	> 50	8.1	> 50
acrolein-spermidine **7**	4.6	> 50	6.8	> 50
spermine	2.5	> 50	2.8	> 50
acrolein-spermine **8**	0.14	> 50	3.0	> 50

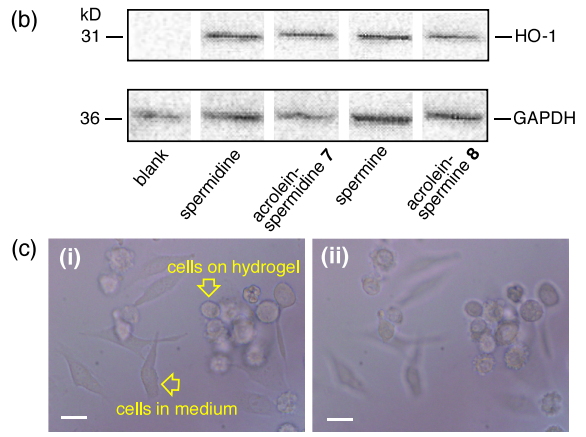
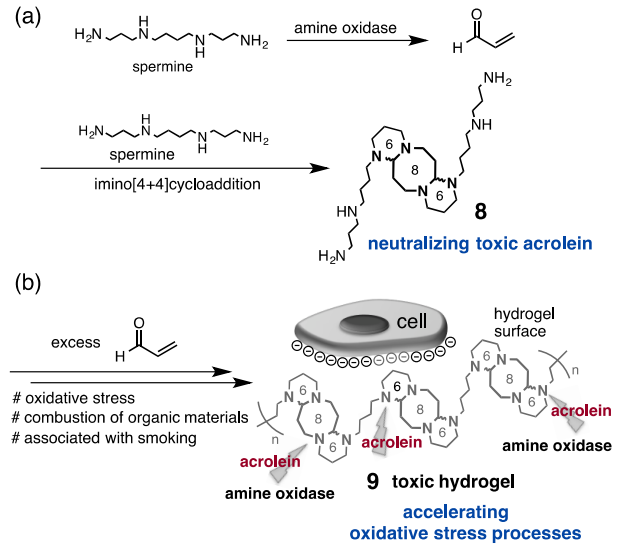


Fig. 2 (a) Cytotoxic activities of the polyamines, i.e., the 1,5-diazacyclooctanes, **7** and **8**. HeLa and A549 cells were treated with the compounds for 72 h at 37 °C, and the cytotoxicities were evaluated using the MTS method. AG: 1 mM aminoguanidine was used as the amine oxidase inhibitor. NA: Not available. (b) Western blots of the HeLa cell lysates using anti-HO-1 (upper) and anti-GAPDH (lower) antibodies after treatment with **7** and **8** in the absence of aminoguanidine. HO-1: heme oxygenase-1. GAPDH: glyceraldehyde-3-phosphate dehydrogenase. (c) HeLa cells treated with the hydrogel **9** in medium at 37 °C for 1 min. Images were focused (i) on live cells in the medium and (ii) on dead cells adhered to the hydrogel. The scale bars indicate 10 μm.

The polyamines, i.e., spermine and spermidine, are present in cells at concentrations of a few mM and mainly bind to RNA.[42,45-47] Polyamines are essential for cell growth, and their expression levels inside mammalian cells are precisely regulated by biosynthesis, degradation, and transport.[48] Once the cells are damaged, however, e.g., under oxidative stress conditions, polyamines are released from RNA, exit the cells,[49] and are oxidized by serum amine oxidase to generate acrolein. The reactive and toxic acrolein then further accelerates cell damage. Comprehensive toxicity investigations by Igarashi and co-workers revealed that acrolein is more toxic to cells[9] than reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) or hydroxy radicals (·OH), the major oxidative stress factors that lead 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 and cytotoxic properties of 1,5-diazacyclooctanes, the previously unrecognized acrolein-modified polyamines, suggested a new mechanism for acrolein-mediated oxidative stress (Scheme 6).

Once acrolein was produced through the oxidation of polyamine or through other sources, the diazacyclooctanes were immediately produced through a reaction with nearby polyamines (Scheme 6a). The cytotoxicity test revealed that the diazacyclooctanes themselves were not toxic. The formation of the eight-membered product via the newly identified chemical reaction appeared to effectively neutralize the toxic acrolein. The acrolein-neutralizing effects of the FDP derivatives have been suggested previously.[9]

As large quantities of polyamines are released from disordered cells, polyamines and cyclic products are readily oxidized by serum amine oxidase to continuously produce acrolein (Scheme 6b). Acrolein then mediates a sequential [4+4] imino-polymerization reaction with polyamines to yield a diazacyclooctane hydrogel. Finally, the resulting diazacyclooctane hydrogel, which is a highly cationic and conformationally flexible material,[41] strongly adheres to the negatively charged cell surfaces and leads to immediate cell death. The presumed oxidase-mediated production of large quantities of acrolein near cell adhesion regions on the diazacyclooctane polymeric materials may account for the high cellular toxicity of acrolein.



Scheme 6 A new mechanism underlying acrolein-mediated oxidative stress.

It should be noted that acrolein is generated not only extracellularly by serum amine oxidase, as discussed above, but is also found in cigarettes and other environmental sources.[1] Acrolein produced during the combustion of organic materials, such as during smoking, could produce the toxic diazacyclooctane hydrogel, leading to cell damage. Amine oxidase levels are elevated inside diseased cells under oxidative stress. Increased intracellular levels of polyamine oxidase and acrolein are consistently found to provide good markers of chronic organ failure and brain stroke.[29,50,51] Although we have not examined the intracellular effects of the diazacyclooctane or the hydrogel, these previously unrecognized diazaheterocyclic products could damage cells, if formed inside the cells, due to the high reactivity and toxicity profiles of the cyclic products reported here.

Conclusions

In conclusion, we discovered that the exclusive products of the acrolein modification of biologically relevant amines, including sphingosine, noradrenaline, and polyamines, were 2,6,9-triazabicyclononanes or 1,5-diazacyclooctanes, rather than the tetrahydropyridine-type FDP biomarkers. Our study showed that some of 2,6,9-triazabicyclononanes were subsequently metabolized into FDP derivatives. The facile formation of eight-membered azaheterocycles, i.e., the formation at μM concentrations under physiological conditions, has been overlooked until now. This could be because (i) the eight-membered diazaheterocycles are present in equilibrium with the starting imines, or (ii) these heterocycles are unstable under the biological conditions, i.e., in the presence of amine oxidase, which facilely oxidizes and metabolizes these products. Furthermore, (iii) these products may be transformed into a variety of unidentified compounds upon exposure to acidic, basic, or heated conditions, and they are most likely undetectable using standard analytical methods (chromatographic separation conditions, including exposure to silica gel or LC-MS techniques). The FDP derivatives are the only derivatives that have been examined in depth, as these compounds are stable under standard analytical conditions used to examine both chemical and biological samples.[7,9,10] We succeeded in identifying a novel polyamine modification by acrolein during a basic research study, and the reactivity profiles of the unsaturated imines were explored. The eight-membered diazaheterocycles were efficiently produced in larger quantities compared with FDP; therefore, they provide new candidate biomarkers of oxidative stress, when stabilized through an additional chemical transformation.

It should be noted that these biologically relevant and reactive diazaheterocycles could display unexplored and previously overlooked biological functions. As an example, we suggest that the formation of diazacyclooctane from a polyamine is involved in the mechanism underlying acrolein-mediated oxidative stress. Explorations of the biological significance of eight-membered heterocycles outside of acrolein-mediated oxidative stress processes are underway in our laboratory.

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Notes and references

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