Nitrosative Cytosine Deamination. An Exploration of the
Chemistry Emanating from Deamination with
Pyrimidine Ring-Opening

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A discussion of nitrosative deamination of cytosine 1 is presented that argues for the formation of 6 by diazotization of 1 to cytosinediazonium ion 2 and its electrostatic complex 3, diazoniation to 4 ↔ 5, and amide-bond cleavage to 6. The reaction channels available to 6 include hydrolytic deglycation to 3-isocyanatoacrylonitrile 7, water addition to carbamic acid 9 with the possibility for re-closure to uracil 13, water addition to carbamic acid 9, and decarboxylation to 3-aminoacrylonitrile 10. With a view to the instability of the carbamic acid 9, the carbamate models ethyl (Z)-2-cyanovinylcarbamate 14 and (Z)-2-cyano-1-tert-butyvl-

nylcarbamate 20 were studied. Acid-catalyzed hydrolysis of 14 leads to 2-amino-carbonylphe-

nylcarbamate 15, and its cyclization yields the benzo-fused uracil quinazoline-2,4-dione 16. In contrast to the aromatic system 14, acid-catalyzed cyclization cannot compete with oligomer-

ization in the case of 20, and 5-tert-buty luracil 22 is accessible only with base-catalysis. It is shown that 23, the parent of 10, also easily polymerizes. The experimental results provide a rationale as to why 9, 10, and 12 would have escaped detection in in vitro studies: they would have oligomerized. In contrast to the in vitro experiments, the oligomerizations of 9, 10, or 12 clearly are not relevant in vivo because of low monomer concentrations. With the exclusion of recyclization and of oligomerization in vivo, attention thus needs to focus on (Z)-3-aminoacry-

lonitrile 10 as the most likely deamination product of cytosine aside from uracil.

Introduction

The high fidelity of the genome relies to a great extent on the inherent stability of the chemical makeup of DNA. Any damage to the DNA can have deleterious effects via mutagenesis, cell transformation, and cell death (1). Nitrosating reagents represent one important class of DNA damaging chemicals, and they may cause a variety of lesions. It is well-known that nitrous acid and nitric oxide cause nucleobase deamination and interstrand cross-link formation, and such DNA damage, if left unrepaired, causes a variety of diseases. Cytosine was discovered by Kossel and Steudel (2) in 1903. Kossel and Steudel also discovered uracil shortly thereafter, and there was the question from the start as to whether uracil might be the product of nitrosative cytosine deamination (3). Indeed, cytosine deamination to uracil may occur without or with enzyme catalysis (Figure 1). DNA cytosine methyltransferases methylate and/or deaminate cytosine (C) and form 5-methylcytosine (5meC), thymine (T), and uracil (U) (4), and these interconversions are important for health maintenance and also can trigger disease (5). In some organisms, there also exists an enzymatic path for the conversion of thymine to uracil (6). C-to-T damage can be repaired by very short patch (VSP) repair (7) and C-to-U damage can be repaired via enzymatic base excision by uracil glycosylase (8) and subsequent cytosine reproduction. If left unrepaired, the C-to-U transformation results in the G:C → A:T mutation (9), which was linked to several diseases including hemophilia (10), Alzheimer’s (11), colon cancer (12), retinoblastoma (13), and Gerstmann-Sträussler syndrome (14).

The mechanisms of spontaneous (nonenzymatic) hydrolytic and of nitrosative deamination of cytosine have been discussed (15). In the nonenzymatic hydrolytic deamination, protonated cytosine is thought to undergo direct nucleophilic ipso-substitution by water, while the nitrosative deamination is thought to involve the hydrolysis of cytosinediazonium ion (15). The spontaneous deamination is a slow reaction with a measured half-life for cytosine residues on the order of 30 000 years in
double-stranded DNA (16). While cytosine deamination has been studied qualitatively, it appears that no study accounted quantitatively for all of the cytosine and its reaction products and, at this time, not even all reaction products might be known. For example, in a study of the deamination of 2′-deoxycytidine and 2′-deoxycytidine 5′-monophosphate (dC and dCMP) by NO at pH = 7.4, the ratio between unreacted cytosine and slowly formed uridine (dU and dUMP) was reported as about 9:1 (17), and no information was given as to how much material was unaccounted for by the time this ratio was determined by HPLC analysis.

We have been studying the mechanisms of the nitrosative deamination of DNA bases by theoretical (18–20) and experimental (21–23) methods and with focus on guanine deamination. Our theoretical studies (18) revealed that the cytosinediazonium ion 2 is not a viable species on the potential energy surface and the weakly bound electrostatic complex 3 (E_b = 4.3 kcal/mol) is formed instead (Figure 2). If water is readily available to replace the dinitrogen as it leaves, then a heteroaromatic nucleophilic substitution occurs to form uracil. The nucleophilic attack of water might involve any species along the path from 2 to 3. On the other hand, a more or less free ion–neutral complex 3 might be formed, and this complex contains the ion 4 which is stabilized by hyperconjugation of the electron-deficient carbon center by the β,γ-NC σ-bond. In fact, one has every reason to describe ion 4 as a cyclic nitrilium ion 5 with a dative bond between the nitrile N-atom and the carbonyl C-atom. This insight lead us to examine the stability of this dative bond, and we found it to be extremely weak. The ring-opened cation 6 is 5 kcal/mol more stable than the cyclic ion, and there is hardly any kinetic hindrance; E_A(MP2/6-31G**) = 2.5 kcal/mol (24). The same situation occurs for the cation generated by dediazoniation of adeninediazonium ion (25).

The acyclic cation 6 is an interesting intermediate because of its high reactivity, its multifunctionality (isocyanate, nitrile, alkene), and its polarity (cation, donor–acceptor substituted alkene), and some reaction possibilities are described in Figure 2. Cation 6 could undergo hydrolytic deglycation forming an abasic site (26) and releasing (Z)-2-isocyanocyrolonitrile 7 (27). As an unsaturated isocyanate (28), 7 is toxic (29) and, in addition to forming adducts with nucleobases via amine addition, 7 has the potential to form adducts and interstrand cross-links (30, 31). Alternatively, 6 can add water and form carbamic acid 9 (32). The water addition to 6 is diffusion-controlled, while the deglycation is an activated process and unlikely to complete (20). Since carbamic acids easily decarboxylate (33), 9 is a precursor to (Z)-3-aminoacrylonitrile, 10. The push–pull activation renders 10 highly susceptible to base-catalyzed nucleo-
philic addition to the C=C bond (34) or the C=N bond (35), respectively, and this chemistry also might lead to DNA adducts. Similarly, 10 also is highly reactive under acidic conditions (36), and in the present paper, this issue is discussed for the parent of 10, 3-aminoacrylonitrile 23.

There is a remote chance that the carboxylic acid 9 might add water to form 12 and 12 then might recyclize to uracil 13. The synthesis of uracil derivatives by addition of amides to carbamate esters does have precedent with aromatic substrates (37, 38), and we wondered whether such ring closures might be possible for aliphatic 12 and whether they might perhaps even occur for these substrates under milder conditions as compared to the aromatic substrates. The geometry of 12 positions the amide-N well for approach to the carboxyl-C (Figure 3).

The direct examination of the hypothesis that 9 has the chemical competence to form uracil 13 is not possible since ds-oligonucleotides containing 9 or 12 are not accessible. Thus, we have to learn from model studies, and we report here on the chemistry of the ethyl (Z)-2-cyanovinylcarbamates 14, 17, and 20 and their cyclization to the respective uracils (Figure 4).

**Materials and Methods**

**General Procedures.** All chemicals were purchased from Aldrich. All moisture-sensitive reactions were carried out in oven-dried glassware, and the reagents were transferred with oven-dried syringes. Tetrahydrofuran was dried by distillation over sodium/benzophenone. Diethyl-carbonate and benzene were oven-dried syringes. Tetrahydrofuran was dried by distillation over sodium/benzophenone. Diethyl-carbonate and benzene were oven-dried glassware, and the reagents were transferred with oven-dried syringes. Tetrahydrofuran was dried by distillation over sodium/benzophenone. Diethyl-carbonate and benzene were oven-dried glassware, and the reagents were transferred with oven-dried syringes. Tetrahydrofuran was dried by distillation over sodium/benzophenone. Diethyl-carbonate and benzene were oven-dried glassware, and the reagents were transferred with oven-dried syringes. Tetrahydrofuran was dried by distillation over sodium/benzophenone. 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filtration, the solvent was evaporated, and the crude product was recrystallized in ethanol/water to obtain 2.36 g of 14, 83% yield. 1H NMR (300 MHz, CDCl3): δ 8.22 (d, H), 7.58–7.52 (m, 2H), 7.07–7.12 (m, 2H), 4.24 (q, 2H), 1.32 (t, 3H). 13C NMR (300 MHz, CDCl3): 152.82 (CO), 140.91 (C–(NH)), 134.13 (CH), 132.22 (CH), 123.05 (CH), 119.33 (CH), 116.26 (CN), 100.98 (C–(CN)), 61.95 (CH3), 14.32 (CH3).

Quinazoline-2,4-dione 16. Carbamate 16 (23 g, 120 mmol) was refluxed overnight in 9 mL concentrated HCl and 12.5 mL of liquid ammonia, and the reaction was carried out at 100 °C in nitrogen. The reaction mixture was stirred for 3 h and worked up after 15 h by addition of a saturated solution of sodium bicarbonate to neutralize the HBr produced. The reaction was filtered off, and the methanol was evaporated to obtain (Z)-3-amino-2-tert-buty lacrylonitrile 28 (7.3 g, 72%). The 1H NMR of the crude product agreed with the literature. 1H NMR (300 MHz, CDCl3): δ 6.58–6.66 (t, 1H), 4.20–4.50 (br, NH2), 1.12 (s, 9H). 13C NMR (500 MHz, CDCl3): δ 142.32 (CH2), 119.30 (CN), 92.54 (C, olefinic), 31.91 (C, tert-buty1), 29.84 (CH3, tert-buty1), MS (ESI), m/z: 195 [M + H]+. MS (EI) m/z: 124 [M]+, 104 [M+– CH3].

Ethyl (Z)-2-Cyano-3,3-dimethylbut-1-enylcarbamate 20. To a stirred solution of (Z)-3-amino-2-tert-buty lacrylonitrile 28 (2.0 g, 16 mmol) in freshly distilled benzene (15 mL) was added sodium hydride (0.77 g, 32 mmol). After 5 min, freshly distilled diethyl carbonate (0.04 mol) was added to the reaction mixture. The reaction mixture was allowed to warm to room temperature. The color of the reaction mixture changed to reddish orange. TLC (25% EA/hexane) indicated the completion of the reaction after 1 h. The reaction was worked up by the slow addition of 1:1 ethanol/water mixture until all of the unreacted sodium hydride was gone. The reaction mixture was filtered over Celite, and the filtrate was extracted with ethyl acetate and evaporated to obtain the product. The product was purified by column chromatography (1.5 g, 47% yield). 1H NMR (250 MHz, CDCl3): δ 7.14–7.18 (d, 2H), 4.18–4.26 (q, 2H), 2.31–2.33 (t, 3H), 1.17 (s, 9H). 13C NMR (300 MHz, CDCl3): δ 152.57 (CO), 134.74 (CH2), 116.29 (CN), 101.89 (C, olefinic), 62.57 (O–CH3), 33.00 (C, tert-buty1), 29.24 (CH3, tert-buty1), 14.33 (CH3). MS (ESI) m/z: 195 [M + H]+.

Ethyl (Z)-2-Aminocarbonyl-1-tert-buty1vinylcarbamate 21 and 5-tert-Butyluracil 22 from Ethyl (Z)-2-Cyano-1-tert-buty1vinylcarbamate 20. Potassium carbonate (66 mg, 0.48 mmol) was added to a suspension of LAH (1 g, 26 mmol) in ether/THF (4:1, 120 mL) under nitrogen. The reaction mixture was stirred for 3 h and washed successively with water (2.5 mL), 20% NaOH (2.5 mL), and water (7.5 mL). Excess water was used to dissolve any unreacted salt. The ether layer was dried over K2CO3. The reaction mixture was allowed to warm to room temperature. The color of the reaction mixture changed to reddish orange. TLC (25% EA/hexane) indicated the completion of the reaction after 1 h. The reaction was worked up by the slow addition of 1:1 ethanol/water mixture until all of the unreacted sodium hydride was gone. The reaction mixture was filtered over Celite, and the filtrate was extracted with ethyl acetate and evaporated to obtain the product. The product was purified by column chromatography (0.24 g, 1.2 mmol) in DMSO (1.2 mL). Hydrogen peroxide (0.198 mL) was then added slowly, and the reaction mixture was allowed to warm to room temperature. TLC indicated the formation of spots more polar than the starting material after 24 h. Some solidification occurred when the reaction progressed. More hydrogen peroxide was added. The reaction was continued for 4–5 days and quenched by the addition of water which resulted in a white precipitate. The reaction mixture was filtered and the residue dissolved in methanol. The filtrate was subjected to vacuum distillation to remove water and DMSO. Both the residue and the filtrate indicated the presence of amide 21 and starting material. The amide was separated on preparative TLC (eluent 60% EA/hexane) in 10% yield, 26 mg. Trace amounts of 5-tert-buty1uracil 22 were formed.

5-tert-Butyluracil 22 from Ethyl (Z)-2-Aminocarbonyl-1-tert-buty1vinylcarbamate 21. Potassium t-butoxide (28 mg, 0.252 mmol) was dissolved in DMSO (5 mL), and the solution was stirred for 30 min. To this solution was added 21 (36 mg, 0.168 mmol) dissolved in DMSO (5 mL). The reaction mixture was stirred at 70 °C for 15 days. DMSO was removed by distillation under vacuum. The residue was separated by column chromato graphy. 5-tert-Butyluracil 22 was isolated in (4 mg) 14% yield. 1H NMR (500 MHz, CD3OD): δ 7.11 (s, 1H), 1.27 (s, 9H). 13C NMR (500 MHz, CD3OD): δ 165.77 (CONH), 153.59 (CO), 137.41 (CH2), 122.35 (C, olefinic), 33.48 (C, tert-buty1), 29.05 (CH3, tert-buty1). MS (+APCI) m/z: 201 [M + H]+ + CH3OH, 169 [M + H]+.

Results

Synthesis, Properties, and Oligomerization of β-Aminocarboxilnitrile. We synthesized (Z)-β-aminocarboxilnitrile-
Sieveking and Lüttke succeeded with the separation of dimer 24 (because of their larger scale reaction while we worked with liquid ammonia according to Peeters, Prange, and Vogt are competitive.

yield (Luettke, 1992). Oligomerization. ESI-MS analysis showed peaks at m/z = 137 and m/z = 205 corresponding to the protonated dimer 24 and trimer 25, respectively. It is likely that Sieveking and Lüttke succeeded with the separation because of their larger scale reaction while we worked at microscale. We did succeed in the synthesis of pure (Z)-23 by autoclave reaction of 3-ethoxycarbonyl nitrile in liquid ammonia according to Peeters, Prange, and Vogt (1974). While the isomer mixture of 23 was made in about 80% yield, the yield decreased to 45% during fractional distillation, and we observed the formation of a solid during the distillation.

We recently reported an ab initio study of 10 and its protonated derivatives (36). It was found that the ions are generated by C2-protonation and the nitrilium ions are competitive (Figure 5), while the ammonium ions are all high in energy. The results suggest that acrylonitrile and its 3-aminoo derivative differ not merely quantitatively but that there are significant qualitative differences. An addition to the alkene moiety of acrylonitrile proceeds by 1,4-addition to form the keteneimine and subsequent tautomerization. This analogous path remains possible for 10, but 10 also can behave like an enamine by way of C2-protonation and direct C-C 1,2-addition. The proton affinity of 10 is much higher than that of acrylonitrile and suggests a much higher reactivity of 10 in acid-catalyzed reactions.

This chemistry informs about the possible fate of any 10 that might be formed by way of decarboxylation of 9 (Figure 2). The experiments provide evidence for the ease of thermal and of acid-catalyzed oligomerization of 23, and the theoretical study of the protonation of 10 explains this ease for oligomerization because of the possibility for C2-protonation. We suggest that this ease for oligomerization is the major reason as to why ring-opened cytosine derivatives have not been observed.

Cyclization of the Benzo-Analogue: Preparation of Ethyl (Z)-2-Cyanophenylcarbamate 14 and its Cyclization to Quinazoline-2,4-dione. The synthesis of uracils by addition of amides to carbamate esters has precedent. Ishikawa et al. showed that the reaction of 5-ethoxycarbonyl amino-4-indancarbonitrile with HCl in the presence of urea affords quinazoline-2,4-dione (37); nitrile hydrolysis is followed by ring-closure. Hegarty et al. demonstrated the cyclization of phenyl N-methyl-N-(o-carbamoylphenyl) carbamate to 2-(N-methyl)-4-(1H,3H)-quinazolinedione in basic media (38). These ring-closures involve acidic or basic conditions, respectively. We wondered whether such ring-closures could be achieved under milder conditions and whether aliphatic substrates would react in the same fashion. Hence, we synthesized ethyl 2-cyanophenylcarbamate 14 and studied its hydrolysis to ethyl 2-aminocarbonylphenylcarbamate, 15, and cyclization to quinazoline-2,4-dione, 16 (Figure 4).

Ethyl 2-cyanophenylcarbamate 14 was prepared from 2-aminobenzonitrile and ethyl chloroformate (40). The cyclization of 14 to 16 was first studied at pH values of 3.7 (37 and 51 °C), 2.2 (37 and 46 °C), and 1.2 (reflux) with 0.1 M solutions of 14. In all cases, the starting material was found unreacted in the reaction mixture after several hours. The cyclization of 14 to 16 was achieved under the conditions employed by Ishikawa (pH = −0.9), and this cyclization is possible without the addition of urea. Amide 15 can be isolated at low temperature; the cyclization to 16 requires reflux conditions (Figure 4). Hence, the hydrolysis of the nitrile 14 and its subsequent cyclization can only be achieved under extremely acidic conditions.

Preparation, Oligomerization, and Hydrolysis of Ethyl (Z)-2-Cyano-1-tert-butyl-carbamate 20. With a view to the propensity of 10 for thermal and acid-catalyzed oligomerization (vide supra), there is little hope to prepare and isolate 17 and to study its reaction to 18 and 19. We studied 20 instead, because the tert-butyl group provides a strong disincentive for any reaction with sp2-sp3 rehybridization at C2 and should slow the oligomerization of 20. The synthesis of carbamate 20 is outlined in Figure 6. The cyclization of 20 to 22 was attempted under the conditions employed for the cyclization of 14 to 16 and failed. Even with the bulky substituent, the hydrolysis of the aliphatic system 20 to 21 cannot compete with acid-catalyzed oligomerization under these conditions (pH = −0.9).

The preparation of 21 under nonacidic conditions was attempted to explore whether 21 might cyclize to uracil. Many methods are available for nitrile hydrolysis under nonacidic conditions; we tried the reagents H2O2/NaOH in MeOH (48), H2O2/NaOH in CH3Cl with phase transfer catalysis (49), H2O2/PEG employing microwave irradiation (50), TMSiOK in THF and in toluene (51), and KOH/ t-BuOH (52), and none worked. Microwaves cleave the carbamate ester to 28, and the other reagents did not react. Sawaki and Ogata reported that the rate of nitrile hydrolysis is accentuated in DMSO solution (53). Therefore, the hydrolysis of 20 was carried out in the presence of H2O2/K2CO3 and DMSO (54). This method affords the highly selective hydrolysis of the nitrile group in the presence of the carbamate ester. The results by Hegarty et al. (38) suggest that the hydrolysis of the nitrile group of 20 under basic conditions will be immediately followed by ester hydrolysis of the carbamate and results in 5-t-butyluracil 22. K2CO3·1.5H2O was added to the stirred ice-cold solution of 20 in DMSO, and hydrogen peroxide was added dropwise. The reaction was extremely slow, monitored by TLC, and continued for 4–5 days. Reaction products were separated on a preparative TLC plate. Three spots with Rf values of 0.24–0.29 (A), 0.33–0.37 (B), and 0.44–0.49 (C, major) were extracted with methanol and analyzed by HPLC. The photodiode array detector indicated A to be similar to uracil and/or thymine, and the optical spectra of B and C resembled each other. The LC-MS studies (APCI) showed [A + H]+ with m/z = 169 and identified A as 5-tert-butyluracil 22, [B + H]+ at m/z = 216 and identified B as the product of complete nitrile hydrolysis, and [C + H]+ at m/z = 215 identified C as the amide 21. Pure 21 was obtained in

**Figure 5.** C2-Protonation and nitrilium ion formation of 10 are competitive.
larger quantities and characterized by $^1$H and $^{13}$C NMR and (+)APCI/MS.

Hegarty et al. (38) reported that phenyl N-methyl-N-(o-carbamoylphenyl)carbamate cyclizes to 2-(N-methyl)-4-(1H,3H)-quinazolinedione and the rate of this cyclization increases with pH. Since 21 and phenyl N-methyl-N(o-carbamoylphenyl)carbamate are structurally similar, we tried to cyclize 21 to 22 in analogy at pH = 12. Yet, amide 21 did not cyclize after 24 h, even when its solubility was improved (potassium phosphate buffer at pH = 12 containing small amounts of dioxane, or NaOH, and dioxanewater mixture, or KOH/DMF). The cyclization of 21 to 22 was accomplished with potassium $t$-butoxide in DMSO (55, Figure 6). The uracil 22 was separated by column chromatography ($R_f$ = 8.23 min) and purified by HPLC, and $^1$H and $^{13}$C NMR spectra were recorded.

Discussion

A mechanistic hypothesis for nitrosative cytosine deamination has been stated that involves pyrimidine ring-opened intermediates (Figure 2). This hypothesis was formulated on the basis of results from theoretical studies, it is corroborated by some known chemistry, and it incorporates insights from studies of nitrosoative guanine deamination. The hypothesis provides ideas about possible reaction channels that were not previously considered. One of these reaction channels suggests an explanation as to why the products of the acyclic intermediates were not previously observed, or even considered, and this chemistry has been explored. The hypothesis brings up a remote possibility for uracil formation via a sequence of ring-opening, 2-fold water addition, and reclosure by condensation, and this reaction channel has been explored by experiment as well.

2-Aminoacrylonitrile was prepared and found to have a high propensity for thermal and acid-catalyzed oligomerization. This reactivity is consistent with the theoretical finding that iminium ion formation is competitive with nitrilium ion formation (36).

Extremely acidic conditions (pH $< 0$) and high temperatures are required for the cyclization of ethyl 2-cyanophenylcarbamate 14 to uracil 16. Even under these extreme conditions, ethyl (Z)-2-cyano-1-tert-butylvinylcarbamate 20 does not cyclize to 22. Instead, acid-catalysis triggers oligomerization of 20. While the cyclization of 20 to 22 cannot compete under acidic conditions, we have shown (merely for completeness) that it can be accomplished under extremely basic conditions (pH $> 11$).

The pH dependencies of the cyclizations of the esters 14 $\rightarrow$ 15 $\rightarrow$ 16 and 20 $\rightarrow$ 21 $\rightarrow$ 22 in vitro suggest that the cyclization of the acid 9 $\rightarrow$ 12 $\rightarrow$ 13 do not occur under physiological conditions. Present knowledge neither excludes nor suggests the possibility of in vivo enzymatic catalysis of the reaction 9 $\rightarrow$ 12 $\rightarrow$ 13. Microorganisms (56) feature nitrile hydratases for the conversion of nitriles to amides (57–59), and human nitrile hydratases might exist, but they are not known at present.

In analogy to $\beta$-aminoacrylonitrile 23, acid-catalyzed oligomerization presents a possible channel for any 10 that might be formed by in vitro nitrosative cytosine deamination. In analogy to 20 and 21, acid-catalyzed oligomerization presents possible reaction channels for any of 9 or 12 that might be formed by in vitro nitrosative cytosine deamination.

In contrast to the in vitro experiments, the acid-catalyzed oligomerizations of 9, 10, or 12 clearly are not relevant in vivo because of low monomer concentrations. With the exclusion of cyclization and oligomerization in vivo, attention thus needs to focus on the glycoside of (Z)-3-aminoacrylonitrile 10 as the most likely deamination product of cytosine. The reactivity of 10 exhibits a propensity for C=C and C=N additions, and these may lead to the formation of adducts and/or cross-links in DNA and/or proteins. No such adducts and/or cross-links have been discovered as yet, and there have not been any reasons to search for such cross-links. However, our results suggest and justify the search for such adducts and cross-links caused by nitrosative DNA deamination. The exploration of all the options and the complete understanding of the mechanism of cytosine deamination will not only provide, but it is essential to, a better understanding of the disease processes in the human body, and it will assist in the search for new toxins and new modes of DNA modifications. Nitrosative deamination of cytosine in DNA remains a challenging problem in chemical toxicology.

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References

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