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**, dediazoniation 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*-butylvinylcarbamate **20** were studied. Acid-catalyzed hydrolysis of **14** leads to 2-amino-carbonylphenylcarbamate **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 oligomerization in the case of **20**, and 5-*tert*-butyluracil **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-aminoacrylonitrile **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*).

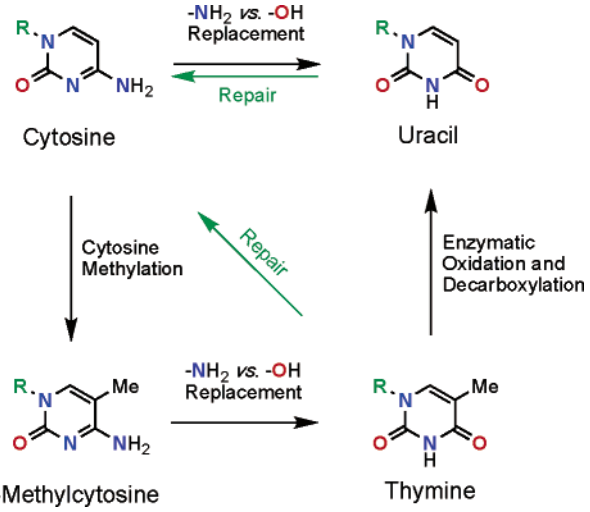

Figure 1. Cytosine deamination to uracil and thymine.

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

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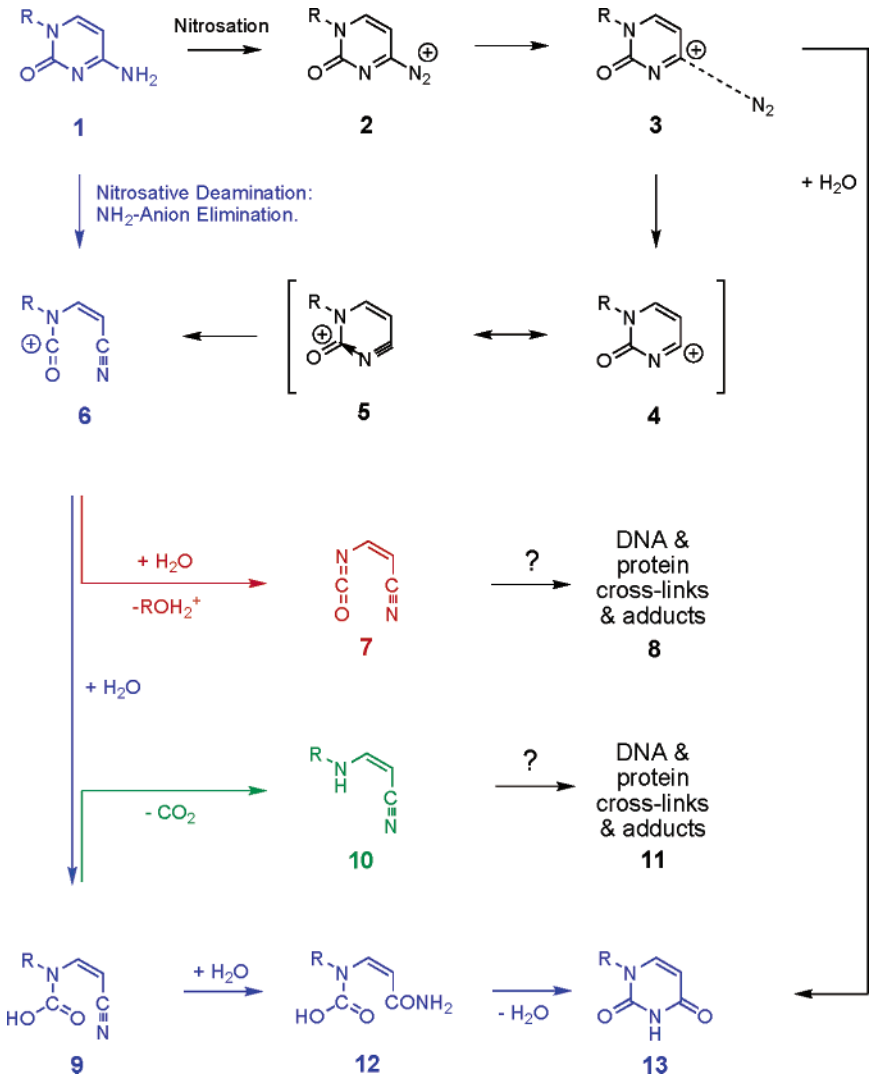

Figure 2. Putative intermediates discussed for nitrosative cytosine deamination.

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-isocyanoacrylonitrile **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-

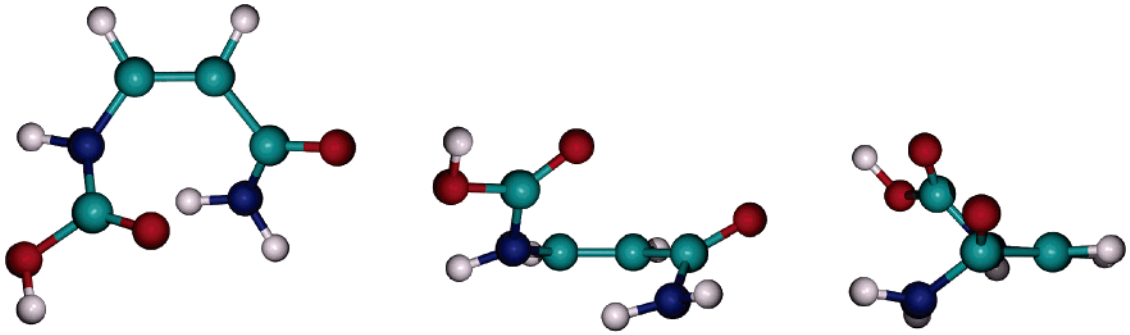

Figure 3. The structure of **12** (MP2/6-31G*) is well-suited for cyclization.

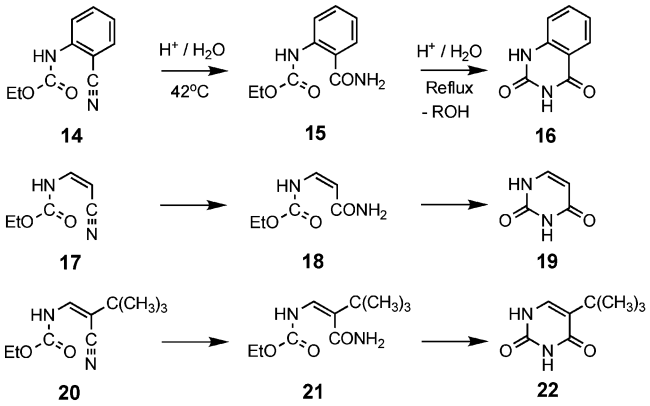

Figure 4. Scope of the cyclization study.

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 carbamic 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 freshly distilled before use for the carbamate synthesis. Pd-C used was 10 wt %. HPLC-grade water and methanol were purchased from Fischer. The HPLC-grade water was filtered through 0.45 μm filter paper under reduced pressure before use. Thin-layer chromatography was carried out on 5−17 μm silica gel plates and UV-light was used as a visualizing agent. Preparative TLC purifications were performed on 20 cm × 20 cm silica gel plates with a layer thickness of 1 mm. Standard column chromatography was performed using 200−425 mesh silica gel.

High-Pressure Liquid Chromatography. The HPLC analyses were performed on a Shimadzu LC system that consisted of a LC-10ATvp pumping system, a CTO-10Avp column oven, and a SPD-M10Avp photodiode array detector. The temperature of the column oven was set at 30 °C. The samples were injected with a SIL 10A autosampler, and the mobile phase was an isocratic 60:40 water/methanol mixture. For analytical purposes, a Supelcosil octadecylsilane column (250 × 4.6 mm i.d., 5 μm particle size) was used, and the flow rate was set at 1 mL/min. The volume of sample injected was 10 μL. For the semipreparative HPLC work, a Supelcosil octadecylsilane column (250 × 10 mm i.d., 5 μm particle size) was used and the flow rate was maintained at 3.73 mL/min. The volume of the sample injected was 37.3 μL. The fractions were collected in multiple runs with the FRC-10A fraction collector.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 250, 300, or 500 MHz on Bruker spectrometers. Chemical shifts and coupling constants are reported in parts per million and Hertz, respectively. The peaks in the ^{13}C NMR were assigned based on increment systems (*39*).

Mass Spectrometry. Electron impact mass spectra of ethyl 2-aminocarbonylphenyl-carbamate **15** and quinazoline-2,4-dione **16** were recorded on a ZAB-SE mass spectrometer (VG Analytical, Manchester, U.K.) operating with MTIMS data system (Mommers Tech., Inc., Ottawa, Ontario, Canada). The ionization energy was 70 eV, and the ion source temperature was 220 °C. EI mass spectra were averaged over at least 20 scans. Mass spectra of the oligomers of 3-aminoacrylonitriles **23** were recorded with a TSQ 7000 triple-quadrupole mass spectrometer (Thermoquest, San Jose, CA) operated in positive ion electrospray ionization mode. Samples were introduced using a syringe pump at a flow rate of 10 μL/min. The temperature of the heated capillary was 250 °C, and an electrospray voltage of 4.5 kV was applied. Collision-induced dissociation of mass selected ions was performed using Ar as a target (pressure ~1.9 mTorr) at the normal collision energy of 25 eV.

Liquid Chromatography/Mass Spectrometry. The LC component consisted of a Finnigan P4000 pump, an AS3000 autosampler, and a UV6000 LP detector. The mobile phase was an isocratic 60:40 mixture of 0.1% HCOOH in water and MeOH. The flow rate was 1 mL/min, and the injection volumes were 20 μL. The separations were carried out on a Supelcosil octadecylsilane column (250 × 4.6 mm i.d., 5 μm particle size). The LC was coupled to a TSQ 7000 triple-quadrupole mass spectrometer which was operated in positive ion atmospheric pressure chemical ionization mode. The temperature of the heated capillary was 250 °C.

Ethyl 2-Cyanophenylcarbamate 14. Carbamate **14** was synthesized in analogy to 2-ethoxycarbonylamino-4-hydroxybenzonitrile (*40*). Aminobenzonitrile (1.78 g, 15 mmol) was dissolved in ethyl acetate (15 mL), and ethyl chloroformate (1.63 g, 15 mmol) was added. After the mixture was boiled for 30 min, another 15 mmol of ethyl chloroformate was added, and the solution was boiled for another 30 min. After cooling and

filtration, the solvent was evaporated, and the crude product was recrystallized in ethanol/water to obtain 2.36 g of **14**, 83% yield. ^{1}H NMR (300 MHz, $CDCl_3$): 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). ^{13}C NMR (300 MHz, $CDCl_3$): 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 (CH_2), 14.32 (CH_3).

Quinazoline-2,4-dione 16. Carbamate **14** (23 g, 120 mmol) was refluxed overnight in 9 mL concentrated HCl and 12.5 mL water. Some solid formed during refluxing. The reaction mixture was cooled, and the solid was filtered. ^{1}H NMR, ^{13}C NMR, and mass spectra confirmed the formation of **16** (16.6 g, 85% yield). In a deviation from the literature (*41*), this ring-closure was accomplished in the absence of urea.

This reaction was also attempted at low temperature (42 °C). After 96 h, the cyano group hydrolyzed to the amide, ethyl 2-(aminocarbonyl)phenylcarbamate **15** (13.7 g, 55% yield), which was insoluble. The temperature was then increased, and the reaction mixture was refluxed, the solid dissolved, and a new solid precipitated upon cooling. The solid was filtered off, and NMR indicated the formation of **16**. **15**, ^{13}C NMR (300 MHz, $CDCl_3$): δ 170.82 (CO−NH_2), 152.95 (CO−OEt), 139.92 (C−NH), 132.44 (CH), 128.71 (CH), 121.53 (CH), 118.61 (C−CO), 118.45 (CH), 60.57 (CH_2), 14.42 (CH_3). MS (EI) *m/z* (%): 208.0 (55) [M^+]. **16**, ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.23 (d, 2H), 7.87 (dd, H), 7.62 (td, H), 7.14−7.18 (m, 2H). ^{13}C NMR (300 MHz, $CDCl_3$): δ 162.79 (NH−CO−NH), 150.26 (CH−CO−NH), 140.83 (C−NH), 134.92 (CH), 126.92 (CH), 122.29 (CH), 115.28 (CH), 114.31 (C−CO). MS (EI) *m/z* (%): 162.0 (100) [M^+].

3-Aminoacrylonitriles 23, Method I. Malononitrile (2 g, 30 mmol) dissolved in ether/THF (1:2, 10 mL) 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 precipitated salt. The ether layer was dried over K_2CO_3. The NMR spectrum of the crude mixture indicated the presence of three products, the (*E*)- and (*Z*)-isomers of 3-aminoacrylonitriles, **23**, and 2-aminonicotinonitrile. The separation of the (*E*)- and (*Z*)-isomers was attempted by column chromatography using 5% CH_3CN/CH_2Cl_2, and it was unsuccessful. The compounds decomposed when exposed to air at room temperature but were stable in the refrigerator for several weeks. (*Z*)-3-aminoacrylonitrile **23**, ^{1}H NMR (250 MHz, CD_3COCD_3): δ 6.83−6.95 (d, J = 8.27 Hz, H, CH attached to NH_2), 5.70−6.30 (br, NH_2), 3.92−3.95 (d, J = 8.28 Hz, H, CH attached to CN). (*E*)-3-aminoacrylonitrile **23**, ^{1}H NMR (250 MHz, CD_3COCD_3): δ 7.01−7.15 (d, J = 13.67 Hz, H, CH attached to NH_2), 5.70−6.30 (br, NH_2), 4.21−4.27 (d, J = 13.79 Hz, H, CH attached to CN). The coupling to NH_2 protons was observed in both isomers as with the 3-amino acrylic esters (*42*). 2-Aminonicotinonitrile, (250 MHz, CD_3COCD_3): δ 8.20−8.23 (d, H), 7.78−7.82 (d, H), 6.67−6.72 (dd, H), 5.57−5.59 (br s, NH_2).

3-Aminoacrylonitriles 23, Method II. A total of 9.7 g (0.1 mol) of 3-ethoxyacrylonitrile was mixed with 17 g (1 mol) of liquid ammonia, and the reaction was carried out at 100 °C in an autoclave for 4 h. Fractional distillation gave 3 g (45% yield) of (*Z*)-3-acrylonitrile, **23**. ^{1}H NMR (250 MHz, $CDCl_3$): δ 6.66−6.78 (m, H), 5.03 (s, 2H, NH_2), 3.81 (d, H). ^{13}C NMR (250 MHz, $CDCl_3$): δ 150.17 (C−NH_2), 118.58 (CN), 62.55 (CH).

***t*-Butylmalonodinitrile 27 (*43*).** The reaction was carried out under nitrogen. To the solution of aluminum chloride (14.5 g, 100 mmol) in 40 mL of nitromethane was added malonodinitrile **26** (6.6 g, 100 mmol). The temperature of the reaction mixture was kept between −15 and −20 °C, and *tert*-butyl bromide (22 g, 160 mmol) was added over a period of 30−45 min. The reaction mixture was allowed to warm to 0−5 °C and was maintained at that temperature. The reaction was monitored by TLC; the eluent was 30% EA/hexane. More *tert*-butyl bromide was added to complete the reaction. The reaction was worked up after 15 h by addition of a saturated solution of sodium bicarbonate to neutralize the HBr produced. The reaction mixture was extracted with methylene chloride. The organic layer was dried over anhydrous sodium sulfate and concentrated over vacuum. The **27** obtained (8.5 g, 70% yield) can be used for the next step without purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.42(s, H), 1.24 (s, 9H).

(*Z*)-3-Amino-2-*tert*-butylacrylonitrile 28 (*44*). *t*-Butylmalonodinitrile **27** (1 g, 8.2 mmol) was dissolved in methanol (5 mL) and hydrogenated using Pd-C (300 mg) as catalyst. The hydrogen gas pressure was kept at 60 psi. The reaction was very slow and completed after 6 days. The reaction was monitored by TLC (30% EA/hexane). The catalyst was filtered off, and the methanol was evaporated to obtain (*Z*)-3-amino-2-*tert*-butylacrylonitrile **28** (0.73 g, 72%). The ^{1}H NMR of the crude product agreed with the literature. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.58−6.66 (t, 1H), 4.20−4.50 (br, NH_2), 1.12 (s, 9H). ^{13}C NMR (500 MHz, $CDCl_3$): δ 142.32 (CH), 119.30 (CN), 92.54 (C, olefinic), 31.91 (C, *tert*-butyl), 29.84 (CH_3, *tert*-butyl). MS (ESI), *m/z*: 195 [$M - H]^-$. MS (EI) *m/z*: 124 [M^+], 104 [$M^+ - CH_3$].

Ethyl (*Z*)-2-Cyano-3,3-dimethylbut-1-enylcarbamate 20 (*45*). To a stirred solution of (*Z*)-3-amino-2-*tert*-butylacrylonitrile **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 pruduct **20** was purified by column chromatography (1.5 g, 47% yield). ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.14−7.18 (d, 1H), 4.18−4.26 (q, 2H), 1.26−1.31 (t, 3), 1.17 (s, 9H). ^{13}C NMR (300 MHz, $CDCl_3$): δ 152.57 (CO), 134.74 (CH), 116.29 (CN), 101.89 (C, olefinic), 62.57 (O−CH_2), 33.00 (C, *tert*-butyl), 29.24 (CH_3, *tert*-butyl), 14.33 (CH_3). MS (ESI) *m/z*: 195 [$M - H]^-$.

Ethyl (*Z*)-2-Aminocarbonyl-1-*t*-butylvinylcarbamate 21 and 5-*t*-Butyluracil 22 from Ethyl (*Z*)-2-Cyano-1-*t*-butylvinylcarbamate 20. Potassium carbonate (66 mg, 0.48 mmol) was added to a solution of **20** (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 as 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*-butyluracil **22** were formed.

5-*t*-Butyluracil 22 from Ethyl (*Z*)-2-Aminocarbonyl-1-*t*-butylvinylcarbamate 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 chromatography. 5-*tert*-Butyluracil **22** was isolated in (4 mg) 14% yield. ^{1}H NMR (500 MHz, CD_3OD): δ 7.11 (s, 1H), 1.27 (s, 9H). ^{13}C NMR (500 MHz, CD_3OD): δ 165.77 ($CONH_2$), 153.59 (CO), 137.41 (CH), 122.35 (C, olefinic), 33.48 (C, *tert*-butyl), 29.05 (CH_3, *tert*-butyl). MS (+APCI) *m/z*: 201 [$M + H^+ + CH_3OH$], 169 [$M + H]^+$.

Results

Synthesis, Properties, and Oligomerization of β-Aminoacrylonitrile. We synthesized (*Z*)-β-aminoacry-

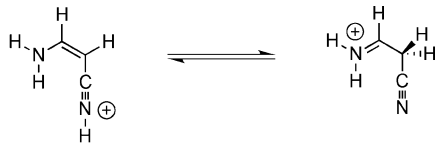

Figure 5. C2-Protonation and nitrilium ion formation of **10** are competitive.

lonitrile **23**. According to one report by Sieveking and Lüttke, **23** should be accessible from malononitrile in 40% yield (*46*). Yet, our attempts gave mixtures of (*Z*)- and (*E*)-**23** along with small amounts of 2-aminonicotinonitrile. Vacuum distillation damaged the product, and column chromatography with acidic silica gel caused 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-ethoxyacrylonitrile in liquid ammonia according to Peeters, Prange, and Vogt (*47*). 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 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-amino 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-(1*H*,3*H*)-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 sp^2-sp^3 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 H_2O_2/NaOH in MeOH (*48*), H_2O_2/NaOH in CH_2Cl_2 with phase transfer catalysis (*49*), H_2O_2/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 H_2O_2/K_2CO_3 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**. $K_2CO_3 \cdot 1.5H_2O$ 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 R_f 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 $[\mathbf{A} + H]^+$ with *m/z* = 169 and identified **A** as 5-*tert*-butyluracil **22**, $[\mathbf{B} + H]^+$ at *m/z* = 216 and identified **B** as the product of complete nitrile hydrolysis, and $[\mathbf{C} + H]^+$ at *m/z* = 215 identified **C** as the amide **21**. Pure **21** was obtained in

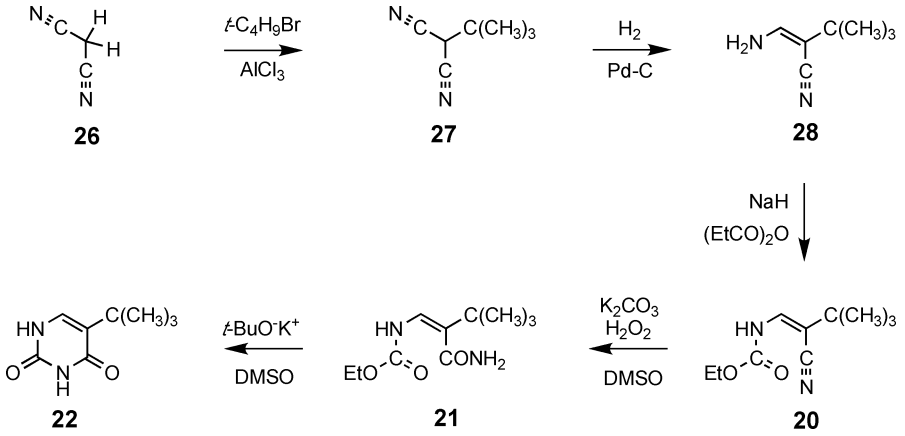

Figure 6. Synthesis of ethyl (*Z*)-2-cyano-1-*tert*-butylcarbamate **20**, of (*Z*)-2-carboxamido-1-*tert*-butylcarbamate **21**, and of 5-*t*-butyluracil **22**.

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-(1*H*,3*H*)-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 dioxane/water 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_T = 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 nitrosative 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 experimentation 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** → **15** → **16** and **20** → **21** → **22** in vitro suggest that the cyclization of the acid **9** → **12** → **13** do not occur under physiological conditions. Present knowledge neither excludes nor suggests the possibility of in vivo enzymatic catalysis of the reaction **9** → **12** → **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 β-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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