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Cytosine Catalysis of Nitrosative Guanine Deamination and Interstrand Cross-Link Formation

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Abstract: Effects are discussed of the anisotropic DNA environment on nitrosative guanine deamination based on results of an ab initio study of the aggregate **3** formed by guaninediazonium ion **1** and cytosine **2**. Within **3**, the protonation of **2** by **1** is fast and exothermic and forms **6**, an aggregate between betaine **4** (2-diazonium-9*H*-purin-6-olate) and cytosinium ion **5**. Electronic structure analysis of **4** shows that this betaine is not mesoionic; only the negative charge is delocalized in the π-system while the positive charge resides in the σ-system. Potential energy surface exploration shows that both dediazoniation and ring-opening of betaine **4** in aggregate **6** are fast and exothermic and lead irreversibly to *E*-**11**, the aggregate between (*E*)-5-cyanoimino-4-oxomethylene-4,5-dihydroimidazole *E*-**10** and **5**. The computed pair binding energies for **3**, **6**, and *E*-**11** greatly exceed the GC pair binding energy. While **1** can be a highly reactive intermediate in reactions of the "free nucleobase" (or its nucleoside and nucleotide), the cyanoimine **10** emerges as the key intermediate in nitrosative guanine deamination in ds-DNA and ds-oligonucleotides. In essence, the complementary nucleobase cytosine provides base catalysis and switches the sequence of deprotonation and dediazoniation. It is argued that this environment-induced switch causes entirely different reaction paths to products as compared to the respective "free nucleobase" chemistry, and the complete consistency is demonstrated of this mechanistic model with all known experimental results. Products might form directly from **10** by addition and ring closure, or their formation might involve water catalysis via 5-cyanoamino-4-imidazolecarboxylic acid **12** and/or 5-carbodiimidyl-4-imidazolecarboxylic acid **13**. The pyrimidine ring-opened intermediates **10**, **12**, and **13** can account for the formations of xanthosine, the pH dependency and the environment dependency of oxanosine formation, the formation of the classical cross-link **dG(N^2)-to-dG(C2)**, including the known sequence specificity of its formation, and the formation of the structure-isomeric cross-link **dG(N1)-to-dG(C2)**.

Introduction

DNA oxidizing chemicals are of interest because of their antibiotic, antitumor, carcinogenic, and mutagenic properties,[1] and nitrosating reagents comprise an important class of DNA damaging agents.[2] Disorders in cell function result when the DNA damage exceeds the repair capabilities and may lead to miscoding or a blocking lesion during replication.[3,4] A variety of disorders in people are thought to result from DNA base deamination and interstrand cross-link (ICL) formation due to reaction with HNO_2 or NO.[5] Guanine deamination has been studied the most (Table 1), and it had been generally assumed, basically since Strecker's 1861 article,[6,7] that the reaction of HNO_2 and guanine leads to the formation of the guaninediazonium ion as the common intermediate. In this mechanistic model, the hydrolysis of guaninediazonium ion gives xanthine by nucleophilic heteroaromatic substitution of N_2 by water, traces of hypoxanthine are formed by S_NAr reaction with nitrite ions, and the cross-link **dG-to-dG**[8−11] is formed if the NH_2 group of a neighboring **dG** on the opposite strand acts as the nucleophile (Scheme 1). The discovery of 2′-deoxyoxanosine as a product of nitrosation of 2′-deoxyguanosine, oligodeoxynucleotide, and calf thymus[12] with nitrous acid required a closer examination of the reaction mechanism (vide infra). The recognition that endogenous nitric oxide[13] causes nitrosation[14] and that this process is accelerated by chronic inflammatory diseases[15] led to several studies of guanosine deamination by

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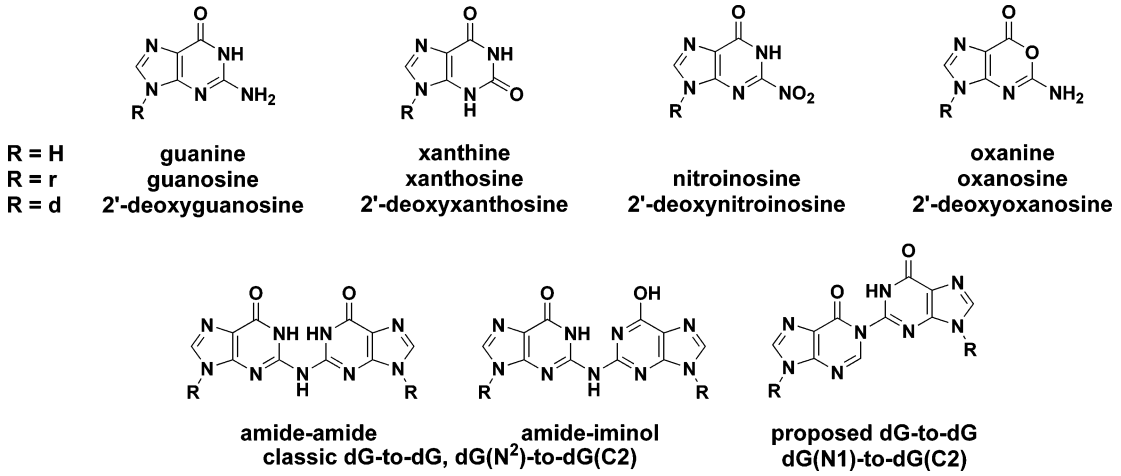
Table 1. Overview of Experimentally Observed Products in Various Reaction Environments

			yield (%)			reference	
pH	reagent	substrate	X	O	dG-to-dG		
4.2	HNO_2	ds-DNA			<0.17	Shapiro 1977	8
4.1−4.5	HNO_2	ds-ON			<3.2	Hopkins 1991 and 1992	9
3.7	HNO_2	dG	58.7	21.5		Suzuki 1996	12a
		ss-dTGTT		24.7			
		ds-DNA		29.4			
3.7	HNO_2	rG	79.3	17.9		Suzuki 2000	12d
2.9	NO	dG, dpG	obsd			Keefer 1991	16
∼7	NO (no O_2)	yeast RNA	80			Tannenbaum 1992	17
7.4	NO and O_2	dG		obsd		Suzuki 1997	12c
7.4	NIAN	dG	obsd	obsd		Shuker 1999	18a
7.4	NIAN	ds-ON			0.35	Shuker 2001	18b
7.4	biol. concd NO and O_2	ds-DNA	∼25−35	absent	∼2	Dedon 2003	19

Scheme 1



NO, by NO-releasing compounds (e.g., 1-nitroso-indole-3-acetonitrile, NIAN), and by NO/O_2. Kiefer et al.[16] and Tannenbaum et al.[17] reported the formation of xanthosine, and Suzuki[12c] and Shuker[18] also detected oxanosine under these conditions. On the other hand, oxanosine was absent in experiments by Dedon,[19] which mimicked physiological NO concentrations.

The mechanistic hypotheses invoking a guaninediazonium ion as the reactive species in guanosine deamination and cross-link formation are deductions based on product analyses and analogy to the chemistry of aromatic primary amines. Yet, while benzenediazonium ions are well-characterized stable compounds,[20] no guaninediazonium ion has ever been observed directly. Ab initio studies in our group showed that free guaninediazonium ion **1** intrinsically is kinetically and thermodynamically unstable toward loss of N_2, and moreover, the studies showed that the unimolecular dediazoniation of the ion occurs with concomitant pyrimidine ring-opening.[21,22] The ease of this ring-opening provided a straightforward explanation for the formation of oxanosine via protonated **10**. Properties and reactions of **10** and **10**+H^+ were reported,[23,24] and the nitrosative guanosine deamination was studied with labeling techniques to learn whether *only oxanosine* is formed via **10**+H^+ or whether *all products* are formed via this same intermediate. The labeling studies established that oxanosine was formed via 5-cyanoamino-4-imidazolecarboxylic acid **12** or its tautomer 5-carbodiimidyl-4-imidazolecarboxylic acid **13** and that xanthosine was formed by S_NAr chemistry.[25] The S_NAr chemistry under "free nucleobase" conditions also accounts for the traces of nitroinosine. We reported the synthesis and purification of the respective amide, 5-cyanoamino-4-imidazolecarboxamide **14**,[26] studied its cyclization chemistry (possibly via **15**),[26] and reported on the formation of the cross-links **dG(N^2)-to-dG(C2)** and **dG(N1)-to-dG(C2)** by addition of **dG** to **14** or **15**.[27] Note that

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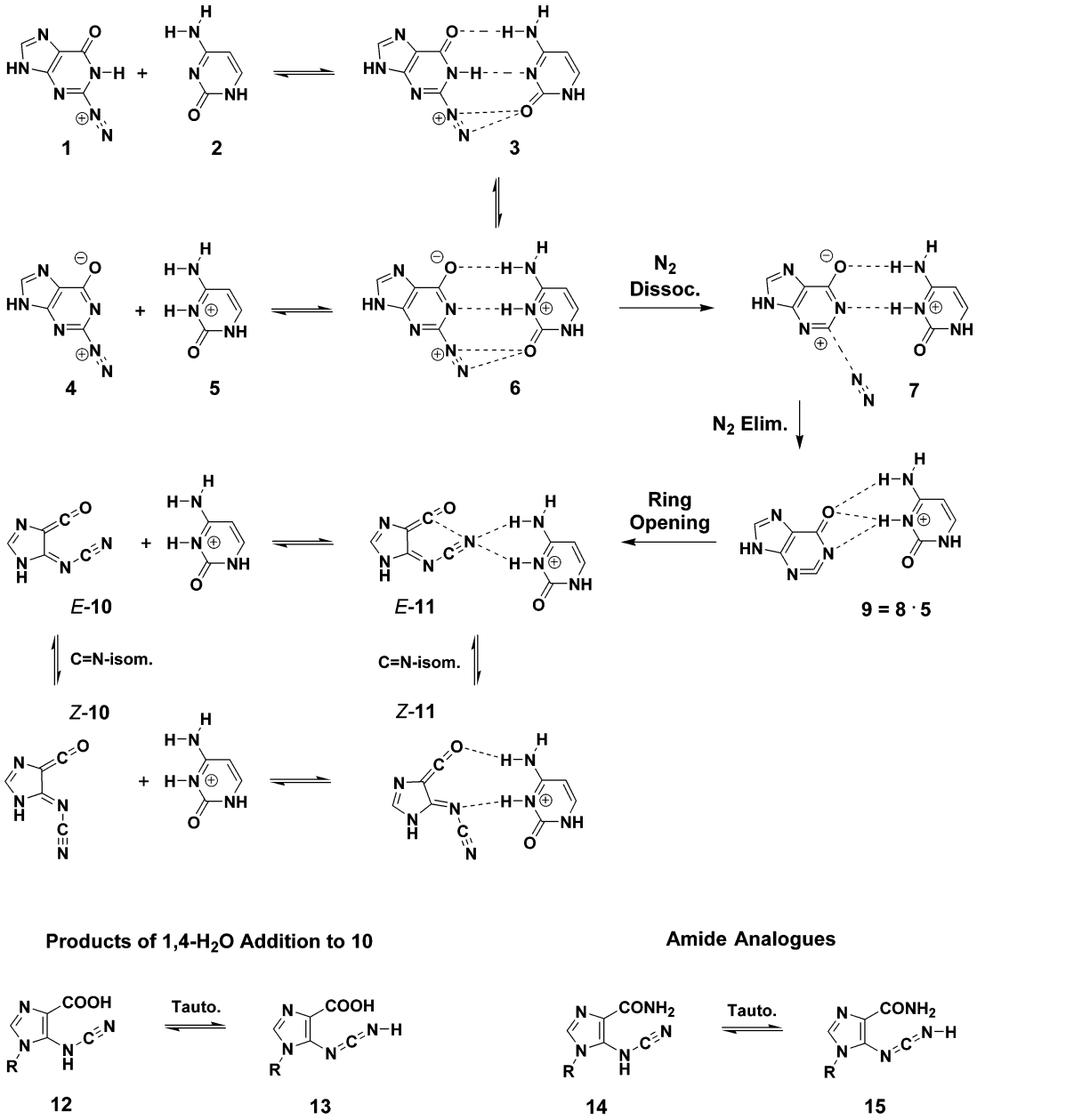
Scheme 2



dG(N^2)-to-dG(C2) usually is written as the amide−amide tautomer, while recent theoretical and experimental studies indicate a preference for the amide−iminol tautomer.[27,28]

It thus seemed possible to explain all the guanosine deamination chemistry with the same mechanistic model irrespective not only of pH and nitrosating reagent, but also as to whether it was chemistry of the "free nucleobase" (or of its nucleoside, nucleotide, or of ss-DNA or ss-oligonucleotides) or the "GC base pair" (in ds-DNA or ds-oligonucleotides). However, the recent report by Dedon[19] did reveal a significant environmental effect: *Oxanosine is not formed in the NO-nitrosation of ds-DNA*, while the groups of Suzuki[12c] and Shuker[18a] reported oxanosine formation by NO-nitrosation of the nucleoside at the same pH, 7.4. Hence, there must be an effect of the ds-environment. The situation is further complicated because the 1996 Suzuki experiments show that oxanosine is formed in ds-DNA under acidic conditions (pH = 3.7).[12a] It will be shown that all of these observations can be accounted for by cytosine catalysis.

Studies of the aggregate **3** formed between guaninediazonium ion **1** and cytosine **2** showed that spontaneous single-proton transfer results in the thermodynamically more stable complex **6** formed between the electronically interesting betaine **4**, 2-diazonium-9*H*-purin-6-olate, and cytosinium ion **5**.[29] Dediazoniation of **6** to **11** (Scheme 2) would lead to the aggregate between **5** and 5-cyanoimino-4-oxomethylene-4,5-dihydroimidazole **10**. Note that cyano-N-protonated **10**, **10**+H$^+$, is the product of dediazoniation of **1**. Knowledge about the process **6** to **11** is of fundamental significance to the discussion as to whether **1** might or might not exist in the aggregate with cytosine. If dediazoniation of **6** requires activation, then **3** might

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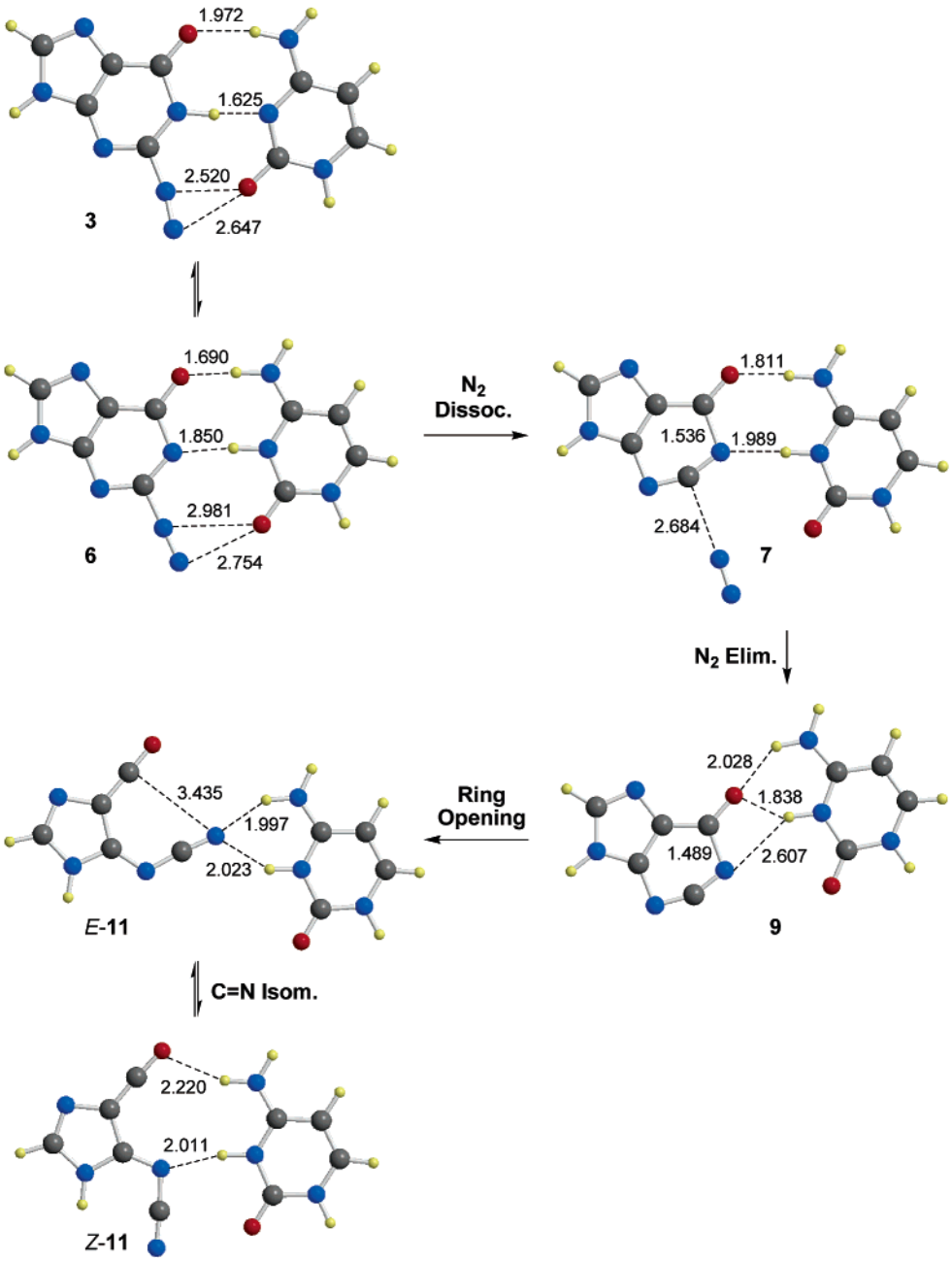

Figure 1. MP2(full)/6-31G* structures of the complexes formed between the guanine derivatives and cytosine or cytosinium ion, respectively.

exist and **1** might be reactive species. On the other hand, if the dediazoniation of **6** is facile, then one would have to conclude that all chemistry emanates from **11** and that **1** is not the central reactive species. The binding energies were determined of the complexes **3**, **6**, and **11**. These binding energies influence the kinetics of the equilibrium between **3** and **6**; a fast forward reaction requires that **1** and **2** are sufficiently bound in **3**, and insufficient binding between **4** and **5** would impede the reversion of **6** to **3**. The stability of **11** informs about stereochemical constraints for reactions of the central intermediate in guanine deamination, **10**. The relevance of the theoretical study of the simple model is established by thorough and thoughtful connection between the experimental results and the conceptual insights derived from the theoretical study. Hence, a discussion is presented as to how the chemistry of **10** can account for the formations of xanthosine and oxanosine, the sequence-specific formation of the classical cross-link **dG(N^2)-to-dG(C2)**, and the formation of the structure-isomer **dG(N1)-to-dG(C2)**, and we state several predictions that will be tested by experimentation.

Computational Methods

In the communication about the proton transfer from **3** to **6**, geometries, vibrational properties, and relative energies computed at RHF/6-31G* were reported together with relative energies determined with density functional theory, B3LYP/6-31G*,[30] that were based on the RHF/6-31G* structures. Density functional theory is a cost-effective method to account for parts of the electron correlation effects in a semiempirical fashion,[31] and the results often are considered more

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Table 2. Energies, Enthalpies, and Gibbs Free Enthalpies (kcal·mol^{-1})

		ΔE	ΔE_0	ΔH_{298}	ΔG_{298}
relative energies	**3** vs **6**	9.53	8.64	8.67	8.71
	7 vs **6**	−2.76	−5.08	−3.57	−8.69
	9 + N_2 vs **6**	1.66	−1.29	−0.58	−12.14
	E-**11** + N_2 vs **6**	−12.12	−16.04	−14.61	−27.78
	Z-**11** + N_2 vs **6**	4.60	0.17	1.73	−11.21
C═N isomerization	*Z*-**10** vs *E*-**10**	1.34	1.36	1.38	1.16
	Z-**10** vs **TS**(**10**)	−18.30	−17.82	−17.54	−18.04
	E-**10** vs **TS**(**10**)	−19.64	−19.18	−18.92	−19.19
	Z-**11** vs *E*-**11**	16.72	16.21	16.34	16.56
pairing energies	**1** + **2** → **3**	50.33	49.43	49.31	37.52
	4 + **5** → **6**	46.12	44.17	44.23	32.13
	E-**10** + **5** → *E*-**11**	29.42	28.36	28.41	17.26
	Z-**10** + **5** → *Z*-**11**	14.05	13.52	13.45	1.85
dissociation energies	**6** → **7**	−2.76	−5.08	−3.57	−8.69
	9 → *E*-**11**	−13.78	−14.75	−14.02	−15.64
	9 → *Z*-**11**	2.93	1.45	2.31	0.92

reliable than the results of perturbation calculations.[32] As the studies progressed,[23] however, the importance of van der Waals bonding and the inability of the B3LYP method to account for dispersion[33] was recognized more fully. Consequently, all of the results reported in the present study employ second-order Møller Plesset perturbation theory.[34] All geometry optimizations and frequency determinations were carried out at the MP2(full)/6-31G* level with the program Gaussian03.[35] Structures along the paths for dediazoniation and for pyrimidine ring-opening of **6** were determined by the coordinate driving method.[36] Atom charges for **6** were calculated with the natural bond orbital method at the MP2(full)/6-31G* level.[37]

Total energies *E* (in hartrees), vibrational zero-point energies VZPE (kcal·mol^{-1}), thermal energies TE (kcal·mol^{-1}, 298.15 K), and entropies *S* (cal·mol^{-1}·K^{-1}) are reported as part of the Supporting Information. In Table 2, ΔE, $\Delta E_0 = \Delta E + \Delta$VZPE, $\Delta H_{298} = \Delta E + \Delta$TE + ΔRT, and $\Delta G_{298} = \Delta H_{298} - 0.29815 \cdot \Delta S$ are reported. Molecular models of the optimized complexes are shown in Figure 1, and coordinates of **1**−**11** are provided as part of the Supporting Information. The NBO charges of **6** are reported in Figure 2.

Results and Discussion

Preference for 6 over 3. The (N1−H)-to-(N3) proton transfer in the **GC** base pair is rather endothermic (17.9 kcal·mol^{-1} at MP2/6-31G**//HF/6-31G*).[38] While the proton transfer in the **GC** base pair *creates* ions, the proton transfer in the deaminated system merely converts one aggregated cation **3** into another aggregated cation **6**. There is no a priori reason for the proton transfer to be endothermic, and in fact, it is substantially exothermic. On the ΔE_0 surface at the MP2(full)/6-31G* level, **6** is 8.6 kcal·mol^{-1} more stable than **3**, and this value agrees closely with the preference energy of ΔE_0(B3LYP/6-31G*//RHF/6-31G*) = 9.2 kcal·mol^{-1}. Proton-transfer energies in aggregates are not just a matter of the acidities and basicities of the free components, and this complicates their explanation. It is well-known that the proton transfer from guanine radical cation to cytosine is almost thermoneutral; theoretical studies show a slight endothermicity for the cytosine aggregate of guanine radical cation,[39] and experimental measurements showed a high rate of proton transfer from guanine radical cation to cytosine in double-stranded DNA.[40]

Proton transfer between proximate donor and acceptors generally requires hardly any activation barrier,[41] and this is true here. The activation barrier for the reaction of **3** to **6** is only ΔE_0(RHF/6-31G*) = 5.1 kcal·mol^{-1} or ΔG_{298}(RHF/6-31G*) = 2.1 kcal·mol^{-1}, respectively, and the transition state disappears on the B3LYP/6-31G*//RHF/6-31G* ΔE_0 and ΔG_{298} surfaces.[42] The proton transfer is tightly coupled to the internal motions of the base pair in the DNA environment, and its precise description would require ab initio direct dynamics studies. For the present purpose, it suffices to know that any activation barrier for the proton transfer from **3** to **6** would be very small and that the formation of guaninediazonium ion **1** in the presence of cytosine immediately leads to **6**.

Intramolecular Bonding in Betaine 4. Betaine **4** formally is a *mesomeric betaine* (aka *mesoionic* compound) that is defined by IUPAC[43] as "dipolar five- (possibly six-) membered heterocyclic compounds in which *both the negative and the positive charge* [emphasis ours] are delocalized, for which a totally covalent structure cannot be written, and which cannot be represented satisfactorily by any one polar structure." Sydnones[44a] and münchnones[44b] are early examples of such betaines with exocyclic negative charges, and diazopyrazole is the classical example[45] with exocyclic positive charge. The diazonium and

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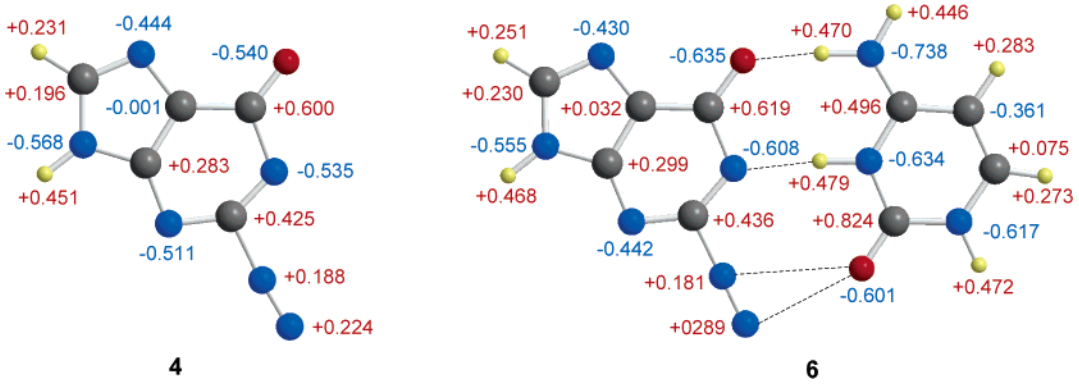
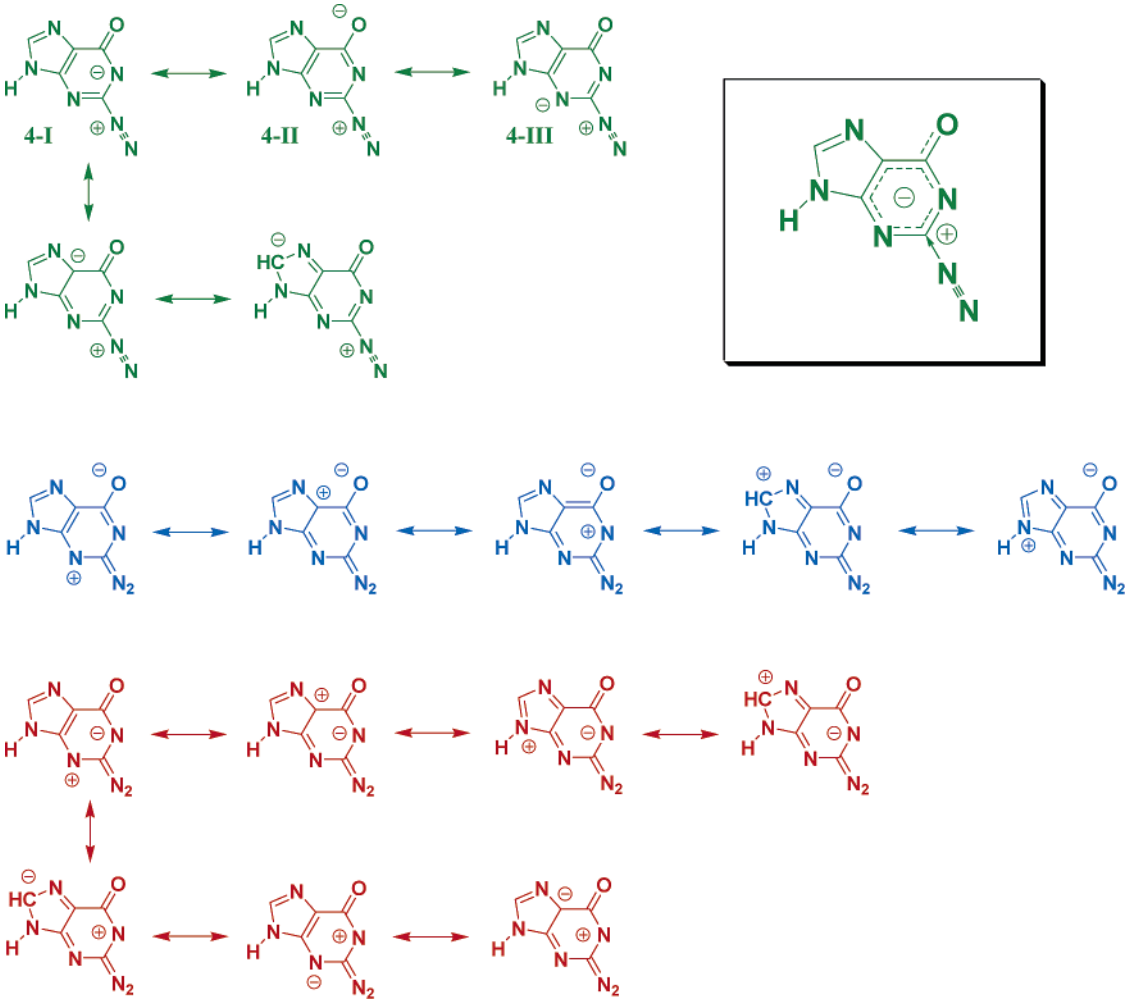


Figure 2. NBO charges for **4** and **6**.

Scheme 3. Delocalization in Betaine **4**[a]



[a] Green: exocyclic (+)-charge; blue: endocyclic (+)- and exocyclic (−)-charge; red: endocyclic (+)- and endocyclic (−)-charge.

diazo resonance forms are shown in Scheme 3. The results of the NBO analyses (Figure 2) clearly show that only **I**−**III** are important for **4** ($q(O^6) = -0.540$; $q(N1) = -0.535$; $q(N3) = -0.511$) and that cytosinium ion complexation increases the contributions of **4-I** and **4-II** in **6** ($q(O^6) = -0.635$; $q(N1) = -0.608$; $q(N3) = -0.442$).

Here is a case that is not well served by its formal description as a mesomeric betaine; *both charges* would have to be delocalized in a mesomeric betaine, and only the negative charge is delocalized in **4**.[46] We have shown in a series of articles[47] that diazonium ions are best described by a bonding model invoking C←N σ-dative bonding between the σ-donor N_2 and the σ-cation. This model applies with minor modification to the heteroaromatic diazonium ion **4** as well, and two differences are noteworthy. While the N_2 group is almost neutral in

(45) Ollis, W. D.; Stanforth, S. P.; Ramsden, C. A. *Tetrahedron* **1985**, *41*, 2239.

(46) Diazocyclohexa-2,4-dienone: Lowe-Ma, C. K.; Nissan, R. A.; Wilson, W. S.; Houk, K. N.; Wang, X. *J. Chem. Res., Synop.* **1988**, *7*, 214.

(47) (a) Glaser, R.; Horan, C. J. *J. Org. Chem.* **1995**, *60*, 7518. (b) Glaser, R.; Horan, C. J.; Zollinger, H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2210. (c) Glaser, R.; Horan, C. J.; Lewis, M.; Zollinger, H. *J. Org. Chem.* **1999**, *64*, 902.

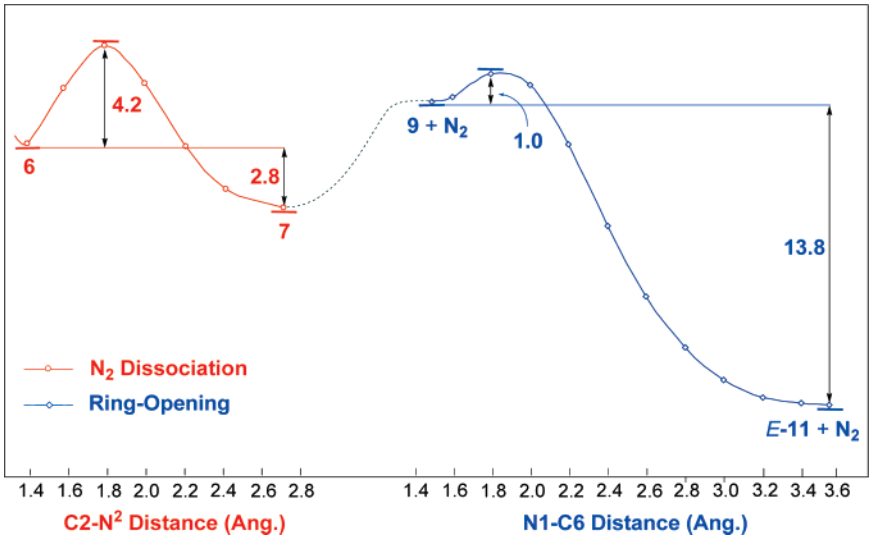


Figure 3. ΔE potential energy surfaces. Dediazoniation and ring-opening require hardly any activation and are overall exothermic.

benzenediazonium ion, it is slightly positive in **4** (+0.412) and **6** (+0.470). This stronger C←N σ-dative bonding is the expected consequence of the higher electronegativity of the pyrimidine ring as compared to benzene itself.[48] The other difference concerns N_α; this atom is slightly *positive* in **4** while it is *negatively* charged in benzenediazonium ion. With stronger C←N σ-dative bonding and the overall loss of N_2 electron density in **4**, the cause for the internal N_2 polarization diminishes and positive charge delocalization over C_{ipso}, N_α, and N_β results. Hence, betaine **4** does *not* have a positive charge in the π-system at all! Only if the N_2 group would engage in much *weaker* C←N σ-dative bonding would π-donation from the heterocycle-diazo resonance forms become an option.

Binding Energies for Aggregates 3 and 6. The binding energy of the Watson−Crick guanine−cytosine (WCGC) base pair provides the appropriate reference for the discussion of the binding energies for **3** and **6** (Table 2). The gas-phase **GC** binding enthalpy $\Delta H_{298} = 21.0$ kcal·mol^{-1} was measured.[49] **GC** pair binding enthalpies of 25.8 and 26.5 kcal·mol^{-1}, respectively, were reported at the levels B3LYP/6-31G** and MP2/6-31G**//HF/6-31G**, respectively.[50] The consideration of the DNA environment on the WCGC base pair stability with the AMBER, CHARM, MSC1, and MSC2 force fields resulted in binding enthalpies of 22.7−23.7 kcal·mol^{-1}.[51] A WCGC binding enthalpy of 21.9 kcal·mol^{-1} was calculated using the Langevin dipoles solvation model taking into account vertical stacking of DNA base pairs.[52] These studies show that the DNA environment affects the binding energy of the WCGC base pair by less than 5 kcal·mol^{-1}, and thus, the binding energy of the free base pair is a good first approximation of the binding energy of the base pair embedded in DNA.

Binding enthalpies ΔH_{298} of 49.3 and 44.2 kcal·mol^{-1} were computed for **3** and **6**, respectively, and the Gibbs free enthalpies ΔG_{298} of 37.5 and 32.1 kcal·mol^{-1} for **3** and **6**, respectively, are about 12 kcal·mol^{-1} lower. The entropy calculations for the model systems refer to the free components, and the entropy loss associated with aggregation in this model is much higher than it would be in the DNA environment. Hence, the ΔG_{298} pair binding free enthalpies present lower limits. Both **3** and **6** are tightly bound, and the *nitrosative derivatization of the* ***GC*** *base pair approximately doubles the pair binding energy*.

The dediazoniation of guanine results in a protonated cytosine fragment, **5**. The site of protonation, N3, is expected from the pK_b values of aniline and pyridine and was confirmed experimentally for cytosine[53] and cytidine.[54] Proton affinities of 225.8 and 233.7 kcal·mol^{-1} were measured for cytosine and cytidine in gas phase, respectively, and N3 was found to be the preferred site of protonation.[53−55] Theoretical studies have shown that N3 and O^6 have similar proton affinities.[56] Protonated cytosine is highly electrophilic, prone to various reactions, and able to provide acid catalysis to reactions of **10**.

Dediazoniation and Ring-Opening of Betaine 4 in Aggregate 6. The N_2 dissociation from fragment **4** in complex **6** was studied by driving the C2−N^2 bond length stepwise (0.2 Å, C_s symmetry), and in Figure 3, the relative energy is shown as the dissociation progresses to **7**, a van der Waals complex between **9** and N_2 with d(C2···N^2) = 2.684 Å. The activation barrier separating **6** and **7** is only 4.2 kcal·mol^{-1}, and **7** is $\Delta G_{298} = 8.7$ kcal·mol^{-1} more stable than **6**.

Complete removal of N_2 yields **9**, a complex between cytosinium ion **5** and the cyclic carbodiimide **8**. Optimization of **9** in C_s symmetry rotates fragment **4** and results in H-bonding involving the carbonyl-O and N7 of **4**, respectively, and the H_2N−C4 group and H−N3 in **5**, respectively. In DNA, this type of rotation is impeded by the sugar−phosphate backbones which would replace the H-atoms at N9(**4**) and N1(**5**). The appropriate model **9** (Figure 1) was obtained by optimization

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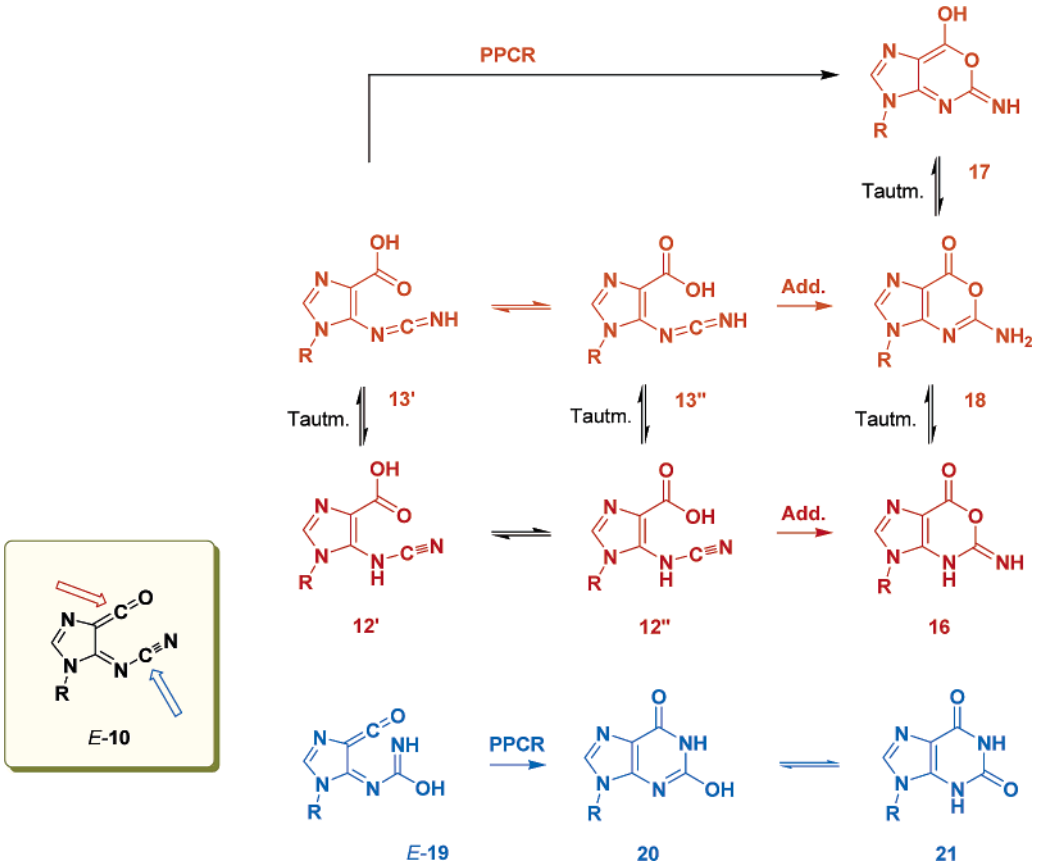
(56) (a) Podolyan, Y.; Gorb, L.; Leszczynski, J. *J. Phys. Chem. A* **2000**, *104*, 7346. (b) Russo, N.; Toscano, M.; Grand, A.; Jolibois, F. *J. Comput. Chem.* **1998**, *19*, 989. (c) Gorb, L.; Leszczynski, J. *Int. J. Quantum Chem.* **1998**, *70*, 855. (d) Colominas, C.; Luque, F. J.; Orozco, M. *J. Am. Chem. Soc.* **1996**, *118*, 6811.

Scheme 4. Paths to Xanthine and Oxanosine from **10**



with the distance between the H-atoms at N9(**4**) and N1(**5**) fixed to the distance found optimal in **7** (10.413 Å). The complete removal of N_2 requires $\Delta E = 4.2$ kcal·mol^{-1} (Figure 3, Table 2).

We are aware of only one report about a cyclic carbodiimide, 2*H*-imidazol-2-ylidene, which was generated in matrix by photolysis of 2-diazo-2*H*-imidazole.[57] The free carbodiimide **8** does not correspond to a minimum on the MP2(full)/6-31G* potential energy surface; all attempts to find **8** resulted in the ring-opening structure **10**. In aggregate **9**, however, cyclic **8** is stabilized and the N1−C6 bond length in fragment **8** of complex **9** was driven. Driving this intrinsic reaction coordinate results in the more stable aggregate *E*-**11** with *d*(N1−C6) = 3.435 Å. The ring-opening reaction is hardly hindered; the barrier is merely $\Delta E_A = 1.0$ kcal·mol^{-1}. There is a small *E*-preference for **10** itself and its *E*/*Z*-isomerization is fast (Table 2). Geometrical isomerization of C-push−N-pull substituted imines occurs readily even at low temperature.[58,59] The intrinsic *E*-preference is greatly enhanced in aggregate **11**; *E*-**11** is preferred over *Z*-**11** by more than 15 kcal·mol^{-1}.

The reaction **6** → *E*-**11** is exothermic, and there are hardly any kinetic barriers (Figure 3). The reaction is exothermic by $\Delta E = 12.1$ kcal·mol^{-1} and $\Delta H_{298} = 14.6$ kcal·mol^{-1}, and the reaction is driven even more by entropy so that the overall reaction is exergonic by $\Delta G_{298} = 27.8$ kcal·mol^{-1}. The dediazoniation and the ring-opening were computed as separate elemental steps. We realize that these processes are coupled and that a large transition state region might exist, but it also is clear that all possible paths feature early N_2 loss and late N1−C6 bond cleavage. Most importantly, the results of the calculations provide compelling evidence in firm support of the conclusion that **6** does not have any significant lifetime and it follows that **3** also does not have any significant lifetime either. In other words, the formation of guaninediazonium ion **1** in the presence of cytosine immediately within less than a nanosecond leads to **6** and on to **11**. In the presence of cytosine, guaninediazonium ion **1** cannot be considered a "reactive species" because it has essentially no lifetime. Hence, one must seek to explain the formations of all the products of nitrosative guanine deamination in ds-DNA or ds-oligonucleotides (ds-ON) based on the chemistry of **10**.

Oxanosine *and* Xanthosine Formations from 10. Water addition to the ketene moiety of **10** leads to the acids **12** and **13** that react on to oxanosine **18**, and this chemistry has been demonstrated.[25] Oxanosine can be formed by intramolecular addition from **12″** and **13″** or by pseudopericyclic reaction from **13′** via **17** (Scheme 4). Water addition to the cyanoamine moiety of **10** gives *E*- and *Z*-**20**, and *E*-**20** can react on to **21**. Both oxanosine and xanthosine can thus be formed by way of pseudopericyclic reactions. Birney's theory of pseudopericyclic reactions suggests that such reactions have hardly any activation barrier.[60] In "free nucleobase" chemistry, the conformational

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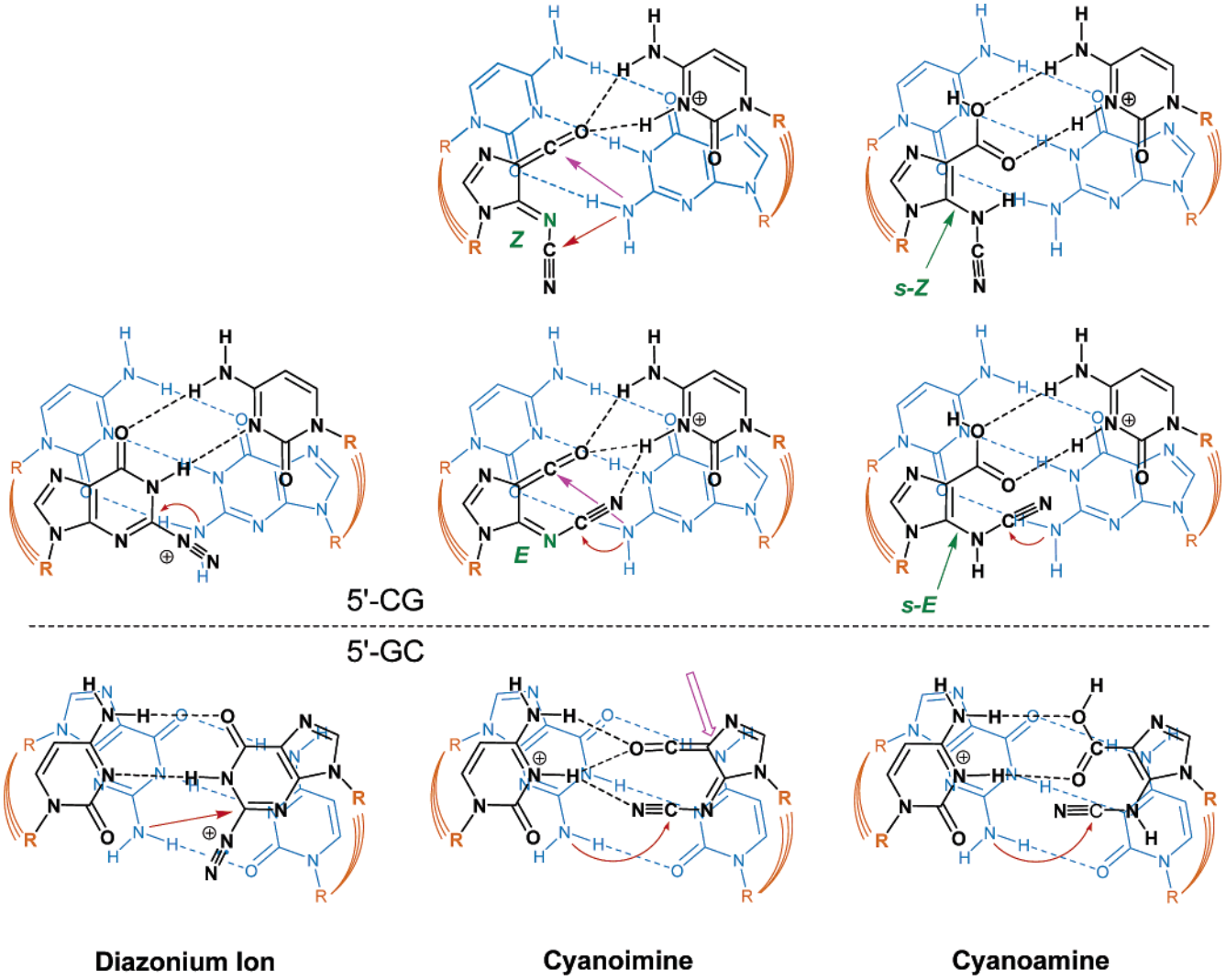
(60) (a) Zhou, C.; Birney, D. M. *J. Am. Chem. Soc.* **2002**, *124*, 5231. (b) Birney, D. M. *J. Am. Chem. Soc.* **2000**, *122*, 10917. (c) Birney, D. M.; Ham, S.; Unruh, G. R. *J. Am. Chem. Soc.* **1997**, *119*, 4509.

Scheme 5. Proximity Effects To Rationalize Sequence Specificities in 5′-**CG** and 5′-**GC** ds-Oligonucleotides and ds-DNA



equilibrium between **12′** and **12″** is very fast, and **12″** is available for ring closure. In DNA chemistry, the water approach occurs in the molecular plane, only **12′** is formed, and the rotation to **12″** is slowed because of base pairing. There is time for **12′** to tautomerize to **13′**, there is time for **12′** and **13″** to form primary cross-links (vide infra), and not all of the initially formed **12′** must react to oxanosine!

Sequence-Specific dG-to-dG Cross-Link Formation via Ring-Opened Guanosine Derivatives. Hopkins[61] reported a 5′-**CG** sequence preference for ICL formation as compared to the sequence 5′-**GC** and rationalized this selectivity based on the sequence-dependent distance between C2(**1**) and the NH_2 group of the **C**-flanking guanine in the opposite strand. Richards tested this hypothesis with QM/MM studies.[62] The two guanines were described by quantum mechanics (QM), and everything else was described by molecular mechanics (MM). Reaction profiles were computed, and a lower barrier was found for the 5′-**CG** reaction than for the 5′-**GC** reaction. There are several methodological deficiencies with this QM/MM study, and they concern both the use of semiempirical QM theory and the selection of the model's QM part. The AM1 parametrization is known to overestimate the stabilities of diazonium ions;[63] this problem was recognized, and it is a minor issue. However, AM1 fails completely to even indicate any propensity for pyrimidine ring-opening even at long C−N_2 distances. This problem became evident only by the time of the ab initio study of guaninediazonium ion.[21,22] The QM/MM calculations did not allow for proton transfer since the cytosine base was described by MM. The force field is not able to describe the zwitterionic product of proton transfer, and more fundamentally, it is not a *reactive* force field[64] and does not allow for any change in *constitution*. Again, this methodological problem only was revealed by ab initio study.[29] Nevertheless, the magnitude of the QM/MM results should have caused some pause because activation barriers of 30−50 kcal·mol^{-1} would make it absolutely impossible for ICL formation to compete with hydrolysis to form xanthine.

In Scheme 5, the proximity considerations by Hopkins are reproduced for **1** (left column) together with alternative scenarios that invoke either the cyanoimine **10** (center) or the cyanoamine **12** (or its carbodiimide tautomer **13)** as reactive species. For

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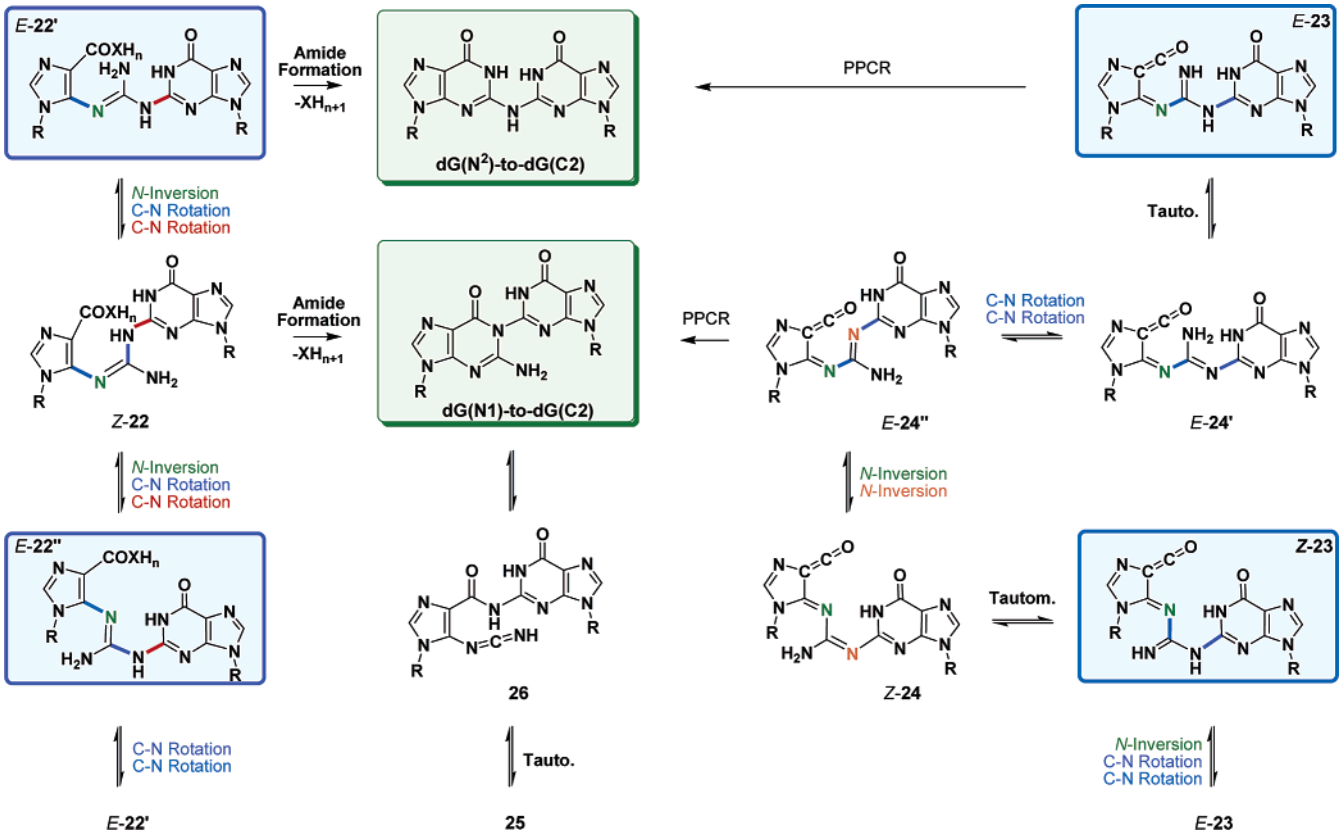
(62) Elcock, A. H.; Lyne, P. D.; Mulholland, A. J.; Handra, A.; Richards, W. G. *J. Am. Chem. Soc.* **1995**, *117*, 4706.
(63) (a) Ford, G. P. *J. Am. Chem. Soc.* **1986**, *108*, 5104. (b) Ford, G. P.; Scribner, J. D. *J. Am. Chem. Soc.* **1983**, *105*, 349.
(64) van Duin, A. C. T.; Dasgupta, S.; Lorant, F.; Goddard, W. A. *J. Phys. Chem. A* **2001**, *105*, 9396.

Table 3. Anisotropic Effects of the DNA Environment on **dG-to-dG** Cross-Link Formation[a]

G derivative	1° ICL	motions and reactions	2° ICL	cycl.	stable ICL	after enzyme workup	free base	DNA
acids **12** or **13**	*E*-**22′**			AF	classical	classical	YES	YES
	E-**22″**	rotations	*E*-**22′**	AF	classical	classical	maybe	NO
	E-**22′**	rotations inversion	*Z*-**22**	AF	new	new	maybe	NO
	E-**22″**	rotations inversion	*Z*-**22**	AF	new	new	maybe	NO
ketene **10**	*E*-**23**			PPCR	classical	classical	YES	YES
	E-**23**	TR rotations	*E*-**24″**	PPCR	new	new	YES	NO
	Z-**23**	TR inversions	*E*-**24″**	PPCR	new	new	YES	YES
	Z-**23**	inversions rotations	*E*-**23**	PPCR	classical	classical	maybe	NO
ketene **10**	**26**			CDIAA	new	new	NO	YES?
	25	TR	26	CDIAA	new	new	NO	YES?

[a] AF = amide formation; PPCR = pseudopericyclic reaction; CDIAA = carbodiimide amine addition; TR = tautomerization reaction.

Scheme 6. Mechanistic Hypotheses for **dG-to-dG** ICL Formation



the 5′-**CG** case, *E*- and *Z*-**10** are considered. The respective structures of **12** (and **13**) are conformations, and they are referred to as *s*-*E*-**12** and *s*-*Z*-**12** ("*s*" for single bond). It is known from ab initio studies that *s*-*Z*-**12** is preferred over *s*-*E*-**12**, and it is known from molecular dynamics studies that the rotational barrier is so low that *s*-*E*-**12** is accessible.

Hopkins' rationale for the 5′-**CG** preference is a reasonable distance criterion: the distance between the guanine C2-amino group and C2(**1**) is shorter in 5′-**CG** as compared to that of the 5′-**GC** case (red arrows in Scheme 5). The sequence preference can be explained by **1**, but the statement is not commutative and does not imply in any way that **1** has to be the reactive species. It will be shown in the following that this same distance criterion applies to considerations of **10** or **12**, and Table 3 gives an overview of options.

Mechanistic hypotheses for ICL formation via ketene **10** or acids **12** or **13** are described in Scheme 6. The possible paths are discussed for the formations of the classical cross-link **dG(N^2)-to-dG(C2)** and its structure-isomer **dG(N1)-to-dG(C2)**; these cross-links are highlighted in green.[27] The cross-links shown on the corners in Scheme 6 are the postulated primary addition products formed by **dG** addition to either a cyanoamine or a cyanomine moiety in the pre-reaction scenarios of Scheme 5. The **dG** addition to the ketene moiety is straightforward and is possible only from one pre-reaction scenario. We start with a consideration of the scenarios involving acid **12** (or **13**), which was explored by experimentation.[26,27]

dG Addition to Cyanoamine 12 or Carbodiimide 13 (5′-CG Only). The structures *E*-**22′** and *E*-**22″** shown on the left (purple frames) in Scheme 6 are conformers of geometrical

isomer *E*-**22** of guanidine **22**, *N*″-(4-imidazolecarboxamide-5-yl)-*N*-(1*H*-purin-6(9*H*)-one-5-yl)-guanidine. Guanidine **22** most likely is the product of **dG** addition to **13**, and there also is the possibility of **dG** addition to **12** and subsequent tautomerization to conjugated **22**. Considering the C−N conformations of **12** and because of the constraints imposed by the double helix, *E*-**22**′ is accessible from *s*-*E*-**12** (Scheme 5, center right), conformation *E*-**22**″ might be formed from *s*-*Z*-**12** (Scheme 5, right top), and the direct formation of isomer *Z*-**22** in the DNA environment is not likely. Both conformers of *E*-**22** can be converted into *Z*-**22** by N-inversion and two C−N rotations, and this conversion is possible for free **22**. In DNA, however, the *E*,*Z*-isomerization of **22** becomes impossible because the C−N rotations are impossible. Similarly, the conversion between *E*-**22**′ and *E*-**22"** requires C−N rotations, and they are possible for free **22** but they are not possible in DNA.

We recently discovered the formations of both **dG(N^2)-to-dG(C2)** and **dG(N1)-to-dG(C2)** from **14** (XH_n = NH_2) and assume that they are formed via the guanidines *E*-**22** and *Z*-**22**, respectively (Scheme 6). Both *E*-**22** and *Z*-**22** can be formed directly in "free nucleobase" chemistry. However, *in DNA only E-22′ can be formed directly and then cyclize to the classical cross-link. E*-**22**″ could form and result in a stable cross-link in DNA. Any such *E*-**22**″ would escape detection in DNA analyses involving enzymatic cleavage with phosphodiesterases; under those conditions *E*-**22**″ would convert to *E*-**22**′ and *Z*-**22**, respectively, and form the classical and the new **dG-to-dG** cross-links, respectively.

dG Addition to Cyanoimine Moiety of 10 (5′-CG only). Guanidines *E*-**23** and *Z*-**23** (Scheme 6, blue frames) are geometrical isomers of **23**, *N*′-(4-imidazole-carboxamide-5-ylen)-*N*-(1*H*-purin-6(9*H*)-one-5-yl)-guanidine. Guanidine **23** results from **dG** addition to cyanoimine **10**. Considering the C═N configurations of **10** and the double-helix structure, *E*-**23** is accessible from *E*-**10** (Scheme 5, center) and the formation of isomer *Z*-**23** from *Z*-**10** (Scheme 5, center top, red arrow) is much less likely. *E*-**23** is perfectly set up to form the classical cross-link by pseudopericyclic reaction. The formation of the structure-isomeric cross-link requires tautomerization of **23** to **24**. In Scheme 6, two conformations, *E*-**24**′ and *E*-**24**″, are shown of *E*-**24** and one conformation is shown of *Z*-**24**. *E*-**24**″ can form the nonclassical **dG-to-dG** cross-link via pseudopericyclic reaction. In nucleoside chemistry, *E*-**24**″ is accessible from *E*-**24**′ and from *Z*-**24**. In DNA, however, the only path to *E*-**24**″ is via double N-inversion of *Z*-**24**.

dG Addition to Ketene Moiety of 10 (5′-CG Only). Paths to the nonclassical cross-link are provided by **dG** addition to **10** in the pre-reaction scenarios shown in the center and center-top of Scheme 5 (purple arrows). Such an addition would form **25** and **26**, the *N*-(1*H*-purin-6(9*H*)-one-5-yl)-substituted analogues of amides **14** and **15**, and the nonclassical **dG-to-dG** ICL forms by fast cyclization.[27]

Overview of Options. In "free nucleobase" chemistry the water addition is fast because [H_2O] ≫ [dG] and acids **12** and **13** are formed. Under these conditions, there is one direct path to the classical ICL and there are three indirect paths to the structure-isomeric **dG-to-dG** ICL. The inversions and rotations delay the formation of the structure-isomeric **dG-to-dG** in the "free nucleobase" chemistry, and this is consistent with our experimental findings for the model system.[27] In DNA, the rotations all are impeded and only the classical **dG-to-dG** ICL can be formed.

On the other hand, there is no reason to assume that water addition to **10** is faster than the formation of a primary cross-link *in a DNA environment* because the bimolecular hydrolysis competes with the de facto unimolecular and template-assisted primary ICL formations. Under these conditions, there are paths from **23** to *both* cross-links in "free nucleobase" and "base pair" chemistry. Moreover, the formation of the new ICL via **25** and **26** becomes an entirely plausible option in the DNA template.

Effects of Cytosine Catalysis on Reaction Mechanisms of Guanosine Deamination. The DNA environment fundamentally changes the mechanism for xanthosine formation, whereas the paths for the formations of oxanosine and of the cross-links are affected by changes in the way of the formation of the key common intermediate (Scheme 7).

The proton affinity of water[65,66] is about 167 kcal·mol^{-1} and much lower than the proton affinity of cytosine[53,54] of about 230 kcal·mol^{-1}. The low basicity of water makes the deprotonation of guaninediazonium ion impossible, and **1** can be a highly reactive intermediate in "free nucleobase" chemistry in aqueous solution at neutral or acidic pH. The product formation depends on the precise mechanism of the dediazoniation. We recently showed that the "unimolecular hydrolysis" of benzenediazonium ion actually proceeds by a bimolecular S_N2Ar mechanism with a very loose transition state structure.[67] Xanthosine is formed if water assists in the dediazoniation of **1** in an analogues fashion (Scheme 7). On the other hand, every time the dediazoniation proceeds without a water molecule "right there" to replace the leaving dinitrogen, the ring opens and protonated cyanoimine **10**+H^+ is formed. The proton affinity for the imine-N protonation of **10** is higher than 200 kcal·mol^{-1} and greatly exceeds the proton affinity of water;[23] hence, **10**+H^+ must add water to form **13**. The chances that an amine is in the solvent shell of **10**+H^+ are practically nil since [H_2O] ≫ [dG]. The water addition results in an O-protonated system, and deprotonation now becomes possible in aqueous media. *At this point, the chemistry changes from cation chemistry (diffusion controlled, unselective) to acid-catalyzed chemistry of neutrals (nucleophile selectivity).* After this point, there is a possibility for amines to compete in the addition to the carbodiimide,[68] and guanidines[27] are formed and not ureas.[69] It is known from labeling experiments that **13** does not add a second water; it either cyclizes to oxanosine or forms the two **dG-to-dG** cross-links. Under "free nucleobase" conditions, these considerations suggest that oxanosine *and the two cross-links* are formed via **10**+H^+ (and not from **1**), while all xanthosine is formed via **1** (not via **13** because of labeling results)!

In the presence of cytosine, the evidence is compelling that guaninediazonium ion **1** merely exists for nanoseconds, a lifetime that is much too short for **1** (or **3**) to qualify as an intermediate in the usual mechanistic sense. There is no time for *any* nucleophile to engage in aromatic substitution of **1**

(65) Collyer, S. M.; McMahon, T. B. *J. Phys. Chem.* **1983**, *87*, 909.
(66) This intrinsic difference is too large to be made up by multiple hydration: (a) Cunningham, A. J.; Payzant, J. D.; Kebarle, P. *J. Am. Chem. Soc.* **1972**, *94*, 7627. (b) Kebarle, P.; Searles, S. K.; Zolla, A.; Scarborough, I.; Arshid, M. *J. Am. Chem. Soc.* **1967**, *89*, 6393.
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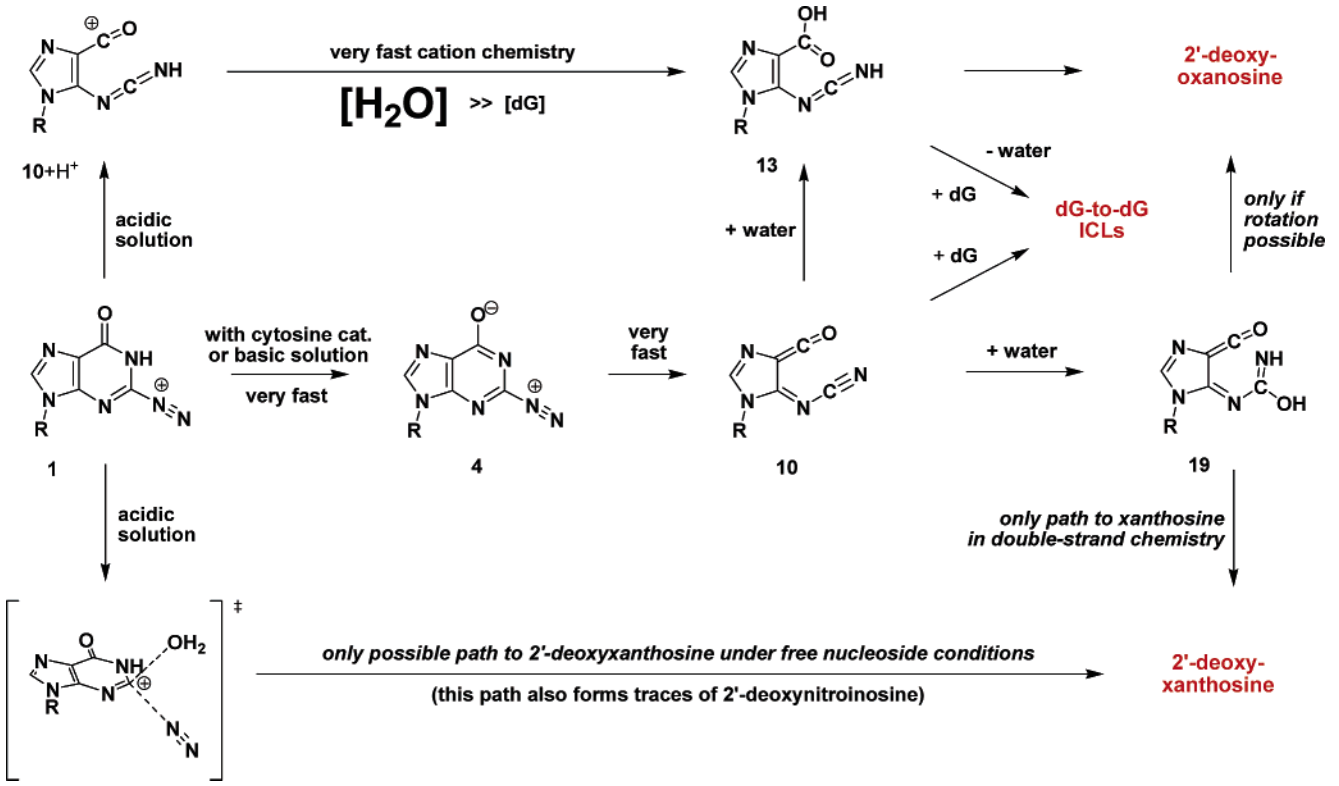
(68) Glaser, R.; Lewis, M.; Wu, Z. *J. Phys. Chem. A* **2002**, *106*, 7950.
(69) Lewis, M.; Glaser, R. *Chem.−Eur. J.* **2002**, *8*, 1934. (b) Lewis, M.; Glaser, R. *J. Am. Chem. Soc.* **1998**, *120*, 8541.

Scheme 7. Entirely Different Paths for Formation of Xanthosine; Different Paths to Common Intermediate for Formations of Oxanosine and Interstand Cross-Links



before the protonation of cytosine **2** by **1** forms **6**, an aggregate between betaine **4** and cytosinium ion **5**. The present results show that both dediazoniation and ring-opening of **4** are fast and exothermic and lead irreversibly to cyanoimine **10** (Scheme 7). The cyanoimine **10** emerges as the key intermediate in nitrosative guanine deamination in the presence of cytosine (e.g., in ds-DNA and ds-oligonucleotides).

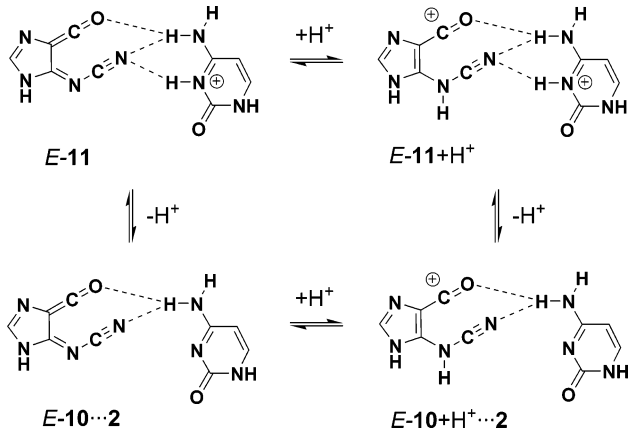
Cross-link formation might be the result of direct **dG** addition to **10** and ring closure by cycloaddition, or it may involve water catalysis via **12** or **13** and subsequent ring closure with water condensation. All these ICL formations via pyrimidine ring-opened intermediates are consistent with Hopkins' sequence preference and can be rationalized by proximity arguments (Schemes 5 and 6). Under these conditions, the only path to xanthosine involves water addition to **19** and pseudopericyclic reaction (Scheme 4).

The chemistry of **10** in **11** presents an interesting situation of acid catalysis (Scheme 8). The simple fact that xanthosine is the main product in ds-DNA deamination shows that *E*-**11** creates an advantage for water addition to the cyanoimine! A lowering of the pH might simply shift the pH-dependent equilibrium *E*-**10**···**5** + H_2O ⇌ *E*-**10**···**2** + H_3O^+, it might allow in addition for some tautomerization of *E*-**11** by way of protonation of *E*-**10**···**2** at *E*-**10**, or it might even lead to some protonation of *E*-**11** to *E*-**11** + H^+ (Scheme 8). All of these effects provide an advantage for the water addition to ketene.

Conclusion

Guanine nitrosation is a complex process and depends greatly on the environment in which it occurs. While the same products are observed in different environments, their formations involve

Scheme 8. Deprotonation, Deprotonation and Reprotonation, and Protonation of **11**



different mechanisms and changed product ratios. In particular, the mechanistic scheme shows that all the results summarized in Table 1 can be explained in a consistent fashion and, hence, allows for the reconciliation of apparently conflicting data. The results of the present study have broad implications on the way one ought to think of the nitrosative guanine deamination.

(1) Historically, the products **dX** and **dG-to-dG** of nitrosative deamination were explained via nucleophilic heteroaromatic substitution of the guanosinediazonium ion. This model is incomplete as it cannot explain the formation of **dO**.

(2) The discovery of oxanosine required the *additional consideration* of a ring-opened intermediate **10**+H^+ to explain **dO** formation. In this mechanistic model, the ratio between [**dX** plus **dG-to-dG**] and [**dO**] informs about the competition between S_N2Ar and pure S_N1Ar chemistry of the guanosinediazonium ion.

(3) In "free nucleobase" chemistry (e.g., nucleobase, nucleoside, nucleotide, and ss-oligonucleotides), our work suggests that it is the ratio between [**dX**] and [**dO** plus **dG-to-dG**] that informs about the competition between S_N2Ar and pure S_N1Ar chemistry of the guanosinediazonium ion (e.g., both **dG-to-dG** cross-links are formed after the ring-opening to cation **10**+H^+).

(4) The discoveries that (a) cytosine promotes the instantaneous deprotonation of **1** to **4** and that (b) dediazoniation and ring-opening of **4** are fast and irreversible require the *replacement* of any consideration of **1** by the consideration of the ring-opened intermediate **10** to explain the formations of all products in "base pair" chemistry (e.g., ds-DNA and ds-ON). Consistent explanations were provided for the formations of **dX**, **dO**, both **dG-to-dG** cross-links, and the sequence-specific formation of the classical cross-link. On the basis of all currently available experimental and theoretical evidence, any hypothesis invoking a reactive guanosinediazonium ion in a double-stranded environment must be rejected.

(5) The simple fact that xanthosine is the main product in all experiments shows that cytosine catalysis at near-neutral pH provides for a preference for water addition to the cyanoimine moiety as compared to the ketene moiety of **10** (e.g., the formation of **13** cannot compete with the formation of **19**).

(6) The different outcomes of the experiments by Dedon[19] (ds-DNA) and by Suzuki[12c] and Shuker[18a] (nucleosides) at near-neutral pH are caused by environmental effects. The Dedon experiment is "base pair" chemistry with cytosine catalysis. Oxanosine formation under those conditions requires either a path via **13** or a path via **19**. The C−N rotation in **19** is not possible in double-stranded DNA. The absence of oxanosine in the Dedon experiment thus provides a second line of evidence in support of conclusion (5). The nucleoside chemistry is cation chemistry, and oxanosine is formed via **10**+H^+.

(7) The different outcomes of the experiments with ds-DNA by Dedon[19] (pH = 7.4) and by Suzuki[12a] (pH = 3.7) reflect events after the formation of *E*-**11**. The cytosine catalysis mechanisms can account for an increase in oxanosine formation at lower pH (Scheme 8). This hypothesis can be tested by comparative analysis of ss- and ds-DNA and oligonucleotides as a function of pH value. In addition, these equilibria should be sensitive to the replacement of cytosine by 5-methylcytosine.

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Supporting Information Available: A table with total energies and thermochemical data, Cartesian coordinates of **1**−**11**, and complete citations of refs 16 and 35. This material is available free of charge via the Internet at http://pubs.acs.org.

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