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Substituents on Quinone Methides Strongly Modulate Formation and Stability of Their Nucleophilic Adducts

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Abstract

Electronic perturbation of quinone methides (QM) greatly influences their stability and in turn alters the kinetics and product profile of QM reaction with deoxynucleosides. Consistent with the electron deficient nature of this reactive intermediate, electron-donating substituents are stabilizing and electron-withdrawing substituents are destabilizing. For example, a dC N3-QM adduct is made stable over the course of observation (7 days) by the presence of an electron-withdrawing ester group that inhibits QM regeneration. Conversely, a related adduct with an electron donating methyl group is very labile and regenerates its QM with a half-life of approximately 5 hr. The generality of these effects is demonstrated with a series of alternative quinone methide precursors (QMP) containing a variety of substituents attached at different positions with respect to the exocyclic methylene. The rates of nucleophilic addition to substituted QMs measured by laser flash photolysis similarly span five orders of magnitude with electron rich species reacting most slowly and electron deficient species reacting most quickly. The reversibility of QM reaction can now be predictably adjusted for any desired application.

Introduction

Developing reagents for selective reaction with cellular components is often dismissed due to an unnecessary assumption that covalent processes are limited to irreversible chemistry. Reversible covalent reaction has the potential to overcome kinetic traps that limit both potency and selectivity. Reactive species may be regenerated repeatedly in a reversible process to escape trapping with non-targeted materials and even overcome cellular repair processes. The ability of the natural product Ecteinascidin 743 (Et-743) to bind covalently to duplex DNA and yet still migrate between various nucleotide sequences, for example, also allows Et-743 to reassociate with DNA after its initial adduct has been removed by excision repair (Scheme 1).[1] In contrast, reagents such as nitrogen mustards react irreversibly with strong nucleophiles in DNA, most notably the N7 of guanine (Scheme 2) and other cellular compounds.[2–4] The most significant effect of the mustards, DNA cross-linking, only proceeds if a second nucleophile is proximal to the initial site of attachment. However, this second nucleophile is not typically present and reaction is quenched before the mustard can migrate to a site in DNA that would support cross-linking.

Reversible alkylating agents may also react with a range of cellular components, but after multiple regenerations they may ultimately associate with their thermodynamically favorable targets (e.g., Et-743 with duplex DNA, Scheme 1). Reversibility then extends the effective

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lifetime of electrophiles and consequently enhances their potential for selective biological activity. Such activity has been investigated with carcinogens such as malondialdehyde[5–8] and a variety of anticancer drugs candidates based on cyclopropylpyrroloindole.[9,10] For two closely related drug candidates, duocarmycin SA (duoSA) is twenty-fold more cytotoxic than duocarmycin A (duoA). This result is likely a function of the slower reaction of duoSA vs. duoA with nucleophiles and its faster release from adducts formed by nucleophilic coupling.[11,12] A direct correlation between reversibility of DNA adducts and cytotoxicity was also extended to a number of additional cyclopropylpyrroloindole derivatives as well.[13]

Our laboratories and others have applied the reversibility of quinone methide (QM) generation for efficient cross-linking and target-promoted alkylation of DNA.[14–18] Related QMs had previously been implicated in the biological activation 2,6-di*tert*-butyl-4-methylphenol,[19] tamoxifen[20] and a variety of natural products including mitomycin[21] and certain diterpenone catechols.[22] Initial model studies suggested a selectivity of QM for weak nucleophiles and an ability of some adducts to form reversibly.[19,23–26] Further investigation revealed that this unusual selectivity was a function of QM adduct stability rather than initial product formation. [27–29] In contrast to earlier assumptions, the strongest nucleophiles of DNA reacted most quickly with a simple *ortho*-QM model to form kinetic products. However, these products are not stable and regenerate the *ortho*-QM for ultimate transfer to weaker nucleophiles that react irreversibly and form thermodynamic products (Scheme 3).[27,30]

Computational studies on this same *ortho*-QM model anticipated the experimental results with remarkable accuracy.[28,29] A low value for $\Delta G^\ddagger$ was calculated for both adduct formation and QM regeneration with strong nucleophiles such as dC N3, dA N1 and dG N7 (Scheme 2) whereas $\Delta G^\ddagger$ values for addition of the *exo*-amino groups (dG N^2 and dA N^6) to *ortho*-QM were considerably higher and essentially irreversible. The free energy of activation should also be sensitive to variations in the electronic properties of the intermediate QMs. A relatively electron-rich derivative may be more stable and hence more readily generated than its electron-poor counterpart. Hence, electron withdrawing and donating groups attached directly on the QM should influence the rate of QM formation and adduct stability. A series of such derivatives has now been prepared to test this possibility and determine the sensitivity of the QM to a variety of substituents. A method for predictably manipulating QM stability will be indispensable for optimizing the kinetics and selectivity of target promoted alkylation of DNA[15] and will additionally facilitate use of QM for mechanism-based inactivation of enzymes[31] and drug release by self-immolative, or cascade-release, dendrimers.[32–34] Quinone methides are also used as intermediates in organic synthesis[35] and can be stabilized by metal coordination.[36–39]

Results and Discussion

A limited number of studies previously began to explore substituent effects on QM reactivity, [40–43] but no data was available to estimate structural effects influencing the kinetics or product distribution of deoxynucleoside reaction. Electron donating and withdrawing groups have now been placed *para* and *meta* to the QM methylene to avoid complications from steric effects. The products of reversible reaction were expected to be most susceptible to changes in the QM stability since their presence depends on the rates of both nucleophilic addition to the QM as well as subsequent elimination to regenerate the QM. In contrast, the products formed irreversibly should depend on only the relative strength of their nucleophilic components to compete for initial addition to the QM.

Formation and Decomposition of dC Adducts Generated from QMs of Varying Reactivity

Our initial investigation to determine the extent to which QM reactivity can be controlled by aromatic substituents was performed with dC since this deoxynucleoside forms a single adduct

upon incubation with **QM1**.[44] The quinone methide precursor (**QMP1**) originally used to generate the unsubstituted **QM1** was also modified alternatively with a methyl (**QMP2**) and methyl ester (**QMP3**) group to examine the effect of electron donating and withdrawing groups, respectively (Scheme 4). As described previously, fluoride was used to initiate deprotection of the QMPs.[44] Subsequent elimination of acetate generated the QMs for dC alkylation. Both **QM2** and **QM3** also generated only a single adduct with dC under the same conditions used previously to form the dC N3 adduct of **QM1**.[44] ^{1}H NMR data for these new derivatives are consistent in each case with linkage through the N3 of dC. The chemical shifts of the benzylic methylene protons are quite characteristic of QM adduct structure[25,26] and benzylic signals for the dC N3-QM adducts of **QM1**, **QM2** and **QM3** differ by no more than approximately 0.1 ppm (Table 1).

Kinetic measurement of dC addition to the QMs was performed under conditions identical to those first used to follow reaction of **QM1**.[30] The maximum yield of adduct formed in each case was similar, but their rates of formation and stability varied greatly (Figure 1). The dC N3 adduct of the electron-rich **QM2** formed most rapidly, and a maximum yield was achieved in less than 1 hr. Decomposition of this adduct was also very rapid with a half-life of approximately 5 hr and resulted in release of dC and presumably quenching of **QM2** with water. In contrast, the unsubstituted **QMP1** examined previously generated a maximum yield of dC N3-**QM1** adduct after 4 – 10 hr. This product exhibited moderate stability under the reaction conditions and decomposed with a half-life of approximately 24 hr. Finally, the electron-poor **QM3** formed very slowly over 40 hr, but the dC N3 adduct that resulted from its reaction persisted without change over a minimum of 72 hrs. No decomposition of the dC N3-**QM3** adduct was even detected after an extended incubation of 7 days.

The rate of adduct formation primarily reflects the rate of initial QM formation by elimination of the benzylic acetate after rapid desilylation. Electron donation of a single methyl substituent stabilizes **QM2** sufficiently to promote its formation by at least 4-fold relative to formation of **QM1** (Figure 1 and Supporting Information). Similarly, **QM1** forms perhaps 10-fold more rapidly than **QM3** that contains an electron withdrawing group. This same trend is apparent for QM regeneration from each of the dC N3 adducts. Thus, small changes in the electronics of the QMs affect their reactivity and adduct stability dramatically and now also predictably.

Deoxynucleoside Competition with an Electron-rich QM

The consequences of QM stability on the kinetics of forming all deoxynucleoside adducts was next examined with a competitive equimolar mixture of dA, dG, dC and T in analogy to earlier studies with the unsubstituted **QM1**.[30] Product standards were first isolated after reaction with individual deoxynucleosides, and each product was characterized by ^{1}H NMR, high-resolution mass spectrometry (HRMS) and UV absorbance. Once again, the chemical shift of the benzylic protons was diagnostic of the linkage between the QMs and deoxynucleosides (Table 1). Characteristic changes in the UV absorbance maxima further confirmed the assignments (Supporting Information). Reaction of **QM2** with dA yielded two adducts linked through its N1 and N^6 positions. Only the N^6 adduct was stable enough for isolation and characterization. However, the UV maximum of the second product was recorded during HPLC analysis and is consistent with an N1 adduct (Supporting Information). Three products were generated by **QM2** and dG and identified as adducts of its N1, N^2 and N7 positions. Both the N1 and N^2 adducts were characterized as their dG derivatives. The N7 adduct of dG was evident during chromatographic separation but spontaneously deglycosylated to form its guanine derivative. This secondary product was stable and subject to standard analysis. Similar results had previously been observed with reaction of dG and **QM1**.[26,30] No adducts of T were observed under the range of conditions examined in this work.

Monitoring the competitive reaction of dA, dC, dG and T with **QMP2** over time resulted in the same trends observed previously with **QMP1** except the timeframe of the evolving products was significantly compressed (Figure 2). The initial two hrs were dominated by reaction of the strong nucleophiles, dA N1 and dC N3. However, the resulting adducts decomposed with half-lives of less than 4 hrs. The weaker nucleophiles in turn formed adducts more slowly over 8 hr and most likely as a result of **QM2** regeneration from the adducts of dA N1 and dC N3. **QM2** generation from its initial **QMP2** precursor is nearly complete within the first 2 hrs (Supporting Information). This again illustrates the ability of reversible adducts to extend the effective lifetime of an otherwise highly transient intermediate. The leaving group abilities of dA N^6, dG N1 and dG N^2 are not sufficient to regenerate the QM, and thus their adducts remained stable over the course of observation.

Initial reaction at dG N7 is less than might be expected from its strong nucleophilicity. However, its adduct has the unique ability to deglycosylate as well as eliminate **QM2**. At the deoxynucleoside level, partitioning between deglycosylation and QM regeneration had previously been observed to occur at equivalent rates.[30] The deglycosylation product from reaction with **QM2** accumulated over approximately 15 hrs and remained stable since guanine N7 does not share the strong leaving group ability of dG N7. Although the kinetics of alkylation with **QM2** were significantly faster than with **QM1**, all of the irreversible adducts were stable for at least six days beyond the observations of Figure 2. The nucleophilic sites used to create these adducts therefore still lacked the leaving group ability to eliminate from a QM with the added electron density provided by a pendent methyl group. This group was, however, sufficient to accelerate QM regeneration from adducts that had previously exhibited lability with **QM1**.[30] Finally, the overall yields of stable adducts were similar for **QM1** and **QM2**, and only minor differences in their relative formation was detected.

Deoxynucleoside Competition with an Electron-poor QM

Individual deoxynucleosides were also treated with the electron-poor **QMP3** in order to generate synthetic standards for structural and chromatographic markers. Incubation of dA with **QMP3** and fluoride resulted in adducts formed by its N1 and N^6 positions. This is consistent with reactions of both **QM1**[27] and **QM2**. Additionally, the ^{1}H NMR signals for the benzylic positions were again diagnostic of their structures (Table 1). Comparable reaction of **QMP3** with dG yielded only two products. These were isolated and identified as adducts of guanine N7 and dG N^2. The dG N7 adduct did not accumulate since its deglycosylation is likely faster than its formation due to the slow generation of **QM3**. The product of dG N1 alkylation was also not initially apparent but could later be formed when the concentration of dG was raised from the standard 0.25 mM to 5.0 mM. Computational studies for **QM1** indicated that reaction at dG N1 has an activation energy approximately 3 kcal/mol higher than that of dG N^2 as modeled with 9-methylguanine in water.[29] This can be used to explain the greater yield of the dG N2 adduct over the yield of the dG N1 adduct with **QM1** and **QM3**. Surprisingly, **QM2** exhibited the opposite selectivity. The origin of this difference is currently subject to additional computational analysis. Once again, no reaction of T was detected.

Reaction of **QMP3** with dNs was significantly slower than reaction with **QMP1** due to electronic destabilization of **QM3** from the attached methyl ester (Figure 3). Maximum accumulation of the dC N3 adduct required more than 24 hr and reflected a slow generation of **QM3** from its desilylated derivative of **QMP3** (Supporting Information). Additionally, this adduct was stable well beyond the 120 hr period of observation (see above). The leaving group ability of dC N3 was thus insufficient to overcome the barrier to regenerate **QM3** in contrast to its behavior with the more electron-rich **QM1** and **QM2** derivatives. The change in electronics between **QM2** and **QM3** did not greatly effect the maximum yield of the dC N3 adduct despite its conversion from a reversible to irreversible adduct, respectively.

Formation of the dA N1 adduct similarly reflected the slow generation of **QM3** (Figure 3). A maximum yield for this adduct required approximately 10 hrs with **QMP3** while less than 0.5 hr was necessary with the electron rich **QMP2**. Reversibility of the dA N1 adduct persisted despite the electron deficiency of **QM3**, although regeneration of **QM3** (half-life > 40 hr, Figure 3) was greatly suppressed relative to comparable regeneration of **QM2** (half-life < 2 hr, Figure 2). Behavior of the dC N3 and dA N1 adducts is consistent with the relative strength of dC N3 and dA N1 as leaving groups.[30] The slow formation of the dA N1 adduct combined with its remaining lability prevented its accumulation to the same level as the dC N3 adduct or related adducts with **QM2**. The guanine N7 adduct resulting from QM alkylation and deglycosylation also formed slowly with **QMP3**, but its yield was similar with both **QM2** and **QM3**.

Destabilization of the QM affects both the kinetics of its formation and the resulting product profile as evident by the stability of the dC N3-**QM3** adduct and the complete absence of a dG N1 adduct. Adducts of dG N^2 and dA N^6 were still generated by **QM3** in similar yields and approach the levels of accumulation measured with **QM2** (Figure 2). However, these **QM3** adducts continue to increase for more than 120 hrs (rather than 10 hr for **QM2**). The original source of **QM3** is mostly consumed by 30 hrs, and thus continued generation of the dG N^2 and dA N^6 adducts likely derives from the regeneration of **QM3** from its dA N1 adduct.

Effects of Aromatic Substituents on QM Generation

A methoxy group was originally attached to the model **QM1** in order to examine a broad range of substituent effects on deoxynucleoside adduct stability. Preliminary study of the adduct formed between this electron-rich intermediate and dC N3 indicated that it was too labile for convenient analysis. Thus, the weaker electron-donating methyl group was used in place of the MeO group as described above. As a complement to the range of derivatives available for deoxynucleoside study, a series of *N*-morpholino derivatives were prepared to extend correlations between QM stability and its rate of (re)generation. The morpholino-QM adducts were chosen to replace deoxynucleoside-QM adducts based on the pKa of morpholine's conjugate acid. This property reflects the nucleophile's leaving group ability that, along with QM stability (see above), determines the rate of adduct collapse and QM regeneration (Scheme 3).[30,45] For nucleophiles with conjugate acids exhibiting pKa's between approximately 4 and 8, QM regeneration has been detected over hours under physiological conditions.[30] Morpholine has a conjugate acid pKa of 8.4 which is at the high end of this acidity range and should consequently provide some stability to even highly electron-rich QM precursors. Accordingly, morpholino adducts provided a basis for determining electronic effects of QM reversibility over a greater range of electronic perturbations than available with the deoxynucleosides.

The desired morpholino adducts of substituted QMs were synthesized by a Mannich reaction of 4-methylenemorpholinium halide and the appropriate hydroxylated aromatic prescursor. Substituents influencing QM stability ranged from the strongly electron-donating -OMe group to the electron-withdrawing -COOMe group (Table 2). These groups were placed at the 5-position like **QMP2** and **QMP3** as well as at the 4-position to compare positional differences. The overall trend of adduct stability (**QMP4**-**QMP8**) was monitored by conversion of the morpholino adducts to their water adducts (benzylic alcohols, QMA). The range of reactivities observed for this series was even more dramatic than that observed for the deoxynucleoside adducts above. An aqueous solution of the electron-poor adduct **QMP4** was fully stable after incubation at 100 °C for 3 hrs (Table 2). No consumption of **QMP4** or formation of **QMA3** was detected by HPLC after this harsh treatment. This suggests that **QM3** was not generated under these conditions nor was **QMP4** subject to direct substitution through a S_N2 mechanism. This alternative process should not be significantly influenced by remote aromatic substitution,

and thus QMA formation from the other adducts is indicative of QM generation as detected in a number of related systems[14,15,25,45–47] rather than direct substitution. Similarly, an alternative S_N1 mechanism is not consistent with the lack of reactivity of *O*-silyl QMPs and their *O*-methyl derivatives compared to that of their parent phenols[14,48] since these substituent should all promote such a process almost equally.[49]

An electron-withdrawing –COOMe group placed at the 4-position was not as effective at destabilizing its QMP (**QMP5**) as the same group placed at the 5-position (**QMP4**, Table 2). **QMP5** was slowly converted to its benzylic alcohol **QMA5** albeit heating to 100 °C was still necessary. The unsubstituted parent **QMP6** was somewhat more labile. Formation of **QMA1** from trapping of the transient **QM1** was observed after heating to only 50 °C. In sharp contrast, the electron rich derivatives containing methoxy substituents (**QMP7**, **QMP8**) were very labile with half-lives of only minutes at 22 °C. The sensitivity of QM generation to electronic effects that was first observed for deoxynucleoside adducts is consequently quite general and remarkable for other QM-producing adducts as well. Furthermore, the site of substitution within the QM adduct also plays an important role in its stability. The *ortho*-QMs characterized in this study were more strongly influenced by substitution at the 5- than 4-position (**QMP4** vs. **QMP5** and **QMP8** vs. **QMP7**). Inductive and resonance effects from both positions apparently modulate QM stability, but only the 5-position can participate directly in resonance with the nascent exocyclic methylene.

Substituent Effects on Nucleophilic Addition to Quinone Methides

Substituents used to tune the stability of QM adducts should concurrently effect their electrophilicity and lifetime. The magnitude of these perturbations has now been quantified by laser flash photolysis (LFP). Photochemical activation of hydroxybenzyl alcohols and phenolic Mannich bases (Scheme 5, L = OH and NMe_2, respectively) has been well described as a mild source of QMs by Wan,[50–52] Kresge[53–55] and Saito.[56] Recently, one of our laboratories has developed an alternative photochemical precursor to QMs, benzyl ammonium salts of phenolic Mannich bases (Scheme 5, L = $^+NMe_3I^-$).[45] In contrast to Mannich bases, these salts are not nucleophilic and do not react with the transient QM. Also, these salts display higher photochemical quantum yields than the corresponding alcohols for QM generation.[45] The quaternary ammonium salts are therefore ideal for generating a series of *o*-QMs and monitoring their consumption over time in the presence of various nucleophiles.

While Wan,[50–52] Kresge,[53–55,57] Richard[58–60] and our laboratories[30,45] have investigated the kinetic behavior of the parent *o*-QM (**QM1**), its *para* isomer (*p*-QM) and a few of their derivatives under aqueous conditions, no systematic analysis of substituent effects on QM reactivity has yet been published to our knowledge. Consequently, **QMP9**-**QMP14** were synthesized to examine a diverse range of electronic perturbations, and QM reaction was tested with three prototypical nucleophiles.

Each QM intermediate was generated from its corresponding QMP (0.2 mM) by flash photolysis at 266 nm. QM consumption was then monitored at its λ_{max} under aqueous conditions (pH 7) in the absence and presence of 0.2 – 50 mM morpholine (buffered at pH 9 with Na_2CO_3) and 2-mercaptoethanol (buffered at pH 7 with sodium phosphate), alternatively (Table 3). Second order decay rate constants (k_2) were calculated from the concentration dependence of pseudo-first order kinetic measurements. The rate constants determined under each of the three conditions exhibit parallel trends that are consistent with the behavior of the deoxynucleoside and morpholino adducts. Electron-donating groups significantly stabilize QM. Such groups facilitate QM regeneration from their adducts (Figures 1–3, Table 2) and are now shown to slow nucleophilic addition to the nascent QM as well (Table 3). In a complementary manner, electron-withdrawing groups suppress QM regeneration and strongly promote nucleophilic addition to the nascent QMs.

The presence of an increasingly strong electron-withdrawing group (5-Cl, 5-COOMe, 5-CN and 5-NO$_2$) progressively decreased QM lifetime as evident by the increasing values of k$_2$. The 5-NO$_2$ (**QM14**) in particular resulted in a k$_2$ for the hydration reaction that was several orders of magnitude greater than that of the parent **QM1**. In contrast, the electron-donating ability of the 5-MeO group decreased the rate of its QM (**QM7**) consumption vs. the parent **QM1** by 4- to 5-fold. Although the rates of QM decay were much faster in the added presence of strong nucleophiles represented here by morpholine and 2-mercaptoethanol, their general sensitivity to electron-withdrawing and donating substituents remains nearly equivalent. Still, the relatively selectivity for the individual nucleophiles decreases with increasing reactivity of the QM. Such data is consistent with the much maligned reactivity-selectivity principle despite the lack of reaction controlled by diffusion.[61]

The sensitivity of QM reaction to substituents was also examined by its free energy relationship [ln(ky/k$_H$)] for addition of morpholine (Figures 4a,a′), water (Figure 4b,b′) and 2-mercaptoethanol (Figure 4c,c′). Linear correlations were slightly better with Hammett σ$_p^-$ constants (Figures 4a,b,c) than with Hammett σ$_p$ constants (Figures 4a′,b′,c′). The σ$_p^-$ constants were originally set by changes in the pKa of *para* substituted phenols and are now used for systems with strong resonance interaction between substituent and reaction center. Such a case is proposed for reactive QMs that appear to develop a degree of aromaticity in the transition-state (TS) with negative charge efficiently stabilized by delocalization over the former oxygen carbonyl group and Y substituent (Scheme 6). Such resonance contributions are likely even greater for substituents at the 5-position since they may influence the exocyclic methylene of the QM more directly. Preliminary support for this prediction is evident by the greater influence of substituents at the 5- vs. 4-position as summarized in Table 2.

Hansch had previously noted that σ$_p^-$ constants often yield better correlations than σ$_p$ when resonance interaction develops between the substituent and the reaction center, and the resulting magnitude of ρ has provided insight into reaction mechanisms.[49] For QM addition, the linear free energy correlations are positive and large. The values of ρ equal 3.0, 5.9 and 5.4 for morpholine, water and 2-mercaptoethanol, respectively (Figure 4a,b,c). In comparison, ρ values for the equilibrium acidity of phenol in water and DMSO are 2.1 and 5.3, respectively.[62,63] The equilibrium acidity of *p*-substituted benzyltriphenylphosphonium cations to form the corresponding ylide in DMSO exhibited a ρ of 4.78.[64] Much larger values of ρ (12 and 10) were determined for substituent effects on the acidity of a benzylic hydrogen in toluene and 10-substituted-9-methylanthracene derivatives in DMSO, respectively.[65,66] The intermediate values of ρ for QM addition in water is then suggestive of significant anionic character in the TS. The lower value for the phosphonium salt vs. toluene was considered by Bordwell to indicate a diminished charge delocalization on the salt due to coulombic and polarization contributions by the triphenylphosphonium group.[64] The smaller ρ value of morpholine vs. those of water and 2- mercaptoethanol may also be explained by the electrostatics of the developing positive charge on the nitrogen nucleophile.

Conclusions

Formation of QMs had previously demonstrated a dependence on the strength of the leaving group attached to the benzylic position of its precursor when studying a model *o*-QM in the presence of biological nucleophiles (Scheme 3).[30,45] Now, both the formation and subsequent reaction of QMs have also been shown to be highly responsive to the presence of electron-withdrawing and donating groups. All trends in reactivity are consistent with the electron deficient nature of the QM intermediate. Electron donating groups greatly facilitate initial QM generation and its subsequent regeneration from adducts formed by the nucleophiles of deoxynucleosides that also function as good leaving groups. Conversely, electron withdrawing groups greatly suppress initial formation of QM and suppress its regeneration from the

reversible deoxynucleoside adducts. These properties similarly extend to nucleophiles and leaving groups beyond those of DNA and provide a foundation for predicting the general behavior of QM stability and selectivity. In a complementary manner, electron-rich QMs react much more slowly but more selectively with nucleophiles than do the electron-poor QMs. These fundamental characteristics provide the basis for explaining the product profiles observed after DNA has been exposed to various QMs. In the future, these same characteristics may be used to fine-tune the stability and reactivity of QM conjugates for target-promoted and gene-specific alkylation.[15] An equivalent approach may also be used to optimize the kinetics and sensitivity of polymers based on QMs to disassemble for drug release.[32–34]

Experimental Procedures for Quinone Methide Reaction

Kinetic Studies with Individual Deoxynucleosides

To a aqueous solution (70 μL) of 4 mM phenol, 0.5 mM deoxynucleoside, 10 mM potassium phosphate pH 7, 500 mM KF was added the quinone methide precursor (**QMP1**, **QMP2** and **QMP3** alternatively) in DMF (30 μL) yielding a final concentration of 25 mM. The reactions were incubated at 37 °C and, at the indicated times, analyzed directly by reverse phase HPLC (C-18, Varian, Microsorb-MV 300, 5 μm particle size, 250 mm × 4.6 mm) using a gradient of 3 % CH_3CN, 9.7 mM TEAA, to 25 % CH_3CN, 7.5 mM TEAA at 1 mL/min over 66 min. For **QMP1** reaction, TEAA was adjusted to pH 4, and for **QMP2** and **QMP3**, the TEAA was adjusted to pH 6. Product formation was monitored by integrating the elution profile at 260 nm generated by a diode array detector and normalizing its signal by the relative ε_{260} of each product and absorbance of an internal standard (phenol).

Competition Studies in the Presence of all Deoxynucleosides

To a aqueous solution (70 μL) of 4 mM phenol, 0.25 mM of dA, dC, dG, and T (1 mM total dN), 10 mM potassium phosphate pH 7, 500 mM KF, was added the quinone methide precursor (**QMP1**, **QMP2** and **QMP3** alternatively) in DMF (30 μL) yielding a final concentration of 25 mM. Reactions were incubated at 37°C and analyzed as described above.

Kinetics of Quinone Methide Regeneration and Benzylic Alcohol Formation from the Morpholino-quinone Methide Adducts

A stock solution of **QMP6** in acetonitrile was diluted with water:acetonitrile (1:1) to yield final concentration of 1.0 mM, and anisole (0.6 mM) was added as internal standard. The resulting solution was incubated for 240 min at 50 °C. Every 5 min, an aliquot (200 μL) was removed and cooled at −20 °C in a ice-salt bath prior to analysis by reverse phase HPLC (C-18, Intersil ODS-2, 5 μm, column dimension: φ = 4.6 mm, length = 250 mm, φ = 10.0 mm, length = 250 mm) with 50% aq CH_3CN (1 mL/min). The benzylic alcohol (**QMA1**) was detected with a variable-wavelength detected the alcohol and compared to an authentic sample. Reaction of the adducts (**QMP4**, **QMP5**, **QMP7** and **QMP8**) was monitored similarly although reaction time and temperature as indicated in Table 1 to detect the benzylic alcohols (**QMA3**, **QMA5, QMA7** and **QMA8).**

Laser Flash Photolysis (LFP) to Determine the Second Order Rate Constants for Addition of Water, Morpholine and 2-Mercaptoethanol to a Range of Quinone Methides

Kinetic studies of nucleophilic addition to QMs were carried out at 25 °C, in pure water for the hydration reaction and in the added presence of 2-mercaptoethanol (pH 7) and morpholine (adjusted to pH 9 by addition of Na_2CO_3) alternatively. The pH of each solution was measured using an Orion SA520 pH meter with a 8102 Ross electrode. **QM1, QM5, QM8, QM11, QM13 and QM14** were generated by flashing a dilute solutions of **QMP9-QMP14** ($2-5 \times 10^{-4}$ M). Disappearance of the QMs was followed under pseudo first-order conditions by monitoring

the absorbance decrease at the maximum wavelength reported in Table 3. Pseudo-first-order rate constants (k_{obsd}) were obtained from the fitting the absorbance data to a single exponential function and were reproducible to +/− 5%. The second-order rate constants k_2 (M^{-1} s^{-1}) for addition of nucleophiles to the QMs were determined as the least-squares linear slopes of k_{obsd} vs total concentration of the nucleophile varied between 5×10^{-2} to 2×10^{-4} M.

Laser Flash Photolysis Apparatus

The laser pulse photolysis apparatus consisted of a Nd:YAG laser used at the fourth harmonic of its fundamental wavelength. It delivers a maximum power of 10 mJ at 266 nm with 10 ns pulse duration. The monitoring system was arranged in a cross-beam configuration and consisted of a 275 W Xe arc lamp, an F/3.4 monochromator, and a five-stage photomultiplier supplied by Applied Photophysics. Signals were captured by a Hewlett-Packard 54510A digitizing oscilloscope, and the data was processed on a 486-based computer system using software developed in-house. Solutions for analysis were placed in a fluorescence cuvette (d=10 mm) and adjusted to an absorbance of 1.5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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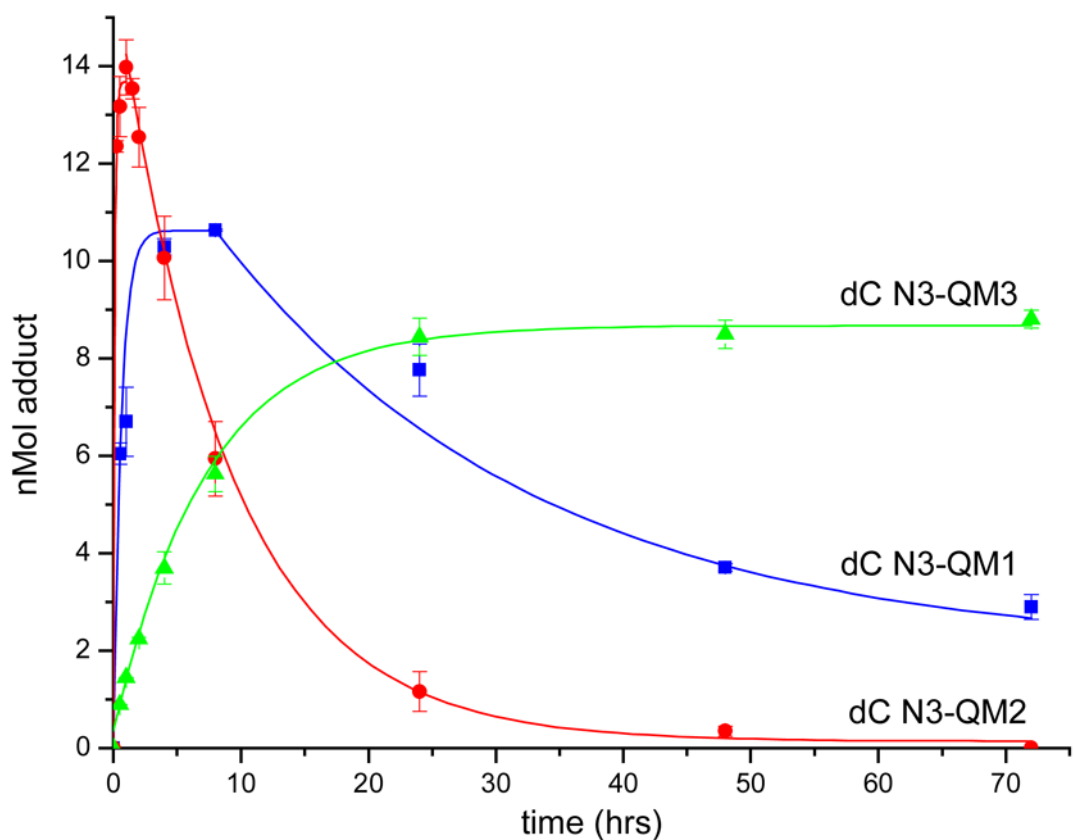
Figure 1.
Formation and decomposition of quinone methide adducts of dC N3. Reaction conditions and product analysis are described in the Experimental Procedures. Each point represents the average of at least three independent determinations and was fit to exponential processes for highlighting the net trends of the data. The indicated error derives from the standard deviations.
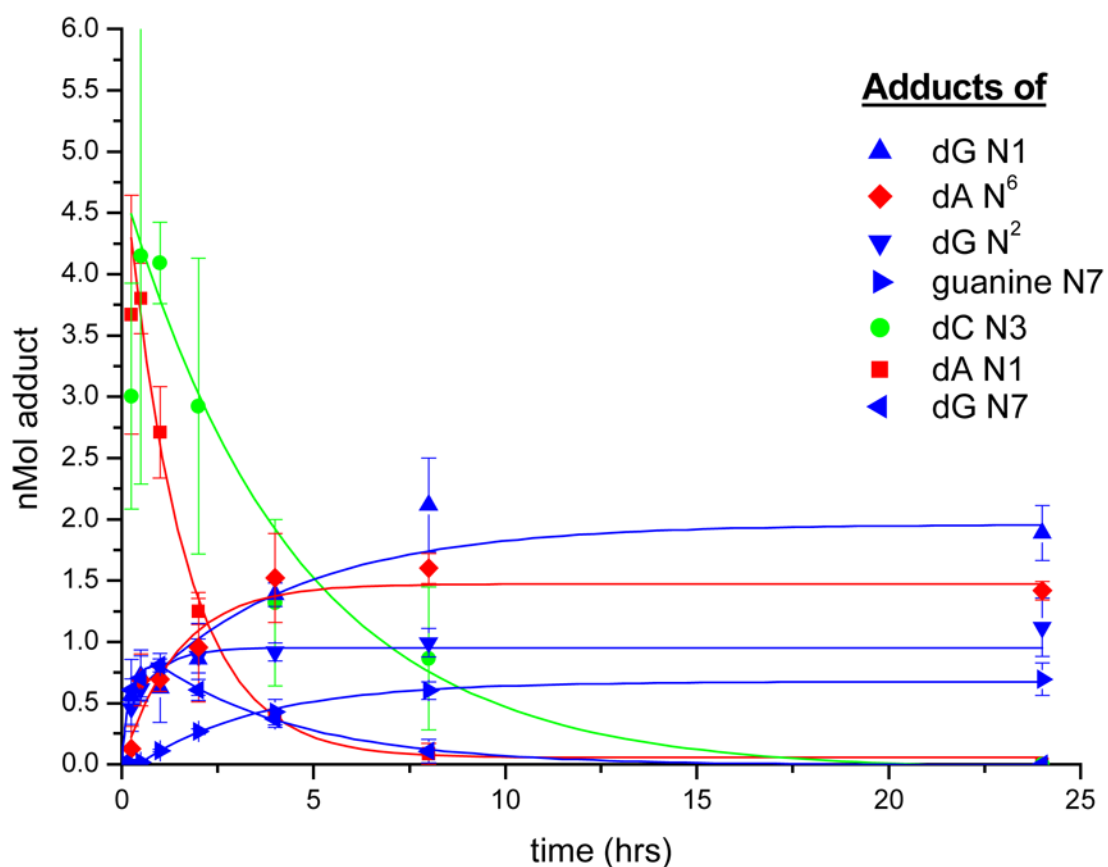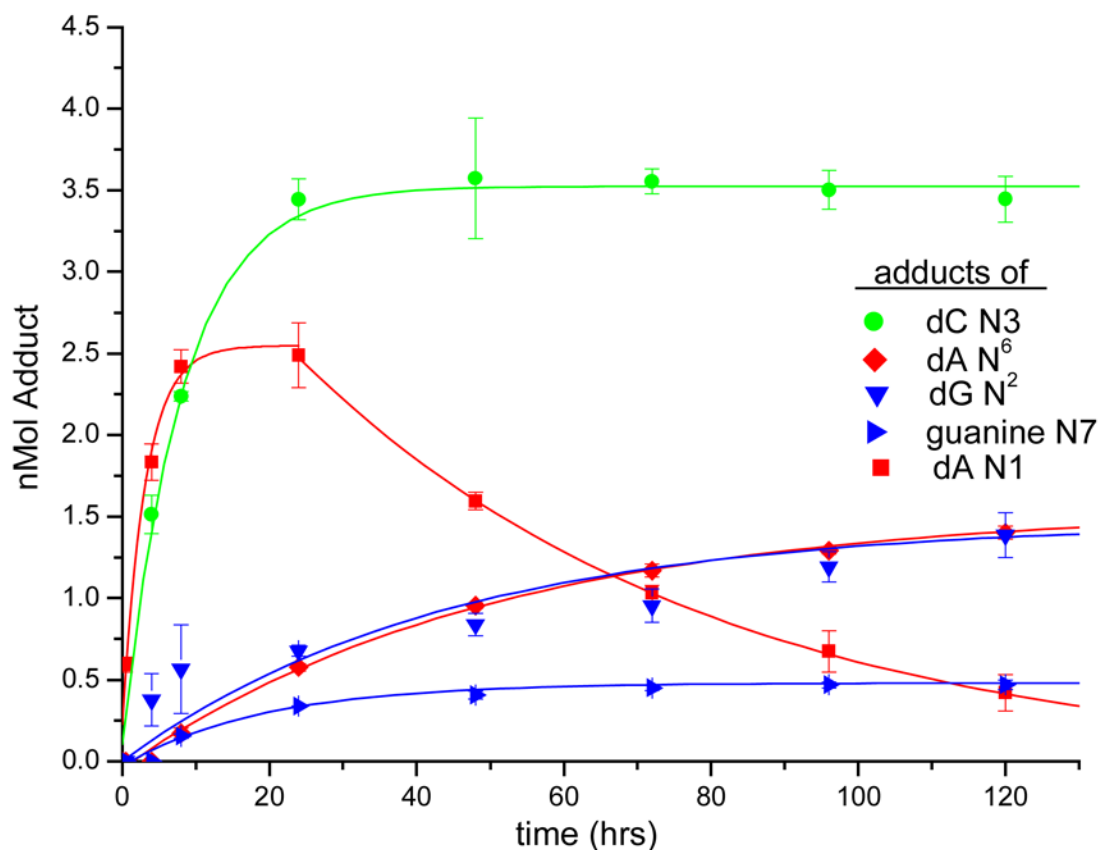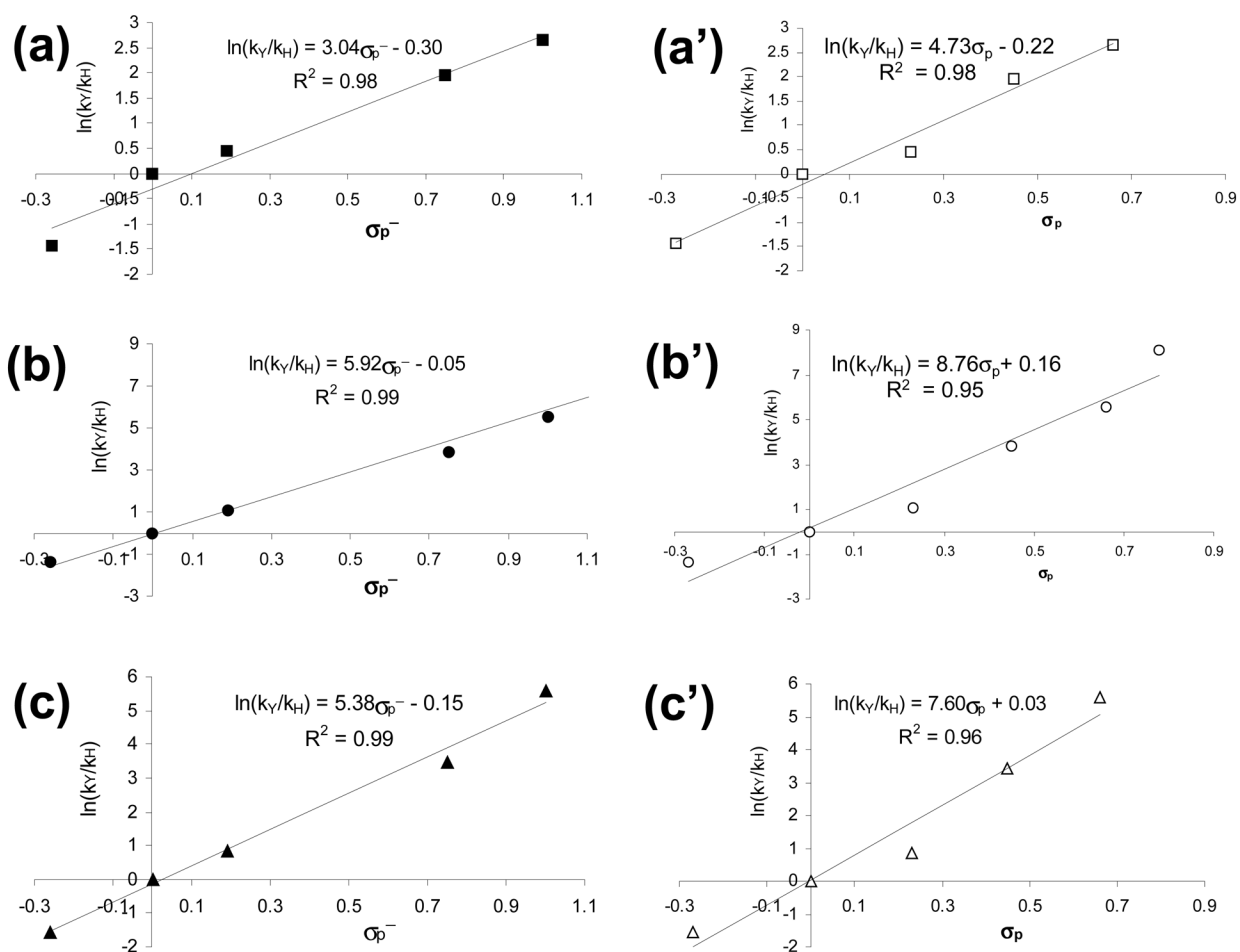
Figure 2.
Time-dependent profile of adducts formed by the electron-rich **QM2** and deoxynucleosides. Reaction conditions and product analysis are identical to those used for Figure 1. Again, each point represents the average of at least three independent determinations and was fit to exponential processes for highlighting the net trends of the data. The indicated error derives from the standard deviations.

Figure 3.
Time-dependent profile of adducts formed by the electron-poor **QM3** and deoxynucleosides. Reaction conditions and product analysis are identical to those used for Figure 1. Again, each point represents the average of at least three independent determinations and was fit to exponential processes for highlighting the net trends of the data. The indicated error derives from the standard deviations.
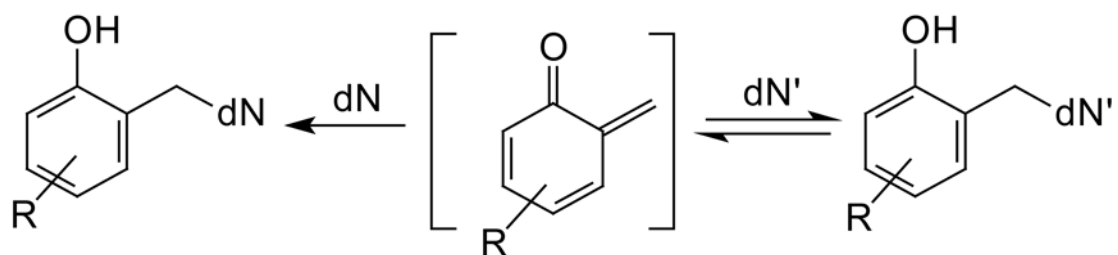
Figure 4.
Free energy relationships $\ln(k_y/k_H)$ vs Hammet σ_p^- (a,b,c) and Hammet σ_p (a′,b′,c′) for the addition reactions of morpholine (a, a′), water (b, b′) and thiol (c, c′).
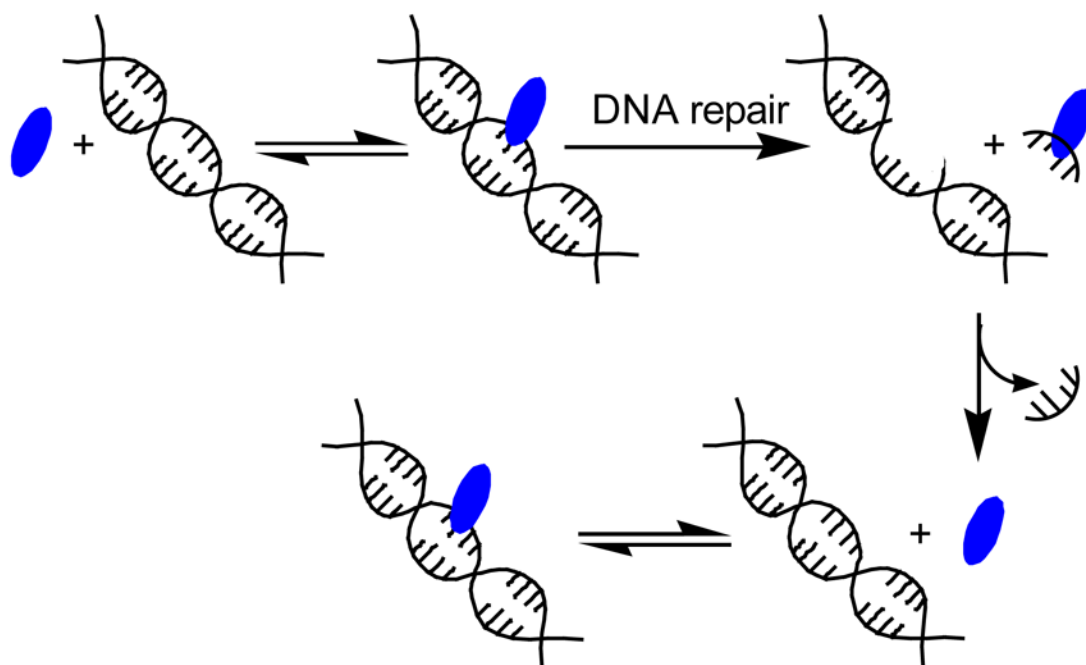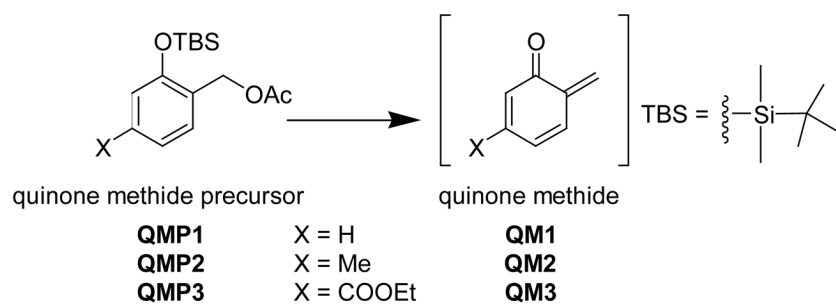
Scheme 1.
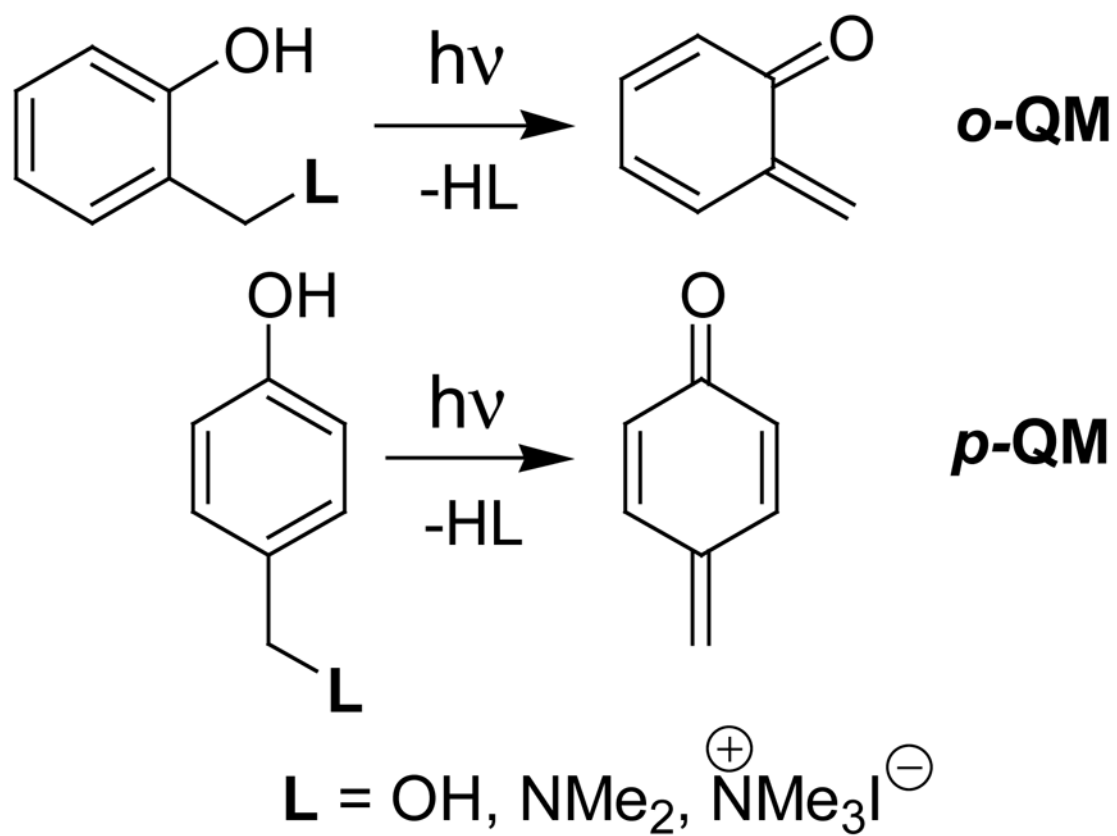Repair and Reformation of a DNA Adduct
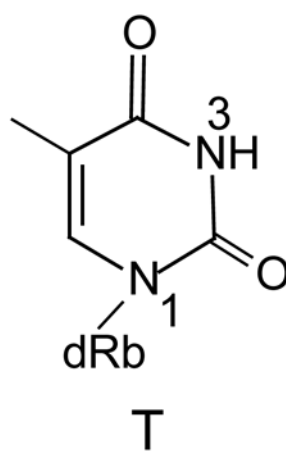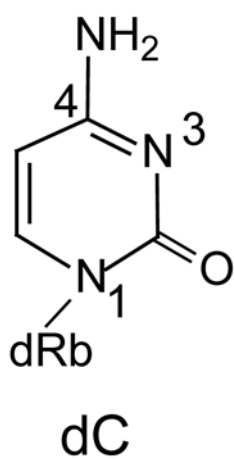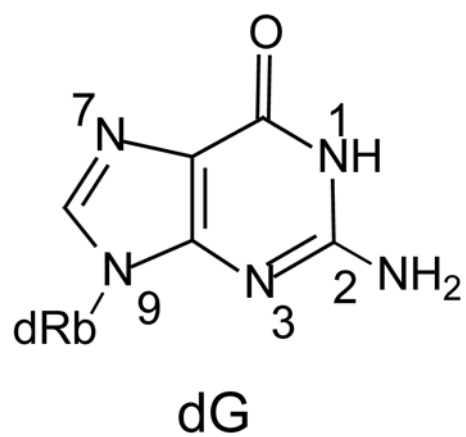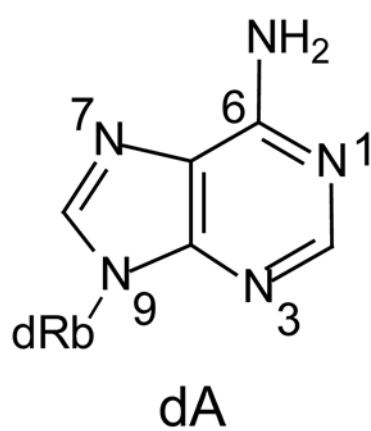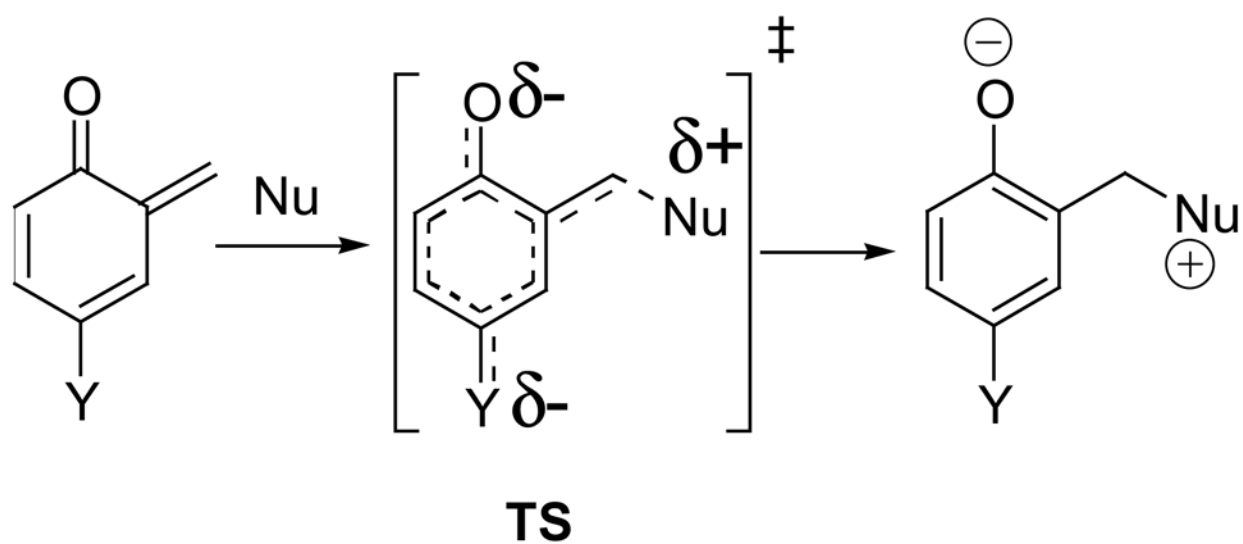
Scheme 2.
Nucleobase Structure

Scheme 3.
Reversible and Irreversible Formation of QM Adducts

Scheme 4.
QM generation from Silyl-protected Precursors

Scheme 5.
Photogeneration of QM

Scheme 6.
Aromaticity Develops in the Transition-state of QM Addition

Table 1

Benzylic proton shifts of substituted QM adducts.

Adducts formed by	dC N3	dA N1	dA N^6	dG N1	dG N^2	guanine N7
QM1	4.96	5.26	4.76	5.28	4.58	5.49
QM2	5.03	ND	4.72	5.24	4.33	5.30
QM3	5.11	5.16	4.69	5.09	4.43	5.40

Table 2

quinone methide precursor quinone methide benzylic alcohol

QMP	X	Y	QM	QMA	T/°C	$t_{1/2}$ of QMP (min) in water[a]
QMP4	COOMe	H	QM3	QMA3	100	Stable[a]
QMP5	H	COOMe	QM5	QMA5	100	149
QMP6	H	H	QM1	QMA1	50	115
QMP7	H	OMe	QM7	QMA7	22	11
QMP8	OMe	H	QM8	QMA8	22	4

[a] No benzylic alcohol **QMA3** was detected by HPLC.

Table 3

Quaternary ammonium salts	Y	Quinone Methide	λ_{max}/nm	$k_2(H_2O)$/$M^{-1}s^{-1}$	k_2(morpholine)[a]/$10^5 M^{-1}s^{-1}$	k_2(RSH)/$10^5 M^{-1}s^{-1}$
QMP9	OMe	**QM7**	410	1.9	5.5	0.40
QMP10	H	**QM1**	400	7.8	23	1.9
QMP11	Cl	**QM11**	400	22.8	36	4.4
QMP12	COOMe	**QM5**	420	363	160	59
QMP13	CN	**QM13**	415	1999	505	501
QMP14	NO_2	**QM14**	435	25400	_b	_b

[a]Keeping pH 9.0 with Na2CO3.

[b]The transient absorbance of QM bearing the strongest NO2 electron withdrawing substituent in the presence of morpholine and thiol were completely quenched. This could be due to an electron transfer mechanism from the nucleophile to the QM (work in progress).