ABSTRACT

Title of Document: REVERSIBLE QUINONE METHIDE ALKYLATION OF DNA

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Alkylation of DNA has been found to cause cancer and also to serve as its treatment. Quinone methides (QMs) are highly electrophilic molecules implicated in numerous metabolism processes. Studies of QM's reversible reaction with nucleophiles of DNA are important to understand the mechanism of its biological activity.

Reversible alkylation of QMs can extend their lifetime under aqueous conditions. The repeated capture and release of QM from dA adduct can help QM equivalents escape the irreversible trapping and extend QM's lifetime by 100-fold. This effect of dA saturates at a concentration of about 6 mM. In contrast, dG, dC, and dT do not have the ability to preserve QM under aqueous conditions.

Oligonucleotides can also preserve QM equivalents by forming labile intrastrand adducts. An oligonucleotide has now been shown to transfer bisQM to its

complementary sequences to form interstrand crosslinking. Non-complementary sequences can not be alkylated by bisQM-oligonucleotide adducts. The nucleotide composition of oligonucleotides affects their ability to transfer QM as well. A G rich sequence showed a strong ability for crosslinking a complementary sequence. However, C rich and A rich sequences did not have such an ability. Excess alkylation of C rich and A rich oligonucleotides relative to that of G rich oligonucleotide may interrupt the hybridization of complementary sequences and suppress the formation of DNA crosslinking.

The reversibility of crosslinking by QM within duplex DNA has been demonstrated by a strand displacement system. The reversible QM-DNA bond does not prevent strand displacement and allows bisQM to migrate within a series of changing DNA structures by forming crosslinking. The reactivity of bisQM is preserved beyond 11 days in duplex DNA by forming labile DNA cross-links under aqueous conditions. The migration of QM is found to be under thermodynamic control and bisQM preferentially retain cross-links in the most stable DNA duplexes.

REVERSIBLE QUINONE METHIDE ALKYLATION OF DNA

By

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Table of Contents

List of Tables

List of Figures

List of Schemes

Abbreviations

$\Delta G^{\ddagger}$ - Gibbs free energy of activation

dA – 2'-deoxyadenosine

dC – 2'-deoxycytidine

dG – 2'-deoxyguanine

DMF – N, N-dimethylformamide

DMSO – dimethylsulfoxide

dN – 2'-deoxynucleosides

DNA – 2'-deoxyribonucleic acid

dRb – 2'-deoxyribose

dsDNA – double stranded DNA

ESI – electrospray ionization

Et743 – ecteinascidin 743

EtOAc – ethyl acetate

g – gram

H_2O – water

H_2SO_4 – sulfuric acid

HCl – hydrochloric acid

HMM – hexamethylmelamine

HPLC – high pressure liquid chromatography

NaOH– sodium hydroxide

KOH – potassium hydroxide

L - liter

M – molar

9-MeA - 9-methyladenine

9-MeG - 9-methylguanine

1-MeC- 1-methylcytosine

mg - milligram

mL - milliliter

mmol - millimoles

MS – mass spectrometry

nmol - nanomoles

NMR – nuclear magnetic resonance

o – ortho

p – para

pH– -log([H+])

QM – quinone methide

QMP – quinone methide precursor

bisQMP– bi-functional quinone methide precursor

ssDNA – single stranded DNA

T – thymidine

TBDMS – *tert*-butyldimethylsilyl

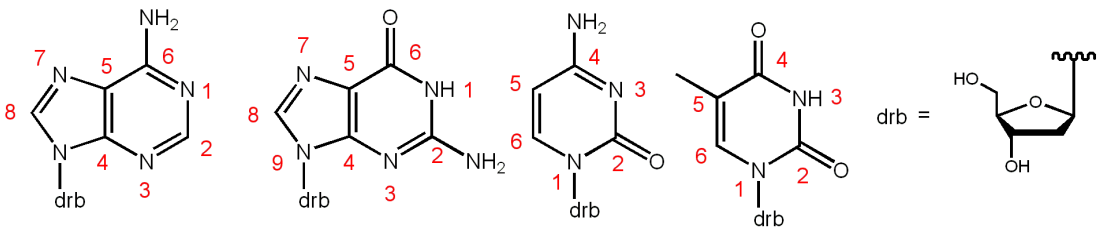
TBDMS-Cl – *tert*-butyldimethylsilylchloride

TEAA – triethyl ammonium acetate

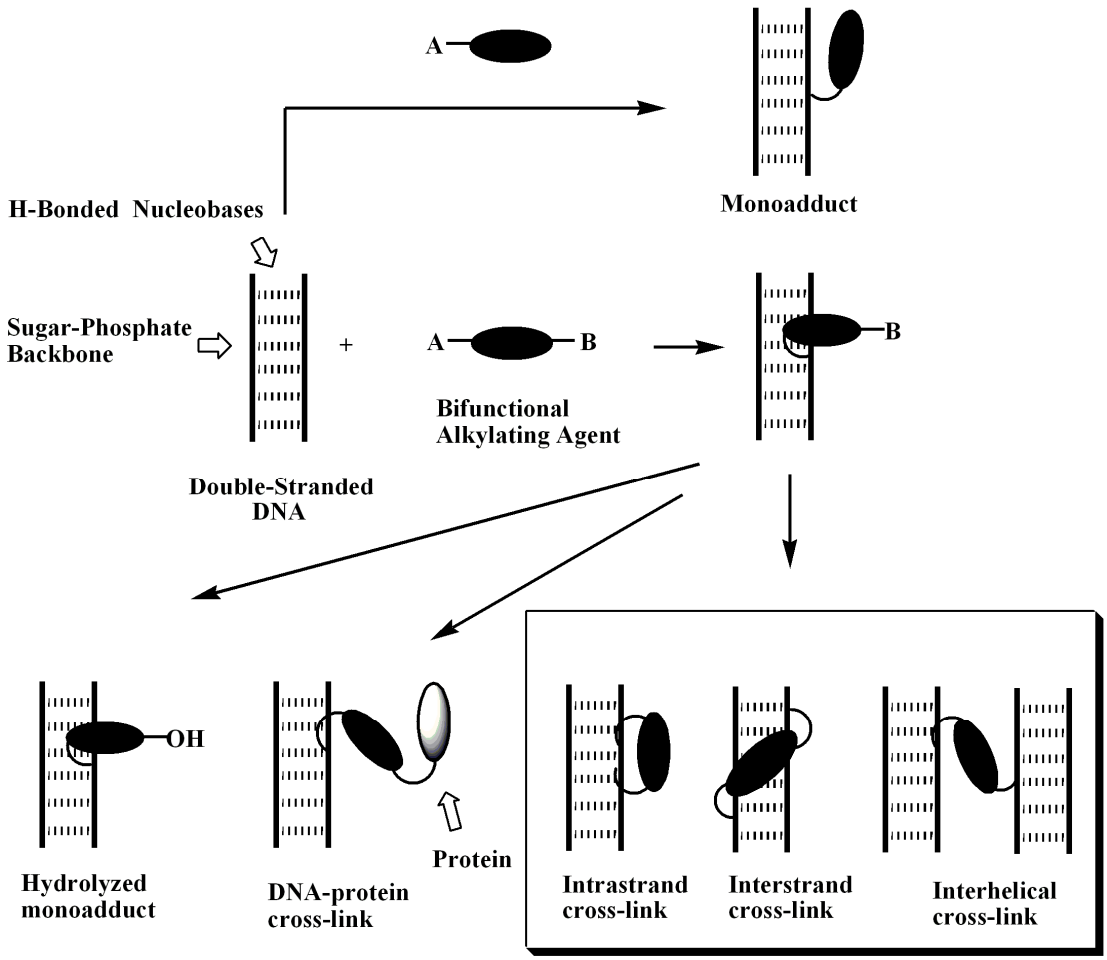
Chapter 1. Background and significance

1.1. Structure of DNA and types of DNA alkylation

Deoxyribonucleic acid (DNA) contains the genetic information used in the development and function of all known living organisms and some viruses.[1] DNA is composed of four bases, adenine, cytosine, guanine and thymine. There are multiple nucleophilic sites on nucleobases, including oxygen and nitrogen atoms, and they can react with a variety of electrophilic agents. Among the nitrogen nucleophiles, the N7 of dG is typically considered as the most nucleophilic site of DNA.[2] The N1 position of dA and N3 position of dC are also considered strong DNA nucleophiles. The N1 and N^2 positions of dG and N^6 position of dA are regarded as weak nucleophiles (Scheme 1.1).[2]



Scheme 1.1. Structures of nucleosides of DNA.



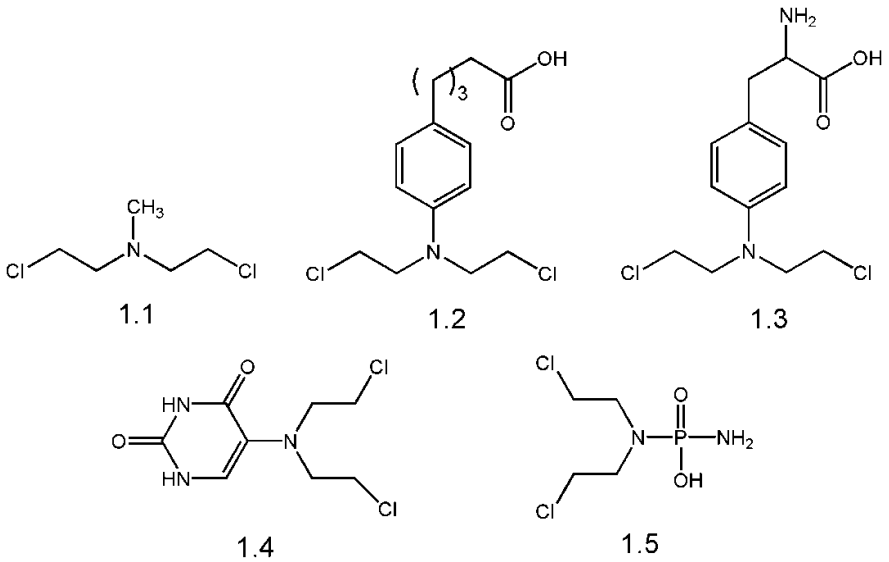
Scheme 1.2. Types of DNA alkylation.[3] A and B represent two reactive centers.

DNA alkylating agents are generally electrophilic compounds and react with nucleophiles of DNA. There are three major types of DNA alkylation: mono-alkylation, intrastrand crosslink, and interstrand crosslink (Scheme 1.2). Since DNA is associated with protein in chromosome, bi-functional DNA alkylating agents may also cause DNA-protein cross-links. Among the three types of DNA alkylation, interstrand crosslinking is the most toxic because it can completely shut down the replication process and is usually hard to repair in cells.[4-6] The formation of DNA cross-links depends on the sequence of duplex DNA and the structure of

crosslinking agent. After DNA reacts with the first reactive center of a bi-functional alkylating agent, nucleophiles of DNA must be available within the reach of the second reactive center of alkylating agent to cause crosslinking. Otherwise, the second reactive center is likely to be hydrolyzed by forming mono-alkylation adducts. If nucleophiles of DNA are available to the second reactive center, interstrand or intrastrand crosslinking could happen depending on which strand the DNA nucleophile comes from.

1.2. Irreversible and reversible alkylation by DNA alkylating agents

DNA alkylating agents derive from a variety of compounds with different sizes and types of reactive moieties. The nitrogen mustards represent the earliest and most intensively studied chemotherapeutic DNA interstrand crosslinking agents.[3, 7] A series of nitrogen mustards derivatives have been developed with varied reactivity towards electrophiles (Scheme 1.3).



Scheme 1.3. Structures of nitrogen mustards and derivatives. [7]

Mechlorethamine (**1.1**) and chlorambucil (**1.2**) are two of the most used clinical anticancer agents.[8] Their high degree of cytotoxicity is caused by their ability to generate interstrand DNA cross-links and therefore inhibit replication. The site specificity of mechlorethamine was assigned as the 5'GNC3' (N=dA, dG, dC or dT) sequence within B-form DNA.[9, 10] The mechanism of nitrogen mustards to crosslink DNA starts with the formation of an azridinium intermediate (**1.6**), which is followed by alkylation, and then the second reactive center repeats the cycle to ultimately afford crosslinking product as shown in Figure 1.1.[11, 12]

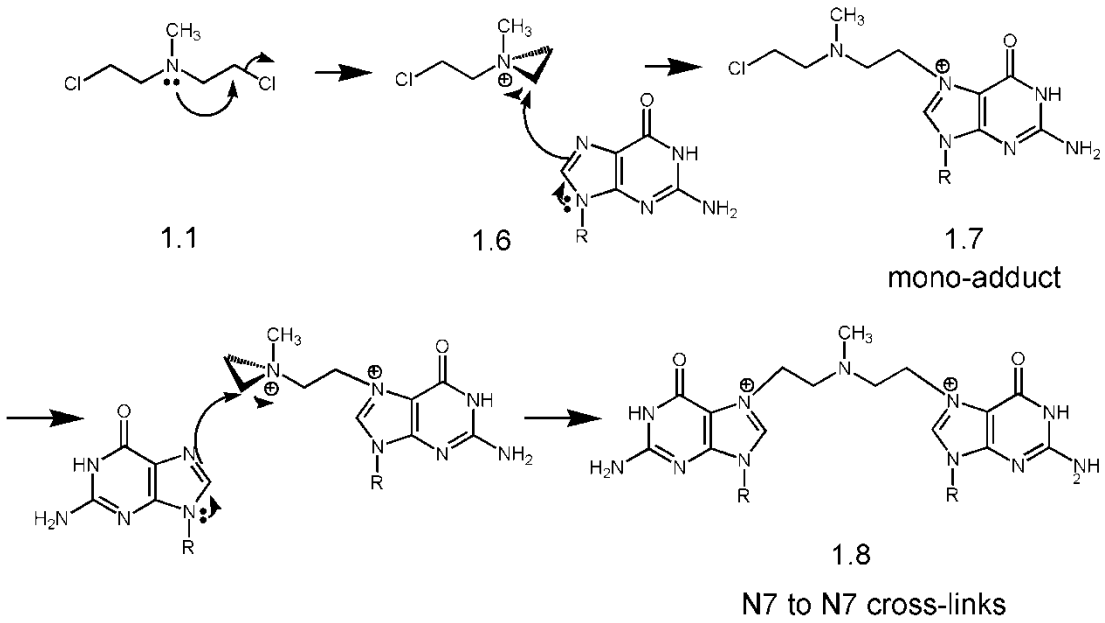


Figure 1.1. Proposed mechanism of DNA-DNA interstrand cross-links formation by mechlorethamine (**1.1**).[11]

Some alkylating agents are not reactive in their original form and need activation to transform into active compounds.[13-16 17] One example is antitumor agent hexamethylmelamine (HMM) (**1.9**), which requires hepatic oxidation by cytochrome P-450 to generate reactive species (Figure 1.2).[18] The proposed mechanism of its activation and DNA alkylation starts with stepwise hydroxylation of two methyl

groups in HMM, which generates compound (**1.11**). Subsequent dehydration of the oxidation product (**1.11**) generates the imminium intermediate (**1.12**), which is a potent electrophile and can alkylate DNA. Dehydration and nucleophilic addition can repeat at another hydroxyl group (**1.13**) and form DNA crosslinking irreversibility (**1.15**).

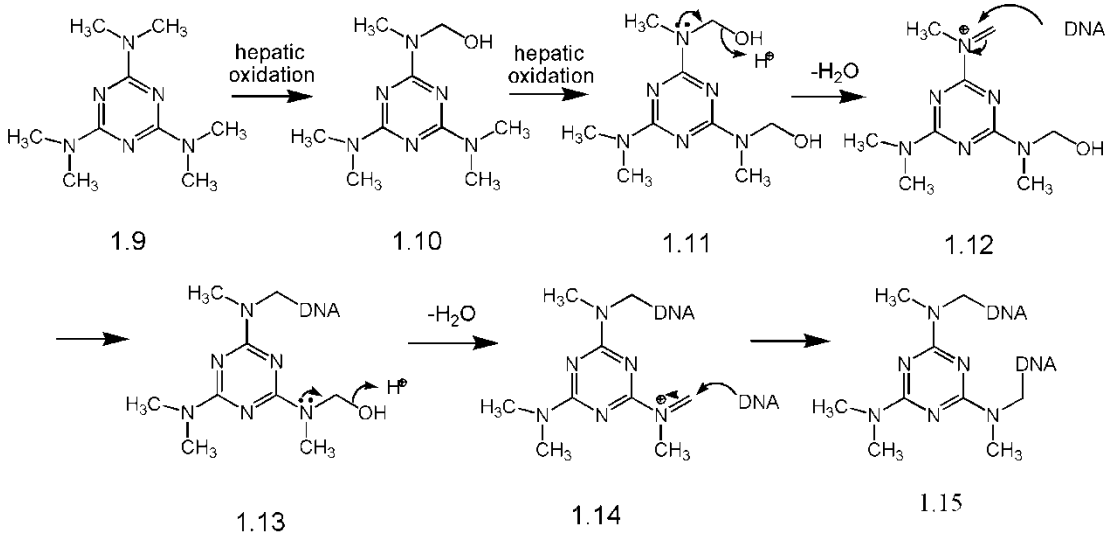
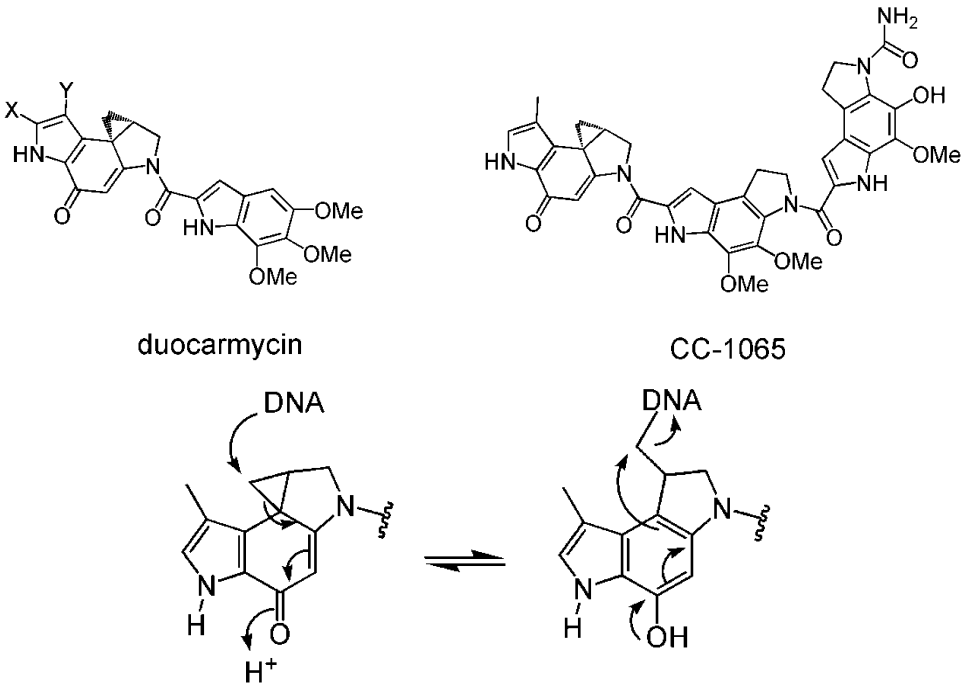


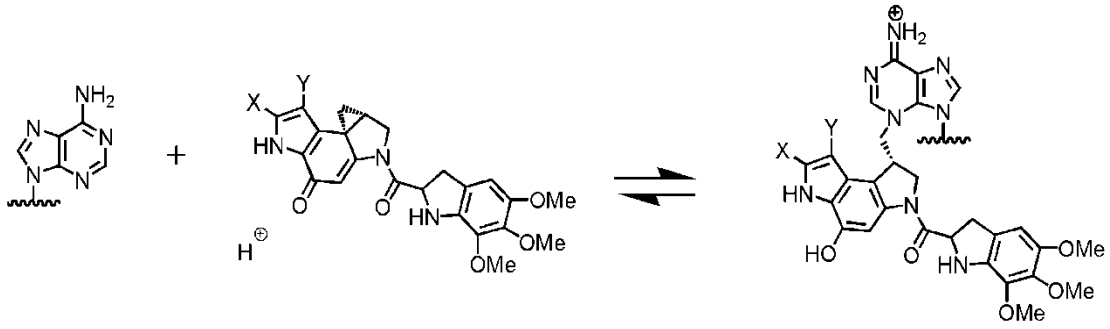
Figure 1.2. Mechanism of activation of hexamethylmelamine and subsequent DNA modifications.[19]

Although most DNA alkylating agents react with DNA irreversibly by forming stable covalent adducts, some compounds alkylate DNA reversibly. CC-1065 and duocarmycin derivatives with a central cyclopropylpyrrolindole core are among the first discovered alkylation agents to act reversibly (Scheme 1.4).[20-22] These compounds bind to minor groove of duplex DNA and target the nitrogen nucleophiles selectively.[20, 22-24] DNA becomes alkylated by attacking the cyclopropylpyrrolindole core of CC-1065 and the reversal of alkylation is initiated by deprotonation of phenol in alkylation products (Scheme 1.4).



Scheme 1.4. Structures of CC-1065 and duocarmycin and the reversible DNA alkylation by a central cyclopropylpyrrolindole.

Interestingly, studies revealed a correlation between the cytotoxicity and the reversibility of duocarmycin (DUM)-DNA adducts.[25] DUM-DNA adducts that have a stronger ability to release integral DUM exhibited stronger cytotoxicity (Table 1.1). When the efficiency of DUM's release was increased from 30% to 90% in 100 hr by alternating the substitutions on indole ring, IC_{50} value of DUM- DNA adduct decreased by 50-fold (Table 1.1). It is very likely that DUMs continue to alkylate DNA after their release. Therefore, the reversible reaction could extend the lifetime of DUMs for DNA alkylation and cause repeated DNA damage.[25]



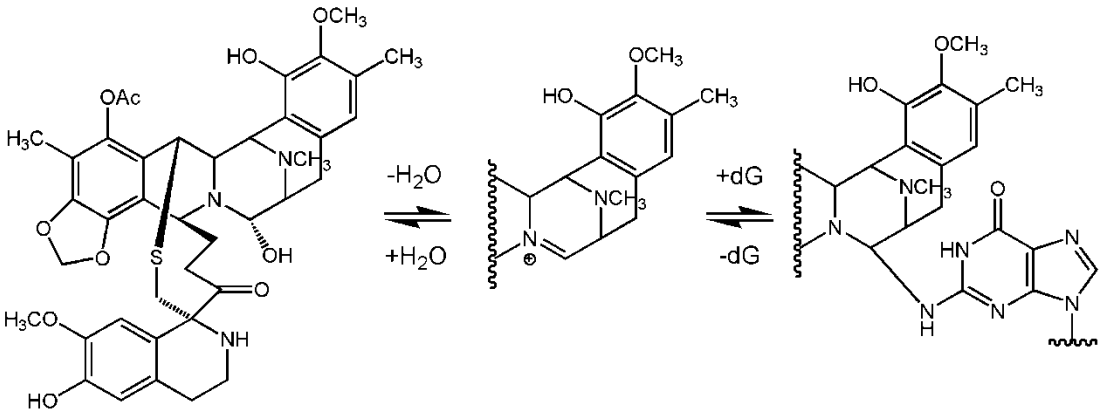
Cytotoxicity of DUMs and their DNA adducts to HeLa S3 cells.

Compounds	IC_{50} adduct (nM)	IC_{50} free (nM)	IC_{50} adduct/ IC_{50} free	Release Rate (% in 100 hr)
DUM 1 (X=-CO_2Me, Y=-H)	150	1.3	115	30
DUM 2 (X=-Me, Y=CO_2Me)	14	0.2	70	45
DUM 3 (X=-Me, Y=-H)	3	0.4	7.5	96

Table 1.1. Cytotoxicity of DUM-DNA adducts correlates to the reversibility of DUM-DNA adducts.[25](source: modified from reference 25)

Ecteinascidin 743 (Et743) can also alkylate DNA reversibly by reacting with the 2-amino group of guanine (Scheme 1.5). Dehydration of Et743 generates an imminium intermediate (**1.17**) and nucleophilic attack by dG N^2 forms corresponding adducts (**1.18**). The dehydration of Et743 is believed to be facilitated by an intramolecular acid catalyzed mechanism.[26] In addition to the potent antitumor activity, Et743 has been shown to "walk" along DNA by reversible reaction from its kinetic site to its thermodynamic site. Et743 can alkylate both 5'-AGT and 5'-AGC sequences with similar initial alkylation rates. However, the reverse reaction from 5'-AGT sequence is found to be faster than from 5'-AGC sequence.[26] The differential rate of reversibility between two target sequences is thought to be caused by their structural difference. Et743-AGT adduct is less stable than Et743-AGC adduct because Waston-Crick base pairing is more disrupted to the 5' side of the target

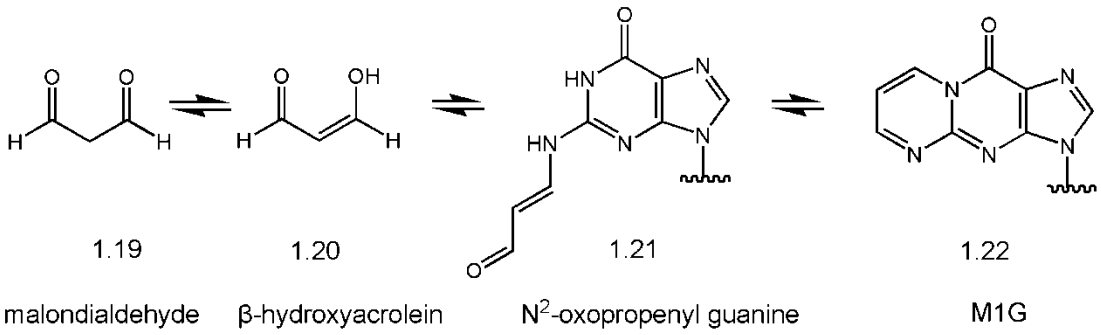
guanine in Et743-AGT adduct. Therefore, the thermal stability of Et743-AGT adduct is reduced.[26] Since Et743-AGT adducts cause distortion of base pairing and form a more open structure, the covalent linkage is more accessible to the nucleophilic attack from water, which can reverse the alkylation of Et743. The dealkylation will release Et743 from its AGT adduct and react with DNA at a different site. As a consequence, Et743-AGC adduct accumulates along time as a more stable adduct and Et743 is translocated through reversible alkylation. Water, which usually consumes alkylating agents, facilitates the migration of Et743 by catalyzing the reverse reaction in this case.[26] Et743's migration may involve repeated capture and release of Et743 by DNA because Et743 binds to target sequences by hydrogen bonding and it is very likely to re-alkylate the original site before released from DNA into solution.[26] The reversible property of Et743 may contribute to its strong antitumor activity by providing a mechanism to escape the DNA repair process. Even after Et743 is excised from chromosome, it could be regenerated and cause further DNA alkylation.



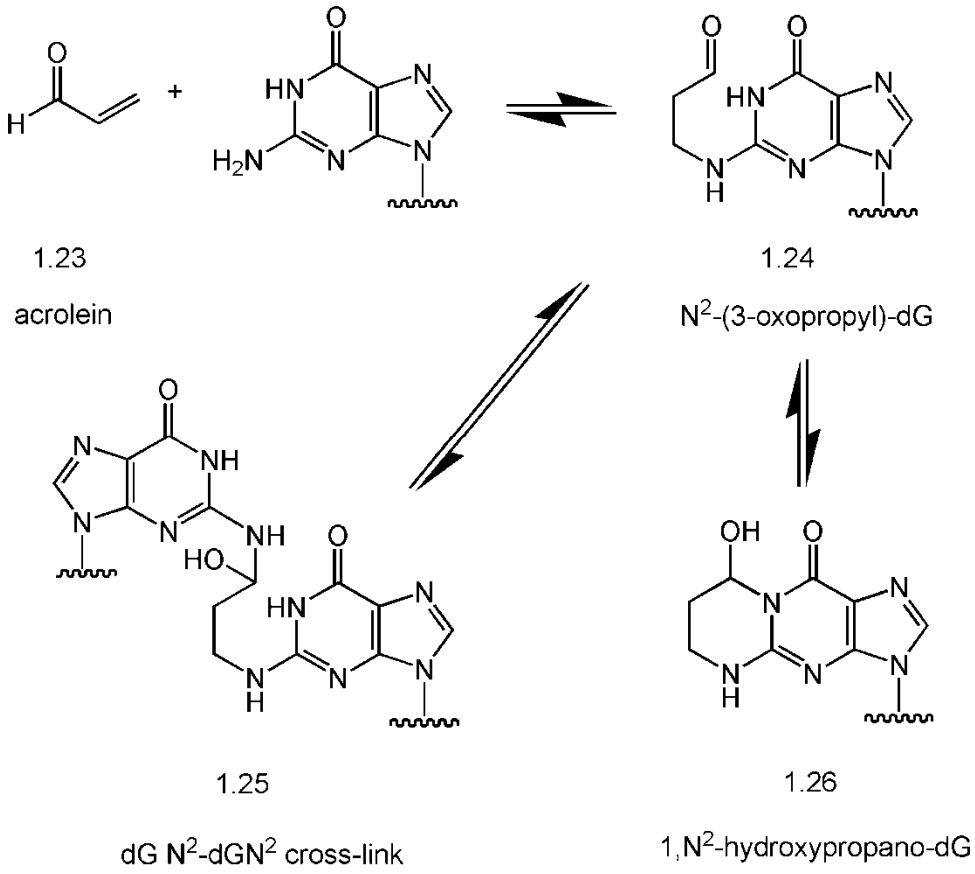
1.16 **Et743** 1.17 1.18

Scheme 1.5. The reversible reaction between Et 743 and DNA.[26]

Malondialdehyde (**1.19**) and acrolein (**1.23**) can form a variety of DNA adducts reversibly as well. Malondialdehyde (**1.19**) appears to first react with the 2-amino group of dG by forming an 3-oxo-1-propenyl adduct (**1.21**), which is followed by subsequent cyclization by reacting with dG N1 (Scheme 1.6). All steps in this process are reversible and the equilibrium is controlled by the environment.[27] Acrolein (**1.23**) can react with 2-amino group of dG initially and form a variety of DNA adducts, including interstrand crosslinking, reversibly (Scheme 1.7). Multiple alkylation adducts caused by malondialdehyde and acrolein, including mono-adducts (**1.21**, **1.24**), cyclization adducts (**1.22**, **1.26**), crosslinking (**1.25**) will make the DNA damage repair difficult in cell.



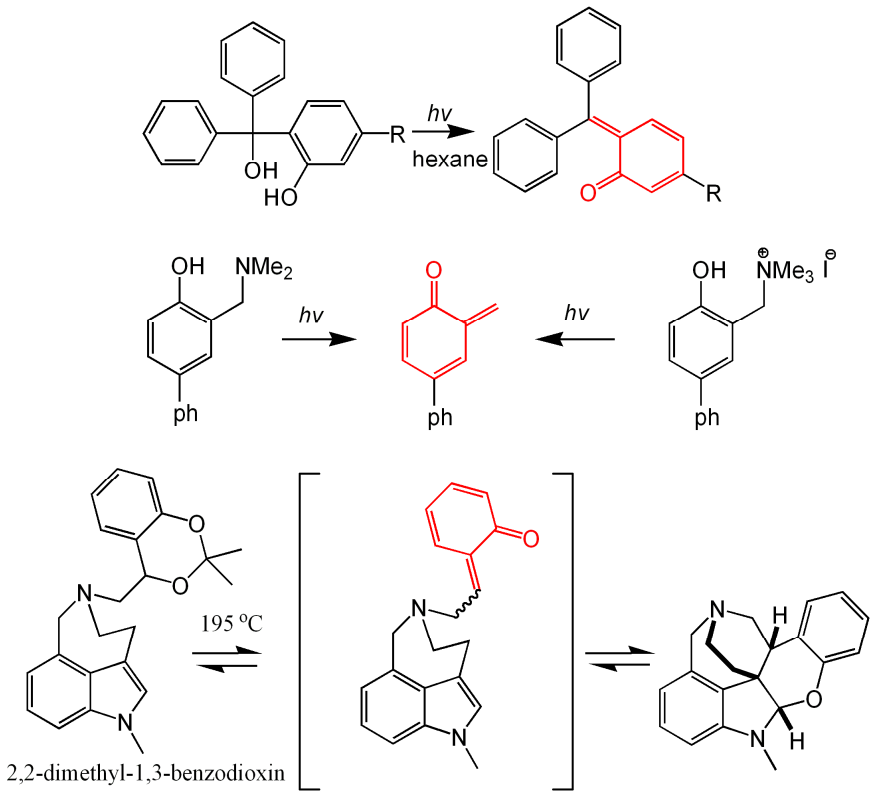
Scheme 1.6. Reversible reaction between malondialdehyde and DNA.



Scheme 1.7. Reversible reactions between acrolein and DNA.

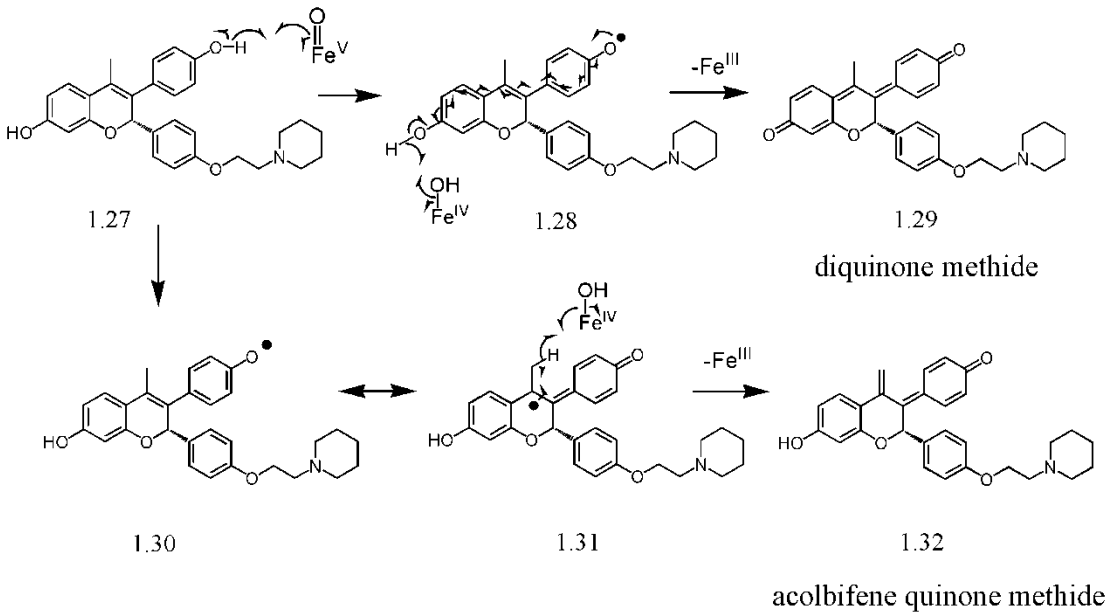
1.3. Quinone Methides and their reversible alkylation of DNA

Quinone methides (QMs) are highly electrophilic and transient intermediates that are implicated in alkylation of DNA by drugs and some natural products and play an important role in bioorganic chemistry and medicinal chemistry.[28-32] Quinone methides can be generated by photodehydration,[33, 34] photoelimination of quaternary ammonium salts,[35] and thermal generation as shown in Scheme 1.8.

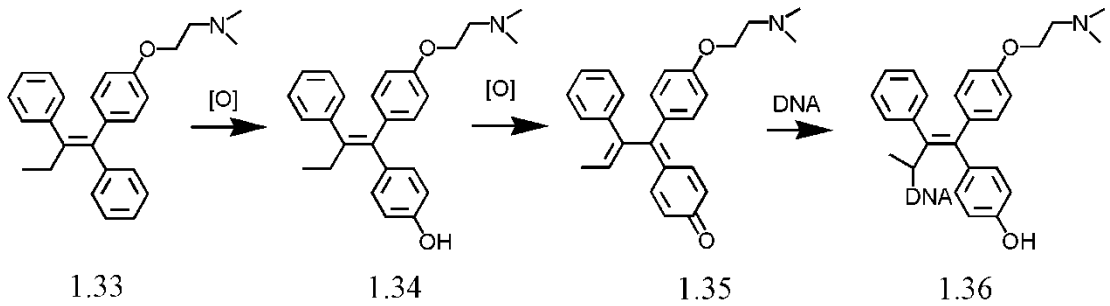


Scheme 1.8. Methods to generate quinone methide intermediates.

Quinone methides can also be generated by enzymes.[36-39] Acolbifene (1.27, Scheme 1.9) is a fourth-generation selective estrogen receptor modulator and can form two kinds of quinone methides intermediates through enzymatic oxidation (Scheme 1.9).[39] One was a classic acolbifene quinone methide (1.33) and it was formed by oxidation at the methyl group of compound (1.32). The other was a diquinone methide (1.30) involving stepwise oxidation of two phenol groups (Scheme 1.9).



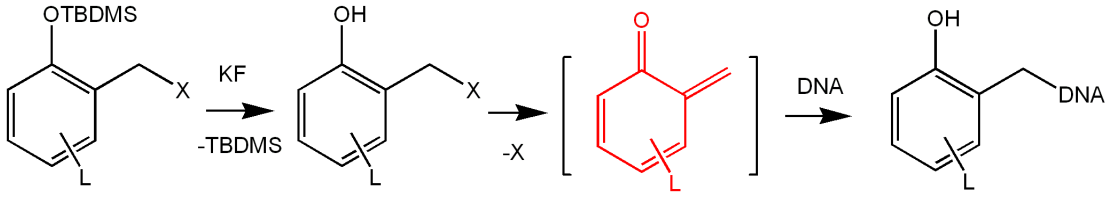
Scheme 1.9. Formation of diquinone methide and classical acolbifene quinone methide.[39]



Scheme 1.10. Formation of quinone methide by oxidation of tamoxifen.

Tamoxifen (1.34) is an antagonist of the estrogen receptor in breast tissue and one of the examples that quinone methide is generated during metabolism of some drugs. It has been the standard endocrine (anti-estrogen) therapy for hormone-positive early breast cancer.[40] However, research also showed that tamoxifen increases the chance of endometrial cancer in women.[41] One metabolic pathway of tamoxifen causing genotoxicity to humans may involve in oxidation of 4-hydroxytamoxifen (1.35) and then further oxidized to quinone methide (1.36) (Scheme 1.10). The quinone methide (1.36) can react with DNA by forming

covalent adducts and contribute to the tumorigenic effect of tamoxifen. [41]

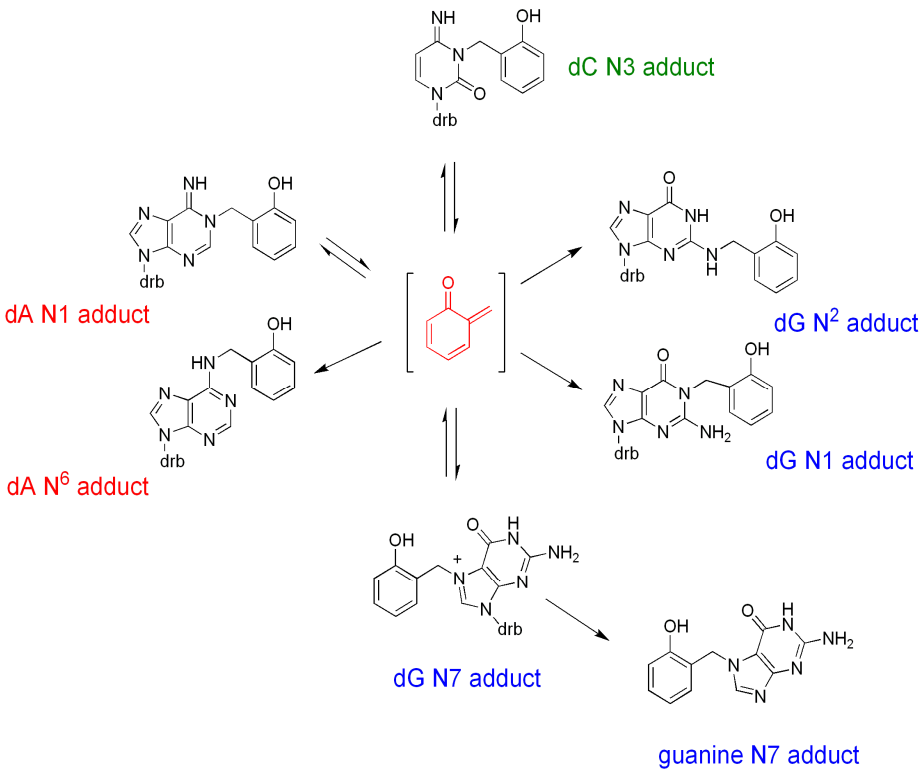


1.37 1.38 1.39 1.40

TBDMS = $SiMe_2tBu$, X=Br, AcO

Scheme 1.11. Structure of an *ortho*-quinone methide developed in the Rokita lab and its reaction with DNA.

Quinone methide can react with DNA reversibly by forming unstable adducts.[42,43] By using an *ortho*-quinone methide developed in the Rokita lab (Scheme 1.11), adducts formed by QM and strong nucleophiles of DNA (dG N7, dC N3, and dA N1) are unstable and decompose by regenerating QM intermediates. However, weak nucleophiles of DNA (dG N1, dG N^2, and dA N6) can form stable adducts with QM (Scheme 1.12).[42, 43]



Scheme 1.12. Structures of QM-nucleoside adducts (Modified from reference 43).

Time-dependent profile of dN's alkylation (N=A, G, C, T) by an unsubstituted QM showed that strong nucleophiles of DNA react with QM under kinetic control by forming unstable adducts (Figure 1.3). However, these unstable adducts will decompose and regenerate QM over time. The regenerated QM will partition among strong and weak nucleophiles of DNA. Eventually, stable adducts formed by weak nucleophiles of DNA will accumulate and dominate under thermodynamic control.[43] As shown in Figure 1.3, QM adducts of dC N3 and dA N1 formed quickly and reached maximum with in 10 hr. These adducts then gradually decompose and no dA N1 adduct was detected by HPLC after 40 hr. In contrast, adducts of dA N6, for example, formed very slowly initially but kept accumulating. The QM adducts of weak DNA nucleophiles dominated after a 3 days reaction.[43]

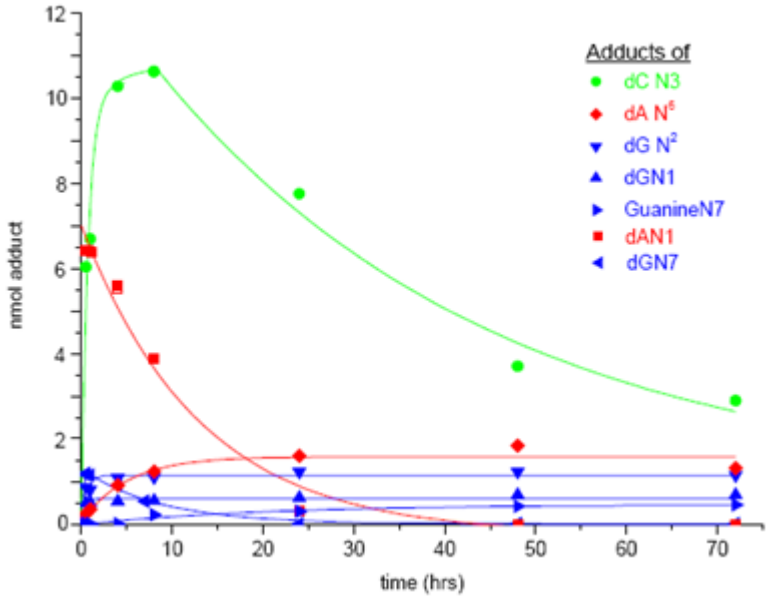


Figure 1.3. Time-dependent evolution of dN's (N=A, G, C, T) alkylation by an unsubstituted quinone methide (modified from reference 43).

Computations performed by Freccero group agree with observed kinetic preference of adduct formation and their stability.[44] The calculated activation free energies of QM alkylation at dA N1and dC N3 are 14.5 kcal/mol and 14.2 kcal/mol, respectively (Table 1.2). These values are significantly lower than the activation free energy of the rest of nucleophilic sites, which is about 20 kcal/mole (Table 1.2). Therefore, dA N1 and dC N3 are the kinetically favored sites to react with under aqueous conditions and their adducts dominate initially (Figure 1.3). However, the reverse of dA N1 and dC N3 adducts only requires activation free energy of 19.7 kcal/mole and 21.4 kcal/mol, respectively, which are energetically accessible at 37℃. Therefore, dA N1 and dC N3 adducts can readily decomposed and regenerate QM intermediate. In contrast, the free activation energies for reversal reaction of dA N6, dG N1, and dG N2 adducts are all above 30 kcal/mol, which is too high for regeneration of QM under conditions studied (Table 1.2).[44] Thus, QM adducts of

weak DNA nucleophiles are stable under the tested conditions.

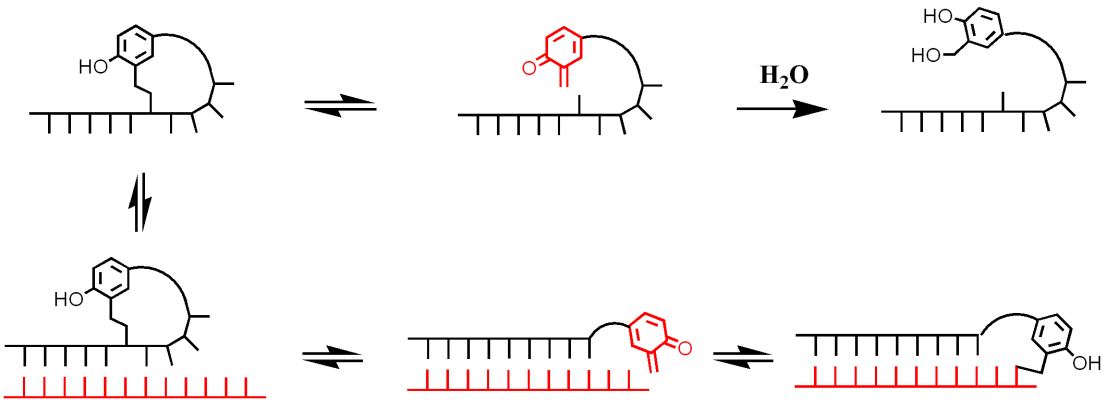
Table 1.2. Theoretical calculation of forward and reverse QM alkylation reactions.

Adduct	**$\Delta G^{\ddagger}_{aq}$(kcal/mol)**	**ΔG_{aq}(kcal/mol)**	**$\Delta G^{\ddagger}_{rev\text{-}aq}$(kcal/mol)**
QM- 9-MeA N1	14.5	-5.2	19.7
QM- 9-MeA N^6	22.4	-10.7	33.1
QM- 9-MeG N1	22.9	-7.4	30.3
QM- 9-MeG N^2	19.8	-11.7	31.5
QM- 9-MeG N7	20.1	-2.8	22.9
QM- 1-MeC N3	14.2	-7.2	21.4

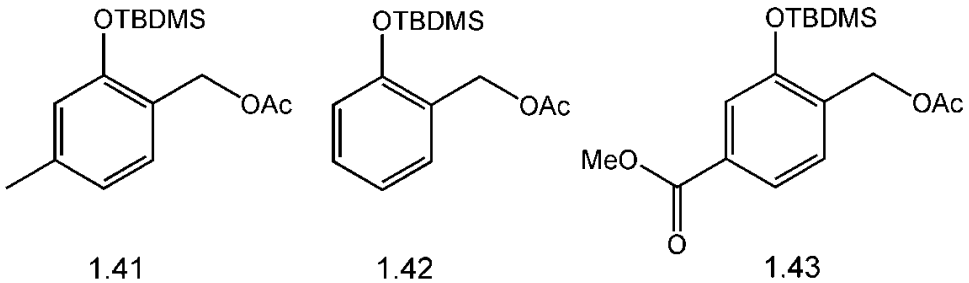
($\Delta G^{\ddagger}_{aq}$=activation free energy of QM alkylation in aqueous solution, ΔG_{aq}= free energy of QM alkylation in aqueous solution, $\Delta G^{\ddagger}_{rev\text{-}aq}$= activation free energy of the decomposition of QM adduct in aqueous solution. All calculations were based on B3LYP/6-31G (d) model.[44])

QM's reversible reaction with DNA is also found in a 2'-oligodeoxy-nucleotide-QM self-adduct, which is designed to alkylate single-stranded DNA in a sequence specific manner.[45] The QM-oligonucleotide self-adduct can regenerate the QM intermediate by reversible reaction and alkylation of its complementary sequence (Scheme 1.13). This ability of target alkylation remains even after incubating the self-adduct for 8 days under aqueous conditions, which indicates that the intramolecular alkylation is highly favored and can compete efficiently with irreversible trapping of water. [45] Multiple release and capture of QM from its oligonucleotide self-adduct during incubation are likely because the lifetime of

QM-nucleobases is hours, which is significantly shorter than 8 days. [45] A thermodynamic driving force of base pairing to the target is required to transfer a QM equivalent to generate interstrand crosslinking. [45]



Scheme 1.13. QM-oligonucleotide self-adduct and its target promoted DNA alkylation.[45]



1.41 AcQMP-Me 1.42 AcQMP-H 1.43 AcQMP-Est

Scheme 1.14. Structures of QM precursors.[46]

The reactivity of QM intermediate and the stability of QM-DNA adducts are sensitive to the aromatic substituents. Electron-donating groups on aromatic ring will stabilize QM and facilitate its generation.[46] In contrast, electron-withdrawing groups will destabilize QM and retard its generation.[46] An *ortho*-quinone methide precursor was modified by either methyl group or methyl ester at the meta position of phenol (Scheme 1.14).[46] The reactions between those three QMPs and dC N3 have been studied and taken as an example here to demonstrate the effect of aromatic

substituents on regulating QM's reactivity (Figure 1.4). Electron-rich AcQMP-Me formed dC adduct quickly and reached maximum amount of adduct in 30 min. QMP with no substituent reacted with dC slower than AcQMP-Me and formed a maximum amount of adduct after 5 hr.[46] The decomposition of AcQM-Me-dC adduct is moderately faster than AcQM-dC adduct. In contrast, AcQMP-Est reacted with dC very slow and did not reach maximum alkylation in 24 hr. More interestingly, AcQM-Est-dC adduct do not decompose over time and regenerate QM as the other two QMPs. This indicates that aromatic substituent cannot only affect kinetics of adduct formation, but also alternate the stability of AcQM-DNA adducts. Electron-donating substituents increase the reactivity of QMP and accelerate the regeneration of QM. Electron-withdrawing substituents decrease the reactivity of QMP and slow down the regeneration of QM from its DNA adduct. When the electron-withdrawing effect is strong enough, unstable DNA adduct can be converted into stable DNA adduct, such as AcQM-Est-dC adduct. [46]

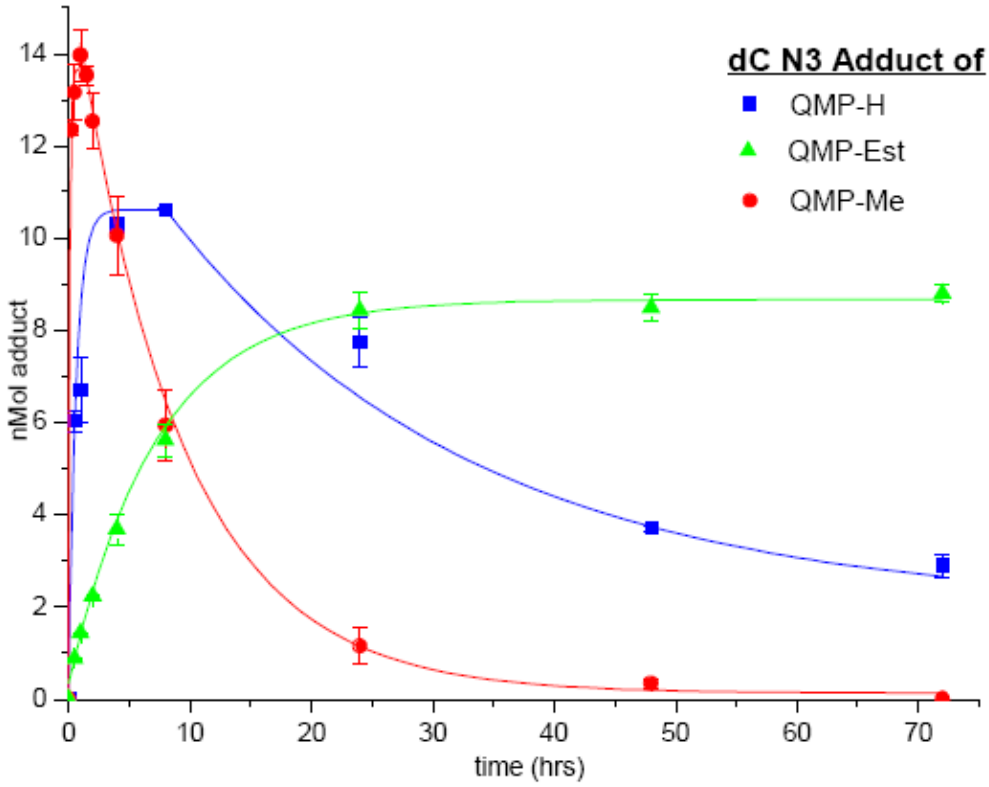


Figure 1.4. Formation and decomposition of QM adducts of dC N3 (source: copied from reference 46).

1.4. Reversible DNA alkylation is both troublesome and beneficial

A number of alkylating agents, such as acrolein and duocarmycin, have been reported to react with DNA in a reversible manner as described above. The reversible DNA alkylation can lead to a re-distribution of initial products formed under kinetic control. Reversible alkylation could potentially impact the cytotoxicity of alkylating agents by regenerating integral drug and extending its lifetime for DNA alkylation. Some small molecules, such as acrolein, can produce multiple adducts, which potentially make it difficult for cells to repair DNA damage. Benefits could also be seen when reversible alkylating agents are conjugated to target recognition elements for sequence selective alkylation. Target promoted alkylation can achieve

DNA alkylation in a sequence specific manner. However, reversible DNA alkylation also causes trouble for adduct analysis. Conventional methods for analyzing DNA adducts require enzyme digestion, dialysis and separation, which can take days to complete. Information of initial labile adducts can be lost during these conventional procedures.

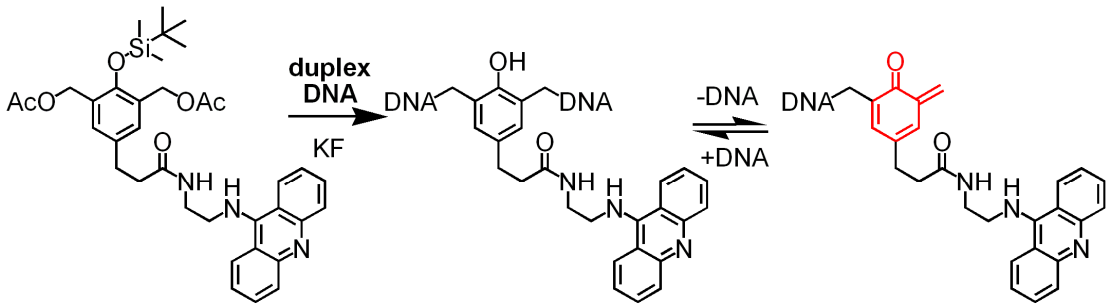
Consequences of QM's reversible reaction with oligonucleotide and duplex DNA remain unknown. Therefore, the goal of this dissertation will focus on the reversible alkylation of QM with free nucleosides, single-stranded DNA and duplex DNA based on a bi-functional quinone methide precursor acridine conjugate (bisQMP). These experiments should provide an insightful view of QM's reversible reaction with nucleophiles of DNA and therefore serve as basis to understand and control QM's toxicity.

Chapter 2. The lifetime of a bi-functional QMP-acridine conjugate for crosslinking duplex DNA can be extended by reversible reaction with strong nucleophiles of nucleosides.

2.1. Introduction

DNA alkylating agents used to treat cancer or other diseases often express a low efficiency for target alkylation and poor selectivity towards DNA.[3, 47, 48] Numerous cellular components other than DNA, for example, protein and water, can also act as nucleophiles and consume alkylating agents before they were able to associate with DNA. In our efforts to improve alkylation efficiency of quinone methide, we have focused modifying quinone methide precursors to be more intrinsically selective to DNA over other nucleophiles.

Pre-association of quinone methide (QM) precursors with dsDNA can improve the target selectivity and alkylation efficiency. Guanine N7, which is accessible in the major groove of DNA, is one of the strongest nucleophiles of DNA.[2] Therefore, major groove of DNA appears as a promising target for alkylation and crosslinking.[3, 49] A bi-functional quinone methide precursor (bisQMP) had been conjugated onto acridine (Scheme 2.1), which is a cationic DNA intercalator. Acridine can direct QM into the major groove of duplex DNA and retain the kinetically favored but labile alkylation adducts.[49] Once both benzylic positions of bisQM are covalently linked to DNA and form interstrand crosslinking, there will always be one covalent anchor to locate bisQM in duplex DNA because only one QM-DNA bond could possibly disassociate at any one time (Scheme 2.1).



BisQMP acridine

Scheme 2.1. BisQMP causes DNA crosslinking.

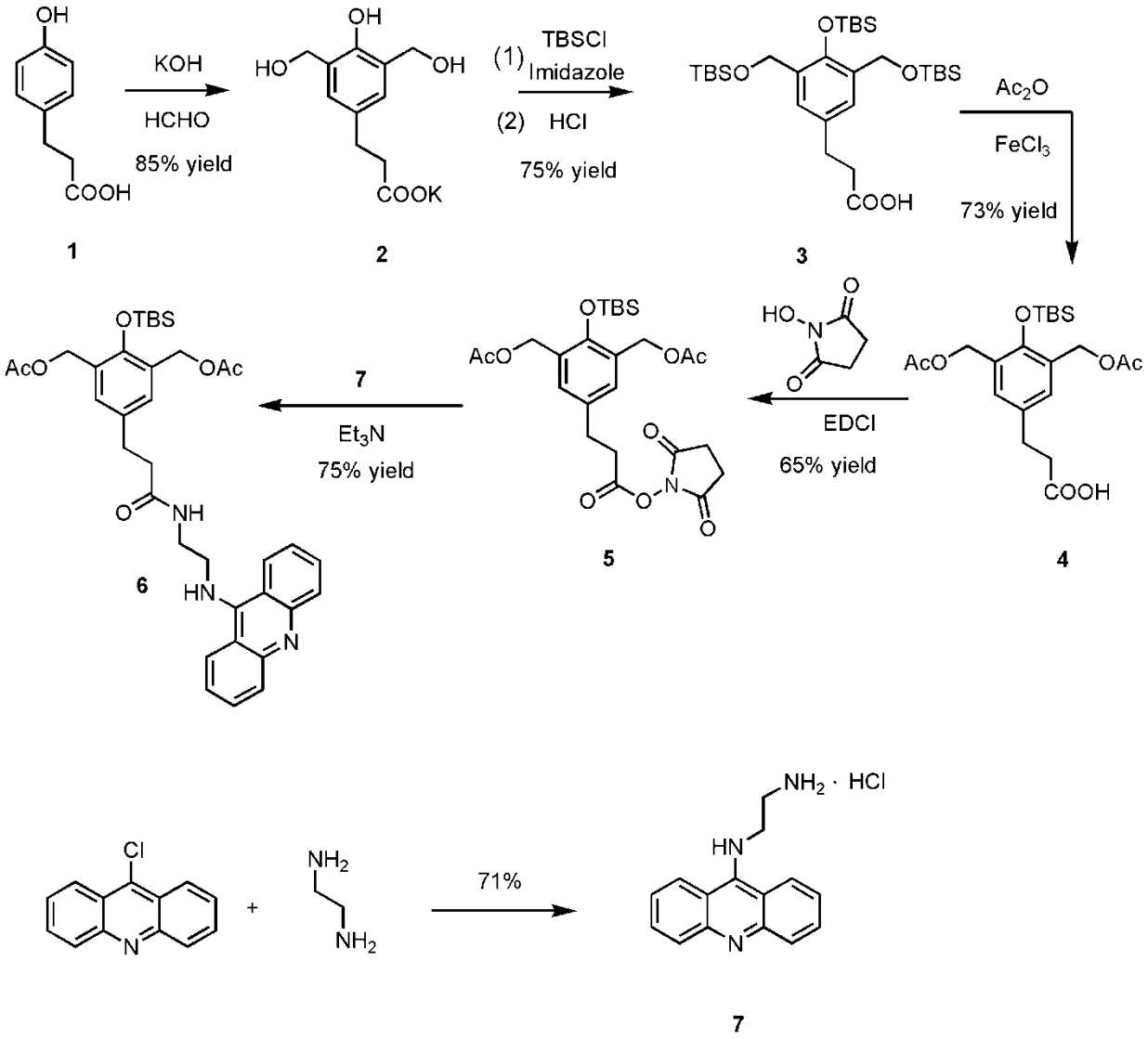
Irreversible trapping by nucleophiles other than DNA can consume significant amount of QM before its diffusion to DNA target. However, reversible trapping of QM could have the opposite effect and extend QM's lifetime. Labile adducts between QM and strong nucleophiles of DNA have the ability to regenerate QM intermediates and the newly released QM intermediates could further alkylate other nucleophiles in the solution (Figure 2.1). The reversible reactions between QM and strong nucleophiles of DNA may have the potential to extend the lifetime of QM precursor by repeated capture and release. However, this assumption has not been evaluated yet. Thus, the bisQMP-acridine conjugate was used to study the longevity of QM in the presence of strong nucleophiles of DNA.

2.2. Results and discussion

2.2.1. Synthesis of bisQMP acridine conjugate and derivatives and their ability to crosslink duplex DNA.

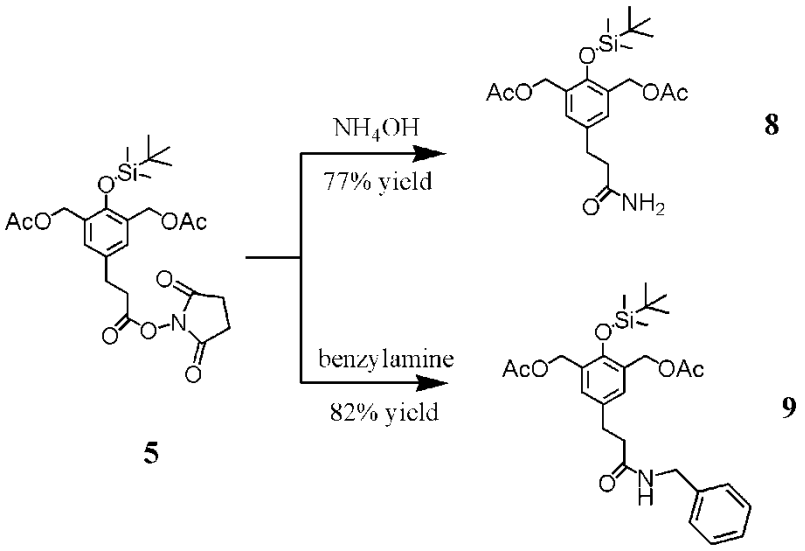
The bisQMP acridine conjugate was synthesized previously in our laboratory.[49] However, the previous synthetic procedure suffered from low yield and difficulty in product purification, especially in the first step. Previous literature indicated that

under mild alkaline condition, the reaction between hydroxylphenylpropionic acid **1** and formaldehyde was run for 17 hours before quenched by adding in HCl to neutralize KOH.[49] However, after 17 hour reaction, ^{1}H NMR analysis of crude products showed that only about 50% of **1** was converted to di-substituted compound **2** and the rest was mono-substituted byproduct. To push this reaction to completion, the reaction temperature was raised from 55 ^{o}C to 65 ^{o}C and the reaction time was extended to 2 days. ^{1}H NMR analysis of crude products indicated that after 2 days' reaction, 85% of starting material **1** was converted to compound **2**. Longer reaction time beyond 2 days did not change this ratio significantly. Crude **2** was then silylated with an excess of TBS-Cl (5 equivalents) and imidazole (8 equivalents) to afford **3** in 75% yield. Following the synthesis of **3**, the acetoxy groups were selectively introduced at the benzylic positions by a method established by Ganem and Small.[50] This method applied acetic anhydride and a catalytic quantity of ferric chloride to provide **4** in 73% yield. The carboxylic acid **4** was then converted to the N-hydroxysuccinimidyl ester **5** in 65% yield. This activated ester was coupled with synthesized acridine derivative **7** in a 75% yield.



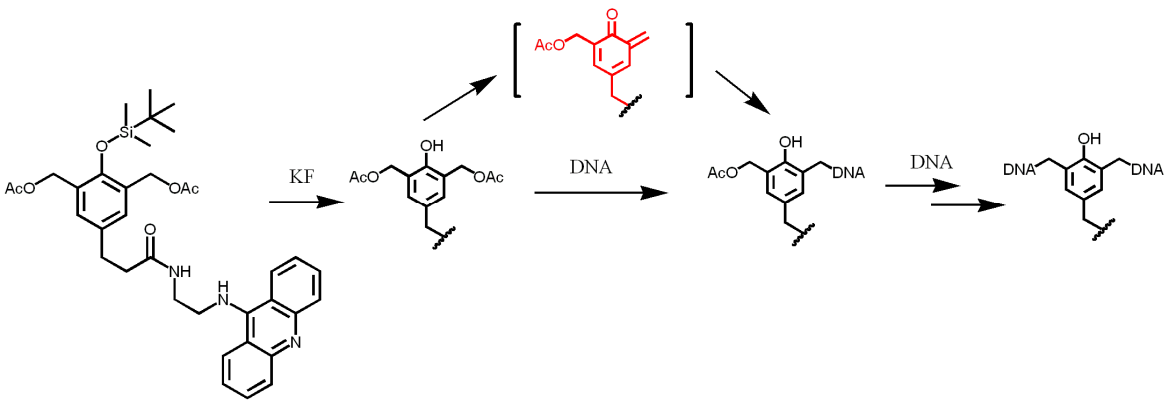
Scheme 2.2. Synthesis of the bisQMP-acridine conjugate **6**.[49]

Two bisQMP derivatives without attached acridine, **8** and **9**, were synthesized by coupling the activated ester **5** with ammonium hydroxide and benzylamine, respectively (Scheme 2.3).



Scheme 2.3. BisQMP analogues without attached acridine.

The ability of bisQMP to crosslink duplex DNA was confirmed by incubating bisQMP **6** and complementary oligonucleotide sequences (**OD1** and **OD2**) of different lengths (Figure 2.1).[49] The cross-linking species and alkylation products can be distinguished by denaturing gel electrophoresis. A cross-link species will show equivalent migration on gel because they share the same components. Alkylation products of each oligonucleotide should migrate differently through denaturing gel because of their length difference.[49] Reaction of bisQMP **6** (10 equivalents, 30 μM, 24 hr, room temperature) with duplex DNA (**OD1**/**OD2**, 3 μM) and potassium fluoride (10 mM) produced bands corresponding to crosslinking species in 64% yield after 24 hr by denaturing gel electrophoresis (Figure 2.1).



Scheme 2.3. BisQMP acridine conjugate cross-links duplex DNA.

OD1 5'-CAGATTACGCGCAGAAAAAAAGGATCTCAAG-3'
OD2 3'-AATGCGCGTCT TT TT TTCCTAGAGTTC -5'

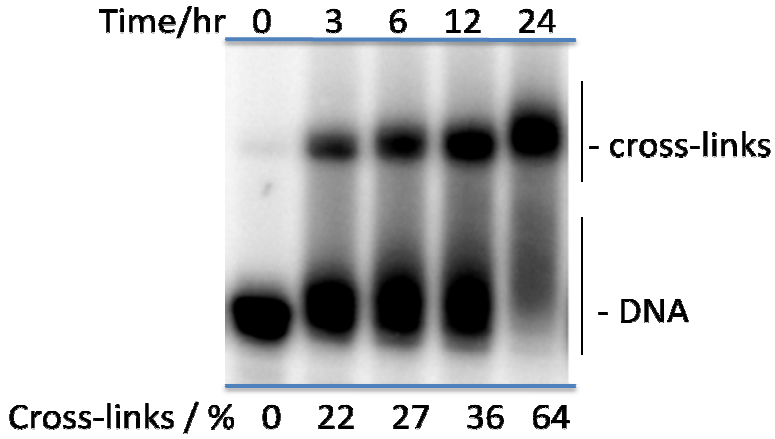


Figure 2.1. DNA crosslinking by bisQMP **6**. Double stranded DNA (5'-[^{32}P]-**OD1**/**OD2**, 30 nCi, 3.0 μM) was incubated with **6** (30 μM) for 0 to 24 hr at room temperature in 20% aqueous acetonitrile (10 mM MES, pH 7) in the presence of potassium fluoride (10 mM). Samples were analyzed by 20% denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

The importance of acridine's binding to duplex DNA was significant when compared to the crosslinking efficiency of bisQMP-benzylamine conjugate **9**. Under equivalent conditions, 10 mM bisQMP-benzylamine was only able to produce about 45% crosslinking after 24 hr reaction (Figure 2.2) whereas 300 fold lower concentration of bisQMP-acridine conjugate **6** (30 μM) was able to produce 64%

crosslinking. Acridine, a strong intercalator of DNA, can enhance bisQMP's crosslinking efficiency by increasing the effective concentration of two reaction components.

The high concentration of bisQMP-benzylamine **9** produced broad bands which appeared to be modestly higher molecular weight than single stranded DNA and crosslinking with lower bisQM does. The broadness of the bands was consistent with multiple alkylation events per DNA strand (Figure 2.2).[49]

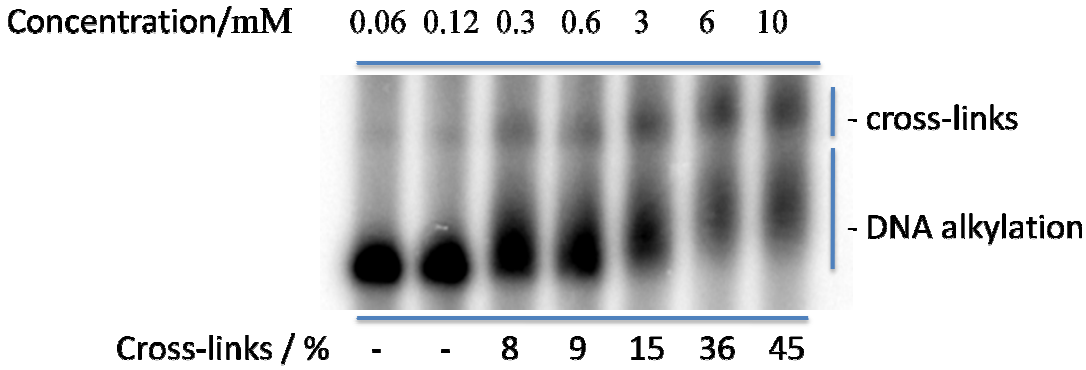
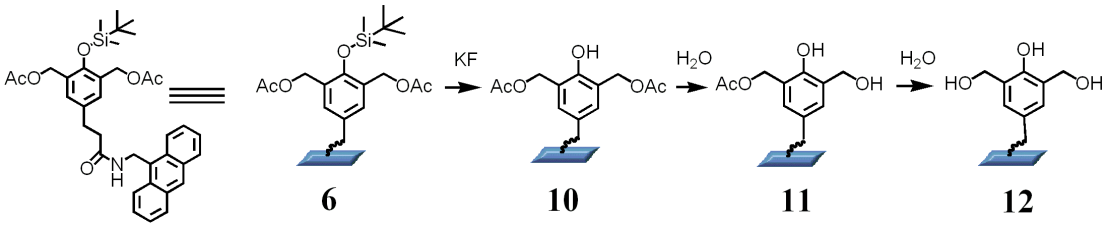


Figure 2.2. BisQMP-benzylamine conjugate's ability to crosslink duplex DNA. Double stranded DNA (5'-[^{32}P]-**OD1**/**OD2**, 30 nCi, 3.0 μM) was incubated with the bisQMP-benzylamine conjugate **9** for 24 hr at room temperature in 20% aqueous acetonitrile (pH 7, 10 mM MES). Potassium fluoride (50 mM) was added to initiate reaction. Samples were analyzed by 20% denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

2.2.2. Lifetime of bisQMP to crosslink duplex DNA is short under aqueous conditions.

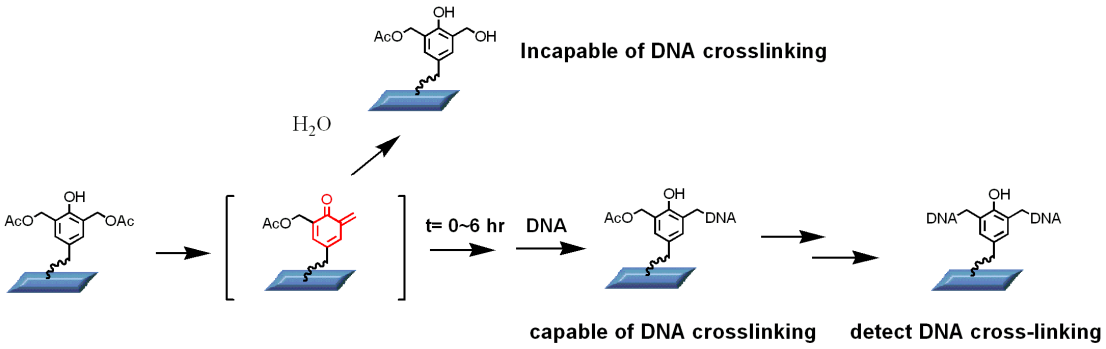
BisQMP can generate two equivalents of QM intermediates and both benzylic positions of bisQM must link to DNA strands in order to cause crosslinking (Scheme

2.1). BisQM will lose its cross-linking ability if either of its benzylic positions is quenched by water irreversibly. In the absence of DNA, bisQMP will be converted into its hydration products after initiation of reaction by KF (Scheme 2.4).



Scheme 2.4. The hydration of bisQMP under aqueous conditions.

The loss of bisQMP's crosslinking ability was monitored by pre-incubating bisQMP with KF under aqueous conditions for various times. Then, duplex DNA was added to detect the amount of bisQM equivalents that persisted to crosslink DNA (Scheme 2.5).



Scheme 2.5. The lifetime of bisQMP as a DNA cross-linking agent is limited by irreversible water trapping.

The effective longevity of the QM equivalents under aqueous conditions was measured by time-dependent loss of cross-linking after pre-incubation (Figure 2.3). As expected, DNA cross-linking by the bisQMP acridine conjugate **6** was maximum (25%) when the reaction was initiated in the presence of duplex DNA (**OD1/OD2**) and irreversible trapping was minimized. However, the yield of DNA cross-linking

dissipated quickly when the QM was incubated with water prior to addition of duplex DNA. The irreversible trapping by water consumed QM equivalent for DNA crosslinking during pre-incubation. The half-life for this quenching is about half an hour (Figure 2.4), and no cross-linking was detected after a pre-incubation of 2 hours (Figure 2.3).[51]

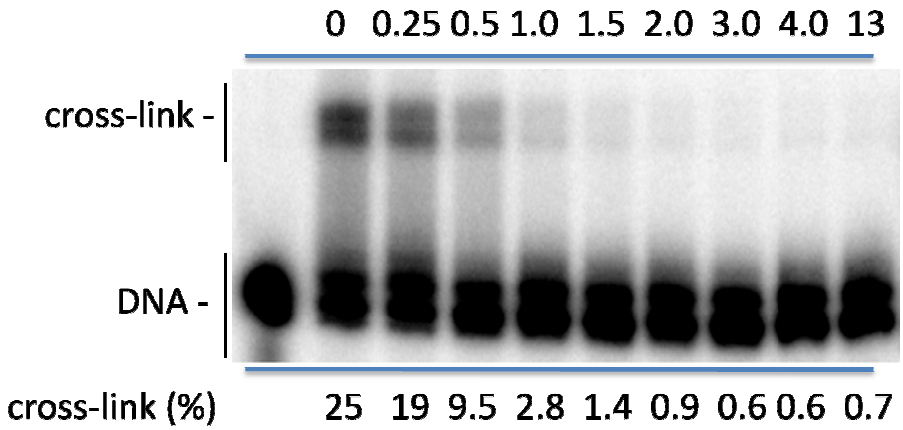


Figure 2.3. The lifetime of bisQMP as a DNA cross-linking agent is short under aqueous conditions. [51] (A) Reaction of the bisQMP-acridine conjugate (100 μM) was initiated by addition of KF (100 mM) in 10 mM MES, pH 7 and 20% acetonitrile under ambient conditions for the indicated time. The persistent ability of these samples to cross-link DNA was measured by subsequent addition of **OD1**/5'-[^{32}P] **OD2** (3.0μM, 30 nCi annealed with 10% excess **OD1**). Samples were then further incubated for 48 hr under ambient conditions. Each solution was then frozen, lyophilized and analyzed by denaturing 20% polyacrylamide gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA.

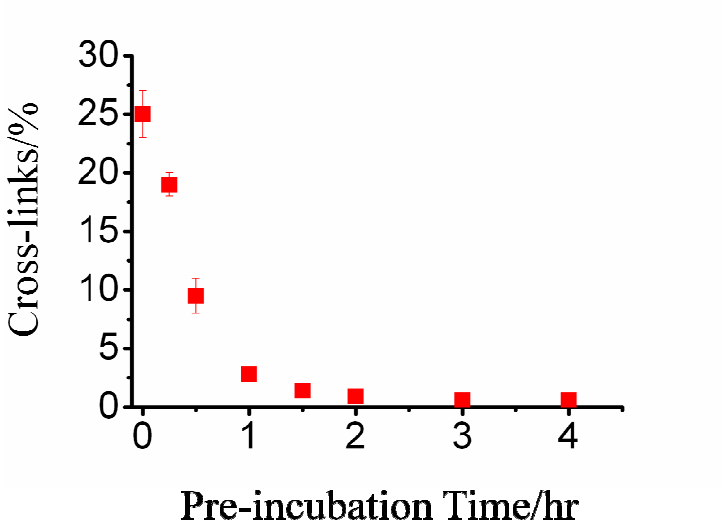


Figure 2.4. Time-dependent loss of DNA crosslinking during pre-incubation under aqueous conditions. Cross-linked products were quantified by phosphoimage analysis and reported (%) relative to total DNA. Data represent the average of two independent experimental values and their range is indicated by the error bars.

BisQMP's hydration during pre-incubation under aqueous conditions was also monitored by reverse-phase HPLC. Initial attempts were made by injecting pre-incubation samples of bisQMP **6** into HPLC and monitoring the formation of proposed stepwise water adducts (Scheme 2.4). However, it was found that the acridine attachment made it extremely difficult to elute bisQM-acridine conjugate **6** from HPLC column. Even after 1 hr washing by 100% acetonitrile, no peak corresponding to bisQMP acridine conjugate **6** was observed by HPLC. The elution time of bisQM and its derivatives must be short if they are going to be observed since the transient hydration products **10** and **11** will spontaneously transform into their next hydration products. Therefore, bisQMP analogues **8** and **9** were synthesized for optimized elution time and resolution in HPLC studies (Scheme 2.4).

Hydrophilic group $-NH_2$ was used in place of acridine in compound **8** in order to facilitate its elution through HPLC column. However, it was difficult to separate

compound **8** from its hydration products by HPLC. Less hydrophilic bisQMP-benzylamine conjugate **9** demonstrated a better separation of the four stepwise hydration products (Figure 2.5).

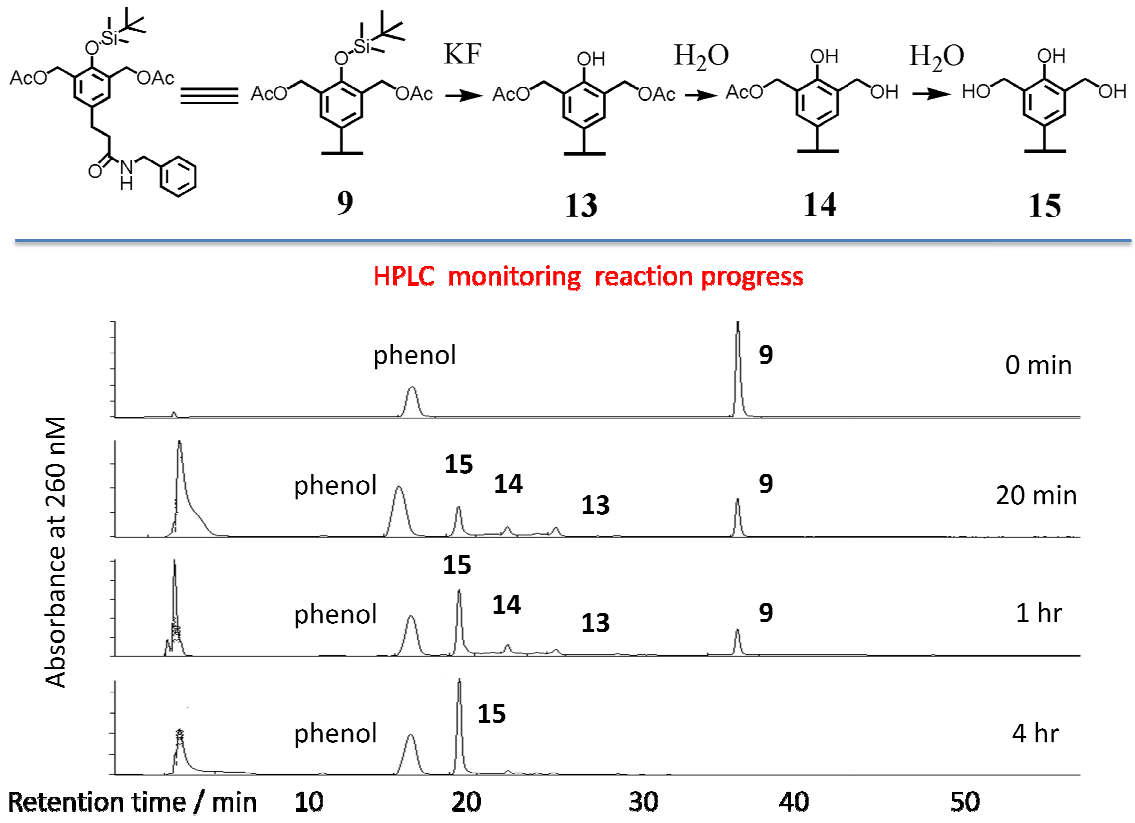


Figure 2.5. BisQMP's hydration under aqueous conditions.
BisQMP benzylamine conjugate **9** (3 μM) was incubated with KF (100 mM) in 10 mM MES, pH 7 and 20% acetonitrile under ambient conditions for the indicated time. Then samples were injected into reverse-phase HPLC without further treatment. Phenol was added as internal standard before injection to HPLC. The peaks of compound **9** and **15** were collected from HPLC and identified by ESI-MS. Two minor peaks between **9** and **15** were tentatively assigned as compound **13** and **14**, respectively, based on their hydrophobic properties.

BisQMP **9** was converted to its final hydration product quickly. After 1 hr incubation, 70% of bisQMP **9** converted to its final hydration product **15** and after 4 hr incubation, all bisQMP **9** was consumed and only compound **15** was detected by HPLC (Figure 2.5). The rates of compounds **9**'s consumption fitted first order decay,

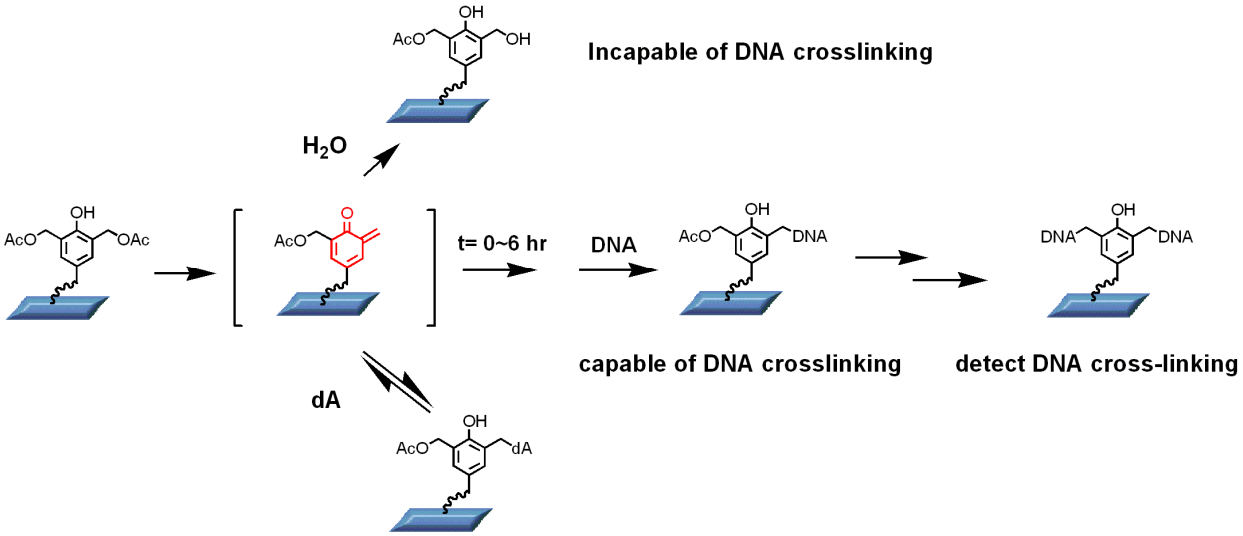
which was consistent with the assumption that desilylation of compound **9** was the rate determining step (Figure 2.5). These results were consistent with the DNA assay (Figure 2.4) that bisQMP-acridine conjugate's ability to crosslink DNA decreased rapidly due to the irreversible quenching of water.

Very low accumulation of compound **13** and **14** was detected by HPLC (Figure 2.5). This suggested that **13** and **14** were transient products and therefore the desilylation step, instead of the generation of bisQM intermediate, was the rate determining step of bisQMP's hydration. Previous studies on unsubstituted *ortho*-QM suggested that the generation of QM intermediate was the rate determining step (Scheme1.11).[52] The formation of oligonucleotide-QM self-adduct also showed the slow conversion from QM's desilylation product to QM's oligonucleotide adducts by HPLC.[45] However, desilylation of compound **9** appeared as the rate determining step for its hydration according to HPLC studies. This was consistent with the fact that electron-donating substituents can facilitate the formation of QM by stabilizing the electron-deficient intermediate.[46] The formation of bisQM intermediate was accelerated by two alky substituents and therefore no longer the rate determining step.

2.2.3. Lifetime of bisQMP to crosslink duplex DNA is greatly prolonged in the presence of dA under aqueous conditions

One major drawback of QMs as a DNA alkylating agent is that QMs always suffer from trapping by nucleophiles other than DNA, such as water. However, QMs

could potentially escape trapping by forming reversible adducts. Continual capture and release of QM from its transient adducts should extend the effective lifetime for DNA alkylation (Scheme 2.6).



Scheme 2.6. The lifetime of bisQMP-acridine conjugate is extended by reacting with dA reversibly.

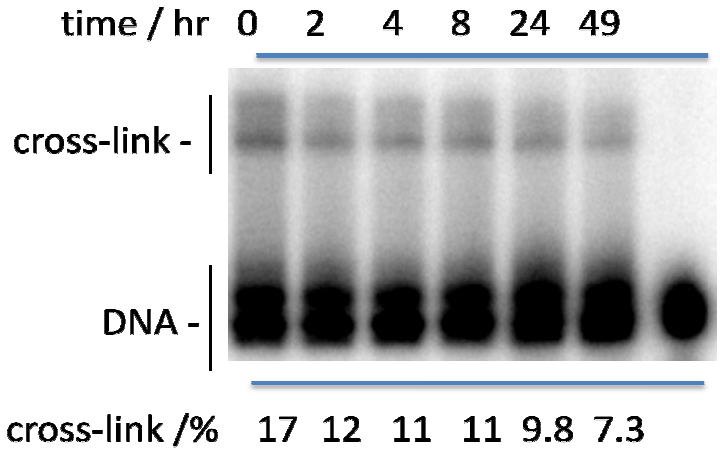


Figure 2.6. The lifetime of bisQMP as a DNA cross-linking agent is extended by dA under aqueous conditions. Reaction of the bisQMP-acridine conjugate (100 μM) was initiated by addition of KF (100 mM) in 10 mM MES, pH 7 and 20% acetonitrile in the presence of 20 mM dA under ambient conditions for the indicated time. The persistent ability of these samples to cross-link DNA was measured by subsequent addition of **OD1**/5'-[^{32}P] **OD2** (3.0 μM, 30 nCi annealed with 10% excess **OD1**). Samples were then further incubated for 48 hr under ambient conditions. Each solution was then frozen, lyophilized and analyzed by denaturing 20% polyacrylamide gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

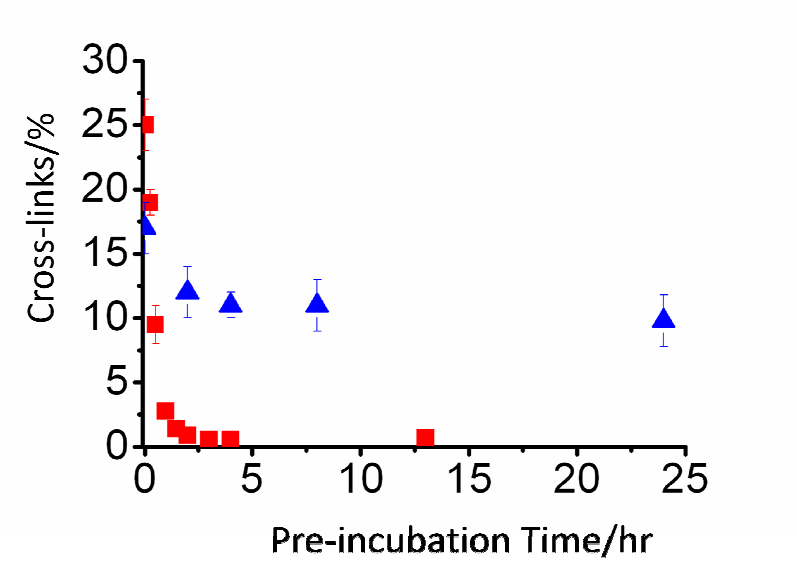
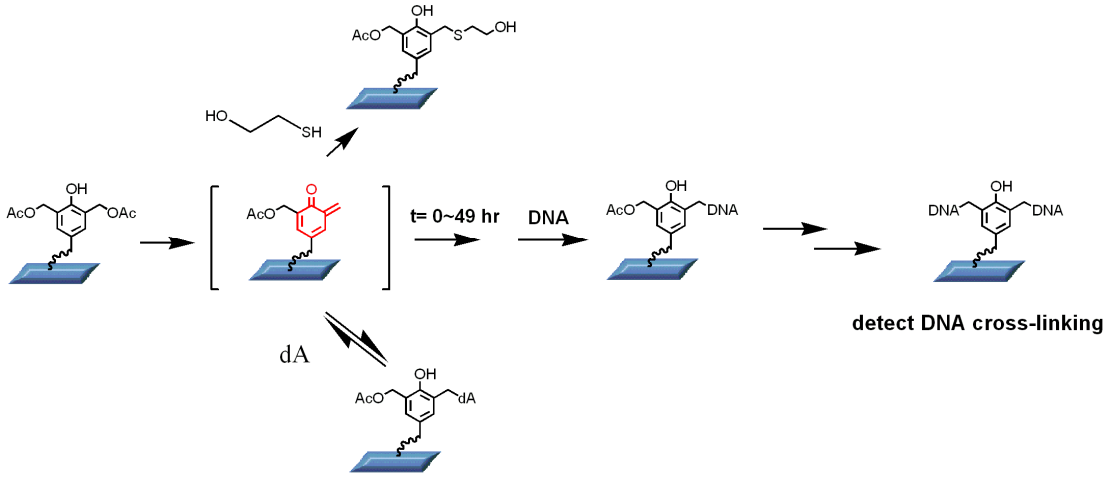


Figure 2.7. Time-dependent loss of DNA crosslinking during pre-incubation under aqueous conditions in the absence (red) and presence (blue) of dA. Data represent the average of two independent experimental values and their range is indicated by the error bars.

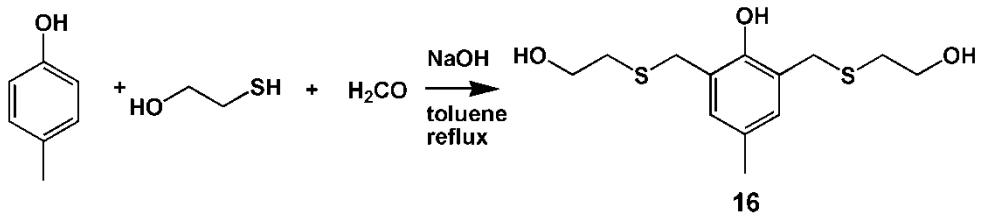
dA (20 mM) competed with DNA for reaction with the QM intermediate and lowered the maximum yield of DNA cross-linking to 17% (Figure 2.6) from 25% in its absence (Figure 2.4). However, the resulting dA adducts also provided a continual source of the QM intermediate (Scheme 2.6). The cross-linking activity of the bisQMP-acridine conjugate was preserved beyond at least 49 hr under aqueous conditions in the presence of dA. The effective lifetime of bisQM for DNA crosslinking was consequently increased by almost 100-fold because of the ability of dA to forestall irreversible trapping by water. This observation was consistent with the efficient and reversible reaction of dA N1.[42-44] The symmetric dA N1 bisQM adduct had been synthesized and confirmed by two-dimensional NMR spectroscopy before[51], which confirmed the ability of dA to preserve both benzylic positions of bisQM. Previous studies also suggested that the half-life of the reversible dA N1

adduct was only approximately 2 hr under aqueous conditions.[42] Thus, dA N1 likely captured and released the bisQM-acridine conjugate repeatedly in order to maintain cross-linking activity throughout incubations of 49 hr prior to addition of duplex DNA (Scheme 2.6).



Scheme 2.7. A strong and irreversible nucleophile such as β-mercaptoethanol can compete with dA efficiently and diminish the effect of a reversibly acting nucleophile.

Trapping the QM irreversibly with an alternative nucleophile such as thiol can counteract the effect of dA and suppressed cross-linking (Scheme 2.7). One requirement for QM trapping agents is their ability to form stable adducts that are unable to regenerate QM intermediate, however, the stability of QM-thiol adducts had not been examined yet. Therefore, bisQM's β-mercaptoethanol adduct **10** was synthesized and incubated under identical conditions described in Figure 4.3 for 7 days and analyzed by reverse phase HPLC (Scheme 2.8). No degradation of adduct **10** was observed in 7 days, which confirmed that QM's thiol adduct is stable (Appendix Figure 1).



Scheme 2.8. Synthesis of bisQM-mercaptoethanol adduct **16**.

The steady state between QM intermediate and QM-dA adduct could be regulated by QM trapping agents and dA can not compete with thiol to preserve bisQM equivalents for DNA crosslinking during pre-incubation. In the presence of dA (20 mM), 5 mM β-mercaptoethanol decreased crosslinking by 90% when experiment in Figure 2.6 was repeated with thiol (Figure 2.8, lane 1 vs. Figure 2.6). BisQMP's ability of crosslinking DNA was fully suppressed in the presence of 20 mM β-mercaptoethanol (Figure 2.8, lanes 11-15). DNA alkyaltion was not observed either, indicating DNA was not able to compete with thiol by reacting with QM.

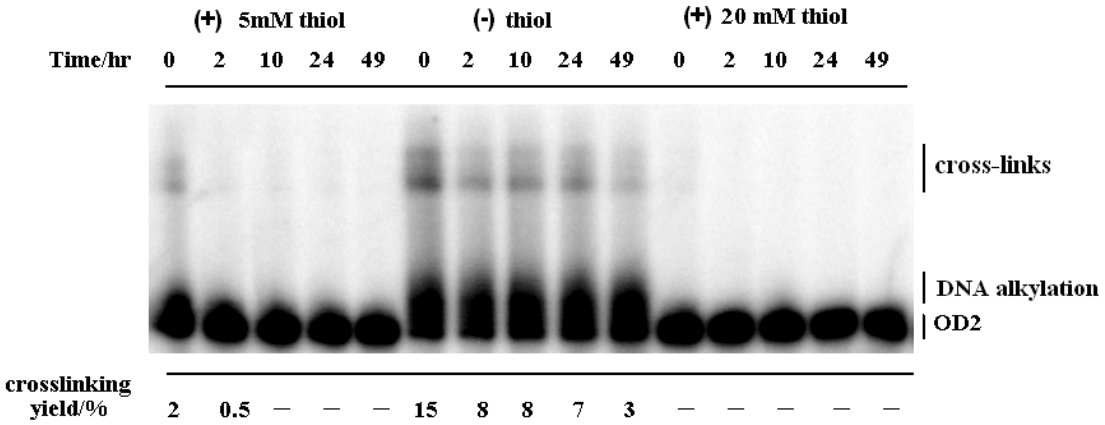


Figure 2.8. A thiol counteracts the ability of dA to prolong the activity of a quinone methide. The bisQMP-acridine conjugate (100 μM) and dA (20 mM) in 10 mM MES pH 7 20% acetonitrile were incubated under ambient conditions in the presence (lanes 1-5, 11-15) and absence (lanes 6-10) of β-mercaptoethanol (lanes 1-5, 5 mM; lanes 11-15, 20 mM). Reaction was initiated by addition of KF (100 mM.) After the indicated time, **OD1**/5'-[^{32}P]-**OD2** (3.0 μM, 30 nCi annealed with 10% excess **OD1**) was added, and the resulting mixture was incubated for an addition 48 hr prior to electrophoretic analysis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

2.2.4. dA's ability to extend bisQM's lifetime is concentration dependent.

To preserve bisQMP's ability of crosslinking DNA, higher concentration of dA may help compete more efficiently with water's irreversible trapping by forming QM-dA adduct. The cross-linking yield of DNA increased from none to about 15% when the concentration of dA was increased from 0 to 6 mM after 8 hr pre-incubation (Figure 2.9). Persistence of the crosslinking activity saturated at approximately 6 mM under tested conditions (Figure 2.10). The crosslinking yield decreased slightly to 12% when raising dA's concentration to 20 mM (Figure 2.9). The higher concentration of dA may compete with DNA to react with regenerated bisQM intermediate and thus lower the crosslinking.

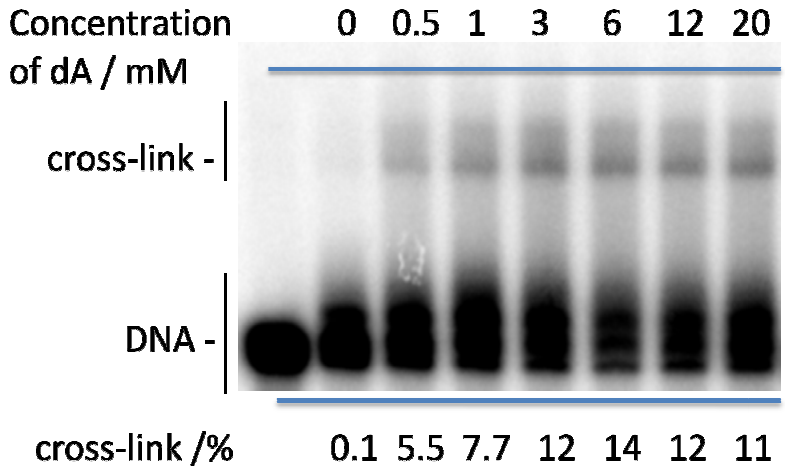


Figure 2.9. Concentration dependence of dA's ability to preserve the quinone methide reactivity.[51] The bisQMP acridine conjugate (100 μM) in 10 mM MES pH 7 20% acetonitrile were incubated with varying concentrations of dA under ambient condition for 8 hr after addition of 100 mM KF to initiate reaction. Duplex DNA (**OD1**/5'-[^{32}P]-**OD2**, 3.0 μM, 30 nCi annealed with 10% excess **OD1**) was then added, and the mixture was incubated under ambient conditions for another 48 hr before analysis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

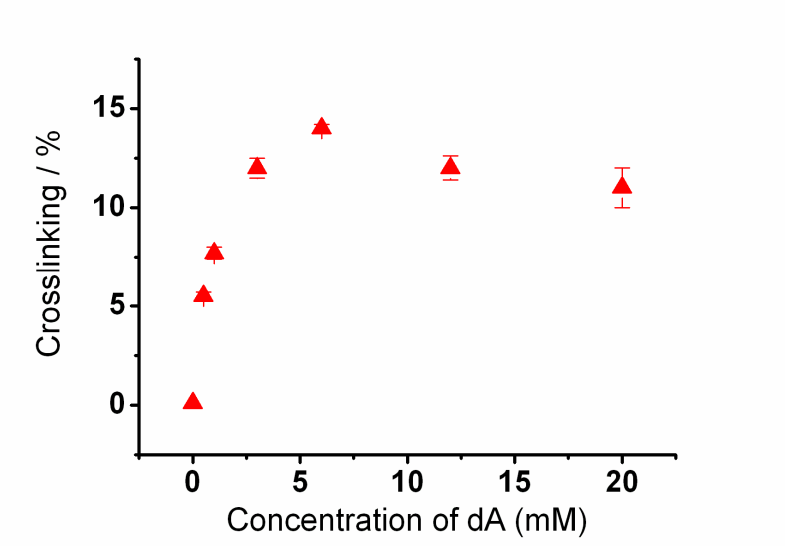


Figure 2.10. Concentration dependence of dA's ability to preserve the quinone methide's reactivity. Data represent the average of two independent experimental values and their range is indicated by the error bars.

An adenine derivative was selected as QM carrier in these experiments because dA N1 position is both accessible and abundant in vivo in the form of ATP, NAD(P)H, mRNA etc. Concentrations of ATP in human cells typically range from 1 mM and 5 mM but can rise as high as 9 mM.[53] ATP levels also tend to be greater in tumor than normal cells and thus it may selectively prolong the biological lifetime of electrophiles acting reversibly in these cells.[53]

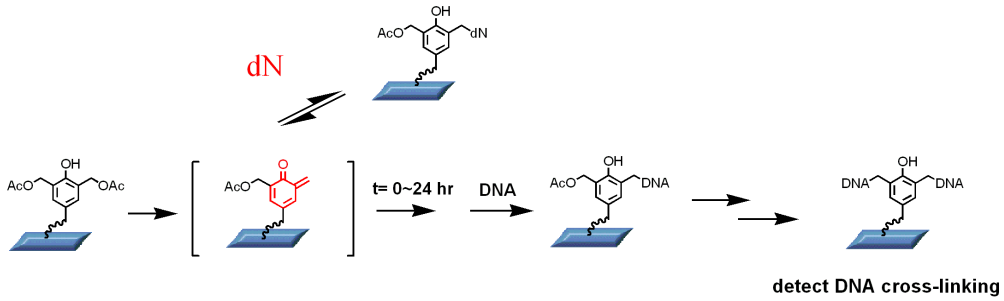
2.2.5. dC, dG and dT have no ability to extend bisQMP's lifetime for DNA crosslinking.

Efficient capture and release of QM is the major requirement for strong nucleophiles to extend QM's lifetime under aqueous conditions. dC N3 and dG N7, similar to dA N1, are also strong nucleophiles of DNA and react with QM reversibly under kinetic control.[43, 52] dT does not react with QM. Parallel experiments to

compare dG, dC, dA and thymine's abilities to preserve bisQM were conducted under the same conditions described in the dA experiments above (Figure 2.11A).

Interestingly, the presence of dC and dG did not show any effect on bisQM's persistence under aqueous conditions (Figure 2.11 B). The half-life for the quenching of QM in the presence of dG and dC is about half an hour and crosslinking is no longer detected after a pre-incubation of 2 hours (Figure 2.11 B), which is almost identical to the situation when no nucleotides were present during pre-incubation (Figure 2.4). dT did not have any impact on bisQM's lifetime as expected because it does not react with *ortho*-QM. The presence of dG and dC did not lower the maximum cross-linking yield either, suggesting that dC and dG were not able to compete with DNA to react with bisQM.

(A)



(B)

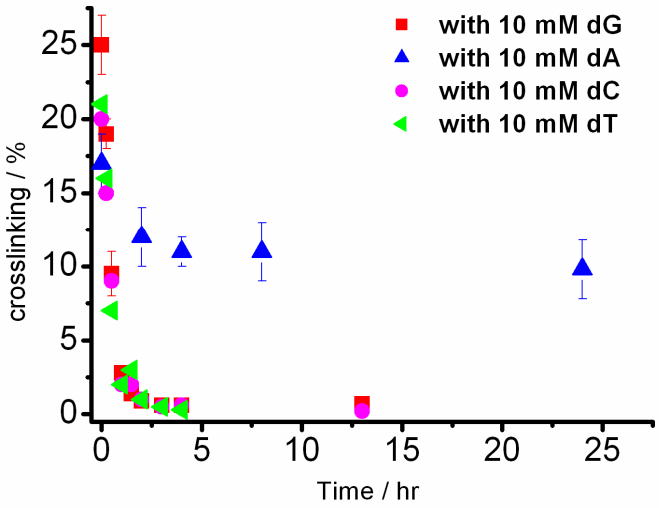


Figure 2.11. dN's (N=G, C, A,T) effect on extending the effective lifetime of bisQM for cross-linking DNA under aqueous conditions. (A) Schematic assay to test dN's effect on bisQM's lifetime. (B) Reaction of the bisQMP-acridine conjugate (100 μM) was initiated by addition of KF (100 mM) in 10 mM MES pH 7 and 20% acetonitrile under ambient conditions in the presence of 10 mM dN (N=G, C, A) and thymine. The persistent ability of these samples to cross-link DNA was measured by addition of **OD1**/5'-[^{32}P]-**OD2** (3.0 μM, 30 nCi annealed with 10% excess **OD1**) after the indicated time. Samples were then further incubated for 48 hr under ambient conditions. Each solution was then frozen, lyophilized, and analyzed by denaturing 20% polyacrylamide gel electrophoresis. Cross-linked products were quantified by phosphoimage analysis and reported (%) relative to total DNA. Data represent the average of two independent experimental values and their range is indicated by the error bars.

Previous studies on kinetic profiles of dN's reactions with an unsubstituted ortho-QM might explain dC and dG's failure to preserve bisQM (Figure 1.3).

Kinetic competition studies showed that dA N1 and dC N3 reacted with QM predominantly and reached their maximum in less than 30 min and 10 hr, respectively.[43] Then dA N1 and dC N3's QM adducts decomposed with a half-life about 10 hr and 30 hr, respectively. Therefore, dC N3 is not as good a nucleophile as dA N1 both in capturing and releasing QM efficiently. dG N7 could only generate low amount of QM adduct when competing with other nucleophiles, which suggested its weak ability to capture QM. The regeneration of QM from its dG N7 adduct was significantly slower than dA N1-QM adduct (half-life about 3 hr) and also suffered from the deglycosylation, which yielded QM-guanine N7 adduct.[43] Thus, the inabilities of dG and dC to capture and release QM intermediate as efficiently as dA likely explained their failure to extend bisQM's effective lifetime to crosslink DNA.

2.3. Conclusions.

The ability of nucleotides to prolong the lifetime of bisQMP- acridine conjugate has been examined. The lifetime of bisQMP in aqueous to crosslink DNA is short due to the irreversible trapping from water. dA, as a strong nucleophile of DNA, can capture and release QM intermediate efficiently and repeatedly and therefore help bisQM equivalents escape water's trapping during pre-incubation. By reversible reaction, dA is able to extend bisQMP's effective lifetime for crosslinking DNA by more than 100 fold. dA's ability to preserve bisQM has also been found to be concentration dependant and this effect saturates at 6 mM. Trapping the QM irreversibly with an alternative nucleophile such as thiol has the opposite effect of dA

and suppresses crosslinking.

dG, dC do not have the ability to prolong bisQM's lifetime to cross-link DNA. dT has not impact on bisQM's lifetime. This is likely due to their lack of ability to capture and release QM efficiently.

The reversibility of QM reactivity significantly expands the potential biological activity of this intermediate based on its repeated capture and release to forestall irreversible trapping. This would increase the complexity and highlight the need to understand the biological effects and toxicology of compounds which can form reversible adducts with cellular components.

2.4. Materials and Methods.

Materials. Solvents, starting materials, and reagents of the highest commercial grade were used without further purification. All denaturated solvents for NMR spectroscopy were purchased from Cambridge Isotope Laboratories. All aqueous solutions were prepared with distilled, deioinized water with a resistivity of 17.0 MΩ. Silica gel (230- 400 mesh) for column chromatography was purchased from EM Sciences. ^{1}H and ^{13}C spectra were recorded on a DRX 400 spectrometer (^{1}H, 400.13 Mhz; ^{13}C, 100.62 MHz). All NMR chemical shifts (δ) are reported in parts per million (ppm) and were determined relative to the standard values for solvent. Coupling constants (J) are reported in Hertz (Hz). High resolution mass spectra were determined with a JEOL SX102 mass spectrometer. Oligonucleotides were purchased and used from IDT without further purification. Oligonucleotides were radiolabeled at their 5' terminus with 5'-[γ-^{32}P]-ATP (PerkinElmer, Inc.) as directed

with T4 kinase purchased from New England BioLabs Inc.

General Methods. Analytical HPLC were performed on both a Jasco PU-908/MD1510 diode array instrument and a Jasco PU-2080 PLUS/UV-2077 PLUC fixed wavelength instrument. Analytical samples used a reverse phase C-18 analytical column (Varian, Microsorb-MV 300, 5 μm particle size, 250 mm, 4.6 mm) with a flow rate of 1 mL/min.

UV-vis spectra were measured on an HP 8543 series sprectrophotometer. Adduct formation was quantified using HPLC. Areas of the bisQM-water adduct was compared at λ_{260} relative to an internal standard (phenol) at λ_{260}. DNA reactions were analyzed using 20% polyacrylamide (19: 1 acrylamide: bisacryl-amide) gel electrophoresis under denaturing conditions (7 M urea). Gels were analyzed by phosphor-imaging with a Molecular Dynamics phosphor screen and phosphorimager.

Synthetic procedures

3-[4'-Hydroxy-3',5'-bis(hydroxymethyl)phenyl]propionic Acid (2). This procedure was modified from the original preparation in the literature.[49] Cold aqueous 5 M KOH was added to 3-(4'-hydroxyphenyl)propionic acid (2.0 g, 12 mmol) to adjust pH to 11, and the resulting solution was combined with formaldehyde (37%, 6 mL). The reaction was stirred at 65° C for 2 days, cooled (5° C), and combined with acetone (100 mL). The resulting orange oil was collected, mixed with methanol (15 mL). This solution was poured into acetone (150 mL) to form a white

precipitate. The solid was collected and washed with acetone to yield **2** as its sodium salt (1.98 g, 63%). ^{1}H NMR (CD_3OD) δ 2.39 (t, J=7.8, 2H), 2.80 (t, J= 7.8, 2H), 4.66 (s, 4H), 7.00 (s, 2H). ^{1}H NMR is consistent with literature report.[49]

3-[4'-*tert*-Butyldimethylsilyloxy-3',5'-bis(*tert*-butyldimethylsilyloxymethyl)] propionic Acid (3) .[49] Imidazole (3.30 g, 48.5 mmol) was added to a solution of *tert*-butyldimethylsilyl chloride (TBDMSCl, 3.30 g, 21.9 mmol) and the sodium salt of **2** (1.00 g, 4.03 mmol) in 15 mL of DMF. The reaction mixture was stirred at room temperature for 2 days, diluted with brine (100 mL), and extracted with ether. The organic phases were combined, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was re-dissolved in methanol (50 mL) and potassium carbonate (2.00 g) was added. The solution was stirred for 3 h and neutralized with 2 M HCl. The mixture was then diluted with water and extracted with ether. The organic phases were combined, washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography (hexanes: ethyl acetate= 5:1) to yield **3** as a colorless solid. ^{1}H NMR ($CDCl_3$) δ 0.08 (s, 12H), 0.13 (s, 6H), 0.91 (s, 18H), 0.99 (s, 9H), 2.64 (t, J = 8.0, 2H), 2.91 (t, J = 8.0, 2H), 4.66 (s, 4H), 7.17 (s, 2H). ^{1}H NMR is consistent with literature report.[49]

3-[4'-*tert*-Butyldimethylsilyloxy-3', 5'-bis(acetoxymethyl) phenyl]- propionic Acid (4).[49] Solid ferric chloride (10 mg, 0.62 mmol) was added to a solution of **3** (0.20 g, 0.80 mmol) in acetic anhydride (10 mL) at 0 °C under nitrogen. The

reaction mixture was stirred for 30 min and then diluted with ether. The combined organic phases were washed with water and saturated $NaHCO_3$, dried with $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to silica gel flash chromatography (hexane: ethyl acetate, 3:1) and yielded **4** (60 mg, 73%) as a colorless liquid. ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H), 1.00 (s, 9H), 2.07 (s, 6H), 2.74 (m, 2H), 2.91 (m, 2H), 5.06 (s, 4H), 7.13 (s, 2H). ^{1}H NMR is consistent with literature report.[49]

***N*-Succinimidyl-3-(4'-*tert*-butyldimethylsiloxy-3',5'-bis(acetoxymethyl)phenyl) propionate (5).[49]** *N*-Hydroxysuccinimide (0.010 g, 0.080 mmol) was added to a DMF solution (1.0 mL) of **4** (0.017 g, 0.050 mmol). This mixture was cooled to 0 °C and combined with 1-ethyl- 3-(3-dimethylaminopropyl) carbodiimide (EDC, 0.012 g, 0.06 mmol). The mixture was then stirred for 20 h at 4 °C, diluted with brine, and extracted with ether. The organic phase was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The resulting residue was subjected to silica gel flash chromatography (hexane: ethyl acetate, 3:1) to yield **5** (0.02 g, 65%) as a viscous colorless liquid. ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H), 1.00 (s, 9H), 2.08 (s, 6H), 2.80 (s, 4H), 2.91(m, 2H), 2.95 (m, 2H), 5.05 (s, 4H), 7.14 (s, 2H). ^{1}H NMR is consistent with literature report .[49]

***N*-(*N*'-Acridinyl-2'-aminoethyl)-3-(4''-*tert*-butyldimethylsilyoxy-3'',5''-bis(aceto xy-methyl)phenyl)propionamide (6).[49]** Triethylamine (40 mg) was added to acridine-linker **7** (30 mg) in methanol (10 mL). Once this was homogeneous, **5** (30

mg) in acetonitrile (0.5 mL) was added dropwisely over 10 min. Reaction was maintained for 35 min at room temperature and then acetic acid was added. Solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2. This solution was washed with water and brine, dried, and concentrated under reduced pressure. The solid residue was recrystallized using methylene chloride and diethyl ether to yield **6a** (0.05 g, 75%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H), 1.01 (s, 9H), 2.08 (s, 6H), 2.78 (t, *J* =7.4, 2H), 3.04 (t, *J* = 7.4, 2H), 3.91 (m, 2H), 4.32 (m, 2H), 5.05 (s, 4H), 7.12 (bs, 1H), 7.20 (s, 2H), 7.46 (t, *J*= 7.8, 2H), 8.11 (d, *J* =8.5, 2H), 8.20 (d, *J* = 8.5, 2H), 8.48 (t, *J*= 7.8, 2H), 9.28 (bs, 1H). ^{1}H NMR is consistent with literature report.[49]

***N*-Benzyl-3-(4'-*tert*-butyldimethylsiloxy-3',5'-bis(acetoxymethyl)phenyl) propionamide (8).** Triethylamine (40 mg) was added to benzylamine (30 mg) in methanol (10 mL). Once this was homogeneous, **5** (30 mg) in acetonitrile (5 mL) was added dropwisely over 10 min. Reaction was maintained for 35 min at room temperature and then acetic acid was added. Solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2. This solution was washed with water and brine, dried, and concentrated under reduced pressure. The solid residue was recrystallized using methylene chloride and diethyl ether to yield **8** (34 mg, 65%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H), 1.01 (s, 9H), 2.08 (s, 6H), 2.78 (t, *J* =7.4, 2H), 3.04 (t, *J* = 7.4, 2H), 3.91 (m, 2H), 4.32 (m, 2H), 5.05 (s, 4H), 7.12 (bs, 1H), 7.20 (d, 2H), 7.46 (t, *J*= 7.8, 2H).

3-(4'-*tert*-Butyldimethylsiloxy-3',5'-bis(acetoxymethyl)phenyl) propionamide (9). Ammonium hydroxide (1ml) was added dropwisely into **5** (30 mg) in acetonitrile (5 mL) over 10 min. Reaction was maintained for 2 hr at room temperature and then acetic acid was added. Solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2. This solution was washed with water and brine, dried, and concentrated under reduced pressure. The solid residue was recrystallized using methylene chloride and diethyl ether to yield **9** (21 mg, 63%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H), 1.01 (s, 9H), 2.08 (s, 6H), 2.78 (t, J =7.4, 2H), 3.04 (t, J = 7.4, 2H), 3.91 (m, 2H), 4.32 (m, 2H), 5.05 (s, 4H). ^{1}H NMR is consistent with literature report. [1]

2,6-Bis((2-hydroxyethylthio)methyl)-4-methylphenol (16). *p*-cresol (2.0 g, 15 mmol) was added into a toluene solution (20 ml) of formaldehyde (37%, 12 ml) and β-mercaptoethanol (2 ml). NaOH was added to adjust pH to 12. The mixture was then refluxed for 2 hr, cooled to room temperature. Solvent was removed under reduced pressure. The resulting residue was subjected to silica gel flash chromatography (hexane: ethyl acetate, 3:1) to yield **16** (1.5 g, 65%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 2.35 (s, 3H), 2.63 (t, J =7.4, 4H), 3.70 (t, J =7.4, 4H), 3.90 (t, J = 7.4, 4H), 6.50 (m, 2H).

DNA Reactions.

Formation of 5'-[^{32}P] radiolabeled and unlabeled oligonucleotide duplexes

OD1/OD2. Typically, 1.0 equivalent of the 5'-[^{32}P] radiolabeled oligonucleotide and

1.1 equivalent of its unlabeled complementary strand were annealed in a solution of 2-(*N*-morpholino)ethanesulfonic acid (MES) (10 mM, pH 7) by placing the mixture in a microcentrifuge tube. The tube was placed in 90℃ water bath and was allowed to cool to room temperature over 4 hr to afford a 10 μM solution of labeled duplex DNA.

Alkylation and crosslinking of duplex DNA by bisQMP-acridine conjugate. Typically, 4 μL of a duplex DNA solution of 5'-[^{32}P] radiolabeled **OD1/OD2** (4.5 μM, 15 mM MES, pH 7) was mixed with an acetonitrile solution of bisQMP conjugate (1.2 μL) and aqueous KF solution (0.8 μL). The resulting reaction solutions (6.0 μL, 10 mM MES, pH 7) of DNA duplex (3 μM) reactants (specified in each figure) and KF (10 mM) in 20% acetonitrile aqueous solution were incubated at room temperature for indicated time (see figures). Reactions were quenched by addition of 2-mercaptoethanol (2 μL), frozen and lyophilized. The residue was dissolved in 10 μL of formamide loading solution (0.05 % bromophenol blue and 0.05 % xylene cyanol FF in formamide) and analyzed by denaturing gel electrophoresis (20% polyacrylamide). Radiolabeled DNA was detected using a Molecular Dynamics PhosphorImager and quantified with ImageQuant software to determine DNA crosslinking.

BisQMP's reaction with dN (N= G, T, A, C) and dsDNA in studies of bisQM's longevity under aqueous conditions. Typically, 100 μM bisQM was incubated with 20 mM dN in 10 mM MES buffer, pH 7, 20% acetonitrile aqueous solution. 34 mM

KF was used to initiate the reaction. Samples were then incubated for indicated time (see figures) and 3.0 µM dsDNA was added. Samples were further incubated for 48 hr (see figures). Reactions were quenched by addition of 2-mercaptoethanol (0.3 µL/µL reaction), frozen and lyophilized. The residue was dissolved in 10 µL of formamide loading solution (0.05 % bromophenol blue and 0.05 % xylene cyanol FF in formamide) and analyzed by denaturing gel electrophoresis (20% polyacrylamide). Gel was detected using a Molecular Dynamics PhosphorImager and quantified with ImageQuant software to determine DNA crosslinking.

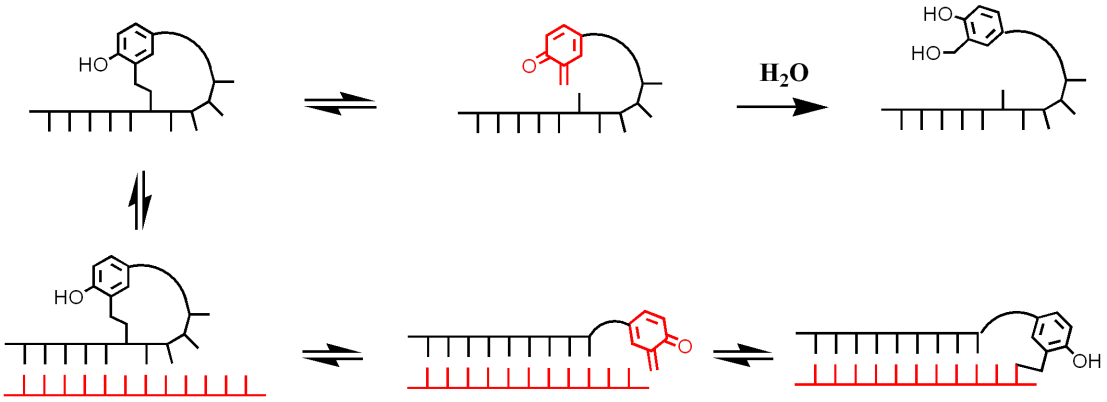
Chapter 3. Oligonucleotides can trap and transfer bisQM selectively to their complementary sequences by producing interstrand cross-links

3.1 Introduction

A major problem associated with DNA alkylating agents as therapeutic agents is their lack of specificity. Most small alkylating agents have very limited recognition of target sites. Conjugating recognition and reactive elements together is one strategy to achieve target or sequence specific modification of nucleic acids. Multiple recognition elements have been developed to target nucleic acids for this purpose, including intercalators, minor groove binding polyamides,[54-56] duplex forming antisense oligonucleotides,[57] major groove binding antigene oligonucleotides[58] and DNA mimic[59].

Target-promoted DNA alkylation was designed based on reversible reaction between DNA and QM in our laboratory (Scheme 3.1). An intramolecular adduct was formed by an oligonucleotide-quinone methide conjugate.[45] This self-adduct adduct was able to preserve reactive QM equivalent beyond 8 days under aqueous conditions and alkylate its complementary sequence selectively by DNA hybridization. Non-complementary sequence can not be alkylated by the self-adduct. The studies on QM's reversible reactions with free nucleotides supported the assumption that QM underwent repeated capture and release from its oligonucleotide adduct.[43] The intramolecular alkylation in the self-adduct was so dominant that external nucleophiles, such as water and thiols, were not able to compete efficiently. The

thermodynamic driving force of complementary base pairing is thought to be required to accumulate interstrand crosslinking.[45]

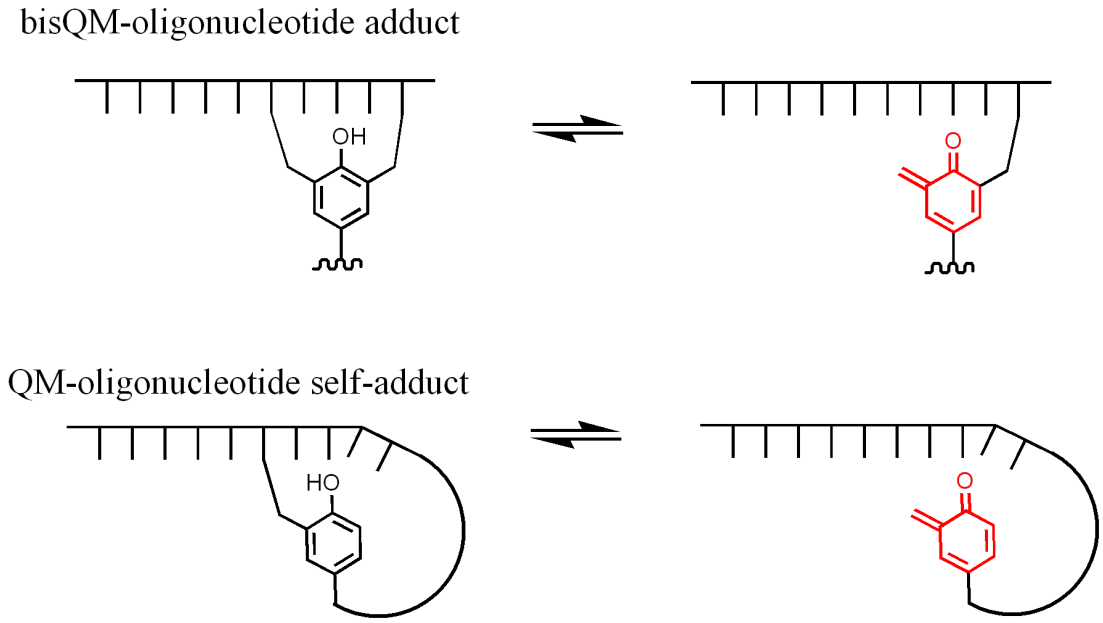


Scheme 3.1. Quinone methide-oligonucleotide conjugate self-adduct and its target promoted DNA alkylation.[45]

In addition to the high selectivity, another advantage of using a QM-oligonucleotide self-adduct to alkylate DNA is that no external signal or activation is necessary. Typical methods to activate reactive elements of other oligonucleotide conjugates include irradiation with ultraviolet light,[56, 60, 61] addition of an oxidant to activate[62, 63] and addition of a reductant. [38, 64] These techniques offered the possibility of great controls but also create a barrier for biological applications, where the addition of external chemicals or light could be toxic or technically impossible to be accomplished in living systems. In contrast, the function of QM-oligonucleotide self-adduct can alkylate target sequence without external signals.[45]

A bi-functional quinone methide precursor (bisQMP) acridine conjugate has shown strong ability to crosslink duplex DNA[49] and to react with deoxynucleotides reversibly (Chapter 2). BisQMP's alkylation of single-stranded DNA was even more efficient than its alkylation of double stranded DNA since nucleophiles of DNA

were more accessible in single-stranded DNA.[49] A number of nucleophilic sites, such as dA N1 and dC N3, are hidden inside duplex structure and their nucleophilicity is weakened by hydrogen bonding.



Scheme 3.2. Structural similarity between a bisQM-oligonucleotide adduct and QM-oligonucleotide self-adduct.

BisQM-oligonucleotide adducts were structurally similar with, yet not as well defined as, QM-oligonucleotide self-adduct (Scheme 3.2). In both cases, QM molecules were linked to DNA by two covalent bonds. The intermediates of bisQM-oligonucleotide adduct and QM-oligonucleotide self-adduct also shares great similarity. Although both bisQM-DNA bonds have the potential to dissociate, only one can dissociate and regenerate QM intermediate at a time (Scheme 3.2). Both bisQM-DNA dissociating at the same time is chemically impossible. In a QM-oligonucleotide self-adduct , QM is conjugated to oligonucleotide by an amide linkage to hold the QM intramolecular.[45] Thus, both intermediates of bisQM-oligonucleotide adduct and QM-oligonucleotide self-adduct had a QM

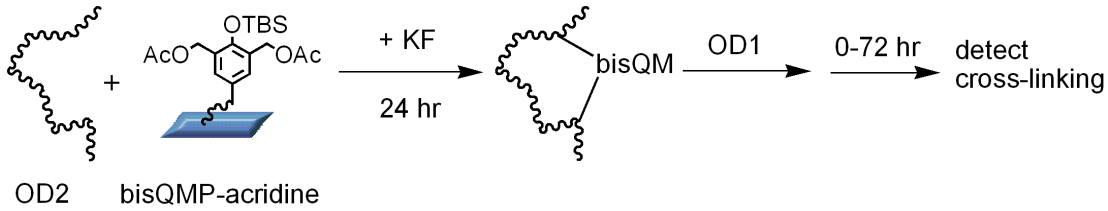
intermediate linked to DNA strand by a covalent bond. Based on the structural similarity, bisQM-oligonucleotide adducts might also be able to transfer QM to its complementary sequences and cause alkylation or cross-linking selectively.

3.2. Results and discussion

3.2.1. Oligonucleotide-bisQM adducts can alkylate complementary sequence by causing crosslinking

In order to examine the ability of oligonucleotide-bisQM adducts to alkylate complementary sequence, single-stranded DNA 5'-[^{32}P]-**OD2** was pre-incubated with 20 fold excess of bisQMP- acridine conjugate in the presence of KF for 24 hr to allow the formation of OD2-bisQM intrastrand adducts. Then a complementary sequence **OD1**was added and the mixture was further incubated for up to 72 hr (Figure 3.1). An accumulation of DNA cross-linking was detected by denaturing gel and the maximum yield (15%) was achieved after 3 days' incubation.

(A)



(B)

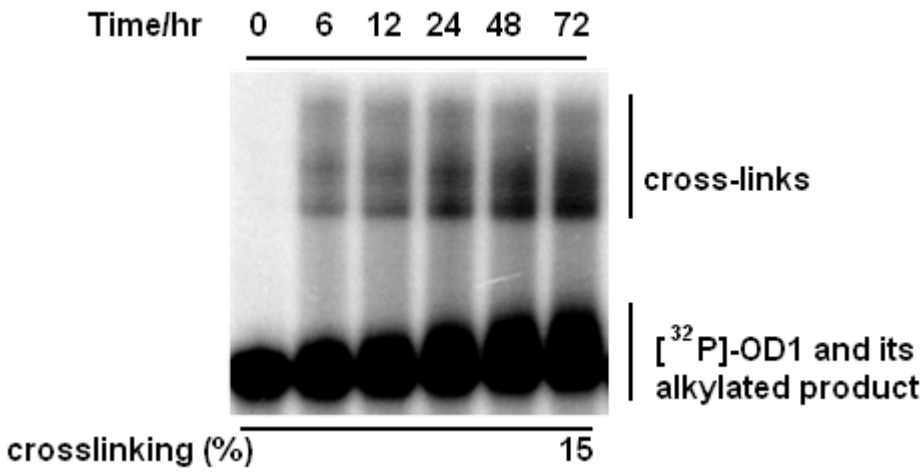
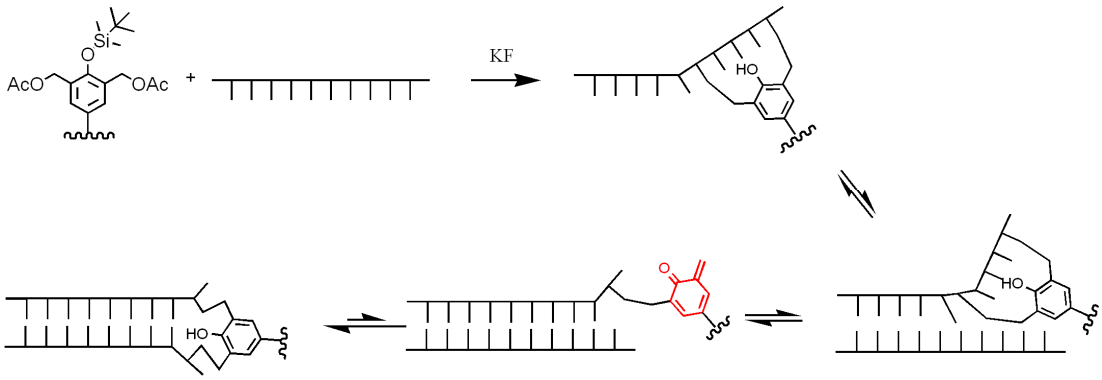


Figure 3.1. ssDNA can capture and transfer QM to its complementary sequence by forming crosslinking.[51] The bisQMP-acridine conjugate (30 μM) and **OD2** (3.0 μM) were combined with 10 mM MES pH 7 20% acetonitrile. Reaction was initiated by addition of KF (10 mM) and the mixture was incubated under ambient conditions for 24 hr. 5'-[^{32}P]-**OD1** (3.0 μM, 30 nCi) was then added and reaction progress was analyzed after the indicated times. Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

The formation of **OD1**/**OD2** cross-linking indicated that adducts formed between the bisQMP-acridine conjugate and single-stranded DNA **OD2** did not prevent strand hybridization to form duplex DNA nor block interstrand transfer of the bisQM to form DNA cross-linking. The proposed mechanism includes initial reaction of QM and **OD2** by forming intrastrand adducts during pre-incubation. Then alkylated **OD2** was able to hybridize with **OD1** by forming duplex structure. When bisQM

intermediate was regenerated from its bisQM-**OD2** adducts, it could alkylate **OD1** by forming crosslinking. The overall consequence was that one QM equivalent was transferred from **OD2** to its complementary strand **OD1** by producing DNA cross-links (Scheme 3.3). Multiple alkylation sites in oligonucleotide **OD2** are possible and Scheme 3.3 only represents one of the possibilities.

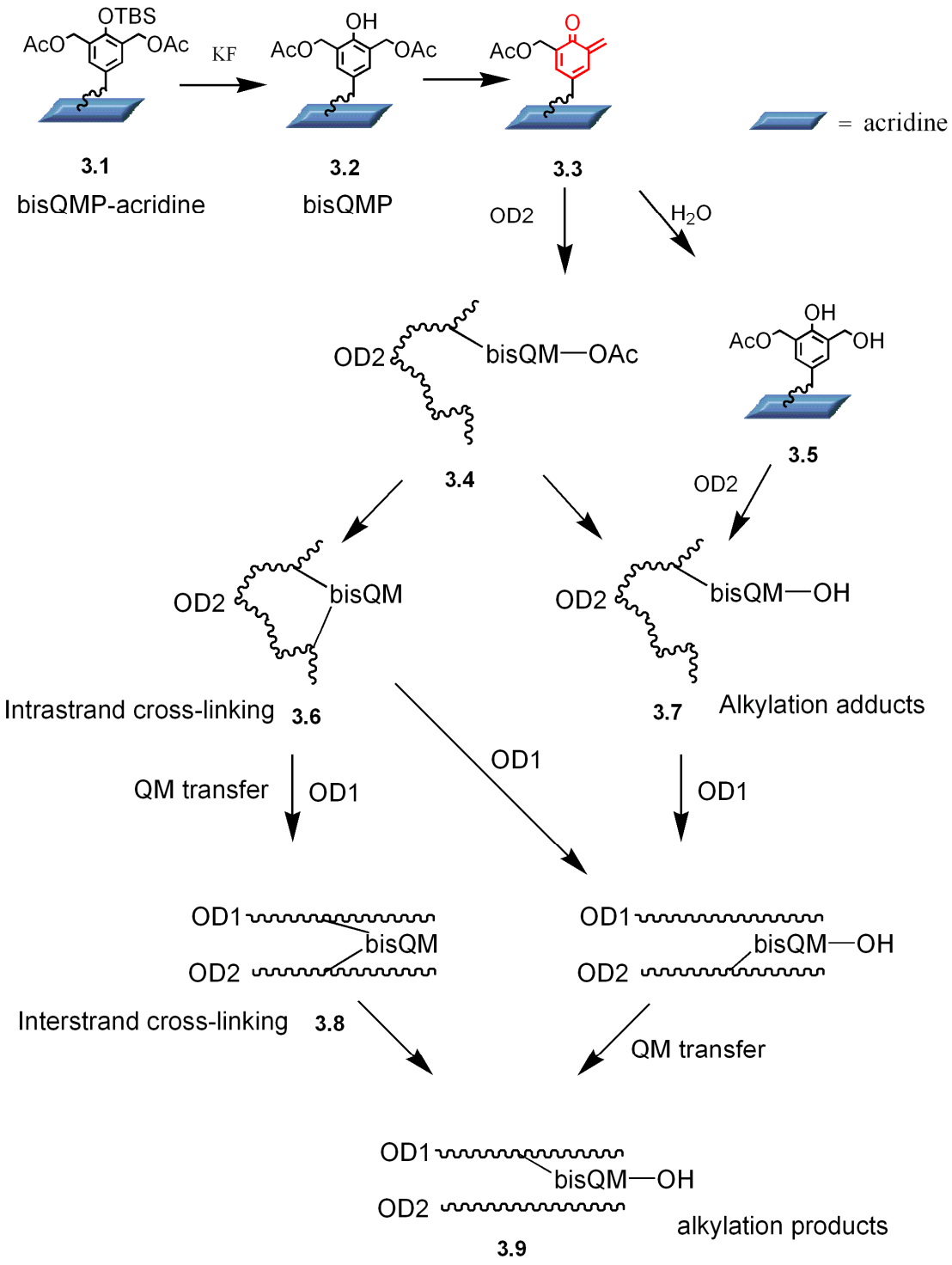


Scheme 3.3. Proposed mechanism of bisQM's transfer.

The maximum crosslinking was achieved in 3 days upon the addition of **OD1** into bisQM-**OD2** adducts (Figure 3.1). In comparison, the QM-oligonucleotide self-adduct (Scheme 3.2) required 8 days to attain the maximum alkylation in the presence of complementary strands of similar concentration.[45] The concentration of OD1 was 3.0 μM in Figure 3.1 and the target DNA in QM-oligonucleotide self-adduct experiment was 2.5 μM.[45] According to the proposed mechanism, the rate of QM's transfer from intrastrand adducts to interstrand crosslinking was decided by the rate of QM's regeneration (Scheme 3.3). The hybridization of DNA requires a few minutes and lifetime of regenerated QM intermediate is only milliseconds.[30, 35, 65, 66] The regeneration of QM from its DNA adduct, however, can take hours depending the specific alkylated nucleotide.[43] BisQM contains an extra methyl group comparing

with QM in oligonucleotide-QM conjugate and therefore is more electron-rich (Scheme 3.2). Electron-donating group can promote the regeneration of QM by stabilize the electrophilic intermediate.[46] Therefore, the electron-rich bisQM must be more easily regenerated from its DNA adduct and the achievement of maximum crosslinking was accelerated significantly compared to oligonucleotide-QM self-adduct.

BisQM can cause both alkylation and crosslinking of DNA (Scheme 3.4). When **OD1** was radiolabeled, a minor shift of single-stranded DNA to higher molecular weight level was observed on denaturing gel (Figure 3.1). This was identified as bisQM alkylation of **OD1** but not DNA cross-linking. The formation of DNA alkylation is expected because bi-functional alkylating agents usually generate multiple types of alkylation products.



Scheme 3.4. DNA alkylation and cross-linking caused by bisQMP.

BisQMP acridine conjugate (**3.1**) is first converted into its bisQMP intermediate (**3.2**) after deprotection (Scheme 3.4). This allows for spontaneous loss of acetate to form a QM intermediate (**3.3**). The QM intermediate should then alkylate **OD2** by

forming DNA adducts (**3.4**) or react with water to form (**3.5**). Formation of water adduct (**3.5**) will diminish bisQM's ability to cause DNA crosslinking. When the second acetate in (**3.4**) is lost and another bisQM intermediate is generated, it can react with either water or nucleophile from DNA to form **OD2**-bisQM intrastrand crosslinking (**3.6**). The addition of **OD1** leads to the formation of **OD1**/**OD2** duplex DNA. When QM is reformed spontaneously from **OD2**-bisQM intrastrand cross-links (**3.6**), **OD1**/**OD2** cross-links (**3.8**) could be formed. Alkylation adduct (**3.7**) could also transfer bisQM-OH to **OD1** by causing alkylation (**3.9**), which was observed as a minor shift of radiolabeled **OD1**. **OD1** alkylation products (**3.9**) can also be derived from **OD1**/**OD2** cross-links (**3.8**) during incubation. QM can be trapped by water after its regeneration from DNA cross-links (**3.8**). This too would contribute to the alkylation but not crosslinking of **OD1**. DNA crosslinking could also be converted into alkylation products under denaturing conditions during gel electrophoresis for the same reason. Hydrogen bonding between DNA strands will be disassociated under denaturing conditions and DNA strands will be separated. For DNA cross-links caused by bisQM, two strands are covalently linked until one of the QM-DNA bonds is broken by spontaneous QM regeneration. Then two DNA strands will be separated immediately after the loss of covalent linkage and QM will react with water by forming DNA alkylation product. This process during gel electrophoresis can also contribute to the formation of **OD1** alkylation (**3.9**). Partial loss of DNA cross-links is a source of data error and one limitation of using denaturing gel electrophoresis to analyze and quantify DNA crosslinking caused by

bisQM. The DNA crosslinking yield gained from denaturing gel should be lower than the real value and can only represent a minimum yield that can be detected.

The alkylation products of the reaction between **OD2** and bisQMP were likely heterogeneous because of multiple nucleophilic sites in DNA and multiple alkylation per strand. Structural characterization of oligonucleotide-bisQM adducts is important for understanding the efficiency of oligonucleotides to trap and transfer bisQM to target DNA. The site specificity of alkylation by bisQM depended on the nucleophilicity of nucleobases and also their accessibility. Strong nucleophiles of DNA would react with bisQM under kinetic control. But it was almost impossible to predict the accessibility of nucleobases in single-stranded DNA molecule under aqueous conditions. The acridine attachment can intercalate into the major groove of duplex DNA, however, its association with the compact structure of single-stranded DNA is non-specific.[67, 68]

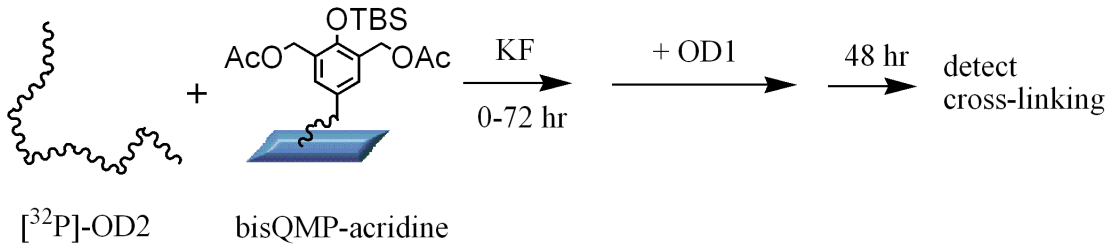
3.2.2. Oligonucleotides can preserve bisQM's ability to cross-link DNA for days

The lifetime of bisQMP under aqueous conditions is short due to the water trapping of the transient QM intermediate (Chapter 2). However, DNA cross-linking was generated from bisQM-**OD2** adducts even after a pre-incubation period of 24 hr before the addition of a complementary sequence **OD1** (Figure 3.1). This suggests that bisQM equivalents were preserved by oligonucleotide **OD2** in the form of bisQM-DNA adducts to survive pre-incubation and alkylate complementary

sequences.

In the presence of 5'-[^{32}P]-**OD2**, the ability of bisQMP to cross-link DNA was preserved for more than 72 hr (Figure 3.2). The bisQM-**OD2** intrastrand adducts likely remained in equilibrium with its high energy QM intermediate because the most reactive nucleophiles of DNA add reversibly to the QM.[43] Similar to dA's effect on prolonging bisQM's effective lifetime for crosslinking DNA (chapter 2), the persistence of cross-linking activity with **OD2** again suggested that QM intermediates were trapped and released from oligonucleotide multiple times during pre-incubation period because 72 hr is significantly longer than the half-life of labile bisQM-DNA adducts. For example, the half-life of the reversible dA N1 adduct is only approximately 2 hr under aqueous conditions.[42] Intramolecular addition to reform intrastrand adducts compete efficiently with irreversible trapping of water, and thus bisQM's ability to cause crosslinking could be preserved for days.

(A)



(B)

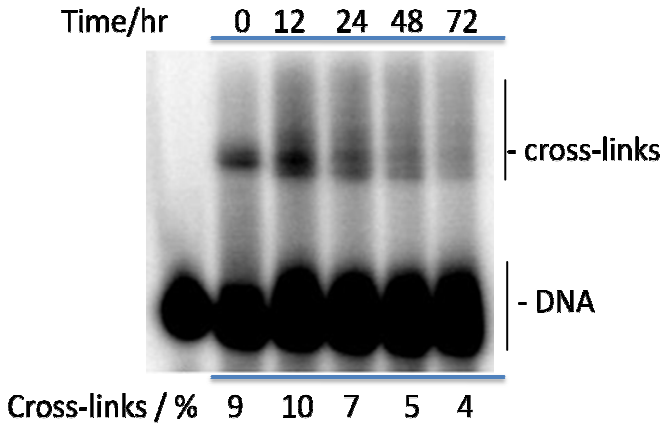


Figure 3.2. Single-stranded DNA **OD2** extends the lifetime of bisQMP and promotes its interstrand transfer for crosslinking DNA under aqueous conditions.[51] Reaction of the bisQMP–acridine conjugate (30 μM) was initiated by addition of KF (10 mM) in 10 mM MES (pH 7), 20% acetonitrile, and 5'-[^{32}P]-**OD2** (3.0 μM, 30 nCi) under ambient conditions. After the indicated time, the complementary strand **OD1** (3.3 μM) was added and the samples were further incubated for an additional 48 hours under ambient conditions. Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

BisQM equivalents were preserved more efficiently by **OD2** than by dA for crosslinking DNA even though less amount of bisQMP was available. While dA (20 mM) might have appeared to extend the longevity of cross-linking to a greater extent than **OD2**, this phenomenon is concentration dependent. A low concentration of dA, for example 0.5 mM, only had a weak effect and helped to maintain a DNA cross-linking yield of only 6 % after a pre-incubation for 8 hr (Chapter 2). However, this concentration was still ten times greater than the equivalents in the

oligonucleotide assay (Figure 3.3). An **OD2** concentration of 3 μM corresponded to a 45 μM solution of nucleophiles (dA N1, dC N3, dG N7) that can act reversibly. Additionally, 100 μM bisQMP–acridine conjugate was used in dA assay whereas only 30 μM bisQMP was added in oligonucleotide assay. Therefore, oligonucleotide **OD2** has a stronger ability to preserve bisQM for crosslinking of duplex DNA than dA. This is at least partially because **OD2** has a strong ability to regain regenerated bisQM than dA. After the regeneration of QM intermediate, one covalent anchor always exist in bisQM-**OD2** adducts and intramolecular molecular alkylation is dominant due to the high effective concentration (Scheme 3.3). In contrast, the reaction between dA and QM intermediate suffers from water's trapping seriously because water has a much higher concentration than dA under aqueous conditions.

Cross-linking observed by gel electrophoresis was confirmed by the equivalency of product mobility when either strand was radiolabeled (Figure 3.3). The dispersed bands of DNA crosslinking products suggested that DNA cross-links generated from bisQM transfer was heterogeneous (Figure 3.2, Figure 3.3). As previously discussed, bisQM-**OD2** adducts were likely heterogeneous due to multiple alkylation sites and multiple bisQM addition to DNA.[49] Therefore, the crosslinking caused by bisQM-**OD2** adducts would also likely be heterogeneous. Crosslinking at different positions of dsDNA by QM can result in different DNA conformation in denaturing gel and therefore affect the mobility of DNA cross-links (Scheme 3.5).[69-72] DNA cross-links that can adopt linear conformation are likely to go through the gel matrix faster than star-shaped DNA cross-links (Scheme 3.5). Varied equivalents of bisQM

addition on DNA strands can also change their mobility in denaturing gel by adding molecular weight. Therefore, the band of crosslinking appeared widely smeared (Figure 3.3).

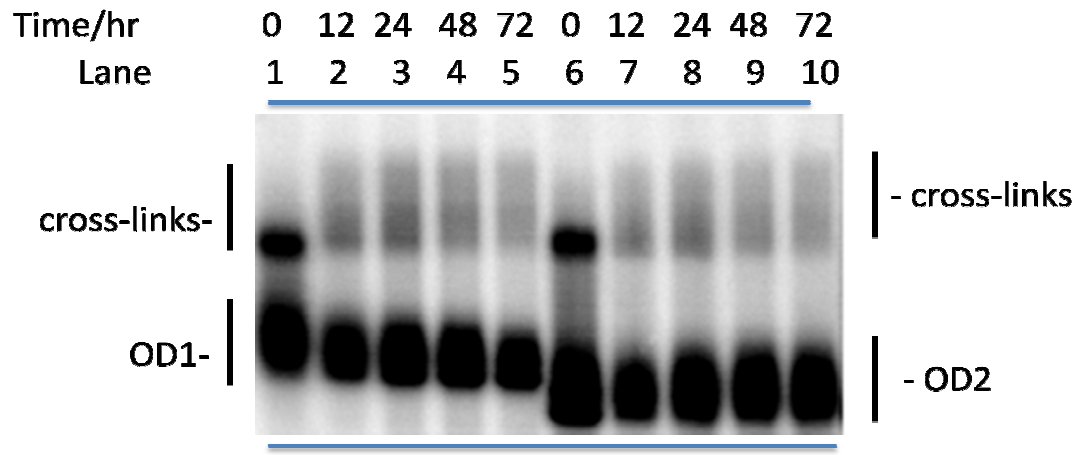
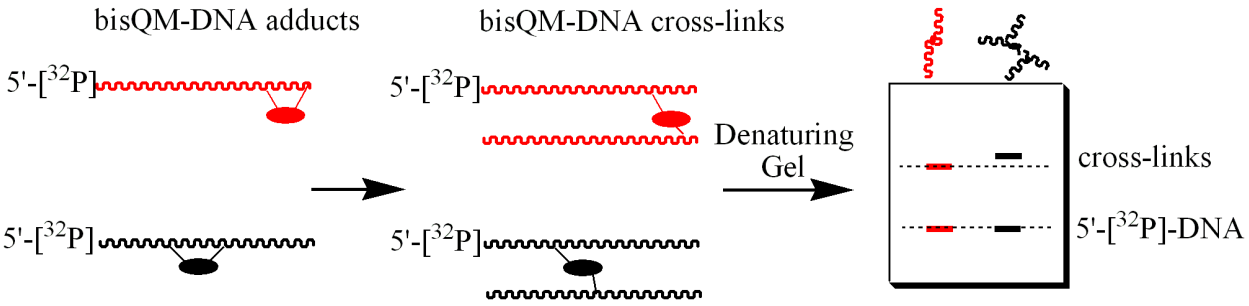


Figure 3.3. Cross-linking of DNA is confirmed by the equivalent gel mobility of products formed when either strand is radiolabeled.[51] The bisQMP-acridine conjugate (100 μM) and **OD2** were combined in 10 mM MES pH 7 20% acetonitrile. Reaction was initiated by addition of KF (10 mM), and the mixtures were incubated for the indicated time under ambient conditions. The complementary strand **OD1** was then added and incubation was continued for another 48 hr before the products were analyzed. For lanes 1-5, 5'-[^{32}P]-**OD1** (3.3 μM) was used with **OD2** (3.0 μM), and for lanes 6-10, **OD1** (3.0 μM) was used with 5'-[^{32}P]-**OD2** (3.3 μM). Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.



Scheme 3.5. Schematic demonstration of differing migration for crosslinking of duplex DNA under denaturing conditions used in gel electrophoresis. With the same DNA components, linear (black) conformation of DNA crosslinking migrates faster than star-shaped (red) conformation due to its less contact with gel matrix.

3.2.3. The bisQM transfer process is sequence selective.

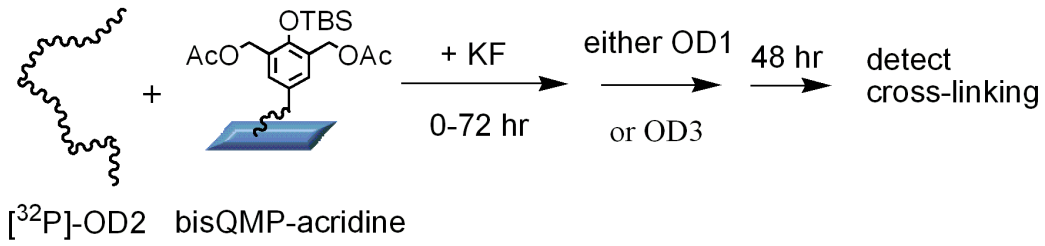
The efficiency of intrastrand trapping of the bisQMP-acridine conjugate by **OD2** may in part be driven by the nonspecific association between the attached acridine and the compact structure of single-stranded DNA.[67] However, subsequent transfer of QM from a species such as (3.6) in Scheme 3.4 to form interstrand cross-linking required specific association between complementary strands of DNA (Scheme 3.3).

QM adducts formed between **OD2** and bisQMP-acridine conjugate during pre-incubation period of 0-72 hr produced cross-linking upon addition of its complementary sequence **OD1** (Figure 3.4). However, no cross-linking beyond background levels was observed upon the addition of **OD3** 5'-d (GGTACACATAGAGATAGAGAGATACACACAC)-3', which had the same deoxynucleotides composition, but not sequence, as **OD1**. Non-complementary sequence **OD3**'s failure to generate cross-linking confirmed that the hybridization of DNA strands was a requirement for bisQM transfer from its DNA-adducts to complementary DNA.

DNA hybridization can contribute to the efficient transfer of bisQM. dA preserves bisQM equivalents by storing QM in the form of its dA adduct, which is able to release free bisQM intermediate back into solution. However, the newly released bisQM intermediate would need to diffuse to DNA for alkylation and could suffer from irreversible trapping of water prior to this. Acridine may help locate bisQM-dA adduct around duplex DNA, however, the bulky size of bisQM-dA adducts could potentially prevent the intercalation of acridine. Oligonucleotide bisQM-**OD2**

adducts can deliver bisQM selectively to complementary sequence **OD1**. Upon hybridization, bisQM is localized inside duplex DNA by covalent anchor and skips the diffusion step. Thus, bisQM can alkylate **OD1** efficiently due to the high effective concentration between bisQM and **OD1** resulting from duplex formation.

(A)



(B)

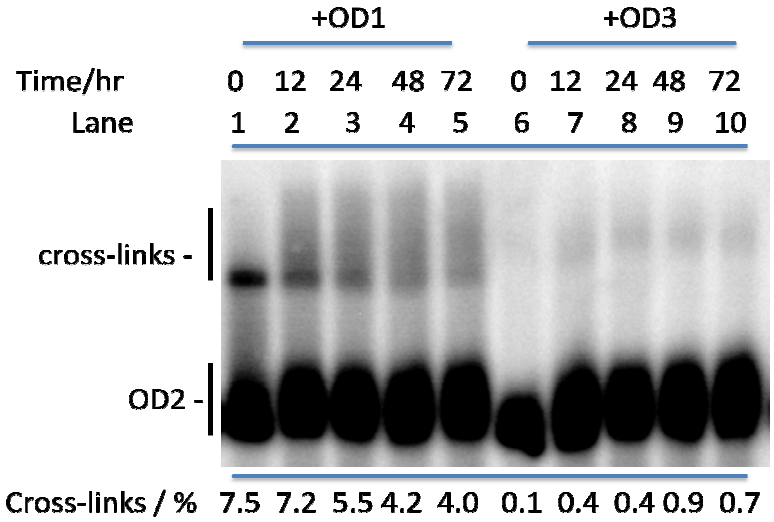
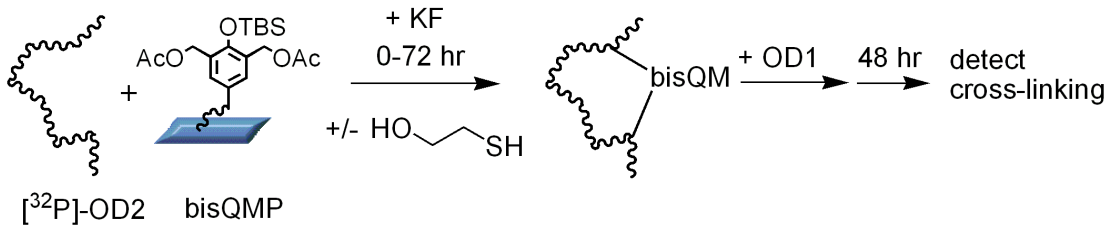


Figure 3.4. Single-stranded DNA extends the lifetime of a quinone methide and promotes its selective interstrand transfer for crosslinking DNA under aqueous conditions.[51] Reaction of the bisQMP–acridine conjugate (30 mM) was initiated by addition of KF (10 mM) in 10 mM MES (pH 7), 20% acetonitrile, and 5'-[^{32}P]-OD2 (3.0 mM, 30 nCi) under ambient conditions. After the indicated time, either the complementary strand OD1 (3.3 mM) (Figure 3.4B, lanes 1–5) or a noncomplementary strand OD3 (3.3 mM) (Figure 3.4B, lanes 6–10) was added, and the samples were further incubated for an additional 48 hr under ambient conditions. Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

3.2.4. BisQM's transfer from intrastrand adduct to interstrand crosslinking is weakly sensitive to the presence of strong nucleophiles.

Initial formation of adducts between the bisQMP and oligonucleotide **OD2** was found to be sensitive to the presence of other strong nucleophiles, such as β-mercaptoethanol. Thiols represent the strongest and abundant nucleophiles in mammalian cells.[73] Without addition of thiol, 30 μM bisQMP-acridine conjugate can cause crosslinking of duplex DNA (Figure 3.5B, lane 1). However, the presence of 5 mM β-mercaptoethanol during pre-incubation quenched the bisQMP's ability to cause crosslinking completely (Figure 3.5B, lanes 2-6). Oligonucleotide **OD2** was not able to preserve any bisQM equivalent for cross-linking under these conditions.

(A)



(B)

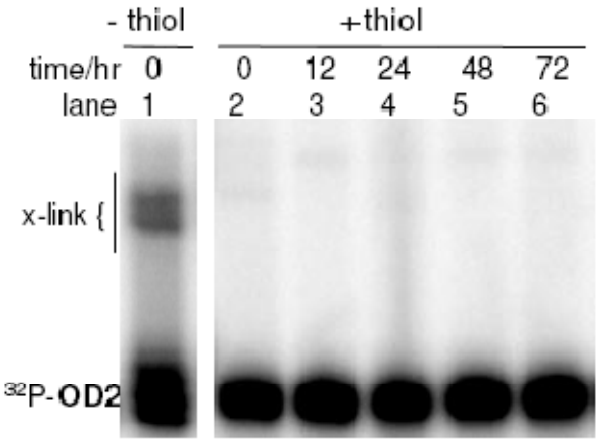
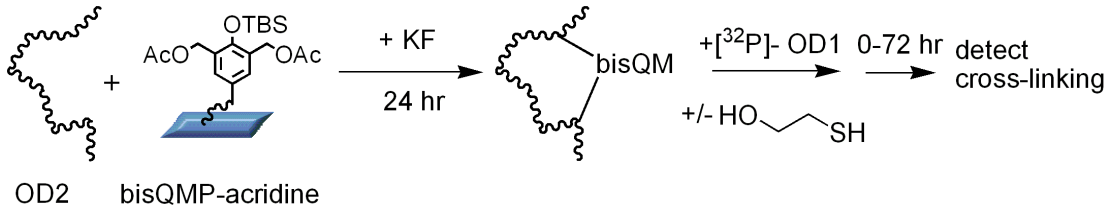


Figure 3.5. A thiol counteracts the ability of single-stranded DNA to prolong the activity of a quinone methide.[51] The bisQMP-acridine conjugate (30 μM) and **OD2** (3 μM) were combined with 10 mM MES pH 7 20% acetonitrile in the absence (lane 1) or presence of 2-mercaptoethanol (5 mM, lanes 2-6). Reaction was initiated by addition of KF (100 mM), and the mixtures were incubated under ambient conditions for the indicated time before 5'-[^{32}P]-**OD1** (3.0 μM, 30 nCi) was added. Incubation was then continued for another 48 hr prior to analysis. Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

(A)



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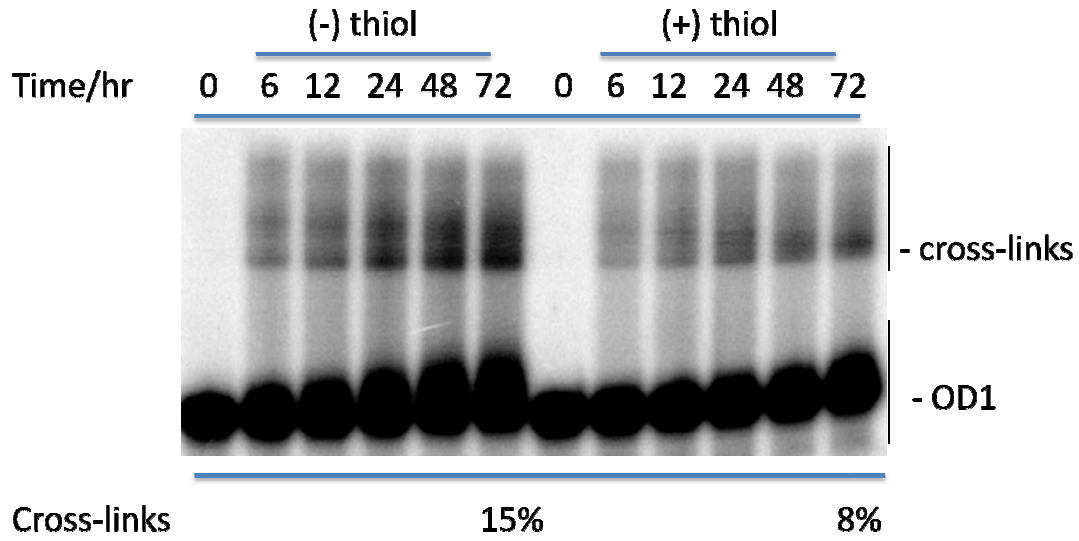
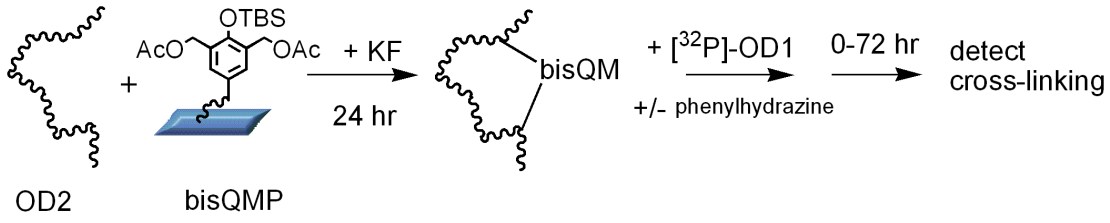


Figure 3.6. Interstrand transfer of quinone methide is only moderately sensitive to the presence of a thiol.[51] The bisQMP-acridine conjugate (30 μM) and **OD2** (3 μM) were combined with 10 mM MES pH 7 20% acetonitrile. Reaction was initiated by addition of KF (10 mM) and the mixture was incubated under ambient conditions for 24 hr. 5'-[^{32}P]-**OD1** (3.0 μM, 30 nCi) (lanes 1-6) or a mixture of 5'-[^{32}P]-**OD1** (3.0 μM, 30 nCi) and 3 mM β-mercaptoethanol (lanes 7-12) was then added and reaction progress was analyzed after the indicated times. Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

Interstrand transfer of bisQM to form DNA cross-links, however, was surprisingly insensitive to nucleophilic competition by other strong nucleophiles including β-mercaptoethanol and phenylhydrazine. After incubating bisQMP and **OD2** for 24 hr, OD1 was added either with or without thiol and the mixture was further incubated for up to 72 hr to allow the formation of DNA crosslinking. The

maximum crosslinking formation in the presence and absence of thiol was 8% and 15%, respectively. 3 mM β-mercaptoethanol suppressed the formation of crosslinking by only 50 % when the thiol was added at the same time with **OD1** (Figure 3.6).

Alternative strong nucleophile phenylhydrazine too can only moderately quench the formation of DNA crosslinking caused by bisQM's transfer. After incubating bisQMP and **OD2** for 24 hr, **OD1** was added either with or without phenylhydrazine and the mixture was further incubated for up to 72 hr to allow the formation of DNA crosslinking. 20 fold excess of phenylhydrazine suppressed the maximum formation of DNA crosslinking by only about 40% (Figure 3.7). 6 mM phenylhydrazine, which was 200 fold excess of DNA, decreased the cross-linking yield from 16 % to 5 % (Figure 3.8, Lane 6 and Lane 12). Raising the concentration of phenylhydrazine to 8 mM caused serious DNA degradation. Therefore, phenylhydrazine, similar to β-mercaptoethanol, can only moderately suppress the formation of DNA cross-links caused by interstrand QM transfer.



Scheme 3.6. Experiment to examine the ability of phenylhydrazine to quench the formation of DNA crosslinking caused by QM transfer.

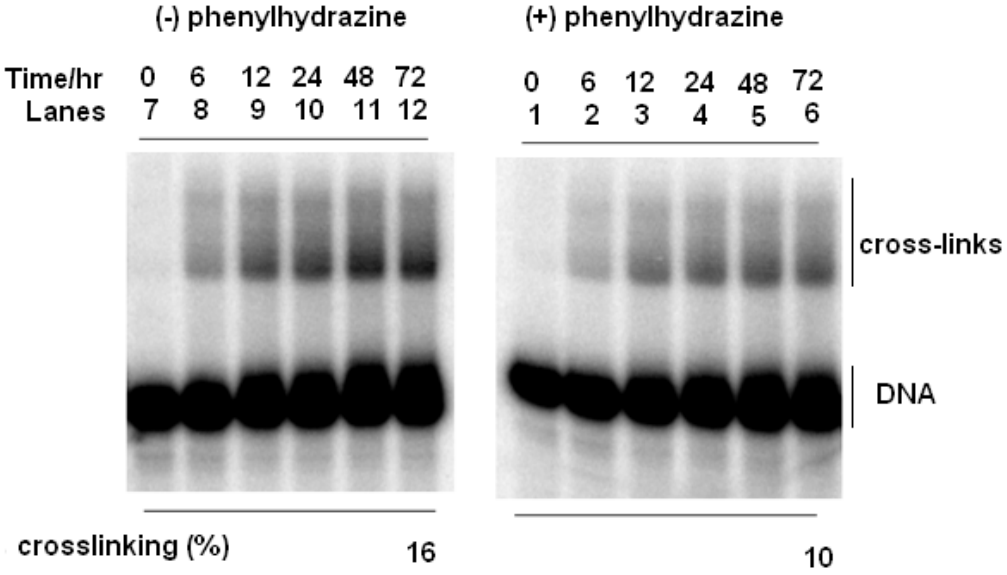


Figure 3.7. Interstrand transfer of quinone methide is only moderately sensitive to the presence of phenylhydrazine. Single-stranded DNA **OD2** (3.0 μM) was first incubated at room temperature with 10 fold excess of BisQMP (30 μM) in 20% aqueous acetonitrile. Addition of KF (10 mM) was used to initiate reaction. After 24hr incubation, 5'-[^{32}P]-**OD1 (**3.0 μM) was added as its complementary strand and mixture was incubated for indicated time in absence of trapping agent (lanes 1-6) and in presence of 0.6 mM phenylhydrazine (lanes 7-12). Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

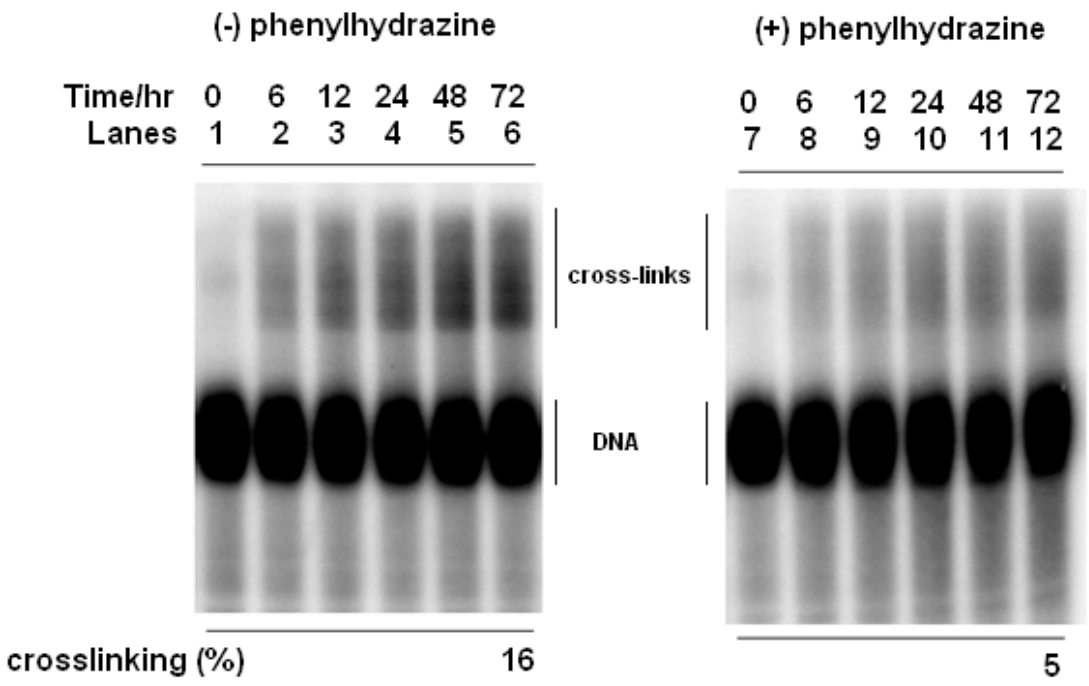


Figure 3.8. Interstrand transfer of quinone methide is only moderately sensitive to the presence of phenylhydrazine. Single-stranded DNA 5'-[^{32}P]-**OD2** (3.0 μM) was first incubated at room temperature with bisQMP (30 μM) in 20% aqueous acetonitrile,10 mM MES, pH 7 . 10 mM KF was used to initiate reaction. After 24 hr incubation, **OD1 (**3.3 μM) was added as its complementary strand and mixture was then incubated for indicated time in absence of trapping agent (lanes 1-6) and in presence of 6 mM phenylhydrazine (lanes 7-12). Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

The fact that interstrand transfer of bisQM to form DNA cross-links was insensitive to nucleophilic competition again showed similarity with the thiol resistance of oligonucleotide-QM self adduct (Scheme 3.1).[45] After **OD2**-bisQM adducts hybridized with **OD1**, regenerated bisQM intermediate was located around duplex DNA by covalent anchor, which raised the effective concentration of **OD1** and QM, and thus facilitated the formation of crosslinking.

The success of heterogeneous bisQM-**OD2** adducts to recognize and cross-link its complementary DNA offered us a convenient strategy to alkylate DNA in a

sequence specific manner. One potential advantage of bisQM-oligonucleotide adducts as alkylating agents might be their simplicity of preparation. QM-oligonucleotide self-adduct described previously required several steps of preparation: synthesis of QM, conjugation of QM onto a modified DNA (-NH_2 in DNA available for conjugation), generation of self adduct and further purification. Multiple steps of preparation tended to be time-consuming and required relatively high stability of QM precursor, which potentially limited the diversity of application for QMs. BisQM-oligonucleotide adducts were generated by simply incubating bisQMP and DNA for more than 4 hr without further treatment. In addition, bisQM-oligonucleotide adducts could generate two equivalents of QM intermediate. This could potentially enhance the ability of bisQM-DNA adducts to crosslink complementary sequences by carrying more QM equivalents.

3.2.5. Sequence effect on oligonucleotides' ability to capture and transfer bisQM

BisQM-oligonucleotide adducts exhibited the ability to alkylate complementary sequences by forming cross-links, however, the crosslinking yield was low and not satisfactory for further studies in Chapter 4 (Figure 3.1). Using the same sequences (**OD2/OD1**), attempts were made to optimize the cross-linking yield by varying the dose of bisQMP and pre-incubation time. However, crosslinking efficiency was not improved significantly. The number of particular dNs (N=A, T, G, C) of oligonucleotide should have played an important role in QM's transfer. Strong nucleophiles of DNA can capture QM dominantly under kinetic control and later

release QM efficiently for crosslinking.[43] The availability of proper strong nucleophiles in oligonucleotide would have a significant impact on DNA's ability to transfer QM and therefore efforts were made to optimize the composition of DNA for QM's transfer.

Three different sequences were employed to study how the composition of DNA could affect its ability to transfer bisQM and cause crosslinking (Table 3.1). In those sequences, 19 nucleotides N (N= A, C, G) were interspersed with 11 thymine (T), which does not react with QM (Table 3.1). Thymine was inserted into the poly (N) carefully in order to avoid possible stable secondary structures under aqueous conditions. G rich sequences tend to form stable secondary structures, such as G quartet and G quadruplex.[74, 75] In those secondary structures, the strong nucleophilic site dG N7 is hidden by hydrogen bonding, which could potentially reduce guanine's reactivity over bisQM intermediate.[76] Unlike previous experiments (Chapter 2), NaF was used to generate QM intermediate instead of KF. This is because that K cation tends to stabilize G quartet and G quadruplex structures.[77, 78]

The design of those three sequences was to examine the function of each nucleotide in a mixed sequence for bisQM's interstrand transfer. The role of oligonucleotides includes both (1) initial trapping and later releasing bisQM and (2) target recognition and hybridization of DNA strands by Waston-Crick base pairing. Since the role of each residue within an oligonucleotide cannot be separately distinguished, an oligonucleotide containing only one DNA nucleophile that reacts with QM reversibly was created along with the inert thymine. Studies based on

those simplified sequences are expected to provide some insight into nucleotide's function in transferring bisQM among DNA strands. In the following table, oligonucleotides used in this section are named as OD(N). The general sequence is OD(N): 5'-TNN TNT NNT NNT TNN NTN TNT NNT NNN TNN-3' (N= A, C or G). Their complementary sequences are named as OD(N)comp. The detailed sequences of those three oligonucleotides and their complementary sequences are listed in Table 3.1.

OD(A): 5'-TAA TAT AAT AAT TAA ATA TAT AAT AAA TAA-3'

OD(A)comp:3'-ATT ATA TTA TTA ATT TAT ATA TTA TTT ATT -5'

OD(C): 5'-TCC TCT CCT CCT TCC CTC TCT CCT CCC TCC-3'

OD(C)comp: 3'-AGG AGA GGA GGA AGG GAG AGA GGA GGG AGG-5'

OD(G): 5'-TGG TGT GGT GGT TGG GTG TGT GGT GGG TGG-3'

OD(G)comp: 3'- ACC ACA CCA CCA ACC CAC ACA CCA CCC ACC-5'

Table 3.1. OD(N) and their complementary sequences used to study the sequence effect on bisQM's transfer.

In a typical experiment, after incubating OD(N) sequences with 20 fold excess of bisQMP under equivalent conditions in Figure 3.1, the appropriate complementary sequences of OD(N) were added and samples were further incubated for 0- 72 hr. Surprisingly, OD(G) exhibited very strong ability to preserve bisQM and form cross-linking (Figure 3.11), whereas OD(A) and OD(C) caused no cross-linking beyond background level (Figure 3.9 and Figure 3.10). In Figure 3.9, there is seemingly some high molecular weight species observed on gel. Those species is

identified as DNA impurity in purchase OD(A) sample.

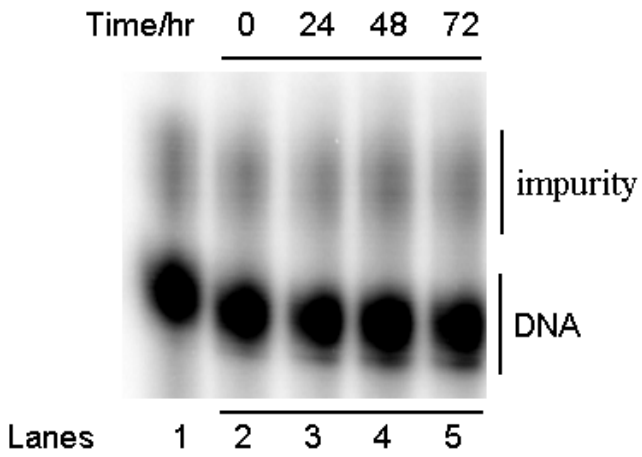


Figure 3.9. OD(A)'s ability to preserve bisQM and transfer bisQM to its complementary sequence. 3.0 μM 5'-[^{32}P]- OD(A) was first incubated with 30 μM bisQMP were in 20% aqueous acetonitrile (10 mM MES buffer, pH 7) for 24 hr at room temperature. 10 mM NaF was added to initiate the reaction. Then 3.3 μM OD(A)comp was added and the mixture was further incubated for indicated time at room temperature (Lanes 2-5). Single stranded 5'-[^{32}P]-OD(A) was incubated with 60 μM bisQMP and 10 mM NaF for 24 hr (Lane 1). Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

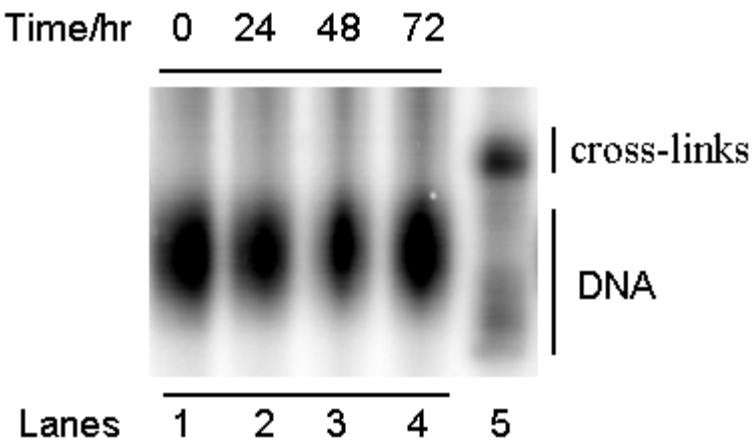


Figure 3.10. OD(C)'s ability to preserve bisQM and transfer bisQM to its complementary sequence. Identical procedure in Figure 3.9 was followed but OD(C) and OD(C)comp were used instead. 3.0 μM dsDNA annealed with 5'-[^{32}P]-QT(C) and QT(C)comp was incubated with 60 μM bisQMP and 10 mM NaF for 24 hr to crosslinking (Lane 5). Samples were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

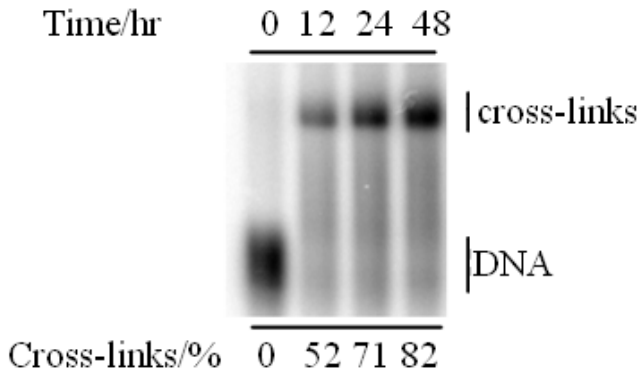


Figure 3.11. OD(G) was able to trap bisQM and transfer bisQM to its complementary sequence by forming crosslinking. 3.0 μM 5'-[^{32}P]- OD(G) was first incubated with 60 μM bisQMP in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature. 10 mM NaF was added to initiate the reaction. Then 3.3 μM OD(G)comp was added and the mixture was further incubated for indicated time and then analyzed by 15% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified in the indicated area by phosphoimagery analysis and reported (%) relative to total DNA.

In Figure 3.11, 15% polyacrylamide denaturing gel was used instead of 20% polyacrylamide gel. This is because in practice G rich sequence OD(G) showed similar mobility with its crosslinking products in 20% polyacrylamide gel. Therefore, 15% polyacrylamide gel was used in this case for better separation of DNA from cross-links.

Previous studies on free nucleosides indicated that dA had strong ability to preserve bisQM for cross-linking because of its strong ability to capture and release QM intermediate.[51] dC and dG, however, did not show any effect on bisQM's effective lifetime (Chapter 2). However, G rich sequence OD(G) showed very strong ability to deliver bisQM to its complementary sequence and cause crosslinking (> 80%), whereas OD(A) and OD(C) showed no such ability. These seemingly conflicted results suggested that there must be factors controlling bisQM's transfer other than kinetics of capturing and releasing QM intermediate.

DNA alkylation, but not crosslinking, of OD(A) and OD(C) were observed on gel (Figure 3.9 and Figure 3.10). These results suggested OD(C) and OD(A) were able to react with QM during pre-incubation period. The average number of bisQM captured by single-stranded DNA OD(N) was determined by UV-Vis measurement. OD(N) was incubated with 10 fold excess of bisQMP under equivalent conditions for 24 hr. The excess bisQM derivatives that did not react with DNA were filtered out by P-6 spin column, which is a size exclusion column and only molecules bigger than 5 base pairs can be eluted according to product description. BisQMP-acridine conjugate and its water adducts can not go through column. After eluting bisQMP's

aqueous solution through P-6 spin column, the resulting elution was measured by UV-Vis spectrum and no characteristic absorbance of bisQMP was observed, which indicated that all bisQMP was filtered out from the aqueous solution. The ratio of DNA and bisQM-acridine conjugate was determined by UV-Vis based on the DNA's absorbance at 260 nm and acridine's absorbance at 415 nm and the results are listed in Table 3.2.

Sequence	Average bisQM per strand determined by UV-Vis spectrum	Cross-links formed by bisQM's trasnsfer (%) determined by gel electrophoresis
OD(G)	2.5±0.4	>80 %
OD(C)	7.8±0.1	<1%
OD(A)	6.1±0.1	<1%

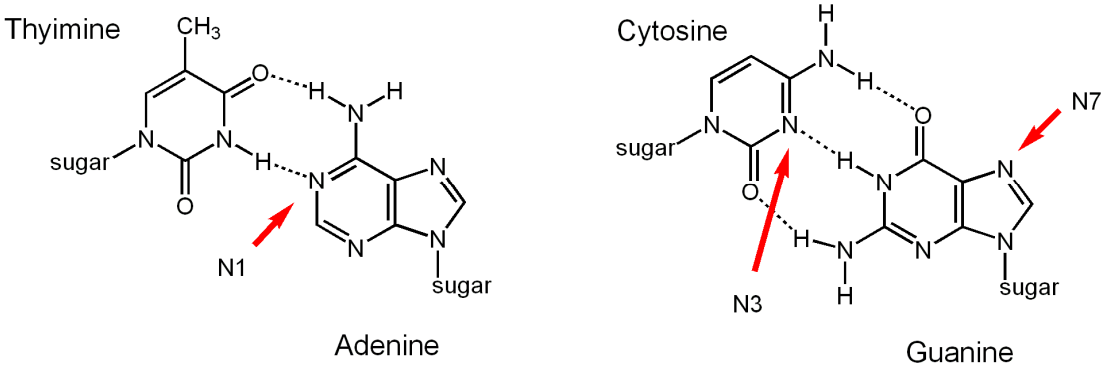
Table 3.2 Average number of bisQM equivalents attached to per DNA strand. 3.0 μM OD(N) was first incubated with 60 μM bisQMP in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature. 10 mM NaF was added to initiate the reaction. Then the sample was filtered by P-6 spin column and measured by UV-Vis spectrum. The average number of QM per strand was calculated based on the DNA's absorbance at 260 nm and acridine's absorbance at 415 nm. Crosslinking yields are determined by gel electrophoresis in Figure 3.9-3.11. The date represents the average value of two independent determinations and the indicated error represents the range of two determinations.

Interestingly, G rich sequence OD(G) trapped only 2 equivalents of bisQM per strand on average, which was much less than OD(A) and OD(C) (Table 3.2). These results agreed with the observation of high level alkylation of OD(A) and OD(C) observed on gel electrophoresis (Figure 3.9 and Figure 3.10) and the data from kinetic

competition studies based an unsubstituted QM and nucleotides,[43] where dA N1and dC N3 formed QM adducts dominantly under kinetic control but dG N7 only formed limited amount of QM adduct.

Over alkylation apparently decreased the ability of oligonucleotides to transfer QM and causing crosslinking in the preliminary studies described above. It was easy to assume that the oligonucleotides which preserved more equivalents of bisQM may provide more chance to regenerate QM intermediate and alkylate their complementary sequences. However, QM alkylation of single-stranded DNA could also potentially interrupt the hybridization of DNA and therefore suppress bisQM's transfer. dG N7, dA N1 and dC N3 were primary alkylation sites of DNA due to their strong nucleophilicity.[43] Among those three nucleophilic sites, only alkylation on dG N7 would not interrupt DNA hybridization (Scheme 3.7). Alkylation on dA N1and dC N3 would destabilize duplex structures by preventing efficient base pairing. Among OD(N) sequences, OD(G) was modified by only 2 equivalents of bisQM after pre-incubation and since alkylation on dG N7 would not prevent DNA hybridization, it could successfully form duplex structure and transfer bisQM to its complementary sequence. OD(A) and OD(C) carried more than 6 equivalents of bisQM after pre-incubation. If all bisQM formed intrastrand cross-linking by reacting with DNA at both benzylic positions, 12 out of 30 deoxynucleotides were modified in OD(A) and OD(C) and more than one third of base pairing would be interrupted. This would greatly destabilize the duplex structures necessary for bisQM's transfer. The multiple intrastrand crosslinking by bisQM might further distort the necessary

conformation of DNA for target recognition and duplex formation. Therefore, OD(A) and OD(C)'s failure to recognize and form duplex structures with their complementary sequences likely explained their inability to transfer bisQM and cause DNA crosslinking. More studies are needed to gain more insight of the sequence effect on QM's transfer process.



Scheme 3.7. Alkylation on cytosine N3 and adenine N1 interrupts the base pairing of DNA whereas alkylation on guanine N7 does not.

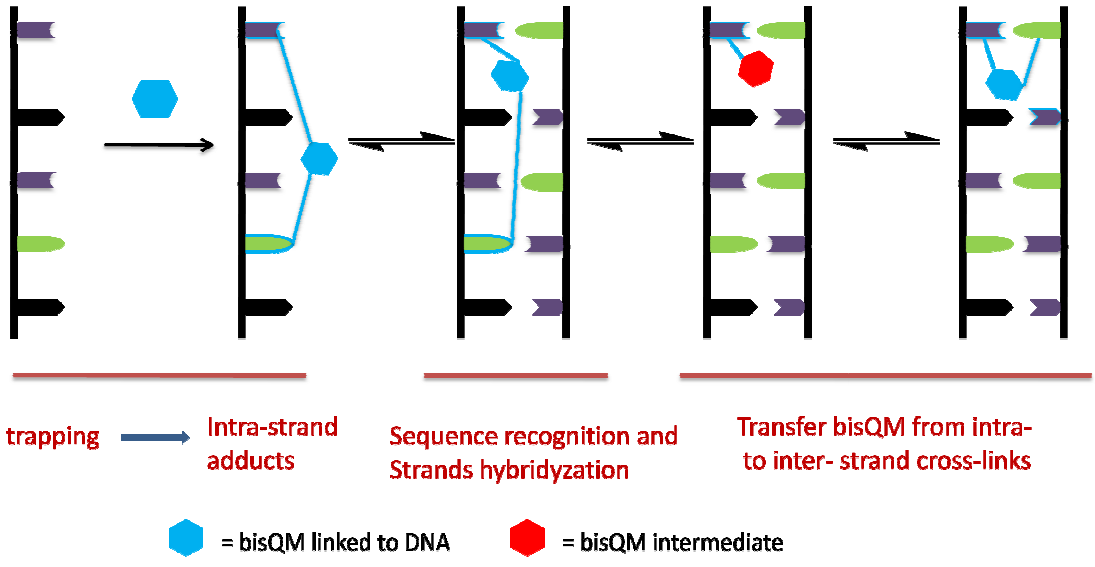
3.3. Conclusions

Oligonucleotide **OD2** was able to preserve and transfer bisQM to its complementary sequence OD1 by forming crosslinking in a sequence specific manner. BisQM's effective lifetime was extended by forming reversible adducts with oligonucleotide **OD2**. The bisQM-OD2 adducts provides us a convenient strategy to alkylate DNA in a sequence specific manner based on QM's reversible alkylation.

The initial reaction between **OD2** and bisQM was sensitive to the presence of thiols. However, bisQM's interstrand transfer was only weakly sensitive to external strong nucleophiles, such as β-mercaptoethanol and phenylhydrazine. QM-oligonucleotide adducts were not reactive with external nucleophiles and

therefore could potentially preserve its ability for DNA alkylation for extended time in *vivo*.

Composition of oligonucleotides had a great impact on its ability to transfer QM. Ability of oligonucleotide to capture QM initially is necessary; however, over-alkylation during pre-incubation will interrupt the necessary sequence recognition and the formation of duplex structure. Failure of annealing with complementary sequences may stop QM's transfer process. The proposed mechanism of bisQM's transfer from intrastrand adducts to interstrand cross-linking requires three steps: initial trapping of bisQM, sequence recognition and strand hybridization, regeneration and transfer of bisQM (Scheme 3.8). The transfer of QM from intrastrand to interstrand adducts suggests that the reversible QM-DNA bonds may allow QM to move among DNA strands, which is important to understand the toxicity of quinone methide based DNA alkylating agents.



Scheme 3.8. Schematic demonstration of bisQM's transfer process.

The detailed product distribution of single-stranded DNA- bisQM adducts remained unclear. More insightful understanding could be gained if labile DNA-QM adducts could be converted into stable adducts and then precise analysis of product distribution would be possible with minimum information loss.

3.4. Materials and Methods.

Materials. Reagents and solvents were purchased were purchased as ACS grade or higher and used without purification unless noted. Oligonucleotides were purchased as desalting samples from IDT Inc. without further purification.

Methods. DNA reactions were analyzed using 20% or 15% polyacrylamide (19:1 acrylamide: bis-acrylamide) gel electrophoresis under denaturing conditions (7 M urea) as specified in figures. Gels were analyzed by phosphorimaging with a Molecular Dynamics phosphor screen and phosphorimager. Cross-linked products were reported (%) relative to total DNA.

DNA reactions. Procedures and conditions of DNA reactions are specified in Figures.

Measurement of the average number of bisQM-acridine conjugate linked to each oligonucleotide. Typically, 3.0 μM OD(N) was first incubated with 60 μM bisQMP in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature. 10 mM NaF was added to initiate the reaction. After incubation, reaction mixture was lyophilized and dissolved in 80 μL ddH_2O and then the sample

was filtered by P-6 spin column. The collected elution was diluted to 1000 μL and the absorbance at 260 nm and 415 nm was measured by UV/Vis spectrometer. The extinction coefficients of acridine at 260nm and 415 nm were determined by UV/VIS: ε(260 nm) = 34641, ε(415 nm) = 7620

3.0 μM OD(T) (5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3') was incubated with bisQMP and measured by UV/Vis spectrometer following identical procedure to experiments of OD(N) described above. Absorbance of resulting OD(T) solution at 260 nm and 415 nm serves as negative control for OD(N) experiments. The absorbance of OD(N) samples shown in the table below are the values after deducing the absorbance of OD(T) samples.

DNA	Abs_{260}	Abs_{415}	Acridine (μM)	DNA (μM)	Acridine/DNA
OD(A)	1.07830	0.10535	10.82	1.77	6.0
OD(C)	0.67354	0.090930	8.93	1.15	7.9
OD(G)	0.76101	0.052713	3.91	1.82	2.1

Table 3.3. UV/Vis measurement of OD(N)-bisQM adduct samples and the average number of bisQM attached to per DNA strand. The experiment has been repeated twice.

Chapter 4. Migration of bisQM among DNA strands

4.1. Introduction

Most DNA alkylating agents react with DNA by forming stable covalent bonds. However, some compounds like acrolein and duocarmycin react with DNA reversibly by forming labile adducts.[20-22] Quinone methides (QMs) react with strong nucleophiles of DNA reversibly under kinetic control by forming labile adducts. Those adducts can later regenerate QM intermediates and eventually thermodynamically stable adducts between QM and weak nucleophiles of DNA will dominate.[43]

The covalent, yet reversible, bond between QM and DNA is beneficial for its biological function in some cases. Reversible reactions between QMs and strong nucleophiles of DNA have a significant impact on QM's lifetime under aqueous conditions (chapter 2). By repeated capture and release of bisQM intermediate, dA was able to extend bisQM's lifetime for crosslinking by 100-fold. Reversible reactions between QMs and oligonucleotides also preserve QM from irreversible trapping of nucleophiles other than DNA and prolong their lifetime for DNA alkylation.[45, 51] QM-oligonucleotide intrastrand adducts were able to alkylate complementary sequences selectively even after days of incubation under aqueous conditions.

The reversible DNA alkylation of QM may also help QM to escape the DNA repair and enhance its biological function. When a damaged DNA segment is excised from the chromosome and hydrolyzed, QMs may be regenerated and return to

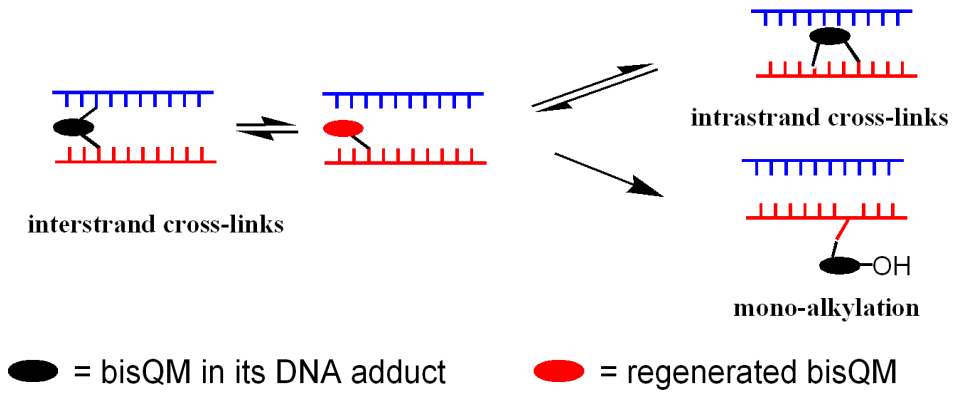
the chromosome by forming new alkylation adducts. The repeated alkylation by QM with extended lifetime could cause continuous DNA damage in cell, which could increase the likelihood of cell apoptosis.

The reversibility of QM-DNA also poses a significant problem to understanding the alkylation selectivity of QM-based compounds for dsDNA. The QM-DNA adducts would convert from kinetically favored products into thermodynamically stable adducts readily. The half life of QM-DNA adducts is usually only hours long.[43] The general procedure to determine reaction specificity of alkylating agents includes incubating the alkylation agent with DNA, followed by dialysis to remove any organic reagents that may interfere with the subsequent enzymatic digestion. The alkylated DNA is then digested and analyzed by HPLC or LC-MS. Days of dialysis and enzymatic digestion would allow adduct distribution to change appreciably and the information of initial QM adducts would be partially lost. Therefore, conventional analytical methods fail to provide a true accounting of all adducts between QM and DNA due to the reversible alkylation.

Interstrand crosslinking of duplex DNA is more toxic than DNA alkylation because crosslinking is more difficult for cell to repair than mono-alkylation.[4, 79] Therefore, DNA interstrand crosslinking agents comprise an important class of clinical agent in the treatment of various cancers.[80, 81] A bisQMP-acridine conjugate was designed to be a DNA crosslinking agent and has shown strong ability to cause DNA crosslinking (Chapter 2). Its reversible reaction with free nucleosides and oligonucleotides were previously discussed in chapter 2 and chapter 3 and the

consequences of its reversible alkylation inside duplex DNA is addressed in this chapter.

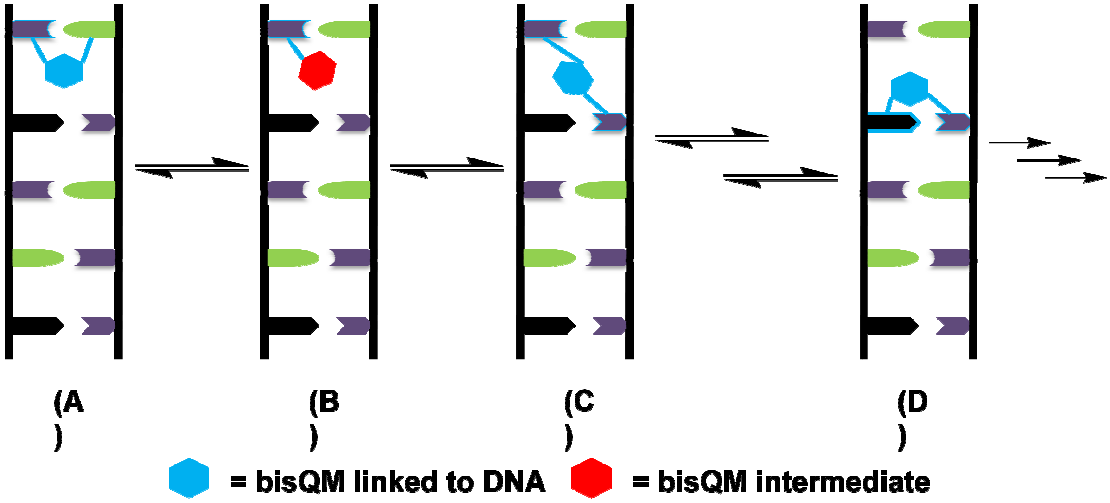
The regeneration of bisQM from DNA cross-links could lead to loss of crosslinking if irreversible trapping by water occurs. Once bisQM is regenerated, DNA cross-links will lose its covalent bridge. The regenerated bisQM has three possible reactions: (1) react with the second DNA strand and regain crosslinking; (2) react with the same DNA strand to form intrastrand crosslinking and lose interstrand crosslinking; (3) react with water and lose crosslinking (Scheme 4.1). Intrastrand bisQM adducts may still remain in dynamic equilibrium with the QM intermediate and possibly regain the interstrand crosslinking by alkylating the second strand (Chapter 3). However, once bisQM is quenched by water, DNA crosslinking will be lost permanently.



Scheme 4.1. Possible reaction pathways of bisQM intermediate in duplex DNA.

The reversible alkylation of bisQM in DNA cross-links can also result in re-distribution of adducts. Mono-functional QM will be released into solution after its regeneration and thus the next alkylation site on DNA by this regenerated QM is unpredictable and irrelevant to the initial adduct. However, since bisQM-DNA

crosslinking adduct can only generate one equivalent of QM at one time, a covalent anchor to DNA always exists after bisQM's regeneration from DNA cross-links (Scheme 4.2 B). Acridine attachment also helps keep bisQM in the major groove of duplex DNA. Therefore, regenerated bisQM is always located in its initial alkylation site and is only able to alkylate the nucleotides nearby (Scheme 4.2C). As demonstrated in Scheme 4.2, the regenerated bisQM can change its crosslinking sites by reacting with another nucleotide in duplex DNA. Since QM intermediate can be generated at both benzylic positions, the repetition of bisQM's regeneration and DNA alkylation at different sites could lead to a stepwise migration along duplex DNA (Scheme 4.2) and therefore a product re-distribution.



Scheme 4.2. Proposed bisQM's migration along duplex DNA in a stepwise manner.

Detection of bisQM's migration along duplex DNA will be difficult in one duplex structure. BisQM's migration only changes the position of the covalent bridge and the nucleotides that are alkylated. Conventional methods to analyze DNA adducts could not provide precise information of the product distribution due to

the lability of QM adducts. Therefore, it is not feasible to monitor the migration of bisQM by analyzing which nucleotides are alkylated over time. No direct observation of stepwise migration of reversible DNA cross-linker has been reported so far. To understand the cytotoxicity of a DNA alkylating agent, investigation into the action mechanism and product distribution is necessary. Therefore, a strand displacement system was developed in order to demonstrate the existence of bisQM's dynamic motion in duplex DNA by reversible alkylation (Scheme 4.3).

DNA strand exchange and displacement between single-stranded DNA and duplex DNA are important processes in the repair of DNA damage and genetic recombination in *vivo*.[82-84] Strand-displacement is also one of the most frequently utilized structural transitions for constructing DNA devices.[85, 86] Strand exchange process involved tri-strand intermediates that may be only marginally stable due to the electrostatic repulsion of phosphate anions (Figure 4.1 A).[82, 87] Therefore, strand exchange between single-stranded DNA and duplex DNA is extremely slow at room temperature.

A toehold of sufficient length can stabilize the intermediates and help initiate strand exchange (Figure 4.1 B).[85, 86, 88] A toehold generally refers to a single-stranded segment overhang in a duplex DNA structure, which is able to anneal with complementary segment in a third sequence by base pairing. Pre-association can stabilize the tri-strand assembly and raise the effective molarity of DNA strands. In one report, a toehold of 10 nucleobases can efficiently accelerate strand displacement reaction by up to 6 orders of magnitude.[88] The strand displacement

process is usually completed in minutes when a toehold of proper length is present. A toehold can also drive the equilibrium of strand displacement towards the duplex structure containing more base pairing.[88] If the leaving strand and incoming strand are identical, the strand exchange process is thermodynamically neutral and equilibrium will be reached when 50% of DNA strands are exchanged assuming their equivalent concentration. When the incoming strand has an extra toehold, the resulting duplex structure will be thermodynamically favored due to extra base pairing. Therefore, a toehold cannot only kinetically facilitate the initiation of strand displacement reaction, but also drive the equilibrium by generating an extended duplex structure.

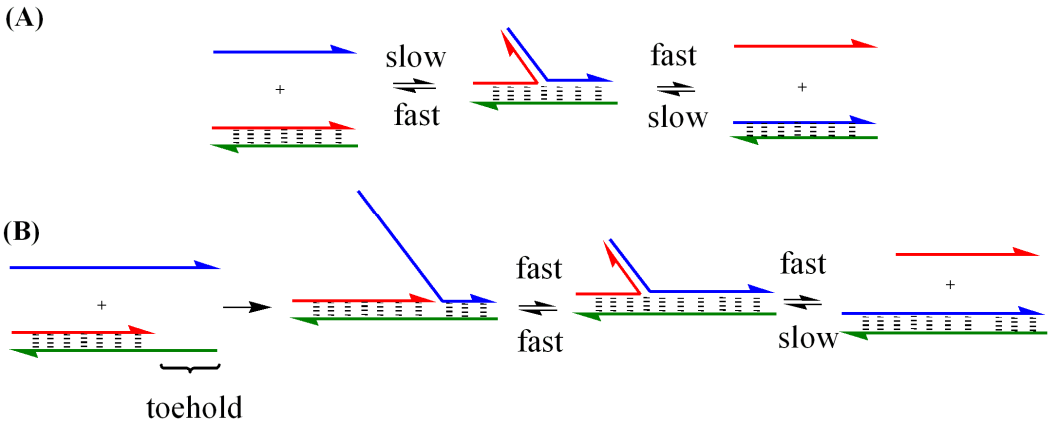
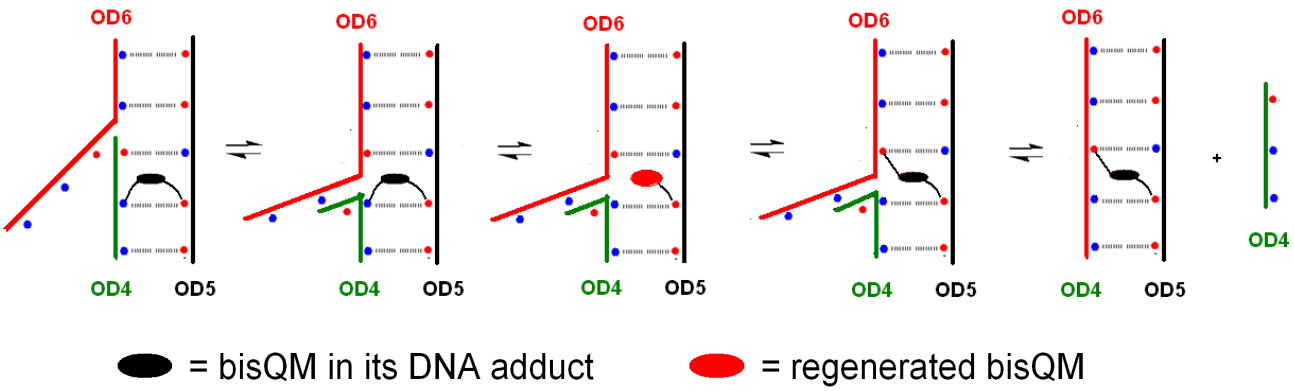


Figure 4.1. Strand exchange and strand-displacement process. (A) Strand exchange without a toehold. (B) Strand displacement process with a toehold. The colored arrows indicate the backbones of DNA strands in the 5' to 3' direction. The incoming strand (blue) hybridizes to the unpaired toehold of initial duplex structure (green/red) to form a branched structure. The branching point randomly migrates up- and downstream, eventually replacing the leaving strand (red) and form a new duplex structure.

To date, investigation of the reversible QM alkylation inside DNA cross-links is limited due to the lack of reliable method to differentiate the alkylated nucleotides before and after bisQM's migration. The advantage of a strand displacement system

to study this migration is that the nucleotides alkylated before and after QM's migration may come from different DNA strands. Therefore the migration of QM can result in formation of DNA cross-links with different strands, which can be analyzed by denaturing gel electrophoresis. The incoming DNA strand therefore acts as a probe of QM migration. Formation of DNA cross-links containing the incoming strand will demonstrate the reversibility of bisQM in the original DNA cross-links. In the proposed assay, the strand displacement assay starts with **OD4/OD5** DNA cross-links with a toehold presented in one strand **OD5** (Scheme 4.3). **OD4/OD5** cross-links can be generated by sequence selective QM transfer from **OD4**-bisQM adducts to **OD5** in high yield (Chapter 3). BisQM can only crosslink **OD4** and **OD5** in duplex regions, leaving the toehold segment unmodified. Incoming strand **OD6** is fully complementary to **OD5** and will pre-associate with the toehold region by forming a tri-strand DNA complex. Through a strand displacement, **OD6** can readily replace **OD4** by base-pairing with **OD5**. If bisQM is regenerated from **OD4/OD5** cross-links and then alkylates **OD6** after strand displacement, this reversible alkylation of QM will be detected by the formation of **OD6/OD4** crosslinking and concurrent loss of **OD4/OD5** crosslinking when either **OD4** or **OD6** is radiolabeled.

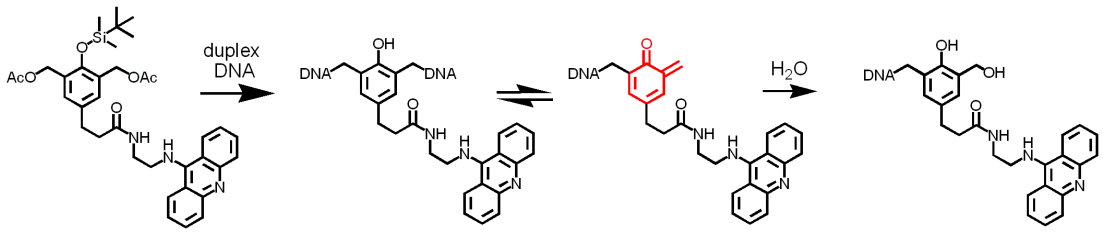


Scheme 4.3. A strand displacement system was used to demonstrate the dynamic property of bisQM in DNA cross-links.

4.2. Results and discussion.

4.2.1. Loss of DNA crosslinking due to reversible alkylation of bisQM

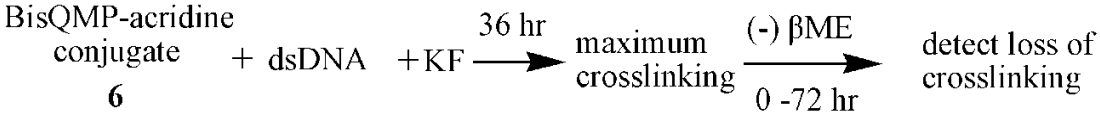
For interstrand DNA crosslinking caused by bisQMP, irreversible trapping of the regenerated bisQM by water or other nucleophile, such as β-mercaptoethanol, will result in a loss of crosslinking (Scheme 4.4). The covalent linkage between duplex strands will be broken and cross-linked DNA will convert into either unmodified DNA or DNA alkylation products. In both cases, the yield of DNA cross-links will decrease over time. The rate of bisQM's regeneration and trapping can therefore be monitored by loss of DNA crosslinking over time.



Scheme 4.4. Quenching of regenerated bisQM intermediate by water leads to loss of crosslinking.

Previous studies described in Chapter 2 showed that using a 10-fold excess of bisQMP-acridine conjugate **6** relative to duplex DNA, the yield of cross-links reached maximum after 36 hr. Thus, the decomposition of DNA cross-links was studied after the initial 36 hr that allowed for formation of maximum crosslinking (Figure 4.2). Samples were further incubated either in the absence or presence of strong nucleophile β-mercaptoethanol (3.8 M) for 0-72 hr and analyzed by denaturing gel electrophoresis (Figure 4.2 and Figure 4.3).

(A)



(B)

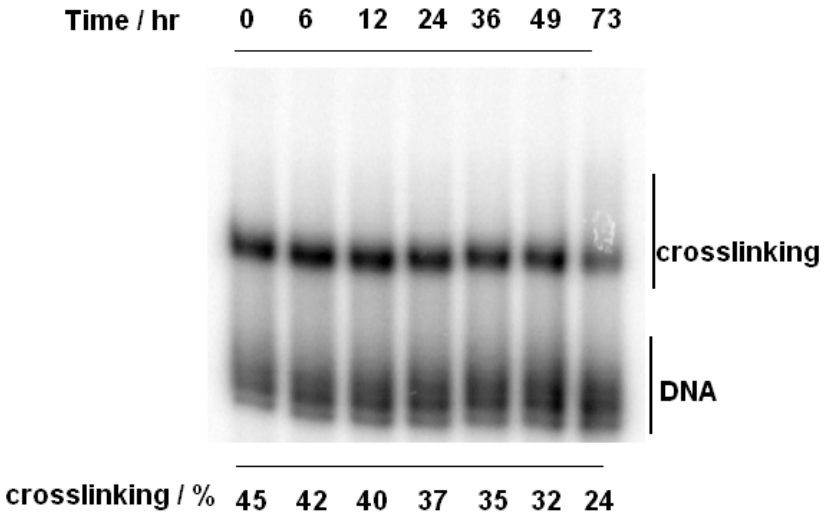
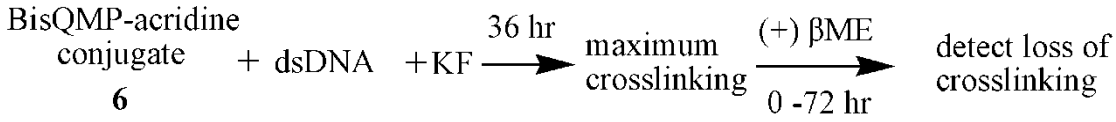


Figure 4.2. Time dependent reversal of DNA cross-links due to the reversible quinone methide alkylation of DNA. (A) Scheme of experiment. (B) Duplex DNA formed by 5'-[^{32}P]-**OD1** (3.0 μM) and **OD2** (3.3 μM) was first incubated at room temperature with bisQMP (30 μM) in 20% aqueous acetonitrile 10 mM MES pH 7. 10 mM KF was added to initiate reaction. After 36 hr incubation, 30% (v/v %) water was added and the mixture was further incubated for indicated time at room temperature. Samples were analyzed by 20% denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA.

(A)



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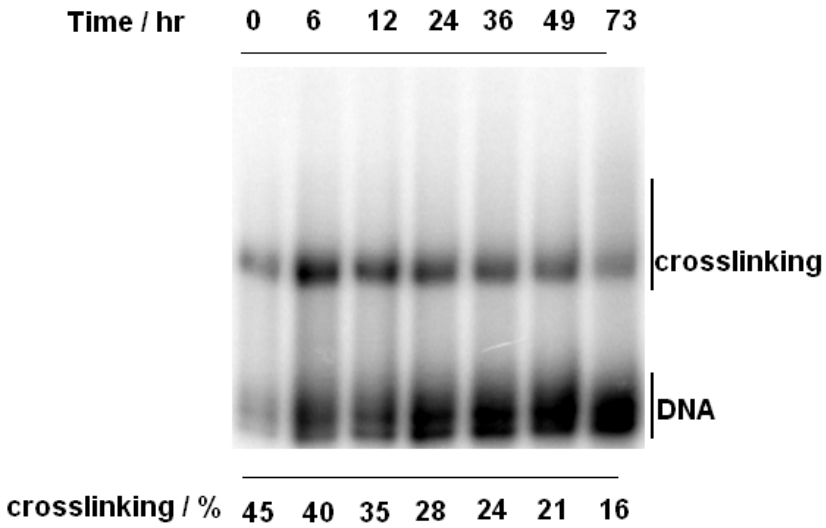


Figure 4.3. Time dependent reversal of DNA cross-links due to the reversible quinone methide alkylation of DNA in the presence of β-mercaptoethanol. Duplex DNA formed by 5'-[^{32}P]-**OD1** (3.0 μM) and **OD2** (3.3 μM) was first incubated at room temperature with bisQMP (30 μM) in 20% aqueous acetonitrile 10 mM MES pH 7. 10 mM KF was added to initiate reaction. After 36 hr incubation, 30% (v/v %) β-mercaptoethanol was added to a final concentration of 3.8 M and the mixture was further incubated for indicated time at room temperature. Samples were analyzed by 20% denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA.

Time dependent reversal of DNA crosslinking was observed either in the absence (Figure 4.2) or presence (Figure 4.3) of thiol. In 72 hr, DNA crosslinking was decreased from 45% to 24% in absence of thiol and with thiol, the final crosslinking yield was only 16%. The rate of time dependent loss of DNA crosslinking fit a first order decay in both cases (Figure 4.4). A faster decomposition was observed in the presence of strong nucleophile β-mercaptoethanol (Figure 4.4). Thiol is more nucleophilic than water and therefore has a stronger ability to compete

with DNA to trap regenerated QM. The half-life of DNA cross-links in the absence and presence of thiol were about 72 hr and 36 hr, respectively, which were significantly longer than the half-life of QM-deoxynucleotide adducts, which is within only hours under comparable conditions.[43, 49] This indicated that reversible alkylation between QM and DNA likely occurred multiple times before it was stopped by irreversible trapping. The dynamic intramolecular alkylation between QM and duplex DNA is weakly sensitive to the presence of external nucleophiles since 3.8 M β-mercaptoethanol only moderately accelerates the reversal of DNA crosslinking.

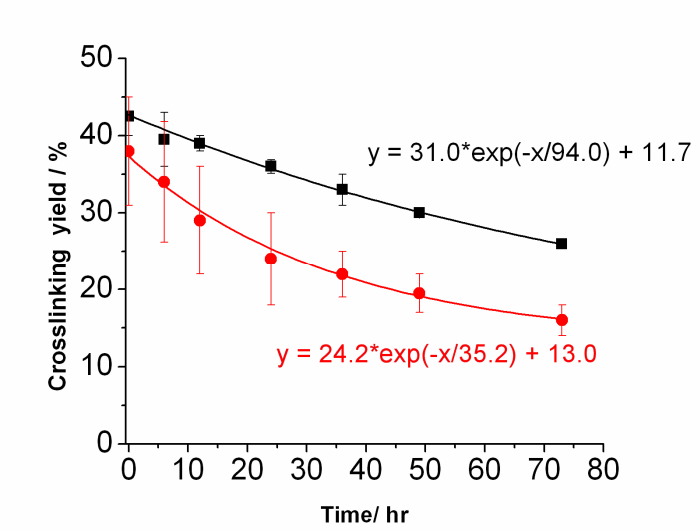


Figure 4.4. The reversal of DNA crosslinking due to reversible alkylation of bisQM in presence and absence of strong nucleophile β-mercaptoethanol. Line in red represents loss of cross-links in the presence of β-mercaptoethanol and line in black represents loss of cross-links in absence of β-mercaptoethanol. Cross-linked products were quantified by phosphoimage analysis and reported (%) relative to total DNA. Data represent the average of two independent experimental values and their range is indicated by the error bars.

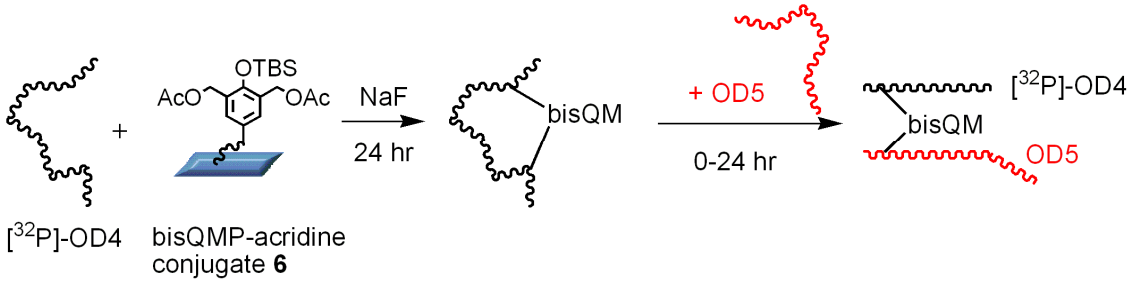
4.2.2. Visualization of bisQM's migration among DNA strands

The reversal of DNA crosslinking provided the first indication of the reversible alkylation of QM inside duplex DNA cross-links. Regeneration and alkylation of

bisQM may repeat multiple times before bisQM is trapped irreversibly by weak nucleophiles of DNA or external nucleophiles such as water and thiol during days of incubation.

The strand displacement assay starts with the formation of **OD4**/**OD5** DNA cross-links in high yield (Figure 4.5). **OD5** is a complementary sequence of **OD4**, which contains a toehold of 16 bases at its 5' end (Figure 4.5 C). The G rich sequence **OD4** has a strong ability to capture and transfer bisQM to **OD5** by forming crosslinking in high yield (Figure 4.5 B). Since bisQM transfer is sequence selective, bisQM can only cross-link **OD5** in **OD4**'s complementary segment but not the toehold region (Chapter 3) (Figure 4.5 A). In addition, after the formation of **OD4/OD5** crosslinking, bisQM does not migrate to the toehold region and cause further reaction in toehold region (Figure 4.9, section 4.2.3). Therefore, the toehold segment in **OD5** will remain unmodified during the formation of DNA crosslinking, which allows for efficient association with new coming strands.

(A)



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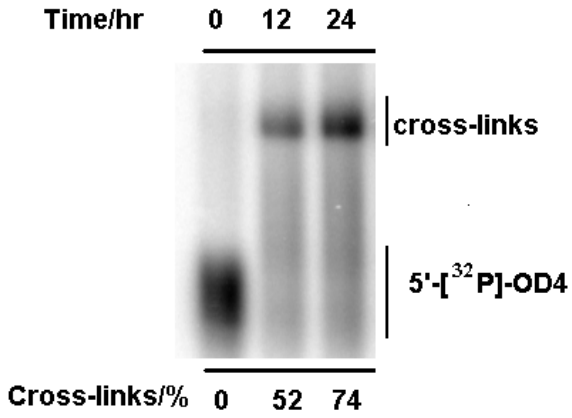


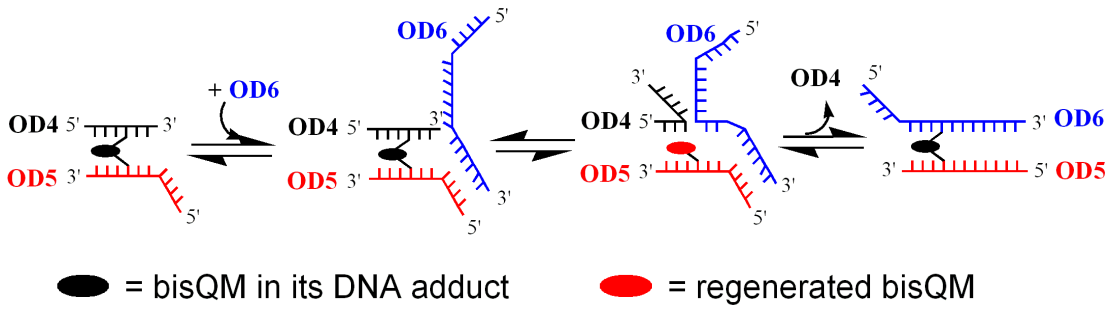
(C)

OD4: 5'-(TGG TGT GGT GGT TGG GTG TGT GGT GGG TGG)-3'
OD5: 5'-(TTT ACC TCT TCA ACC GCC ACC CAC CAC ACA CCC AAC CAC CAC ACC A)-3'

Figure 4.5. Recognition and transfer of QM to a complementary strand. (A) The formation of **OD4**/**OD5** cross-links by bisQM's transfer in a sequence specific manner. (B) 3.0 μM **5'-[^{32}P]-OD4** was first incubated with 60 μM bisQMP in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature. 50 mM NaF was added to initiate the reaction. Then 3.3 μM **OD5** was added and the mixture was further incubated for indicated time and analyzed by 15% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA. (C) Sequences used in experiment.

Addition of a third sequence **OD6** will initiate the strand displacement process. **OD6** is fully complementary to **OD5** and will pre-associate with the toehold segment by forming a tri-strand DNA assembly and **OD6** will displace **OD4** by base-pairing

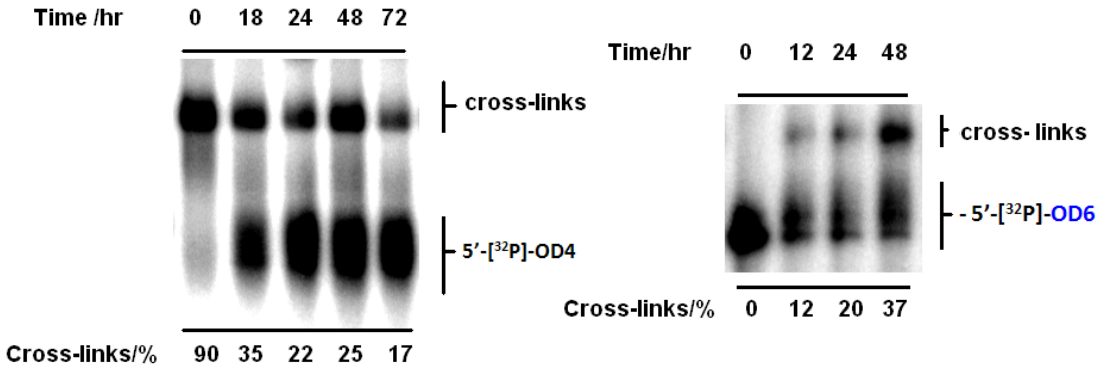
with **OD5** (Scheme 4.5). When bisQM is regenerated by breaking its covalent bond with **OD4** during the strand displacement process, it could either alkylate **OD4** by regaining the **OD4**/**OD5** cross-links, or react with **OD6** by forming new **OD5**/**OD6** crosslinking (Scheme 4.5). This partition of bisQM should result in a decreasing amount of **OD4/OD5** crosslinking and an accumulation of **OD5/OD6** crosslinking.



Scheme 4.5. BisQM's migration among DNA strands by strand displacement process.

The migration of bisQM among DNA strands was demonstrated by the concurrent formation of new **OD5/OD6** crosslinking and loss of original **OD4/OD5** crosslinking (Figure 4.6 A, B). When **OD4** was radiolabeled, it was observed that 5'-[^{32}P]-**OD4** was released from initial **OD4/OD5** cross-links as single-stranded DNA under denaturing conditions. The decomposition rate of 5'-[^{32}P]-**OD4/OD5** was fast upon the addition of **OD6** and the crosslinking yield dropped from 90% to 35% in the first 35 hr. This yield kept decreasing gradually and after 72 hr only 17% of the **OD4/OD5** crosslinking remained. The formation of new **OD5/OD6** crosslinking kept accumulating and reached a maximum of 45% after about 48 hr (Figure 4.6).

(A) **(B)**



(C)

OD4: (5'-TGG TGT GGT GGT TGG GTG TGT GGT GGG TGG-3')

OD5: (5'-TTT ACC TCT TCA ACC GCC ACC CAC CAC ACA CCC AAC CAC CAC ACC A-3')

OD6: (5'-CTA CTG ACG TCG TGG TGT GGT GGT TGG GTG TGT GGT GGG TGG CGG TTG AAG AGG TAA A-3')

Figure 4.6. BisQM inside duplex DNA remained reactive and was able to alkylate a third strand by forming corresponding cross-links after strand displacement. (A) and (B) 3.0 μM **OD4** was first incubated with bisQMP (60 μM) in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature and 50 mM NaF was added to initiate reaction. The mixture was further incubated with 3.0 μM **OD5** for 24 hr. Then 3.0 μM **OD6** was added and incubated further for indicated time and analyzed by 15% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA. **OD4** was radiolabeled in (A) and **OD6** was radiolabeled in (B). (C) Sequences used in experiments in these experiments.

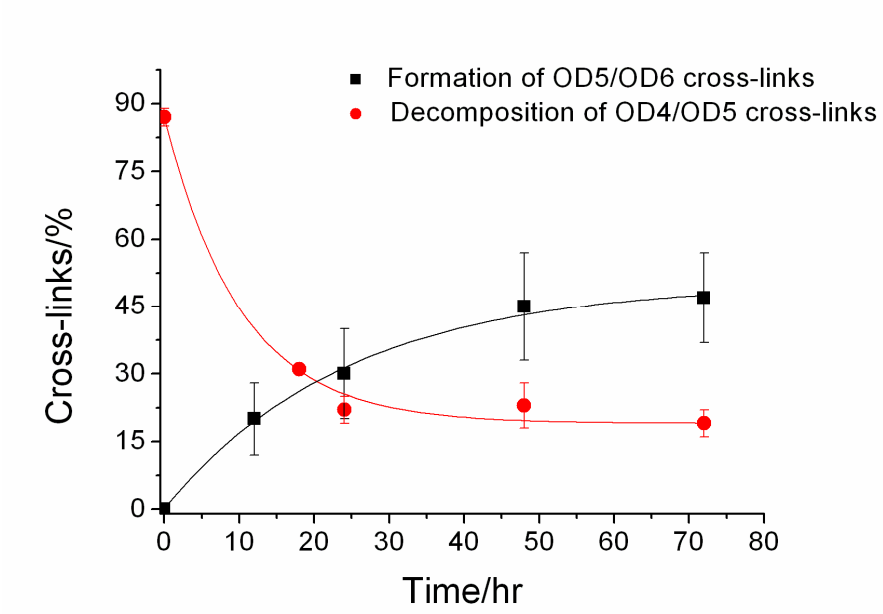
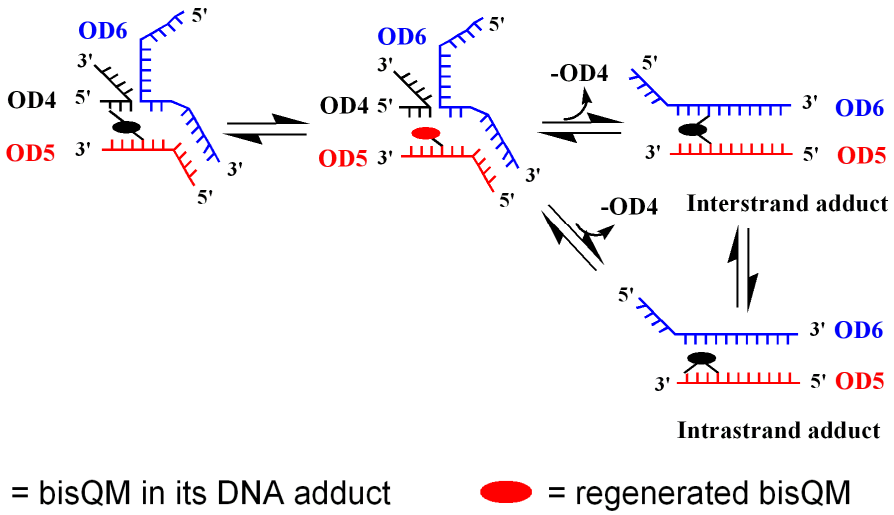


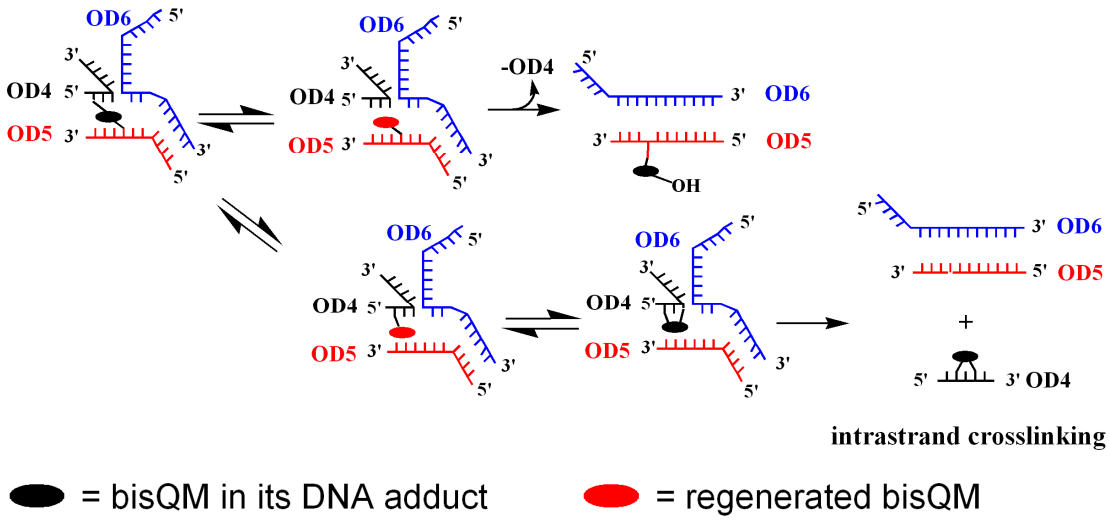
Figure 4.7. The formation of **OD5/OD6** cross-links and decomposition of **OD4/OD5** cross-links. Cross-linked products were quantified by phosphoimage analysis and reported (%) relative to total DNA. Data represent the average of two independent experimental values and their range is indicated by the error bars. Lines represent the trend of crosslinking change.

The initial formation rate of **OD5/OD6** crosslinking was slower than the decomposition of **OD4/OD5**, especially during the first 24 hr (Figure 4.7). This delay of **OD5/OD6** cross-links formation suggested that some bisQM intermediate did not alkylate **OD6** by forming new crosslinking directly after its regeneration. As demonstrated in Scheme 4.6, there are multiple reaction pathways for QM and one productive pathway is that QM first alkylates **OD5** by forming intrastrand adduct and then get transferred to **OD6** by forming the new crosslinking species (Scheme 4.6). This extra step may account for the slower formation of final **OD5/OD6** cross-links comparing to the decomposition of **OD4/OD5** cross-links because it requires bisQM to be regenerated one more time from its **OD5** intrastrand cross-links to alkylate **OD6**, which takes hours.



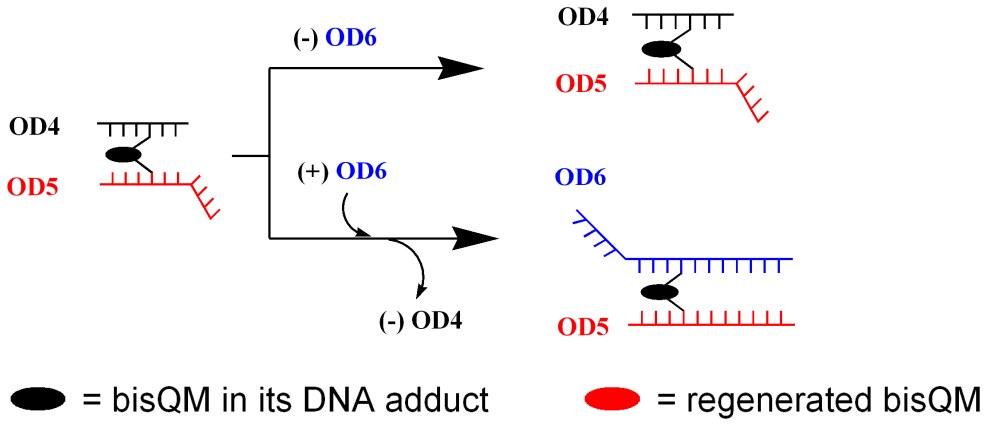
Scheme 4.6. The formation of bisQM-**OD5** intrastrand crosslinking delayed the formation **OD5/OD6** interstrand crosslinking.

Not all bisQM released from **OD4/OD5** cross-links migrated to **OD5/OD6** cross-links. After 72 hr, 73% of **OD4/OD5** cross-links was lost and only 50% of **OD5/OD6** cross-links was formed. About 23% of released bisQM was non-productive. Several non-productive pathways exist in the strand displacement system (Scheme 4.7). Irreversible trapping of water could terminate bisQM's migration and lead to the loss of DNA crosslinking. The bisQM intermediate can also be regenerated by breaking QM-**OD5** bond and subsequently form **OD4**-bisQM interstrand crosslinking. When **OD4** strand is displaced from the DNA complex, bisQM will leave the duplex in the form of a single-stranded DNA bis-adduct, which causes the loss of total amount of DNA crosslinking (Scheme 4.7).



Scheme 4.7. Non-productive reaction pathways of QM lead to loss of quantitative transfer of DNA crosslinking.

The **OD4/OD5** cross-linking was stable under aqueous conditions and its decomposition was only initiated by the addition of **OD5**'s complementary sequence **OD6** (Scheme 4.8). Without addition of **OD6**, the **OD4/OD5** cross-links remained stable over 72 hr and no degradation was detected by denaturing gel (Figure 4.8). This confirmed that bisQM's migration followed a strand displacement mechanism (Scheme 4.5).



Scheme 4.8. The decomposition of **OD4/OD5** is only initiated by the addition of **OD6**.

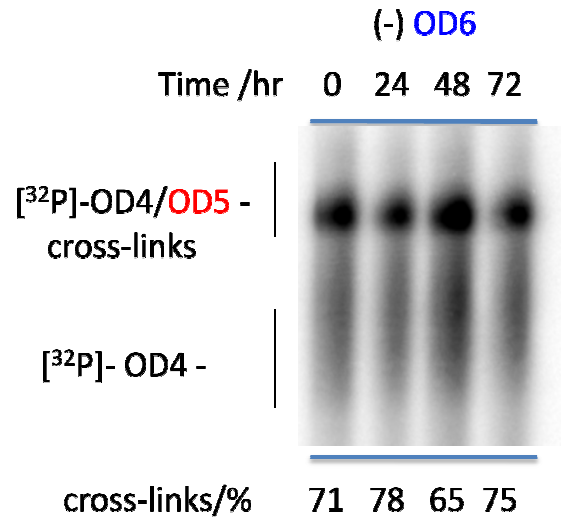
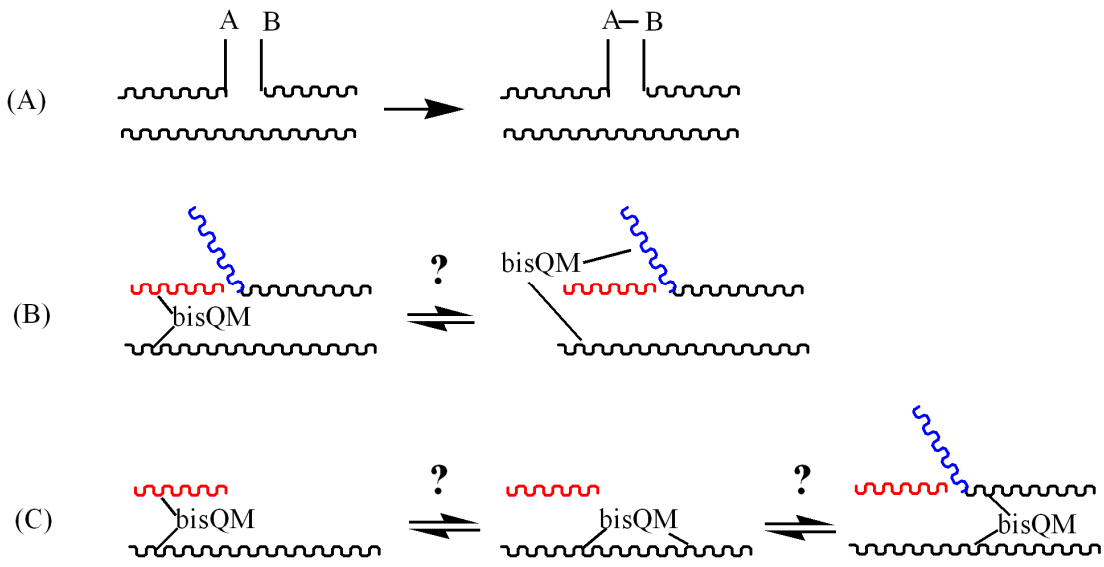


Figure 4.8. **OD4/OD5** cross-links are stable under aqueous conditions in the absence of **OD6**. 3.0 μM 5'-[^{32}P]-radiolabeled **OD4** was first incubated with bisQMP (60 μM) in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature and 50 mM NaF was added to initiate the reaction and the mixture was further incubated with 3.3 μM **OD5** for 24 hr. The sample was further incubated for indicated time and analyzed by 15% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA.

4.2.3. BisQM's migration requires complementary sequences

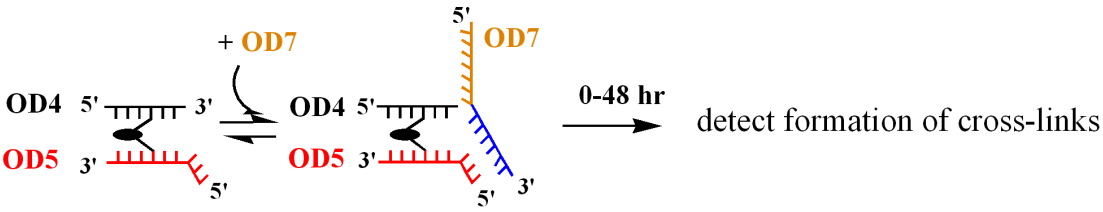
DNA duplex formation can provide remarkable control over the effective molarity of DNA-linked reactants. For many DNA-templated reactions, products form efficiently even when reactive groups are separated by large distances on the template ("distance- independent synthesis") (Scheme 4.9A).[89] Such reactions have been utilized for organic synthesis in the past decade.[89-91]



Scheme 4.9. DNA templated reaction and possible QM's migration among non-complementary DNA strands.

DNA templated reactions may provide a scheme to explain bisQM's migration among DNA strands alternative to a strand displacement process (Scheme 4.6). If the high effective molarity of the QM and a third sequence was the major requirement of QM's migration, a fully complementary sequence would not be necessary since the toehold is already able to stabilize the association with the DNA template (Scheme 4.9 B). Another possible mechanism is described in Scheme 4.10C. After the formation of initial DNA cross-links, bisQM could move to the toehold region by reversible alkylation and form intrastrand crosslinking. The bisQM could then be transferred to a third strand and form cross-links in the toehold region. In order to confirm that bisQM's migration followed a strand displacement mechanism instead of DNA template catalysis, a non-complementary sequence **OD7** (5'-CTT GAG ATA CTT TTT TTC TGC GCG TCG TTG AAG AGG TAA A-3') was used to examine the necessity of a sequence fully complementary to **OD5** (Figure 4.9).

(A)



(B)

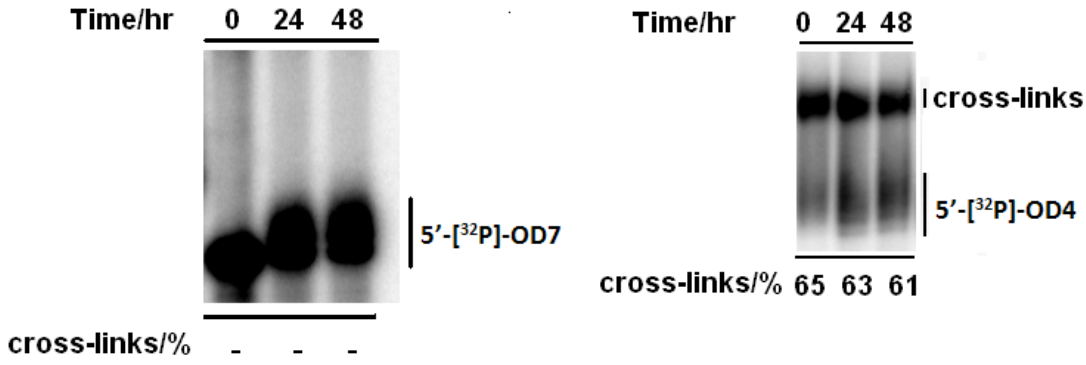


Figure 4.9. Non-complementary sequence **OD7** can not trigger bisQM's migration. (A) 3.0 μM **OD4** was first incubated with bisQMP (60 μM) in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature and 50 mM NaF was used to initiate reaction. The mixture was further incubated with 3.0 μM **OD5** for 24 hr. Then 2.7 μM 5'-[^{32}P]-radiolabeled **OD7** was added and samples were incubated for indicated time and analyzed by 15% polyacrylamide denaturing gel electrophoresis. (B) **OD4** was radiolabeled instead of **OD7**and experiment followed identical procedure to (A). Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA.

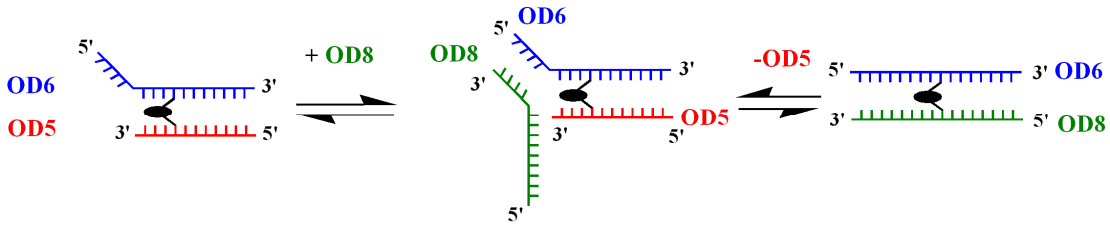
OD7 also contains a 16 base segment (Figure 4.9 A, blue segment) that is complementary to the toehold region in **OD5** and therefore was able to associate with **OD4/OD5** cross-links. However, the rest of **OD7** (Figure 4.9 A, yellow segment) was not complementary to **OD5** and could not initiate strand displacement process. **OD7** was incubated with the **OD4/OD5** cross-links for up to 48 hr under identical conditions to Figure 4.4 and no **OD7/OD5** crosslinking formation was detected by denaturing gel (Figure 4.9 B), which indicated that bisQM was not able to form cross-links with the non-complementary sequence **OD7**. In meantime, no

degradation of **OD4/OD5** was observed in the presence of **OD7**. This confirmed that the presence of non-complementary sequence **OD7** did not interrupt original DNA cross-links. Although the association between **OD7** and **OD5** can increase the effective molarity between bisQM and **OD7**, nucleophiles of **OD4** are more accessible to the regenerated QM because bisQM is always located in original duplex structure without strand displacement process. The fact that **OD7** was not cross-linked also indicated that bisQM did not migrate from **OD4/OD5** duplex structure to a joint duplex structure formed by **OD7** and the toehold of **OD5** directly. Result in Figure 4.9 also eliminated the possible action of bisQM described in Scheme 4.9 C. BisQM therefore can not move from original cross-links to toehold region and crosslink **OD7**. Therefore, bisQM's migration was sequence specific and must have followed a strand displacement mechanism.

4.2.4. BisQM can remain dynamic through multiple steps of migration by strand displacement

When the G rich sequence **OD6** reacted with regenerated QM from **OD4**/**OD5** cross-links, dG N7 should be the primary alkylation sites because of its strong nucleophilicity.[43] Similarly, bisQM should have alkylated **OD5** primarily at the strong nucleophilic site dC N3 and dA N1by forming reversible bonds. Therefore, bisQM should remain reversible in the newly formed **OD5/OD6** cross-links and have the potential to migrate for a second time because QM-**OD5** and QM-**OD6** bonds could both be reversible. In meantime, the longevity of bisQM will be extended in

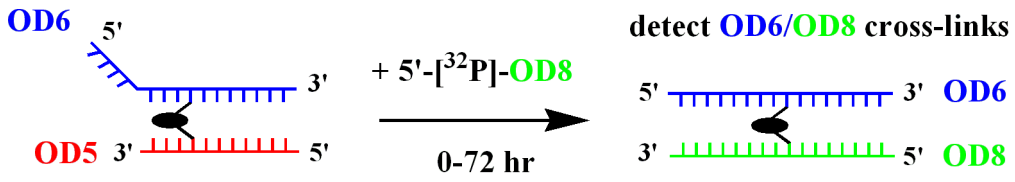
the form of reversible DNA cross-links.



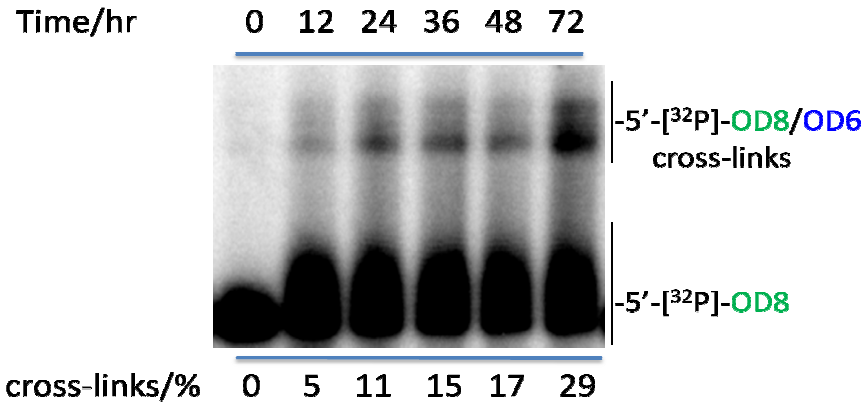
Scheme 4.10. BisQM can migrate to a fourth strand in a strand displacement system.

OD6 was designed to contain an extension beyond its complement to **OD5** and offers a single-stranded toehold region beyond the **OD5**/**OD6** duplex structure (Scheme 4.10). Therefore, addition of **OD8** will initiate strand displacement after associating with the toehold of **OD6**. When the bisQM is regenerated by breaking its QM-**OD5** bond, strand displacement is possible and **OD8** could react with bisQM intermediate to form **OD8**/**OD6** cross-links (Scheme 4.10).

(A)



(B)

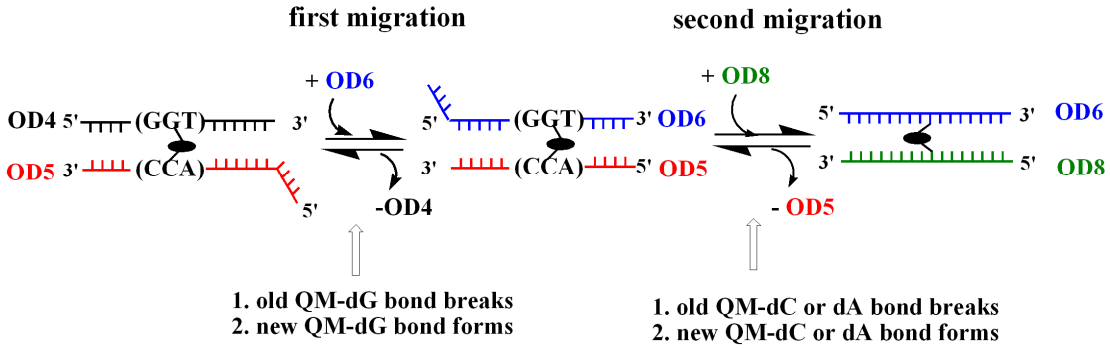


(C) OD8:5'-TTT ACC TCT TCA ACC GCC ACC CAC CAC ACA CCC AAC CAC CAC ACC ACG ACG TCA GTA G-3

Figure 4.10. Formation of **OD6/OD8** cross-links upon addition of [^{32}P]-radiolabeled **OD8**. (A) Formation of **OD6/OD8** cross-links. (B) After formation of **OD5/OD6** cross-links using the same procedure in Figure 4.4, 3.0 μM 5'-[^{32}P]-radiolabeled **OD8** was added into reaction mixture and incubated for the indicated time. Samples were analyzed by 15% denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA. (C) Sequence of **OD8**.

Incubation of **OD5/OD6** cross-links and radiolabeled **OD8** generated a new crosslinked species, which was assigned as **OD8/OD6** crosslinking (Figure 4.10 B). After 72 hr incubation, 29% crosslinking of **OD6/OD8** was detected in denaturing gel, which is formed from 50% of **OD6** that was cross-linked with **OD5**. The formation of **OD8/OD6** indicated that bisQM remained dynamic in DNA cross-links even after its previous migration and was able to migrate again by reversible alkylation in a

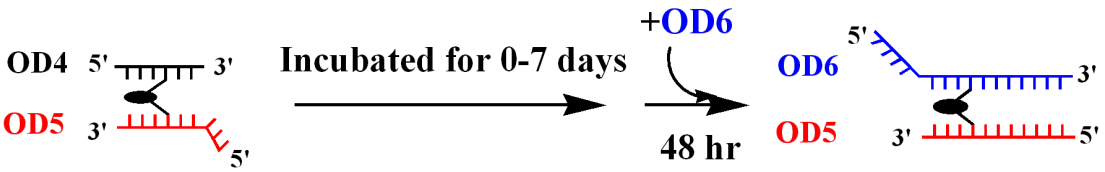
strand displacement system. After the second QM migration, both benzylic positions of bisQM have shown the ability to regenerate the QM intermediate from **OD4** and **OD5** adducts.



Scheme 4.11. BisQM's first and second migration in strand displacement system.

The lifetime of QM for migration in its DNA cross-links was examined by incubating **OD4/OD5** cross-links for up to 7 days before the initiation of strand displacement reaction (Figure 4.11). Radiolabeled **OD6** was added to detect reactive bisQM in the strand displacement system. The formation of **OD8/OD6** cross-links gradually decreased from 51% to 10 % when incubation time was extended from 0 to 7 days (Figure 4.11). This decrease indicated the loss of QM's reactivity due to irreversible reactions, both from water and weak nucleophiles of DNA that could form irreversible adducts. BisQM equivalents were preserved by duplex DNA and the half life of bisQM for migration in **OD4/OD5** cross-links was estimated as about 3-4 days.

(A)



(B)

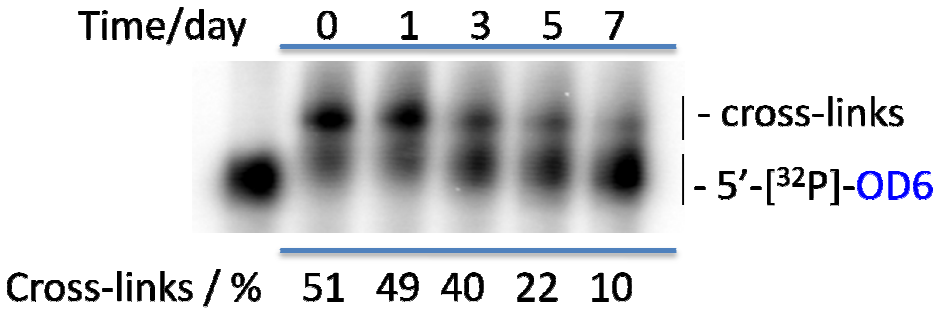
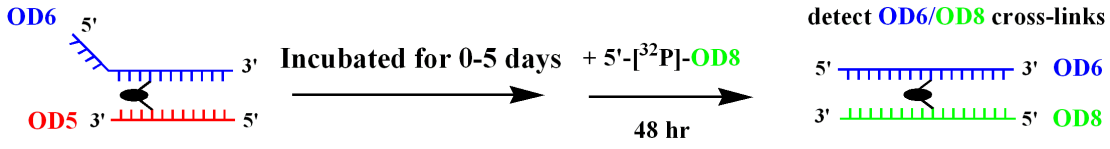


Figure 4.11. BisQM remains reactive for up to 7 days inside **OD4/OD5** and can continue to alkylate **OD6**. After formation of **OD4/OD5** cross-links following the same procedure in Figure 4.4, the reaction mixture was incubated for indicated time before addition of 3.0 μM 5'-[^{32}P]-radiolabeled **OD6**. The reaction was further incubated for another 48 hr at room temperature and analyzed by 15% denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA.

The persistence of bisQM's reactivity in DNA cross-links was again examined by incubating **OD5/OD6** crosslinking after the first QM migration (Figure 4.12). Radiolabeled **OD8** was added to detect reactive bisQM in the strand displacement system. The formation of **OD8/OD6** cross-links gradually decreased from 30% to 17 % when the incubation time was extended from 0 to 5 days (Figure 4.12 B). BisQM equivalents were still well preserved by **OD5/OD6** duplex DNA and after incubation of 5 days, more than 50% of bisQM's reactivity remains for further alkylation. BisQM's half-life in **OD4/OD5** duplex and **OD5/OD6** duplex for DNA crosslinking was both about 5 days (Figure 4.11, 4.12). BisQM seemingly has

similar lifetime in duplex DNA with similar sequences.

(A)



(B)

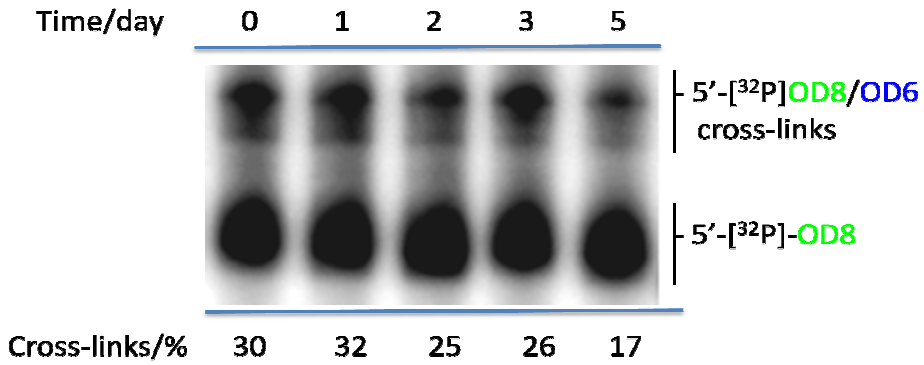
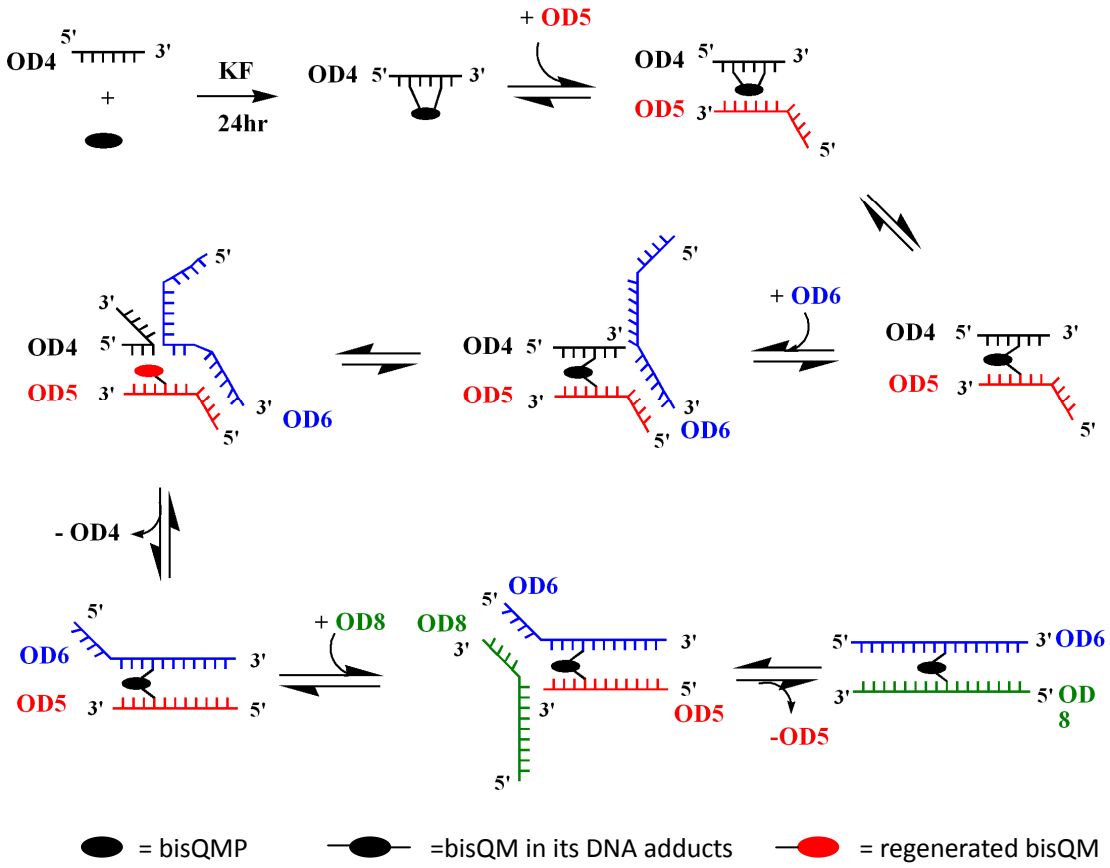


Figure 4.12. BisQM remains reactive for up to 5 days inside **OD5/OD6** cross-links and can continue to alkylate **OD8**. After formation of **OD5/OD6** cross-links following the same procedure in Figure 4.4, the reaction mixture was incubated for the indicated time before addition of 3.0 μM 5'-[^{32}P]-**OD8**. The reaction was further incubated for another 48 hr at room temperature and analyzed by 15% denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA.

To summarize, bisQM's migration among DNA strands has been demonstrated in a strand displacement system (Scheme 4.12). Starting with single-stranded DNA **OD4** and bisQMP-acridine conjugate, an intrastrand crosslinking of **OD4** was formed. This **OD4**-bisQM adduct then associated with **OD5** to generate interstrand cross-links by QM's sequence selective transfer (step 1). Addition of a third sequence **OD6** initiated the strand displacement process and bisQM was able to migrate to **OD5/OD6** duplex by forming corresponding cross-links (step 2). A fourth sequence **OD8** triggered bisQM's migration for a second time by replacing **OD5** and generating

OD6/OD8 crosslinking (step 3). The whole process involved at least 5 alkylation events and 3 times of QM regeneration. Both benzylic position of bisQM was substituted at least twice. The lifetime of QM-nucleoside adducts is usually only a few hours. For example, the half-life of dG N7-QM adduct is about 2~3 hours.[43] Thus, 5 alkylation events and 3 times of QM regeneration are only minimal numbers of QM's reversible alkylation during more than days of incubation. BisQM was transferred among DNA strands for 3 times and complete the migration from one duplex DNA structure (**OD4/OD5**) to another completely different duplex DNA (**OD6/OD8**) (Scheme 4.12). The extraordinary long lifetime of bisQM inside its DNA cross-links may enhance its function in biological systems involving strand displacement process, such as the repair of DNA damage. The cross-links caused by bisQM could be very hard to repair due to its ability to migrate among DNA strands. BisQM alkylation and crosslinking of DNA could cause lasting pressure on cell because of its long lifetime.



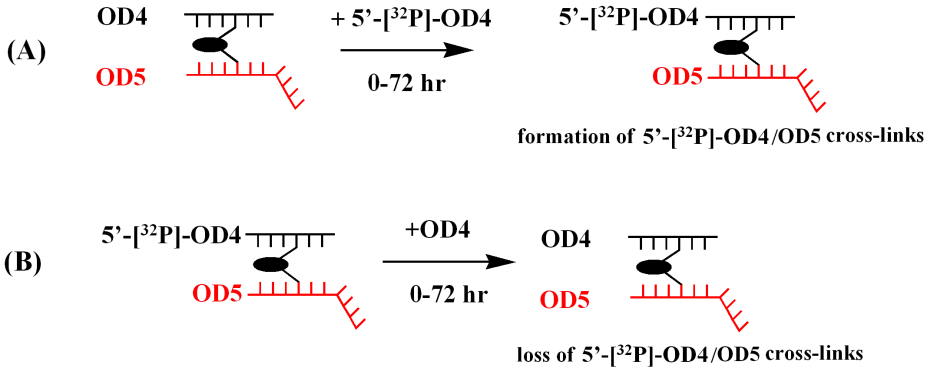
Scheme 4.12. BisQM's migration among DNA strands by reversible alkylation in a strand displacement system.

4.2.5. The role of toehold during bisQM's migration in a strand displacement system

A short single-stranded toehold is often used to initiate the strand displacement reaction.[92-94] The exchange of DNA strands is usually very slow but the presence of toehold can significantly accelerate this process. In one example, a toehold of 10 nucleotides was able to accelerate strand displacement reaction by 6 orders of magnitude.[94] Previous studies on the kinetics of strand displacement reactions also observed an exponential dependence of kinetics on the length of the toehold.[95] This is because longer toeholds can facilitate the assembly of DNA reactants and stabilize the tri-strand DNA assembly.[95] However, this effect saturates when the toehold

reaches sufficient length, which varies in different reaction networks.[86, 94] With a toehold of sufficient length, strand displacement reactions are very fast and usually complete in a few minutes.[86, 92, 94] The regeneration of bisQM intermediates usually takes hours and migration of bisQM within duplex DNA takes days to complete. In addition, the presence of toehold makes the formation of new duplex structure thermodynamically favored because more base pairing will be formed after strand displacement (Figure 4.1B). Therefore, it is difficult to predict whether a toehold can facilitate the strand displacement kinetically or thermodynamically.

An experiment was conducted to examine the thermodynamic effects of toehold on QM's migration. In Figure 4.5, a strand displacement experiment was performed in the presence of a 16 base toehold. In contrast, a strand exchange experiment without a toehold should show how the formation of new DNA cross-links was effected by the absence of toehold (Figure 4.11). After the formation of **OD4/OD5** crosslinking, 0.9 equivalent of 5'-[^{32}P]-**OD4** was added and strand exchange between **OD4** in DNA cross-links and 5'-[^{32}P]-**OD4** was expected without the assistance of toehold (Scheme 4.13).



Scheme 4.13. Strand exchange experiments to study the role of toehold during QM's migration.

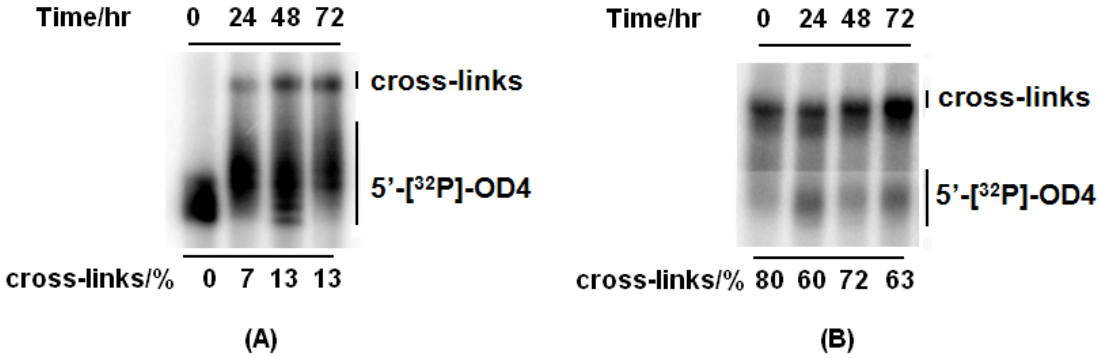
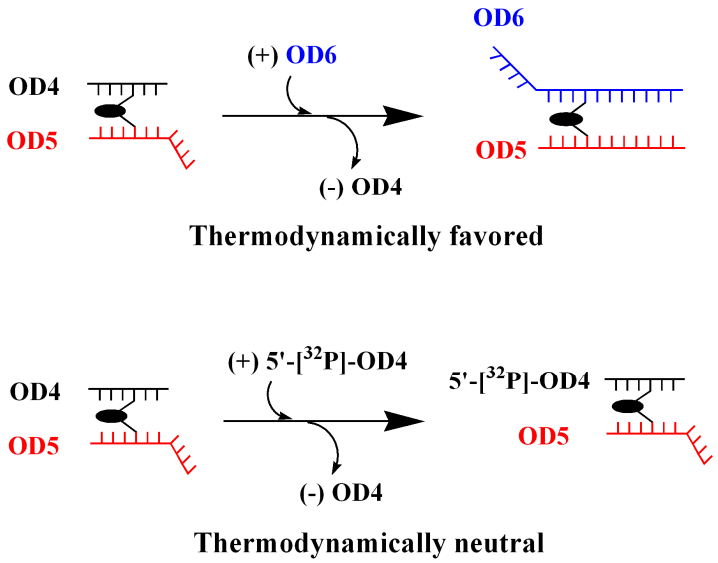


Figure 4.13. The formation of **OD5/OD6** is decreased in a strand dispalcement system without a toehold. (A) 3.0 μM **OD4** was first incubated with bisQMP (60 μM) in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature after 50 mM NaF was added to initiate the reaction. The mixture was further incubated with 3.0 μM **OD5** for 24 hr. Then 2.7 μM 5'-[^{32}P]-radiolabeled **OD4** was added and incubated further for indicated time and analyzed by 15% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA. (B) Experiment started with 5'-[^{32}P]-radiolabeled **OD4** and then non-radiolabeled **OD9** was added in an identical procedure to that described in (A).

After incubating **OD4/OD5** cross-links and 5'-[^{32}P]-**OD4**, the accumulation of 5'-[^{32}P]-**OD4/OD5** crosslinking was detected by denaturing gel and the maximum crosslinking was achieved at 13% after 48 hr (Figure 4.13A). Concurrently, the initial DNA cross-links decomposed over time and the crosslinking yield deceased from 80% to 63% after 72 hr when **OD4** was diluted with equivalent non-radiolabeled **OD4** (Figure 4.13B). Without a toehold, the maximum crosslinking caused by QM migration was much lower than the system which contained a 16 bases toehold (Figure 4.4). The toehold segment provided extra base pairing and therefore the formation of **OD5/OD6** cross-links was thermodynamically favored (Scheme 4.14). **OD4** was expected to be replaced by **OD6** completely and the ideal maximum formation of **OD5/OD6** cross-links would be 100%, which meant that all bisQM

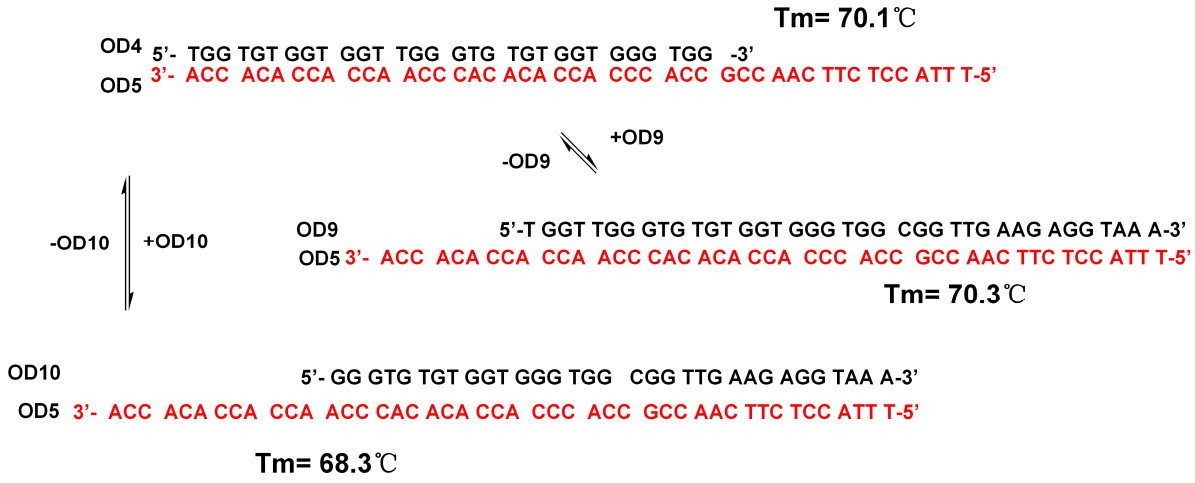
migrate from original **OD4/OD5** cross-links to **OD5/OD6** duplex (Scheme 4.14). In contrast, the strand exchange between **OD4** and 5'-[^{32}P]-**OD4** was thermodynamically neutral. Only 50% of **OD4** in **OD4/OD5** cross-links could be replaced by incoming 5'-[^{32}P]-**OD4** in maximum (Scheme 4.14). Therefore, the formation of **OD5/OD6** cross-links should be twice as much as the formation of 5'-[^{32}P]-**OD4/OD5** cross-links (Scheme 4.14). However, the formation of **OD5/OD6** was about 50% (Figure 4.5), which is 3 fold more than the formation of 5'-[^{32}P]-**OD4/OD6** cross-links (Figure 4.13). This indicated that the formation of new DNA cross-links was under thermodynamic control. Since 2-3 days were required to reach maximum crosslinking despite the presence or absence of a toehold (Figure 4.5, Figure 4.13), no apparent acceleration of QM's migration was observed when a toehold was present. Preliminary results suggested the presence of a toehold increased the maximum yield of new crosslinking species under thermodynamic control but did not accelerate QM's migration.



Scheme 4.14. Thermodynamics of strand displacement reactions with and without a toehold.

4.2.6. Crosslinking caused by QM migration is under thermodynamic control.

Preliminary results illustrated in Figure 4.5 and Figure 4.11 suggested that the toehold can facilitate the formation of new DNA crosslinking by providing a thermodynamic driving force. QMs seemingly prefer to migrate towards the thermodynamically stable duplex structure by forming the corresponding DNA cross-links. To confirm a correlation between crosslink migration and thermodynamic stability of duplex structures, single-stranded DNA **OD9** and **OD10** were compared to **OD6** for promoting strand displacement process and DNA crosslinking reactions.

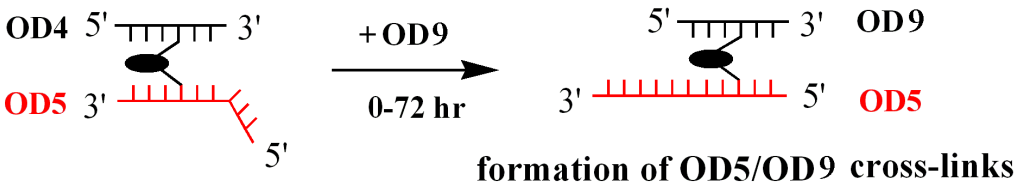


Scheme 4.15. Duplex DNA structures with different thermal stabilities formed by **OD5** and **OD4**, **OD9**, **OD10**. Melting temperatures of duplex DNA were determined by Oligoanalyzer 3.1 software provided by IDT Inc using the same ionic conditions and oligonucleotide concentration as in Figure 4.14.

OD9 and **OD10** are sequences of different length but both contain the same toehold segment to initiate the strand displacement reaction with **OD4**/**OD5** duplex DNA, whose melting temperature is 70.1℃ (Scheme 4.15). The melting temperature of **OD5**/**OD9** and **OD5**/**OD10** duplex structures are 70.3℃ and 68.3℃, respectively, as determined by Oligoanalyzer 3.1 software provided by IDT Inc using

the same ionic conditions and oligonucleotide concentration as in Figures. Therefore, the equilibrium between **OD4/OD5** duplex and **OD5/OD9** duplex structures is almost thermodynamically neutral. In comparison, the formation of **OD5/OD10** duplex is thermodynamically unfavored. **OD9** and **OD10** were alternatively incubated with cross-linked **OD4/OD5** and QM's migration was measured by the formation of **OD5/OD9** or **OD5/OD10** cross-links.

(A)



(B) (C)

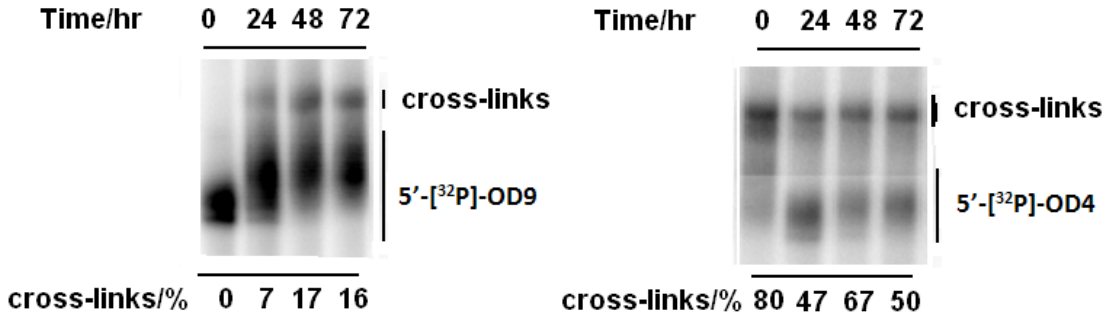
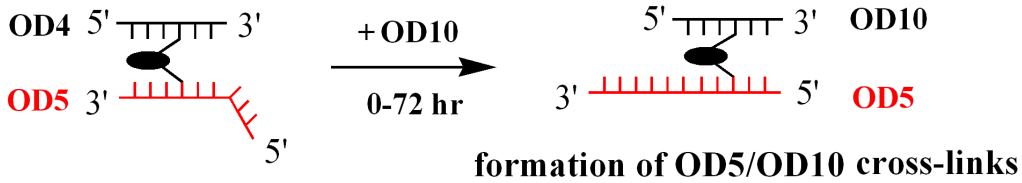


Figure 4.14. The formation of **OD5/OD9** and loss of **OD4/OD5** crosslinking caused by bisQM migration in a strand dispalcement system with toehold. (A) **OD4** was displaced by an incoming strand **OD9** by forming **OD5/OD9** cross-links. (B) 3.0 μM **OD4** was first incubated with bisQMP (60 μM) in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature and 50 mM NaF was added to initiate the reaction. The mixture was further incubated with 3.0 μM **OD5** for 24 hr. Then 2.7 μM 5'-[^{32}P]-radiolabeled **OD9** was added and incubated further for indicated time and analyzed by 15% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA. (C) Experiment started with 5'-[^{32}P]-radiolabeled **OD4** and then non-radiolabeled **OD9** was added in an identical procedure to that described in (B).

By incubating **OD9** and **OD4/OD5** cross-links, formation of **OD5/OD9** cross-links was detected by denaturing gel. The maximum crosslinking was achieved at 17% after 48 hr incubation (Figure 4.14). The time dependent formation of new crosslinking and the maximum crosslinking yield are almost identical to results in Figure 4.13. Strand displacement reactions in Figure 4.11 and Figure 4.12 are both thermodynamically neutral. **OD9** as an incoming strand can associate with toehold region in **OD5**, whereas **OD4** can not. Therefore, **OD9** is expected to facilitate the initiation and completion of strand displacement. However, the rate of **OD5/OD9** crosslinking formation was almost the same as the rate of 5'-[^{32}P]-**OD4/OD5** crosslinking formation in Figure 4.13. This result supported the assumption that a toehold did not accelerate the formation of new crosslinking and the maximum of QM migration was driven by thermodynamic equilibrium.

When **OD10** was the incoming strand to displace **OD4** from **OD4/OD5** cross-links, the migration of QM was examined following identical procedure in Figure 4.12. By incubating 5'-[^{32}P]-**OD10** with cross-linked **OD4/OD5** for up to 72 hr, less than 5% **OD5/OD10** crosslinking was detected by denaturing gel (Figure 4.15 B). The original **OD4/OD5** cross-links barely decomposed during the incubation period (Figure 4.15 C). The formation of **OD5/OD10** duplex is unfavored in strand displacement reaction because it has a melting temperature of 68.3℃, which is 2℃ lower than the starting **OD4/OD5** duplex DNA. The thermodynamic unfavored strand displacement reaction resulted in a very low formation of new crosslinking and thus bisQM preferred to form of DNA cross-links in more stable duplex structure.

(A)



(B) (C)

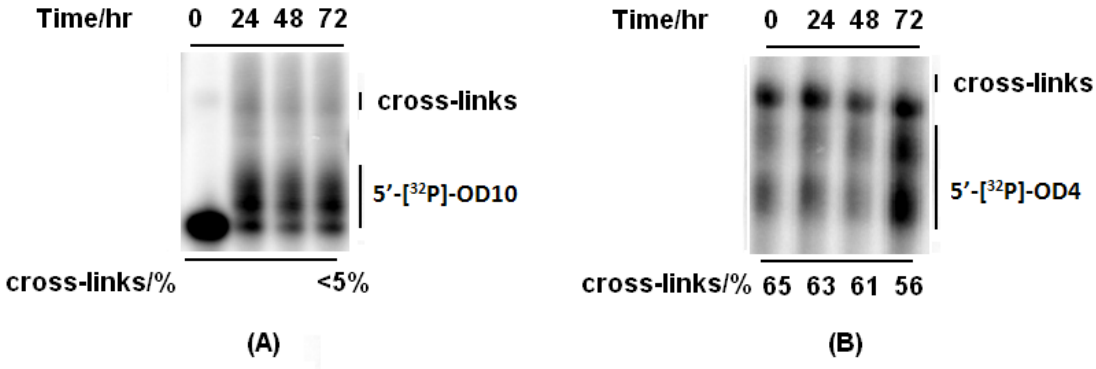


Figure 4.15. The formation of **OD5/OD10** and loss of **OD4/OD5** crosslinking caused by bisQM migration in a strand dispalcement system. (A) Experimental scheme of strand displacement reaction. (B) 3.0 μM **OD4** was first incubated with bisQMP (60 μM) in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature and 50 mM NaF was added to initiate the reaction. The mixture was further incubated with 3.0 μM **OD5** for 24 hr. Then 2.7 μM 5'-[^{32}P]-radiolabeled **OD10** was added and incubated further for indicated time and analyzed by 15% polyacrylamide denaturing gel electrophoresis. Cross-linked products were quantified by phosphoimagery analysis in the indicated area and reported (%) relative to total DNA. (C) Experiment started with 5'-[^{32}P]-radiolabeled **OD4** and then non-radiolabeled **OD9** was added in an identical procedure to that described in (B).

To summarize, the maximum formation of new crosslinking caused by QM migration is determined by the thermodynamic preference of the strand displacement reaction. When forward strand displacement is thermodynamically favored by forming more base-pairing, bisQM prefers to migrate into new DNA duplex by causing new DNA cross-links (Figure 4.5). When forward strand displacement is

unfavored, bisQM prefers to remain in original DNA duplex and does not migrate (Figure 4.15).

4.3. Conclusions

The proposed QM migration among DNA strands caused by its reversible DNA alkylation has been demonstrated by a strand displacement system. The dynamic QM-DNA bond allows the strand exchange process. BisQM remains reactive inside its DNA cross-links for up to 11days and continues to alkylate incoming DNA strand after strand displacement process. Both benzylic positions of bisQM have shown ability to regenerate QM intermediate. The reversibility of QM in duplex DNA can lead to the loss of DNA crosslinking because the regenerated QM intermediate can be trapped by external nucleophiles irreversibly and therefore lose its ability to cross-link DNA. Thiol has a stronger ability to trap QM regenerated from DNA cross-links than water because of its stronger nucleophilicity.

The maximum formation of new DNA crosslinking caused by QM migration is under thermodynamic control of the strand displacement reaction. BisQM prefers to form DNA cross-links in more stable duplex structure. The toehold in strand displacement system does not accelerate the migration of QM. The success of strand displacement reaction in DNA cross-links could be applied to the reversible alkylation of a variety of DNA alkylation agents.

4.4. Materials and Methods

Materials. Reagents and solvents were purchased were purchased as ACS grade or higher and used without purification unless noted. Oligonucleotides were purchased as desalting samples from IDT Inc. without further purification.

Methods. DNA reactions were analyzed using 20% or 15% polyacrylamide (19:1 acrylamide: bis-acrylamide) gel electrophoresis under denaturing conditions (7 M urea) as specified in figures. Gels were analyzed by phosphorimaging with a Molecular Dynamics phosphor screen and phosphorimager. Cross-linked products were reported (%) relative to total DNA.

DNA reactions

Formation of 5'-[^{32}P] radiolabeled and unlabeled oligonucleotide duplexes

OD1/OD2. Same procedure as described in Chapter 2.

Time-dependent loss of DNA crosslinking due to reversible alkylation of bisQM. Duplex DNA formed by 5'-[^{32}P]-**OD1** (3.0 μM) and **OD2** (3.3 μM) was first incubated at room temperature with bisQMP (30 μM) in 20% aqueous acetonitrile 10 mM MES pH 7. 10 mM KF was added to initiate reaction. After 36 hr incubation, 30% (v/v %) β-mercaptoethanol (3.8 M) or water was added and the mixture was further incubated for a period of time specified in figures at room temperature. Samples were analyzed by 20% denaturing gel electrophoresis. Results were

quantified by phosphoimagery using IMAGEQUANT software.

Strand displacement reactions. 3.0 μM **OD4** was first incubated with bisQMP (60 μM) in 20 % acetonitrile aqueous solution, 10 mM MES pH 7, for 24 hr at room temperature. 50 mM NaF was added to initiate reaction. The mixture was further incubated with 3.0 μM **OD5** for 24hr to allow the maximum formation of **OD4/OD5** cross-links. Then 3.0 μM of a third single-stranded DNA was added and incubated further for a period of time. The sequence of the third DNA strand is specified in each DNA reaction. Samples were analyzed by 15% polyacrylamide denaturing gel electrophoresis. The gel was pre-heated to 45℃ before loading of samples and the temperature of gel was maintained between 40℃ to 45℃ during gel electrophoresis. The results were quantified by phosphoimagery using IMAGEQUANT software.

Chapter 5. Conclusions

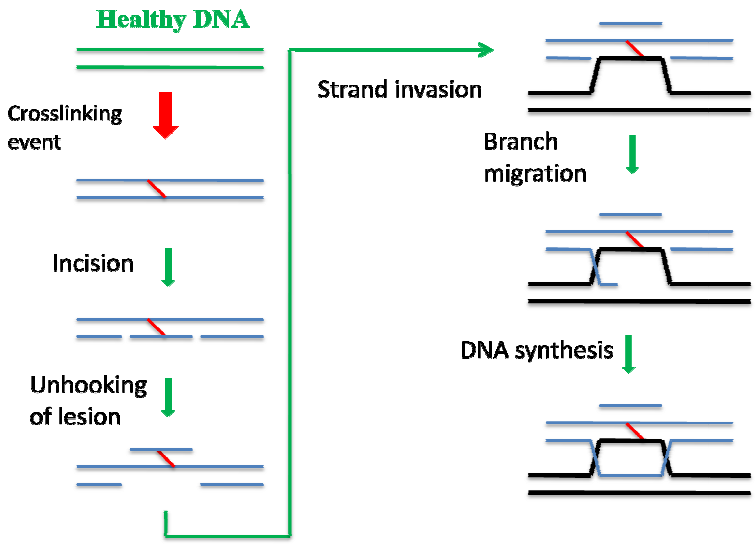
DNA alkylating agents comprise an important category of antitumor drugs as well as carcinogens. For DNA alkylating agents that react with DNA irreversibly, their covalent adducts can be analyzed by conventional methods involving dialysis, enzymatic digestion and adduct separation. However, reversible DNA alkylating agents pose a problem for analysis because a significant change of product distribution occurs during the days required for dialysis and digestion due to the reversibility of some of their adducts. On the other hand, reversible DNA alkylation can also be beneficial in some cases. The reversible reaction can extend the lifetime of DNA alkylating agents by their regeneration. It is also likely that reversible DNA alkylating agents could escape the DNA repair process after their excision by regeneration and subsequent reaction with DNA. This dissertation has focused on investigating the reversible reaction between quinone methide intermediates and nucleophiles of DNA that extends the longevity of those intermediates.

Previous studies focused on the reversible reactions between nucleosides and mono-functional QM. This dissertation extended these studies using a bi-functional QMP acridine conjugate. dA has demonstrated the ability to prolong bisQM's effective lifetime for DNA crosslinking by reversible alkylation using a bi-functional quinone methide precursor (bisQMP) conjugated to an acridine. The lifetime of bisQMP is short under aqueous conditions due to irreversible trapping of water. The repeated capture and release of bisQM from dA adduct can help QM equivalents

escape the irreversible trapping and extend bisQM's lifetime by 100-fold. This effect of dA saturates at a concentration of about 6 mM. Since there is abundant source of adenine derivatives in cell, QM's reversible alkylation can help QM escape irreversible trapping from nucleophiles other than DNA and expand the potential biological activity of QM intermediate in *vivo* by prolonging the lifetime of the fleeting intermediate.

Oligonucleotides have also shown an ability to capture and transfer bisQM to its complementary sequences selectively by forming crosslinking. The dynamic covalent bond between QM and oligonucleotide allows QM to transfer from intrastrand adducts to interstrand crosslinking. Non-complementary sequences can not be alkylated by bisQM-oligonucleotide adducts. The bisQM-oligonucleotide adducts, similar to a previously reported QM-oligonucleotide self-adduct, provide a strategy for sequence specific DNA alkylation based on the reversible alkylation of QM. The convenient preparation of bisQM-oligonucleotide adducts is an advantage for its application in practice. The sequences of oligonucleotides have an impact on their ability to transfer bisQM. G rich sequences have a strong ability to preserve bisQM equivalents and cause interstrand crosslinking. A and C rich sequences did not show an ability to transfer bisQM to complementary strands since alkylation on dA and dC likely interrupted the formation of duplex DNA. Further examination on A and C rich sequences' ability to transfer bisQM can be done by reducing the average number of bisQM linked to each DNA strand during preincubation, which may allow the formation of duplex DNA.

The reversibility of bisQM inside DNA cross-links has been demonstrated by a strand displacement system. This is the first study on the dynamics of a DNA cross-linker so far. The reversible QM-DNA bond allows the strand displacement process and bisQM can migrate in a series of changing DNA structures by forming crosslinking. Previous studies suggested that dG is an important site for DNA crosslinking due to the accessibility and reactivity of dG N7. However, the exchanging C rich sequences in the strand displacement system now suggest that dC in oligonucleotides is also important for DNA crosslinking and deserves further examination. The reactivity of bisQM is preserved beyond 11days in duplex DNA by forming labile DNA cross-links and QM can continue to alkylate DNA in the strand displacement system under aqueous conditions. This discovery of QM migration supports the assumption that the reversible alkylation of QM could lead to a product re-distribution over time. BisQM's biological function as a DNA cross-linker may be enhanced by its reversible alkylation because of its ability to cause repeated DNA crosslinking in changing DNA structures could counteract the repair process for DNA crosslinking in cells (Scheme 5.1).



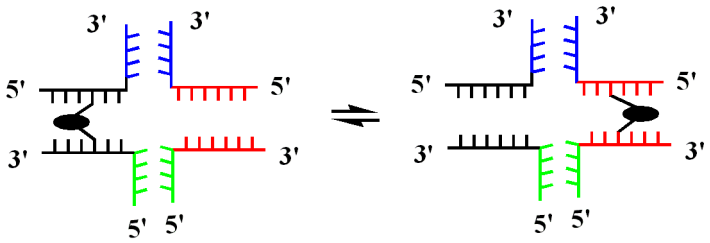
Scheme 5.1. BisQM can alkylate a third DNA strand during DNA repair after the initial crosslinking event.

The extraordinary long lifetime of bisQM in duplex DNA can cause lasting stress on cells and increase the possibility of apoptosis. This work also expands our knowledge on the action of QMs in their DNA adducts by demonstrating their stepwise migration. The mechanism of QM's migration might be applied to other reversible DNA alkylating agents with similar functionality.

The detection and analysis of the formation of DNA cross-links reply on denaturing gel electrophoresis. However, due to the reversible nature of QM reactivity, DNA cross-links may dissociate during gel electrophoresis under denaturing conditions. Once QM is regenerated from DNA cross-links, two DNA strands will be separated and appear as single strand species on gel. Therefore crosslinking yields determined by gel electrophoresis may be lower than the actual value and the crosslinking yield obtained from denaturing gel electrophoresis only represents the minimal amount of DNA cross-links.

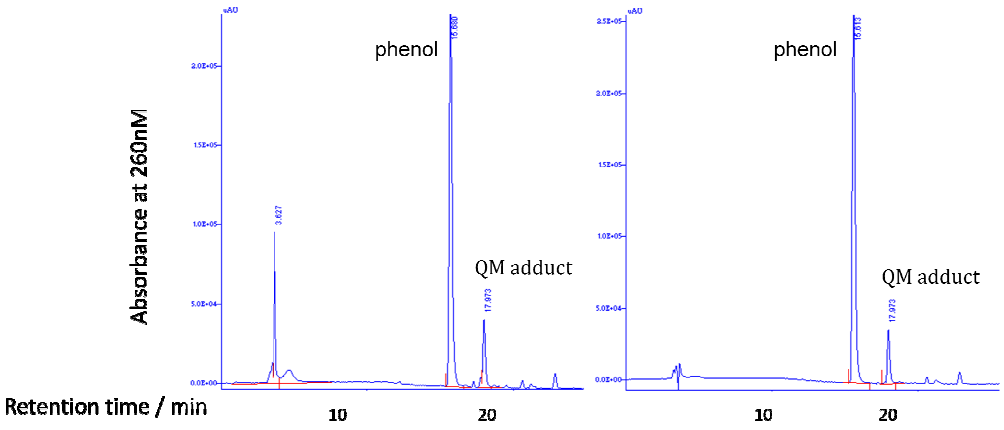
The discovery of bisQM's ability to transfer among DNA strands will encourage

us to further explore its reversible nature and potential utility. BisQM's migration in duplex DNA might be demonstrated by a "kissing" DNA complex (Scheme 5.2). Two duplex DNA of proper design can form a four-strand complex as shown in Scheme 5.2. BisQM's migration from one duplex to a second duplex DNA can be detected by radiolabeling two duplex DNA alternatively. The reversible bisQM-DNA bond can be used to stabilize strained complex DNA architectures and also allows further structural change because of the reversible bond does not prevent strand displacement.



Scheme 5.2. BisQM's migration from one duplex DNA to another duplex DAN in a "kissing" complex.

BisQM-oligonucleotide adducts can also be used as sequence specific DNA alkylating agents. A variety of oligonucleotide sequences have the ability to transfer QM and allow for selective alkylation. Therefore, it could provide a powerful method to control a variety of genes. Furthermore, the dynamic migration of bisQM as a DNA cross-linker among DNA strands is demonstrated for the first time and it could serve as a mode for other reversible alkylating agents with similar functionality. This discovery suggests the possibility of using reversible DNA alkylating agents to counteract the DNA repair process in cell.



(A) (B)

Figure 1. QM adduct compound **16** is stable for up to 7 days under 20% acetonitrile aqueous conditions at room temperature. (A) Compound **16** before incubation. (B) Compound **16** after incubation of 7 days at room temperature. Phenol was added as an internal standard. The sample were analyzed by reverse phase HPLC under analytical conditions (3% CH_3CN, 9.7 mM TEAA, pH 4, to 40% CH_3CN, and 5.2 mM TEAA, pH 4, over 35 min at 1 mL/min)

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