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Synthesis of Structurally Diverse Major Groove DNA Interstrand Crosslinks

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Abstract of the Dissertation

Synthesis of Structurally Diverse Major Groove DNA Interstrand Crosslinks

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DNA Interstrand Cross-links (ICLs) are extremely cytotoxic lesions caused by a variety of endogenous and exogenous agents. ICLs prevent the separation of two DNA strands and hence block essential cellular functions such as DNA transcription and replication. ICL causing agents are widely used in cancer chemotherapy and the removal of ICLs is associated with the resistance to anticancer agents. Despite the clinical importance of ICLs, the details of the pathways by which these lesions are repaired remain poorly understood.

This dissertation focuses on the synthesis of structurally diverse major groove DNA ICLs that induce different degrees of distortion into the DNA. We synthesized a three-carbon alkyl-aldehyde precursor and used it along with one and two carbon precursors to form a range of ICLs with different amines using the strategy of post-synthetic reductive amination. The newly formed ICLs induce little, moderate or no distortion into the DNA and provide insights into the structure and reactivity parameters of ICL formation by double reductive amination. These ICLs will be valuable substrates for exploring structure-activity relationships in ICL repair.

Additionally, we synthesized a 7-deazaguanosine moiety having an alkyl-amino linker and coupled it to an oligonucleotide having an aldehyde precursor to form an ICL connected by a single base. We also generated ICLs that were used to in collaborative efforts to study the role of structure-specific endnonucleases in ICL repair.

To my Parents

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List of Abbreviations

ICL: interstrand crosslink

NM: nitrogen mustard

MMC: mitomycin C

CENU: chloroethylnitrosourea

FA: Fanconi anemia

BER: base excision repair

NER: nucleotide excision repair

HR: homologous recombination

TLS: translesion synthesis

MMR: mismatch repair

ERCC1: excision repair cross complementing group 1

XPF: xeroderma pigmentosum

ss-DNA: single stranded DNA

ds-DNA: double stranded DNA

DSB: double strand break

DPC: DNA-protein crosslink

HDR: homology dependent repair

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Publications

1. **Mukherjee, S**., Guainazzi, A. and Schärer, O.D. (2014**)** Synthesis of Structurally Diverse Major Groove DNA Interstrand Crosslinks Using Three Different Aldehyde Precursors. ***Nucleic Acids Res***., Published online April 29, *doi: 10.1093/nar/gku328*.

2. Hodskinson, M.R., Silhan, J., Crossan, G.P., Garaycoechea, J.I., **Mukherjee, S**., Johnson, C.M., Schärer, O.D. and Patel, K.J. (2014**)** Mouse SLX4 is a tumor suppressor that stimulates the activity of the nuclease XPF-ERCC1 in DNA crosslink repair. ***Mol. Cell***, **54**, 472-484.

3. Wickramaratne, S., **Mukherjee, S.**, Villalta, P., Schärer, O.D. and Tretyakova, N. (2013) Synthesis of site-specific DNA-protein crosslinks that mimic N7-guanine lesions via a reductive amination strategy. ***Bioconjugate Chem***., **24***,* 1496-1506.

Chapter 1

General Introduction

Introduction

Genomic DNA is exposed to damage and chemical modifications by endogenous as well exogenous sources. One type of damage is DNA interstrand crosslinks (ICLs) - an extremely cytotoxic class of lesions that block essential functions of DNA such as replication and transcription and, if not repaired, leads to cell death. Studies have shown that 20-40 unrepaired ICLs in mammalian cells can be lethal (1). This cytotoxic effect of ICLs is utilized in anticancer chemotherapy and ICL forming agents such as cisplatin, nitrogen mustard, mitomycin C, chloroethylnitrosoureas are among the frontline chemotherapeutic drugs (2). ICLs can also form as a result of normal cellular processes such as oxidation of lipids that produce unsaturated aldehydes.

ICLs, like other DNA lesions, are removed from DNA by specific repair pathways. Studies of DNA repair pathways have taken advantage of cell lines that are sensitive to crosslinking agents. The importance of ICL repair is demonstrated by the existence of the disorder Fanconi Anemia (FA). Cells from patients suffering from this disorder are extremely sensitive to crosslinking agents. While cells have evolved mechanisms to counteract the effect of such lesions, it has been shown that repair of ICLs in tumor cells can lead to resistance against chemotherapeutic agents, providing a motivation to understand ICL repair pathways. The repair of ICLs is a complex process and mechanism of the individual steps as well as the proteins involved have been studied extensively (3). One of the major challenges involved in studying the repair process has been the difficulty of preparing site-specific ICLs in good yield that can serve as substrates. A major breakthrough in the field of ICL repair has been the development of strategies to synthesize site-specific ICLs in high yields and their subsequent use in cell-free biochemical system to study replication-dependent ICL repair (4-8). It is therefore of importance

to generate a range of structurally diverse ICLs that will provide more insights into the structure-function relationships of ICL repair. This will ultimately contribute to the understanding of resistance of tumor cells to therapeutic agents and eventually lead to the development of more effective anti-cancer chemotherapeutic drugs.

In the following sections, some of the most important ICL forming agents are discussed, followed by the ICL repair pathways and strategies of how this knowledge might be used in developing more effective anti-tumor agents. Finally, the different strategies employed to synthesize ICLs are reviewed focusing on recent developments in the generation site-specific ICLs.

Nitrogen Mustards

The ability of mechlorethamine (NM agent) to form ICLs in vitro was first reported by Brookes and Lawley in 1961 (9). The structure formulated by them was confirmed by molecular modeling studies showing the formation of NM ICL at a 5'-d(GC) position which would be the expected product (10). However, it was shown later that the actual site of nitrogen mustard crosslink is 5'-d(GNC) (11). This gave rise to considerable interest because the five atom tether is not long enough to bridge the distance between the N7 atoms of guanine in the 5'-d(GNC) sequence without inducing some sort of distortion in the DNA. It was then shown that NM ICLs induce a bend in the DNA to accommodate the alkyl chain tether (12).

NM form 1,3-GNC DNA ICLs by alkylation reaction of N,N-bis(2-chloroethyl)amine to a N7 of the guanine residues at 5'-d(GNC) sites (11,13,14). The mechanism involves the intramolecular cyclization of one of the two chloroethyl moieties of the NM (**1)** to produce an aziridinium ion (**2)** that can react with the N7 of the guanine.

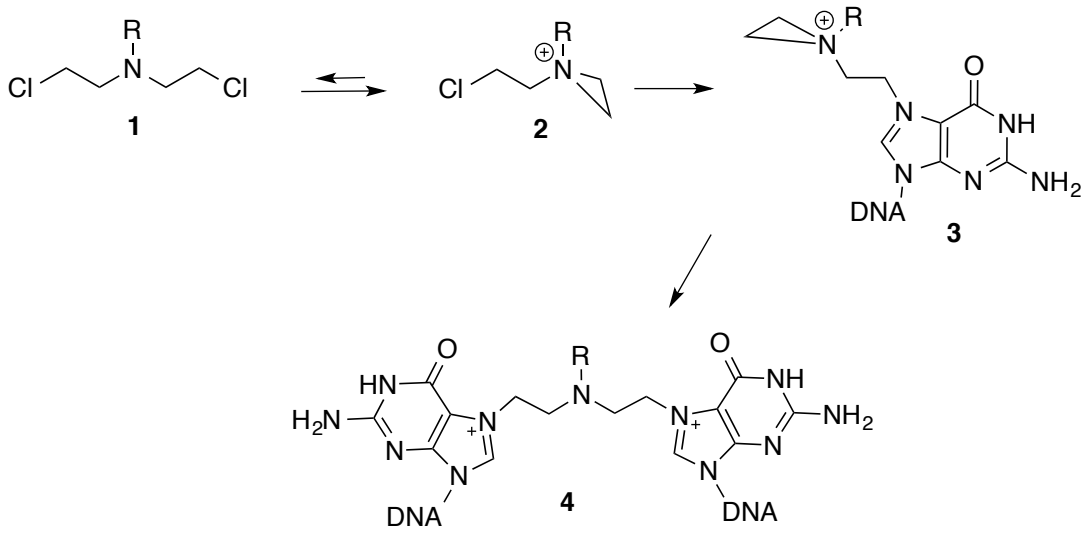

Figure 1.1: Mechanism of NM ICL formation

Cyclization of the other moiety produces a second aziridium ion that in turn reacts with a second guanine located on the opposite strand of DNA to give rise to the N7G-alkyl-N7G interstrand crosslink **4**. NM ICLs were initially generated by treating dsDNA with a nitrogen mustard that resulted in a small fraction of the desired ICL with mono-alkylated adducts being the predominant products. Another problem with NM is their instability on a laboratory time scale (few hours) that have hindered elaborate characterization studies (15-17). One stable way to generate NM ICLs is to treat the crosslink with a base to form the more stable formamidopyrimidine or ring opened form of N7-alkyl guanine (11). This treatment generated a crosslinked DNA that could be purified and inserted into a plasmid (18).

Our laboratory has developed a method to synthesize stable analogs of NM ICLs in a sequence specific manner with high yields. Our approach involved the incorporation of aldehyde moiety on the 7-position of a deazaguanine in complementary oligonucleotides and then using a double

reductive amination to form the ICL in a site-specific manner with high yields (19,20). The use of deazaguanine removed the inherent instability that is associated with a regular NM ICL. This approach has allowed us to synthesize major groove ICLs that induce different degrees of distortion into the DNA. As it is becoming increasingly clear that structure of ICL plays an important role in the repair of such lesions, these substrates would be valuable in advancing studies to understand the structure-function relationships in ICL repair.

Cisplatin

Cisplatin [cis-diamminedichloroplatinum(II)] is one of the most widely used anticancer drugs and has been successfully used to treat tumors of the neck, ovaries, lungs and testis. It was approved by the FDA in 1978 and has led to a cure rate of over 90% for testicular cancer when tumors are promptly diagnosed (21). Cisplatin **5** is a neutral, square planar coordination complex of platinum (II) with the two chloride ligands in *cis*-position. While the ammonia ligands remain strongly attached to platinum (II), the chloride ligands can be easily replaced by nucleophiles. Inside the cell, the chloride ligands are substituted by water molecules giving rise to an activated form of cisplatin **6** that can then form complexes with DNA, RNA or proteins (22).

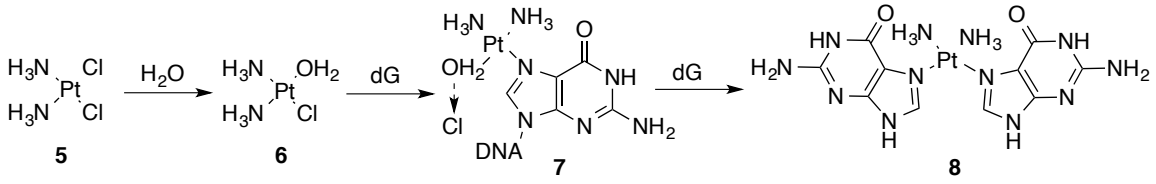

Figure 1.2: Mechanism of cisplatin ICL formation

With respect to DNA, adducts are formed through binding to the N7 atom of a guanine base giving rise to monoadducts **7** and subsequent crosslinks **8** (Figure 2). The various adducts formed by cisplatin are 1,2-intrastrand crosslinks in a 5'-d(GG) (47 - 50%) and 5'-d(AG) (23 - 28%) sequences followed by 1,3-intrastrand (GNG) and interstrand crosslinks (8 - 10%) and another 2 – 3% being monoadducts bound to guanine (22). The interstrand crosslink formed by cisplatin induces severe distortion into the DNA as shown by NMR (23,24) and X-ray crystallography (25). The cytosines that are Watson-Crick base paired to the crosslinking guanines, are flipped out into an extrahelical conformation. The helix is bent by 45° towards the minor groove and results in the groove being slightly enlarged (26). Cisplatin ICLs have been prepared majorly by hybridization directed crosslinking (27). An oligonucleotide having a unique 5'-d(GC) sequence is first allowed to react with the activated form of cisplatin (cis-$[Pt(NH_3)_2(H_2O)Cl]$). The product is purified on FPLC followed by annealing to the complementary strand to form the ICL. This strategy has been utilized in our laboratory to synthesize cisplatin ICLs that were incorporated into plasmids to study ICL repair (28). A derivative of a cisplatin ICL has also been synthesized in which one of the amines was conjugated to a photoreactive benzophenone group and the substrate was used to identify the difference in the pattern of nuclear proteins binding to intra- vs interstrand crosslinks (29).

Chloroethylnitrosoureas

Chloroethylnitrosoureas (CENUs) are highly active anticancer agents with broad antitumor spectrum known to modify both DNA and proteins (30). They decompose in water producing carbamolyating and alkylating species. It is the alkylating species that are responsible for the formation of DNA ICLs. CENUs **9** in aqueous solution decompose to generate a highly

reactive carbonium ion that transfers a chloroethyl group to the O6 position of deoxyguanosine **10** (31). Intramolecular cyclization of **11** with the N1 atom of deoxyguanosine results in removal of chloride ion to form a reactive tricyclic intermediate **12** that is then attacked by the N3 of a cytosine from the opposite strand to form the ethyl bridge crosslink **13** (Figure 3).

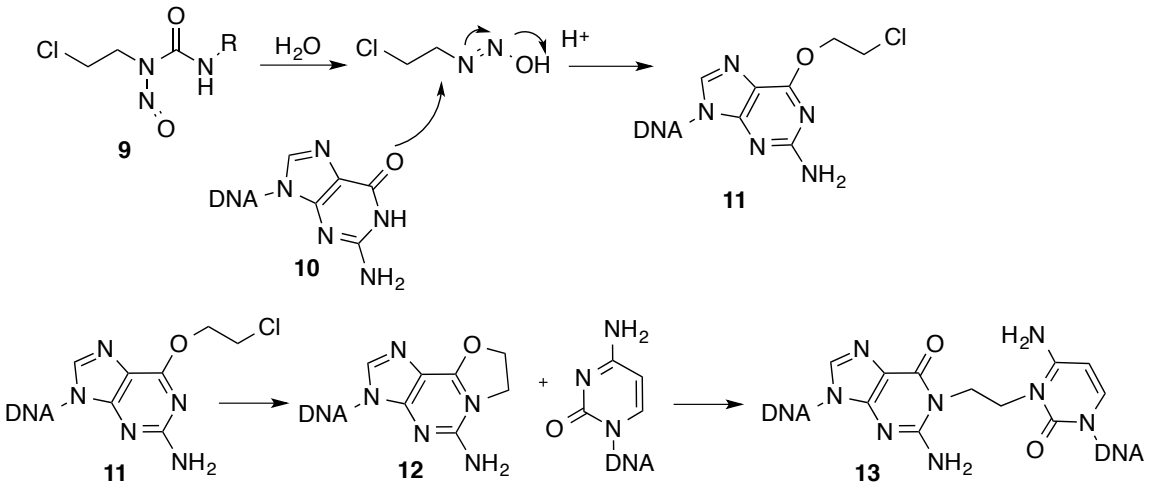

Figure 1.3: Mechanism of CENU ICL formation

CENU-like ICLs have been formed by either using a chloroethyl modified thymine (32) or by the incorporation of crosslinked nucleotide dimer into DNA using solid phase synthesis (33-35). The later strategy has been utilized to synthesize crosslinks that mimic the ones formed naturally by CENUs with varying bridge lengths and structure. A recent breakthrough was achieved by Luedtke and co-workers who used a photocaged nucleobase as an ICL precursor to form the ICL that formed by the biologically active BCNU. The photolabile protecting group on the ICL precursor was selectively removed after its incorporation into duplex DNA, triggering ICL formation (36).

Mitomycin C

Mitomycin C (MMC), a product of the mold *Streptomyces caespitosus*, is a chemotherapeutic agent that is used to treat a wide variety of tumors. It is particularly effective in the treatment of gastrointenstinal tumors, pancreatic, colorectal and anal cancer (37). MMC is relatively inert and requires chemical or enzymatic reduction of its quinone group to alkylate DNA with the preferred sequence being 5'-CG-3'. Like other bifunctional alkylating agents, the ICL formed is only a small fraction of the total number of adducts formed with DNA but the ICL fraction is higher than for cisplatin or CENU. Other adducts include a monoadduct and an intrastrand crosslink (38-41). The mechanism of ICL formation initiates via a two electron reduction of the quinone ring **14** to that facilitates the loss of methoxy group, leading to the formation of hydroquinone phenolic intermediate **15**. This is followed by a tautomerization and reaction with the exocyclic–NH_2 of guanine **16**. Elimination of the carbamoyl group gives the hydroquinone intermediate **17**, which then alkylates the guanine on the opposite strand of DNA, and after oxidation produces the ICL **18** (Figure 4) (42) ICLs with MMC have been synthesized by utilizing either a synthetic DNA duplex having a unique –CG- site and reacting it with MMC or hybridization directed method (43,44). The crosslink is formed in the minor groove of DNA and molecular modeling studies have shown that it induces minimum perturbation in the DNA (42). NMR studies on the mono adduct (45) and the ICL (46) have also shown no disruption of Watson-Crick base pairing with the only effect being a moderate widening of the minor groove to accommodate the MMC heterocycle. MMC ICLs have been used in a number of studies to understand ICL repair pathways as well as for biochemical purposes (47-49).

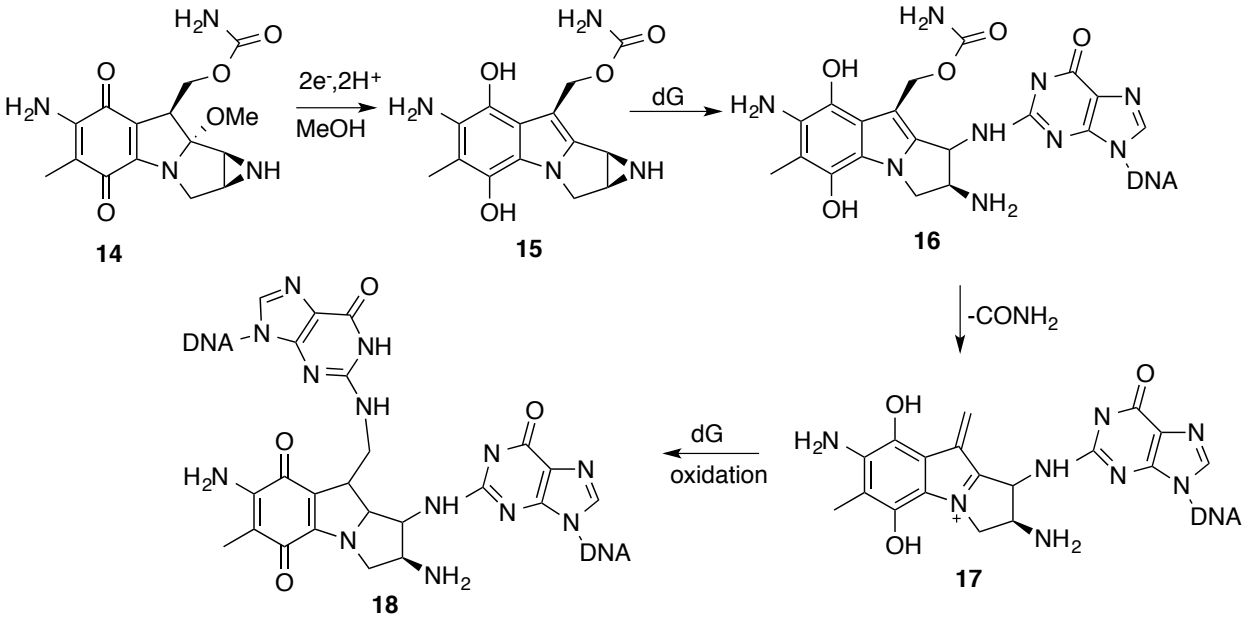

Figure 1.4: Mechanism of MMC ICL formation

Psoralens

Psoralens are linear furocoumarins found in leafy vegetables and plants and are used in the treatment of skin disease like vitiligo and psoriasis. They interact with DNA by intercalation and can form covalent adducts by irradiation with long wavelength (320-410 nm) UV light with thymines in 5'-TA-3' or 5'-AT-3' sequences (50,51). When intercalated in the DNA, ICL formation is initiated by UVA irradiation resulting in a [2 + 2] cycloaddition between the furan or pyrone ring of **19** and the adjacent thymine, leading to the formation of a monoadduct **20** linking the psoralen and thymine through a cyclobutane ring. The furan-side monoadduct can undergo a second cycloaddition between the pyrone double bond and thymine on the opposite strand, resulting in the formation of ICL **21** (Figure 5**)** (42).

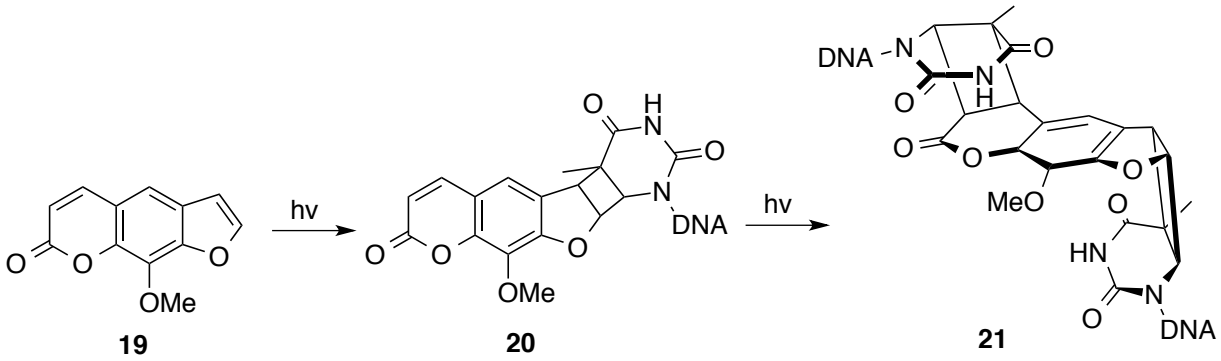

Figure 1.5: Mechanism of psoralen ICL formation

The use of high-intensity lasers to specifically produce the first monoadduct and solid-phase synthesis of the monoadduct with subsequent incorporation into the DNA have improved the efficiency of ICL formation (52,53). NMR and X-ray crystallography studies of psoralen ICLs have revealed that they produce local distortion at the site of DNA damage but have no effect on the overall structure of DNA (54-56). Psoralen ICLs are relatively easy to synthesize compared to nitrogen mustard and mitomycin C and are also chemically more stable. They have also been synthesized in living cells using psoralen conjugates (57). As such, they have been extensively used to study recruitment of repair proteins to psoralen lesions in cells (58) and for biochemical studies (59).

Endogenous ICL forming agents

DNA ICLs are also formed by endogenous agents arising from normal cellular metabolism. A major source of endogenous ICL forming agents are aldehydes that are produced as a result of the lipid peroxidation process, with the α, β-unsaturated ones being the most cytotoxic. The major aldehyde by-products of lipid peroxidation are 4-hydroxynoneal, acrolein,

malondialdehyde **22** and crotonaldehyde- all of which react with the exocylic amino groups of the guanine to produce DNA ICLs (60-62).

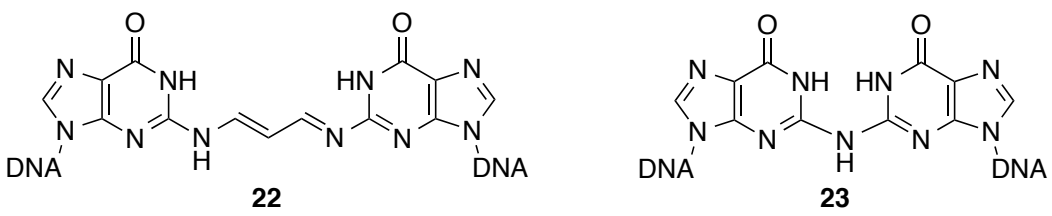

Figure 1.6: Structure of ICLs formed by reactive aldehydes **22** and nitrous acid **23**

However, these ICLs have are intrinsically reversible, which has made their detection and study difficult. To circumvent this problem Harris and coworkers have synthesized duplexes with a trimethylene ICL mimic in a 5'-GC-3' or 5'-CG-3' sequence and characterized it using NMR. The 5'-GC-3' was found to cause minimal distortion while the 5'-CG-3' destabilized the duplex and induced a distortion in the DNA duplex (63,64).

Another common endogenous agent that can form ICL is nitrous acid. Studies have shown nitrous acid forms stable ICLs at 5'-GC-3' or 5'-CG-3' sites (65). The diazotization of the exocyclic amino of a guanine followed by a nucleophilic attack on C2 of the diazotized guanine by the exocyclic amino group of neighboring guanine leads to the formation of the ICL (**23**, Figure 6). Using solid-phase strategy this type of ICLs have been incorporated into oligonucleotides as dimers (66,67). NMR studies have shown that these ICLs they induce distortion whereby the cytosines bound to the guanines are flipped out into the minor groove (67).

Abasic sites are another frequently found endogenous lesions caused by spontaneous hydrolysis of the glycosidic bond in DNA (68,69). This arises from oxidation of DNA under oxygen deficient conditions. Greenberg and co-workers have synthesized such an ICL between an adenine and a modified thymine (70-72).

Repair of DNA Interstrand Crosslinks

DNA ICLs pose a serious problem for the cell due to its unique feature of covalently linking two strands of DNA. This prevents essential cellular processes such as DNA replication and transcription. They also provide a serious problem for the cellular DNA repair machinery as ICLs block both strands of DNA an intact template is not available to restore the original DNA sequence, as is the case with monoadducts, which are repaired by nucleotide excision repair (NER) or base excision repair (BER). The first studies of ICL repair was carried out in *E. coli* by Cole and showed that the coordinated action of NER and homologous recombination (HR) is required to repair ICLs (73,74). Further studies carried out in *S. cerevisiae* showed the ICLs lead to the formation of DSBs during replication and that mutants defective in NER and HR were extremely sensitive to ICL forming agents (75-78). In eukaryotes, ICL repair is more complex and can take place in various stages of the cell cycle (during DNA replication or outside S/G2 phase). In addition, the involvement of proteins from pathways such as FA, translesion synthesis (TLS) and mismatch repair (MMR) make the repair process more challenging to understand. In the following sections, the two major pathways of ICL repair in mammalian cells, replication-dependent and replication-independent ICL repair, are discussed.

Replication-Dependent ICL Repair Pathway

ICL repair in mammalian cells were initially studied when DNA polymerase encountered ICLs during replication and their removal suggested a mechanism for repair of these lesions (79). Later experiments with psoralen ICLs suggested that repair of ICLs take place in the S phase of cell cycle (79). An important breakthrough in understanding ICL repair was achieved by the establishment of a biochemical system using plasmids having site-specific ICLs in cell free *Xenopus* egg extracts (80-82). Incubation of the plasmid with the egg extracts allows replication to happen under physiological conditions. In the S-phase of cell cycle (Figure 7), it is believed that the leading strand of the replication fork approaches the ICL and stalls 20-40 nucleotides away from the lesion. After a delay, one of the strands is extended to one nucleotide away from the ICL. Simultaneously, activation of FA core complex leads to the ubiquitination of FANCD2/FANCI proteins. The incision of the ICL is brought about by two structure-specific endonucleases. Current knowledge suggests that the 3' incision is brought about by ERCC1-XPF- that is recruited to the active site by SLX4 (83) while the exact nuclease making the 5'-incision remains to be determined. Dual incision leads to the unhooking of the ICL and creates a DSB in the lagging strand and an unhooked ICL in the leading strand. The unhooked ICL becomes a template for TLS, which bypasses the lesion and allows full extension of the leading strand. REV1 and Pol ζ are the key TLS polymereases in the repair pathway (80). The exact polymerase inserting the dNTP opposite the ICL and extending it past the lesion has been shown to be dependent on the structure of the ICL (62). Following TLS the unhooked ICL may be removed by NER and the leading strand serves as a template for HR-mediated DSB repair to generate a lesion free DNA sequence.

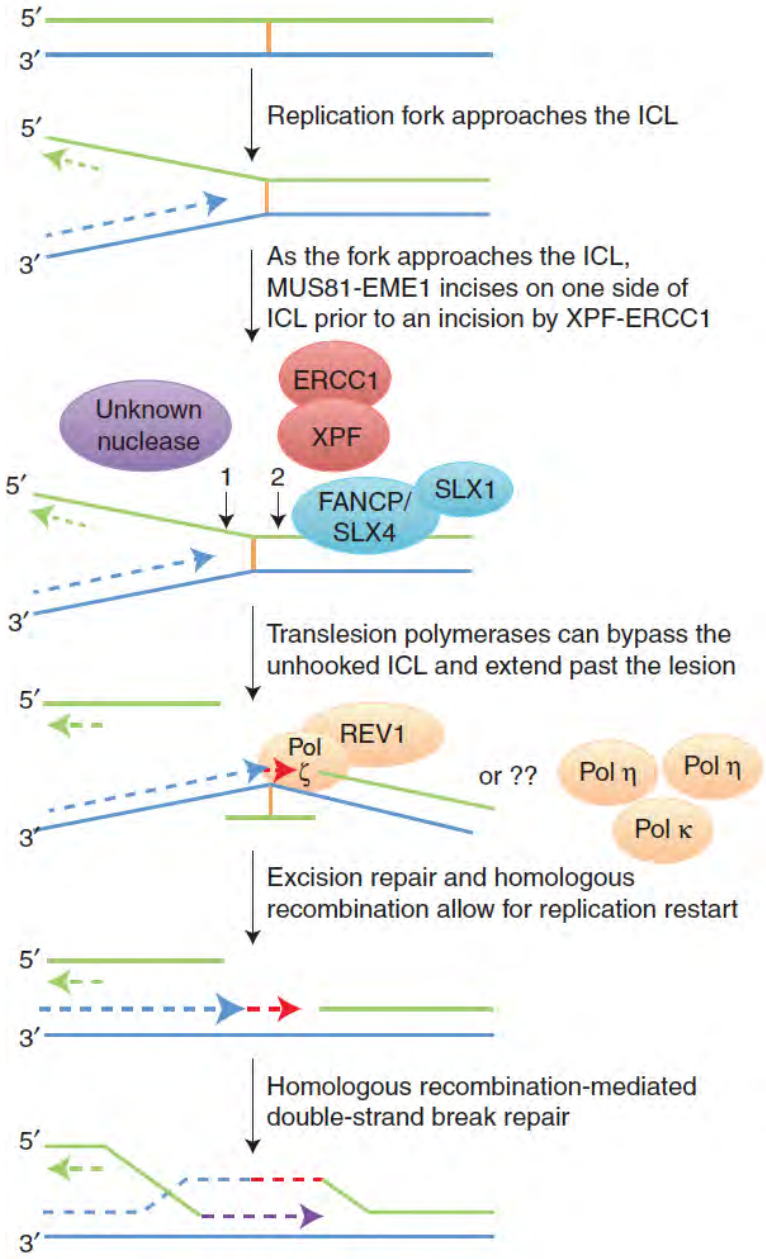

Figure 1.7: Model for replication-dependent ICL repair pathway. Replication fork stalls 20-40 nucleotides away from ICL. Dual incisions take place on lagging strand template by structure-specific endonucleases. Translesion synthesis polymerase extend leading strand past the ICL. HR and NER complete the process to generate an intact DNA sequence. The figure is taken from (3).

Fanconi anemia Proteins in replication dependent repair pathway

Fanconi anemia (FA) is a rare inherited genetic disorder and FA patients have developmental abnormalities, progressive bone marrow failure and an increased chance of developing cancer, especially acute myeloid leukemia and squamous cell carcinomas of the head and neck. To date 16 genes have been identified majorly by complementation analysis and cell fusion experiments: *FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM*, *FANCN*, *FANCO*, *FANCP* and *FANCQ*. A characteristic of cells derived from FA patients is their severe hypersensitivity to crosslinking agents such as MMC, cisplatin and diepoxybutane (84). Eight of the Fanconi proteins (A, B, C, E, F, G, L and M) form a large nuclear core complex (85), in which FANCL acts as a E3 ubiquitin ligase monoubiquitinating FANCD2 and FANCI following DNA damage. Monoubiquitination of FANCD2 and FANCI is an important step in ICL repair pathway (86). Removing FANCD2/FANCI or a mutation in FANCD2 leads to loss of the unhooking and TLS steps of ICL repair in the xenopus egg extract system (81). Deubiquitination of FANCD2/FANCI is needed to complete ICL repair (87). The two core complex proteins that have catalytic activities are FANCL and FANCM. As discussed above FANCL is a ubiquitin ligase and is required for the monoubiquitination of FANCD2-FANCI. FANCM along with FAAP24 and MHF1-2 (88-90) is believed to bind DNA when a replication fork stalls at an ICL and lead to the remodeling of the stalled replication fork. This process does not require the presence of core complex. Some other FA proteins such as FANCJ, FANCN act downstream of the ICL repair process and play a role in homologous recombination (91).

Roles of structure-specific endonucleases in replication dependent repair pathway

An important step in replication dependent ICL repair is the formation of double stranded breaks that take place at stalled replication forks. This enables the leading strand to continue DNA synthesis past the lesion (92) . The identity of the exact nuclease that brings about the incisions is not clear, but the key ones among these are XPF-ERCC1, MUS81-EME1 and SLX4-SLX1.

XPF-ERCC1 cleaves ds-DNA adjacent to a 3' single strand region. *XPF* has also been identified as a complementation group of FA (93). Cells defective in these enzymes have been shown to be sensitive to crosslinking agents (94,95). The absence of XPF-ERCC1 leads to accumulation of DSBs in response to an ICL (94-97) and also affects chromatin localization of FANCD2 (96,98). Current data suggests that XPF-ERCC1 is required to make one of the incisions near the ICL for the continuation of tranlesion synthesis and HR-mediated replication (99).

MUS81-EME1 is also a conserved endonuclease and the deletion of either protein causes hypersensitivity of crosslinking agents. DSBs formation in response to crosslinking agents is also dependent on MUS81-EME1 (100,101). This protein has been found to play a role in Holliday junction resolution and has a preference for complex DNA junctions (102). Thus it might suggest that MUS81-EME1 has a role to play in converting stalled replication forks at ICLs to DSBs for the start of HR-mediated repair.

SLX4-SLX1 is an endonuclease that interacts with both XPF-ERCC1 and MUS81-EME1. SLX4 knocked down cells are extremely sensitive to crosslinking agents and are deficient in HR (103). SLX4 is recruited to sites of DNA damage foci via interaction with

monoubiquitinated FANCD2 (104-106). The key function of SLX4 is thought to be that of a scaffold and recruit the nucleases to the site of DNA damage.

Role of TLS polymerases in replication dependent repair pathway

An important step in mammalian ICL repair is translesion synthesis (TLS). As suggested in the model of replication dependent pathway, TLS polymerases bypass the unhooked ICL from one of the two crosslinked strands. In humans, a number of DNA polymerases, including Pol ζ, Pol η, Pol ι, Pol κ, Pol ν and REV1 have been found to bypass various DNA damages (107,108). Among them Pol ζ (REV3-REV7) and REV1 have been found to be important in ICL repair (109,110). Chicken DT40 cells that are deficient in REV3 and cell lines deficient in REV1 are extremely sensitive to crosslinking agents (109,111,112). Studies in *Xenopus* egg extracts have also shown a role of Pol ζ and REV1 in replication-dependent ICL repair (80). Current understanding suggests that dual actions of Pol ζ and REV1 are needed to insert a nucleotide opposite the unhooked ICL and allow for the extension to restore one of the two sister chromatids. REV1 and Pol ζ also bypass psoralen, MMC or cisplatin ICLs in replication-independent ICL repair (28,113).

The role of other TLS polymerases in ICL repair is not well understood, and cells deficient in their genes are less sensitive to ICL forming agents than those lacking Rev1 and Pol ζ. Pol η has a role in the repair of MMC and psoralen ICLs (114-117) and can also bypass non-distorting ICLs (118). Pol κ can bypass minor groove trimethylene ICLs (62), while it has less activity with major groove ICLs such as cisplatin and nitrogen mustard ICLs (118). Pol ν has been found to bypass major groove ICLs efficiently and poorly with minor groove ones (119,120).

Overall, this suggests that the structure of the ICL plays an important role in the choice of polymerases that is involved in their repair.

Replication-independent ICL Repair Pathway

There is increasing evidence that ICL repair also takes place in G0/G1 phase of cell cycle in the absence of replication. These studies have been carried out yeast systems (121) and by using site-specific ICLs in reporter plasmids that have no replication origin present (28,49,113,122). Both NER and TLS were found to be essential in this pathway. In mammalian cells, the studies have been done by having site-specific ICLs in oligonucleotides and ligating them into the promoter region of a gene of a plasmid. ICLs formed by psoralen, MMC and cisplatin were found to be repaired using NER and TLS proteins (28,49,113,122). Similar to replication-dependent pathway, the TLS polymerases REV1 and Pol ζ play an important role in the bypass of ICL lesions (113).

The ubiquitination of PCNA by Rad 18 is important for the recruitment of these polymerases to the ICLs(121). Studies have shown that most ICLs are recognized by global genome NER(49,122) except for cisplatin ICL that was repaired only by transcription-coupled NER(28).

The exact role of NER proteins in this pathway is not clearly understood. In the repair of lesions affecting one strand of DNA, NER proteins separate the two DNA strands around the lesion, and this would appear not to be feasible with an ICL. It is therefore unclear how NER endonucleases might incise ICLs. In line with this, it has been shown that cisplatin ICLs are not incised by NER (123) and that with psoralen and alkyl ICL dual incisions take place on one side of a ICL (124-126).

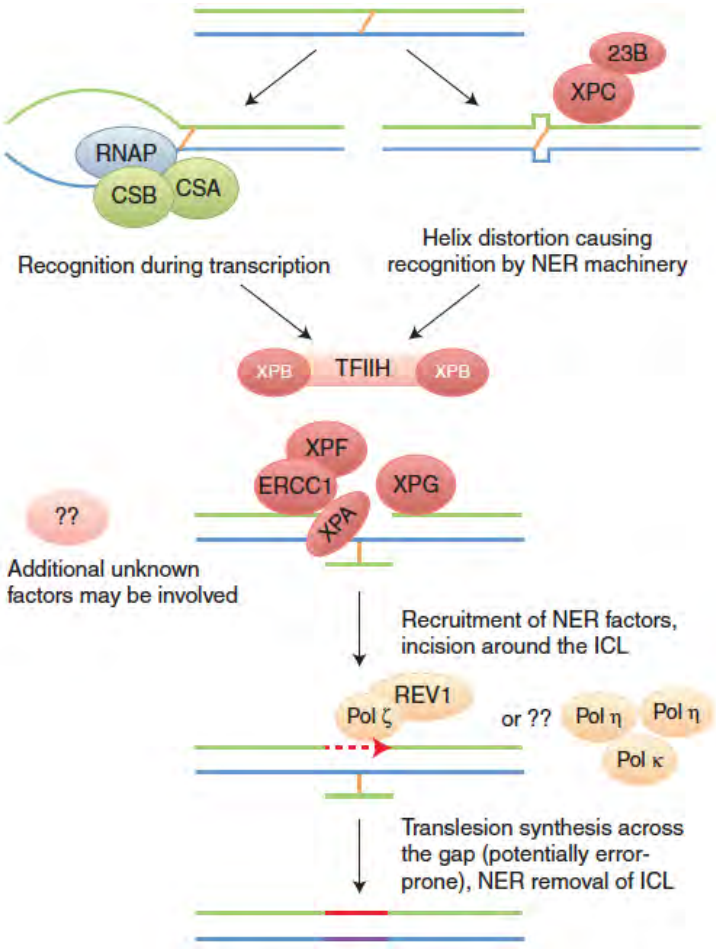

Figure 1.8: Model for replication-indepdendent ICL repair pathway. In G0/G1 stage of cell cycle, ICL may be detected either during transcription or by helix distortion, recognized by NER. NER proteins can unhook the lesion and TLS polymerases bypass the lesion to generate a ds-DNA free of ICL. The figure is taken from (3).

Studies using psoralen ICLs, where the repair takes place in G1 phase of cell cycle, has shown that NER proteins such as XPC, XPB, XPA and XPF are recruited to the damaged sites (127). The mismatch repair protein MutSβ has also been suggested to play a role in recognition of ICLs with or without NER (128).

Thus, even though the precise role of NER machinery in this pathway remains to be elucidated, the studies discussed have shown that replication-independent ICL repair pathway

remains evolutionary conserved and is important for cell survival following damage by ICL agents.

Targeting ICL repair proteins for chemotherapeutic potential

ICL-forming alkylating agents and cisplatin continue to represent some of the most successful chemotherapeutic agents. The ability to overcome resistance of tumor cells to treatment with these drugs could have long-term therapeutic implications. It has been shown that removal of ICLs from tumor cells is associated with resistance to treatment with cisplatin and NMs (129,130). It has been shown that in ovarian and testicular cancer, resistance to the treatment with cisplatin correlates with elevated levels of XPF-ERCC1, MUS81-EME1 and FA proteins (131-135). Sensitivity to cisplatin has been observed in lymphoma and non-small-cell lung cancer models where Rev1 or Rev3 levels were depleted using shRNA. Therefore, selective inhibition of TLS polymerases may be used to sensitize cancer cells to ICL forming agents (136,137).

Among the various pathways in ICL repair, the FA pathway is a promising one as FA-deficient cells are extremely sensitive to crosslinking agents. Studies have shown that disruption of ubiquitin-mediated signal transduction can influence the FA pathway. Disruption of FANCD2 monoubiquitination has also been observed by disruption of FA core complex proteins FANCC and FANCG in adenocarcinoma cell lines (138,139). The siRNA-mediated suppression of FANCF has been found to sensitize multiple myeloma cells to melphalan (140). Using a cell-based screening assay for small molecule inhibitors of the FA pathway, four inhibitors that prevent FANCD2 foci formation were identified, among which three were protein kinase inhibitors (141,142). Similar studies to screen for small molecules that prevent FANCD2

ubiquitination were carried out by Landais et. al using *Xenopus laevis* cell-free extracts (143). A screen to identify molecules selectively toxic to FA-deficient cells has also resulted in the isolation of lead compounds (139). Overall, a better understanding of the mechanisms of the FA proteins in ICL repair pathway will lead to the development of additional inhibitors that will target specific FA proteins.

The endonucleases ERCC1-XPF and SLX4 are also promising targets for development of specific inhibitors. A mouse xenograft model of melanoma with normal level of ERCC1 was found to be resistant to treatment with cisplatin, while the ERCC1-deficient one responded to treatment after two doses of cisplatin (144). The disruption of interaction between XPF and SLX4 is also a potential drug target as this would cause cells to become sensitive to crosslinking agents without disrupting the role of ERCC1-XPF in NER (145,146).

Strategies to synthesize ICLs

A major challenge in studying the ICL repair pathway is the availability of DNA ICLs at a defined site in a DNA duplex. Traditionally, ICLs have been generated by the treatment of ds-DNA with a crosslinking agent. This process usually gives rise to a mixture of products that include: a) monoadducts that are formed on either strand of the duplex, b) intrastrand crosslink as a result of reaction of two base residues on same strand of the duplex and c) the desired interstrand crosslink (Figure 9A). The typical yield of the ICL in such cases is between 1-5%, with the monoadducts being the major products (2,42). ICLs have also been synthesized using hybridization directed crosslink formation.

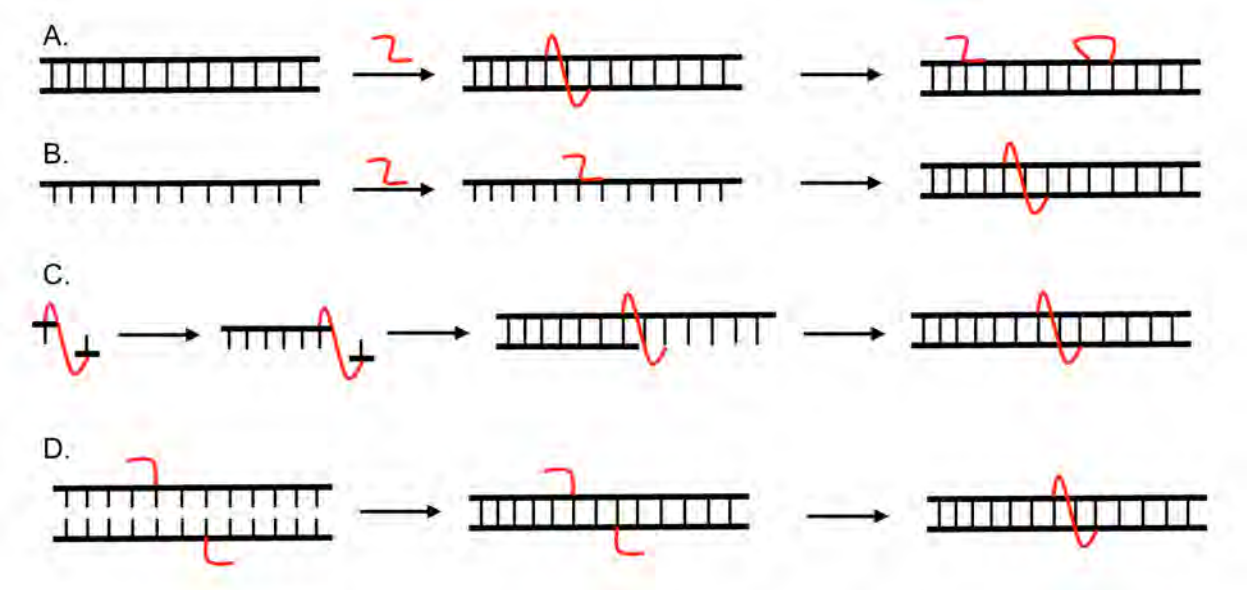

Figure 1.9: Various strategies to synthesize DNA ICLs a) Reaction of ds-DNA with crosslinking agent; b) Reaction of ss-DNA with a crosslinking agent followed by annealing to complementary strand; c) Insertion of crosslinked nucleotide dimer into ds-DNA during oligonucleotide synthesis; d) Incorporation of crosslink precursors on ss-DNA, followed by annealing and site-specific coupling reaction.

A monoadduct is formed on one strand of the duplex either during solid-phase synthesis or postsynthetically which is then purified and annealed to the complementary strand to initiate crosslink formation (Figure 9B). This method is superior in the amount of ICL obtained than treating ds-DNA with crosslinking agents. However, the success of this method depends on the annealing between the oligonucleotides that are being crosslinked. This procedure has been used to synthesize platinum, psoralen and MMC crosslinks (43,53,147).

Another strategy for generating ICLs has been achieved by synthesizing the crosslink as a nucleotide dimer and incorporating it into the DNA using solid-phase DNA synthesis (Figure 9C). Since the chemistry used to introduce the crosslink is highly specific, this procedure yields fewer unwanted side products and gives an overall good yield of the crosslink (33,34,148). An improved approach consists of site-specific incorporation of post-synthetically modifiable crosslink precursors on complementary strands of DNA, annealing of the two strands and

subsequent use of a specific coupling reaction to form the ICL (Figure 9D). This methodology has been used to generate ICLs with psoralen, malondialdehyde and alkyl linkages (63,71,147,149,150). Our laboratory employed this strategy to synthesize major groove DNA ICLs based on the ones formed naturally by NMs (19,20). This has allowed us to synthesize structurally diverse NM ICL analogs that will be valuable tools to understand the structure-function relationships in ICL repair.

Preview

The work presented in this dissertation focuses on synthesizing structurally diverse major groove DNA ICLs based on the ones formed by nitrogen mustard using a strategy that involved a specific post-synthetic reductive amination reaction. In Chapter 2, using aldehyde precursors of different lengths, we have synthesized ICLs that induce different degrees of distortion into the DNA. Moving forward, we synthesized an alkyl amino precursor molecule (Chapter 3) that was used to generate an ICL between a single nucleoside covalently linked to an oligonucleotide. We also demonstrate the importance of our strategy to generate site-specific ICLs in the context of synthesizing such substrates for our collaborators (Chapter 4) that have been useful in understanding the role of structure-specific endonucleases in the ICL repair pathway. The availability of such structurally diverse ICLs will be crucial in carrying out structure-function relationship studies of ICL repair using biochemical and cell biological studies approaches.

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Chapter 2

Synthesis of Structurally Diverse Major Groove DNA Interstrand Crosslinks Using Three Different Aldehyde Precursors

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Abstract

DNA interstrand crosslinks (ICLs) are extremely cytotoxic lesions that block essential cellular process such as replication and transcription. Crosslinking agents are widely used in cancer chemotherapy and form an array of structurally diverse ICLs. Despite the clinical success of these agents, resistance of tumors to crosslinking agents, for example through repair of these lesions by the cellular machinery remains a problem. We have previously reported the synthesis of site-specific ICLs mimicking those formed by nitrogen mustards to facilitate the studies of cellular responses to ICL formation. Here we extend these efforts and report the synthesis of structurally diverse major groove ICLs that induce severe, little or no distortion in the DNA. Our approach employs the incorporation of aldehyde precursors of different lengths into complementary strands and ICL formation using a double reductive amination with a variety of amines. Our studies provide insight into the structure and reactivity parameters of ICL formation by double reductive amination and yield a set of diverse ICLs that will be invaluable for exploring structure-activity relationships in ICL repair.

INTRODUCTION

DNA interstrand crosslinks (ICLs) are extremely cytotoxic lesions that covalently connect two complementary strands of a DNA duplex. As DNA strand separation is essential to cellular processes such as transcription and replication, ICLs are highly cytotoxic (1-3) Bifunctional electrophiles such as cisplatin, nitrogen mustards, chloro ethyl nitroso ureas and mitomycin C form ICLs and are a mainstay as frontline chemotherapeutic drugs (4). Despite the clinical success of these drugs, resistance mechanisms including the removal of ICLs from DNA by cellular proteins contributes to the resistance of tumor cells to treatment with crosslinking agents (5). ICLs are also formed by endogenous bifunctional agents such as malondialdehyde and formaldehyde (6-8), and such agents have likely been the evolutionary drivers for the cellular responses to ICL formation. The importance of these repair pathways is underscored by the existence of the hereditary cancer prone disorder Fanconi anemia (FA) (9,10). Cells from FA patients display exquisite sensitivity to ICL-forming agents and exposure to crosslinking agents serves as the definitive clinical diagnosis for FA.

The repair of ICLs is a complex process and the pathways involved have been subject of intense recent studies (11). These efforts have been critically dependent on the ability to generate site-specific ICL (reviewed in (12)). Breakthroughs in the synthesis of site-specific ICLs have been achieved by a number of laboratories by incorporating ICL precursors into DNA as phosphoramidites by solid phase DNA synthesis. Such approaches made use of the incorporation of nucleosides crosslinked outside of the DNA (7,13-15)and the incorporation of ICL precursors on one or two strands of DNA and the use of specific reaction to induce ICL formation (16-26). These efforts have provided access to structurally diverse ICLs formed in the major and minor grooves, on the Waston-Crick base pairing face or stacked between the two bases. ICLs induce a

variety of alterations in DNA duplex structures, due to differences in the attachment sites on the DNA bases and the chemical composition of the crosslink. Recent studies have revealed that the biological responses triggered by ICL can vary depending on how they influence duplex structure (27-32) .

Our own studies have been mainly concerned with major groove ICLs (23,25), the site where adducts are formed by the clinically important cisplatin and nitrogen mustards or the environmental pollutant diepoxybutane. The length of the crosslink agent bridging two N7 atoms of dG residues on opposite strands ICLs formed in the major groove lead to different degrees of degrees of distortion in DNA, dictated by length of the ICL linking the two bases (33-36)

We have synthesized ICLs using precursor molecules containing reactive aldehyde groups masked as diols that were incorporated into complementary strands of a duplex and by using a double reductive amination reaction (Figure 1). Using aldehyde precursors connected to the base through one or two carbon alkyl chains and using a variety of amines, we found that the yield of ICL formation dramatically depended on the length of the ICL formed, and the reactivity of the amine used (23,25). Here we extend these studies through the synthesis of a new ICL precursor and a systematic assessment of reaction conditions for ICL formation, providing access to a variety of NM ICL mimics inducing moderate, mild or no distortion in the DNA duplex. We expect that these structurally diverse ICLs will be invaluable for structure-function relationships of ICL repair, extending previous studies that have started to reveal important differences in how ICLs of different structures are processed in replication-dependent and -independent repair (28,37,38).

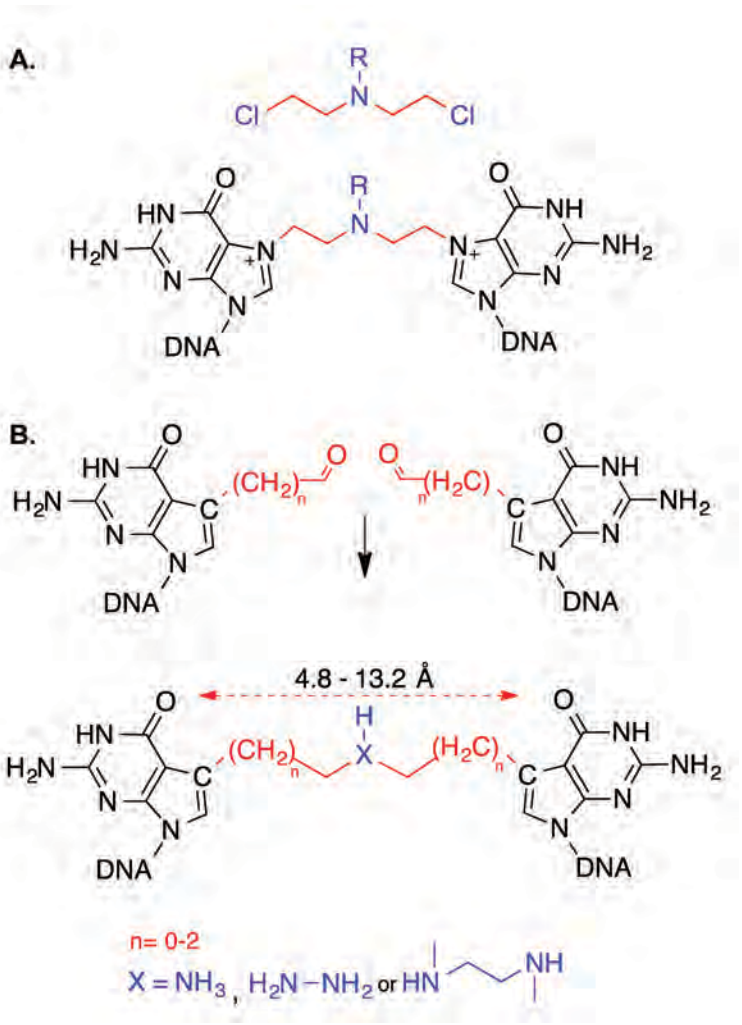


Figure 2.1: Native and synthetic nitrogen mustard ICLs. A. Nitrogen mustard and the DNA ICL formed by reaction with duplex DNA. The structure of the side chain R, varies with the type of nitrogen mustard for example "CH_3" is for mechlorethamine, the first clinically used antitumor drug. **B**. Our approach to forming ICLs having possible linker lengths from 4.8 – 13.2 Å uses a combination of ICL precursors, a 7-alkylaldehyde-7-deazaguanine with C1, C2 or C3 alkyl chains in complementary strands of a DNA duplex and a double reductive amination reaction with ammonia, hydrazine or DMEDA. Note that guanine residues at the ICL are replaced with 7-deazaguanine to facilitate ICL synthesis and increase stability.

RESULTS and DISCUSSION

Strategy for the synthesis of structurally diverse ICLs

We have previously reported that NM-like ICLs can be synthesized by incorporation of two aldehyde precursors on complementary strands of DNA and crosslink formation using a double reductive amination reaction (23,25). In the course of these studies, we noted that the efficiency of ICL formation is dependent upon the length of the ICL as well as the reactivity of the amine used in the reaction (Figure 1B). This is illustrated by our attempts to synthesize an ICL isosteric to those formed by NMs that contain a 5 atom chain in the crosslink and induce a bend of about 20° in the DNA (n=1, Figure 1). We observed that formation of this crosslink was unsuccessful with two aldehyde precursors with a C2 alkyl chain and ammonia as the amine, while it could be formed with a C2 and C1 alkyl and hydrazine as an amine. By contrast, an ICL that does not induce a distortion can be formed with a number of different amines (17,22). These examples show that the length of the ICL to be formed and the reactivity of the amine (hydrazine is more nucleophilic than ammonia) determine the success of ICL formation in our approach. We wished to more fully exploit our approach to generate structurally diverse major grove ICLs by double reductive amination and to study the parameters of reactivity that govern crosslink formation.

To be able to access ICLs spanning a broad range of distances across the major groove, we set out to synthesize a new ICL precursor with a three-carbon alkylaldehyde (C3) precursor. Together with the previously synthesized C1 and C2 aldehyde precursors and using ammonia, hydrazine and N,N'-dimethylethylenediamine (DMEDA) this should allow us to generate a set of structurally diverse ICLs. We define here the length of an ICL based on the fully extended alkylamine chain spanning the distance between the two crosslinked deazaguanine residues

(illustrated in Figure 2). The distance between the atoms at the 7 position of dG where the ICL is attached is approximately 8.9Å in B-form DNA, so any ICL that spans a length of 8.9Å or less will induce some form of distortion in the DNA.

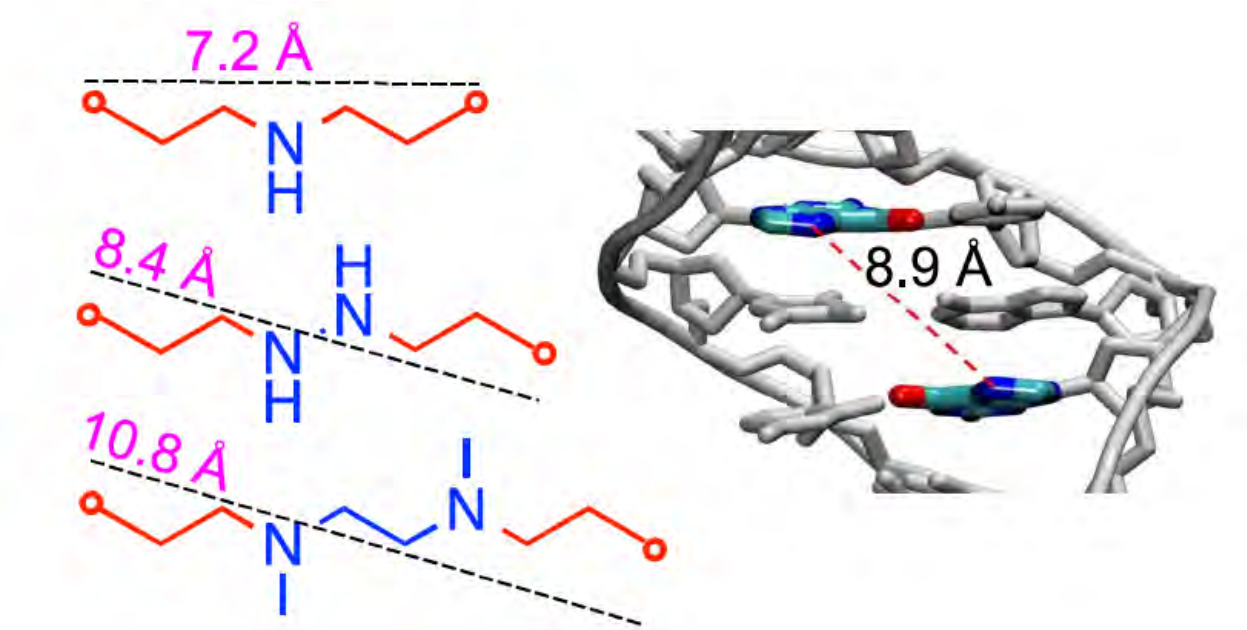


Figure 2.2: **Distance between N7 position on opposing strand of two G residues in B-form DNA and the theoretical length of the alkylamine chain linker in ICLs**. The lengths of the alkyl chains are indicated over the dotted lines represent the maximum possible length in a fully extended all *anti* conformation. The actual distance after ICL formation may be different from the calculated value.

Synthesis of a three-carbon alkyl aldehyde chain precursor

The synthesis of the C3 precursor **31** started with a Sonogashira cross-coupling reaction between 7-Iodo-7-deazaguanosine **28** (23) with the protected alkynediol **27** (Figure 3). **27** was synthesized starting from the protected (*S*)-glyceraldehyde **24**, which was converted to the dibromo-olefin **25** (39) and reprotected as TBDMS-ether **26**, compatible for solid phase DNA synthesis. Treatment of **26** with EtMgBr gave acetylene **27** (40). The alkyne group in **29** was

reduced by passing hydrogen over palladium catalyst. Subsequently, protecting groups on the 5'- and 3'- positions of the sugar were exchanged using standard reagents and conditions to generate the phosphoramidite **31**. Using solid phase DNA synthesis, **31** was incorporated in two complementary 20-mer strands containing a 5'-**G***NC sequence, where **G*** denotes the aldehyde-bearing residue. The oligonucleotides were deprotected - first with concentrated NH_3 to remove the standard base and phosphate protecting groups and then with TEA•3HF to remove the TBDMS groups, generating the free diol. The oligonucleotides were purified by HPLC and the presence of the diol in the modified residues was confirmed by MALDI-TOF measurements (see supplementary data). In addition, 20-mer oligonucleotides with the C1 and C2 aldehyde precursors on complementary strands were also synthesized and purified according to our earlier published protocol (23,25).

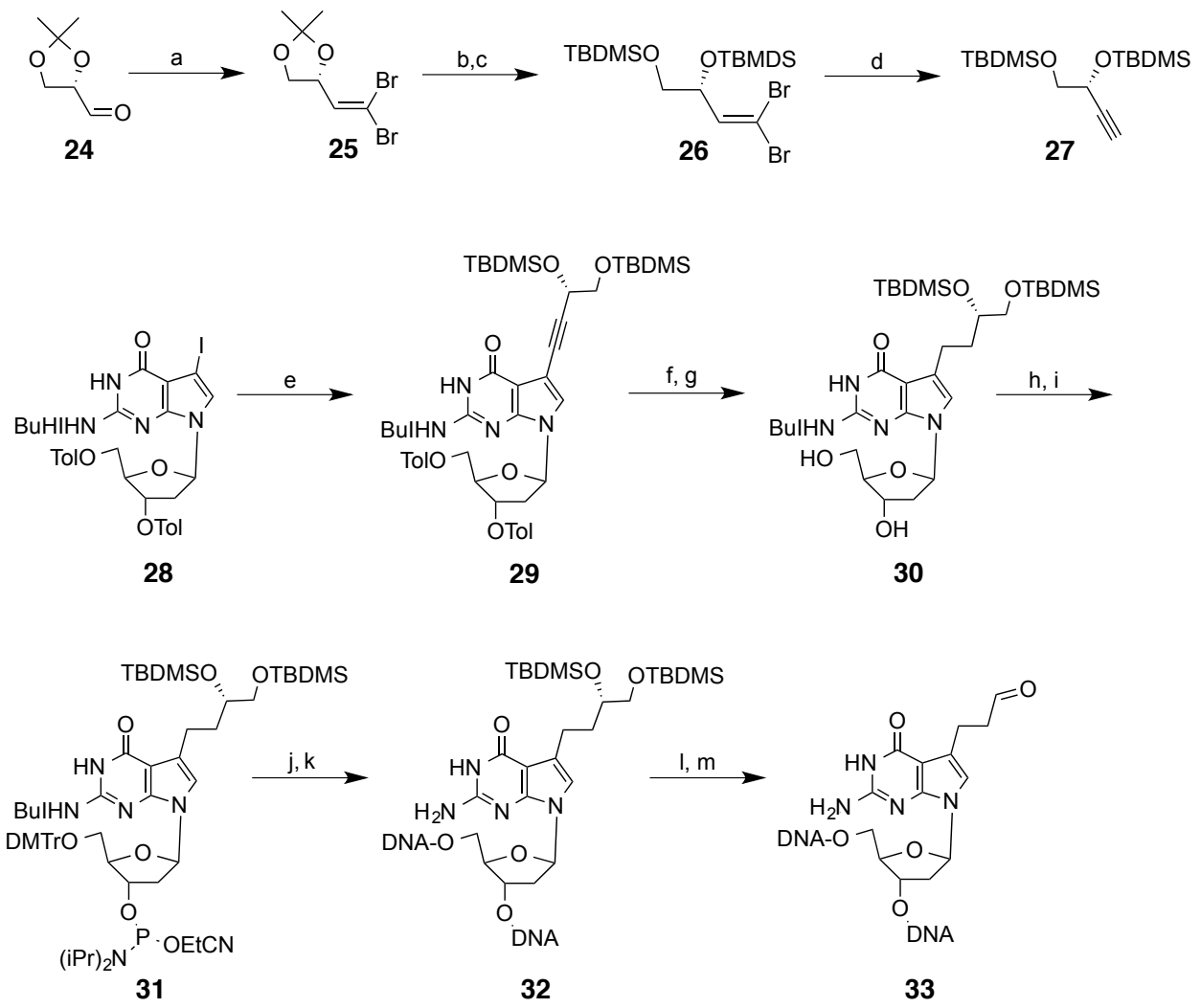


Figure 2.3: Scheme showing the synthesis of the C3 alkyl chain ICL precursor phosphoramidite 8. Reagents, conditions and yields: a) PPh_3, CBr_4, CH_2Cl_2, 3 h, 84%; b) Dowex-50W, MeOH, 12 h, 50°C, 95%; c) TBDMSCl, Imidazole, DMF, 12 h, 83%; d) EtMgBr, THF, 3 h, 90%; e) **4**, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, 24 h, 60°C, 59%; f) H_2, Pd/C, EtOAc/MeOH, 42h, 84%; g) NaOMe, THF, 6 h, 90%; h) DMTrCl, Py, 1.5 h, 72% i) $(iPr)_2NP(Cl)OEtCN$, CH_2Cl_2, 1.5 h, 71%; j) solid phase DNA synthesis; k) NH_3, 16 h, 50°C; l) TEA.3HF, 40°C, o.n.; m) $NaIO_4$, pH=5.4

ICL Formation using 3 different length alkyl aldehyde precursors

We studied ICL formation using the three different length precursors (C1, C2 and C3) and three amines (ammonia, hydrazine and DMEDA) in a systematic manner (Figure 4). ICLs were in 5'-G*NC sequences, the preferred site of ICL formation by nitrogen mustards (41,42). As previously discussed, the efficiency of ICL formation is dependent on both the reactivity of the amine as well as the distortion induced in the DNA. Duplex bearing diol-containing ICL precursors were annealed, the diol oxidized to the aldehyde using sodium periodate and ICL formation induced by incubation with one of the three amines in the presence of sodium cyanoborohydride. The identity of the ICLs formed was confirmed by MALDI-TOF measurements. With a duplex having C1 precursors on each strand, ICL formation, indicated by the appearance of a band with slower mobility on a denaturing polyacrylamide gel, was only observed with DMEDA, yielding a 8.4 Å linkage (Figure 4, lanes 3). By contrast, we did not observe the formation of the 4.8 Å or 6.0 Å ICLs with two C1 precursors and ammonia and/or hydrazine, respectively (Figure 4, lanes 1-2). ICL formation was dependent on the presence of the reducing agent and the amine and omission of either one prevented ICL formation (Figure 4, lanes 4-5). In a duplex containing a C1 and a C2 aldehyde precursor on complementary strands, ICL formation was observed with hydrazine and DMEDA (Figure 4, lanes 7 & 8), but not with ammonia (Figure 4, lane 6), as previously reported (25). The 7.2 Å ICL containing hydrazine is the shortest ICL that we were able to form in our approach and it is isosteric to the native nitrogen mustard ICL. Having two C2 precursors gave ICLs with hydrazine and DMEDA (Figure 4, lanes 17 and 18), but not with ammonia (Figure 4, Lane 16). These results confirmed that an ICL isostructural to that formed by NM with a linker length of

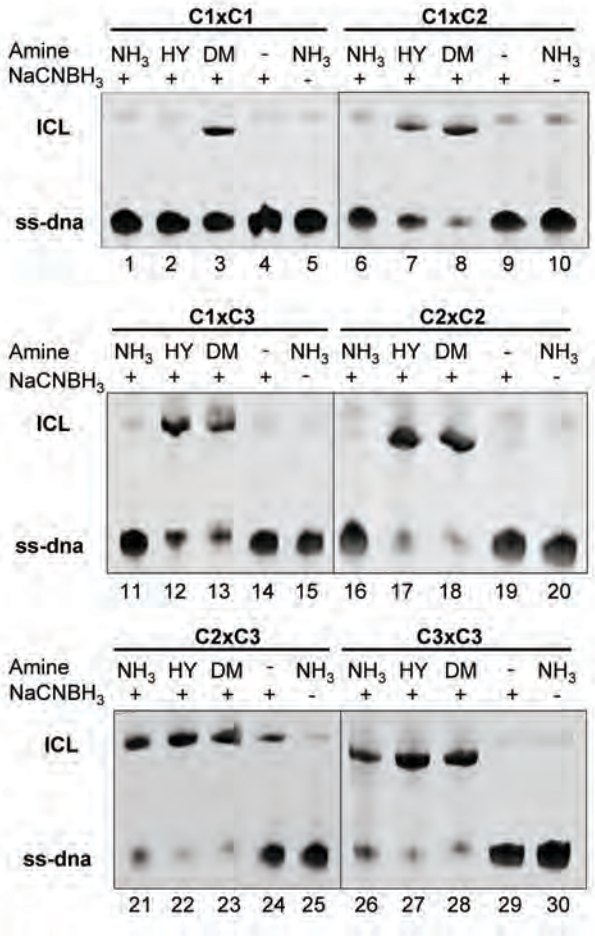


Figure 2.4: ICL formation by reductive amination using C1, C2 and C3 precursors.
Analysis of ICL formation of the reaction of duplexes containing C1, C2 and C3 precursors in the presence of amines and $NaBH_3CN$. Reactions were analyzed by denaturing PAGE gel and stained with methylene blue. The sequences used were 5'- d(GTCACTGGTA**G***ACAGCATTG) and 5'-d(CAATGCT**G***TCTACCAGTGAC) where **G*** represents the modified guanine having seither C1, C2 or C3 diol. As a control for each set of ICL formation, reactions in which the amine or $NaBH_3CN$ was omitted were performed. The precursor and amine used are indicated over each gel. NH_3 = Ammonia; HY = hydrazine, DM = DMEDA.

7.2 Å and inducing a bend in the DNA of about 20° can be formed by double reductive amination with hydrazine, but not ammonia, in line with the higher reactivity of hydrazine.

The reactivity patterns of ICL formation were further investigated with the newly synthesized C3 aldehyde ICL precursor paired to a complementary strand with C1 or C2 precursor. As was expected from the results with the C2/C2 precursors (Figure 4, lanes 16-18), ICL formation with the C1/C3 pair did not yield an ICL upon reaction with ammonia, showing that a 7.2 Å ICL cannot be formed with ammonia, irrespective of the position of carbon and nitrogen atoms in the ICL. As expected, ICLs were formed with C1/C3 using hydrazine and DMEDA (Figure 4, lanes 12 & 13) as an amine.

The question then arose whether the combination of C3 and C2 precursors would be able to yield an ICL with NH_3, with an 8.4 Å linkage. This was indeed the case, and ammonia, hydrazine and DMEDA all formed ICLs with the C2/C3 oligonucleotides (Figure 4,lanes 21-23). This result shows that the minor distortion in the DNA of the 8.4 Å ICL allowed the reductive amination to occur with ammonia. A band of lesser intensity comigrating with the ICL band was also formed in the absence of an amine with the C2/C3 duplex (Figure 4, lane 24). Although the identity of this band remains to be determined, we speculate that it is the result of a reductive amination reaction of one of the aldehyde group with an exocyclic amine in the complementary strand of DNA. As expected based on these observations, reactions of the duplex containing two C3 aldehyde precursors, yielded ICL with all of the three amines with lengths of 9.6 Å and more (lanes 26-28, Figure 4).

Summary and conclusion: Distance-dependent ICL formation

We used oligonucleotides with 7-deazaguanine residues having alkyl aldehyde chains of different lengths (C1, C2, C3) at the 7 position and studied ICL formation with ammonia, hydrazine and DMEDA using a reductive amination reaction. The efficiency of ICL formation was found to be correlated with the length of the ICL and the reactivity of the amine (summarized in Figure 5). We were able to form ICLs with bridge lengths ranging from 7.2 Å, for which our molecular modeling studies predict a bend of about 20° in the DNA duplex (22), to those of 10.8 Å and more, which our preliminary NMR experiments show are free of distortion. ICL formation was found to be most efficient with non-distorting ICLs (10.8-13.2 Å), followed by those with minor (8.4-9.6 Å) and moderate distortion (7.2-8.4 Å) (Figure 5). The higher nucleophilicity and reactivity of hydrazine allowed for the formation of more distorted ICLs. The major-groove ICLs reported here inducing no, minor and moderate distortion in DNA duplexes (Figure 6) will be invaluable for advancing studies elucidating structure-function relationships in ICL repair.

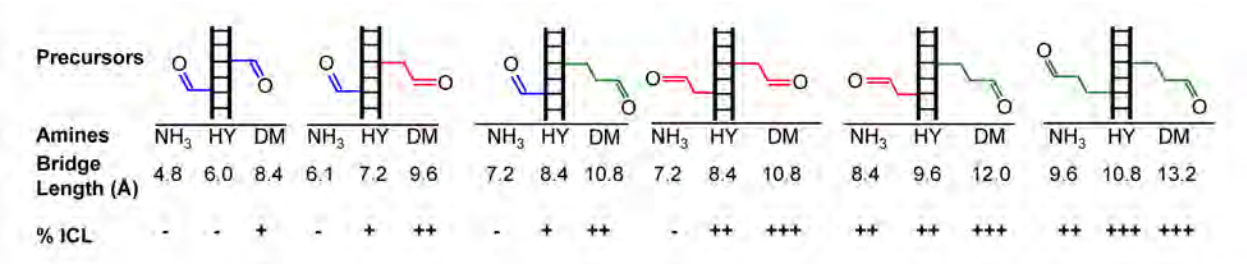


Figure 2.5: Overview of the reaction with of all the ICL reactions tested. Reactions with the C1, C2 and C3 precursors and the three different amines (NH_3, HY and DMEDA), the theoretical bridge lengths and relative qualitative yields are indicated. '+' low yield, '++' moderate yield and '+++' for high yield. As staining with methylene blue does not allow for the quantitative determination of the ratio of ss- vs ds-DNA, qualitative yields are indicated.

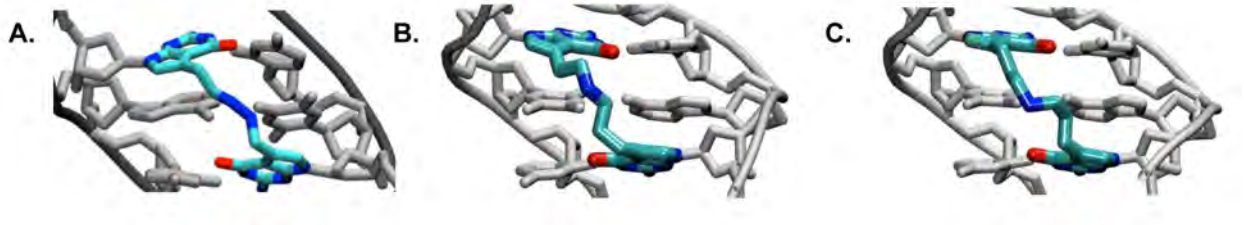


Figure 2.6: Models of structures of nitrogen mustard ICLs. **A.** C1/C2/HY ICL isosteric to a native nitrogen mustard ICL with a bridge length of 7.2 Å inducing a 20° bend in the DNA as predicted by molecular modeling studies (22); **B.** C2/C3/NH_3 ICL with a bridge length of 8.4 Å, inducing a distortion in the DNA. **C**) C3/C3/NH_3 ICL with a bridge length of 9.6 Å without DNA distortion. The structure in A. was calculated using molecular dynamic simulations (25), B. and C. were manually generated using VMD 1.9 (43) .

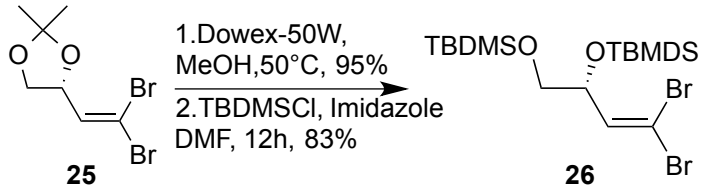
Experimental Section

General Information

6-chloro-7-deaza-7-iodo-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine was prepared according to published procedures (23). 4,4'-Dimethoxytrityl chloride was purchased from Syngen (USA). 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite was purchased from ChemGenes (USA). Protected 2'-deoxyribonucleoside-3'-phosphoramidites and all other reagents necessary for automated DNA synthesis were purchased from Glen Research (USA). All other reagents and solvents were purchased from Sigma Aldrich (USA), EMD (USA) or Alpha Aesar. Dry solvents (acetonitrile, dichloromethane, dioxane, DMF, ethyl acetate, methanol, pyridine and THF) were purchased from EMD and used without further purification. NMR Spectra were recorded on Varian 400 MHz spectrometer. HR-MS was recorded on Thermo LTQ Orbitrap XL Ion Trap Mass Spectrometer. MALDI-TOF was recorded on Voyager-DE STR. All reactions were carried out under an inert atmosphere of nitrogen or argon. Chemical shifts (δ) are in ppm. J values are in Hz. **24** and **25** were synthesized according to published literature procedures (39).

Synthesis of phosphoramidite 31

(*R*)-3,4-di-(O-tertbutyl-dimethylsilyl)but-(1,1-dibromo)-ene (26)



To a solution of (*R*)-4-(2,2-Dibromoethenyl)-2,2-dimethyl-1,3-dioxolane (4 g, 13.9 mmol) in 40 mL methanol was added Dowex-50W (4.4 g) and the resulting mixture was refluxed at 50 °C for 12 h. Dowex-50W was filtered off and methanol was evaporated. The resulting liquid was dissolved in DMF (45 mL) and treated with TBDMSCl (50% w/w in toluene, 14 ml, 40 mmol) and imidazole (5.4 g, 80 mmol). The reaction was incubated at room temperature for 12 h, diluted with 400 mL ethyl acetate, washed with 5% HCl (400mL), sat. $NaHCO_3$ (400 mL), and brine (300 mL), dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure. Purification of the residue by silica gel column chromatography (hexane/ethyl acetate 4:1) gave the product as a colorless liquid (6 g, 12.6 mmol, 90%).

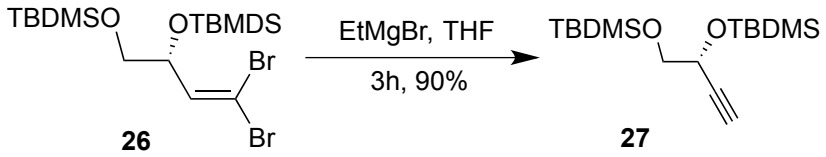
R_f : 0.30 (hexane/ethyl acetate 4:1)

^{1}H NMR ($CDCl_3$): δ 6.33 (d, 1H, C(2)-H, J = 8.4), 4.34 (m, 1H, C(4)-H_a), 3.56 (dd, 1H, C(3)-H, J = 10.4, 6), 3.48 (dd, 1H, C(4)-H_b, J = 10.4, 6), 0.89 & 0.87 (2s, 18H, tBu-Me), 0.08 & 0.07 (2s, 6H, Si-Me), 0.05 (s, 6H, Si-Me).

^{13}C NMR: ($CDCl_3$): δ 140.0 (C), 89.9 (C), 74.4 (CH_2), 66.2 (CH), 25.9 (CH_3), 25.7 (CH_3), 18.3 (C), 18.1 (C), - 4.5 (CH_3), - 5.3 (CH_3).

ESI-MS: $[M+H]^+$ m/z calculated for $C_{10}H_{20}Br_2OSi$ 341.96, observed 341.0 (fragmented during MS).

(*R*)-3,4-di-(O-tertbutyl-dimethylsilyl)but-1-yne (27)



To a solution of (*R*)-3,4-di-(O-tertbutyl-dimethylsilyl)but-(1,1-dibromo)-ene (3 g, 6.32 mmol) in THF (10 mL) cooled in an ice bath was added EtMgBr (1M in THF, 12.6 mL, 12.6 mmol) dropwise. The ice bath was removed and the reaction mixture was stirred at room temperature for 3 h. Solid NH_4Cl (0.8 g) was added to quench excess of EtMgBr, and the reaction mixture was diluted with ethyl acetate (40 mL), washed with brine (40 mL), dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure. Purification of the residue by silica gel column chromatography (hexane/ethyl acetate 4:1) gave the product as colorless liquid (1.4 g, 4.4 mmol, 70%).

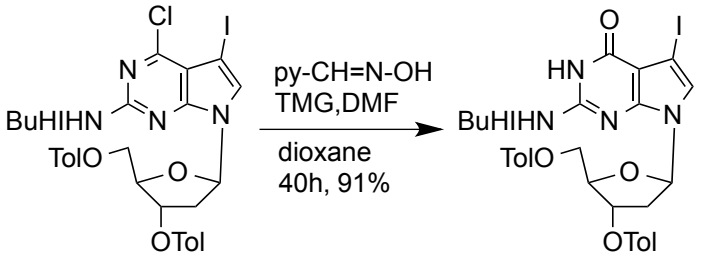
R_f : 0.65 (hexane/ethyl acetate 4:1)

^{1}H NMR ($CDCl_3$): δ 4.37 (m, 1H, C(4)-H_a), 3.69-3.66 (m, 2H, C(4)-H_b & C(3)-H), 2.36 (d, 1H, C(1)-H, J = 2.4), 0.91 & 0.90 (2s, 18H, tBu-Me), 0.14 & 0.12 (2s, 6H, Si-Me), 0.08 & 0.07 (2s, 6H, Si-Me).

^{13}C NMR: ($CDCl_3$): δ 83.7 (C), 72.9 (CH), 68.1 (CH_2), 64.7 (CH), 26.1 (CH_3), 25.9 (CH_3), 18.5 (C), 18.4 (C), - 4.6 (CH_3), -5.1 (CH_3).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{16}H_{35}O_2Si_2$ 315.2170, observed 315.2169.

7-deaza-7-iodo-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (28)



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A solution of 6-chloro-7-deaza-7-iodo-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (3.7 g, 5.16 mmol), pyridine-2-carboaldoxime (3.15 g, 25.8 mmol) and 1,1,3,3-tetramethylguanidine (3.26 mL, 25.8 mmol) in DMF (60 mL) and dioxane (60 mL) was stirred for 40 h at room temperature. The reaction mixture was diluted with ethyl acetate (200 mL) and washed with 5% HCl (200 mL), sat. $NaHCO_3$ (200 mL) and brine (100 mL), dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure. Purification of the residue by silica gel column chromatography (hexane/ethyl acetate 2:1) gave the product as a light yellow solid (3.3 g, 4.72 mmol, 91 %).

R_f : 0.75 (hexane/ethyl acetate 1:1)

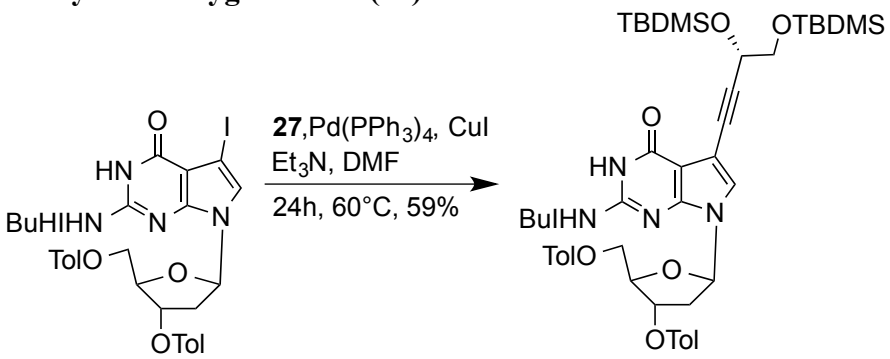
^{1}H NMR ($CDCl_3$): δ 11.64 (s, 1H, N(1)-H), 8.64 (s, 1H, C2-(NH)), 7.93-7.87 (dd, 4H, Tol-H, J = 6.8, 2), 7.27-7.23 (dd, 4H, Tol-H, J = 10, 7.6), 6.92 (s, 1H, C(8)-H), 6.23 (dd, 1H, C(1')-H, J = 14, 7.6), 5.79 (dd, 1H, C(3')-H, J = 6, 2.8), 4.99 (dd, 1H, C(5')-H_a, J = 10.8, 6.8), 4.62- 4.57 (m, 2H, C(5')-H_b & C(4')-H), 3.05-2.98 (m, 1H, C(2')-H_a), 2.67 (sept, 1H, iBu-CH, J = 6.8), 2.59 (ddd, 1H, C(2')-H_b, J = 14.4, 6.4, 2.8), 2.43 & 2.41 (2s, 6H, Tol-Me), 1.31-1.28 (d, 6H, iBu-Me, J = 7.2).

^{13}C NMR: ($CDCl_3$): δ 178.2 (C), 166.5 (C), 165.7 (C), 156.9 (C), 147.0 (C), 146.4 ©, 144.2 (C), 129.5 (CH), 129.5 (CH), 129.3 (CH), 129.2 (CH), 129.1 (CH), 126.4 (CH), 126.2 (CH), 105.9

(C), 85.0 (CH), 81.9 (CH), 74.7 (CH_2), 63.4 (CH_2), 55.1 (CH), 37.2 (CH_2), 36.1 (CH_2), 21.5 (CH_3), 18.8 (C).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{31}H_{32}O_7N_4I$ 699.1310, observed 699.1306.

7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butynl)-N(2)-isobutryl-3'-5'-di-O-p-toluoyl-2'-deoxyguanosine (29)



28 **29**

To a solution of 7-deaza-7-iodo-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (1 g, 1.43 mmol), $Pd[(PPh)_3]_4$ (160 mg, 0.14 mmol), CuI (50 mg, 0.28 mmol), Et_3N (0.4 mL, 2.86 mmol) in DMF (16 mL) was added **4** (1.35 g, 4.29 mmol) and the reaction mixture was stirred at 60 °C for 24 h, diluted with ethyl acetate (150 mL) and washed with 5% HCl (150 mL), sat.$NaHCO_3$ (150 mL), brine (100 mL), dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure. Purification of the residue by silica gel column chromatography (hexane/ethyl acetate 3:1) gave the product as a yellow solid (750 mg, 0.84 mmol, 59%).

R_f: 0.80 (hexane/ethyl acetate 1:1)

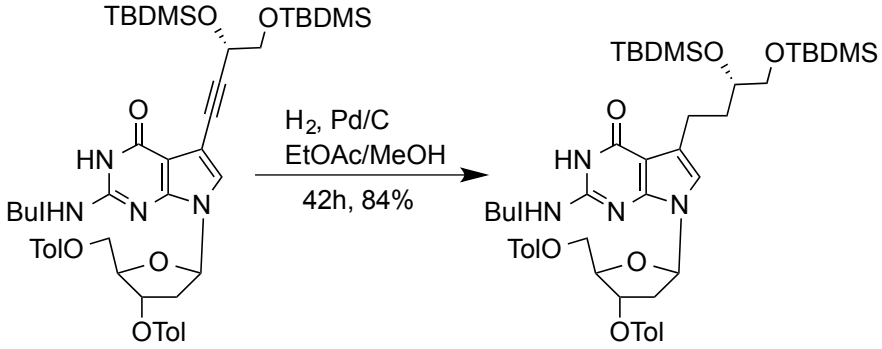
^{1}H NMR ($CDCl_3$): δ 11.62 (s, 1H, N(1)-H), 8.70 (s, 1H, C2-(NH)), 7.91 (d, 2H, Tol-H, J = 8.8), 7.86 (d, 2H, Tol-H, J = 8.4), 7.27-7.21 (dd, 4H, Tol-H, J = 16.8, 8.4), 6.99 (s, 1H, C8-(H)), 6.19 (t, 1H, C(1')-H, J = 6.8), 5.81 (dd, 1H, C(3')-H, J = 6, 2.4), 5.06 (dd, 1H, C(5')-H, J = 10.8, 5.6), 4.62 (m, 3H, C(5')H_b, C(4')H, C(7)-C≡C-C(*H*)-O-), 3.78 (dd, 1H, C(7)-C≡C-$CH_{2(a)}$, J = 10.4, 4),

3.70 (dd, 1H, C(7)-C≡C-$CH_{2(b)}$, J = 10.4, 8), 3.10 (dd, 1H, C(2')H_a, J = 14.4, 6.4), 2.71 (sept, 1H, iBu-CH, J = 6.8), 2.56 (ddd, 1H, C(2')H_b, J = 14.4, 6.4, 2.8), 2.43 (s, 3H, Tol-Me), 2.40 (s, 3H, Tol-Me), 1.30 (dd, 6H, iBu-Me, J = 8, 6.8), 0.92 & 0.89 (2s, 18H, t-BuMe), 0.18 & 0.17 (2s, 6H, Si-Me), 0.07 (2s, 6H, Si-Me).

^{13}C NMR: ($CDCl_3$): δ 178.5 (C), 165.8 (C), 156.5 (C), 146.7 (C), 146.3 (C), 144.3 (C), 129.6 (C), 129.2 (C), 129.1 (C), 105.8 (C), 100.6 (C), 90.1 (C), 86.0 (CH), 81.8 (CH), 74.8 (CH), 68.1 & 65.48 (-C≡C-), 63.5 (CH_2), 36.8 (CH_2), 36.3 (CH), 25.9 (CH_3), 25.8 (CH_3), 18.9 (C), 18.3 (C), - 4.6 (CH_3), - 4.8 (CH_3), -5.2 (CH_3), -5.3 (CH_3).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{47}H_{65}O_9N_4Si_2$ 885.4284, observed 885.4290.

7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butyl)-N(2)-isobutryl-3'-5'-di-O-p-toluoyl-2'-deoxyguanosine



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To a solution of 7-deaza-7 -(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butynl)-N(2)-isobutryl-3'-5'-di-O-p-toluoyl-2'-deoxyguanosine (450 mg, 0.5 mmol) in 10 mL EtOAc and 10 mL MeOH was added 10% Pd/C (100 mg). The reaction was monitored using mass spectrometry for complete reduction of the triple bond. After the reduction was complete (42 h), the solution was

filtered over Celite and solvent evaporated under reduced pressure. Purification of the residue by silica gel column chromatography (hexane/ethyl acetate 2:1) gave the product as a light yellow solid (380 mg, 0.42 mmol, 84 %).

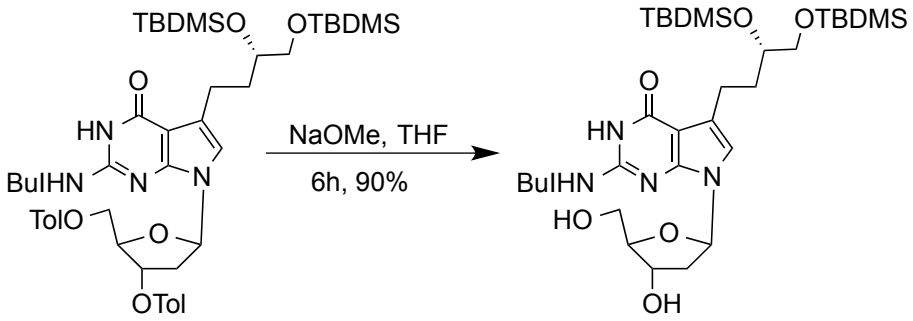
R_f : 0.57 (hexane/ethyl acetate 2:1)

^{1}H NMR ($CDCl_3$): δ 11.53 (s, 1H, N(1)-H), 8.58 (s, 1H, C2-(NH)), 7.93 (d, 2H, TolH, J = 8), 7.88 (d, 2H, TolH, J = 8), 7.27-7.21 (dd, 4H, J = 12.8 ,8), 6.56 (s, 1H, C8-(H)), 6.24 (dd, 1H, C(1')-H, J = 7.6, 6.4), 5.80 (t, 1H, C(4')-H, J = 2.8), 5.04 (dd, 1H, C(5')-H_a, J = 10.0, 4.4), 4.60 (m, 2H, C(5')-H_b & C(3')-H), 3.73 (m, 1H, C(7)-$(CH)_2$-C(*H*)-O-), 3.55-3.47 (m, 2H, C(7)-$(CH)_2$-C$H_{2(a\&b)}$), 3.09 (dd, 1H, C(2')-H_a, J = 14.4, 6.4), 2.88-2.66 (m, 3H, C(7)-$CH_{2a\&b}$, iBu-CH), 2.51 (ddd, 1H, C(2')-H_b J = 14.4, 6.4, 2.8), 2.43&2.40 (2 s, 6H, Tol-Me), 1.95-1.86 (m, 1H, C(7)-$(CH_2)_2$-CH_a), 1.73-1.64 (m, 1H, C(7)-$(CH_2)_2$-CH_b), 1.28 (dd, 6H, iBu-Me, J = 16, 9.2), 0.88 & 0.87 (s, 9H, tBu-Me), 0.07 & 0.06 (2s, 6H, Si-Me), 0.03 (s, 6H, Si-Me).

^{13}C NMR: ($CDCl_3$): δ 178.3 (C), 166.7 (C), 165.8 (C), 157.7 (C), 146.9 (C), 145.7 (C), 144.2 (C), 129.6 (C), 129.1 (C), 126.5 (C), 121.7 (C), 116.5 (C), 105.2 (C), 85.5 (CH), 81.5 (CH), 75.0 (CH), 73.1 (CH), 63.7 (CH_2), 67.5 (CH_2), 36.6 (CH_2), 36.3 (CH), 34.4 (CH_2), 25.9 (CH_3), 21.6 (CH_3), 18.9 (C), 18.3 (C), - 4.2 (CH_3), - 4.6 (CH_3), - 5.3 (CH_3).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{47}H_{69}O_9N_4Si_2$ 889.4597, observed 889.4602.

7-deaza-7-(*S*)-(2,3-di-(O-tertbutyl-dimethylsilyl)butyl)-N(2)-isobutryl-2'-deoxyguanosine (30)



30

To a solution of 7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butyl)-N(2)-isobutryl-3'-5'-di-O-p-toluoyl-2'-deoxyguanosine (300 mg, 0.34 mmol) in 10 mL THF was added 1M NaOMe solution in MeOH (0.34 mL) in an ice bath. The ice bath was removed and the reaction mixture was allowed to run at room temperature for 6 h, quenched with 20 µL acetic acid and the solvent evaporated under reduced pressure. Purification of the residue by silica gel column chromatography (dichloromethane/methanol 20:1) gave the product as a white solid (200 mg, 0.30 mmol, 90%).

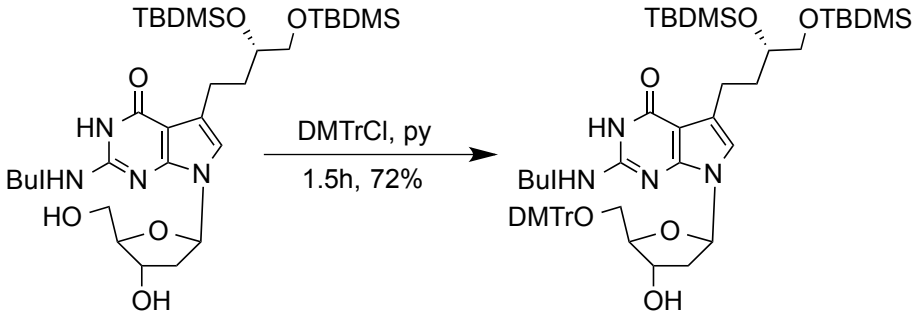
R_f : 0.71 (dichloromethane/methanol 10:1)

^{1}H NMR ($CDCl_3$): δ 11.59 (s, 1H, N1-(H)), 8.17 (s, 1H, C2-(NH)), 6.53 (s, 1H, C8-(H)), 6.09 (dd, 1H, C(1')-H, J = 9.2, 5.6), 4.65 (d, 1H, C(4')-H, J = 5.6), 4.12 (d, 1H, C(7)-(CH_2)-C(H)-$CH_{2(a)}$, J = 2), 3.90 (dd, 1H, C(7)-$(CH_2)_2$-CH-$CH_{2(b)}$, J = 12, 2.4), 3.73 (m, 2H, C(5')-H_a & C(3')-H), 3.54 (dd, 1H, C(5')-H_b, J = 10.4, 6), 3.47 (dd, 1H, C(7)-$(CH_2)_2$-C(*H*)-O-, J = 10, 5.6), 2.72 (m, 3H, iBu-CH & C(7)-C$H_{2(a\&b)}$), 2.56 (dd, 1H, C(2')-H_a, J = 13.6, 6.8), 2.23 (ddd, 1H, C(2')-H_b, J = 13.6, 5.6, 1.6), 1.89 (m, 1H, C(7)-CH_2-CH_{2a}), 1.66 (m, 1H, C(7)-CH_2-CH_{2b}), 1.24 (dd, 6H, iBu-Me, J = 6.8, 5.2), 0.89 & 0.88 (2 s, 18 H, tBu-Me), 0.07 & 0.06 (2s, 6H, Si-Me), 0.04 & 0.03 (2s, 6H, Si-Me).

^{13}C NMR: ($CDCl_3$): δ 179.2 (C), 158.1 (C), 147.1 (C), 146.1 (C), 121.4 (C), 116.7 (C), 104.9 (C), 87.1 (CH), 85.1 (CH), 72.9 (CH), 72.4 (CH), 67.5 (CH_2), 63.5 (CH_2), 39.9 (CH_2), 36.0 (CH_2), 34.5 (CH), 25.9 (CH_3), 21.9 (CH_3), 19.0 (C), 18.1 (C), - 4.2 (CH_3), - 4.6 (CH_3), - 5.3 (CH_3).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{31}H_{57}O_7N_4Si_2$ 653.3760, observed 653.3760.

7-deaza-7-(*S*)-(2,3-di-(O-tertbutyl-dimethylsilyl)butyl)-5'-O-(4,4'-dimethoxytrityl)-N(2)-isobutryl-2'-deoxyguanosine



To a solution of 7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butyl)-N(2)-isobutryl-2'-deoxyguanosine (150 mg, 0.23 mmol) in 6 mL pyridine was added 4,4'-dimethoxytrityl chloride (90 mg, 0.26 mmol). The reaction was allowed to run at room temperature for 1.5 h, quenched with 60 μL of MeOH and pyridine evaporated under reduced pressure. Purification of the residue by silica gel column chromatography (hexane/ethyl acetate 1:1, 0.5% Et_3N) gave the product as a white solid (160 mg, 0.16 mmol, 72%).

R_f : 0.63 (hexane/ethyl acetate 1:2)

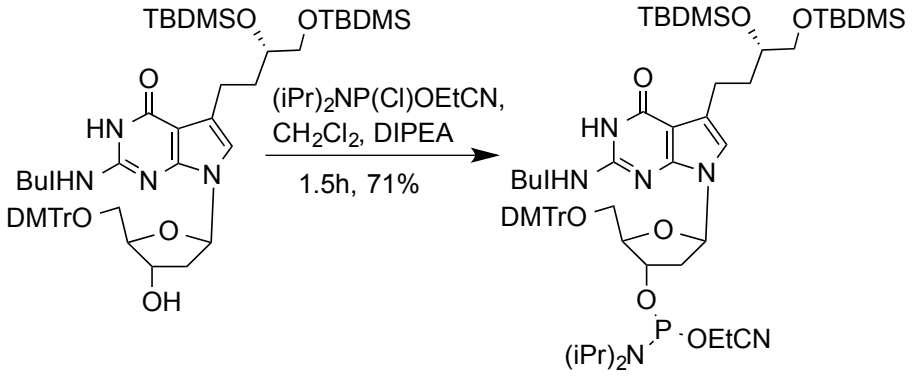
^{1}H NMR ($CDCl_3$): δ 11.52 (s, 1H, N(1)-H, 8.08 (s, 1H, C2-(NH)), 7.41 (dd, 2H, DMT-H, J = 6.4, 1.2), 7.31-7.20 (m, 7H, DMT-H), 6.80-6.77 (m, 4H, DMT-H), 6.55 (s, 1H, C8-(H)), 6.38 (dd, 1H, C(1')-H, J = 8, 5.6), 4.55 (m, 1H, C(4')-H), 4.04 (m, 1H, C7-$(CH_2)_2$-CH(O)-$CH_{2(a)}$),

3.76 (s, 6H, DMT-OMe), 3.70 (m, 1H, C7-(CH_2)-CH(O)-$CH_{2(b)}$), 3.52-3.43 (m, 2H, C(5')-H_a & C7-(CH_2)$_2$-C(*H*)-O), 2.79 (m, 1H, iBu-CH), 2.69 (m, 1H, C(2')-H_a), 2.57 (m, 1H, C7-$CH_{2(a)}$), 2.29-2.21 (m, 2H, C(2')-H_b & C7-$CH_{2(b)}$), 1.86-1.80 (m, 1H, C7-CH_2-CH_{2a}), 1.67-1.61 (m, 1H, C7-CH_2-CH_{2b}), 1.07 (dd, 6H, iBu-Me, J = 10.8, 6.8), 0.86 & 0.85 (2s, 18H, t-BuMe), 0.04 (s, 6H, Si-Me), 0.01 (s, 6H, Si-Me).

^{13}C NMR: ($CDCl_3$): δ 178.9 (C), 158.5 (C), 157.6 (C), 147.7 (C), 146.3 (C), 144.3 (C), 135.4 (C), 129.9 (C), 128.0 (C), 127.8 (C), 122.4 (CH), 114.4 (CH), 113.1 (CH), 104.1 (CH), 86.6 (C), 85.6 (CH), 82.6 (CH), 73.2 (CH), 67.6 (CH), 64.2 (CH), 55.0 (C), 39.6 (CH), 36.0 (CH_2), 34.7 (CH), 25.9 (CH_3), 22.2 (CH_2), 18.4 (CH_3), 18.3 (C), 18.1 (C), -4.2 (CH_3), -4.6 (CH_3), -5.3 (CH_3).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{52}H_{75}O_9N_4Si_2$ 955.5067, observed 955.5069.

7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butyl)-5'-O-(4,4'-dimethoxytrityl)-N(2)-isobutryl-2'-deoxyguanosine-3'-[(2-cyanoethyl)N,N-diisopropylphosphoramidite] (31)



31

To a solution of 7-deaza-7-(*S*)-(2,3-di-(O-tertbutyl-dimethylsilyl)butyl)-5'-O-(4,4'-dimethoxytrityl)-N(2)-isobutryl-2'-deoxyguanosine () (160 mg, 0.17 mmol) in 4 mL CH_2Cl_2 and

N-ethyl-diisopropylamine (117 μL, 0.67 mmol) was added 2-cyanoethyl-N,N-diisopropyl-chlorophosphoramidite (75 μL , 0.33 mmol). The reaction was stirred at room temperature for 1.5 h, quenched with 400 μL methanol and solvent evaporated under reduced pressure. Purification of the residue by silica gel column chromatography (hexane/ethyl acetate 3:1, 0.5% Et_3N) gave the product as a white solid (140 mg, 0.12 mmol, 71%).

R_f : 0.75 (hexane/ethyl acetate 1:2)

^{1}H NMR ($CDCl_3$): 11.51 (s, 1H, N(1)-H), 8.29 & 7.90 (2s, 1H, C2-(NH)), 7.47-7.43 (m, 2H, DMT-H), 7.35-7.19 (m, 7H, DMT-H), 6.80-6.76 (m, 4H, DMT-H), 6.59 (2 peaks, C8-(H)), 6.36-6.28 (m, 1H, C(1')-H), 4.71-4.60 (m, 1H, C7-$(CH_2)_2$-CH(O)-$CH_{2(a)}$), 3.88-3.80 (m, 1H, C7-$(CH_2)_2$-CH(O)-$CH_{2(b)}$), 3.77 & 3.76 (2s, 6H, DMT-OMe), 3.65-3.55 (m, 2H, iPr-CH), 3.54-3.44 (m, 2H, C(5')-H_a & C(3')-H), 3.37-3.22 (m, 2H, C(5')-H_b & C7-$(CH_2)_2$-C*H*), 2.86-2.77 (m, 1H, iBu-CH), 2.75-2.65 (m, 2H, C(2')-H_a & NC-CH_2), 2.63-2.51 (m, 1H, C7-$CH_{2(a)}$), 2.47 (t, 1H, NC-CH_2), 2.36-2.18 (m, 2H, C(2')H_b & C7-$CH_{2(b)}$), 1.88-1.82 (m, 1H, C7-$(CH_2)_2$-$CH_{2(a)}$), 1.69-1.58 (m, 1H, C7-$(CH_2)_2$-$CH_{2(b)}$), 1.19-1.05 (m, 18H, iPr-Me & iBu-Me), 0.86 (s, 18H, tBu-Me), 0.04 & 0.01 (2s, 12H, Si-Me).

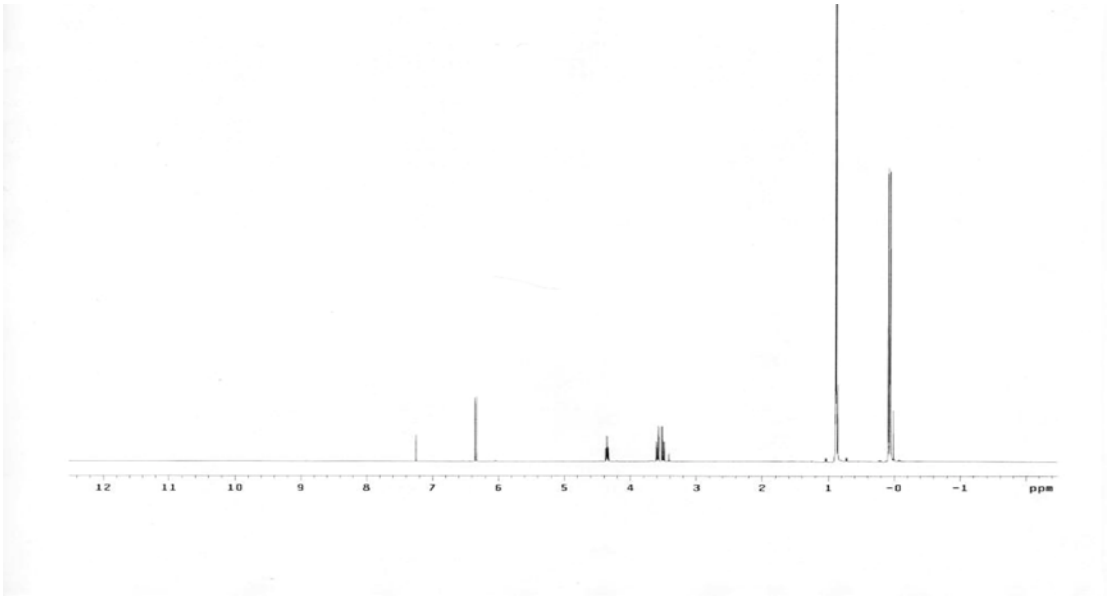
^{31}P NMR ($CDCl_3$): 149.35, 149.22, 149.09, 148.93.

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{61}H_{92}O_{10}N_6PSi_2$ 1155.6145, observed 1155.6149.

NMR Spectra

Figure 2.7: ^{1}H & ^{13}C NMR Spectra of (*R*)-3,4-di-(O-tertbutyl-dimethylsilyl)but-(1,1-dibromo)-ene (26)

^{1}H



^{13}C

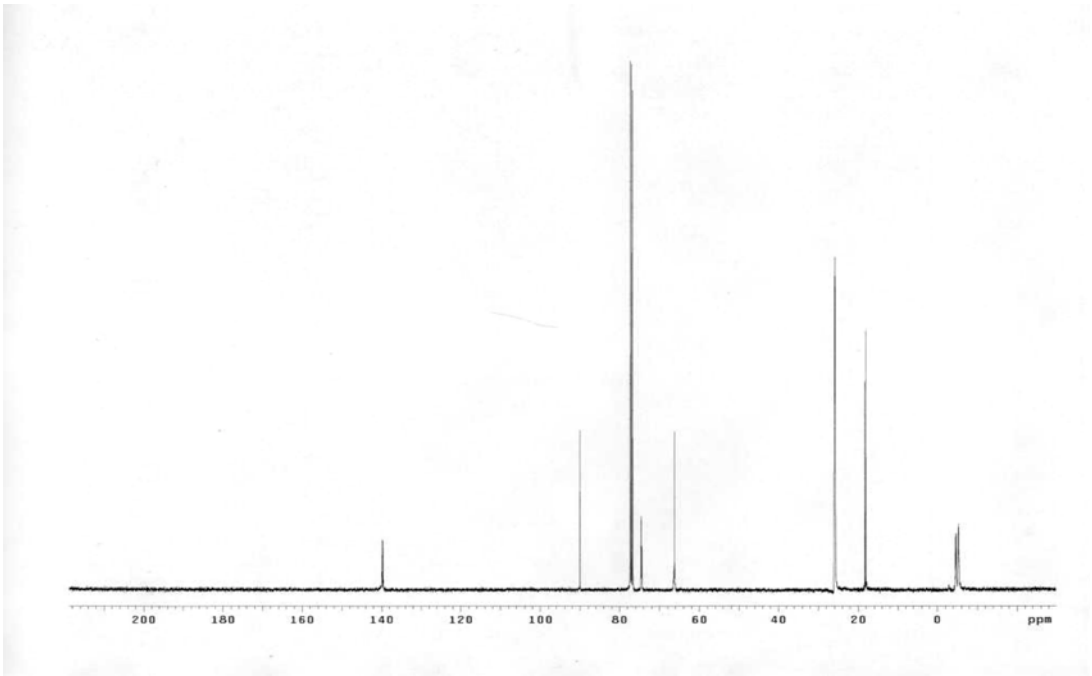
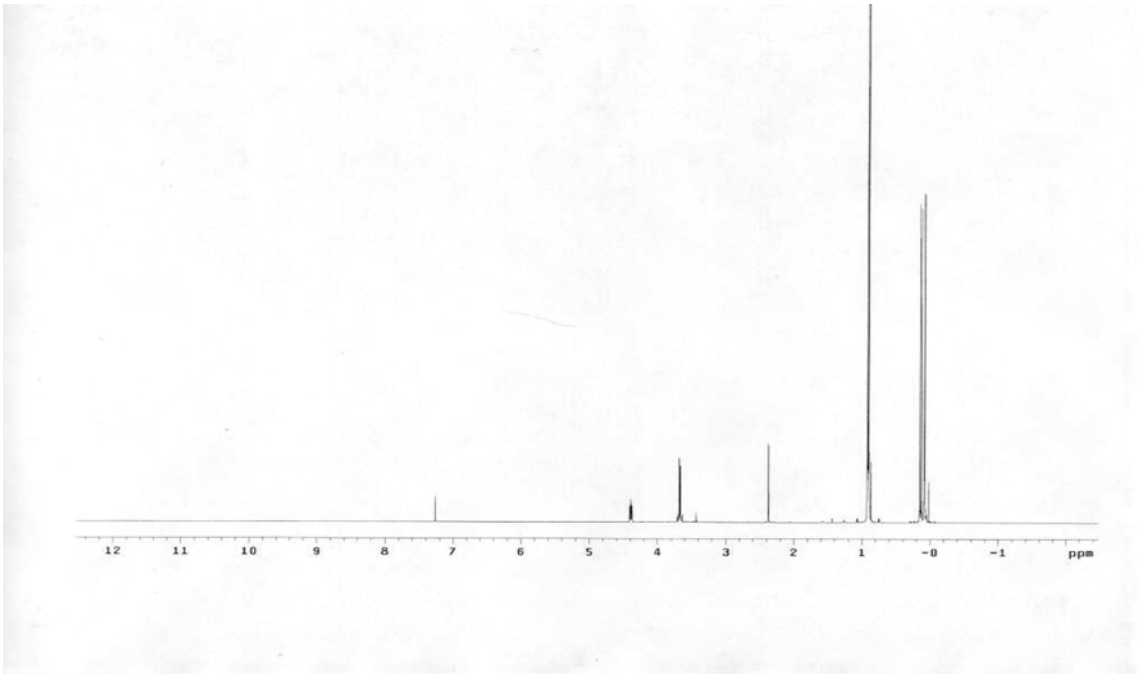


Figure 2.8: ^{1}H & ^{13}C NMR Spectra of (*R*)-3,4-di-(O-tertbutyl-dimethylsilyl)but-1-yne (27)

^{1}H



^{13}C

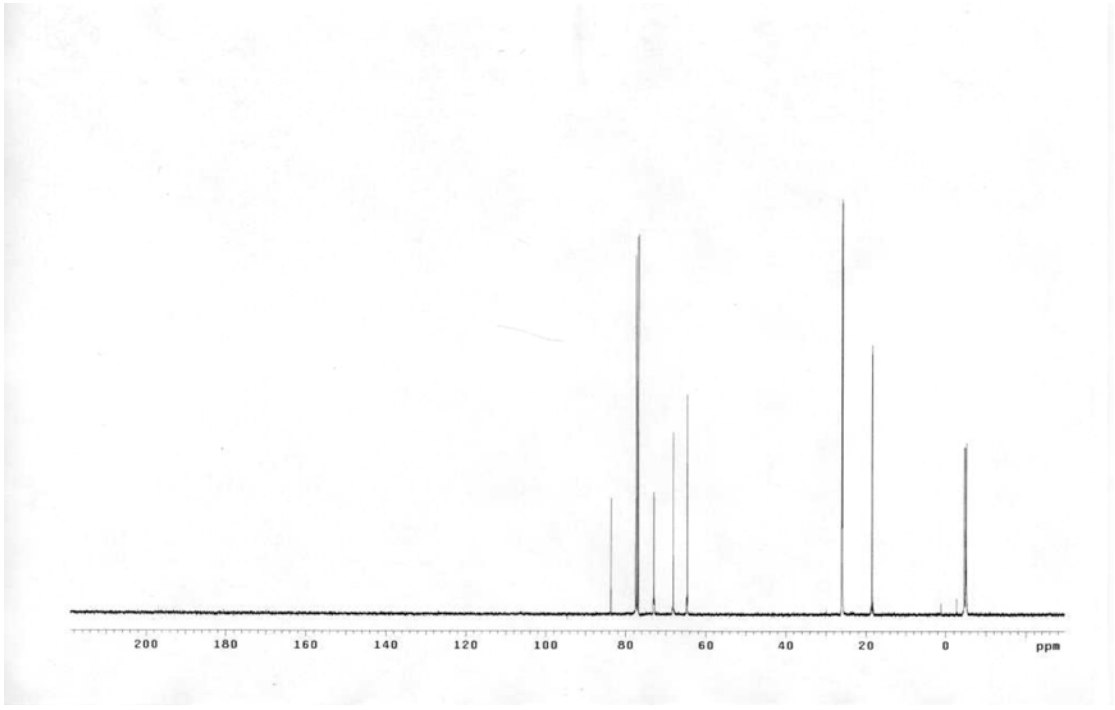
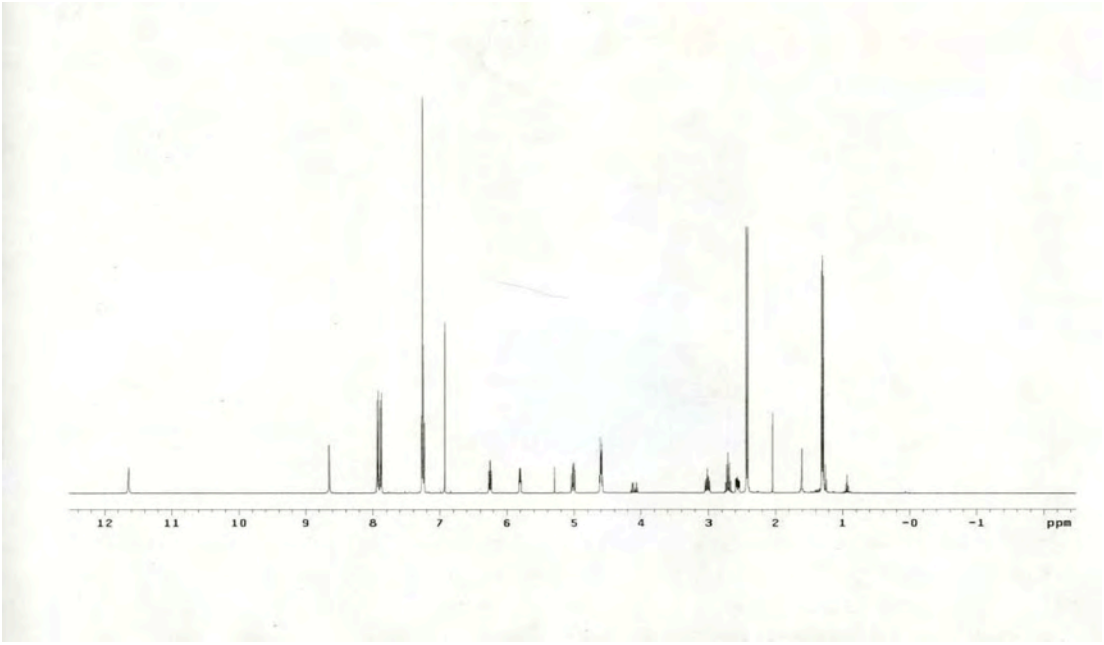


Figure 2.9: ^{1}H & ^{13}C NMR Spectra of 7-deaza-7-iodo-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (28)

^{1}H



^{13}C

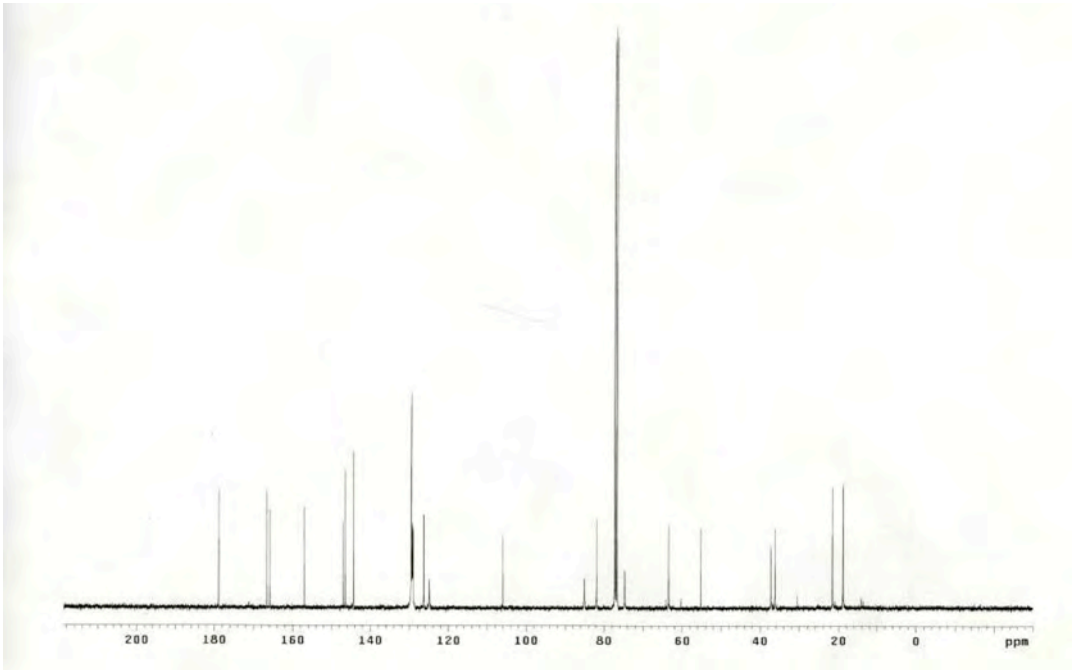
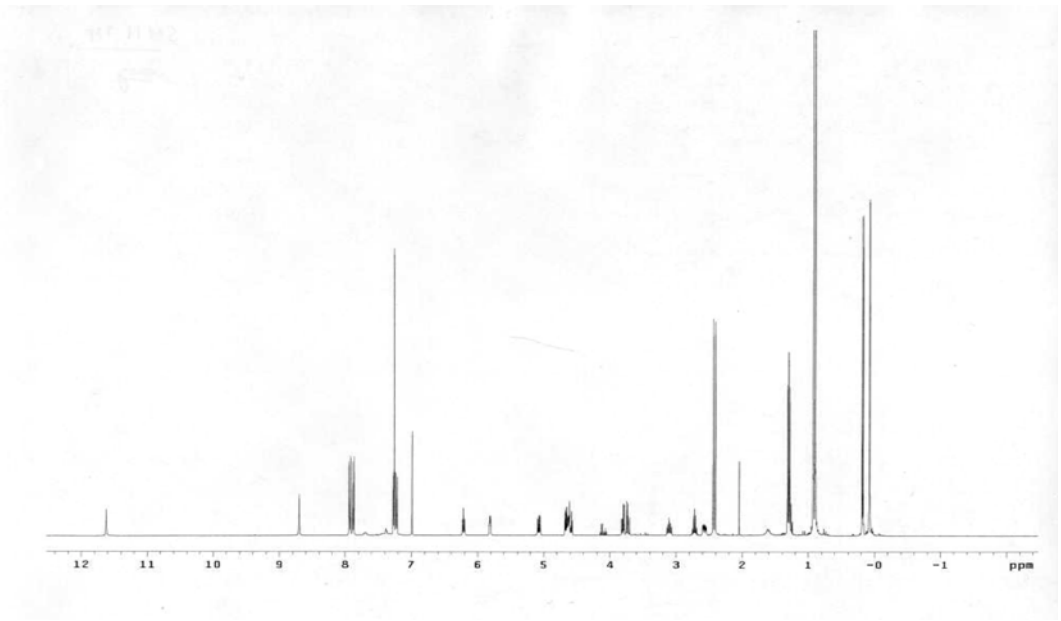


Figure 2.10: ^{1}H & ^{13}C NMR Spectra of 7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butynl)-N(2)- isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (29)

^{1}H



^{13}C

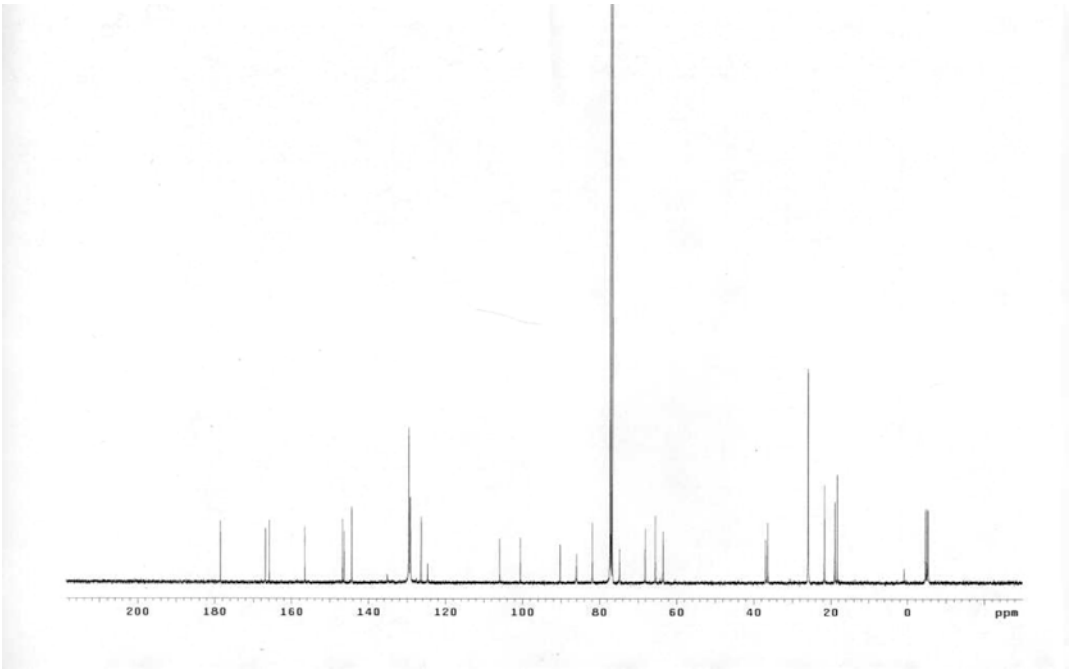
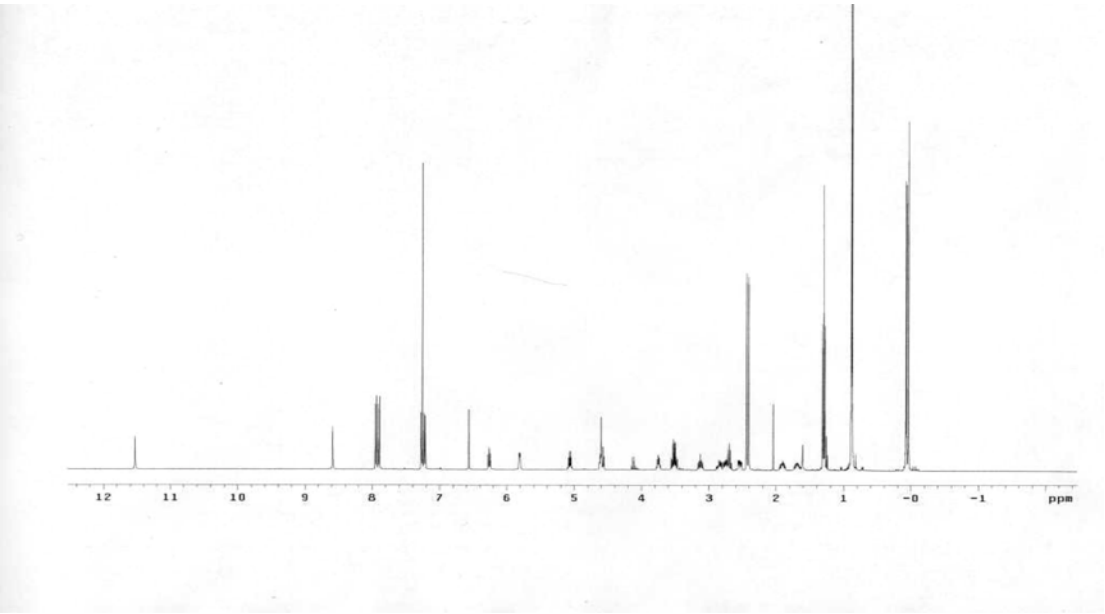


Figure 2.11: ^{1}H & ^{13}C NMR Spectra of 7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butyl)-N(2)- isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine

^{1}H



^{13}C

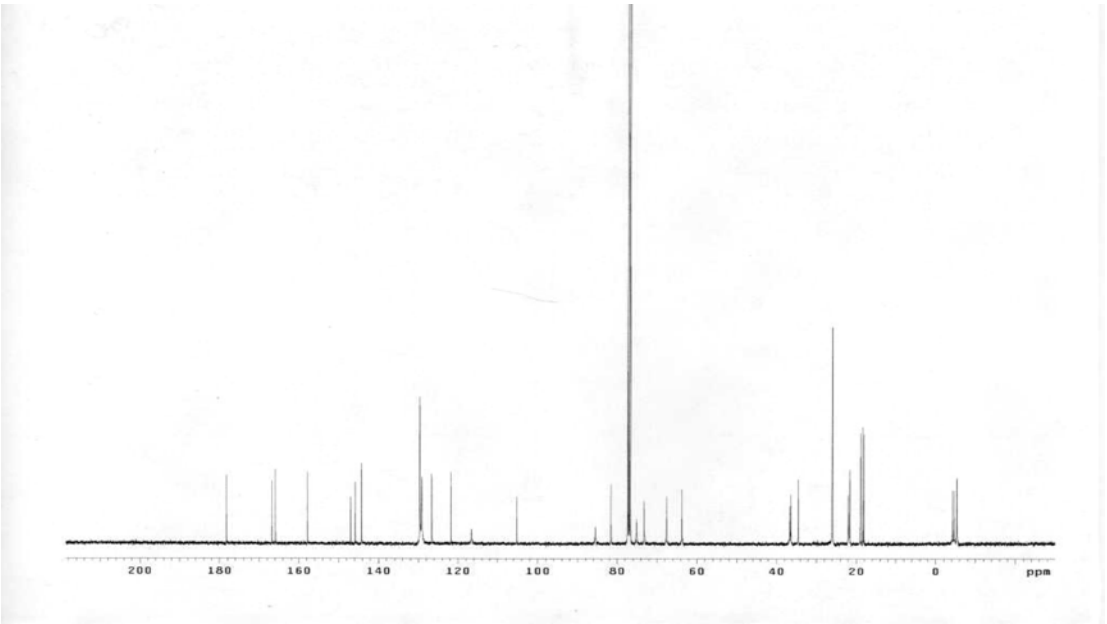
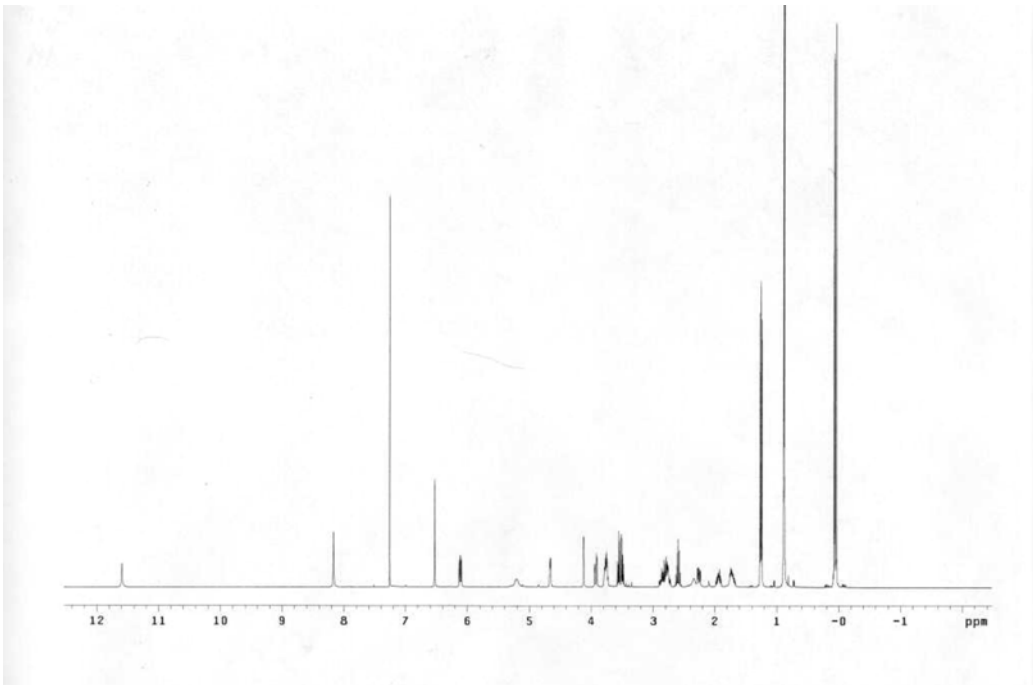


Figure 2.12: ^{1}H & ^{13}C NMR Spectra of 7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butyl)-N(2)- isobutyryl-2'-deoxyguanosine (30)

^{1}H



^{13}C

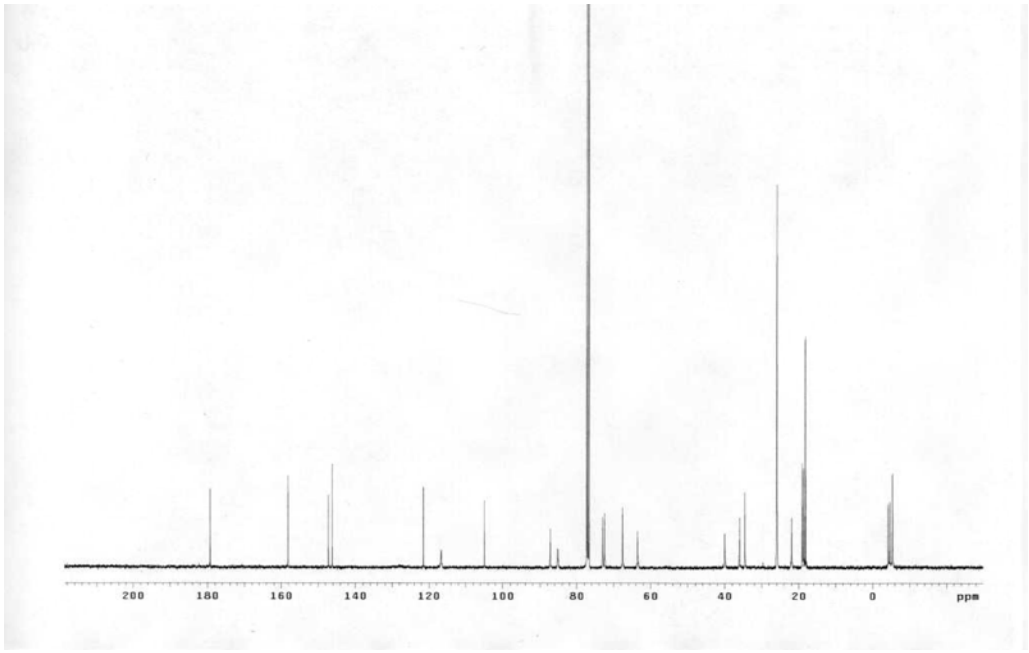
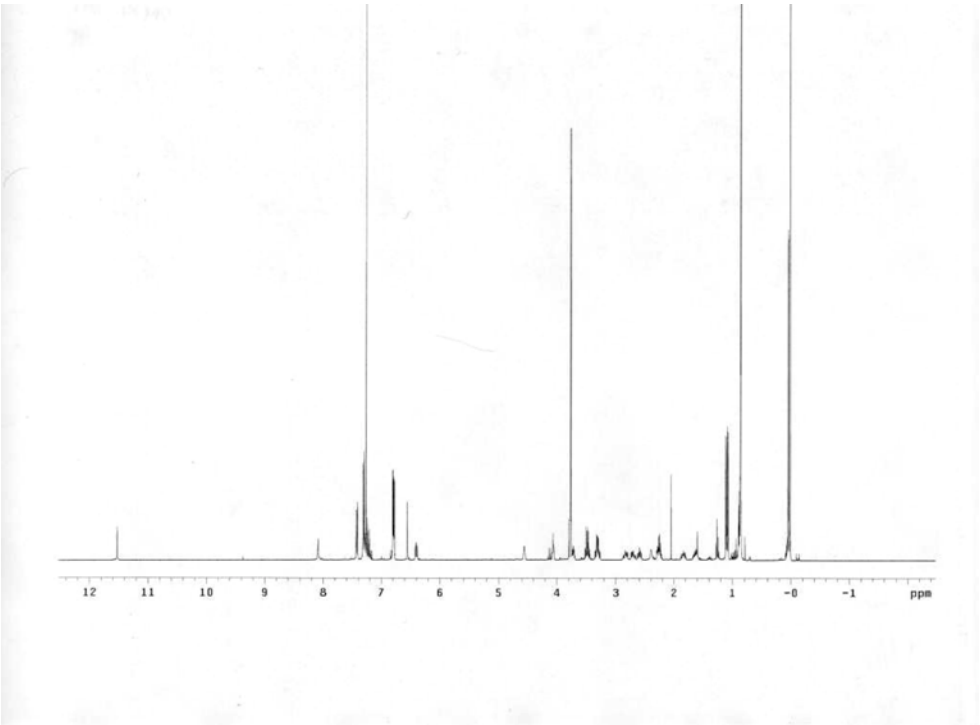


Figure 2.13: ^{1}H & ^{13}C NMR Spectra of 7-deaza-7-(*S*)-(2,3-di-(O-tertbutyl-dimethylsilyl)butyl)-5'-O-(4,4'-dimethoxytrityl)-N(2)-isobutryl-2'-deoxyguanosine

^{1}H



^{13}C

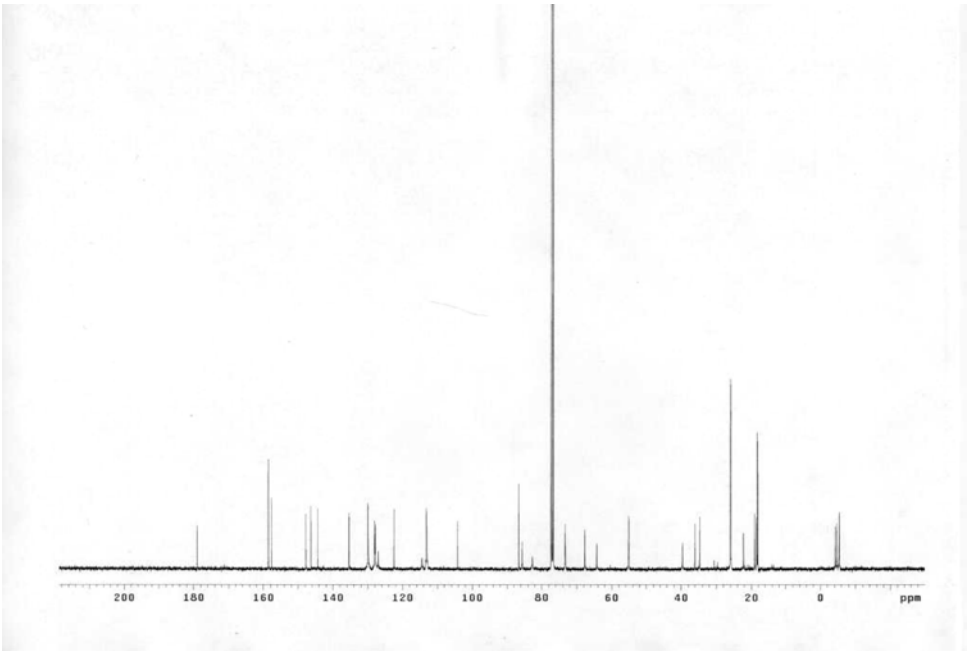
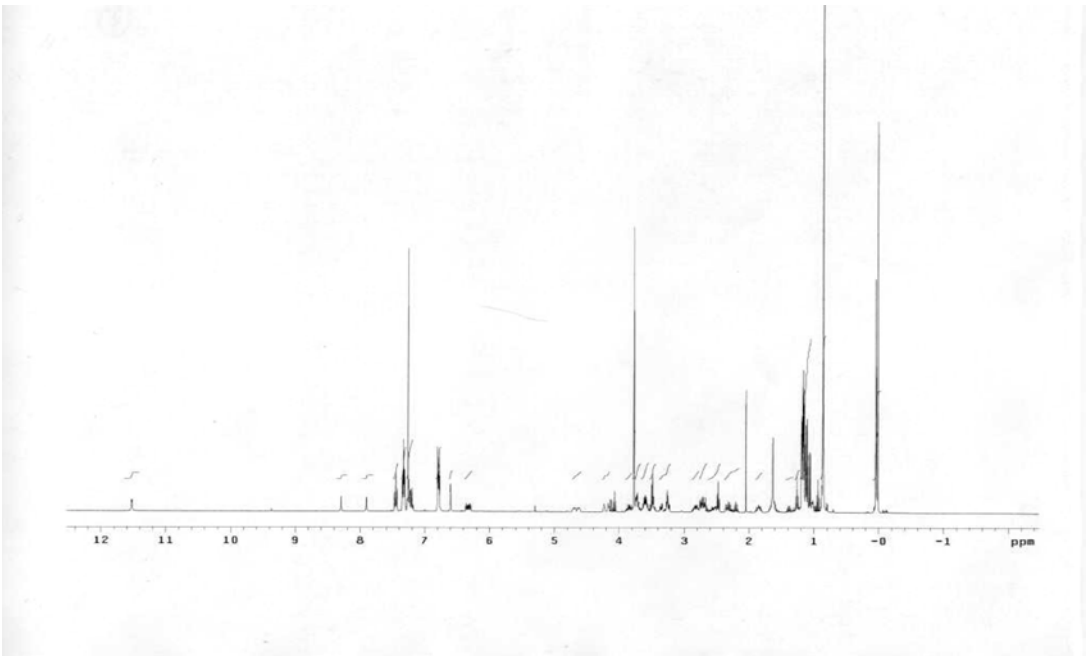
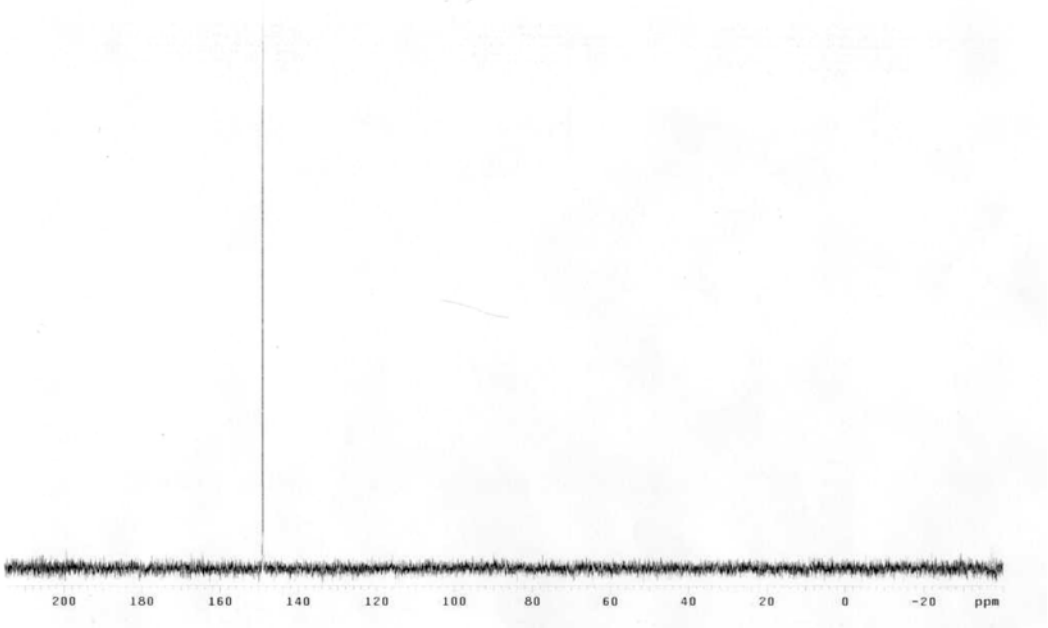


Figure 2.14: ^{1}H & ^{13}C NMR Spectra of 7-deaza-7-(*S*)-(3,4-di-(O-tertbutyl-dimethylsilyl)butyl)-5'-O-(4,4'-dimethoxytrityl)-N(2)-isobutryl-2'-deoxyguanosine-3'-[(2-cyanoethyl)N,N-diisopropylphosphoramidite (31)

^{1}H



^{31}P



Oligonucleotide Synthesis and Purification

Oligonucleotide synthesis was carried out on an Expedite 8909 Nucleic Acid Synthesis System (Applied Biosystems) using 1 μmol 1000 Å CPG –dG and –dC column cartridges (Biosearch Technolgies). An extended coupling time of 15 min was used with all the modified phosphoramidite building blocks. The following sequences were synthesized: 5'-d(GTCACTGGTA**G***ACAGCATTG) and 5'-d(CAATGCT**G***TCTACCAGTGAC) where **G*** is the modified phosphoramidite having either C1, C2 or C3 linker. The trityl-on oligonucleotides were deprotected by treatment with concentrated NH_4OH solution at 50°C for 12 h, then purified and detritylated using 1 μmol TOP-cartridges (Agilent Technologies) following the manufacturer's protocol. The TBDMS group in the oligonucleotides containing the C3 aldehyde precursor was removed by treating with TEA•3HF overnight at 40°C followed by precipitation using 1-butanol. Subsequently, oligonucleotides were purified by HPLC on a C18 column (Phenomenex Clarity 5μ Oligo-RP 50 x 10 mm) using the following elution gradient: linear 2.5-15% B over 21 min, linear 15-90% B over 25 min, isocratic 90% B till 26 min, linear 90-2.5 % B till 28 min, isocratic 2.5 % B till 30 min; (eluent A: 0.1 M TEAA (pH 7); eluent B: CH_3CN).

Formation of ICLs by reductive amination

A solution containing two single stranded oligonucleotides (25 nmols in 125 μL 100 mM NaCl) was heated to 95°C and allowed to cool over a period of 5 h for annealing to take place. For oxidation, 10 μL 50 mM $NaIO_4$ and 15 μL 1 M sodium phosphate buffer (pH 5.4) was added and mixture was kept at 4°C overnight. Excess $NaIO_4$ was removed by centrifugation through Microcon columns with a 3K cutoff (Millipore). The crosslink was formed by adding 10 μL 5 mM aqueous solution of the amine (ammonium acetate, hydrazine or N,N'-dimethylethylene

diamine (DMEDA)) and 10 μL of a 0.5 M solution of sodium cyanoborohydride and allowing to react in the dark at room temperature overnight. ICL formation was analyzed by gel electrophoresis on a 20% denaturing polyacrylamide gel (7 M urea). To purify and isolate the ICL-containing oligonucleotide the gel was visualized by UV shadowing and the crosslinked oligonucleotide was excised from the gel and the DNA was extracted by electroelution using the Elutrap device (Schleicher & Schuell). After purification the isolated yields of ICLs were in the range from 5-12 nmols.

Table 1.1: MALDI-TOF data of single stranded and ICL oligonucleotides

S1: 5'-GTCACTGGTA**G*** ACAGCATTG-3', **S2**: 5'-CAATGCT**G*** TCTACCAGTGAC-3'

G*= modified phosphoramidite with either C1/C2/C3 alkyl chain linker

C1, C2, C3: 1, 2 or 3 carbon alkyl chain linker with diol

HY: Hydrazine, DMEDA: N,N'-dimethylethylenediamine

Oligo Sequence	Amine	Calculated m/z	Observed m/z
S1C3	-	6244	6238
S2C3	-	6164	6158
S1C2 x S2C3	NH_3	12315	12331
S1C2 x S2C3	HY	12330	12326
S1C2 x S2C3	DMEDA	12386	12385
S1C3 x S2C3	NH_3	12329	12333
S1C3 x S2C3	HY	12344	12342
S1C3 x S2C3	DMEDA	12400	12391
S1C1 x S2C3	HY	12316	12379
S1C1 x S2C3	DMEDA	12372	12372

MALDI-TOF Images of single stranded and ICL oligonucleotides

Figure 2.15: MALDI-TOF of ss-oligo S1C3 (5'-GTCACTGGTA**G***ACAGCATTG-3')

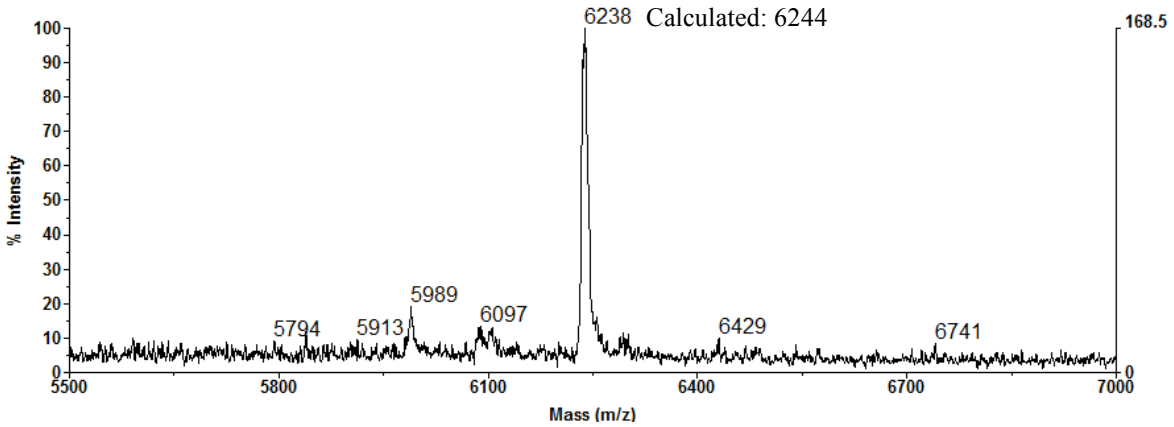


Figure 2.16: MALDI-TOF of ss-oligo S2C3 (5'-CAATGCT**G***TCTACCAGTGAC-3')

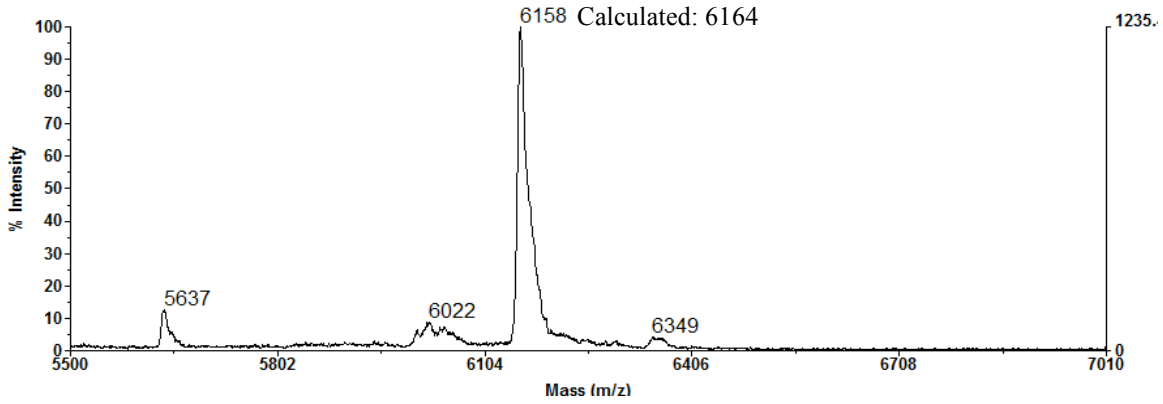


Figure 2.17: MALDI-TOF of ICL S1C2 x S2C3 + NH_3

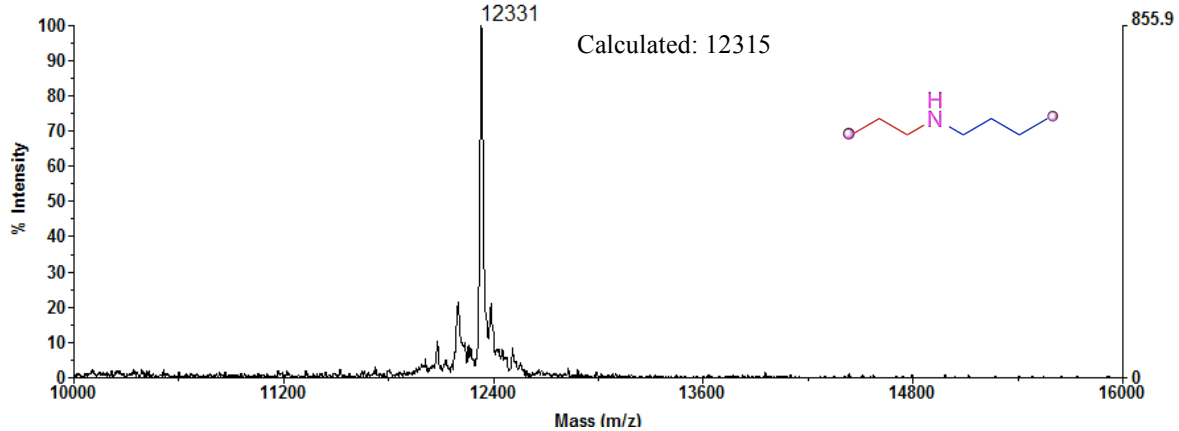


Figure 2.18: MALDI-TOF of ICL S1C2 x S2C3 + HY

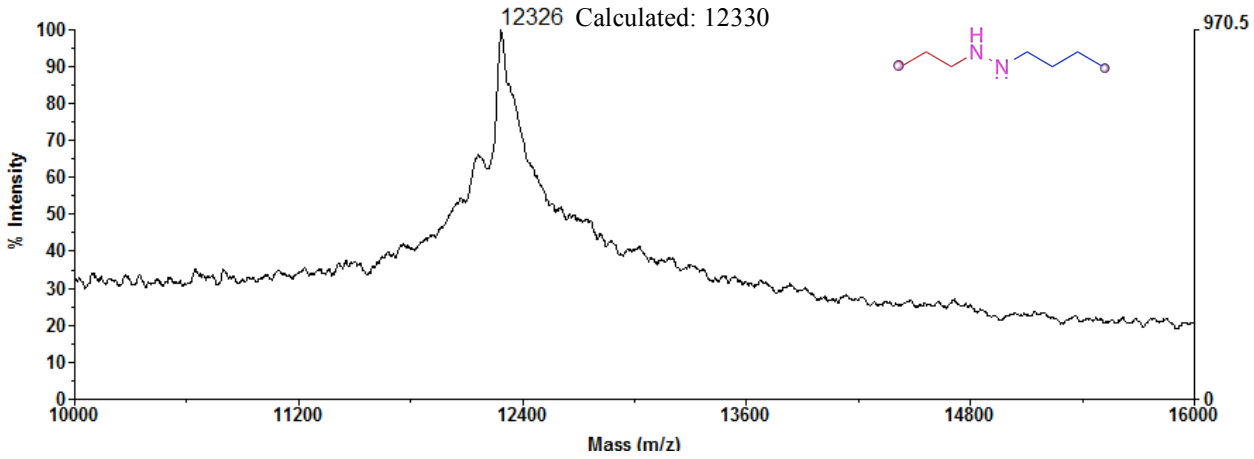


Figure 2.19: MALDI-TOF of ICL S1C2 x S2C3 + DM

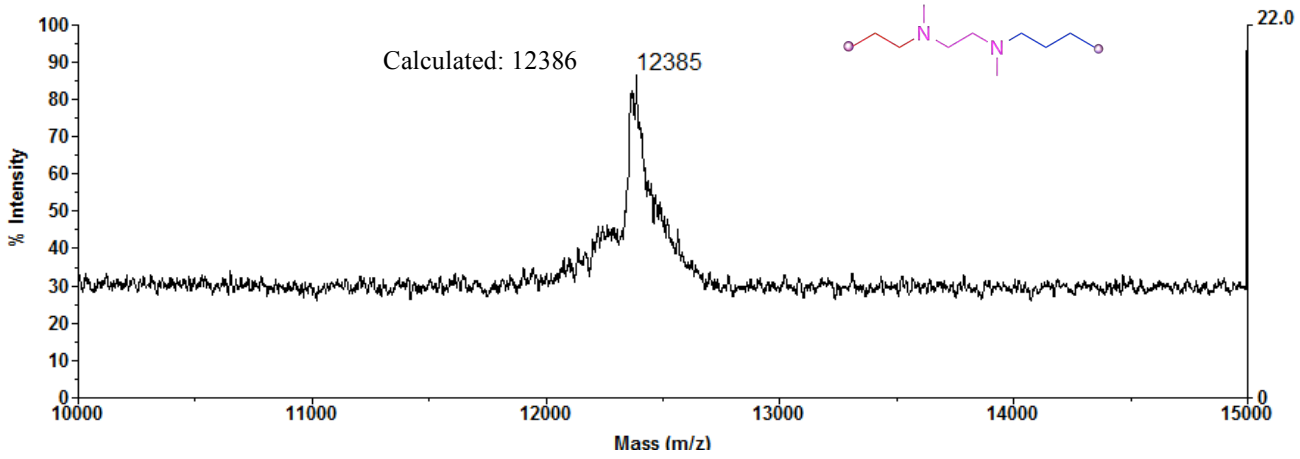


Figure 2.20: MALDI-TOF of ICL S1C3 x S2C3 + NH_3

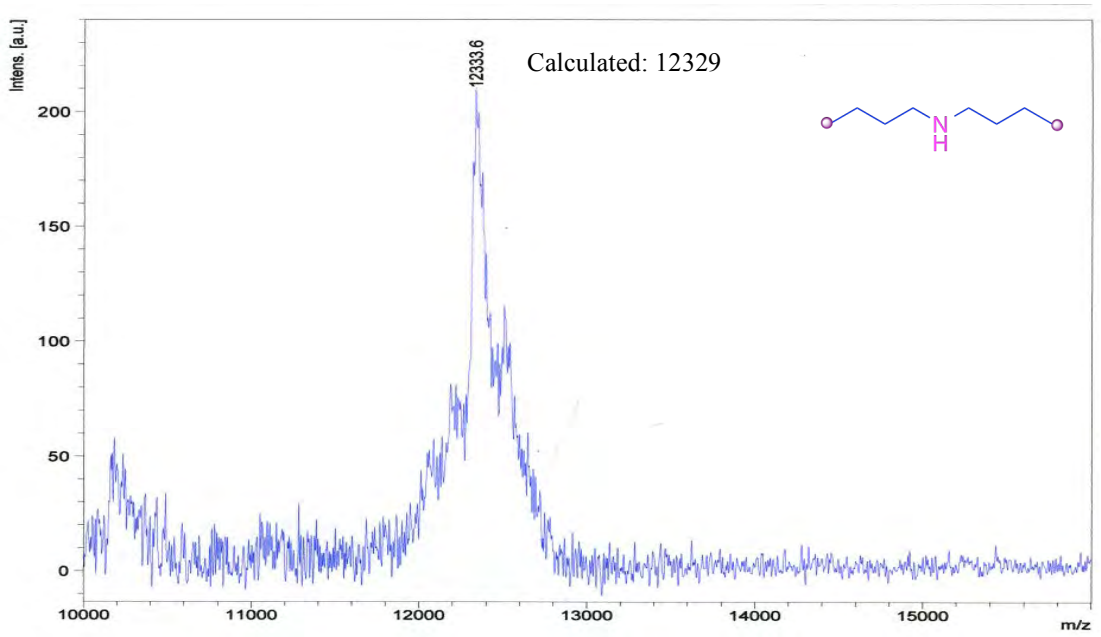


Figure 2.21: MALDI-TOF of ICL S1C3 x S2C3 + HY

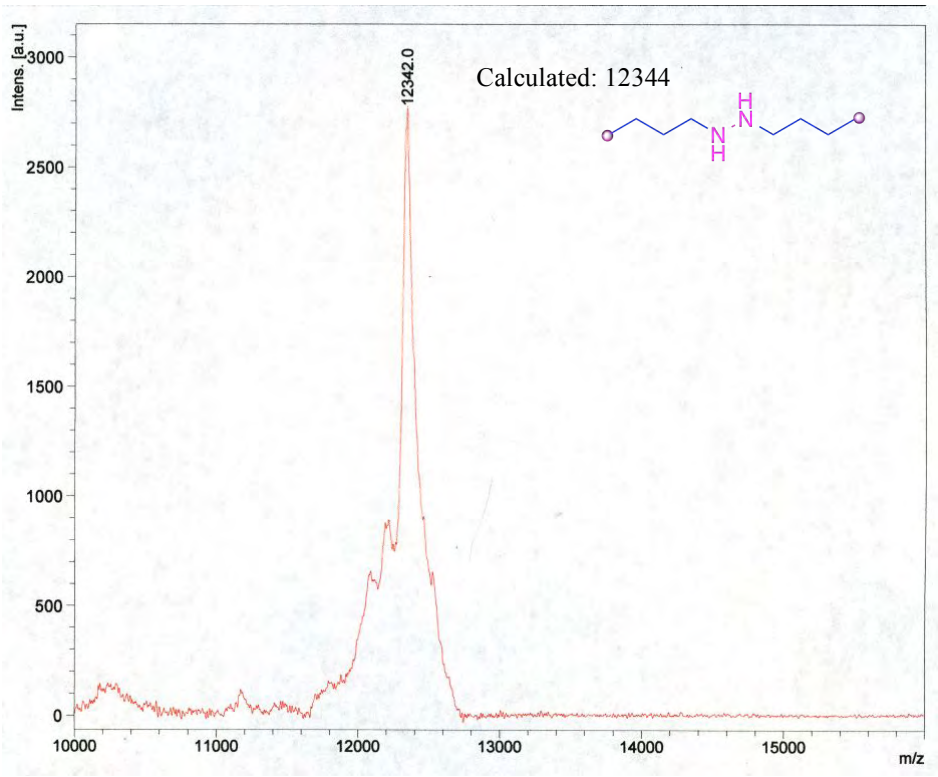


Figure 2.22: MALDI-TOF of ICL S1C3 x S2C3 + DM

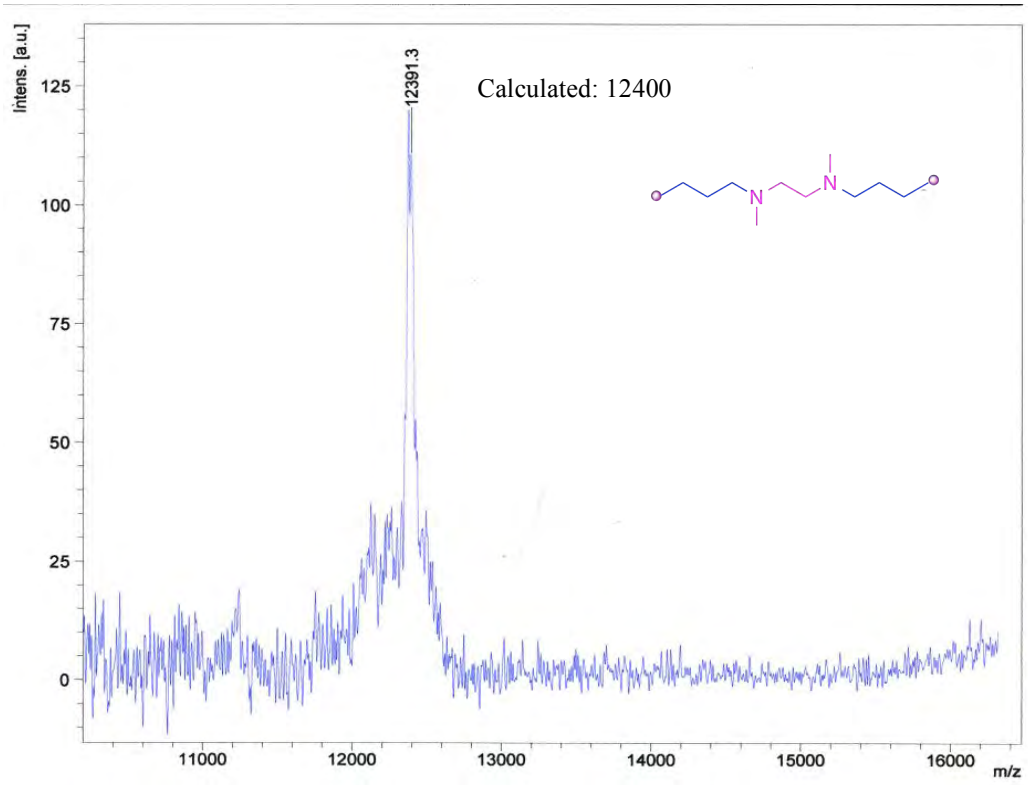


Figure 2.23: MALDI-TOF of ICL S1C1 x S2C3 + HY

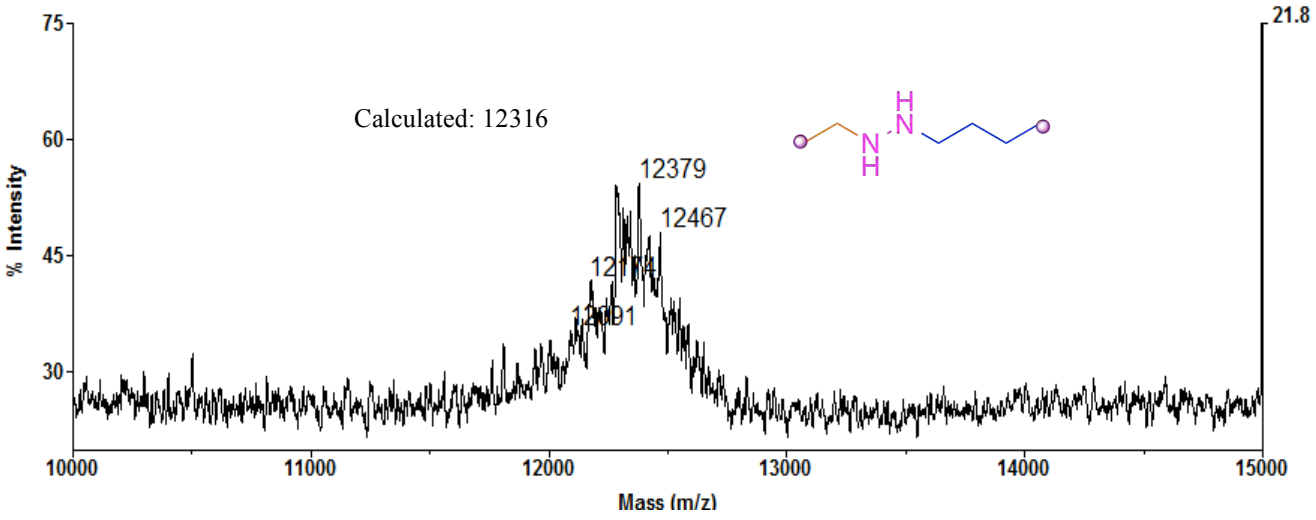
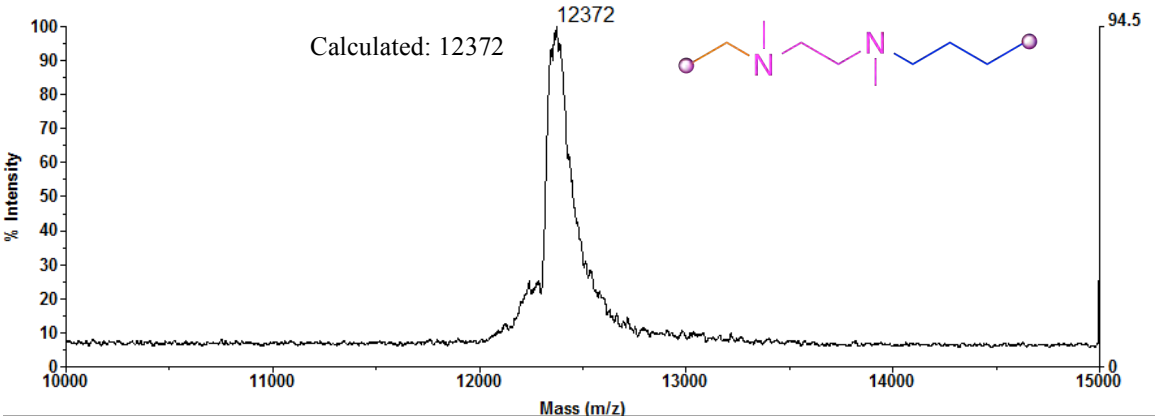


Figure 2.24: MALDI-TOF of S1C1 x S2C3 + DM



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Chapter 3

Synthesis of a Single Nucleotide DNA Interstrand Crosslink

Abstract

Translesion Synthesis (TLS) is an important step in replication-dependent and replication-independent ICL repair. This step is carried out by a specialized set of DNA polymerases known as TLS polymerases that help in the bypass of an ICL that is unhooked from one of the two crosslinked strands. Studies have shown that the activity of TLS polymerases is greatly influenced by the length of double stranded DNA surrounding the ICL. Here we describe our efforts to generate an ICL that consists of a single nucleotide connected to a single stranded oligonucleotide through the synthesis of a new precursor molecule and subsequent ICL formation by post-synthetic reductive amination. The development of such a substrate will allow us to further study the effect of TLS polymerases on partial duplex ICL substrates.

Introduction

An important step in ICL repair pathway is translesion synthesis (TLS) carried out by specialized DNA polymerases. In replication-dependent ICL repair, following dual incisions, TLS polymerases insert a base opposite to the crosslinked base and fully extend the nascent past the unhooked ICL. This is important to generate an intact template for the homologous recombination (HR) machinery to repair the double strand break (DSB) (Figure 7, chapter 1). In the replication-independent repair pathway, TLS polymerases bypass the unhooked ICL and fully restore one of the two strands (1). Consistent with this fact a number of human polymerases, such as Pol η, Pol κ, Pol ι, Pol ν and REV1, have been found to insert a base opposite an ICL or to bypass it. In mammals, REV1 and Pol ζ are key players in the repair pathway. Cells deficient in either of these genes are profoundly sensitive to cross-linking agents (2,3). In replication-dependent ICL repair pathway the role of REV1 and Pol ζ have been shown using the *Xenopus* egg extract system (4) while in a replication-independent pathway the same has been demonstrated by using a psoralen, MMC or cisplatin site-specific ICLs in a *nonreplicating* plasmid-based reporter assay (5,6). The role of other DNA polymerases in the replication-dependent repair pathway is not well understood. The activity of TLS polymerase on ICL substrates is dictated by two main factors: the length of the ICL bridge and the amount of ds-DNA surrounding the ICL. *In vitro* studies with Pol η, Pol κ, Pol ι on N^2-N^2 dG ICL adducts have shown that reducing the length of ds-DNA surrounding the lesion remarkably enhanced the bypass by the polymerases (7-9). Our own studies with Pol ζ, Pol η, Pol κ, and Pol ι showed that resection of ds-DNA around the ICL leads to the bypass by the polymerases (10). In the course of this study, it was also observed that the

distortion of the DNA caused by the ICL allows for more efficient strand displacement synthesis.

Our strategy of using post-synthetic reductive amination is a powerful tool in generating structurally diverse ICLs (11,12). Here we extend this strategy through the synthesis of a new precursor molecule to synthesize an ICL that consists of a single base connected to a ss-DNA oligonucleotide. The availability of such a substrate would allow us to address the question on how the length of ds-DNA surrounding the ICL influences the activity of TLS polymerases on ICLs. As ICL remnants with single nucleotide maybe formed during ICL repair (4), this substrate would provide us with a mimic of what might be encountered under physiological conditions.

RESULTS and DISCUSSION

The activity of TLS polymerases around an ICL is greatly influenced by the length of ds-DNA surrounding the ICL. Shortening the duplex significantly enhances lesion bypass by TLS polymerases. Our laboratory has used the strategy of incorporating uracil residues to partially cleave one of the strands, thereby shortening the length around the ICL (10) . However, since the activity of the enzyme uracil DNA glycolyase was inhibited closer to the ICL, this method only allows the generation of ICL embedded in duplex containing around five nucleotides . We reasoned that the synthesis of a 7-deazaguanosine molecule having an alkylamino linker **38** and it's subsequent coupling to an oligonucleotide with an aldehyde precursor using reductive amination strategy would generate a single base ICL.

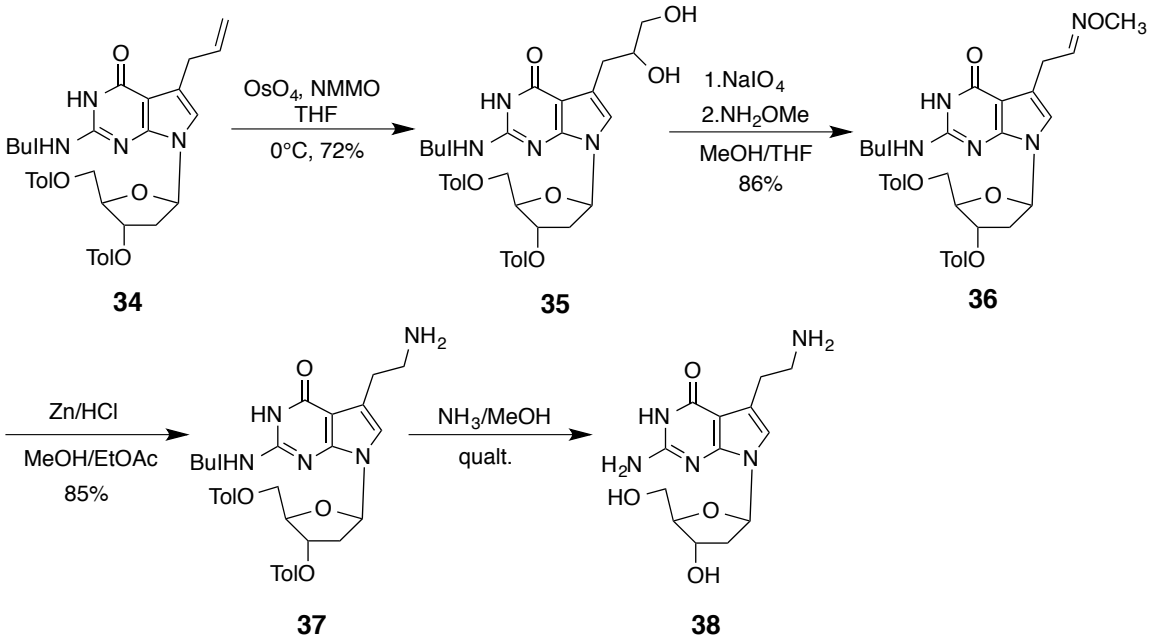


Figure 3.1: Scheme showing the synthesis of 7-(2-aminoethyl)-7-deazaguanosine **38**

The route for the synthesis of **38** was developed by our former group member Dr. Todor Angelov. Starting with the previously synthesized **34** (11), the allyl group was converted to the dihydroxypropyl moiety **35** by treatment with OsO_4 and N-methylmorpholine-N-oxide. **35** was transformed into the oxime nucleoside **36** by sequential reaction with $NaIO_4$ and O-methyl hydroxylamine. The O-methyloxime group in **36** was reduced using zinc over HCl to furnish **37** having a two-carbon alkyl-amino linker. The protecting groups on exocyclic $–NH_2$ and the 5'- and 3'- positions of sugar were removed by treatment with NH_3 in methanol to generate **38** having a two-carbon alkyl amino linker.

For ICL formation, an 11-mer single stranded oligonucleotide with a modified 7-deazaguanine with C2 diol precursor was oxidized with $NaIO_4$. After removal of excess $NaIO_4$, ICL formation was initiated by the addition of 7-(2-aminoethyl)-7-deazaguanine and $NaCNBH_3$ (Figure 2A).

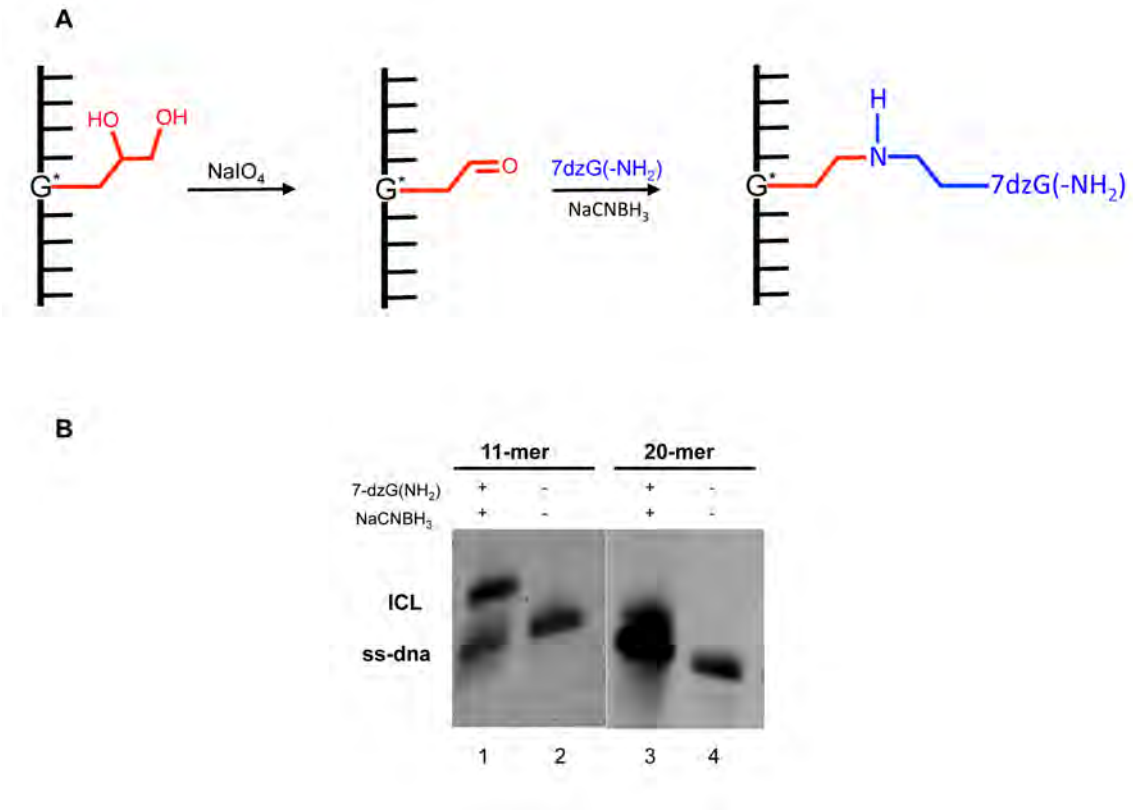


Figure 3.2: A. Scheme showing the synthesis of single nucleotide ICL; B. Analysis of ICL reaction on 11- and 20-mer oligonucleotide with 7-(2aminoethyl)-7-deazaguanosine (7dzG-(NH_2)) and $NaCNBH_3$. Reactions were analyzed by 20% denaturing PAGE gel followed by staining with SYBR Gold. The sequences used were 5'-d(CGTA**G***TTCATGC) (11-mer) and 5'-d(GTCACTGGTA**G***ACAGCATTG) (20-mer) with **G*** being the modified guanine having C2 diol.

ICL formation was detected by the presence of a slower moving band in the reaction mixture when compared to the control 11-mer oligonucleotide (Figure 2B, lane 1 vs 2). To analyze the product formed, the oligomer was excised from the gel by UV-shadowing and DNA extracted and desalted using the crush and soak method. The identity of the ICL was confirmed by MALDI-TOF mass spectrometry (calculated $[M+H]^+$ 3683, observed 3676).

We next investigated ICL formation on a longer oligonucleotide (20 mer) having a C2 diol linker using the procedure outlined above. The ICL band in this case was found to migrate very close to the control single strand-oligonucleotide (Figure 2B, lane 3 vs 4). As such, band separation and further quantification was not possible with the 20-mer oligonucleotide. To further expand our methodology in forming ICLs with longer oligonucleotides (20-mer or more), a different route needs to be employed. Having a trityl group on the 5'- position of **37** might be useful as the ICL can be purified using reverse phase HPLC.

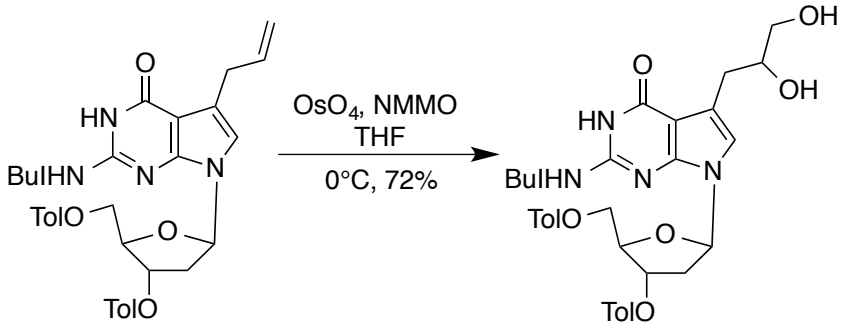
In conclusion, we have developed an effective strategy to generate an ICL connected by a single nucleotide. Further optimization of the strategy would allow us to have a more complete set of partial duplexes that would be valuable in understanding how the activity of TLS polymerases on ICLs is dependent on the length of ds-DNA surrounding the ICL.

Experimental Section

General Information

6-chloro-7-deaza-7-iodo-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine was prepared according to published procedures (1). All other reagents and solvents were purchased from Sigma Aldrich (USA), EMD (USA) or Alpha Aesar. Dry solvents (acetonitrile, dichloromethane, dioxane, DMF, ethyl acetate, methanol, pyridine and THF) were purchased from EMD and used without further purification. NMR Spectra were recorded on Bruker 400 MHz spectrometer. MALDI-TOF were recorded on Voyager-DE STR. All reactions were carried out under an inert atmosphere of nitrogen or argon. Chemical shifts (δ) are in ppm. J values are in Hz.

7-Deaza-7-(2,3-dihydroxypropyl)-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (35)



34 **35**

To a solution of 7-allyl-7-deaza-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (410 mg, 0.7 mmol) in a mixture of THF (10 mL) and H_2O (1 mL) was added a 2.5% solution of OsO_4 in t-BuOH (1.8 mL, 0.14 mmol) at 0°C. 4-methylmorpholine-4-oxide

monohydrate (180 mg, 0.16 mmol) was added to the reaction mixture and it was stirred at 0°C for 4 h under argon atmosphere. The reaction mixture was diluted with EtOAc (30 mL) and washed with 5% HCl (30 mL), sat. $NaHCO_3$ (30 mL) and brine (30 mL), dried over Na_2SO_4 and evaporated to dryness under reduced pressure. Purification of the residue by silica gel column chromatography (dichloromethane/methanol 20:1) gave the product as a light yellow solid (310 mg, 72%).

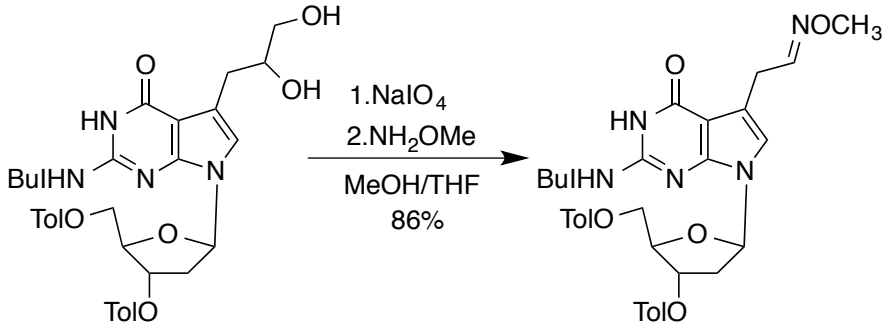
R_f: 0.76 (CH_2Cl_2/MeOH 10:1)

^{1}H NMR ($CDCl_3$): δ 11.89 (s, 1H, N(3)-H), 9.14 (2s, 1H, C2-(NH)), 7.94 (d, 2H, Tol-H, J= 8), 7.92 (d, 2H, Tol-H, J= 8.2), 7.24 (dd, 4H, Tol-H, J= 11.2, 6.6), 6.67 & 6.65 (2s, 1H, C8-H), 6.32 (m, 1H, C1'-H), 5.79 (dd, 1H, C3'-H, J= 5.8, 2.2), 4.98 (m, 1H, C4'-H), 4.61- 4.59 (m, 2H, C5'-H_2), 3.75 (m, 1H, C(*H*)-OH), 3.51 (m, 2H, C7-CH_2), 3.05- 2.92 (m, 2H,C7-CH_2), 2.89- 2.87 (m, 2H, C*H*$_2$OH, C2'-H_1), 2.78- 2.69 (m, 1H, iBu-CH), 2.64- 2.57 (m, 1H, C2'-H_2), 2.44 & 2.43 (2s, 6H, Tol-CH_3), 1.28 (dd, 6H, iBu-CH_3, J= 4, 2).

^{13}C NMR ($CDCl_3$): δ 178.7 (C), 166.7 (C), 165.9 (C), 159.4 (C), 147.3 (C), 145.7 (C), 144.4 & 144.3 (C), 129.7 (CH), 129.6 (CH), 129.2 (CH), 129.1 (CH), 126.5 (CH), 126.4 (CH), 119.6 & 119.3 (CH), 115.9 & 115.8 (C), 105.3 (C), 85.7 & 85.5 (CH), 81.9 & 81.8 (CH), 75.1 (CH), 72.7 & 72.6 (CH), 65.1 & 65.0 (CH_2), 37.0 (CH_2), 36.9 (CH_2), 36.3 (CH_2), 29.6 (CH_3), 27.7 (CH_3), 26.8 (CH_3), 21.6 (CH_3), 18.9 (CH_3).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{34}H_{39}O_9N_4$ 647.2712, observed 647.2717.

7-deaza-7-(2-methoxyiminoethyl)-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (36)



35 **36**

To a solution of 7-deaza-7-(2,3-dihydoxypropyl)-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (310 mg, 0.5 mmol) in a mixture of MeOH (10 mL) and THF (10 mL) was added 0.5 M aqueous solution of $NaIO_4$ (3 mL). The reaction mixture was stirred for 1.5 h at room temperature, quenched with 0.5 M aqueous solution of Na_2SO_3 (2.2. mL) and the resulting precipitate was filtered. To the clear filtrate was added 0.75 M methanol solution of O-methylhydroxylamine acetate (3.3 mL, 2.5 mmol) and the reaction was allowed to run for 1.5 h. It was subsequently concentrated under reduced pressure and the resulting oil was dissolved in EtOAc (30 mL), washed with H_2O (30 mL), sat. $NaHCO_3$ (30 mL) and brine (30 mL), dried over Na_2SO_4 and evaporated to dryness under reduced pressure. Purification of the residue by silica gel column chromatography (dichloromethane/methanol 20:1) gave the product as a yellow solid (280 mg, 86%).

R_f: 0.85 (CH_2Cl_2/MeOH 10:1)

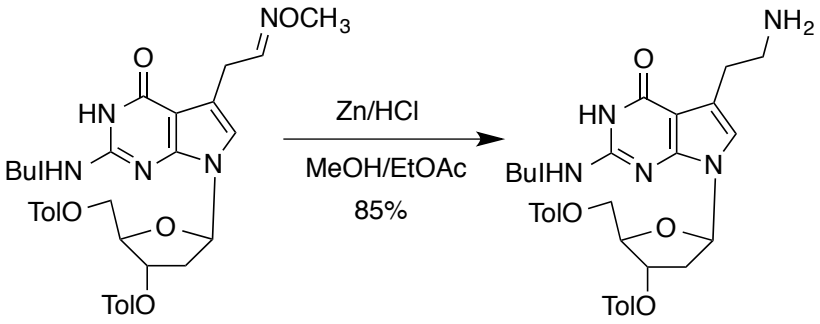
^{1}H NMR ($CDCl_3$): δ 11.61 (s, 1H, N(3)-H), 8.61 & 8.57 (2s, 1H, C2(NH)), 7.93 (dd, 2H, Tol-H, J= 8.1, 1.6), 7.88 (dd, 2H, Tol-H, J= 8.3, 2.1), 7.63 (t, 1H, N=C-H, J= 6.4), 7.28

(d, 2H, Tol-H, J= 8.4), 7.22 (d, 2H, Tol-H, J= 8.0), 6.89 (t, 0.3H, N=C-H), 6.64 & 6.62 (2s, 1H, C8-H), 6.22 (dd, 1H, C1'-H, J= 14.2, 7.8), 5.80 (dt, 1H, C3'-H, J= 5.8, 2.8), 5.04 (m, 1H, C5'-H), 4.56 (m, 2H, C5'-H & C4'-H), 3.89 & 3.81 (2s, 3H, N-OCH_3), 3.75 (d, 1H, C7-CH_2, J= 5.2), 3.63 (dd, 1H, C7-CH_2, J= 6.2, 0.8), 3.05 (m, 1H, C2'-H_1), 2.67 (m, 1H, iBu-CH), 2.51 (m, 1H, C2'-H_2), 2.44 & 2.41 (2s, 6H, Tol-CH_3), 1.29 (t, 6H, iBu-CH_3, J= 8.1).

^{13}C NMR ($CDCl_3$): δ 178.5 (C), 166.7 & 166.6 (C), 165.8 (C), 157.8 & 157.7 (C), 149.4 & 149.2 (C), 146.1 & 146.0 (C), 144.2 (C), 129.6 (CH), 129.5 (CH), 129.2 (CH), 129.1 (CH), 126.5 (C), 126.4 (C), 118.0 (CH), 115.3 (CH), 104.8 (CH), 85.5(CH), 81.7 (CH), 74.9 (CH), 63.6 (CH_2), 61.5 (CH_3), 36.8 (CH_2), 36.5 (CH), 26.3 (CH_2), 21.6 (CH_3), 18.8 (CH_3).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{34}H_{38}O_8N_5$ 644.2715, observed 644.2722.

7-(2-Aminoethyl)-7-deaza-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (37)



36 **37**

To a solution of 7-deaza-7-(2-methoxyiminoethyl)-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (280 mg, 0.43 mmol) in a mixture of MeOH (10 mL) in EtOAc (10 mL) was added Zinc powder (1.4 g, 20.7 mmol) and 37% aqueous HCl (1.5 mL, 15 mmol) diluted in 3 mL MeOH. The reaction mixture was stirred for 1.5 h at room temperature and then neutralized with 25% NH_4OH (2 mL). Zinc powder was filtered off and the reaction mixture was evaporated to dryness under reduced pressure. Purification by silica gel column chromatography (dichloromethane/methanol 20:1) gave the product as a light brown solid (225 mg, 85%).

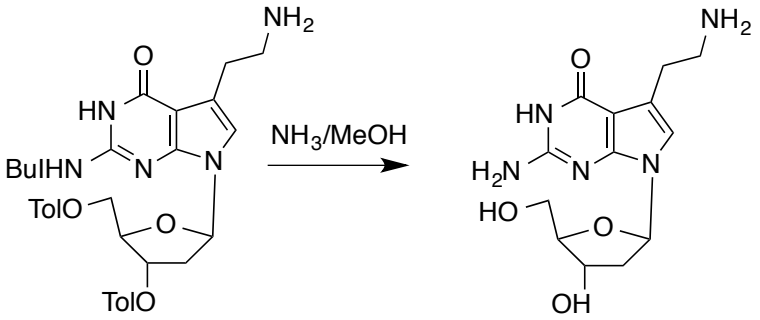
R_f: 0.22 (CH_2Cl_2/MeOH 20:1)

^{1}H NMR (CD_3OD): δ 7.88 (d, 2H, Tol-H, J= 8.2), 7.83 (d, 2H, Tol-H, J= 8.2), 7.26 (d, 2H, Tol-H, J= 8.0), 7.22 (d, 2H, Tol-H, J= 8.0), 6.99 (s, 1H, C8-H), 6.50 (dd, 1H, C1'-H, J= 8.1, 5.68 (dt, 1H, C3'-H, J= 6, 2.3), 4.62 (dd, 1H, C5'-H_1, J= 11.7, 4.6), 4.52 (dd, 1H, C5'-H_2, J= 11.8, 5.0), 4.45 (m, 1H, C4'-H), 3.26 (m, 2H, C7-CH_2), 3.17 (t, 2H, C7-CH_2, J= 6.8), 2.91 (t, 2H, C7-CH_2, J= 7.0), 2.82 (sept, 1H, iBu-CH, J= 8.0), 2.69- 2.61 (m, 2H, C2'-$H_{1\&2}$), 2.35 & 2.37 (2s, 6H, Tol-CH_3), 1.15 (dd, 6H, iBu-CH_3, J= 6.8, 0.7).

^{13}C NMR (CD_3OD): δ 181.7 (C), 167.8 & 167.4 (C), 145.9 & 145.7 (C), 130.8 & 130.7 (CH), 130.4 (CH), 128.1 & 128.0 (C), 119.9 (CH), 116.1 (CH), 104.6 (C), 85.4 (CH), 83.5 (CH), 76.8 (CH), 65.5 (CH_2), 41.6 (CH_2), 38.3 (CH_2), 37.0 (CH_2), 25.4 (CH_3), 21.8 (CH_3), 19.4 (CH_3).

HR ESI-MS: $[M+H]^+$ m/z calculated for $C_{33}H_{38}O_7N_5$ 616.2766, observed 616.2768.

7-(2-Aminoethyl)-7-deaza-deoxyguanosine (38)



37 **38**

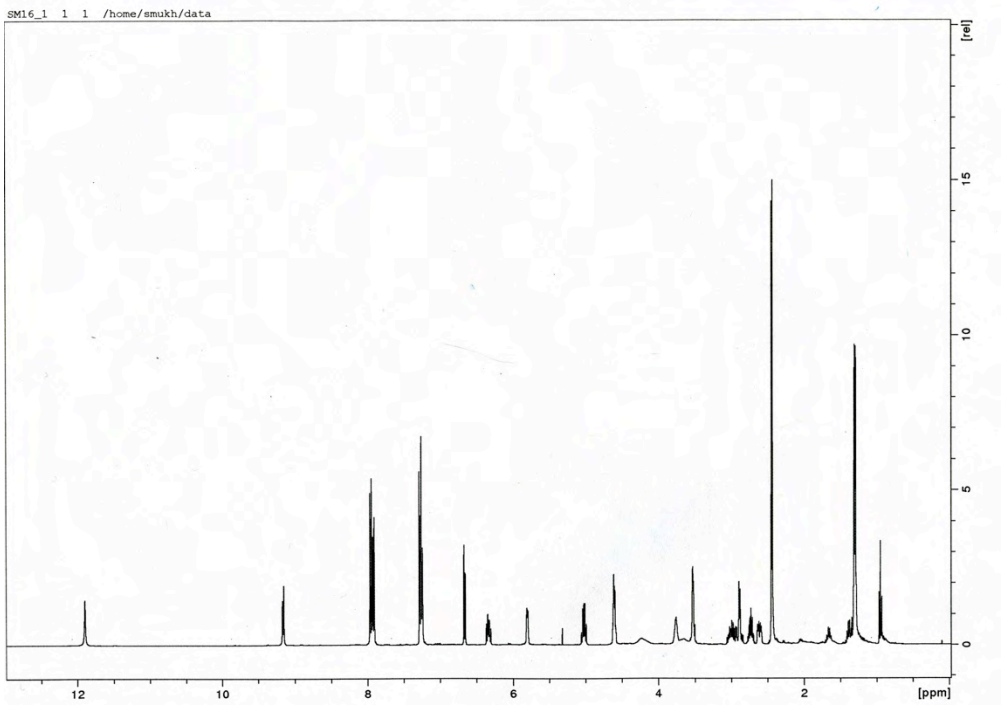
To a solution of 7-(2-Aminoethyl)-7-deaza-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (10 mg, 0.016 mmol) in 5 mL methanol was added 2 mL of 25% NH_4OH and the reaction allowed to run overnight. The sample was dried under reduced pressure and re-dissolved in 2 mL chloroform and water. The aqueous layer was pipetted out, lyophilized to dryness and the identity of the sample confirmed by mass spectrometry.

ESI-MS: $[M+H]^+$ m/z calculated for $C_{13}H_{19}N_5O_4$ 309.3, observed 310.1.

NMR and MS Spectra

Figure 3.3: ^{1}H and ^{13}C NMR Spectra of 7-deaza-7-(2,3-dihydoxypropyl)-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (35).

^{1}H



^{13}C

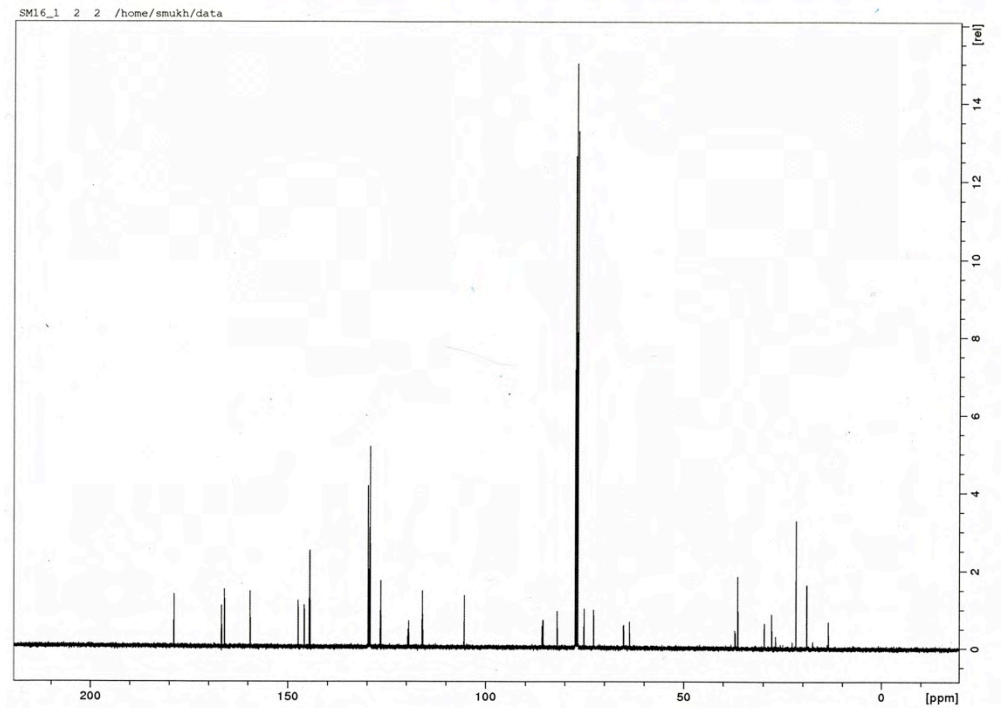
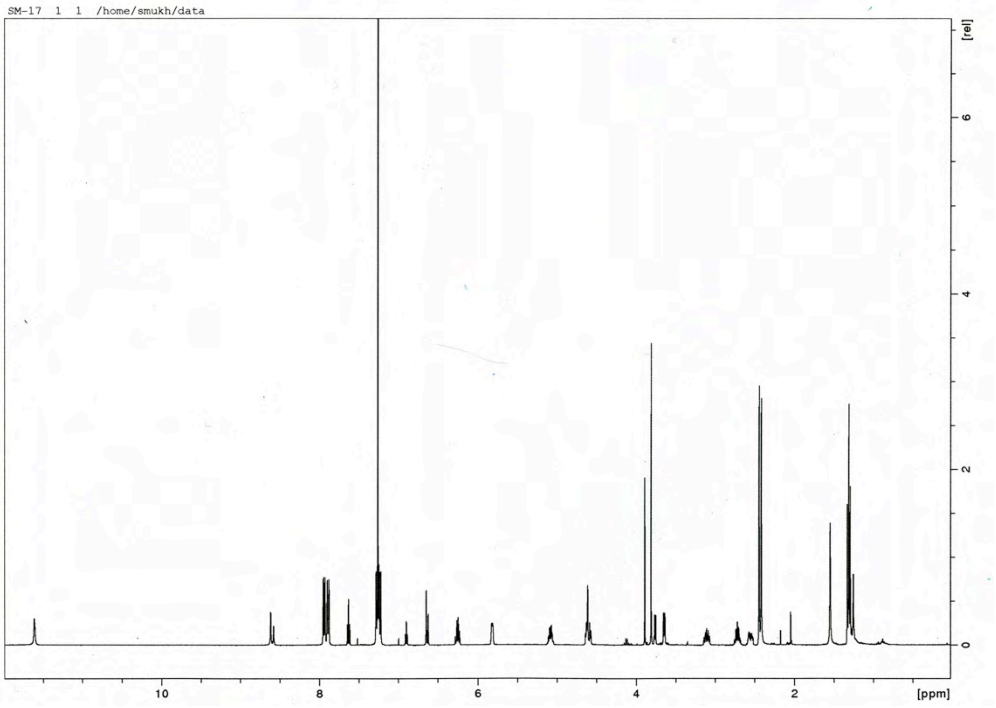


Figure 3.4: ^{1}H and ^{13}C NMR Spectra of 7-deaza-7-(2-methoxyiminoethyl)-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (36).

^{1}H



^{13}C

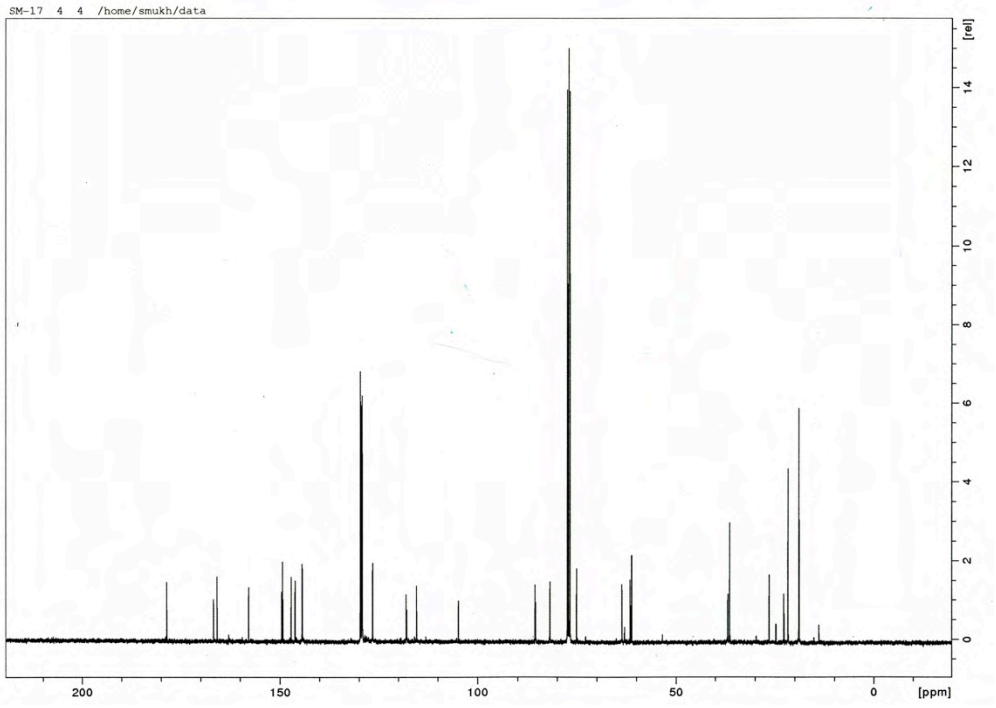
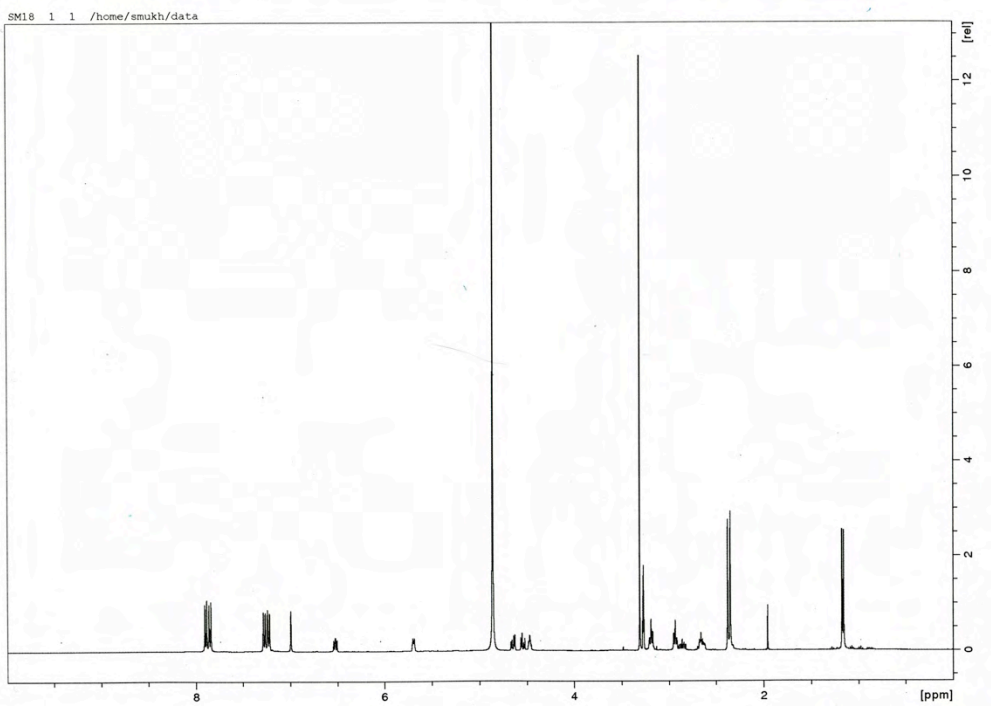


Figure 3.5: ^{1}H and ^{13}C NMR Spectra of 7-(2-Aminoethyl)-7-deaza-N(2)-isobutyryl-3',5'-di-O-p-toluoyl-2'-deoxyguanosine (37).



^{1}H

^{13}C

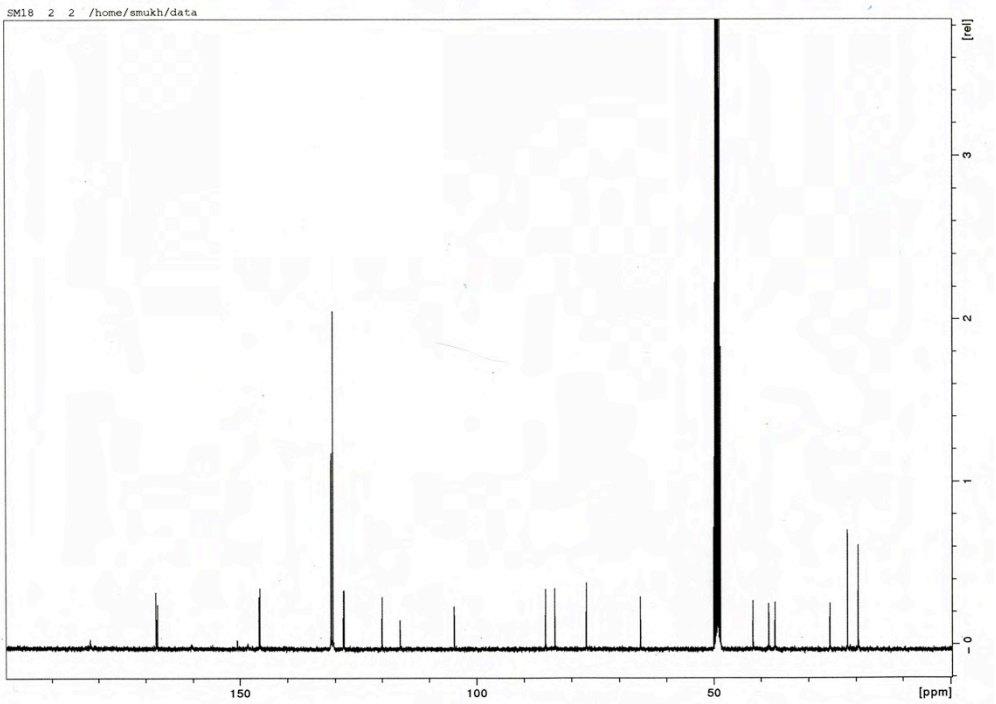
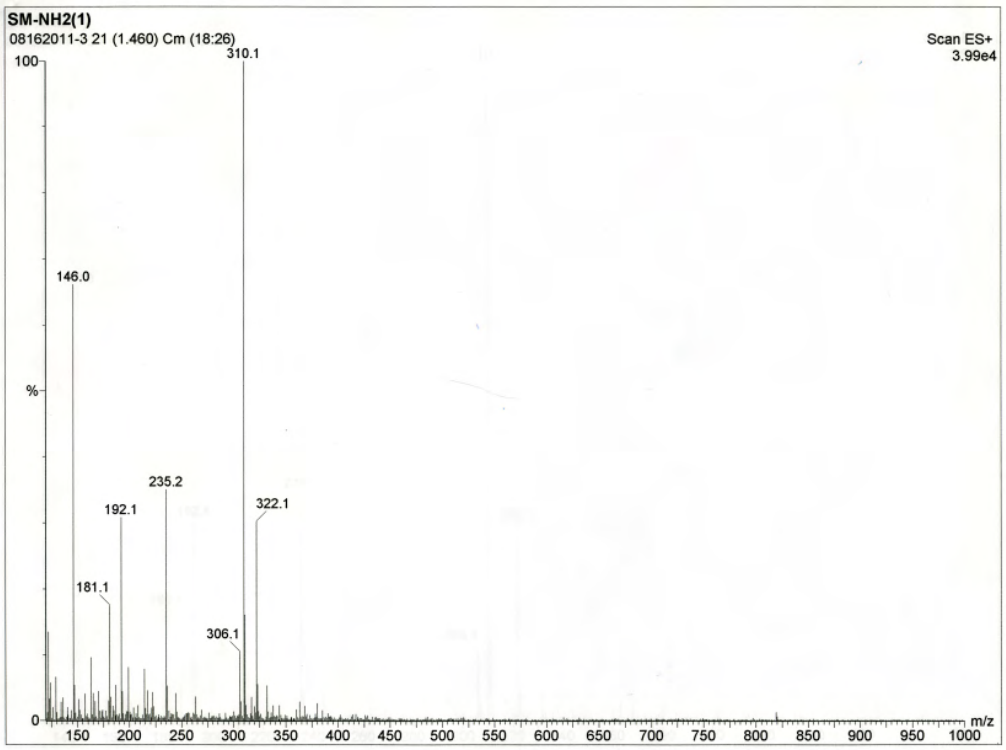


Figure 3.6: ESI-MS spectra of 7-(2-Aminoethyl)-7-deazaguanosine (38).



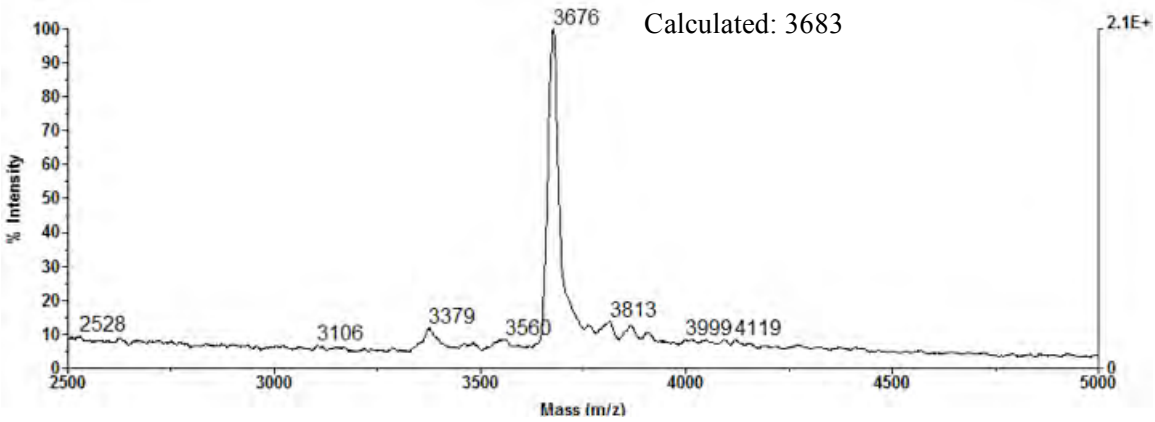
Oligonucleotide Synthesis and Purification

Oligonucleotide synthesis was carried out according to the procedure outlined in Chapter 2. The 11- and 20-mers having C2 aldehyde precursors were deprotected by treating with a 1:1 AMA solution (50% of 25% NH_4OH solution/50% CH_3NH_2) at room temperature for 2 h, then purified and detritylated using 1 μmol TOP-cartridges (Agilent Technologies) following the manufacturer's protocol. The identity of the oligonucleotides was verified by MALDI-TOF spectrometry.

ICL Formation by reductive amination

A solution of single stranded oligonucleotide (20 nmols, 100 μL) (5'-CGTA**G*** TCATGC-3') was treated with 10 μL 50 mM $NaIO_4$ and allowed to stand overnight in the dark at 4°C. Excess $NaIO_4$ was removed by centrifugation through Microcon columns with a 3K cutoff (Millipore). The ICL was formed by adding 10 μL of 0.5 M solution of 7-(2-aminoethyl)-7-deazaguanosine **38** and 10 μL of 0.5 M $NaCNBH_3$ and the reaction mixture left overnight in the dark at room temperature. It was subsequently run on a 20% denaturing PAGE gel and the ICL band was excised under UV-light. The band was extracted with 0.5 M NH_4OAc using the crush and soak method. The identity of the ICL band was confirmed using MALDI-TOF mass spectrometry.

Figure 3.7: MALDI- TOF of ICL between 11-mer oligonucleotide and 7-(2-aminoethyl)-7-deazaguanine 38



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Chapter 4

Synthesis of DNA Interstrand Crosslinks for various biochemical studies

Abstract

A number of different DNA ICLs were synthesized using the strategy of post-synthetic reductive amination that were used in the laboratories of our collaborators for various biochemical experiments in relation to ICL repair. We also synthesized modified single stranded oligonucleotides that were used to generate DNA-protein conjugates.

Introduction

Our strategy for synthesizing modified 7-deazaguanine nucleosides with different linker lengths and subsequent formation of ICLs via post-synthetic reductive amination has enabled a number of studies of various aspects of ICL repair in the laboratories of our collaborators. In the following sections, the design and generation of different ICLs and/or the single stranded oligonucleotides for various biochemical studies is described. The availability of site-specific and stable ICLs in high yields made it possible to synthesize diverse ICL-containing oligonucleotides and eliminated the formation unwanted side products formed when DNA duplexes are treated with crosslinking agents such as nitrogen mustards.

Synthesis of ICL for studying structure-specific endonucleases in ICL repair

SLX4-SLX1 is a key endonuclease in ICL repair pathway. SLX4 is believed to have a scaffold function in recruiting other endonucleases (XPF-ERCC1, MUS81-EME1) to the incision sites in ICL repair (1,2). It has also been shown that the interaction of SLX4 with XPF-ERCC1 is absolutely essential for ICL repair (3).

Our laboratory prepared ICL substrates that were used by the group of KJ Patel (MRC Cambridge, UK) to study the combined effect of SLX4-ERCC1-XPF on ICL substrates. These substrates had a structure that mimics a stalled replication fork. We used a C2/C2 combination of crosslink precursor with N,N'-dimethylethylenediamine as the amine (linker length 10.8 Å) to generate the ICL (Figure 1A).

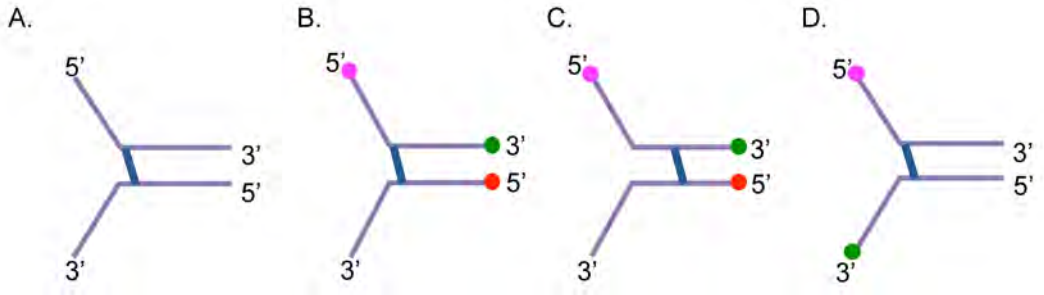


Figure 4.1(A-D): Structure of ICLs mimicking replication fork. The position of the crosslink and the dyes are indicated. The dyes attached on 5'- ends are HEX (5'-Hexachloro Fluorscein) and Cy3 (Cyanine 3) and 6-FAM (6-Fluorscein) on 3'-ends.

This substrate was used to demonstrate that SLX4 significantly stimulates the dual incisions activity of ERCC1-XPF on the synthetic replication fork mimic (4). In addition, we also prepared similar replication fork mimic ICL substrates that have various fluorescent dyes attached at either 5'- or 3'- ends on one/both strands (Figure 1 B-D). For the preparation of the dye labeled ICL substrates, we used HEX (5'-Hexachloro Fluorescein phosphoramidite, λ_{em}=556 nm) and Cy3 (Cyanine 3 phosphoramidite, λem=563 nm) on the 5'- ends and 6-FAM (6-Fluorescein on CPG support, λ_{em}=525 nm) on the 3'- end of the oligonucleotide. The dyes were chosen in a manner that they have different emission maximum. The treatment of synthetic replication fork mimic with the endonucleases SLX4/XPF-ERCC1 generate incised products and labeling of the strands with the dyes will be helpful in tracking them during the reaction for future studies.

Synthesis of oligonucleotides for the formation of DNA-Protein crosslinks

Proteins can get covalently bound to DNA giving rise to DNA-protein crosslinks (DPCs). This can happen through a variety of exogenous processes such as exposure to environmental toxins, carcinogens and chemotherapeutic agents (cisplatin, mechlorethamine) or endogenous exposure to reactive oxygen species and lipid peroxidation products (5). DPCs are bulky lesions and

compromise genetic stability by interfering with normal DNA-protein interactions. An important challenge in studying the biological response to DPC lesions is the availability of stable DPCs at specific sites of DNA. This is specifically important for studying DPCs formed by bis-electrophiles, which alkylate N7-position of guanine. The inherent instability of the glycosidic bond following N7-alkylation of guanine has prevented the formation N7 guanine conjugated DPCs.

To circumvent this problem, the laboratory of our collaborator Dr. Natalia Tretyakova (University of Minnesota) have developed a strategy to synthesize stable site-specific mimics of N7 guanine conjugated DPCs by carrying out reductive amination reaction between lysine and arginine side chains of proteins with DNA having aldehyde precursors on 7-deazaguanine (Figure 2) (6). The use of 7-deazaguanine provides a structural mimic to the naturally occurring lesion and also eliminates the inherent instability associated with guanine.

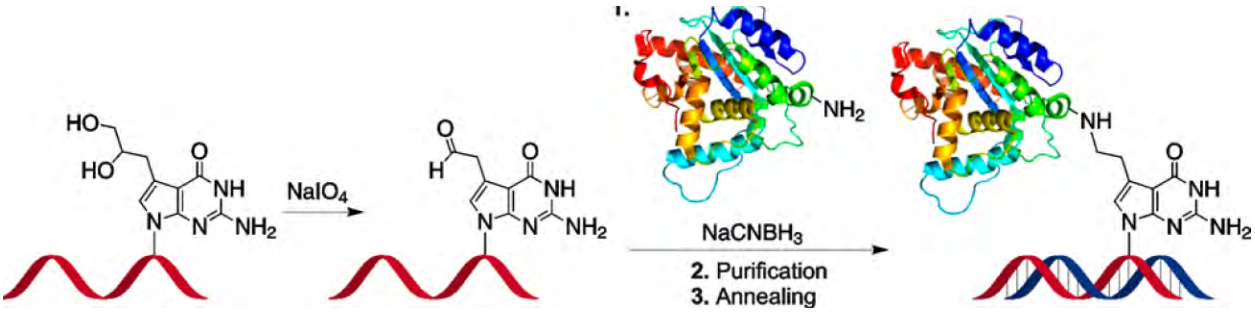


Figure 4.2: Scheme showing the formation of DPCs. The figure is taken from (5)

We synthesized a number of single stranded oligonucleotides having a modified 7-deazaguanine with a two-carbon aldehyde (C2) precursor. These oligonucleotides were used to synthesize site-specific DPCs that are structurally similar to the ones formed naturally by antitumor drugs or environmental toxins (Figure 2) (6).

In addition, we also synthesized oligonucleotides that have a biotinylated thymidine along with the modified C2 phosphoramidite. The biotin can be coupled to streptavidin-containing beads that can block proteins from approaching DNA-protein conjugates and will be helpful in pulldown experiments.

ICL substrates for studying FAN1 nuclease activity

FAN1 (Fanconi anemia-associated nuclease 1) is a structure-specific endonuclease and 5'-exonuclease (7-9). Cells depleted in FAN1 have been shown to be sensitive to crosslinking agents and the protein co-localizes with FANCD2 and FACI through its UBZ domain (8,10). These results point to the role of FAN1 in ICL repair. However, the exact step in which FAN1 plays a role is currently not clear.

To further understand and explore the role of FAN1 in ICL repair, we synthesized ICL substrates for our collaborator Dr. Josef Jiricny (IMCR Zurich, Switzerland). The designed substrates were a mimic of the replication fork with the ICL at the junction or linear with ICL in the middle of the duplex. We expect that these substrates would be useful in elucidating the exact location of FAN1 in ICL repair.

ICL substrate for studying mechanism of DNA damage tolerance

DNA lesions can lead to the stalling of replication forks. For replication to restart, there are two main damage tolerance mechanisms that are used by cells- translesion DNA synthesis (TLS) and homology dependent repair (HDR) (11,12). To address the question of tolerance between the two processes, an assay was developed in the laboratory of Dr. Zvi Livneh (Weizmann Institute of

Science, Israel) (13). We have synthesized an ICL lesion to be used by them for simultaneous analysis of TLS and HDR. The ICL substrate has a mismatched base opposite the crosslinking base. This would help in understanding the tolerance mechanism among accurate/mutagenic TLS and HDR.

Conclusions

We were successful in synthesizing DNA ICLs and single stranded oligonucleotides having a modified phosphoramidite for our collaborators.

The DNA ICL mimicking the replication fork was used in studies to show that presence of SLX4 influences the dual incision activity of ERCC1-XPF. The labeling of the strands with dyes will be useful for tracking the incised products that are generated during the reaction.

The single stranded oligonucleotides having a modified phosphoramidite were used to synthesize stable DNA-protein conjugates via the strategy of reductive amination.

In addition, we have also synthesized DNA ICLs that would be useful in elucidating the role of FAN1 in ICL repair pathway and DNA damage tolerance assays.

Experimental Section

Synthesis of ICL for studying structure-specific endonucleases in ICL repair

The C2 precursor phosphoramidite was synthesized according to published literature procedure (14). The modified phosphoramidite was introduced at position **G*** of the sequence as indicated. HEX (Hexachloro Fluorescein phosphoramidite), 6-FAM (6-Fluorescein) and Cy3 (Cyanine 3 phosphoramidite) dyes were purchased from Glen Research and dissolved in MeCN. HEX and Cy3 dyes were attached to the 5'-ends of oligonucleotides while 6-FAM was on a solid CPG support and attached on 3'-end. Oligonucleotide synthesis was carried out by automated DNA synthesis on an Expedite 8909 Nucleic Acid Synthesis System (Applied Bioscience) using 1 μmol 1000Å CPG column cartridge (Glen Research) and standard reagents. For the modified C2 phosphoramidite an extended coupling time of 15 min was used. The coupling time for the dyes on the 5'-ends (Hex and Cy3) was done as per Glen Research protocol. The single stranded oligonucleotides were deprotected with 1:1 AMA solution (50% of 25% solution NH_4OH/50% CH_3NH_2) and purified by running on a 15% denaturing PAGE gel.

ICL Formation by reductive amination

A solution of complementary single stranded oligonucleotides (25 nmols) in 125 μL 100 mM NaCl was heated to 95°C for 5 mins and allowed to cool over 6 h for annealing to take place. For oxidation, 10 μL 50 mM $NaIO_4$ and 15 μL 1M PO_4 (pH 5.4) buffer was added and the reaction mixture was kept overnight at 4°C. Excess $NaIO_4$ was removed by centrifugation using Amicon columns (3K cutoff). ICL reaction was initiated by adding 10 μL 5 mM N,N'-dimethylethylenediamine and 10 μL 0.5 M $NaCNBH_3$ and allowing the reaction to take place overnight in the dark. The reaction mixture was run on a 15% denaturing PAGE gel and the ICL

band was excised under UV-light. It was subsequently extracted with 0.5 M NH_4OAc and desalted using Amicon columns. The purity of the ICLs were confirmed by running on a 15% PAGE gel followed by exposure to Typhoon 9400 Phosphoimager (shown in Figure 4.3).

Sequences:

KJ 1: 5'-ATG CCT GCA CGA ATT AA**G*** CCA TTC GTA ATC ATG GT-3'
KJ 2: 3'-CAA GCA GTC CTA AGG TTC G**G***T AAG CAT TAG TAC CA-5'

KJ 3: 5'H-ATG CCT GCA CGA ATT AA ATG CCT TA**G*** CCT CAC TCG ACC-F-3'
KJ 4: 3'-GTA CGA GTC CTA AGG TG TAC GGA ATC G**G***A GTG AGC TGG- Cy-5'

KJ 5: 5'H-ATG CCT GCA CGA ATT AA AT**G*** CCT TAG CCT CAC TCG ACC-F-3'
KJ 6: 3'-GTA CGA GTC CTA AGG TG TAC G**G***A ATC GGA GTG AGC TGG- Cy-5'

KJ 7: 5'H-ATG CCT GCA CGA ATT AA AT**G*** CCT TAG CCT CAC TCG ACC-3'
KJ 8: 3'F-GTA CGA GTC CTA AGG TG TAC G**G***A ATC GGA GTG AGC TGG-5'

The dyes [HEX (H), 6-FAM (F), Cy (Cy3)] are color-coded according to their emission.

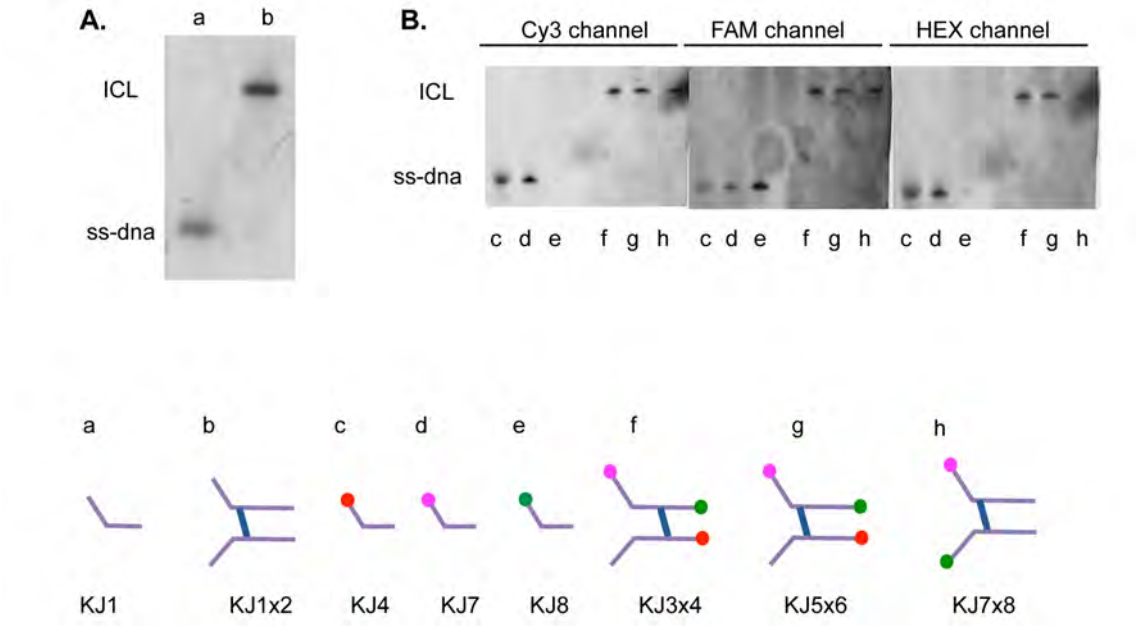


Figure 4.3: Analysis of the purity of ICLs with or without dyes. Each of the ICLs were analyzed by running on a 15% PAGE gel followed by staining with SYBR Gold (A) or exposure to Typhoon 9400 Phosphoimager (B) in various channels. The ss-dna and ICLs are marked as shown above. The dyes are color-coded according to their emission. KJ4: ss-dna with Cy3, KJ7: ss-dna with HEX, KJ8: ss-dna with 6-FAM.

Synthesis of oligonucleotides for the formation of DNA-Protein crosslinks

The modified C2 phosphoramidite, synthesized according to published literature procedure (14), was inserted at position **G*** as shown in the sequences below (NT1 – 5).

The sequences having Biotin dT (Glen Research) is indicated with T* (structure shown below). Oligonucleotide synthesis was performed by automated DNA synthesis as described above. The oligonucleotides were deprotected with 1:1 AMA solution and purified by reverse phase HPLC using the following elution gradient: linear 2.5-15% B over 21 min, linear 15-90% B over 25 min, isocratic 90% B till 26 min, linear 90-2.5 % B till 28 min, isocratic 2.5 % B till 30 min; (eluent A: 0.1 M TEAA (pH 7); eluent B: CH_3CN). The identity of the oligonucleotides was confirmed by MALDI-TOF mass spectrometry.

Sequences:

NT 1: 5'-AGG GTT TTC CCA **G***TC ACG ACG TT-3'
NT 2: 5'-GTC ACT GGT A**G***C AAG CAT TG-3'
NT 3: 5'-AGG GT*T* TTC CCA **G***TC ACG ACG TT-3'
NT 4: 5'-CCA T*GG TG**G*** CTT TAC CAA CAG T*A-3'
NT 5: 5'-CCA TGG TG**G*** CTT TAC CAA CAG TA-3'
where T* is BiotindT

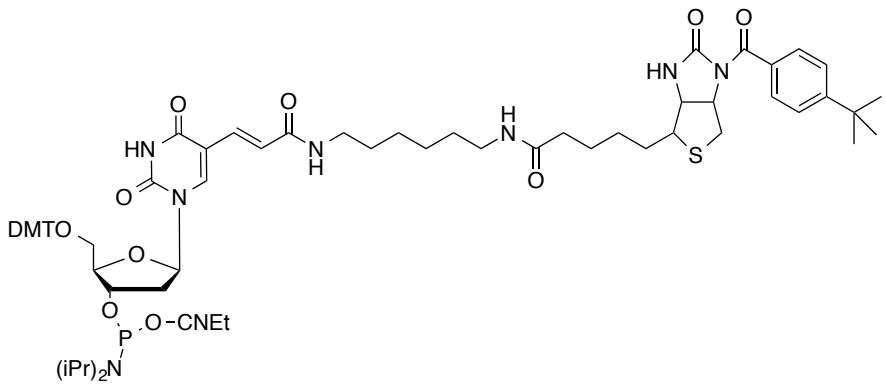


Figure 4.4: Structure of BiotindT

NT 3 and NT 4 single stranded oligonucleotides were given to our collaborator on CPG solid support.

Figure 4.5: MALDI-TOF of NT1

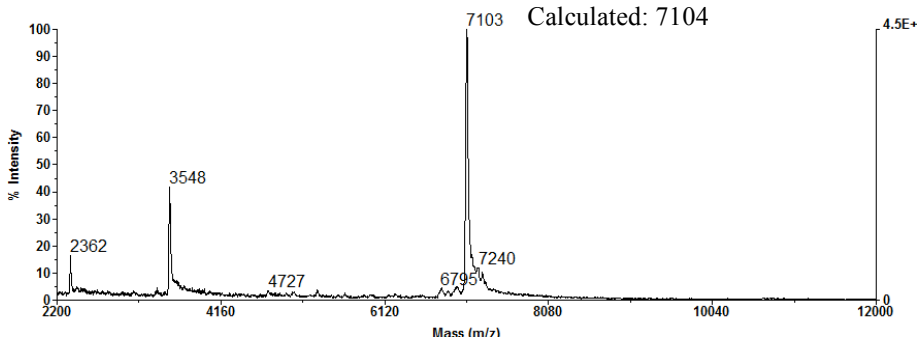


Figure 4.6: MALDI-TOF of NT2

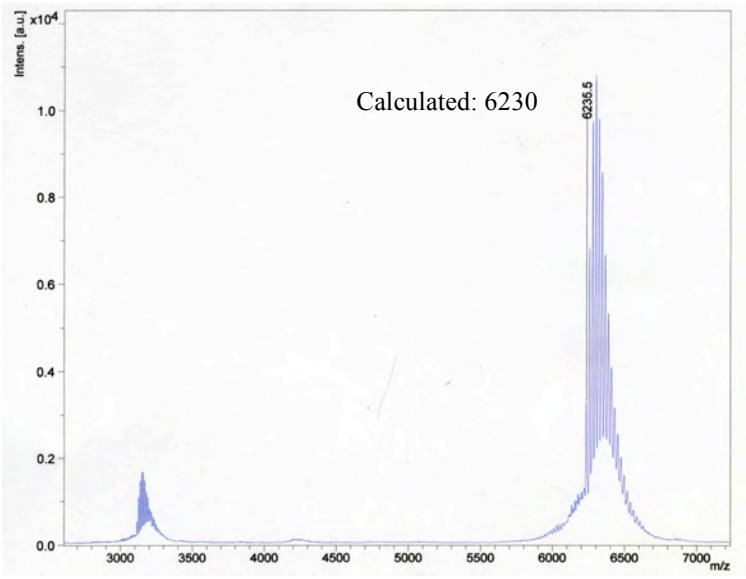
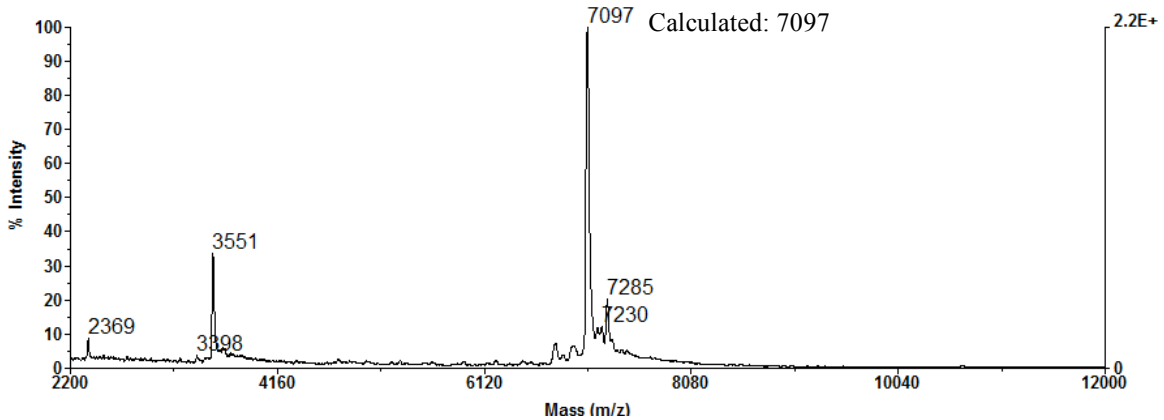


Figure 4.7: MALDI-TOF of NT5



ICL substrate for the study of FAN 1 nuclease activity

The modified C2 phosphoramidite, synthesized according to published literature procedure (TA), was inserted at position **G*** as shown in the sequences below (JJ 1 - 4). Oligonucleotide synthesis was performed by automated DNA synthesis as described above. The oligonucleotides were deprotected with 1:1 AMA solution and the 24-mers were purified by TOP column according to manufacturer's protocol. The 35 and 37-mer was purified by 15% PAGE gel and isolated using the crush and soak method. ICL formation was initiated with N,N'-dimethylethylenediamine/$NaCNBH_3$ and the crosslink purified as described above.

Sequences:

JJ 1: 5'- ATG CCT GCA CGA ATT AA**G*** CCA TTC GTA ATC ATG GT-3'
JJ 2: 3'- CAA GCA GTC CTA AGG TTC G**G***T AAG CAT TAG TAC CA **TA**-5'

JJ 3: 5'-CCGG GTC ACT GGT A**G***A CAG CAT TG-3'
JJ 4: 3'-CAG TGA CCA TCT **G***TC GTA AC TTAA- 5'

ICL substrate for studying mechanism of DNA damage tolerance

The modified C2 phosphoramidite, synthesized according to published literature procedure (14), was inserted at position **G*** as shown in the sequences below (ZL 1 - 4). The sequences ZL 2 and 4 have the terminal 5'-phosphate group retained. The 5'-phosphate group was introduced by addition of Chemical Phosphorylation Reagent (Glen Research) at 5'-end during solid-phase oligonucleotide synthesis. Oligonucleotide synthesis and ICL formation was done as described above.

Sequences:

ZL1: 5'P-CTCTCTCTTTCTTCCTTCCCTCTTCT**G***TTCTTCTTTC-3'
ZL 2: 3'-TGGTGAGAGAGAAAGAAGGAAGGGAGAAGATA**G***GAAGAAAGGTAA-P5'
ZL 3: 3'-TGGTGAGAGAGAAAGAAGGAAGGGAGAAGAGA**G***GAAGAAAGGTAA-P5'

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