Development of Methodologies to Prepare Interstrand Cross-Links in Oligonucleotides

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Abstract

Development of Methodologies to Prepare Interstrand Cross-Links in Oligonucleotides

Gang Sun, Ph.D.

Concordia University, 2014

Cellular DNA is susceptible to damage by chemical agents that causes modifications which includes interstrand cross-links (ICL). ICLs covalently attach the complementary DNA strands, and interfere with replication and transcription by preventing strand separation. The deliberate formation of ICL in DNA by bi-functional alkylating chemotherapeutic agents leads to the death of cancer cells. Development of tumors resistant to these agents is a factor in the lack of response in some patients, with removal of ICL believed to play a role in resistance. In mammalian cells, the precise role of excision repair in eliminating ICL is not completely understood. For better understanding of the repair pathways involved in removing ICL damage, ICL DNA duplexes containing well-defined modified moieties are required to mimic the lesions induced by chemotherapeutic agents.

This thesis describes two major approaches that have been investigated to synthesize ICL DNA. The first describes a method to prepare DNA duplexes containing cross-linked *N*3-butylene-*N*3 thymidines that enables the preparation of asymmetric nucleotide

sequences around cross-linked sites. Protective groups for the 3'- and 5'-hydroxyl moieties were screened for compatibility of subsequent extensions from a cross-linked thymidine dimer incorporated in a support bound oligonucleotide by automated DNA synthesis. Two cross-linked dimer phosphoramidites were prepared, one with dimethoxytrityl (DMT) and allyloxycarbonyl (Alloc) protective groups at 5'-*O* positions and a 3'-*O*-*t*-butyldimethylsilyl (TBS) group which enabled the production of completely asymmetric ICL DNA duplexes in good yields. After coupling of the cross-linked phosphoramidite to a linear strand assembled on the solid support, the DMT group was cleaved on the synthesizer to allow for the synthesis of the second arm of the duplex. The Alloc group was then removed *via* an off-column strategy to expose the 5'-hydroxyl group to complete assembly of one strand of the duplex to form a "Y-shaped" intermediate. Final removal of a 3'-*O*-TBS group off column followed by coupling with deoxynucleoside 5'-phosphoramidites yielded ICL DNA duplexes containing completely asymmetric nucleotide composition around the cross-link site. The identity and composition of the ICL duplexes were confirmed by mass spectrometry (ESI-TOF) and enzymatic digestion. The synthesized ICL duplexes displayed characteristic features of a B-form duplex and had stabilities that were higher than those of the unmodified controls assessed by circular dichroism (CD) spectroscopy and UV thermal denaturation experiments.

The second major project describes approaches to prepare a 7-deaza-2'-deoxyguanosine cross-linked dimer where the C7 atoms are attached by an alkylene linker. The chemical instability of alkylated *N*7 2'-deoxyguanosine (dG) represents a major challenge for preparing ICL DNA containing an alkylene linkage between the *N*7

atoms. The incorporation of a C7-alkylene cross-linked dimer of 7-deaza-2'-deoxyguanosine in DNA would allow for the preparation of a chemically stable ICL which mimic lesions formed by bifunctional alkylating agents (i.e. mechlorethamine and hepsulfam). Two synthetic methods were explored to prepare 7-deazaguanine (and other 7-deazapurines). These involved two cyclization strategies to prepare these molecules starting from a pyrimidine or a pyrrole to produce the purine. In both synthetic methods it was challenging to purify some of the intermediates. All intermediates in the synthetic method starting from the pyrimidine precursors to produce the 7-deazapurines were more stable while the production of the 7-deazapuines from the pyrroles resulted in higher yields.

An attempt to produce a C7 cross-linked dimer of 7-deaza-2'-deoxyguanosine containing a heptamethylene linker is described. Starting from 7-deazaguanine, 7-iodo-7-deaza-2'-deoxyguanosine was prepared in good yield. This nucleoside was converted to 5'-*O*-DMT-7-iodo-7-deaza-2'-deoxyguanosine and the Sonogashira reaction used with 1,6-heptadiyne to introduce the heptamethylene linker. Unfortunately, multiple challenges were encountered with the dimerization and hydrogenation reactions which did not allow for the synthesis of the desired dimer for solid-phase synthesis of the ICL DNA.

Acknowledgments

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Thanks to those who I forget to mention. I wish good luck to all of you.

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List of Compounds

List of Abbreviations

A	adenosine
Alloc	allyloxycarbonyl
Ar	aromatic
br s	broad singlet
C	cytidine
cal	calculated
CD	circular dichroism
dCMP	2'-deoxycytidine-5'-monophosphate
DCM	dichloromethane
DMAP	*N*,*N*-dimethylaminopyridine
DMF	dimethyl formamide
DMT	dimethoxytrityl
DNA	deoxyribose nucleic acid
ESI	electrospray ionization
EtOAc	ethyl acetate
FAPY	formamidopyrimidine
G	guanosine

hAGT human O^6-alkylguanine-DNA alkyltransferase

HPLC High-performance liquid chromatography

***i*Bu** isobutyryl

ICL interstrand cross-link

Lev levulinoyl

m multiplet

MeOH methanol

MS mass spectrometry

NMR Nuclear Magnetic Resonance

NOC N-nitroso compound

O^6-MedG O^6-methyl 2'-deoxyguanosine

Pac phenoxyacetyl

PAGE Polyacrylamide gel electrophoresis

RNA Ribonucleic acid

RT room temperature

s singlet

T thymidine

TBS *tert*-butyldimethylsilyl

THF tetrahydrofuran

TLC thin layer chromatography

T_m thermal denaturation

TMS trimethylsilyl

TOF time of flight

Tol toluoyl

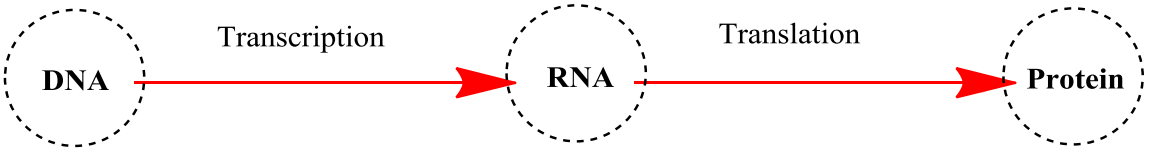
U uridine

Chapter 1. Introduction

1.1 General introduction

Nucleic acids are linear bioactive macromolecules with a number of important roles and functions including the storage of genetic information and serving as a template for the process of replication by which they can duplicate themselves with the aid of proteins. Together with proteins, nucleic acids are found in abundance in all living cells, where they function in encoding, transmitting and expressing genetic information.

There are two classes of nucleic acids found in living cells. 2'-Deoxyribonucleic acid (DNA) is found mainly in the nucleus of the cell, while ribonucleic acid (RNA) is found mainly in the cytoplasm of the cell although it is usually synthesized in the nucleus. In cells, genetic information is encoded in the sequences of nucleic acids, and DNA serves as the instructor for the construction of an entire organism. DNA contains the genetic codes to make RNA and the RNA in turn then contains the codes for the primary sequence of amino acids to make proteins. This process is described as central dogma of biology (**Scheme 1-1**).



Scheme 1-1. Central dogma of biology.

For more than half a century the structures of DNA and RNA were recognized as polymers of four nucleotides, which afford a huge number of sequences. Each nucleotide consists of three components: A pentose sugar, phosphate group and heterocycle (nucleobase). The sugar and phosphate groups form the structural framework of nucleic acids, including DNA and RNA. This backbone is composed of alternating sugar and phosphate units, and defines the directionality of the molecule.

Two types of pentose sugars are found in nucleic acids. The pentose sugar in DNA (2′-deoxyribose) differs from the sugar in RNA (ribose) by the absence of a hydroxyl group (−OH) at the 2′ carbon of the sugar ring. Through a glycosidic bond, nucleobases are linked to the 1'-carbon of pentose sugar, which is referred as the anomeric position. Without an attached phosphate group, the moiety of a pentose sugar and a nucleobase is known as a nucleoside. The phosphate group connects successive sugar residues by bridging the 5′-hydroxyl group on one sugar to the 3′-hydroxyl group of the adjacent sugar along the chain. These nucleoside linkages are linked by phosphodiester groups in both RNA and DNA.

Nucleobases consist of two types of heterocycles: purines or pyrimidines. The purines are bicyclic with fused six and five membered rings whereas the pyrimidines have a single six member ring. There are two primary purines (adenine (A) and guanine (G)) and three primary pyrimidines (cytosine (C), uracil (U) and thymine (T)). Their structures are shown in **Figure 1-1** and **Figure 1-2**. Among these five nucleobases, normally four kinds are found in either DNA or RNA. A, G and C are found in both DNA and RNA. A major difference between DNA and RNA is that DNA contains thymine, but not uracil, whereas RNA contains uracil but not thymine.

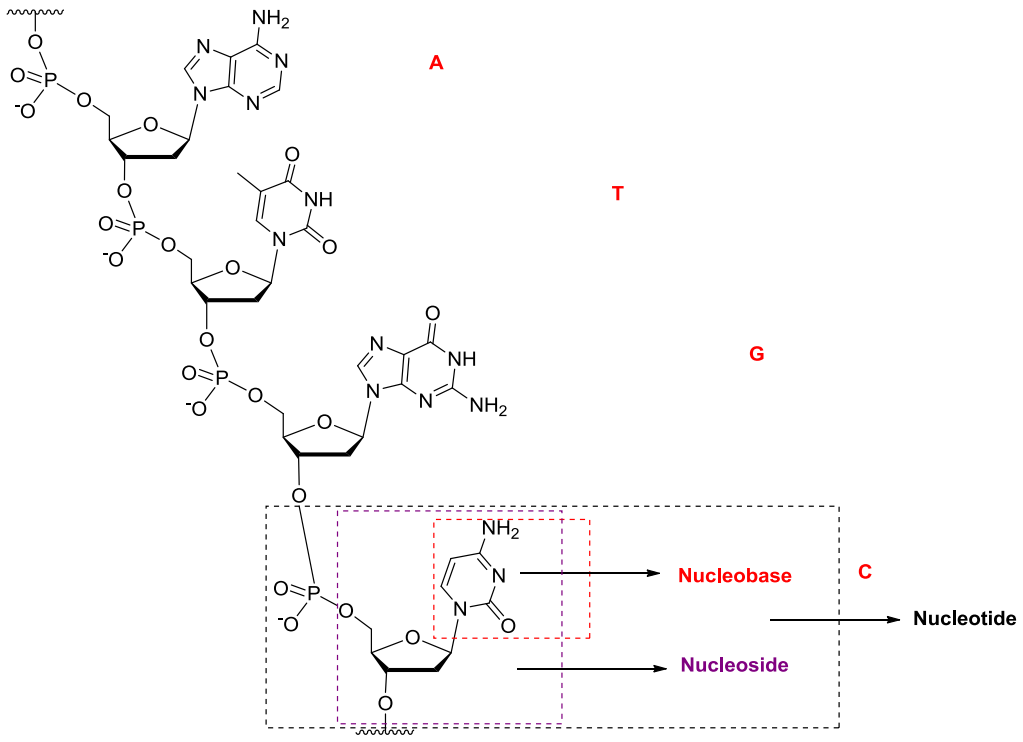


Figure 1-1. Structure of nucleotide, nucleoside and nucleobases of DNA.

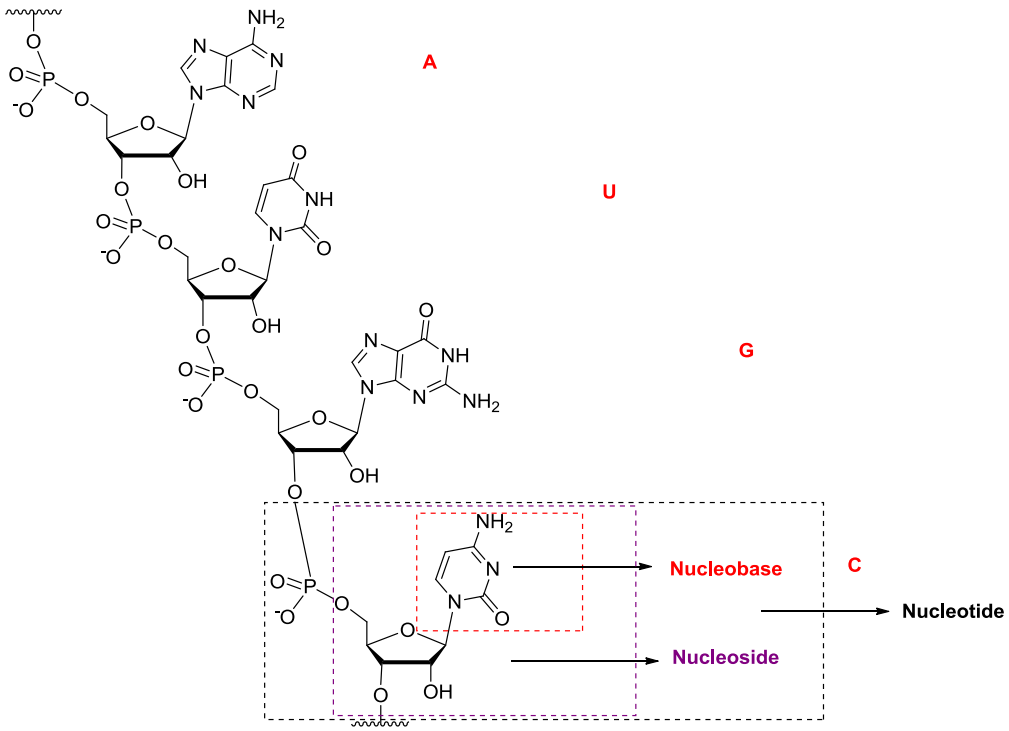


Figure 1-2. Structure of nucleotide, nucleoside and nucleobases of RNA.

The four nucleotides A, C, G, and T are the building units for DNA molecules, in which the nitrogen-containing nucleobases have potential to form hydrogen bonds between them. In Watson-Crick base pairing A always pairs with T through two hydrogen bonds, and G always pairs with C through three hydrogen bonds. The C1'-C1' distance spans of A:T and G:C hydrogen-bonded pairs are nearly identical, allowing them to bridge the sugar-phosphate chains uniformly (see **Figure 1-3**). In a DNA molecule, the two strands coil around each other to form a double helix through an extensive network of hydrogen bonds. The three-dimensional structure for the DNA double helix was first proposed by

James D. Watson and Francis H. C. Crick in 1953.[1, 2] The two DNA strands run in opposite directions as determined by the orientation of the 5′ to 3′ phosphodiester bond. The sugar-phosphate backbone runs along the outside of the helix, and the nucleobases are embedded inside. Within DNA duplex the nucleobase pairs (A:T and G:C) on the two complementary strands interact through hydrogen bonds.

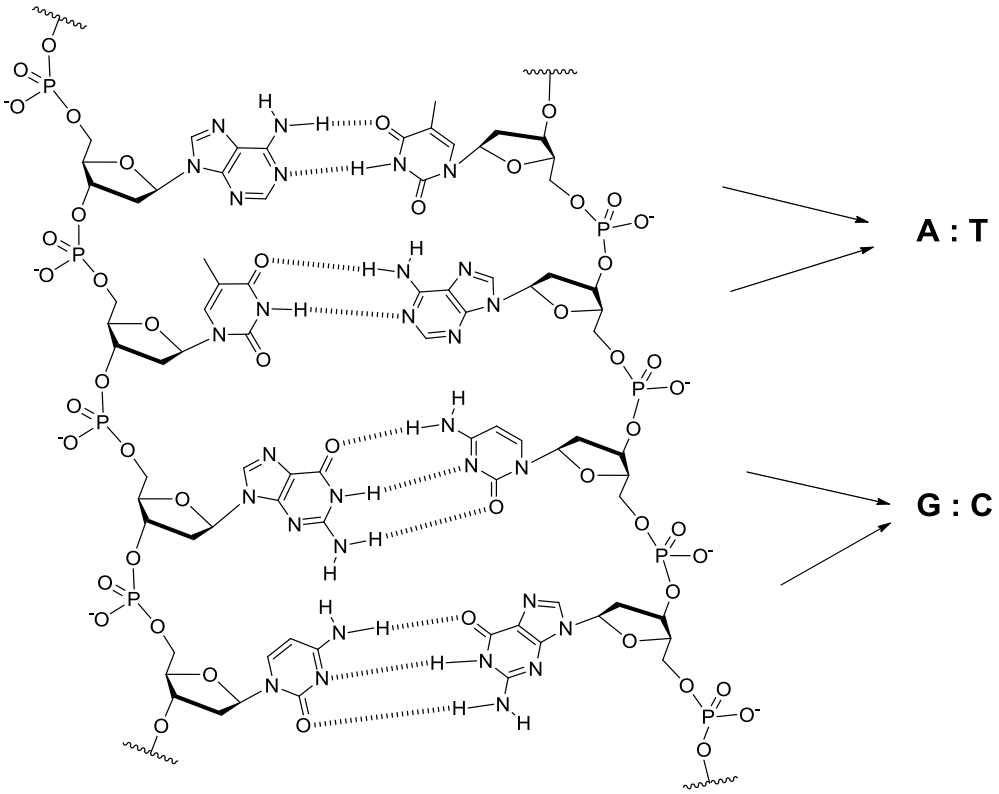
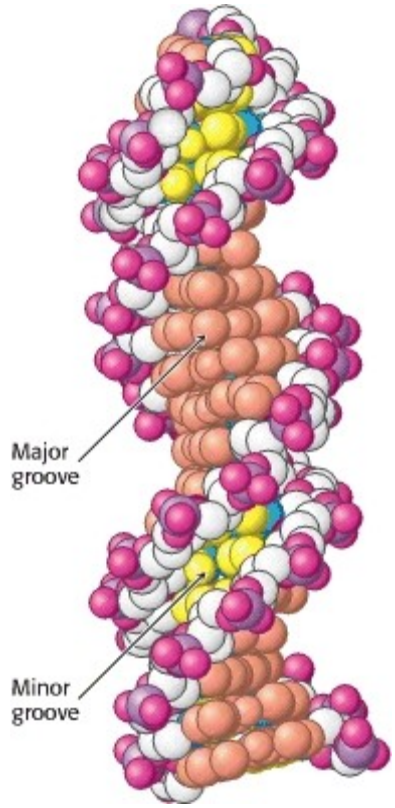


Figure 1-3. Watson-Crick base pairing pattern in DNA.

DNA in the cell exists as a right handed double-stranded helix with what is known as a B-form DNA structure, which was first solved by Franklin and Gosling in 1953.[3, 4] In this structure, there are approximately 10 base pairs per turn (each turn at 3.4 nm), and the distance between two adjacent base pairs is 0.34 nm. The intertwined strands form a right-handed helix with two grooves of different widths, referred to as the major groove (2.2 nm wide) and the minor groove (1.2 nm wide).[5] The interactions between proteins and nucleic acids play a crucial role in central biological processes, ranging from the replication, transcription and recombination to enzymatic events utilizing nucleic acids as substrates.[6-8] Proteins can bind to the major groove of DNA where they can make sequence-specific contacts with the nucleobases.[9-12] In addition, some proteins have preference to interact with nucleic acids via the minor groove.[11-17] The B-form structure is shown in **Figure 1-4**.



The major groove is depicted in orange, and the minor groove is depicted in yellow. The carbon atoms of the backbone are shown in white.[18]

Figure 1-4. Structure of B-DNA showing the location of the major and minor grooves.

In a solution with a high salt concentration, a different right-handed DNA structure may be formed known as A-form. Compared to B-DNA, the A-DNA duplex is a wider right-handed helix, with a shallow, wide minor groove and a narrower, deeper major groove. The A-form of DNA makes one helical turn every 2.5 nm with 11 base pairs per turn.[19-21]

Another DNA structure that is known is referred to as Z-form. Different from the two forms above, Z-form DNA is a left-handed helix. One turn spans 4.6 nm, comprising 12 base pairs. DNA containing alternating G-C sequences in alcohol or high salt concentration can form such structures.[22-27]

B-form DNA is commonly formed in cell, so the following contents mainly focus on this form without further description.

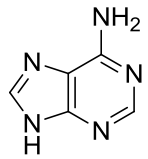
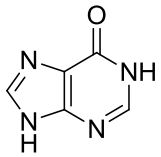
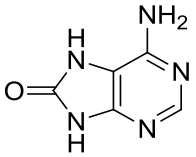
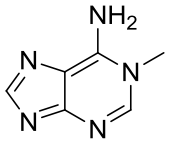
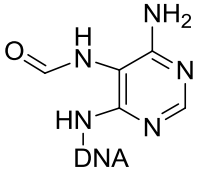
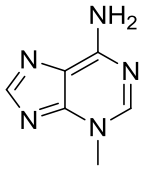
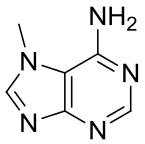
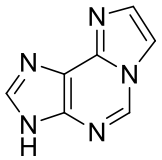
1.2 DNA damage and Repair

DNA is the repository of genetic information, thus its integrity and stability are essential to life. Although DNA is the carrier of genetic information, it has limited chemical stability, and subject to damage from both environmental factors and normal metabolic processes inside the cell.

The degree and spectrum of DNA damage depends on the source and the type of environment to which it is exposed. Some types of damage are ubiquitous and potentially present in all extracted DNA, while other types of damage are caused by exposure to one or more specific sources. It has been estimated that DNA damage occurs at a rate of 1,000 to 1,000,000 molecular lesions per cell per day.[28, 29]

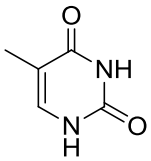
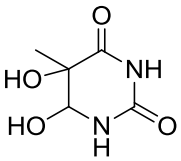
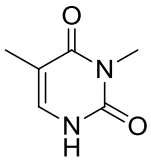
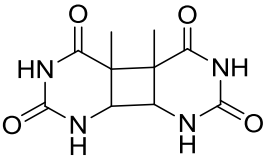
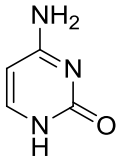
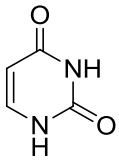
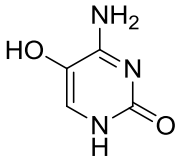
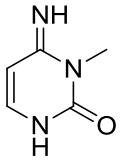
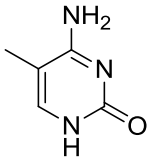
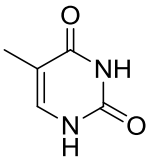
DNA may be modified in a variety of ways, such as hydrolysis, oxidation and nonenzymatic methylation (some examples are shown in **Table 1-1**).[30] If left unrepaired, these DNA modifications in critical genes may impede a cell's ability to carry out its bioactive functions, and ultimately lead to mutations and genomic instability. Links between DNA damage and the increase of mutagenesis, ageing, carcinogenesis and some other diseases have been demonstrated.[31] For example, skin cancer may be caused by excessive exposure to UV radiation from sunlight. Another example is the damage caused by tobacco smoke, which can lead to mutations in lung cells and subsequent cancer of the lung. Besides the exposure to exogenous chemicals, DNA is also subject to oxidative damage from byproducts of metabolism, such as free radicals.

Table 1-1. Common Types of Damaged Bases Found in DNA

Normal base	Examples of common base modifications			
	Deamination	Oxidation	Alkylation	UV
 Adenine	 hypoxanthine	 8-oxoadenine	 *N*1-methyladenine	
		 4,6-Diamino-5-formamidopyrimidine	 *N*3-methyladenine	
			 *N*7-methyladenine	
			 *N*1,*N*^{6}-ethenoadenosine	

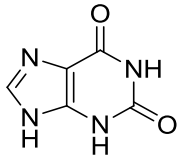
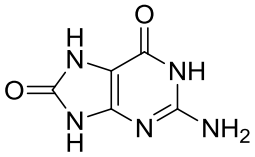
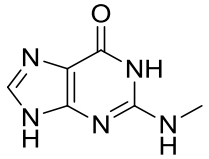
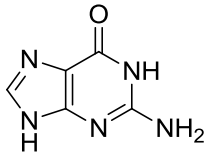
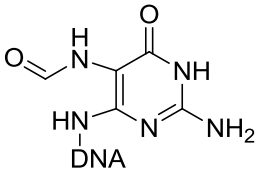
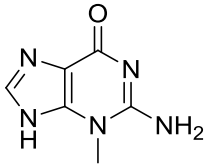
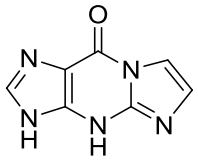
Adapted from reference [30]

Table 1-1. Common Types of Damaged Bases Found in DNA (continued)

Normal base	Examples of common base modifications			
	Deamination	Oxidation	Alkylation	UV
 Thymine		 thymine glycol	 *N*3-methylthymine	 cyclobutane thymine dimer
 Cytosine	 uracil	 5-hydroxycytosine	 *N*3-methylcytosine	
 5-Me-cytosine	 thymine			

Adapted from reference [30]

Table 1-1. Common Types of Damaged Bases Found in DNA (continued)

Normal base	Examples of common base modifications			
	Deamination	Oxidation	Alkylation	UV
	 xanthine	 8-oxoguanine	 N^2-methylguanine	
 Guanine		 2,6-diamino-4-hydroxy-5-formamidopyrimidine	 N3-methylguanine	
			 N1,N^2-Ethenoguanine	

Adapted from reference [30]

Some alkylating agents have been used for chemotherapy of diseases like cancer by modifying specific DNA site(s). However, cells have also developed repair functions for DNA damage, and reverse the cytotoxicity of many clinically used DNA-damaging

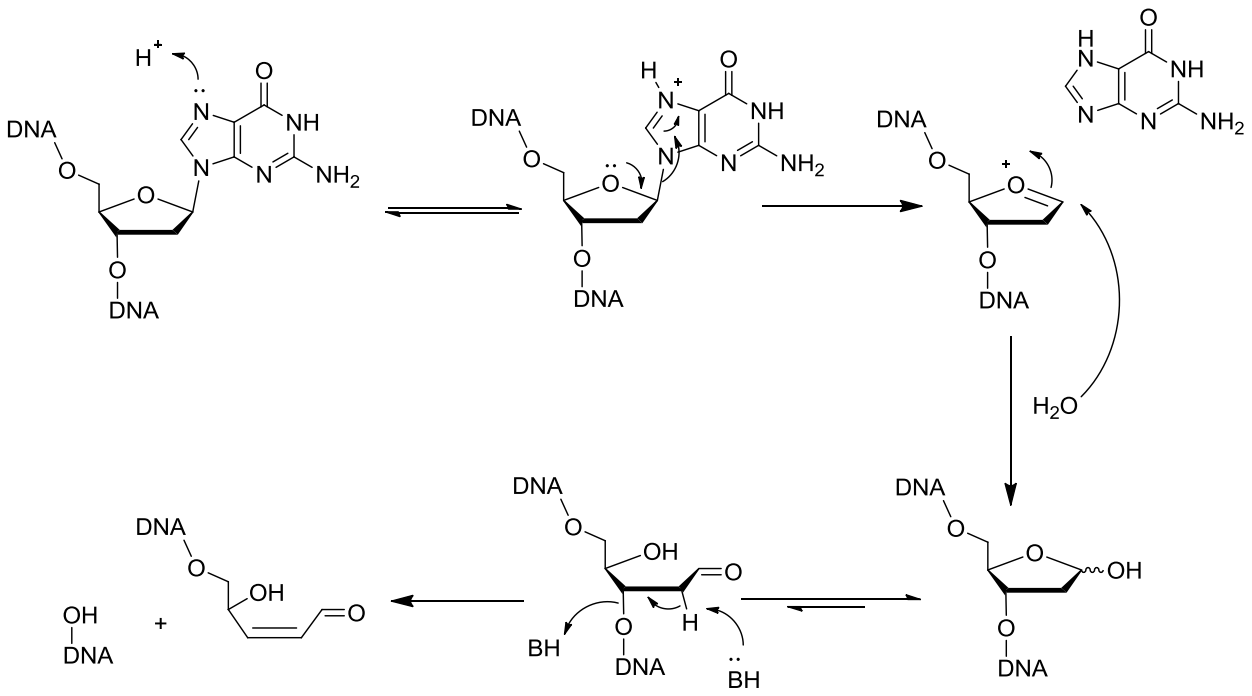
agents. Better understanding of the interaction between DNA damage and repair could lead to the improvement in the effectiveness of existing anticancer drugs, and the development of novel and effective molecules to treat various diseases.[32]

1.2.1 Hydrolysis of DNA

The process of hydrolysis of DNA involves either deamination or total removal of individual bases. The hydrolysis of the phosphodiester bonds is a thermodynamically favored process ($\Delta G^{\circ\prime} = -5.3$ kcal/mol), but extremely slow in the DNA backbone.[33, 34] Phosphodiester linkages that join the nucleotides of DNA are highly resistant to spontaneous hydrolysis, and studies with model compounds indicates that the half-life of phosphodiester hydrolysis is approximately 30 million years in the absence of nucleases.[34-37] This means that spontaneous hydrolysis of the phosphodiester linkages in DNA can be ignored under biological conditions.

In contrast to RNA in which the phosphodiester bond in the backbone is the least stable under physiological conditions, the most labile bond in DNA is the *N*-glycosyl bond that attaches the base to the deoxyribose backbone. Loss of a DNA nucleobase will produce an apurinic or apyrimidinic (AP) site. The mechanisms and reactivities of purine and pyrimidine nucleosides to glycosidic bond hydrolysis depend on the relative acidities of various functional groups on the leaving group bases.[30] Lindahl et al. compared the

rates of depyrimidination and depurination, and found the former occured with rate constants of 1.5×10^{-12} s^{-1} ($t_{1/2}$ = 14,700 y) while the reaction is faster for the latter with rate constants of 3.0×10^{-11} s^{-1} ($t_{1/2}$ = 730 y).[38, 39] This accounts for why hydrolytic cleavage of the glycosidic bonds in DNA is often referred to as depurination. Kinetic studies indicate that hydrolysis of 2'-deoxypurines proceeds *via* an acid-catalyzed S_N1 reaction mechanism where equilibrium protonation increases the leaving group ability of the base and facilitates unimolecular, rate-limiting C-N bond cleavage that generates the free nucleobase and an oxocarbenium ion (shown in **Scheme 1-2**).[30, 40] The oxocarbenium ion undergoes subsequent hydration to yield an AP site. It is found that depurination occurs about four times faster in single-stranded DNA than it does in double-stranded DNA. Some damage caused by free radicals and alkylating agents lead to promotion of DNA hydrolysis of the bases as some damaged nucleobases generate better leaving groups and destabilize the *N*-glycosyl bond.[41, 42]



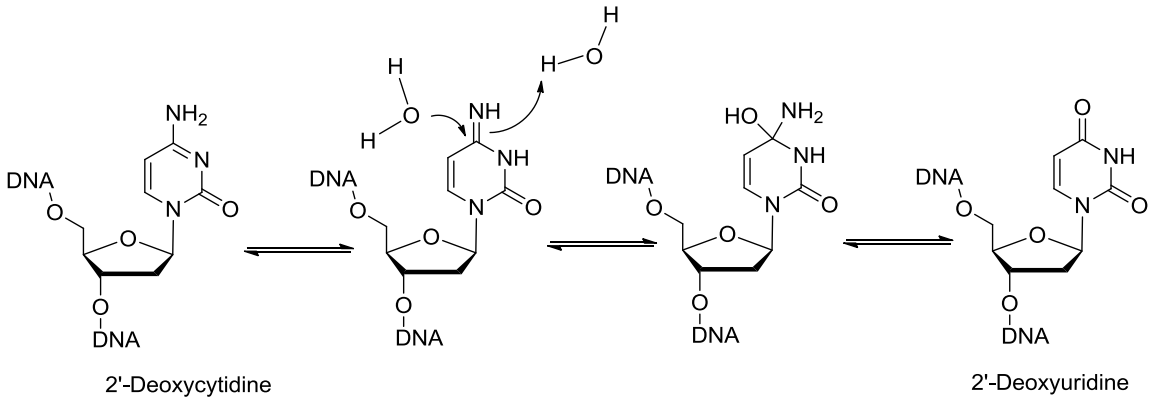
Scheme 1-2. Depurination process [30, 40]

Because the reactive species is H_2O, AP sites may occur along all DNA strands. Under metabolically active conditions it is estimated that approximately 2,000-10,000 AP sites are generated in genomic DNA of mammalian tissues per cell per day, and steady state levels of 10,000–50,000 abasic sites have been detected in cells. [31, 39, 43, 44] Besides the biochemical reaction from various metabolites, hydrolytic damage may also result from the overabundance of reactive oxygen species. The presence of AP sites in a DNA

sample is problematic due to the fact that the AP site may lead to base-pairing failure during DNA replication.

To counteract the deleterious action of AP sites, organisms have developed multiple and robust DNA repair mechanisms including base excision repair (BER), nucleotide excision repair (NER), recombination and translesion DNA synthesis (TLS).[30, 41, 45-47] In eukaryotic cells, the deleterious action of AP sites is also prevented by the action of a multiplicity of DNA repair and tolerance pathways.[47]

Another type of hydrolytic DNA damage that occurs under physiological conditions is the hydrolysis of cytosine to form uracil (shown in **Scheme 1-3**).[31, 48-51] Sequencing studies on DNA extracted from ancient samples have determined that this is the major damage complicating data analysis.[52, 53] Interestingly, cytosine is prone to deamination *via* attack of hydroxide on the neutral nucleobase or attack of water on the *N*3-protonated base, which results in the displacement of its exocyclic amino group to afford uracil. Uracil does not exist in DNA, and it can be effectively detected and removed by repair enzymes. However, if not detected and repaired, it can lead to base mispairing by interacting with adenine. In a subsequent round of replication, the adenine in turn would specify thymine, and as a result this process generates a mutated daughter strand (shown in **Figure 1-5**).



Scheme 1-3. Deamination of 2'-deoxycytidine into 2'-deoxyuridine

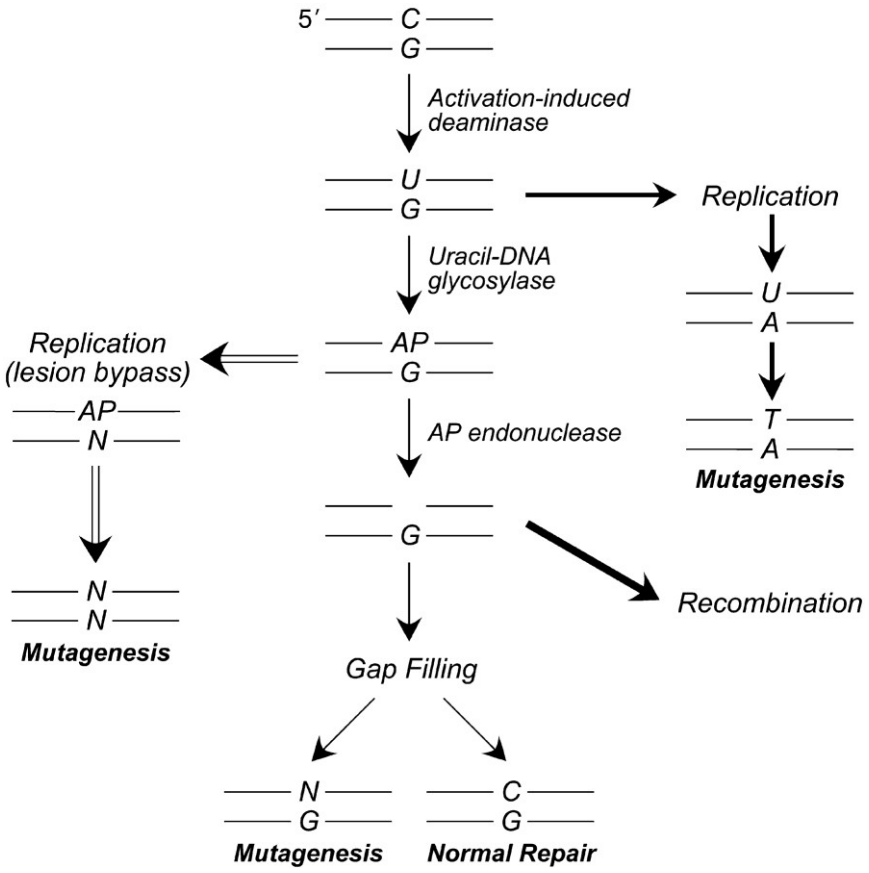


Figure 1-5. Mutagenic effect of deamination of 2'-deoxycytidine.[54]

Cytosine deamination may be caused by hydrolysis. Unlike nucleobase loss in DNA strands, it has been established that cytosine deamination in single-stranded DNA occurs at a rate 140 times faster compared to double-stranded DNA and it is known that mispaired cytosines deaminate at a rate between these processes.[55-57]

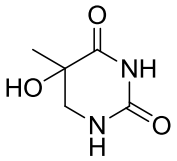
1.2.2 Oxidation of DNA

Reactive oxygen species (ROS), which can be generated by both normal cellular metabolism (endogenous) and ionizing or ultraviolet radiation (exogenous), may lead to oxidative DNA damage.[58] These ROS include superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) and they can oxidize DNA, which can lead to several types of DNA damage, including oxidized bases and single- and double-strand breaks. DNA damage produced by ROS is the most frequently occurring damage.[28, 59-64]

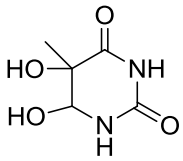
The highly reactive hydroxyl radical (•OH) reacts with DNA by addition to the double bonds of the DNA bases and by abstraction of an H atom from the methyl group of thymine and each of the C-H bonds of 2'-deoxyribose. Addition to the C5-C6 double bond of pyrimidines may lead to the formation of C5-OH and C6-OH radicals while H

atom abstraction from C5-methyl of thymine results in the allyl radical. Adduct radicals differ in terms of their redox properties, with C5-OH adduct radicals being reducing and C6-OH adduct radicals oxidizing.[65] Oxidation may also occur to purine and pentose sugar moieties. Numerous products are produced by a variety of mechanisms. [66-70] DNA base products of interaction with reactive oxygen species are shown in **Figure 1-6**.

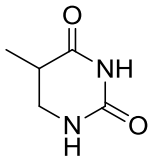
Among the library of adducts formed with reactive oxygen species, 8-oxo-2'-deoxyguanosine (8-oxo-dG) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) (molecular structures shown in **Figure 1-7**) are the most common marker for oxidative DNA damage and can be measured in virtually any species.[61, 71-74] It is formed and enhanced most often by chemical carcinogens. Similar oxidative damage occurring in RNA form 8-OHG (8-hydroxyguanosine) and has been implicated in various neurological disorders.[75-77] 8-Oxo-dG is a mutagenic product that forms a base pair with adenine.[78-80] Such damage is prevalent in mitochondria and may be one of the factors in the ageing process.[81, 82]



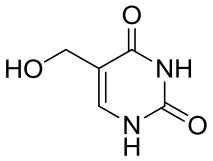
5-hydroxy-5-hydrothymine



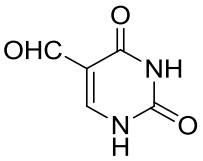
thymine glycol



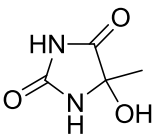
5,6-dihydro-thymine



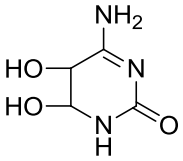
5-hydroxymethyl-uracil



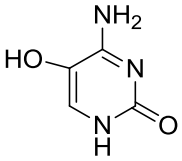
5-formyluracil



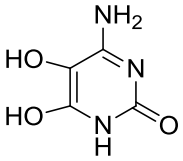
5-hydroxy-5-methyl-hydantoin



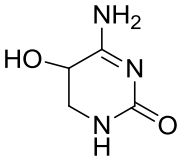
cytosine glycol



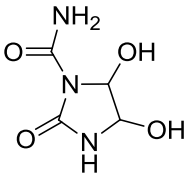
5-hydroxycytosine



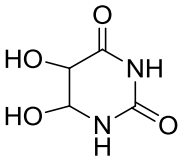
5,6-dihydroxy-cytosine



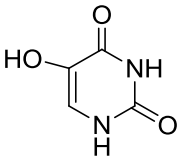
5-hydroxy-6-hydrocytosine



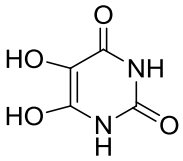
trans-1-carbamoxyl-2-oxo-4,5-dihydroxyimidazolidine



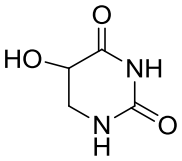
uracil glycol



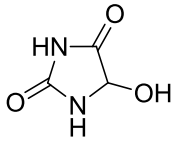
5-hydroxy-uracil



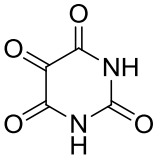
5,6-dihydroxy-uracil



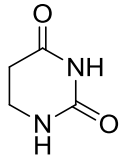
5-hydroxy-6-hydrouracil



5-hydroxy-hydantoin



Alloxan



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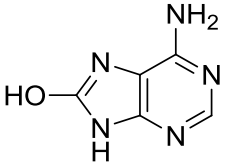
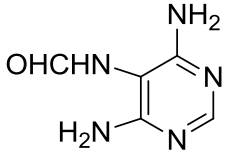
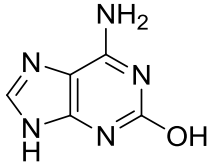


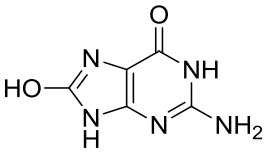
8-hydroxy-adenine



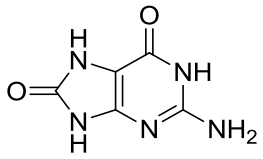
4,6-diamino-5-form-amino-pyrimidine



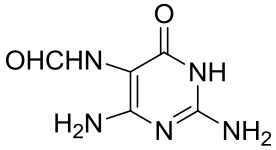
2-hydroxy-adenine



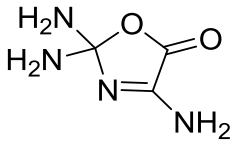
8-hydroxyguanine



8-oxo-guanonine

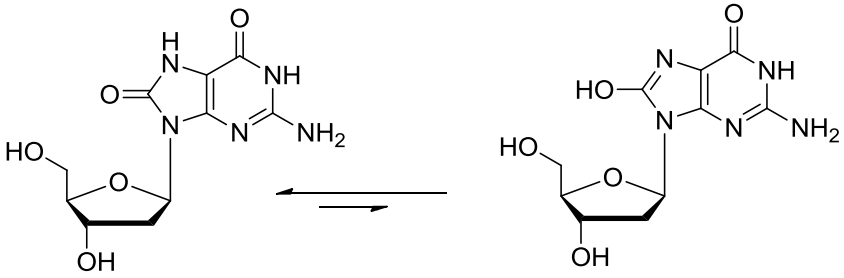


2,6-diamino-4-hydroxy-5-form-amidopyrimidine



oxazoione

Figure 1-6. DNA base products of interaction with reactive oxygen and free radical species.[58, 83, 84]



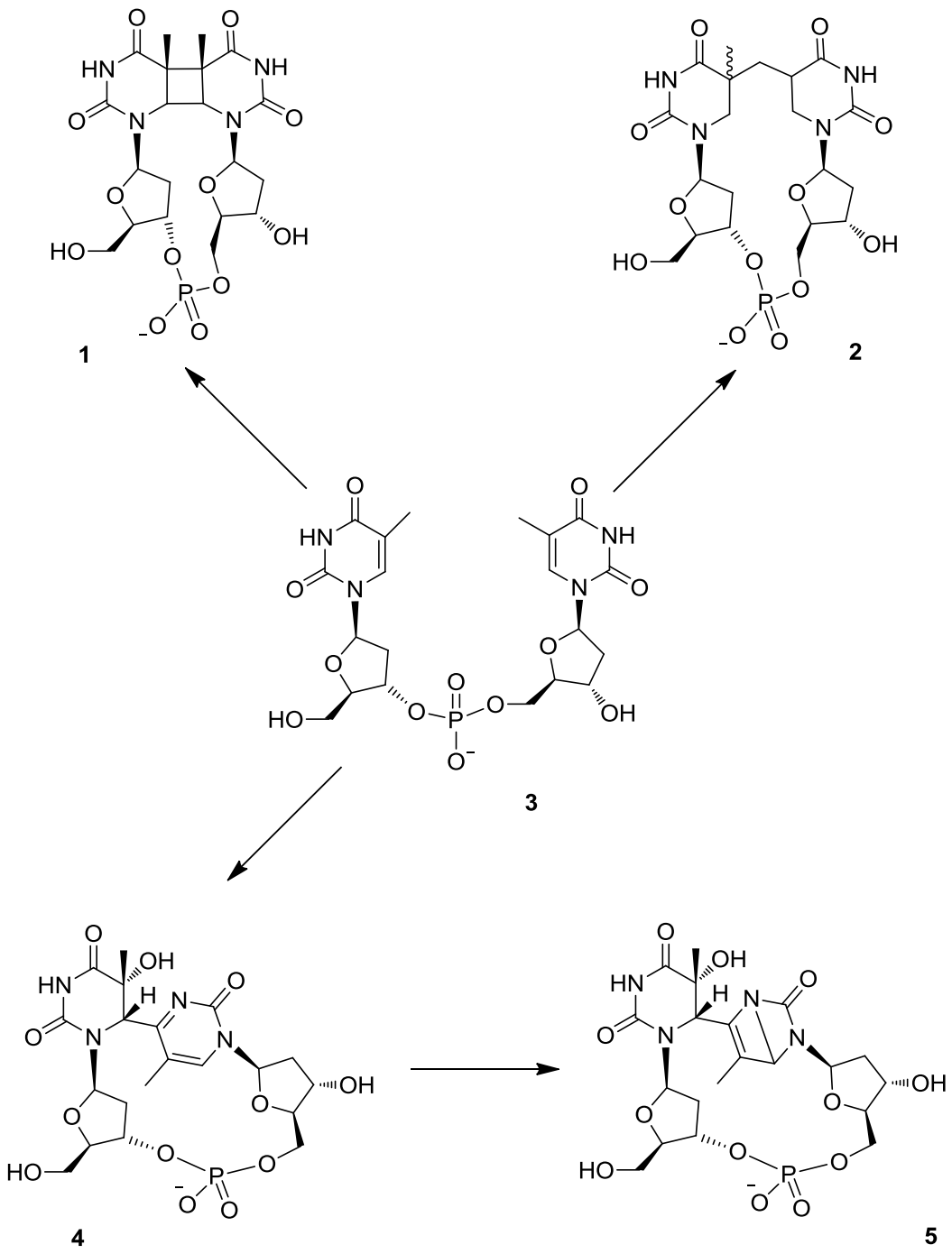
8-oxo-2'-deoxyguanosine (8-oxo-dG)

8-hydroxy-2'-deoxyguanosine (8-OHdG)

Figure 1-7. Equilibrium between two oxidized adducts, 8-oxo-2'-deoxyguanosine (8-oxo-dG) and 8-hydroxy-2'-deoxyguanosine (8-OHdG).[85]

1.2.3 UV-induced damage

Ultraviolet light and other types of radiation can do damage to DNA in organisms *via* photochemical reactions. Solar UV rays may lead to a variety of toxic DNA lesions along genomic DNA strands. When exposed to UV radiation, covalent cross-links may occur in adjacent cytosine and thymine residues to produce pyrimidine dimers. Among them cyclobutane pyrimidine dimers (CPD) and pyrimidine–pyrimidone-(6-4)-photoproducts (6-4PP) are found most commonly.[86-93] The formation of CPD and 6-4PP (as well as its Dewar valence isomers) are shown in **Scheme 1-4**.[92-96] CPD lesion **1** and 6-4PP lesion **4**, caused by these reactions, and their secondary products the Dewar valence isomers (Dewar lesion) **5**, are the most abundant UV lesions and are responsible for much of the destructive effect of UV light.



1: CPD, **2**: spore photoproduct, **4**: 6-4PP photoproduct, **5**: Dewar valence isomer of **4**.

Scheme 1-4. Depiction of the four main pyrimidine–pyrimidine photolesions**.**[92-96]

Due to UV-B (315-280 nm) and UV-C (< 280 nm) irradiation dimerization may occur through electrocyclic ring formation between two adjacent pyrimidines along DNA strands in cells (**Scheme 1-4**). Triggered by exposure to UV light, the cis-syn cyclobutane pyrimidine dimer (CPD) is formed between two C5=C6 double bonds of two adjacent pyrimidine bases (usually a pair of thymines) by [2 + 2] cycloaddition, whereas a cycloaddition between the C5=C6 double bond of the 5′ nucleoside and the C4 carbonyl group of the 3′ nucleoside, named the Paternó–Büchi reaction, leads to 6-4PP lesions.[93] Even though UV-A (400-315 nm) does not directly affect the nucleobases, it may lead to the formation of CPD lesions by the excitation of photo sensitizers and triplet energy transfer.[93]

Of the three categories of solar UV radiation, only UV-A and UV-B are able to penetrate Earth's atmosphere. Thus, these two types of UV radiation are of greatest concern to humans and other creatures, especially as continuing depletion of the ozone layer causes higher levels of this radiation to reach the planet's surface. The lesions formed by UV irradiation will lead to distortion of DNA's structure, introducing bends or kinks and thereby impeding transcription and replication. Relatively flexible areas of the DNA double helix, for example, the mutated oncogene the *p53* gene, are most susceptible to damage.[97]

To counteract the DNA damage caused by UV, cells have developed a number of repair mechanisms. Cooperating with the enzyme photolyase, photoreactivation is found as an efficient repair pathway in bacteria and several other organisms.[98, 99] During photoreactivation, photolyase binds pyrimidine dimer lesions; in addition, a second molecule known as a chromophore converts light energy into the chemical energy required to directly revert the damaged site of DNA to its intact form. Photolyases are found in numerous organisms, including fungi, plants, invertebrates such as fruit flies, and vertebrates including frogs. However, there is no indication for their existence in human cells.[100]

Excision repair including base excision repair (BER) and nucleotide excision repair (NER) plays an important role in DNA repair of these dimers in several organisms with the aid of glycosylases and polymerases. In contrast to photoreactivation, these pathways are much more complex and do not directly reverse DNA damage but instead replace the damaged DNA with new, undamaged nucleotides.[101-106]

In addition, mutagenic repair or dimer bypass, recombinational repair, cell-cycle checkpoints, apoptosis and certain alternative repair pathways are found to be operative in various organisms.[100]

1.2.4 Alkylating agents

Alkylation lesions can be formed along DNA and RNA strands when exposed to alkylating agents that result from endogenous compounds, [107, 108] environmental agents [109-111] and alkylating drugs. Alkylating agents are mutagenic and genotoxic.

DNA alkylating agents can be monofunctional or bifunctional. Bifunctional alkylating agents can introduce interstrand and intrastrand cross-links along DNA strands, and these cross-links are responsible for the inhibition of DNA synthesis and for cytotoxicity (more details in **Sections 1.3** and **1.4**). Cytotoxicity of monofunctional alkylating agents is related to single strand breaks in DNA or to damaged nucleobases.

Depending on the source of the alkylating agents, they can be classified as endogenous and exogenous (including chemotherapy drugs).

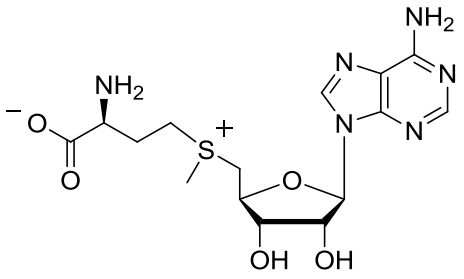
1.2.4.1 Endogenous alkylating agents

There are many possible sources of endogenous alkylating agents and several compounds have been identified.

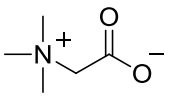
S-Adenosyl methionine (SAM, molecular structure shown in **Figure 1-8**) is a weak chemical methylating agent involved in methyl group transfers in biochemical reactions. SAM was first discovered by Cantoni in 1952.[112] It is made from adenosine triphosphate (ATP) and methionine by methionine adenosyltransferase. The methyl group

bonded to the methionine sulfur atom in SAM is chemically reactive, and allows methyl donation to bioactive molecules in cells, such as nucleic acids, proteins, lipids and secondary metabolites. It is shown to induce mutations in DNA and mainly generates *N*7-methylguanine (*N*7-meG) and *N*3-methyladenine (*N*3-meA), and to a reduced extent O^6-methylguanine (O^6-meG).[107, 113-115]

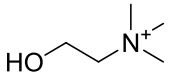
Quaternary ammonium compounds, such as betaine and choline (molecular structures shown in **Figure 1-8**) are alkylating agents. Betaine occurs in numerous vertebrate tissues as an osmolyte, ensuring osmoprotection. Although choline originates from exogenous sources such as diet, tobacco smoke or environmental contaminates, some may also be formed endogenously from cellular precursors.[116] These act as methyl group donors in transmethylation reactions in organisms.



S-Adenosyl methionine (SAM)



Betaine

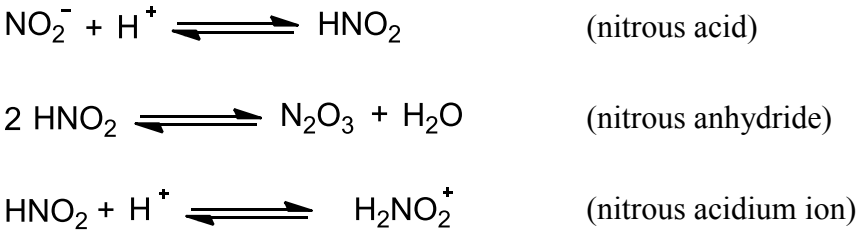


Choline

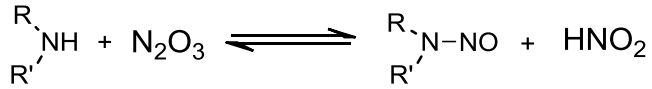
Figure 1-8. Molecular structures of some endogenous alkylating agents

Another important class of alkylating agents are *N*-nitroso compounds (NOCs), which originate from the diet in foods such as smoked meat, bacon and fish. They can also be formed *in vivo* by acid-catalyzed and bacterial nitrosation in the stomach and *via* nitric oxide (NO) formation during inflammation.[117-126] Based on their chemical structures, NOC are divided into two major groups: *N*-nitrosamines and *N*-nitrosamides. Both are formed by the reaction of a nitrite compound with amines or amides. Under acidic conditions, nitrite forms intermediate nitrosating species such as nitrous anhydride and the nitrous acidium ion (N_2O_3, $H_2NO_2^+$, respectively) (see Equation (1) in **Scheme 1-5**). [125, 127, 128] *In vitro*, the nitrosation of amines is accelerated by thiocyanates; the nitrosation of amides is catalyzed by citrate and other organic acids.[129, 130] Under neutral conditions, nitrosation occurs *via* bacteria catalyzed processes in the hypochlorhydric stomach in individuals with chronic gastritis.[125] The reaction between secondary amines and nitrite affords *N*-nitrosamines (Equation (2) in **Scheme 1-5**) while nitrosation of amides produces *N*-nitrosamides (Equation (3) in **Scheme 1-5**). These NOCs act as alkylating agents that react along nucleic acid strands. The alkylation mechanism is similar to exogenous *N*-nitroso compounds, and will be introduced in **Section 1.2.4.2**.

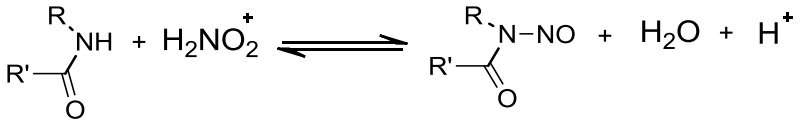
Equation 1: Formation of the nitrosating species



Equation 2: Formation of *N*-nitrosamines



Equation 3: Formation of *N*-nitrosamides



Scheme 1-5. Schematic diagram of NOC formation [125, 127]

Employing the endogenous alkylating agents, the most frequent effect of DNA methylation is the generation of *N*7-meG and *N*3-meA. *N*7-meG is relatively harmless, because this modification does not alter the coding specificity of the base. However, alkylation on *N*7 of 2'-deoxyguanosine will accelerate the hydrolysis leading to the generation of mutagenic apurinic (AP) sites and imidazole ring opening of *N*7-meG, which results in the halting of DNA replication.[131, 132] *N*3-meA is a cytotoxic DNA lesion that blocks replication. All living cells have an efficient DNA glycosylase that removes 3-meA from DNA, generating an AP site. SAM also generates the minor

pyrimidine lesions *N*3-methylthymine (*N*3-meT) and *N*3-methylcytosine (*N*3-meC). *N*3-meT blocks DNA replication *in vivo* while *N*3-meC is a strong inhibitor of DNA synthesis and could lead to mutagenesis.[133-135]

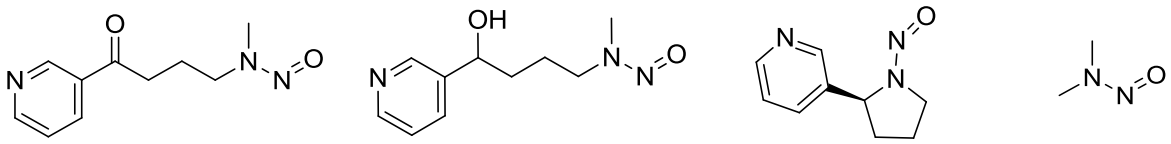
Some other alkylated and highly mutagenic DNA lesions of endogenous origin are O^6-meG, O^4-methylthymine (O^4-meT) and O^4-ethylthymine (O^4-etT), which may lead to GC→AT and TA→CG transitions during DNA replication.[28, 136-138]

1.2.4.2 Exogenous alkylating agents

There are many different kinds of exogenous alkylating agents, most of which are electrophilic compounds. The simplest are halocarbons generated from industrial production, terrestrial (e.g. by plants and fungi) and marine environments.[110, 111] These compounds may lead to alkylated DNA lesions which are mutagenic and possibly carcinogenic.[139-141] However, no adequate evidence shows the link between carcinogenicity and exposure to halocarbons in animals and humans.[142, 143]

N-nitroso compounds (NOC) may be generated from tobacco smoke and in the diet as well as other environmental sources.[125, 144-146] In animal dietary intake experiments *N*-nitroso compounds showed their potent carcigonenicity as the precursors of cancer development.[145, 147] Tobacco smoke contains both tobacco-specific *N*-nitroso compounds and several other nitroso compounds, e.g. *N*-nitrosodimethylamine. Tobacco-

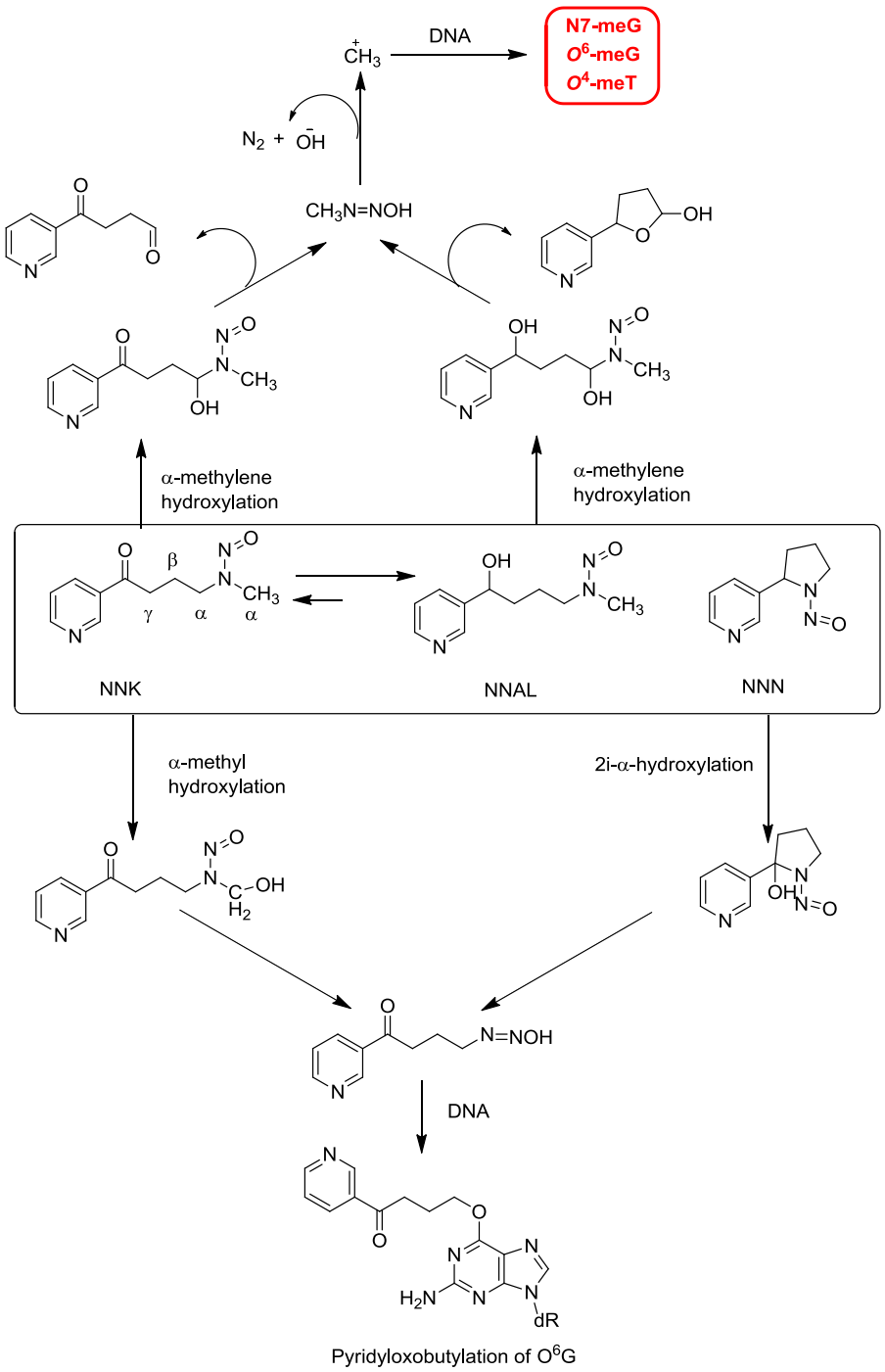
specific nitrosamines have shown carcinogenicity in laboratory animals, and some known molecules are identified with the molecular structures shown in **Figure 1-9**. Employing tobacco-specific nitrosamines will afford a series of alkylated adducts along DNA strands, and their formation is shown in **Scheme 1-6**.[148-151] In the diet, the key role of nitrite and nitrogen oxides in forming *N*-nitroso compounds by interaction with secondary and tertiary amino compounds (see **Scheme 1-5**) has led to the examination worldwide for the presence of *N*-nitroso compounds in food.[125, 145, 152-154]



A B C D

A) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); B) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL); C) *N*-nitrosonornicotine (NNN); D) *N*-nitrosodimethylamine (NDMA)

Figure 1-9. Molecular structures of *N*-nitroso compounds in tobacco.[155-157]



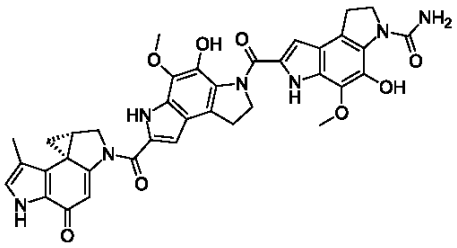
Scheme 1-6. Overview of metabolism and DNA adduct formation by the tobacco-specific nitrosamines NNK, NNAL and NNN.[109]

Mustard gas, alkyl sulfate-ester and sulfonic-esters can also act as alkylating agents. All these alkylating agents are electrophilic compounds, which can transfer active alkyl groups to nucleophilic positions of the DNA nucleobases. Those with two alkylating functional groups can react with two positions of DNA to form cross-links within a DNA strand (intrastrand), between the two DNA strands (interstrand) or between DNA and protein. The damaged DNA, especially interstrand cross-linked DNA, can be mutagenic and are potentially cytotoxic. Some alkylating agents have been used in chemotherapy. Alkylating drugs are mostly methylating agents (e.g. temozolomide and streptozotocin, an antibiotic) or chloroethylating agents (e.g. carmustine, lomustine and fotemustine). The effects of these drugs are strongly modulated by DNA repair processes. Understanding and controlling repair processes may therefore allow development of new therapies by protecting normal tissues or by potentiating effects in target tissues.[158-161] More details related to bifunctional alkylating agents are discussed in **Section 1.3**.

Ethylene oxide is classified as a known human carcinogen. The formation of alkylated DNA adducts, mainly *N*7-(2-hydroxyethyl)-2'deoxyguanosine (7-BEG) in DNA, is considered as an important early event in the carcinogenic process of ethylene oxide.[162-165]

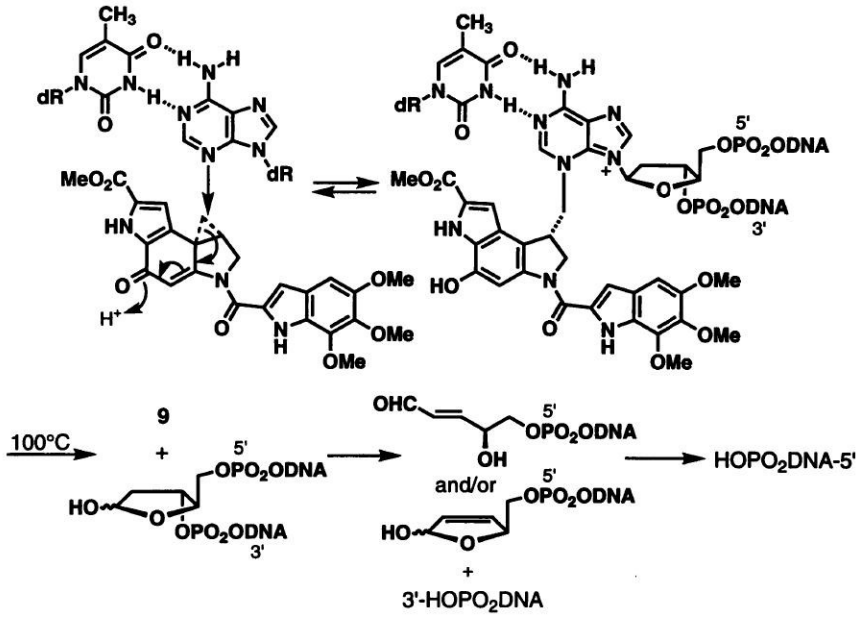
Some natural substances also exhibit potent antitumor properties, such as CC-1065, duocarmycin A, and duocarmycin SA (molecular structures shown in **Figure 1-10**). [166]

These substances alkylate mainly in the minor groove of DNA, with *N*3-alkyl 2'-deoxyadenosine (*N*3-alkylA) as the main adduct (the example of duocarmycin SA action on DNA is shown in **Scheme 1-7**).[167] The binding affinity, selectivity and reaction mechanism involving DNA alkylation or cleavage, is currently being studied to explore analogs (i.e. adozelesin, bizelesin, and carzelesin) as agents for cancer chemotherapy.



CC-1065 (+)-Duocarmycin A Duocarmycin SA

Figure 1-10. Molecular structures of some naturally occurring alkylating substances.



Scheme 1-7. Binding pattern of Duocarmycin SA with DNA and thermally induced depurination. [167]

Alkylating agents containing conjugates such as *N*-methylpyrrole (Py)-*N*-methylimidazole (Im) polyamides and 1,2,9,9a-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI) with a 5-amino-1*H*-indole-2-carbonyl linker have been synthesized and exhibited highly efficient sequence-specific DNA alkylation. These agents are under development towards application as antitumor agents.[168, 169]

1.2.4.3 Alkylating sites

There are a variety of nucleophilic sites along nucleic acid strands, as a result alkylated lesions may be introduced at almost all O- and N-atoms in the nucleobases, as well as at

O-atoms in phosphodiesters. The alkylation pattern depends on the number of reactive sites within the alkylating agent (monofunctional versus bifunctional), its particular chemical reactivity (S_N1 versus S_N2-type nucleophilic substitution), the type of alkyl group addition (methyl, chloroethyl, etc.) and the DNA substrate (double- or single-stranded).[170] Alkylation damage in RNA generally follows the same pattern as for single-stranded DNA.[171] Exposure to methylating agents such as methyl methanesulfonate (MMS), *N*-methyl-*N*-nitrosurea (MNU), tobacco-specific nitrosamines (*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG)) and drugs like temozolomide (TMZ), streptozotocin (STZ) or dacarbazine (molecular structures shown in **Figure 1-11**), results in a broad spectrum of alkylated adducts. The alkylation pattern for DNA duplexes may occur in major and minor grooves with differential preference (see **Figure 1-12**).[172-175]

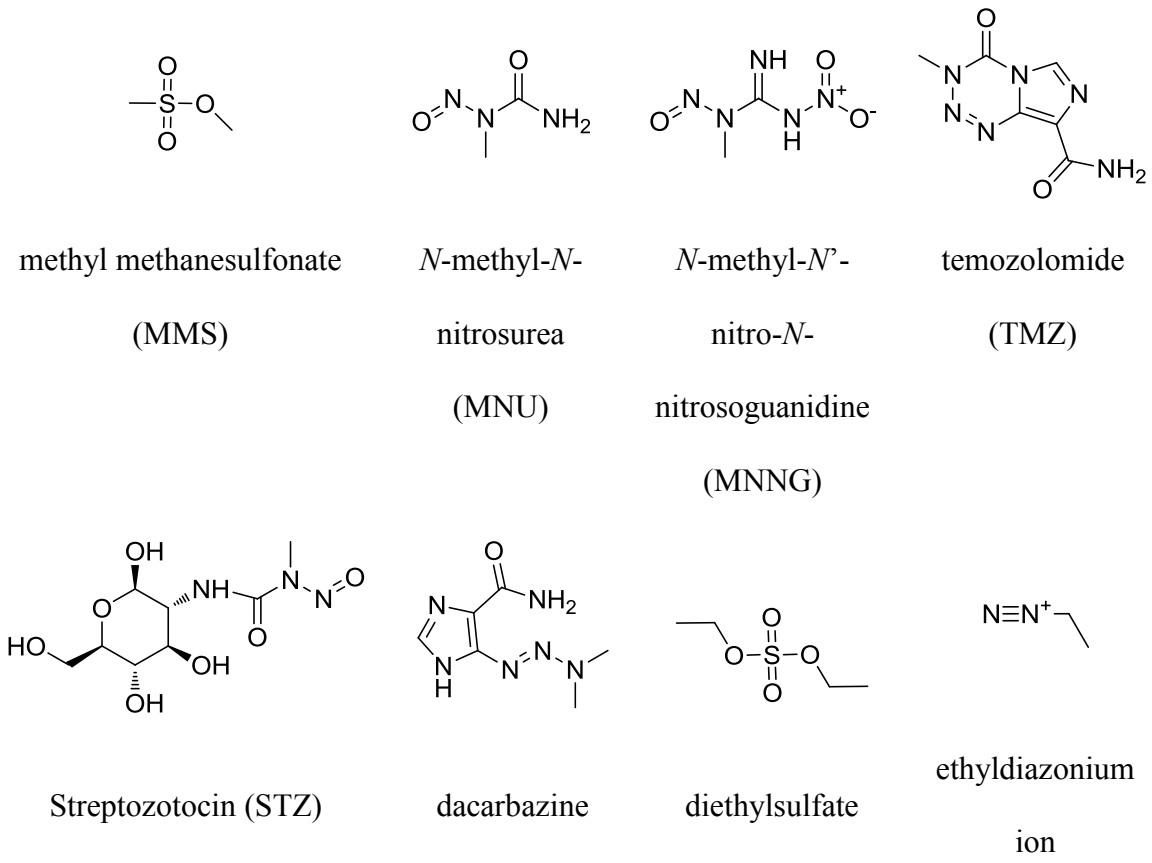


Figure 1-11. Molecular structures of alkylating agents

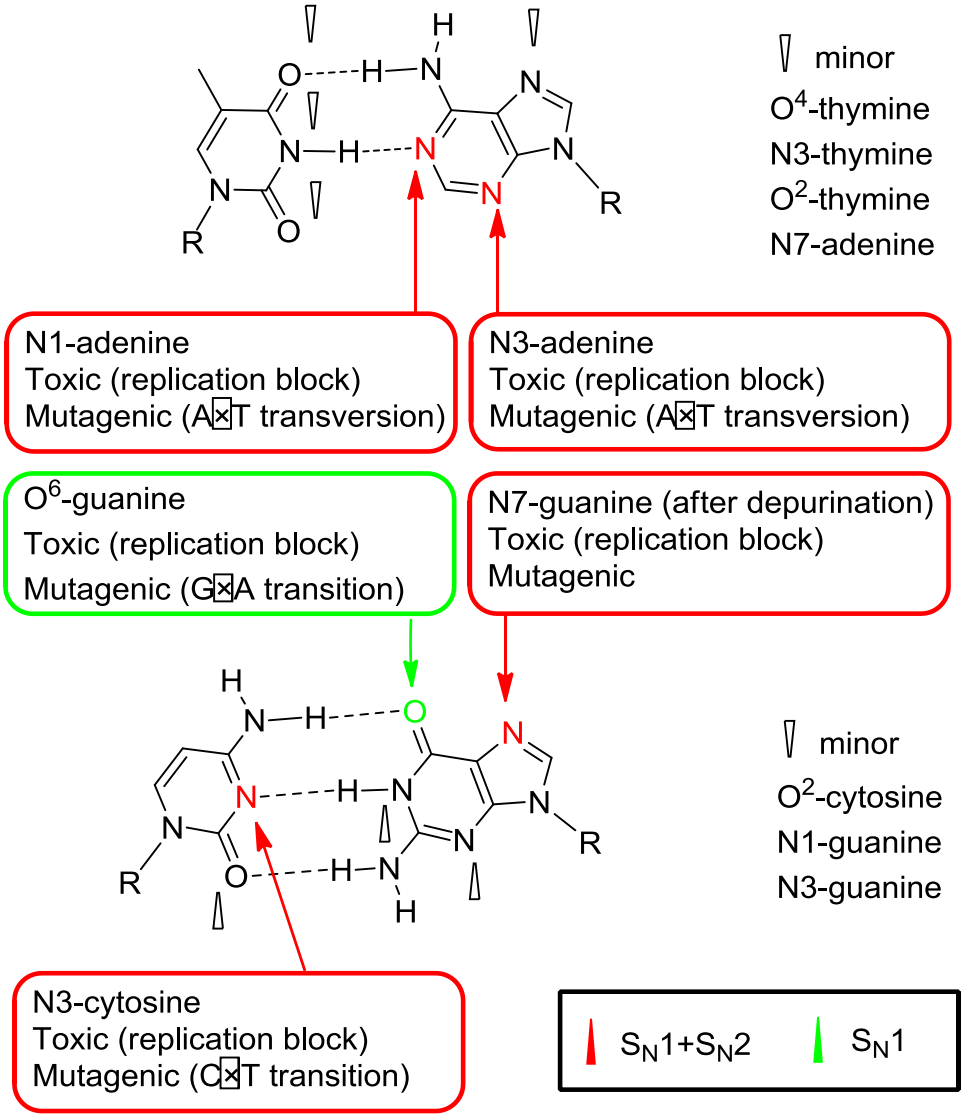


Figure 1-12. Position of the reactive sites of some DNA alkylators on G-C or A-T base pairs [172-175]

The S_N1 pathway is dependent on the stability of the formed carbocations, generated prior to their attack on DNA residues with low preference between nitrogen or oxygen.

Thus, alkylating agents of the S_N1-type (e.g. MNU) alkylate both oxygens and nitrogens in nucleic acids. By contrast, both the properties of alkylating agents and nucleophilicity of targeted sites are important in the S_N2 pathway. The main adducts with S_N2 alkylating agents (e.g. MMS) occur on nitrogen due to the fact that nitrogen has higher nucleophilicity than oxygen.[176-180]

Alkylations on nucleobases of nucleic acids are both genotoxic and cytotoxic. In general, *O*-alkylations (O^6-alkylG and O^4-alkylT) are highly mutagenic and genotoxic, whereas *N*-alkylations (e.g. *N*3-alkylA and *N*1-alkyl adenine (*N*1-alkylA)) are cytotoxic, but relatively less mutagenic.[181-184]

The preferred alkylation sites in duplex DNA depends on the nature of the alkylating agent(s). For example, employing diethylsulfate (structure shown in **Figure 1-11**) the preferred sites of reaction follow the order: *N*7G >> P-O > *N*3A >> *N*1A ~ *N*7A ~ *N*3G ~ *N*3C >> O^6G. In contrast, the preferred sites for alkylation by ethyldiazonium ion (structure shown in **Figure 1-11**) follow the order: P-O >> *N*7G > O^2T > O^6G > *N*3A ~ O^2C > O^4T > *N*3G ~ *N*3T ~ *N*3C ~ *N*7A.[176-178] Two more examples of methylation patterns in single-stranded and double-stranded nucleic acids employing alkylating agents MMS and MNU are shown in **Table 1-2**. [148] Examples of alkylated DNA nucleobases are shown in **Figure 1-13**.[185]

Table 1-2. Methylation patterns of single-stranded vs. double-stranded nucleic acids upon reaction with MMS and MNU[a,b] [148]

Site of methylation	MMS			MNU		
	ssDNA/RNA[c]	RNA[d]	dsDNA	ssDNA/RNA[c]	RNA[d]	dsDNA
Adenine						
*N*1-	18	11	3.8	2.8	2	1.3
*N*3-	1.4	1.5	10.4	2.6	nd[e]	9
*N*7-	3.8	nd	1.8	1.8	nd	1.7
Guanine						
*N*3-	~1	nd	0.6	0.4	1	0.8
O^6-	–	0.2	0.3	3	4	6.3
*N*7-	68	72	83	69	80	67
Uracil/thymine						
O^2-	nd	nd	nd	nd	nd	0.11
*N*3-	nd	0.4	nd	nd	nd	0.3
O^4-	nd	nd	nd	nd	nd	0.4
Cytosine						
O^2-	nd	Nd	–	nd	nd	0.1
*N*3-	10	7	<1	2.3	1.5	0.6
Diester	2	Nd	0.8	~10	nd	17

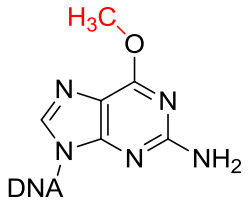
[a] Adapted from refs.[171, 176]

[b] Expressed as percent of the total methylation.

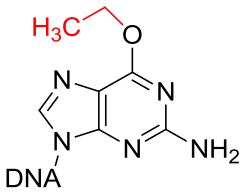
[c] The values are from data compilation by ref. [176], and originate from experiments with the ssDNA phage M13, and RNA from TMV, yeast, HeLa cells, animal ribosomes, and μ2 phage.

[d] These data are from the original work of ref. [171] and indicate methylation pattern in RNA from bacteriophage R17. The values are percent of the total radioactivity analysed for methylation. 92% (MMS) and 89% (MNU) of the total radioactivity were recovered as identifiable and detectable residues (>0.5% of total radioactivity).

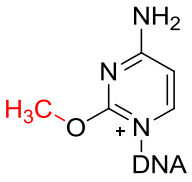
[e] nd: not determined.



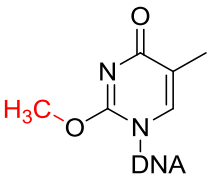
O^6MeG



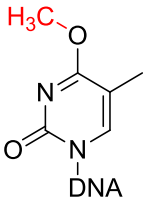
O^6EtG



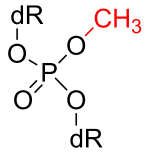
O^2MeC



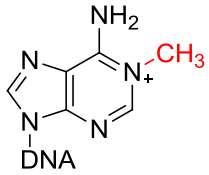
O^2MeT



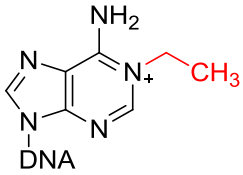
O^4MeT



MePT

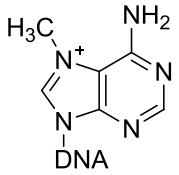


1MeA

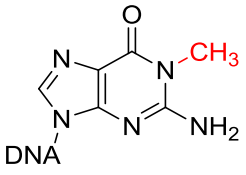


1EtA

3MeA



7MeA



1MeG

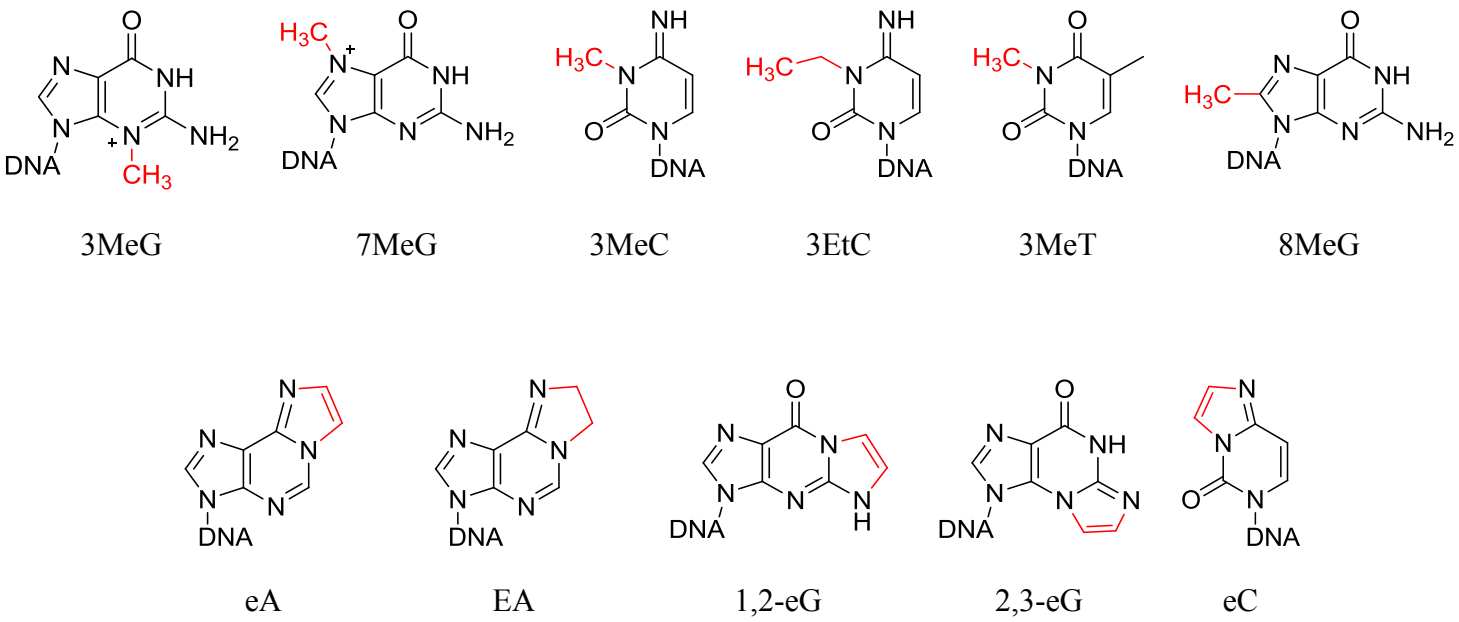


Figure 1-13. Structures of DNA alkylation lesions. (Adapted from reference [185])

The data in **Table 1-2** clearly shows the high nucleophilic reactivity of the *N*7-position of guanine in DNA, with the formation of *N*7-alkyl guanine (*N*7-alkylG) as the predominant alkylation adduct, which does not possess any mutagenic or cytotoxic properties in itself. However, it is subject to rapid spontaneous depurination and enzymatic removal, thus creating cytotoxic abasic sites.[148, 178, 186-188]

Compared to other DNA adducts, *N*7-guanine adducts are less chemically stable. The instability of *N*7-guanine adducts is caused by an additional positive charge on the guanine ring system. In general, larger alkyl groups promote depurination in dsDNA.[177] This has been demonstrated under physiological conditions (pH 7.4, 37°C).[189] In addition, *N*7-guanine adducts, which accumulate in DNA with continuous

exposure or treatment, usually reach a steady state of biological equilibrium after 7 to 10 days.[190-192] In contrast, alkylated adducts such as O^4-etT and O^6-meG are more persistent. Compared to intact 2'-deoxyguanosine, *N*7-meG depurinates 106 times faster under the conditions of pH7 at 37 °C.[193] Besides depurination, other reactions occur to *N*7-guanine adducts, for example ring opening to yield 5-*N*-alkyl-2,6-diamino-4-hydroxyformamidopyrimidine (alkyl-FAPy), loss of the C-8 proton and rearrangement to C8 adducts.

The presence of *N*7-meG and other *N*7-guanine adducts (which originate from endogenous and exogenous sources) has been demonstrated ubiquitously in DNA of human cells.[116, 194-201] Despite the ubiquitous nature of *N*7-guanine adducts, their application as a biomarker of exposure in larger molecular epidemiology studies is not common practice due to the chemical instability.

In addition to *N*7-alkylG, *N*3-alkylA is generated as another primary *N*-alkylation product.[148] In contrast to the relatively innocuous *N*7-alkyl G lesions, *N*3-alkyl A lesions are highly cytotoxic and the presence of this lesion in DNA is thought to halt DNA polymerase, which then initiates a cascade of events including cell death.[202-205]

Alkylation (mainly methylation) at the guanine O^6 position confers the greatest mutagenic and carcinogenic potential.[206-208] The formation of this damage may lead to a G:C–A:T mutation. This lesion can not only generate point mutations but can also initiate mismatch repair-mediated DNA recombination and cell death.[181, 209] The

relative preference for incorporation of T and C opposite an O^6-meG lesion varies somewhat with polymerase and sequence context.[210]

In the absence of polymerase, the structure of DNA duplexes containing O^6-meG·T or O^6-meG·C base pairs indicates that O^6-meG·C forms a wobble pair under neutral conditions (**Figure 1-14 *a***) while this base pairing is weaker than canonical C·G pairings by 4–5 kcal/mol.[211, 212] O^6-meG·T mispairs are relatively unstable, forming only one hydrogen bond, but adopt a shape similar to Watson–Crick pairs (**Figure 1-14 *b***).[213, 214] The Watson–Crick-like conformation of O^6-meG·T mispairs has been postulated to allow preferential incorporation of T opposite O^6-meG by high-fidelity polymerases but cannot account for the fact that the polymerase only weakly discriminates against O^6-meG·C.[215, 216] Repair pathways to revert this damage have been studied by a couple of research groups including ours.[217-221]

In single-stranded DNA, the *N*1-position of adenine and the *N*3-position of cytosine are also subject to methylation by monofunctional methylating agents to generate the replication blocking and mispairing lesions, *N*1-methyladenine (*N*1-meA) and *N*3-meC).[185] In double-stranded DNA, these sites are protected due to base pairing, but they can be transiently exposed during replication, transcription or recombination.

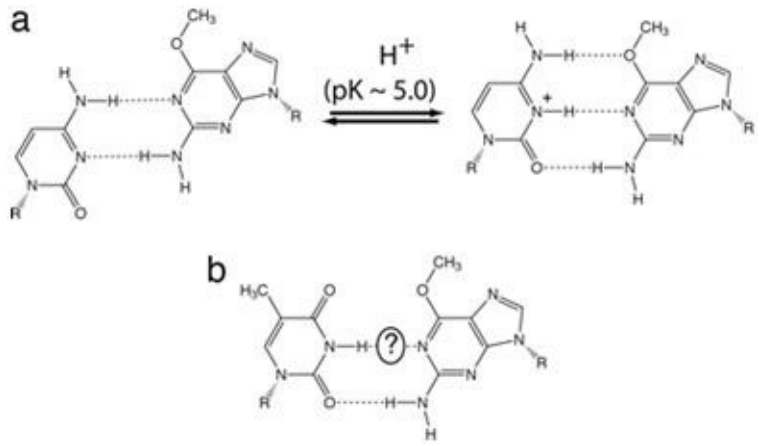


Figure 1-14. Structures of O^6-meG·C (a) and O^6-meG·T (b) pairs in DNA duplexes, unbound by protein. Hydrogen bonds are shown as dashed lines. The presence or absence of the H bond indicated by the "?" has been the subject of some controversy.[216]

Numerous cellular pathways, including direct DNA damage reversal, base excision repair (BER), nucleotide excision repair (NER: long/short-patch, transcription coupled/global genome), mismatch repair (MMR), homologous recombination (HR) or nonhomologous end-joining (NHEJ), have evolved as DNA repair pathways to defend against alkylation-induced cell death or mutation.[148, 222]

Alkylating agents modify DNA in different patterns, which produce lethal and mutagenic lesions to both healthy and diseased cells without proper discrimination. Maintaining a proper balance of alkylating potentials and DNA repair pathways is one of the important factors in chemotherapy to modulate alkylating agent toxicity.[180]

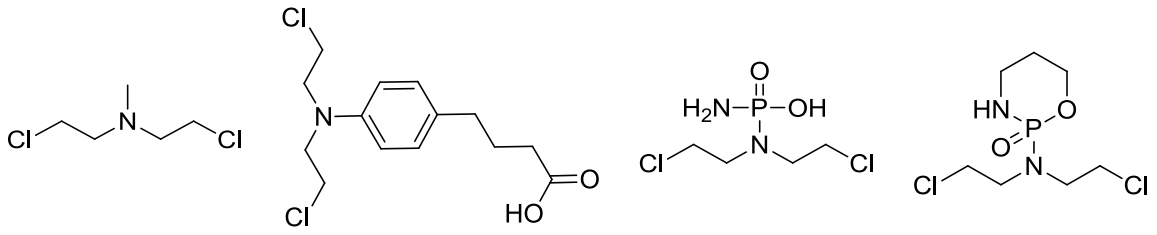
1.3 Bifunctional alkylating agents and other cross-linking agents

Alkylating drugs have been used as anticancer drugs for decades and continue to play an important role in the treatment of several types of cancers.[159, 223-227] Most alkylating drugs are monofunctional methylating agents (More details in **Section 1.2**) or bifunctional alkylating agents such as nitrogen mustards (e.g., chlorambucil and cyclophosphamide), or chloroethylating agents (e.g., nimustine [ACNU], carmustine [BCNU], lomustine [CCNU], and fotemustine). Employing bifunctional alkylating agents, interstrand cross-links (ICLs) may be generated between two complementary DNA strands (DNA-protein crosslinks may be formed as well). Formation of an ICL covalently linking the opposite strands of double helix results in severe disruptions of normal DNA functions, such as replication, transcription, and recombination. Compared to DNA damage with monoalkylating agents, ICLs are more highly cytotoxic and have more complicated effects on the biological process in damaged cells.[148, 222, 228-232] In the bacterial or mammalian genome 20 ICLs, if left unrepaired, will be lethal to cells. [233, 234]

A description of several types of bifunctional alkylating agents, according to their source and molecular moieties, are categorized below.

1.3.1 Nitrogen Mustards

Nitrogen mustards are bifunctional alkylating agents containing *N*,*N*-bis-(2-chloroethyl)amine functional groups. Mustards are extensively used in cancer chemotherapy while they are highly carcinogenic.[235, 236] Clinically important examples for this category include mechlorethamine, chlorambucil and phosphamide mustard with their molecular structures shown in **Figure 1-15**.



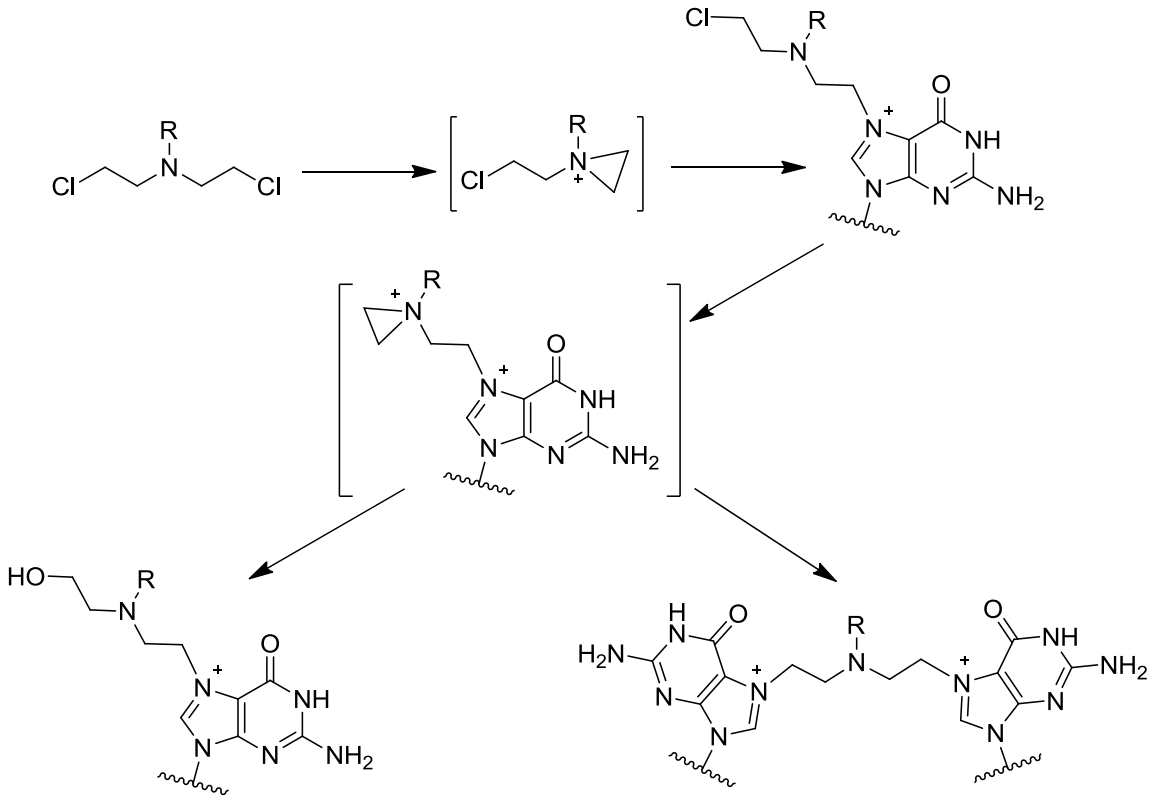
mechlorethamine chlorambucil phosphoramide mustard cyclophosphamide

Figure 1-15. Molecular structures of clinical mustards

Employing nitrogen mustards, a lesion will be formed *via* the aziridinium intermediate (see **Scheme 1-8**) to produce monofunctional guanine-*N*7 adducts and interstrand cross-linked adducts involving two guanines in GNC· GNC (5′ → 3′ / 5′ ← 3′) sequences as the main adducts.[235-243] Computer-modelling to investigate the preference of the 1,3- versus 1,2-cross-linkage indicated that the former linkage was favored both thermodynamically and kinetically. Compared to the 1,2 linkage, the dG-to-dG *N*7-to-*N*7

1,3 linkage was much more flexible, which leads to a more entropic state for this linkage.[241]

Due to the introduction of a five-atom linkage with these mustards, a distortion of the DNA helix is generated. Studies have shown that the linkage distance span (5.1 Å) is shorter than the minimal distance between the guanines (6.8 Å), which leads to static bend of 12.4-16.8^{o} per lesion in B-form DNA.[244-246]



Scheme 1-8. Formation of mono-alkyl and ICL *N*7 adduct employing nitrogen mustards.[247]

Nitrogen mustards are genotoxic, and produce a wide array of mutations, including base substitutions at both G·C and A·T base pairs, intragenic as well as multilocus deletions and chromosomal rearrangements.[237]

Besides the main adduct involving *N*7-guanine, melphalan and chlorambucil also induce substantial alkylation at adenine *N*3 while cyclophosphamide forms phosphotriesters with relatively high frequency.[237] The modification of side chains of this category of alkylating agents may alter their binding patterns with both region- and sequence-selectivity. For example, employing nitrogen mustards attached to a variety of carrier molecules (intercalators, polypyrroles, polyimidazoles, bis(benzimidazoles), anilinoquinolinium salts and polybenzamides) the generated adducts range from reaction primarily at most guanine *N*7 sites in the major groove (chlorambucil) to selected adenine *N*3 sites at the 3'-end of poly(A/T) sequences in the minor groove (tallimustine).[248]

1.3.2 Chloroethylnitrosourea

Among nitrosourea compounds used in chemotherapy, the 2-chloroethylnitrosoureas (CENUs) are employed as bifunctional alkylating agents which may generate cross-links within DNA strands.[249-251]

Numerous CENUs have been developed, including bis(chloroethyl) nitrosourea (BCNU, carmustine), 1-(2-chloroethyl)-3-cyclohexyl)-1-nitrosourea (CCNU, lomustine), 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (methyl-CCNU, semustine) and chlorozotocin. Their structures are shown in **Figure 1-16**.

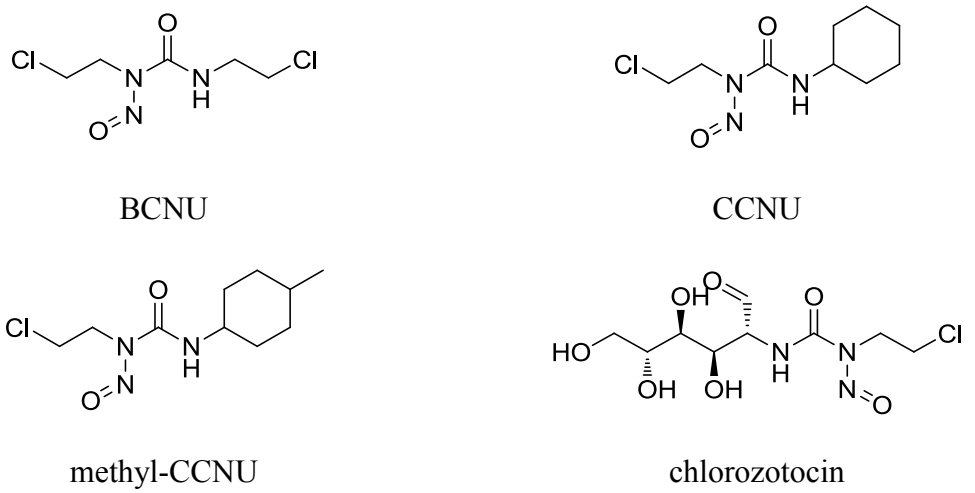


Figure 1-16. Molecular structures of BCNU, CCNU, methyl-CCNU and chlorozotocin

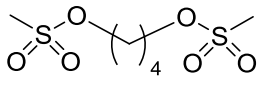
These bifunctional alkylating agents share the same core moieties, 2-chloroethylnitrosourea, which may degrade to form the 2-chloroethyl carbonium ion.[252-254] This is a strong electrophile capable of alkylating guanine, cytidine, and adenine. The chloride atom at the terminal may be displaced to provide another lesion to form DNA cross-links (either intrastrand or interstrand).[249] Interstrand cross-links are considered to be associated with the cytotoxicity of nitrosoureas. Of these four

nitrosoureas, chlorozotocin has low carbamoylation activity.[255-257] Methyl-CCNU is known to be a human carcinogen and the other three are listed as reasonably anticipated human carcinogens.

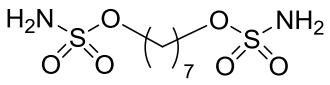
Chloroethylnitrosoureas (CENUs) are clinically important chemotherapeutic agents, forming an ethylene-bridge ICL lesion between *N*1-G and *N*3-C. CENUs may also generate alkylation at the *N*7- and O^6- positions of guanine, with the latter lesion being the precursor to the interstrand cross-link.[258]

1.3.3 Bifunctional alkylene sulfonates and sulfamates

Busulfan and hepsulfam (structures shown in **Figure 1-17**) are major representatives of this family, and they have been investigated and used for treatment of some diseases.[259-265] Sulfonate is a good leaving group accounting for the electrophilicity of these agents. As a result, busulfan and hepsulfam have a higher reactivity than nitrogen mustards, which accounts for their higher cytotoxic properties.



busulfan



hepsulfam

Figure 1-17. Structures of busulfan and hepsulfam

Busulfan is the drug of preference in treatment of chronic myelogenous or granulocytic leukemia because its cytotoxic activity results in primary damage or destruction of hematopoietic cells.[266] The butylene lesion mainly occurs at the *N*7 of guanine. In 1961 P. D. Lawley suggested the formation of *N*7-diguanyl cross-links with busulfan, and in 1980 was confirmed through a chromatographic study while no distinction has been made as to whether this bridge represents an inter- or intrastrand cross-link.[245, 267] Later study showed busulfan mainly leads to the formation of an intrastrand cross-link within DNA duplex, preferentially to generate double-base lesions at 5′-GA-3′ sites. To a lesser extent, cross-linking lesions between the *N*7 atoms of two guanines occurs at 5'-GG-3' sites. [268]

Hepsulfam is a bisulfamic ester which is similar in structure to busulfan while more hydrophilic than busulfan.[265] It is employed as a bifunctional alkylator inducing both DNA-DNA and DNA-protein cross-links.[269] Compared to busulfan, hepsulfam generates ICL DNA mainly on *N*7 of two guanines, and as a result exhibits higher toxicity and cytotoxicity.[264]

Sequence-specificity studies were performed with dimethylsulfonates of different carbon-length to investigate cross-linking patterns. Intrastrand cross-linking was favored for 5- and 6-methylene linkage containing agents and the main adduct occurred at *N*7 of guanine in 5'-GNC sites. Random cross-linking is observed in 5'-GNC and 5'-GC with an 8-methylene linkage. This is not consistent with general patterns observered for other

cross-linking agents (i.e. nitrogen mustards). It was proposed that the sequence specificity involved the local disruption of initial monofunctional cationic *N*7-alkylguanine prior to closure of the interstrand linkage in the second covalent reaction. Based on molecular modeling calculations, a nitrogen mustard could involve a noncovalent association for this step. This difference may account for this inconsistency.[270]

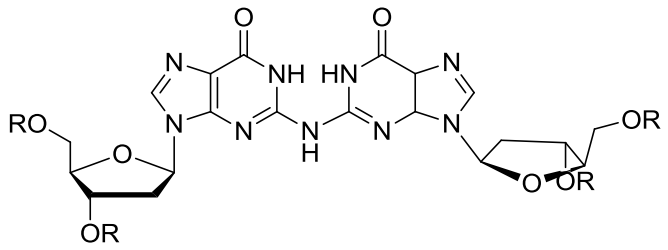
1.3.4 Nitrous Acid

Nitrous acid mostly originates from nitrites which are common food additives, and under acidic conditions in the stomach produces this reactive intermediate. Nitrous acid is a mutagenic agent that induce ICLs in the DNA duplex, preferentially at d(CpG) steps. The occurrence of nitrous acid leads to deamination and cross-linking in the cell, and these two kinds of damage proceed by diazotization of an exocyclic amino group.[271, 272] Displacement by water results in deamination while nucleophilic substitution from the amino group of another DNA residue generates a cross-link. It has been estimated that for every four deaminations of 2'-deoxyguanosine, the most readily deaminated DNA component, one ICL is formed.[273, 274]

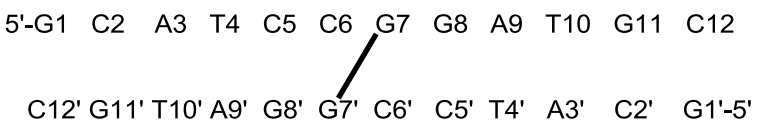
Nitrous acid-induced DNA ICLs form preferentially between two 2'-deoxyguanosine (dG) residues in [5'-d(CG)]$_2$ sequences, forming a cross-link lesion in which the guanines

share a common exocyclic amino group (Shown in **Figure 1-18**). It has been proposed that this sequence preference is due to the close proximity of an exocyclic amine of dG on one DNA strand to a diazonium ion on the other strand.[274-276]

A



B



(**A**) Structure of the nitrous acid induced DNA interstrand cross-link. (**B**) Schematic representation of the cross-linked dodecamer duplex with the residue numbering scheme. Note in particular the cross-linked guanines (G7 and G7′), and the cytosines preceding the cross-link, C6 and C6′, which would base pair with G7′ and G7, respectively, in normal B-DNA.

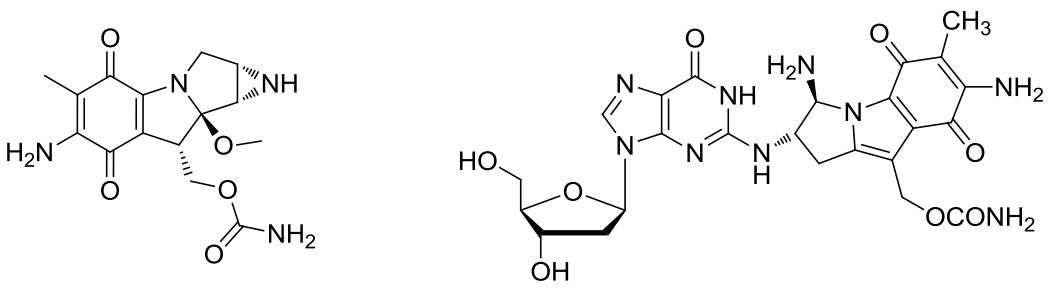
Figure 1-18. ICL formation with nitrous acid.(Adapted from reference [277])

Molecular modeling studies suggest that the resulting cross-linked lesion can be accommodated with minimal structural reorganization in B-form DNA, despite a severe propeller twist of the cross-link lesion.[275] Structural studies of nitrous acid induced DNA interstrand cross-links indicated that the cross-linked guanines formed a nearly

planar, covalently linked 'G:G base pair', stacked on the 3' side of guanines of the spatially adjacent G:C base pairs. The complementary partner cytidines, which normally form a base pair with the cross-linked guanines, are flipped out of the helix, adopting well defined extrahelical positions in the minor groove. The identity of the flanking base pairs significantly altered the stacking patterns and phosphate backbone conformations.[278]

1.3.5 Mitomycin C

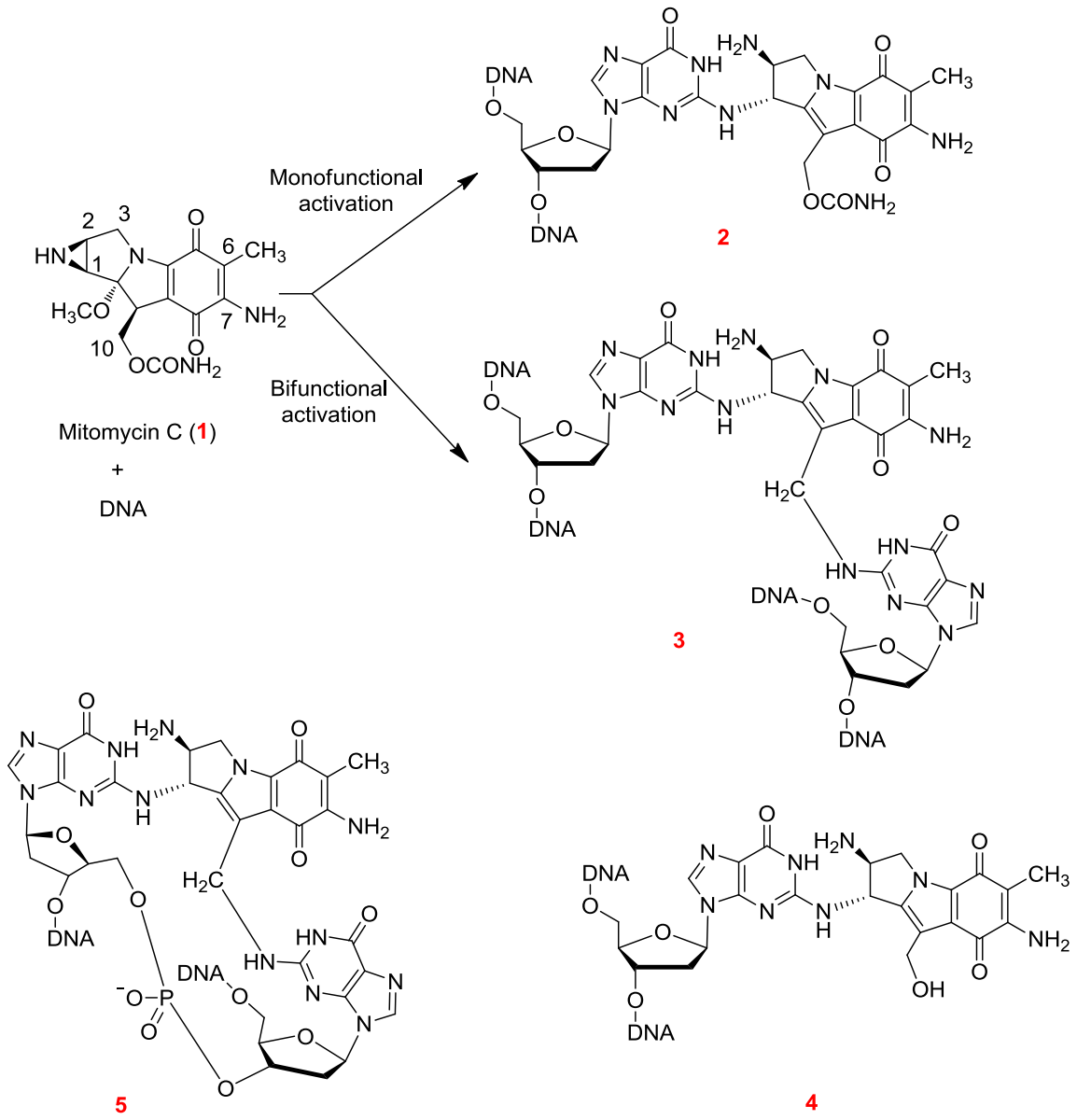
Mitomycin C (MC, shown in **Figure 1-19**), discovered in the 1950s in fermentation cultures of the microorganism *Streptomyces caespitosis*, is a bifunctional alkylating agent used as an antitumor antibiotic.[279-281] A number of close structural variants of MC have since been synthesized and isolated, collectively called mitomycins.[282-286] In mammalian cells alkylation of DNA with MC will lead to selective inhibition of DNA synthesis and mutagenesis. It has therefore been widely used as a tool to study such phenomena and widely used as important antitumor drug in the clinic.[279, 287, 288]



Mitomycin C MC-mono-dG adduct

Figure 1-19. Mitomycin C and MC-mono-dG adduct.[289]

Employing MC, damage occurs with a guanine residue to form MC-mono-dG adduct (principally at N^2 of guanine), a G-G interstrand cross-link at CpG sites, or a G-G intrastrand cross-link at GpG sites.[290] Different from other bifunctional alkylating agents such as the nitrogen mustards, MC reacts with guanine residues of 5′-CG-3′ sequences through the minor groove of DNA. MC itself is relatively inert to form covalent adducts with DNA, and requires sequential chemical or enzymatic reductions of its quinone ring to initiate alkylation. The adducts of MC alkylation of DNA molecules are shown in **Scheme 1-9**.[279, 290-292]



Activation of only one of the alkylating functions gives monoadduct 2 and 4, whereas activation of both alkylating functions can give products 3 and 5.

Scheme 1-9. Adducts of MC and DNA formed by reductive activation. (Adapted from reference [293])

Employing MC as an alkylating agent, a structural study found that the formation of the intrastrand cross-link led to a distortion of B-form DNA with a 14.6±2.0° bend per lesion (minimum value). The bend may be generated in a considerable deviation from parallel of the normals to the best planes of the intrastrand cross-linked guanines, due to the formation of the MC intrastrand lesion requiring a shorter distance between their N^2 atoms than normal. The observed bending may account for the increased flexibility of MC-modified DNA, localized to distinct regions, as observed in earlier work by hydrodynamic methods and electron microscopy. The MC adduct-caused DNA bend may serve as a recognition site for certain DNA-binding proteins. [289, 294, 295]

1.3.6 Psoralen

Psoralen and its derivatives (molecular structures shown in **Figure 1-20**) are planar, tricyclic, semiaromatic compounds. They are bifunctional photoreagents that efficiently intercalate in double stranded DNA. Psoralen preferentially forms ICL DNA at d(TpA) dinucleotides upon exposure to light. This agent initially intercalates noncovalently between base-pairs of the DNA duplex. Upon absorption of a single long wavelength photon (ultraviolet light of 320-410 nm), either the furan-side or pyrone-side of the drug reacts with a pyrimidine base to form a cyclobutanyl monoadduct. Furan-side

monoadducts can absorb a second photon to cross-link the thymine base on the complementary strand of the DNA duplex.[296-299] Psoralens have been investigated as photoactive probes of nucleic acid structure and function.[243, 244, 247-249] As mutagenic agents, they have a long history being employed as photoactive drugs against dermatological diseases.

psoralen 4’-hydroxymethyl-4,5’,8-trimethylpsoralen (HMT)

Figure 1-20. Molecular structure of psoralen and 4’-hydroxymethyl-4,5’,8-trimethylpsoralen (HMT).

The formation of the ICL lesion with psoralen leads to a significant distortion of B-form DNA structure. One example of a structural study was conducted with 4’-hydroxymethyl-4,5’,8-trimethylpsoralen (HMT) as the cross-linking agent. The d(T·A) base-pair with the thymine on the DNA furan-side (the strand with the furano portion linked to DNA is designated as F-strand) showed the least distortion, and there existed strong π-stacking interactions between the adenine base and the HMT pyrone ring. By contrast, the most

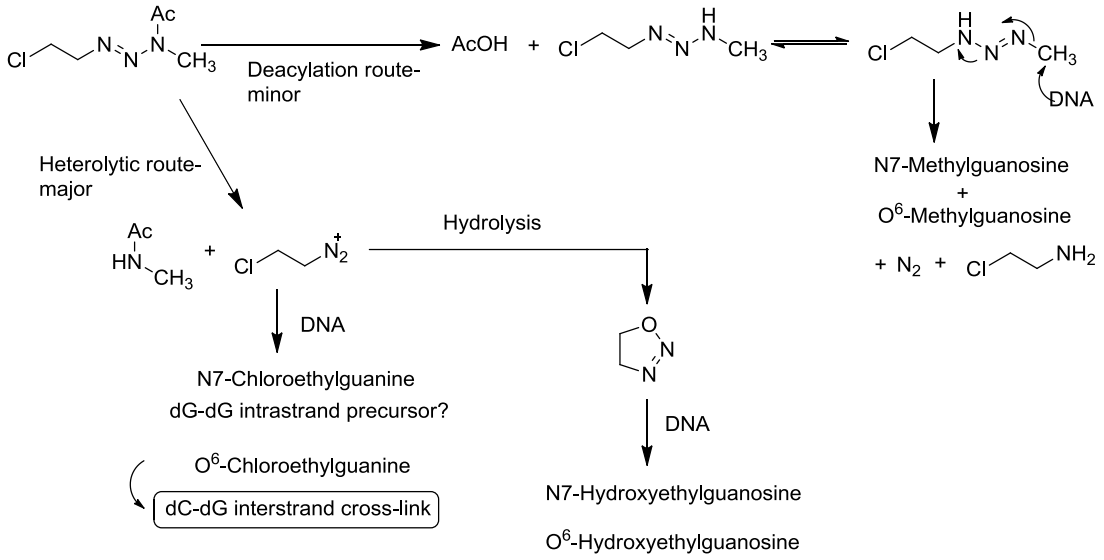
dramatic distortion in the d(T·A) base-pair with the thymine on the pyrone-side (the strand with the pyrone portion linked to DNA is designated as P-strand) resulted from a steric repulsion between the O^4 keto oxygen of the thymine base and the HMT pyrone ring. There was virtually no stacking interaction between the adenine of this base-pair and the HMT furan ring. The perturbations on P-strand also had effect on the direction of the phosphoribose backbone, and accounted for the destabilization of the DNA duplex at the P-strand in the HMT adduct as compared to the native B-form DNA.[298]

1.3.7 Triazene

The 1,3-dialkyl-3-acyltriazenes, including 1-(2-chloroethyl)-3-methyl-3-carbethoxy-(CMC), 1-(2-chloroethyl)-3-methyl-3-acetyl-(CMA), 1-(2-hydroxyethyl)-3-methyl-3-carbethoxy-(HMC), 1-(2-hydroxyethyl)-3-methyl-3-acetyl-(HMA) and 1,3-dimethyl-3-acetyl-(DMA), can undergo degradation to produce a bifunctional alkylating agent.[300-302]

Two degradative routes have been demonstrated for these compounds (shown in **Scheme 1-10**). The process of deacylation and tautomerization will generate an active alkanediazonium intermediate. Generation of the diazonium followed by DNA alkylation or hydrolysis (also giving rise to dG adducts) ensues and completes the major pathway of triazene decomposition. In the case where the *N*1 alkyl group is chloroethyl, quenching of

the diazonium yields chloroethylation at *N*7 or O^6 of guanine. As with the nitrosoureas, O^6 chloroethylation ultimately leads to the dG-dC interstrand cross-link while *N*7 chloroethylation at *N*7 may proceed to form a dG-dG intrastrand lesion. [300, 303]

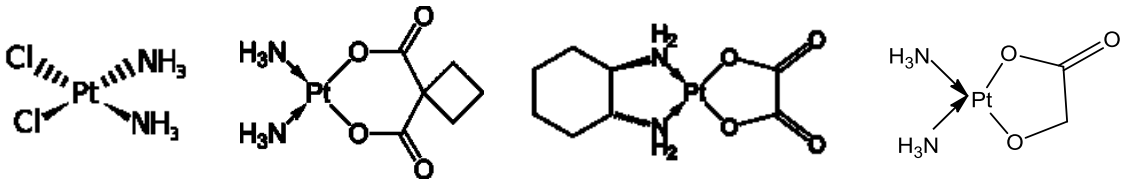


Scheme 1-10. Degradation of 1,3-dialkyl-3-acyltriazenes to afford DNA alkylation adducts. [244, 300]

1.3.8 Platinum complexes

Platinum complexes, such as cisplatin [*cis*-diamminedichloroplatinum(II)], carboplatin [(1,1-cyclobutanedicarboxylato)platinum(II)], oxaliplatin [*(trans*-L-diaminocyclohexane)oxalatoplatinum(II)] and nedaplatin [*cis*-Diammine-glycoloato-*O*,*O*'-platinum(II)] are used in chemotherapy for testicular and ovarian cancers, among

others.[304-306] The molecular structures of these agents are shown in **Figure 1-21**. These drugs enter the cell by diffusion or with the aid of a transporter, become activated by forming aqua complexes, and bind to nuclear DNA, which in eukaryotes is packaged in highly condensed chromatin structures comprising nucleosome building blocks. Cisplatin reacts well with both free and nucleosomal DNA, showing a slight preference for the linker region of the latter.[307]



cisplatin Carboplatin oxaliplatin nedaplatin

Figure 1-21. Molecular structures of cisplatin, carboplatin and oxaliplatin

In the reaction between cellular DNA and cisplatin, different bifunctional adducts are formed including intrastrand and interstrand cross-links (including ICL DNA and cross-linked DNA-protein).[304, 308] The most prevalent adduct is the 1,2-intrastrand cross-link to generate *cis*-$[Pt(NH_3)_2]^{2+}$ 1,2-d(GpG). In this adduct, the platinum is covalently bound to the *N*7 position of adjacent purine bases, preferentially to the *N*7 of guanine. Monofunctional adducts and 1,3-interstrand cross-link (and even longer range) adducts

are also found after the administration of platinum complex(es), each of which gives rise to a distinct structural element that interacts with DNA differently.[304]

The ability of cisplatin to bind to DNA and distort its structure suggested that it would interfere with the normal functioning of this important cellular component. Among all the formed adducts, the bioactivity of cisplatin is generally attributed to the formation of ICL DNA by sequential platination of two nucleophilic sites within the DNA duplex. The formation of the 1,2-intrastrand *cis*-$[Pt(NH_3)_2]^{2+}$ cross-link leads to structural distortion of B-form DNA, which is unwound and bent towards the major grove.[227, 304, 309-313] Compared to the main 1,2-intrastrand cross-linked adducts at 1,2-d(GpG) sites, the solution structure of a short ICL duplex at the GC:GC site indicated that the 2'-deoxyguanosine-bridging *cis*-diammineplatinum(II) lies in the minor groove, and the complementary 2'-deoxycytidines are extrahelical. The double helix is locally reversed to a left-handed form, and the helix is unwound and bent toward the minor groove. The NMR structure differs markedly from previously proposed models but accounts for the chemical reactivity, the unwinding, and the bending of cisplatin interstrand cross-linked DNA and may be important in the formation and repair of these cross-links in chromatin.[314] Crystal structures of ICL adducts show the double-helix is bent and unwound but with significantly different angle values. The platinum coordination is nearly square and the platinum residue is embedded into a cage of nine water molecules

linked to the cross-linked guanines, the two ammine groups, and to the phosphodiester backbone through other water molecules.[315]

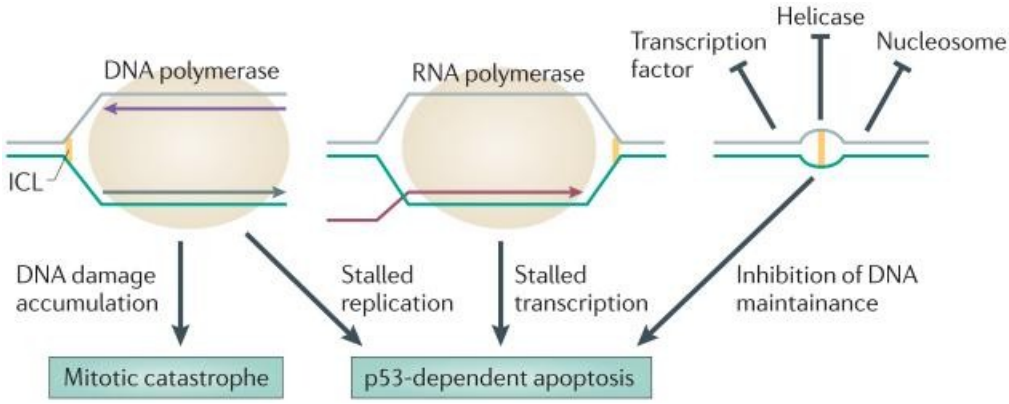
Despite its use in the clinic, cisplatin has several disadvantages including severe toxicity (i.e. nephrotoxicity, neurotoxicity, and emetogensis) and limited solubility in aqueous solution (only allowing for administration intravenously). Furthermore, cisplatin is employed as a chemotherapy agent to a relatively narrow range of tumors. Some tumors have natural resistance to cisplatins, while others develop resistance after the initial treatment. Compared to cisplatin, carboplatin is less toxic and can be administered at a much higher dose. However, carboplatin is only active in the same range of tumors as cisplatin and is still administered intravenously. The same limitations also apply to oxaliplatin and nedaplatin. The exploration of less toxic, orally active compounds that are non-cross-resistant with cisplatin and carboplatin is needed.[305]

1.4 Interstrand cross-links and DNA repair

Alkylating agents, especially bifunctional alkylating agents, are widely used in chemotherapy regimens for cancer treatments. A variety of bifunctional alkylating agents, i.e. chlorambucil, cyclophosphamide, CCNU, melphalan, procarbazine, cisplatin, BCNU, busulfan and mitomycin C, are administrated for the treatment of patients with malignant

diseases. Bifunctional alkylating agents form monoadducts, intrastrand cross-links, and interstrand cross-links (ICLs) in DNA (and DNA-protein cross-links as well).[316]

ICL lesions covalently connect the strands of DNA, and therefore disturb cell cycle progression interfering with replication, transcription, and any other processes requiring strand separation. Compared with other forms of DNA damage, the repair of ICLs is a laborious challenge. As a result, ICLs are extremely toxic, particularly to dividing cells, and this likely explains their efficacy as chemotherapy agents (see **Figure 1-22**).[317-319]



DNA replication and its transcription to RNA can be blocked by ICLs. ICLs may also cause the distortion of chromatin structure, which prevent the access of DNA-interacting proteins. The introduction of ICLs into tumor cells will lead to their death *via* mitotic catastrophe or p53-ligand-dependent apoptosis.

Figure 1-22. ICLs killing of tumor cells.[319]

Although they might differ in their clinical activity, all alkylating agents share the same mechanism of action. These agents act directly on DNA, resulting in its cross-linking and causing DNA strand breaks, leading to abnormal base pairing and inhibiting cell division. When DNA is altered in this manner, desired cellular activities come to a halt and the cell cannot multiply. Alkylating anticancer drugs are effective during all phases of the cell cycle and are used to treat a wide variety of cancers.[320]

Simple methylating agents form adducts at the *N*- and *O*-atoms in DNA bases. *N*-methylation adducts comprise more than 80% of methylated bases. Since there are a variety of nucleophilic sites (*N*- and *O*-atoms) within DNA duplexes, the introduction of bifunctional alkylating agents usually generates a broad spectrum of ICL adducts while some containing intrastrand cross-links may also be formed.[321] It has been demonstrated that some cross-linking agents (i.e. nitrous acid, formaldehyde and mitomycin C) have preference to form 1,2 ICLs while some others (i.e. nitrogen mustards and hepsulfam) generate 1,3 ICL DNA moieties as major products.[238, 240, 246, 274, 322-334]

In **Table 1-3** the main adducts formed by various bifunctional alkylating agents are presented.[244]

Table 1-3. Summation of DNA cross-linking agents and their interaction within DNA duplex. (Adapted from reference [244])

Agent or class thereof	ICL specificity	DNA groove occupancy
N-mustard	dG *N*7-dG *N*7	major
chloroethylnitrosoureas (CNU)	dC *N*3-dG *N*1	major
1,3-dialkyl-3-acytriazene	dC *N*3-dG *N*1	major
Busulfan	dG *N*7-dG *N*7	major
Clomesone	dC *N*3-dG *N*1	major
Glycidaldehyde	not known	not known
Diepoxybutane	dG *N*7-dG *N*7	major
carzinophilin/azinomycin B	dG *N*7-dG *N*7	major
	dG *N*7-dA *N*7	
Cisplatin	dG *N*7-dG *N*7	major
Isochrysohermidin	not known	not known
cyclopropylpyrroloindole dimers	dA *N*3- dA *N*3	minor
Bizelesin	dA *N*3- dA *N*3	minor
pyrrolobenzodiazepine dimers	dG N^2- dG N^2	minor
dinuclear cis-DDP dimers	dG N7- dG N7	major
8-methoxypsoralen	T C5,6- T C5,6	major
4,5',8-trimethylpsoralen	T C5,6- T C5,6	major
Cyclophosphamide	dG *N*7- dG *N*7	major

Hexamethylmelamine	not known	minor
pyrrolizidine alkaloids	dG N^2- dG N^2	minor
RSU-1069	not known	not known
aza aromatic N-mustards	dG *N*7- dG *N*7	major
Nitromin	dG *N*7- dG *N*7	major
chlorambucil N-oxide	dG *N*7- dG *N*7	major
Co(III) mustards	dG *N*7- dG *N*7	major
Daunorubicin	dG N^2- dG N^2	minor
Doxorubicin	dG N^2- dG N^2	minor
Cyanomorphilinodoxorubicin	dG N^2- dG N^2	minor
AZQ/DZQ	dG *N*7- dG *N*7	major
mitomycin C	dG N^2- dG N^2	minor
FR-900482	dG N^2- dG N^2	minor
bioxalomycin α_2	dG N^2- dG N^2	minor

As shown in the table above, a majority of the lesions formed by the bifunctional alkylating agents to produce ICL DNA occur in major groove at the *N*7 of guanine, and the preference of these as the main adducts are consistent with the better accessibility and higher nucleophilicity at this site. It is of great interest to understand the relationships between *N*7-guanine adducts and exposure, mutagenesis, and other biological endpoints. ICL *N*7-guanine adducts do not affect Watson Crick base pairing and pose a challenge to

study as they are chemically unstable and may lead to the production of FAPY and depurination products, which are potentially toxic in biological process of the cell. *N*7-guanine adducts have been shown to be excellent biomarkers for internal exposure to direct acting and metabolically activated carcinogens.[189]

There are also some examples of ICL lesions that protrude in the minor groove. Agents that alkylate DNA in the minor groove are potent cytotoxins, and represent a class of cytotoxic antitumor agents whose DNA sequence specificity may lead to a high selectivity of action.[335] Together with their potential sequence selectivity of interaction with DNA, they are of great interest as potential anticancer drugs.

These alkylated DNA adducts exhibit different stabilities. *N*7-alkylG adducts are chemically unstable caused by the formal placement of an additional positive charge on the guanine ring system.[336] In double-stranded DNA (dsDNA) the half lives range from as little as 2 to 150 h, and larger alkyl groups generally promote depurination in dsDNA.[189] By contrast, O^6-alkylated-guanines (O^6-alkylG), which accounts for a lower proportion of alkylated DNA adducts, are more stable and persist in the absence of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT). In general, *O*-alkylations (e.g., O^6-alkylG and O^4-alkylT) are highly mutagenic and genotoxic, whereas *N*-alkylations (e.g., *N*3-alkylA and *N*1-alkylA) are cytotoxic, but less mutagenic.[222]

The cellular responses to ICLs, in particular DNA repair, are of importance not only for maintaining genome stability, but also for tumor resistance against chemotherapeutic agents. DNA repair is essential for maintaining the integrity of genomic DNA, and their loss is associated with cancer predisposition syndromes.[337] The current applications of ICL repair are mainly explored for the use of ICL-inducing chemicals in biochemical or genetic analysis of cells and cell lines as well as their anticancer chemotherapeutics.[316, 338] This is an efficient strategy for the study of specific DNA damage induced by bifunctional alkylating agents. Several classes of bifunctional alkylating agents have been employed in cancer therapies, such as cisplatin (with intrastrand cross-links as main adduct around 5–8% ICL),[319, 339-341] bis(2-chloroethyl)methylamine and nitrogen mustards (1–5% ICLs),[246, 319, 342] bis(2-chloroethyl)nitrosurea (BCNU or carmustine, around 8% ICLs),[343] Mitomycin C (MMC, 5–14% ICLs),[344] and Pyrrolo[2,1-*c*][1,4]benzodiazepines (PBD, 440-fold higher ICL formation activity than the nitrogen mustards).[345, 346]

ICL repair mechanisms are more complicated compared to those of mono-alkylated damage. An ICL represents a formidable block to the DNA replication machinery and requires a combination of Fanconi anemia (FA) repair, NER, translesion synthesis (TLS), and HR repair for efficient repair.[148, 319, 347] The repair of ICL remains an active field of study while genetic studies indicated that various classes of proteins are involved

in this biological process.[348-350] On the other hand, the study of DNA repair pathways may attribute to the therapeutic effects of alkylating agents (particularly bifunctional alkylating agents), therefore, characterization of the repair pathways is essential for developing new treatments.[222]

1.5 Synthesis of ICL oligonucleotides for repair studies

DNA, which carries genetic information, is subjected to various chemical reactions resulting in damage within the cell. The chemical reactions occurring to DNA may generate lesions within DNA duplexes, which interferes with the bioactivities of DNA and induces mutations in the DNA sequences. If left unrepaired and allowed to accumulate, these damages will lead to carcinogenesis and cell death.

ICL lesions are toxic and result in havoc in the damaged cells as stated above. The repair of this category of lesions to restore genomic integrity is more complicated, and understanding the mechanism(s) involved continues to be challenging, particularly in mammalian cells. The processing of ICL, introduced by bifunctional alkylating drugs employed during chemotherapy against diseases such as cancers, can reduce the efficacy of this group of drugs. A better understanding of the relationship between ICL and DNA repair might enable the development of improved therapeutic agents. The pursuit of

understanding the repair of interstrand cross-links by repair systems has necessitated the synthesis of sufficient quantities of such damaged DNA.[351]

Formerly, DNA from bacteria and bacteriophages and DNA fragments treated with bifunctional alkylating agents (i.e. to form ICLs) were used in biochemical studies. There are a variety of nucleophilic sites within DNA duplexes, which leads to a wide spectrum of alkylated adducts. Employing bifunctional alkylating agents, monoadducts, intrastrand and ICL adducts can usually be generated. For example, the approach to generate ICL DNA duplexes by treatment of DNA with nitrogen mustards, cisplatin, BCNU or mitomycin C, is very inefficient, yielding only about 1–5% of ICL adducts, with monoadducts and intrastrand cross-links making up the majority of products.[247, 352] Thus, it is very challenging to isolate the expected DNA sequences containing the desired ICL lesion(s).

Another strategy for the preparation of ICL DNA involves the solution and solid-phase synthesis of modified oligonucleotides. Oligonucleotides containing modified moieties are commonly used for a wide range of biochemical and biophysical studies. Some modified oligonucleotides have been investigated as therapeutic and diagnostic agents, such as antisense oligonucleotides to block gene expression by binding with complementary sequences of target mRNA. They are also employed as molecular probes to better understand the interaction between proteins and nucleic acids at molecular level,

which in fact is responsible for all aspects of cellular or viral gene expression.[351, 353-358] A combined approach (solution synthesis and solid-phase synthesis) enables the site specific incorporation of a modification at a defined position within any sequence context.

This method for the preparation of modified DNA oligonucleotides normally consists of two synthetic parts. A phosphoramidite of a modified nucleoside is first prepared in solution followed by incorporation into an oligonucleotide by a solid-phase DNA synthesizer.

Solid-phase synthesis (SPS), invented initially for peptide synthesis in the 1960s by Bruce Merrifield, is a method in which target compounds are synthesized step-by-step on a solid support in a reactant solution. This approach has also been applied for the preparation of other molecules including DNA and RNA.[359] SPS is carried out on a solid support held between filters, in columns that enable all reagents and solvents to pass through freely. Compared to solution synthesis, SPS has a number of advantages.

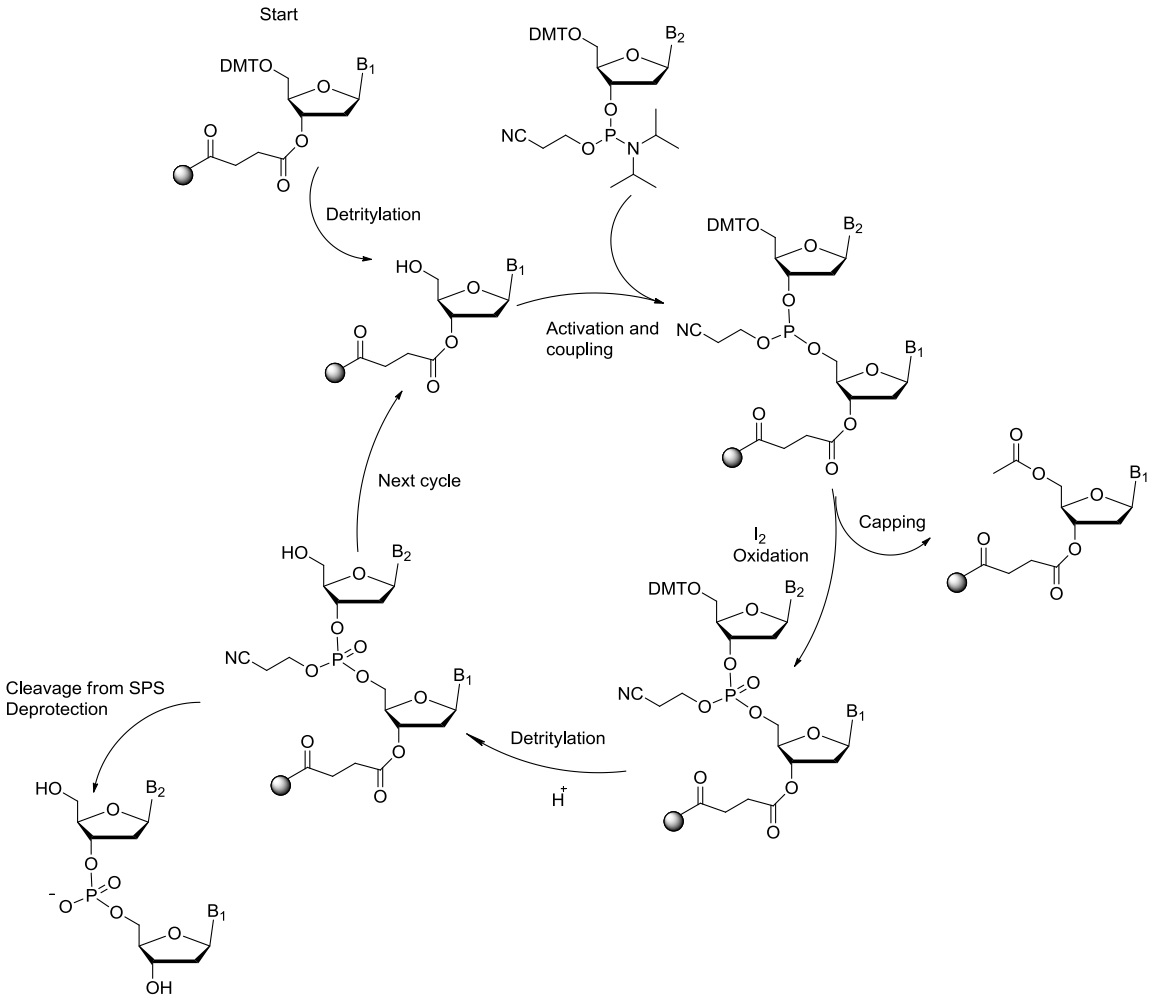
a) Excess reagents are used to drive reactions quickly to completion.

b) Impurities and excess reagents are easily washed away, and no purification is required after each step.

c) The process can be operated automatically and is suitable for parallel synthesis.

Resins are widely employed as solid support. They are the insoluble particles, typically 50-200 μm in diameter, to which the oligonucleotide is bound during synthesis. Many types of solid support have been used, but controlled pore glass (CPG) and polystyrene have proved to be the most useful.

Opposite to the 5′- to 3′-direction of DNA biosynthesis during the process of DNA replication, the synthesis of oligonucleotides on the solid-phase synthesizer usually proceeds in the 3′- to 5′-direction. One nucleoside is added per SPS cycle while the phosphoramidite DNA synthesis cycle consists of a series of steps outlined in **Scheme 1-11**.[359-361]



Scheme 1-11. SPS cycle for oligonucleotides

As shown above, the first 5'-*O*-DMT-nucleoside (4,4′-dimethoxytrityl) is attached to the solid-support through a linker. The DMT protective group is sensitive to acids, and can be cleaved from the 5'-OH to afford a new reaction site. This step is called detritylation, and its mechanism is shown in **Figure 1-23**.

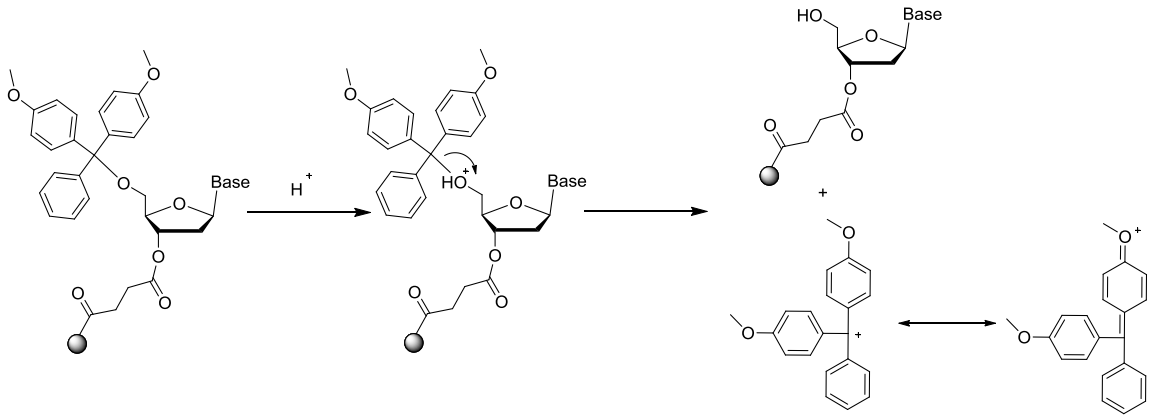


Figure 1-23. Mechanism of acid-catalyzed detritylation

Following detritylation, the support-bound nucleoside is ready to react with a nucleoside phosphoramidite. The nucleoside phosphoramidite, which is activated with tetrazole, enters the reaction cycle. With the aid of the soft base diisopropylamine, the phosphoramidite is attacked by the 5′-hydroxyl group of the support-bound nucleoside to form a support-bound phosphite triester (**Figure 1-24**).

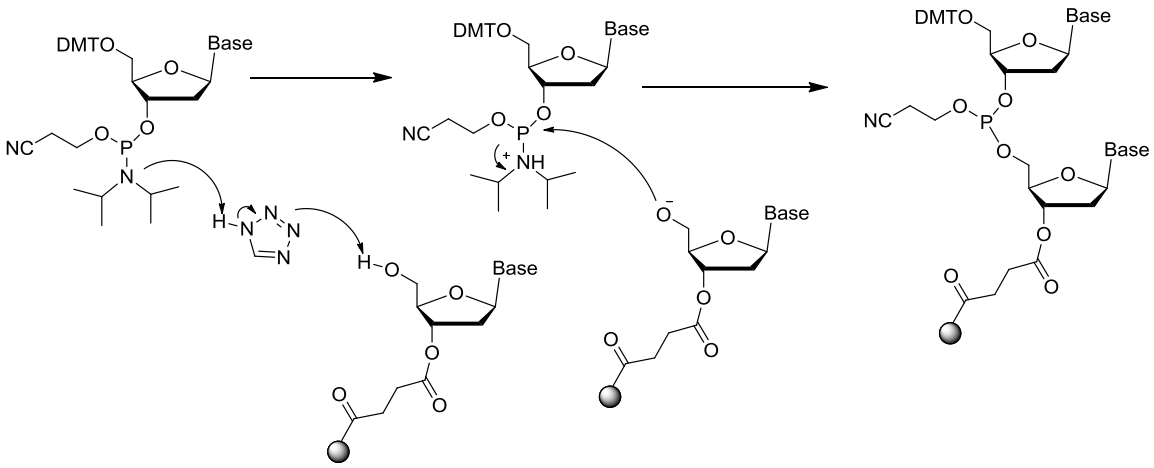


Figure 1-24. Mechanism of coupling reaction of nucleoside phosphoramidite

It is inevitable that after each coupling step some unreacted 5′-hydroxyl groups remain on the resin-bound oligonucleotide as a result of failure of the phosphoramidite to couple. If these are present, their 5′-hydroxyl groups would compete in the next coupling step, reacting with the incoming phosphoramidite. One extra step is needed to block the unreacted 5′-hydroxyl groups with capping reagents. These capped oligomers will no longer react during subsequent coupling steps in the DNA synthesis cycle, resulting in truncated sequences.

After the coupling step, the formed phosphite-triester is oxidized with iodine to generate a stable phosphate-triester (**Figure 1-25**).

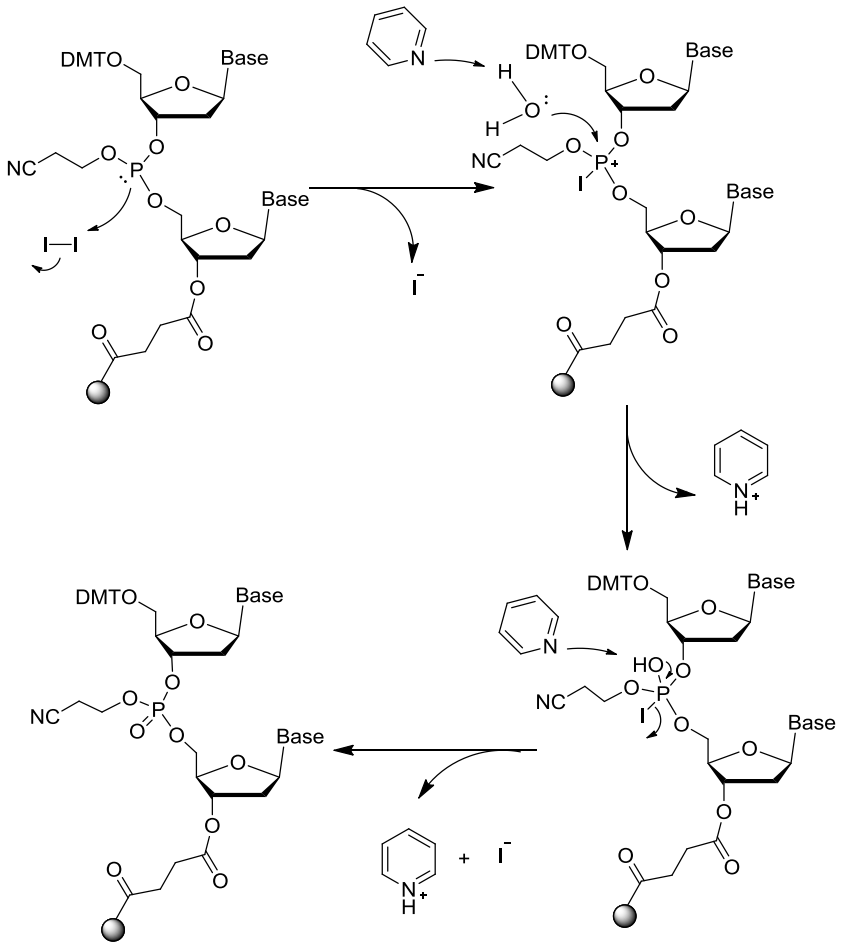


Figure 1-25. Mechanism of oxidation

The DMT protective group at the 5′-end of the resin-bound DNA chain is removed with acid to free a new primary hydroxyl group, which enters the next reaction cycle.

The reaction cycle is repeated with the appropriate nucleoside phosphoramidites (including the modified nucleoside phosphoramidite) until the desired oligonucleotide is produced on the solid support. In contrast to the synthesis of oligonucleotides with

standard DNA nucleobases, oligonucleotide synthesis with specialized monomers often requires modified synthetic or deprotection conditions.[353]

A cleavage procedure is performed to remove the synthesized oligonucleotide from the solid support as well as the protective groups on the nucleobases and 2-cyanoethyl group on the phosphotriester backbone (the deprotection of phosphodiester backbone shown in **Figure 1-26**).

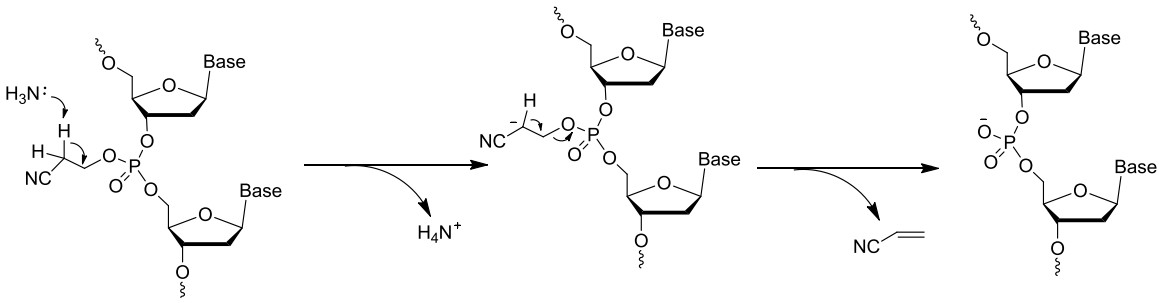


Figure 1-26. Cyanoethyl phosphodiester deprotection with ammonia

Depending on the efficiency of synthesis and wash steps, the amount of failure sequences may vary, and as a result a mixture of oligonucleotides including the expected sequence and failure sequences are generated. Purification is required to separate the desired oligonucleotide from such failure sequences. The most common methods for purification

are high-performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE).

A combination of solution synthesis and solid-phase synthesis enables the introduction of modified residues into DNA and the preparation of ICL DNA duplexes. The synthesis of ICL DNA duplexes will be introduced with some examples in **Chapter 2**.

ICL lesions can be introduced either in form of a cross-linked dimer or as ICL precursors that can undergo a specific coupling reaction after incorporation into complementary strands and annealing.[355, 362]

1.6 Purification and characterization methods of oligonucleotides

The lengths of oligonucleotides produced by automated solid-phase synthetic process typically range from 10 to 80 nucleotides, and the synthesis scales vary from nanograms to kilograms, depending on the application. Although the synthesis efficiencies are very high, the overall yields of the products decrease as the impurities accumulate with the increasing number of synthetic cycles. Truncated oligonucleotide sequences and other process-related modifications and impurities are often artifacts of synthesis. Coupling and capping failures may lead to a series of deletion sequences, which constitutes the major impurities in solid-phase synthesis. Additional impurities may result from incomplete detritylation and incomplete oxidation as well as from the starting materials and post-

synthesis processing. It is important to analyze synthetic products to determine whether the purity is sufficient or if further purification is required. In addition, the analysis of intermediates and final products is also necessary for quality control and design study. To obtain final products of good quality, the impurities need to be removed to ensure that the purity of synthetic oligonucleotides meets the requirements for the experiments to be performed.

Oligonucleotide purification is typically performed either by HPLC and PAGE. HPLC is an efficient method for the analysis and purification of synthetic oligonucleotides. It can be used for the separation of oligomers of similar size. The technique depends on the structure and length of the oligonucleotides, the presence or absence of the trityl group, and the nature of any modifications. The purification of synthetic oligonucleotides is usually challenging because the small differences in size, charge and hydrophobicity between the full-length product and impurities may lead to co-elution. HPLC systems typically consist of the following components: an injector, pumps for delivery of solvents through the column, an interchangeable HPLC column, a column oven, a solvent mixing system, and a detector (normally UV/visible). Reversed-phase (RP-HPLC) and strong anion-exchange (SAX-HPLC) are typically employed in this field. Following HPLC purification, gel filtration is typically needed to remove salts from oligonucleotides.

1.6.1 Reversed-phase HPLC

Crude oligonucleotides containing the desired products, failure sequences and other impurities may vary in hydrophobicity. Reversed-phase HPLC separates oligonucleotides and contaminants on the basis of differences in hydrophobicity. The mixture of oligonucleotides is injected onto a column of hydrocarbon chains (normally C_8 or C_{18}) which are chemically bound to a silica support of defined pore size, typically about 300 Å. Elution with a mobile phase such as aqueous ammonium acetate forces the oligonucleotide to interact strongly with the reversed-phase column. Further elution with a gradient of organic solvent such as acetonitrile in aqueous ammonium acetate causes the individual components in the mixture to enter the mobile phase, which becomes more hydrophobic as the percentage of acetonitrile increases. Molecules with a greater degree of hydrophobicity elute more slowly (for example if a DMT is retained on the desired oligonucleotide), and this leads to separation of the components in the mixture.

Although reversed-phase HPLC is very powerful and routinely employed for the purification of oligonucleotides, there are certain limitations. It still remains challenging to separate long oligonucleotides from those failure sequences of a similar length. For those composed of stable secondary structures (e.g. hairpin loops), HPLC separation may lead to failure due to elution as a broad peak or even a series of peaks. Higher

temperature (60 °C) is needed to run HPLC separation by temporarily denaturing the secondary structures.

1.6.2 Anion-exchange HPLC

Anion-exchange HPLC separates oligonucleotides according to the charge differences. Each oligonucleotide is a polyanion and has a particular net negative charge based on the number of phosphodiester groups in the molecule. The anion-exchange purification method is performed with a salt-gradient elution on a quaternary ammonium stationary phase column or a similar structure (cationic). Separation of the crude mixture is accomplished by slowly increasing the ionic strength of the mobile phase to weaken the ionic interactions between the oligonucleotide (polyanion) and the stationary phase (cationic amine). The longer oligonucleotides with higher charge elute later than the shorter ones. This technique is effective in separating oligonucleotides of 5 to 50 bases in length from failure sequences at smaller quantities, and can be coupled with purification by RP-HPLC.

Similar to RP-HPLC, anion-exchange HPLC is also limited by length (usually up to 40mers). The separation of longer oligonucleotides usually results in lower resolution on the anion-exchange HPLC column, and lower purity of the target oligonucleotides.

1.6.3 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is a technique that separate molecules based on their electrical charge and hydrodynamic properties, which are a function of chain length for DNA and RNA. PAGE purification can be used for oligonucleotides, up to nucleotide lengths of 100 and longer. Visualization using UV detection or intercalating staining techniques (STAINS-ALL™) can be used on oligonucleotides to enable a more sensitive level of detection with an increasing length of the oligonucleotide. Preparative PAGE is an alternative to HPLC separation however it is not commonly used due to lower recovery, lower mass loading as well as being more time-consuming and labor intensive.

As with preparative HPLC, resolution decreases when performing preparative PAGE highlighting the need for verification of the purity prior to further steps.

1.7 Biophysical Analysis of Oligonucleotides

1.7.1 Mass Spectrometry

Mass spectrometry (MS) is employed for the measurement of the mass-to-charge ratio of molecules. Molecules must be ionized before their introduction into the vacuum system of the mass spectrometer. A number of ionization methods (i.e. electron impact

ionization, chemical impact ionization, fast atom bombardment and atmospheric pressure chemical ionization) have been explored and used for MS analysis. Compared with gaseous or volatile samples, thermally labile analytes require so-called soft ionization methods such as field desorption and electrospray ionization for the production of ionized particles with little or no fragment-ion content.[363-365]

Among the existing soft ionization methods, electrospray ionization (ESI) is widely performed for accurate mass measurement, particularly for thermally labile, high molecular mass substances (ie. proteins, oligonucleotides, synthetic polymers, etc.).[366-371] In this ionization method, a solution is nebulized under atmospheric pressure and exposed to a high electrical field which creates a charge on the surface of the droplet. Droplets rapidly become much smaller through vaporization of solvent when moving towards an analyzer.[371-377]

1.7.2 Enzymatic Digests

Exonucleases are a category of enzymes that degrade DNA or RNA to generate distinct cleavage products useful for the study of oligonucleotide sequences.[378] There are two classes of exonucleolytic enzymes, one specifically attacking at 3'-hydroxyl terminus (3'→5', 3'-exonuclease) and another at the 5'-phosphoryl terminus (5'→3', 5'-exonuclease).[379]

Snake venom phosphodiesterase, also called snake venom exonuclease, is a 3'-

exonuclease and attacks both DNA and RNA to produce 5' monophosphate esters.[380, 381] Calf intestinal phosphatase is a type of alkaline phosphatase that catalyzes the hydrolysis of 5′-phosphate groups from DNA, RNA, and ribo- and deoxyribonucleoside triphosphates.[382-385] The combination of snake venom phosphodiesterase and calf intestinal phosphatase results in digestion of an oligonucleotide to the nucleosides. This combination of enzymes is useful in determining the nucleoside composition of synthesized ICL DNA duplexes as the percentage of four native nucleosides (2'-deoxycytidine, 2'-deoxyadenosine, 2'-deoxyguanosine and thymidine) and cross-linked nucleoside when analyzed by RP HPLC.

1.7.3 UV Thermal Denaturation Studies

The stability of DNA duplexes depends on a number of factors such as hydrogen bonding and base stacking. The process to separate double-stranded helical DNA duplexes into single strand is referred to as denaturation. The denaturation process is generally reversible (the reverse process is known as renaturation), and is commonly caused by heating while some other factors, i.e. pH change, lowering ionic strength by decreasing the dielectric constant of the aqueous medium (with alcohol, ketones, amides, urea et al.) can also lead to denaturation of DNA duplexes.[386-390]

UV thermal denaturation is normally employed for this analysis. It was found that the absorption spectrum of native DNA was much lower than that of calculated constituent nucleotides at the characteristic ultraviolet wavelength near 260 nm. Heating double-strand nucleic acids may lead to the formation of single-stranded oligonucleotides by breaking hydrogen bonds and disrupting the ordered base stacking. Thermal denaturation of double-stranded duplexes usually occurs following a sigmoidal curve, monitoring absorbance at 260 nm with a relatively smooth increase in UV absorbance as the double strands unwind to single strands (hyperchromic effect).[391-393] The mid-point of this transition is measured as melting temperature (T_m) at which 50% of DNA is denatured into random-coil state. The T_m is dependent on a number of factors, i.e. the length and sequence of the DNA molecule and others.[394-401]

1.7.4 Circular Dichroism (CD) Spectroscopy

Circular dichroism (CD) has been employed for structural study of optically active molecules, with numerous examples in the literature.[402-408] CD spectroscopy has applications for the study of structure and interaction of biomacromolecules such as proteins and nucleic acids.[409-422]

Compared to nuclear magnetic resonance (NMR) and X-ray crystallography which may genenerate structural information of more direct and detailed residue-specificity, CD spectroscopy provides alternative measurements which can be performed with small amount of substrates in physiological buffers while monitoring structural changes adapting to environmental conditions (i.e. pH, temperature, ionic strength and interaction of other molecules).[423] CD spectroscopy is now widely used to provide structural information of nucleic acids, particually for the study of secondary structure of nucleic acids ranging from single-stranded to triplex and quadruplex forms.[418, 424]

In this thesis, CD spectroscopy is conducted with the modified DNA duplexes. This can provide the conformational information for the structural intergrity due to the formation of ICL lesions.

Chapter 2. Project Outline

2.1 Background and Significance of ICL Oligonucleotide Synthesis

2.1.1 Current State of Knowledge

There are a number of bifunctional alkylating agents which react with DNA including dialdehydes that are produced in the cell and compounds that have both been employed as therapeutics (i.e., BCNU and mechlorethamine) or studied in clinical trials (i.e. hepsulfam) (structure shown in **Figure 2-1**).[161, 189, 235, 317, 333, 425] Treatment of DNA duplexes with these bifunctional alkylating agents often results in a broad spectrum of alkylated DNA products, with only a small percentage of ICL duplex produced. Of all the nucleophilic sites in DNA, the *N*7-position of guanine is the most reactive with electrophiles.[144, 189, 426-430] Hundreds of DNA adducts have been identified addressing a number of issues such as the relationships between DNA adducts produced by exposure to chemical agents with mutagenesis and other biological endpoints. The covalent binding to DNA and the ability to form *N*7-guanine adducts has been considered as evidence of genotoxicity for several drugs and chemicals.

(1a) (1b) (1c)

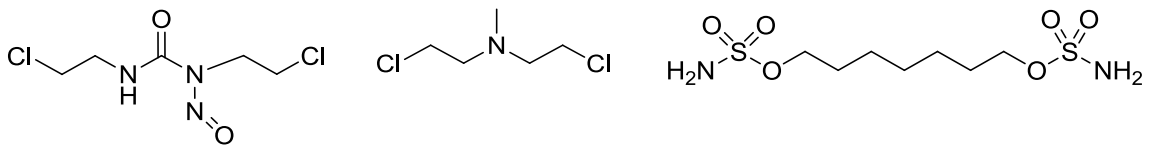


Figure 2-1. The molecular structures of BCNU (1a), mechlorethamine (1b) and hepsulfam (1c)

To counteract the damage of ICLs, cells have developed mechanisms to repair these lesions, and recover the integrity of their intact sequences. The failure to repair such lesions may cause inherited disorders. In contrast, the repair of ICLs in tumor cells leads to resistance against anti-tumor agents, which are introduced in cells to generate ICL lesions during chemotherapy. Therefore better understanding of the relationship between ICL damage and cell repair should not only significantly contribute to our understanding of the molecular basis of human disease but also to the mechanisms of resistance of tumor cells to chemotherapeutic agents. This knowledge will facilitate the development of new targets for tumor chemotherapy.

Although significant progress has been made in studying various DNA repair pathways, the mechanisms underlying the repair of ICLs remain poorly understood, particularly in mammalian cells. As stated above, employing bifunctional alkylating agents adducts

containing alkylation at the *N*7 of guanine are usually generated as the major products. The investigation of the role these adducts plays is important in investigating DNA repair. The main obstacle in studying the mutagenic potency of *N*7-guanine adducts is their chemical instability which has prevented systematic investigation of the lesions in site-directed mutagenesis studies. The instability of *N*7-guanine adducts is created by the formal placement of an additional positive charge on the guanine ring system. The alkylated guanines undergo a further reaction resulting in cleavage of the *N*-glycosyl bond (**Figure 2-2**). This depurination creates an abasic site in the DNA and in the case of bifunctional alkylating agents, effectively removes the ICL formed. Alternatively, the imidazole ring of the alkylated guanine can undergo hydrolysis to produce a FAPY derivative, which is relatively resistant to further chemical reaction (**Figure 2-2**).[132, 185, 189, 193, 235, 322, 336, 431-435]

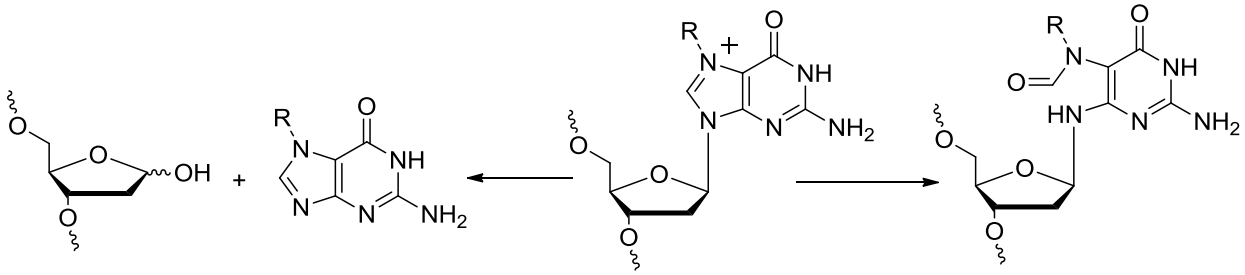
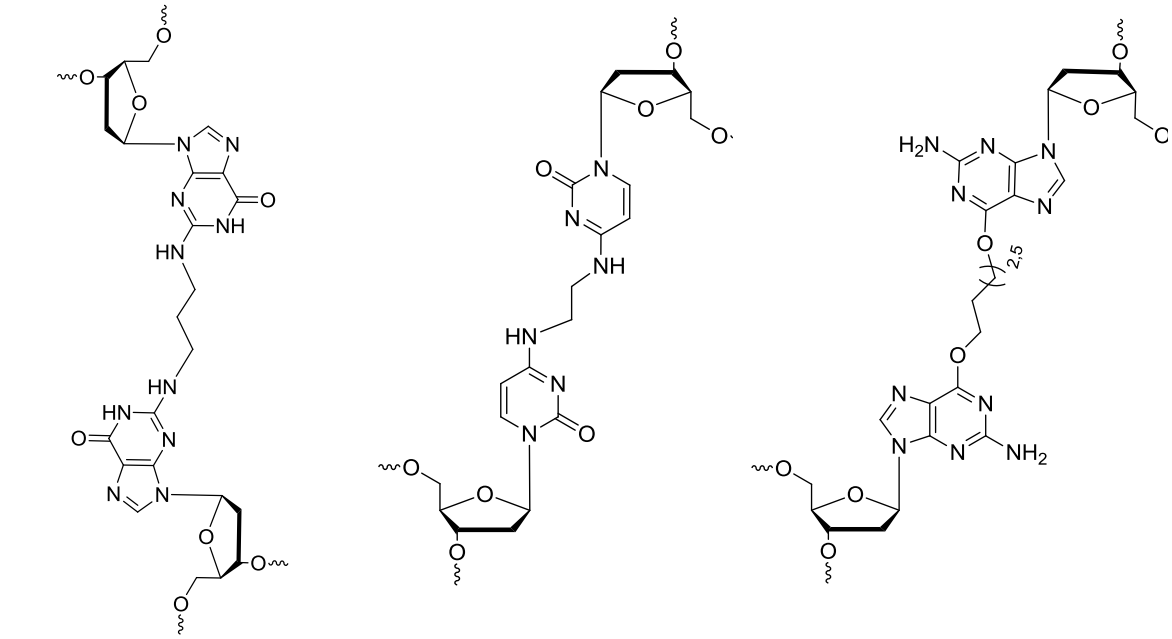


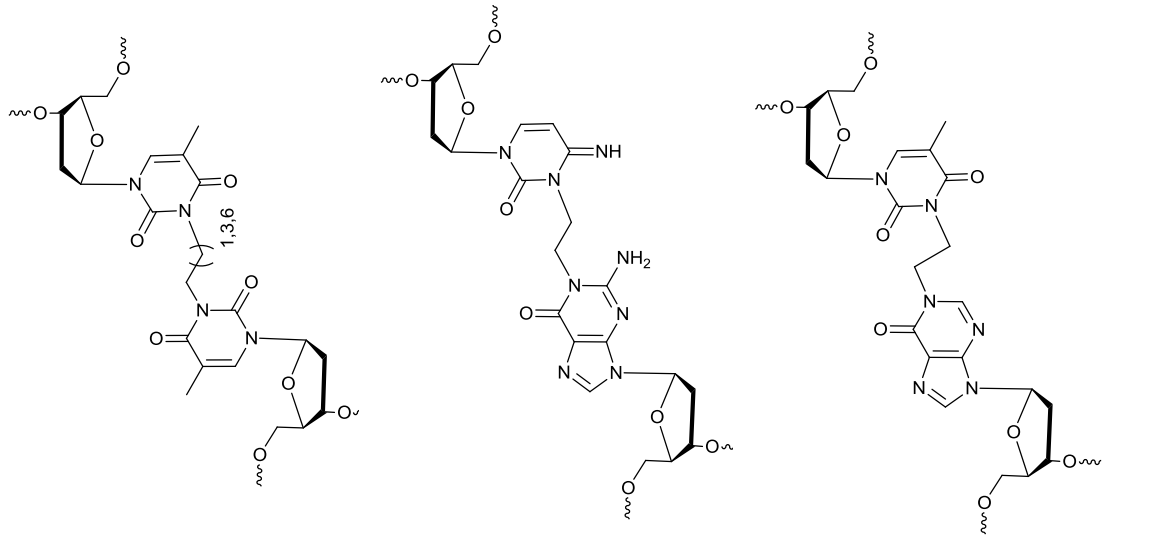
Figure 2-2. Potential products formed by alkylation of the *N*7 atom of 2'-deoxyguanosine; depurination to form abasic sites (left) or FAPY (right).

2.1.2 Examples of synthesized alkylene linked ICL oligonucleotides

Understanding how ICL are repaired has been and continues to be a challenge. A better understanding of the mechanism(s) involved is not only of academic interest but may be relevant to cancer chemotherapy as well. Oligonucleotides functionalized by conjugation with a variety of molecules have been employed as substrates for various studies for a few decades with aliphatic linkers frequently used for tethering these modifications. The introduction of aliphatic linkers to synthesize ICL oligonucleotides via chemical synthesis has been performed by several groups with numerous examples including: N^2-dG-alkylene-dG-N^2 ICL (Harris, **Figure 2-3a**),[436-438] N^4-dC-alkylene-dC-N^4 ICL (Miller, **Figure 2-3b**),[439-442] O^6-dG-alkylene-dG-O^6 ICL (Wilds, **Figure 2-3c**),[443-445] *N*3-T-alkylene-T-*N*3 (Miller and Wilds, **Figure 2-3d**),[446] *N*1-dG-ethylene-dC-*N*3 (mimic BCNU lesion, Luedtke, **Figure 2-3e**),[447] and *N*1-dI-ethylene-T-*N*3 (mimic 1,3-bis-(2-chloroethyl)-1-nitrosourea, Wilds, **Figure 2-3f**) [351] for DNA repair studies.



a **b** **c**



e **e** **f**

Figure 2-3. Chemically synthesized ICL duplexes from the Harris, Miller, Luedtke and Wilds' groups

Recently, Yasuo Komatsu et al. applied amino linkers containing a carbamate structure for the preparation of covalent cross-linking of apurinic/apyrimidinic sites (AP pair) on opposite positions of complementary strands (**Figure 2-4**). Two aldehyde groups of the AP pair are linked with a bifunctional cross-linker having two aminooxyacetyl groups.[448, 449]

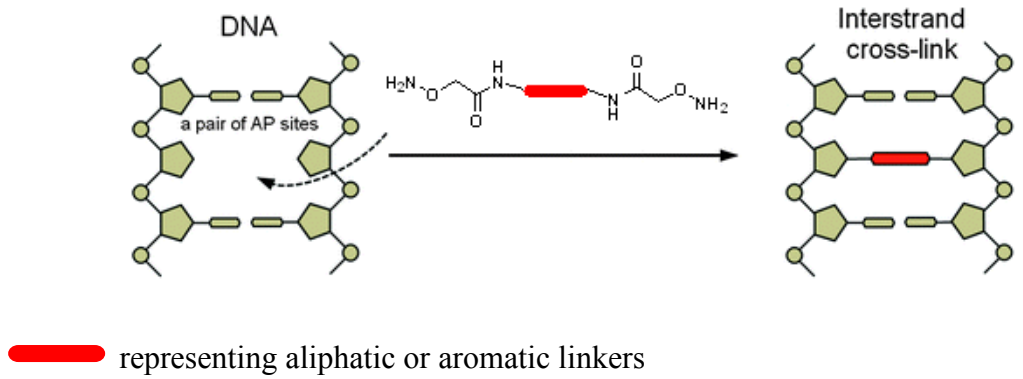


Figure 2-4. ICL reaction of an AP pair

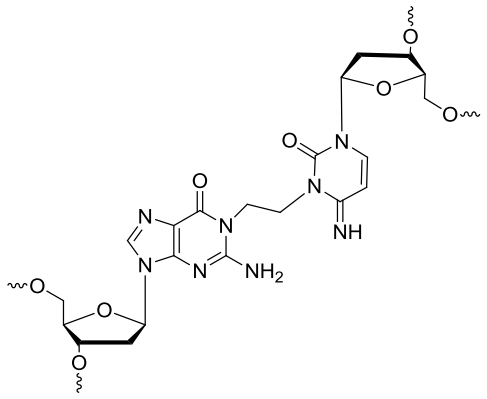
The development of chemically stable substrates that mimic the lesions formed by clinically used bifunctional alkylating agents is of scientific interest.

2.2 Statement of the Problem

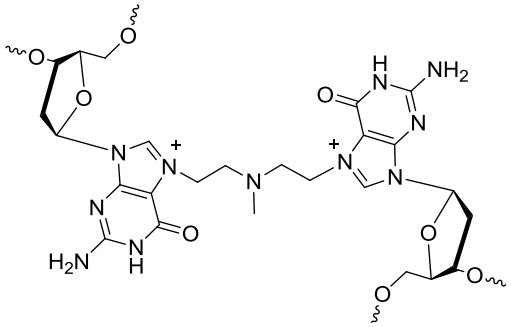
Cellular DNA is susceptible to damage by endogenous and environmental agents that may cause modifications such as cross-links. This can either occur on the same strand (intrastrand cross-link) or on the opposite strands of DNA (interstrand cross-link). Interstrand cross-links (ICL) in DNA exert significant biological effects by preventing replication and transcription, which cause cell death if the cross-link is not repaired.[235, 317, 350, 425, 450, 451]

ICL can also be introduced in DNA as a consequence of the action of cancer chemotherapeutic agents that kill cancer cells. Alkylating drugs such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU or carmustine in **Figure 2-1a**) and mechlorethamine (**Figure 2-1b**) react to form ICL at different positions in DNA duplexes (**Figure 2-3** and **Figure 2-5**).[161, 235, 425]

(a)



(c)



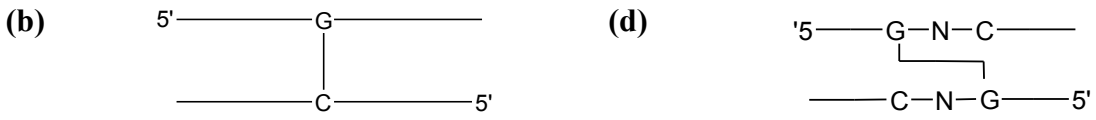


Figure 2-5. Examples of ICL linkages introduced by alkylating drugs and major sequence motifs (a/b): *N*1-dG-alkylene-*N*3-dC; (c/d): *N*7-dG-alkylene-*N*7-dG).

Development of tumors, resistant to these agents is a factor in the lack of response in some patients, with removal of ICL believed to play a role in resistance. These lesions are eliminated from DNA by a mechanism involving excision repair and recombination (for example in *E. coli* and in yeast).[450, 451] In eukaryotic cells, the precise role of excision repair in eliminating cross-links is not yet clearly understood.[235, 317]

The investigation of ICL removal by repair enzymes requires access to substrates containing this damage. One way in which ICL containing DNA can be prepared involves direct treatment of DNA with bi-functional alkylating agents. However, this approach is not optimal for practical reasons. Alkylating agents lack target specificity and can react with numerous nucleophilic sites within DNA. As a result, a huge variety of alkylated adducts will be formed during this process.[235] As stated in **Chapter 1**, the preference of alkylating sites depends on several factors when they are employed for the introduction ICL along DNA complementary strands. Among all the discovered and developed bifunctional alkylating agents, the modified adducts containing *N*7-alkylated

guanine are predominant due to the higher nucleophilic reactivity at this site.[116, 194-201] Even though less mutagenic or cytotoxic, the main ICL adducts containing *N*7-alkylated guanine exhibit important biological properties (i.e. hepsulfam, NSC 3296801 shown in **Figure 2-6**).[333] Due to the chemical instability some studies of *N*7-guanine adducts are challenging, in particular structural and DNA repair experiments.

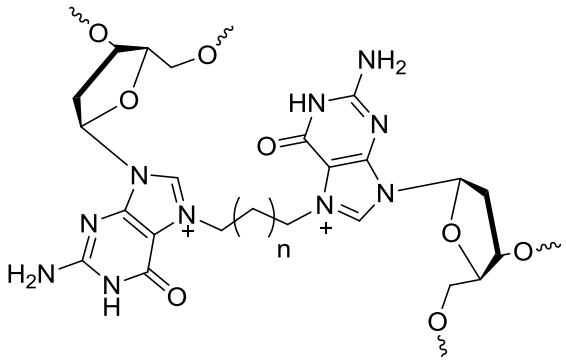


Figure 2-6. Structure of *N*7-dG-alkylene-dG-*N*7 ICL (n= 2~5)

Another approach to investigate ICL repair involves the use of chemically synthesized substrates of well defined structure. Such substrates are challenging to prepare in quantities sufficient for biochemical and structural studies. Employing a combination of solution and solid-phase synthesis, ICL DNA that are mimics of clinically relevant lesions formed during chemotherapy can be produced.

Given the chemical instability of the *N*7-adducts of 2'-deoxyguanosine, we have had interest in the preparation of ICL containing the heterocycle 7-deazaguanine, whose C7 atoms in complementary DNA strands are attached by an alkylene linker. One major goal of this thesis is the development of procedures to prepare 7-deazaguanine and ICL linking the C7 atoms to address some problems outlined above. 7-Deazaguanine (with the major tautomer shown in **Figure 2-7**), is an ideal analog of guanine. In comparison to guanine, 7-deazaguanine lacks the *N*7 atom, and thus loses a hydrogen-bond acceptor. As such, a Watson-Crick base pair may be formed between 7-deazaguanine and cytosine similar to guanine and cytosine (see **Figure 2-8)** while there is no Hoogsteen base pair (see **Figure 2-9**).[452]

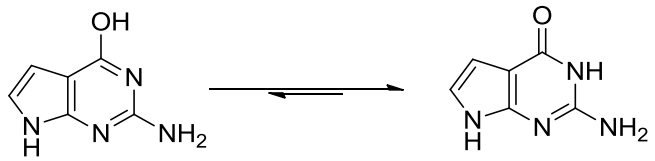


Figure 2-7. Tautomeric equilibrium of 7-deazagaunine

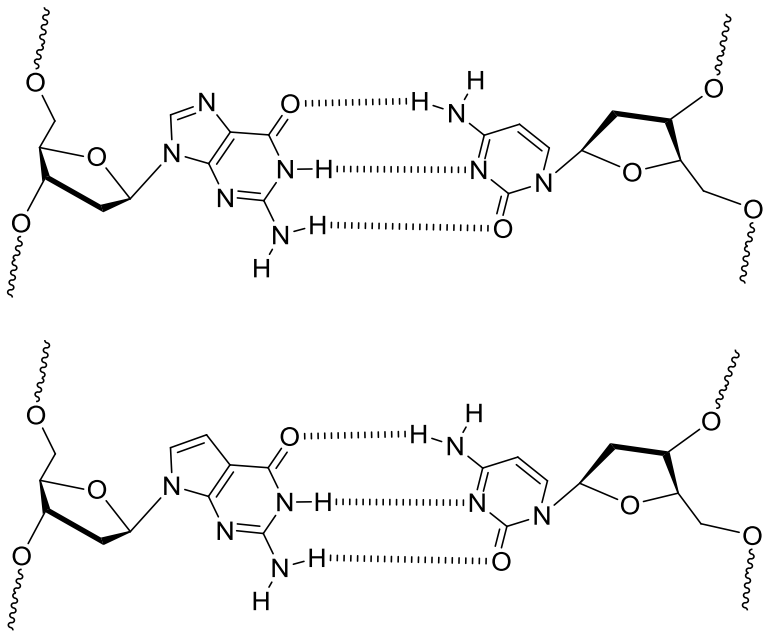
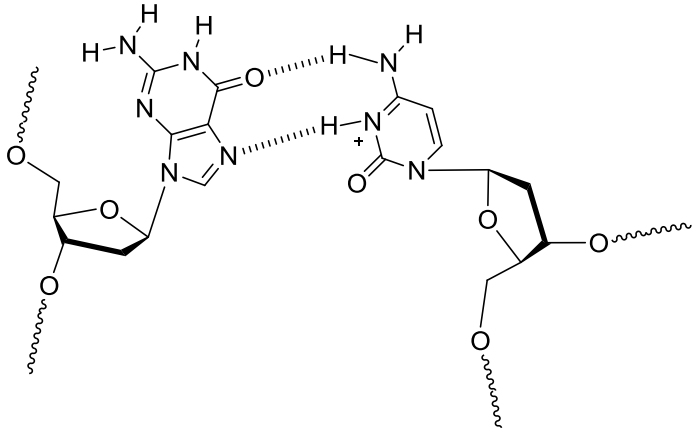


Figure 2-8. Watson-Crick base pairs in G:C (top) and deazaG:C (bottom)



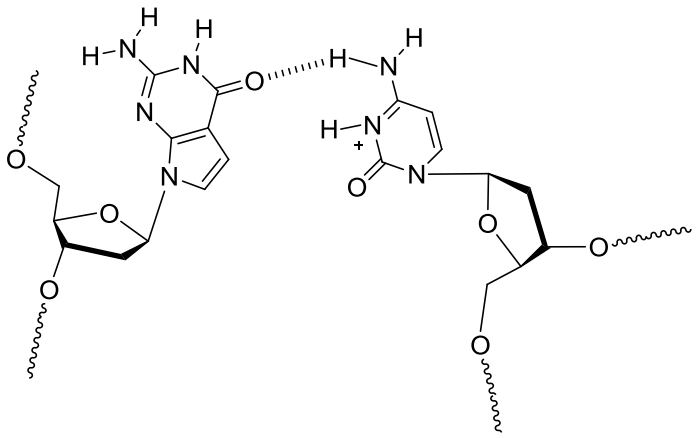


Figure 2-9. Hoogsteen base pair in G:C (top) while no such base pair is possible in deazaG:C (bottom)

The five-member heterocyclic pyrroles consist of six π electrons which can delocalize over the five ring atoms, and the delocalization of *p*-orbital electrons of nitrogen affords an electron-rich system. By comparison, the six-member heterocyclic pyrimidine is electron-poor (deficient) due to the two nitrogens which act as electon-withdrawing groups. According to the structure of bicyclic 7-deazaguanine, a pyrimidine nucleus is fused to a pyrrole as an electron- dragging-pushing aromatic system. The fused structure shows the typical reactivity with reduced electron density in the five- member ring and increased electron density in the six-membered ring. A large range of pharmacological properties are attributed to the angular as well as linear polycyclic condensed pyrrolo-pyrimidine moieties.[453-458]

We proposed to prepare via organic synthesis a chemically stable ICL that mimics the alkylene lesions formed in duplex DNA as a result of treatment with alkylating drugs such as mechlorethamine and hepsulfam.[333, 425] These structural analogs would link the C7 atoms of two 7-deaza-2'-deoxyguanosine (C7-dG) nucleosides with an alkylene linker and will be incorporated into DNA by solid-phase synthesis (**Figure 2-10**).

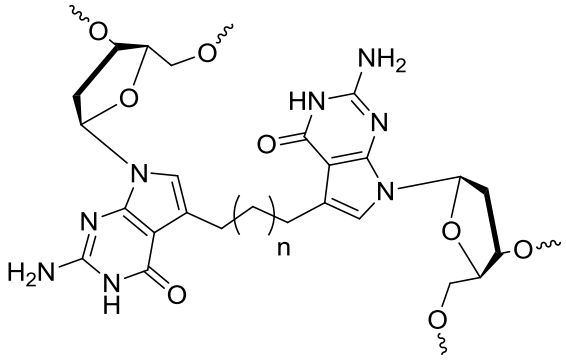


Figure 2-10. The *C*7-dG-alkylene-dG-*C*7 ICL oligonucleotides (n= 2~5)

The similarity between guanine and 7-deazaguanine made the attempt to syntheize this ICL of interest (see **Figure 2-6** and **Figure 2-10**). Compared with previously synthesized ICL duplexes by our group, these proposed substrates containing cross-linked 7-deazaguanosine would have the following merits.

1) The molecular structures are more similar to ICL formed by bi-functional alkylating agents.

2) The use of 7-deazaguanine should stabilize the introduction of alkylene linkers at this site in DNA.

3) ICL oligonucleotides containing alkylene-linked 7-deazagaunine should not affect Watson-Crick base pairs between the two complementary strands, similar to the Watson-Crick base pairs of the cross-linked guanine and cytosine (shown in **Figure 2-11**).

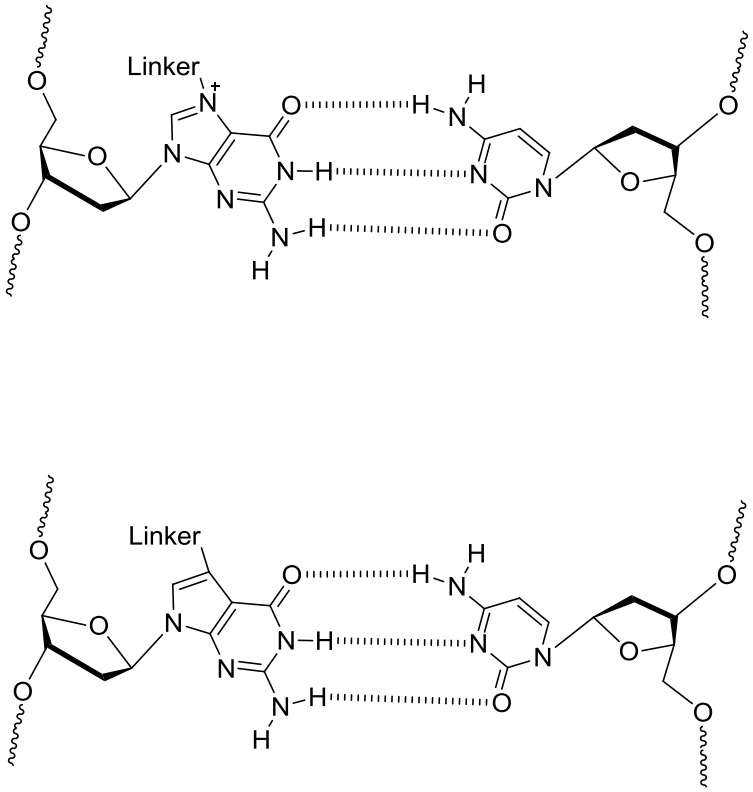
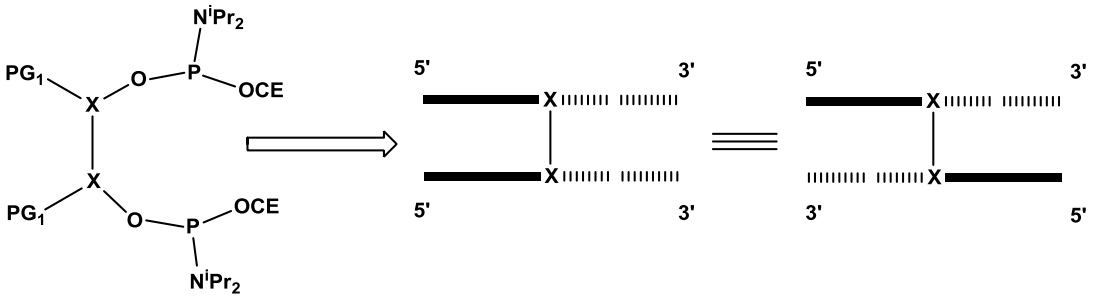


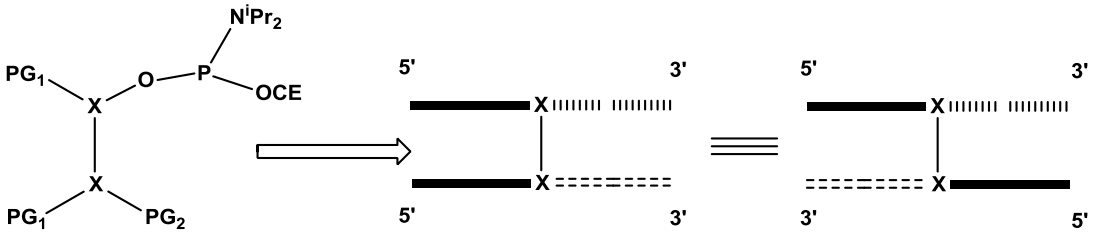
Figure 2-11. Watson-Crick base pairs between cross-linked G:C (top) and cross-linked deazaG:C (bottom)

An additional goal of this thesis is to explore the use of various protective groups around the cross-linked nucleoside dimers (which are converted to phosphoramidites and then used in solid-phase synthesis) to enable the preparation of oligonucleotides containing different nucleotide compositions around the cross-linked site. This has been a limitation in our lab in preparing various ICL containing oligonucleotides. To date, due to the protective groups that have been explored, nucleotide sequences around the cross-linked sites are either partly or completely symmetrical (see **Figure 2-12a** and **2-12b**).

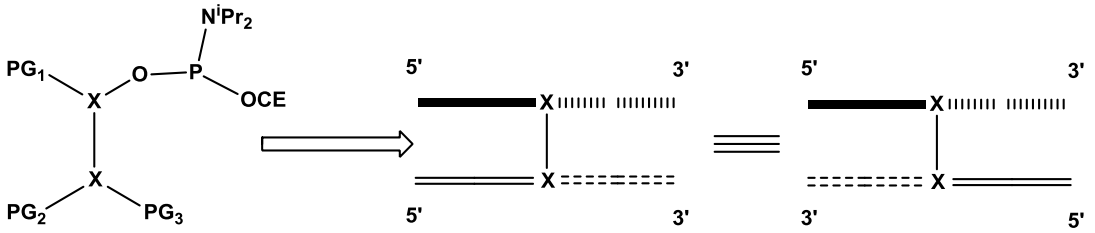
a)



b)



c)



Xs are the cross-linked nucleosides. PG_1, PG_2 and PG_3 represent different protective groups.

Figure 2-12. Synthetic strategies for preparation of symmetrical (**a**), partially asymmetrical (**b**) and completely asymmetrical sequences (**c**).

For the preparation of ICL via strategy (**c**), four compatible protective groups are needed to prepare a cross-linked tripartite phosphoramidite, which enables ICL oligonucleotides to be prepared on a solid-phase synthesizer (see **Figure 2-12c**). The three protective groups will be removed individually, followed by chain elongation by phosphoramidite coupling, from the support bound oligonucleotide until ICL synthesis is complete.

Ultimately, these strategies will employed to produce ICL DNA substrates that will be used in biophysical, structural and biochemical studies in order to examine their interaction with DNA repair enzymes. These results will provide us with insights as to whether these lesions are recognized and repaired, thus establishing a relationship between DNA repair with chemotherapeutic resistance to these drugs which could aid in

the development of more effective alkylating therapeutics that could induce lesions that may resist repair.

2.3 Specific Aims

The overall goals of this thesis are:

a) Optimize protective group and deprotection strategies to prepare ICL containing completely asymmetrical nucleotide sequences around the cross-linked site. These studies are performed using a system containing a *N*3-thymidine-butylene-*N*3-thymidine interstrand cross-linked DNA, given the challenges that were forseen in preparing alkylene linked 7-deaza-2'-deoxyguanosine ICL (**Chapter 3**).

b) To optimize methods to prepare the heterocycle 7-deazaguanine (**Chapter 4**).

c) To prepare dimers of 7-deaza-2'-deoxyguanosine linked by a C7-alkylene linker employing the Sonogashira reaction and Pd-catalyzed hydrogenation to prepare ICL containing cross-linked 7-deaza-2'-deoxyguanosines (**Chapter 5**).

Chapter 3. Preparation of *N*3-thymidine-butylene-*N*3-thymidine interstrand cross-linked DNA *via* an orthogonal deprotection strategy

3.1 Introduction

Cellular DNA, on exposure to various environmental and chemotherapeutic agents, can undergo damage with numerous modifications identified including interstrand cross-links (ICLs). ICLs threaten genomic and cellular integrity because they present formidable blocks to essential metabolic processes that require obligate strand separation (replication and transcription). Even though cells must efficiently detect and remove damage for survival, ICL-inducing agents continue to be employed as chemotherapeutic treatments for many cancers.[247, 459] However, resistance to ICL-inducing agents due in part to efficient DNA repair, can result in recurrent malignancies which are unresponsive to treatment,[460, 461] in addition to possibly increasing the risk of secondary cancers likely due to their mutagenic processing in normal cells.[462] Despite the substantial progress in the fields of ICL repair in bacteria and yeast which occur primarily *via* a combination of processes including nucleotide excision repair (NER), homologous recombination (HR), and translesion synthesis (TLS),[348, 463-465] a complete understanding of such repair in mammalian systems is still unclear.[350, 352, 466-471]

In mammalian cells, ICL repair is thought to proceed by the coordination of proteins from several pathways, including NER,[472, 473] base excision repair (BER),[474] mismatch repair (MMR),[475, 476] HR,[477, 478] TLS,[479] and proteins involved in Fanconi anemia (FA).[480] Therefore, efforts to better understand the cellular responses to and repair of ICL-inducing lesions in mammalian cells offers the potential to improve the efficacy of these drugs in cancer therapy.

One continuing approach to investigate the role that repair pathways play in removing ICL is to use chemically synthesized oligonucleotides which contain adducts that represent the lesions introduced by ICL-inducing agents. Differing strategies have been developed to obtain ICL-containing DNA. One avenue to ICL synthesis involves the incorporation of stable ICL forming precursors into single stranded DNA using standard DNA synthesis after which hybridization triggers ICL formation.[447, 481] We and others have established an approach (mono and bidirectional) to synthesize cross-linked nucleosides to directly introduce the ICL, which enables exact placement of the desired ICL, using a combination of solution and solid-phase synthesis.[272, 329, 351, 443, 445, 446, 482-485] However, several of these model ICL systems to date have contained symmetrical nucleotide sequence composition (either complete or partial) around the cross-linked site.

We have previously reported the synthesis of *N*3-thymidine-butylene-*N*3-thymidine (*N*3T-butylene-*N*3T) ICL duplexes with either partial or complete symmetry around the site of the cross-link using nucleoside dimers containing DMT and TBS protecting groups on the 5' and 3'-*O* functionalities using mono- and bis-phosphoramidite approaches.[446, 483] In order to accomplish the synthesis of asymmetric sequences it is imperative to design the dimer phosphoramidite to contain three different protective groups around the 5'- and 3'-*O* functionalities that are compatible with each other for selective removal without compromising the ICL lesion to introduce the cross-link into the desired ICL duplexes (shown in **Figure 3-1**).[486]

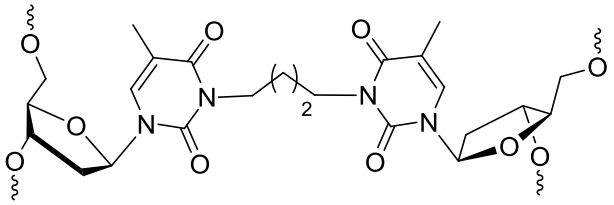
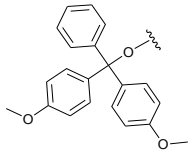
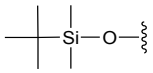
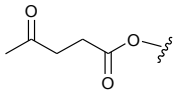
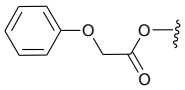
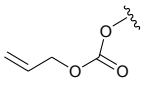


Figure 3-1. Chemical structure of the *N*3T-butylene-*N*3T ICL.

In order to construct completely asymmetrical nucleotide sequences around the ICL site, it was necessary to design the dimer phosphoramidites with protective groups that were compatible with each other (introduction as well as their removal) and more importantly their compatibility with solid-phase synthesis when being assembled within the desired

ICL duplexes.[486] The five functional groups that were investigated in the synthesis of the dimers and consequently the ICL duplexes were the DMT, TBS, Pac, Alloc and Lev groups. The cleavage conditions that allow for their selective removal are shown in **Table 3-1**.[443, 482, 487-496]

Table 3-1. Protective groups investigated for asymmetrical ICL synthesis

Protective group	Molecular structure	Cleavage conditions
DMT		Acid
TBS		TBAF
Lev		NH_2NH_2
Pac		Base
Alloc		Pd-catalyzed

Preliminary study for the compatibility between Pac and Lev protecting groups was performed with synthesized 3'-*O*-phenoxyacetyl-*5'*-*O*-levulinoyl-thymidine (compound **3-2**), and the result indicated these two groups were not suitable as protecting groups together in the synthesis of the cross-linked dimers. Considering the higher lability of Pac under basic conditions, four functional groups, DMT, TBS, Alloc and Lev were investigated in the synthesis of the dimers, which were employed in the preparation of the cross-linked dimer phosphoramidites (compounds **3-11** and **3-16**) to generate ICL DNA duplexes containing completely asymmetrical sequences.

3.2 Experimental Procedures

The syntheses and characterization of dimers **3-11 and 3-16** and precursors are described in **Chapter 6.1**.

3.2.1 Solid-phase assembly of cross-linked DNA duplexes, purification and characterization

The cross-linked duplexes **CJW201**, **CJW202**, **CJW216** and **CJW217**, whose sequences are shown in **Figure 3-2**, were assembled using an Applied Biosystems Model 3400 synthesizer on a 1 μmole scale employing standard β-cyanoethylphosphoramidite cycles

supplied by the manufacturer with slight modifications to coupling times described below. **CJW201** and **202** were prepared for ligation into larger DNA for single molecule biophysical studies. The other two ICL DNA duplexes were employed as substrates containing a directly opposed 1-1 cross-link (**CJW216**) and a 1-2 staggered cross-link (**CJW217**).

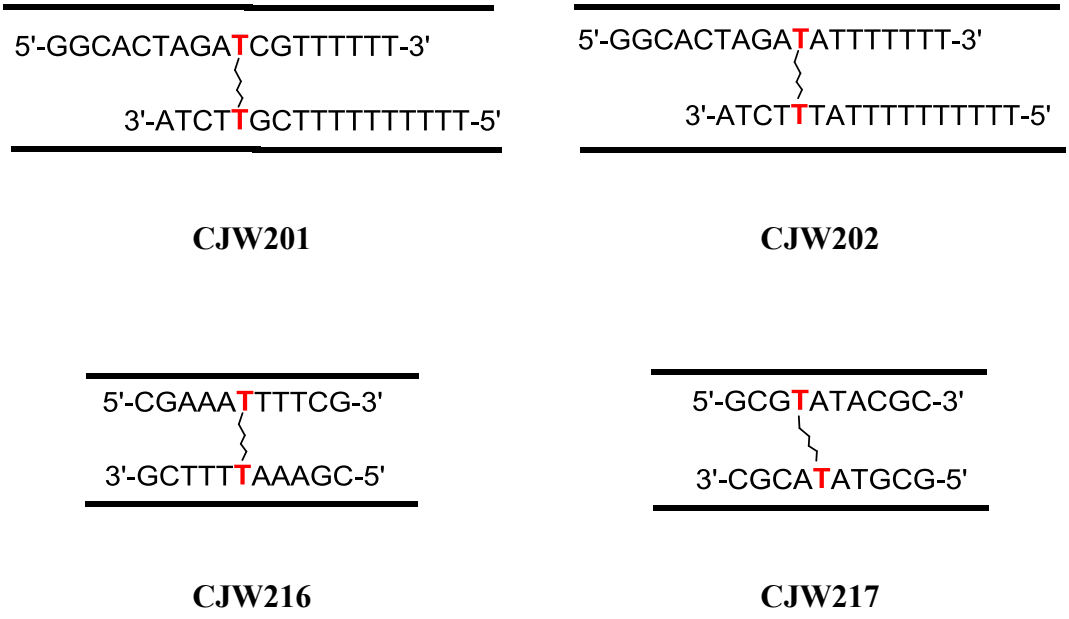


Figure 3-2. The sequences of ICL duplexes containing *N*3T-butylene-*N*3T

Solutions of the nucleoside phosphoramidites containing standard protecting groups were prepared in anhydrous acetonitrile at a concentration of 0.1 M for the 3'-*O*-2'-deoxyphosphoramidites, 0.15 M for the cross-linked phosphoramidites (**3-11** and **3-**

16) and 0.2 M for the 5'-*O*-2'-deoxyphosphoramidites. Assembly of sequences first involved detritylation (3% trichloroacetic acid [TCA] in CH_2Cl_2), followed by nucleoside phosphoramidite coupling with commercially available 3'-*O*-2'-deoxyphosphoramidites (2 min), 5'-*O*-2'-deoxyphosphoramidites (3 min) or cross-linked phosphoramidites **3-11** or **3-16** (10 min); Subsequent capping with acetic anhydride/pyridine/tetrahydrofuran (1:1:8, v/v/v; solution A, and *N*-methyl-imidazole/tetrahydrofuran 16:84 w/v; solution B) and oxidation (0.02 M iodine in tetrahydrofuran/water/pyridine 2.5:2:1) followed every coupling phosphoramidite. To form the Y intermediate first the cyanoethyl groups were removed from the polystyrene-linked oligomers by treating the support with 1 mL of anhydrous triethylamine (TEA) for at least 12 h. The support was then washed with 30 mL of anhydrous acetonitrile (ACN) followed by anhydrous THF. The 5'-*O*-alloc group was removed from the partial duplex by treating the support with $Pd(PPh_3)_4$ and PPh_3 (10 : 20 eq. to the support bound oligonucleotide) in a buffer solution of butylamine-formic acid (1:1, 100 eq. the support bound oligonucleotide) for 3 h at 35°C. The support was then washed with 30 mL each of anhydrous THF and ACN followed by drying via high vacuum (30 min). The support was then subjected to a Pd scavenger treatment using a solution of ACN saturated with sodium *N*,*N*-diethydithiocarbamate solution (3 mL x 2) for a total of 1 h at RT.[497-499] This was followed by washing with 30 mL each of DCM and ACN followed by drying via high vacuum (30 min).

Chain assembly was continued using 3'-*O*-2'-deoxyphosphoramidites followed by detrilylation and an additional capping step for the 5'-OH group. Then, the *tert*-butyldimethylsilyl (TBS) group was removed from the partial duplex by treating the support with 2 x 1 mL triethylamine trihydrofluoride (TEA•3HF) for a total of 1 h. The support was then washed with 30 mL each of anhydrous THF and ACN followed by drying via high vacuum (20 min). The final extension of the cross-linked duplex was then achieved using 5'-*O*-2'-deoxyphosphoramidites with a total detritylation exposure of 130 seconds and removal of the 3'-terminal trityl group on the synthesizer to yield duplexes **CJW201, CJW202, CJW216** and **CJW217** on the solid support.[446]

The oligomer-derivatized polystyrene beads were transferred from the reaction column to screw cap microfuge tubes fitted with teflon lined caps and the oligomer released from the support and protecting groups removed by treatment with a mixture of concentrated ammonium hydroxide/ethanol (0.3 mL:0.1 mL) for 4 h at 55ºC. The cross-linked final products were separated from pre-terminated products by strong anion exchange (IEX) HPLC using a Dionex DNAPAC PA-100 column (0.4 cm x 25 cm) purchased from Dionex Corp, (Sunnyvale, CA) with a linear gradient of 20–55% buffer B over 30 min (buffer A: 100 mM Tris HCl, pH 7.5, 10% acetonitrile and buffer B: 100 mM Tris HCl, pH 7.5, 10% acetonitrile, 1 M NaCl) at 40°C. The columns were monitored at 260 nm for analytical runs or 280 nm for preparative runs.

The purified oligomers were desalted using C-18 SEP PAK cartridges (Waters Inc.) as previously described.[445, 500]

3.2.2 Enzymatic digests

The cross-linked oligomers (0.1 A_{260} units) were characterized by enzymatic digestion (snake venom phosphodiesterase: 0.28 units and calf intestinal phosphatase: 5 units, in 10 mM Tris, pH 8.1 and 2 mM magnesium chloride) for a minimum of 36 h at 37°C as previously described.[447] The resulting mixture of nucleosides was analyzed by reversed phase HPLC carried out using a Symmetry® C-18 5μm column (0.46 x 15 cm) purchased from Waters Inc, Milford, MA. The C-18 column was eluted with a linear gradient of 0-60% buffer B over 30 min (buffer A, 50 mM sodium phosphate, pH 5.8, 2% acetonitrile and buffer B, 50 mM sodium phosphate, pH 5.8, 50% acetonitrile). The resulting peaks were identified by co-injection with the corresponding standards and eluted at the following times: dC (4.6 min), dG (6.8 min), dT (7.4 min), dA (7.9 min), and cross-linked dimer (14.2 min) and the ratio of nucleosides was determined. The molar extinction coefficient of *N*3-thymidine-butylene-*N*3-thymidine was estimated to be the average of a dT and a previously reported dT-dT adduct (13156 liter/(mol.cm)).

3.2.3 Mass Spectrometry

ESI mass spectra for small molecules were recorded at the McGill University Department of Chemistry Mass Spectrometry Facility with a Finnigan LCQ DUO mass spectrometer in MeOH or acetone. ESI mass spectra for oligonucleotides were obtained at the Concordia University Centre for Biological Applications of Mass Spectrometry (CBAMS) using a Micromass Qtof2 mass spectrometer (Waters) equipped with a nanospray ion source. The mass spectrometer was operated in full scan, negative ion detection mode.

3.2.4 UV thermal denaturation

Molar extinction coefficients for the oligonucleotides were calculated from those of the mononucleotides and dinucleotides according to nearest neighbor approximations.[501] Non-cross-linked duplexes were prepared by mixing equimolar amounts of the interacting strands and lyophilizing the mixture to dryness. The resulting pellet (both controls and cross-linked duplexes) were then re-dissolved in 90 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA buffer (pH 7.0) to give a final concentration of 2.8 μM for control and cross-linked duplexes. The solutions were then heated to 90 ^{o}C for 10 min, cooled slowly to RT, and stored at 4 ºC overnight before measurements. Prior to the thermal run, samples were degassed by

placing them in a speed-vac concentrator for 2 min. Denaturation curves were acquired at 260 nm at a rate of heating of 0.5 ^{o}C/min, using a Varian CARY Model 3E spectrophotometer fitted with a 6-sample thermostated cell block and a temperature controller. The data were analyzed in accordance with the convention of Puglisi and Tinoco[272] and transferred to Microsoft Excel™.

3.2.5 Circular Dichroism (CD) spectroscopy

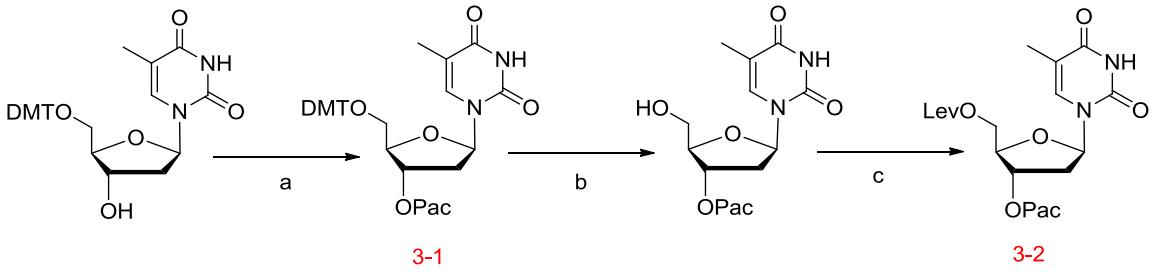
Circular dichroism spectra were obtained on a Jasco J-815 spectropolarimeter equipped with a Julaba F25 circulating bath. Samples were allowed to equilibrate for 5-10 min at 10ºC in 90 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA (pH 7.0), at a final concentration of 2.8 μM for the cross-linked duplexes and ca. 2.8 μM for control duplexes. Each spectrum was an average of 5 scans. Spectra were collected at a rate of 100 nm/min, with a bandwidth of 1 nm and sampling wavelength of 0.2 nm using fused quartz cells (Starna 29-Q-10). The CD spectra were recorded from 350 to 200 nm at 10ºC. The molar ellipticity was calculated from the equation $[\phi] = \varepsilon/Cl$, where ε is the relative ellipticity (mdeg), C is the molar concentration of oligonucleotides (moles/L), and l is the path length of the cell (cm). The data were processed on a PC computer using Windows™ based software supplied by the manufacturer (JASCO, Inc.) and transferred into Microsoft Excel™ for presentation.

3.3 Results and discussion

3.3.1 Synthesis of cross-linked dimer phosphoramidites

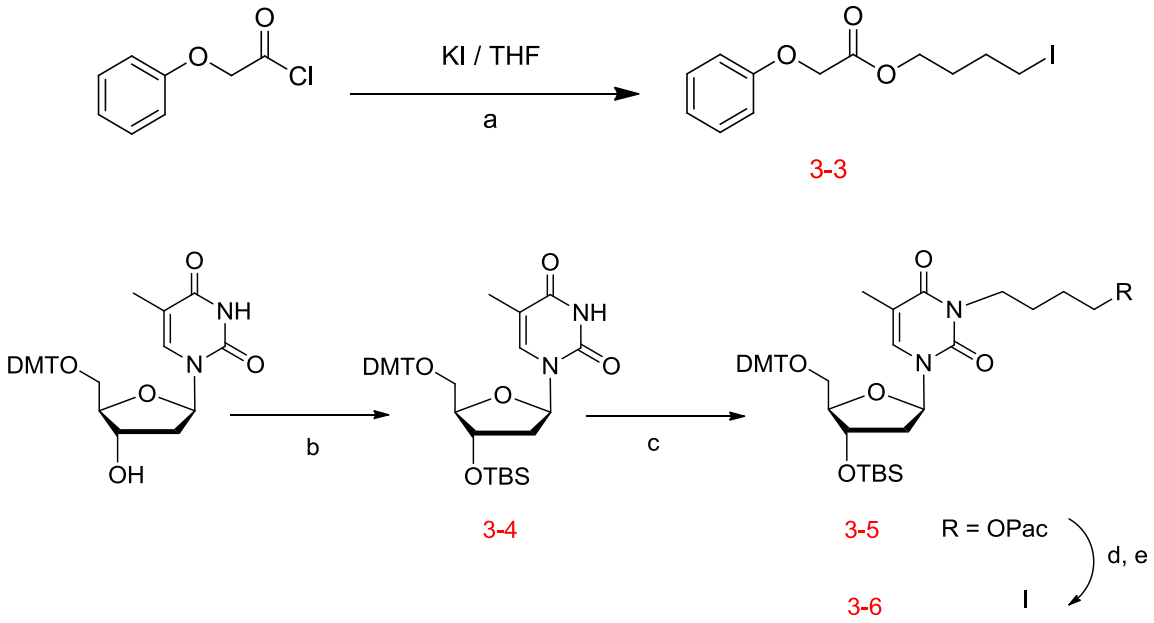
In the process of screening out four compatible protective groups for this strategy, preliminary attempts to use the Lev and Pac groups as in the precursor 3'-*O*-phenoxyacetyl-*5'*-*O*-levulinoyl-thymidine (**3-2**), which was prepared through the synthesis route in **Scheme 3-1**, were investigated but ultimately shown to be incompatible.[502, 503] Treatment of precursor **3-2** with 0.01 mol/L Na_2CO_3 or 0.001 mol/L Na_2CO_3 in CH_3OH/DCM [504, 505] and analysis by TLC revealed that both the Pac and Lev groups were removed under these conditions making them unsuitable as protecting groups together in the synthesis of the cross-linked dimers. The four functional groups that were investigated in the synthesis of the dimers and consequently the ICL duplexes were the DMT, TBS, Alloc and Lev groups.

The linker 4-iodobutyl 2-phenoxyacetate (**3-3**) was prepared as shown in **Scheme 3-2** by reacting 2-phenoxyacetyl chloride and potassium iodide in tetrahydrofuran at 95% yield.



a) 1.0 eq. 5'-*O*-DMT-thymidine, 1.5 eq. phenylacetyl chloride, 1.6 eq. triethylamine in anhydrous THF at RT overnight, 96%; b) 2.2 eq. *p*-TsOH, in DCM/CH_3OH (9/1) at RT for 30 min. 76%. c) 2.0 eq. Levulinic acid, 2.0 eq. EDC, in pyridine at RT for 18 h. 95%.

Scheme 3-1. Synthesis scheme of 5'-*O*-levulinoyl-3'-*O*-phenoxyacetyl-thymidine (**3-2**).



a) 1.0 eq. phenoxyacetyl chloride, 2.5 eq. KI, in anhydrous THF at RT for 24 h., 95%. b) 1.0 eq. 5'-*O*-DMT-thymidine, 2.2 eq. TBSCl, 4.4 eq. imidazole, in anhydrous DMF overnight, 99%. c)

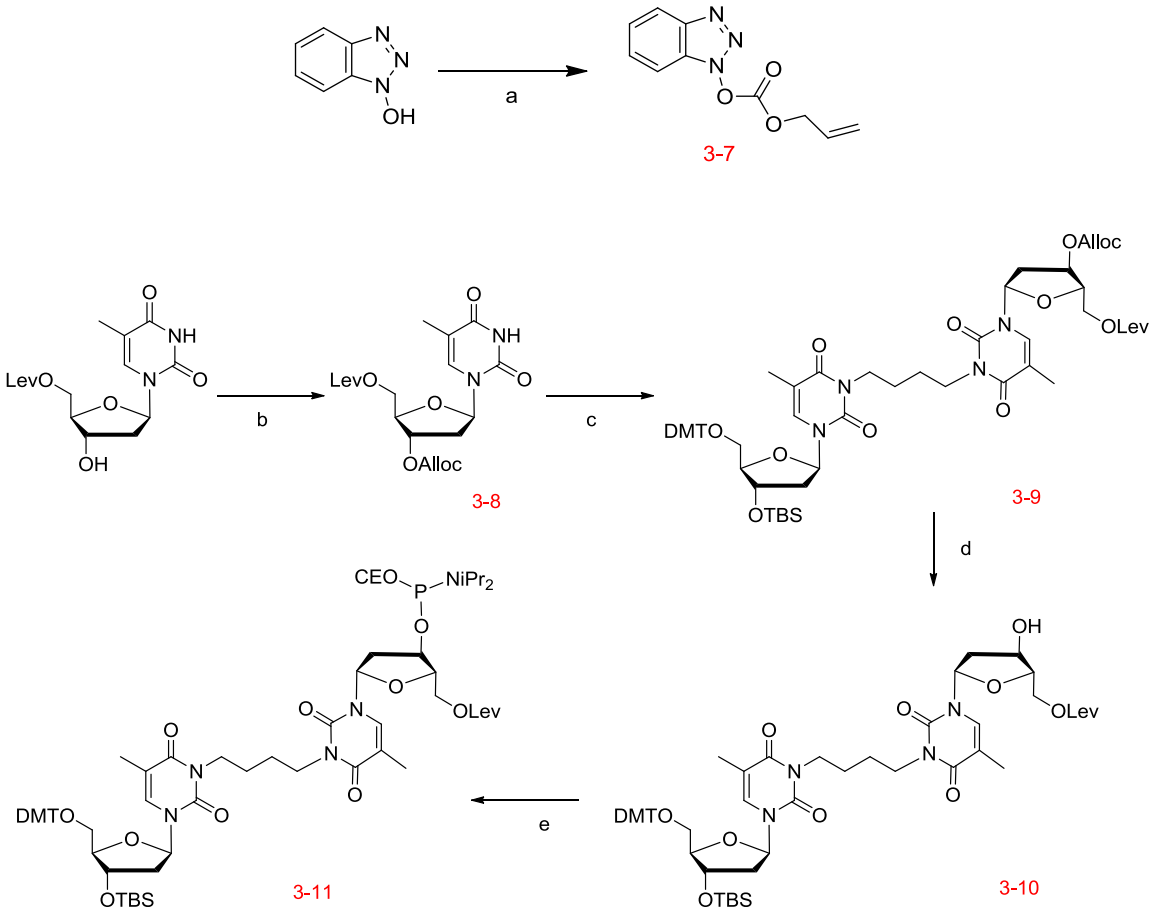
2.0 eq. 4-iodobutyl-1-phenoxyacetate (**3-3**), 2.5 eq. DBU, in in CH_3CN at RT for 24 h. 83%. d) 3 mL propylamine in 10 mL DCM/1 mL CH_3OH at RT for 6 h., 75%. e) 6.0 eq. imidazole, 3.0 eq. PPh_3, 3.0 eq. iodine in anhydrous THF at RT for 6 h. 95%.

Scheme 3-2. Synthesis route of *N*3-(4-Iodobutyl)-5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine (**3-6**)

Starting from commercially available 5'-*O*-dimethoxytrityl-thymidine the free 3'-alcohol was protected as a TBS group using TBSCl to produce 5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine (**3-4**) in quantitative yields. Linker (**3-3**) was then coupled at the *N*3 of thymidine in the presence of 1,8-diazabicyclo-(5.4.0)-undec-7-ene (DBU), a strong hindered base, to form the monoadduct *N*3-(4-(2-phenoxyacetate)butyl)-5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine (**3-5**) in 87% yield. Removal of the Pac group on the linker moiety using propanamine at RT for at least 6 h (75%) followed by its conversion to *N*3-(4-iodobutyl)-5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine (**3-6**) with elemental iodine and Ph_3P (95% yield) (**Scheme 3-2**) set the stage for the critical dimerization reaction with 5'-*O*-levulinoyl-3'-*O*-allyloxycarbonyl-thymidine (**3-8**).

Two asymmetric phosphoramidites of the *N*3T-butylene-*N*3T dimer were synthesized, numbered as **3-11** and **3-16**. **Schemes 3-3** and **3-4** illustrate the synthesis of these two

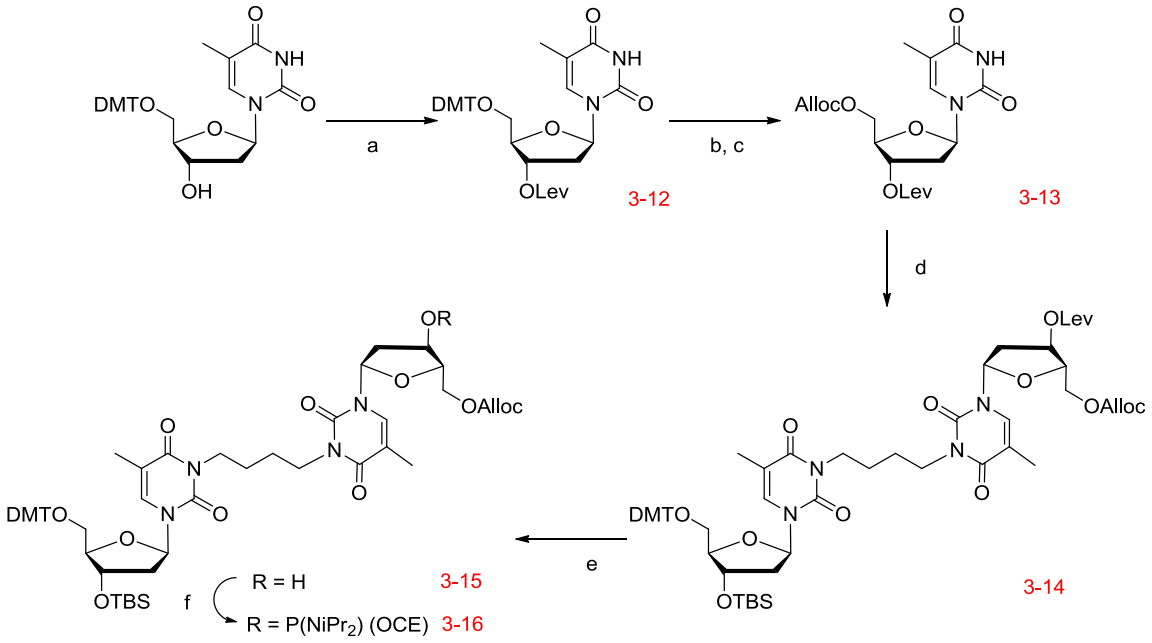
nucleoside dimers, which allow for an orthogonal deprotection to assemble ICL DNA containing sequences of asymmetric nucleotide composition around the cross-linked site.



a) 1.0 eq. 1-hydroxybenzotriazole hydrate, 1.04 eq. allyl chloroformate and 1.12 eq. DIPEA in anhydrous THF at RT for 30 min. 79%. b) 1.33 eq. Alloc-OBt **(3-7)**, 0.2 eq. DMAP, in pyridine/THF (9/1) at RT for 24 h. 66%. c) 1.0 eq *N*3-(4-iodobutyl)-5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine **(3-6)**, 2.0 eq. DBU, in in CH_3CN at RT for 24 h. 68%. d) 0.1

eq. $Pd(PPh_3)_4$, 0.2 eq. PPh_3, in 1-butanamine/formic acid (50/50) at RT for 2.5 h. 100%. e) 1.2 eq. $ClP(OCE)(NiPr_2)$, 1.5 eq. DIPEA, in THF at RT for 0.5 h. 71%.

Scheme 3-3. Synthesis route of phosphoramidite **3-11**.



a) 2.0 eq. Levulinic acid, 2.0 eq. EDC, 0.01 eq. DMAP, in 1,4-dioxane at RT for 12 h. Quantitative yield. b) 2.2 eq. *p*-TsOH, in DCM/CH_3OH (4/1) at RT for 0.5 hour. 69%. c) 1.33 eq. Alloc-OBt (**3-7**), 0.2 eq. DMAP, in THF/pyridine (9/1) at RT for 24 h. 85%. d) 1.0 eq. *N*3-(4-iodobutyl)-5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine (**3-6**), 2.0 eq. DBU, in

CH_3CN at RT for 48 h. 55%. e) 0.5M hydrazine hydrate, in pyridine/acetic acid (9/1) at RT for 10 min. 96%. f) 1.2 eq. ClP(OCE)(N*i*Pr_2), 1.5 eq. DIPEA, in THF at RT for 1 h. 71%.

Scheme 3-4. Synthesis route of phosphoramidite **3-16**.

Two asymmetric phosphoramidites of the *N*3T-butylene-*N*3T dimer were synthesized, numbered as **3-11** and **3-16**. **Schemes 3-3** and **3-4** illustrate the synthesis of these two nucleoside dimers, which allow for an orthogonal deprotection to assemble ICL DNA containing sequences of asymmetric nucleotide composition around the cross-linked site.

The precursors **3-10** and **3-15** of phosphoramidites **3-11** and **3-16** differ with respect to the placement of the Alloc and Lev protective groups at either the 5' or 3' position of one thymidine in the dimer.

Compound **3-8** is itself synthesized from commercially available 5'-*O*-levulinoyl-thymidine which is reacted with Alloc-OBt (**3-7**) in pyridine at RT overnight (78% yield). The dimerization reaction between compound **3-6** and **3-8 (**which is in slight excess) is accomplished with DBU overnight to produce the dimer **3-9** in 68% yield. The Alloc group of dimer **3-9** was removed with $Pd(PPh_3)_4$ in a butylamine-formic acid buffer solution to give the free 3'-hydroxyl in **3-10** (100%), which was then converted to the cross-linked phosphoramidite **3-11** using a slight excess of *N*,*N*-diisopropylamino

cyanoethyl phosphonamidic chloride in the presence of Hunigs base. This phosphoramidite was isolated by hexane precipitation in 71% yield.

The synthetic route involving amidite **3-16** varied as follows. The Lev group was introduced at the 3'-OH of 5'-*O*-dimethoxytrityl-thymidine with levulinic acid, in the presence of EDC and DMAP in dioxane at RT in quantitative yield to produce compound **3-12**. This step required longer reaction times as it was sluggish in general. The 5'-*O*-DMT group was removed with *p*-TsOH to release a free 5'-OH (77%) followed by the introduction of the Alloc group as described above, producing compound **3-13** (85%). A similar coupling reaction of compound **3-13** with **3-6** in the presence of DBU yielded the second precursor **3-14** in 55% yield. The Lev protective group was removed by treatment with 0.5M hydrazine in a pyridine: acetic acid buffer (v/v 9/1) for 10 min at RT to yield the precursor **3-15** (96%). This was then phosphitylated as described above on the newly released free 3'-OH to produce the cross-linked phosphoramidite **3-16** in 71% yield.

The isolated phosphoramidites **3-11** and **3-16** were analyzed by mass spectrometry and were found to have the expected molecular masses (1275.5786 and 1261.5638). ^{31}P NMR analysis of these phosphoramidites revealed the presence of two signals for **3-11** (148.39 and 148.61ppm) and **3-16** (146.71 and 146.97 ppm) in the region diagnostic for a phosphoramidite.

3.3.2 Design, purification and characterization of interstrand cross-linked DNA duplexes

The ICL duplexes containing these dimers were synthesized on an ABI 3400 solid-phase DNA synthesizer using polystyrene (PS) rather than controlled-pore glass (CPG) solid-support due to the incompatibility of triethylamine-trihydrofluoride (TEA•3HF) with the latter. As an added precaution, the cyanoethyl protecting groups were removed using triethylamine (TEA) as it has been observed that prolonged fluoride treatment using tetrabutylammonium fluoride (TBAF) could lead to chain cleavage.[471] The assembly of ICL containing duplex CJW201 utilizing phosphoramidite **3-11** is illustrated in **Scheme 3-5** (left). The U shaped intermediate **CJW201-U** was assembled on solid support in one step by coupling 0.15M of **3-11** directly to a short linear segment of DNA for 10 min, followed by removal of the DMT group from the ICL dimer **3-11,** followed by chain elongation. Chain assembly in the 3' to 5' direction proceeded smoothly using 3'-*O*-2'-deoxyphosphoramidites (0.1M). A higher concentration and longer coupling time was necessary for **3-11** to ensure a high coupling efficiency due to its larger size compared to the standard 3'-*O*-2'-deoxyphosphoramidites. This step was followed by capping on the synthesizer to prevent undesired chain growth in subsequent steps. At each stage of this assembly 1-2 mg of solid support was deprotected with ethanolic

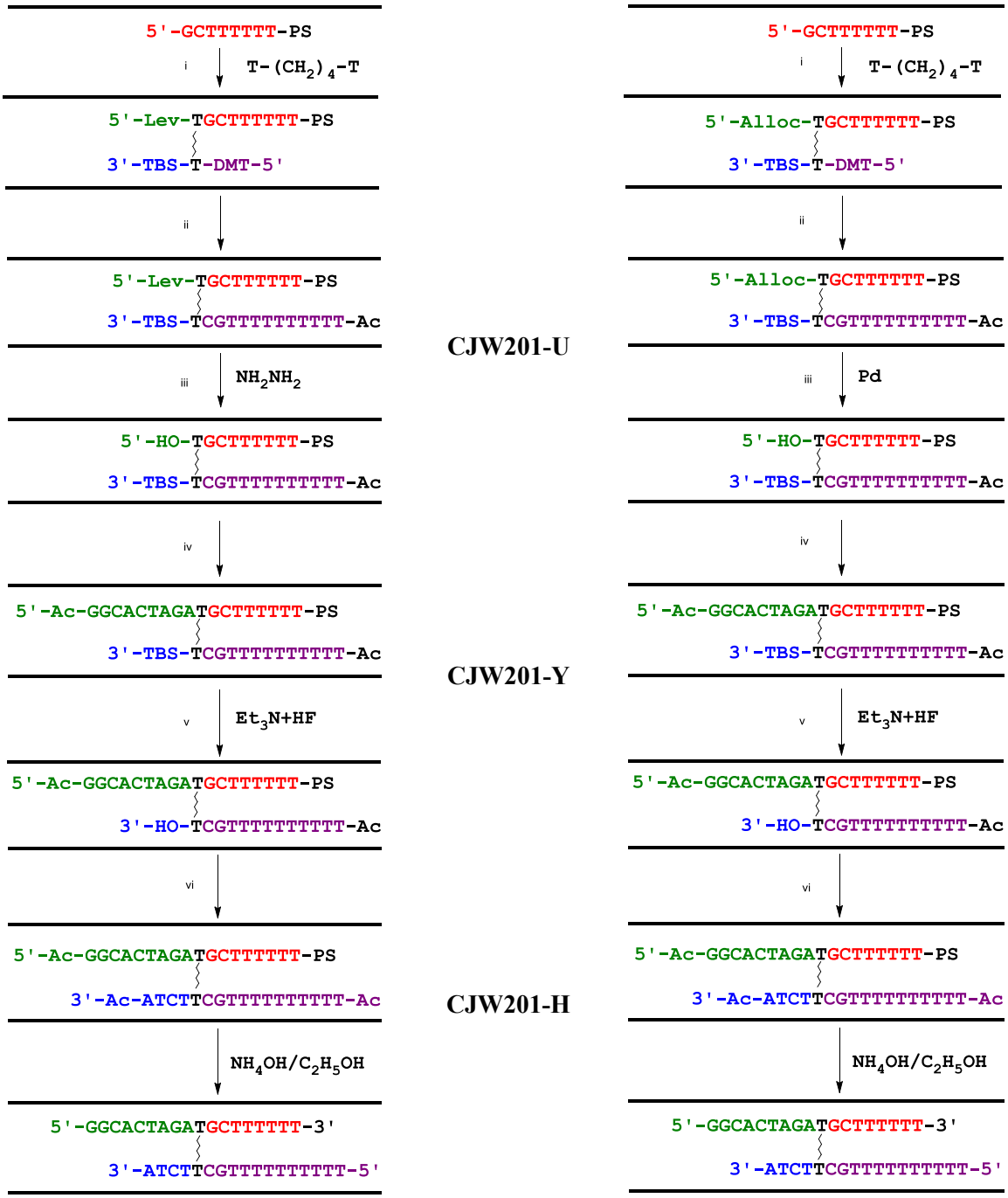
ammonia (1:3 v/v, 0.5mL) at 55°C for 4 h. The success of either coupling or removal of a specific protecting group at each stage was then monitored using Ion exchange (IEX) HPLC. From **Figure 3-3**, it is clear that coupling of the dimer phosphoramidite **3-11** was highly satisfactory where the major species at *ca*.18 min was that of the desired U intermediate. After subjecting the oligo-bound support to TEA treatment as described above the Lev protective group was removed using hydrazine in an acetic acid: pyridine mixture (1:1), for 30 min at RT. Unfortunately, the extent of removal of the Lev group cannot be monitored by direct deprotection and IEX HPLC analysis as above. Extension from the free OH group serves as an indirect indicator for cleavage success. This is because the Lev group is cleaved by the strong base NH_4OH itself. **Scheme 3-5** (left) illustrates the removal of the Lev protective group followed by chain extension producing oligonucleotide **CJW201-Y**. It is clear that there are a number of failure products suggesting that the deprotection conditions with hydrazine were not optimal for ICL synthesis while being retained on solid support.[500] Although it has been reported that the Lev protecting groups are compatible with solid-phase synthesis and are removed completely using hydrazine treatment, these earlier studies involved only oligothymidylates. These findings were confirmed with a test oligothymidylate bearing 5'-*O*-Lev-thymidine group, which on removal of the Lev group and subsequent extension proved successful for linear T rich sequences (Data not shown). One possible reason for unsuccessful extension of the ICL could be due to steric hindrance at the cross-link site

making removal of the Lev group incomplete and /or oligonucleotide extension more difficult. It was assumed that alternatively mixed base oligomers that contain benzoyl protected cytidine residues could undergo transamination at the N^4 site in the presence of hydrazine or could be removed and this can cause undesired growth to form several failure products.[506-509] The 3'-*O*-TBS group was then removed from **CJW201-Y** by treating the support with anhydrous triethylamine overnight followed by TEA•3HF twice for 30 min at RT. C-18 reversed-phase HPLC analysis of the deprotected intermediate revealed complete removal of the silyl group with the shift of the major peak from 13.0 to 9.5 min in the case of the cross-link. Continued synthesis with repetitive coupling of 5'-*O*-2'-deoxyphosphoramidites at the 3'-end of intermediate **CJW201-Y** gave the full length **CJW201-H**. Because of the challenges faced with the Lev group this route to synthesize ICL duplexes was not pursued, and instead amidite **3-16** was used as the second pathway in our attempts to synthesize asymmetric ICL duplexes.

The assembly of ICL utilizing phosphoramidite **3-16** is illustrated in **Scheme 3-5** (right). The U shaped intermediate **CJW201-U** was assembled on polystyrene solid support in one step by coupling **3-16** directly to a short linear segment of DNA followed by capping on the synthesizer as described above. Analysis by IEX HPLC revealed that coupling of the dimer phosphoramidite was highly satisfactory (**Figure 3-4**). After subjecting the oligo-bound support to TEA treatment overnight as described above the Alloc protective

group was removed with $Pd(PPh_3)_4$ in a butylamine-formic acid buffer solution for 3 h at 35°C to free the 3'-hydroxyl (**Scheme 3-5** right and **Figure 3-5**).[489, 499, 510] As in the case of the Lev group the successful removal of the Alloc groups has to be determined indirectly through subsequent extension of the Y oligo from the free OH group. It is extremely essential that all traces of remnant Pd be removed using a Pd scavenger namely sodium *N*, *N*-diethyldithiocarbamate to enable the successful synthesis of the Y intermediate of **CJW201.**[497-499] Failure to perform this Pd scavenger step leads to oligonucleotide degradation as illustrated by IEX HPLC analysis of oligonucleotide **CJW201-U** with and without scavenger-washing (**Figure 3-5**).

After capping the **CJW 201-Y**, the TBS group was removed as described above, starting with overnight treatment of the support with TEA followed by TEA•3HF. Formation of the complete **201-H** duplex was accomplished with 5' reverse phosphoramidites as described above. IEX analysis of **CJW201-Y** and **H** are shown in **Figure 3-6a** and **3-6b**, respectively.



(i) Growth of the linear segment with 3'-*O*-2'-deoxyphosphoramidites; (ii) coupling of phosphoramidites **3-11**/**3-16** followed by extension with 3'-*O*-2'-deoxyphosphoramidites (to afford **CJW201-U**); (iii) removal of the Lev protective group;[511] in a or in a removal of the

Alloc protective group;[497-499, 512] in b (iv) extension with 3'-*O*-2'-deoxyphosphoramidites (to give **CJW201-Y**); (v) removal of the TBS protective group; (vi) extension with 5'-*O*-2'-deoxyphosphoramidites followed by cleavage from the solid support and deprotection of the **CJW201-H** with NH_4OH/C_2H_5OH.

Scheme 3-5. Solid-phase synthesis procedure of oligonucleotide **CJW201** involving *N*3T-butylene-*N*3T phosphoramidite **3-11** (left) and **3-16** (right).

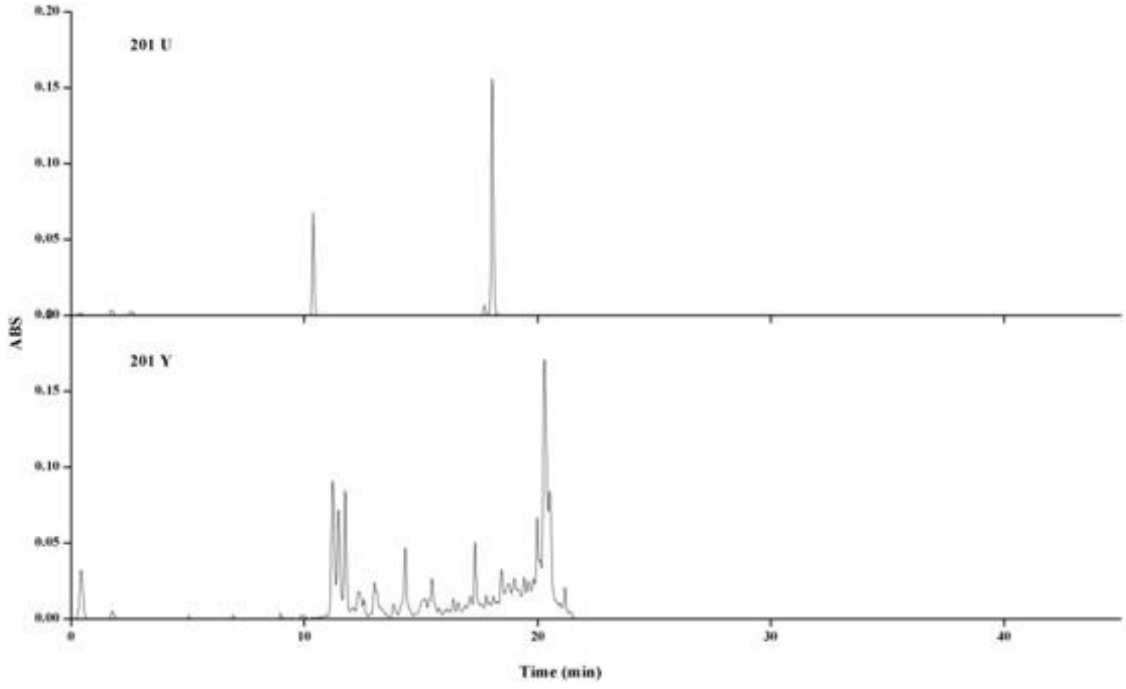


Figure 3-3. IEX analysis of **CJW201-U** (top) and **CJW201-Y** (bottom) prepared with phosphoramidite **3-11** (the column was monitored at 260 nm, and eluted at RT with a

gradient of 0 to 70% buffer B in 30 min. Buffer A: 10% acetonitrile, 100mM Tris-HCl (pH 7.8); Buffer B: 10% acetonitrile, 100mM Tris-HCl (pH 7.8), 1M NaCl).

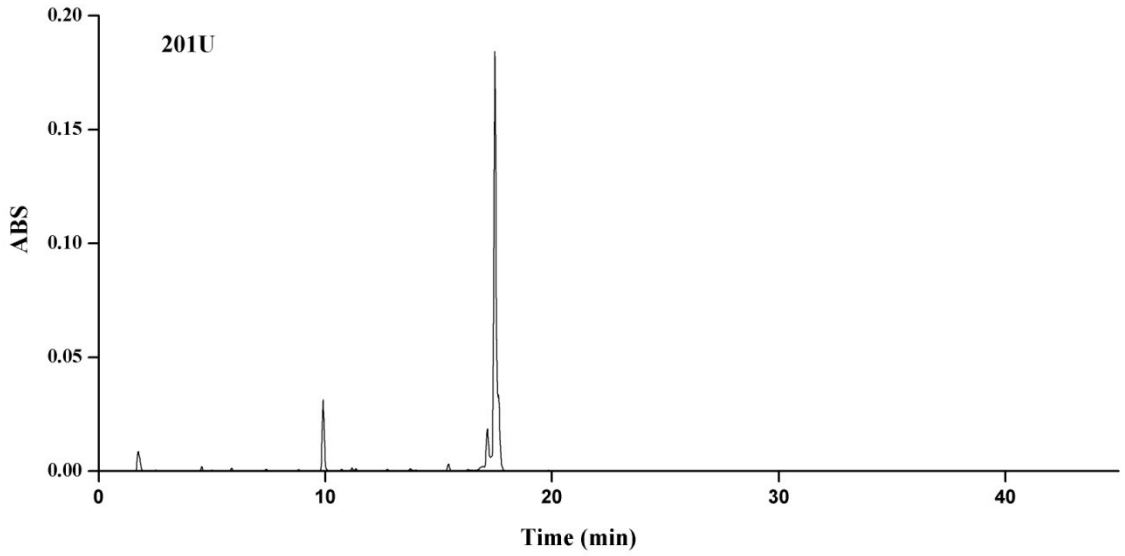


Figure 3-4. IEX HPLC chromatogram of oligonucleotide **CJW201-U** using phosphoramidite **3-16** (HPLC conditions are as described in the **Figure 3-3** legend).

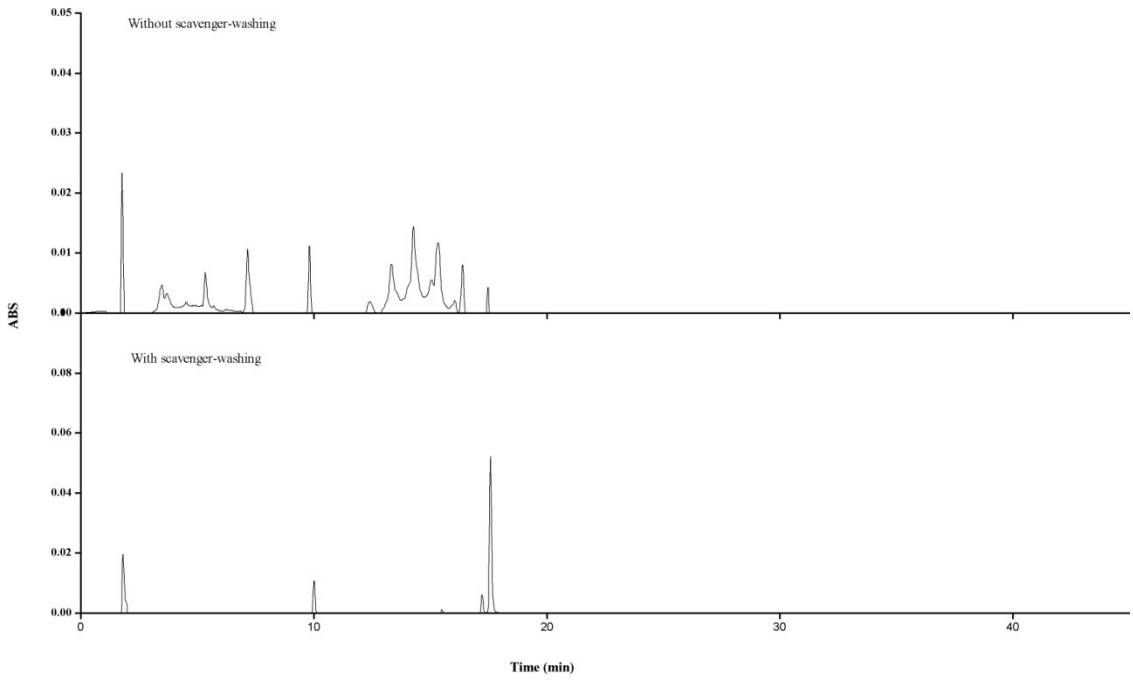


Figure 3-5. The effect of using a Pd scavenger in the synthesis of oligonucleotide **CJW201-U** with phosphoramidite **3-16** (**CJW201-U** (prepared with phosphoramidite **3-16**) was treated with $Pd(PPh_3)_4$ at 35 °C for 3.5 h, and deprotected from solid support with ammonia/ethanol (3/1) at 55 °C overnight. HPLC conditions are as described in the **Figure 3-3** legend)**.**

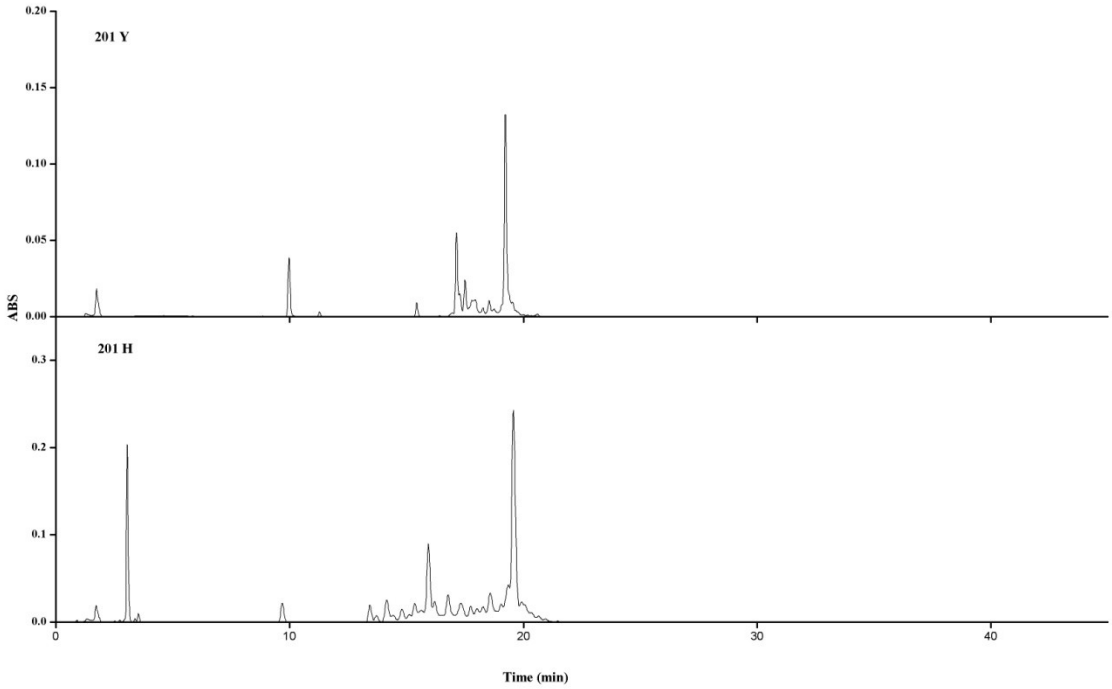


Figure 3-6. IEX HPLC chromatograph of **CJW201-Y** and **CJW201-H** prepared with phosphoramidite **3-16** (HPLC conditions are as described in the **Figure 3-3** legend except a gradient of buffer B from 0.2 to 0.55 M over 30 min at 40 °C was used for **201H).**

It is clear that the assembly of ICL using phosphoramidite **3-16** was superior to that of **3-11**. Thus this route was chosen for solid-phase assembly of all subsequent cross-linked duplexes reported here. These include the three additional ICL sequences, **CJW202, CJW216 and CJW217**. The coupling of this dimer and removal of the protecting groups to form the various intermediates were all found to be similar to those of **CJW 201**. The full-length cross-linked H-oligomers were cleaved from the solid support and deprotected

by treating the support with a mixture of concentrated ammonium hydroxide/ethanol 0.5mL, (3:1) at 55 °C, overnight as previously reported.[513] The crude oligomers were purified by IEX HPLC, using a gradient buffer of 20% to 55% B in 30 min, followed by desalting to afford the pure cross-linked duplexes **CJW201/202/216/217** in yields of 11-25 %. These ICL duplexes were digested to the constituent nucleosides with a combination of snake venom phosphodiesterase and calf intestinal phosphatase in a buffer containing 10 mM Tris (pH 8.1) and 2 mM magnesium chloride at 37 ^{o}C for 16 h and analyzed by C-18 reversed phase HPLC. The extinction coefficient of *N*3T-butylene-*N*3T was assumed to be 13156 liter/(mol.cm), as described earlier. In addition to the four standard 2'-deoxynucleosides, one additional peak was observed with a retention time of 14 min for the dimer (shown in Figures **S48-51** in **Supporting Information**). This additional peak had a retention time identical to the completely deprotected dimer of **3-11** and **3-16**. As shown in Table **3-2**, the ratios of the component 2'-deoxynucleosides and cross-linked nucleosides were consistent with the theoretical composition of ICL duplexes **CJW201/202/216/217.** The molecular weight of the cross-linked duplexes as determined by mass spectrometry (ESI-TOF) were in close agreement with the expected theoretical values 10663.0, 10661.1, 6716.4 and 6110.0 Da respectively (shown in **Table 3-2**).[513]

Table 3-2. Retention times, nucleoside ratios and MS data for the cross-linked duplexes

Cross-linked duplexes	Retention time (min)	Nucleoside composition	Nucleoside Ratios [c]		Mass	
			Expected	Observed	Expected	Observed
		dC	1.00	1.00		
		dG	1.00	1.06		
CJW201	22.98 [a]	dT	3.80	3.71	10663.2	10663.0
		dA	0.80	0.83		
		dT-dT [d]	0.20	0.21		
		dC	1.00	1.00		
		dG	1.00	1.06		
CJW202	23.09 [a]	dT	7.00	7.47	10661.2	10661.1
		dA	2.00	2.06		
		dT-dT [d]	0.33	0.35		
		dC	1.00	1.00		
		dG	1.00	1.00		
CJW216	18.06 [b]	dT	1.50	1.42	6716.7	6716.4
		dA	1.50	1.48		
		dT-dT [d]	0.25	0.25		

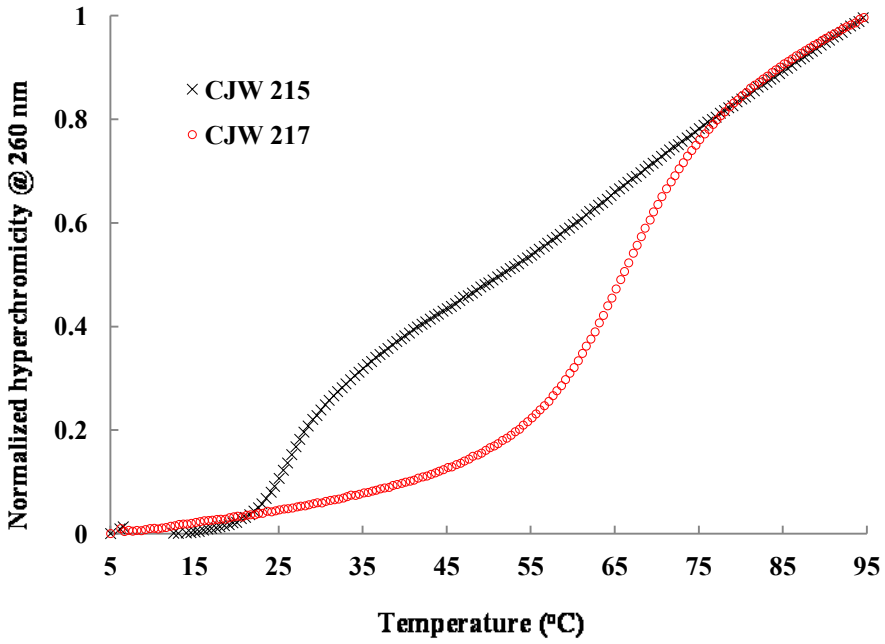
		dC	1.00	1.00		
		dG	1.00	1.05		
CJW217	15.94 [b]	dT	0.33	0.34	6110.1	6110.0
		dA	0.67	0.67		
		dT-dT [d]	0.17	0.17		

a) Retention time of the ICL oligonucleotide **CJW201-H** with IEX. HPLC conditions are as described in the **Figure 3-6** legend. b) Retention time of the ICL oligonucleotide **CJW201-H** with IEX. HPLC conditions are as described in the **Figure 3-3** legend except a gradient of buffer B from 0.2 to 0.55 M over 30 min was used. c) Nucleoside ratio analysis was run with 0 to 60% of mobile buffer B in 30 min on RPLC. Buffer A: 2% acetonitrile, 50 mM sodium phosphate, pH 5.8; Buffer B: 50% acetonitrile, 50 mM sodium phosphate, pH 5.8. d) The extinction coefficient for dT-dT was assumed to be 13156 liter/(mol.cm).

3.3.3 UV thermal denaturation and Circular Dichroism spectra (CD) of ICL duplexes

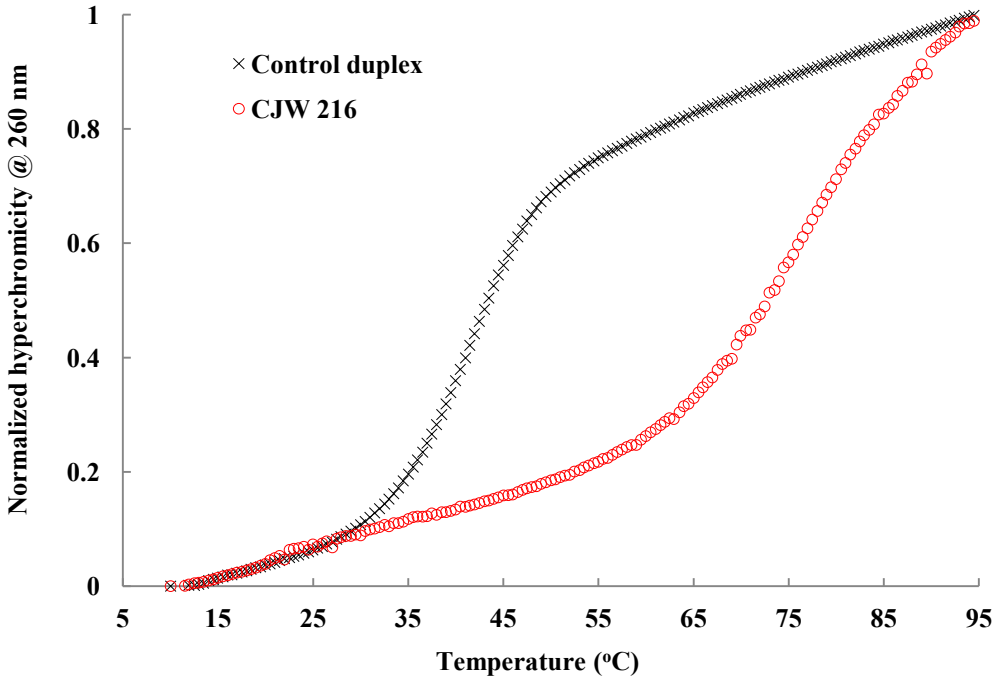
The stability of the cross-linked duplexes (both symmetrical and asymmetrical) was assessed by the ultraviolet thermal denaturation experiments, as illustrated in **Figure 3-7** to **3-9** and reported in **Table 3-3**. The profiles for duplexes **CJW216** and **CJW 217** were

sigmoidal indicating cooperativity with values of approximately 73° and 65°C, respectively. The control duplexes for **CJW216** and **CJW217 (**sequences shown in **Figure 3-10)** were prepared by solid-phase synthesis for this study. Single-strand DNA oligonucleotides were prepared, and the complementary oligonucleotides were hybridized for T_m studies. The biophysical study of ICL DNA duplexes **CJW217** was perfomed and compared to its control duplex **CJW215**.[483]



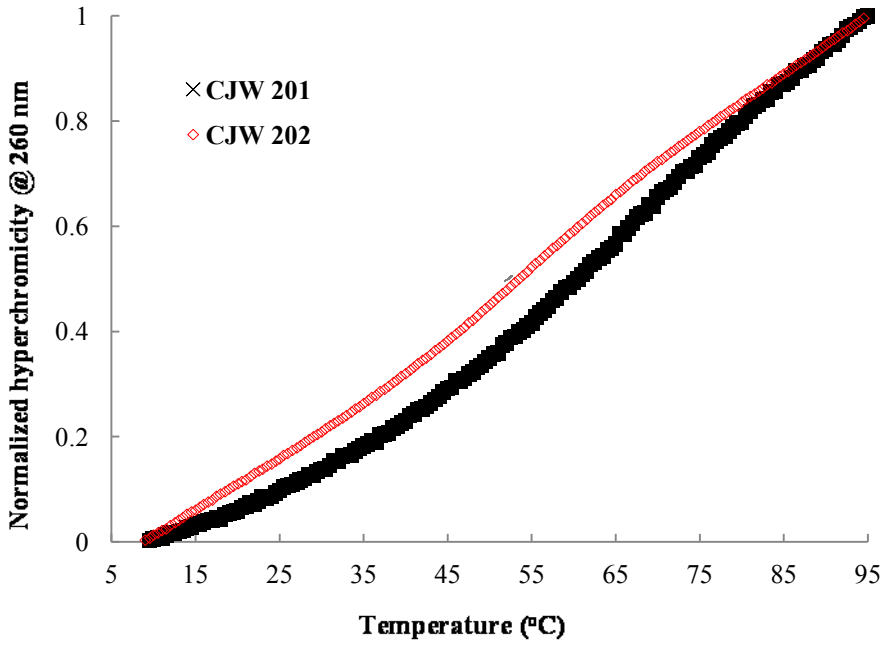
Solutions contained a duplex concentration of 2.8 μM in 90 mM sodium chloride, 10 mM sodium phosphate and 1 mM *EDTA* buffer, pH 7.0. The rate of data acquisition was 0.5 °C/min.

Figure 3-7. Absorbance (A_{260}) versus temperature profiles of **CJW215** (control) and cross-linked duplex **CJW217**.



Conditions are as described in the legend for **Figure 3-7**.

Figure 3-8. Absorbance (A_{260}) versus temperature profiles of cross-linked duplex **CJW216** and the non-cross-linked control.



Solutions contained a duplex concentration of 1.7 μM in 90 mM sodium chloride, 10 mM sodium phosphate and 1 mM *EDTA* buffer, pH 7.0. The rate of data acquisition was 0.5 °C/min.

Figure 3-9. Absorbance (A_{260}) versus temperature profiles of cross-linked duplexes **CJW201** and **CJW202**.

5'-CGAAA**T**TTTCG-3'	5'-GCG**T**ATACGC-3'
3'-GCTTT**T**AAAGC-5'	3'-CGCA**T**ATGCG-5'
Control duplex for **CJW216**	CJW215 (Control duplex for **CJW217**)

Figure 3-10. Sequences of control duplexes for **CJW216** and **CJW217**

Table 3-3. Thermal denaturation of cross-linked duplexes

Duplexes	T_m (°C)		ΔT_m (°C) [a]
	Control	ICL	
CJW216	43.5	73.5	30
CJW217	53.5	65.5	12

a) The temperature difference between cross-linked duplex and control. $\Delta T_m = T_m^{ICL} - T_m^{control}$.

In contrast the profiles for **CJW201** and **CJW202** were broad which were not unexpected due to the smaller amount of complementary segments only on one side of the cross-linked dimer (shown in **Figure 3-9**).

The CD spectra of cross-linked duplexes as well as the non-cross-linked control were recorded at 10ºC (spectra are shown **Figure 3-11**). In all cases, the CD spectra of the duplexes exhibited signatures characteristic of B-form DNA with a positive maximum peak centered around 275 nm, a negative peak at approximately 250 nm and a cross over around 260 nm.[514] The CD spectra of non-cross-linked controls revealed some minor differences, particularly a reduction of the signal at 275 nm as shown in **Figure 3-11**. In

the case of cross-linked duplex **CJW217**, the CD spectra suggested that when the cross-link is in the 1-2 orientation there is a greater distortion induced in the duplex.[471, 515]

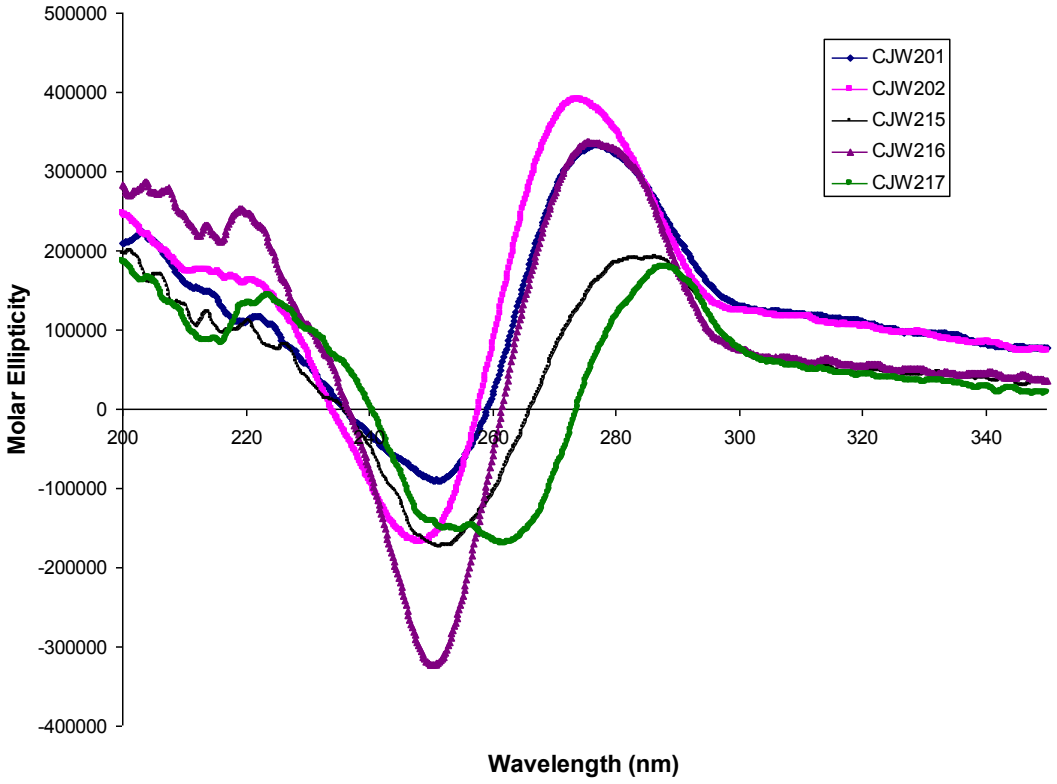


Figure 3-11. Circular dichroism spectra of cross-linked duplexes **CJW201**, **CJW202**, **CJW216** and **CJW217** (The analysis was conducted in 90 mM sodium chloride, 10 mM sodium phosphate and 1 mM EDTA (pH 7.0) with 0.5 OD of oligonucleotides. The CD spectra were recorded from 350 to 200 nm at 10^{o}C).

Previously, Webba da Silva et al. have shown that **CJW 216** displays dramatic widening of the major groove of the B-DNA stem without disruption of Watson-Crick base pairing due to the dominant contribution of cooperative and cumulative base stacking to the stability of the ApT compared to the TpT step suggesting that the latter is more deformable within a DNA stem. The configurational constraints of the butylene tether perturb the structure, resulting in accommodation in a region between major and minor grooves that is in a staggered fashion and that deviates from being perpendicular to the stem axis. However the canonical B-DNA local structure throughout the stem is observed.[516]

3.4 Conclusions

In this chapter, the compatibility of protective groups for solution and solid-phase synthesis to produce ICL oligonucleotide duplexes was studied. Five kinds of protective groups, dimethoxytrityl (DMT), *tert*-butyldimethylsilyl (TBS), levulinoyl (Lev), phenoxyacetyl (Pac) and allyloxycarbonyl (alloc) were evaluated for the masking of the 3'- and 5'- hydroxyls of thymidine. Incompatibility of Pac and Lev protective groups was observed by the preliminary studies with 5'-*O*-levulinoyl-3'-*O*-phenoxyacetyl-thymidine.

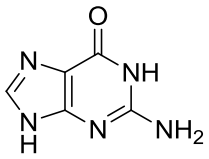
DMT, TBS, Lev and Alloc protective groups were employed in this study. Two novel cross-linked dimer phosphoramidites **3-11** and **3-16** were prepared by multistep synthesis

in solution, and the assembly of ICL duplexes by solid-phase synthesis were studied for each extension to form the final products. By comparison, phosphoramidite **3-16** (with DMT and Alloc protective groups for the two 5'-*O* positions and one TBS for the 3'-*O* position) afforded better conversion of key extension steps (from U-shaped to Y-shaped oligonucleotide intermediates).

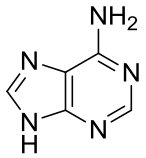
Two partially asymmetrical and two completely asymmetrical ICL duplexes containing *N*3T-butylene-*N*3T have been successfully synthesized using phosphoramidite **3-16**. The duplexes display characteristics typical of B-form as observed by circular dichroism spectroscopy. The formation of expected ICL duplexes was further confirmed with enzymatic digestion and MS analysis. Ultimately, this protective group combination may find applications in the synthesis of other ICL duplexes.

Chapter 4. Development of two cyclization strategies to synthesize 7-deazapurine and related analogs

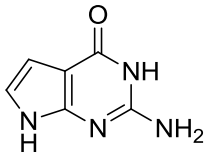
4.1 Introduction



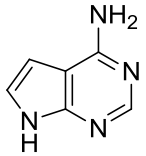
Guanine



Adenine



7-Deaza guanine



7-Deaza adenine

Figure 4-1. Structures of guanine, adenine and their 7-deaza analogos

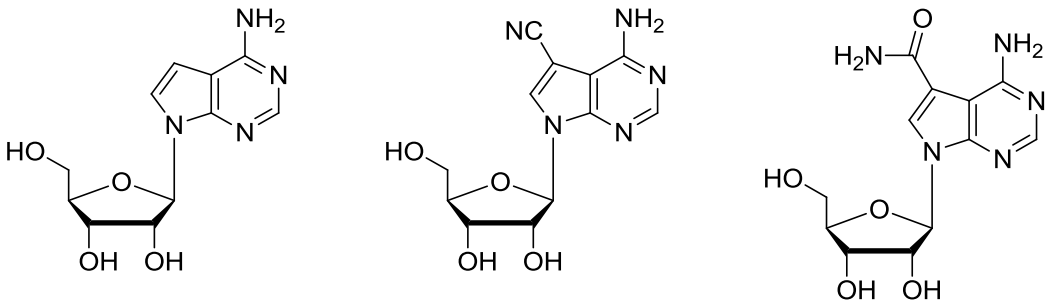
Derivatives of 7-Deazapurine (pyrrolo[2,3-*d*]-pyrimidine, structures shown in **Figure 4-1**), which can be converted to nucleosides and nucleotides, have been investigated as probes for various physical and biochemical studies in the field of nucleic acids.[452] The imine nitrogen at the 7 position of purine nucleosides (in guanine and adenine) is

located in the major groove of B-form DNA and can act as a hydrogen bond acceptor by Hoogsteen base pairing. 7-Deazapurine nucleobases such as 7-deaza-guanine and 7-deaza-adenine are able to form Watson-Crick base pairs, maintaining pairing specificity of the respective natural nucleobases. The absence of the *N*7 atom of these 7-deazapurines disrupts the formation of Hoogsteen hydrogen bonding. The modification at the 7-position alters the electronic properties of the heterocycle and eliminates a major groove cation-binding site that could affect the organization of salts and water in the major groove. These analogs can easily be incorporated in oligonucleotides, either by enzymatic means or by automated solid-phase synthesis.[517-519] When the nucleotides containing 7-deazapurines replace their natural counterparts in PCR reactions, the modified DNA displays improved properties, such as lower melting temperature of the duplex. They can be employed in DNA sequencing reactions, for PCR of GC-rich templates and to improve the DNA bands in polyacrylamide gel electrophoresis. Increased stability of nucleic acids containing the 7-deazapurines (7-deaza-adenosine and 7-deaza-guanosine) over their 7-aza analogs has been shown by precluding protonation at the 7-position.[520] It was reported that oligonucleotides possessing at least one substituted 7-deazapurine nucleobase would hybridize to form duplexes with significantly more stability than unmodified oligonucleotides.[521] Further studies have been undertaken to evaluate the influence of alkyl, alkynyl as well as halogeno substituents at the 7 position of oligonucleotides containing 7-deazaadenine and 7-

deazaguanine. The modified DNA duplexes were observed for alternating, consecutive, or randomly distributed 7-substituted 7-deazaadenine residues to produce the same increase in stability. The increased stability of the duplex structures can be explained by hydrophobization of the modified DNA**,** stronger H-bonding, and/or more favorable stacking interactions.[522, 523]

Absence of the nitrogen atom at the 7-position in deazapurines is also utilized for the syntheses of sequence-specific DNA in the study of chemotherapy and cell repair.[524-532] 7-Deaza-adenosine is a potent and selective inhibitor of hepatitis C virus.[533, 534] 7-deaza-2'-deoxyguanosine exhibits extraordinarily chemical, physical and biological properties compared with its corresponding 7-aza analog. The incorporation of 7-deazaguanine modifications into DNA is frequently used to probe protein recognition of H-bonding information in the major groove of DNA. [526, 527, 535, 536]

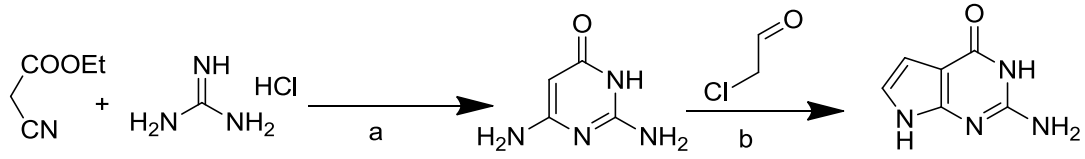
Several naturally occurring 7-deazapurine ribonucleosides, such as tubercidin, toyocamycin and sangivamycin, exhibit a broad spectrum of biological activity (structures shown in **Figure 4-2**). The frequent natural occurrences and the biological properties of this class of compounds have promoted ample studies toward the synthesis, biological activity, and incorporation in oligonucleotides as well as the chemically designed analogues. The 7-position of 7-deazapurine is an ideal site for modifications that may lead to increasing antiviral activity.[528-532, 536-545]



Tubercidin Toyocamycin Sangivamycin

Figure 4-2. Structures of tubercidin, toyocamycin and sangivamycin

Though the 7-deazapurine analogs are of great interest, their efficient syntheses are still challenging. For example, the preparation of 7-deazaguanine was reported following the synthetic route shown in **Scheme 4-1**.

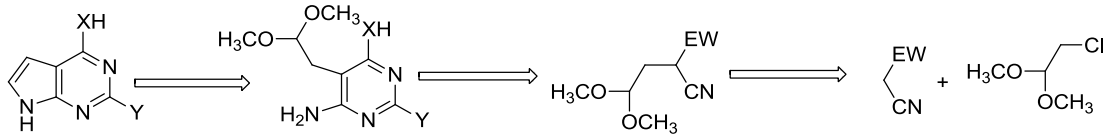


a) Sodium methoxide (2.0 eq.) in MeOH refluxed for 4 h or overnight; b). Sodium acetate trihydrate (2.0 eq.), in water (or DMF) at RT to 80 ^{o}C for 2 h to 2 days. (20% to 86%).

Scheme 4-1. Reported synthesis of 7-deazaguanine

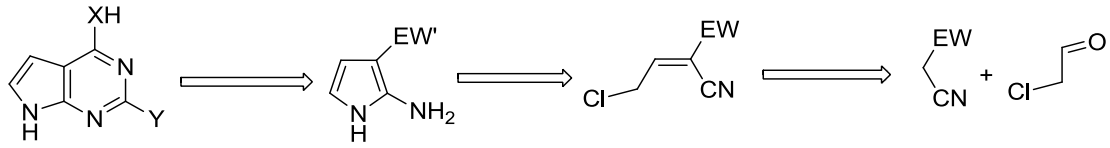
In this approach, the pyrimidine ring is prepared first in moderate yield followed by another cyclization to produce the purine with yields that vary significantly (ranging from 20% to 86%). Attempts to synthesize this compound by our lab resulted in yields ranging between 20 to 40%).[526, 527, 543, 546-551]

Thus, it is worthwhile to develop methods for the chemical syntheses of the 7-deazapurines. To this end, we have explored two synthetic routes to afford a series of 7-deazapurines, which are shown to first form the pyrimidine followed by conversion to purine (shown in **Scheme 4-2)** and from the formation of a pyrrole followed by transformation to purine (shown in **Scheme 4-3**). In the design of the two synthetic methods, synthesis begins with commercially available reagents containing a methylene group adjacent to two electron-withdrawing groups (carboxylate and/or nitrile). In **Scheme 4-2** a reaction between dimethylchloroacetal and ethyl 2-cyanoacetate or malononitrile would afford precursors to enable formation of the pyrimidine ring. Subsequent cyclization would produce 7-deazapurine analogs containing electron-withdrawing groups found in the starting reagents. For the second synthetic method (**Scheme 4-3**) the condensation intermediates from chloroacetaldehyde and ethyl 2-cyanoacetate or malononitrile would undergo cyclization to generate 2-aminopyrroles, which are then converted into the purines.



EW = CO_2Et or CN; X = O or NH; Y = OH, NH_2 or SH

Scheme 4-2. Approach A for preparation of 7-deazapurines



EW = CO_2Et or CN; EW' = $CONH_2$ or $CNHNH_2$; X = O or NH; Y = OH, NH_2 or SH

Scheme 4-3. Approach B for preparation of 7-deazapurines

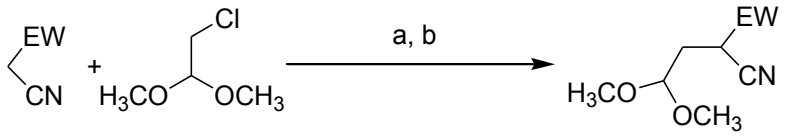
4.2 Experimental

Details of the synthesis and characterization of all compounds in this chapter are described in **Chapter 6.2**.

4.3 Results and Discussion

4.3.1 Pyrimidine Approach for 7-Deazapurine Analogs (Method A)

The precursors, ethyl 2-cyano-4,4-dimethoxybutanoate (**4-1**) and 2-(2,2-dimethoxyethyl)malononitrile (**4-2**) were prepared with modifications to reported procedures (**Figure 4-3**).[534, 536, 552-554] A mixture of ethyl 2-cyanoacetate or malononitrile with sodium methoxide was stirred vigorously at RT for 4 h. The solvent was removed, and the powder was dried in high vacuum. A solution of dimethylchloroacetal in anhydrous THF was added to the solution and refluxed for another 20 hours. The compounds were obtained (EW: 55% for CO_2Et; 61% for CN as yields), and no further purification was performed for the following cyclization steps.



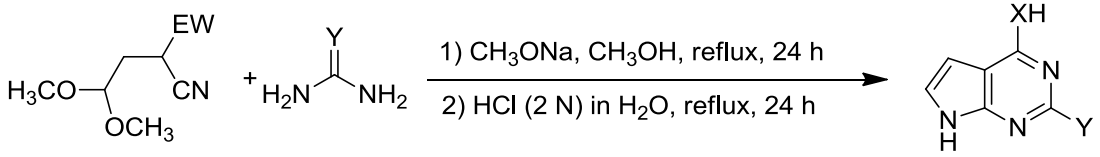
EW = CO_2Et or CN

a) 1.10 eq. sodium hydride in anhydrous DMF at RT for 1 to 4 h; b) 1.10 eq. dimethylchloroacetal in anhydrous DMF, reflux for 20 h.

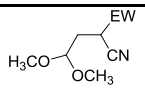
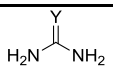
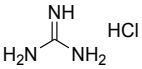
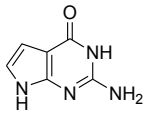
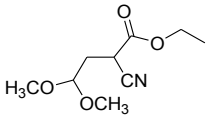
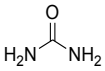
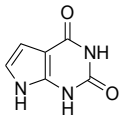
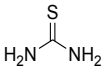
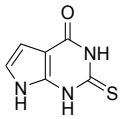
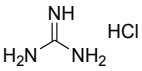
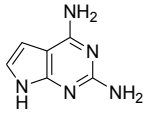
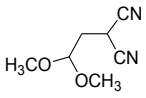
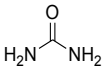
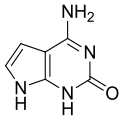
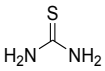
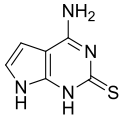
Figure 4-3. Synthesis of ethyl 2-cyano-4,4-dimethoxybutanoate (**4-1**, EW = CO_2Et) and 2-(2,2-dimethoxyethyl)malononitrile (**4-2**, EW = CN)

The formation of pyrimidine precursors was conducted employing ethyl 2-cyano-4, 4-dimethoxybutanoate or 2-(2,2-dimethoxyethyl)malononitrile with 1.0 eq. of guanidine, urea or thiourea and 1.0 eq. of sodium methoxide (2.0 eq. of sodium methoxide for guanidine hydrochloride) in anhydrous MeOH. The solution was refluxed overnight to generate the pyrimidine intermediates.[552] After cooling down to RT, the intermediates were filtered through a short silica gel column to remove the inorganic salts, and then hydrochloric acid (2 N) was added to the solution. The reaction was continued under acidic conditions under reflux for another 24 hours. After cooling down to RT, the brown solution was run through a short silica gel column to yield the various 7-deazapurines. The structures and yields after the two cyclization steps are shown in **Table 4-1**.

Table 4-1. Formation of 7-deazapurines from ethyl 2-cyano-4,4-dimethoxybutanoate and 2-(2,2-dimethoxyethyl)malononitrile

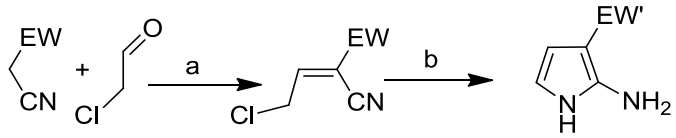


EW = CO_2Et or CN; X = O or NH, Y = OH, NH_2 or SH

Entry			Product	Yield
1			 **4-7**	86%
2	 **4-1**		 **4-8**	81%
3			 **4-9**	89%
4			 **4-10**	79%
5	 **4-2**		 **4-11**	76%
6			 **4-12**	82%

4.3.2 Pyrrole Approach for 7-Deazapurine Analogs (Method B)

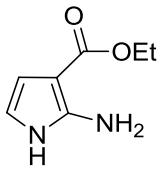
An alternative approach was explored to prepare 7-deazapurines involving the preparation of pyrrole heterocycles that would undergo further conversion into the bicyclic products. Condensation between chloroacetaldehyde and ethyl 2-cyanoacetate or malononitrile was conducted with the catalyst piperidine and acetic acid at RT to reflux for 16 h.[555-566] The product was extracted with EtOAc from aqueous solution, and then concentrated under high vacuum. The reaction of the condensation intermediates in saturated ammonia solution resulted in unsuccessful synthesis of ethyl 2-amino-1H-pyrrole-3-carboxylate and 2-amino-1H-pyrrole-3-carbonitrile (structures shown in **Figure 4-4**). We also employed different concentrations of aqueous ammonia in MeOH for pyrrole cyclization, however, the desired products were not formed. When these syntheses were attempted in a saturated solution of ammonia in anhydrous MeOH at RT for 16 h following with reflux for another 24 h the desired intermediates were obtained as brown powders in good yields (EW: 67% for CO_2Et; 59% for CN). NMR analysis of the products revealed that the electron-withdrawing groups at C3 of pyrrole underwent ammonolysis to afford the amide precursor (structures shown in **Figure 4-5**). Even though the synthesized precursor was different from the expected product (ethyl 2-amino-1H-pyrrole-3-carboxylate), it could still be employed for the following purine formation.



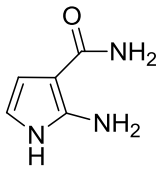
EW = CO_2Et or CN; EW' = $CONH_2$ or CN

a) 0.1 eq. piperidine, 0.1 eq. acetic acid in anhydrous DCM at RT to reflux for 12 h; b) saturated NH_3 (gas) in MeOH, RT 16 hours then reflux for 24 h.

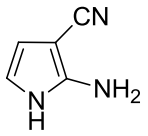
Figure 4-4. Synthesis of 2-amino-1H-pyrrole-3-carboxamide (**4-5**) and 2-amino-1H-pyrrole-3-carboximidamide (**4-6**)



Ethyl 2-amino-1H-pyrrole-3-carboxylate



2-Amino-1H-pyrrole-3-carboxamide (**4-5**)

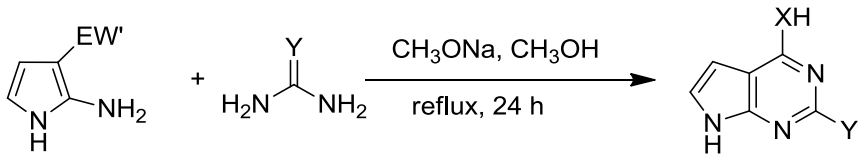


2-Amino-1H-pyrrole-3-carbonitrile (**4-6**)

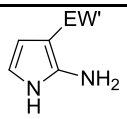
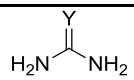
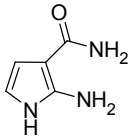
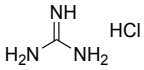
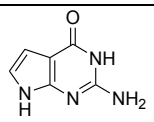
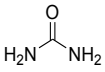
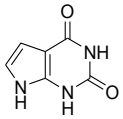
Figure 4-5. Structures of ethyl 2-amino-1H-pyrrole-3-carboxylate, 2-amino-1H-pyrrole-3-carboxamide and 2-amino-1H-pyrrole-3-carbonitrile

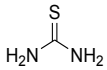
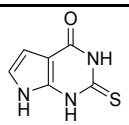
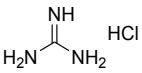
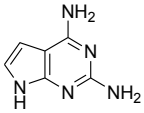
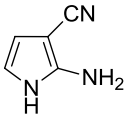
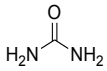
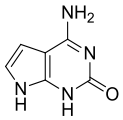
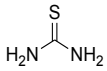
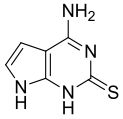
The two intermediates, 2-amino-1H-pyrrole-3-carboxamide (**4-5**) and 2-amino-1H-pyrrole-3-carbonitrile (**4-6**) were then used in reaction with guanidine, urea or thiourea (1.0 eq.) in anhydrous MeOH under reflux for 24 h to generate desired 7-deazapurines. The formed products as well as corresponding isolated yields are shown in Table **4-2**.

Table 4-2. Formation of 7-deazapurines 2-amino-1H-pyrrole-3-carboxamide and 2-amino-1H-pyrrole-3-carboximidamide



EW' = $CONH_2$ or CN; X = O or NH; Y = OH, NH_2 or SH

Entry			Product	Yield
1	 **4-5**		 **4-7**	90%
2			 **4-8**	82%

3			 **4-9**	92%
4			 **4-10**	86%
5	 **4-6**		 **4-11**	79%
6			 **4-12**	86%

4.3.3 Comparison of Pyrimidine (Method A) versus Pyrrole (Method B) Approach for 7-Deazapurine Analogs

Overall, the yields of the products for Method **A** (from pyrimidine to purine in **Scheme 4-2**) were lower compared to the yields obtained for the procedure for the purine cyclization step from the pyrrole precursors (Method **B** shown in **Scheme 4-3**).

In Method **A**, anhydrous solvents such as DMF and MeOH were required for most steps. The final cyclization of the pyrimidines to form the purines required aqueous acid. By

comparison, all the synthetic steps in Method **B** occurred in anhydrous MeOH. Some of the reaction conditions in Method **A** require a higher degree of caution, particularly the step involving sodium hydride (which can ignite in air) which is required for the preparation of intermediates **4-1** or **4-2** whereas the reagents involved in Method **B** were milder.

Throughout the course of this study, the intermediates involved in Method **A** were found to be more stable compared with those in Method **B**. For example, ethyl 4-chloro-2-cyanobut-2-enoate (**4-3**) and 2-(2-chloroethylidene)malononitrile (**4-4**) were prone to polymerization. Decomposition of the two substituted pyrroles, 2-amino-1H-pyrrole-3-carboxamide (**4-5**) and 2-amino-1H-pyrrole-3-carbonitrile (**4-6**) were observed during storage (occurring over a few weeks). These intermediates had to be prepared and used relatively quickly.

Base and acid are employed in both approaches **A** and **B** for the two cyclization steps. In approach **A** the first cyclization to form intermediates **4-1** or **4-2** was conducted under basic conditions to afford the pyrimidines. The formation of the purines from the pyrimidines employed acid. The conditions are reversed in approach **B**. Cyclization from intermediate **4-5** or **4-6** was conducted under acidic conditions first, followed by saturated ammonia in anhydrous MeOH. The formation of purines from pyrroles employed base.

Some of the intermediates and products in both methods were challenging to purify. In approach **A** the purification to obtain the non-cyclic intermediates **4-1** or **4-2** by column chromatography required a gradient of mixtures of solvents as eluent since TLC analysis revealed similar R_f values of substituted intermediates and unreacted starting reagents. In particular, the purification of purines **4-7** to **4-12** was difficult as the R_f values were similar to the unreacted pyrrole starting materials in approach **B**.

4.4 Summary

In summary, two efficient and convergent synthetic routes for the preparation of a series of 7-deazapurines were explored. These molecules were synthesized based on two cyclization strategies, either from pyrimidine to purine or from pyrrole to purine to enable the preparation of a wide range of 7-deazapurine analogs.

Chapter 5. Synthesis of cross-linked nucleosides containing 7-deaza-2'-deoxyguanosine

5.1 Introduction

Cellular DNA is susceptible to damage by endogenous and environmental agents that may cause modifications such as interstrand cross-links. This can either occur on the same strand (intrastrand cross-link) or on the opposite strands of DNA (interstrand cross-link). Interstrand cross-links (ICL) in DNA exert significant biological effects by preventing replication and transcription, which cause cell death if the cross-link is not repaired.[235, 316, 317, 319, 350, 425, 450, 451, 567-571]

ICL can also be introduced in DNA as a consequence of the action of cancer chemotherapeutic agents that kill cancer cells. There are a number of bifunctional alkylating agents which react with DNA including dialdehydes that are produced in the cell and chemotherapeutics, i.e., 1,3-bis(2-chloroethyl)-1-nitrosourea (also known as BCNU or carmustine in **Figure 5-1a**), mechlorethamine (**Figure 5-1b**) and hepsulfam. Treatment of DNA duplexes with these bifunctional alkylating agents often results in a broad spectrum of alkylated DNA products, with only a small percentage of ICL duplex produced. These alkylating agents react to form ICL at different positions in DNA duplexes (**Figure 5-1c-1f**). [189, 235, 270, 317, 333, 425]

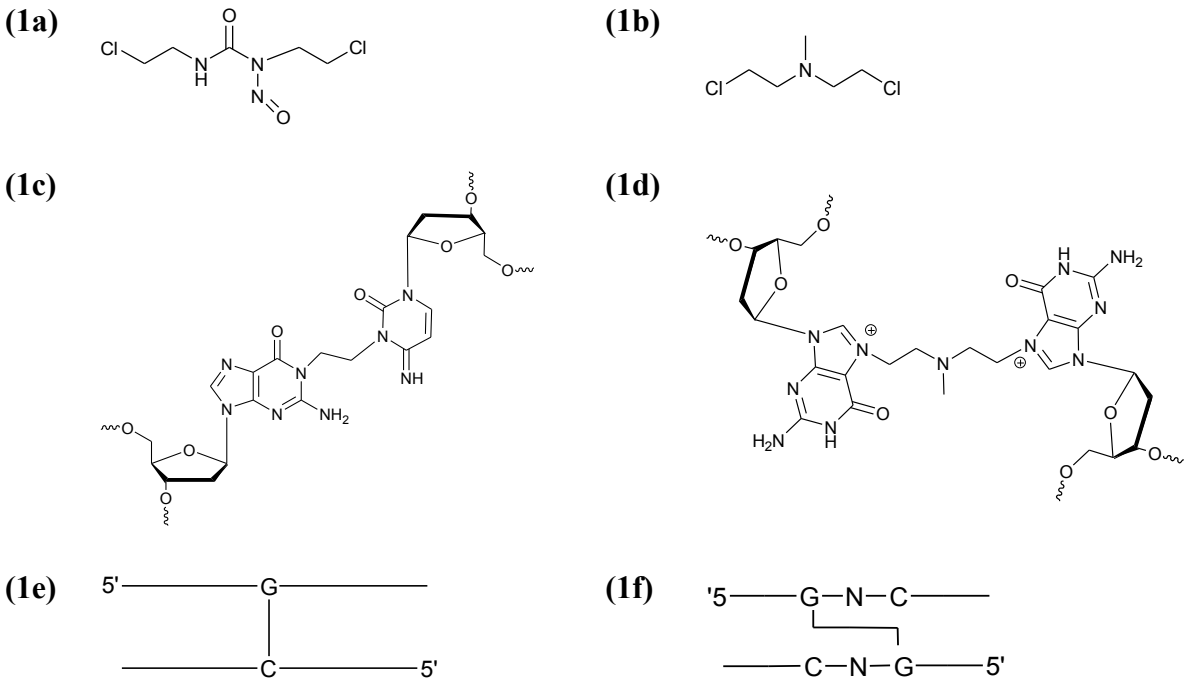


Figure 5-1. The molecular structures of BCNU (1a), mechlorethamine (1b) and the relevant ICL oligonucleotides ((1c/1e): *N*1-dG-alkylene-*N*3-dC; (1d/1f): *N*7-dG-alkylene-*N*7-dG).

Development of tumors, resistant to these agents is a factor in the lack of response in some patients, with removal of ICL believed to play a role in resistance. These lesions are eliminated from DNA by a mechanism involving excision repair and recombination (for example in *E. coli* and in yeast).[450, 451] In eukaryotic cells, the precise role of excision repair in eliminating cross-links is not yet clearly understood.[235, 317, 471, 568-571]

While alkylating agents react at numerous positions in DNA, to our knowledge, the *N*7-position of guanine is the most reactive of all the nucleophilic sites in DNA.[144, 189, 238, 240, 241, 246, 270, 322, 426-430, 530] Hundreds of DNA adducts have been

identified addressing a number of issues such as the relationships between DNA adducts produced by exposure to chemical agents with mutagenesis and other biological phenomena. The covalent binding to DNA and the ability to form *N*7-guanine adducts has been considered as evidence of genotoxicity for several drugs and chemicals.

The main obstacle in studying the mutagenic potency of *N*7-guanine adducts is their chemical instability which has prevented systematic investigation of the lesions in site-directed mutagenesis studies. The instability of *N*7-guanine adducts is created by the formal placement of an additional positive charge on the guanine ring system. The alkylated guanines undergo a further reaction resulting in cleavage of the *N*-glycosyl bond (**Figure 5-2**). This depurination creates an abasic site in the DNA and in the case of bifunctional alkylating agents and effectively cleaves the ICL formed. Alternatively, the imidazole ring of the alkylated guanine can undergo hydrolysis to produce a FAPY derivative, which is relatively resistant to further chemical reaction (**Figure 5-2**).[189, 235, 322, 431-434]

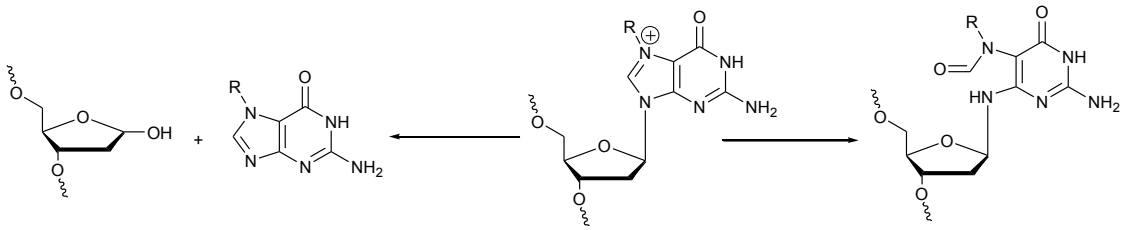


Figure 5-2. Consequences of *N*7-2'-deoxyguanosine alkylation: Depurination (left) and FAPY formation (right).

The investigation of ICL removal by repair enzymes requires access to substrates containing this damage. One way in which ICL containing DNA can be prepared involves direct treatment of DNA with bi-functional alkylating agents. However, this approach is not optimal as: (1) Some DNA adducts formed are difficult to isolate and are chemically unstable, making repair and structural studies challenging. (2) Some alkylating agents lack target specificity and can react with numerous nucleophilic sites within DNA [235].

Another approach to investigate ICL repair involves the use of chemically synthesized substrates of well defined structure. Such substrates are challenging to prepare in quantities sufficient for biochemical and structural studies. Employing a combination of solution and solid-phase synthesis, ICL DNA that are mimics of clinically relevant lesions formed during chemotherapy can be produced.

To overcome the instability of main adduct with the treatment of alkylating agents, we have attempted to produce subtle changes via a synthesis technique combining solution and solid-phase synthesis by introducing a 7-deaza-2'-deoxyguanosine residue. The removal of the nitrogen lone pair electrons should reduce the electrostatic charge in the vicinity of a major groove site with high cation occupancy.[452, 572] Besides, the introduction of alkylene linkers to C7 sites of 7-deaza-2'-deoxyguanosines enables the prepared ICL duplexes containing a chemically stable moiety to mimic the alkylene lesions formed in duplex DNA as a result of treatment with alkylating drugs such as mechlorethamine and hepsulfam [333, 425]. These structural analogs will link the *C*7

atoms of two 7-deaza-2'-deoxyguanosine (*C*7-dG) nucleosides with an alkylene linker and will be incorporated into DNA by solid-phase synthesis (**Figure 5-3**).

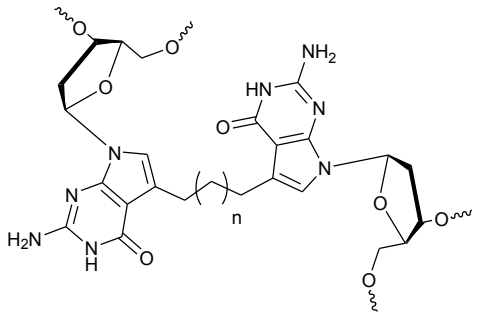


Figure 5-3. The *C*7-dG-alkylene-dG-*C*7 ICL oligonucleotides (n= 2~5).

For the preparation of the cross-linked dimer containing 7-deazaguanine moieties, the precursor 5'-*O*-dimethoxytrityl-7-iodo-7-deazaguanosine needs to be synthesized in adequate quantities. The synthesis started from 7-deazaguanine, and followed **Scheme 5-1** to afford two expected precursors 5'-*O*-dimethoxytrityl(DMT)-7-iodo-7-deaza-2'-deoxyguanosine and 5'-*O*-dimethoxytrityl(DMT)-3'-*O*-*tert*-butyldimethylsilyl(TBS)-7-iodo-7-deaza-2'-deoxyguanosine. The classic approach for preparation of 7-iodo-7-deaza-2'-deoxyguanosine was to convert the 7-deaza-2'-deoxyguanosine to the 6-chloro compound, which was suitable for the better selectivity on C7 than C8 of 7-deaza-2'-deoxyguanosine. The 6-chloro nucleoside was then converted back to the keto derivative by hydrolysis.[573-579] In addition to the extra synthetic steps, the 6-chloro derivatives

had very poor solubility characteristics, which confounded their functionalization.[575, 579-581]

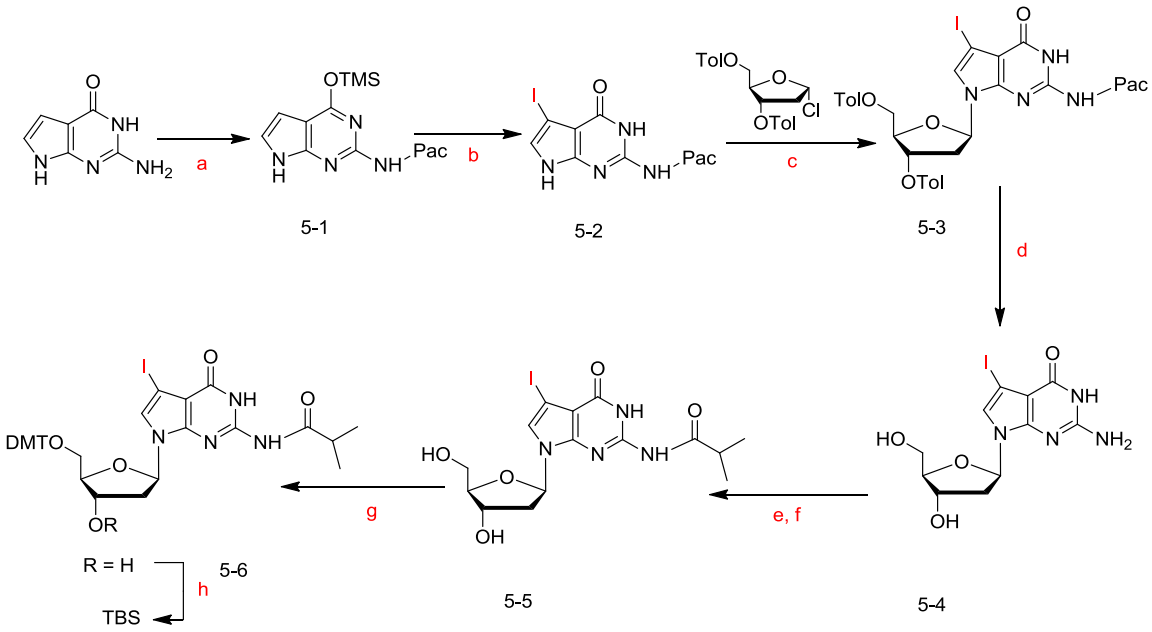
According to **Scheme 5-1**, 7-iodo-7-deazaguanine was prepared by protecting the 6-oxo group with trimethylsilyl chloride and treatment with phenoxyacetyl chloride. After the glycosylation, introduction of protective groups was performed to produce 5'-*O*-dimethoxytrityl-7-iodo-7-deaza-2'-deoxyguanosine and 5'-*O*-dimethoxytrityl-3'-*O*-*tert*-butyldimethylsilyl-7-iodo-7-deaza-2'-deoxyguanosine.

The alkylene linker to be placed between the two 7-deazaguanines was introduced by the palladium-catalyzed Sonogashira reaction followed with hydrogenation to convert the linker from an alkynylene into alkylene functionality. In order to perform the Sonogashira reaction at the C7 of 7-deaza-2'-deoxyguanosine, the C7 site was first activated with an iodo group. To enhance the reactivity and selectivity (competitive with C8) of iodinated substitution at the C7 position of the 7-deazaguanine heterocyclic moiety, the 6-oxo group has to be masked.[523, 582] At this position the labile protective group TMS was introduced while the 2-amine was protected with a phenoxyacetyl (Pac). After the substitution of the iodo atom at the C7 site, the workup procedure to remove the high boiling-point solvent (DMF) resulted in the cleavage of TMS at the 6-oxo position.

In order to synthesize the Sonogashira substrate 5'-*O*-DMT-7-iodo-7-deaza-2'-deoxyguanosine, the modified Robins *N*-glycosylation method was employed. Glycolysation of 7-iodo-7-deazaguanine (*N*Pac) with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-β-D-erythro-pentofuranosyl was performed via a one-pot reaction in anhydrous acetonitrile to afford the β anomer nucleoside at the C1' atom of the

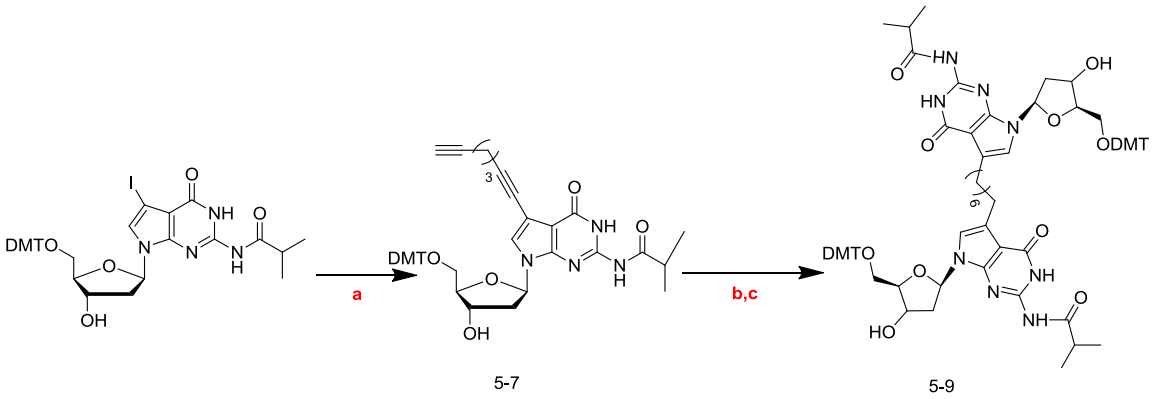
deoxyribofuranose only. Afterwards, the protective toluoyl group was removed with a saturated solution of ammonia in anhydrous MeOH while also removing the Pac group from the amine at the 2-position. Before the attachment of DMT to the 5'-*O* of 7-deaza-2'-deoxyguanosine, the isobutyryl (*i*Bu) protective group was added to the exocyclic amine. A portion of 5'-*O*-DMT-7-iodo-7-deaza-2'-deoxyguanosine was set aside for the synthesis of 5'-*O*-DMT-3'-*O*-TBS-7-iodo-7-deaza-2'-deoxyguanosine.

The introduction of the linker to the C7 site of 7-deaza-2'-deoxyguanosine was performed with the Sonogashira reaction (shown in **Scheme 5-2**). This reaction was conducted with palladium and copper catalyst under mild basic condition to enable the formation of a new sp^2-sp carbon-carbon bond.[535, 583-586] A concern was that the DMT may be removed at higher temperature, so the coupling reaction was carried out at 40 ^{o}C under argon atmosphere. However, it was observed the TBS protective group was removed under the conditions of the Sonogashira reaction. The two precursors 5'-*O*-DMT-7-iodo-7-deaza-2'deoxyguanosine and 5'-*O*-DMT-3'-*O*-TBS-7-iodo-7-deaza-2'-deoxyguanosine produced the same monomer product 5'-*O*-DMT-7-alkynylene-7-deaza-2'deoxyguanosine. This monomer was then reacted with 5'-*O*-DMT-7-iodo-7-deaza-2'-deoxyguanosine via a second Sonogashira reaction, and alkynylene linker was established between the two 7-deaza-2'-deoxyguanosines. Hydrogenation to reduce the triple-bond linker to corresponding alkylene was attempted with catalyst Pd/C under hydrogen atmosphere (60 psi).



a) 3.0 eq. TMSCl, in anhydrous pyridine at RT for 1.5 h, then 1.5 eq. PacCl was added and the reaction proceeded overnight at RT; b) 1.3 eq. iodine in DMF at RT for overnight, 71% for steps a and b; c) NaH (2.5 eq.), anhydrous acetonitrile at RT for 12 h, 66%; d) NH_3/CH_3OH at RT overnight; e) 6.0 eq. TMSCl, anhydrous pyridine at RT for 2 h, then 1.5 eq. *i*BuCl overnight at RT; f) NH_3/CH_3OH (sat.) ice-bath for 30 min. or TBAF/THF (cat.), 10 eq. KF in MeOH for 5 hours; g) 1.2 eq. DMTCl, in anhydrous pyridine at RT overnight, 42% for steps d to g; h) 2.0 eq. TBSCl, 4.0 eq. imidazole, in anhydrous DMF at RT for overnight, 91%.

Scheme 5-1. Synthesis of 5'-*O*-DMT-7-iodo-7-deaza-2'-deoxyguanosine.



a) 4.0 eq. 1,6-heptadiyne, 0.02 eq. $PdCl_2(PPh_3)_2$, 0.1 eq. CuI, 0.1 eq. PPh_3 in Et_3N/DMF at 40 ^oC for 60 h, 86%; b) 0.02 eq. $PdCl_2(PPh_3)_2$, 0.1 eq. CuI , 0.1 eq. PPh_3 in Et_3N/DMF at 40 ^oC for 60 h; c) 60 psi H_2, 100 mg Pd/C (10%) in 30 mL MeOH at RT for 60 h.

Scheme 5-2. Synthesis of C7 cross-linked dimer containing 5'-*O*-DMT-7-deaza-2'-deoxyguanosine.

5.2 Experimental Procedures

Details of the synthesis and characterization of all compounds in this chapter are described in **Chapter 6.3**.

5.3 Results and discussion

In this chapter a synthetic strategy to prepare a cross-linked dimer containing two 7-deaza-2'-deoxyguanosines was explored. A key intermediate connects the C7 atoms of 7-deaza-2'-deoxyguanosines with sp^2-sp^3 bonds to an alkylene chain. This adduct would enable further synthesis of a cross-linked dimer phosphoramidite which would be

incorporated into DNA to produce interstrand cross-linked oligonucleotides by solid-phase synthesis to mimic the lesions formed in DNA by some chemotherapeutic agents.

Starting from 7-deazaguanine prepared in **Chapter 4**, a phenoxyacetyl group was attached to the 2-amino group while the 6-oxo position was protected with TMS before iodination of the C7 site. The lability of TMS on the 6-oxo group resulted in *N*-(5-iodo-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-2-phenoxyacetamide as the product obtained after workup. This substrate was coupled with 1-chloro-3,5-di-*O*-toluoyl-2-deoxy-α-D-ribofuranose at C1' in anhydrous acetonitrile which produced the β anomer only. Toluolyl protective groups on both 3'-*O* and 5'-*O* atoms were removed with a saturated solution of ammonia in anhydrous MeOH, and three more synthetic steps were conducted before the attachment of the DMT protective group at the 5'-*O* position.

Overall, the multi-step procedure to produce protected nucleoside (5'-*O*-DMT-7-iodo-7-deaza-2'-deoxyguanosine) gave the expected products at each step in moderate yields. To our knowledge, the Sonogashira reaction is more sensitive requiring inert atmosphere compared to the Heck, Stille, Suzuki and Hiyama coupling reactions. The reaction system was purged with argon three times to remove the trace oxygen/air to avoid side reactions. Normally the coupling reactions are performed at higher temperature for better conversions, however, thermal instability of the DMT group was a concern when the linker was introduced at the C7 of 7-iodo-7-deaza-2'-deoxyguanosine. The yields to attach the alkylene linker to 7-iodo-7-deaza-2'-deoxyguanosine by Sonogashira coupling were lower compared with other synthetic strategies (such as nucleophilic substitution reaction, Mitsunobu reaction explored by our group and others) to introduce ICL.

Two substrates 7-iodo-7-deaza-5'-*O*-DMT-2'-deoxyguanosine (*Ni*Bu) and 7-iodo-7-deaza-5'-*O*-DMT-3'-*O*-TBS-2'-deoxyguanosine (*Ni*Bu), were employed for the Sonogashira coupling reaction. In **Chapter 3** we studied the synthetic strategy to produce ICL duplexes containing assymetric nucleotide composition around a cross-linked site with the selective removal of compatible protective groups (DMT, TBS and Alloc) and sequential nucleotide extensions. 5'-*O*-DMT-3'-*O*-TBS-7-iodo-7-deaza-2'-deoxyguanosine (*Ni*Bu) was first explored for the coupling with the diyne linker. A cross-linked dimer with different compatible protective groups on the 3'-*O* and 5'-*O* groups would enable the preparation of ICL duplexes containing partially or completely asymmetric sequences around cross-linked sites. In this chapter, we planned to synthesize a cross-linked dimer containing two different protective groups on 5'-*O* and 3'-*O* (two DMT on 5'-*O* and one TBS on 3'-*O*). It has been reported that silyl protective groups can be cleaved under the conditions of Pd-catalyzed hydrogenation.[587-590] We observed that the TBS protective group was unstable under the Sonogashira reaction conditions. The Sonogashira coupling reaction was first conducted with the catalyst system of bis(triphenylphosphine)palladium(*II*) dichloride, copper (I) iodide under the conditions of Et_3N/DMF (v/v, 0.5:2). NMR characterization of the product isolated using 5'-*O*-DMT-3'-*O*-TBS-7-iodo-7-deaza-2'-deoxyguanosine (*Ni*Bu) as starting material indicated that the TBS group was cleaved. The attempt to reduce the alkalinity of the reaction system (the ratio of Et_3N to DMF was 0.1:2) still resulted in the removal of TBS. The same product was obtained from both 5'-*O*-DMT-3'-*O*-TBS-7-iodo-7-deaza-2'-deoxyguanosine (*Ni*Bu) and 5'-*O*-DMT-2'-7-iodo-7-deaza-2'-deoxyguanosine (*Ni*Bu).

The attempt to form the cross-linked dimer by a second Sonogashira coupling reaction was not successful. The conversion of the Sonogashira coupling performed between the two monomers 5'-*O*-DMT-7-iodo-7-deaza-2'deoxyguanosine (*Ni*Bu) (**5-6**) and 5'-*O*-DMT-2'-deoxy-7-(1,6-heptadiynyl)-7-deazaguanosine (**5-7**) did not proceed in high yield for the formation of the dimer. One possible reason is the steric hindrance of the moieties (both containing DMT protective groups) which may have resulted in lower accessibility of palladium catalyst to the C7 sites. To form the linkage by the Sonogashira coupling reaction, the palladium (0) core would be centered between four bulky groups, two triphenylphosphine and two 5'-*O*-DMT-7-deaza-2'deoxyguanosines in the transition state. NMR analysis of what was isolated from the reaction indicated that monomer(s) and dimer co-existed in the final product.

We attempted to proceed with the synthesis of phosphoramidite(s) with the hydrogenated compound(s), however ^{31}P NMR data also showed the mixture of one monomer and dimer from 5 peaks around 148 ppm.

5.4 Summary

In this chapter, 7-iodo-7-deazaguanine was coupled with 1-chloro-3,5-di-*O*-toluoyl-2-deoxy-α-D-ribofuranose. After the removal of the toluoyl protective groups, the precursor 5'-*O*-DMT-7-iodo-7-deaza-2'-deoxyguanosine was produced in moderate yield. The Sonogashira coupling reaction was performed on 7-iodo-7-deazaguanosine to introduce the C7-alkynyl linker to form the monomer. Attempts to form a cross-linked 7-

deazaguanosine dimer containing a heptamethylene linker *via* a second Sonogashira reaction followed by palladium-catalyzed hydrogenation were not successful.

Chapter 6. Experimental Procedures

6.1 Experimental procedure for preparation of the *N*3-thymidine-butylene-*N*3-thymidine dimer

6.1.1 Materials and General Methods

5'-*O*-Levulinoyl-thymidine, 5'-*O*-dimethoxytrityl-thymidine, 3'-*O*-dimethoxytrityl-2'-deoxyribonucleoside-5'-*O*-(β-cyanoethyl-*N*,*N*'-diisopropyl) phosphoramidites and *N*,*N*-diisopropylamino cyanoethyl phosphonamidic chloride were purchased from ChemGenes Inc. (Wilmington, MA). 5'-*O*-Dimethoxytrityl-2'-deoxyribonucleoside-3'-*O*-(β-cyanoethyl-*N*,*N*'-diisopropyl)phosphoramidites, protected 2'-deoxyribonucleoside-polystyrene supports and ancillary reagents for solid-phase synthesis were purchased from Glen Research (Sterling, Virginia). Allyl chloroformate, *n*-butylamine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), formic acid, 1-hydroxybenzotriazole hydrate, hydrazine hydrate, levulinic acid, phenoxyacetyl chloride, *tert*-butyldimethylsilyl chloride (TBS-Cl), *p*-toluene sulfonic acid (*p*-TsOH), diisopropylethylamine (DIPEA), 4-dimethylaminopyridine (DMAP), triphenylphosphine, sodium *N*,*N*-diethydithiocarmbamate, tetrakis(triphenyl phosphine)palladium(0) and all other chemicals and solvents for synthesis were purchased from the Aldrich Chemical Company (Milwaukee, WI).

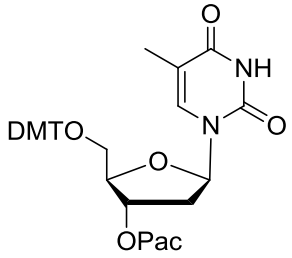
5'-*O*-Dimethoxytrityl-3'-*O*-phenoxyacetyl-thymidine, 5'-*O*-dimethoxy trityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine, and *N*3-(4-iodobutyl)-5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-

butyldimethylsilyl)-thymidine were all prepared with minor modifications to published procedures.[425]

Flash column chromatography was performed using silica gel 60 (230–400 mesh) obtained from Silicycle (Quebec City, QC). Thin layer chromatography (TLC) was performed using precoated TLC plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) purchased from EMD Chemicals Inc. from (Gibbstown, NJ). All solvents for column chromatography were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). ^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 MHz NMR spectrometer at RT at frequencies of 499.9 and 125.7 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts were reported in parts per million downfield from tetramethylsilane. ^{31}P NMR spectra (^{1}H decoupled) were recorded at a frequency of 202.4 MHz with H_3PO_4 used as an external standard.

6.1.2 Synthesis of Cross-Linked Phosphoramidites

Compound 3-1. 5'-*O*-Dimethoxytrityl-3'-*O*-phenoxyacetyl-thymidine



The title compound was prepared as previously described with the yield 96%.[425]

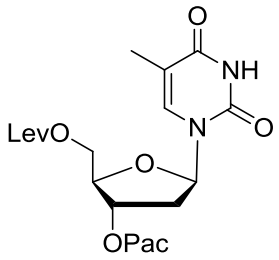
R_f (SiO_2 TLC): 0.42 in MeOH/DCM (1:19).

^{1}H NMR ($CDCl_3$, ppm): δ 1.31 (s, 3H, C5-CH_3), 2.36-2.40 (ddd, *J* = 14.5, 8.0, 5.5 Hz, 1H, 2'-H), 2.39-2.44 (ddd, *J* = 14.5, 6.0, 5.5 Hz, 1H, 2"-H), 3.37-3.41 (dd, *J* = 10.5, 8.0 Hz, 1H, 5'-H), 3.41-3.44 (dd, *J* = 10.5, 2.5 Hz, 1H, 5"-H), 3.72 (s, 6H, -OCH_3 of DMT), 4.04-4.07 (ddd, J = 5.5, 5.5, 5.5 Hz, 1H, 3'-H), 4.59 (s, 2H, CH_2 of Pac), 5.48-5.49 (ddd, 8.0, 5.5, 2.5, 1H, 4'-H), 6.33-6.35 (dd, *J* =8.0, 6.0 Hz, 1H, 1'-H), 6.76-6.94 (m, 7H, Phs of DMT and Pac), 7.17-7.31 (m, 11H, Phs of DMT and Pac), 7.53 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): δ 11.65, 37.82, 55.27, 63.58, 65.18, 76.41, 83.94, 84.27, 87.28, 111.80, 113.35, 114.63, 122.03, 127.27, 128.07, 128.11, 129.68, 130.06, 130.11, 135.10, 135.15, 135.25, 144.16, 150.45, 157.54, 158.80, 150.82, 163.58, 168.54.

ESI-MS ($M+Na^+$): consistent with the reported value. [425]

Compound 3-2. 5'-*O*-Levulinoyl-3'-*O*-phenoxyacetyl-thymidine



5'-*O*-Dimethoxytrityl-3'-*O*-phenoxyacetyl-thymidine (1.738 g, 2.560 mmol, 1.0 eq.) was dissolved in a mixture of DCM/CH_3OH (4:1, 50 mL). Then, *p*-TsOH (1.071 g, 5.632 mmol, 2.2 eq.) was added at RT. After 30 min, the reaction was diluted with DCM (200 mL) and then washed with aqueous $NaHCO_3$ (5%, 100 mL). The organic layer was washed with distilled water (100 mL), and dried over sodium sulfate and concentrated to

give a colorless gum. The gum was then dissolved in pyridine (16 mL). EDC (0.697 g, 3.648 mmol, 2.0 eq.) and levulinic acid (0.424 g, 3.648 mmol, 2.0 eq.) were added at RT. After 18 h, the solvent was removed *in vacuo*, the crude product was taken up in DCM (50 mL) and the solution was washed with aqueous $NaHCO_3$ (3%, 50 mL). The organic layer was dried over sodium sulfate and concentrated to give a yellowish gum. The crude product was purified by silica gel column chromatography using hexane/EtOAc (49:1) as eluent to afford 0.822 g (95%) of product as a colorless foam.

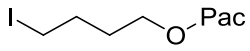
R_f (SiO_2 TLC): 0.28 in hexane/EtOAc (1:1).

^{1}H NMR ($CDCl_3$, ppm): δ 1.87 (s, 3H, C5-CH_3), 2.12 (s, 3H, CH_3 of Lev), 2.17-2.22 (ddd, J = 14.5, 5.5, 1.5 Hz, 1H, H2'), 2.39-2.44 (ddd, J = 14.5, 8.0, 6.5 Hz, 1H, H2"), 2.50-2.53 (t, J = 6.5 Hz, 2H, CH_2 of Lev), 2.72-2.75 (t, J = 6.5 Hz, 2H, CH_2 of Lev), 4.16-4.18 (dd, J = 12.0, 3.0 Hz, 1H, H5'), 4.24-4.27 (ddd, J = 9.0, 3.5, 3.0 Hz, 1H, H4'), 4.38-4.42 (dd, J = 12.0, 3.5 Hz, 1H, H5"), 4.62 (s, 2H, CH_2 of Pac), 5.27-5.30 (ddd, J = 9.0, 6.5, 1.5 Hz, 1H, H3'), 6.20-6.23 (dd, J = 8.0, 5.5 Hz, 1H, H1'), 6.84-6.96 (m, 3H, Ph of Pac), 7.20-7.25 (m, 2H, Ph of Pac), 7.28 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): 12.60, 27.81, 29.72, 37.21, 37.83, 63.71, 65.12, 75.20, 82.12, 84.71, 111.67, 114.64, 122.05, 129.69, 134.78, 150.46, 157.53, 163.77, 168.59, 172.26, 206.46.

ESI-MS ($M+Na^+$): 497.1540 (calcd. 497.1541).

Compound 3-3. 4-Iodobutyl-1-phenoxyacetate



Phenoxyacetyl chloride (3.412 g, 20 mmol, 1.0 eq.) and potassium iodide (8.300 g, 50 mmol, 2.5 eq.) were dissolved in THF (50 mL) at RT. After 24 h, $NaHCO_3$ (1.681g, 20 mmol, 1.0 eq.) was added slowly to the solution. The solvent was removed *in vacuo*, the crude product was taken up with DCM (100 mL) and the reaction quenched with aqueous $NaHCO_3$ (5%, 100 mL). The organic layer was washed with distilled water (100 mL × 2), dried over sodium sulfate and concentrated to give a viscous liquid. The crude product was purified by silica gel column chromatography using hexane/EtOAc (1:1) as eluent to afford 4.26 g (95%) of product.

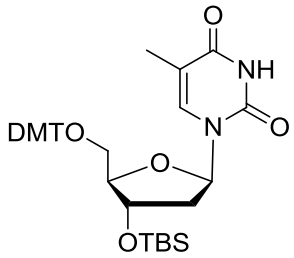
R_f (SiO_2 TLC): 0.76 in hexane/EtOAc (1:1).

^{1}H NMR ($CDCl_3$, ppm): δ 1.63-1.71 (tt, J = 8.0, 6.5 Hz, 2H, CH_2), 1.72-1.79 (tt, J = 8.0, 6.5 Hz, 2H, CH_2), 3.08-3.10 (t, J = 6.5 Hz, 2H, CH_2), 4.15-4.17 (t, J = 6.5 Hz, 2H, CH_2), 4.56 (s, 2H, OCH_2 of Pac), 6.83-6.85 (dd, J = 8.5, 0.5 Hz, 2H, Ph of Pac), 6.93-6.94 (dd, J = 7.0, 0.5 Hz, 1H, Ph of Pac), 7.19-7.23 (dd, J = 8.5, 7.0 Hz, 2H, Ph of Pac).

^{13}C NMR ($CDCl_3$, ppm): 5.9, 29.4, 29.8, 64.0, 65.3, 114.6, 114.6, 121.8, 129.6, 129.6, 157.8, 169.0.

ESI-MS ($M+K^+$): 372.9655 (calcd. 372.9703).

Compound 3-4. 5'-*O*-Dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine



The title compound was prepared as previously described in quantitative yield. [425]

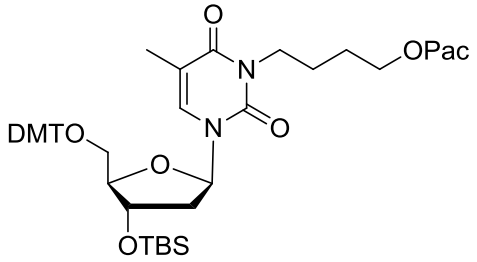
R_f (SiO_2 TLC): 0.44 in hexane/EtOAc (1:1).

^{1}H NMR ($CDCl_3$, ppm): δ 0.00, 0.05 (2s, 6H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 1.53 (s, 3H, C5-CH_3), 2.24-2.28 (ddd, J = 13.0, 6.5, 6.5 Hz, 1H, 2'-H), 2.34-2.36 (ddd, J = 13.0, 6.5, 3.0 Hz, 1H, 2"-H), 3.29-3.31 (dd, J = 10.5, 3.0 Hz, 1H, 5'-H), 3.49-3.52 (dd, J = 10.5, 3.0 Hz, 1H, 5"-H), 3.82 (s, 6H, OCH_3 of DMT), 4.00-4.01 (ddd, J = 6.5, 6.5, 3.0 Hz, 1H, 3'-H), 4.55-4.56 (ddd, J = 6.5, 3.0, 3.0 Hz, 1H, 4'-H), 6.38-6.40 (dd, J = 6.5, 6.5 Hz, 1H, 1'-H), 6.86-6.88 (m, 4H, Ph of DMT), 7.13-7.45 (m, 9H, Ph of DMT), 7.69 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): -4.87, -4.67, 11.89, 17.94, 25.72, 41.56, 55.25, 62.94, 72.10, 84.90, 86.80, 86.84, 111.01, 113.29, 127.96, 128.14, 130.05, 130.07, 135.46, 135.50, 135.63, 144.35, 150.31, 158.76, 163.84.

MS: consistent with the reported value. [425]

Compound 3-5. *N*3-[4-(Phenoxyacetyl)butyl]-5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine



The title compound was prepared as previously described with a yield of 84%.[425]

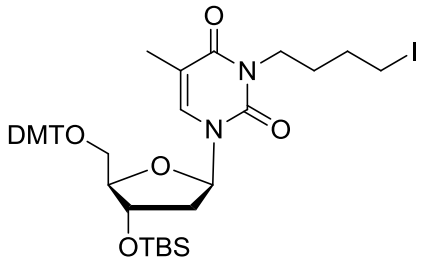
R_f (SiO_2 TLC): 0.63 in hexane/EtOAc (1:1).

^{1}H NMR ($CDCl_3$, ppm): δ 0.00, 0.06 (2s, 6H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 1.21-1.24 (m, 2H, CH_2 of butyl), 1.56 (s, 3H, C5-CH_3), 1.71-1.74 (m, 2H, CH_2 of butyl), 2.22-2.28 (ddd, *J* = 14.5, 6.5, 6.5 Hz, 1H, 2'-H), 2.33-2.38 (ddd, *J* = 14.5, 3.0, 3.0 Hz, 1H, 2"-H), 3.29-3.32 (dd, *J* = 10.5, 2.5 Hz, 1H, 5'-H), 3.47-3.50 (ddd, *J* = 6.5, 3.0, 3.0 Hz, 1H, 3'-H), 3.49-3.51 (t, *J* = 7.5 Hz, 2H, CH_2 of butyl), 3.80 (s, 6H, OCH_3 of DMT), 3.98-4.01 (t, *J* = 7.5 Hz, 2H, CH_2 of butyl), 4.24-4.27 (dd, *J* = 10.5, 5.5 Hz, 1H, 5"-H), 4.54-4.56 (ddd, *J* = 5.5, 3.0, 2.5 Hz, 1H, 4'-H), 4.64 (s, 2H, OCH_2 of Pac), 6.33-6.45 (dd, *J* = 6.5, 3.0 Hz, 1H, 1'-H). 6.85-6.94 (m, 4H, Ph of DMT), 6.97-7.00 (m, 3H, Ph of Pac), 7.26-7.33 (m, 9H, Phs of DMT and Pac), 7.34-7.46 (m, 2H, Ph of DMT), 7.69 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): -4.87, -4.65, 12.69, 15.30, 17.93, 24.20, 25.72, 40.72, 41.62, 55.23, 62.90, 64.89, 65.27, 72.07, 85.48, 86.71, 86.82, 110.20, 113.25, 113.27, 114.58, 114.66, 121.64, 127.12, 127.98, 128.13, 129.54, 129.59, 130.05, 130.08, 133.64, 135.43, 135.48, 144.37, 150.82, 157.83, 158.72, 163.42, 168.98.

ESI-MS ($M+Na^+$): consistent with the reported value. [425]

Compound 3-6. *N*3-(4-Iodobutyl)-5'-*O*-dimethoxytrityl-3'-*O*-(*tert*-butyldimethylsilyl)-thymidine



The title compound was prepared as previously described with a yield of 71%.[425]

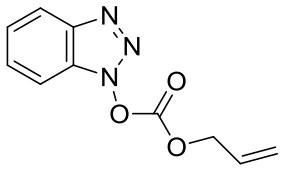
R_f (SiO_2 TLC): 0.71 in hexane/EtOAc (1:1).

^{1}H NMR ($CDCl_3$, ppm): δ 0.00, 0.06 (2s, 6H, $SiCH_3$), 0.87 (s, 9H, $SiC(CH_3)_3$), 1.60 (s, 3H, C5-CH_3), 1.78-1.82 (m, 2H, CH_2 of butyl), 1.89-1.93 (m, 2H, CH_2 of butyl), 2.22-2.26 (ddd, *J* = 13.0, 6.5, 6.5 Hz, 1H, 2'-H), 2.36-2.39 (ddd, *J* = 13.0, 7.0, 6.5 Hz, 1H, 2″-H), 3.24-3.27 (t, *J* = 7.0 Hz, 2H, CH_2 of butyl), 3.28-3.31 (dd, *J* = 11.0, 3.0 Hz, 1H, 5'-H), 3.48-3.51 (dd, *J* = 11.0, 3.0 Hz, 1H, 5″-H), 3.83 (s, 6H, OCH_3 of DMT), 3.98-4.02 (ddd, *J* = 6.5, 6.5, 6.5,Hz, 1H, 3'-H), 4.00-4.03 (t, *J* = 7.0, 2H, CH_2 of butyl), 4.53-4.55 (ddd, *J* = 6.5, 3.0, 3.0 Hz, 1H, 4'-H), 6.40-6.42 (dd, *J* = 7.0, 6.5 Hz, 1H, 1'-H), 6.86-6.88 (m, 4H, Ph of DMT), 7.26-7.35 (m, 7H, Ph of DMT), 7.44-7.45 (m, 2H, Ph of DMT), 7.68 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): -4.87, -4.66, 5.96, 12.69, 17.93, 25.71, 28.72, 30.90, 40.08, 41.62, 55.26, 62.90, 72.05, 85.50, 86.72, 86.80, 110.21, 113.24, 113.26, 127.11, 127.97, 128.12, 130.04, 130.07, 133.62, 135.43, 135.48, 144.34, 150.82, 158.70, 163.45.

ESI-MS ($M+Na^+$): consistent with previously reported value. [425]

Compound 3-7. Allyl 1-hydroxybenzotriazole carbonate



1-Hydroxybenzotriazole hydrate (6.06 g, 39.6 mmol, 1 eq.) was dissolved in THF (10 mL) followed by the removal of the solvent *in vacuo*. The same operation was repeated twice. The white powder was dissolved in THF (30 mL). DIPEA (5.76 g, 44.6 mmol, 1.12 eq.) was added, followed by the addition of allyl chloroformate (5.12 g, 41.2 mmol, 1.04 eq.). After 30 min, the white precipitate was filtered off and washed with THF (20 mL×2). The combined organic layer was collected and concentrated *in vacuo*.[2] The crude product was purified by silica gel column chromatography using hexane/EtOAc (1:1) as eluent to give 6.85 g (79%) of product.

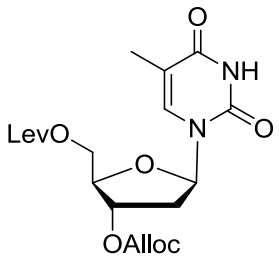
R_f (SiO_2 TLC): 0.40 in hexane/EtOAc (1:1).

^1H NMR ($CDCl_3$, ppm): δ 5.03-5.04 (d, J = 6.0 Hz, 2H, OCH_2 of Allyl), 5.42-5.44 (dd, J = 11.0, 0.5 Hz, 1H, CH of Allyl), 5.54-5.57, (dd, J = 16.0, 0.5 Hz, 1H, CH of Allyl), 6.05-6.13 (ddt, J = 16.0, 11.0, 6.0 Hz, 1H, CH of Allyl), 7.55-7.58 (m, 1H, Ph), 7.78-7.80 (m, 1H, Ph), 8.03-8.04 (m, 1H, Ph), 8.22-8.24 (m, 1H, Ph).

^{13}C NMR ($CDCl_3$, ppm): 69.43, 115.10, 115.81, 120.96, 126.37, 126.43, 130.09, 132.78, 133.41, 147.17.

ESI-MS ($M+Na^+$): consistent with the commercial reagent's data.

Compound 3-8. 5'-*O*-Levulinoyl-3'-*O*-allyloxycarbonyl-thymidine



5'-*O*-Levulinoyl-thymidine (1.000 g, 2.938 mmol, 1 eq.), allyl 1-hydroxy benzotriazole carbonate (0.858 g, 3.908 mmol, 1.33 eq.) and DMAP (0.072 g, 0.588 mmol, 0.2 eq.) were dissolved in THF/pyridine (9:1, 100 mL) at RT. After 24 h, the solvent was removed *in vacuo* and the crude was taken up in DCM (50 mL). The organic layer was washed with aqueous $NaHCO_3$ (5%, 50 mL) and dried over sodium sulfate. After being concentrated *in vacuo*, the crude product was purified by silica gel column chromatography using hexane/EtOAc (3:7) as eluent to afford 0.824 g (66 %) of product.

R_f (SiO_2 TLC): 0.47 in hexane/EtOAc (3:7).

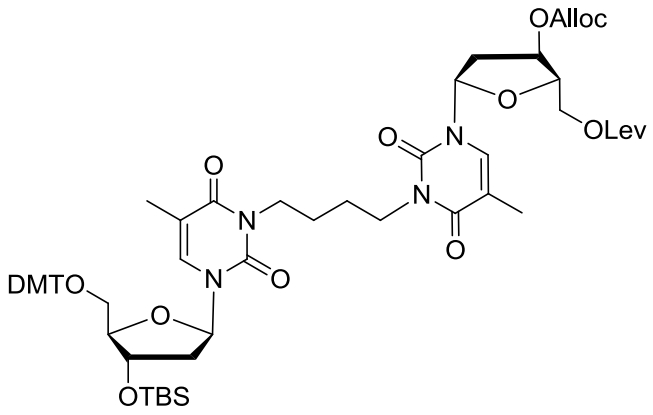
^{1}H NMR ($CDCl_3$, ppm): δ 1.86 (s, 3H, C5-CH_3), 2.12 (s, 3H, CH_3 of Lev), 2.17-2.22 (ddd, J = 14.5, 8.5, 6.5 Hz, 1H, H2'), 2.47-2.51 (ddd, J = 14.5, 5.5, 1.5 Hz, 1H, H2″), 2.51-2.53 (t, J = 6.0 Hz, 2H, CH_2 of Lev), 2.73-2.75 (t, J = 6.0 Hz, 2H, CH_2 of Lev), 4.25-4.27 (ddd, J = 3.5, 3.0, 3.0 Hz, 1H, H4'), 4.26-4.29 (dd, J = 12.0, 3.5 Hz, 1H, H5'),

4.38-4.41 (dd, J = 12.0, 3.0 Hz, 1H, H5"), 4.57-4.58 (d, J = 5.5 Hz, 2H, CH_2 of Alloc), 5.09-5.11 (ddd, J = 6.5, 3.0, 1.5 Hz, 1H, H3"), 5.22-5.25 (dd, J = 10.5, 1.0 Hz, 1H, CH of Alloc), 5.30-5.33 (dd. J = 17.0, 1.0 Hz, 1H, CH of Alloc), 5.82-5.90 (ddt, J = 17.0, 10.5, 5.5 Hz, 1H, CH of Alloc), 6.25-6.27 (dd, J = 8.5, 5.5 Hz, 1H, H1'), 7.29 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): 12.58, 27.80, 29.71, 37.36, 37.80, 63.91, 68.98, 77.63, 82.01, 84.79, 111.50, 119.53, 131.00, 134.86, 150.40, 154.17, 163.90, 172.26, 206.40.

ESI-MS ($M+Na^+$): 447.1373 (calcd. 447.1380).

Compound 3-9. 1-{*N*3-[5'-*O*-(Dimethoxytrityl)-3'-*O*-(*tert*-butyldimethylsilyl)-thymidinyl]}-4-{*N*3-[5'-*O*-(levulinoyl)-3'-*O*-(allyloxycarbonyl)-thymidinyl]}butane



Compound **3-6** (0.841 g, 1.000 mmol, 1 eq.) and compound **3-8** (0.468 g, 1.100 mmol, 1.1 eq.) were dissolved in acetonitrile (30 mL) at RT. Then DBU (0.305 g, 2.000 mmol, 2.0 eq.) was added dropwise. After 24 h, the solvent was removed *in vacuo*, the crude product was taken up in DCM (100 mL) and the solution was washed with three portions of aqueous $NaHCO_3$ (5%, 100mL). The organic layer was dried over sodium sulfate, and

the crude product was purified by silica gel column chromatography using hexane/EtOAc (1:1 to 3:7) as eluent to afford 0.773 g (68%) of product.

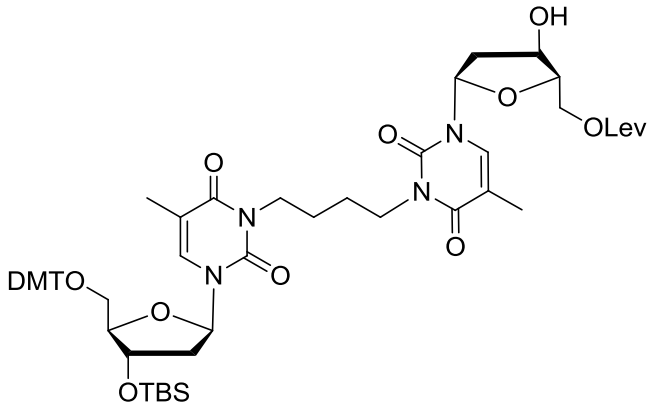
R_f (SiO_2 TLC): 0.69 in hexane/EtOAc (3:7).

^{1}H NMR ($CDCl_3$, ppm): δ 0.00 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 1.53 (s, 3H, C5-CH_3), 1.72-1.74 (m, 4H, CH_2 of butyl), 1.98 (s, 3H, C5-CH_3), 2.23 (s, 3H, CH_3 of Lev), 2.23-2.25 (m, 2H, 2 x H2'), 2.26-2.29 (m, 2H, 2 x H2"), 2.62-2.66 (t, *J* = 6.0 Hz, 2H, CH_2 of Lev), 2.82-2.85 (t, *J* = 6.0 Hz, 2H, CH_2 of Lev), 3.28-3.30 (dd, *J* = 14.0, 2.5 Hz, 1H, H5'), 3.50-3.52 (dd, 14.0, 2.5 Hz, 1H, H5"), 3.82 (s, 6H, OCH_3 of DMT), 3.97-3.99 (m, 1H, H4'), 3.98-4.01 (t, *J* = 7.0 Hz, 4H, CH_2 of butyl), 4.35-4.39 (m, 2H, H4' and H5'), 4.53-4.57 (m, 2H, H3' and H5"), 4.67-4.69 (d, *J* = 5.5 Hz, 2H, OCH_2 of Alloc), 5.18-5.21 (m, 1H, H3'), 5.32-5.35 (dd, *J* = 10.5, 0.5 Hz, 1H, CH of Alloc), 5.40-5.44 (dd, *J* = 17.0, 0.5 Hz, 1H, CH of Alloc), 5.97-6.01 (ddt, *J* = 17.0, 10.5, 5.5, Hz, 1H, CH of Alloc). 6.38-6.41 (m, 2H, 2 x H1'), 6.86-6.89 (m, 4H, Ph of DMT), 7.27-7.44 (m, 9H, Ph of DMT), 7.46 (s, 1H, H6), 7.69 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): -4.90, -4.65, 12.67, 13.37, 17.92, 25.30, 25.71, 27.82, 29.73, 37.52, 37.81, 41.01, 41.15, 41.64, 55.25, 62.83, 63.92, 68.96, 71.94, 77.68, 81.89, 85.39, 85.41, 86.60, 86.76, 110.18, 110.64, 113.23, 113.26, 119.53, 127.08, 127.97, 128.14, 130.03, 130.07, 131.02, 132.70, 133.51, 135.45, 135.52, 144.34, 150.74, 150.83, 154.19, 158.68, 163.23, 163.47, 172.23, 206.22.

ESI-MS ($M+Na^+$): 1159.4924 (calcd. 1159.4923).

Compound 3-10. 1-{*N*3-[5'-*O*-(Dimethoxytrityl)-3'-*O*-(*tert*-butyldimethylsilyl)-thymidinyl]}-4-{*N*3-[5'-*O*-(levulinoyl)-thymidinyl]}butane



Compound **3-9** (0.436 g, 0.384 mmol, 1.0 eq.), triphenylphosphine (0.020 g, 0.077 mmol, 0.20 eq.) and tetrakis(triphenylphosphine)palladium(0) (0.044 g, 0.038 mmol, 0.10 eq.) were dissolved in THF (10 mL) at RT, followed by the addition of buffer composed of *n*-butylamine (50 eq.) and formic acid (50 eq.). After 2.5 h, the solvent was removed *in vacuo*, the crude residue was taken up in DCM (100 mL) and the solution was washed with aqueous NaCl (sat., 100 mL). The organic layer was dried over sodium sulfate and concentrated for silica gel column chromatography using hexane/EtOAc (3:7 to 1:9) as eluent. The product was obtained as colorless foam in a yield of 0.400 g (100%).

R_f (SiO_2 TLC): 0.32 in hexane/EtOAc (2:8).

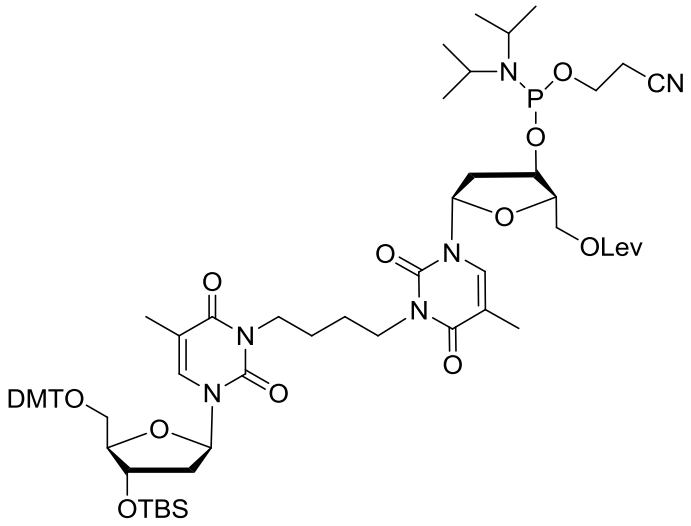
^{1}H NMR ($CDCl_3$, ppm): δ 0.00 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 1.22-1.25 (m, 2H, CH_2 of butylene), 1.53 (s, 3H, C5-CH_3), 1.71-1.73 (m, 4H, CH_2 of butylene), 1.96 (s, 3H, C5-CH_3), 2.15-2.21 (m, 1H, H2'), 2.22 (s, 3H, CH_3 of Lev), 2.23-2.28 (m, 1H, H2"), 2.35-2.38 (m, 1H, H2'), 2.44-2.47 (m, 1H, H2"), 2.60-2.63 (t, J = 6.5 Hz, 2H, CH_2 of Lev), 2.81-2.84 (t, J = 6.5 Hz, 2H, CH_2 of Lev), 3.28-3.31 (dd,

J = 14.0, 3.0, 1H, H5'), 3.50-3.53 (dd, *J* = 14.0, 7.0, 1H, H5"), 3.82 (s, 6H, OCH_3 of DMT), 3.96-3.98 (m, 1H, H4'), 3.97-4.01 (2 t, *J* = 7.0 Hz, 4H, CH_2 of butyl), 4.12-4.14 (m, 1H, H4'), 4.29-4.32 (m, 2H, H5' and H5"), 4.39-4.45 (m, 1H, H3'), 4.54-4.57 (m, 1H, H3'), 6.36-6.41 (m, 2H, 2 x H1'), 6.86-6.88 (m, 4H, Ph of DMT), 7.32-7.59 (m, 9H, Ph of DMT), 7.69 (s, 1H, H6), 7.71 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): -4.9, -4.7, 12.7, 13., 17.9, 25.3, 25.4, 25.7, 27.9, 29.8, 37.9, 40.3, 41.0, 41.6, 55.3, 62.8, 63.8, 70.9, 71.9, 84.0, 85.3, 85.4, 86.6, 86.8, 110.2, 112.9, 113.2, 127.1, 127.6, 127.8, 128.0, 128.1, 128.3, 128.5, 128.6, 129.7, 130.0, 130.1, 131.7, 132.0, 132.1, 133.3, 133.6, 135.4, 135.5, 144.3, 150.7, 150.8, 158.7, 163.4, 163.5, 172.6, 206.7.

ESI-MS ($M+Na^+$): 1075.4720 (calcd. 1075.4712).

Compound 3-11. 1-{*N*3-[5'-*O*-(Dimethoxytrityl)-3'-*O*-(*tert*-butyldimethylsilyl)-thymidinyl]}-4-{*N*3-[5'-*O*-(levulinoyl)-thymidinyl-3'-*O*-(β-cyanoethyl *N*,*N*'-diisopropyl) phosphoramidite]}butane



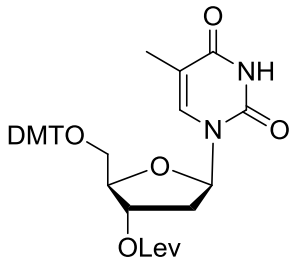
Compound **3-10** (0.291 g, 0.276 mmol, 1.0 eq.) was dissolved in THF (1.25 mL) followed with the dropwise addition of DIPEA (0.054 g, 0.414 mmol, 1.5 eq.) and *N*,*N*-diisopropylamino cyanoethyl phosphonamidic chloride (0.078 g, 0.331 mmol, 1.2 eq.) at RT. After 30 min, the reaction was diluted with EtOAc (50 mL). The organic layer was washed twice with aqueous $NaHCO_3$ (3%, 100 mL) followed by aqueous NaCl (sat., 100 mL), then dried over sodium sulfate. After concentration *in vacuo*, the gum was precipitated from hexane to yield 0.246 g (71%) of product.

R_f (SiO_2 TLC): 0.82 in hexane/EtOAc (2:8).

^{31}P NMR (Acetone-d_6, ppm): 148.39, 148.61.

ESI-MS ($M+Na^+$): 1275.5786 (calcd. 1275.5790).

Compound 3-12. 5'-*O*-Dimethoxytrityl-3'-*O*-levulinoyl-thymidine



5'-*O*-Dimethoxytrityl-thymidine (5.446 g, 10.00 mmol, 1.0 eq.), EDC (3.820 g, 20.00 mmol, 2.0 eq.), DMAP (0.012 g, 0.10 mmol, 0.01 eq.) and levulinic acid (2.322 g, 20.00 mmol, 2.0 eq.) were dissolved in 1,4-dioxane (60 mL) at RT. After 12 h the solvent was removed *in vacuo*, the residue was taken up in DCM (100 mL) and the solution was washed with aqueous $NaHCO_3$ (5%, 100 mL). The organic layer was dried over sodium sulfate, and concentrated for purification by silica gel column chromatography. The product was isolated as a colorless foam in a yield of 6.42 g (99%).

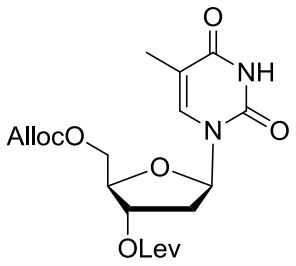
R_f (SiO_2 TLC): 0.40 in hexane/EtOAc (1: 9).

^{1}H NMR ($CDCl_3$, ppm): δ 1.29 (s, 3H, C5-CH_3), 2.12 (s, 3H, CH_3 of Lev), 2.32-2.38 (ddd, *J* = 13.5, 7.0, 6.0 Hz, 1H, H2'), 2.58-2.62 (ddd, *J* = 13.5, 5.5, 4.5 Hz, 1H, H2"), 2.63-2.66 (t, *J* = 6.5 Hz, 2H, CH_2 of Lev), 2.67-2.70 (*J* = 6.5 Hz, 2H, CH_2 of Lev), 3.35-3.38 (dd, *J* = 13.0, 2.5 Hz, 1H, H5'), 3.39-3.42 (dd, *J* = 13.0, 2.5 Hz, 1H, H5"), 3.72 (s, 6H, OCH_3 of DMT), 4.06 (ddd, *J* = 5.0, 2.5, 2.5, Hz, 1H, H4'), 5.38-5.40 (ddd, *J* = 6.0, 5.0, 4.5 Hz, 1H, H3'), 6.36-6.38 (dd, *J* = 7.0, 5.5 Hz, 1H, H1'), 6.75-6.77 (m, 4H, Ph of DMT), 7.17-7.31 (m, 9H, Ph of DMT), 7.53 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): 11.58, 27.96, 29.79, 37.79, 37.86, 55.26, 63.73, 75.67, 83.98, 84.32, 87.19, 111.62, 113.32, 127.22, 128.03, 128.13, 130.08, 130.12, 135.15, 135.24, 135.46, 144.21, 150.40, 158.76, 158.77, 163.60, 172.24, 206.30.

ESI-MS ($M+Na^+$): 665.2483 (calcd. 665.2475).

Compound 3-13. 5'-*O*-Allyloxycarbonyl-3'-*O*-levulinoyl-thymidine



Compound **3-12** (2.310 g, 3.594 mmol, 1.0 eq.) was dissolved in a mixture of DCM/CH_3OH (4:1, 75 mL). Then, *p*-TsOH (1.505 g, 7.910 mmol, 2.2 eq.) was added at RT. After 30 min, the solution was diluted with DCM (300 mL) and then washed with aqueous $NaHCO_3$ (5%, 150 mL). The organic layer was washed with distilled water (150 mL), and dried over sodium sulfate and concentrated to give a colorless gum. The residue was purified by silica gel column chromatography using CH_3OH/DCM (1:9) as eluent to afford the pure intermediate. This intermediate (0.620 g, 1.822 mmol, 1.0 eq.) was then dissolved in a mixture of pyridine/THF (9:1, 70 ml) to which was added allyl 1-hydroxybenzotriazole carbonate **3-7** (0.531 g, 2.423 mmol, 1.33 eq.) and DMAP (0.045 g, 0.364 mmol, 0.2 eq.) at RT. After 24 h, the solvent was removed *in vacuo*, the residue was taken up in DCM (100 mL) and the solution was washed with aqueous $NaHCO_3$

(5%, 100 mL). The organic layer then was dried over sodium sulfate. The concentrated residue was purified by silica gel column chromatography using hexane/EtOAc (7:3) as eluent. The isolated product was obtained with a yield of 1.295 g (85%).

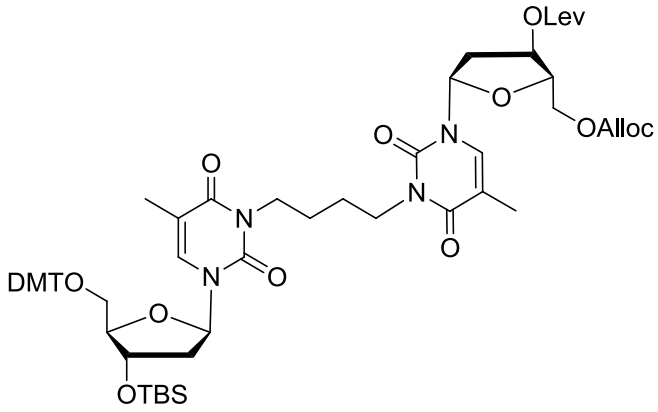
R_f (SiO_2 TLC): 0.72 in hexane/EtOAc (7:3)

^{1}H NMR ($CDCl_3$, ppm): δ 1.93 (s, 3H, CH_3-C5), 2.21 (s, 3H, CH_3 of Lev), 2.22-2.27 (ddd, *J* = 14.0, 8.5, 6.5 Hz, 1H, H2'), 2.41-2.45 (ddd, *J* = 14.0, 5.5, 1.5 Hz, 1H, H2"), 2.58-2.61 (t, *J* = 6.5Hz, 2H, CH_2 of Lev), 2.77-2.80 (t, *J* = 6.5Hz, 2H, CH_2 of Lev), 3.76-3.81 (dd, *J* = 11.5, 2.5 Hz, 1H, H5'), 4.24-4.26 (ddd, *J* = 5.0, 2.5, 2.5 Hz, 1H, H4'), 4.41-4.44 (dd, *J* = 11.5, 2.5 Hz, 1H, H5"), 4.66-4.68 (d, *J* = 6.0 Hz, 2H, CH_2 of Alloc), 5.26-5.30 (ddd, *J* = 6.5, 5.0, 1.5 Hz, 1H, H3'), 5.30-5.33 (dd, *J* = 10.5, 1.0 Hz, 1H, CH of Alloc), 5.37-5.40 (dd, *J* = 17.0, 1.0 Hz, 1H, CH of Alloc), 5.90-5.96 (ddt, *J* = 17.0, 10.5, 6.0 Hz, 1H, CH of Alloc), 6.40-6.43 (dd, *J* = 8.5, 5.5 Hz, 1H, H1'), 7.42 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): 12.55, 27.86, 29.74, 37.15, 37.77, 67.34, 69.04, 74.74, 82.08, 84.48, 111.74, 119.73, 131.04, 134.98, 150.62, 154.39, 163.82, 172.40, 206.40.

ESI-MS ($M+Na^+$): 447.1375 (calcd. 447.1380).

Compound 3-14. 1-{*N*3-[5'-*O*-(Dimethoxytrityl)-3'-*O*-(*tert*-butyldimethylsilyl)-thymidinyl]}-4-{*N*3-[5'-*O*-(allyloxycarbonyl)-3'-*O*-(levulinoyl)-thymidinyl]}butane



Compound **3-6** (0.452 g, 0.537 mmol, 1.0 eq.) and compound **3-13** (0.251 g, 0.592 mmol, 1.1 eq.) were dissolved in acetonitrile (20 mL) and DBU (0.164 g, 1.074 mmol, 2.0 eq.) added at RT. After 48 h, the solvent was removed *in vacuo*, the residue was taken up with DCM (100 mL) and the solution was washed with aqueous $NaHCO_3$ (5%, 100 mL). The organic layer was dried over sodium sulfate and concentrated, then purified by silica gel column chromatography with a gradient of hexane/EtOAc (5:5 to 1:9) as eluent. The product was obtained with a yield of 0.336 g (55 %).

R_f (SiO_2 TLC): 0.76 in hexane/EtOAc (1:9).

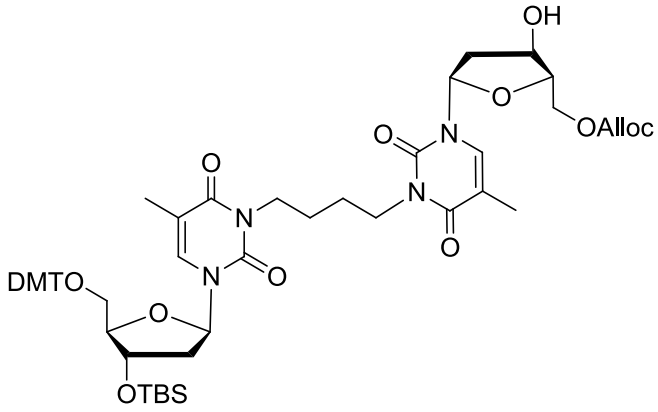
^{1}H NMR ($CDCl_3$, ppm): δ 0.00 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 1.53 (s, 3H, C5-CH_3), 1.71-1.74 (m, 4H, CH_2 of butyl), 1.95 (s, 3H, C5-CH_3), 2.24 (s, 3H, CH_3 of Lev), 2.23-2.25 (m, 1H, H2'), 2.23-2.27 (m, 1H, H2"), 2.36-2.39 (m, 1H, H2'), 2.46-2.50 (m, 1H, H2"), 2.62-2.64 (t, J = 6 Hz, 2H, CH_2 of Lev), 2.80-2.83 (t, J = 6 Hz, 2H, CH_2 of Lev), 3.28-3.30 (dd, J = 10.5, 1.5 Hz, 1H, H5'), 3.50-3.52 (dd, J = 10.5, 1.5 Hz, 1H, H5''), 3.83 (s, 6H, OCH_3 of DMT), 3.99-4.02 (m, 1H, H4'), 3.99-4.02 (t, J = 5.5 Hz, 4H, CH_2 of butyl), 4.21-4.24 (m, 1H, H4'), 4.43-4.48 (m, 2H, H5' and H5"), 4.54-4.57 (m, 1H, H3'), 4.70-4.72 (d, J = 6.0 Hz, 2H, OCH_2 of Alloc),

5.31-5.34 (m, 1H, H3'), 5.33-5.36 (dd, *J* = 10.5, 1.0 Hz, 1H, CH of Alloc), 5.40-5.44 (dd, *J* = 17.0, 1.0 Hz, 1H, CH of Alloc), 5.95-6.02 (ddt, *J* = 17.0, 10.5, 6.0 Hz, 1H, CH of Alloc), 6.38-6.42 (m, 2H, 2 x H1'), 6.87-6.89 (m, 4H, Ph of DMT), 7.28-7.44 (m, 9H, Ph of DMT), 7.46 (s, 1H, H6), 7.67 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): -4.89, -4.66, 12.67. 13.27, 17.92, 25.31, 25.71, 27.90, 29.73, 37.24, 37.79, 41.02, 41.17, 41.64, 55.26, 62.90, 67.30, 69.00, 72.01, 74.76, 82.01, 85.15, 85.45, 86.63, 86.79, 110.18, 110.84, 113.26, 113.29, 119.63, 127.08, 127.96, 128.17, 130.05, 130.08, 131.12, 132.87, 133.48, 135.52, 135.58, 144.38, 150.85, 150.95, 154.46, 158.73, 163.22, 163.46, 172.31, 206.08.

ESI-MS ($M+Na^+$): 1159.4929 (calcd. 1159.4923).

Compound 3-15. 1-{*N*3-[5'-*O*-(Dimethoxytrityl)-3'-*O*-(*tert*-butyldimethylsilyl)-thymidinyl]}-4-{*N*3-[5'-*O*-(allyloxycarbonyl)-thymidinyl]}butane



Compound **3-14** (0.441 g, 0.388 mmol, 1.0 eq.) was treated with 10 mL HPAA (0.5 M hydrazine in a buffer composed of 9 mL pyridine and 1 mL glacial acetic acid) at RT. After 10 min, the solution was diluted with DCM (100 mL) and washed with aqueous $NaHCO_3$ (5%, 100 mL). The water phase was extracted twice with DCM (100 mL). The combined organic layer was dried over sodium sulfate and concentrated, then purified by silica gel column chromatography with a solvent system of hexane/EtOAc (1:9). The product was obtained with a yield of 0.387 g (96%).

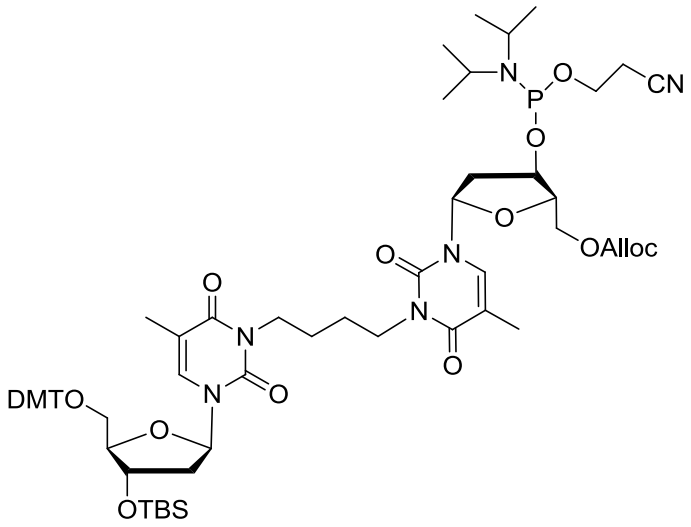
R_f (SiO_2 TLC): 0.56 in hexane/EtOAc (1:9).

^{1}H NMR ($CDCl_3$, ppm): δ 0.00 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 1.53 (s, 3H, C5-CH_3), 1.71-1.73 (m, 4H, CH_2 of butyl), 1.95 (s, 3H, C5-CH_3), 2.23-2.29 (m, 2H, H2' and H2"), 2.35-2.38 (m, 1H, H2'), 2.41-2.45 (m, 1H, H2"), 3.27-3.31 (dd, *J* = 14.0, 3.0 Hz, 1H, H5'), 3.50-3.54 (dd, *J* = 14.0, 3.5 Hz, 1H, H5''), 3.83 (s, 6H, OCH_3 of DMT), 3.97-4.00 (m, 1H, H4'), 3.98-4.00 (t, *J* = 6.0 Hz, 4H, CH_2 of butyl), 4.15-4.18 (m, 1H, H4'), 4.45-4.48 (m, 2H, H5' and H5"), 4.50-4.53 (m, 2H, 2 x H3'), 4.69-4.71 (d, *J* = 6.0 Hz, 2H, OCH_2 of Alloc), 5.52-5.55 (dd, *J* = 10.5, 1.0 Hz, 1H, CH of Alloc), 5.58-5.62 (dd, *J* = 17.0, 1.0 Hz, 1H, CH of Alloc), 5.94-6.01 (ddt, J = 17.0, 10.5, 6.0 Hz, 1H, CH of Alloc). 6.39-6.42 (m, 2H, 2 x H1'), 6.87-6.89 (m, 4H, Ph of DMT), 7.29-7.44 (m, 9H, Ph of DMT), 7.46 (s, 1H, H6), 7.68 (s, 1H, H6).

^{13}C NMR ($CDCl_3$, ppm): -4.89, -4.65, 12.68, 13.29, 15.27, 17.93, 25.31, 25.71, 40.61, 41.05, 41.08, 41.65, 55.27, 62.84, 66.72, 69.02, 71.22, 83.79, 85.32, 85.42, 86.61, 86.77, 110.19, 110.38, 113.24, 113.27, 119.66, 127.10, 127.98, 128.15, 130.04, 130.08, 131.08, 133.24, 133.55, 135.46, 135.53, 144.34, 150.82, 150.83, 154.76, 158.69, 163.40, 163.52.

ESI-MS ($M+Na^+$): 1061.4567 (calcd. 1061.4555).

Compound 3-16. 1-{*N*3-[5'-*O*-(Dimethoxytrityl)-3'-*O*-(*tert*-butyldimethylsilyl)-thymidinyl]}-4-{*N*3-[5'-*O*-(allyloxycarbonyl)-thymidinyl-3'-*O*-(β-cyanoethyl *N*,*N*'-diisopropyl) phosphoramidite]}butane



Compound **3-15** (0.317 g, 0.305 mmol, 1.0 eq.) was dissolved in anhydrous THF (1.25 mL). Then, DIPEA (0.059 g, 0.458 mmol, 1.5 eq.) followed by *N*,*N*-diisopropylamino cyanoethyl phosphonamidic chloride (0.087 g, 0.366 mmol, 1.2 eq.) were added. After 30 min, the reaction was diluted with EtOAc (50 mL). The organic layer was washed with aqueous $NaHCO_3$ (3%, 100 mL ×2) followed by aqueous NaCl (sat., 100 mL), then dried over sodium sulfate. After concentration *in vacuo*, the gum was precipitated from hexane to yield 0.269 g (71%) of product as a colorless foam.

R_f (SiO_2 TLC): 0.81 in hexane/EtOAc (2:8).

^{31}P NMR (Acetone-d_6, ppm): 146.71, 146.97.

ESI-MS ($M+Na^+$): 1261.5638 (calcd. 1261.5634).

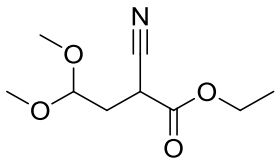
6.2 Experimental procedure for the development of the cyclization strategies to synthesize 7-deazapurine and related analogs

6.2.1 Materials and General Methods

All chemicals and solvents for synthesis were purchased from the Aldrich Chemical Company. 2-Chloroacetaldehyde (50% in H_2O) was distilled to remove the water present prior to the Knoevenagel condensation.

6.2.2 Experimental procedures for syntheses of 7-deazapurines and related analogs

Compound 4-1. Ethyl 2-cyano-4,4-dimethoxybutanoate



To a solution of ethyl 2-cyanoacetate (11.3110 g, 100 mmol, 1.00 eq.) in anhydrous DMF (50 mL) was added sodium hydride (2.6400 g, 110 mmol, 1.10 eq.) in portions. The solution was stirred at RT for 4 h under a drying tube. 2-Chloro-1,1-dimethoxyethane (13.7027 g, 110 mmol, 1.10 eq.) and a crystal of potassium iodide were added to the solution, and stirred at 100 ^{o}C for 20 h under a drying tube. The solution was cooled, and

diluted with distilled H_2O (200 mL). The crude product was extracted with EtOAc (100 mL × 5). The organic phase was washed with brine (50 ml), dried over sodium sulfate, and filtered. The filtrate was concentrated *in vacuo* for the further purification by silica gel column chromatography using a gradient of hexane/EtOAc (9:1 to 7:3) as eluent to afford the product as amber liquid (11.0710 g, 51%).

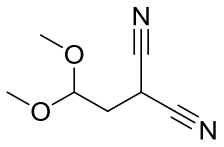
R_f (SiO_2 TLC): 0.15 in hexane/EtOAc (7:3).

^{1}H NMR ($CDCl_3$, ppm): δ 1.34-1.37 (t, J = 7.0 Hz, 3H, CH_3 of OEt), 2.26-2.30, 2.32-2.36 (m, 2H, CH_2 of $CH_2CH(OCH_3)_2$), 3.36 and 3.38 (2s, 6H, CH_3 of $CH(OCH_3)_2$), 4.24-4.26 (q, J = 7.0 Hz, 2H, CH_2 of OEt), 4.28-4.32 (dd, J = 7.5, 7.0 Hz, 1H, CH of $CH(CO_2Et)(CN)$), 4.56-4.58 (dd, J = 6.5, 5.0 Hz, 1H, CH of $CH(OCH_3)_2$).

^{13}CNMR ($CDCl_3$, ppm): δ 13.91, 32.62, 33.36, 54.00, 54.02, 62.87, 101.76, 116.25, 165.76.

ESI-MS ($M+Na^+$): 224.0903 (calc. 224.0899)

Compound 4-2. 2-(2,2-dimethoxyethyl)malononitrile



To a solution of malononitrile (3.3030 g, 50 mmol, 1.00 eq.) in anhydrous DMF (20 mL) was added sodium hydride (1.3200 g, 55 mmol, 1.10 eq.) in portions. The solution was stirred at RT for 1 h under a drying tube. 2-Chloro-1,1-dimethoxyethane (6.8514 g, 55

mmol, 1.10 eq.) and a crystal of potassium iodide were added to the solution, and stirred at 100 ^{o}C for 24 h under a drying tube. The solution was cooled, and diluted with distilled H_2O (50 mL). The crude product was extracted with EtOAc (50 mL × 5). The organic phase was washed with brine (50 ml), dried with sodium sulfate, and filtered. The filtrate was concentrated *in vacuo* for further purification by silica gel column chromatography using a gradient of hexane/EtOAc (7:3 to 5:5) as eluent to afford the product as an amber liquid (3.315 g, 43%).

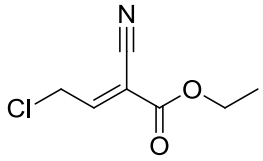
R_f (SiO_2 TLC): 0.36 in DCM/MeOH (19:1).

^{1}H NMR ($CDCl_3$, ppm): δ 2.25-2.29, 2.37-2.41 (m, 2H, CH_2 of $CH_2CH(OCH_3)_2$), 3.42 and 3.44 (2s, 6H, CH_3 of $CH(OCH_3)_2$), 4.14-4.16 (dd, J = 6.0, 6.0 Hz, 1H, CH of $CH(CN)_2$), 4.56-4.58 (dd, J = 6.0, 5.0 Hz, 1H, CH of $CH(OCH_3)_2$).

^{13}C NMR ($CDCl_3$, ppm): δ 31.09, 34.40, 53.55, 54.78, 101.30, 115.48.

ESI-MS ($M+Na^+$): 177.0644 (calc. 177.0640)

Compound 4-3. Ethyl 4-chloro-2-cyanobut-2-enoate



To a solution of 2-chloroacetaldehyde (9.03 g, 115 mmol, 1.15 eq.) and ethyl 2-cyanoacetate (11.3 g, 100 mmol, 1.00 eq.) in anhydrous DCM (50 mL) were added a mixture of piperidine (0.8515 g, 10.0 mmol, 0.1 eq.) and acetic acid (0.6005 g, 10.0 mmol, 0.1 eq.). After being stirred at RT for 15 min., the reaction mixture was heated

under reflux for 12 h. After being cooled down to RT, the solution was concentrated *in vacuo*, and then diluted with diethyl ether (200 mL), and washed twice with water (100 mL). The aqueous phases were extracted with diethyl ether (100 mL) three times. The combined diethyl ether phase was dried over sodium sulfate, and filtered through a short silica gel column. After removing the solvent, the crude products were pure enough to be used in further reactions. Analytically pure samples were obtained by silica gel column chromatography using hexane/EtOAc (19:1 to 9:1) as eluent.

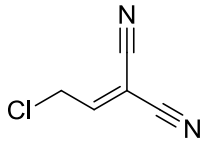
R_f (SiO_2 TLC): 0.66 in hexane/EtOAc (9:1).

^{1}H NMR ($CDCl_3$, ppm): δ 1.33-1.36 (t, J = 7.5 Hz, 3H, CH_3 of OEt), 4.32-4.40 (m, 4H, CH_2 of OEt and CH_2 of CH_2Cl), 7.58-7.59 (t, 7.0 Hz, 1H, CH adjacent to CH_2Cl).

^{13}C NMR ($CDCl_3$, ppm): δ 14.01, 39.38, 63.25, 111.71, 113.47, 154.15, 164.32.

EI-MS (M-1): 172.03 (calc.172.02)

Compound 4-4. 2-(2-Chloroethylidene)malononitrile



To a solution of 2-chloroacetaldehyde (9.03 g, 115 mmol, 1.15 eq.) and malononitrile (6.61 g, 100 mmol, 1.00 eq.) in anhydrous DCM (50 mL) were added a mixture of piperidine (0.8515 g, 10.0 mmol, 0.1 eq.) and acetic acid (0.6005 g, 10.0 mmol, 0.1 eq.). After being stirred at RT for 15 min, the reaction mixture was heated under reflux for 12 h. After being cooled down to RT, the solution was diluted with 150 mL DCM, and

washed with water (100 mL) twice. The aqueous phases were extracted with DCM (100 mL) twice. The combined organic phase was dried over sodium sulfate, and filtered through a short silica gel column. After removing the solvent, the crude products were pure enough to be used in further reactions. Analytically pure samples were obtained by silica gel column chromatography using hexane/EtOAc (9:1 to 7:3) as eluent.

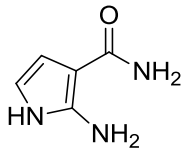
R_f (SiO_2 TLC): 0.61 in hexane/EtOAc (7:3).

^{1}H NMR (d_6-DMSO, ppm): δ 4.18-4.20 (dd, J = 11.0, 5.5 Hz, 1H, CH_2 of CH_2Cl), 4.21-4.23 (dd, J = 11.0, 5.5 Hz, 1H, CH_2 of CH_2Cl), 7.46-7.49 (dd, J = 5.5, 5.5 Hz, 1H, CH adjacent to CH_2Cl).

^{13}C NMR (d_6-DMSO, ppm): δ 36.25, 59.97, 113.00, 113.62, 168.05

EI-MS (M-1): 125.00 (calc. 124.99)

Compound 4-5. 2-Amino-1H-pyrrole-3-carboxamide



To the prepared ethyl 4-chloro-2-cyanobut-2-enoate (**4-3**) was added a solution of hydrogen chloride in ethanol (2 N, 50 mL). The solution was stirred at RT for 10 h, and then dried *in vacuo*. Ice-cold saturated ammonia in MeOH (100 mL) was added. The solution was stirred at RT for 16 h, and then refluxed for another 24 h. The crude product was filtered through a short silica gel column and concentrated *in vacuo*. The residue was

purified by silica gel column chromatography using a gradient of hexane/EtOAc (7:3 to 5:5) then DCM/MeOH (9:1) as eluent to afford the product (8.44 g, 67%).

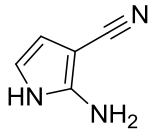
R_f (SiO_2 TLC): 0.19 in DCM/MeOH (17:3).

^{1}H NMR (d_6-DMSO, ppm): δ 3.56, 3.59 (s, 5H, NH_2 and NH), 7.31 (dd, J = 2.5, 2.0 Hz, 1H, CH of pyrrole), 7.62 (dd, J = 2.5, 2.0 Hz, 1H, CH adjacent to NH of pyrrole).

^{13}C NMR (d_6-DMSO, ppm): δ 93.38, 116.74, 117.67, 134.11, 166.75.

ESI-MS ($M+Na^+$): 148.0491 (calc. 148.0487)

Compound 4-6. 2-Amino-1H-pyrrole-3-carbonitrile



To the prepared 2-(2-chloroethylidene)malononitrile (**4-4**) was added a solution of hydrogen chloride in ethanol (2 N, 50 mL). The solution was stirred at RT for 10 h, and then dried *in vacuo*. Ice-cold saturated ammonia in MeOH (100 mL) was added. The solution was stirred at RT for 16 h, and then refluxed for another 24 hours. The crude product was filtered through a short silica gel column and concentrated *in vacuo*. The residue was obtained by silica gel column chromatography using gradient hexane/EtOAc (7:3 to 5:5) then DCM/MeOH (9:1) as eluent to afford the product (7.32 g, 59%).

R_f (SiO_2 TLC): 0.17 in DCM/MeOH (17:3).

^{1}H NMR (d_6-DMSO, ppm): δ 6.93 (dd, J = 2.5, 2.0 Hz, 1H, CH of pyrrole), 7.27 (dd, J = 2.5, 2.0 Hz, 1H, CH adjacent to NH of pyrrole).

^{13}C NMR (d_6-DMSO, ppm): δ 60.20, 115.51, 118.76, 124.35, 136.58.

ESI-MS (M+Na^+): 130.0386 (calc. 130.0381)

General procedure for the preparation of 7-deazapurines (4-7 to 4-12) by Approach A (Scheme 4-2)

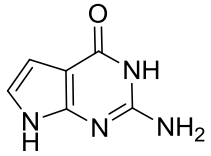
To a solution of ethyl 2-cyano-4,4-dimethoxybutanoate (**4-1**) or 2-(2,2-dimethoxyethyl)malononitrile (**4-2**) (1.0 eq.) was added guanidine hydrochloride, urea or thiourea (1.0 eq.) in anhydrous MeOH (100 mL) followed by sodium methoxide (1.0 excess eq.). The solution was refluxed for 24 h, and cooled down to RT. The solution was concentrated and filtered through a short silica gel column. After removing the solvent, the residue was treated with hydrogen chloride (2 N, 100 mL) at RT for 10 min, and refluxed for 24 h. The solution was concentrated *in vacuo*, and the residue was loaded on a silica gel column for further purification using a gradient of DCM to DCM/MeOH (9:1) as eluent. The corresponding yields are shown in **Table 4-1**.

General procedure for the preparation of 7-deazapurines (4-7 to 4-12) by Approach B (Scheme 4-3)

To a solution of 2-amino-1H-pyrrole-3-carboxamide (**4-5**) or 2-amino-1H-pyrrole-3-carboximidamide (**4-6**) (1.0 eq.) was added guanidine hydrochloride, urea or thiourea (1.0 eq.) in anhydrous MeOH (100 mL) followed by sodium methoxide (1.0 excess eq.).

The solution was refluxed for 24 h, and cooled down to RT. The solution was concentrated *in vacuo*, and the residue was loaded on a silica gel column using a gradient of DCM to DCM/MeOH (9:1). The corresponding yields are shown in **Table 4-2**.

Compound 4-7. 2-Amino-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one



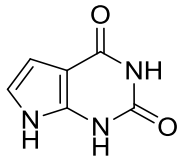
R_f (SiO_2 TLC): 0.23 in DCM/MeOH (17:3).

^{1}H NMR (d_6-DMSO, ppm): δ 6.19 (dd, J = 3.0, 2.5 Hz, 1H, CH of pyrrole), 6.61 (dd, J = 3.0, 2.5 Hz, 1H, CH adjacent to NH of pyrrole), 4.04, .8.19, 12.66 (3s, 4H, H of NH).

^{13}C NMR (d_6-DMSO, ppm): δ 99.88, 101.61, 116.61, 151.80, 152.21, 159.00.

ESI-MS ($M+Na^+$): 173.0443 (calc. 173.0439)

Compound 4-8. 1H-pyrrolo[2,3-d]pyrimidine-2,4(3H,7H)-dione



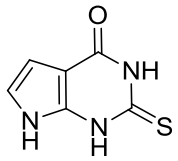
R_f (SiO_2 TLC): 0.23 in DCM/MeOH (17:3).

^{1}H NMR (d_6-DMSO, ppm): δ 6.20 (dd, J = 2.5, 2.0 Hz, 1H, CH of pyrrole), 6.61 (dd, J = 2.5, 2.0 Hz, 1H, CH adjacent to NH of pyrrole), 6.70, 11.36, 12.29 (3s, 3H, H of NH).

^{13}C NMR (d_6-DMSO, ppm): δ 100.18, 108.70, 126.33, 139.84, 151.60, 167.82.

ESI-MS (M+Na$^+$): 174.0283 (calc. 174.0279)

Compound 4-9. 2-Thioxo-2,3-dihydro-1H-pyrrolo[2,3-d]pyrimidin-4(7H)-one



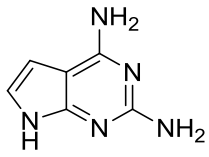
R_f (SiO_2 TLC): 0.32 in DCM/MeOH (17:3).

^{1}H NMR (d_6-DMSO, ppm): δ 6.19 (dd, J = 2.5, 2.0 Hz, 1H, CH of pyrrole), 6.60 (dd, J = 2.5, 2.0 Hz, 1H, CH adjacent to NH of pyrrole). 8.85, 9.05, 11.33 (3s, 3H, H of NH).

^{13}C NMR (d_6-DMSO, ppm): δ 98.46, 108.45, 116.03, 126.60, 167.83, 168.81.

ESI-MS (M+Na$^+$): 190.0055 (calc. 190.0051)

Compound 4-10. 7H-pyrrolo[2,3-d]pyrimidine-2,4-diamine



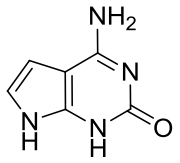
R_f (SiO_2 TLC): 0.27 in DCM/MeOH (17:3).

^{1}H NMR (d_6-DMSO, ppm): δ 6.18 (dd, J = 3.0, 2.5 Hz, 1H, CH of pyrrole), 6.59 (dd, J = 3.0, 2.5 Hz, 1H, CH adjacent to NH of pyrrole), 7.80, 8.94, 9.89 (3s, 5H, H of NH).

^{13}CNMR (d_6-DMSO, ppm): δ 89.14, 101.90, 126.28, 151.46, 155.24, 165.10.

ESI-MS (M+Na$^+$): 172.0604 (calc. 172.0599)

Compound 4-11. 4-Amino-1H-pyrrolo[2,3-d]pyrimidin-2(7H)-one



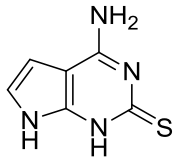
R_f (SiO_2 TLC): 0.25 in DCM/MeOH (17:3).

^{1}H NMR (d_6-DMSO, ppm): δ 6.18 (dd, J = 3.0, 2.5 Hz, 1H, CH of pyrrole), 6.58 (dd, J = 3.0, 2.5 Hz, 1H, CH adjacent to N of pyrrole), 10.61, 13.47 (2s, 3H, NH_2).

^{13}C NMR (d_6-DMSO, ppm): δ 97.98, 107.44, 116.89, 135.18, 159.04, 162.80.

ESI-MS ($M+Na^+$): 173.0443 (calc. 173.0439)

Compound 4-12. 4-Amino-1H-pyrrolo[2,3-d]pyrimidine-2(7H)-thione



R_f (SiO_2 TLC): 0.37 in DCM/MeOH (17:3).

^{1}H NMR (d_6-DMSO, ppm): δ 6.18 (dd, J = 2.5, 2.0 Hz, 1H, CH of pyrrole), 6.59 (dd, J = 2.5, 2.0 Hz, 1H, CH adjacent to NH of pyrrole), 8.74, 8.89, 12.87 (3s, 4H, H of NH)

^{13}C NMR (d_6-DMSO, ppm): δ 98.00, 107.89, 116.90, 134.40, 159.04, 178.25.

ESI-MS ($M+Na^+$): 189.0216 (calc. 189.0211).

6.3 Experimental procedure for synthesis of cross-linked nucleosides containing 7-deaza-2'-deoxyguanosine

6.3.1 Materials and General Methods

Trimethylsilyl chloride (TMSCl), phenoxyacetyl chloride (Pac-Cl), sodium hydride, *tert*-butyldimethylsilyl chloride (TBS-Cl), isobutyryl chloride (*i*BuCl), 4-dimethylaminopyridine (DMAP), triphenylphosphine, sodium *N*,*N*-diethydithiocarmbamate, bis(triphenylphosphine)palladium(*II*) dichloride, copper iodide, 1,6-heptadiyne, diisopropylethylamine (DIPEA) and all other chemicals and solvents for synthesis were purchased from the Aldrich Chemical Company (Milwaukee, WI).

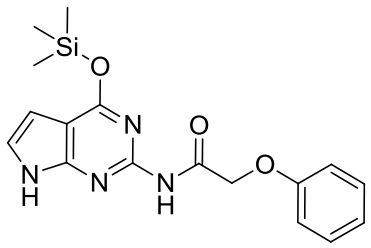
1-Chloro-3,5-di-*O*-toluoyl-2-deoxy-α-D-ribofuranose was purchased from Berry & Associates, Inc. (Dexter, MI). 4,4'-dimethoxytrityl chloride and *N*,*N*-diisopropylamino cyanoethyl phosphonamidic chloride were purchased from ChemGenes Inc. (Wilmington, MA).

Flash column chromatography was performed using silica gel 60 (230–400 mesh) obtained from Silicycle (Quebec City, QC). Thin layer chromatography (TLC) was performed using precoated TLC plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) purchased from EMD Chemicals Inc. from (Gibbstown, NJ). All solvents for column chromatography were obtained from Mallinckrodt Baker, Inc. (Phillipsburg , NJ). ^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 MHz NMR spectrometer at RT at frequencies of 499.9 and 125.7 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts were reported in parts per million downfield from tetramethylsilane. ^{31}P NMR spectra (^{1}H

decoupled) were recorded at a frequency of 202.4 MHz with H_3PO_4 used as an external standard.

6.3.2 Experimental procedure for synthesis of cross-linked 7-deaza-2'-deoxyguanosine

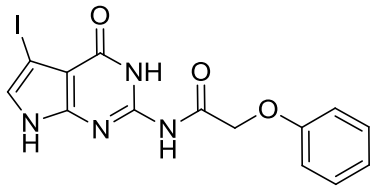
Compound 5-1. 2-phenoxy-*N*-(4-(trimethylsilyloxy)-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)acetamide



To the solution of 7-deazaguanine (3.0 g, 20 mmol, 1.0 eq.) in anhydrous pyridine (20 mL) was added TMSCl (4.35 g, 40 mmol, 3.0 eq.) in portions. The solution was stirred in an ice-bath and allowed to warm to RT for 1.5 h. Phenoxyacetyl chloride (5.1 g, 30 mmol, 1.5 eq.) was added in portion, and the solution stirred at RT for overnight. The solution was concentrated *in vacuo*, and the residue was washed with ice-cold water. Then the residue was run through a silica gel columnn to remove most of the pyridine solvent with hexane/EtOAc (v/v, 9:1 to 5:5) as eluent. The crude product was eluted off the column with DCM/MeOH (v/v, 19:1).

The TMS protective group at the 6-oxo position was labile at higher temperature, and cleavage of TMS at this site occurred during removal of pyridine from the product. The crude product was used for the iodination step directly without further purification.

Compound 5-2. *N*-(5-iodo-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-2-phenoxyacetamide



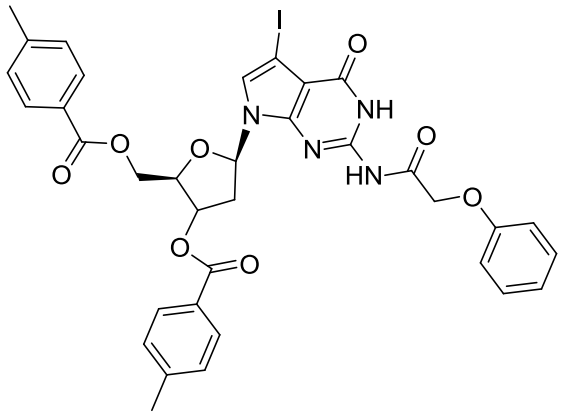
To the solution of compound **5-1** in DMF (20 mL) was added iodine (6.6 g, 26 mmol, 1.3 eq.) in portions. The brown solution was stirred at RT overnight. The solution was concentrated *in vacuo* (water bath at 65 ^{o}C), and the residue was purified by silica gel column chromatography (twice) using a gradient of hexane/EtOAc (v/v, 8:2 to 2:8) to DCM/CH_3OH (v/v, 10:0 to 9:1) as eluent. The product, a brown solid was obtained with a yield of 5.8 g (71%) over two steps.

^{1}H NMR (d_6-DMSO, ppm): δ 4.84 (s, 2H, CH_2 of Pac), 6.89-6.97 (m, 4H, Ph of Pac and H of C8), 7.26-7.31 (m, 2H, Ph of Pac).

^{13}C NMR (d_6-DMSO, ppm): δ 68.93, 113.11, 113.14, 114.67, 114.76, 118.89, 121.10, 129.68, 129.73, 143.98, 149.77, 158.11, 166.40, 175.89.

ESI (M+Na): 432.9779 (calc. 432.9774).

Compound 5-3. (2R,3R,5R)-5-(5-iodo-4-oxo-2-(2-phenoxyacetamido)-3H-pyrrolo[2,3-d]pyrimidin-7(4H)-yl)-2-((4-methylbenzoyloxy)methyl)tetrahydrofuran-3-yl 4-methylbenzoate (7-iodo-7-deaza-3',5'-di-*O*-toluoyl-2'-deoxyguanosine)



To the solution of compound **5-2** (5.0600 g, 12.33 mmol, 2.4 eq.) in anhydrous acetonitrile (50 mL) was added sodium hydride (0.3084 g, 12.85 mmol, 2.5 eq.) in portions. The solution was stirred at RT for 45 min. 1-Chloro-3,5-di-*O*-toluoyl-2-deoxy-α-D-ribofuranose (2.000 g, 5.14 mmol, 1.0 eq.) was added, and the solution was stirred at RT for another 12 h. The solution was concentrated, and the residue was purified by silica gel column chromatography using a gradient of hexane/EtOAc (v/v, 8:2 to 3:7) as eluent to afford the product as a yellow solid (2.0310 g, 66%)

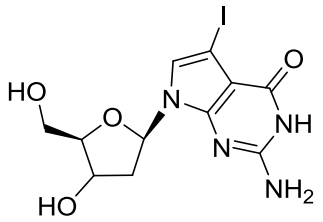
R_f (SiO_2 TLC): 0.61 in hexane/EtOAc (3:7).

^{1}H NMR ($CDCl_3$, ppm): δ 2.67, 2.68 (2s, 6H, CH_3 of Tol), 2.87-2.90 (m, 1H, H2'), 3.14-3.17 (m, 1H, H2"), 4.52-4.54 (m, 1H, H5'), 4.63-4.69 (m, 4H, CH_2 of Pac, H4' and H5"), 5.15-5.18 (m, 1H, H3'), 6.35-6.38 (dd, J = 6.0, 6.0 Hz, 1H, H1'), 7.13-7.33 (m, 9H, Phs of Pac and Tol, H8), 7.90-7.99 (m, 6H, Phs of Tol).

^{13}C NMR ($CDCl_3$, ppm): δ 21.70, 21.75, 34.55, 63.42, 69.20, 74.27, 87.90, 97.97, 105.06, 125.41, 126.14, 127.04, 128.29, 129.07, 129.11, 129.21, 129.37, 129.39, 129.48, 129.62, 129.69, 129.71, 129.81, 129.89, 130.19, 130.23, 140.61, 144.58, 145.10, 146.16, 165.10, 166.14.

ESI (M+Na): 785.1088 (calc. 785.1084).

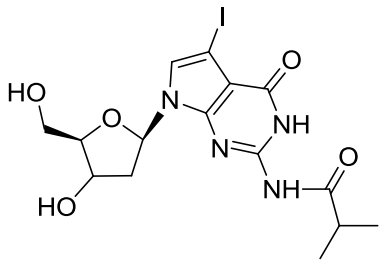
Compound 5-4. 2-Amino-7-(2'-deoxy-β-D-erythro-pentofuranosyl)-5-iodo-pyrrolo[2,3-d]pyrimidin-4-one (7-iodo-7-deaza-2'-deoxyguanosine)



Compound **5-3** (2.000 g, 2.62 mmol, 1.0 eq.) was dissolved in a saturated solution of ammonia in MeOH. The solution was stirred with an ice-bath and allowed to warm to RT for 6 h, then concentrated *in vacuo*. The residue was loaded onto silica gel, and purified by silica gel column chromatography using a gradient of DCM to DCM/MeOH (v/v, 9:1) as eluent. A yellowish solid was obtained, and this precursor was employed directly for following reaction.

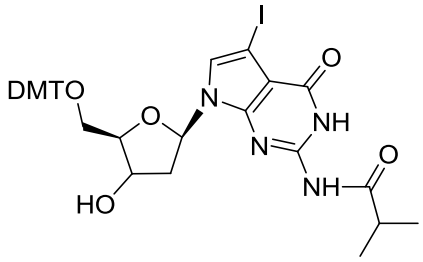
R_f (SiO_2 TLC): 0.22 in DCM/MeOH (17:3).

Compound 5-5. *N*-(7-((2R,4R,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-5-iodo-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)isobutyramide (7-iodo-7-deaza-2'-deoxyguanosine (*Ni*Bu))



To a solution of compound **5-4** (0.7012 g, 1.79 mmol, 1.0 eq.) in anhydrous pyridine (15 mL) was added TMSCl (1.1668 g, 10.74 mmol, 6.0 eq.). The solution was stirred at RT for 2 h followed the addition of isobutyryl chloride (0.2861 g, 2.69 mmol, 1.5 eq.). The solution was stirred at RT overnight, and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography, and then treated with saturated ammonia in MeOH with an ice-bath for 30 min. The solution was again concentrated *in vacuo*, and was purified by silica gel column chromatography to obtain a polar residue. TLC analysis indicated that pyridine was also present, and no further purification was performed for the following step.

Compound 5-6. 7-Iodo-7-deaza-5'-*O*-dimethoxytrityl-2'-deoxydeoxyguanosine (*Ni*Bu)



Compound **5-5** (containing pyridine) was dried on a high vacuum for 2 days, and then dissolved in anhydrous pyridine (3.5 mL). 4,4'-Dimethoxytrityl chloride (0.7280 g, 2.15 mmol, 1.2 eq.) was added in two portions. The reaction was allowed to stir at RT overnight, and then concentrated *in vacuo*. The residue was diluted with aqueous sodium bicarbonate (sat., 50 mL), and then extracted with EtOAc (50 mL) three times. The organic phase was dried over sodium sulfate, and concentrated for silica gel column chromatography using a gradient of DCM to DCM/MeOH (v/v, 19:1) as eluent. An amber gum was obtained with a yield of 0.7520 g (42% for two steps from compound **5-3** to **5-6**).

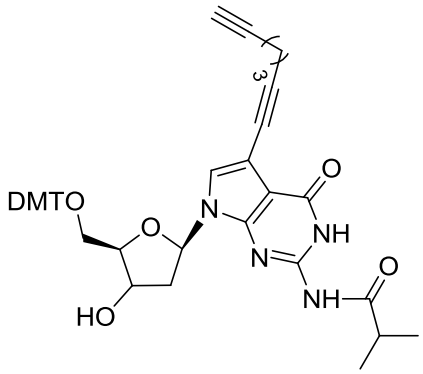
R_f (SiO_2 TLC): 0.29 in DCM/MeOH (19:1).

^{1}H NMR ($CDCl_3$, ppm): δ 1.06-1.08 (d, J = 7.0 Hz, 3H, CH_3 of *i*Bu), 1.10-1.11 (d, J = 6.5 Hz, 3H, CH_3 of *i*Bu), 2.45-2.50 (m, 2H, H2' and H2"), 2.67-2.70 (dd, J = 7.0, 6.5 Hz, 1H, CH of *i*Bu), 3.27-3.33 (m, 2H, H5' and H5"), 3.70, 3.71 (2s, 6H, OCH_3 of DMT), 4.16-4.17 (m, 1H, H4'), 4.72-4.73 (m, 1H, H3'), 6.16-6.19 (dd, J = 7.0, 6.5 Hz, 1H, H1'), 6.71-6.74 (m, 4H, Phs of DMT), 7.12-7.70 (m, 10H, H8 and Phs of DMT).

^{13}C NMR ($CDCl_3$, ppm): δ 17.94, 18.72, 36.24, 40.71, 51.45, 55.23, 55.26, 63.31, 72.46, 84.26, 86.32, 87.03, 113.21, 122.04, 127.01, 127.94, 128.04, 129.93, 129.94, 135.69, 135.87, 137.33, 144.68, 147.26, 148.10, 158.63, 158.64, 162.62, 178.38.

ESI (M+Na): 787.1609 (calc. 787.1605).

Compound 5-7. 7-(1,6-Heptadiynyl)-7-deaza-5'-*O*-dimethoxytrityl-2'-deoxyguanosine (*Ni*Bu)



Compound **5-6** (0.1210 g, 0.16 mmol, 1.0 eq.), bis(triphenylphosphine)palladium(*II*) dichloride (0.0023 g, 0.0032 mmol, 0.02 eq.), copper(I) iodide (0.0030 g, 0.016 mmol, 0.1 eq.) and triphenylphosphine (0.0042 g, 0.016 mmol, 0.1 eq.) were dissolved in anhydrous DMF (2 mL). The solution was purged with argon three times, and 1,6-heptadiyne (0.0583 g, 0.63 mmol, 4.0 eq.) and triethylamine (0.1 mL) were injected into the solution. The reaction was conducted under argon atmosphere at 40 ^{o}C for 60 h. The reaction was quenched with aqueous sodium bicarbonate (sat. 50 mL). The crude product was extracted with EtOAc (50 mL) three times, and the organic phase dried over sodium sulfate. The concentrated residue was purified by silica gel column chromatography using a gradient of hexane/EtOAc (v/v, 9:1 to 3:7) as eluent to afford the product as a yellowish solid (0.1002 g, 86%).

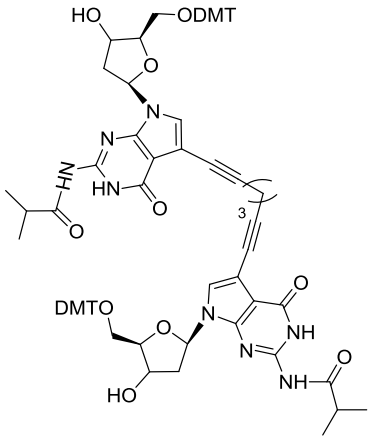
R_f (SiO_2 TLC): 0.29 in hexane/EtOAc (3:7).

^{1}H NMR ($CDCl_3$, ppm): δ 0.96-0.98 (d, J = 6.5 Hz, 3H, CH_3 of *i*Bu), 1.18-1.20 (d, J = 6.5 Hz, 3H, CH_3 of *i*Bu), 1.59-1.61 (m, 2H, CH_2 of heptadiynyl), 2.22-2.25 (m, 1H, H2'), 2.55-2.58 (m, 4H, CH_2 of heptadiynyl), 2.61-2.66 (m, 3H, H2", CH of *i*Bu, CH of of heptadiynyl), 3.15-3.17, 3.36-3.38 (m, 2H, H5' and H5"), 3.78, 3.79 (2s, 6H, OCH_3 of DMT), 4.06-4.08 (m, 1H, H4'), 4.57-4.59 (m, 1H, H3'), 6.18-6.21 (dd, J = 8.5, 5.0 Hz, 1H, H1'), 6.80-6.85 (m, 4H, Phs of DMT), 7.07-7.70 (m, 10H, H8 and Phs of DMT).

^{13}C NMR ($CDCl_3$, ppm): δ 18.96, 21.62, 21.64, 25.80, 31,38, 36.42, 40.95, 55.22. 55.26, 64.32, 65.31, 71.11, 83.87, 84.65, 87.09, 93.87, 94.00, 98.89, 105.42, 113.11, 113.46, 113.48, 126.44, 127.75, 127.82, 127.89, 127.98, 128.20, 128.56, 130.07, 130.11, 132.01, 133.95, 134.05, 141.05, 149.11, 150.13, 158.09, 162.50, 175.69.

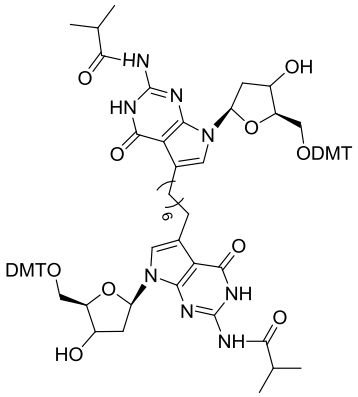
ESI (M+Na): 751.3114 (calc. 751.3108).

Compound 5-8. 1-{C7-[5'-*O*-dimethoxytrityl-N^2-isobutyryl-7-deaza-2'-deoxy guanidinyl]}-7-{C7-[5'-*O*-dimethoxytrityl-N^2-isobutyryl-7-deaza-2'-deoxy guanidinyl]}-1,6-heptadiyne



Compound **5-6** (0.2010 g, 0.28 mmol, 1.0 eq.), compound **5-7** (0.2000 g, 0.28 mmol, 1.0 eq.), bis(triphenylphosphine)palladium(*II*) dichloride (0.0040 g, 0.0056 mmol, 0.02 eq.), copper(I) iodide (0.0054 g, 0.028 mmol, 0.1 eq.) and triphenylphosphine (0.0074 g, 0.028 mmol, 0.1 eq.) were mixed in anhydrous DMF (3 mL). The solution was purged with argon three times, and triethylamine (0.1 mL) was injected into the solution. The reaction was conducted under argon atmosphere at 40 ^{o}C for 60 h. The reaction was then quenched with aqueous sodium bicarbonate (sat. 50 mL). The crude product was extracted with EtOAc (50 mL) three times. The organic phase was treated with aqueous *N*,*N*-diethydithiocarmbamate (0.5 g in 50 mL distilled H_2O) and then dried over sodium sulfate. The concentrated residue was purified by silica gel column chromatography using a gradient of hexane/EtOAc (v/v, 9:1 to 3:7) as eluent to afford the yellowish solid (0.1900 g). The solid was used for hydrogenation.

Compound 5-9. 1-{C7-[5'-*O*-dimethoxytrityl-N^2-isobutyryl-7-deaza-2'-deoxy guanidinyl]}-7-{C7-[5'-*O*-dimethoxytrityl-N^2-isobutyryl-7-deaza-2'-deoxy guanidinyl]}-heptane



To the solution of compound **5-8** (0.1900 g) in MeOH (30 mL) was added 0.1000 g of palladium on activated carbon (Pd/C, 10%). Hydrogenation was performed with a Parr hydrogenator. The solution was purged with hydrogen (20 psi) three times, and then the hydrogen pressure was increased to 60 psi. The solution was shaken at RT for 60 h. The hydrogen was released, and the solution was run though a short silica gel column to remove the Pd/C. The solution was concentrated *in vacuo*, and aqueous sodium bicarbonate (sat. 50 mL) was added. The crude product was extracted with EtOAc (50 mL) three times. The organic phase was treated with aqueous *N*,*N*-diethydithiocarbamate (0.5 g in 50 mL distilled H_2O) and then dried over sodium sulfate. The concentrated residue was purified by silica gel column chromatography using a gradient of hexane/EtOAc (v/v, 9:1 to 3:7) as eluent to afford the slightly yellowish solid (yield 0.1560 g).

R_f (SiO_2 TLC): 0.26 in DCM/MeOH (19:1).

^{1}H NMR ($CDCl_3$, ppm): δ 1.11-1.27 (m, 18H, 4 CH_3 of *i*Bu, 3 CH_2 of $(CH_2)_7$), 1.57-1.59 (m, 4H, 2 CH_2 of $(CH_2)_7$), 2.10-2.12 (m, 2H, H2' and H2''), 2.40-2.60 (m, 6H, H2' and H2'', 2 CH_2 of $(CH_2)_7$), 2.64-2.68 (m, 2H, CH of *i*Bu), 3.37-3.47 (m, 4H, H5' and H5''), 3.78-3.80 (4s, 12H, OCH_3 of DMT), 4.35-4.39 (m, 2H, H4' and H4''), 4.40-4.44 (m, 2H, H3' and H3''), 6.08-6.10 (dd, J = 7.0, 6.5 Hz, 1H, H1'), 6.12-6.15 (J = 8.0, 6.0 Hz, 1H, H1''), 6.76-6.82 (m, 10H, Phs of DMT, H of C8), 7.14-7.38 (m, 18H, Phs of DMT).

^{13}CNMR ($CDCl_3$, ppm): mixture of monomer and dimer.

Chapter 7. Conclusions and Future Work

7.1 Conclusions

A synthetic strategy to produce ICL duplexes containing completely asymmetric nucleotide sequences around the cross-linked site has been developed. Dimethoxytrityl (DMT), *tert*-butyldimethylsilyl (TBS), levulinoyl (Lev), phenoxyacetyl (Pac) and allyloxycarbonyl (alloc) were screened for the compatibility of these protective groups in solution and solid-phase synthesis. By comparison of the solid-phase synthesis process with the two novel cross-linked dimer phosphoramidites, phosphoramidite **3-16** with DMT and Alloc protective groups for two 5'-*O* and one TBS for 3'-*O* enabled preparation of H-shaped ICL duplexes of completely asymmetric nucleotide composition around the cross-linked sites in satisfactory yields.

Four duplexes containing a *N*3T-butylene-*N*3T ICL, two partially asymmetrical and two completely asymmetrical sequences, were synthesized and characterized by circular dichroism which suggested that they formed a B-form DNA duplex. The composition of the expected ICL duplexes was confirmed by enzymatic digestion and mass spectrometry. This approach is now being explored for the synthesis of other ICL DNA systems to produce substrates for various DNA repair experiments.

For the preparation of ICL oligonucleotides containing 7-deazaguanine, a synthetic procedure for important precursor 7-deazaguanine was optimized. Two synthetic routes for the preparation of a series of 7-deazapurines have been developed. These heterocyclic molecules were synthesized based on two cyclization strategies, either from pyrimidine to

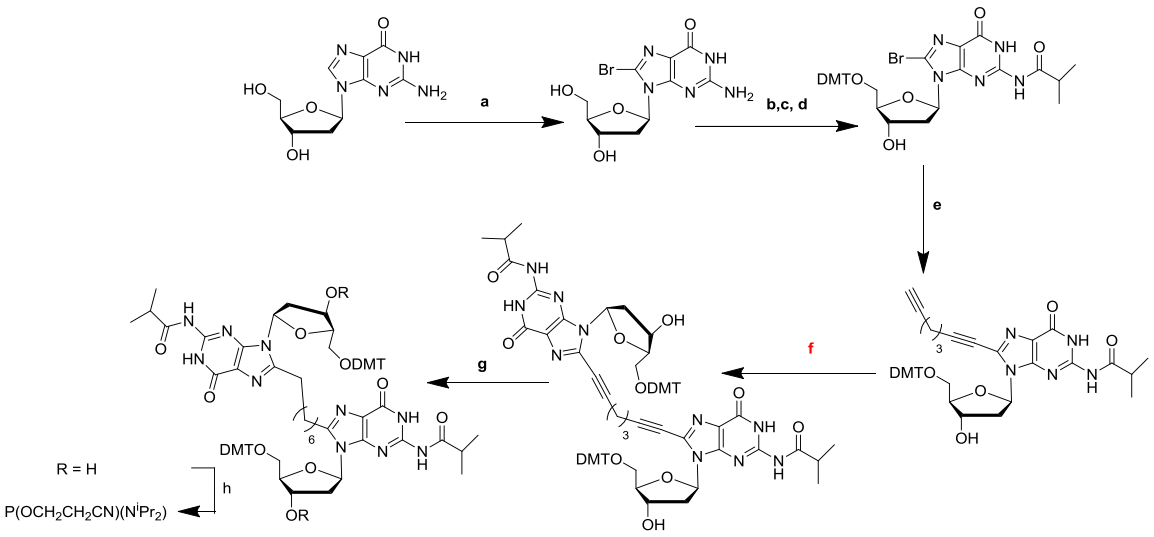
purine or from pyrrole to purine. The merits and shortcomings of these two methods were compared, which provided an efficient and convergent synthetic strategy to generate 7-deazapurines. The compound 7-deazaguanine, prepared by these approaches, was employed in a synthesis to attempt to prepare a cross-linked dimer containing two 7-deaza-2'-deoxyguanosines linked at the C7 atoms by an alkylene linker.

The precursor, *N*-(5-iodo-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-2-phenoxyacetamide was prepared and coupled with 1-chloro-3,5-di-O-toluoyl-2-deoxy-α-D-ribofuranose. Numerous obstacles were encountered in the optimization of various steps after the removal of toluolyl protective group at both 3'-*O* and 5'-*O* positions which generated 7-iodo-7-deaza-2'-deoxyguanosine which was converted into the 5'-*O*-DMT protected nucleoside. A C7 alkynyl linker was introduced to the protected nucleoside by the Sonogashira reaction. Attempts to form a cross-linked dimer with a second Sonogashira coupling reaction were not successful. This Sonogashira coupling reaction was attempted between the two monomers: 7-iodo-7-deaza-5'-*O*-DMT-2'-deoxyguanosine (*Ni*Bu) (**5-6**) and 7-(1,6-heptadiynyl)-7-deaza-5'-*O*-DMT-2'-deoxyguanosine (*Ni*Bu) (**5-7**). It is believed that this coupling was not successful due to steric hinderance of the two bulky DMT moieties at the 5'-*O* position. Nuclear magnetic resonance analysis of what was isolated from the coupling reactions indicated that monomer(s) and dimer co-existed in the final product.

An attempt to proceed forward to prepare the phosphoramidite(s) following hydrogenation revealed 5 major signals by ^{31}P NMR data which is assumed to be a mixture of inseparable monomer and dimer products.

7.2 Future Work

To optimize the preparation of the cross-linked dimer, it is proposed that a C8-halogenated derivative of 2'-deoxyguanosine rather than 7-deaza-2'-deoxyguanosine be explored. For example, 8-bromo-2'-deoxyguanosine could be employed as substrate. By attaching a halogen atom to the C8 position, coupling reactions to introduce a linker between two 2'-deoxyguanosines may be studied. In **Chapter 5**, obstacles were encountered with the second Sonogashira reaction to prepare the dimer. Preliminary reactions can be conducted with 8-bromo-2'-deoxyguanosine to optimize this crucial coupling step. A proposed synthesis is shown in **Scheme 7-1**.



Scheme 7-1. Synthesis of crosslinked dimer phosphoramidite containing C8-heptylene-C8 guanosine

a) *N*-bromosuccinimide, acetonitrile/water 4:1, 1 h, RT. b) TMSCl, pyridine, RT, 1h; c) isobutyryl chloride, pyridine, RT, overnight; d) DMTCl, pyridine, RT, overnight; e) 1,6-heptadiyne, $PdCl_2(PPh_3)_2$, CuI, Et_3N, in

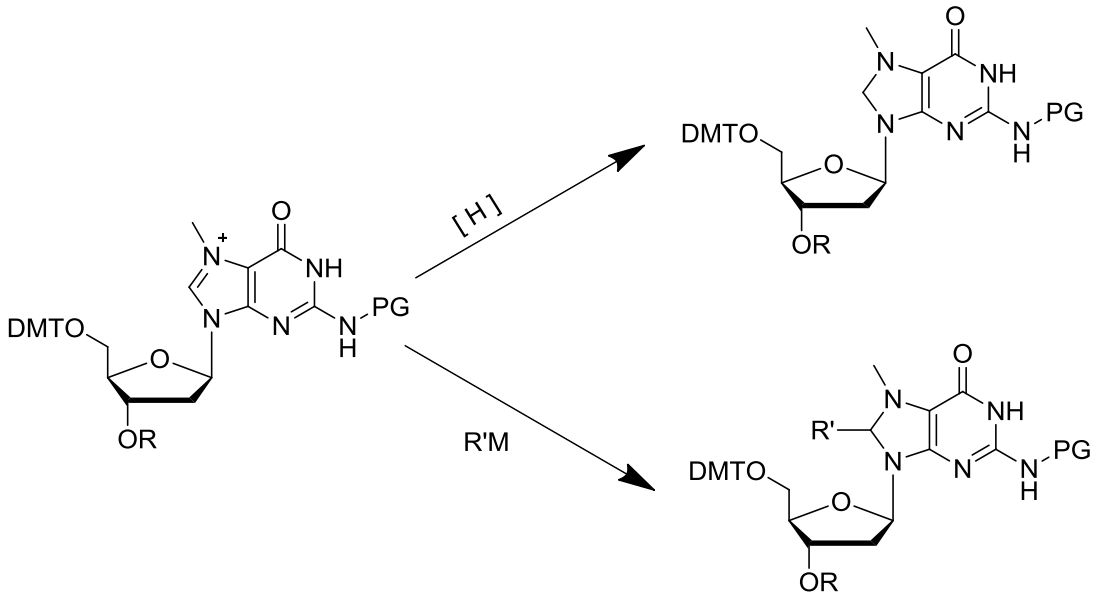
DMF, argon; f) 8-bromo-2'-deoxyguanosine, $PdCl_2(PPh_3)_2$, CuI, Et_3N, in DMF, argon; g) hydrogen, Pd/C in MeOH, rt; h) $ClP(OCH_2CH_2CN)(NiPr_2)$.

To convert 2'-deoxyguanosine into the 8-bromo adduct, 2'-deoxyguanosine can be used as starting material rather than 5'-*O*-DMT-2'-deoxyguanosine.[591-595] After the attachment of DMT protective group at the 5'-*O* position, the first Sonogashira reaction can be performed following the published procedures outlined in **Chapter 5**. The key step, the second Sonogashira reaction, must be optimized to get better conversion and improved isolation of cross-linked dimers from unreacted monomer(s). Afterwards, hydrogenation will produce the precursor that can ultimately be converted into the phosphoramidites required for solid-phase synthesis of ICL DNA.

Once optimized, these conditions may be extended towards the formation of the 7-deaza-2'-deoxyguanosine crosslinked dimer which may ultimately be used for DNA repair studies.

The chemical instability of *N*7-alkyl-2'-deoxyguanosine is caused by the positive charge on the guanine ring system.[336] To mimic the main adduct formed by the introduction of alkylating agents (including bifunctional alkylating agents), another synthetic approach involving modified nucleosides is proposed. This would involve the modification of 7-alkyl-2'-deoxyguanosines to generate chemically stable substrates. After the alkylation of *N*7 of 2'-deoxyguanosine, the product formed is structurally similar to a guaninium salt. This may react as a NHC carbene, coordinating to a transition metal complex such as

xanthinium.[596] When treated with reducing agents, i.e. sodium borohydride, more stable structures containing 8-H-guanine may be generated.[597-601] This may produce molecules that are more stable substrates for various repair and structural studies. Addition of alkyl or aromatic groups on C8 of *N*7-alkyl-guanosine could produce an interesting class of modified nucleosides that may find use in a wide range of applications (see **Scheme 7-2**).



R = H or other protective groups (PG)

R' = alkyl or aromatic

Scheme 7-2. Post-treatment of *N*7-alkyl guanosine with reducing agents or C8 addition

References

1. Watson, J.D. and F.H.C. Crick, *Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid.* Nature, 1953. **171**(4356): p. 737-738.
2. Wilkins, M.H.F., A.R. Stokes, and H.R. Wilson, *Molecular Structure of Nucleic Acids: Molecular Structure of Deoxypentose Nucleic Acids.* Nature, 1953. **171**(4356): p. 738-740.
3. Franklin, R.E. and R.G. Gosling, *The structure of sodium thymonucleate fibres. I. The influence of water content.* Acta Crystallographica, 1953. **6**(8-9): p. 673-677.
4. Franklin, R.E. and R.G. Gosling, *The structure of sodium thymonucleate fibres. II. The cylindrically symmetrical Patterson function.* Acta Crystallographica, 1953. **6**(8-9): p. 678-685.
5. Wing, R., et al., *Crystal structure analysis of a complete turn of B-DNA.* Nature, 1980. **287**(5784): p. 755-758.
6. Kerppola, T.K., *Protein–DNA Interactions: Structure and Energetics*, in *eLS*. 2001, John Wiley & Sons, Ltd.
7. Xu, H. and S.W. Morrical, *Protein Motifs for DNA Binding*, in *eLS*. 2001, John Wiley & Sons, Ltd.
8. Saecker, R.M., *Protein–DNA Interactions: Polyelectrolyte Effects*, in *eLS*. 2001, John Wiley & Sons, Ltd.
9. Xiong, Y. and M. Sundaralingam, *Protein–Nucleic Acid Interaction: Major Groove Recognition Determinants*, in *eLS*. 2001, John Wiley & Sons, Ltd.

10. Hamilton, P.L. and D.P. Arya, *Natural product DNA major groove binders.* Natural Product Reports, 2012. **29**(2): p. 134-143.

11. Pabo, C.O. and R.T. Sauer, *Protein-DNA Recognition.* Annual Review of Biochemistry, 1984. **53**(1): p. 293-321.

12. Strauch, M.A., *Protein–DNA Complexes: Specific*, in *eLS*. 2001, John Wiley & Sons, Ltd.

13. McBryant, S.J., et al., *Assessment of Major and Minor Groove DNA Interactions by the Zinc Fingers of Xenopus Transcription Factor IIIA.* Nucleic Acids Research, 1996. **24**(13): p. 2567-2574.

14. Bewley, C.A., A.M. Gronenborn, and G.M. Clore, *MINOR GROOVE-BINDING ARCHITECTURAL PROTEINS: Structure, Function, and DNA Recognition1*. Annual Review of Biophysics and Biomolecular Structure, 1998. **27**(1): p. 105-131.

15. Biswas, I. and P. Hsieh, *Interaction of MutS Protein with the Major and Minor Grooves of a Heteroduplex DNA.* Journal of Biological Chemistry, 1997. **272**(20): p. 13355-13364.

16. Stella, S., D. Cascio, and R.C. Johnson, *The shape of the DNA minor groove directs binding by the DNA-bending protein Fis.* Genes & Development, 2010. **24**(8): p. 814-826.

17. Tolstorukov, M.Y., R.L. Jernigan, and V.B. Zhurkin, *Protein–DNA Hydrophobic Recognition in the Minor Groove is Facilitated by Sugar Switching.* Journal of Molecular Biology, 2004. **337**(1): p. 65-76.

18. JM, B., T. JL, and S. L., *Biochemistry*. 2002, W H Freeman: New York.

19. Basham, B., G.P. Schroth, and P.S. Ho, *An A-DNA triplet code: thermodynamic rules for predicting A- and B-DNA.* Proceedings of the National Academy of Sciences, 1995. **92**(14): p. 6464-6468.

20. Nevzorov, A.A., S. Moltke, and M.F. Brown, *Structure of the A-Form and B-Form of DNA from Deuterium NMR Line Shape Simulation†.* Journal of the American Chemical Society, 1998. **120**(19): p. 4798-4805.

21. Pastor, N., *The B- to A-DNA Transition and the Reorganization of Solvent at the DNA Surface.* Biophysical journal, 2005. **88**(5): p. 3262-3275.

22. Mitsui, Y., et al., *Physical and Enzymatic Studies on Poly d(I-C).Poly d(I-C), an Unusual Double-helical DNA.* Nature, 1970. **228**(5277): p. 1166-1169.

23. Wang, A.H.J., et al., *Molecular structure of a left-handed double helical DNA fragment at atomic resolution.* Nature, 1979. **282**(5740): p. 680-686.

24. Rich, A., A. Nordheim, and A.H.J. Wang, *The Chemistry and Biology of Left-Handed Z-DNA.* Annual Review of Biochemistry, 1984. **53**(1): p. 791-846.

25. Ho, P.S., *The non-B-DNA structure of d(CA/TG)n does not differ from that of Z-DNA.* Proceedings of the National Academy of Sciences, 1994. **91**(20): p. 9549-9553.

26. Rich, A. and S. Zhang, *Z-DNA: the long road to biological function.* Nat Rev Genet, 2003. **4**(7): p. 566-572.

27. Ha, S.C., et al., *Crystal structure of a junction between B-DNA and Z-DNA reveals two extruded bases.* Nature, 2005. **437**(7062): p. 1183-1186.

28. De Bont, R. and N. van Larebeke, *Endogenous DNA damage in humans: a review of quantitative data.* Mutagenesis, 2004. **19**(3): p. 169-185.

29. Martin, L.J., *DNA Damage and Repair: Relevance to Mechanisms of Neurodegeneration.* Journal of Neuropathology & Experimental Neurology, 2008. **67**(5): p. 377-387 10.1097/NEN.0b013e31816ff780.

30. Stivers, J.T. and Y.L. Jiang, *A Mechanistic Perspective on the Chemistry of DNA Repair Glycosylases.* Chemical Reviews, 2003. **103**(7): p. 2729-2760.

31. Lindahl, T., *Instability and decay of the primary structure of DNA.* Nature, 1993. **362**(6422): p. 709-715.

32. Srinivasan, A. and B. Gold, *Small-molecule inhibitors of DNA damage-repair pathways: an approach to overcome tumor resistance to alkylating anticancer drugs.* Future Medicinal Chemistry, 2012. **4**(9): p. 1093-1111.

33. Dickson, K.S., C.M. Burns, and J.P. Richardson, *Determination of the Free-Energy Change for Repair of a DNA Phosphodiester Bond.* Journal of Biological Chemistry, 2000. **275**(21): p. 15828-15831.

34. Schroeder, G.K., et al., *The time required for water attack at the phosphorus atom of simple phosphodiesters and of DNA.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(11): p. 4052-4055.

35. Williams, N.H. and P. Wyman, *Base catalysed phosphate diester hydrolysis.* Chemical Communications, 2001(14): p. 1268-1269.

36. Cassano, A.G., V.E. Anderson, and M.E. Harris, *Evidence for Direct Attack by Hydroxide in Phosphodiester Hydrolysis.* Journal of the American Chemical Society, 2002. **124**(37): p. 10964-10965.

37. Iché-Tarrat, N., et al., *Theoretical Studies of the Hydroxide-Catalyzed P−O Cleavage Reactions of Neutral Phosphate Triesters and Diesters in Aqueous*

Solution: Examination of the Changes Induced by H/Me Substitution. The Journal of Physical Chemistry B, 2005. **109**(47): p. 22570-22580.

38. Lindahl, T. and O. Karlstrom, *Heat-induced depyrimidination of deoxyribonucleic acid in neutral solution.* Biochemistry, 1973. **12**(25): p. 5151-5154.

39. Lindahl, T. and B. Nyberg, *Rate of depurination of native deoxyribonucleic acid.* Biochemistry, 1972. **11**(19): p. 3610-3618.

40. Zoltewicz, J.A., et al., *Kinetics and mechanism of the acid-catalyzed hydrolysis of some purine nucleosides.* Journal of the American Chemical Society, 1970. **92**(6): p. 1741-1750.

41. Dianov, G.L., et al., *Repair of abasic sites in DNA.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2003. **531**(1–2): p. 157-163.

42. Dutta, S., G. Chowdhury, and K.S. Gates, *Interstrand Cross-Links Generated by Abasic Sites in Duplex DNA.* Journal of the American Chemical Society, 2007. **129**(7): p. 1852-1853.

43. Nakamura, J., et al., *Highly Sensitive Apurinic/Apyrimidinic Site Assay Can Detect Spontaneous and Chemically Induced Depurination under Physiological Conditions.* Cancer Research, 1998. **58**(2): p. 222-225.

44. Nakamura, J. and J.A. Swenberg, *Endogenous Apurinic/Apyrimidinic Sites in Genomic DNA of Mammalian Tissues.* Cancer Research, 1999. **59**(11): p. 2522-2526.

45. Friedberg, E.C., *DNA damage and repair.* Nature, 2003. **421**(6921): p. 436-440.

46. Mol, C.D., D.J. Hosfield, and J.A. Tainer, *Abasic site recognition by two apurinic/apyrimidinic endonuclease families in DNA base excision repair: the 3' ends justify the means.* Mutation Research/DNA Repair, 2000. **460**(3–4): p. 211-229.

47. Boiteux, S. and M. Guillet, *Abasic sites in DNA: repair and biological consequences in Saccharomyces cerevisiae.* DNA Repair, 2004. **3**(1): p. 1-12.

48. Losito, I., et al., *Cytosine to uracil conversion through hydrolytic deamination of cytidine monophosphate hydroxy-alkylated on the amino group: a liquid chromatography – electrospray ionization – mass spectrometry investigation.* Journal of Mass Spectrometry, 2012. **47**(10): p. 1384-1393.

49. Carpenter, M., et al., *Sequence-dependent enhancement of hydrolytic deamination of cytosines in DNA by the restriction enzyme PspGI.* Nucleic Acids Research, 2006. **34**(13): p. 3762-3770.

50. Labet, V., et al., *Proton catalyzed hydrolytic deamination of cytosine: a computational study.* Theoretical Chemistry Accounts, 2008. **120**(4-6): p. 429-435.

51. Gates, K.S., *An Overview of Chemical Processes That Damage Cellular DNA: Spontaneous Hydrolysis, Alkylation, and Reactions with Radicals.* Chemical Research in Toxicology, 2009. **22**(11): p. 1747-1760.

52. Stiller, M., et al., *Patterns of nucleotide misincorporations during enzymatic amplification and direct large-scale sequencing of ancient DNA.* Proceedings of the National Academy of Sciences, 2006. **103**(37): p. 13578-13584.

53. Gilbert, M.T.P., et al., *Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis.* Nucleic Acids Research, 2007. **35**(1): p. 1-10.

54. Poltoratsky, V.P., et al., *Recombinogenic Phenotype of Human Activation-Induced Cytosine Deaminase.* The Journal of Immunology, 2004. **172**(7): p. 4308-4313.

55. Tessman, I., M.A. Kennedy, and S.-K. Liu, *Unusual Kinetics of Uracil Formation in Single and Double-stranded DNA by Deamination of Cytosine in Cyclobutane Pyrimidine Dimers.* Journal of Molecular Biology, 1994. **235**(3): p. 807-812.

56. Shen, J.-C., W.M. Rideout, and P.A. Jones, *The rate of hydrolytic deamination of 5-methylcytosine in double-stranded DNA.* Nucleic Acids Research, 1994. **22**(6): p. 972-976.

57. Petersen-Mahrt, S.K. and M.S. Neuberger, *In Vitro Deamination of Cytosine to Uracil in Single-stranded DNA by Apolipoprotein B Editing Complex Catalytic Subunit 1 (APOBEC1).* Journal of Biological Chemistry, 2003. **278**(22): p. 19583-19586.

58. COOKE, M.S., et al., *Oxidative DNA damage: mechanisms, mutation, and disease.* The FASEB Journal, 2003. **17**(10): p. 1195-1214.

59. Slupphaug, G., B. Kavli, and H.E. Krokan, *The interacting pathways for prevention and repair of oxidative DNA damage.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2003. **531**(1–2): p. 231-251.

60. Tudek, B., *Oxidative DNA damage and its repair: Base excision repair.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2003. **531**(1–2): p. 1-3.

61. Cadet, J., et al., *Oxidative damage to DNA: formation, measurement and biochemical features.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2003. **531**(1–2): p. 5-23.

62. Hazra, T.K., et al., *Oxidative DNA damage repair in mammalian cells: A new perspective.* DNA Repair, 2007. **6**(4): p. 470-480.

63. Cadet, J., et al., *Oxidative damage to DNA: Formation, measurement, and biological significance*, in *Reviews of Physiology Biochemistry and Pharmacology, Volume 131*. 1997, Springer Berlin Heidelberg. p. 1-87.

64. Demple, B. and L. Harrison, *Repair of Oxidative Damage to DNA: Enzymology and Biology.* Annual Review of Biochemistry, 1994. **63**(1): p. 915-948.

65. Steenken, S., *Addition-elimination paths in electron-transfer reactions between radicals and molecules. Oxidation of organic molecules by the OH radical.* Journal of the Chemical Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases, 1987. **83**(1): p. 113-124.

66. Téoule, R., *Radiation-induced DNA Damage and Its Repair.* International Journal of Radiation Biology, 1987. **51**(4): p. 573-589.

67. Stone, M.P., et al., *Chemistry and Structural Biology of DNA Damage and Biological Consequences.* Chemistry & Biodiversity, 2011. **8**(9): p. 1571-1615.

68. Dizdaroglu, M., *Oxidative damage to DNA in mammalian chromatin.* Mutation Research/DNAging, 1992. **275**(3–6): p. 331-342.

69. Breen, A.P. and J.A. Murphy, *Reactions of oxyl radicals with DNA.* Free Radical Biology and Medicine, 1995. **18**(6): p. 1033-1077.

70. Wallace, S.S., *Biological consequences of free radical-damaged DNA bases.* Free Radical Biology and Medicine, 2002. **33**(1): p. 1-14.

71. Iida, T., et al., *Accumulation of 8-oxo-2'-deoxyguanosine and increased expression of hMTH1 protein in brain tumors.* Neuro-Oncology, 2001. **3**(2): p. 73-81.

72. Olinski, R., et al., *Oxidative DNA damage in cancer patients: a cause or a consequence of the disease development?* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2003. **531**(1–2): p. 177-190.

73. Fortini, P., et al., *8-Oxoguanine DNA damage: at the crossroad of alternative repair pathways.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2003. **531**(1–2): p. 127-139.

74. Sova, H., et al., *8-Hydroxydeoxyguanosine: a new potential independent prognostic factor in breast cancer.* Br J Cancer, 2010. **102**(6): p. 1018-1023.

75. Nunomura, A., et al., *Oxidative Damage to RNA in Neurodegenerative Diseases.* Journal of Biomedicine and Biotechnology, 2006. **2006**.

76. NUNOMURA, A., et al., *Oxidative Damage Is the Earliest Event in Alzheimer Disease.* Journal of Neuropathology & Experimental Neurology, 2001. **60**(8): p. 759-767.

77. Abe, T., et al., *Alteration of 8-hydroxyguanosine concentrations in the cerebrospinal fluid and serum from patients with Parkinson's disease.* Neuroscience letters, 2003. **336**(2): p. 105-108.

78. Fromme, J.C., et al., *Structural basis for removal of adenine mispaired with 8-oxoguanine by MutY adenine DNA glycosylase.* Nature, 2004. **427**(6975): p. 652-656.

79. Hayashi, H., et al., *Replication-Associated Repair of Adenine:8-Oxoguanine Mispairs by MYH.* Current biology : CB, 2002. **12**(4): p. 335-339.

80. Sheng, Z., et al., *8-Oxoguanine causes neurodegeneration during MUTYH-mediated DNA base excision repair.* The Journal of Clinical Investigation, 2012. **122**(12): p. 4344-4361.

81. Nakabeppu, Y., et al., *Oxidative damage in nucleic acids and Parkinson's disease.* Journal of Neuroscience Research, 2007. **85**(5): p. 919-934.

82. Weissman, L., et al., *DNA repair, mitochondria, and neurodegeneration.* Neuroscience, 2007. **145**(4): p. 1318-1329.

83. Wiseman, H. and B. Halliwell, *Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer.* Biochem. J., 1996. **313**(1): p. 17-29.

84. Cadet, J. and J.R. Wagner, *DNA Base Damage by Reactive Oxygen Species, Oxidizing Agents, and UV Radiation.* Cold Spring Harbor Perspectives in Biology, 2013. **5**(2).

85. Iwahashi, H., *8-Oxo-7,8-dihydro-2'-deoxyguanosine Forms a Relatively Unstable Tetrameric Structure Compared with 2'-Deoxyguanosine.* Journal of Clinical Biochemistry and Nutrition, 2009. **44**(1): p. 57-61.

86. Torizawa, T., et al., *Investigation of the Cyclobutane Pyrimidine Dimer (CPD) Photolyase DNA Recognition Mechanism by NMR Analyses.* Journal of Biological Chemistry, 2004. **279**(31): p. 32950-32956.

87. Kim, S.-i., S.-G. Jin, and G.P. Pfeifer, *Formation of cyclobutane pyrimidine dimers at dipyrimidines containing 5-hydroxymethylcytosine.* Photochemical & Photobiological Sciences, 2013. **12**(8): p. 1409-1415.

88. Rochette, P.J., et al., *UVA-induced cyclobutane pyrimidine dimers form predominantly at thymine–thymine dipyrimidines and correlate with the mutation spectrum in rodent cells.* Nucleic Acids Research, 2003. **31**(11): p. 2786-2794.

89. Vink, A.A. and L. Roza, *Biological consequences of cyclobutane pyrimidine dimers.* Journal of Photochemistry and Photobiology B: Biology, 2001. **65**(2–3): p. 101-104.

90. Clingen, P.H., et al., *Induction of Cyclobutane Pyrimidine Dimers, Pyrimidine(6-4)pyrimidone Photoproducts, and Dewar Valence Isomers by Natural Sunlight in Normal Human Mononuclear Cells.* Cancer Research, 1995. **55**(11): p. 2245-2248.

91. Bates, S.E., et al., *Repair of cyclobutane pyrimidine dimers or dimethylsulfate damage in DNA is identical in normal or telomerase-immortalized human skin fibroblasts.* Nucleic Acids Research, 2005. **33**(8): p. 2475-2485.

92. Marrot, L. and J.-R. Meunier, *Skin DNA photodamage and its biological consequences.* Journal of the American Academy of Dermatology, 2008. **58**(5, Supplement 2): p. S139-S148.

93. Heil, K., D. Pearson, and T. Carell, *Chemical investigation of light induced DNA bipyrimidine damage and repair.* Chemical Society Reviews, 2011. **40**(8): p. 4271-4278.

94. Setlow, R.B., *Cyclobutane-Type Pyrimidine Dimers in Polynucleotides.* Science, 1966. **153**(3734): p. 379-386.

95. Tuteja, N., et al., *Genotoxic stress in plants: Shedding light on DNA damage, repair and DNA repair helicases.* Mutation Research/Reviews in Mutation Research, 2009. **681**(2–3): p. 134-149.

96. Douki, T. and J. Cadet, *Formation of the spore photoproduct and other dimeric lesions between adjacent pyrimidines in UVC-irradiated dry DNA*. Photochemical & Photobiological Sciences, 2003. **2**(4): p. 433-436.

97. Clancy, S., *DNA damage & repair: mechanisms for maintaining DNA integrity.* Nature Education, 2008. **1**(1).

98. Jans, J., et al., *Powerful Skin Cancer Protection by a CPD-Photolyase Transgene.* Current Biology. **15**(2): p. 105-115.

99. Garinis, G.A., J. Jans, and G.T.J. van der Horst, *Photolyases: capturing the light to battle skin cancer.* Future Oncology, 2006. **2**(2): p. 191-199.

100. Sinha, R.P. and D.-P. Hader, *UV-induced DNA damage and repair: a review.* Photochemical & Photobiological Sciences, 2002. **1**(4): p. 225-236.

101. Lindahl, T. and R.D. Wood, *Quality Control by DNA Repair.* Science, 1999. **286**(5446): p. 1897-1905.

102. Kozmin, S.G. and S. Jinks-Robertson, *The Mechanism of Nucleotide Excision Repair-Mediated UV-Induced Mutagenesis in Nonproliferating Cells.* Genetics, 2013. **193**(3): p. 803-817.

103. Hu, J., et al., *Nucleotide Excision Repair in Human Cells: FATE OF THE EXCISED OLIGONUCLEOTIDE CARRYING DNA DAMAGE IN VIVO.* Journal of Biological Chemistry, 2013. **288**(29): p. 20918-20926.

104. Zotter, A., et al., *Recruitment of the Nucleotide Excision Repair Endonuclease XPG to Sites of UV-Induced DNA Damage Depends on Functional TFIIH.* Molecular and Cellular Biology, 2006. **26**(23): p. 8868-8879.

105. Lombaerts, M., et al., *Removal of cyclobutane pyrimidine dimers by the UV damage repair and nucleotide excision repair pathways of Schizosaccharomyces pombe at nucleotide resolution.* Nucleic Acids Research, 1999. **27**(14): p. 2868-2874.

106. Kienzler, A., et al., *UV-induced Nucleotide Excision Repair (NER) and Photoreactivation Repair (PER) in two trout fish cell lines used in ecotoxicological assessment studies.* Journal of Photochemistry and Photobiology B: Biology, 2013. **125**(0): p. 51-55.

107. Rydberg, B. and T. Lindahl, *Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction.* The EMBO Journal, 1982. **1**(2): p. 211-216.

108. Taverna, P. and B. Sedgwick, *Generation of an endogenous DNA-methylating agent by nitrosation in Escherichia coli.* Journal of Bacteriology, 1996. **178**(17): p. 5105-11.

109. Hecht, S.S., *DNA adduct formation from tobacco-specific N-nitrosamines.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1999. **424**(1–2): p. 127-142.

110. Ballschmiter, K., *Pattern and sources of naturally produced organohalogens in the marine environment: biogenic formation of organohalogens.* Chemosphere, 2003. **52**(2): p. 313-324.

111. Hamilton, J.T.G., et al., *Chloride Methylation by Plant Pectin: An Efficient Environmentally Significant Process.* Science, 2003. **301**(5630): p. 206-209.

112. Cantoni, G.L., *THE NATURE OF THE ACTIVE METHYL DONOR FORMED ENZYMATICALLY FROM L-METHIONINE AND ADENOSINETRIPHOSPHATE1,2.* Journal of the American Chemical Society, 1952. **74**(11): p. 2942-2943.

113. Barrows, L.R. and P.N. Magee, *Nonenzymatic methylation of DNA by S-adenosylmethionine in vitro.* Carcinogenesis, 1982. **3**(3): p. 349-351.

114. Chiang, P.K., et al., *S-Adenosylmethionine and methylation.* The FASEB Journal, 1996. **10**(4): p. 471-80.

115. Holliday, R. and T. Ho, *Gene silencing and endogenous DNA methylation in mammalian cells.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1998. **400**(1–2): p. 361-368.

116. Zhao, C. and K. Hemminki, *The in vivo levels of DNA alkylation products in human lymphocytes are not age dependent: an assay of 7-methyl- and 7-(2-hydroxyethyl)-guanine DNA adducts.* Carcinogenesis, 2002. **23**(2): p. 307-310.

117. Keszei, A.P., et al., *Dietary N-nitroso compounds, endogenous nitrosation, and the risk of esophageal and gastric cancer subtypes in the Netherlands Cohort Study.* The American Journal of Clinical Nutrition, 2013. **97**(1): p. 135-146.

118. Parnaud, G., et al., *Endogenous N-Nitroso Compounds, and Their Precursors, Present in Bacon, Do Not Initiate or Promote Aberrant Crypt Foci in the Colon of Rats.* Nutrition and Cancer, 2000. **38**(1): p. 74-80.

119. Loh, Y.H., et al., *N-nitroso compounds and cancer incidence: the European Prospective Investigation into Cancer and Nutrition (EPIC)–Norfolk Study.* The American Journal of Clinical Nutrition, 2011. **93**(5): p. 1053-1061.

120. Bartsch, H., et al., *Endogenously formed N-nitroso compounds and nitrosating agents in human cancer etiology.* Pharmacogenetics and Genomics, 1992. **2**(6): p. 272-277.

121. Dietrich, M., et al., *A review: dietary and endogenously formed N-nitroso compounds and risk of childhood brain tumors.* Cancer Causes & Control, 2005. **16**(6): p. 619-635.

122. Eichholzer, M. and F. Gutzwiller, *Dietary Nitrates, Nitrites, and N-Nitroso Compounds and Cancer Risk: A Review of the Epidemiologic Evidence.* Nutrition Reviews, 1998. **56**(4): p. 95-105.

123. Magee, P.N., *Nitrosamines and human cancer: introduction and overview.* European Journal of Cancer Prevention, 1996. **5**: p. 7-10.

124. Startin, J.R., *N-nitroso compounds in foods and drinks.* European Journal of Cancer Prevention, 1996. **5**: p. 39.

125. Mirvish, S.S., *Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC.* Cancer Letters, 1995. **93**(1): p. 17-48.

126. Schleifer, S. and K. Tempel, *Formation and Persistence of N7 - and O6 -Methyl-Guanine in DNA of Chick Embryo Brain Cells in ovo Following Administration of N-Nitroso-N-Methylurea.* Journal of Veterinary Medicine Series A, 1996. **43**(1-10): p. 589-598.

127. Mirvish, S.S., *Formation of N-nitroso compounds: Chemistry, kinetics, and in vivo occurrence.* Toxicology and Applied Pharmacology, 1975. **31**(3): p. 325-351.

128. Bartsch, H. and B. Spiegelhalder, *Environmental exposure to N-nitroso compounds (NNOC) and precursors: an overview.* European Journal of Cancer Prevention, 1996. **5**: p. 11-17.

129. Yamamoto, M., et al., *Effects of food components and additives on the formation of nitrosamides.* Food Additives & Contaminants, 1988. **5**(3): p. 289-298.

130. Boyland, E. and S.A. Walker, *Effect of thiocyanate on nitrosation of amines.* Nature, 1974. **248**(5449): p. 601-602.

131. Barbarella, G., V. Tugnoli, and M. Zambianchi, *Imidazole Ring Opening of 7-Methylguanosine at Physiological pH.* Nucleosides and Nucleotides, 1991. **10**(8): p. 1759-1769.

132. Tudek, B., S. Bioteux, and J. Laval, *Biological properties of imidazole ring-opened N7-methylguanine in M13mp18 phage DNA.* Nucleic Acids Research, 1992. **20**(12): p. 3079-3084.

133. Huff, A.C. and M.D. Topal, *DNA damage at thymine N-3 abolishes base-pairing capacity during DNA synthesis.* Journal of Biological Chemistry, 1987. **262**(26): p. 12843-12850.

134. Boiteux, S. and J. Laval, *Mutagenesis by alkylating agents: Coding properties for DNA polymerase of poly (dC) template containing 3-methylcytosine.* Biochimie, 1982. **64**(8–9): p. 637-641.

135. Safihill, R., *Differences in the promutagenic nature of 3-methylcytosine as revealed by DNA and RNA polymerising enzymes.* Carcinogenesis, 1984. **5**(5): p. 691-693.

136. Richardson, K.K., et al., *DNA base changes and alkylation following in vivo exposure of Escherichia coli to N-methyl-N-nitrosourea or N-ethyl-N-nitrosourea.* Proceedings of the National Academy of Sciences, 1987. **84**(2): p. 344-348.

137. Jansen, J.G., et al., *Marked Differences in the Role of O6-Alkylguanine in hprt Mutagenesis in T-Lymphocytes of Rats Exposed in Vivo to Ethylmethanesulfonate, N-(2-Hydroxyethyl)-N-nitrosourea, or N-Ethyl-N-nitrosourea.* Cancer Research, 1995. **55**(9): p. 1875-1882.

138. Burdon, R.H., *Genes and the Environment*. 1999: CRC Press. 233.

139. Bolt, H.M. and B. Gansewendt, *Mechanisms of Carcinogenicity of Methyl Halides.* Critical Reviews in Toxicology, 1993. **23**(3): p. 237-253.

140. Vaughan, P., T. Lindahl, and B. Sedgwick, *Induction of the adaptive response of Escherichia coli to alkylation damage by the environmental mutagen, methyl chloride.* Mutation Research/DNA Repair, 1993. **293**(3): p. 249-257.

141. Van Duuren, B.L., *On the possible mechanism of carcinogenic action of vinyl chloride.* Annals of the New York Academy of Sciences, 1975. **246**(1): p. 258-267.

142. Lee, H.J., et al., *Exposure to Potentially Toxic Hydrocarbons and Halocarbons Released From the Dialyzer and Tubing Set During Hemodialysis.* American journal of kidney diseases : the official journal of the National Kidney Foundation, 2012. **60**(4): p. 609-616.

143. Calabrese, E.J. and R.B. Blain, *The Single Exposure Carcinogen Database: assessing the circumstances under which a single exposure to a carcinogen can cause cancer.* Toxicological Sciences, 1999. **50**(2): p. 169-185.

144. SJ, L., et al., *DNA adduct formation from tobacco-specific N-nitrosamines.* Mutation Research, 1999. **424**(1-2): p. 127-142.

145. Lijinsky, W., *N-Nitroso compounds in the diet.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 1999. **443**(1–2): p. 129-138.

146. Hotchkiss, J.H., *Preformed N-nitroso compounds in foods and beverages.* Cancer surveys, 1989. **8**(2): p. 295-321.

147. Stuff, J.E., et al., *N-Nitroso Compounds: Assessing Agreement between Food Frequency Questionnaires and 7-Day Food Records.* Journal of the American Dietetic Association, 2009. **109**(7): p. 1179-1183.

148. Drabløs, F., et al., *Alkylation damage in DNA and RNA—repair mechanisms and medical significance.* DNA Repair, 2004. **3**(11): p. 1389-1407.

149. Blömeke, B., et al., *Distribution of 7-alkyl-2'-deoxyguanosine adduct levels in human lung.* Carcinogenesis, 1996. **17**(4): p. 741-748.

150. Bartsch, H., et al., *EXPOSURE OF HUMANS TO ENDOGENOUS N-NITROSO COMPOUNDS - IMPLICATIONS IN CANCER ETIOLOGY.* Mutation Research, 1990. **238**(3): p. 255-267.

151. O'Driscoll, M., et al., *The cytotoxicity of DNA carboxymethylation and methylation by the model carboxymethylating agent azaserine in human cells.* Carcinogenesis, 1999. **20**(9): p. 1855-1862.

152. Stuff, J.E., et al., *Construction of an N-nitroso database for assessing dietary intake.* Journal of Food Composition and Analysis, 2009. **22, Supplement**(0): p. S42-S47.

153. Mirvish, S.S., et al., *Ascorbate-Nitrite Reaction: Possible Means of Blocking the Formation of Carcinogenic N-Nitroso Compounds.* Science, 1972. **177**(4043): p. 65-68.

154. Dubrow, R., et al., *Dietary Components Related to N-Nitroso Compound Formation: A Prospective Study of Adult Glioma.* Cancer Epidemiology Biomarkers & Prevention, 2010. **19**(7): p. 1709-1722.

155. Nair, J., et al., *Tobacco-specific and betel nut-specific N-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid.* Carcinogenesis, 1985. **6**(2): p. 295-303.

156. Stepanov, I., et al., *Comparative Analysis of Tobacco-Specific Nitrosamines and Total N-Nitroso Compounds in Moldovan Cigarette Tobacco.* Journal of Agricultural and Food Chemistry, 2005. **53**(20): p. 8082-8086.

157. Hecht, S.S. and D. Hoffmann, *N-nitroso compounds and tobacco-induced cancers in man.* IARC scientific publications, 1991(105): p. 54-61.

158. Hurley, L.H., *DNA and its associated processes as targets for cancer therapy.* Nat Rev Cancer, 2002. **2**(3): p. 188-200.

159. Chaney, S.G. and A. Sancar, *DNA Repair: Enzymatic Mechanisms and Relevance to Drug Response.* Journal of the National Cancer Institute, 1996. **88**(19): p. 1346-1360.

160. Middleton, M.R. and G.P. Margison, *Improvement of chemotherapy efficacy by inactivation of a DNA-repair pathway.* The Lancet Oncology, 2003. **4**(1): p. 37-44.

161. Huang, Y. and L. Li, *DNA crosslinking damage and cancer - a tale of friend and foe.* Translational Cancer Research, 2013. **2**(3): p. 144-154.

162. Wu, K.-Y., et al., *Molecular dosimetry of endogenous and ethylene oxide-induced N7-(2-hydroxyethyl) guanine formation in tissues of rodents.* Carcinogenesis, 1999. **20**(9): p. 1787-1792.

163. Bolt, H.M., M. Leutbecher, and K. Golka, *A note on the physiological background of the ethylene oxide adduct 7-(2-hydroxyethyl)guanine in DNA from human blood.* Archives of Toxicology, 1997. **71**(11): p. 719-721.

164. Bolt, H.M., et al., *Quantitation of 7-(2-Hydroxyethyl)guanine Produced by Endogenous and Environmental Ethylene Oxide in DNA of Lymphocytes*, in *Environmental Hygiene III*, N. Seemayer and W. Hadnagy, Editors. 1992, Springer Berlin Heidelberg. p. 59-62.

165. Liao, P.-C., et al., *Quantitative detection of N7-(2-hydroxyethyl)guanine adducts in DNA using high-performance liquid chromatography/electrospray ionization*

tandem mass spectrometry. Journal of Mass Spectrometry, 2001. **36**(3): p. 336-343.

166. Yasuzawa, T., et al., *STRUCTURES OF DUOCARMYCINS, NOVEL ANTITUMOR ANTIBIOTICS PRODUCED BY STREPTOMYCES SP.* Chemical & pharmaceutical bulletin, 1988. **36**(9): p. 3728-3731.

167. Boger, D.L. and D.S. Johnson, *CC-1065 and the duocarmycins: unraveling the keys to a new class of naturally derived DNA alkylating agents.* Proceedings of the National Academy of Sciences, 1995. **92**(9): p. 3642-3649.

168. Bando, T., et al., *Efficient DNA Alkylation by a Pyrrole-Imidazole CBI Conjugate with an Indole Linker: Sequence-Specific Alkylation with Nine-Base-Pair Recognition.* Bioconjugate Chemistry, 2006. **17**(3): p. 715-720.

169. Bando, T. and H. Sugiyama, *Synthesis and Biological Properties of Sequence-Specific DNA-Alkylating Pyrrole−Imidazole Polyamides.* Accounts of Chemical Research, 2006. **39**(12): p. 935-944.

170. Fu, D., J.A. Calvo, and L.D. Samson, *Balancing repair and tolerance of DNA damage caused by alkylating agents.* Nature reviews. Cancer, 2012. **12**(2): p. 104-120.

171. Shooter, K.V., et al., *The molecular basis for biological inactivation of nucleic acids. The action of methylating agents on the ribonucleic acid-containing bacteriophage R17.* Biochem. J., 1974. **137**(2): p. 303-312.

172. Basu, A.K., et al., *Effect of site specifically located mitomycin C-DNA monoadducts on in vitro DNA synthesis by DNA polymerases.* Biochemistry, 1993. **32**(18): p. 4708-4718.

173. David-Cordonnier, M.-H., et al., *DNA and Non-DNA Targets in the Mechanism of Action of the Antitumor Drug Trabectedin.* Chemistry & Biology, 2005. **12**(11): p. 1201-1210.

174. Friedman, D., et al., *Ecteinascidin-743 Inhibits Activated but not Constitutive Transcription.* Cancer Research, 2002. **62**(12): p. 3377-3381.

175. Kašpárková, J., et al., *Effect of Geometric Isomerism in Dinuclear Platinum Antitumor Complexes on DNA Interstrand Cross-Linking†.* Biochemistry, 1999. **38**(34): p. 10997-11005.

176. Singer, B. and G. D, *Molecular Biology of Mutagens and Carcinogens*. 1983, New York, New York: Plenum Press.

177. Beranek, D.T., *Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1990. **231**(1): p. 11-30.

178. Platz, M.S., R.A. Moss, and J. Maitland Jones, *Reviews of Reactive Intermediate Chemistry*. 2007, Hoboken, New Jersey: John Wiley & Sons. 337-340.

179. Warwick, G.P., *The Mechanism of Action of Alkylating Agents.* Cancer Research, 1963. **23**(8 Part 1): p. 1315-1333.

180. Fu, D., J.A. Calvo, and L.D. Samson, *Balancing repair and tolerance of DNA damage caused by alkylating agents.* Nat Rev Cancer, 2012. **12**(2): p. 104-120.

181. Margison, G.P., M.F. Santibáñez Koref, and A.C. Povey, *Mechanisms of carcinogenicity/chemotherapy by O6-methylguanine.* Mutagenesis, 2002. **17**(6): p. 483-487.

182. Christmann, M., et al., *Mechanisms of human DNA repair: an update.* Toxicology, 2003. **193**(1–2): p. 3-34.

183. Wyatt, M.D., et al., *3-methyladenine DNA glycosylases: structure, function, and biological importance.* BioEssays, 1999. **21**(8): p. 668-676.

184. Krokan, H.E., R. Standal, and G. Slupphaug, *DNA glycosylases in the base excision repair of DNA.* Biochem. J., 1997. **325**(1): p. 1-16.

185. Shrivastav, N., D. Li, and J.M. Essigmann, *Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation.* Carcinogenesis, 2010. **31**(1): p. 59-70.

186. Delft, J.H.M.v., et al., *Determining N7-Alkylguanine Adducts by Immunochemical Methods and HPLC with Electrochemical Detection: Applications in Animal Studies and in Monitoring Human Exposure to Alkylating Agents.* Environmental Health Perspectives, 1993. **99**: p. 25-32.

187. Hemminki, K., K. Peltonen, and P. Vodicka, *Depurination from DNA of 7-methylguanine, 7-(2-aminoethyl)-guanine and ring-opened 7-methylguanines.* Chemico-Biological Interactions, 1989. **70**(3–4): p. 289-303.

188. Shuker, D.E.G. and P.B. Farmer, *Relevance of urinary DNA adducts as markers of carcinogen exposure.* Chemical Research in Toxicology, 1992. **5**(4): p. 450-460.

189. Boysen, G., et al., *The formation and biological significance of N7-guanine adducts.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2009. **678**(2): p. 76-94.

190. Doerge, D.R., et al., *DNA adducts derived from administration of acrylamide and glycidamide to mice and rats.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2005. **580**(1–2): p. 131-141.

191. Walker, V.E., et al., *Biomarkers of exposure and effect as indicators of potential carcinogenic risk arising from in vivo metabolism of ethylene to ethylene oxide.* Carcinogenesis, 2000. **21**(9): p. 1661-1669.

192. Young, J.F., R.H. Luecke, and D.R. Doerge, *Physiologically Based Pharmacokinetic/Pharmacodynamic Model for Acrylamide and Its Metabolites in Mice, Rats, and Humans.* Chemical Research in Toxicology, 2007. **20**(3): p. 388-399.

193. Gates, K.S., T. Nooner, and S. Dutta, *Biologically Relevant Chemical Reactions of N7-Alkylguanine Residues in DNA.* Chemical Research in Toxicology, 2004. **17**(7): p. 839-856.

194. Zhao, C., et al., *Endogenous and background DNA adducts by methylating and 2-hydroxyethylating agents.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1999. **424**(1–2): p. 117-125.

195. Harrison, K.L., et al., *N7-methyldeoxyguanosine levels in DNA isolated from cervical cytology samples are associated with smoking.* International Journal of Cancer, 2006. **119**(4): p. 961-963.

196. Mustonen, R. and K. Hemminki, *7-Methylguanine levels in DNA of smokers' and non-smokers' total white blood cells, granulocytes and lymphocytes.* Carcinogenesis, 1992. **13**(11): p. 1951-1955.

197. Harrison, K.L., et al., *Development and Application of a Sensitive and Rapid Immunoassay for the Quantitation of N7-Methyldeoxyguanosine in DNA Samples.* Chemical Research in Toxicology, 2001. **14**(3): p. 295-301.

198. Harrison, K.L., et al., *No association between N7-methyldeoxyguanosine and 8-oxodeoxyguanosine levels in human lymphocyte DNA.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2006. **600**(1–2): p. 125-130.

199. Saad, A.A., et al., *Bladder Tumor Contains Higher N7-Methylguanine Levels in DNA than Adjacent Normal Bladder Epithelium.* Cancer Epidemiology Biomarkers & Prevention, 2006. **15**(4): p. 740-743.

200. Lewis, S.J., et al., *Associations between smoking, GST genotypes and N7-methylguanine levels in DNA extracted from bronchial lavage cells.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2004. **559**(1–2): p. 11-18.

201. Szyfter, K., et al., *Tobacco smoke-associated N7-alkylguanine in DNA of larynx tissue and leucocytes.* Carcinogenesis, 1996. **17**(3): p. 501-506.

202. Johnson, R.E., et al., *A Role for Yeast and Human Translesion Synthesis DNA Polymerases in Promoting Replication through 3-Methyl Adenine.* Molecular and Cellular Biology, 2007. **27**(20): p. 7198-7205.

203. Shah, D., et al., *Evidence in Escherichia coli that N3-Methyladenine Lesions Induced by a Minor Groove Binding Methyl Sulfonate Ester Can Be Processed by both Base and Nucleotide Excision Repair†.* Biochemistry, 2001. **40**(6): p. 1796-1803.

204. Fronza, G. and B. Gold, *The biological effects of N3-methyladenine.* Journal of Cellular Biochemistry, 2004. **91**(2): p. 250-257.

205. Monti, P., et al., *Mutagenicity of N3-methyladenine: A multi-translesion polymerase affair.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2010. **683**(1–2): p. 50-56.

206. Newbold, R.F., et al., *Mutagenicity of carcinogenic methylating agents is associated with a specific DNA modification.* Nature, 1980. **283**(5747): p. 596-599.

207. Dumenco, L., et al., *The prevention of thymic lymphomas in transgenic mice by human O6-alkylguanine-DNA alkyltransferase.* Science, 1993. **259**(5092): p. 219-222.

208. Jena, N.R. and B. Manju, *Mutagenicity associated with O6-methylguanine-DNA damage and mechanism of nucleotide flipping by AGT during repair.* Physical Biology, 2011. **8**(4): p. 046007.

209. Topal, M.D., J.S. Eadie, and M. Conrad, *O6-methylguanine mutation and repair is nonuniform. Selection for DNA most interactive with O6-methylguanine.* Journal of Biological Chemistry, 1986. **261**(21): p. 9879-9885.

210. Singer, B., et al., *Effect of 3′ flanking neighbors on kinetics of pairing of dCTP or dTTP opposite O6-methylguanine in a defined primed oligonucleotide when Escherichia coli DNA polymerase I is used.* Proceedings of the National Academy of Sciences, 1989. **86**(21): p. 8271-8274.

211. Patel, D.J., et al., *Structural studies of the O6meG.cntdot.C interaction in the d(C-G-C-G-A-A-T-T-C-O6meG-C-G) duplex.* Biochemistry, 1986. **25**(5): p. 1027-1036.

212. Gaffney, B.L. and R.A. Jones, *Thermodynamic comparison of the base pairs formed by the carcinogenic lesion O6-methylguanine with reference both to Watson-Crick pairs and to mismatched pairs.* Biochemistry, 1989. **28**(14): p. 5881-5889.

213. Patel, D.J., et al., *Structural studies of the O6meG.cntdot.T interaction in the d(C-G-T-G-A-A-T-T-C-O6meG-C-G) duplex.* Biochemistry, 1986. **25**(5): p. 1036-1042.

214. Leonard, G.A., et al., *High-resolution structure of a mutagenic lesion in DNA.* Proceedings of the National Academy of Sciences, 1990. **87**(24): p. 9573-9576.

215. Tan, H.-B., P.F. Swann, and E.M. Chance, *Kinetic Analysis of the Coding Properties of O6-Methylguanine in DNA: The Crucial Role of the Conformation of the Phosphodiester Bond.* Biochemistry, 1994. **33**(17): p. 5335-5346.

216. Warren, J.J., L.J. Forsberg, and L.S. Beese, *The structural basis for the mutagenicity of O6-methyl-guanine lesions.* Proceedings of the National Academy of Sciences, 2006. **103**(52): p. 19701-19706.

217. Margison, G.P. and M.F. Santibáñez-Koref, *O6-alkylguanine-DNA alkyltransferase: Role in carcinogenesis and chemotherapy.* BioEssays, 2002. **24**(3): p. 255-266.

218. Pegg, A.E. and T.L. Byers, *Repair of DNA containing O6-alkylguanine.* The FASEB Journal, 1992. **6**(6): p. 2302-10.

219. Shibata, T., et al., *Novel synthesis of O6-alkylguanine containing oligodeoxyribonucleotides as substrates for the human DNA repair protein, O6-methylguanine DNA methyltransferase (MGMT).* Nucleic Acids Research, 2006. **34**(6): p. 1884-1891.

220. Terashima, I., et al., *Substrate Specificity of Human O6-Methylguanine-DNA Methyltransferase for O6-Benzylguanine Derivatives in Oligodeoxynucleotides.* Chemical Research in Toxicology, 1997. **10**(11): p. 1234-1239.

221. Schoonhoven, N.M., et al., *Synthesis, Biophysical and Repair Studies of O6-2'-Deoxyguanosine Adducts by Escherichia coli OGT.* Nucleic Acids Symposium Series, 2008. **52**(1): p. 449-450.

222. Kondo, N., et al., *DNA Damage Induced by Alkylating Agents and Repair Pathways.* Journal of Nucleic Acids, 2010. **2010**.

223. Kohn, K.W., *Beyond DNA Cross-Linking: History and Prospects of DNA-targeted Cancer Treatment—Fifteenth Bruce F. Cain Memorial Award Lecture.* Cancer Research, 1996. **56**(24): p. 5533-5546.

224. Esteller, M., et al., *Inactivation of the DNA-Repair Gene MGMT and the Clinical Response of Gliomas to Alkylating Agents.* New England Journal of Medicine, 2000. **343**(19): p. 1350-1354.

225. Corrie, P.G., *Cytotoxic chemotherapy: clinical aspects.* Medicine, 2011. **39**(12): p. 717-722.

226. Lind, M.J., *Principles of cytotoxic chemotherapy.* Medicine, 2011. **39**(12): p. 711-716.

227. Siddik, Z.H., *Mechanisms of Action of Cancer Chemotherapeutic Agents: DNA-Interactive Alkylating Agents and Antitumour Platinum-Based Drugs*, in *The Cancer Handbook*. 2005, John Wiley & Sons, Ltd.

228. Takata, K.-i., et al., *Human DNA helicase HELQ participates in DNA interstrand crosslink tolerance with ATR and RAD51 paralogs.* Nat Commun, 2013. **4**.

229. Fleer, R. and M. Brendel, *Toxicity, interstrand cross-links and DNA fragmentation induced by 'activated' cyclophosphamide in yeast.* Chemico-Biological Interactions, 1981. **37**(1–2): p. 123-140.

230. Shukla, P., et al., *DNA interstrand cross-link repair: understanding role of Fanconi anemia pathway and therapeutic implications.* European Journal of Haematology, 2013: p. n/a-n/a.

231. Pichierri, P., D. Averbeck, and F. Rosselli, *DNA cross-link-dependent RAD50/MRE11/NBS1 subnuclear assembly requires the Fanconi anemia C protein.* Human Molecular Genetics, 2002. **11**(21): p. 2531-2546.

232. Enoiu, M., J. Jiricny, and O.D. Schärer, *Repair of cisplatin-induced DNA interstrand crosslinks by a replication-independent pathway involving transcription-coupled repair and translesion synthesis.* Nucleic Acids Research, 2012.

233. Lawley, P.D. and D.H. Phillips, *DNA adducts from chemotherapeutic agents.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1996. **355**(1–2): p. 13-40.

234. Murnane, J.P. and J.E. Byfield, *Irreparable DNA cross-links and mammalian cell lethality with bifunctional alkylating agents.* Chemico-Biological Interactions, 1981. **38**(1): p. 75-86.

235. Noll, D.M., T.M. Mason, and P.S. Miller, *Formation and Repair of Interstrand Cross-Links in DNA.* Chemical Review, 2006. **106**(2): p. 277-301.

236. Zuravka, I., et al., *Synthesis and DNA Cleavage Activity of Bis-3-chloropiperidines as Alkylating Agents.* ChemMedChem, 2014: p. n/a-n/a.

237. Povirk, L.F. and D.E. Shuker, *DNA damage and mutagenesis induced by nitrogen mustards.* Mutation Research/Reviews in Genetic Toxicology, 1994. **318**(3): p. 205-226.

238. Millard, J.T., S. Raucher, and P.B. Hopkins, *Mechlorethamine cross-links deoxyguanosine residues at 5'-GNC sequences in duplex DNA fragments.* Journal of the American Chemical Society, 1990. **112**(6): p. 2459-2460.

239. Cullis, P.M., L. Merson-Davies, and R. Weaver, *Conjugation of a Polyamine to the Bifunctional Alkylating Agent Chlorambucil Does Not Alter the Preferred Crosslinking Site in Duplex DNA.* Journal of the American Chemical Society, 1995. **117**(30): p. 8033-8034.

240. Rink, S.M., et al., *Covalent structure of a nitrogen mustard-induced DNA interstrand cross-link: an N7-to-N7 linkage of deoxyguanosine residues at the duplex sequence 5'-d(GNC).* Journal of the American Chemical Society, 1993. **115**(7): p. 2551-2557.

241. Dong, Q., et al., *A structural basis for a phosphoramide mustard-induced DNA interstrand cross-link at 5'-d(GAC).* Proceedings of the National Academy of Sciences, 1995. **92**(26): p. 12170-12174.

242. Bauer, G.B. and L.F. Povirk, *Specificity and Kinetics of Interstrand and Intrastrand Bifunctional Alkylation by Nitrogen Mustards at a G-G-C Sequence.* Nucleic Acids Research, 1997. **25**(6): p. 1211-1218.

243. Golding, B.T., M.J. Kebbell, and I.M. Lockhart, *Chemistry of nitrogen mustard [2-chloro-N-(2-chloroethyl)-N-methylethanamine] studied by nuclear magnetic resonance spectroscopy.* Journal of the Chemical Society, Perkin Transactions 2, 1987(6): p. 705-713.

244. Rajski, S.R. and R.M. Williams, *DNA Cross-Linking Agents as Antitumor Drugs.* Chemical Reviews, 1998. **98**(8): p. 2723-2796.

245. Brookes, P. and P.D. Lawley, *The reaction of mono- and di-functional alkylating agents with nucleic acids.* Biochem. J., 1961. **80**(3): p. 496-503.

246. Rink, S.M. and P.B. Hopkins, *A mechlorethamine-induced DNA interstrand cross-link bends duplex DNA.* Biochemistry, 1995. **34**(4): p. 1439-1445.

247. Noll, D.M., T.M. Mason, and P.S. Miller, *Formation and Repair of Interstrand Cross-Links in DNA.* Chemical Reviews, 2005. **106**(2): p. 277-301.

248. Denny, W.A., *DNA minor groove alkylating agents.* Expert Opinion on Therapeutic Patents, 2000. **10**(4): p. 459-474.

249. Eisenbrand, G., et al., *DNA adducts and DNA damage by antineoplastic and carcinogenic N-nitrosocompounds.* Journal of Cancer Research and Clinical Oncology, 1986. **112**(3): p. 196-204.

250. Kunz, B.A. and J.R.A. Mis, *Mutational Specificities of 1,3-Bis(2-chloroethyl)-1-nitrosourea and Nitrogen Mustard in the SUP4-o Gene of Saccharomyces cerevisiae.* Cancer Research, 1989. **49**(2): p. 279-283.

251. Tong, W.P. and D.B. Ludlum, *Formation of the Cross-Linked Base, Diguanylethane, in DNA Treated with N,N'-Bis(2-chloroethyl)-N-nitrosourea.* Cancer Research, 1981. **41**(2): p. 380-382.

252. Reed, D.J., et al., *2-Chloroethanol Formation as Evidence for a 2-Chloroethyl Alkylating Intermediate during Chemical Degradation of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea and 1-(2-Chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea.* Cancer Research, 1975. **35**(3): p. 568-576.

253. Lown, J.W. and S.M.S. Chauhan, *Mechanism of action of (2-haloethyl)nitrosoureas on DNA. Isolation and reactions of postulated 2-(alkylimino)-3-nitrosooxazolidine intermediates in the decomposition of 1,3-bis(2-chloroethyl)-, 1-(2-chloroethyl)-3-cyclohexyl-, and 1-(2-chloroethyl)-3-(4'-trans-methylcyclohexyl)-1-nitrosourea.* Journal of Medicinal Chemistry, 1981. **24**(3): p. 270-279.

254. Montgomery, J.A., et al., *Decomposition of N-(2-chloroethyl)-N-nitrosoureas in aqueous media.* Journal of Medicinal Chemistry, 1975. **18**(6): p. 568-571.

255. Ahlgren, J.D., et al., *Repair of DNA Alkylation Induced in L1210 Leukemia and Murine Bone Marrow by Three Chloroethylnitrosoureas.* Cancer Research, 1982. **42**(7): p. 2605-2608.

256. Bedford, P. and G. Eisenbrand, *DNA Damage and Repair in the Bone Marrow of Rats Treated with Four Chloroethylnitrosoureas.* Cancer Research, 1984. **44**(2): p. 514-518.

257. Kroes, R.A., et al., *Selective activation of human heat shock gene transcription by nitrosourea antitumor drugs mediated by isocyanate-induced damage and activation of heat shock transcription factor.* Proceedings of the National Academy of Sciences, 1991. **88**(11): p. 4825-4829.

258. Chen, F.-X., et al., *DNA crosslinking, sister chromatid exchange and cytotoxicity of N-2-chloroethylnitrosoureas tethered to minor groove binding peptides.* Carcinogenesis, 1993. **14**(5): p. 935-940.

259. Pacheco, D.Y., N.K. Stratton, and N.W. Gibson, *Comparison of the Mechanism of Action of Busulfan with Hepsulfam, a New Antileukemic Agent, in the L1210 Cell Line.* Cancer Research, 1989. **49**(18): p. 5108-5110.

260. Boland, I., et al., *Busulphan is active against neuroblastoma and medulloblastoma xenografts in athymic mice at clinically achievable plasma drug concentrations.* Br J Cancer, 1999. **79**(5-6): p. 787-792.

261. Marshall, M.V., et al., *In vitro cytotoxicity of hepsulfam against human tumor cell lines and primary human tumor colony forming units.* STEM CELLS, 1993. **11**(1): p. 62-69.

262. Ravdin, P.M., et al., *A Phase I Clinical and Pharmacokinetic Trial of Hepsulfam.* Cancer Research, 1991. **51**(23 Part 1): p. 6268-6272.

263. Eberly, A.L., et al., *Optimal Prevention of Seizures Induced by High-Dose Busulfan.* Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 2008. **28**(12): p. 1502-1510.

264. Pacheco, D.Y., et al., *Mechanisms of toxicity of hepsulfam in human tumor cell lines.* Cancer research, 1990. **50**(23): p. 7555-7558.

265. Marshall, M., et al., *Hepsulfam distribution in blood, plasma and cerebrospinal fluid of baboons.* Investigational New Drugs, 1995. **13**(1): p. 33-36.

266. Bishop, J.B. and J.S. Wassom, *Toxicological review of busulfan (Myleran).* Mutation Research/Reviews in Genetic Toxicology, 1986. **168**(1): p. 15-45.

267. Tong, W.P. and D.B. Ludlum, *Crosslinking of DNA by busulfan Formation of diguanyl derivatives.* Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis, 1980. **608**(1): p. 174-181.

268. Iwamoto, T., et al., *DNA intrastrand cross-link at the 5′-GA-3′ sequence formed by busulfan and its role in the cytotoxic effect.* Cancer Science, 2004. **95**(5): p. 454-458.

269. Larson, R., et al., *Encephalopathy is the dose-limiting toxicity of intravenous hepsulfam: results of a phase I trial in patients with advanced hematological malignancies.* Cancer Chemotherapy and Pharmacology, 1995. **36**(3): p. 204-210.

270. Fan, Y.-H. and B. Gold, *Sequence-Specificity for DNA Interstrand Cross-Linking by α,ω-Alkanediol Dimethylsulfonate Esters: Evidence for DNA Distortion by the Initial Monofunctional Lesion.* Journal of the American Chemical Society, 1999. **121**(51): p. 11942-11946.

271. Glaser, R., et al., *Theoretical Studies of DNA Base Deamination. 2. Ab Initio Study of DNA Base Diazonium Ions and of Their Linear, Unimolecular Dediazoniation Paths†,§.* Journal of the American Chemical Society, 1999. **121**(26): p. 6108-6119.

272. Harwood, E.A., et al., *Chemical Synthesis and Preliminary Structural Characterization of a Nitrous Acid Interstrand Cross-Linked Duplex DNA.* Journal of the American Chemical Society, 1999. **121**(21): p. 5081-5082.

273. Becker Jr, E.F., B.K. Zimmerman, and E.P. Geiduschek, *Structure and function of cross-linked DNA: I. Reversible denaturation and Bacillus subtilis transformation.* Journal of Molecular Biology, 1964. **8**(3): p. 377-391.

274. Kirchner, J.J., S.T. Sigurdsson, and P.B. Hopkins, *Interstrand cross-linking of duplex DNA by nitrous acid: covalent structure of the dG-to-dG cross-link at the sequence 5′-CG.* Journal of the American Chemical Society, 1992. **114**(11): p. 4021-4027.

275. Kirchner, J.J. and P.B. Hopkins, *Nitrous acid cross-links duplex DNA fragments through deoxyguanosine residues at the sequence 5′-CG.* Journal of the American Chemical Society, 1991. **113**(12): p. 4681-4682.

276. Elcock, A.H., et al., *Combined Quantum and Molecular Mechanical Study of DNA Crosslinking by Nitrous Acid.* Journal of the American Chemical Society, 1995. **117**(16): p. 4706-4707.

277. Edfeldt, N.B.F., et al., *Solution structure of a nitrous acid induced DNA interstrand cross-link.* Nucleic Acids Research, 2004. **32**(9): p. 2785-2794.

278. Edfeldt, N.B.F., et al., *Sequence context effect on the structure of nitrous acid induced DNA interstrand cross-links.* Nucleic Acids Research, 2004. **32**(9): p. 2795-2801.

279. Tomasz, M., *Mitomycin C: small, fast and deadly (but very selective).* Chemistry & Biology, 1995. **2**(9): p. 575-579.

280. Stevens, C.L., et al., *Chemistry and Structure of Mitomycin C1.* Journal of Medicinal Chemistry, 1965. **8**(1): p. 1-10.

281. Crooke, S.T. and W.T. Bradner, *Mitomycin C: a review.* Cancer Treatment Reviews, 1976. **3**(3): p. 121-139.

282. Boger, D.L., et al., *DNA alkylation properties of the duocarmycins: (+)-duocarmycin A, epi-(+)-duocarmycin A, ent-(−)-duocarmycin A and epi,ent-(−)-duocarmycin A.* Bioorganic & Medicinal Chemistry Letters, 1992. **2**(7): p. 759-765.

283. Smith, J.A., et al., *The structural basis for in situ activation of DNA alkylation by duocarmycin SA.* Journal of Molecular Biology, 2000. **300**(5): p. 1195-1204.

284. Fukuda, Y., K. Nakatani, and S. Terashima, *Synthesis and cytotoxicity of enantiomeric pairs of duocarmycin a and its 2-epimer.* Bioorganic & Medicinal Chemistry Letters, 1992. **2**(7): p. 755-758.

285. Fukuda, Y., et al., *First total synthesis of dl-duocarmycin A.* Tetrahedron Letters, 1990. **31**(46): p. 6699-6702.

286. Andrez, J.-C., *Mitomycins syntheses: a recent update.* Beilstein Journal of Organic Chemistry, 2009. **5**: p. 33.

287. Bradner, W.T., *Mitomycin C: a clinical update.* Cancer Treatment Reviews, 2001. **27**(1): p. 35-50.

288. Doll, D.C., R.B. Weiss, and B.F. Issell, *Mitomycin: ten years after approval for marketing.* Journal of Clinical Oncology, 1985. **3**(2): p. 276-86.

289. Weng, M.-w., et al., *Repair of mitomycin C mono- and interstrand cross-linked DNA adducts by UvrABC: a new model.* Nucleic Acids Research, 2010. **38**(20): p. 6976-6984.

290. Warren, A.J. and J.W. Hamilton, *Synthesis and Structural Characterization of the N2G−Mitomycin C−N2G Interstrand Cross-Link in a Model Synthetic 23 Base Pair Oligonucleotide DNA Duplex.* Chemical Research in Toxicology, 1996. **9**(7): p. 1063-1071.

291. Tomasz, M., et al., *Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA.* Science, 1987. **235**(4793): p. 1204-1208.

292. Kennedy, K.A., et al., *pH Dependence of Mitomycin C-induced Cross-Linking Activity in EMT6 Tumor Cells.* Cancer Research, 1985. **45**(8): p. 3541-3547.

293. Paz, M.M., A. Das, and M. Tomasz, *Mitomycin C linked to DNA minor groove binding agents: synthesis, reductive activation, DNA binding and cross-linking properties and in vitro antitumor activity.* Bioorganic & Medicinal Chemistry, 1999. **7**(12): p. 2713-2726.

294. Rink, S.M., et al., *Bending of DNA by the Mitomycin C-Induced, GpG Intrastrand Cross-Link.* Chemical Research in Toxicology, 1996. **9**(2): p. 382-389.

295. Kumar, S., W.S. Johnson, and M. Tomasz, *Orientation isomers of the mitomycin C interstrand cross-link in non-self-complementary DNA. Differential effect of the*

two isomers on restriction endonuclease cleavage at a nearby site. Biochemistry, 1993. **32**(5): p. 1364-1372.

296. Takasugi, M., et al., *Sequence-specific photo-induced cross-linking of the two strands of double-helical DNA by a psoralen covalently linked to a triple helix-forming oligonucleotide.* Proceedings of the National Academy of Sciences, 1991. **88**(13): p. 5602-5606.

297. Wellinger, R.E. and J.M. Sogo, *In vivo mapping of nucleosomes using psoralen-DNA crosslinking and primer extension.* Nucleic Acids Research, 1998. **26**(6): p. 1544-1545.

298. Eichman, B.F., et al., *The crystal structures of psoralen cross-linked DNAs: drug-dependent formation of Holliday junctions.* Journal of Molecular Biology, 2001. **308**(1): p. 15-26.

299. Derheimer, F.A., et al., *Psoralen-Induced DNA Interstrand Cross-Links Block Transcription and Induce p53 in an Ataxia-Telangiectasia and Rad3-Related-Dependent Manner.* Molecular Pharmacology, 2009. **75**(3): p. 599-607.

300. Kroeger Smith, M.B., et al., *Base Sequence Selectivity in the Alkylation of DNA by 1,3-Dialkyl-3-acyltriazenes.* Chemical Research in Toxicology, 1996. **9**(1): p. 341-348.

301. Kroeger-Koepke, M.B., C.J. Michejda, and R.H. Smith, *Alkylation of DNA by 1,3-dialkyl-3-acyltriazenes: correlation of biological activity with chemical behavior.* Chemical Research in Toxicology, 1992. **5**(4): p. 541-547.

302. Marchesi, F., et al., *Triazene compounds: Mechanism of action and related DNA repair systems.* Pharmacological Research, 2007. **56**(4): p. 275-287.

303. Smith, R.H., et al., *Novel cross-linking alkylating agents, 1-(2-chloroethyl)-3-methyl-3-acyltriazenes.* The Journal of Organic Chemistry, 1988. **53**(7): p. 1467-1471.

304. Jamieson, E.R. and S.J. Lippard, *Structure, Recognition, and Processing of Cisplatin−DNA Adducts.* Chemical Reviews, 1999. **99**(9): p. 2467-2498.

305. Wong, E. and C.M. Giandomenico, *Current Status of Platinum-Based Antitumor Drugs.* Chemical Reviews, 1999. **99**(9): p. 2451-2466.

306. Wang, D. and S.J. Lippard, *Cellular processing of platinum anticancer drugs.* Nat Rev Drug Discov, 2005. **4**(4): p. 307-320.

307. Lippard, S.J. and J.D. Hoeschele, *Binding of cis- and trans-dichlorodiammineplatinum(II) to the nucleosome core.* Proceedings of the National Academy of Sciences, 1979. **76**(12): p. 6091-6095.

308. Malinge, J.-M., M.-J. Giraud-Panis, and M. Leng, *Interstrand cross-links of cisplatin induce striking distortions in DNA.* Journal of Inorganic Biochemistry, 1999. **77**(1–2): p. 23-29.

309. Ober, M. and S.J. Lippard, *A 1,2-d(GpG) Cisplatin Intrastrand Cross-Link Influences the Rotational and Translational Setting of DNA in Nucleosomes.* Journal of the American Chemical Society, 2008. **130**(9): p. 2851-2861.

310. Moggs, J.G., et al., *Differential Human Nucleotide Excision Repair of Paired and Mispaired Cisplatin-DNA Adducts.* Nucleic Acids Research, 1997. **25**(3): p. 480-490.

311. Farrell, L.R.K.a.N., *Platinum-Based Drugs in Cancer Therapy*. XII ed. Chemistry and Structural Biology of 1,2-Interstrand Adducts of Cisplatin, ed. V. Brabec. 2000, Totowa, NJ: Humana Press Inc. 37-61.

312. Stehlikova, K., et al., *DNA bending and unwinding due to the major 1,2-GG intrastrand cross-link formed by antitumor cis-diamminedichloroplatinum(II) are flanking-base independent.* Nucleic Acids Research, 2002. **30**(13): p. 2894-2898.

313. Kelland, L.R. and N.P. Farrell, *Platinum-Based Drugs in Cancer Therapy*. XII ed. Chemistry and Structural Biology of 1,2-Interstrand Adducts of Cisplatin, ed. L.R. Kelland and N.P. Farrell. 2000, Totowa, NJ: Humana Press Inc. 341.

314. Huang, H., et al., *Solution Structure of a Cisplatin-Induced DNA Interstrand Cross-Link.* Science, 1995. **270**(5243): p. 1842-1845.

315. Coste, F., et al., *Crystal structure of a double-stranded DNA containing a cisplatin interstrand cross-link at 1.63 Å resolution: Hydration at the platinated site.* Nucleic Acids Research, 1999. **27**(8): p. 1837-1846.

316. McHugh, P.J., V.J. Spanswick, and J.A. Hartley, *Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance.* The Lancet Oncology, 2001. **2**(8): p. 483-490.

317. Helleday, T., et al., *DNA repair pathways as targets for cancer therapy.* Nature Reviews Cancer, 2008. **8**(3): p. 193-204.

318. Lee, P.C., et al., *Combination of bifunctional alkylating agent and arsenic trioxide synergistically suppresses the growth of drug-resistant tumor cells.* Neoplasia (New York, N.Y.), 2010. **12**(5): p. 376-387.

319. Deans, A.J. and S.C. West, *DNA interstrand crosslink repair and cancer.* Nat Rev Cancer, 2011. **11**(7): p. 467-480.

320. Ralhan, R. and J. Kaur, *Alkylating agents and cancer therapy.* Expert Opinion on Therapeutic Patents, 2007. **17**(9): p. 1061-1075.

321. Woo, J., S.T. Sigurdsson, and P.B. Hopkins, *DNA interstrand cross-linking reactions of pyrrole-derived, bifunctional electrophiles: evidence for a common target site in DNA.* Journal of the American Chemical Society, 1993. **115**(9): p. 3407-3415.

322. Ojwang, J.O., D.A. Grueneberg, and E.L. Loechler, *Synthesis of a Duplex Oligonucleotide Containing a Nitrogen Mustard Interstrand DNA-DNA Cross-Link.* Cancer Research, 1989. **49**(23): p. 6529-6537.

323. Alley, S.C., K.A. Brameld, and P.B. Hopkins, *DNA Interstrand Crosslinking by 2,5-Bis(1-aziridinyl)-1,4-benzoquinone: Nucleotide Sequence Preferences and Covalent Structure of the dG-to-dG Cross-Links at 5′-d(GNnC) in Synthetic Oligonucleotide Duplexes.* Journal of the American Chemical Society, 1994. **116**(7): p. 2734-2741.

324. Millard, J.T. and M.M. White, *Diepoxybutane cross-links DNA at 5′-GNC sequences.* Biochemistry, 1993. **32**(8): p. 2120-2124.

325. Sawyer, G.A., E.D. Frederick, and J.T. Millard, *Flanking Sequences Modulate Diepoxide and Mustard Cross-Linking Efficiencies at the 5′-GNC Site.* Chemical Research in Toxicology, 2004. **17**(8): p. 1057-1063.

326. Millard, J.T., et al., *The 5'-GNC Site for DNA Interstrand Cross-Linking Is Conserved for Diepoxybutane Stereoisomers.* Chemical Research in Toxicology, 2005. **19**(1): p. 16-19.

327. Romano, K.P., et al., *DNA Interstrand Cross-Linking by Epichlorohydrin.* Chemical Research in Toxicology, 2007. **20**(5): p. 832-838.

328. Alley, S.C. and P.B. Hopkins, *DNA-DNA Interstrand Crosslinking by 2,5-Bis(1-aziridinyl)-3,6-bis(carbethoxyamino)-1,4-benzoquinone: Covalent Structure of the dG-to-dG Cross-Links in Calf Thymus DNA and a Synthetic DNA Duplex.* Chemical Research in Toxicology, 1994. **7**(5): p. 666-672.

329. Huang, H. and P.B. Hopkins, *DNA interstrand cross-linking by formaldehyde: nucleotide sequence preference and covalent structure of the predominant cross-link formed in synthetic oligonucleotides.* Journal of the American Chemical Society, 1993. **115**(21): p. 9402-9408.

330. Huang, H., M.S. Solomon, and P.B. Hopkins, *Formaldehyde preferentially interstrand cross-links duplex DNA through deoxyadenosine residues at the sequence 5'-d(AT).* Journal of the American Chemical Society, 1992. **114**(23): p. 9240-9241.

331. Weidner, M.F., J.T. Millard, and P.B. Hopkins, *Determination at single nucleotide resolution of the sequence specificity of DNA interstrand crosslinking agents in DNA fragments.* Journal of the American Chemical Society, 1989. **111**(26): p. 9270-9272.

332. Weidner, M.F., S.T. Sigurdsson, and P.B. Hopkins, *Sequence preferences of DNA interstrand cross-linking agents: dG-to-dG cross-linking at 5'-CG by structurally simplified analogs of mitomycin C.* Biochemistry, 1990. **29**(39): p. 9225-9233.

333. Streeper, R.T., et al., *Molecular Pharmacology of Hepsulfam, NSC 3296801: Identification of Alkylated Nucleosides, Alkylation Site, and Site of DNA Cross-Linking.* Cancer Research, 1995. **55**(7): p. 1491-1498.

334. Dong, Q., et al., *A structural basis for a phosphoramide mustard-induced DNA interstrand cross-link at 5'-d(GAC).* Proc Natl Acad Sci U S A, 1995. **92**(26): p. 12170-4.

335. Cozzi, P., *A new class of cytotoxic DNA minor groove binders: α-halogenoacrylic derivatives of pyrrolecarbamoyl oligomers.* Il Farmaco, 2001. **56**(1–2): p. 57-65.

336. Christov, P.P., et al., *Site-Specific Synthesis and Characterization of Oligonucleotides Containing an N6-(2-Deoxy-d-erythro-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-N-methylformamidopyrimidine Lesion, the Ring-Opened Product from N7-Methylation of Deoxyguanosine.* Chemical Research in Toxicology, 2008. **21**(12): p. 2324-2333.

337. Boiteux, S. and S. Jinks-Robertson, *DNA Repair Mechanisms and the Bypass of DNA Damage in Saccharomyces cerevisiae.* Genetics, 2013. **193**(4): p. 1025-1064.

338. Grillari, J., H. Katinger, and R. Voglauer, *Contributions of DNA interstrand cross-links to aging of cells and organisms.* Nucleic Acids Research, 2007. **35**(22): p. 7566-7576.

339. Brabec, V. and M. Leng, *DNA interstrand cross-links of trans-diamminedichloroplatinum(II) are preferentially formed between guanine and complementary cytosine residues.* Proceedings of the National Academy of Sciences, 1993. **90**(11): p. 5345-5349.

340. Jones, J.C., et al., *Gene-specific formation and repair of cisplatin intrastrand adducts and interstrand cross-links in Chinese hamster ovary cells.* Journal of Biological Chemistry, 1991. **266**(11): p. 7101-7.

341. Roberts, J.J. and F. Friedlos, *Quantitative estimation of cisplatin-induced DNA interstrand cross-links and their repair in mammalian cells: Relationship to toxicity.* Pharmacology & Therapeutics, 1987. **34**(2): p. 215-246.

342. Balcome, S., et al., *Adenine-Containing DNA−DNA Cross-Links of Antitumor Nitrogen Mustards.* Chemical Research in Toxicology, 2004. **17**(7): p. 950-962.

343. JK, W. and W. J., *Genotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1995. **339**(2): p. 91-119.

344. Seow, H.A., et al., *Bioactivation and Resistance to Mitomycin C*, in *Methods in Enzymology*, S. Helmut and P. Lester, Editors. 2004, Academic Press. p. 221-233.

345. Gregson, S.J., et al., *Linker Length Modulates DNA Cross-Linking Reactivity and Cytotoxic Potency of C8/C8′ Ether-Linked C2-exo-Unsaturated Pyrrolo[2,1-c][1,4]benzodiazepine (PBD) Dimers.* Journal of Medicinal Chemistry, 2004. **47**(5): p. 1161-1174.

346. Gregson, S.J., et al., *Design, Synthesis, and Evaluation of a Novel Pyrrolobenzodiazepine DNA-Interactive Agent with Highly Efficient Cross-*

Linking Ability and Potent Cytotoxicity. Journal of Medicinal Chemistry, 2001. **44**(5): p. 737-748.

347. Muniandy, P.A., et al., *DNA interstrand crosslink repair in mammalian cells: step by step.* Critical Reviews in Biochemistry and Molecular Biology, 2010. **45**(1): p. 23-49.

348. Dronkert, M.L.G. and R. Kanaar, *Repair of DNA interstrand cross-links.* Mutation Research/DNA Repair, 2001. **486**(4): p. 217-247.

349. Niedernhofer, L.J., A.S. Lalai, and J.H.J. Hoeijmakers, *Fanconi Anemia (Cross)linked to DNA Repair.* Cell, 2005. **123**(7): p. 1191-1198.

350. Räschle, M., et al., *Mechanism of Replication-Coupled DNA Interstrand Crosslink Repair.* Cell, 2008. **134**(6): p. 969-980.

351. Wilds, C.J., F. Xu, and A.M. Noronha, *Synthesis and Characterization of DNA Containing an N1-2'-Deoxyinosine-ethyl-N3-thymidine Interstrand Cross-Link: A Structural Mimic of the Cross-Link Formed by 1,3-Bis-(2-chloroethyl)-1-nitrosourea.* Chemical Research in Toxicology, 2008. **21**(3): p. 686-695.

352. Schärer, O.D., *DNA Interstrand Crosslinks: Natural and Drug-Induced DNA Adducts that Induce Unique Cellular Responses.* ChemBioChem, 2005. **6**(1): p. 27-32.

353. Cui, Z., et al., *Characterization of synthetic oligonucleotides containing biologically important modified bases by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.* Analytical biochemistry, 2008. **379**(2): p. 196-207.

354. Venkatesan, N., S.J. Kim, and B.H. Kim, *Novel Phosphoramidite Building Blocks in Synthesis and Applications Toward Modified Oligonucleotides.* Current Medicinal Chemistry, 2003. **10**(19): p. 1973-1991.

355. Iwai, S., *Chemical Synthesis of Oligonucleotides Containing Damaged Bases for Biological Studies.* Nucleosides, Nucleotides and Nucleic Acids, 2006. **25**(4-6): p. 561-582.

356. Wang, H.H., et al., *Modified bases enable high-efficiency oligonucleotide-mediated allelic replacement via mismatch repair evasion.* Nucleic Acids Research, 2011. **39**(16): p. 7336-7347.

357. Verma, S. and F. Eckstein, *MODIFIED OLIGONUCLEOTIDES: Synthesis and Strategy for Users.* Annual Review of Biochemistry, 1998. **67**(1): p. 99-134.

358. Itakura, K., J.J. Rossi, and R.B. Wallace, *Synthesis and Use of Synthetic Oligonucleotides.* Annual Review of Biochemistry, 1984. **53**(1): p. 323-356.

359. Beaucage, S.L. and R.P. Iyer, *Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach.* Tetrahedron, 1992. **48**(12): p. 2223-2311.

360. Reese, C.B., *Oligo- and poly-nucleotides: 50 years of chemical synthesis.* Organic & Biomolecular Chemistry, 2005. **3**(21): p. 3851-3868.

361. Scremin, C.L., et al., *Stepwise Regeneration and Recovery of Deoxyribonucleoside Phosphoramidite Monomers During Solid-Phase Oligonucleotide Synthesis.* The Journal of Organic Chemistry, 1994. **59**(8): p. 1963-1966.

362. Guainazzi, A. and O. Schärer, *Using synthetic DNA interstrand crosslinks to elucidate repair pathways and identify new therapeutic targets for cancer chemotherapy.* Cellular and Molecular Life Sciences, 2010. **67**(21): p. 3683-3697.

363. Lapthorn, C., F. Pullen, and B.Z. Chowdhry, *Ion mobility spectrometry-mass spectrometry (IMS-MS) of small molecules: Separating and assigning structures to ions.* Mass Spectrometry Reviews, 2013. **32**(1): p. 43-71.

364. El-Aneed, A., A. Cohen, and J. Banoub, *Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers.* Applied Spectroscopy Reviews, 2009. **44**(3): p. 210-230.

365. Lin, Y., et al., *Progress of laser ionization mass spectrometry for elemental analysis — A review of the past decade.* Spectrochimica Acta Part B: Atomic Spectroscopy, 2010. **65**(11): p. 871-883.

366. Ho, C.S., et al., *Electrospray ionisation mass spectrometry: principles and clinical applications.* Clin Biochem Rev, 2003. **24**(1): p. 3-12.

367. Smyth, W.F. and P. Brooks, *A critical evaluation of high performance liquid chromatography-electrospray ionisation-mass spectrometry and capillary electrophoresis-electrospray-mass spectrometry for the detection and determination of small molecules of significance in clinical and forensic science.* Electrophoresis, 2004. **25**(10-11): p. 1413-46.

368. Rosenberg, E., *The potential of organic (electrospray- and atmospheric pressure chemical ionisation) mass spectrometric techniques coupled to liquid-phase separation for speciation analysis.* J Chromatogr A, 2003. **1000**(1-2): p. 841-89.

369. Smyth, W.F., *Recent studies on the electrospray ionisation mass spectrometric behaviour of selected nitrogen-containing drug molecules and its application to drug analysis using liquid chromatography-electrospray ionisation mass spectrometry.* J Chromatogr B Analyt Technol Biomed Life Sci, 2005. **824**(1-2): p. 1-20.

370. Pitt, J.J., *Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry.* Clin Biochem Rev, 2009. **30**(1): p. 19-34.

371. Fenn, J., et al., *Electrospray ionization for mass spectrometry of large biomolecules.* Science, 1989. **246**(4926): p. 64-71.

372. Yamashita, M. and J.B. Fenn, *Electrospray ion source. Another variation on the free-jet theme.* The Journal of Physical Chemistry, 1984. **88**(20): p. 4451-4459.

373. Pozniak, B. and R. Cole, *Current measurements within the electrospray emitter.* Journal of the American Society for Mass Spectrometry, 2007. **18**(4): p. 737-748.

374. Olumee, Z., J.H. Callahan, and A. Vertes, *Droplet Dynamics Changes in Electrostatic Sprays of Methanol−Water Mixtures.* The Journal of Physical Chemistry A, 1998. **102**(46): p. 9154-9160.

375. Li, K.-Y., H. Tu, and A.K. Ray, *Charge Limits on Droplets during Evaporation.* Langmuir, 2005. **21**(9): p. 3786-3794.

376. Smith, J.N., R.C. Flagan, and J.L. Beauchamp, *Droplet Evaporation and Discharge Dynamics in Electrospray Ionization†.* The Journal of Physical Chemistry A, 2002. **106**(42): p. 9957-9967.

377. Kebarle, P. and U.H. Verkerk, *Electrospray: From ions in solution to ions in the gas phase, what we know now.* Mass Spectrometry Reviews, 2009. **28**(6): p. 898-917.

378. Bonilla, J.V. and G.S. Srivatsa, *Handbook of Analysis of Oligonucleotides and Related Products*. 2011: Taylor & Francis.

379. Boyer, P.D., *The Enzymes*. 1982: Academic Press.

380. Mackessy, S.P., *Handbook of Venoms and Toxins of Reptiles*. 2009: Taylor & Francis.

381. Iwanaga, S. and T. Suzuki, *Enzymes in Snake Venom*, in *Snake Venoms*, C.-Y. Lee, Editor. 1979, Springer Berlin Heidelberg. p. 61-158.

382. Barnett, C.A., T.J. Schmidt, and G. Litwack, *Effects of calf intestinal alkaline phosphatase, phosphatase inhibitors, and phosphorylated compounds on the rate of activation of glucocorticoid-receptor complexes.* Biochemistry, 1980. **19**(23): p. 5446-5455.

383. Coleman, J.E., *Structure and mechanism of alkaline phosphatase.* Annu Rev Biophys Biomol Struct, 1992. **21**: p. 441-83.

384. Rankin, S.A., et al., *Invited review: The application of alkaline phosphatase assays for the validation of milk product pasteurization.* Journal of Dairy Science. **93**(12): p. 5538-5551.

385. Rittie, L. and B. Perbal, *Enzymes used in molecular biology: a useful guide.* J Cell Commun Signal, 2008. **2**(1-2): p. 25-45.

386. NISHIGAKI, K., et al., *Strand Dissociation and Cooperative Melting of Double-Stranded DNAs Detected by Denaturant Gradient Gel Electrophoresis.* Journal of Biochemistry, 1984. **95**(3): p. 627-635.

387. Rouzina, I. and V.A. Bloomfield, *Force-Induced Melting of the DNA Double Helix 1. Thermodynamic Analysis.* Biophysical Journal. **80**(2): p. 882-893.

388. Rouzina, I. and V.A. Bloomfield, *Force-Induced Melting of the DNA Double Helix. 2. Effect of Solution Conditions.* Biophysical Journal. **80**(2): p. 894-900.

389. Williams, M.C., et al., *Effect of pH on the Overstretching Transition of Double-Stranded DNA: Evidence of Force-Induced DNA Melting.* Biophysical Journal. **80**(2): p. 874-881.

390. Blake, R.D., *Denaturation of DNA*, in *Encyclopedia of Molecular Cell Biology and Molecular Medicine*. 2006, Wiley-VCH Verlag GmbH & Co. KGaA.

391. Breslauer, K.J., *[10] Extracting thermodynamic data from equilibrium melting curves for oligonucleotide order-disorder transitions*, in *Methods in Enzymology*, G.K.A. Michael L. Johnson, Editor. 1995, Academic Press. p. 221-242.

392. Marmur, J. and P. Doty, *Heterogeneity in Deoxyribonucleic Acids: I. Dependence on Composition of the Configurational Stability of Deoxyribonucleic Acids.* Nature, 1959. **183**(4673): p. 1427-1429.

393. Doktycz, M.J., *Nucleic Acids: Thermal Stability and Denaturation*, in *eLS*. 2001, John Wiley & Sons, Ltd.

394. Thomas, R., *The denaturation of DNA.* Gene, 1993. **135**(1–2): p. 77-79.

395. Mandel, M. and J. Marmur, *[109] Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA*, in *Methods in*

Enzymology, K.M. Lawrence Grossman, Editor. 1968, Academic Press. p. 195-206.

396. Breslauer, K.J., et al., *Predicting DNA duplex stability from the base sequence.* Proc Natl Acad Sci U S A, 1986. **83**(11): p. 3746-50.

397. Owczarzy, R., et al., *Predicting sequence-dependent melting stability of short duplex DNA oligomers.* Biopolymers, 1997. **44**(3): p. 217-239.

398. SantaLucia, J., Jr. and D. Hicks, *The thermodynamics of DNA structural motifs.* Annu Rev Biophys Biomol Struct, 2004. **33**: p. 415-40.

399. Torgasin, S. and K.H. Zimmermann, *Algorithm for thermodynamically based prediction of DNA/DNA cross-hybridisation.* Int J Bioinform Res Appl, 2010. **6**(1): p. 82-97.

400. Bommarito, S., N. Peyret, and J. SantaLucia, Jr., *Thermodynamic parameters for DNA sequences with dangling ends.* Nucleic Acids Res, 2000. **28**(9): p. 1929-34.

401. Wienken, C.J., et al., *Thermophoretic melting curves quantify the conformation and stability of RNA and DNA.* Nucleic Acids Research, 2011.

402. Fasman, G.D., *Circular Dichroism and the Conformational Analysis of Biomolecules*. 1996: Springer US.

403. Nakanishi, K., N. Berova, and R. Woody, *Circular Dichroism: Principles and Applications*. 1994: VCH.

404. Bertucci, C., M. Pistolozzi, and A. De Simone, *Circular dichroism in drug discovery and development: an abridged review.* Analytical and Bioanalytical Chemistry, 2010. **398**(1): p. 155-166.

405. Martin, S.R. and P.M. Bayley, *Absorption and circular dichroism spectroscopy.* Methods Mol Biol, 2002. **173**: p. 43-55.

406. Woody, R.W., *Circular dichroism.* Methods Enzymol, 1995. **246**: p. 34-71.

407. Schellman, J.A., *Circular dichroism and optical rotation.* Chemical Reviews, 1975. **75**(3): p. 323-331.

408. Johnson, C.W., *Circular Dichroism Spectroscopy and the Vacuum Ultraviolet Region.* Annual Review of Physical Chemistry, 1978. **29**(1): p. 93-114.

409. Whitmore, L. and B.A. Wallace, *Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases.* Biopolymers, 2008. **89**(5): p. 392-400.

410. Greenfield, N.J., *Using circular dichroism spectra to estimate protein secondary structure.* Nat. Protocols, 2007. **1**(6): p. 2876-2890.

411. Greenfield, N.J., *Using circular dichroism spectra to estimate protein secondary structure.* Nat Protoc, 2006. **1**(6): p. 2876-90.

412. Greenfield, N.J., *Methods to estimate the conformation of proteins and polypeptides from circular dichroism data.* Anal Biochem, 1996. **235**(1): p. 1-10.

413. Greenfield, N.J., *Analysis of the kinetics of folding of proteins and peptides using circular dichroism.* Nat Protoc, 2006. **1**(6): p. 2891-9.

414. Keiderling, T.A., *Protein and peptide secondary structure and conformational determination with vibrational circular dichroism.* Curr Opin Chem Biol, 2002. **6**(5): p. 682-8.

415. Fiedler, S., L. Cole, and S. Keller, *Automated circular dichroism spectroscopy for medium-throughput analysis of protein conformation.* Anal Chem, 2013. **85**(3): p. 1868-72.

416. Greenfield, N.J., *Circular dichroism analysis for protein-protein interactions.* Methods Mol Biol, 2004. **261**: p. 55-78.

417. Kelly, S.M., T.J. Jess, and N.C. Price, *How to study proteins by circular dichroism.* Biochim Biophys Acta, 2005. **1751**(2): p. 119-39.

418. Bishop, G.R. and J.B. Chaires, *Characterization of DNA structures by circular dichroism.* Curr Protoc Nucleic Acid Chem, 2003. **Chapter 7**: p. Unit 7.11.

419. Jaumot, J., et al., *Classification of nucleic acids structures by means of the chemometric analysis of circular dichroism spectra.* Anal Chim Acta, 2009. **642**(1-2): p. 117-26.

420. Norden, B. and T. Kurucsev, *Analysing DNA complexes by circular and linear dichroism.* J Mol Recognit, 1994. **7**(2): p. 141-55.

421. Miyahara, T., H. Nakatsuji, and H. Sugiyama, *Helical Structure and Circular Dichroism Spectra of DNA: A Theoretical Study.* The Journal of Physical Chemistry A, 2012. **117**(1): p. 42-55.

422. Bishop, G.R. and J.B. Chaires, *Characterization of DNA Structures by Circular Dichroism*, in *Current Protocols in Nucleic Acid Chemistry*. 2001, John Wiley & Sons, Inc.

423. Martin, S.R. and M.J. Schilstra, *Circular dichroism and its application to the study of biomolecules.* Methods Cell Biol, 2008. **84**: p. 263-93.

424. Kypr, J., et al., *Circular dichroism and conformational polymorphism of DNA.* Nucleic Acids Research, 2009. **37**(6): p. 1713-1725.

425. Noll, D.M., et al., *Preparation of interstrand cross-linked DNA oligonucleotide duplexes.* Frontiers in Bioscience, 2004. **9**(1): p. 421-437.

426. Ganesan, N., et al., *N-7-Alkyl-2'-Deoxyguanosine as surrogate biomarkers for N-nitrosamine exposure in human lung.* International Journal of Cancer Prevention, 2007. **2**(4): p. 265-277.

427. B, B., et al., *Distribution of 7-alkyl-2'-deoxyguanosine adduct levels in human lung.* Carcinogenesis, 1996. **17**(4): p. 741-748.

428. Ekanayake, K.S. and P.R. Lebreton, *Model transition states for methane diazonium ion methylation of guanine runs in oligomeric DNA.* Journal of Computational Chemistry, 2007. **28**(14): p. 2352-2365.

429. Li, L., et al., *Methylation reaction for four DNA base molecules by methanediazonium ions.* Science in China Series B: Chemistry, 2009. **52**(1): p. 26-30.

430. Thomson, C. and C.A. Reynolds, *A theoretical study of N-nitrosamine metabolites: Possible alkylating species in carcinogenesis by N,N'-dimethyl nitrosamine.* International Journal of Quantum Chemistry, 1986. **30**(6): p. 751-762.

431. Citti, L., et al., *The reaction of 3, 4-epoxy-1-butene with deoxyguanosine and DNA in vitro: synthesis and characterization of the main adducts.* Carcinogenesis, 1984. **5**(1): p. 47-52.

432. Margison, G.P., J.M. Margison, and R. Montesano, *Methylated purines in the deoxyribonucleic acid of various Syrian-golden-hamster tissues after administration of a hepatocarcinogenic dose of dimethylnitrosamine.* Biochemical Journal, 1976. **157**: p. 627-634.

433. King, H.W.S., M.R. Osborne, and P. Brookes, *The in vitro and in vivo reaction at the N7-position of guanine of the ultimate carcinogen derived from benzo[a]pyrene.* Chemico-Biological Interactions, 1979. **24**(3): p. 345-353.

434. Osborne, M. and K. Merrifield, *Depurination of benzo[a]pyrene-diolepoxide treated DNA.* Chemico-Biological Interactions, 1985. **53**: p. 183-195.

435. O'Connor, T.R., S. Boiteux, and J. Laval, *Ring-opened 7-methylguanine residues in DNA are a block to in vitro DNA synthesis.* Nucleic Acids Research, 1988. **16**(13): p. 5879-5894.

436. Lao, Y. and S.S. Hecht, *Synthesis and Properties of an Acetaldehyde-Derived Oligonucleotide Interstrand Cross-Link.* Chemical Research in Toxicology, 2005. **18**(4): p. 711-721.

437. Stone, M.P., et al., *Interstrand DNA Cross-Links Induced by α,β-Unsaturated Aldehydes Derived from Lipid Peroxidation and Environmental Sources.* Accounts of Chemical Research, 2008. **41**(7): p. 793-804.

438. Dooley, P.A., et al., *Structural Studies of an Oligodeoxynucleotide Containing a Trimethylene Interstrand Cross-Link in a 5'-(CpG) Motif: Model of a Malondialdehyde Cross-Link.* Journal of the American Chemical Society, 2001. **123**(8): p. 1730-1739.

439. Swenson, M.C., et al., *Structure of a DNA Repair Substrate Containing an Alkyl Interstrand Crosslink at 1.65 Å Resolution.* Biochemistry, 2007. **46**(15): p. 4545-4553.

440. M, W.d.S., et al., *Solution structure of a DNA duplex containing mispair-aligned N4C-ethyl-N4C interstrand cross-linked cytosines.* Biochemistry, 2002. **41**(51): p. 15181-15188.

441. AM, N., et al., *N(4)C-ethyl-N(4)C cross-linked DNA: synthesis and characterization of duplexes with interstrand cross-links of different orientations.* Biochemistry, 2002. **41**(3): p. 760-771.

442. Miller, P.S., *Syntheses of DNA duplexes that contain a N⁴C-alkyl-N⁴C interstrand cross-link.* Current protocols in nucleic acid chemistry / edited by Serge L. Beaucage ... [et al.], 2011. **Chapter 5**: p. Unit5.10.

443. Wilds, C.J., J.D. Booth, and A.M. Noronha, *Synthesis of oligonucleotides containing an O6-G-alkyl-O6-G interstrand cross-link.* Tetrahedron Letters, 2006. **47**(51): p. 9125-9128.

444. Fang, Q., et al., *Repair of O6-G-alkyl-O6-G interstrand cross-links by human O6-alkylguanine-DNA alkyltransferase.* Biochemistry, 2008. **47**(41): p. 10892-10903.

445. McManus, F.P., et al., *Synthesis and characterization of an O6-2[prime or minute]-deoxyguanosine-alkyl-O6-2[prime or minute]-deoxyguanosine interstrand cross-link in a 5[prime or minute]-GNC motif and repair by human O6-alkylguanine-DNA alkyltransferase.* Organic & Biomolecular Chemistry, 2010. **8**(19): p. 4414-4426.

446. Wilds, C.J., et al., *Mispair-Aligned N3T-alkyl-N3T Interstrand Cross-Linked DNA: Synthesis and Characterization of Duplexes with Interstrand Cross-Links of Variable Lengths*. Journal of the American Chemical Society, 2004. **126**(30): p. 9257-9265.

447. Hentschel, S., et al., *Synthesis of DNA Interstrand Cross-Links Using a Photocaged Nucleobase.* Angewandte Chemie International Edition, 2012. **51**(14): p. 3466-3469.

448. Ichikawa, K., et al., *Interstrand cross-link of DNA by covalently linking a pair of abasic sites.* Chemical Communications, 2012. **48**(15): p. 2143-2145.

449. Kojima, N. and Y. Komatsu, *Synthesis and Application of Highly Reactive Amino Linkers for Functional Oligonucleotides*, in *Current Protocols in Nucleic Acid Chemistry*. 2001, John Wiley & Sons, Inc.

450. Hanada, K., et al., *The structure-specific endonuclease Mus81–Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks.* The EMBO Journal, 2006. **25**(20): p. 4921-4932.

451. Niedernhofer, L.J., et al., *The Structure-Specific Endonuclease Ercc1-Xpf Is Required To Resolve DNA Interstrand Cross-Link-Induced Double-Strand Breaks.* Molecular and Cellular Biology, 2004. **24**(13): p. 5776-5787.

452. Ganguly, M., et al., *A study of 7-deaza-2'-deoxyguanosine–2'-deoxycytidine base pairing in DNA*. Nucleic Acids Research, 2007. **35**(18): p. 6181-6195.

453. Xie, H., et al., *Novel pyrrolopyrimidine analogues as potent dipeptidyl peptidase IV inhibitors based on pharmacokinetic property-driven optimization.* Eur J Med Chem, 2012. **52**: p. 205-12.

454. Selvam, T.P., et al., *A mini review of pyrimidine and fused pyrimidine marketed drugs.* Research in Pharmacy, 2012.

455. Bahashwan, S., et al., *Synthesis and Pharmacological Activities of Some New Triazolo- and Tetrazolopyrimidine Derivatives*. Molecules, 2013. **18**(12): p. 15051-15063.

456. Tarzia, G., et al., *Synthesis and pharmacological properties of derivatives of pyrrolo(3,4-d)pyrimidines and pyrrolo(3,4-b)pyridines (I).* Farmaco Sci, 1976. **31**(2): p. 81-97.

457. Lipson, V.V. and N.Y. Gorobets, *One hundred years of Meldrum's acid: advances in the synthesis of pyridine and pyrimidine derivatives.* Mol Divers, 2009. **13**(4): p. 399-419.

458. McCarty, R.M. and V. Bandarian, *Biosynthesis of pyrrolopyrimidines*. Bioorg Chem, 2012. **43**: p. 15-25.

459. Noll, D.M., et al., *Preparation of interstrand cross-linked DNA oligonucleotide duplexes*. Frontiers in Bioscience, 2004. **9**: p. 421-437.

460. Boulikas, T. and M. Vougiouka, *Cisplatin and platinum drugs at the molecular level.* Oncology Reports, 2003. **10**(6): p. 1663-1682.

461. Sampath, D. and W. Plunkett, *The role of DNA repair in chronic lymphocytic leukemia pathogenesis and chemotherapy resistance.* Current Oncology Reports, 2007. **9**(5): p. 361-367.

462. Wang, S.Q., et al., *Ultraviolet A and melanoma: A review*. Journal of the American Academy of Dermatology, 2001. **44**(5): p. 837-846.

463. Cole, R.S., *Repair of DNA Containing Interstrand Crosslinks in Escherichia coli: Sequential Excision and Recombination.* Proceedings of the National Academy of Sciences, 1973. **70**(4): p. 1064-1068.

464. Magaña-Schwencke, N., et al., *The fate of 8-methoxypsoralen photoinduced crosslinks in nuclear and mitochondrial yeast DNA: comparison of wild-type and repair-deficient strains.* Proceedings of the National Academy of Sciences, 1982. **79**(6): p. 1722-1726.

465. Van Houten, B., et al., *Action mechanism of ABC excision nuclease on a DNA substrate containing a psoralen crosslink at a defined position.* Proceedings of the National Academy of Sciences, 1986. **83**(21): p. 8077-8081.

466. Helleday, T., et al., *DNA repair pathways as targets for cancer therapy.* Nat Rev Cancer, 2008. **8**(3): p. 193-204.

467. Stivers, J.T. and R.D. Kuchta, *Introduction: DNA Damage and Repair.* Chemical Reviews, 2006. **106**(2): p. 213-214.

468. Hanada, K., et al., *The structure-specific endonuclease Mus81-Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks.* EMBO J, 2006. **25**(20): p. 4921-4932.

469. Niedernhofer, L.J., et al., *The Structure-Specific Endonuclease Ercc1-Xpf Is Required To Resolve DNA Interstrand Cross-Link-Induced Double-Strand Breaks.* Molecular and Cellular Biology, 2004. **24**(13): p. 5776-5787.

470. Li, L., et al., *Interstrand Cross-Links Induce DNA Synthesis in Damaged and Undamaged Plasmids in Mammalian Cell Extracts.* Molecular and Cellular Biology, 1999. **19**(8): p. 5619-5630.

471. Zheng, H., et al., *Repair of DNA interstrand cross-links: Interactions between homology-dependent and homology-independent pathways.* DNA Repair, 2006. **5**(5): p. 566-574.

472. Bessho, T., D. Mu, and A. Sancar, *Initiation of DNA interstrand cross-link repair in humans: the nucleotide excision repair system makes dual incisions 5′ to the cross-linked base and removes a 22- to 28-nucleotide-long damage-free strand.* Molecular and Cellular Biology, 1997. **17**(12): p. 6822-30.

473. Batty, D.P. and R.D. Wood, *Damage recognition in nucleotide excision repair of DNA*. Gene, 2000. **241**(2): p. 193-204.

474. Couvé, S., et al., *The Human Oxidative DNA Glycosylase NEIL1 Excises Psoralen-induced Interstrand DNA Cross-links in a Three-stranded DNA Structure.* Journal of Biological Chemistry, 2009. **284**(18): p. 11963-11970.

475. Wu, Q., et al., *Mismatch repair participates in error-free processing of DNA interstrand crosslinks in human cells.* EMBO Rep, 2005. **6**(6): p. 551-557.

476. Zhao, J., et al., *Mismatch repair and nucleotide excision repair proteins cooperate in the recognition of DNA interstrand crosslinks.* Nucleic Acids Research, 2009. **37**(13): p. 4420-4429.

477. Clingen, P.H., et al., *The XPF-ERCC1 endonuclease and homologous recombination contribute to the repair of minor groove DNA interstrand crosslinks in mammalian cells produced by the pyrrolo[2,1-c][1,4]benzodiazepine dimer SJG-136.* Nucleic Acids Research, 2005. **33**(10): p. 3283-3291.

478. De Silva, I.U., et al., *Defining the Roles of Nucleotide Excision Repair and Recombination in the Repair of DNA Interstrand Cross-Links in Mammalian Cells.* Molecular and Cellular Biology, 2000. **20**(21): p. 7980-7990.

479. Wang, X., et al., *Involvement of Nucleotide Excision Repair in a Recombination-Independent and Error-Prone Pathway of DNA Interstrand Cross-Link Repair.* Molecular and Cellular Biology, 2001. **21**(3): p. 713-720.

480. Wang, W., *Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins.* Nat Rev Genet, 2007. **8**(10): p. 735-748.

481. Alzeer, J. and O.D. Schärer, *A modified thymine for the synthesis of site-specific thymine-guanine DNA interstrand crosslinks.* Nucleic Acids Research, 2006. **34**(16): p. 4458-4466.

482. Harwood, E.A., P.B. Hopkins, and S.T. Sigurdsson, *Chemical Synthesis of Cross-Link Lesions Found in Nitrous Acid Treated DNA: A General Method for the Preparation of N2-Substituted 2'-Deoxyguanosines.* The Journal of Organic Chemistry, 2000. **65**(10): p. 2959-2964.

483. Wilds, C.J., E. Palus, and A.M. Noronha, *An approach for the synthesis of duplexes containing N3T-butyl-N3T interstrand cross-links via a bisphosphoramidite strategy.* Canadian Journal of Chemistry, 2007. **85**(4): p. 249-256.

484. Webba da Silva, M., et al., *Solution Structure of a DNA Duplex Containing Mispair-Aligned N4C−Ethyl−N4C Interstrand Cross-Linked Cytosines†.* Biochemistry, 2002. **41**(51): p. 15181-15188.

485. Noll, D.M., A.M. Noronha, and P.S. Miller, *Synthesis and Characterization of DNA Duplexes Containing an N4C−Ethyl−N4C Interstrand Cross-Link.* Journal of the American Chemical Society, 2001. **123**(15): p. 3405-3411.

486. Schelhaas, M. and H. Waldmann, *Protecting Group Strategies in Organic Synthesis.* Angewandte Chemie International Edition in English, 1996. **35**(18): p. 2056-2083.

487. Zatsepin, T.S., et al., *Synthesis of DNA conjugates by solid-phase fragment condensation via aldehyde–nucleophile coupling.* Tetrahedron Letters, 2005. **46**(18): p. 3191-3195.

488. Tsuji, J., *Transition metal reagents and catalysts: innovations in organic synthesis*. 2002, Chichester: John Wiley & Sons Ltd.

489. Hayakawa, Y., et al., *Allyloxycarbonyl group: a versatile blocking group for nucleotide synthesis.* The Journal of Organic Chemistry, 1986. **51**(12): p. 2400-2402.

490. Ortiz Mayo, J.U., et al., *Facile synthesis of a cis–syn thymine dimer building block and its incorporation into oligodeoxynucleotides.* Tetrahedron, 2003. **59**(37): p. 7377-7383.

491. Bergstrom, D.E. and P.W. Shum, *Synthesis and characterization of a new fluorine-substituted nonionic dinucleoside phosphonate analog. P-Deoxy-P-(difluoromethyl)thymidylyl(3' .fwdarw. 5')thymidine.* The Journal of Organic Chemistry, 1988. **53**(17): p. 3953-3958.

492. Díaz-Rodríguez, A., et al., *Novel Chemoenzymatic Protocol for the Synthesis of 3'-O-Dimethoxytrityl-2'-deoxynucleoside Derivatives as Building Blocks for*

Oligonucleotide Synthesis. Organic Process Research & Development, 2006. **10**(3): p. 581-587.

493. de Koning, M.C., et al., *Simple and Efficient Solution-Phase Synthesis of Oligonucleotides Using Extractive Work-Up.* Organic Process Research & Development, 2006. **10**(6): p. 1238-1245.

494. Guibé, F., *Allylic protecting groups and their use in a complex environment part II: Allylic protecting groups and their removal through catalytic palladium π-allyl methodology.* Tetrahedron, 1998. **54**(13): p. 2967-3042.

495. Lee, Y.J., et al., *Acceptor-Dependent Stereoselective Glycosylation: 2′-CB Glycoside-Mediated Direct β-d-Arabinofuranosylation and Efficient Synthesis of the Octaarabinofuranoside in Mycobacterial Cell Wall.* Organic Letters, 2005. **7**(15): p. 3263-3266.

496. Lyttle, M.H., et al., *New Reagents and Methods for the Synthesis of Internal and 3′-Labeled DNA.* Bioconjugate Chemistry, 2002. **13**(5): p. 1146-1154.

497. Asanuma, H., et al., *Postsynthetic modification of DNA via threoninol on a solid support by means of allylic protection.* Tetrahedron Letters, 2008. **49**(35): p. 5144-5146.

498. Spinelli, N., et al., *Use of Allylic Protecting Groups for the Synthesis of Base-Sensitive Prooligonucleotides.* European Journal of Organic Chemistry, 2002. **2002**(1): p. 49-56.

499. Hayakawa, Y., et al., *The allylic protection method in solid-phase oligonucleotide synthesis. An efficient preparation of solid-anchored DNA oligomers.* Journal of the American Chemical Society, 1990. **112**(5): p. 1691-1696.

500. Li, H.-Y., et al., *Synthesis of DNA Oligomers Possessing a Covalently Cross-Linked Watson–Crick Base Pair Model.* Angewandte Chemie International Edition, 2001. **40**(8): p. 1471-1475.

501. Puglisi, J.D. and I. Tinoco Jr, *[22] absorbance melting curves of RNA*, in *Methods in Enzymology*, J.N.A. James E. Dahlberg, Editor. 1989, Academic Press. p. 304-325.

502. Wuts, P.G.M. and T.W. Greene, *Green's protecting groups in organic synthesis*. 2006, Hoboken, New Jersey: John Wiley & Sons, Inc. 1082.

503. Schulhof, J.C., D. Molko, and R. Teoule, *Facile removal of new base protecting groups useful in oligonucleotide synthesis.* Tetrahedron Letters, 1987. **28**(1): p. 51-54.

504. Duynstee, H.I., et al., *Synthesis of Verbascoside: A Dihydroxyphenylethyl Glycoside with Diverse Bioactivity.* European Journal of Organic Chemistry, 1999. **1999**(10): p. 2623-2632.

505. Virta, P., *Solid-phase synthesis of base-sensitive oligonucleotides.* ARKIVOC, 2009. **2009**(iii): p. 54-83.

506. Reynolds, M.A., et al., *Synthesis and Thermodynamics of Oligonucleotides Containing Chirally Pure RP Methylphosphonate Linkages.* Nucleic Acids Research, 1996. **24**(22): p. 4584-4591.

507. Gildea, B. and L.W. McLaughlin, *The synthesis of 2-pyrimidinone nucleosides and their incorporation into oligodeoxynucleotides.* Nucleic Acids Research, 1989. **17**(6): p. 2261-2281.

508. Sinha, N.D., et al., *Synthesis of oligodeoxynucleoside methylphosphonates utilizing the fert-butylphenoxyacetyl group for exocyclic amine protection.* Nucleic Acids Research, 1994. **22**(15): p. 3119-3123.

509. Cassidy, R.A., N. Puri, and P.S. Miller, *Effect of DNA target sequence on triplex formation by oligo-2'-deoxy- and 2'-O-methylribonucleotides.* Nucleic Acids Res, 2003. **31**(14): p. 4099-108.

510. Orain, D., J. Ellard, and M. Bradley, *Protecting Groups in Solid-Phase Organic Synthesis.* Journal of Combinatorial Chemistry, 2001. **4**(1): p. 1-16.

511. Lyttle, M.H., et al., *New Reagents and Methods for the Synthesis of Internal and 3'-Labeled DNA.* Bioconjugate Chemistry, 2002. **13**(5): p. 1146-1154.

512. Hayakawa, Y., et al., *Allyloxycarbonyl Group: A Versatile Blocking Group for Nucleotide Synthesis.* The Journal of Organic Chemistry, 1986. **51**(12): p. 2400–2402.

513. Smeaton, M.B., et al., *Effect of Cross-Link Structure on DNA Interstrand Cross-Link Repair Synthesis.* Chemical Research in Toxicology, 2009. **22**(7): p. 1285-1297.

514. Johnson, W.C., *Circular Dichroism and Its Empirical Application to Biopolymers*, in *Methods of Biochemical Analysis*. 2006, John Wiley & Sons, Inc. p. 61-163.

515. Cleaver, J.E. and E. Crowley, *UV damage, DNA repair and skin carcinogenesis.* Frontiers in Bioscience, 2002. **7**(d): p. 1024-1043.

516. Webba da Silva, M., et al., *Accommodation of Mispair Aligned N3T-Ethyl-N3T DNA Interstrand Cross Link†.* Biochemistry, 2004. **43**(39): p. 12549-12554.

517. Seela, F., Q.H. Tran-Thi, and D. Franzen, *Poly(7-deazaguanylic acid), the homopolynucleotide of the parent nucleoside of queuosine.* Biochemistry, 1982. **21**(18): p. 4338-4343.

518. Seela, F. and H. Driller, *Palindromic oiigonucleotides containing 7-deaza-2′ -deoxyguanosine: solid-phase synthesis of d[(p)GG*AATTCC] octamers and recognition by the endodeoxyribonnclease EcoRI.* Nucleic Acids Research, 1986. **14**(5): p. 2319-2332.

519. Seela, F. and A. Kehne, *Oligomers with alternating thymidine and 2′-deoxytubercidin: duplex stabilization by a 7-deazapurine base.* Biochemistry, 1985. **24**(26): p. 7556-7561.

520. Schneider, K. and B.T. Chait, *Increased stability of nucleic acids containing 7-deaza-guanosine and 7-deaza-adenosine may enable rapid DNA sequencing by matrix-assisted laser desorption mass spectrometry.* Nucleic Acids Research 1995. **23**(9): p. 1570-1575.

521. Frank, S. and H. Thomas, *Modified oligonucleotides, their preparation and their use*, U.S. Patent, Editor. 2002, Aventis Pharma Deutschland Gmbh.

522. Buhr, C.A., et al., *Oligodeoxynucleotides Containing C-7 Propyne Analogs of 7-Deaza-2′ -Deoxyguanosine and 7-Deaza-2′-Deoxyadenosine.* Nucleic Acids Research, 1996. **24**(15): p. 2974-2980.

523. Ramzaeva, N. and F. Seela, *7-Substituted 7-Deaza-2′-deoxyguanosines: Regioselective Halogenation of Pyrrolo[2,3-d]pyrimidine Nucleosides.* Helvetica Chimica Acta, 1995. **78**(5): p. 1083-1090.

524. Pivoňková, H., et al., *Direct Voltammetric Analysis of DNA Modified with Enzymatically Incorporated 7-Deazapurines.* Analytical Chemistry, 2010. **82**(16): p. 6807-6813.

525. Brückl, T., et al., *Efficient Synthesis of Deazaguanosine-Derived tRNA Nucleosides PreQ0, PreQ1, and Archaeosine Using the Turbo-Grignard Method.* European Journal of Organic Chemistry, 2010. **2010**(34): p. 6517-6519.

526. Klepper, F., et al., *Synthesis of the Transfer-RNA Nucleoside Queuosine by Using a Chiral Allyl Azide Intermediate.* Angewandte Chemie International Edition, 2007. **46**(13): p. 2325-2327.

527. Fletcher, T.M., et al., *Inhibition of Human Telomerase by 7-Deaza-2'-deoxyguanosine Nucleoside Triphosphate Analogs: Potent Inhibition by 6-Thio-7-deaza-2'-deoxyguanosine 5'-Triphosphate.* Bioorganic Chemistry, 2001. **29**(1): p. 36-55.

528. Tangeda, S.J. and A. Garlapati, *Synthesis of new pyrrolo[2,3-d]pyrimidine derivatives and evaluation of their activities against human colon cancer cell lines.* European Journal of Medicinal Chemistry, 2010. **45**(4): p. 1453-1458.

529. Guainazzi, A., et al., *Synthesis and Molecular Modeling of a Nitrogen Mustard DNA Interstrand Crosslink.* Chemistry – A European Journal, 2010. **16**(40): p. 12100-12103.

530. Angelov, T., A. Guainazzi, and O.D. Schärer, *Generation of DNA Interstrand Cross-Links by Post-Synthetic Reductive Amination.* Organic Letters, 2009. **11**(3): p. 661-664.

531. Seela, F., et al., *8-Aza-7-deazaguanine nucleosides and oligonucleotides with octadiynyl side chains: synthesis, functionalization by the azide-alkyne 'click' reaction and nucleobase specific fluorescence quenching of coumarin dye conjugates.* Organic & Biomolecular Chemistry, 2009. **7**(7): p. 1374-1387.

532. Gibson, C.L., et al., *The synthesis of 7-deazaguanines as potential inhibitors of guanosine triphosphate cyclohydrolase I.* Tetrahedron, 2004. **60**(4): p. 943-959.

533. Olsen, D.B., et al., *A 7-Deaza-Adenosine Analog Is a Potent and Selective Inhibitor of Hepatitis C Virus Replication with Excellent Pharmacokinetic Properties.* Antimicrobial Agents and Chemotherapy, 2004. **48**(10): p. 3944-3953.

534. Jung, M.-H., et al., *Synthesis of pyrrolo[2,3-d]pyrimidine derivatives and their antiproliferative activity against melanoma cell line.* Bioorganic & Medicinal Chemistry Letters, 2009. **19**(23): p. 6538-6543.

535. Seela, F. and S.A. Ingale, *"Double Click" Reaction on 7-Deazaguanine DNA: Synthesis and Excimer Fluorescence of Nucleosides and Oligonucleotides with Branched Side Chains Decorated with Proximal Pyrenes.* The Journal of Organic Chemistry, 2009. **75**(2): p. 284-295.

536. Reigan, P., et al., *Xanthine oxidase-activated prodrugs of thymidine phosphorylase inhibitors.* European Journal of Medicinal Chemistry, 2008. **43**(6): p. 1248-1260.

537. Seela, F. and X. Peng, *7-Functionalized 7-Deazapurine Ribonucleosides Related to 2-Aminoadenosine, Guanosine, and Xanthosine: Glycosylation of Pyrrolo[2,3-*

d]pyrimidines with 1-O-Acetyl-2,3,5-tri-O-benzoyl-d-ribofuranose. The Journal of Organic Chemistry, 2006. **71**(1): p. 81-90.

538. Reigan, P., et al., *Synthesis and enzymatic evaluation of xanthine oxidase-activated prodrugs based on inhibitors of thymidine phosphorylase.* Bioorganic & Medicinal Chemistry Letters, 2004. **14**(21): p. 5247-5250.

539. Seela, F., S. Menkhoff, and S. Behrendt, *Furanoside-pyranoside isomerization of tubercidin and its 2[prime or minute]-deoxy derivatives: influence of nucleobase and sugar structure on the proton-catalysed reaction.* Journal of the Chemical Society, Perkin Transactions 2, 1986(4): p. 525-530.

540. Gangjee, A., et al., *Synthesis and Discovery of Water-Soluble Microtubule Targeting Agents that Bind to the Colchicine Site on Tubulin and Circumvent Pgp Mediated Resistance.* Journal of Medicinal Chemistry, 2010. **53**(22): p. 8116-8128.

541. Tangeda, S.J. and A. Garlapati, *ChemInform Abstract: Synthesis of New Pyrrolo[2,3-d]pyrimidine Derivatives and Evaluation of Their Activities Against Human Colon Cancer Cell Lines.* ChemInform, 2010. **41**(31): p. no-no.

542. Gangjee, A., H.D. Jain, and R.L. Kisliuk, *Novel 2-amino-4-oxo-5-arylthio-substituted-pyrrolo[2,3-d]pyrimidines as nonclassical antifolate inhibitors of thymidylate synthase.* Bioorganic & Medicinal Chemistry Letters, 2005. **15**(9): p. 2225-2230.

543. Gangjee, A., J. Yu, and R.L. Kisliuk, *2-amino-4-oxo-6-substituted-pyrrolo[2,3-d]pynmidines as potential inhibitors of thymidylate synthase.* Journal of Heterocyclic Chemistry, 2002. **39**(4): p. 833-840.

544. Gangjee, A., N.P. Dubash, and R.L. Kisliuk, *Synthesis of novel, nonclassical 2-amino-4-oxo-6-(arylthio)ethylpyrrolo[2,3-d] pyrimidines as potential inhibitors of thymidylate synthase.* Journal of Heterocyclic Chemistry, 2001. **38**(2): p. 349-354.

545. Gangjee, A., et al., *Potent Dual Thymidylate Synthase and Dihydrofolate Reductase Inhibitors: Classical and Nonclassical 2-Amino-4-oxo-5-arylthio-substituted-6-methylthieno[2,3-d]pyrimidine Antifolates.* Journal of Medicinal Chemistry, 2008. **51**(18): p. 5789-5797.

546. C., M.J., et al., *Novel Compounds and Compositions for Treatment of Breathing Control Disorders or Diseases*, U.p.t. office, Editor. 2012, Galleon Pharmaceuticals, Inc.

547. Wilding, B., et al., *Nitrile Reductase from Geobacillus kaustophilus: A Potential Catalyst for a New Nitrile Biotransformation Reaction.* Advanced Synthesis & Catalysis, 2012. **354**(11-12): p. 2191-2198.

548. Gao, L.-J., et al., *Synthesis and evaluation of novel ligands for the histamine H4 receptor based on a pyrrolo[2,3-d]pyrimidine scaffold.* Bioorganic & Medicinal Chemistry Letters, 2013. **23**(1): p. 132-137.

549. Shi, J., et al., *EC144 Is a Potent Inhibitor of the Heat Shock Protein 90.* Journal of Medicinal Chemistry, 2012. **55**(17): p. 7786-7795.

550. Bourdin, C., et al., *Synthesis and evaluation against hepatitis C virus of 7-deaza analogues of 2'-C-methyl-6-O-methyl guanosine nucleoside and l-Alanine ester phosphoramidates.* Bioorganic & Medicinal Chemistry Letters, 2013. **23**(7): p. 2260-2264.

551. Julian, B., *Heterocyclic GTP Cyclohydrolase 1 Inhibitors For the Treatment of Pain*, W.i.p. organization, Editor. 2011, PABST, Patrea, L.

552. Davoll, J., *26. Pyrrolo[2,3-d]pyrimidines.* Journal of the Chemical Society (Resumed), 1960: p. 131-138.

553. Zepeda, L.G., et al., *Synthese directe d'indoles anti-hypertensifs.* Tetrahedron, 1989. **45**(20): p. 6439-6448.

554. Morales-Ríos, M.S., L.G. Zepeda, and P. Joseph-Nathan, *Synthèse brève de l'indorénate.* Tetrahedron Letters, 1988. **29**(23): p. 2825-2826.

555. Kemme, S.T., T. Šmejkal, and B. Breit, *Combined Transition-Metal- and Organocatalysis: An Atom Economic C3 Homologation of Alkenes to Carbonyl and Carboxylic Compounds.* Chemistry – A European Journal, 2010. **16**(11): p. 3423-3433.

556. Gazit, A., et al., *Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors.* Journal of Medicinal Chemistry, 1989. **32**(10): p. 2344-2352.

557. Paramelle, D., et al., *A Straightforward Approach for Cellular-Uptake Quantification.* Angewandte Chemie International Edition, 2010. **49**(44): p. 8240-8243.

558. Wang, S., et al., *Electric-field-induced layer-by-layer fabrication of stable second-order nonlinear optical films.* Polymer International, 2009. **58**(8): p. 933-938.

559. Mukhopadhyay, C. and A. Datta, *A Simple, Efficient and Green Procedure for the Knoevenagel Condensation of Aldehydes with N-Methylpiperazine at Room*

Temperature under Solvent-Free Conditions. Synthetic Communications, 2008. **38**(13): p. 2103-2112.

560. Tietze, L.F., U. Beifuss, and M. Ruther, *Intramolecular ene reactions. 7. Asymmetric induction in intramolecular ene reactions of chiral 1,7-dienes: a diastereo- and enantioselective synthesis of substituted cyclohexanes.* The Journal of Organic Chemistry, 1989. **54**(13): p. 3120-3129.

561. Robert, A., S. Jaguelin, and J.L. Guinamant, *Syntheses d'esters ou d'acides α-halogenes a partir des gem dicyano epoxydes.* Tetrahedron, 1986. **42**(8): p. 2275-2281.

562. Jiang, Z., et al., *P–C Bond formation via direct and three-component conjugate addition catalyzed by 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD).* Tetrahedron Letters, 2007. **48**(1): p. 51-54.

563. Stevens, C.V., et al., *Synthesis of Substituted Cyclopropylphosphonates by Michael Induced Ring Closure (MIRC) Reactions.* Synlett, 2002. **2002**(07): p. 1089-1092.

564. Tietze, L.F. and U. Beifuß, *Intramolecular ene reactions, III. Diastereoselective formation of cyclohexanes by intramolecular ene reactions of 1,7-dienes.* Liebigs Annalen der Chemie, 1988. **1988**(4): p. 321-329.

565. Tietze, L.F. and U. Beifuß, *Nicht-induziert hochdiastereoselektive intramolekulare En-Reaktionen von 1,7-Dienen zu trans-1,2-disubstituierten Cyclohexanen.* Angewandte Chemie, 1985. **97**(12): p. 1067-1068.

566. Tietze, L.F. and U. Beifuss, *Asymmetric induction in intramolecular ene reactions of 1,7-dienes.* Tetrahedron Letters, 1986. **27**(16): p. 1767-1770.

567. Williams, Hannah L., Max E. Gottesman, and J. Gautier, *Replication-Independent Repair of DNA Interstrand Crosslinks.* Molecular cell, 2012. **47**(1): p. 140-147.

568. Long, D.T., et al., *Mechanism of RAD51-Dependent DNA Interstrand Cross-Link Repair.* Science, 2011. **333**(6038): p. 84-87.

569. Vare, D., et al., *DNA interstrand crosslinks induce a potent replication block followed by formation and repair of double strand breaks in intact mammalian cells.* DNA Repair, 2012. **11**(12): p. 976-985.

570. Kisby, G.E., et al., *DNA repair modulates the vulnerability of the developing brain to alkylating agents.* DNA Repair, 2009. **8**(3): p. 400-412.

571. Powell, S.N. and R.S. Bindra, *Targeting the DNA damage response for cancer therapy.* DNA Repair, 2009. **8**(9): p. 1153-1165.

572. Howerton, S.B., et al., *Locating Monovalent Cations in the Grooves of B-DNA†,‡.* Biochemistry, 2001. **40**(34): p. 10023-10031.

573. Ramasamy, K., et al., *A facile synthesis of tubercidin and related 7-deazapurine nucleosides via the stereospecific sodium salt glycosylation procedure.* Tetrahedron Letters, 1987. **28**(43): p. 5107-5110.

574. Winkeler, H.D. and F. Seela, *Synthesis of 2-amino-7-(2'-deoxy-.beta.-D-erythro-pentofuranosyl)-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one, a new isostere of 2'-deoxyguanosine.* The Journal of Organic Chemistry, 1983. **48**(18): p. 3119-3122.

575. Ramasamy, K., et al., *Total and stereospecific synthesis of cadeguomycin, 2[prime or minute]deoxycadeguomycin, ara-cadeguomycin, and certain related*

nucleosides. Journal of the Chemical Society, Perkin Transactions 1, 1989(12): p. 2375-2384.

576. Ramasamy, K., R.K. Robins, and G.R. Revankar, *Total and stereospecific synthesis of 2[prime or minute]-deoxycadeguomycin.* Journal of the Chemical Society, Chemical Communications, 1989(9): p. 560-562.

577. Seela, F. and X. Peng, *7-Functionalized 7-Deazapurine Ribonucleosides Related to 2-Aminoadenosine, Guanosine, and Xanthosine: Glycosylation of Pyrrolo[2,3-d]pyrimidines with 1-O-Acetyl-2,3,5-tri-O-benzoyl-d-ribofuranose.* The Journal of Organic Chemistry, 2005. **71**(1): p. 81-90.

578. Meng, Q., et al., *Design and Synthesis of a Photocleavable Fluorescent Nucleotide 3'-O-Allyl-dGTP-PC-Bodipy-FL-510 as a Reversible Terminator for DNA Sequencing by Synthesis.* The Journal of Organic Chemistry, 2006. **71**(8): p. 3248-3252.

579. Field, A.K., et al., *(±)-(1α,2β,3α)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine [(±)-BHCG or SQ 33 054]: A potent and selective inhibitor of herpesviruses.* Antiviral Research, 1990. **13**(1): p. 41-52.

580. Dey, S. and P. Garner, *Synthesis of tert-Butoxycarbonyl (Boc)-Protected Purines.* The Journal of Organic Chemistry, 2000. **65**(22): p. 7697-7699.

581. Wang, R.-W. and B. Gold, *A Facile Synthetic Approach to 7-Deazaguanine Nucleosides via a Boc Protection Strategy.* Organic Letters, 2009. **11**(11): p. 2465-2468.

582. Kawate, T., C.R. Allerson, and J.L. Wolfe, *Regioselective Syntheses of 7-Nitro-7-deazapurine Nucleosides and Nucleotides for Efficient PCR Incorporation.* Organic Letters, 2005. **7**(18): p. 3865-3868.

583. Seela, F. and K.I. Shaikh, *Oligonucleotides containing 7-propynyl-7-deazaguanine: synthesis and base pair stability.* Tetrahedron, 2005. **61**(10): p. 2675-2681.

584. Seela, F., et al., *Fluorescent DNA: the development of 7-deazapurine nucleoside triphosphates applicable for sequencing at the single molecule level.* Journal of Biotechnology, 2001. **86**(3): p. 269-279.

585. Seela, F., V.R. Sirivolu, and P. Chittepu, *Modification of DNA with Octadiynyl Side Chains: Synthesis, Base Pairing, and Formation of Fluorescent Coumarin Dye Conjugates of Four Nucleobases by the Alkyne−Azide "Click" Reaction.* Bioconjugate Chemistry, 2007. **19**(1): p. 211-224.

586. Seela, F., et al., *PROPYNYL GROUPS IN DUPLEX, HAIRPIN AND TRIPLEX DNA: 7-DEAZAPURINES AND 9-DEAZAPURINES.* Nucleosides, Nucleotides and Nucleic Acids, 2005. **24**(5-7): p. 851-854.

587. Rotulo-Sims, D. and J. Prunet, *A New, Simple, and Selective Palladium-Catalyzed Cleavage of Triethylsilyl Ethers.* Organic Letters, 2002. **4**(26): p. 4701-4704.

588. Ikawa, T., H. Sajiki, and K. Hirota, *Unexpected deprotection of silyl and THP ethers induced by serious disparity in the quality of Pd/C catalysts and elucidation of the mechanism.* Tetrahedron, 2004. **60**(29): p. 6189-6195.

589. Kim, S., et al., *Silyl group deprotection by Pd/C/H2. A facile and selective method.* Tetrahedron Letters, 2004. **45**(9): p. 1973-1976.

590. Sajiki, H., et al., *A remarkable solvent effect toward the Pd/C-catalyzed cleavage of silyl ethers.* Chemical Communications, 2003(5): p. 654-655.

591. Kuska, M.S., et al., *Influence of Chlorine Substitution on the Hydrolytic Stability of Biaryl Ether Nucleoside Adducts Produced by Phenolic Toxins.* The Journal of Organic Chemistry, 2013. **78**(14): p. 7176-7185.

592. Suzuki, T., E. Kaya, and M. Inukai, *Effects of Halides on Reaction of Nucleosides with Ozone.* Nucleosides, Nucleotides & Nucleic Acids, 2012. **31**(6): p. 461-473.

593. Gubala, V., J.E. Betancourt, and J.M. Rivera, *Expanding the Hoogsteen Edge of 2'-Deoxyguanosine: Consequences for G-Quadruplex Formation.* Organic Letters, 2004. **6**(25): p. 4735-4738.

594. Wang, Y. and Y. Wang, *Synthesis and Thermodynamic Studies of Oligodeoxyribonucleotides Containing Tandem Lesions of Thymidine Glycol and 8-Oxo-2'-deoxyguanosine.* Chemical Research in Toxicology, 2006. **19**(6): p. 837-843.

595. Münzel, M., et al., *Discovery and Synthesis of New UV-Induced Intrastrand C(4−8)G and G(8−4)C Photolesions.* Journal of the American Chemical Society, 2011. **133**(14): p. 5186-5189.

596. Kuhl, O., *Functionalised N-Heterocyclic Carbene Complexes*. 2010: Wiley. 364.

597. In-Sook Han, L., K. Hyun Joo, and J. Young Ran, *Reactivities of acridine compounds in hydride transfer reactions.* Journal of Physical Organic Chemistry, 2007. **20**(7): p. 484-490.

598. Zhu, X.-Q., et al., *Hydride, Hydrogen Atom, Proton, and Electron Transfer Driving Forces of Various Five-Membered Heterocyclic Organic Hydrides and*

Their Reaction Intermediates in Acetonitrile. Journal of the American Chemical Society, 2008. **130**(8): p. 2501-2516.

599. Matsubara, Y., et al., *Thermodynamic and Kinetic Hydricity of Ruthenium(II) Hydride Complexes.* Journal of the American Chemical Society, 2012. **134**(38): p. 15743-15757.

600. Salerno, A., V. Ceriani, and I.A. Perillo, *Reduction of substituted 1H-4,5-dihydroimidazolium salts.* Journal of Heterocyclic Chemistry, 1992. **29**(7): p. 1725-1733.

601. Denisenko, A.V., et al., *Synthesis of 6-Amino-2-bromo-4-hydroxynicotinaldehyde Derivatives.* Synthesis, 2011. **2011**(02): p. 251-256.