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Synthesis of new substrates for the investigation of Nucleotide Excision Repair pathway using fluorescent imaging techniques

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Abstract of the dissertation

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Nucleotide Excision Repair (NER) is a very versatile DNA repair pathway that enables cells to repair UV induced DNA lesions as well as structurally diverse types of adducts formed by carcinogens. Versatility and specificity in NER are achieved through the sequential and highly coordinated action of at least 30 proteins that detect the lesion

and excise a damage-containing oligonucleotide of 24 to 32 nucleotides in length followed by repair synthesis and ligation to restore the DNA sequence to its original state. Reconstituted *in vitro* NER reactions are one of the most powerful ways to investigate this DNA repair pathway. In a typical NER assay, plasmids containing a lesion are incubated with a Hela whole cell extract followed by radioactive labeling of the oligonucleotides after the reaction.

Although this technique has been intensively used and has allowed the collection of much data about the NER mechanism, it is limited in scope. Our aim was to overcome those limitations by developing a new substrate which would allow the monitoring of the NER reaction by fluorescent techniques. Toward this objective, we synthesized a structural analogue of the efficient NER substrate 8-acetylaminofluorene-2'-deoxyguanosine (AAF) containing a ketone linker allowing its site-specific labeling with fluorophores or biotin via oxime formation. Following incorporation into DNA this new substrate will alleviate the need for radioactive labeling in NER by using fluorescence as the method of detection of the oligonucleotides released by the NER reaction. Also, when coupled to a fluorophore, the lesion contains an internal label of the released oligonucleotides, which permits the detection and quantification of all the different products of the NER reaction, which is not possible with current labeling techniques. Together with the use of GFP-tagged NER proteins, this new substrate should also allow the monitoring of the interaction between NER proteins and substrate by fluorescent imaging techniques.

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Chapter 1

General introduction

1.1. DNA DAMAGES AND DNA REPAIR

DNA is the carrier of the genetic information and therefore encodes all of the proteins required for the proper functioning of the cell. Even if some degree of genetic variability is needed to enable evolutionary processes, throughout the lifetime of an organism the integrity of the genomic information is of critical importance for the survival of the species. It has been estimated that the DNA of a single cell undergoes 10^4-10^5 modifications a day (Friedberg et al., 2005). Errors that occur during DNA metabolism such as the incorporation of a wrong base during DNA replication that leads to a base mismatch, are common as are chemical modifications of DNA due to its high reactivity. One typical example is, the loss of purine bases due to the chemical lability of the N-glycosidic bond linking the base to the sugar moiety of the phosphate backbone. Furthermore DNA is highly reactive towards a number of physical agents such as UV-, X- and gamma radiations and a large number of chemical compounds arising both endogenously and exogenously. If unrepaired these modifications of the DNA accumulate in the cell and can interfere with metabolic processes, such as replication, recombination and transcription or even give rise to changes in the DNA coding sequence. In a worst case scenario, changes or inhibition of metabolic processes may

occur, potentially leading to genetic instability, inborn defects, premature aging or cancer (Hoeijmakers, 2001).

The integrity of the genetic information of every living organisms is continuously challenged to such a high degree by the local and external environments that life would not have been possible if cellular DNA repair machineries had not evolved. Indeed all cells possess several different repair systems that are capable of dealing with a variety of DNA lesions and are well conserved across all species (Schärer, 2003). Some pathways are able to repair only a single type of lesion, whereas others have the ability to address many different lesions. Some of them are relatively simple, involving single enzymes and single steps, whereas others operate by more complex mechanisms involving a variety of enzymes that work in concert. The following section presents a brief overview of the main DNA repair pathways and a more detailed description of the mechanism pertinent to this thesis, nucleotide excision repair (NER).

1.1.1. Direct Damage Reversal (DDR)

In higher eukaryotes, two mechanisms have arisen to deal with alkylated bases. Both operate by direct reversal of the damage. For example O^6-alkylguanines are directly repaired by an O^6-alkylguanine transferin (AGT). AGT simply transfers the alkyl group from DNA to one of its conserved cysteine residue to yield the original base. The new bond formed is irreversible and AGT therefore act as a “suicide” protein and is degraded after a single turn over (Margisson et al., 2003). Recently, AGT has also been shown to repair O^4-alkylthymine (Fang et al, 2010). Damaged bases represented by N^1-methyladenine and N^3-methylcytosine are directly repaired by AlkB, a member of the

oxoglutarate-Fe^{2+}-dependent oxygenase superfamily (Aravind and Koonin, 1999). Using Fe^{2+}, O_2 and α-ketoglutarate, AlkB catalyzes the oxidative demethylation of the damaged bases in a mechanism that releases CO_2 and formaldehyde (Falnes et al., 2002; Trewick et al., 2002). Bacteria and lower eukaryotes are additionally equipped with photolyases that directly reverse UV-induced pyrimidine dimers by an electron transfer reaction (Carell et al., 2001).

1.1.2. Base Excision Repair (BER)

Minor modifications to DNA bases results most frequently from alkylation (e.g. 3-methyladenine), oxidation (e.g. 8-oxoguanine and thymine glycol) or deamination (e.g. uracil and hypoxanthine) and are mainly repaired by BER. Although this repair mechanism is always initiated by a DNA glycosylase that hydrolyzes the N-glycosidic bond linking the target base and the deoxyribose sugar to generate an AP-site, two alternative BER pathways have been identified (Schärer, 2003). In the "short-patch BER", a strand-cleavage 5' to the AP-site is performed by an AP endonuclease (APE1). Polymerase β then incorporates a single nucleotide and removes the abasic site by virtue of its AP lyase activity. The nick in the DNA is then sealed by DNA ligase III/XRCC1 (X-ray repair complementing group 1). In the case where the mechanism is initiated by a bifunctionnal glycosylase/AP lyase, the AP lyase activity of polymerase β may not be required. In the so-called long-patch BER, DNA polymerases δ/ε and associated replication factors paste in 2 to 6 nucleotides beyond the abasic site. Endonuclease FEN-1 then excises the oligonucleotide overhang that is generated and DNA ligase I then seals the nick. Until now 11 DNA glycosylases have been identified

and characterized in humans. Each of them excises a subset of damaged or mismatched bases (Wood et al., 2001). Biallelic mutations in the MYH glycosylase, which removes adenine from 8-oxo-dG:dA mispairings has been shown to lead to significant predisposition to colorectal cancer (Cheadle and Sampson, 2003).

1.1.3. Recombinational Repair

Double-strand breaks (DSBs) can be induced by X-rays, radiomimetic chemicals and reactive oxygen species (ROS) generated by the cellular oxidative metabolism. DSBs can also be generated during replication of single-strand breaks during meiosis, V(D)J or immunoglobulin class-switch recombination, as well as during repair of interstrand crosslinks. DSBs can be repaired by two different mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ) (Schärer, 2003). It is noteworthy that a single DSB can ultimately cause cell death. Erroneous rejoining of DNA ends may lead to loss, amplification or translocation of chromosomal material that can lead to tumorigenesis if tumor suppressors genes are lost, or if proto-oncogenes are amplified or deregulated (Khanna and Jackson, 2001). These repair mechanisms therefore are crucial for the maintenance of genome stability and the survival of the cells. The two pathways that have evolved to deal with these kinds of lesions complement each other. Thus, HR predominates in early development and in the late S and G2 phases of the cell cycle, when sister-chromatids are available, whereas NHEJ acts during the G1 phase, and in terminally differentiated cells. In HR, the Rad50/Mre11/NBS1 complex initiates a 5'- to 3'- nucleolytic resection of the DSBs (Jackson, 2002) that allows Rad51 to initiate chain extension on the resulting 3'-single-

stranded fragment to form a nucleoprotein filament with the aid of RPA, Rad52 and the Rad51 paralogues. Assisted by Rad54, the Rad51 nucleofilament then initiates a strand-invasion within the sister chromatid, that is then used as a template by a DNA polymerase to extend the 3'-end of the damaged DNA, followed by a ligation performed by Ligase I. Finally, after branch migration, a protein complex resolves the DNA crossovers, or Holliday junctions. Two different protein complexes have been suggested to be involved in the Holliday junction resolution, one containing Mus81 (Boddy et al., 2001), and another containing Rad51C (Liu et al., 2004).

In NHEJ, the Ku-70/Ku-80 heterodimer recognizes and binds to the DNA termini and recruits the DNA-dependent protein kinase DNA-PKcs. The XRCC4-DNA ligase IV DNA-end-processing complex then religates the two DNA ends. If the DNA ends require processing before ligation, Rad50/Mre11/Nbs1 is also involved. Interestingly the physical presence of Mre11 is required for end-processing but not for its nuclease activity. The nuclease activity has therefore been attributed to an additional nuclease, proven to be the Artemis nuclease (Jackson, 2002). HR is intrinsically accurate whereas NHEJ is error-prone.

1.1.4. Mismatch Repair (MMR)

Replicative DNA polymerases, with their proofreading activity, have an error rate of about 10^{-7}. The 8 possible base mismatches as well as the small insertions and deletions are recognized and corrected by MMR. This lowers the overall mutation frequency by another factor of 10^{2}-10^{3} (Friedberg et al., 2005). Because MMR also recognizes non-damaged nucleotides, a proper discrimination between template and

daughter strand constitutes a key point in its mechanism of action. In *Escherichia coli*, a MutS homodimer recognizes and binds to the mismatch. Upon ATP hydrolysis a repair complex containing MutL and MutH assemble and a bidirectionnal threading of DNA through the complex extrudes the mismatch in a loop with the MMR proteins sitting at its base (Allen et al., 1997). MutS releases the mismatch during this assembly. The strand-discrimination factor MutH nicks the newly synthesized strand at the nearest GATC sequence, not yet dam-methylated. The MutL homodimer mediates the interaction between MutS and MutH (Modrich, 1991). Assisted by the helicase UvrD, one of several exonucleases degrades the daughter-strand past the damage and the missing genetic information is restored by DNA polymerase III and DNA ligase I seals the nick. In eukaryotes, MMR genes have been conserved, but no MutH homologues have been identified. Therefore the mechanism functions in a similar way, the main difference appearing to be in the strand-discrimination step, which is still unknown for eukaryotes. Three MutS homologs (MSH2, MSH3 and MSH6) and three MutL homologues (MLH1, MLH3 and PMS2 (PMS1 in yeast) function as two heterodimers rather than homodimers. The initial step can be performed by either of the two different MutS complexes; MutSα (MSH2 + MSH6) and MutSβ (MSH2 + MSH3). MutSα is more abundant and participates in the repair of base/base mismatches and small strand misalignments whereas MutSβ is responsible for initiating the repair of larger loops. A conformational change in MutSα converts it to a sliding clamp that translocates along the DNA away from the mismatch. Assembly of the MMR repairosome requires then the MutL homologs MLH1/PMS2 (MutLα heterodimer) if repair initiated by MutSα or MLH1/MLH3 (MutLβ heterodimer) in case of initiation by MutSβ. The MutS homologues

MSH6 and MSH3 have been shown to interact directly with PCNA (proliferating cell nuclear antigen), the processivity factor of replicating DNA polymerases (Jiricny, 2006), making a clear link between MMR and the replication machinery.

1.2. NUCLEOTIDE EXCISION REPAIR (NER)

1.2.1. INTRODUCTION

NER is the repair pathway for many structurally unrelated DNA lesions. Lesions formed by environmental and certain chemotherapeutic agents are removed by NER. Very importantly the two types of lesions generated by UV irradiation, the cyclobutane pyrimidine dimmers (CPDs) and the 6-4-photoproducts (6,4-PPs) are corrected by this process. The pool of proteins involved and the basic mechanism of NER are conserved from bacteria to humans and among eukaryotes (Petit and Sancar, 1999). Approximatly 30 proteins are involved in this mechanism in Eukaryotes (Aboussekra et al., 1995; Mu et al., 1995; Araujo et al., 2000). XPC-HR23B, the ten subunits of the basal transcription factor TFIIH, the single-strand DNA binding protein RPA, and XPA are involved in the damage recognition and the unwinding of the DNA around the lesion. The two structure-specific endonucleases, XPF-ERCC1 and XPG, make incisions 5' and 3' to the lesion, respectively. DNA polymerases δ and/or ε, assisted by the sliding clamp PCNA and the clamp loader take charge of the repair synthesis. DNA ligase I seals the nick (Gillet and Schärer, 2006). All of these proteins are required for the repair of the lesions throughout the whole genome in the so-called global genome NER (GG-NER). A sub-pathway of NER, transcription-coupled NER (TC-NER), preferentially repair damaged nucleotides

that are present within actively transcribed genes. TC-NER is not dependent on XPC-HR23B (Venema et al., 1991) and is initiated by a stalled RNA polymerase at the site of the lesion. The two additional factors CSA and CSB that are not required for GG-NER are required for TC-NER (reviewed by Svejstrup, 2002). In both cases, a chemical modification of the DNA by a bulky residue is required. In GG-NER a significant distortion of the DNA helix is a prerequisite for recognition by NER factors (Hess et al., 1997), and the efficiency of repair is proportional to the degree of distortion induced by the damage (Gunz et al., 1996).

1.2.2. HISTORY OF NER

In the early nineteen-sixties several discoveries of critical importance led to the identification of the NER pathway and its elucidation. In 1964, it was shown that thymine dimers were released in the form of short oligonucleotides from UV-irradiated cells in bacteria (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964). In the same year, *de novo* DNA synthesis was observed after UV irradiation both in bacterial and human cells (Pettijohn and Hanawalt, 1964; Rasmussen and Painter, 1964). This phenomenon is now referred to as “unscheduled DNA synthesis” (UDS). Furthermore, it has been observed that cultured fibroblast cells from different XP patients exhibit different survival capabilities upon exposure to UV light (Cleaver, 1968). And that restoration of UDS can be achieved by the fusion of cells from XP patients with defect in various XP genes (de Weerd-Kastelein et al., 1972), what has lead to the identification of different complementation groups designated XP-A to XP-G, each of them carrying mutations in genes coding for NER proteins. Therefore, the UDS observed upon UV

irradiation reflects the repair synthesis step of NER and is the step that is impaired in cells from xeroderma pigmentosum (XP) patients.

In humans, three rare autosomal recessive disorders are intimately associated with defects in nucleotide excision repair: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodistrophy (TTD). All three diseases are characterized by an extreme sensitivity to UV light but differ in many of their genetic and symptomatic aspects.

The term XP was first used in 1874 by Moriz Kaposi to describe the symptoms observed in one of his patients (von Hebra and Kaposi, 1874). XP patients have a more than 1000-fold increased risk of developing skin cancer, especially in areas exposed to sunlight such as the hands, face and neck. The onset of disease occurs at 8 years of age, which is 50 years earlier than that of the general population. In about 18 % of the cases, these symptoms are coupled with a primary neuronal degeneration and loss of neurons (Bootsma et al., 1997). Statistically it has been estimated that XP affects 1 individual in 250,000 in the Western world and about a 6 times greater number of individuals in Japan and North Africa.

In 1936, Edward Alfred Cockayne reported for the first time the syndrome that will later carry his name (Cockayne, 1936). Although CS patients show abnormal sensitivity to UV light they do not display a clear predisposition for skin cancer. The main features that characterize these patients are stunted growth, impaired development of the nervous system caused by dysmyelination, and premature ageing. Hearing loss and ocular abnormalities are other common features, but problems with any or all of the internal organs are possible. Originally, CS was thought to be only

associated with a deficiency in TC-NER (Svejstrup, 2002). However, the fact that many XP patients are defective in both GG-NER and TC-NER but do not show several of the CS symptoms has lead to the conclusion that CS cannot be simply explained by a defect in TC-NER, but may be associated with a more general mild impairment in transcription (van Gool et al., 1997)

TTD was first reported by Price in 1980. In addition to symptoms displayed by CS patients, TTD patients show characteristic sulfur-deficient brittle hair, scaling of the skin, short stature and mental retardation (Price et al., 1980). The disease is nowadays known to be associated with mutations in the genes coding for XPB, XPD and TTDA, subunits of NER/transcription factor TFIIH (Bergmann and Egly, 2001, Giglia-Mari et al., 2004). Is it commonly accepted that TTD reflects more an impairment in transcription than a regular defect in DNA repair and is sometimes referred as a “transcriptional syndrome”.

The purification and biochemical characterization of the different NER factors has allowed the assessment of their role in NER. A significant breakthrough came with the *in vitro* reconstitution of the NER reaction on defined DNA lesions (Aboussekra et al., 1995; Guzder et al., 1995; Mu et al., 1995; Mu et al., 1997), which allowed the determination of the sequential recruiting and association of the individual NER proteins needed to perform NER. *In vivo* studies confirmed that the NER reaction indeed involves the sequential and coordinated assembly of the individual NER protein or sub-complexes at the site of the lesion (Guzler et al., 1995; Mu et al., 1997; Wakasugi and Sancar, 1998; Hoogstraten et al., 2002; Rademaker et al., 2003; Riedl et al., 2003;

Zotter et al., 2006) and not via a pre-assembled NER complex ("the repairosome") as it had been proposed previously (He et al., 1995; Svejstrup et al., 1996).

To summarize, the NER reaction was firmly established mechanistically as a damage recognition followed by the excision of a patch of DNA containing the damage with the repair machinery restoring the original DNA sequence.

1.2.3. LESIONS ADDRESSED BY NER

The main biological role of the NER pathway is to remove the UV-photoproducts from DNA. But due to its remarkable broad substrate specificity, NER also deals with bulky base adducts formed by various environmental mutagens and certain chemotherapeutic agents (Gillet and Schärer, 2006). The structural differences among all the chemical modifications recognized by NER make it the most versatile of all DNA repair pathways, highlighting its important role in the survival of organisms in an environment containing unexpected DNA-damaging agents.

1.2.3.1. Damages of the pyrimidine bases induced by reaction with UV light

Upon UV-irradiation two adjacent pyrimidines can undergo a [2+2] cycloaddition. Depending on the nature of the two pyrimidines coupling, two different photoproducts of the DNA can occur, the so-called cyclobutane pyrimidine dimers (CPD) and the pyrimidine (6-4) pyrimidones (6-4PPs).

CPDs constitute the major UV-induced photoproducts and are mainly formed by the cycloaddition reaction between the C5-C6 double bonds of two adjacent thymine residues. Depending on the wavelength, dose of irradiation and the sequence context,

the three other possible combinations of intrastrand crosslinks between pyrimidines (TC, CT and CC) can also be generated. Considering the different orientation of the reactive pyrimidines, four products can in principle occur. Indeed all combinations of *cis*/*trans* (indicating the relative positions of the bases) and *syn*/*anti* (indicating the relative orientation of the C5-C6 bonds) diastereoisomers can be drawn (figure 1, upper insert). But due to the steric constraints into the DNA, only *syn* isomers can be formed; the *cis*-*syn* representing the large majority in double-stranded DNA (ds-DNA), whereas the *trans*-*syn* can exclusively occur in single-stranded DNA (ss-DNA). Due to its residual absorption capacity below 250 nm, the dimer can be photochemically reversed to yield the original free bases.

6-4PPs are formed by the cycloaddition reaction between the C5-C6 bond of a 5'-pyrimidine residue and the C4 carbonyl group of a 3'-thymine or the 4-imino group of a 3'-cytosine. The respective oxetane or azetidine formed both spontaneously rearrange to yield the 6-4PPs (figure 1, bottom insert). The occurrence of the preferred intra-strand crosslinks formed (TC, CC and TT) depends on the irradiation wavelength and the sequence context. As for CPDs, higher dosage of UVB can potentially lead to a rearrangement leading to the Dewar isomer (Ravanat et al., 2001) but it is not clear that these kinds of structures are really biologically relevant (figure 1). 6-4PPs are distinguished from CPDs by their level of occurrence, about 25-30% of that of CPDs (Mitchell, 1988), as well as the degree of DNA distortion they induce. Whereas CPDs induce only a slight bending of the DNA helix with no significant alteration of the Watson-Crick base pairing (Kim et al., 1995; Lee et al., 2004), 6-4PPs produce a more pronounced distortion of the DNA backbone (bending and unwinding) inducing a loss of

base-pairing at the site of the lesion (Kim et al., 1995). Finally, 6-4PPs have been shown to be repaired much faster and more efficiently by NER than CPDs (Reardon et al., 1993; Szymkowski et al., 1993; Kusumoto et al., 2001; Sugasawa et al., 2001; Reardon and Sancar, 2003), highlighting the importance of the correlation between the degree of DNA distortion induced by a lesion and its suitability as an NER substrate.

In addition to CPD, 6-4PP and Dewar pyrimidones, other minor photoproducts have been identified in heavily UV-irradiated DNA. These lesions, including the cytosine photohydrate, the adenine dehydrodimer or thymine-adenine dimer are very rare and their biological significance in regard to solar UV damage remains to be examined.

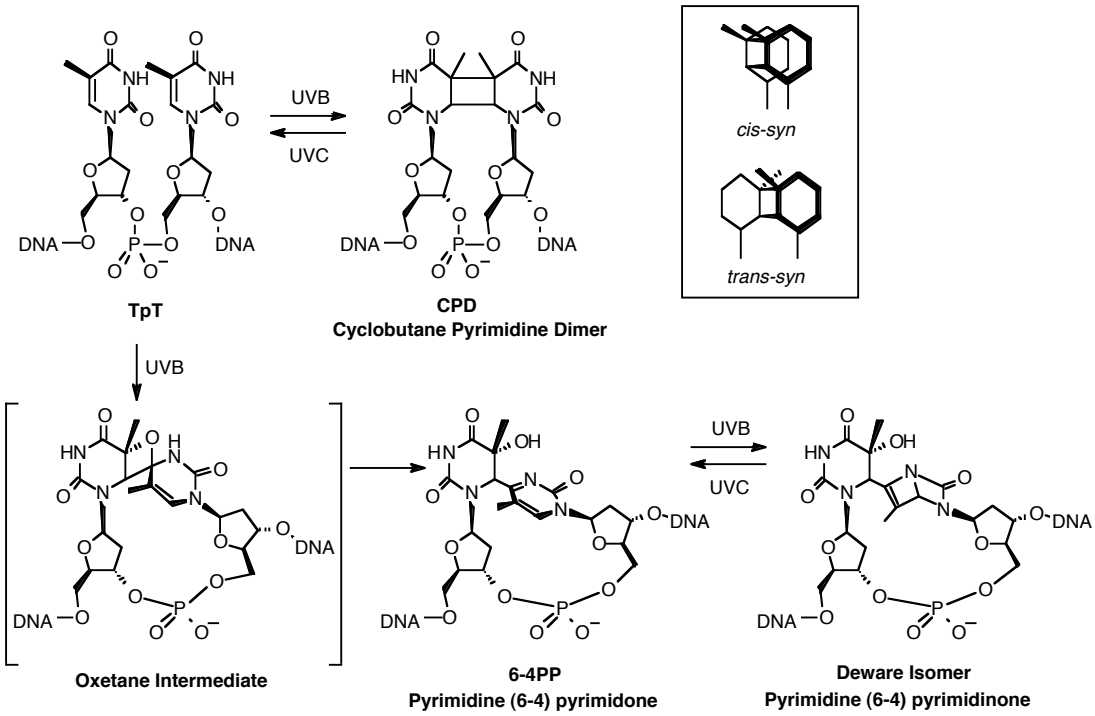

Figure 1. Ultraviolet photoproducts of a TpT in DNA. The figure is from Gillet and Schärer, 2006; the structures of the cyclobutane dimer, the pyrimidine (6-4) pyrimidone and of the Dewar pyrimidinone are originally adapted from Taylor and Corr, 1987. The insert shows the different diastereoisomers of the CPD within DNA. The different wavelengths for the formation and reversion of the photoproducts are indicated.

1.2.3.2. Damage to the purine bases by reaction with carcinogenic compounds

Most carcinogens are electrophilic compounds (ironically often products of metabolic detoxification processes) that show a certain degree of affinity for double-stranded DNA and react with the nucleophilic functions of the phopshodiester linkages and the purinic bases (N7 of guanine, N3 of adenine, and respectively exocyclic amino groups N^6 of adenine and N^2 of guanine). The N7 position has high electronic density and a good accessibility in the major groove of the DNA helix. It therefore is the most reactive site in DNA toward electrophilic attack. Among the most proficient NER adducts are the chemotherapeutic drug cis-diamino-dichloroplatinum (*cis*-DDP) and many aromatic and aminoaromatic compounds present in the environment.

Cis-DDP is one of the most commonly used chemotherapeutic agents in the clinic. It is a square planar complex with platinum(II) coordinated to two relatively inert ammonia groups and two labile chloride ligands in *cis* geometry (Jamieson and Lippard, 1999). The low salt concentration (chloride) of the cytoplasm allows the substitution of the chloride ligands by water or hydroxide ions rendering the complex more electrophylic. The activated cisplatin can then react at sites in the DNA to form monoadducts, intrastrand and interstrand cross-links. Platination of oligonucleotides preferentially leads to N7-N7 intrastrand cross-links between purines (figure 2, insert A); 1,2d(GpG) (up to 65%), 1,2-d(ApG) (25%), 1,3-d(GpNpG) (5 to 10%). Interstrand crosslinks represent only 2% of the lesions formed. 1,2-intrastrand crosslinks generate a pronounced kink in the double helix but retain mostly intact base pairing (Jamieson and Lippard, 1999). By contrast, 1,3-intrastrand cross-links induce a much smaller kink

in the DNA but lead to much more pronounced unwinding and loss of base-pairing. They are therefore much better NER substrates.

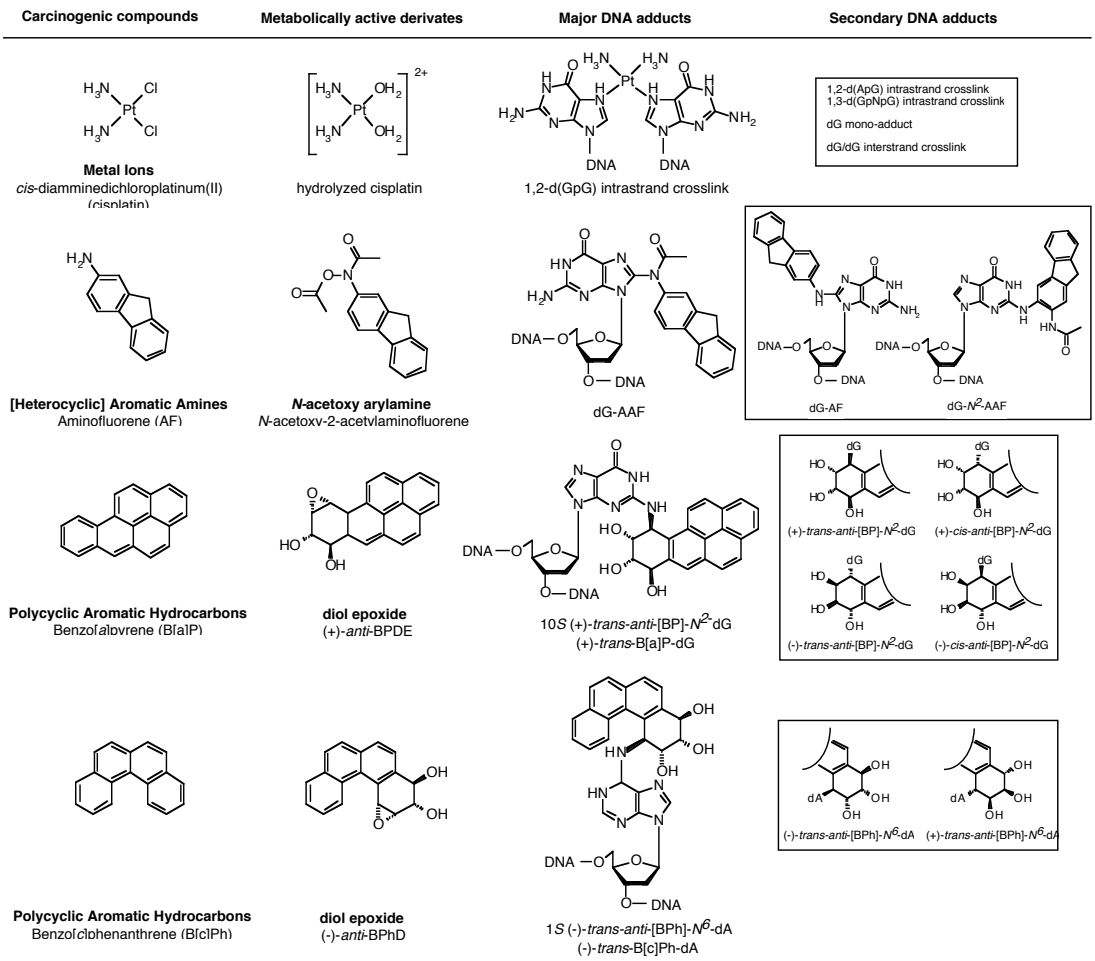

Figure 2. Examples of potent carcinogenic chemicals. Their corresponding active derivatives and their major adducts within DNA are indicated in parallel. The inserts show the main secondary DNA adducts formed by AF, B[a]P and B[c]Ph. Note that the guanine moiety are depicted in *syn* or *anti* conformation to reflect the structures of the adducts within DNA. The picture is from Gillet and Schärer, 2006.

Aromatic carcinogens are a major health concern because they are present in abundance in our daily environment (Beije and Moller, 1988). For example, benzo[a]pyrene and benzo[c]phenanthrene are present in cigarette smoke and car exhaust. These non-polar lesions do not show reactivity toward DNA. Nevertheless, it

has been clearly established that they are activated, mostly in the liver by a detoxification metabolism. Upon metabolic activation, non-polar polycyclic aromatic hydrocarbons are transformed into phenols and dihydrodiols, which are easily excreted. But a fraction of those molecules is converted into epoxides (Geacintov et al., 1997; Friedberg et al., 2005), which can react with the exocyclic amino group of the purine bases following intercalation into the DNA (figure 2, insert B and C). Depending on the isomer formed in this reaction, two major conformations are induced in DNA (Geacintov et al., 1997): the apolar ring of the molecule can either intercalate into the double helix forcing the displacement of the modified base from its normal position, or the aromatic ring can be accommodated in a groove with the conservation of regular base-pairing. Consistent with what is observed for the UV-photoproducts and *cis*-DDP, the loss of base-pairing and the degree of distortion determine the efficiency with which the lesion is repaired.

A second important class of carcinogens are polycyclic aromatic amines that are found in cigarette smoke and cooked food (Tureski, 2002), and include the infamous 2-aminofluorene. Aromatic amines also can be activated during the detoxification processes. This reaction leads to N-hydroxy, N-acetoxy or N-sulfoxy compounds that can undergo N-O bond heterolysis, yielding a very reactive arylnitrenium intermediate that forms adducts at the C8 position of the guanine and to a lesser extent the N^2 position. Interestingly, the activated aminofluorene can generate two different adducts (figure 2, insert 1), 8-(N^2-acetyl-aminofluorene)-2'-deoxyguanosine (dG-AAF) and 8-aminofluorene-2'-deoxyguanosine (dG-AF). Due to allylic strain in the N^8-acetyl derivative, these two adducts adopt a different conformation (Belguise-Valladier and

Fuchs, 1991; Veaute and Fuchs, 1991; Belguise-Valladier and Fuchs, 1995), therefore inducing a different degree of mispairing and helix distortion, while they differ only by one acetyl group in their structure, difference inducing here again a different NER proficiency. While the dG-AF adduct allows the maintenance of Watson-Crick base-pairing, the dG-AAF adduct causes major DNA distortion by intercalating the fluorene ring within the DNA double helix and is therefore a better NER substrate (figure 3). These two last adducts are nowadays intensively used to study carcinogenesis and DNA repair (Heflich and Neft, 1994).

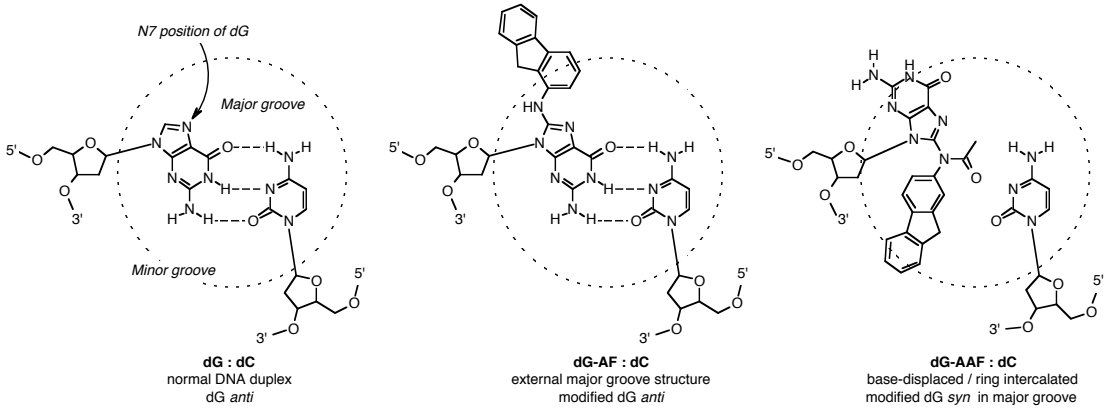

Figure 3. Conformations of dG, dG-AF and dG-AAF within DNA. In normal DNA duplex, dG is in *anti* conformation and fully paired with the complementary dC. Note the accessibility of the nucleophilic N7 position of dG, positioned in the major groove of the DNA. The major conformation of DNA containing the AF modification displays normal Watson-Crick pairing for the modified dG; the fluorene ring is accommodated in the major groove of the DNA helix. The AAF modification causes an *anti* to *syn* rotation of the modified guanine in order to accommodate the fluorene ring inside the helix ("base-displaced, intercalated" conformation). This intercalation produces a severe DNA helix distortion and explains the profound differences in damage recognition, repair efficiency and mutagenicity of these two adducts. The figure is from Gillet and Schärer, 2006; originally adapted from Hoffmann and Fuchs, 1997; and based on structural information from O'Handley et al., 1993 and Mao et al., 1998.

1.2.4. MOLECULAR MECHANISM OF GLOBAL GENOME REPAIR (GGR)

1.2.4.1. Damage recognition: XPC-HR23B and XPE

For almost a decade the identity of the GGR damage recognition factor was a matter of debate for a significant amount of time. Different groups reported that the different factors XPA (Jones and Wood, 1993; Li et al., 1995; Buschta-Hedayat et al., 1999), RPA (He et al., 1995; Burns et al., 1996; Schweizer et al., 1999), XPC-HR23B (Batty et al., 2000; Sugasawa et al., 1998; Sugasawa et al., 2001) and XPE (Keeney et al., 1993; Reardon et al., 1993; Fujiwara et al., 1999) display some preference for binding damaged DNA. However, the binding affinities and specificities for damaged DNA of these proteins were not sufficient to clearly identify any of them as the initial damage recognition factor. Furthermore, data from *in vitro* NER assays in which factors were added in various order suggested either XPC-HR23B (Sugasawa et al., 1998) or XPA/RPA (Wakasugi and Sancar, 1999) as the initial damage recognition factor. Additional studies investigating which factors were required to generate open DNA structures around the lesion supported an early role for XPC and TFIIH (Evans et al., 1997). This observation is in perfect correlation with the observation that XPC cells are only deficient in GG-NER and not TCR where the repair of the damage is initiated by a stalled RNA polymerase.

The development of a new *in vivo* immunofluorescence technique0 allowing the study of the assembly of NER proteins at sites of local UV DNA damages provided important insight into the mechanism of damage recognition (Mone et al., 2001; Volker et al., 2001). XPC was clearly recruited to the sites of UV lesions in XP-A cells whereas XPA was not found at damaged sites in XP-C cells (Volker et al., 2001). Finally, new

biochemical experiments showed XPC to be required for the DNA unwinding and the recruitments of all other NER factors (Riedl et al., 2003; Tapias et al., 2004).

XPC is found in a tight complex with Centrin 2 (Araki et al., 2001) and mostly HR23B, one of the two human homologues of *S. cerevisiae* Rad23 (Masutani et al., 1994). How the lesion is recognized is poorly understood. Its role is not clearly understood yet but Centrin 2 has been shown to stimulate NER (Nishi et al., 2005). HR23B is believed to stabilize XPC and protect it from proteasomal degradation (Ng et al., 2003). Biochemical experiments have shown that the complex binds preferentially ssDNA (Fitch et al., 2003) and helix distortions (Sugasawa et al., 1998; Wakasagi et al., 1999), regardless as to whether they contain a lesion or are just mispaired (Sugasawa et al., 2001). Interestingly non-damaged DNA distortions are not repaired by NER (Sugasawa et al., 2001; Hess et al., 1997) suggesting that even if XPC-HR23B recognizes the helix distortion, the complex is not sufficient by itself to trigger the repair reaction. This indicates the existence of a subsequent damage verification step. The less distorting CPDs are more weakly bound specifically by XPC-HR23B and less efficiently repaired by NER than the more distorting 6-4-PPs (Szymkowski et al., 1993; Batty et al., 2000). Interestingly when a mismatch is placed opposite to the CPD, XPC-HR23B binding affinity and NER processivity is increased (Sugasawa et al., 2001). It has been suggested that XPE (Wittschieben and Wood, 2003) could play a role in the detection of some less distorted lesions. XPE, or UV-damage DNA binding factor (UV-DDB) is a complex composed of two subunits, p127/DDB1 and p48/DDB2. It has indeed been shown that this dimer binds specifically to CPDs and introduces a bend around the lesion (Fujiwara et al., 1999; Tang and Chu, 2002). *In vivo* the presence of DDB2 is

required for the recruitment of XPC to sites of UV damage (Fitch et al., 2003b) and increasing repair of CPDs (Wakasugi et al., 2002). Little is known about the XPE interactions within the lesions.

DDB1 and DDB2 are part of an ubiquitin ligase complex with Roc1 and Cul4A (Groisman et al., 2003). COP9 signalosome (CSN) is associated with the Cul4A E3 ligase and suppresses Cul4A ligase ubiquitininylation. After interaction within the lesion, CSN dissociates from the E3 ligase complex leading to polyubiquitinylation of DDB2 and its subsequent degradation by the proteasome (Rapic-Otrin et al. 2002; Fitch et al., 2003; Groisman et al., 2003).

XPC has also been shown to be polyubiquitinylated by the same ubiquitin ligase complex (Sugasawa et al., 2005; Wang et al., 2005). This ubiquitinylation does not constitute a signal for degradation but instead increased the binding affinity of XPC for both damaged and undamaged DNA (Sugasawa et al., 2005).

1.2.4.2. DNA unwinding and lesion verification: TFIIH

Through protein-protein interactions with XPC-HR23B (Drapkin et al., 1994; van der Spek et al., 1996; Volker et al., 2001; Yokoi et al., 2002), the ten-subunits TFIIH factor is recruited to the site of the lesion (Araujo et al., 2001; Riedl et al., 2003; Uchida et al., 2002). TFIIH is composed of a core complex (XPB, XPD, p63, p52, p44, p34 and p8) and a cyclin-activating kinase (CAK) subunit (Cdk7, cyclin H and Mat1), which are arranged together in a ring-like structure (Schultz et al., 2000). This complex is a basal transcription factor mostly involved in RNA polymerase I (Iben et al., 2002) and II (Gerard et al., 1991) transcription but that can be recruited to take part in the NER

reaction (Hoogstraten et al., 2002). XPB and XPD are helicases with 3' to 5' and 5' to 3' DNA unwinding activity respectively that form an open DNA structure in transcription (Gerard et al., 1991; Iben et al., 2002) and NER (Schaeffer et al., 1993, 1994). In transcription it has been demonstrated that only the helicase activity of XPB is required (Tirode et al., 1999) while the helicase activity of both XPB (Hwang et al., 1996) and XPD (Winkler et al., 2000) are required in NER to open the DNA around the lesion (Evans et al., 1997). But as for transcription, ATP hydrolysis is required for the unwinding to occur (Evans et al., 1997; Riedl et al., 2003; Tapias et al., 2004). Conversely, it has been shown that the XPB ATPase but not its helicase activity is needed for DNA opening in NER (Coin et al., 2006). To summarize, XPC-HR23B recognizes the lesion, initiates a local unwinding around the lesion and recruits TFIIH through protein-protein interactions, allowing it to extend the DNA opening and allow the loading of the subsequent NER factors. The multi-ATP-dependent recognition has been referred to as "kinetic proofreading" (Reardon and Sancar, 2003; Reardon and Sancar, 2004).

TFIIH has two additional roles in NER. First, TFIIH is required for the recruitment of XPG to the NER complex (Dunand-Sauthier et al., 2005; Volker et al., 2001). Second, a role in the damage verification has been proposed for XPD. The helicase activity of Rad3, the XPD homologue in *Saccharomyces cerevisiae* has been shown to be inhibited by bulky DNA lesions (Naegeli et al., 1993) and XPD has been cross-linked at the site of a photoactive psoralen lesion during NER (Reardon and Sancar, 2003). Consistent with the fact that XPB helicase activity is not needed in NER (Coin et al., 2007), XPD naturally appeared to be a good candidate to be involved in the damage

verification step. Accordingly, XPC-HR23B would first recognize the helix-distortion and thermodynamic destabilization and TFIIH would subsequently verify the presence of a chemical DNA modification by stalling the XPD helicase (Hess et al., 1997). Recently, contrasting data showed that an archeal homolog of XPD was not getting stalled by NER lesions (Rudolf et al., 2010), showing that the debate on damage recognition and verification is not yet over. The importance of TFIIH in the damage verification step has been also attributed to its tenth subunit p8 (Giglia-Mari et al., 2004). This small subunit is present into two distinct kinetic pools, one fraction bound to TFIIH while the other one is freely shuttling between the cytoplasm and the nucleus. No perturbation of this equilibrium is observed during transcription but a more stable incorporation of p8 into TFIIH is observed during NER. Interestingly, this shift is not observed for conformations that only provoke abortive-type NER reaction (Giglia-Mari et al., 2006). Finally, together with XPC-HR23B, p8 stimulates the ATPase activity of XPB, allowing the opening of DNA around the lesion and recruitment of XPA (Coin et al., 2006).

1.2.4.3. Assembly of the pre-incision complex: RPA, XPA, XPG

Once THFIIH is engaged at the lesion, the three factors RPA, XPA and XPG (Wakasugi and Sancar, 1998; Volker et al., 2001; Riedl et al., 2003) are recruited to form the stable NER “pre-incision complex”. XPC-HR23B is actually thought to leave this complex upon the arrival of XPG (Wakasugi and Sancar, 1998; Riedl et al., 2003). RPA has been shown to colocalize with the site of UV lesions in the absence of XPA and XPG (Rademakers, 2003), and both XPA and RPA can be recruited in the absence of XPG (Rademakers, 2003). Conversely, recruitment of XPG is independent of the

presence of XPA (Volker et al., 2001). These three NER factors can therefore be recruited independently of each other, likely through protein-protein interactions with TFIIH.

RPA is a trimeric protein (Henricksen et al., 1994) that binds specifically to single-stranded DNA and also plays a central role in replication and recombination (reviewed by Bochkarev and Bochkareva, 2004). RPA was found to be required for both the dual incision (Coverley et al., 1992; Guzder et al., 1995; Mu et al., 1995) and the repair synthesis steps of NER (Coverley et al., 1991; Shivji et al., 1995). With a well defined 5' to 3' polarity (Bochkarev et al., 1997), RPA likely binds to the non-damaged strand after DNA opening. RPA is therefore believed to play an important role in the proper assembly and orientation of the "pre-incision complex" (de Laat et al., 1998).

XPA is a small protein with a zinc-finger domain (Tanaka et al., 1990) that has been shown to be an RPA interaction domain (Lieber, 1997). The specificity of RPA for damaged DNA has been shown to be enhanced by addition of XPA (He et al., 1995; Buschta-Hedayat et al., 1999; Segers-Nolten et al., 2002). XPA may therefore work in a cooperative fashion with RPA assuring its positioning on the non-damaged strand. XPA has a much higher binding affinity for unusual DNA structures than for DNA lesions themselves (Missura et al., 2001). This fact suggests that XPA is potentially probing for non-standard DNA structures that arise during NER. XPA does not only interact with DNA but also with XPC-HR23B (You et al., 2003), RPA (Ikegami et al., 1998; Matsuda et al., 1995; Saijo et al. 1996), TFIIH (Li et al., 1998; Park et al., 1995b; and ERCC1 (Li et al., 1995; Nagai et al., 1995; Park and Sancar, 1994). XPA therefore likely plays a key structural role in the proper assembly of NER pre-incision complex.

XPG belongs to the FEN-1 (flap endonuclease) family of structure-specific endonucleases (Lieber, 1997). It is recruited to the pre-incision complex through its interaction with TFIIH (Araujo et al., 2001). It is able to incise flap or bubble DNA structures with a defined polarity (O'Donovan et al., 1994; Mu et al., 1996; Hohl et al., 2003), cleaving specifically at the junction between the 3' end of single-stranded DNA and the 5' end of double-stranded DNA, consistent with the 3' incision during the NER reaction. XPG not only interacts with TFIIH but also with RPA but to what extent this interaction contributes to the positioning of XPG in the NER complex is not known (He et al., 1995; Matsunaga et al., 1996; de Laat et al., 1999). XPG not only has a catalytic activity but also has a structural function in NER. The presence of XPG, but interestingly not its catalytic activity, is required for the 5' incision by ERCC1-XPF (Wakasugi et al, 1997; Constantinou et al., 1999).

1.2.4.4. Dual incision: XPF-ERCC1

It has been shown both *in vitro* (Riedl et al., 2003; Wakasugi and Sancar, 1998) and *in vivo* (Volker et al., 2001) that XPF-ERCC1 is the final DNA repair component that joins the pre-incision complex. XPF-ERCC1 is an obligate heterodimer and the two proteins are stable only in each other's presence (Biggerstaff et al., 1993; van Vuuren et al., 1993). XPF is an endonuclease that cleaves the 5' side of double-strand/single-strand DNA junctions, in agreement with its 5' activity in NER (Matsunaga et al., 1995; Sijbers et al., 1996). It was shown that binding of RPA to single-strand DNA can stimulate XPF-ERCC1 endonuclease activity on model substrates if the factors are

positioned with a polarity reflective of the one found in the NER reaction (Bessho et al., 1997; de Laat et al., 1998; Matsunaga et al., 1996).

XPF-ERCC1 is recruited to the complex by direct interaction between ERCC1 and XPA (Li et al., 1994; Park et al., 1994; Li et al., 1995; Nagai et al., 1995; Saijo et al., 1996). The addition of XPF-ERCC1 does not appear to induce any conformational changes in the open DNA structure (Evans et al., 1997; Tapias et al., 2004), but addition of ERCC1-XPF to the pre-incision complex triggers the dual incision, releasing a damage-containing DNA segment of 24 to 32 nucleotides in length (de Laat et al., 1999). Uncoupled incision 3' to the lesion by XPG has been observed in the absence of XPF (Mu et al., 1996; Sijbers et al., 1996; Evans et al., 1997), while the presence of XPG, but not its catalytic activity is required for the 5' incision by XPF-ERCC1 (Wakasugi et al., 1997; Constantinou et al., 1999), raising questions as to whether or not the two incisions occur simultaneously or in a defined order. Recently, clear evidence that XPF-ERCC1 exerts its nuclease activity first and that the repair process is even initiated before XPG makes the 3'-incision, has been obtained (Staresincic et al., 2009).

1.2.4.5. Repair synthesis: RPA, RFC, PCNA, Pol δ/ε

Repair synthesis is accomplished by the coordinated action of PCNA (Nichols and Sancar, 1992; Shivji et al., 1992; Aboussekra and Wood, 1995), DNA polymerase δ (Nishida et al., 1988; Hunting et al., 1991; Zeng et al., 1994), and ε (Podust et al., 1992; Shivji et al., 1995) and RPA (Coverley et al., 1991). RPA, PCNA, the clamp loader RFC, and DNA ligase I together with either Pol δ or Pol ε in total have been shown to be

necessary and sufficient for repair synthesis *in vitro* (Shivji et al., 1995; Araujo et al., 2000). The function of these proteins has mostly been studied during replication processes. PCNA is a homotrimeric protein arranged in a ring-shaped clamp (Krishna et al., 1994) that can slide along the DNA, ensuring the processivity of replication (Miyata et al., 2004). RFC is a pentameric clamp loader that catalyse the ATP-dependent loading of PCNA to DNA near the 3'-termini of primers (Majka and Burgers, 2004). Pol δ and Pol ε belong to the B family of DNA polymerases. Their active site has stringent requirements for appropriate Watson-Crick base pairing. Furthermore the 3' to 5' exonuclease activity associated with replicative polymerases allows the removal of any mis-inserted base (Lehmann, 2006).

Transition from dual incision to repair synthesis has to be a tightly coordinated process to avoid the generation of potentially mutagenic and recombinogenic ssDNA intermediates. RPA is necessary for both repair synthesis and dual incision and is potentially responsible for the transition between the two steps. It is involved in the recruitment of both PCNA (Gomes and Burgers, 2001; Riedl et al., 2003) and RFC (Yushakov et al., 1999) during replication. Interestingly, XPG stays bound to the excised oligonucleotide longer than the other NER pre-incision factors (Riedl et al., 2003). It also interacts with PCNA (Miura et al., 1996; Gary et al., 1997) and has therefore been hypothesized to play an important role in the coupling between repair-synthesis and dual incision in NER (Gary et al., 1997). Finally, the existence of this crucial link between the two steps is highlighted even more by the fact that PCNA has been shown to stimulate the dual incision (Nichols and Sancar, 1992) and by the similar kinetics of

both excision and repair synthesis steps (Riedl et al., 2003). The current model of NER based on these data is presented on figure 4.

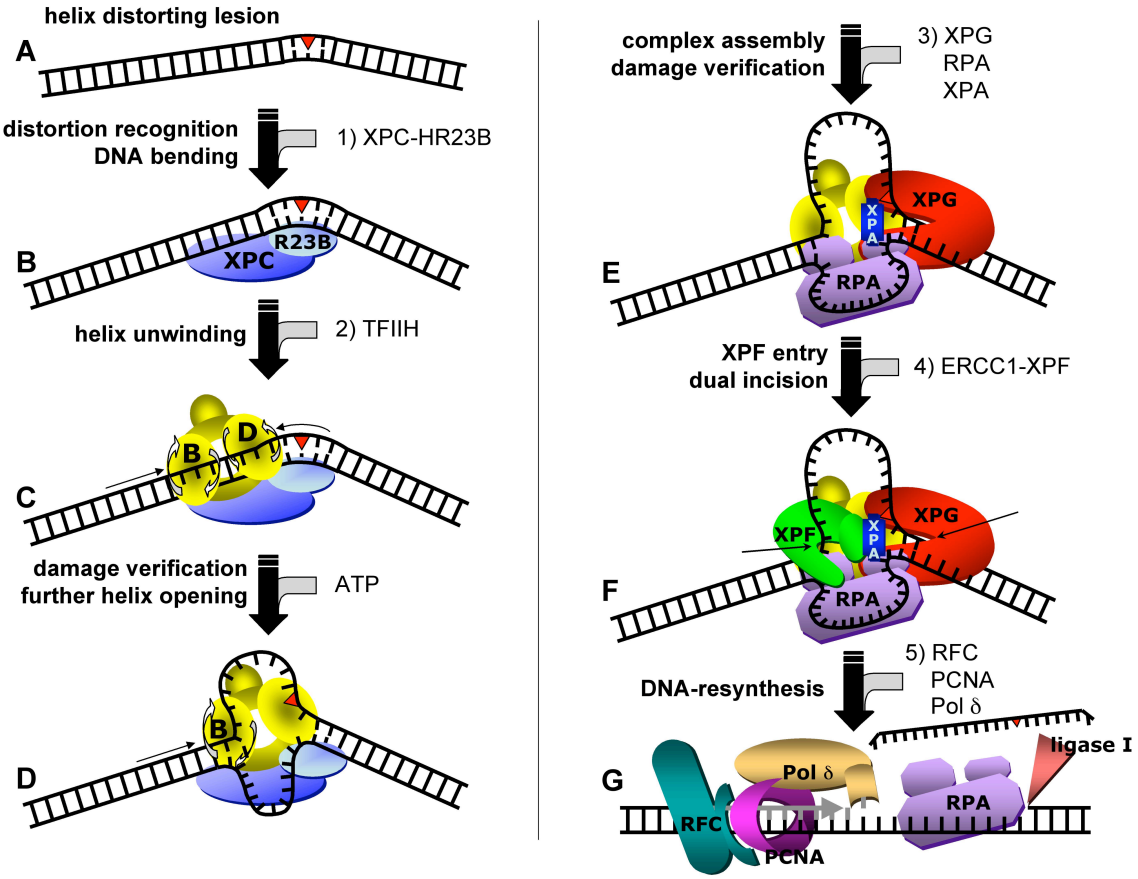

Figure 4. Model of the NER mechanism. A. A lesion induces DNA helix distortion; B. XPC-HR23B detects the helix distortion and stabilizes the DNA bend; C. XPC-HR23B recruits TFIIH at the site of the lesion; D. upon ATP addition, TFHII unwinds the DNA helix, until one of its helicase subunit (here XPD) encounters a chemically modified base; the second helicase subunit (here XPB) goes on unwinding the DNA to create a 20 bp opened "bubble" structure; E. TFIIH recruits RPA, XPA and XPG which assemble to form the "preincision" complex; F. ERCC1-XPF joins the complex and the dual incision (5' by ERCC1-XPF and 3' by XPG) occurs; G. RPA recruits RFC and PCNA; Polδ (or ε) performs the repair resynthesis; RPA covers the non-damaged strand until it gets displaced by the polymerase machinery; ligase I seals the final nick.

1.3. PREVIEW

Since its existence, the Schärer's group has been combining synthetic chemistry and biological techniques to develop innovative ways of investigating the nucleotide excision repair pathway. Here, we disclose the synthesis of a new type of proficient NER susbtrate that allows the development and application of new techniques to take a closer look at the operative mechanisms of NER.

The foundation of the work herein presented was laid by a former graduate student, Ludovic Gillet, who developed a general synthetic route for the synthesis and introduction into oligonucleotides of 8-(N-acetyl-2-aminofluorene)-2'-deoxyguanosine (dG-AAF) and related lesions into DNA. The work presented in this manuscript expands this approach to introduce a structural analogue of the dG-AAF adducts into DNA by solid-support DNA synthesis, functionalized with a linker that allows its selective labeling at the oligonucleotide level with biotin-based and fluorescent probes.

It is necessary to understand the importance of such adducts. Significantly, using fluorescence as the method of detection of the oligonucleotides released by the NER reaction will obviate the need for radioactive labeling and make the NER assays easier and faster to accomplish. In addition, an adduct coupled to a fluorophore constitutes an internal label of the oligonuclotides released by the dual incision This will allow not only the detection and the quantification of the oligonucleotides produced by the successful dual incisions but all the products of the NER reaction, which is not possible with current labeling techniques. Finally, selective *in situ* fluorescent labeling of this adduct will allow the direct monitoring of the interaction of the lesion with NER proteins tagged with the

Green Fluorescent Protein (GFP) and/or its diverse mutants by Föster Resonance Energy Transfer (FRET).

The ensuing chapter presents the synthesis of this new substrate and some preliminary data.

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Chapter 2

Synthesis of the new substrate and preliminary data

2.1. INTRODUCTION

Many aromatic-amino and -nitro compounds are known to produce C8-arylamine adducts of 2'-deoxyguanosine (Heflich et al., 1985; Heflich and Neft, 1994; Schut and Snyderwine, 1999). As explained in the previous chapter, these DNA chemical modifications can challenge the integrity of the encoded genetic information, the DNA metabolic reactions and potentially induce mutagenesis and subsequently cancer. Among these DNA adducts, 8-(N^2-acetylaminofluorene)-2'-deoxyguanosine (dG-AAF), has been used for many years as a model compound to study mutagenesis, carcinogenesis, and DNA repair (Heflich and Neft, 1994). A very important feature of this lesion is the dramatic importance of the acetyl at the N^8-position, as highlighted by the different physicochemical properties and biological effects compared to the 8-(N-aminofluorene)-2'-deoxyguanosine (dG-AF). Indeed, the presence of this acetyl causes a much more severe local distortion of DNA (O'Handley et al., 1993; Heflich and Neft, 1994) and dG-AAF is therefore a much more efficient blocking agent to replication and transcription than is dG-AF (Heflich and Neft, 1994; Shibutani et al., 1998). It is also a much better DNA substrate for repair enzymes (Gunz et al., 1996; Batty and Wood, 2000).

In the past, the standard procedure to prepare site-specifically modified oligonucleotides containing an AAF adduct at the C8 position of dG consisted in treating an oligonucleotide containing a unique potentially reactive guanine with a concentrated solution of the activated 2-(N-acetoxy-N-acetyl)aminofluorene (N-AAAF). This method was successful but limited by the number of sequences that could be modified. One group (Zhoo and Romano, 1993) later described an alternative method consisting in the incorporation of the corresponding modified nucleotide during solid-phase DNA synthesis. The preparation of this modified 2'-deoxyguanosine was also involving reaction with the activated N-AAAF. These two pathways have been intensively used and employed with different N-hydroxy-, N-acetoxy- or N-sulfoxy- aromatic amines to prepare various C8 adducts of dG (Famulok and Boche, 1989; Famulok et al., 1989; Zhoo et al., 1994; Novak and Kennedy, 1995; Kennedy et al., 1997; McClelland et al, 1999). Due to the instability of some of these activated aromatic amines, this still did not represent a general synthetic method for C8-arylamine derivatives of 2'-deoxyguanosine. Many groups have therefore been interested in trying to find a more general synthetic route to synthesize these types of substances.

Two major issues needed to be addressed to achieve this goal. First, the N^8-acetyl group of dG-AAF is really base labile and therefore known to be unstable upon the standard ammonium hydroxide (NH_4OH) oligonucleotide deprotection step commonly used after solid-phase DNA synthesis (Stöhrer et al., 1983). Therefore, protecting groups that can be removed under conditions compatible with the maintenance of the N^8-acetyl group were required.

Zhou and Romano have reported a solution to this problem using an Fmoc protecting group strategy and milder deprotection conditions that ensured the stability of the dG-AAF (Zhoo and Romano, 1993; Zhoo and Romano, 1994). A limitation of this strategy, however, is that Fmoc-protected nucleotide phosphoramidites are not commercially available. Second, the syntheses of C8- arylamine and acetylarylamine adducts of dG have traditionally been based on the modification of nucleosides and oligonucleotides with the corresponding N-hydroxy or N-acetoxy arylamine derivates. This reaction is severely limited in yield and scope (Zhoo and Romano, 1993; Schut and Snyderwine, 1999) ; in particular, a single site-specific modification cannot be introduced into oligonucleotides that contain multiple guanines, therefore restricting the sequence context in which dG-AAF can be introduced.

An alternative strategy for obtaining these adducts is a Buchwald-Hartwig coupling reaction (Louie and Hartwig, 1995; Hartwig, 1998; Wolfe et al., 1998; Wolfe and Buchwald, 2000) of protected 8-bromo-2'-deoxyguanosine (Br-dG) derivates with aromatic amines. Indeed the Buchwald-Hartwig coupling reaction has been reported for the formation of N^6 adducts of dA (Lakshman et al., 1999), N^2 adducts of dG (De Riccardis et al., 1999; Hartwood et al., 2000), C8 adducts of dA (Schoffers et al., 2001) and dG (Wang and Rizzo, 2001; Meier and Gräsl, 2002).

Previous syntheses of C8-arylamine adducts of dG have shown that protections of the hydroxyl groups of the deoxyribose and of the N^2 and O^6 positions of the base of Br-dG were required for the palladium-mediated coupling reactions. Depending on the amine to be coupled, Wang and Rizzo used either bis-BOC or STABASE protection for the N^2 position (Wang and Rizzo, 2001), while Meier and Gräsl reported that an N^2-

isobutyryl protecting group allowed the coupling of a number of simple amines (Meier and Gräsl, 2002). Although the isobutyryl group is the standard N^2 protecting group for dG in DNA synthesis, the conditions required for its removal are incompatible with the base sensitive N^8-acetyl group of dG-AAF. Despite these advances, an efficient method for the synthesis of N^8-acetyl arylamine adducts of dG and a protecting group strategy compatible with their incorporation into DNA was still missing.

A previous student of our group, Ludovic Gillet addressed successfully these issues and developed a general synthetic route that could be applied to any aromatic amines (Gillet and Schärer, 2002). The use of a transient 4,4'-dimethoxytrityl (DMTr) protecting group for the N^2 position of Br-dG derivatives allowed the efficient coupling of a wide variety of amines under Buchwald-Hartwig conditions with good yields due to its stability towards the basic conditions required to perform such cross-coupling reactions. The products of this coupling reaction were subsequently selectively acetylated at the N^8 position due to the steric hindrance of the DMTr. The subsequent replacement of this protective group with the N^2-isopropylphenoxyacetyl (iPrPac) group yielded monomers suitable for solid-phase DNA synthesis in which the integrity of the base labile N^8 acetyl group could be preserved by using the commercial "ultramild" phosphoramidites and reagents to perform the incorporation of the xenonucleotide into oligonucleotides by solid-phase DNA synthesis. Indeed these oligonucleotides could be deprotected in a very mild basic solutions without observing loss of the precious N^8-acetyl, due to the dramatic base lability of the iPrPac protective group (Schulhof et al., 1987). His approach thus provided a general strategy for the synthesis of C8-arylamine and acetylarylamine adducts of dG suitably protected for their incorporation into DNA.

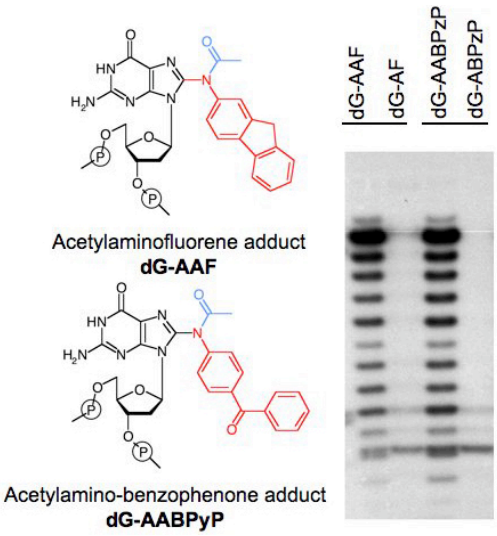

Figure 5. Autoradiogram of a denaturing PAGE analysis of the *in vitro* NER assay of pBluescript II plasmids containing either the dG-AAF or the dG-AF adduct. The 24- to 32-mer excised products of the NER reaction were indirectly detected with a "fill-in" method: the excised fragments were annealed to a complementary oligonucleotide presenting a 5'-GpGpGpG overhang, providing 3'- recessed ends that were "filled-in" with radiolabeled [α-^{32}P]dCTP in the presence of sequenase enzyme. The 24- to 32-mer oligonucleotides containing the lesion were thus extended, depending on the exact excision positions, to 28- to 36-mer in the typical pattern that can be observed by autoradiography.

Among the various synthesized adducts, Ludovic Gillet prepared a mimetic of dG-AAF, the 8-(N-acetylaminobenzophenone)-2'-deoxyguanosine adduct (dG-AABzP). This adduct was expected to be as good as a proficient NER substrate than the AAF adduct due to its structural analogy and was designed to develop a new type of photocrosslinking assays to investigate different aspects of NER. dG-AABzP was then shown by Ludovic Gillet to be recognized and repaired by NER analogous to that of dG-AAF (figure 5). Interestingly, as in the case of the AAF adduct, the deacetylated analogue of dG-AABzP (dG-ABzP) appeared not to be repaired (figure 5). Therefore dG-AABzP appears to have the same NER pattern as dG-AAF, highlighting once again the general importance of the N^8-acetyl in this kind of structure to allow recognition and repair by NER. Given that the C8-benzophenone adduct has never been found to occur

in biological organisms, it is therefore a good and pertinent NER substrate for *in vitro* assays.

2.2. AIM AND FUNDAMENTALS OF THE WORK PRESENTED

As introduced in the preview of the previous chapter, the purpose of the project presented in this manuscript was to synthesize a NER proficient substrate that would contain a functionalized linker allowing its site-specific labeling at the oligonucleotide level with a biotin-based or fluorescent probe. Such a new NER substrate would then permit its interactions within the NER machinery to be examined by applying isolation and spectroscopic techniques that could not be applied to unlabelled NER substrates.

At the beginning of this project, we chose to synthesize a new AFF adduct containing an amine linker that would allow its selective conjugation with probes functionalized with an N-hydroxysuccinimidyl ester group. This approach is extensively used in bioconjugation (Hermanson, 1996). With regards to our synthetic route, the first step of the synthesis was to functionalize 2-amino-7-bromofluorene with the protected amino-linker. Rapidly, this approach gave rise to two majors concerns. First, the choice of the protective groups for the aliphatic amine appeared to be really challenging in terms of ensuring a good yield of the Buchwald-Hartwig coupling. Many group already had issues to find a good protective group for the N^2 position of the 2'-deoxyguanosine to serve two major goals of the synthesis of the C8-arylamine adduct. The use of the 4-4'-dimethoxytrityl could certainly have led to a satisfying yield for the cross-coupling reaction but the aliphatic amine would still have been accessible for acetylation,

therefore raising the challenge of performing the selective N^8-acetylation at the next step. Secondly, 2-amino-7-bromofluorene is prohibitively expensive.

Because almost all of the commercially available probes are either functionalized with a succinimidyl ester, an hydrazide or an hydroxylamine linker, we naturally turned our efforts to the synthesis of an adduct containing a ketone linker that could later be selectively conjugated with probes functionalized either with an hydrazide or an hydroxylamine by hydrazone or oxime formation, an approach already used on modified oligonucleotides functionalized with aldehydes (Kubo et al., 1992; Ida et al., 1993; Forget et al., 2001; Olivier et al., 2002). This approach was more reasonable in term of minimizing the synthetic difficulties since no extra protective group was required along our synthetic route, therefore minimizing protection issues. Finally, due to the low cost of preparation of 4-bromo-4'-nitrobenzophenone and the previous data from Ludovic Gillet showing that dG-AABzP is as a good proficient NER substrate as dG-AAF, we decided to base our new substrate on the skeleton of the benzophenone.

Here then, we disclose the synthesis of a 8-(N-acetyl-4-amino-4'-(3-oxobutyl)-benzophenone)-2'-deoxyguanosine and its incorporation into oligonucleotides by solid support DNA synthesis leading to site-specifically modified oligonucleotides (figure 10, compounds 10). We also demonstrate that this susbtrate is NER proficient and disclose the efficiency of its conjugation with hydroxylamine species.

2.3. RESULTS AND DISCUSSION

2.3.1. Synthesis of the xenonucleotide phosphoramidite

As described by Ludovic Gillet (Gillet and Schärer, 2002), our synthesis started with the bromination of dG with N-bromosuccinimide and precipitation of the product in acetone. This was followed by silylation of the 5'- and 3'-hydroxyl groups, protection of the O^6 position as the benzyl ether using standard methods, and finally protection of the exocyclic amine with the 4-4'-dimethoxytrityl group (figure 6) to obtain the fully protected 2'-deoxyguanosine (compound 1). All products were obtained in good yield on a multigram scale, using chromatography on mildly basic aluminium oxide to purify the N^2-dimethoxytritylated final product to prevent loss of this acid-labile group on silica gel.

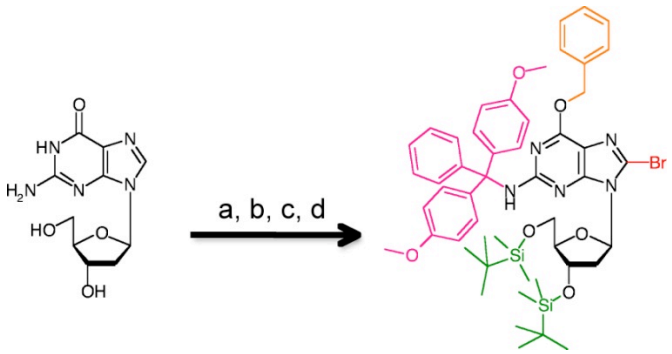

Figure 6. Preparation of the fully protected Br-dG. Reaction conditions: (**a**) NBS, water, acetonitrile (80%); (**b**) tBDMS-Cl, imidazole , DMF (98%); (**c**) Bn-OH, PPh3, DIAD, dioxane (78%); (**d**) DMTr-Cl, pyridine (94%).

The arylamine required in the coupling step with the fully protected guanosine was synthesized in three steps by simple and well known reactions (compound 4, figure 7). Friedel-Craft coupling (Friedel and Crafts, 1877) between 4-nitrobenzoyl chloride and bromobenzene leads to the 4-bromo-4'-nitrobenzophenone (compound 2, figure 7) which was subsequently coupled to Methyl Vinyl Ketone by a Heck reaction (Heck and

Nolley, 1972). This led to the 4-nitro-4'-(3-oxobut-1-enyl)benzophenone (compound 3, figure 7) with a 67% overall yield. Finally, co-hydrogenation of the nitro and the alkene group produced the saturated arylamine (compound 4, figure 7).

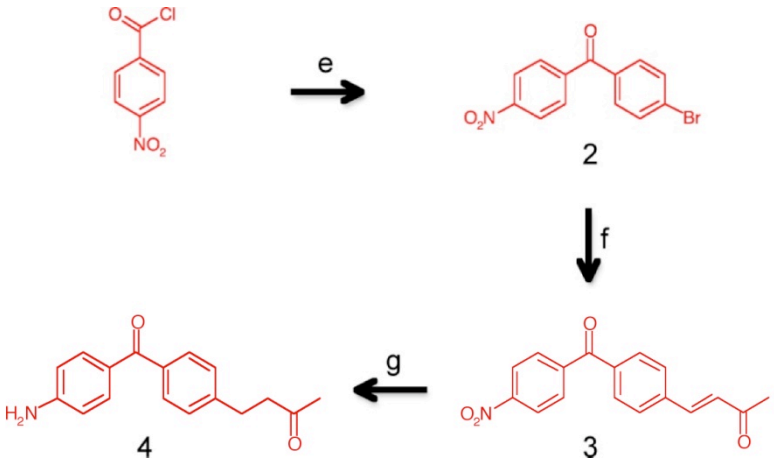

Figure 7. Preparation of the aminobenzophenone to coupled. Reaction conditions: (e) 4-NO_2-ArCOCl, ArBr, $AlCl_3$ (88%); $Pd(PPh_3)_4$,TEA, $Bu_4NH_3^+I^-$, Methyl Vinyl Ketone, DMF (76%); (g) Bn-OH, PPh_3, DIAD, dioxane (78%).

The fully protected 2'-deoxyguanosine (compound 1, figures 5 and 8) and the synthetic 4-aminobenzophenone (compound 4, figure 7 and 8) were then coupled via Buchwald-Hartwig reaction (Louie and Hartwig, 1995; Hartwig, 1998; Wolfe et al., 1998; Wolfe and Buchwald, 2000) to obtain the new 8-(N-aminobenzophenone)-2'-deoxyguanosine (compound 5, figure 8). As expected, the Buchwald-Hartwig coupling reaction gives a poor but reasonable yield due to the poor electron density of the 4-aminobenzophenone, and potential dimerisation and/or polymerization of the aminobenzophenone by imine formation.

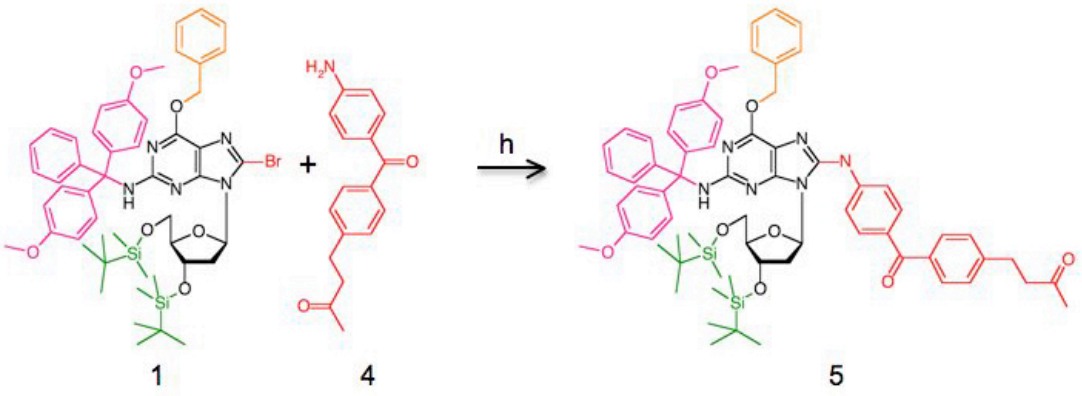

Figure 8. Buchwald-Hartwig coupling reaction. Reaction conditions: (**h**) $Pd_2(dba)_3$, BINAP, NaOtBu, toluene (59 %).

The selective N^8 acetylation was easily achieved, the bulky dimethoxytrityl group preventing further acetylation reaction at the N^2 position due to its steric hindrance. The DMT group was then smoothly removed in a one-pot reaction with a very dilute hydrochloric solution to prevent any depurination, leading to the 8-(N-acetylaminobenzophenone)-2'-deoxyguanosine (compound 6, figure 9).

Following the method of Ludovic Gillet and to satisfy the purpose of incorporating this new xenonucleotide into oligonucleotides by solid support DNA synthesis, the N^2 position was then reprotected by reaction with isopropylphenoxyacetyl chloride, a protective group that has been shown to be cleavable under very mild conditions without compromising the integrity of the baso-labile N^8-acetyl group (Gillet et al., 2005).

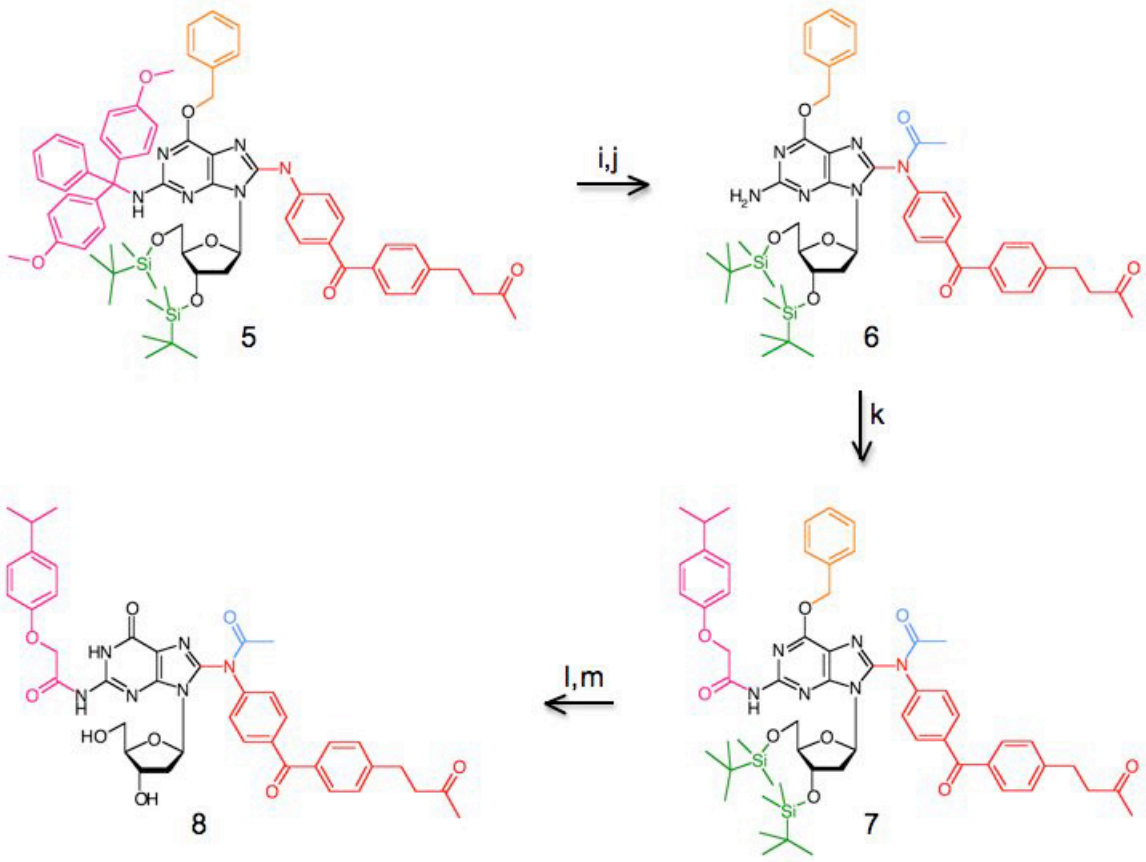

Figure 9. Selective acetylation and deprotection of the dG-BzP. Reaction conditions: (**i**) AcOAc, TEA, DMAP, pyridine; (**j**) HCl 0.001 N, MeOH (76% for **i**-**j**); (**k**) iPrPac-Cl, pyridine (84%); (**l**) CH_3COOH, TBAF, THF; (**m**) H_2, Pd/C (70% for **l**-**m**).

The nucleotide obtained (compound 7, figure 9) was then deprotected to generate the targeted xenonucleotide (compound 8, figure 9) ready for incorporation into oligonucleotides. The benzyl group was removed by hydrogenolysis and the silyl protective groups were removed by TBAF buffered with acetic acid to preserve the base-sensitive N^8-acetyl group.

The diol was then functionalized in readiness for its use in solid-support DNA synthesis by conventional method. The 5'-hydroxyl is protected as its 4,4'-dimethoxytrityl ether derivative (compound 9, figure 10). Then the 3'-hydroxyl was allowed to react with the appropriate phosphitylating agent to generate the final

nucleotide phosphoramidite to be used for incorporation into oligonucleotides (compound 10, figure 10).

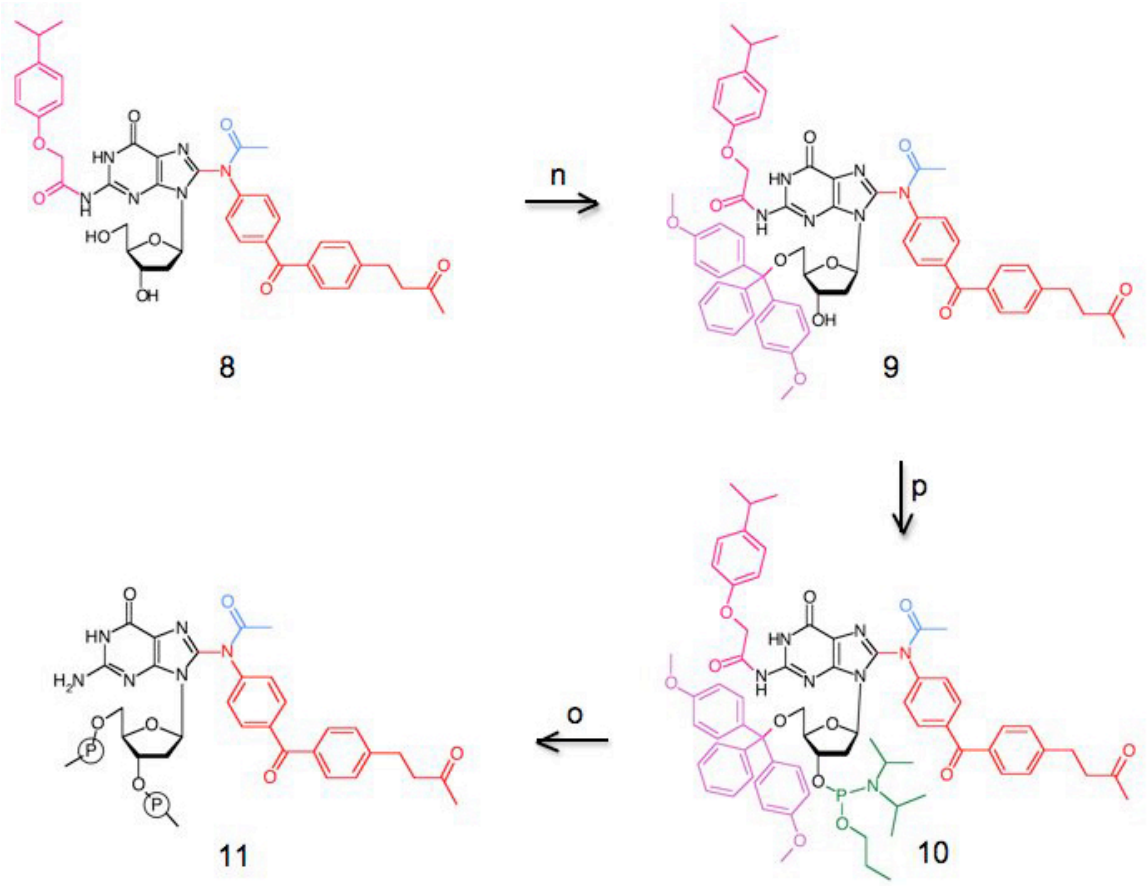

Figure 10. Preparation of nucleotide for solid support DNA synthesis. Reaction conditions: (**n**) DMTr-Cl, pyridine (76%); (**o**) phosphitylating agent, DIPA, DCM (81%); (**p**) solid support DNA synthesis.

2.3.2. Incorporation into oligonucleotide by solid-support DNA synthesis

Due to the enhanced sensitivity to depurination of the C8-arylamine-2'-deoxyguanosines and the base-sensitivity of the N^8-acetyl group, the new xenonucleotide was incorporated into oligonucleotide by the mild solid support DNA synthesis methods and the 'ultra mild' deprotection conditions previously established by Ludovic Gillet.

Typically, for such oligonucleotide synthesis, our modified 2'-deoxyguanosine phosphoramidite (compound 10, figure 10) was dissolved to 0.1 M in dichloromethane, which provides improved stability and coupling efficiency over the acetonitrile that is normally used (Gillet et al., 2005). The activator 5-(ethylthio)1*H*--tetrazole was used rather than tetrazole to avoid precipitation and to improve the coupling yield.

Since the final deprotection of the oligonucleotide obtained at the end of the DNA synthesis cycle must be deprotected under mild conditions to avoid loss of the precious N^8-acetyl group of the adduct to maintain a good NER proficiency, the non-modified purine bases used during the synthesis were the commercially available N^2-(isopropylphenoxyacetyl)-2'-deoxyguanosine (iPrPac-dG) phosphoramidite and N^2-(isopropylphenoxyacetyl)-2'-deoxyadenosine (Pac-dA) phosphoramidites. As a capping agent, phenoxyacetic anhydride was used rather than acetic anhydride to avoid transamidation reactions on the Pac/iPrPac protected purine (Zhou et al., 2001).

To avoid any depurination during the DNA synthesis procedure, 3% dichloroacetic acid in methanol was used as the deblocking solution instead of the commonly used 3% tricloroacetic acid solution.

Finally, the synthetic oligonucleotides were deprotected with 10% isopropylamine in methanol at 50°C. Despite being known to allow the cleavage of all the exocyclic protective groups present in synthetic oligonucleotides without inducing loss of the N^8-acetyl of the N^8-acetylaminoaryl adducts of the 2'-deoxyguanosine (Gillet et al., 2005), these conditions were not suitable for an efficient cleavage of the oligonucleotides from the commonly used solid support containing a succinate linker. To avoid therefore having only a marginal recovery of the synthesized oligonucleotides during their

cleavage at the deprotection stage and to maximize the yield, we used the more labile hydroquinone-based “Q-support” (Pon and Yu, 1997).

	Modified "Ultra-mild" protocol
Solid support	hydroquinone based ("Q-column")
Phosphoramidites	dT, Ac-dC, Pac-dA, iPrPac-dG (0.1 M in CH_3CN)
Modification	iPrPac-dG-AAF (0.1 M in CH_2Cl_2) • coupling time extended to 12 min
Activator	5-(ethylthio)-1H-tetrazole (0.25 M in CH_3CN)
Oxidizer*	I_2 (0.1 M in THF/pyridine/H_2O)
Capping A	iPrPac anhydride (0.5 M in THF) • capping time extended to 6 s • delivered double volume of solution
Capping B*	1-methylimidazole (2 M in THF)
Deblock	DCA (3% v/v) in CH_2Cl_2
Deprotection	iPr_2NH (10% v/v) in MeOH • 7 to 8 treatment at 55ºC

Table 1. Protocol for the modified "ultra-mild" DNA synthesis. *: Note that these solutions are regular reagents for standard solid-phase DNA synthesis and are repeated here for the record.

All the conditions used to introduce our modification into oligonucleotides are summarized in table 1.

We synthesized oligonucleotides containing 9 and 24 nucleotides in lengths having a site specific C8-(N-acetyl-4-amino-4’-(3-oxobutyl)-benzophenone adduct designed to be complementary to the (+) strand of pBluescript II SK. Our xenonucleotide phosphoramidite coupled as efficiently as an unmodified nucleotide phosphoramidite during the DNA synthesis cycle (figure 11).

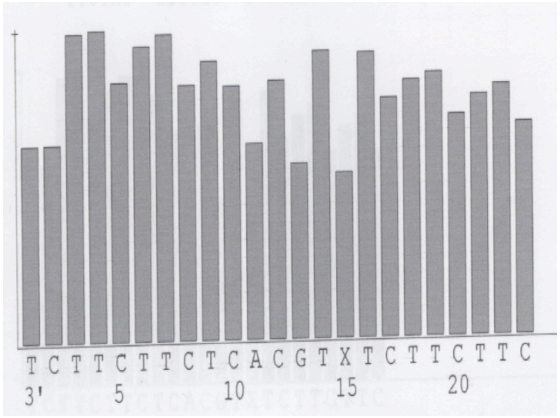

Figure 11: Trityl viewer of the DNA synthesizer. The bar standing for X shows the efficiency of the coupling of the xenonucleotide during the extension of the oligonucleotide being synthesized.

The HPLC trace revealed the homogeneity and the purity of the synthesized modified oligonucleotides. Nano-ESI analyses confirmed their identity and purity (table 2).

Oligonucleotide	calculated mass	measured
9-BzP	3046	3040
24-AABzP	7484	7482
24-AABzP-biotin	7797	7797

Table 2. Mass spectroscopic analyses of the synthesized oligonucleotides. The actual spectra are provided in the supplementary materials.

2.3.3. Test of the NER proficiency

To determine if our new adducted oligomers were efficient NER substrate, we performed the so-called NER assay. This assay consists in mixing a plasmid containing a single lesion with NER proficient extract. If the lesion is recognized and repaired by NER, the excision reaction releases a pool of short oligonucleotides from 24 to 32 nucleotides in length containing the lesion (de Laat et al., 1999; Petty and Sancar, 1999; Batty and Wood, 2000). With the proper labeling, these oligonucleotides can be resolved and detected by gel electrophoresis (Shivji et al., 1999).

We therefore incorporated our new 24-mer oligonucleotide containing the 4'-(3-oxobutyl)benzophenone adduct into a plasmid by annealing with single-stranded pBluescript II SK(+), followed by primer extension by T4 DNA polymerase, then ligation with T4 DNA ligase and finally purification over a cesium chloride/ethidium bromide density gradient as described by Shivji (Shivji et al., 1999). This provided the corresponding double-stranded plasmid containing the site-specific adduct.

The plasmid containing the lesion was then incubated with HeLa whole cell extract. The excised products of the NER reaction were then annealed to a complementary oligonucleotide having a 5'-d(GpGpGpG) overhang, thus providing 3'-recessed ends that were "filled-in" with radiolabeled [α-^{32}P]dCTP in the presence of sequenase. The reaction was then analyzed on a 14% denaturing polyacrylamine sequencing gel, using the very proficient NER *cis*-platinum adduct (*cis*-Pt) for comparison.

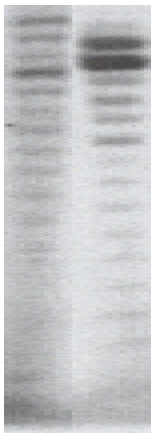

Figure12. Autoradiogram of a denaturing PAGE analysis of the *in vitro* NER assay of pBluescript SK+ plasmids containing either the dG-AABzP (**left band**) or the *cis*-Pt adduct (**right band**). The 24- to 32-mer excised products of the NER reaction were indirectly detected with a "fill-in" method: the excised fragments were annealed to a complementary oligonucleotide presenting a 5'-GpGpGpG overhang, providing 3'- recessed ends that were "filled-in" with radiolabeled [α-^{32}P]dCTP in the presence of sequenase enzyme. The resolved characteristic bands of NER are detected by radiography.

The bands on the gel in figure 12 show clearly that our new substrate is well recognized and repaired by NER and is therefore a proficient NER substrate.

2.3.4. Conjugation with a hydroxylamine probe at the oligonucleotide level

Because the main purpose of synthesizing this new adduct was to be able to prepare oligonucleotides containing a NER proficient DNA lesion that would allow conjugation with hydrazide- or hydroxylamine-functionalized probes, we tested the actual possibility to accomplish such a site-specific labeling at the lesion site.

The AABzP-containing 24-mer was then successfully conjugated with biotinylated hydroxylamine ARP in a slightly acidic phosphate buffer solution (pH 6.8) to facilitate the reaction. No depurination was observed.

HPLC analysis of the product revealed the homogeneity and the purity of the biotinylated 24-mer-AABzP. Nano-ESI analyses confirmed its identity and purity (table 2), demonstrating the feasibility of our approach and its high efficiency.

2.4. CONCLUDING REMARKS

We have synthesized a new 8-(N-acetylarylamine)-2'-deoxyguanosine that can easily be incorporated into oligonucleotides and plasmids. This adduct has been demonstrated to be an efficient substrate in NER. The presence of the ketone group in the adduct allows its functionalization at the oligonucleotide level with hydroxylamine-functionalized probes.

We have extended the synthesis of the C8-modified 2'-deoxyguanosine to the point where not only can we prepare oligonucleotides containing a site-specific NER proficient substrate but it is possible also to perform interesting site-specific labeling at this DNA lesion. This is an exciting development and should allow more precise investigations into mechanisms of NER, an area where further elucidation of lesion processing will yield interesting discoveries in this life-preserving vital process.

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Chapter 3

Experimental procedures

3.1. SYNTHESIS OF THE NEW XENONUCLEOTIDE

All the reagents, including the solvents, were purchased from VWR (United States). Exception for tris(dibenzylideneacetone)-dipalladium, rac-2,2'-Bis(diphenylphosphino)-1,1'-binaphtyl, dimethylformamide dimethyl acetal and 10% palladium on charcoal which were obtained from SigmaAldrich (United States), whereas 2'-deoxyguanosine was supplied by SynGen (United States).

NMR spectra were recorded on a 400 MHz Varian Inc. NMR instrument. ESI-MS and HR-MS were recorded on a Thermo Fisher Scientific TSQ Quantum Access spectrometer.

The "ultra-mild" phosphoramidites were available from Glen Research and the 1000 Å "Q-columns" from Biosearch Technologies. DNA syntheses were performed on a PerSeptive Biosystems Expedite 8900. HPLC analysis and purifications were performed on a JASCO system equipped with a semi-preparative Clarity column from Phenomenex. The C18-SepPak cartridges were from Micropore. Snake venom phosphodiesterase I (SVPD) was purchased from Worthington Biochemical, and calf intestine phosphatase (CIP) from New England Biolabs. The sequenase enzyme (T7

DNA polymerase) was purchased from USB and the radiolabeled [α-^{32}P]dCTP was obtained from Amersham.

4-Bromo-4'-nitrobenzophenone (2)

To a suspension of 4-nitrobenzoyl chloride (10 g, 53.89 mmol) in bromobenzene (20 mL) was slowly added a finely divided powder of anhydrous aluminium chloride (10 g, 75 mmol) at room temperature. The reaction mixture was heated under reflux for 5 hours, then cooled to 0°C and quenched with 1 M hydrochloric acid. The organic phase was then separated and washed with saturated sodium bicarbonate, water and then brine. The desired product was recovered by precipitation with hexane to provide 15 g (49.03 mmol, 88%) of a fine white powder (2). This was used for the next step without further purification.

Rf = 0.43 (toluene)

^{1}H NMR (DMSO-d_6) δ **(ppm) 8.33** (d, 2H, BzP-H), **7.95** (d, 2H, BzP-H), **7.80** (d, 2H, BzP-H), **7.70** (d, 2H, BzP-H)

^{13}C (DMSO-d_6) δ 193.6, 149.5, 142.0, 135.0, 131.8, 131.7, 130.7, 127.6, 123.6

HR m/z calculated for $C_{13}H_8BrNO_3+H^+$ 305.9766, found 306.9850

4-Nitro-4'-(3-oxobut-1-enyl)benzophenone (3)

4-Bromo-4'-nitrobenzophenone (7 g, 22.87 mmol), methyl vinyl ketone (15 mL, 184.9 mmol), tetrakis(triphenylphospine)palladium (4 g, 3.4 mmol) and tetrabutylammonium iodide (9.3 g, 25.16 mmol) were mixed together in dimethylformamide (15 mL) and the reaction mixture was stirred at 100°C for 48 hours, until no starting material could be

detected by TLC. The reaction mixture was then concentrated by distillation under vaccum, resolubilized in ethyl acetate and the solution extracted with water and brine. The organic phase was concentrated and purified by chromatography on silica gel (hexanes:EtOAc 20:1) to give 5.46 g (18.50 mmol, 76%) of the product (3) as a white powder.

Rf = 0.45 (hexanes:EtOAc 4:1)

^{1}H NMR (DMSO-d_6) δ **(ppm) 8.36** (d, 2H, BzP-H), **7.95** (d, 2H, BzP-H), **7.90** (d, 2H, BzP-H), **7.80** (d, 2H, BzP-H), **7.70** (d, 1H, BzP-CH=C-CO-C), **6.94** (d, 1H, BzP-C=CH-CO-C), **2.36** (s, 2H, BzP-C=C-CO-CH3)

^{13}C (DMSO-d_6) δ 194.4, 184.8, 150.3, 142.5, 138.1, 133.6, 131.5, 130.8, 124.4, 124.3, 90.6, 88.0, 33.4

HR m/z calculated for $C_{17}H_{13}NO_4+H^+$ 296.0923, found 296.0925

4-Amino-4'-(3-oxobutyl)benzophenone (4)

To 4-Nitro-4'-(3-oxobut-1-enyl)benzophenone (5 g, 16.95 mmol) dissolved in tetrahydrofuran/ethanol 1/1 v/v was added a 10% powder of Pd/C (2.5 g). The reaction mixture was stirred at room temperature for 2 hours under a hydrogen atmosphere, until no starting material could be detected by mass spectrometry. The catalyst was then removed by filtration through Celite and the filtrate concentrated to dryness. The product was then purified by chromatography on silica gel (DCM:MeOH 10:1) to provide 3.54 g (13.22 mmol, 78%) of the desired product (4) as an orange solid.

Rf = 0.52 (DCM:MeOH 10:1)

^{1}H NMR (DMSO-d_6) δ **(ppm) 7.53** (d, 2H, BzP-H), **7.50** (d, 2H, BzP-H), **7.33** (d, 2 H, BzP-H), **6.11** (s, 2H, BzP-NH_2), **2.84** (m, 4 H, BzP-CH_2-CH_2-CO-C), **2.12** (s, 3H, BZP-C-C-CO-CH_3)

^{13}C (DMSO-d_6) δ 193.755, 184.171, 149.620, 141.834, 137.433, 132.955, 130.838, 130.186, 123.743, 123.675, 89.997, 87.356, 32.756

HR m/z calculated for $C_{17}H_{17}NO_2$+H^+ 268.1332, found 268.1324

8-(4-Amino-4'-(3-oxobutyl)benzophenone)-*O*^{6}-benzyl-3',5'-O-bis(*tert*-butyldimethylsilyl)-*N*^{2}-(4,4'-dimethoxytrityl)-2'-deoxyguanosine (5)

O^6-Benzyl-8-bromo-3'-5'-O-bis(tert-butyldimethylsilyl)-*N*^{2}-(4,4'-dimethoxytrityl)-2'-deoxyguanosine* (3 g, 2,60 mmol), 4-amino-4'-(3-oxobutyl)benzophenone (1 g, 3.04 mmol), tris(dibenzylideneacetone)-dipalladium (150 mg, 165 µmol) and rac-2,2'-Bis(diphenylphosphino)-1,1'-binaphthyl (300 mg, 480 µmol) were preheated in degassed toluene at 90°C (15 mL). After 40 min, sodium *tert*-butoxide (450 mg, 4.65 mmol) was added and the reaction mixture was stirred 1 additional hour at 100°C, until no more starting material could be detected by TLC. The reaction mixture was then cooled, diluted with diethyl ether and filtered. The filtrate was concentrated and purified by chromatography on aluminium oxide (hexanes:EtOAc 20:1) to provide 1.77 g (1.53 mmol, 59%) of the product (5) as a faintly yellow foam.

Rf = 0.51 (hexanes:EtOAc 2:1)

^{1}H NMR (DMSO-d_6) δ **(ppm) 9.33** (s, 1H, *N*^{8}-H), **7.67-7.74** (m, 6H, BzP-H), **7.63** (m, 1H, BzP-H), **7.51-7.56** (m, 2H, BzP-H+Bn-H), **7.45-7.51** (m, 2H, BzP-H+Bn-H), **7.36-7.41** (m, 2H, BzP-H+Bn-H), **7.33** (m, 1H, Bn-H), **6.33** (dd, J=7.0, 7.0, 1H, C_1'-H), **6.13** (s, 2H,

N^2-H_2), **5.50** (AB quart, J= 12.2, 2H, Bn-CH_2), **4.64** (ddd, J=2.9, 2.9, 5.8, 1H, C_3'-H), **3.80-3.86** (m, 2H, C_5'-H+C_4'-H), **3.67** (dd, J= 4, 9.5, 1H, C_5'-H), **3.51** (ddd, J=6.6, 6.6, 13.2, 1H, C_2'-H), **2.14** (ddd, J=3.3, 6.6, 13.2, 1H, C_2'-H), **0.90** (m, 9H, tBu-H), **0.81** (m, 9H, tBu-H), **0.13** (s, 6H, 2xCH_3), **-0.01** (s, 3H, CH_3), **-0.03** (s, 3H, CH_3)

^{13}C (DMSO-d_6) δ 208.0, 194.5, 158.3, 157.3, 156.4, 153.8, 146.7, 146.4, 146.3, 145.8, 138.7, 138.6, 137.2, 136.3, 131.9, 130.4, 130.2, 130.0, 129.1, 129.0, 128.9, 128.6, 128.1, 126.9, 117.3, 113.5, 112.6, 87.5, 83.8, 73.3, 70.1, 55.6, 44.2, 30.4, 29.6, 26.4, 26.3, 18.6, 18.5, -4.0, -4.2, -4.8, -4.9

HR m/z calculated for $C_{67}H_{80}N_6O_8Si_2$+H^+ 1153.5654, found 1153.5670

*O^6-benzyl-8-bromo-3'-5'-O-bis(tert-butyldimethylsilyl)-N^2-(4,4'-dimethoxytrityl)-2'-deoxy-guanosine (**1**) was synthesized according to a published procedure developed previously in our laboratory (Gillet, L. and Schärer, O., 2002).

8-(N-Acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-O^6-benzyl-3',5'-O-bis(*tert*-butyldimethylsilyl)-2'-deoxyguanosine (6)

To a solution of 8-(4-amino-4'-(3-oxobutyl)benzophenone)-O^6-benzyl-3',5'-O-bis(*tert*-butyldimethylsilyl)-N^2-(4,4-dimethoxytrityl)-2'-deoxyguanosine (1.6 g, 1.31 mmol) in pyridine (25 mL) were added triethylamine (900 µL, 6.55 mmol), 4-dimethylaminopyridine (100 mg, 0.80 mmol) and acetic anhydride (750 µL, 8 mmol). After stirring for 5 hours at room temperature, the mixture was evaporated, diluted in ethyl acetate and extracted with water. The organic layer was dried over anhydrous magnesium sulfate, evaporated to dryness and treated with a 0.001 M solution of hydrochloric acid in methanol (20 mL). The mixture was stirred overnight at room

temperature, purified by chromatography on silica gel (hexanes:EtOAc 10:1) to provide 1 g (1.22 mmol, 76%) of the product (6) as slightly yellow foam.

Rf = 0.21 (hexanes:EtOAc 2:1)

^{1}H NMR (DMSO-d_6) δ **(ppm) 7.60-7.80** (m, 6H, BzP-H_6), **7.44-7.59** (m, 4H, Bn-H_2+BzP-H_2), **7.30-7.58** (m, 3H, Bn-H_1+BzP-H_2), **6.48** (s, 2H, N^2-H_2), **5.92** (m, 1H, $C_{1'}$-H), **5.49** (AB quart, J=12.6, 2H, Bn-CH_2), **4.64** (m, 1H, $C_{3'}$-H), **3.70-3.81** (m, 2H, $C_{5'}$-H+$C_{4'}$-H), **3.65** (m, 1H, $C_{5'}$-H), **3.23** (m, 1H, $C_{2'}$-H), **2.73-2.88** (m, 4H, BzP-CH_2-CH_2-CO-C), **2.07** (s, 3H, N^8-Ac), **1.90** (m, 1H, $C_{2'}$-H), **1.72** (s, 3H, BzP-C-C-CO-CH_3), **0.85** (s, 9H, tBu-H), **0.75** (s, 9H, tBu-H), **0.02-0.11** (m, 6H, 2×CH_3), **0.08** (br s, 6H, 2xCH_3)

^{13}C (DMSO-d_6) 208.0, 194.5, 171.4, 156.4, 153.8, 146.7, 146.4, 146.3, 145.8, 138.7, 138.6, 131.9, 130.4, 128.9, 128.8, 128.5, 128.1, 126.9, 117.3, 113.5, 87.5, 83.8, 73.3, 767..1, 63.3, 44.2, 30.4, 29.6, 26.4, 26.3, 18.6, 18.5, -4.0 -4.2, -4.8

HR m/z calculated for $C_{48}H_{64}N_6O_7Si_2$+H^+ 893.4453, found 893.4451

8-(N-Acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-*O*^{6}-benzyl-3',5'-O-bis(*tert*-butyldimethylsilyl)-*N*^{2}-isopropylphenoxyacetyl-2'-deoxyguanosine (7)

To a solution of 8-(N-acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-*O*^{6}-benzyl-3',5'-O-bis(*tert*-butyldimethylsilyl)-2'-deoxyguanosine (900 mg, 1 mmol) in pyridine (25 mL) was added isopropylphenoxyacetyl chloride** (850 mg, 4 mmol) and the reaction mixture was stirred for 1 hour, until no starting material could be detected by TLC. The reaction mixture was then concentrated , diluted in ethyl acetate and extracted with water. The organic layer was dried over anhydrous magnesium sulfate, concentrated and purified

by chromatography over silica gel (hexane:EtOAc 20:1) to provide 898 mg (0.84 mmol, 84%) of the product (7) as a pale yellow foam.

Rf = 0.21 (hexane:EtOAc 2:1)

^{1}H NMR (DMSO-d_6) δ **(ppm) 10.60** (s, 1H, N^2-H), **7.62-7.80** (m, 6H, BzP-H_6), **7.45-7.83** (m, 4H, Bn-H_2+BzP-H_2), **7.30-7.44** (m, 3H, Bn-H_3), **7.12** (d, J=7.8, 2H, Pac-H_2), **6.86** (d, J=7.8, 2H, Pac-H_2), **6.19** (m, 1H, $C_{1'}$-H), **5.63** (AB quart, J=12.5, 2H, Bn-CH_2), **4.79-4.99** (m, 3H, Pac-CH_2+$C_{3'}$-H), **3.65-3.83** (m, 3H, $C_{4'}$-H+$C_{5'}$-H_2), **3.24** (m, 1H, $C_{2'}$-H), **3.00-3.15** (m, 4H, BzP-CH_2-CH_2-CO-C), **2.80** (sept, J=6.9, 1H, iPr-H), **2.12** (s, 3H, N^8-Ac), **2.06** (m, 1H, $C_{2'}$-H), 1.82 **1.16** (s, 3H, iPr-CH_3), **1.14** (s, 3H, iPr-CH_3), **0.81** (br s, 9H, tBu-H), **0.71** (br s, 9H, tBu-H), **0.01** (m, 6H, 2×CH_3), **-0.14** (br s, 6H, 2xCH_3)

^{13}C (DMSO-d_6) δ 207.3, 193.8, 171.4, 159.7, 153.0, 147.3, 146.0, 145.7, 145.6, 138.9, 138.6, 135.6, 131.2, 129.7, 128.2, 128.0, 127.8, 127.4, 126..2, 112.8, 111.9, 86.8, 83.1, 72.6, 69.4, 66.4, 62.6, 43.5, 29.7, 28.9, 24.6, 17.9, 17.8, -4.9, -5.5, -5.6

HR m/z calculated for $C_{59}H_{76}N_6O_9Si_2$+H^+ 1069.5291, found 1069.5294

**Isopropylphenoxyacetic acid was obtained from isopropylphenol according to a published procedure (Sarges, R. et al. 1996) and allowed to react with 1.2 equivalents of $SOCl_2$. After distillation of the product (BP=104°C at 4mm Hg), isopropylphenoxyacetyl chloride was obtained in 75% yield.

8-(N-Acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-N^2-isopropylphenoxyacetyl-2'-deoxyguanosine (8)

To a solution of 8-(N-acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-O^6-benzyl-3',5'-O-bis(*tert*-butyldimethylsilyl)-N^2-isopropylphenoxyacetyl-2'-deoxyguanosine (800 mg, 0.75

mmol) in tetrahydrofuran/ethanol 1/1 v/v (100mL) was added a 10% Pd/C catalyst (100 mg). The reaction mixture was stirred at room temperature for 2 hours under a hydrogen atmosphere, until no starting material could be detected by mass spectrometry. The catalyst was then filtered off under Celite and the filtrate concentrated to dryness.

A 1M solution of tetrabutylammonium fluoride in tetrahydrofurane (2.2 mL, 2.1 mmol) and acetic acid (250 µL, 4.37 mmol) were added to the crude product redissolved in tetrahydrofuran. The reaction was allowed to reach room temperature then stirred overnight. After concentration, the residue was purified by chromatography over silica gel (DCM:MeOH 20:1) to provide 394.4 mg (0.52 mmol, 70%) of the product (8) as a white powder.

Rf = 0.51 (DCM:MeOH 5:1)

^{1}H NMR (DMSO-d_6) δ **(ppm) 11.99** (s, 1H, N_1-H), **11.56** (s, 1H, N^2-H), **7.82** (m, 2H, BzP-H_2), **7.74** (m, 2H, BzP-H_2), **7.43-7.69** (m, 4H, BzP-H_4), **7.18** (d, J=8.5, 2H, Pac-H_2), **6.92** (d, J=8.5, 2H, Pac-H_2), **6.20** (m, 1H, $C_{1'}$-H), **5.25** (m, 1H, $C_{3'}$-OH), **4.89** (br s, 2H, Pac-CH_2), **4.73** (m, 1H, $C_{5'}$-OH), **4.49** (m, 1H, $C_{3'}$-H), **3.85** (m, 1H, $C_{4'}$-H), **3.66** (m, 1H, $C_{5'}$-H), **3.55** (m, 1H, $C_{5'}$-H), **3.09** (m, 1H, $C_{2'}$-H), **2.89-3.00** (m, 4H, BzP-CH_2-CH_2-CO-C), **2.82** (sept, J=6.9, 1H, *i*Pr-H), **2.13** (br s, 3H, N^8-Ac), **2.05** (m, 1H, $C_{2'}$-H), **1.8** (s, 1H, BzP-C-C-CO-CH_3), **1.17** (s, 3H, *i*Pr-CH_3), **1.14** (s, 3H, *i*Pr-CH_3)

^{13}C (DMSO-d_6) δ 207.3, 194.4, 171.0, 155.7, 154.5, 148.1, 147.3, 146.8, 143.0, 142.3, 141.4, 134.5, 130.7, 129.9, 128.5, 127.2, 119.3, 114.4, 88.0, 83.8, 70.714, 66.5, 61.851, 43.5, 36.7, 32.6, 29.7, 29.0, 24.0, 22.9

HR m/z calculated for $C_{40}H_{42}N_6O_9$+H^+ 751.3092, found 751.3089

8-(N-Acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-N^2-isopropylphenoxyacetyl-5'-O-(4,4-dimethoxytrityl)-2'-deoxyguanosine (9)

8-(N-Acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-N^2-isopropylphenoxyacetyl-2'-deoxyguanosine (280 mg, 0.37 mmol) was dissolved in pyridine (5 mL) with 4,4-dimethoxytrityl chloride (190 mg,0.56 mmol) and the reaction mixture was stirred for 2 hours, until no starting material could be detected by TLC. The mixture was concentrated and purified by chromatography on silica gel (CH_2Cl_2:MeOH 20:1) to yield 290 mg (0.28 mmol,74%) of the product (9) as a faintly yellow powder.

Rf = 0.61 (CH_2Cl_2:MeOH 10:2)

^{1}H NMR (DMSO-d_6) δ **(ppm) 10.64** (s, 1H, N^2-H), **7.61-7.83** (m, 7H, BzP-H_7), **7.49-7.57** (m, 4H, Bn-H_2+BzP-H_2), **7.36-7.44** (m, 3H, Bn-H_3), **7.16** (d, J=8.8, 2H, Pac-H_2), **6.88** (d, J=8.8, 2H, Pac-H_2), **6.26** (dd, J=6.5, 7.2, 1H, $C_{1'}$-H), **5.62** (m, 2H, Bn-CH_2), **5.23** (d, J=4.6, 1H, $C_{3'}$-OH), **4.98** (s, 2H, Pac-CH_2), **4.60** (m, 2H, $C_{5'}$-OH+$C_{3'}$-H), **3.81** (m, 1H, $C_{4'}$-H), **3.67** (m, 1H, $C_{5'}$-H), **3.53** (m, 1H, $C_{5'}$-H), **3.24** (m, 1H, $C_{2'}$-H), **2.82** (sept, J=6.9, 1H, *i*Pr-H), **2.10** (m, 4H, N^8-Ac+$C_{2'}$-H), **1.17** (s, 3H, *i*Pr-CH_3), **1.15** (s, 3H, *i*Pr-CH_3)

^{13}C (DMSO-d_6) δ 207.9, 194.5, 171.4, 158.2, 157.3, 156.4, 155.8, 146.4, 146.0, 145.8, 138.7, 138.6, 137.3, 136.3, 131.9, 130.4, 130.2, 130.0, 129.1, 129.0, 128.9, 128.9, 128.8, 128.5, 128.1, 126.9, 117.3, 113.5, 112.6, 87.8, 83.8, 73.3, 70.1, 67.1, 63.3, 55.6, 44.2, 30.4, 29.6, 26.4, 26.3, 18.6, 18.5

HR m/z calculated for $C_{61}H_{60}N_6O_{11}+H^+$ 1053.4398, found 1053.4394

8-(N-Acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-3'-O-(2'-cyanoethoxy-diisopropylaminophosphino)-5'-O-(4,4-dimethoxytrityl)-*N*^{2}-isopropylphenoxyacetyl-2'-deoxyguanosine (10)

8-(N-Acetyl-4-amino-4'-(3-oxobutyl)benzophenone)-*N*^{2}-isopropylphenoxyacetyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyguanosine (200 mg, 0.19 mmol) was dissolved in dichloromethane (3 mL) with added N-ethyldiisopropylamine (130 µL, 0.76 mmol) and 2-cyanoethoxydiisopropylaminochlorophosphine (85 µL, 0.38 mmol). The reaction mixture was stirred for 2 hours at room temperature, concentrated to dryness and redissolved in the minimum amount of dichloromethane. This mixture was then added dropwise to 40 mL of hexane under smooth stirring and the product was allowed to precipitate for 2 hours at -20°C. The clear supernatant was then removed and the residue was purified by chromatography on silica gel (hexane:THF) to provide 193 mg (0.15 mmol, 81%) of a white powder (10).

Rf = 0.55 (CH_2Cl_2:MeOH 10:1)

^{1}H NMR (DMSO-d_6) δ **(ppm) 11.95** (s, 1H, N_1-H), **10.50** (s, 1H, *N*^{2}-H), **7.61-7.83** (m, 7H, BzP-H_7), **7.53-7.60** (m, 2H, BzP-H_2), **7.17** (d, J=8.5, 2H, Pac-H_2), **6.90** (d, J=8.5, 2H, Pac-H_2), **6.20** (m, 1H, $C_{1'}$-H), **5.22** (d, J=5.0, 1H, $C_{3'}$-OH), **4.87** (s, 2H, Pac-CH_2), **4.67** (t, J=5.7, 1H, $C_{5'}$-OH), **4.46** (m, 1H, $C_{3'}$-H), **3.79** (m, 1H, $C_{4'}$-H), **3.62** (m, 1H, $C_{5'}$-H), **3.49** (m, 1H, $C_{5'}$-H), **3.10** (m, 1H, $C_{2'}$-H), **2.84** (sept, J=6.9, 1H, *i*Pr-H), **2.08** (m, 4H, *N*^{8}-Ac+$C_{2'}$-H), **1.18** (s, 3H, *i*Pr-CH_3), **1.16** (s, 3H, *i*Pr-CH_3);

^{31}P-NMR {^{1}H} NMR (DMSO-d_6) δ (ppm) 150.4 (s), 150.0 (s)

HR m/z calculated for $C_{70}H_{77}N_7O_{12}P+H^+$ 1253.5477, found 1253.5473

3.2. INCORPORATION INTO OLIGONUCLEOTIDES

3.2.1. Solid supported DNA synthesis

The sequences prepared, with the exception of the 9-mer, were designed to be complementary to the (+) strand of pBluescript II SK and are as follows. 9-mer: 5'-d(CGATXCAGT)-3'; 24-mer: 5'-d(GTATCGATAAXCTTGATATCGAAT)-3' where X denotes dG (unmodified) or the modified dG-BzP. All DNA syntheses were performed on a 1 μM scale, on 1000 Å "Q-columns". The "ultra-mild" phosphoramidites (T, Ac-dC, Pac-dA, iPrPac-dG) were dissolved to 0.1 M in CH_3CN. The iPrPac-dG-AA-BzP phosphoramidite (**7**) was dissolved to 0.1 M in CH_2Cl_2 and its coupling time during the DNA synthesis extended to 12 min. A 0.25 M solution of 5-(ethylthio)-1H-tetrazole in CH_3CN was used as the "activator". A 0.5 M solution of phenoxyacetyl anhydride, in THF was used as the "capping A" solution with an extended capping time of 6 seconds and double the amount of reagent delivered on the solid support in comparison to the standard 1 μM scale DNA synthesis protocol. A 3% (v/v) dichloroacetic acid solution in CH_2Cl_2 was used in the deblocking step. The terminal 5'-DMTr protective group was retained for all the syntheses ('DMTr-ON' synthesis). After completion of the synthesis, the solid support was dried and treated overnight at 55ºC with a solution containing 10% (v/v) of diisopropylamine (iPr_2NH) in MeOH. The supernatant containing the released oligonucleotide was decanted and concentrated, taken up in 1 ml of 1M triethylamonium acetate (TEAA) buffered at pH 7, passed through a 0.45 μm filter and purified by HPLC.

3.2.2. Oligonucleotides purification

All HPLC elutions were performed at a flow rate of 1 mL/min, with the following gradient: linear 5-20% B over 15 min, linear 20-75% B until 30 min, isocratic 75% B until 35 min, linear 75-5% B until 36 min, isocratic 5% B until 40 min; buffer A: 0.1 M TEAA (pH=7); buffer B: CH_3CN. The peak of the 'DMTr-ON' oligonucleotide was eluted between 20 and 22 min– was collected, concentrated and treated with an 80% acetic acid solution for 40 min at room temperature to remove the 5'-DMTr group. After concentration, the resulting oligonucleotide was redissolved in 1ml of 1M TEAA buffered at pH 7 and repurified on HPLC. The major peak that was eluted between 15 and 17 min was collected, concentrated, redissolved in 0.1 M TEAA (pH 7), desalted on a C18-SepPak cartridge and lyophilized. The lyophilizate was redissolved in 300-400 µl of milli-Q water to typically yield concentrations of 100-600 pmol/µl (µM). The solution was then neutralized with 1M HCl, directly desalted on a C18-SepPak cartridge then lyophilized.

3.3. INCORPORATION INTO PLASMID AND NER ASSAY

120 pmol of 24-AABzP oligonucleotide were 5'-phosphorylated by incubation with 20 units of T4 PNK enzyme and 2 mM of ATP for 2 h. After annealing with 30 pmol of single-stranded pBluescript II SK(+), further incubation with dNTPs, T4 DNA polymerase and T4 DNA ligase (Shivji et al., 1999) yielded to covalently closed circular DNA containing a single BzP adduct. The closed circular DNA was isolated after cesium chloride/ethidium bromide density gradient centrifugation, purified by consecutive butanol extraction and concentrated on a Centricon YM-30. The dG-AABzP containing

plasmids were divided into aliquots and stored at -80 °C. HeLa whole cell extracts were prepared as described (Shivji et al., 1999), separated into aliquots and stored at -80ºC. The *in vitro* NER assay was performed as described (Shivji et al., 1999) and the excised NER products were detected after annealing with the following complementary oligonucleotide: 5'-d(GGGGGATATCAAGCTTATCGATACCGTCGACCTCG-Pho)-3'. Subsequent fill-in reaction of [α-^{32}P]dCTP was performed in the presence of sequenase enzyme as described (Shivji et al., 1999). The reactions were analyzed on a 14% denaturing polyacrylamine sequencing gel (migrating for 2h at 50W) and exposed overnight t on a BioMax MS (Kodak) radiographic film at -80ºC.

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Shivji, M.K., Moggs, J.G., Kuraoka, I. and Wood, R.D. (1999). Dual-incision assays for nucleotide excision repair using DNA with a lesion at a specific site. Methods Mol Biol, 113**,** 373-392.

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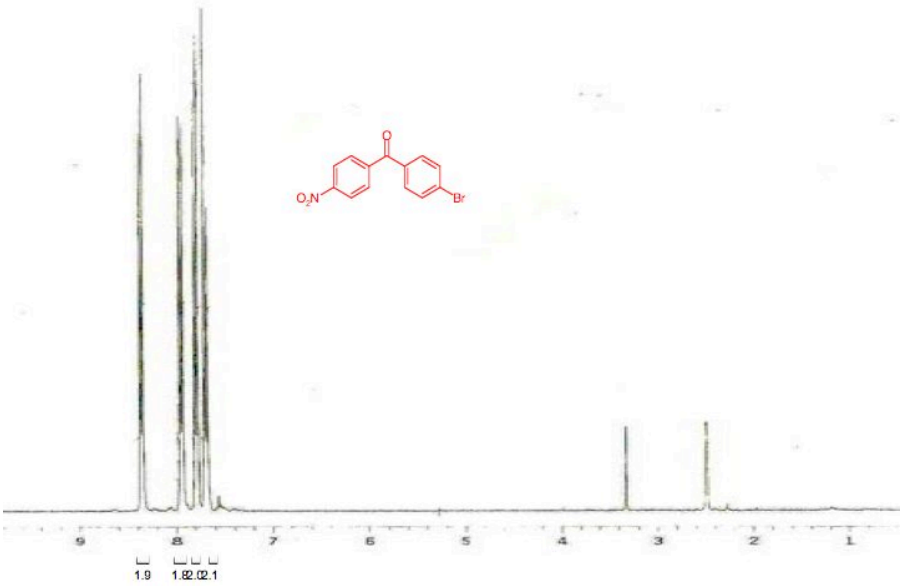

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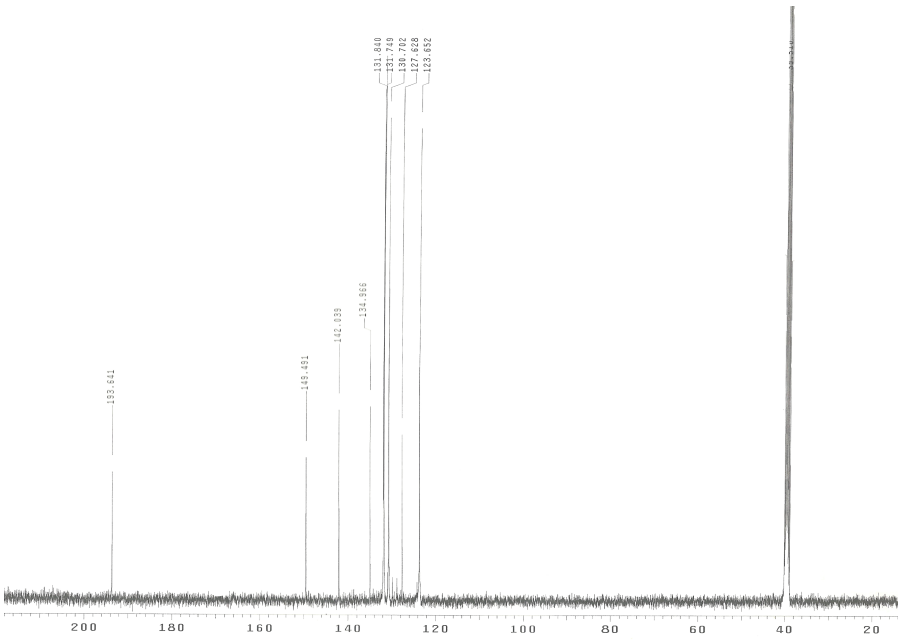

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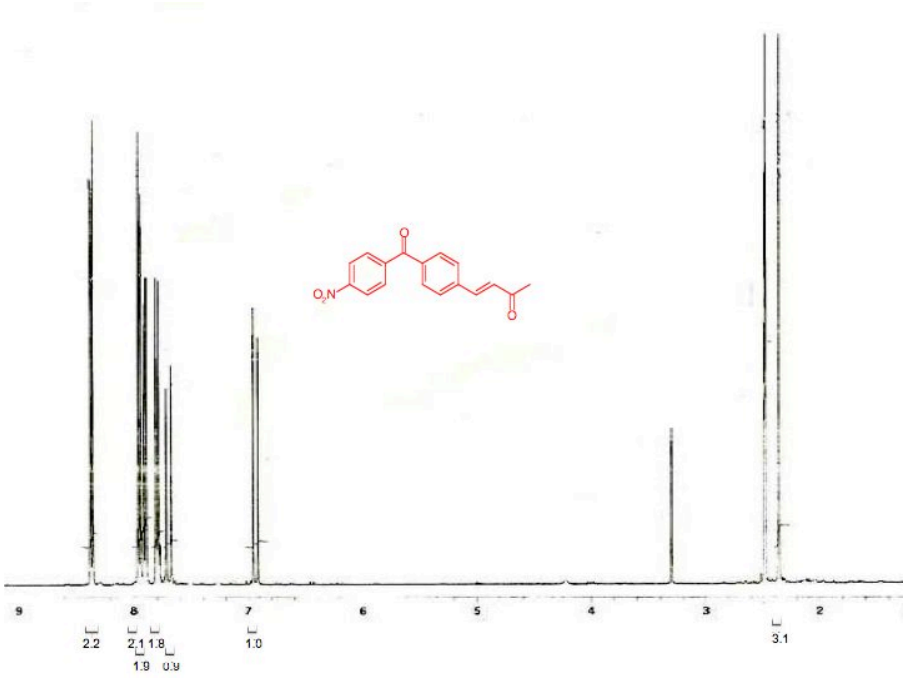

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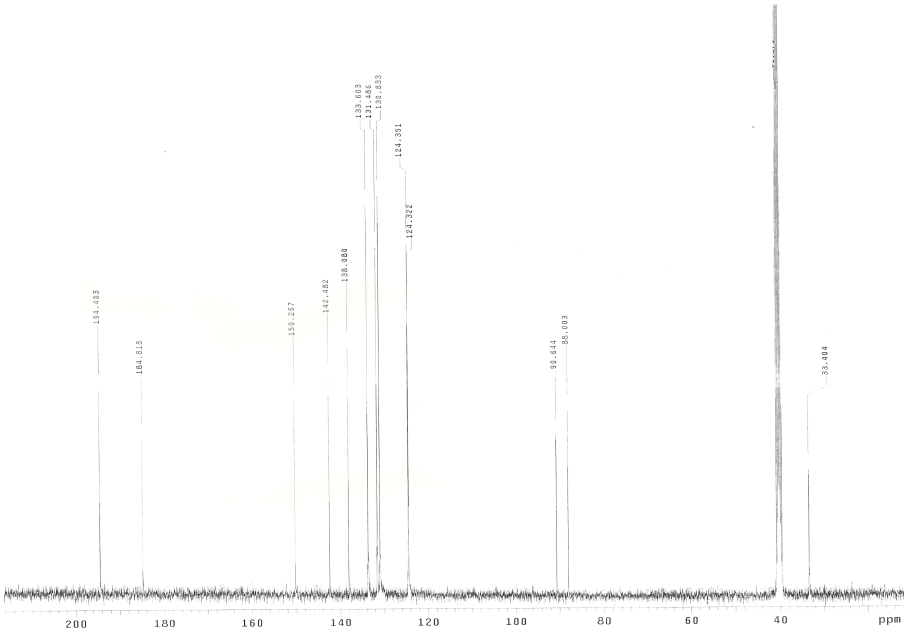

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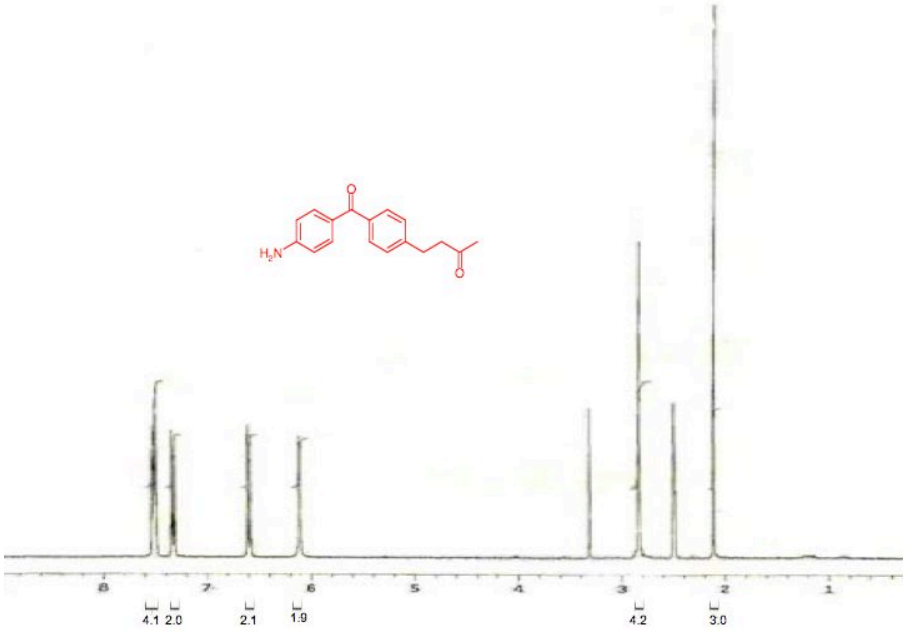

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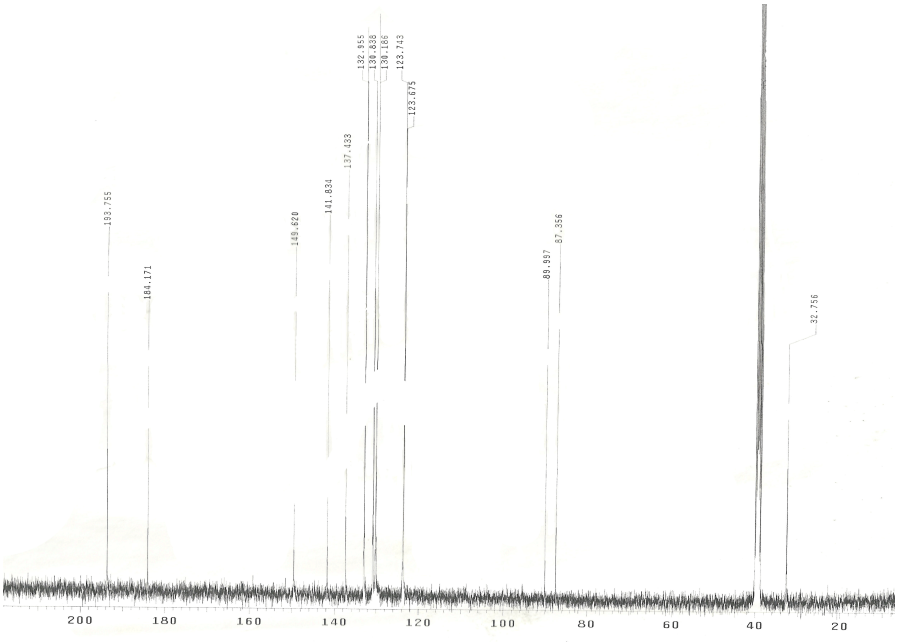

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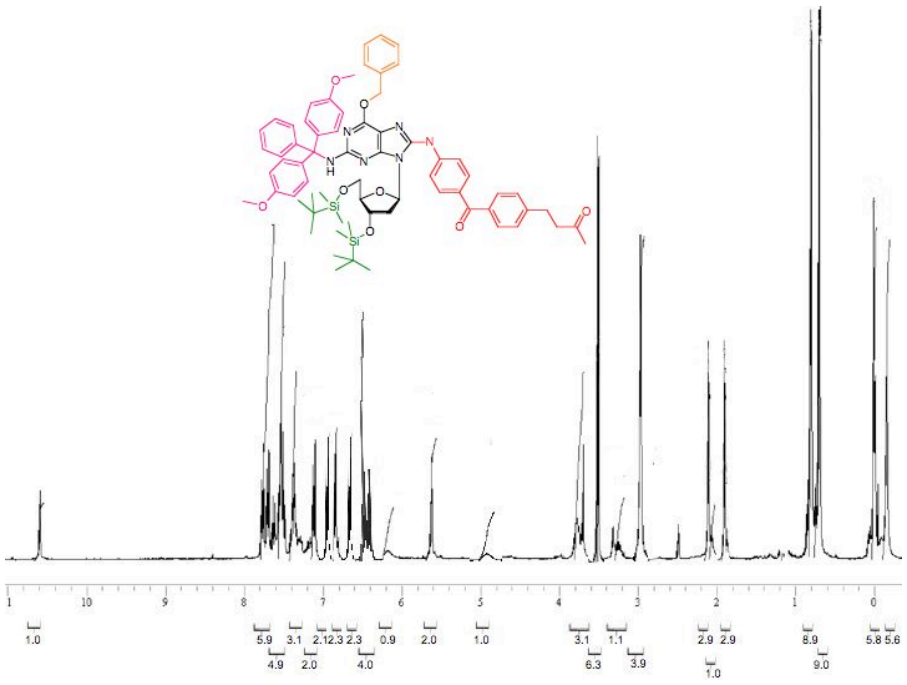

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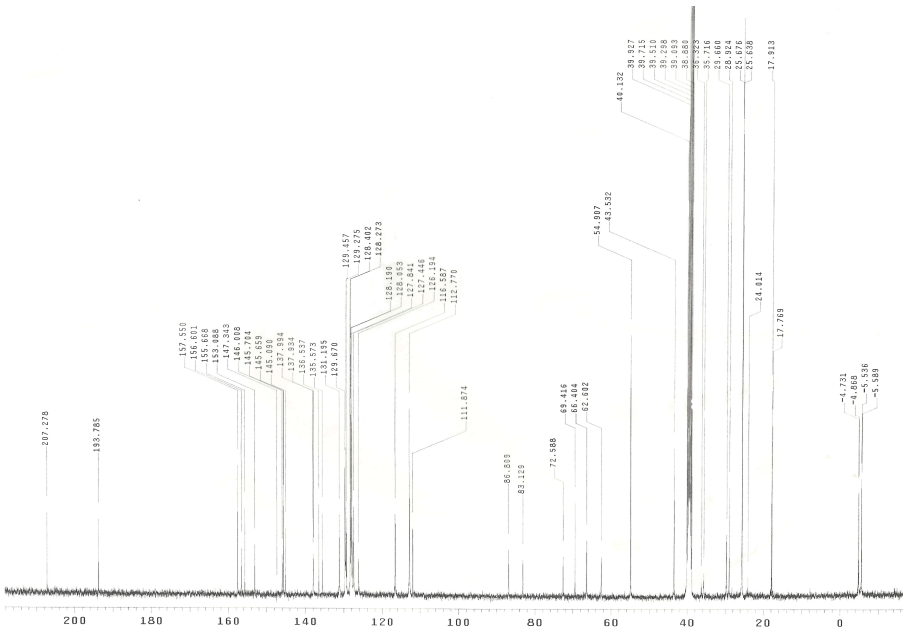

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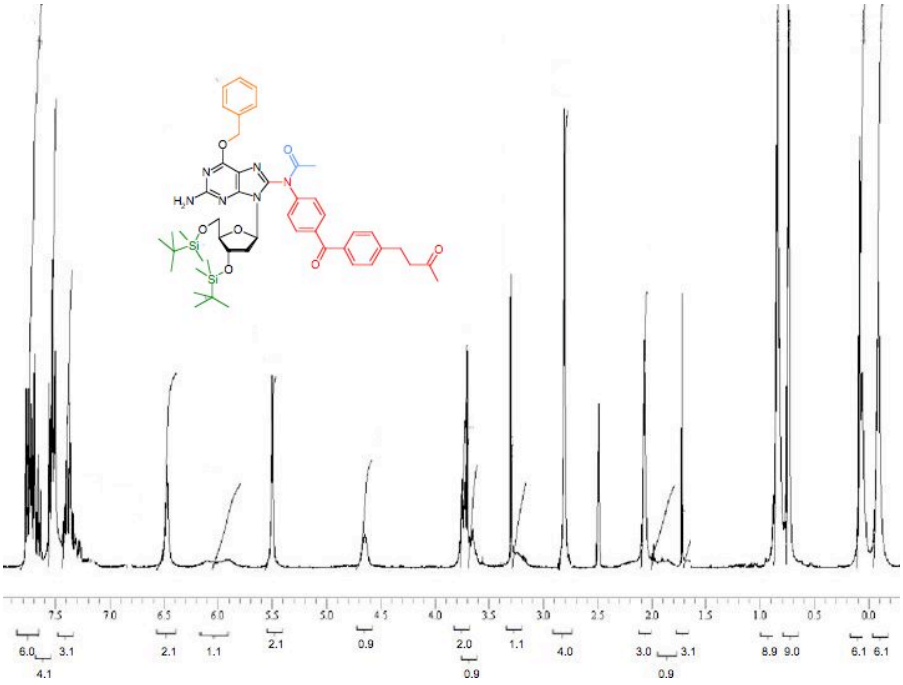

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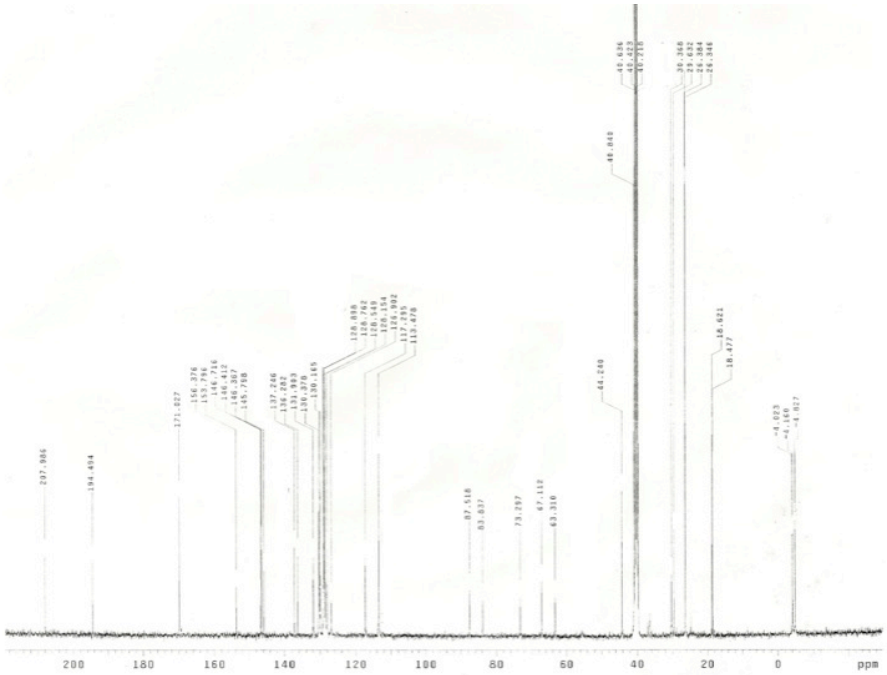

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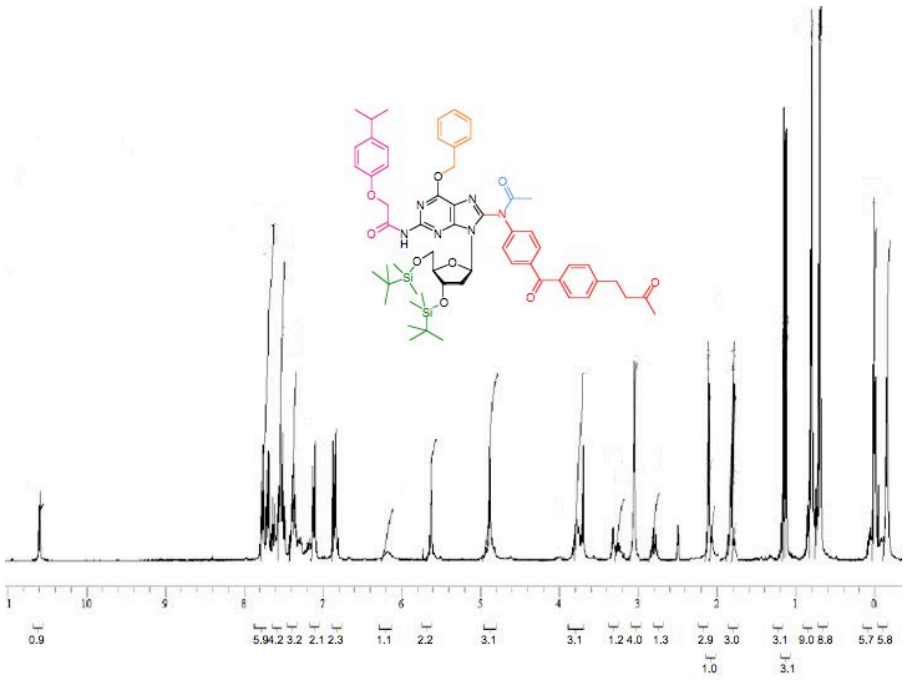

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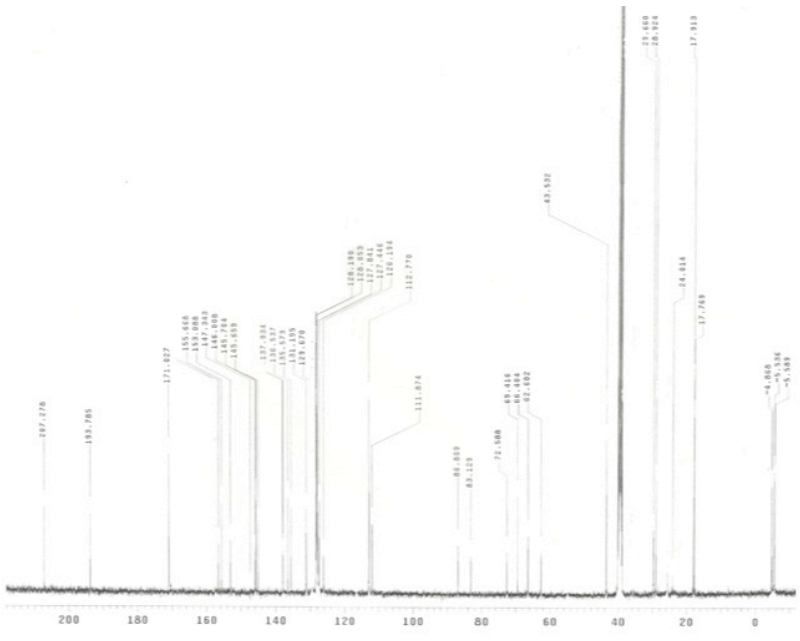

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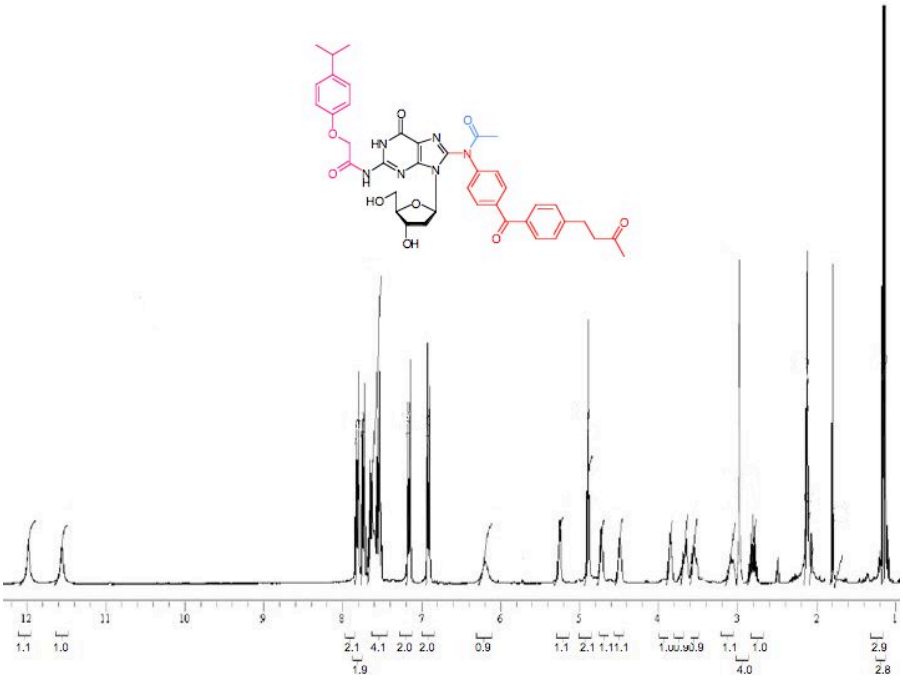

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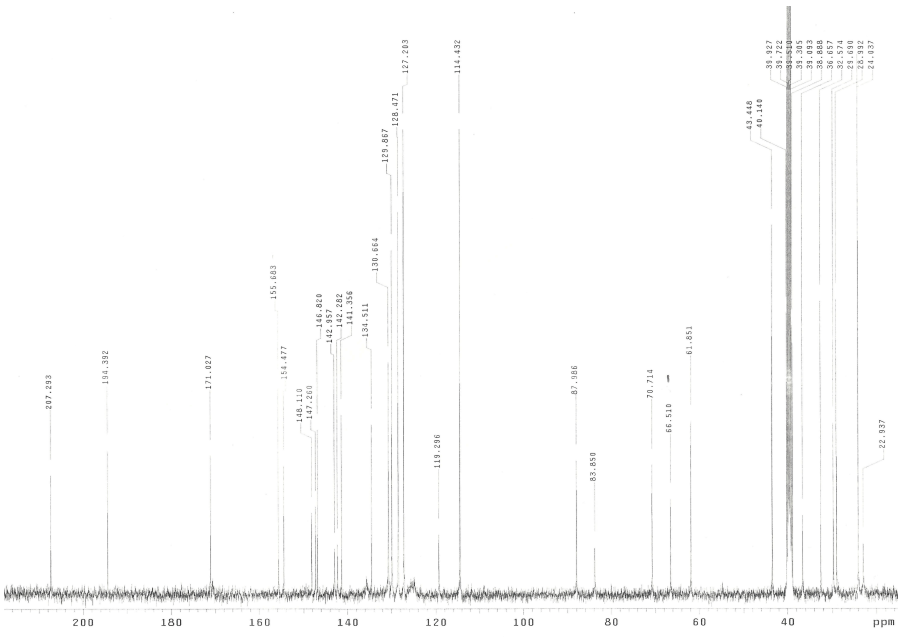

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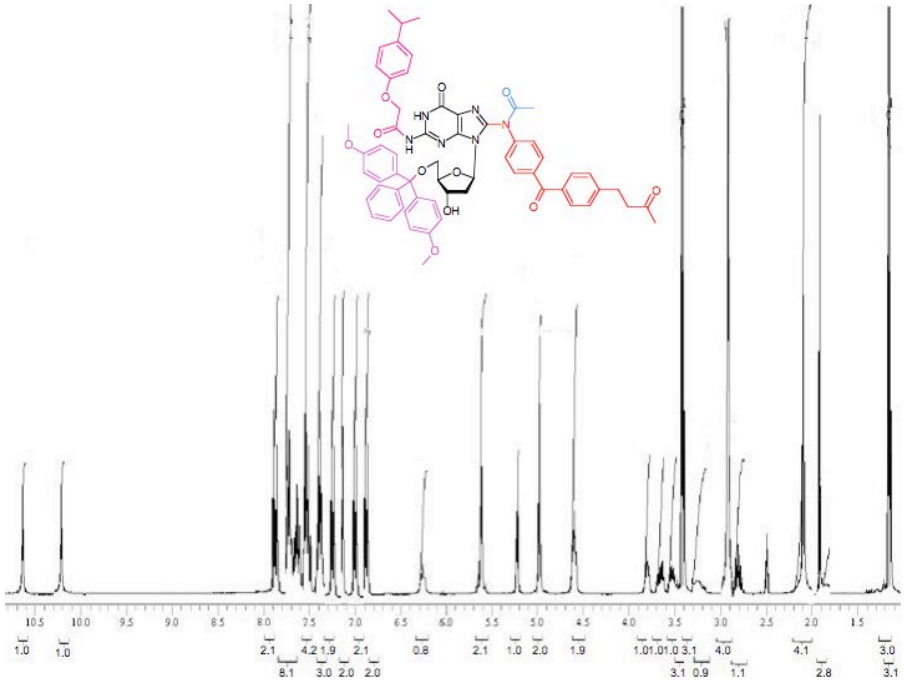

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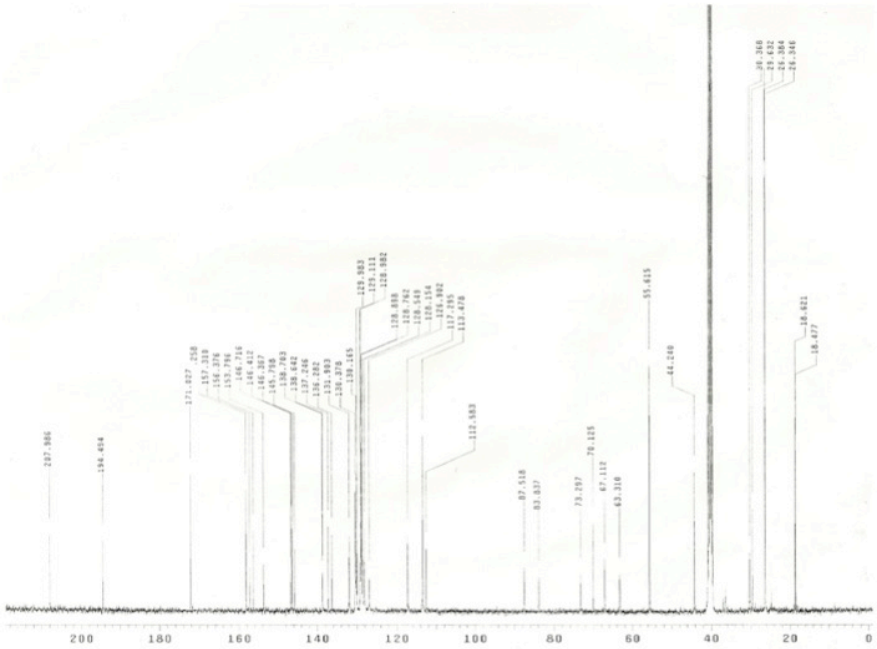

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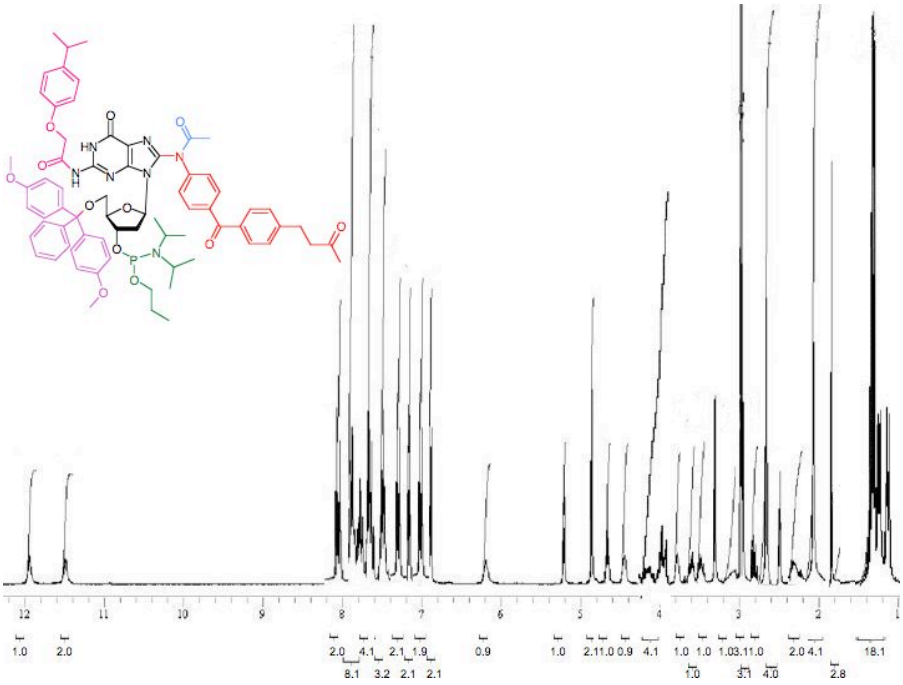

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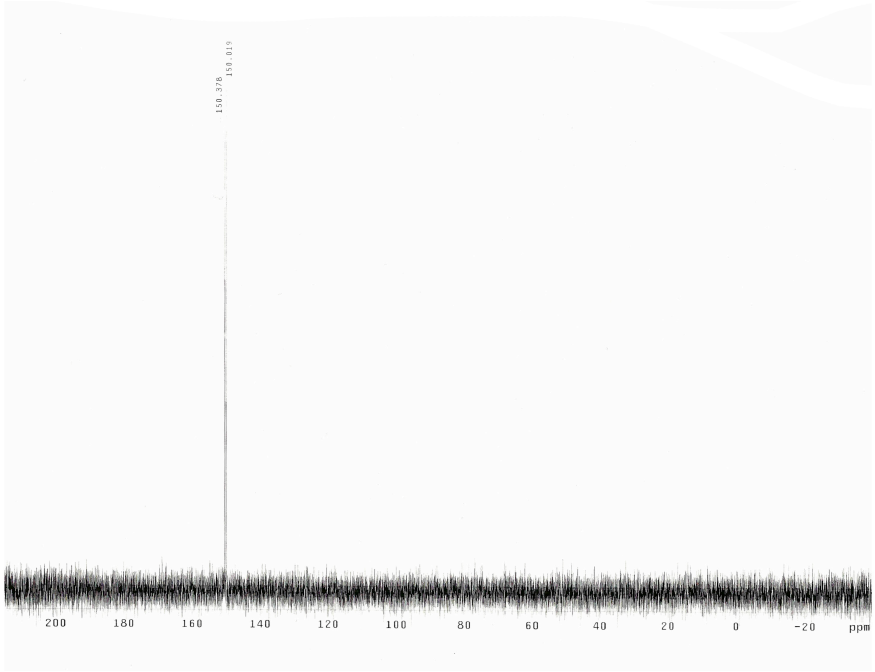
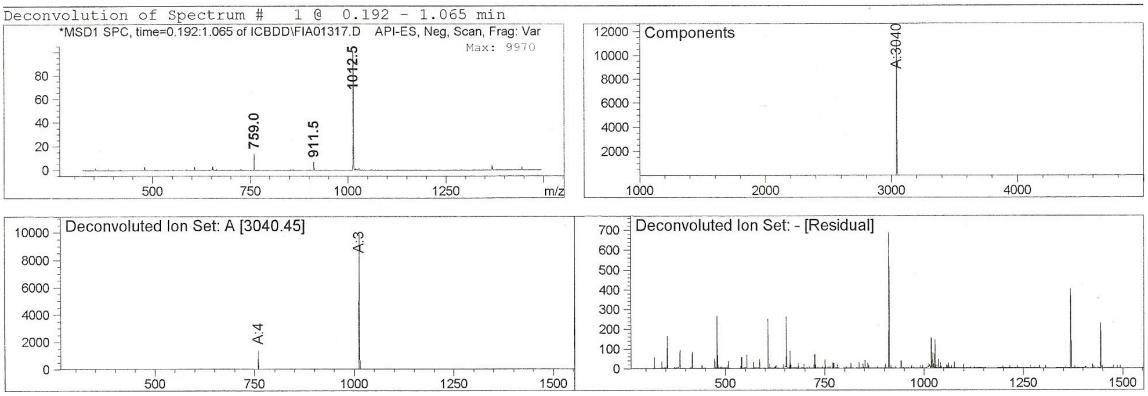


Deconvulated ESI-MS: 9-mer AABzP



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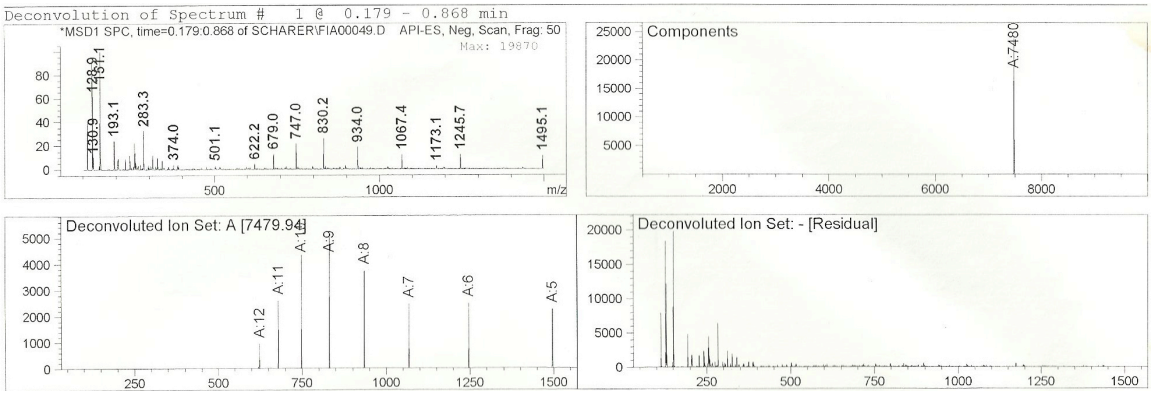
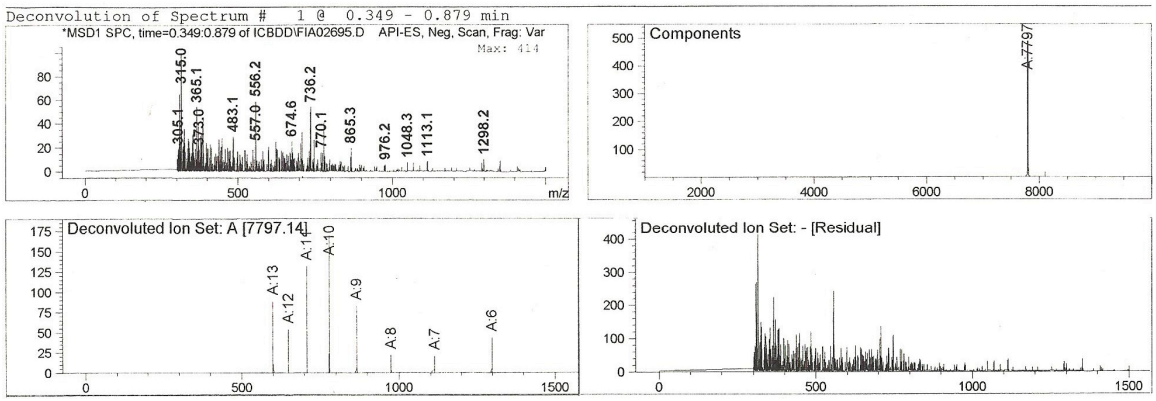


Deconvulated ESI-MS: biotylated 24-mer AABzP



Conclusion and perspectives

In the present work, we extended the chemistry developed in our laboratory by demonstrating the feasibility of preparing a new N-8-acetylarylamine adduct of 2'-deoxyguanosine and of site-specifically incorporating it into oligonucleotides, which were later incorporated into plasmids. This new lesion has been proven to be an efficient NER substrate. Due to its original functionality for such a lesion, we showed that this lesion can be site-specific conjugated with interesting probes commonly used in modern biological techniques, opening the window to a new horizon in the investigation of NER.

To go further on the chemical aspects of this project, it will certainly be interested to test the conjugation efficiency of the ketone linker with different probes to see if it can become a common labeling method for NER substrate or if the reactivity of the ketone linker presents some limitations in the choice of probe being used.

We are confident that this new substrate has excellent potential. Not only does it allow the replacement of radioactive labeling, which represents a considerable technical advance, but it should certainly allow the development of new NER assays based on fluorescent spectroscopy and imaging. The internal labeling of the oligonucleotides released by the NER reaction represent already by itself a considerable improvement in

the amount of information we can obtain from the product of the NER reaction by offering their full detection and quantification.

Föster Resonance Energy Transfer measurements of the lesion within GFP tagged NER proteins will allow the monitoring in real time of the specific steps of the NER mechanism and access to new structural information about the incision complex.