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Reactions of Chlorambucil and its main metabolite, Phenylacetic Acid Mustard, with 2'-deoxyribonucleosides and Calf Thymus DNA

by

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ABSTRACT

Chlorambucil is an anticancer agent used in the treatment of a variety of cancers, especially in chronic lymphocytic leukemia, and autoimmune diseases. Nevertheless, chlorambucil is potentially mutagenic, teratogenic and carcinogenic.

The high antitumor activity and high toxicity of chlorambucil and its main metabolite, phenylacetic acid mustard, to normal tissues have been known for a long time. Despite this, no detailed chemical data on their reactions with biomolecules in aqueous media have been available.

The aim of the work described in this thesis was to analyze reactions of chlorambucil with 2'-deoxyribonucleosides and calf thymus DNA in aqueous buffered solution, at physiological pH, and to identify and characterize all adducts by using modern analyzing methods. Our research was also focused on the reactions of phenylacetic acid mustard with 2'-deoxynucleosides under similar conditions.

A review of the literature consisting of general background of nucleic acids, alkylating agents and ultraviolet spectroscopy used to identify the purine and pyrimidine nucleosides, as well as the results from experimental work are presented and discussed in this doctoral thesis.

PREFACE

This thesis is based on experimental work carried out at the Laboratory of Organic Chemistry and Chemical Biology at the Department of Chemistry, University of Turku during the years 2002-2008. The Graduate School of Organic Chemistry and Chemical Biology, Medical Research Fund of the Tampere University Hospital and Magnus Ehrnrooth Foundation are gratefully acknowledged for financial support.

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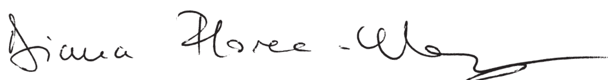

Diana Florea-Wang

Turku, September 2009

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LIST OF ORIGINAL PUBLICATIONS

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I. Diana Florea-Wang, Elina Haapala, Jorma Mattinen, Kristo Hakala, Juhani Vilpo and Jari Hovinen. Reactions of *N,N*-bis(2-chloroethyl)-*p*-aminophenylbutyric Acid (Chlorambucil) with 2'-Deoxyadenosine. *Chem. Res. Toxicol.* **2003**, *16*, 403-408.

II. Diana Florea-Wang, Elina Haapala, Jorma Mattinen, Kristo Hakala, Juhani Vilpo and Jari Hovinen. Reactions of *N,N*-bis(2-chloroethyl)-*p*-aminophenylbutyric Acid (Chlorambucil) with 2'-Deoxycytidine, 2'-Deoxy-5-methylcytidine, and Thymidine. *Chem. Res. Toxicol.* **2004**, *17*, 383-391.

III. Diana Florea-Wang, Inna Ijäs, Kristo Hakala, Jorma Mattinen, Juhani Vilpo and Jari Hovinen. Reactions of {4-[Bis(2-chloroethyl)amino]phenyl}acetic Acid (Phenylacetic Acid Mustard) with 2'-Deoxyribonucleosides. *Chem. & Biodiv.* **2007**, *4*, 406-423.

IV. Diana Florea-Wang, Agnieszka Pawlowicz, Jari Sinkkonen, Leif Kronberg, Juhani Vilpo and Jari Hovinen. Reactions of *N,N*-Bis(2-chloroethyl)-*p*-aminophenylbutyric acid (Chlorambucil) with Calf Thymus DNA. *Chem. & Biodiv.* **2009**, *6*, 1002-1013.

ABBREVIATIONS

Ade	adenine
alkylA	alkyladenine
alkylG	alkylguanine
alkylT	alkylthymine
CLB	4-[*N*,*N*-bis(2-chloroethyl)-*p*-aminophenyl]butyric acid; chlorambucil
Cyt	cytosine
COSY	correlation spectroscopy
dAdo	2'-deoxyadenosine
dCtd	2'-deoxycytidine
dGuo	2'-deoxyguanosine
dMeCtd	2'-deoxy-5-methylcytidine
Gua	Guanine
IARC	International Agency for Research on Cancer
NOESY	Nuclear Overhauser effect spectroscopy
PAM	{4-[bis(2-chloroethyl)amino]phenyl}acetic acid; phenylacetic acid mustard
Thd	thymidine
Thy	thymine
Ura	uracil
UV	ultraviolet

1. INTRODUCTION

1.1. The structure and biological significance of deoxyribonucleic acid

Structure of deoxyribonucleic acid (DNA). DNA is a nucleic acid, a long-chain polymer made up of a linear array of monomers called nucleotides.[1] Each nucleotide is constructed from a heterocyclic nitrogen *base*, a *pentose* sugar, and one or more *phosphate* residues. The most common *bases* are monocyclic pyrimidines and bicyclic purines. The DNA contains two purines, adenine (Ade) and guanine (Gua), and two pyrimidines, cytosine (Cyt) and thymine (Thy) (Chart 1).[2]

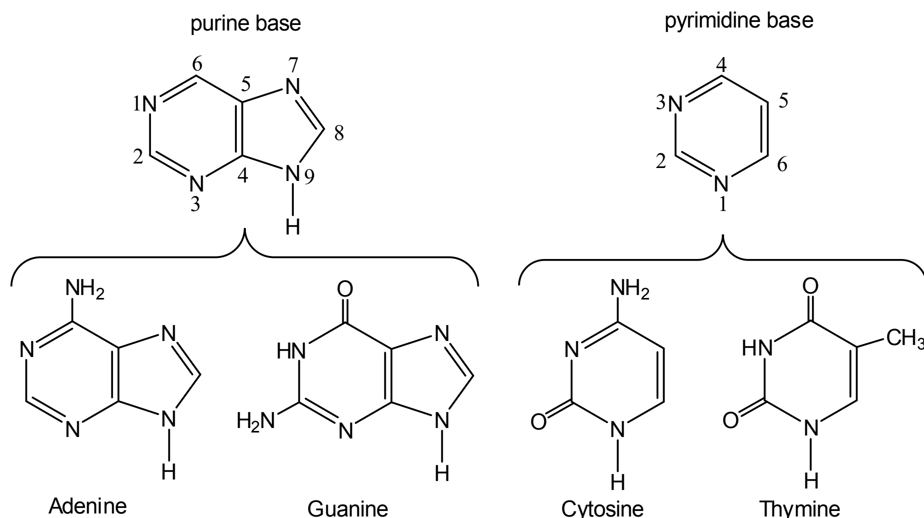


Chart 1. Structures, numbering of atoms and names of DNA bases.

The structure of the nucleosides consists of the *base* bonded to carbon-1 of the *pentose* sugar molecule by a *N*-β-glycosidic bond. The sugar component of DNA is in furanose form and it is a *β*-D-2-deoxyribose. Successive monomer units in nucleic acids are connected through a phosphate residue which is attached to the hydroxyl on the carbon-5' of one unit and the hydroxyl on the carbon-3' of the next unit. Thus, the nucleic acids are assembled on a backbone made up of the pentose units linked by phosphate esters (Chart 2).[3]

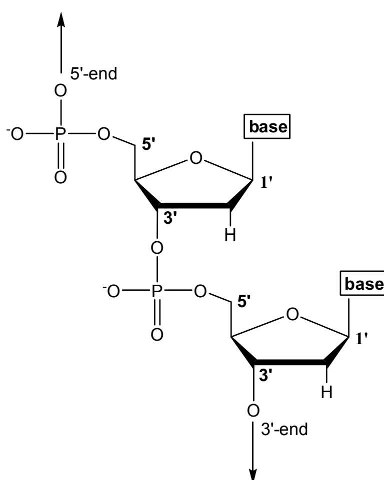


Chart 2. Phosphate ester backbone: from carbon-5’ of a pentose to the carbon-3’ of the next pentose, and further.

The genetic information is encoded in DNA and transmitted from a parent cell to its daughter cells through hydrogen-bonding interaction between specific pairs of nucleobases, known as Watson-Crick base-pairing. Bases which contain amino groups and carboxyl groups are ideal for hydrogen bonding. Every purine base forms a stable hydrogen-bonded pair with a specific pyrimidine base: adenine forms a base pair through two hydrogen bonds to thymine, while guanine forms a base pair through three hydrogen bonds to cytosine (Chart 3).

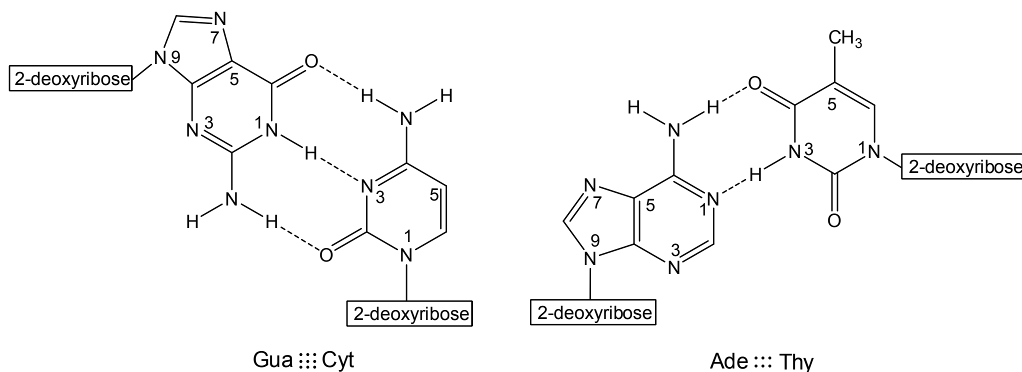


Chart 3. Watson-Crick base-pairing in DNA.

The three-dimensional model of DNA structure consists of two complementary polynucleotide chains held together by hydrogen bonds between the paired bases (Figure 1). The two strands are anti-parallel: one strand is arranged 3’ ⟶ 5’ from left to right, while the other goes in the opposite direction, 5’ ⟶ 3’ from left to right.[2,4]

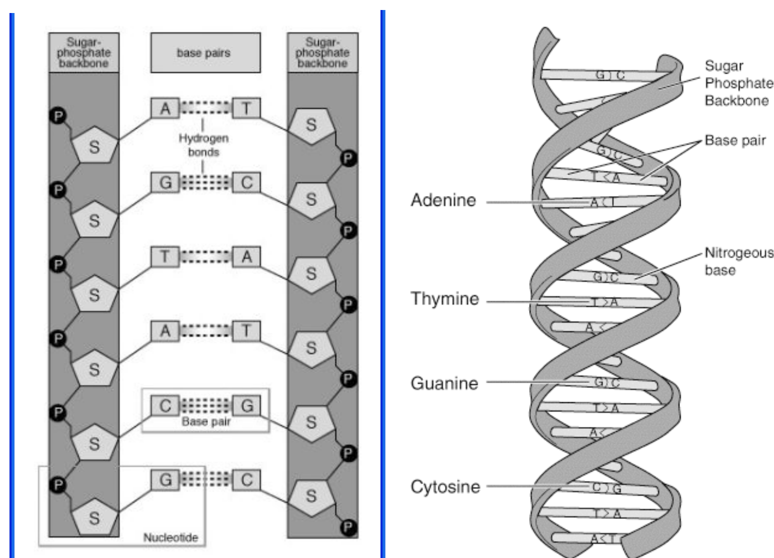


Figure 1. DNA double helix model.

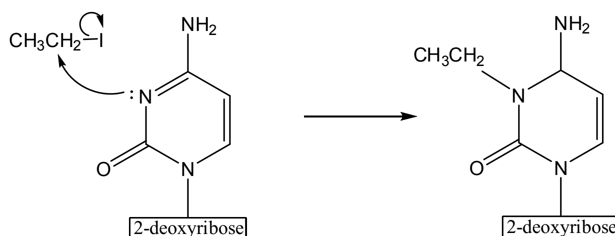
Biological relevance of DNA. The biological properties of the DNA are based on the precise interstrand hydrogen bonding described above. Deoxyribonucleic acids give essential functions in all living organisms, wherein the most vital functions are the long-term storage and transmission of genetic information with high fidelity from one generation to the next.[5,6]

The DNA is exposed to a large variety of harmful physical and chemical agents that have a constant damaging effect. There are estimations of 10000 lesions per day taking place in human genes in every cell.[7] Ultraviolet component of sunlight, X-rays, genotoxins present in foods or cigarette smoke, chemotherapeutic agents (*i.e.* alkylating agents), oxygen radicals resulted from the normal cellular metabolism may direct to mutations that increase the threat of cancer.[8]

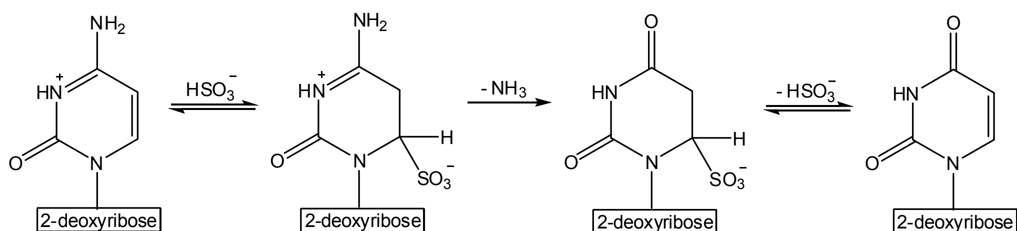
1.2. Alkylating agents and alkylation of purine and pyrimidine nucleosides

Alkylation is the transfer of a reactive alkyl group from one molecule to another molecule where electron density is high.

Nucleic acid bases are prone to structural modification by nucleophiles as well as by electrophiles and these modifications represent the basis of chemical carcinogenesis. In most of the alkylation reactions, the nucleobases play the role of the nucleophile that will react with electrophiles, such as alkyl halides (R-X), alkanesulfonates ($R'-SO_2-OR''$) and dialkyl sulfates ($R'O-SO_2-OR''$) (Scheme 1); in this case, purines are alkylated more readily than pyrimidines. Alternatively, pyrimidines may also serve as electrophiles when strong nucleophiles, such as hydrazines (H_2N-NHR), alkoxyamines (H_2NOR) and bisulfite ion (HSO_3^-),[9] attack their C-6 position (Scheme 2).



Scheme 1. Reaction of 2'-deoxycytidine with ethyl iodide.[10]



Scheme 2. Reaction of 2'-deoxycytidine with bisulfite ion.[9]

As discussed above, alkylating agents are reactive electrophiles. They can react with the nucleophilic (electron-rich) sites of cellular macromolecules, such as DNA and proteins, causing DNA damage by strand breaks, DNA-DNA crosslinks and DNA-protein crosslinks.[11] Alkylating agents are important research topic due to their controversy: promising anticancer drugs, but also possible carcinogens, mutagens and teratogens.

1.2.1. Classification of alkylating agents

The classification of alkylating agents is not an easy task due to the many different aspects used in classifying them, such as their provenience, number of functional groups, mode of action, nucleophilic or electrophilic character/the mechanism of their action, chemical and structure similarities.

There are *endogenous* and *environmental* alkylating agents, as well as *alkylating drugs*. *Endogenous* agents are formed during the metabolism. *S*-Adenosylmethionine,[12] that is a coenzyme implicated in methyl group transfer in biochemical reactions, and nitrosoamines and related compounds are few examples of the endogenous alkylating agents.

Environmental alkylating agents are found in the air, water and foods. Humans are most exposed to *N*-nitroso compounds formed in tobacco smoke. [13] Different halocarbons are present in the air in small but detectable concentrations. Chloromethane gas is generated by plants, fungi, industrially and it is one of the halocarbons present in the highest percentage in the atmosphere.[13] Bromo-compounds (*i.e.* bromoethane) are more abundant in marine environment. There are also different food mutagens, such as aflatoxins, polycyclic aromatic hydrocarbons, and heterocyclic amines.[14] The oldest anti-cancer drugs are alkylating agents and they are still important in the treatment of different types of cancer.[15]

According to the number of the functional groups, alkylating agents are divided into three groups: *monofunctional* alkylating agents, such as methanesulfonates[16,17,18] [*i.e.* methyl methanesulfonate, MMS (**1**)] and methylnitrosourea[19,20] (**2**); *bifunctional* alkylating agents, as for example bis(chloroethyl)nitrosourea[21,22,23] (**3**), bischloroethyl sulfide[24] (**4**), bis(chloromethyl) ether[25] (**5**), epichlorohydrin[26] (**6**), nitrogen mustards; and *cyclic* alkylating agents, such as 2-chlorooxirane[27,28] (**7**) (Chart 4). There are not strict limits between these three categories of alkylating agents (*see* compound **6** that is both, bifunctional and cyclic). However, most of mutagens and carcinogens are simple mono- or bi-functional alkylating agents.

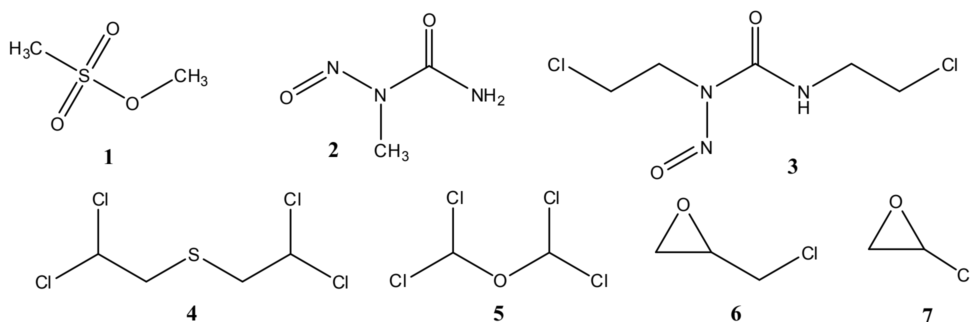


Chart 4. Structural formula of different types of alkylating agents.

Nitrogen mustards, such as 4-[*N*,*N*-bis(2-chloroethyl)-*p*-aminophenyl]butyric acid (chlorambucil, CLB, **8**), cyclophosphamide[29] (**9**), melphalan[30] (**10**) and mechlorethamine (**11**) (Chart 5), are typical bifunctional alkylating agents and they are few of the oldest anticancer agents in clinical use.[31] They have the tendency especially to form interstrand crosslink between two dGuo molecules in their *N*7 position.[32]

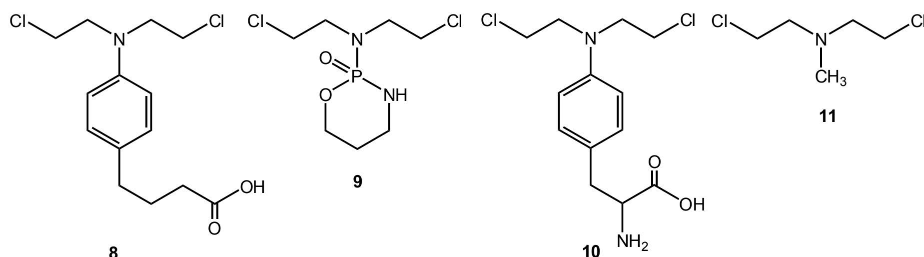


Chart 5. Structures of nitrogen mustards: chlorambucil (**8**), cyclophosphamide (**9**), melphalan (**10**) and mechlorethamine (**11**).

Alkylating agents can be divided also by their mode of action into two categories: alkylating agents that react *directly* with nucleic acids and are known as primary carcinogens; and alkylating agents that react after *metabolic activation*.[33] Most of the alkylating agents are directly acting carcinogens, such as dimethyl sulfate (DMS, **12**), MMS (**1**), 2-methylaziridine (**13**), 1,3-propanesultone (**14**), epichlorohydrin[34] (**6**), and old anticancer agents as myleran (**15**), CLB (**8**) and cyclophosphamide (**9**) (Chart 6).

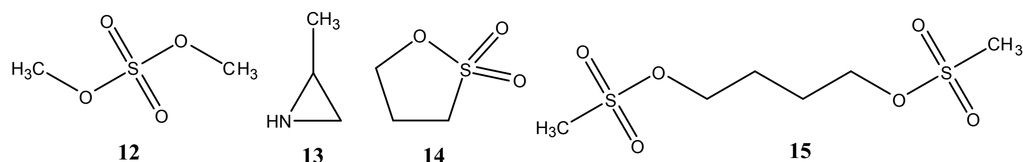
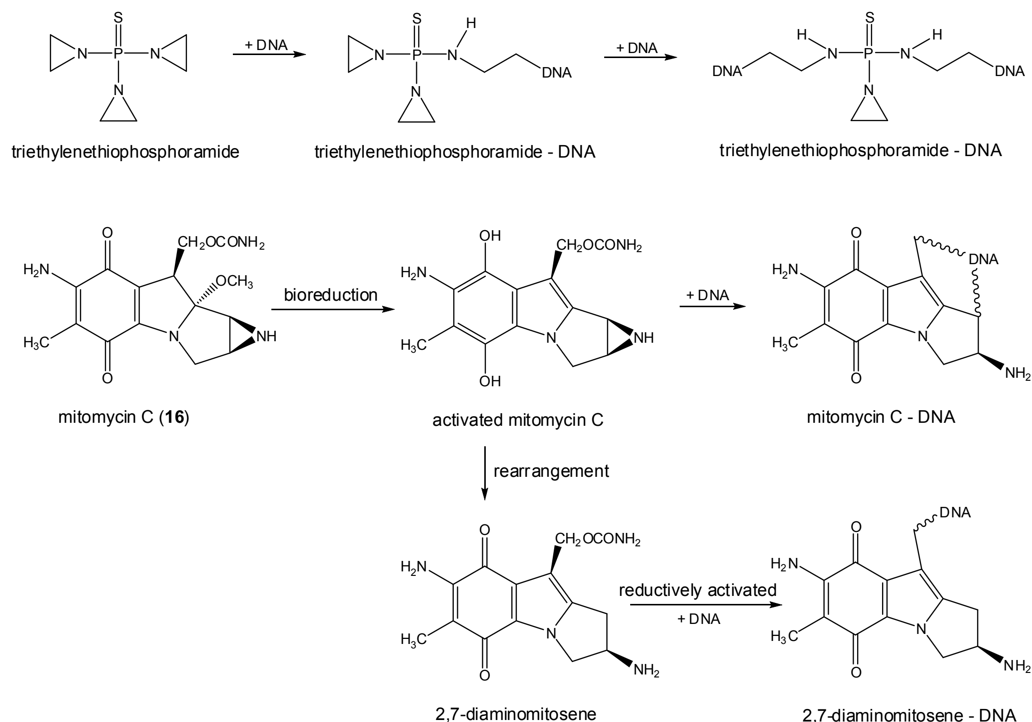


Chart 6. Structures of directly acting agents.

Quinone methides are known as bioreductive alkylating agents.[35,36] A quinine methide precursor is activated, forming a very reactive intermediate that can react with DNA. The antibiotic mitomycin C[37] (**16**, Scheme 1) is a known example of this class due to its high anti-tumor activity; **16** binds covalently to DNA upon reductive activation[38] and it is the only quinone-containing alkylating agent that was approved for general use.

Scheme 1 shows example of reactions of direct acting alkylating agent, triethylenethiophosphoramide,[31] and metabolically activated alkylating agent, mitomycin C,[39,40,41] with DNA.

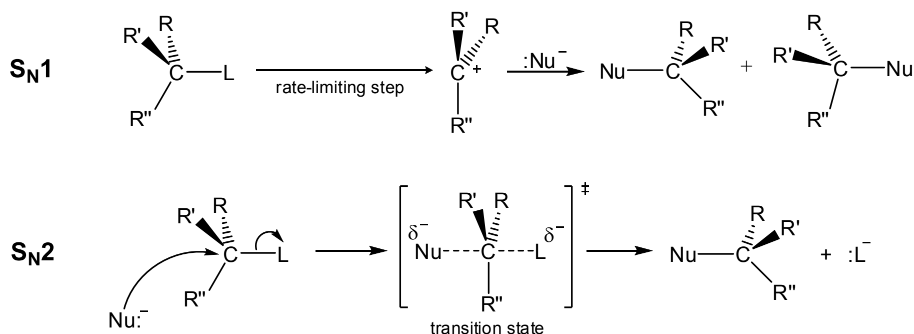


Scheme 1. Reaction pathways of direct acting and metabolically activated alkylating agents.

Dialkylnitrosamines are other examples of alkylating agents that are metabolically activated to reactive species.[42]

Alkylating agents are *electrophiles* that are able to react at a variety of sites on DNA molecules following mainly the rules of electrophilicity and nucleophilicity.[33] There are

S_N1 and S_N2 alkylating agents, depending on which mechanism they react with DNA. Two basic mechanisms of alkylation are generally accepted: the first-order nucleophilic substitution (S_N1) and the second-order nucleophilic substitution (S_N2). S_N1 is referring to unimolecular reaction, while S_N2 is corresponding to bimolecular reaction. In a nucleophilic substitution, an electron-rich attacking nucleophile replaces a leaving group from a carbon atom, using its lone pair of electrons to form a new bond to the carbon atom. A typical S_N1 and S_N2 reaction mechanism is shown in Scheme 2.



Scheme 2. A representative depiction of the S_N1 and S_N2 reaction mechanism, in which L is the leaving group and Nu is the nucleophile.

In S_N1 substitution reaction, the first step is the formation of an electrophilic planar carbocation intermediate; this takes place slowly and it represents the rate-limiting step. The covalently bonded adduct is rapidly formed from this intermediate and the nucleophile. The stability of the carbocation and/or nature of the leaving group determine the reactivity of the electrophile.[33]

Strong electrophiles, as for example methyl iodide, dimethylsulfate, are very reactive and they react by following the S_N2 mechanism. The S_N2 reaction involves an attack of the electrophilic carbon atom by a nucleophile from the opposite site of the leaving group. These types of reactions are dependent on steric accessibility.[33]

The electrophiles can be divided into hard electrophiles and soft electrophiles. Hard electrophiles that have large dipole moments react at the oxygens of the DNA bases, while soft electrophiles that have small dipole moment react at nitrogens of the DNA bases. Hard electrophiles, as for example *N*-methyl-*N*-nitrosourea, are S_N1-like alkylating agents, whereas soft electrophiles, as for example DMS, MMS, alkyl halides, react preferably with endocyclic sp^2-hybridized nitrogen atoms of nucleosides[43] in a S_N2-like fashion.

Platinum compounds are also an important class of chemotherapeutic alkylating agents. Cisplatin (**17**) and carboplatin (**18**) are few examples of these types of compounds (Chart 7). They are neutral complexes that move freely into the cells until their two chlorides are exchange by water and form bi-positive charged molecules that are stuck into the cells. They can form intrastrand crosslink *N*7 dGuo – *N*7 dGuo.

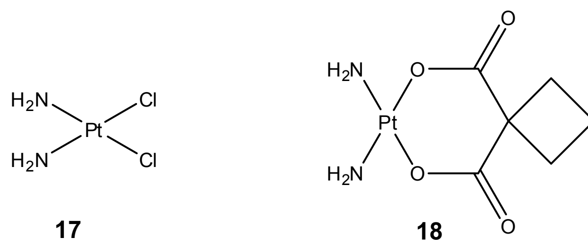
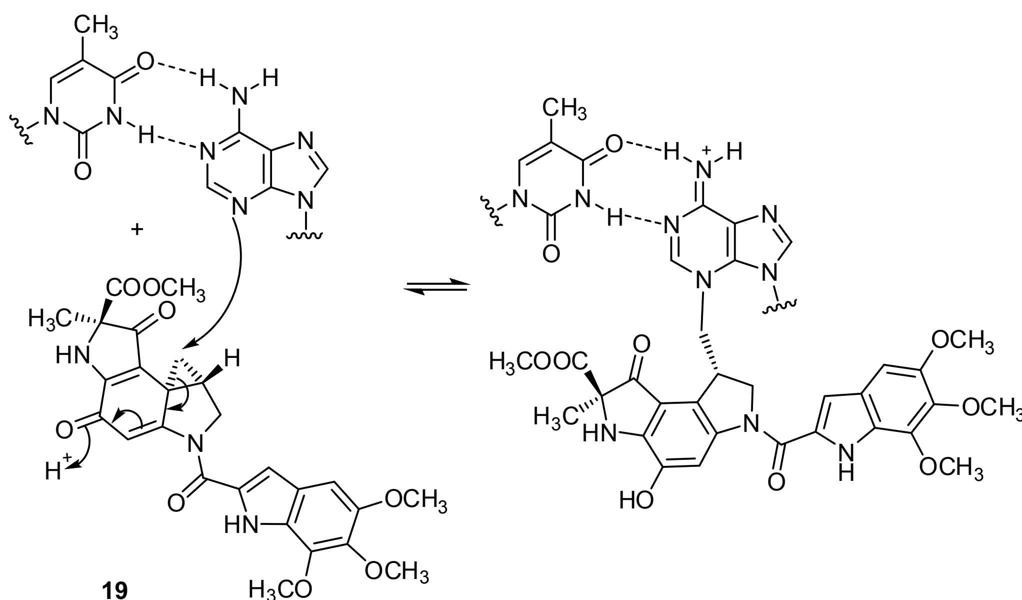


Chart 7. Chemical structures of platinum compounds.

Cyclopropylpyrroleindoles derivatives, such as duocarmycin A[44] (**19**), are a class of alkylating agents and antitumor antibiotics that exhibit their biological effects through a reversible, sequence-selective minor groove alkylation of DNA.[45,46] The reversibility of drug – DNA adducts might be the main reason for which these antitumor alkylation agents act selectively on the tumor cells. Scheme 3 shows the regeneration of intact drugs from their covalent DNA adducts.[47,48]



Scheme 3. Reversible DNA alkylation reaction of duocarmycin A.

1.2.2. DNA crosslink

The antitumor activity of bifunctional alkylating agents is associated to their capability to induce DNA-DNA crosslinks within the DNA duplex.[49] Bifunctional alkylating agents can also react with the nucleophilic sites within proteins, giving rise to DNA-protein crosslinks. [50] These crosslinks are also responsible for the large cytotoxic potential of the alkylating agents.[51,52]

The alkylation reaction on DNA generates different types of products: monoalkylated adducts and crosslinks, which can be intrastrand crosslinks and interstrand crosslinks (Figure 2).

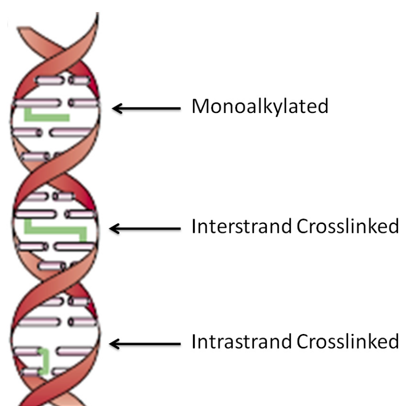
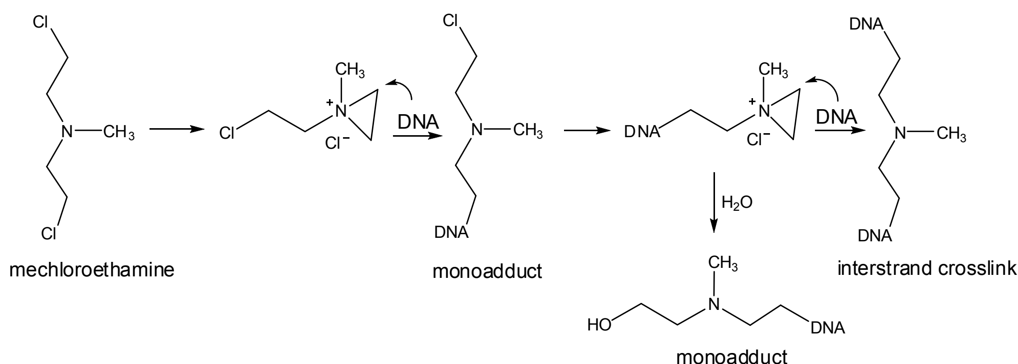


Figure 2 . Type of products in DNA alkylation.[15]

The interstrand crosslink arises from the covalent binding of the alkylating agent to both strands of the double helix (Scheme 4) and it is considered to be the most toxic lesion.[53] In order to form DNA crosslink, alkylating agents have to contain two or more reactive sites (to be multifunctional) and two reactive nucleophilic sites of DNA must be present in close proximity. Monoalkylated adducts and intrastrand crosslinked products may be formed when the alkylating agent is smaller than the width of the minor groove of the DNA strand, while the interstrand product may be formed when the alkylating agent is longer than the width of the minor groove of the DNA strand. Monoalkylated adducts appear when an alkylating agent that already has reacted with DNA additionally reacts with an external nucleophile, such as water or a buffer component. Thus, the type of product that is formed depends on the nucleophiles present in the system as well as on the structure of the alkylating agent and DNA.



Scheme 4. Example of DNA interstrand crosslink formation in the case of mechloroethamine.[54,55] Chloride is the leaving group and DNA holds the nucleophilic site.

The DNA interstrand cross-linking may disrupt crucial cellular processes including the DNA replication and transcription[53,55] and that may have a lethal influence on cells.

1.2.3. Sites of alkylation in purine and pyrimidine deoxynucleosides and DNA

Alkylating agents can react at any of the nucleophilic sites (endocyclic and exocyclic nitrogens, exocyclic oxygens) of the purine and pyrimidine bases of nucleic acids in aqueous solution at neutral pH[56,57] (Chart 8). Furthermore, the oxygen atoms of the phosphate internucleotide linkages are prone to alkylation.[58] However, there are two exceptions: the nitrogen in position 9 of the purines and the nitrogen atom in position 1 of pyrimidines.

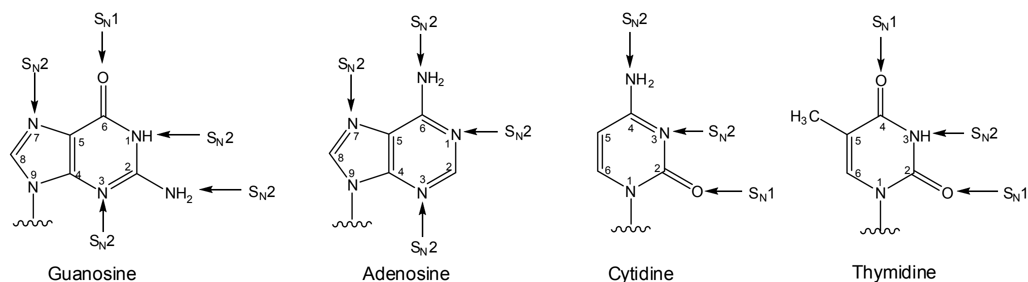


Chart 8. Alkylation sites of the nucleic acid bases and the main type of substitution at each nucleophilic site.

It is generally known that the base-paired positions of nucleic acids are shielded from modifications.[59] This is basically the case with the nitrogen atoms but not with the oxygens.[60] Base-paired oxygens, such as O^2 of Cyt, O^6 of Gua, O^4 of Thy, possess an extra pair of electrons that is free to react even in dsDNA.[61,60]

In the case of cytidine, O^2-alkylation was considered to be impossible due to the instability of the resulting adduct.[62] In 1976, Singer[62] reported for the first time the O^2-alkylcytidine derivatives that were obtained as major products in the reaction of cytidine with *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea at neutral pH, in aqueous media. It was also observed that weaker carcinogens, such as dimethylsulfate, do not alkylate O^2 position of cytidine.

A very important factor in determining the site of alkylation is the nature of the alkylating group (*i.e.* ethyl *vs.* methyl).[10] The *N*7 of guanine base is the principal site of alkylation of nucleic acids bases with alkylating agents.[10]

1.2.4. Basics of carcinogenesis and introduction to alkylating agents as carcinogens

Based on extensive empirical observations,[63] as well as on mathematical model,[64] human carcinogenesis is a multistep process in which three phases, *initiation*, *promotion* and *progression*, follow each other in a sequential manner. The nature of these stages is very complex and they are described in a simplistic mode in the following text. However, we have to bear in mind that carcinogenesis is far from a simple process, but it consists of multiple genetic alterations of cells, involves multiple steps, and is affected by multiple environmental factors.[65]

There are many different endogenous and exogenous carcinogenic factors to which humans are exposed: chemicals, physical agents, radiation, viruses and bacteria.[66] The identification of carcinogens is a complicate process that involves the scientific evaluation of human epidemiological studies, animal bioassays, and mechanistic and other relevant data.[67] According to the IARC Monographs, there are more than 400 agents classified as carcinogenic to humans, probably carcinogenic to humans or possibly carcinogenic to humans.[68]

The exposure to mutagens is followed by the *initiation* step which starts with the alterations of DNA due to spontaneous or carcinogen-induced genetic changes, epigenetic modifications, or inherent genetic mutations.[66] The alterations in specific genes will cause the change in the initiated cell's response to its microenvironment, giving a possible growth advantage compared to normal cells.[69] In initiation phase, the genetic cellular modifications take place very slowly and with small or unobservable changes in the cellular or tissue morphology. In this stage, a permanent inclination to develop cancer exists and it increases by time, and further tumor will develop only if the environmental conditions changes in such a way that further evolution of tumor is favored.

The initiation is followed by the *promotion* stage in which a clonal expansion of initiated cells takes place. The formation of tumor is stimulated by a non-mutagenic external factor, as for example wounding or inflammation.[70] A non-malignant tumor is formed and, without additional stimulus, the tumor may regress.[64] This step is associated with hyperproliferation, apoptosis, tissue remodeling and inflammation.[71]

In the *progression* phase, the tumor goes through a malignant transformation with unlimited and invasive growth. Progression does not involve external stimulus usually. This stage is associated with alterations in gene expression and supplementary genetic damage because of progressive genomic instability.[72]

Carcinogens can be classified into directly acting agents and metabolically activated agents. The direct carcinogens are reacting with nucleic acids without enzymatic activation, while the metabolic activated carcinogens, known also as procarcinogens, require metabolic activation. The last DNA-reactive carcinogenic species of procarcinogens are electophilic and this is a general characteristic of all procarcinogens' metabolic activation.[73] Additionally, the damage of DNA can be caused by many directly acting carcinogens through their electrophilic intermediates.[74]

A further classification will divide the directly acting compounds into nonalkylating and alkylating agents. Nonalkylating agents change base pairing by deamination or a shift in tautomeric equilibria, while the alkylating agents substitute a proton with an alkyl moiety. Alkylating agents, including melphalan, chlorambucil, chlornaphazine, mustard gas, muleran and cyclophosphamide, are few of the known human carcinogens.[75,76]

Alkylating agents are used widely in cancer chemotherapy. However, they may damage the DNA and be mutagenic and carcinogenic. They are known to alkylate DNA at various sites on bases, sugars and phosphate groups. The O^6 position of guanine is the major mutagenic and lethal lesion among all the alkylation sites; the O^6-alkylguanine is predominantly repaired by O^6-alkylguanine-DNA alkyltransferase.[77]

1.2.5. Stability of alkylated DNA adducts

Under physiological conditions, many alkylating agents bind to various heteroatoms, such as nitrogens and oxygens, of genomic DNA.[33] The DNA adducts are formed by the covalent binding of alkyl groups to nucleophilic sites on DNA and they show different levels of stability. On one hand, for example, 3-alkylpurine nucleosides and 7-alkylpurine nucleosides are easily depurinated at neutral pH because of the lability of their glycosidic bond. On other hand, same and other adducts can also be removed enzymatically by different pathways: by glycosylase mediated excision repair or by transferase enzymes specific for the removal of only the alkyl group.[58,78,79]

It was observed that only a small percentage of adducts are stable and stay bound to DNA for a long time[33] and also that adducts formed *in vitro* are usually more stable than adducts formed *in vivo*.[78]

1.2.6. DNA-alkylation repair processes

It is generally believed that the alkylating agents/drugs bind covalently to DNA and lead to misreading of the DNA code, cross-linking of DNA,[80] and single-stranded and double-stranded breaks of DNA.[81] Most of the modifications of nucleic acid bases can be repaired *in vivo* by repair enzymes, but not all, and cell may die without a proper repair of the lesion.[82]

In living cells, there are two types of basic repair processes of lesions/damages induced by DNA alkylation. The first process consists of the direct base repair in which the modifying alkyl group is transferred directly to the repair protein. For example the O^6-methylguanine-DNA methyltransferases and 3-methyladenine-DNA glycosylases protect the cells from the killing effect of alkylating agents.[80] In the second type of repair, the modified base is removed by a glycosylase; an apurinic or apyrimidinic site is formed and this will be repaired by an excision-repair process.[33] There is base excision repair and, in some extend, the nucleotide excision repair (NER). Bifunctional alkylating agents, known anti-cancer drugs, cause complex DNA lesions that involve complex repair mechanism that depends on the type of lesion.[7,13] For example, NER is repairing the DNA damages caused by UV light, while base excision repair finds minor damages that take place at nucleobases and sugar moieties.[7] However, other repair systems do exist.

1.2.7. Biological activity of DNA-alkylation adducts

Since *O*^{6} site of guanine was suggested to be important for mutation induction,[83] many scientific groups started to analyze the biological activity of DNA-adducts. It is considered that *O*-alkylations, such as *O*^{6}-alkylG and *O*^{4}-alkylT, are highly mutagenic and genotoxic.[84] *O*^{6}-Alkylguanine and *O*^{4}-alkylthymidine are of great biological importance[85,86] and show promutagenic potential in both RNA and DNA in vitro assays[58] and they were found also *in vivo*.[87,88] *O*^{6}-AlkylG pairs with thymine producing G ⟶ A transitions, while *O*^{4}-alkylT pairs with guanine producing T ⟶ A transitions.[57] The alkylation of the *O*^{2} position of all deoxypyrimidines causes a high destabilization of the glycosidic bond;[89] these adducts are much more labile than their parent compounds.

In the case of DNA methylation, probably the most significant products are *O*^{6}-methylguanine and 3-methyladenine. The first is a miscoding base and the second is a cell-killing lesion.[90] *N*-alkylations, as for example 1-alkylA and 3-alkylA, are less mutagenic, but they are cytotoxic.[91] In general, 1-alkylpurines and 3-alkylpyrimidines were found to inhibit DNA synthesis and to contribute to cytotoxicity.[58]

7-AlkylG is the most observed lesion in DNA and RNA and itself is not harmful. The situation changes when 7-alkylG goes through spontaneous depurination and enzymatic removal, giving arise to cytotoxic abasic sites.[13]

S_N1 alkylating agents are highly mutagenic due to their ability to react with the DNA sites containing oxygens and therefore causing the formation of mispairing adduct, especially *O*^{6}-alkylguanine and *O*^{4}-alkylthymine.[92]

1.3. Ultraviolet spectroscopy used in characterization of purine and pyrimidine nucleoside adducts

There are several types of molecular excitations, but four of them are especially important to chemists because they give information related to the molecular structure of a particular compound. These are the absorption of infrared radiation (IR), which tells about the type of functional group that is present in the molecules, the absorption of ultraviolet and visible light (UV), which proves the compounds that contain π bonds, nuclear magnetic resonance (NMR), which gives information about the carbon skeletons of molecules and the number of hydrogen atoms present on each carbon atom, and mass spectrometry (MS), which elucidates the elemental composition and the structure of a molecule. UV spectroscopy is especially useful in determining the site of base modifications because of the extensive literature data available that can be used as a reference material. In addition, the method is experimentally very easy.

Molecular absorption in the ultraviolet region of the spectrum depends on the electronic transitions of the molecule. [93,94,95,96,97] Most UV absorptions by organic molecules are attributed to transitions involving the excitation of an electron from

the highest occupied molecular orbital to the next higher energy orbital, the lowest unoccupied orbital.

Absorption spectra of nucleosides and their derivatives. It is known that the sugar and phosphate components of nucleotides do not have any significant UV absorption above 230 nm, which means that the nucleotides and nucleosides have UV spectra alike to those of their constituent bases.[98] Nucleic acid bases and their derivatives show a strong UV absorption in the range from 240 to 300 nm,[99] which greatly facilitates the detection and quantification of nucleosides.

In nucleic acid studies, three different parameters of absorption spectroscopy may be utilized: molar absorptivity, wavelength of maximum absorption, and hypochromicity.[96] The absorption maximum is close to 260 nm [100] and the molar absorptivity to 10^4 $M^{-1}cm^{-1}$. Hypochromic effect appears when a nucleic acid base is inserted into a polymeric structure and its absorptivity is reduced.

Nucleosides have pK_a-values in the normal pH-range from 0 to 14 (Ado at 3.7, Guo at 2.0 and 9.5, Ctd at 4.3, and Thd at 9.7) and the wavelengths of the absorption maxima (λ_{max}) and the respective molar absorptivities (ε-values) are changed on passing these pH values.[99] However, at pH 7 all nucleosides are neutral.

UV spectroscopy used in the characterization of adducts. UV spectroscopy is a useful tool for detection of base modifications and characterization of the adducts that are formed. Characterization of the adducts is usually verified by other spectroscopic techniques, such as mass spectroscopy and nuclear magnetic resonance spectroscopy.

Figure 3 includes examples of UV spectra of nucleosides and their modified counterparts. Usually, knowing the value of maximum absorption (λ_{max}) of a compound, it is easy to observe if a modification took place on that compound by checking the λ_{max} of the new formed products. If the value is different, an adduct has been formed. Sometimes also the shape of the spectra may indicate the presence of the modification.

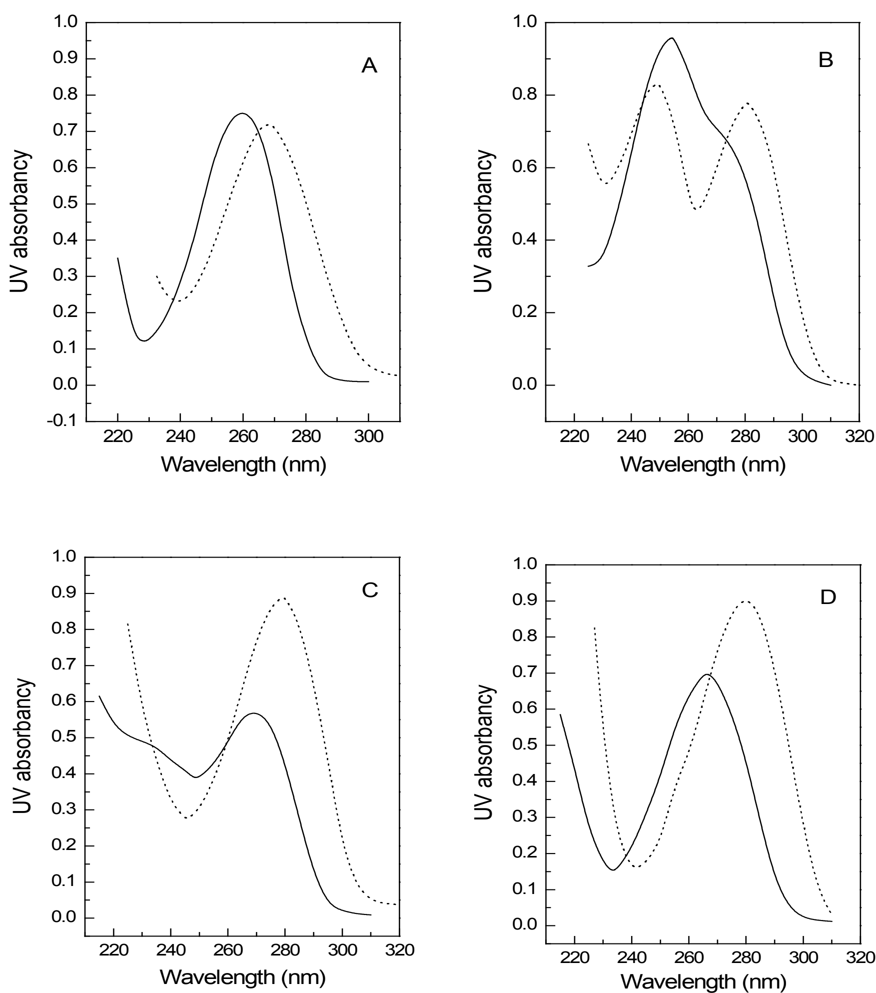


Figure 3. Neutral UV spectra of (**A**) adenosine (λ_{max} = 259, —) and N^6-ethyladenosine (λ_{max} = 268, ...), (**B**) guanosine (λ_{max} = 253, —) and N^6-ethylguanosine (λ_{max} = 248, 280 ...), (**C**) cytidine (λ_{max} = 271, —) and *N*3-ethylcytidine (λ_{max} = 279, ...), and (**D**) thymidine (λ_{max} = 267, —) and O^4-ethylthymidine (λ_{max} = 279, ...) recorded in H_2O. Spectra adapted from *Ref.* [101].

2. AIMS OF THE THESIS

Chlorambucil (CLB) is an aromatic nitrogen mustard and an alkylating agent originally synthesized by Everett *et al.* in 1953.[102] It has been mainly used in the chemotherapy of chronic lymphocytic leukemia.[103,104,82,105] Other clinical applications include Hodgkin's lymphoma, non-Hodgking's lymphoma,[106] Waldenström's macroglobulineamia,[107] trophoblastic neoplasms,[108] polycythemia vera,[109] ovarian carcinoma,[110] breast cancer and some other tumors. It can also be used as an immunosuppressive drug for autoimmune and inflammatory conditions, as for example nephrotic syndrome.[111]

Like other alkylating agents, CLB binds covalently to DNA,[112] RNA and proteins. The covalent binding of CLB to DNA may cause misreading of the DNA code, cross-linking of DNA, single-stranded and double-stranded breaks of DNA,[81] and death of the cell.[82] It is known that therapeutic alkylating agents, including CLB, may generate second tumors in patients who have taken these drugs as a treatment for their primary cancer.[113] CLB, although used extensively in cancer chemotherapy, is itself potentially mutagenic,[114,115] teratogenic,[116] and carcinogenic, [54,77] and an increased incidence of acute leukemias and other secondary malignancies has been reported in patients who have received this drug.[117] However, exhaustive chemical data on CLB reactions with biomolecules in aqueous solution have not existed.

The main aim of the thesis was to identify, characterize and quantify adducts formed in the reactions of chlorambucil with calf thymus DNA in aqueous solution at physiological pH. Therefore, for an easier understanding, the reactions of chlorambucil with nucleosides were followed, alkylation products were characterized and quantified and the results were used further as references for the reaction of chlorambucil with single- and double-stranded DNA. Also the reactions of phenylacetic acid mustard (PAM), chlorambucil's main metabolite, with nucleosides were studied.

The goals were achieved by detailed study on the reactions of chlorambucil with nucleosides[118,119] and single-stranded and double-stranded DNA,[120] and the reaction of phenylacetic acid mustard with nucleosides.[121] Adducts were isolated and purified by chromatography; then they were characterized by chromatographic, spectroscopic and spectrometric methods. The identification of the products formed in DNA treated with chlorambucil is essential to be able to understand better the properties of chlorambucil as a human carcinogen, the molecular mechanism of its antitumor effect and to make a better use of it in clinical chemotherapy.

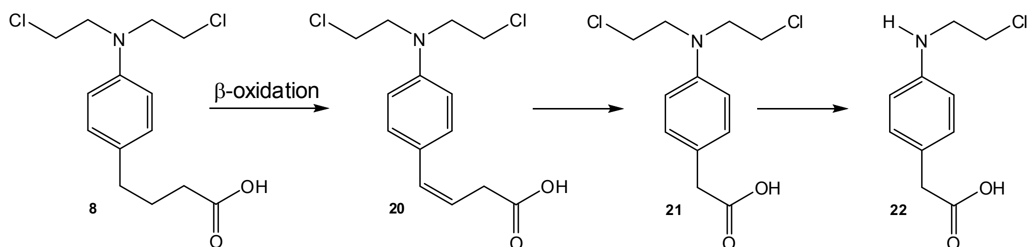
3. RESULTS AND DISCUSSION

This section deals with general knowledge related to chlorambucil and phenylacetic acid mustard and with the results obtained and described in the original publications.[118,119,120,121] CLB and PAM behave similarly in their reactions with nucleosides and give same type of products. Therefore, the reactions of CLB at physiological pH were studied in the presence and absence of 2'-deoxyribonucleosides and used as examples, being mainly described in the following text; exception makes the case of dGuo, in which its reaction with PAM will be described.

3.1. Chlorambucil versus phenylacetic acid mustard

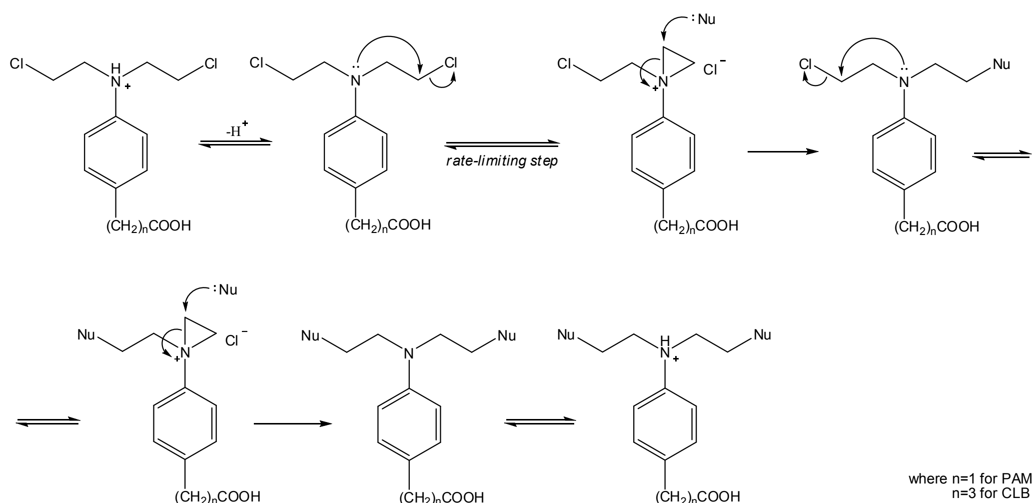
CLB is an orally administrated drug that is in clinical use for several decades and yet its optimal use is not established.[122] CLB is a drug used often as a reference when new chemotherapeutical drugs, especially purine analogues as fludarabine,[123,124] cladribine[125,126] and pentostatin,[127,128] are developed and tested clinically. CLB can be purchased from different chemical suppliers. Phenylacetic acid mustard is the metabolite of CLB and it was synthesized by different methods for many years;[102,129,130] PAM is not commercially available.

Chlorambucil undergoes fast gastrointestinal absorption and it is nearly entirely metabolized. CLB is metabolized mainly by β-oxidation of the butyric acid side chain[131] to {4-[bis(2-chloroethyl)amino]phenyl}acetic acid (PAM, **21**) through the intermediate (*E*)-4-{4-[bis(2-chloroethyl)amino]phenyl}but-3-enoic acid (**20**) (Scheme 5).[132,133] PAM is further metabolized to its monodechloroethylated derivative (**22**).[134]



Scheme 5. Metabolism of chlorambucil

As other aromatic and aliphatic nitrogen mustards, CLB and PAM decompose in aqueous media and form covalent bonds with a variety of nucleophiles. The mechanism of decomposition of CLB and PAM is described in Scheme 6 and it consists of an intramolecular, rate-determining attack of an unprotonated *N*-atom to form an aziridinium ion intermediate, followed by attack of an external nucleophile.[135,136,137,138,139]



Scheme 6. Mechanism of decomposition of CLB and PAM

The rate-limiting step (Scheme 6) shows that the reactivity of CLB/PAM is not influenced by the external nucleophiles, but by the internal nucleophile which is the nitrogen atom. The external nucleophile then traps the cyclic aziridinium ion intermediate obtained.

It was observed that the rate of decomposition of CLB and PAM is significantly slower in the presence of chloride ion in the solution, [131,140] which is in accordance to the behavior of other aromatic nitrogen mustards.[137,139,141] Thus, chloride ion has a stabilizing effect on CLB/PAM. An obvious stabilizing effect on CLB is encountered in the case of high concentration of H^+ (lower pH) and chloride in human gastric juice.[131]

Effects of the fluid matrices on the decomposition of CLB and PAM, as well as the cellular incorporation and protein binding of the drug were studied *in vitro*.[131,140] In the *absence of nucleophiles* (*i.e.* unbuffered water, cacodylic acid buffer and perchloric acid solution), the main reaction is CLB/PAM hydrolysis yielding monohydroxy derivative as an intermediate and dihydroxy derivative as the stable end product. In the *presence of nucleophiles* (*i.e.* phosphate-buffered solution, formic acid and acetic acid buffers), extra products are formed from the ions (*i.e.* phosphate, or acetate, or formate) present in the reaction mixtures with the aziridinium ions derived from CLB/PAM.

It is known that approximately 99 % of CLB binds immediately to plasma protein.[142] Albumin is the main plasma protein that participates in this binding in human blood.[143] CLB binds non-covalently to the hydrophobic pockets of albumin, which explains the decrease of decomposition (hydrolysis) of the drug in plasma and the considerable stabilization effect on the drug. The decrease in the decomposition of CLB due to the binding to albumin is proven also by the fact that the aziridinium ion formation is not favorable in hydrophobic surroundings and that the cellular incorporation of CLB increases when the concentration of plasma protein decreases.[140]

CLB and PAM have positive effect, as killing the tumor cells, and negative effects, as being mutagenic, teratogenic and carcinogenic. However, the manner of their action is unclear.[80]

It was revealed that the pharmacological activity of CLB, its analogs and metabolites depends of a variety of facts and some controversy is present.[134] Some studies show approximately twice stronger acute toxicity of PAM compared to CLB in mice.[132,134,144,145] More disputed is the antitumor activity of PAM that is similar to CLB[132] or two-fold higher than CLB.[134,144] In addition, PAM has similar cytotoxic activity as CLB against the human tumor cells in vitro,[146] but higher teratogenic and cytotoxic effect in a rat-embryo model.[147] However, the therapeutic index of the metabolite is similar[134] to or two-fold lower[132] than the therapeutic index of the parent drug. A reason for the lower therapeutic index may be the type of metabolism involved.[148] The bifunctional alkylators, including CLB and PAM, are known to exert their cytotoxic action through the DNA-cross linking.[149]

CLB and PAM are S_N2 type of alkylators, which means that they react mainly with the endocyclic nitrogens of the purine and pyrimidine nucleosides. The half life of PAM in aqueous solution at neutral pH is slightly shorter than the one of CLB[140, 150] and suggests that PAM is slightly more reactive compared to CLB.

Based on their chemical structure, PAM is slightly more soluble in water than CLB.

3.2. Reactions of chlorambucil and phenylacetic acid mustard in the absence of 2'-deoxyribonucleosides

Since the predominant reaction of CLB and PAM in aqueous solution is their hydrolysis, the identification of the major products of hydrolysis was necessary prior to studies on the reactions of CLB and PAM with 2'-deoxyribonucleosides.

CLB and PAM were allowed to react in a nonnucleophilic buffer (cacodylic acid, 50 % base) at pH 6.8 and 37 °C for 24 hours and the reactions were followed by HPLC techniques. The products of CLB and PAM hydrolysis, respectively, were characterized by HPLC-MS as well as by spiking with authentic samples synthesized according to the literature.[140,151]

Products of CLB and PAM hydrolysis are shown in Chart 9. In the case of CLB, the major product was *N*,*N*-bis(2-hydroxyethyl)-*p*-aminophenyl butyric acid (**23**) with a molecular ion of 268. The second most abundant hydrolysis product was an ester (**25**) with a molecular ion of 517. Other products formed were esters **26** and **27** with a molecular ion of 780, and an ether derivative **24** with a molecular ion of 517; **24** was stable under basic conditions, which proved this structure to consist of an ether bond. The structures of **25-27**, containing an ester linkage, were confirmed by saponification: all of them formed compound **23**.

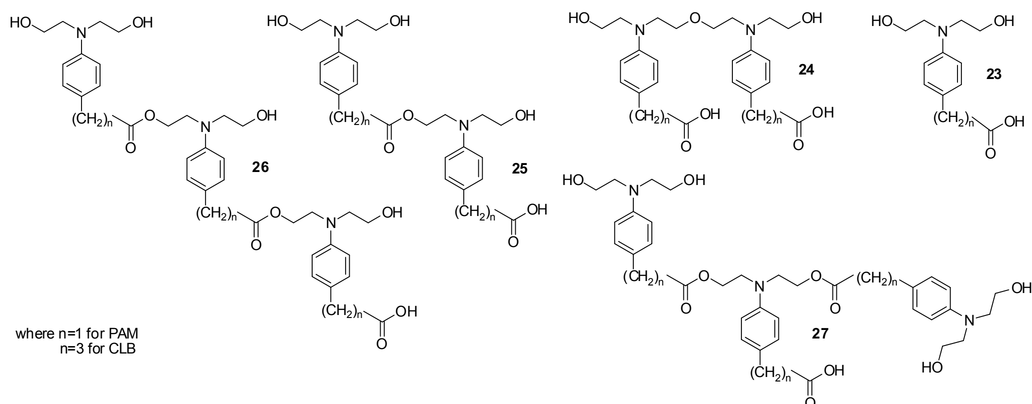


Chart 9. Main products of the hydrolysis of CLB and PAM

The pseudo-first-order rate constants were determined based on the hydrolysis of CLB and PAM at physiological conditions. The rate of decomposition of the two mustards were similar, as the k_{obs} for the disappearance of CLB and PAM were 5.84×10^{-4} s^{-1} and $4{,}44\times10^{-4}$ s^{-1}, respectively. The effect of an external nucleophile, imidazole, on the product distribution was also studied. The results are incorporated in Table 1.

Table 1. Pseudo first-order rate constants (k_{obs}) of hydrolysis and mole fraction (χ) of the diols of CLB and PAM as the function of [imidazole]; where diols refer to 4-(4-(bis(2-hydroxyethyl)amino)phenyl)butanoic acid and 2-(4-(bis(2-hydroxyethyl)amino)phenyl) acetic acid for CLB and PAM, respectively.

Alkylating agent	k_{obs} [10^{-4} s^{-1}]	χ [%] of diol in the presence of 0.05 M imidazole	χ [%] of diol in the presence of 0.10 M imidazole
CLB	5.86 ± 0.11	0.51	0.21
PAM	4.44 ± 0.03	0.52	0.21

3.3. *Reactions of chlorambucil and phenylacetic acid mustard with 2’-deoxyribonucleosides*

2.5 mM CLB (**8**) and PAM (**21**), respectively, were allowed to react in the presence of 16.1 mM 2’-deoxynucleosides in 0.2 M cacodylic acid buffer (50 % base, pH 6.8) for 24 hours at 37 ˚C. However, the concentration of CLB in the reaction with 2’-deoxyadenosine (dAdo) was 0.6 mM. The nucleosides used in these studies were dAdo, 2’-deoxyguanosine (dGuo), 2’-deoxycytidine (dCtd), 2’-deoxy-5-methylcytidine (dMeCtd), and thymidine (Thd). CLB and PAM reacted with various heteroatoms of the nucleosides and numerous adducts were obtained, although the main reaction observed was the hydrolysis of CLB and PAM.

The reactions were followed by HPLC techniques. After the reactions were completed, the reaction mixtures were injected directly onto the column and analyzed by analytical HPLC. Then the products from the reaction mixtures were

separated by semipreparative HPLC and the obtained fractions were collected and characterized by means of UV, HPLC-MS, ESI-MS and NMR.

3.3.1. Reactions of chlorambucil and phenylacetic acid mustard with 2'-deoxyadenosine

The HPLC trace on the reaction of CLB with dAdo after 24 hours is shown in Figure 4. The substances marked as **28-34** are CLB - dAdo adducts, compounds **23** and **25** are products of CLB hydrolysis, while the peaks marked with asterisks are unidentified impurities.

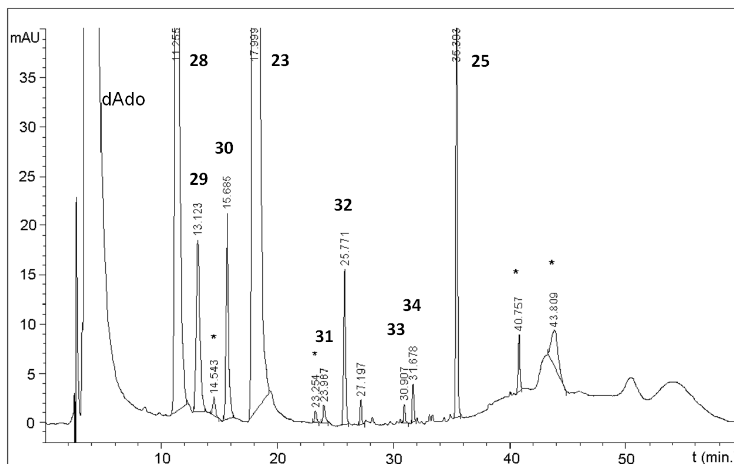


Figure 4. HPLC trace of the reaction of 0.6 mM CLB with 16 mM dAdo in 0.2 M cacodylic acid buffer (50 % base) after 24 hours at 37 °C.

Adducts **28** and **32** (Chart 10) gave the same molecular ion of 501 that referred to the structures were the alkylation had taken place at one site of dAdo. The presence of the fragment ion 385.3 in both adducts indicated the loss of the carbohydrate moiety and proved that the site of alkylation was at the adenine moiety. Several facts were taken into account to clarify whether the alkylation had occurred at the endocyclic or exocyclic nitrogen atom (*N*1 or N^6, respectively) of dAdo: a) the intensity of 385.3 ion was larger in the MS spectrum of **32** than in the spectrum of **28**; b) in the ultraviolet absorption spectra, the maximum absorption of the **28** is red-shifted comparing to **32** at neutral pH (Figure 5), which means that λ_{max} value of **28** is lower than of **32;** this is in accordance with previous literatures related to the UV spectral properties[152, 101] for endocyclic and exocyclic *N*-atoms, respectively.

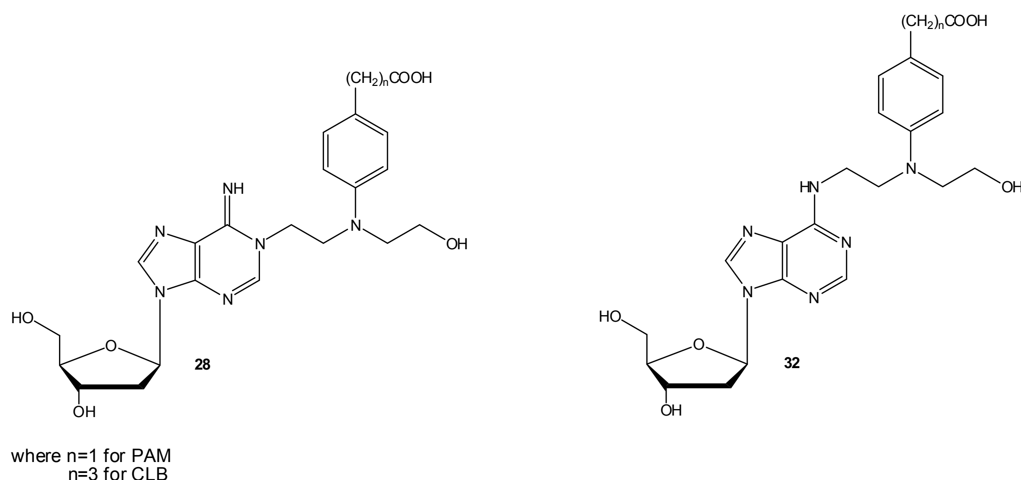


Chart 10. Structures of *N*1- and *N*^{6}-alkylated adduct of CLB/PAM – dAdo reactions.

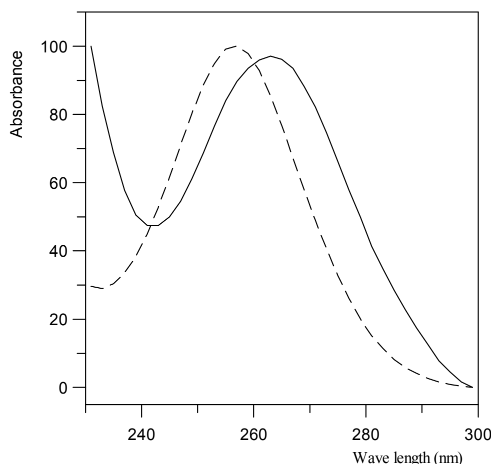


Figure 5. UV spectra of substance **28** marked with dashed line and substance **32** marked with solid line.

The site of alkylation of compounds **28** and **32** was verified by comparison to the available literature data on ^{1}H NMR.[153] The resonance of the adenine-linked methylene protons is different when the alkylation is on endocyclic nitrogen or exocyclic nitrogen of adenine. In the case of CLB, the chemical shift of the proton from Ade-CH_2 was shifted from 4.42 ppm for *N*1-alkylated adduct to 3.80 ppm for *N*^{6}-alkylated derivative.

Based on the facts described above, **28** was assigned as an *N*1-adduct and **32** as an *N*^{6}-adduct of dAdo. Substance **28** can be also assigned as *N*1-alkylated product based on the Dimroth rearrangement[154,155,156] of **28** to **32** when treated with aqueous base, reaction that is characteristic for *N*1-alkyladenine nucleosides. This transformation is a base-catalyzed reaction.

It is known that different alkylating agents alkylate adenosine at *N*1 and N^6 site.[157] N^6 adduct of the reaction of CLB with dAdo was the result of the direct alkylation of N^6 site of the nucleoside, and not a result of the Dimroth rearrangement of the *N*1 adduct; that was demonstrated by the very slow rearrangement of *N*1 to N^6 derivative under the reaction conditions employed.

Compound **31** (Chart 11) had the same molecular ion of 501, as **28** and **32**, in the case of reaction of dAdo with CLB. This adduct was identified as a carbohydrate derivative based on its mass spectra that shows a fragment ion of 366, which represents the loss of adenine base. A similar spectrum was observed previously[150] for an adduct of 2'-deoxyguanosine in which the alkylation had taken place at the 2'-deoxyriboside moiety. Because of steric reasons, substance **31** was tentatively assigned as 5'-*O*-alkylated adduct, and not as 3'-*O* adduct.

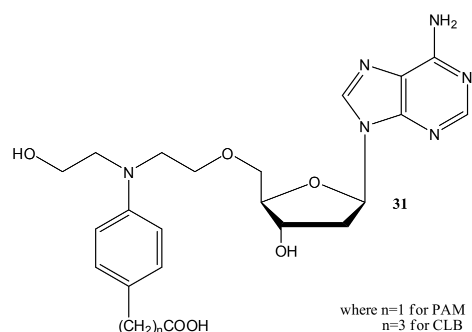


Chart 11. Structures of 5'-*O*-alkylated adduct of CLB/PAM – dAdo reactions.

Adducts **29** and **30** (Chart 12) had the same molecular ion mass that referred to an adduct where the alkylation had taken place at only one site of the adenine base. In the case of CLB – dAdo reactions, the MH^+ was 385 for compounds **29** and **30**. This information and the fact that the alkylation at *N*3 or *N*7 of 2'-deoxyadenosine labilizes the *N*-glycosidic bond[81, 101, 158] led us to the believe that **29** and **30** were *N*3- and *N*7-alkylated derivative.

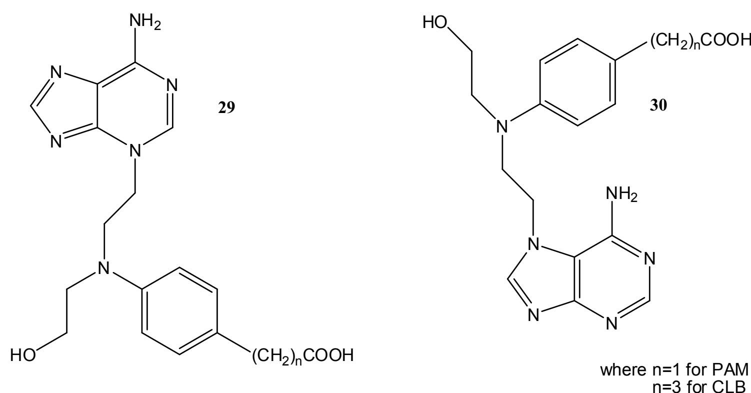


Chart 12. Structures of *N*3- and *N*7-alkylated adduct of CLB/PAM – dAdo reactions

The final assignment was based on NMR experiments (^{1}H- and ^{13}C-NMR, COSY, NOESY, HSQC, HMBC) which were carried out to assign unequivocally the site of alkylation within these two adducts. Spectra were recorded in D_2O or DMSO-d_6 at 25 °C. Figure 6 shows the ^{1}H NMR spectra of **29** and **30** in DMSO-d_6. According to literature,[159,160] the $\Delta\delta$ of the chemical shifts of the H-2 and H-8 protons is larger with 3-alkyladenine than with the corresponding 7-alkyladenine. In the current case, $\Delta\delta$ of **29** was 0.36 ppm and $\Delta\delta$ of **30** 0.13 ppm. The carbon chemical shifts in the ^{13}C NMR spectra for **29** and **30** also showed clear differences between the alkylation at *N*3 or *N*7 of adenine.

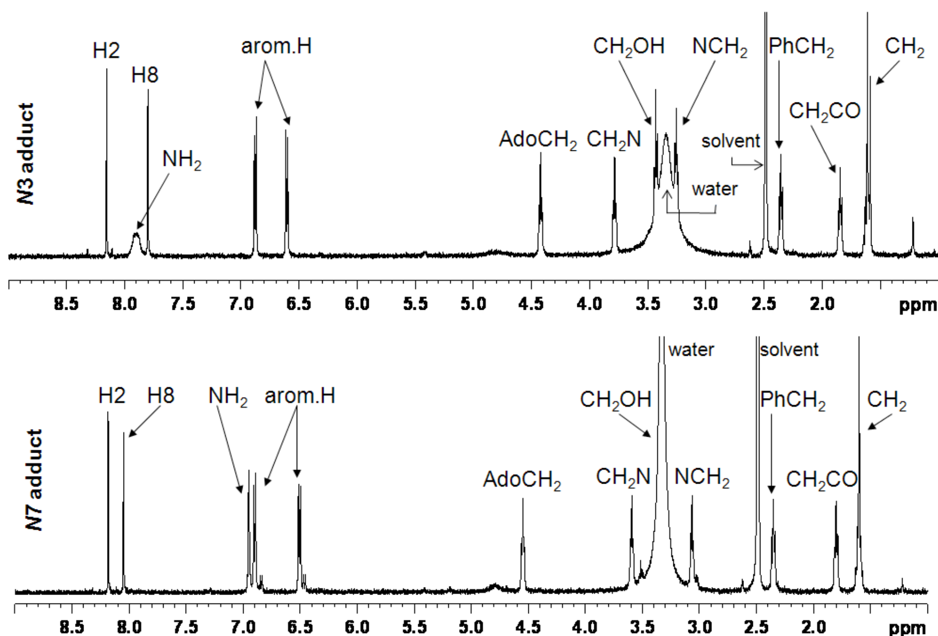


Figure 6. ^{1}H NMR of *N*3-alkylated adenine (up) and *N*7-alkylated adenine (down); run in DMSO-d_6 at 25 °C.

While the ^{1}H- and ^{13}C-NMR experiments together provided substantial evidence for structural characterization of the *N*3- and *N*7-adducts, the NOE correlation brought the most conclusive information for the identification of the alkylation site. It was crucial that the NOESY experiment was done in DMSO-$d_{6,}$ so that the NH_2-protons and the correlation with them could be observed. Substance **30** showed correlation between the protons of NH_2 of adenine and the protons of Ade-CH_2 due to their vicinity in space (Figure 7) and it was assigned as *N*7-alkylated adduct, while substance **29** did not present this type of correlation and it was assigned as the *N*3-alkylated adduct.

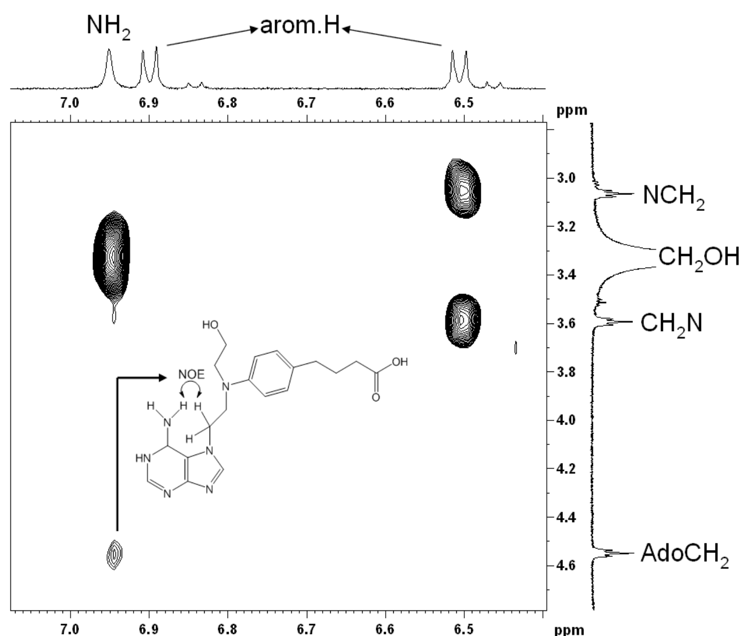


Figure 7. The significant NOE correlation in *N*7-alkylated adenine (**30**).

There are many reactive chlorambucil dimers, such as monochloro, dichloro and trichloro derivatives of **25**, in the reaction mixtures and this could be an explanation for the appearance of esters in the reaction mixture. Substances **33** and **34** (Chart 13) were tentatively assigned as the esters of *N*1 adduct of 2'-deoxyadenosine based on their mass spectra and their lability under basic conditions due to hydrolysis of the ester function (saponification) present in their structure.

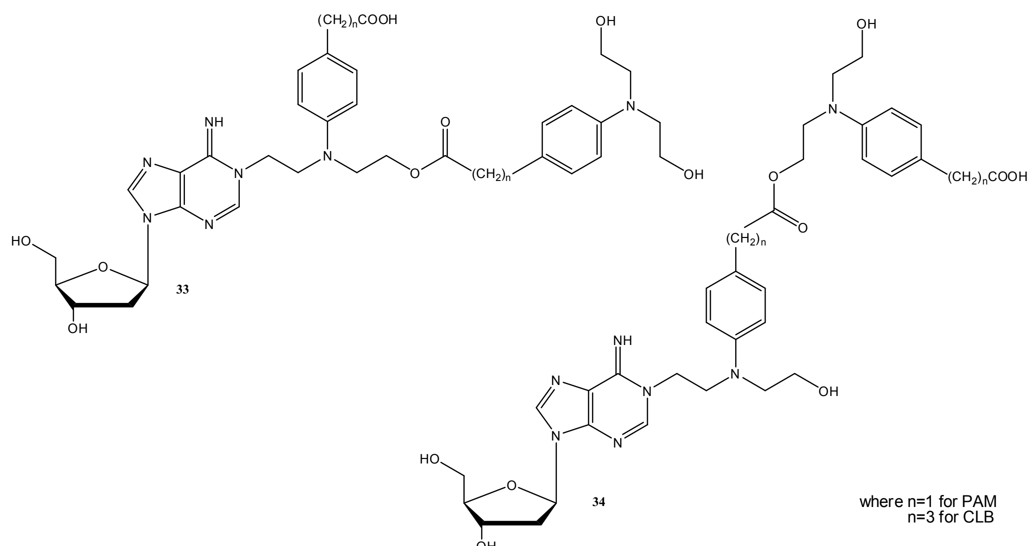


Chart 13. Structures of *N*1-adducts derived from the reaction of CLB dimers/PAM dimers with *N*1-adduct of dAdo.

Cross-links, in which one chlorambucil molecule bridged two dAdo molecules, were not observed.

One extra minor adduct was produced by the reactions of PAM and CLB with dAdo. Its proposed structure is shown in Chart 14 and, based on the isotopic distribution observed in MS data, it might be *N*1-alkylated adduct of dAdo that still has a chlorine atom attached to ethyl chain of the molecule. It is not known why the second chlorine atom was not displaced.

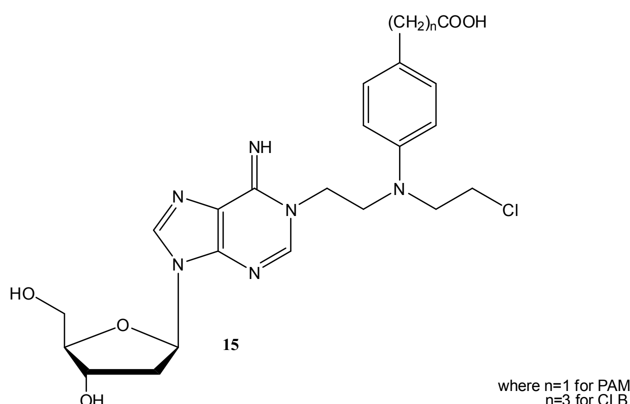


Chart 14. Structure of *N*1-adduct with a Cl-atom left present in PAM-dAdo reaction.

Hence, many adducts were detected and, as expected,[31] the *N*1-alkylated derivative was the main adduct among them. Other alkylation sites observed in the reaction of CLB/PAM with dAdo were N^6, *N*7, *N*3 and 5'-*O*.

3.3.2. Reactions of chlorambucil and phenylacetic acid mustard with 2'-deoxyguanosine

The reaction of CLB with dGuo had been in the attention of our laboratories before.[150] In this thesis work, the reaction of phenylacetic acid with 2'-deoxyguanosine was studied and the products characterization was based on the previous literature.[150]

The HPLC trace for the reaction of PAM with dGuo after 24 hours is shown in Figure 8. Several adducts (**35-45**) were identified and characterized by means of HPLC-MS and ^{1}H NMR.

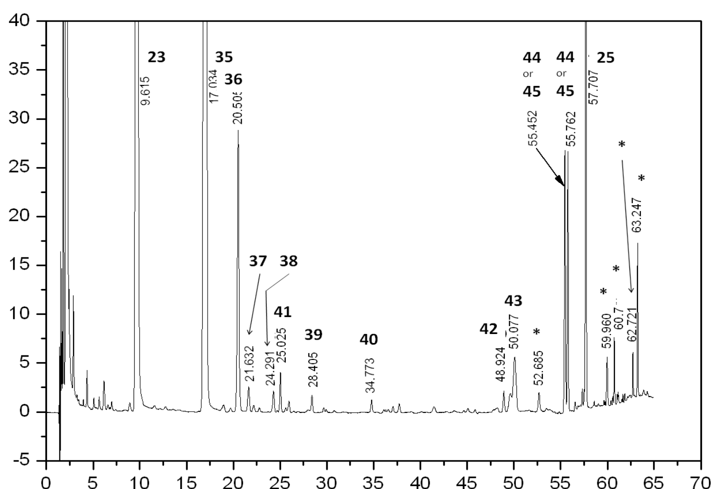
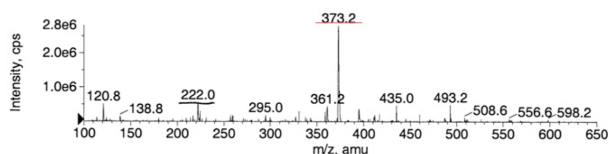
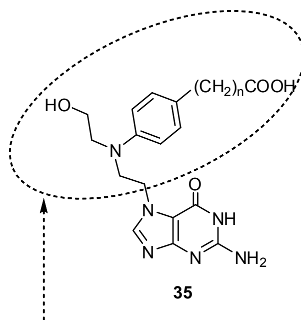


Figure 8. HPLC trace of the reaction of 2.5 mM PAM with 16.1 mM dGuo in 0.2 M cacodylic acid buffer (50 % base) after 24 hours at 37 ˚C. Note: **23**, **25** are products of PAM hydrolysis and peaks marked with * are impurities.

The most abundant adduct formed was **35** (Chart 15). Its structure was analyzed by MS which showed the molecular ion 373 and a fragment ion 222 that corresponds to the mass of the CME substituent (Chart 15). The presence of ion 222 indicates the alkylation on guanine base. ^{1}H NMR analyses proved that **35** is the *N*7-adduct of guanine. This is as expected[31] because the *N*7 site is the most nucleophilic site of guanine base and thus, the site most prone to alkylation.





where n=1 for PAM
n=3 for CLB

CME is 2-((4-(carboxymethyl)phenyl)(2-hydroxyethyl)amino)-ethyl substituen, when n=1
CPE is 2-((4-(3-carboxypropyl)phenyl)(2-hydroxyethyl)amino)-ethyl substituent, when n=3

Chart 15. MS spectra and structure of *N*7-alkylated guanine from the reaction of PAM with dGuo.

The second most abundant adduct was **36** that had a molecular ion of 506 (Chart 16) and it was assigned as a deglycosylated dimer based on its MS data.

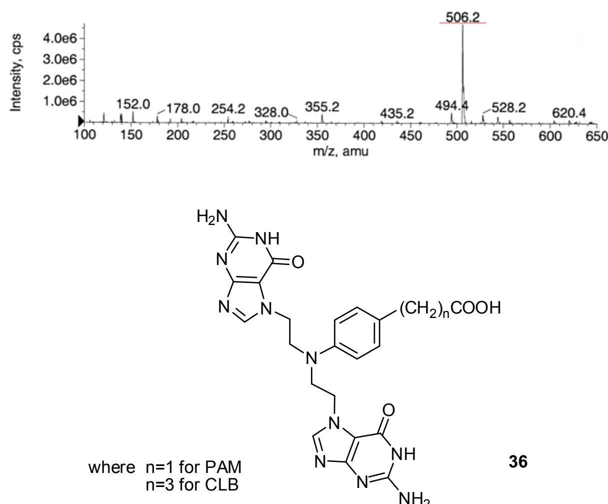


Chart 16. MS spectra and structure of *N*7,*N*7-bis alkylated guanine from the reaction of PAM with dGuo.

Four products (**37-40**) had the same molecular ion of 489 (Chart 17). They were tentatively identified according to their MS data (Figure 9 a-d). Compound **37** had a fragment ion of 338 that indicated the loss of unmodified guanine base. This means that the reaction with the alkylating agent had taken place at the carbohydrate moiety, most likely at 5'-*O*. Substance **37** was identified as the 5'-*O*-alkylated adduct.

Fragment ion 373 is present in the mass spectra of compounds **38**, **39** and **40**, indicating the loss of the sugar moiety, and that the alkylation had taken place at the guanine base. Thus, the alkylation site could to be *N*1, N^2, *N*3 or O^6 site of guanine base. Compounds **39** and **40** had a fragment ion 222 indicating the cleavage of the CME group. Based on our observations, the absence of that fragment was characteristic to the compounds were the alkylation had taken place at their endocyclic *N* atom, while its presence was an indication that the alkylation had taken place at one of the exocyclic heteroatoms of the guanine base. In the case of **39**, the intensity of the signal at 222 was much higher than with **40** and, on the basis of the previous studies of CLB with dGuo,[150] that could be characteristic to the O^6-alkylated compound which is more labile. The final identification of **39** as an O^6-alkylated adduct and **40** as an N^2-alkylated adduct was based additionally on the lability of **39** under basic conditions, which was observed previously in the case of *O*-alkyl groups.[89] Substance **38** was assigned as an *N*1-alkylated derivative based on its fragment ion 373 and the missing fragment ion 222. The *N*3-alkylated adduct was detected neither in PAM-dGuo reaction nor in CLB-dGuo reaction and one may speculate that this might be due to the large size of PAM/CLB molecule that make difficult for such molecule to react with the sterically hindered *N*3 site of dGuo.

However, knowing that PAM/CLB reacts with the *N*3 position of dAdo (*see* section 3.3.1.) and that dAdo has same large size as dGuo, probably additional factors beside steric effect are involved.

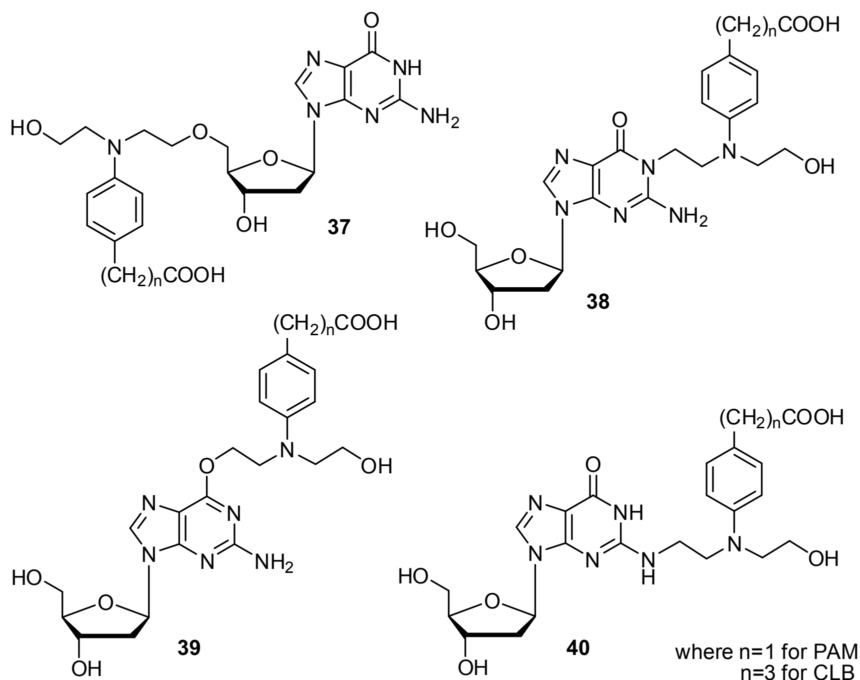


Chart 17. Structures of 5'-*O*- (**37**), *N*1- (**38**), O^6- (**39**), and N^2- (**40**) alkylated derivatives of reactions between dGuo and PAM/CLB.

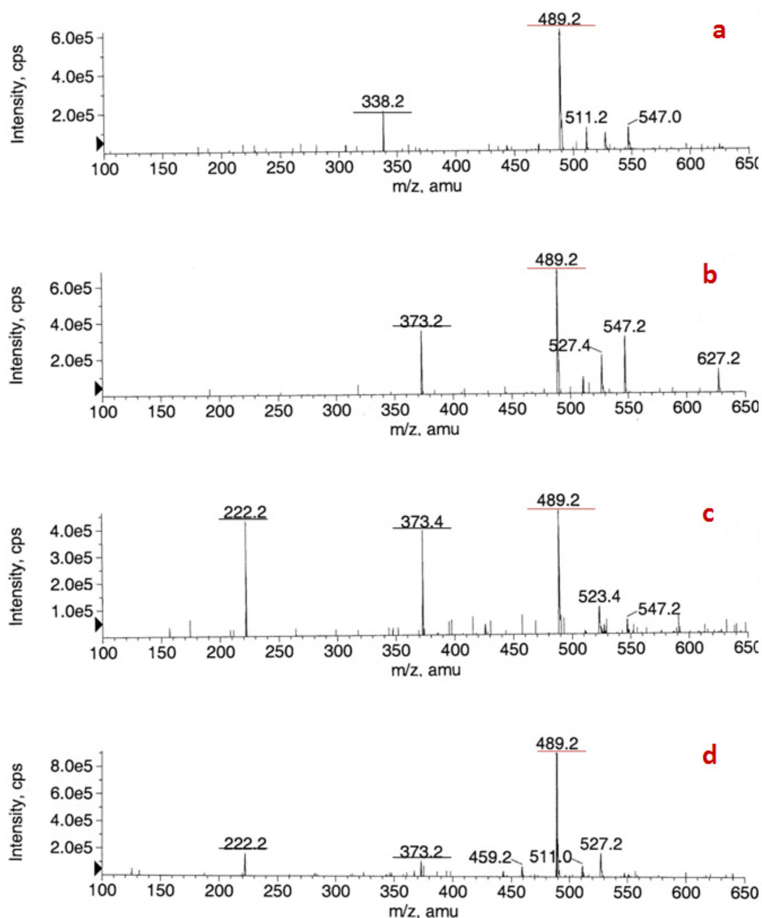


Figure 9. MS spectra of 5'-*O* adduct (**a**), *N*1 adduct (**b**), *O*[6] adduct (**c**), and *N*[2] adduct (**d**).

Substances **41-45** (Chart 18) all showed the same molecular ion of 594 in the case of reaction of dGuo with PAM. Compound **41** was identified as a *N*7,*N*9-bis adduct. **44** and **45** were tentatively assigned as the *N*7 adducts derived from the reactions of two different PAM dimers with *N*7 of guanine; the corresponding derivatives were not observed in the reaction of dGuo with CLB. The formation of *N*7,*N*9-bis alkylated adduct was expected, since its presence had previously been reported.[161] Compounds **42** and **43** could not be identified on the basis of their MS data, but they might be the isomeric adducts of **44** and **45** or positively charged dialkylated guanine analogs of **41**.

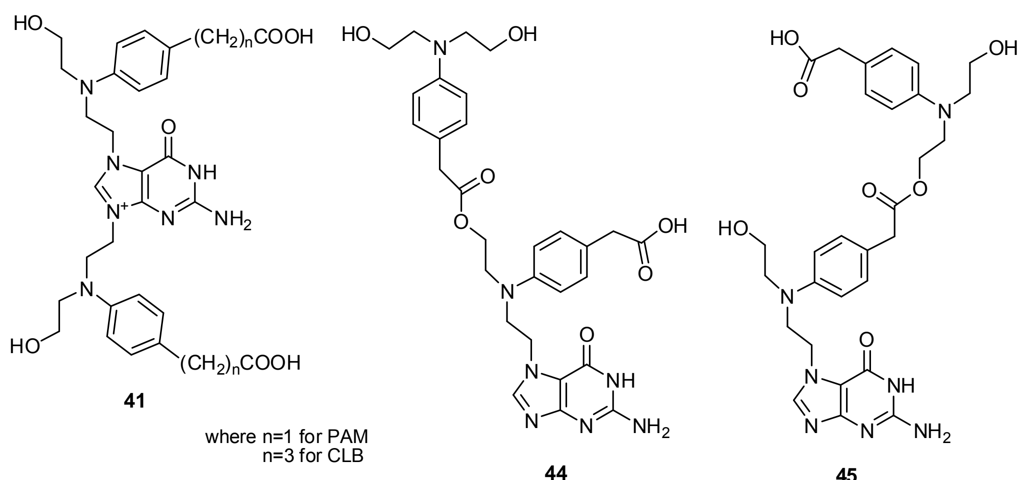


Chart 18. Structures of *N*7,*N*9-bis alkylated adduct (**41**), and esters of *N*7-alkylated guanine derivatives (**44**, **45**) formed by reactions between dGuo and CLB, dGuo and PAM.

Thus, many adducts were detected. *N*7-alkylated derivative was the main adduct among them. Other alkylation sites observed in the reaction of CLB/PAM with dGuo were 5'-*O*, *N*1, O^6, N^2 and *N*9.

3.3.3. Reactions of chlorambucil and phenylacetic acid mustard with 2'-deoxycytidine and 2'-deoxy-5-methylcytidine

The HPLC trace of the reaction of 2'-deoxycytidine with chlorambucil after 24 hours is presented in Figure 10 and the corresponding HPLC trace of the reaction of 5-methyl-2'-deoxycytidine with chlorambucil is shown in Figure 11. dCtd and dMeCtd, which differ from one to another only by a methyl group in C-5 position, exhibit quite similar alkylation sites in their reactions with CLB/PAM. The structures of these adducts can be seen in Chart 19. The reaction of CLB with dCtd and dMeCtd is taken as an example.

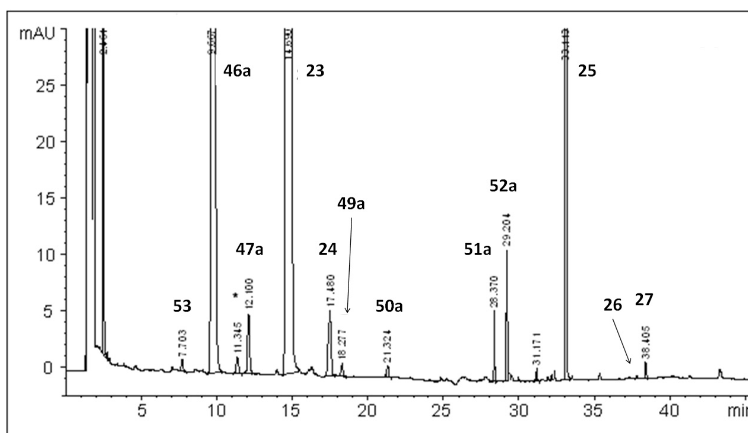


Figure 10. HPLC trace of the reaction of 2.5 mM CLB with 16.1 mM dCtd in 0.2 M cacodylic acid buffer (50 % base) after 24 hours at 37 ˚C.

Four of the detected adducts (**46a**, **47a**, **49a** and **53**) gave the same molecular ion 477, which referred to a monomeric adduct of dCtd. The alkylation on the cytosine base of **46a** is proven by the presence of the fragment ion of 361, which shows the loss of the sugar moiety. Further, the ^{1}H resonance of the methylene group linked to the pyrimidine moiety exhibits values (4.37 and 4.08 ppm) comparable to those cases where the alkylation had occurred at the endocyclic nitrogen.[153] Hence, compound **46a** was assigned as the *N*3 derivative.

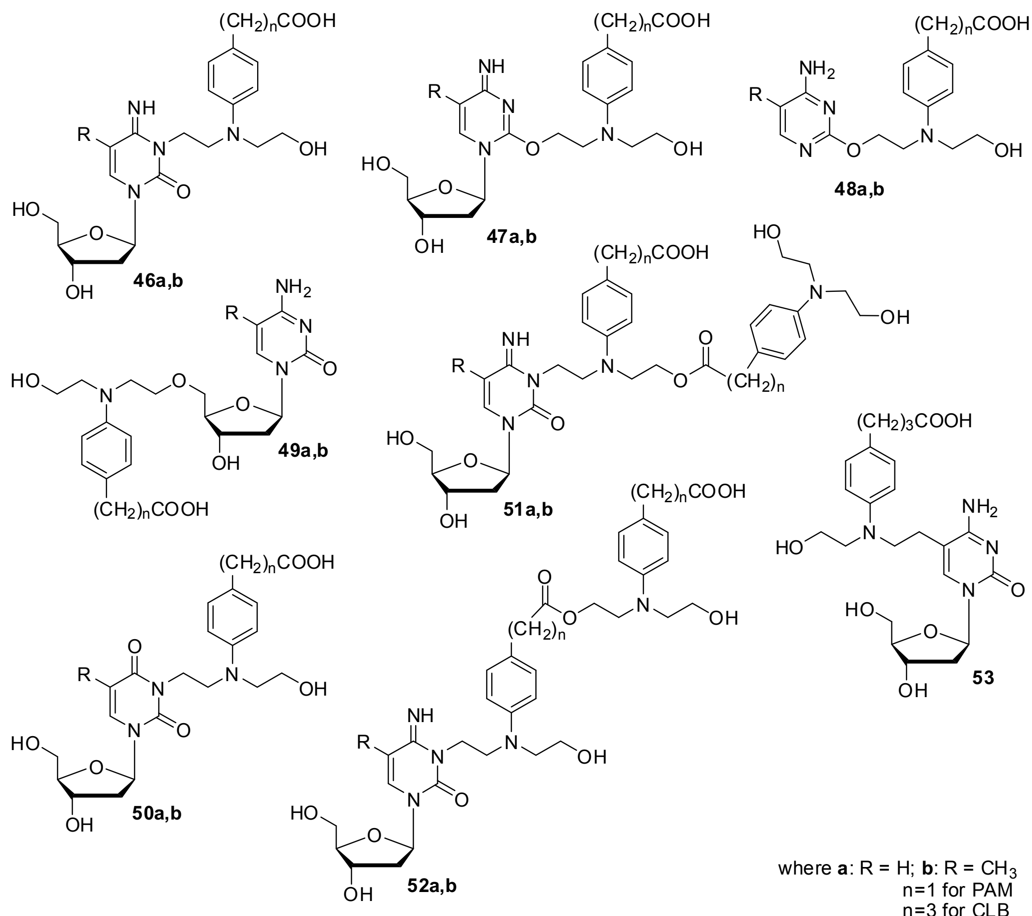


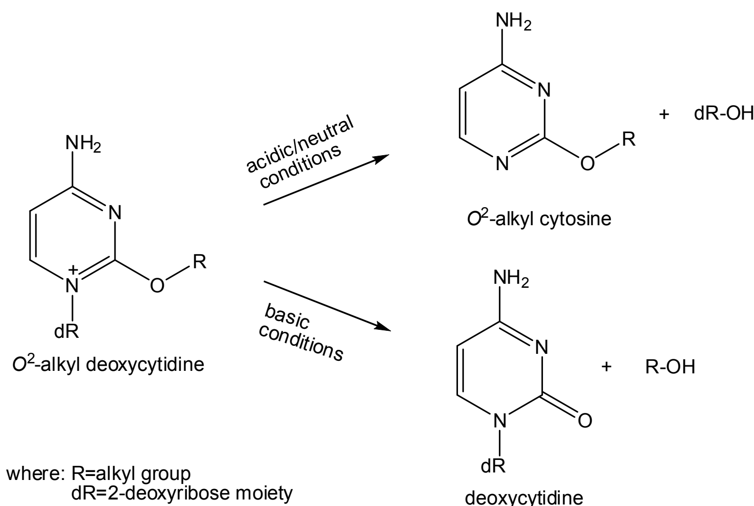
Chart 19. Structures of the products of the reactions between dCtd and CLB (**46a** to **52a, 53**), and dMeCtd and CLB (**46b** to **52b**).

Substance **47a** was characterized by the MS data. The mass spectrum showed a strong fragment ion 250, which indicated that the alkylation had occurred on the exocyclic oxygen[150] (*see* **3.3.2.**). **47a** additionally had the fragment ions 361 and 366, which referred to the loss of alkylated cytosine base and alkylated sugar moiety, respectively. This could be explained by alkylation of O^2 and migration of the alkyl group from O^2 to 5'-*O* of the deoxyribose moiety in the spectrometer during the MS analysis. The corresponding O^2-derivative was not observed in the reaction of PAM with dCtd.

Substance **49a** was characterized on the basis of the MS data that showed a fragment ion 366, referring to the loss of unmodified cytosine base. **49a** was an adduct in which the alkylation had occurred at deoxyriboside moiety.

Substance **53** was present in a very small amount. A tentative assignment was made on the basis of the molecular ion of 477 and a fragment ion of 361. In other words, a strong covalent bond between the alkyl moiety and the cytosine base did not brake further during ionization. This tentative assignment was further confirmed by the fast elution of **53** from the reverse-phase column which is specific to nucleosides with no alkylation on their heteroatoms. Even more, this type of adduct was not observed in the case of dMeCtd and Thd reaction with CLB, where *C*5 position is occupied by a methyl group. Thus, compound **53** was assigned as *C*5 adduct. The corresponding derivative was not detected in the reaction mixtures of PAM and pyrimidine nucleosides. While the reactions of *C*5-*C*6 double bond of pyrimidine nucleosides are well known,[162] the reactions of a carbon electrophile of an alkylating agent with *C*5 has not been mentioned before, to the best of our knowledge.

The structural assignment of **47a** and **48a** was based on the MS data and on the fact that **47a** in acidic and neutral solutions gives rise to **48a** as a major product, due to the destabilization of the *N*-glycosidic bond,[89, 163] and to diol **23** as a minor product. By contrast, under basic conditions, only the O^2-alkyl group is cleaved from **47a** (Scheme 7). This verifies that **47a** is O^2-alkylated deoxycytidine and **48a** is O^2-alkylated cytosine. The O^2-alkyl pyrimidines are difficult to assign because of their instability.[62] However, the identity of **48a** was further proven by ^{1}H NMR spectroscopy of the corresponding deglycosylated O^2 adduct formed in the reaction of PAM with dCtd.



Scheme 7. General scheme showing the behavior of O^2-alkyl deoxycytidine in neutral, acidic and basic conditions.

Compound **50a** showed a molecular ion of 478, which is typical for a deaminated adduct where *N*3 of the base moiety has been alkylated. This assignment was also

supported by the coelution of **50a** with the product raised from the treatment of pure **46a** with basic solution.

Based on their molecular ion of 726 and lability under basic conditions, substances **51a** and **52a** were tentatively assigned as esters formed from the reaction of CLB dimer with *N*3 of dCtd.

The HPLC trace of the reaction of chlorambucil with 5-methyl-2'-deoxycytidine is shown in Figure 11. The products formed in this reaction are similar to those obtained by the reaction of CLB with dCtd (Chart 19).

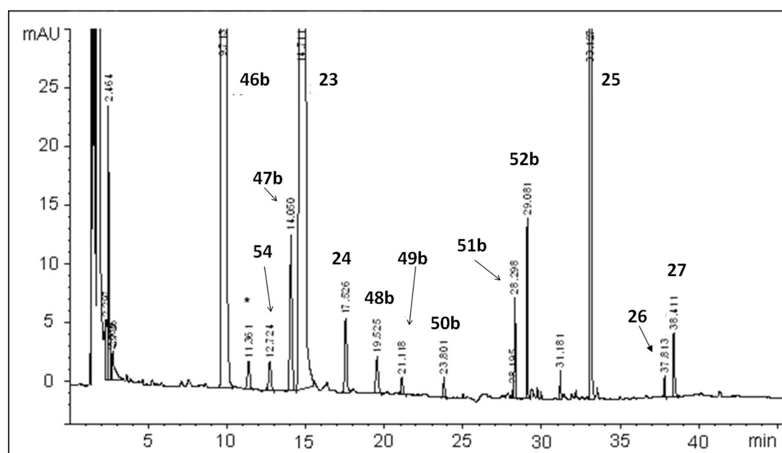


Figure 11. HPLC trace of the reaction of 2.5 mM CLB with 16.1 mM dMeCtd in 0.2 M cacodylic acid buffer (50 % base) after 24 hours at 37 °C.

The major product was the *N*3 adduct **46b**, as judged by ^{1}H NMR and MS analyses. The carbohydrate derivative **49b**, the deaminated *N*3 adduct **50b**, and the *N*3 esters **51b** and **52b** were present in comparable quantities in the reaction mixtures. The O^2 adducts, including O^2-alkylated nucleoside and the corresponding nucleobase, were present in higher amount than in the reaction with dCtd: the relative abundance of (**47b**+**48b**) was 7.5 % compared to the relative abundance of (**47a**+**48a**) that was 2.4 %; no explanation could be given for this difference.

The relative abundance of the O^2-alkylated products in the reaction of dMeCtd with PAM was also much higher than in the case of dCtd: 8.3 % *vs.* 2.6 %. The deaminated derivative, in turn, was present in a very small amount. It even was very difficult to be detected.

No cross-linked derivative where a single CLB or PAM molecule was connected to two dCtd or two dMeCtd molecules could be detected. No alkylation at N^4 of pyrimidine nucleosides was observed. In this respect the results differ from those of the earlier studies of cytidine with ethylating agents[10] or 2'-deoxycytidine with epoxides,[164] in which the N^4-alkylated adduct was formed at neutral pH.

The *N*3-alkylated dCtd and dMeCtd were relatively stable under the reaction conditions employed. Even so, they deaminated considerably to their corresponding

derivatives **50a** and **50b** at neutral pH, especially in the case of CLB. This was surprising because the spontaneous deamination of cytosine nucleoside is known to be slow. [162,165] Possibly the N3-alkylation enhances the deamination since the rates of deamination were similar for the *N*3 derivatives in the reactions of dCtd and dMeCtd with CLB; in this situation, the corresponding *N*3 derivative of dCtd-PAM deaminated more readily than the corresponding dMeCtd-PAM. This is also unexpected because it is known that dMeCtd is more susceptible to deamination than dCtd.[162]

*N*3 derivatives of dCtd and dMeCtd were treated in aqueous base and only the deaminated products were observed, in contrast to other cytosine derivatives[166] which gave also products of Dimroth rearrangement.

Compound **54** is probably a nonnucleosidic impurity, as suggested by its UV and MS spectrum.

3.3.4. Reactions of chlorambucil and phenylacetic acid mustard with thymidine

Thymidine is the least reactive nucleoside among the purine and pyrimidine nucleosides studied in this thesis work. The HPLC trace of the reaction of chlorambucil with thymidine is shown in Figure 12 and the products formed in this reaction can be seen in Chart 20.

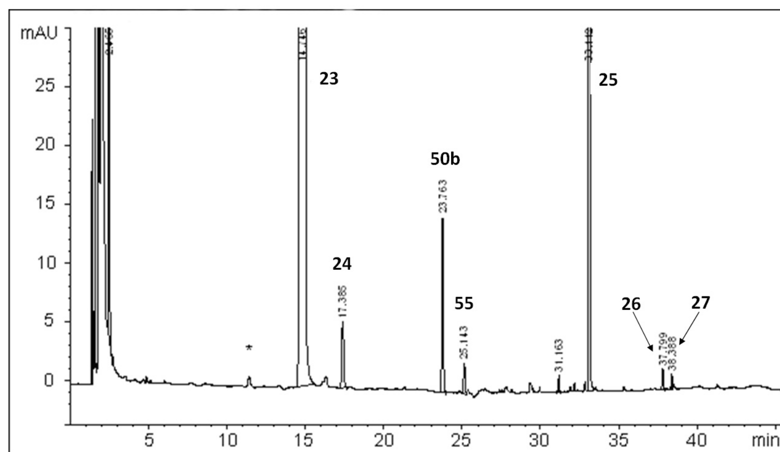


Figure 12. HPLC trace of the reaction of 2.5 mM CLB with 16.1 mM Thd in 0.2 M cacodylic acid buffer (50 % base) after 24 hours at 37 ˚C.

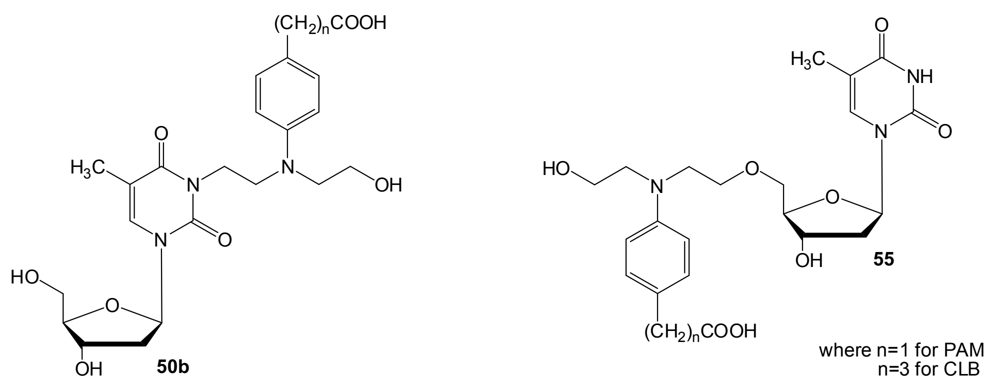


Chart 20. Structures of products of thymidine – chlorambucil reaction

Product **50b** was characterized by means of ^{1}H NMR, MS/MS, HPLC-MS analyses and by coelution with the deaminated *N*3 derivative of 5-methyl-2'-deoxycytidine. **50b** is the *N*3 alkylated derivative and it is the main alkylation site of thymidine, as expected.[167]

The second abundant adduct, **55,** was assigned as a carbohydrate derivative on the basis of the HPLC-MS analysis. No other adducts were detected in the case of thymidine.

3.4. Reactions of chlorambucil with calf thymus DNA

The reactions of CLB with single-stranded and double-stranded calf thymus DNA were performed in 0.2 M cacodylic acid (50 % base) at pH 6.8, at 37 °C for 24 hours. Then, the DNA was enzymatically hydrolyzed to 2'-deoxynucleosides. On the basis of NMR, UV, HPLC, LC-ESI-MS/MS studies, as well as by spiking with standard samples obtained in our previous work (**I**, **II**), the major DNA-CLB adducts were assigned as *N*1 adduct of 2'-deoxyadenosine (**28**), *N*3 adduct of 2'-deoxyadenosine (**29**), *N*3 adduct of 2'-deoxycytidine (**46a**) and *N*7 base adduct of 2'-deoxyguanosine (**56**) (Figure 13, Chart 21).

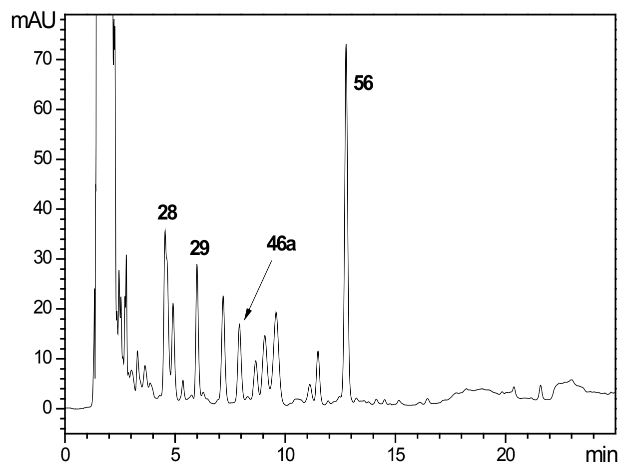


Figure 13. HPLC trace of the reaction of chlorambucil (2.5 mM) with calf thymus single-stranded DNA in 0.2 M cacodylic acid buffer (50 % base) after 24 hours at 37 °C.

Several minor products were detected and characterized by coelution with authentic samples and by MS. Among these adducts were N^6 adduct of 2’-deoxyadenosine (**32**), *N*7-alkylated nucleoside (**58**) and *N*7,*N*7-bis (**57**) adducts of 2’-deoxyguanosine, deaminated *N*3 (**50a**) and O^2-alkylated (**47a**) adducts of 2’-deoxycytidine, and *N*3 adduct of thymidine (**50b**). All these products were present in the hydrolysates of both, single-stranded and double-stranded DNA. **57** was the only crosslink detected.

Other adducts (**60**, **31**, **49a**) were tentatively characterized as “sugar” derivatives based on their MS data. They might be 3’-*O*-alkylated nucleoside, knowing that the 5’-*O* of DNA is phosphorylated. On other hand, the DNA does not have many free 3’-hydroxy groups and these products may be artifacts.

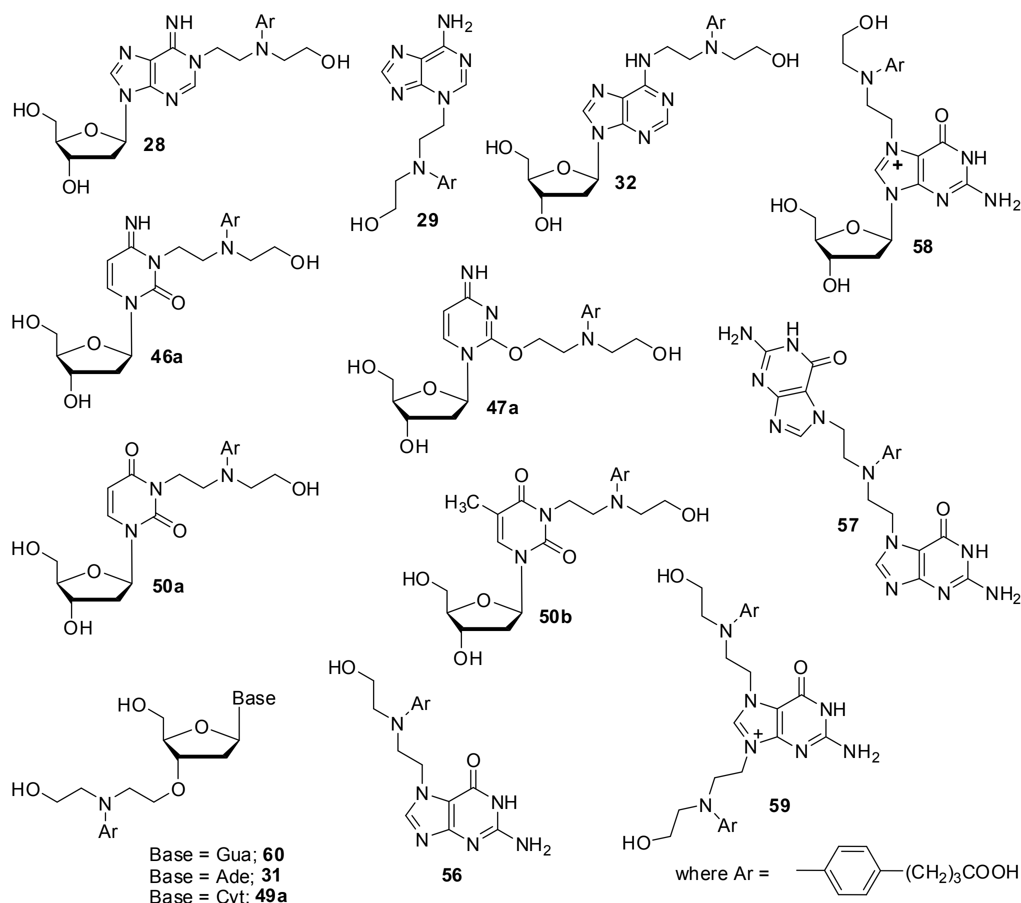


Chart 21. Structures of products of reactions of CLB with ssDNA and dsDNA

*N*7,*N*9-Bis alkylated derivative of guanine (**41**) was also observed, as well as other minor adducts.

These reactions were performed in non-nucleophilic buffer, which means that a CLB molecule has only two options: to react with water (hydrolyses) or to react with DNA. Important to remember is also that the reactivity and concentration of nucleophile determines the site of reaction.

Single-stranded DNA was approximately 3.4 times more reactive than double-stranded DNA. The reactivity order in ssDNA and dsDNA was partially different than in the case of nucleobases (Table 2). The most reactive site in ssDNA was *N*1 of Ade, then *N*7 of Gua, *N*3 of Cyt and *N*3 of Ade. The order of reactivity, from most reactive site to less reactive site, in dsDNA was: *N*3 of Ade, *N*7 of Gua, *N*3 of Cyt and *N*1 of Ade, last two being approximately equally reactive.

Table 2. The order of reactivity of the nucleophilic sites of nucleobases with CLB.

	more reactive ←		→	**less reactive**
Nucleosides	Gua-N7	Ade-N1	Cyt-N3	Ade-N7 / Ade-N3
ssDNA	Ade-N1	Gua-N7	Cyt-N3	Ade-N3
dsDNA	Ade-N3	Gua-N7	Cyt-N3 / Ade-N1	Cyt-N3 / Ade-N1

Surprising was that *N*3 of Ade was the most reactive site in dsDNA, and not *N*7 of Gua. It is known from different studies on the reaction of chlorambucil with DNA that the principal site of alkylation is *N*7 of guanine[168,169,170] and the second site is *N*3 of adenine.[171] The reason for this situation is that guanine *N*7 is situated in the major groove and easily accessible for different alkylating agents, while adenine *N*3 is accommodated with very little distortion of the DNA helix.[172]

Also surprising was that *N*1 of Ade was the most reactive site in ssDNA, and *N*7-alkylated adduct of Ade could not be detected in ssDNA or in dsDNA.

It is known that the nucleophilic sites of nucleobases participating to the base pairing in dsDNA are partly protected from alkylation. That could be observed also in our case, where the *N*3 of Cyt and *N*1 of Ade were less reactive in dsDNA than in ssDNA. Exception is offered by the O^2-alkylated adduct of dCtd which was stable enough to be found also in the hydrolysate of dsDNA.

The molar yields of the main adducts were calculated from the original amount of 2-deoxyribonucleosides (Table 3).

Table 3. Yields of the major adducts present in the DNA hydrolysate.

Adducts mol/10^6 mol of normal nucleotides	**Gua-*N*7**	**dAdo-*N*1**	**Ade-*N*3**	**dCtd-*N*3**
ssDNA	320	390	224	234
dsDNA	90	3	245	4

4. SUMMARY AND CONCLUSIONS

CLB (**8**) was found to be hydrolytically slightly less stable than its metabolite PAM (**21**). CLB and PAM reacted with different heteroatoms of the 2'-deoxyribonucleosides, even though the predominant reaction observed was the hydrolysis of **8** and **21**. The overall reactivity of CLB towards dGuo,[150] dAdo, dCtd, dMeCtd, and Thd was approximately 24, 7, 10, 10, and < 1 %, respectively, based on the decrease of the products of CLB hydrolysis. PAM's overall reactivity towards dGuo, dAdo, dCtd, dMeCtd, and Thd was *ca.* 25, 10, 10, 10, and < 1 %, respectively, based on the decrease of the products of PAM hydrolysis. One difference in the reactivity of CLB and PAM is that the *N*3 dCtd-PAM adduct was deaminated easier than the corresponding CLB derivative.

The order of reactivity of the nucleophilic sites of nucleobases with chlorambucil is in accordance with previous data,[173] while the situation was different in the case of ssDNA and dsDNA: *nucleosides* ⟶ Gua-N7 > Ade-N1 > Cyt-N3 > Ade-N7 = Ade-N3, *ssDNA* ⟶ Ade-N1 > Gua-N7 > Cyt-N3 > Ade-N3, and *dsDNA* ⟶ Ade-N3 > Gua-N7 > Cyt-N3 = Ade-N1.

This thesis work was concentrated at first on the analyses of the nucleosides' reactions with CLB and PAM and the data obtained from them was used as example for the reactions of DNA with CLB. Many adducts were detected and characterized in our work and they may be of biological importance. The medical implication of DNA damage caused by alkylation is becoming more and more important.[13] For example, in the clinical chemotherapy, identifying the CLB adducts formed with DNA, as well as the system used in their repair, help in understanding the drug resistance and its side effects.[174] CLB and PAM have similar chemical behavior, as we have showed,[121] and this is in accordance with the previous suggestions that these two mustards cause DNA lesions with similar effectivines.[80,175]

5. EXPERIMENTAL SECTION

5.1. General

2'-Deoxyribonucleosides (dGuo, dAdo, dCtd, dMeCtd, and Thd), cacodylic acid, *N*,*N*-bis(2-chloroethyl)-*p*-aminophenylbutyric acid, calf thymus DNA (Type I: sodium salt, highly polymerized), nuclease P1 from *Penicillium citrinum*, alkaline phosphatase (*Escherichia coli* Type III), acid phosphatase (wheat germ Type I) and bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane hydrochloride (Bis-tris) buffer were purchased from Sigma, and they were used as received. *N*,*N*-Bis(2-hydroxyethyl)-*p*-aminophenylbutyric acid and {4-[bis(2-chloroethyl)amino] phenyl}acetic acid were synthesized as described previously.[130,140] All inorganic reagents were of ACS grade or better.

5.2. Chromatographic methods

Analytical HPLC analyses were performed on a Hewlett-Packard 1090 Instrument equipped with a diode array detector and autosampler. The reversed-phase column in use was Hypersil ODS2 C18, 4.6 mm × 250 mm with particle size of 5 µm (**I**), or Waters Symmetry C18, 4.6 mm × 150 mm with particle size of 3.5 µm (**II**, **III**, **IV**). The columns were eluted with different gradients of acetonitrile in 10 mM ammonium acetate containing 0.1 % of acetic acid, and with a flow rate of 1 mL min^{-1}. The solvents for chromatography were filtered and degassed prior use. The reaction mixtures were injected directly to the HPLC.

Preparative HPLC analyses (isolation of adducts) were performed on a Merck-Hitachi instrument consisting of a L-6200A Intelligent pump, L-4000 UV detector, and D-2500 Chromato-Integrator. Hypersil C18 reversed-phase column with dimensions of 10 mm × 250 mm, and particle size of 8 µm (**I**, **II**), or Hypersil ODS C18 column, 10 × 250 mm, and particle size of 5 µm (**III**), or Waters SymmetryPrep C18 with diamentions of 7.8 mm × 150 mm and particle size of 7 µm (**III**, **IV**) were used for these analyzes. Same buffers as in analytical HPLC were used. The flow rate of the eluents (buffers) was 4 mL min^{-1} (**I**, **II**), or 2 mL min^{-1} (**III**, **IV**), or 2.5 mL min^{-1} (**IV**).

5.3. Spectroscopic and spectrometric methods

All adducts were characterized by nuclear magnetic resonance spectroscopy, ultraviolet spectroscopy and mass spectrometry.

NMR analyses were performed on JEOL Alpha 500 instrument operating at 500.0 MHz for ^{1}H (**I**), Bruker Avance 600 spectrometer (**II**, **III**) and Bruker Avance 500 spectrometer (**IV**) equipped with BBO-5mm-Zgrad probe operating at 500.13 and 125.77 MHz for ^{1}H and ^{13}C, respectively. Isolated pure compounds were dissolved in D_2O and the signal of HDO was used as an internal reference in **I**, **II**, **III**. DMSO-d6

was used as a solvent for the NMR samples in **IV** and its instrument's value was used as an internal reference.

Depending on the amount of isolated product and of how much information was required from that specific sample, different NMR experiments were performed: ^{1}H NMR, ^{13}C NMR, COSY, NOESY, HSQC, HMBC.

The HPLC instrument was equipped with a diode-array detector that recorded the ***UV spectra*** of adducts as the compounds eluted from the reversed-phase liquid chromatography column.

The ***MS analyses*** were performed on several mass-spectrometry instruments: a Perkin-Elmer Sciex API-365 Triple-Quadrupole LC/MS/MS system, equipped with a PE 200 Micro pump and a PE Series 200 Autosampler (**I**); a Perkin-Elmer Sciex API-365 Triple-Quadrupole LC/MS/MS system, equipped with two PE 200 Micro pumps, a PE Series 200 Autosampler, and an Applied Biosystems 783A UV detector connected to a LKB 2210 two channel recorder (**II**); an Applied Biosystems API-4000 Triple-Quadrupole LC/MS/MS system, equipped with a Shimadzu-Prominence HPLC system and an Applied-Biosystems-783A UV detector connected to an LKB-2210 two-channel recorder (**III**); an Agilent 1100 Series LS/MSD Trap SL instrument, equipped with an electrospray source (**IV**); and Micro LC triple-quadrupole mass spectrometer, equipped with an electrospray interface (**IV**).

Same RP-HPLC columns and buffers used in the analytical HPLC analyses were used also in the MS analyses.

A variety of mass-spectrometry studies were done in order to obtain a consistent characterization of products: HPLC/MS, MS/MS, HPLC/ESI-MS, HPLC/ESI-MS/MS.

5.4. Quantitative product analyses

Overall reactivity of chlorambucil and phenylacetic acid mustard with nucleosides. CLB was allowed to react in the presence and absence of 2'-deoxyribonucleosides over night in cacodylic acid buffer at 37 ˚C. The reaction mixtures were analyzed by analytical HPLC and the amount of nonnucleosidic products **23-27** was quantitated by HPLC at 267 nm (isosbestic point of the reaction of CLB and PAM with nonchromophoric nucleophiles[139]) in the absence of nucleosides. Then the amount of 2'-deoxyribonucleosides alkylation was calculated based on the decrease of the mole fraction of **23-27** in the presence of nucleosides. Similar procedure was used in the case of PAM too.

CLB and PAM were also allowed to react in the presence and absence of 1*H*-imidazole in cacodylic acid buffer (50 % base, 1.0 M $NaClO_4$, pH 7.4), in the presence of 2'-deoxyribonucleosides. The mole fraction of all products (CLB and PAM with imidazole, CLB and PAM with nucleosides) were calculated as described above.

Relative adduct levels. The relative levels of the most-relevant adducts formed by reaction of CLB or PAM with nucleosides were calculated considering that all

adducts of each nucleoside have same extinction coefficient. Reaction mixtures were run on analytical HPLC and the yield of the most abundant adduct of each nucleoside was considered as 100 %; other adducts were calculated by comparison with it.

Determination of adducts' yields with calf thymus DNA. The yields of the major adducts of CLB reaction with ssDNA and dsDNA where determined based on the quantitative ^{1}H NMR analysis.[176,177,178]

Dry isolated adducts were dissolved in a known amount of D_2O in the case of CLB-dAdo and CLB-dCtd, and in DMSO-d_6 in the case of CLB-dGuo. A known amount of a 5 % triethylamine solution in D_2O and DMSO-d_6, respectively was added to each of the adduct sample as an internal standard and ^{1}H NMR was run. The concentration of adduct in each NMR sample was calculated by comparing the integral of one proton signal of adduct to the integral of the CH_3 signal of triethylamine, of which concentration was known. An exact volume of NMR sample was diluted with a suitable volume of water and run on a HPLC instrument equipped with a diode array detector. The UV detection used was at 267 nm and the quantitative determination of adducts was done by comparing the peak area of these HPLC standard samples with the peak area of adducts in the reaction mixtures. The original amount of 2'-deoxyribonucleosides in the reaction mixture was used to calculate the molar yields of adducts.

5.5. Stability of the end products

CLB and PAM were allowed to react with nucleosides in 0.2 M cacodylic acid buffer, 37˚C for 24 hours. The products were isolated by preparative HPLC. The collected fractions were concentrated and then dissolved in 0.1 M NaOH, 0.1 M HCl, or 0.2 M cacodylic acid (50 % base); after 24 hours at 37˚C, samples were analyzed by HPLC.

5.6. Dimroth rearrangement

CLB and PAM were allowed to react with 2'-deoxyadenosine overnight in the 0.2 M cacodylic acid buffer (50 % base, pH 6.8) at 37 ˚C. The *N*1 adduct was separated by semipreparative HPLC from the reaction mixture, diluted 10-fold with cacodylic acid buffer or 0.1 M NaOH and kept overnight at 37 ˚C (**I**) or room temperature (**III**). HPLC was used to follow the reactions. The mixture was neutralized with aqueous HCl prior HPLC analysis.

5.7. Kinetic measurement

Reactions were carried out in a nonnucleophilic buffer (0.2 M cacodylic acid buffer, 50 % base, pH 6.8) at 37 ˚C for 24 hours in the presence and absence of dAdo, dGuo, dCtd, dMeCtd, and Thd (16.1 mM). The initiation of the reactions was done by adding a stock solution of CLB or PAM in acetonitrile so that a final concentration of

0.6 mM CLB (**I**) or 2.5 mM CLB (**III**) and 2.5 mM PAM (**III**) was obtained. The final reaction mixtures included 1 % (v/v) acetonitrile.

In the case of PAM, the ionic strength of the cacodylic acid buffer was adjusted to 1.0 M with $NaClO_4$.

Reactions were performed in stoppered bottles and aliquots withdrawn at suitable intervals from the reaction mixture were cooled down in ice-water bath, then stored at -20 ˚C and melted before the high-performance liquid chromatography analysis. Same RP-HPLC columns, buffers, gradients and flow rate as in the analytical HPLC analyses were used.

5.8. Reactions of chlorambucil and phenylacetic acid mustard with 2'-deoxyribonucleosides

Chlorambucil (0.6 mM in **I**, 2.5 mM in **II**) and phenylacetic acid mustard (2.5 mM in **III**) were allowed to react overnight in a nonnucleophilic buffer (aqueous 0.2 M cacodylic acid, 50 % base, pH 7.4) at 37 °C in the presence of dGuo, dAdo, dCtd, dMeCtd, and Thd (16.1 mM), respectively. The reaction mixtures were analyzed by HPLC techniques after 24 hours and main adducts formed were isolated by preparative HPLC, then characterized and identified based on their UV spectra, HPLC/MS, HPLC/ESI/MS, HPLC/MS/MS data and/or ^{1}H NMR spectra.

5.9. Reactions of chlorambucil with calf thymus DNA

The reactions of CLB with calf thymus DNA were performed in similar conditions to the reactions of CLB with nucleosides, with one exception: concentration of DNA was different than the concentration of nucleosides.

Single-stranded (ss) DNA was obtained from double-stranded (ds) DNA by heating the solution of dsDNA to 100 °C for 10 min and then cooling it down rapidly on ice.[179] Chlorambucil was allowed to react with ssDNA and dsDNA, respectively in 0.2 M cacodylic acid buffer (50 % base) overnight at physiological pH and 37 °C. The modified DNA was enzymatically hydrolyzed to 2'-deoxyribonucleosides by following the methods described in the literature.[178] The precipitation of DNA was done by adding 5 M NaCl solution, cold ethanol and cooling the DNA solution to -20 ˚C. The mixtures were centrifuged and the recovered DNA was washed with cold 70 % ethanol, cold ethanol and then dissolved in water. The DNA was reprecipitated from the solution adding cold ethanol, cooling to -20 ˚C and recovered by centrifugation. The recovered DNA was enzymatically hydrolyzed by dissolving it in 100 mM Bis-tris-$MgCl_2$ buffer, adding Nuclease P1, bacterial alkaline phophatase, wheat germ acid phosphatase and incubating for several hours at 37 ˚C. The enzymatically digested mixtures were filtered while centrifuging, then the ultrafiltrates were collected, evaporated to dryness, reconstituted in 20 % methanol solution and analyzed by HPLC and LC-ESI-MS/MS.

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