Synthesis and Transdermal Penetration of Cytarabine and Selected Amide, Ester and Carbamate Derivatives

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Preface

This thesis was written in article format. The candidate (Lesetja Jan Legoabe) was the principal and corresponding author of the three articles included in this thesis and performed the experimental work under supervision and assistance of all promoters. The articles were submitted to the following journals:

Drug development and Industrial Pharmacy (Chapter 3; Status: submitted, Manuscript ID: LDDI-2009-0447).

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The article manuscripts were formatted according to a standard format chosen for the thesis. However, the reference style of the specific journal was maintained. The link to each journal's website with instructions to/guidelines for authors is given directly following the references.

Abstract

Cancer is reported to be one of the top ten leading causes of death worldwide and its treatment poses a number of challenges. Cytarabine is a deoxycytidine analogue commonly used in the treatment of haematological malignant diseases. Its clinical utility, however, is severely limited by its short plasma half-life due to the catabolic action of nucleoside deaminases. Due to the cell cycle (S-phase) specificity of cytarabine, a prolonged exposure of cells to cytarabine's cytotoxic concentrations is essential to achieve maximum activity and is often achieved by more invasive and inconvenient modes of administration such as continuous intravenous infusion. Transdermal drug delivery systems (TDDS), on the other hand, have the potential to achieve this sustained release which is useful for drugs with short biological half-lives without the inconvenience associated with intravenous infusion. However, not all the drugs are suited for TDDS. Owing to good barrier function of skin mainly due to its lipophilic outermost layer, the stratum corneum, most drugs with hydrophilic structures permeate the skin too slowly to be of therapeutic benefit. This is reported to be due to hydrogen-bonding functionality on the permeant which drastically retard skin permeation. Cytarabine is known for its high hydrophilicity and plurality of polar functional groups capable of hydrogen bonding. Therefore, it becomes apparent that cytarabine would not easily permeate the skin. The disadvantages of TDDS include skin irritation, which is one of the possible side effects. Prodrug approach could be used to circumvent these setbacks. This approach has been investigated to enhance dermal and transdermal penetration of drugs with unfavourable intrinsic properties and it showed promising outcomes. Increased skin penetration of the drug could be achieved if delivered via its derivative with better physicochemical properties for transdermal penetration.

The aims of this study were to determine the transdermal penetration of cytarabine and its synthesized amide, ester and carbamate derivatives and to establish a correlation, if any, between transdermal penetration and selected physicochemical properties.

The alkylamide (5 compounds), alkylester (6 compounds) and carbamate (6 Compounds) derivatives of cytarabine were synthesized by standard chemical procedures, their structures confirmed by NMR and MS and they were evaluated for transdermal penetration using human epidermis as a model. The transdermal flux values of these derivatives were determined *in vitro* using Franz diffusion cell methodology. Quantification of compounds was achieved by using HPLC. Selected physicochemical properties (aqueous and lipid solubility; melting point and log D) of cytarabine derivatives were determined and assessed for any correlation with transdermal parameters of these compounds.

The steady-state flux value of cytarabine was found to be 3.7 nmol.cm^{-2}.h^{-1}. In the N4-methoxypoly(ethylene glycol) homologous series, the first member, N4-methoxyethanol-cytarabine carbamate, with a log D value of -1.20 exhibited the highest flux. In this series, no significant increase in transdermal delivery of cytarabine by its derivatives was observed. Moreover, no clear relationship between lipid and aqueous solubility, molecular weight and transdermal flux values was observed.

In the alkylester and alkylamide homologous series, octanol solubility values increased whereas aqueous solubility decreased as the alkyl chain lengthened. As a consequence, the log D increases as the chain lengthens. Generally, the flux values of cytarabine and its derivatives are very low compared to those of compounds that are clinically administered by transdermal delivery system such as nicotine and scopolamine.

Statistically significant skin penetration enhancement of cytarabine was achieved by N4-hexanoylcytarabine and cytarabine-5'-butanoate with log D values of 0.91 and -0.26 respectively. These compounds exhibited the highest flux values in their respective series. In comparison to the other members of their homologous series, they showed relatively good balance between lipid and aqueous solubilities. These findings highlight the importance of biphasic properties of compounds in optimisation of their skin penetration.

Opsomming

Kanker is, na berig word, wêreldwyd een van die tien belangrikste oorsake van sterftes en die behandeling daarvan skep verskeie uitdagings. Sitarabien is 'n deoksisitidienanaloog wat dikwels gebruik word vir die behandeling van maligne hematologiese siektes. Die kliniese gebruik daarvan word egter drasties beperk deur die kort plasmahalfleeftyd, wat toegeskryf kan word aan die kataboliese werking van nukleosieddeaminases. As gevolg van die selsilkus-spesifisiteit (S-fase) van sitarabien, is 'n verlengde blootstelling van selle aan sitotoksiese konsentrasies van sitarabien noodsaaklik om maksimum aktiwiteit te verkry en dit kan dikwels slegs bereik word deur ongerieflike toedieningsmetodes soos kontinue intraveneuse infusie. Transdermale geneesmiddelafleweringsisteme (TDGS), daarenteen, kan potensieel volgehoue geneesmiddelvrystelling bewerkstellig word, wat nuttig is vir geneesmiddels met kort biologiese halfleeftye en dit skakel die ongerief van intraveneuse infusie uit. Alle geneesmiddels is egter nie geskik vir TDGS nie. Omdat die vel so 'n effektiewe skans vorm, vanweë sy biofisiese buitenste laag, die stratum corneum, penetreer die meeste hidrofiele verbindings die vel te stadig om enige terapeutiese voordeel te behaal. Laasgenoemde verskynsel is, na berig word, die gevolg van die eienskap van die aktief om waterstofbindings te vorm wat veldeurlaatbaarheid drasties inkort. Die sterk hidrofiele karakter en meervoudige polêre funksionele groepe van sitarabien het tot gevolg dat dit maklik waterstofbindings vorm. Dit is dus voor die hand liggend dat sitarabien nie maklik deur die vel sal dring nie. Een van die nadele van TDGS is velirritasie, wat dus ook 'n moontlike newe-effek kan wees. Aangesien 'n progeneesmiddelbenadering moontlik hierdie negatiewe aspekte mag omseil, is hierdie benadering ondersoek ten einde dermale en transdermale penetrasie van geneesmiddels met ongunstige intrinsieke eienskappe te verbeter. Gunstige resultate is behaal en verhoogde velpenetrasie is verkry met derivate wat oor beter fisies-chemiese eienskappe vir transdermale penetrasie beskik.

Die doel van hierdie studie was om die transdermale penetrasie van sitarabien en die gesintetiseerde amied-, ester- en karbamaatderivate daarvan te bepaal en vas te stel of daar 'n verwantskap tussen die transdermale penetrasie van dié verbindings en geselekteerde fisies-chemiese eienskappe bestaan.

Die alkielamied-, alkielester- en karbamaatderivate van sitarabien is met standaard chemiese metodes gesintetiseer, hul strukture is deur KMR en MS bevestig en hul transdermale penetrasie is met menslike epidermis as model bepaal. Transdermale flukswaardes van die sitarabienderivate is *in vitro* met die Franz-diffusieselmetode bepaal. Die verbindings is met HPLC gekwantifiseer. Geselekteerde fisies-chemiese eienskappe (water- en lipiedoplosbaarheid, smeltpunt en log D-waardes), van die sitarabienderivate is bepaal en

geassesseer om vas te stel of daar enige korrelasie met die transdermale parameters van dié verbindings bestaan.

Die gelykvlak-flukswaardes van sitarabien was 3.7 $nmol.cm^{-2}.h^{-1}$. In die N4-metoksipoli(etileenglikool)homoloog-reeks, het die eerste verbinding, N4-metoksietanol-sitarabienkarbamaat, met 'n log D-waarde van -1.20, die hoogste fluks vertoon. In hierdie reeks was daar nie 'n beduidende verhoging in die transdermale aflewering van sitarabien deur sy derivate nie. Hierbenewens is geen duidelike verwantskap tussen lipied- en wateroplosbaarheid, molekulêre massa en transdermale flukswaardes aangetoon nie.

In die alkielester- en alkielamiedhomoloog-reeks, het oplosbaarheid in oktanol verhoog maar wateroplosbaarheid verlaag met 'n verlenging van die alkielketting. Dus verhoog log D soos wat die ketting verleng word. In die algemeen is die flukswaardes van sitarabien en sy derivate baie laag vergeleke met dié van verbindings, soos nikotien en skopolamien, wat klinies deur transdermale sisteme toegedien word.

Statisties beduidende verhoogde velpenetrasie van sitarabien is met N4-heksanoielsitarabien en sitarabien-5'-butanoaat verkry, met log D-waardes van 0.91 en -0.26 onderskeidelik. Hierdie verbindings het die hoogste flukswaardes in hul onderskeie reekse vertoon. In vergelyking met ander komponente van hul homoloog-reekse, het hierdie verbindings relatief goeie balans tussen hul lipied- en wateroplosbaarheid vertoon. Hierdie bevindings beklemtoon die belang van die bifasiese eienskappe van verbindings vir die optimalisering van hul velpenetrasie.

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"Many are the plans in a man's heart, but it is the Lord's purpose that prevail"

Proverbs 19:21

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CHAPTER 1

1 Introduction and Problem Statement

1.1 Introduction

Cytarabine is a deoxycytidine analogue commonly used in the treatment of haematological malignant diseases. This pyrimidine nucleoside analogue is one of the most active single agents in the treatment of myleloid leukaemia (Galmarini *et al.,* 2002). However, its clinical utility is severely limited by the catabolic action of nucleoside deaminases which are widely distributed in both normal and tumour tissues, which give rise to the inactive metabolite 1-(β-D-arabinofuranosyl)uracil (ara-U) (Hadfield & Sartorelli, 1984). As a result, cytarabine exhibit a very short plasma half-life. Due to its cell cycle (S-phase) specificity, a prolonged exposure of cells to cytarabine's cytotoxic concentrations is essential to achieve maximum activity (Hamada *et al.,* 2002; Rustum & Raymakers, 1992). In practice, it is administered by repetitive schedules or continuous intravenous infusion in order to achieve sustained supply. These regimens however are associated with adverse effects such as myelosuppresion, vomiting and stomatitis at conventional dose (Galmarini *et al.,* 2002; Frei *et al.,* 1969; Bolwell *et al.,* 1988). Because of these shortcomings, cytarabine has been a subject of many studies aiming to circumvent these problems. In particular, many prodrugs approaches have been explored with varied degree of success (Fadl *et al.,* 1995; Silverman, 2004).

In comparison with more conventional drug delivery strategies, transdermal drug delivery systems (TDDS) offers several important advantages over more traditional dosage forms. These include the potential for sustained release which is useful for drugs with short biological half-lives requiring frequency oral or parenteral administration and controlled input kinetics which are particularly indispensable for drugs with narrow therapeutic indices (Naik *et al.,* 2000). To date, however, transdermal drug delivery received a scanty attention in a quest to improve pharmacokinetics of cytarabine.

Despite the many advantages of the skin as site of drug delivery, only a small number of drugs currently in the market are delivered transdermally, e.g. include clonidine, estradiol, nitroglycerine, fentanyl, testosterone, scopolamine, nicotine and oxybutinin. The most important reason for this is the low permeability of drugs through the stratum corneum which is affected by the physicochemical properties of the permeant (Honeywell-Nguyen & Bouwstra, 2005). For instance,

many drugs with hydrophilic structures permeate the skin too slowly to be of therapeutic benefit. Hydrogen-bonding functionality on the permeant is reported to drastically retard permeation (du Plessis *et al.,* 2002; Roberts *et al.,* 1995; Pugh *et al.,* 2000). Against that background, cytarabine with its inherent high hydrophilicity and plurality of hydrogen bonding functionalities would not easily penetration the skin. Prodrug approaches could be used to transiently modify the physicochemical properties of a therapeutic agent for optimum transdermal penetration and reduced skin irritation.

1.2 Aims and objectives

The aims of this study were to determine transdermal absorption of cytarabine and its synthesized amide, ester and carbamate derivatives and to establish a correlation, if any, with selected physicochemical properties.

The following objectives were set in order to achieve the goals:

- Synthesise 5'-alkylesters, N4-alkylamides and N4-methoxypoly(ethylene glycol) carbamates of cytarabine and confirm their structures by NMR and MS.
- Experimentally determine the aqueous solubility and the partition coefficient for synthesized derivatives
- Experimentally determine the transdermal flux of cytarabine and its derivatives.
- Find whether a correlation exists between the aqueous solubility, partition coefficient and transdermal flux data of the cytarabine derivatives.

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CHAPTER 2

2 Anticancer nucleosides and transdermal drug delivery

2.1 Cancer

Cancer is a term used for a class of diseases in which a group of cells display uncontrolled growth, invasion and sometimes metastasis. It is a very complicated life-threatening disease which affects millions of South Africans. The most common cancer in South Africa is skin cancer with about 20 000 reported new cases each year (Cancer Association of South Africa (CANSA), 2008). According to South African Medical Research Council (2008), one in four South Africans will develop a cancer in their lifetime (Stein, 2008). Based on these statistics, it is clear that cancer continues to pose a threat to the well-being of the people. It is therefore essential that, better prevention and treatment methods are found.

2.2 Nucleosides

Nucleosides are the fundamental building blocks for biological systems. Their analogues have a wide variety of biological activities including anticancer, immunosuppressive and antiviral activities. Cytotoxic nucleoside analogues were among the first chemotherapeutic agents to be introduced for the medical treatment of cancer (Secrist, 2005). Over the years, nucleosides analogues have been in and out of favour as potential anticancer drugs due to concerns about their toxicity; and concern that perhaps new nucleoside analogues would not be sufficiently different from those already known. Research and clinical progress with nucleosides however, made it clear that even modest structural changes on nucleosides can have a significant effect on mechanism of action, toxicity and clinical indications (Secrist, 2005).

The anticancer nucleosides include several analogues of physiological pyrimidine and purine nucleosides and nucleobases. The two main pyrimidine-based deoxynucleosides analogues used in clinical settings are cytarabine and gemcitabine; and the purines deoxycytidine derivatives are cladribine and fludarabine. Cytarabine (1) is extensively used in the treatment of both acute and chronic myeloblastic leukaemias while its analogue gemcitabine, also has activity in various solid tumours; (Secrist, 2005; Galmarini *et al.,* 2002).

2.2.1 Action mechanisms and metabolism of nucleosides analogues

Anticancer nucleoside analogues (NA) are antimetabolites that interfere with the synthesis of nucleic acids. These agents can exert cytotoxic activity by being incorporated into and altering the DNA and RNA macromolecules themselves, by interfering with various enzymes involved in synthesis of nucleic acids, or by modifying the metabolism of physiological nucleosides (figure 2.1).

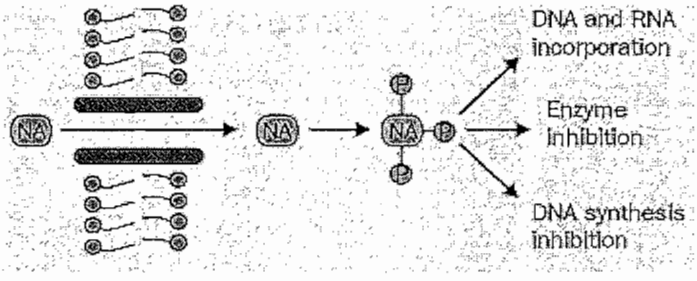

Figure 2.1: Common characteristics in metabolism and drug-target interactions of nucleoside analogues (Galmarini *et al.,* 2002).

The nucleoside analogues share common characteristics including transport mediated by membrane transporters, activation by intracellular metabolic steps that retain the nucleotide residues in the cell, and the formation of the active phosphate derivatives (Galmarini *et al.,* 2001). Nucleoside analogues are generally hydrophilic molecules, and require specialised nucleoside transporter proteins to enter the cell. There is emerging evidence that the abundance and tissue distribution of nucleoside transport proteins contributes to cellular specificity and sensitivity to nucleoside analogues (Mackey *et al.,* 1998). However, each of nucleoside analogues also has unique drug-target interactions that help explain their differences in activity in various diseases. For instance, the cytotoxic effects of the purine analogues fludarabine and cladribine on non-dividing cells may be explained by interaction with targets involving DNA repair rather than replication and direct or indirect effects on mitochondria (Galmarini *et al.,* 2002).

2.2.2 Nucleosides transporters

Several nucleoside transporters have been identified over the years, and are categorised into two families, equilibrative nucleoside transporters (ENT) and concentrative nucleoside transporters (CNT). The members of each family differ in substrate specificity and sensitivity to inhibition by different molecules. There is an increased knowledge about specificity and tissue distribution of each nucleoside transporters in recent times, which can now be used for the development of new

drugs with high affinity for tumour tissues as compared to tissues in which toxicity occurs (Jordheim & Dumontet, 2007). The expression of the human equilibrative nucleoside-transport-facilitating protein 1 (hENT1) is the rate-limiting factor for cytarabine uptake in treatment regimens. The importance of hENT1 was seen clinically. Patients who have myeloblasts with low expression of this transporter have shown poor clinical outcomes.

2.2.3 Deoxycytidine kinase

To exert their effects inside the cell deoxynucleoside analogue need to be converted by intracellular metabolic steps to their active triphosphate derivatives. Deoxynucleoside kinases catalyze the first and rate-limiting step of this process. Of the four human kinases, deoxycytidine kinase (dCK) is the main protein involved in this process and represents a key enzyme in the activation of these molecules (Jordheim & Dumontet, 2007; Eriksson *et al.,* 2002).

2.2.4 Deaminases and 5'-nucleotidase

Metabolism of deoxynucleosides analogues also involves catabolizing enzymes such as extra- and intracellular deaminases and cytoplasmic 5'-nucleotidases. Deaminases, and in particular cytidine deaminase (CDA) and deoxycytidilate deaminase (dCMP-DA) catalyze the production of inactive uridine derivatives of deoxynucleosides and monophosphorylated deoxynucleosides. Cytarabine is a substrate of CDA and the deaminating reaction results in the production of the corresponding inactive uracil derivatives araU. The action of 5'-nucleotidases lead to dephosphorylation of monophosphorylated deoxynucleoside analogues and thus involved in their overall mechanism of action (Jordheim & Dumontet, 2007; Bianchi & Spychala, 2003).

2.2.5 Resistance to deoxynucleoside analogues

Resistance development is one of the drawbacks in clinical use of nucleosides. There are three possible mechanisms of resistance to deoxynucleoside analogues. Firstly, mutation of genes involved in the steps of production and intracellular accumulation of the active molecules, kinases, 5'-nucleotidases, deaminases and membrane efflux pumps could result in resistance.

Secondly, the alterations of proteins interacting with deoxynucleoside analogues in order to induce the cytotoxicity, such as DNA polymerases, ribonucleotide reductase and CTP synthase could also lead to resistance. Thirdly, modifications in the cellular response to the stress triggered by the cytotoxic molecules, involving for example proteins recognizing DNA breaks, involving in DNA repair or in apoptotic machinery could be the basis of some resistance (Jordheim & Dumontet,

2007). Better understanding of mechanism of resistance to deoxynucleoside analogues are being used in the rational design of new molecules that may be even more powerful anticancer drugs. This approach led to the development of analogues such as clofarabine, troxacitabine, tezitabine, T-ara-C and 4'-thio-FAC (Jordheim & Dumontet, 2007).

2.3 Cytarabine

The two main pyrimdine-based deoxynucleosides analogues used in clinical settings are cytarabine and gemcitabine. Cytarabine (fig. 2.2) is the main focus of our current study. Cytarabine [1-(β-D-arabinofuranosyl)cytosine] is a deoxycytidine analogue commonly used in the acute and chronic treatment of human leukaemias.

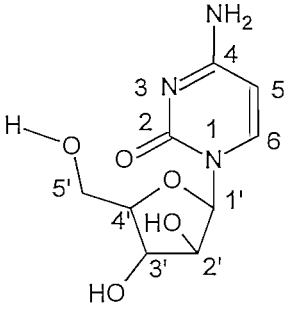

Figure 2.2: Cytarabine

Over the years, cytarabine has been one of the most active single agents in the treatment of acute myeloid leukaemia. However, this drug has a very short plasma half-life and low oral bioavailability due to its low permeability across the intestine and extensive metabolism to its non-toxic metabolite uracil arabinoside (Ara-U) by cytidine deaminase which is widely distributed in both normal and cancerous tissue (Galmarini *et al.,* 2002; Camiener & Smith, 1965; Capizzi *et al.,* 1983). As a consequence, cytarabine is administered by intravenous infusion (which requires clinic setting) regimen which are associated with adverse effects such as myelosuppresion, vomiting and stomatitis at conventional dose (Galmarini *et al.,* 2002; Frei *et al.,* 1969; Bolwell *et al.,* 1988). A prolonged exposure of tumour cells to cytotoxic levels of cytarabine is critical to achieve maximum activity because cytarabine is an S-phase-specific drug (Graham & Whitmore, 1970; Hamada *et al.,* 2002). Other limitations of cytarabine's cytotoxicity activity include low affinity for deoxycitidine kinase, and rapid elimination of the triphosphate derivative (Galmarini *et al.,* 2002).

Intracellular penetration of cytarabine is dependent on the plasma concentration and the expression of the human equilibrative nucleoside-transport-facilitating protein 1 (hENT1) is the rate-limiting factor for cytarabine uptake in regimens that include conventional doses of cytarabine (plasma concentrations of 0·5–1 μM). Once cytarabine is inside the cell, the rate-limiting step in intracellular anabolism is conversion to arabinosyl CMP by deoxycytidine kinase (Plunkett *et al.,* 1987). Cytarabine monophosphate can be dephosphorylated by cytoplasmic 5′-nucleotidases. Its cytotoxicity mechanism involves direct inhibition of DNA polymerases and incorporation of arabinosyl CTP into DNA, which leads to chain termination and DNA synthesis arrest. A low degree of incorporation of arabinosyl CTP into the DNA of blast cells *in vitro* is predictive of an adverse outcome in patients with AML (Acute myelogenous leukemia) who receive cytarabine-based therapy (Galmarini *et al.,* 2002; Raza *et al.,* 1992).

2.3.1 Strategies to improving efficacy of cytarabine

Many different strategies have been tried to improve efficacy of this compound by increasing its plasma stability and therefore its half-life. Prodrug strategies have been explored with varied degrees of success. Carboxylic and phosphate ester derivatives of cytarabine have been extensively examined, but few have led to an approved product (Hadfield & Sartorelli, 1984; Wipf & Li, 1994). N4-acyl derivatives were also examined in an attempt to protect N4 primary amino group from cytidine deaminase. Saturated and monounsaturated C18 and C20 long-chain N4-acyl derivatives of gemcitabine (cytarabine analogue) and all N4-amide derivatives showed better cytotoxic activity than the parent compound (Myhren *et al.,* 1998). However, due to the ease of enzymatic hydrolysis of the acyl functionality connected to the highly electron withdrawing cytosine terminus, acylation of the N4-amino group led to very labile amide or carbamate prodrug forms (Plunkett *et al.,* 1987; Storniolo & Allerheiligen, 2002). In other studies N4-alkyl derivatives of cytarabine, considered to be susceptible to hydrolysis were examined. N4-octadecyl(NOAC)-cytarabine and N4-hexadecyl(NHAC) cytarabine exhibited impressive anticancer activity against various solid tumours (Horber *et al.,* 1995).

The amino acids ester prodrugs of cytarabine have been studied and showed deeply modified pharmacokinetics and increased oral bioavailability, decreased deamination and intracellular cleavage by carboxylesterases (Cheon & Han, 2007). Many more strategies were explored and led to a number of patents, but none led to a product that entirely circumvents all clinical limitations of cytarabine.

2.4 Transdermal drug delivery

Transdermal drug delivery system (TDDS) has been in existence for a long time. In the past, the most commonly applied systems were topically applied creams and ointments for dermatological disorders (Shreeraj, 2008). In the recent past, there has been a great interest in development of transdermal delivery systems for therapeutic use because of its better safety profile, better bioavailability, and better patient compliance compared to oral delivery. These drug delivery systems are designed for controlled release of drug through the skin into systemic circulation maintaining consistent efficacy and reducing dose of the drug and its related side effects (Samad *et al.,* 2009). TDDS can be divided into two categories: the active and passive transdermal systems. The active TDDS uses active assisting means, including ultrasound (Sonoporation), laser, iontophoresis and electroporation, to push the drug through the skin. The passive TDDS allows the active pharmaceutical ingredient (API) to diffuse through the skin layers to achieve drug delivery (Banga & Chien, 1993; Guy & Hadgraft, 2002; Ferry, 1995).

2.4.1 Advantages of transdermal drug delivery

In comparison to more conventional drug delivery strategies such as oral delivery, transdermal drug delivery systems (TDDS) offer several important advantages. These include the potential for sustained release which is useful for drugs with short biological half-lives requiring frequent oral or parenteral administration and controlled input kinetics which are particularly indispensable for drugs with narrow therapeutic indices (Naik *et al.,* 2000). The steady permeation of drugs across the skin allows for more consistent plasma levels, which is often a goal of therapy. Intravenous infusion can achieve consistent plasma levels, but it is more invasive than transdermal drug delivery. Lack of peaks in plasma concentration can reduce the risk of side effects. For instance, transdermal clonidine, nitroglycerin and fentanyl patches exhibited fewer adverse effects than the conventional oral dosage form (Creamer & Saks, 1994). If toxicity were to develop from a drug administered transdermally, the effects could be limited by simply removing the patches (Wilkosz & Bogner, 2003). TDDS can be used as an alternative route of administration to accommodate patients who cannot tolerate oral dosage form. It is of great advantage in patients who are nauseated or unconscious. Drugs that cause gastrointestinal upset can be good candidates for transdermal delivery because this method avoids direct effects on the stomach and intestines. Drugs that are degraded by the enzymes and acids in the gastrointestinal system may also be good targets. First pass metabolism, an additional limitation to oral drug delivery, can be avoided with transdermal administration (Wilkosz & Bogner, 2003). For example, transdermal estradiol patches are used by over a million patients per year and in contrast to oral formulations, are not associated with lever

damage. TDDS can also lead to better patient compliance through simplified dosage regimen (Micheal & David, 2003) and reduced frequency of administration through sustained release.

Although many (more than 200) TDDS patents have been granted world-wide, a relatively small number of drugs (approximately 16 active ingredients) have been approved for use globally (Shreeraj, 2008; Samad *et al.,* 2009). The most important reason for this is the low permeability of drugs in the stratum corneum (Honeywell-Nguyen & Bouwstra, 2005).

Table 2.1: Marketed Products of Transdermal Patches

Brand Name	Drug	Manufacturer	Indications
Nicotinell®	Nicotine	Novartis	Pharmacological smoking cessation
Matrifen®	Fentanyl	Nycomed	Pain relief patch
Ortho Evra™	Norelgostromin/ Ethinyl Estradiol	ORTHO-McNEIL	Postmenstrual syndrome
NuPatch 100	Diclofenac diethylamine	Zydus Cadila	Anti Inflammatory
Neupro®	Rigotine	UCB and Schwarz Pharma	early-stage idiopathic Parkinson's disease
Alora	Estradiol	TheraTech/Proctol and Gamble	Postmenstrual syndrome
Nicoderm®	Nicotine	Alza/GlaxoSmithKline	Smoking cessation
Estraderm	Estradiol	Alza/Norvatis	Postmenstrual syndrome
Climara	Estradiol	3M Pharmaceuticals/Berlex Labs	Postmenstrual syndrome
Androderm	Testosterone	TheraTech/GlaxoSmithKline	Hypogonadism in males
Nitrodisc	Nitroglycerin	Roberts Pharmaceuticals	Angina pectoris
Transderm-Scop®	Scopolamine	Alza/Norvatis	Motion sickness
Nuvelle TS	Estrogen/Progesterone	Ethical Holdings/Schering	Hormone replacement therapy

Deponit	Nitroglycerin	Schwarz-Pharma	Angina pectoris
Nitro-dur	Nitroglycerin	Key Pharmaceuticals	Angina pectoris
Catapres TTS^{R}	Clonidine	Alza/Boehinger Ingelheim	Hypertension
FemPatch	Estradiol	Parke-Davis	Postmenstrual syndrome
Minitran Climaderm	Nitroglycerin Estradiol	3M Pharmaceuticals Ethical Holdings/Wyeth-Ayerest	Angina pectoris Postmenstrual syndrome
Duragesic^{R}	Fentanyl	Alza/Janssen Pharmaceutical	Moderate/severe pain
Estraderm	Estradiol	Alza/Norvatis	Postmenstrual syndrome
Fematrix	Estrogen	Ethical Holdings/Solvay Healthcare Ltd.	Postmenstrual syndrome
Transderm-Nitro^{R}	Nitroglycerin	Alza/Norvatis	Angina pectoris
Testoderm TTS^{R}	Testosterone	Alza	Hypogonadism in males
Oxytrol^{R}	oxybutynin	Watson Pharma	Overactive bladder
Prostep	Nicotine	Elan Corp./Lederle Labs	Smoking cessation

Adapted from (Shreeraj, 2008)

2.4.2 Disadvantages of transdermal drug delivery

Despite the many advantages of transdermal delivery systems, they are not without disadvantages. Disadvantages of transdermal delivery include; the possibility that a local irritation will develop at the site of application. Drug, the adhesive or other excipients in the formulation can cause erythema, itching and local oedema. While for some patients, site rotation can minimize irritation, severe allergic reaction in other patients leaves no option other than discontinuation of the therapy (Wilkosz & Bogner, 2003; Prochazka, 2000). Other limitations of TDDS include relatively high manufacturing costs and less than ideal cosmetic appearance (Thomas & Finnin, 2004). Given the excellent diffusion resistance offered by stratum corneum, the daily drug dose can be systemically delivered through a reasonable patch-sized area remains in the <10 mg range. This requires transdermal candidate drugs to be pharmacologically potent, and have therapeutic blood concentration in the $ng.ml^{-1}$ range or less

(Naik *et al.,* 2000). Another disadvantage is lag time from application to therapeutic effect (Berti & Lipsky, 1995)

2.4.3 Skin as a barrier to transdermal absorption

To understand drug delivery through the skin, one should first become familiar with the skin barrier (Hadgraft, 2001). Human skin consists of four layers: stratum corneum (non-viable epidermis) the remaining layers of the dermis (viable epidermis), dermis and subcutaneous tissues. The barrier function of skin is accomplished entirely and quite remarkably by stratum corneum (Naik *et al.,* 2000). Skin is the most accessible and probable the most extensive organ (Lundi, 1994). It is the largest organ of the body, accounting for more than 10% of the body mass, and it covers an average area of 1.7 m^2. It is the body part that interacts most intimately with its environment (Walter & Roberts, 2002; Williams, 2003). Skin's average thickness is about 0.5 mm (Foldvari, 2000). The human skin surface is known to contain on average, 3 blood vessel, 10 hair follicles, 12 nerves, 15 sebaceous gland and 100 sweat glands on every square centimetre (Asbill & Michniak, 2000).

2.4.3.1 Stratum corneum

The stratum corneum (or horny layer) is the outermost layer of the skin, and is the major source of resistance to the permeation of the skin by drug molecules. This layer is compositionally and morphological unique biomembrane (Scheuplein & Blank, 1971). This thin (approximately one hundredth of an mm) membrane is comprised of keratin-filled corneocytes (terminally differentiated keratinocytes) anchored in a lipophilic matrix (Naik *et al.,* 2000). Stratum corneum's composition (ceramides, free fatty acids and cholesterol) is unique among biological membranes due to *inter alia* the absence of phospholipids (Wertz *et al.,* 1987). Despite the absence of polar bilayer-forming lipids, stratum corneum lipids exist as multilamellar sheets. The predominantly saturated, long-chain hydrocarbon tails facilitate a highly ordered, interdigitated configuration and the formation of gel-phase membrane domains as opposed to the more usual liquid crystalline membrane systems (Naik *et al.,* 2000). Although stratum corneum is recognized as the major rate-limiting step in the diffusion process of a drug permeating across the skin, other components can contribute to the overall barrier resistance especially for lipophilic solutes (Roberts *et al.,* 2002).

2.4.3.2 Viable epidermis

The living cells of the epidermis are located immediately below the stratum corneum. In drug delivery considerations, it is often regarded as a single stratum of living cellular tissue, although histologically it is multilayered. It is primarily aqueous in nature and its diffusional resistance

resembles an aqueous protein gel. The permeant has to cross stratum lucidum, stratum granulosum (granular layer), stratum spinosum (spinous layer) and the stratum basale (or basale). These viable layers may metabolise a drug or activate a prodrug (Barry, 2001). If the stratum corneum is damaged or if extremely lipophilic drugs are being used, the viable epidermis can act as a rate-limiting factor in transdermal absorption (Walters, 1990). The cellular structure of viable epidermis is predominantly hydrophilic throughout its various layers, and substances can be transported in its intercellular fluids. For polar substances, resistance to penetrate is considerably lower than in the stratum corneum; because the tightly packed alternating hydrophilic and lipophilic layers are no longer present (Wiechers, 1989).

2.4.3.3 Hypodermis

Hypodermis or subcutaneous fatty layer is sandwiched between the dermis and the underlying body constituents; acts as a heat insulator and a shock absorber. The principal blood vessels and nerves are carried to the skin in this layer (Walter & Roberts, 2002).

2.4.3.4 Dermis

The dermis (or corium) is typically 3-5 mm thick and is the major component of the human skin. It is composed of a network of connective tissue, predominantly collagen fibrils providing support and elastic tissue providing flexibility, embedded in the mucopolysaccharides gel. In terms of transdermal drug delivery, this layer is often viewed as essentially gelled water and thus provides a minimal barrier to delivery of most polar drugs, although the dermal barrier may be significant when highly lipophilic compounds is delivered (Williams, 2003).

The dermis has numerous structures embedded within it; blood and lymphatic vessel, nerve endings, pilosebaceous units (hair follicles and sebaceous glands), and sweat glands (eccrine and appoccrine) (Williams, 2003). The blood supply is very rich, with a flow rate of 0.05 ml min^{-1} per cm^3 of skin, and reaches to within 0.2 mm of the skin surface (Scheuplein & Blank, 1971). The rich blood supply is needed to regulate temperature and pressure of the skin, deliver nutrients to the skin, and remove waste products. This excellent blood flow usually functions as a 'sink' with respect to the diffusing molecules which reach it during the process of transdermal absorption (Barry, 1983). The lymphatic vessels may also remove permeated molecules from the dermis, hence maintaining a driving force for permeation (Williams, 2003). Cross and Roberts (1993) showed that whilst dermal blood flow affect the clearance of relatively small solutes, such as lidocaine, lymphatic flow was a significant determinant for clearance of larger molecules such as interferon (Cross & Roberts, 1993).

2.4.3.5 Skin appendages

Skin appendages (hair follicles and sweat glands), break the continuity of the epidermal and dermal layers throughout most of the surface of the body. Hair follicles extend through the epidermis into the dermis, where the base of the follicle is well vascularized. Sebaceous glands attached to the sides of the follicles secrete sebum, a lipid mixture, into the region between the hair and the sheath. The sweat glands consist of tubes extending from the dermis, where the tube is coiled and vascularized, to the skin surface where a watery mixture (sweat) is excreted to provide thermal regulation (Ho, 2002). They essentially offer pores that bypass the barrier of stratum corneum. However, their opening occupy about 0.1% (On average, 40 to 100 hair follicles and 210 to 220 sweat ducts exist per square centimetre of skin) of the total surface area (Scheuplein, 1967) and hence their contribution to the total drug flux at pseudo-steady state is generally regarded as being insignificant (Scheuplein & Blank, 1971).

2.4.4 Transdermal absorption process

Transdermal absorption can be defined as the uptake of a compound into the systemic circulation after dermal application, and it describes movement through the various layers of the skin with respect to both rate and extent. This process can be divided into penetration, permeation and absorption steps (Schaefer *et al.,* 1982). Transdermal absorption of compounds through the skin can be influences by the structure of the skin, physicochemical characteristics of the permeant, physicochemical characteristics of the vehicle and the dosing conditions (Wiechers, 1989).

The molecules can cross stratum corneum by intercellular, transcellular and appendageal routes (fig. 2.3). The tortuous intercellular pathway is widely believed to provide the principal route for drug permeation (Scheuplein & Blank, 1971; Wallace & Barnaett, 1978; Stoughton, 1989). The appendageal route usually contributes negligibly to steady state drug flux (Hadgraft, 2001). Fractional appendageal area available for transport is only about 0.1%. This route, however, may be important for penetration of ions and large polar molecules that struggle to cross intact stratum corneum. Appendages may also provide shunts, important at short times prior to steady state diffusion. Additionally, polymers and colloidal particles can target the follicle (Barry, 2001).

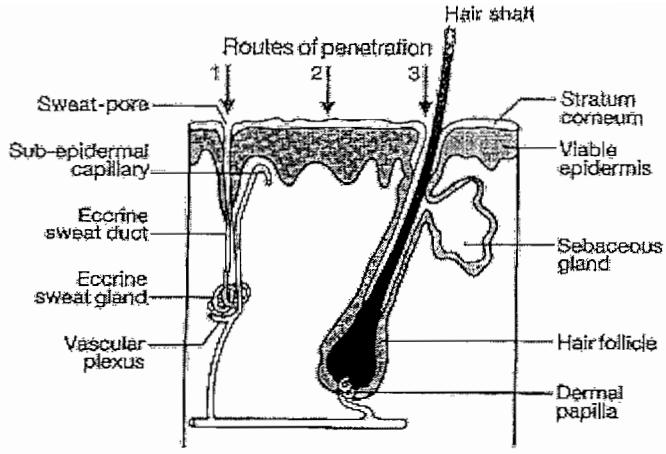

Figure 2 3: Simplified diagram of skin structure and macroroutes of drug penetration: (1) via the sweat ducts; (2) across the continuous stratum corneum or (3) through the hair follicles with their associated sebaceous glands (Barry, 2001).

2.4.5 Physicochemical factors influencing transdermal absorption

The passage of a permeant through the skin is the process of diffusion and partition; and it is often described by Fick's law of diffusion. The main factors affecting this penetration are the properties of the drug, the vehicle and the skin. The physical and chemical nature of each of these components and their collective interactions all influence the rate at which the drug penetrates the skin (Katz & Poulsen, 1971).

The physicochemical properties of a drug substance are very important determinants of its permeation through the skin (Hadgraft & Wolff, 1993). It is generally accepted that, for a molecule to efficiently penetrate stratum corneum, it should have the following properties:

- Low molecular weight (<600 Da), when its diffusion coefficient tend to be high
- Adequate solubility in oil and water- so that the membrane concentration gradient (the driving force for diffusion) can be high.
- A high, but balanced (optimal), partition coefficient (too large a value of K could inhibit clearance by viable tissues)
- Low melting point, correlating with ideal solubility

These requirements are illustrated by nicotine transdermal patches (Barry, 2001).

2.4.5.1 Drug solubility in the stratum corneum

Drug solubility is an important parameter in the design of transdermal drug delivery systems. In order to permeate through the skin, the molecules need to partition from the vehicle to the

stratum corneum. The degree to which drugs partition into outer layer of the stratum corneum is controlled by the amount of drug applied and the solubility limit in the stratum corneum. Subsequently, the molecule need to partition out of the stratum corneum into the essentially aqueous viable epidermis. These layers exhibit variable resistance to penetration by permeates of different chemical nature. For lipophilic molecules, the rate limiting step is the partition of the drug into viable epidermis, whereas for hydrophilic molecules, it is penetration into the stratum corneum. Optimum skin permeation is therefore reached with molecules having biphasic solubility properties (Hadgraft & Wolff, 1993; Surber *et al.,* 1993; Flynn & Yalkowsky, 1972).

2.4.5.2 Solubility parameters

Solubility is one of indexes expressing energetics of molecules interaction, namely, higher miscibility can be realised when two solubility parameter of the components are closer in the binary system. By using the solubility parameter, the solubility of solute in the solvent is almost predictable (Ohta *et al.,* 1999).

The solubility parameter is defined as the square root of the cohesive energy density. The cohesive energy of a material is the energy that holds that substance together and is therefore the net effect of all the intermolecular interactions. It is the amount of energy required to separate the constituent atoms or molecules of the material to an infinite distance and therefore, it is a direct measurement of the attraction that atoms of molecules have for one another (Hilderbrand & Scott, 1950). The solubility parameter of an organic solute (δ_2) in the stratum corneum can be estimated using equation 2.1. If the solubility of the solute in a non-polar organic solvent (like hexane) is known, as well as the solute's heat of fusion, the melting point, and the solubility parameter of the solvent (hexane) is expressed as follows: (Hilderbrand *et al.,* 1970).

$$\ln X_2 = \frac{-\Delta H_f}{RT}\left(\frac{T_f - T}{T_f}\right) + \frac{\Delta C_p}{R}\left[\frac{T_f - T}{T} - \ln\frac{T_f}{T}\right] - \frac{V_2 \varphi_1^{\,2}}{RT}(\delta_1 - \delta_2)^2 \qquad \text{Equation 2.1}$$

Where:

X_2 is the solute's mole fraction solubility in hexane

ΔH_f is the heat of fusion of a solid,

R is the gas constant

T_f is the melting point of the solid (Kelvin)

T is experimental temperature < T_f

ΔCp is the difference in heat capacity between the solid form and hypothetical super cooled liquid form of the compound, both at the same temperature

V_2 is the molar volume of liquid solute

ϕ_1 is the volume fraction of the solvent

δ_1 is the solubility parameter or square root of the cohesive energy density of the solvent (hexane) and

δ_2 is the solubility of parameter of square root of the cohesive energy density of the solute.

The theory states that for crystalline solids in regular solution, the permeability, and hence the partition coefficient between the skin and solvent may be related to the solubility parameter for the solute in the system The solubility parameter of the skin has been estimated as ~10 and therefore drugs, which possess similar values, would be expected to dissolve more readily in the stratum corneum. However in practice, the results are less clear (Rosado, 2000; Liron & Cohen, 1984; Roy & Flynn, 1989).

2.4.5.3 Aqueous solubility and lipophilicity

Since stratum corneum, the main barrier of transdermal penetration of drugs is lipophilic; one would expect compounds with higher lipophilicity to show higher flux. However, it appears that actually the balance between lipid and aqueous solubilities is essential to optimize flux (Sloan, 1989).

For more lipophilic series of prodrugs, the most aqueous soluble member of the series usually exhibits the highest fluxes (Sloan & Wasdo, 2003). In a homologous series of prodrugs with similar lipophilicity compared to a parent drug, the highest flux through the skin is achieved by the derivatives which exhibit the highest aqueous solubility (Sloan, 1989; Bonina *et al.,* 2001).

2.4.5.4 Diffusion coefficient

Diffusion coefficient (D) is a measure of how easily a molecule diffuses through the stratum corneum. It is defined as the number of moles of drug that diffuse across a membrane or within the various strata of a given area per time unit, and it is influenced by molecular size of the drug and the viscosity of the surrounding medium (Barry, 1988; Idson, 1983). The movement of chemical across the stratum corneum into the epidermis occurs primarily by passive diffusion driven by the applied concentration of drug on the surface of the skin. Because of the dense nature of the stratum corneum, values of the diffusion coefficients in these tissues are 1000 times smaller than elsewhere in the skin. This factor contributes to a high resistance and low

permeability (Flynn, 1987). The movement of chemicals across the stratum corneum is best expressed using Fick's Law of diffusion which state that the steady state of drug flux across the membrane can be expressed as:

$$J = K_p.\Delta C = \frac{D.K.\Delta C}{L} \qquad \text{Equation 2.2}$$

Where:

J is the steady state flux of the penetrant across the stratum corneum ($\mu g.cm^{-2}.h$)

K_p is the permeability coefficient of the permeant through stratum corneum ($cm.h^{-1}$)

ΔC is the concentration in the vehicle (C_v) based on the definition of K_p as J/C_v.

D is the diffusion coefficient of the permeant in the stratum corneum ($cm^2.h^{-1}$).

K is the apparent partition coefficient of the permeant between the stratum corneum and vehicle and L is the length of the pathway through the stratum corneum (cm).

$$D = A.V_m^{-1/3} \qquad \text{Equation 2.3}$$

$$K = \frac{Ph}{D} \qquad \text{Equation 2.4}$$

$$T_{lag} = \frac{L^2}{6D} \qquad \text{Equation 2.5}$$

2.4.5.5 Partition coefficient

Partition coefficient determines the ability of the drug to gain access to the diffusion pathway. The partition coefficient is normally determined experimentally by measuring octanol/water partition or lipid/water partition (Riviere & Papich, 2001; Zatz, 1993).

Essentially, the stratum corneum barrier is lipophilic, with the intercellular lipids lamellae forming a conduit through which drugs must diffuse in order to reach the underlying vascular infrastructure and to ultimately access the systemic circulation. For this reason, lipophilic drugs are better accepted by the stratum corneum. A molecule must first be liberated from the formulation and partition into the uppermost stratum corneum layer, before diffusing through the entire thickness, and must then repartition into the more aqueous viable epidermis beneath.

Ideally, a drug must possess both lipoidal and aqueous solubilities. If too hydrophilic, the drug molecule will be unable to transfer into the stratum corneum; if too lipophilic, the drug will tend to remain in the stratum corneum layers. It is generally accepted that the optimal log octanol/water partition coefficient for a drug to penetrate the stratum corneum, is approximately two (Naik *et al.,* 2000; Riviere & Papich, 2001).

2.4.5.6 Hydrogen bonding

Not all the drugs are suited for TDDS. Drugs with hydrophilic structures permeate the skin too slowly to be of therapeutic benefit. Hydrogen-bonding functionality such as alcohols, phenols, ketones, carboxylic acids and ether on the permeants are reported to drastically retard permeation (du Plessis *et al.,* 2002; Roberts *et al.,* 1995; Pugh *et al.,* 2000). In the study on ketorolac oligoethylene esters skin penetration, it was observed that as polyethylene chain lengthened the penetration enhancement decreased with the longest member exhibiting penetration lower than the parent drug. This was partly attributed to its high molecular weight and its increased ability to form hydrogen bonds (owing to more oxyethylene groups) reducing the permeation rate through the skin (Puglia *et al.,* 2006). By transiently masking polar hydrogen bonding functional groups on a permeant, increased topical delivery of the parent drug could be achieved (Sloan & Wasdo, 2003).

Hydrogen-bonding donor (α) and acceptor (β) parameters are generally derived from substructure summation and have been successfully used to predict transdermal permeability. Relation of log K_p value to solute structure, using hydrogen bond descriptors through the linear free energy relationship is expressed as follows (Abraham *et al.,* 1997):

$$\text{Log}Kp = c + rR_2 + spi_2^H + a\Sigma\alpha_2^H + b\Sigma\beta_2^H + vV_x \qquad \text{Equation 2.6}$$

Where log Kp is the permeability coefficient
R_2 is excess molar refraction
Pi_2^H is the dipolarity/polarizability
$\Sigma\alpha_2^H$ is the effective hydrogen-bond acidity
$\Sigma\beta_2^H$ is the effective hydrogen-bond basicity
V_x is the McGowan characteristic volume.

Anderson & Raykar (1989) suggested that the stratum corneum barrier microenvironment resembled an H-bonding organic solvent (Anderson & Raykar, 1989). The diffusion of a series of phenols across simple silicone membrane saturated with octanol to mimic the polar/hydrogen

bonding environment of the stratum corneum lipid barrier was studied. In that study, it was found that for octanol impregnated membrane, the diffusion coefficient decreased significantly with the number of H-bonding groups (Du Plessis *et al.*, 2001).

2.4.5.7 Melting point

The melting point of a substance is related to its relative hydrophobia associated with low crystalline interactions. Drug crystallinity, or melting point, influences permeability and was found to be inversely proportional to lipophilicity (log K_{oct}). The melting point of a permeant is often considered to be indicative of the maximum flux attainable through the skin (Calpena *et al.*, 1994; Cleary, 1993).

2.4.5.8 Ionisation

The role of pH in transdermal transport is obvious since contributions of ionisation, solubility, lipophilicity and pH are interrelated (Singh *et al.*, 2005). As biological membranes are selectively permeable to the free base or uncharged (unionized) form of the drug, higher flux values are obtained at the pH under which more of the drug is uncharged (Abdul, 1989). Numerous drugs are weak organic electrolytes, the ionization of which depends on the delivery medium pH. The activity coefficient of the molecular form of such drugs is rapidly changing as a function of pH (Barr, 1962). The impact of pH change on transdermal penetration was demonstrated in the study of penetration enhancement of acidic drugs by 1-menthol–ethanol systems at different pH values (Katayama *et al.*, 2001).

Weak bases and acids are dissociated to different degrees, depending on the pH and pK_a or pK_b of the diffusant. The concept of pK_a is derived from the Handerson-Hasselbach equation (Ansel, 1981).

For an acid:

$$\log\frac{[A^-]}{[HA]} = pH - pK_a \qquad \text{Equation 2. 7}$$

For a base:

$$\log\frac{[BH^+]}{[B]} = pK_a - pH \qquad \text{Equation 2 8}$$

According to pH-partition theory, only unionized forms of drugs are able to permeate through the phospholipids. However, there is increasing evidence that the ionized species can contribute to transdermal absorption of drugs (Flynn, 1987; Wallace *et al.,* 1978; Oakley & Swarbrick, 1987). When penetrating species exists in both ionized and unionized forms, it is the unionized ones that permeate faster through the lipid regions while the ionized penetrate slower through the aqueous regions.

According to Hadgraft &Valenta (2000), the transport of the permeant can be describes by the permeability of the ionized and ionized species and the respective concentrations K_{ion} , $K_{pnion,}$ c_{ion} and c_{ion} (Hadgraft & Valenta, 2000).

$$J_{tot} = Kp_{union} X\ C_{union} + Kp_{ion} X\ C_{ion} \qquad \text{Equation 2.9}$$

Consideration of this pH, as well as of the drug dissociation constant (pK_a), allow some degree of absorption to be predicted and controlled by varying of delivery medium.

Martinez-Pla *et al.* (2004) showed that biopartitioning micellar chromatography (BMC) can be useful in predicting the effect of pH of the skin permeation of drugs (Martinez-Pla *et al.,* 2004). BMC methodology is fast, reproducible, simple and economical and provides similar results than the conventional *in vivo* approaches that use human and rat skin for compounds studied. By using this method it is possible to estimate the permeability constant of the ionized and unionized forms of drugs.

2.4.5.9 Molecular weight and size

Considering the fact that stratum corneum is a compact membrane and that diffusing molecules follows a tortuous path through it. It might seem obvious that the diffusion coefficient would be inversely related to molecular weight or some other measure of molecular size (Naik *et al.,* 2000; Zatz, 1993). The larger the molecule, the more difficult it is to move about and the lower the diffusivity. Diffusivity is a kinetic term, and is a rough measure of the ease with which a molecule can move about within a medium (in this case, the skin). Compounds of small molecular size may penetrate through the aqueous pathway easily than larger molecules which penetrate through the lipoidal pathway more readily (Zatz, 1993).

According to Potts & Guy (1995), increasing the molecular volume increases the hydrophobic surface area and will increase partitioning into, and hence permeability through the membrane (Potts & Guy, 1995). Conversely larger molecules diffuse more slowly, since they require more space to be created in the medium, and this in turn leads to diminished permeability. Although small molecules penetrate more rapidly than larger molecules, within a narrow range of

molecular weight there is little correlation between size and penetration rate (Liron & Cohen, 1984). The following equation relates diffusion (D) to size:

$$D = D_o(MW)^b \qquad \text{Equation 2.10}$$

Where D_o is the diffusion coefficient and b refers to the mass selectivity coefficient. For diffusion across membranes, apparent values of b from -3 to -5 indicate a strong dependence of diffusion on molecular weight (Lieb & Stein, 1969).

For the stratum corneum and other lipid membranes, it is suggested that the functional dependence of permeant diffusivity on molecular volume is exponential. A model of compounds ranging in molecular weight from 18 to >750 and log K_{oct} from -3 to -6 was introduced by Potts & Guy (1992). They found that Equation 2.14 could predict the permeability through human skin:

$$\log K_p = -2.7 + 0.71 \times \log K_{oct} - 0.0061 \times MW \qquad \text{Equation 2.11}$$

Where K_p is the permeability coefficient ($cm.sec^{-1}$), K_{oct} is the octanol/water partition and MW is the molecular weight. It was found that the substitution of molecular weight for molecular volume provides an equivalent fit in the model. In conclusion, the apparent sigmoidal dependence of log K_p upon log K_{oct} suggests a non-linear relationship between these parameters. However, when molecular volume is taken into consideration, data lies on a three-dimensional surface defined by log K_p, log K_{oct} and molecular volume (Potts & Guy, 1992).

Further research (Pugh *et al.,* 2000) confirmed the direct relationship between log K_p and log K_{oct}, but found that the relationships between log Kp and MW is also direct and not inverse as found by Potts and Guy.

2.4.5.10 Influence of alkyl chain length on skin penetration

Drug's physicochemical properties are very important in determining its biological and pharmaceutical characteristics (Yalkowsky *et al.,* 1972). By the understanding of the manner in which these characteristics change within a homologous series, i.e. with additions of methylene units, a derivative with optimum properties can be chosen (Yalkowsky *et al.,* 1972). In the homologous series, water-lipid partition coefficient increases exponentially with increasing chain length. The improvement in skin penetration of a drug could be achieved by the design of

transient derivatives that show good hydrophilic/lipophilic balance in their solubility (Beall *et al.*, 1993).

Flynn & Yalkowsky (1972) showed that the relationship can be drawn for the influence of chain length on partition coefficient and solubility. Partition coefficients of member of a homologous series between the immiscible polar and non-polar phases increase by a constant factor as the series ascent.

2.4.6 Biological factors influencing transdermal drug permeability

Biological factors such as diseases, skin age and skin hydration influence transdermal permeation of drugs. These factors must be regarded when developing a transdermal drug candidate, as they may influence the drug concentration ultimately absorbed into the systemic bloodstream (Pefile & Smith, 1997).

2.4.6.1 Skin hydration

Skin hydration plays an important role in the extent and rate of transdermal absorption. Increasing stratum corneum hydration alters barrier function, hence often increasing transdermal absorption *in vitro* (Lambert *et al.,* 1989). However, *in vivo* transdermal studies by Bucks and Maibach (1999) suggest that, increased hydration does not necessarily increase transdermal absorption of hydrophilic compounds (Bucks & Maibach, 1999).

2.4.6.2 Skin variations

The most widely investigated physiological factor influencing transdermal drug delivery is skin ageing. Ageing changes the skin in several ways, which impact on transdermal drug absorption. Changes in skin structures could be caused by both chronological skin aging and sun-exposed skin aging (Kaestli *et al.,* 2008). There is a significant variation (approximately 40%) in permeation between the same body parts on different individuals (Williams, 2003). Furthermore, inter- and intra-individual variability increases with increasing age (Kaestli *et al.,* 2008).

2.4.6.3 Skin metabolism of drugs

The cutaneous metabolic activity has been identified and widely studied and review in recent years (Zhang *et al.,* 2009; Hikima *et al.,* 2005). The active enzymes in viable skin tissues have the capacity of bio-transforming topically applied compounds.

2.5 Prodrug Design for transdermal delivery

Prodrug is a transient chemical modification of a parent that is either inactive or less potent (1000 times) than the parent drug, and is metabolized either chemically or enzymatically in a controlled and predictable manner to its parent, active drug (Sloan & Wasdo, 2003).

2.5.1 Choice of prodrug carriers

The choice of carriers in transdermal prodrugs depends on *inter alia*; whether increased lipophilicity or hydrophilicity is aimed at. Generally, lipophilicity of the parent drug could be increased by conjugation with an aliphatic or aromatic promoiety, whereas for increased hydrophilicity, promoieties containing free hydrophobic groups such as hydroxyl, carboxylic and amino groups (in addition to a group involved in conjugation) are employed (Silverman, 2004). With appropriate choice of polyethylene glycol promoieties, in particular, both lipophicility and hydrophilicity could be enhanced (Bonina *et al.,* 2001; Puglia *et al.,* 2006). This is attributed to the amphiphilic nature of polyethylene glycol.

2.5.2 Choice of prodrug linkers

The choice of linkers in transdermal prodrugs depends on among others;

- Chemical functional groups on the parent drug amenable to derivatization,
- Stability of envisaged prodrug in delivery vehicle and biological fluids,
- Whether increased lipophilicity or hydrophilicity is aimed at.

Various linkers have been used in transdermal prodrugs studies which include: amides, esters, carbamates, ureas, N-mannich bases and soft alkyl derivatives (Sloan & Wasdo, 2003; Sloan *et al.,* 1984). In other studies, it was found that by using carriers of different alkyl chains and bearing various terminal (ω) functionalities, variable chemical stability of linkers (carbonates, esters and carbamates) between prodrug moiety and the parent drug, could be achieved (Vlieghe *et al.,* 2001). For carbonates and ester prodrugs, the influence of terminal groups on the sensitivity to enzymatic hydrolysis, was found to be in the following stability order: carboxylic acid>> *tert*-butyl>amine ≈ alcohol. These terminal groups are thought to be involved in rearrangement through an intramolecular cyclic process during enzymatic hydrolysis of carbonate prodrugs. The chain length of promoiety had a crucial effect on mechanism of intramolecular rearrange and half-lives ($t_{1/2}$).

In transdermal delivery studies, the stability of prodrug linkers in various vehicles is an important aspect to consider. For prodrugs which are more labile in protic solvents such propylene glycol, ethanol and water, other aprotic vehicle such as isopropyl myristate (IPM) could be used. For instance, for 1-alkycarbonyl derivatives of 5-fluorouracil (1-AC-5-FU) which are very unstable in water with the $t_{1/2}$s of only 3-5 min, IPM was used as vehicle in diffusion cell experiments (Beall

et al., 1996; Beall *et al.*, 1996; Beall & Sloan, 1996). Other classes of compounds known to be commonly too labile in aqueous solutions include Schiff bases [imines] (Silverman, 2004) and N-Mannich bases (Sloan *et al.*, 1984; Bundgaard & Johansen, 1981).

The chemical lability of prodrug linkers doesn't necessarily translate to enzymatic lability. It is therefore important to find linker with optimum *in vivo/in vitro* lability ratio. The prodrug should be more stable in formulation to have reasonably long shelf life, but be hydrolyzed relatively easier *in vivo* to have reasonable short plasma $t_{1/2}$. According to Silverman (2004), ideally the prodrug should have *invivo/in vitro* lability ratio on the order of 10^6. Although transiently masking a polar functional group, which is capable of donating a hydrogen bond, invariable leads to some increase in topical delivery of the parent drug, the greatest increase in topical delivery is achieved by the members of a homologous series that are more water soluble (Sloan & Wasdo, 2003).

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CHAPTER 3

3. Synthesis and Transdermal permeation of Novel N4-methoxypoly(ethylene glycol) carbamates of cytarabine

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Abstract

Cytarabine is a deoxycytidine analogue commonly used in the treatment of haematological malignant diseases. Its clinical utility, however, is severely limited by its short plasma half-life due to the catabolic action of nucleoside deaminases. In the current study, N4-carbamate derivatives of cytarabine (**1**) were synthesized and evaluated for transdermal penetration as this mode of administration may circumvent its limitations. The synthesis of these compounds was achieved in a two step process. Firstly, the methoxypoly(ethylene glycol) (mPEG) was activated by *p*-nitrophenyl chloroformate. Secondly, the activated intermediates were reacted with cytarabine in the presence of *N*-hydroxysuccinamide (HOSu) to give the N4-methoxypoly(ethylene glycol) carbamate derivatives. The transdermal flux values of the N4-carbamates of cytarabine were determined *in vitro* using Franz diffusion cell methodology. Aqueous solubility and log D (pH 7.4) values were determined and assessed for correlation with transdermal flux values. The synthesized carbamates, particularly, (**9**)–(**13**), showed increased solubility in both aqueous and lipid media. Log D values decreased as the oxyethylene chain lengthened.

Although none of the derivatives showed a significantly higher transdermal penetration than cytarabine (**1**), it should be mentioned that the mean for (**8**) was 10 times higher and mean was 2 times higher.

Keywords: Transdermal delivery systems, cytarabine, methoxypoly(ethylene glycol) (mPEG), derivatives, percutaneous absorption, carbamate, physicochemical properties, skin penetration, log D

3.1 Introduction

Cytarabine is a deoxycytidine analogue commonly used in the treatment of haematological malignant diseases. This pyrimidine nucleoside analogue is one of the most active single agents in the treatment of myleloid leukaemia (Galmarini, Mackey, & Dumontet, 2002). However, its clinical utility is severely limited by the catabolic action of nucleoside deaminases which are widely distributed in both normal and tumour tissues, which gives rise to the inactive metabolite 1-(β-D-arabinofuranosyl)uracil (ara-U) (Hadfield & Sartorelli, 1984). As a result, cytarabine (**1**) exhibits a very short plasma half-life. Due to its cell cycle (S-phase) specificity, a prolonged exposure of cells to cytarabine's cytotoxic concentrations is essential to achieve maximum activity (Hamada, Kawaguchi, & Nakano, 2002; Rustum & Raymakers, 1992). In practice it is administered intravenously by repetitive schedules or continuous infusion in order to achieve sustained supply. These regimens, however, are associated with adverse effects such as myelosuppresion, vomiting and stomatitis at conventional dose (Bolwell, Cassileth, & Gale, 1988; Frei et al., 1969; Galmarini et al., 2002). Because of these shortcomings, cytarabine has been a subject of many studies aiming to circumvent these problems. In particular, many prodrug approaches have been explored with varied degrees of success (Boyer & Erion, 2004; Brachwitz et al., 1998; Fadl et al., 1995; Kluge & Schott, 1997; Wang, Chen, Lu, & Lin, 2005).

Over the years, the prodrug approach for transdermal delivery has been explored with some success stories (Sloan, 1989). When compared to more conventional drug delivery strategies, transdermal drug delivery (TDD) offers several important advantages over more traditional dosage forms. These include the potential for sustained release, which is useful for drugs with short biological half-lives requiring frequent oral or parenteral administration, and controlled input kinetics which is particularly indispensable for drugs with narrow therapeutic indices (Naik, Kalia, & Guy, 2000). To date, however, transdermal drug delivery received scanty attention in a quest to improve pharmacokinetics of cytarabine. Despite the many advantages of the skin as site of drug delivery only a small number of drugs are currently in a transdermal delivery system on the market, *inter alia* clonidine, estradiol, nitroglycerine, fentanyl, testosterone, scopolamine, nicotine and oxybutinin. The most important reason for this is the low permeability of drugs through the stratum corneum which acts as a barrier and penetration is affected by the physicochemical properties of the permeant and the possible skin sensitization reactions which can be caused by the drug (Honeywell-Nguyen & Bouwstra, 2005). For instance, many drugs with hydrophilic structures permeate the skin too slowly to be of therapeutic benefit. Hydrogen-bonding functionality on the permeant is reported to drastically retard permeation (du Plessis, Pugh, Judefeind, & Hadgraft, 2002; Pugh, Degim, & Hadgraft, 2000; Roberts, Pugh, Hadgraft, & Watkinson, 1995). Against this background, cytarabine with its inherent high hydrophilicity and plurality of hydrogen bonding functionalities, would not easily penetrate the skin. Prodrug

approaches could be used to transiently modify the physicochemical properties of a therapeutic agent for optimum transdermal penetration (Sloan & Wasdo, 2003). The success of this depends on factors including the inherent nature of a drug and choices of promoieties and linkers. It is known that the balance between lipid and aqueous solubility is essential to optimize flux (Sloan, 1989). The choice of functional groups as carriers in transdermal prodrugs will of course depends on among others whether increased lipophilicity or hydrophilicity is aimed at. Generally, lipophilicity of the parent drug could be increased by conjugation with an aliphatic or aromatic promoiety, whereas for increased hydrophilicity promoieties containing free hydrophilic groups such as hydroxyl, carboxylic and amino groups (in addition to a group involved in conjugation) are employed (Silverman, 2004). With appropriate choice of poly(ethylene glycol) (PEG) promoieties, in particular, both lipophicility and hydrophilicity could be enhanced (Bonina et al., 2001; Puglia et al., 2006).

The poly(ethylene glycols) (PEGs) posses a unique set of advantageous properties, including absence of toxicity, immunogenicity, and antigenicity, low-mass dependent elimination *via* the kidney, and high amphiphilicity (solubility in water and organic media) (Ballico, Cogoi, Drioli, & Bonora, 2003). The choice of mPEG as a promoiety is due to its amphiphilic nature, since water solubility as well as enhanced lipid solubility is important factors to consider in any attempt to optimize a particular derivative approach to enhancing transdermal penetration and hence topical delivery (Cleary, 1993; Fadl et al., 1995). It is generally accepted that compounds with *inter alia* low molecular weight (<600 Da) penetrate the skin better than high molecular weight permeants (Hadgraft & Wolff, 1993). The choice of mPEG with higher molecular weights (350, 550 and 750) was to determine what the influence of these groups on the transdermal penetration would be given that previous research has found that compounds with high molecular weight could be delivered by passive diffusion through the skin (Jordan, 2008; Li et al., 2008).

In the present study N4-carbamate methoxypoly(ethylene glycol) derivatives of cytarabine were synthesized and evaluated for transdermal penetration. The log D and solubility of these compounds in water and octanol were determined and assessed for correlation with transdermal flux values.

3.2 Materials and methods

3.2.1 Materials

Cytarabine (**1**) (fig 3.1) was purchased from Jingma Chemicals Ltd. China. para-Nitrophenyl chloroformate (*p*-NPCF) was purchased from Sigma-Aldrich (Johannesburg, South Africa). Methoxyethanol, di- and tri(ethylene glycol) monomethyl

ethers and poly(ethylene glycol) monomethyl ethers (mPEG) (average molecular weight 350, 550 and 750) were purchased from Fluka (Johannesburg, South Africa). HPLC grade methanol was obtained from Labchem South Africa Ltd. All other reagents were of analytical grade and were used without further purification.

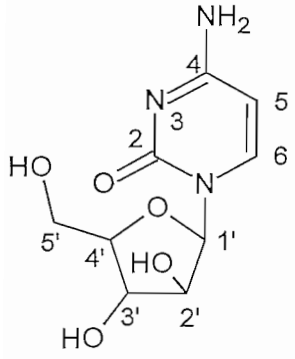


Figure 3.1: Cytarabine

3.2.2 General procedures

The ^{1}H, ^{13}C, COSY, HSQC and HMBC spectra were recorded on a Bruker 600 spectrometer, using deuterated DMSO as solvent. The ^{1}H and ^{13}C spectra were recorded at frequencies of 600.17 and 151.92 MHz respectively. All the chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane ($\delta = 0$). The splitting pattern abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), bs (broad singlet) and m (multiplet). The melting points of solid products were determined by Shimadzu DSC-60A using TA60 (Version 2.11) software. MS spectra were recorded on an analytical VG 7070E mass spectrometer using fast atom bombardment (FAB) as ionization technique. Thin-layer chromatography was performed using silica gel plates (60F254 Merck) and flash column chromatography on silica gel (70 - 240 mesh, G60 Merck). Mobile phases were mixed in a volume to volume ratio.

3.2.3 High pressure liquid chromatography (HPLC)

The high performance liquid chromatography (HPLC) system consisted of a Hewlett Packard (HP) Agilent 1100 series auto sampler, HP Agilent 1100 series variable wavelength detector (VWD) and HP Agilent 1100 series pump (Agilent, Palo Alto, CA). A Phenomenex (Luna C-18, 150 x 4.60 mm, 5 µm) column was used with a Securityguard pre-column (C-18, 4x 3 mm) insert (Phenomenex, Terronce, CA) in order to prolong column life. Elution was accomplished with gradient at a flow rate of 1 ml min^{-1} with a mobile phase consisting of methanol (A) and 0.005 M heptane sulphonic acid-Na in water adjusted to pH 3.5 with orthophosphoric acid (B).

The gradient was started with 30% A, then increased linearly to 95% A in 8 min, held until 11 min, where after the column was re-equilibrated at the starting conditions.

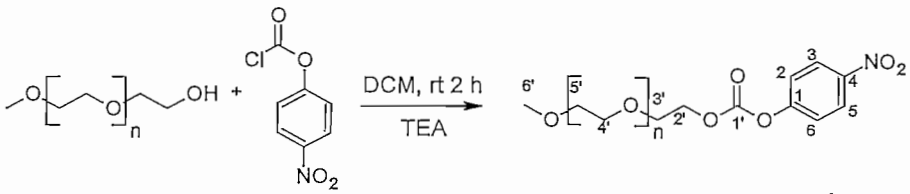
3.2.4 LC-MS analysis

LC–MS was performed using a Hewlett Packard HP1100 series HPLC with binary gradient pump, autosampler and vacuum degasser, coupled to an Applied Biosystems API 2000 triple quadrupole mass spectrometer and analyte data acquisition and analysis software. The column was a Gemini C-8, 150 x 2 mm, 5 mm (Phenomenex). The gradient consisted of 90% A (0.1% formic acid in water) and 10% B (0.1% formic acid in acetonitrile) initially for up to 3 min, then 10% A / 90% B up to 6 min, followed by 90% A / 10% B up to 10 min. The flow rate was 250 ml/min and the injection volume 5 ml. The mass spectrometer used atmospheric pressure electron ionisation (turbo ion spray source) in positive-ion mode. The full scan from 100 to 200 amu was performed in 1 s. The declustering, focusing and entrance potentials were 80, 400 and 10 V, respectively; the ion spray voltage was 5500 V. The curtain gas and ion source gases 1 and 2 were all used at a flow rate of 20 l/h; the temperature was 300°C.

3.2.5 Chemical synthesis

The syntheses of the compounds were generally achieved in a two step process. Firstly, methoxypoly(ethylene glycol)s were activated by *p*-nitrophenyl chloroformate (*p*-NPCF). Secondly, the activated intermediates were reacted with cytarabine in the presence of *N*-hydroxysuccinamide (HOSu) to give N4-methoxypoly(ethylene glycol) carbamates of cytarabine.

3.2.5.1 Activation of mPEGs



n	compound
0	(2)
1	(3)
2	(4)
7	(5)
11	(6)
16	(7)

Scheme 3.1: Activation of mPEGs

In order to react with the N4-amino group on pyrimidine ring of cytarabine, terminal hydroxyl groups of monomethyl ethylene glycol oligomers and mPEGs were converted to their corresponding active *p*-nitrophenyl carbonate derivatives (2)-(7) (Bodansky, 1955). For simplicity only the synthesis of methoxyethylene glycol-(*p*-nitrophenyl carbonate) (2) is described here: Methoxyethylene glycol (22.32 mmol, 1.7 g) and *p*-nitrophenyl chloroformate (*p*-NPCF) (24.80 mmol, 5.0 g) were dissolved in 50 ml anhydrous DCM. To this solution triethylamine (24.80 mmol, 3.4 ml) was added and the mixture was stirred at room temperature for 2 h. The solvent was removed under vacuum. The residue was suspended in 100 ml of diethyl ether to precipitate triethylammonium chloride salt and filtered. The filtrate was concentrated and separated by silica column chromatography using DCM:EtOAc (20:1) to yield the product as a white crystals (18.03 mmol, 4.4 g).

3.2.5.1.1 Methoxyethylene glycol-(*p*-nitrophenyl carbonate) (2)

Carbonate (**2**) was purified by flash silica gel column chromatography eluting with DCM:EtOAc (10:1) and obtained as white crystals in 4.4 g (81%) yield after crystallization in hexane:EtOAc (5:1). mp. 47.8 °C, $C_{10}H_{11}NO_6$. ^{1}H NMR δ (ppm) 3.40 (s, 3H, O*CH*$_3$), 3.66 - 3.69 (m, 2H, H-3'), 4.39-4.30 (m, 2H, H-2'), 7.37 (d, 2H, H-3 & -5, J = 9.26 Hz), 8.25 (d, 2H, H-2 & -6, J = 9.26 Hz). ^{13}C NMR δ (ppm): 59.02 (C-6'), 68.10 (C-5), 69.83 (C-2'), 115.53 (C-4), 121.68 (C-5), 125.47 (C-3), 126.09 (C-2), 145.37 (C-5), 152.45 (C-1), 155.47 (C-1'). MS FAB 242 ($M+H^+$) 70%), 289 (10%), 210 (20%), 165 (12%), 154 (90%), 137 (100%), 123 (32%), 119 (20%), 107 (44%).

3.2.5.1.2 Methoxydiethylene glycol-(*p*-nitrophenyl carbonate) (3)

Compound (**3**) was purified by flash silica gel column chromatography eluting with DCM:EtOAc (10:1) and obtained as yellowish oil in 4.9 g (77%) yield. $C_{12}H_{15}NO_7$. ^{1}H NMR δ (ppm): 3.36 (s, 3H, O*CH*$_3$), 3.50-3.54 (m, 2H, H-5'), 3.55 - 3.60 (m, 2H, H-4'), 3.8 - 3.85 (m, 2H, H-3'), 4.40 - 4.50 (m, 2H, H-2'), 7.36 (d, 2H, H-3 & -5, J = 9.13 Hz), 8.25 (d, 2H, H-6 & -2, J = 9.25 Hz). ^{13}C NMR δ (ppm): 57.60 (C-6'), 68.25 (C-5'), 68.37 (C-4'), 69.35 (C-3'), 71.50 (C-2'), 121.57 (C-4), 124.85 (C-5), 126.09 (C-3), 144.95 (C-6), 152.25 (C-2), 154.97 (C-1'). MS FAB 286 (($M+H^+$) 40%), 210 (72%), 165 (12%), 154 (22%), 137 (42%), 123 (22%), 119 (16%), 107 (26%), 103 (100%).

3.2.5.1.3 Methoxytriethylene glycol-(*p*-nitrophenyl carbonate) (4)

Purification by flash silica gel column chromatography eluting with DCM:EtOAc (10:1) afforded 3.7 g (57%) of carbonate (**4**) as yellowish oil. $C_{14}H_{19}NO_8$. ^{1}H NMR δ (ppm):

3.28 (s, 3H, H-6''), 3.44 - 3.47 (m, 2H, end chain H-5''), 3.55 - 3.63 (m, 6H, H-4'', mid-chain H-5''), 3.71 - 3.74 (m, 2H, H-3'), 4.33 - 4.36 (m, 2H, H-2'), 7.37 (d, 2H, H-3 & -5, J = 9.31 Hz), 8.25 (d, 2H, H-6 & -2, J = 9.23 Hz). ^{13}C NMR δ (ppm): 58.69 (C-6'), 68.07 (C-5'), 68.37 (C-4'), 70.32 (C-3'), 71.69 (C-2'), 121.55 (C-4), 125.04 (C-5), 126.09 (C-3), 145.15 (C-6), 152.20 (C-2), 155.33 (C-1'). MS FAB 330 ((M+H$^+$) 30%), 392 (30%), 210 (66%), 167 (18%), 154 (34%), 149 (100%), 137 (42%), 103 (50%).

3.2.5.1.4 Methoxyoctaethylene glycol-(*p*-nitrophenyl carbonate) (5)

Compound (**5**) was purified by flash silica gel column chromatography eluting with DCM:EtOAc (5:1) and EtOAc, successively, and obtained as yellowish oil in yield of 3.5 g (29%). $C_{24}H_{39}NO_{13}$. ^{1}H NMR δ (ppm): 3.33 (s, 3H, H-6'), 3.49 - 3.52 (m, 2H, end chain H-5'), 3.62 (bs, 26H, H-4', mid-chain H-5'), 3.75 - 3.79 (m, 2H, H-3'), 4.38 - 4.41 (m, 2H, H-2'), 7.37 (d, 2H, H-3 & -5, J = 9.31 Hz), 8.26 (d, 2H, H-6 & -2, J = 9.31 Hz). ^{13}C NMR δ (ppm): 58.45 (C-6''), 68.17 (C-5'), 68.32 (C-3'), 70.22 (C-4'), 71.65 (C-2'), 121.45 (C-4), 125.14 (C-5), 126.01 (C-3), 145.12 (C-6), 152.25 (C-2), 155.32 (C-1'). MS *m/z* 342 (9), 298 (9), 210 (72), 147 (46), 133 (10), 122 (26), 102 (100).

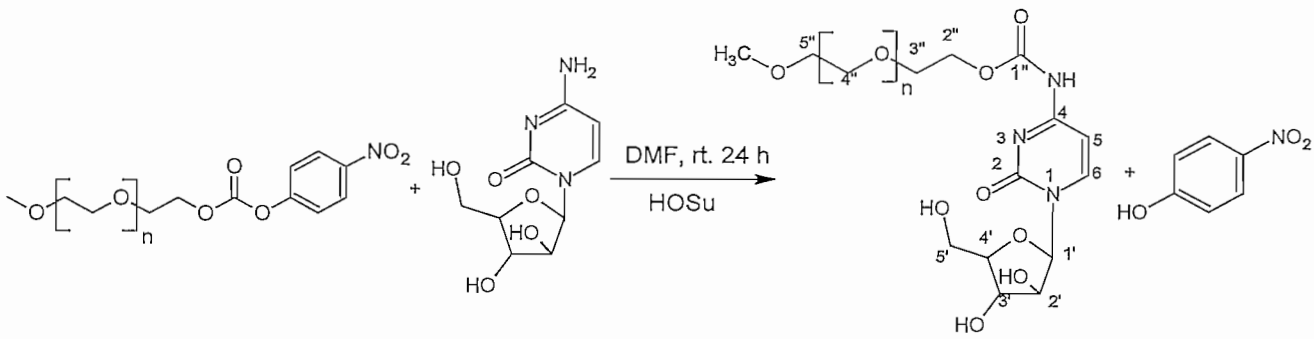
3.2.5.1.5 Methoxydodecaethylene glycol-(*p*-nitrophenyl carbonate) (6)

Purification by flash silica gel column chromatography eluting with DCM:EtOAc (3:1) and EtOAc, successively, afforded 5.1 g (31%) of carbonate (**6**) as yellowish oil. $C_{32}H_{55}NO_{17}$. ^{1}H NMR δ (ppm) 3.28 (s, 3H, H-6'), 3.44 - 3.46 (m, 2H, end chain H-5'), 3.54 (bs, 42H, H-4', mid-chain H-5'), 3.70 - 3.73 (m, 2H, H-3'), 4.32 - 4.36 (m, 2H, H-2'), 7.30 (d, 2H, H-3 & -5 , J =9.29 Hz), 8.18 (d, 2H, H-6 & -2, J =9.31 Hz). ^{13}C NMR δ (ppm): 58.72 (C-6'), 68.09 (C-5'), 68.38 (C-3'), 70.32 (C-4'), 71.69 (C-2'), 121.55 (C-4), 125.04 (C-3'), 126.09 (C-3), 145.15 (C-6), 152.22 (C-1), 155.33 (C-1'). MS *m/z* 664 (14), 648 (20) 342 (10), 279 (14), 210 (70), 191 (18), 167 (33), 147 (54), 133 (30), 102 (100).

3.2.5.1.6 Methoxyheptadecaethylene glycol-(*p*-nitrophenyl carbonate) (7)

Carbonate (**7**) was purified by flash silica gel column chromatography eluting with DCM:EtOAc (1:3) and EtOAc, successively, and obtained as yellowish oil in yield of 4.3 g (21%). $C_{42}H_{75}NO_{22}$. ^{1}H NMR δ (ppm) 3.19 (s, 3H, H-6'), 3.37 - 3.44 (m, 2H, end chain H-5'), 3.46 (bs, 60H, H-4', mid-chain H-5'), 3.60 - 3.70 (m, 2H, H-3'), 4.20 - 4.30 (m, 2H, H-2'), 7.23 (d, 2H, H-3 & -5, J = 9.31 Hz), 8.10 (d, 2H, H-6 & -2, J = 9.28 Hz). ^{13}C NMR δ (ppm): 58.42 (C-6'), 67.83 (C-5'), 69.12 (C-3'), 70.06 (C-4'), 71.44 (C-2'), 121.37 (C-4), 125.04 (C-5), 126.09 (C-3), 145.15 (C-6), 152.22 (C-1), 155.33 (C-1'). MS *m/z* 211 (84), 149 (49), 137 (19), 115 (25), 103 (100).

3.2.5.2 Synthesis of N4-mPEG-cytarabine carbamates



n	compound
0	(8)
1	(9)
2	(10)
7	(11)
11	(12)
16	(13)

Scheme 3.2: Conjugation of mPEG with cytarabine

The methoxy(polyethylene glycol) N4-carbamates of cytarabine were synthesized according to the method as depicted in Scheme 3.2 and described as follows to exemplify the preparation of carbamate (**8**). To a solution of (**2**) (6.26 mmol, 1.5 g, 1 equiv) and cytarabine (6.91 mmol, 1.7 g, 1.1 equiv) dissolved in 20 ml anhydrous dimethylformamide (DMF), was added hydroxysuccinamide (HOSu) (0.59 mmol, 0.07 g, 0.9 equiv) and reaction was stirred at room temperature. The reaction progress was monitored by silica TLC using DCM:MeOH (8:1). After 48 h of stirring at room temperature the solvent was removed *in vacuo* to yield a yellow residue. The residue was purified by flash chromatography with silica gel as stationary phase and DCM:MeOH (8:1) as an eluent to give 1.4 g of product as white powder. The structure of the product was elucidated by NMR and MS data presented below.

3.2.5.2.1 Cytarabine N4-methoxyethyleneoxycarbamate (8)

Compound (**8**) was purified by flash silica gel chromatography eluting with DCM:MeOH (8:1) to yield 1.4 g (64%) of white powder. m.p. 121.3°C. ^{1}H NMR (600 MHz, DMSO) δ 3.27 (s, 3H, O*CH*$_3$), 3.57 – 3.52 (m, 2H, H-3"), 3.61 (t, *J* = 5.3, 2H, H-5'), 3.82 (td, *J* = 5.4, 2.9, 1H, H-4'), 3.92 (dd, *J* = 6.0, 2.7, 1H, H-3'), 4.05 (t, *J* = 5.5, 1H, H-2'), 4.30 – 4.17 (m, 2H, H-2"), 5.07 (t, *J* = 5.4, 1H, OH-5'), 5.48 (d, *J* = 4.1, 1H, OH-2'), 5.50 (d, *J* = 5.3, 1H, OH-3'), 6.04 (d, *J* = 3.9, 1H, H-1'), 7.00 (d, *J* = 7.4, 1H, H-5), 8.04 (d, *J* = 7.5, 1H, H-6), 10.68 (s, 1H, HNCOO). ^{13}C NMR (151 MHz, DMSO) δ 58.02 (C-ω), 60.99 (C-5'), 64.21 (C-2"), 69.72 (C-3"), 74.56 (C-2'), 76.09 (C-3'),), 85.68 (C-4'), 86.85 (C-1'), 93.19 (C-5), 146.31 (C-6), 154.28 (C-2), 162.58 (C-4), 165.29 (C-1"). MS FAB 345.7 $(M+H)^+$, 368.0 $(M+Na)^+$, 11.8, 214.5.

3.2.5.2.2 Cytarabine N4-methoxydiethyleneoxycarbamate (9)

Carbamate (9) was purified by column chromatography using DCM:MeOH (8:1) as a mobile phase to give1.8 g (75%) of white powder. m.p. 132.9°C. ^{1}H NMR (600 MHz, DMSO) δ 3.24 (s, 3H, H-ω), 3.46 – 3.41 (m, 2H, H-5"), 3.58 – 3.52 (m, 2H, H-4"), 3.62 (ddd, *J* = 13.1, 7.8, 4.3, 4H, H-5' & 3"), 3.82 (td, *J* = 5.4, 2.9, 1H, H-4'), 3.92 (dd, *J* = 6.2, 2.7, 1H, H-3'), 4.05 (dd, *J* = 7.5, 4.0, 1H, H-2'), 4.23 (dd, *J* = 5.4, 3.8, 2H, H-2"), 5.06 (t, *J* = 5.5, 1H, OH-5'), 5.48 (d, *J* = 4.1, 1H, OH-3'), 5.50 (d, *J* = 5.4, 1H, OH-2'), 6.04 (d, *J* = 3.9, 1H, H-1'), 7.01 (d, *J* = 7.4, 1H, H-5), 8.04 (d, *J* = 7.5, 1H, H-6), 10.69 (s, 1H, HNCOO). ^{13}C NMR (151 MHz, DMSO) δ 58.04 (C-ω), 60.99 (C-5"), 64.50 (C-2"), 68.25 (C-3"), 69.56 (C-4"), 71.21 (C-5'), 74.56 (C-2'), 76.10 (C-3'), 85.68 (C-4'), 86.84 (C-1'), 93.20 (C-5), 146.29 (C-6), 153.20 (C-1"), 154.27 (C-2), 162.58 (C-4). MS FAB $(M+H)^+$ 390.0, 279.9, 258.0, 137.6.

3.2.5.2.3 Cytarabine N4-methoxytriethyleneoxycarbamate (10)

Carbamate (**10**) was purified by column chromatography using DCM:MeOH (10:1) as a mobile phase to give1.5 g (53%) of yellowish viscous oil. ^{1}H NMR (600 MHz, DMSO) δ 3.23 (s, 3H, H-ω), 3.45 – 3.39 (m, 2H, end-chain H-5"), 3.53 – 3.48 (m, 4H, mid-chain H-5" & end-chain H-4"), 3.55 (td, *J* = 3.9, 0.9, 2H, mid-chain H-4"), 3.61 (t, *J* = 5.5, 2H, H-5'), 3.67 – 3.63 (m, 2H, H-3"), 3.82 (td, *J* = 5.4, 2.9, 1H, H-4'), 3.92 (dd, *J* = 6.3, 2.6, 1H, H-3'), 4.08 – 4.01 (m, 1H, H-2'), 4.23 (dd, *J* = 5.4, 3.8, 2H, H-2"), 5.07 (t, *J* = 5.6, 1H, OH-5'), 5.48 (d, *J* = 4.1, 1H, OH-3'), 5.50 (d, *J* = 5.4, 1H, OH-2'), 6.04 (d, *J* = 3.9, 1H, H-1'), 7.01 (d, *J* = 7.4, 1H, H-5), 8.04 (d, *J* = 7.5, 1H, H-6), 10.68 (s, 1H, HNCOO). ^{13}C NMR (151 MHz, DMSO) δ 58.00 (C-ω"), 60.99 (C-5'), 64.49 (C-2"), 68.27 (C-3"), 69.55 (C-4"), 69.72 (C-5"), 69.77 (end chain C-4"), 71.23 (end-chain C-5"), 74.55 (C-2'), 76.10 (C-3'), 85.68 (C-4'), 86.85 (C-1'), 93.19 (C-5), 146.29 (C-6"), 153.21 (C-1"), 154.26 (C-2), 162.58 (C-4). MS FAB $(M+H)^+$ 433.8, 323.7, 301.8, 229.9, 186.6, 137.9.

3.2.5.2.4 Cytarabine N4-methoxyoctaethyleneoxycarbamate (11)

Carbamate (**11**) was purified by column chromatography using DCM:MeOH (8:1) as a mobile phase to give1.0 g (41%) of light yellow oil. ^{1}H NMR (600 MHz, DMSO) δ 3.23 (s, 3H, H-ω), 3.45 – 3.40 (m, 2H, end-chain H-5"), 3.54 – 3.46 (m, 30H, H-5" & mid-chain 4" & mid-chain 5" & end-chain 4"), 3.56 (dd, *J* = 5.9, 3.4, 2H, start–chain H-4"), 3.61 (t, *J* = 5.4, 2H, H-5'), 3.67 – 3.63 (m, 2H, H-3"), 3.82 (dd, *J* = 8.1, 5.3, 1H, H-4'), 3.92 (s, 1H, H-3'), 4.05 (s, 1H, H-2'), 4.23 (d, *J* = 2.8, 2H, H-2"), 5.07 (t, *J* = 5.5, 1H, OH-5'), 5.49 (d, *J* = 4.1, 1H, OH-3'), 5.50 (d, *J* = 5.5, 1H, OH-2'), 6.04 (d, *J* = 3.9, 1H, H-1', 7.01 (d, *J* = 7.2, 1H, H-5), 8.04 (d, *J* = 7.5, 1H, H-6), 10.68 (s, 1H, HNCOO). ^{13}C NMR (151 MHz, DMSO) δ 58.01 (C-5), 60.99 (C-4'), 64.51 (C-3'), 68.26 (C-2'), 69.53 (C-end-chain 5"), 69.73 (C- H-5" & mid-chain 4" & mid-chain 5" & end-chain 4"), 71.23 (C-4"), 74.54 (C-3"), 76.09 (C-2"), 85.69 (C-5'), 86.85 (C-ω). LC-MS, M^+ (*m/z* 653).

3.2.5.2.5 Cytarabine N4-methoxydodecaethyleneoxycarbamate (12)

Carbamate (12) was purified by column chromatography using DCM:MeOH (8:1) as a mobile phase to give 2.9 g (35%) of light yellow oil. ^{1}H NMR (600 MHz, DMSO) δ 3.24 (s, 3H, H-ω), 3.44 – 3.40 (m, 2H, end-chain H-5"), 3.54 – 3.46 (m, 48H, H-5" & mid-chain 4" & mid-chain 5"& end-chain 4"), 3.56 (dd, *J* = 5.9, 3.3, 2H, start-chain H-4"), 3.61 (t, *J* = 5.2, 2H, H-5'), 3.68 – 3.63 (m, 2H, H-3"), 3.82 (td, *J* = 5.4, 2.9, 1H, H-4'), 3.92 (s, 1H, H-3'), 4.04 (d, *J* = 5.5, 1H, H-2'), 4.27 – 4.19 (m, 2H, H-2"), 5.08 (t, *J* = 5.4, 1H, OH-5'), 5.49 (t, *J* = 5.4, 2H, OH-2' & 3'), 6.04 (d, *J* = 3.9, 1H, H-1'), 7.00 (d, *J* = 7.4, 1H, H-5), 8.04 (d, *J* = 7.5, 1H, H-6), 10.59 (s, 1H, HNCOO), LC-MS, M^+ (*m/z* 830).

3.2.5.2.6 Cytarabine N4-methoxyheptadecaethyleneoxycarbamate (13)

Carbamate (**13**) was purified by column chromatography using DCM:MeOH (8:1) as a mobile phase to give 1.2 g (30%) of light yellow oil. ^{1}H NMR (600 MHz, DMSO) δ 3.24 (s, 3H, H-ω), 3.46 – 3.40 (m, 2H, end-chain H-5"), 3.54 – 3.46 (m, 43H, H-5" & mid-chain 4" & mid-chain 5" & end-chain 4"), 3.58 – 3.54 (m, 2H, start-chain H-4"), 3.61 (t, *J* = 5.4, 2H, H-5'), 3.67 – 3.63 (m, 2H, H-3"), 3.82 (d, *J* = 2.8, 1H, H-4'), 3.93 (s, 1H, H-3'), 4.05 (s, 1H, H-2'), 4.23 (d, *J* = 2.9, 2H, H-2"), 5.09 (t, *J* = 5.5, 1H, OH-5'), 5.50 (t, *J* = 4.4, 2H, OH-2' & 3'), 6.04 (d, *J* = 3.9, 1H, H-1'), 7.01 (s, 1H, H-5), 8.04 (d, *J* = 7.5, 1H, H-6), 10.85 – 10.53 (m, 1H, HNCOO). LC-MS, M^+ (*m/z* 1094).

3.2.6 Physicochemical properties

3.2.6.1 Solubility

The aqueous solubility of solid compounds (**1**), (**8**) and (**9**) was determined by preparing saturated solutions in phosphate buffer solution (PBS) at pH 7.4. The slurries were stirred with magnetic bars in a water bath at 32 °C for 24 h. It was ensured that an excess of solute is present at all times to provide saturation. The solutions were filtered through 0.2 µm filter, diluted appropriately in PBS (7.4) and analysed by HPLC to determine the concentration of dissolved solutes in the PBS solution. The experiment was done in triplicate. Carbamates (**10**)-(**13**) were water-miscible oils and therefore experimental aqueous solubility could not be determined. The water solubility of these compounds was estimated employing the theoretical method reported by Yalkowsky and co-workers (Yalkowsky, Valvani, & Roseman, 1983). The water solubility of the oils is calculated from $\log S_w = -1.072 \log D + 0.672$ and that of solids from $\log S_w = -\log D - 0.01\ mp + 1.05$, where mp. is the melting point. The solubility in octanol was calculated by substituting S_w and D data in $S_{OCT} = D \times S_w$. These equations are specific to non-electrolytes. Although the investigated compounds are all weak acids, at experimental pH 7.4 they behave as non-electrolytes. The routinely used and well-documented experimental method

(Gerber, Breytenbach, & du Plessis, 2008; Kiptoo et al., 2008), which involves adding an excess quantity of compound to the aqueous medium could not be applied for all the compounds, some being crystalline while others are water-miscible oils (see Table 3.1).

Table 3.1: Oxyethylene unit (n), molecular weight (Mw), melting point (Mp), partition coefficient D (n-octanol:phosphate buffer, pH 7.4) aqueous solubility (S_w), octanol solubility (S_{OCT}), steady-stead flux (Jss) of cytarabine and its N4-carbamates (8)-(13)

Compounds	n	Mw (g/mol)	Mp (°C)	Log D[a]	SD	Log S_w (mM)	Log S_{OCT} (mM)[e]	J_{ss} (nmol/cm^2/h)[f]	SD	J_{ss} (nmol/cm^2/h)[h]	p-value[I]	p-value[J]
1		243.2	212.0	-1.93	0.02	0.86[b] (2.86[c])	-1.07	3.70[f]	1.37	3.70		
8	0	345.3	173.6	-1.20	0.03	0.51[b] (2.36[c])	-0.16	35.18[f]	59.88	8.30	0.019	1.000 (1-8)[k]
9	1	389.4	132.9	-1.38	0.02	1.10[b] (2.52[c])	-0.28	nd[g]	-	nd	-	-
10	2	433.4	oil	-1.66	0.01	2.46[d]	0.79	nd[g]	-	nd	-	-
11	7	653.7	oil	-1.83	0.02	2.65[d]	0.80	3.88[f]	3.20	3.15	0.019	1.000 (1-11)[k]
12	11	829.9	oil	-1.90	0.01	2.71[d]	0.81	1.43[f]	0.90	1.20	0.019	0.853 (1-12)[k]
13	16	1050.2	oil	-1.94	0.02	2.75[d]	0.81	2.23[f]	0.51	2.10	0.019	1.000 (1-13)[k]

[a]Means ± SD (n = 5 experiments); [b]log S_W for aqueous compounds was calculated from log S_w = -log D - 0.01MP + 1.05; [c]Determined experimentally; [d]log S_w for oils was calculated from log S_w = -1.072log D + 0.672; [e]Calculated from S_{OCT} = D X S_W; [f]Each experiment was run on three different cells; values are means. SD = Standard deviation; [g]Each experiment was run on six different cells. [h]Flux medians; [I]Kruskal-Wallis p-value; [J]Multiple Comparison p-value; nd = not detected. [k]not statistically significant

3.2.6.2 Experimental log D

The partition coefficients were determined by a method reported by Taylor and Sloan (Taylor & Sloan, 1998). Equal volumes of *n*-octanol and phosphate buffer solution of pH 7.4 were saturated with each other under vigorous stirring for at least 24 h. Accurately weighed 30 mg of (**1**) as well as of each derivative (**8**)-(**13**) was dissolved in 3 ml of pre-saturated *n*-octanol, stoppered and agitated for 10 min in a 10 ml graduated tube (0.5 ml division). Subsequently 3 ml of pre-saturated buffer was transferred to the tubes containing before-mentioned solutions. The tubes were stoppered and agitated for 45 min then centrifuged at 4000 rpm for 30 min. The volume ratio (octanol:buffer) was not discernable different from 1. The octanol and buffer phases were each diluted with methanol and the concentrations of (**8**)-(**13**) were measured by HPLC. The log D values were calculated as logs of the concentration ratios in the two phases. The results expressed as means are listed in Table 3.1.

3.2.7 *In vitro* skin permeation

3.2.7.1 Preparation of donor phase

The effect of drug concentration and solubility has been thoroughly studied for transdermal drugs. Generally the flux is concentration dependent and increases as the concentration of the drug in formulation increases (Bonate & Howard, 2004) up to saturation. In order to compare the flux of different members of a homologous series a standard concentration at which all members are in solution had to be used. Donor solutions (0.2 M) of compounds were prepared by dissolving each compound in PBS buffer at pH 7.4 and 32°°C. All the compounds were in solution at this concentration.

3.2.7.2 Skin preparation

Caucasian female human abdominal skin used for permeation studies was obtained (Human ethics approval reference number 04D08, North-West University) with written informed consent after cosmetic abdominoplasty at Sunwardpark Clinic (Boksburg, South Africa). The skin was kept in a cooler box during transportation and stored in a freezer (at -20 °C) until time of use. A scalpel was used to separate the skin from the fat layer. The epidermis was removed by first immersing the skin in 60 °C HPLC water for 60 s (Kligman & Christophers, 1963). The epidermis was then gently teased away from the skin with forceps. The epidermis was placed in a bath with HPLC water, with the outer side facing up and carefully set on Whatman® filter paper, left to dry at room temperature and wrapped in foil. The foil containing epidermis was stored in a freezer at -20 °C and was used within 3 months. Prior to use, the epidermis was

thawed and examined with a light microscope for any defects, before mounting on the Franz diffusion cells.

3.2.7.3 Skin permeation

Vertical Franz diffusion cells with 2.0 ml receptor compartments and 1.0751 cm^3 effective diffusion areas were used for permeation studies. The epidermis skin layer prepared above was placed on the lower half of the Franz cell with the stratum corneum facing upwards, the upper half of the cell was mounted ensuring no possible leakage(by applying a grease around the junction) with the epidermis separating the donor and receptor compartments. The receptor compartment was filled with PBS (pH 7.4) taking special care that no air bubbles came between the receptor vehicle and epidermis, as this would reduce the effective diffusion area. The donor compartment was filled with 1.0 ml PBS and the system was equilibrated at 32 °C for one hour in water bath. Only the receptor compartments were submerged in water and were equipped with magnetic stirrers. After a period of one hour, 1 ml of freshly prepared solutions (0.2 M) was added to each donor compartment, which was immediately covered with Parafilm® to prevent evaporation of any constituent within the solution for the duration of the experiment. On scheduled times (i.e. after 2, 4, 6, 8, 10 and 12 h), the total receptor phase was withdrawn and replaced with 32 °C fresh buffer solution (pH 7.4) to mimic sink conditions as they occur in the human body. The withdrawn samples were immediately analyzed by HPLC to determine the concentrations of the compounds that permeated the epidermis. The flux was determined by plotting cumulative drug permeating per unit area versus time to determine the slope of the steady state region. The experiments were performed in triplicate. The flux values are summarized in Table **3.1**.

3.2.7.4 Data analysis

The following statistical procedures were used to test if there were statistical significant differences between the parent compound's median flux value and that of the newly synthesized derivatives. The reason for comparing the medians instead of the means of the flux values was that nonparametric tests were done, because normality of the distribution of the data of this study could not be assumed. The usual parametric test to compare means in the case of samples of less than thirty items can only be applied when normality is assured (Steyn, Smit, Du Toit, & Strasheim, 1996).

The nonparametric Kruskal-Wallis test was therefore done with Statistica (StatSoft Inc, 2007) to test the statistical significance of differences between the medians of different compounds on a 5% level. Multiple comparisons on the mean ranks of individual groups were performed to

determine where the differences occurred. Note that the compounds where no fluxes were detected were not included in the statistical analysis, because the conclusion that they were much less efficient skin penetrant than the parent compound is trivial.

3.3 Results and Discussion

3.3.1 Chemistry

The carbamates (**8**)-(**13**) were successfully prepared in 30-75% yields. Carbamates (**8**)-(**10**) have oligomeric ethylene moieties while (**11**)-(**13**) are polymeric. All the carbamates of cytarabine were synthesized by the method as described by Bodansky (Bodansky, 1955) with modifications.

Compounds (**8**) and (**9**) were obtained as solids while (**10**)-(**13**) were oils. This reaction shows selectivity to the N4-amino group of cytosine in cytarabine as the major product is the N4 substituted derivative. The structures of the compounds were confirmed by NMR and MS. Comparison of the intergral of peak at 3.20 ppm assignable to OCH_3 (H-ω) of mPEG moiety with the intergral of the peak at 6.04 ppm assignable to H-1' that belongs to the drug moiety showed 3:1 ratio. This indicates that one molecule of the native drug is linked to one molecule of the mPEG. The carbamate linker was confirmed by the peak at 165 ppm assignable to carbamate carbonyl carbon (C-1"). Due to the electro-withdrawing nature of carbamate linker, H-5 and H-6 signals were deshielded from 5.60 and 7.50 ppm to 7.00 and 8.00 ppm, respectively. This confirms that acylation took place at N4. The ^{1}H NMR spectra of all the carbamates showed resonances in the 3.41-3.65 ppm region characteristic of methylene protons, $-OCH_2-CH_2O-$, of the mPEG part of the molecule. The MS data for the compounds confirmed the presence of molecular ions (M^+) at 345.3, 389.4 and 433.4 corresponding to the molecular formulae $C_{13}H_{19}N_3O_8$ (**8**), $C_{15}H_{23}N_3O_9$ (**9**), and $C_{17}H_{27}N_3O_{10}$ (**10**), respectively which was confirmed with accurate mass values of 345.3, 389.4 and 433.4 MS. These formulae in turn indicate the number of oxyethylene unit(s) (n) in (**8**), (**9**) and (**10**) to be 1, 2 and 3 respectively. LC–MS analysis also showed the presence of molecular ions of (**11**) (m/z 653), (**12**) (m/z 829) and (**13**) (m/z 1050). Thus, the correlation with ^{1}H NMR data revealed (n) values for (**11**), (**12**) and (**13**) to be 7, 11 and 16 respectively.

3.3.2 Hydrophilicity and lipophilicity

Generally, prodrugs with enhanced biphasic (both lipophilic and hydrophilic) solubilities are reported to show better transdermal penetration than the parent drugs (Sloan, 1989; Guy & Hadgraft, 1992). To assess solubility parameters of carbamates (**8**)-(**13**), log D

and aqueous solubility were determined. Cytarabine carbamates (9)-(10) exhibited increased hydrophilicity and lipid solubility compared with the parent compound with the increasing number of oxyethylene units (see fig 3.1 & 3.2).

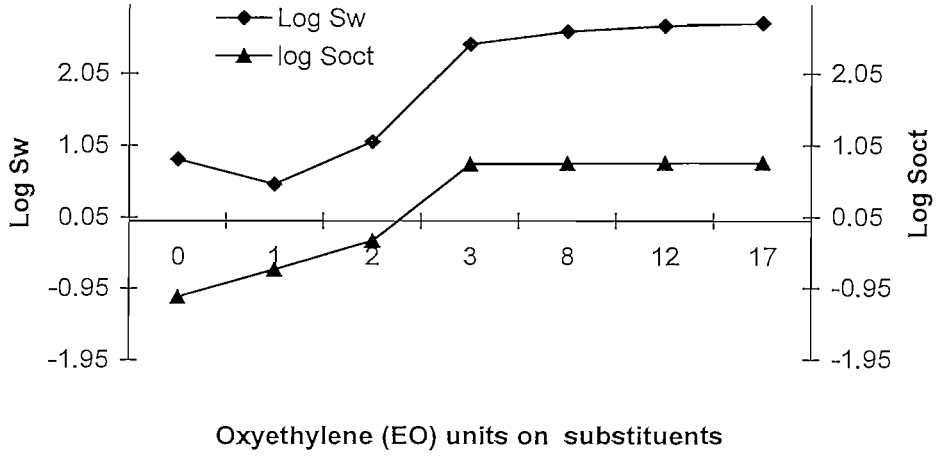


Fig. 3.1. Relationship between the octanol (▲) and water (■) solubility and number of oxyethylene (EO) units in the carbamate series

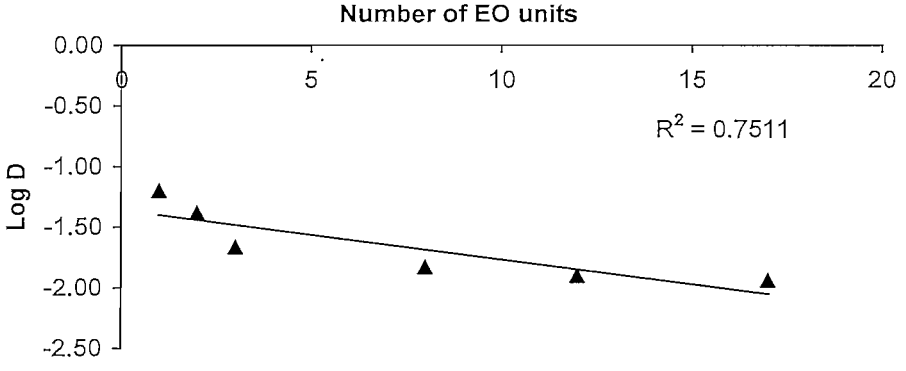


Fig. 3.2. Relationship between log D (n-octanol : PBS, pH 7.4) and the number of oxyethylene (EO) in the carbamate series

The trend was expected because mPEG is expected to impose its properties as the polymeric index increases. As the chain length increases beyond 3 oxyethylene units, the change in both lipophilicity and hydrophility becomes marginal. In the oligomeric series (8)-(10), log D decreases abruptly as oxyethylene chain lengthens whereas in polymeric series (11)-(13)

the change is marginal. It appears that the mPEG moiety imposed its amphiphilic nature by increasing both aqueous and lipid solubility of the carbamates. All the carbamate (8)-(13) prodrugs are more lipophilic than the parent drug cytarabine. Moreover, carbamate (8), the first member of the homologous series, exhibited greater solubility in octanol than cytarabine, which is in accordance with a previous report (Taylor & Sloan, 1998) that, generally, the initial increase in lipid solubility exhibited by members of a homologous series occurs because the promoiety masks a hydrogen bond donor, in this case N4-amino group of cytarabine. As may be seen in Fig. **3.1**, the aqueous solubilities increase as the ethylene oxide chain lengthens but the octanol solubilities remain relatively constant. The hydrophilicity increases as result of increased association of water molecules with the intrachain oxygen atoms of mPEG (Solomons, 1995). Although a lipophilic ethylene group was added with each oxygen atom, the additional associated water molecules mask the lipophilic ethylene group from association with the lipophilic octanol.

3.3.3 Skin permeation

The first member of the series (**8**) was found to have a higher flux than others. Compounds (**9**) and (**10**) were not detected in the receptor phase. There is no clear trend between flux values and molecular weights of compounds. Compounds (**11**)-(**13**) penetrated the skin much better than (**9**) and (**10**), despite having higher molecular weights. This corroborates previous findings in which some members of homologous series with higher molecular weights penetrated the skin better than members with lower molecular weights (Bonina et al., 1995; Bonina et al., 2001). Drug derivatives with enhanced biphasic (both lipophilic and hydrophilic) solubilities are reported to show better transdermal penetration than the parent drugs (Guy & Hadgraft, 1992; Sloan & Wasdo, 2003; Sloan, 1989). The use of amphiphililic promoieties such as polyethylene glycol and methoxypolyethylene glycol have been reported with variable success (Bonina et al., 1995; Bonina et al., 2001; N'Da & Breytenbach, 2009). In the current study, however, no significant transdermal penetration enhancement was found by using the N4-methoxypoly(ethylene glycol) carbamates of cytarabine (**1**). As compared to ketoprofen, naproxen and diclofenac for which polyoxyethylene glycol was successfully used as a promoiety for transdermal prodrug design (Bonina et al., 2001), cytarabine (**1**) and its carbamate derivatives in this study are very polar and have more hydrogen bonding functional groups. The hydrogen bonding functional groups in the permeant tend to slow down transdermal diffusion due to their interaction with polar head groups of the intercellular lipids present in the skin (du Plessis et al., 2002; Pugh et al., 2000). Although the apparent molecular weights of N4–oligomeric ethylene carbamates (**8**)-(**10**), ranging from 243.2 to 433.4 g/mol, fell in the appropriate range for transdermal delivery (Goldsmith, 1991), no significant enhancement in transdermal penetration of the parent compound was observed (Table **3.1**). This could be

attributed to *inter alia* the fact that PEG moieties in solution have higher effective MW than is apparent from the molecular formula as a result of the association of water molecules with the oxyethylene chain. Studies of PEG moieties in solution have shown that each oxyethylene unit is tightly associated with water molecules (Goldsmith, 1991).. Beize et al.(1994) used neutron diffraction to show that an average of 6 six water molecules can be packed on the surface of a monomer.

3.4 Conclusion

The N4-methoxypolyethylene glycol carbamates of cytarabine were successfully synthesized and their structures elucidated by MS and NMR spectroscopy. Lipid solubility, aqueous solubility and transdermal fluxes of the carbamates were determined. There was no significant increase in transdermal delivery of cytarabine by its derivatives and no clear relationship between lipid and aqueous solubility and transdermal flux values was observed. mPEG as a promoiety exhibited the ability to increase both aqueous and lipid solubility of carbamates of cytarabine (**1**).

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Conflict if interest

The authors declare that they have no conflict of interest to disclose.

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CHAPTER 4

4 Transdermal penetration of cytarabine and its 5'-O alkyl ester derivatives

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Abstract

The purpose of this study was to synthesize and determine the *in vitro* transdermal penetration of cytarabine and its 5'-alkyl esters and to establish a correlation, if any, with selected physicochemical properties. The *n*-alkyl esters were synthesized by acylation of cytarabine (**1**) at its 5'-OH. The transdermal flux values of (**1**) and its esters were determined *in vitro* using Franz diffusion cell methodology. Aqueous solubility and log D (pH 7.4) values were determined and assessed for correlation to transdermal flux. An inverse relation was observed between the water solubility (S_w) and log D values. Of all esters, (**4**) exhibited the highest flux value of 22.2 $nmol.cm^{-2}.h^{-1}$, which is significantly different to that of the parent drug cytarabine (3.70 $nmol.cm^{-2}.h^{-1}$). No trend was found between water solubility and flux values.

Keywords

Transdermal penetration, cytarabine, promoiety, esters, physicochemical properties, log D. aqueous solubility, skin.

4.1 Introduction

Cytarabine [1-(β-D-arabinofuranosyl)cytosine] (**1**) is a deoxycytidine analogue commonly used in the treatment of acute and chronic human leukaemia. This drug is one of the most active single agents in the treatment of acute myeloid leukaemia. It, however, has a very short plasma half-life and exhibit low oral bioavailability due to its low permeability across the intestine and extensive metabolism to its non-toxic metabolite uracil arabinoside (ara-U) by cytidine deaminase in liver, plasma and kidney [1,2]. Consequently, cytarabine is administered by intravenous infusion or by repetitive high dose regimen, which at conventional dose of 100 to 200 mg per square meter of body surface area per day for seven days as an intravenous (IV) medication is associated with adverse effects such as myelosuppresion, vomiting and stomatitis [2-4]. Since cytarabine is an S-phase-specific drug, a prolonged exposure of tumour cells to its cytotoxic levels is critical to achieve maximum activity [5,6]. To achieve this, more consistent plasma levels of cytarabine should be attained. Intravenous infusion can achieve this, but it is invasive and inconvenient.

In comparison to more conventional drug delivery strategies (such as oral tables and intravenous infusion), transdermal drug delivery (TDD) offers several important advantages over more traditional dosage forms. These include the potential for sustained release (without inconveniences of intravenous infusion) that is useful for drugs such as cytarabine with short biological half-lives requiring frequent oral or parenteral administration and controlled input kinetics, which are particularly indispensable for drugs with narrow therapeutic indices [7]. However, not all the drugs are suited for TDD. Drugs with hydrophilic structures permeate the skin too slowly to be of therapeutic benefit. Hydrogen-bonding functionality on the permeant is reported to drastically retard permeation [8-10].

Cytarabine (**1**) is known for its high hydrophilicity and plurality of polar functional groups capable of hydrogen bonding. Therefore, it becomes apparent that cytarabine would not easily permeate the skin. However, the prodrug approach could be used to circumvent these setbacks. This approach has been investigated to enhance dermal and transdermal penetration of drugs with unfavourable intrinsic properties and it showed promising outcomes [11,12].

In this study, *n*-alkyl-ester derivatives of cytarabine were synthesized and evaluated for transdermal penetration with the aim to enhance the transdermal delivery of cytarabine. The esters were characterized for log D, aqueous solubility and meting points and these physicochemical properties were assessed for any correlation with the flux values.

4.2 Materials and methods

4.2.1 Materials

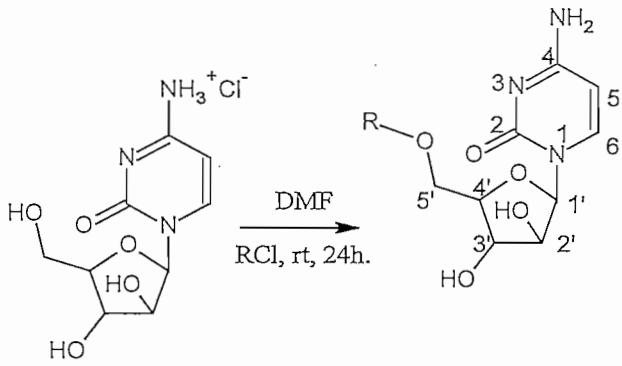
Cytarabine was purchased from Jingma Chemicals Ltd. China and acid chlorides were purchased from Sigma-Aldrich South Africa Ltd. HPLC grade methanol was obtained from Labchem South Africa Ltd. All other reagents were of analytical grade and were used without further purification.

4.2.2 General procedures

The ^{1}H and ^{13}C spectra were recorded at frequencies of 600.17 and 151.92 MHz respectively on a Bruker 600 spectrometer using deuterated DMSO as solvent. All the chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (δ = 0). The splitting pattern abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), bs (broad singlet) and m (multiplet). DSC thermograms were recorded with a Shimadzu DSC-60A using TA60 (Version 2.11) software. Approximately 2-4 mg of each sample was weighed and heated in closed aluminium crucibles. Samples were heated at 10°C/min. Nitrogen gas was used as inert atmosphere. The MS spectra were recorded on an analytical VG 7070E mass spectrometer using fast atom bombardment (FAB) as ionization technique. Thin-layer chromatography was performed using silica gel plates (60F254 Merck). Flash column chromatography was carried out on silica gel (70 -240 mesh, G60 Merck).The high performance liquid chromatography (HPLC) system consisted of a HP (Hewlett Packard) Agilent 1100 series auto sampler, HP Agilent 1100 series wave detector (VWD) and HP Agilent 1100 series pump (Agilent, Palo Alto, CA). A Phenomenex (Luna C-18, 150 x 4.60 mm, 5µm) column was used together with a Securityguard pre-column (C-18, 4x 3 mm) insert (Phenomenex, Terronce, CA) in order to prolong column life.

Elution was accomplished with a gradient consisting of methanol (A) and 0.005 M heptane sulphonic acid-Na in water (B) adjusted to pH 3.5 with orthophosphoric acid. The flow rate was 1 ml.min^{-1}. The gradient was started with 30% A, then increased linearly to 95% A in 8 min, held until 11 min, after which the column was re-equilibrated at the starting conditions.

4.2.3 Chemical synthesis



Scheme. 4.1. Compounds (**1**)-(**7**).

The synthesis of (**5**)-(**7**) was acheived by using a general method previously reported [13], and described as follows: Cytarabine hydrochloride salt (1 mmol) was dissolved in anhydrous DMF (20 mL) and the carboxylic acid chloride (1.1 mmol) was added. The reaction mixture was stirred at room temperature for 24 h. The solvent was removed under high vacuum at 80°C to yield viscous oil. This residue was thoroughly triturated several times with Et_2O:EtOAc (1:1). The solid was mixed with saturated $NaHCO_3$ solution until no further bubbles formed in order to neutralise the carboxylic acid formed as by-product. The mixture was filtered off to collect the product, which was rinsed with a minimum amount of water. The product was dried and recrystallized from a suitable solvent.

For the synthesis of compounds (**2**)-(**4**), the above described general method was used with slight modification. Thus, cytarabine (**1**) (1 mmol) was dissolved in anhydrous DMF (20 mL) and reacted with the carboxylic acid chloride (2 mmol). The reaction mixture was stirred at room temperature for 24 h. The solvent was removed under high vacuum at 80°C to dryness. The residue was purified by silica gel column chromatography to yield the target compound.

4.2.3.1 4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one-5'-acetate (2)

Ester (**2**) was purified by flash silica gel column chromatography eluting with DCM/MeOH/25% NH_3 (4:1:0.1). A yield of 1.93 g (27%) white crystalline compound was obtained. m.p. 81°C. ^{1}H NMR (600 MHz, DMSO) δ 2.04 (s, 3H, -COO*CH*$_3$), 3.89 (s, 1H, H-3'), 3.90 – 3.94 (m, 1H, H-4'), 3.98 (d, *J* = 2.0, 1H, H-2'), 4.18 (dd, *J* = 11.6, 4.3, 1H, H-5'b), 4.27 (dd, *J* = 11.6, 7.7, 1H, H-5'a), 5.60 (d, *J* = 4.6, 1H, OH-2'), 5.62 (s, 1H, OH-3'), 5.73 (d, *J* = 7.5, 1H, H-5), 6.08 (d, *J* = 3.9, 1H, H-1'), 7.32 (s, 2H, NH_2), 7.52 (d, *J* = 7.5, 1H, H-6). ^{13}C NMR (151 MHz, DMSO) δ 20.64 (, -

$COOCH_3$), 63.86 (C-5'), 74.26 (C-2'), 76.07 (C-3'), 81.83 (C-4'), 86.17 (C-1'), 92.64 (C-5), 143.17 (C-6), 154.31 (C-4), 164.93 (C-2),170.26 ($COOCH_3$). MS FAB 285.9 ($M+H^+$), 112.0, 133.9, 238.8.

4.2.3.2 4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one-5'-propionate (3)

Ester (**3**) was purified by flash silica gel column chromatography eluting with DCM/MeOH/25% aq.NH_3 (3:1:0.1). A yield of 1.25 g (39%) white crystalline compound was achieved. m.p. 113°C. ^1H NMR (600 MHz, DMSO) δ 1.06 (t, *J* = 7.5, 3H, -$COOCH_2CH_3$), 2.35 (q, *J* = 7.5, 2H, -$COOCH_2$-), 3.88 – 3.94 (m, 2H, H-3'& 4'), 4.01 (s, 1H, H-2'), 4.22 (dd, *J* = 11.6, 4.1, 1H, H-5'a), 4.29 (dd, *J* = 11.6, 7.0, 1H, H-5'b), 5.44 (d, *J* = 4.5, 1H, OH-3'), 5.48 (d, *J* = 3.4, 1H, OH-2'), 5.70 (d, *J* = 7.4, 1H, H-5), 6.10 (d, *J* = 4.0, 1H, H-1'), 6.94 (s, 2H, NH_2), 7.49 (d, *J* = 7.4, 1H, H-6). MS FAB 313.7 ($M+H^+$), 335.8 ($M+Na^+$), 122.1, 134.0.

4.2.3.3 4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one-5'-butyrate (4)

Ester (**4**) was purified by flash silica gel column chromatography eluting with DCM/MeOH/25% aq.NH_3 (5:1:0.1). A yield of 1.41 g (42%) white crystalline compound was achieved. m.p. 135°C. ^1H NMR (600 MHz, DMSO) δ 0.89 (t, *J* = 7.4, 3H, -CH_2CH_3), 1.56 (h, *J* = 7.3, 2H, -$COOCH_2CH_2$-), 2.30 (t, *J* = 7.2, 2H, -$COOCH_2$-), 3.82 – 3.94 (m, 2H, H-4'&3'), 3.99 (s, 1H, H-2'), 4.20 (dd, *J* = 11.6, 4.3, 1H, H-5'b), 4.29 (dd, *J* = 11.6, 7.4, 1H, H-5'a), 5.31 – 5.52 (m, 2H, OH-2'&3'), 5.67 (d, *J* = 7.4, 1H, H-5), 6.09 (d, *J* = 3.9, 1H, H-1'), 6.90 (s, 2H, NH_2), 7.47 (d, *J* = 7.4, 1H, H-6). ^{13}C NMR (151 MHz, DMSO) δ 13.08 (-CH_2CH_3), 17.68 (-$COOCH_2CH_2$-), 35.16 (-$COOCH_2$-), 63.43 (C-5'), 74.36 (C-2'), 76.75 (C-4'), 81.56 (C-3'), 85.94 (C-1'), 92.28 (C-5), 142.60 (C-6), 154.89 (C-4), 165.44 (C-2), 172.38 (-$COOCH_2$-). MS FAB 335.8 ($M+H^+$), 313.8 ($M+Na^+$), 112.1.

4.2.3.4 4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one-5'-hexanoate (5)

Ester (**5**) was recrystallized from methanol and a yield of 2.03 g (55%) white crystalline compound was achieved. m.p. 136°C. ^1H NMR (600 MHz, DMSO) δ 0.85 (t, *J* = 6.9, 3H, -CH_2CH_3), 1.20 – 1.32 (m, 4H, -$CH_2CH_2CH_3$), 1.50 – 1.57 (m, 2H, H-3), 2.32 (t, *J* = 7.4, 2H,-$COOCH_2$-), 3.85 – 3.89 (m, 1H, H-3'), 3.89 – 3.93 (m, 1H, H-4'), 3.96 (dd, *J* = 6.3, 4.4, 1H, H-2'), 4.18 (dd, *J* = 11.6, 4.2, 1H, H-5'b), 4.30 (dd, *J* = 11.5, 7.7, 1H, H-5'a), 5.57 (dd, *J* = 7.1, 4.4, 2H, OH-2'&3'), 5.66 (d, *J* = 7.4, 1H, H-5), 6.09 (d, *J* = 3.8, 1H, H-1'), 7.09 (s, 2H, NH_2), 7.47 (d, *J* = 7.4, 1H, -CH_2CH_3). ^{13}C NMR (151 MHz, DMSO) δ 13.76 (-CH_2CH_3), 21.74 (-CH_2CH_3), 24.10 (-

CH$_2$CH$_2$CH$_3$), 30.55 (-COOCH$_2$*CH*$_2$-), 33.34 (- CO*OCH*$_2$-), 63.64 (C-5'), 74.26 (C-2'), 76.69 (C-4'), 81.72 (C-3'), 86.11 (C-1'), 92.49 (C-5), 142.78 (C-6), 155.05 (C-4), 165.56 (C-2), 172.82 (-*C*OOCH$_2$-). MS FAB 341.7 (M+H$^+$), 134.2, 112.0.

4.2.3.5 4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one-5'-octanoate (6)

Ester (**6**) was recrystallized from methanol and a yield of 1.70 g (55%) white crystalline compound was achieved. m.p. 148°C. ^{1}H NMR (600 MHz, DMSO) δ 0.85 (t, *J* = 7.0, 3H, -CH$_2$*CH*$_3$), 1.25 (-(*CH*$_2$)$_4$CH$_3$), 1.53 (dd, *J* = 18.4, 11.3, 2H, -COOCH$_2$*CH*$_2$-), 2.32 (t, *J* = 7.3, 2H, -COO*CH*$_2$-), 3.87 (t, *J* = 2.4, 1H, H-3'), 3.88 – 3.93 (m, 1H, H-4'), 3.96 (dd, *J* = 3.8, 2.3, 1H, H-2'), 4.18 (dd, *J* = 11.6, 4.2, 1H, H-5'b), 4.30 (dd, *J* = 11.6, 7.8, 1H, H-5'a), 5.62 (s, 2H, H-2'&3'), 5.66 (d, *J* = 7.4, 1H, H-5), 6.09 (d, *J* = 3.9, 1H, H-1'), 7.08 (s, 2H, NH$_2$), 7.47 (d, *J* = 7.4, 1H, H-6).^{13}C NMR (151 MHz, DMSO) δ 13.89 (-CH$_2$*CH*$_3$), 21.97 (-*CH*$_2$CH$_3$), 24.43 (-*CH*$_2$CH$_2$CH$_3$), 28.29 (-COOCH$_2$CH$_2$*CH*$_2$*CH*$_2$-), 33.39 (-COO*CH*$_2$-), 31.06 (-COOCH$_2$*CH*$_2$-), 63.66 (C-5'), 74.28 (C-2'), 76.69 (C-4'), 81.70 (C-3'), 86.08 (C-1'), 92.48 (C-5), 142.81 (C-6), 155.05 (C-4), 165.55 (C-2), 172.82 (-*C*OOCH$_2$-). MS FAB 392.0 (M+Na$^+$), 355.6, 265.7, 239.2, 133.9.

4.2.3.6 4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one-5'-decanoate (7)

A yield of 1.31 g (31%) white crystalline compound was achieved. m.p. 120°C.^{1}H NMR (600 MHz, DMSO) δ 0.85 (t, *J* = 7.0, 3H, -CH$_2$*CH*$_3$), 1.18 – 1.30 (m, 12H, -(*CH*$_2$)$_6$CH$_3$), 1.52 (dd, *J* = 14.1, 7.1, 2H, -COOCH$_2$*CH*$_2$-), 2.32 (t, *J* = 7.3, 2H, -COO*CH*$_2$-), 3.87 (s, 1H, H-3'), 3.89.– 3.93 (m, 1H, H-4'), 3.96 (s, 1H, H2'), 4.17 (dd, *J* = 11.6, 4.2, 1H, H-5'a), 4.30 (dd, *J* = 11.5, 7.8, 1H, H-5'a), 5.58 (s, 2H, OH-2'&3'), 5.66 (d, *J* = 7.4, 1H, H-5), 6.08 (d, *J* = 3.8, 1H, H-1'), 7.08 (s, 2H, NH$_2$), 7.47 (d, *J* = 7.4, 1H, H-6). ^{13}C NMR (151 MHz, DMSO) δ 13.92 (-CH$_2$*CH*$_3$), 22.04 (-*CH*$_2$CH$_3$), 24.43 (-*CH*$_2$CH$_2$CH$_3$), 31.22 (-COOCH$_2$*CH*$_2$-), 33.38 (-COO*CH*$_2$-), 63.66 (C-5'), 74.25 (C-2'), 76.69 (C-4'), 81.75 (C-3'), 86.13 (C-1'), 92.49 (C-5), 142.78 (C-6), 155.05 (C-4), 165.55 (C-2), 172.82 (-*C*OOCH$_2$-).

4.2.4 Physicochemical properties

4.2.4.1 Solubility

The aqueous solubility of the alkyl esters was obtained by preparing saturated solutions in phosphate buffer solution PBS (10^{-2}M, pH 7.4). The slurries were stirred with magnetic bars in a water bath at 32°C for 24 h. It was ensured that an excess of solute was present at all times to provide saturation. The solutions were filtered through 0.2 μm acrodisc filters, appropriately

diluted with PBS (10^{-2}M, pH 7.4) and analysed by HPLC to determine the concentration of dissolved solutes.

4.2.4.2 Log D

Equal volumes of *n*-octanol and PBS at pH 7.4 were saturated with one another by mixing and vigorously stirring for at least 24 h. The two phases were allowed to partition and then separated. An excess of the compounds was dissolved in 1 mL pre-saturated *n*-octanol and 1 mL pre-saturated PBS buffer, stoppered, shaken for 60 min and then centrifuged at 25°C and 4000 rpm for 30 minutes. Excess solutes were present at all times. The aqueous and the *n*-octanol phases were separated diluted appropriately by PBS and methanol respectively, prior to HPLC analysis. The *n*-octanol–PBS partition coefficients (log D) were calculated as logarithmic ratios of the solutes concentrations in the *n*-octanol phase to the concentrations in the PBS. These experiments were done in triplicate. Log P values were also calculated by KowWin [14] and ACD/ChemSketch [15] softwares. All the results are reported in Table **4.1**.

Table 4.1: Aqueous solubility, partition coefficient, melting point and transdermal data for compounds (**1**)-(**7**)

Compound	Mr	Mp (°C)	S_w (±SD) (μmol/mL)[a]	S_w(±SD) (mg.mL^{-1})	Log D[a]	Log S_w (μmole/mL)	Log S_{OCT} (μmole/mL)[b]	J_{ss} (nmol.cm^{-2}.h^{-1})[c]	J_{ss} (nmol.cm^{-2}.h^{-1})[f]	SD	p-value[g]	p-value[h]	R_t
1	243.2	212.0	738.18 ± 33.79	179.54 ± 8.22	-1.9 ± 0.020	2.86	0.93	3.70 [d]	3.70	1.37			4.4
2	285.3	81.1	153.82 ± 7.19	43.88 ± 2.05	-1.2 ± 0.020	2.19	1.02	nd[e]	nd				5.4
3	299.3	112.5	74.79 ± 0.80	22.38 ± 0.24	-0.83 ± 0.02	1.87	1.04	3.60 [d]	3.65	1.45	1.000 (1-3)	0.044	6.1
4	313.3	135.0	48.46 ± 1.14	15.18 ± 0.36	-0.26 ± 0.00	1.69	1.43	22.20 [d]	21.81	2.72	0.075 (1-4)	0.044	3.7
5	341.4	135.4	15.02 ± 0.40	5.13 ± 0.14	0.83 ± 0.01	0.71	1.54	nd[e]	nd		-	-	8.8
6	369.4	147.9	0.56 ± 0.02	0.2 1± 0.01	1.96 ± 0.06	-0.25	1.71	nd[e]	nd		-	-	8.8
7	397.5	120.2	0.02 ± 0.00	0.01 ± 0.00	3.25 ± 0.09	-1.79	1.46	nd[e]	nd		-	-	9.7

[a]Determined experimental; [b]Calculated from $S_{OCT} = D \times S_W$; [c]Median steady-state flux;

[d]Each experiment was run on three different cells; [e]Each experiment was run on six different cells;

[f]Mean steady-state flux; [g]Multiple comparison p-value; [h]Krusal-Wallis p-value; R_t, retention time; nd, not detected

4.2.5 *In vitro* skin permeation

4.2.5.1 Preparation of the donor phase

Donor solutions were obtained by preparing saturated solutions of compounds (**1**)-(**7**) in PBS (10^{-2}M, pH 7.4) at 32°C. To ensure saturation, the slurries were stirred in a water bath at 32°C for 24 h.

4.2.5.2 Skin preparation

Female Caucasian human abdominal skin used for permeation studies was obtained from Sunwardpark Clinic (Boksburg, South Africa) after cosmetic procedure. The skin was stored in a cooler box during transportation and stored in a freezer at (-20°C) until time of use (less than 3 months). A scalpel was used to separate the skin from the fat layer. Afterward the epidermis was removes by first immersing the skin in 60°C HPLC water for 60 s [16]. The epidermis was then gently teased away from the skin with forceps. The epidermis was placed in a bath filled with HPLC water, with the outer side facing up and carefully set on Whatman® filter paper, left to dry at room temperature and wrapped in a foil. The foil containing epidermis was stored in a freezer at -20°C and was used within 3 months. Prior to use, the epidermis was thawed and thoroughly examined using light microscope for any defects, before mounting on the Franz diffusion cells.

4.2.5.3 Skin permeation

Vertical Franz diffusion cells with 2.0 mL receptor compartments and 1.0751 cm^2 effective diffusion areas were used for permeation studies. The epidermis skin layer prepared above was mounted on the lower half of the Franz cell with the stratum corneum facing upwards. Subsequent to that, the upper half of Franz cell was mounted and vacuum grease applied around the junction of upper and lower components of Franz cell to prevent possible leakage. A clamp was used to fasten the upper and lower parts of the Franz cell together, with the epidermis separating the donor and receptor compartments. The receptor compartment was filled with PBS (10^{-2}M, pH 7.4). Special care was taken to ensure that no air bubbles came between the receptor vehicle and epidermis, as this would reduce effective diffusion area. The donor compartment was filled with 1.0 mL PBS, equilibrated at 32°C for one hour in water bath. Only the receptor compartments were submerged in a water bath and were equipped with magnetic stirring bars. After a period of one hour, 1 mL of freshly prepared saturated solutions of the analytes were added to each donor compartment, which were immediately covered with Parafilm® to prevent evaporation of any constituent within the solution for the duration of the

experiment. Excess of solutes were present in the donor phase at all times during the experimental procedure to ensure that the donor solutions remained saturated. After, 2, 4, 6, 8, 10 and 12 h the receptor phase was withdrawn and replaced by fresh buffer (32°C) to mimic sink conditions as they occur in the human body. The withdrawn samples were immediately analyzed by HPLC to determine the concentrations of the compounds which permeated through the epidermis. These experiments were done in three different cells. The steady-state flux (J_{ss}) was determined as the slope of the plot of cumulative drug permeating per unit area *versus* time in the steady-state.

4.2.5.4 Statistical Methods

The following statistical procedures were used to tests if there were statistical significant differences between the parent compound's median flux value and the new designed compounds' median flux values. The reason for comparing the medians of the flux values instead of the mean flux values was that nonparametric tests were done, because normality of the distribution of the data could not be assumed in the case of this study's data. The usual parametric test to compare means in the case of samples less than thirty can only be applied when normality is assumed [17]. Thus mean values were only reported in table for completeness' sake.

The nonparametric Kruskal-Wallis test was therefore done with Statistica [18] to test the statistical significance of differences between the medians of different compounds on a 5% level. Multiple comparisons on the mean ranks of individual groups were performed to determine where the differences occurred. Note that the compounds where no fluxes were detected were not included in the statistical analysis, because the conclusion that they were much less efficient than the parent compound is trivial.

4.3 Results and discussion

4.3.1 Synthesis of cytarabine derivatives

The alkyl esters were successfully synthesized and their structures confirmed by NMR and MS data.[13]C NMR spectra of the esters confirmed the presence of carbonyl carbon indicated by a signal at 172 ppm. The 5'-H peak appeared in the ^{1}H NMR spectra of the esters, as two doublets of doublets shifted down field to *ca* 1-1.2 ppm, in comparison to that of the native compound due to linkage to the electron-withdrawing carbonyl group. MS spectra confirmed the molecular weights of (**2**), (**3**), (**4**), (**5**), (**6**) and (**7**) to be 285.2, 299.2; 313.3; 341.3; 369.4 and 397.4, respectively.

4.3.2 Physicochemical properties

Physicochemical properties such as partition coefficient and aqueous solubility have an influence on transdermal penetration of chemical compounds including drugs [19]. Physicochemical properties of (**1**) and its esters (**2**)-(**7**) are presented in Table (**4.1**). As can been in Table (**4.1**), compound (**2**), the first member of the ester homologues series exhibited greater solubility in octanol than the native compound (**1**), which is in accordance with previous reports that, generally, the initial increase in lipid solubility exhibited by members of a homologous series occurs because the promoiety masks a hydrogen bond donor, in this case 5'-OH of cytarabine [20]. In this homologous series, octanol solubility values increased whereas aqueous solubility decreased as the alkyl chain lengthened (see Fig. **4.1 & 4.2**). As a consequence, the log D, increases as the chain lengthens (Table **4.1**). This corroborates previous findings in the literature that by increasing non-polar portion of a molecule by extending the length of the chain produces certain characteristic features, such as elevation of boiling point, decreased aqueous solubility and increased partition coefficient [21]. It is noteworthy that the trend lines for $\log S_{oct}$ and $\log S_w$ cross for compound (**4**), suggesting that the best balance of $\log S_{oct}$ and $\log S_w$ is obtained for compound (**4**)

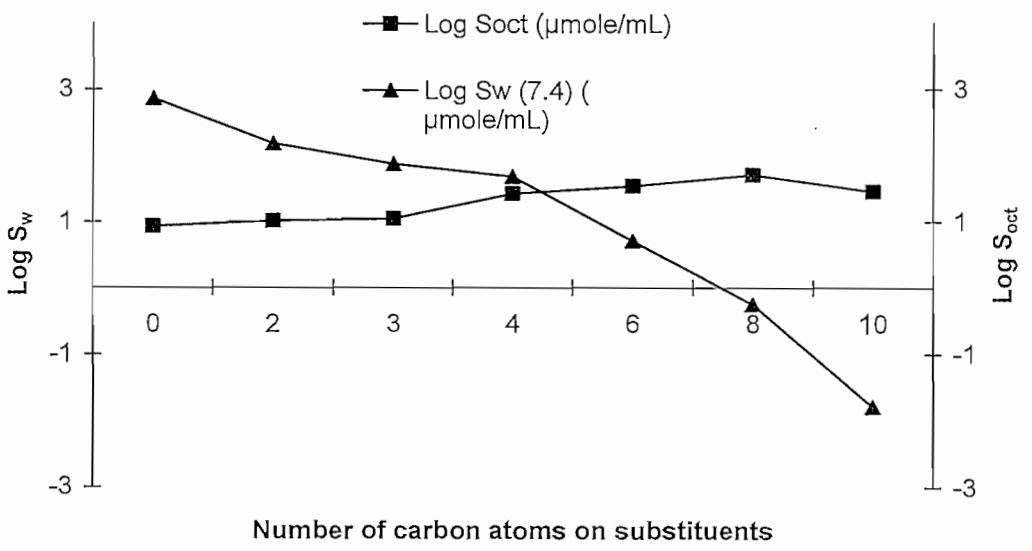


Fig. 4.1. Relationship between lipids and aqueous solubility parameters and number of carbons in the ester series

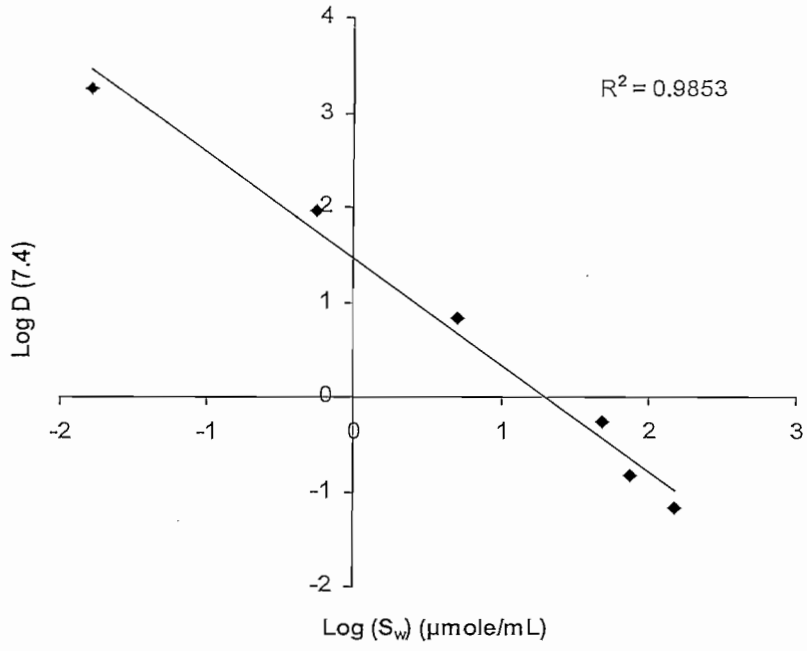


Fig. 4.2. Log D (7.4) versus log S_w of (**1**)-(**7**)

The melting points of ester compounds (**2**)-(**7**) were lower than that of cytarabine. The S_w of compounds decreases of as the melting point increases in the series except for compound (**7**). It is known that the solubility of a solid solute in water is dependent on two factors: the crystallinity of the solute and the ability of the solute to interact with water [22]. Generally, compounds with lower meting points are associated with higher solubility in water and organic solvent and are known to permeate the skin better than those with higher melting points [23]. In this study, however, no clear relationship was observed between melting points and aqueous solubility. Dispite having higher melting point, cytarabine exhibited the highest aqueous solubility than its ester derivatives. It is interesting to compare experimentally determined log D values with those predicted by ACD and KowWin software. As shown in Fig. (**4.3**), there is a linear relationship between predicted and experimental values for (**1**) and its derivatives. ACD predicted values are very close to experimental ones. KowWin predicted values were generally higher the experimental ones.

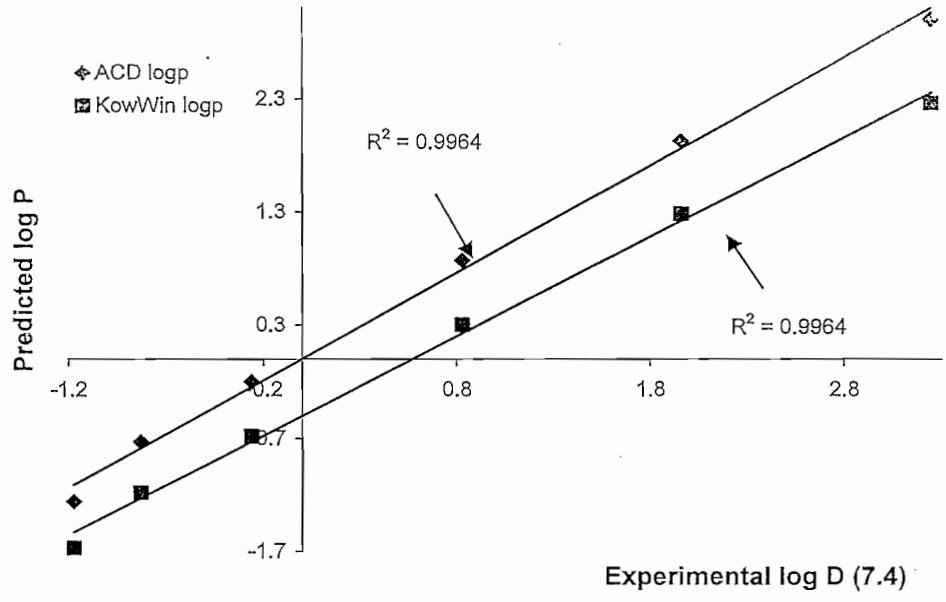


Fig. (3). Experimental log D (7.4) versus calculated log P (ACD version 11 & KowWin) for 5'-alkyl ester derivatives of cytarabine

4.3.3 *In vitro* skin permeation study

The diffusion experiments showed that there is a varying degree of permeation between (**1**) and its 5'-alkyl esters. There is no clear trend between flux values and molecular weights of compounds. Compounds (**3**)-(**4**) penetrated the skin much better than the first member of the series (**2**), despite having higher molecular weights. These corroborate previous findings in which some members of homologous series with higher molecular weights penetrated the skin better than members with lower molecular weights The steady-state flux value of (**1**) was found to be 3.7 nmol.cm^{-2}.h^{-1}. The flux of cytarabine is very low in comparison to that of chemically related nucleoside zalcitabine, with a reported flux of 442 nmol.cm^{-2}.h^{-1} [26]. This could be attributed to *inter alia* physicochemical properties, its high hydrophilicity, since many drugs with hydrophilic structures permeate the skin too slowly due to hydrogen-bonding functionalities on the permeants which drastically retard their skin permeation through their interaction with polar head groups of the intercellular lipids present in the skin [8-10]. The only structural difference between these two drugs is that, (**1**) has extra two hydroxyl groups at C-2' and C-3'. Zalcitabine has a reported flux value of 442 nmol.cm^{-2}.h^{-1} from PBS (10^{-2}M, pH 7.4) [26], which is 120-fold that of (**1**). It is noteworthy that the presence of only extra two hydrogen bonding groups makes a huge difference.

Of all these esters, compound (4) clearly exhibited the highest flux of 21.8 nmol.cm^{-2}.h^{-1}, which is a significant enhancement compared to (1). This is could be attributed to its relative good balance between aqueous and lipid solubility (Fig. **4.1**). This finding corroborates the previous finding that the balance between lipid and aqueous solubility is essential to optimize flux [12]. Generally, the flux values of (**1**) and its alkyl esters are very low compared to compounds that are clinically administered by transdermal delivery system such as nicotine and scopolamine [23]. The fact that compound (**1**), and its alkyl esters contain numerous hydrogen bond functionalities, could justify their poor skin penetration. It is generally accepted that for optimum transdermal permeation, the hydrogen bonding groups on permeants should not exceed 2 [8,27].

4.4 Conclusion

The *n*-alkyl esters of cytarabine were synthesized and characterized for aqueous solubility, lipophilicity and transdermal penetration. The chemical structures were confirmed by NMR and MS spectroscopy. The highest flux value of (**4**) is attributable to better balance between its hydrophilicity and lipophilicity than others. The generally low flux values of cytarabine and its esters could be due to their numerous hydrogen-bonding groups, which are known to impede transdermal penetration of drugs.

4.5 Acknowledgments

The authors acknowledge the financial support provided by North-West University and the National Research Foundation of South Africa.

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4.7 Website address of Medicinal Chemistry

http://www.bentham.org/mc/MSandI.htm

CHAPTER 5

5 *In vitro* transdermal penetration of cytarabine and its N4-alkylamide derivatives

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Abstract

Objectives The aim of this study was to synthesise and determine the transdermal penetration of cytarabine alkylamide derivatives and assess the correlation of flux with physicochemical properties.

Methods The alkylamide derivatives of cytarabine were synthesised by acylation at N4-amino group by the mixed anhydride method. The *in vitro* permeation studies were performed using Franz diffusion cell methodology. Furthermore, partition coefficients (*n*-octanol-water) and aqueous solubility of N4-alkylamide derivatives of cytarabine were determined in order to obtain information about their lipophilicity and hydrophilicity.

Key findings Alkylamides (**2**)-(**6**) showed decreased hydrophilicity and increased lipophilicity. The log D values of the alkylamides were higher than that of the parent compound and increased linearly as the alkyl chain lengthened. Compound (**4**) showed the highest median steady-state flux (J_{ss}) of 89.0 nmol/cm^2/h in the series, which shows a high statistical difference with the parent compound flux value (3.70 nmol/cm^2/h).

Conclusions The prodrug approach appears to be a promising strategy for the enhancement of transdermal penetration of cytarabine.

Keywords: Akylamides; cytarabine; transdermal penetration; *in vitro*

5.1 Introduction

Cytarabine is a nucleoside analogue of deoxycytidine which is extensively used in the treatment of both acute and chronic myeloblastic leukaemias. It's a geometric isomer of cytidine and differs in that the 2'-hydroxyl group is oriented in the *trans* position.[1] The major impediments to a broad clinical use of cytarabine include the rapid metabolism of the drug in plasma to its inactive metabolite ara-U by the enzyme deoxycytidine deaminase and its cell cycle (S-phase) specificity.[2] A prolonged exposure of cells to cytarabine's cytotoxic concentrations is essential to achieve maximum activity since it is a cell-cycle specific drug. In practice it is administered by repetitive schedules or continuous intravenous infusion in order to achieve sustained supply.[1] These modes of administration are inconvenient and invasive, which could contribute to increased patient non-compliance.

Transdermal drug delivery (TDD) offers several advantages over more traditional dosage forms such as oral and intravenous infusion. These include the potential for sustained release which is useful for drugs with short biological half-lives requiring frequency oral or parenteral administration and controlled input kinetics which are particularly indispensable for drugs with narrow therapeutic indices.[3] TDD can achieve consistent plasma concentrations similar to intravenous infusion without the inconvenience. Due to its unfavourable physicochemical properties, cytarabine would not easily permeate the skin without chemical modifications.

Many approaches have been explored to protect cytarabine from deamination which include chemical modifications. Among others, N4-acylation has been shown to prevent inactivation by cytidine deaminase. [4, 5] N4-acylation of cytarabine with long chain fatty acids such as stearic acid and behenic acid lead to lipophilic amide derivatives of cytarabine which exhibited greater antileukaemia activity than native cytarabine.[6]

In studies done on a structurally related compound (gemcitabine), the amide linkage was shown to have extreme chemical stability in pH range 4-9 and yet were bioreversable.[7] Therefore an amide would be a plausible linker for N4–prodrugs of cytarabine.

The objective of this study was to synthesise and investigate the transdermal flux of N4-alkylamides of cytarabine. Physicochemical properties were determined and assessed for correlations to transdermal flux values.

5.2 Material and Methods

5.2.1 Materials

Cytarabine was purchased from Jingma Chemicals Ltd. China. The carboxylic acid anhydrides and ethylchlorocarbonate were purchased from Sigma-Aldrich South Africa Ltd. HPLC grade methanol was obtained from Labchem South Africa Ltd. All other reagents were of analytical grade and were used without further purification.

5.2.2 General procedures

The ^{1}H, ^{13}C, COSY, HSQC and HMBC spectra were recorded on a Bruker 600 spectrometer, using deuterated DMSO as solvent. The ^{1}H and ^{13}C spectra were recorded at frequencies of 600.17 and 151.92 MHz respectively. All the chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane ($\delta = 0$). The splitting pattern abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), bs (broad singlet) and m (multiplet). The meting points of solid products were determined by Shimadzu DSC-60A using TA60 (Version 2.11) software. MS spectra were recorded on an analytical VG 7070E mass spectrometer using fast atom bombardment (FAB) as ionization technique. Thin-layer chromatography was performed using silica gel plates (60F254 Merck) and flash column chromatography on silica gel (70 -240 mesh, G60 Merck).

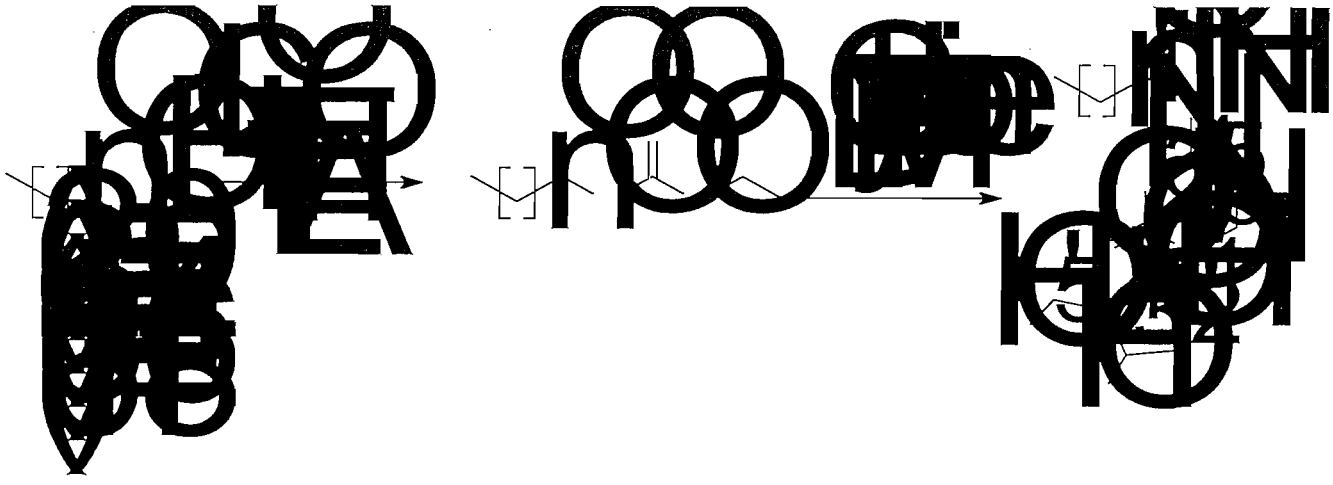
5.2.3 High pressure liquid chromatography (HPLC)

The high performance liquid chromatography (HPLC) system consisted of a Hewlett Packard (HP) Agilent 1100 series auto sampler, HP Agilent 1100 series variable wavelength detector (VWD) and HP Agilent 1100 series pump (Agilent, Palo Alto, CA). A Phenomenex (Luna C-18, 150 x 4.60 mm, 5 µm) column was used with a Securityguard pre-column (C-18, 4x 3 mm) insert (Phenomenex, Terronce, CA) in order to prolong column life. Elution was accomplished with a flow rate of 1 $ml.min^{-1}$ with a mobile phase consisting of methanol (A) and 0.005 M heptane sulphonic acid-Na in water adjusted to pH 3.5 with orthophosphoric acid (B). The gradient was started with 30% A, then increased linearly to 95% A in 8 min, held until 11 min, whereafter the column was re-equilibrated at the starting conditions.

5.2.4 Chemical synthesis

The N4-alkylamide derivatives of cytarabine were synthesised by a mixed anhydride method.[8] Aliphatic carboxylic acids were linked with amino group of cytarabine in two step process: by reacting ethylchlorocarbonate (chloroformic acid ethyl ester) and the carboxylic acid in the presence of triethylamine (TEA) to obtain the corresponding very reactive mixed-anhydride; and

by adding cytarabine solution to the reactive mixture (without isolating the mixed anhydrides) to give the N4-alkylamide derivatives.



Scheme 5.1. Synthesis of N4-alkylamide derivatives of cytarabine

Ethylchlorocarbonate (0.56 mmol, 60.8 mg) was added drop-wise to an anhydrous THF solution of the lipophilic acid (0.56 mmol) and TEA (0.56 mmol, 56.7 mg). The reaction was maintained under stirring at ± 15°C for 20 min. A solution of cytarabine (0.56 mmol, 136.2 mg) in anhydrous dimethylformamide (DMF) was added under stirring at –15°C. The reaction mixture was maintained at 5-10°C with stirring until the end of reaction. The reaction mixture was concentrated to dryness under high vacuum and the residue purified by silica column chromatography using mixture of organic solvents as eluting agents.

5.2.4.1 N4-acetyl-4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one (2)

Compound (**2**) was purified by column chromatography using DCM:MeOH (8:1,v/v) as a mobile phase to obtain 1.7 g (36%) of white powder. m.p. 183°C. ^{1}H NMR (600 MHz, DMSO) δ 2.10 (s, 3H, -HNCO*CH*$_3$), 3.63 (t, J = 5.3, 2H, 5'), 3.80 – 3.86 (m, 1H, H-4'), 3.94 (s, 1H, H-3'), 4.07 (dd, J = 7.6, 4.1, 1H, H-2'), 4.92 (t, J = 5.5, 1H, 5'), 5.33 (d, J = 4.3, 1H, OH-3'), 5.35 (d, J = 5.6, 1H, OH-2'), 6.06 (d, J = 4.0, 1H, H-1'), 7.13 (d, J = 7.4, 1H, H-5), 8.03 (d, J = 7.5, 1H, H-6), 10.63 (s, 1H,-HNCO). ^{13}C NMR (151 MHz, DMSO) δ 24.07 (-HNCO*CH*$_3$), 60.92 (C-5'), 74.59 (C-3'), 76.19 (C-2'), 85.52 (C-4'), 86.72 (C-1'), 93.98 (C-5), 146.35 (C-6), 154.27 (C-4), 161.94 (C-2), 170.57 (C-1"). MS FAB 244.0 (M+H$^+$), 175.9, 153.8, 122.2, 102.0, 74.0, 57.9

5.2.4.2 N4-butyryl-4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one (3)

Amide (3) was purified by column chromatography using DCM:MeOH (8:1) as a mobile phase to obtain 1.2 g (23%) of white powder. m.p. 193°C. ^{1}H NMR (600 MHz, DMSO) δ 0.90 (t, *J* = 7.4, 3H, -CH_2CH_3), 1.59 (h, *J* = 7.3, 2H, -CH_2CH_3), 2.39 (t, *J* = 7.3, 2H, HNCOCH_2-), 3.64 (s, 2H, H-5'), 3.85 (td, *J* = 5.2, 3.1, 1H, H-4'), 3.95 (d, *J* = 1.8, 1H, H-3'), 4.09 (s, 1H, H-2'), 4.94 (s, 1H, OH-5'), 5.35 (s, 2H, OH-2'&3'), 6.07 (t, *J* = 4.9, 1H, H-1'), 7.18 (d, *J* = 7.4, 1H, H-5), 8.05 (d, *J* = 7.5, 1H, H-6), 10.60 (s, 1H, -HNCO). MS FAB (M^+) 313.9, $(M+Na)^+$ 335.9.

5.2.4.3 N4-hexanoyl-4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one (4)

Compound (**4**) was purified by column chromatography using DCM:MeOH (12:1) as a mobile phase to obtain 3.5 g (63%) of white powder. m.p. 130°C. ^{1}H NMR (600 MHz, DMSO) δ 0.88 (t, *J* = 7.0, 3H, -CH_2CH_3), 1.19 – 1.38 (m, 4H, -$CH_2CH_2CH_3$), 1.50 – 1.65 (m, 2H, -$COCH_2CH_2$-), 2.40 (t, *J* = 7.4, 2H, -HNCOCH_2-), 3.64 (t, *J* = 4.5, 2H, H-5'), 3.82 – 3.87 (m, 1H, H-4'), 3.95 (s, 1H, H-3'), 4.08 (s, 1H, H-2'), 4.93 (s, 1H, OH-5'), 5.35 (t, *J* = 4.8, 2H, OH-2'&3'), 6.07 (d, *J* = 4.0, 1H, H-1'), 7.18 (d, *J* = 7.4, 1H, H-5), 8.04 (d, *J* = 7.5, 1H, H-6), 10.60 (s, 1H, HNCO). MS FAB 341.7 (M^+), 231.7, 210.0, 148.7, 112.0.

5.2.4.4 N4-octanoyl-4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one (5)

Amide (**5**) was purified by column chromatography using DCM:MeOH (10:1) as a mobile phase to obtain 2.8 g (43%) of white powder. m.p. 145°C. ^{1}H NMR (600 MHz, DMSO) δ 0.87 (t, *J* = 7.0, 3H, -CH_2CH_3), 1.28 (dd, *J* = 7.5, 3.7, 8H, -$(CH_2)_4CH_3$, 1.48 – 1.67 (m, 2H, -$COCH_2CH_2$-), 2.40 (t, *J* = 7.4, 2H, HNCOCH_2-), 3.64 (t, *J* = 4.6, 2H, H-5'), 3.81 – 3.87 (m, 1H, H-4'), 3.91 – 4.00 (m, 2H, H-3'), 4.09 (s, 1H, H-2'), 4.93 (s, 1H, OH-5'), 5.35 (t, *J* = 4.5, 2H, OH-2'& 3'), 6.07 (d, *J* = 4.0, 1H, H-1'), 7.18 (d, *J* = 7.5, 1H, H-5), 8.04 (d, *J* = 7.5, 1H, H-6), 10.60 (s, 1H, HNCO). MS FAB 370.2 ($M+H^+$), 260.0, 238.0, 112.1

5.2.4.5 N4-decanoyl-4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one (6)

Amide (**6**) was purified by column chromatography using DCM:EtOAc:MeOH (6:2:1) as a mobile phase to obtain 1.8 g (28%) of white powder. m.p. 153°C. ^{1}H NMR (600 MHz, DMSO) δ 0.86 (t, *J* = 7.0, 3H, -CH_2CH_3), 1.21 – 1.33 (m, 14H, -$(CH_2)_6CH_3$), 1.52 – 1.60 (m, 2H, $COCH_2CH_2$-), 2.39 (t, *J* = 7.4, 2H, HNCOCH_2-), 3.64 (s, 2H, H-5'), 3.81 – 3.88 (m, 1H, H-4'), 3.91– 3.99 (m,

1H, H-3'), 4.09 (s, 1H, H-2'), 4.93 (s, 1H, OH-5'), 5.35 (s, 2H, OH-2'&3'), 6.07 (d, J = 4.0, 1H, H-1'), 7.18 (d, J = 7.5, 1H, H-5), 8.04 (d, J = 7.5, 1H, H-6), 10.60 (s, 1H, HNCO). MS FAB 397.9 (M^+), 390.9, 288.1, 265.8, 265.8, 243.9, 112.3, 60.3.

5.2.5 Physicochemical properties

5.2.5.1 Solubility determination

The aqueous solubility of the alkylamides was obtained by preparing saturated solutions in PBS (10^{-2}M, pH 7.4). The slurries were stirred with magnetic bars in a water bath at 32°C for 24 h. It was ensured that an excess of solute was present at all times to provide saturation. The solutions were filtered through 0.2 µm acrodisc filters, diluted appropriately in PBS (7.4) and analysed by HPLC to determine the concentration of dissolved solutes in the PBS.

5.2.5.2 Experimental log D

The partition coefficients were determined by a method reported by Taylor and Sloan.[9] Equal volumes of *n*-octanol and phosphate buffer solution of pH 7.4 were saturated with each other with vigorous stirring for at least 24 h. Accurately weighed 30 mg of the amides (**2**)-(**6**) was dissolved in 3 mL of pre-saturated *n*-octanol, stoppered and agitated for 10 min in a 10 mL graduated tube (0.5 mL division). Subsequently 3 mL of pre-saturated buffer was transferred to the tubes containing the before-mentioned solutions. The tubes were stoppered and agitated for 45 min then centrifuged at 4000 rpm for 30 min. The volume ratio (octanol:buffer) was not discernably different from 1. The octanol and buffer phases were each diluted with methanol and the concentrations of (**2**)-(**6**) were measured by HPLC. The log D values were calculated as logs of the concentration ratios in the two phases. These experiments were done in triplicate. The results expressed as means are listed in Table **5.1**.

Table 5.1. Transdermal data and physicochemical properties of N4-amide derivatives of cytarabine

Compound	n	Mp (°C)	Mr	S_w (±SD) (mg/mL)	S_w (±SD) µmol/mL	Log S_{oct} (µmole/ml)[a]	Log D (7.4) (*n*-octanol-PBS)	J_{ss} (nmol/cm^2/h)[b]	J_{ss} (nmol/cm^2/h)[e]	SD	R_t	p-value[f]	p-value[g]
1		212	243.2	179.54 ± 8.22	738.18 ± 33.79	0.93	-1.9±0.02	3.70[c]	3.70	1.37	4.4		
2	0	183	285.3	12.10 ± 0.35	42.43 ± 1.2	0.36	-1.27±0.02	19.72[c]	16.60	10.79	2.2	0.005*	0.127(1-2)
3	2	193	313.3	7.95 ± 0.08	25.38 ±0.26	1.24	-0.17±0.01	nd[d]	nd		4.6		
4	4	130	341.4	4.96 ± 0.24	14.53 ± 0.69	2.07	0.91±0.05	95.93[c]	89.0	55.87	7.1	0.005*	0.005 (1-4)
5	6	145	369.4	0.45 ± 0.00	1.22 ± 0.01	2.23	2.15±0.02	nd[d]	nd		8.8		
6	8	153	397.5	0.03 ± 0.00	0.07 ± 0.01	2.14	3.28±0.06	nd[d]	nd		10.0		

[a]Calculated from $S_{OCT} = D \times S_W$; [b]flux mean; [c]each experiment was run on three different cells; [d]each experiment was run on six different cells; [e]flux medians; [f]Kruskal-Wallis p-value; [g]Multiple Comparison p-value; nd = not detected; *statistically significant

5.2.6 *In vitro* skin permeation experiments

5.2.6.1 Preparation of donor phase

Donor solutions were obtained by preparing saturated solutions of compounds (**2**)-(**6**) in PBS (10^{-2}M, pH 7.4) at 32°C. To ensure saturation, the slurries were stirred in a water bath at 32°C for 24 h.

5.2.6.2 Skin preparation

Female Caucasian human abdominal skin used for permeation studies was obtained from Sunwardpark Clinic (Boksburg, South Africa) after cosmetic procedure. The skin was stored in a cooler box during transportation and stored in a freezer at (-20°C) until time of use (less than 3 months). A scalpel was used to separate the skin from the fat layer. Afterwards, the epidermis was removes by first immersing the skin in 60°C HPLC water for 60 s.[10] The epidermis was then gently teased away from the skin with forceps. The epidermis was placed in a bath filled with HPLC water, with the outer side facing up and carefully set on Whatman® filter paper, left to dry at room temperature and wrapped in a foil. The foil containing epidermis was stored in a freezer at -20°C and was used within 3 months. Prior to use, the epidermis was thawed and examined by light microscope for any defects, before mounting on the Franz diffusion cells.

5.2.6.3 Skin permeation determination

Vertical Franz diffusion cells with 2.0 mL receptor compartments and 1.0751 cm^2 effective diffusion area was used for permeation studies. The epidermis skin layer prepared above was mounted on the lower half of the Franz cell with the stratum corneum facing upwards. Subsequent to that, the upper half of Franz cell was mounted and vacuum grease applied around the junction of upper and lower components of Franz cell to prevent possible leakage. A clamp was used to fasten the upper and lower parts of the Franz cell together, with the epidermis separating the donor and receptor compartments. The receptor compartment was filled with PBS (pH 7.4). Special care was taken to ensure that no air bubbles came between the receptor vehicle and epidermis, as this would reduce effective diffusion area. The donor compartment was filled with 1.0 mL PBS, equilibrated at 32°C for one hour in water bath. Only the receptor compartments were submerged in a water bath and were equipped with magnetic stirring bars. After a period of one hour, 1 mL of freshly prepared saturated solution was added to each donor compartment, which was immediately covered with Parafilm® to prevent evaporation of any constituent within the solution for the duration of the experiment. Excess of solutes were present in the donor phase at all times during the experimental procedure to ensure that the donor solutions remained saturated. After, 2, 4, 6, 8, 10 and 12 hours the

receptor phase was withdrawn and replaced by fresh buffer (32°C, to mimic sink conditions as they occur in the human body. The withdrawn samples were immediately analyzed by HPLC to determine the concentrations of the compounds which permeated through the epidermis. These experiments were performed in triplicate. The steady-state flux (J_{ss}) was determined by plotting cumulative drug permeating per unit area *versus* time and determining the slope of the steady-state

5.2.6.4 Statistical methods

The following statistical procedures were used to test if there were statistical significant differences between the parent compound's median flux value and that of the newly synthesised compounds. The reason for comparing the medians of the flux values instead of the mean flux values was that Nonparametric tests were done, because normality of the distribution of the data of this study could not be assumed. The usual parametric test to compare means in the case of samples less than thirty, can only be applied when normality is assumed. [11]

The nonparametric Kruskal-Wallis test was therefore done with Statistica [12] to test the statistical significance of differences between the medians of different compounds on a 5% level. Multiple comparisons on the mean ranks of individual groups were performed to determine where the differences occurred. Note that the compounds where no fluxes were detected were not included in the statistical analysis, because the conclusion that they were much less efficient than the parent compound is trivial.

5.3 Results

5.3.1 Synthesis

The N4-alkylamide derivatives of cytarabine (**2**)-(**6**) with molecular weight ranging from 285.3 to 397.47 were synthesised by mixed anhydride method in 23-63% yield.

5.3.2 Hydrophilicity and lipophilicity

The physicochemical data of cytarabine amide derivatives are presented in Table **5.1**. The inter-relation between octanol solubility, aqueous solubility and partition coefficient are presented in Fig. **5.1** and Fig. **5.2.**

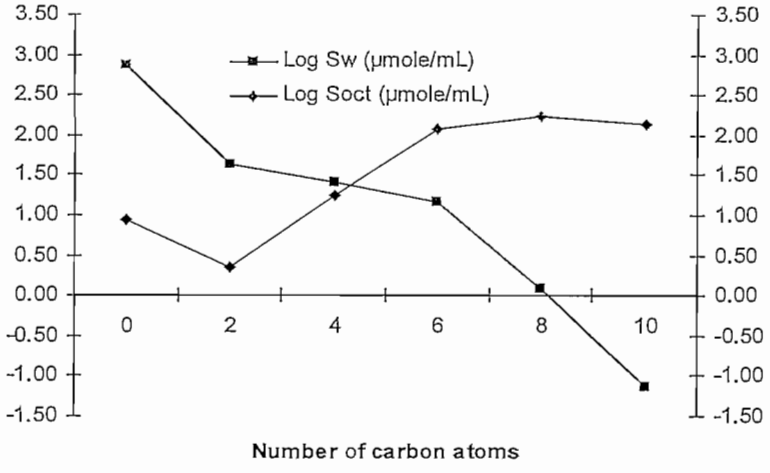


Figure 5.1. Relationship between aqueous and lipid solubility versus number of carbon atoms on the alkyl substituent

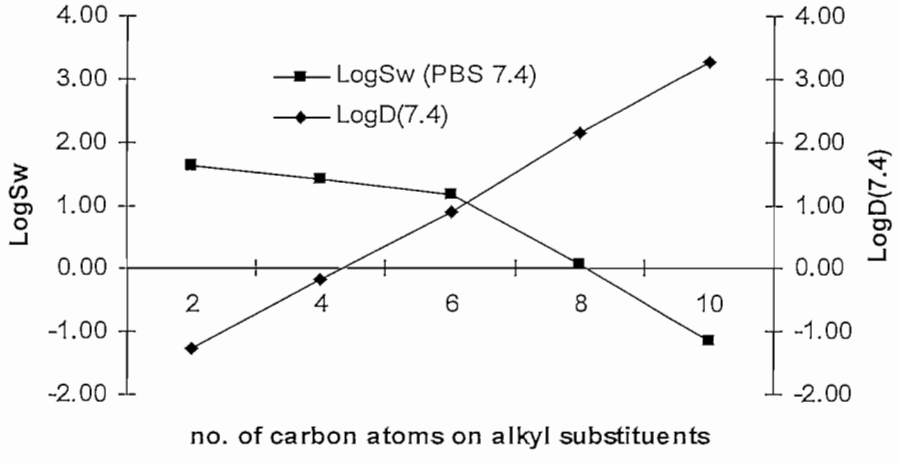


Figure 5.2. Relationship between no. of Cs on N4-alkylamide of cytarabine, Log D(7.4) and Log S_w

5.3.3 Skin permeation

The transdermal penetration parameters of cytarabine and its N4-alkylamide derivatives were determined *in vitro* and are summarised in Table **5.1.**

5.4 Discussion

5.4.1 Synthesis of cytarabine derivatives

The ^{13}C NMR spectra of amides exhibit the resonance of carbonyl carbons at around 170 ppm. Due to the electron-withdrawing capacity of amide linker, H-5 and H-6 signals were deshielded from 5.6 and 7.5 ppm to 7.1 and 8.0 ppm respectively. This confirms that the acylation took place at N4-amino group. The deshielding of signal at 7.1 ppm assignable to N4-amino group to around 10.7 ppm suggested that N4-amino group is adjacent to an electron withdrawing carbonyl group. Mass spectra confirmed the molecular weights of (**2**), (**3**), (**4**), (**5**) and (**6**) as 244.0; 313.3; 341.7; 370.2 and 397.9, respectively.

5.4.2 Physicochemical properties

Physicochemical parameters such as aqueous solubility and lipophilicity have been reported to influence membrane permeation, therapeutic activity and pharmacokinetic profile of medicines. [13] The physicochemical data of cytarabine amide derivatives are presented in Table **5.1**. In this homologous series, octanol solubility values increased whereas aqueous solubility decreased as the alkyl chain lengthened (see Fig. **5.1**). As a consequence, the log D, increases as the chain lengthens (Fig. **5.2**). This corroborates previous findings in the literature that by increasing non-polar portion of a molecule by extending the length of the chain produces certain characteristic features, such as elevation of boiling point, decreased aqueous solubility and increased partition coefficient. [14] It is noteworthy that compound (**2**) exhibited lower solubility in both water and lipid than the parent compound. Moreover, amide (**2**), the first member of the homologous series, exhibits lower solubility in octanol than cytarabine, which is contrary to previous report [9] that, generally, the first member of a homologous series exhibit increased lipid solubility, thought to be due the masking of hydrogen bond donor. All amides showed lower melting points than the parent compound. Generally, compounds with lower meting points are associated with higher solubility and known to permeate the skin better than those with higher melting points.[15]

5.4.3 *In vitro* skin permeation study

The diffusion experiments showed that there is a varying degree of transdermal permeation between (**1**) and its N4-alkylamides (Table **5.1**). There is no clear trend between flux values and molecular weights of compounds. Compound (**4**) penetrated the skin much better than (**2**) and (3), despite having higher molecular weight. These corroborate previous findings in which some

members of homologous series with higher molecular weights penetrated the skin better than members with lower molecular weights. [16, 17]

Compound (**4**) exhibited a significant enhancement on transdermal penetration of cytarabine. It showed a median steady-state flux of 89.0 nmol/cm^2/h^1, which is a significant enhancement. This enhancement could be attributable to the solubility of (**4**) in both aqueous and lipid media (Fig. **5.1**). A good balance between lipid and aqueous solubility values is reported to be essential to optimize transdermal flux.[18] Other compounds (**3**), (**5**) and (**6**) were not detected in the receptor phase, which means they did not permeate the epidermis. Epidermis, especially its outermost layer is the major source of resistance to the permeation of the skin by drug molecules.[3] It has been reported that the optimal log octanol/water partition coefficient for a drug to penetrate the stratum cormeum, is approximately 2. [19] However, in this study compound (**5**) with log D of 2.15, exhibited poor percutaneous penetration. This could be due to *inter alia* hydrogen-bonding phenomenon. The hydrogen bonding functional groups in the permeant tend to slow down transdermal diffusion to due to their interaction with polar head groups of the intercellular lipids present in the skin.[20, 21]

5.5 Conclusion

The alkylamide derivatives of cytarabine were successfully prepared and their structures were confirmed by NMR and MS techniques. The transdermal data showed no trend with melting point, lipophilicity and hydrophilicity. The amide (**4**), characterised by a good balance lipophilicity and hydrophilicity, exhibited the highest enhancement in transdermal penetration of cytarabine.

5.6 Acknowledgments

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CHAPTER 6

6 Summary and Conclusion

Cancer is a very complicated life-threatening disease which affects millions of South Africans. South African Medical research council (2008) reported that one in four South Africans will develop a cancer in their lifetime (Stein, 2008). According to World health Organisation (WHO) cancer accounts for about 13% of all human deaths worldwide (WHO, 2007). These numbers are projected to continue rising, with an estimated 12 million deaths in 2030 (Cancer Research UK, 2007). With these statistics it is clear that cancer continues to pose a threat to the well-being of the people. It is therefore essential that, better prevention and treatment methods are found.

Cytarabine is one of the most active single agents used in the treatment of cancer (myleloid leukaemia) (Galmarini *et al.,* 2002). The clinical utility of nucleosides such as cytarabine is severely limited by their short half-lives due to the catabolic action of nucleoside deaminases (Hadfield & Sartorelli, 1984). As a consequence, to achieve the required sustained therapeutic plasma levels, cytarabine is currently administered by repetitive schedules or continuous intravenous infusion. These regimens, however, are associated with adverse effects such as myelosuppresion, vomiting and stomatitis at conventional doses (Galmarini *et al.,* 2002; Frei *et al.,* 1969; Bolwell *et al.,* 1988).

Transdermal drug delivery systems (TDDS) have been reported to have a potential to achieve sustained drug release similar to continuous intravenous infusion with less pain and inconvenience (Naik *et al.,* 2000).

The aims of the current study were to determine transdermal absorption of cytarabine and its synthesized alkylamide, alkylester and carbamate derivatives and to establish a correlation, if any, with selected physicochemical properties.

The following objectives were set in order to achieve the goals:

- Synthesize 5'-alkylesters, N4-alkylamides and N4-methoxypoly(ethylene glycol) carbamates of cytarabine and confirm their structures by NMR and MS.
- Experimentally determine the aqueous solubility and the partition coefficient for 5'-alkylesters, N4-alkylamides and N4-carbamates of cytarabine.
- Experimentally determine the transdermal flux of cytarabine and its derivatives.
- Find whether a correlation exists between the aqueous solubility, partition coefficient and transdermal flux data of the cytarabine derivatives.

The N4-alkylamide, 5'-alkylester and N4-ethylene glycol carbamate derivatives were successful synthesized and their chemical structures were confirmed by NMR and MS spectroscopy. The alkyl moieties were chosen to increase lipophilicity while methoxypoly(ethylene glycol) moieties were incorporated for their amphiphilic nature. All alkyl (i.e., esters and amides) derivatives exhibited lower aqueous solubility compared to the parent compound, which decreased further as the alkyl chain lengthened in the homologous series. On the other hand, their octanol solubility values were higher than that of the parent drug and increases as the alkyl chain lengthens. This corroborates previous findings that by increasing non-polar portion of a molecule by extending the length of the chain produces certain characteristic features, such as elevation of boiling point, decreased aqueous solubility and increased partition coefficient (Abdul, 1989). The N4-mPEG carbamate derivatives, exhibited increased solubility in both aqueous and lipid media. These features were expected since; PEG promoieties are known to have the ability to increase both lipophicility and hydrophilicity of drugs (Puglia *et al.,* 2006; Bonina *et al.,* 2001). It is noteworthy that log p values predicted by ACD were very close to those determined experimentally for alkylester homologous series. Although Log p values predicted by kowWin were different from experimental ones, they followed the same trend.

In alkylamide and alkylester homologous series, the compounds (N4-hexanoylcytarabine and cytarabine-5'-butanoate) with the relatively good balance between aqueous and lipid solubilities, exhibited the highest flux values in their respective series. This finding corroborates the previous reports that a good balance between lipid and aqueous solubilities is essential to optimize transdermal flux (Sloan & Wasdo, 2003). Generally, the flux values of cytarabine and its derivatives are very low compared to compounds that are clinically administered by transdermal delivery system such as nicotine and scopolamine (Cleary, 1993). The fact that cytarabine and its derivatives contain numerous hydrogen bond functionalities, could justify their low skin penetration. It is generally accepted that for optimum transdermal permeation, the hydrogen bonding groups on permeants should not exceed 2 (Thomas & Finnin, 2004; du Plessis *et al.,* 2002). The findings of this study corroborate the role of hydrogen bonding in transdermal penetration of drugs.

N4-octanoylcytarabine and cytarabine-5'-octanoate, despite having log D values close to 2, which is reported to be optimum for a drug to penetrate stratum corneum (Riviere & Papich, 2001), were not detected in the receptor phase. This could be due to their low solubility in the vehicle used (PBS 7.4). Further studies could be done on N4-hexanoylcytarabine and cytarabine-5'-butanoate to increase transdermal permeation further, by other approaches such as chemical and physical penetration enhancement strategies. The use of these techniques in tandem could lead to synergistic enhancement effect.

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7 Appendix

7.1 MS spectra for carbamate derivatives of cytarabine

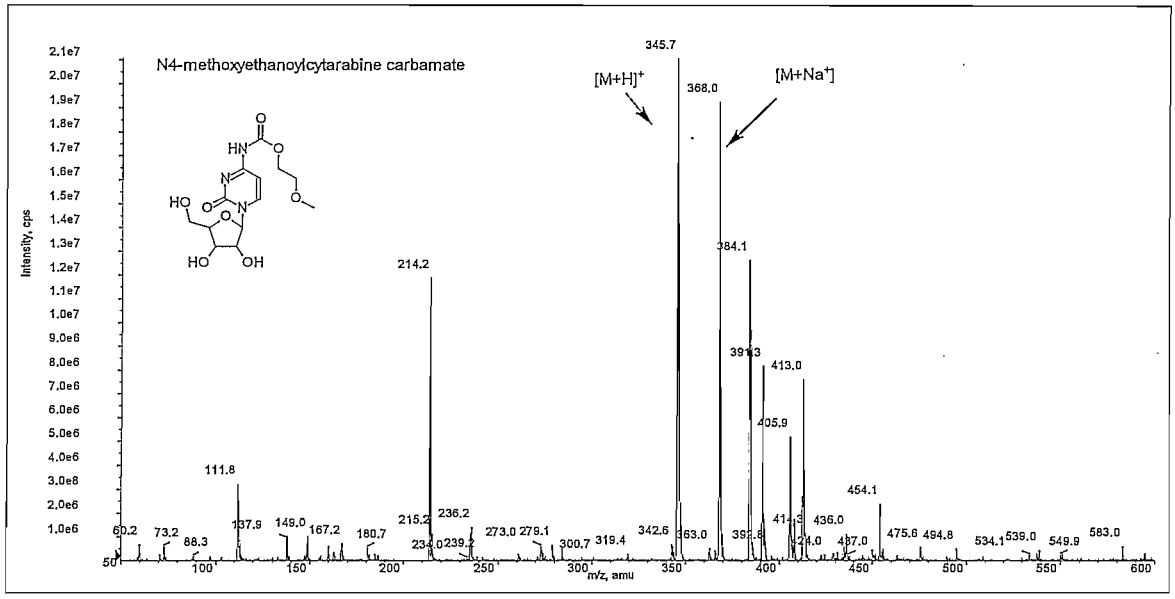


Figure A.1: Mass spectrum of N4-methoxyethanoylcytarabine carbamate

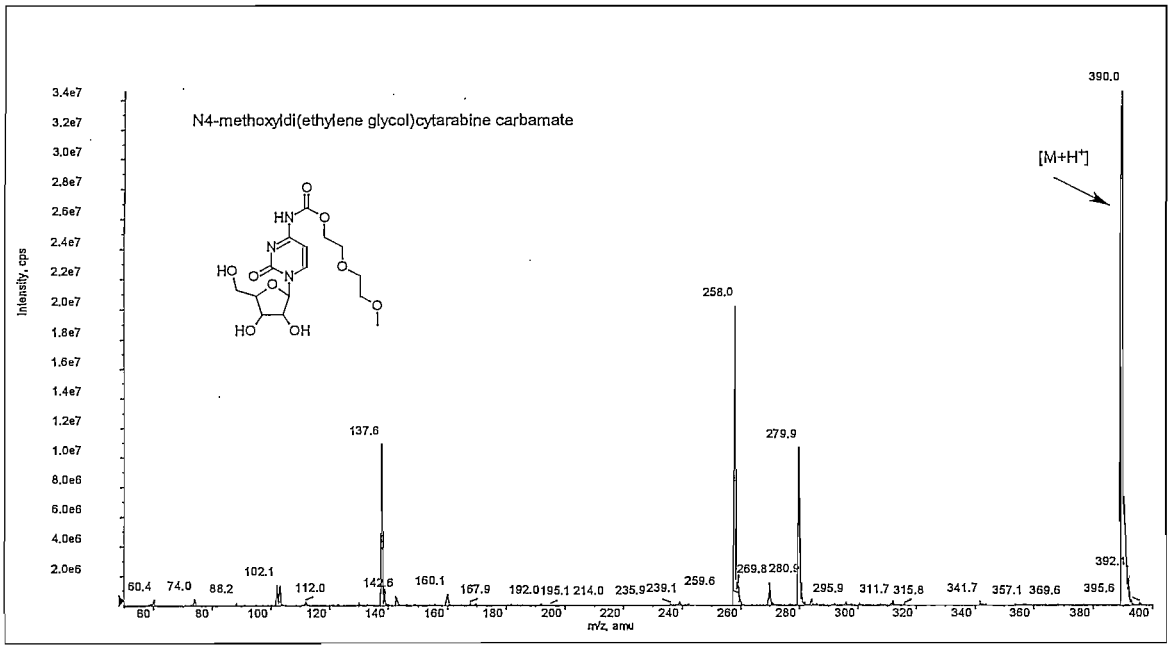


Figure A.2: Mass spectrum of N4-methoxydi(ethylene glycol)cytarabine carbamate

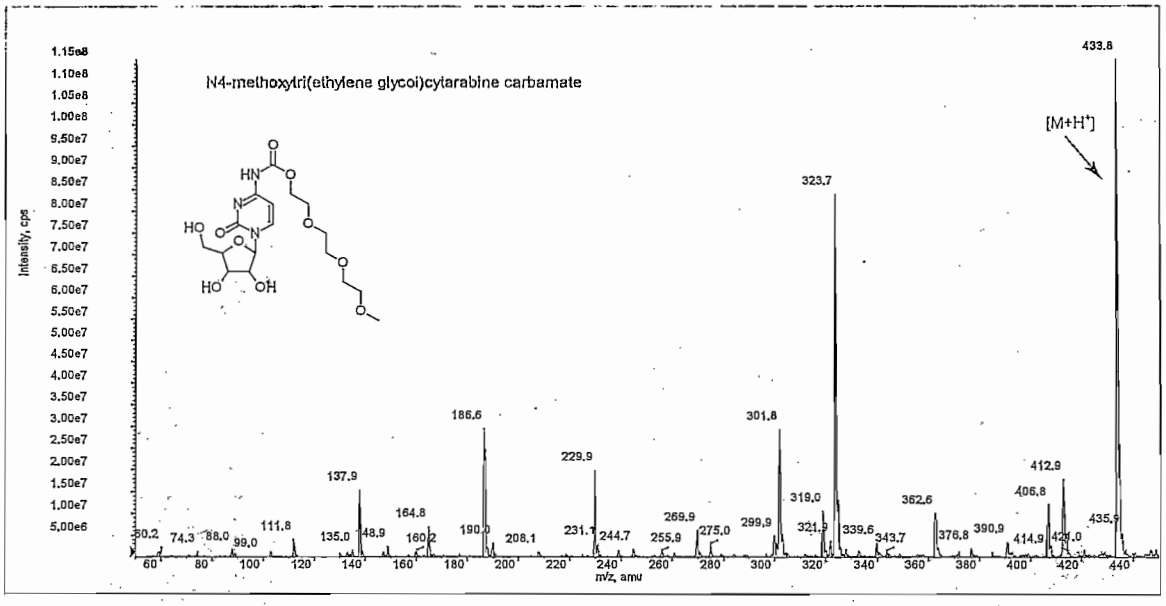


Figure A.3: Mass spectrum N4-methoxytri(ethylene glycol)cytarabine carbamate

7.2 MS spectra for ester derivatives of cytarabine

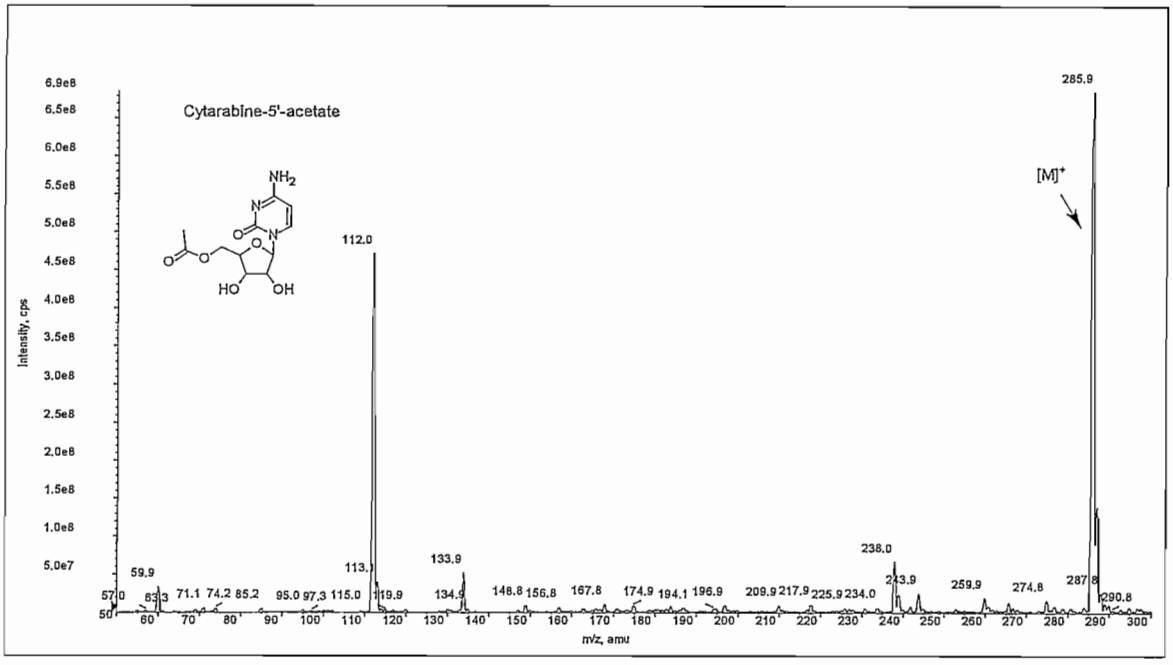


Figure A.4: Mass spectrum of cytarabine-5'-acetate

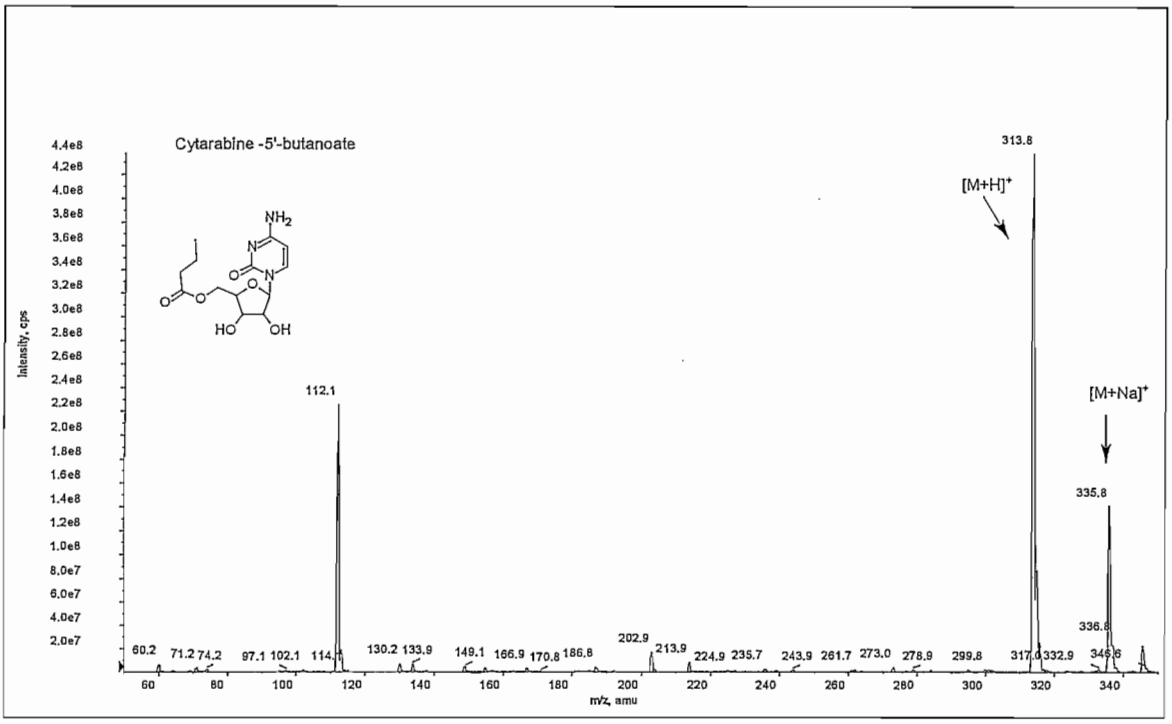


Figure A.5: Mass spectrum of cytarabine-5'-butanoate

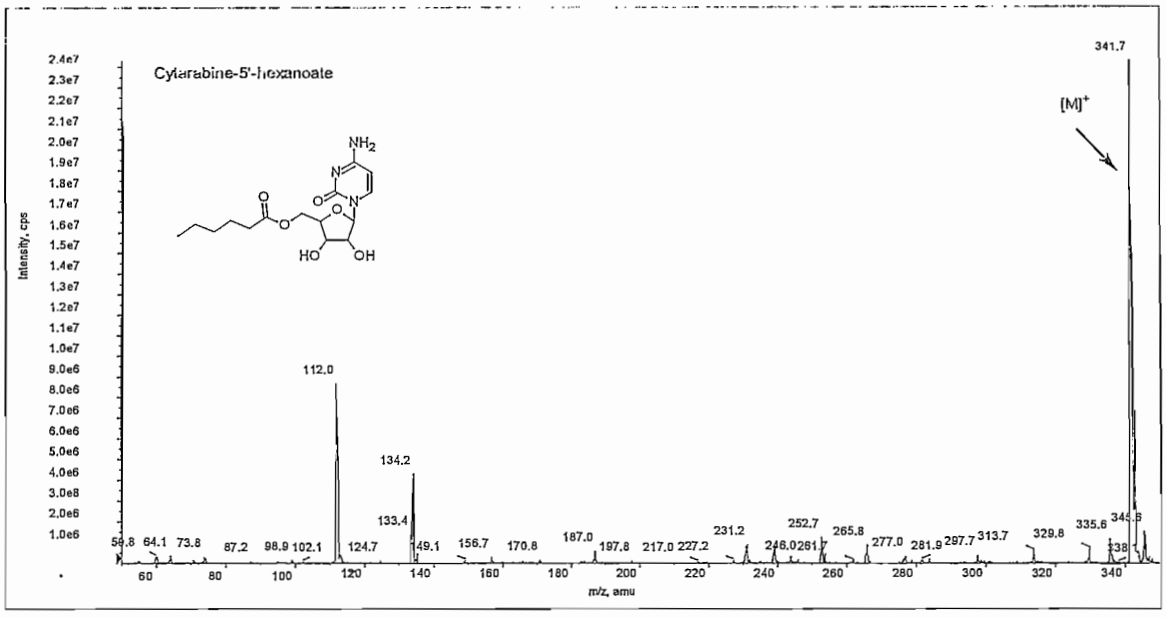


Figure A.6: Mass spectrum of cytarabine-5'-hexanoate

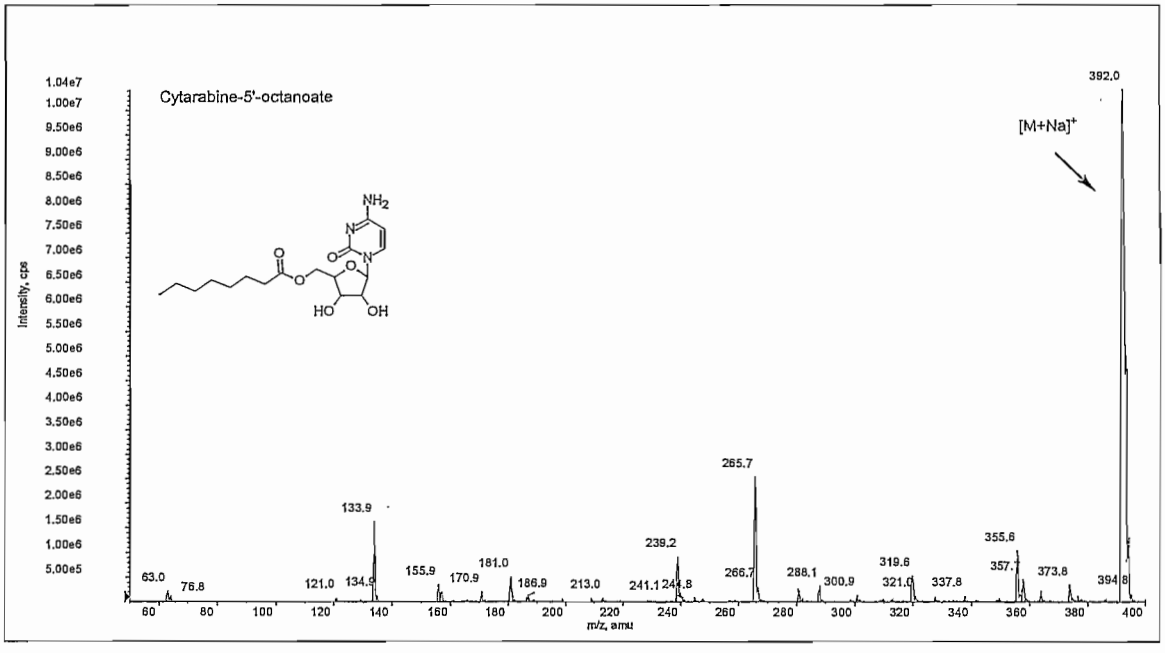


Figure A.7: Mass spectrum of cytarabine-5'-octanoate

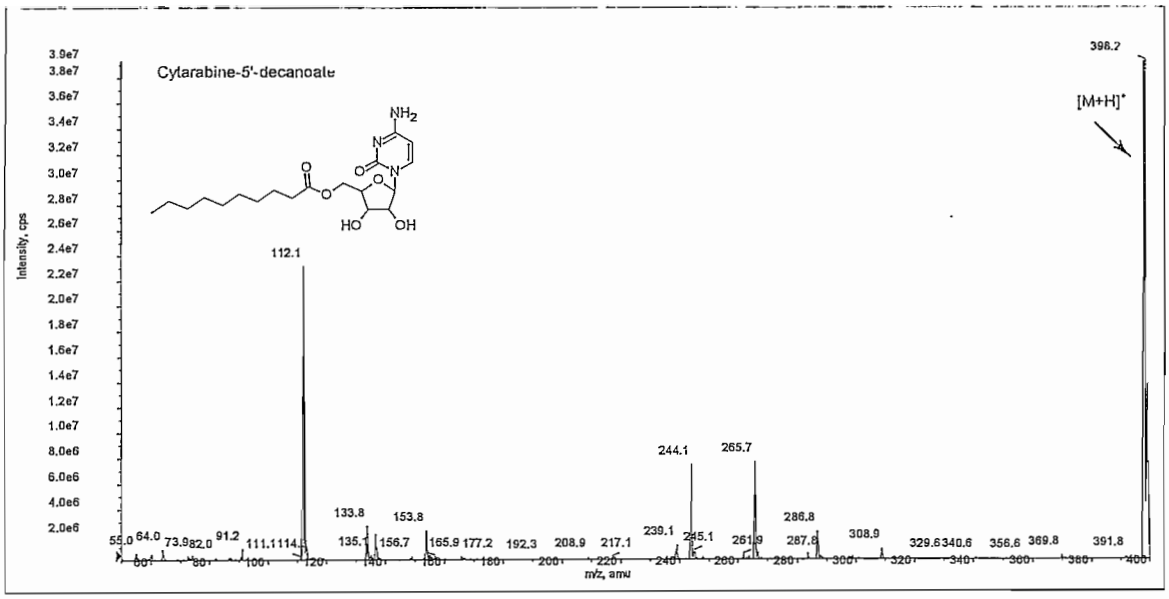


Figure A.8: Mass spectrum of cytarabine-5'-decanoate

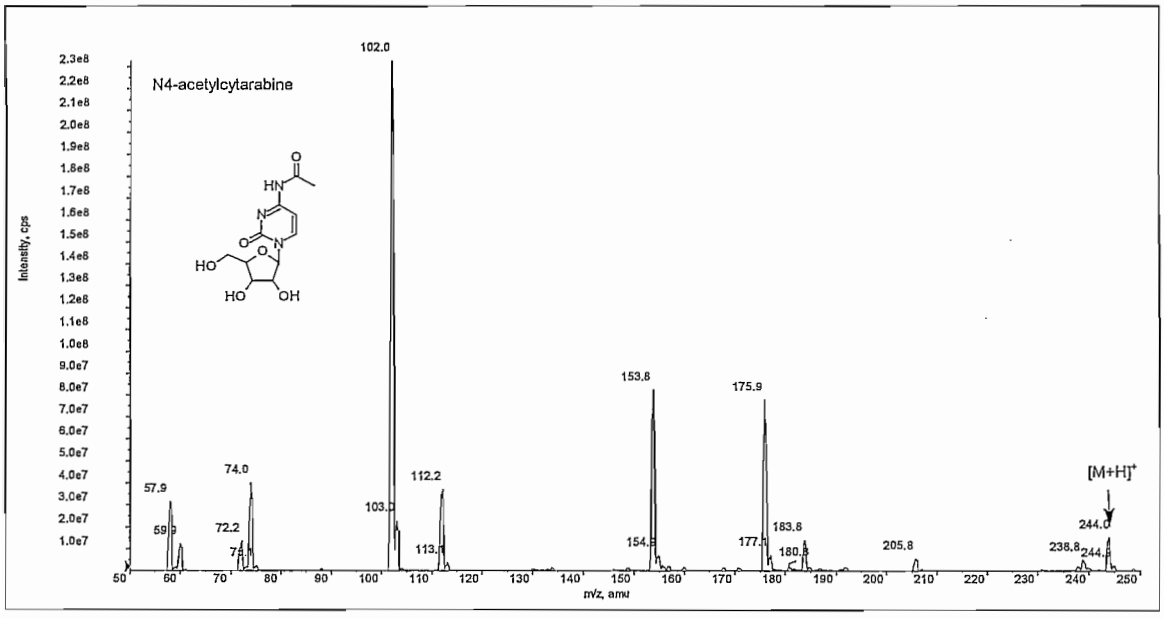


Figure A.9: Mass spectrum of N4-acetylcytarabine

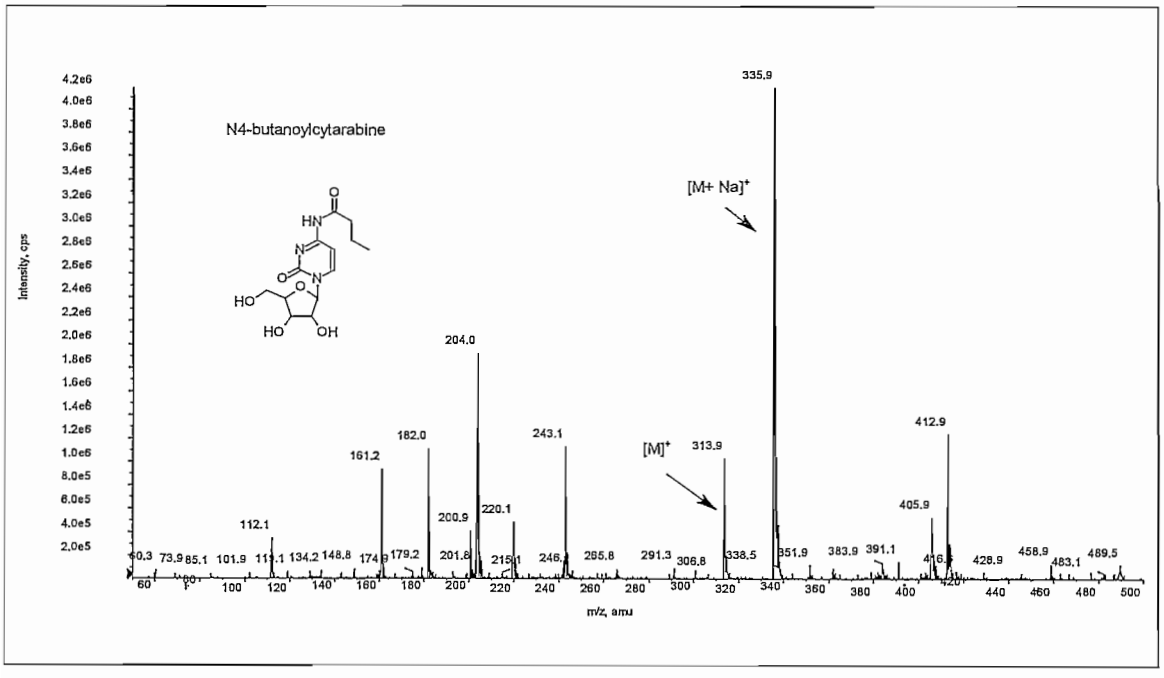


Figure A.10: Mass spectrum of N4-butanoylcytarabine

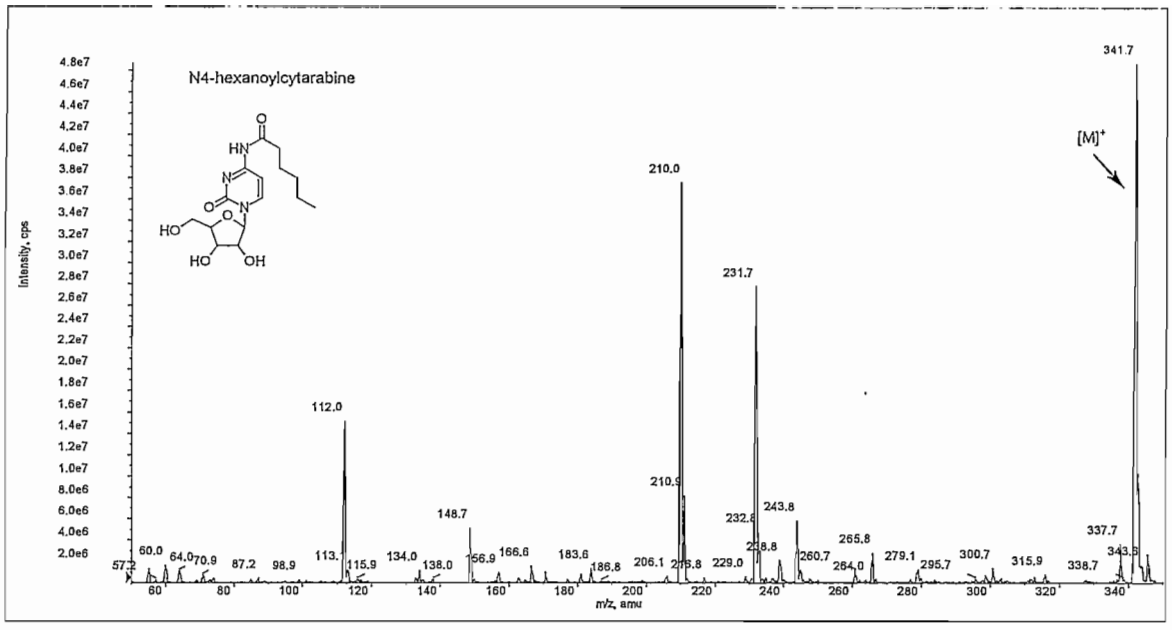


Figure A.11: Mass spectrum of N4-hexanoylcytarabine

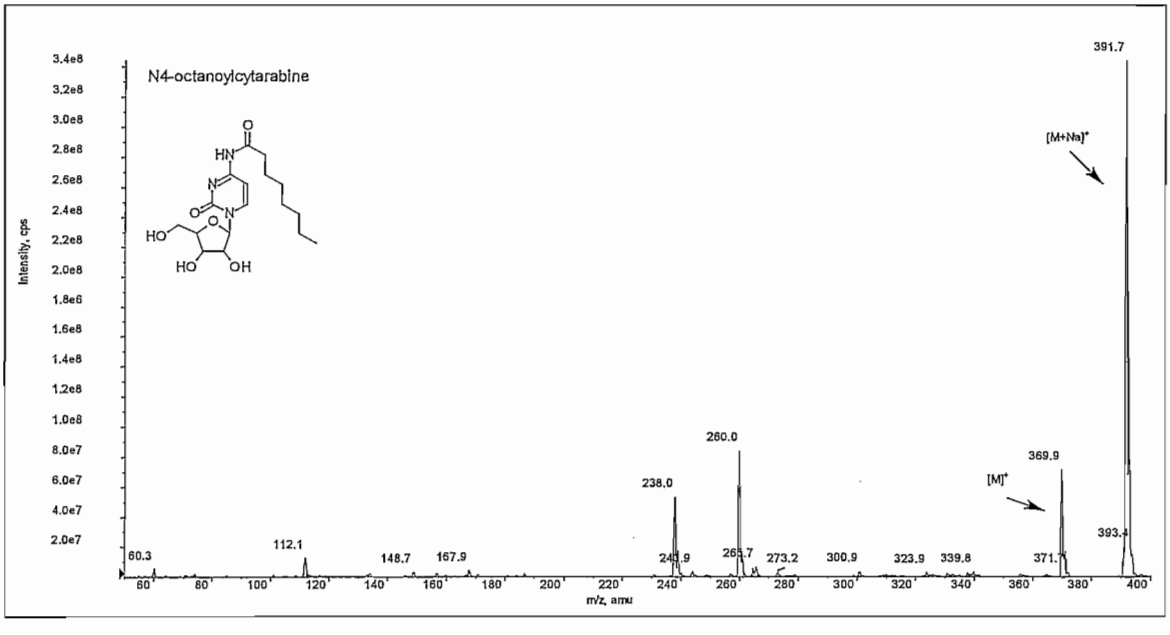


Figure A.12: Mass spectrum of N4-octanoylcytarabine

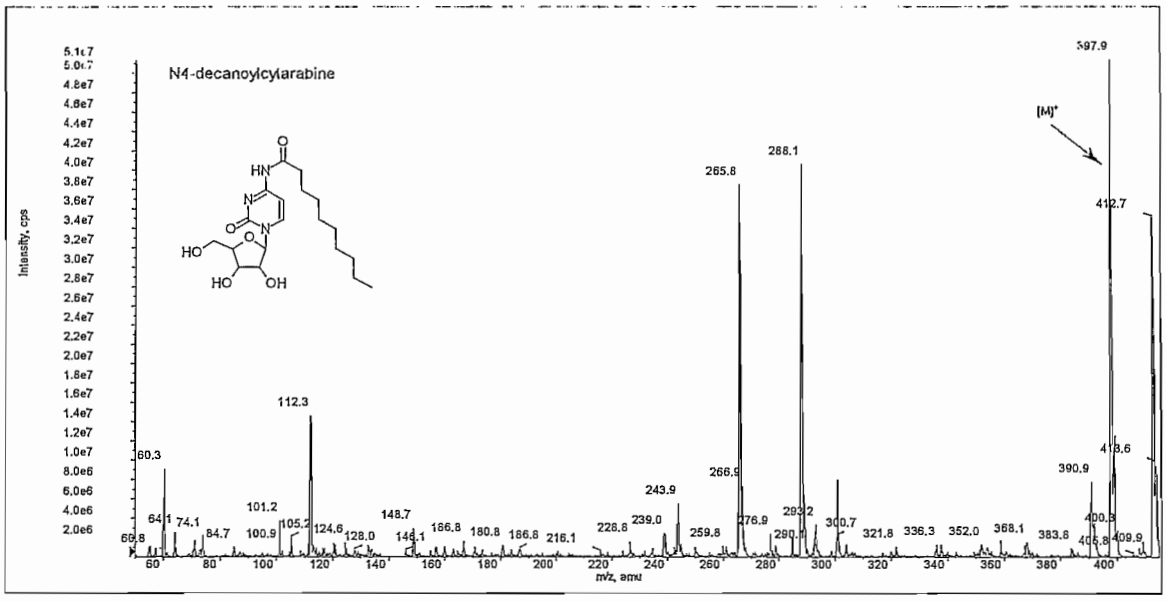


Figure A.13: Mass spectrum of N4-decanoylcytarabine

7.4 NMR spectra of carbamate derivatives of cytarabine

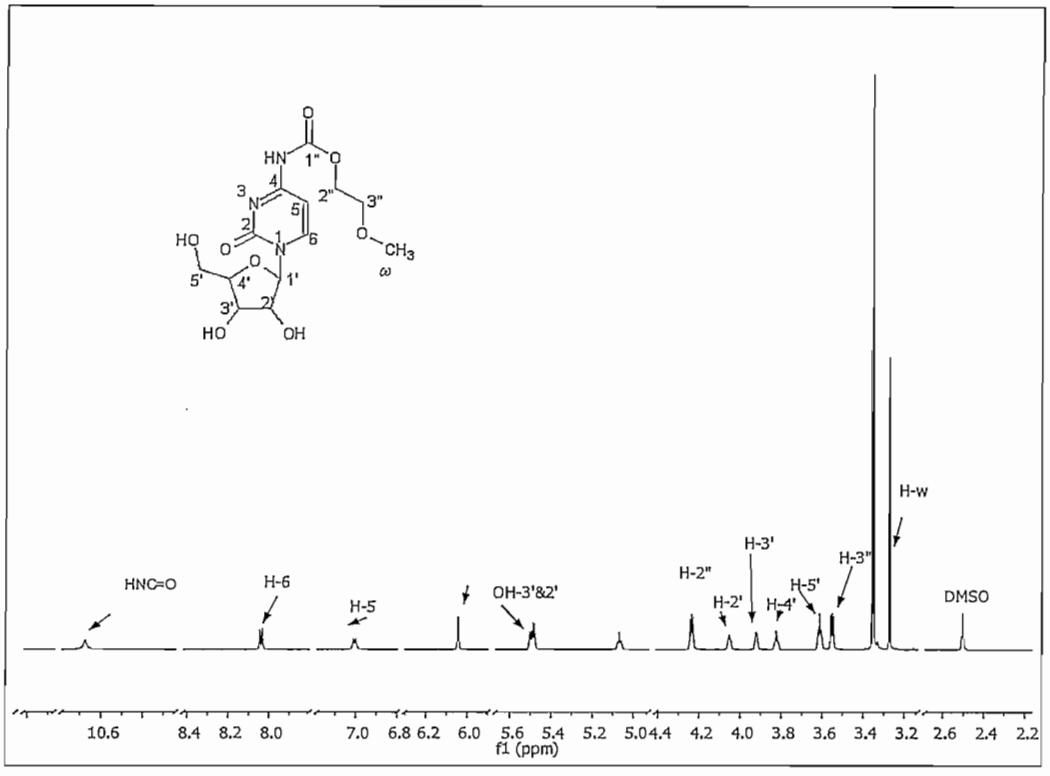


Figure A.14: ^{1}H NMR spectrum of N4-methoxyethanoylcytarabine

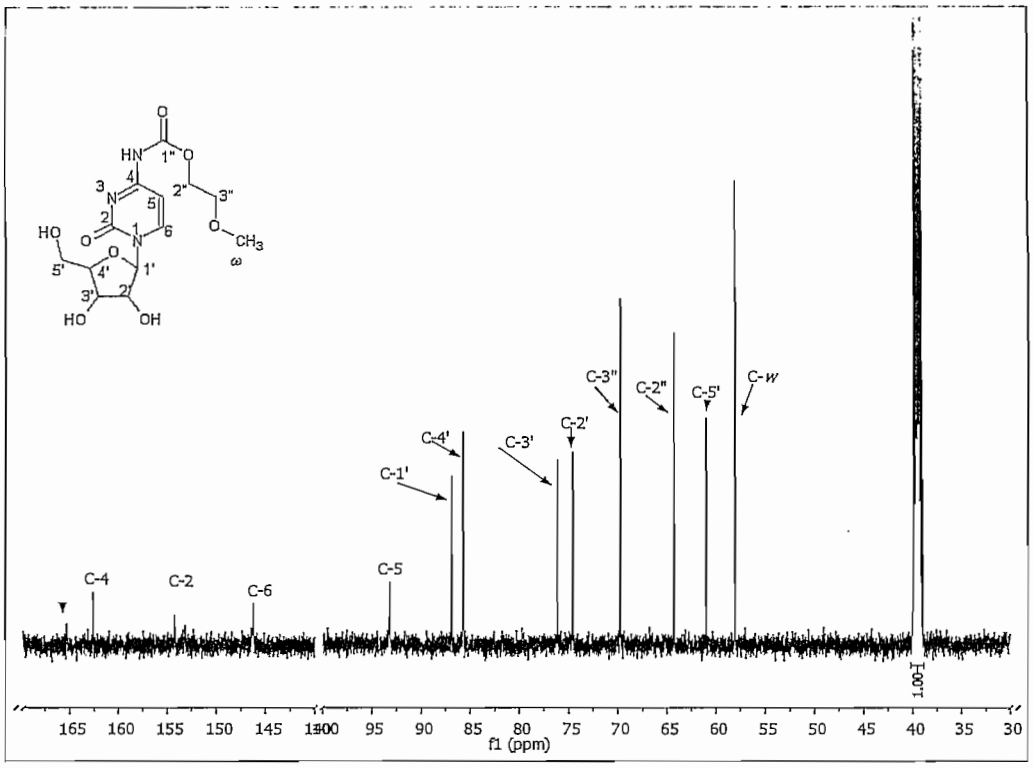


Figure A.15: ^{13}C NMR spectrum of N4-methoxyethanoylcytarabine

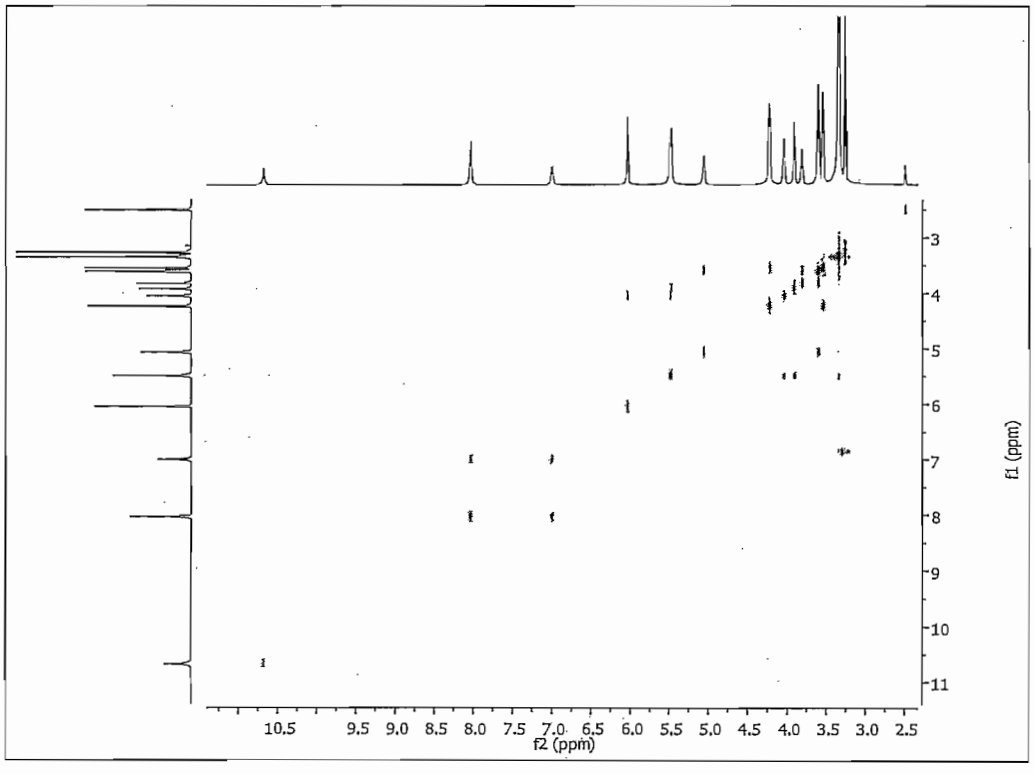


Figure A.16: COSY NMR spectrum of N4-methoxyethanoylcytarabine

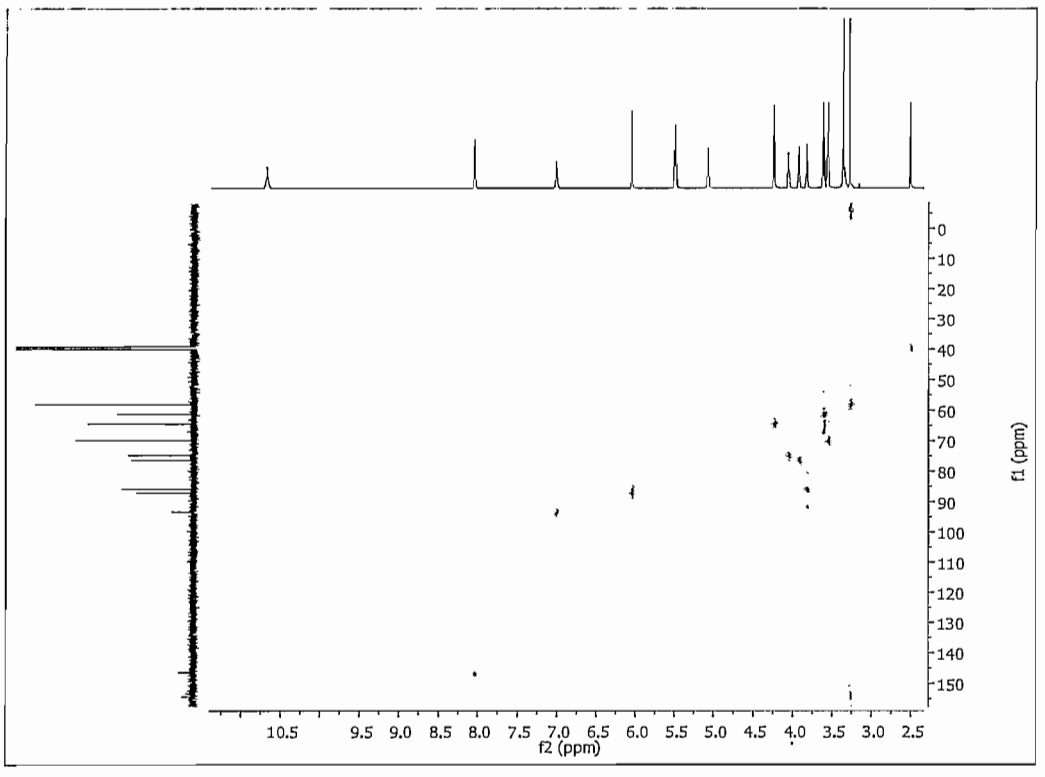


Figure A.17: HSQC spectrum of N4-methoxyethanoylcytarabine

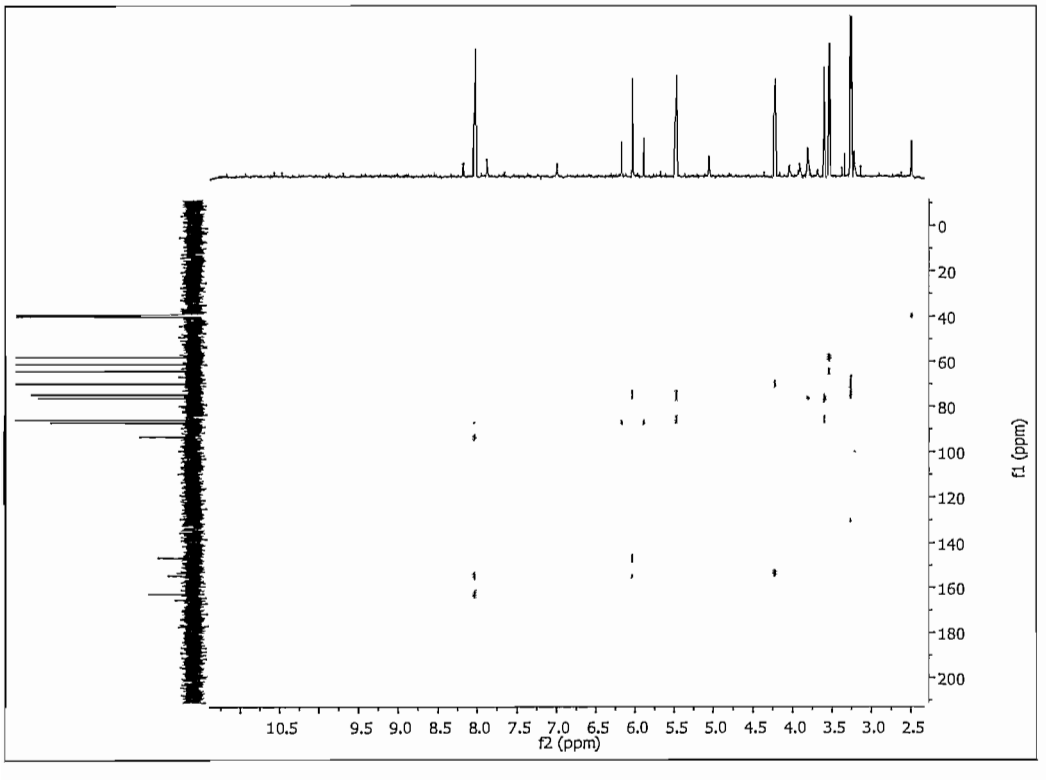


Figure A.18: HMBC spectrum of N4-methoxyethanoylcytarabine

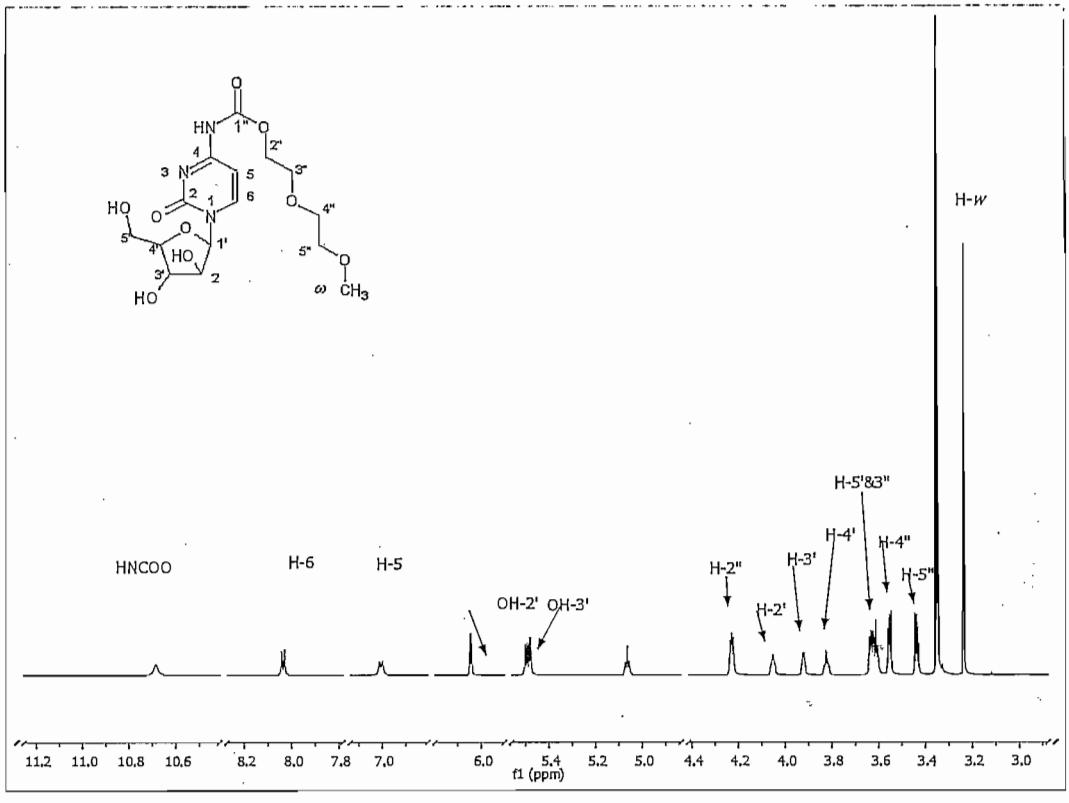


Figure A.19: ^{1}H NMR spectrum of N4-methoxydi(ethylene glycol) cytarabine carbamate

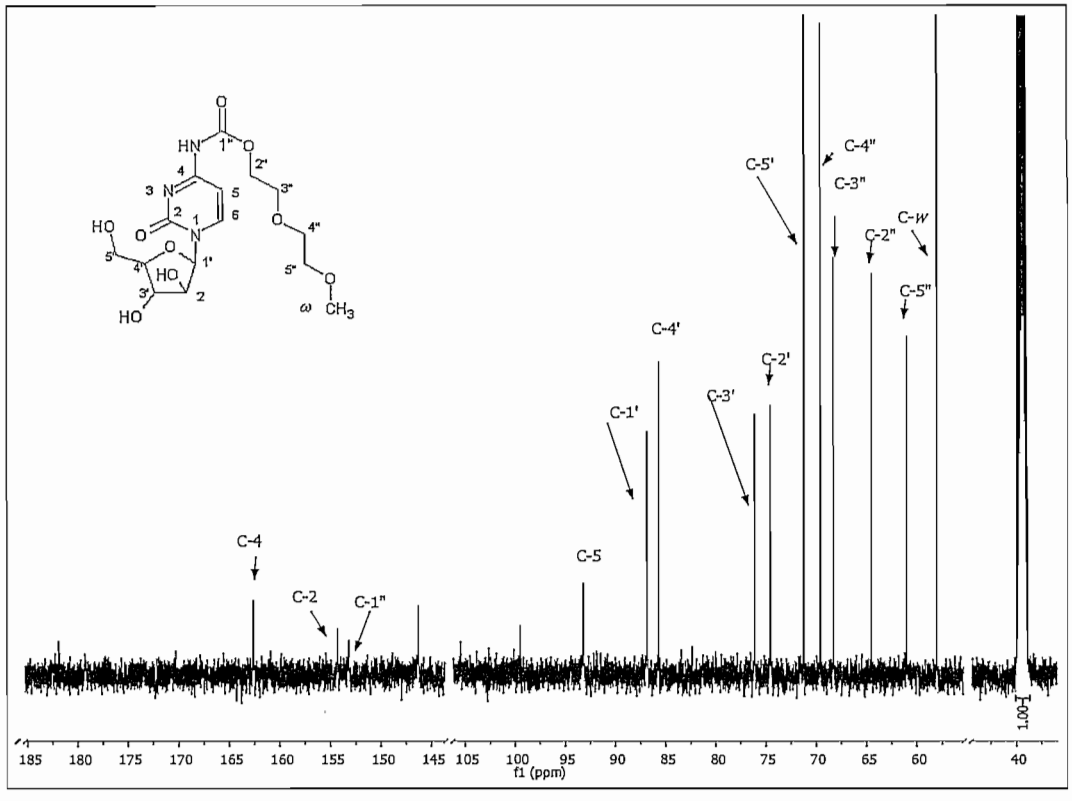


Figure A.20: ^{13}C NMR spectrum of N4-methoxydi(ethylene glycol) cytarabine carbamate

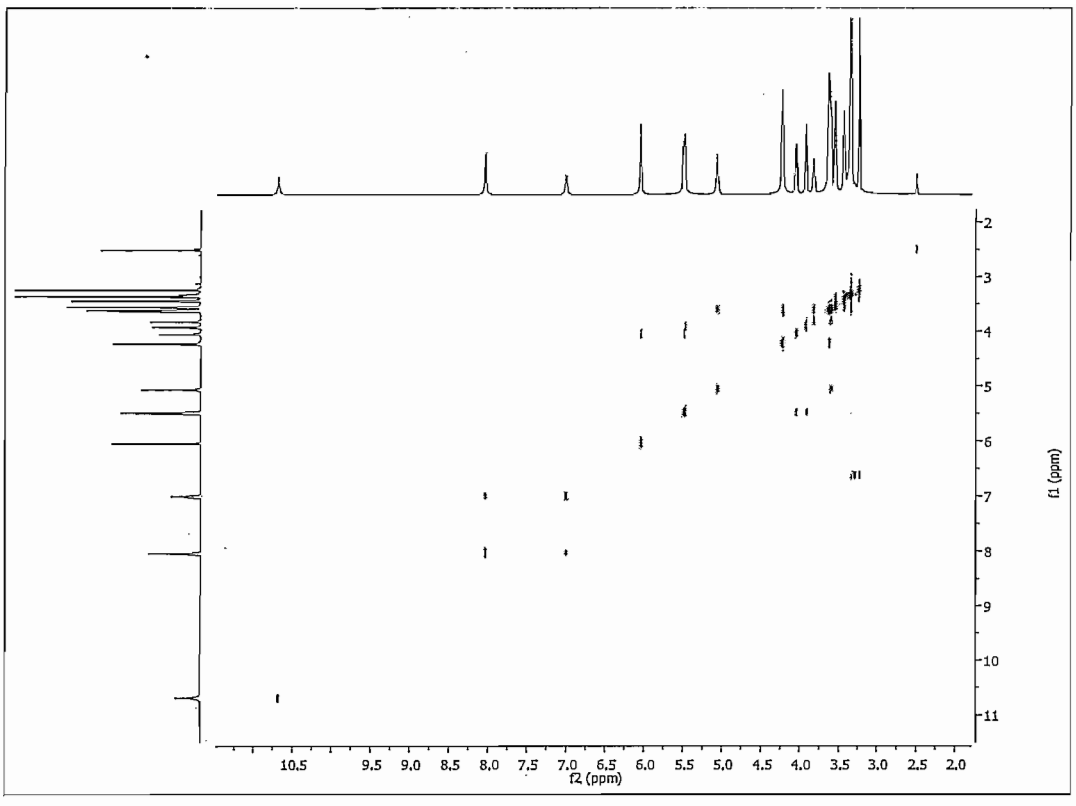


Figure A.21: COSY NMR spectrum of N4-methoxydi(ethylene glycol) cytarabine carbamate

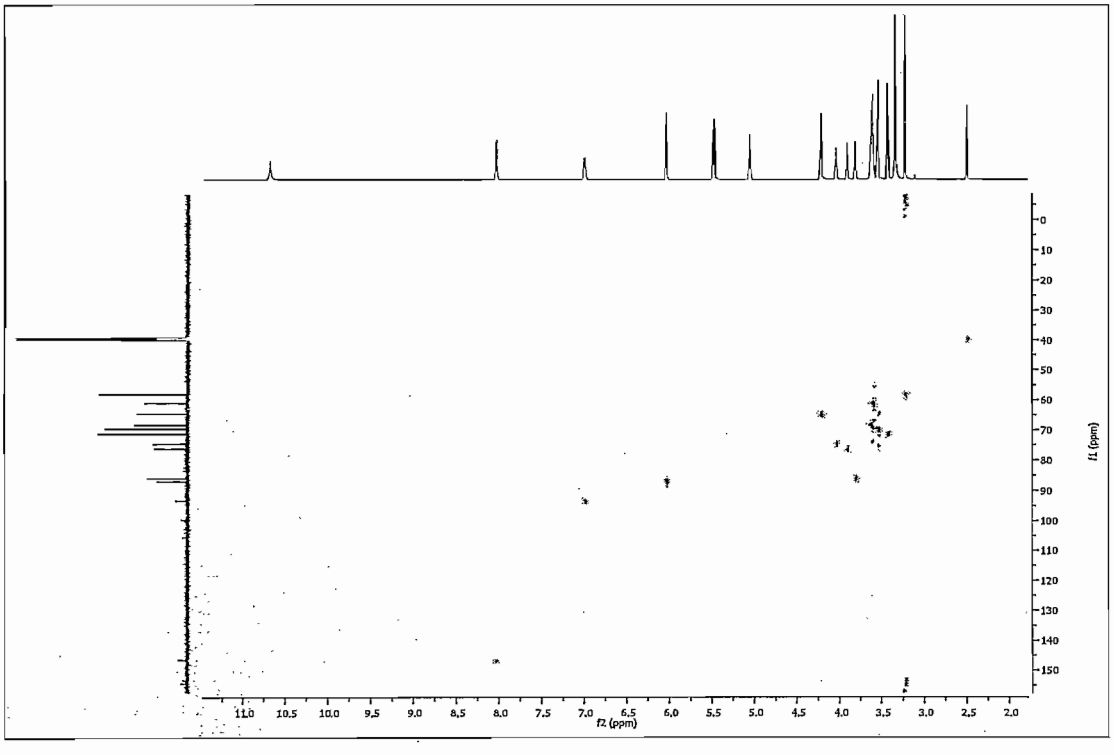


Figure A.22: HSQC NMR spectrum of N4-methoxydi(ethylene glycol) cytarabine carbamate

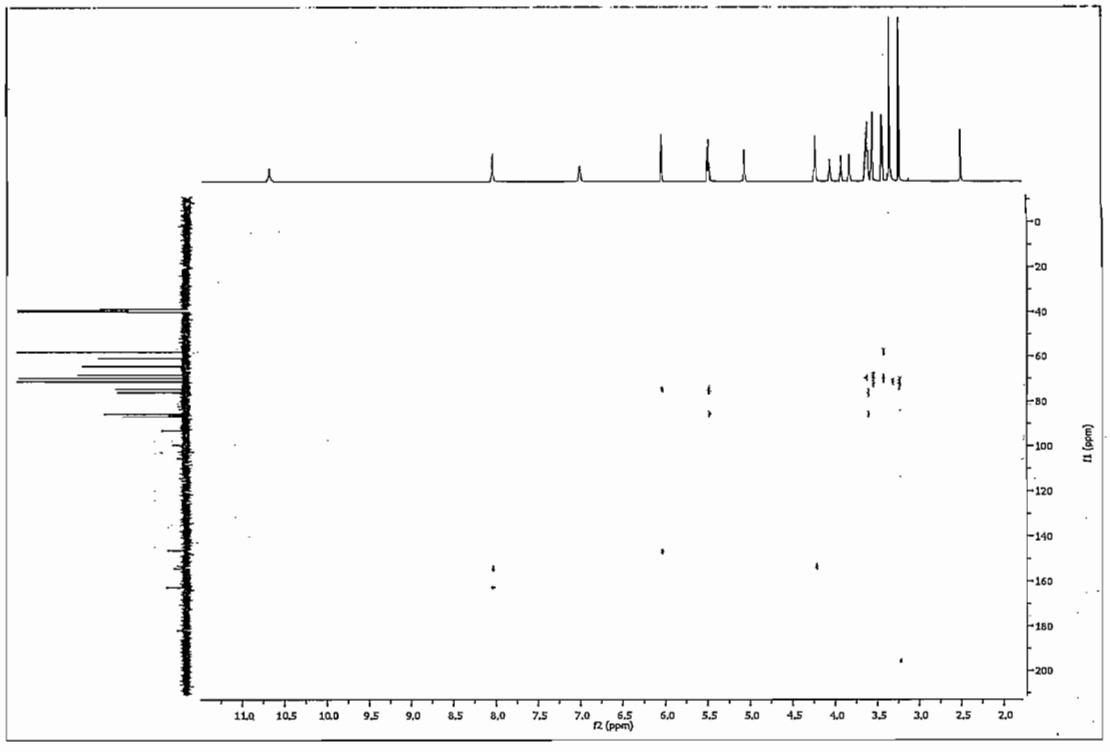


Figure A.23: HMBC NMR spectrum of N4-methoxydi(ethylene glycol) cytarabine carbamate

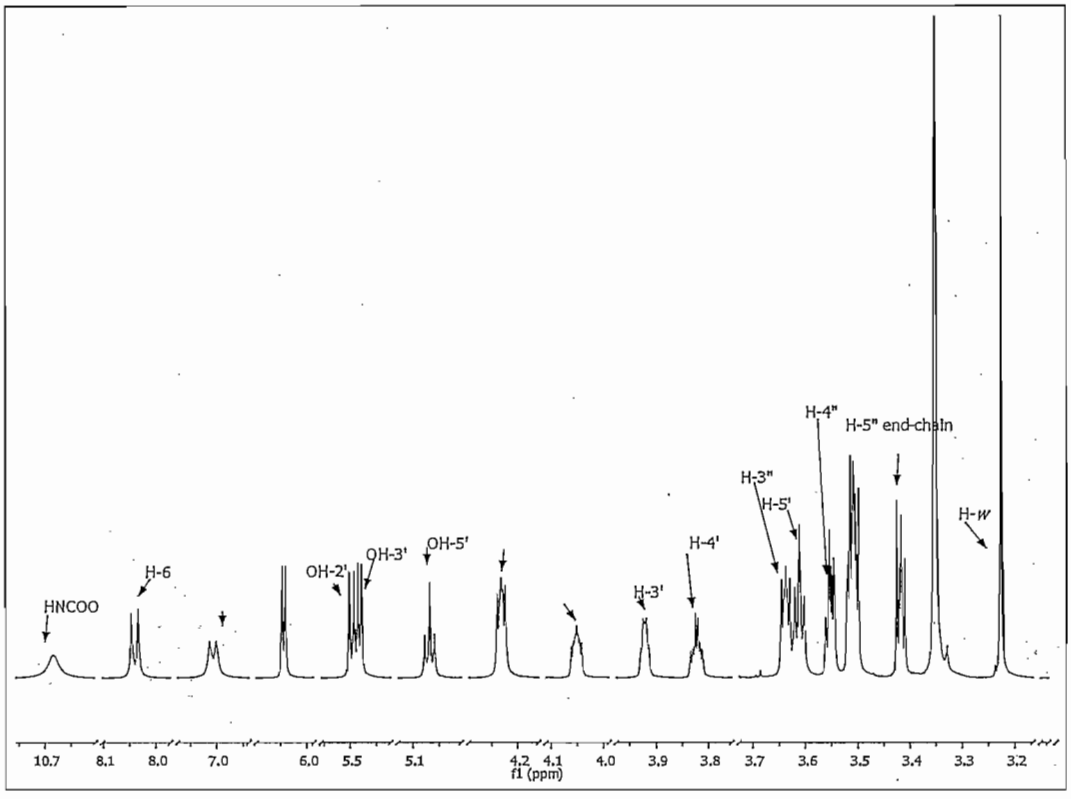


Figure A.24: ^{1}H NMR spectrum of N4-methoxytri(ethylene glycol) cytarabine carbamate

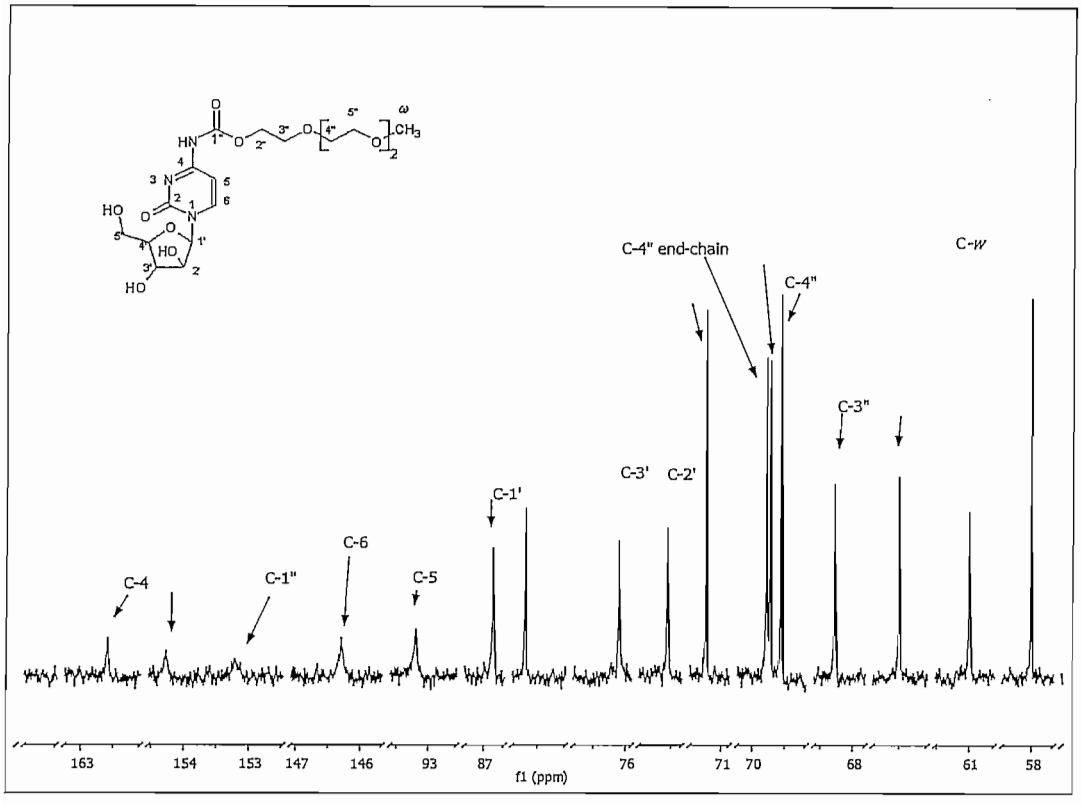


Figure A.25: ^{13}C NMR spectrum of N4-methoxytri(ethylene glycol) cytarabine carbamate

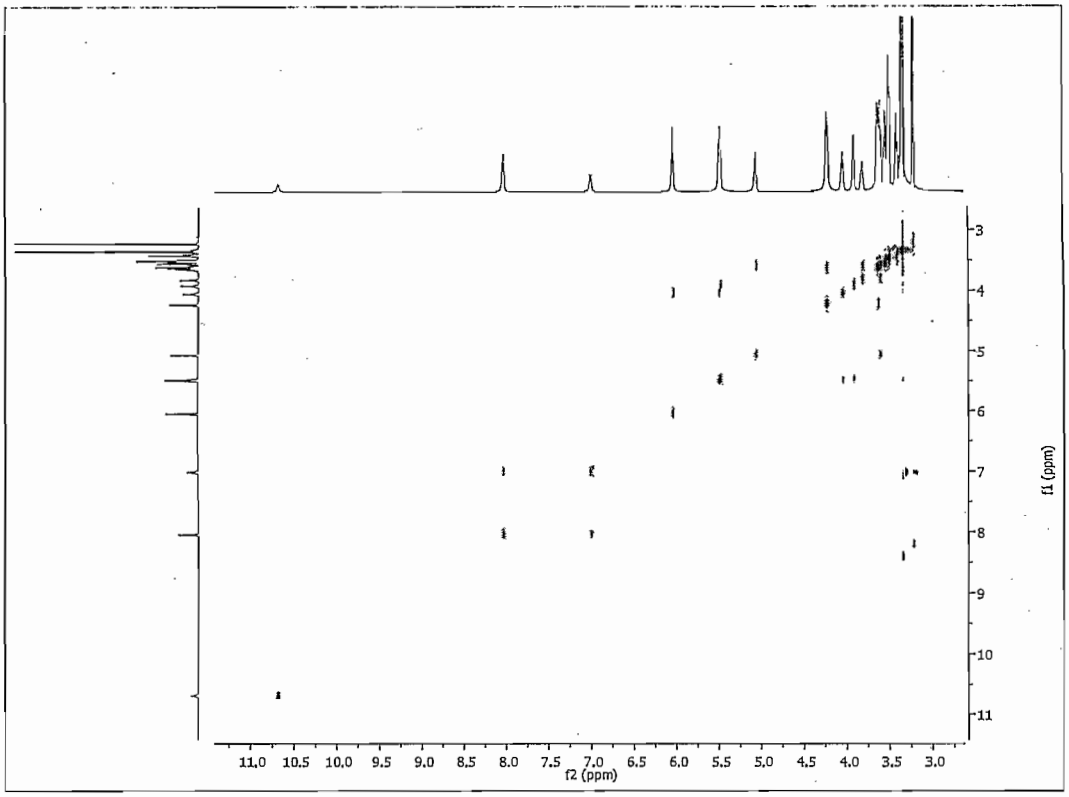


Figure A.26: COSY NMR spectrum of N4-methoxytri(ethylene glycol) cytarabine carbamate

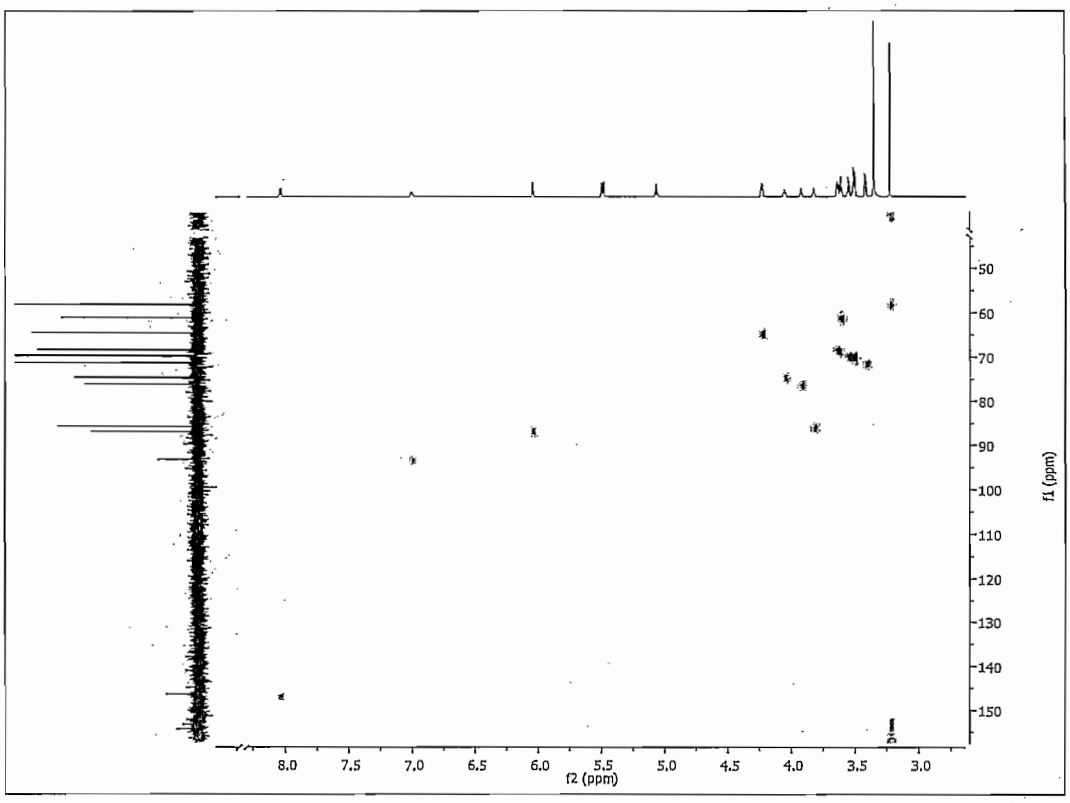


Figure A.27: HSQC NMR spectrum of N4-methoxytri(ethylene glycol) cytarabine carbamate

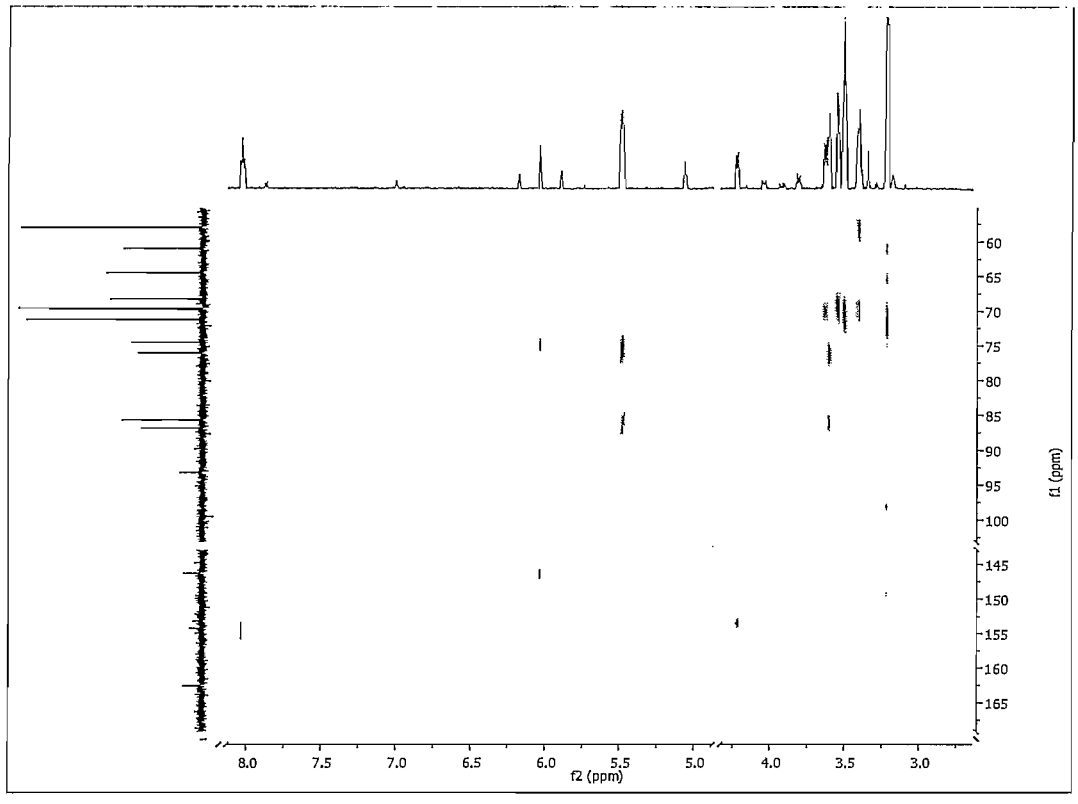


Figure A.28: HMBC NMR spectrum of N4-methoxytri(ethylene glycol) cytarabine carbamate

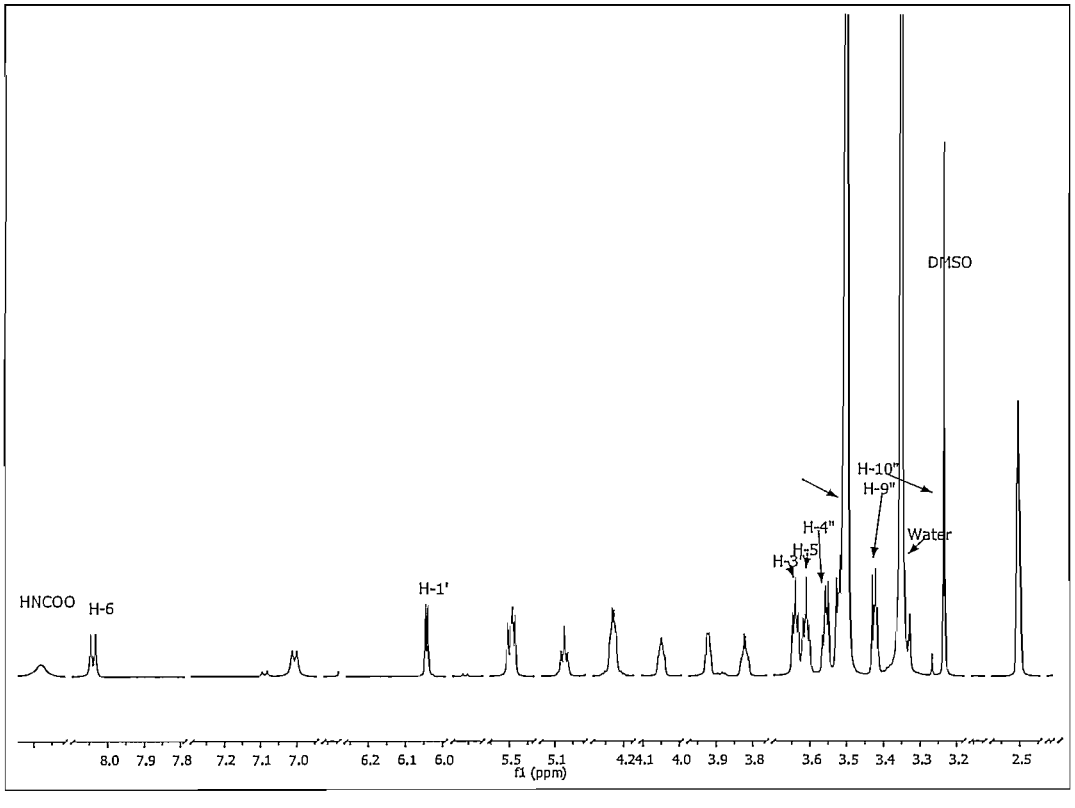


Figure A.29: ^{1}H NMR spectrum of N4-mPEG350-cytarabine carbamate

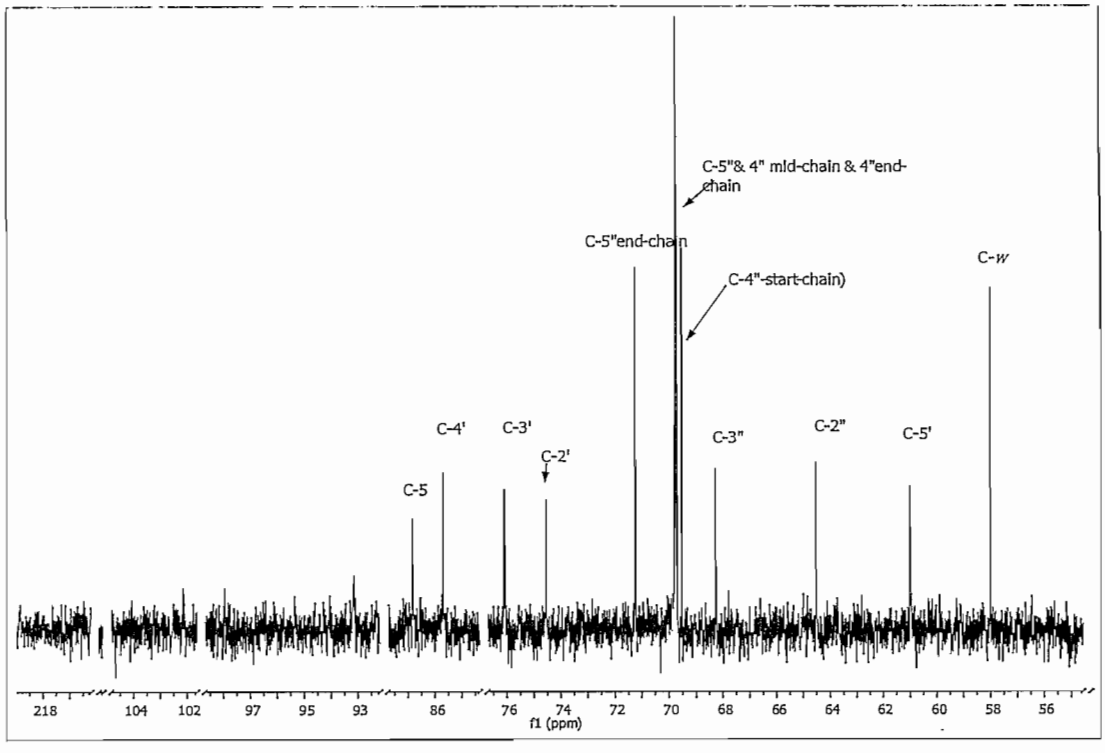


Figure A.30: ^{13}C NMR spectrum of N4-mPEG350-cytarabine carbamate

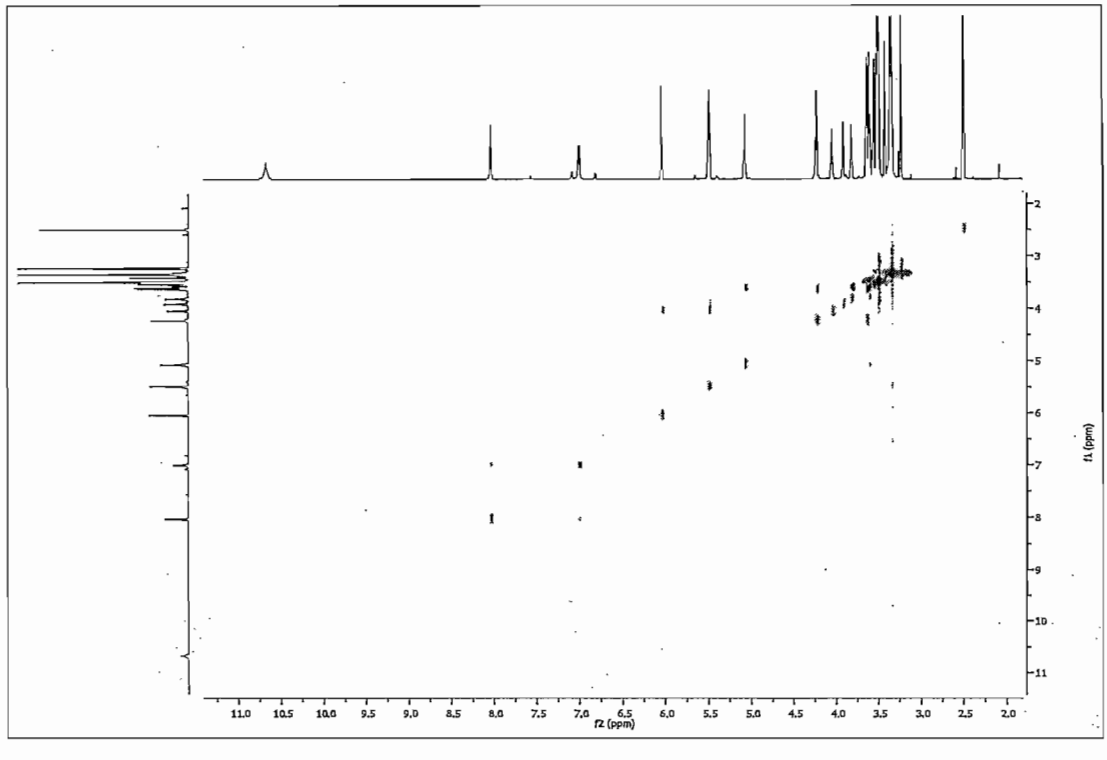


Figure A.30: COSY NMR spectrum of N4-mPEG350-cytarabine carbamate

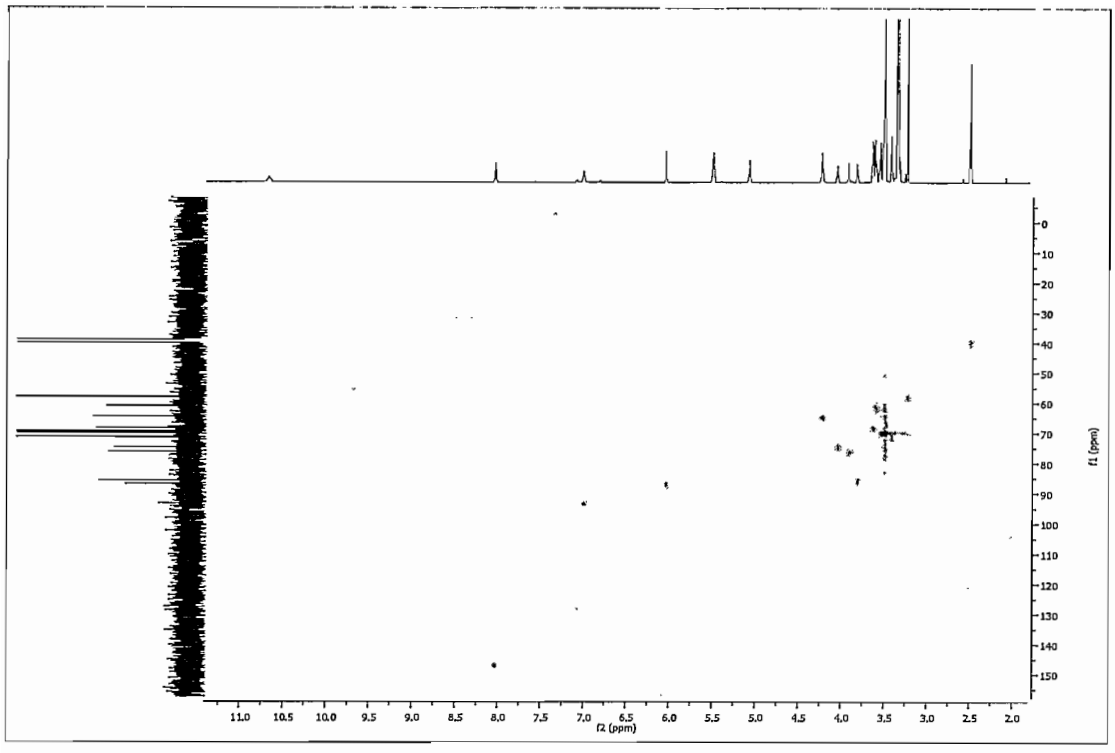


Figure A.32: HSQC NMR spectrum of N4-mPEG350-cytarabine carbamate

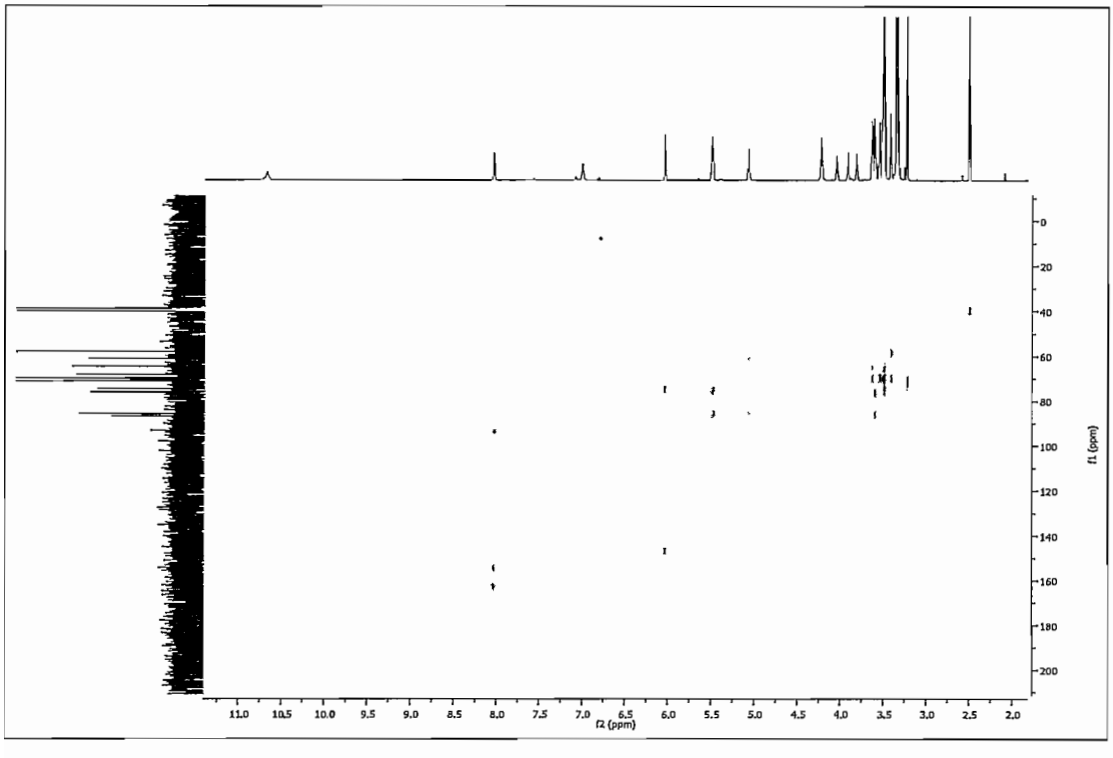


Figure A.33: HMBC NMR spectrum of N4-mPEG350-cytarabine carbamate

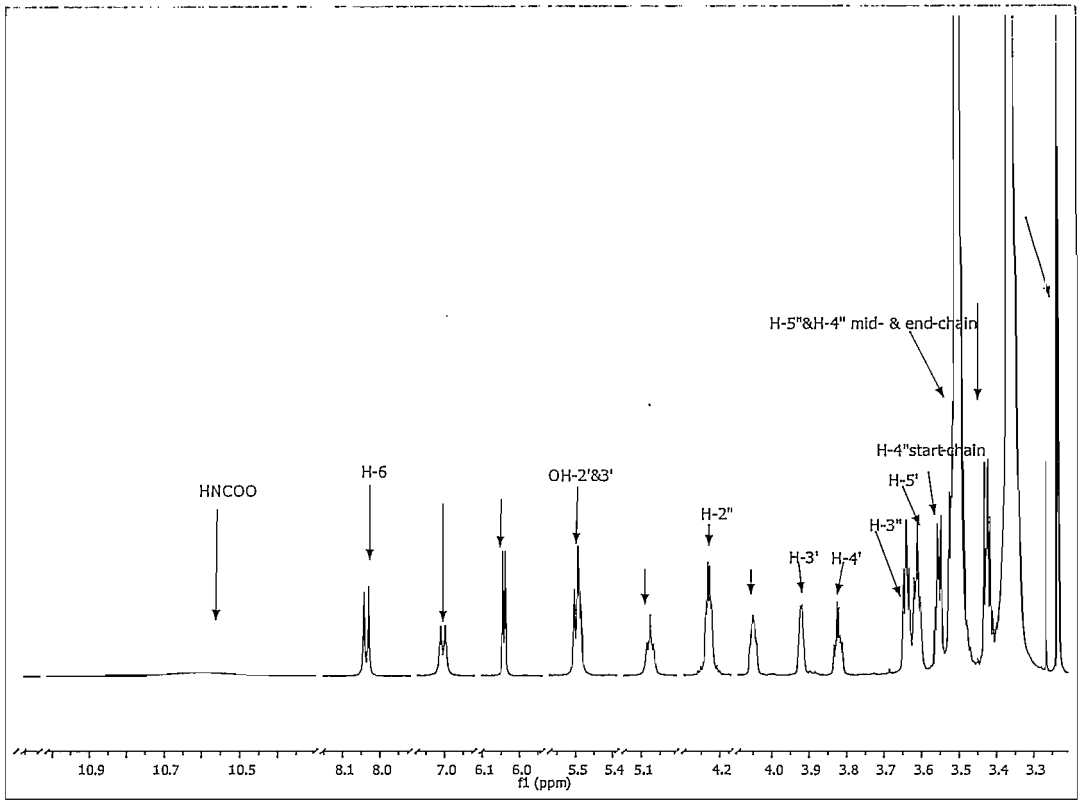


Figure A.34: ^{1}H NMR spectrum of N4-mPEG550-cytarabine carbamate

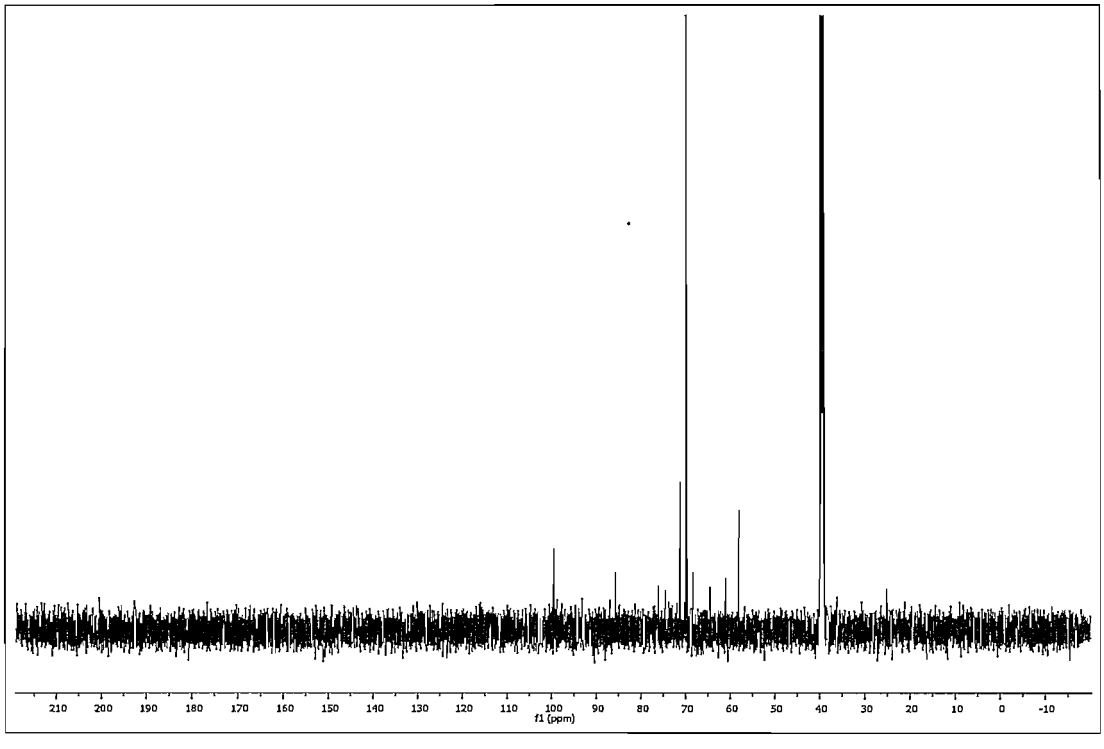


Figure A.35: ^{13}C NMR spectrum of N4-mPEG550-cytarabine carbamate

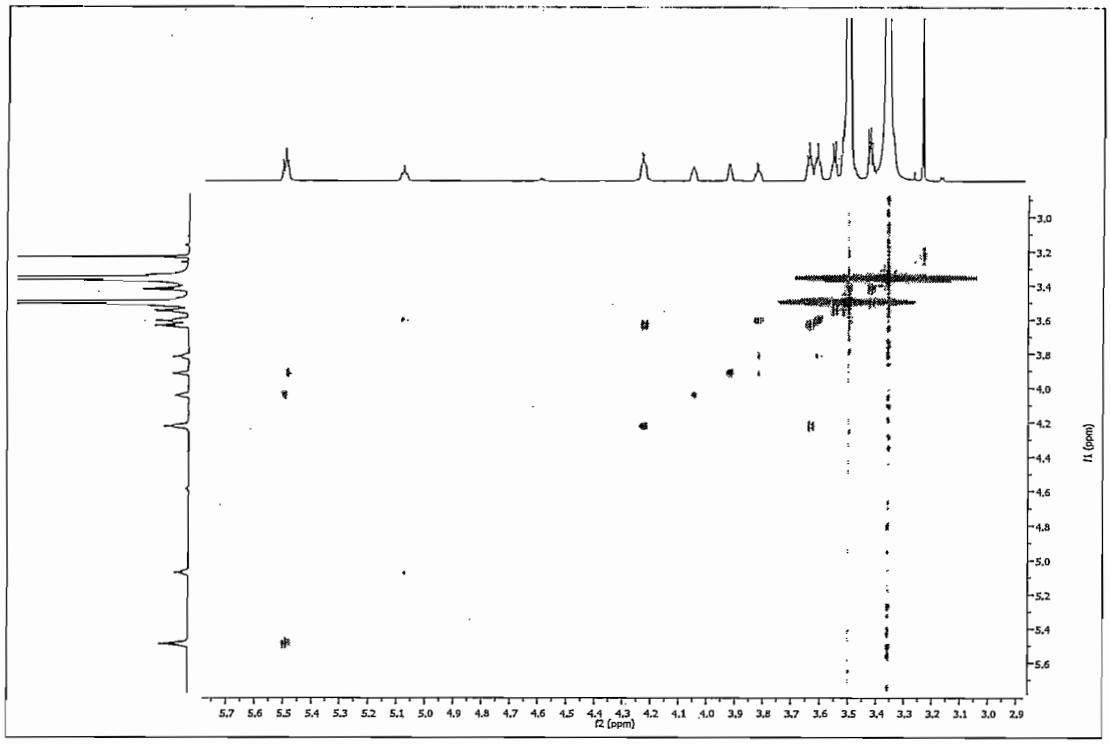


Figure A.36: COSY NMR spectrum of N4-mPEG550-cytarabine carbamate

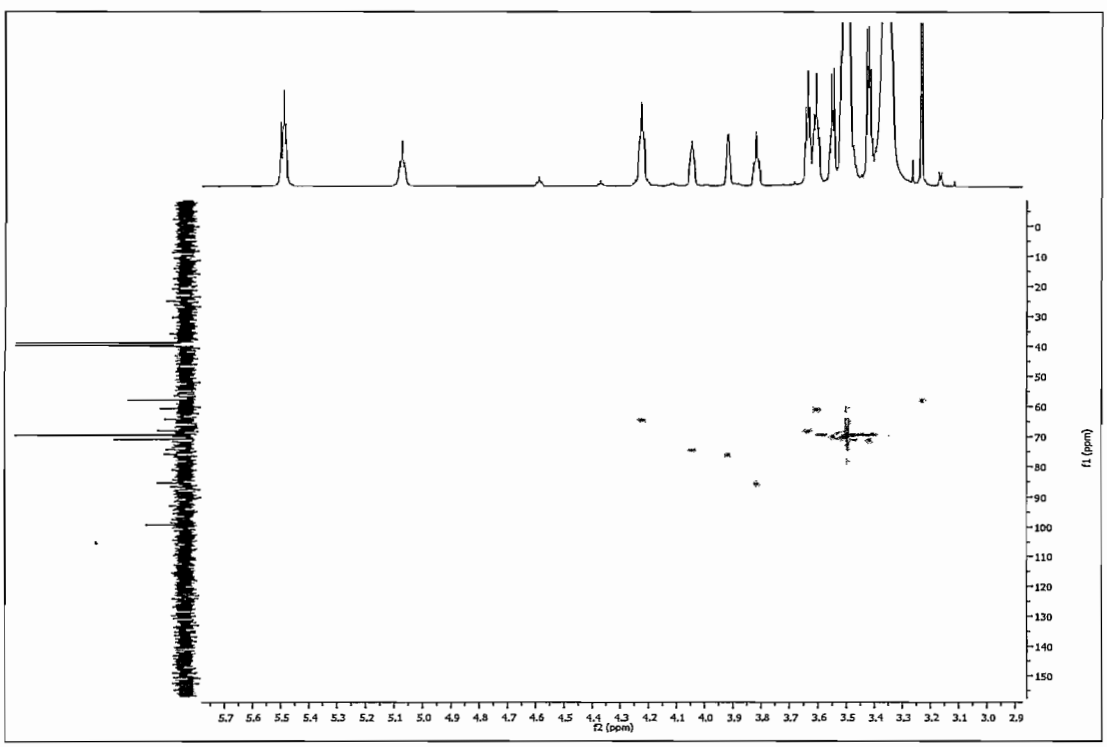


Figure A.37: HSQC NMR spectrum of N4-mPEG550-cytarabine carbamate

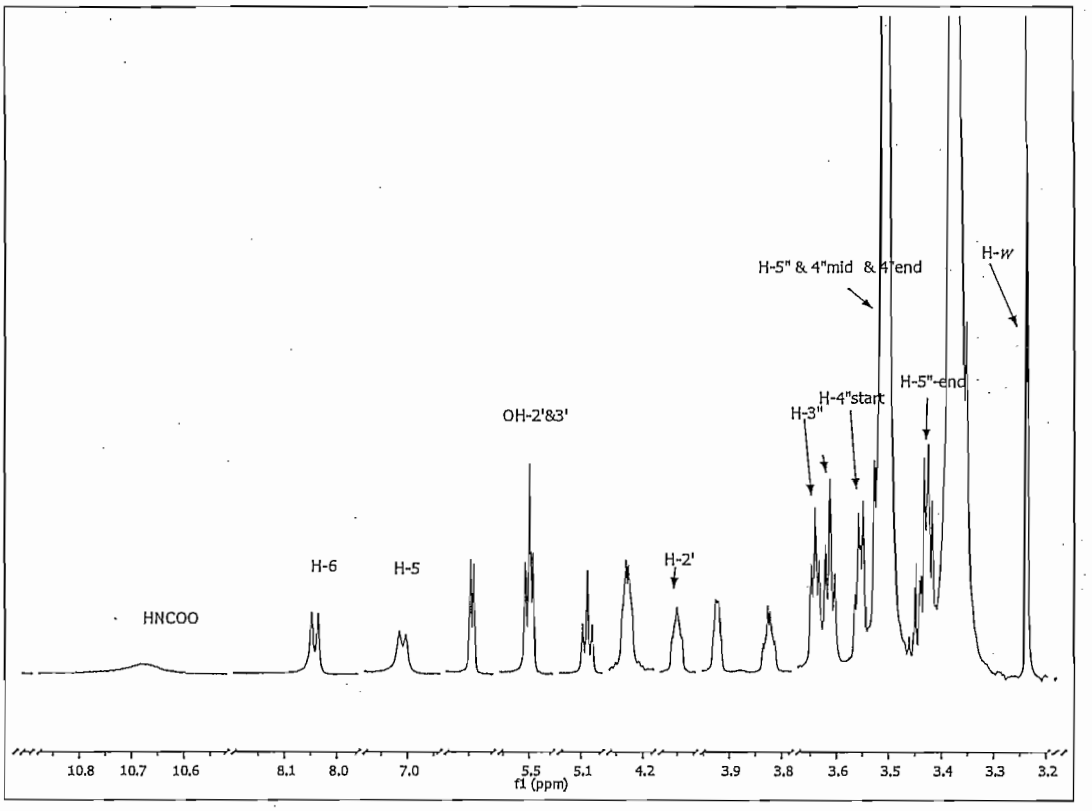


Figure A.38: ^{1}H NMR spectrum of N4-mPEG750-cytarabine carbamate

7.5 NMR spectra of ester derivatives of cytarabine

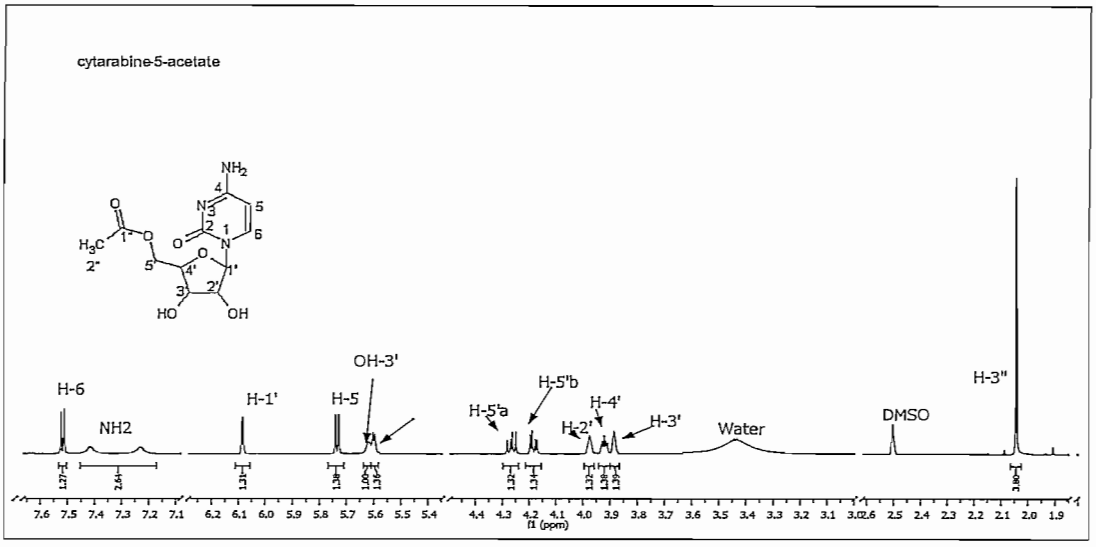


Figure A.39: ^{1}H NMR spectrum of cytarabine-5'-acetate

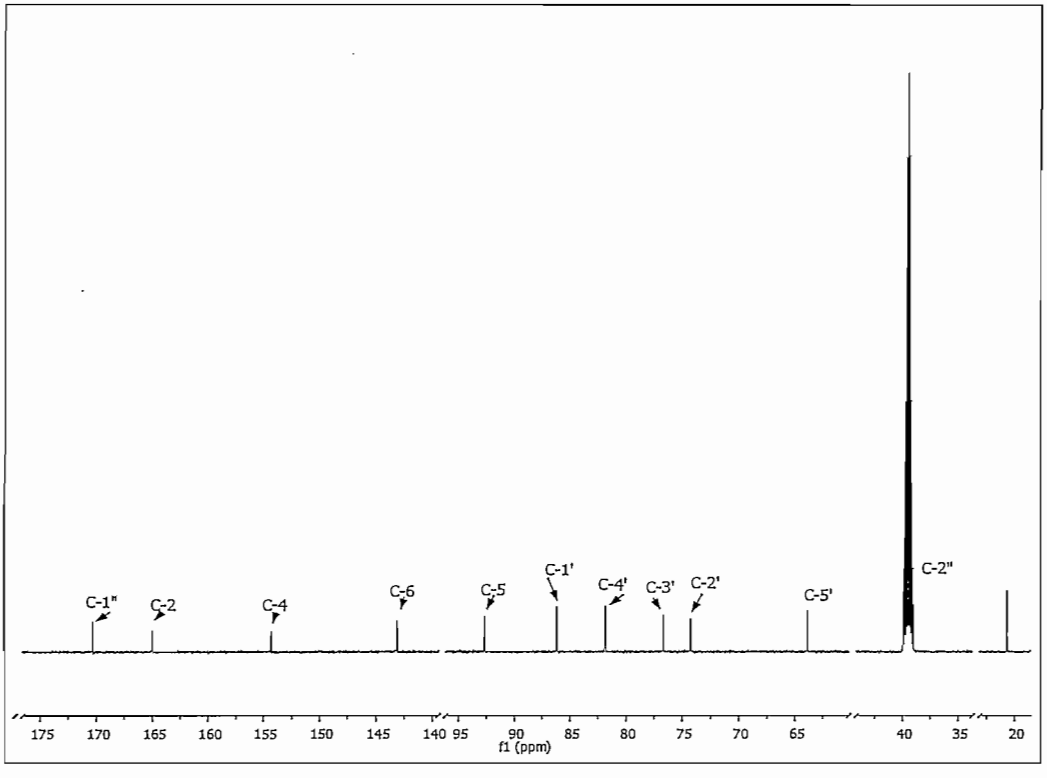


Figure A.40: ^{13}C NMR spectrum of cytarabine-5'-acetate

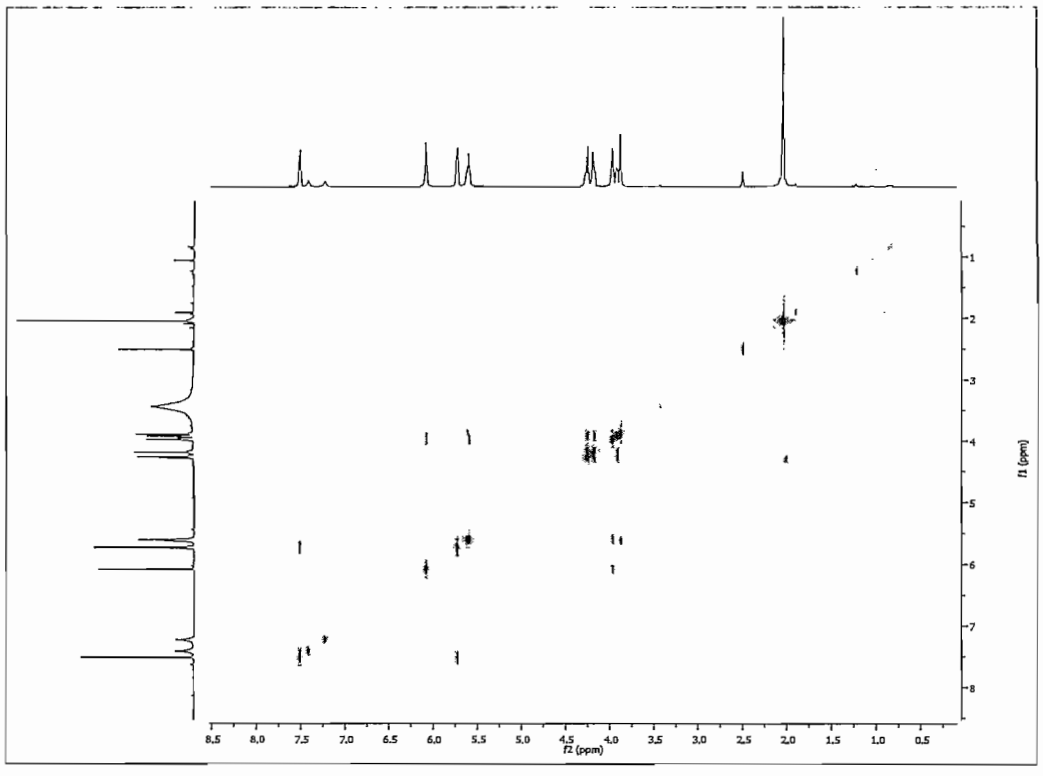


Figure A.41: COSY NMR spectrum of cytarabine-5'-acetate

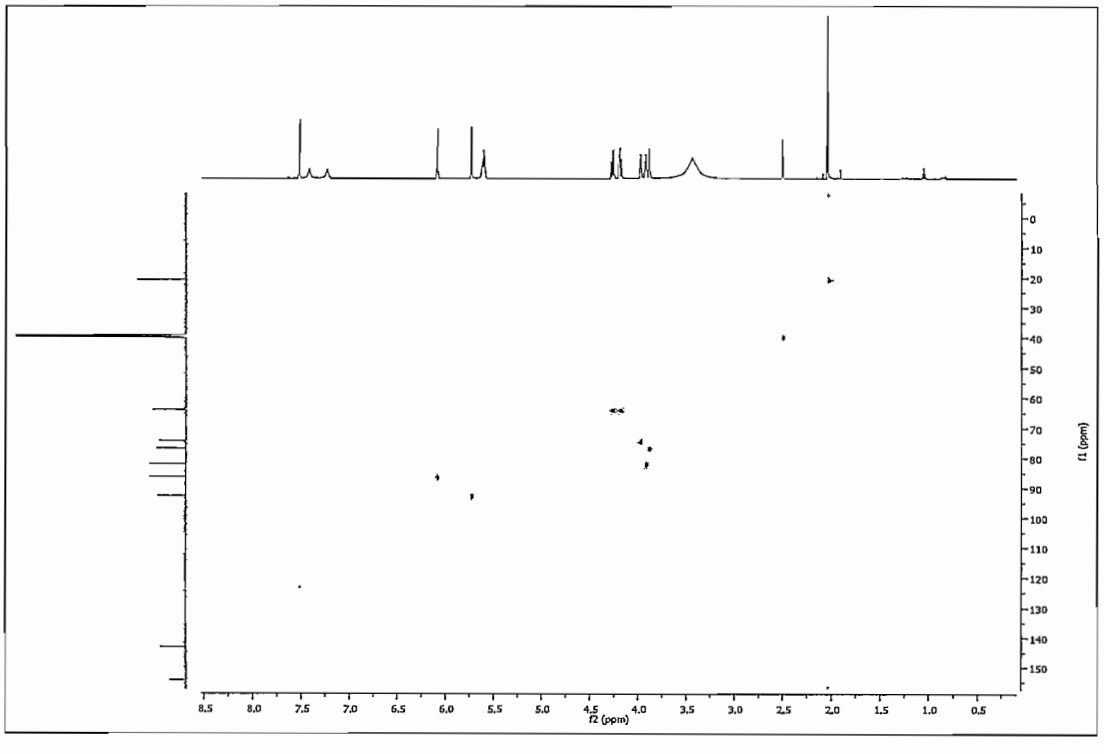


Figure A.42: HSQC NMR spectrum of cytarabine-5'-acetate

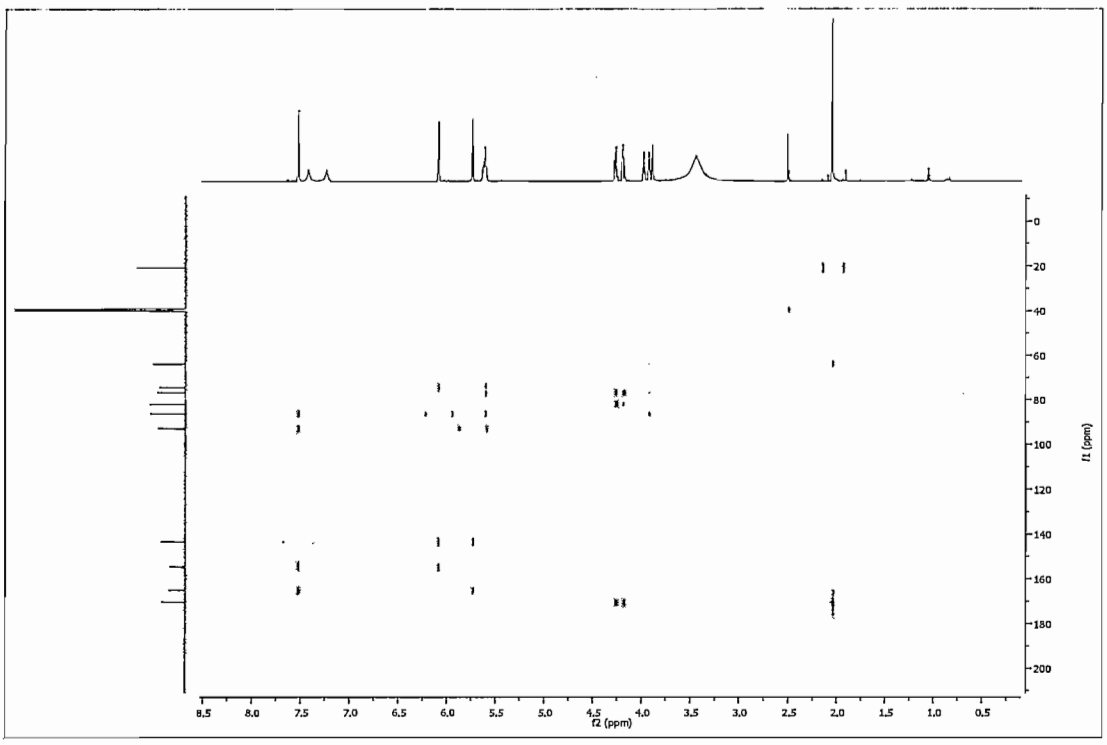


Figure A.43: HMBC NMR spectrum of cytarabine-5'-acetate

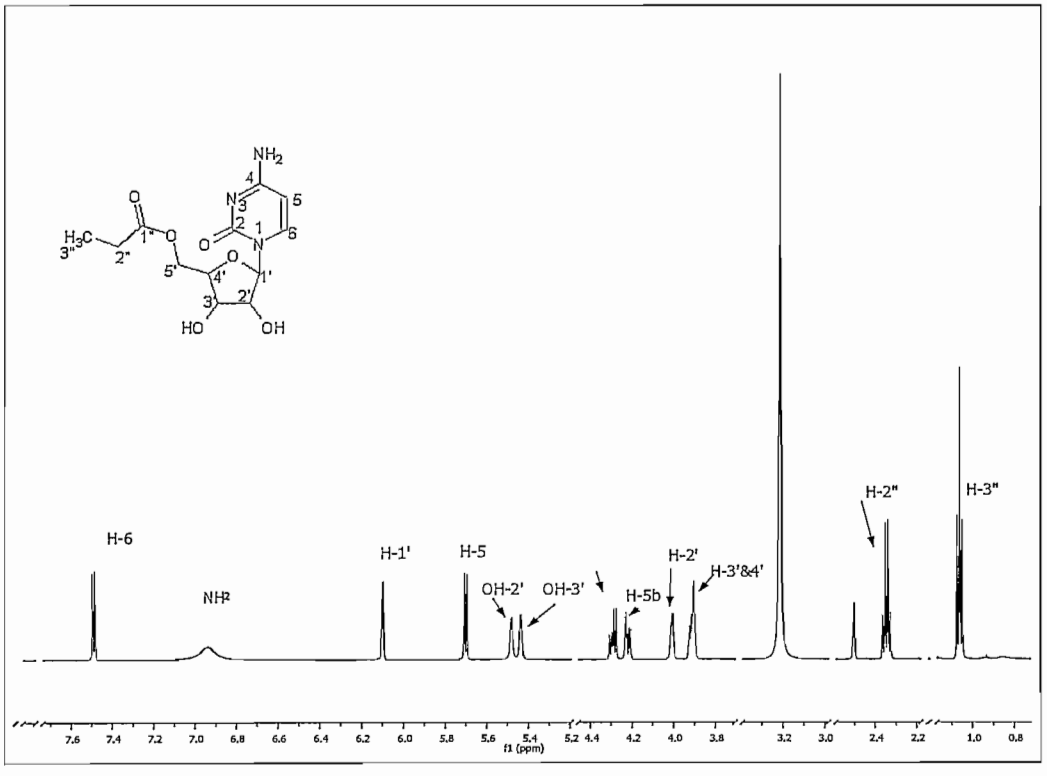


Figure A.44: ^{1}H NMR spectrum of cytarabine-5'-propionate

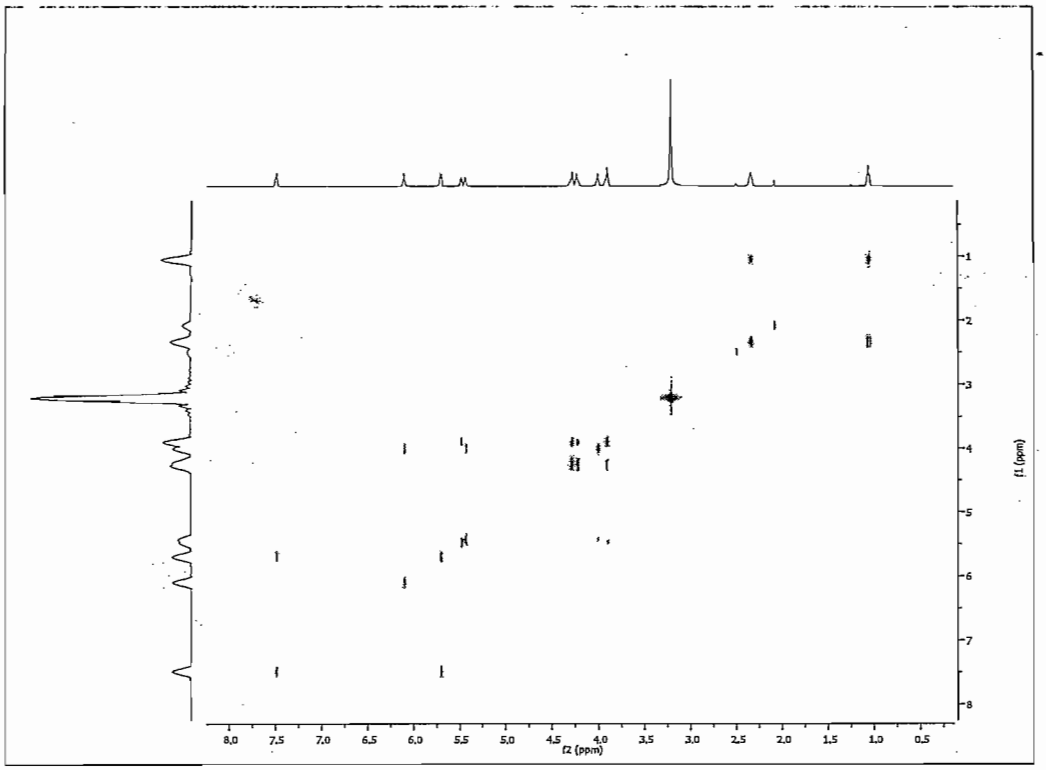


Figure A.45:COSY NMR spectrum of cytarabine-5'-propionate

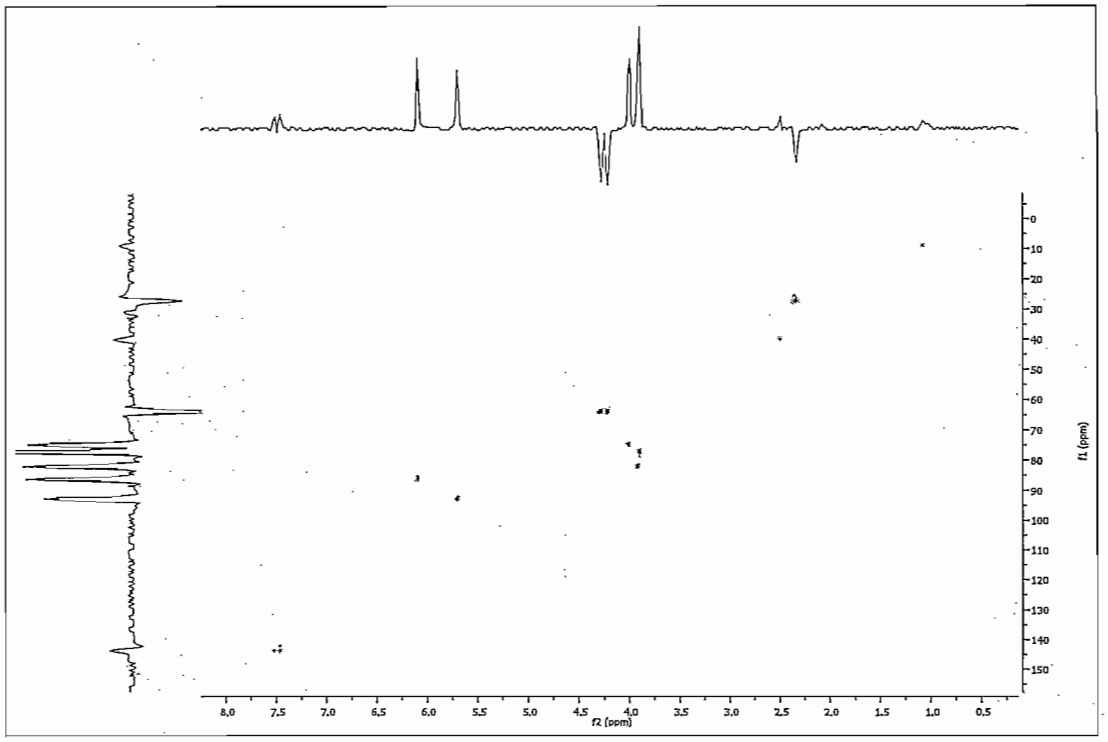


Figure A.46: HSQC NMR spectrum of cytarabine-5'-propionate

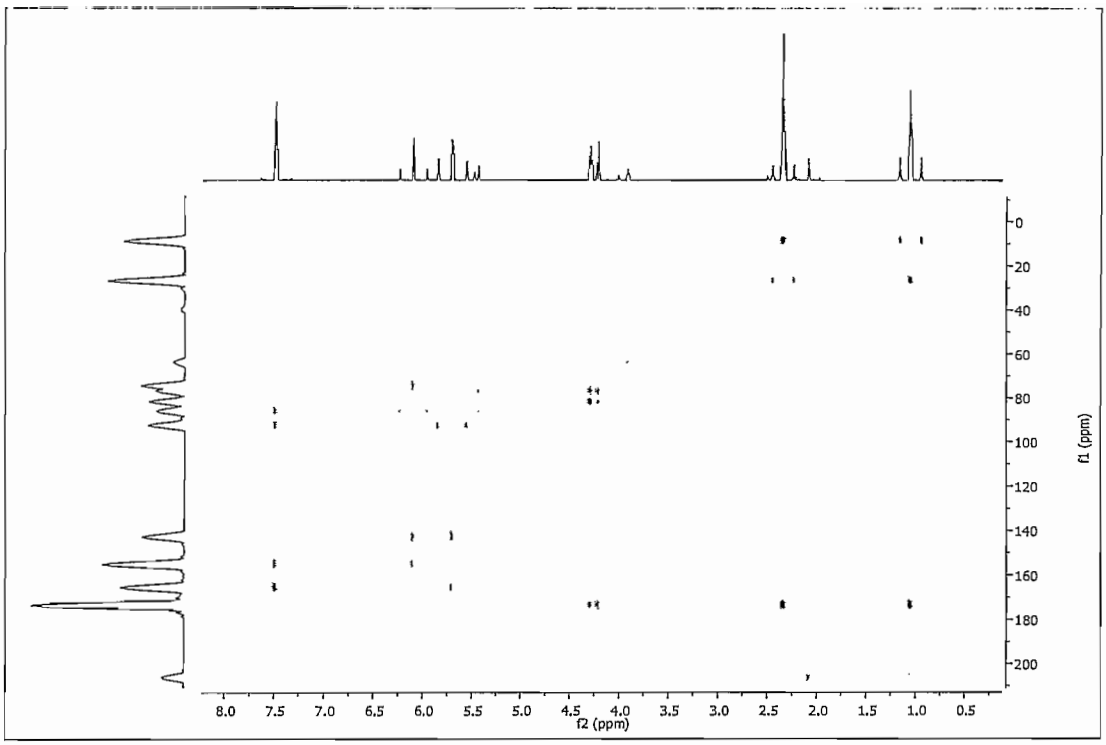


Figure A.47: HSQC NMR spectrum of cytarabine-5'-propionate

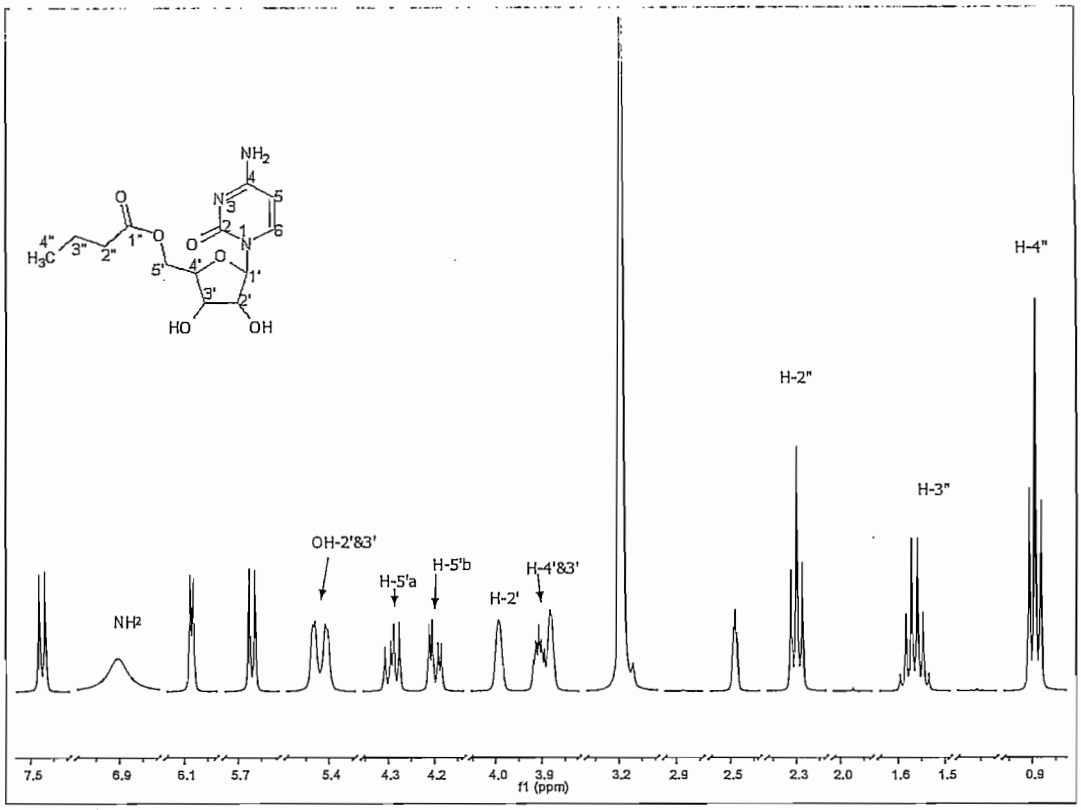


Figure A.48: ^{1}H NMR spectrum of cytarabine-5'-butanoate

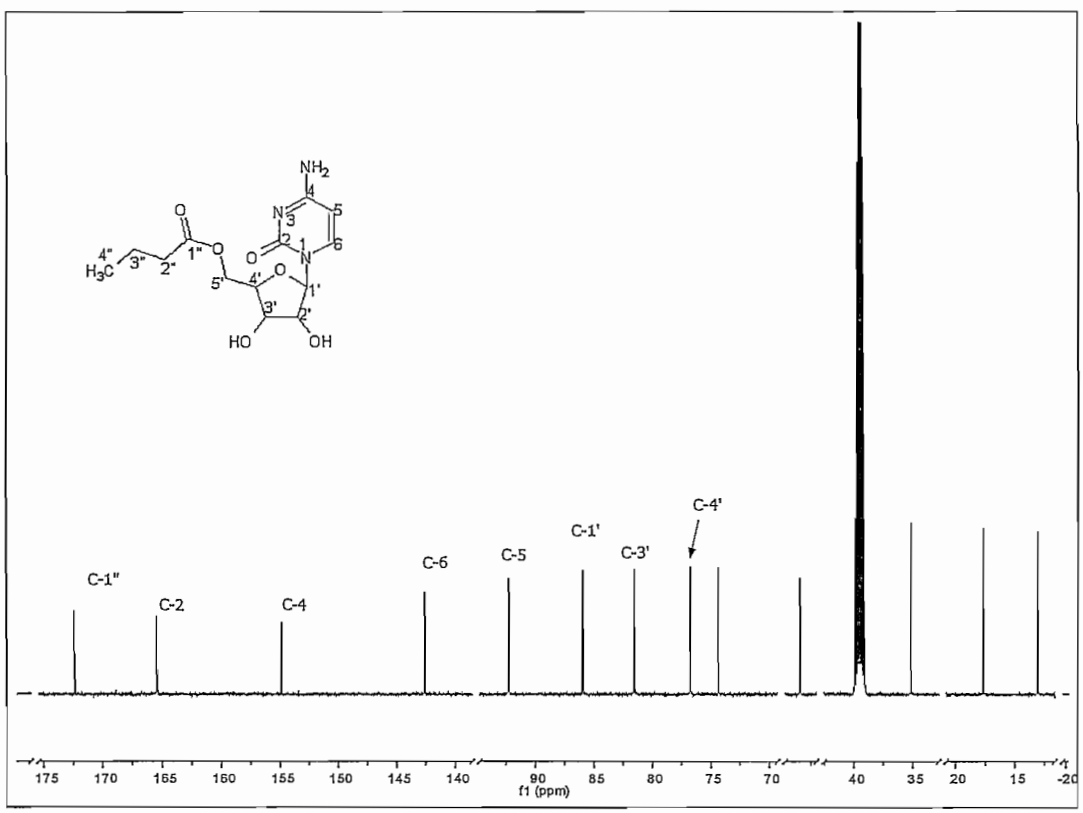


Figure A.49: ^{13}C NMR spectrum of cytarabine-5'-butanoate

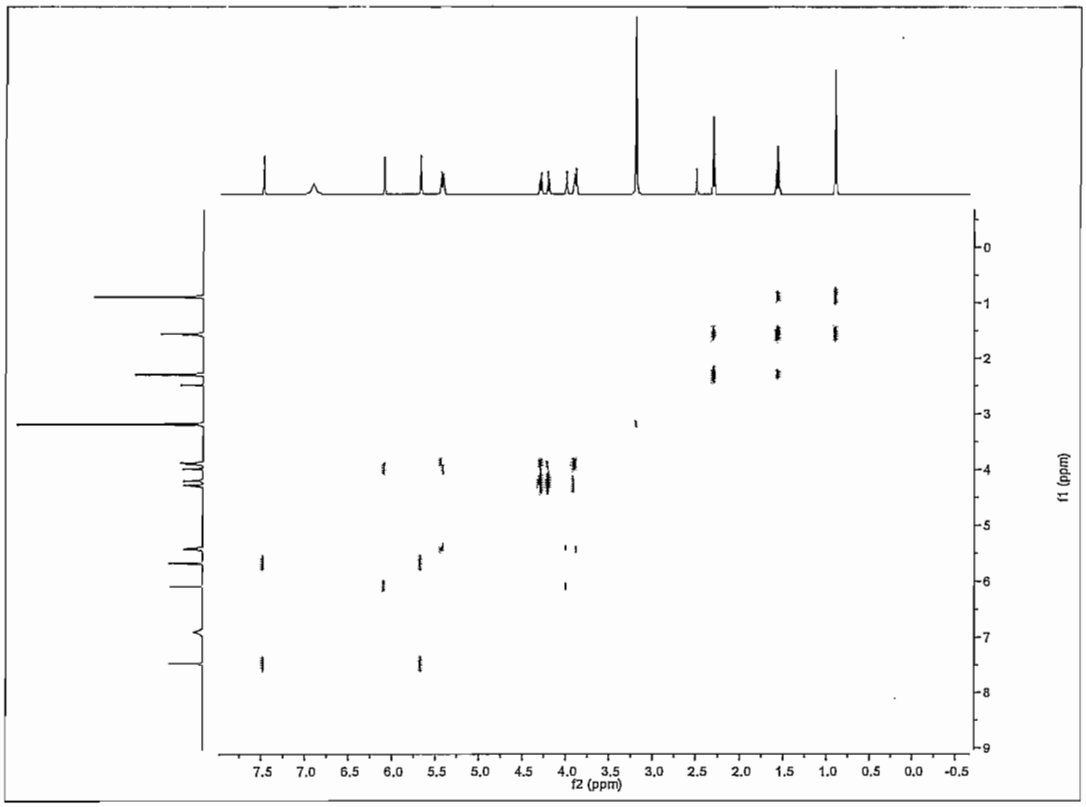


Figure A.50: COSY NMR spectrum of cytarabine-5'-butanoate

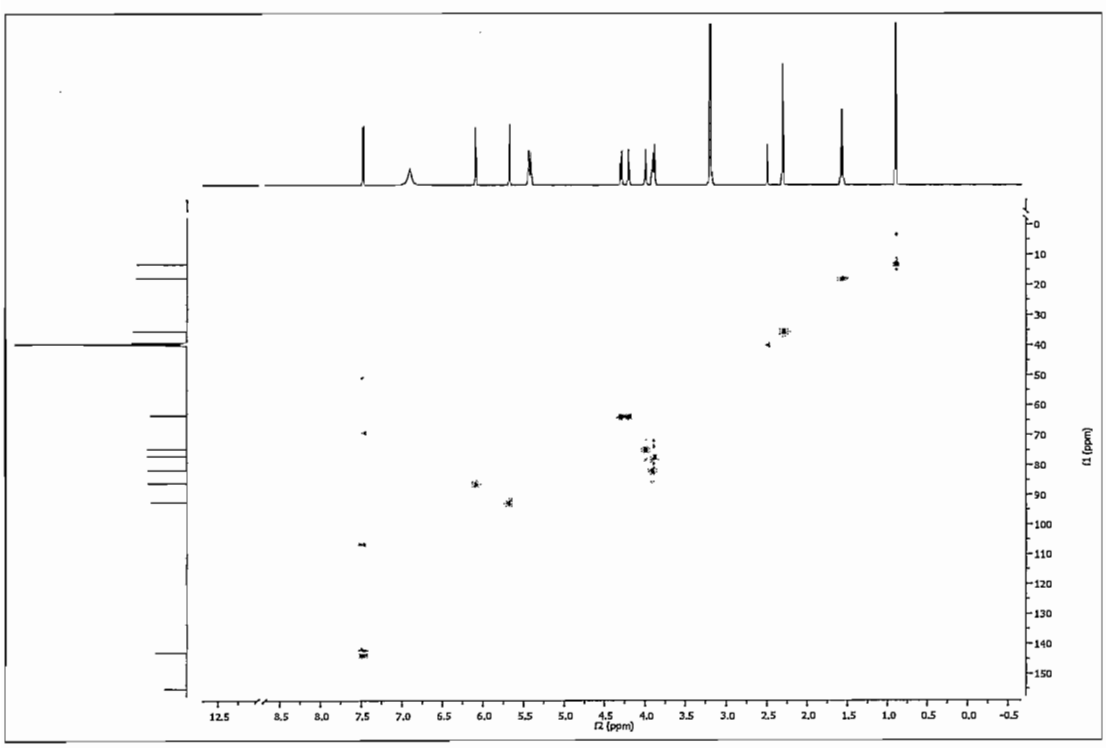


Figure A.51: HSQC NMR spectrum of cytarabine-5'-butanoate

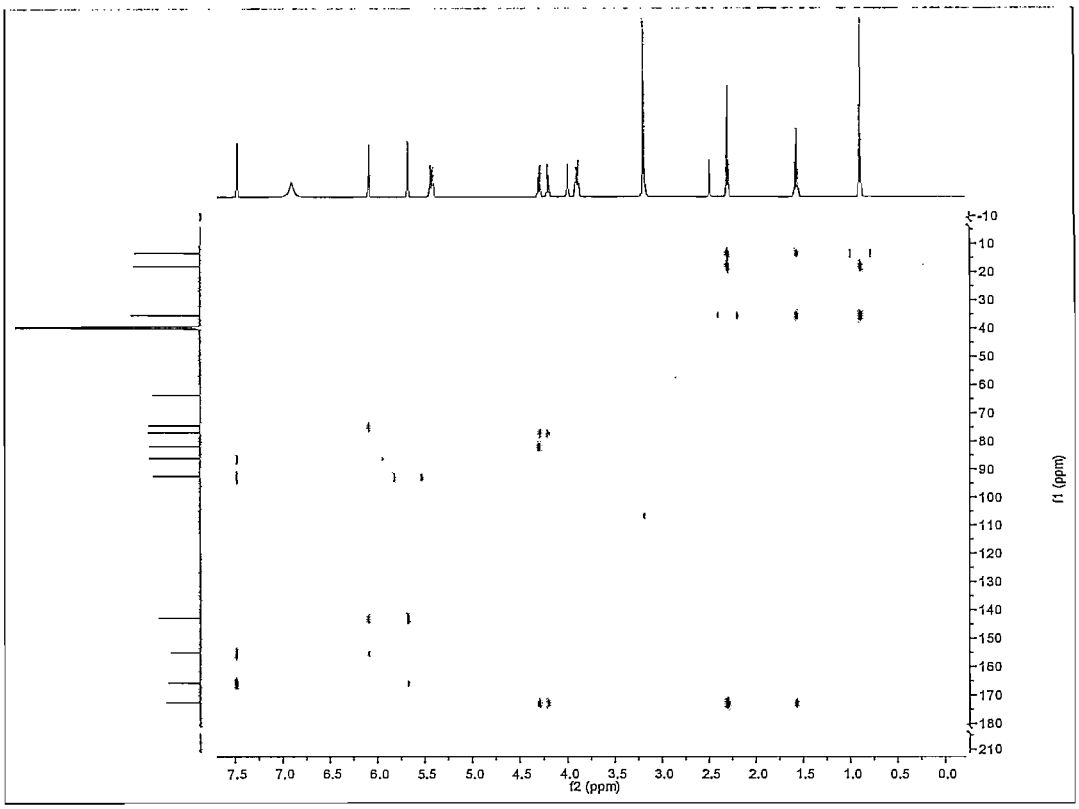


Figure A.52: HMBC NMR spectrum of cytarabine-5'-butanoate

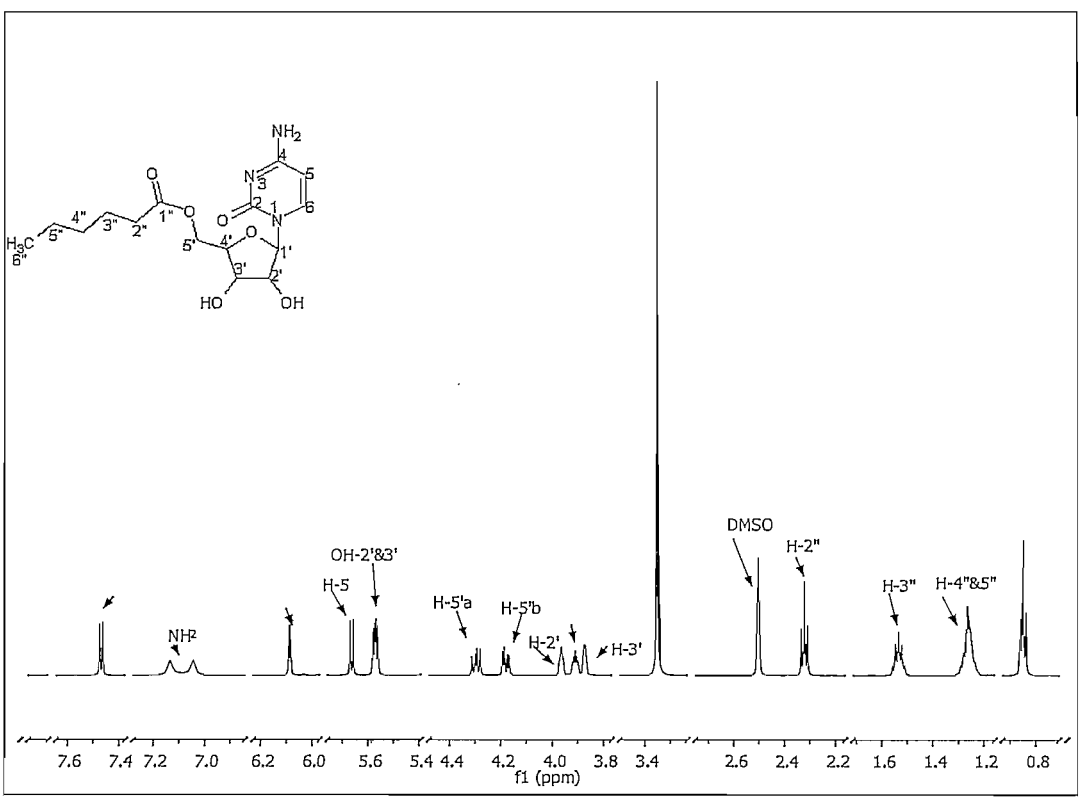


Figure A.53: ^{1}H NMR spectrum of cytarabine-5'-hexanoate

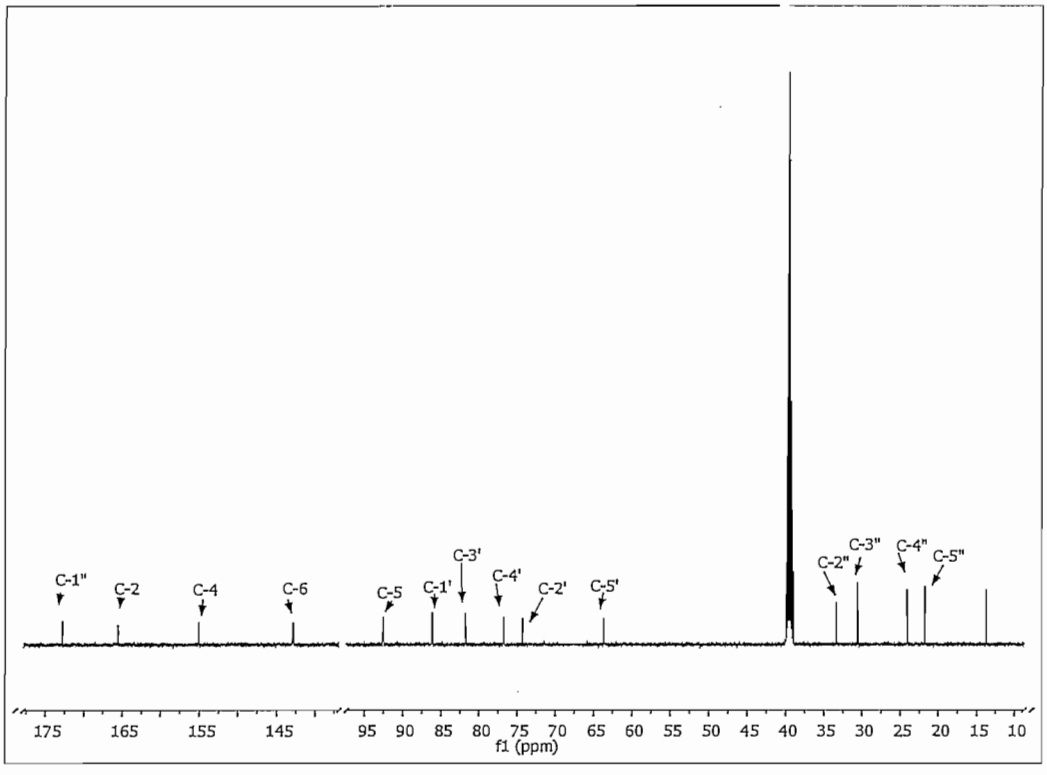


Figure A.54: ^{13}C NMR spectrum of cytarabine-5'-hexanoate

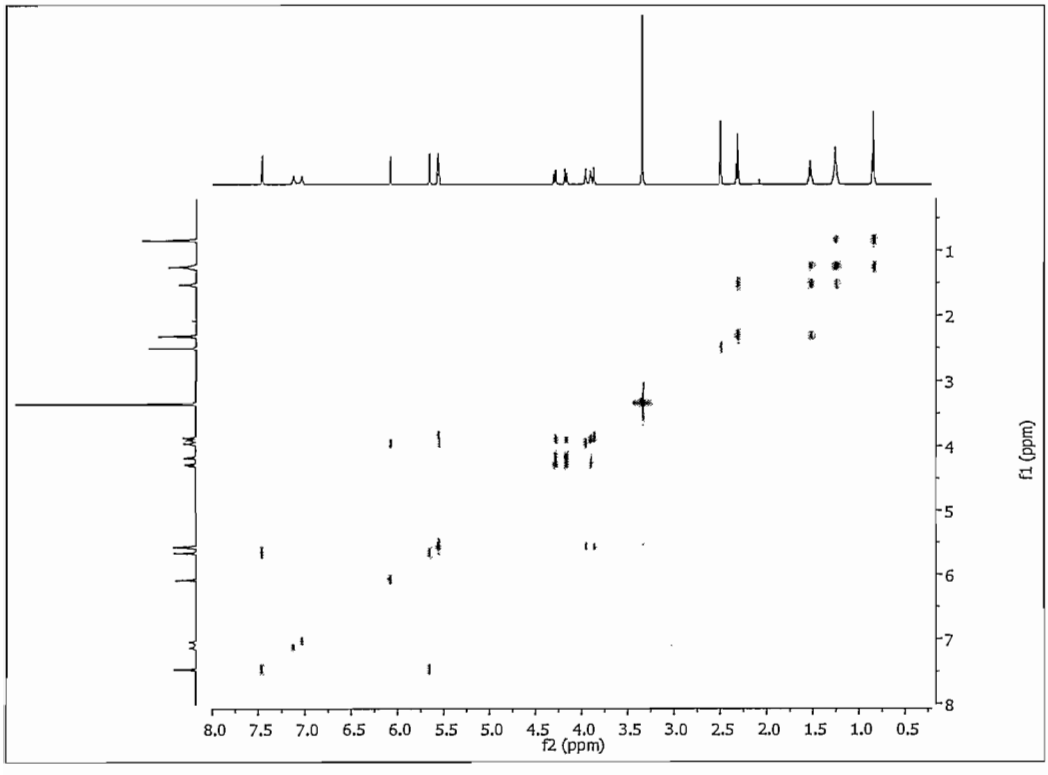


Figure A.55: COSY NMR spectrum of cytarabine-5'-hexanoate

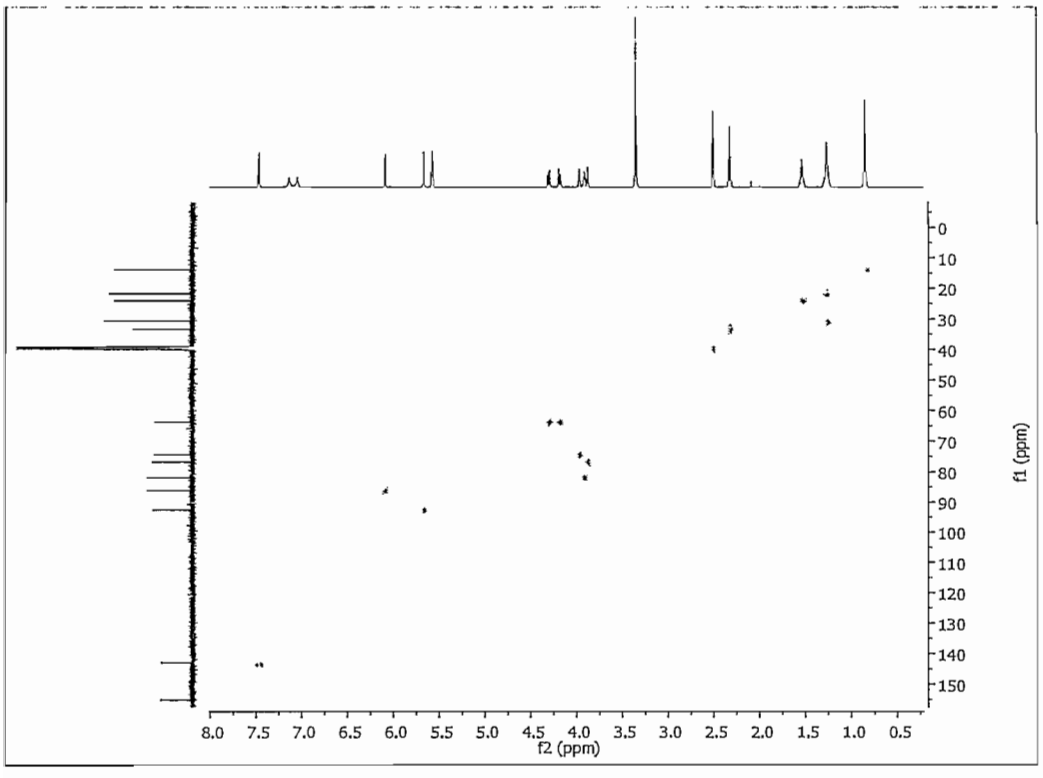


Figure A.56: HSQC NMR spectrum of cytarabine-5'-hexanoate

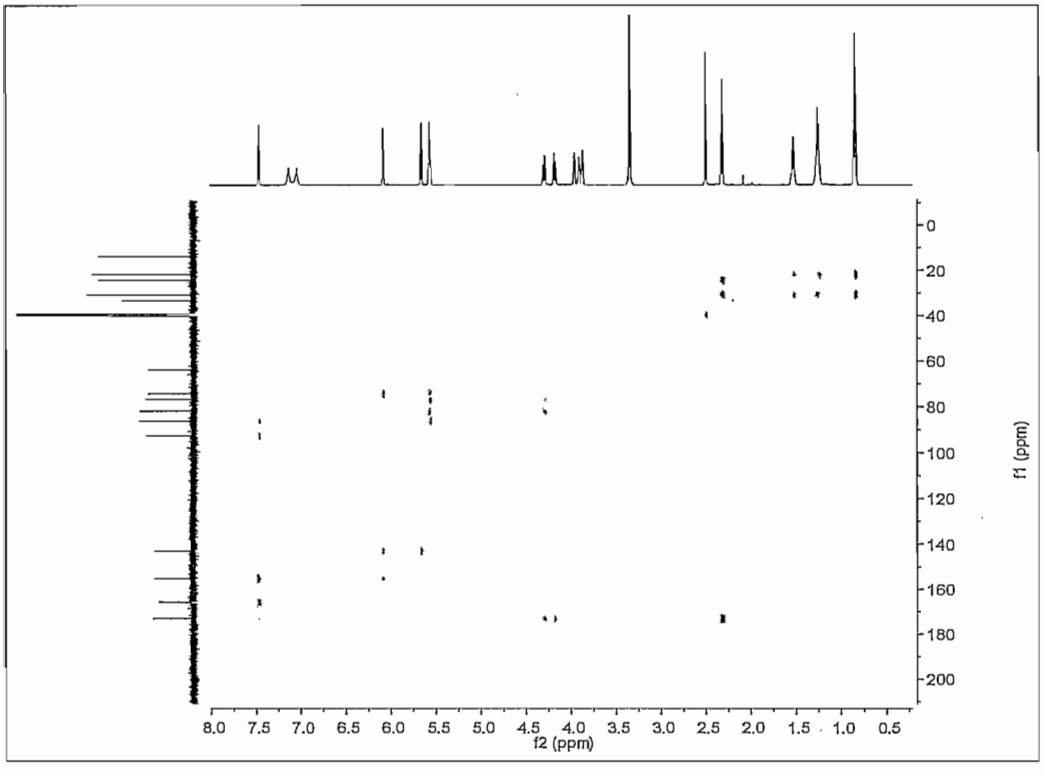


Figure A.57: HBMC NMR spectrum of cytarabine-5'-hexanoate

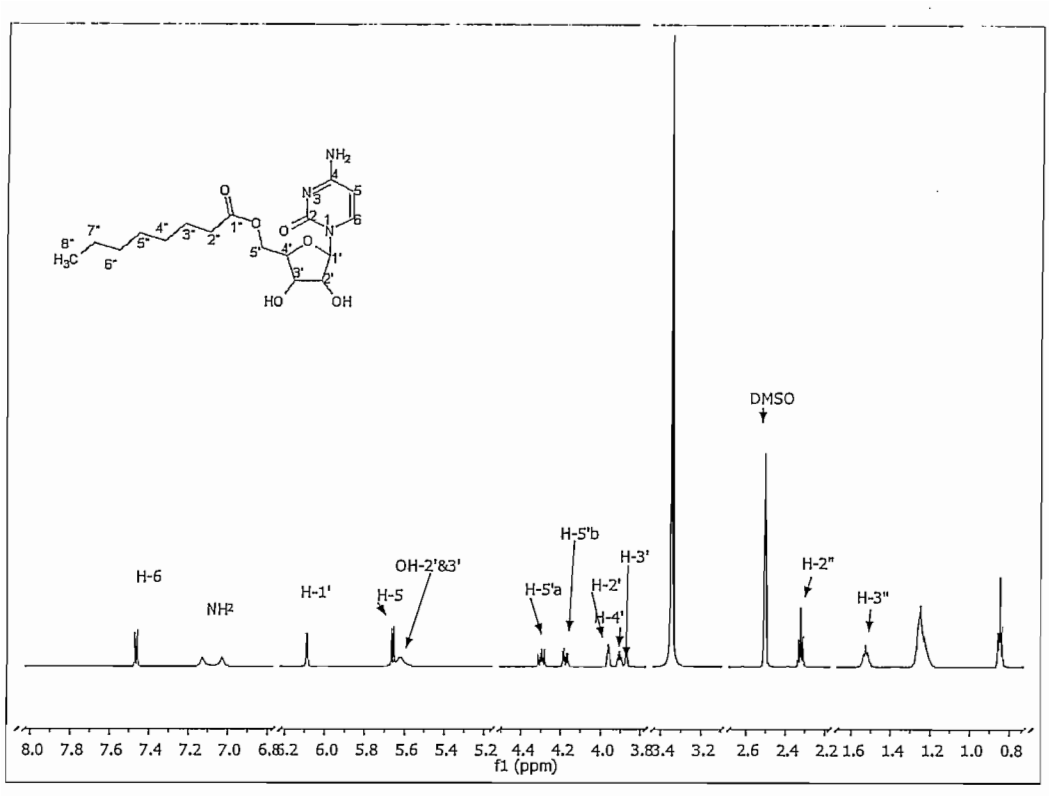


Figure A.58: ^{1}H NMR spectrum of cytarabine-5'-octanoate

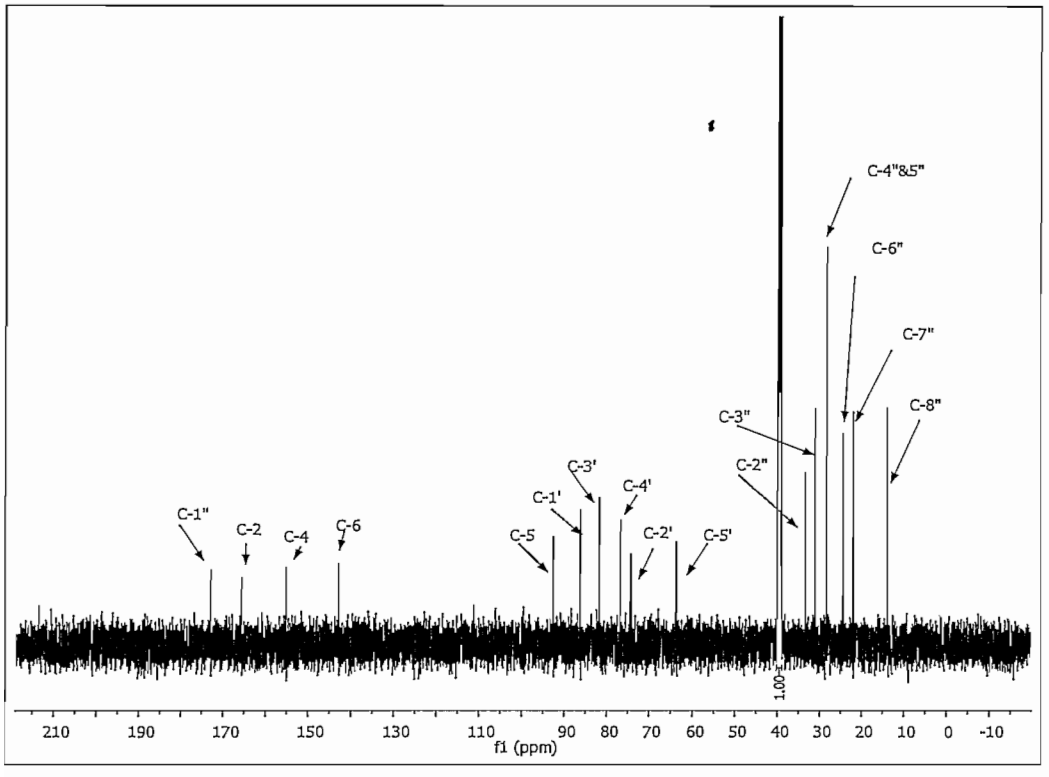


Figure A.59: ^{13}C NMR spectrum of cytarabine-5'-octanoate

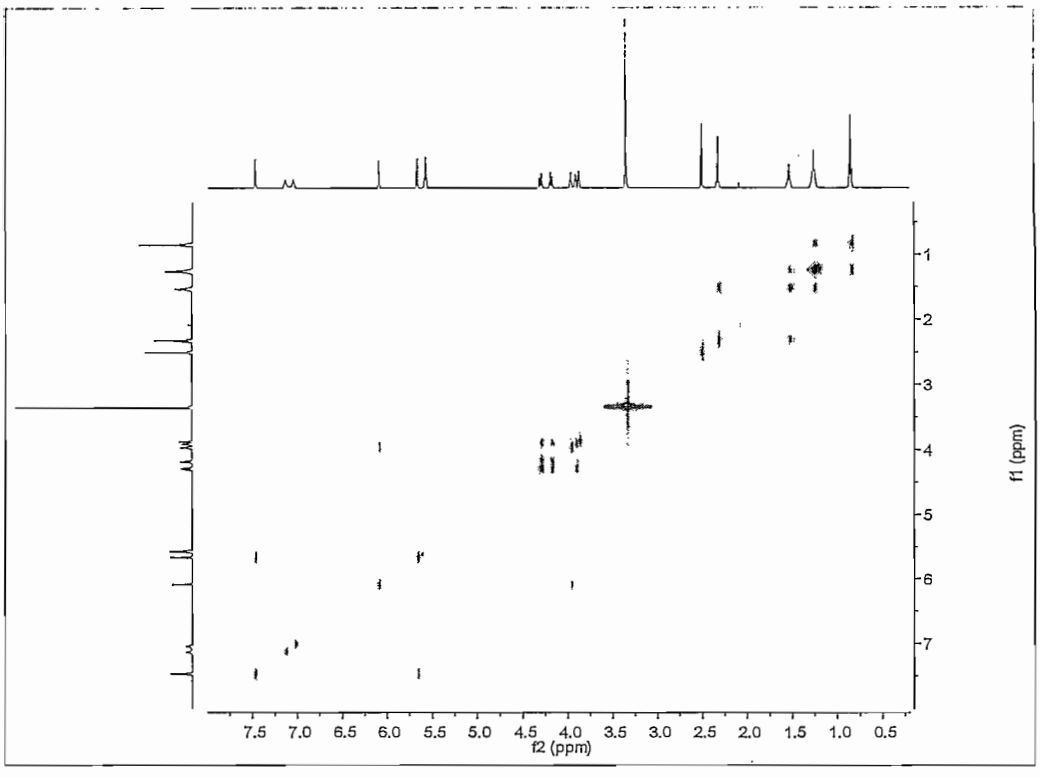


Figure A.60: COSY NMR spectrum of cytarabine-5'-octanoate

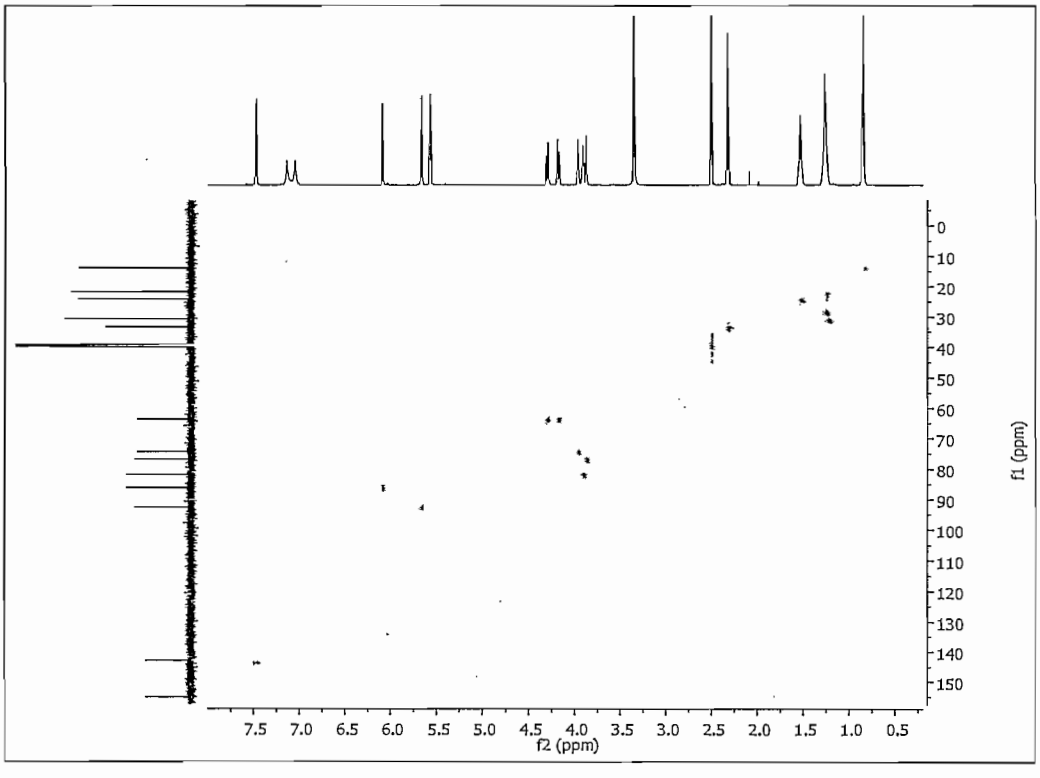


Figure A.61: HSQC NMR spectrum of cytarabine-5'-octanoate

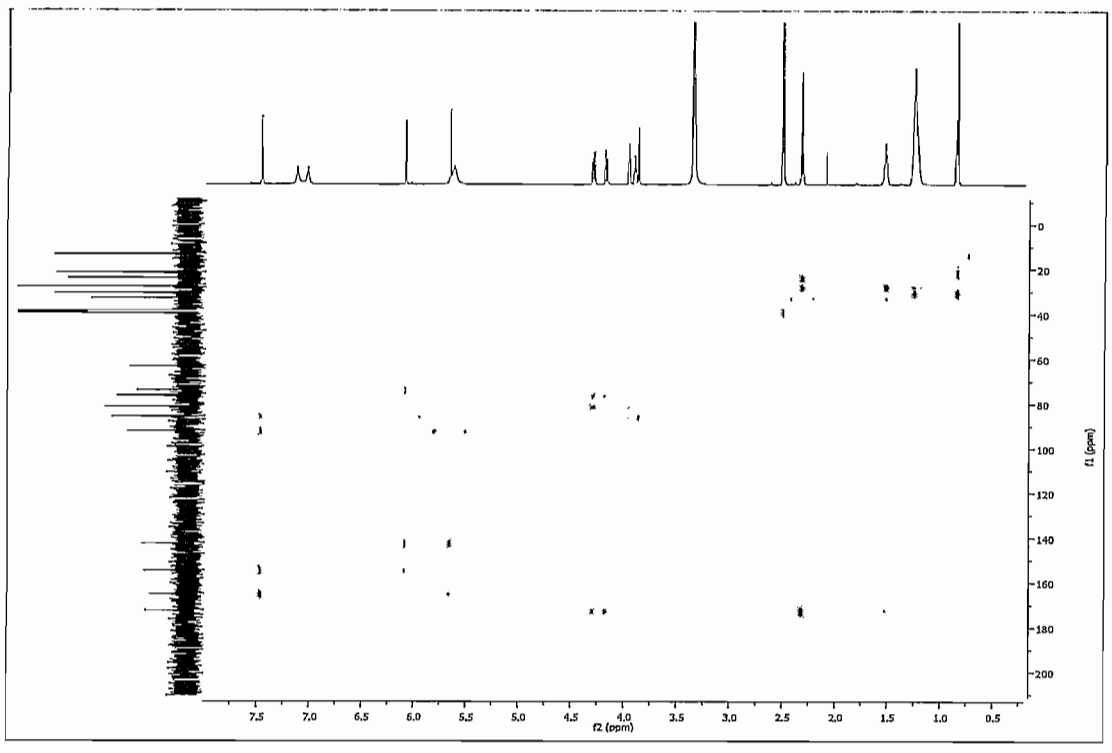


Figure A.62: HMBC NMR spectrum of cytarabine-5'-octanoate

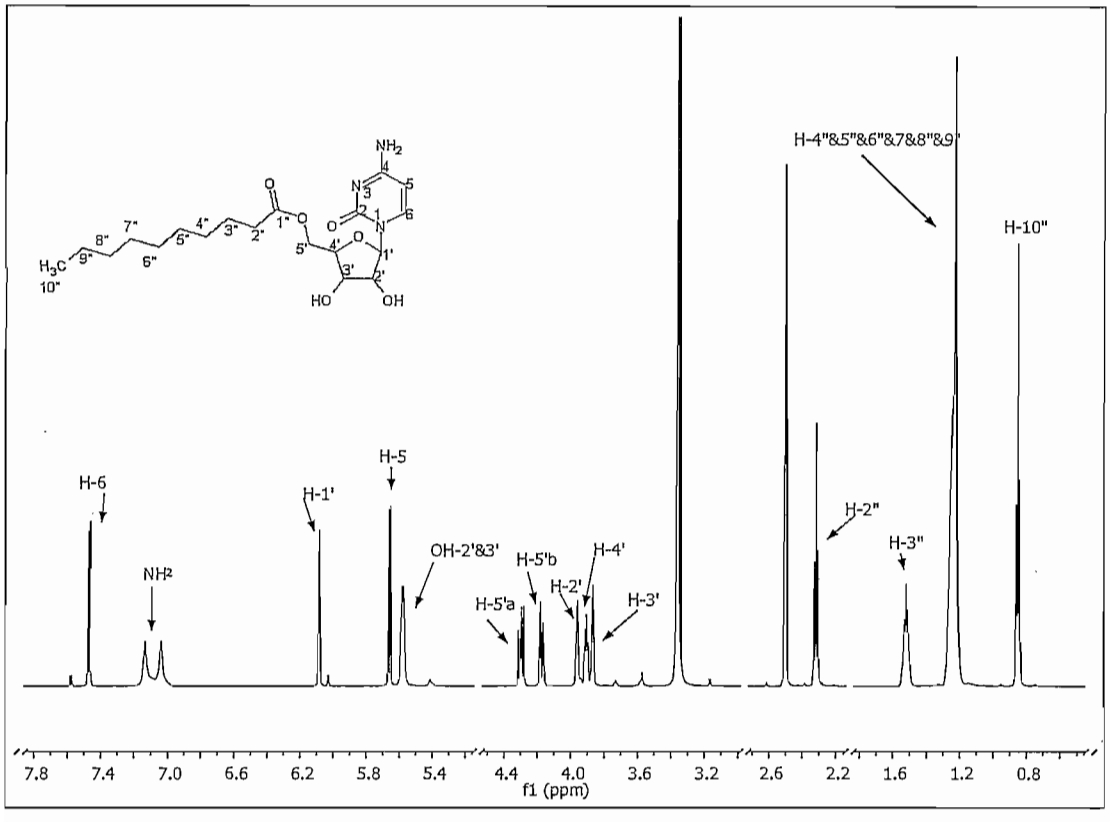


Figure A.63:[1]H NMR spectrum of cytarabine-5'-decanoate

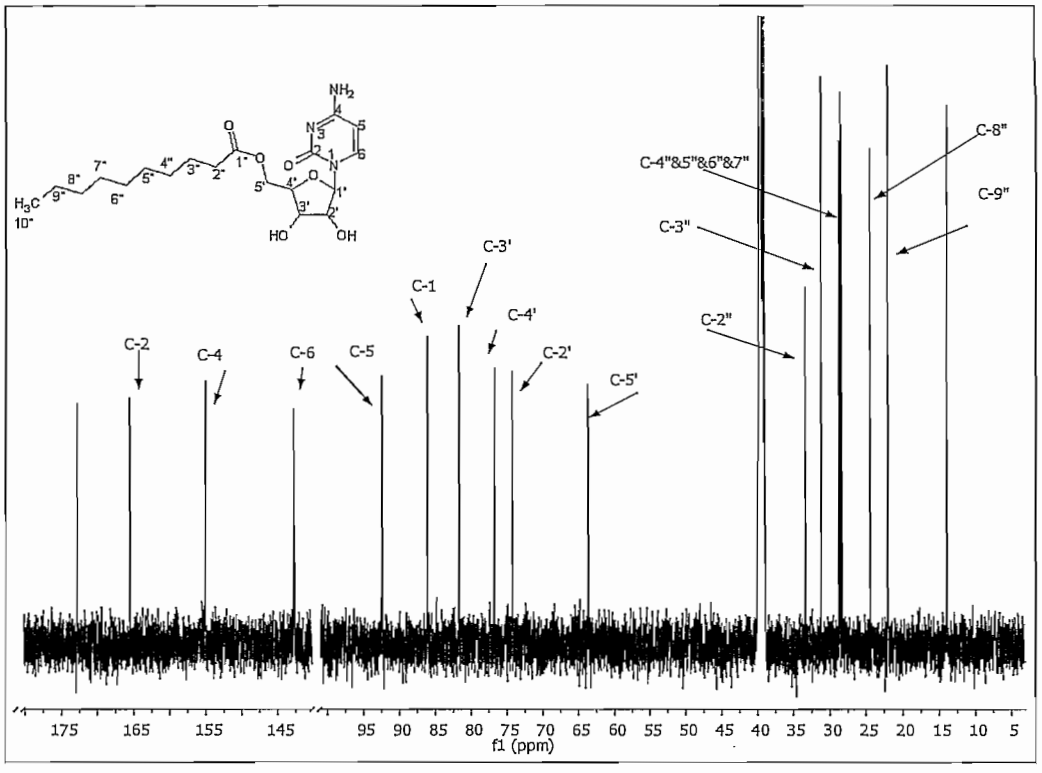


Figure A.64: ^{13}C NMR spectrum of cytarabine-5'-decanoate

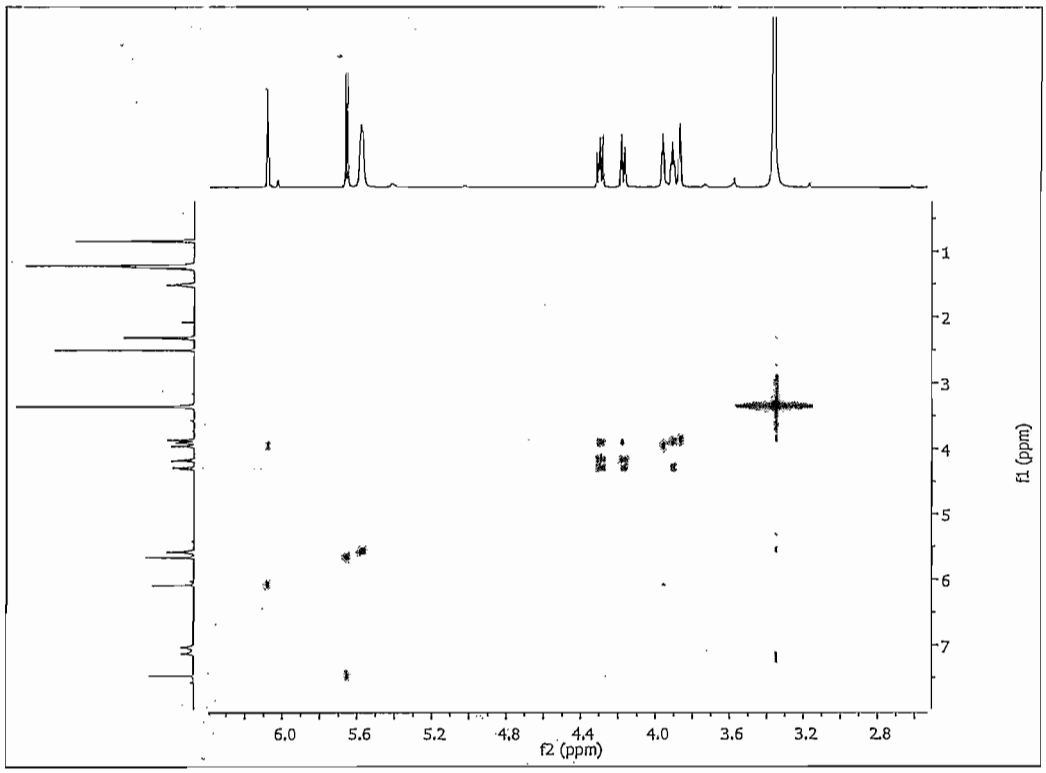


Figure A.65: COSY NMR spectrum of cytarabine-5'-decanoate

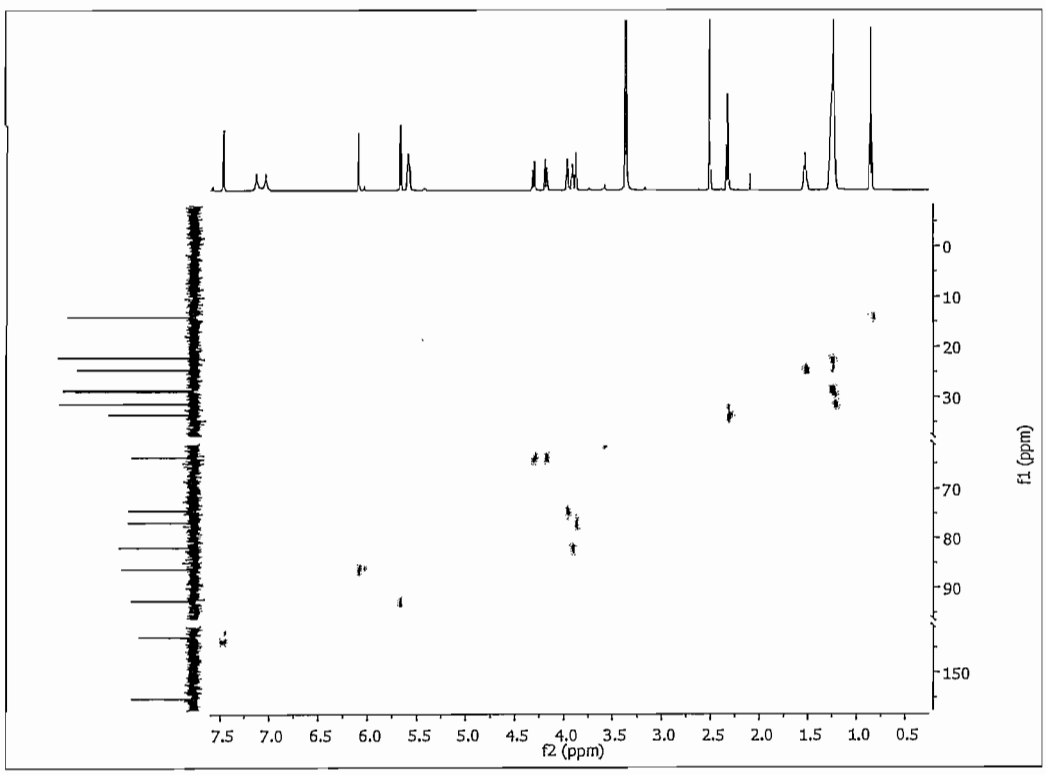
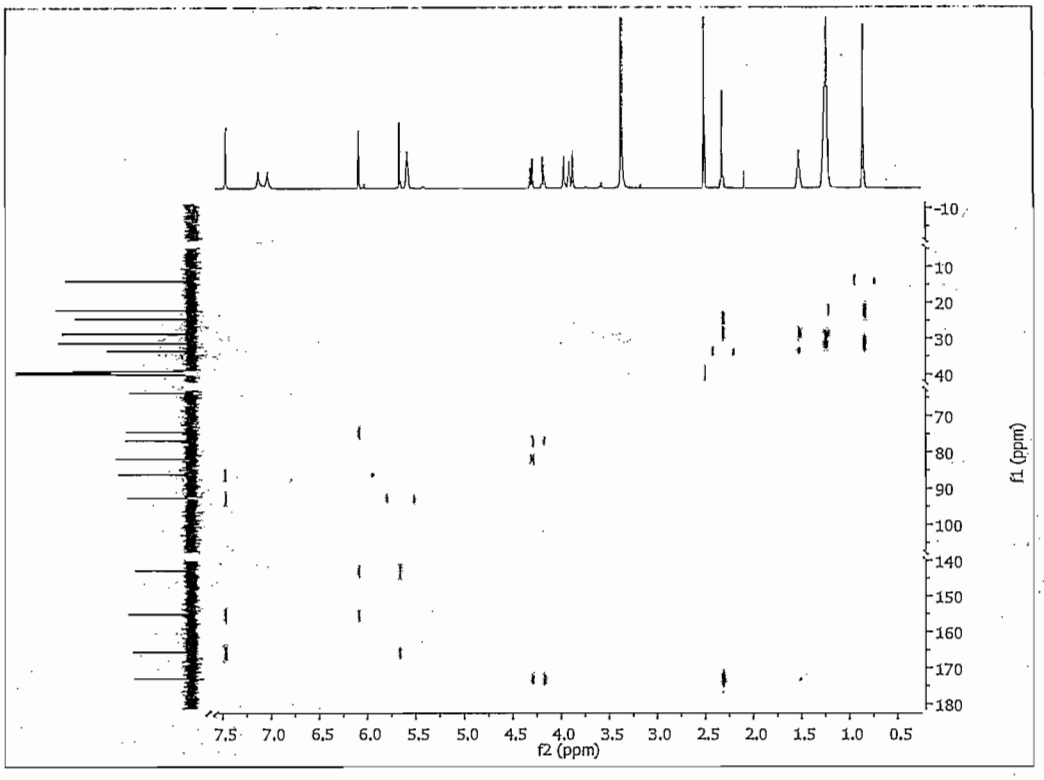


Figure A.66: HSQC NMR spectrum of cytarabine-5'-decanoate



FigureA.67: HMBC NMR spectrum of cytarabine-5'-decanoate

7.6 NMR spectra of amide derivatives of cytarabine

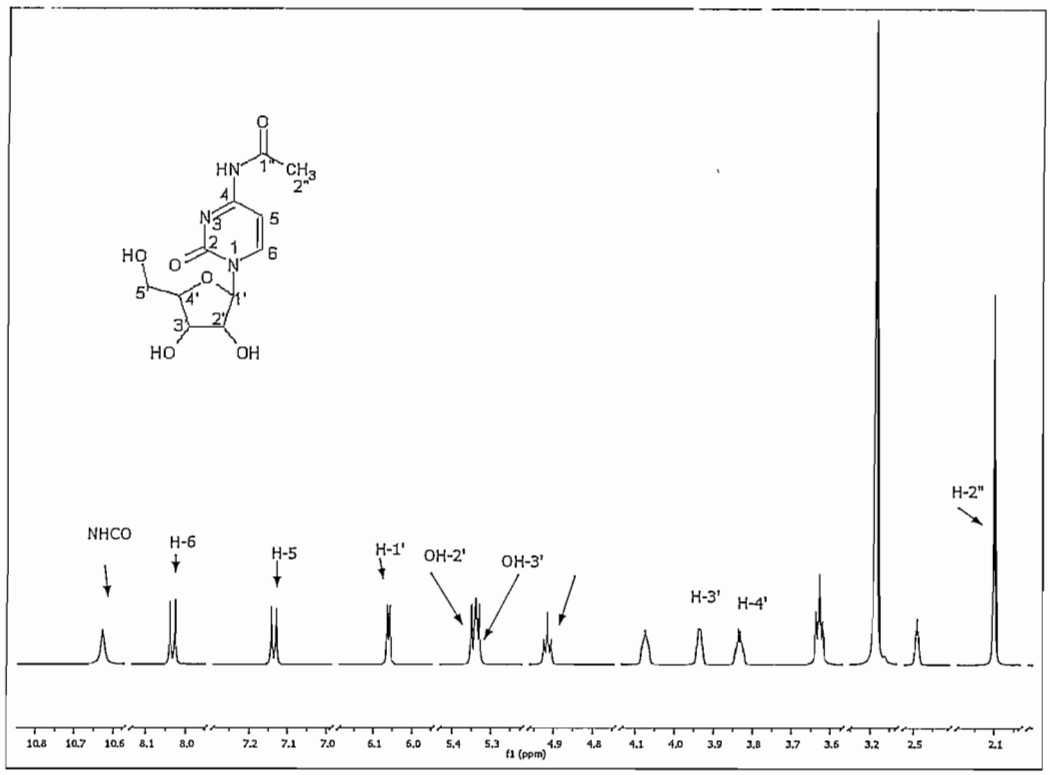


Figure A.68: ^{1}H NMR spectrum of N4-acetylcytarabine

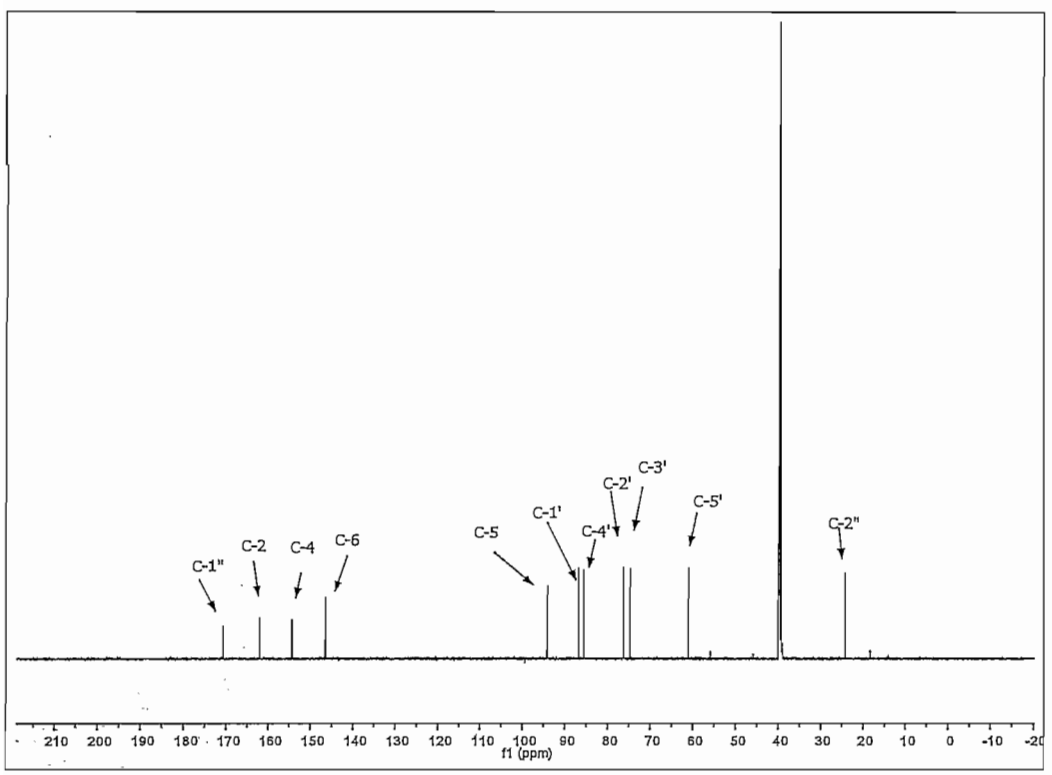


Figure A.69: ^{13}C NMR spectrum of N4-acetylcytarabine

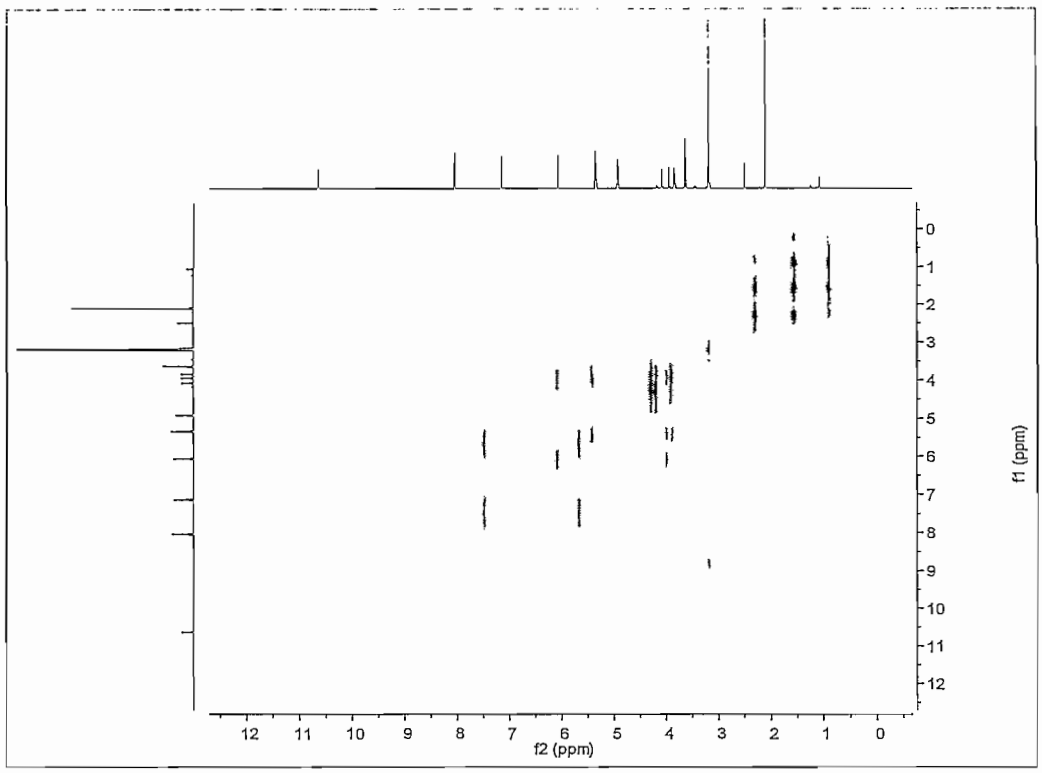


Figure A.70: COSY NMR spectrum of N4-acetylcytarabine

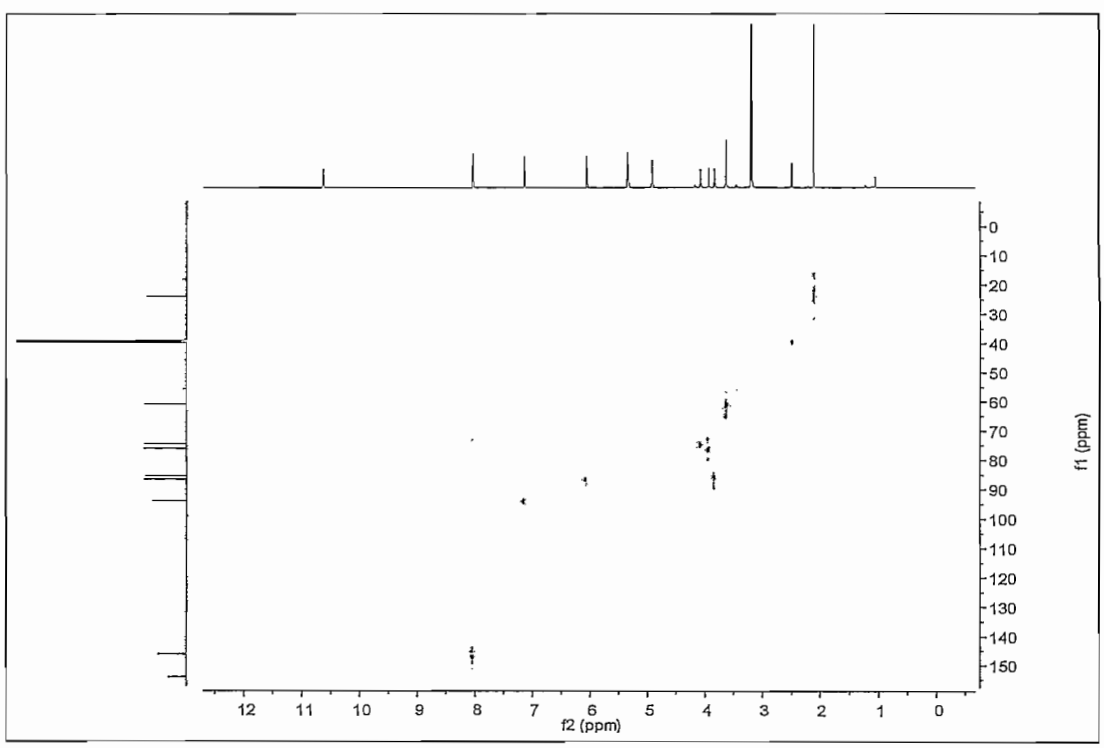


Figure A.71: HSQC NMR spectrum of N4-acetylcytarabine

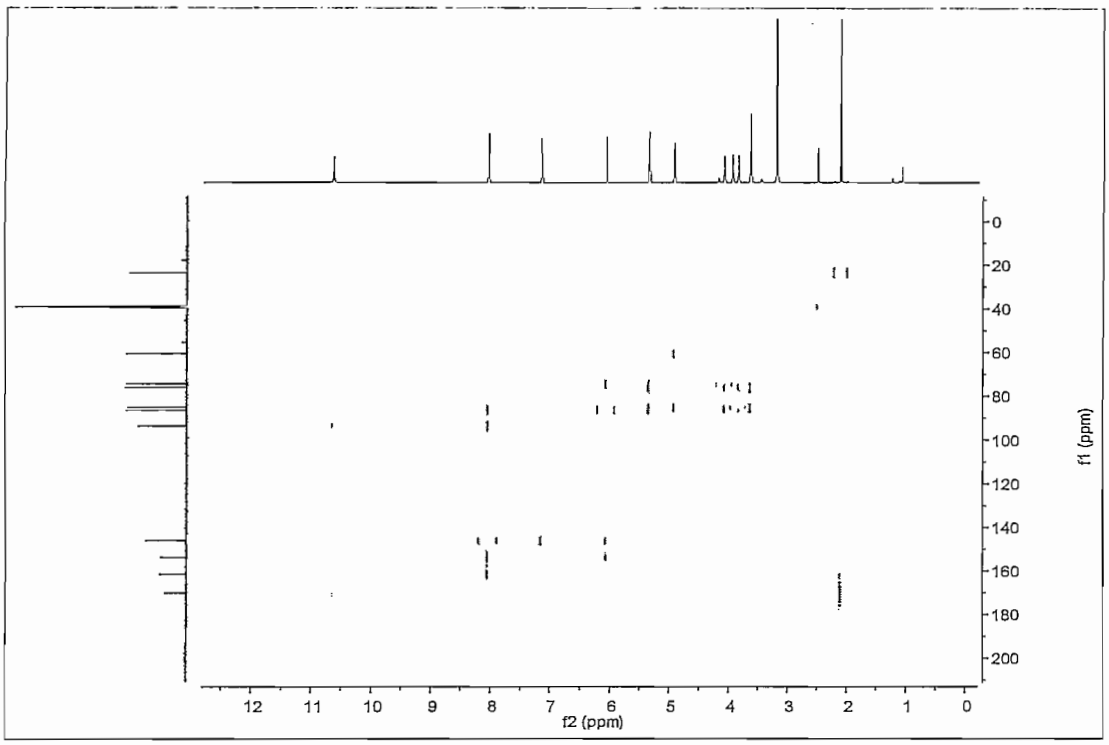


Figure A.72: HMBC NMR spectrum of N4-acetylcytarabine

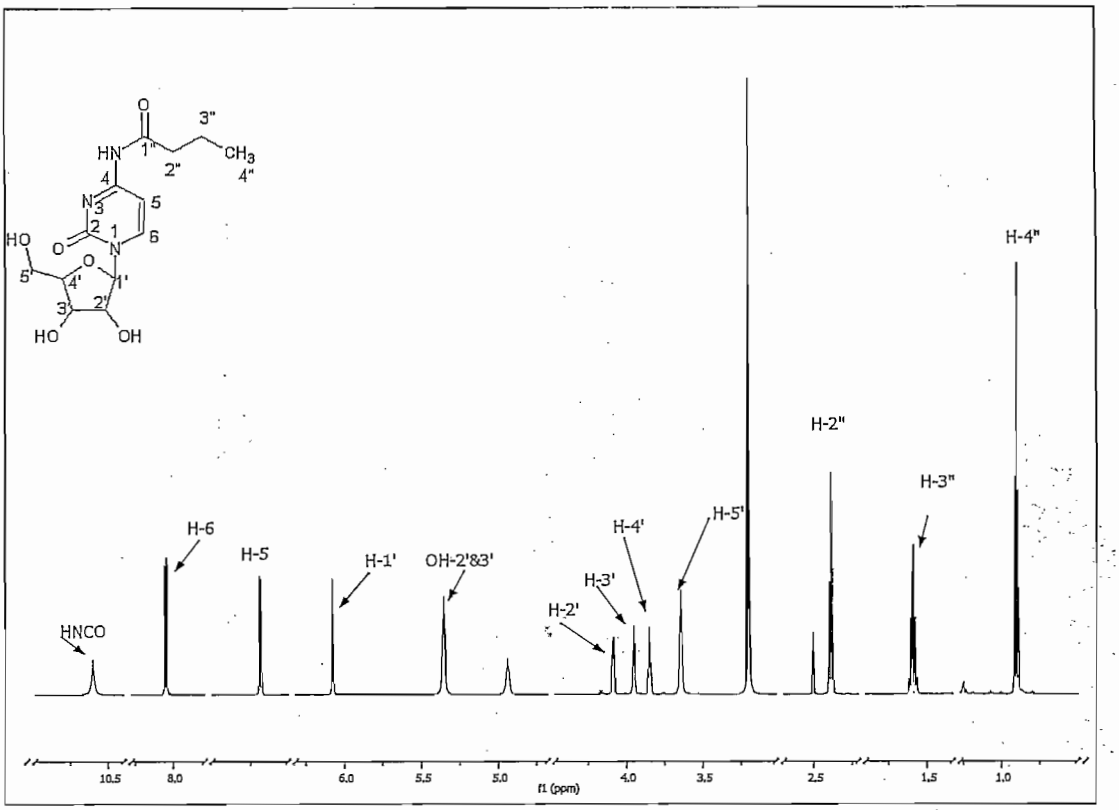


Figure A.73: ^{1}H NMR spectrum of N4-butanoylcytarabine

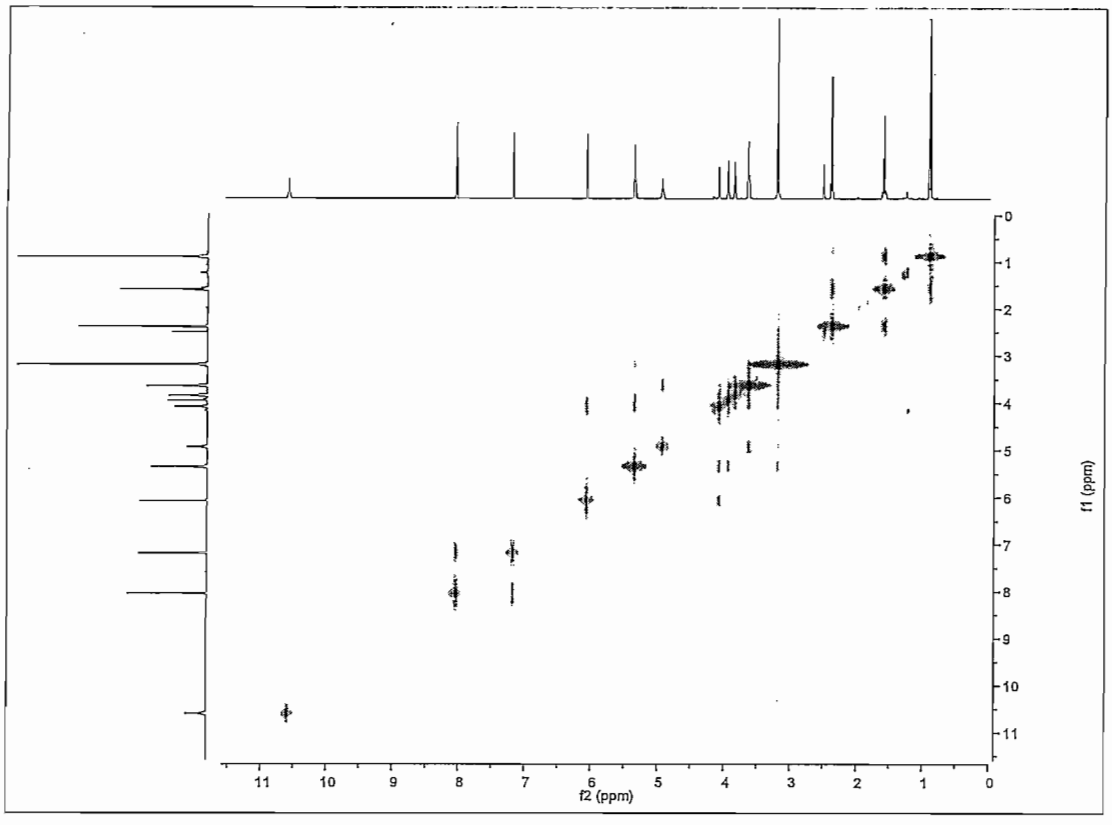


Figure A.74: COSY NMR spectrum of N4-butanoylcytarabine

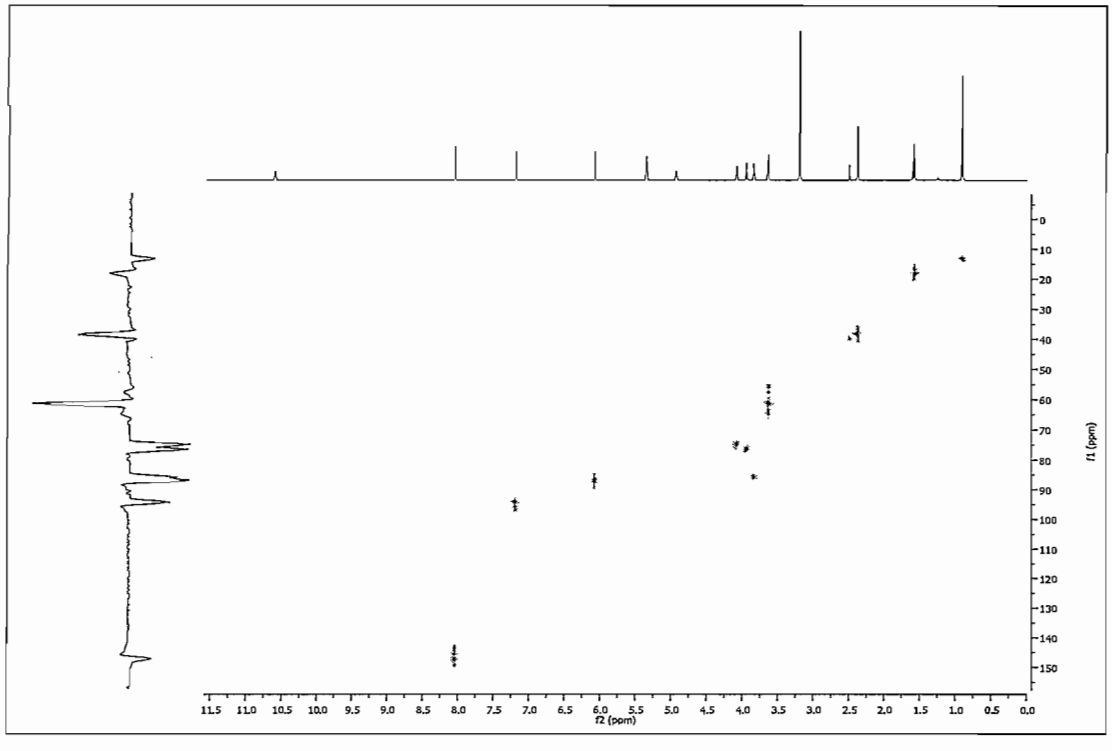


Figure A.75: HSQC NMR spectrum of N4-butanoylcytarabine

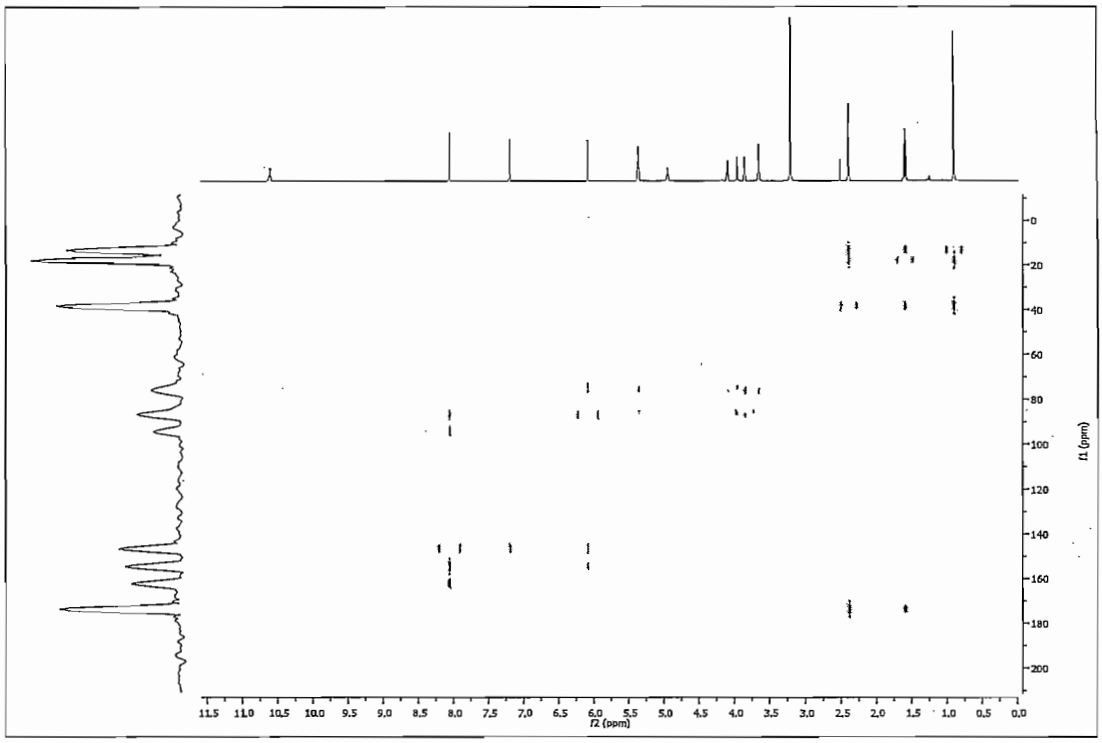


Figure A.76: HMBC NMR spectrum of N4-butanoylcytarabine

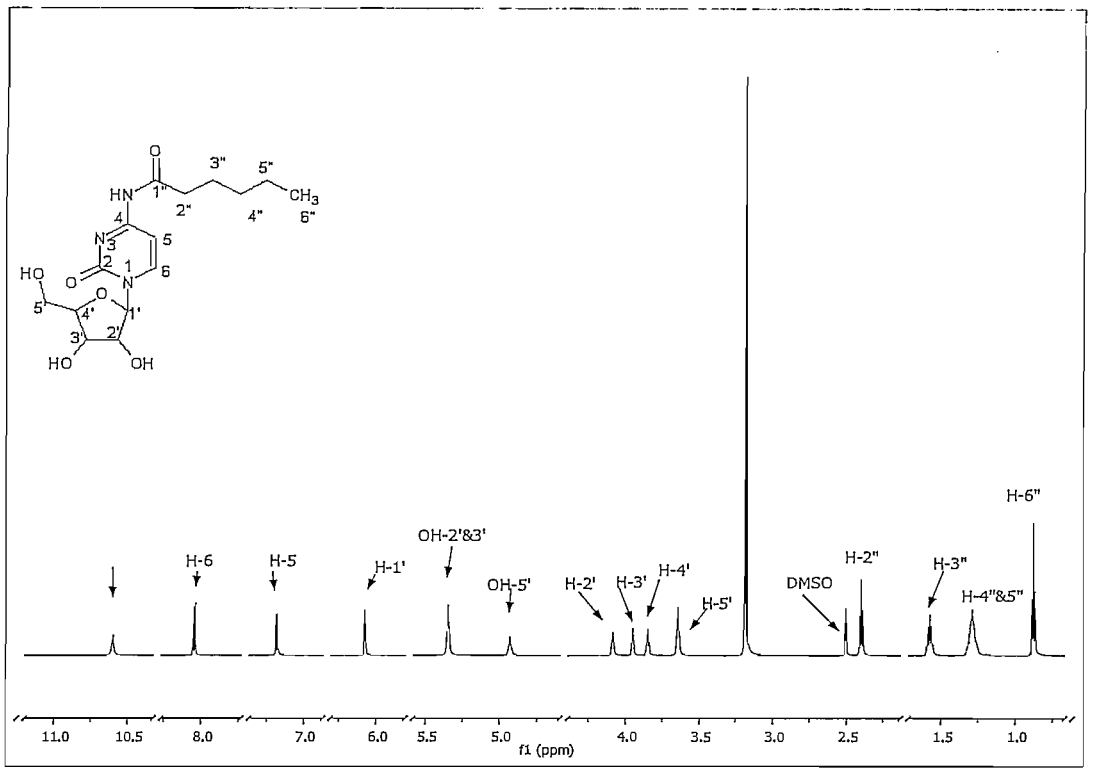


Figure A.77: ^{1}H NMR spectrum of N4-hexanoylcytarabine

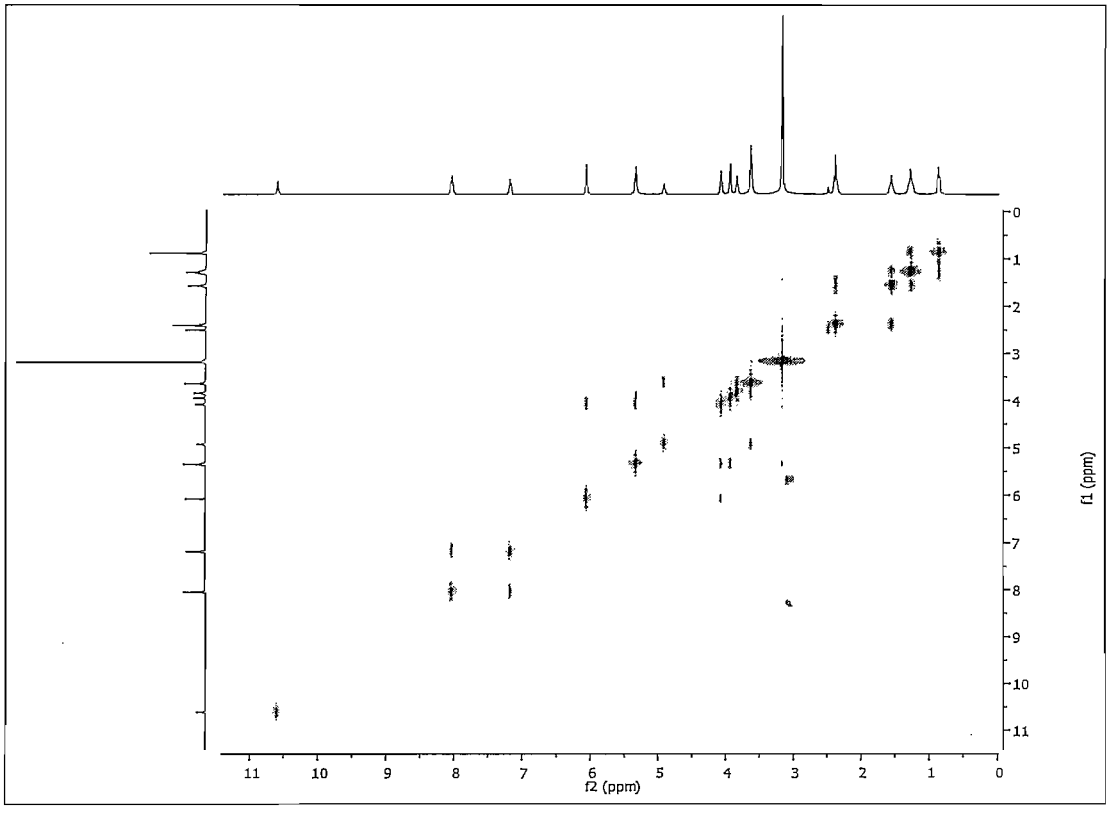


Figure A.78: COSY NMR spectrum of N4-hexanoylcytarabine

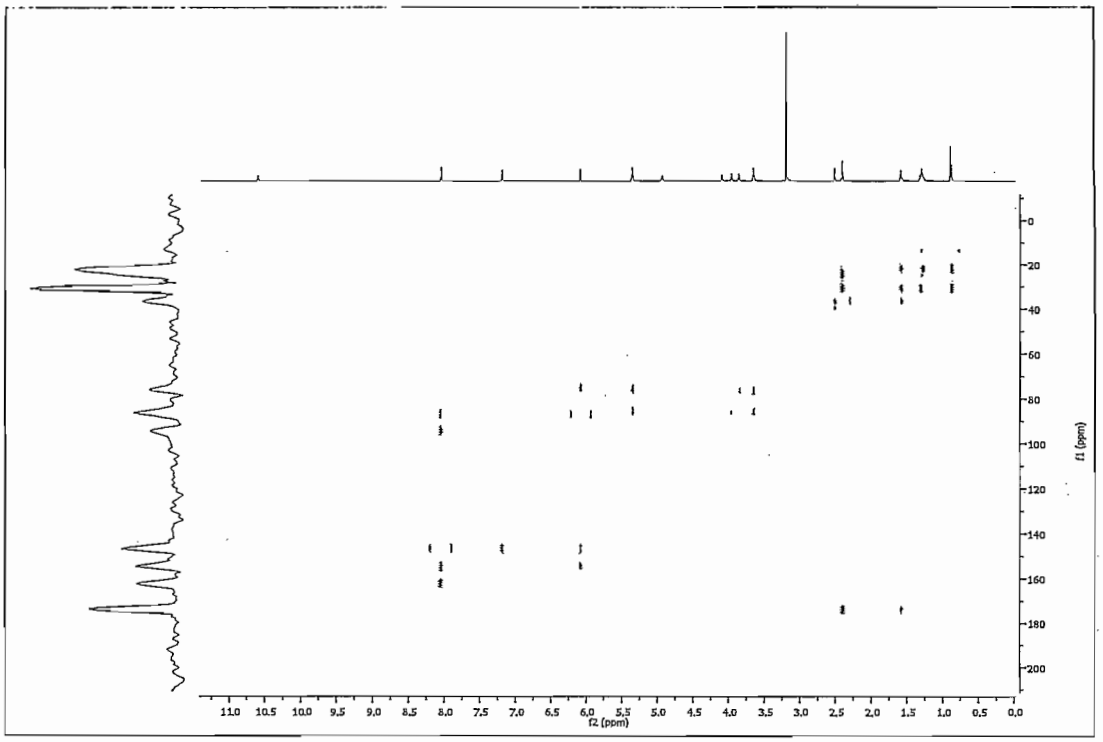


Figure A.79: HMBC NMR spectrum of N4-hexanoylcytarabine

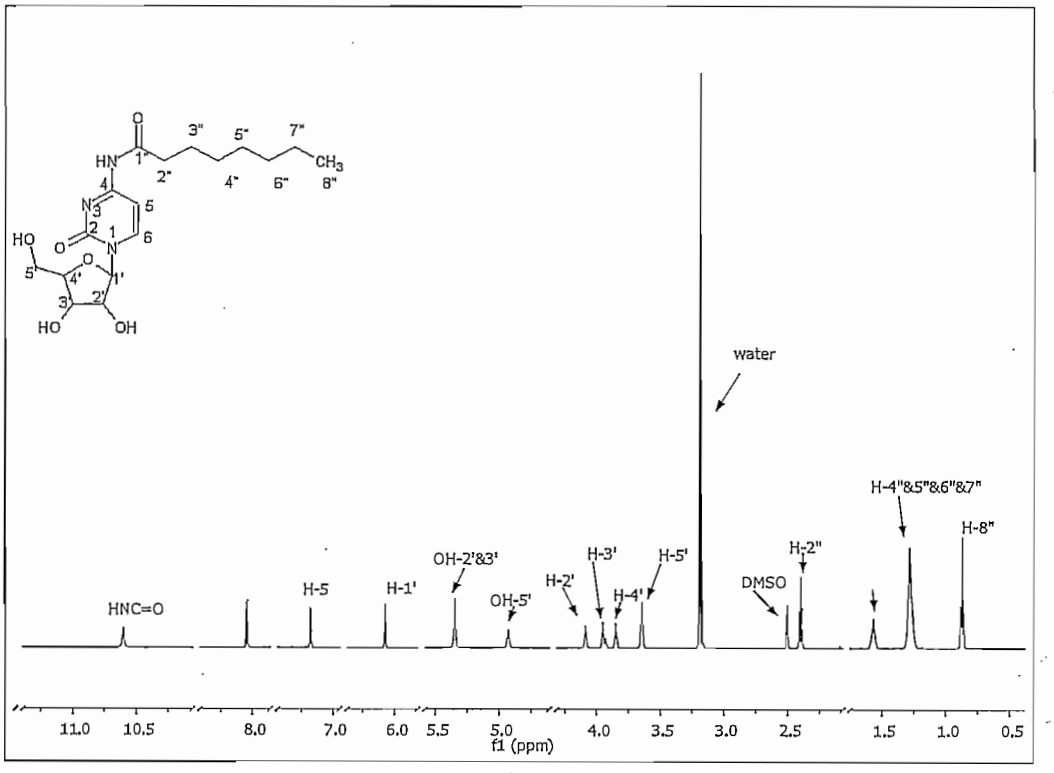


Figure A.80: ^{1}H NMR spectrum of N4-octanoylcytarabine

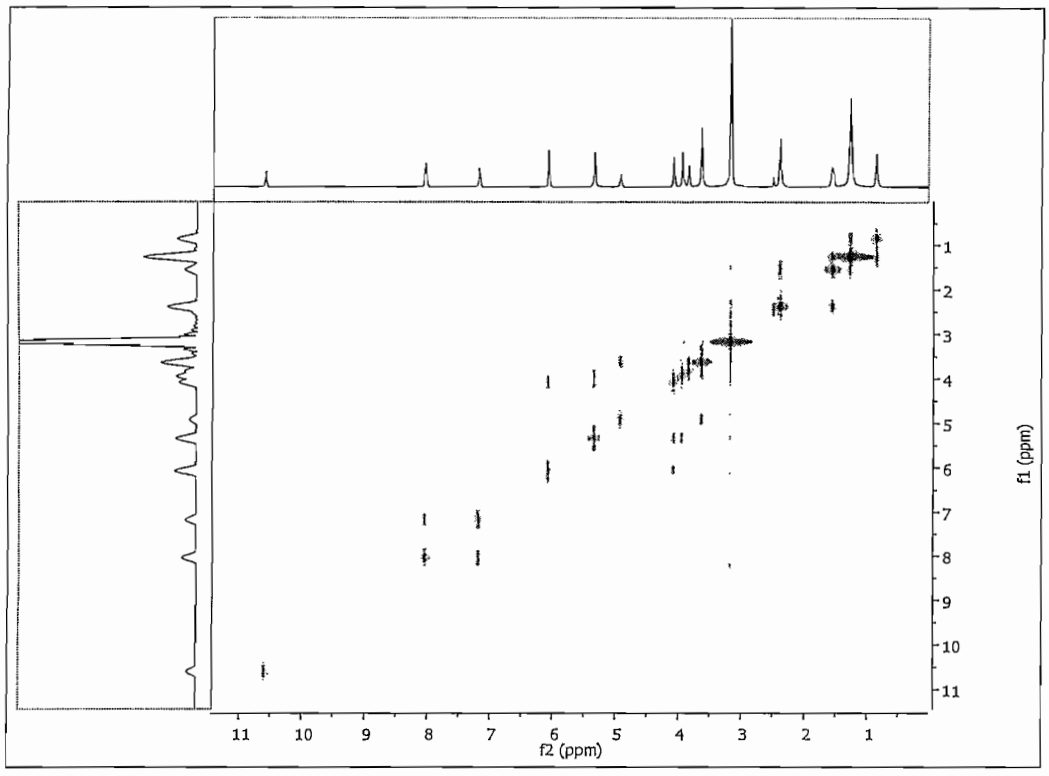


Figure A.81: COSY NMR spectrum of N4-octanoylcytarabine

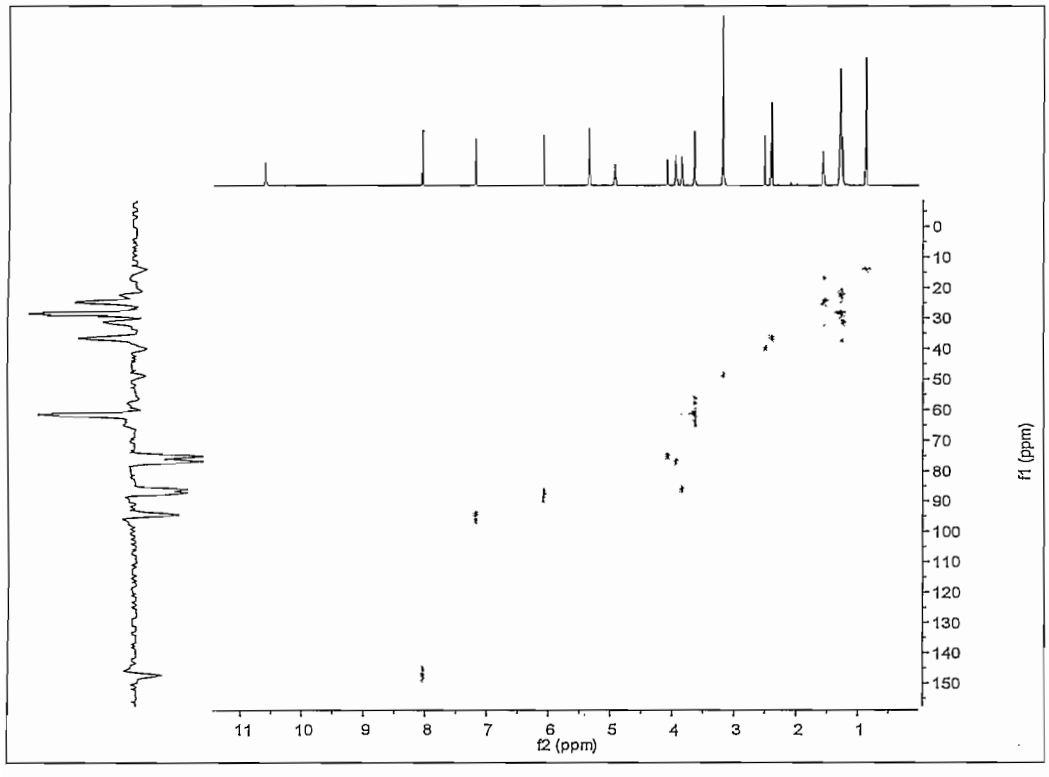


Figure A.82: HSQC NMR spectrum of N4-octanoylcytarabine

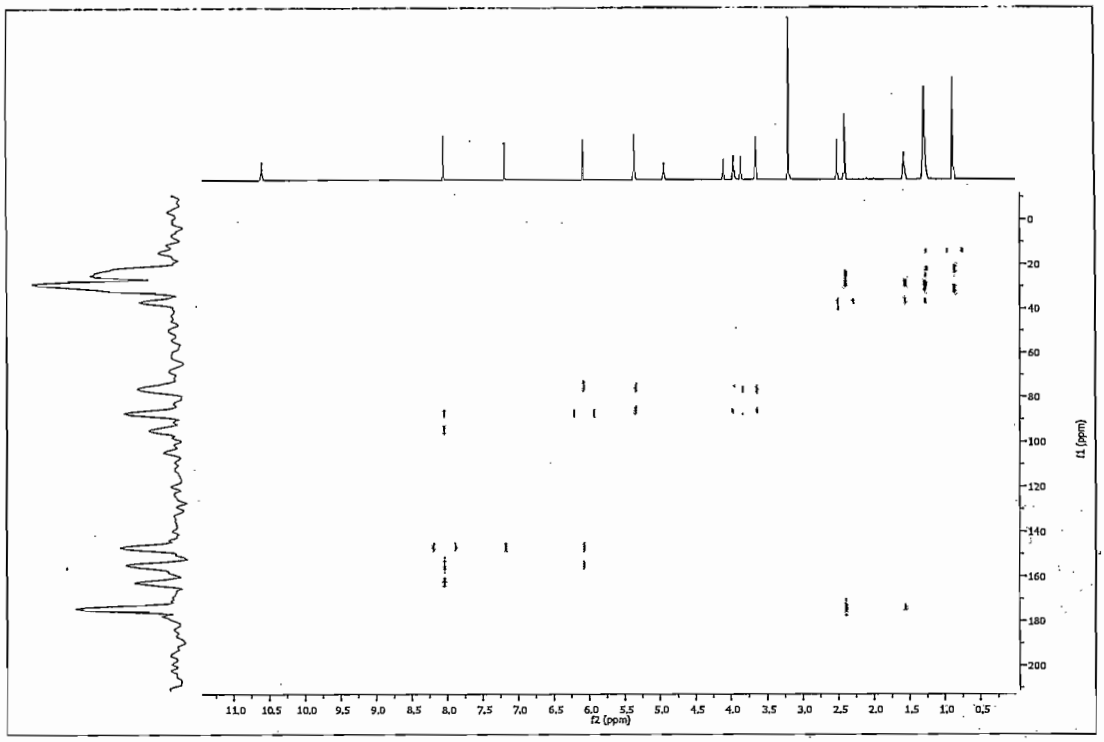


Figure A.83: HMBC NMR spectrum of N4-octanoylcytarabine

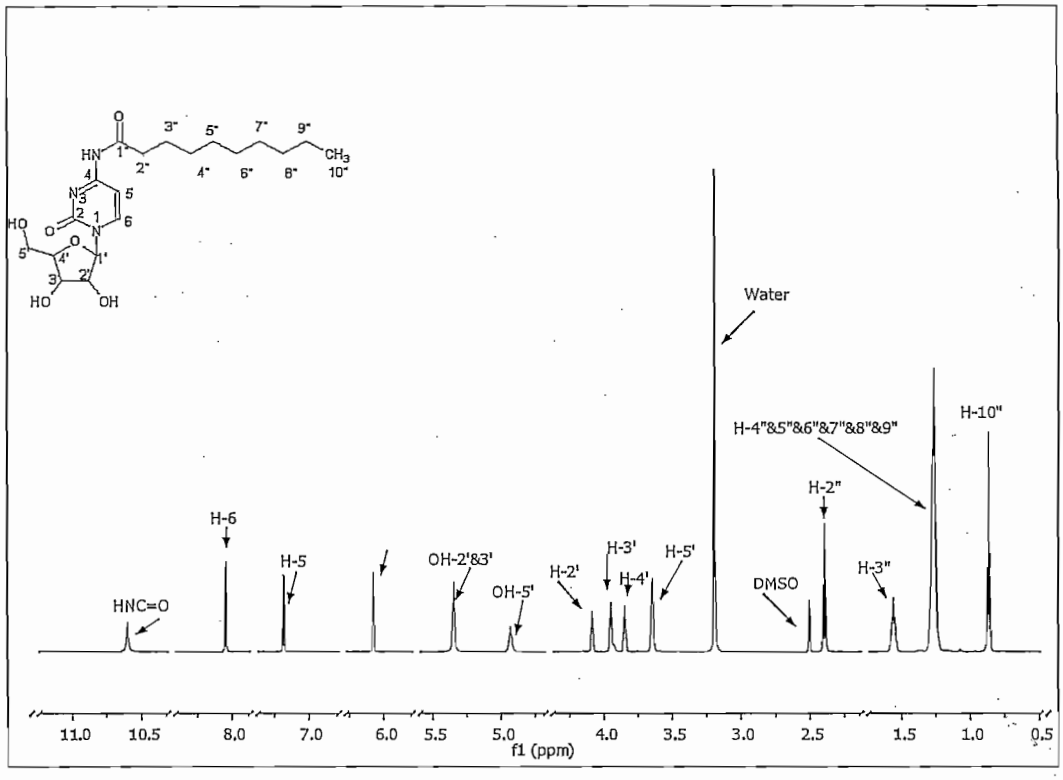


Figure A.84: ^{1}H NMR spectrum of N4-decanoylcytarabine

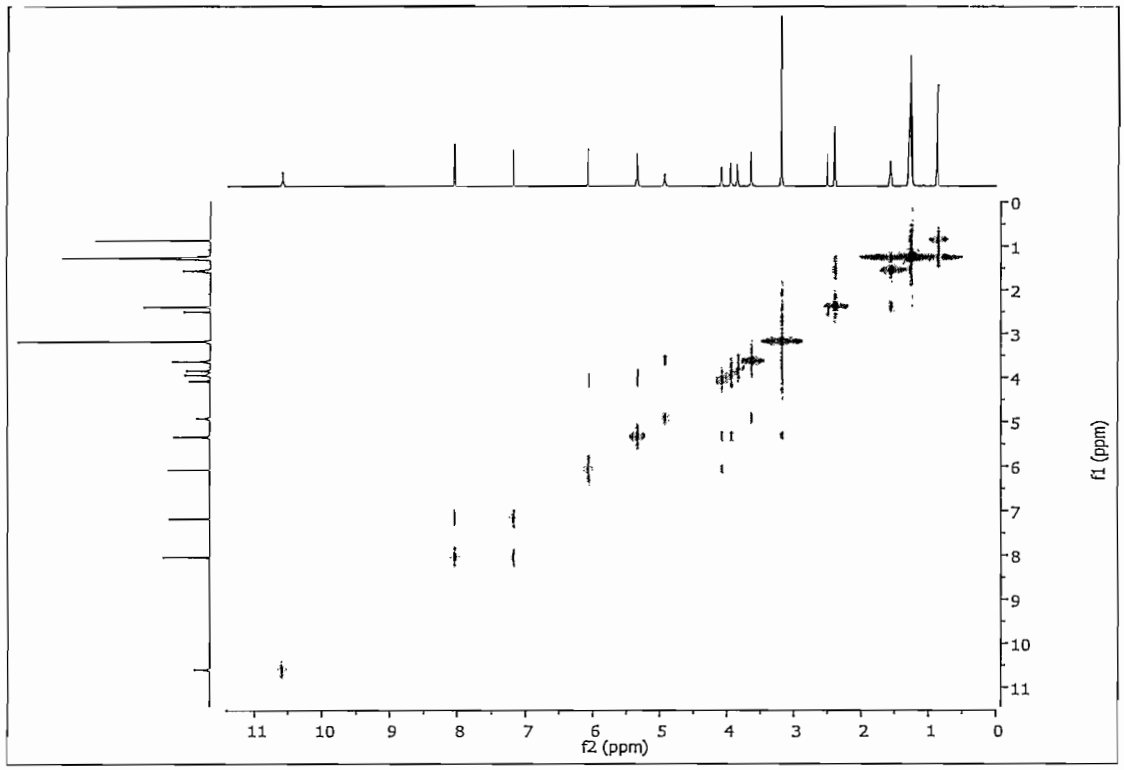


Figure A.85: COSY NMR spectrum of N4-decanoylcytarabine

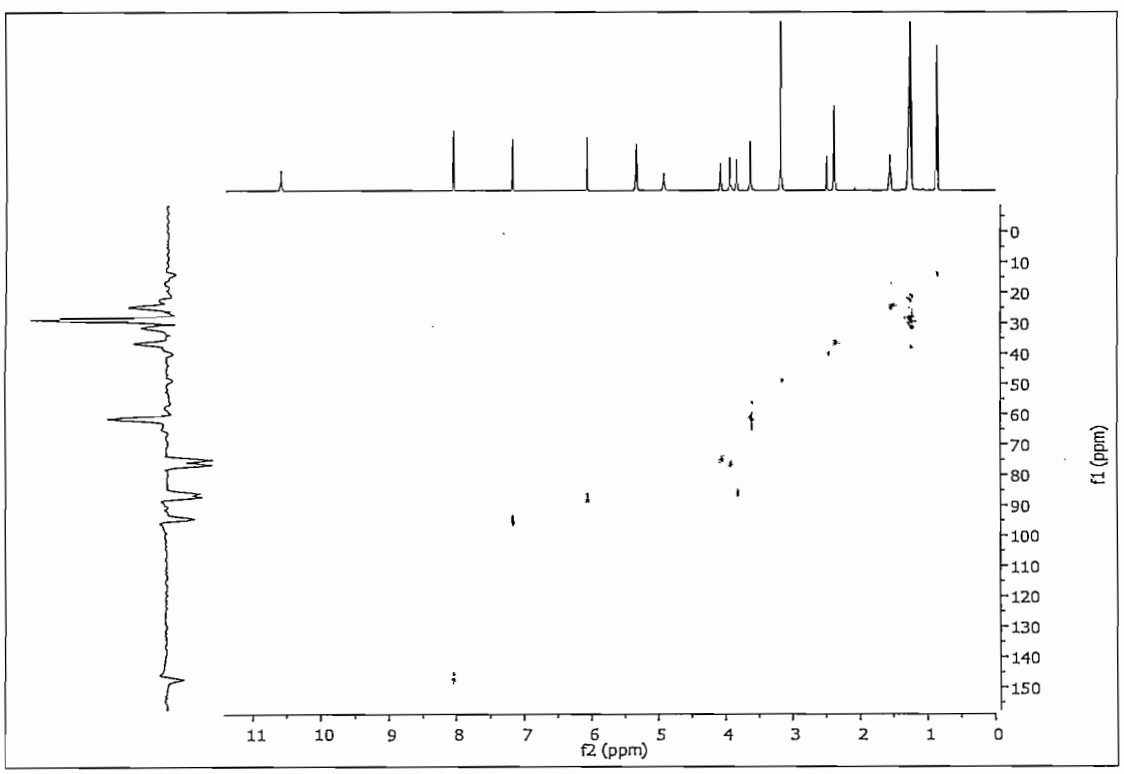


Figure A.86: HSQC NMR spectrum of N4-decanoylcytarabine

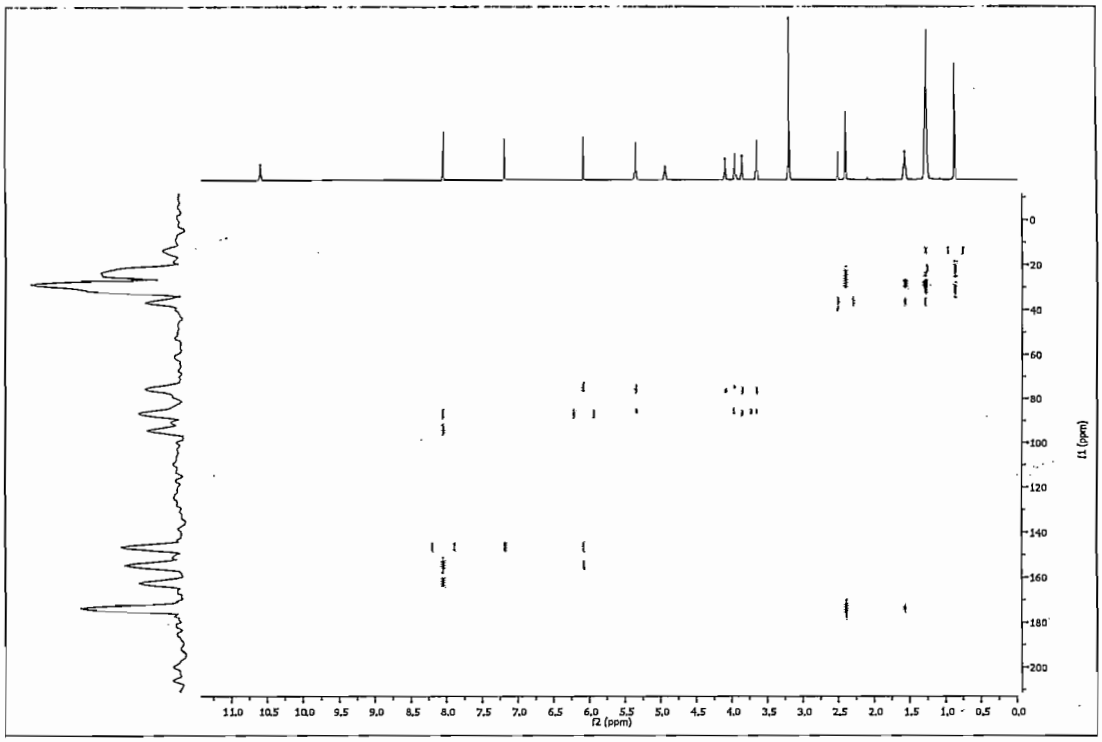


Figure A.87: HMBC NMR spectrum of N4-decanoylcytarabine