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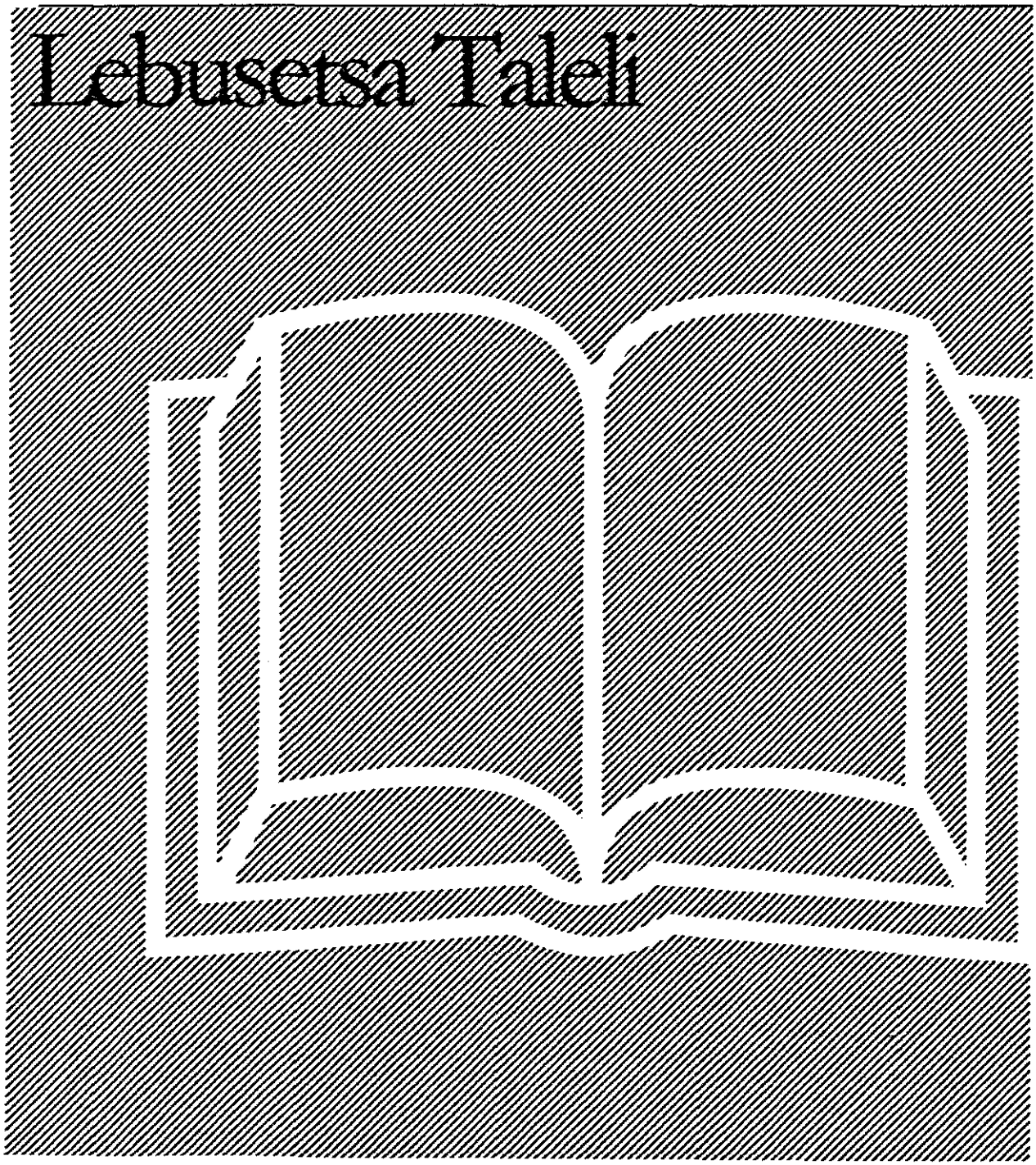
MASTERS THESIS

RADIOSYNTHESIS OF VARIOUS PYRIMIDINE DERIVATIVES AND DETERMINING THEIR UPTAKE INTO CELLS

Lebusetsa Taleli



Cape Peninsula
University of Technology

RADIOSYNTHESIS OF VARIOUS PYRIMIDINE DERIVATIVES AND DETERMINING THEIR UPTAKE INTO CELLS

by

LEBUSETSA TALELI

Thesis submitted in fulfilment of the requirements for the degree Master of Technology in CHEMISTRY

in the Faculty of APPLIED SCIENCES at the Cape Peninsula University of Technology

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The undersigned external supervisor, appointed by CAPE PENINSULA UNIVERSITY OF TECHNOLOGY, have examined the thesis entitled,

RADIOSYNTHESIS OF VARIOUS PYRIMIDINE DERIVATIVES AND DETERMINING THEIR UPTAKE INTO CELLS

presented by LEBUSETSA TALELI

a candidate of for the degree of Master of Technology in Chemistry

and hereby confirm that in his opinion is worthy of acceptance.

Dr D.D. Rossouw

DECLARATION

I, Lebusetsa Taleli, declare that the contents of this thesis represent my own work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed _____ **Date** 17/12/2009

This work is submitted for the re-examination on 12/07/2010.

Abstract

N^3-substituted pyrimidine nucleoside derivatives containing either an iodovinyl moiety or an allyl group, i.e. [^{123}I]-N^3-(3-iodoprop-2-en-1-yl)thymidine, [^{123}I]-***7a*** and [^{123}I]-N^3-(prop-2-en-1-yl)-5-iodo-2′-deoxyuridine, [^{123}I]-***10***, were synthesised and preliminarily evaluated by determining their uptake into CHO-K1 cells. Compound [^{123}I]-***7a*** was designed to be a delivery vector of ^{123}I into the DNA of the cells, while [^{123}I]-***10*** was designed to serve as a control. Compound [^{123}I]-***7a*** was also intended to have a higher metabolic radiochemical stability than 5-iodo-2′-deoxyuridine ([^{123}I]-IUdR) for the therapeutic use in cancer. The synthesis of the N^3-substituted intermediate precursors ***4a***, ***5*** and ***9*** was achieved by N-alkylation of suitably protected thymidine and 5-iodo-2′-deoxyuridine analogues, respectively. The immediate precursors for radiolabelling, ***4a*** and ***11***, were obtained by incorporating a trialkylstannyl group at the respective labelling positions prior to radioidination. [^{123}I]-***7a*** was recovered from [^{123}I]-***6a*** after acid-hydrolysis of the protecting groups and ***10*** was obtained from direct oxidative iodination of ***11***. The radiochemical yields ranged from 73% to 91% at the end of the synthesis and radiochemical purities were in excess of 99% after HPLC purification.

The cell-uptakes of the radiotracers were carried out and assessed by a direct comparison with the gold standard [^{123}I]-IUdR, which is known to be phosphorylated and incorporated into the DNA of cells during the cells S-phase. The cell-uptake results of [^{123}I]-***7a*** and [^{123}I]-***10*** were roughly 4% and 3% relative to [^{123}I]-IUdR, respectively. The poor cell-uptake of [^{123}I]-***10*** suggested that the uptake into the cells is not influenced by the position of the iodine atom in the molecule, but most probably by the availability of the N^3-position in its non-substituted form. As a result of its poor incorporation into cells, it was concluded that the synthesised radiotracer [^{123}I]-***7a*** would be a poor candidate for use in the eradication of malignant cells.

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I am grateful to all iThemba LABS radionuclide production members for being friendly, and for whom we shared joy and cheerlessness. To my family: *Ke leboha batsoali ka tlotlofatso. Phahamisang lihloho tsa lona menyako ea ke mehla.*

You aint a painter and got not brush to paint a poetry of love, neither can a pilot fly one through the heavens above. Losi, the courage I ever receive, stimulates me daily. A solemn thankfulness and appreciation is passed to Tiro James dan Modisane for his cordial reception and hospitality during my entire stay in Cape Town. Your ever growing love and support has been very valuable. I am really indebted champ *God will let me know what best it shall be.*

I listened to James Joyce (1882-1941) telling me a man's errors are his portals of discovery.

Longing fulfilled is like a tree of life and keen insight wins favour, but instruction from the wise is like a life-giving fountain.

Dedicated to my family

PREFACE

This thesis is divided into five chapters. Chapter 1 motivates and explains why a research topic of this thesis is important. This chapter elaborates on radiopharmaceutical drug development and explicitly mention the research questions this work investigates. Chapter 2 is review of the literature, comprising the biochemistry background, radiolabelling methods and therapeutic radionuclides relevant to this study. This section gives a review of previous research work in the subject area related to this research project. In Chapter 3, specific results and crucial experimental findings have been interpreted and discussed. Its entire discussion section stresses important experimental findings in line with the research objectives. The biological study performed in this work was intended to assess the potential of the synthesised radiotracers to be internalised into cells. In order to avoid diminishing the chemical aspect of this thesis, discussion of the biological assay is catered to only marry the outcome of this study with what is researched in this field, and not primarily to formulate reasons for the outcome. Chapter 4 gives detailed experimental methods used in this work, yields and purities of compounds, as well as spectroscopic and spectrometric data. The net results, findings and implications of this work are mentioned in Chapter 5. This chapter also acknowledges the limitation of the scope of the research project. Finally, the appendices are primarily composed of raw data and pictorial illustrations of the spectral data acquired during experimental work.

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CHAPTER 1
Introduction and research objectives

1.1 General introduction

Radiolabelled pharmaceutical compounds, commonly known as radiopharmaceuticals, are widely used in nuclear medicine for several applications. These applications are mainly diagnostic and therapeutic in nature. In order for a radiopharmaceutical to be used in either application, it should have an affinity for a specific biological target. This affinity largely depends on its chemical structure. Radiopharmaceuticals are designed according to the same principles that are applied to the development of their non-radioactive counterparts, one of which is a relationship between structure and biological activity. Another factor that is taken into consideration when designing new radiopharmaceuticals is the choice of a radionuclide which is used to label them. A wide range of properties of the radionuclide, such as mode of decay and the energy associated with it, are crucial when deciding on the type of application for the radiopharmaceutical.

Radioiodines are commonly used in the synthesis of radiopharmaceuticals due to the relative ease with which iodine can react with many organic compounds. Of the radioiodines, ^{123}I, ^{125}I and ^{131}I have been the most exploited. The former is produced at iThemba LABS, South Africa and is mostly used in diagnostic applications due to its suitably emitted gamma ray energy. As will be discussed later, ^{123}I can also be used as an Auger electron emitter in the radiotherapy of cancer to destroy cancer cells when deposited into the nucleus of the cells.

Radionuclide therapy is a form of radiotherapy based on the use of radionuclides which are known to possess cytotoxic ionising radiation for the eradication of malignant cells. Target-directed drug development, therefore, is fundamentally not a new invention; several cancer therapy modalities, such as external radiotherapy and/or tumour-targeted internal therapy have been standard tumour therapies for a long time (Thurston, 2007). Auger electron emitters are low energy electron emitters resulting in the release of highly localised energy in an extremely small volume around a decay site, causing the cellular molecules in the immediate vicinity of the decaying atoms to be irradiated by these atoms. If this decay occurs in a cell nucleus, DNA strands will break and cell death may follow (Kassis, 2003).

Radionuclides that decay by electron capture (EC) and/or internal conversion (IC) demonstrate an Auger effect. In order to deposit the radionuclidic source of Auger electrons (*e.g.* radioiodine) into its biological target, the desired radionuclide must be chemically bound to a bioactive compound that can act as a DNA targeting vector. This process is called radiolabelling and involves the formation of a stable covalent bond between a carbon atom on the pharmaceutical, or bioactive compound, and the radionuclide. The choice of bioactive compound is critical. It should have a high target binding and rapid excretion of metabolised components without redistribution to non-target tissues (Eary and Brenner, 2007). The match between the target tissue residence time and the biodistribution of the pharmaceutical with the physical half-life of the radionuclide results in high therapeutic efficiency (Eary and Brenner, 2007).

The use of pyrimidine nucleoside analogues for studying metabolic pathways of pyrimidine nucleoside incorporation into DNA in order to measure cell proliferation dates back more than 30 years (Tjuvajev *et al.*, 1994). Pyrimidines are six-membered heterocyclic aromatic rings (nitrogenous bases) containing two nitrogen atoms separated by a carbon atom in the ring. All nucleosides are the condensation products of a nitrogenous base and a pentose sugar ring. Nucleotides are biological molecules that possess phosphate esters of nucleosides. Generally, both nucleotides and nucleosides are the components of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). The phosphate groups link different nucleosides together in the formation of the DNA molecule. The sugar molecules form the backbone of the DNA molecule and the nitrogenous bases contain the actual code of genetic information (Leninger, 2007). The common pyrimidine nucleosides are derived from their corresponding bases. For example, Figure 1.1-1 illustrates a thymine base nucleotide structure, also known as thymidine.

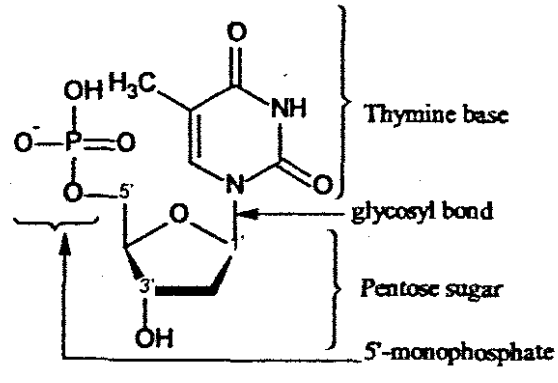

Figure 1.1-1 Chemical structure of a thymidine nucleotide unit

There is compelling evidence from loco-regional administration studies that radiolabelled 5-iodo-2'-deoxyuridine (IUdR), a thymidine analogue in which the 5-methyl group of thymidine (dT) is replaced by an iodine atom, is effective for the treatment of tumours characterised by high proliferation kinetics, such as the liver tumours resulting from metastases of colorectal cancer and high grade malignant gliomas (Kassis *et al.*, 1998; Mariani *et al.*, 1996). [^{123}I]-IUdR, in particular, has been useful in demonstrating the cytotoxicity of Auger electron emitters in the nucleus of cells. Like many cell-cycle dependent radiopharmaceuticals, IUdR is incorporated into a cell during the S-phase to synthesise DNA in preparation for cell duplication (Yang *et al.*, 2008). Once it has been incorporated into the DNA of a cell, however, IUdR is retained for the lifetime of the cell. The sum of the energy deposited by Auger electrons results in DNA double strand breaks with an efficacy of approximately one break per disintegration (Painter *et al.*, 1974). Before it is safely incorporated into the DNA of a cell IUdR is, however, susceptible to catabolism or de-halogenation by enzymes (Spencer *et al.*, 1987). IUdR that is unincorporated into cells is quite unstable *in vivo*. Obtaining high uptake of IUdR by tumour cells and high tumour to non-tumour (T/NT) ratios, after intravenous administration, remains a challenge (Semnani *et al.*, 2005). The short half-life of IUdR *in vivo*, its rapid de-halogenation in the liver, and its cellular uptake in the S-phase of the cell-cycle are limiting factors in the use of this compound (Hampton and Eidinoff, 1961; Prusoff, 1963).

There is a desire for radiotracers which are phosphorylated and taken into nuclear DNA of cells with high rates in combination with high *in vivo* stability. Iodovinyl moieties have been used in the development of new radiopharmaceuticals (Hadley and Wilbur, 1989). The vinylic carbon-iodide bond (268 – 297 kJ mol^{-1}) is chemically and biologically stronger than the aliphatic carbon-iodide (222 kJ mol^{-1}) bond. The biological and chemical stabilities of vinyl iodides are comparable to those of aryl iodides (Coenen *et al.*, 1983). An iodovinyl group is relatively small and impart less steric bulkiness and lipophilicity to a molecule to which it is attached. Such groups are favourable for the radioiodination of small molecules, since minimal perturbation of structural physicochemical parameters increases the likeliness of preserving the favourable properties of the parent compound (Musachio and Lever, 1992). A reasonable alternative for the incorporation of an iodine atom into an organic molecule in a stable manner, therefore, is the introduction of the iodovinyl group.

The introduction of an iodovinyl group into a 2'-deoxyuridine molecule has been documented in several published literature articles. In all these cases the iodovinyl group has been inserted

at the 5-position of the base ring that normally houses a methyl group (in the case of thymidine) or an iodine atom (in the case of IUdR). For example, deoxyuridine analogues containing a halovinyl unit in the 5-position of the base ring, 5-(2-radiohalovinyl)-2'-deoxyuridines, have been reported as tracers for monitoring cancer gene therapy with PET (Yu *et al.*, 2003). (*E*)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU), a highly effective and selective inhibitor of HSV-1 in both cell-culture and mice, has also been reported (Balzarini *et al.*, 1990). The synthesis of a few 5-iodovinyl-uracil nucleoside substrates were also reported (Dougan *et al.*, 1994; Morin *et al.*, 1997). From a chemistry point-of-view, the introduction of an iodovinyl group into the 5-position requires fairly complicated synthetic procedures, resulting in low yields. In addition, the presence of a 5-halovinyl substituent may result in deglycosylated products. The driving force behind this process could be the formation of imide tautomers, one of which is presumably favoured by the halovinyl substituent due to resonance stabilisation (Yu *et al.*, 2003).

1.2 Research objectives

Other than the 5-position, an alternative position to introduce an iodovinyl moiety into a deoxyuridine or thymidine molecule would be at the N^3 atom, which is prone to alkylation by 'soft' electrophiles such as alkyl halides. The presence of a nitrogen lone pair makes the process of creating a new carbon-nitrogen bond very facile with most electrophiles. The influence of N-alkylation on the biological properties of deoxyuridines is, however, not entirely clear. Several N^3-substituted deoxyuridine compounds have been synthesised and biologically tested, with conflicting results (see literature review). Amongst these were non-radiolabelled N^3-fluoroethyl thymidine (N^3-FET) and N^3-fluoropropyl thymidine (N^3-FPrT). The synthesis of similar N^3-substituted radioiodine-labelled deoxyuridine or thymidine analogues has apparently not been reported, neither that of N^3-substituted iodovinyl analogues such as N^3-(3-iodopropen-2-yl)-2'-deoxyuridine or N^3-(3-iodopropen-2-yl)-thymidine, despite the relative ease with which such compounds could be synthesised. With this in mind, the following objectives for this study were, therefore, formulated:

a) To synthesise N^3-substituted thymidine and deoxyuridine analogue precursors suitable for radiolabelling.
b) To radiolabel the above mentioned precursors with the radionuclide ^{123}I.
c) To determine the uptake of these radioiodinated analogues into cells (Chinese hamster ovarian epithelial cells).

d) To compare the respective cell-uptakes to that of the conventional gold standard [^{123}I]-5-iodo-2'-deoxyuridine.

The main theme of this work involves organic synthetic chemistry, radiolabelling chemistry and the *in vitro* cell-uptake testing of the radiolabelled pyrimidine nucleoside analogues. The *in vitro* cell-uptake studies should further assist in verifying the application of thymidine-based N^3-substituted compounds in cancer therapy. If the N^3-substitution has no detrimental influence on the cell-uptake of radioiodinated pyrimidine nucleoside analogues, it would follow that an iodovinyl moiety could be attached to the more accessible N^3-position, rather than the 5-position. It would then also pave the way to establish, by means of following *in vivo* animal studies, whether radioiodinated thymidine analogue containing an N^3-substituted iodovinyl moiety exhibits increased metabolic radiochemical stability, as opposed to [^{123}I]-5-iodo-2'-deoxyuridine.

CHAPTER 2

Literature review: biochemistry background, therapeutic radionuclides and radiolabelling methods

The biochemistry background provided in this chapter reveals the biosynthetic role of nucleosides in cells. This chapter also summarises previous studies on nucleosides used in radiotherapy. The applications of therapeutic radionuclides possessing ionising radiation will also be reviewed and radiolabelling methods of pharmaceuticals with radionuclides, in particular ^{123}I, will also be discussed.

2.1 Biosynthesis and cell-uptake of nucleosides

The two main constituents of a cell are the cytoplasm, which supports all metabolic functions within the cell, and the nucleus, which contains the genetic information. Cells propagate through cell division to produce new daughter cells, which propagate cell division processes up to the next cell-cycle process.

In the cytoplasm of most eukaryotic cells, pyrimidine nucleosides are normally synthesised as the starting materials for DNA, which is critically important for the use of these nucleosides as delivery vectors of therapeutic radionuclides in cancer therapy. The two main pathways leading to the formation of these nucleotides are the *de novo* and salvage pathways (Leninger, 2007). These synthetic processes are important in cell division and in enabling the uptake of nucleosides into mammalian and human cells. The cellular pools of nucleotides are probably inadequate to synthesise the DNA of the cells, therefore, the cells must continue to synthesise nucleosides. In some cases nucleoside synthesis may limit the rate of DNA replication (Leninger, 2007).

Free nucleosides are not intermediates in the salvage pathways as in the *de novo* pathways. The salvage pathways require the presence of specific kinases to promote conversion of the nucleoside to a monophosphate by a process known as phosphorylation. Phosphorylation is performed by a class of enzymes called nucleoside monophosphate kinases. Thymidine

kinase, TK, and other kinases are located in the cytoplasm of most cells and these enzymes are specific to a particular base (Stryer *et al.*, 2002).

In virus-infected cells, the viral thymidine kinases play the role of thymidine kinases because the life-cycle of a virus or a viral-infected cell has a combination of its own enzymes and those of a cell it hosts (Blackburn *et al.*, 2006). Other viral cell kinases are known to phosphorylate thymidine derivatives that are metabolically trapped in cells and the process leads to intracellular accumulation (Byun *et al.*, 2004).

The precise mechanism(s) by which nucleosides are internalised into the cells, regardless of structural modifications, may not be fully understood, however, the nucleosides are phosphate acceptors in phosphorylation reactions and must be located inside the cells where the phosphoryl group donors are abundant (Stryer *et al.*, 2002).

2.2 Model cell-cycle in the formation of cancerous cells

A series of events in the cell-cycle is of great importance in availing nucleosides as substantial participants in proliferating tissues. It is necessary to have a thorough understanding of the cellular processes involved in the development and treatment of cancer. The events of DNA replication and cell-cycle are limited and controlled; protein kinases and protein phosphorylation are a cellular checkpoint that determine and modulates the entry and progression of cell division. Healthy tissues in most parts of the body tend not to grow, but to maintain a steady number of cells (Leninger, 2007).

Protein kinases and protein phosphorylation help to regulate cellular proliferation and determine all the fundamental stages of mammalian cell-cycle. The phases are as follows: mitotic (M) nuclear division of cells; a dormant gap (G_0 and G_1) phase where cell division stops but other metabolic processes are taking place; the synthesis (S) phase where DNA is synthesised and replicated; and, lastly, another DNA dormancy gap (G_2) phase before another cell-cycle resumes. The cell proliferation time, therefore, can be well defined by the two time periods: the M division and the S-phase. The cell-cycle of malignant cells is shorter than that of normal tissue cells, due to a series of multiple cell divisions (Hall and Giaccia, 2005). It is within the S-phase of cell division where nucleotides demands are high for the synthesis of DNA. It is hypothesised that uptake of nucleosides is relatively high during the S-phase of the

proliferating cells. These cells are most radiosensitive in the dividing M phase and G_2 phase and most resistant in the late S-phase (Hall and Giaccia, 2005).

When the controlling mechanism of cell growth is defective, the formation of abnormal tissue mass known as neoplasm or tumour may occur, leading to a cell proliferation (Thurston, 2007). The formation of these tumour cells can be subdivided into two main groups, namely, benign or malignant (Macdonald *et al.*, 2004). Benign tumours are rarely life-threatening, they grow within a well defined capsule which limits their size and maintains the characteristics of the cell of origin and are, thus, well differentiated. Malignant tumours are caused by the spreading of a primary tumour to one or more other sites of the body (by a process called metastasis), which makes intervention of several therapeutic methods impossible (Thurston, 2007). The metastasis process is often life-threatening, since spreading of primary tumours obstructs vessels or organs in different parts of the body. At this stage one has developed cancer. The stages of the cell life-cycle in eukaryotic cells are illustrated by a pie chart in Figure 2.2-1.

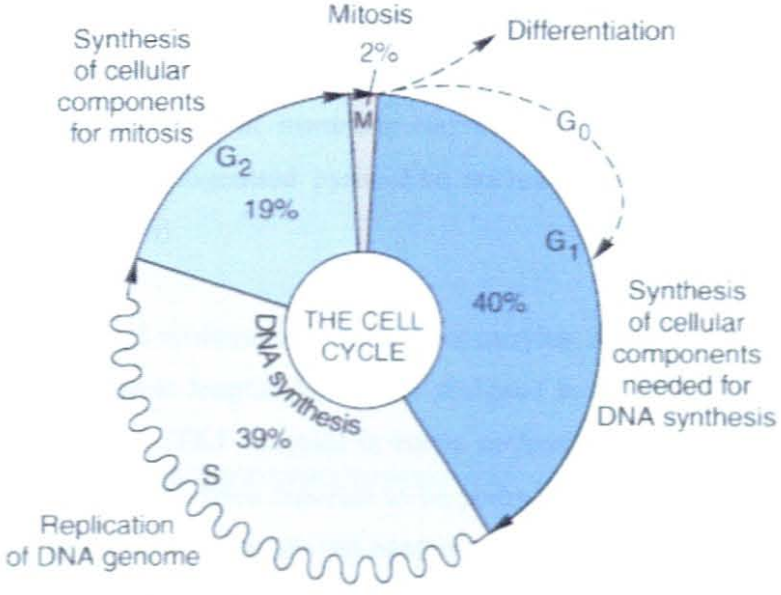

Figure 2.2-1 The stages of the cell life-cycle in eukaryotic cells (Katzung, 2007).

2.3 Previous studies on nucleosides

The molecular mechanisms of cancer therapy are defined in a number of ways depending on the central target of anti-cancer drugs, such as the direct interaction with DNA, DNA replication or interaction with signalling pathways to control cell proliferation and differentiation (Schulz, 2007). Previous investigators demonstrated that pharmaceuticals such

compounds provided the evidence for the degree of efficiency of the radiotracers for PET imaging of tumours, which warrants a further detailed investigation of the N^3-substituted thymidine analogues.

Non-radiolabelled N^3-fluoroethyl thymidine (N^3-FET) and N^3-fluoropropyl thymidine (N^3-FPrT) have been previously investigated using *in vitro* enzymatic assays. The apparent rates of accumulation in tumours of N^3-FET and N^3-FPrT have demonstrated that both are substrates for TK1 with phosphorylation rates of 47% and 26%, as compared to thymidine, respectively (Toyoharaa *et al.*, 2006). The same group of investigators did the *in vivo* studies with radiolabelled [^{18}F]-N^3-FET for cellular imaging proliferation with PET. Conversely, the animal imaging studies demonstrated that [^{18}F]-N^3-FET does not accumulate in the tumour tissue. This negated their previous *in vitro* enzyme assay results. Although many biological explanations have been provided by the authors for the conflicting results, preliminary PET imaging of the same [^{18}F]-labelled N^3-substituted analogues done by Mukhopadhyay *et al.*, 2008, were in agreement with the *in vitro* enzymatic assay data reported earlier by Toyoharaa *et al.*, 2006.

Previously, an N^3-methyl analogue of IVDU was studied for enhancing IVDU diffusion across the blood brain barrier (BBB) on the basis of increasing its lipophilicity so that it can be metabolically trapped in herpes simplex virus (HSV) infected cells (Tandon *et al.*, 1989). The biological phosphoryl transfer enzyme assay with recombinant human TK1 of N^3-position substituted thymidine derivatives exhibits higher phosphorylation rates than any other C-5 substituted thymidine analogues (Byun *et al.*, 2004; Toyoharaa *et al.*, 2006). TK1, which is a cytoplasmic enzyme, is responsible for converting dT and IUdR to the corresponding 5'-monophosphates in the S-phase of rapidly dividing cells such as cancer cells (Byun *et al.*, 2004). The rapid division process of cancer cells necessitates the presence of nucleosides in large quantities to maintain replication and growth of cells. Provided N^3-3-iodopropenyl derivatives of thymidine are substrates of TK1, these analogues could also be phosphorylated and accumulate in cancer cells under the influence of DNA polymerase, following conversion to nucleotides in cells.

Finally, it should not be ignored that the N^3-alkyl substituted thymidine analogues could undergo catabolism mediated by some yet undetermined enzymes, which could produce radiolabelled metabolites that may rapidly and selectively accumulate in tumour tissue (Mukhopadhyay *et al.*, 2008). This is possible in spite of the results reported by Toyoharaa *et*

al., 2006, demonstrating the resistance of N^3-substituted thymidine analogues to thymidine phosphorylase catabolism.

2.4 Studies on therapeutic radionuclides

In eradication of fast spreading malignancies, the use of radionuclides in cancer therapy appears to be a better choice than several other methods employed because the cytotoxic radionuclides with ionising radiation are delivered to targeted metastatic tumours (Thurston, 2007). Radionuclides exhibiting cytotoxic effects can be categorised into three main groups: halogens ($^{123/125/131}I$, ^{211}At); metals (^{90}Y, ^{67}Cu, ^{213}Bi, ^{212}Bi); and transitional elements (^{186}Re). Radionuclides can be further categorised into four types of cytotoxic agents: pure beta (β) emitters (^{67}Cu, ^{90}Y); alpha (α) emitters (^{213}Bi, ^{211}At); beta emitters that emit gamma (γ) radiation (^{177}Lu, ^{186}Re, ^{131}I); and Auger emitters and radionuclides that decay by electron capture ($^{123/125}I$ and ^{67}Ga).

Radioisotopes used in therapy are predominantly β- or α-emitters. The β-particle emitting radionuclides are utilised for their ability to penetrate tissues. Lower energy β-particles can travel a few cell diameters and these may be useful in microscopic targets and, thus, reducing normal tissue damage. Higher energy β-particles (emitted by e.g. ^{32}P and ^{90}Y) penetrate beyond several millimetres and are utilised when a high homogeneous dose to a large target is being delivered (Brenner, 2007). α-Particles (helium nuclei) can also deliver high dose, but they have a very short mean free path of 50 – 80 μm (Vaidyanathan *et al.*,1996). This results in a high amount of energy being deposited in a relatively small volume of tissue and, consequently, increases relative biological efficiency. The problems associated with recoil following alpha decay (biodistribution and systemic toxicity) and the production of non-metal daughters, however, critically hampers the therapeutic use of these particles (Eary and Brenner, 2007).

Auger electron emitters have been proposed as an attractive alternative to energetic β-emitters for use in cancer therapy (Semnani *et al.*, 2005; De Jong *et al.*,2002). The biological effect induced by Auger electrons is comparable to that of alpha particle radiation and are, thus, classified as radiation with high linear energy transfer (Goddu *et al.*, 1994). Auger electron emitters are excellent candidates to achieve high specific cytotoxicity, in combination with a low degree of side effects when they are delivered to the DNA of a tumour cell (Fischer *et al.*, 2008). ^{125}I is predominantly (93%) IC decay after EC with a mean production of 20 Auger

electrons per decay, while ^{123}I produces approximately 11 Augers per decay (Kassis, 2003). No significant lethal effects are produced when the decaying atom is situated within the cellular cytoplasm, at the cell plasma membrane or outside the cells (Kassis *et al.*, 1987).

In the past decade, most work on Auger electrons focused on the use of ^{125}I, but the potential alternatives such as ^{123}I are now well known. ^{125}I exhibits a higher Auger electron yield than ^{123}I, but ^{125}I is not readily available in Southern Africa like ^{123}I. In addition, the relatively long half-life of ^{125}I (60 days) imposes severe limitations in terms of radiation protection. The shorter half-life of ^{123}I (13.2 hours) makes it a favourable radionuclide in the therapy of malignant cells. Studies also suggest that ^{123}I is more efficient than ^{125}I in producing DNA damage, although ^{125}I is more potent on a per disintegration basis (O' Donoghue, 1996).

For *in vitro* or *in vivo* therapeutic study applications, it is important to have a product of high specific activity. Specific activity is defined as the amount of radioactivity, expressed in Becquerel (Bq) or curies, per mole of a radiotracer. The use of a high specific activity radiotracer will ensure that chemical toxicity is reduced and that non-radioactive substances of a tracer will not interfere with the latter's biological properties. iThemba LABS produces no-carrier-added ^{123}I with a high specific activity. It is produced through proton bombardment of a sodium iodide target, following the nuclear reaction:

$$^{127}I\,(p,5n)^{123}Xe \longrightarrow {}^{123}I$$

Radionuclides produced via this route are referred to as no-carrier-added because they are obtained from the decay of a different element (^{123}Xe).

2.5 Methods for labelling pharmaceuticals with radioiodine.

Several methods exist for the incorporation of radiohalogens into both aliphatic and aromatic organic substrates. The substitution of atoms or groups in molecules is the most frequently used. Two types of substitution can occur, namely, electrophilic or nucleophilic substitution (Baldwin, 1986; Coenen *et al.*, 1983; Seevers and Counsell, 1982). Depending on the molecular structure of the precursor, the first decision to be made is whether a nucleophilic or electrophilic approach should be used. Feasible reaction conditions and reliable labelling yields are factors to consider in selecting an appropriate method.

Electrophilic substitution can either involve direct or indirect labelling reactions. Direct electrophilic substitution of an aromatic hydrogen atom with electrophilic iodine is mostly

suited for labelling substrates that are substituted with very strong activating electron-donating groups (e.g. the amino and hydroxyl groups in aniline and phenol, respectively). These activating substituents trigger electrophilic attack on substrates through resonance stabilisation of the formed intermediate carbocations. The labelling in this case is, however, not always regioselective and could result in the formation of isomers and mixtures of isomers. The indirect electrophilic iodination approach is also used in carbon substrates of aromatic or vinyl moieties without substituents that activate or have a directing effect in the substitution of an incoming radioiodine electrophile. In these cases the labelling proceeds via iodo-demetallation. In order to apply this labelling procedure, a suitable labelling precursor containing an organometallic group at the site of labelling should firstly be synthesised. Examples of such groups are trimethyl- and tributylstannyl groups. These groups substitute halogen atoms in aromatic rings or add to alkyne structures by way of reducing triple bonds. The former will result in an aryltrialkylstannyl and the latter in a vinyltrialkylstannyl compound. Radiolabelling vinyl- and aryl-containing trialkylstannyl groups via oxidative iodination is well researched. The radioiodide is converted to an oxidation state of +1 or 0 through the action of suitable oxidising agents, such as chloramine-T or peracetic acid. This is followed by an electrophilic attack on the carbon-tin bond of the precursor and iodine is, subsequently, regio-specifically incorporated.

The use of electrophilic halogen-demetallation reactions is advantageous, due to regio- and stereo-specific halogenation under mild conditions. Radio-destannylations are fast and permit radioiodination in high yields (Musachio and Lever, 1992). This radioiodination method is crucial in ensuring radiochemical and metabolic stability of the formed radiolabelled compound (Hanson *et al.*, 1989; Wilbur *et al.*, 1989). In addition, it yields radiolabelled products with relatively high specific activities, as the former can be separated from their non-radioactive precursors using chromatographic methods.

Isotopic or halogen substitution for radioiodine (the Finkelstein reaction) commonly employs a different labelling technique referred to as nucleophilic substitution. When aliphatic alkyl halides are the substrates, the reaction involves a bimolecular nucleophilic substitution (S_N2) mechanism. In the case of aromatic halide substrates, the S_NAr mechanism is followed. This radiolabelling technique could result in compounds with lower specific activity than those obtained via electrophilic reactions, as the chromatographic separation of the labelled compound and its precursor could be either difficult or entirely impossible.

CHAPTER 3

Results and discussion

3.1 Reaction schemes

3.1.1 Synthesis of N^3-substituted thymidine derivatives

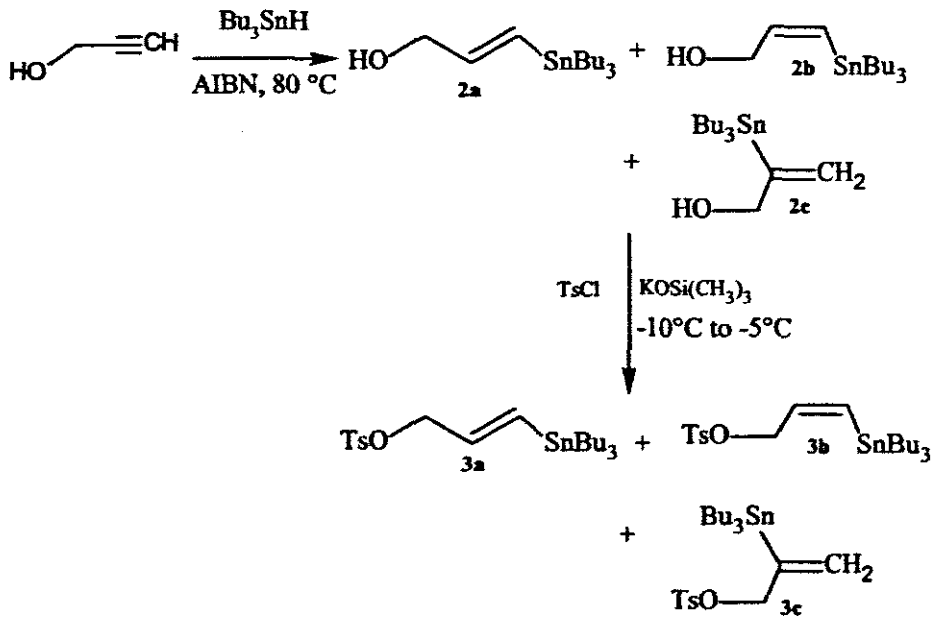

Figure 3.1-1 Preparation of alkylating agents

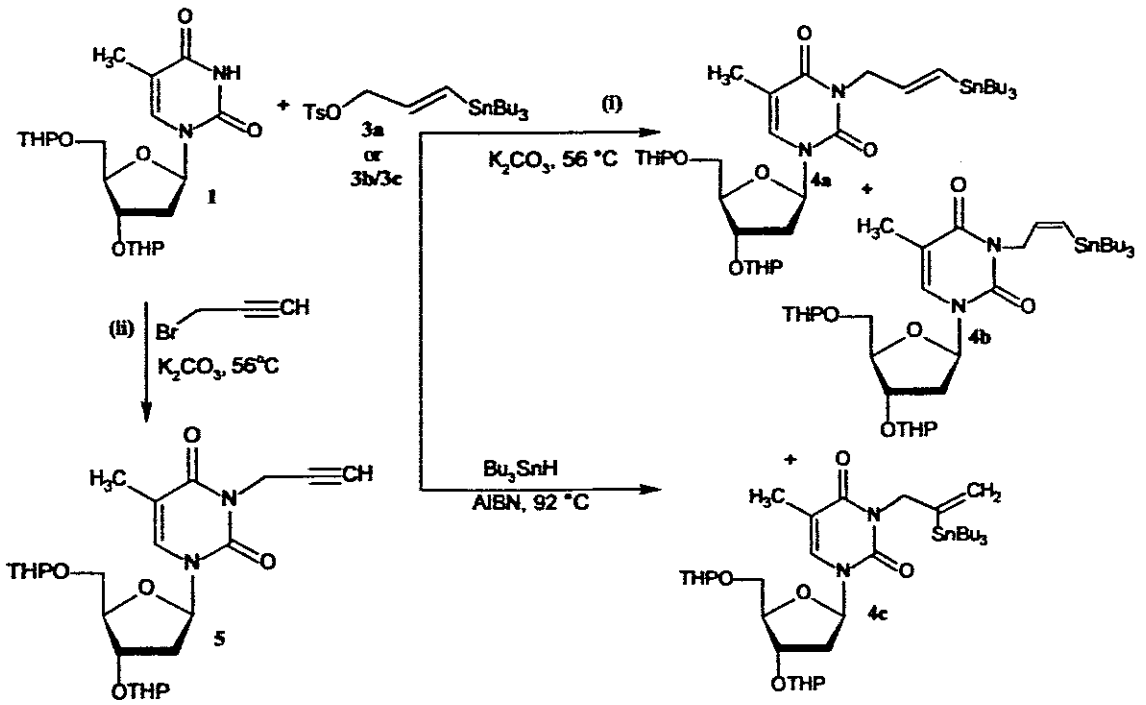

Figure 3.1-2 N^3-alkylation of thymidine THP ether

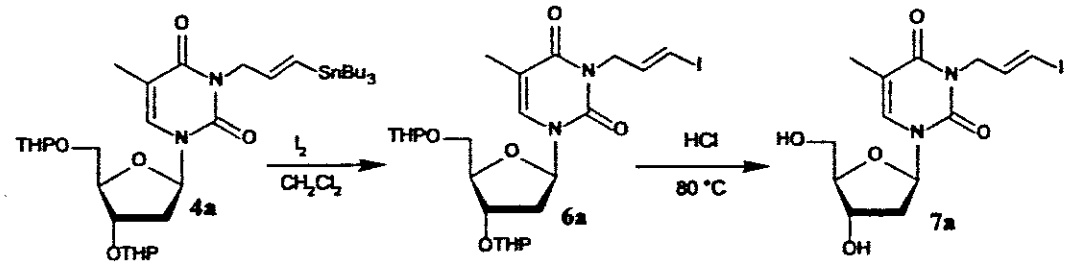

Figure 3.1-3 Iodination and hydrolysis of N^3-alkylated thymidine THP ether

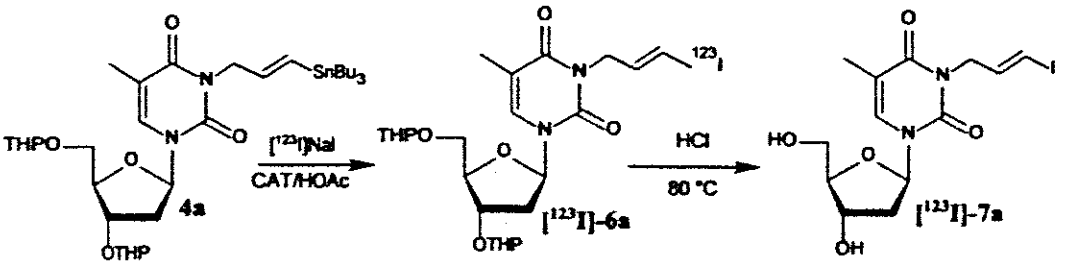

Figure 3.1-4 Radioiodination and hydrolysis of N^3-alkylated thymidine THP ether

3.1.2 Synthesis of N^3-substituted 5-iodo-2'-deoxyuridine derivatives

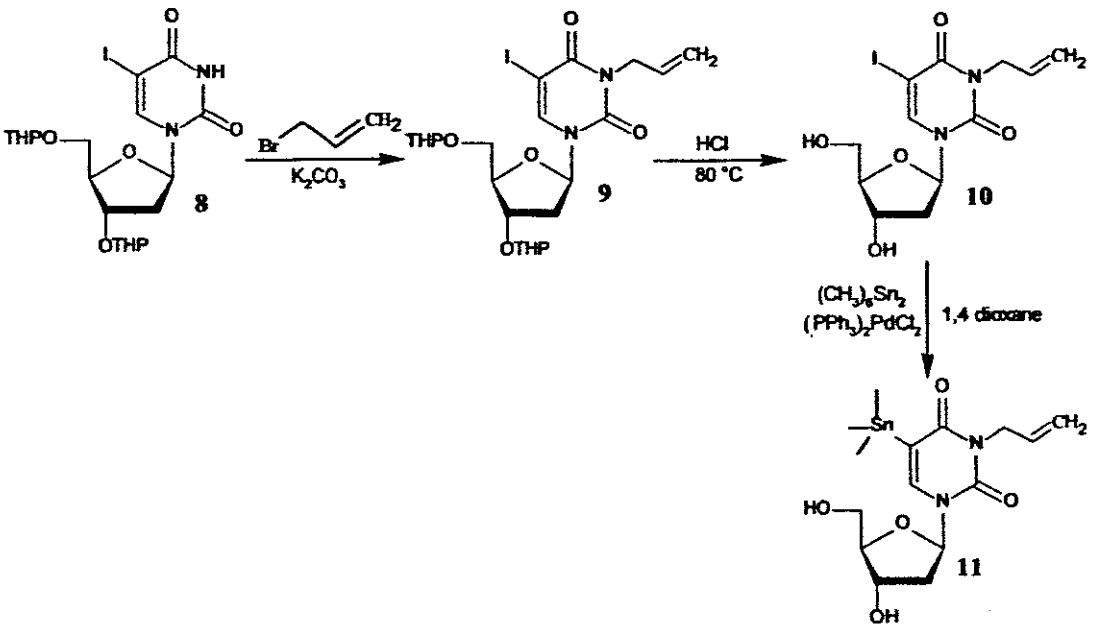

Figure 3.1-5 Preparation of N^3-allyl-5-iodo-2'-deoxyuridine precursors

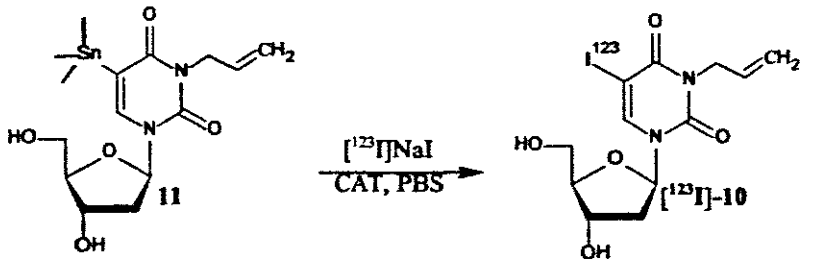

Figure 3.1-6 Radio-iododestannylation of N^3-allyl-5-trimethyltin-2'-deoxyuridine

3.2 Chemical synthesis of N^3-substituted thymidine derivatives

3.2.1 Protection of the hydroxyl groups

Before the synthesis of the N^3-alkylated pyrimidine derivatives, the physiologically important 3'- and 5'-hydroxyl groups had to be protected from undergoing side-reactions during synthetic procedures. Conversion of a deoxyribose sugar to its corresponding ether is a well known method for protecting hydroxyl groups. Tetrahydropyranyl (THP) ether was used as a protecting group, due to its stability under strongly basic reaction conditions, and in the presence of organometallic reagents. Preparations of ***1*** and ***8*** were acid catalysed by *p*-toluenesulphonic acid under anhydrous conditions at room temperature according to the reaction mechanism below.

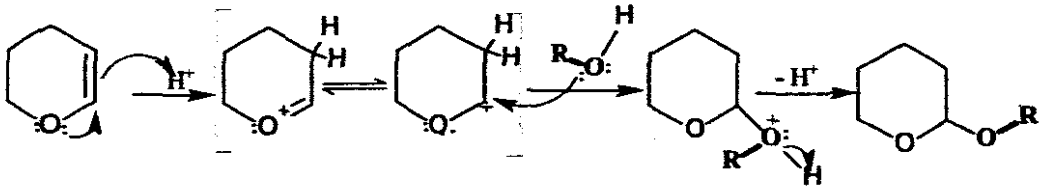

Figure 3.2-1 Conversion of a hydroxyl to ether using THP protective group
RO-H is a pyrimidine nucleoside.

Thymidine and its derivatives have three chiral centres. Additional chiral centres were generated during protection by the tetrahydropyranyl ether. This resulted in a mixture of diastereomers, resulting in the complex ^{1}H NMR spectra. The anomeric C-1′ proton in the deoxyribose unit has two C-2′ neighbouring protons and appears as a triplet in the ^{1}H NMR, without spatial interaction (Jacobsen, 2007). It appeared considerably downfield (6.38 ppm - 6.32 ppm) of the pair of C-2′ protons due to the inductive pull of electron density from the hydrogen atom by the oxygen and nitrogen atoms. However, the proton signal was split into a multiplet as a result of diastereomers besides the C-2′ adjacent protons. The C_6 proton appeared as four singlets resonating at higher frequency of 7.61 ppm to 7.54 ppm in the aromatic region, confirming a diastereomeric mixture of four. Likewise, the methyl protons were split into three singlet peaks, possibly due to magnetic non-equivalence or spatial interaction effects of the protecting groups. The THP protons resonated at chemical shift values of C_3-H; 1.85 ppm -1.68 ppm and $C_{4\&5}$-H; 1.64 ppm – 1.48 ppm. The supplementary information about the diastereomic thymidine THP ether was gathered from the ^{13}C NMR proton-decoupled spectrum. The carbon signals of C-6 (~ 136 ppm) and THP C-2 (100.2 ppm to 98.62 ppm) were a cluster of singlets instead of single singlets as a result of the diastereomic product.

3.2.2 N^3-alkylation

Thymidine THP ether derivatives were N^3-alkylated without experiencing side reactions. Two synthetic routes for the introduction of substituted propenyl groups into the molecules were followed. These involved nucleophilic substitution reactions in which an alkynyl- or alkenyl halide or alkenyl tosylate (*p*-toluenesulphonate) is attacked by the nucleophilic N^3-nitrogen atom of THP-protected *dT* or *IUdR* to produce a nitrogen-carbon bond. The synthetic pathways, illustrating the two different synthetic routes, (i) and (ii), are shown in Figure 3.1-2.

Following route (ii), 3-bromoprop-1-yne was coupled with *1* under base catalysis in a 1:1 mixture of acetone-DMSO to produce *5*. After chromatographic isolation of *5* in 82% quantitative yield, the ^{1}H and the ^{13}C NMR structural elucidations were done. The chemical shift value of the acetylene proton was 2.14 ppm and it appeared as a finely split triplet due to slight allylic coupling. The N^3-CH_2- protons resonated at 4.6 ppm - 4.7 ppm and overlapped with the C-4′ proton peak. Similarly, the carbon-13 spectrum confirmed and displayed acetylene carbons (78.48 ppm and 70.69 ppm for the internal and the terminal carbons, respectively), together with N^3-C_1 resonating in the up-field region at 30.58 ppm.

In addition to NMR, structural elucidation of *5* was also done by means of high resolution electrospray ionisation mass spectrometry (ESI-MS). The protonated molecular ions, $[M + H]^+$, were the dominant species although results showed that they have been accompanied by salt adducts. The salt adducts, mainly sodium (Mr = 23 Da), originate from MS instrument calibration with NaF before the samples were analysed. The tallest peak on the spectrum (usually with the highest abundance) was arbitrarily given 100%, which is equivalent to a base peak. This, however, may not necessarily be a molecular ion peak, since nucleosides fragment so easily that no molecular ion may be observed even at 70 eV in an ESI^+ spectrum. The obtained experimental mass of *5* was 449.229 Da (with 17% relative abundance) and correlated well with a theoretical mass ion of $[M + H]^+$ = 449.517 Da. A base peak belonged to an adduct, however, formed by compounding *5* with sodium-23 to produce a peak of m/z 471.192 Da. Daughter ions produced were mainly fragmentised by a loss of THP protecting groups and glycosylic bond cleavage. This fundamentally provided fragments of m/z 365.165 Da (with 50% abundance) to account for one THP molecule, m/z 281.114 Da (with 81% abundance) for two THP molecules and m/z 165.064 (with 84% abundance) peak for the cleavage of the glycosyl bond. From these data, it can be inferred that thymidine THP ether nucleosides are easily cleaved under soft ionisation (+35 eV), in particular, THP protecting groups and the glycosyl bond of thymidine. The ionisation pattern is summarised in the following Figure 3.2-2.

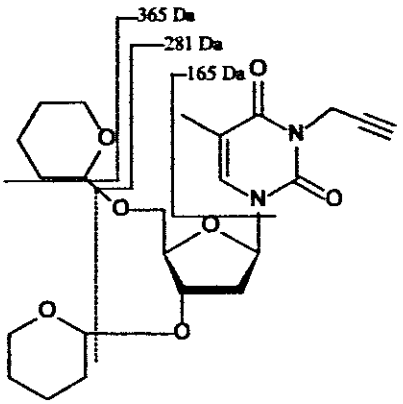

Figure 3.2-2: The MS fragmentation pattern of ***5***.

3.2.3 Hydrostannylation of *5*.

In the preparation of the vinyl stannane isomers, ***4a*** and ***4b***, the triple bond of ***5*** had to be reduced to an alkene derivative, substituted with a trialkyltin group via hydrostannylation under free radical addition conditions. The tributyltin radical ($Bu_3Sn^{\cdot}$) was initiated by the catalyst azoisobutyronitrile (AIBN). The mechanism of the reaction is illustrated in Figure 3.2-3.

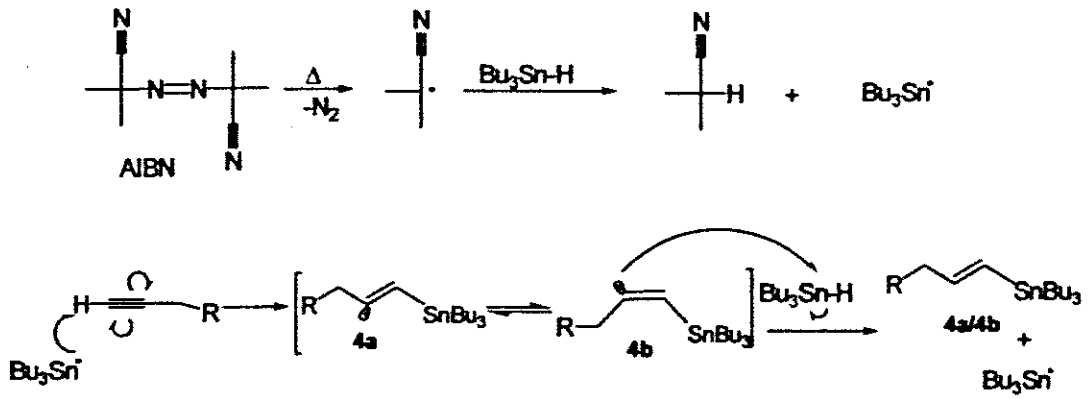

Figure 3.2-3 AIBN radical initiation and addition of tributyltin radical addition reaction
R = thymidine THP ether

Upon heating, there is a facile generation of relatively stable and reactive carbon-centered isopropylnitrile radicals ($NCMe_2C^{\cdot}$) that abstracts a hydrogen atom from Bu_3SnH. Because the tin hydrogen bond (Sn-H: 310 kJ/mol) is significantly weaker than the carbon hydrogen bond (C-H: 414 kJ/mol), $NCMe_2C^{\cdot}$ selectively abstracts a hydrogen from Bu_3SnH in the presence of a variety of organic compounds, including a solvent (Chatgilialoglu *et al.*, 1983; Neumann, 1987). The newly formed $Bu_3Sn^{\cdot}$ radical adds to unsaturated carbon-carbon bonds,

thereby, relocating a radical electron. Finally, a free radical vinyl carbon promotes the second homolytic bond-breaking of the Sn-H bond in Bu_3Sn-H and the hydrogen radical attaches to the carbon radical to give the anti-Markovnikov product.

Radical addition of $Bu_3Sn^{\bullet}$ is not fully stereoselective. The geometric *E*-isomer is advantaged over the *Z*-isomer at higher temperatures, i.e. the *E*-stereoisomer is a major, but not the exclusive, product. This is because an intermediate vinyl radical formed has a very low (~2 Kcal) barrier to inversion energy and the induction period of *E/Z* equilibration reactions is very short (Singer and Kong, 1966). Under experimental conditions, at 92°C in toluene and in inert atmosphere, ***4a*** was obtained in 76% yield, while other possible isomers only contributed 12% to the total yield. Once the products have been formed the resulting structures retain their configuration and are not easily inter-converted, due to a high barrier-to-rotation energy in most alkenes (>50 kcal/mol). The *E*- and the *Z*-isomers have dissimilar physical properties and are, therefore, readily separable on TLC and by means of silica gel chromatography. The *Z*-isomer, however, was often not easily obtained as a pure product due to its intimate polar properties with another regioisomer, which is reviewed later in this chapter.

From the ^{13}C NMR spectrum, the vinyl carbons of ***4a*** or ***4b*** were resonating in the downfield region of 131 ppm – 134 ppm. The spectrum displayed higher intensity signals of *n*-tributyltin carbons in the low frequency regions (the internal CH_2 carbons were resonating at 29 ppm - 27 ppm and the terminal CH_3 carbons at ~14 ppm). In the ^1H NMR spectrum the chemical shifts of the vinylic protons, appearing as doublets of triplets, were in the order of 5.98 ppm – 5.93 ppm for the *E* isomer and 5.89 ppm – 5.71 ppm for the *Z* isomer. The stereochemistry of the isomers ***4a*** and ***4b*** was elucidated by the vinyl stannane protons coupling constants across a double bond in the ^1H NMR spectrum [***4a*** (*E*); J = 19.5 Hz and ***4b*** (*Z*); J = 12.56 Hz]. Generally, the coupling constants of protons with *cis* or *Z*-configuration are smaller (J = 12 Hz - 14 Hz) than those with *trans* configuration (J = 18 Hz - 20 Hz) (Leusink *et al.*, 1968; Seevers and Counsell, 1982).

One of the isomers, ***4a***, was subjected to a high resolution MS. The results displayed a similar fragmentation pattern similar to ***5*** and peaks were readily assigned. The base peak, however, did not correspond to the parent compound, but to a daughter ion without a protecting group, ($[M + H]^+ - [Mr_{thp}] = 657.291$ Da) which compared to a theoretical value of 657.462 Da.

3.2.4 Significance of the stereochemistry of a radiopharmaceutical

The stereochemistry of radiopharmaceutical compounds often plays a significant role with regard to *in vivo* binding to receptors (a receptor may be defined as the specific constituent of the cell with which a radiopharmaceutical interacts to produce a physiological effect). Many radiopharmaceutical compounds exhibit stereoisomerism in their chemical structures, which have remarkable selectivity and specificity towards receptors. Most often one form binds more strongly to a receptor than its stereoisomer or the other may be inactive or even toxic. The two geometric isomers synthesised in this study might display different biological properties.

3.2.5 Preparation of alkylating agents

As mentioned before, hydrostannylation reactions do not always lead to the exclusive formation of a single geometric isomer. The synthesis of either ***4a*** or ***4b*** may, however, be possible by using priorly synthesised and isolated isomeric vinyl stannane alkylating agents. The alkylating agent (*E*)-1-chloro-3-(tri-*n*-butylstannyl)prop-2-ene, has been prepared before (Hanson, 1989). Attempts in preparing a similar alkylating agent by direct hydrostannylation of propargyl bromide, however, under free radical addition reaction conditions, possibly led to de-bromination (Togo, 2004). The de-bromination reaction is exothermic and proceeds via carbon-bromine cleavage even at lower temperatures. The rationale behind the selection of a bromo-derivative was that the bromine atom would be more easily displaced in the N-alkylation reaction than a chlorine atom. The better leaving group ability of sulphonic acid esters produced by conversion of an alcohol to an alkyl tosylsulphonate, therefore, propelled the preparation of alkylating agents by hydrostannylation of propargyl alcohol, followed by tosylation. The hydrostannylation reaction was performed at 80°C as outlined in Figure 3.1-1. The compounds ***2a*** and ***2b/2c*** were visualised under UV light after staining the developed TLC plate with iodine. According to Musachio and Lever (1992), the more polar component on TLC corresponds to the *E*-isomer and the less polar component would be a mixture of ***2b/2c***. The same scenario applied in preparations of ***4a*** or ***4b***, although the ***2c*** isomer was never characterised. The quantitative yield of ***2a*** was low (17%) compared to ***4a*** (76%), prepared from *5*.

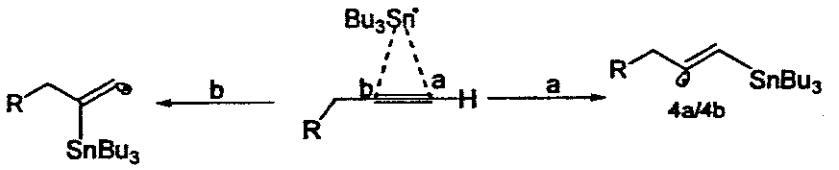

Figure 3.2-4 Competing reactions following radical addition to an alkyne moiety.

Formation of the *E/Z*-isomers is favoured by route (a) in Figure 3.2-4, where $Bu_3Sn^{\bullet}$ adds to a terminal position. After a successful column chromatographic separation of stereoisomers ***2a*** and ***2b***, the sulphonates ***3a*** or ***3b*** were separately synthesised with *p*-toluenesulphonyl chloride in diethyl ether, in the presence of potassium trimethylsilanolate ($KOSiMe_3$) catalyst below -5°C. Potassium salts were washed from the reaction mixture with deionised water and products were extracted in diethyl ether and subjected to further column chromatographic purification before coupling to the thymidine THP ether.

3.2.6 The use of tosyl sulphonate reagents in N^3-alkylation.

Tosyl sulphonates were selectively displaced in the alkyl coupling reaction with thymidine THP ether (see Figure 3.1-2, route (i)). Products ***4a*** and ***4b***, prepared via route (i), each displayed similar HPLC retention times and TLC R_f values to those of the same products prepared via route (ii). The overall quantitative yields of ***4a***, with respect to thymidine THP ether, from either reaction route was almost equal, being 60% and 62% for route (i) and (ii), respectively. Route (i) is advantageous in controlling the stereochemistry and availability of either *E*- or *Z*-isomer for the end product. This reaction route, however, may not be ideal due to reaction temperature constraints of -20°C in the tosylation step during the synthesis of the alkylating agents, as recommended in literature (Musachio and Lever, 1992). The alternative synthetic route (ii) for aromatic N-alkylation was convenient and productive because the attached triple bond could be selectively reduced while leaving the carbonyl groups or the double bonds of the heterocyclic base intact. Careful consideration, however, must be made not to elevate temperatures higher than 100°C when using aromatic substrates in free radical hydrostannylation, since $Bu_3Sn^{\bullet}$ addition to double bonds may occur and aryls can often act as radical stabilisers at higher temperatures (Knochel, 2005).

3.2.7 Iododestannylation

In order to positively identify a radiopharmaceutical compound, its non-radioactive ('cold') counterpart is prepared or labelled with a stable nuclide and characterised as a reference standard. Tributyltin precursor, ***4a***, was labelled with 'cold' iodine via destannylation of tributyltin to produce ***6a***. The reaction was performed at room temperature in the presence of excess iodine to recover a product (82% quantitative yield) in 5 minutes. On the basis of HPLC gradient elution on a C-18 column, which allows detection of a mixture of closely polar isomers, iododestannylation produced a single product. In order to provide evidence for a successful iododestannylation, the structure of ***6a*** was characterised by ^{1}H and ^{13}C NMR spectroscopy and backed up by mass spectrometry (MS).

Substitution of the tributyltin group on the terminal vinylic carbon atom with an iodine atom caused a lesser shielding effect on the neighbouring hydrogen atoms, resulting in downfield shifting of the N-alkenyl protons in the ^{1}H NMR spectrum. The characteristic shifting was as follows: vicinal shift of doublet of triplets (5.98 ppm – 5.93 ppm) to (6.64 ppm – 6.58 ppm) and a doublet from 6.15 ppm to 6.47 ppm. Conversely, in the ^{13}C NMR up-field shifting was observed for the carbon atom attached to the iodine atom (HC=<u>C</u>Sn; 132.5 ppm, to HC=<u>C</u>HI; 83.98 ppm) as a result of a crowding shielding effect caused by the large iodine atom. Migration of the signal as a result of the presence of the iodine atom was close to the chemical environments of the anomeric C-1′ and C-4′. Furthermore, a decrease in the coupling constant (from J = 19.5 Hz for the *trans* vinyl protons in ***4a*** to J = 14.2 Hz for the vinyl *trans* protons in ***6a***) was as a result of substitution of a tributyl group with the more electronegative iodine atom. In the ^{1}H NMR spectrum, the coupling constants for the *cis* protons in vinyl iodides (J = 7 Hz - 8 Hz) are smaller than those of the *trans* protons (J = 13 Hz – 15 Hz) (Leusink *et al.*, 1968). This further illustrated retention in stereochemistry of ***4a*** upon iododestannylation. The molecular mass of ***6a*** and its molecular portions were assigned in detail from the MS spectrum. The sodium adduct of ***6a*** was found in 92% relative abundance, the molecular ion was 17% abundant and the base peak was assigned to the molecular fragment of ***6a*** without a deoxyribose portion.

The final step in the synthesis was the cleavage of the protecting THP groups. Compound ***6a*** was hydrolysed in 0.15 M HCl at 76°C to afford ***7a*** (68% quantitative yield) in 6 minutes. Removal of the THP protecting groups assured a simplified proton spectrum, as compared to other THP protected compounds studied. The four singlets of C_6-H appeared as a single

singlet at 7.38 ppm and the N^3-CH_2- protons were transformed into a doublet at 4.45 ppm. As expected, the anomeric proton was split into a triplet as a result of vicinal interaction with the C-2'-protons. The observed hydroxyl groups signal bulged at 2.62 ppm – 2.46 ppm as a twin of broad singlets. The bumpy shape of hydroxyl signals was probably due to proton exchange.

The ESI mass spectral data indicated that ***7a*** was strongly fragmented after 60 eV ionisation. The molecular ion peak, detected at 409.07 Da, was 16% abundant relative to the base peak with m/z value of 292.97 Da, matching a daughter ion formed due to the glycosylic bond cleavage of ***7a***.

3.3 Chemical synthesis of N^3-substituted 5-iodo-2'-deoxyuridine derivatives

In pursuit of the investigation on the impact of the position of the radiolabel on *in vitro* cell-uptake, combined with the effect of N^3-substitution, N^3-allyl substituted 5-iodo-2'-deoxyuridine (***10***) was also synthesised. Its labelling precursor, N^3-allyl-5-trimethylstannyl-2'-deoxyuridine (***11***) was prepared in three steps from THP-protected IUdR (***8***) as shown in Figure 3.1-5.

After the preparation of ***9***, structural assignments were made by ^1H and ^{13}C NMR spectroscopy, and supported by MS. In the ^1H NMR spectrum of **9**, the C_6-proton was split into four singlets, similarly to the corresponding proton in ***1***, but resonated more downfield at 8.24 ppm - 8.17 ppm because of the deshielding effect of the iodine atom in the 5-position. Internal allylic proton chemical shift corresponded to that of a vinyl proton, disclosing the similarity of the chemical environments of vinylic and allyllic protons. The allyllic proton in the β-position to N^3 atom gave a very complex multiplet, due to coupling amongst the four proton environments (ABC system). In spite of this, terminal allylic protons are both magnetic and chemically non-equivalent. This was proven by the separate resonance position of their individual doublets at 5.29 ppm (J = 17.2 Hz) and 5.21 ppm (J = 10.2 Hz), respectively. The coupling constants in terminal allylic protons are analogous to *cis/trans* coupling of protons in which protons in a *trans* configuration have a larger coupling constant than the *cis* configuration. The defragmentation pattern of the mass spectrum of ***9*** was not different from those of thymidine derivatives. The base peak of m/z 479.07 Da matched a daughter ion without THP substituents; the molecular ion had a 54% abundance, while the sodium adduct was only 24% abundant.

The protecting groups of ***9*** were removed, similarly to those in ***6a***, and a reference standard compound for the radiolabelled ***10*** was obtained. The ^{1}H NMR spectrum of ***10*** displayed the anomeric proton as a non-overlapped triplet downfield at 6.21 ppm, relative to its non-equivalent neighbouring C-2′ protons in the up-field region of 2.43 ppm – 2.38 ppm and 2.36 ppm – 2.30 ppm. N^3-CH_2- protons appeared as a broad and distorted doublet, while the 3′- and 5′-OH protons overlapped to produce a broad singlet at 2.17 ppm, pointing towards a successful hydrolysis reaction.

At this stage, ***10*** could be radiolabelled directly by a direct halogen exchange reaction, however, chromatographic separation of the radiolabelled compound from the non-radiolabelled precursor would not be possible. As a result, the good leaving group trimethyltin was introduced at the 5-position of ***10***, following a Stille cross coupling reaction under palladium catalysis, to form ***11***. Radiolabelling of this precursor would make the chromatographic separation between precursor and radiolabelled product possible. In the ^{1}H NMR spectrum, a high intensity singlet integrated for 9 protons represented the trimethyltin protons at 0.21 ppm downfield to TMS. A proton shift from 8.22 ppm to 7.21 ppm upon substitution of an iodine atom with trimethyltin was also observed for the adjacent C_6-H. In the ^{13}C NMR a downfield shift of the C-5 signal from a lower frequency of 68.74 ppm to a higher frequency of 112.5 ppm was observed upon substitution of the iodine atom with a trimethyltin group.

3.4 Radiochemistry

3.4.1 Radiochemical labelling

Both [^{123}I]-***6a*** and [^{123}I]-***10*** were synthesised by means of electrophilic iododestannylation from their corresponding precursors using choramine-T hydrate as an oxidising agent. Radiolabelling reactions were performed using [^{123}I]-NaI as the source of radioiodine. The isolation of radiotracers was done by means of HPLC purification to afford radiochemical purities greater than 99%. The reference 'cold' standard compounds were co-injected with the corresponding radioiodinated compound on HPLC and simultaneous analysis of the radioactive and the reference was done on two integrator channels, i.e. the radioactive and the UV channels. The UV channel, connected to the UV detector, displayed the stable reference compound, while the radioactivity channel, connected to the radio-detector, displayed the radiolabelled compound approximately 40 seconds later. The delay time is a consequence of a series connection between the UV and the radio-detector.

Radiochemical yields of [^{123}I]-*6a* and [^{123}I]-*7a* at the end of synthesis were good to exceptionally high (73% - 91%) under optimised reaction conditions. The radiolabelling of the hydroxyl protected thymidine analogues was followed by hydrolysis (de-protection) of the radiotracer at 80°C. In recent radiolabelling procedures heat treatment has been avoided for a number of reasons, including radiotracer stability (Hanson, 1989). In this study, the hydrolysis step was found to improve the radiochemical purity of the final radiotracer. Radio-impurities that were formed during radiolabelling, especially where the pH was adjusted by acetic acid, were eliminated at this stage and made the isolation of the pure product easier.

3.4.2 Radiochemical labelling optimisation for [^{123}I]-*6a*

The purpose of these experiments was to optimise radiolabelling yields for [^{123}I]-*6a*, taking the following variables into account: quantity of precursor, quantity of oxidising agent and acetic acid content. The radiolabelling procedure used for the preparation of [^{123}I]-IUdR was adapted for [^{123}I]-*10*, therefore, no optimisations were done for the synthesis of the latter. The solvent volume was restricted to less than 50 µL to match the volume of the HPLC injection loop when conducting HPLC purification. Figure 3.4-1 displays a sample chromatogram of the un-hydrolysed reaction mixture, representing optimised labelling conditions, and shows the retention times of free iodine (2.9 min), a radiochemical impurity (9.9 min) and [^{123}I]-*6a* (12.916 min), respectively. Figure 3.4-2 displays a chromatogram of the hydrolysed reaction mixture and shows the retention time of [^{123}I]-*7a* (8.25 min).

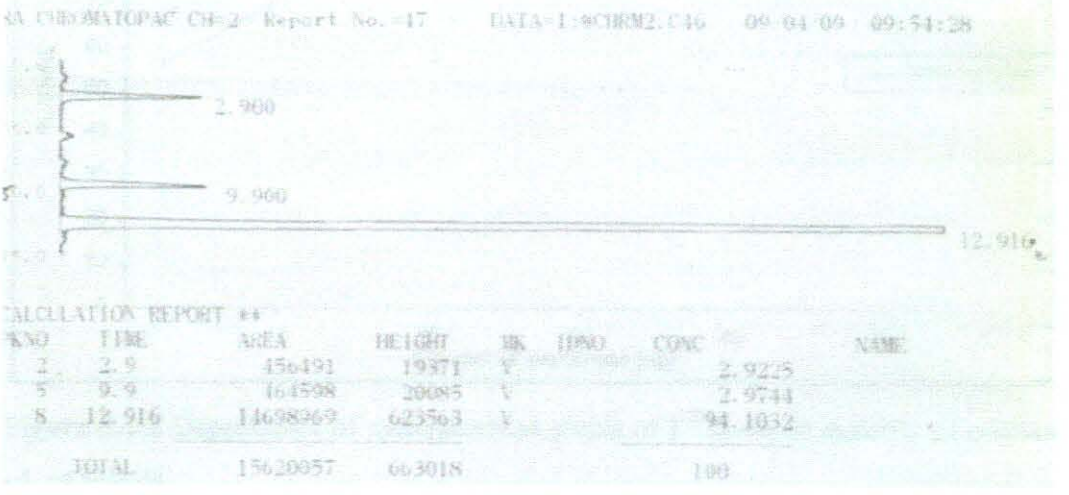

Figure 3.4-1 Radio-HPLC chromatogram for optimised labelling of *6a* before hydrolysis.

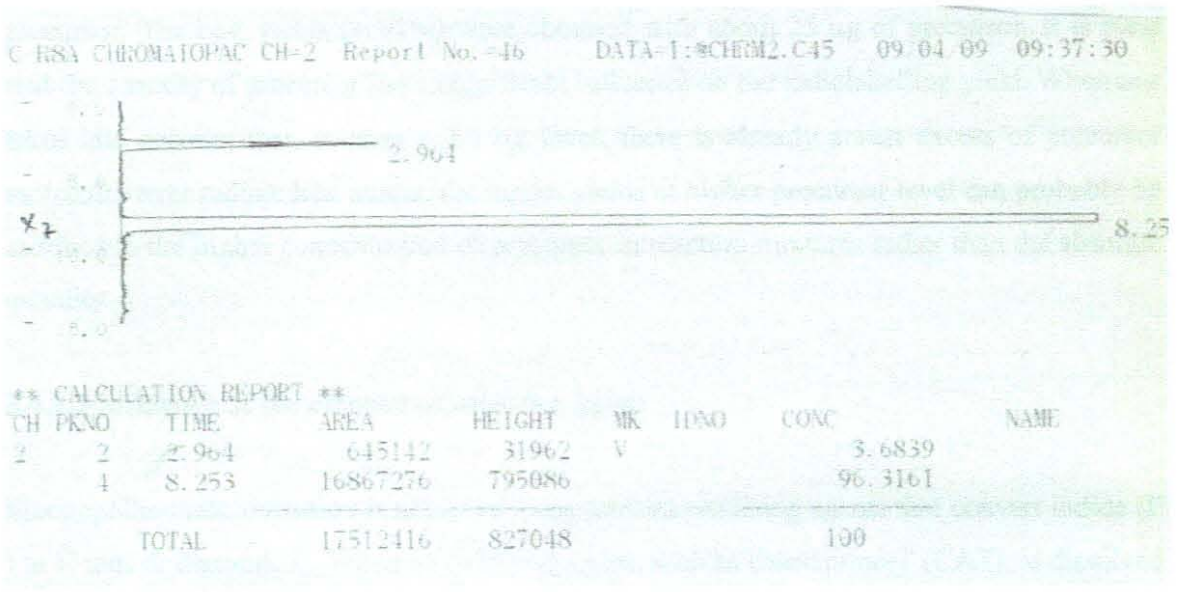

Figure 3.4-2 Radio-HPLC chromatogram for labelled ***7a*** after hydrolysis.

3.4.2.1 Influence of the precursor quantity

To improve the yield of [^{123}I]-***6a***, an effort was made to determine the impact of precursor quantity on radiolabelling yield. Figure 3.4-3 graphically demonstrates the results for a range of 2.5 µg – 50 µg precursor as assessed by analytical HPLC.

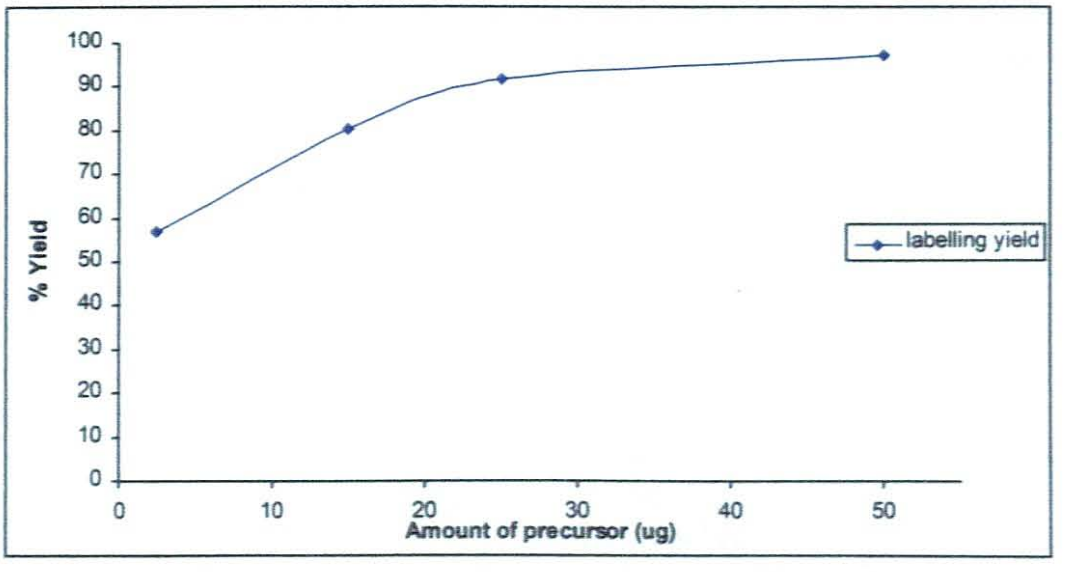

Figure 3.4-3 Dependence of radiochemical yields of [^{123}I]-***6a*** on quantity of precursor in 25 µL of MeOH

The data indicate that radiolabelling yield increased sharply, starting from a precursor mass of 2.5 µg up to approximately 20 µg. Thereafter, there was a slower increase in yield up to 50 µg

precursor. The best yields (> 91%) were obtained with about 25 μg of precursor. It is clear that the quantity of precursor has a significant influence on the radiolabelling yield. When one takes into account that, even at a 2.5 μg level, there is already a vast excess of precursor molecules over radioiodine atoms, the higher yields at higher precursor level can probably be ascribed to the higher concentration of precursor in reaction mixtures rather than the absolute quantity.

3.4.2.2 Influence of the amount of oxidising agent

Electrophilic radioiodination is achieved using various oxidising agents that convert iodide (I^-) to I^+ ions or diatomic I_2. When an oxidising agent, such as chloramine-T (CAT), is dissolved in a partial aqueous solution, such as MeOH:H_2O = 80/20, hypochlorous acid is released and oxidises iodide to a protonated hypoiodous ion (H_2OI^+) (Coenen *et al.*, 2006). Among many oxidising agents, CAT has a higher oxidising strength compared to iodogen or peracetic acid for iododestannylation reactions (Foulon *et al.*, 1996). The following order of reactivity has also been suggested by other investigators: chloramine-T > iodogen > peracetic acid (Zea-Ponce *et al.*, 1993). As a result, chloramine-T trihydrate was selected and its influence on radiochemical yields was investigated.

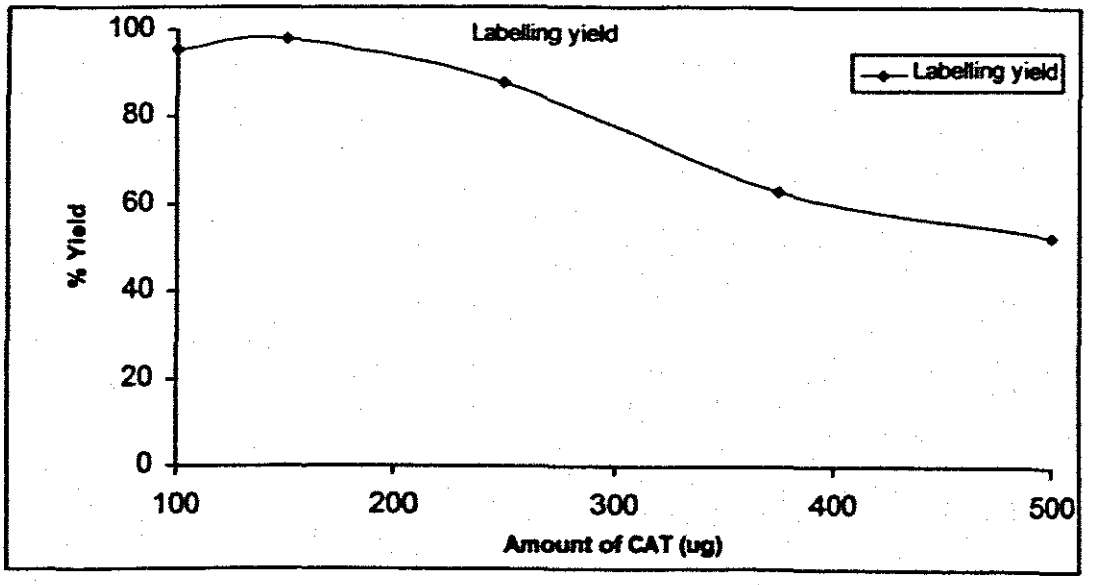

Figure 3.4-4 Dependence of radiochemical yields of [^{123}I]-*6a* on the quantity of chloramine-T trihydrate oxidising agent

Maximum radiolabelling yields were achieved using 100 µg – 150 µg of CAT. Higher quantities of CAT caused the formation of unidentified radio-impurities and resulted in a decreased overall radiochemical yield. The identities of these impurities were not examined. A disadvantage of using CAT has been linked to substrate oxidative side reactions and, in many cases, with prolonged radiolabelling reaction times in which interhalogen species are formed (El-Mohty *et al.*, 1995). The high oxidation potential of CAT at optimal levels, however, is beneficial to short-lived radionuclides - like ^{123}I - where electrophilic radioiodination time is minimal.

3.4.2.3 Influence of the amount of acetic acid

Electrophilic radioiodination is also affected by the pH of the labelling medium. The radioiodine solution, [^{123}I]-NaI, is received in sodium hydroxide (~ 0.03 M NaOH) and the labelling pH should be adjusted accordingly using an acid such as acetic acid, or a strong buffer such as sodium phosphate. Due to the small volumes of labelling mixtures, the pH could not be directly measured and labelling yields were, therefore, determined rather as a function of the amount of acetic acid added. Maximum labelling yields at the end of radiolabelling and hydrolysis were obtained for an acetic acid content of 1.5 µL - 2.5 µL per 5 µL of radioiodine solution.

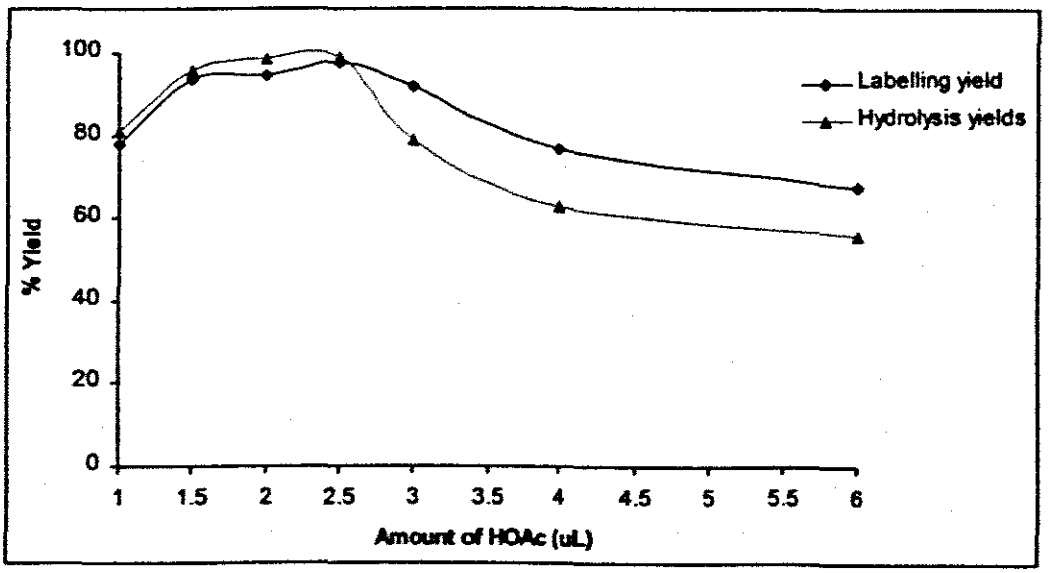

Figure 3.4-5 Dependence of radiochemical yields of [^{123}I]-*7a* on acetic acid content per 5 µL of radioiodine solution

Hydrolysis and/or heating destroyed radioimpurities in the reaction mixture formed during labelling. Upon the addition of more acetic acid, labelling yields dropped to about 65% for 6 μL acetic acid, but no significant by-products were observed. During a follow-up hydrolysis step, however, there was a consistent formation of a new radioimpurity in strong competition with the authentic product (see Figure 3.4-6). The identity of this impurity (retention time is 10.46 min) could not be confirmed, because no "cold" reference standard was available.

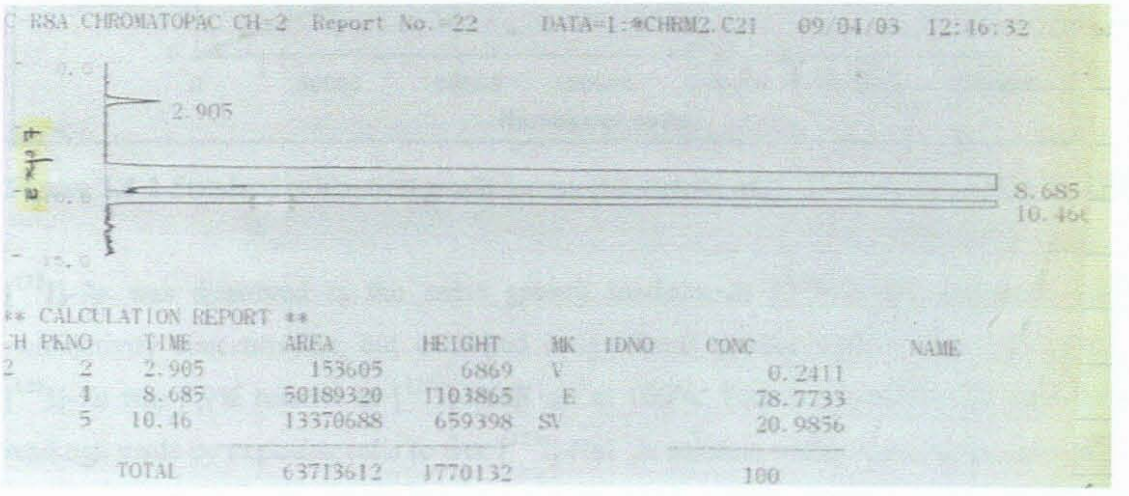

Figure 3.4-6 HPLC chromatogram of [^{123}I]-*7a* post hydrolysis, in the presence of excess acetic acid during radiolabelling

3.5 *In vitro* DNA incorporation studies.

The aim of the *in vitro* cell-uptake studies was to perform a preliminary evaluation for the potential application of radioiodinated N^3-iodovinyl thymidine analogues in radionuclide therapy by determining and comparing their uptakes into CHO-K1 cells with the gold standard [^{123}I]-IUdR, synthesised in a separate study (unpublished results). CHO-K1 cells have a short doubling time of about 11 hours. This has the benefit that a relatively large fraction of cells in the culture is undergoing DNA synthesis (S-phase) at a given time. [^{123}I]-IUdR, which incorporates into the DNA of cells, showed a clear increase in radioactivity readings with the increase in cell numbers ($r^2 \cong 0.99$, see Figure 3.5-1).

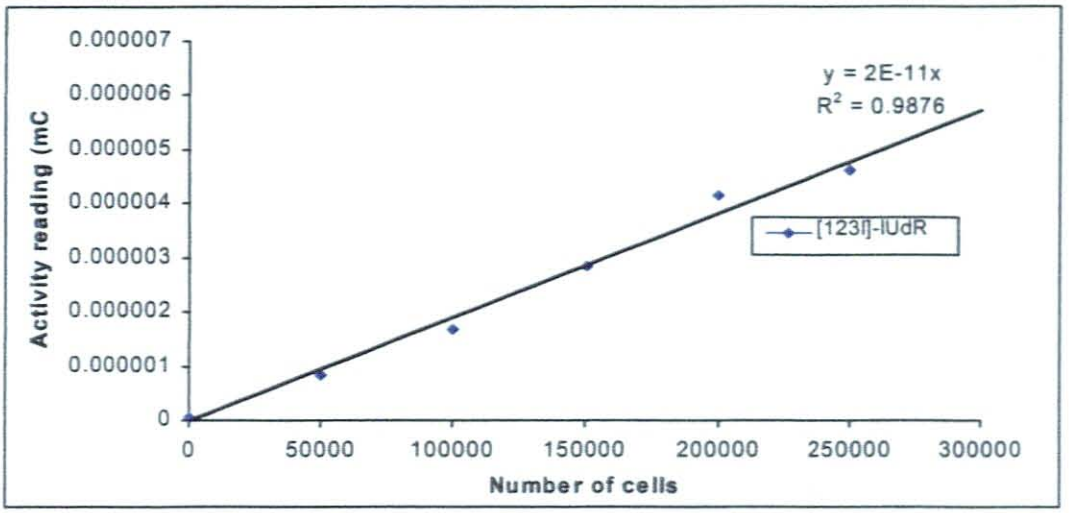

Figure 3.5-1 Standard calibration graph for the cell-uptake of [^{123}I]-IUdR into CHO-K1 cells

[^{123}I]-***7a*** was dissolved in the same growth medium as [^{123}I]-IUdR, and with similar radioactivity concentration, but exhibited insignificant cellular uptake. The cell-uptake of [^{123}I]-***7a*** was 3.9% relative to [^{123}I]-IUdR set at 100%. This is practically the same as the readings made by exposing cells to free [^{123}I]-NaI in solution which is not taken up by CHO-K1 cells (Slabbert *et al.*, 1999). Figure 3.5-2 illustrates cellular uptake results of [^{123}I]-***7a*** in comparison with [^{123}I]-IUdR and free [^{123}I]-NaI.

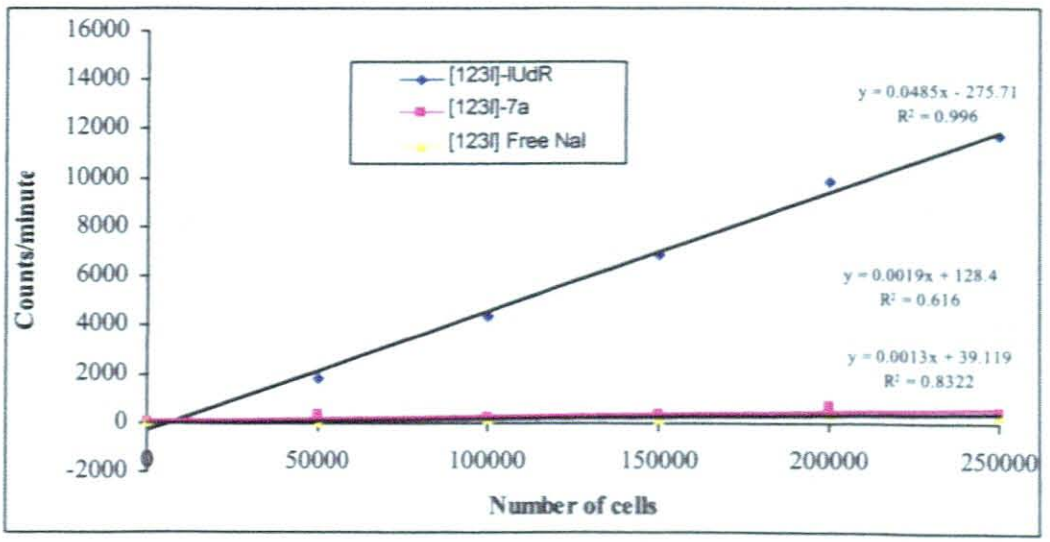

Figure 3.5-2 Comparison of the cell-uptake results of [^{123}I]-IUdR, [^{123}I]-***7a*** and free [^{123}I]-NaI in CHO-K1 cells, respectively.

In order to eliminate the possibility that the low uptake results might have been caused by chemical toxicity induced by [^{123}I]-***7a*** to the cells, growth-dependent cell-uptake studies were

carried out for 30 hours. Under these conditions, CHO-K1 cells were manipulated to a range of proliferations (from actively dividing phase to dormancy phase). Cell-uptake was determined in parallel cell-cultures (growth in [^{123}I]-IUdR, [^{123}I]-***7a***, and non-subculture control medium such as RPMI 1640 with 10% foetal calf serum). The results obtained are shown in Figure 3.5-3.

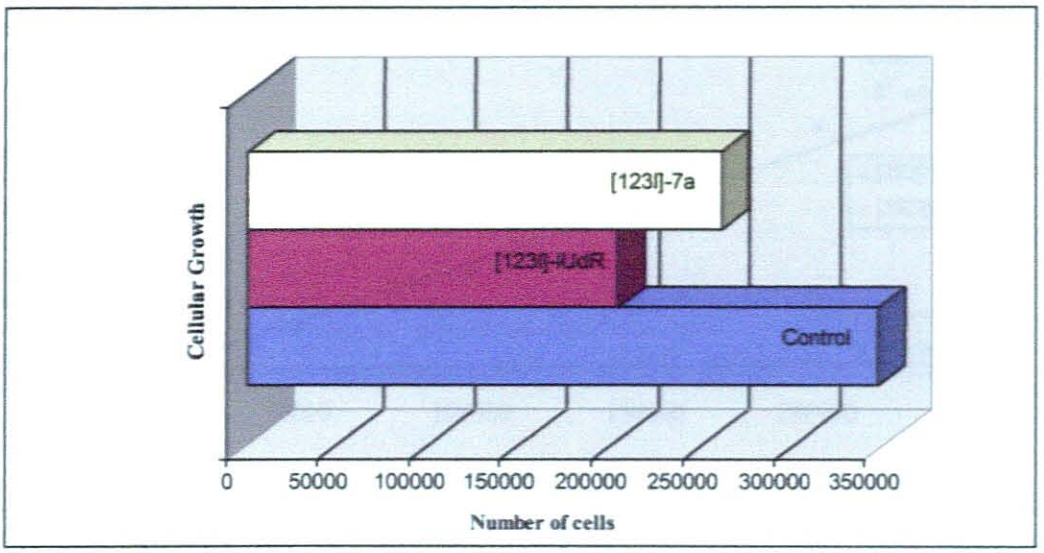

Figure 3.5-3 CHO-K1 growth-dependent cell-uptake of substrates in 30 hrs

From the growth-dependent cell-uptake in Figure 3.5-3, the cell culture showed continual growth in all media, including the medium with [^{123}I]-***7a***. The number of cells after 30 hours incubation was established to be 1.28% higher in the cell culture containing [^{123}I]-***7a*** than the cell culture containing [^{123}I]-IUdR. This slightly higher growth rate of cells in [^{123}I]-***7a*** medium proved that its low uptake was not as a result of chemical toxicity effects induced to the cells. As a result of the very low DNA incorporation of [^{123}I]-***7a***, the *Z*-isomer cell binding study was not performed, as it was assumed that the geometric location of the iodine atom on either isomer is unambiguous and would have little effect upon the biological activity of the compound.

In order to establish whether the position of the radioiodine label had an influence on the inferior uptake of [^{123}I]-***7a***, an N^3-allyl thymidine analogue, [^{123}I]-***10*** was prepared in which the radioiodine label was placed in a similar location in the molecule (5-position) to that of [^{123}I]-IUdR. The rationale behind this selection was that [^{123}I]-***10*** and [^{123}I]-***7a*** are both N^3-substituted with a similar prop-2-enyl group, while the methyl group of [^{123}I]-***7a*** was replaced by a similarly sized radioiodine label in [^{123}I]-***10*** (see Figures 3.1-3 and 3.1-4). In addition, [^{123}I]-***10*** would also mimic an N^3-substituted propargyl analogue of thymidine which has been

reported to exhibit as high as a 98% phosphorylation rate compared to thymidine (Byun *et al.*, 2004). Despite the placement of the radioiodine label in a similar position to the one in [^{123}I]-IUdR, **[^{123}I]-*10***, however, did not show improved uptake into cells (see Figure 3.5-4). Its uptake was established to be only 2.5% relative to the gold standard [^{123}I]-IUdR.

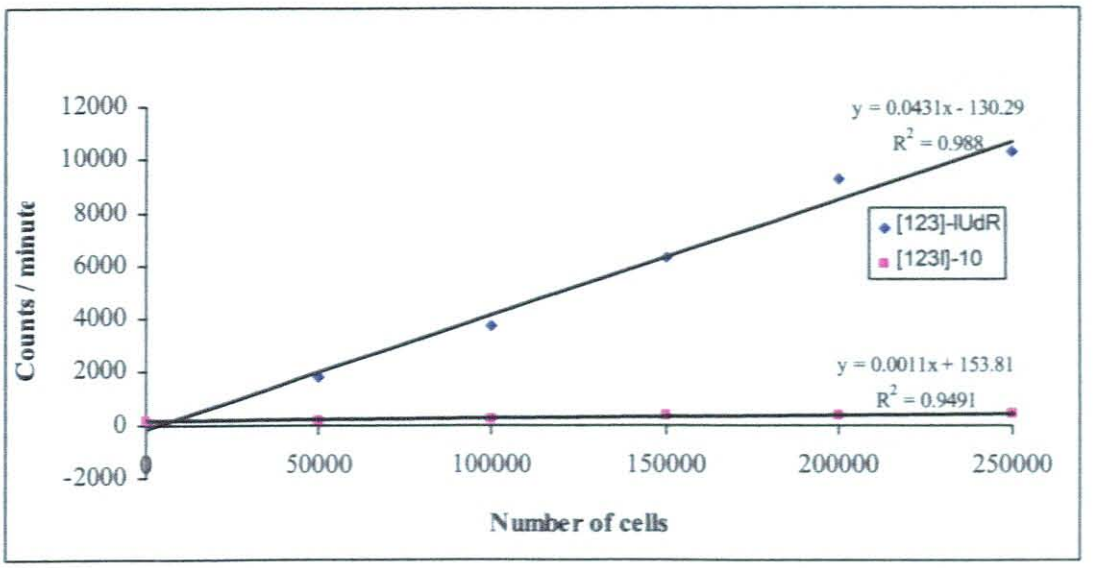

Figure 3.5-4 Comparison of the cell-uptake results of [^{123}I]-IUdR and **[^{123}I]-*10*** in CHO-K1 cells

If one assumes that the uptake of the radiotracers into the cell nucleus, and therefore into the DNA, is regulated only by TK1 enzyme action on the compound, their low uptake values are, to some extent, contradicted by some literature results, but also supported by a few others. The low uptake of **[^{123}I]-*10*** is, for example, contradictory to the earlier mentioned finding by Byun *et al.*, 2004. Some N^3-alkylated thymidine analogues having alkyl carbon spacer lengths equivalent to that of **[^{123}I]-*7a*** have been reported to be effective substrates of TK1 with medium to high enzymatic phosphorylation rates (Al-Madhoun *et al.*, 2004; Byun Y *et al.*, 2005). The *in vitro* properties of **[^{123}I]-*7a*** would be expected to mimic some of these compounds. The reported biological studies, however, were done using the *in vitro* enzyme assay method, as opposed to the *in vitro* nucleosides cell-uptake method performed in this study. Previously, a study on the *in vitro* cell-uptake of N^3-methyl substituted IVDU in rabbit kidney cells, infected by TK^+ herpes simplex virus, revealed that this compound had substantially less metabolic trapping in the cells than that of un-substituted IVDU (Tandon *et al.*, 1989). Results on the *in vivo* PET imaging animal studies of [^{18}F]-N^3-(2-fluoroethyl)-thymidine also showed lack of accumulation in tumours implanted in mice (Toyoharaa *et al.*,

2006). These latter results are in agreement with the results obtained in this study. The difference in uptake tendencies could probably be ascribed to different uptake mechanisms.

3.6 Preliminary conclusions

[^{123}I]-N^3-(prop-2-en-1-yl)-5-iodo-2'-deoxyuridine and [^{123}I]-N^3-[(*E* and *Z*)-(3-iodopropen-2-yl]thymidine analogues have been synthesised with high radiochemical yields and purities. The net *in vitro* cellular binding of these N^3-substituted thymidine analogues were very low and did not match the uptake of [^{123}I]-IUdR into cells. The pronounced differences in cell-uptake for the synthesised N^3-substituted thymidine analogues and [^{123}I]-IUdR can probably be attributed to the fact that [^{123}I]-IUdR, due to its ability to behave like thymidine, is phosphorylated and can enter the DNA of cells, whereas the N^3-substituted thymidine analogues appear to be excluded from the nucleus. Taking into consideration that no significant lethal effects are produced to cancer cells when the decaying Auger atom is situated within cellular cytoplasm, at the plasma membrane or outside the cells (Kassis *et al.*, 1987), it is concluded that N^3-substituted iodovinyl derivatives of thymidine do not have sufficient cellular uptake into the DNA of cells for therapeutic consideration. The precise reason(s) for these poor cellular uptakes is not entirely clear, but could possibly be linked to a lack of enzymatic action on the N^3-substituted compounds. Final conclusions and recommendations will be given in Chapter 5.

CHAPTER 4
Experimental methods and analysis

4.1 Materials and methods

4.1.1 General

Chemicals and solvents were reagent grade and used as received from commercial sources unless otherwise stated. Reactions were monitored qualitatively by thin layer chromatography (TLC) plates pre-coated with silica gel (Alugram Sil G-UV_{254}) and visualised using either a multi-band UV-254/366 nm lamp alone or in combination with iodine staining. Purification and isolation of target compounds were done on a column bed prepared using silica gel 60 with particle size of 0.063 – 0.2 mm (70 – 230 mesh ASTM).

4.1.2 Instrumentation

4.1.2.1 High Performance Liquid Chromatography (HPLC) system

The HPLC system set-up for reaction monitoring and quality control consisted of a Perkin Elmer 200 lc series binary pump equipped with a Rheodyne injector. A Phenomenex Luna C18 5μ analytical column (250 x 4.6 mm) was used for reversed phase and a Phenomenex Luna silica 10μ column with identical dimensions was used for normal phase chromatography. The column outlet was connected to a fixed wavelength (254 nm) Waters Series 440 UV Detector. A Hewlett-Packard model HP-3394 integrator set at an attenuation factor of 6 was used to record and integrate chromatograms.

Alternatively, a quaternary Agilent Technologies HPLC system was used for chemical and radiochemical purity assurance of cold preparations and radiochemical products using the same column(s) as in the above system. The set-up was composed of an Agilent 1100 Series pump, fitted with a programmable HP 1100 Series Control module for gradient elution, a Rheodyne injector, a Carroll & Ramsey model 105S-1 radio detector with a CsI (Tl) detector probe in a series with a variable wavelength Spectra Series UV100 detector and a Chromatopac C-R8A integrator from Shimadzu. In either systems the retention time and the chemical or the radiochemical purity of the compounds were displayed on a printed chromatogram.

Quality control of all preparations was done using acetonitrile/water (MeCN/H_2O) gradient solvent system with a flow rate of 1 ml/min. Non-radioactive as well as radioactive compounds were analysed using the illustrated procedure below. The percentage composition ratio is given with respect to volume of solvent.

Program	Time / minutes	%Composition (v/v)	
		H_2O	MeCN
Equilibration	10	25	75
Step 1	5	25	75
Step 2	5	0	100
Step 3	20	0	100

The same HPLC procedure was also used for the purification of the hydrolysed radiolabelled product. For this purpose, the program method was modified by inserting 100% water composition for the initial 3 minutes of step 1 to enhance elution of excess free ^{123}I from the radiolabelled compounds. All the HPLC data given in this work was obtained from the Agilent Technologies HPLC system although the Perkin Elmer HPLC was also used to monitor reactions during preliminary 'cold' synthesis work.

4.1.2.2 NMR experiments

^1H and ^{13}C NMR spectra were obtained on respective frequencies displayed for each preparation sample on a Varian Unity *Inova* NMR spectrometer with a 5 mm dual broad band and a probe temperature of 25°C. ^1H NMR Spectrum chemical shifts were reported as parts per million, δ units, downfield from Me_4Si (TMS) or a residual $CDCl_3$ singlet peak at 7.26 ppm.^{13}C NMR spectra were obtained or referenced to a solvent $CDCl_3$ triplet centre peak at 77 ppm. Abbreviations used for the multiplicity of signals are as follows:

s	singlet	dd	doublet of doublets
d	doublet	br	broadened
t	triplet	m	multiplet

4.1.2.3 MS experiments

High resolution mass spectra were obtained on a Waters API Q-TOF Ultima calibrated using NaF. Using solvent as a matrix, positive electrospray ionisation (ESI^+) technique was used on

compounds dissolved in either methanol or acetonitrile to obtain molecular ions, $[MH]^+$, and fragmentised molecular portions that were readily assignable. Electrospray ions are reported and the relative intensities are given in parentheses.

4.1.2.4 Radioactivity measurements

An Isocal MODEL Radionuclide Assay calibrator instrument was used to carry out radioactivity measurements. The vial containing the activity was put inside the well on a Teflon holder, a pre-determined calibration factor, which is linked to the volume of the activity and radioisotope was keyed in, and the radioactivity was read in millicuries (mCi). A millicurie is equal to one thousandth of a curie, which is a unit of ionizing radiation and equal to 3.7×10^{10} nuclear transformations in one second. Each radioactivity reading is corrected for decay using a known exponential decay equation as follows:

$$A_t = A_o\, e^{(-0.693\, t/t_{1/2})}$$

Here A_t is the expected activity reading at the time t different from the initial activity measurement at a specific time o (in hours); e is a natural log and in parenthesis, t is the time difference between time t and time o; and $t_{1/2}$ is a radionuclide half-life (13.27 hours in the case of ^{123}I).

4.2 Synthesis of precursors

4.2.1 Preparation of 3', 5'-O-bis-(2-tetrahydropyranyl)thymidine (or thymidine THP ether) *1*.

The compound was prepared according to a literature method described by Alauddin and Conti, 1994. Thymidine (199.8 mg, 0.825 mmol) in a glass vial was dissolved in anhydrous tetrahydrofuran (THF, 5 mL). *p*-Toluenesulphonic acid (15.3 mg) was added to the reaction vial followed by addition of 3,4-dihydro-2H-pyran (1.1 mL, 12 mmol). The reaction mixture was stirred at room temperature for 2.5 hours until TLC showed no remaining starting material. The reaction was quenched by adding 1 drop of triethylamine. The solvent was evaporated and the crude product was applied to a silica gel (7 g) column and purified and eluted with *n*-hexane/acetone (70:30, 60:40) respectively. The fractions were combined and evaporated to dryness, yielding a colourless oil that solidified to a white powder upon storage, (313.0 mg, 92% quantitative yield). The chemical purity (96%) of *1* was confirmed on reverse phase HPLC. ^1H NMR ($CDCl_3$) 600 MHz δ: 8.45 (s, 1H, NH), 7.61, 7.57, 7.56, 7.54 (4s, 1H, C_6H), 6.38 - 6.29 (m, 1H, 1'H), 4.72 - 3.47 (m, 10H, 3' - 5'H and THP), 2.52 - 2.28

(m, 2H, 2'H), 1.93, 1.91, 1.89 (3s, 3H, CH_3), 1.85 - 1.68 (m, 4H, THP), 1.64 - 1.48 (m, 8H, THP). ^{13}C NMR ($CDCl_3$) 150 MHz δ: 163.8 (C-4), 150.4 (C-2), 135.9 (C-6), 110.9 (C-5), 100.2 (C-2; THP), 98.62 (C-2; THP), 85.35 (C-4'), 83.95 (C-1'), 67.96 (C-3'), 63.52 (C-5'), 62.67 (C-6; THP), 62.16 (C-6; THP), 38.23 (C-2'), 30.88 (C-3; THP), 30.73 (C-3; THP), 25.65 (C-5; THP), 25.40 (C-5; THP), 20.38 (C-4; THP), 19.35 (C-4;THP), and 12.57 (CH_3).

4.2.2 Preparation of alkylating reagent

Preparation of the vinylstannylated alkylating agent was done in two steps: propargyl alcohol multiple bonds were reduced with tributyltin hydride followed by converting the hydroxyl substituent to a tosylate.

4.2.2.1 Preparation of (*E/Z*)-(3-tri-*n*-butylstannyl)prop-2-en-1-ol , *2a* and *2b*.

Propargyl alcohol (200 mg, 3.568 mmol) was added to a mixture of tributyltin hydride (1.069 mL, 3.675 mmol) and α-α'-azo*bis*isobutyronitrile (60 mg, 0.365 mmol). The mixture was heated at 80°C for 3 hours under a nitrogen atmosphere when TLC showed no starting material remained. The crude product was successfully purified and collected into fractions on a silica gel (7 g) column using *n*-hexane/ethyl acetate (98:2) and (95:5) in a stepwise elution. The fractions were monitored on TLC (*n*-hexane/ethyl acetate, 90:10). The developed TLC plates were observed under a UV lamp after spray coating/ staining with iodine. The separated products were composed of a joint mixture of *2b* and a regioisomer *2c* in the ratio of 1:1 (375.2 mg, 30% yield), and the more polar product was the *E*-isomer *2a*, which was readily separable and was obtained as a colourless oil in 218.8 mg and 17% overall yield. The isomers retention pattern on TLC was in agreement with those found in literature (Musachio and Lever, 1992) and no further structural elucidations were conducted.

4.2.2.2 Preparation of *(E/Z)*-(3-tri-*n*-butylstannyl)prop-2-en-1-yl-4-methylbenzenesulphonate, *3a* or *3b/3c*.

p-Toluenesulphonyl choride (35 mg, 0.072 mmol) was added to a solution of *2b/2c* mixture (60 mg, 0.067 mmol) in diethyl ether (1.5 mL) cooled to -5°C, and potassium trimethylsiloanolate (110 mg, 0.311 mmol) was added to a mixture in portions over 30 minutes. The reaction mixture was stirred between -5°C and -10°C over about 6.5 hours until TLC showed insignificant starting material remained. The reaction mixture was washed with water and extracted with diethyl ether. The organic solvent was dried with Na_2SO_4 and evaporated to dryness. The product (74.3 mg, 62% yield) was obtained after purification on a

silica gel (2.5 g) column using 10% ethyl acetate in *n*-hexane as the eluant. The product, ***3b/3c***, was obtained and the isomeric mixture was retained with a ratio of 1:1 as confirmed on HPLC.

3a was prepared from ***2a*** using the same procedure for the synthesis of ***3a/3c*** and it was obtained in a quantitative yield of 60% with a chemical purity of 87%.

4.2.3 N^3-alkylation of thymidine THP ether

The coupling of the alkylating agents to ***1*** was carried out with two different alkylating agents: ***1*** was coupled to the prepared tosylate derivatives, ***3a*** or ***3b/3c***, or alkylated with propargyl bromide.

4.2.3.1 Preparation of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-[(*E* and *Z*)-(3-tri-*n*-butylstannyl prop-2-en-1-yl]thymidine, *4a* and *4b*

The preparation of ***4a*** was performed following a coupling method by Alauddin *et al.*, 2006 with modifications. ***1*** (24.9 mg, 0.061 mmol) was dissolved in DMSO (600 µL) and a solution of ***3a*** (32 mg, 0.061 mmol) in acetone (600 µL) was added. Potassium carbonate monohydrate (41.3 mg, 0.299 mmol) was added as a base and the reaction mixture was heated at 54°C for approximately 27 hours until TLC showed no remaining starting material. The product was filtered, evaporated to dryness and re-dissolved in CH_2Cl_2 (6 mL). The solution was washed with distilled water (3 x 6 mL) and the organic solvent was dried over $MgSO_4$, and evaporated to dryness. The crude product was purified on silica gel (5 g) column using 20% acetone in *n*-hexane. The eluted fractions were monitored on TLC using *n*-hexane/acetone (70:30). Pure ***4a*** was recovered in fractions, 27.1 mg, 60% quantitative yield and a chemical purity of 95%. ^{1}H NMR ($CDCl_3$) 600 MHz δ: 7.54, 7.48, 7.45, 7.43 (4s, 1H, C_6H), 6.42 - 6.33 (m, 1H, 1'H), 6.07 (d, 1H, J = 19.5 Hz, SnC<u>H</u>=CH), 5.98 - 5.93 (dt, 1H, J_{trans} = 19.5 Hz, J = 5.6 Hz, SnCH=C<u>H</u>), 4.73 - 3.49 (m, 12H, 3'-5'H, N^3C_1H and THP), 2.54 - 2.48 (m, 1H, 2'H), 2.42 - 2.37 (m, 1H, 2'H), 1.96 - 1.90 (2s, 3H, CH_3), 1.85 - 1.67 (m, 4H, THP), 1.63 - 1.50 (m, 8H, THP), 1.50 - 1.42 (m overlapping, 6H, *n*Bu), 1.30 - 1.24 (m, 7H, *n*Bu), 0.89 - 0.83 (m,14H, *n*Bu). ^{13}C NMR ($CDCl_3$) 150 MHz δ: 163.1 (C-4), 150.7 (C-2), 140.8 (C-6), 133.5 (HC=<u>C</u>HSn), 131.8 (H<u>C</u>=CHSn), 109.09 (C-5), 98.86 (C-2; THP), 97.27 (C-2; THP), 85.92 (C-4'), 84.37 (C-1'), 67.67 (C-3'), 63.05 (C-5'), 62.55 (C-6; THP), 62.34 (C-6; THP), 46.15 (N^3CH_2), 39.28 (C-2'), 30.68 (C-3; THP), 30.62 (C-3; THP), 29.05 ($SnCH_2\underline{C}H_2$), 27.32 ($SnCH_2CH_2\underline{C}H_2$), 25.31 (C-5; THP), 25.13 (C-5; THP), 19.59 (C-4; THP), 19.03 (C-4; THP),

13.34 (CH_3), 13.66 ($SnCH_2$ $CH_2CH_2\underline{C}H_3$), 9.44 ($SnCH_2$). MS ESI$^+$: 764.34 (M + Na, 11), 741.35 (M + H, 84), 657.29 (100), 655.26 (76), 653 (40), 457.19 (21), 363.98 (50).

A separate preparation of stereoisomer ***4b*** was done using a similar procedure and starting from the ***3b/3c*** (32.4 mg, 0.06 mmol) mixture and ***1*** (25 mg, 0.06 mmol). A silica gel chromatographic purification using 40% acetone in *n*-hexane as a mobile phase yielded 35 mg of a product. The isocratic HPLC analysis (10 µm silica gel column) using *n*-hexane/ethyl acetate (80:20) as mobile phase, displayed a mixture of two isomers (***4b/4c***, ~1:1 ratio) with very close retention times. In order to isolate ***4b***, the isomeric mixture was re-separated on a silica gel (4 g) bed using *n*-heptane / ethyl acetate (80:20). ***4b*** was successfully isolated (19 mg, 94% chemical purity). The identity of ***4b*** was matched to a parallel preparation of the same compound on the basis of retention time on HPLC. Thus ^1H and ^{13}C NMR spectral characteristics were observed for ***4b*** and are presented in section **4.2.4.1** for the preparation of the same product following route (ii) from ***5*** (refer to Figure 3.1-2).

4.2.3.2 Preparation of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-(prop-2-yn-1-yl)thymidine *5*.

Compound ***5*** was prepared by coupling propargyl bromide to thymidine THP ether following a literature method (Ghosh *et al.*, 2007). Compound ***1*** (201.6 mg, 0491 mmol) was dissolved in dimethyl sulphoxide (DMSO, 5 mL) and to the solution was added potassium carbonate (339.3 mg, 2.45 mmol). 3-Bromoprop-1-yne (36.9 µL, 0.491 mmol) in acetone (5 mL) was added and the resulting reaction mixture was stirred at 56°C for 24 hours until TLC showed that all the starting material was consumed. The reaction mixture was concentrated by evaporating the acetone and the base was filtered off. The crude product, in DMSO, was taken up in dichloromethane (23 mL) and washed with water (3 x 23 mL). The organic phase was dried over magnesium sulphate ($MgSO_4$) and evaporated to dryness. The remaining product was purified on a silica gel (6 g) column using 25% and 30% acetone in *n*-hexane in a stepwise elution. The pure product of 181 mg (82% quantitative yield) was recovered and a chemical purity of 95% was obtained. ^1H NMR ($CDCl_3$) 400 MHz δ: 7.67, 7.62, 7.60, 7.56 (4s, 1H, C_6H), 6.41 - 6.33 (m, 1H, 1'H), 4.71 - 3.47 (m, 12H, 3' - 5'H, N^3C_1H and THP), 2.56 - 2.48 (m, 1H, 2'H), 2.43 - 2.37 (m, 1H, 2'H), 2.17 - 2.14 (br t, 1H, N^3C_3H), 1.96, 1.95, 1.92, 1.91 (4s, 3H, CH_3), 1.86 - 1.45 (m, 12H, THP). ^{13}C NMR ($CDCl_3$) 100 MHz δ: 162.7 (C-4), 150.4 (C-2), 134.3 (C-6), 110.2 (C-5), 100.1 (C-2; THP), 99.07 (C-2; THP), 98.32 (C-2), 86.18 (C-4'), 84.72 (C-1'), 78.48 ($N^3CH_2\underline{C}CH$), 70.69 ($N^3CH_2C\underline{C}H$), 67.89 (C-3'), 63.46 (C-5'), 62.83 (C-6; THP), 62.62 (C-6; THP), 39.55 (C-2'), 30.92 (C-3; THP), 30.86 (C-3; THP),

30.58 (N^3CH_2), 25.52 (C-5; THP), 25.44 (C-5; THP), 20.28 (C-4; THP), 19.55 (C-4; THP), and 13.26 (CH_3). MS ESI^+: 471.19 (M + Na, 100), 449.23 (M + H, 17), 365.17 (59), 281.10 (86), 165.06 (84).

4.2.4 Hydrostannylation

4.2.4.1 Preparation of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-[(*E/Z*)-(3-tri-*n*-butylstannyl prop-2-en-1-yl)]thymidine, *4a* or *4b* via hydrostannylation.

The preparation of a compound was performed according to the procedure of Waterhouse *et al.*, 1996, with minor modifications. *n*-Tributyltin hydride (70 mg, 0.241 mmol) was added to a solution of *5* (90 mg, 0.201 mmol) in anhydrous toluene (3 mL) and a catalytic amount of α-α'-azo*bis*isobutyronitrile (AIBN; 10 mg, 0.061 mmol) was added. The reaction mixture was heated at 92°C for 9 hours under nitrogen until TLC displayed complete consumption of the starting material. The solvent was evaporated and the product was purified on silica gel (3 g) column using *n*-hexane/acetone (80:20). The product was collected in fractions and monitored on TLC using *n*-hexane/ethyl acetate (70:30). The following products were obtained: ***4a*** was 113 mg, 76% quantitative yield and a chemical purity of 92%, ***4b/4c*** was 18 mg and a 12% quantitative yield. Separation of ***4b/4c*** was done as described earlier on section **4.2.3.1**.

The structure of ***4a*** had already been assigned on ^1H and ^{13}C NMR and MS from a previous preparation (see **4.2.3.1**). Thus another stereoisomer, ***4b***, was characterised on ^1H and ^{13}C NMR and its chemical structure assignments details are provided as shown below. The third regioisomer, ***4c***, was regarded as a by-product and was not characterised. ^1H NMR ($CDCl_3$) 600 MHz δ: 7.54, 7.48, 7.45, 7.43 (4s, 1H, C_6H), 6.42 – 6.31 (m, 1H, 1'H), 6.07 (d, 1H, J = 12.5 Hz, SnC<u>H</u>=CH), 5.89 - 5.71 (dt, 1H, J = 12.5 Hz, J = 5.6 Hz, SnCH=C<u>H</u>), 4.72 - 3.49 (m, 12H, 3' - 5'H, N^3C_1H and THP), 2.52 – 2.46 (m, 1H, 2'H), 2.41 – 2.35 (m, 1H, 2'H), 1.92 (2s, 3H, CH_3), 1.85 – 1.41 (m, 12H, THP), 1.39 – 1.21 (m, 6H, *n*Bu), and 1.07 - 0.82 (m, 21H, *n*Bu). ^{13}C NMR ($CDCl_3$) 150 MHz δ: 162.2 (C-4), 149.7 (C-2), 141.6 (C-6), 132.5 (HC=<u>C</u>HSn), 131.6 (H<u>C</u>=CHSn), 108.9 (C-5), 98.08 (C-2; THP), 96.40 (C-2; THP), 84.66 (C-4'), 82.59 (C-1'), 66.65 (C-3'), 62.00 (C-5'), 61.54 (C-6; THP), 61.35 (C-6; THP), 44.43 (N^3CH_2), 38.25 (C-2'), 29.71 (C-3; THP), 29.61 (C-3; THP), 28.18 ($SnCH_2\underline{C}H_2$), 26.31 ($SnCH_2CH_2\underline{C}H_2$), 25.83 (C-5; THP), 24.24 (C-5; THP), 18.92 (C-4; THP), 18.14 (C-4; THP), 16.13 (CH_3), 12.17 ($Sn(CH_2)_3\underline{C}H_3$), 9.3 ($Sn\underline{C}H_2$).

4.2.5 Iododestannylation

4.2.5.1 Preparation of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-[(*E*)-(3-iodoprop-2-en-1-yl)thymidine, *6a*.

The compound was prepared by displacement of *n*-tributyltin by iodine in a single step procedure at room temperature according to a slightly modified method by Waterhouse *et al.*, 1996. A solution of iodine (36.8 mg, 0.145 mmol) in dichloromethane (1 mL) was added dropwise to *4a* (50 mg, 0.07 mmol) dissolved in 2 mL dichloromethane in a separate vial. The reaction mixture was stirred in the absence of light for 5 minutes until the reaction mixture did not decolourise further upon additional iodine drops. The reaction mixture was further stirred for another 5 minutes in excess iodine which was reduced by addition of 200 µL aqueous sodium metabisulphite (25 mg/mL; 0.026 mmol). The reaction mixture was diluted with distilled water (4 mL) and the product was extracted into dichloromethane (3 x 4 mL). The organic extracts were dried ($MgSO_4$), the solvent evaporated to dryness to obtain a pale yellow oil. The product was further purified on a silica gel column (2 g) using a stepwise elution with 20% and 30% acetone in petroleum ether. The pure product, *6a*, was obtained in 32 mg (82% quantitative yield) with a chemical purity of 94%. ^{1}H NMR ($CDCl_3$) 600 MHz δ: 7.66, 7.61, 7.56, 7.54 (4s, 1H, C_6H), 6.64 - 6.58 (dt, 1H, J_{trans} = 14.2 Hz, J = 6.6 Hz, HC=CHI), 6.49 - 6.46 (d, 1H, J = 14.2 Hz, HC=CHI), 6.39 - 6.30 (m, 1H, 1'H), 4.72 - 3.48 (m, 12H, 3' - 5'H, N^3C_1H and THP), 2.54 - 2.48 (m, 1H, 2'H), 2.42 - 2.37 (m, 1H, 2'H), 1.95, 1.90 (2s, 3H, CH_3), 1.84 - 1.68 (m, 4H, THP), 1.63 - 1.51 (m, 8H, THP). ^{13}C NMR ($CDCl_3$) δ: 163.1 (C-4), 150.7 (C-2), 139.1 (C-6), 134.1 (HC=CHI), 110.1 (C-5), 100.1 (C-2; THP), 98.26 (C-2; THP), 86.21 (C-4'), 84.7 (C-1'), 82.98 (HC=CHI), 67.87 (C-3'), 63.38 (C-5'), 62.79 (C-6; THP), 62.61 (C-6; THP), 44.61 (N^3CH_2), 38.21 (C-2'), 30.91 (C-3; THP), 30.87 (C-3; THP), 25.51 (C-5; THP), 25.42 (C-5; THP), 20.43 (C-4; THP) 19.27 (C-4; THP), 13.25 (CH_3). MS ESI$^+$: 599.11 (M + Na, 92), 577.14 (M + H, 17), 494.09 (17), 493.08 (78), 409.01 (92), 293.98 (30), 292.96 (100), and 165.07 (12).

4.2.6 Hydrolysis

4.2.6.1 Preparation of N^3-[(*E*)-(3-iodoprop-2-en-1-yl)]thymidine, *7a*.

The compound was prepared from *6a* via acid hydrolysis of THP protecting groups in HCl. Hence, *6a* (23 mg, 0.04 mmol) was dissolved in methanol (1 mL) and hydrochloric acid (80 µL of 1 M HCl in methanol) was added to the solution. The reaction mixture was refluxed for 6 minutes, cooled to room temperature and the solvent evaporated. The crude product was

purified on a silica gel column (2 g) using a stepwise elution of 30% and 40% acetone in petroleum ether as eluants. The pure compound was recovered in fractions, 11mg, 68% quantitative yield and a chemical purity of 96%. ^{1}H NMR ($CDCl_3$) 600 MHz δ: 7.38 (s, 1H, C_6H), 6.61 - 6.56 (dt, 1H, J_{trans} = 14.3 Hz, J = 5.8 Hz, HC=CHI), 6.49 - 6.46 (d, 1H, J = 14.3 Hz, HC=CHI), 6.19 (t, 1H, J = 6.70 Hz, 1'H), 4.58 - 4.55 (m, 1H, 3'H), 4.45 (d, 2H, J = 6.70 Hz, N^3C_1H), 3.99 (m, 1H, 4'H), 3.91 - 3.83 (m, 2H, 5'H), 2.62 - 2.46 (br s, 2H, 3'OH and 5'OH), 2.41 - 2.36 (m, 1H, 2'H), 2.33 - 2.30 (m, 1H, 2'H), 1.92 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) 150 MHz δ: 163.1 (C-4), 150.7 (C-2), 139.1 (C-6), 134.1 (HC=CHI), 110.1 (C-5), 87.21 (C-4'), 84.61 (C-1'), 82.48 (HC=CHI), 72.00 (C-3'), 62.85 (C-5'), 44.92 (N^3CH_2), 40.45 (C-2'), 13.62 (CH_3). MS ESI$^+$ (CH_3CN): 409.03 (M + H, 16), 293.98 (13), 292.97 (100), 165.07 (9).

4.2.7 Synthesis of N^3-allyl-5-trimethylstannyl-2'-deoxyuridine, N^3-allyl-5-TMS-UdR, precursor.

This compound was prepared in a four step procedure from 5-iodo-2'-deoxyuridine (IUdR).

4.2.7.1 Preparation of 3',5'-O-bis(2-tetrahydropyranyl)-5-iodo-2'-deoxyuridine, IUdR THP ether, *8*.

The compound was prepared from IUdR (200 mg, 0.565 mmol) following the procedure used for preparation of *1*.

4.2.7.2 Preparation of 3',5'-O-bis(2-tetrahydropyranyl)-N^3-(prop-2-en-1-yl)-5-iodo-2'-deoxyuridine, N^3-allyl-IUdR THP ether, *9*.

The compound was prepared from *8* (261 mg, 0.500 mmol) and 3-bromoprop-1-ene (6.2 mg, 0.512 mmol) following the procedure similar to the preparation of *5*. After silica gel (2.5 g) column purification, *9* was quantitatively obtained with a quantitative yield of 264 mg, 94% and a chemical purity of 98%. ^{1}H NMR ($CDCl_3$) 600 MHz δ: 8.24, 8.20, 8.18, 8.17 (4s, 1H, C_6H), 6.37 - 6.34 (m, 1H, 1'H), 5.90 - 5.82 (m, 1H, N^3CH_2CH), 5.29 (d, 1H, J_{trans} = 17.2 Hz, $N^3CH_2CH=CH_2$), 5.21 (d, 1H, J_{cis} = 10.2 Hz, $N^3CH_2CH=CH_2$), 4.72 - 3.48 (m, 12H, 3'-5'H, N^3C_1H and THP), 2.61 - 2.55 (m, 1H, 2'H), 2.49 - 2.40 (m, 1H, 2'H), 2.23 - 1.48 (m, 12H, THP). ^{13}C NMR (CDCl3) 150 MHz δ: 159.7 (C-4), 150.4 (C-2), 143.0 (C-6), 131.3 (N^3CH_2CH-), 119.0 ($N^3CH_2CHCH_2$), 100.2 (C-2; THP), 98.65 (C-2; THP), 86.97 (C-4'), 84.55 (C-1'), 67.79 (C-3'), 63.31 (C-5), 62.67 (C-6; THP), 45.04 ($N^3C_1H_2$), 38.91 (C-2'),

31.01 (C-3; THP), 30.82 (C-3; THP), 25.25 (C-5; THP), 20.25 (C-4; THP), and 19.31 (C-4; THP). MS ESI$^+$ (CH_3CN): 585.11 (M + Na, 24.29), 563.13 (M + H, 54.28), 479.07 (100), 395.01 (54.43), and 363.02 (12.86).

4.2.7.3 Preparation of N^3-(prop-2-en-1-yl)-5-iodo-2'-deoxyuridine, N^3-allyl IUdR, *10*.

The compound was prepared by direct acid hydrolysis of the THP protecting groups of *9* (230 mg, 0.409 mmol) according to the procedure used for the preparation of *6a* with modifications. After neutralising the reaction mixture with $NaHCO_3$, excess methanol was evaporated; the crude product was diluted with water (8 mL), and extracted into diethyl ether (3 x 8 mL). The organic extracts were dried by $MgSO_4$ and the solvent was evaporated to dryness to obtain a colourless oil. The crude product was further purified on a silica gel (2 g) column using 30% ethyl acetate in *n*-heptane to recover the pure product in fractions. The product, *10*, was obtained in 113 mg, 70.12% quantitative yield and a chemical purity of 99%. ^{1}H NMR ($CDCl_3$) 600 MHz δ: 8.22 (s, 1H, C_6H), 6.22 (t, 1H, J = 6.7, 1'H), 5.89 - 5.81 (m, 1H, $N^3CH_2C\underline{H}$), 5.29 (d, 1H, J_{trans} = 17.1 Hz, $N^3CH_2CH{=}C\underline{H_2}$), 5.20 (d, 1H, J_{cis} = 10.2 Hz, $N^3CH_2CH{=}C\underline{H_2}$), 4.58 - 4.56 (m, 3H, 3'H and N^3-C_1H), 4.05 (m, 1H, 4'H), 3.98 - 3.88 (m, 2H, 5'H), 2.43 - 2.38 (m, 1H, 2'H), 2.36 - 2.31 (m, 1H, 2'H), 2.18 (br s, 2H, 3'OH and 5'OH). ^{13}C NMR (CDCl3) 150 MHz δ: 160.1 (C-4), 150.9 (C-2), 144.4 (C-6), 131.7 ($N^3CH_2\underline{C}H$), 119.6 ($N^3CH_2CH\underline{C}H_2$), 87.97 (C-4'), 87.71 (C-1'), 71.96 (C-3'), 68.74 (C-5), 62.78 (C-5'), 45.53 ($N^3\underline{C}H_2$), and 41.73 (C-2'). MS ESI$^+$ (CH_3CN): 395.02 (M + H, 42.8), 369.19 (5.71), 279.97 (10.00), 278.96 (100), 252.95 (2.86), and 238.93 (3.57).

4.2.7.4 Preparation of N^3-(prop-2-en-1-yl)-5-trimethyltin-2'-deoxyuridine, *11*.

The compound was prepared from *10* using a modified method of Wigerinck *et al.*, 1993. Hence, N^3-allyl-IUdR, *10* (41 mg, 0.104 mmol) was dissolved in 1,4-dioxane (1.5 mL) and bis(triphenylphosphine)palladium(II) dichloride (7 mg, 0.01 mmol) was added. Hexamethyl*di*tin (42 mg, 0.125 mmol) was added to the reaction mixture. The reaction mixture was refluxed for about 4 hours under a nitrogen atmosphere. The reaction mixture was cooled and a brownish-black solid residue was filtered off on a glass filter fitted with a cotton wool plug and washed with chloroform. The organic solvent was evaporated and the product was further purified on silica gel (2.5 g) column using *n*-heptane/ethyl acetate (30:70) as an eluant. *11* was obtained in 12 mg, 27% quantitative yield and a chemical purity of 97% after second purification on a silica gel column. ^{1}H NMR ($CDCl_3$) 600 MHz δ: 7.20 (s, 1H, C_6H), 6.10 (t, 1H, J = 6.7 Hz, 1'H), 5.85 - 5.78 (m, 1H, $N^3CH_2C\underline{H}$), 5.18 (d, 1H, J_{trans} = 16.9

Hz, $N^3CH_2CH=C\underline{H}_2$), 5.10 (d, 1H, J_{cis} = 10.4 Hz, $N^3CH_2CH=C\underline{H}_2$), 4.55 - 4.44 (m, 3H, 3'H and N^3-C_1H), 3.95 (m, 1H, 4'H), 3.85 - 3.76 (m, 2H, 5'H), 2.60 (m, 1H, 2'H), 2.45 - 2.36 (m, 2H, 2'H & 3'OH overlapping), 2.28 - 2.22 (m, 1H, 5'OH), and 0.21 (s, 9H, CH_3). ^{13}C NMR (CDCl3) 150 MHz δ: 165.6 (C-4), 151.5 (C-2), 143.4 (C-6), 132.2 ($N^3CH_2\underline{C}H$), 118.1 (N^3C-3), 112.5 (C-5), 88.35 (C-4'), 87.19 (C-1'), 71.81 (C-3'), 62.69 (C-5'), 43.54 (N^3CH_2), 40.37 (C-2'), -9.04 ($SnCH_3$).

4.3 Radiochemistry and labelling.

4.3.1 Preparation of [^{123}I]-N^3-[(*E*)-(3-iodoprop-2-en-1-yl)]thymidine, [^{123}I]-*7a*.

Preparations and labelling of [^{123}I]-*7a* were optimised following a procedure by Waterhouse *et al.*, 1996. Hence, acetic acid (2.5 µL) was added to a solution of sodium [^{123}I]-iodide (5 µL) in aqueous sodium hydroxide (0.03 M) in a glass vial. To this solution, 5 µL of chloramine-T trihydrate (125 µg, 25 µg/µL) dissolved in methanol/water (80:20) was added, followed by immediate addition of 25 µL of the precursor (*4a*; 50 µg, 0.088 µmol) dissolved in methanol. The reaction mixture was mixed on a vortex mixer for 3 minutes before addition of sodium metabisulphite (2µL, 1N). After 1 minute further mixing on a vortex mixer, a solution of 3.1 M HCl in methanol (2 µL) was added to the reaction mixture, followed by heating in the oil bath at 80°C for 5 minutes. The product was isolated by means of HPLC purification; pure [^{123}I]-*7a* was obtained by collecting the relevant HPLC fraction (3.2 mL) in a glass vial. The solvent was evaporated to dryness under nitrogen to yield a product of approximately 2 mCi, 63% radiochemical yield with greater than 99% radiochemical purity. The dried residue was re-dissolved in a solution of water/ethanol/0.1 M phosphate buffer pH 7 (85/5/10) to get the effective concentration of 1.7 mCi/mL (equivalent to a standard [^{123}I]-IUdR solution).

4.3.2 Preparation of [^{123}I]-5-iodo-N^3-(prop-2-en-1-yl)-2'-deoxyuridine, [^{123}I]-*10*.

[^{123}I]-*10* was prepared according to a procedure by Semnani *et al.*, 2005 with modifications. Hence, *11* (50 µg, 0.114 µmol), dissolved in ethanol (10 µL) was added to 15 µL of 0.15 M phosphate buffer (pH = 7.4), 5 µL of freshly prepared chloramine-T trihydrate (25 µg/µL, H_2O/ MeOH = 80:20), and 5 µL of 3.51 mCi [^{123}I]-NaI in ~0.03 M sodium hydroxide. The reaction mixture was homogenised on a vortex mixture at room temperature for 2 minutes and the reaction was quenched by the addition of 2 µL $Na_2S_2O_5$ (1.5 µg/µL). The product was isolated by HPLC purification as described before. Purified [^{123}I]-*10* was obtained by collecting HPLC fractions (4.6 mL) in a glass vial. The solvent was evaporated to dryness on

a hot plate under nitrogen. The recovered radioactivity of [^{123}I]-*10* was 90% at the end of synthesis and the radiochemical purity was over 99%. [^{123}I]-*10* was re-dissolved in water/ethanol/0.1 M phosphate buffer pH 7 (85/5/10) to get an effective concentration of 1.0 mCi/mL.

4.3.3 Preparation of [^{123}I]-5-iodo-2'-deoxyuridine ([^{123}I]-IUDR)

The preparation of this reference compound and its labelling precursor did not form part of this work and was conducted as part of another study. The precursor, 5-(trimethylstannyl)-2'-deoxyuridine was synthesised from 5-iodo-2'-deoxyuridine according to the method of Wigerinck *et al.*, 1993. The radiodination was carried out according to a slightly modified method of Semnani *et al.*, 2005, using chloramine-T as oxidant. The radioiodinated product was purified by means of reversed phase HPLC, using a mixture of water/ethanol/0.1 M phosphate buffer pH 7 = 85/5/10 (v/v/v) as mobile phase and a flow rate of 1 ml/min. The fraction containing the desired product was used as it is for the cell-uptake studies.

4.4 *In Vitro* cell-uptake studies

4.4.1 Method for the cell-uptake studies

Chinese Hamster Ovarian epithelial cells (CHO-K1), fast growing cell lines were used for the cell-uptake of the radiolabelled thymidine derivatives studied. The characteristic phases of the cell are as follows: M = 1hour, S = 6 hours, G_1 = 1hour, G_2 = 3 hours.

Cellular uptake and growth-dependent cell-uptake studies were performed by iThemba LABS Radiation Biophysics Group. In summary, plated monolayer cell seeding was harvested from 24-well plates in multiples of 50 x 10^3 (< 3 x 10^5 to ensure a state of exponential growth in S-phase). Cells were incubated in RPMI 1640 with 10% foetal calf serum in 5% CO_2-humidified atmosphere at 37°C for 4 hours prior to the cell study. The incubated cells were treated with a radiotracer (1.5 µCi in phosphate buffer saline per well) and incubation continued for 60 minutes for the cell-uptake. After 60 minutes, the incubation medium was removed and cells were washed twice with ice-cold PBS. 500 µL of 1 M NaOH was added on washed cells for 10 minutes to break cells open and release radioactivity (cell lysis). Lysate (500 µL) was transferred and measured in a multi-channel radioactivity scintillator to obtain uptake into cells. Cell counting for each seeding (number of cells per well) was done in replicates of 4 following cell lysis.

4.4.2 Method for evaluating chemical toxicity of radiotracer(s) in CHO-K1 cells

To determine the toxicity of [^{123}I]-*6*, the number of live cells in all cell-cultures were counted by means of a haemocytometer. The cell cycle activity of the CHO-K1 cells was evaluated and examined by culturing the cells in different media with radiotracers and a control (RPMI 1640 with10% foetal calf serum) over a period of 30 hours. The cells were seeded and harvested similarly to the experimental conditions in the *in vitro* cell binding studies. The harvested cell colonies were incubated with [^{123}I]-*6*, gold standard [^{123}I]-IUdR, and the control medium. After 30 hours, the incubated cells were transferred and counted under a haemocytometer chamber.

Chapter 5

Summary, final conclusions and recommendations

5.1 Summary of study results

The primary objectives of this work were achieved. The synthesis of N^3-substituted radioiodinated pyrimidine derivatives was successfully accomplished. It was started by preparing suitable radiolabelling precursors. It was shown that the introduction of a (3-tri-*n*-butylstannyl)prop-2-en-1-yl group at the N^3 position of THP-protected thymidine can be easily accomplished by following two different routes. The first route comprised a one-step N^3-alkylation with a pre-synthesised (*E*)-3-tributylstannyl)prop-2-en-1-yl-4-tosylate, while the second route comprised the initial introduction of a prop-2-yn-1-yl group at the N^3-position, followed by a hydrostannylation reaction to introduce the tributylstannyl group at the terminal carbon atom. Both routes yielded identical end-products and the spectrometric and spectroscopic data obtained suggested that the precursors and reference compounds had the correct chemical structures. The first route had the advantage of directly establishing the desired stereochemistry around the vinyl carbon bond without having to separate the isomers, but this advantage is cancelled out by the need to pre-synthesise the alkylation agent. Given the similar overall yields obtained by the two routes (60% versus 62%), none of the routes has a significant advantage over each other. Besides, the stereochemistry of the final compound did not feature in the biological investigations and was, therefore, irrelevant. The synthesis of the deoxyuridine precursor, in which the tributylstannyl group was introduced at the C-5 position of the base ring and an allyl group at the N^3-position, also proved to be straightforward, however, a very low yield was obtained with the palladium-catalysed Stille cross coupling reaction.

Radiolabelling of the synthesised precursors with radioiodine was done under mild reaction conditions and achieved within a reasonably short period. De-protection of the intermediate radioiodinated compound ***6a*** proved to be facile. The similar HPLC retention times of authentic reference standards and radioiodinated products confirmed the authenticity of the latter. The most outstanding features of the optimised radiolabelling method and HPLC

purification was the ability to obtain products with high radiochemical yields (73% - 91%) and excellent radiolabelling purities (up to 99%). Additionally, the radiolabelled compounds showed less than 1% deiodination when analysed 24 hours post synthesis.

A disappointing outcome of this study was the apparent lack of any significant cell-uptake of the radioiodinated N^3-substituted thymidine and deoxyuridine analogues. The successful uptake of the benchmark control product, [^{123}I]-IUdR, suggested that the chosen CHO-K1 cells were in the correct S-phase and, therefore, functioning correctly. Cell growth studies in various media have also shown that the cells were viable. The lack of uptake of the N^3-substituted 5-iododeoxyuridine ***10*** also suggested that the uptake is not influenced by the position of the iodine atom in the molecule, but most probably by the availability of the N^3 atom in its free, non-substituted form. Uptake could also not have been affected by the size and/or lipophilicity of the N^3-substituent, as the allyl group in compound ***10*** is smaller and less lipophilic than the iodoallyl group in compound ***7a***. As this work is chemistry-orientated, one can but only speculate on the possible biological reasons for these results. The main reason could be a lack of enzymatic action on the N^3-substituted compounds. This latter action ensures that the compound will be phosphorylated and enter the cell nucleus to be taken up into the DNA (internalised). Repeated washing of cells sample monolayers will remove intracellular radioactivity if not bound to DNA. Even if the compound is taken up by the cytoplasm, for example, but not phosphorylated by the relevant enzyme, it will not enter the cell DNA. As mentioned in the introductory chapters, enzymatic assay studies performed by various investigators on N^3-substituted thymidine analogues have shown that for the abilities of these compounds to be effective substrates for TK1, one of the main enzymes present in cells must be variable. In the current work, enzymatic assay studies were not performed, but cell-uptake abilities had been directly measured. The assay method could also have an effect on results. In any case, the cellular uptake mechanism of nucleosides is, most probably, a highly complicated process which is perhaps not only regulated by enzyme action but also by other factors. A more in-depth investigation into these possible factors falls beyond the scope of this study.

5.2 Final conclusions and recommendations

One of the main objectives of this study was to synthesise a radioiodinated nucleoside (thymidine) in which the iodine atom is placed at a stable vinylic position. It was hoped that such a compound would show favourable cell-uptake characteristics comparable to, or even

better than that of, [^{123}I]-IUdR. It has been shown in this study that the introduction of an iodovinyl moiety into thymidine via the insertion of an iodoallyl group at the N^3-position is feasible from a chemistry point-of-view. Unfortunately, its cell-uptake was poor and such a compound, therefore, will not be suitable as a carrier of Auger electrons into cells. This result also does not justify following the *in vivo* metabolic radiochemical stability studies as proposed in the research objectives. Despite the more complicated chemistry involved, as well the risk of forming deglycosylated products as postulated by Yu *et al.*, 2003, the 5-position of the base ring might still be a better location to introduce an iodovinyl group, as has already been shown in other studies with (*E*)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU). Comparative *in vitro* cell-uptake studies of [^{123}I]-IVDU and [^{123}I]-IUdR could also be a fruitful area for investigation, as this has apparently not been done before.

Appendix I

^{1}H NMR and ^{13}C NMR spectra

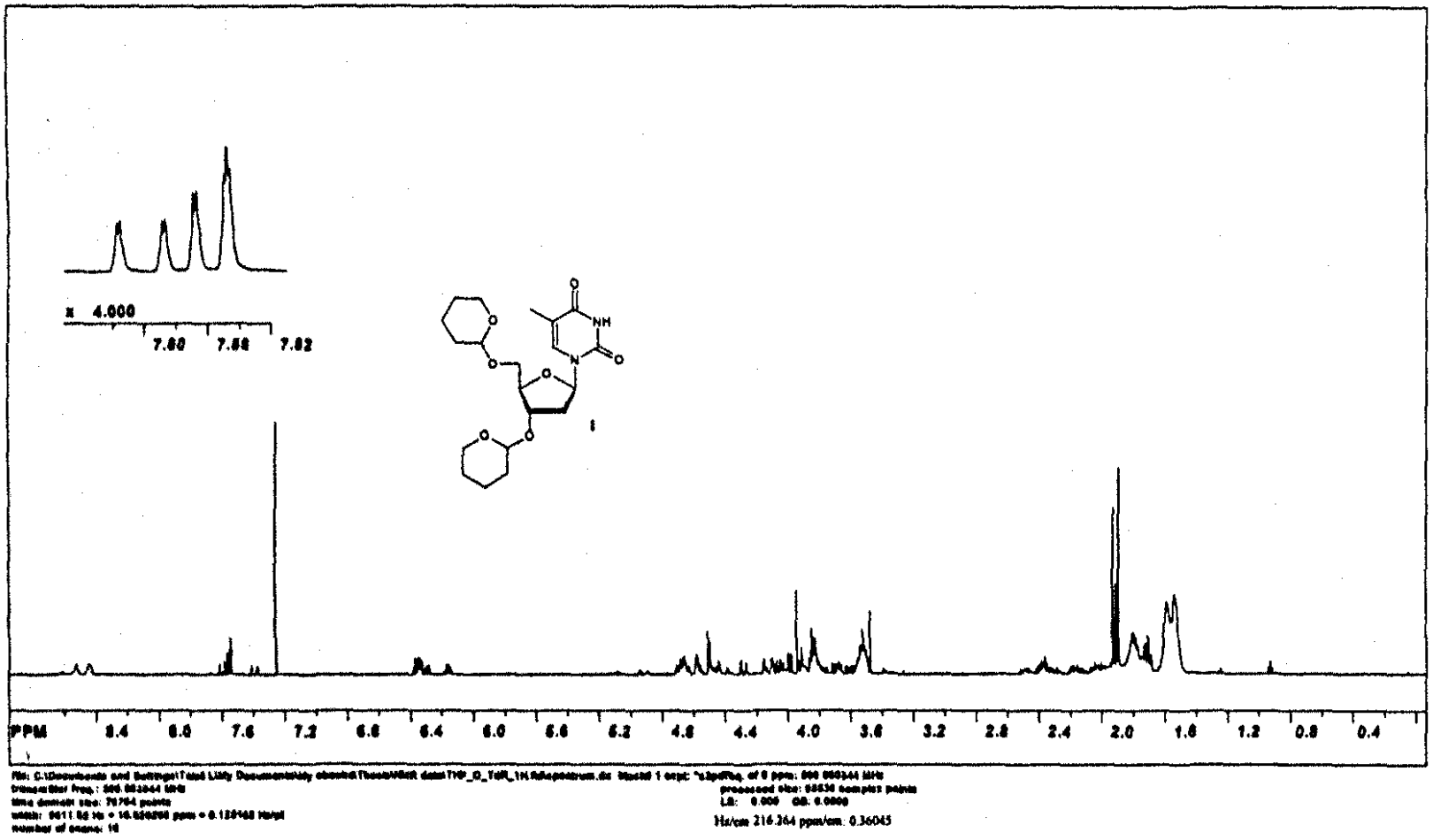

Figure I-1 The ^{1}H NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)thymidine

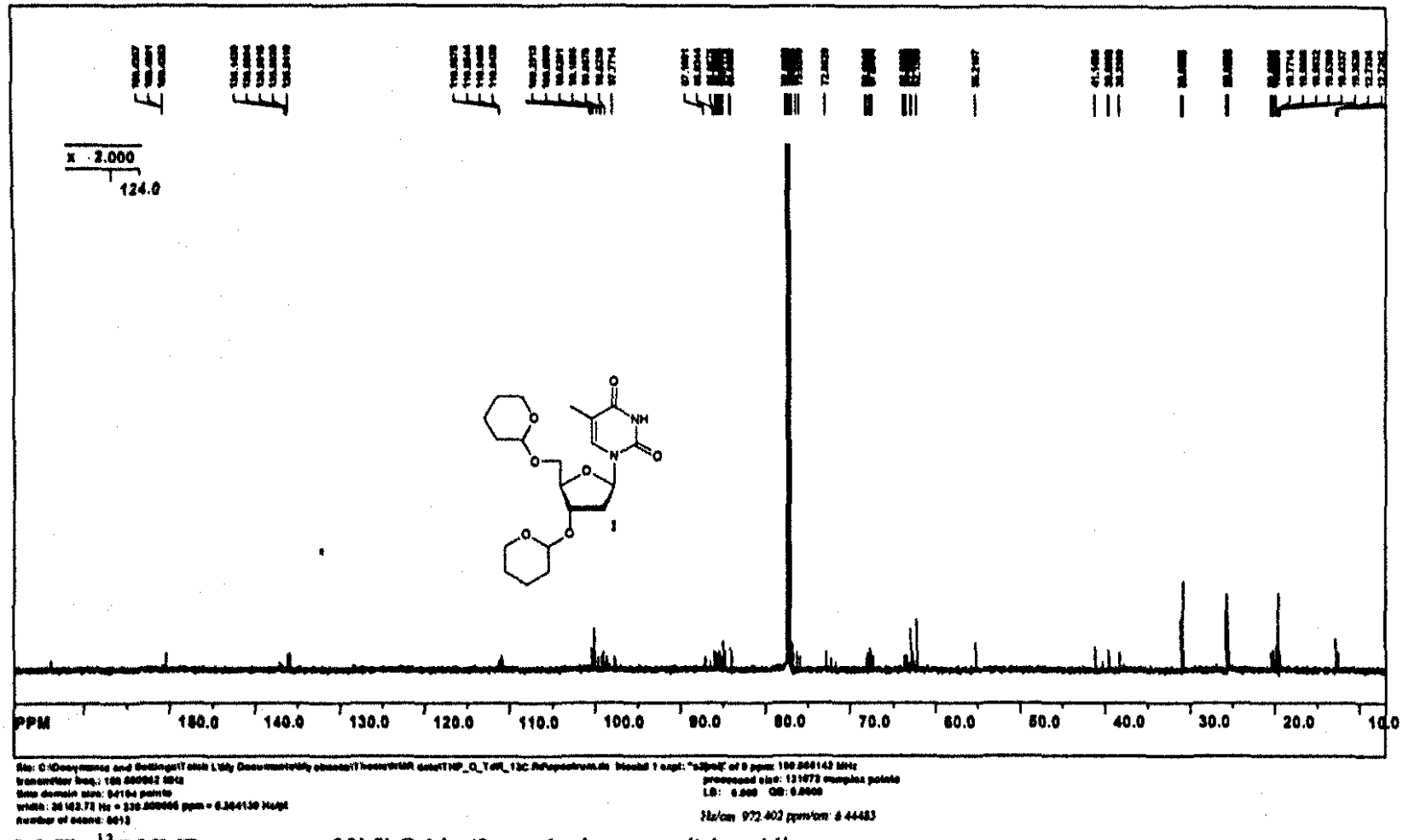

Figure I-2 The ^{13}C NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)thymidine

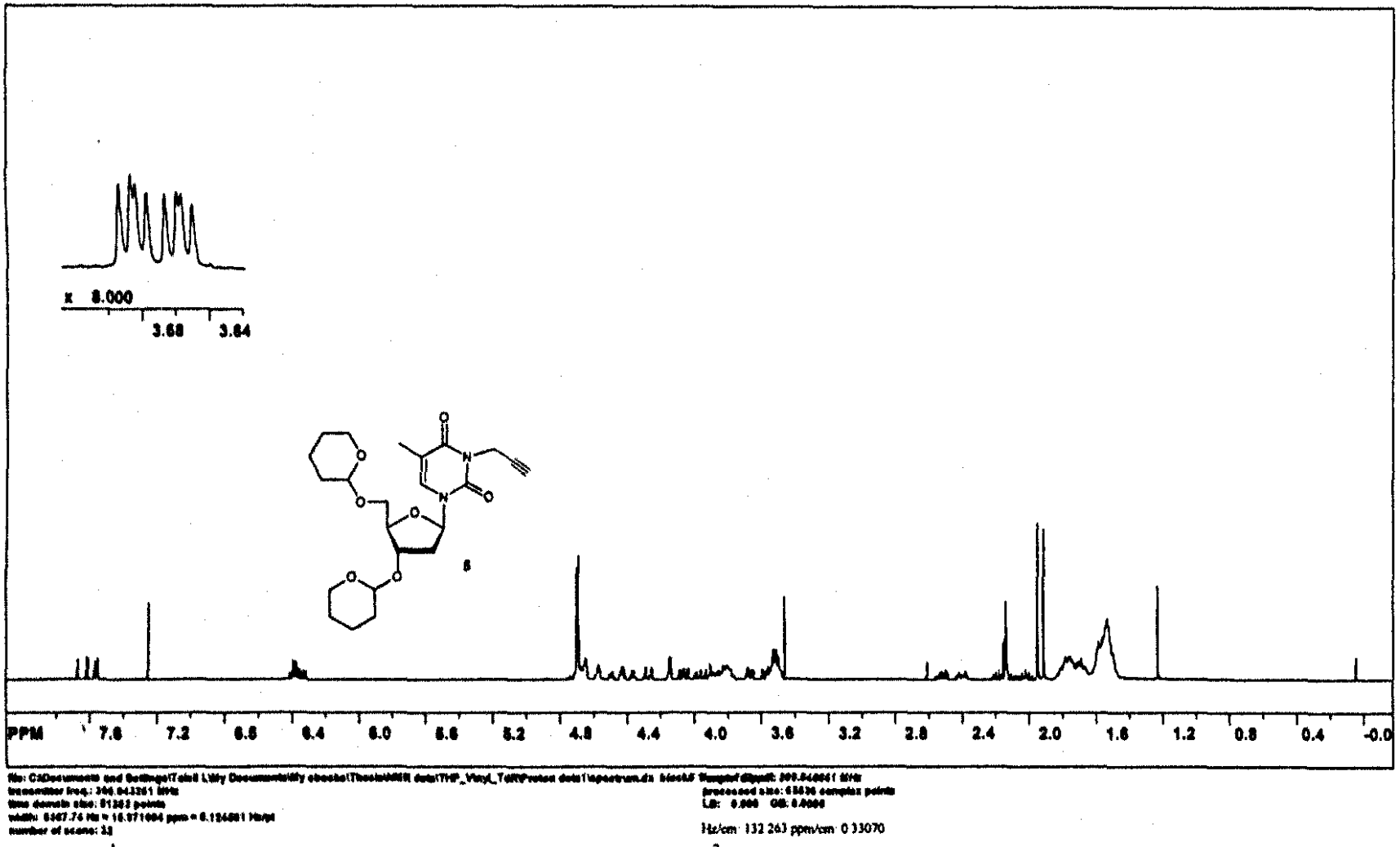

Figure I-3 The ^{1}H NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^{3}-(prop-2-yn-1-yl)thymidine

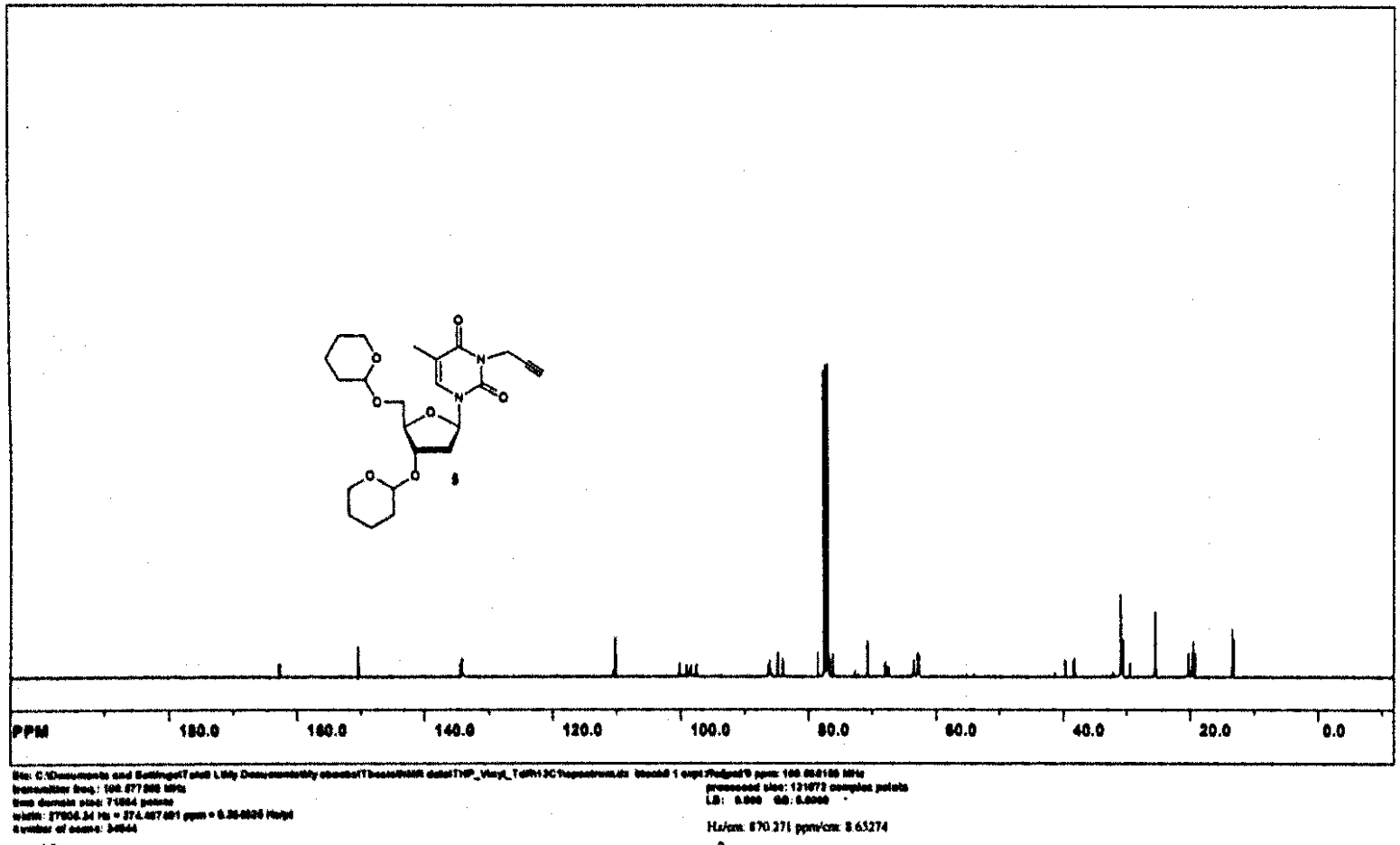

Figure I-4 The ^{13}C NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-(prop-2-yn-1-yl)thymidine

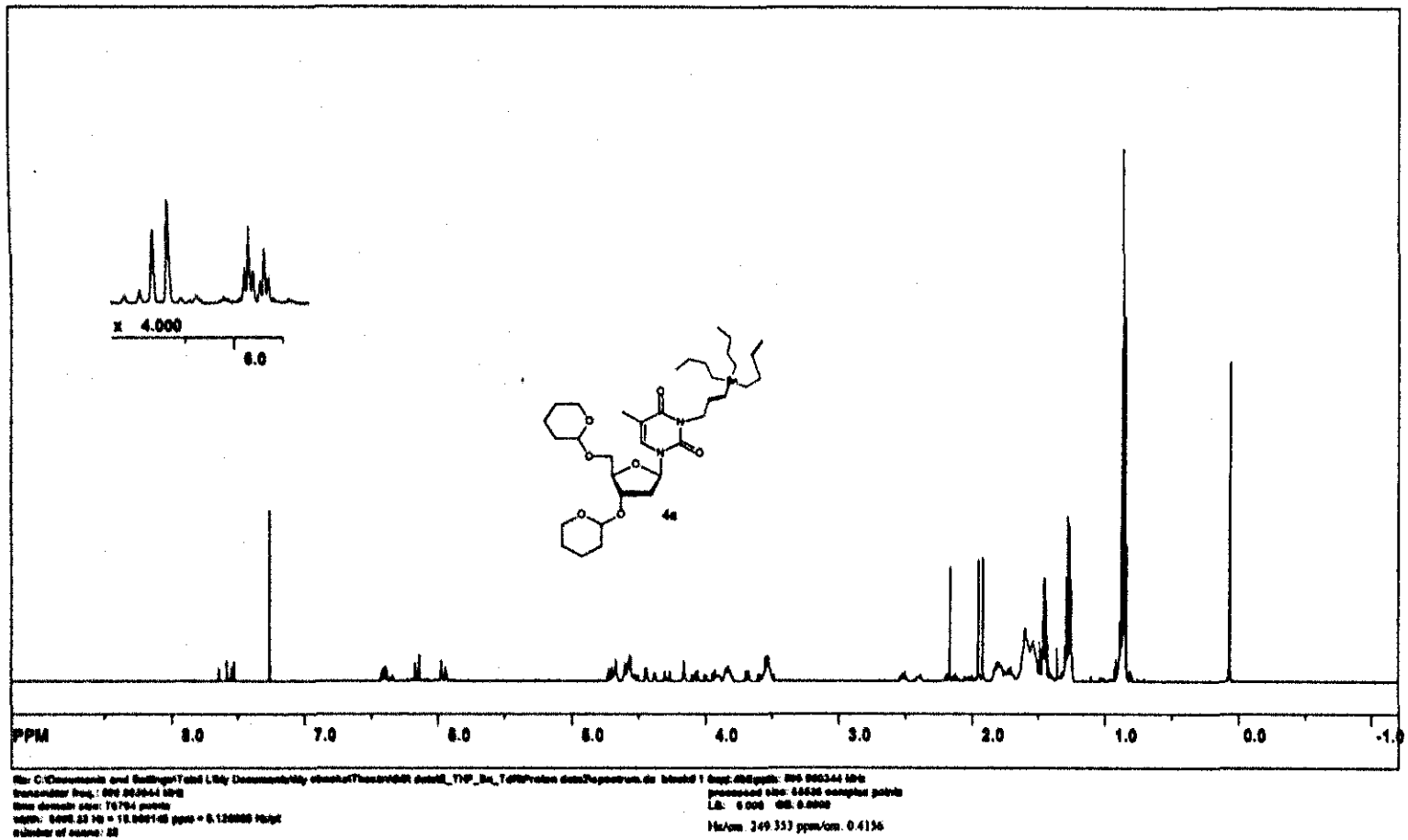

Figure I-5 The ^{1}H NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^{3}-[(*E*)-(3-tri-*n*-butylstannyl)prop-2-en-1-yl]thymidine

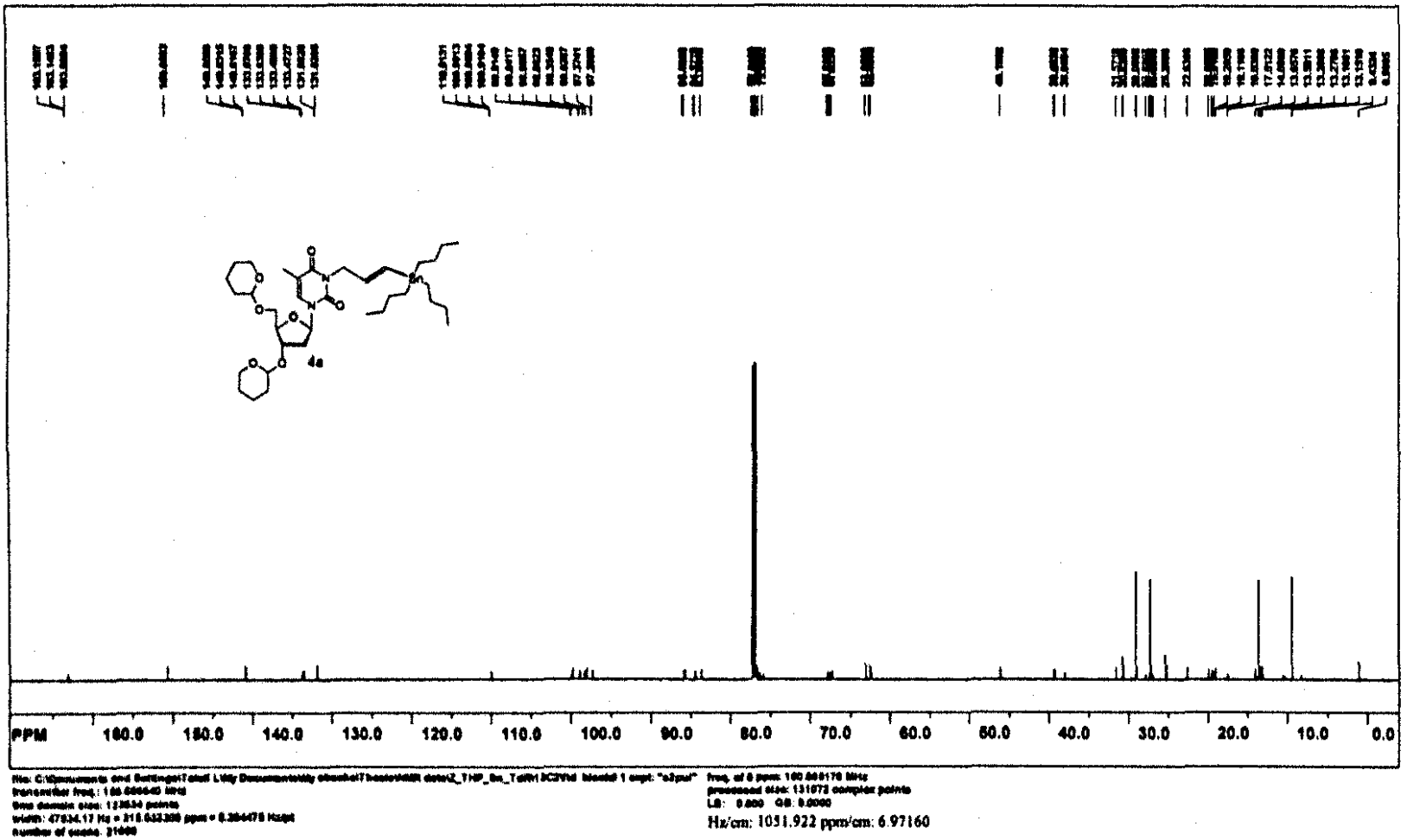

Figure I-5 The ^{13}C NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-[(*E*)-(3-tri-*n*-butylstannyl)prop-2-en-1-yl]thymidine

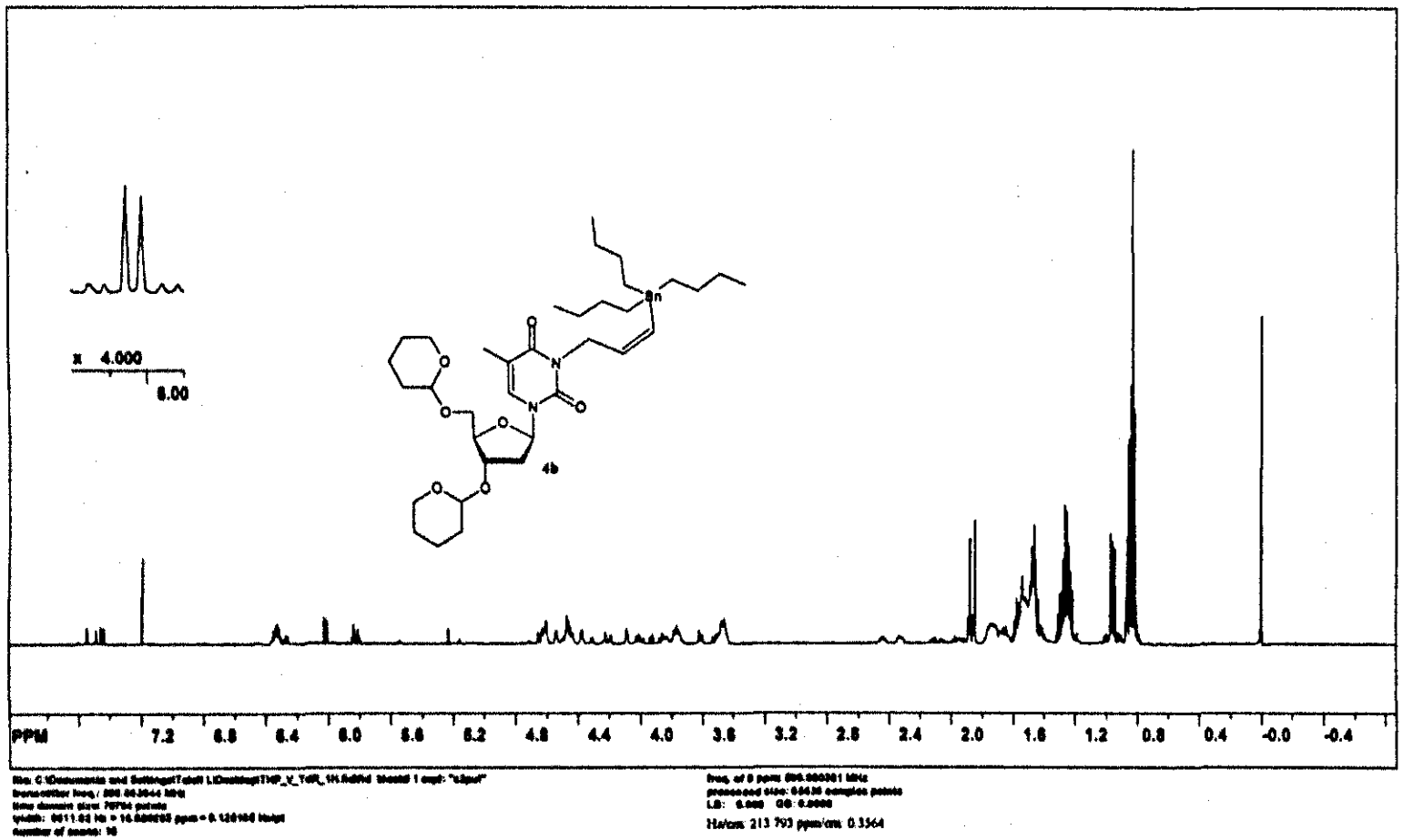

Figure I-7 The ^{1}H NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^{3}-[(*Z*)-(3-tri-*n*-butylstannyl)prop-2-en-1-yl)thymidine

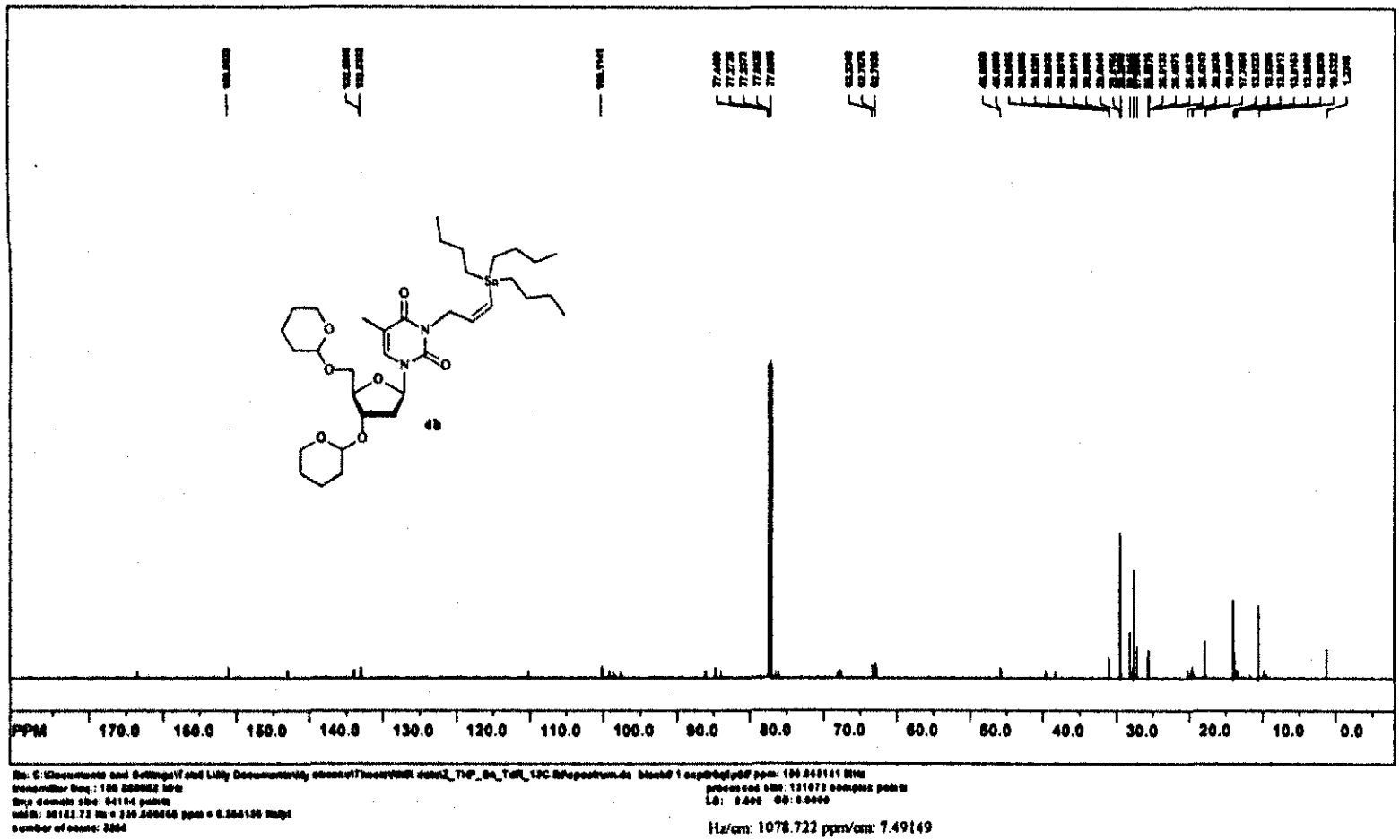

Figure I-8 The ^{13}C NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-[(*Z*)-(3-tri-*n*-butylstannyl)prop-2-en-1-yl]thymidine

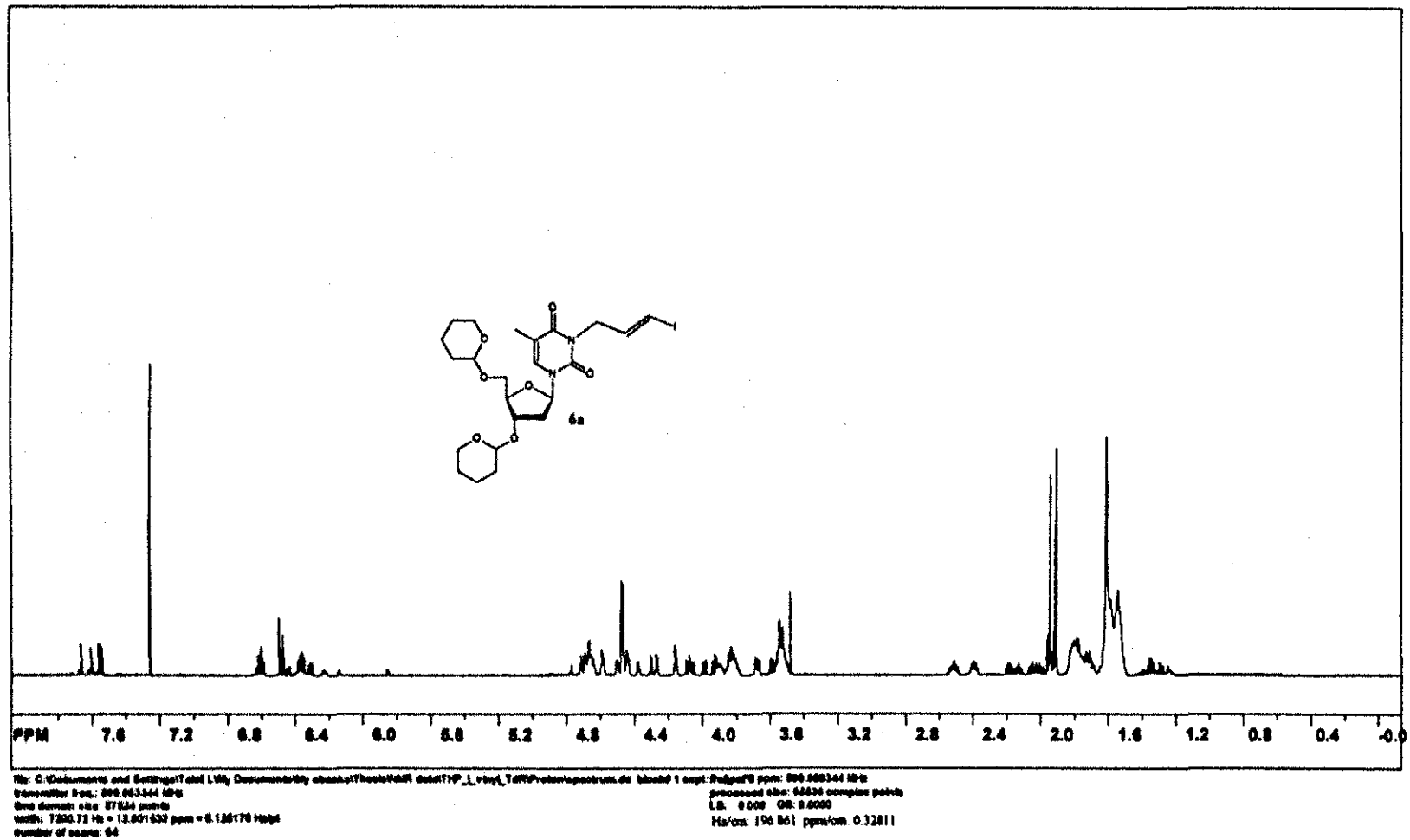

Figure I-9 The ^{1}H NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^{3}-[(*E*)-3-iodoprop-2-en-1-yl]thymidine

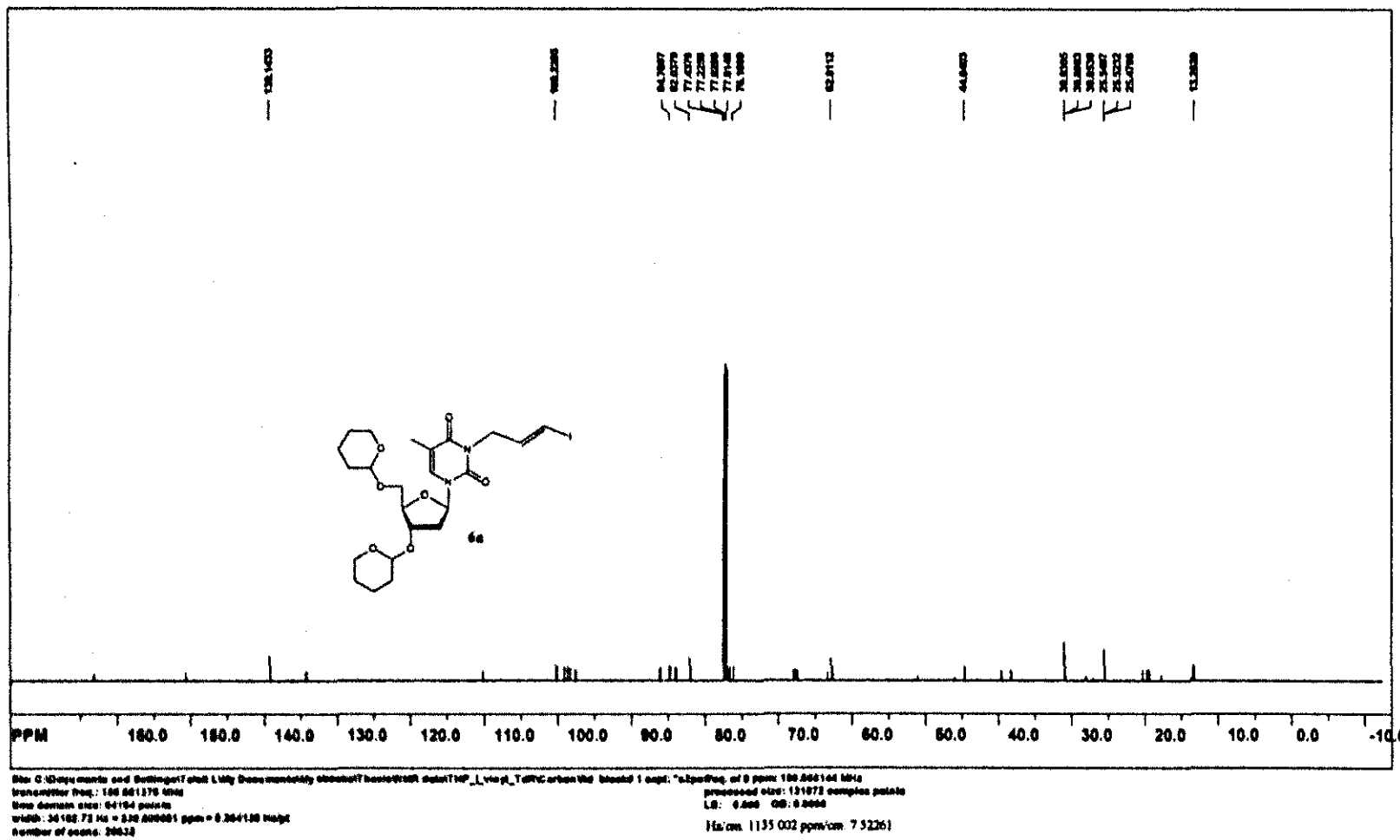

Figure I-10 The ^{13}C NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-[(*E*)-3-iodoprop-2-en-1-yl]thymidine

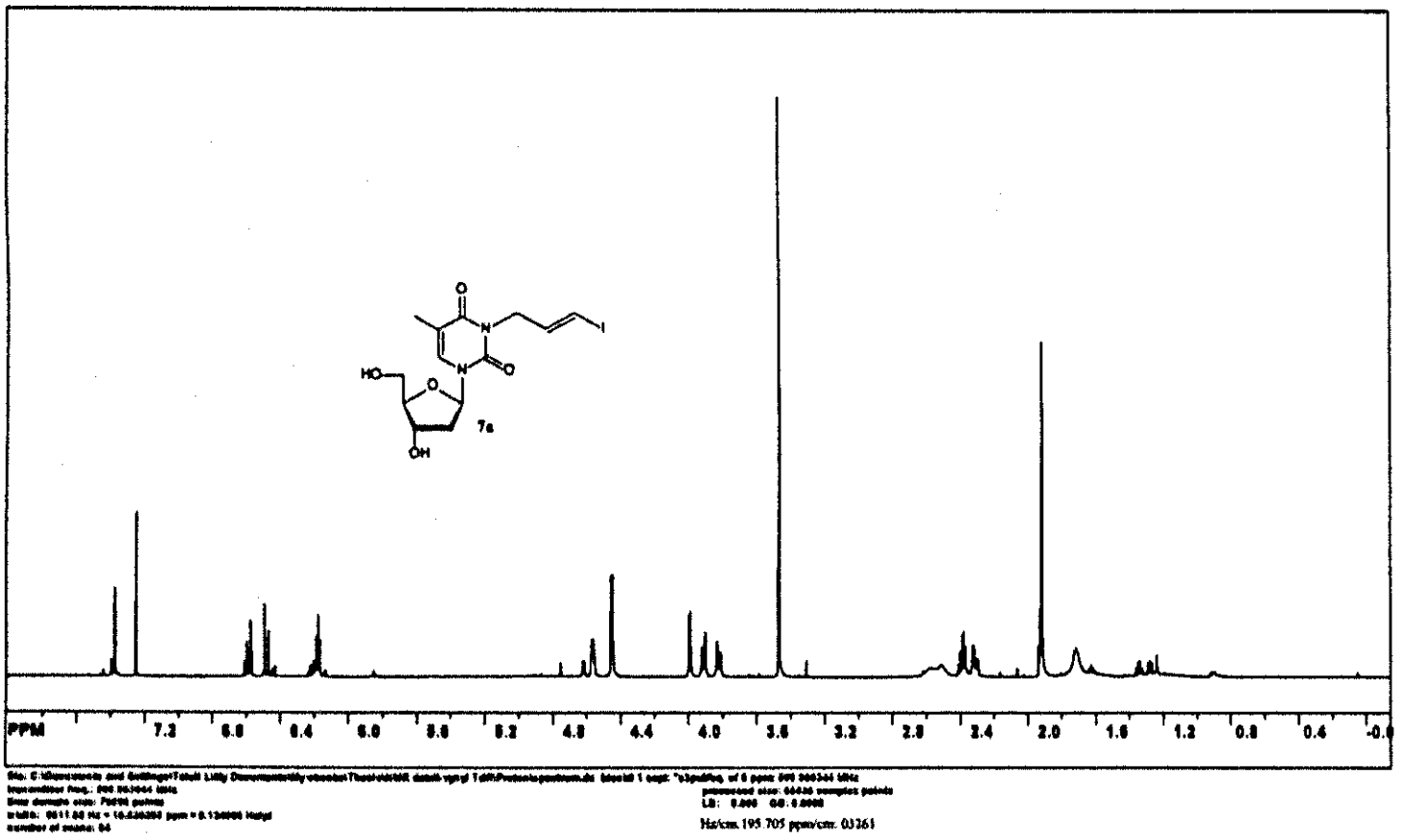

Figure I-11 The ^{1}H NMR spectrum of N^{3}-[(*E*)-3-iodoprop-2-en-1-yl]thymidine

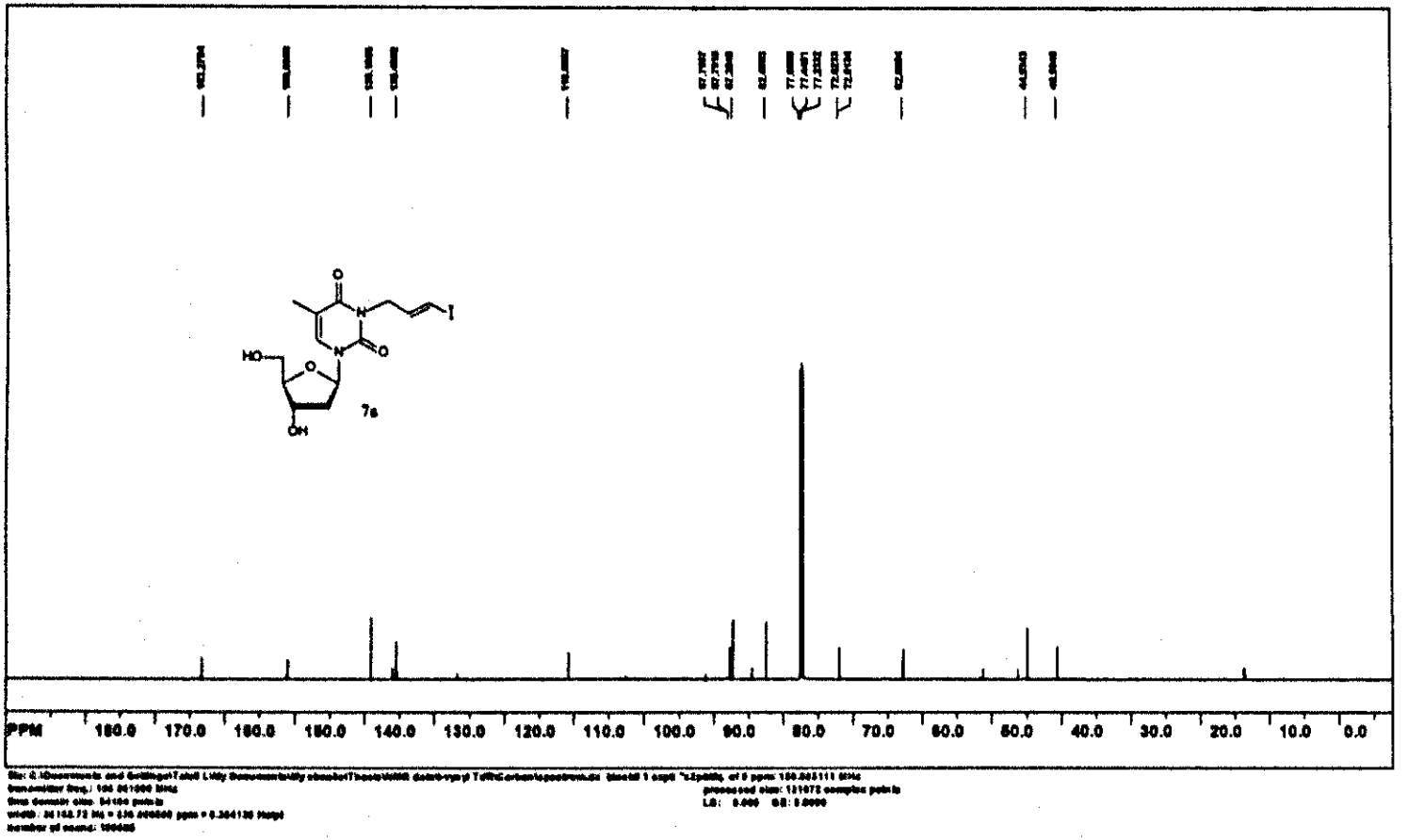

Figure I-12 The ^{13}C NMR spectrum of N^3-[(*E*)-3-iodoprop-2-en-1-yl]thymidine

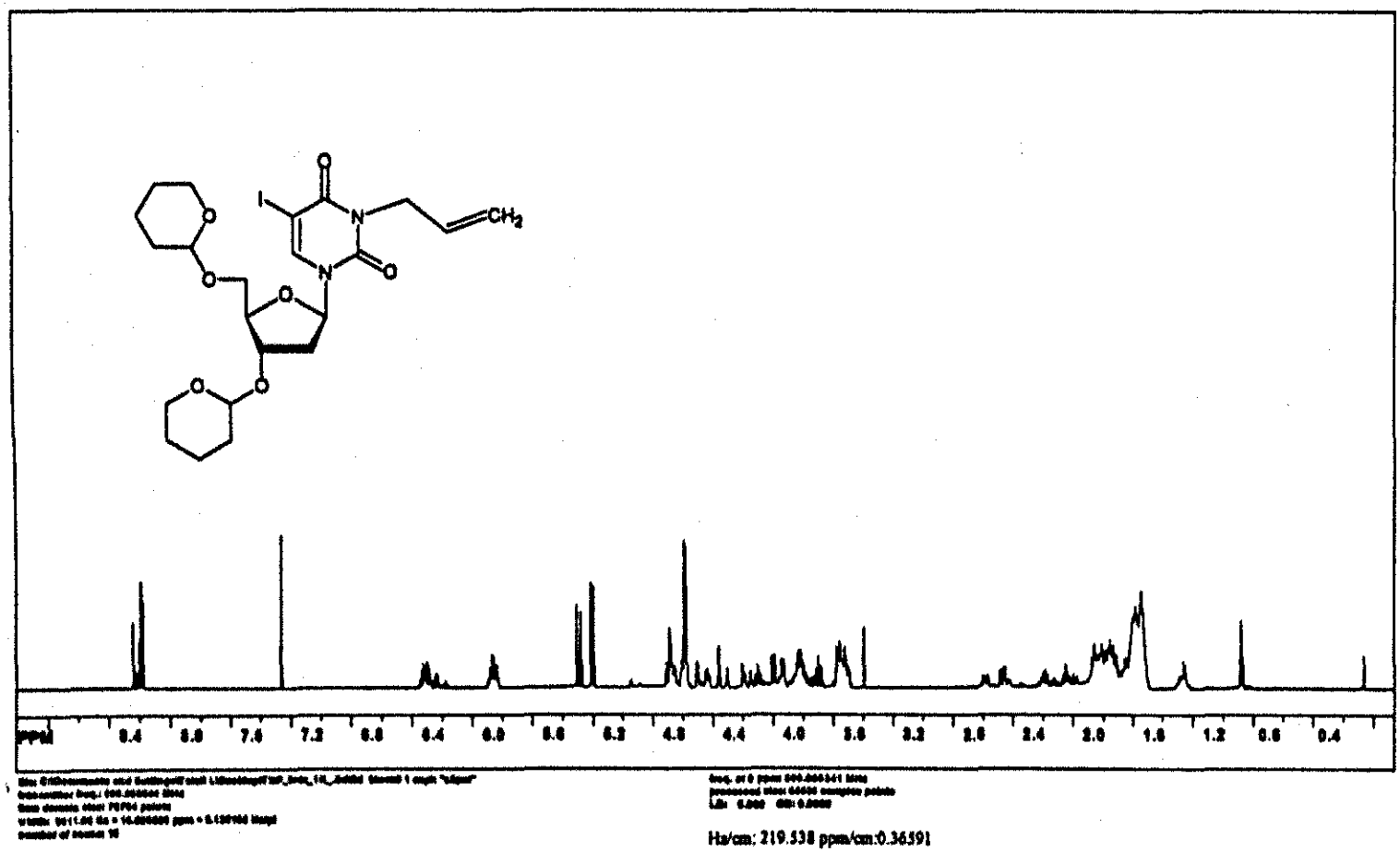

Figure I-13 The ^{1}H NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^{3}-allyl-5-iodo-2'-deoxyuridine

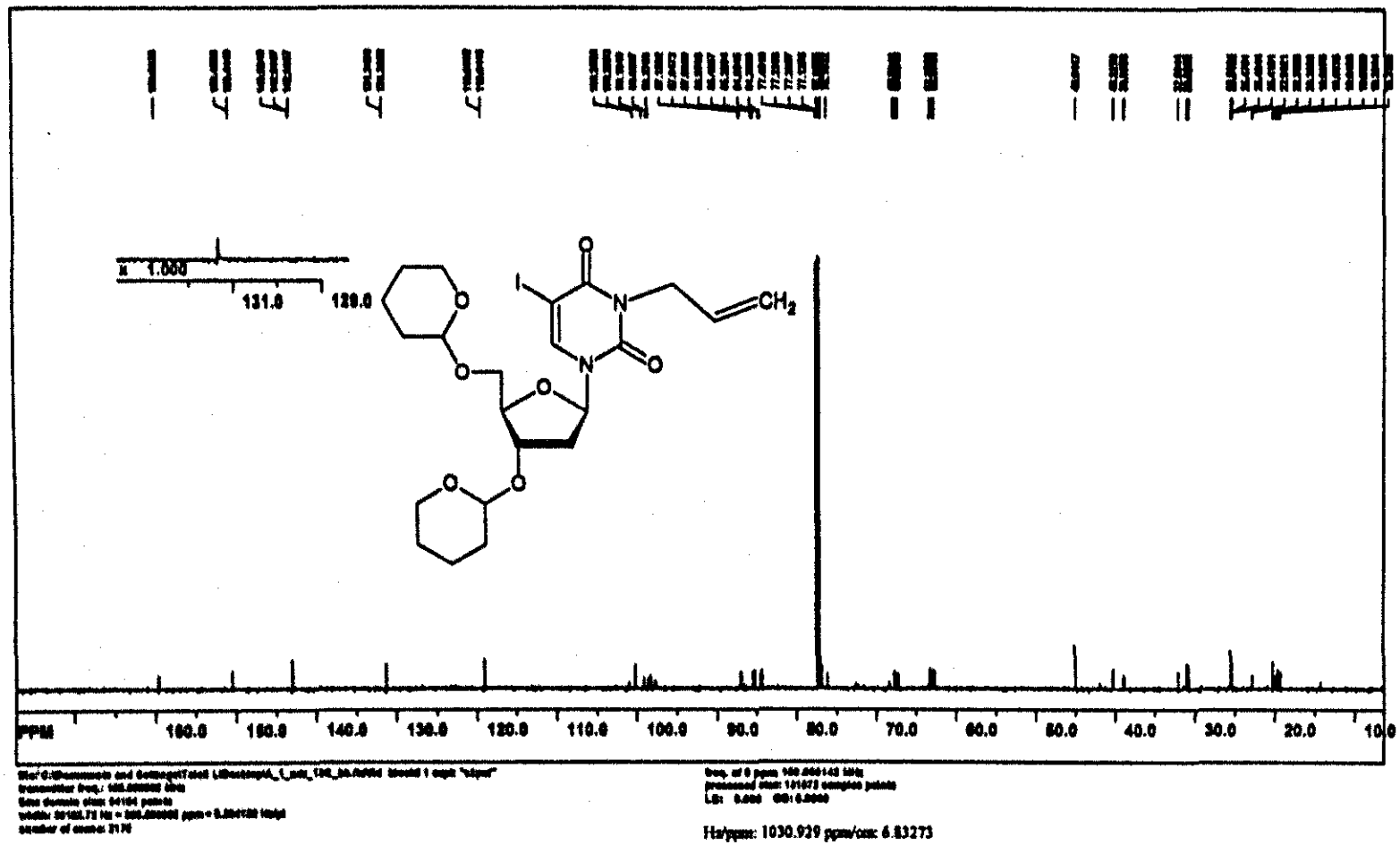

Figure I-14 The ^{13}C NMR spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-allyl-5-iodo-2'-deoxyuridine

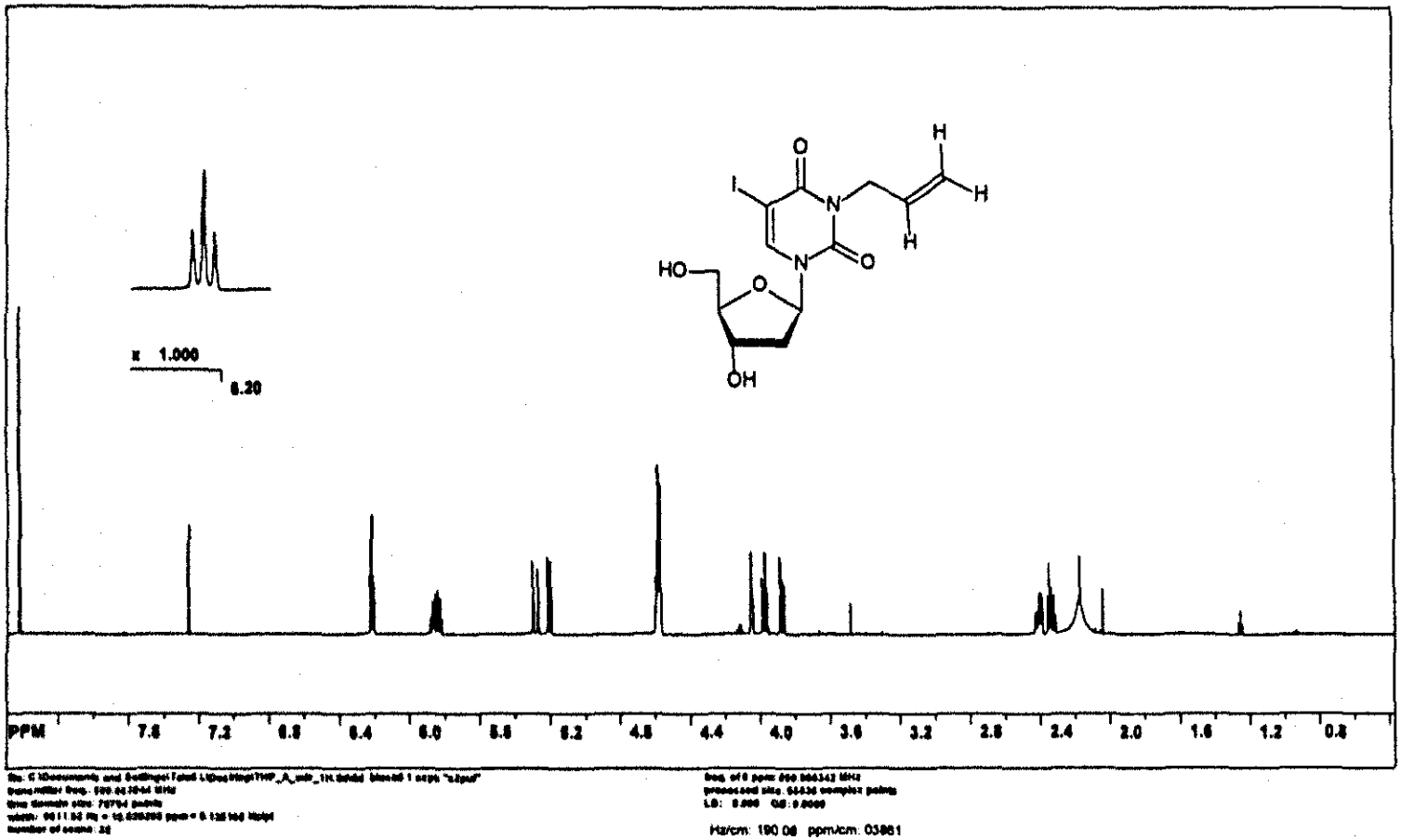

Figure I-15 The ^{1}H NMR spectrum of N^{3}-allyl-5-iodo-2'-deoxyuridine

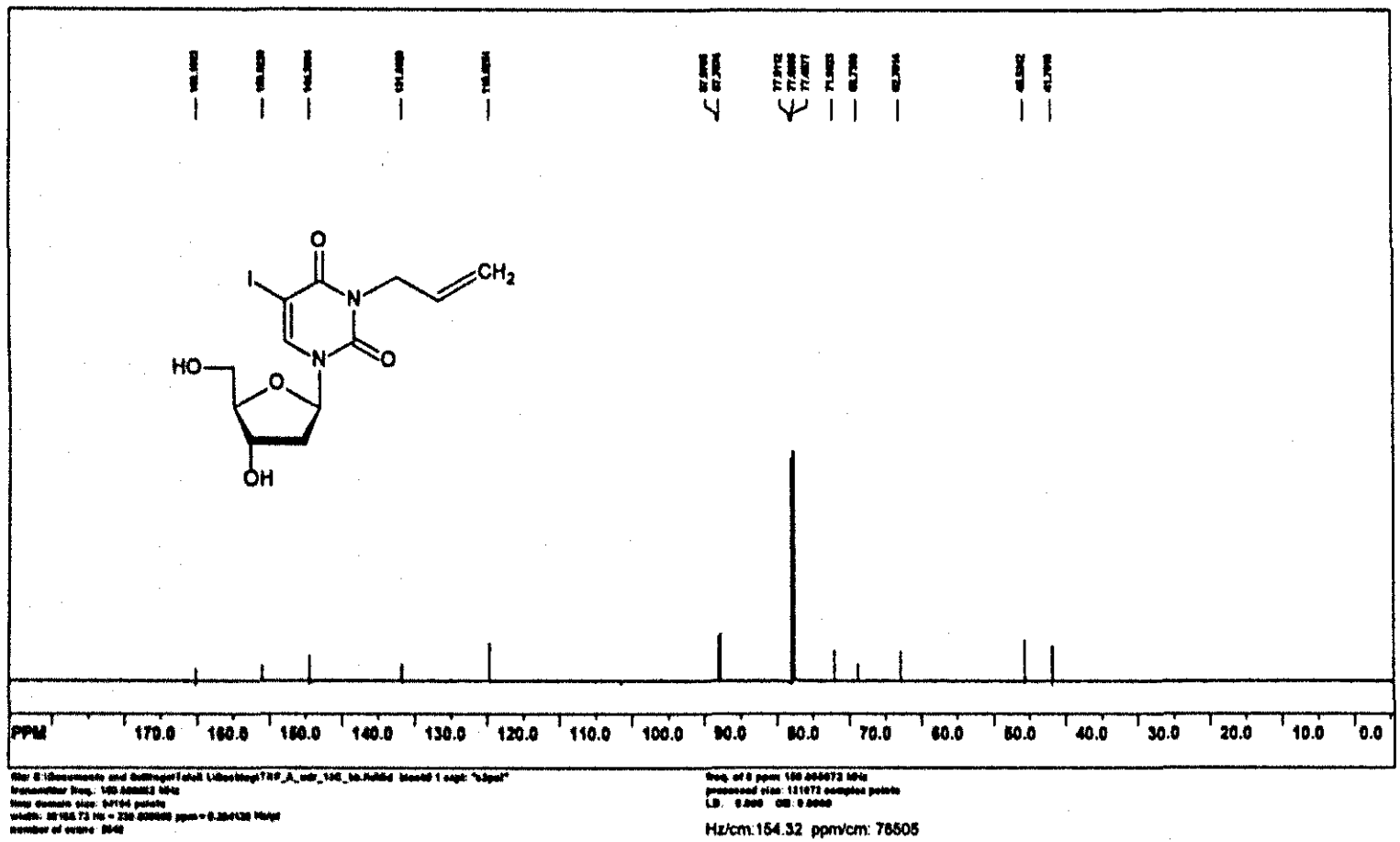

Figure I-16 The ^{13}C NMR spectrum of N^3-allyl-5-iodo-2'-deoxyuridine

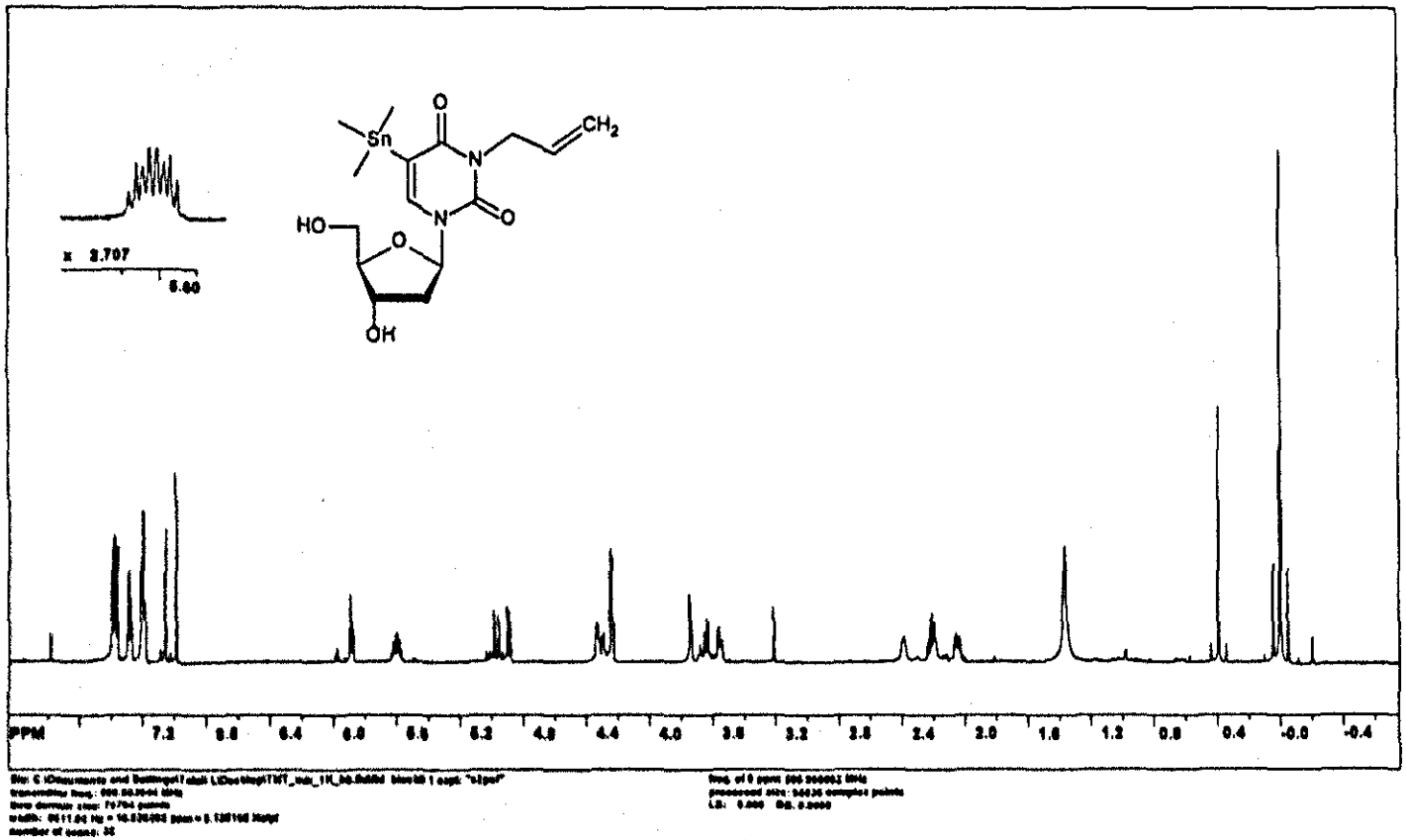

Figure I-17 The ^{1}H NMR spectrum of N^{3}-allyl-5-trimethyltin-2'-deoxyuridine

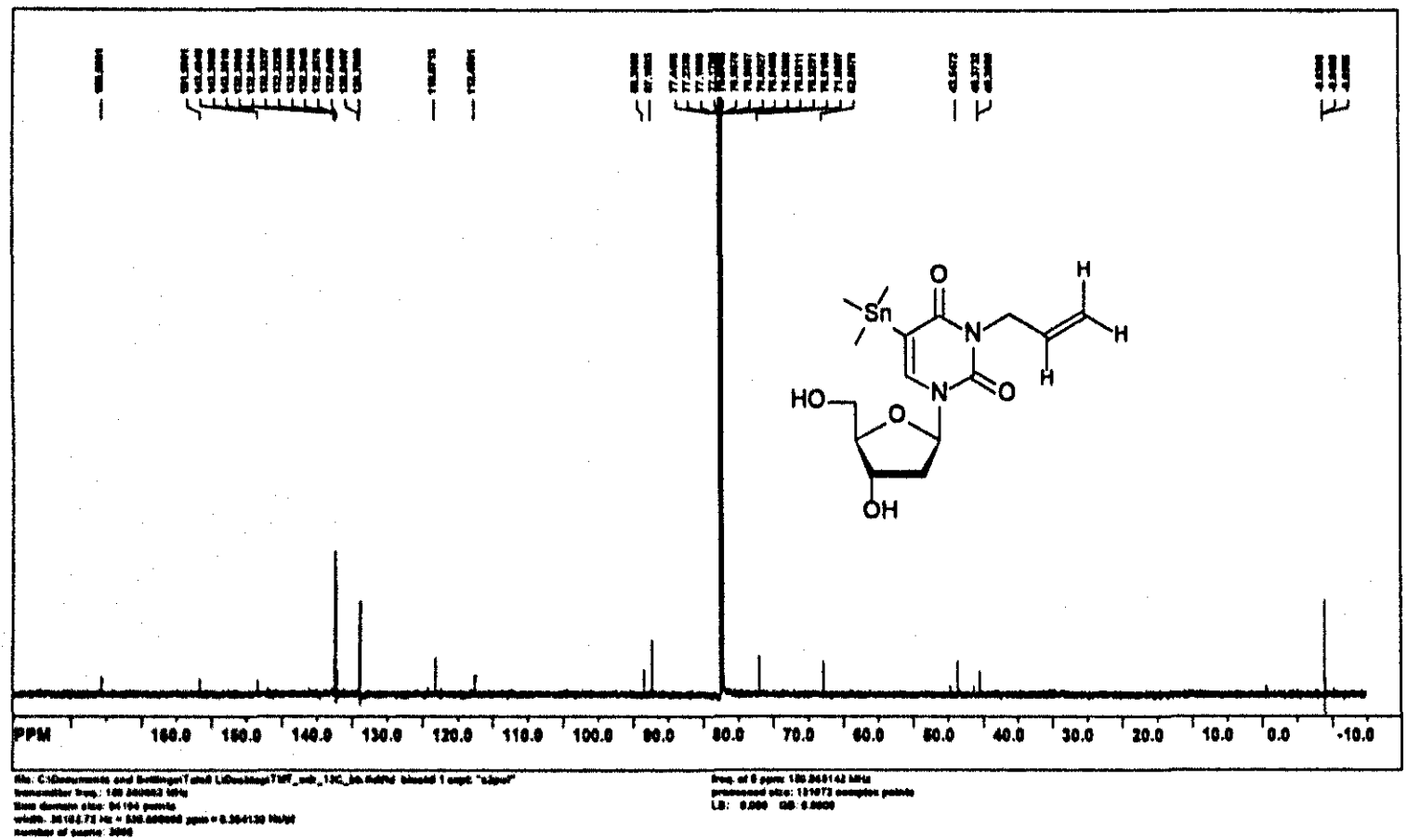

Figure I-18 The ^{1}H NMR spectrum of N^{3}-allyl-5-trimethyltin-2'-deoxyuridine

Appendix II

The mass spectra

Table II-1 Mass Spectrometry method used for analysis of the nucleoside analogues

Instrument	**Waters API Q-TOF Ultima**
Ionisation method	Electro-spray ionization $(M+nH)^{n+}$
Calibration	NaF
Tune File	W mode
Capillary (Ionisation) voltage	+3.5 kV
Cone Voltage (Energy Deposition)	+35 (soft method fragmentation)or +60 (strong fragmentation)
Resolution factor	1:40
Source	100°C
Desolvation Temperature	350 °C
Desolation Gas	350 L/h
Cone (nebulisation) gas	50L/h
Sample introduction (Injector)	300 µl /min Waters UPLC
Sample size	2 µl
Sample type	Solution
Matrix	Solvent (Methanol/acetonitrile)
Mass range	100-1500 Da
Reference	Pure and Applied chemistry 63, 975-90 (1991)

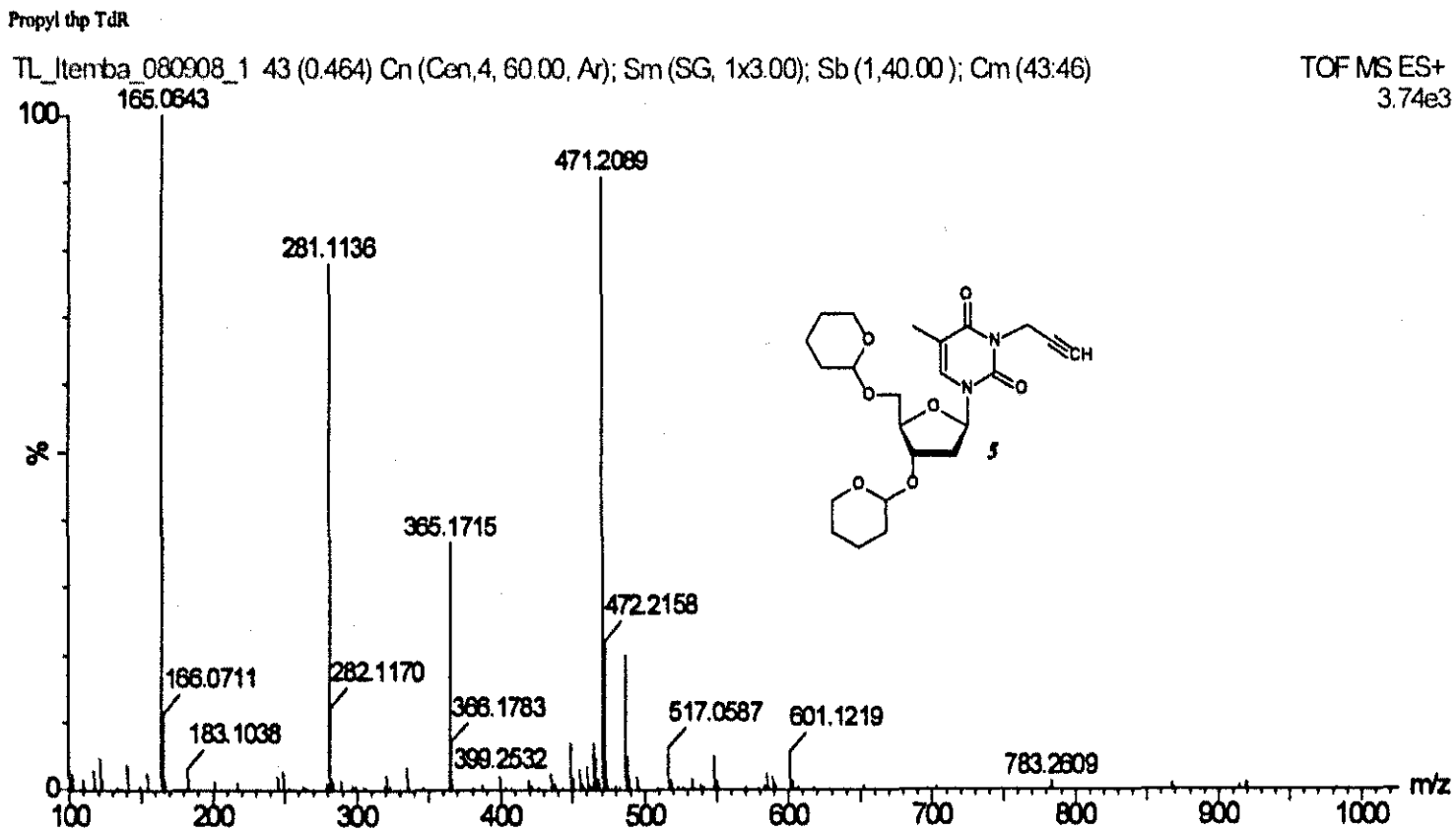

Figure II-1 The mass spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)thymidine.

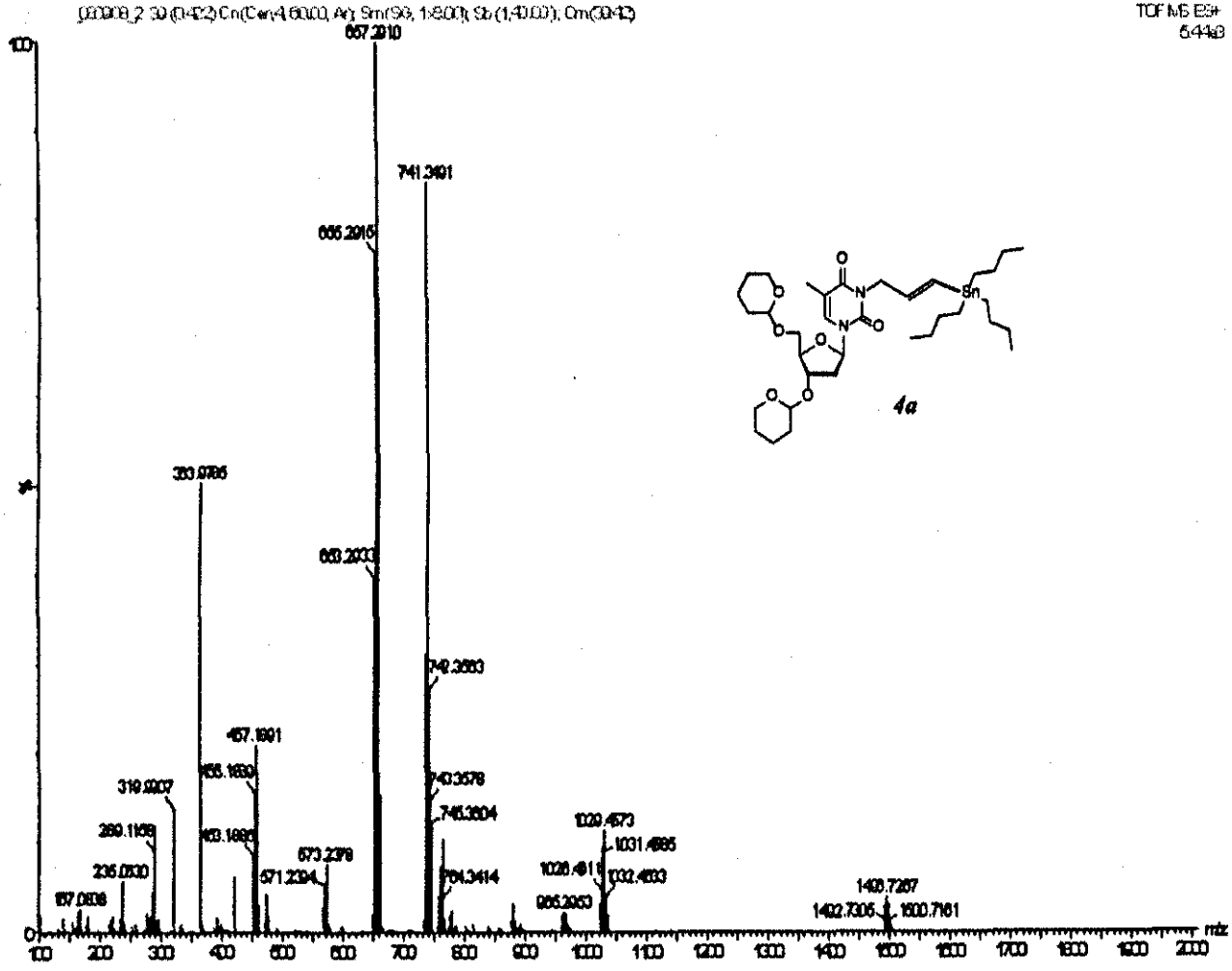

Figure II-2 The mass spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-[(*E*)-(3-tributylstannyl)prop-2-en-1-yl]thymidine

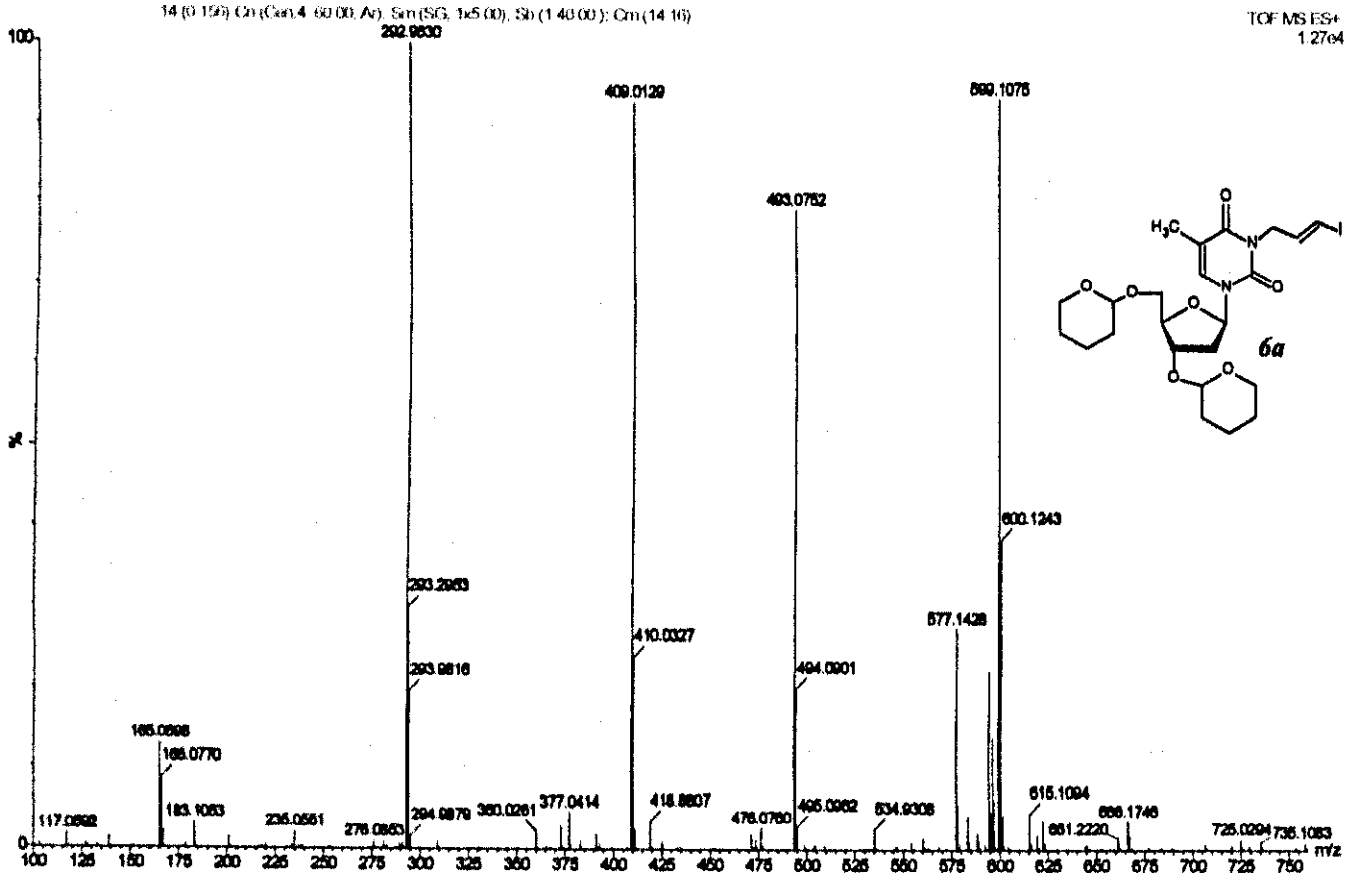

Figure II-3 The mass spectrum of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-[(*E*)-3-iodoprop-2-en-1-yl]thymidine

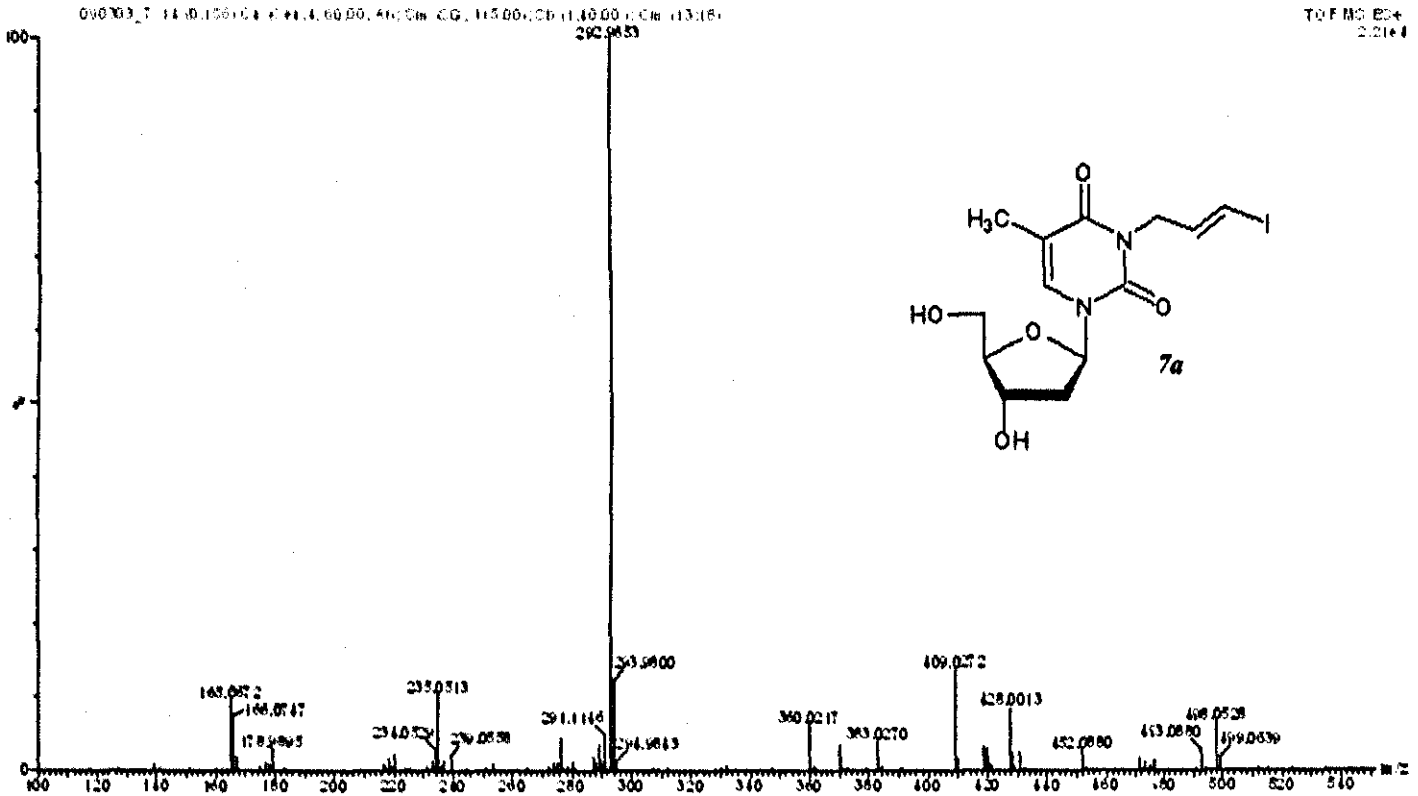

Figure II-4 Mass spectra of N^3-[(*E*)-3-iodoprop-2-en-1-yl]thymidine

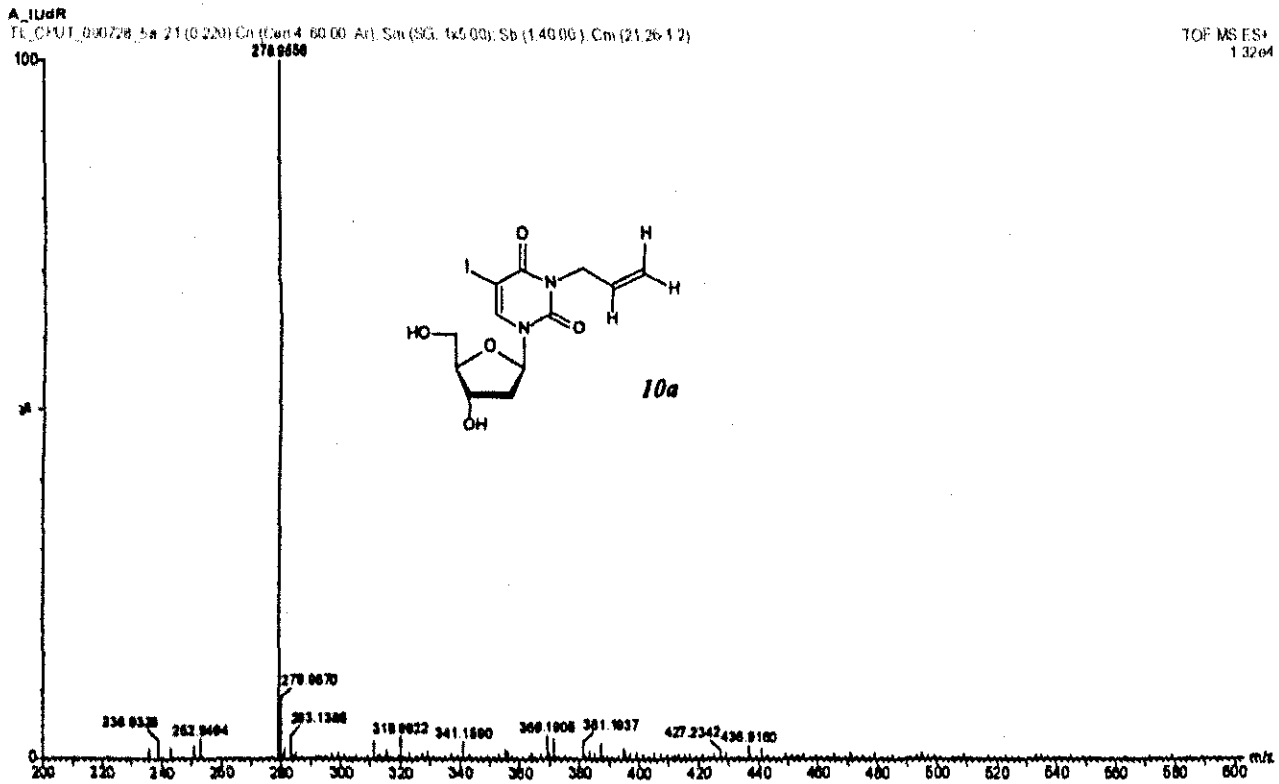

Figure II-5 Mass spectra of N^3-allyl-5-iodo-2'-deoxyuridine

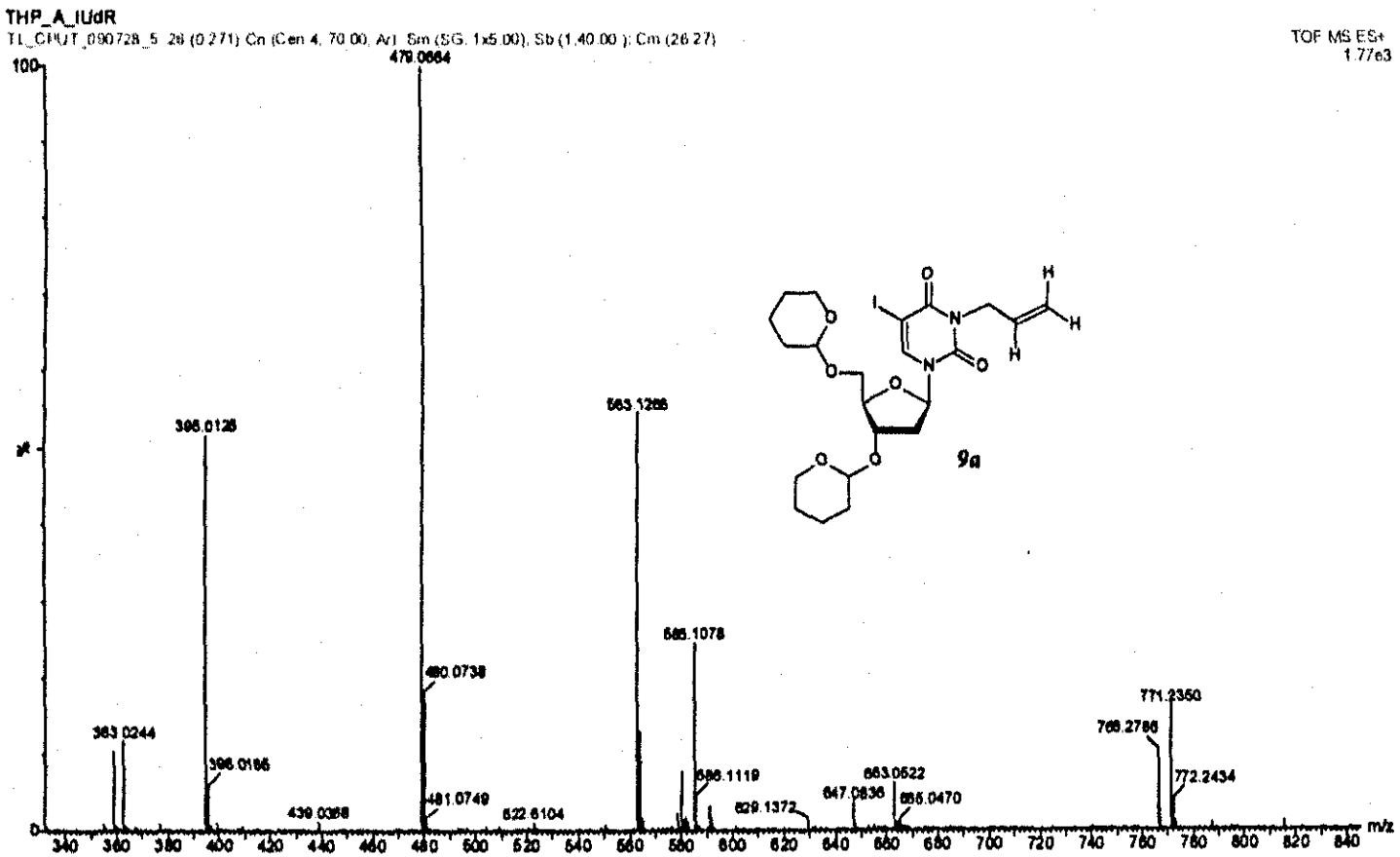

Figure II-6 Mass spectra of 3',5'-O-bis-(2-tetrahydropyranyl)-N^3-5-iodo-2'-deoxyuridine

Appendix III

In vitro cell-uptake data

Table III-1 Radioactivity counting of [^{123}I]-*7a* (per minute) in CHO-K1 cells

		Radioactivity of a substrate per seeding/4		
Cell seeding	**# of Counts**	**[^{123}I]-NaI**	**[^{123}I]-IUdR**	**[^{123}I]-*7a***
Background	1	42	23	44
	1	30	14	8
	1	52	115	33
	1	60	31	42
5 000	1	48	1881	200
	1	24	1548	219
	1	82	1998	1010
	1	104	2011	89
10 000	1	206	4775	235
	1	118	4492	230
	1	151	3808	227
	1	368	4358	215
15 000	1	76	7121	297
	1	196	7141	366
	1	221	6794	328
	1	236	6510	526
20 000	1	739	9324	414
	1	235	9425	400
	1	319	10315	1415
	1	210	100218	567
25 000	1	277	11267	396
	1	203	10053	485
	1	571	12346	585
	1	203	13257	412

Table III-2 CHO-K1 cells count rate data for radiolabelled substrates after 30 hours incubation time

		Growth-dependent cell-uptake per substrate ± (Average deviation)		
Cell seeding	**Replicates**	**Control**	**[^{123}I]-IUdR**	**[^{123}I]-*7a***
Mean cell growth /10 000	16	36.625 ± 5.328	20.313 ± 3.351	26.000 ± 4.706

Table III-3 Radioactivity counting of [^{123}I]-*10* (per minute) in CHO-K1 cells

		Radioactivity of a substrate per seeding/4	
Cell seeding	**# of Counts**	[^{123}I]-IUdR	[^{123}I]-*10*
Background	1	42	78
	1	44	48
	1	48	61
	1	82	75
5 000	1	1008	78
	1	739	70
	1	601	87
	1	623	73
10 000	1	1674	85
	1	1621	98
	1	1542	87
	1	1187	121
15 000	1	2658	125
	1	2616	155
	1	2469	121
	1	2453	167
20 000	1	4635	186
	1	4128	159
	1	3489	140
	1	3539	141
25 000	1	4362	182
	1	4637	125
	1	2750	151
	1	3412	193

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