Evaluation of N,N'-disubstituted 1,3-Diaminopropan-2-ols for anti-HIV activity

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To my Dad

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ABSTRACT

The acquired immunodeficiency syndrome (AIDS) pandemic continuous to be a medical, social and economic challenge of staggering proportions. The human immunodeficiency virus (HIV) has been identified as the etiologic agent of this disease.

The HIV-1 protease enzyme is one of the specific targets for anti-retroviral therapy. It was chosen for this study because of its well described crystal structure and based on previous research and results.

A novel series of protease inhibitors were designed, and evaluated for possible anti-HIV activity. 1,3-Diaminopropan-2-ol derivatives containing benzofuran-2-carboxylic acid/-aldehyde and indole-2-carboxylic acid/-aldehyde moieties were included in this study and evaluated for possible protease interaction. A structure search performed on compounds containing the core structure 1,3-diaminopropan-2-ol guided the selection of the published structure, (S,S,R)-1-[2-(N-t-Butoxycarbonyl)amino-1-benzyl-2-hydroxypropyl]-N-methyl-N-benzylaminobenzoylimide (AQ148) or 3AID (PDB ID) of the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB). The premise for this study was that the novel series is structurally similar to AQ148, containing the hydroxyethylamine isostere, with affinity for the HIV protease enzyme.

Molecular modelling was performed using a SGI Fuel computer, with Insight II software to determine the feasibility for further evaluation of candidate compounds. Root mean square (RMS) fitting values were obtained by superimposing the proposed compounds on AQ148. The results of this RMS fit of candidate compounds on AQ148 indicated the likelihood of interaction of the proposed compounds at the protease active site. The hydrogen bond network formed in the enzyme was also used as an indication for possible inhibitory activity. H-bonds from the substrate to residues of the active site in the enzyme, Asp 25 and Asp '25 as well as H-bonds to the water molecule, which is bound to the flap residues, Ile 50 and Ile '50 was taken as essential interactions. The molecular modelling study indicated that the novel compounds were reasonable candidates for further investigation in view of their interaction profiles at the HIV protease active site.

Compounds were synthesised by using either direct coupling or activation chemistry. Direct coupling of 1,3-diaminopropan-2-ol with benzofuran-2-carboxaldehyde and indole-3-carboxaldehyde was done respectively. Activation chemistry, using *N,N'*-carbonyldiimidazole

(CDI) was used to synthesise the other compounds. Difficulties were experienced with the yields, purification and isolation of the compounds due to solubility properties and multiple reactions taking place. Selected, compounds were included in the biological evaluation against HIV-1.

The pure compounds namely **SVZ-2** and **4** were evaluated by measuring T-cell line adapted HIV-1 strain neutralisation in MT-2 cells. MN and IIB virus strains were used in this study, as well as a positive control, IBU21 (a sample isolated from an HIV-1 infected individual with neutralising activity).

The biological evaluation showed no meaningful inhibition by the novel compounds, but was of experimental value in this kind of study/research. The insolubility of the compounds in aqueous solutions could have reduced their bioavailability, which in turn would account for their lack of inhibitory activity.

This study prompts further investigation into small substituted molecules for HIV-1 protease inhibition. This first exploration into the possible anti-HIV properties of this series could thus pave the way for further in depth anti-HIV studies on this class of compounds and derivatives thereof.

OPSOMMING

Die verworwe immuniteitsgebreksindroom (VIGS) pandemie is nog steeds 'n medies, sosiale en ekonomiese probleem met verbysterende gevolge. Die menslike immuun gekompromitteerde virus is geïdentifiseer as die etiologiese oorsaak van die siekte.

Die HIV-protease ensiem is een van die spesifieke teikens vir anti-retrovirale terapie. Dié ensiem is geselekteer vir die studie a.g.v. sy goed beskryfde en welbekende kristalsktruktuur asook weens vorige gepubliseerde navorsingsbevindinge.

'n Nuwe reeks HIV protease inhibeerders is ontwerp en geëvalueer vir moontlike anti-HIV aktiwiteit. 1,3-Diaminopropan-2-ol derivate wat bensofuraan-2-karboksaldehied/karboksielsuur en indool-2-karboksaldehied/karboksielsuur eenhede bevat is ingesluit in die studie en geëvalueer vir moontlike protease inhiberende aktiwiteit. 'n Struktuursoektog is gedoen op verbindings wat die kern struktuur 1,3-diaminopropan-2-ol bevat, wat aanleiding gegee het tot die seleksie van 'n gepubliseerde struktuur, (S,S,R)-1-[2-(N-t-Butoksiekarboniel)amino-1-bensiel-2-hidroksiepropiel]-N-metiel-N-bensielaminobensoielimied (AQ148) of 3AID (PDB ID) van die "Reasearch Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB)". Die basis van die studie is dat die nuwe reeks strukturele ooreenkomste het met AQ148, wat 'n hidroksi-etielamien isosteer bevat wat 'n affiniteit het vir die HIV protease ensiem.

Molukulêre modulering is gedoen op 'n SGI Fuel rekenaar met Insight II sagteware, om te bepaal of dit sinvol sou wees om die voorgestelde verbindings te sintetiseer en te evalueer. Kleinste kwadraatpassingwaardes is verkry deur passing van die voorgestelde verbindings op AQ148. Die resultate van die passing op AQ148 was 'n indikasie dat daar 'n goeie moontlikheid is vir interaksie tussen die voorgestelde verbindings op die protease aktiewe setel. Die waterstofbindingnetwerk was ook 'n indikasie vir moontlike inhiberende aktiwiteit. H-bindings tussen die substraat en die residus van die aktiewe setel in die ensiem, Asp 25 en Asp '25, sowel as H-bindings tussen die substraat en die strategiese water molekule, wat op sy beurt gebind is aan Ile 50/'50 van die flap residue, is as essensieël geag in die ondersoek. Die moduleringstudie het gewys dat die nuwe verbindings geskikte kandidate is vir verdere navorsing na aanleiding van hul interaksies by die HIV-protease ensiem.

Die verbindings is gesintetiseer deur of direkte koppeling of aktiverende chemie te gebruik. Direkte koppeling is gebruik om 1,3-diaminopropan-2-ol met bensofuraan-2-karboksaldehied en indool-3-karboksaldehied respektiewelik te sintetiseer. *N,N*-Karbonieldiimidasool is as

aktiveringsagent gebruik om die res van die verbindings te sintetiseer. Probleme is ondervind met die isolering en suiwering van die verbindings a.g.v hul oplosbaarheidseienskappe en meervoudige reaksies wat plaasgevind het. Geselekteerde verbindings is ingesluit in die biologiese evaluasie teen HIV-1.

Die suiwer verbindings is getoets deur die neutralisering van die MT-2 selle te meet wat deur T-sel gemodifiseerde HIV-1 stamme geïnfekteer is. MN en IIIB virusstamme is gebruik in die studie, sowel as 'n positiewe kontrole, IBU21 ('n plasmamonster met neutraliserings aktiwiteit geïsoleer uit 'n HIV-1 geïnfekteerde persoon).

Die biologiese evaluering het geen betekenisvolle inhiberende aktiwiteit vir die nuwe reeks verbindings getoon nie, maar die resultate is van eksperimentele belang in die tipe van studie en navorsing. Die onoplosbaarheid van die verbindings in waterige oplossing, kon gelei het tot lae biobeskikbaarheid wat 'n negatiewe invloed kon gewees het in die bepaling van aktiweit.

Die studie kan dien as motivering vir verdere ondersoek na klein gesubstitueerde molekules vir HIV-1 protease inhibering. Hierdie eerste ondersoek na moontlike anti-HIV eienskappe in die reeks, kan lei na verdere in-diepte anti-HIV navorsing van die groep verbindings en derivate daarvan.

Chapter 1

Introduction and Aims

HIV/AIDS has developed as one of the major life threatening diseases of today with an increasing number of patients dying due to the secondary consequences of HIV infection. In the following chapter the background to, and statistics of, the human immunodeficiency virus infection are described and the aims and objectives of this study are stated.

1.1 BACKGROUND

It is generally accepted that HIV (human immunodeficiency virus) causes AIDS (acquired immunodeficiency syndrome). According to statistics of World Health Organisation (WHO; 2003) 3 million adults and children have died due to AIDS in 2003 globally and 5 million adults and children have been newly infected in the same year. In Sub-Saharan Africa the total death toll in 2003 due to AIDS was 2.2 million of which 370 000 were South Africans. The estimate globally for people living with HIV/AIDS is 40 million of which 25 – 28 million resides in Sub-Saharan Africa (WHO, 2004).

The origin of HIV infection has however not yet been identified. HIV/AIDS is a relatively new disease and was first described in 1981. Two main types of HIV strains have since been identified (Hooper, 1997; Beil, 1999; Kanabus & Allen, 2004):

- HIV-1
- HIV-2

It is widely accepted that HIV-2's origin can be traced back to the sooty mangabey monkey (an

African species) in West Africa. This type does not cause the disease as quickly, nor is it as virulent as HIV-1, and thus far it has remained mostly in Africa. This disease's earliest evidence can be traced back to 1965 (Hooper, 1997; Daniel *et al.,* 1987).

HIV-1 can be divided into two groups: HIV-1 group M ('main') and HIV-1 group O ('outlier'). The earliest confirmed case of HIV-1 group O is that of a Norwegian sailor who died in 1976. This type is also mainly restricted to West Africa (Hooper, 1997; Gao *et al.,* 1999; Lachman, 1999).

The major cause of AIDS is however HIV-1 group M. Over the years, HIV-1 sequences have diverged substantially and can be classified into subtypes A-J. The earliest evidence of this infection is from 1959. A stored blood sample of an African man was genetically analysed and was found to be seropositive for HIV-1. Its viral sequence was placed near the ancestral node of subtypes B and D (Lachman, 1999).

POSTULATED MECHANISMS TO EXPLAIN THE BRIDGING OF THE SPECIES BARRIER BY HIV

A virus similar to HIV, namely simian immunodeficiency virus (SIV) can be found in chimpanzees and monkeys. The African green monkeys (AGM) are believed to be asimptomatic carriers (Daniel *et al.,* 1987). Between 1950 and 1960, primary kidney-cell cultures of the AGM were used for polio vaccine preparation. It is possible that the polio vaccine stocks were contaminated with this virus and thus transmitted to humans (Hooper, 1997; Lachman, 1999). Another possibility is that the simian viruses may have been transferred to humans during the skinning and butchery of chimpanzees – the hunters were exposed to vast amounts of blood and allowed the virus to enter into the human population (Gao *et al.,* 1999; Kanabus & Allen, 2004).

THE EFFECT OF HIV ON THE IMMUNE SYSTEM

The human immunodeficiency virus targets and destroys the helper T-cell (CD4) lymphocytes in the body, which forms an integral part of the immune system. As the number of T-cells declines, the normal immune control mechanism breaks down. When an infection occurs, suppressor factors released by suppressor T-cells inhibit an immune response before the few surviving helper T-cells can stimulate the formation of cytotoxic T-cells or plasma cells in adequate numbers (Martini, 1998). HIV's effect on the immune system is explained in more detail in the following chapter under section 2.2.

1.2 AIMS AND OBJECTIVES

Several anti-retroviral drugs have been introduced and are currently available for treatment, either as single therapy or in combination therapy. Some of the challenges associated with HIV/AIDS treatments are resistance to drug treatment and the cost of anti-retroviral therapy. It is thus imperative that new drugs become available to replace those against which resistance have developed, preferably at a lower cost.

The primary aim of this study was to explore a series of novel compounds that may elicit HIV-1 protease activity. The protease enzyme was singled out because of its specificity and the fact that the crystal structure, complexed with several inhibitors, is available and has been well described (Kempf *et al.*, 1990). A series of compounds having the 1,3-diaminopropan-2-ol structure as a core (fig. 1.1) and containing benzofuran-2-carboxylic acid and indole-2-carboxylic acid moieties were synthesised and evaluated, *in silico* and *in vitro*, for anti-HIV activity.

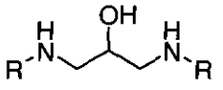


Figure 1.1: 1,3-Diaminopropan-2-ol as a core structure in the novel series of compounds.

1.3 DESIGN

To achieve the aim the following approaches were used:

- Design and molecular modelling of the compounds to evaluate the proposed interaction with the enzyme;
- Synthesis and characterisation of compounds with promising in silico properties;
- Biological evaluation of characterised compounds using *in vitro* anti-HIV screening.

This first exploration into the possible anti-HIV properties of this series could thus pave the way for further in depth anti-HIV studies on this class of compounds and derivatives thereof.

Chapter 2

HIV and Drugs

It is generally accepted that HIV/AIDS has caused much trauma and fatalities around the world, and there is an ongoing need for new medication. In the following chapter, the structure of the human immunodeficiency virus, lifecycle and transmission of HIV is described, as well as current medication available for treatment and those still under investigation.

2.1 THE HUMAN IMMUNODEFICIENCY VIRUS

The virus belongs to a subgroup of the retroviruses known as lentiviruses. The course of infection with these viruses is characterised by a long interval between initial infection and the onset of serious symptoms. Other lentiviruses infect nonhuman species e.g. the simian immunodeficiency virus (SIV) that infects monkeys and other nonhuman primates. Like HIV in humans, these animal viruses primarily infect immune system cells, often causing immunodeficiency and AIDS-like symptoms (NIAID, 2001; Daniel *et al.*, 1987).

HIV has genes composed of ribonucleic acid (RNA) while human genes contain a related molecule, deoxyribonucleic acid (DNA). Like all viruses, HIV can only replicate once inside a cell (NIAID, 2001). DNA usually produces RNA, but not in this case. The retrovirus undergoes certain biochemical changes in which the genetic material, in the form of a single-strand RNA, is converted into a double-strand DNA. The enzyme responsible for this conversion is reverse transcriptase (NIAID, 2001; Joshi & Joshi, 1996).

2.1.1 The Structure Of HIV

The virus has a circular shape and it consists of a viral envelope and a viral core.

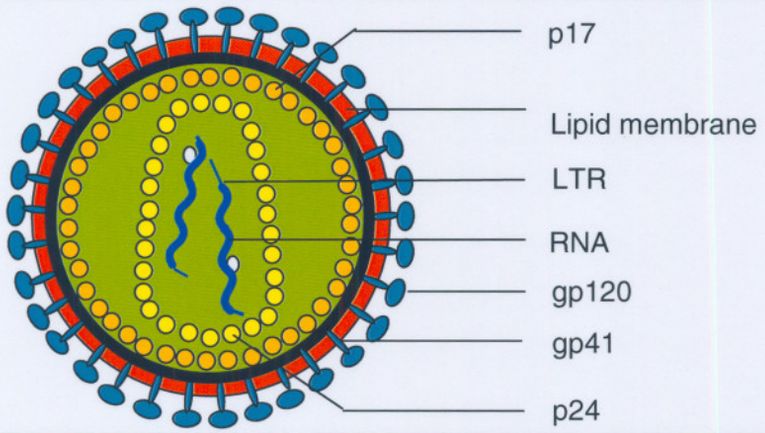


Figure 2.1: The structure of HIV (NIAID, 2001).

VIRAL ENVELOPE:

The viral envelope is composed of two layers of lipids, taken from the membrane of a human cell when a newly formed virus particle buds from the cell. Proteins from the host cell are embedded in the viral envelope, as well as 72 copies (on average) of a complex HIV protein that protrudes from the envelope surface. This protein (*env)* consists of a cap of glycoprotein 120 (gp120), and a stem of gp41. These projections (gp120) have an attraction to certain target cells, especially CD4 receptor sites (NIAID, 2001, Aloia *et al.,* 1988).

VIRAL CORE:

Within the envelope of a mature HIV particle is a bullet shaped core or capsid, made of viral proteins, called p24. The capsid surrounds two single strands of HIV RNA, each of which contain a copy of the virus's nine genes. Three of these genes, namely: *gag, pol,* and *env,* contain information needed to make structural proteins for new virus particles (Mervis *et al.,* 1988). The ends of each strand contain a RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or host cell.

The core also contains six regulatory genes, *tat, rev, nef, vif, vpr* and *vpu.* These genes contain information needed for the production of proteins that control the ability of HIV to infect a cell, produce new copies of virus or cause disease (Joshi & Joshi, 1996).

The core also includes the p7 protein, the HIV nucleocapsid protein, and three enzymes that perform the later steps in the virus's life cycle: reverse transcriptase, integrase and protease. Another HIV protein namely p17, or the HIV matrix protein, lies between the viral core and the viral envelope (NIAID, 2001; Joshi & Joshi, 1996).

2.1.2 The Life Cycle of HIV

There are eight basic steps in the virus's life cycle (steps 1-8 in fig. 2.2) and therapeutic intervention may target one or more of them.

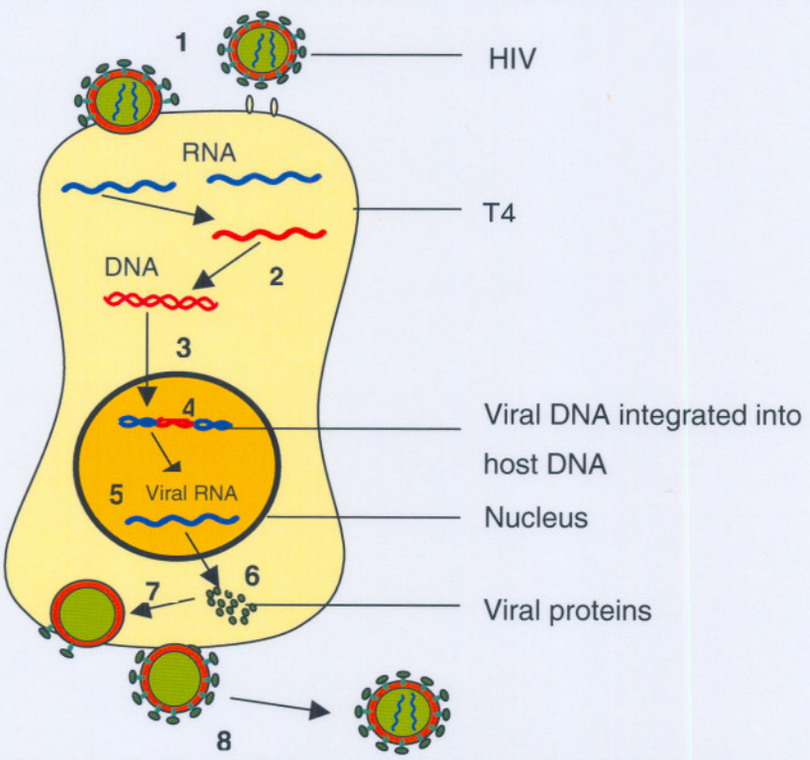


Figure 2.2: The life cycle of the HIV virus (NIAID, 2001).

The following footnotes are explanatory of these steps and therapeutic interventions.

1. **Entry:** After binding to the CD4 receptor site, the membranes of the virus and the cell fuse. The virus's RNA, proteins and enzymes are released into the cell.
2. **Reverse transcription:** In the cytoplasm of the cell, HIV reverse transcriptase converts viral RNA into DNA.
3. **Transport:** The newly made HIV DNA is then transported to the nucleus.
4. **Integration:** There it is spliced into the host's DNA with the help of HIV integrase. Once incorporated into

the cell's genes, HIV DNA is called a "provirus."

5. **Transcription:** For a provirus to produce new viruses, RNA copies must be made. These copies are called messenger RNA (mRNA), and the production of mRNA is called transcription, a process that involves the host cell's own enzymes.
6. **Translation:** After HIV mRNA is processed in the cell's nucleus; it is transported to the cytoplasm. In the cytoplasm, the virus co-operates with ribosomes to make long chains of viral proteins and enzymes, using HIV mRNA as a template.
7. **Assembly and budding:** Newly made HIV core proteins, enzymes and RNA gather just inside the cell's membrane, while the viral envelope proteins aggregate within the membrane. An immature viral particle forms and pinches off from the cell, acquiring an envelope that includes both cellular and HIV proteins from the cell membrane. During this part of the viral life cycle, the core of the virus is immature and the virus is not yet infectious. The long chains of proteins and enzymes that make up the immature viral core are now cleaved into smaller pieces by a viral enzyme called protease. This step results in infectious viral particles.
8. The virus is then released into the bloodstream, matures and infect other cells (NIAID, 2001).
9. In this process, the host cells (such as CD4 T lymphocytes) are damaged and destroyed (Joshi & Joshi, 1996).

2.2 HIV INFECTION AND AIDS

2.2.1 Effect On The Immune System

Lymphocytes play a major role in the immune system and are composed of 2 complementary arms:

CELL-MEDIATED IMMUNITY refers to T-cells, which consists of:

- Cytotoxic (killer) cells (CD8) that attack foreign cells or body cells infected by viruses.
- Helper T-cells (CD4) that stimulate the activation and function of both T- and B cells.
- Supressor T-cells inhibit the activation and function of both T- and B cells (Platt, 1993).

HUMORAL IMMUNITY refers to B cells, which are:

- Antibody-secreting cells that secretes antibodies that function by inactivating antigens (foreign organisms) and marking them for engulfment and digestion by phagocytic cells, such as macrophages (Platt, 1993).

After entering the body, HIV attaches to the CD4 receptors. These receptors are present on various types of blood cells including: lymphocytes, such as CD4 (helper) T lymphocytes, macrophages, monocytes, tissue cells (such as dendritic cells present in the genital tract and ano-rectal region), certain brain cells (glia cells) and some other cells (Cook *et al.*, 1994; Sharpless *et al.*, 1992).

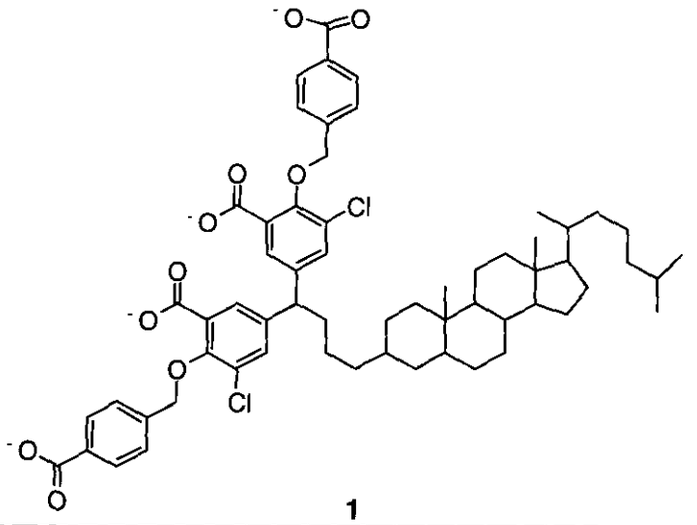
The CD4 helper cells protect the body from invasion by certain bacteria, viruses, fungi and parasites. The T helper cells remove cancer cells and are involved in the production of interleukins and interferons. They also influence the development and function of monocytes and macrophages, which act as scavenger cells in the immune system. Destroying these cells thus means that some of the most important cells of the body's immune or defence system are immobilised. It takes the virus a number of years to sufficiently deplete the immune system to cause immunodeficiency and immune-incompetence (Evian, 2000).

2.3 TREATMENT OF HIV

HIV can be effectively treated with anti-retroviral drugs that target specific steps in the life cycle of HIV (fig. 2.2; De Clercq, 2002).

2.3.1 Virus Adsorption Inhibitors (gp120 inhibitors)

The virus adsorption inhibitors include a variety of polyanionic compounds that interfere with the binding of the virus to the cell surface (step 1 in fig. 2.2): i.e. polysulfates, polysulfonates, polycarboxylates, polyphosphates, polyphosphonates, polyoxometalates, etc. Cosalane analogues (**1**; fig. 2.3) containing the polycarboxylate pharmacophore, as well as the sulfated polysaccharides extracted from sea algae, are also included in this class of compounds (De Clercq, 2002).

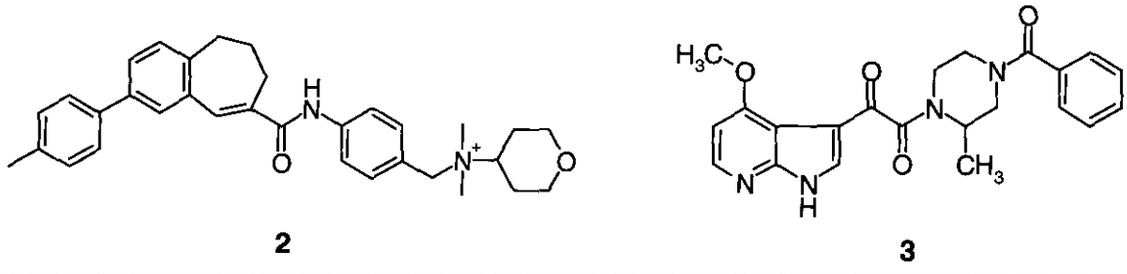


1

Figure 2.3: Cosalane analogue as a virus adsorption inhibitor (gp 120 inhibitor).

2.3.2 Viral Co-Receptor Antagonists

After the binding of the virus to the CD4 cell (step 1 in fig. 2.2), the HIV-1 particles have to interact through the gp120, with the CXCR4 co-receptor or CCR5 co-receptor in order to enter the cell. CXCR4 is the co-receptor for HIV-1 strains that infect T-cells and the CCR5 for HIV-1 strains that infect macrophages. TAK-779 (**2**; fig. 2.4) was the first non-peptidic molecule that has been described to block replication of M-tropic R5 HIV-strains at the CCR5 level (Baba *et al.*, 1999). BMS-378806 (**3**; fig. 2.4) is an investigational drug that blocks the HIV entry process. These compounds specifically inhibit gp120 binding to the cellular CD4 receptors, functioning independently of viral co-receptors. As such, this class of attachment inhibitors appears suitable for advancement into clinical development (Lin *et al.*, 2003).

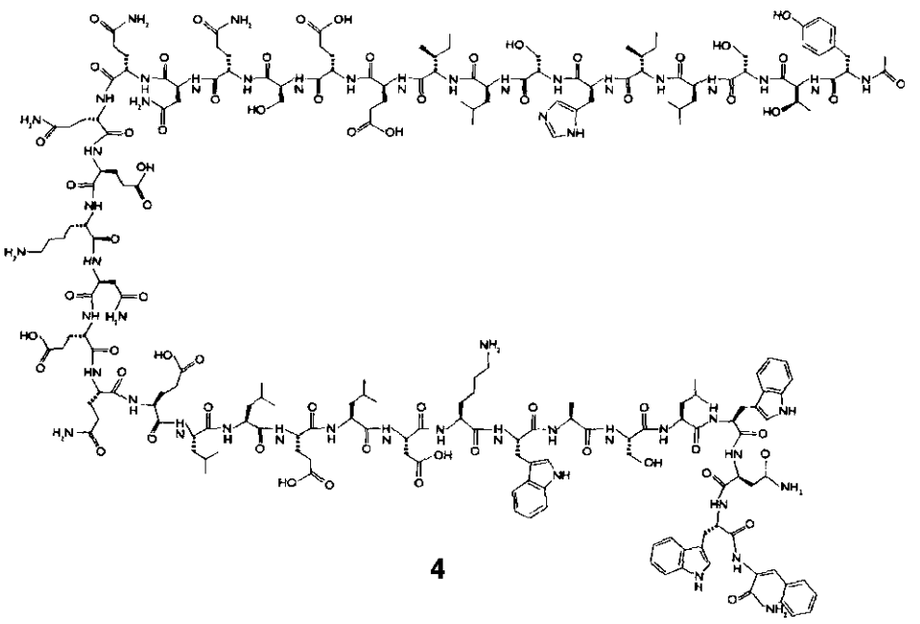


2 **3**

Figure 2.4: TAK-779 (**2**) as a CCR5 co-receptor antagonist and BMS-378806 (**3**) as a gp120 inhibitor.

2.3.3 Viral Fusion Inhibitors (gp41 inhibitors)

After the interaction described above under paragraph 2.3.2, the gp41 (fig. 2.1) anchors itself to the membrane of the target cell, by a spring-loaded action of the protein through the amino terminus ('fusion peptides') into the target cell membrane (step 1 in fig. 2.2). This initiates the fusion of the lipid layer of the viral envelope with that of the cellular plasma membrane. The first described compound of this class is enfuvirtide (**4**; fig. 2.5). It is a novel 36-amino-acid synthetic peptide that binds to the HR-1 region of HIV-1 gp41 and inhibits the fusion of the virus with CD4 cells (Rockstroh & Mauss; 2004).

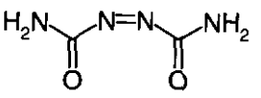


4

Figure 2.5: Enfuvirtide (**4**), as a viral fusion inhibitor.

2.3.4 Nucleocapsid Protein Targeted Agents (ncp7)

The two zinc fingers [Cys-X_2-Cys-X_4-His-X_4-Cys (CCHC), where X = any amino acid] in the nucleocapsid (NCp7) protein comprise the proposed molecular target for zinc-ejecting compounds. These compounds should be able to interfere with both early (uncoating) and late phases (packaging) of retrovirus replication (step 1 in fig. 2.2). ADA (azadicarbonamide) (**5**; fig. 2.6) was the first compound in this group to proceed to phase I/II clinical trials in advanced AIDS patients at the time of this study (De Clercq, 2002).



5

Figure 2.6: ADA (**5**) as a nucleocapsid protein inhibitor (NCp7 Zn-finger inhibitor).

2.3.5 Nucleoside Reverse Transcriptase Inhibitors

Nucleoside reverse transcriptase inhibitors (NRTIs), targets the substrate binding site (step 2 in fig. 2.2). These drugs include zidovudine (**6**; fig. 2.7), lamivudine (**7**; fig. 2.7), didanosine, zalcitabine, stavudine and abacavir. Emtricitabine (**8**; fig. 2.7) has recently been approved by the FDA for use by adults in combination therapy (Anon; 2003a). Tenofovir disoproxil fumurate (**9**; fig. 2.7) was the first nucleotide reverse transcriptase inhibitor (NtRTIs) approved for use in combination therapy (Fung *et al.*, 2002).

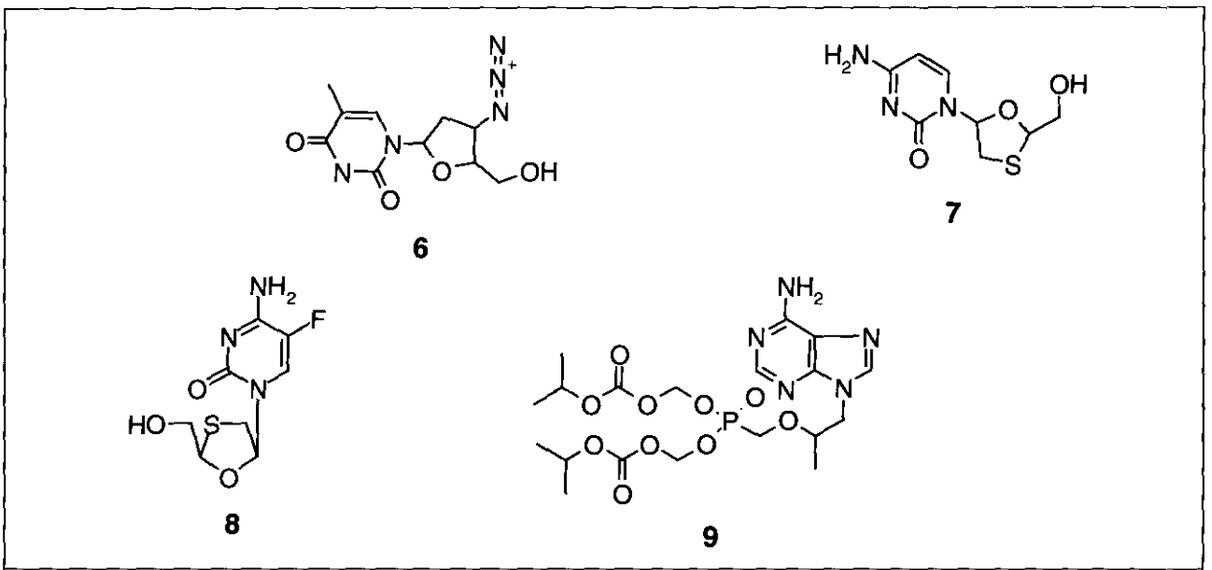
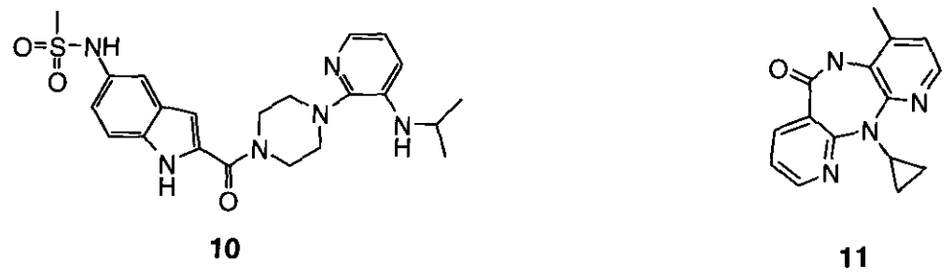


Figure 2.7: Nucleoside reverse transcriptase inhibitors and **9** as a nucleotide reverse transcriptase inhibitor.

2.3.6 Non-nucleoside Reverse Transcriptase Inhibitors

Several compounds that show inhibition of HIV-1 replication and targeted at a nonsubstrate binding site of the reverse transcriptase, have been identified as non-nucleoside reverse transcriptase inhibitors (NNRTIs). Delavirdine (**10**; fig. 2.8), nevirapine (**11**; fig. 2.8) and efavirenz have so far been formally licensed for clinical use (Costi *et al.*, 2004).

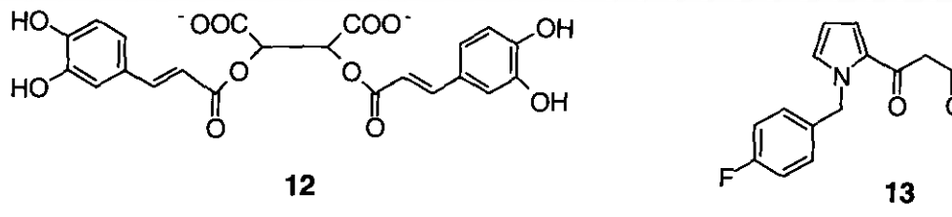


10 11

Figure 2.8: Non-nucleoside reverse transcriptase inhibitors.

2.3.7 HIV Integrase Inhibitors

HIV is unable to replicate without integration into a host chromosome (step 4 in fig. 2.2) and this is the target for the action of L-chicoric acid (**12**; fig. 2.9). Numerous integrase inhibitors have been described. The problem with integrase inhibitors is that while they might be effective in an enzyme-based assay, their effectivity *in vivo* might be compromised by their cytotoxity. Starting from L-731,988 (**13**; fig. 2.9) various derivates have been reported as integrase inhibitors in trials (King *et al.*, 2003).

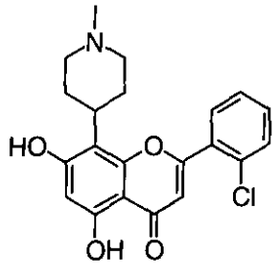


12 13

Figure 2.9: HIV integrase inhibitors L-chicoric acid (**12**) and L-731,988 (**13**).

2.3.8 Transcription Inhibitors

Compounds have been designed to interfere with the transcription process, and thus inhibit the replication of the virus. HIV gene expression might be inhibited by compounds that interact with cellular factors that bind to the LTR promoter (fig. 2.1), but greater specificity, however, can be expected from compounds that specifically inhibit the transactivation of the LTR promoter by the viral *tat* protein (step 5 in fig. 2.2). Flavopiridol (**14**, fig. 2.10), which was in clinical trial for the treatment of cancer, was found to block *tat* and also inhibited HIV replication (Sadaie *et al.*, 2004).



14

Figure 2.10: Transcription inhibitor flavopiridol (**14**).

2.3.9 HIV Protease Inhibitors

HIV protease is the enzyme responsible for the processing of the polyproteins to structural proteins and enzymes essential for the viral maturation and infectivity. Inhibition of protease prevents maturation and replication of the virus and thus results in the production of immature, non-infectious virons (Kohl *et al.*, 1988; Le Grice *et al.*, 1988; Peng *et al.*, 1989; Gottlinger *et al.*, 1989; Chen *et al.*, 2003).

Drug discovery of HIV-1 protease inhibition started in the 1980's when renin inhibitors were used as lead compounds (Richards *et al.*, 1989). The recombinant crystal structure of the HIV-1 protease enzyme was designed and inhibitors were developed using this structure (Navia *et al.*, 1989). Inhibitors were also designed based upon the chemically synthesised protease enzyme (Wlodawer *et al.*, 1989) of which the inhibitor MVT-101 was one of the first (Miller *et al.*, 1989). The structure of the HIV-1 protease enzyme has been elucidated crystallographically and used for the design of inhibitors. The C_2-symmetry was an attractive characteristic to be used in drug discovery and design. A hydroxyethyleneamine containing moiety has been used in most designed protease inhibitors as a core structure (Kempf *et al.*, 1993).

Protease inhibitors have been part of the highly active anti-retroviral therapy (HAART), thus used in combination with other drugs. The problem with this regime is the cross-resistance that occurs. Mutant strains have become resistant to current medication and therefore new drugs are needed (Fatkenheuer *et al.*, 1997; Prabu-Jeyabalan *et al.*, 2003).

There are currently seven peptidomimetic protease inhibitors available for therapeutic use, namely saquinavir (**15,** fig. 2.11), ritonavir, indinavir, amprenavir (**16**, fig. 2.11) nelfinavir (**17**, fig. 2.11) and lopinavir (Costi *et al.*, 2004). Atazanavir (**18**, fig. 2.11) is the most recent protease

inhibitor approved for HIV treatment (Anon, 2003b).

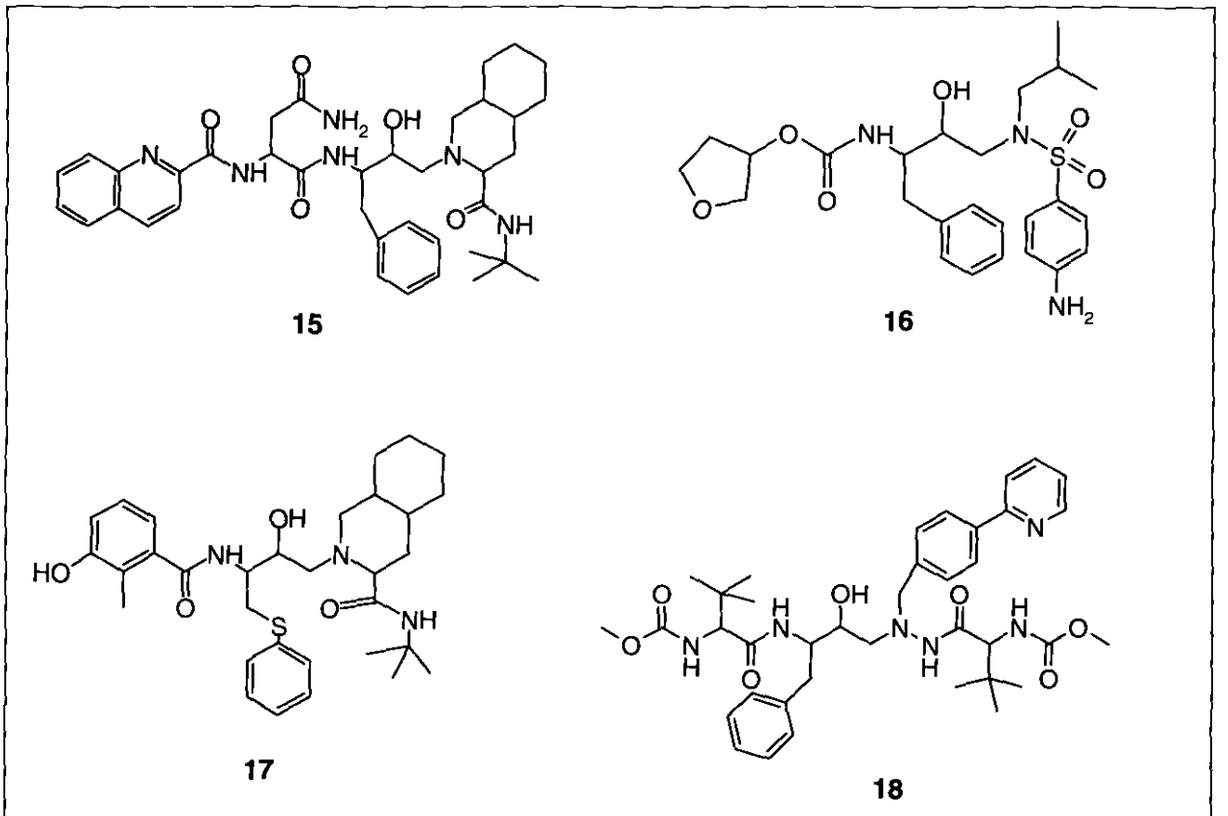


Figure 2.11: Peptidomimetic protease inhibitors.

Non-peptidic protease inhibitors that have been designed, but which are still under investigation, includes: mozenavir (DMP-450) (**19**, fig. 2.12), and tipranavir (**20**, fig. 2.12) (Moyle, 2002; Plosker & Figgitt, 2003).

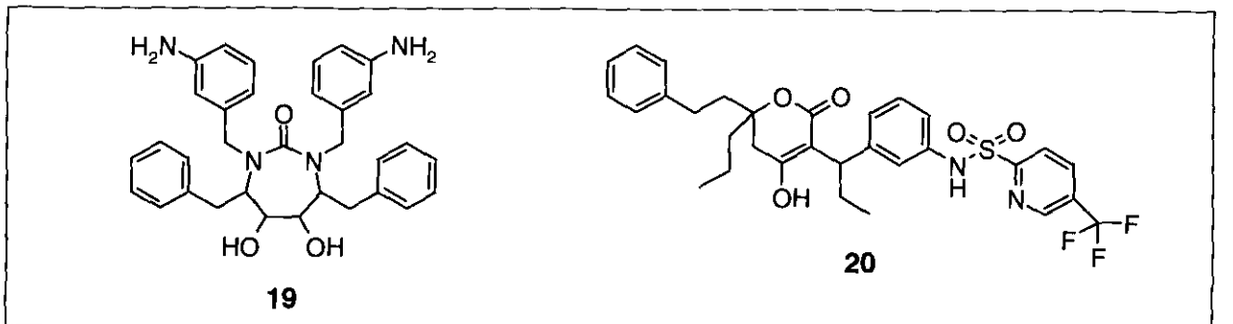


Figure 2.12: Non-peptidic protease inhibitors DMP-450 (**19**) and tipranavir (**20**).

2.4 DISCUSSION AND CONCLUSION

The human immunodeficiency virus is an intracellular parasite that uses the CD4 cells of the human body as a host to reproduce more virons. The life cycle offers opportunities for drug targeting as described in Figure 2.2 (De Clercq, 2002). The current available data indicate that the therapeutic interventions thus far showed some benefit in treatment of HIV/AIDS and had to some extend caused a decrease in mortality rate. Combination therapy has been developed and has been part of highly active anti-retroviral therapy (HAART) that has assisted to suppress the replication of the virus. One of the major difficulties however is the development of drug resistance as well as cross-resistance, therefore continuous new development in anti-HIV treatment is necessary (Fatkenheuer *et al.,* 1997; Prabu-Jeyabalan *et al.,* 2003).

The HIV-1 protease enzyme as a specific target has been selected for this study. This enzyme is well described and has been known since the late 1980's. Development and discovery of aspartic acid protease inhibitors have been described since the early 1980's. It started with the research that was conducted on renin inhibitors as a lead compound (Richards *et al.,* 1989), the discovery of the crystal structure of the native HIV protease (Wlodawer *et al.,* 1989), the structures of complexes between HIV-1 protease and hundreds of inhibitors at the end of the 1980's (Miller *et al.*, 1989) and then the C_2-symmetry in the 1990's etc (Kempf *et al.,* 1993). HIV protease is an attractive target for the design and discovery of new protease inhibitors. Novel protease inhibitors are needed that could inhibit the mutant strains that are resistant to the current protease inhibitors. In chapter 3 the HIV protease enzyme is described and a novel series of inhibitors introduced.

Chapter 3

HIV Protease

New anti-HIV drugs needs to be developed urgently with the vast increase in HIV infections, and the drug resistance that occurs. This study was narrowed down to focus on HIV protease, an attractive target for therapeutic intervention. In this chapter the protease enzyme and possible ligands are discussed.

3.1 HIV PROTEASE ENZYME

Retroviral proteases were tentatively assigned to the aspartic proteinase family in 1985 (Toh *et al,*. 1985) and were hypothesised to function as C_2-symmetric homodimers in which each monomer contributes one of the two conserved aspartates to the active site (Pearl & Taylor, 1987). The hypothesis was verified by the recombinant (Navia *et al.*, 1989) and chemically synthesised HIV-1 protease enzyme of which MVT-101 was one of the inhibitors designed and is used as an illustration later in this chapter (Miller *et al.*, 1989; Wlodawer *et al.*, 1989). Figure 3.1 shows the C_2-symmetry of the HIV-1 protease enzyme.

The sequence of each monomer of the dimer of the protease enzyme is as follows: PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMSLPGRWKPKMIGGIGGFIKVRQYDQILIEICGHKAIG TVLVGPTPVNIIGRNLLTQIGCTLNF, retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank.

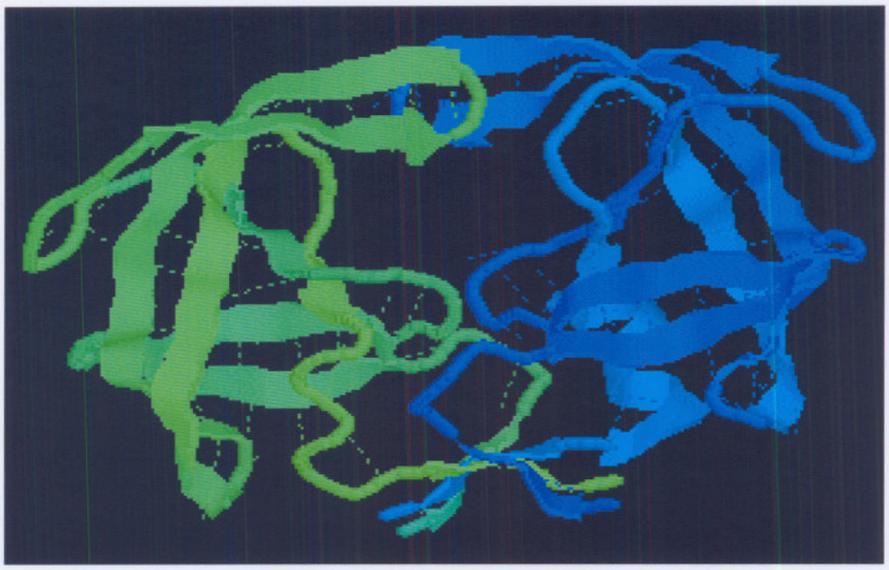


Figure 3.1: HIV Protease enzyme adapted from the RCSB Protein Data Bank, with its C_2-symmetry clearly visible (1M0B).

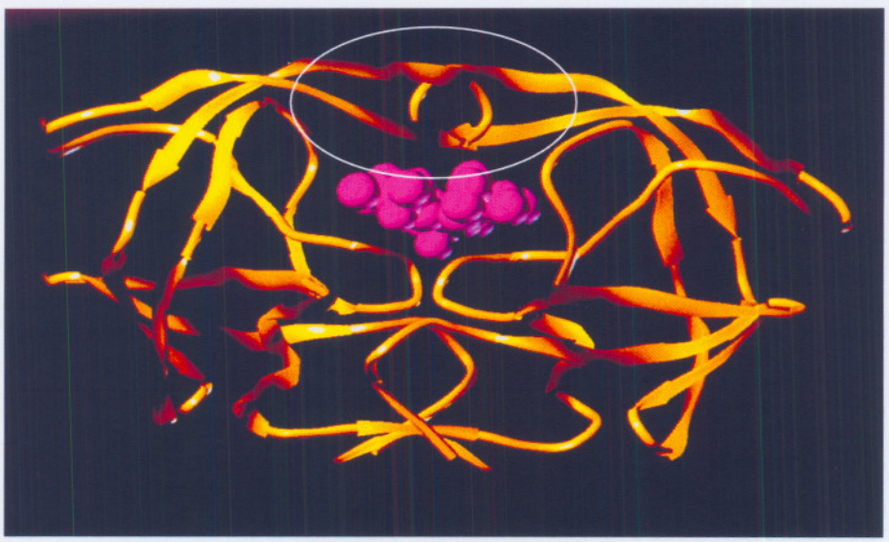
As was earlier mentioned, HIV protease is responsible for the cleaving of the polyproteins $p55_{gag}$ and $p160_{gag\text{-}pol}$ into structural proteins (p17, p24, p7, p6, p2, p1) and enzymes (protease, reverse transcriptase and integrase), which is necessary for viral maturation and infectivity. The protease inhibitor thus prevents the maturation and viral replication of the virus and leads to immature non-infectious virons (Kohl *et al.,* 1988; Le Grice *et al.,* 1988; Peng *et al.,* 1989; Gottlinger *et al.,* 1989; Oscarsson *et al.,* 2003).

The proteases contain an extended β-hairpin structure, or so-called flap, that tightly embraces the substrate in the active site. This active site (residues 25 to 27) consists of a catalytic triplet Asp-Thr-Gly. The two flaps lie almost parallel to the inhibitor, and form two short β-sheets. It contains residues 42 to 58, which closes jaw like over the substrate and thus locking the inhibitor in the binding cleft (James *et al.,* 1982; Toh *et al.*, 1985; Gustchina & Weber, 1990). The β-sheets are separated near the scissile bond where the two flaps overlap, and there are hydrogen bond interactions with a water molecule (Gustchina & Weber, 1990). This water molecule is bound to Ile50 and Ile'50 in the flaps by H-bonds and to the carbonyl of the substrate when in the binding pocket, thus stabilising the protease in its 'closed' conformation (Huang *et al.,* 2002).

Both the binding of the substrate or inhibitor to the enzyme and the release of products thus require a substantial movement of the flaps. Preliminary modelling suggests that the flaps must

move by about 15 Å from their position in the inhibitor complex in order to allow the polyprotein to enter the active site. The inhibitor-protease complex is further stabilised by additional interactions with the two flexible flaps (Gustchina & Weber, 1990). Figure 3.2 is a representation of the flaps in its closed conformation (in the presence of an inhibitor) and in its open conformation without a substrate (Collins *et al.,* 1995, Nicholson *et al.,* 1995). Figure 3.3 **A** shows the binding mode of an inhibitor (MVT-101) to the HIV protease involving the two Ile moieties from the flaps and a water molecule and **B** a network of hydrogen bonds between the protease enzyme and MVT-101 (Molecular Conceptor, 1996).

A.



B.

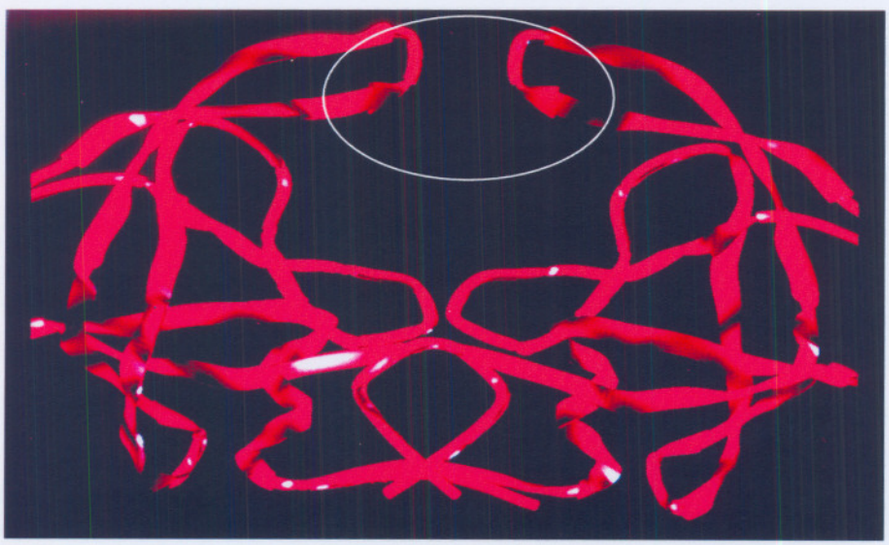
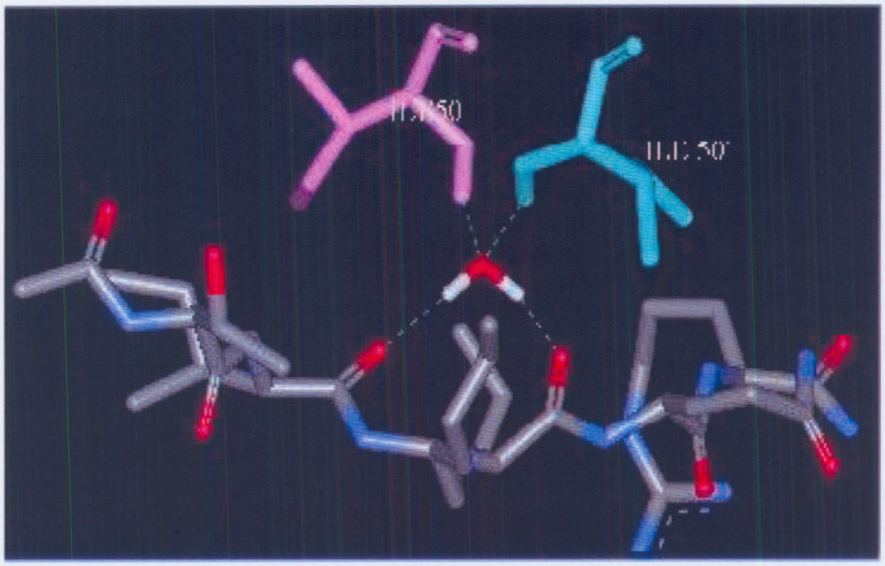


Figure 3.2: **A.** The closed conformation of the HIV-1 protease enzyme in the presence of a substrate (purple). **B.** HIV-1 protease enzyme without an inhibitor, with the flap in an open conformation.

A.



B.

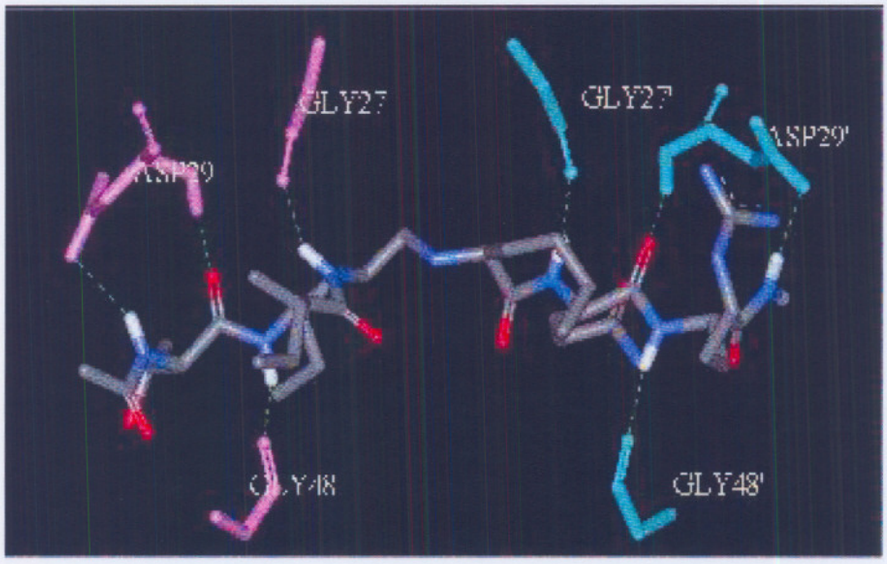


Figure 3.3: **A.** Inhibitor MVT-101 bound to the active site and through a water molecule to Ile 50 and Ile '50. **B.** A network of hydrogen bonds of MVT-101 to the protease enzyme (Molecular Conceptor, 1996).

Asp 25 and Asp '25, which form the pair of catalytic aspartic acids are situated in the centre of the binding site which is hidden under the flaps. The inhibitor has favourable interaction if it can also bind to these two residues (fig. 3.4, Molecular Conceptor, 1996).

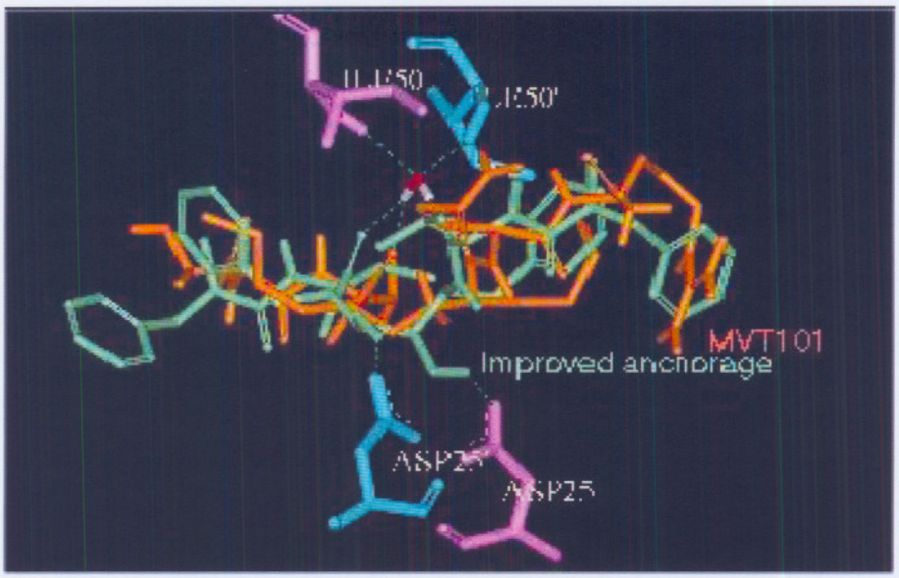


Figure 3.4: Binding of MVT-101 to Asp 25 and Asp '25 (Molecular Conceptor, 1996).

The active site of the enzyme gives an improved anchorage and includes a hydrophobic cavity, with 6 hydrophobic pockets P1-P3; P'1-P'3 around the active site (the hydrophobic residues are almost inside the inner part of the molecule). Based on previous results, it was suggested that the inhibitor should have side chains that could fill these pockets for activity (Molecular Conceptor, 1996). The outer surface of the enzyme is more hydrophilic as can be seen from the Molecular Lipophilicity Potential (MLP) of the HIV protease complexed with an inhibitor (fig. 3.5; Laguerre *et al.*, 1997).

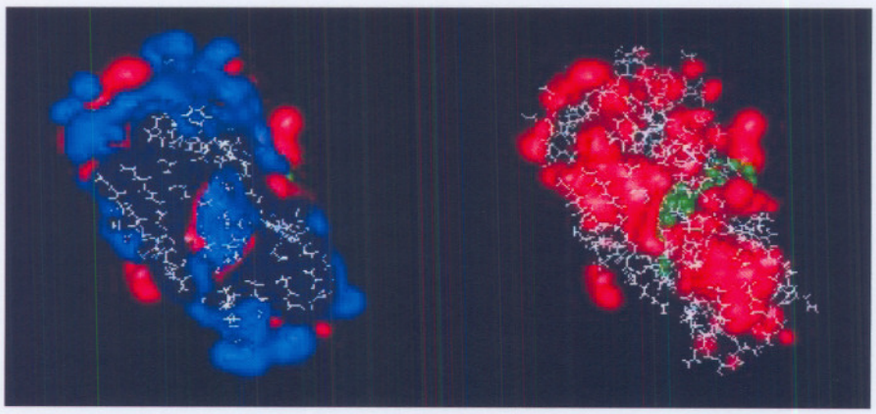


Figure 3.5: MLP of protease complexed with an inhibitor (Laguerre *et al.*, 1997). On the left is the hydrophilic site in blue with the polar residues mostly on the outer surface. On the right is the hydrophobic pattern in red with the inhibitor in green.

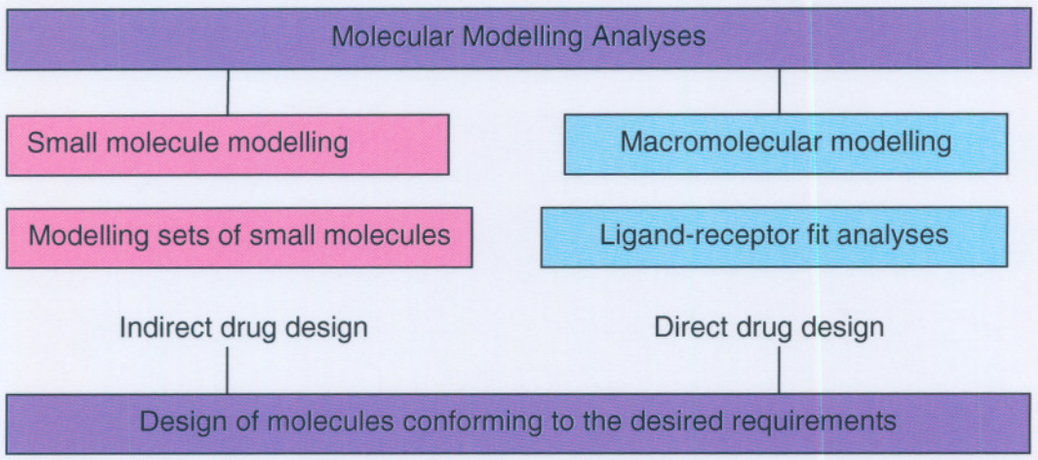


Figure 4.1: Conceptual frame used in molecular modelling and drug design (Cohen, 1996).

4.1.2 Superimposition

To evaluate properties of molecules, one technique is to compare it to its homologous series. This comparison involves aligning or superimposing the molecules so that their differences become obvious (fig. 4.2). Electrostatic calculations can be performed to define positive, neutral and negative regions of a molecule and sites can then also be superimposed on this basis (Gund, 1996). When compounds are superimposed, a RMS (root-mean-square) value is calculated. These RMS values give insight into the global conformation similarity or difference and the lower the RMS-value, the better the fit and similarity. An RMS value of 1.0 Å is considered to be good and 1.5 Å to be acceptable (Martin & Lin, 1996).

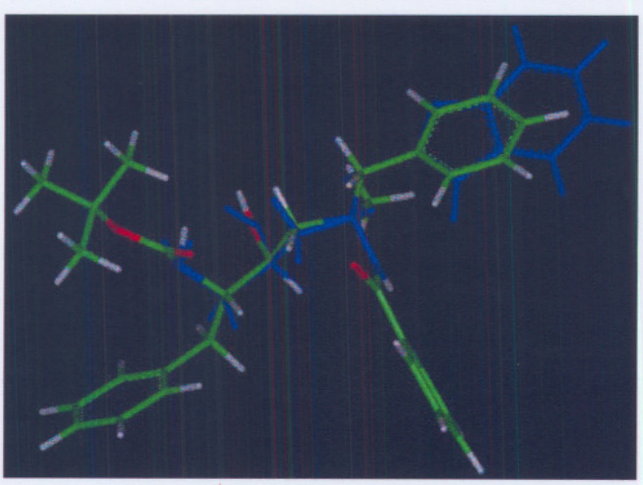


Figure 4.2: SVZ-3 superimposed on AQ148.

4.1.3 Energy Calculations

An essential property of a molecule is its minimum energy conformations as a lower energy level is an indication of a more stable molecule. Molecular mechanics is one of the approaches of computing energy levels and is probably one of the fastest methods as well. The method employs fundamental principles of vibrational spectroscopy as well as the idea that bonds have natural lengths and angles and molecules will adopt geometries that can best reach these natural values. If the natural value cannot be achieved it results in strain, and molecular mechanics methods have van der Waals potential functions that can measure this amount of strain energy. This set of functions is called a force field and it contains adjustable parameters that are optimised to obtain the best fit of calculated and experimental properties of molecules, such as geometries, conformational energies, heats of formation and other properties. Molecular mechanics energy minimisation involves successive iterative computations where an initial conformation is submitted to full geometry optimisation. All parameters defining the geometry of the system are modified by small increments until the overall structural energy reaches a local minimum. The local minimum, however, may not be the global minimum and searching methods are then employed to search other conformations with different energy minima (Gund, 1996).

4.1.4 The HIV Protease Binding Site

The HIV protease enzyme's active site contains the amino acid triad Asp-Thr-Gly (Kempf *et al.,* 1993). The pair of catalytic aspartic acids (residues Asp 25 and Asp '25), are situated in the centre of the binding site, which is hidden under the flaps, that insures better anchorage of the inhibitor to the enzyme (Skálová *et al.*, 2003; Molecular Conceptor, 1996). The presence of an inhibitor or substrate stabilises the HIV-1 protease enzyme dimer in a closed flap conformation (Collins *et al.,* 1995; Nicholson *et al.,* 1995). It has been shown that the flaps are in an open conformation when there is no substrate present and closed in the presence of a substrate thus locking the inhibitor in the binding cleft (Gustchina & Weber, 1990). A network of hydrogen bonds in the catalytic site also plays a vital role in the binding affinities of HIV protease (Huang *et al.,* 2002). The two β-sheets are separated near the scissile bond where the two flaps overlap. Hydrogen bond interactions occur with a water molecule bound to Ile 50 and Ile '50 (Gustchina & Weber, 1990), which is necessary for protease inhibition (Molecular Conceptor, 1996).

Figure 4.3 shows a diagram of the hydrogen bond network between (S,S,R)-1-[2-(N-t-Butoxycarbonyl)amino-1-benzyl-2-hydroxypropyl]-N-methyl-N-benzylaminobenzoylimide (AQ148) and HIV protease.

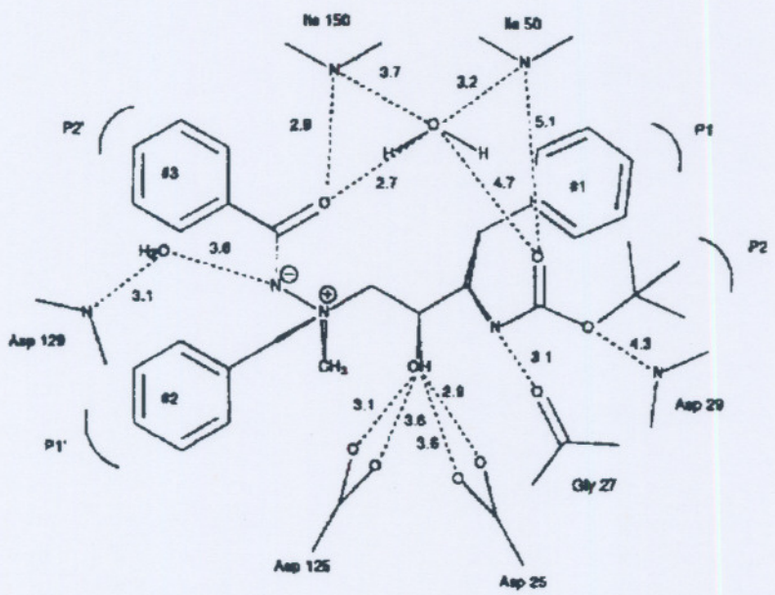


Figure 4.3: The hydrogen bond network of AQ148 with the HIV protease enzyme (Rutenber *et al.*, 1996).

4.2 EXPERIMENTAL

Ten compounds were selected for molecular modelling (**SVZ-1 – SVZ-10**). A structure search was done to find a compound that contained the same core structure as that of the novel series and with favourable properties (fig. 4.4), to do superimposition with. (S,S,R)-1-[2-(N-t-Butoxycarbonyl)amino-1-benzyl-2-hydroxypropyl]-N-methyl-N-benzylaminobenzoylimide (AQ148) was selected and recovered from RCSB Protein Data Bank.

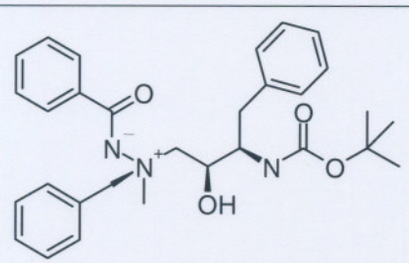


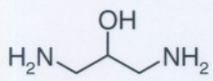
Figure 4.4: AQ148 retrieved from RCSB Protein Data Bank.

4.2.1 General Procedure

The modelling was conducted on a Silicon Graphics Fuel computer using Insight II software (Accelrys Inc; 2002). The 3D structure code of AQ148, selected for superimposition, was retrieved from RCSB Protein Data Bank (fig. 4.4).

The compounds were built on the computer, using the Builder module. The structures were optimised using the default optimisation function of Insight II. AQ148 complexed with the HIV protease enzyme were retrieved from the RCSB Protein Data Bank. The neutral form of the protease enzyme was used throughout the molecular modelling study. The enzyme was then undisplayed with just AQ148 displayed. The novel compounds were superimposed on AQ148 in the cavity. The pharmacophore used for superimposition was the 1,3-diaminopropan-2-ol moiety (fig. 4.5A). The following elements of the pharmacophore were used: NH, CH, OH, NH (fig. 4.5B). The RMS values obtained from these superimpositions were an indication of the 'fit' of the novel compounds on the pharmacophore of AQ148 in the cavity. RMS values of 1 Å and below indicate a favourable fit (Martin & Lin, 1996). A favourable fit however, may not necessarily mean it will exhibit effective anti-HIV activity, because other requirements are also important, such as the network of hydrogen bonds and the interactions of the compounds with the enzyme.

A.



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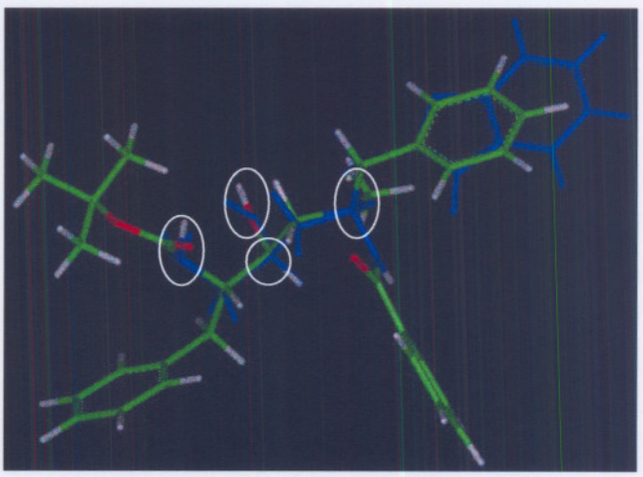


Figure 4.5: **A.** 1,3-Diaminopropan-2-ol used as pharmacophore. **B.** **SVZ-3** superimposed on AQ148, with the pharmacophore elements marked in white.

After superimposing of the novel compounds, the enzyme was displayed and AQ148 was removed. Using the DISCOVER module the compound and the enzyme were defined as an assembly and the backbone of the enzyme was fixed. The default force field (CVFF) was selected with the number of automatic parameters set on 20. Each assembly was minimised, using the default conjugate gradient method. Non-bonded interactions were cut off at distance 9.5 Á for van der Waals and Coulomb forces. The computations were performed with dielectric constant $\epsilon = 1$, as default parameter.

In some instances where minimised structures showed unfavourable fit into the active site, molecular dynamic calculations followed in order to sample conformational space that may show conformations with a better fit. These conformations were again minimised in the active site. Different dynamic runs, with different temperatures and iterations were used in order to explore the conformational space and to obtain the best fit.

The process (minimisation, dynamic runs, minimisation) was repeated 15 times with each compound and the most favourable was recorded. The hydrogen bonds that formed of al ten compounds were compared to that of AQ148 with the HIV-1 protease enzyme (fig. 4.3). The criteria used to select the most promising conformations of the compounds include the lowest energy calculations for the assembly and the formation of hydrogen bonds with Asp 25/Asp '25 and with the water molecule that binds to Ile 50 and/or Ile '50. The hydrogen bonds formed were calculated as a function in the DISCOVER module and were measured in Å.

The results of this study are presented in the following paragraphs with figures showing the network of hydrogen bonds and the distances of the bonds measured.

4.3 RESULTS AND DISCUSSION

4.3.1 *N-(3-Amino-2-hydroxypropyl)-1-benzofuran-2-carboxamide (SVZ-1)*

Superimposition of **SVZ-1** on AQ148 in the protease cavity, had an RMS value of 0,148 Å. Minimisation was done with 3 000 iterations, and a movement limit of 0,2 Å. The maximum derivative was 0.009528 kcal/Å. The temperature was set at 298 K. The total energy of the assembly calculated was 2 009 kcal/mol.

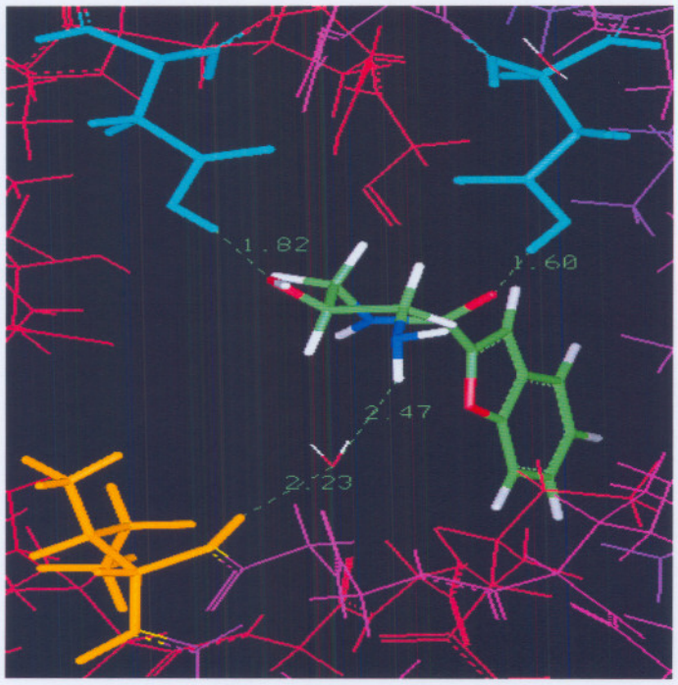


Figure 4.6: **SVZ-1** incorporated into the enzyme. H-bonds formed between Asp 25, '25 (blue) and the inhibitor. **SVZ-1** was bound to the H_2O molecule, which in turn was bound to the flap residue Ile 50 (gold). Distances were measured in Å.

In Figure 4.6 it is clear that there were favourable interactions between **SVZ-1** and the HIV-1 protease enzyme. The hydroxyl group of **SVZ-1** formed a hydrogen bond with Asp 25' while the carbonyl oxygen of the inhibitor was bound to Asp 25. A hydrogen bond also formed between the –NH group of the inhibitor with the structural water molecule, which in turn was bound to Ile 50. These calculations as well as the energy values match the criteria set for possible anti-HIV activity.

4.3.2 N-{3-[(1-Benzofuran-2-carbonyl)amino]-2-hydroxypropyl}-1-benzofuran-2-carboxamide (SVZ-2)

A RMS value of 0,148 Å was calculated for the fit of **SVZ-2** on AQ148. After the default optimisation of **SVZ-2** in complex with the protease enzyme, no meaningful bonds formed in the assembly. Molecular dynamic runs were performed at a temperature of 298 K, with a temperature window of 10 K. The default integration method used was velocity verlet. After the dynamic runs were performed the assembly was minimised, using the conjugate gradient method, with 5 000 iterations and a movement limit of 0,2 Å. The maximum derivative was 1,58 kcal/Å. The total energy measured for the assembly was 2 068 kcal/mol.

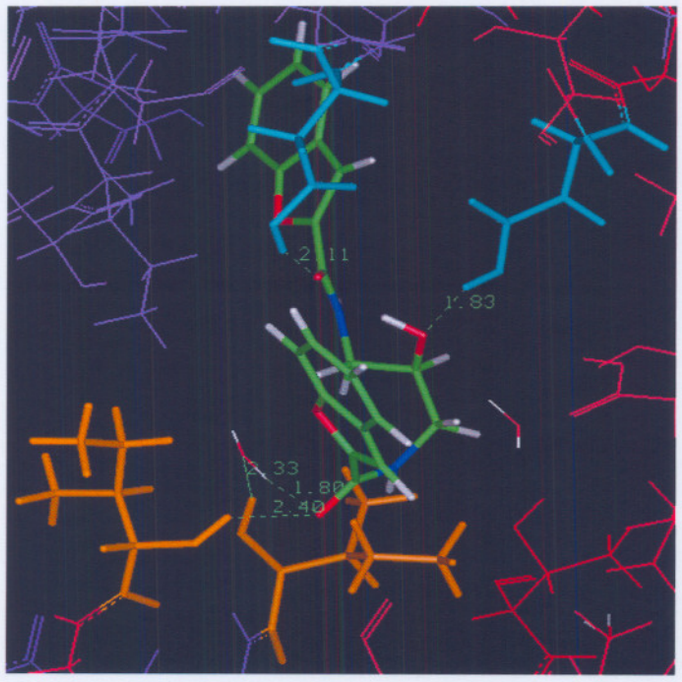


Figure 4.7: **SVZ-2** incorporated into the HIV-1 protease enzyme, with H-bonds forming between Asp 25; '25 (blue); Ile '50 (gold) and the H_2O molecule, which in turn formed a bond with Ile 50 (gold). Distances are measured in Å.

The carbonyl oxygen of **SVZ-2** formed a hydrogen bond with Asp 25 while Asp '25 was bound to the hydroxyl group of the test compound. A bond formed directly between Ile '50 and a carbonyl oxygen of **SVZ-2**. A second bond formed between that same carbonyl and the water molecule, which was bound to Ile 50. The bonds formed here indicate possibility for protease inhibiting activity.

4.3.3 1-Amino-3-[(1-benzofuran-3-methyl)amino]propan-2-ol (SVZ-3)

An RMS fit of 0.109 Å were obtained from **SVZ-3** with AQ148 inside the cavity. A series of dynamic runs yielded an improved conformation of the inhibitor complex. The series of runs were done at a temperature of 298 K, with a temperature window of 10 K. 5 000 iterations were done and the integration method used was velocity verlet. The minimisation was performed with 5 000 iterations and a movement limit of 0.2 Å. The maximum derivative was 0.008861 kcal/Å. The conjugate gradient method was used to do the minimisation. The total energy for the assembly was 2 045 kcal/mol.

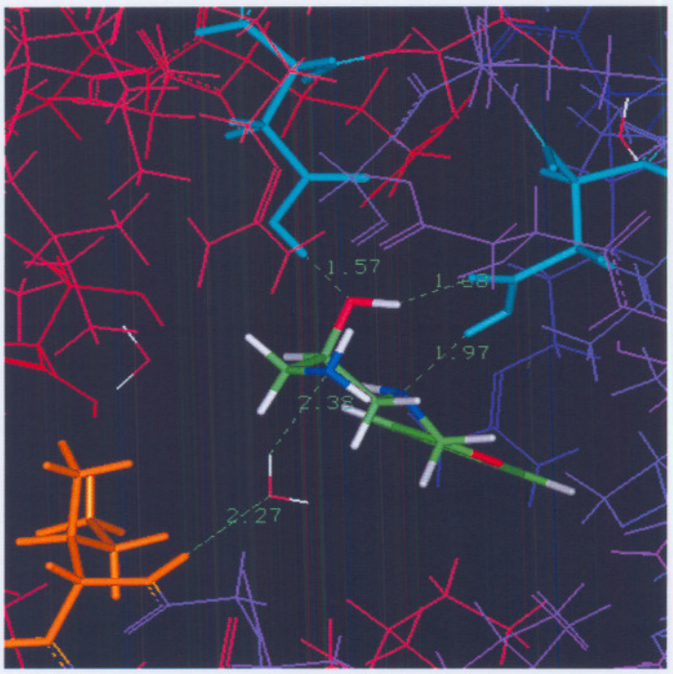


Figure 4.8: **SVZ-3** was incorporated into HIV-1 protease enzyme. H-bonds formed between Asp 25; '25 (blue) and the H_2O molecule, which in turn was bound to Ile 50 (gold). Distances are measured in Å.

In this case the presence of 3 H-bonds with the essential aspartates indicates an increased probability for HIV interaction. The hydroxyl group of **SVZ-3** was bound to Asp '25 and to Asp 25 with distances respectively of 1.57 Å and 1.68 Å. The –NH group, nearest to the aromatic ring of the inhibitor also formed a bond with Asp 25. The other –NH group of the inhibitor was bound to the vital water molecule, which in turn formed a bond with Ile 50. The RMS value of 0.109 Å showed a favourable fit for **SVZ-3** with AQ148 in the cavity.

4.3.4 1,3-Bis[(1-benzofuran-2-methyl)amino]propan-2-ol (SVZ-4)

SVZ-4 had an RMS fit with AQ148 of 0.110 Å. The conjugate gradient method was used to do the minimisation of 5 000 iterations with a default movement limit of 0.2 Å. The maximum derivative was 0.007462 kcal/Å. The temperature was set on 298 K and total energy measured for the assembly was 2 096 kcal/mol.

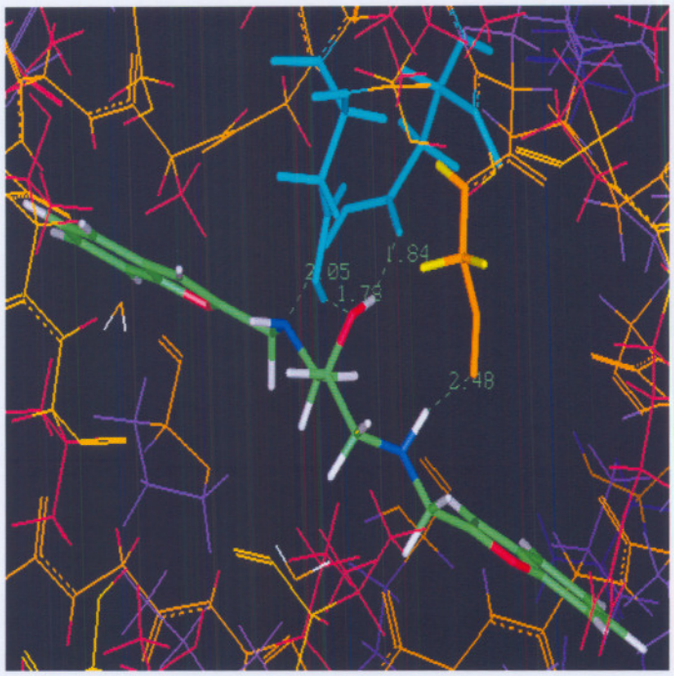


Figure 4.9: SVZ-4 incorporated into the protease enzyme. H-bonds formed between Asp 25; '25 (blue); Gly 27 (yellow) and the inhibitor. Distances were measured in Å.

The hydroxyl group of **SVZ-4** formed two hydrogen bonds with Asp 25 and Asp '25, with distances measure of 1.84 Å and 1.78 Å respectively. Asp '25 also had a bond with one of the –NH groups of the inhibitor while the other –NH group formed a bond with Gly 27 of the active site. It was interesting to notice that the disubstituted compound formed bonds with the two aspartates 25 and '25, but not with the strategically placed water molecule. It was also expected that the disubstituted compound would have more hydrogen bonds than the monosubstituted one, and that a hydrophobic pocket of the enzyme could be filled. A decrease in the interaction of the compound was thus suspected.

4.3.5 N-(3-Amino-2-hydroxypropyl)-1H-indole-2-carboxamide (SVZ-5)

Minimisation on **SVZ-5** was done using the conjugate gradient method, with 3 000 iterations and a movement limit of 0.2 Å. The maximum derivative was 0.0040 kcal/Å. A favourable RMS fit of 0.106 Å were calculated. A total energy of 2 075 kcal/mol was calculated for the assembly.

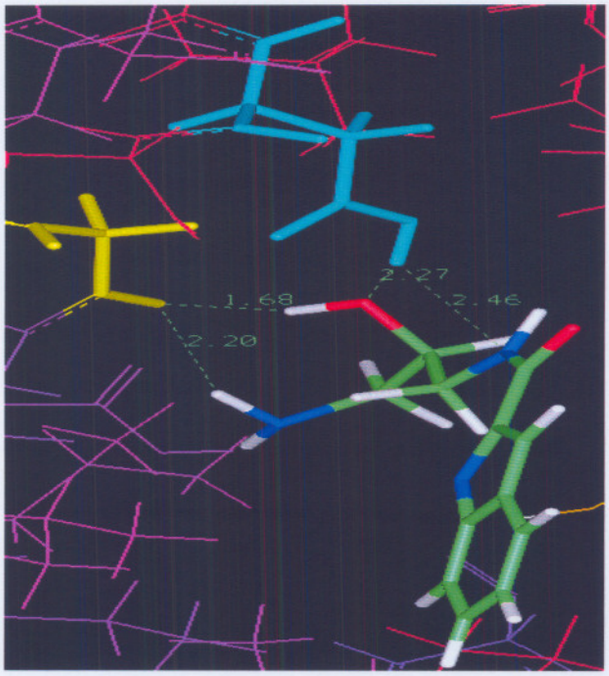


Figure 4.10: **SVZ-5** incorporated into the HIV-1 protease enzyme. H-bonds formed between Asp '25 (blue); Gly 27 (yellow) and the inhibitor. Distances were measured in Å.

For **SVZ-5** the Asp '25 formed two hydrogen bonds, one with the hydroxyl group and the other with the –NH group nearest to the ring. The hydroxyl group formed a second bond with Gly 27 of the active site, at a distance of 1.68 Å. The $-NH_2$ group also formed a bond with Gly 27. It's important to notice the absence of H-bonds with the essential water molecule and Ile 50/'50. A decrease in interaction was thus expected.

4.3.6 N-{2-Hydroxy-3-[(1H-indole-2-carbonyl)amino]propyl}-1H-indole-2-carboxamide (SVZ-6)

SVZ-6 had a RMS value of 0.668 Å in the protease cavity with AQ148. Minimisation was done with 5 000 iterations and with a default movement limit of 0.2 Å. The conjugate gradient method was used and a maximum derivative of 0.000923 kcal/Å was measured. The minimisation was done at a temperature of 298 K with a total energy calculated of 2 125 kcal/mol.

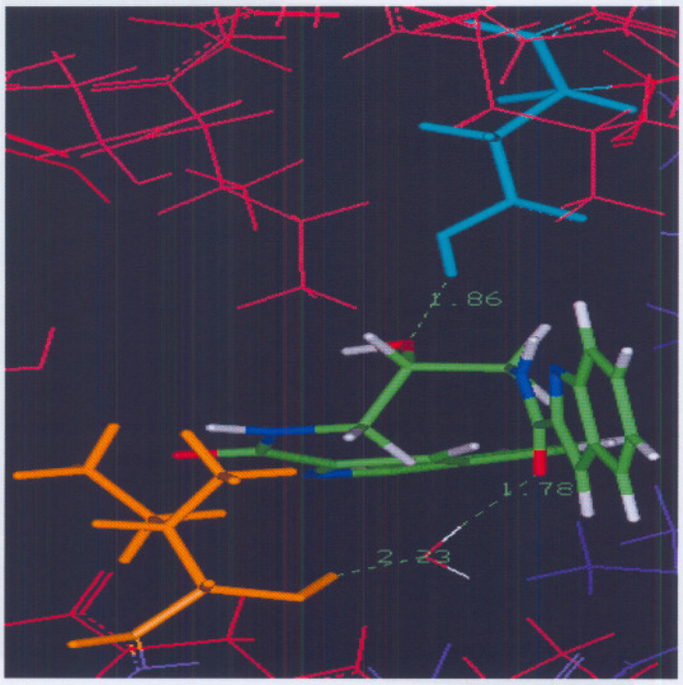


Figure 4.11: SVZ-6 incorporated into the protease enzyme. H-bonds formed between the ligand and Asp '25 (blue) and the H_2O molecule that was bound to Ile 50 (gold). Distances were measured in Å.

Only one hydrogen bond formed between Asp '25 and the hydroxyl oxygen. One carbonyl group formed a hydrogen bond with the strategically placed water molecule, which in turn was bound to Ile 50. Neither the carbonyl oxygen nor the –NH group of the disubstituted compound had hydrogen bonds with the aspartates or the water molecule as was expected. The RMS value was 0.668 Å, which showed a relatively favourable overlap, but a decrease in potency of the inhibitory effects was expected.

4.3.8 1,3-Bis[1H-indole-2-methyl)amino]propan-2-ol (SVZ-8)

A RMS value of 0.186 Å was calculated for **SVZ-8** and AQ148 in the cavity. Minimisation was done using the conjugate gradient method with 5 000 iterations and a movement limit of 0.2 Å at a temperature of 298 K. The maximum derivative was 0.000978 kcal/Å. The total energy of the assembly was 2 114 kcal/mol.

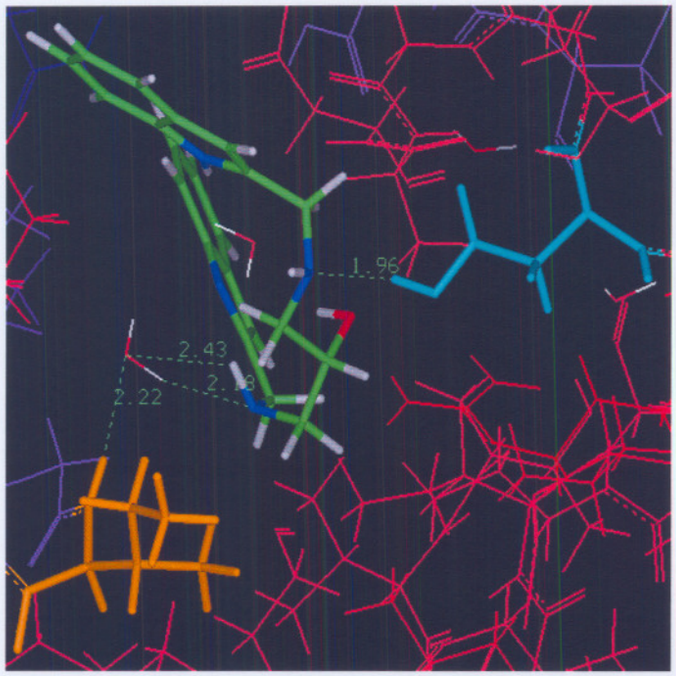


Figure 4.13: **SVZ-8** in complex with the HIV-1 protease enzyme. Bonds formed between Asp '25 (blue) and the inhibitor. **SVZ-8** was bound to a water molecule, which in turn was bound to Ile '50 (gold). Distances were measured in Å.

Only one of the active site's aspartates formed a hydrogen bond with **SVZ-8**. The –NH group of the inhibitor was bound to Asp '25. There was no bond with the hydroxyl group of **SVZ-8** to the enzyme. Only one Ile residue of the flaps was bound to the compound via the water molecule. The necessary water molecule was bound with two bonds to the other –NH group of the inhibitor as well as to Ile '50.

4.3.9 1-Amino-3-[(1H-indole-3-methyl)amino]propan-2-ol (SVZ-9)

The superimposition of **SVZ-9** with AQ148 resulted in an RMS value of 0.765 Å, which indicates a favourable fit. Minimisation of the assembly was done using the conjugate gradient method with 5 000 iterations and a movement limit of 0.2 Å. The maximum derivative was 0.000975 kcal/Å. The minimisation was done at 298 K. The total energy calculated for the assembly was 2 067 kcal/mol.

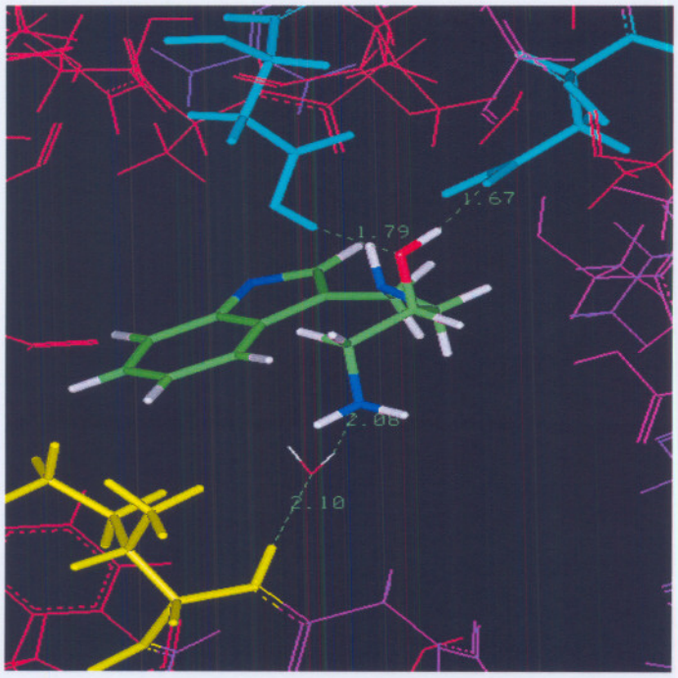


Figure 4.14: **SVZ-9** was incorporated into the HIV protease enzyme. H-bonds formed to Asp 25, '25 (blue) and the H_2O molecule, which was bound to Ile50 (gold). Distances were measured in Å.

In this case, both the aspartates of the active site were involved in hydrogen bonds with the compound **SVZ-9**. The hydroxyl group of **SVZ-9** formed two hydrogen bonds, one with Asp 25 at a distance of 1.67 Å and one bond with Asp '25 at a distance of 1.79 Å. The $-NH_2$ of the compound was bound to the water molecule, which was bound to Ile 50. Favourable interaction with HIV protease was expected.

4.3.10 1,3-Bis[1H-indole-3-methyl)amino]propan-2-ol (SVZ-10)

Minimisation was done using the conjugate gradient method with 1000 iterations and a movement limit of 0.2 Å. The maximum derivative was 4.2094 kcal/Å. The minimisation was done at 298 K and the total energy for the assembly calculated was 2 110 kcal/mol.

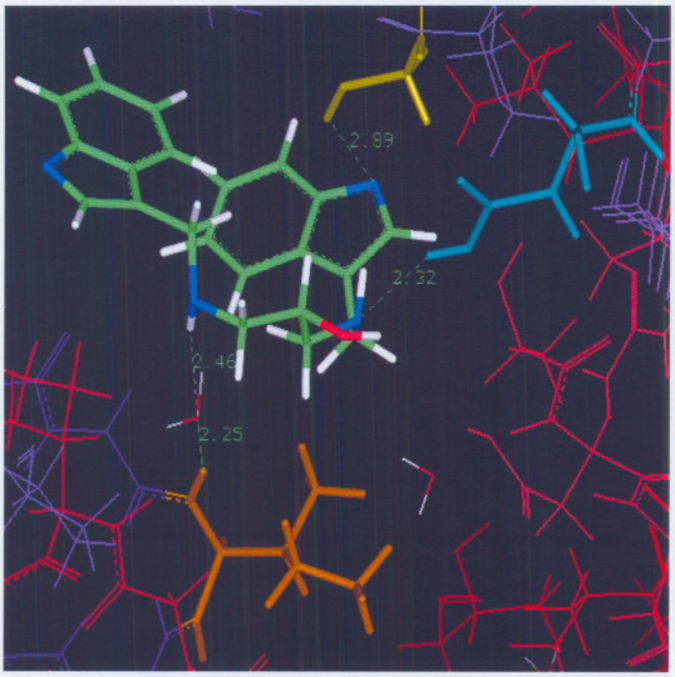


Figure 4.15: SVZ-10 complexed with HIV-1 protease. H-bond network formed between the inhibitor and Asp '25 (blue), Gly 27 (yellow) and the H_2O molecule, which in turn was bound to Ile 50 (gold). Distances were measured in Å.

Only one aspartate residue of the active site was involved in hydrogen bonds with the compound. Asp '25 was bound to one of the amines of the inhibitor. An extra bond formed between a residue of the active site, Gly 27 and the –NH group of the indole moiety. The other –NH group of **SVZ-10** was bound to the water molecule, which in turn formed a bond with Ile 50. There was no bond visible between the hydroxyl group of the compound with the enzyme. The inhibitor had a satisfyingly overlap with AQ148 with a RMS value of 0.871 Å and may have interactions at the active site.

The unique symmetry of the HIV protease structure offers a unique opportunity for the design of C_2-symmetric inhibitors that match the characteristics of the enzyme structure, making HIV protease a very attractive target for therapeutic intervention. The first inhibitors, based on its C2-symmetry were designed by Kempf *et al.* (1990) and Erickson *et al.* (1990). Highly potent and selective peptidomimetic inhibitors have been designed, and it has proven to be a very effective strategy. Clinical studies showed that these inhibitors drastically reduced viral replication and improved the CD4 lymphocyte count in HIV infected patients (Chen *et al.*, 2003, Palella *et al.*, 2003).

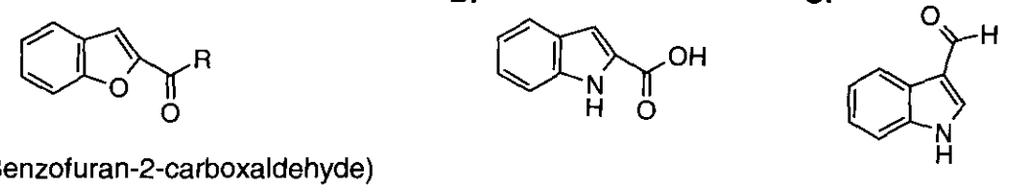
Protease inhibitors are often combined with other drugs to form a highly active antiretroviral therapy (HAART), which has been responsible for a meaningful decrease in the death rate from AIDS since its introduction. However, after 24 weeks of treatment, the success rates are reduced by a variety of factors including compliance issues, short- and long term toxicity of antiretroviral agents, unfavourable pharmacokinetic profiles and differences in potency (Marcelin *et al.*, 2004). As a result, in clinical practice virological failure rates up to 50 % are common within the first 2 years after initiation of HAART (Fatkenheuer *et al.*, 1997; Prabu-Jeyabalan *et al.*, 2003). The peptidomimetic inhibitors are characterised by low oral bioavailability/high pill load, rapid biliarly excretion, high molecular weight and high lipophilicity (Mitsuya, 1992; Medou *et al.*, 1998; Tomasselli & Heinrikson, 2000).

These findings prompted the design of non-peptidic or pseudopeptidic inhibitors with a C_2-symmetry that may be expected to inhibit the mutant strains of HIV-1 that have become resistant to peptidomimetic protease inhibitors. This strategy has been proven to be effective for the design of compounds with desired pharmacological properties. Compounds, which contain a C_2-symmetry would present a good molecular complement within the enzyme and would be less recognisable by the endogenous proteases, which should result in better bioavailability (Wlodawer & Vondrasek, 1998; Sham *et al.*, 2001; Tossi *et al.*, 2003).

A few of the symmetric inhibitors that have been designed, presented in Figure 3.6 include LC (Marastoni *et al.*, 1997), A-80987 (Kempf *et al.*, 1994) and A-74704 (Erickson *et al.*, 1990).

Recent studies have shown that indole derivatives are more potent than their pyrrole counterparts and some of these compounds showed interesting protease inhibition (Di Santo *et al.*, 2002). Indole-2-carboxylic acid (**B**; fig. 3.8) and Indole-3-carboxaldehyde (**C**; fig. 3.8) were thus also incorporated into the proposed structures containing 1,3-diaminopropan-2-ol.

A. **B.** **C.**



R = H (Benzofuran-2-carboxaldehyde)

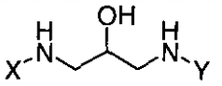
R = OH (Benzofuran-2-carboxylic acid)

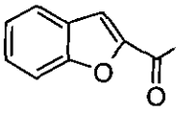
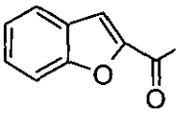
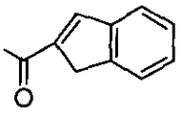
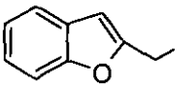
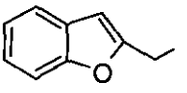
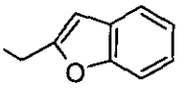
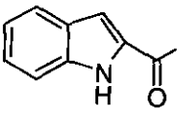
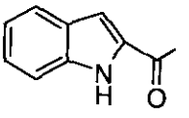
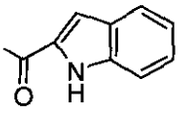
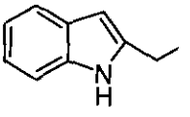
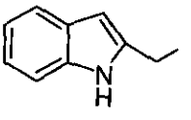
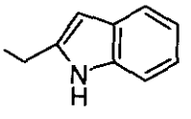
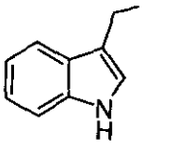
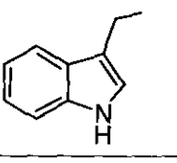
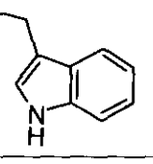
Figure 3.8: A. Benzofuran-2-carboxylic acid/-aldehyde. **B.** Indole-2-carboxylic acid. **C.** Indole-3-carboxaldehyde.

In this study the aim was thus to design and synthesise new compounds that includes mono- and disubstituted compounds, containing the above moieties.

In view of this discussion the following structures were proposed for this study:

Table 3.1: Proposed structures in this study.



Nr.	Name	X	Y
SVZ-1	N-(3-amino-2-hydroxypropyl)-1-benzofuran-2-carboxamide		H
SVZ-2	N-{3-[(1-benzofuran-2-carbonyl)amino]-2-hydroxypropyl}-1-benzofuran-2-carboxamide		
SVZ-3	1-amino-3-[(1-benzofuran-2-methyl)amino]propan-2-ol		H
SVZ-4	1,3-bis[(1-benzofuran-2-methyl)amino]propan-2-ol		
SVZ-5	N-(3-amino-2-hydroxypropyl)-1*H*-indole-2-carboxamide		H
SVZ-6	N-{2-hydroxy-3-[(1*H*-indole-2-carbonyl)aminopropyl}-1*H*-indole-2-carboxamide		
SVZ-7	1-amino-3-[(1*H*-indole-2-methyl)amino]propan-2-ol		H
SVZ-8	1,3-bis[(1*H*-indole-2-methyl)amino]propan-2-ol		
SVZ-9	1-amino-3-[(1*H*-indole-3-methyl)amino]propan-2-ol		H
SVZ-10	1,3-bis[(1*H*-indole-3-methyl)amino]propan-2-ol		

3.2 DISCUSSION AND CONCLUSION

The focus of the current study was the design of compounds interacting with the HIV protease enzyme. The HIV enzyme has been described as a homodimer with an Asp-Thr-Gly catalytic active site, with a rotational C_2-symmetry (Pearl & Taylor, 1987). The enzyme contains a flap region, i.e. residues 42 – 58 that tightly embrace the substrate. The flap is flexible in its movements and can 'open' or 'close' to accommodate the substrate (Gustchina & Weber, 1990). Essential parts of the flaps are residues Ile 50 and Ile '50 that form a hydrogen bond with a water molecule that stabilises the enzyme in the closed position (Huang *et al.*, 2002). It is generally accepted that hydrogen bonds with residues Asp 25 and Asp '25 is necessary for substrate binding with interaction. It was also suggested that a hydrogen bond to the water molecule, which is bound to Ile 50 and Ile '50 is necessary for HIV-1 protease inhibition (Molecular Conceptor, 1996).

Protease inhibition proved to be an effective approach of HIV-1 treatment in combination with other drugs. After 24 weeks of treatment, however resistance as well as cross-resistance occurs (Marcelin *et al.*, 2004) and virological failure rates of up to 50 % in the first two years of the HAART regime occur (Fatkenheuer *et al.*, 1997; Prabu-Jeyabalan *et al.*, 2003). Therefore it is clear that novel protease inhibitors are needed in the treatment of HIV-1.

In view of previous studies, the current program focussed on 1,3-diaminopropan-2-ol as a core structure, which have been used, throughout HIV-1 protease inhibitor research. In view of these findings, ten novel compounds have been designed and submitted for computer modelling.

Chapter 4

Computer Aided Molecular Modelling

In this chapter molecular modelling is described as a method of drug design/discovery. It is a relatively new method of drug discovery and is still developing in numerous ways.

4.1 MOLECULAR MODELLING

4.1.1 Introduction

One of the most difficult challenges for medicinal chemists today is the rational drug design of new therapeutic compounds. In the years past, medicinal chemists developed new drugs based on the structure of a lead compound. The medicinal chemists used their experience and chemical intuition, to find an analogue that exhibits good biological properties. This procedure involved many trial and error cycles, not to mention patience. It is also very expensive, difficult and time-consuming. Computer modelling represents one of the more recent developments in medicinal chemistry and has established itself in pharmaceutical industry and academic research as a useful tool in drug discovery research (Cohen, 1996).

Drug design has a more direct approach by the understanding of the molecular processes involved in the underlying disease with the aid of computer aided molecular modelling. The first step is the understanding of the molecular target (receptor, enzyme) and it became possible with the developments in X-ray crystallography and the information it provided, to see the

molecular architecture of protein targets. This aids the understanding of the 3D structure of receptors and ligands and precisely how it is controlled in the regulation of the life process. New compounds can be designed either on the basis of similarities to already known structures or on the basis of their complementarily to known 3D active sites. In combination with X-ray crystallography or NMR data, molecular modelling implies the use of theoretical methods like ab initio, semi empirical, or molecular mechanics to evaluate and predict the minimum energy conformation and other physical and chemical properties of the molecule. Molecular modelling assists us in understanding this process in a qualitative but also sometimes quantitative way. It can be described as a series of computerised techniques based on theoretical chemistry methods and experimental data that can be used either to analyse molecules and molecular systems or to predict molecular and biological properties (Cohen, 1996).

Molecular modelling in drug design can be applied in two ways: *Direct* and *Indirect*. The *direct* design can be used when the three-dimensional (3D) features of the target molecule (receptor, enzyme) is known and considered for the design of lead structures. The medicinal chemist is able to build the molecule, based on the structure determined by X-ray crystallography, with the help of the computer. He can then optimise the fit of the ligand molecule to its host molecule and establish the best geometric and electronic complementarities between the ligand and its receptor. The structure of the ligand, its substituents and its conformation can be modified and the most favourable conditions for interaction can be simulated on the screen (Wermuth, 1996).

Indirect design is an alternative approach. Here the analysis is based on the comparison of the stereochemical and physicochemical features of a set of known active/inactive molecules. Lead structures are then designed on the basis of the pharmacophore model obtained by such analysis. This can be done by superimposing proposed compounds on a known active. If a favourable overlap is found for the test compound on an active, one could speculate that the chosen compounds would mimic the activity of the compound used of template (Wermuth, 1996).

4.4 DISCUSSION AND CONCLUSION

All the proposed compounds formed hydrogen bond networks in the active site of the enzyme but the critical bonds to the catalytic aspartates and to the isoleucine residues in the flaps (through the water molecule) were not always evident.

The results of the modelling study are presented containing the RMS-fit with AQ148, the number of hydrogen bonds formed between the test compounds and the HIV protease enzyme (without and through the water molecule) and the energy of the compound-HIV protease enzyme assembly.

Table 4.1: Summary of the results obtained.

CODE	**NAME**	**RMS** Å	**NO. OF BONDS** Without H_2O to Ile 50/'50	**NO. OF BONDS** Through H_2O to Ile 50/'50	**ENERGY** kcal/mol
SVZ-1	N-(3-amino-2-hydroxypropyl)-1-benzofuran-2-carbozamide	0.148	3	1	2 009
SVZ-2	N-{3-[(1-benzofuran-2-carbonyl)amino]-2-hydroxypropyl}-1-benzofuran-2-carboxamide	0.148	4	1	2 068
SVZ-3	1-amino-3-[(1-benzofuran-2-methyl)amino]propan-2-ol	0.109	4	1	2 045
SVZ-4	1,3-bis[(1-benzofuran-2-methyl)amino]propan-2-ol	0.110	4	0	2 096
SVZ-5	N-(3-amino-2-hydroxypropyl)-1*H*-indole-2-carboxamide	0.106	4	0	2 075
SVZ-6	N-{2-hydroxy-3-[(1*H*-indole-2-carbonyl)aminopropyl}-1*H*-indole-2-carboxamide	0.668	2	1	2 125

SVZ-7	1-amino-3-[(1*H*-indole-2-methyl)amino]propan-2-ol	0.198	3	1	2 030
SVZ-8	1,3bis[(1*H*-indole-2-methyl)amino]propan-2-ol	0.186	3	1	2 114
SVZ-9	1-amino-3-[(1*H*-indole-3-methyl)amino]propan-2-ol	0.765	3	1	2 067
SVZ-10	1,3-bis[(1*H*-indole-3-methyl)amino]propan-2-ol	0.871	3	1	2 110

In the current molecular modelling study, **SVZ-2** as a disubstituted compound was the only compound that formed hydrogen bonds with both the aspartates of the active site, Asp 25 and Asp '25, as well as two bonds with both the residues in the flap region, Ile 50 and Ile '50. Compounds **SVZ-1, 3, 7** and **9** were bound to both the aspartates Asp 25 and Asp '25, but had only one bond with Ile 50 or Ile '50. **SVZ-4** was bound to both the aspartates Asp 25 and Asp '25, but not to the strategically positioned water molecule that was bound to Ile 50/'50. It did however form a bond to another residue of the active site, Gly 27. The other three compounds of the novel series **SVZ-6, 8** and **10** had only one bond with an aspartate, either Asp 25 or Asp '25 and to the essential water molecule that was bound to either Ile 50 or Ile '50. **SVZ-5** had a bond with one aspartate, Asp '25 and to another residue of the active site, Gly 27. **SVZ-4** and **5** were the only compounds that were not bound to the water molecule. Eight out of the ten novel compounds showed bonds between the enzyme and the hydroxyl group of the inhibitor. **SVZ-8** and **10** didn't have any bonds involving the hydroxyl group.

Four of the five compounds that were bound to both the aspartates and the water molecule, were monosubstituted structures and three of the compounds that were bound to one aspartate and the water molecule were disubstituted structures.

There was no meaningful difference in terms of the energy levels between the monosubstituted and disubstituted compounds. The RMS values were all satisfactory and ranged between 0.106 Å (**SVZ-5**) and 0.871 Å (**SVZ-10**), which was still acceptable considering that it is below 1 Å. All the compounds that were bound to both the aspartates Asp 25 and Asp '25 and the water molecule had RMS values in the range of 0.1 Å with the exception of **SVZ-9** with a value of 0.765 Å.

From the data of the modelling study it can be concluded that the novel compounds appears to be reasonable candidates for further investigations in view of their interaction profiles at the HIV protease active site. In view of all the observations made, it was deemed necessary to synthesise the compounds and to evaluate them biologically for HIV protease interaction.

Chapter 5

Synthesis

Synthesising non-peptidic, symmetrical protease inhibitors was not without some obstacles. The low yields and purification of the products posed significant challenges. In the following chapter the synthesis of the novel compounds are described, as well as the general procedures of the synthetic routes used.

5.1 EXPERIMENTAL METHODS

5.1.1 Instrumentation

MASS SPECTROMETRY (MS):

An analytical VG 70-70E mass spectrometer with ionisation by electron impact (EI) was used. In some instances ionisation by fast atom bombardment (FAB) was employed. Relevant spectra are given in annexure 1.

INFRARED (IR) ABSORPTION SPECTROPHOTOMETRY:

IR spectra were recorded on a Nicolet 470 FT-IR spectrophotometer, applying the samples either as film or incorporated in KBr pellets. Relevant spectra are given in annexure 1.

MELTING POINT:

Melting points were performed in capillary tubes using a Gallenkamp melting point apparatus.

NUCLEAR MAGNETIC RESONANCE SPECTROMETRY (NMR):

A Varian VXR300 spectrometer was used to obtain ^{1}H and ^{13}C spectra. ^{1}H spectra were recorded at a frequency of 300.075 MHz and ^{13}C spectra at 75.462 MHz, in a 7 Tesla magnectic field with tetramethylsilane (TMS) as internal standard and a bandwidth of 100 Hz at 24 kG applied for ^{1}H and ^{13}C decoupling. All chemical shifts are reported in parts per million (ppm) relative to TMS ($\delta = 0$). The following abbreviations are used to indicate multiplicities of signals: s – singulet, bs – broad singulet, d – doublet, t – triplet, and m – multiplet. Spectra of selected compounds are given in annexure 1.

5.1.2 Chromatographic Methods

Mobile phases used were prepared on a volume-to-volume basis (v/v). Snyder's selectivity triangle (Poole & Dias, 2000) and polarity index (Kok ChemWare, 2002) were used to determine suitable mobile phases. A constitution of dichloromethane: petroleum ether: methanol (6:3:1) was used. Ultraviolet light (UV 254 nm)) and 5 % ninhydrine solution was used for visualisation on thin layer chromatography.

THIN LAYER CHROMATOGRAPHY (TLC):

TLC was used to monitor all chemical reactions and progress throughout the experiments. Aluminium silica gel sheets Merck® were used.

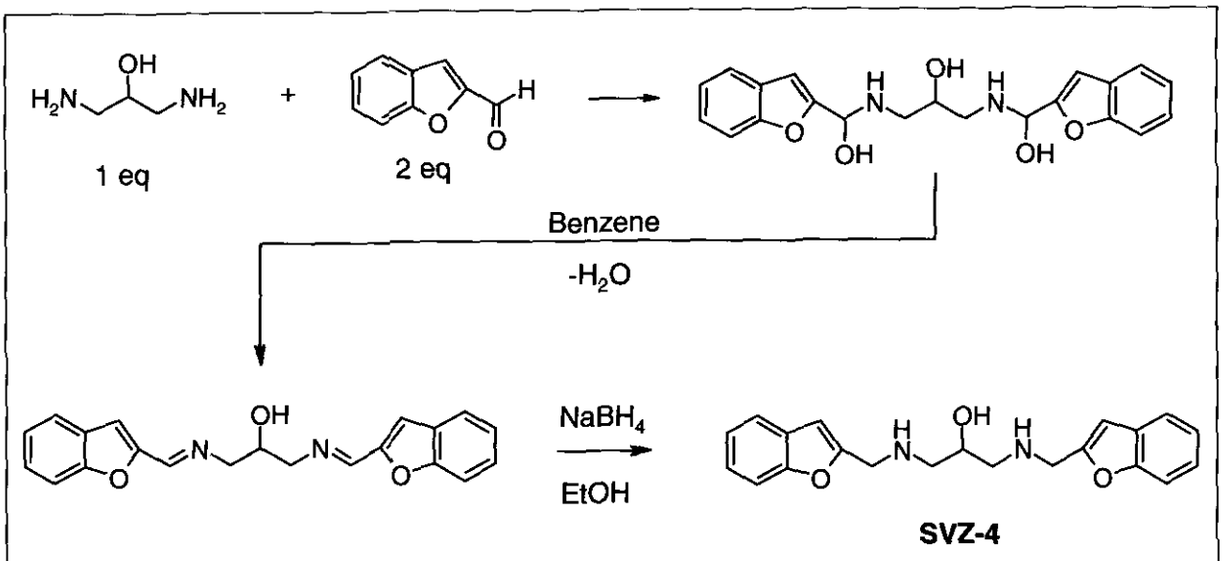
COLUMN CHROMATOGRAPHY:

Separation and purification of product mixtures were done using flash chromatography (Still *et al.*, 1978) on glass columns filled with silica gel Merck® (0.063-0.200 mm).

5.2 SYNTHESIS

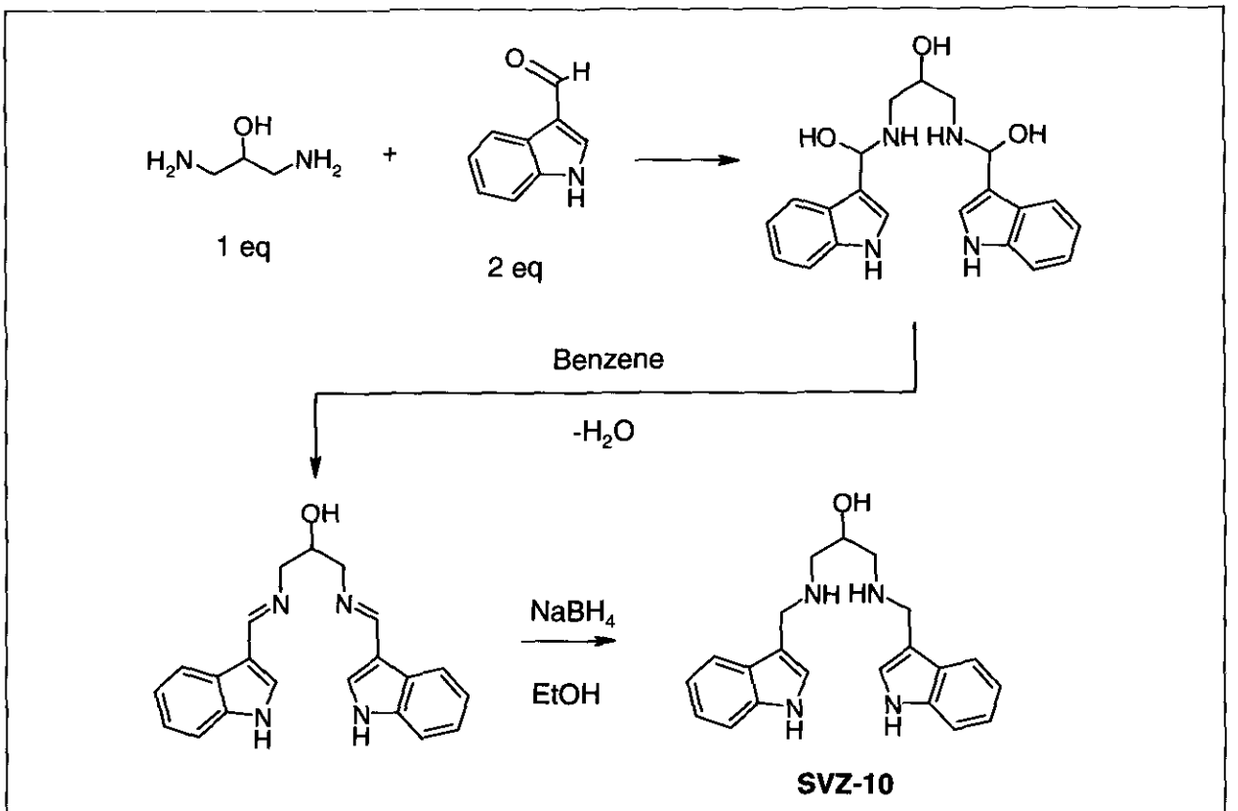
5.2.1 General Route Using Direct Coupling

The basic route of synthesis of **SVZ-3, 4** and **SVZ-9, 10** is given in Figure 4.1 and Figure 4.2 respectively (Medou *et al.*, 1999). Direct coupling of 1,3-diaminopropan-2-ol with benzofuran-2-carboxaldehyde and indole-3-carboxaldehyde was done respectively. Water was removed by Dean-Stark dehydration. The crude diimine was reduced with $NaBH_4$ in anhydrous ethanol to produce the final compound.



For **SVZ-3,** 1 equivalent carboxaldehyde was used

Figure 5.1: General synthesis of **SVZ-3** and **4.**



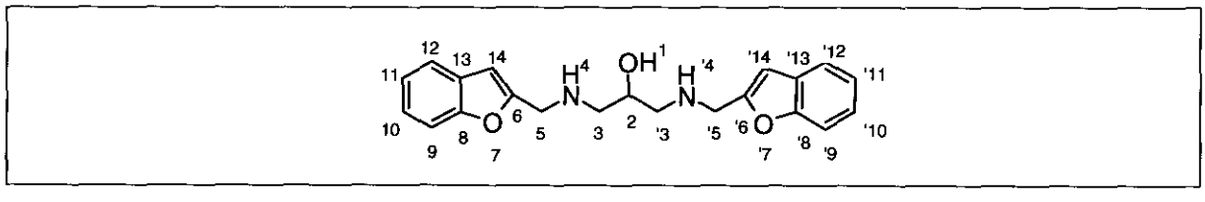
For **SVZ-9,** 1 equivalent carboxaldehyde was used

Figure 5.2: General synthesis of **SVZ-9** and **SVZ-10.**

5.2.1.1 1,3-Bis-[(benzofuran-2-methyl)-amino]-propan-2-ol (**SVZ-4**)

SYNTHETIC PROCEDURE:

1,3-Diaminopropan-2-ol (0,31 g; 3,45 mmol) was dissolved in 30 ml anhydrous ethanol and stirred at room temperature. 1 g (6,85 mmol) benzofuran-2-carboxaldehyde was added to the solution and stirred overnight (18 hours) at room temperature. The ethanol was removed *in vacuo*, and the mixture was then refluxed in 60 ml sodium-dried benzene (Dean-Stark) to remove the water. The benzene was removed *in vacuo* after no more water collected in the trap. The mixture was dissolved in 30 ml anhydrous ethanol and 0,19 g (5,177 mmol) $NaBH_4$ was added in intervals and then stirred overnight at room temperature. The ethanol was removed under reduced pressure, and the residue suspended in 30 ml water (pH=2) and extracted with (3 x 60 ml) dichloromethane. The acidic water was neutralised to pH=7, with sodium hydrogen carbonate and extracted again with dichloromethane (3 x 60 ml). The combined organic fractions of dichloromethane (pH7) were washed with pure distilled water and then dried over sodium sulphate and evaporated under reduced pressure to produce a yellowish oil, which solidified upon standing. Purification of the product was done with flash column chromatography with a yield of 11 %.



PHYSICAL DATA:

$C_{21}H_{22}N_2O_3$: MS (spectrum 1) m/z = 351(M^+), 131; **IR** (spectrum 4) ν_{MAX} = 3442.85, 2919.49, 2832.43, 1454.15,750.79; **^{1}H NMR** (spectrum 7, CD_3OD) δ_H = 7.51 (d, 2H, *J* = 6.98 Hz, H-12, '12) 7.39 (d, 2H, *J* = 5.97 Hz,H-9, '9), 7.19 (m, 4H, H-10, 11, '10, '11), 6.65 (s, 2H, H-14, '14), 4.00 (m, 5H, H-5, 2, '5), 2.70 (m, 4H, H-3, '3, 4, '4); **^{13}C NMR** (spectrum 10, CD_3OD) δ_C = 157.15 (s, C-8, '8), 156.36 (s, C-6, '6), 129.78 (s, C-13, '13), 125.01 (d, C-10, '10), 123.77 (d, C-12, '12), 121.84 (d, C-11, '11), 111.82 (d, C-14, '14), 105.27 (d, C-9, '9), 69.71 (d, C-2), 54.02 (t, C-5, '5), 46.92 (t, C-3, '3).

For **SVZ-3** the same method was used, but with only one equivalent carboxaldehyde instead of two. This compound could not be isolated and purified satisfactorily.

5.2.1.2 1,3-Bis[(1*H*-indole-3-methyl)amino]propan-2-ol (**SVZ-10**)

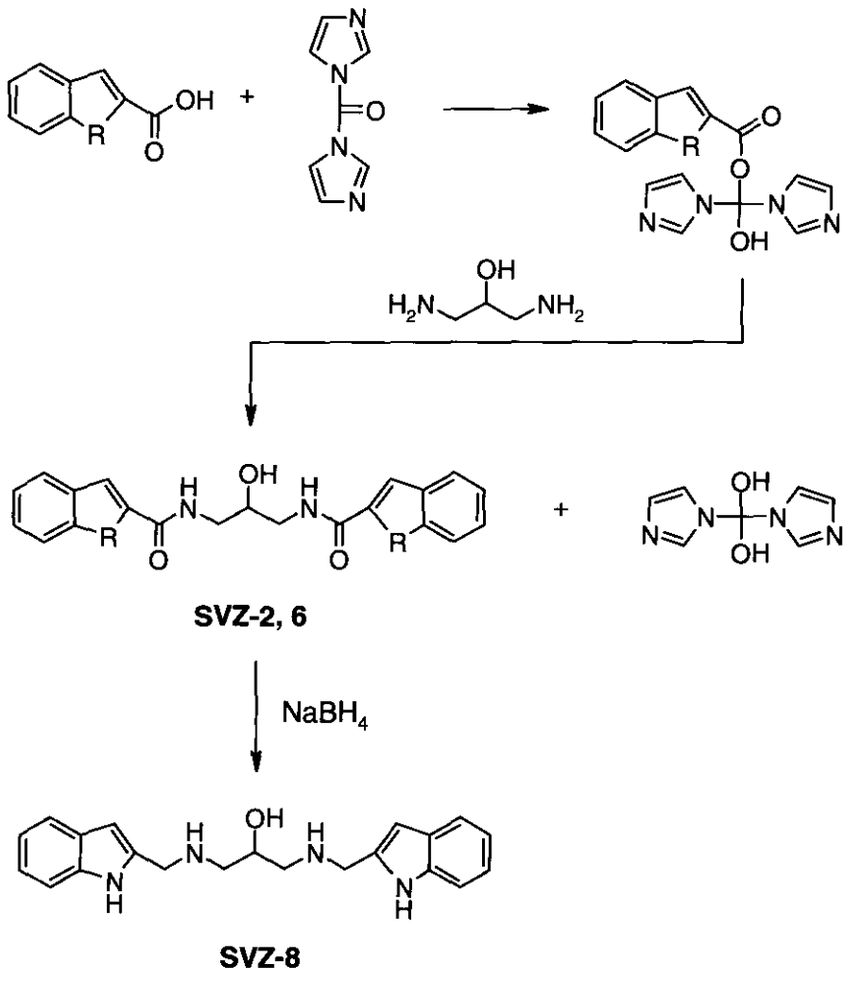
SYNTHETIC PROCEDURE:

0,31 g (3,45 mmol) of 1,3-Diaminopropan-2-ol was dissolved in 30 ml anhydrous ethanol and stirred. 1 g (6,89 mmol) indole-3-carboxaldehyde was added in portions and stirred overnight at room temperature. It was then refluxed for 8 hours and the ethanol was evaporated *in vacuo*. The mixture was then refluxed in 60 ml sodium-dried benzene (Dean-Stark) to remove the water. The excess benzene was removed under reduced pressure when no more water collected in the trap. The mixture was then dissolved in 30 ml anhydrous ethanol and 0,19 g (5,177 mmol) $NaBH_4$ was added portion wise for the reduction process. The mixture was stirred overnight at room temperature. After the ethanol was removed *in vacuo*, the residue was suspended in 30 ml water (pH=2) and extracted with (3 x 60 ml) dichloromethane. The acidic water was neutralised to pH=7, with sodium hydrogen carbonate and extracted again with dichloromethane (3 x 60 ml). The combined organic fractions of dichloromethane (pH=7) were washed with distilled water and then dried over sodium sulphate and evaporated under reduced pressure to produce a yellowish oil, which solidified upon standing. Purification of the product proved to be difficult, and no pure compound could be isolated.

For **SVZ-9** 1 equivalent indole-3-carboxaldehyde was used. The same difficulties with the purification procedures were experienced.

5.2.2 General Route Using Activation Chemistry

Activation chemistry, using *N,N'*-carbonyldiimidazole (CDI) was used to synthesise **SVZ-1, 2, 5, 6, 7, 8** (Miyashita *et al.*, 1992). The CDI was reacted with the indole-2-carboxylic acid (ICA) and benzofurane-2-carboxylic acid (BCA) respectively to give the acylurea. 1,3-Diaminopropan-2-ol was added to yield the resulting amides. Compounds **SVZ-7** and **SVZ-8** were treated with $NaBH_4$ to produce the resulting amines.



R = O (Benzofuran-2-carboxylic acid) **SVZ-1, 2**

R = N (Indole-2-carboxylic acid) **SVZ-5 6**

For compounds **SVZ-1**, **SVZ-5** and **SVZ-7**, 1 equivalent carboxylic acid was used.

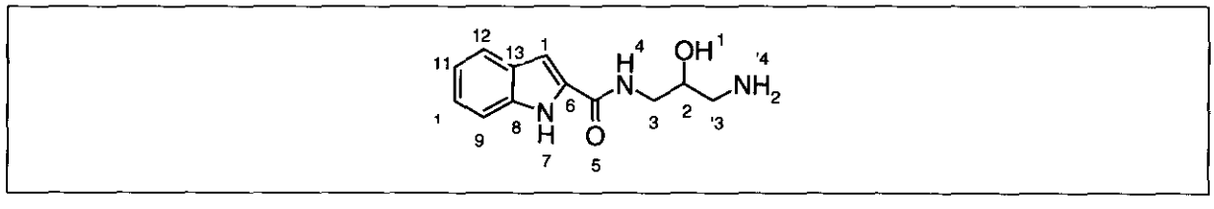
For compounds **SVZ-2, 6** and **8**, 2 equivalents of carboxylic acid were used.

Figure 5.3: General synthesis of **SVZ-1, 2, 5, 6, 7** and **SVZ-8** by using activation chemistry.

5.2.2.1 N-{2-Hydroxy-3-[(1*H*-indole-2-carbonyl)amino]propyl}-1*H*-indole-2-carboxamide (**SVZ-5**)

SYNTHETIC PROCEDURE:

1 g (6,17 mmol) CDI was dissolved in 30 ml sodium-dried dichloromethane and put on ice. The ICA (1 g, 6,17 mmol) was added in portions while stirred at 0 – 5 °C. After about an hour the mixture was brought to room temperature. 1,3-Diaminopropan-2-ol (0,3 g, 3.08 mmol) was added in aliquots and the mixture was stirred at room temperature for an hour and then refluxed for 2 hours. The solvent was evaporated under reduced pressure, to give a yellowish oil which solidified upon standing. The resulting product mixture was purified with flash column chromatography and yielded 2.3 %.



PHYSICAL DATA:

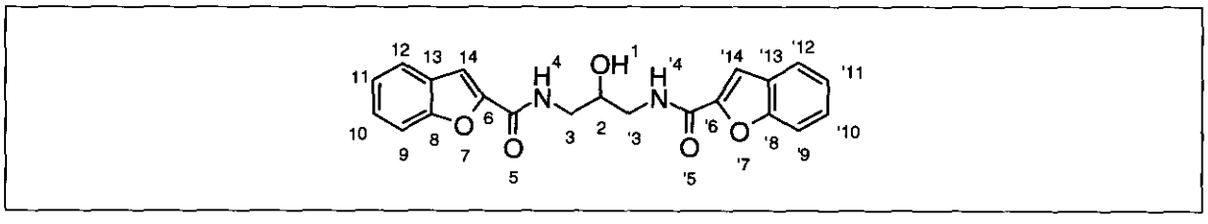
$C_{21}H_{20}N_4O_3$: MS (spectrum 2) m/z = 376, 307, 289, 273, 234, 212, 154,136; **IR** (spectrum 5) ν_{MAX} = 3421.32, 2924.61, 2852.92, 1634.14, 1547.08, 748.22; **^{1}H NMR** (spectrum 8 , CD_3OD) δ_H = 7.61 (d, 1H, *J* = 6.07 Hz, H-9), 7.44 (d, 1H, *J* = 5.63 Hz, H-12), 7.21 (t, 1H, *J* = 1.21 Hz, H-11), 7.06 (t + s, 2H, *J* = 1.07 Hz, H-10, 14), 3.84 (m, 1H, H-2), 3.48 (m, 2H, H-3), 2.84 (m, 1H, H-'3a), 2.69 (m, 1H, H-'3b).

For **SVZ-6**, two equivalents carboxylic acid was used. The product couldn't be isolated in pure form to obtain physical data.

5.2.2.2 *N*-{3-[(1-Benzofuran-2-carbonyl)amino]-2-hydroxypropyl}-1-benzofuran-2-carboxamide (**SVZ-2**)

SYNTHETIC PROCEDURE:

CDI (1 g, 6,17 mmol) was dissolved in 30 ml sodium-dried dichloromethane and stirred on ice. 1 g (6,17 mmol) BCA was added to the solution and stirred for an hour at 0 - 5 °C. The mixture was then stirred at room temperature for an hour and refluxed overnight. The solvent was evaporated *in vacuo*. 30 ml anhydrous (sodium-dried) dichloromethane was added to the CDI-BCA complex and put on ice. 0,28 g (3,08 mmol) 1,3-Diaminopropan-2-ol was added in aliquots while stirred at 0 – 5 °C. The mixture was then stirred at room temperature for an hour and then refluxed overnight. The mixture was filtered and concentrated *in vacuo* to result in a yellow-brownish oil, which solidified upon standing. The product mixture was purified with flash column chromatography and obtained as white crystals with a yield of 27 %.



PHYSICAL DATA:

$C_{21}H_{18}N_2O_5$: MP 190 °C – 210 °C; **MS** m/z (spectrum 3) = 379(M^+), 204, 175, 145, 68; **IR** (spectrum 6) ν_{max} = 3431.58, 3318.92, 2919.49, 2858.04, 1629.02, 1588.05, 743.10; **^{1}H NMR** (spectrum 9, CD_3OD) δ_H = 7.72 (d, 2H, *J* = 5.85 Hz, H-12, '12), 7.59 (d, 2H, *J* = 6.64 Hz, H-9, '9), 7.51 (s, 2H, H-14, '14), 7.44 (t, 2H, *J* = 7.67 Hz, H-11, '11), 7.32 (t, 2H, *J* = 7.83 Hz, H-10, '10), 4.10 (m, 1H, H-2), 3.60 (m, 4H, H-3, '3, 4, '4); **^{13}C NMR** (spectrum 11, CD_3OD) δ_C = 161.65 (s, C-5, '5), 156.47 (s, C-8, '8), 149.84 (s, C-6, '6), 128.79 (s, C-13, '13), 128.25 (d, C-10, '10), 124.90 (d, C-12, '12), 123.75 (d, C-11, '11), 112.80 (d, C-14, '14), 111.48 (d, C-9, '9), 70.13 (d, C-2), 44.00 (t, C-3, '3).

For **SVZ-1**, one equivalent carboxylic acid was used. The product couldn't be isolated in pure form to obtain physical data.

5.2.2.3 1,3-Bis[(1*H*-indole-2-methyl)amino]propan-2-ol (**SVZ-8**)

SYNTHETIC PROCEDURE:

1 g (6,17 mmol) CDI was dissolved in 30 ml sodium-dried dichloromethane and stirred at 0 °C. BCA (1 g, 6,17 mmol) was added to the solution and stirred overnight at 0 – 5 °C. The mixture was brought to room temperature and refluxed overnight. The solvent was removed *in vacuo*. The BCA-CDI complex was dissolved in 30 ml anhydrous dichloromethane and 0,28 g (3.08 mmol) 1,3-diaminopropan-2-ol was added in aliquots while stirred at 0 – 5 °C. The mixture was stirred at room temperature for an hour then refluxed overnight. The mixture was filtered and then concentrated *in vacuo*. The amide was then treated with $NaBH_4$ (0,12 g 3,08 mmol) in 30 ml anhydrous ethanol. The mixture was refluxed overnight and filtered. The ethanol was removed under reduced pressure to give a yellowish oil, which solidified upon standing. The product was not isolated to obtain physical data.

For **SVZ-7**, one equivalent carboxylic acid was used. Purification could not be achieved during this study.

5.3 DISCUSSION AND CONCLUSION

Although some success have been achieved with the synthesis of some of these compounds, its purification, solubility and the multiple reactions taking place made it difficult to obtain the compounds in pure form for characterisation.

With the synthesis of the indole compounds, more difficulties were experienced than with the benzofuran. More success was achieved with the synthesis of the latter compound since it was moderately less hydrophyllic than the indoles.

SVZ-2 and **4** were evaluated for anti-HIV activity.

Chapter 6

Biological Evaluation

The purified compounds namely **SVZ-2** and **4** were biologically evaluated to explore their HIV interaction. The testing was conducted at the National Institute For Communicable Diseases (NICD; Private Bag X4, 2131 Sandringham, South Africa) and involved the determination of the cell toxicity of the compounds in the absence of the virus and an anti-HIV activity evaluation.

6.1 NEUTRALISATION ASSAY IN MT-2 CELLS

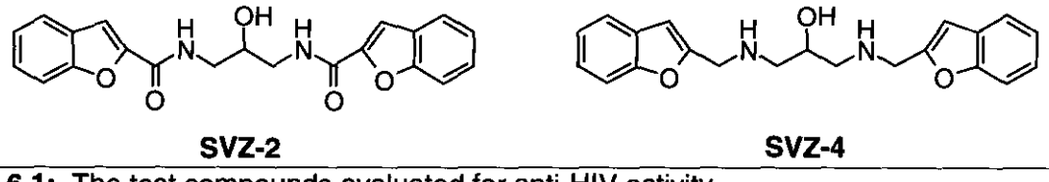
6.1.1 Introduction

Neutralising antibodies and cytotoxic T lymphocytes are the major weapons of the host defence system against viruses and play a vital role in preventing the spread of cell-free virus *in vivo* (Parren *et al.*, 1999; Musey *et al.*, 1997). The method used in this study was the vital dye staining approach and uses a 96-well microtiter infection assay for HIV. The assay utilises human T-cell lymphotropic virus type I-immortalised MT-2 cells as targets for infection. Cytolysis was quantitated by vital dye uptake of poly-L-lysine (PLL) adhered cells as an endpoint indication for infection. The assay's efficacy was proven by the sensitive and accurate assessment of several known anti-HIV agents incl. zidovudine and zalcitabine. The assay measured neutralisation as a function of a reduction in viral cythopathic effects in MT-2 cells in the presence of a test sample relative to a negative control sample (Montefiori *et al.*, 1988; Bures *et al.*, 2000; Zhang *et al.*, 2003; Peçanha *et.al.*, 2003).

6.1.2 Approach

In this neutralising antibody assay of the HIV-1 main group, virus strains IIIB and MN, both from subtype B were used. The IIIB isolate is generally used in laboratories around the world and is one of the first isolates of the HIV-1 virus (Wünschmann & Stapleton, 2000). The MN isolate has been known to be a better representative of the HIV-1 strains found in North Americans and Europeans (NIAID, 2001b).

Structures **SVZ-2** and **4** (fig. 6.1) were assayed against both TCLA (T-cell line adapted) strains namely IIIB and MN. The MT-2 assay was set-up with the test compounds and IBU21 as a positive control (this is a plasma sample isolated from a HIV-1 infected individual with neutralising activity).



SVZ-2 **SVZ-4**

Figure 6.1: The test compounds evaluated for anti-HIV activity.

Two tests were performed on the test compounds:

- Cell cytotoxicity of test compounds (in absence of the virus);
- Anti-HIV activity of the test compounds (in presence of the virus);

The anti-HIV activity assay is based on the staining of poly-L-lysine coated plates with neutral red, in the presence of the test compounds and the HIV-1 virus strains.

The cell cytotoxicity test was monitored visually for the viability of the cells as well as by the cell count of the plates, at the highest concentration of the test compounds, which was then compared to the cell control. The cell count was done by using a haemocytometer. The anti-HIV activity test could also be monitored visually by the appearance of syncytium formation in the cultures and by its absorbance reading (OD values) at 540 nm (A_{540}). Syncytium formation in MT-2 cells is clearly observable, since the giant cells typically form large balloon-like structures. These structures may differ in nature, depending on the strain of the virus.

6.1.3 Preparations

CELLS AND VIRUS CULTURE:

The vital dye staining can be used for neutralisation of laboratory T-cell line adapted (TCLA) HIV-1strains, rather than for primary HIV-1 isolates. The growth medium was RPMI 1640 containing 10% heat-inactivated foetal bovine serum and acid alcohol (50% ethanol in 1% acetic acid). The 50 % tissue culture infectious dose ($TCID_{50}$) of the virus stock was determined by endpoint titration on TCLA HIV-1 strains.

POLY-L-LYSINE (PLL) COATED PLATES:

Poly-L-lysine (PLL) coated plates were prepared for the staining. A solution of 50 µg/ml PLL in phosphate buffered saline (PBS), pH=7.3 were prepared. The solution (100 µl) was added to all the plates and it was then incubated at room temperature for 1 hour. The PLL solution was then aspirated, and the wells were washed twice with PBS. It was allowed to drain and stored in a refrigerator at 4 °C until used.

TEST COMPOUNDS:

Samples were centrifuged and subsequently heat-inactivated at 56°C by placing it in a water bath for 45 minutes. **SVZ-2** and **4** were dissolved in phosphate buffered saline (PBS) (low solubility) and then diluted to a concentration of 1mg/ml (stock) in RPMI-1640.

6.1.4 Method

CELL CYTOTOXICITY TEST:

Growth medium (100 µl) was placed in columns 1-7. The volume of column 1 (cell control) was adjusted to 150 µl. Growth medium was added to the bottom wells of columns 2-7. The desired amount of **SVZ-2** was added to the bottom wells of columns 2-4, as was done for **SVZ-4** in columns 5-7. The test compounds and the growth medium were mixed and a dilution was made of 1:1 to give a concentration of 100 µg/ml. The plates were then incubated for 1 hour at 37 °C under 5 % CO_2 in a humidified incubator. Cell suspension (100 µl/50 000 MT-2 cells) was added per well to columns 1-7. The plates were then incubated at 37 °C under 5 % CO_2 and 95 % air for 3 days. The cell count was done 3 days after the setup.

ANTI-HIV ACTIVITY TEST:

Growth medium (100 µl) was placed in columns 1-11. The volume of column 1 (cell control) was adjusted to 150 µl and 250 µl of growth medium/well was placed in column 12 (blank). Growth medium was added to the bottom wells of columns 3 –11. The desired amount of **SVZ-2** was added to the bottom wells of columns 3-5, as was done for **SVZ-4** in columns 6-8, and for IBU21 in columns 9-11. The samples and the growth medium was mixed and serial dilutions were made (1:1; 1:4; 1:16; 1:64; 1:256; 1:1024; 1:4096; 1:16384); starting at the bottom (row H) and progressing to the top (row A). 50 µl of the virus dilution (5 000 $TCID_{50}$ per well) was added to all the wells in columns 2-11 (column 2 is the virus control and gets no serum). A high $TCID_{50}$ is used to minimize the number of replication cycles required to induce a cythopathic effect. The plates were incubated for 1 hour at 37 °C under 5 % CO_2 in a humidified incubator. 100 µl of cell suspension (50 000 MT-2 cells) was then added per well to columns 1-11.

The plates were again incubated at 37 °C under 5 % CO_2 and 95 % air until syncytium formation and cythopathic effects were observed microscopically in the virus control wells (column 2). Approximately 80 % of the cells should be involved in syncytium formation or appear dead before it is ready to be stained. A positive control serum with known titer was used to judge when plates were ready to stain and to assure they were stained at an optimal time point.

The cell suspension (100 µl) was transferred to the PLL-coated plates and stained with 100 µl of Finter's natural red (diluted 1:10 in media). The plates were then incubated for 75 minutes at 37 °C. The media was removed and it was washed twice with 150 µl of PBS. 100 µl of acid alcohol was added and it was left for 30 minutes. The wavelength for the colorimeter reading was set at 540 nm. Protection rate (%) was determined by the following formula:

$$\frac{\text{Absorption of test wells}_{(\text{cells + test compounds + virus})} - \text{Absorption of virus control wells}_{(\text{cells + virus})}}{\text{Absorption cell control wells}_{(\text{cells})} - \text{Absorption of virus control wells}_{(\text{cells + virus})}} \times 100$$

Neutralising antibody titers were expressed as the reciprocal of the serum dilution required to protect 50 % of the cells from virus-induced cell death.

6.2 RESULTS

6.2.1 Cell Cytotoxicity Test

SVZ-2:

After 3 days the cell count in presence of **SVZ-2** at a concentration of 100 µg/ml was 78 x 10^4, compared to the cell control well of 80 x 10^4. The cells were thus viable and no cellular toxicities were observed for the test compound.

SVZ-4:

The cell count in presence of **SVZ-4** at a concentration of 100 µg/ml was 72 x 10^4, which correlated with the cell control well of 80 x 10^4. The cells were thus viable and no cellular toxicities were observed for **SVZ-4.**

6.2.2 Anti-HIV Activity Test

SVZ-2 and **4** were tested in a cell death assay against two HIV-1 strains namely IIIB and MN isolates. The assays were repeated on three consecutive days. The vital dye uptake of the stained cells is represented as OD values (absorbance) in the graphs (fig. 6.2 and 6.4). There was extensive cell death in the virus control wells resulting in low OD readings, whilst a higher OD value in the cell control (CC) well indicated that MT-2 cells were healthy and viable. The percentage inhibition used in the graphs (fig. 6.3 and 6.5), was calculated by the formula given above. Each graph represents one assay as an example.

IIIB VIRUS:

Both **SVZ-2** and **4** showed minimal inhibition of cell death (fig. 6.2). The maximum OD values for **SVZ-2** and **4** were at a concentration of 0.16 µg/ml with a colorimetric reading of 0.1746 and 0.1783 respectively but this was still not strong enough to warrant further investigation. There was meaningful syncytium formation in all wells containing test compounds, which was similar to the virus control (VC) wells, indicating that the two compounds evaluated had no anti-HIV activity against this strain. Inhibition of cell death was observed for IBU21 at higher concentrations (1 – 100 µg/ml).

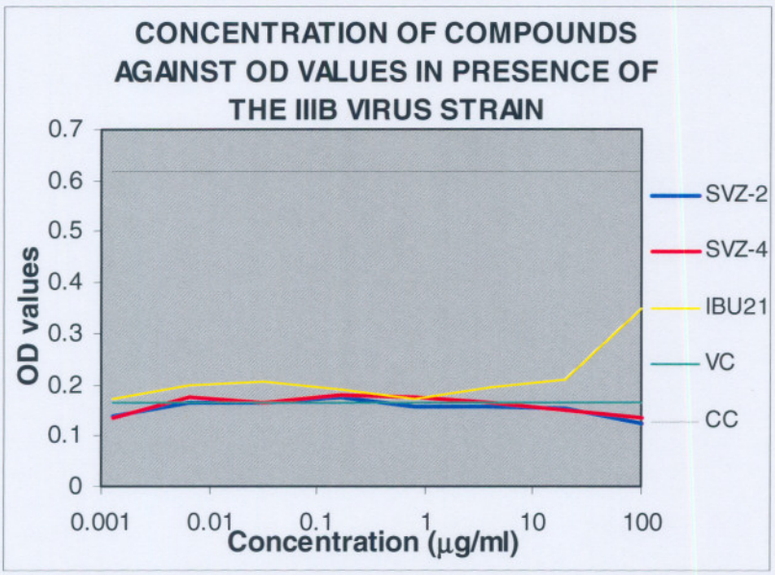


Figure 6.2: **SVZ-2** and **4** with IBU21, cell control (CC) and virus control (VC) plotted against the colorimetric reading (A_{540}).

The percentage inhibition calculated from the OD values are presented in Figure 6.3. A weak (5 %) inhibition was observed for **SVZ-2** at some dilutions but **SVZ-4** showed no inhibition of the IIIB virus strain. IBU21 showed inhibition of 60 % at a dilution of 1:20.

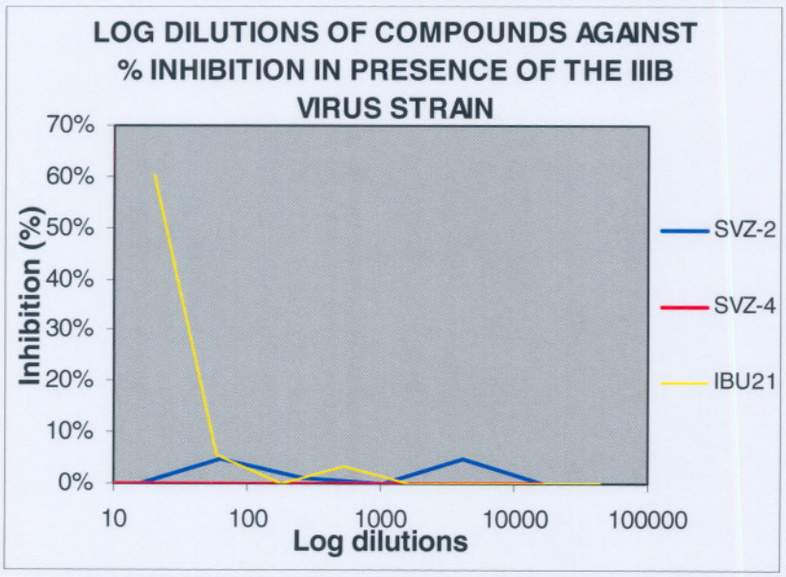


Figure 6.3: Inhibition of compounds against Log dilutions.

MN VIRUS:

Figure 6.4 represents the activity of the test compounds against the MN virus strain. **SVZ-2** and **4** had maximum OD values at a concentration of 0.16 µg/ml with colorimetric readings of 0.2543 and 0.213 respectively, which was slightly higher than with the IIIB virus strain, but still too weak to warrant further investigation. Syncytium formation was clearly visible with these compounds, indicating that the two compounds evaluated had no meaningful anti-HIV activity. Inhibition of cell death was observed for IBU21 at concentrations of 0,1 – 100 µg/ml.

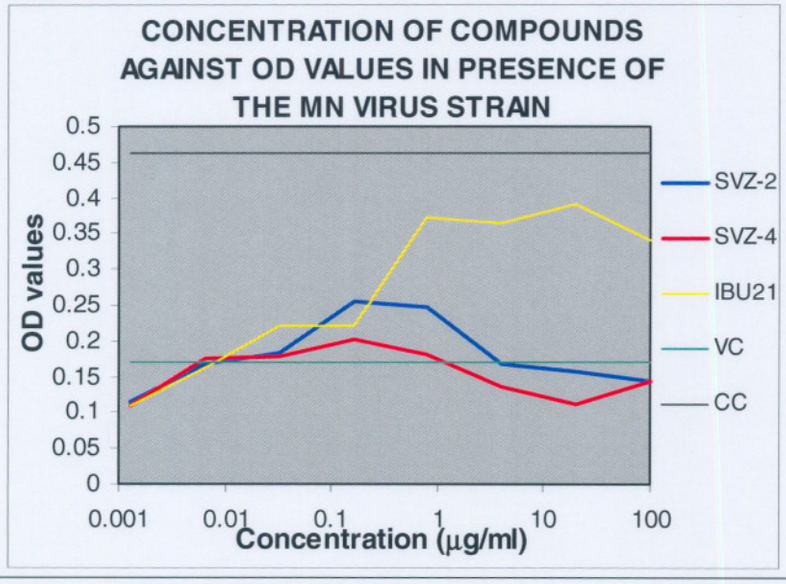


Figure 6.4: SVZ-2 and **4** against the MN virus strain.

The percentage inhibition obtained on the MN virus strain (fig. 6.5) may indicate some weak inhibition of the virus at higher concentrations (around 20% for **SVZ-2** and 10% for **SVZ-4**). This weak inhibition however is meaningless. IBU21 showed inhibition of 87 % at a dilution of 1:60.

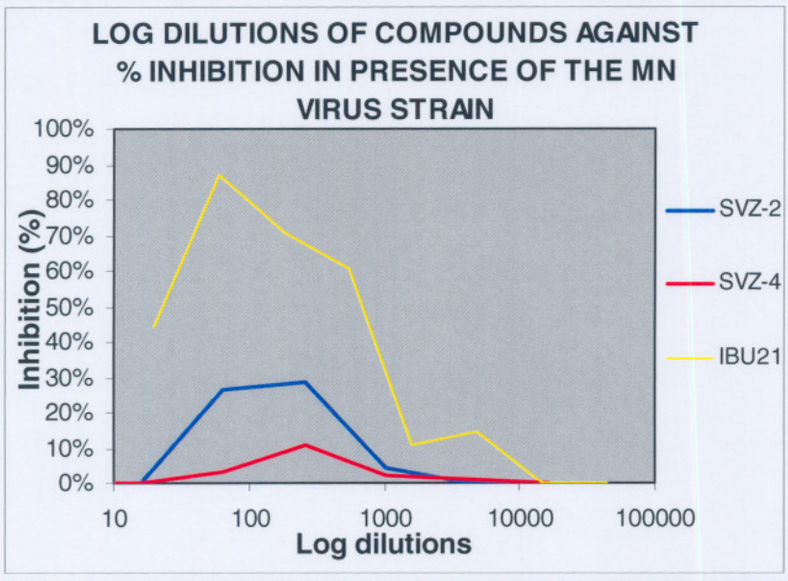


Figure 6.5: Inhibition of the compounds versus Log dilutions in the MN virus.

6.3 DISCUSSION AND CONCLUSION

The evaluation of **SVZ-2** and **4** was based on the neutralisation of MT-2 cells infected with the MN and IIIB virus strains. The assay measured neutralisation as a function of a reduction in viral cythopathic effects in MT-2 cells in the presence of a test sample relative to a negative control sample. A positive control with neutralising antibodies, IBU21 was also used in this assay. Anti-HIV activity was monitored by virus replication both, visually (appearance of syncytium in cultures) and by staining with neutral red.

In all the wells syncytium formed which indicated that no meaningful inhibition of the HIV-1 virus effects occurred, except in the positive control well. The low solubility of the test compounds in aqueous solution could have influenced the observed activities negatively. It appears that the disubstituted amide, **SVZ-2,** exhibited a higher percentage inhibition than the disubstituted amine, **SVZ-4** (29 % vs. 11 %). This may indicate that the carbonyl group of the amides play a role in the binding of the inhibitor to the enzyme (fig. 6.6).

In view of the results in particular of the MN virus strain indicating some although weak activity, may suggest that the interactions at the protease enzyme is still sufficient and that compounds should be designed that could show increased interaction with HIV protease. Furthermore the inclusion of HIV protease inhibitor in clinical use in the biostudy would have given more insight for comparison purposes at the protease enzyme and the degree of inhibition of the enzyme in

relationship to the *in vitro* anti-HIV activity.

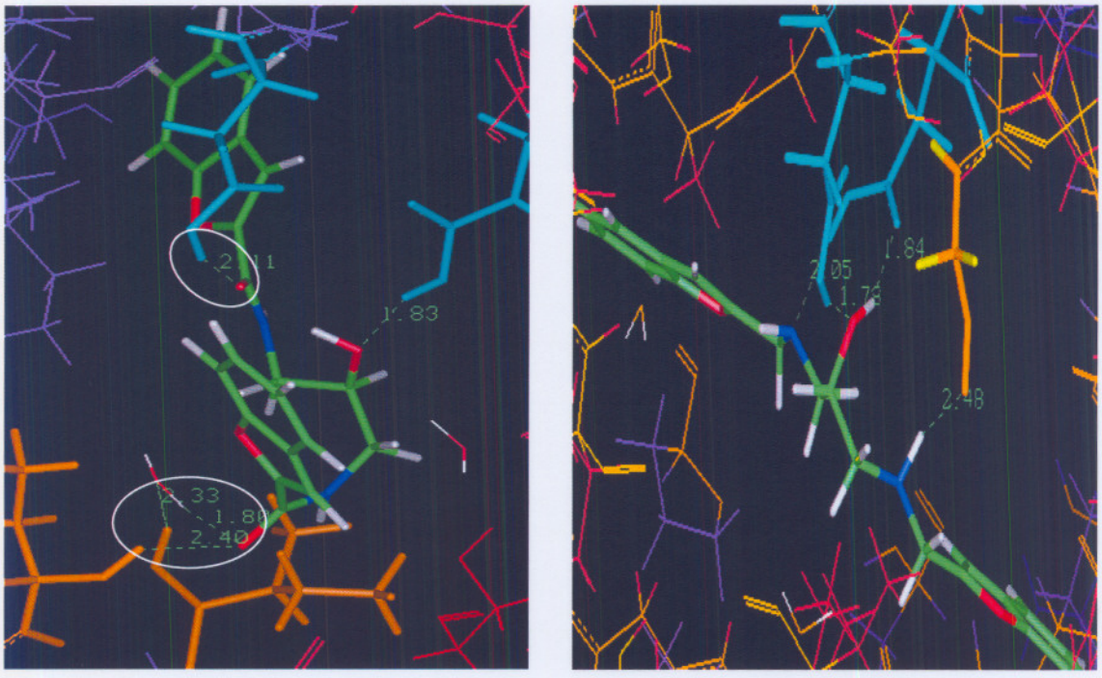


Figure 6.6: **SVZ-2** (left) with its hydrogen bonds from the carbonyl groups to the protease enzyme. **SVZ-4** (right) with its hydrogen bonds to the protease enzyme.

Chapter 7

Summary, Discussion and Conclusion

This chapter provides a summary, discussion and conclusion of the different phases of the current study on the design of new drug candidates for HIV, targeting the HIV protease enzyme.

7.1 SUMMARY

7.1.1 Molecular Modelling

Molecular modelling was performed on a series of compounds to identify potential drug candidates for the HIV protease as target. An *indirect design* approach was followed to achieve the above mentioned. This approach was employed by superimposing or fitting the novel series on the database structure, AQ148, retrieved from the RCSB Protein Data Bank. RMS values were calculated and these RMS values gave an insight into the global conformational similarity or difference between the control compound and the newly proposed structures. The relatively good RMS values for the compounds indicated a reasonable possibility for interaction with the protease active site. Each of the compounds in the novel series was then made an assembly, with the protease enzyme in neutral form, the backbone was fixed and the assembly was optimised. Dynamic studies were conducted on the assembly in order to sample 3D conformational space and to identify possible conformations for favourable interactions. There was no distinction in energy levels or the total number of bonds that formed between the ligand and the receptor, or between the mono- and disubstituted compounds. Compound **SVZ-2** was the only disubstituted compound that showed H-bonds with both the aspartate residues and the

water molecule, which was bound to both isoleucine residues. Monosubstituted compounds **SVZ-1, 3, 7** and **9** were bound to both the aspartates Asp 25 and Asp '25, but had only one bond with Ile 50 or Ile '50. It was expected that the disubstituted compounds should exhibit more intermolecular interactions with the HIV active site. The molecular modelling study clearly suggests that compounds of this type show potential to interact and inhibit the HIV protease enzyme with subsequent inhibition of the enzyme.

7.1.2 Synthesis

The series of compounds were synthesised by using either direct coupling or activation chemistry. Direct coupling of 1,3-diaminopropan-2-ol with benzofuran-2-carboxaldehyde and indole-3-carboxaldehyde respectively was done. Activation chemistry, using *N,N'*-carbonyldiimidazole (CDI) was used to synthesise **SVZ-1, 2, 5, 6, 7, 8.** Multiple reactions and solubility problems made purification of the compounds extremely difficult. Flush column chromatography was mostly used in the purification of these compounds. Other methods applied included: isolation on preparative plates, recrystallisation, acid-base extractions and C_{18} preparative plate chromatography. Only 3 compounds could be adequately purified and characterised of which two were obtained in sufficient quantities for biological evaluation.

7.1.3 Biological Evaluation

The selected compounds were tested for anti-HIV activity according to a method of neutralising the MT-2 cells. The assay measured neutralisation of the cells as a function for the reduction of viral cythopathic effects in MT-2 cells in the presence of a test sample relative to a negative control sample. The *in vitro* activity of **SVZ-2** and **4** in MT-2 cells, infected with viruses MN and IIIB (X4-using viruses) were evaluated. A positive control, IBU21 was also used as a control of inhibition for anti-HIV activity. Anti-HIV activity was monitored by virus replication both visually by the appearance of syncytium formation in the cultures as well as by the absorbance values of the cells stained with neutral red. Only the positive control, IBU21 showed meaningful inhibition of cell death and weak but meaningless inhibition was observed for **SVZ-2** and **4.** Solubility problems were also observed during biological screening and the low solubility of the test compounds in aqueous solution could have influenced the observed activities negatively. It was also noteworthy that **SVZ-2**, a disubstituted amide, showed some weak activity compared to the inactive **SVZ-4**, a disubstituted amine. It may be possible that the amide, with its carbonyl

group had some improved interaction with the HIV-1 protease enzyme at the active site than the amine. None of the compounds exhibited any cell toxicity.

7.2 DISCUSSION AND CONCLUSION

The urgent need for novel agents in the treatment of HIV/AIDS is clearly emphasised by the increasing concern over the development of HIV strains that are resistant to current drugs in clinical use. This is of particular concern in view of the importance placed on the use of combination therapy in the management of HIV even for the cases of mother to child transmission. The current study, explored the design of structurally simple C_2-symmetric molecules with the potential to interact with the HIV protease enzyme. The study successfully utilised a molecular modelling approach to examine various mono- and disubstituted derivatives of 1,3-diaminopropan-2-ol. These modelling studies indicated that symmetrical, disubstituted molecules filling the hydrophobic pockets of the protease enzyme, should be explored as potential drug candidates. The synthesis of the suggested series of compounds proved to be difficult and challenging, particularly with respect to yields and purification. From the difficulties and problems observed here, it is recommended that alternative synthetic routes that will have better yields and more effective isolation and purification procedures be explored. The compounds tested showed no meaningful inhibition of HIV-1 activity but from the results obtained some important observations could however be made which suggests further investigation. From these observations it is evident that future compounds should include amide moieties and adequate substituents to effectively interact with the hydrophobic pockets of the protease enzyme.

In conclusion the aims and objectives of this study to design and evaluate a novel series of compounds for possible anti-HIV activity, was achieved to a certain degree, paving the way for future studies to be conducted in order to urgently address the HIV/AIDS pandemic.

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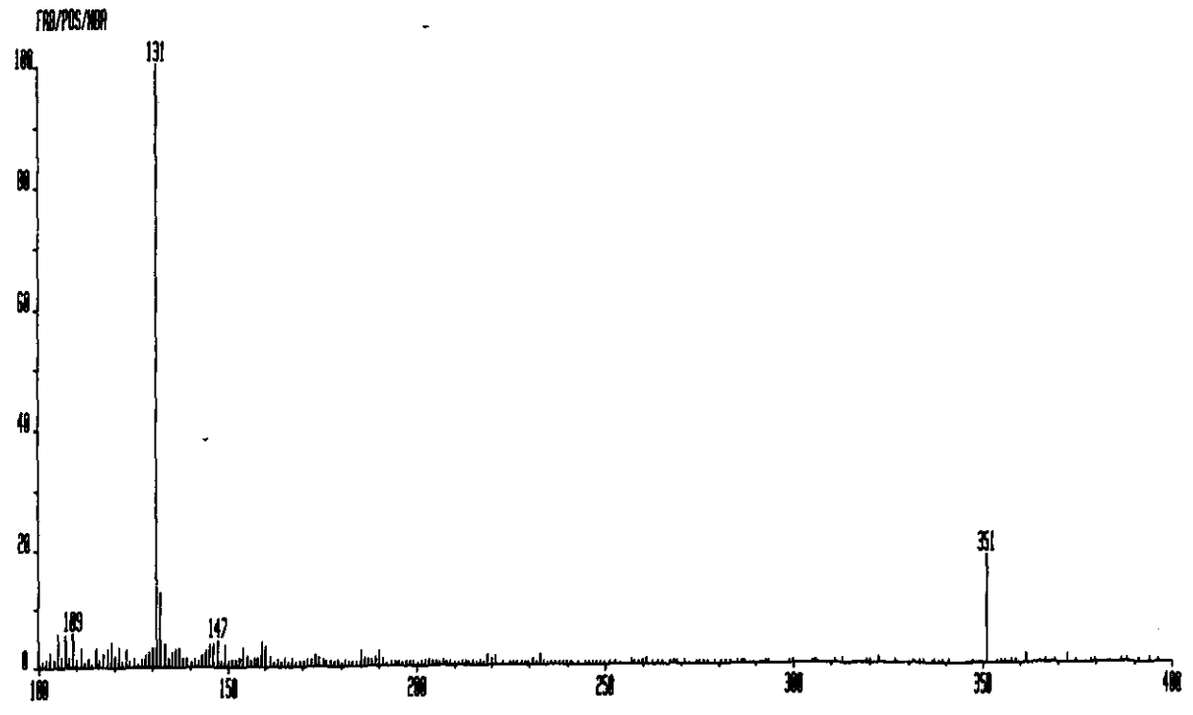
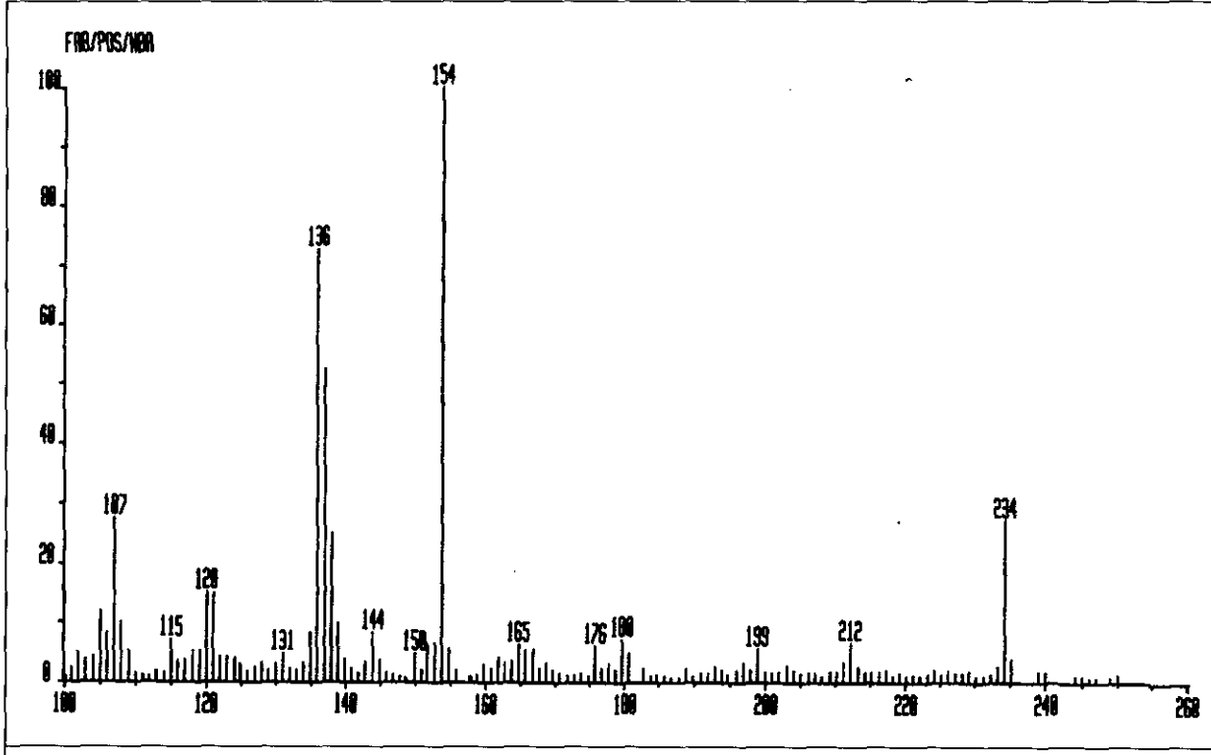
ANNEXURE 1



Spectrum 1: SVZ-4; Mass Spectrometry (FAB).



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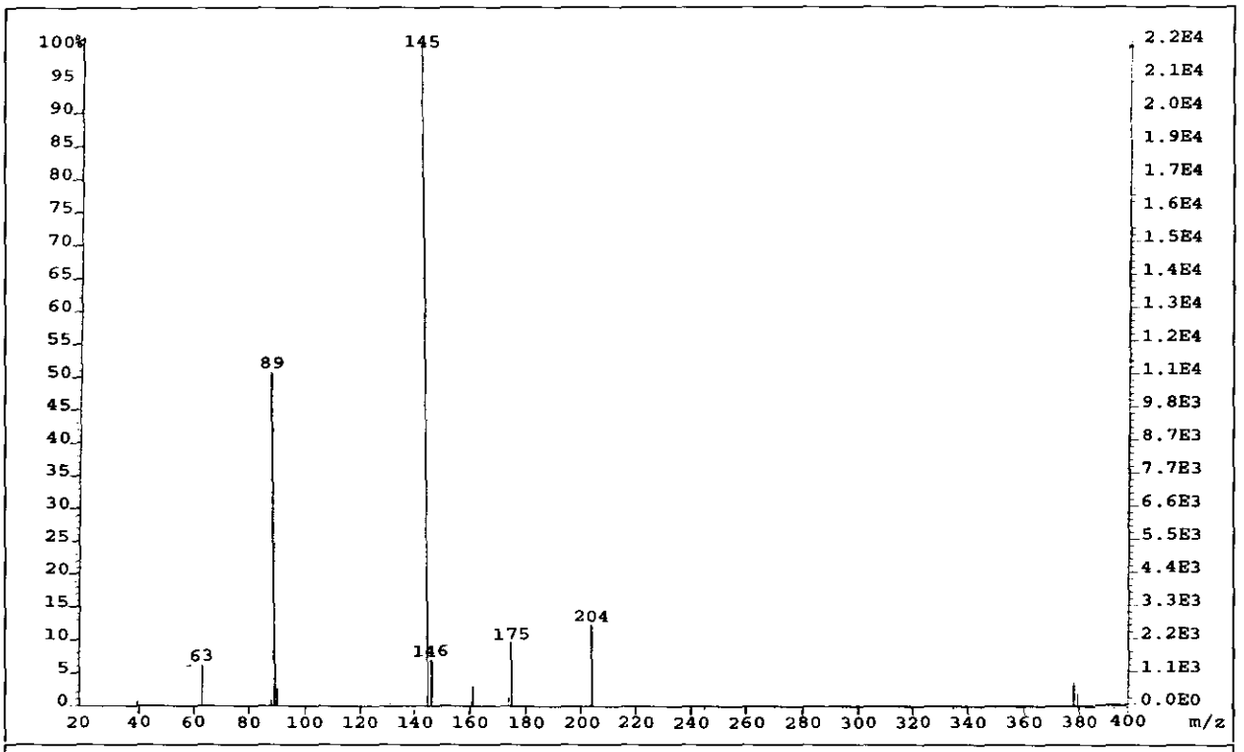

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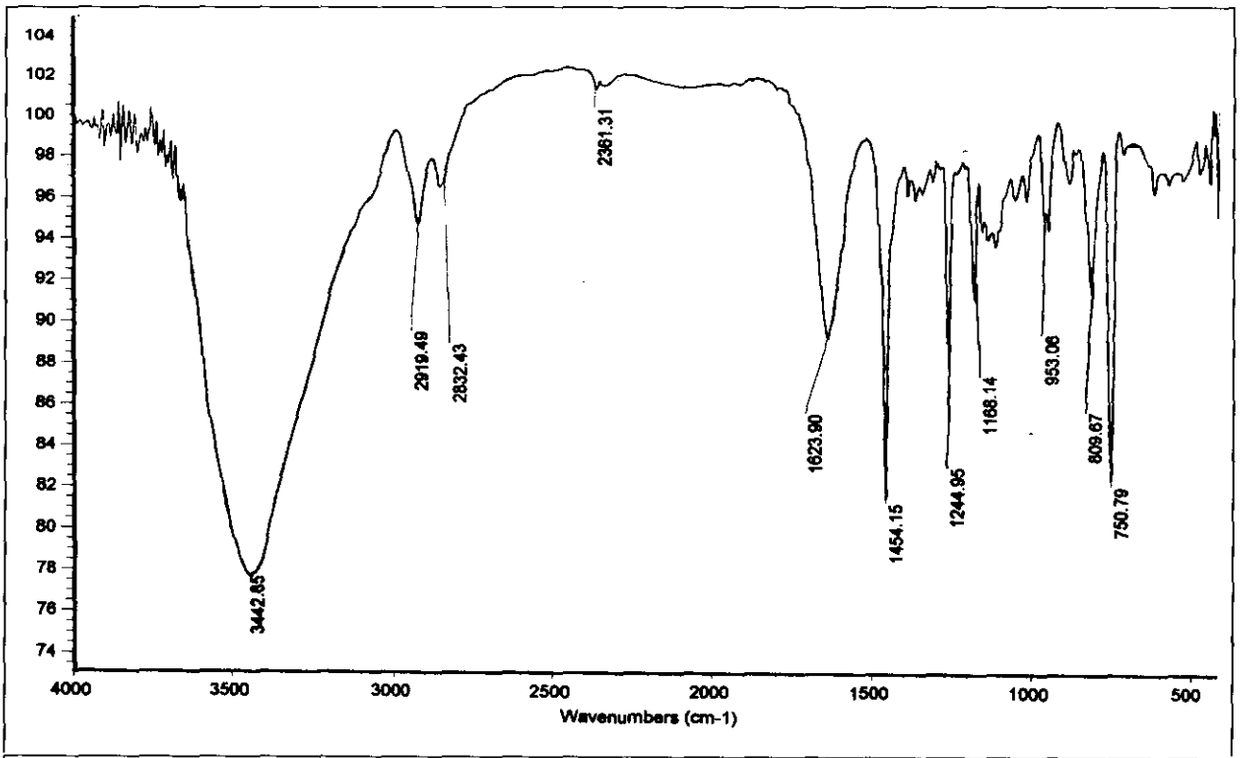
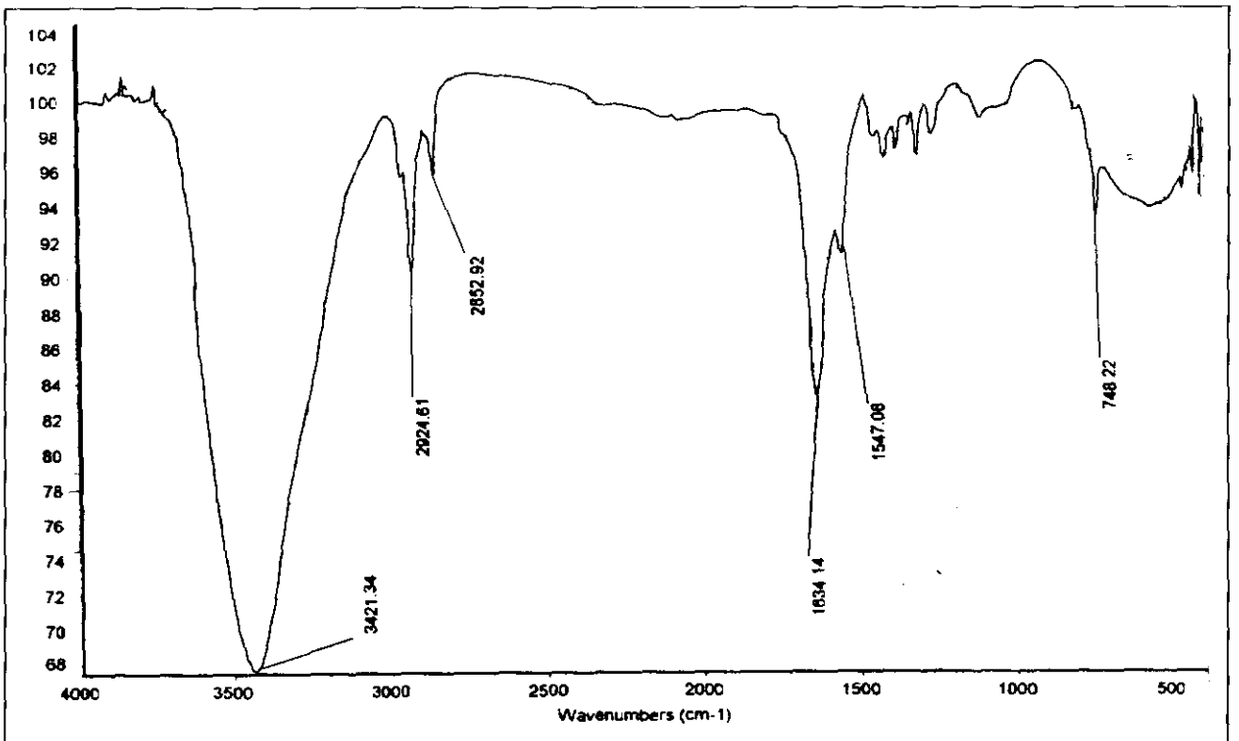
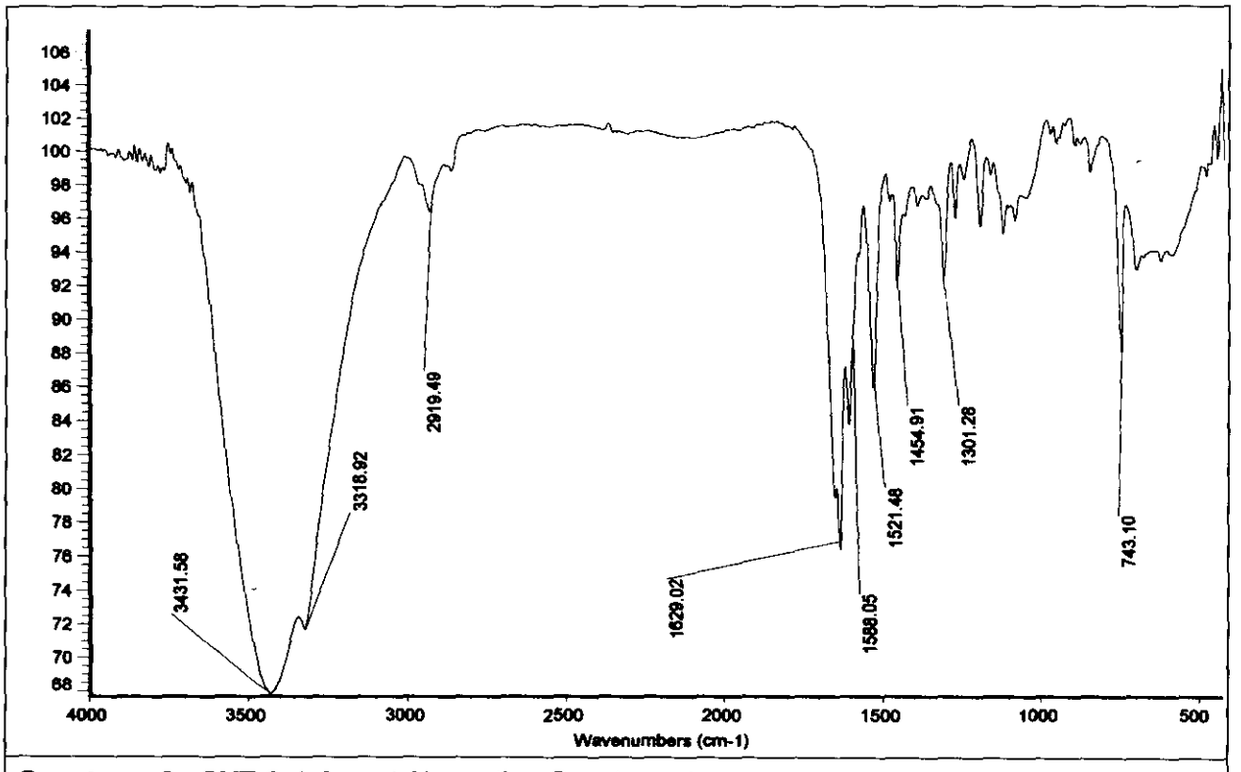


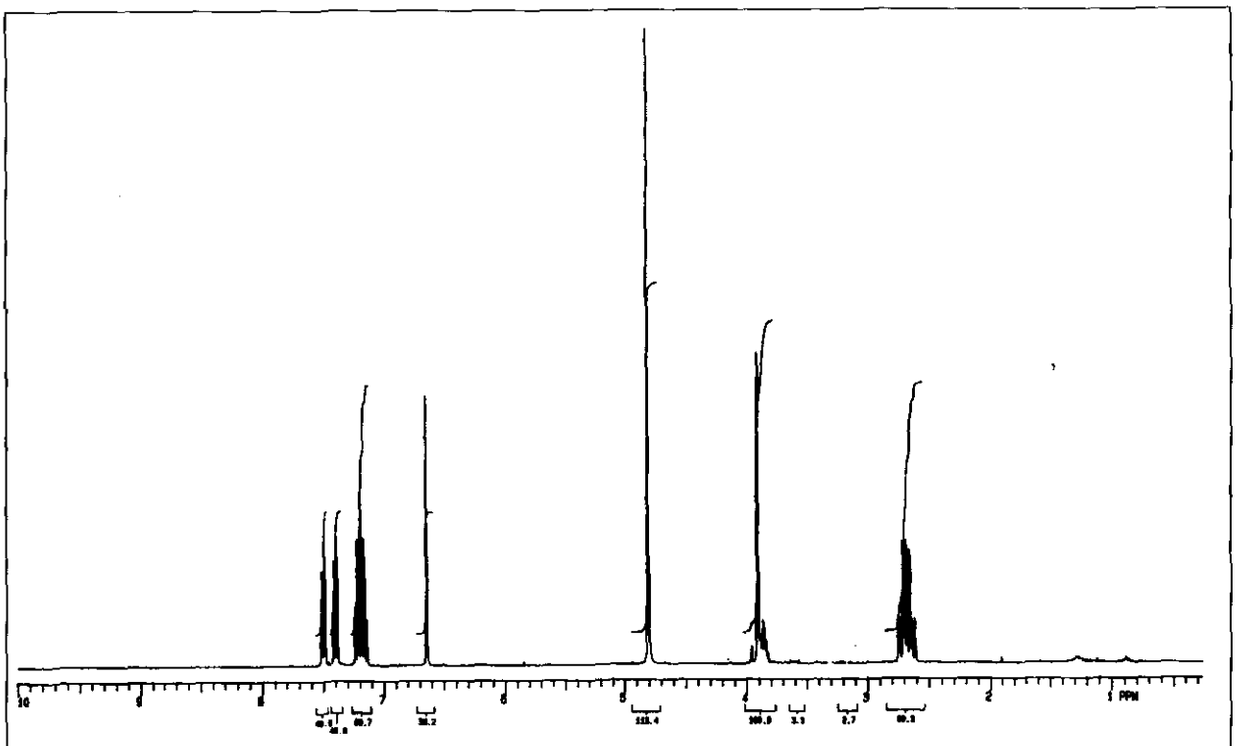
Spectrum 4: SVZ-4; Infrared Absorption Spectrometry.



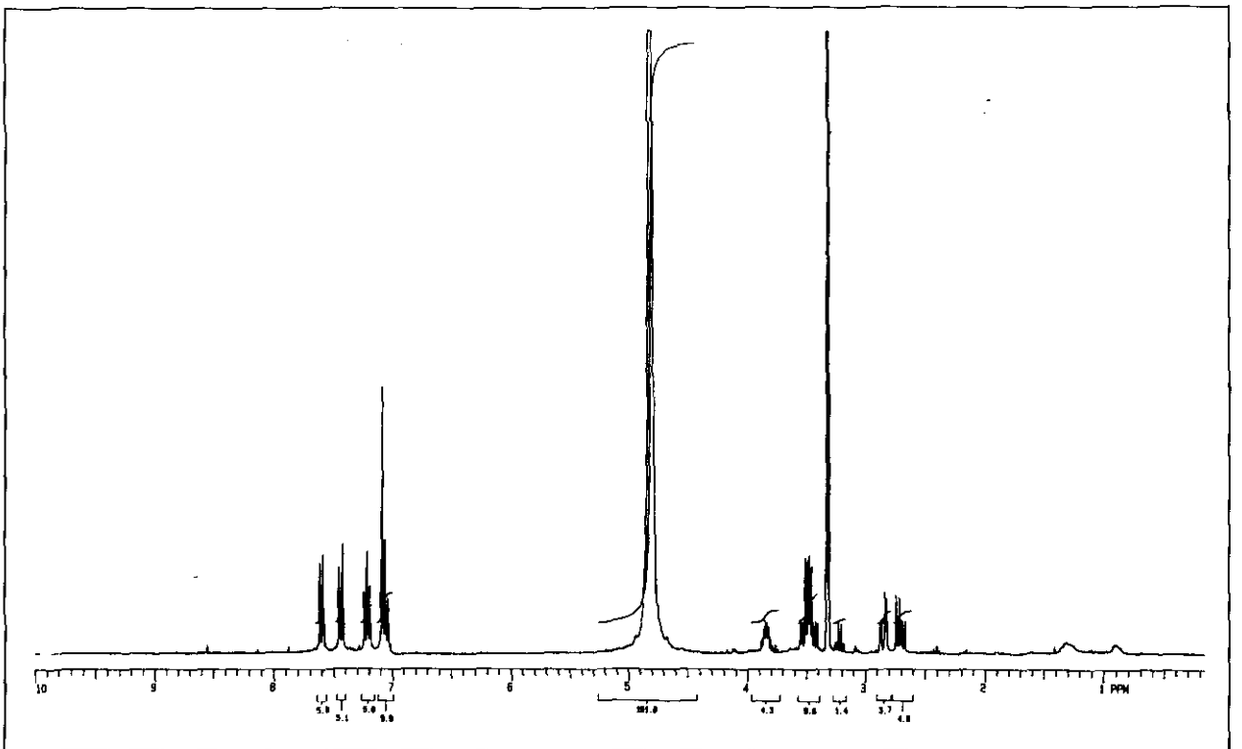
Spectrum 5: SVZ-5; Infrared Absorption Spectrometry.



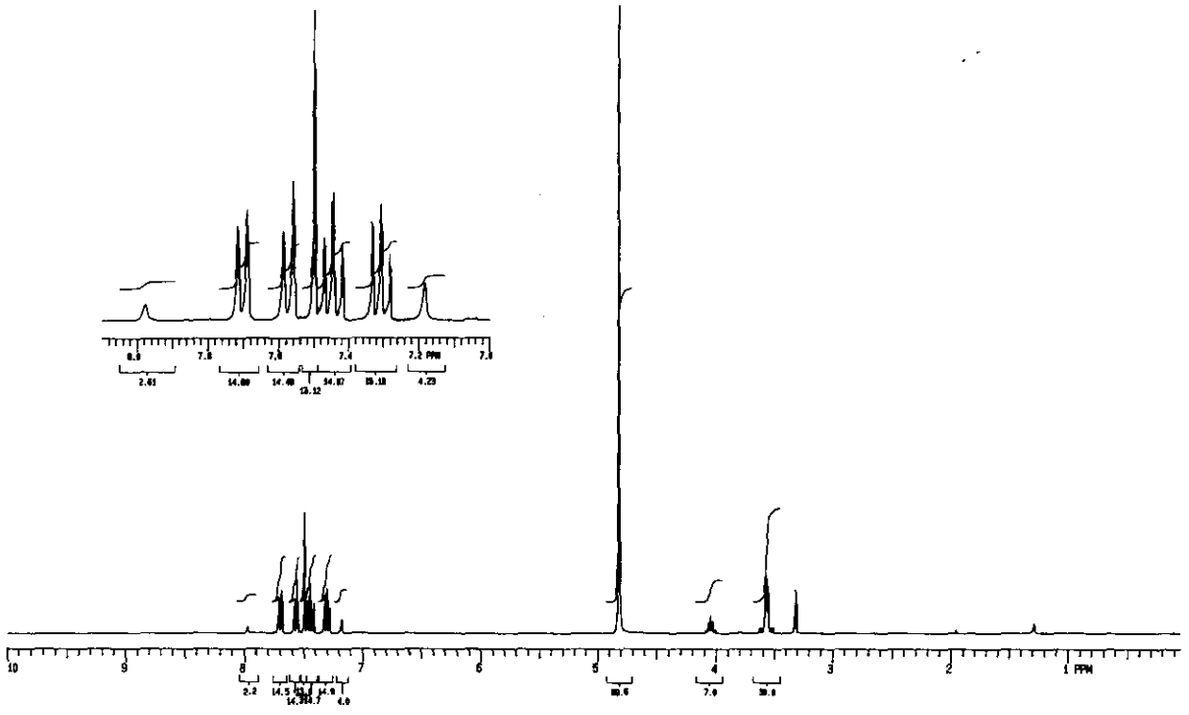
Spectrum 6: SVZ-2; Infrared Absorption Spectrometry.



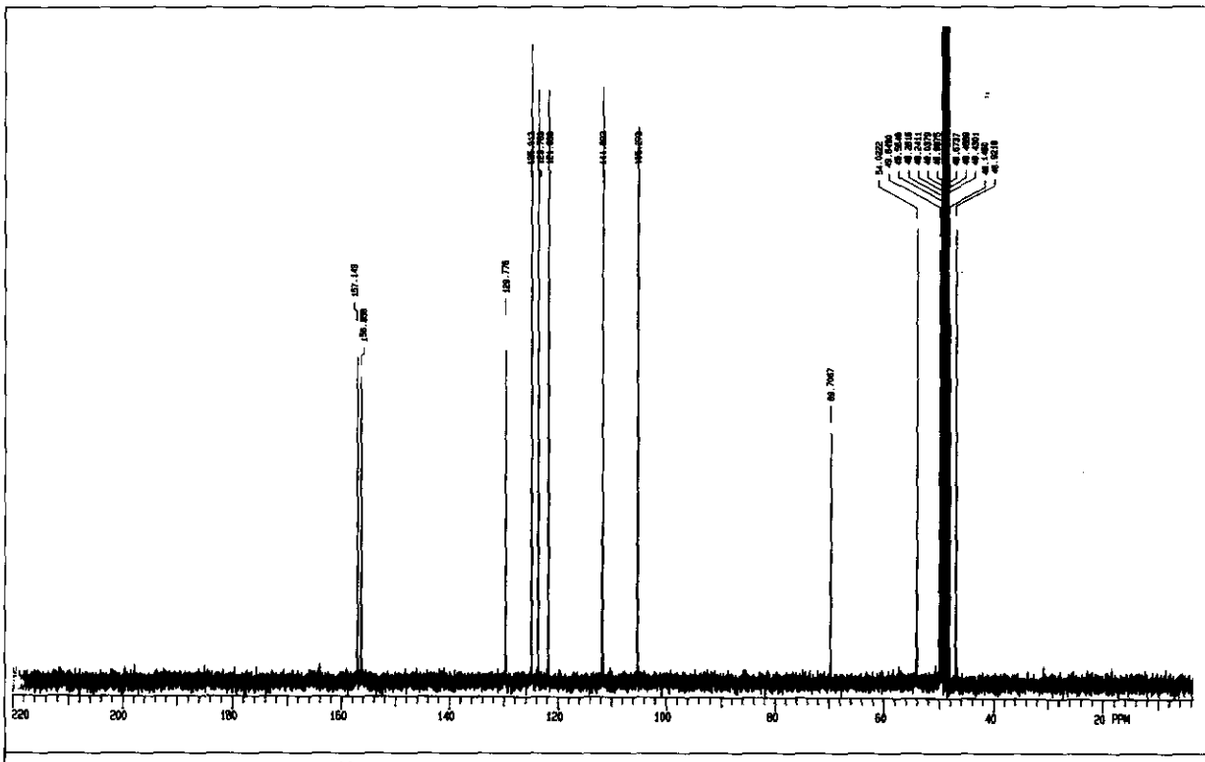
Spectrum 7: SVZ-4; H^1 Nuclear Magnetic Resonance Spectrometry.



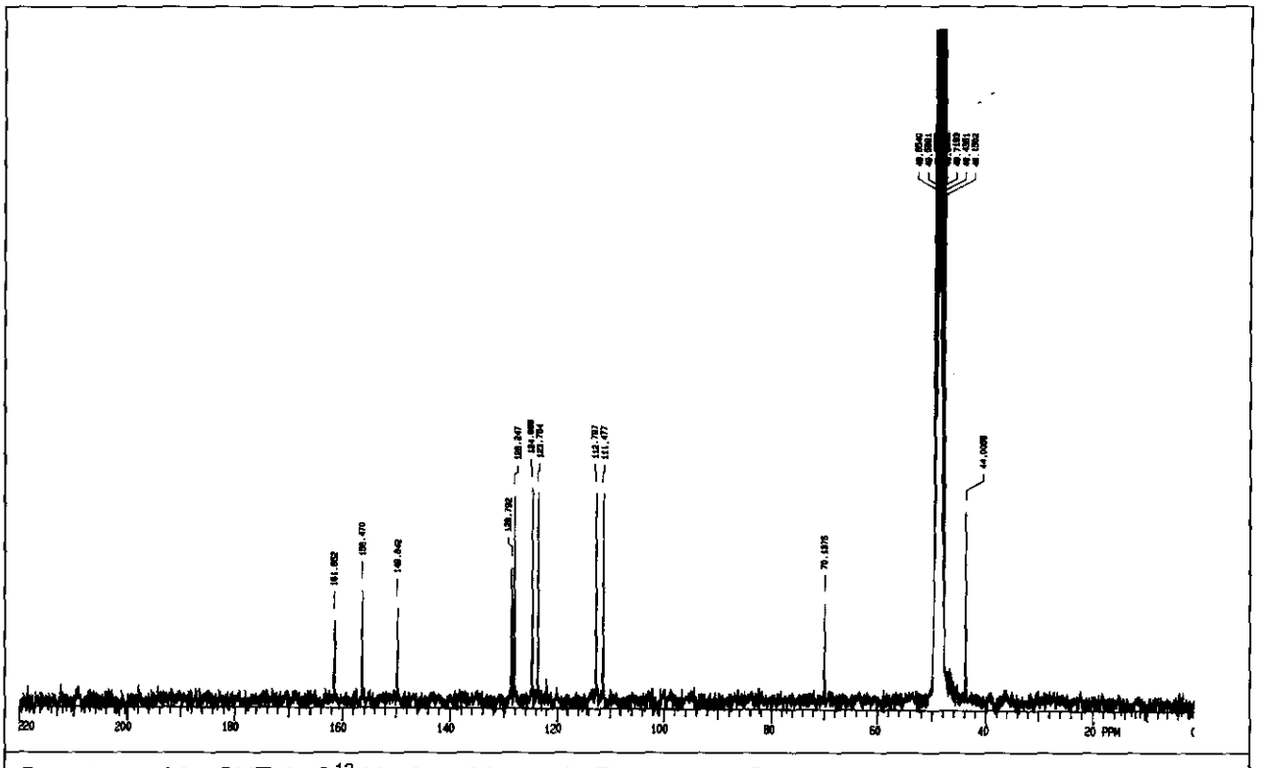
Spectrum 8: SVZ-5; H^1 Nuclear Magnetic Resonance Spectrometry.



Spectrum 9: SVZ-2; H^1 Nuclear Magnetic Resonance Spectrometry.



Spectrum 10: SVZ-4; C^{13} Nuclear Magnetic Resonance Spectrometry.



Spectrum 11: SVZ-2; C^{13} Nuclear Magnetic Resonance Spectrometry.

ANNEXURE 2

A poster presented at the 24th Annual Congress of the Academy of Pharmaceutical Sciences, 7 –10 September 2003 at Durban, South Africa.

Synthesis of N,N-disbustituted 1,3-diaminopropan-2-ol derivatives for HIV-1 protease inhibition

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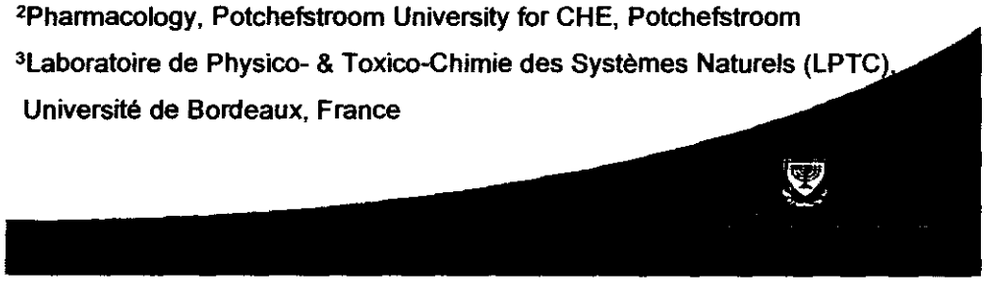

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