UNIVERSITY OF EAST ANGLIA

FACULTY OF SCIENCE

School of Pharmacy

Synthesis of proteasome inhibitors: analogues of nelfinavir

by

Anna Dysko

Supervisor: Prof. Ganesan

Advisor: Dr. C. Hamilton

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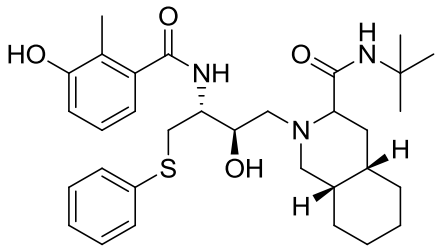
Abstract

Nelfinavir **1** has been found to be a very successful nonpeptidic HIV-protease inhibitor.



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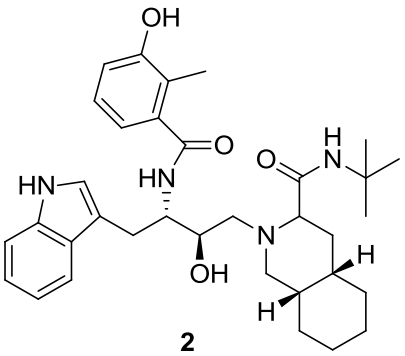
According to a previous investigation, nelfinavir **1** proved to have an inhibitory effect on chymotrypsin-like activity of 20S proteasome. Previous studies also show the promotion of acceleration of the apoptosis process within cancer cells by inhibition of proteasome activity. Separate experiments proved nelfinavir **1** to be involved in the inhibition of Akt pathway – an enzymatic pathway that results in arrest of apoptosis and prolongation of cell survival, which is also a very common mechanism in many types of cancers. These characteristics made nelfinavir **1** a very promising target for the anticancer therapy development. In this project, the synthesis of two nelfinavir **1** analogues was attempted, based on the replacement of the thiophenyl group by indole or phenyl group to give **2** and **3** respectively.



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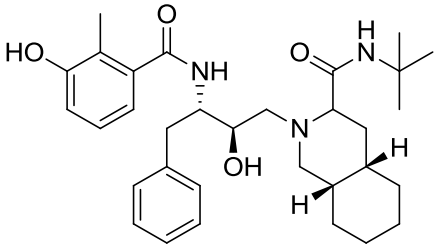

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The analogues were prepared in seven to eight steps from amino acids. Six of the synthesised compounds have been tested biologically following the MTS colourimetric assay procedure on THP-1 leukemia cells.

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Declaration of Authorship

This thesis is submitted to the University of East Anglia for the Degree of Master in Research and has not been previously submitted at this or any university for assessment or for any other degree. Except where stated, and reference and acknowledgment is given, this work is original and has been carried out by the author alone.

Signed: ……..

Date:……..

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Abbreviations

Ac ..... Acetyl

AIDS ..... Acquired Immunodeficiency Syndrome

AMP ..... Adenosine monophosphate

ATP ..... Adenosine triphosphate

Boc ..... *tert*-butoxycarbonyl

Cbz ..... Carboxybenzyl

CD4 ..... Cluster of Differentiation 4

DCC ..... Dicyclohexylcarbodiimide

DCM ..... Dichloromethane

DMSO ..... Dimethylsulfoxide

ESI ..... Electrospray ionisation

HIV ..... Human Immunodeficiency Virus

HOBt 1-Hydroxybenzotriazole

Ms ..... Mesyl (methanesulfonyl)

MS ..... Mass Spectrometry

mRNA ..... Messenger RNA

NMR ..... Nuclear Magnetic Resonance spectroscopy

PGPH ..... Peptidyl-Glutamyl Peptide-Hydrolysing

PP_i ..... Pyrophosphate

Pyz ..... Pyrazinoic acid

T-cells/T-lymphocytes ..... Thymus derived cells/lymphocytes

TFA ..... Trifluoroacetic acid

THF ..... Tetrahydrofuran

Ts ..... Tosyl (4-toluenesulfonyl)

1. INTRODUCTION

1.1. THE PROTEASOME

In eukaryotes, archaea and some bacteria, proteasomes play a crucial role in destroying misfolded and superfluous proteins and producing cell cycle control modifying proteins by proteolysis in specific places. They are very large, multimeric protein complexes that are located in the nucleus and the cytoplasm of cells.[1] The proteasomes that are present in eukaryotes and archaea are the 20S proteasomes. 20S is a proteolytic core unit, linked with two regulatory 19S pieces (placed on both sides of 20S) to build the whole 26S complex.[2] It has been examined in yeast that the 20S proteasome is a cylindrical particle, that contains in its structure two outer rings and two inner rings. These consist of 14 different subunits, which co-operate with each other. The outer rings are made of 7 different α-subunits, with the inner rings consisting of 7 different subunits in each case with the exception of the β-type. β1, β2 and β5 subunits characterise the catalytic activity. All three are based on a similar mechanism but are distinguished according to their substrate specificity: trypsin-like, chymotrypsin-like and peptidyl-glutamyl peptide-hydrolyzing activity.[3] The enzyme involved in the proteolysis process is dependent on the peptide residues that are to be cleaved. Trypsin preferably breaks amide bonds, where the carboxylic acid side of the peptide is lysine or arginine. Chymotrypsin performs proteolysis on peptides, which on the carboxylic end of the structure comprise amino acids containing aromatic side chains, phenylalanine, tryptophan and tyrosine. This feature ensures access of the peptide to the hydrophobic pocket of the enzyme. Finally, peptidyl-glutamyl peptide-hydrolyzing domain cleaves proteins ending with aspartic acid and glutamic acid on the carboxylic side.

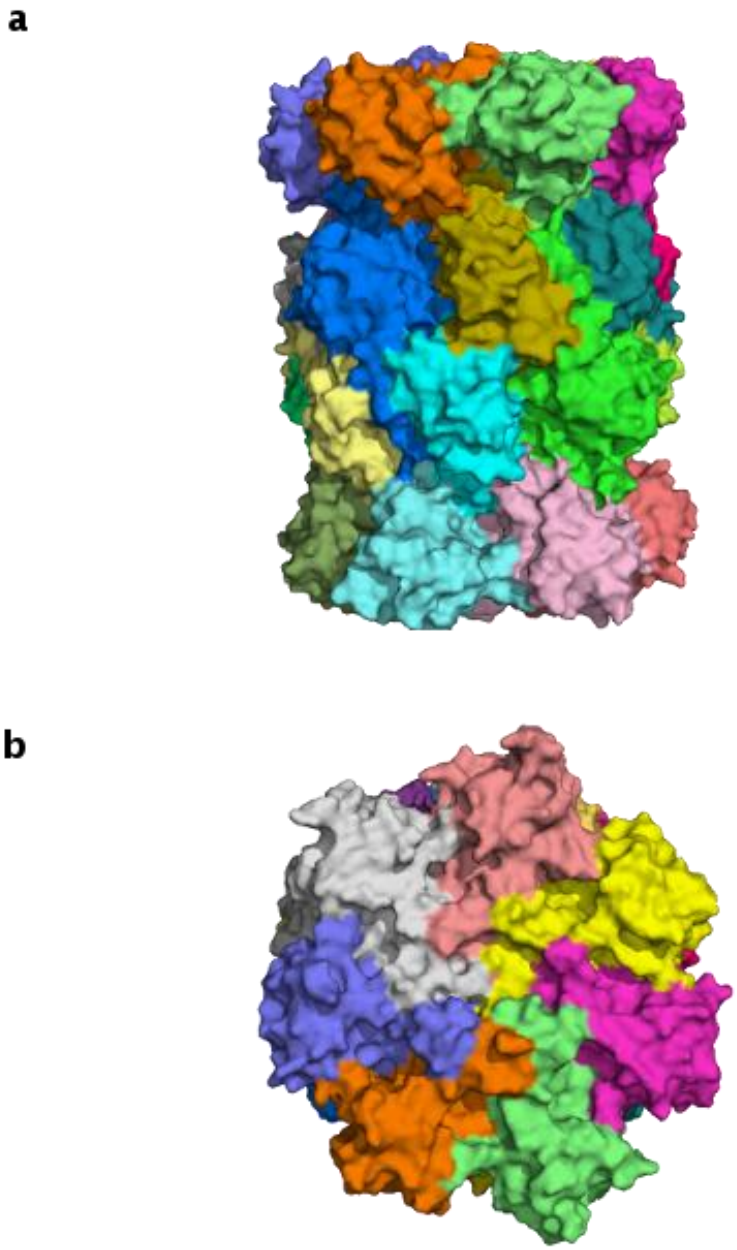
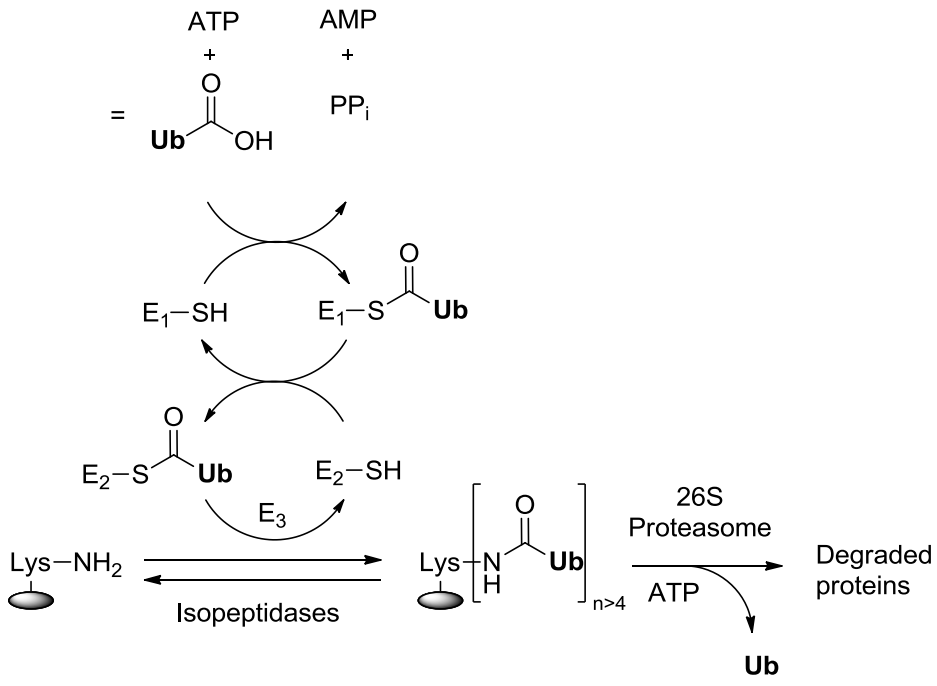


Figure 1 - Models of the 20S proteasome from *Saccharomyces cerevisiae* showing the α-β-β-α ring pattern (a) and the seven α-units in the outer ring (b).[4]

The starting point of the protein digestion process is the attachment of ubiquitin - a small protein which itself consists of 76 amino acids. Looking deeper at the mechanism of ubiquitylation and targeting, it is an enzymatic process, where the *C*-terminal tail of glycine, from activated ubiquitin, binds via an amide bond with the *N*-terminal tail of lysine residue which has been attached to the superfluous protein. This happens in a

three step process. First ubiquitin is activated, forming an active thioester by an ubiquitin-activating enzyme (E1). This reaction requires an energy supply, which comes from ATP hydrolysis. The next protein is carried to an ubiquitin-conjugating enzyme (E2), leaving E1. Finally one of many kinds of ubiquitin ligases (E3) catalyses translocation of ubiquitin to the lysine residue of the specific substrate.[5] This tagged protein gives a signal to form a polyubiquitin chain by activating other ligases and adding the next ubiquitin units. This chain on the protein binds to 9 (out of 19) proteins that are incorporated in the 19S unit. The other 10 subunits of 19S bind to the outer ring in 20S. Protein degradation to requires binding of ATP to ATPase subunits, placed on 19S.[6] This enables the protein to unfold and the 'gate' in the 20S to open. Thanks to this process, labelled protein can enter the narrow channels of the 20S core but also prevent uncontrolled crossing through this barrier and the degradation of cellular proteins. It is worth noting that all the ubiquitin proteins; after fulfilling their purpose, are recovered and reused in the next tagging process.[7] To highlight the importance of ubiquitin protein function, those involved in the elucidation of the process were awarded the Nobel Prize for chemistry in 2004.[1]



Scheme 1 - The attachment of ubiquitin to proteins for degradation by the proteasome.[7]

There are many cellular processes where the ubiquitination mechanism plays an important role. These include apoptosis, antigen processing or DNA transcription or repair.[7] Cyclins, for example, are a group of proteins involved in cell development during the cell cycle and are also disregulated in many tumor cells. They are not enzymes themselves, but have an active site that activates cyclin-dependent kinase enzymes (Cdk).[9] There are different types of cyclins controlling different stages of the cell cycle. To complete the process, those proteins have to be digested by proteasome, which is where the ubiquitin-proteasome system plays an important role.[10]

Apoptosis is programmed cellular death which requires a supply of ATP, protein synthesis and gene transcription. It depends on the activity of caspases (group of cysteine proteases), p53 and p27 proteins, NF-$_K$B (protein complex used for the DNA transcription) and I$_K$B (inhibitors of NF-$_K$B).[7] Later research showed the levels of ubiquitinated proteins increase while E1, E2 and E3 (enzymes involved in the ubiquitination process) levels decrease, prior to the completion of apoptosis. It has also been found that the ubiquitin-proteasome pathway destroys numerous important apoptosis proteins, some of which have been proven to have proteasome-inhibitory activity toward programmed cell death (which might have a major influence in tumor cell development).

Proteasomes also have an impact on the immune system, being involved in the adaptive immune system. This is the system that allows the organism to have a stronger immune response by recognising pathogens by their specific antigens. These antigens are peptides that are produced by the proteasome in the process of degradation of proteins coming from pathogens attacking the organism.[11] The proteasome also activates NF-$_K$B, an anti-apoptotic and pro-inflammatory factor, which makes it part of inflammatory and autoimmune diseases, such as systemic lupus erythematosus or rheumatoid arthritis.[11]

There have been several proteasome inhibitors discovered to induce apoptosis, such as salinosporamide A (**4**), bortezomib (**5**) and MG132 (**6**) (Figure 2).

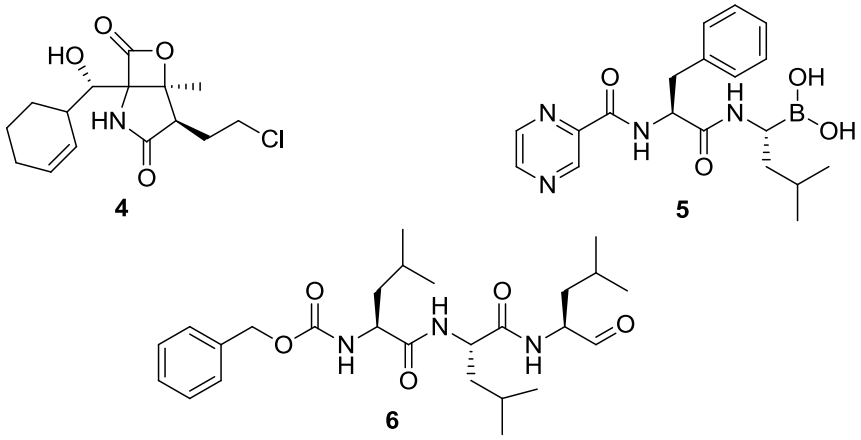
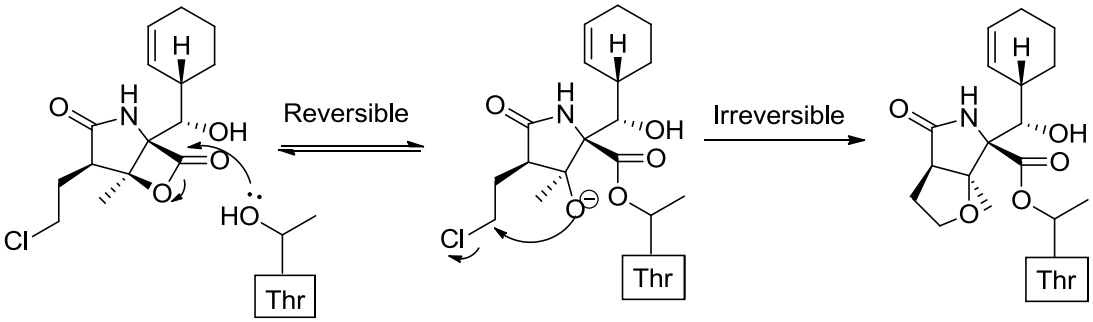


Figure 2 - Structures of the proteasome inhibitors: salinosparamide A (4), bortezomib (5), MG132 (6)

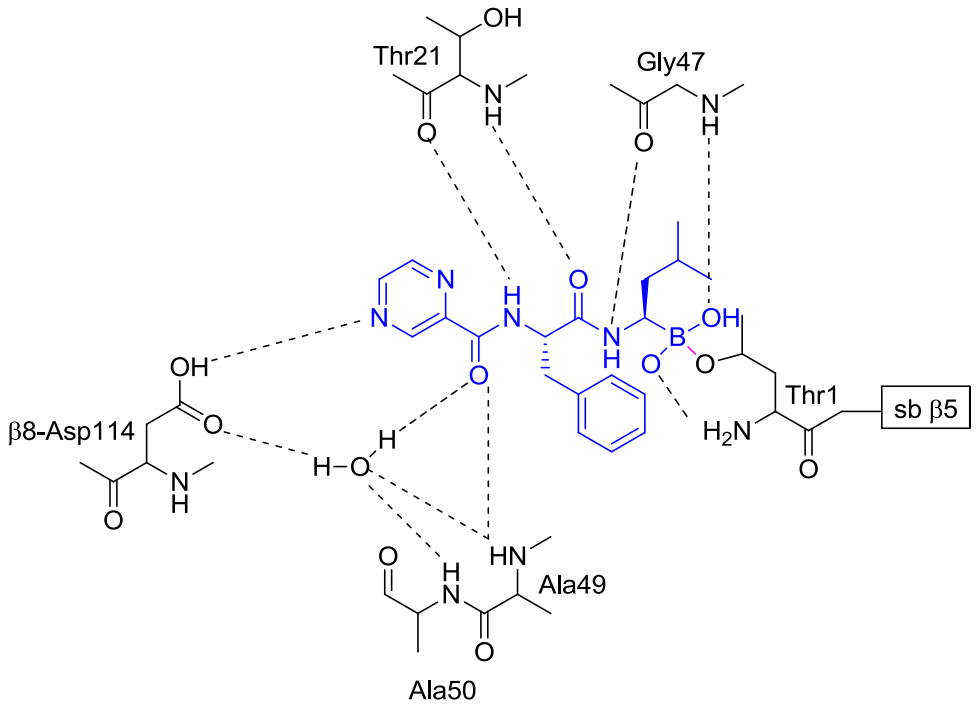
Salinosporamide A (**4**) is a novel marine natural product isolated from an extract of the marine bacterium *Salinispora tropica*. It is a potent and selective anticancer agent[12] and inhibits proteasome activity through covalent bonding to the active site threonine residue of 26S (Scheme 2). Formation of a five-membered ring in the second step, blocks the approach of water, which prevents a breakdown of the inhibitor – proteasome complex.



Scheme 2 – Mechanism of action for salinosporamide A (4)

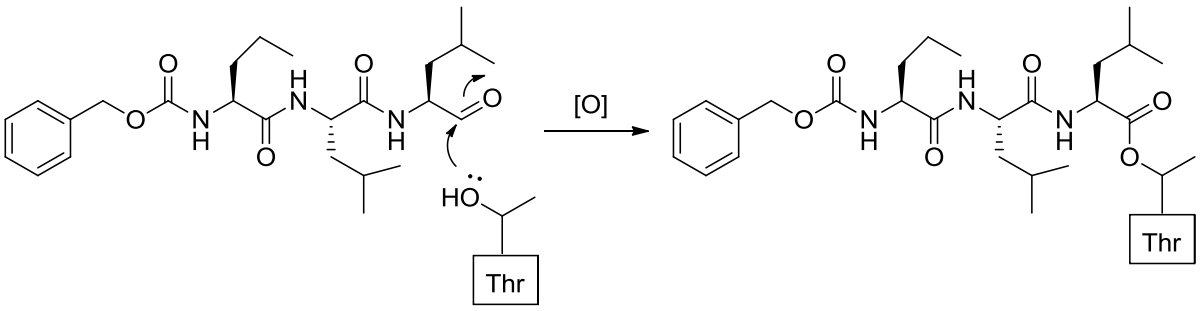
Bortezomib (**5**), marked as Velcade® is a dipeptide – Pyz-Phe-boroLeu with a boronic acid at the leucine residue instead of a carboxylic acid, and is used for relapsed multiple myeloma and mantle cell lymphoma treatment.[13] It is the first proteasome inhibitor that has reached and successfully progressed through clinical trials.[14] One principal activity

is to inhibit the breakdown of the NF-κB inhibitor IκB. A critical interaction of bortezomib **5** with the proteasome is its binding with the chymotryptic-like and the caspase-like active sites. The mechanism of action is based on the covalent linkage of the inhibitor's boron atom with nucleophilic threonine and several hydrogen bonds between inhibitor and residues of the active site (Scheme 3).



Scheme 3 – Interaction of bortezomib (5) with chymotryptic-like active site of the 20S proteasome

MG132 (**6**) is an aldehyde proteasome inhibitor and reversibly forms a covalent bond to the threonine residue of the β subunit in the 26S proteasome (Scheme 4). It also inhibits NF-$_K$B activation by arresting proteolysis of their inhibitor IκB and has agonistic effects on JNK1 kinase that initiates apoptosis.[15]



Scheme 4 – Mechanism of action of MG132 (6)

1.2. HIV-PROTEASE AND NELFINAVIR

Acquired Immunodeficiency Syndrome (AIDS) was first identified and reported by the US Center for Diseases Control (CDC) in1981 and only a few years later was discovered to be caused by the human immunodeficiency virus (HIV).[16] As AIDS became a huge problem all over the world, finding antiviral therapies against HIV type 1 was of primary importance among many drug discovery research groups. By gaining knowledge about the virus' life cycle, researchers could work on discovering drugs that would inhibit the effects of HIV in different stages of its life cycle.

HIV is a member of the retrovirus group and its genetic information is presented in RNA form. It attacks T cells (cells belonging to a group of lymphocytes, important in many immunological processes) characterised by CD4 glycoprotein that is expressed on the T cell surface.[17] In its life cycle, the virus first enters a cell and reverse-transcribes its RNA to DNA via the reverse transcriptase enzyme (RT). In this form, the virus gets to the cell nucleus and there integrates with the genetic material of the infected cell, thanks to the integrase enzyme activity. The viral DNA is then transcribed to mRNA and transferred outside the nucleus to the cell cytoplasm, where it is translated into the viral proteins. Finally, the viral protein needs to divide into smaller, separate pieces, made possible by HIV protease that cleaves amide bonds and forms mature proteins. As a result, the cell is destroyed and releases new virions ready to infect other cells.[18]

By developing further knowledge about the HIV life cycle, scientists have come up with some theories behind the prevention of AIDS. One such theory reflects the replication stage in virus life. These drugs inhibit the reverse transcriptase enzyme RT (for instance: AZT, ddI or nevirapine and delavirdine). Another strategy is based on the inhibition of HIV-protease, which prevents hydrolysis of polypeptide bonds to individual mature proteins (for example nelfinavir **1**, ritonavir **7**, saquinavir **8**).[19]

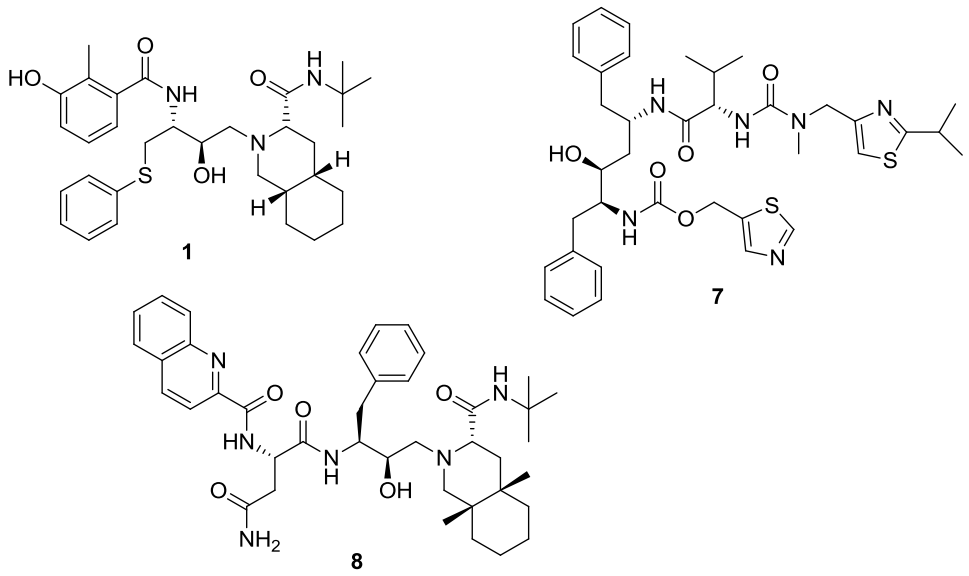


Figure 3 - Structures of HIV-protease inhibitors: nelfinavir (1), ritonavir (7), saquinavir (8)

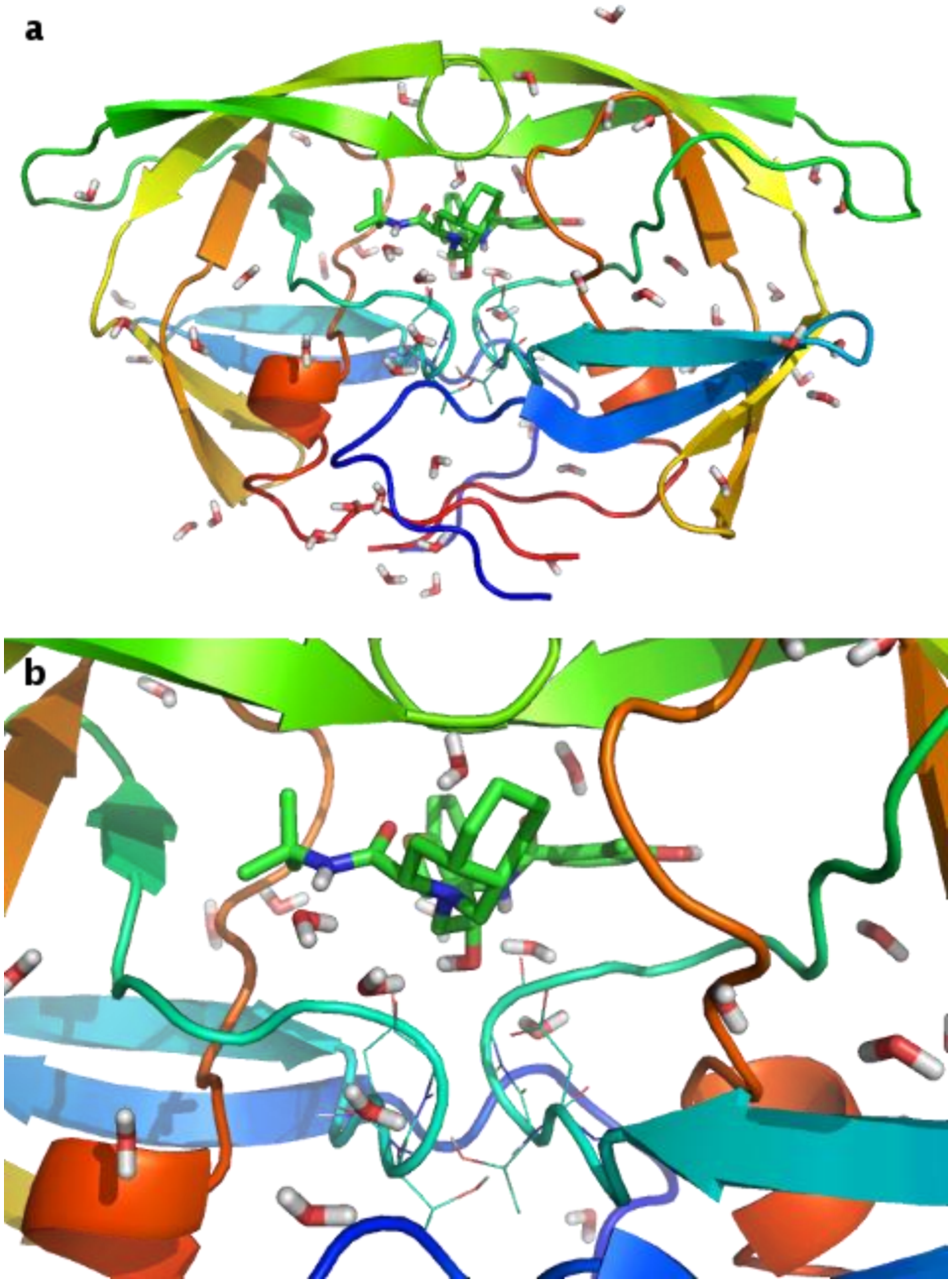


Figure 4 - Binding of nelfinavir 1 - in green - to HIV-1 protease, showing the C2-symmetric homodimer structure of the enzyme (a) and the secondary alcohol bound to the Asp-Thr-Gly region (cyan sticks, b). (PDB: 1OHR).[20]

Kent and co-workers published the first crystal structure of HIV-protease. Analysis of the structure indicates it is a C_2-symmetric homodimer with 99 amino acids in both subunits.[21] There is also the specific amino acid sequence Asp-Thr-Gly in both monomers, which is characteristic of aspartic proteases.[22] This amino acid triad is also a part of the two active sites of the enzyme, one on each monomer.

Proteases can be divided into two groups depending on the mechanism that activates their catalytic activity. In the first class of proteases amide bond hydrolysis is initiated by the nucleophilic atom on the amino acid side chain, which is first activated by another amino side chain. In the second class of enzymes, a nucleophile that attacks the carbonyl group on the amide bond is activated by water. The mechanism for water activation is, as of yet, not very well understood. It is known that the two active sites play an important role in this process, and studies prove that HIV-protease is one of the enzymes belonging to this class of proteases.[18]

Nelfinavir **1** (formally AG1343 and marketed as Viracept®), as mentioned above, is one of the most successful HIV-protease inhibitors. It is an antiviral drug widely prescribed for the treatment of HIV infection used in combination with HIV reverse transcriptase inhibitors.[23] It was approved for therapeutic use in 1997, and after only one year in the United States gained 30% of the market share, which is growing yearly.[24]

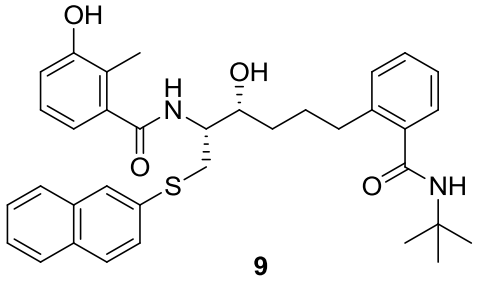


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Figure 5 – Nelfinavir precursor 9

The discovery of nelfinavir **1** started from another compound **9** that showed tight binding with HIV-protease but its poor antiviral activity and poor water solubility made it an unattractive drug. According to previous results, the *S*-aryl substituent improves the inhibitory activity of the enzyme. Nelfinavir **1**, an analogue of **9** with S-phenyl instead of S-naphthyl proved to be a potent inhibitor of HIV-protease. For a better understanding of the binding affinity, studies on the co-crystal structure of the nelfinavir complex with the protease were performed.[25] The study showed that the *S*-phenyl group of nelfinavir binds to the S1 pocket and has some additional interaction with the S3 pocket, with the decahydroisoquinoline occupies the S1' hydrophobic

pocket and the *tert*-butylcarboxyamide residue occupies the S2' region of the enzyme. The structure also proved that the 2-methyl-3-hydroxy-benzamide of the compound fits in the S2 pocket, where its 3-phenol group binds with the Asp-30 via hydrogen bonding, and the 2-methyl group interacts with two other amino acids Val-32 and Ile-84.[26]

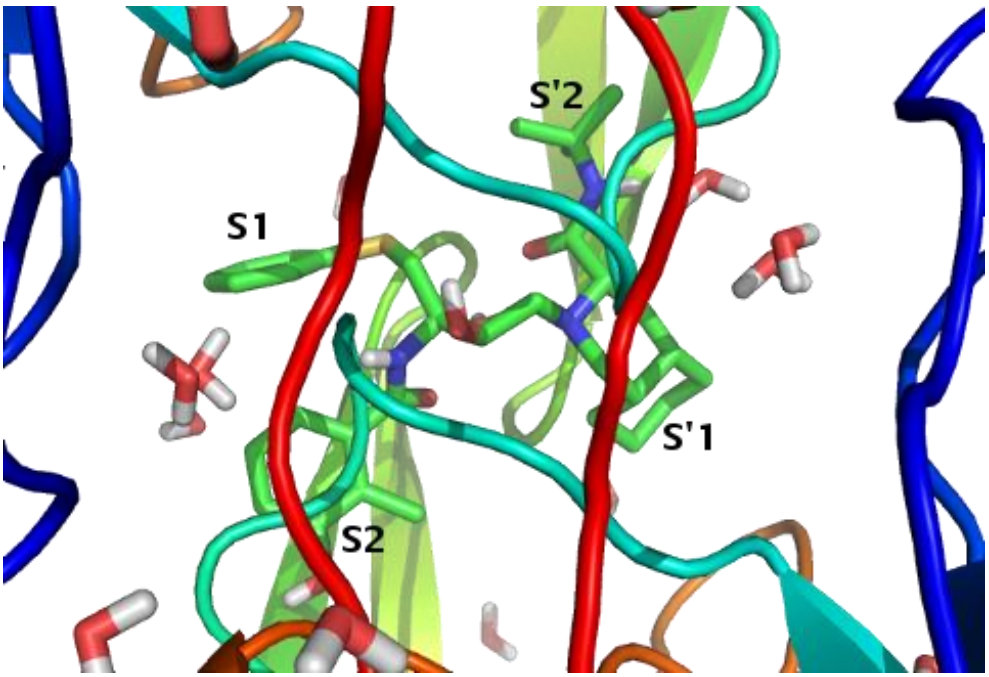

Figure 6 - Arrangement of nelfinavir 1 in the HIV-1 protease active site looking through the catalytic region (cyan ribbons) with labelled substrate pockets. (PDB: 1OHR).[27]

Besides these two compounds, phenylalanine analogue **3** was also tested, showing much better binding affinity of nelfinavir **1** comparing to **3**, making the former the lead compound.[26]

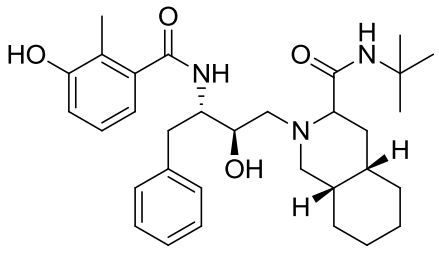

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Figure 7 – Nelfinavir analogue 2.

It has been discovered that nelfinavir **1** causes several side effects, such as diarrhea, abdominal pain, flatulence and less commonly, excessive urination, fatigue, hepatitis

and rashes. It has also been found that it can inhibit the insulin degrading enzyme and block oral epithelial cell DNA synthesis.[28,29] Despite these disadvantages, nelfinavir **1** still remains one of the most effective and most widely used anti-HIV drugs.

1.3. NELFINAVIR AS A POTENTIAL ANTICANCER DRUG

The approximate cost of a new cancer drug development is in the region of 1 billion dollars, with the time required to go from concept to Food and Drug Administration approval estimated to be around 15 years. One strategy to reduce the costs of this process is by repositioning molecules already approved for other indications.[30] Making use of already available toxicity and pharmacokinetic data would then accelerate the progress of drug development.[31]

During HIV resistance studies it has been found that the number of CD4 cells (co-receptor, placed on the T cells, mainly attacked by HIV) increase even though the inhibitory activity of the antiretroviral drug had stopped due to acquired viral resistance. This observation suggested the possibility of HIV-protease inhibitors having effects on other enzymes.[32]

During the late nineties, research groups examined mice infected with lymphocytic choriomeningitis virus and treated with ritonavir **7** (a HIV-protease inhibitor), to find if it caused effects other than that of its antiviral activity. As a result, they observed a significant inhibitory impact against antiviral cytotoxic T lymphocyte (CTL) activity by stopping the presentation of antigens on infected cells to CTL cells. This process causes incorrect activity of T lymphocyte cells, which may contributing to AIDS therapies and other viral diseases.[33] It has also been found throughout these studies that ritonavir inhibits the chymotrypsin-like activity of 20S proteasome, which at the same time caused massive growth of trypsin-like activity.[33] Harbouring these interesting results, scientists extended the group of compounds tested in the same direction to other HIV-protease inhibitors, among which *N*-acetyl-leucyl-leucyl-norleucinal (LLnL) **10** showed similar potency to ritonavir.[34] Considering previous investigation in this area, which proved that proteasome inhibitors stop tumour growth by acceleration of the apoptosis process in cancerous cells only, scientists suggested that inhibition of proteasomes with protease inhibitors such as nelfinavir **1**, is a very promising lead in the development of anticancer therapies.

Serine/threonine protein kinases called Akt are another factor crucial to the process of cell multiplication (making them involved in cancer development). Three different genes (Akt1, Akt2, Akt3) code for three different enzymes which are part of the non-specific serine/threonine – protein kinase family and stop the apoptotic proccess.[35] Akt1 takes part in the pathway of cellular survival, and so by blocking apoptosis, these enzymes extend cell survival. As a result of this feature, Akt1 has been recognized as a key factor in many types of cancers. Inhibiting this enzyme may provide a pathway towards cancer treatment by facilitating apoptosis in cancer cells.[35]

Scientists noticed that toxicities caused by HIV-protease inhibitors are similar to those observed in Akt inhibition, which led them to believe that HIV-protease inhibitors may be working in the same or a very similar way to Akt inhibitors. As mentioned before, the Akt enzymatic pathway is a common activity in many types of cancers and many researchers are working on developing anticancer agents based on the phoshphoinositode 3-kinase (PI3K)/Akt pathway. After several studies – screening six clinically approved HIV protease inhibitors, it has been found that three of them ritonavir **7**, saquinavir **8** and nelfinavir **1** inhibited growth at clinical concentrations of the drug. From these results, we can see that the most effective was nelfinavir **1** and suggests its development as a prospective anticancer agent.[36]

Studies made into the effects of **1**, **7** and **8** on the basic level of Akt activation showed that nelfinavir **1** and saquinavir **8** decreased Akt phosphorylation. Other studies showed that nelfinavir can stop signalling to Akt from two types of tyrosine kinase receptors (IGF-IR and EGFR). Thus, it suggests that this HIV protease inhibitor is capable of acting at the level of the plasma membrane to inhibit growth factor receptor activation, which next inhibits the PI3K/Akt pathway.[36] Nelfinavir was tested in a panel of drug-sensitive and drug-resistant breast cancer cell lines. All tested lines (with one exception) were inhibited by at least 60%. This research proved that **1** has activity against cancer cells that obtain resistance to therapies such as tamoxifen – which is another step toward successful anti-cancer therapy methodology.

2. AIMS

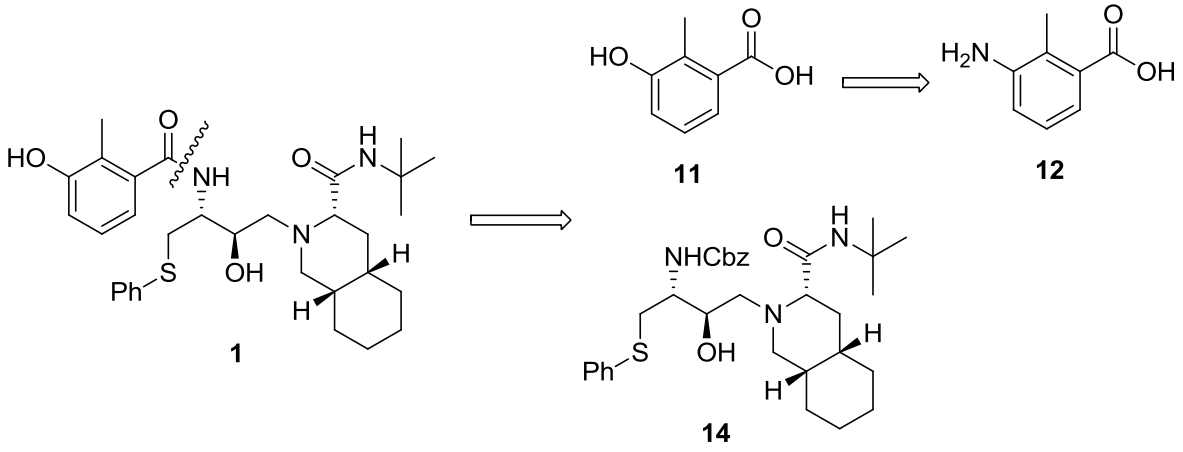
Although nelfinavir is a successful antiviral drug, it has not been optimized for anticancer activity. It is likely that analogues of nelfinavir will be better than the parent molecule against this new indication. In order to study the structure – activity relationship (SAR) of nelfinavir **1** with the proteasome, the aim of this project is to synthesise a series of analogues and examine their biological activity.

3. RESULTS AND DISCUSSION

2.1 THE PREVIOUS SYNTHESIS OF NELFINAVIR

Looking at nelfinavir **1** retrosynthetically, this compound can be broken into three different elements: a perhydroisoquinoline derivative **13**, a benzoic acid derivative **11** and central 4-carbon core.

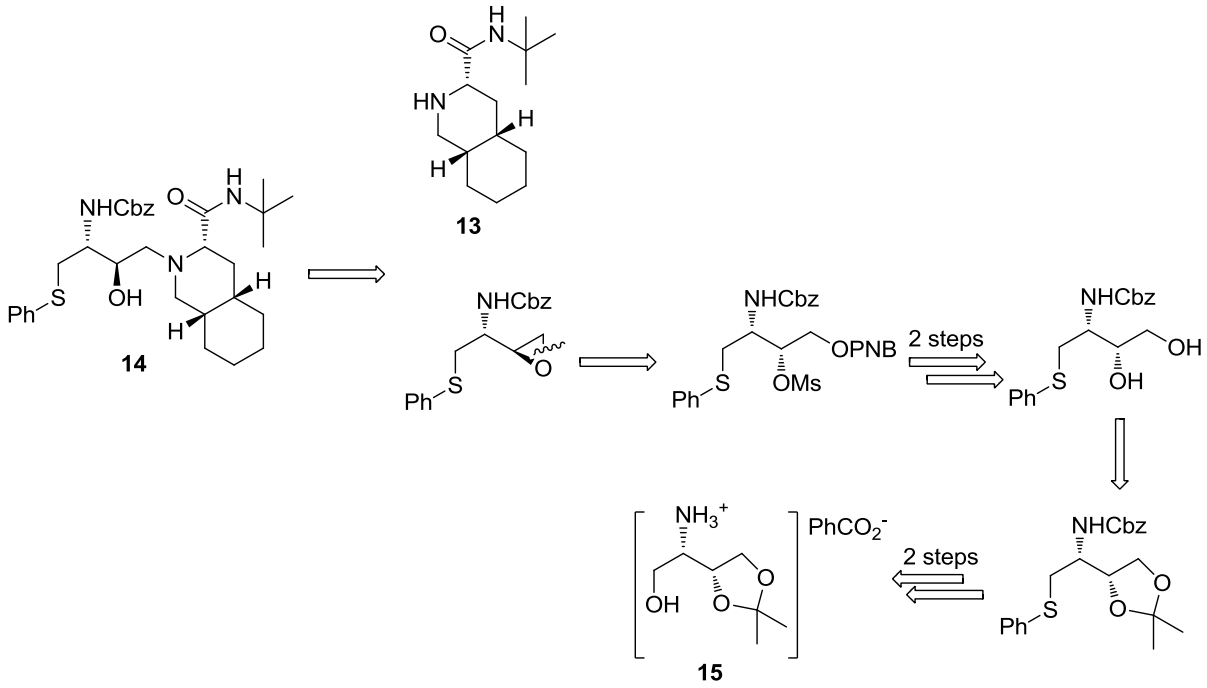
There were many different synthetic routes for nelfinavir **1** published in the past two decades. In the first five disconnections presented below nelfinavir **1** is made from two main precursors: **3**-hydroxy-2-methylbenzoic acid **11** (which can be synthesised from commercially available 3-amino-2-methylbenzoic acid **12** through diazotisation reaction) and amine **14** (Scheme 5). Although there were different methods published to obtain 14. Different research groups have investigated a variety of routes to find the best way for nelfinavir **1** synthesis (disconnection A – E) using these intermediates.[1,37,38,39,40]



Scheme 5 – Retrosynthesis of nelfinavir to disconnections A, B, C, D and E.

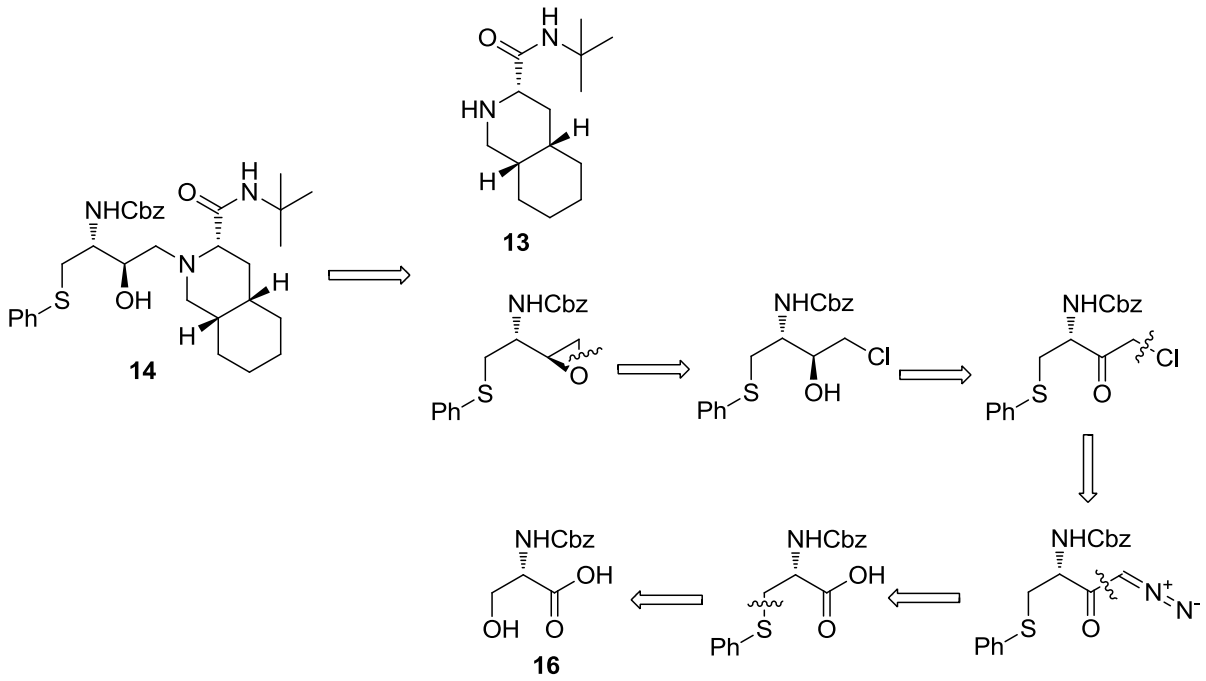
Disconnection A – One of the routes to synthesis intermediate epoxide was proposed by Tahashi Inaba.[37] His work concentrated on making a general compound that can produce a number of protease inhibitors. The general method requires many regioselective protection and deprotection reactions, to assure that primary and

secondary hydroxyl groups react separately and with the correct stereochemistry. To afford the desired epoxide in the first step, amine **15** is protected with Cbz group and then treated with thiophenyl anion to produce the sulfide. Addition of HCl in MeOH removes the acetonide protection. Next protection of the primary hydroxyl group was performed with 4-nitrobenzoyl chloride (PNBCl) followed by treatment with MsCl to convert the secondary hydroxyl group to a leaving group. After basic treatment of such prepared product, PNB group is hydrolysed and spontaneous ring closure gives the epoxide, which then reacts with amine **13** giving desired fragment **14** (Scheme 6).[37]



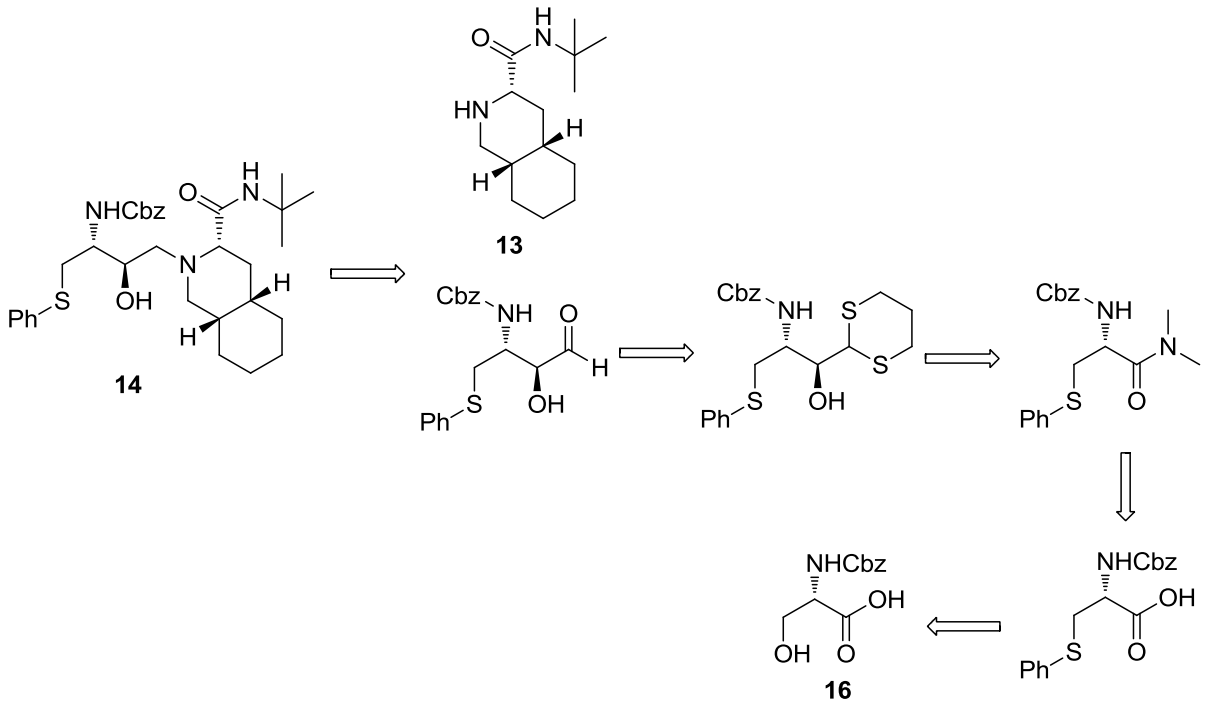
Scheme 6 – Retrosynthetic analysis of nelfinavir (1): Disconnection A.

Disconnection B – This original method for nelfinavir **1** uses as a starting material *N*-Cbz-L-serine **16**. Reaction with sodium aryl thiolate gives the protected unnatural amino acid, which is converted to a chloroketone. This is reduced to the corresponding chloro alcohol by treatment with sodium borohydride. Lastly, alkali-initiated cyclisation of the alcohol gives the epoxide which then is opened by treatment with amine **13** forming desired fragment **14** (Scheme 7).[1]



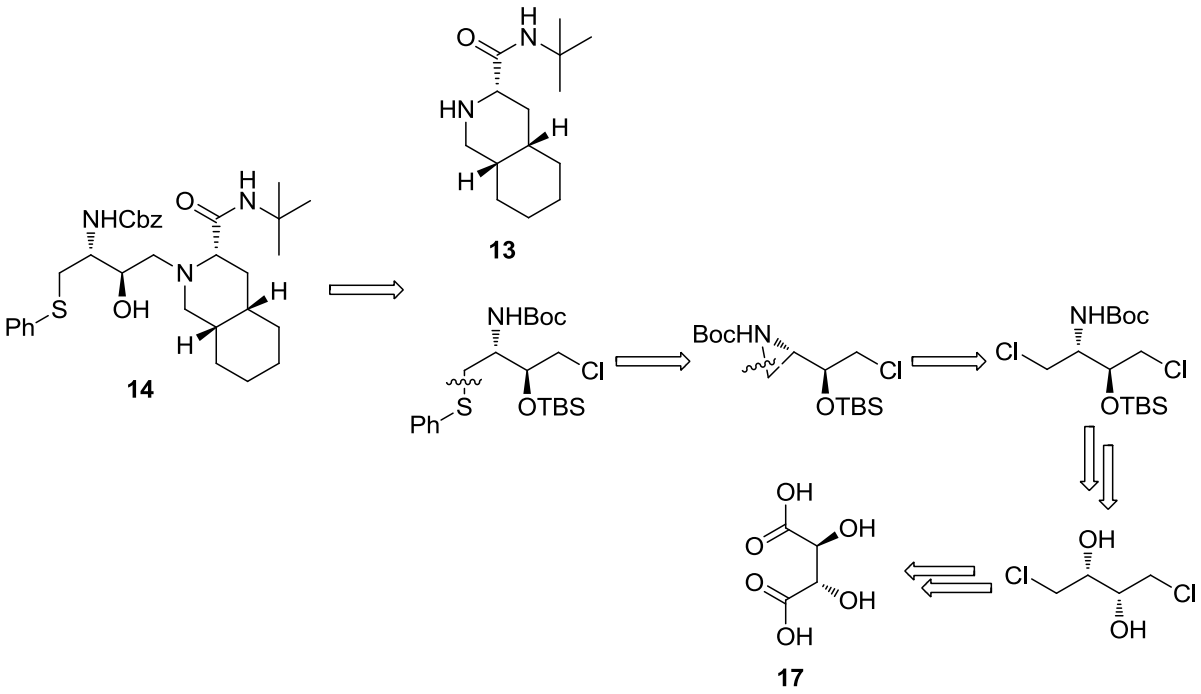
Scheme 7 – Retrosynthetic analysis of nelfinavir (1): Disconnection B.

Disconnection C – In this third method published by Rieger, L-serine **16** is also used as a starting material, like in the disconnection B.[38] The amino acid is easily transformed to *N*-Cbz-*S*-phenyl-L-cysteine. Further formation of the amineand addition of 2-lithio-1,3-dithiane (made from 1,3-dithiane and *n*-BuLi) gives a ketone, which is reduced with sodium borohydride to provide the dithiane alcohol. To complete the synthesis of nelfinavir **1**, the dithianyl group is removed to give α-hydroxy aldehyde, which can be aminated with **13** to achieve desired compound. By this method nelfinavir **1** is obtained by one step less than the previous route (Scheme 8).[38]



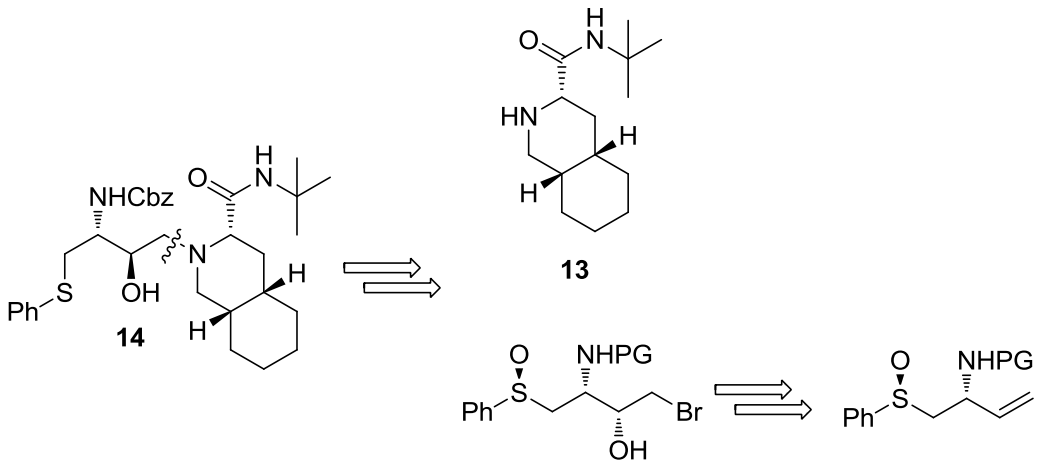
Scheme 8 - Retrosynthetic analysis of nelfinavir (1): Disconnection C.

Disconnection D – This route requires D-tartaric acid **17** as a starting material. This is converted to acetonide diester, reduced and transformed into a dichlorodiol. After several protection and deprotection reactions, the protected amino alcohol is obtained, which upon reaction with sodium hydride forms desired aziridine. This functionality allows the addition of thiophenoxide by opening of the aziridine to create *S*-phenyl moiety with desired stereochemistry. This intermediate can be used for the synthesis of nelfinavir **1** by the deprotection of oxygen and formation of epoxide (Scheme 9).[39]



Scheme 9 – Retrosynthetic analysis of nelfinavir (1): Disconnection D.

Disconnection E – This route uses sulfoxide as a starting material that undergoes stereoselective bromohydrin formation, which then can be converted to desired *S*-phenyl derivative with a very good yield 82% (Scheme 10).[40]

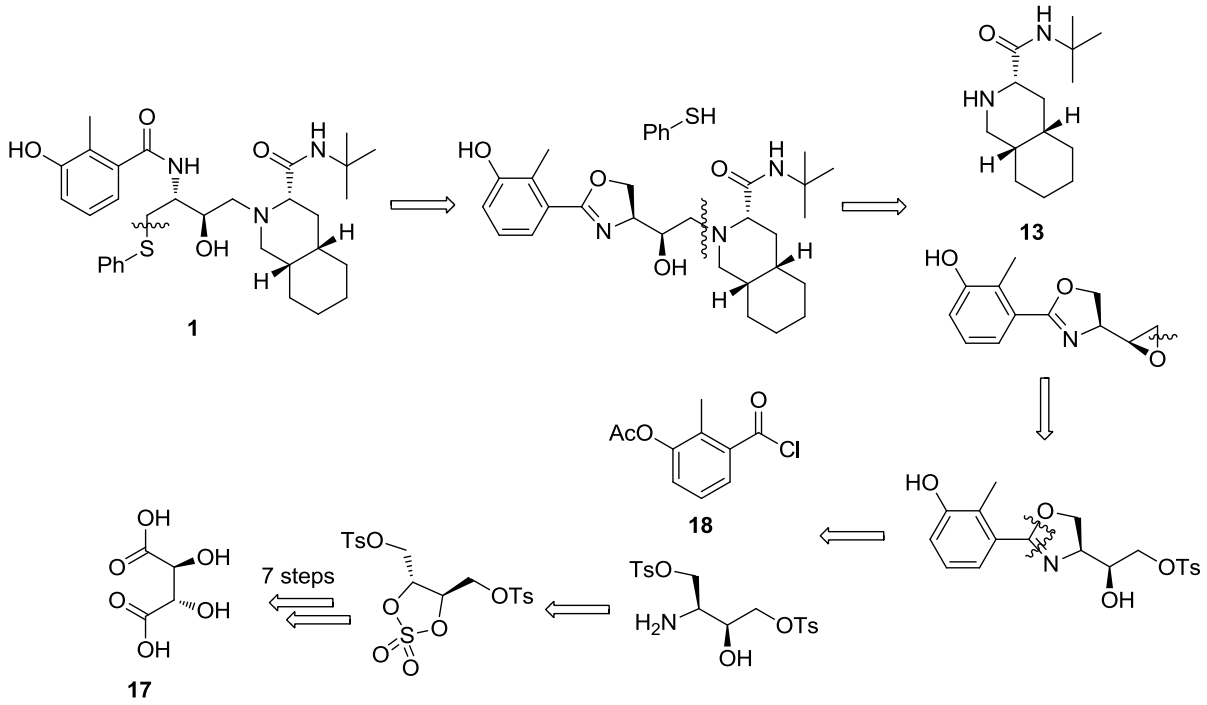


Scheme 10 – Retrosynthetic analysis of nelfinavir (1): Disconnection E.

There are two published synthetic routes where thiophenyl group undergoes disconnection. In both cases it proceeds through the ring-opening of an aryl oxazoline and use of 3-acetoxy-2-methyl benzoyl chloride **18** - differently to most routes used by

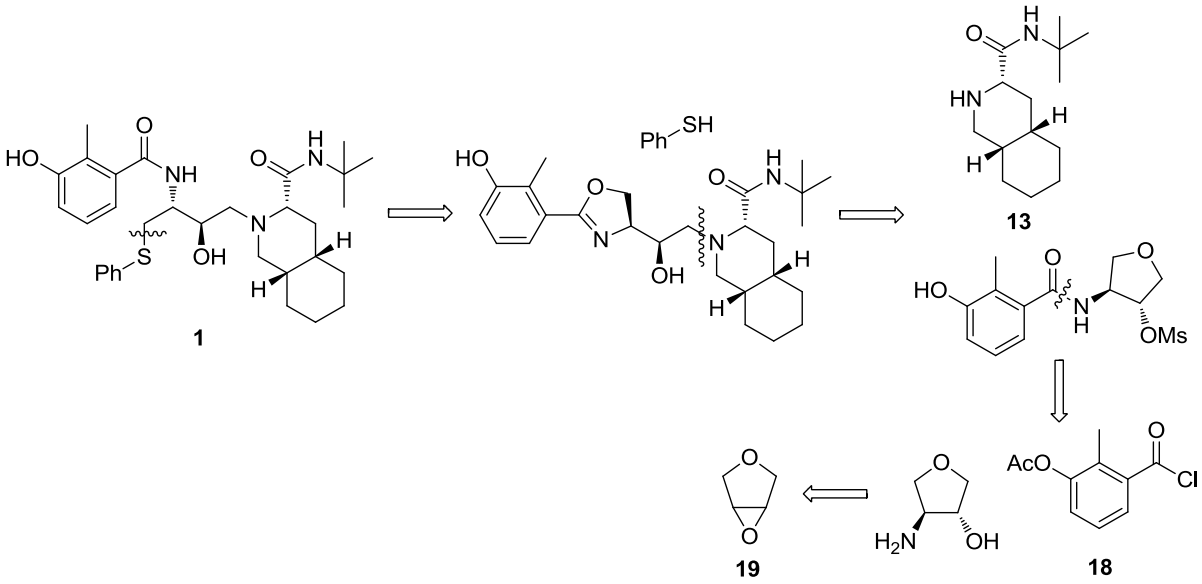
other groups, where 3-hydroxy-2-methylbenzoic acid **11** was used, (Scheme 11 and 12).[41,42]

Disconnection F – In the work of Albizati *et al.*, the starting material is again unnatural D-tartaric acid **17**. This acid through seven straightforward protecting and deprotection steps forms a cyclic sulfate. The nitrogen nucleophile opens the cyclic sulfate giving directly the amino alcohol intermediate**.** Next epoxy-oxazoline is synthesised by addition of 3-acetoxy- 2-methylbenzoyl chloride **18**. Epoxide ring is opened by introducing perhydroisoquinoline **13** and then ring-opening of an oxazoline by introduction of the thiophenol moiety. This gives the final product nelfinavir **1** (Scheme 11).[41]



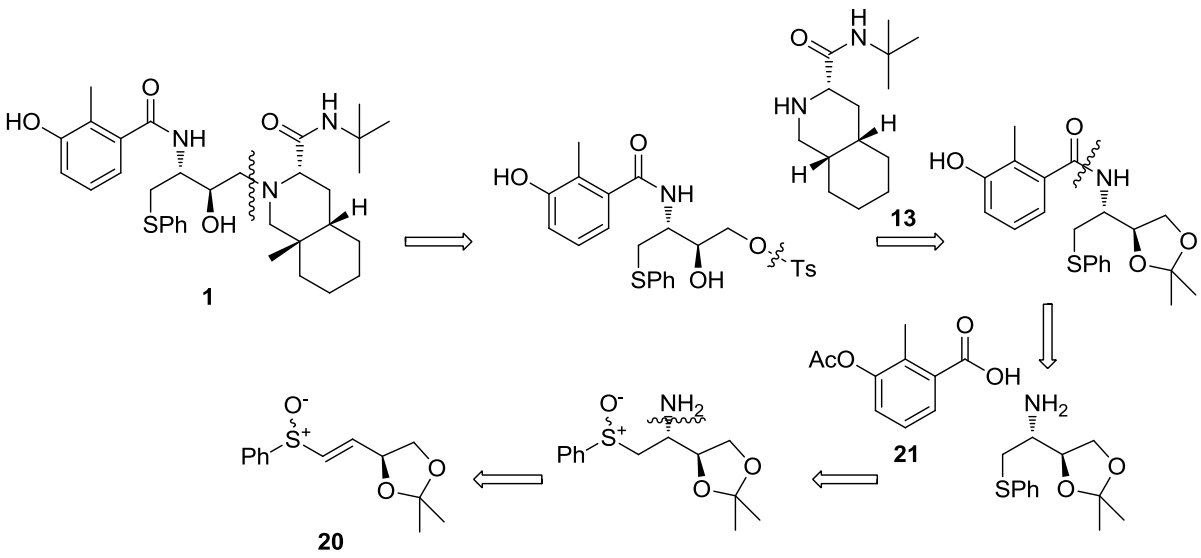
Scheme 11 - Retrosynthetic analysis of nelfinavir (1): Disconnection F.

Disconnection G – In this synthesis the required point is to synthesise amino alcohol, which is available from symmetric *meso*-epoxide **19** that is desymmetrised to afford azide, followed by reduction and hydrolysis. This prepared amino alcohol is treated with aryl acid chloride **18** forming amide and the desired oxazoline is obtained after cyclisation reaction on the amide. Following treatment of oxazoline with the perhydroisoquinoline **13** and then thiophenol group, gives nelfinavir **1** (Scheme 12).[42]



Scheme 12 – Retrosynthetic analysis of nelfinavir (1): Disconnection G.

Disconnection H – One synthetic method presented as a last step reaction with decahydroisoquinoline **13**, while the 3-hydroxy-2-methybenzoic acid moiety **11** was attached already. The group tried to obtain the required stereochemistry by attachment of amines to α,β-unsaturated sulfoxide **20**, using the Michael addition reaction. Then reduction of sulfoxide to sulfide, coupling with 3-acetoxy-2-methybenzoic acid **21** and hydrolysis afforded the diol. Final treatment of this diol with tosyl chloride followed by coupling with the amine **13** gave nelfinavir **1** (Scheme 13).[43]



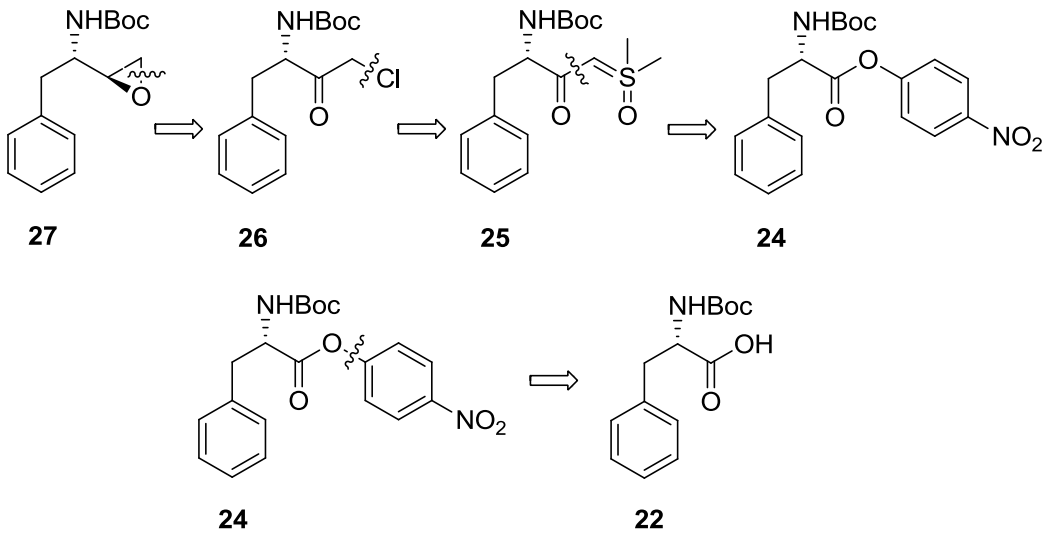
Scheme 13 – Retrosynthetic analysis of nelfinavir (1): Disconnection H.

3.2 APPROACH TOWARDS THE SYNTHESIS

Most of the approaches described above to obtain nelfinavir suffered from a few drawbacks. These included the need for expensive reagents and starting materials, a complex synthesis which required numerous steps and reactions that are not fully controlled in terms of regio- and stereochemistry.[40] Added to this, we required the replacement of the thiophenyl group with an indole and phenyl group in order to reach the desired targets **2** and **3**. In this project, to replace the thiophenyl group of nelfinavir **1** the two starting materials L-phenylalanine and L-tryptophan have been chosen considering the knowledge of the chymotrypsin-like activity. It is known that this enzyme in its active site has hydrophobic pocket where it binds to the particular amino acids phenylalanine and tryptophan which in their sidechain contain hydrophobic aromatic rings – phenyl and indole ring respectively. This transformation might increase the binding strength between compound and enzyme active site and increase the inhibitory effect compared to the nelfinavir **1**.

This change would be only a slight modification to most of the aforementioned syntheses but in others could be much more difficult. Upon consideration, it was decided that the most suitable method to achieve the desired targets is the one similar to the original epoxide-based route.[1] It has proven before to be successful in the use of protected L-phenylalanine amino acid as a starting material, which would be crucial during the early stages of this work.

In the aforementioned disconnection B (Scheme 7), diazomethane is used as one of the reagents.[1] A big advantage of our chosen route by Wang and Nugent is that there is no need to use this chemical, as it is a highly hazardous toxic and explosive gas.[44] They reported that activated *N*-Boc-protected amino acid L-phenylalanine **22**, by treatment with 4-nitrophenol **23** provided Boc-L-phenylalanine–4-nitrophenol ester **24** that can then react with a sulfoxonium ylide, affording the dimethylsulfoxonium butylide **25**, through nucleophilic attack.[44] It has been shown reacting a product such as **25** with an acid chloride gives chloroketone **26**. Using a bulky hydride donor they formed the desired anti-α-amino epoxide **27** with good yield and diastereoselectivity (Scheme 14).[44]



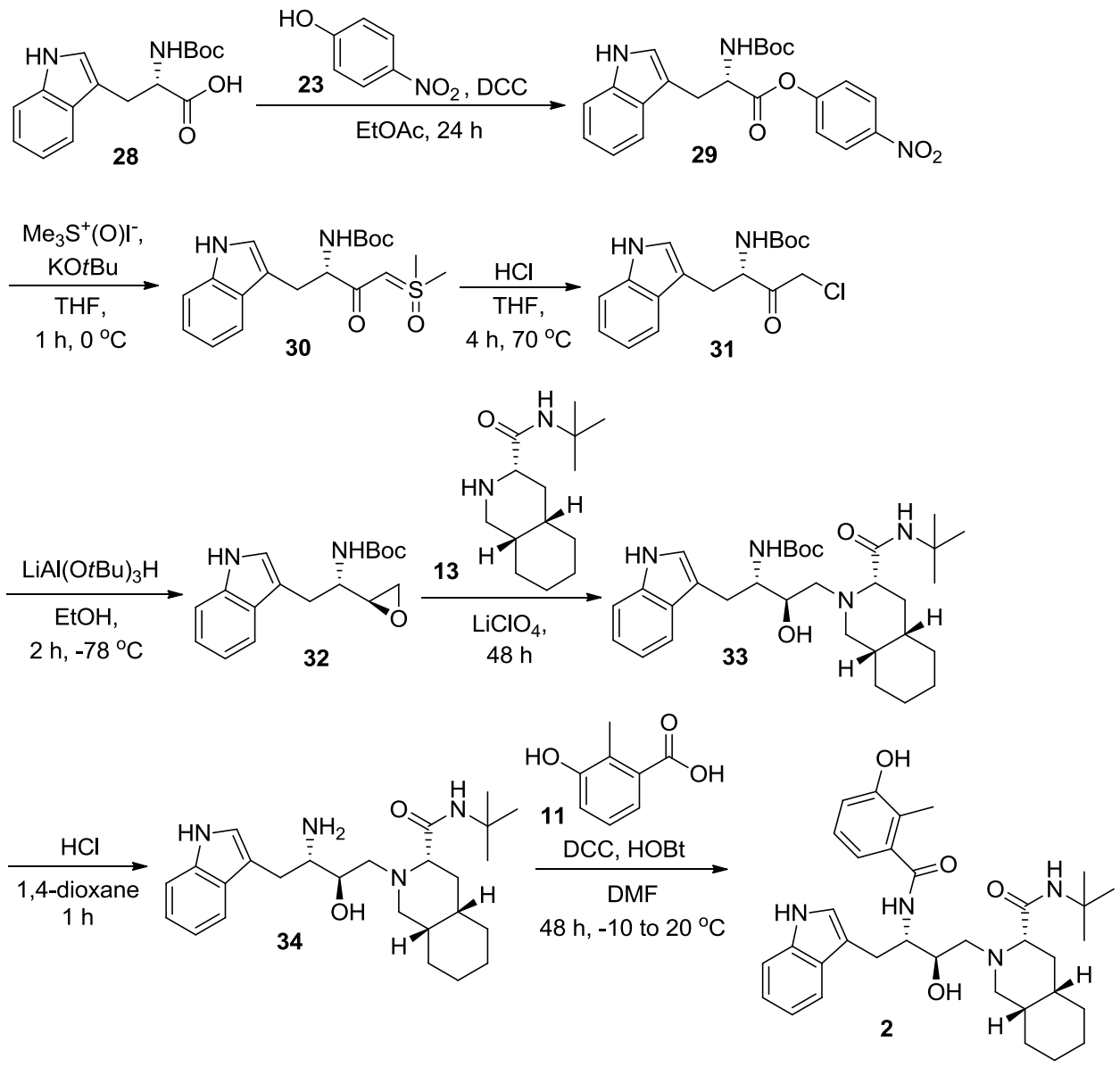
Scheme 14 – Retrosynthesis of anti-α-amino epoxide 27 by Wang and Nugent and esterification of *N*-Boc-L-phenylalanine (22) with *p*-nitrophenol (23).[44,57]

In order to prepare target compound **2**, *N*-Boc-L-tryptophan amino acid **28** was to be used instead of *N*-Boc-L-phenylalanine **22**.

The epoxide can be then be opened with decahydroisoquinoline derivative **13** (commercially available) that results in the formation of tertiary amine **33**. Subsequent Boc removal with 4 M HCl in 1,4-dioxane affords the free amine **34** ready for final amide **35** formation. This last step can proceed according to original work for nelfinavir **1** coupling amine with acid 3-hydroxy-2-methylbenzoic acid **11** (Scheme 15).[1]

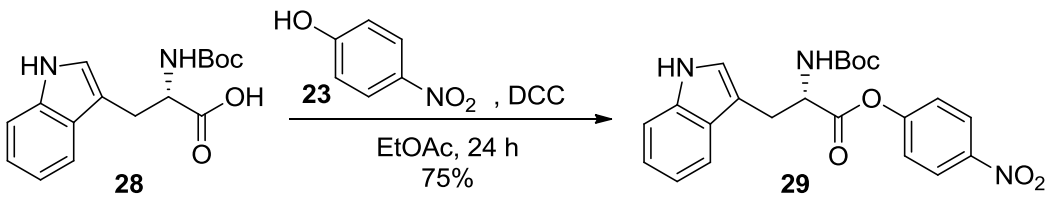
As an idea for making a broad range of nelfinavir analogues, different amines and acids (or acid derivatives) other than decahydroisoquinoline derivative **13** and 3-hydroxy-2-methylbenzoic acid **11** respectively can be used.

The whole process can be divided into three stages; the synthesis of the anti- α-amino epoxide, the opening of the epoxide with different amines, and finally, the coupling of different acids to afford a variety of amides.



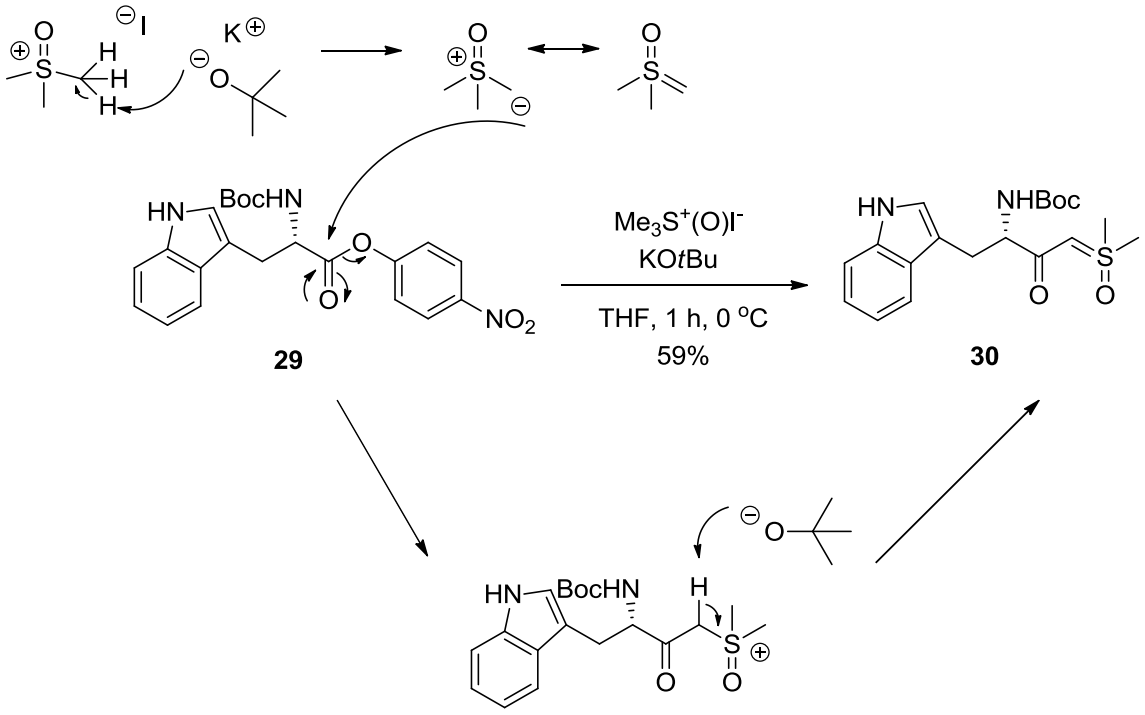
Scheme 15 – Proposed synthesis of nelfinavir analogue 2.

The method used in this first step of the synthesis is an ester formation using DCC as a coupling reagent, which gave very good yield (scheme 16).[44] The reaction mixture was allowed to stir for at least five times longer (7.5 h vs 1.5 h) than that reported in the paper, as after several trials it was found to give a better yieldConsidering the subsequent reactions and reagents which were to be used, an electron-withdrawing group such as Boc seemed to be the most suitable, as it needs acidic conditions to be removed. In this coupling, a by-product is produced: dicyclohexyl urea, which is an insoluble solid, removable by filtration.



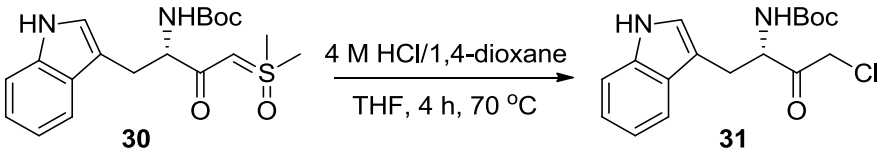
Scheme 16 – Synthesis of the ester 29

In the second step (Scheme 17), to prepare **30**, trimethylsulfoxonium iodide is deprotonated in the presence of KO*t*Bu in THF while refluxing for 2 hours producing dimethylsulfoxonium methylide.[44] This intermediate, after being cooled, attacks the ester **29** to form, a carbon – carbon bond, which is the key point to this reaction, as the mechanism indicates below. The product is a stable ylide while removal of co-product is accomplished by extraction with ethyl acetate (leaving excess nitrophenol from the previous step in the aqueous layer) and washing with distilled water, $NaHCO_3$ and brine. Attempted purification by flash chromatography led to degradation of the product, so the product was used in the next step without further purification. The yield for this reaction is not very accurate due to impurities such as nitrophenol **23**, difficult to remove completely by only extraction.



Scheme 17 - Synthesis of the dimethylsulfoxonium 30

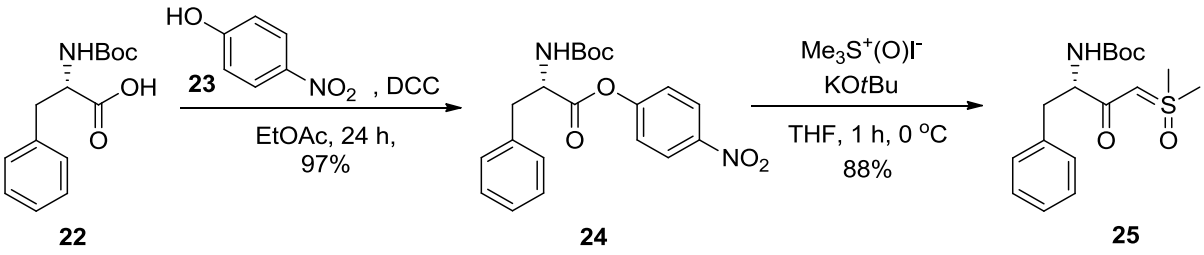
In the next step, the formation of chloroketone **31** (Scheme 18) by nucleophilic substitution was attempted. The hydrochloric acid treatment of dimethylsulfoxonium butylide **30** was supposed to form α-chloroketone **31** and as co-product DMSO.[44] However during this reaction our compound decomposed. The possible reason is that the literature method is based on phenylalanine whereas we have the electron rich indole ring present in tryptophan.[44] This reaction failed to give desired product.



Scheme 18 – Attempted synthesis of the chloroketone 31

In accordance with this decision we decided to carry on this general idea for the project with a different amino acid as a starting material – phenylalanine **22**.

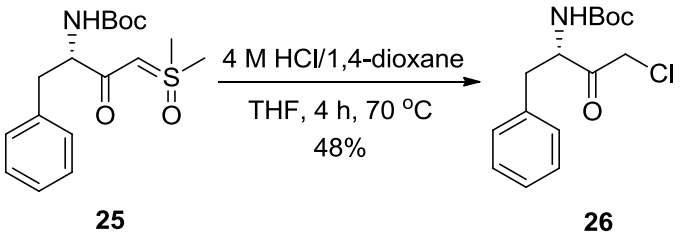
The first two steps (Scheme 19) proceeded in the same way as for the tryptophan compound, forming **24** and **25**. After the first reaction to achieve the *p*-nitrophenyl ester, recrystallisation from ethanol was used to obtain pure compound.[44]



Scheme 19 – Synthesis of the ester 24 and the dimethylsulfoxonium ylide 25

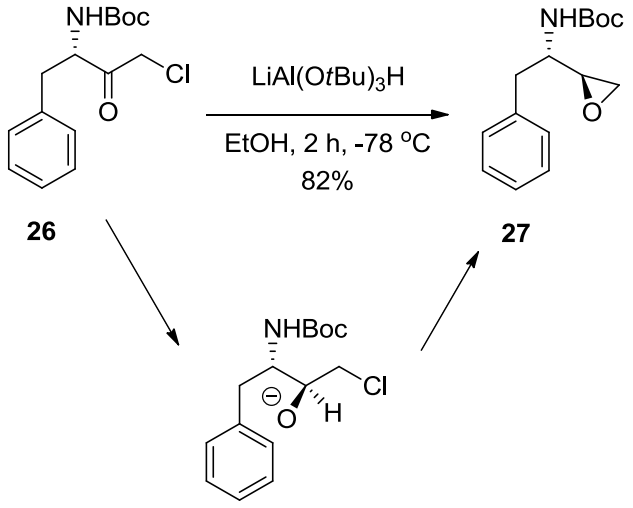
Unfortunately after the second step, compound **25** was impossible to purify completely and was used crude in the next reaction. Formation of α-chloroketone **26** (Scheme 20) by treatment of **25** with acid in this case was successful.[44] To remove DMSO from the mixture extraction with ethyl acetate and hexane (1:2) and further washing with distilled water, $NaHCO_3$ and brine were employed. The resulting product was

recrystallized from hot hexane.[44] In the conversion of **25** to **26**, similar acidic conditions used for Boc deprotection are employed, but in this case deprotection does not occur. It is probably because the chloroketone group is more reactive and the reaction is stopped right after completion, monitored by TLC, so that no further reactions can take place. Further Boc deprotection during this reaction might explain the low yield of this reaction.



Scheme 20 – Synthesis of the chloroketone 26

Then, the α-chloroketone was reduced to form epoxide **27** (Scheme 21) with a good yield (82%). Running this reaction with LiAl(O*t*Bu)$_3$H ensures high *anti*-selectivity, due to its bulky structure.



Scheme 21 – Synthesis of the epoxide 27

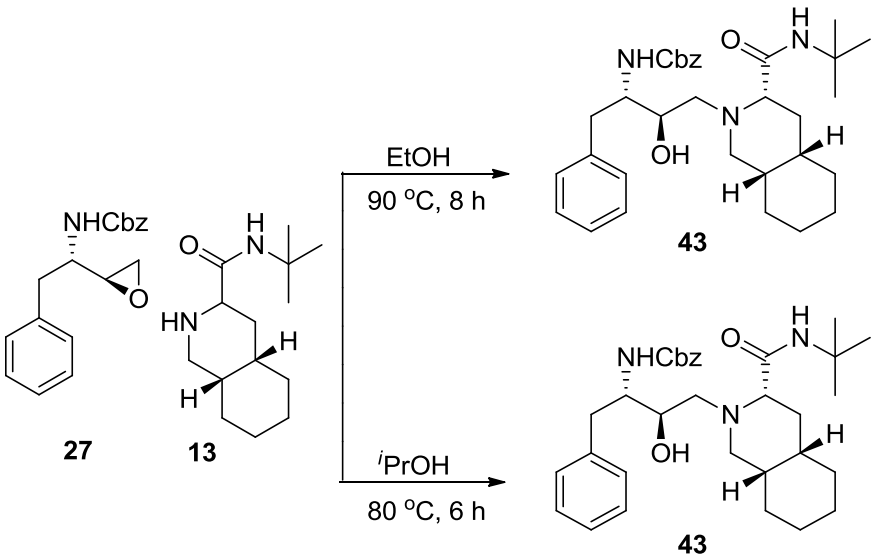
As the mechanism indicates, the reaction proceeds via formation of the intermediate chlorohydrin. Final epoxide-ring closure happens during work up with use of basic Rochelle salt solution. Substitution of this solution with water will result in obtaining

chlorohydrin intermediate.[44] It is also important to use dry ethanol for this reaction as LiAl(O*t*Bu)H forms hydroxides upon reaction with water.[47] Also as this reagent is added dropwise, very slowly at -78 °C, ice can stop homogenous stirring and mixing of the reaction solution. In addition, Rochelle salt solution is used to break aluminium-containing co-products to simplify work-up by extraction.[44]

It is important to notice that this product is acid sensitive. It is impossible to purify it by flash column chromatography and it can only be purified by recrystallization. Yields obtained were similar to those reported by Wang and Nugent.[44]

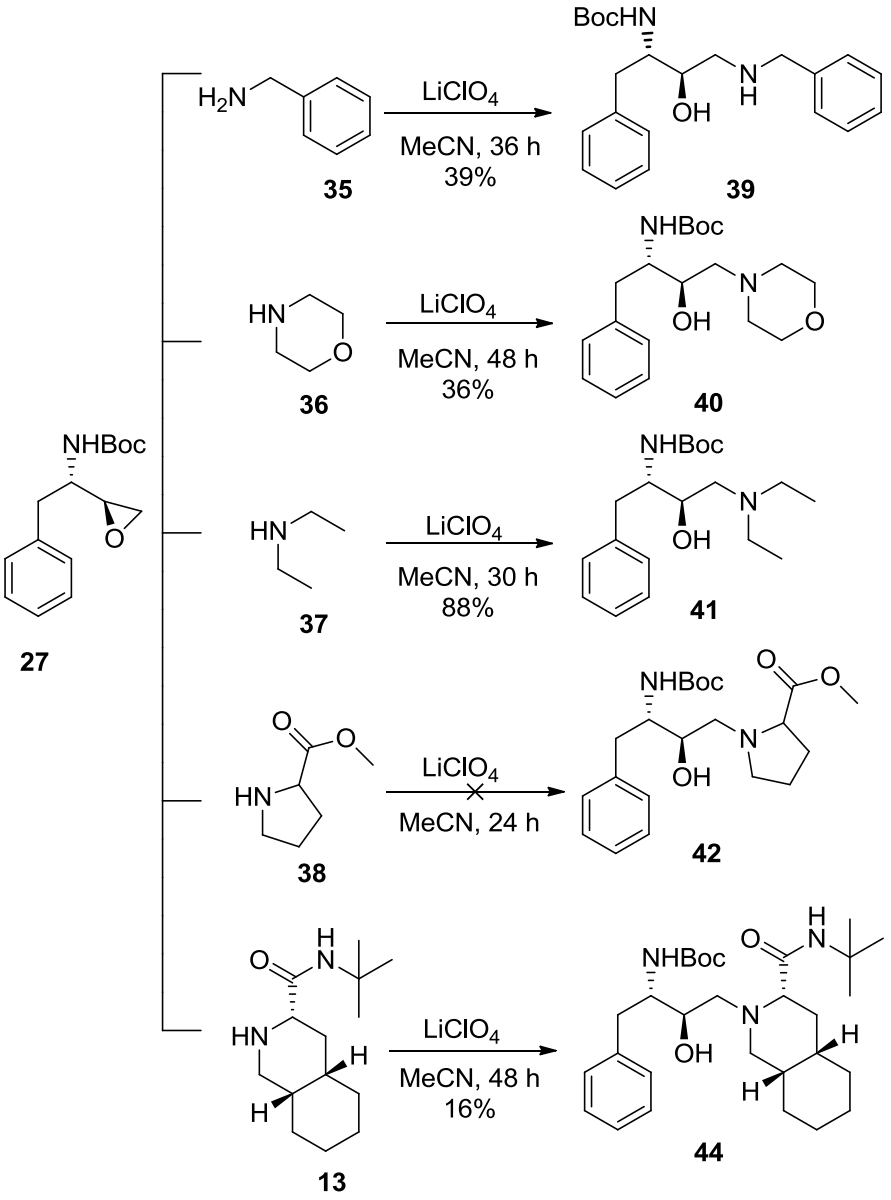
Successful completion of the first stage of the project – having a substantial amount of desired epoxide compound **27**, moved us to the second stage - the opening of the epoxide with different amines. This is a very good way to make a library of diverse analogues, as it gives us a wide variety of choices between different amines that could give us bigger spectrum of different biological results in the end.

There were different methods used to synthesise **43** in previous publications. One of the original methods involves 8 hours of refluxing epoxide **27** and decahydroisoquinoline **13** solution in ethanol or 6 hours of reflux of the same substrates in 2-propanol (Scheme 22).[1,48] These reactions were performed though with a different protecting group attached to the amine – carboxybenzyl (Cbz).



Scheme 22 – Previously published synthesis of 43[1, 48].

Initially, it had been decided to use: decahydroisoquinoline **13**, benzylamine **35,** morpholine **36**, diethylamine **37** and L-proline methyl ester **38** to achieve respectively **44, 39, 40, 41** and **42** (Scheme 23).



Scheme 23 – The opening of epoxide 27 reactions with different amines 35, 36, 37, 38 and 13

Work of R. T. Lum *et al.* on the synthesis of new potent inhibitors of the S20 proteasome has shown the *C*-terminal benzyl amide derivative of the non-selective, traditional serine protease inhibitor - ALLN (Ac-Leu-Leu-Nle-H), resulted in very selective

competitive inhibitors of the chymotrypsin-like activity of the 20 proteasome.[49] This makes the choice of benzylamine **35** a suitable nucleophile for opening of the epoxide in this work.

Different methods of the epoxide **27** opening were published in the literature. In work presenting the synthesis of the C_2-symmetric aminodiol protease inhibitor drugs, solution of epoxide **27** and benzylamine **35** in acetonitrile was stirred with $LiClO_4$ at room temperature for 22 hours.[50] In my case, the reaction was not going to completion in the suggested time, and the reaction mixture was stirred for another 12 hours obtaining desired **39**. The product did not require any further purification as it precipitatedduring quenching with distilled water. The yield obtained in this case was not as good as the one published in article (39% vs 96% respectively) but this might Happen because the recrystallization was not optimazed. [50]

To test the utility of this method used for benzylamine **35**, it has been decided to perform three other reactions with amines **36**, **37**, and **38** following the same procedure. Synthesis of compound **40** and compound **41** were never attempted before to the best of our knowledge. Morpholine and diethylamine were stirred in acetonitrile with $LiClO_4$. In both cases, a long reaction time (over 30 hours) was needed to bring the reaction to completion. (Scheme 23) The advantage is that no additional purification was needed for both compounds. Morpholine derivative **40** was simply collected by filtration, as the desired product **40** crashed out, in a pure form, from the reaction solution upon quenching with distilled water and a little addition of diethyl ether. Diethylamine derivative **41** also crashed out while quenching the reaction with distilled water, giving an off yellow solid. Yield of the reaction producing **41** can be considered as absolutely satisfactory – 88%. This cannot be said about morpholine derivative **40** with moderate yield – 33%. The other disadvantage is the excessive (5 equivalent) use of the required amines. To assure the highest yield of the reactions, five equivalents of each amine is required, which increases the cost of production.

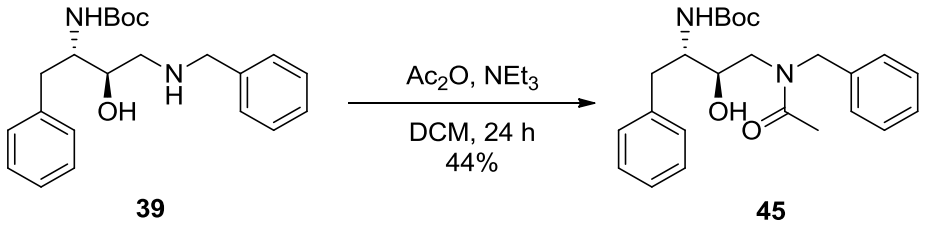
These three experiments, giving compounds **39**, **40** and **41** proved to be successful for the epoxide ring opening reaction, with both primary and secondary amines. To keep cohesion in methodology and for simplification of the previously mentioned methods (Scheme 22) without need of reflux, the same procedure was used for *N*-Boc aminol **44** synthesis (which also has never been attempted via this approach). To avoid

unnecessary use of very expensive secondary amine **13**, only 1.5 equivalents were used in the reaction (Scheme 23). Also unreacted excess of amine **13** can be recovered from the mixture by flash column chromatography or by acidic extraction. This reaction gives fairly good yield – 51% of crude product but unfortunately only 16% yield of pure product after flash column chromatography.

One more attempt was performed following the $LiClO_4$ method, with L-proline methyl ester hydrochloride **38**. As this ester is stored as a salt, it had to be neutralised using a base before the epoxide ring opening was then carried out. Surprisingly this trial did not give the expected product (Scheme 23)**.** The reason for this failure could be the need for neutralisation of the ester **38** before putting the reagent to the reaction, while here it was attempted to be neutralised in the reaction solution by additional input of 1 equivalent of base. This reaction was not repeated due to lack of time.

Epoxide **27** ring opening reaction with benzylamine **35** (Scheme 23) gave aminol **39** with a secondary amine. It is necessary to protect this amine before proceeding with deprotection of the Boc, to prevent undesired reactions in the following steps, by competition with free primary amine. To do so, acetylation of secondary amine group on a similar compound, reported, was repeated to obtain novel compound **45**, with yield 44% (Scheme 24).[51,86]

This compound can undergo deprotection reaction, however, due to a lack of time, this has not been done.



39 **45**

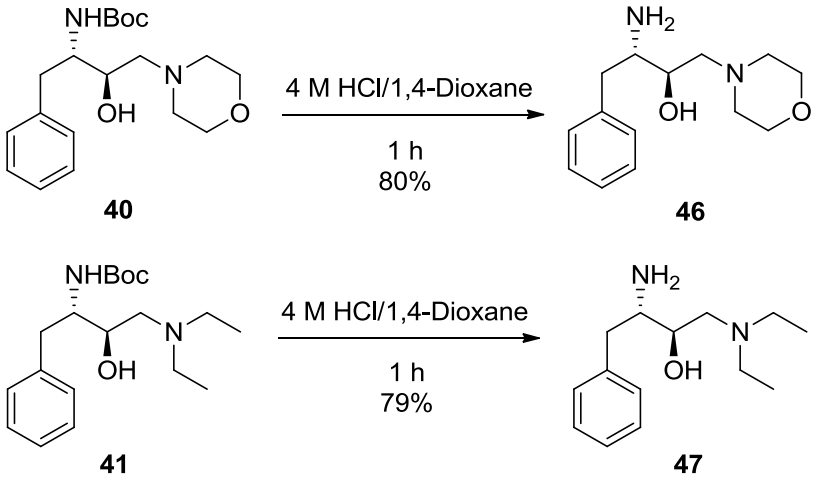
Scheme 24 – Synthesis of the acetylated benzylamine derivative 45

Boc is a stable protecting group toward most nucleophiles and bases, cleaveable under strongly acidic conditions, producing *tert*-butyl cation as a co-product. The most common ways for deprotection are the use of 4 M solution of HCl in 1,4-dioxane or trifluoroacetic acid (TFA).[52,53] It has been shown before that deprotection through

reflux very often leads to by-products with sensitive compounds, so it is more favoured to use methods that do not require heating.

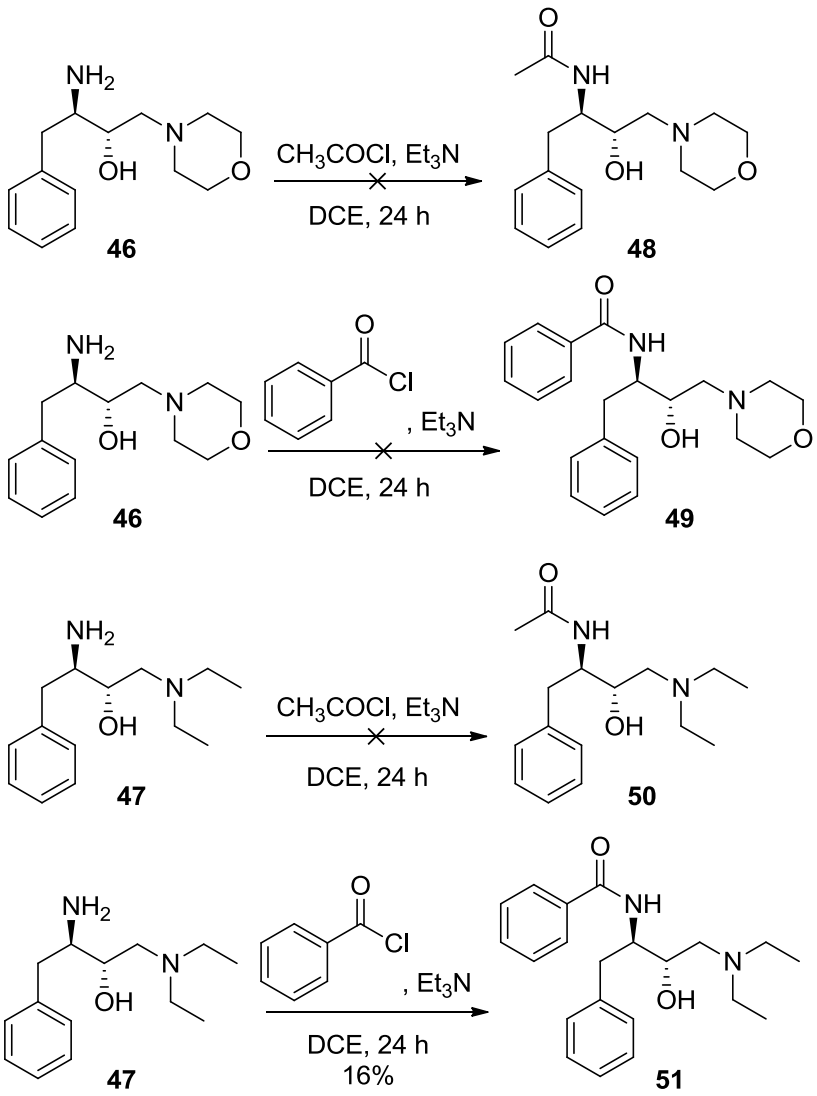
The method we used is a classic procedure for Boc-group deprotection used in many peptide syntheses.[54] Protected amine is stirred with 4 M HCl solution in 1,4-dioxane until Boc group is entirely removed from the compound, as observed by TLC. Boc cleavage has been worked up in two ways. After completion of the reaction, mixture was evaporated and then co-evaporated with acetonitrile and chloroform, followed by basic extraction to neutralise the formed HCl salt to give the free amine. In the other approach, product after evaporation was put straight to the next reaction. This requires the addition of extra base in the next reaction in order to neutralise the formed salt and to enable the reaction to proceed.

Reactions on the prepared compounds **40** and **41** were carried out according to the explained procedure, affording pure amines **46** and **47**, with 80% and 79% yield respectively (Scheme 25).



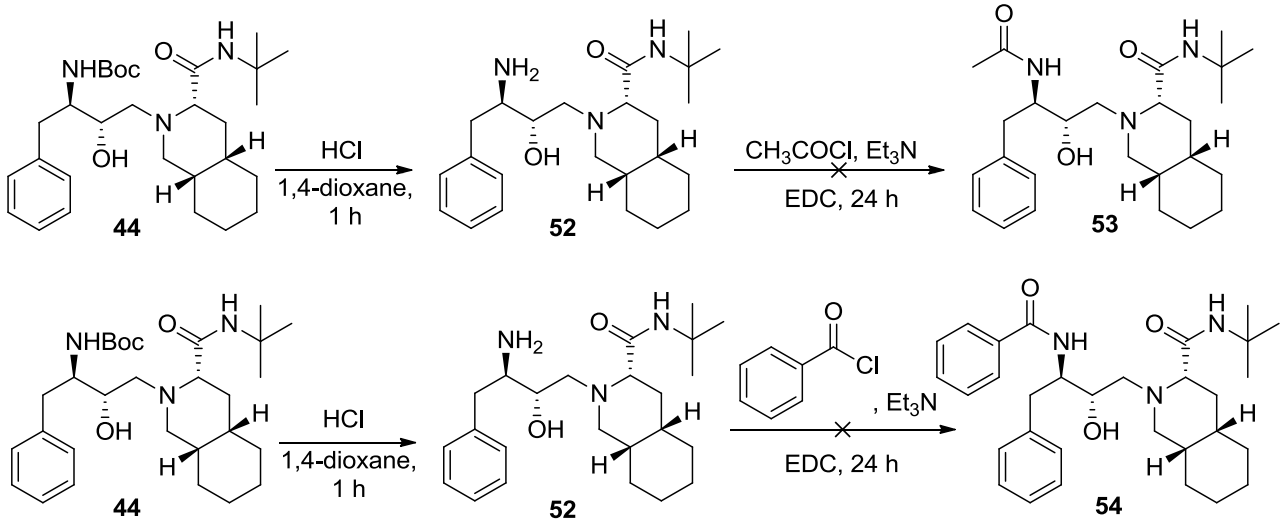
Scheme 25 – Deprotection reactions on compounds 40 and 41.

The final step of the synthesis was coupling of the deprotected aminols **46** and **47** with carboxylic acid derivatives. It has been chosen to use acetyl chloride and benzoyl chloride to complete these final steps. Unfortunately only one of them – the coupling of **47** with benzoyl chloride gave the desired product **51**, while three remaining reactions to produce **48**, **49** and **50** failed (Scheme 26).



Scheme 26 – Attemps to synthesis of nelfinavir analogues amides 48, 49, 50 and 51

Further steps on **44** were also attempted. *N*-Boc aminol **44** was deprotected in HCl conditions and straight after freeing the amine group from *N*-Boc protection (monitored by TLC), the coupling of amine **52** was attempted, also with acetyl chloride and benzoyl chloride. These two trials also didn't seem to give any of desired compounds **53** and **54** respectively (Scheme 27).



Scheme 27 – Attemps to synthesis nelfinavir analogues amides 53 and 54

Due to the pressure of time, no more syntheses were performed. Regarding all these reactions that did not happen to work and produce final amides, there are different methods that could be used to achieve desired compounds. Acyl chlorides and benzoyl chloride could be replaced with anhydride derivatives, for example acetic anhydride and benzoyl anhydride which are also very reactive reagents. All of them are unstable in presence of water, being hydrolysed to their carboxylic acids – acetic acid and benzoic acid respectively, which makes dry reaction conditions a priority.

Also in original work for nelfinavir **1** the final step of amide formation is performed with the carboxylic acid **11** using DCC and 1-hydroxybenzotriazole hydrate (HOBt) as coupling reagents in DMF.[1] For this work these conditions could be used also for the amide formation with acetic acid and benzoic acid giving more chances for the successful synthesis.

3.3 BIOLOGICAL TESTING OF NELFINAVIR ANALOGUES

In medicinal chemistry and drug discovery, after identification of potential drug targets, synthesis and characterisation, biological assays for therapeutic efficacy take place. These are the experiments to assess the effect of the synthesised chemicals on the target and give information on the validity of the strategy used.

To complete this project, an MTS colourimetric assay was performed using THP-1 human leukemia cells. It is a proliferation assay to measure the number of viable cells after their interaction with applied compounds. The test is based on the bioreduction by cells of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] into formazan product, a coloured compound which is soluble in cell culture medium (Figure 8). For the clarity of this method the number of living cells in culture is directly proportional to the quantity of formazan product (measured by the absorbance).[55]

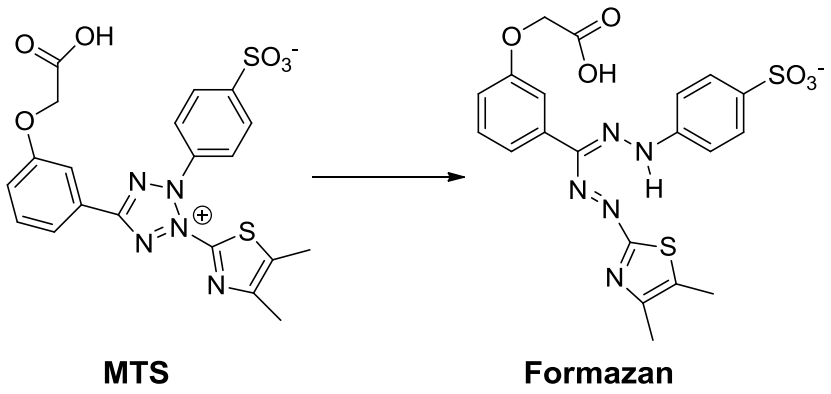


Figure 8 - Structures of MTS tetrazolium and its formazan product.

The results from the MTS assay showed that the morpholine derivative **40** and acetylated benzylamine derivative **45** have no anti-proliferative activity (>1 mM) in THP-1 cells. Moderate activity was found with benzylamine derivative **39**, diethylamine derivative **41** and decahydroisoquinoline derivative **44**, giving an IC_{50} of 34 μM, 37 μM

and 33 μM respectively. Interestingly the only compound without *N*-Boc protecting group tested in this assay showed the best result – 2.8 μM. This would appear to be a key characteristic in finding potent compounds in future work (Table 1).

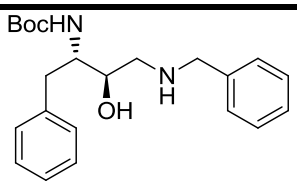
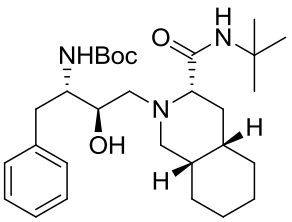
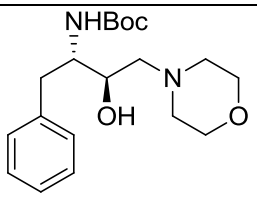
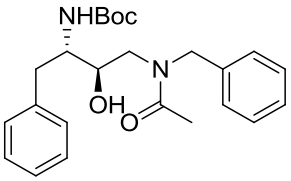
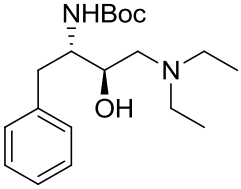
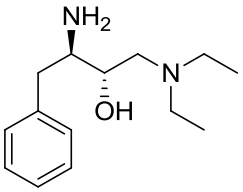
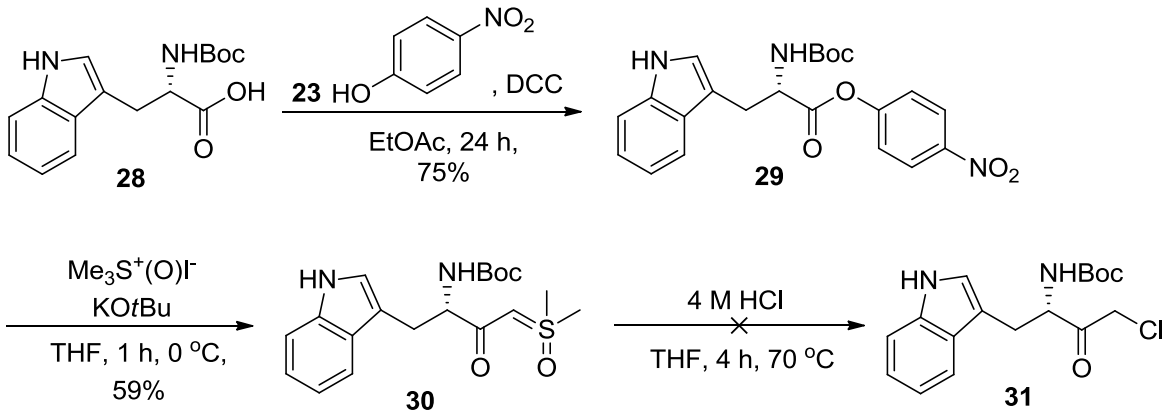
Compound	Structure	IC_{50}	Compound	Structure	IC_{50}
39		**34 μM**	**44**		**33 μM**
40		**>1 mM**	**45**		**>1 mM**
41		**37 μM**	**47**		**2.8 μM**

Table 1 – Results of MTS assay – IC_{50} of compounds 39, 40, 41, 44, 45 and 47.

4. CONCLUSIONS

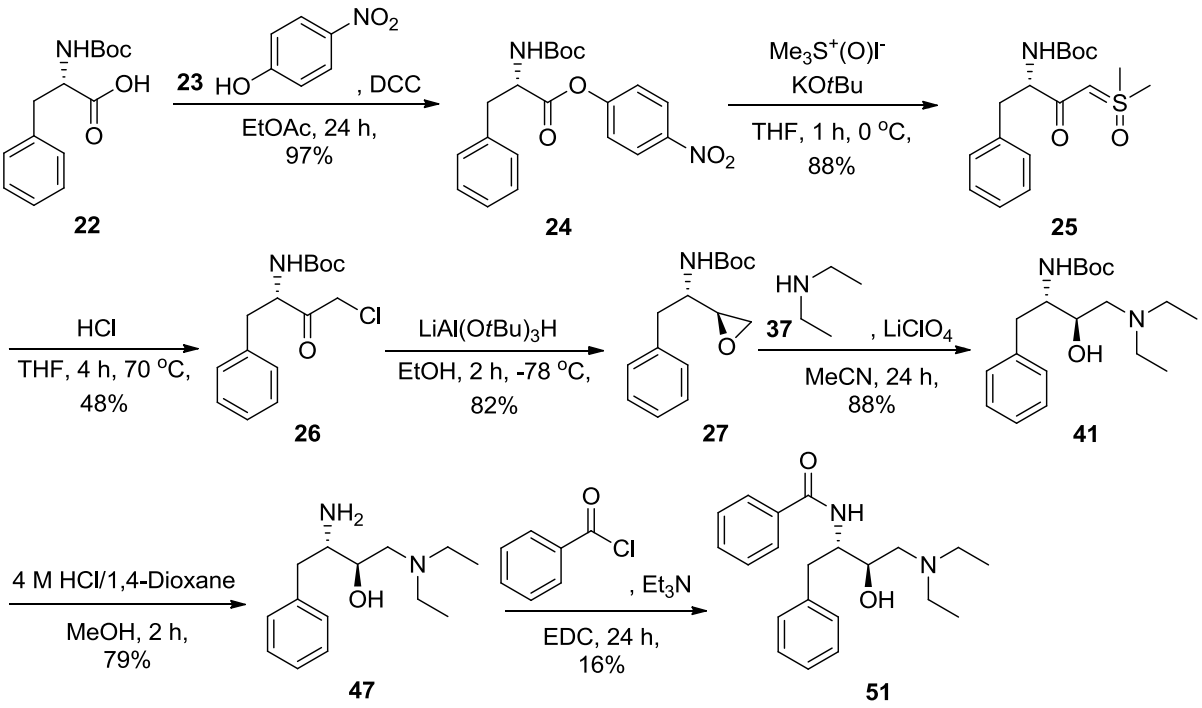
A preliminary attempt to synthesise nelfinavir **1** derivative **2** failed during the early stages of the synthetic route. After successfully obtaining the first two products: 4-nitrophenol ester **29** and dimethylsulfoxonium derivative **30,** synthetic investigations to produce the chloroketone derivative **31** did not yield the desired product. This is possibly due to the substitution phenyl group with indole (Scheme 28).[44]



Scheme 28 – Unsuccessful approach to Wang and Nugent method used for tryptophan-based analogue 2.

The synthesis towards the second derivative of nelfinavir **1** – compound **3,** proved this methodology to be more successful, finally forming the amide **51** (Scheme 29). The esterification reaction between phenylalanine **22** and *p*-nitrophenol **23** yielded the desired 4-nitrophenol ester **24**, with a very pleasing yield of 97%. The next 3 steps, following the procedures suggested by Wang and Nugent, proved this method to be useful, giving the desired dimethylsulfoxonium derivative **25**, chloroketone derivative **26** and finally the epoxide **27**, all with very satisfactory yields – 88%, 48% and 82% respectively.[44] The next stage of the synthesis was the epoxide ring opening using different primary and secondary amines. All the attempted reactions were performed according to the same lithium perchlorate-mediated method, not used previously for this group of compounds.[50] Out of the five trials, using the benzylamine **35**, morpholine

36, diethylamine **37**, L-proline methyl ester **38** and finally decahydroisoquinoline derivative **13**, four gave the expected products, while that with **38** failed.



Scheme 29 – Overall synthetic route for the formation of nelfinavir analogue 51.

The protection of the secondary amine group of benzylamine derivative **39** was achieved with an acetylation reaction, giving novel compound in this synthesis.[51]

Boc deprotection was successfully performed on compounds **40**, **41** and **44**, providing next novel compound **47** to the best of our knowledge in this work.

For the final stage of this synthesis, six different coupling reactions between synthesised aminols **46**, **47** and **52** and two carboxylic acid derivatives acetyl chloride and benzoyl chloride were performed. Unfortunately only one out of all six produced the expected product **51**. Further methods were not attempted due to the lack of sufficient time.

As a final study for this project, a biological assay was carried out on six synthesised compounds **39**, **40**, **41**, **44**, **45** and **47** (Figure 9). TheMTS colourimetric assays on THP-1 leukaemia cells, gave interesting results; especially for deprotected diethylamine derivative **47**, showing its IC_{50} – 2.8 μM, which is a very promising outcome and places particular attention on developing this structure for future work.

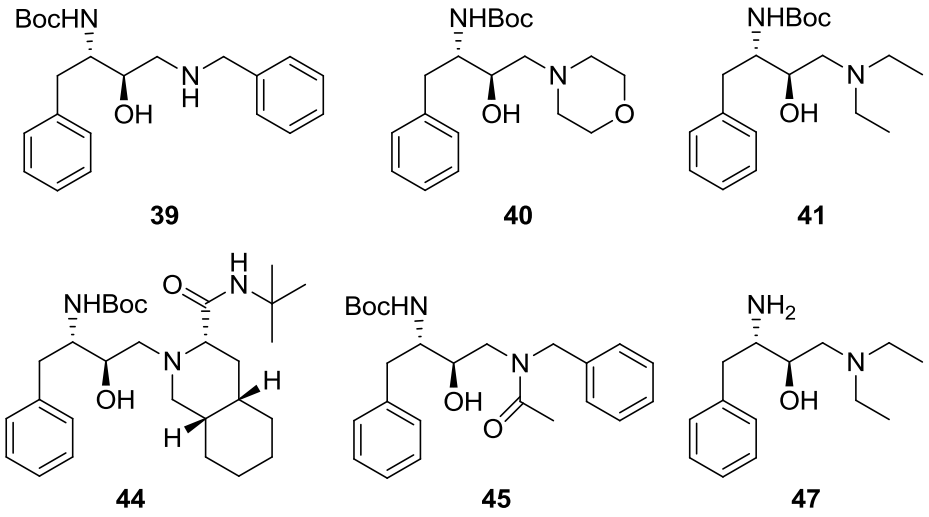


Figure 9 – Structures of compounds 39, 40, 41, 44, 45 and 47 tested biologically in MTS assay.

5. FUTURE PERSPECTIVES

The final, unsuccessful stage of the synthesis performed in this work using two types of acyl chloride – the acetyl chloride and the benzoyl chloride, places importance on developing other methods to perform these reactions in future as they are the key point throughout the synthesis. Utilisation of different acid derivatives such as acid anhydrides, which are very reactive reagents, might bring the reaction to completion and yield the desired amides. Use of other methods for coupling carboxylic acids with the amine group, such as the application of dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole hydrate (HOBt) or benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP) could give expected compounds in future. Also implementation of different carboxylic acids or acid derivatives would produce a more diverse range of compounds ready for screening.

During the second stage of the synthesis – the epoxide ring opening – a wide range of different amines could be used to extend the variety of formed structures. Different alkyl, cyclic, aromatic or asymmetric amines as well as others with aromatic heterocycles, would be an excellent idea to broaden the library of nelfinavir **1** analogues.

To protect the secondary amine group formed by the attachment of benzylamine **35** to the epoxide **27**, an acetylation reaction was performed. This nitrogen could be also protected through methylation, producing another derivative.

Different amino acids could also be employed in the first step of this synthesis, to widen the diversity of analogue compounds. Considering the existing knowledge of the proteasome structure it would be preferable to choose amino acids with a hydrophobic character such as isoleucine or leucine to increase the possibility of good interaction.

The MTS assay on THP-1 cell line with six of the synthesised compounds in this project showed the most attractive results for the aminol **47**, the only one having undergone *N*-Boc deprotection. For this reason it would be interesting to do more tests on deprotected compounds. It also seems sensible to develop the synthesis of **47** for future biological tests, as the diethylamine attached instead of decahydroisoquinoline

derivative which would make the compound much smaller in molecular weight which is a desirable feature in drug discovery. As nelfinavir **1** has not been tested on this type of cell, it would be worthwhile to check its activity in this cell line for pure comparison and assessment of worthiness of the synthesis for the future continuation of drug discovery.

6. EXPERIMENTAL

6.1 GENERAL EXPERIMENTAL

The reagents and solvents were purchased from Sigma-Aldrich, Fisher Scientific, Alfa Aesar and Novabiochem. The anhydrous solvents were bought from Aldrich as sure-seal bottles. Anhydrous THF was freshly distilled from sodium.

Reactions were monitored by thin layer chromatography (TLC) using pre-coated aluminium backed sheets of silica. The spots were visualised with UV light and a potassium permanganate stain. Column chromatography was carried out using silica gel (MN Kieselgel 60, 40-63 μm 230-400 mesh ASTM).

Melting points were determined using an Electrothermal melting point apparatus.

Optical rotation analysis was performed on a polarimeter Optical Rotation PolAAr 3001 instrument or polarimeter ADP220 (Bellingham and Stanley) in HPLC grade methanol.

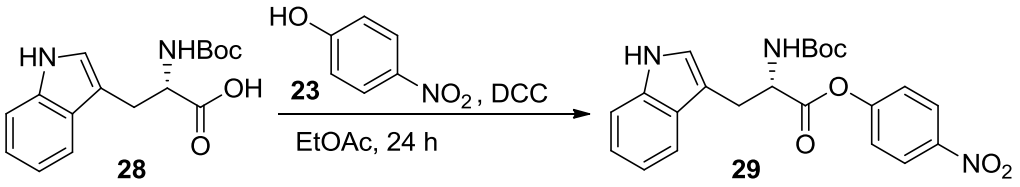
Infrared spectra were recorded using a Thermo Nicolet 380 FT-IR spectrometer with a Smart Orbit Golden Gate attachment. Absorptions are reported in wavenumbers (cm^{-1}).

NMR spectra were taken on a Bruker AV-300 or Bruker DPX-400 instrument (300 or 400 MHz ^{1}H, 75 MHz ^{13}C), in $CDCl_3$ unless otherwise noted. The chemical shifts data are reported in ppm. Multiplicity is described by: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; m, multiplet; app., apparent; r, roofing and br, broad.

Electrospray ionisation MS (ESI-MS) was undertaken on a Micromass Platform II instrument with a quadropole detector, using acetonitrile as solvent.

6.2 EXPERIMENTAL

(2*S*)-4-Nitrophenyl 2-((*tert*-butoxycarbonyl)amino)-3-(1*H*-indol-3-yl)propanoate (29)

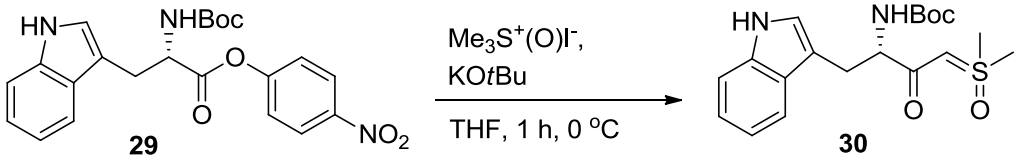


To *N*–Boc–L–tryptophan **28** (2.29 g, 7.5 mmol, 1.0 eq.) in EtOAc (30 mL) was added *p*-nitrophenol **23** (1.05 g, 7.5 mmol, 1.0 eq.) and dicyclohexyl carbodiimide (1.64 g, 7.9 mmol, 1.1 eq.). The reaction mixture was stirred for 15 hours and then filtered through Celite. The resulting clear solution with added hexane (55 mL) was washed sequentially with water (30 mL), $NaHCO_3$ solution (30 mL x 3) and brine (30 mL x 3), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting solid was azeotroped from chloroform, yielding **29** as a pale yellow solid (2.38 g, 75%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (9H, s), 3.33-3.54 (1H, m), 4.87 (1H, d, *J* = 6.6 Hz), 5.18 (1H, d, *J* = 7.0 Hz), 7.01 (2H, d, *J* = 1.0 Hz), 7.09-7.19 (2H, m), 7.21-7.26 (1H, m), 7.42 (1H, d, *J* = 8.1 Hz), 7.61 (1H, d, *J* = 8.1 Hz), 8.19 (2H, d, *J* = 9.1 Hz), 8.24 (1H, br s).

The spectroscopic data are consistent with that reported in the literature.[56]

(3*S*)-*Tert*-butyl (4-(dimethylsulfoxonium)-1-(1H-indol-3-yl)-3-oxobutan-2-yl)carbamate (30)

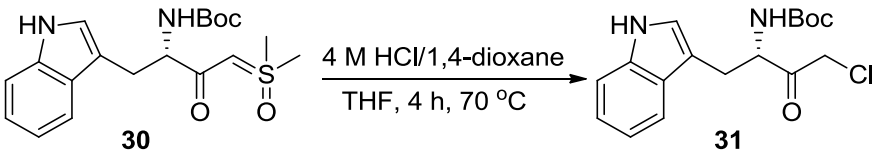


A 250 mL round bottom flask was charged with trimethyl sulfoxonium iodide (4.20 g, 19.2 mmol, 3.0 eq.) in anhydrous THF (11 mL) under a nitrogen atmosphere. To this, a solution of potassium *tert* – butoxide (2.15 g, 19.2 mmol. 3.0 eq.) dissolved in 11 mL of anhydrous THF was added. The flask was equipped with a condenser and heated to 70 ^{o}C for 2 hours. The solution was then cooled to 0 ^{o}C. Boc–L–Trp–4–nitrophenol **29** (2.72 g, 6.4 mmol, 1.0 eq.) in 7 mL of anhydrous THF was added dropwise to the cooled mixture over 15 min and allowed to stir for another hour. Then distilled water was added and stirred for a further 15 min and the solution was brought to room temperature. After filtration through Celite, the filtrate was concentrated *in vacuo*. The residue was next rinsed with distilled water (15 mL) and extracted with EtOAc (15 mL and 2 x 7 mL). The organic extract was washed with distilled water (2 x 5 mL), followed by $NaHCO_3$ solution (4 x 5 mL) and brine (4 x 5 mL) and concentrated *in vacuo*. The resulting solid was azeotroped from chloroform to yield **30** as yellow solid (1.43 g, 59%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.43 (s, 9 H), 3.04 (s, 1 H), 3.10 - 3.30 (m, 1 H), 3.22 (s, 1 H), 3.47 (s, 1 H), 4.38 (m, *J*=7.7 Hz, 1 H), 4.32 (s, 1 H), 5.36 (d, *J*=8.1 Hz, 1 H), 7.00 - 7.10 (m, 2 H), 7.15 (t, *J*=7.7 Hz, 1 H), 7.30 - 7.37 (m, 1 H), 7.63 (d, *J*=8.1 Hz, 1 H), 8.34 (br. s., 2 H).

The spectroscopic data are consistent with that reported in the literature.[56]

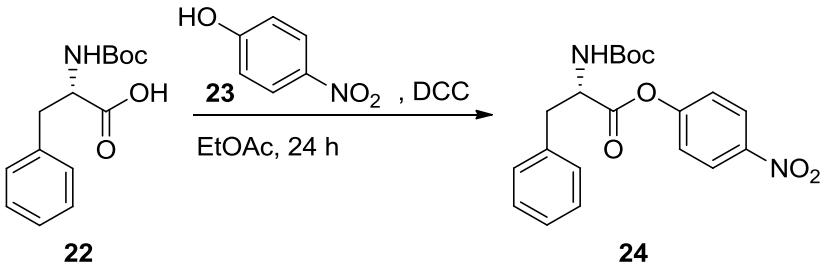
(2*S*)-*Tert*-butyl (4-chloro-1-(1H-indol-3-yl)-3-oxobutan-2-yl)carbamate (31)



30 31

In a 100 mL round bottom flask under nitrogen, were placed compound **30** (1.42 g, 3.8 mmol, 1.0 eq.) and THF (25 mL). A solution of hydrochloric acid in 1,4-dioxane (4.0 M, 0.62 mL, 4.4 mmol, 1.2 eq.) – was added dropwise over 5 minutes. The mixture was then allowed to reflux (70 °C), stirring for 4 hours. The reaction mixture was then cooled to room temperature, and mixed with hexane (11 mL) and EtOAc (5 mL) and washed with water (2 x 11 mL), followed by $NaHCO_3$ solution (5 mL), water (4 x 11 mL) and brine (5 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*, afforded a dark brown oil that turned out to be not the expected product.

(2*S*)-4-Nitrophenyl 2-((1,1-dimethylethoxy)methanamido)-3-phenyl propanoate (24)



22 24

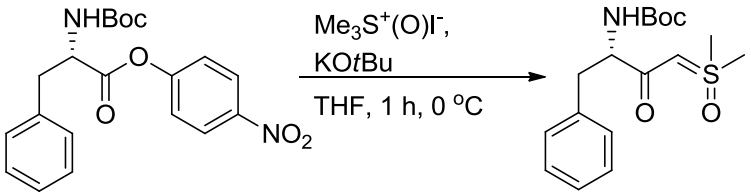
To *N*–Boc–L–phenylalanine **22** (6.00 g, 22.6 mmol, 1.0 eq.) in AcOEt (30 mL) were added *p*-nitrophenol **23** (3.15 g, 22.6 mmol, 1.0 eq.) and dicyclohexyl carbodiimide (4.91 g, 23.8 mmol, 1.1 eq.). The reaction mixture was stirred for 14 hours and then filtered through Celite. The resulting clear solution with added hexane (55 mL) was washed sequentially with water (30 mL), $NaHCO_3$ solution (30 mL x 3) and brine (30 mL x 3),

dried over $MgSO_4$, filtered and concentrated. The resulting solid was azeotroped from chloroform yielding **24** as an off white solid (8.50 g, 97%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.47 (s, 9 H), 3.24 (d, *J*=5.9 Hz, 1 H), 4.81 (d, *J*=1.0 Hz, 1 H), 5.05 (d, *J*=1.0 Hz, 1 H), 7.12 - 7.19 (m, 2 H), 7.23 - 7.29 (m, 3 H), 7.32 - 7.40 (m, 2 H), 8.27 (d, *J*=9.1 Hz, 2 H).

The spectroscopic data are consistent with that reported in the literature.[57]

(3*S*)-Dimethylsulfoxonium 3-(1,1-dimethylethoxy)methanamido)-2-oxo-4-phenylbutylide (25)



24 **25**

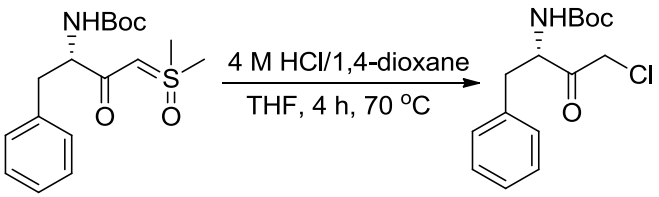
A 250 mL round bottom flask was charged with trimethyl sulfoxonium iodide (14.50 g, 65.8 mmol, 3.0 eq.) and anhydrous THF (66 mL) under nitrogen atmosphere. A solution of potassium *tert* – butoxide (7.40 g, 65.8 mmol, 3.0 eq.) dissolved in 66 mL of anhydrous THF was added to first solution. The flask was equipped with a condenser and heated to 70 °C for 2 hours. The solution was then cooled to 0 °C. A solution prepared by dissolving Boc–L–Phe–4–nitrophenol **24** (8.50 g, 22.0 mmol, 1.0 eq.) in 50 mL of anhydrous THF was added to the cooled mixture dropwise over 15 min and allowed to stir for another hour. Then distilled water was added and stirred further 15 min and the solution was brought to room temperature. After filtration through Celite, filtrate was concentrated *in vacuo*. The residue was next rinsed with distilled water (45 mL) and extracted with EtOAc (45 mL and 2 x 20 mL). The organic extract was washed with distilled water (2 x 15 mL), $NaHCO_3$ solution (4 x 15 mL) and brine (4 x 15 mL) and

concentrated *in vacuo*. Remaining solid was azeotroped from chloroform and evaporated to yield **25** as a light yellow solid (6.55 g; 92%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.41 (s, 9 H), 3.00 (d, *J*=1.0 Hz, 2 H), 3.26 (s, 3 H), 3.35 (s, 3 H), 4.25 - 4.38 (m, 1 H), 4.27 (s, 1 H), 5.23 (d, *J*=8.1 Hz, 1 H), 7.13 - 7.33 (m, 5 H).

The spectroscopic data are consistent with that reported in the literature.[40]

(2*S*)-4-Chloro-3-oxo-1-phenylbutan-2-yl 1,1-dimethylethyl carbamate (26)



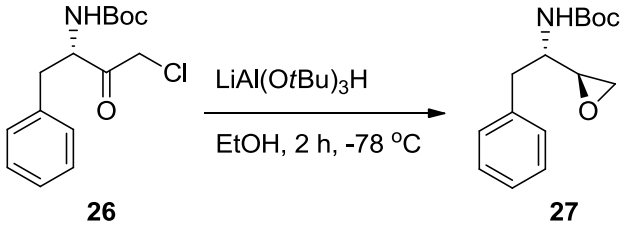
25 **26**

In a 100 mL round bottom flask under nitrogen were placed compound **25** (6.55 g, 19.4 mmol, 1.0 eq.), 120 mL of THF and a solution of hydrochloric acid in 1,4-dioxane (4.0 M, 5.6 mL, 22.4 mmol, 1.2 eq.) – was added dropwise over 5 minutes. The mixture was then allowed to reflux (70 °C), stirring for 4 hours. After cooling the solution to room temperature, it was mixed with hexane (95 mL), and EtOAc (50 mL) and washed with water (2 x 100 mL), $NaHCO_3$ solution (50 mL), water (4 x 100 mL) and brine (55 mL). After drying organic layer over $MgSO_4$, filtration and concentration, afforded solid was recrystallised from minimum of hot hexane and dried to yield **26** as an off-white solid (2.76 g, 48%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.41 (s, 9 H), 2.94 - 3.17 (m, 2 H), 4.08 (d, *J*=1.0 Hz, 2 H), 4.67 (q, *J*=7.0 Hz, 1 H), 5.05 (d, *J*=6.6 Hz, 1 H), 7.13 - 7.38 (m, 5 H).

The spectroscopic data are consistent with that reported in the literature.[44]

(2*S*,3*S*)-3,4-Epoxy-1-phenylbutan-2-yl 1,1-dimethylethyl carbamate (27)



26 **27**

Compound **26** (2.76 g, 9.3 mmol, 1.0 eq.) dissolved in anhydrous ethanol (73 mL) under nitrogen was cooled to -78 °C followed by slow addition (20 min) of solution of lithium tri-*tert*-butoxyaluminium hydride in THF (1 M, 19.4 mmol, 19.4 mL, 2.1 eq.). Mixture was allowed to stir for 2 hours. After raising the temperature to -20 °C, reaction was quenched with Rochelle salt solution (18 mL) and stirred for another 15 min. Resulting mixture was then partially evaporated to get two liquid phases of around 10 mL. Mixture with extra Rochelle's salt solution (73 mL) added was extracted with EtOAc (180 mL), after which organic layer was washed with water (2 x 70 mL) and brine (70 mL), dried over $MgSO_4$, filtered and evaporated. Solid was recrystallised from hot mixture of EtOAc (3 mL) and hexane (1 mL) to afford desired product **27** as a pale yellow solid (2.00 g, 82%).

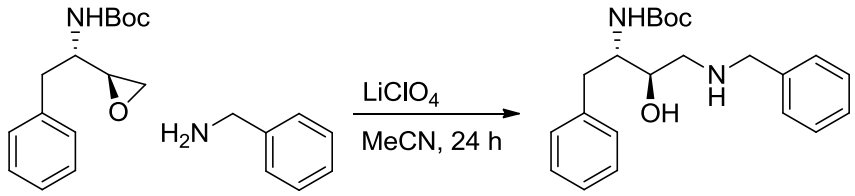
^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.31 (s, 9 H), 2.63 - 2.97 (m, 5 H), 3.62 (br. s., 1 H), 4.37 (br. s., 1 H), 7.03 - 7.33 (m, 5 H).

The spectroscopic data are consistent with that reported in the literature.[44]

General procedure A – *Epoxide opening*

A round-bottomed flask is charged with a magnetic stirrer bar, epoxide **27** (1.0 eq.), lithium perchlorate (1.0 eq.), CH_3CN dried over $MgSO_4$ and an amine (5.0 eq. unless written differently) and purged with Ar/N_2. The solution is magnetically stirred under Ar/N_2 for 30 hours. Then the reaction is quenched with addition of H_2O and transferred to a separating funnel. The solution is partitioned with three portions of diethyl ether and then organic extract dried over $MgSO_4$ and concentrated *in vacuo*. The crude material is purified by flash column chromatography (various eluent systems) to afford the desired *N*-Boc aminols.

(2*S*,3*R*)-3-Hydroxy-1-phenyl-4-(*N*-(phenylmethyl)amino)butan-2-yl 1,1-dimethylethyl carbamate (39)



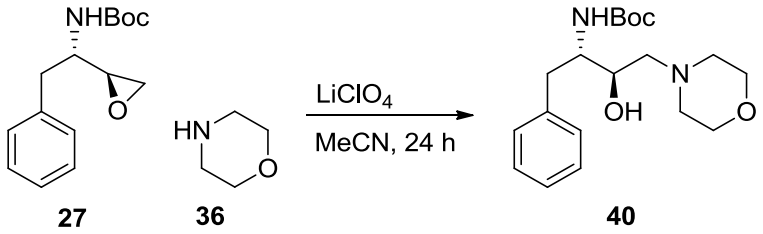
27 **35** **39**

Using the general procedure A, epoxide **27** (188 mg, 0.71 mmol, 1.0 eq.), lithium perchlorate (75 mg, 0.71 mmol, 1.0 eq.) and benzylamine **35** (392 mg, 0.4 mL, 3.60 mmol, 5.0 eq.) in CH_3CN (2 mL) were stirred for 36 hours and then quenched with water. The precipitate was rinsed with hexane and water and then collected by filtration giving secondary amine **39** as an off white solid (102 mg, 39%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.31 (s, 9 H) 2.68 (d, *J*=4.90 Hz, 2 H) 2.79 - 3.00 (m, 2 H) 3.38 - 3.50 (m, 2 H) 3.77 (td, *J*=1.00 Hz, 1 H) 4.63 (d, *J*=8.29 Hz, 1 H) 7.11 - 7.32 (m, 5 H).

The spectroscopic data are consistent with that reported in the literature.[50]

(2*S*,3*R*)-3-Hydroxy-4-morpholino-1-phenylbutan-2-yl 1,1-dimethylethyl carbamate (40)



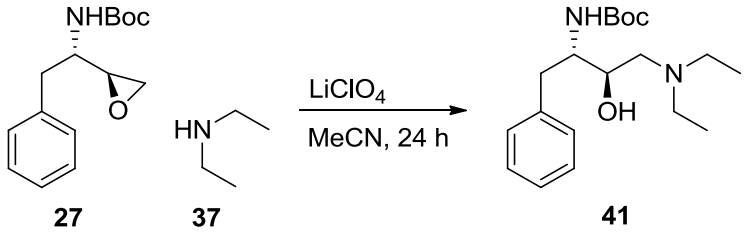
27 **36** **40**

Using the general procedure A, epoxide **27** (188 mg, 0.713 mmol, 1 eq.), lithium perchlorate (75 mg, 0.71 mmol, 1.0 eq.) and morpholine **36** (249 mg, 0.25 mL, 2.67 mmol, 5.0 eq.) in 2 mL of CH_3CN were stirred for 48 hours and then quenched with water. The precipitate, after addition of a small amount of diethyl ether, was rinsed with hexane and water and then collected by filtration, giving **40** (89 mg, 36%).

mp: 121 – 125 ^{o}C; **^{1}H NMR (300 MHz, $CDCl_3$) δ ppm** 1.36 (s, 9 H), 2.35 - 2.50 (m, 4 H), 2.58 - 2.68 (m, 2 H), 2.91 (d, *J*=4.4 Hz, 1 H), 2.98 (dd, *J*=1.0 Hz, 1 H), 3.60 - 3.68 (m, 3 H), 3.69 - 3.75 (m, 3 H), 4.57 (d, *J*=7.7 Hz, 1 H), 7.21 - 7.34 (m, 5 H); **^{13}C δ:** 28.3 ($3CH_3$), 36.3 (CH_2), 53.7 ($2CH_2$), 54.4 (CH), 61.5 (CH_2), 67.0 ($2CH_2$), 67.8 (CH), 79.4 (C), 126.3 (CH), 128.4 (2CH), 129.6 (2CH), 139.5 (C), 155.6 (CH); **ES$^+$ MS m/z:** 351.2 ($[M+H]^+$)

$[\alpha]_D^{25}$ and IR tests had not been performed due to lost sample.

(2*S*,3*R*)-4-(*N*,*N*-Diethylamino)-3-Hydroxy-1-phenylbutan-2-yl 1,1-dimethylethyl carbamate (41)

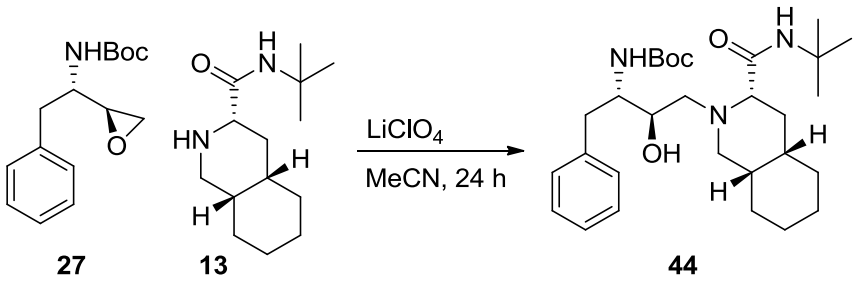


27 **37** **41**

Using general procedure A, epoxide **27** (180 mg, 0.68 mmol, 1.0 eq.), lithium perchlorate (72 mg, 0.68 mmol, 1.0 eq) and diethylamine **37** (249 mg, 0.35 mL, 3.40 mmol, 5.0 eq.) in CH_3CN (2 mL) were stirred for 30 hours and then quenched with water (4 mL). The crashed out solid was rinsed with hexane and water and then collected by filtration giving of expecting product **41** (202 mg, 88%).

$[\alpha]_D^{25}$: 65 (*c* 0.48, CH_3OH); **mp:** 84 – 86 °C**; ^{1}H NMR (400 MHz, $CDCl_3$) δ** 0.94 (t, *J*=7.2 Hz, 6 H), 1.27 (s, 9 H), 2.27 - 2.48 (m, 4 H), 2.48 - 2.61 (m, 2 H), 2.70 - 2.86 (m, 1 H), 2.86 - 2.99 (m, 1 H), 3.44 (br. s., 1 H), 3.71 (br. s., 1 H), 4.46 (d, *J*=7.4 Hz, 1 H), 7.07 - 7.29 (m, 5 H); **^{13}C δ:** 12.0 ($2CH_3$), 28.3 ($3CH_3$), 36.4 (CH_2), 47.0 ($2CH_2$), 54.5 (CH), 56.4 (CH_2), 68.4 (CH), 79.2 (C), 126.2 (CH), 128.3 (CH), 129.6 (CH), 138.0 (C), 155.5 (C); **ES$^+$ MS m/z:** 337.1 ($[M+H]^+$).

(3*S*,4a*S*,8a*S*)-2-((2*S*,3*R*)-3-(1,1-Dimethylethoxy)methanamido-2-hydroxy-4-phenylbutyl)decahydroisoquinoline 3-(*N*-(1,1-dimethylethyl))carboxamide (44)



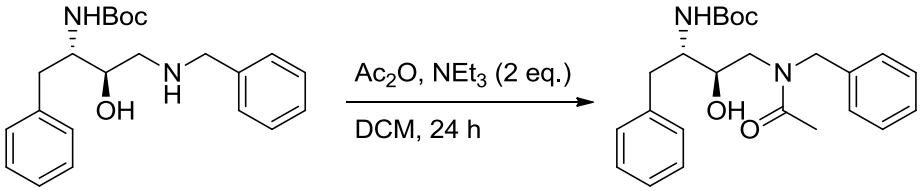
Using the general procedure A, epoxide **27** (200 mg, 0.76 mmol, 1.0 eq.), lithium perchlorate (80.85 mg, 0.76 mmol, 1.0 eq.) and **13** (272 mg, 1.14 mmol, 1.5 eq.) in 2 mL of CH_3CN were stirred for 48 hours and then quenched with water. After triple partition with diethyl ether, drying organic layer over $MgSO_4$, and concentration *in vacuo*, flash column chromatography (EtOAc/petroleum ether 1:4) afforded expected **44** as transparent/slightly white foam (62 mg, 16%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (s, 9 H), 1.28 (s, 9 H), 1.45 (td, *J*=8.8, 4.5 Hz, 6 H), 1.60 - 1.64 (m, 1 H), 1.64 - 1.81 (m, 4 H), 1.83 - 1.94 (m, 1 H), 2.15 - 2.26 (m, 2 H), 2.54 (dd,

J=10.9, 3.2 Hz, 1 H), 2.59 (dd, *J*=13.4, 6.1 Hz, 1 H), 2.78 - 2.94 (m, 3 H), 3.67 - 3.86 (m, 2 H), 4.82 (d, *J*=7.0 Hz, 1 H), 5.86 (s, 1 H), 6.50 (br. s., 1 H), 7.10 - 7.26 (m, 5 H).

The spectroscopic data are consistent with that reported in the literature.[47]

(2*S*,3*R*)-4-(*N*-Ethanoyl-*N*-(phenylmethyl)amino)-3-hydroxy-1-phenylbutan-2-yl 1,1-dimethylethyl carbamate (45)



39 **45**

Benzylamine derivative compound **39** (130 mg, 0.35 mmol, 1.0 eq.) was added to the solution of acetic anhydride (35.62 mg, 32.5 µL, 0.35 mmol, 1.0 eq.) and triethylamine (71 mg, 97.5 µL, 0.70 mmol, 2.0 eq.) in 1 mL of CH_2Cl_2 and stirred for one hour at 0 °C.

Ethyl acetate was added to the solution and washed with aqueous hydrogen chloride solution (0.5 M), saturated aqueous sodium bicarbonate solution and brine and then dried over $MgSO_4$, filtered and dried *in vacuo* to afford off yellow solid.

After addition of diethyl ether white solid crushed out and was collected by filtration and washed with hexane, affording **45** (64 mg, 44%).

*Acetylation mixture: To a solution of acetic anhydride (2.70 mmol, 27.4 mg, 0.25 mL) in DCM (20 mL) was added triethylamine (5.40 mmol, 54.6 mg, 0.75 mL). The solution was magnetically stirred and 1 mL taken for this reaction.

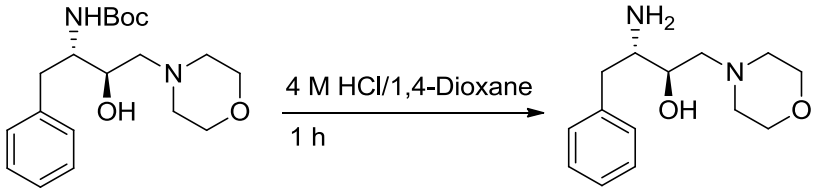
$[\alpha]_D^{25}$: - 35 (*c* 0.51, CH_3OH); **mp:** 152 – 156 °C; **IR:** 2971, 2932, 2823, 1604, 1494, 1452, 1259, 1202 cm^{-1}; **^{1}H NMR (300 MHz, $CDCl_3$) δ ppm** 1.17 - 1.28 (9 H, s), 2.11 (3 H, s) 2.65 - 2.83 (1 H, m), 2.89 (1 H, dd, *J*=13.9, 4.8Hz), 3.30 (1 H, d, *J*=12.4 Hz), 3.46 - 3.84 (3 H, m), 4.42 (1 H, d, *J*=8.4 Hz), 4.51 (1 H, s), 6.97 - 7.43 (10 H, m) **^{13}C δ:** 21.7 (CH_2), 28.2

(3CH_3), 36.1 (CH_2), 51.1 (CH_2), 53.9 (CH_2), 71.5 (CH), 73.6 (CH), 79.5 (C), 126.4 (CH), 127.8 (2CH), 128.4 (2CH), 128.7 (CH), 129.0 (2CH), 129.7 (2CH), 136.0 (C), 137.7 (C), 155.8 (C), 174.1 (C); **ES^+ MS m/z:** 313.1 ($[M+H]^+$), 335.1 ($[M+Na]^+$);

General Procedure B – *N-Boc deprotection*

A round-bottomed flask is charged with a magnetic stirrer bar, *N*-Boc aminol (1.0 eq) and 4 M HCl in dioxane (1.5 mL per 100 mg of *N*-Boc aminol). The reaction mixture was stirred for 2 hours under Ar/N_2. After completion of reaction solvent was evaporated *in vacuo*, and then co-evaporated two times with $CHCl_3$ (or CH_2Cl_2) and two times with acetonitrile.

(2*S*,3*R*)-3-Amino-1-morpholino-4-phenylbutan-2-ol (46)



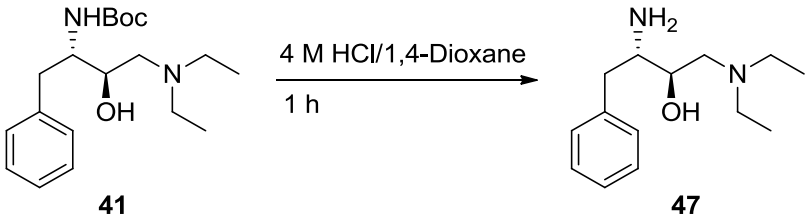
40 **46**

Using general procedure B, *N*-Boc aminol **40** (120 mg, 0.34 mmol) and HCl in dioxane (1.8 mL) were stirred under Ar for 90 minutes. Solvent was evaporated, and crude material co-evaporated with $CHCl_3$ and CH_3CN. Then product was extracted with CH_2Cl_2 (10 mL) and aqueous NaOH solution (2 M, 10 mL), dried over $MgSO_4$ and concentrated *in vacuo*, affording **46** (69 mg, 80%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.30 - 2.65 (7 H, m), 2.87 (1 H, dd, *J*=13.4, 4.0 Hz), 3.09 (1 H, dt, *J*=9.6, 4.6 Hz), 3.54 - 3.72 (5 H, m), 7.03 - 7.33 (5 H, m).

The spectroscopic data are consistent with that reported in the literature.[58]

(2*S*,3*R*)-Amino-1-(diethylamino)-4-phenylbutan-2-ol (47)

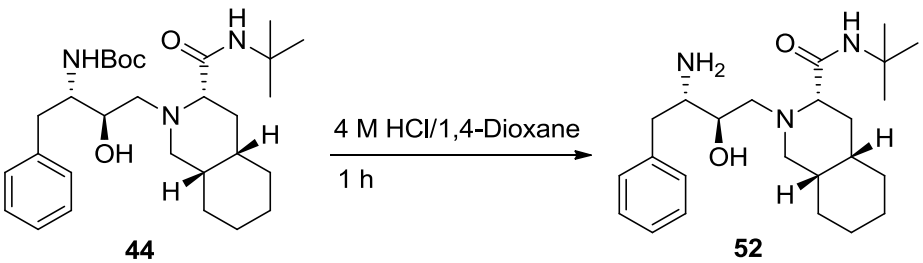


41 **47**

Using general procedure B, aminol **41** (200 mg, 0.59 mmol) and HCl in dioxane (3 mL) were stirred under Ar for 2 hours. Solvent was evaporated, and crude material co-evaporated with $CHCl_3$ and CH_3CN. Then product was extracted with CH_2Cl_2 (10 mL) and aqueous NaOH solution (2 M, 10 mL), dried over $MgSO_4$ and concentrated *in vacuo*, affording **47** (110 mg, 79%).

$[\alpha]_D^{25}$: +85 (*c* 0.42 , CH_3OH); **mp:** 68 – 72 °C; **IR:** 2968, 1716, 1497, 1366, 1259, 1169, 1015 cm^{-1}; **^{1}H NMR (400 MHz, $CDCl_3$) δ ppm** 1.08 (6 H, t, *J*=7.0 Hz), 2.42 - 2.79 (7 H, m), 3.01 (1 H, d, *J*=13.4 Hz), 3.16 (1 H, br. s.), 3.56 (1 H, d, *J*=5.8 Hz), 7.18 - 7.38 (5 H, m); **^{13}C δ:** 11.9 ($2CH_3$), 39.8 (CH_2), 47.1 ($2CH_2$), 54.9 (CH), 55.9 (CH_2), 69.6 (CH), 126.3 (CH), 128.5 (2CH), 128.9 (2CH), 135.4 (C); **ES$^+$ MS m/z:** 237.1 ([M+H]+).

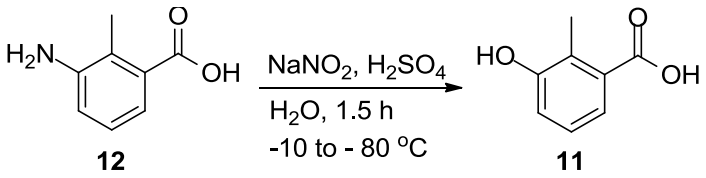
(3*S*)-2-((2*S*,3*R*)-Amino-2-hydroxy-phentylbutyl)-*N*-(*tert*-butyl)decahydroisoquinoline-3-carboxamide (52)



44 **52**

Using general procedure B, *N*-Boc aminol **44** (30 mg, 0.06 mmol) and 4 M HCl in 1,4-dioxane (0.45 mL) were stirred under Ar for 2 hours. Solvent was evaporated, and crude material co-evaporated with $CHCl_3$ and CH_3CN. Such produced product **52** was used immediately in next step.

3-Hydroxy-2-methylbenzoic acid (11)

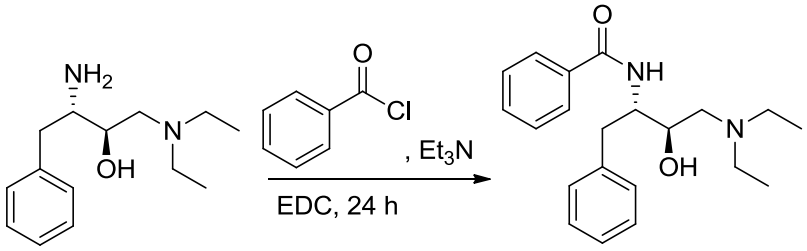


Concentrated sulfuric acid (5.3 g, 2.6 mL, 56 mmol, 3.7 eq.) was added to the solution of water (20 mL) with 3-amino-2-methylbenzoic acid (2.25 g, 15 mmol, 1.0 eq.) and then cooled to -10 °C. Sodium nitrite was added gradually (to not exceed 7 °C) to the flask and stirred for 30 minutes. Second solution was prepared by adding concentrated sulfuric acid (24.5 g, 12 mL, 280 mmol, 18.7 eq.) to distilled water (60 mL) and then combined with the first solution. After slow heating the mixture to 80 °C, it was stirring for one hour and then cooled again (after no further gas was produced) to the room temperature. The reaction mixture was extracted with five portions of EtOAc and then organic layer was extracted with $NaHCO_3$ solution. The aqueous extracts were acidified to pH 2 by the dropwise addition of HCl, then extracted with a further four measures of EtOAc. The organic layers were combined and washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*, affording **11** as an off white solid (1.88 g, 74%).

^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 2.35 (s, 3 H), 7.01 (d, J=1.0 Hz, 1 H), 7.11 (t, J=7.9 Hz, 1 H), 7.23 (d, J=1.0 Hz, 1 H), 9.64 (br. s., 1 H), 12.72 (br. s., 1 H).

The spectroscopic data are consistent with that reported in the literature.[1]

***N*-((2*S*,3*R*)-4-(Diethylamino)-3-hydroxy-1-phenylbutan-2-yl)benzamide**



47 **51**

A round-bottomed flask was charged with a magnetic stirrer bar, (2S,3R)-amino-1-(diethylamino)-4-phenylbutan-2-ol **47** (50 mg, 0.21 mmol, 1.0 eq.), benzoyl chloride (59.2 mg, 0.05 mL, 0.42 mmo, 2.0 eq.l), triethylamine (0.043 mg, 0.059 mL, 0.42 mmol, 2.0 eq.) and 1,2-dichloroethane (2 mL). The reaction mixture was stirred for 40 hours (first 10 min at 0 ^{o}C). After completion of the reaction, ethyl acetate was added to mixture and the product was extracted with the aqueous KOH solution (2 M) and brine. The organic layers was combined, dried over Mg_2SO_4 and concentrated *in vacuo* and purified by flash column chromatography (EtOAc/petroleum ether 2:3) to afford the **51** as an yellow oil (12 mg, 16%).

$[\alpha]_D^{25}$: 15 (*c* 0.37, CH_3OH)**; IR:**2977, 2928, 2481, 1677, 1622, 1417, 1240, 1160, 1009 cm^{-1}; **ES$^+$ MS m/z:** 341.1 ($[M+H]^+$), 363.1 ($[M+Na]^+$); **^{1}H NMR (300 MHz, $CDCl_3$) δ ppm** 0.86 (6 H, t, *J*=7.2 Hz), 2.46 - 2.59 (4 H, m), 2.68 - 2.80 (2 H, m), 2.95 - 3.11 (2 H, m), 4.72 (1 H, t, *J*=6.3 Hz), 5.16 (1 H, d, *J*=4.8 Hz), 7.30 - 7.95 (10 H, m), 8.57 (1 H, d, *J*=6.8 Hz).

^{13}C NMR test has not been performed due to lost sample.

6.3 BIOLOGICAL TESTING OF NELFINAVIR ANALOGUES

Cell culture:

THP-1 is a human monocytic leukemia cell line, which was purchased from ECACC (Porton Down, UK). THP-1 cells were derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia. Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10 % foetal calf serum, 2 mM L^{-1} -glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (PAA). They were passaged twice weekly and maintained between 2.5-6 x 10^5 cells/ml at 37 ^oC and 5% CO_2.

Cell counting:

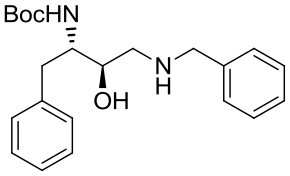
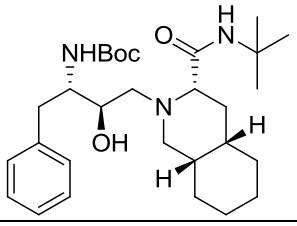
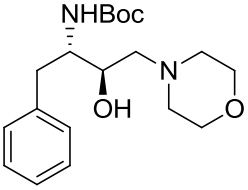
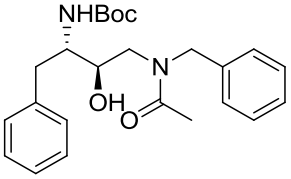
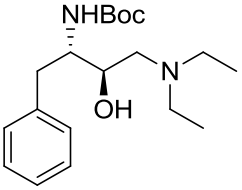
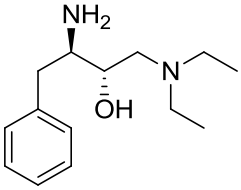
Cells were counted using a Neubauer haemacytometer and 0.4% Trypan Blue solution, according to the manufacturer's instructrions (purchased from Sigma-Aldrich). Procedure: 20 μL cells were added to 80 μl 0.4% Trypan Blue solution, left for 5 min and added to the chamber of the haemacytometer. The average cell count in each corner was calculated and the following calculation applied:

Cell count per ml = average of four corners x 5 (dilution factor) x 10^4/mL.

Proliferation assay:

Proliferation was determined by the MTS assay using the CellTiter 96 Aq_{ueous} One Solution Cell Proliferation Assay (purchased from Promega) according to the manufacturer's instructions.[55]

Procedure: THP-1 cells (3 x 10^4/100μL) were seeded in 96-well plates and left untreated or treated with DMSO (vehicle control, Sigma), doxorubicin or compounds **39**, **40**, **41**, **44**, **45** and **47** (with concentrations ranging from 100 pM – 1 mM) in triplicate for 72 hr at 37 °C with 5% CO_2. Following this, 10 μL MTS assay reagent was added for 4 hours and the absorbance was measured at 490 nm using the Polarstar Optima microplate reader (BMG Labtech). IC_{50} values were calculated using GraphPad Prism Version 5.0 software.

Compound	Structure	IC50	Compound	Structure	IC50
39		34µM	44		33µM
40		>1mM	45		>1mM
41		37µM	47		2.8µM

Results of MTS assay – IC50 of compounds 39, 40, 41, 44, 45 and 47.

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