3.1- Introduction

3.1.1- Proteases as Chemotherapeutic Targets for Malaria Treatment

Protozoan parasites are an extremely diverse group of unicellular organisms of the kingdom Protista. A small number of these have evolved to parasitize humans and have had a profound effect on shaping the course of human history and evolution[1]. Despite the great advances in combating infectious diseases over the past century, these parasites continue to inflict a tremendous social and economic burden on human societies, particularly in tropical and subtropical regions of the world. Malaria, currently one of the most deadly infectious diseases of humans, is caused by such an organism. *P. falciparum* is the most lethal strain of the four parasitic species. Control of malaria is challenged by increasing resistance to available drugs, and new antimalarial drugs, ideally directed against new targets, are an urgent need. In parasitic protozoa organisms, proteases carry out "housekeeping" tasks common to many eukaryotes, as well as functions highly specific to the parasite life cycles. Thus, proteases belonging to the malaria parasite arose as potential new targets for malaria chemotherapy. In this field of action, a great deal of knowledge can be obtained from other biological systems, where hundreds of proteases have been well characterized[2] and potent inhibitors have been developed, and proven to be of great value in fighting other diseases. Four of the major catalytic classes have now been described from *Plasmodium falciparum*, leading to an increasing interest in the potential for protease inhibition as antimalarial chemotherapy is firmly established. As protein characterization and purification techniques advance, so does the identification of optimal chemotherapeutic targets and the development of inhibitors of these proteases as antimalarial drugs.

3.1.2- Life Cycle of Malaria Parasites: Protease Participation

Malaria parasites are protozoans from the large and diverse phylum Apicomplexa, with complex life cycles that include the infection of anopheline mosquitoes, functioning as vector, and animals, as the host (see Figure 2 in chapter one for analysis of the complete malaria parasite life cycle: mosquito, human liver and human blood stages). Human infection follows the injection of sporozoites by mosquitoes, which, after a brief circulation, invade hepatocyte cells. During the asymptomatic hepatic stage of infection merozoites are produced, which rapidly invade erythrocytes. It is during the erythrocytic cycle of infection that all clinical manifestations occur. The intraerythrocytic parasites develop from small ring-stage organisms to larger, more metabolically active trophozoites and then to multinucleated schizonts.

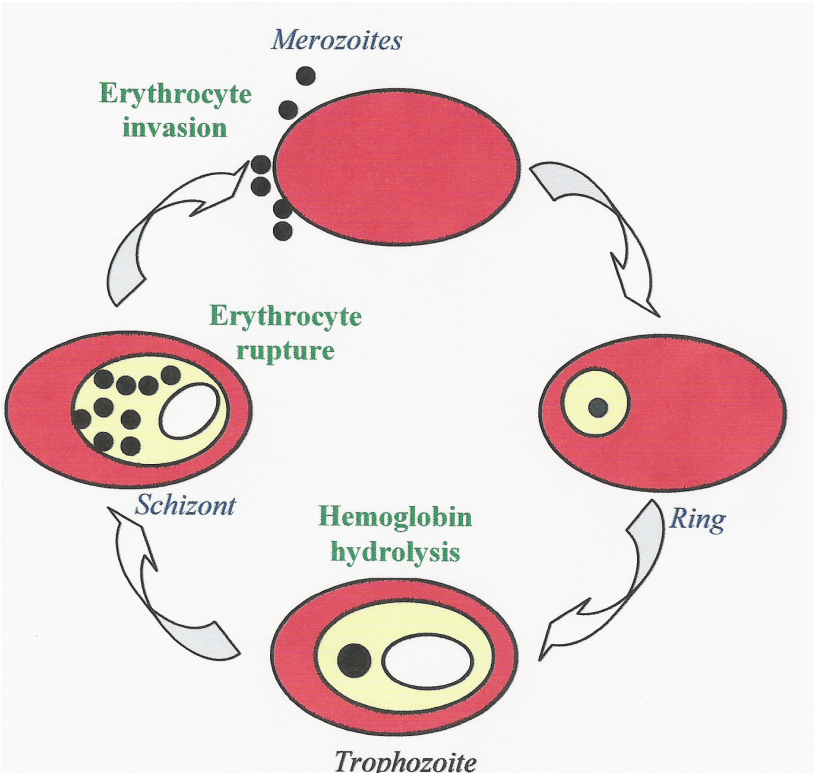


Figure 35. Erythrocytic life cycle of malaria parasites.[3]

As shown in figure 35, there are at least three processes during the erythrocytic stage of infection where erythrocyte proteins are apparently hydrolyzed by infecting malaria parasites: rupture and subsequent reinvasion of erythrocytes by merozoite–stage parasites and degradation of hemoglobin by intraerythrocytic trophozoites. All of these require protease activity and thus are potential targets for protease inhibitors, as studies in the field using known peptide protease inhibitors have demonstrated (Table 2).

In a brief description of these biological events one can say that, firstly, during invasion of red blood cells by merozoites, cytoskeletal proteins are hydrolyzed by parasite proteases released from merozoite secretory organelles, in order to allow movement of the parasite into the erythrocyte. Secondly, in particular during the trophozoite phase, but also in much of the whole erythrocytic stage, parasites take up and hydrolyse large quantities of hemoglobin as a source of amino acids. Thirdly, at the complexion of the cycle, mature schizonts cause rupture of the erythrocyte, and parasites egress the erythrocyte in the form of free merozoites, through cleavage of cytoskeletal proteins by plasmodial proteases.

Life-cycle stage	*Biological process*	*Effective inhibitors*	*Protease class implicated*[a]
Trophozoite	Hemoglobin hydrolysis	Leupeptin, E-64 Pepstatin	Cysteine Aspartic
Schizont	Erythrocyte rupture	Chymostatin Leupeptin	Serine Cysteine
Merozoite	Erythrocyte invasion	Chymostatin	Serine

Table 2. Effects of Peptide Protease Inhibitors on the life cycle of Malaria Parasites[3] ([a]Principal class inhibited, although some of the compounds also inhibit other classes).

Some proteases from Plasmodium falciparum are well characterized and their erythrocytic substrates and putative roles have been established (Table 3). Among the 3 referred processes, one that has been better characterised is the hemoglobin hydrolysis. Many more uncertainties exist related to the clarification of the erythrocyte invasion and rupture processes.

Protease	*Class*	*Substrate(s)*	*Putative role(s)*
Falcipains	Cysteine	Hemoglobin, Ankyrin	Hemoglobin hydrolysis, Erythrocyte rupture
Plasmepsins	Aspartic	Hemoglobin, Spectrin	Hemoglobin hydrolysis, Erythrocyte rupture
Falcilysin	Metalloprotease	Globin fragments	Hemoglobin hydrolysis
Aminopeptidase	Metallo-aminopeptidase	Globin fragments	Hemoglobin hydrolysis
Pfsub-1 and Pfsub-2	Serine	Membrane parasite proteins?	Erythrocyte invasion

Table 3. Proteases of *Plasmodium falciparum*[4] and their main roles within the erythrocyte.

3.1.2.1- Proteases and Hemoglobin Hydrolysis

During the intraerythrocytic phase of its life cycle, malaria parasites take up cytosol (the internal fluid, rich in hemoglobin[5]) from the host erythrocyte, through a specialised organelle, the cytostome (or cell mouth), which transports the cytosol to a sophisticated organelle, the acidic food vacuole, where hemoglobin degradation occurs. As

hemoglobin is processed, its heme component is converted into hemozoin pigment, and globin is hydrolysed to its constituent amino acids. The result of this process has deep implications for more directions in antimalarial chemotherapy, namely related with the action of chloroquine and other quinolines (effects on the disposition of free heme) and with the action of artemisinin (effects also at the interaction with heme level).This metabolic process is thought to be necessary not only to provide amino acids for the parasite to be able to synthesize its proteins[6] and feed itself, but also to maintain the osmotic stability of malaria parasites[7] and to provide space for the growing intraerythrocytic parasite[8] through diffusion of the resulting amino acids into the erythrocyte. Thus, hemoglobin catabolism is clearly essential for the parasite's survival. Proteases are deeply involved with the degradation of hemoglobin, as this appears to be the result of a cooperative process involving proteases of multiple catalytic classes (Table 3), including cysteine (falcipains), aspartic (plasmepsins), metalloproteases (falcilysin) and at least one metalloaminopeptidase. Available evidence suggests an ordered and compartimentalized overall process (Figure 36), though this is not fully consensual[9].

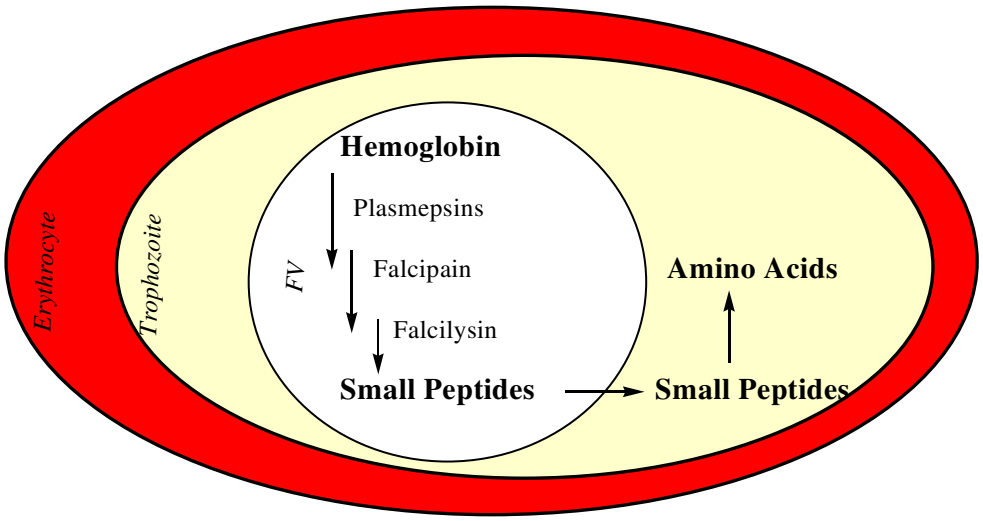


Figure 36. Proposed order and compartimentalization of hemoglobin degradation[10].

Within the food vacuole, hemoglobin is degraded first by the plasmepsins, followed by falcipain and then falcilysin, generating smaller peptides. These peptides are transported out of the food vacuole, to the parasite cytoplasm, where they are terminally degraded to amino acids by exopeptidases.

Aspartic Proteases: plasmepsins

Aspartic proteases are proteases that have catalytic aspartic acid residues in their active sites. These proteases play a critical role in *P. falciparum*, since they are involved in the early events of hemoglobin degradation in the food vacuole. Two closely related aspartic proteases, and the best characterized ones are plasmepsin I and II which initiate hemoglobin degradation by hydrolysing the peptide bond between residues Phe33 and Leu34 in the α chain of native hemoglobin[11,12], being the other cleavage sites distinct between the enzymes. This initial cleavage is thought to promote unfolding and release of the heme moiety and possibly allows unravelling, exposing other peptide bonds to hydrolysis by other proteases. *In vivo* evidence for this semi-ordered hemoglobin degradation pathway, beginning with aspartic proteases, comes from the observation that brief treatment of parasites with the aspartic protease inhibitor Ro 40-4388 prevents accumulation of the heme-binding antimalarial chloroquine, which suggests that heme release has been blocked[13]; no such effect was observed with the cysteine protease inhibitor leupeptin[14].

Cysteine Proteases: falcipains

Cysteine proteases are proteolytic enzymes that function by action of a catalytic cysteine, which mediates protein hydrolysis via nucleophilic attack on the carbonyl carbon of a susceptible peptide bond. Trophozoite extracts were found to contain

abundant cysteine protease activity[15], and studies using cysteine protease inhibitors have shown dramatic swelling of the food vacuole of the parasite, arresting parasite growth due to blockage of the hemoglobin degradation process[16,17]. This led to a search for the cysteine proteases responsible for this process. Initially it was though that falcipain 1 was the cysteine protease involved. However, it is currently clear that two other papain-family cysteine proteases, falcipain 2 and falcipain 3, have a more prominent role.

Metalloprotease: falcilysin

Incubation of hemoglobin with food extract revealed cleavage sites that could not be accounted for by the known substrate specificities of plasmepsins or falcipains[18]. The *P. falciparum* zinc metalloprotease falcilysin was recently purified from food vacuoles using fluorescence techniques[19]. This protease was unable to hydrolyse either native hemoglobin or denaturated globin, but was capable of cleaving 10-20 amino-acid peptides derived from the concerted action of plasmepsins and falcipain-2, at acidic pH. This enzyme is also robustly active at neutral pH, but with a substantially different substrate specificity. This suggests that this protease may function as two different proteases, in its two locations[20].

Aminopeptidase

No free amino acids have been found in the parasite food vacuole, suggesting that the small hemoglobin fragments are completely hydrolysed elsewhere. Aminopeptidase activity is big in the trophozoite phase, in the parasite cytosol, which should then be the place where the ultimate steps of hemoglobin degradation occur[19,21]. Aminopeptidase inhibitors were indeed found to block the development of cultured parasites, and did not lead to accumulation of hemoglobin in the food vacuole[21,22].

3.1.2.2- Hydrolysis of Erythrocyte Cytoskeletal Proteins: invasion and rupture of red blood cells by the parasite

In one part of the life cycle, inside the host's red blood cells, malaria parasites replicate asexually, forming a short lived form of the parasite: the merozoites. Mature merozoites burst out the depleted erythrocyte host and rapidly enter new red blood cells. The erythrocyte's cytoskeleton is a physical barrier that needs to be transgressed, in both cases. Merozoite invasion is a complex, ordered process, with some of the many proteins involved having been identified; however much is yet to be clarified concerning the cellular and molecular biology of the process. A tentative model of merozoite invasion includes: attachment of the merozoites to specific erythrocyte receptors, reorientation to bring the apical end of the parasite in contact with the RBC surface, release of the contents of specialized secretory organelles, and concomitant formation of a parasite-red cell junction through which the merozoites enter[23]. The observation that some protease inhibitors block invasion suggests that some proteases should play a critical role in this process[24,25]. Merozoite release from the host erythrocyte also appears to be a stepwise process, beginning with release from the FV, rather than from the red cell[13]. Recent reports have begun to characterize specific proteases that play a role in these processes, and surprisingly, it appears that some of the same proteases that hydrolyze hemoglobin also degrade cytoskeletal proteins.

Cysteine Proteases: falcipain-2

Cytoskeleton-cleaving cysteine protease activity has recently been described. Analysis of the effects of parasite extracts on cytoskeletal proteins identified a neutral ankyrin

and a 4,1-cleaving protease as erythrocyte membrane skeletal proteins. Surprisingly, it was shown that *P. falciparum*-derived cysteine protease falcipain-2 was also responsible for this activity, cleaving these host erythrocyte membrane skeletal proteins at neutral pH[26,27] and inducing membrane instability, as evidenced by an increased rate of membrane fragmentation. At first glance it might seem odd, to have a protease that cleaves hemoglobin at PV acidic pH and also cytoskeletal proteins at neutral pH. However, assessment of the malaria parasite genome sequence suggests that the repertoire of cysteine proteases is somewhat limited, thus allowing the parasite to use one enzyme for more than one function[4].

Aspartic Proteases: plasmepsin II

It was recently shown that purified recombinant plasmepsin II cleaved spectrin, another erythrocyte skeletal component (and also actin and protein 4.1), at near neutral pH[28]. Also, plasmepsin II was shown to localize both inside and outside the erythrocytic schizonts, supporting its activity against more than one target, as seems to happen also with cysteine protease falcipain-2.

Serine Proteases: Pfsub-1 and Pfsub-2

Merozoite surface protein-1 (MSP-1) is an abundant protein on the surface of the invading merozoite. Although the precise role of this protein is not clear, the observation that anti-MSP-1 antibodies are capable of blocking invasion has elicited great interest in this molecule[29]. The search for the MSP-1 processing enzyme has led to the discovery of a pair of *P. falciparum* serine proteases, Pfsub-1[30] and Pfsub-2[31]. These subtilisin-like proteases are both located in secretory organelles released from merozoites at the time of the erythrocyte invasion.

In conclusion:

- Protease inhibitors have demonstrated to be of great value in other diseases, and have therefore attracted the interest of the scientific community as potential antimalarial agents. Although progress in the area of structural and functional characterization of proteases has been fairly slow, recent advances have brought new insights into the abilities of these fascinating catalytic machines, promoting the development of selective inhibitors of key proteases.
- Although the available studies suggest clear evidences for the involvement of all four classes of proteases in the life-cycle of the parasite, the investigation described within this thesis was focused on the design and synthesis of cysteine protease falcipain-2 inhibitors.

3.2- Falcipain Cysteine Proteases: the Rationale Supporting this Project

Cysteine proteases are so-named due to the function of a catalytic cysteine, which mediates protein hydrolysis via nucleophilic attack on the carbonyl carbon of a susceptible peptide bond. There are three structurally different classes of cysteine proteases that serve as targets for drug design[32]: papain-like family, ICE-like (caspases) family and *picornaviridae* family. The best characterized *Plasmodium* cysteine proteases are the falcipains, a family of four papain-family enzymes of *P. falciparum.*

3.2.1 The Genome Sequencing Data and Biochemical Characterization

The genome sequence shows that there are four falcipains: falcipain-1, which is encoded on chromosome 14, and three other proteases whose genes are located within chromosome 11. These proteases are called falcipain-2, falcipain-2' (which has 99% homology with falcipain-2 in the catalytic domain) and falcipain-3. The falcipains differ most notably in that homology between falcipain-1 and the other falcipains is relatively low; on the contrary, falcipain-2 and falcipain-3 are much more similar in sequence (68% identity), share similar sized prodomains, and include an unusual amino-terminal extension of the catalytic domain that is not found in falcipain-1[33,34]. It seems consensual to consider two distinct groups of falcipains, the falcipain-1 and the falcipain-2/3 sub-families.

Biochemical characterization of falcipain-2 and 3 has been expedited by the development of efficient systems for the heterologous expression of these two proteases in *Escherichia coli*, followed by refolding of the active enzymes. The biochemical

features[9,35] of the two enzymes are very similar, but not identical, in that both falcipains have acidic pH optima, consistent with activity in the food vacuole, though falcipain-2 processing appears to occur earlier in the transport pathway allowing also activity at the cytosolyc neutral pH. Both prefer peptydil substrates with a leucine residue on the pocket-2 position, though falcipain-2 appears to be much more active.

Recent studies have definitely discarded the hypothesis that falcipain-1 mediated hemoglobin hydrolysis, since disruption of the falcipain-1 gene had no obvious effect on erythrocytic parasites[36]; on the contrary, disruption of falcipain-2 gene confirmed the critical role in the hemoglobin degradation process exhibited by this protease[37]. It has also been demonstrated very recently, that *P. falciparum* contains two nearly identical copies of the falcipain-2 gene, located on chromosome 11, encoding two distinct enzymes, namely, falcipain-2 and falcipain-2'[38]. These enzymes are 97% identical at the amino acid level and recent results have confirmed that falcipain-2' is, like falcipain-2, an active hemoglobinase playing a similar role in erythrocytic parasites. Thus, it is anticipated that specific inhibitors targeting falcipain-2 and falcipain-3 will also inhibit falcipain-2'[38].

3.2.2- Falcipain Inhibitors

Initial studies with generic peptide cysteine protease inhibitors including leupeptin (99, Figure 37) and E64 (100, Figure 37) were followed by more specific protease inhibitors. Several peptide-based FP-2 inhibitors have been identified over the past 20 years, specifically, peptidyl fluoromethyl ketones[16,39] (101, IC_{50}= 59 nM; 102, IC_{50}= 4 nM, Figure 38), vinyl sulfones and sulfides[40-42] (103, IC_{50}= 4 nM; 104, IC_{50}= 1.6 nM, Figure 38), aldehyde and α-ketoamide inhibitors[43].

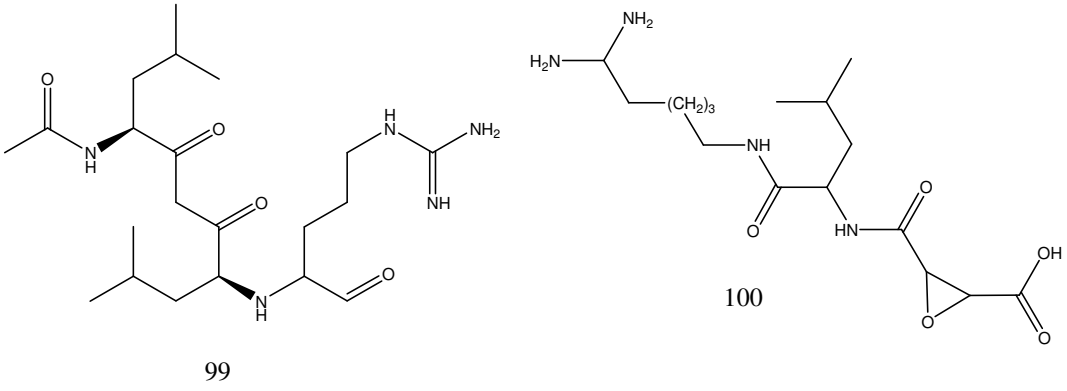


Figure 37. Generic peptide cysteine inhibitors.

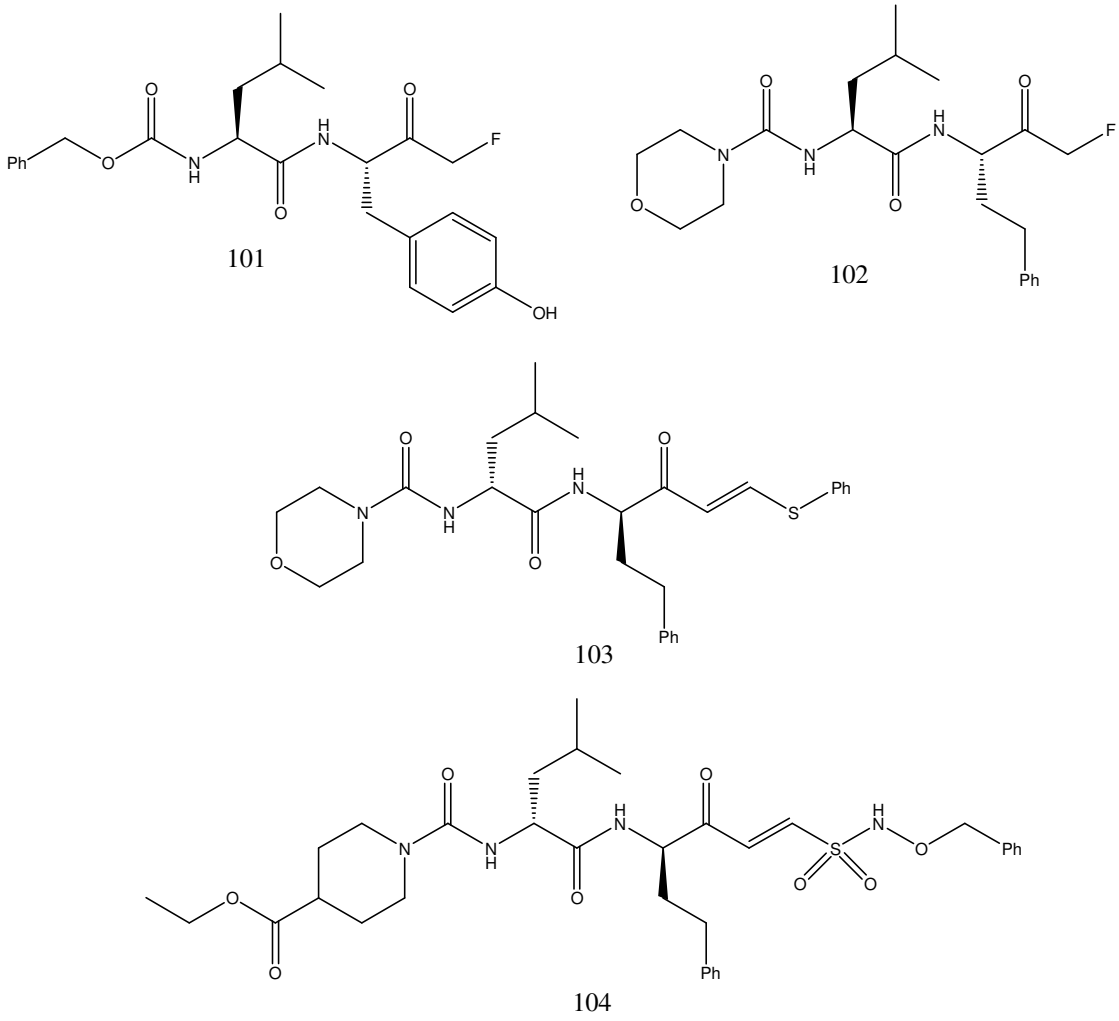


Figure 38. Peptidyl fluoromethyl ketones and vinyl sulfides or sulfones

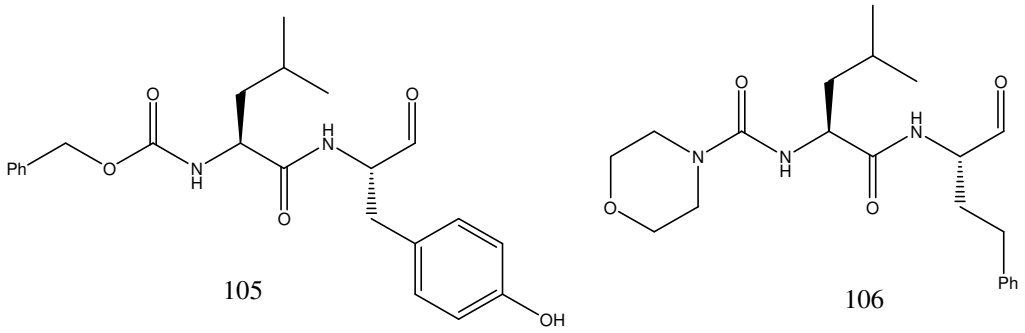


Figure 39. Peptidyl aldehyde inhibors.

A number of low nanomolar concentration peptidyl-aldehyde inhibitors blocked parasite development at low to mid nanomolar concentrations (compound 105, IC_{50}= 40 nM; compound 106, IC_{50}= 10 nM, Figure 39), the same holding for amide 107 (IC_{50}= 10 nM, Figure 40). These small-molecule inhibitors have also shown very similar activities against both falcipain-2 and falcipain-3[43].

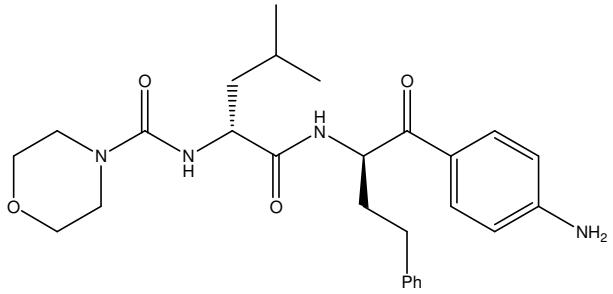
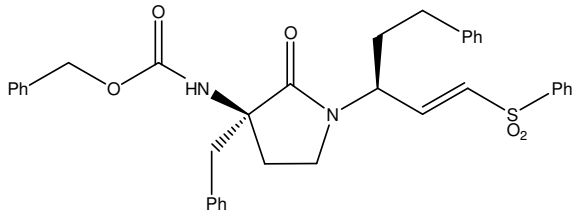


Figure 40.

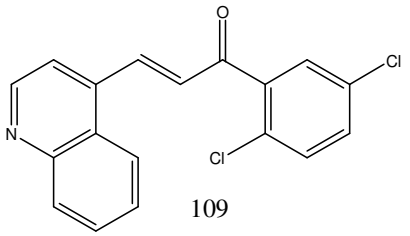
Also, a conformationally constrained vinyl sulfone has been prepared (compound 108, IC_{50}= 2 μM, Figure 41), which can accommodate a favourable conformation within the proposed active site of FP-2 and has demonstrated high FP-2 inhibition[44].



108

Figure 41.

Peptidyl cysteine protease inhibitors have also demonstrated antimalarial activities *in-vivo*[40,43,45] using mice infected with otherwise lethal malaria infections, although these have not been as robust as might have been anticipated on the *in vitro* findings. However one explanation defended by many investigators for this limitation in activity might be the differences in actions against *P. falciparum* and rodent parasite targets[43]. Some *non-peptide-based falcipain inhibitors* have shown antiparasitic activity, though more modest [46-48]. Chalcones (compound 109, IC_{50}= 0.2 μM, Figure 42) and phenothiazines (compound 110, IC_{50}= 10 μM, Figure 42) inhibited FP-2 and stopped the development of cultured parasites at nanomolar to micromolar concentrations.



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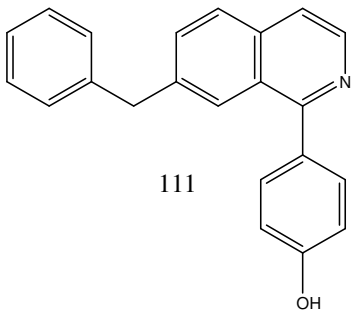

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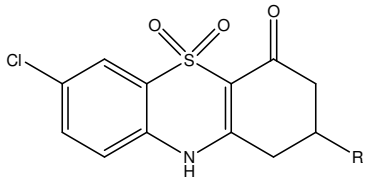


110. R= 2,3-$(CH_3O)_2C_6H_3$

Figure 42.

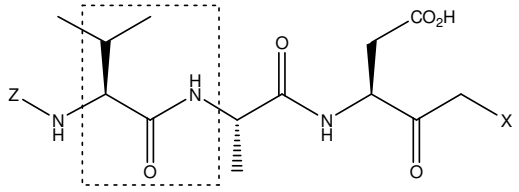
Non-peptide inhibitors with a isoquinoline motif have also been designed, synthesized and assayed for inhibition of FP-2 (compound 111, IC_{50}= 8 μM, Figure 42)[49].

With nearly all of the potent inhibitors, antimalarial activity is accompanied by a blockage of parasite hydrolysis of hemoglobin, resulting in accumulation of intact hemoglobin in the food vacuole, evidenced by the appearance of a swollen, dark-stained FV. This morphological abnormality confirms the specific activity of these compounds on falcipain targets.

3.2.2.1- The peptidomimetic approach

Peptides display a diverse range of biological properties, but their use as drugs is usually compromised by their poor pharmacokinetic profiles, because the amide bonds are susceptible to cleavage by other proteases[50]. Their irreversible mode of action may increase toxicity resulting from the formation of complexes that stimulate autoimunne or other toxic reactions. Also, these compounds are relatively non-selective, and toxicity might be engendered by the inhibition of human host proteases[10]. In the field of peptidomimetics, incorporation of a nonpeptidic scaffold into an amino-acid sequence and of unnatural amino acids has potential advantages, including increased potency and selectivity by stabilizing a biologically active conformation, increased membrane permeability, enhancement of oral bioavailability and stability toward degradation by enzymes[51]. A valuable amount of work has also been carried out in the field of cysteine protease inhibitors, where non-peptidic replacements in a variety of inhibitors has had considerable success. Leading examples include the studies by Sanofi's group on

interleukin 1β-converting (ICE) inhibitors[52]. The discovery of novel classes of peptide-based ICE inhibitors (compound 112, Figure 43), led to the attempt to design a surrogate for a portion of their inhibitors, allowing removal of much of the associated peptide character. It was found that the 5-aminopyrimidin-6-one system was a mimetic which satisfied their criteria (Figure 43, compound 113). In fact, this was a mimetic that had demonstrated previous success in the design of human leukocyte elastase inhibitors[53,54].



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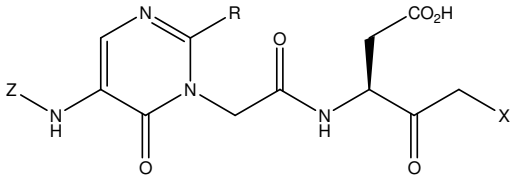
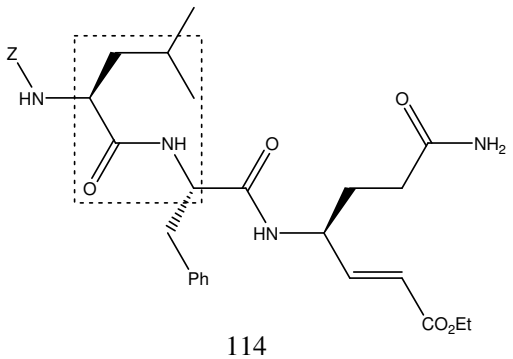


113

Figure 43. Design features for the pyrimidine mimetic 113 versus the tripeptide-based inhibitor.

Also, studies by Dragovich concerning human rhinoviruses HRVs (members from the cysteine protease picornavirus family and cause of human cold) inhibitors have demonstrated that 2-pyridone-containing peptidomimetic inhibitors (compound 115, figure 44), displayed inhibition activities and antiviral properties when compared with related tripeptidyl inhibitors (compound 114, Figure 44).



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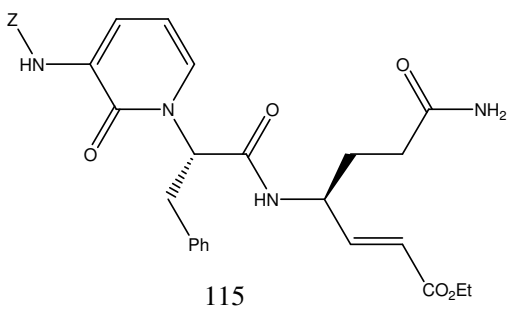

115

Figure 44.

More significantly, studies by Lin at the Walter Reed Army Institute of Research, have shown that peptidomimetic analogues can be designed with potent antimalarial activity *in vitro*. As part of their malaria chemotherapy project, they undertook the synthesis and antimalarial evaluation of a novel class of pyrimidinyl peptidomimetics. The core structure of the new agents comprises, as a peptidomimetic segment, a substituted 5-aminopyrimidone ring, for recognition and binding to the target enzyme, and an active group, which could be considered a Michael acceptor for enzyme alkylation (compound 116, Figure 45). They've also evaluated the potential human toxicity for this class of new peptidomimetic compounds and the results were encouraging, since only weak to moderate cytotoxicity was exhibited.

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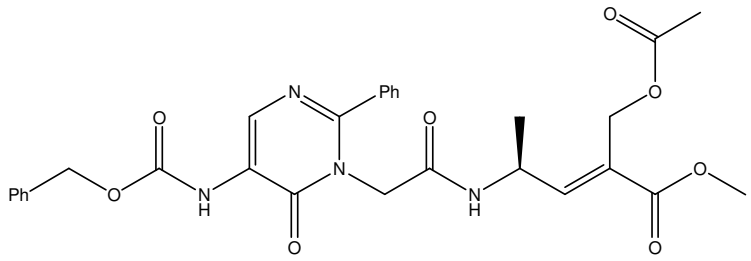


116

Figure 45. Pyrimidinil peptidomimetic 116 (IC_{50}= 9 ng/mL against D6 clone and IC_{50}= 10 ng/mL against W2 clone, in comparison with chloroquine as the control: 6 ng/mL against D6 clone and 14 ng/mL against W2 clone).

3.2.3 Substrate Mapping and Inhibitor Profiling of falcipains

Clues to how specific proteases selectively recognize small molecules most often come from peptide substrates for proteases. Protease inhibitors have been traditionally developed by natural product screening as lead compounds with subsequent

optimization or by empirical substrate-based methods involving truncating polypeptides to short peptides.

Most proteases are sequence-specific, the size and hydrophobicity/hydrophilicity of enzyme sites defining possible binding amino acid chains of polypeptide substrates. The standard nomenclature used to designate substrate/inhibitor residues (e.g. P3, P2, P1, P1', P2', P3') that bind to corresponding enzymes subsites (S3, S2, S1, S1', S2', S3') is shown in Figure 46.

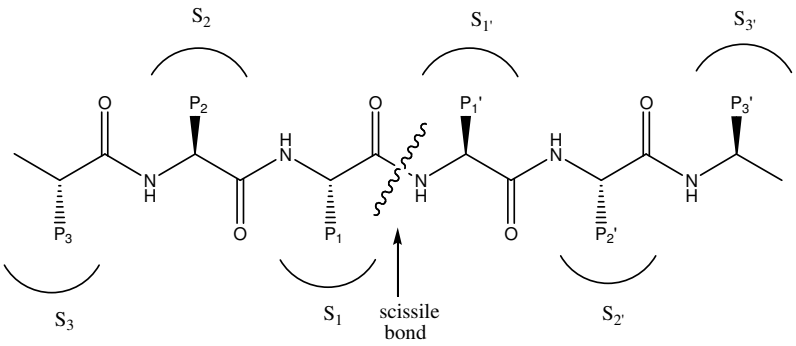


Figure 46. Standard nomenclature for substrate residues and their corresponding binding sites[55].

Analysis of the specificity of FP-2 indicated that, at least with peptide substrates, the amino acid at the P2 position plays a key role in mediating substrate specificity. FP-2 showed a preference for the bulky amino acids leucine and phenylalanine over smaller hydrophobic (valine) or charged (arginine, glutamic acid) P2 amino acids. These findings distinguish FP-2 from cathepsin L but show more similarity to cathepsin K and cathepsin S, two other mammalian papain cysteine proteases[9]. The substrate-based drug design has been substantially improved in recent years with the availability of three-dimensional structural information for proteases, permitting receptor-based design. Finally, and very recently, a crystal structure of mature FP-2 has been presented[56]

(Figure 47), revealing novel structural features of the FP-2 subfamily. This lack of knowledge has been until now, what most impeded progress towards the rational discovery of potent, selective and efficacious inhibitors.

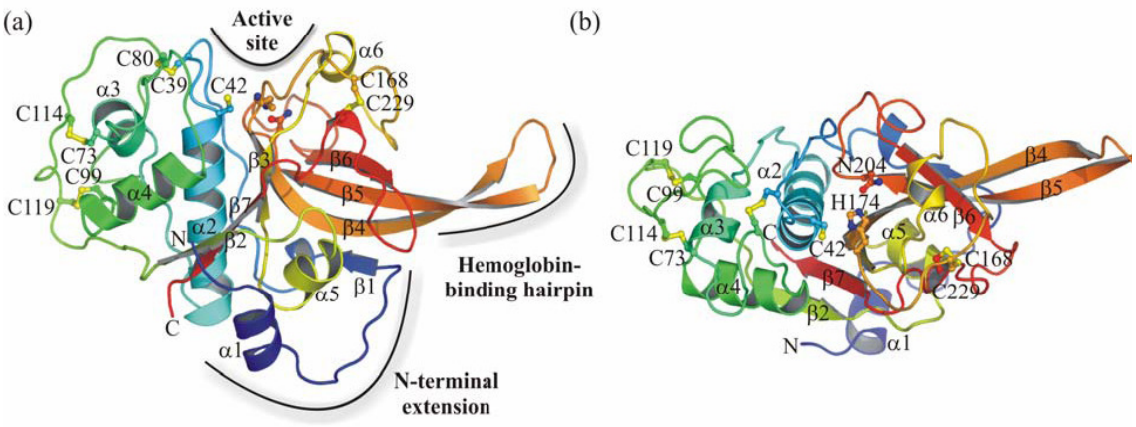


Figure 47. Overall view of falcipain-2. (a) Ribbon representation of FP-2 with the L-domain on the left, the R-domain on the right, and the active site cleft located on top of the molecule. The spectral colour coding is according to sequence, from dark blue (amino terminus) to dark red (carboxy terminus). Disulfide bonds and active site residues are shown in ball-and-stick. (b) An orthogonal view relative to panel (a) generated by a ~90º rotation towards the viewer.

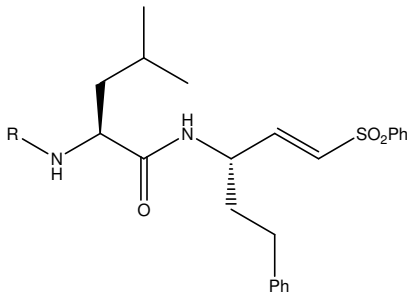
3.3- Design and Synthesis of New CPI Inhibitors

3.3.1- Aim of the project

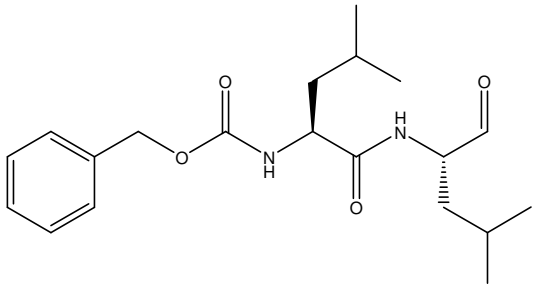
Available information related to structure-activity correlations, mainly from studies by Rosenthal and co-workers, show a consistent trend throughout the investigation: inhibitors containing Leu at the P2 position are about an order of magnitude more potent than those with Phe at this position[43] and the unnatural amino-acid Homo-Phe at the P1 site of a series of vinyl sulfone inhibitors (117, Figure 48) showed to be the preferred structural features for maximal inhibition. Also, it has been shown that FP-2 is homologous to the Trypanosomal enzyme cruzain. Several X-ray structures of cruzain containing covalently bound Z-Phe-Ala-fluoromethyl ketone (compound 101, Figure 48) provided clues as to the important bonding actions at the active site[57]. Given the improved activity observed by the incorporation of a homophenyl alanine at the P1 (-Me for -Hphe) position, it was clear that an additional hydrophobic pocket was available in FP-2 for accommodation of this substituent. Thus, we intended to include the unnatural amino acid homophenylalanine in our inhibitors in line with the observed beneficial effects of this substituent in available inhibitors.

A common feature of virtually all cysteine protease inhibitors is an electrophilic functionality, such as a carbonyl or a Michael acceptor, which can react with the nucleophilic cysteine residue. This is the case with peptidyl aldehydes (118, Figure 48), which were, as mentioned before, the first class of reversible inhibitors to show a good antimalarial profile. However, the inherent reactivity of the aldehyde pharmacophore to nucleophilic attack and oxidation is a considerable liability for attaining good pharmacokinetics. In order to overcome the drawbacks predicted by the use of

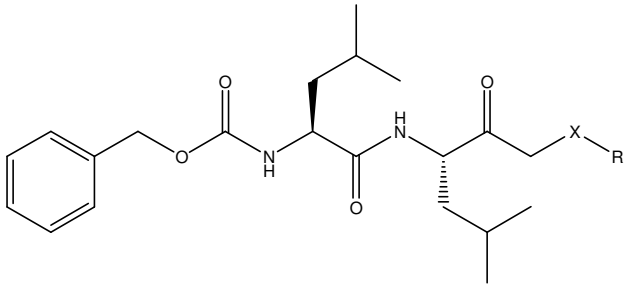
aldehydes[58] in this class of compounds we planned to incorporate an electron-poor moiety in the form of a reactive carbonyl "thiol acceptor". This ketone-based pharmacophore is expected to be chemically more stable and also to present the advantage of being able to display functionality on both sides of the carbonyl, providing the potential to achieve enhanced specificity through multiple interactions with the active site. Indeed, Veber and co-workers have recently reported several examples of reversible peptidyl ketones with nanomolar inhibition towards cathepsin K (119, Figure 48).



117. R= CBz, $PhSO_2$



118



119. R= Ar, X= O, S

Figure 48. Known cysteine protease inhibitors.

The aim of this project is to examine the structure-activity relationships of a series of novel *alpha* phenoxy carbonyl inhibitors of FP-2 of general structure 120 (Figure 49).

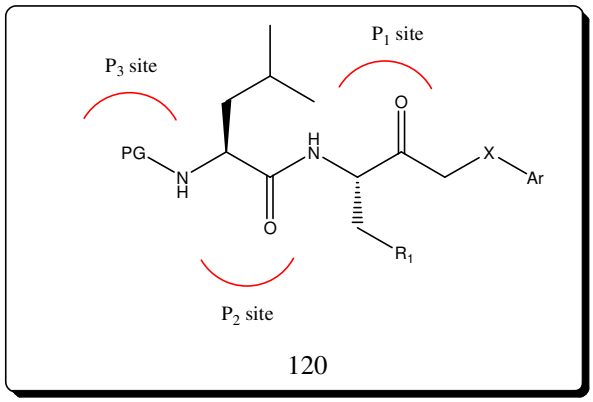


Figure 49. Target peptidyl inhibitor template

Considering the theoretical criticism that any peptide based inhibitor might be subjected to host amidases, and although the presence of the Homo-Phe residue in existing analogues has been proposed to reduce host proteolysis, the peptidic nature of target template 120 remains a concern. With this fact in mind, a second class of carbonyl-based inhibitors was also designed and synthesized as a peptidomimetics analogue of known inhibitors (121, Figure 50), where the pyridone ring system replaces part of the peptide backbone (template 122, Figure 50).

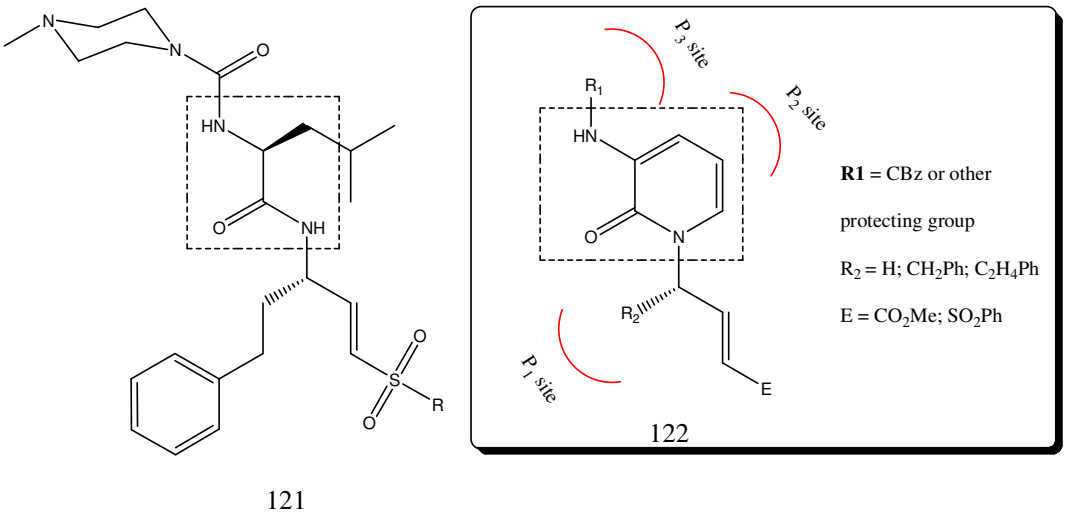


Figure 50. Target peptidomimetic inhibitor template

Most CP inhibitors bind to the enzyme in an extended conformation and the pyridone heterocycle should enable such an arrangement.

Peptides and unconstrained peptide inhibitors generally exist as a mixture of conformers in solution. Limiting the number of possible conformations a molecule can adopt, by designing conformationally constrained inhibitors, could, in theory, improve binding energies, by lowering the entropic contribution to the binding of a particular conformation[59].

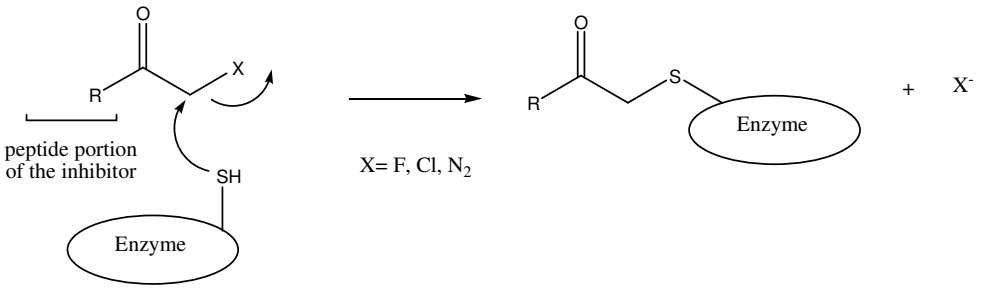
3.3.2- Proposed Mechanism of Action

The compounds described in this chapter act by inhibiting a particular enzyme, resulting in its inactivation. Most of the inhibitors of cysteine proteases reported in the literature depend on the chemical interaction of an electrophilic "warhead" with the cysteine thiolate anion of the active site. This inactivation of the enzyme can, in principle, be either reversible, when temporary and based in interactions other than permanent covalent bonding, as is the case for the interaction with aldehyde inhibitors[60] and nitriles[61], or irreversible, when a covalent bond between the enzyme and the inhibitor is formed, as is the case of interaction with α-halomethyl ketones[62], diazomethylketones[63] and vinyl sulfides and sulfones[42].

Peptidyl aldehydes are potent reversible inhibitors that form transition-state-like hemithioacetals with the active site of the cysteine protease.

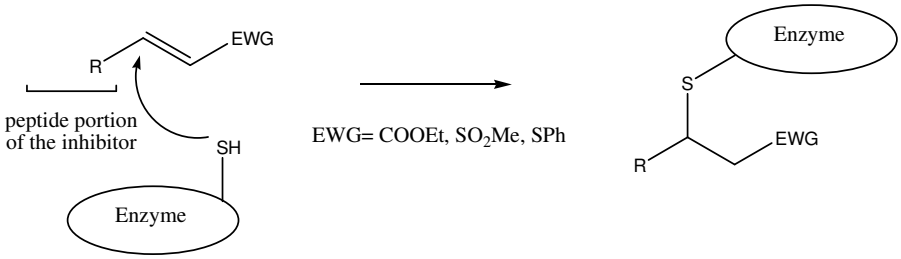
Irreversible inhibition of cysteine proteases rely upon several methods: (i) direct alkylation of the active site thiol group through nucleophilic attack at the electron-

deficient position of the carbonyl inhibitor followed by displacement of the nucleofuge, as is the case of inhibition by peptide α-fluoromethyl ketones or diazomethylketones (Scheme 12):



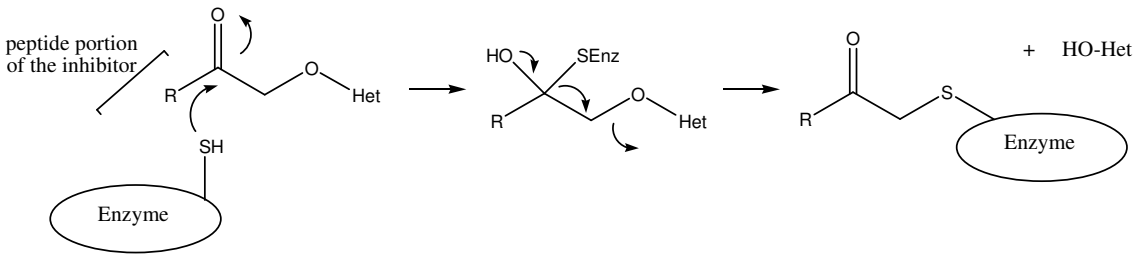
Scheme 12. Enzyme alkylation via an S_N2 mechanism.

(ii) Irreversible inhibition via formation of a covalent bond between the nucleophilic thiol group of the enzyme and the inhibitor bearing a Michael acceptor group through a 1,4-conjugate addition mechanism (Scheme 13):



Scheme 13. Enzyme alkylation via a 1,4 conjugate addition.

(iii) Irreversible inactivation via formation of a covalent bond between an activated carbonyl with a suitable α-leaving group and the enzyme, through carbonyl attack by the cysteine thiolate anion to give a hemiacetal intermediate, which collapses via a 1,2-thermal migration of the thiolate and subsequent intramolecular displacement of the α-keto-leaving group (Scheme 14):



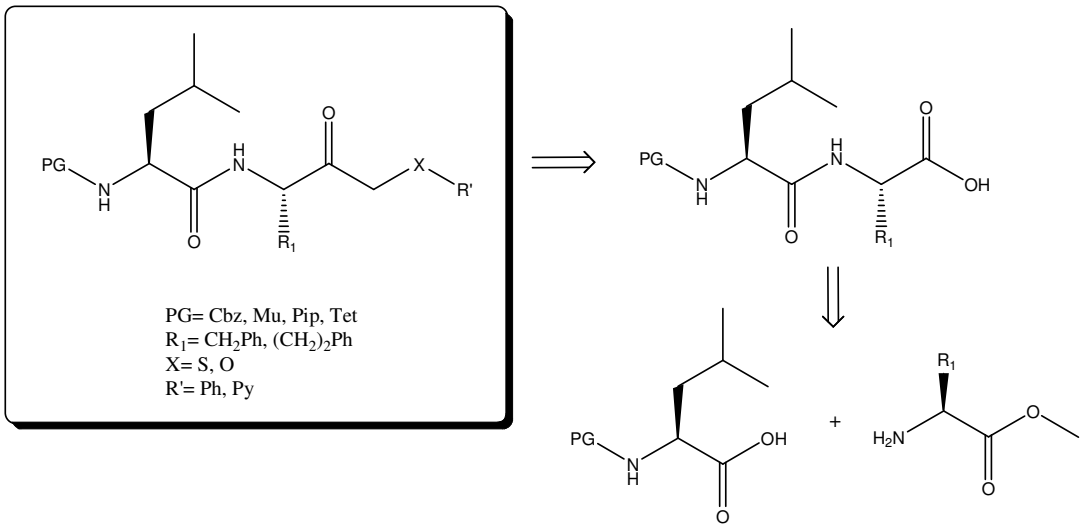
Scheme 14. 1,2-Thermal migration with displacement of α-leaving group.

The usefulness of these inhibitors on inactivating the enzyme depends therefore not only of the "lock and key" fit of the peptide portion, but also on the reactivity of the bond holding the α-leaving group and selectivity of the process, that is, the leaving group should only be reactive to the intramolecular displacement via the 1,2-migration of sulphur in the breakdown of the hemithioacetal intermediate.

3.3.3- Chemistry Developed

3.3.3.1- Peptide-based Inhibitors

The proposed retrosynthetic route towards the target dipeptidyl cysteine protease inhibitors is depicted in scheme 15:

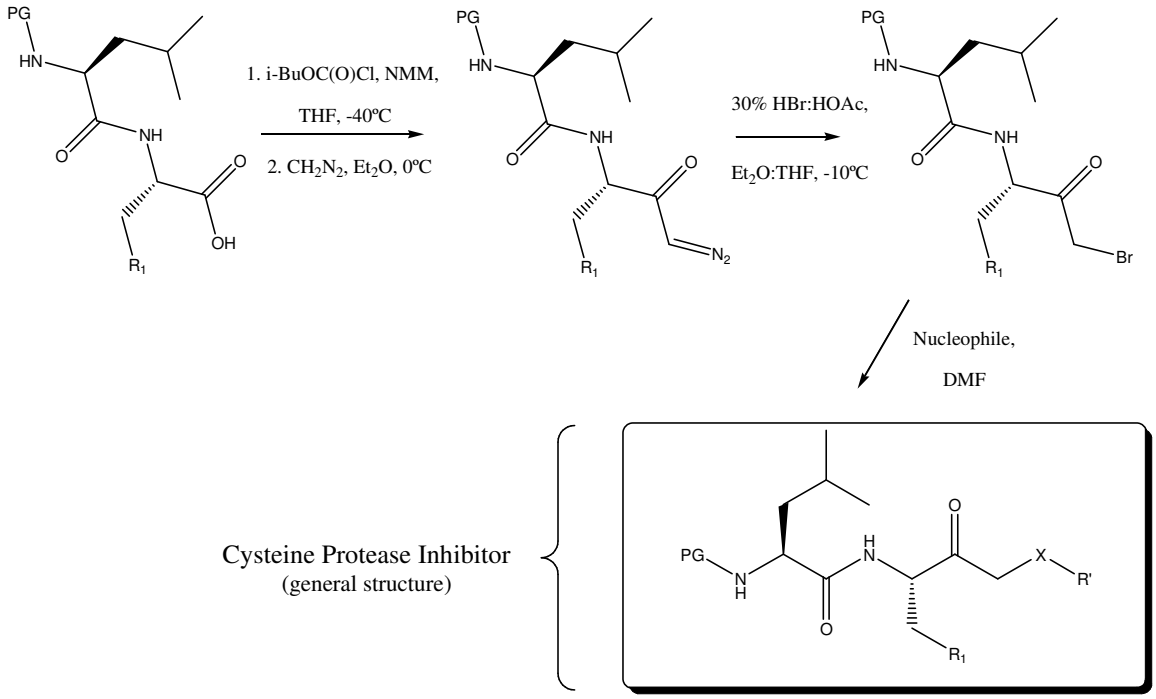


Scheme 15

The initial steps of the chemistry developed involve solely the preparation of the dipeptide. The first chemical transformations in the synthesis described were related to the blocking of the free amine and carboxylic-termini of the chosen amino acids by several protecting groups. The chosen amino acids for this synthesis were, as sustained in the rationale of this project, leucine for P2 position and homo-phenylalanine for the P1 position. Nevertheless, the whole synthetic route was firstly tried and optimised using phenylalanine for the P1 position, and only after proof of efficacy was the

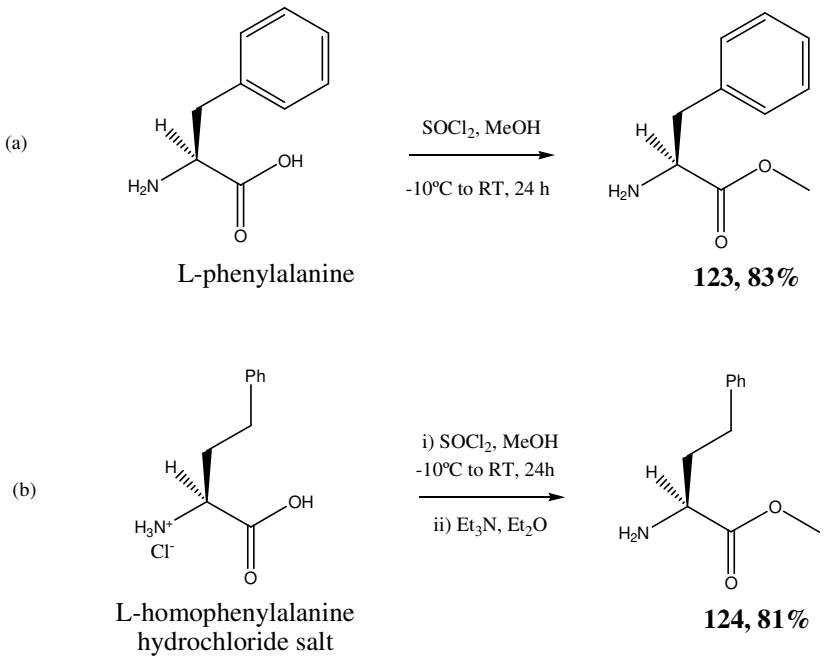
synthesis performed with the unnatural amino acid Homo-phe, due to availability and cost of materials.

From the dipeptide, the designed chemical route allowed formation of the desired cysteine protease inhibitors in only 3 steps (Scheme 16). Purification of intermediates and target molecules was often possible by precipitation of the product in a suitable solvent system



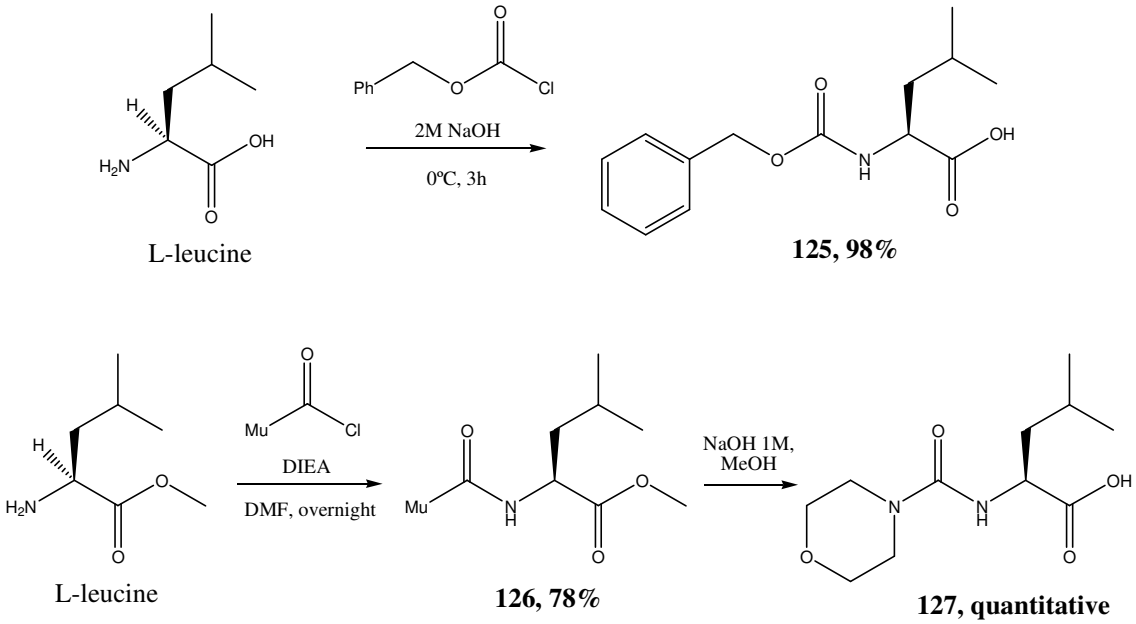
Scheme 16

From scheme 15, protection of the carboxylic acid termini of the second amino acid was achieved by treating the amino acid with thionyl chloride in methanol, at -10ºC. As *L*-homophenylalanine was used as the hydrochloride salt, addition of triethylamine in ether was needed in order to release the free amino group. Both protections afforded the desired products (Scheme 17, reactions (a) and (b)) in excellent yields.



Scheme 17.

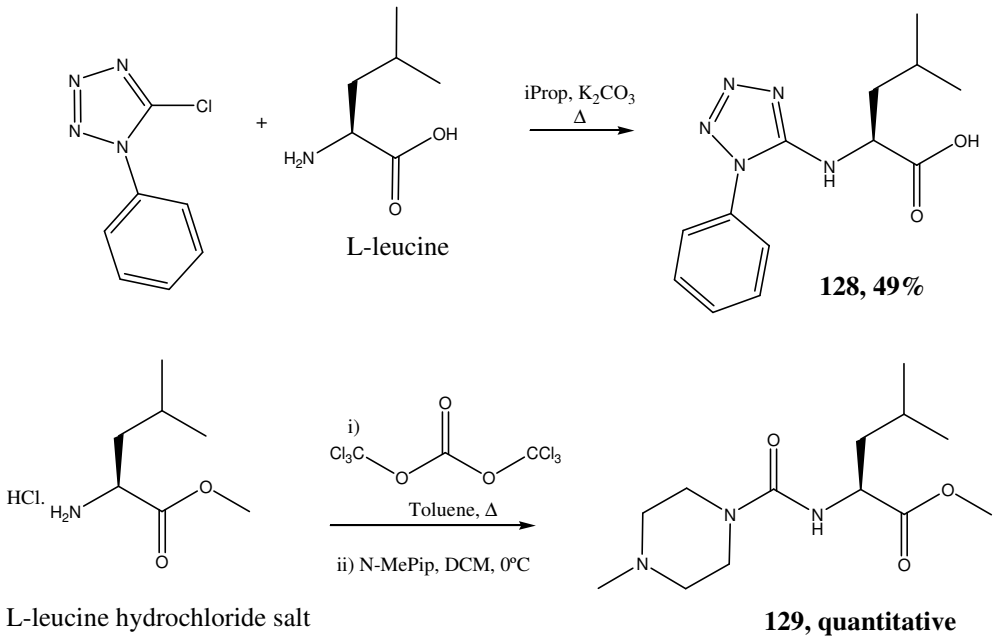
We developed several protections for the amino-terminal group of leucine, as this was a blocking group designed to be incorporated in the final target inhibitors. Though it seems that changing this group at the P3 position doesn't change significantly the compound's activity, it is certainly an available place for structural modifications, which may have influence on the overall compound's solubility. Bearing in mind that the main aim of this project, was the development of an efficient synthetic approach to FP-2 cysteine protease inhibitors, we selected the benzyloxycarbonyl, Cbz, and the morpholine-urea group, Mu, as the constituents of our final molecules (Scheme 18). We considered morpholine-urea to be a suitable alternative to carbobenzyloxy protection, in order to have inhibitors more easily soluble in aqueous solutions. Nevertheless, other protections were also assayed, as depicted in scheme 19.



Scheme 18

The benzyloxycarbonyl protection[64] was achieved quantitatively for the *L*-leucine substrate, by addition of benzyl chloroformate in a sodium hydroxide solution (125). Different procedures for morpholine urea protection were tried, but the use of N-morpholine carbonyl chloride in the presence of DIEA as the base, at RT, was the most successful, being easy to carry and high yielding[61]. A standard ester hydrolysis[64] of compound 126 afforded the desired product 127, quantitatively.

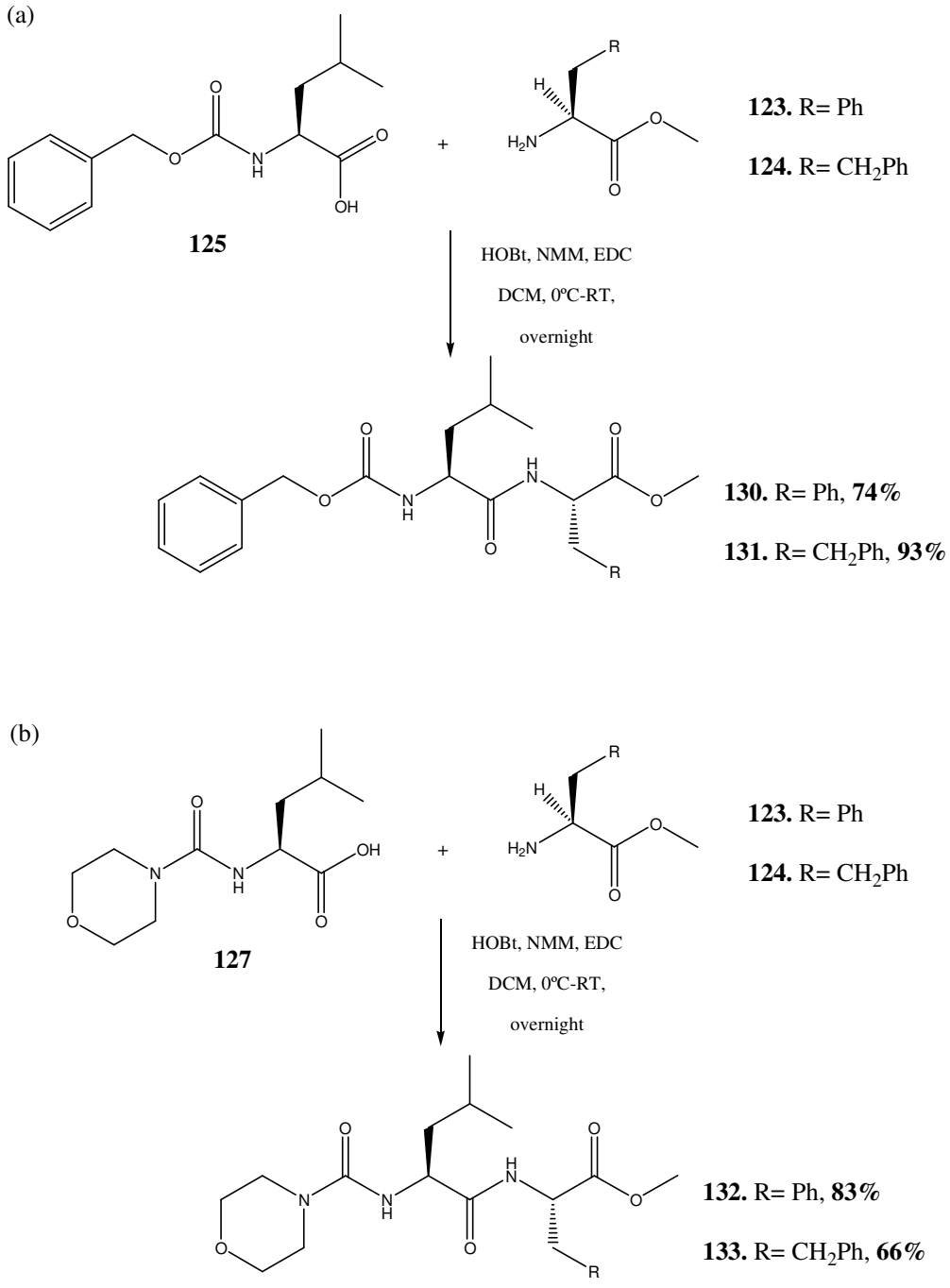
The piperazinyl protection[65] was also achieved quantitatively (129), by activating the amino-termini of *L*-leucine with triphosgene, at high temperature and then adding N-methylpiperazine at 0ºC. The phenyl tetrazole protection[66] was however achieved with lower yield (128), probably caused by conversion of 5-chloro-1-phenyltetrazole to 1-phenyl-tetrazol-5-one in the alkaline media used. Substrates 128 and 129 (Scheme 19) have a more accentuated hydrophilic character than compounds 125 and 127 (scheme 18).



Scheme 19

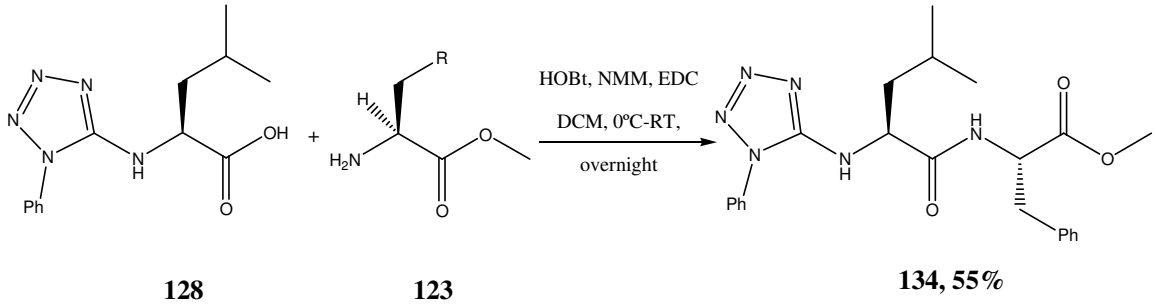
The coupling step was performed using a methodology selected from those described in the literature relative to this type of chemistry. The major criteria for selection being the good results reported in matters of avoiding racemization. Treatment of the amino-protected leucine and the carboxylic acid-protected phenylalanine and homo-phealanine with a mixture of hydroxybenzotriazole (HOBt), *N*-methylmorpholine (NMM) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), at 0ºC, followed by stirring at RT overnight, afforded the desired dipeptides, after column chromatography, in very good yields (Scheme 20). The carbodiimide EDC is generally used as a carboxyl activating agent[67] for the coupling of primary amines to yield amide bonds. As such, it is used here to activate the free carboxylic acid of the protected leucine towards formation of the amide bond. It reacts with the water formed, forming a stable urea. Hydroxybenzotriazole is an additive used to decrease side reactions and improve yields; dichloromethane was the solvent of choice, due to its low dielectric

constant, which also minimizes the occurrence of side reaction leading to formation of undesired products.



Scheme 20

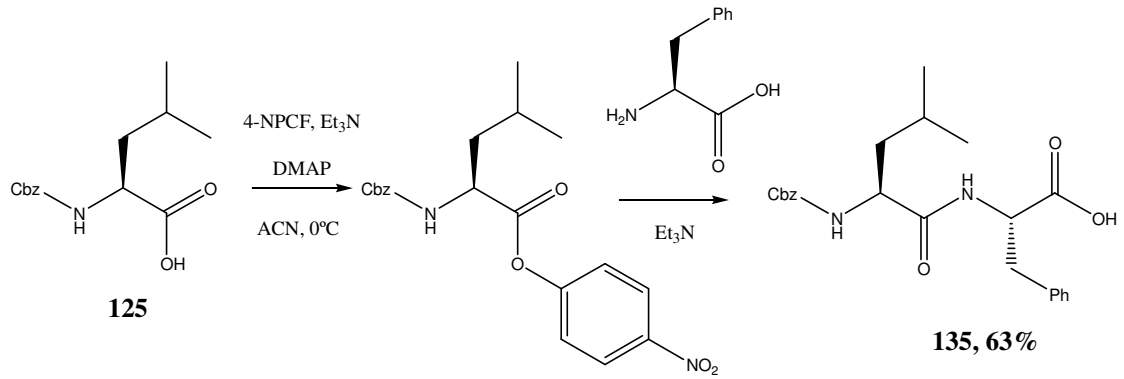
We also tried the coupling procedure using one of the more polar P3 protections, the tetrazole-protected leucine derivative (Scheme 21).



128 **123** **134, 55%**

Scheme 21

A different chemical route for the amino acid coupling had been tried previously within the group. Following this approach, the obtained product was the unprotected carboxylic acid-dipeptide, ready to use in further chemistry (Scheme 22). The coupling step was achieved by treating Cbz-protected *L*-leucine with triethylamine, 4-nitrophenyl chloroformate and 4-dimethylaminopyridine, at 0ºC; after formation of the activated carboxylic acid (TLC monitoring), a mixture of an excess of *L*-phenylalanine in water, with triethylamine was then added, and the desired dipeptide 135 obtained in moderate yield.

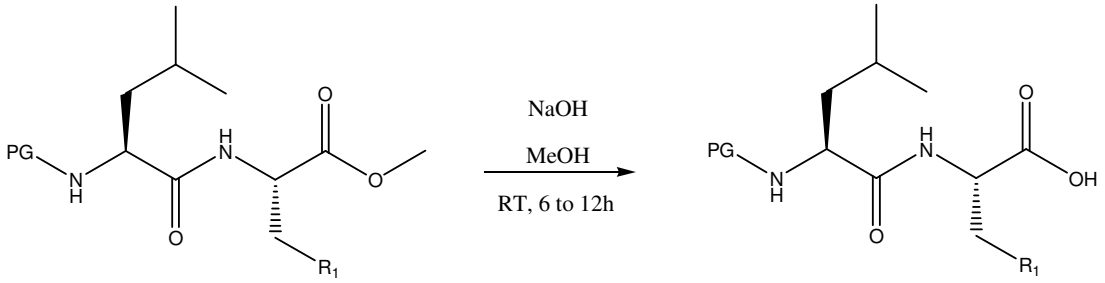


125 **135, 63%**

Scheme 22

Although representing a synthetic improvement to the proposed chemical route herein used, it was lower yielding. We felt that, before studying this new route more carefully, it would be better to use a well-proven procedure, in order to go on with the previously devised synthetic strategy.

For the subsequent transformations we needed to have the terminal carboxylic group available, thus, we hydrolysed the terminal ester function of the synthesised dipeptides, with very good yields, as shown in scheme 23.



130. PG= Cbz, R_1= Ph

131. PG= Cbz, R_1= CH_2Ph

132. PG= Mu, R_1= Ph

133. PG= Mu, R= CH_2Ph

136. PG= Cbz, R_1= Ph, **85%**

137. PG= Cbz, R_1= CH_2P, **99%**

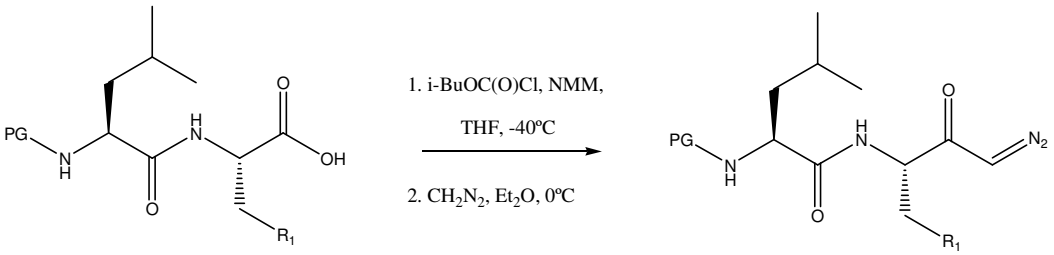
138. PG= Mu, R_1= Ph, **90%**

139. PG= Mu, R= CH_2Ph, **97%**

Scheme 23

The generated free dipeptide acids were then converted into the corresponding α-diazoketones[68,69] in yields about 40%, by formation of the mixed anhydride with *iso*-butylchloroformate, followed by removal of the precipitated salts and trapping with freshly distilled diazomethane (Scheme 24). Diazomethane was generated *in-situ* from commercially available *N*-methyl-*N*-nitroso-*p*-toluenesuphonamide (Diazald^{TM}) using a custom-made apparatus considered appropriate for small amounts of distillation[70]. Risks highlighted in the literature were circumvented by adhering to simple safety measures

related to handling of precursors and products, preparation of diazomethane and neutralization of any product in excess. α-Diazoketone 140 was obtained pure following precipitation in a mixture of diethyl ether and hexane, in a yield comparable to those obtained for the pure compounds, after column chromatography.



136. PG= Cbz, R_1= Ph

137. PG= Cbz, R_1= CH_2Ph

138. PG= Mu, R_1= Ph

139. PG= Mu, R_1= CH_2Ph

140. PG= Cbz, R_1= Ph, **38.5%**

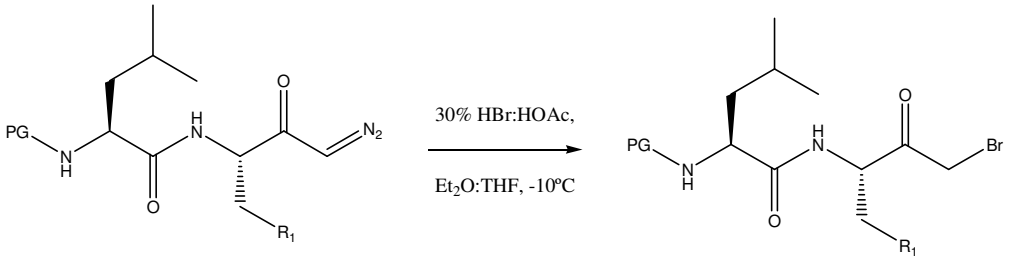
141. PG= Cbz, R_1= CH_2Ph, **40%**

142. PG= Mu, R_1= Ph, **81% crude**

143. PG= Mu, R_1= CH_2Ph, **33%**

Scheme 24.

Treatment of the α-diazoketones with an excess of a solution of HBr (30%) in acetic acid, afforded the corresponding bromomethylketones (Scheme 25).



140. PG= Cbz, R_1= Ph

141. PG= Cbz, R_1= CH_2Ph

142. PG= Mu, R_1= Ph

143. PG= Mu, R_1= CH_2Ph

144. PG= Cbz, R_1= Ph, **47%**

145. PG= Cbz, R_1= CH_2Ph, **68%**

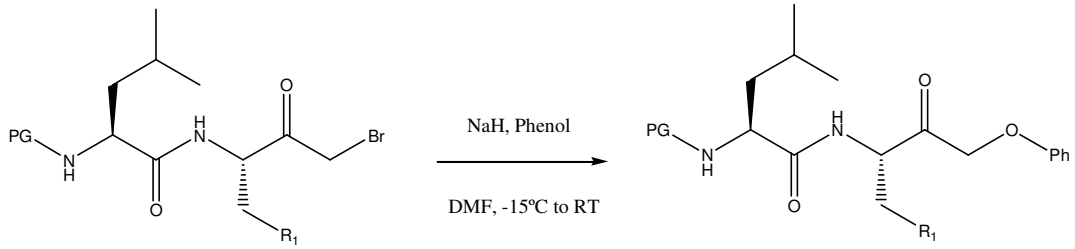
146. PG= Mu, R_1= Ph, **34%**

147. PG= Mu, R_1= CH_2Ph, **87%**

Scheme 25

Products 144 to 147 were obtained pure after purification by column chromatography and are stable compounds. The yields varied quite a bit, appearing that compounds bearing the unnatural amino acid homo-phenylalanine, with the benzyl substituent, are more susceptible to bromination under these conditions than those resonance-stabilised by the phenyl. Isolation of these brominated compounds represented an improvement to previous work within the group, where no purification seemed possible.

Displacement of bromine by a nucleophile allowed the introduction of a functionality on one side of the ketone. Reaction of the bromomethyl ketones with sodium phenoxide in dimethylformamide, at -15ºC, afforded the desired target phenoxymethyl ketones 148, 149 and 150 in reasonable yields (Scheme 26).



144. PG= Cbz, R_1= Ph

145. PG= Cbz, R_1= CH_2Ph

146. PG= Mu, R_1= Ph

147. PG= Mu, R_1= CH_2Ph

148. PG= Cbz, R_1= Ph, **43%**

149. PG= Cbz, R_1= CH_2Ph, **58%**

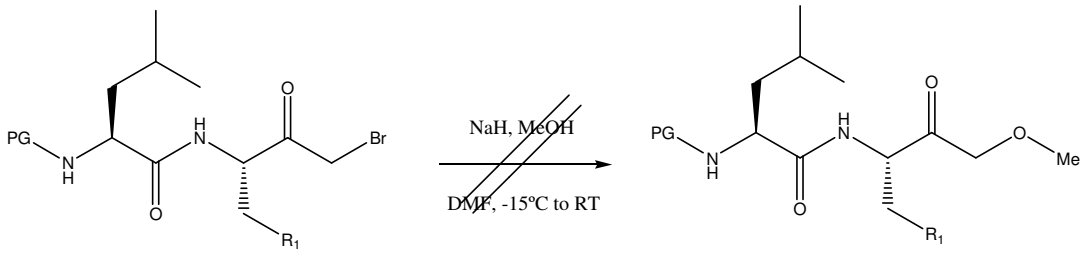
150. PG= Mu, R_1= Ph, **54%**

Scheme 26

Unfortunally, bromomethylketone 147 was left at room temperature for a couple of weeks and, though stable at low temperature, degraded under storage conditions, making the product of this last step impossible to purify due to the very small amounts involved at this point of the synthesis. Nevertheless, compound 150 was successfully synthesised and, according to results from earlier steps, we can trust that synthesis of the homophe-corresponding target molecule should also be possible. However, an

unexpected result came from applying these conditions to bromoderivative 146: though starting material 146 seemed pure (all analysis seemed clear and the compound came out as single peak from the hplc column), when passing target compound 150 through the hplc column, we could isolate two fractions in a 1:1 proportion. These fractions were characterized and showed slight differences in their NMR spectra.

We tried to obtain the same target molecules, but with a methoxy substituent, in order to gain insight into structure-activity relationships. Replacement of a phenyl group by a methyl group would give us some information about the more appropriate degree of bulkiness for fitting the enzyme's S1' pocket. However, following the experimental procedure (same as depicted in scheme 26), the desired target molecules (scheme 27) were not produced. In fact, from analysis of the characterization data, the reaction lead to formation of the α-hydroxy ketones, probably caused by the presence of water in the medium.



i. PG= Cbz, R_1= Ph

ii. PG= Cbz, R_1= CH_2Ph

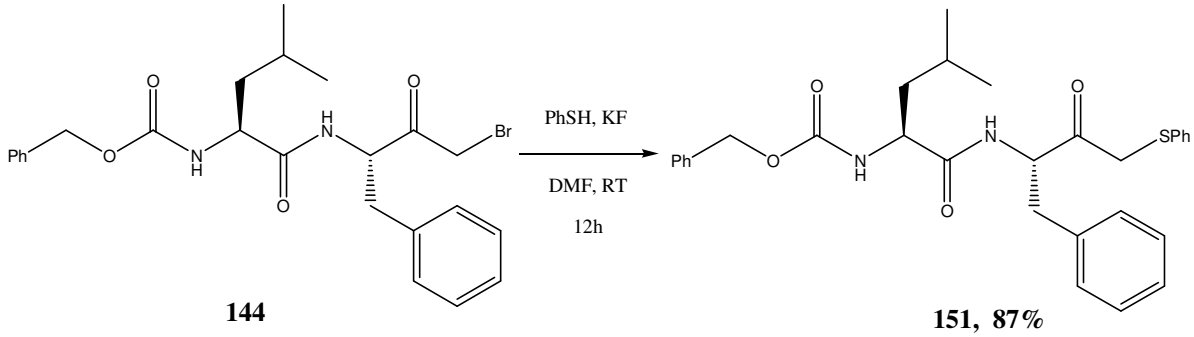
i'. PG= Cbz, R_1= Ph

ii'. PG= Cbz, R_1= CH_2Ph

Scheme 27

The incorporation of sulphur instead of oxygen is an option well sustained within our chemical route, since both atoms have nucleophilic properties. Moreover, mercaptomethyl ketones have shown to be efficient inhibitors of the cysteine protease cruzain[71], with improved potency over other selected substituted methyl ketones, such

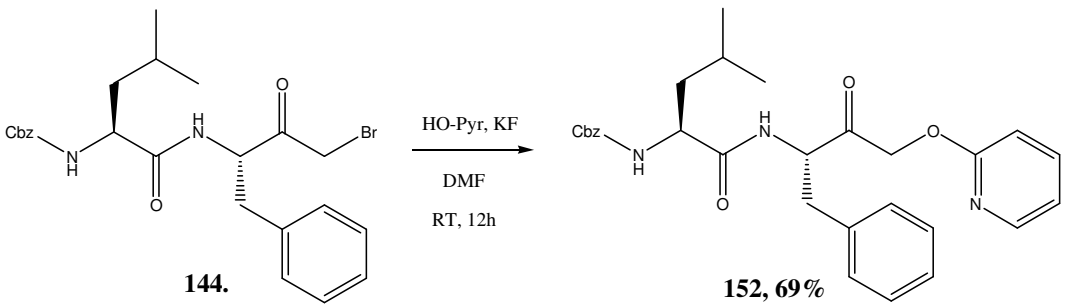
as amidomethylketones. So, we have decided to introduce this functionality. Thiomethyl ketone 151 was synthesized by treatment of bromomethyl ketone 144 with thiophenol and KF in dimethylformamide at room temperature[68]. The required product was obtained in 87% yield. (Scheme 28).



144 **151, 87%**

Scheme 28

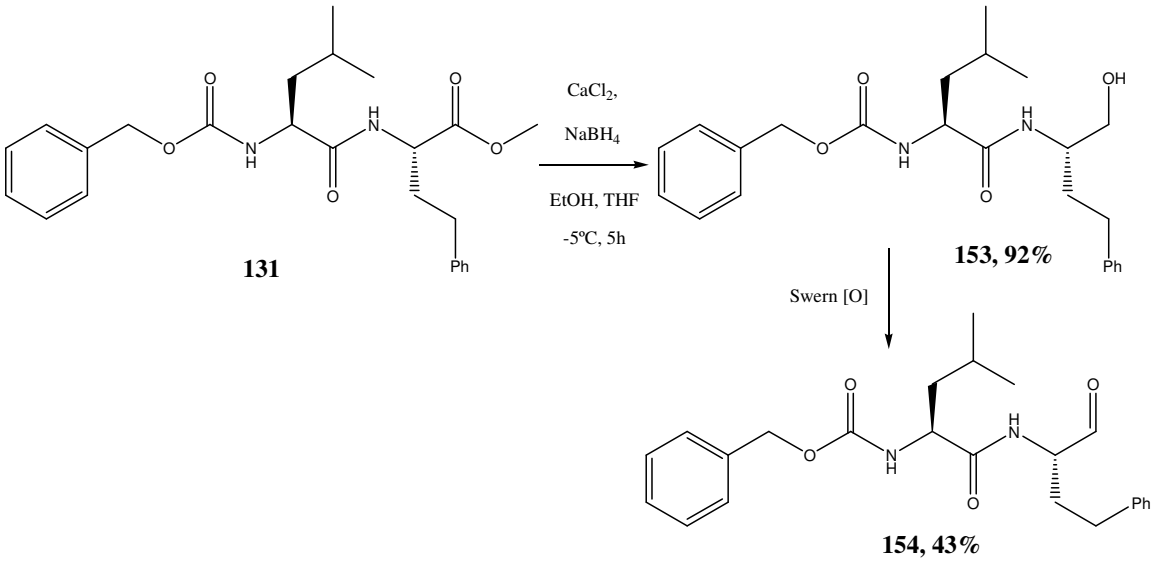
The number of hydrophobic phenyl rings in the inhibitor structures was a source of concern, since good solubility in aqueous solutions is an important requirement for the drug's success (increase of bioavailability). Replacement of the Cbz group by a morpholinecarbonyl group was one of the strategies used to circumvent this question; another could be to functionalize the methyl ketone with a substituent less hydrophobic, such as an hydroxyl pyridine ring. The above described synthetic procedure was used, as in scheme 28, and compound 152 was easily obtained, pure, by precipitation from a mixture of diethyl ether/hexane, in good yield.



144. **152, 69%**

Scheme 29

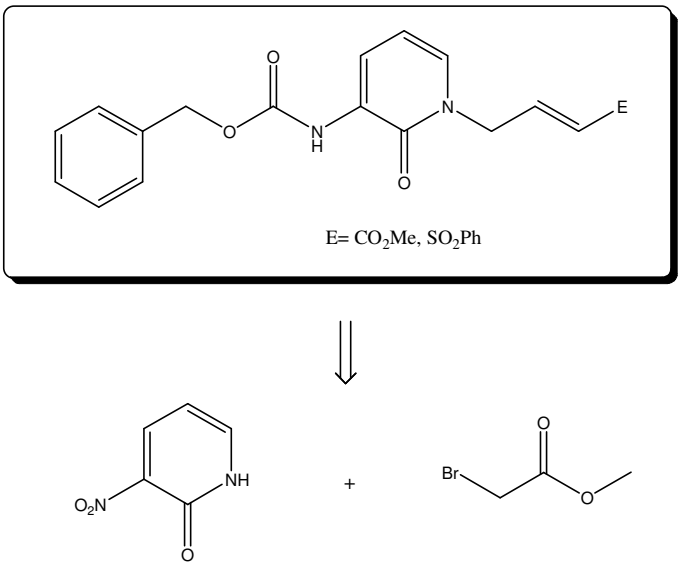
Target molecules bearing the unnatural amino-acid homophenylalanine were the most promising, as stated before in the rationale of the project. In order to have a control compound for comparison, upon *in vitro* testing, we decided to synthesize the dipeptide aldehyde (Figure 51) analogous to our compounds, previously synthesized and tested[43] by Rosenthal and co-workers. The synthetic route towards the dipeptidyl aldehyde cysteine protease inhibitor 154 is depicted in scheme 30. Given the sensitivity of the substrate involved, we adopted an indirect form of obtaining the aldehyde: first we reduced the dipeptide 131 to the alcohol 153, in the presence of calcium chloride using sodium borohydride as the reducing agent[44]. Reduction was achieved in high yield and the resulting alcohol was then selectivity oxidized to the target dipeptidyl aldehyde 154, *via* a standard Swern oxidation. This last step wasn't very effective, given the poor yield obtained. Other synthetic strategies could be employed, as alternative routes to obtain the aldehyde; however, we believe that our experimental conditions for the Swern oxidation might not have been fully optimised, requiring further work.



Scheme 30. Z-Leu-Hphe-al *in-vitro* testing results for comparison: IC_{50} for falcipain-2: 2 nM; IC_{50} for inhibition of parasite development: 30 nM.

3.3.3.2- Peptidomimetic Inhibitors

The proposed retrosynthetic route towards the 2-pyridone-containing cysteine protease peptidomimetic inhibitors is depicted below (schemes 31 and 32), with respect to the presence or absence of a chiral centre, at the P1 site. From a synthetic viewpoint, the original aim in this project was to develop a synthetic approach to target molecules with the general structure depicted in scheme 31.



Scheme 31

Later, the synthetic strategy was altered, in order to introduce chirality at the P1 site. There are two commonly used ways by which chiral compounds can be synthesized in the form of a single enantiomer: by doing an asymmetric synthesis (an enantioselective synthetic method) or by beginning the synthesis with a single stereoisomer, and then follow a synthetic pathway that does not affect the chiral centre (Figure 51). We decided to start the synthesis from available optically pure starting materials with the desired substituent.

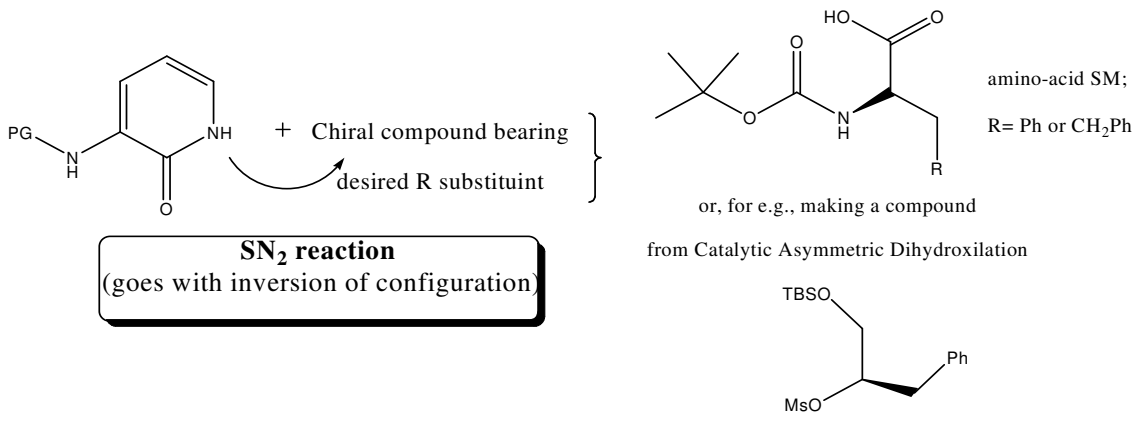
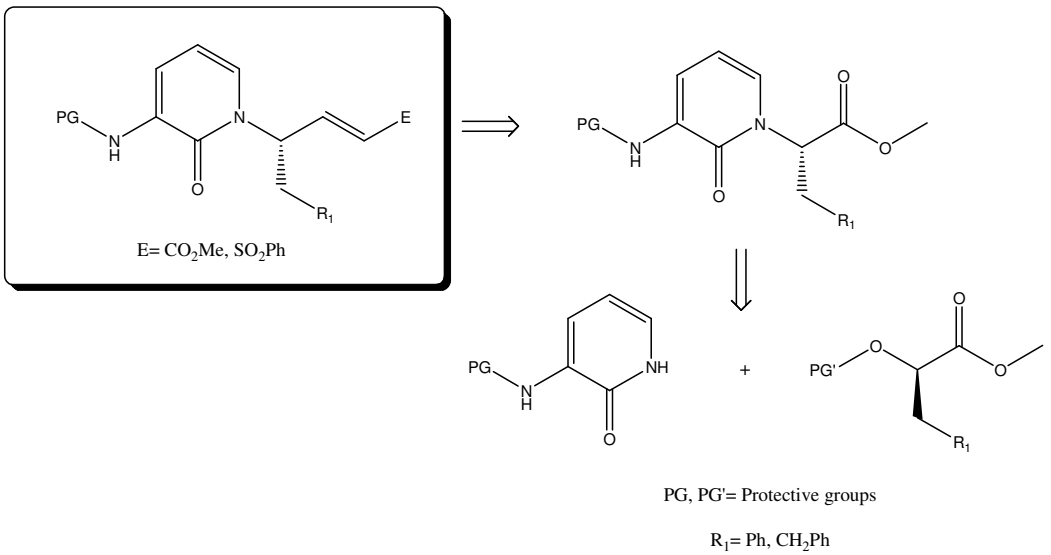


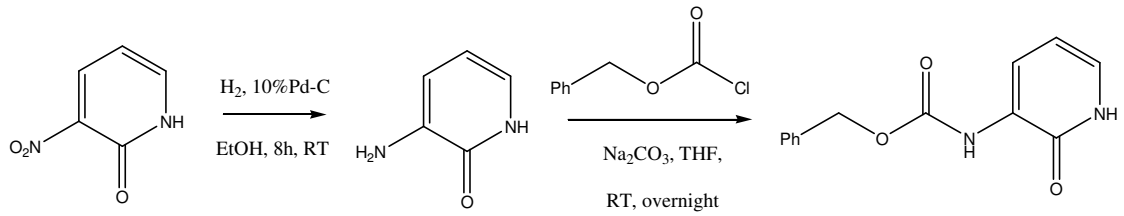
Figure 51

As stated in the rationale of this chapter, the unnatural amino acid homophenylalanine is the preferred substituent for this position in falcipain-2 protease inhibitors; as such, a new synthetic approach was applied, taking this into account (scheme 32). We started initially from D-phenylalanine, a fairly non-expensive alternative, in order to optimize the conditions, and later moved on to use D-homophenylalanine. The same Michael acceptor terminal endings were prepared, for both starting amino acids. The final Michael acceptors were obtained from the intermediate aldehydes, which, in turn, were also themselves seen as target inhibitors within the scope of this project.



Scheme 32

Commercially available 3-nitropyrid-2-one, 155, was reduced quantitatively to the corresponding 3-aminopyrid-2-one 156, via palladium catalyzed reduction in an hydrogen atmosphere. This crude material was then dissolved in THF, and benzyl chloroformate added dropwise, in the presence of sodium carbonate, allowing protection of the primary amino group of the pyridone ring by the carboxybenzyloxy group, Cbz. The required benzyl carbamate was obtained in moderate yield, by crystallization (157, Scheme 33).

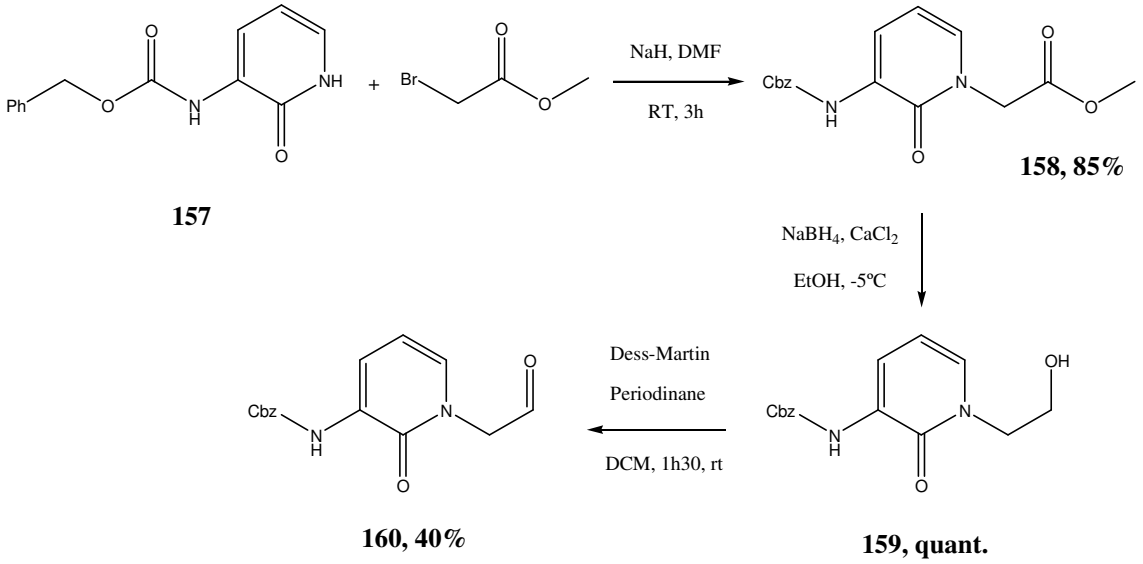


155 **156, 98%** **157, 50%**

Scheme 33

The anion derived from compound 157 upon treatment with sodium hydride, was alkylated at the ring nitrogen by methyl bromoacetate, to give compound 158, in very good yield. Methyl ester reduction to the primary alcohol by $NaBH_4$ afforded the desired compound 159 in quantitative yield, after work-up and without any further purification. The crude alcohol 159 was then dissolved in dichloromethane, at room temperature, and Dess-Martin periodinane was added, in order to selectively obtain the desired aldehyde 160, which was recovered in low yield (Scheme 34). Dess-Martin periodinane, or 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one, is a chemical reagent used to oxidize alcohols to aldehydes and secondary alcohols to ketones[72,73] and it was chosen for this reaction because of its advantages over other oxidants. One of the reasons for its effectiveness is due to its high selectivity for the hydroxyl group, which allows alcohols to rapidly perform ligand exchanges, the first step in the

oxidation reaction. This reagent can be synthesised in the laboratory from 2-iodobenzoic acid, using potassium bromate and acetic anhydride. Better results are obtained when the oxidant is freshly used.

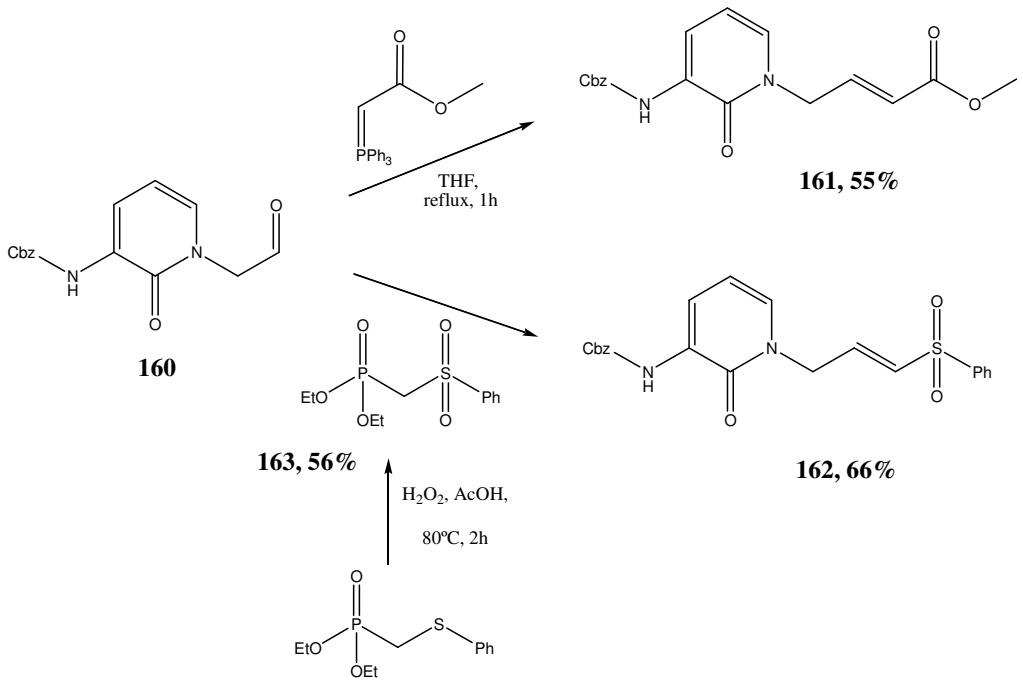


Scheme 34

The devised synthetic strategy to obtain compound 158 involved alkylation of the pyridone ring after reduction and Cbz-protection of the nitro group (schemes 33 and 34) in quantitative yield. A different approach can be found in the literature, where the first step is alkylation of the commercially available 3-nitropyridin-2-one at the NH position, followed by reduction of the nitro group and protection of the amine functionality. This approach could represent a useful modification to the early steps of our synthetic pathway, as it might facilitate the purification of compounds in cases where the chosen pyridone protecting group has a more polar character. However, since when using Cbz as the protection group, both the yields obtained and the simplicity of procedures were very satisfactory, we saw no advantages on changing the strategy.

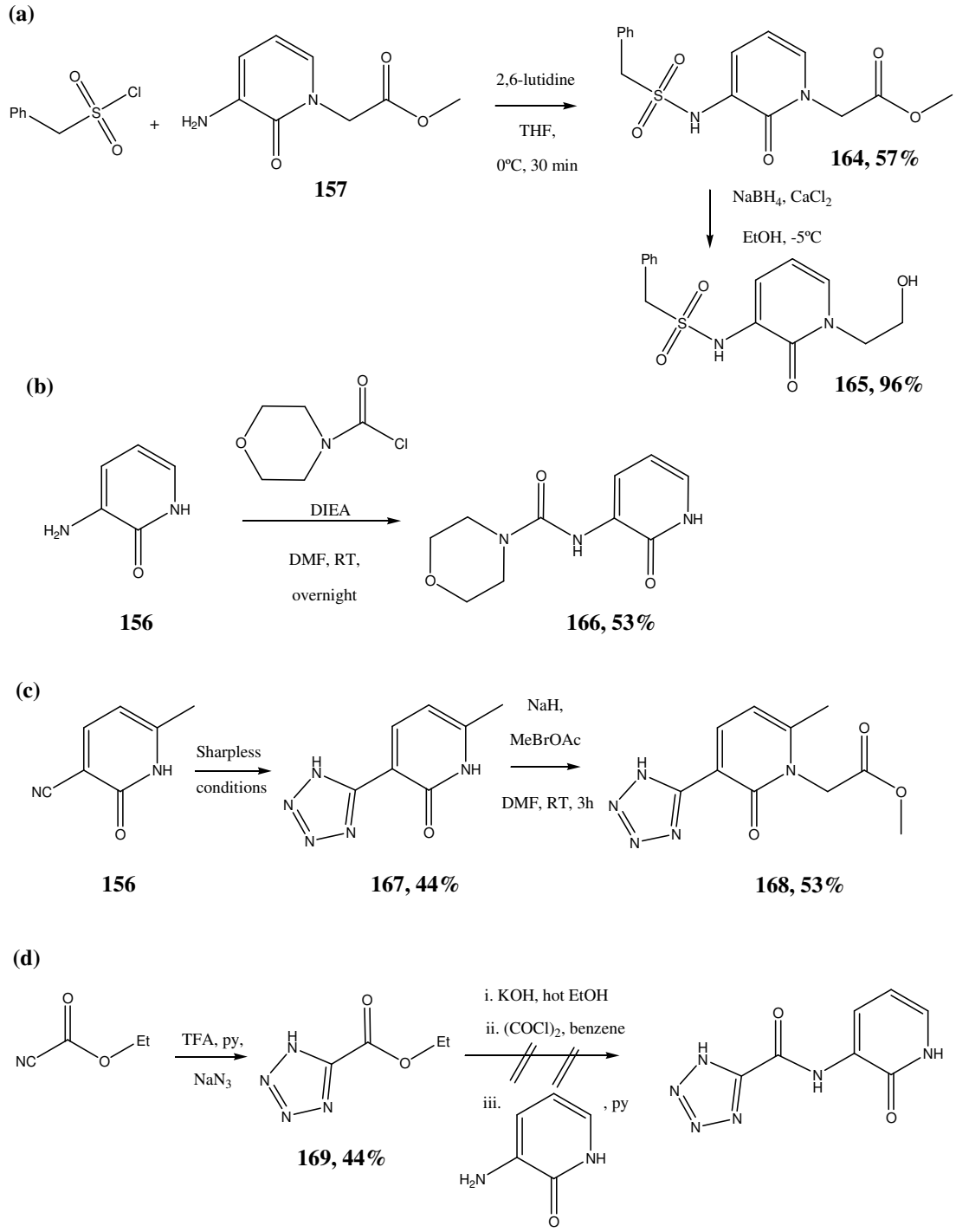
Carrying on with the devised methodology, a key step for the synthesis is the preparation of olefins from the synthesised aldehyde. Olefination of aldehyde 160 with commercially available (methoxycarbonylmethylene)-triphenylphosphorane, in THF, provided one of the desired Michael acceptors, the α,β-unsaturated methyl ester 161, in moderate yield. As was encountered during previous synthesis of peptidyl and peptidomimetic inhibitors[74], this reaction afforded the desired *trans* isomer, with no significative formation of the *cis* isomer[75], as determined by NMR analysis. The Wittig reaction is a classical method for the preparation of unsaturated esters, and like the related reactions of stabilized ylides with aldehydes, shows a preference for the formation of the more stable E-olefins[75].

The other desired Michael acceptor, vinyl sulfone 162, was obtained in good yield, by the Horner-Wadsworth-Emmons reaction of aldehyde 160 with phosphonoester 163, a versatile intermediate commonly used in synthesis and, particularly, as the phosphonate donor for this kind of olefinations[76] (Scheme 35).



Scheme 35

Having obtained the first set of target peptidomimetics, compounds 160, 161 and 162, we decided to investigate some modifications to the designed compounds by having different protections on the pyridone ring, aiming at the preparation of targets with different polarity and solubility characteristics (Scheme 36).

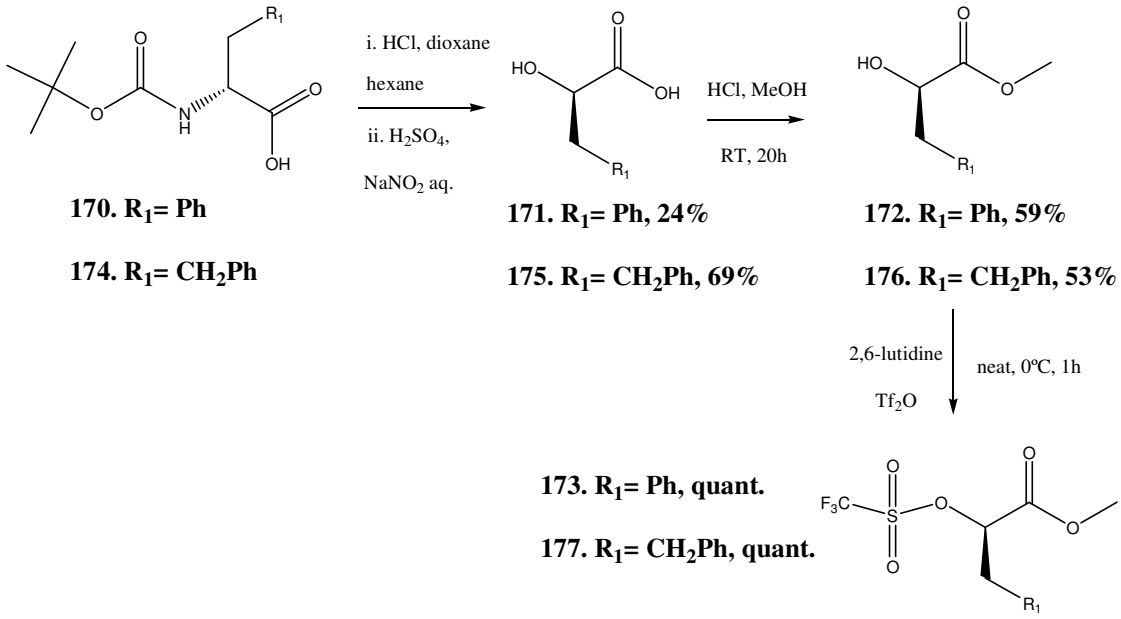


Scheme 36

Compounds 166 and 168 were synthesised at this point with the goal of verifying the chemical stability of the chosen protecting group and alsoto gain insight into the purification techniques needed to apply to compounds bearing these groups.

At this point we had devised a reasonable synthesis of peptidomimetics, though without having a substituent next to the pyridone ring, in the P1 site. The chemistry devised below represents our efforts aimed at obtaining the improved target peptidomimetics shown in scheme 32.

The protected 2-hydroxy acids 173 and 177, required for the coupling reaction with the protected pyridone ring, are obtained from the corresponding commercially available starting materials, Boc-D-phenylalanine and Boc-D-homophenylalanine, 170 and 174 by a two step process: deprotection of the Boc moiety under mildly acidic conditions (by protonation, loss of t-butyl cation and decarboxylation) followed by nitrous deamination, afforded α-hydroxy acid intermediates 171 and 175 with variable yield.



170. R_1= Ph

174. R_1= CH_2Ph

171. R_1= Ph, 24%

175. R_1= CH_2Ph, 69%

172. R_1= Ph, 59%

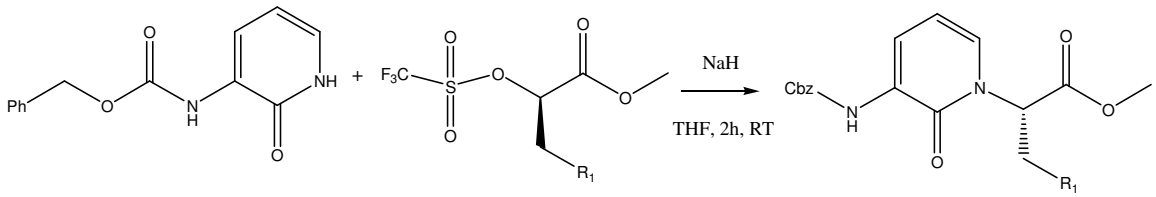
176. R_1= CH_2Ph, 53%

173. R_1= Ph, quant.

177. R_1= CH_2Ph, quant.

Scheme 37

We think that this difference in yields is due to an heavy loss of the phenylalanine derivative upon filtration, during the experimental procedure followed. This method is analogous to that generally utilized for the conversion of α-amino acids to the corresponding α-hydroxy acids with retention of optical purity[77]. Synthesis of the ester intermediates 172 and 176 under acidic conditions was achieved in moderate yields, with some difficulty upon purification, since these compounds are hardly seen by TLC staining or under UV light. These materials were converted to the corresponding triflates 173 and 177 by dropwise addition of trifluoromethane sulfonic anhydride, in quantitative yield (Scheme 37). Triflate is an excellent leaving group, extremely reactive in SN2 reactions. Thus, condensation with the protected pyridone occurred smoothly with good yields, after purification on silica gel (Scheme 38).



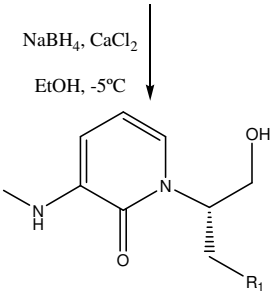
157

173. R_1= Ph

177. R_1= CH_2Ph

178. R_1= Ph, 76%

179. R_1= CH_2Ph, 67%



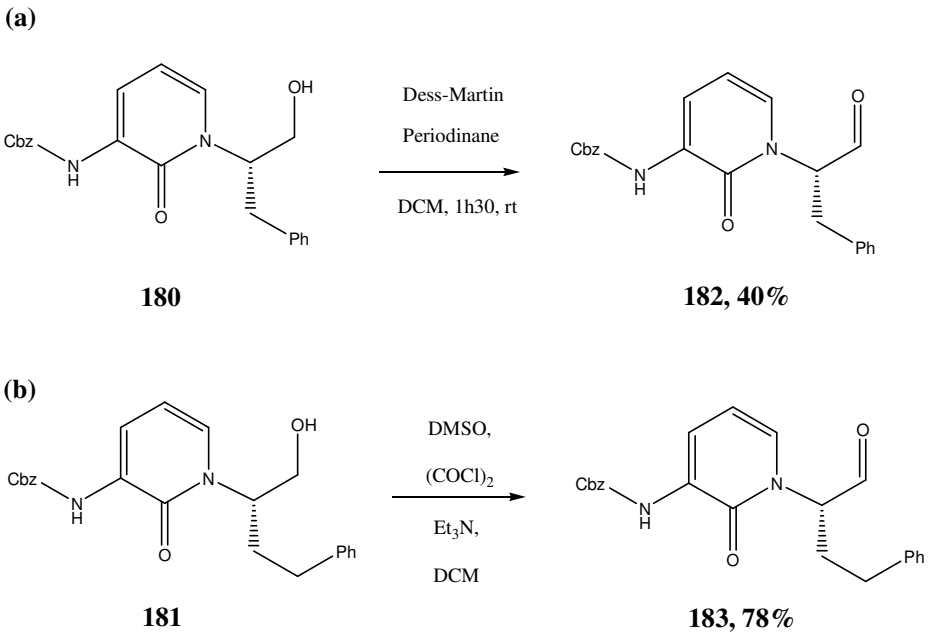
180. R_1= Ph, quant.

181. R_1= CH_2Ph, quant.

Scheme 38

Since the substrate under nucleophilic attack is chiral, this strategy leads to an inversion of stereochemistry, allowing preparation of mimetic intermediates 178 and 179, that can

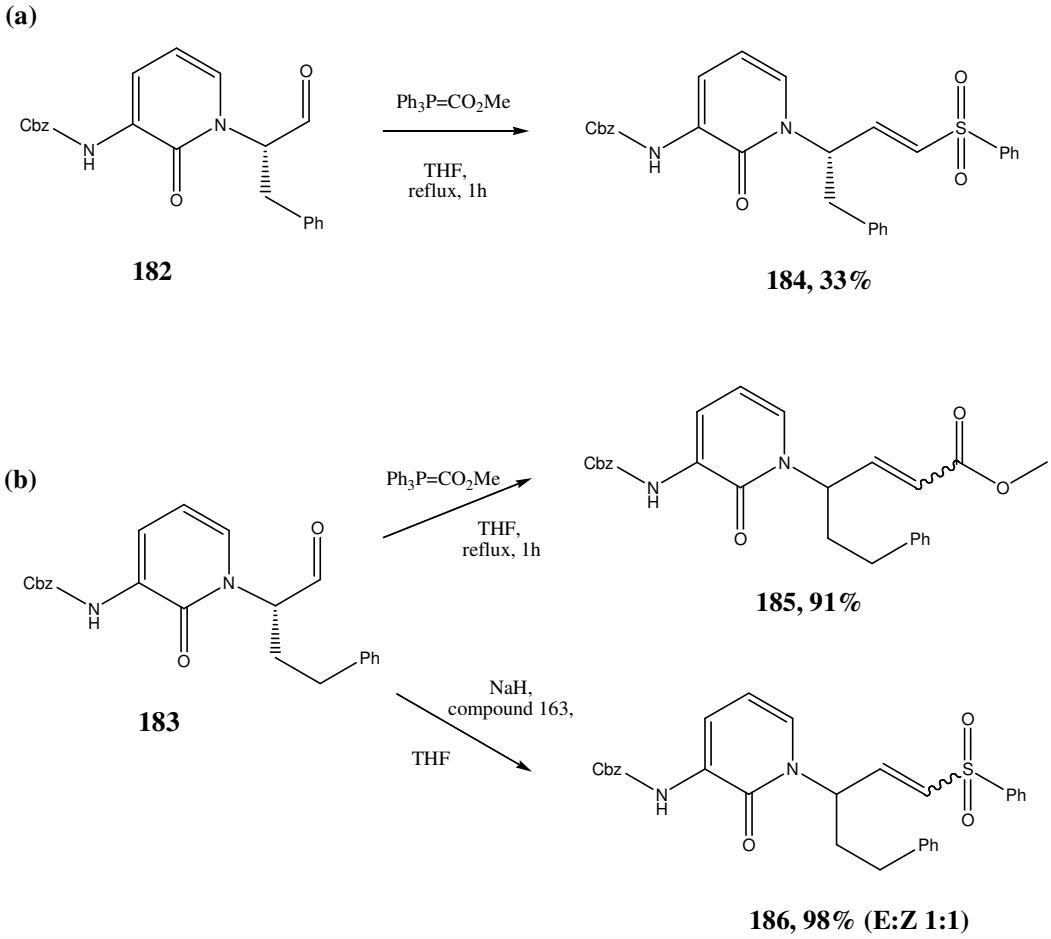
be reduced to the primary alcohols 180 and 181 quantitatively (only minor impurities were observed). Crude alcohol 180 was dissolved in dichloromethane and reacted with the Dess-Martin periodinane oxidant (a, Scheme 39) but, again, as observed previously for the synthesis of aldehyde 160, the yield was somehow disappointing. Therefore it was decided to try a Swern oxidation on substrate 181 (b, Scheme 39). The Swern conditions[78,79] required using oxalyl chloride, dimethyl sulfoxide and triethylamine, as the organic base, to afford aldehyde 183 in very good yield. The Swern oxidation is a reaction known for its mild character and wild tolerance to functional groups, preventing further oxidation to the carboxylic acid[80,81]. Though the formed by-product, dimethyl sulfide, has an unpleasant odour, taking the appropriate required precautions, this seemed like a better alternative and an improvement to our previous oxidation strategy.



Scheme 39

Due to the small amount of compound 182, we tried only one of the olefinations strategies, the Horner-Emmons-Wadsworth olefination (a, Scheme 40); compound 184

was isolated after purification by column, although in low yield. By analysis of the chemical shifts and coupling constant values for the vinyl and methionine protons, we believe that the *E*-olefin is the formed product. This process has been reported as a reliable criterion for the stereochemical assignment of α,β-unsaturated sulfonates.[82,83] We applied the Wittig reaction and the Horner-Emmons-Wadsworth conditions to peptidomimetic 183, and obtained the α,β-unsaturated methyl ester 185 and the α,β-unsaturated phenyl sulfone 186 (b, Scheme 40) in excellent yields.



Scheme 40

The desired Michael acceptor 185 was obtained in excellent yield, although contaminated with an impurity that we considered to be the cis-isomer (<10%);

compound 186 was also obtained in excellent yield, however, a more detailed analysis of its NMR spectra, made us believe that a mixture of E and Z-olefins was present (approximately 1:1 proportion; H and H' proton signalling is used in the experimental section of this chapter). The fact that we had a substituent (homophenylalanine) and the greater bulkiness of the phenyl sulfone group introduced, might have influenced the course of the reaction, and allowed formation of the less thermodynamically stable Z-olefin isomer. In fact, this might also explain the poor yield observed upon isolation of compound 184, because probably one of the impurities not collected was the other isomer.

3.3.4 Results and Discussion

3.3.4.1- Peptide-based Inhibitors

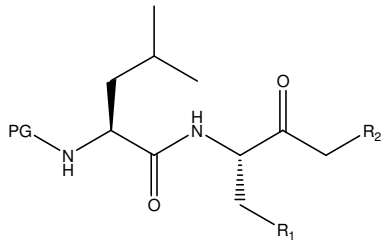


Table 4. *In-vitro* testing results for the peptide-based inhibitors

Inhibitor	*Structural Substituents*	*IC_{50} (μM)*[a]
148	PG = Cbz R_1 = Ph R_2 = OPh	2.93
151	PG = Cbz R_1 = Ph R_2 = SPh	4.60
152	PG = Cbz R_1 = Ph R_2 = OPy	2.80
150	PG = Mu R_1 = Ph R_2 = OPh	6.15
145	PG = Cbz R_1 = CH_2Ph R_2 = Br	0.43
147	PG = Mu R_1 = CH_2Ph R_2 = Br	8.74
149	PG = Cbz R_1 = CH_2Ph R_2 = OPh	6.65
105	PG = Cbz R_1 = CH_2Ph Control CHO	8.78
ART[b]		0.002

a: testing performed by the School of Tropical Medicine

b: ART= Artemisinin

The *in vitro* testing results for the synthesized peptide-based inhibitors within this project are intriguing and raise new questions. The analogues prepared are less active than expected and this also questions some of the considerations taken as proved previously, related to SAR.

From analysis of table 4, we can see that the best compound from this series, active in the nanomolar region, is compound 145, the α-bromo ketone, with the Cbz protecting group and homophenylalanine at P2 position. Its analogue, but with the morpholine-urea as protection, compound 147, was about ten times less potent, which seems to indicate that Cbz is preferred to Mu; this is also true for the pair 148/150, where the compound bearing Cbz as protecting group, compound 148, is more potent than its analogue 150, with Mu as the P3 component. This seems to indicate a preference for Cbz group as protecting group for leucine. In fact, compound 148, with Cbz as protecting group and phenylalanine at the P1 position was our third best inhibitor, right after compound 152, with identical structural choices. Compound 149, with Cbz protection and homophenylalanine as the second amino acid was less potent than its analogue 150, with the shorter phenylalanine substituent. Other compounds from the synthesized series should be part of this testing, in order to have more information available, such as compound 144, with Cbz and phenylalanine. The data is not sufficient to support a SAR trend. Dipeptidyl aldehyde 154 was used in this experiment as the control inhibitor, but showed equally poor inhibitory effect, though in the same range as the other tested compounds; nevertheless, this same compound has been reported in the literature as having an IC_{50} of 2 nM gainst FP-2[43] and we believe our results must be related to the low stability of the compound.

3.3.4.2- Peptidomimetic inhibitors

Following the *in vitro* testing procedures described in the experimental section of this chapter (page 158), we tested our own peptidomimetics in the School of Tropical Medicine in Liverpool. Counting tables with growth percentages calculated and their graphic results are presented respectively in appendix ii and iii (pages 245 and 253).

Table 5: *In vitro* testing results using different parasite strains from *P. falciparum*

Parasite strain tested	*3D7*	*TM6*	*W2* [b)]
IC_{50} results: / Compound:	[a)] IC_{50} ± Std. Err.	[a)] IC_{50} ± Std. Err	IC_{50}
Chloroquine	23.3 ± 1.0 nM	158.4 ± 11.5 nM	[c)]
	20.4 ± 2.7 nM	143.2 ± 24.3 nM	
	3.3 ± 0.6 nM	38.9 ± 1.8 nM	
186	5.0 ± 1.4 µM	8.2 ± 0.5 µM	16.2 µM
	6.0 ± 0.5 µM	10.0 ± 0.9 µM	
	6.1 ± 0.9 µM	8.7 ± 0.6 µM	
185	27.3 ± 2.1 µM	35.2 ± 5.1 µM	23.7 µM
	24.9 ± 2.8 µM	35.6 ± 5.8 µM	
	31.7 ± 5.7 µM	35.2 ± 5.1 µM	
183	27.1 ± 1.4 µM	16.3 ± 1.0 µM	26.9 µM
	33.7 ± 2.5 µM	26.1 ± 2.0 µM	
	32.1 ± 5.3 µM	25.7 ± 3.3 µM	
184	35.8 ± 6.1 µM	34.5 ± 5.7 µM	[c)]
	38.3 ± 8.0 µM	37.3 ± 8.8 µM	
	33.0 ± 5.8 µM	36.2 ± 7.6 µM	
182	34.4 ± 3.6 µM	34.5 ± 5.7 µM	[c)]
	38.2 ± 5.7 µM	37.3 ± 8.8 µM	
	39.3 ± 7.5 µM	36.2 ± 7.6 µM	
ALLN[d)]	8.2 ± 0.8 nM	11.9 ± 1.3 nM	39.2 nM
	17.2 ± 1.2 nM	12.2 ± 3.1 nM	
	7.6 ± 0.6 nM	15.3 ± 1.3 nM	

a) See attachments for complete table countings, calculations and corresponding graphics, needed to obtain IC_{50} results.

b) Tests performed by Dr Jiri Gut, from Phil Rosenthal's group, in the US.

c) Compounds not tested.

d) ALLN is a peptidyl aldehyde inhibitor of calpain and other neutral cysteine proteases; it was used also as a control in this study, given its similarity with our compounds.

The vinyl sulfone peptidomimetic inhibitor 186 was the best inhibitor of this series, followed by the α,β unsaturated methyl ester 185, with very close activity to the one presented by aldehyde 183. As expected, the analogues 184 and 182, respectively, the vinyl sulfone and the aldehyde, with the phenylalanine substituent at P2 position, were less active than their counterparts bearing the longer unnatural segment of homophenylalanine. These results follow what we expected, though we were aiming at having higher activities. In fact, few potent non-peptide inhibitors of the parasite cysteine proteases have been reported so far[84], and identification of novel and chemically diverse inhibitors provides initial lead for optimization into more potent and efficacious drug candidates to treat malaria. Reporting to the activity found in our compounds, we believe we have achieved this goal, by comparison with other inhibitory results of peptidomimetic cysteine proteases found in the literature. M. Avery and co-workers performed a virtual screening of The Available Chemical Directory (consists of nearly 355 000 compounds)[84] against the homology models of plasmodial FP-2 and FP-3, in order to identify structurally diverse inhibitors, other then peptidic. The investigation led to the identification of only 18 compounds with activity, though not noticeable for up to a concentration of 20 μM, with the sole exception of a compound exhibiting activity with an IC_{50} of 9.5 μM. Very recently, N. Micale and M. Hanspal

have reported the synthesis of a new class of peptidomimetics[85], based on a 1,4-benzodiazepine framework introduced internally to a peptide sequence, which mimics the fragment D-Ser-Gly and provides inhibitory activity with IC_{50} values varying from 8 to 26 μM.

We feel that the results obtained from our tests are trustable, since those obtained for the controls used were as expected: CQ was more effective against parasite strain 3D7, the sensitive one and the CQ resistant parasite strain TM6, showed higher IC_{50} results for CQ; we also tested a known peptidyl aldehyde inhibitor of calpain proteases, ALLN, or N-acetylleucylleucylnorleucinal, and have obtained interesting results: these compound is more effective than chloroquine, and shows the same activity both for CQ-resistant strains, as for CQ-sensitive strains.

Inhibitors of the protease calpain are known to have selectively toxic effects on *Plasmodium falciparum*. Only a few papers describe the effect of calpain inhibitors on P. falciparum. The first[86], described the effect of calpain inhibitors on the invasion of erythrocytes. The authors found that the inhibitors used were about 100 times as potent as the other protease inhibitors tested (like leupeptine, pepstatain A). Erythrocytes normally contain only calpain 2 and it wasn´t clear, at the time, if the effect of these inhibitors was a result of inhibition of the parasite's and/or of the erythrocyte's calpain. This was later clarified by Hanspal *et al*[87] who reinvestigated this effect in calpain 2 knock-out mice. The mouse erythrocytes were shown to have no detectable calpain activity but still supported the invasion and growth of P. falciparum in culture. Calpain inhibition again prevented re-invasion. Another paper[88] has shown that the removal of Ca^{2+} from the parasite's growth medium results in growth arrest in the late trophozoite stage and failure to invade erythrocytes. These findings are consistent with a role for calpain in the parasite life cycle. Recent studies show that a calpain gene is present in

the genome of the malaria parasite[89], thus, it might be worth exploring this enzyme further as a potential drug target for malaria chemotherapy. Our *in vitro* testing results are supportive of this idea.

Screening results of our 3 best peptidomimetics against purified enzymes recombinant FP-2 and recombinant FP-3, performed in collaboration with Phil Rosenthal's group, are presented in Table 6.

Table 6: Screening results against both recombinant enzymes FP-2 and FP-3.

Purified Enzymes: / *Compounds:*	*rec FP-2* [a)]	*rec FP-3* [a)]
186	>250 µM [b)]	>250 µM [b)]
185	190 µM	>250 µM
183	109 µM	>250 µM
ALLN	0.000124 nM	0.000413 nM

a) Tests performed by Dr Jiri Gut, from Phil Rosenthal's group, in the US.

b) Some precipitation occurred.

From analysis of this table we can say that these compounds are weekly active against the purified enzymes, especially towards FP-3. The best inhibition is achieved by aldehyde 183, followed by α,β unsaturated methyl ester 185. Nevertheless, these results are surprisingly low. Finding an optimized conformation of the inhibitor regarding recognition by the enzyme was the leading objective of our work toward the development of these conformationally restricted peptidomimetic inhibitors; however, by decreasing the flexibility of our initially synthesized peptide-based inhibitors, we

have also diminished their activity. The fact that our inhibitors were less active against the enzyme FP-3 than with FP-2, supports previous observations that falcipain-2 has much greater activity against a range of peptidyl substrates[35], though Rosenthal's inhibitors show similar inhibition against both recombinant plasmodial cysteine proteases[43].

3.3.5 Summary and Future Work

New synthetic routes were devised and applied to the synthesis of cysteine protease inhibitors, with fairly good yields and simplicity of experimental procedures.We have prepared a new set of peptidyl and peptidomimetic irreversible cysteine protease inhibitors active at low micromolar concentrations and also have improved and shown some alternative synthetic procedures toward the synthesis of α-substituted dipeptidyl ketones. Also, we modified the side-chains, used a non-native amino acid, tried different protecting groups in the amino terminus and different leaving groups for reacting with the enzyme's thiolate anion, in order to gather information on SAR.

A new mimetic scaffold was tested, the unsubstituted pyridone ring, for the amino acid segment Leucine, at the P2 position of the inhibitors. However, based on our in-vitro testing results, this conformational restricted option is not the most adequate, since these inhibitors are less active than their analogues having the free linear conformation[42].

We have performed *in vitro* testing procedures and analysed *in-vitro* data, completing the peptidomimetic part of this project.

In vitro testing results for the peptide-based inhibitors showed intriguing results: not only the inhibitors were less active than we predicted, but also they didn't show the expected activity enhancement from the use of homophenylalanine in place of

phenylalanine (though this seems to be an important replacement for *in-vivo* selectivity), or from the replacement of the carbobenzyloxy leucine protecting group by the more water-soluble morpholine-urea (though, it is believed that the difference *in-vivo* should be more noticeable, due to differences in the oral bioavailability of the compounds).

Future investigation should start by doing a more complete testing for the whole set of synthesised dipeptides with phenylalanine at P1 position and the several P'1 terminations tried (thiophenyl and pyridine), and only then, compare results with the homophenylalanine replacement. Also, given the short half-life of the aldehyde control and possibly of some of our target molecules, the IC_{50} testing should be performed with less delay than this time.

Also, we should check the selectivity toward other cysteine proteases, like for instance, recombinant human caspases, to verify if these inhibitors do not show inhibitory effects, or towards other plasmodial cysteine proteases, like aspartic protease plasmepsin. Different Michael acceptor's terminations could easily be incorporated in our inhibitors (extended systems), as also different α-ketone groups (less bulkier than OPh, or with a more accentuated leaving-group character). A more deep study of the possibility of racemization and formation of less stable olefin conformations is required.

Glaxo-Smith-Kline has agreed to model our compounds against the very recently clarified crystal structure of FP-2. This will certainly bring new highlights toward interpretation of these results and serve as guidance for future work within the purpose of this project.

Optimization of these cysteine proteases can lead to their use per se, but they can be also incorporated into endoperoxide pro-drugs, thereby encompassing the rationale of combination chemotherapy as a strategy aimed at reducing the development of resistance by the parasite.

3.4- Experimental Section- Preparation of CP Inhibitors

3.4.1- Experimental Details

Solvents, reagents and compounds were purified as explained before in the experimental section of chapter 2- pages 71 to 74-, as well as the characterization techniques used also in this chapter. However, the determination of the IC_{50} values of the synthesized peptidomimetic species within this project, required additional experimental techniques now explained.

3.4.1.1- *In Vitro* Testing Proceedings for *P. falciparum*

Biology: *In Vitro Plasmodium falciparum* Drug Sensitivity Assay

- *Reagents*

  [^{3}H] Hypoxanthine was purchased from Amersham. Tissue solubilizer was purchased from BDH. All other reagents were purchased from Sigma-Aldrich Chemical Company.

- *Parasite and drug sensitivity assays*

  P. falciparum strain TM6 (chloroquine resistant) was obtained from Mahidol University, Bangkok, Thailand. *P. falciparum* strain 3D7 (chloroquine sensitive) was obtained from Prof. D. Walliker, Edinburgh University, Edinburgh, United

Kingdom. Parasites were maintained in continuous culture using the method of Jansen and Trager[1] and synchronised using standard techniques.

The sensitivity of *P. falciparum* infected erythrocytes was determined using the [^{3}H] hypoxanthine incorporation method[2]. IC_{50}s were calculated using the four parameter logistic method (GraFit program, Erithacus Software, Surrey, United Kingdom).

- *In vitro testing protocol for Antimalarial Activity*

Two strains of P. falciparum were used in this study: (a) the TM6 strain which is known to be CQ resistant and (b) the 3D7 strain which is CQ sensitive. Parasites were synchronized previously to the day of the assay and at the ring stage; the percentage of ring stage parasites was then evaluated by analysing under the microscope a stained thin smear. A solution of red blood cells 1% (v/v) in complete media with parasitemia of 2% was prepared (if the parasitemia was lower or higher then 2%, infected parasites or uninfected red blood cells needed to be added). The separated red blood cells were obtained after whole blood was spun down and supernatant removed.

Cultures were grown in flasks containing human erythrocytes with parasitemia of 2% and complete media: RPMI 1640 medium, supplemented with 1M HEPES, gentamycin (10 mg/ml) and 10% pooled serum. Cultures were gassed with a mixture of 3% O_2, 4% CO_2 and 93% N_2. Antimalarial activity was assessed with an adaptation of the 48 hours sensitivity assay of Desjardins et al. using [^{3}H] hypoxanthine incorporation as an assessment of parasite growth[2]. Stock drug solutions were prepared in 100% dimethyl sulfoxide and diluted to the appropriate concentration using complete medium (the usual concentration range used in the laboratory was altered from the lower 0.46 nM to 1μM to the wider range 23 nM to

50 μM, after preliminary assays with the studied drugs). Assays were performed in sterile 96-well microtiter plates, and each plate contained 200μL of parasite culture (2% parasitemia, 1.0% haematocrit) with or without 10 μL drug dilutions. Each drug was tested in triplicate and parasite growth compared to control wells (using CQ as the drug in action and just with DMSO). After 24-h incubation at 37 °C inside incubated gassing chambers, 0.5μCi hypoxanthine was added to each well. Cultures were gassed and incubated for a further 24 h before they were harvested onto filter-mats, dried for 10 minutes at 55 °C, and counted using a Wallac 1450 Microbeta Trilux Liquid scintillation and luminescence counter. IC_{50} values were calculated by interpolation of the probit transformation of the log dose-response curve.

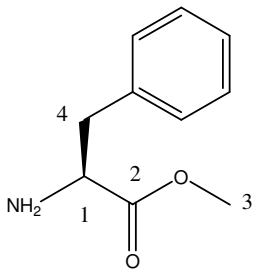
3.4.1.2-Bibliography

(1) W. Trager, J. B. Jenson. *Science* **1976***, 193*, 673-675.
(2) R. E. Desjardins, C. J. Canfield, J. D. Haynes and J. D. Chulay. *Antimicrob. Agents. Chemother.* **1979***, 16*, 710-718.

3.4.2- Preparation of Peptidic CPI

1-General procedure: Protection of the carboxylic terminal

Methyl 2-amino-3-phenylpropanoate (representative procedure 1.1)



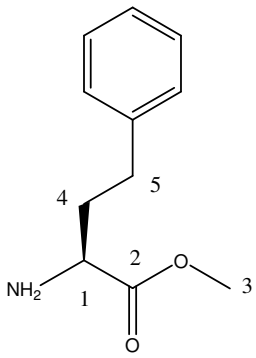
123.

Thionyl chloride (3.4 ml, 4.6 x10^{-2} mol) was added dropwise to a solution of *L*-phenylalanine (2.0 g, 1.2x10^{-2} mol) in anhydrous MeOH (20 ml) at -10ºC. The final mixture was allowed to warm to room temperature and stirred for 24 hours. After that period it was washed with water and the required product extracted with DCM. The organic phase was dried over magnesium sulphate, filtered, and the solvent evaporated to dryness. The product was recristallised from diethyl ether to give a white solid (1.81 g, 83%).

ν_{max} (KBr)/cm^{-1} 3477 (-NH_2), 2885 (C-H), 2625 (C-H), 2478 (C-H), 1745 (C=O), 1601 (C=C, Ar), 1496, 1446, 1292, 1242, 1145, 1084, 989, 935, 864. (Ph), 810 (Ph). $[\alpha]_D^{24}$ = 38.5 º (*c* 0.4 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.39-7.25 (m, 5H, Ar), 4.79

(brs, 3H, C(3)H_3), 4.32 (t, *J*= 7.2 Hz, 1H, C(1)H), 3.30 (m, 2H, NH_2), 3.29-3.15 (m, 2H, C(4)H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 170. (C(2)), 135.7 (Ar), 130.8 (Ar), 130.6 (Ar), 129.4 (Ar), 55.7 (C(1)), 54.0 (C(3)), 37.8 (C(4)). MS *m/z* (CI, +ve) 180 ($[M+H]^+$, 100). Found $[M+H]^+$ 180.1022, $C_{10}H_{14}NO_2$ requires 180.1024.

Methyl 2-amino-4-phenylbutanoate (representative procedure 1.2)



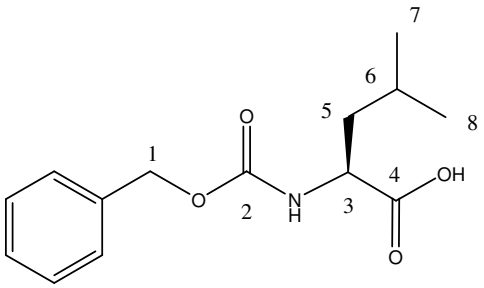
124.

To a solution of homophenylalanine hydrochloride salt 1.5 g (6.95 mmol) in MeOH (20 ml), at -10ºC, was added thionyl chloride (1.52 ml, 20.8 mmol) dropwise. The reaction mixture was allowed to warm up to room temperature and stirred for 24 hours. Then, it was washed with water and the organic product extracted with DCM affording a white solid, after evaporation of the solvent. This solid was dissolved in diethyl ether and triethylamine (1.6 ml) was added. The mixture was stirred at room temperature for 2 hours. The solid in suspension was filtered off the reaction mixture and the filtrate subjected to a small chromatographic column (silica gel, 95% dichloromethane/ 5% methanol), affording a yellow transparent oil, in 81% overall yield.

ν_{max} (neat)/cm^{-1} 338, 3316 (-NH_2), 3026, 2951, 2859 (-CH_2, –CH_3), 1736 (-C=O), 1602, 1496, 1454, 1436, 1197, 1174 (C-N), 743, 700 (Ph). $[\alpha]_D^{24}$ = -14.4 º (*c* 0.14 in $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H 7.32-7.18 (m, 5H, Ar), 3.71 (s, 3H, C(3)H_3), 3.46 (dd, *J*= 8.2 Hz, *J*= 4.7 Hz, 1H, C(1)H), 2.73 (m, 2H, C(5)H_2), 2.10-1.76 (m, 2H, C(4)H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 176.8 (C(2)), 141.6 (Ar), 128.6 (Ar), 126.4 (Ar), 54.1 (C(1)), 52.0 (C(3)), 36.6 (C(5)), 32.1 (C(4)). MS m/z (CI, +ve) 194 ([M+H]$^+$, 100).

2- General procedure: Protection of the amino terminal

2-Benzyloxycarbonylamino-4-methyl-pentanoic acid (representative procedure 2.1)



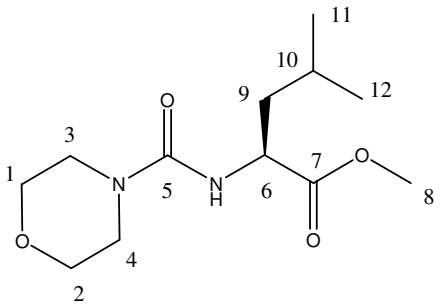
125.

To a stirring solution of *L*-leucine (1.0 g, 7.6 mmol) in a 2M sodium hydroxide solution (4.0 ml, 7.6 mmol) at 0ºC were added simultaneously benzyl chloroformate (1.09 ml, 7.6 mmol) and 2M NaOH solution (4.0 ml, 7.6 mmol) dropwise. The reaction mixture was left stirring for 3 hours at 0ºC and then washed with diethyl ether (5 ml). The aqueous phase was acidified with 2M hydrochloric acid solution and extracted with ethyl acetate (3 x 10 ml). The combined organic phases were dried over magnesium

sulphate, filtered and the solvent removed under reduced pressure to furnish the pure product as a transparent oil (1.99 g, 98%).

ν_{max} (neat)/cm^{-1} 3321 (-OH), 3065, 3034, 2958.5, 2871 (C-H), 1716 (C=O), 1633 (NHCO), 1469, 1455, 1414, 1345, 1266, 1230 (C-O-), 1050, 737 (Ph), 697 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.76 (br s, 1H, OH), 7.33 (br s, 5H, Ar), 5.24 (d, *J*= 8.4 Hz, 1H, NH), 5.11 (s, 2H, C(1)H_2), 4.40 (br s, 1H, C(3)H), 1.72-1.55 (m, 3H, C(5)H_2, C(6)H), 0.94 (d, *J*= 5.2 Hz, 6H, C(7)H_3, C(8)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 178.3 (C(4)), 156.6 (C(2)), 67.6 (C(1)), 65.7, 52.9 (C(3)), 41.9 (C(5)), 25.2 (C(7)), 23.2 (C(8)), 22.1 (C(6). MS *m/z* (CI, +ve) : 266 ([M+H]+, 37), 283 ($[M+NH_3]^+$, 100). Found $[M+H]^+$ 266.1392, $C_{14}H_{20}NO_4$ requires 266.1392.

4-Methyl-2-(morpholine-4-carboxamido) methyl pentanoate (representative procedure 2.2)



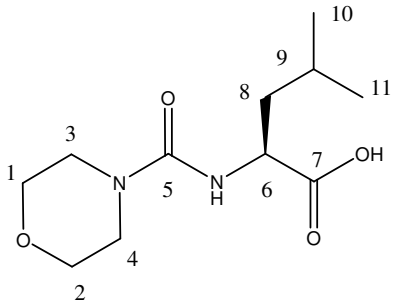
126.

L-Leucine methyl ester hydrochloride (4.0 g, 22.0 mmol) was dissolved in dimethylformamide (10 ml). *N, N*-diisopropylethylamine (11.5 ml, 66.0 mmol) was then added and the mixture was stirred under nitrogen for 15 min. After that period of time *N*-morpholine carbonyl chloride (3.80 ml, 33.0 mmol) was added and the final mixture

was stirred overnight. The solution was diluted with 200 ml of ethyl acetate, washed with water (3 x 100 ml) and then with a saturated sodium chloride solution (100 ml). The organic phase was dried over magnesium sulphate, filtered and concentrated to dryness to give 5.04 g of a light yellow solid as the crude product. Purification by flash chromatography (SiO_2, 80 % EtOAc/ n-hexanes) gave a beige solid (4.44 g, 78%).

ν_{max} (neat)/cm^{-1} 3334 (CONHR), 2957 (C-H), 2866 (C-H), 1740 (C=O), 1669 (C=O), 1533, 1439, 1389, 1268, 1203, 1117, 1018, 869, 830, 668. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 4.81 (d, *J*= 8.0 Hz, 1H, NH), 4.53 (dt, *J*= 14.0 Hz, *J*= 8.6 Hz, 1H, C(6)H), 3.74 (s, 3H, C(8)H_3), 3.69 (t, *J*= 4.9 Hz, 4H, C(1)H_2, C(2)H_2), 3.38 (m, 4H, C(3)H_2, C(4)H_2), 1.73-1.67 (m, 1H, C(10)H), 1.66-1.59 (m, 1H, C(9)H), 1.55-1.48 (m, 1H, C(9)H'), 0.96 (d, *J*= 6.4 Hz, 3H, C(11)H_3), 0.95 (d, *J*= 6.4 Hz, 3H, C(12)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 174.9 (C(7)), 157.2 (C(5)), 66.5 (C(1), C(2)), 52.3 (C(8)), 52.1 (C(6)), 44.0 (C(3), C(4)), 42.0 (C(9)), 24.9 (C(10)), 22.8 (C(11)), 22.1 (C(12)). MS *m/z* (CI, +ve) 259 ($[M+H]^+$, 100). Anal. Calc. for $C_{12}H_{22}N_2O_4$: C, 55.80, H, 8.58, N, 10.84 %; found: C, 56.01, H, 8.58, N, 10.86 %.

4-Methyl-2-(morpholine-4-carboxamido)pentanoic acid (representative procedure 2.3)



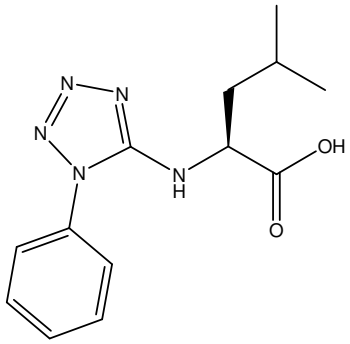
127.

Methyl [4-methyl-2-(morpholine-4-carboxamido)] pentanoate (4.4 g, 17.0 mmol) was dissolved in methanol (40 ml), and aqueous sodium hydroxide solution 1N (21 ml) added. The reaction was left stirring at room temperature for 2 hours. The reaction mixture was concentrated through removal of methanol under reduced pressure and the resulting concentrate extracted with dichloromethane (50 ml) and the organic phase separated. The aqueous phase was acidified with hydrochloric acid 1N (25 ml), and then extracted with dichloromethane (2 x 80 ml). The organic phases were combined, washed with HCl 1N (9 ml), then brine (9 ml), dried over magnesium sulphate, filtered and concentrated under reduced pressure to afford a transparent viscous oil, pure by TLC (1.91 g, quantitative yield).

ν_{max} (neat)/cm^{-1} 3381 (OH), 2950 (C-H), 2862 (C-H), 1721 (C=O), 1628, 1535, 1442, 1413, 1266 (C-N), 1232 (C-O), 1115. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.11 (brs, 1H, OH), 5.06 (d, *J*= 7.80 Hz, 1H, NH), 4.42 (m, 1H, C(6)H), 3.70 (t, *J*= 5.0 Hz, 4H, $C(1)H_2$, $C(2)H_2$), 3.48-3.34 (m, 4H, $C(3)H_2$, $C(4)H_2$), 1.79-1.67 (m, 2H, C(8)H, C(9)H), 1.61-1.54 (m, 1H, C(8)H'), 0.96 (d, *J*= 5.3 Hz, 3H, $C(10)H_3$), 0.95 (d, *J*= 5.3 Hz, 3H, $C(11)H_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 177.0 (C(7)), 158.4 (C(5)), 67.7 (C(1),

C(2)), 52.9 (C(6)), 44.5 (C(3), C(4)), 41.3 (C(8)), 25.3 (C(9)), 23.3 (C(10)), 22.3 (C(11)). MS *m/z* (CI, +ve) 245 ($[M+H]^+$, 13), 262 ($[M+NH_4]^+$, 0.2), 88 (100), 175 (39), 227 (31). Found $[M+Na]^+$ 267.1318, $C_{11}H_{20}N_2O_4{}^{23}Na$ requires 267.1321.

2-(1-Phenyl-1*H*-tetrazol-5-yl-amino)-4-methylpentanoic acid (representative procedure 2.4)



128.

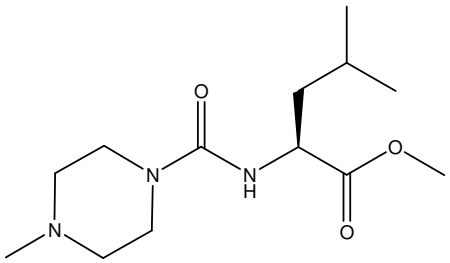
A suspension of 5-chloro-1-phenyl-1*H*-tetrazole (2.0 g, 11.1 mmol), *L*-leucine (2.4 g, 18.3 mmol) and potassium carbonate (4.59 g, 33.2 mmol) in isopropanol (15 ml) was heated to reflux for 3 hours. The mixture was then cooled and partitioned between ethyl acetate and aqueous hydrochloric acid 1M. The organic phase was washed with brine and the solvent removed under reduced pressure. The product was purified by flash chromatography (SiO_2, ethyl acetate/ n-hexanes (1:1) and a drop of acetic acid) and obtained as a viscous yellow oil (1.54 g, 49%).

ν_{max} (neat)/cm^{-1} 3302 (OH), 2958, 2929, 2871 (C-H), 1726 (C=O), 1603 (C=C, Ar), 1585, 1520, 1504, 1460, 1275 (C-N), 1159, 1128, 1093, 1072, 762 (Ph), 688 (Ph). ^1H NMR (400 MHz, $CDCl_3$) δ_H 7.72-7.52 (m, Ar, 5H), 5.94 (brs, 1H), 4.80 (d, *J*= 9.3 Hz, 1H), 4.65 (dt, *J*= 9.4 Hz, *J*= 4.6 Hz, 1H), 1.46-1.26 (m, 3H), 0.99 (d, *J*= 6.1 Hz, 3H),

0.98 (d, *J*= 6.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 176.5, 154.2, 130.4, 130.2, 124.1, 68.2, 38.7, 23.7, 23.0, 22.8. Found $[M+H]^+$ 276.1466, $C_{13}H_{18}N_5O_2$ requires 276.1461.

Methyl [2-(4-methylpiperazine-1-carboxamido)-4-methyl] pentanoate

(representative procedure 2.5)



129.

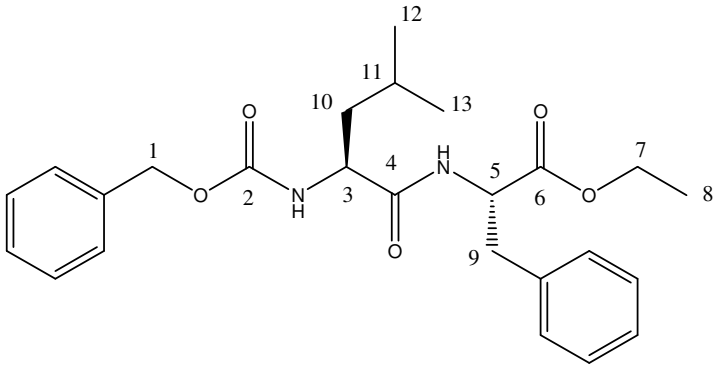
To (*S*)-methyl 2-amino-4-methylpentanoate hydrochloride salt (1.0 g, 5.5 mmol) in toluene (30 ml) was added bis-(trichloromethyl) carbonate (0.82 g, 2.8 mmol). The reaction mixture was heated at 100ºC for 3 hours and then concentrated under vacuum to afford a semi-solid residue, which was dissolved in methylene chloride (20 ml) and cooled to 0ºC. *N*-Methylpiperazine was then added (0.55 g, 5.5 mmol) and the mixture was stirred for 1 h, concentrated under reduced pressure and the residue purified (silica gel, eluting with 4% MeOH in $CHCl_3$) to afford the required product as an orange oil (1.56 g, quantitative yield).

ν_{max} (neat)/cm^{-1} 3383 (CONHR), 2956 (C-H), 2925 (C-H), 2862 (C-H), 1738 (C=O), 1630 (C=O), 1538, 1630, 1537, 1462 (C-N), 1410 (C-N), 1271 (C-O), 1215, 1171, 1001, 978, 870, 770. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 4.94 (d, *J*= 8.0 Hz, 1H), 4.50 (dt, *J*= 13.6 Hz, *J*= 8.4 Hz, 1H), 3.74 (s, 3H), 3.56 (brs, 4H), 2.61 (brs, 4H), 1.73-1.49 (m,

3H), 0.95 (d, *J*= 6.4 Hz, 6H)). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 174.9, 157.0, 54.2, 52.2, 52.2, 45.4, 43.1, 41.9, 24.9, 22.8, 22.0. MS *m/z* (CI, +ve) 272 ($[M+H]^+$, 100).

3-General procedure: Peptide Coupling

2-(2-Benzyloxycarbonylamino-4-methyl-pentanoylamino)-3-phenyl propionic acid ethyl ester (representative procedure 3.1)

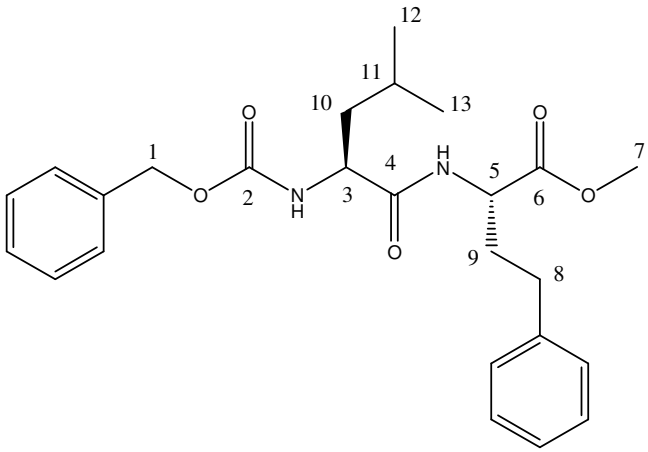


130.

Hydroxybenzotriazole (0.86 g, 5.63 mmol), NMM (1.45 ml, 13.13 mmol) and EDC (0.90 g, 4.69 mmol) were added to a stirring solution of 2-benzyloxycarbonylamino-4-methyl-pentanoic acid (1.24 g, 4.69 mmol) and ethyl 2-amino-3-phenylpropanoate (0.91 g, 4.69 mmol) in dichloromethane (60 ml) at 0ºC. The reaction was allowed to warm up to room temperature and left stirring for 24 hours. Evaporation of the solvent under reduced pressure afforded the crude material, which was subjected to flash chromatography (25% ethyl acetate in hexanes). The purified dipeptide was obtained as a white solid (1.42 g, 74%).

ν_{max} (KBr)/cm^{-1} 3317, 3278 (-CONH-), 3087, 3066, 3033, 2954, 2924, 2871 (C-H), 1736 (C=O), 1682 (C=O), 1666 (C=O), 1535 (-CONH-), 1454, 1373, 1288 (C-O), 1039, 794 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.29 (d, *J*= 7.6 Hz, 1H, NH), 7.36-7.20 (m, 10H, Ar), 5.01 (brs, 3H, C(1)H_2, NH)), 4.43 (m, 1H, C(5)H), 4.07 (m, 1H, C(3)H), 4.01 (q, *J*= 7.0 Hz, 2H, C(7)H_2), 2.98 (m, 2H, C(9)H_2), 1.58 (m, 1H, C(11)H), 1.38 (m, 2H, C(10)H_2), 1.08 (t, *J*= 7.2 Hz, 3H, C(8)H_3), 0.85 (d, *J*= 10.4 Hz, 3H, C(12)H_3), 0.83 (d. *J*= 10.0 Hz, 3H, C(13)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 172.8 (C(4)), 171.7 (C(6)), 156.1 (C(2)), 137.4, 137.4, 129.5, 128.7, 128.5, 128.1, 128.0, 126.8 (Ar), 65.7 (C(1)), 60.8 (C(7)), 53.9 (C(5)), 53.2 (C(3)), 41.1 (C(10)), 36.9 (C(9)), 24.4 (C(12)), 23.3 (C(13)), 21.9 (C(11)), 14.2 (C(8)). Anal. Calc. for $C_{25}H_{32}N_2O_5$: C, 68.16, H, 7.32, N, 6.36 %; found: C, 68.19, H, 7.32, N, 6.33 %.

2-(2-Benzyloxycarbonylamino-4-methyl-pentanoylamino)-4-phenyl-butanoic acid methyl ester



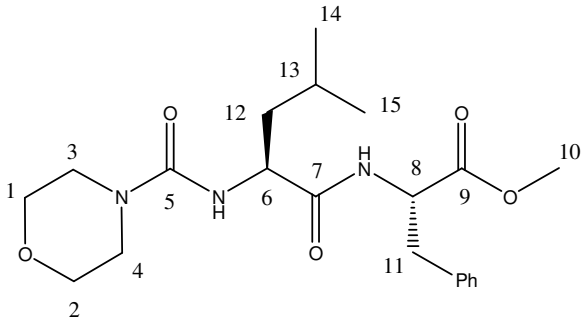
131.

Hydroxybenzotriazole (0.674 g, 4.40 mmol), NMM (1.13 mL, 10.3 mmol) and EDC (0.704 g, 3.67 mmol) were added to a stirring solution of 2-benzyloxycarbonylamino-4-

methyl-pentanoic acid (0.97, 3.67 mmol) and methyl 2-amino-4-phenylbutanoate (0.709, 3.67 mmol) in dichloromethane (45 ml) at 0ºC. Representative procedure 3.1 was applied and the desired dipeptide was obtained as a white crystalline solid (1.50 g, 93%).

ν_{max} (neat)/cm^{-1} 3308 (N-H), 3063, 3029, 2955, 2868 (C-H), 1745 (C=O), 1696 (C=O), 1659 (C=O), 1539 (NHCO), 1455, 1367, 1239 (C-O-), 1172, 1117, 1043, 739 (Ph), 698 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.33-7.14 (m, 10H, Ar), 6.46 (d, *J*= 6.8 Hz, 1H, NH), 5.11 (s, 2H, C(11)H_2), 5.08 (brd s, 1H, NH), 4.62 (dt, *J*= 7.6 Hz, *J*= 2.4 Hz, 1H, C(3)H), 4.18 (brs, 1H, C(5)H), 3.71 (s, 3H, C(7)H_3), 2.62 (t, *J*= 7.6 Hz, 2H, C(8)H_2), 2.19 (m, 1H, C(9)H), 2.0 (m, 1H, C(9)H'), 1.71-1.62 (m, 2H, C(10)H, C(11)), 1.49 (m, 1H, C(10)H'), 0.94 (d, *J*= 6.0 Hz, 6H, C(12)H_3, C(13)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 172.7 (C(6)), 172.2 (C(4)), 156.6 (C(2)), 141.1, 136.6, 128.9, 128.8, 128.6, 128.4, 127.4, 126.6 (Ar), 67.5 (C(1)), 54.0 (C(3)), 52.8 (C(5)), 52.4 (C(7)), 41.6 (C(10)), 34.2 (C(9)), 31.9 (C(8)), 25.1 (C(13)), 23.3 (C(12)), 22.4 (C(11)). Found $[M+Na]^+$ 463.2193, $C_{25}H_{32}N_2O_5{}^{23}Na$ requires 463.2209.

2-(4-Methyl-2-(morpholine-4-carboxamido)pentanoylamino)-3-phenyl-propionic acid methyl ester (representative procedure 3.2)



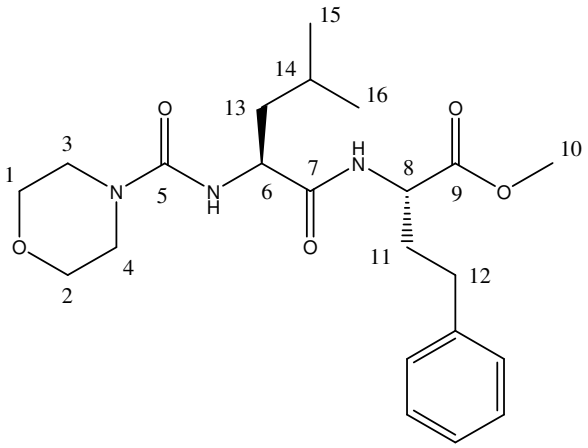
132.

Hydroxybenzotriazole (0.89 g, 5.80 mmol), NMM (1.50 m, 1.36 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide(0.93 g, 4.87 mmol) were added to a stirring solution of 4-methyl-2-(morpholine-4-carboxamido)pentanoic acid (1.19 g, 4.87 mmol) and methyl 2-amino-3-phenylpropanoate (0.87 g, 4.87 mmol) in dichloromethane (60 ml) at 0ºC. The reaction was allowed to warm up to room temperature after 30 min and left stirring overnight. The solvent was removed under reduced pressure and the residue was re-suspended in a mixture of dichloromethane (450 ml) and hydrochloric acid 1N (50 ml). The organic phase was separated and washed with hydrochloric acid 0.5N (50 ml), with saturated solution of sodium hydrogen carbonate (50 ml), and brine (50 ml). The organic solution was dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. The pure product was obtained as a white solid (1.63 g, 83%).

ν_{max} (neat)/cm^{-1} 3331 (N-H), 2954 (C-H), 2857 (C-H), 1738 (C=O), 1661 (C=O), 1625 (C=O), 1543 (NHCO), 1435, 1409, 1366, 1268 (C-O-C), 1203, 1174, 1117, 1072, 1020, 999, 864, 747 (Ph), 700 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.30-7.23 (m, 3H, Ar),

7.13- 7.11 (m, 2H, Ar), 6.59 (d, *J*= 7.8 Hz, 1H, NH), 4.82 (m, 2H, NH, C(8)H), 4.33 (dt, *J*= 14.0 Hz, *J*= 8.3 Hz, 1H, C(6)H), 3.71 (s, 3H, $C(10)H_3$), 3.67 (t, *J*= 4.9 Hz, 4H, $C(1)H_2$, $C(2)H_2$), 3.38-3.28 (m, 4H, $C(3)H_2$, $C(4)H_2$), 3.14 (dd, *J*= 13.9 Hz, *J*= 5.8 Hz, 1H, C(11)H), 3.06 (dd, *J*= 13.9 Hz, *J*= 6.4 Hz, 1H, C(11)H'), 1.70-1.59 (m, 2H, C(12)H, C(13)H), 1.51-1.43 (m, 1H, C(12)H'), 0.92 (d, *J*= 6.4 Hz, 3H, $C(14)H_3$), 0.72 (d, *J*= 6.2 Hz, 3H, $C(15)H_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 173.1 (C(9)), 172.1 (C(7)), 157.7 (C(5)), 136.2, 129.7, 128.9, 127.4 (Ar), 66.8 (C(1), C(2)), 53.6 (C(8)), 53.2 (C(6)), 52.6 (C(10)), 44.4 (C(3), C(4)), 41.9 (C(12)), 38.3 (C(11)), 25.2 (C(13)), 23.2 (C(14)), 22.7 (C(15)). Found $[M+Na]^+$ 428.2159, $C_{21}H_{31}N_3O_5Na$ requires 428.2161.

2-(4-Methyl-2-(morpholine-4-carboxamido)pentanoylamino)-3-phenyl-butanoic acid methyl ester



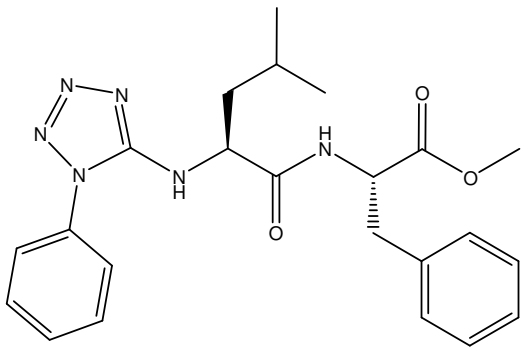
133.

Hydroxybenzotriazole (1.04 g, 6.78 mmol), NMM (1.47 ml, 15.8 mmol) and EDC (1.08 g, 5.65 mmol) were added to a stirring solution of 4-methyl-2-(morpholine-4-carboxamido)pentanoic acid (1.45, 5.65 mmol) and methyl 2-amino-4-phenylbutanoate

(1.09 g, 5.65 mmol) in dichloromethane (70 ml) at 0ºC. The reaction mixture was allowed to warm up to room temperature and left stirring for 24 hours. By following on representative procedure 3.2, the pure product was obtained as a white solid (1.58 g, 66%).

ν_{max} (neat)/cm^{-1} 3284 (N-H), 3063, 3027, 2960, 2926, 2860 (-CH_2, -CH_3), 1746 (C=O), 1661 (C=O), 1622 (C=O), 1538 (NHCO), 1454, 1436, 1409, 1367, 1268 (C-O-C), 1203, 1172, 1118, 1071, 1019, 1000, 913, 864, 733 (Ph), 701 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.27-7.13 (m, 5H, Ar), 7.13- 7.11 (m, 2H, Ar), 6.86 (d, *J*= 7.6 Hz, 1H, NH), 5.03 (d, *J*= 8.0 Hz, 1H, NH), 4.56 (dt, *J*= 2.4 Hz, *J*= 7.6 Hz, 1H, C(8)H), 4.40 (dt, *J*= 2.4 Hz, *J*= 7.6 Hz, 1H, C(6)H), 3.71 (s, 3H, C(10)H_3), 3.65- 3.62 (m, 4H, C(1)H_2, C(2)H_2), 3.36- 3.33 (m, 4H, C(3)H_2, C(4)H_2), 2.62 (t, *J*= 8.0 Hz, 2H, C(12)H_2), 2.21-2.12 (m, 1H, C(11)H), 2.03- 1.94(m, 1H, C(11)H'), 1.75-1.62 (m, 2H, C(13)H, C(14)H), 1.52 (m, 1H, C(13)H'), 0.95 (d, *J*= 6.4 Hz, 3H, C(15)H_3), 0.94 (d, *J*= 6.4 Hz, 3H, C(16)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 173.5 (C(9)), 172.8 (C(7)), 157.8 (C(5)), 141.0, 128.9, 128.8, 126.6 (Ar), 66.8 (C(1), C(2)), 53.3 (C(10)), 52.7 (C(6)), 52.7 (C(8)), 44.5 (C(3), C(4)), 41.9 (C(13)), 34.2 (C(11)), 31.9 (C(12)), 25.2 (C(14)), 23.3 (C(15)), 22.7 (C(16)). Found $[M+Na]^+$ 442.2332, $C_{22}H_{33}N_3O_5Na$ requires 442.2318.

(*S*)-2-[(*S*)-4-Methyl-2-(1-phenyl-1*H*-tetrazol-5-ylamino)-pentanoylamino]-3-phenyl-propionic acid methyl ester



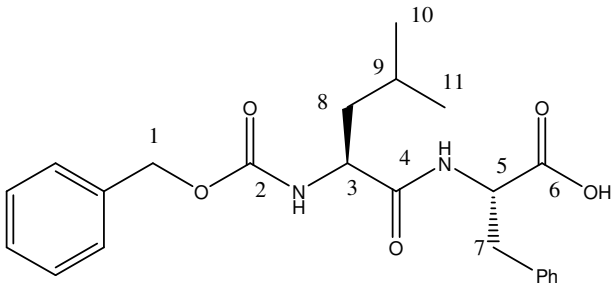
134.

Hydroxybenzotriazole (0.87 g, 5.7 mmol), NMM (1.45 ml, 1.3 mmol), and EDC (0.90 g, 4.7 mmol) were added to a stirring solution of 2-(1-phenyl-1H-tetrazol-5-yl-amino)-4-methylpentanoic acid (1.3 g, 4.7 mmol) and methyl 2-amino-3-phenylpropanoate (0.84 g, 4.7 mmol) in dichloromethane (60 ml) at 0ºC. Work up as in representative procedure 3.1 gave the desired product as a yellowish foam (1.13 g, 55 %).

ν_{max} (neat)/cm^{-1} 3282 (RCONHR'), 3064, 3032, 2956, 2870 (C-H), 1745 (C=O), 1662 (C=N), 1603 (C=C, Ar), 1583, 1549, 1504, 1458, 1439, 1360, 1284, 1213, 1174 (C-N), 1134, 1088, 1018. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.60-7.45 (m, Ar, 5H), 7.25-7.07 (m, Ar, 5H), 6.55 (d, *J*= 8.0 Hz, 1H), 4.81 (d. *J*= 9.1 Hz, 1H), 4.88-4.80 (m, 1H), 4.51- 4.45 (m, 1H), 3.75 (s, 3H), 3.167 (dd, *J*= 13.9 Hz, *J*= 5.4 Hz, 1H), 3.03 (dd, *J*= 13.9 Hz, *J*= 6.8 Hz, 1H), 1.72- 1.55 (m, 3H), 0.93 (d, *J*= 6.2 Hz, 3H), 0.92 (d, *J*= 6.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 171.6, 171.5, 154.2, 135.4, 133.0, 130.3, 129.9, 130.0, 128.6, 127.2, 123.9, 56.5, 53.4, 52.5, 41.7, 37.9, 24.7, 22.7, 22.7, 22.2. MS *m/z* (CI, +ve) 437 ($[M+H]^+$, 100). Found $[M+Na]^+$ 459.2119, $C_{23}H_{28}N_6O_3$ requires 459.2121.

2-(2-Benzyloxycarbonylamino-4-methyl-pentanoylamino)-3-phenyl-propionic acid

(representative procedure 3.3)



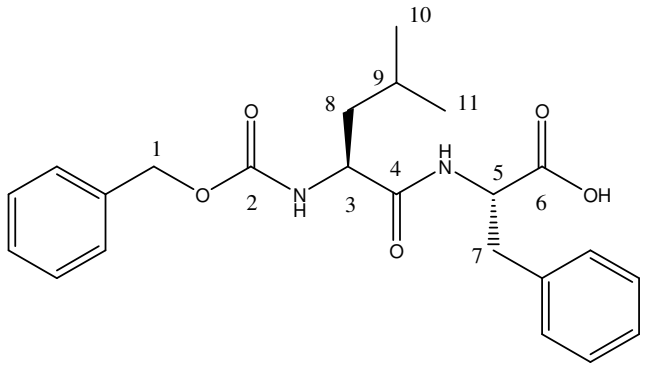
135.

To a stirring solution of 2-benzyloxycarbonylamino-4-methyl-pentanoic acid (0.80 g, 3.03 mmol) in acetonitrile (150 ml) was added triethylamine (0.46 ml, 3.33 mmol) and the mixture cooled down to 0ºC. 4-Nitrophenyl chloroformate (0.67 g, 3.33 mmol) was added and the reaction left stirring for 5 min, after which, DMAP (0.04 g, 0.30 mmol) was added and the mixture left stirring for 50 minutes at 0ºC. After that period of time, the reaction mixture was removed from the ice-bath and a solution of 2-amino-3-phenylpropanoic acid (2.0 g, 12.1 mmol) in water (150 ml) and triethylamine (1.68 ml, 12.1 mmol) was added drop wise to the reaction during 1 hour, and left stirring for another 2 hours. ACN was then removed under pressure, 5 mL of conc. HCl were added and the mixture extracted with dichloromethane. The organic phase was dried over $MgSO_4$ and the solvent removed under reduced pressure to afford the crude product as 1.99 g of a pale yellow foam, which was subjected to flash chromatography (SiO_2, 40%EtOAc/hexane), affording a white crystalline solid (0.78 g, 63% yield).

ν max (nujol)/cm^{-1} 3319, 2725, 2484, 1982, 1803, 1782, 1716, 1693, 1664, 1597, 1537, 1462, 1450, 1377, 1290, 1267, 1242. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.37-7.18 (m, 10H, Ar), 5.01 (s, 2H), 4.42 (m, 1H), 4.04 (m, 1H), 3.05 (dd, *J*= 13.9 Hz, *J*= 5.3 Hz, 1H), 2.91 (dd, *J*= 13.9 Hz, *J*= 8.7 Hz, 1H), 1.56 (m, 1H), 1.42-1.24 (m, 2H), 0.85 (d, *J*= 6.5 Hz, 3H), 0.82 (d, *J*= 6.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 171.5, 170.9, 154.5, 136.2, 135.8, 127.9, 127.1, 126.9, 126.5, 126.4, 125.1, 39.5, 35.4, 22.9, 21.7, 20.2. Found $[M+Na]^+$ 435.1881, $C_{23}H_{29}N_2O_5Na$ requires 435.1896.

4- General procedure: Carboxylic acid deprotection

2-(2-Benzyloxycarbonylamino-4-methyl-pentanoylamino)-3-phenyl-propionic acid (representative procedure 4.1)



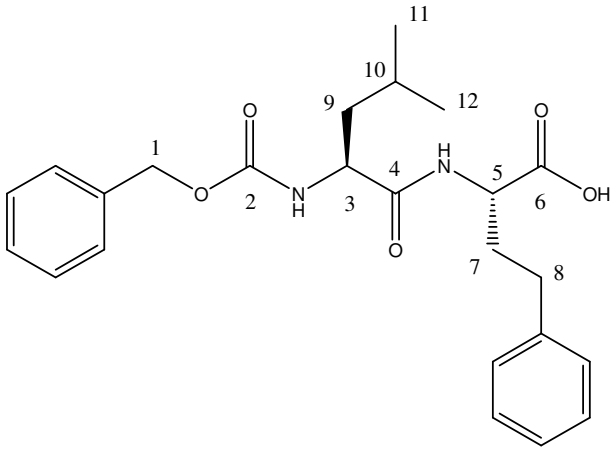
136.

To a solution of 2-(2-benzyloxycarbonylamino-4-methyl-pentanoylamino)-3-phenyl propionic acid ethyl ester (2.06 g, 4.68 mmol) in MeOH (22 ml), at room temperature, was added a solution of sodium hydroxide 2 N (18 ml) and the final mixture was stirred overnight. The organic solvent was removed under reduced pressure and the product

extracted from the concentrate with EtOAc (2x60 ml). The organic phase was dried over magnesium sulphate, filtered and the solvent evaporated under vacuum affording the pure product as a viscous yellow oil (1.63 g, 85%).

^{1}H NMR (400 MHz, MeOD) δ_H 7.37-7.13 (m, 10H, Ar), 4.95 (s, 2H, C(1)H_2), 4.66 (brs, 1H, OH), 4.48 (dd, *J*= 6.4 Hz, *J*= 4.8 Hz, 1H, C(5)H), 4.12 (dd, *J*= 9.6 Hz, *J*= 5.6 Hz, 1H, C(3)H), 3.23 (dd, *J*= 13.6 Hz, *J*= 4.8 Hz, 1H, C(7)H), 3.03 (dd, *J*= 13.6 Hz, *J*= 7.2 Hz, 1H, C(7)H'), 1.64 (m, 1H, C(9)H), 1.50-1.42 (m, 2H, C(8)H_2), 0.93 (d, *J*= 6.8 Hz, 3H), 0.89 (d, *J*= 6.8 Hz, 3H). ^{13}C NMR (100 MHz, MeOD) δ_C 174.5 (C(6)), 158.6 (C(2)), 139.1, 138.1, 130.7, 129.5, 129.2, 128.8, 128.0, 127.4 (Ar), 67.8 (C(1)), 57.0 (C(5)), 55.2 (C(3)), 41.9 (C(8)), 39.0 (C87)), 25.8 (C(10)), 23.5 (C(11)), 21.8 (C(9)). Found $[M+Na]^+$ 435.1913, $C_{23}H_{29}N_2O_5Na$ requires 435.1896.

2-(2-Benzyloxycarbonylamino-4-methyl-pentanoylamino)-4-phenyl-butanoic acid



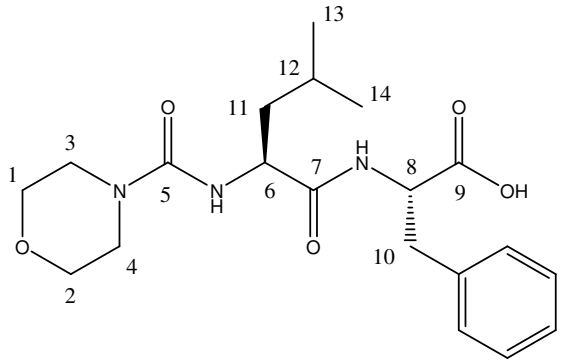
137.

To a solution of 2-(2-benzyloxycarbonylamino-4-methyl-pentanoylamino)-4-phenyl-butanoic acid methyl ester (1.5 g, 3.4 mmol) in MeOH (15 ml) was added 2N sodium hydroxide (13 ml) and the reaction was left stirring at room temperature overnight.

Following representative procedure 4.1 (but using chloroform to extract the product), a pale yellow crystalline solid was obtained in quantitative yield.

ν_{max} (neat)/cm^{-1} 3400 (O-H), 3304 (N-H), 3063, 3029, 2957, 2870 (C-H), 1700 (C=O), 1652 (C=O), 1599 (C=O), 1538 (NHCO), 1455, 1265, 1173, 1121, 1046, 1028, 737 (Ph), 698 (Ph). $[\alpha]_D^{24}$ = -15 º (*c* 2.6 in $CHCl_3$). ^{1}H NMR (400 MHz, MeOD) δ_H 7.37-7.12 (m, 10H, Ar), 5.12 (s, 2H, C(1)H_2), 4.28 (dd, *J*= 7.8 Hz, *J*= 4.6 Hz, 1H, C(3)H), 4.23 (dd, *J*= 9.2 Hz, *J*= 5.6 Hz, 1H, C(5)H), 2.64 (m, 2H, C(8)H_2), 2.16 (m, 1H, C(7)H), 1.97 (m, 1H, C(7)H'), 1.72-1.53 (m, 3H, C(10)H, C(9)H_2), 0.97 (d, *J*= 6.4 Hz, 3H), 0.95 (d, *J*= 6.8 Hz, 3H).^{13}C NMR (100 MHz, MeOD) δ_C 178.4 (C(6)), 174.6 (C(4)), 158.7 (C(2)), 143.6, 138.2, 129.5, 129.3, 129.0, 128.8, 128.3, 128.0, 126.7 (Ar), 68.2 (C(1)), 65.7, 56.3 (C(5)), 55.5 (C(3)), 42.1 (C(9)), 36.3, 34.86, 32.9 (C(7)), 31.7 (C(8)), 26.1 (C(12)), 23.9 (C(11)), 22.3 (C(10)). Found $[M-H]^-$ 425.2062, $C_{24}H_{29}N_2O_5$ requires 425.2076.

2-(4-Methyl-2-(morpholine-4-carboxamido)pentanoylamino)-3-phenyl-propionic acid (representative procedure 4.2)

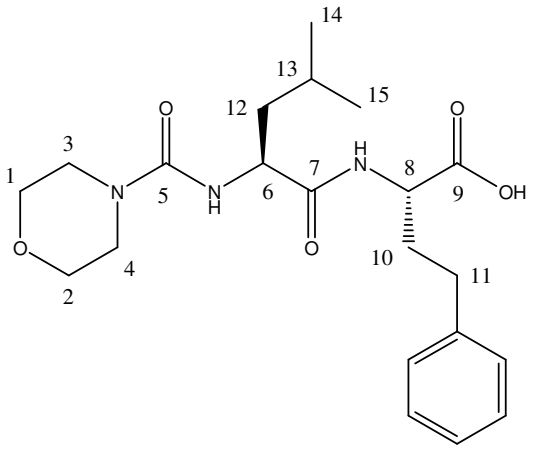


138.

2-(4-Methyl-2-(morpholine-4-carboxamido)pentanoylamino)-3-phenylpropionic acid methyl ester_(1.59 g, 3.92 mmol) was dissolved in methanol (20 ml), and sodium hydroxide aqueous solution 1N (7 ml) was added. The reaction was left stirring at room temperature for 7 hours. After that period of time, methanol was removed under reduced pressure and ethyl acetate (5 ml) was added. The layers were separated and the aqueous phase acidified with hydrochloric acid 1N (8 ml) and extracted with ethyl acetate (70 ml). The combined organic phases were washed with hydrochloric acid 1N (8 ml), dried over magnesium sulphate, filtered and the solvent removed under reduced pressure to afford the pure product as pale yellow foam which solidified after a while (1.38 g, 90%).

ν_{max} (neat)/cm^{-1} 3610 (OH), 3330 (N-H), 2959 (-CH_2, -CH_3), 2868 (-CH_2, -CH_3), 1732 (C=O), 1620 (C=O), 1537 (C=C, Ar), 1455, 1412, 1268 (C-O-C), 1116, 1071, 1000, 912, 865, 733 (Ph), 701 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 9.40- 8.60 (brs, 1H, OH), 7.26- 7.14 (m, 6H, 5H, Ar, 1H, NH), 5.43 (d, *J*= 8.4 Hz, 1H, NH), 4.79 (dt, *J*= 13.1 Hz, *J*= 7.5 Hz, 1H, (C(8)H), 4.48 (dt, *J*= 14.5 Hz, *J*= 8.3 Hz, 1H, C(6)H), 3.65 (t, *J*= 5.0 Hz, 4H, C(1)H_2, C(2)H_2), 3.39- 3.27 (m, 4H, C(3)H_2, C(4)H_2), 3.18 (dd, *J*= 14.0 Hz, *J*= 5.4 Hz, 1H, C(10)H), 2.97 (dd, *J*= 14.0 Hz, *J*= 6.6 Hz, 1H, C(10)H'), 1.64- 1.52 (m, 2H, C(11)H, C(12)H), 1.50- 1.42 (m, 1H, C(11)H'), 0.89 (d, *J*= 9.6 Hz, 3H, C(13)H_3), 0.88 (d, *J*= 9.6 Hz, 3H, C(14)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 173.8 (C(9), C(7)), 157.8 (C(5)), 136.5, 129.9, 129.8, 129.0, 128.8, 127.2 (Ar), 66.8 (C(1), C(2)), 53.9 (C(8)), 53.2 (C(6)), 44.4 (C(3), C(4)), 41.6 (C(11)), 38.0 (C(10)), 25.1 (C(12)), 23.1 (C(13)), 22.7 (C(14)). MS *m/z* (CI, +ve) 392 ([M+H]$^+$, 51), 88 (100), 305 (61), 322 (38), 374 (15).

2-(4-Methyl-2-(morpholine-4-carboxamido)pentanoylamino)-3-phenyl-butanoic acid



139.

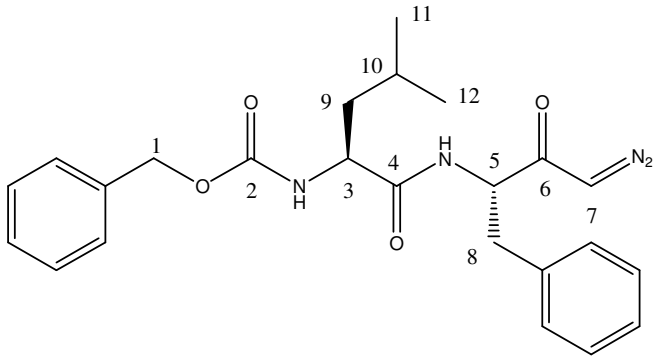
To a solution of 2-(4-methyl-2-(morpholine-4-carboxamido)pentanoylamino)-3-phenyl-butanoic acid methyl ester (1.58 g, 3.75 mmol) in methanol (20 ml) was added 1M sodium hydroxide (6 ml) and the reaction was left stirring at room temperature overnight. Application of general procedure 4.2, afforded the pure product as a white crystalline solid (1.47 g, 97%).

ν_{max}(neat)/cm^{-1} 3301 (N-H), 3067, 3022, 2957, 2926, 2865 (-CH_2, -CH_3), 1716 (C=O), 1664 (C=O), 1625 (C=O), 1538 (NHCO), 1455, 1413, 1365, 1268 (C-O-C), 1216, 1180., 1117., 1070., 1021., 1000, 863, 755 (Ph), 700 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.48 (d, *J*= 8.0 Hz, 1H, NH), 7.20-7.13 (m, 3H, Ar), 7.04- 7.03 (m, 2H, Ar), 5.58 (d, *J*= 8.4 Hz, 1H, NH), 4.61 (m, 1H, C(8)H), 4.53 (m, 1H, C(6)H), 3.64 (t, *J*= 4.0 Hz, 4H, C(1)H_2, C(2)H_2), 3.42- 3.32 (m, 4H, C(3)H_2, C(4)H_2), 2.60 (m, 2H, C(11)H_2), 2.14 (m, 1H, C(10)H), 1.92 (m, 1H, C(10)H'), 1.72-1.58 (m, 2H, C(12)H, C(13)H), 1.51 (m, 1H, C(12)H'), 0.95 (d, *J*= 6.4 Hz, 3H, C(14)H_3), 0.91 (d, *J*= 6.4 Hz, 3H, C(15)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 174.7 (C(9)), 174.2 (C(7)), 158.0 (C(5)), 141.1, 128.8,

128.8, 126.5 (Ar), 66.8 (C(1), C(2)), 53.6 (C(6)), 52.5 (C(8)), 44.4 (C(3), C(4)), 41.4 (C(12)), 34.5 (C(10)), 31.8 (C(11)), 25.1 (C(13)), 23.0 (C(14)), 22.8 (C(15)). Found $[M+Na]^+$ 428.2143, $C_{21}H_{31}N_3O_5Na$ requires 428.2161.

5- General Procedure: Generation of α-diazo-ketones

[(*S*)-1-((*S*)-1-Benzyl-3-diazo-2-oxo-propylcarbamoyl)-3-methyl-butyl]-carbamic acid benzyl ester (representative procedure 5.1)



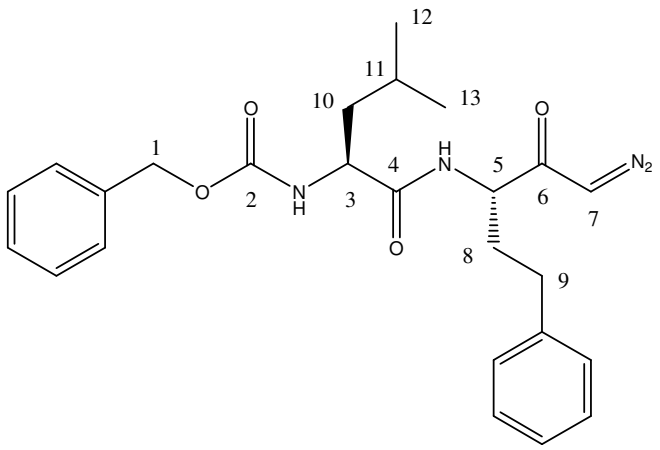
140.

Diazald® (5.0 g, 3.5 equiv.) was dissolved in 45 ml of diethyl ether and added to a 0ºC solution of potassium hydroxide (5 g) in water (8 ml) and ethanol (10 ml). The ice bath was removed and the reaction temperature increased to about 48ºC and left at that temperature while the diazomethane in ether was collected in a receiver at 0ºC. To a -40ºC solution of 2-(2-benzyloxycarbonylamino-4-methyl-pentanoylamino)-3-phenyl-propionic acid (1.85 g, 4.48 mmol) in 40 ml of freshly distilled tetrahydrofuran was added N-methylmorpholine (0.49 ml, 4.48 mmol) followed by drop wise addition of isobutyl chloroformate (0.59 ml, 4.48 mmol). The reaction was allowed to activate for

30 min and then filtered into a cold flask to remove precipitated salts. To the filtered solution was added an excess of the previously prepared diazomethane solution and the mixture was allowed to stand at 0ºC for 16 hours. The reaction mixture was then diluted with 50 ml of diethyl ether and washed successively with water (2x40 ml), a saturated solution of sodium hydrogen carbonate (40 ml) and brine (40 ml). The organic phase was then dried over magnesium sulphate, filtered and evaporated to dryness to afford 1.41 g of a yellow viscous oil. Addition of a mixture of diethyl ether/hexane (30:60) and overnight standing in the fridge caused precipitation of the pure product as a pale yellow solid, which was filtered and collected (0.75 g, 38.5%).

Anal. Calc. for $C_{24}H_{28}N_4O_4$: C, 66.04, H, 6.47, N, 12.84 %; found: C, 66.58, H, 6.62, N, 12.50 %. $[\alpha]_D^{24}$ = -20 º (*c* 0.7 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.33-7.14 (m, 10H, Ar), 6.61 (d, *J*= 7.2 Hz, 1H, NH), 5.21 (brs, 1H, C(7)H), 5.09 (s, 2H, $C(1)H_2$), 5.01 (d, *J*= 8.0 Hz, 1H, NH), 4.69 (brs, 1H, C(5)H), 4.13 (brs, 1H, C(3)H), 3.09-2.96 (m, 2H, $C(8)H_2$), 1.67-1.54 (m, 2H, C(9)H, C(10)H), 1.40 (m, 1H, C(9)H'), 0.90 (d, *J*= 6.8 Hz, 6H, $C(11)H_3$, $C(12)H_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 192.8 (C(6)), 172.3 (C(4)), 156.6 (C(2)), 136.5, 129.7, 129.0, 129.0, 128.9, 128.7, 128.6, 128.4, 128.4, 127.4, 127.0 (Ar), 67.6 (C(1)), 57.5 (C(5)), 55.1 (C(7)), 54.1 (C(3)), 41.6 (C(9)), 38.5 (C(8)), 25.1 (C(10)), 23.3 (C(11)), 22.2 (C(12)). Found $[M+Na]^+$ 459.1986, $C_{24}H_{28}N_4O_4{}^{23}Na$ requires 459.2008.

[(*S*)-1-((*S*)-3-Diazo-2-oxo-1-phenethyl-propylcarbamoyl)-3-methyl-butyl]-carbamic acid benzyl ester



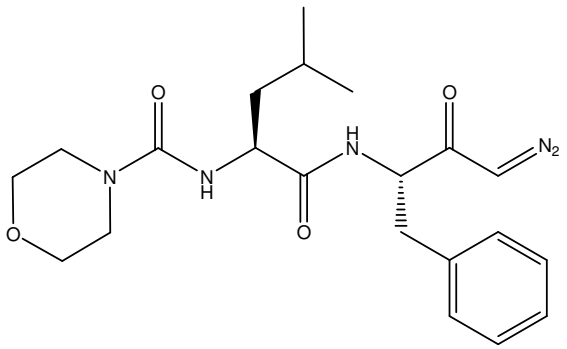
141.

Following representative procedure 5.1 a diazomethane solution in ether was prepared and isobutyl chloroformate (0.44 ml, 3.40 mmol) and *N*-methylmorpholine (0.37 ml, 3.40 mmol) were added to a solution of 2-(2-benzyloxycarbonylamino-4-methyl-pentanoylamino)-4-phenyl-butanoic acid (1.46 g, 3.40 mmol) in 40 ml of tetrahydrofuran. Addition of the prepared diazomethane solution to the activated mixture and following work-up afforded the pure product as a light yellowish solid (0.56 g, 40%) after purification by flash chromatography (SiO_2, 30% ethyl acetate in hexane).

ν_{max} (neat)/cm^{-1} 3300 (N-H), 3063, 3029, 2956, 2869 (C-H), 2108 (C=N_2), 1707 (C=O), 1653 (C=O), 1534 (NHCO), 1454, 1368, 1240, 1121, 1044, 739 (Ph), 699 (Ph). $[\alpha]_D^{24}$ = -12 ° (*c* 1 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.37-7.13 (m, 10H, Ar), 6.56 (d, *J*= 8.0 Hz, 1H, NH), 5.63 (brs, 1H, C(7)H), 5.12 (s, 2H, C(1)H_2), 5.00 (d, *J*= 6.4 Hz, 1H, NH), 4.49 (brs, 1H, C(5)H), 4.15 (brs, 1H, C(3)H), 2.63 (t, J= 8.0 Hz, 2H,

C(9)H_2), 2.21-2.12 (m, 1H, C(8)H), 1.94-1.85 (m, 1H, C(8)H'), 1.69-1.60 (m, 2H, C(10)H, C(11)H), 1.50-1.43 (m, 1H, C(10)H'), 0.93 (d, *J*= 6.4 Hz, 6H, C812)H_3, C(13)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 193.0 (C(6)), 172.3 (C(4)), 156.6 (C(2)), 141.2, 136.5, 129.0, 128.9, 128.9, 128.8, 128.7, 128.5, 126.9, 126.5 (Ar), 67.6 (C(1)), 56.2 (C(5)), 55.9, 54.6 (C(7)), 54.1 (C(3)), 41.6 (C(10)), 34.1 (C(8)), 33.1 (C(8)), 31.9 (C(9)), 25.6, 25.1 (C(11)), 23.5, 23.3 (C(12)), 22.2 (C(13)), 21.8. Found $[M+Na]^+$ 473.2188, $C_{25}H_{30}N_4O_4Na$ requires 473.2165.

Morpholine-4-carboxylic acid [(*S*)-1-((*S*)-1-benzyl-3-diazo-2-oxo-propylcarbamoyl) -3-methyl-butyl]-amide

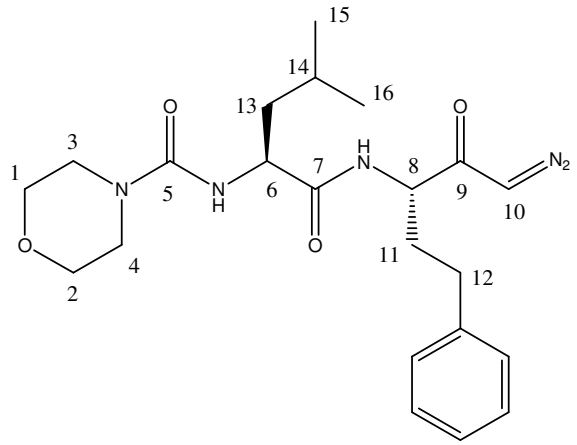


142.

Diazomethane was produced following representative procedure 5.1. To a -48ºC solution of 2-(4-methyl-2-(morpholine-4-carboxamido)pentanoylamino)-3-phenyl propionic acid (2.09 g, 5.34 mmol) in 40 ml of freshly distilled THF was added *N*-methylmorpholine (0.59 ml, 5.34 mmol) followed by dropwise addition of isobutyl chloroformate (0.70 ml, 5.34 mmol). After subjecting the resulting crude product to flash chromatography, a pale orange foam was obtained, which seemed still a mixture

of two compounds (1.78 g, 81%). This fraction was used directly to subsequent chemistry without any further purification.

Morpholine-4-carboxylic acid [(S)-1-((S)-3-diazo-2-oxo-1-phenethyl-propyl carbamoyl) -3-methyl-butyl]-amide



143.

Following representative procedure 5.1 a diazomethane solution in ether was prepared and isobutyl chloroformate (0.34 ml, 2.61 mmol) and *N*-methylmorpholine (0.29 ml, 2.61 mmol) were added to a solution of 2-(4- methyl-2- (morpholine-4- carboxamido) pentanoylamino) -3- phenyl butanoic acid (1.12 g, 2.61 mmol) in 40 ml tetrahydrofuran. Addition of the prepared diazomethane solution to the activated mixture and following work-up afforded the pure product as a yellow foam (0.36 g, 33%) after purification by flash chromatography (SiO_2, 30% ethyl acetate in hexanes).

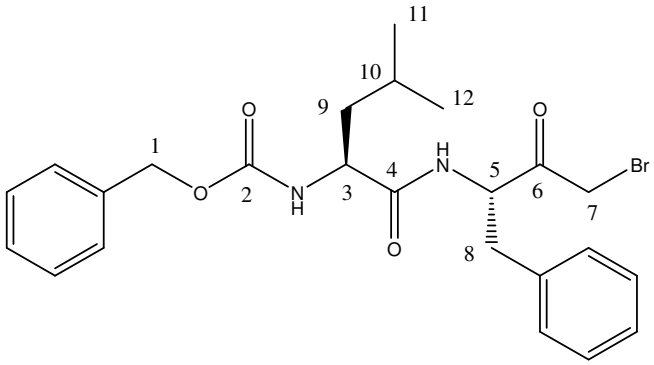
ν_{max}(neat)/cm^{-1} 3282 (N-H), 3062, 2956, 2925, 2859 (C-H), 2107 (C=N_2), 1624 (C=O), 1538 (NHCO), 1454, 1366, 1267 (C-O-C), 1118, 863, 737 (Ph), 700 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.28- 7.14 (m, 5H, Ar), 6.95 (d, *J*= 8.0 Hz, 1H, NH), 5.42 (s, 1H, C810)H, 4.91 (d, *J*= 7.6 Hz, 1H, NH), 4.45 (brs, 1H, C(8)H), 4.37 (m, 1H, C(6)H),

3.69- 3.61 (m, 4H, C(1)H_2, C(2)H_2), 3.40- 3.30 (m, 4H, C(3)H_2, C(4)H_2), 2.63 (t, *J*= 8.0 Hz, 2H, C(12)H_2), 2.20- 2.11 (m, 1H, C(11)H), 1.95- 1.85 (m, 1H, C(11)H'), 1.71-1.61 (m, 2H, C(14)H, C(13)H), 1.56- 1.48 (m, 1H, C(13)H'), 0.94 (m, 6H, C(15)H_3, C(16)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 193.2 (C(9)), 173.5 (C(7)), 157.8 (C(5)), 141.2, 128.9, 128.8, 126.6 (Ar), 66.8 (C(1), C(2)), 56.25 (C(8)), 54.43 (C(10)), 53.43 (C(6)), 44.5 (C(3), C(4)), 41.8 (C(13)), 34.1 (C(11)), 31.9 (C(12)), 25.3 (C(14)), 23.3 (C(15)), 22.6 (C(16)). Found $[M+Na]^+$ 452.2290, $C_{22}H_{31}N_5O_4{}^{23}Na$ requires 452.2274.

6- General procedure: α-bromination of diazo-ketones

Benzyl 1-(4-bromo-3-oxo-1-phenylbutan-2-ylcarbamoyl)-3-methylbutylcarbamate

(representative procedure 6.1)



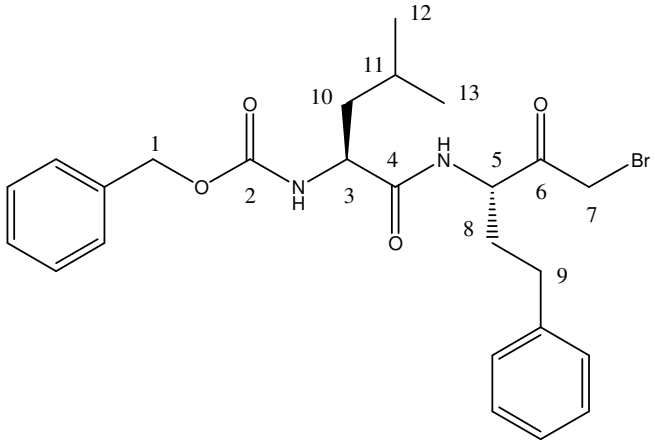
144.

[(*S*)-1-((*S*)-1-Benzyl-3-diazo-2-oxo-propylcarbamoyl)-3-methyl-butyl]-carbamic acid benzyl ester (0.30 g, 0.69 mmol) was dissolved in diethyl ether (12 ml) and terahydrofuran (12 ml) and stirred at -10ºC. An excess of 30% hydrogen bromide in acetic acid solution (0.5 ml) was added slowly and drop wise. The reaction mixture was

kept stirring at -10 ºC for 30 min. and then 15 min. at room temperature. Then it was washed with an equal volume of brine and the phases separated. The aqueous phase was again extracted with diethyl ether and the combined organic extracts washed carefully with saturated aqueous sodium bicarbonate and brine. The organic solution was dried over magnesium sulphate, filtered and evaporated to give the crude product as a light yellow solid. The crude was chromatographed (SiO_2, 20% EtOAc: hexane) to yield the pure product as a clear pale yellow solid (0.158 g, 47%).

$[\alpha]_D^{24}$ = -20 º (*c* 0.6 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.38-7.12 (m, 10H, Ar), 6.63 (brs, 1H, NH), 5.09 (s, 2H, C(1)H_2), 5.02 (d, *J*= 7.6 Hz, 1H, NH), 4.95 (q, *J*= 8.0 Hz, C(5)H), 4.13 (brs, 1H, C(3)H), 3.90 (d, *J*= 12.0 Hz, 1H, C(7)H), 3.79 (d, *J*= 12.0 Hz, 1H, C(7)H'), 3.13 (dd, *J*= 14.0 Hz, *J*= 6.8 Hz, 1H, C(8)H), 3.00 (dd, , *J*= 14.0 Hz, *J*= 8.0 Hz, 1H, C(8)H), 1.65-1.53 (m, 2H, C(9)H, C(10)H), 1.40 (brs, 1H, C(9)H'), 0.91 (m, 6H, C(11)H_3, C(12)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 200.4 (C(6)), 172.5 (C(4)), 136.4, 136.0, 129.5, 129.3, 129.0, 128.7, 128.5, 127.8 (Ar), 67.7 (C(1)), 57.7 (C(5)), 53.9 (C(3)), 41.3 (C(9)), 38.0 (C(8)), 33.0 (C(7)), 25.1 (C(12)), 23.2 (C(11)), 22.3 (C(10)). Found $[M+Na]^+$ 511.1230, $C_{24}H_{28}N_4O_4{}^{23}Na^{79}Br$ requires 511.1208; Found $[M+Na]^+$ 513.1193, $C_{24}H_{28}N_4O_4{}^{23}Na^{81}Br$ requires 513.1188.

Benzyl 1-(1-bromo-2-oxo-5-phenylpentan-3-ylcarbamoyl)-3-methylbutylcarbamate

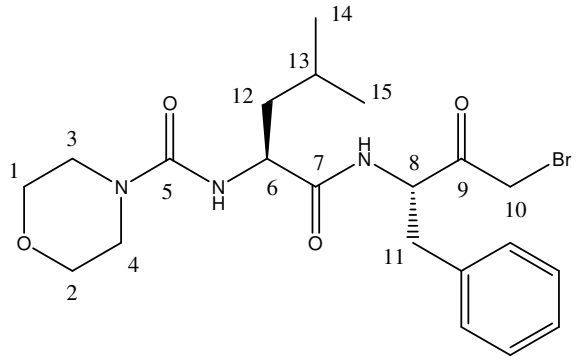


145.

[(*S*)-1-((*S*)-3-Diazo-2-oxo-1-phenethyl-propylcarbamoyl)-3-methyl- butyl]-carbamic acid benzyl ester (0.39 g, 0.87 mmol) was dissolved in diethyl ether (10 ml) and tetrahydrofuran (10 ml) and representative procedure 6.1 followed. From purification by flash chromatography, the product was obtained pure as a white solid (0.30 g, 68%). $[\alpha]_D^{24}$ = -7.9 º (*c* 1.2 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.37-7.13 (m, 10H, Ar), 6.60 (brs, 1H, NH), 5.11 (s, 2H, C(1)H_2), 5.01 (d, *J*= 6.0 Hz, 1H, NH), 4.78 (dt, *J*= 8.0 Hz, *J*= 4.4 Hz, C(5)H), 4.16 (brs, 1H, C(3)H), 3.98 (d, *J*= 13.2 Hz, 1H, C(7)H), 3.92 (d, *J*= 13.2 Hz, 1H, C(7)H'), 2.63 (t, *J*= 8.0 Hz, C(9)H_2) 2.30-2.22 (m, 1H, C(8)H), 1.99-1.90 (m, 1H, C(8)H'), 1.69-1.61 (m, 2H, C(10)H, C(11)H), 1.51-1.44 (m, 1H, C(10)H'), 0.94 (d, *J*= 8.0 Hz, 6H, C(13)H_3, C(12)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 200.5 (C(6)), 172.6 (C(4)), 140.7, 136.4, 129.1, 129.0, 128.8, 128.7, 128.5, 128.0, 127.4, 126.8 (Ar), 67.7 (C(1)), 65.8, 56.6 (C(5)), 54.0 (C(3)), 41.3 (C(10)), 33.3 (C(9)), 32.1 (C(7)), 32.0 (C(7)) 25.1 (C(12)), 23.3 (C(11)), 22.3 (C(13)). Found $[M+Na]^+$

525.1367, $C_{24}H_{28}N_4O_4{}^{79}Br^{23}Na$ requires 525.1365; Found $[M+Na]^+$ 527.1349, $C_{24}H_{28}N_4O_4{}^{81}Br^{23}Na$ requires 527.1344.

1-(4-Bromo-3-oxo-1-phenylbutan-2-ylcarbamoyl)-3-methylbutyl)morpholine-4-carboxamide



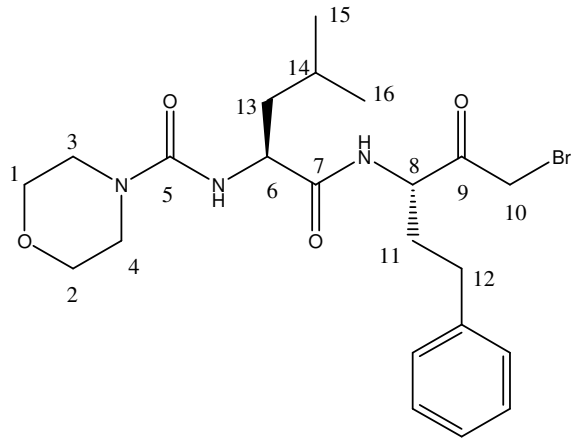
146.

Representative procedure 6.1 was followed, using morpholine-4-carboxylic acid [(*S*)-1-((*S*)-1-benzyl-3-diazo-2-oxo-propylcarbamoyl) -3-methyl-butyl]-amide (1.48 g, 3.55 mmol) in 20 ml diethyl ether and 20 ml tetrahydrofuran, to which was added 2.0 ml of the hydrogen bromide in acetic acid solution. The obtained crude was subjected to flash chromatography (SiO_2, 50% EtOAc: hexanes) to yield the pure product as a yellow foam (0.56 g, 34%).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.32- 7.25 (m, 3H, Ar), 7.17- 7.16 (m, 2H, Ar), 6.92 (d, *J*= 6.4 Hz, 1H, NH), 4.91 (dd, *J*= 14.4 Hz, *J*= 7.6 Hz, 1H, C(8)H), 4.69 (d, *J*= 7.6 Hz, 1H, NH), 4.29 (m, 1H, C(6)H), 3.93 (d, *J*= 14.0 Hz, 1H, C(10)H), 3.81 (d, *J*= 13.6 Hz, 1H, C(10)H'), 3.67 (t, *J*= 4.0 Hz, 4H, $C(1)H_2$, $C(2)H_2$), 3.37- 3.26 (m, 4H, $C(3)H_2$, $C(4)H_2$), 3.15 (dd, *J*= 13.6 Hz, *J*= 6.8 Hz, 1H, C(11)H), 3.02 (dd, *J*= 13.6 Hz, *J*= 7.6 Hz, 1H, C(11)H'), 1.67- 1.56 (m, 2H, C(12)H, C(13)H), 1.48- 1.42 (m, 1H, C(12)H'),

0.93 (d, *J*= 6.8 Hz, 3H, C(14)H_3), 0.89 (d, *J*= 6.6 Hz, 3H, C(15)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 200.4 (C(9)), 173.6 (C(7)), 157.7 (C(6)), 136.2, 129.5, 129.2, 127.7 (Ar), 66.8 (C(1), C(2)), 57.8 (C(8)), 53.1 (C(6)), 44.4 (C(3), C(4)), 41.4 (C(12)), 37.8 (C(10)), 33.4 (C(11)), 25.2 (C(13)), 23.2 (C(14)), 22.6 (C(15)). Found $[M+Na]^+$ 490.1320, $C_{21}H_{30}N_3O_4{}^{23}Na^{79}Br$ requires 490.1317; Found $[M+Na]^+$ 492.1287, $C_{21}H_{30}N_3O_4{}^{23}Na^{81}Br$ requires 492.1297.

N-((*S*)-1-((*S*)-1-Bromo-2-oxo-5-phenylpentan-3-ylcarbamoyl)-3-methylbutyl) morpholine - 4-carboxamide



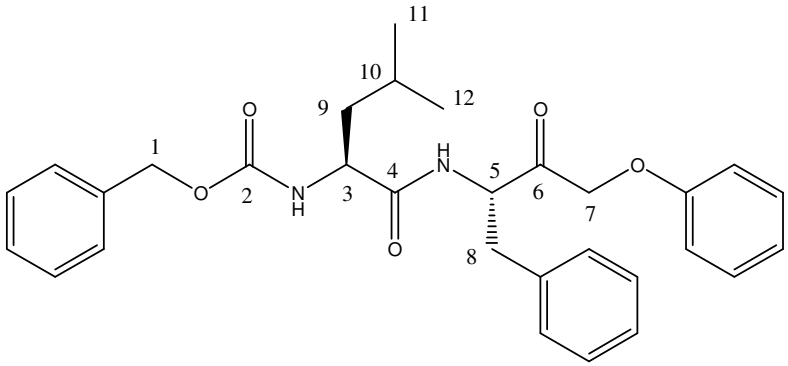
147.

To morpholine-4-carboxylic acid [(*S*)-1-((*S*)-3-diazo-2-oxo-1-phenethyl-propyl carbamoyl) -3-methyl-butyl]-amide (0.31 g, 0.72 mmol) dissolved in diethyl ether (7 ml) and in tetrahydrofuran (7 ml) was added carefully a solution of hydrogen bromide in acetic acid solution (0.6 ml). In accordance with representative procedure 6.1 the crude material was obtained and purified by flash chromatography (SiO_2, 3% methanol in dichloromethane) to yield the pure product as a yellowish foam (0.30 g, 87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.49 (brs, 1H, NH), 7.28-7.12 (m, 5H, Ar), 5.29 (brs, 1H, NH), 4.64 (m, 1H, C(8)H), 4.43 (m, 1H, C(6)H), 4.03 (d, *J*= 13.6 Hz, 1H, C(10)H), 3.96 (d, *J*= 13.2 Hz, 1H, C(10)H'), 3.62 (brs, 4H, $C(1)H_2$, $C(2)H_2$), 3.34 (brs, 4H, $C(3)H_2$, $C(4)H_2$), 2.69- 2.58 (m, 2H, $C(12)H_2$), 2.44-2.15 (m, 1H, C(11)H), 1.98- 1.88 (m, 1H, C(11)H'), 1.71-1.52 (m, 3H, $C(13)H_2$, C(14)H), 0.94 (brs, 6H, $C(14)H_3$, $C(15)H_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 200.6 (C(9)), 174.3 (C(7)), 158.0 (C(6)), 140.8, 129.0, 128.8, 126.8 (Ar), 66.7 (C(1), C(2)), 56.6 (C(8)), 53.4 (C(6)), 44.5 (C(3), C(4)), 41.5 (C(13)), 33.1 (C(11)), 32.6 (C(10)), 32.1 (C(12)), 25.3 (C(14)), 23.3 (C(15)), 22.7 (C(16)). Found $[M+Na]^+$ 504.1456, $C_{22}H_{32}N_3O_4{}^{23}Na^{79}Br$ requires 504.1474; Found $[M+Na]^+$ 506.1449, $C_{22}H_{32}N_3O_4{}^{23}Na^{81}Br$ requires 506.1453.

7- General procedure: obtaining the α-substituted targets

Benzyl1-(3-oxo-4-phenoxy-1-phenylbutan-2-ylcarbamoyl)-3-methylbutyl Carbamate (representative procedure 7.1)

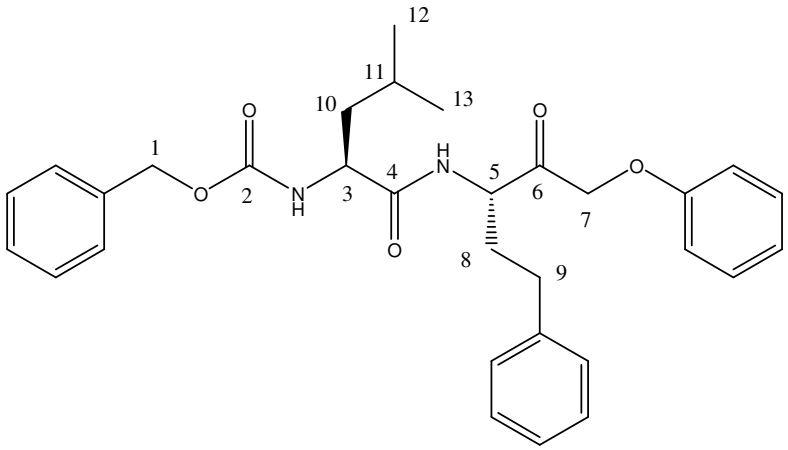


148.

Phenol (58.0 mg, 0.61 mmol) and sodium hydride (60% dispersion in oil, 15.0 mg, 0.61 mmol) were stirred in dimethylformamide (2 ml) at -15ºC, under a nitrogen atmosphere for 15 min. Benzyl 1-(4-bromo-3-oxo-1-phenylbutan-2-ylcarbamoyl)-3-methylbutylcarbamate (300 mg, 0.61 mmol) in DMF (2 ml) was added and the reaction mixture stirred for 30 min, at -15ºC, then at room temperature, for an additional 2h. After that period of time, the mixture was neutralized with 0.3 M solution of potassium bisulfate and the solvent removed with vacuum. The obtained residue was partitioned between ethyl acetate and water and the organic extracts were washed with brine. The solution was dried over magnesium sulphate, filtered and evaporated to give a crude residue, which was dissolved in a mixture of diethyl ether and hexane (60:40) and left standing overnight in the fridge, affording the next day, by filtration, the purified product as a white solid (0.132 g, 43%).

ν_{max} (nujol)/cm^{-1} 3304, 3269 (-CONH-), 3049, 2953 (-CH_3, -CH_2), 1735 (C=O), 1682 (C=O), 1656 (C=O), 1594 (Ph), 1529 (-CONH-), 1494, 1384, 1366, 1314, 1261, 1235, 1169, 1116, 1042, 1020, 752., 695, 669. $[\alpha]_D^{24}$ = 3.8 º (*c* 0.6 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.33-7.23 (m, 10H, Ar), 7.14 (d, *J*= 7.6 Hz, 2H, Ar), 6.99 (t, *J*= 7.2 Hz, 1H, Ar), 6.85 (d, *J*= 8.4 Hz, 2H, Ar), 6.51 (brs, 1H, NH), 5.11 (m, 1H, C(5)H), 5.08 (s, 2H, C(1)H_2), 5.00 (brs, 1H, NH), 4.67 (d, *J*= 17.2 Hz, 1H, C(7)H), 4.56 (d, *J*= 16.8 Hz, 1H, C(7)H'), 4.12 (brs, 1H, C(3)H), 3.20 (brs, 1H, C(8)H), 3.02 (brs, 1H, C(8)H'), 1.58-1.51 (m, 2H, C(9)H, C(10)H), 1.42- 1.37 (m, 1H, C(9)H'), 0.87 (d, *J*= 5.6 Hz, 3H, C(11)H_3), 0.86 (d, *J*= 6.4 Hz, 3H, C(12)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 203.4 (C(6)), 170.9 (C(4)), 156.5 (C(2)), 134.6, 128.6, 128.2, 127.8, 127.6, 127.2, 127.1, 126.3, 120.9, 113.7 (Ar), 71.0 (C(7)), 66.2 (C(1)), 55.4 (C(5)), 52.5 (C(3)), 40.1 (C(9)), 36.1 (C(8)), 23.7 (C(10)), 21.8 (C(11)), 20.9 (C(12)). Found $[M+Na]^+$ 525.2348, $C_{30}H_{34}N_2O_5$23Na requires 525.2365.

Benzyl 1-(2-oxo-1-phenoxy-5-phenylpentan-3-ylcarbamoyl)-3-methylbutyl carbamate



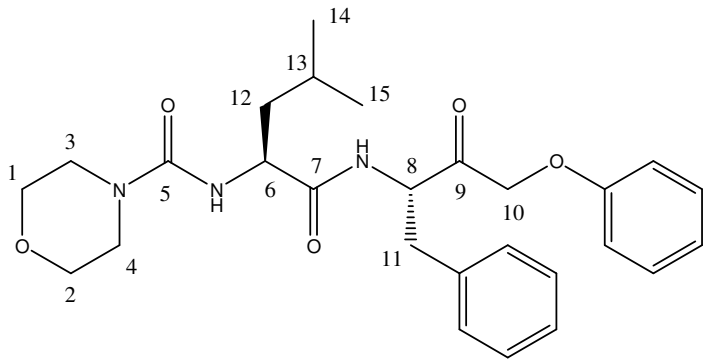
149.

Phenol (0.020 g, 0.21 mmol) and sodium hydride (60% dispersion in oil, 0.009 g, 0.21 mmol) were stirred in dimethylformamide (2 ml) at -15ºC. Following representative procedure 7.1, benzyl 1-(1-bromo-2-oxo-5-phenylpentan-3-ylcarbamoyl)-3-methylbutylcarbamate (0.107g, 0.21 mmol) in DMF (1 mL) was added. After work up, flash chromatography (20% ethyl acetate in hexanes) afforded the pure product as a white solid (0.064 g, 58%)

$[\alpha]_D^{24}$ = -6.6 º (*c* 0.6 in $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ_H 7.32-7.19 (m, 10H, Ar), 7.13 (d, *J*= 7.2 Hz, 2H, Ar), 6.98 (m, 1H, Ar), 6.84-6.76 (m, 2H, Ar), 6.63 (d, *J*= 6.0 Hz, 1H, NH), 5.15 (d, *J*= 8.0 Hz, 1H, NH), 5.10 (s, 2H, $C(1)H_2$), 4.99- 4.93 (m, 1H, C(5)H), 4.62 (s, 2H, $C(7)H_2$), 4.21 (brm, 1H, C(3)H), 2.64 (m, 2H, $C(9)H_2$), 2.32-2.22 (m, 1H, C(8)H), 1.97-1.88 (m, 1H, C(8)H'), 1.72-1.61 (m, 2H, C(11)H, C(10)H), 1.55-1.45 (m, 1H, C(10)H'), 0.93 (m, 6H, $C(12)H_3$, $C(13)H_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 205.4, 205.3 (C(6)), 172.7, 172.5 (C(4)), 157.9 (C(2)), 140.9, 140.8, 130.1, 129.0,

128.9, 128.9, 128.9, 128.6, 128.5, 126.8, 126.7, 122.4, 122.3, 115.0 (Ar), 71.7 (C(7)), 67.6 (C(1)), 55.9, 55.4 (C(5)), 54.0 (C(3)), 41.6 (C(10)), 33.1 (C(9)), 32.0 (C(8)), 25.21, 25.1 (C(11)), 23.3, 23.3 (C(12)), 22.3 (C(13)). Found $[M+Na]^+$ 463.2202, $C_{30}H_{34}N_2O_5{}^{23}Na$ requires 463.2209.

_N_-(1-(3-Oxo-4-phenoxy-1-phenylbutan-2-ylcarbamoyl)-3-methylbutyl)morpholine-4-carboxamide



150.

Phenol (96.0 mg, 1.02 mmol) and sodium hydride (60% dispersion in oil, 21.0 mg, 1.02 mmol) were stirred in dimethylformamide (3.5 ml) at -15ºC, under a nitrogen atmosphere for 15 min. *N*-((*S*)-1-((*S*)- 4-bromo- 3-oxo-1- phenylbutan-2-ylcarbamoyl)-3-methylbutyl)morpholine-4-carboxamide (0.48 g, 1.02 mmol) in DMF (3.5 ml) was added. Representative procedure 7.1 was followed but the crude residue was purified by flash chromatography (45% ethyl acetate/n-hexane) affording a pale yellow solid, which by TLC appeared to be a pure compound (0.26 g, 54%).

$[\alpha]_D{}^{24}$ = -9.5 º (*c* 0.2 in $CHCl_3$). ν_{max} (neat)/cm^{-1} 3279 (-CONH-), 3063, 2956, 2924, 2856 (-CH_2, -CH_3), 1734 (C=O), 1653 (C=O), 1620 (C=O), 1540 (-CONH-), 1496,

1456, 1268, 1247 (C-O), 1117, 1000, 864, 752 (Ph), 691 (Ph). MS *m/z* (CI, +ve) 482 ($[M+H]^+$, 13), 88 (100), 227 (36), 303 (44), 395 (20), 412 (12). Found $[M+Na]^+$ 504.2460, $C_{27}H_{35}N_3O_5Na$ requires 504.2474.

After injecting the compound in the HPLC column, separation in two fractions (1:1 proportion) was observed. The fractions were collected, their NMR spectra analysed and optical rotation measured.

1st fraction

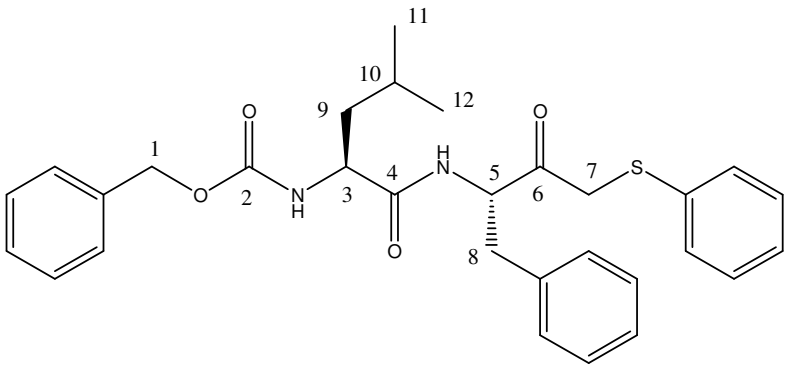
$[\alpha]_D^{24}$ = - 9.6 º (*c* 0.8 in $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ_H 7.31- 7.22 (m, 5H, Ar), 7.17 (m, 2H, Ar), 6.98 (t, *J*= 7.4 Hz, 1H, Ar), 6.86 (d, *J*= 8.0 Hz, 1H, Ar), 6.79 (d, *J*= 7.6 Hz, 1H, NH), 5.08 (m, 1H, C(8)H), 4.76 (d, *J*= 7.6 Hz, 1H, NH), 4.72 (d, *J*= 17.2 Hz, 1H, C(10)H), 4.58 (d, *J*= 17.2 Hz, 1H, C(10)H'), 4.28 (m, 1H, C(6)H), 3.64 (t, *J*= 9.2 Hz, 4H, $C(1)H_2$, $C(2)H_2$,), 3.33- 3.30 (m, 4H, $C(3)H_2$, $C(4)H_2$), 3.23 (dd, *J*= 14.0 Hz, *J*= 6.0 Hz, 1H, C(11)H), 3.00 (dd, J= 14.0 Hz, J= 7.6 Hz, 1H C(11)H'), 1.56- 1.43 (m, 2H, C(12)H, C(13)H), 1.44- 1.35 (m, 1H, C(12)H'), 0.86 (d, *J*= 6.0 Hz, 3H, $C(14)H_3$), 0.85 (d, *J*= 6.0 Hz, 3H, $C(15)H_3$).(100 MHz, $CDCl_3$) δ_C 204.9 (C(9)), 173.6 (C(7)), 157.8 (C(5)), 136.0, 130.0, 129.6, 129.2, 127.6, 122.2, 115.1 (Ar), 72.2 (C(10)), 66.7 (C(1), C(2)), 57.1 (C(8)), 53.2 (C(6)), 44.4 (C(3), C(4)), 41.6 (C(12)), 37.2 (C(11)), 25.2 (C(13)), 23.1, (C(14)), 22.7 (C(15)).

2nd fraction

$[\alpha]_D^{24}$ = - 2.4 º (*c* 0.8 in $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ_H 7.30-7.21 (m, 5H, Ar), 7.17 (m, 2H, Ar), 6.98 (t, *J*= 7.2 Hz, 1H), 6.84 (m, 3H, 2H (Ar), 1H (NH)), 5.08 (q, *J*= 6.8 Hz, 1H, C(8)H), 4.74 (d, *J*= 7.2 Hz, 1H, NH), 4.67 (d, *J*= 17.2 Hz, 1H, C(10)H), 4.51 (d, *J*= 17.1 Hz, 1H, C(10)H'), 4.33 (m, 1H, C(6)H), 3.66 (t, *J*= 4.8 Hz, 4H, $C(1)H_2$, $C(2)H_2$), 3.37- 3.26 (m, 4H, $C(3)H_2$, $C(4)H_2$), 3.19 (dd, *J*= 14.0 Hz, *J*= 6.8 Hz, 1H, C(11)H), 3.03 (dd, *J*= 14.0 Hz, *J*= 7.2 Hz, 1H, C(11)H'), 1.67- 1.63 (m, 2H,

C(12)H, C(13)H), 1.49- 1.43 (m, 1H, C(12)H'), 0.91 (d, *J*= 6.0 Hz, 3H, $C(14)H_3$), 0.90 (d, *J*= 6.0 Hz, 3H, $C(15)H_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 204.8 (C(9)), 173.5 (C(7)), 158.1 (C(5)), 136.2, 130.1, 129.6, 129.2, 127.6, 122.2, 115.1 (Ar), 72.5 (C(10)), 66.8 (C(1), C(2)), 57.0 (C(8)), 53.1 (C(6)), 44.5 (C(3), C(4)), 41.7 (C(12)), 37.4 (C(11)), 25.0 (C(13)), 23.0, (C(14)), 22.0 (C(15)).

Benzyl 1-(3-oxo-1-phenyl-4-(phenylthio)butan-2-ylcarbamoyl)-3-methylbutyl carbamate (representative procedure 7.2)

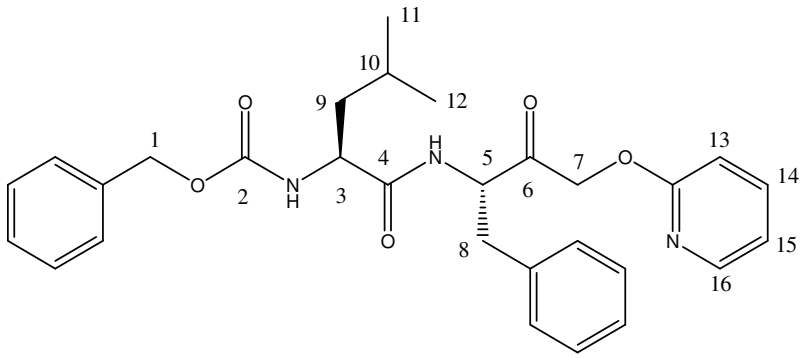


151.

Thiophenol (25µl, 0.24 mmol) was added to a solution of benzyl 1-(4-bromo-3-oxo-1-phenylbutan-2-ylcarbamoyl)-3-methylbutylcarbamate (142 mg, 0.29 mmol) in dimethylformamide (1.0 ml) at room temperature. Then solid potassium fluoride (19.0 mg, 0.32 mmol) was added and the reaction mixture was left stirring overnight. Concentration of the reaction mixture in vacuum followed by column chromatography (SiO_2, 20% ethyl acetate/n-hexane) of the residue gave the title compound as a white solid (0.130 g, 87%).

$[\alpha]_D^{24}$ = -47 ° (*c* 0.2 in $CHCl_3$). ν_{max} (neat)/cm^{-1} 3301 (-CONH-), 3062, 3031, 2957, 2929, 2870 (-CH_3, -CH_2), 1718 (C=O), 1654 (C=O), 1603 (Ph), 1534 (-CONH-), 1454, 1386, 1267, 1122, 1073, 1042 (C-S), 1027, 739 (Ph), 698 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.33-7.07 (m, 15H, Ar), 6.61 (d, *J*= 4.8 Hz, 1H, NH), 5.07 (s, 2H, C(1)H_2), 5.02 (m, 1H, C(5)H_2), 4.21 (m, 1H, C(3)H), 4.12 (brs, 1H, NH), 3.62 (s, 2H, C(7)H_2), 3.05 (dd, *J*= 13.5 Hz, *J*= 6.6 Hz, 1H, C(8)H), 2.94 (m, 1H, C(8)H'), 1.68 (m, 1H, C(9)H), 1.61-1.26 (m, 2H, C(9)H', C(10)H), 0.87 (d, J= 6.8 Hz, 6H, C(11)H_3, C(12)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 202.6 (C(6)), 171.9 (C(4)), 167.7, 156.0 (C(2)), 136.1, 135.8, 134.2, 132.4, 130.8, 130.1, 129.1, 128.7, 128.5, 128.2, 128.0, 127.1 (Ar), 68.1 (C(1)), 67.1(C(5)), 57.4, 53.5 (C(3)), 42.5 (C(7)) , 41.1 (C(9)), 38.7, 37.6 (C(8)), 30.3, 29.6, 28.9, 24.6, 23.7 (C(10)), 22.9, 22.7 (C(11)), 21.9 (C(12)), 14.0, 10.9. Found $[M+Na]^+$ 541.2134, $C_{30}H_{34}N_2O_4S^{23}Na$ requires 541.2137.

Benzyl 1-(3-oxo-1-phenyl-4-(pyridin-2-yloxy)butan-2-ylcarbamoyl)-3-methylbutyl carbamate (representative procedure 7.3)



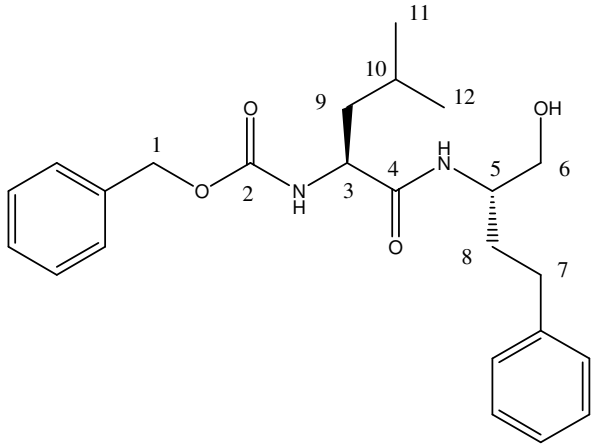
152.

Benzyl 1-(4-bromo-3-oxo-1-phenylbutan-2-ylcarbamoyl)-3-methylbutylcarbamate (0.26 g, 0.53 mmol), potassium fluoride (0.12 g, 2.1 mmol), and 2-hydroxypyridine (0.20 g, 2.1 mmol) were dissolved in dimethylformamide (14 ml). The reaction mixture was left stirring under nitrogen overnight. The solvent was removed under vacuum and dichloromethane added. The organic phase was then washed with water, dried over magnesium sulphate, filtered and the solvent evaporated. Dissolution of the crude product in a mixture of diethyl ether/n-hexane allowed precipitation of the product as a white/silvery solid (0.184 g, 69%).

$[\alpha]_D^{21}$ = -95.8 º (*c* 0.8 in $CHCl_3$). ν_{max} (neat)/cm^{-1} 3286 (-CONH-), 3062, 3031, 2956, 2869 (-CH_3, -CH_2) , 1716 (C=O), 1658 (C=O), 1583 (-C=N-, conjugated cyclic system), 1538 (-CONH-), 1455, 1387, 1340, 1263 (C-O), 1175, 1148, 1122, 1044, 1028, 736 (Ph), 699 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.39-7.11 (m, 12H, Ar, C(14)H, C(16)H), 6.92 (brs, 1H, NH), 6.54 (d, *J*=9.2 Hz, 1H, C(13)H), 6.19 (t, *J*= 6.8 Hz, 1H, C(15)H), 5.05 (s, 2H, C(1)H_2), 4.98 (brs, 1H, C(5)H), 4.56 (d, *J*= 16.4 Hz, 1H, NH), 4.11 (brs, 1H, C(3)H), 3.30 (dd, *J*= 14.0 Hz, *J*= 5.2 Hz, 1H, C(8)H), 2.98 (dd, *J*= 14.0 Hz, *J*= 8.4 Hz, 1H, C(8)H'), 1.57-1.49 (m, 2H, C(9)H, C(10)H), 1.36 (m, 1H, C(9)H'), 0.88 (m, 6H, C(11)H_3, C(12)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 201.9 (C(6)), 173.0 (C(4)), 156.0 (C(2)), 147.8 (C(16)), 140.8 (Ar), 138.9 (C(14)), 136.7, 129.7, 129.1, 128.9, 128.7, 128.5, 127.4 (Ar), 120.9 (C(13)), 106.7 (C(15)), 67.7 (C(1)), 58.6 (C(7)), 56.1 (C(5)), 41.3 (C(9)), 36.7 (C(8)), 25.1 (C(10)), 23.3 (C(11)), 22.1 (C(12)). Found $[M+Na]^+$ 526.2327, $C_{29}H_{33}N_3O_5$23Na requires 526.2318.

8- Synthesis of the Control Compound

Benzyl 1-(1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamate (representative procedure 8.1)



153.

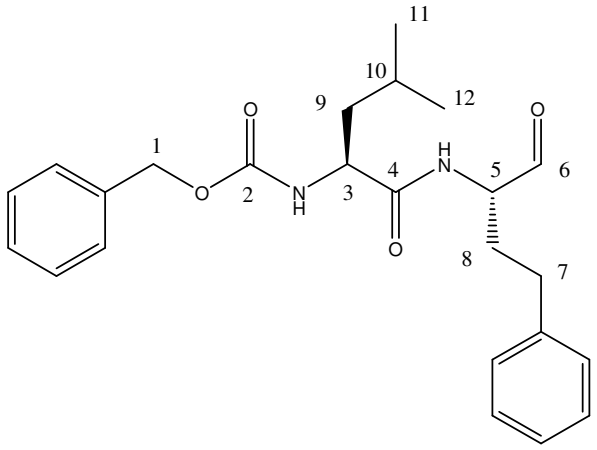
To a stirring solution of 2-(2-benzyloxycarbonylamino-4-methyl-pentanoylamino)-4-phenyl-butanoic acid methyl ester (0.52 g, 1.2 mmol) in ethanol (10 ml) at -5ºC was added a suspension of calcium chloride (0.26 g, 2.4 mmol) in tetrahydrofuran (5 ml). Sodium borohydride (0.18 g, 4.7 mmol) was then added to the mixture and the reaction was left stirring for 5 hours. The mixture was then carefully poured into an iced citric acid solution, extracted with chloroform and washed with brine, affording a white crystalline solid as the product (0.45 g, 92%).

$[\alpha]_D^{24}$ = -35.1 º (*c* 0.6 in $CHCl_3$). ν_{max} (neat)/cm^{-1} 3400 (OH), 3307 (N-H), 3063, 3052, 2951, 2870 (C-H), 1683 (C=O), 1652 (C=O), 1532 (NHCO), 1454, 1278, 1241, 1039, 749 (Ph), 699 (Ph).^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.36-7.14 (m, 10H, Ar), 6.29 (d, *J*=

6.4 Hz, 1H, NH), 5.16 (d, *J*= 8.0 Hz, 1H, NH), 5.09 (s, 2H, C(1)H_2), 4.12 (brs, 1H, C(3)H), 3.96- 3.89 (m, 1H, C(5)H), 3.65 (brs, 1H, C(6)H), 3.56 (dd, *J*= 11.2 Hz, *J*= 5.2 Hz, 1H, C(6)H'), 2.63 (t, *J*= 8.0 Hz, 2H, C(7)H_2), 1.91-1.77 (m, 2H, C(8)H_2), 1.70-1.62 (m, 2H, C(10)H, C(9)H), 1.52-1.44 (m, 1H, C(9)H'), 0.93 (t, *J*= 6.0 Hz, 6H, C(12)H_3, C(11)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 173.1 (C(4)), 156.8 (C(2)), 141.8, 136.5, 129.1, 128.9, 128.9, 128.8, 128.8, 128.8, 128.5, 128.5, 126.4 (Ar), 67.6 (C(1)), 65.6 (C(6)), 54.3 (C(3)), 52.2 (C(5)), 41.5 (C(9)), 33.0 (C(7)), 32.7 (C(8)), 25.2 (C(10)), 23.3 (C(11)), 22.3 (C(12)). Found $[M+Na]^+$ 435.2266, $C_{24}H_{32}N_2O_4{}^{23}Na$ requires 435.2260.

Benzyl 1-(1-formyl-3-hydroxypropylcarbamoyl)-3-methylbutyl carbamate

(representative procedure 8.2)



154.

Dimethyl sulfoxide (0.15 ml, 2.18 mmol) was added to a solution of oxalyl chloride (0.12 ml, 1.31 mmol) in dichloromethane (0.4 ml) at -78ºC. The reaction mixture was stirred for 5 minutes and a solution of benzyl 1-(1,4-dihydroxybutan-2-ylcarbamoyl)-3-

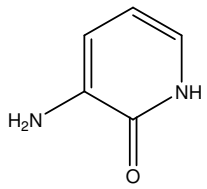
methylbutylcarbamate (0.30 g, 7.3 mmol) in dichloromethane was added. The mixture was stirred for 15 minutes at -78ºC and triethylamine (0.98 ml, 5.09 mmol) was added. The reaction mixture was allowed to warm up to 0ºC and kept at that temperature for 1 hour and 30 minutes. Water (5 ml) was added to the reaction and the reaction mixture was washed with aqueous saturated sodium chloride solution, followed by aqueous saturated sodium hydrogen carbonate. The organic extract was dried over magnesium sulphate and the solvent removed under reduced pressure. The desired product was obtained as transparent oil (0.15 g, 50%) after purification by flash chromatography (SiO_2, 30 to 45% ethyl acetate / in hexane).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 9.47 (d, *J*= 11.6 Hz, 1H), 7.34-7.13 (m, 10H, Ar), 6.86 (s, NH), 6.70 (s, NH), 5.29 (brs, NH), 5.24 (brs, NH), 5.11 (s, 2H, C(1)H_2), 4.45 (brs, 1H, C(5)H), 4.25 (brs, 1H, C(3)H), 2.64 (t, *J*= 8.0 Hz, 2H, C(7)H_2), 2.23 (m, 1H, C(8)H), 2.03 (m, 1H, (C(8)H'), 1.68-1.57 (m, 2H, C(10)H, C(9)H), 1.57-1.48 (m, 1H, C(9)H'), 0.94 (m, 6H, C(12)H_3, C(11)H_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 199.0 (C(6)), 173.0 (C(4)), 157.0 (C(2)), 140.9, 129.0, 128.9, 128.8, 128.8, 128.7, 128.6, 128.4, 126.8, 126.8 (Ar), 67.6 (C(1)), 58.8 (C(5)), 53.0 (C(3)), 42.0 (C(9)), 31.8 (C(7)), 30.8 (C(8)), 25.1 (C(10)), 23.3 (C(11)), 22.4 (C(12)). Found $[M^+Na^+CH_3OH]^+$ 465.2372, $C_{25}H_{34}N_2O_5{}^{23}Na$ requires 465.2365.

3.4.3- Preparation of Peptidomimetic CPI

1- Nitro group reduction: general procedure

3-Aminopyridin-2(1*H*)-one (representative procedure 1.1)

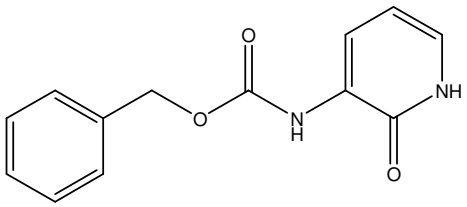


156.

Ethanol (75 ml) was added to a mixture of 10% (w/w) palladium on charcoal (0.25g) and 3-nitropyrid-2-one (2.5 g, 17.8 mmol). The mixture was hydrogenated at room temperature under an atmospheric pressure of H_2 for 8 hours. The catalyst was then removed by filtration, over celite and washed with ethanol (2x 50 ml). The ethanol solution was evaporated to dryness to give the amine as a light brown crystalline solid (1.99 g, 98%). Anal. Calc. for $C_5H_6N_2O$: C, 54.54 , H, 5.49, N, 25.44 %; found: C, 54.67, H, 5.47, N, 25.33 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ_H 6.83 (dd, *J*= 6.5 Hz and *J*= 1.7 Hz, 1H), 6.66 (dd, *J*= 7.2 Hz, *J*= 1.7 Hz, 1H), 6.17 (t, *J*= 6.8 Hz, 1H), 4.15 (brs, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 159.7, 138.0, 122.2, 115.1, 108.0. MS *m/z* (CI, +ve) 111 ($[M+H]^+$, 100), 128 ($[M+NH_4]^+$, 5).

2-Amino group protection: general procedure

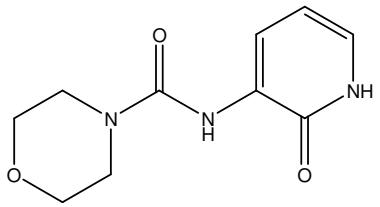
Benzyl 1,2-dihydro-2-oxopyridin-3-yl-carbamate



157.

Benzyl chloroformate (1.42 ml, 9.97 mmol) was added dropwise to a stirred suspension of Na_2CO_3 (2.11 g, 39.9 mmol) and 3-aminopyridin-2(1*H*)-one (1.0 g, 9.1 mmol) in tetrahydrofuran (10 ml). The reaction mixture was stirred overnight and poured into ethyl acetate (100 ml), washed with saturated aqueous sodium hydrogen carbonate (25 ml), dried with magnesium sulphate, filtered and evaporated. The resulting pink crystalline solid residue was purified by crystallization from methanol to afford the pure product as a white crystalline solid (1.1 g, 50 %). Anal. Calc. for $C_{13}H_{12}N_2O_3$: C, 63.93 , H, 4.95, N, 11.47 %; found: C, 63.91, H, 4.89, N, 11.45%. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 11.97 (brs, 1H), 8.12 (d, *J*= 7.2 Hz, 1H), 7.78 (s, 1H), 7.42-7.31 (m, 5H, Ar), 6.98 (dd, *J*= 6.8 Hz, *J*= 2.0Hz, 1H), 6.32 (t, *J*= 6.8 Hz, 1H), 5.21 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 158.8, 153.7, 136.3, 129.9, 128.7, 128.6, 125.9, 122.4, 107.9, 67.5. MS *m/z* (CI, +ve) 245 ($[M+H]^+$, 41), 262 ($[M+NH_4]^+$, 1), 137 (100), 154 (64).

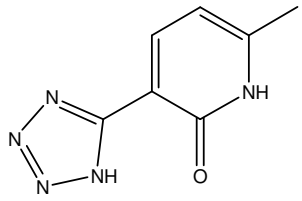
N-(1,2-Dihydro-2-oxopyridin-3-yl)morpholine-4-carboxamide



166.

3-Aminopyridin-2(1*H*)-one (1.22 g, 11.1 mmol) was dissolved in dimethylformamide and *N,N*-diisopropylethylamine (5.80 ml, 33.3 mmol) added, following representative procedure 1.2 (previous chapter). *N*-morpholine carbonyl chloride (1.91 ml, 16.6 mmol) was added and the final mixture was stirred overnight. After work up, the crude product was purified by flash chromatography (SiO_2, 10% methanol in dichloromethane) and the required product was isolated as a white solid (1.10 g, 53%). Anal. Calc. for $C_{10}H_{13}N_3O_3$: C, 53.80, H, 5.87, N, 18.82 %; found: C, 53.43, H, 5.78, N, 18.05 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.23 (dd, *J*= 7.2 Hz, *J*= 1.6 Hz, 1H), 7.74 (brs, 1H), 6.92 (dd, *J*= 6.8 Hz, *J*= 1.6 Hz, 1H), 6.31 (t, *J*= 7.2 Hz, 1H), 3.74 (t, *J*= 4.8 Hz, 4H), 3.51 (t, *J*= 5.2 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 164.2, 124.6, 122.0, 108.3, 66.9, 44.5. MS *m/z* (CI, +ve) 224 ($[M+H]^+$, 15), 154 (82), 137 (68), 88 (100).

6-Methyl-3-(1*H*-tetrazol-5-yl)pyridin-2(1*H*)-one (representative procedure 2.1: Sharpless conditions)

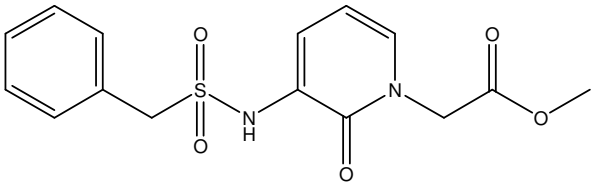


167.

To a 50 ml round bottomed flask was added 1,2-dihydro-6-methyl-2-oxopyridine-3-carbonitrile (1.0 g, 7.45 mmol), sodium azide (0.533 g, 8.2 mmol), zinc bromide (1.68 g, 7.45 mmol) and water (10ml). The reaction mixture was refluxed for 24 hours with vigorous stirring. A solution of hydrochloric acid 3N (10 ml) and ethyl acetate (30 ml) were added, with vigorous stirring, until the residue was completely dissolved, and the pH of the aqueous layer reached about 1. The organic layer was isolated and the aqueous layer extracted with ethyl acetate (2x 30 ml). The combined organic layers were evaporated to dryness. A solution of sodium hydroxide 0.25 M (45 ml) was added to the solid residue and the mixture stirred for an additional 30 minutes while a suspension of zinc bromide was formed. This suspension was filtered, and the solid washed with aqueous sodium hydroxide 1M (2x 5.0 ml). To the filtrate was added hydrochloric acid 3N (10 ml) and the organic product was extracted with chloroform. Evaporation of the solvent under reduced pressure afforded a beige solid (0.99 g, 75%). ν_{max} (KBr)/cm^{-1} 3338, 2966, 2825, 1664, 1622, 1579, 1535, 1398, 1307, 1221, 1192, 1034, 1009. Anal. Calc. for $C_7H_7N_5O$: C, 47.46, H, 3.98, N, 39.54 %; found: C, 47.55,

H, 3.96, N, 39.47 %. MS m/z (CI, +ve) 178 ($[M+H]^+$, 100), 197 ($[M+NH_4]^+$, 19). Found $[M+H]^+$ 178.0734, $C_7H_8N_5O$ requires 178.0729.

(2-Oxo-3-phenylmethanesulfonylamino-2*H*-pyridin-1-yl)-acetic acid methyl ester

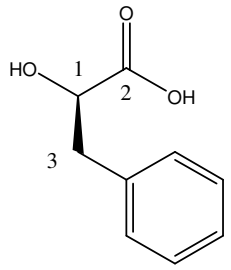


164.

Methyl 2-(3-amino-2-oxopyridin-1(2*H*)-yl) acetate (0.500 g, 2.74 mmol) was dissolved in tetrahydrofuran (5 ml) at 0ºC and with stirring. 2,6-Lutidine (0.64 ml, 5.48 mmol) was then added, followed by dropwise addition of a solution of phenylmethanesulfonyl chloride (0.522 g, 2.74 mmol) in tetrahydrofuran (3 ml). The final reaction mixture was stirred for 30 minutes at 0ºC, then diluted with ethyl acetate (15 ml), washed with hydrochloric acid 1.0N (until aqueous layer is at pH 1), water (5 ml), saturated solution of sodium bicarbonate (10 ml) and finally brine (5ml). Drying over magnesium sulphate, filtration and evaporation of the solvent afforded the product as a brown foam (0.529 g, 57%). Anal. Calc. for $C_{15}H_{16}N_2SO_5$: C, 53.56, H, 4.79, N, 8.33 %; found: C, 53.27, H, 4.95, N, 8.53 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.40-7.27 (m, 6H), 6.93 (dd, *J*= 6.9 Hz, *J*= 1.5 Hz, 1H), 6.15 (t, *J*= 7.2 Hz, 1H), 4.69 (s, 2H), 4.43 (s, 2H), 3.83 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 167.5, 157.0, 131.4, 131.2, 129.5, 129.4, 129.2, 121.2, 106.7, 58.8, 53.2, 51.2. MS m/z (CI, +ve) 337 ($[M+H]^+$, 31), 354 ($[M+NH_4]^+$, 20), 183 (100).

3- Preparation of the D- aminoacid derivatives: general procedure

(*R*)-2-Hydroxy-3-phenylpropanoic acid (representative procedure 3.1)

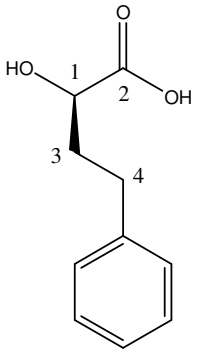


171.

Boc-*D*-Phenylalanine (2.0 g, 7.54 mmol) was stirred for 4 hours in a solution of hydrochloric acid in 1,4-dioxane (4.0 M, 11.5 ml). Hexane was then added (11.5 ml), and the mixture was stirred for an additional 20 min. The resulting solid was collected by filtration and air-dried, then dissolved in a solution of sulphuric acid solution 1M (32 ml) and cooled to –2 ºC. A solution of sodium nitrite (40 % aq., 3.75 ml) was added dropwise, keeping the temperature below 10ºC. After addition was complete, the reaction mixture was held at 0 ºC for 3 hours, then allowed to warm to 23 ºC and stirred for an extra 16 hours. The final mixture was extracted with *tert*-butyl methyl ether (3x30 ml) and the combined organic extracts dried over magnesium sulphate, filtered and concentrated under reduced pressure to afford a yellow oil (1.0 g). The crude product was purified by flash chromatography using dichloromethane with a drop of acetic acid as the eluent (300 mg, 24 %). ν_{max} (neat)/cm^{-1} 3490 (OH), 3029, 2927 (C-H)), 1718 (C=O), 1496 (C=C, Ar), 1453 (C=C, Ar), 1287, 1238, 1184, 1088, 1065, 1030, 911, 794, 740 (Ph), 700 (Ph). ^{1}H NMR (250 MHz, $CDCl_3$) δ_H 7.28-7.12 (m, 5H,

Ar), 4.53 (m, 1H), 3.22 (dd, *J*= 14.0 Hz, *J*= 6.9 Hz, 1H, C(3)H), 2.99 (dd, *J*= 14.0 Hz, *J*= 3.8 Hz, 1H, C(3)H'). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 170.6 (C(2)), 135.34, 129.8, 128.9, 127.5 (Ar), 53.6 (C(1)), 36.1 (C(3)). MS *m/z* (CI, +ve) 166 ($[M]^+$, 6), 184 ($[M+NH_4]^+$, 100).

(*R*)-2-hydroxy-4-phenylbutanoic acid



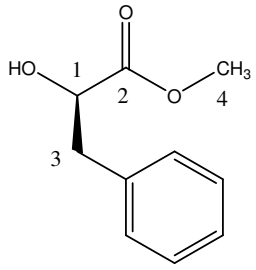
175.

Boc-*D*-Homophenylalanine (2.0 g, 7.2 mmol, 1.0 equiv) was stirred for 4 hours in a solution of hydrochloric acid in 1,4-dioxane (4.0 M, 10.8 ml). Following representative procedure 3.1, addition of hexane (11 ml) allowed formation of a solid which was separated and dissolved in a solution of sulphuric acid 1 M (30 ml) and cooled to 0 ºC. A solution of sodium nitrite (40 % aq., 3.58 ml) was then added dropwise. After stirring overnight the reaction mixture was extracted with *tert*-butyl methyl ether (3x30 ml) and the combined organic extracts dried over magnesium sulphate and filtered. The filtrate was concentrated under reduced pressure to afford a yellow oil. The product was purified by flash chromatography (95%dichloromethane /5% methanol and a drop of acetic acid) to give a yellow oil (0.89 g, 69 %). ν_{max} (neat)/cm^{-1} 3447. (COOH), 3400

(O-H), 2949, 2922, 2850 (C-H)), 1725 (C=O), 1453 (C=C, Ar), 1172, 1121, 1095, 1072, 742 (Ph), 695 (Ph). ^{1}H NMR (250 MHz, $CDCl_3$) δ_H 7.32-7.20 (m, 5H, Ar), 4.27 (q, *J*= 4.1 Hz, 1H, C(1)H), 4.51 (brs, 1H), 2.80 (t, *J*= 8.2 Hz, 2H, C(4)H_2), 2.24-1.94 (m, 2H, C(3)H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 171.0 (C(2)), 141.3, 131.4, 129.2, 128.9, 126.2 (Ar), 67.2 (C(1)), 38.3 (C(3)), 30.9 (C(4)). MS *m/z* (CI, +ve) 181 ($[M+H]^+$, 1), 198 ($[M+NH_4]^+$, 100).

4- Carboxylic acid protection: general procedure

(*R*)-Methyl 2-hydroxy-3-phenylpropanoate (representative procedure 4.1)

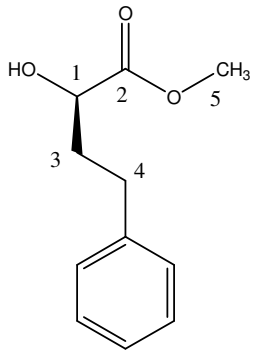


172.

A solution of hydrochloric acid in 1,4-dioxane (4.0 M, 0.16 ml) and (*R*)- 2-hydroxy-3-phenylpropanoic acid (0.22 g, 1.3 mmol) were added to methanol at 23 ºC and maintained at that temperature for 20 hours. After that period of time the reaction mixture was evaporated under reduced pressure and the crude product purified by flash chromatography (silica gel, 20% ethyl acetate/ hexane) to give a yellow oil (0.14 g, 59 %). ν_{max} (neat)/cm^{-1} 3474 (O-H), 3029 (C-H), 2954 (C-H), 1738, 1604 (C=C, Ar), 1497 (C=C, Ar), 1455 (C=C, Ar), 1275 (C-O), 1219 (C-O), 1096, 1031, 747 (Ph), 700

(Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.25 (m, 5H, Ar), 4.45 (m, 1H, C(1)H), 3.76 (s, 3H, C(4)H), 3.12 (dd, *J*= 13.9Hz, *J*= 4.4Hz, 1H, C(3)H), 2.96 (dd, *J*= 13.9 Hz, *J*= 6.8 Hz, 1H, C(3)H'), 2.74 (d, *J*= 6.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 174.9 (C(2)), 136.8, 129.9, 129.0, 127.3, 71.7 (C(1)), 52.8 (C(4)), 41.0 (C(3)). MS *m/z* (CI, +ve) 181 ($[M+H]^+$, 13), 198 ($[M+NH_4]^+$, 100). Found $[M+NH_4]^+$ 198.1123, $C_{10}H_{16}NO_3$ requires 198.1130.

(*R*)-Methyl 2-hydroxy-4-phenylbutanoate



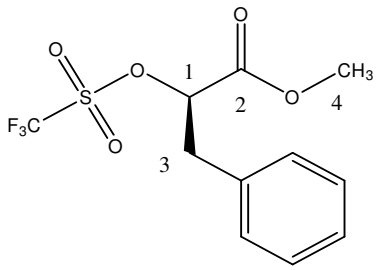
176.

A solution of HCl in 1,4-dioxane (4.0 M, 0.59 ml) and (*R*)-2-hydroxy-4-phenylbutanoic acid (0.89 g, 4.9 mmol) were added to methanol (30 ml) at 23ºC following representative procedure 4.1. The product was purified by flash chromatography (35 % ethyl acetate/ 65% hexane) to give a yellow oil (0.51 g, 53 %). ν_{max} (neat)/cm^{-1} 3438 (O-H), 3051, 2954, 2858 (C-H), 1755 (C=O), 1604 (C=C, Ar), 1504 (C=C, Ar), 1454, 1437, 1275, 1199 (C-O). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.31-7.18 (m, 5H, Ar), 4.20 (td, *J*= 4.0Hz, *J*= 1.2 Hz, 1H, (C(1)H), 3.75 (s, 3H), 2.83-2.74 (m, 2H, $C(4)H_2$), 2.17-2.08 (m, 1H, C(3)H), 2.00-1.91 (m, 1H, C(3)H'). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 176.1

(C(2)), 141.4, 129.0, 128.8, 126.5 (Ar), 70.0 (C(1)), 53.0 (C(5)), 36.3 (C(3)), 31.4 (C(4)). MS *m/z* (CI, +ve) 194 ($[M+H]^+$, 1), 212 ($[M+NH_4]^+$, 100).

5- Alcohol protection: general procedure

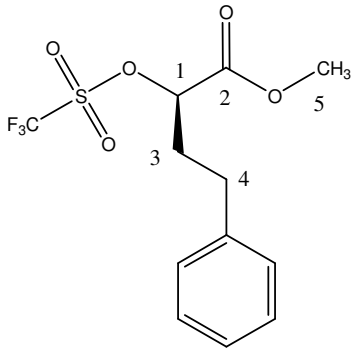
(*R*)-Methyl-3-phenyl-2-trifluoromethanesulfonylpropanoate (representative procedure 5.1)



173.

A solution of (*R*)-methyl 2-hydroxy-3-phenylpropanoate (0.14 g, 0.78 mmol) and 2,6-lutidine (0.15 ml, 1.3 mmol) was cooled to 0 ºC. Trifluoromethane sulfonic anhydride (0.21 ml, 1.24 mmol) was added dropwise and the reaction mixture was stirred for 1 hour at 0 ºC. The final mixture was then diluted with MTBE (165 ml), washed with hydrochloric acid 1 M (37 ml) and brine (2 x 37 ml). The liquid product was obtained quantitatively and used with no further purification. ν_{max} (neat)/cm^{-1} 3067 (C-H), 2959 (C-H), 1744 (C=O), 1653, 1498 (C=C, Ar), 1457, 1438, 1417 (C=C, Ar), 1384, 1320 (S=O), 1211 (C-O), 1143, 1030 (S=O), 940, 799, 760, 701 (Ph), 639 (Ph). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H 7.36-7.20 (m, 5H, Ar), 5.26 (m, 1H, C(1)H), 3.84 (s, 3H), 3.36 (dd, *J*= 14.8 Hz, *J*= 4.3 Hz, 1H, C(3)H), 3.20 (dd, *J*= 14.4 Hz, *J*= 8.8 Hz, 1H, C(3)H'). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 167.4 (C(2)), 133.7, 132.9, 128.8, 128.6, 125.2 (Ar), 53.1 (C(1)), 51.3 (C(4)), 29.3 (C(3)).

(R)-1-(methoxycarbonyl)-4-phenylpropyl trifluoromethanesulfonate



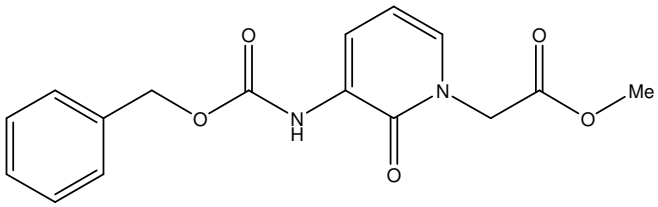
177.

In accordance to representative procedure 5.1, to a solution of (*R*)-methyl 2-hydroxy-4-phenylbutanoate (0.50 g, 2.6 mmol) and 2,6-lutidine (0.51 ml, 4.4 mmol) was added dropwise trifluoromethane sulfonic anhydride (0.70 ml, 4.12 mmol). After stirring for 1h at 0ºC, the reaction mixture was diluted with MTBE (500 ml), washed with 1M HCl (120 ml) and brine (2 x 120 ml). The required liquid product was obtained quantitatively and used with no further purification. ν_{max} (neat)/cm^{-1} 2954, 2933, 2860 (C-H), 1747 (C=O), 1651, 1446, 1417.4 (C=C, Ar), 1383, 1284, 1211 (C-O), 1172, 1132, 1030 (S=O). ^{1}H NMR (250 MHz, $CDCl_3$) δ_H 7.32-7.18 (m, 5H, Ar), 5.17 (t, *J*= 6.5 Hz, 1H, C(1)H), 3.82 (s, 3H), 2.78 (t, *J*= 8.2 Hz, 2H, C(4)H), 2.38-2.32 (m, 2H, C(3)H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 168.1 (C(2)), 134.4, 128.9, 128.9, 126.5, 69.9 (C(1)), 67.0 (C(5)), 34.3 (C(3)), 31.4 (C(4)).

6- NH alkylation on the pyridinium ring: general procedure

(3-Benzyloxycarbonylamino-2-oxo-2*H*-pyridin-1-yl)-acetic acid methyl ester

(representative procedure 6.1)

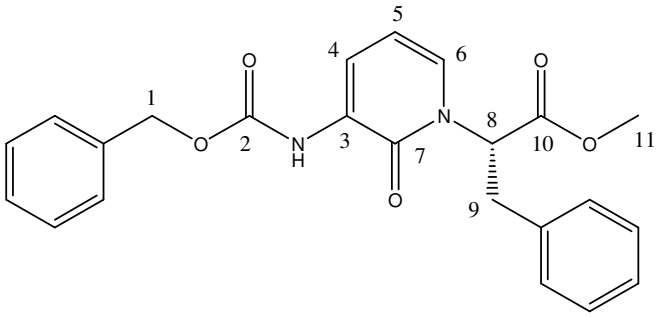


158.

A suspension of benzyl 1,2-dihydro-2-oxopyridin-3-yl-carbamate (1.94 g, 7.94 mmol) in dimethylformamide (15 ml) was added to a suspension of sodium hydride (0.381 g, 9.53 mmol) in dimethylformamide (15 ml) maintaining the reaction temperature between 15 ºC and 20ºC. After the mixture had stirred for 20 min., methyl bromoacetate (0.94 ml, 9.93 mmol) was added dropwise, maintaining the temperature below 20 ºC. The mixture was stirred at room temperature for 3 hours, then poured over iced hydrochloric acid (100 ml), and extracted with ethyl acetate (2 x 200 ml). The combined organic layers were washed with brine (2 x 100 ml), dried over magnesium sulphate, filtered and the solvent evaporated to give the residue as a pink oil, which after cooling in the freezer overnight formed crystals of the pure compound. Nevertheless, a chromatographic column was required to purify the remaining crude product (ethyl acetate/ dichloromethane 2:98). The pure compound was obtained as a light pink solid (2.13 g, 85%).

Anal. Calc. for $C_{16}H_{16}N_2O_5$: C, 60.76, H, 5.10, N, 8.86 %; found: C, 60.88, H, 5.05, N, 8.79%. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.05 (d, *J*= 6.8 Hz, 1H), 7.83 (s, 1H), 7.38-7.26 (m, 5H, Ar), 6.87 (dd, *J*= 6.8 Hz, *J*= 1.6 Hz, 1H), 6.26 (t, *J*= 6.8 Hz, 1H), 5.20 (s, 2H), 4.67 (s, 2H), 3.78 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 168.1, 157.6, 153.6, 136.3, 129.9, 129.7, 128.9, 128.6, 128.4, 120.8, 107.1, 67.4, 62.1, 53.0, 51.1. MS *m/z* (CI, +ve) 317 ($[M+H]^+$, 54), 335 ($[M+NH_4]^+$, 0.2), 183 (17), 209 (100).

(*S*)-2-(3-Benzyloxycarbonylamino-2-oxo-2*H*-pyridin-1-yl)-3-phenyl-propionic acid methyl ester (representative procedure 6.2)

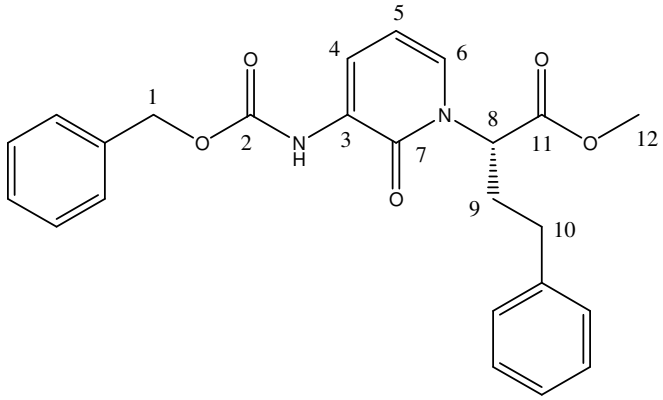


178.

A solution of benzyl 1,2-dihydro-2-oxopyridin-3-yl-carbamate (0.26 g, 1.06 mmol) in tetrahydrofuran (10 ml) was treated with sodium hydride (24 mg, 0.06 mmol) and left stirring for 30 minutes at 23 ºC, while gas evolution ceased. Then, a solution of compound **11** (0.30 g, 0.96 mmol) in tetrahydrofuran (10 ml) was added. The resulting reaction mixture was stirred for 2 hours at room temperature. After that period of time, the reaction mixture was diluted with MTBE (230 ml), and the organic phase separated and washed with brine (2 x 45 ml), dried over magnesium sulphate, filtered, and the

filtrate concentrated under reduced pressure to yield 500 mg of an yellow oil. The crude product was purified by flash chromatography (silica gel, 35% ethyl acetate in hexane) and the pure product was isolated as an orange oil (0.298 g, 76 %). ν_{max} (neat)/cm^{-1} 3378 (CONHR), 3030 (C-H), 2952 (C-H), 1741 (C=O), 1650 (C=O), 1601 (C=C, Ar), 1565, 1513 (C=C), 1455 (CONHR), 1387, 1359, 1265, 1199 (C-O), 1162, 1096, 1073, 1050, 1011, 913, 746 (Ph), 699 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.96 (d, *J*= 6.1 Hz, 1H), 7.81 (brs, 1H), 7.38-7.03 (m, 10H, Ar), 6.69 (dd, *J*= 7.0 Hz, *J*= 1.7 Hz, 1H), 6.12 (t, *J*= 7.2 Hz, 1H), 5.36 (dd, *J*= 10.2 Hz, *J*= 5.2 Hz, 1H, C(8)H), 5.19 (s, 2H), 3.70 (s, 3H), 3.54 (dd, *J*= 14.2 Hz, *J*= 5.2 Hz, 1H, C(9)H), 3.31 (dd, *J*= 14.2 Hz, *J*= 10.3 Hz, 1H, C(9)H'). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 173.1, 158.0, 153.7, 135.0, 129.3, 129.1, 129.0, 128.4, 127.5, 120.4, 106.7, 67.4, 63.0, 53.2, 37.8. MS *m/z* (CI, +ve) 407 ($[M+H]^+$, 85), 424 ($[M+NH_4]^+$, 0.1), 137 (54), 154 (46), 299 (100). Found $[M+H]^+$ 407.1611, $C_{23}H_{23}N_2O_5$ requires 407.1607.

(S)-2-(3-Benzyloxycarbonylamino-2-oxo-2H-pyridin-1-yl)-4-phenyl-butyric acid methyl ester

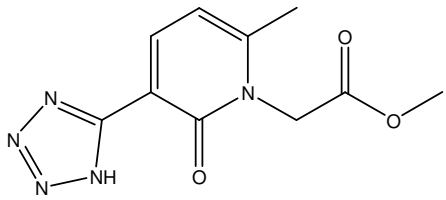


179.

A solution of benzyl 1,2-dihydro-2-oxopyridin-3-yl-carbamate (0.98 g, 4.0 mmol) in tetrahydrofuran (35 ml) was treated with sodium hydride (60% dispersion in mineral oil, 88.0 mg, 3.65 mmol) in accordance with representative procedure 6.2. A solution of compound 12 (1.19 g, 3.65 mmol) in tetrahydrofuran (35 ml) was added. After work up following procedure 6.2, evaporation of the organic solvent afforded 2.68 g of a dark brown liquid which was subjected to purification by flash chromatography (silica gel, ethyl acetate/hexane (30:70)) to give the pure product as a very viscous yellow oil (1.03 g, 67 %). ν_{max} (neat)/cm^{-1} 3379 (CONHR), 3030, 2941, 2854 (C-H), 1738 (C=O), 1651 (C=O), 1603 (C=C, Ar), 1512 (C=C, Ar), 1454, 1360, 1199 (C-O), 1066. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.02 (d, *J*= 7.2 Hz, 1H), 7.90 (s, 1H), 7.41-6.97 (m, 10H, Ar), 6.86 (dd, *J*= 7.2 Hz, *J*= 2.0 Hz, 1H), 6.29 (t, *J*= 7.2 Hz, 1H), 5.40 (m, 1H, C(8)H), 5.21 (s, 2H), 3.73 (s, 3H), 2.58 (m, 2H, $C(10)H_2$), 2.22 (m, 2H, $(C(9)H_2)$. ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 170.5 (C(11)), 157.6 (C(7)), 153.7 (C(2)), 140.2, 136.4, 129.8, 129.0, 128.7, 128.69, 128.5, 127.3, 126.8 (Ar), 120.3 (C(5)), 107.2 (C(4)), 67.4 (C(1)), 59.1 (C(8)),

53.1 (C(12)), 32.4 (C(10)), 32.32 (C(9)). Found $[M+H]^+$ 421.1757, $C_{24}H_{25}N_2O_5$ requires 421.1763.

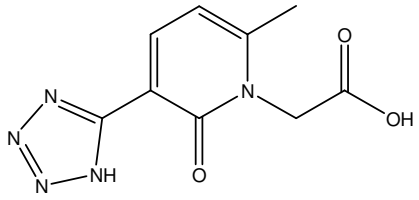
Methyl 2-(6-methyl-2-oxo-3-(1*H*-tetrazol-5-yl)pyridin-1(2*H*)-yl)acetate



168.

Following representative procedure 6.1, sodium hydride (60% dispersion in mineral oil, 33 mg, 1.36 mmol) in dimethylformamide (2 ml) was added to a suspension of 6-methyl-3-(1H-tetrazol-5-yl)pyridin-2(1H)-one (0.20 g, 1.13 mmol) in the same solvent and methylbromoacetate (0.13 ml, 1.41 mmol) was added. Washing with brine and extraction with ethyl acetate afforded a crude mixture from which the pure product was isolated by flash column chromatography (SiO_2, 5% methanol in dichloromethane) as a light yellow crystalline solid (0.151 g, 44%). ν_{max} (KBr)/cm^{-1} 3478.9, 3008.4, 2831.0, 1758.8, 1658.5, 1619.9, 1575.6, 1220.7, 1004.7. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.39 (d, *J*= 7.6 Hz, 1H), 6.27 (d, *J*= 7.2 Hz, 1H), 5.53 (s, 2H), 3.81 (s, 3H), 2.46 (s, 3H). Found $[M+Na]^+$ 272.0760, $C_{10}H_{11}N_5O_3{}^{23}Na$ requires 272.0760.

2-(6-Methyl-2-oxo-3-(1*H*-tetrazol-5-yl)pyridin-1(2*H*)-yl)acetic acid

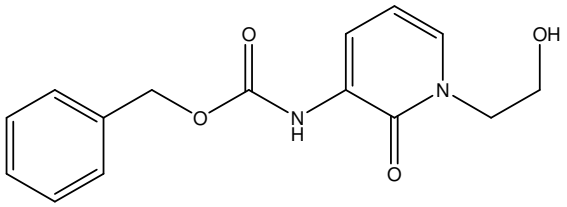


169.

In accordance to representative procedure 2.1, a mixture of methyl 2-(3-cyano-6-methyl-2-oxopyridin-1(2*H*)-yl)acetate (0.500 g, 2.42 mmol), sodium azide (0.173 g, 2.67 mmol), zinc bromide (0.546 g, 2.42 mmol) and water was refluxed for 24 hours. Evaporation of the solvent from the mixture under reduced pressure, after work up, afforded the pure product as a white solid (0.280 g, 50%). ν_{max} (nujol)/cm^{-1} 3500, 1644, 1556, 1203, 1150, 1034, 842, 779. Anal. Calc. for $C_9H_9N_5O_3$: C, 45.96, H, 3.86, N, 29.78 %; found: C, 46.10, H, 3.86, N, 29.44 %. ^{1}H NMR (400 MHz, DMSO) δ_H 8.34 (d, *J*= 7.2 Hz, 1H), 6.50 (d, *J*= 7.2 Hz, 1H), 4.84 (s, 2H), 2.41 (s, 3H). MS m/z (CI, +ve) 236 ($[M+H]^+$, 100), 255 ($[M+NH_4]^+$, 7). Found $[M+H]^+$ 236.0787, $C_9H_{10}N_5O_3$ requires 236.0783.

8- Ester reduction to the primary alcohol: general procedure

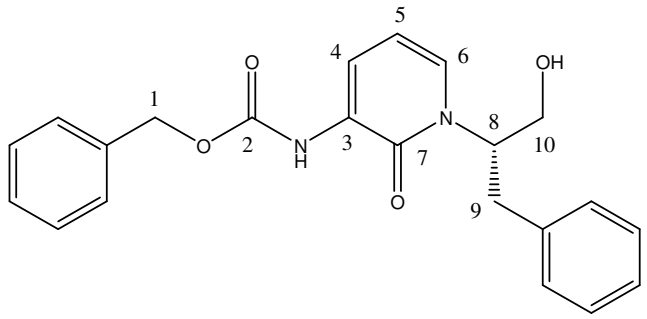
Benzyl 1,2-dihydro-1-(2-hydroxyethyl)-2-oxopyridin-3-yl-carbamate (representative procedure 8.1)



159.

To a stirred –5 ºC solution of (3-benzyloxycarbonylamino-2-oxo-2*H*-pyridin-1-yl)-acetic acid methyl ester (0.55 g, 1.7 mmol) in ethanol (10 ml) was added a suspension of calcium chloride (0.39 g, 3.5 mmol) in tetrahydrofuran (5 ml), followed by addition of sodium borohydride (0.26 g, 7.0 mmol). After 3 hours, the reaction mixture was poured into an excess of iced citric acid and extracted exhaustively with ethyl acetate. The combined organic extracts were washed with a saturated solution of sodium hydrogen carbonate, dried with magnesium sulphate, filtered and evaporated to the pure product as a pink oil, which crystallized with freezing (0.50 g, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.06 (d, *J*= 7.4 Hz, 1H), 7.87 (s, 1H), 7.40- 7.30 (m, 5H, Ar), 6.99 (dd, *J*= 6.8 Hz, *J*= 1.8 Hz, 1H), 6.26 (t, *J*= 7.2 Hz, 1H), 5.20 (s, 2H), 4.17 (t, *J*= 4.5 Hz, 2H), 3.95 (t, *J*= 4.5 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 158.00, 153.38, 135.98, 130.09, 129.55, 128.62, 128.35, 128.15, 120.65, 106.83, 67.12, 61.88, 53.74. MS *m/z* (CI, +ve) 289 ($[M+H]^+$, 16), 137 (17), 181 (100). Found $[M+Na]^+$ 289.11923, $C_{15}H_{17}N_2O_4$ requires 289.11884.

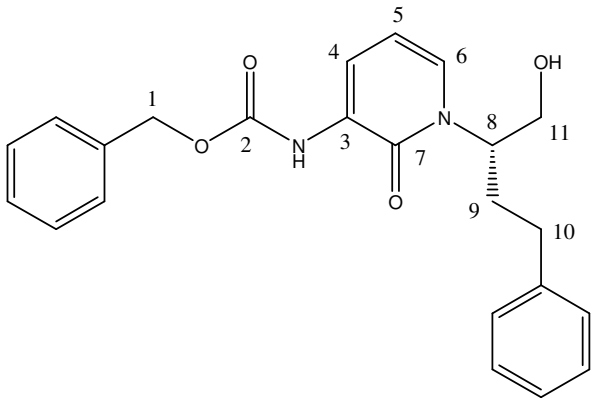
Benzyl 1,2-dihydro-1-((*S*)-1-hydroxy-3-phenylpropan-2-yl)-2-oxopyridin-3-yl-carbamate



180.

Following representative procedure 8.1, to a solution of (*S*)-2-(3-benzyloxycarbonylamino-2-oxo-2*H*-pyridin-1-yl)-3-phenyl-propionic acid methyl ester (298 mg, 0.73 mmol) in ethanol (3.0 ml) was added a suspension of calcium chloride (163 mg, 1.47 mmol) in tetrahydrofuran (1.5 ml), followed by addition of sodium borohydride (111 mg, 2.93 mmol). After work-up as described and with no further purification, the product was obtained as a pale yellow foam (349 mg, quantitative). ν_{max} (neat)/cm^{-1} 3400 (broad, O-H), 3375 (CONHR), 3022 (C-H), 2922 (C-H), 1727 (C=O), 1641 (C=O), 1587 (C=C, Ar), 1560, 1506 (C=C, Ar), 1456 (CONHR), 1392, 1352, 1261, 1198 (C-O), 1157, 1066, 1053, 1030, 912, 740 (Ph), 695 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.97 (d, *J*= 6.8 Hz, 1H), 7.89 (s, 1H), 7.40-7.11 (m, 10H, Ar), 6.86 (dd, *J*= 7.2 Hz, *J*= 1.4 Hz, 1H), 6.15 (t, *J*= 7.2 Hz, 1H), 5.19 (s, 2H), 4.89 (brs, 1H), 3.95-3.90 (m, 2H), 3.27 (brs, 1H), 3.21-3.12 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 158.0 (C(7)), 153.8 (C(2)), 137.4, 136.4, 129.8, 129.3, 129.1, 129.0, 128.7, 128.5, 128.2 (Ar), 127.3 (C(6)), 120.5 (C(5)), 107.1 (C(4)), 67.4 (C(1)), 63.8 (C(8), C(10)), 35.8 (C(9)). MS m/z (CI, +ve) 379 ($[M+H]^+$, 51), 245 (46), 271 (100).

Benzyl 1,2-dihydro-1-((S)-1-hydroxy-4-phenylbutan-2-yl)-2-oxopyridin-3-ylcarbamate

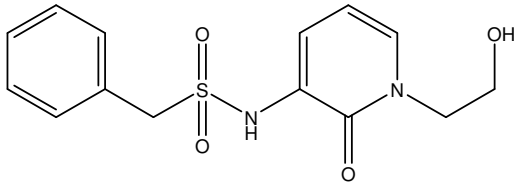


181.

Following representative procedure 8.1, to a solution of (S)-2-(3-benzyloxycarbonylamino-2-oxo-2H-pyridin-1-yl)-4-phenyl-butyric acid methyl ester, (1.0 g, 2.4 mmol) in ethanol (4.7 ml) was added a suspension of calcium chloride (0.53 g, 4.8 mmol) in tetrahydrofuran (2.9 ml), followed by addition of sodium borohydride (0.36 g, 9.5 mmol). A very viscous yellow oil, which proved to be the pure product, was obtained upon work-up and used with no further purification (0.88 g, 95 %). [α]D22 = -88 º (c 1.4 in CH2Cl2). ν max (neat)/cm-1 3400 (broad, O-H), 3379 (CONHR), 3087, 3062, 3012, 2891, 2873 (C-H), 1728 (C=O), 1645 (C=O), 1589 (C=C, Ar), 1564, 1512 (C=C, Ar), 1454 (CONHR), 1356, 1199, 1066. 1H NMR (400 MHz, CDCl3) δH 8.04 (d, J= 7.6 Hz, 1H), 7.91 (s, 1H), 7.41-6.95 (m, 10H, Ar), 6.96 (dd, J= 7.2 Hz, J= 1.6 Hz, 1H), 6.28 (t, J= 7.2 Hz, 1H), 5.21 (s, 2H), 4.75 (brs, 1H), 3.90 (dd, J= 6.0 Hz, J= 2.8 Hz, 2H), 2.65-2.53 (m, 2H), 2.24-2.19 (q, J= 7.6 Hz, 2H), 1.60 (brs, 1H). 13C NMR (100 MHz, CDCl3) δC 157.9 (C(7)), 153.4 (C(2)), 140.5, 136.0, 129.6, 128.6, 128.6, 128.3, 128.3, 128.1, 127.2, 126.3 (Ar), 120.0 (C(5)), 107.0 (C(4)), 67.1 (C(1)), 64.5 (C(11)),

61.0 (C(8)) 32.1 (C(10)), 30.8 (C(9)). Found [M+H]+ 393.1811, C23H25N2O4 requires 393.1814.

***N*-[1-(2-Hydroxy-ethyl)-2-oxo-1,2-dihydro-pyridin-3-yl]-*C*-phenyl-methane sulfonamide**



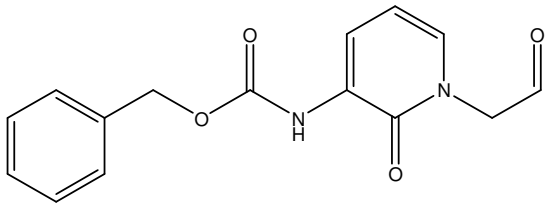
165.

Following representative procedure 8.1, to a solution of (2-Oxo-3-phenylmethanesulfonylamino-2*H*-pyridin-1-yl)-acetic acid methyl ester (0.427 g, 1.27 mmol) in ethanol (5 ml), was added a suspension of calcium chloride (0.282 g, 2.54 mmol) in tetrahydrofuran (1.5 ml), followed by sodium borohydride (0.192 g, 5.08 mmol). After work-up and with no further purification, the product was obtained as a white solid (0.393 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.65 (s, 1H), 7.39-7.31 (m, 6H), 7.16 (dd, *J*= 7.2 Hz, *J*= 1.6 Hz, 1H), 6.14 (t, *J*= 7.2 Hz, 1H), 4.58 (s, 2H), 4.43 (s, 2H), 3.99 (t, J= 6.0 Hz, 2H), 3.68-3.63 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 157.0, 135.0, 131.3, 129.8, 128.6, 128.5, 128.2, 126.0, 104.6, 58.9, 58.4, 52.3. MS m/z (CI, +ve) 309 ($[M+H]^+$, 42), 326 ($[M+NH_4]^+$, 4), 155 (100).

9- Oxidation to the aldehyde: general procedure

[2-Oxo-1-(2-oxo-ethyl)-1,2-dihydro-pyridin-3-yl]-carbamic acid benzyl ester

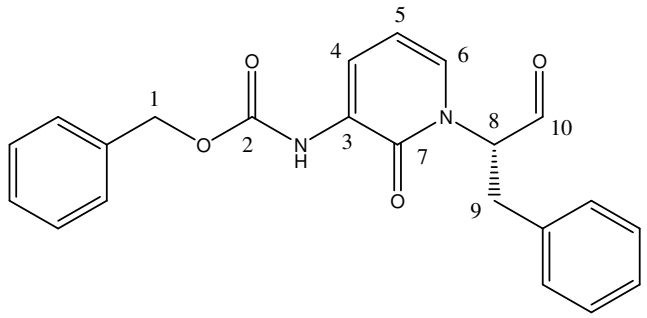
(representative procedure 9.1)



160.

To a solution of the primary alcohol (0.50 g, 1.7 mmol) in dichloromethane (30 ml) was added Dess-Martin Periodinane (5.4 g of 15% wt solution in dichloromethane, 0.810 g, 1.9 mmol,). After stirring at room temperature for 1h 30min, aqueous 5% $Na_2S_2O_3$ (35 ml) was added, and the final mixture stirred for another 15 min. The organic layer was separated, washed with water, saturated sodium hydrogen carbonate, brine, and dried over magnesium sulphate. The solution was then filtered and the filtrate concentrated under reduced pressure. The crude product obtained was purified by flash chromatography (SiO_2, 40% ethyl acetate/ 60% hexane) yielding the pure product as a pink solid (0.20 g, 40%). ν_{max} (neat)/cm^{-1} 1738 (C=O), 1651 (C=O), 1603, 1512 (C=C, Ar), 1454 (C-N), 1388, 1360, 1199 (C-O). Anal. Calc. for $C_{15}H_{14}N_2O_4$: C, 62.94, H, 4.93, N, 9.79 %; found: C, 62.44, H, 4.99, N, 9.68%. ^{1}H NMR (250 MHz, $CDCl_3$) δ_H 9.66 (s, 1H), 8.12 (d, *J*= 7.4 Hz, 1H), 7.84 (m, 5H, Ar), 6.84 (dd, *J*= 6.8 Hz, *J*= 1.8 Hz, 1H), 6.30 (t, *J*= 7.2 Hz, 1H), 5.21 (s, 2H), 4.74 (s, 2H). MS *m/z* (CI, +ve) 287 ($[M+H]^+$, 100), 304 ($[M+NH_4^+]$, 1).

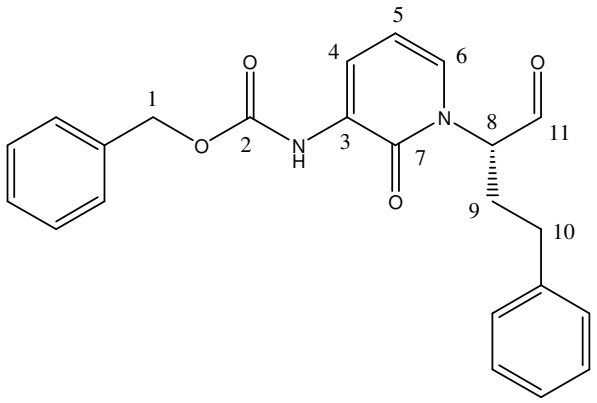
[1-((*S*)-1-Benzyl-2-oxo-ethyl)-2-oxo-1,2-dihydro-pyridin-3-yl]-carbamic acid benzyl ester



182.

Dess Martin Periodinane (0.440 g, 1.04 mmol) was added to a solution of benzyl 1,2-dihydro-1-((*S*)-1-hydroxy-3-phenylpropan-2-yl)-2-oxopyridin-3-yl-carbamate (0.300 g, 0.80 mmol) in dichloromethane, as described in representative procedure 9.1. After work up, evaporation of the organic solvent afforded the crude product which was purified by flash column chromatography (SiO_2, 2 % methanol in dichloromethane) to yield a clear oil (0.120 g, 40 %). $[\alpha]_D^{22}$ = -55 º (*c* 0.4 in CH_2Cl_2). ν_{max} (neat)/cm^{-1} 3374 (CONHR), 3030 (C-H), 2924 (C-H), 1730 (C=O), 1646 (C=O), 1594 (C=C, Ar), 1560, 1512 (C=C, Ar), 1454 (CONHR), 1381, 1359, 1260, 1199 (C-O), 1163, 1071, 1029, 917, 744 (Ph), 699 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.98 (d, *J*= 6.4 Hz, 1H), 7.89 (s, 1H), 7.40-7.10 (m, 10H, Ar), 6.87 ((dd, *J*= 6.8 Hz, *J*= 1.2 Hz, 1H), 6.15 (t, *J*= 7.2 Hz, 1H), 5.18 (s, 2H), 4.90 (brs, 1H, C(8)H), 3.92 (brs, 2H, C(10)H, O-H), 3.48 (brs, 1H, O-H), 3.16 (m, 2H, C(9)H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 158.1 (C(7)), 153.7 (C(2)), 137.4, 136.4, 129.8, 129.1, 129.0, 128.7, 128.5, 128.3, 127.3, 120.5, 107.1, 67.4 (C(1)), 63.9 (C(8)), 35.8 (C(9)). MS *m/z* (CI, +ve) 377 ($[M+H]^+$, 2), 137 (23), 245 (37), 271 (100). MS found $[M+Na+CH_3OH]^+$ 431.1577, $C_{23}H_{24}N_2O_5{}^{23}Na$ requires 431.1583.

[1-((*S*)-1-Formyl-3-phenyl-propyl)-2-oxo-1,2-dihydro-pyridin-3-yl]-carbamic acid benzyl ester (representative procedure 9.2)



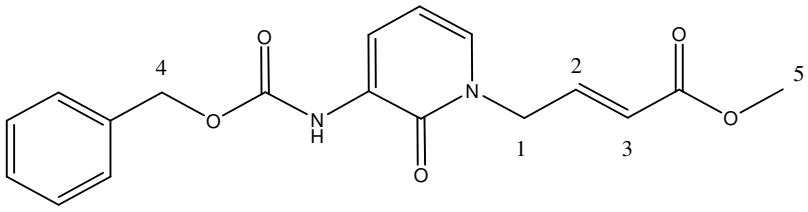
183.

Dimethyl sulfoxide (0.214 ml, 3.02 mmol) was added to a solution of oxalyl chloride (0.158 ml, 1.81 mmol) in dichloromethane (0.55 ml) at -78 ºC. The reaction mixture was stirred for 5 minutes, then a solution of benzyl 1,2-dihydro-1-((S)-1-hydroxy-4-phenylbutan-2-yl)-2-oxopyridin-3-yl-carbamate (0.394 g, 1.01 mmol), in dichloromethane (15 ml) was added. The mixture was stirred for 15 minutes at –78 ºC, and triethylamine (0.98 ml, 7.05 mmol) was added. The reaction mixture was allowed to warm up to 0 ºC and kept at that temperature until TLC analysis indicated consumption of starting material. The reaction mixture was diluted with water, then washed with brine, and a saturated solution of sodium hydrogen carbonate. The required product was extracted with dichloromethane dried over magnesium sulphate, filtered and the organic solvent removed under reduced pressure. The desired pure product was obtained as a yellow oil (0.308 g, 78 %), after purification by flash column chromatography (45 % ethyl acetate in hexane). [α]D22 = - 184 ° (c 0.8 in CH2Cl2). ν max (neat)/cm-1 3386 (N-H), 1732 (C=O), 1649 (C=O), 1597 (C=C, Ar), 1564, 1512, 1454, 1381, 1360, 1261,

1199 (C-O), 1160, 1066. 1H NMR (400 MHz, CDCl3) δH 9.60 (s, 1H, (C(11)H), 8.08 (d, J= 7.2 Hz, 1H), 7.82 (s, 1H), 7.40-7.14 (m, 10H, Ar), 6.75 (dd, J= 7.2 Hz, J= 1.6 Hz, 1H), 6.30 (t, J= 7.2 Hz, 1H), 5.21 (s, 2H), 4.75 (dd, J= 9.6 Hz, J= 4.0 Hz, 1H, C(8)H), 2.70-2.58 (m, 3H, C(10)H2, C(9)H), 2.34-2.22 (m, 1H, C(9)H'). 13C NMR (100 MHz, CDCl3) δC 195.7 (C(11)), 157.1 (C(7)), 153.3 (C(2)), 139.7, 135.9, 129.9, 128.7, 128.6, 128.4, 128.1, 128.1, 126.6, 120.5, 107.3, 67.4 (C(8)), 67.2 (C(1)), 29.7 (C(10)), 29.4, 300 (C(9)). MS found [M+H]+ 391.1663, C23H23N2O4 requires 391.1658.

10- Wittig reaction: general procedure

(*E*)-4-(3-Benzyloxycarbonylamino-2-oxo-2*H*-pyridin-1-yl)-but-2-enoic acid methyl ester (representative procedure 10.1)

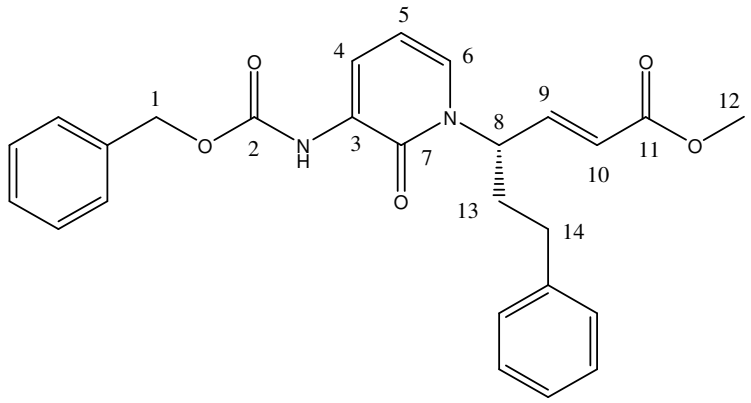


161.

[2-Oxo-1-(2-oxo-ethyl)-1,2-dihydro-pyridin-3-yl]-carbamic acid benzyl ester (0.17 g, 0.59 mmol), was dissolved in dry tetrahydrofuran (8.7 ml) and (methoxycarbonylmethylene)-triphenylphosphorane (0.28 g, 0.83 mmol) added. The reaction mixture was refluxed for 1 hour then left stirring at room temperature, overnight. The crude product obtained was purified by flash chromatography (SiO_2,40%

ethyl acetate/60% hexanes) affording the pure product as a clear oil (0.11 g, 55%). Anal. Calc. for $C_{18}H_{19}N_2O_5$: C, 63.75, H, 5.30, N, 8.18 %; found: C, 62.80, H, 5.19, N, 8.04 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.04 (d, *J*= 6.7 Hz, 1H), 7.87 (s, 1H), 7.41-7.33 (m, 5H, Ar), 6.97 (dt, *J*= 15.7 Hz, *J*= 5.2 Hz, 1H, C(2)H), 6.86 (dd, *J*= 6.9 Hz, *J*= 1.7 Hz, 1H), 6.26 (t, *J*= 7.2 Hz, 1H), 5.77 (dt, *J*= 15.2 Hz, *J*= 2.0 Hz, 1H, C(3)H), 5.21 (s, 2H), 4.74 (dd, *J*= 5.2 Hz, *J*= 2.0 Hz, 1H, C(1)H_2), 3.72 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 143.0 (C(2)), 129.0, 128.8, 128.7, 128.5, 123.6 (C(3)), 120.5, 107.5, 67.5 (C(4)), 52.1 (C(5)), 50.2 (C(1)). MS *m/z* (CI, +ve) 343 ($[M+H]^+$, 100), 360 ($[M+NH_4]^+$, 13). Found $[M+H]^+$ 343.1295, $C_{18}H_{19}N_2O_5$ requires 343.1294.

(*E*)-(*S*)-4-(3-Benzyloxycarbonylamino-2-oxo-2*H*-pyridin-1-yl)-6-phenyl-hex-2-enoic acid methyl ester



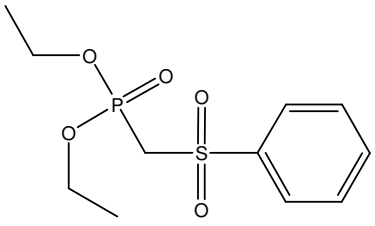
185.

(Methoxycarbonylmethylene)-triphenylphosphorane (0.174 g, 0.52 mmol) was added to [1-((*S*)-1-formyl-3-phenyl-propyl)-2-oxo-1,2-dihydro-pyridin-3-yl]-carbamic acid benzyl ester (0.145 g, 0.37 mmol), dissolved in tetrahydrofuran in accordance to representative procedure 10.1. The crude material was subjected to flash column

chromatography (45 % ethyl acetate in hexanes) from which the product was isolated as a viscous yellow oil (0.150 g, 91 %). ν_{max} (neat)/cm^{-1} 3377, 1728, 1649, 1601, 1512, 1454, 1437, 1389, 1356, 1263, 1198, 1066. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.02 (d, *J*= 7.2 Hz, 1H, C(6)H), 7.91 (s, 1H, NH), 7.41-7.10 (m, 10H, Ar), 6.99 (dd, *J*= 15.6 Hz, *J*= 5.2 Hz, 1H, C(9)H), 6.85 (dd, *J*= 7.2 Hz, *J*= 2.0 Hz, 1H, C(4)H), 6.29 (t, *J*= 7.2 Hz, 1H, C(5)H), 5.82 (dd, *J*= 15.6 Hz, *J*= 2.0 Hz, 1H, C(10)H), 5.77 (m, 1H, C(8)H), 5.21 (s, 2H, $C(1)H_2$), 3.72 (s, 3H, $C(12)H_3$), 2.68-2.60 (m, 1H, C(13)H), 2.56-2.49 (m, 1H, C(13)H'), 2.29-2.12 (m, 2H, $C(14)H_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 166.0 (C(11)), 157.1 (C(7)), 153.4 (C(2)), 145.1 (C(9)), 140.1, 136.0, 129.6, 128.6, 128.3, 128.3, 128.1, 126.4, 125.2 (Ar), 123.2 (C(10)), 119.5 (C(5)), 107.3 (C(4)), 67.1 (C(1)), 55.8 (C(8)), 51.8 (C(12)), 34.7 (C(13)), 32.0 (C(14)), 30.9. MS found $[M+Na]^+$ 469.1722, $C_{26}H_{26}N_2O_5{}^{23}Na$ requires 469.1739.

11- Phosphonate oxidation: general procedure

Diethyl (phenylsulfonyl)methylphosphonate

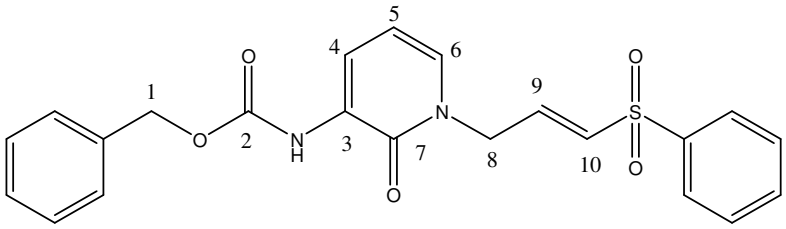


163.

A 50 ml three necked round bottom flask was equipped with a large magnetic stirrer bar, a thermometer and a condenser, and charged with 8 ml of acetic acid and 2 g of diethylphenylthiomethylphosphonate. The mixture was heated with stirring at 50 ºC (internal temperature). Hydrogen peroxide (2.4 ml, 30 % solution) was then added slowly, using a syringe, ensuring that the internal temperature didn't rise above 80 ºC. The final reaction mixture was heated to 85 ºC and stirred for an additional 2h 30min. Then, the mixture was cooled to room temperature, transferred to a beaker and diluted with iced distilled water (30 ml). A solution of 10 M NaOH (10 M) was added dropwise, in order to adjust the pH to 8 or 9. The reaction mixture was then separated and the aqueous phase extracted with dichloromethane (5 x 15 ml). The combined organic extracts were washed with a solution of sodium hydrogen sulfite (2ml), until no oxidizing reagent remained. The organic portion was then dried over magnesium sulphate, filtered and the filtrate concentrated under reduced pressure to afford a white crystalline solid (1.29 g, 56 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.00 (m, 2H, Ar), 7.68 (m, 1H, Ar), 7.58 (m, 2H), 4.20-4.12 (m, 4 H), 3.77 (d, J= 16.8 Hz, 2H), 1.30 (td, J= 6.8 Hz, *J*= 0.4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 140.2, 134.5, 134.1, 129.7, 129.5, 128.7, 128.6, 127.7, 63.8, 63.7, 54.8, 53.5, 16.6, 16.6. MS *m/z* (CI, +ve) 293 ($[M+H]^+$, 47), 310 ($[M+NH_4]^+$, 100). MS found $[M+H]^+$ 293.0602, $C_{11}H_{18}SO_5$ requires 293.0612.

12- Horner- Emmons-Wadsworth reaction: general procedure

Benzyl 1,2-dihydro-2-oxo-1-((*E*)-3-(phenylsulfonyl)allyl)pyridin-3-yl-carbamate (representative procedure 12.1)

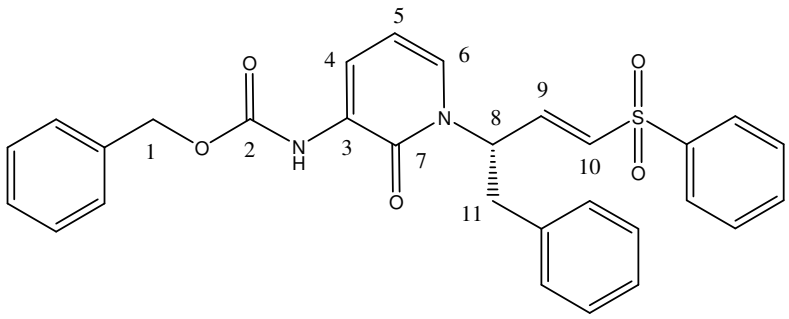


162.

To a solution of diethyl (phenylsulfonyl)methylphosphonate (87 mg, 0.30 mmol) in tetrahydrofuran (3 ml), at -10 ºC, was added sodium hydride (60% dispersion in mineral oil, 7.8 mg, 0.32 mmol). After gas evolution had ceased (approx. 30 min), a solution of [2-oxo-1-(2-oxo-ethyl)-1,2-dihydro-pyridin-3-yl]-carbamic acid benzyl ester (70 mg, 0.24 mmol), in tetrahydrofuran (3 ml), at 0 ºC, was added, under nitrogen and with stirring. The reaction mixture was allowed to warm to 25 ºC, with continuous stirring. After 1h 30 min the reaction mixture was diluted with diethyl ether (15 ml) and then poured into brine (10ml). The layers were separated and the organic portion was dried over magnesium sulphate, filtered and the filtrate concentrated under vacuum to yield 200 mg of a crude pink oil. The product was purified by flash chromatography (40% ethyl acetate in n-hexane) and obtained as a pink solid (67 mg, 66 %). ν_{max} (neat)/cm^{-1} 1727.9, 1650.8, 1602.6, 1515.8, 1319.08, 1309.4, 1201.4, 1147.4, 1085.7, 1070.3. Anal. Calc. for $C_{22}H_{20}N_2SO_5$: C, 62.25, H, 4.75, N, 6.60%; found: C, 62.40, H, 4.82, N, 6.54 %. ^{1}H NMR (250 MHz, $CDCl_3$) δ_H 8.04 (d, *J*= 6.7 Hz, 1H, C(6)H), 7.87-7.35 (m, 11 H,

Ar, NH), 7.03 (dt, *J*= 15.5 Hz, *J*= 7.6 Hz, 1H, C(9)H), 6.83 (dd, *J*= 6.8 Hz, *J*= 1.7 Hz, 1H, C(4)H), 6.30-6.22 (m, 2H, C(5)H, C(10)H), 5.20 (s, 2H, $C(1)H_2$), 4.78 (dd, *J*= 4.8 Hz, *J*= 1.7 Hz, 2H, $C(8)H_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 157.09 (C(7)), 153.63 (C(2)), 139.93, 139.77, 136.17, 134.15, 133.18, 130.17, 129.82, 129.01, 128.78, 128.64, 128.56, 128.21, 120.57, 107.79, 67.56 (C(1)), 49.26 (C(8)). MS found $[M+H]^+$ 425.11799, $C_{22}H_{21}N_2SO_5$ requires 425.11713.

Benzyl 1,2-dihydro-2-oxo-1-((*S,E*)-1-phenyl-4-(phenylsulfonyl)but-3-en-2-yl)pyridin-3-ylcarbamate

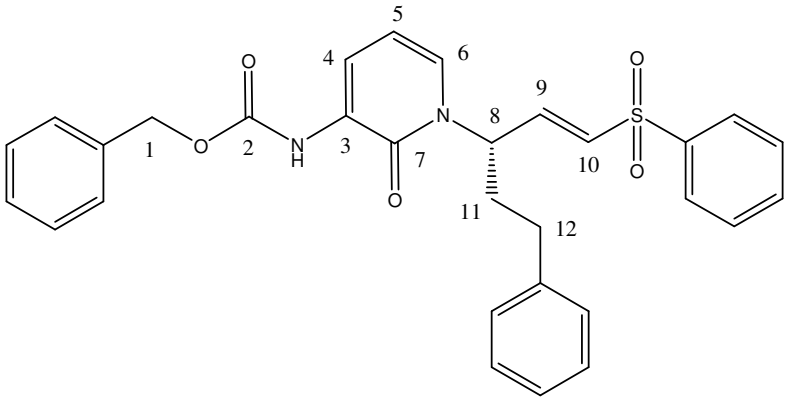


184.

Sodium hydride (60% dispersion in mineral oil, 21 mg, 0.88 mmol) was added to a solution of diethyl (phenylsulfonyl)methylphosphonate (0.257 g, 0.88 mmol) in tetrahydrofuran, accordingly to representative procedure 12.1. A solution of [1-((*S*)-1-benzyl-2-oxo-ethyl)-2-oxo-1,2-dihydro-pyridin-3-yl]-carbamic acid benzyl ester (0.255 g, 0.69 mmol), in tetrahydrofuran was then added. After dilution with diethyl ether, the organic phase was washed with brine, dried over magnesium sulphate and concentrated in vacuum affording the crude product. The product was purified by flash chromatography (35 % ethyl acetate in hexane) to yield a pink oil (116 mg, 33%).

$[\alpha]_D^{22}$ = - 32 º (*c* 1.0 in CH_2Cl_2). ν_{max} (neat)/cm^{-1} 3377 (CONHR), 3063 (C-H), 2925 (C-H), 1729 (C=O), 1648 (C=O), 1599 (C=C, Ar), 1559 (C=C, trans), 1507 (C=C, Ar), 1447 (CONHR), 1387, 1357, 1306 (S=O), 1198, 1148 (C-O), 1086 (S=O), 1069 (S=O), 745 (Ph), 688 (Ph). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.86 (m, 2H, C(6)H, C(Ar)H)), 7.73-6.98 (m, Ar, C(9)H, NH), 6.74 (dd, *J*= 7.2 Hz, *J*= 1.6 Hz, 1H, C(4)H), 6.18 (dd, *J*= 15.0 Hz, *J*= 2.0 Hz, 1H, C(10)H), 6.15 (t, *J*= 7.2 Hz, 1H, C(5)H), 5.90-5.85 (m, C(8)H), 5.10 (s, 2H, C(1)H_2), 3.17-3.03 (m, 2H, C(11)H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 157.1 (C(7)), 153.6 (C(2)), 140.0 (C(9)), 134.0, 133.5 (C(10)), 129.9-127.7 (Ar), 125.84 (C(4)), 120.0 (C(6)), 107.6 (C(5)), 73.3, 68.4, 67.5 (C(1)), 61.6, 57.6 (C(8)), 53.8, 44.8, 39.6 (C(11)), 32.0, 30.1, 25.7. Found $[M+Na]^+$ 537.1453, $C_{29}H_{26}N_2O_5S^{23}Na$ requires 537.1460 and $[M+K]^+$ 553.1220, $C_{29}H_{26}N_2O_5S^{39}K$ requires 553.1200.

Benzyl 1,2-dihydro-2-oxo-1-((*S*,E)-5-phenyl-1-(phenylsulfonyl)pent-1-en-3-yl) pyridin -3-yl-carbamate



186.

Sodium hydride (60% dispersion in mineral oil, 9.5 mg, 0.39 mmol) was added to a solution of diethyl (phenylsulfonyl)methylphosphonate (107 mg, 0.37 mmol) in

tetrahydrofuran, accordingly to representative procedure 12.1. A solution of [1-((*S*)-1-formyl-3-phenyl-propyl)-2-oxo-1,2-dihydro-pyridin-3-yl]-carbamic acid benzyl ester (255 mg, 0.69 mmol), in tetrahydrofuran was then added. After dilution with diethyl ether, the organic phase was washed with brine, dried over magnesium sulphate, filtered and concentrated in vacuum. The crude product was purified by flash chromatography (35 % ethyl acetate in hexane) to yield a colourless oil (157 mg, 98%).

$[\alpha]_D^{22}$ = - 137 ° (*c* 0.3 in CH_2Cl_2). ν_{max} (neat)/cm^{-1} 3375, 1728, 1649, 1604, 1512, 1504, 1446, 1358, 1308, 1252, 1199, 1145, 1084, 902., 872. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.05-7.01 (m, Ar, Ar', C(6)H, C(9)H, C(10)H, C'(6)H, C'(9)H, C'(10)H), 6.66 (dd, *J*= 7.2 Hz, *J*= 2.0 Hz, C(4)H), 6.36 (dd, *J*= 6.8 Hz, *J*= 2.0 Hz, C'(4)H), 6.19 (t, *J*= 7.2 Hz, C(5)H), 6.12 (t, *J*= 6.8 Hz, C'(5)H), 5.73 (dd, *J*= 10.8 Hz, *J*= 5.2 Hz, C'(8)H), 5.55 (t, *J*= 8.4 Hz, C(8)H), 5.21 (s, $C(1)H_2$), 5.20 (s, $C(1)H_2$), 3.83 (d, *J*= 8.4 Hz, $C(11)H_2$), 3.66 (m, C'(11)H), 3.48 (dd, *J*= 14.4 Hz, *J*= 10.8 Hz, C'(11)H'), 3.05(s), 2.80-2.72 (m), 2.67 (t, *J*= 8.4 Hz, $C(12)H_2$), 2.43 (t, *J*= 7.6 Hz, $C'(12)H_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 156.3, 155.8, 153.3, 153.2, 149.3, 146.7, 139.8, 139.7, 138.6, 138.6, 135.9, 135.8, 134.1, 133.9, 133.6, 129.5, 129.3, 128.6, 128.5, 128.3, 128.2, 128.1, 127.3, 126.4, 120.2, 120.1, 116.9, 114.7, 106.5, 106.5, 67.1, 55.3, 55.1, 44.4, 36.1, 32.4, 32.40, 31.6, 30.6, 25.3. Found $[M+H]^+$ 551.1601 , $C_{30}H_{28}N_2SO_5{}^{23}Na$ requires 551.1617 .

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