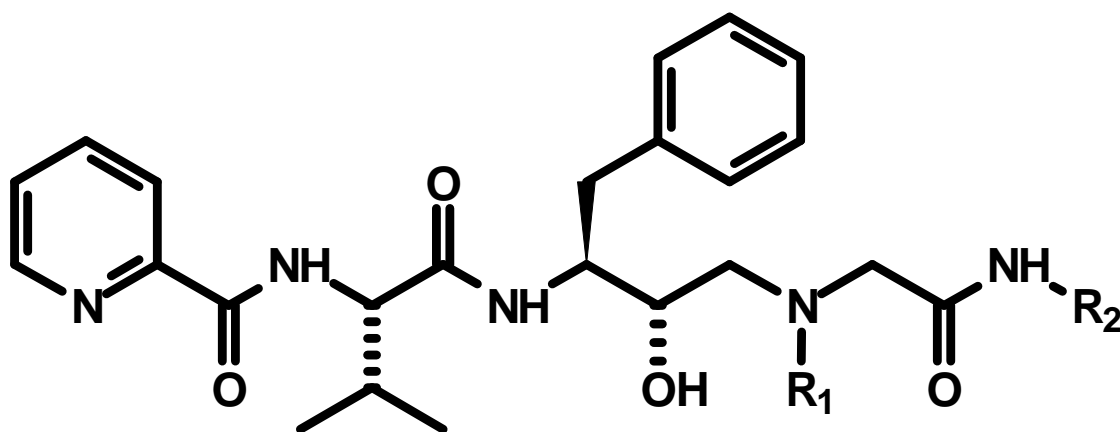


Synthesis of some Hydroxyethylamine Isostere Inhibitors of Plasmeprin II from Plasmodium Falciparum

Master Thesis in Organic Chemistry

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This work has been done under the supervision by
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Abstract

This work presents the synthesis of some drug candidates aiming to be inhibitors of Plasmeprin II, a vital enzyme for the malaria parasite. The inhibitors are based on the hydroxyethyl amine isostere. Special attention has been focused on selectivity achievement and pharmacokinetic aspects of the design. Efforts have been made to optimise each step in matter of high yield and purity as well as fast and robust processes. The synthesis is overall successful and allows the introduction of a diversity of variable groups.

Epidemiology of malaria

Malaria is a mosquito borne disease distributed in the tropical and subtropical parts of the world. The disease causes between one and two million deaths annually and is thus one of the major mortal diseases in the world (1). It is also responsible for a staggering amount of chronic ill health. Thus, it contributes to the deaths caused by other diseases. Four species of malaria exist: *Plasmodium Falciparum*, *Plasmodium Vivax*, *Plasmodium Ovale* and *Plasmodium Malariae*, of which *P Falciparum* is by far the most dangerous and also the most common form.

Targets for antimalarial therapy

Malaria is caused by protozoa transmitted from anophelin mosquitoes during a bite. The malaria parasite has a complex life cycle including a multiple of different forms. It undergoes a sexual reproduction cycle in the mosquito but proliferates in a non-sexual way in vertebrates. The parasite is transferred to the vertebrate in the shape of *sporozoites*, which causes a

primary infection in the liver shortly after its entrance into the body. The sporozoites then develop to *schizonts* and also proliferate dramatically. This phase is termed the pre-erythrocytic phase. After 10-14 days the parasites are released into the bloodstream in the form of *merozoites*.

The merozoites bind to and subsequently enter the erythrocytes and the *erythrocytic phase* starts. Inside the erythrocyte the merozoites form motile *trophozoites* that remodel the host cell by producing proteins expressed from the parasite's genome. Amino acids for the protein synthesis and proliferation in the erythrocytes are harvested from the erythrocyte hemoglobin, a process that takes place in the parasite food vacuole. The heme moiety is converted to a polymeric pigment called haemozoin that can be spotted as isolated crystals in a microscope.

Following mitotic replication to produce *schizonts*, and after another round of replication, *merozoites* are formed. The infected erythrocytes then rupture in a simultaneous fashion to release the merozoites that infect new erythrocytes. During the rupture of erythrocytes, which happens about every two days, the well-known periodical episodes of fever develops.

P Vivax and *P Ovale* may form *hypnozoites* in the liver that can survive for long times and cause a chronic infection that might develop into a new disease. Thus, the hypnozoites have to be depleted in order to cure the disease. *Gametocysts* are formed in the erythrocytes and released into the bloodstream where they might transmit the parasite to healthy mosquitoes.

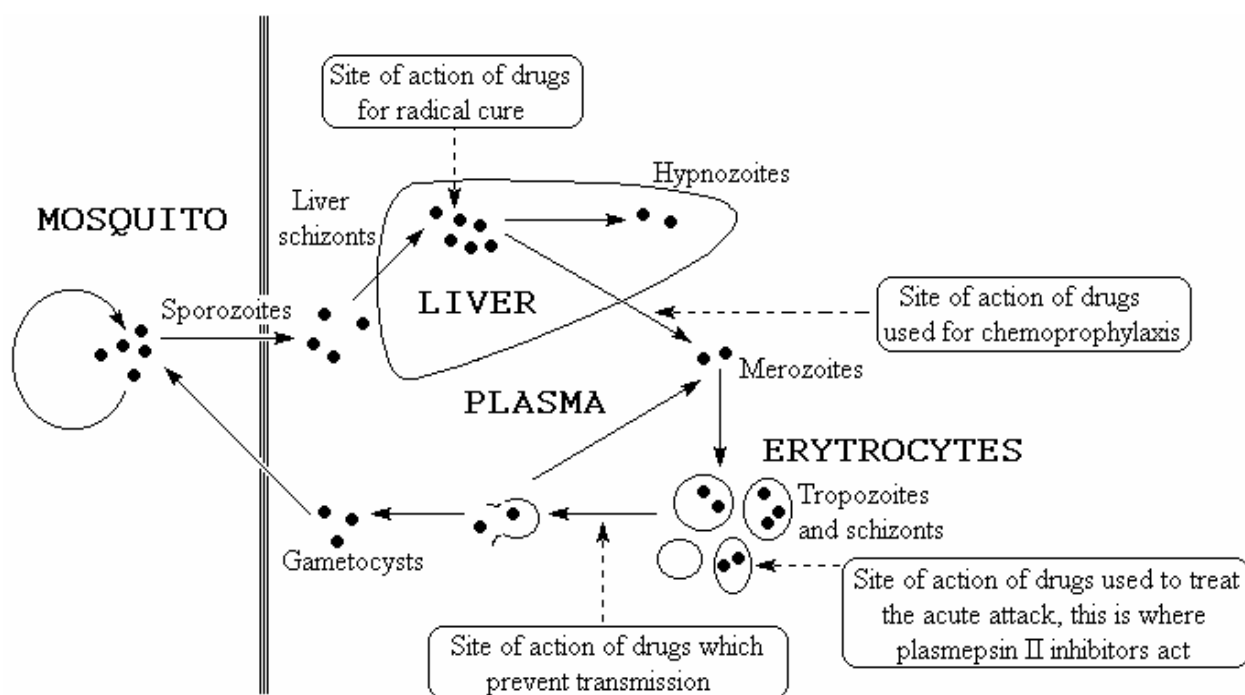


Fig. 1. The life cycle of the malaria parasite and sites of action for antimalarial therapy.

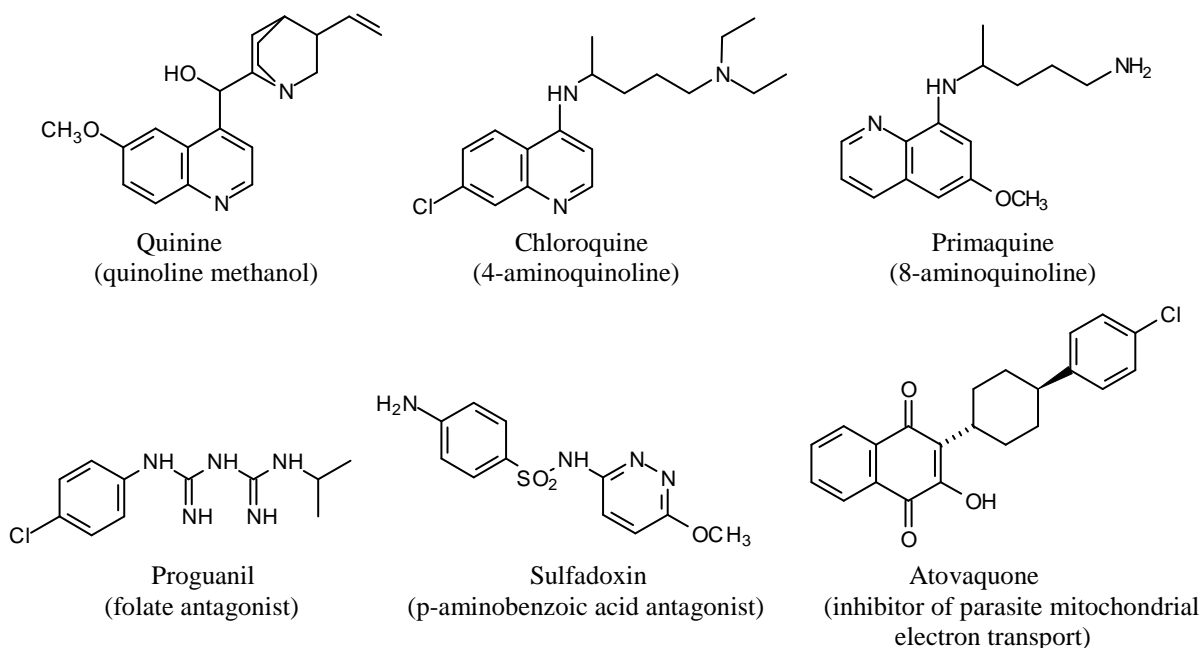


Fig. 2. Some of the most common representatives of the major groups of antimalarial drugs. The substance group belonging in brackets.

Radical cure from the disease requires that the liver forms of the parasite are eliminated. The only drugs that exhibit this effect are the 8-aminoquinolines. These are also effective against gametocytes and thus reduce the spread of infection. Drugs that kill erythrocytic forms can prevent acute attacks. Usually drug regimens that combine quinoline methanols, 4-aminoquinolines and inhibitors of synthesis and utilisation of folate are used. Lower doses of the attack preventing drugs are also utilised for prophylaxis.

The quinoline-derived drugs are all claimed to act by complexing with haemozoin to form a toxic product. The mechanism is however far from clear and other mechanisms have also been proposed to be of importance such as complexation with hemoglobin and thus prevention of hemoglobin degradation. Interference with RNA and DNA has also been suggested.

Folic acid is required for the production of nucleic acids, which is highly demanded for DNA production during the proliferation. Certain drugs that inhibit production and utilisation of folic acid have successfully been invented for the treatment of malaria, several of those showing a selectivity for parasite enzyme forms over vertebrate. The most common representatives being pyrimetamin and proguanil. Atovaquone represents a new class of antimalarial drugs. It is an inhibitor of mitochondrial electron transport in the parasites.

The trophozoites are equipped with two enzymes of the class aspartic proteinases termed Plasmeprin I and II to open up the hemoglobin structure for further digestion. Inhibition of these enzymes has been demonstrated to completely inhibit the breakdown of hemoglobin in cell cultures. However no such inhibitors have yet been synthesised that do not

inhibit certain vital aspartic proteinases in vertebrates. Thus, the inhibitors synthesised so far have not been found promising as antimalarial drugs. The search for aspartic proteinases that show enough selectivity for plasmepsins is still going on. This work presents one attempt.

Aspartic proteases and aspartic protease inhibitors

Aspartic proteases are a huge class of enzymes that catalyse the hydrolysis of peptide bonds. Inhibitors of aspartic proteases have successfully been invented for the treatment of HIV infections. During this search important experiences have also been gained that can be useful in the design of inhibitors of other proteases (2).

All recognised aspartic proteases share some structural aspects. The active site is made up of a groove in which the substrate is bound by hydrogen bonds, van der Waals interactions and electrostatic interactions. Usually 6-10 amino acids are bound. The residues on the substrate or artificial ligand are assigned with numbers starting from P1, which is the residue to the left of the cleavage position, continuing with P2, P3, and P4 towards the N-terminal. On the right side of the cleavage position the residues are assigned with the corresponding prime numbers: P1', P2', P3' etc. The spaces in the enzymes substrate groove that bind the residues are given numbers in a similar way although assigned with the letter S: S2, S1, S1' etc (fig 3) (2).

Aspartic proteases have a globular shape consisting of domains of well-defined β -sheet structures. They consist of one polypeptide chain (although some enzymes occur as dimers) which is in most cases divided into two domains termed the N-

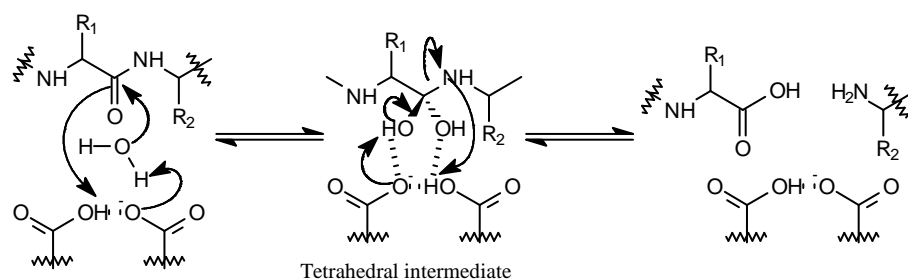


Fig. 3 (above). The mechanism for the peptide breaks catalysed by aspartic proteinases. The carboxylic acid groups in the lower part of the structure belong to the enzymes catalytic aspartic acid residues.

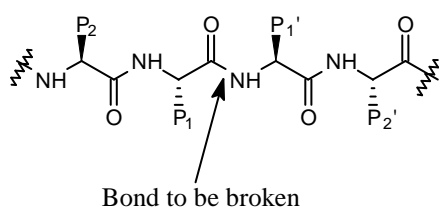


Fig. 4 (left). Assignment of the residues on the substrate.

terminal domain and the C-terminal domain. In most of the aspartic proteases a *flap* is formed from parts of the enzymes polypeptide chain which close down on top of the substrate during bonding further adding to inhibitor-protease interactions.

The term aspartic protease comes from two aspartic acid residues in the active site that are responsible for the catalytic action. The *general acid-base mechanism* has been proposed to explain how the catalysis of the amide hydrolysis is performed. The carboxylic groups from two aspartic acid side chains are positioned close to the amide carbonyl group with a water molecule arranged in between. A concomitant series of nucleophilic attacks between the aspartic acid carboxylic groups, the water molecule and the substrate generates a tetrahedral intermediate identical to what is seen during a traditional acid catalysed amide hydrolysis. The intermediate subsequently collapses into products (fig. 4).

An obvious requirement for a competitive protease inhibitor is that the substance cannot be hydrolysed at the position located near the aspartate residues. This is prevented by replacing the area around the peptide bond with a structure that has a similar physical extension and offers the same opportunities for interaction with the receptor all over the surface, a so called *isostere*. Some drug designers have suggested structures reminding of the substrate as possible inhibitors, where others have suggested structures reminding more of one of the products. The most successful inhibitors are however mimicking the tetrahedral transition state. Thus, most of the inhibitors reported today are based on a transition state isostere (fig. 5).

The first recognised aspartic protease inhibitors were found by natural product screening. To make inhibitors suitable for approval as drugs certain modifications were necessary. New inhibitors were synthesised with an already identified structure as a template by truncation, random structural

modifications and introduction of non-cleavable isosters. The last decade's development in combinatorial chemistry has increased the capacity for random screening. New knowledge about the receptors three-dimensional extension gained by X-ray crystallography has also been important in the design of new inhibitors. By transferring the images of the receptor to a computer, new suggested structures can be evaluated for its ability to fit into the active site.

The ability to inhibit an enzyme is usually measured as the inhibitor's *inhibition constant*, K_i , defined as the quota $[E] \times [I] / [EI]$, where $[E]$ denotes the concentration of enzyme, $[I]$ the concentration of inhibitor and $[EI]$ the concentration of enzyme-inhibitor complex at equilibrium. A strong inhibitor

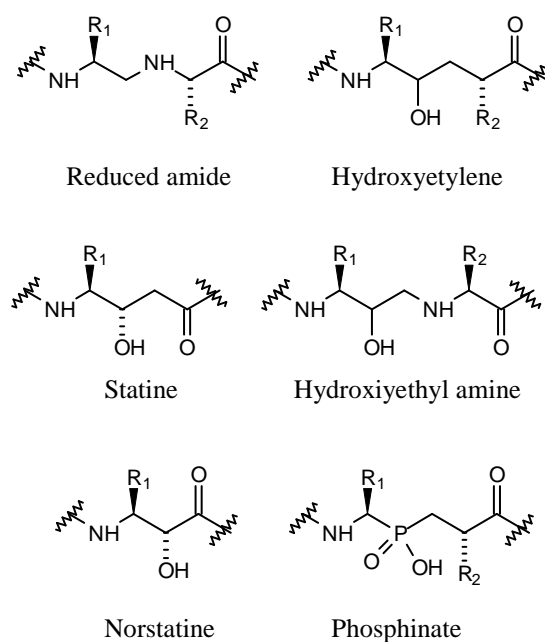


Fig. 5. Some peptide bond isosters used as transition state mimetics.

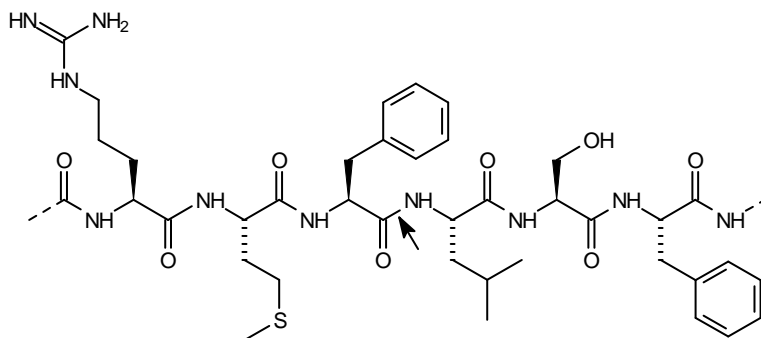


Fig. 6. Hemoglobin at the site of cleavage marked with an arrow.

thus has a low value. Generally inhibitors with K_i in the nanomolar range are regarded as strong inhibitors.

The plasmepsins

Inside the erythrocytes the malaria parasite absorbs vesicles of erythrocyte cytoplasm which are transported to and fused with the *digestive vacuole*, a proteolytic compartment within the parasite with a pH slightly below 5 (3, 4). Hemoglobin is enzymatically broken down here.

The initial step in the degradation of hemoglobin is the cleavage between Phe33 and Leu34 catalysed by plasmepsin I and II (5). The plasmepsins catalyse the same reaction although they differ in some aspects. Expression of plasmepsin I starts immediately after entrance into the erythrocyte and continues until the production of new merozoites begins whereas the expression of plasmepsin II starts when trophozoites have been formed (6). The substrate specificity is also different. Plasmepsin I appears to be specifically adapted for hemoglobin proteolysis whereas plasmepsin II has wider substrate specificity. It has been hypothesised that plasmepsin I might act when the food vacuoles have not yet been fully developed whereas plasmepsin II is more suitable for degradation of hemoglobin which has already been affected by the inhospitable environment in the food vacuole.

The plasmepsins show a high degree of identity to the human endogenous aspartic proteases. About 30% of the amino acid sequence in plasmepsin II are conserved in the human enzymes cathepsin D, cathepsin E, renin, pepsin and gastrin (7). Cathepsin D is showing the highest sequential identity with plasmepsin II and is inhibited by every plasmepsin II inhibitor reported so far. This is one of the major concerns during the search for drug candidates working by plasmepsin II inhibition since cathepsin D is presumed to play a role in the intracellular protein degradation.

Hydrophobic residues are required in the P1-P3 positions (8-11). Oligopeptide substrates with different hydrophobic residues in P2 and P3 have

been cleaved effectively, although peptides with acidic or basic side chains are poor substrates (12). The preference for hydrophobic residues in P2 has been attributed to the enzyme residues 287-289 in the close proximity to P2, which is Ile-Gly-Leu in plasmepsin II (7). Most human aspartic proteases have polar residues in these positions and therefore prefer more polar residues in the near parts of the substrate, only Cathepsin D has a similar demand for

hydrophobic residues in P2 and P3. The residue at position 13 in aspartic proteases is known to be highly important in determining the P3 subsite interactions (13). It is a Met in plasmepsins whereas it is a Gln in cathepsin D offering a possible source to selectivity. Unambiguous evidence confirms that a Phe residue is by far the best choice for P1 among the naturally occurring amino acids.

The P1' position interacts with the catalytic aspartic acid residues and the two following residues in the aspartic proteases. In most aspartic proteases including cathepsin D, it is made up of AspSerGly, compared to AspThrGly in the plasmepsins (7). The hydroxyl residue in the Ser or Thr offers hydrogen bondings to the catalytic carboxylic acid groups. This difference can therefore be of importance for the interaction with an inhibitor. Among the reported libraries of inhibitors the most successful structures contain a leucine or leucine like residue in S1'.

Overall the plasmepsins have a more open structure than cathepsin D exposing a larger area towards the surroundings (8). Plasmepsin I differs from plasmepsin II mainly by having a larger S3' pocket and a more hydrophobic S3 pocket (8).

Plasmepsin inhibitors

Only a very limited number of inhibitors for plasmepsin II have been reported so far. The first plasmepsin II inhibitors reported were derived from pepstatin A (fig. 7a), a general aspartic protease inhibitor of microbiological origin showing an inhibition constant of 6 pM (8). Silva *et al* reported inhibition data for eight structures derived from pepstatin A (8). Because of this limited amount of compounds; few conclusions can be drawn. One compound (fig. 7, b) did however show extraordinary good selectivity having a 40-fold higher K_i for cathepsin D than for plasmepsin II. An S2-S3' bridged compound was showing some affinity giving an indication of the assumed close proximity between S2 and S3' in the enzyme when the substrate is bound.

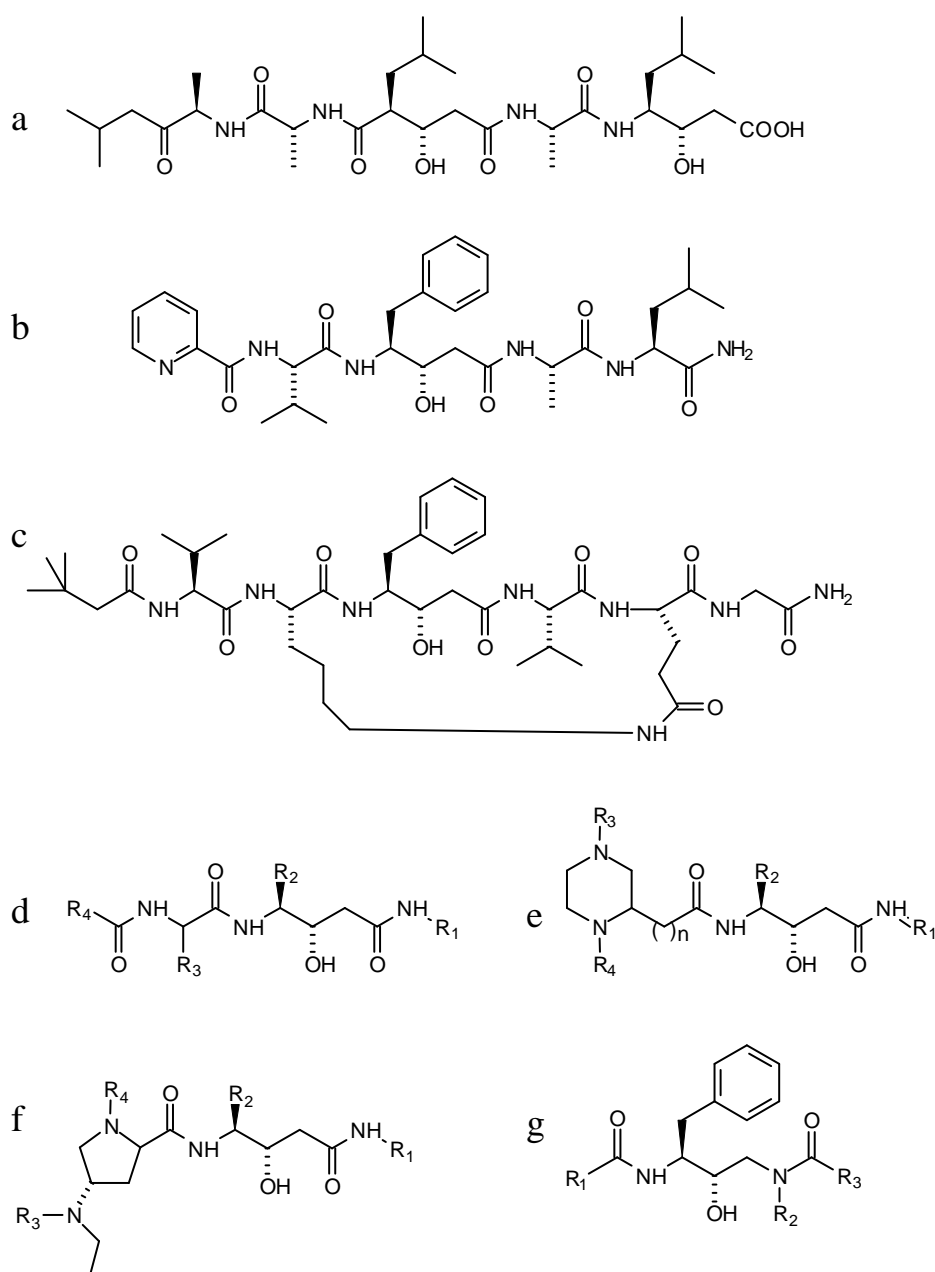


Fig. 7. Some inhibitors of plasmepsin II.

Carroll *et al* reported a 13 020 member library based on the statine isostere (fig 7, d) (9). The library confirmed that large hydrophobic substituents were necessary in P1 for any affinity to plasmepsins or cathepsin D at all. Among some alkyl, ω -phenyl alkyl and pyridyl N-capping groups (R_1) for P2', the large hydrophobic substituents without pyridine moieties were showing highest affinity. A β -branched hydrophobic amino acid was preferred in the P2 position, otherwise the affinity for plasmepsin II as well as the selectivity against cathepsin D decade. Ten of twenty possible substituents representing both hydrophobic, basic and acidic residues were showing considerable affinity to plasmepsin II indicating a high tolerance in the P3 position. The selectivity was most sensitive to changes in R_4 although selectivity altered seemingly by chance upon alteration.

A library of 18 900 similar compounds (fig 7, e and f) was reported later by the same group with a piperazine or proline residue in the P2 pocket (10). Cathepsin D demonstrated an equal preference for the piperazine based compounds (e) with $n=0$ or 1 versus the proline based compounds (f). In contrast, plasmepsin II accepted only the piperazine substituent with $n=1$. Large hydrophobic substituents in R_3 and R_4 were most successful for both enzymes. Those positions were also demonstrated to be highly selectivity determining. The most selective compound in the library had a 90-fold selectivity for plasmepsin II over cathepsin D.

Haque *et al.* reported a library of plasmepsin II inhibitors (fig. 7, g) based on the hydroxyethylamine isostere (11). Possible R-groups for all three positions were listed. Groups that showed too poor qualities in

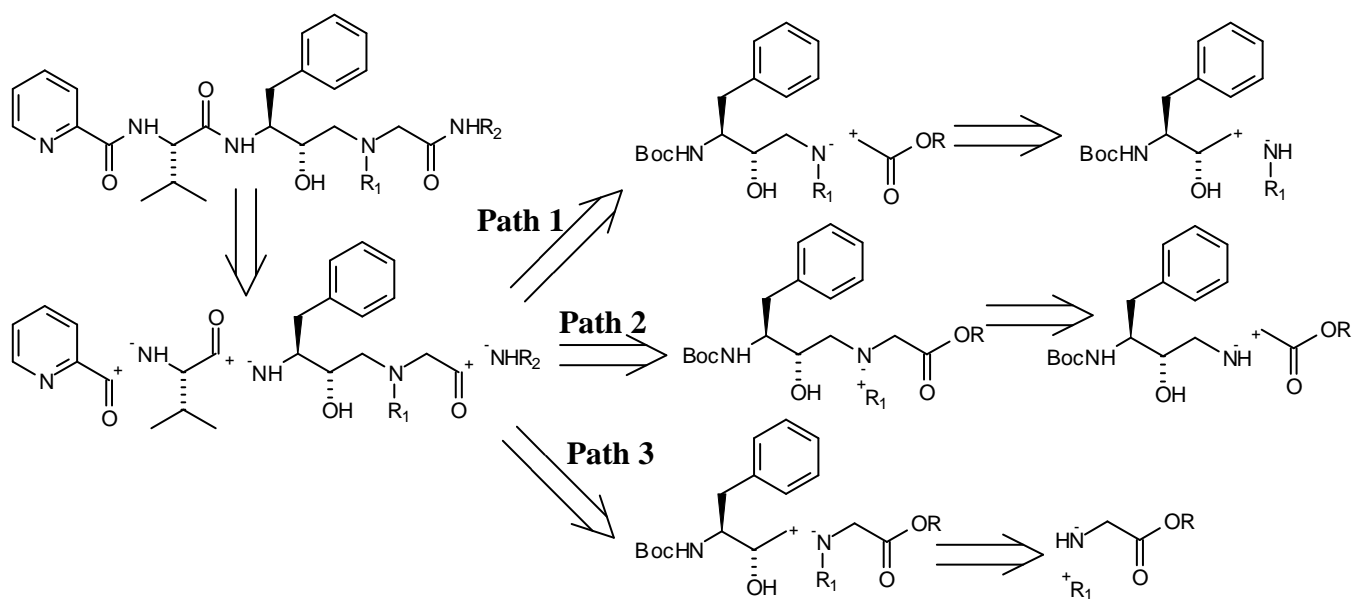


Fig. 10. The initial retrosynthetic analysis.

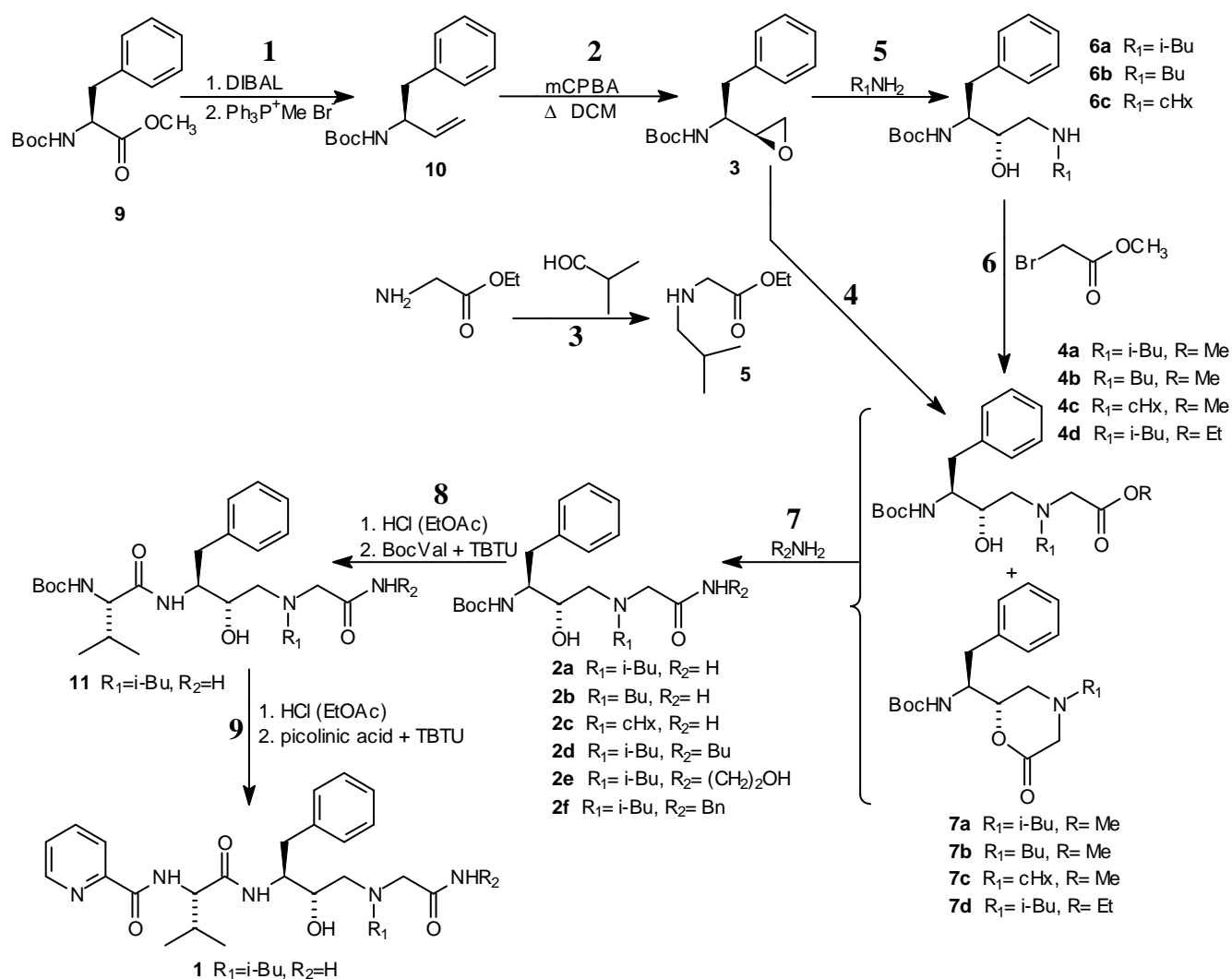


Fig. 11. Compressed reaction scheme.

a computer-modelling program were excluded from the library. The three R-groups were varied one at a time. Finally, groups showing lowest inhibition constants from all three varied positions were simultaneously combined. Much attention was focused on making the inhibitors suitable from a pharmacokinetic point of view. The entry with highest selectivity for plasmepsin II was showing ~15 times lower K_i for plasmepsin II over cathepsin D.

Drug design – our approach

The design of our potential protease inhibitors is based on one of the most selective inhibitors of plasmepsin II versus cathepsin D (fig. 7, b) reported so far (8). However this compound appears to have poor pharmacokinetic characteristics to be suitable for medical treatment. The proposed basic structure

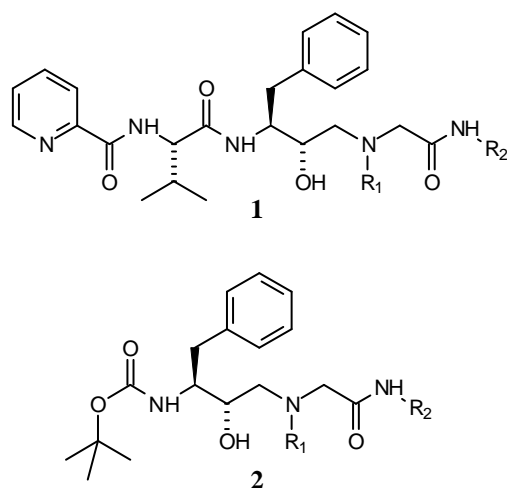


Fig. 8. The suggested structure for this work, **1**, and a synthetic intermediate to be evaluated as an indicator for the final compounds affinity, **2**.

presented in this work (fig. 8) has a less peptide-like character.

The acid environment of the food vacuoles where the plasmepsin is located might trap basic substances because of its charge in the acid form. Such trapping has been suggested to be of importance for chloroquine, which concentrates in the food vacuoles. This discovery has led us to include an amine functionality in the proposed structure.

In order to save time and resources the synthetic intermediate (fig. 8, b) used in the synthesis of the

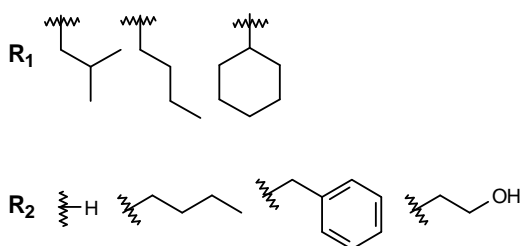


Fig. 9. The suggested variable groups.

final structure was also prepared for K_i determination in order to investigate if this could give a hint of the K_i for the final product. The variable groups (fig. 9) were chosen without cautious considerations or computer based design.

Synthetic strategy

The work has focused on the development of a synthesis for the compound with R_1 =isobutyl and R_2 =H, assuming that it will be possible to apply the same method to the synthesis of compounds with other substituents. Since R_2 was introduced by amidation of an ester with the corresponding amine, amidation with amines could not be considered as easy as with ammonia. Special attention was focused on the introduction of butylamine as a representative of amine introduction.

The initial retrosynthetic analysis (fig. 10) started by disconnecting the R_2 -group leaving an amine and an ester. In such a way, one batch could be made and split up before introduction of R_2 , saving a lot of time when making a library with constant R_1 . Subsequently the picolinic acid and valine was disconnected leaving the N-alkyl-N-alkoxy acetic acid substituted isostere, which could be disconnected at all three bondings to the nitrogen. An obvious starting material for the synthesis was epoxide **3** (fig. 11), which is frequently employed for the synthesis of hydroxyethylamine isostere inhibitors.

Results and discussion

The investigated synthetic routes are pictured in fig. 11. Epoxide **3** was prepared according to a literature procedure (19). Two methods for the preparation of compound **4** (reactions 3-6, fig. 11) were tested.

Preparation of **5** was performed by reductive amination using the hydrochloride salt of glycine ethylester with sodium cyanoborohydride as reducing agent. The strongly electron-withdrawing cyano

| Deviation from standard conditions | Yield |
|--|-----------------------|
| Standard conditions | 57%, 1.4% dialkylated |
| No TsOH added | 2,5% |
| Na ₂ CO ₃ used for extraction. | 15% |
| 1.3 eq. Na(OAc) ₃ BH used instead of NaCNBH ₃ , 1,2-dichloroethane was used as solvent. No TsOH added. | 42% |
| No extraction before chromatography | 4.3% |

Table 1. Reductive aminations tested under different conditions. During standard conditions 1 eq. isobutyraldehyde and 2 eq. glycine ethyl ester hydrochloride and 1-1.1 eq. NaCNBH₃ was dissolved in dry methanol. TsOH was added during 15 minutes and the mixture was allowed to react for 30 minutes. See experimental section for details.

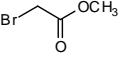
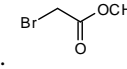
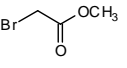
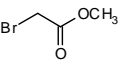
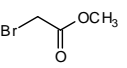
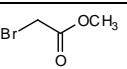
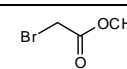
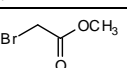
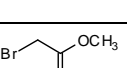

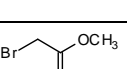

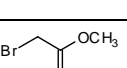
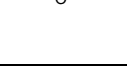
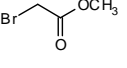

| | Alkylating agent | Solvent & base | Quenching & cyclization catalysis | Temp. | Reaction time (h) | Yield (%) | Comment |
|-----|--|--|---|-------|-------------------|---------------------------------|---|
| 1. |  1 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH | RT | 30 min | ~ 61% | Mostly ester |
| 2. |  1.5 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH | RT | 30 min | ~ 68% | Mostly ester |
| 3. |  1 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH | 0 °C | 30 min | ~ 40% | Mostly ester |
| 4. |  1 eq. | DMF pyridine | 5 min Et ₂ NH | RT | 30 min | ~ 48% | Mostly ester |
| 5. |  1 eq. | THF K ₂ CO ₃ | 5 min Et ₂ NH | RT | 30 min | ~ 47% | Mostly ester |
| 6. |  1 eq. | MeCN K ₂ CO ₃ | 5 min Et ₂ NH | RT | 30 min | ~ 53% | Mostly ester |
| 7. |  1.2 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH | RT | over night | 78-85% | |
| 8. |  2 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH | RT | over night | 89-97% | Standard conditions Mixture of ester and lactone. |
| 9. |  2 eq. | DMF K ₂ CO ₃ | Added 3 ml MeOH, 72h treatment. | RT | over night | 77-84% | Mixture of ester and lactone. Quota didn't change noticeable after MeOH treatment. |
| 10. |  2 eq. | DMF K ₂ CO ₃ | 3h with Et ₃ N | RT | over night | 110-119%, traces of DMF left | Mixture of ester and lactone. Quota didn't change remarkable after amine treatment. |
| 11. |  2 eq. | DMF K ₂ CO ₃ | Et ₂ NH overnight | RT | over night | 77-84% | Mixture of ester and lactone. TLC spot for ester about double as intense. |
| 12. |  2 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH 3 ml 1M HCl (MeOH) → pH=3, 4 days treatment Added 1 ml 4M HCl (EtOAc), 1 days treatment | RT | over night | 34% | Lactone spot about double as intense as ester spot on TLC. |
| 13. |  2 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH 3 ml 1M HCl in H ₂ O → pH=1 | RT | over night | 40% | No ester spotted on TLC or NMR. |
| 14. |  2 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH, K ₂ CO ₃ filtered off, Et ₂ NH removed by suction. Added 0.35M HCl (MeOH), 30 min treatment. | RT | over night | ~ 55% | No ester spotted on TLC. |
| 15. |  2 eq. | DMF KHCO ₃ | No quenching | RT | over night | 97-106% | Mixture of ester and lactone. Not remarkable different from standard conditions. |
| 16. |  2 eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH | RT | over night | 55% | Lactone hardly recognisable on TLC |

Table 3 continue on next page

group makes the agent a milder reducing agent than sodium borohydride and the nucleophilicity of the donated hydride is therefore considerably smaller. Acid usually have to be added to protonate the imine making it more susceptible for reduction (15, 16).

The yield (table 1) was poor compared to reductive aminations between molecules of comparable size, sterical properties and charge distribution found in the literature (17).

The acidity of the protonated glycine ethylester hydrochloride was probably not strong enough to activate the imine. Sodium triacetoxyborohydride, a somewhat stronger reducing agent than sodium cyanoborohydride, could be used in a similar manner without addition of acid. This, however, didn't increase the yield even though sodium triacetoxyborohydride usually gives yields >90% (8) for reductive aminations between non-bulky aldehydes and primary amines. Higher yields could perhaps be gained by adding acid. Or maybe, the acidity of the reaction mixture became high enough to reduce isobutyraldehyde to isobutanol.

An important problem when working with small batches was that the product **5** only could be extracted to an organic phase by a tremendously lot of extractions. Adjustment of waterphase to pH 11 with Na₂CO₃ in order to deprotonate the amine resulted in negligible yield, presumably because of ester hydrolysis.

The ester group is susceptible for nucleophilic attack by another molecule of the glycine ester which may form polyamides or cyclise to produce diketopiperazines in concentrated solutions of free amine (19). To avoid this the primary and secondary amino acid esters were kept as hydrochlorides. Glycine ethylester easily formed some products on heating of the free amine, although the N-isobutyl glycine ethylester did not.

Free amine was needed for reaction 4. Neutralisation of the amine **5** hydrochloride by extraction after addition of base turned out to be difficult when working with small samples. Bases stronger than NaHCO₃ caused rapid hydrolysis of the ester. A high quota between the phase volumes in favour of the organic phase was needed to completely deprotonate **5** when using NaHCO₃. This was

difficult to do when working with small preparations.

Instead, the free amine was achieved by letting the hydrochloride pass through a layer of solid anhydrous Na₂CO₃. The lack of water allowed carbonate to be utilised as base without the risk of hydrolysis. Bubbles were formed in the interface between the Na₂CO₃ and amine hydrochloride and passed to the surface as long as hydrochloride could be spotted as a second indication of remaining hydrochloride. The choice of elution solvent was sensitive if plugging of the column should be avoided. The Na₂CO₃ had to be washed with pure ether before elution started, otherwise the resistance became to high.

The result for reaction 4 was poor (table 2). Although vigorous conditions were used, yields were low. Cleavage of epoxide **3** with morpholine was considerably more efficient (20) indicating that steric effects rather than electronic are responsible.

Refluxing in solvents with boiling temperatures above 100°C resulted in decreasing yields, presumably because of decomposition of product or reactant. Recovered epoxide could have been isolated and quantified, although this wasn't done. Thin layer chromatography was showing several byproducts of higher polarity than the product.

Epoxide ring opening with primary amines (reaction 5, fig. 11) were performed in almost quantitative yield. When using isobutylamine, recrystallisation was the only purification necessary after removal of excessive amine by vacuum. When a less volatile amine was used, it had to be removed for example by extraction before the product could be recrystallized.

Alkylation of compounds **6a-c** could be accomplished in high yield (table 3), although concurrent lactonisation of the ester **4** occurred to some extent forming lactone **7**. Efforts have been made to either prevent lactonisation or enhance its formation to completion.

The reaction was initially considered to be fast. The first experiments were therefore performed under 30 minutes. Optimisation was first performed by varying the amount of alkylating agent (table 3, entry 1-2), temperature (entry 3) and choice of base (entry 4) and solvent (entry 5-6). Quaternary amine was

| Solvent | Boiling-point | Reaction time (h) | Volume | Yield (%) | Comment |
|-------------------|---------------|-------------------|--------|-----------|---|
| iPrOH | 82°C | 18h | 10 ml | - | No product spot on TLC after 18h. |
| tBuOH | 83 °C | 18h | 5 ml | 16% | |
| | | 45h | 3 ml | 18% | |
| | | 98h | 3 ml | 22% | |
| BuOH | 118°C | 100h | 5 ml | ~18% | Partly transesterificated product isolated. |
| Neopentyl-alcohol | 113°C | 110h | 3 ml | 12% | |
| Dioxan | 101°C | 96h | 3 ml | 6% | |

Table 2. Different tested conditions for reaction 4.

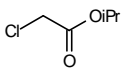
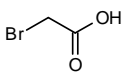
| | Alkylating agent | Solvent & base | Quenching & cyclization catalysis | Temp. | Reaction time (h) | Yield (%) | Comment |
|-----|--|---------------------------------------|-----------------------------------|-------|-------------------|-----------|---|
| 17. |  2 eq. | DMF KHCO ₃ | 5 min Et ₂ NH | RT | over night | 59% | Lacton not spotted on TLC even though strong ester spot was seen. |
| 18. |  2.eq. | DMF K ₂ CO ₃ | 5 min Et ₂ NH | RT | over night | 13.5% | Pure lacton |

Table 3. Different tested conditions for reaction 6. For mixtures almost entirely consisting of ester, the yield have been accounted on the molecular weight for the ester and for mixtures almost entirely consisting of lactone the yield have been calculated on the molecular weight for the lactone. The yield of mixtures of ester and lactone has been presented as an interval.

| No. | Amine | R ₁ | Ester | Solvent | Treatment | Reaction time (h) | Yield (%) | Comment |
|-----|---|----------------|-------|---------|-------------------------------|-------------------|-----------|----------------------------------|
| 1. | NH ₃ | iBu | Me | MeOH | Treatment at room temperature | 1h | quant. | |
| 2. | NH ₃ | Bu | Me | MeOH | Treatment at room temperature | 1h | 95% | |
| 3. | NH ₃ | cHx | Me | MeOH | Treatment at room temperature | 1h | 104% | |
| 4. | BuNH ₂ | iBu | Me | THF | Reflux | 3h | 82% | |
| 5. | BnNH ₂ | iBu | Me | THF | Reflux | 4h | 35%* | |
| 6. | HO(CH ₂) ₂ NH ₂ | iBu | Me | THF | Reflux | 24h | 73% | |
| 7. | NH ₃ | iBu | iPr | MeOH | Treatment at room temperature | 24h | 49% | |
| 8. | BuNH ₂ | iBu | iPr | THF | Reflux | 48h | 18.5% | 72.5% of the ester was recovered |

Table 4. Transamidation of esters 6 and lactones 7 or mixtures.

* This result could be a coincidence since it only was performed once.

formed upon treatment of pure product **4a** with methyl bromoacetate, only at elevated temperature. Reaction was therefore performed over night giving nearly quantitative yields (entry 7-8).

Efforts were made to encourage lactonisation so that pure lacton could be isolated. The attempts included addition of methanol to dissolve K₂CO₃ promoting basic hydrolysis (entry 9), and addition of base (entry 10-11) or acid (entry 12-14). The only successful attempts suffered from severe losses in yield. Use of bromoacetic acid as alkylating agent

(entry 18) in order to make completely esterfree lactone also failed. Bromoacetic acid was not stable towards K₂CO₃ in DMF. It is also likely that the bromoacetate ion was less susceptible to nucleophilic attack by the amine because of repulsion or that nucleophilic attacks from one bromoacetate carboxylic group on another's methylene explain the failure.

Attempts were also made to prevent lactonisation. This was done only in order to gain a chemically pure substance. The original plans to perform the peptide

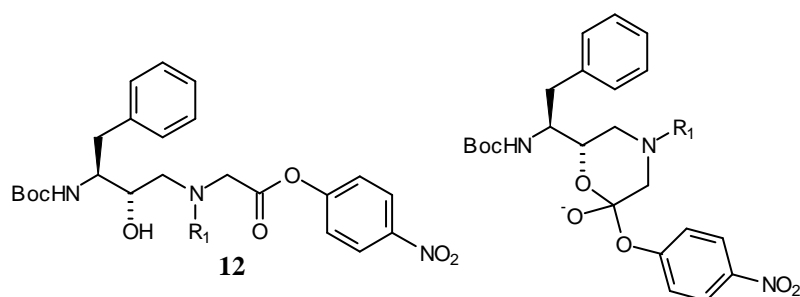


Fig. 12. *P*-nitrophenol withdraws electrons from the ester group in compound **12** making it more susceptible for an attack by the hydroxygroup. To the right the transition state during the reformation of the carbonyl group. *P*-nitrophenolate is by far the weakest base and thus the most likely species to leave besides the entropy effects generated by cyclisation.

couplings before introduction of the amide capping group was not considered possible since the base that had to be used in the peptide couplings would most likely open the lactone. The use of KHCO₃ as a base during the reaction and skipping the quenching could not completely prevent lactonisation. The introduction of an isopropyl ester instead of methyl could prevent lactonisation if KHCO₃ was used as base

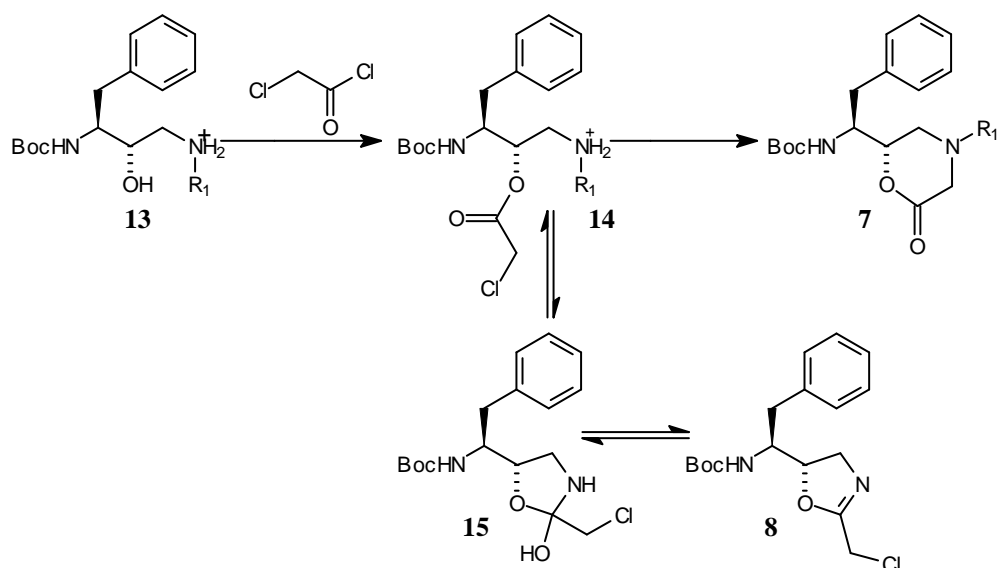


Fig. 13. Acylation of the hydroxyl group in acid environment followed by subsequent cyclisation.

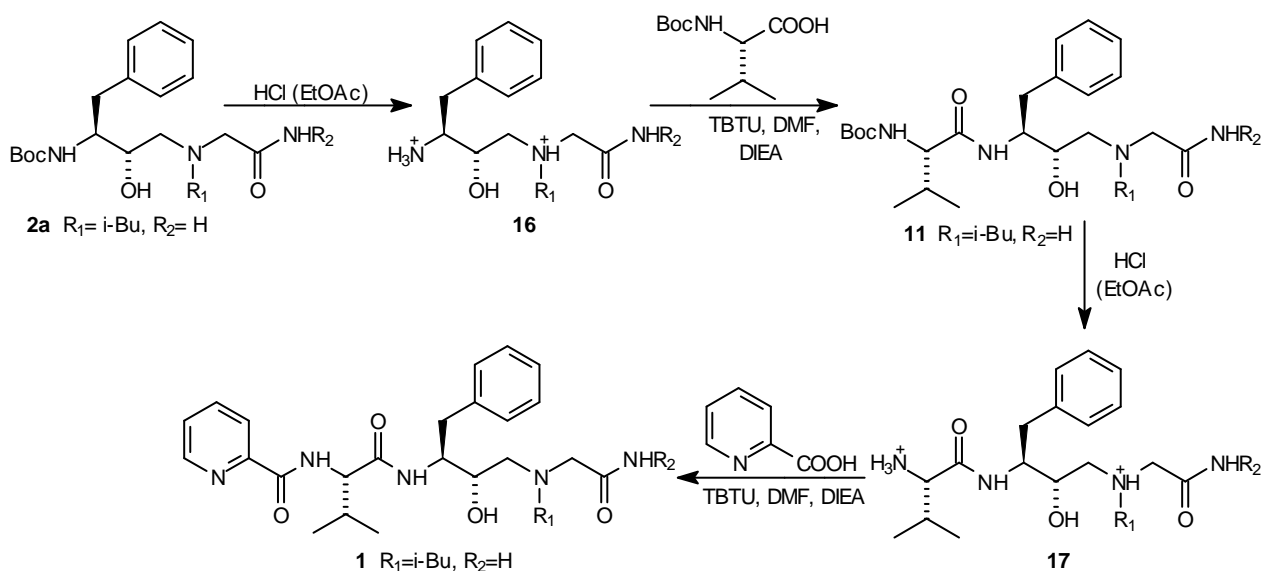


Fig. 15. The peptide couplings.

(entry 17). The isopropyl ester was unfortunately too resistant to amine formation to be used in the following step.

Several ways to produce pure lactone are waiting to be investigated. Similar problems with methyl 4-hydroxy carboxylic acids that only partly form lactone have been reported (21, 22). In cases with a very chemically inert compound, high concentrations of acid or base have generated pure lactone after hours of treatment, eventually upon heating. Some more fragile compounds have formed lactone almost completely upon treatment with lipase (23).

Since the isopropyl ester was less susceptible to lactonisation, one way to encourage lactonisation could be to introduce an alcohol that is a better leaving group than methoxide. The increased stability of the isopropyl ester was probably due to higher steric hindrance and a less positively charged

carbonyl group preventing the attack by the hydroxyl group, and possibly the more basic isopropoxide ion which is less prone to leave the transition state. By using a more electron withdrawing alcohol as leaving group, the lactonisation could possibly be encouraged. *p*-nitrophenol is a possible candidate. Its application as an ester activator has been reported earlier (26, 27).

Another possible way is to begin the preparation of lactone by formation of the coming lactone's ester group (fig. 13). By acylation of the amine **6** in acidic environment the amine will hopefully not be acylated. After acylation of the hydroxyl group remaining bromoacetyl bromide is quenched and base is added to promote cyclisation. Formation of 4,5-dihydrooxazoles has been described for 2-acetylaminoethanols (26). Because of the high entropy associated with its formation it is also likely

to be formed in this case. However in an acidic environment the reaction is probably reversible.

Amides **2** could be formed in seemingly the same high yields either from ester **6a**, lactone **7a** or a mixture (table 4). Amides derived from ammonia were formed by simply treating lactone with saturated ammonia in methanol for one hour.

Amines were inserted by refluxion in THF for 3 h. A lot of lactone could still be spotted on TLC after 3 h reaction using ethanolamine, which was not the case for the other amines. A suggestion is that the hydroxigroup is directed towards the aminogroup and thus stabilises its free electrons by a hydrogen bounding, which decreases its nucleophilicity (fig. 14). After another 20 hours 73% yield could be isolated although the reactant spot was still visible. It is therefore possible that a similar yield could be achieved already after 3h. Benzylamine was producing extraordinary low yield which might be a coincidence since its introduction only was performed once. The isopropylester of **4a** was obviously not reactive enough neither towards ammonia nor amines.

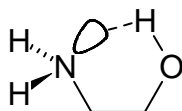


Fig. 14. Hypothesised hydrogen bonding in ethanolamine

The peptide couplings **8** and **9** were performed using TBTU as a coupling agent after removal of the Boc protective groups with HCl in EtOAc (fig. 15).

Experimental section

TLC analyses were performed on precoated silica gel 60 F-254 plates (0.25 mm, E. Merck) with visualisation by UV and/or by spraying with 2% ethanolic ninhydrin solution. NMR spectra were recorded on a JEOL JNM-EX270 spectrometer at 270 MHz. ^1H and ^{13}C signals are reported in ppm from tetramethyl silane. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Chromatography was performed using Silica gel 60 (0.04-0.063 mm) from E. Merck as stationary phase.

(5S, 6R)-3-aza-6-(picolinyl-L-valinyl)-amino-3-isoheptyl-5-hydroxi-7-phenylheptanoyl amide (**1**)

Compound **9** (0.10g, 0.203 mmol) was dissolved in 5 ml of ethyl acetate and under vigorous stirring 20 ml HCl in ethyl acetate was added. After 1h the solvent was evaporated and 20 ml saturated NaHCO_3 was added. The mixture was extracted with 2×10 ml ethyl acetate. The organic phases were combined, dried, and evaporated at reduced pressure. The residue was mixed with picolinic acid (25.0 mg, 0.203 mmol) and TBTU (65.2 mg, 0.203 mmol) and dissolved in 5 ml DMF. DIEA (diisopropyletylamine) (70.7 μl , 0.41 mmol) was added and the mixture was

stirred for 1h. 10 ml saturated NaHCO_3 was added and the mixture was extracted with 10×10 ml ethylacetate. The organic phases were combined, dried and evaporated. The residue was purified using a column (washed out impurities with ethylacetate : isohexan (1:2), eluated compound with methanol : ethyl acetate (1:10)) to give compound **9** (73.7g, 73%). M. p. 95-97°C; $^1\text{H-NMR}$ (CDCl_3 , 270 MHz) δ 0.75-0.96 (m, 12H, CH_3), 1.69-1.73 (m, 1H, $(\text{CH}_3)_2\text{CH}$), 2.03-2.40 (m, 5H, NHCH_2CH , NHCHCO , NCH_2CO), 2.74-3.02 (m, 2H, $\text{CH}(\text{OH})\text{CH}_2\text{NH}$), 3.54 (t, 1H, $\text{CH}(\text{OH})$), 4.32-4.56 (m, 3H, PhCH_2 , NHCHBn), 6.24 (s, 1H, CONH_2), 7.05-7.30 (m, 6H aromatic), 7.44 (dd, pyridine), 7.81-7.90 (m, pyridine), 8.01-8.22 (m, pyridine), 8.36 (d, 1H, CONH), 8.50-8.58 (m, pyridine); $^{13}\text{C-NMR}$ (CDCl_3 , 270 MHz) δ 17.87, 18.25 ($(\text{CH}_3)_2\text{CHCH}$), 20.75, 20.96 ($(\text{CH}_3)_2\text{CH}$), 25.47 ($(\text{CH}_3)_2\text{CHCH}_2$), 30.38 ($(\text{CH}_3)_2\text{CHCH}$), 37.85, 38.89 (PhCH_2), 52.96 (CONHCH), 59.66 ($\text{NHCH}_2\text{CH}(\text{CH}_3)_3$), 60.11 ($\text{CH}(\text{OH})\text{CH}_2$), 60.58 (NHCHCO), 64.64 (NCH_2CO), 68.43 ($\text{CH}(\text{OH})$), 122.75, 128.84, 128.84, 129.63, 137.60, 148.58, 148.58, 149.82 (aromatic), 165.18, 167.20, 168.78 ($\text{CH}(\text{=N})\text{CONH}$, CONH_2), 171.47 (CHCONHCH).

(5S, 6R)-3-aza-6-(tert.-butoxycarbonyl)amino-3-isoheptyl-5-hydroxi-7-phenylheptanoyl amide (**2a**)

The ester-lactone mixture **4a/7a** (0.10g, ~0.25 mmol) was dissolved in 30 ml saturated ammonia in methanol and stirred for 1h. The solution was evaporated and purified using column (eventual byproducts washed through with ethyl acetate : toluene 1:4, eluation of product with ethyl acetate). Amide was isolated in quantitative yield (0.098g, 0.25 mmol). If an oil was achieved, crystallisation was induced by addition of some chloroform. $^1\text{H-NMR}$ (CDCl_3 , 270 MHz) δ 0.82 (d, 6H, $(\text{CH}_3)_2\text{CH}$), 1.34 (s, 1H, OH), 1.37 (s, 9H, $(\text{CH}_3)_3\text{C}$), 1.63 (he, 1H, $(\text{CH}_3)_2\text{CH}$), 2.06-2.26 (he, 2H, NHCH_2CH), 2.45-2.55 (m, 2H, NCH_2CO), 2.87 (m, 2H, $\text{CH}(\text{OH})\text{CH}_2\text{NH}$), 3.02 (m, 2H, PhCH_2), 3.69 (t, 1H, $\text{CH}(\text{OH})$), 3.76 (pent, 1H, NHCHBn), 5.11, 5.48 (d, 1H, CONH), 5.81, 6.11 (s, 1H, CONH_2), 7.15-7.32 (m, 6H aromatic); $^{13}\text{C-NMR}$ (CDCl_3 , 270 MHz) δ 21.00 ($(\text{CH}_3)_2\text{CH}$), 26.78 ($(\text{CH}_3)_2\text{CH}$), 28.65 ($(\text{CH}_3)_3\text{C}$), 39.30 (PhCH_2), 54.14 (CONHCH), 59.77 ($\text{NHCH}_2\text{CH}(\text{CH}_3)_3$), 60.15 ($\text{CH}(\text{OH})\text{CH}_2$), 64.62 (NCH_2CO), 68.61 ($\text{CH}(\text{OH})$), 79.75 ($(\text{CH}_3)_3\text{C}$), 126.71, 128.78, 129.66, 138.57 (aromatic), 156.36 (CONH), 175.08 (CONH_2).

(5S, 6R)-3-aza-6-(tert.-butoxycarbonyl)amino-3-butyl-5-hydroxi-7-phenylheptanoyl amide (**2b**)

Similar to compound **2a**. The product was acquired as an oil. $^1\text{H-NMR}$ (CDCl_3 , 270 MHz) δ 0.84 (t, 3H, CH_3), 1.12-1.45 (m, 13H, $(\text{CH}_3)_3\text{C}$, CH_2CH_2), 2.41 (t, 2H, NHCH_2CH), 2.53 (s, 2H, NCH_2CO), 2.82-2.94 (m, 2H, $\text{CH}(\text{OH})\text{CH}_2\text{NH}$), 3.06 (m, 2H, PhCH_2), 3.56 (s, 1H, OH), 3.67 (t, 1H, $\text{CH}(\text{OH})$), 3.82 (q, 1H,

NHCHBn), 5.10, 5.41 (d, 1H, CONH), 5.85, 6.06 (s, 1H, CONH₂), 7.02-7.32 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 14.26 (CH₃CH₂), 20.69 (CH₃CH₂), 28.65 ((CH₃)₃C), 29.17 (CH₃CH₂CH₂), 39.29 (PhCH₂), 53.91 (CONHCH), 55.93 (NCH₂), 59.00, 59.18 (CH(OH)CH₂ NCH₂CO), 68.45 (CH(OH)), 79.71 ((CH₃)₃C), 126.70, 128.77, 129.61, 138.58 (aromatic), 156.33 (CONH), 175.00 (CONH₂).

(5S, 6R)-3-aza-6-(tert.-butoxycarbonyl)amino-3-cyclohexyl-5-hydroxi-7-phenylheptanoyl amide (2c)

Similar to compound **2a**, although cyclohexylamine was removed from reaction the mixture by extraction with 3×30 ml ethyl acetate after addition of 10 ml water. Organic phases were combined, dried and evaporated before chromatography. The product was acquired as an oil. ¹H-NMR (CDCl₃, 270 MHz) δ 0.95-1.20 (m, 6H, cyclohexyl), 1.34 (s, 9H, (CH₃)₃C), 1.59 (d, 1H, OH), 1.73 (d, 4H, cyclohexyl), 2.06-2.26 (he, 2H, NHCH₂CH₂(CH₂)), 2.45-2.70 (m, 2H, NCH₂CO), 2.89 (m, 2H, CH(OH)CH₂NH), 3.08 (s, 2H, PhCH₂), 3.61 (t, 1H, CH(OH)), 3.83 (q, 1H, NHCHBn), 5.22, 5.47 (d, 1H, CONH), 5.99, 6.12 (s, 1H, CONH₂), 7.15-7.32 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 26.09, 26.22 (CH₂CH₂CH₂CHN), 28.61 ((CH₃)₃C), 29.20 (CH₂CH₂CHN), 39.30 (PhCH₂), 53.84 (CONHCH), 55.09 (CH₂CHN), 55.90 (NCH₂CH₂(CH₂)), 60.70 (CH(OH)CH₂), 61.90 (NCH₂CO), 68.78 (CH(OH)), 79.53 ((CH₃)₃C), 126.57, 128.69, 129.62, 138.75 (aromatic), 156.29 (CONH), 176.35 (CONH₂).

(5S, 6R)-3-aza-6-(tert.-butoxycarbonyl)amino-3-isobutyl-5-hydroxi-7-phenylheptanoyl butylamide (2d)

The ester-lactone mixture **4a/7a** (0.10g, ~0.25 mmol) was dissolved in 3 ml THF and butylamine (0.25 ml, 2.5 mmol) was added. The mixture was refluxed for 3h, evaporated and exposed to vacuum over night to remove butylamine. The residue was purified by column (ethyl acetate : toluene 1:4, elution of product with ethyl acetate). ¹H-NMR (CDCl₃, 270 MHz) δ 0.83 (t, 6H, (CH₃)₂CH), 0.91 (t, 3H, CH₂CH₃), 1.25-1.52 (s, 13H, (CH₃)₃C, CH₂CH₂CH₃), 1.65 (he, 1H, (CH₃)₂CH), 2.09 (1H, CONHCH₂), 2.22 (1H, CONHCH₂), 2.46 (d, 2H, NHCH₂CH), 2.80-2.98 (m, 2H, NCH₂CO), 3.03 (d, 2H, CH(OH)CH₂NH), 3.22 (q, 2H, PhCH₂), 3.60-3.72 (m, 2H, CH(OH), NHCHBn), 5.03 (d, 1H, CONH), 7.15-7.30 (m, 6H aromatic), 8.12 (s, 1H, CONHBu); ¹³C-NMR (CDCl₃, 270 MHz); δ 14.06 (CH₃CH₂CH₂), 20.44 (CH₃CH₂CH₂), 21.00 ((CH₃)₂CH), 21.12 (CH₂CH₂NH), 26.71 ((CH₃)₂CH), 28.61 ((CH₃)₃C), 31.85 (CH₂NHCO), 39.14 (PhCH₂), 54.49 (CONHCH), 60.06 (CH(OH)CH₂ NHCH₂CH(CH₃)₃), 64.75 (NCH₂CO), 68.70 (CH(OH)), 79.71 ((CH₃)₃C), 126.70, 128.75, 129.57, 138.57 (aromatic), 156.34 (OCONH), 171.65 (CONHCH₂).

(5S, 6R)-3-aza-6-(tert.-butoxycarbonyl)amino-3-isobutyl-5-hydroxi-7-phenylheptanoyl ethanamide (2e)

Similar to compound **2d**, although it was refluxed for 24h. Cyclohexylamine was removed from the reaction mixture by extraction with 3×30 ml ethyl acetate after addition of 10 ml water. Organic phases were combined, dried and evaporated before chromatography. The product was acquired as an oil. [α]_D²² -48.9° (13.6 mg/ml, CHCl₃); ¹H-NMR (CDCl₃, 270 MHz) δ 0.82 (d, 6H, (CH₃)₂CH), 1.30 (s, 1H, OH), 1.36 (s, 9H, (CH₃)₃C), 1.63 (he, 1H, (CH₃)₂CH), 2.09-2.35 (he, 2H, NHCH₂CH), 2.50 (bs, 2H, NCH₂CO), 2.86 (t, 2H, CH(OH)CH₂NH), 3.05 (m, 2H, PhCH₂), 3.40 (dd, 2H, CONHCH₂CH₂OH), 3.68(s, 4H, CH(OH), NHCHBn, CHO), 4.05-4.40 (m, 2H, CONHCH₂CH₂OH), 7.15, 5.30 (d, 1H, OCONH), 7.13-7.32 (m, 6H aromatic), 7.91 (s, 1H, CONH); ¹³C-NMR (CDCl₃, 270 MHz) δ 21.03, 21.10 ((CH₃)₂CH), 26.84 ((CH₃)₂CH), 28.63 ((CH₃)₃C), 38.94 (PhCH₂CH), 42.13 (PhCH₂NH), 54.70 (CONHCH), 62.00 (NHCH₂CH(CH₃)₃), 65.52 (NCH₂CO), 69.11 (CH(OH)), 60.23, 60.65 (CONHCH₂CH₂OH), 80.04 ((CH₃)₃C), 126.75, 128.78, 129.56, 138.40 (aromatic), 156.52 (OCONH), 172.66 (CONH).

(5S, 6R)-3-aza-6-(tert.-butoxycarbonyl)amino-3-isobutyl-5-hydroxi-7-phenylheptanoyl benzylamide (2f)

Similar to compound **2d**, although cyclohexylamine was removed from the reaction mixture by extraction with 3 × 30 ml ethyl acetate after addition of 10 ml water. Organic phases were combined, dried and evaporated before chromatography. The product was acquired as an oil. [α]_D²² -32.6° (4.4 mg/ml, CHCl₃); ¹H-NMR (CDCl₃, 270 MHz) δ 0.75 (t, 6H, (CH₃)₂CH), 1.25-1.44 (m, 10H, (CH₃)₃C, CH(OH)), 1.60 (he, 1H, (CH₃)₂CH), 2.00-2.30 (he, 2H, NHCH₂CH), 2.47 (s, 2H, NCH₂CO), 2.83 (t, 2H, CH(OH)CH₂NH), 3.00-3.40 (m, 2H, PhCH₂CH), 3.62 (t, 1H, CH(OH)), 3.68 (d, 1H, NHCHBn), 4.42 (d, 2H, CH₂Ph), 4.91 (s, 1H, CONHBn), 7.12-7.38 (m, 12H, aromatic), 7.58 (s, 1H, CONHBn); ¹³C-NMR (CDCl₃, 270 MHz) δ 20.91, 21.05 ((CH₃)₂CH), 26.69 ((CH₃)₂CH), 28.63 ((CH₃)₃C), 39.09 (PhCH₂CH), 43.44 (PhCH₂NH), 54.40 (CONHCH), 60.06 (NHCH₂CH(CH₃)₃), 64.76 (NCH₂CO), 68.75 (CH(OH)), 79.80 ((CH₃)₃C), 126.73, 127.72, 128.10, 128.78, 128.94, 129.56, 138.45, 138.56 (aromatic), 156.31 (CONH), 171.65 (CONHBn).

(2R,3S)-3-[N-(tert-Butoxycarbonyl)amino]-1, 2-epoxy-4-phenylbutane (3)

To a solution of olefin **10** (0.30 g, 1.21 mmol) in dichloromethane (15 ml), mCPBA was added (1.67 g of paste containing 50% water/50% mCPBA, 4.84 mmol). The reaction mixture was then refluxed for 20h. The solution was cooled on an icebath and a

saturated and freshly prepared solution of Na₂SO₃ was added dropwise to the solution under continuous stirring until no further heat evolved on addition. After addition of 15 ml ether the solution was extracted twice with saturated Na₂SO₃ and five times with large volumes of 0.5 M NaHCO₃, dried with anhydrous MgSO₄ and evaporated under reduced pressure. After silica gel chromatography (ethyl acetate : toluene 1:6), the pure epoxide (0.20 g, 63%) was obtained as a white semi-solid. ¹H-NMR (CDCl₃, 270 MHz) δ 1.39 (s, 9H, tBu), 2.58 (s, 1H, CH₂), 2.69 (t, 1H, CH₂), 2.8-3.4 (m, 3H, CH₂, CH), 4.08-4.16 (br m, 1H, CH), 4.45-4.54 (br m, 1H, NH), 7.31-7.26 (m, 6H, aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 28.61 ((CH₃)₃C), 40.15 (PhCH₂), 44.86, 47.25, 50.68 (epoxide), 52.89 (NHC(Bn)), 79.91 ((CH₃)₃C), 126.95, 128.85, 129.73, 137.64 (aromatic), 155.77 (NHCOO).

Mixture of: (5S, 6R)-Methyl-3-aza-6-(tert.-butoxycarbonyl)amino-3-isobutyl-5-hydroxi-7-phenylheptanoate (4a) and (5S)-3-aza-5-[[1-(tert.-butoxycarbonyl)amino-2-phenyl]-1-etyl]-3-isobutyl-S-valerolactone (7a)

Substance **5a** (0.10g, 0.297 mmol) was dissolved in 1.5 ml dry DMF and added K₂CO₃ (0.041g, 0.30 mmol) and methyl bromoacetate (56.2 μl, 0.594 mmol). The reaction was stirred for 18h at ambient temperature. Diethylamine (50 μl, 0.48 mmol) was added and the mixture was stirred for another 5 minutes. The mixture was added 10 ml water. When the K₂CO₃ had dissolved, the mixture was extracted with 2 × 10 ml ethyl acetate. The organic phase was dried with MgSO₄ and evaporated. The crude product was purified by chromatography (ethyl acetate : isopentane 1:4) to give compound **7a** with traces of compound **4a** in quantitative yield. Lacton: ¹H-NMR (CDCl₃, 270 MHz) δ 0.79 (q, 6H, (CH₃)₂CH), 1.31 (d, 1H, OH), 1.37 (s, 9H, (CH₃)₃C), 1.65 (s, 1H, (CH₃)₂CH), 1.94-2.14 (m, 2H, NHCH₂CH), 2.18-2.30 (m, 1H, NCH₂CO), 2.68-3.82 (m, 2H, CH(O)CH₂NH), 2.88 (m, 2H, PhCH₂), 3.52 (d, 1H, NCH₂CO), 3.91 (q, 1H, NHCH₂Bn), 4.42 (dd, 1H, CH(O)), 4.73 (d, 1H, CONH), 7.15-7.32 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 20.78, 21.03 ((CH₃)₂CH), 25.56 ((CH₃)₂CH), 28.61 ((CH₃)₃C), 38.60 (PhCH₂), 52.60 (CONHCH), 53.21 (CH(OH)CH₂), 55.87 (NHCH₂CH(CH₃)₃), 66.04 (NCH₂CO), 79.17 ((CH₃)₃C), 80.16 (CHO(CO)), 127.02, 128.89, 129.73, 137.50 (aromatic), 155.90 (CONH), 168.63 (COO). Ester: ¹H-NMR (CDCl₃, 270 MHz) δ 0.86 (q, 6H, (CH₃)₂CH), 1.32 (d, 1H, OH), 1.37 (s, 9H, (CH₃)₃C), 1.63 (s, 1H, (CH₃)₂CH), 2.04-2.30 (hep, 2H, NHCH₂CH), 2.35-2.65 (m, 2H, CH(OH)CH₂NH), 2.85-2.98 (m, 2H, PhCH₂), 3.30 (s, 2H, NCH₂CO), 3.52-3.57 (m, 1H, CH(OH)), 3.92 (dd, 1H, NHCH₂Bn), 4.99 (d, 1H, CONH), 7.15-7.32 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 20.71, 20.96 ((CH₃)₂CH), 27.12 ((CH₃)₂CH), 28.70 ((CH₃)₃C), 39.79 (PhCH₂), 52.04 (CONHCH), 53.54

(CH(OH)CH₂), 56.25 (OCH₃), 59.91 (NHCH₂CH(CH₃)₃), 63.83 (NCH₂CO), 67.19 (CH(OH)), 79.41 ((CH₃)₃C), 126.54, 128.64, 129.82, 138.81 (aromatic), 156.22 (CONH), 172.50 (COO).

Mixture of: (5S, 6R)-Methyl-3-aza-6-(tert.-butoxycarbonyl)amino-3-butyl-5-hydroxi-7-phenylheptanoate (4b) and (5S)-3-aza-5-[[1-(tert.-butoxycarbonyl)amino-2-phenyl]-1-etyl]-3-butyl-S-valerolactone (7b)

Prepared similar to **4a/7a**. Lacton: ¹H-NMR (CDCl₃, 270 MHz) δ 0.82-0.91 (m, 3H, CH₃), 1.1-1.5 (m, 13H, (CH₃)₃C, CH₂CH₂), 2.24-2.42 (m, 4H, NCH₂CO, NCH₂CH₂), 2.81, 2.86 (m, 2H, CH(O)CH₂NH), 2.91-2.98 (m, 2H, PhCH₂), 3.63 (d, 1H, NCH₂CO), 3.99 (d, 1H, NHCH₂Bn), 4.44 (q, 1H, CH(O)), 4.93 (d, 1H, CONH), 7.15-7.32 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 14.13 (CH₃), 20.53 (CH₃CH₂), 28.52 ((CH₃)₃C), 28.74 (NCH₂CH₂), 38.48 (PhCH₂), 52.17 (CONHCH), 53.10 (CH(OH)CH₂), 55.51 (NCH₂CH₂), 57.42 (NCH₂CO), 78.98 ((CH₃)₃C), 80.02 (CHO(CO)), 126.91, 128.87, 129.63, 137.39 (aromatic), 155.84 (CONH), 168.44 (COO).

Mixture of: (5S, 6R)-Methyl-3-aza-6-(tert.-butoxycarbonyl)amino-3-cyclohexyl-5-hydroxi-7-phenylheptanoate (4c) and (5S)-3-aza-5-[[1-(tert.-butoxycarbonyl)amino-2-phenyl]-1-etyl]-3-cyclohexyl-S-valerolactone (7c)

Prepared similar to **6a/7a**. Lacton: ¹H-NMR (CDCl₃, 270 MHz) δ 0.98-1.28 (m, 6H, cyclohexyl), 1.40 (s, 9H, (CH₃)₃C), 1.75 (bs, 4H, cyclohexyl), 2.24 (t, 1H, NCH₂CO), 2.44 (t, 1H, NCH₂CO), 2.86 (d, 2H, CH(O)CH₂NH), 2.94 (d, 2H, PhCH₂), 3.18 (d, 1H, NCH), 3.62 (d, 1H, NCH₂CO), 3.98 (q, 1H, NHCH₂Bn), 4.39 (q, 1H, CH(O)), 4.94 (d, 1H, CONH), 7.18-7.35 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 25.54 (CH₂CH₂CH₂CHN), 26.17 (CH₂CH₂CHN), 28.52 ((CH₃)₃C), 28.69 (CH₂CHN), 38.48 (PhCH₂), 48.43 (NCHCH₂), 51.95 (CONHCH), 53.27 (CH(OH)CH₂), 62.32 (NCH₂CO), 79.25 ((CH₃)₃C), 79.98 (CHO(CO)), 126.88, 128.85, 129.64, 137.44 (aromatic), 155.86 (CONH), 169.07 (COO).

(5S, 6R)-Ethyl-3-aza-6-(tert.-butoxycarbonyl)amino-3-isobutyl-5-hydroxi-7-phenylheptanoate (4d)

A pipette was sealed in the end with a plug of glass wool. The pipette was filled with 0.5 g Na₂CO₃ of fine particle size and the hydrochloride of **4** (0.15 g, 0.767 mmol) was added in a layer above the Na₂CO₃. The following solutions were poured into the top of the pipette and allowed to rinse through the amine layer and thus eluate the amine and neutralise it when passing through the Na₂CO₃ layer: 5 ml ether, 3 ml methanol:ether 1:9, methanol:ether 1:5 until all hydrochloride had disappeared, 5 ml ether. Gentle pressure was applied to speed up the eluation. The

solution was evaporated under reduced pressure to give the free amine. Epoxide **3** (0.10 g, 0.38 mmol) and 3 ml of *tert*-butanol was added and refluxed for 96h. The solvent was evaporated under reduced pressure and the residue was purified by column. HOAc:ethyl acetate:toluene (3:10:87) was used to wash out the remaining epoxide. After the last traces of epoxide had been eluted, the mobile phase was changed to ethyl acetate:toluene (1:4). Fractions containing substance were pooled and washed with 30 ml saturated NaHCO₃. The organic phase was dried with K₂CO₃ and solvent was evaporated to give compound **6d** (35.3 mg, 22%). ¹H-NMR (CDCl₃, 270 MHz) δ 0.85 (6H, d, (CH₃)₂CH), 1.23-1.42 (10H, s, OH, (CH₃)₃CH), 1.62 (1H, m, (CH₃)₂CH), 2.35-2.62 (m, NHCH₂CH, CH(OH)CH₂NH), 3.30 (s, 2H, NCH₂CO), 3.52 (d, 1H, CH(OH)), 3.57-3.69 (m, PhCH₂, NHCH₂Bn), 4.99 (d, 1H, CONH), 7.15-7.32 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 14.53 (CH₃CH₂O), 20.69, 20.94 ((CH₃)₂CH), 27.12 ((CH₃)₂CH), 28.69 ((CH₃)₃C), 39.79 (PhCH₂), 53.50 (CONHCH), 56.54 (CH(OH)CH₂), 59.95 (NHCH₂CH(CH₃)₃), 61.06 (CH₃CH₂O), 63.87 (NCH₂CO), 67.19 (CH(OH)), 79.40 ((CH₃)₃C), 126.50, 128.60, 129.81, 139 (aromatic), 156 (CONH), 172 (COO).

Ethyl *N*-isobutyl-aminoacetate hydrochloride (hydrochloride of compound **5**)

Ethyl glycine hydrochloride (0.700g, 5.0 mmol) was dissolved in 12 ml methanol, isobutyraldehyde (0.227 ml, 2.5 mmol) and molecular sieves (0.50 g) were added and the mixture was stirred for 1h before addition of sodium cyanoborohydride (0.157 g, 2.5 mmol). Immediately after the addition of sodium cyanoborohydride, paratoluenesulfonic acid monohydrate (0.523 g, 2.75 mmol) dissolved in 3 ml methanol was added dropwise during 15 minutes under continuous stirring. The reaction mixture was stirred for another half hour. The mixture was evaporated at reduced pressure and the remaining solid was dispersed in 20 ml saturated NaHCO₃ and extracted with 5 × 30 ml ethyl acetate. The combined organic phases were dried with MgSO₄, filtrated and evaporated. The remaining solid was purified by chromatography (ethyl acetate:toluene 1:4) and the fractions containing pure product were combined, 5 ml 1M HCl in ether was added and the mixture was evaporated under reduced pressure after 15 minutes. The product (0.28g, 57%) was crystallised from ethyl acetate to give a wool like cohesive powder. ¹H-NMR (CDCl₃, 270 MHz) δ 1.10 (d, 6H, CH(CH₃)₂), 1.30 (t, 3H, CH₃CH₂-), 2.26-2.30 (m, 1H, CH(CH₃)₂), 2.94 (q, 2H, CHCH₂NH₂⁺), 3.84 (t, 2H, H₂N⁺CH₂CO), 4.27 (q, 2H, OCH₂CH₃), 9.70 (br s, 2H, NH₂⁺); ¹³C-NMR (CDCl₃, 270 MHz) δ 13.97 (CH₂CH₃), 20.44 [CH(CH₃)₂], 25.97 (⁺NH₂CH₂CH), 47.50 [(CH₃)₂CH], 55.35 (OCH₂CH₃), 62.45 (H₂N⁺CH₂CO), 165.76 (CO).

(2*S*, 3*S*)-3-(*tert*-butoxycarbonyl)amino-1-isobutylamino-2-butanol (**6a**)

Epoxide **3** (0.100 g, 0.380 mmol) was dissolved in 10 ml isopropanol and added isobutylamine (0.38 ml, 3.8 mmol) was added in one portion. The container was stirred at 55°C overnight. The solvent was evaporated at reduced pressure and the crude product was dried under vacuum for 24h to remove excess isobutylamine. The product was recrystallized from hexane to give compound **5a** (0.123g, 96%). [α]_D²² – 36.6 (10.9 mg/ml, CHCl₃), M. p. 89°C; ¹H-NMR (CDCl₃, 270 MHz) δ 0.87 (d, 6H, (CH₃)₂CH), 1.35 (s, 1H, OH), 1.39 (s, 9H, (CH₃)₃C), 1.67 (he, 1H, (CH₃)₂CH), 2.36 (he, 2H, NHCH₂CH), 2.50-2.70 (m, 2H, CH(OH)CH₂NH), 2.85-2.95 (m, 2H, PhCH₂), 3.52-3.57 (m, 1H, CH(OH)), 3.72 (dd, 1H, NHCH₂Bn), 5.01 (d, 1H, CONH), 7.15-7.32 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 20.78 ((CH₃)₂CH), 20.85 ((CH₃)₂CH), 28.70 ((CH₃)₃C), 39.59 (PhCH₂), 52.58 (CONHCH), 53.98 (CH(OH)CH₂), 57.85 (NHCH₂CH(CH₃)₃), 68.39 (CH(OH)), 79.48 ((CH₃)₃C), 126.55, 128.67, 129.79, 138.80 (aromatic), 156.22 (CONH).

(2*S*, 3*S*)-3-(*tert*-butoxycarbonyl)amino-1-butylamino-2-butanol (**6b**)

Prepared similar to **6a**.

¹H-NMR (CDCl₃, 270 MHz) δ 0.89 (t, 3H, CH₃), 1.25-1.50 (m, 14H, (CH₃)₃C, CH₃CH₂CH₂, OH), 2.48-2.64 (m, 2H, CH(OH)CH₂NH), 2.85-2.94 (dd, 1H, NHCH₂Bn), 3.19 (bs, 2H, CH₂CH₂NH), 3.59 (t, 1H, CH(OH)), 3.72 (q, 1H, NHCH₂Bn), 5.04 (d, 1H, CONH), 7.16-7.30 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 14.26 (CH₃), 20.58 (CH₂CH₃), 28.69 (C(CH₃)₃), 32.17 (NHCH₂CH₂), 39.48 (PhCH₂), 49.46 (NHCH₂CH₂), 52.51 (CONHCH), 54.06 (CH(OH)CH₂NH), 68.30 (CH(OH)), 79.53 (C(CH₃)₃), 126.57, 128.69, 129.77, 138.70 (aromatic), 156.24 (CONH).

(2*S*, 3*S*)-3-(*tert*-butoxycarbonyl)amino-1-cyclohexylamino-2-butanol (**6c**)

Prepared similar to **6a**.

¹H-NMR (CDCl₃, 270 MHz) δ 0.92-1.30 (m, 4H, cyclohexyl), 1.36 (s, 1H, OH), 1.39 (s, 9H, (CH₃)₃C), 1.55-1.92 (m, 6H, cyclohexyl), 2.32-2.36 (m, 1H, NHCH), 2.48-2.68 (m, 2H, CH(OH)CH₂NH), 2.89 (pent, 2H, PhCH₂), 3.47 (d, 1H, CH(OH)), 3.73 (d, 1H, NHCH₂Bn), 5.08 (d, 1H, CONH), 7.20-7.32 (m, 6H aromatic); ¹³C-NMR (CDCl₃, 270 MHz) δ 25.24 (CH₂CH₂CH₂CH), 26.26 (CH₂CH₂CH), 28.64 (C(CH₃)₃), 33.83, 34.08 (CH₂CH), 39.43 (PhCH₂), 49.74 (CONHCH), 54.13 (CH(OH)CH₂NH), 56.88 (NCHCH₂(CH₂)), 68.81 (CH(OH)), 79.35 (C(CH₃)₃), 126.45, 128.57, 129.70, 138.74 (aromatic), 156.15 (CONH).

(3S)-3-tert-Butoxycarbonylamino-4-phenyl-1-butene (10).

t-BuOK (1.06 g, 9.49 mmol) was added to a solution of methyltriphenylphosphoniumbromide (3.39 g, 9.49 mmol) in 15 ml dry THF. The mixture was then stirred for 1h prior to use. To a $-78\text{ }^{\circ}\text{C}$ stirred solution of the protected amino ester **9** (1.32 g, 4.74 mmol) in 15 ml dry toluene, DIBAL (6.32 ml of 1.5 M solution in toluene, 9.48 mmol) was added dropwise over 30 min. This mixture was then stirred for 1h at $-78\text{ }^{\circ}\text{C}$, before addition of the ylide/THF solution in one portion. The reaction mixture was allowed to attain room temperature and then warmed to $50\text{ }^{\circ}\text{C}$ and stirred overnight. Water (10 ml) and 1M hydrochloric acid (20 ml) were added, the organic layer was separated and the aqueous phase was extracted twice with 50 ml ethyl acetate. Before the second extraction 30 ml toluene was added, more in good phase separation was not achieved. The combined organic layers were then washed with saturated brine and saturated NaHCO_3 , dried with anhydrous MgSO_4 , filtered and evaporated. The product was purified by silica gel flash chromatography (toluene : ethyl acetate 39:1) to give compound **10** (0.67 g, 57%) as white crystals. $^1\text{H-NMR}$ (CDCl_3 , 270 MHz) δ 1.41 (s, 9H, t-Bu), 2.83 (d, 2H, PhCH_2), 4.43 (s, 1H, NHCHBn), 5.10 (t, 2H, vinyl H-1), 5.74-5.88 (m, 1H, vinyl H-2), 7.17-7.34 (m, 5H, aromatic); $^{13}\text{C-NMR}$ (CDCl_3 , 270 MHz) δ 28.70 ($(\text{CH}_3)_3\text{C}$), 41.86 (PhCH_2), 54.02 (NHCHBn), 79.50 ($(\text{CH}_3)_3\text{C}$), 115.07 (vinyl), 126.80, 128.66, 129.90, 138.40 (aromatic), 155.20 (NHCOO).

(5S, 6R)-3-aza-6-[(tert.-butoxycarbonyl)-L-valinyl]-amino-3-isohexyl-5-hydroxi-7-phenylheptanoyl amide (11)

Compound **2a** (0.10g, 0.25 mmol) was dissolved in 5 ml of ethyl acetate. Under vigorous stirring 20 ml saturated HCl in ethyl acetate was added quickly. The mixture was stirred for 1h before the solvent was evaporated. The residue was dissolved in 3 ml DMF and Boc-L-valine (54.3 mg, 0.25 mmol), TBTU (N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methyl-methanaminium tetrafluoroborate N-oxide) (79.9 mg, 0.25 mmol), and diisopropylethylamine (87.1 μl , 0.50 mmol) was added. The mixture was stirred for 1h at ambient temperature. 10 ml of saturated NaHCO_3 was added and the mixture was extracted with 2×10 ml ethyl acetate. The organic phases were combined, dried, evaporated and purified with chromatography (eventual byproducts washed through with ethyl acetate : isohexan (1:4), elution of product with ethyl acetate) to give compound **9** in quantitative yield (123 mg). $^1\text{H-NMR}$ (CDCl_3 , 270 MHz) δ 0.77-0.93 (m, 12H, CH_3), 1.44 (s, 1H, OH), 1.46 (s, 9H, $(\text{CH}_3)_3\text{C}$), 1.66 (he, 1H, $(\text{CH}_3)_2\text{CH}$), 2.03-2.18 (m, 2H, NHCH_2CH), 2.27 (bs, 1H, NHCHCO), 2.51 (d, 2H, NCH_2CO), 2.87-2.98 (m, 2H, $\text{CH}(\text{OH})\text{CH}_2\text{NH}$), 3.06-3.24 (m, 2H, PhCH_2), 3.74 (t, 1H, $\text{CH}(\text{OH})$), 3.90 (pent, 1H, NHCHBn),

5.20 (d, 1H, CONH), 6.16 (s, 1H, CONH_2), 6.91 (d, 1H, OCONH), 7.15-7.32 (m, 6H aromatic); $^{13}\text{C-NMR}$ (CDCl_3 , 270 MHz) δ 17.82, 19.67 ($(\text{CH}_3)_2\text{CHCH}$), 20.89, 21.03 ($(\text{CH}_3)_2\text{CH}$), 26.66 ($(\text{CH}_3)_2\text{CHCH}_2$), 28.60 ($(\text{CH}_3)_3\text{C}$), 30.91 ($(\text{CH}_3)_2\text{CHCH}$), 38.73, 38.89 (PhCH_2), 52.96 (CONHCH), 59.66 ($\text{NHCH}_2\text{CH}(\text{CH}_3)_3$), 60.11 ($\text{CH}(\text{OH})\text{CH}_2$), 60.58 (NHCHCO), 64.64 (NCH_2CO), 68.43 ($\text{CH}(\text{OH})$), 80.16 ($(\text{CH}_3)_3\text{C}$), 126.70, 128.71, 129.57, 138.38 (aromatic), 156.20 (OCONH), 171.98 (CHCONHCH), 175.03 (CONH_2).

Acknowledgements

Besides my supervisors i wish to thank Dr Johan Hultén for valuable advice and all other members of the protease group at the department who have been helpful during my work.

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