Chemo-enzymatic peptide synthesis and derivatization using Subtilisin A in anhydrous organic solvents

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Chemo-enzymatic peptide synthesis and derivatization using Subtilisin A in anhydrous organic solvents

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(met een samenvatting in het Nederlands)

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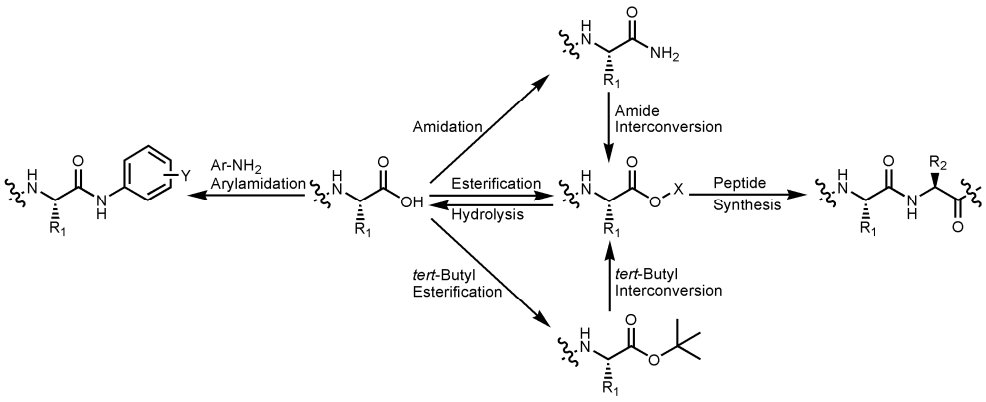

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1.1 Peptides

Peptides, from simple dipeptides to complexly folded globular proteins, are the workhorses of every single living organism. They perform a plethora of functions including catalysis, transportation, recognition and signaling and they regulate vital processes such as DNA transcription, immune response and energy balance.[1] Due to the ever increasing research efforts, a manifold of the processes performed by peptides have been elucidated during the past few decades. Application of these peptides for their use as a therapeutic or prodrug,[2] nutritional additive or cosmetic ingredient is gaining importance.[3] For instance, there are currently over 75 peptide drugs (containing less than 50 amino acids) on the market and more than 45 proteins (over 50 amino acids) targeting 29 different diseases. Looking forward, over 300 peptides are in clinical development targeting a staggering 64 diseases.[4] Despite these promising numbers, even more peptides could have reached the market if their isolation from natural resources or their chemical synthesis was not such an expensive and time consuming process. This is particularly true in case of long peptides consisting of more than 30 amino acids. Hence, only some diseases, *e.g.* AIDS and diabetes-2 are treated with long peptide therapeutics (Fuzeon and Exenatide, respectively), where the benefits outweigh the huge production costs. Driven by these limitations, there is an increased effort being spent on the development of robust, broadly applicable, economically attractive synthetic methods for the production of peptides. Nowadays, there are four commonly used approaches for the synthesis of peptides, namely, fermentation, solid-phase or solution-phase chemical peptide synthesis, and chemo-enzymatic peptide synthesis.[5]

1.2 Peptide synthesis

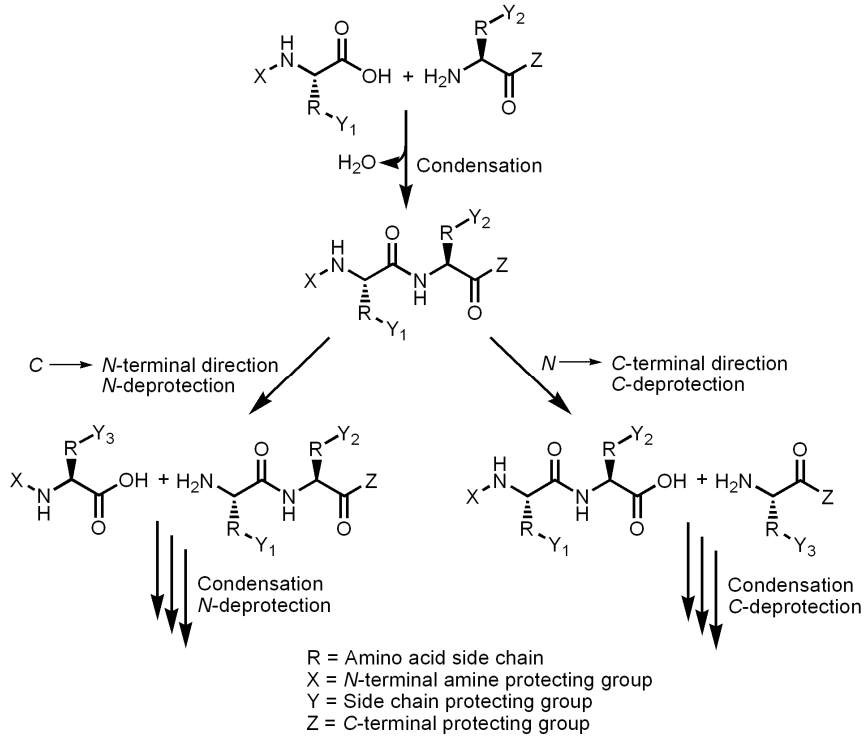
1.2.1 *Peptide fermentation*[6-10]

Peptide fermentation, wherein the natural synthetic machinery of a microorganism is used, is -if possible- by far the cheapest and environmentally benign way to produce therapeutic peptides and proteins. There are, unfortunately, quite some limitations. First, many research and development efforts are needed for every single peptide to identify the most suitable host and production conditions. Peptides are often not secreted by the organism and titer yields are often quite low, typically in the range of a few milligrams per liter culture broth. Furthermore, peptides are often degraded by endogenous microbial proteases. Therefore, generally, only large (over 50 amino acids in length), tightly folded peptides, *i.e.* proteins, can be produced via fermentative means. Another drawback is that non-proteinogenic amino acids or D-amino acids are difficult to incorporate and peptide modifications, such as the introduction of a *C*-terminal amide functionality, are tedious or even impossible. Last but not least, the few milligrams of peptide per liter that have been produced by the microorganism have to be purified from a very complex biological matrix. Therefore, most peptides are synthesized by chemical means, in academia, as well as in industry.

1.2.2 *Solution-phase peptide synthesis*[11-14]

The advantage of chemical peptide synthesis is that almost any desired peptide sequence and modification can be realized. Solution phase peptide synthesis relies on the stepwise

condensation of an *N*-protected amino acid with a *C*-protected amino acid building block, as shown in Scheme 1.



Scheme 1. Solution phase peptide synthesis in the *C* → *N* and the *N* → *C* terminal direction.

To avoid side reactions, the side chain functionalities of the amino acids should preferably be protected resulting in a fully protected dipeptide after condensation. Then, either the *N*-terminal protecting group (for synthesis in the *C* → *N* direction) or the *C*-terminal protecting group (for synthesis in the *N* → *C* direction) should be removed for the next condensation reaction with a protected amino acid building block. The *N* → *C* strategy is preferred because amino acid building blocks containing a *C*-terminal protecting group, such as a carboxamide or *tert*-butyl (^{t}Bu) ester, are cheaper to produce than *N*-protected amino acids. Unfortunately, upon chemical activation of a peptide *C*-terminal acyl amino acid residue, racemization occurs, except if *C*-terminal Gly or Pro residues are used. Therefore the *C* → *N* strategy is more commonly used. An important drawback of solution phase peptide synthesis is that after each elongation and deprotection step, the product should be purified. Especially when the peptide chain becomes longer, purification of the intermediates becomes a difficult and time-consuming process (several days for a coupling and purification step on large scale). Therefore, only short peptides are produced on large scale via chemical synthesis in solution. For longer peptides one has to rely on other chemical methods such as solid-phase peptide synthesis (SPPS).

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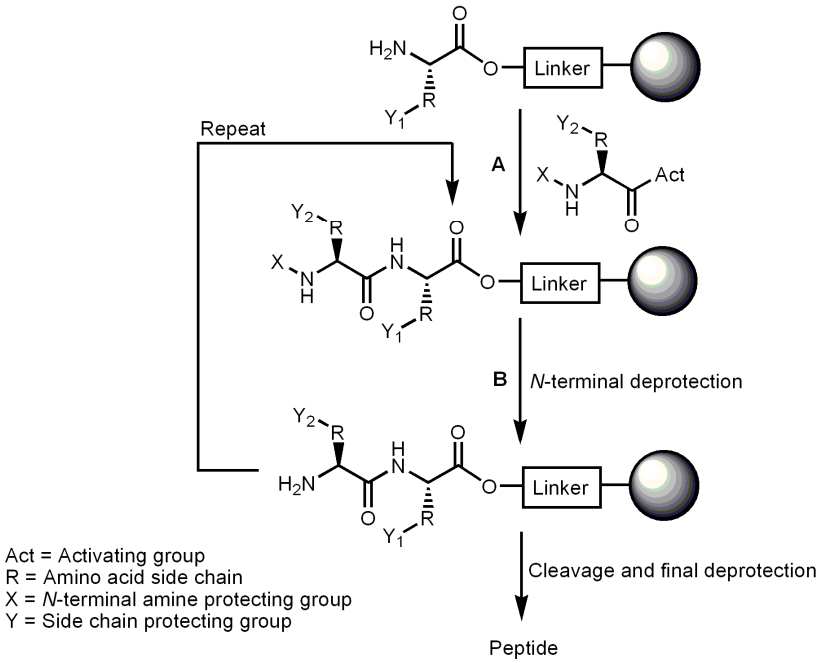
1.2.3 *Solid-phase peptide synthesis*[15-19]

Since its development by Merrifield,[15] solid-phase peptide synthesis (SPPS) has grown to become *the* method of choice for the synthesis of medium-sized to large peptides, generally between 10 and 50 amino acids, including large scale production processes. Small, porous beads, *i.e.* resins, are used as the solid phase and decorated with functional units (linkers) on which the peptide chains can be built. In general, SPPS consists of repetitive cycles of coupling and deprotection steps wherein the peptide is elongated from the *C* → *N* terminal direction in a stepwise manner, as depicted in Scheme 2.



Scheme 2. The various process steps of SPPS.

In SPPS the free *N*-terminal amine function of the resin bound amino acid is coupled to an *N*-protected amino acid building block (Scheme 2, **A**). After selective cleavage of the *N*-terminal protecting group from the dipeptide (Scheme 2, **B**), the two steps -deprotection followed by coupling- are repeated to elongate the peptide sequence until the complete peptide sequence has been synthesized. The main advantages of this method are that the excess of reagents can be conveniently washed away after coupling and deprotection, that the coupling of the amino acid residue is generally racemization free, and that a long peptide can be obtained in a relatively short time.

There are two main approaches of SPPS, namely, the Fmoc/^{t}Bu and the Boc/Bzl based strategy, referring to the *N*-terminal Fmoc or Boc protected amino acids used in each elongation step. The original SPPS as developed by Merrifield relied on the *N*-Boc protection which is cleaved with trifluoroacetic acid (TFA), while the cleavage of the peptide from the solid phase and side chain deprotection is performed with HF. Today, the Boc strategy is rarely used on industrial scale due to the use of hazardous HF, the special

equipment that is required and the lability of the side chain protecting groups and linkers towards the repetitive acid treatments. The Fmoc-based SPPS is nowadays the most often applied method in academia as well as in industry. This method relies on milder *N*-deprotection conditions using a base, usually piperidine, with fully orthogonal acid labile side chain protection and linker moieties. In the past two decades Fmoc-based SPPS has been extensively optimized leading to standardized coupling, deprotection and cleavage protocols using industrially available Fmoc-amino acid building blocks. Following these protocols, a high synthesis yield and purity can be obtained for almost any desired peptide.

Despite these promising developments, there are still a number of drawbacks when using SPPS. Most importantly, extremely high yields should be realized during each coupling and deprotection cycle. For instance, if the yield of each cycle (coupling and deprotection) in the synthesis of a 10-mer peptide is 95%, a crude yield of only 37% would be obtained, as shown in Figure 1. The other 63% are smaller peptides with very similar sequences to the target peptide; obviously purification of the desired peptide from this mixture is difficult. More as a rule than exception, an expensive preparative HPLC purification is required for peptides synthesized by SPPS. As a result, often more than 50% of the production costs of peptides are invoked by the purification and downstream processing. Hence, a high crude peptide yield is an essential factor for economically feasible production (for large scale peptide synthesis reviews see references 20-23).

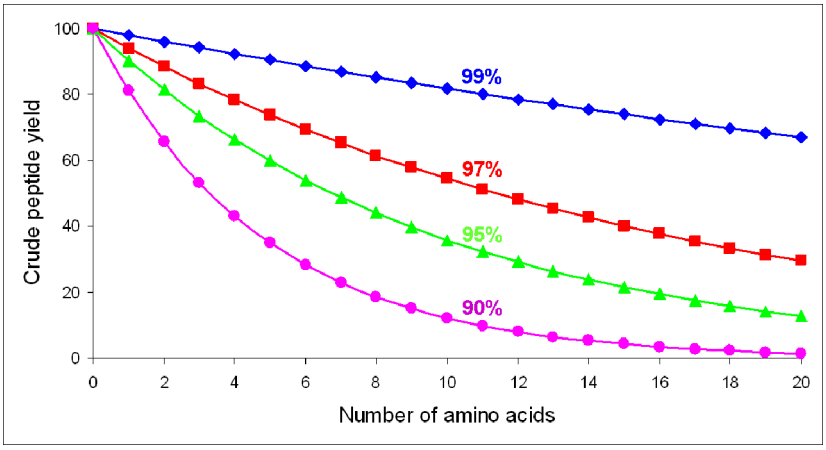


Figure 1. Crude peptide yield is strongly dependent on the yield per step during SPPS

In order to achieve high yields for each SPPS cycle, a large excess of reagents is generally needed to drive coupling and deprotection reactions to completion, resulting in extensive washing procedures after each deprotection and coupling. As much as 150 liter of solvent is needed per kilogram of resin for each coupling and deprotection cycle. Therefore, besides amino acid building blocks and coupling reagents, also solvents significantly contribute to the overall costs.

Due to the decreasing crude peptide yield with an increasing number of amino acids it is often impossible to produce peptides over 10 amino acids in length on large scale with high purity. Moreover, protected peptides attached to the solid phase tend to form tertiary

structures by a phenomenon which is called “hydrophobic collapse”[24] resulting in very troublesome coupling and deprotection cycles. In such a case, the synthesis time is increased due to prolonged deprotection and coupling cycles. Additionally, the couplings are often performed twice, resulting in the use of an even larger amount of reagents, to obtain acceptable crude peptide yields. Therefore, peptides which form such tertiary structures, in practice most peptides between 10 and 15 amino acids in length, are almost impossible to produce via SPPS on large scale.

1.2.4 *Hybrid approach*[11-12, 25]

Taking into account the drawbacks of both SPPS and solution phase peptide synthesis, it is generally impossible to synthesize, for instance, a 30-mer peptide solely by one of these two strategies. To remedy these limitations, a hybrid approach (also called fragment assembly or convergent synthesis) can be used wherein protected peptide fragments are synthesized by means of SPPS and subsequently coupled in solution, as shown in Figure 2.

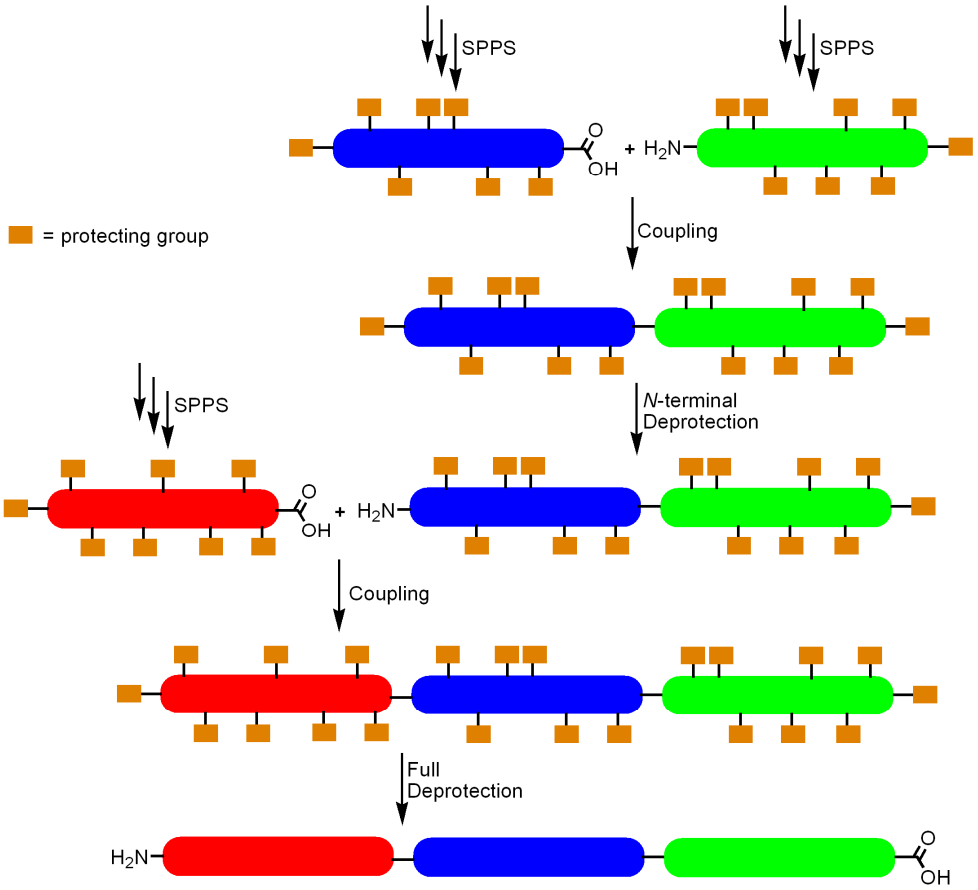


Figure 2. Synthesis of a 30-mer peptide using SPPS and solution phase fragment condensation.

On paper, the “ideal” approach for the synthesis of a 30-mer peptide would be a fully symmetric (convergent) fragment condensation strategy wherein the length of the protected peptides synthesized by SPPS does not exceed 10 amino acids, *e.g.*, a 10 + 10 + 10 strategy. However, if the desired peptide does not contain Gly or Pro residues at the *C*-terminal coupling position of the fragments, racemization is inevitable. Therefore, a convergent approach is most often not feasible and one has to adapt the peptide fragment length to the positions of the Gly and Pro residues, if they are present at all. This leads to the SPPS synthesis of undesirably short (< 5 amino acids) or long (> 10 amino acids) protected peptide fragments, which are often very poorly soluble in organic solvents. Alternatively, other coupling positions are chosen and racemization is taken for granted. As a consequence, a difficult preparative HPLC purification is required due to the similar retention times of the two diastereoisomers leading to (partial) overlap of peaks. Mixed fractions have to undergo a second round of preparative HPLC purification leading to greater expenses and a lower product yield.

1.3 Chemo-enzymatic peptide synthesis[5, 26-29]

Although, with chemical peptide synthesis almost any desired peptide sequence is accessible, racemization occurs quite often which invokes high purification costs. In sharp contrast to this, chemo-enzymatic peptide synthesis, wherein the peptide bond is formed enzymatically, is never accompanied by racemization.

1.3.1 *Proteases*

Van 't Hoff predicted in 1898 that,[30] based on the equilibrium constant of a reversible chemical reaction, along with the function of a catalyst (including biocatalysts) for accelerated achievement of the equilibrium, that enzyme-catalyzed peptide synthesis would be possible. According to this theory, an enzyme able to hydrolyze a peptide bond, should also be able to generate a peptide bond. Therefore, virtually every successful enzymatic peptide synthesis strategy relies on the use of proteases, which are designed by Nature to cleave peptide bonds. Still, about 40 years elapsed before the first experimental proof of Van 't Hoff's prediction became evident through the first protease catalyzed synthesis of an amide bond.[31] The overall process of peptide bond cleavage is identical in all classes of proteases and differences between the catalytic mechanisms are rather subtle. Generally, the four classes of proteases (serine[32], cysteine[33], aspartic[34] and metallo-proteases[35]) only differ in the functional groups that perform nucleophilic attack, general base catalysis, and electrophilic assistance.[36] There are two different approaches for protease catalyzed peptide synthesis, namely the thermodynamically and the kinetically controlled synthesis.

1.3.2 *Thermodynamically controlled enzymatic peptide synthesis*

The equilibrium-controlled enzymatic peptide synthesis, or thermodynamic approach, represents the direct reversal of hydrolysis, as shown in Figure 3. Consequently, all proteases, independent of their catalytic mechanisms, can be used.

Major drawbacks of this approach are that i) the amount of product when the equilibrium is reached is low because the preferred state of the starting materials is the unreactive ionized form;[37-38] ii) the reaction rate is low;[39] and finally iii) large amounts of protease are required.[40] To obtain a high condensation yield, the equilibrium should be shifted to favor

product formation, *e.g.* by precipitation of the product,[41-42] by using a large excess of one of the starting materials or by lowering the amount of free water which is present in the system.[43] Still, especially when longer peptides are to be coupled, the low reaction rates lead to undesired hydrolytic side reactions within the peptide fragments, making the thermodynamically controlled peptide synthesis a non-viable approach.

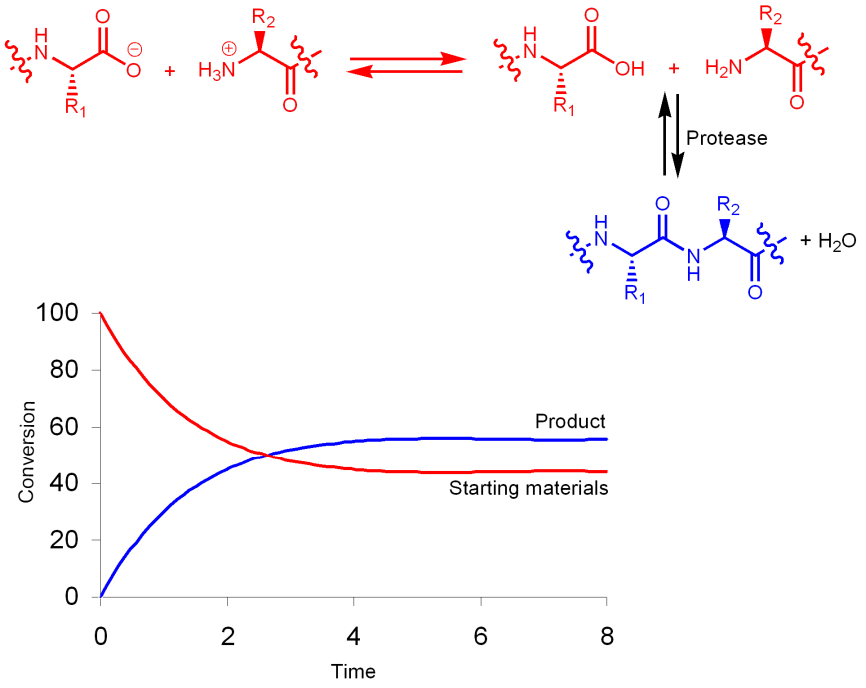


Figure 3. Thermodynamically controlled peptide synthesis.

1.3.3 *Kinetically controlled enzymatic peptide synthesis*

In contrast to the thermodynamically controlled approach, the protease catalyzed kinetically controlled peptide synthesis requires much less enzyme and the reaction time to reach maximal product yield is significantly shorter.[44] Kinetic control means that the product being formed with the highest rate and disappearing with the lowest rate would accumulate. Whereas the thermodynamically controlled approach ends with a true equilibrium, in the kinetic approach the product concentration goes through a maximum before the slower hydrolysis of the product becomes significant. If the reaction is not stopped after the acyl donor ester has been consumed, the same equilibrium position would be obtained as with the thermodynamically controlled approach,[45] as shown in Figure 4.

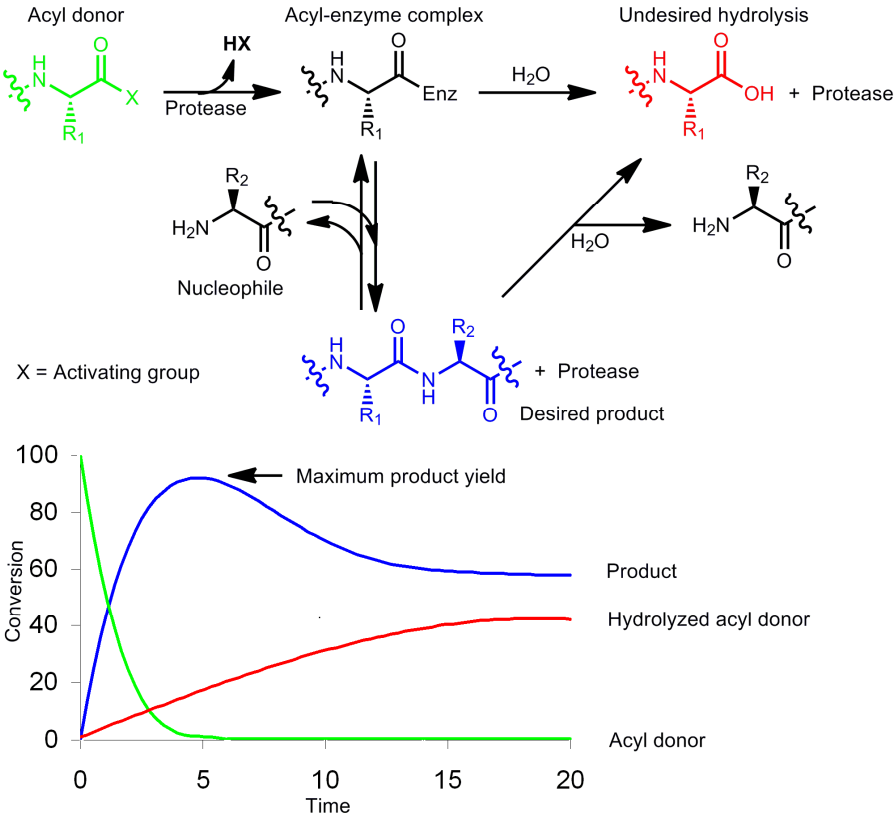


Figure 4. Kinetically controlled peptide synthesis.

The kinetic approach requires the use of an acyl donor ester, referred to as “activated ester”, as the carboxyl component and is limited to proteases which form an acyl-enzyme intermediate, *i.e.* serine and cysteine proteases.[46] In contrast to the thermodynamically controlled synthesis, where both starting materials are mainly present in their non-reactive ionized forms, the acyl donor is fully present in its reactive form in the kinetically controlled approach. The protease acts as a transferase catalyzing the transfer of the acyl moiety to the amino acid- or peptide-derived amine component (“the nucleophile”). Specifically, the acyl-enzyme complex reacts, in competition with water, with the nucleophilic amine component to form the peptide bond.[47] The ratio of aminolysis and hydrolysis (“synthesis/hydrolysis ratio”) is of decisive importance for successful peptide synthesis. An important factor determining the synthesis/hydrolysis ratio is the substrate specificity of the protease. The efficiency of the nucleophilic attack of the amine component depends essentially on an optimal binding within the active site of the protease, as shown in Figure 5.

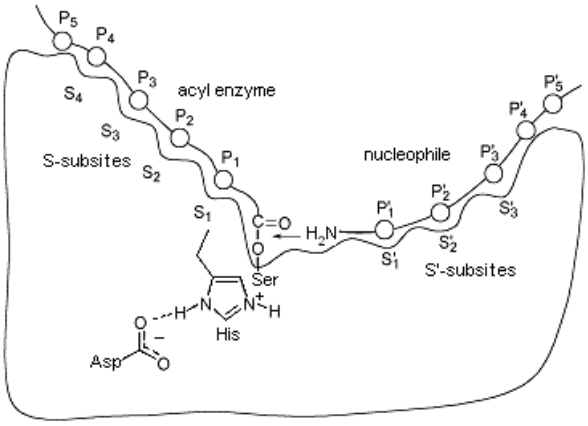


Figure 5. Schematic representation of subsite-substrate interactions of a serine protease[26]

Clearly, the efficiency of peptide synthesis will depend on the interactions of the peptide acyl donor with the S-binding sites of the protease and with the S'-binding sites in case of the nucleophile.[48-51] Therefore, knowledge of the amino acid preferences of the protease binding sites, which can be obtained by systematic acyl transfer studies using libraries of peptide active esters and peptide amines, is essential.

1.3.4 *Increasing the yield of kinetically controlled enzymatic synthesis*

There are several methods known to improve the product yield in kinetically controlled chemo-enzymatic peptide synthesis. Some of them will be discussed in more detoal in the next sections.

1.3.4.1 *Organic solvents*

Although the availability of water in the reaction mixture can dramatically influence the yield of the reaction, unfortunately, an aqueous solution is always the ideal medium for enzyme activity.[52] To lower the amount of available water, water-miscible organic co-solvents can be added, which often also promote the solubility of *N*- and *C*-terminally protected starting compounds.[53] Furthermore, the dielectric constant of the medium is reduced[54] and thus the basicity of the amino group of the nucleophilic amine is also reduced.[55-56] Another option is the use of biphasic aqueous-organic systems, which leads to preservation of enzyme activity (in the water phase) and allows simple product separation.[57-60] These advantages are counteracted by prolonged reaction times due to mass transport between the two phases. The general use of biphasic systems is mostly limited by the solubility of the starting components in the nonpolar organic phase. The ultimate way of preventing undesired hydrolytic side reactions in the course of peptide synthesis is offered by monophasic organic solvents.[61] The coupling reactions can often be driven to completion, with minimal hydrolysis of the peptide products or starting compounds, which results not only in higher yields but also in an easier purification. Often, small amounts of water, between 1 to 5 vol%, are required to maintain the catalytic activity of the enzyme. Besides organic solvents, the enzymatic synthesis of peptides has also been described in supercritical CO_2 and in ionic liquids.[62-65] However, only a few proteases are known which are stable and active enough in

pure organic solvents.[66] Often large amounts of enzyme and a large excess of one of the coupling partners is required to achieve a satisfying conversion.

1.3.4.2 *Immobilization*

To increase its activity and stability in organic solvents, immobilization of the protease can be applied.[67-69] The efforts made to immobilize an enzyme are mostly compensated by the possibility of its repeated use and by an easier work-up of the reaction mixture. The protease is covalently linked or physically adsorbed to an insoluble gel or resin, or a combination of these. Another option is precipitation of the enzyme followed by cross-linking, *e.g*. of the amino groups using glutaraldehyde to obtain so called cross-linked enzyme aggregates (CLEAs).[70] For instance CLEAs of the protease Subtilisin A (Alcalase-CLEA) have been applied for synthesis reactions in neat organic solvents and showed good stability compared to the native enzyme.[71]

1.3.4.3 *Enzyme engineering*

Another option to improve the enzyme is by genetic engineering, which comprises a range of techniques from chemical modification to remodeling a wild-type enzyme by gene technology.[72-73] For instance, so-called peptide ligases (having an improved synthesis/hydrolysis ratio) can be generated by the conversion of the active site serine into a cysteine, via site-directed mutagenesis or via chemical modification.[74-75]

1.3.4.4 *Substrate engineering*

If undesired side reactions occur during kinetically controlled peptide synthesis, such as transamidation or hydrolysis within the peptide fragments or end product, the specificity of the protease for the acyl donor ester is not significantly higher than its specificity for the peptide product or the starting fragments. Since the sequence of the starting components cannot be changed, another option, besides using anhydrous reaction conditions to suppress such competitive reactions is to use highly activated esters as the acyl donor.[76] It was shown for a number of protease catalyzed reactions, that esters containing strong electron withdrawing groups are superior to the commonly used methyl, ethyl or benzyl esters. For instance, good results have been obtained with the highly activated 2,2,2-trifluoroethyl (Tfe),[77] acetoxime[78] and cyanomethyl[79] esters, as shown in Figure 6.

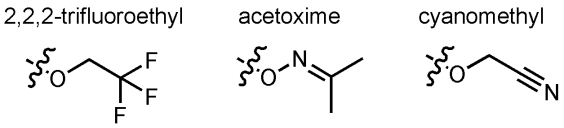


Figure 6. Strong electron withdrawing moieties result in highly activated esters.

A drawback of highly activated peptide *C*-terminal esters is that they are rather difficult to synthesize chemically without the risk of partial racemization.

Another option is to replace the peptide amide bond in the backbone, which is functionally cleaved by an endoprotease (Figure 7, **A**), by an ester bond (Figure 7, **B**). In this way, an additional recognition area for the S'-pockets is created and thereby the affinity for the peptide esters is increased.

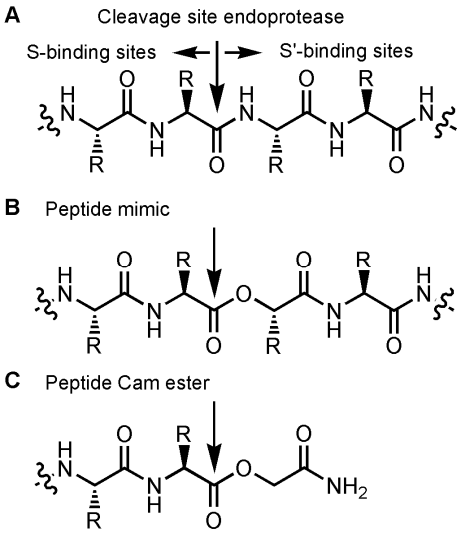


Figure 7. Schematic representation of subsite-substrate interactions of a serine endoprotease.

The specific amino acid residues present in the leaving group can be optimized for the protease of choice. An example of a leaving group in this respect is the carbamoylmethyl ester, which essentially is a mimic of glycine amide (Figure 7, **C**).[80]

A third option to suppress competitive reactions is the use of “substrate mimetics”. The interactions of the S’-binding sites of proteases are often less specific than of the S-binding sites, especially those of the primary S_1 pocket. In other words, proteases often cleave a peptide after a specific amino acid residue. Therefore, esters have been developed which mimic the side chain of the S_1 binding amino acid,[81-84] such as the guanidinophenyl ester which mimics the side chain of arginine,[85-86] as shown in Figure 8.

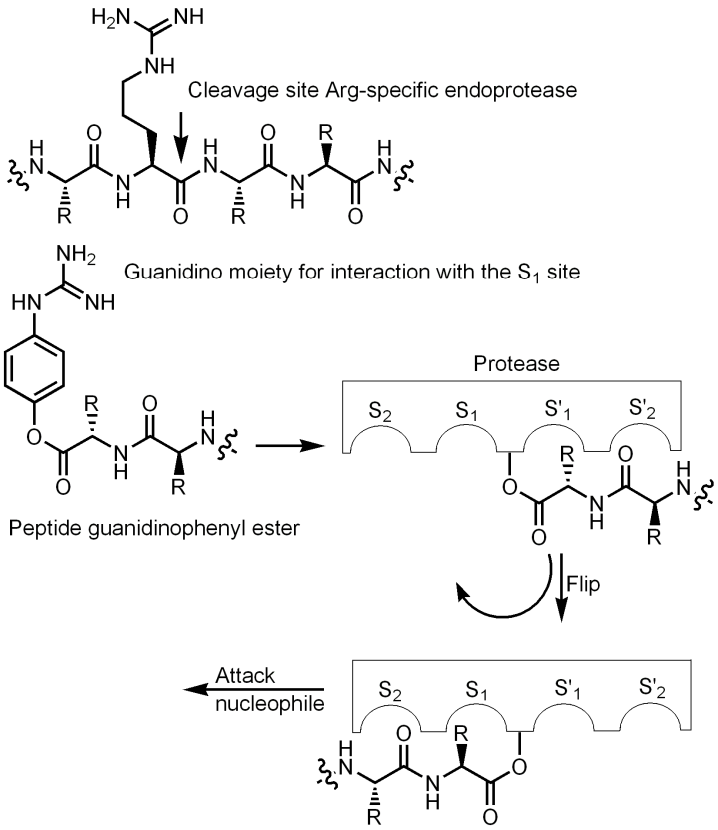
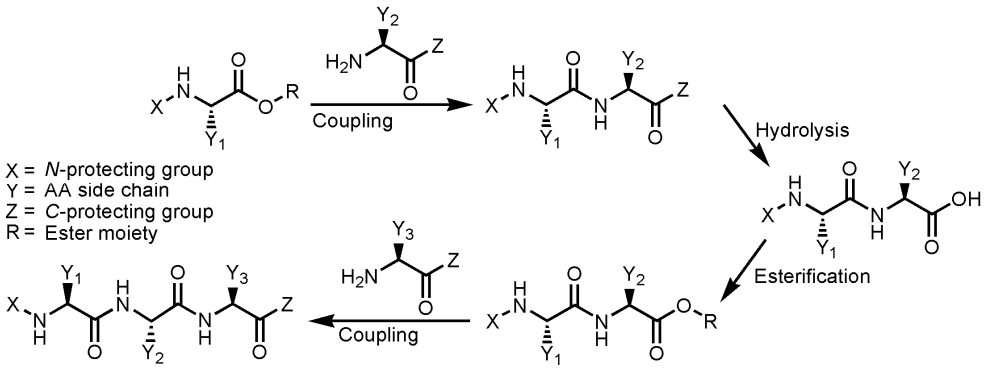


Figure 8. The guanidinophenyl ester or "substrate mimetic" and the peptide "flip".

According to Bordusa *et. al.*,[81] the substrate mimetics form an acyl-enzyme complex with the peptide guanidinophenyl ester directed in the reversed binding orientation. After cleavage of guanidinophenol, the peptide "flips" back to the normal substrate binding orientation followed by a nucleophilic attack of the other peptide fragment (amine component). The great advantage of substrate mimetics is that the newly synthesized peptide bond is not hydrolyzed since it does not contain the specific amino acid residue which is recognized by the protease (*e.g.* arginine). If this specific amino acid is not present in the peptide sequence of interest, almost quantitative product yield can be obtained. The major drawback of such substrate mimetics is that they are difficult to synthesize chemically without substantial racemization.

1.3.5 *Stepwise chemo-enzymatic peptide synthesis*

In contrast to solution phase stepwise chemical peptide synthesis, stepwise (chemo-) enzymatic peptide synthesis can be performed in the *C* → *N* terminal direction as well as in the *N* → *C* terminal direction.[87]



Scheme 3. *N* → *C*-directed (chemo-)enzymatic peptide synthesis.

The *N* → *C* directed synthesis (see Scheme 3), which is chemically not feasible due to racemization, is preferred due to the cheaper amino acid building blocks. Furthermore, the nucleophile is most often used in an excess, which is here a cheap amino acid building block, instead of the growing peptide in the case of the *C* → *N* synthesis approach. Because proteases are very selective for the α-amino and carboxy groups, minimal to no side chain protection of the amino acid building blocks is required. After the peptide coupling, a selective removal of the *C*-terminal protecting group should be at hand.
A drawback of stepwise enzymatic peptide synthesis in the *N* → *C* terminal direction is that after *C*-deprotection, subsequent activation of the peptide *C*-terminal carboxylic acid is required for the next enzymatic elongation step. Chemical esterification of peptide *C*-terminal carboxylic acids often lacks selectivity, can cause racemization or requires too harsh conditions in case of labile substrates.[88] Although the enzymatic esterification of *N*-protected amino acid building blocks has been described extensively, only few reports can be found in the literature describing the *C*-terminal enzymatic esterification of peptides.[89]

1.3.6 *Fragment condensation*

The economically most attractive method for the enzymatic synthesis of *e.g.* an octamer peptide is not via the stepwise approach, but via fully convergent synthesis using peptide fragment condensation,[90-91] as shown in Figure 10.

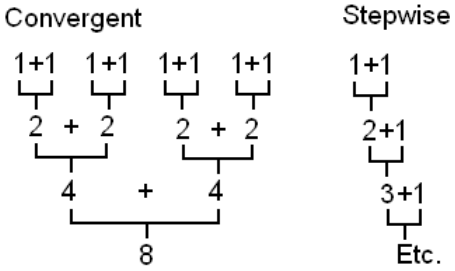


Figure 10. Convergent versus stepwise peptide synthesis.

Although the number of peptide couplings is equal in both strategies, the purification of the intermediates is much easier in case of the convergent synthesis approach, especially when the peptide length increases. Furthermore, *enzymatic* fragment condensation can be conveniently combined with classical chemical peptide synthesis, *e.g.* racemization-free solution phase synthesis of the dipeptide starting materials or SPPS of larger peptide fragments. Despite the huge potential of enzymatic peptide fragment condensation, there is only a limited number of examples reported in the literature.[92] Moreover, the reported methods lack general applicability and additionally these methods rely on condensation of side chain unprotected peptides in an aqueous reaction medium which inevitably leads to undesired hydrolysis.

Reaction schemes for enzymatic fragment condensation are often complicated. For instance, to obtain the peptide *C*-terminal activated ester, generally a fully protected peptide is synthesized by SPPS, followed by a chemical fragment condensation with a single amino acid activated ester. Subsequently, a selective side chain deprotection is required.[93] Finally, the enzymatic fragment condensation can be performed in aqueous solution followed by *N*- and *C*-terminal deprotection, as shown in Figure 11.

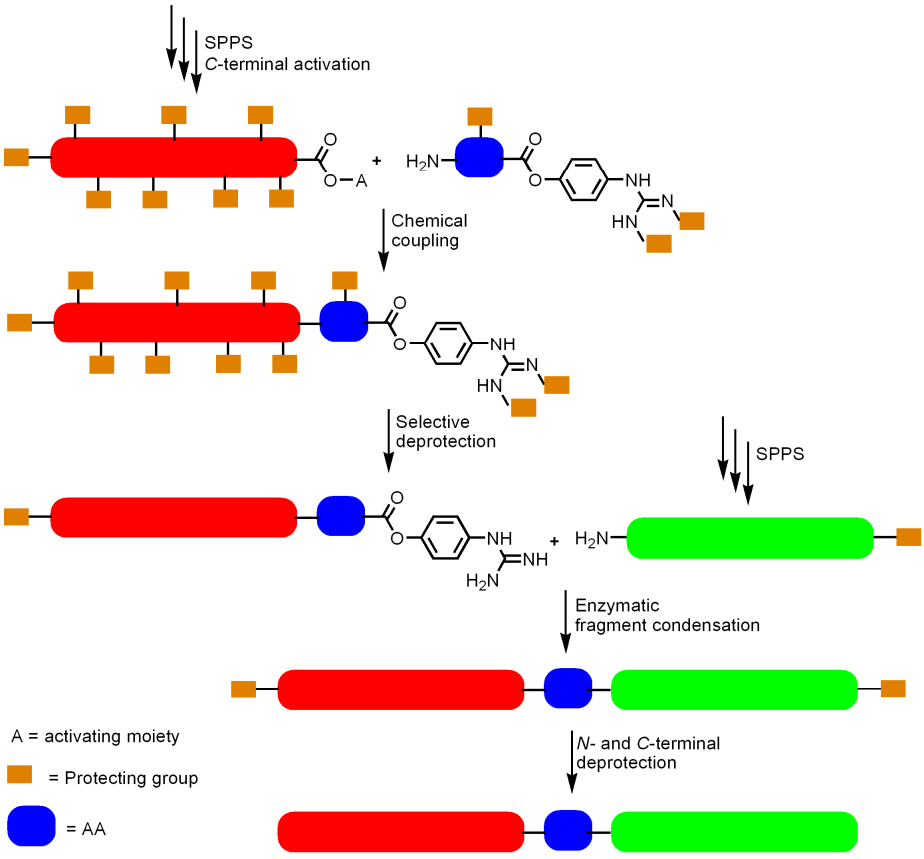


Figure 11. Overview of peptide *C*-terminal guanidinophenyl ester synthesis followed by enzymatic fragment condensation.

Despite the limitations of enzymatic peptide synthesis and the fact that chemical methods are most popular for the synthesis of peptides, a large number of papers has been published in recent decades dealing with enzymatic peptide synthesis, enzymatic manipulation of protecting groups and enzymatic modification of peptides. Some examples of which will be discussed in more detail in the next paragraph.

1.3.7 *Examples of enzymatic peptide synthesis*

A classical example of a kinetically controlled synthesis starting from the *N*-terminus using the protease CPD-Y for all coupling steps is the synthesis of [Met]enkephalin (Tyr-Gly-Gly-Phe-Met).[94] Bz-Arg-OEt was coupled with H-Tyr-NH_2 giving Bz-Arg-Tyr-NH_2 in 85% yield. The CPD-Y-catalyzed deamidation provided Bz-Arg-Tyr-OH in 90% yield. After chemical esterification with EtOH/HCl, the resulting dipeptide Bz-Arg-Tyr-OEt was coupled with H-Gly-OEt to furnish the protected tripeptide derivative (yield: 60%), followed by the successive enzymatic coupling of the other amino acid derivatives in the same manner. Amino acid amides were preferred as the amine components, since amino acid carboxylates only give low yields and amino acid esters give rise to side reactions that are difficult to control. Finally, the *N*-terminal Bz-Arg moiety was easily removed with Trypsin.

A second stepwise peptide synthesis in the *N* → *C*-terminal direction was described for the model tetrapeptide H-Lys-Tyr-Arg-Ser-OH using Clostripain and Chymotrypsin (Figure 12). The synthesis was performed in aqueous solution without side chain protection for all the trifunctional building blocks and the only non-enzymatic reaction was the final catalytic hydrogenation for cleavage of the *N*- and *C*-terminal protecting groups.

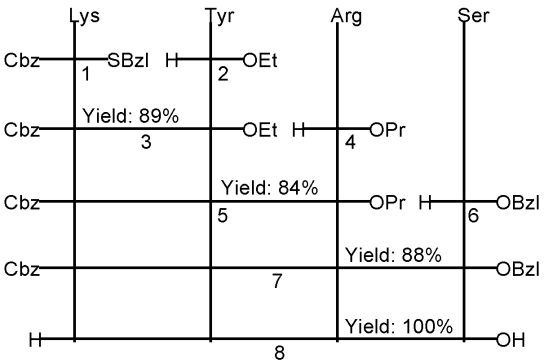


Figure 12. Synthesis of H-Lys-Tyr-Arg-Ser-OH from N- to C-terminus. 1 + 2 and 5 + 6, Clostripain; 3 + 4, Chymotrypsin; 7 → 8, catalytic hydrogenation.

Another typical example using enzymatic stepwise synthesis in combination with enzymatic fragment condensation is the synthesis of the *tert*-butyl ester of Leu-enkephalin[95] using both thermodynamically and kinetically controlled coupling steps. In order to obtain the unprotected Leu-enkephalin, the *C*-terminal protecting group was cleaved by chemical means, as shown in Figure 13.

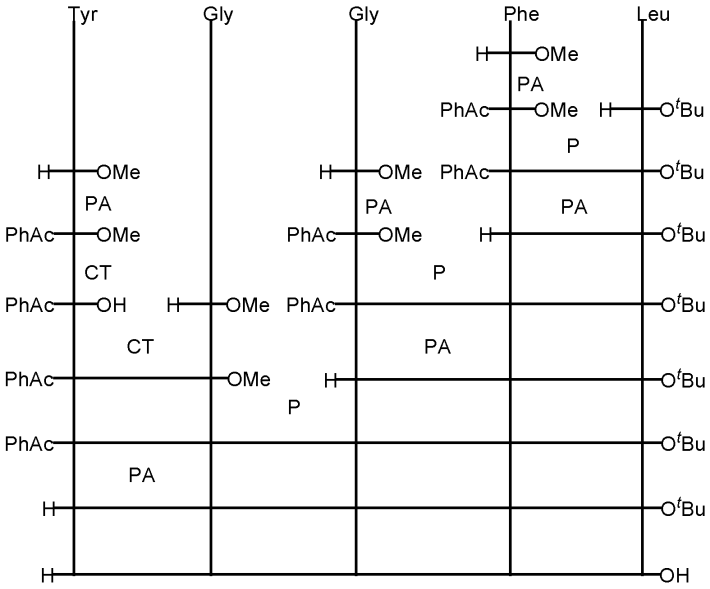


Figure 13. Enzymatic synthesis of [Leu]enkephalin *tert*-butyl ester. PA, Penicillin-G acylase; CT, Chymotrypsin; P, Papain; PhAc, phenylacetyl.

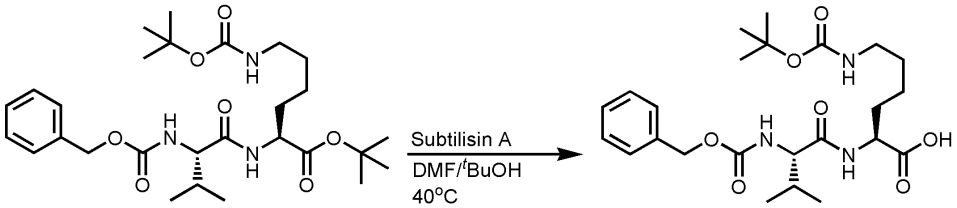
Besides these examples, a number of other peptides has been synthesized via (chemo-) enzymatic approaches using stepwise synthesis, or fragment condensation or a combination thereof.[96-135] Although in principle it is possible to perform fully enzymatic synthesis of peptides, in practice a combination of chemical and enzymatic steps is preferred. For chemo-enzymatic synthesis of peptides and even small proteins, the optimal approach is usually chemical synthesis of fragments for enzymatic conjugation in an overall convergent strategy. Herein, it is necessary to divide the total sequence into segments containing favorable combinations of amino acids which permit an protease catalyzed segment coupling, according to the elucidated S'-subsite specificity. However, until now, (chemo)enzymatic peptide synthesis is not commonly applied on industrial scale since the methods lack general applicability, the synthesis of the peptide *C*-terminal ester starting materials is complicated and the condensations are performed in an aqueous environment, which inevitably leads to undesired hydrolysis and thus high purification costs.

1.3.8 Enzymatic modification of amino acids and peptides

Besides peptide bond formation, also the protection, deprotection and (α-*C*-terminal) modification reactions of amino acids and peptides mediated by enzymes has similar advantages over chemical conversions such as lack of racemization, the mild reaction conditions and the high stereo- and regio-specificity. For instance, chemical synthesis of highly activated esters as acyl donors requires the same toxic coupling reagents as used for chemical peptide bond formation.[136] Another example is the deprotection of *tert*-butyl groups using trifluoroacetic acid.[137] Furthermore, biologically active peptides are often decorated at specific positions with *e.g.* fatty acids, polyethyleneglycol chains or arylamide groups, of which the chemical introduction usually requires complicated protection/deprotection

strategies. Orthogonality of protecting groups is pivotal for robust and reliable synthetic strategies.

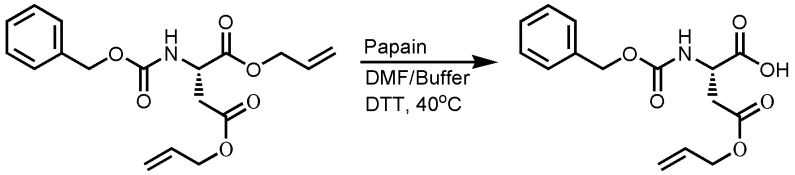
The selective enzymatic cleavage of *N*- and *C*-terminal protecting groups is one of the oldest applications in this respect, and the easiest to perform. Enzymatic hydrolysis of peptide *N*-terminal protecting groups includes, among others, the deprotection of acetyl, formyl, phenylacetyl, phthaloyl, *tert*-butoxycarbonyl and benzyloxycarbonyl groups.[138] Enzymatic hydrolysis of amino acid and peptide *C*-terminal protecting groups[138] includes the deprotection of methyl, benzyl, allyl, and primary amidegroups.[139] A more recent example is the enzymatic hydrolysis of peptide *C*-terminal *tert*-butyl esters using the serine protease Subtilisin A, as shown in Scheme 4.[140]



Scheme 4. Subtilisin A catalyzed hydrolysis of peptide *C*-terminal *tert*-butyl esters

Despite these reported results, protecting group hydrolysis is often accompanied with undesired hydrolysis of peptide bonds, especially when longer peptides are used. Enzymes lacking the ability to cleave peptide bonds are often very selective and as a consequence only applicable for a very small number of peptide sequences.

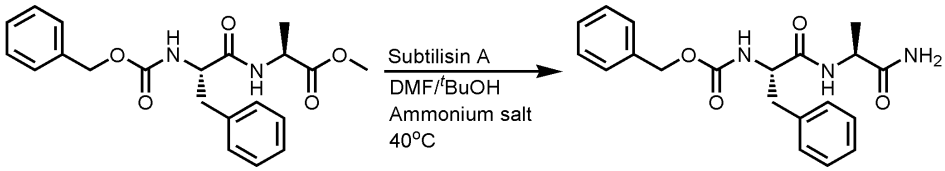
When single amino acid starting materials are used, hydrolytic side reactions are of less importance. Many examples are known for the resolution of racemic mixtures of amino acid esters wherein the ester of the L-amino acid enantiomer is selectively hydrolyzed (kinetic resolution).[141] Another example is the selective hydrolysis of the α-ester of Cbz-Asp(OAll)-OAll using Papain, as shown in Scheme 5.[142]



Scheme 5. Papain catalyzed hydrolysis of peptide Cbz-Asp(OAll)-OAll

The product, Cbz-Asp(OAll)-OH, is a versatile building block for peptide synthesis, whereas chemical discrimination between the two carboxylic acid functionalities is almost impossible. Unfortunately, Papain does not allow the use of other *N*-terminal protecting groups.

A variety of *N*- and *C*-terminal protecting groups[138] can also be enzymatically introduced. Recently, the synthesis of peptide *C*-terminal amides from the corresponding peptide *C*-terminal methyl esters using Subtilisin A was described (Scheme 6).[143]



Scheme 6. Subtilisin A catalyzed synthesis of peptide *C*-terminal carboxamides

The *C*-terminal amide functionality is not only important for the biological activity of many peptides, but also increases their stability against exoproteases. Unfortunately, however, the synthetic strategies generally used to obtain the peptide *C*-terminal methyl ester starting materials have almost the same disadvantages as the direct chemical synthesis of the *C*-terminal amide functionality. Furthermore, the reaction proceeds rather slowly, which results in undesired side reactions such as transamidation of the peptide backbone.

Besides protection and deprotection, a number of important enzymatic *C*-terminal modification reactions of amino acids and peptides has been described, such as the synthesis of amino acid and peptide *C*-terminal thioacids and thioesters using Subtiligase.[144-145] Peptide *C*-terminal thioesters are the pivotal building blocks for protein synthesis using native chemical ligation[146] and are notoriously difficult to synthesize chemically. Therefore, this ligation strategy is still not applied on industrial scale. The enzymatic thioesterification could be a breakthrough, but, Subtiligase is not commercially available and its industrial application is hindered since its use is restricted by several patents.[147]

Furthermore, several enzymes are able to PEGylate,[148] glycosylate[149] or acylate[150] peptides (with fatty acids) on specific sites giving them the desired biological activity. Additionally, there are many modifications of peptides which stabilize them against protease activity. One of the most important examples is via head to tail cyclization, which can be performed mildly by enzymes.[151] Other stabilizing modifications[152] include methylation, halogenation, and oxidation. A major drawback of enzymatic modification of peptides is the lack of general applicability, *e.g.* many enzymes can only glycosylate the threonine residue at certain positions within a peptide. Furthermore, most enzymatic peptide modifications give low yields. Therefore, there remains a need for robust, broadly applicable, high yielding and economically attractive enzymatic methods for the protection, deprotection and modification of amino acids and peptides.

1.4 Outline of this thesis

This thesis describes the synthesis of peptides and amino acid derivatives by using enzymes, in particular the protease Subtilisin A (Alcalase), in anhydrous organic solvents. The study entails applications towards the orthogonal protection of amino acids (chapters 2 and 3), the *C*-terminal derivatization of peptides (chapters 4 and 5), the interconversion of peptide *C*-terminal protecting groups (chapters 6 and 7), and finally, the synthesis of peptides (chapters 8, 9 and 10), as summarized in Figure 14. Finally, a summary, discussion and some future perspectives of enzymatic peptide synthesis are given (chapter 11).

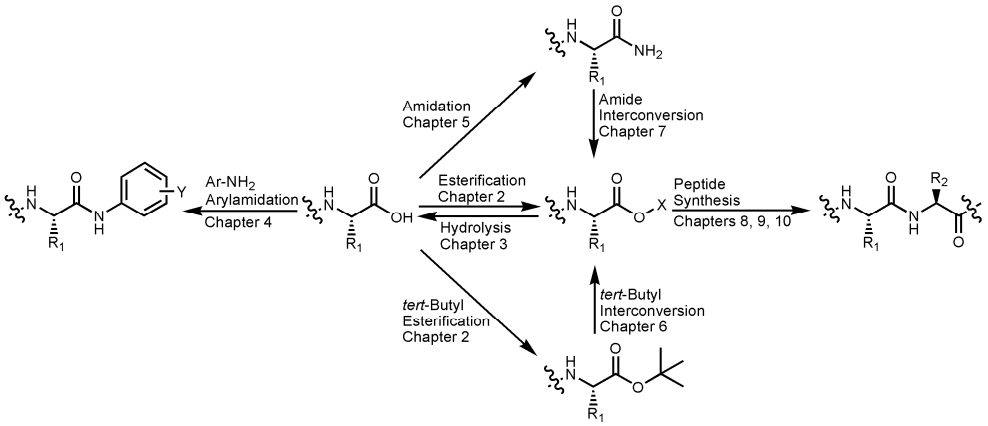


Figure 14. Schematic overview of the investigated reactions

In **Chapter 2**[153] a novel method for the synthesis of amino acid and peptide *C*-terminal α-carboxylic esters is described. Using Subtilisin A and the appropriate alcohol, the synthesis of methyl, ethyl, benzyl, allyl, trimethylsilylethyl, and *tert*-butyl esters of *N*-terminal protected amino acids and peptides is shown. Furthermore, this selective α-esterification is applied to various amino acids, containing unprotected side chain functionalities such as β/γ-carboxyl (Asp/Glu) and hydroxyl (Ser) groups. Moreover, it is demonstrated that side chain protected peptides are accepted as substrates and can be smoothly converted into the corresponding *C*-terminal *tert*-butyl esters.

Chapter 3[154] describes two versatile, high yielding and efficient chemo-enzymatic approaches for the synthesis of β-protected Asp and γ-protected Glu derivatives. The first method is based on the α-selective Subtilisin A catalyzed hydrolysis of aspartic acid and glutamic acid diesters. The second method comprises a three step protocol using i) α-selective enzymatic methyl esterification as described in Chapter 2, ii) chemical β-esterification, and finally iii) α-selective enzymatic methyl ester hydrolysis.

Chapter 4[155] reports on the Subtilisin A mediated synthesis of *C*-terminal arylamides of amino acids and peptides from the corresponding *C*-terminal carboxylic acids, methyl or benzyl esters, in high chemical, and enantiomeric or diastereomeric purity. It is demonstrated that complete *C*-terminal α-carboxylic acid selectivity can be obtained in the presence of various unprotected side chain functionalities such as β/γ-carboxyl, hydroxyl and guanidino groups. Also by applying the cysteine protease Papain and the *Candida antarctica* lipase B to the preparation of amino acid *C*-terminal anilides is demonstrated. It is proven that the chemo-enzymatic synthesis of arylamides is completely free of racemization, in contrast to the state-of-the-art chemical methods.

In **Chapter 5**,[156] a high yielding enzymatic approach for the conversion of semi-protected amino acid and peptide *C*-terminal α-carboxylic acids into the corresponding carboxamides is revealed, using *Candida antarctica* lipase-B or Subtilisin A. It is shown that by application of the ammonium salt of the α-carboxylic acid rather than a separate ammonia source, the

enzymatic amidation reaction proceeds much faster while transamidation or hydrolysis are almost completely absent, in contrast to common literature procedures.

In **Chapter 6**,[157] a novel Subtilisin A catalyzed interconversion of *C*-terminally protected peptide α-*tert*-butyl esters into primary alkyl esters is described. The obtained primary alkyl esters can be directly used in the next enzymatic peptide elongation step with another amino acid *tert*-butyl ester building block. This fully enzymatic *N* → *C* elongation strategy by *C*-terminal *tert*-butyl ester interconversion is applied towards the synthesis of three biologically active peptides up to the pentamer level.

Chapter 7[158] discloses a novel Subtilisin A mediated interconversion reaction of peptide *C*-terminal α-carboxamides into primary alkyl esters yielding another, fully enzymatic, peptide synthesis strategy. Amino acid α-carboxamides are used as building blocks for peptide elongation and the obtained peptide *C*-terminal carboxamides are interconverted to the corresponding primary alkyl esters, which are enzymaticaly condensed with another amino acid amide building block. This fully enzymatic *N* → *C* elongation strategy is applied towards the synthesis of two biologically active peptides.

Chapter 8[159] demonstrates that carboxamidomethyl or trifluoroethyl esters are very useful in Subtilisin A mediated peptide synthesis in the case of sterically demanding and non-proteinogenic amino acids as acyl donors, as well as poor nucleophiles, and combinations thereof. Furthermore, these esters can be efficiently synthesized by using *Candida antarctica* lipase B or Subtilisin A. Finally, it is shown that ester synthesis by *Candida antarctica* lipase B and subsequent peptide synthesis by Subtilisin A can be performed simultaneously using a two-enzyme-one-pot approach with glycolamide or 2,2,2-trifluoroethanol as additive.

In **Chapter 9**,[160] a series of novel glycine esters is evaluated for their efficiency in Subtilisin A catalyzed peptide synthesis. The reactivity of the easily accessible carboxamidomethyl ester is further enhanced by elongating it with an amino acid residue and thereby creating more recognition sites for Subtilisin A.

Chapter 10[161] describes the enzymatic condensation of side chain protected peptide fragments using Subtilisin A. A screening with dipeptide Cbz-Val-Xxx carboxamidomethyl esters with H-Xxx-Val-NH_2 nucleophiles is performed, wherein Xxx stands for every (side chain protected) amino acid residue, to investigate the scope and limitations of the enzymatic fragment condensation strategy. Finally, it is demonstrated that it is feasible to enzymatically condense larger peptide fragments (up to the 10-mer level) bearing multiple side chain protecting groups with very high conversion.

In **Chapter 11**, a summary of this thesis is given, as well as a discussion of the results and some future perspectives of enzymatic peptide synthesis.

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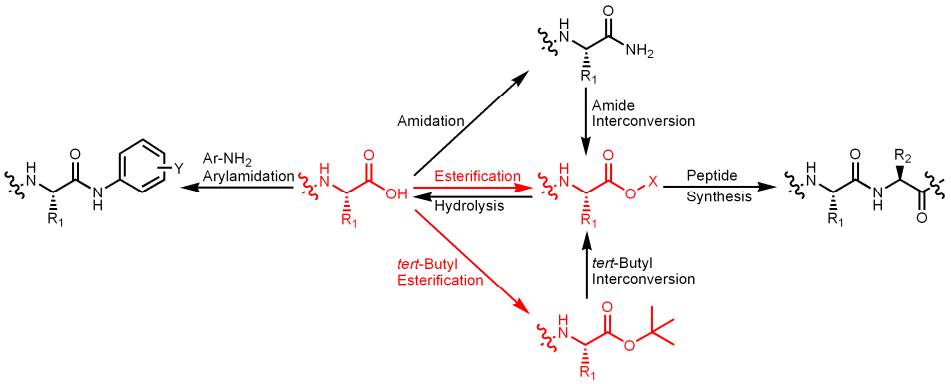
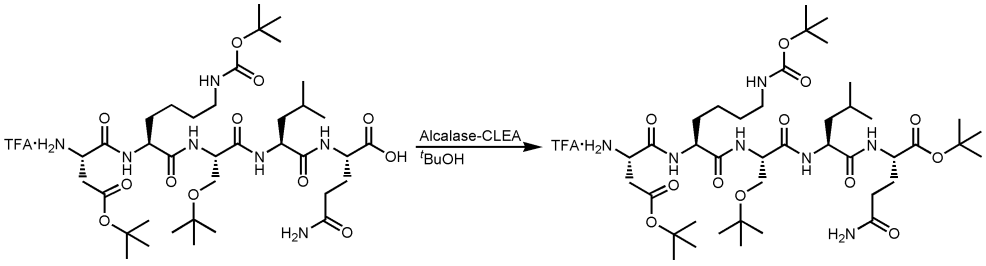
Chapter 2

Enzymatic *C*-terminal α-esterification of amino acids and peptides



In this chapter, a mild chemo-enzymatic method for the synthesis of amino acid and peptide *C*-terminal α-carboxylic esters using Subtilisin A is described. *C*-Terminal α-carboxylic acid methyl, ethyl, benzyl, allyl, trimethylsilylethyl, and *tert*-butyl esters of *N*-terminally protected amino acids and peptides could be synthesized, even in the presence of various unprotected side chain functionalities such as β/γ-carboxyl and hydroxyl groups. Besides this, side chain protected peptides were accepted by Subtilisin A as substrates and smoothly converted to their corresponding *C*-terminal *tert*-butyl esters. The isolated yield ranged between 78% and 92%.



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2.1 Introduction

2

There is a great demand for easily accessible amino acid and peptide building blocks for their use in peptide synthesis. For different applications, a large variety of side chain, α-amino and/or α-carboxylic acid protected amino acids and *C*-terminal protected peptides is required. Orthogonality of the different protecting groups used is pivotal for conventional peptide synthesis protocols. Whereas the α-amino functions are most often protected as carbamates, the α-carboxylic acid functions are most often protected as esters. Esters can be synthesized by mineral acid catalysis using for instance HCl or H_2SO_4 in the presence of the (unprotected) amino acid and the corresponding alcohol. However, this method is restricted to *n*-alkyl esters such as methyl (Me), ethyl (Et) or benzyl (Bn) and for a subset of amino acids. More difficult to synthesize, but very commonly used as protecting groups in Fmoc- or Boc-based solid phase peptide synthesis, are the orthogonal esters such as the *tert*-butyl (^{t}Bu), allyl (All) or trimethylsilylethyl (TMSE) esters. TMSE esters, cleavable with TBAF,[1] and All esters, cleavable with Pd(0),[2] are orthogonal to other commonly used carboxylic acid protecting groups allowing selective deprotection and modification of certain carboxylic acids during peptide synthesis. Of special interest are α-carboxyl protected aspartic acid (Asp) or glutamic acid (Glu) building blocks, since these amino acids also contain a β- or γ-carboxyl moiety, respectively. These selectively protected esters are used for (Fmoc- and Boc-based) on-resin synthesis of head-to-tail cyclic peptides,[3] side chain to side chain lactam peptides[4] and branched peptides.[5]

Besides amino acid building blocks, another interesting group are *C*-terminal (and side chain) protected peptides with a free amine functionality, which can be used for peptide fragment condensation. Side chain protected peptides are relatively easy to synthesize via solid phase peptide synthesis (SPPS) techniques. *C*-terminal protection, however, for instance with a ^{t}Bu group remains very challenging.

In chemo-enzymatic peptide synthesis, *C*-terminal α-carboxylic acid esters are not only used for protection but also, and more importantly, for activation in case of a kinetically controlled process. Most often, non-sterically hindered esters, among others Me, Et or Bn derivatives, are used as activated substrates.[6]

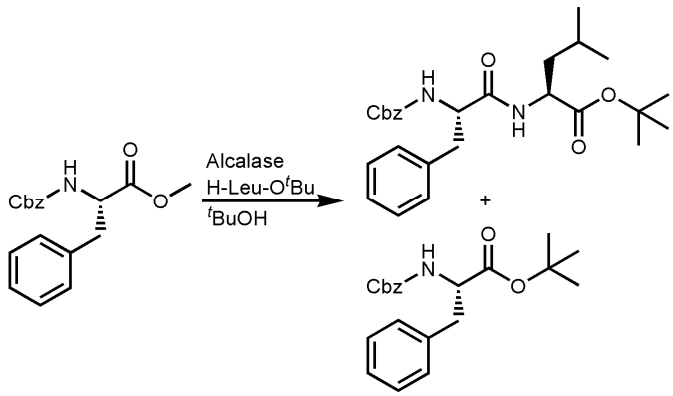
Chemical synthesis of peptide or amino acid esters of which the ester moiety is sterically demanding often requires harsh reaction conditions which may be a limitation in case of sensitive substrates. For instance, ^{t}Bu esters are often synthesized using a strong mineral acid with isobutene under high pressure,[7] which is laborious and incompatible with other acid labile protecting groups. Furthermore, the selective chemical α-carboxyl protection of Asp and Glu derivatives is difficult due to the comparable reactivity of the β- and γ-carboxyl functionality. Although several one step methods have been described for (semi-)selective side chain esterification,[8] α-carboxylic acid protection of Asp and Glu usually needs multi-step and/or low yielding protocols. For instance, the cyclic anhydride method[9] and the chloroformate method[10] both give approximately 3:2 mixtures of α-carboxy versus β/γ-carboxy esterification.

To overcome the selectivity issue and to avoid racemization, as is often encountered in chemical esterification, *N*-protected amino acids and peptides have been esterified using enzymes.[11] However, the amount of water that is liberated during the reaction and the water

required for enzyme activity prohibits a favorable position of the esterification equilibrium resulting in low to moderate yieds.[12] High yields were reported using esterases or lipases in dry organic solvents, but these enzymes are usually not selective for the α-carboxylic acid moiety. Proteases such as papain[13], chymotrypsin[14] and α-chymotrypsin[15] are α-selective but only have demonstrated esterification with sterically undemanding alcohols such as methanol, ethanol or benzylalcohol. Esterification of amino acids to aliphatic esters with Subtilisin A has, to our knowledge, only been described for Boc-Phe-OEt (65% in 48h).
This chapter describes the application of the cheap industrial protease Subtilisin A[16] (available from Novozymes as Alcalase in which Subtilisin A is the main constituent, 10 wt% solution, 6.75 €/kg) to catalyze the esterification of a wide range of *N*-protected proteinogenic, as well as non-proteinogenic amino acids, and also peptides in organic solvents at low water content with a high selectivity toward the *C*-terminal α-carboxylic acid functionality.

2.2 Results and Discussion

During an investigation of the Alcalase catalyzed peptide coupling between Cbz-Phe-OMe and H-Leu-O^{t}Bu in ^{t}BuOH at low water content,[17] a small percentage of by-product (Scheme 1) namely, Cbz-Phe-O^{t}Bu was found. LC/MS and NMR analysis proved this by-product to be the ^{t}Bu ester of Cbz-Phe-OH.

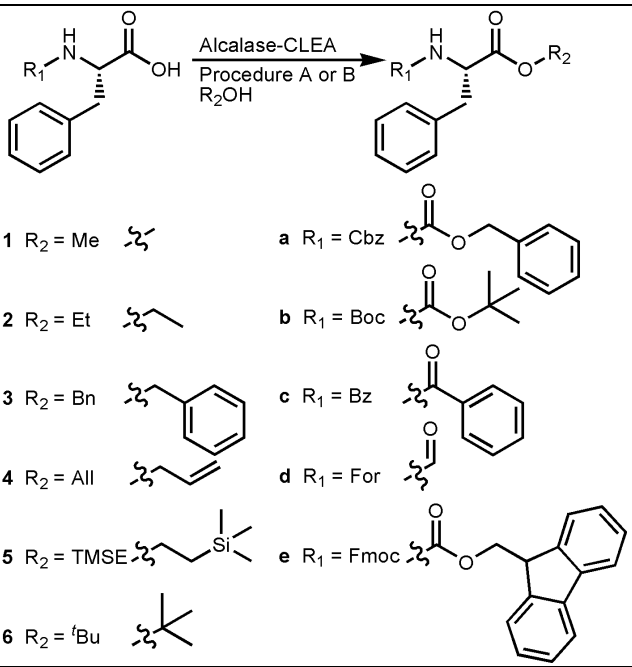


Scheme 1. Alcalase-catalyzed peptide coupling and transesterification side reaction.

To the best of our knowledge, this transesterification towards a ^{t}Bu ester had never been reported before. More surprisingly however, ^{t}BuOH is the solvent of choice for protease-catalyzed peptide synthesis, and enzymes tend to have a very good stability in this solvent.[18]
Further investigation of the ^{t}Bu ester formation showed that, in the absence of the amino acid nucleophile H-Leu-O^{t}Bu, in neat ^{t}BuOH 75% of the ^{t}Bu ester could be obtained. Upon addition of 4 Å molecular sieves, 96% of the Me ester was converted to the ^{t}Bu ester (after 48 h at 50°C), indicating that the amount of water and methanol was critical for the reaction outcome. Most strikingly, equally high yield could be obtained starting from the free

carboxylic acid Cbz-Phe-OH (96% after 16 h at 50°C) using 3 Å molecular sieves whereas in the absence of molecular sieves only a low yield (37% after 48 h) was obtained. Apparently, continuous removal of water shifts the equilibrium towards product formation, while the enzyme remains active. It was therefore decided to investigate the scope of the methodology using *N*-terminal protected phenylalanine (Phe) as the model substrate (Table 1). Alcalase cross-linked enzyme aggregates (CLEAs)[19] were used since it could be easily dried and recovered after the reaction by filtration. Due to the well-known inactivation of Alcalase by methanol and polar aprotic solvents (such as DMSO and DMF), apolar co-solvents were used (*i.e. n*-heptane, toluene or methyl *tert*-butyl ether (MTBE)) in the esterification reactions. All reported esterification reactions with primary alcohols were performed using a mixture of the appropriate alcohol with MTBE (1:14, v:v, respectively).

Table 1. Esterification of various *N*-protected Phe derivatives.



Entry[a]	Product		Yield (%)[b]	Procedure
1	Cbz-Phe-OMe	**1a**	83	A
2	Cbz-Phe-OEt	**2a**	85	A
3	Cbz-Phe-OBn	**3a**	90	A
4	Cbz-Phe-OAll	**4a**	86	A
5	Cbz-Phe-OTMSE	**5a**	87	A
6	Cbz-Phe-O^{t}Bu	**6a**	85	B
7	Boc-Phe-O^{t}Bu	**6b**	85	B
8	Bz-Phe-O^{t}Bu	**6c**	90	B
9	For-Phe-O^{t}Bu	**6d**	92	B
10	Fmoc-Phe-O^{t}Bu	**6e**	88	B

[a] Conditions: Alcalase-CLEA, R_2OH/MTBE (1/14, v/v), 3 Å molecular sieves, 50°C, 16 h (Procedure A); Alcalase-CLEA, ^{t}BuOH, 3 Å molecular sieves, 50°C, 16 h (Procedure B); [b] Isolated yield based on the acyl donor starting material.

As shown in Table 1, Cbz-protected Phe was smoothly converted into a variety of esters in very high yield (**1a-6a**). The choice of *N*-terminal protection (**6a-e**) did not influence the esterification reaction.

As is known from the literature, Alcalase accepts a wide variety of proteinogenic (**7a-e**) and non-proteinogenic (**7f**) amino acids, which was also demonstrated for the ^{t}Bu ester synthesis (Table 2). Only amino acids with a tertiary β-carbon, *e.g.* threonine, isoleucine and valine were not efficiently accepted by Alcalase. The dipeptide Cbz-Phe-Leu-OH proved to be a good substrate for esterification reactions (**8-10**). Under the conditions used, no hydrolysis nor alcoholysis of the peptide bond was observed.

Table 2. Esterification of various amino acids and peptides.

Entry[a]	Product		Yield (%)[b]	Procedure
1	Cbz-Ala-O^{t}Bu	**7a**	86	B
2	Cbz-Leu-O^{t}Bu	**7b**	88	B
3	Cbz-Ser-O^{t}Bu	**7c**	72	B
4	Cbz-Met-O^{t}Bu	**7d**	82	B
5	Cbz-Lys(Cbz)-O^{t}Bu	**7e**	90	B
6	Cbz-DOPA-O^{t}Bu	**7f**	90	B
7	Cbz-Phe-Leu-OMe	**8**	88	A
8	Cbz-Phe-Leu-OBn	**9**	80	A
9	Cbz-Phe-Leu-O^{t}Bu	**10**	90	B

[a] Conditions: Alcalase-CLEA, ROH/MTBE (1/14, v/v), 3 Å molecular sieves, 50°C, 16 h (Procedure A); Alcalase-CLEA, ^{t}BuOH, 3 Å molecular sieves, 50°C, 16 h (Procedure B); [b] Isolated yield based on the acyl donor starting material.

Peptide *C*-terminal ^{t}Bu esters are highly versatile building blocks for chemical or enzymatic peptide fragment condensation, however, their chemical synthesis is rather difficult. For chemical fragment condensation, besides the *C*-terminal protection, all other (side chain) reactive groups should be protected to avoid undesired side reactions. Therefore, we tested the enzymatic ^{t}Bu esterification strategy was tested on peptides containing side chain protected hydroxyl, amine and carboxylic acid functionalities, as shown in Table 3. These protected peptides as starting materials, containing a free *C*-terminal carboxylic acid functionality, can be conveniently synthesized on the industrial applicable 2-chlorotritylchloride (CTC) resin using standard SPPS protocols.[20]

Table 3. ^{t}Bu esterification of two side chain protected pentapeptides using Alcalase.

Entry[a]	Starting material	^{t}Bu ester product		Yield (%)[b]
1	Fmoc-Asp(O^{t}Bu)-Lys(Boc)-Ser(^{t}Bu)-Leu-Gln-OH	Fmoc-Asp(O^{t}Bu)-Lys(Boc)-Ser(^{t}Bu)-Leu-Gln-**O^{t}Bu**	**11**	68
2	TFA.H-Asp(O^{t}Bu)-Lys(Boc)-Ser(^{t}Bu)-Leu-Gln-OH	TFA.H-Asp(O^{t}Bu)-Lys(Boc)-Ser(^{t}Bu)-Leu-Gln-**O^{t}Bu**	**12**	73

[a] Conditions: Alcalase-CLEA, DMF/^{t}BuOH (17.5/82.5, v/v), 3 Å molecular sieves, 37°C, 16 h (Procedure C); [b] Isolated yield based on the acyl donor starting material.

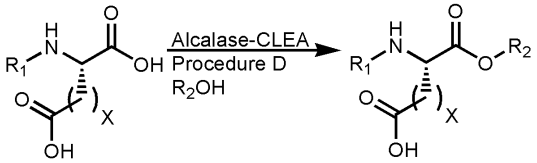
Gratifyingly, even side chain protected peptides were recognized by Alcalase and were smoothly converted to their corresponding ^{t}Bu esters in good yield. After esterification, the *N*-terminal Fmoc-protecting group (**11**) can be selectively removed using a base giving

access to the fully protected peptide with an *N*-terminal free amine, which can be used for the next coupling reaction. However, it was shown that temporary *N*-terminal protection is not necessary because the trifluoroacetate salt of the amine (**12**), which is formed after acidic cleavage of the peptide from the CTC resin using 2.5 vol% TFA, could be esterified while amide formation was absent.

Since the use of molecular sieves is inconvenient on large scale, azeotropic water removal was investigated. Gratifyingly, the ^{t}Bu esters were formed in high yield (>95%) when ^{t}BuOH, which forms an azeotrope with water, was continuously evaporated and dry ^{t}BuOH was simultaneously added to keep the reaction volume constant. Alcohols, which do not form an azeotrope with water, such as allylic alcohol and TMSEOH, could be combined with the azeotropic solvent toluene (1/14, v/v, respectively), under reduced pressure, to obtain the corresponding ester in a yield of up to 98%. The Alcalase-CLEA could be recycled with minimal activity loss: after filtration, new starting materials were added and only 1% activity loss occurred after five esterification cycles.

To investigate the α-carboxylic acid selectivity of Alcalase it was decided to apply the conditions as for Phe, on Asp and Glu derivatives with various *N*-protecting groups (Table 4).

Table 4. Esterification of various Asp and Glu derivatives.



13 R_1 = Cbz X = 1 **a** R_2 = Allyl
14 R_1 = Cbz X = 2 **b** R_2 = TMSE
15 R_1 = Boc X = 1
16 R_1 = Boc X = 2
17 R_1 = Fmoc X = 1
18 R_1 = Fmoc X = 2

Entry[a]	Product		Yield (%)[b]
1	Cbz-Asp-OAll	**13a**	84
2	Cbz-Asp-OTMSE	**13b**	86
3	Cbz-Glu-OAll	**14a**	88
4	Cbz-Glu-OTMSE	**14b**	88
5	Boc-Asp-OAll	**15a**	89
6	Boc-Asp-OTMSE	**15b**	88
7	Boc-Glu-OAll	**16a**	87
8	Boc-Glu-OTMSE	**16b**	85
9	Fmoc-Asp-OAll	**17a**	88
10	Fmoc-Asp-OTMSE	**17b**	85
11	Fmoc-Glu-OAll	**18a**	89
11	Fmoc-Glu-OTMSE	**18b**	86

[a] Conditions: Alcalase-CLEA, 3 Å molecular sieves, MTBE/R_2OH (14/1, v/v), 0.1 vol% HCOOH, 50°C, 16 h (Procedure D); [b] Isolated yield based on the acyl donor starting material.

Gratifyingly, all Asp and Glu derivatives were converted to their corresponding mono-esters (**13a-18b**) in very high yield with complete α-selectivity as was judged by HPLC and NMR analysis and by comparison with commercially available reference compounds or literature data.

In conclusion, in this chapter, a general and versatile method was described for the enzymatic α-carboxylic acid selective esterification of *N*-protected amino acids and peptides. In the presence of Alcalase, a variety of esters were obtained in very high to excellent yield. Even esters which are difficult to synthesize chemically, *e.g.* ^{t}Bu esters, were obtained in high yield. Furthermore, amino acids with an additional carboxylic acid functionality in the side chain, *e.g.* Asp and Glu, were esterified with a complete selectivity for the α-carboxylic acid moiety. The processes appeared amenable to scale-up since water formed during ester bond formation could also efficiently be removed by azeotropic distillation and the Alcalase-CLEA could be recycled with minimal loss of activity.

2.3 Experimental

General:

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. The 2-chlorotritylchloride resin, Fmoc-amino acids and reagents used for SPPS were purchased from GL Biochem (Shanghai, China) and all peptide grade solvents were from Biosolve (Valkenswaard, The Netherlands). Before use, Alcalase-CLEA was dried as follows: 3 g Alcalase-CLEA (CLEA technologies, 650 AGEU/g, 3.5 wt% H_2O), was suspended in 100 mL ^{t}BuOH and crushed with a spatula. After filtration, the enzyme was resuspended in 50 mL MTBE followed by filtration and the solid was dried for 1 min at ambient temperature. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm), DMSO-d_6 (2.50 ppm for ^{1}H or 39.9 ppm for ^{13}C) or $CDCl_3$ (77.0 ppm for ^{13}C). Thin layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} plates (Merck); spots were visualized using UV light, ninhydrin or $KMnO_4$/K_2CO_3 solution. Molecular sieves (3 Å, 8 to 12 mesh, Acros) were activated under reduced pressure at 200°C and ^{t}BuOH was stored on these activated molecular sieves. ^{t}BuOH was pre-heated to liquid (45°C) before use. Column chromatography was carried out using silica gel, Merck grade 9385 60 Å. Analytical HPLC was performed on an HP1090 Liquid Chromatograph, using a reversed-phase column (Inertsil ODS-3, C18, 5 μm particle size, 150 × 4.6 mm internal diameter) at 40°C. UV detection was performed at 220 nm using a UV-VIS 204 Linear spectrometer (Varian). The gradient program was: 0-25 min linear gradient from 5% to 98% eluent B and from 25.1-30 min 5% eluent B (eluent A: 0.5 mL/L methane sulfonic acid (MSA) in H_2O; eluent B: 0.5 mL/L MSA in CH_3CN). The flow was 1 mL/min from 0-25.1 min and 2 mL/min from 25.2-29.8 min, then back to 1 mL/min until stop at 30 min. Injection volumes were 20 μL. The flow-injection analysis (FIA) experiments to determine the exact mass were performed on an Agilent 1100 LC-MS system (Agilent, Waldbronn, Germany), which consists of a binary pump, degasser, autosampler, column oven, diode-array detector and a time-of-flight MS. The ESI-MS was run in positive ionization mode, with the following conditions: *m/z* 50-3200, 175 V fragmentor, 0.94 cycl/s, 350°C drying gas temperature, 10 L N_2/min drying gas, 45 psi

nebuliser pressure and 4 kV capillary voltage. The exact mass was determined using an internal reference (purine at 121.050873 Da and hexakis-(1*H*,1*H*,3*H*-tetrafluoro-pentoxy)phosphazene at 922.009798 Da) to recalibrate the m/z axis for each measurement, which were continuously injected into the electrospray ion source equipped with a dual-sprayer mechanism. The samples were directly introduced into the ESI by injection of 5 µL into the eluent flow of 0.5 mL/min (MeOH/H_2O, 1:1, v:v). Preparative HPLC was performed on a Varian PrepStar system using a stationary-phase column (Pursuit XRs, C18, 10 µm particle size, 500 × 41.4 mm internal diameter) at room temperature. UV detection was performed at 220 nm and 254 nm using a UV-VIS Varian ProStar spectrometer. The isocratic program was 85% eluent B and 15% eluent A (eluent A: 0.1 mL/L TFA in H_2O; eluent B: 0.1 mL/L TFA in CH_3CN) with a flow rate of 80 mL/min, injection volume of 10 mL and stoptime after 30 min. Pure fractions were pooled and lyophilized. Lyophilization was performed on a VaCo 5 (II) lyophilizer from Zirbus technologies.

General Procedure A: Enzymatic esterification with primary alkyl alcohols

Alcalase-CLEA (300 mg) and 3 Å molecular sieves (200 mg) were added to *N*-protected amino acid (1.67 mmol) which was dissolved in a mixture of MTBE (2.8 mL) and the appropriate alcohol (200 µL). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. After filtration, the solid enzyme particles were washed by resuspension in EtOAc and removed by filtration (20 mL, 3×). The combined organic filtrates were washed with sat. aq. $NaHCO_3$ (40 mL, 2×), 0.1 N HCl (40 mL, 2×), brine (40 mL), dried (Na_2SO_4), filtered over basic alumina and subsequently concentrated *in vacuo* and the residue was co-evaporated with toluene (20 mL, 2×) and $CHCl_3$ (20 mL, 2×). According to analytical HPLC analysis most compounds proved >95% pure. Additional preparative HPLC was required for Cbz-Phe-OBn (**3**) and Cbz-Phe-Leu-OBn **(9).**

General Procedure B: Enzymatic esterification with ^{t}BuOH of amino acids

Alcalase-CLEA (300 mg) was added to a solution of *N*-protected amino acid (1.67 mmol) in ^{t}BuOH (neat, 3.0 mL) in the presence 3 Å molecular sieves (200 mg). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. After filtration, the solid enzyme particles were washed by resuspension in EtOAc followed by filtration (20 mL, 3×). The combined organic layers were concentrated *in vacuo* and the resulting oil was redissolved in EtOAc (50 mL). This solution was washed with sat. aq. $NaHCO_3$ solution (40 mL, 2×), 0.1 N HCl (40 mL, 2×) and brine (40 mL), dried (Na_2SO_4), filtered over basic alumina and subsequently concentrated *in vacuo* and the residue was co-evaporated with 20 mL toluene (2×) and 20 mL $CHCl_3$ (2×). According to analytical HPLC analysis most compounds proved >95% pure. Additional column chromatography (*n*-heptane/EtOAc, 1/1, v/v) was required for Cbz-Ser-O^{t}Bu (**7c**).

General Procedure C: Enzymatic esterification with ^{t}BuOH of protected peptides

Alcalase-CLEA (3 g) was added to a solution of protected peptide (1 g) in DMF/^{t}BuOH (100 mL, 17.5/82.5, v/v) in the presence of 3 Å molecular sieves (2 g). The obtained reaction mixture was shaken at 37°C with 150 rpm for 16 h. After filtration, the solid enzyme particles were washed by resuspension in CH_2Cl_2 followed by filtration (100 mL, 3×). The combined

organic layers were concentrated *in vacuo* and the resulting oil was purified by preparative HPLC.

General Procedure D: Enzymatic α-selective esterification of *N*-protected Asp and Glu
Alcalase-CLEA (3 g) and 3 Å molecular sieves (2.0 g) were added to *N*-protected amino acid (0.5 g) which was dissolved in a mixture of MTBE (28.0 mL) and the appropriate alcohol (2.0 mL). In case of *N*-Fmoc-protected derivatives 0.1 vol% of acetic acid was added to the reaction mixture. The obtained reaction mixture was shaken at 50°C with 200 rpm for 16 h. After filtration, the solid enzyme particles were washed by resuspension in 0.1 N HCl followed by filtration (50 mL, 3×) and finally by resuspension in EtOAc followed by filtration (50 mL, 3×). The combined EtOAc and aqueous HCl phases were separated and the organic phase was washed with aq. HCl (100 mL, 0.1 N), dried (Na_2SO_4) and subsequently concentrated *in vacuo*. The resulting oil was redissolved in CH_2Cl_2/MeOH/AcOH (20 mL, 89.9/10/0.1, v/v/v) and this solution was filtered over a short silica plug. The mixture was concentrated *in vacuo* and co-evaporated with toluene (50 mL, 2×) and $CHCl_3$ (50 mL, 2×).

General Procedure E: Solid phase synthesis of protected peptide starting materials
2-Chlorotritylchloride resin (1 g, loading = 1.2 mmol/g) was reacted with Fmoc-Gln-OH (2 mmol) and diisopropylethylamine (DIPEA, 5 mmol) in CH_2Cl_2 (10 mL) for 30 min followed by washing with DMF (10 mL, 2×). Afterwards, the unreacted tritylchloride was capped with MeOH/CH_2Cl_2/DIPEA (10 mL, 15/85/5, v/v/v) followed by washing steps with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and NMP (10 mL, 2 min, 3×). Further Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[20] The desired peptide sequences were cleaved from the resin using 2.5 vol% TFA in CH_2Cl_2 (50 mL, 20 min). After filtration, the resin was washed with CH_2Cl_2 (50 mL) and the combined filtrates were concentrated *in vacuo* to half of the original volume. Subsequently, ^{i}PrOH/H_2O (50 mL, 1/3, v/v) was added and the solution was concentrated *in vacuo* to one third of the original volume. The precipitated protected peptide was filtered off and subsequently washed with H_2O (20 mL, 2×) followed by lyophilization from CH_3CN/H_2O (3/1, v/v). To ensure high yield for the enzymatic reactions the protected peptides were purified by preparative HPLC followed by lyophilization from CH_3CN/H_2O (3/1, v/v).

Cbz-Phe-OMe (1a)[21a]
433 mg; 83% yield; R_f(HPLC) 18.60 min, Purity 97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.57; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.99-3.04 (m, 2H), 3.62 (s, 3H), 4.53-4.65 (m, 1H), 5.00 (s, 2H), 5.19 (d, *J* = 7.5 Hz, 1H), 7.01 (d, *J* = 7.2 Hz, 2H), 7.16-7.24 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 38.1, 52.2, 54.8, 66.9, 127.0, 128.0, 128.1, 128.4, 128.5, 129.2, 135.7, 136.2, 155.6, 171.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{18}H_{19}NO_4Na$: 336.1206; found: 336.1214.

Cbz-Phe-OEt (2a)[21b]
465 mg; 85% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.15 (t, *J* = 7.3 Hz, 3H), 2.99-3.05 (m, 2H), 4.05-4.12 (q, *J* = 8.7 Hz, 2H), 4.55-4.58 (m, 1H), 5.02 (s, 2H), 5.22 (d, *J* = 7.3, 1H), 7.03 (dd, *J* = 2.3 and 7.8 Hz, 2H), 7.15-7.31 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 14.6, 38.7,

55.2, 61.9, 67.3, 127.5, 128.5, 128.9, 129.7, 136.2, 136.7, 156.0, 171.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{19}H_{22}NO_4$: 328.1543; found: 328.1535.

Cbz-Phe-OBn (3a)[21c]

582 mg; 90% yield; R_f(HPLC method 1) 21.90 min, Purity >99%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.58; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.94-3.00 (m, 2H), 4.53-4.65 (m, 1H), 4.96 (s, 2H), 5.00 (2 x dd, 2H), 5.18 (d, *J* = 8.4 Hz, 1H), 6.88-6.90 (m, 2H), 7.03-7.25 (m, 13H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 37.9, 54.6, 66.7, 66.9, 126.7, 127.8, 127.9, 128.2, 128.3, 129.0, 134.8, 135.3, 136.0, 155.3, 171.0; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{24}H_{23}NO_4Na$: 412.1535; found: 412.1540.

Cbz-Phe-OAll (4a)[21d]

488 mg; 86% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.94-3.08 (m, 2H), 4.48 (d, *J* = 5.7 Hz, 2H), 4.53-4.60 (m, 1 H), 5.03 (s, 2H), 5.15-5.21 (m, 2H), 5.28 (d, *J* = 7.7 Hz, 1H), 5.67-5.80 (m, 1H), 7.01 (dd, *J* = 2.3 and 8.0 Hz, 2H), 7.16-7.23 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 29.5, 55.2, 66.5, 67.1, 139.2, 127.0, 128.4, 128.5, 128.8, 128.9, 130.1, 131.0, 136.0, 136.5, 171.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{20}H_{22}NO_4$: 340.1543; found: 340.1603.

Cbz-Phe-OTMSE (5a)

581 mg, 87% yield; R_f(HPLC) 23.87 min; Purity >97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.90; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.05 (s, 9H), 0.94-0.89 (m, 2H), 3.09-3.05 (dd, *J* = 6.0 Hz, 2H), 4.07-4.20 (m, 2H), 4.57-4.60 (dd, *J* = 3.3 and 9.0 Hz, 1H), 5.06 (s, 2H), 5.23 (d, *J* = 9.0 Hz, 1H), 7.06 (dd, *J* = 3.3 and 5.7, Hz, 2H), 7.19-7.34 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = -1.2, 17.2, 29.1, 55.1, 64.0, 67.1, 127.2, 128.2, 128.6, 129.6, 136.0, 136.5, 155.8, 171.8; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{22}H_{29}NO_4NaSi$: 422.1758; found: 422.1757.

Cbz-Phe-O^{t}Bu (6a)

505 mg; 85% yield; R_f(HPLC) 20.93 min; Purity >98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.55; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.30 (s, 9H), 2.98-3.05 (m, 2H), 4.41-4.56 (m, 1H), 5.07 (s, 2H), 5.22 (d, *J* = 7.5 Hz, 1H), 7.11 (dd, *J* = 3.0 and 8.1 Hz, 2H), 7.15-7.31 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 28.0, 38.3, 55.3, 66.9, 82.4, 127.1, 128.2, 128.7, 129.5, 136.0, 136.6, 155.7, 170.7; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{21}H_{25}NO_4Na$: 378.1675; found: 378.1657.

Boc-Phe-O^{t}Bu (6b)[21e]

453 mg; 85% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.33 (s, 9H), 1.35 (s, 9H), 2.98-3.03 (m, 2H), 4.35-4.42 (m, 1H), 4.92 (d, *J* = 6.6 Hz, 1H), 7.12 (dd, *J* = 3.3 and 8.2 Hz, 2H), 7.15-7.25 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.6, 28.1, 38.8, 55.0, 79.8, 82.1, 127.0, 128.5, 129.7, 136.6, 136.0, 155.5, 171.0; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{18}H_{27}NO_4Na$: 344.1832; found: 344.1849.

Bz-Phe-O^{t}Bu (6c)[21f]

490 mg; 90% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.30 (s, 9H), 3.20 (d, *J* = 6.1 Hz, 2H), 4.76-4.85 (m, 1H), 6.55 (d, *J* = 7.8 Hz, 1H), 7.11 (dd, *J* = 7.3 Hz, 2H), 7.42-7.50 (m, 6H),

7.70 (d, *J* = 8.3 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 17.9, 38.0, 53.8, 82.6, 127.1, 128.3, 128.7, 129.6, 130.1, 133.8, 166.8, 170.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{21}H_{26}NO_3$: 340.1907; found: 340.1925.

For-Phe-O^{t}Bu (6d)

382 mg; 92% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.33 (s, 9H), 3.02 (d, *J* = 5.6 Hz, 2H), 4.75 (m, 1H), 4.99 (d, *J* = 7.3 Hz, 1H), 7.22-7.07 (m, 5H), 8.04 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 18.1, 38.7, 51.8, 80.6, 127.6, 128.5, 128.9, 136.8, 165.7, 172.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{14}H_{20}NO_3$: 250.1438; found: 250.1437.

Fmoc-Phe-O^{t}Bu (6e)[21g]

648 mg; 88% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.42 (s, 9H), 3.06-3.10 (m, 2H), 4.11-4.21 (m, 1H), 4.30-4.42 (m, 2H), 4.47-4.53 (m, 1H), 5.24 (d, *J* = 7.5 Hz, 1H), 7.12 (dd, *J* = 3.0 and 7.3 Hz, 2H), 7.23-7.40 (m, 7H), 7.51-7.55 (m, 2H), 7.75 (d, *J* = 7.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.7, 38.1, 47.2, 55.2, 66.6, 82.0, 119.6, 125.0-129.3, 136.0, 141.2, 143.6, 155.2, 170.6; FIA-ESI(+)-TOF-MS: m/z $[M + H]^+$ calcd for $C_{28}H_{30}NO_4$: 444.2169; found: 444.2142.

Cbz-Ala-O^{t}Bu (7a)[21h]

399 mg; 88% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ =1.29 (d, *J* = 7.1 Hz, 3H), 1.38 (s, 9H), 4.16-4.20 (m, 1H), 5.03 (s, 2H), 5.29 (d, *J* = 6.0 Hz, 1H), 7.31-7.18 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 18.8, 27.8, 50.2, 66.7, 71.9, 128.2, 128.6, 136.4, 155.6, 172.2; FIA-ESI(+)-TOF-MS: m/z $[M + Na]^+$ calcd for $C_{15}H_{21}NO_4Na$: 302.1362; found: 302.1387.

Cbz-Leu-O^{t}Bu (7b)

474 mg; 88% yield; R_t(HPLC) 20.30 min; Purity >98%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.86 (dd, *J* = 6.3 Hz, 6H), 1.38 (s, 9H), 1.39-1.70 (m, 3H), 4.19-4.22 (m, 1 H), 5.03 (s, 2 H), 5.12 (d, *J* = 7.2 Hz, 1H), 7.24-7.28 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 22.6, 25.4, 28.6, 42.5, 53.7, 67.4, 82.4, 128.2, 128.7, 137.0, 156.5, 172.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{15}H_{21}NO_5Na$: 318.1311; found: 318.1311.

Cbz-Ser-O^{t}Bu (7c)[21i]

352 mg; 72% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.41 (s, 9H), 3.09 (s, 1H), 3.86 (m, 2H), 4.25 (m, 1H), 5.06 (s, 2H), 5.60 (d, *J* = 6.6 Hz, 1H), 7.20-7.25 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.8, 55.0, 63.4, 67.1, 82.5, 128.3, 128.4, 128.7, 136.1, 156.3, 169.8; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{15}H_{21}NO_5Na$: 318.1311; found: 318.1311.

Cbz-Met-O^{t}Bu (7d)

466 mg; 82% yield; R_t(HPLC) 15.42 min; Purity >96%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.39 (s, 9H), 1.80-1.89 (m, 2H), 2.01 (s, 3H), 2.40-2.48 (m, 2H), 4.28-4.30 (m, 1H), 5.03 (s, 2H), 5.33 (d, *J* = 7.5 Hz, 1H), 7.26-7.29 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 22.2, 23.0, 28.2, 42.1, 53.3, 67.0, 82.0, 128.2, 129.1, 136.6, 156.1, 171.4; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{17}H_{25}NO_4NaS$: 362.1396; found: 362.1334.

Cbz-Lys(Cbz)-O^{t}Bu (7e)

709 mg; 90% yield; R_f(HPLC) 23.75 min; Purity >98%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.25-1.45 (m, 13H), 1.46-1.71 (m, 2H), 3.01-3.12 (m, 2H), 4.13-4.19 (m, 1H), 4.81-4.85 (m, 1H), 5.00 (s, 2H), 5.01 (s, 2H), 5.42 (d, *J* = 7.2 Hz, 1H), 7.18-7.26 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 22.4, 28.2, 29.9, 32.6, 40.9, 54.3, 66.8, 67.1, 82.3, 128.2, 128.7, 136.6, 136.8, 156.2, 156.7, 171.7; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{26}H_{34}N_2O_6Na$: 493.2309; found: 493.2216.

Cbz-DOPA-O^{t}Bu (7f)

580 mg; 90% yield; R_f(HPLC) 17.20 min; Purity >98%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.34 (s, 9H), 2.83-2.88 (dd, *J* = 9.3 and 4.2 Hz, 2H), 4.37-4.40 (dd, *J* = 13.0 and 4.5 Hz, 1H), 5.00 (s, 2H), 5.25 (d, *J* = 8.1 Hz, 1H) 5.90-6.05 (m, 2H), 6.45 (d, *J* = 7.2 Hz, 1H), 6.58-6.65 (m, 2H), 7.19-7.30 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 28.2, 38.0, 55.7, 67.3, 82.8, 115.4, 116.5, 121.9, 127.2, 128.2, 128.4, 128.7, 136.3, 143.2, 144.0, 156.2, 171.2; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{21}H_{25}NO_6Na$: 410.1574; found: 410.1530.

Cbz-Phe-Leu-OMe (8)[21j]

626 mg; 88% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.81 (d, *J* = 6.6 Hz, 6H), 1.33-1.55 (m, 3H), 2.99 (d, *J* = 6.6 Hz, 2H), 3.61 (s, 3H), 4.37-4.51 (m, 2H), 5.00 (s, 2H), 5.45 (d, *J* = 8.0 Hz, 1H), 6.35 (d, *J* = 7.9 Hz, 1H), 7.09-7.27 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 21.8, 22.6, 24.7, 38.4, 41.3, 50.7, 52.2, 56.1, 67.1, 127.2, 128.2, 128.3, 128.7, 128.8, 129.5, 136.1, 155.9, 170.6, 172.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{24}H_{30}N_2O_5Na$: 449.2046; found: 449.2037.

Cbz-Phe-Leu-OBn (9)

668 mg; 80% yield; R_f(HPLC) 22.58 min, Purity 99%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.56; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.78 (d, *J* = 6.0 Hz, 6H), 1.37-1.57 (m, 3H), 2.96-3.01 (dd, *J* = 6.3 Hz, 2H), 4.41-4.49 (dd, *J* = 12.0 and 3.3 Hz, 1H), 4.49-4.55 (m, 1H), 5.00 (s, 2H), 5.05 (s, 2H), 5.25 (d, *J* = 7.8 Hz, 1H), 6.12 (d, *J* = 8.1 Hz, 1H), 7.07-7.29 (m, 15H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 21.9, 22.6, 24.7, 38.2, 41.7, 51.2, 56.3, 67.2, 127.2, 128.1, 128.2, 128.5, 128.7, 128.9, 129.4, 135.5, 136.4, 156.1, 170.7, 172.3; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{30}H_{34}N_2O_5Na$: 525.2359; found: 525.2324.

Cbz-Phe-Leu-O^{t}Bu (10)

701 mg; 90% yield; R_f(HPLC) 22.03 min, Purity 98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.52; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.80 (d, *J* = 6.0 Hz, 6H), 1.36-1.50 (m, 12H), 3.00 (d, *J* = 6.3 Hz, 2H), 4.32-4.39 (m, 2H), 5.00 (s, 2H), 5.29 (d, *J* = 5.4 Hz, 1H), 6.23 (d, *J* = 6.9 Hz, 1H), 7.09-7.25 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 22.1, 22.6, 24.8, 28.0, 38.4, 41.8, 51.4, 56.0, 67.0, 81.9, 127.0, 128.0, 128.1, 128.7, 128.8, 129.5, 136.3, 155.8, 170.3, 171.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{27}H_{36}N_2O_5Na$: 491.2516; found: 491.2525.

Fmoc-Asp(O^{t}Bu)-Lys(Boc)-Ser(^{t}Bu)-Leu-Gln-O^{t}Bu (11)

710 mg; 68% yield; R_f(HPLC) 23.58 min, Purity 99%; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 0.84 (dd, *J* = 6.6 and 1.5 Hz, 6H), 1.09 (s, 9H), 1.15-1.95 (m, 36 H), 2.09-2.14 (m, 2H), 2.41-

2.68 (m, 2H), 2.82-2.87 (m, 2H), 3.15-3.23 (m, 2H), 4.01-4.08 (m, 1H), 4.19-4.42 (m, 6H), 6.69-6.77 (m, 2H), 7.24-7.44 (m, 5H), 7.66-7.72 (m, 4H), 7.90-7.99 (m, 4H), 8.10 (d, *J* = 6.9 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ = 21.5, 23.0, 24.1, 27.0, 27.5, 27.6, 28.1, 32.3, 46.5, 48.7, 52.3, 72.7, 80.0, 80.3, 120.0, 125.1, 126.9, 127.5, 140.6, 143.6, 143.7, 155.6, 169.0, 169.1, 170.6, 171.4, 171.6, 173.2; ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{56}H_{85}N_7NaO_{14}$: 1102.6047; found 1102.6059.

TFA.H-Asp(O^{t}Bu)-Lys(Boc)-Ser(^{t}Bu)-Leu-Gln-O^{t}Bu (12)

771 mg; 73% yield; R$_t$(HPLC) 12.33 min, Purity 99%; ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ = 0.88 (m, *J* = 6.6 Hz, 6H), 1.12 (s, 9H), 1.18-1.96 (m, 36 H), 2.10-2.16 (m, 2H), 2.50-2.65 (m, 2H), 2.72-2.92 (m, 2H), 3.42-3.48 (m, 2H), 4.03-4.10 (m, 2H), 4.23-4.45 (m, 4H), 6.70-6.82 (m, 2H), 7.28 (s, 1H), 7.65-7.73 (m, 3H), 7.82 (d, *J* = 8.1 Hz, 1H), 8.05 (d, *J* = 7.8 Hz, 1H), 8.18 (d, *J* = 8.1 Hz, 1H), 7.52 (d, *J* = 7.8 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ = 21.6, 23.0, 23.8, 27.0, 27.5, 27.6, 28.1, 31.1, 50.4, 52.3, 53.3, 72.7, 80.4, 81.3, 168.6, 168.9, 170.6, 171.0, 171.6, 173.2; FIA-ESI(+)-TOF-MS: *m/z* [M + H]$^+$ calcd for $C_{41}H_{76}N_7O_{12}$: 858.5546; found: 858.5512.

Cbz-Asp-OAll (13a)[21k]

481 mg; 84% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.83 (dd, *J* = 4.1 and 17.1 Hz, 1H), 2.96 (dd, *J* = 3.9 and 16.7 Hz, 1H), 4.55-4.63 (m, 3H), 5.09 (s, 2H), 5.22-5.30 (m, 2H), 5.74 (d, *J* = 8.6 Hz, 1H), 5.79-5.85 (m, 1H), 7.23-7.32 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 36.5, 50.4, 66.7, 67.4, 119.1, 128.2, 128.4, 128.6, 131.5, 136.2, 156.2, 170.4, 175.5; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{15}H_{17}NO_6Na$: 330.0948; found: 330.0961.

Cbz-Asp-OTMSE (13b)

593 mg; 86% yield; R$_t$(HPLC) 20.74 min; Purity >97%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = -0.02 (s, 9H), 0.92-1.01 (m, 2H), 2.85 (dd, *J* = 4.5 and 17.4 Hz, 1H), 2.90 (dd, *J* = 4.2 and 16.8 Hz, 1H), 4.18-4.25 (m, 2H), 4.58-4.64 (m, 1H), 5.10 (s, 2H), 5.84 (d, *J* = 8.1 Hz, 1H), 7.30-7.39 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = -1.5, 17.3, 36.7, 50.4, 53.5, 64.5, 128.0, 128.7, 136.2, 159.9, 170.9, 175.4; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{17}H_{25}NO_6Na$: 390.1343; found: 390.1336.

Cbz-Glu-OAll (14a)

500 mg; 88% yield; R$_t$(HPLC) 14.11 min; Purity >98%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.91-2.20 (m, 2H), 2.38-2.45 (m, 2H), 4.37-4.45 (m, 1H), 4.60 (d, *J* = 5.7 Hz, 2H), 5.07 (s, 2H), 5.20-5.26 (m, 2H), 5.54 (d, *J* = 7.5 Hz, 1H), 5.80-5.91 (m, 1H), 7.23-7.30 (m, 5H), 8.95 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.8, 30.2, 53.2, 66.6, 67.6, 119.6, 128.5, 128.6, 128.9, 131.7, 136.5, 171.9, 178.1; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{16}H_{19}NO_6Na$: 344.1104; found: 344.1134.

Cbz-Glu-OTMSE (14b)

598 mg; 88% yield; R$_t$(HPLC) 21.30 min; Purity >98%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = -0.04 (s, 9H), 0.91-1.00 (m, 2H), 1.93-2.00 (m, 1H), 2.10-2.18 (m, 1H), 2.31-2.41 (m, 2H), 4.10-4.22 (m, 2H), 4.31-4.37 (m, 1H), 5.07 (s, 2H), 5.42 (d, *J* = 8.1 Hz, 1H), 7.23-7.31 (m, 5H);

^{13}C NMR (75 MHz, $CDCl_3$) δ = -1.4, 17.5, 27.9, 30.5, 53.5, 63.0, 64.2, 128.2, 128.3, 128.6, 136.4, 156.0, 172.9, 176.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{18}H_{27}NO_6NaSi$: 404.1505; found: 404.1507.

Boc-Asp-OAll (15a)

511 mg; 89% yield; R_t(HPLC) 12.43 min; Purity >98%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.43 (s, 9H), 2.88 (dd, *J* = 4.5 and 17.1 Hz, 1H), 3.00 (dd, *J* = 3.9 and 16.8 Hz, 1H), 4.45-4.64 (m, 3H), 5.24-5.34 (m, 2H), 5.60 (d, *J* = 8.4 Hz, 1H), 5.82-5.92 (m, 1H), 8.89 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 28.4, 36.8, 50.1, 66.0, 80.7, 119.0, 131.7, 155.9, 171.1, 175.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{12}H_{19}NO_6Na$: 296.1134; found: 296.1117.

Boc-Asp-OTMSE (15b)[21l]

630 mg; 88% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = -0.05 (s, 9H), 0.92-0.99 (m, 2H), 1.43 (s, 9H), 2.79 (dd, *J* = 4.5 and 17.4 Hz, 1H), 3.00 (dd, *J* = 3.9 and 17.0 Hz, 1H), 4.19-4.25 (m, 2H), 4.50-4.52 (m, 1H), 5.54 (d, *J* = 8.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = -1.3, 17.2, 28.3, 37.0, 50.3, 64.5, 80.5, 155.9, 171.5, 175.3; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{14}H_{27}NO_6NaSi$: 356.1499; found: 356.1500.

Boc-Glu-OAll (16a)[21m]

506 mg; 87% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.42 (s, 9H), 1.94-2.03 (m, 1H), 2.15-2.21 (m, 1H), 2.41-2.47 (m, 2H), 4.34-4.36 (m, 1H), 4.61 (d, *J* = 5.7 Hz, 2H), 5.26-5.35 (m, 3H), 5.83-5.94 (m, 1H), 9.44 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.1, 28.2, 30.1, 52.9, 66.0, 80.1, 118.9, 131.5, 155.5, 172.0, 177.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{13}H_{21}NO_6Na$: 310.1261; found: 310.1266.

Boc-Glu-OTMSE (16b)[21n]

599 mg; 85% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = -0.05 (s, 9H), 0.94-1.00 (m, 2H), 1.39 (s, 9H), 1.85-1.92 (m, 1H), 2.11-2.17 (m, 1H), 2.38-2.44 (m, 2H), 4.15-4.21 (m, 2H), 4.25-4.28 (m, 1H), 5.11 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = -1.3, 17.7, 28.0, 29.5, 30.2, 53.1, 54.3, 80.3, 156.7, 172.4, 178.8; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{15}H_{29}NO_6NaSi$: 370.1656; found: 370.1678.

Fmoc-Asp-OAll (17a)[21o]

491 mg; 88% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.90 (dd, *J* = 3.9 and 17.0 Hz, 1H), 3.00 (dd, *J* = 3.9 and 16.7 Hz, 1H), 4.17-4.21 (m, 1H), 4.29-4.43 (m, 2H), 4.58-4.64 (m, 3H), 5.18-5.31 (m, 2H), 5.81-6.02 (m, 2H), 7.23-7.73 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 36.7, 47.3, 50.6, 66.7, 67.6, 119.0, 120.2, 125.3, 127.3, 128.0, 131.5, 141.5, 143.8, 156.3, 170.8, 175.3; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{22}H_{21}NO_6Na$: 418.1261; found: 418.1277.

Fmoc-Asp-OTMSE (17b)

542 mg; 85% yield; R_t(HPLC) 23.56 min; Purity >97%; ^{1}H NMR (300 MHz, $CDCl_3$) δ = -0.03 (s, 9H), 0.80-0.96 (m, 2H), 2.91 (dd, *J* = 3.9 and 16.8 Hz, 1H), 3.06 (dd, *J* = 3.6 and 16.8 Hz, 1H), 4.19-4.57 (m, 6H), 5.79 (d, *J* = 7.8 Hz, 1H), 7.22-7.73 (m, 8H); ^{13}C NMR (75 MHz,

$CDCl_3$) δ = -1.3, 17.5, 30.0, 36.6, 47.4, 64.9, 67.7, 120.3, 125.4, 127.3, 128.1, 141.6, 144.0, 156.1, 170.3, 175.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{24}H_{29}NO_6NaSi$: 478.1656; found: 478.1651.

Fmoc-Glu-OAll (18a)[21p]

495 mg; 89% yield; ^1H NMR (300 MHz, $CDCl_3$) δ = 1.90-2.00 (m, 1H), 2.15-2.23 (m, 1H), 2.38-2.43 (m, 2H), 4.09-4.18 (m, 1H), 4.35-4.41 (m, 3H), 4.60 (d, *J* = 3.3 Hz, 2H), 5.20-5.31 (m, 2H), 5.43 (d, *J* = 6.4 Hz, 1H), 5.81-5.90 (m, 1H), 7.21-7.72 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.4, 30.0, 47.3, 53.4, 66.3, 67.1, 119.2, 120.0, 124.7, 127.1, 127.7, 131.3, 141.3, 143.7, 156.1, 170.5, 177.5**;** FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{23}H_{23}NO_6Na$: 432.1417; found: 432.1442.

Fmoc-Glu-OTMSE (18b)

545 mg; 86% yield; R_t(HPLC) 23.98 min; Purity >98%; ^1H NMR (300 MHz, $CDCl_3$) δ = -0.04 (s, 9H), 0.86-1.02 (m, 2H), 1.91-2.06 (m, 1H), 2.17-2.28 (m, 1H), 2.39-2.45 (m, 2H), 4.18-4.21 (m, 3H), 4.35-4.42 (m, 3H), 5.42 (d, *J* = 6.6 Hz, 1H) 7.23-7.74 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = -1.3, 17.4, 22.3, 27.9, 47.5, 53.8, 63.4, 64.4, 67.5, 120.0, 125.2, 127.2, 128.3, 141.4, 144.2, 156.4, 171.5, 177.4; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{25}H_{31}NO_6NaSi$: 492.1812; found: 492.1824.

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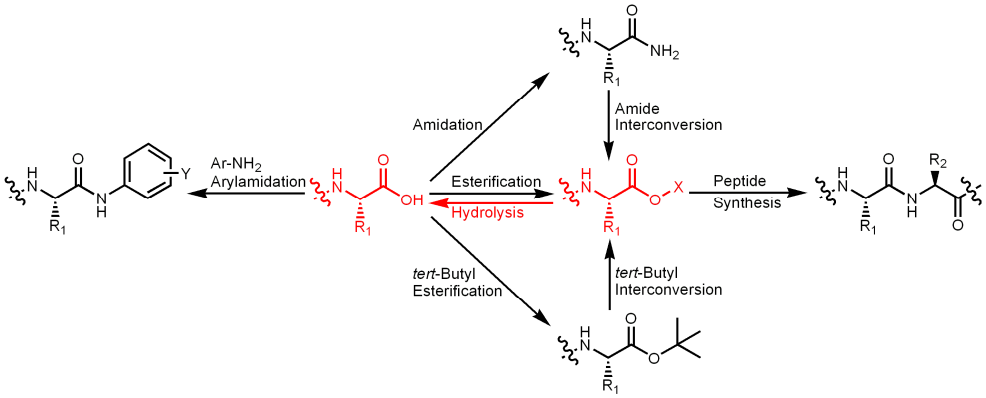
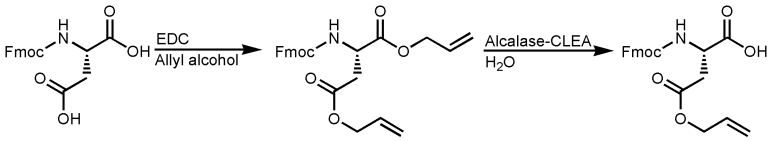
Chapter 3

Enzymatic synthesis of β-protected aspartic acid and γ-protected glutamic acid derivatives



In this chapter, two versatile, high yielding and efficient chemo-enzymatic methods for the synthesis of β-protected Asp and γ-protected Glu derivatives using Subtilisin A are described. The first method is based on the α-selective enzymatic hydrolysis of aspartyl and glutamyl symmetrical diesters. The second method comprises a three step protocol using i) α-selective enzymatic methyl esterification, ii) chemical β-esterification, and finally iii) α-selective enzymatic methyl ester hydrolysis. The isolated yield of the β- and γ-esters ranged between 77% and 91%



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3.1 Introduction

Protecting groups play a pivotal role in organic chemistry and especially in peptide, carbohydrate and nucleic acid synthesis. In peptide synthesis, amine functionalities are usually protected as carbamates, while carboxylic acid moieties are most often protected as esters. A vast variety of esters have been developed to protect *C*-terminal and side chain carboxylic acids for different applications. Orthogonality of these protecting groups and resistance to coupling and deprotection reaction conditions is a key factor for high yield and easy workup. For instance allyl (All) esters, cleavable with Pd(0),[1] and trimethylsilylethyl (TMSE) esters, cleavable with TBAF,[2] are orthogonal to other commonly used protecting groups such as *tert*-butyl (^{t}Bu) or methyl (Me) esters allowing selective deprotection and modification of certain carboxylic acids during peptide synthesis. Of special interest are selectively protected aspartic acid (Asp) or glutamic acid (Glu) building blocks, which are protected either at their α-carboxylic acid functionality or at their β- (Asp) or γ-(Glu) carboxylic acid moiety. These esters find widespread application in on-resin synthesis of head-to-tail cyclic peptides[3] (using both Fmoc/^{t}Bu- and Boc/Bzl SPPS approaches), side chain lactam peptides[4] and branched peptides.[5] In chapter 2, the selective α-carboxylic acid esterification of *N*-protected amino acids, including Asp and Glu residues, using the industrial enzyme Subtilisin A was described.[6] Selective synthesis of β-protected Asp and γ-protected Glu derivatives however, remains a challenge.

A number of synthetic strategies have been disclosed for (semi-)selective β- and γ-protection of Asp and Glu derivatives, respectively.[7] The most commonly used methods rely on the intramolecular anhydride formation of *N*-protected Asp/Glu derivatives using a condensing reagent followed by moderately selective ring opening with a nucleophile.[8] Whereas with Glu residues a relatively good selectivity of 9/1 of γ- over α-ester can be obtained, selective β-protection of Asp residues remains difficult and is often low yielding.

Only a few chemo-enzymatic approaches towards aspartyl β-esters have been reported in the literature. These approaches are based on the α-selective hydrolysis of Asp diesters. However, the reported α-selective hydrolysis with porcine liver esterase is only applicable for *N*-terminal unprotected aspartic acid derivatives.[9] Most proteases proved to be unselective when *N*-unprotected aspartyl diesters were used. Hydrolysis of *N*-protected aspartyl diesters with papain,[10] is limited to Cbz-Asp(OAll)-OAll. The very common *N*-terminal Fmoc/Boc-protected β-esters of Asp and γ-esters of Glu have not been prepared enzymatically.

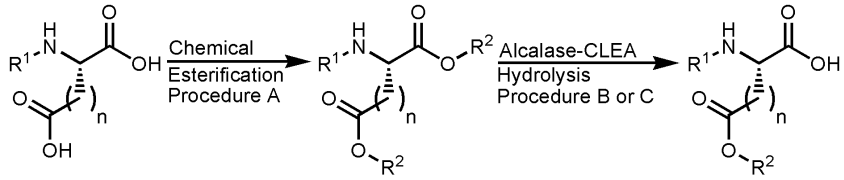
This chapter describes the α-selective hydrolysis of a wide range of *N*-protected Asp and Glu symmetrical diesters with the cheap industrial protease Subtilisin A (commercially available as Alcalase in which Subtilisin A is the main constituent).[11] In addition, a versatile and high yielding three-step protocol for the synthesis of *N*-protected β-aspartyl esters is described.

3.2 Results and Discussion

Alcalase is often used for the hydrolysis and/or resolution of amino acid esters.[12] In chapter 2,[6] a versatile synthetic method for various *N*-protected amino acid esters (*i.e.* methyl, ethyl, benzyl, *tert*-butyl, allyl and trimethylsilylethyl) using Alcalase cross-linked enzyme

aggregates (CLEAs)[13] in dry organic solvents was described. The opposite reaction, namely the hydrolysis of these esters proved to be easy and quantitative. Encouraged by these results, it was decided to focus on the α-selective hydrolysis of *N*-protected aspartyl and glutamyl diesters (Table 1).

Table 1. Chemical diester synthesis and selective enzymatic hydrolysis of the α-carboxyl ester.



#	R^1	R^2	n	#	R^1	R^2	n
1	Cbz	All	1	**8**	Cbz	All	1
2	Cbz	All	2	**9**	Cbz	All	2
3	Boc	All	1	**10**	Boc	All	1
4	Boc	All	2	**11**	Boc	All	2
5	Fmoc	All	1	**12**	Fmoc	All	1
6	Fmoc	All	2	**13**	Fmoc	All	2
7	Cbz	TMSE	1	**14**	Cbz	TMSE	1

Entry[a]	Di-ester		Product		Yield (%)[b]	Procedure
1	Cbz-Asp(OAll)-OAll	**1**	Cbz-Asp(OAll)-OH	**8**	87	B
2	Cbz-Glu(OAll)-OAll	**2**	Cbz-Glu(OAll)-OH	**9**	85	B
3	Boc-Asp(OAll)-OAll	**3**	Boc-Asp(OAll)-OH	**10**	87	B
4	Boc-Glu(OAll)-OAll	**4**	Boc-Glu(OAll)-OH	**11**	86	B
5	Fmoc-Asp(OAll)-OAll	**5**	Fmoc-Asp(OAll)-OH	**12**	77	C
6	Fmoc-Glu(OAll)-OAll	**6**	Fmoc-Glu(OAll)-OH	**13**	78	C
7	Cbz-Asp-(OTMSE)-OTMSE	**7**	Cbz-Asp-(OTMSE)-OH	**14**	85	B

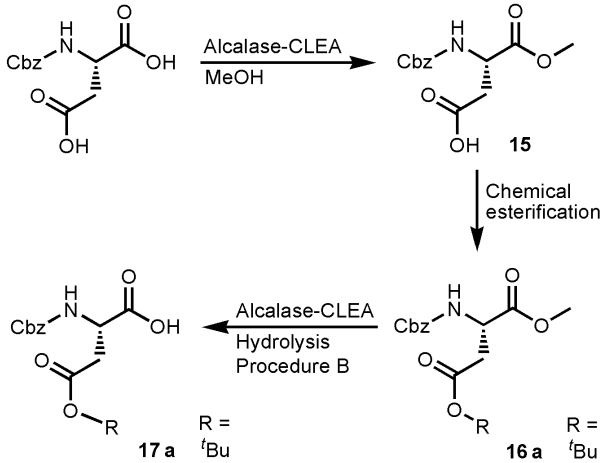
[a] Conditions: CH_2Cl_2, EDC.HCl, R_2OH, rt, 4 h (Procedure A); Alcalase-CLEA, mixture of ^{t}BuOH, 1,4-dioxane or DMF with phosphate buffer, 37°C, 16 h (Procedure B); Alcalase-CLEA, mixture of ^{t}BuOH, 1,4-dioxane or DMF with H_2O, 0.1 vol% acetic acid, 37°C, 24 h (Procedure C); [b] Isolated yield based on the acyl donor starting material.

N-protected aspartyl (n = 1) and glutamyl (n = 2) diesters **1**-**7** were synthesized using 1-ethyl-3-(3-dimethylamino propyl)carbodiimide hydrochloride (EDC·HCl) and the appropriate alcohol. These symmetrical diesters were hydrolyzed using Alcalase-CLEA in water at pH 7.5 using ^{t}BuOH, 1,4-dioxane or dimethylformamide (DMF) as a co-solvent.

Gratifyingly, all the hydrolytic reactions were completely α-selective, *i.e*. no simultaneous β- or γ-ester hydrolysis could be observed by HPLC and reference compounds. As shown in Table 1, very high yield of β-aspartyl and γ-glutamyl esters were obtained using a variety of *N*-protecting groups (**8-14**). Surprisingly, even the sterically demanding trimethylsilylethyl (TMSE) esters of Asp were easily and α-selectively hydrolyzed by Alcalase-CLEA. The newly synthesized esters were analyzed by NMR and were found to be identical to those reported in the literature or to commercially available samples, which clearly proved the selective α-hydrolysis of all the diesters used in this study.

However, as also reported by others,[14] the di-^{t}Bu ester derivatives of Asp and Glu were not easily hydrolyzed enzymatically. It was envisioned that, by combining α-selective synthesis of *N*-protected Asp esters with chemical β-esterification followed by an α-selective hydrolysis of the resulting diesters, a feasible approach towards β-protected Asp derivatives could be obtained. Additionally, this alternative strategy avoids the use of two equivalents of an expensive (*e.g.* TMSEOH) alcohol and coupling reagent, by preparation of a cheap methyl (Me) ester as a temporary protected intermediate of the α-carboxylic acid followed by chemical esterification of the β- or γ-carboxylic acid moiety (Table 2).

Table 2. Three step chemo-enzymatic approach towards β-protected Asp derivatives.



Entry[a]	Product		Yield (%)[b]
1	Cbz-Asp-OMe	**15**	93
2	Cbz-Asp(O^{t}Bu)-OMe	**16a**	94
3	Cbz-Asp(OAll)-OMe	**16b**	92
4	Cbz-Asp(O^{t}Bu)-OH	**17a**	91
5	Cbz-Asp(OAll)-OH	**8**	89

[a] Conditions: Alcalase-CLEA, MTBE/MeOH, 3 Å molecular sieves, 50°C, 16 h (**15**); CH_3CN/^{t}BuOH, Boc_2O, pyridine, DMAP, rt, 24 h (**16a**); EtOAc, EDC.HCl, DiPEA, All-OH, rt, 24 h (**16b**); Alcalase-CLEA, mixture of ^{t}BuOH, 1,4-dioxane or DMF with phosphate buffer, 37°C, 16 h (Procedure B); [b] Isolated yield based on the acyl donor starting material.

As shown in Table 2, the α-methyl esterification proceeded smoothly and the chemical β-esterification, by activation with either EDC for the All ester or with Boc_2O for the ^{t}Bu ester, furnished the desired esters in a high yield. Gratifyingly, subsequent Alcalase-CLEA-mediated enzymatic hydrolysis of **16** (R = All or ^{t}Bu) proceeded completely α-selective giving the desired β-protected Asp derivatives **8** and **17a** in a high yield.[24] Although this approach required three reaction steps, the overall yield (76% for Cbz-Asp(OAll)-OH) was considerably higher compared to the overall yield obtained via hydrolysis of the diesters

(57%) or those obtained by chemical means (around 50%). Even more importantly, this method allowed the synthesis of aspartyl β-esters which are not available *via* the diester hydrolysis method, *e.g.* Cbz-Asp(O^{t}Bu)-OH.
To conclude, this chapter demonstrates, via two attractive approaches, that the complete α-selectivity of Alcalase-CLEA in the synthesis and hydrolysis of various esters of *N*-protected Asp and Glu derivatives, can be utilized to prepare β-esters of *N*-protected aspartic acid or γ-esters of *N*-protected glutamic acid in high yield and purity. These derivatives are very useful as building blocks for the synthesis of peptides and peptide derivatives.

3.3 Experimental

General:
General experimental information is identical to chapter 2 with the following exception. For analytical HPLC chromatography a Phenomenex (C18, 5 μm particle size, 250 × 4.6 mm) column was used.

General Procedure A: Diallyl and diTMSE ester synthesis of *N*-protected Asp and Glu;
To a solution of *N*-protected amino acid (2.0 g) in a mixture of CH_2Cl_2 (100 mL) and the appropriate alcohol (4 mL), EDC.HCl (2.1 equiv) was added. The obtained reaction mixture was stirred at ambient temperature for 4 h and subsequently concentrated *in vacuo*. The resulting oil was partitioned between EtOAc (100 mL) and sat. aq. $NaHCO_3$ (100 mL). The organic phase was washed with sat. aq. $NaHCO_3$ (100 mL), 0.1 N HCl (100 mL, 2×), brine (100 mL), dried over Na_2SO_4 and concentrated *in vacuo*. When necessary, the resulting oil was purified by preparative HPLC.

General Procedure B: Enzymatic α-hydrolysis of diprotected Asp and Glu derivatives
Alcalase-CLEA (3 g) was added to a solution of diprotected amino acid (0.5 g) in a mixture of ^{t}BuOH (15 mL), 1,4-dioxane (15 mL) or DMF (15 mL) with phosphate buffer (10 mL, pH 7.5, 50 mM). The obtained reaction mixture was shaken at 37^{o}C at 200 rpm for 16 h. After filtration, the solid enzyme particles were resuspended in sat. aq. $NaHCO_3$ (50 mL) and removed by filtration. This enzyme particle washing procedure was repeated with H_2O (50 mL, 2×) and with EtOAc (50 mL, 2×). The combined organic phase was washed with H_2O (50 mL, 2×). The combined aqueous phases were acidified to pH 1 with 3 N HCl followed by extraction with EtOAc (100 mL, 3×). The combined organic layers were washed with brine (250 mL), dried over Na_2SO_4, concentrated *in vacuo* and dried by co-evaporation with toluene (50 mL, 2×) followed by $CHCl_3$ (50 mL, 2×). The purity of the β- or γ-ester of the *N*-protected Asp or Glu derivatives was > 95% as determined by analytical HPLC.

General Procedure C: Enzymatic α-hydrolysis of *N*-Fmoc diallyl-protected Asp and Glu derivatives
Alcalase-CLEA (3 g) was added to a solution of Fmoc diallyl-protected amino acid (0.5 g) in a mixture of ^{t}BuOH (15.0 mL), 1,4-dioxane (15.0 mL) or DMF (15.0 mL), with H_2O (10 mL) containing 0.1 vol% acetic acid. The obtained reaction mixture was shaken at 37^{o}C at 200 rpm for 24 h. After filtration, the solid enzyme particles were resuspended in EtOAc and

removed by filtration (50 mL, 2×). The combined organic phase was washed with brine (100 mL), dried over Na_2SO_4, concentrated *in vacuo* and dried by co-evaporation with toluene (50 mL, 2×) and $CHCl_3$ (50 mL, 2×). Finally, column chromatography with EtOAc/*n*-heptane (1/2, v/v) was used, and the β- or γ-ester of the *N*-Fmoc protected Asp or Glu derivatives were obtained > 98% pure as determined by analytical HPLC.

Cbz-Asp(OAll)-OAll (1)[15a]

1.76 g; 68% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.87 (dd, *J* = 4.3 and 17.0 Hz, 1H), 2.93 (dd, *J* = 3.9 and 16.8 Hz, 1H), 4.59 (d, *J* = 3.0 Hz, 2H) 4.65-4.70 (m, 3H), 5.14 (s, 2H), 5.23-5.29 (m, 4H), 5.76 (d, *J* = 8.3 Hz, 1H), 5.82-5.93 (m, 2H), 7.27-7.37 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 36.8, 50.6, 65.8, 66.6, 67.3, 118.9, 119.0, 128.3, 128.4, 128.7, 131.6, 131.8, 136.3, 156.1, 170.5, 170.6; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{18}H_{21}NO_6Na$: 370.1261; found: 370.1279.

Cbz-Glu(OAll)-OAll (2)

1.59 g; 62% yield; R_f(HPLC) 20.40 min; Purity >97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.59; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.93-2.05 (m, 2H), 2.22-2.27 (m, 2H), 2.38-2.43 (m, 2H), 4.41-4.47 (m, 1H), 4.58 (d, *J* = 5.7 Hz, 2H), 4.58 (d, *J* = 6.0 Hz, 2H), 5.12 (s, 2H), 5.22-5.37 (m, 4H), 5.42 (d, *J* = 7.8 Hz, 1H), 5.84-5.93 (m, 2H), 7.27-7.36 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.9, 28.4, 30.4, 53.1, 65.5, 66.2, 118.6, 119.1, 128.2, 128.7, 131.7, 132.2, 155.5, 162.4, 172.0, 172.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{19}H_{23}NO_6Na$: 384.1423; found: 384.1427.

Boc-Asp(OAll)-OAll (3)[15b]

1.52 g; 57% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.39 (s, 9H), 2.78 (dd, *J* = 3.9 and 17.0 Hz, 1H), 3.00 (dd, *J* = 3.7 and 16.7 Hz, 1H), 4.52-4.59 (m, 5H), 5.16-5.29 (m, 4H), 5.42 (d, *J* = 8.1 Hz, 1H), 5.76-5.92 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 28.2, 37.0, 50.2, 65.8, 66.4, 80.3, 118.8, 131.7, 131.8, 155.5, 170.7, 170.8; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{15}H_{23}NO_6Na$: 336.1417; found: 336.1424.

Boc-Glu(OAll)-OAll (4)

1.95 g; 74% yield; R_f(HPLC) 20.16 min; Purity >97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.39; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.45 (s, 9H), 1.91-2.07 (m, 1H), 2.19-2.25 (m, 1H), 2.42-2.46 (m, 2H), 4.35-4.38 (m, 1H), 4.59 (d, *J* = 5.7 Hz, 2H), 4.64 (d, *J* = 5.7 Hz, 2H), 5.12 (d, *J* = 8.9 Hz, 1H), 5.26-5.37 (m, 4H), 5.87-6.00 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 28.0, 28.5, 30.4, 53.2, 65.5, 66.2, 80.2 118.5, 119.1, 131.7, 132.2, 156.4, 172.0, 172.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{16}H_{25}NO_6Na$: 350.1574; found: 350.1594.

Fmoc-Asp(OAll)-OAll (5)

1.68 g; 69% yield; R_f(HPLC) 22.94 min; Purity >97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.87; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.91 (dd, *J* = 4.3 and 17.1 Hz, 1H), 3.10 (dd, *J* = 3.9 and 16.8 Hz, 1H), 4.23-4.27 (m, 1H), 4.34-4.48 (m, 2H), 4.60-4.71 (m, 5H), 5.25-5.37 (m, 4H), 5.82 (d, *J* = 8.9 Hz, 1H), 5.84-6.02 (m, 2H), 7.27-7.76 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 36.8, 47.3, 50.7, 65.9, 66.6, 67.5, 118.9, 119.1, 120.2, 125.3, 127.2, 127.9, 131.6, 131.8, 141.5,

143.9, 144.0, 156.9, 170.8, 171.1; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{25}H_{25}NO_6Na$: 458.1574; found: 458.1587.

Fmoc-Glu(OAll)-OAll (6)

1.76 g; 72% yield; R_f(HPLC) 23.56 min; Purity >97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.74; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.97-2.06 (m, 1H), 2.19-2.27 (m, 1H), 2.39-2.46 (m, 2H), 4.06-4.13 (m, 1H), 4.17-4.23 (m, 3H), 4.56 (d, *J* = 4.9 Hz, 2H), 4.61 (d, *J* = 5.5 Hz, 2H), 5.18-5.34 (m, 4H), 5.45 (d, *J* = 6.0 Hz, 1H), 5.80-5.95 (m, 2H), 7.23-7.75 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.8, 30.4, 47.4, 53.6, 65.5, 66.4, 67.3, 118.6, 119.3, 120.2, 125.3, 125.6, 127.3, 127.9, 131.4, 132.0, 141.5, 143.9, 131.6, 132.2, 156.1, 171.7, 172.5; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{26}H_{27}NO_6Na$: 472.173; found: 472.1737.

Cbz-Asp(OTMSE)-OTMSE (7)

2.71 g; 78% yield; R_f(HPLC) 26.78 min; Purity >97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.84; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.02 (s, 18H), 0.94-1.02 (m, 4H), 2.77-3.04 (m, 2H), 4.14-4.27 (m, 4H), 4.55-4.62 (m, 1H), 5.12 (s, 2H), 5.75 (d, *J* = 8.4 Hz), 7.31-7.37 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = -1.6, 17.3, 36.8, 50.5, 63.3, 64.3, 67.0, 128.1, 128.2, 128.5, 136.2, 155.9, 170.8, 170.9; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{22}H_{37}NNaO_6Si_2$: 490.2052; found: 490.2077.

Cbz-Asp(OAll)-OH (8)[15a]

386 mg; 87% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.90 (dd, *J* = 4.1 and 17.1 Hz, 1H), 3.00 (dd, *J* = 3.9 and 17.0 Hz, 1H, 4.49 (d, 2H, *J* = 3.0 Hz) 4.55-4.63 (m, 1H), 5.02 (s, 2H), 5.10-5.22 (m, 2H), 5.71-5.86 (m, 1H), 5.90 (d, *J* = 8.1 Hz, 1H), 7.21-7.25 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 36.7, 50.5, 66.0, 67.5, 118.8, 128.4, 128.5, 128.7, 131.5, 136.2, 156.4, 170.8, 175.2; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{15}H_{17}NO_6Na$: 330.0948; found: 330.0991.

Cbz-Glu(OAll)-OH (9)

375 mg; 85% yield; R_f(HPLC) 16.75 min; Purity >96%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.27; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.94-1.99 (m, 1H), 2.10-2.15 (m, 1H), 2.33-2.39 (m, 2H), 4.24-4.27 (m, 1H), 4.35 (d, *J* = 6.0 Hz, 2H), 5.01 (s, 2H), 5.13-5.25 (m, 2H), 5.75 (d, *J* = 7.9 Hz, 1H), 5.74-5.85 (m, 1H), 7.19-7.26 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.2, 39.9, 53.4, 65.7, 67.5, 118.7, 128.3, 128.5, 128.7, 132.0, 136.1, 156.4, 177.4, 178.4; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{16}H_{19}NO_6Na$: 344.1104; found: 344.1125.

Boc-Asp(OAll)-OH (10)[15c]

379 mg; 87% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.37 (s, 9H), 2.80 (dd, *J* = 4.1 and 17.3 Hz, 1H), 3.00 (dd, *J* = 3.9 and 16.8 Hz, 1H), 4.37-4.54 (m, 3H), 5.18-5.27 (m, 2H), 5.58 (d, *J* = 7.9 Hz, 1H), 5.78-5.97 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 28.6, 36.7, 50.0, 65.8, 80.6, 118.7, 131.8, 155.8, 170.9, 175.4; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{12}H_{19}NO_6Na$: 296.1104; found: 296.1134.

Boc-Glu(OAll)-OH (11)[15d]

374 mg; 86% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.37 (s, 9H), 1.93-1.98 (m, 1H), 2.11-2.20 (m, 1H), 2.38-2.43 (m, 2H), 4.18-4.32 (m, 1H), 4.53 (d, *J* = 6.1 Hz, 2H), 5.15-5.28 (m, 3H), 5.77-5.89 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.4, 28.3, 30.3, 53.0, 65.5, 118.5, 132.0, 132.2, 156.4, 172.0, 175.7; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{13}H_{21}NO_6Na$: 310.1261; found: 310.1292.

Fmoc-Asp(OAll)-OH (12)[15c]

351 mg; 77% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.97 (dd, *J* = 4.1 and 17.0 Hz, 1H), 2.98 (dd, *J* = 3.9 and 17.0 Hz, 1H), 4.06-4.14 (m, 1H), 4.26-4.32 (m, 2H), 4.50 (d, *J* = 6.0 Hz, 2H), 4.59-4.64 (m, 1H), 5.11-5.23 (m, 2H), 5.73-5.83 (m, 1H), 5.88 (d, *J* = 7.2 Hz, 1H), 7.15-7.65 (m, 8H), 9.75 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 36.6, 47.0, 50.3, 65.8, 67.6, 118.8, 120.3, 125.1, 127.7, 128.2, 131.3, 140.8, 143.6, 156.2, 170.7, 175.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{22}H_{21}NO_6Na$: 418.1261; found: 418.1297.

Fmoc-Glu(OAll)-OH (13)[15c]

352 mg; 78% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.94-2.03 (m, 1H), 2.15-2.20 (m, 1H), 2.33-2.40 (m, 2H), 4.12-4.17 (m, 1H), 4.30-4.38 (m, 3H), 4.51 (d, *J* = 4.5 Hz, 2H), 5.14-5.27 (m, 2H), 5.52 (d, *J* = 7.2 Hz, 1H), 5.80-5.95 (m, 2H), 7.19-7.70 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 27.6, 30.3, 47.3, 53.6, 65.6, 67.1, 118.6, 120.1, 125.2, 127.2, 127.9, 132.2, 141.5, 143.8, 156.3, 172.5, 207.1; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{23}H_{23}NO_6Na$: 432.1423; found: 432.1432.

Cbz-Asp(OTMSE)-OH (14)

350 mg; 85% yield; R_f(HPLC) 20.75 min; Purity >98% R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.42; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.00 (s, 9H), 0.92-0.98 (dd, *J* = 8.7 Hz, 2H), 2.77-3.05 (m, 2H), 4.13-4.19 (dd, *J* = 8.7 Hz, 2H), 4.62-4.68 (m, 1H), 5.10 (s, 2H), 5.80 (d, *J* = 8.4 Hz), 7.23-7.32 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = -1.6, 17.3, 36.6, 50.3, 63.7, 67.3, 128.1, 128.2, 128.5, 136.0, 156.1, 171.3; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{17}H_{26}NO_6Si$: 368.1524; found: 368.1522.

Cbz-Asp-OMe (15)[15e]

Alcalase-CLEA (3 g) was added to a mixture of Cbz-Asp-OH (0.5 g) in methyl *tert*-butyl ether (MTBE, 28 mL), MeOH (2 mL) and 3 Å molecular sieves (2 g). The reaction mixture was shaken at 50^{o}C at 150 rpm for 16 h. After filtration, the enzyme was washed thoroughly with 0.1 N HCl (50 mL, 3×) and EtOAc (3× 50 mL) and removed by filtration. The combined organic layers were washed with 0.1 N HCl (100 mL), dried (Na_2SO_4) and concentrated *in vacuo*. The resulting oil was redissolved in CH_2Cl_2/MeOH/acetic acid (20 mL, 89.9/10/0.1, v/v/v) and passed through a short silica gel column. The effluent was concentrated *in vacuo* followed by co-evaporation with toluene (50 mL, 2×) and $CHCl_3$ (50 mL, 2×). 485 mg; 93% yield; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.55-2.78 (m, 2H), 3.33 (s, 3H), 4.38-4.46 (m, 1H), 5.04 (s, 2H), 7.29-7.40 (m, 5H), 7.76 (d, *J* = 8.1 Hz, 1H), 12.5 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 35.8, 50.4, 52.1, 65.5, 127.6, 127.8, 128.3, 136.7, 155.7, 171.3, 171.6.

Cbz-Asp(O^{t}Bu)-OMe (16a)

Cbz-Asp-OMe (281 mg, 1.0 mmol) was dissolved in CH_3CN/^{t}BuOH (60 mL, 2/1, v/v). Subsequently, di-*tert*-butyl dicarbonate (218 mg, 1.0 mmol), pyridine (81 µL, 1.0 mmol) and (*N,N*)-dimethylaminopyridine (5 mg) were added. The reaction mixture was stirred at rt for 24 h and subsequently concentrated *in vacuo*. The residue was dissolved in EtOAc (50 mL) and the obtained solution was washed with sat. aq. $NaHCO_3$ (50 mL), 0.1 N HCl (50 mL, 2×), brine (50 mL), filtered over basic alumina, dried (Na_2SO_4) and concentrated *in vacuo*. 314 mg; 94% yield; R_t(HPLC) 24.23 min; Purity >96%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.73; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.35 (s, 9H), 2.63-2.90 (m, 2H), 3.68 (s, 3H), 4.49-4.55 (m, 1H), 5.06 (s, 2H), 5.66 (s, *J* = 8.1 Hz, 1H), 7.19-7.29 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 28.0, 37.9, 50.5, 52.6, 67.1, 81.8, 128.1, 128.2, 128.5, 136.2, 156.0, 169.9, 171.4. FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{17}H_{23}NNaO_6$: 360.1418; found: 360.1433.

Cbz-Asp(OAll)-OMe (16b)

Cbz-Asp-OMe (281 mg, 1.0 mmol) was dissolved in of EtOAc (50 mL) and to this solution EDC.HCl (209 mg, 1.1 mmol), *N,N*-diisopropylethylamine (192 µL, 1.1 mmol) and All-OH (2.0 mL) were added. The obtained reaction mixture was stirred for 24 h at rt. Then, the EtOAc solution was washed with sat. aq. $NaHCO_3$ (50 mL), 0.1 N HCl (50 mL, 2×), brine (50 mL), filtered over basic alumina, dried (Na_2SO_4) and concentrated *in vacuo*. 292 mg; 92% yield; R_t(HPLC) 23.55 min; Purity >98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.70; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.69-2.90 (m, 2H), 3.63 (s, 3H), 4.44-4.56 (m, 3H), 5.04 (s, 2H), 5.17-5.33 (m, 2H), 5.82-5.95 (m, 2H), 7.30-7.37 (m, 5H), 7.84 (d, *J* = 8.1 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 35.7, 50.3, 52.2, 64.7, 65.6, 117.7, 127.6, 127.8, 128.3, 132.3, 136.7, 155.7, 169.4, 171.3. FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{16}H_{19}NNaO_6$: 344.1105; found: 344.1087.

Cbz-Asp(O^{t}Bu)-OH (17a)[15f]

435 mg; 91% yield; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 1.37 (s, 9H), 2.55-2.73 (m, 2H), 4.30-4.38 (m, 1H), 5.04 (s, 2H), 7.30-7.39 (m, 5H), 7.60 (d, *J* = 8.7 Hz, 1H), 12.8 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 27.5, 37.2, 50.5, 65.4, 80.2, 127.6, 127.7, 128.2, 136.9, 155.7, 169.1, 172.4.

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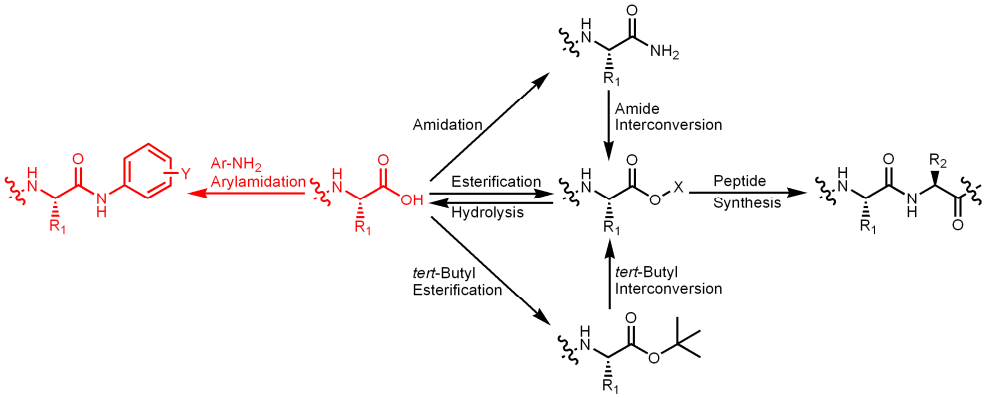
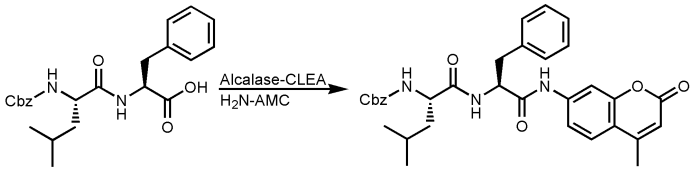
Chapter 4

Enzymatic synthesis of amino acid and peptide *C*-terminal α-arylamides



In this chapter, a mild and cost-efficient chemo-enzymatic method for the synthesis of *C*-terminal arylamides of amino acid and peptides is described using Subtilisin A. *C*-terminal arylamides of *N*-Cbz protected amino acids and peptides could be obtained from the corresponding *C*-terminal carboxylic acids, methyl or benzyl esters, in high chemical, enantio- and diastereomeric purity. The isolated yield ranged between 50% and 95% depending on the size of the aryl substituents and the presence of electron withdrawing substituents. Complete *C*-terminal α-carboxylic acid selectivity could be obtained even in the presence of various unprotected side chain functionalities such as β/γ-carboxyl, hydroxyl and guanidino groups. Also the use of the cysteine protease Papain and the lipase *Candida antartica* lipase B gave anilides in high yield. The chemo-enzymatic synthesis of arylamides proved to be completely free of racemization, in contrast to the state-of-the-art chemical methods.



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4.1 Introduction

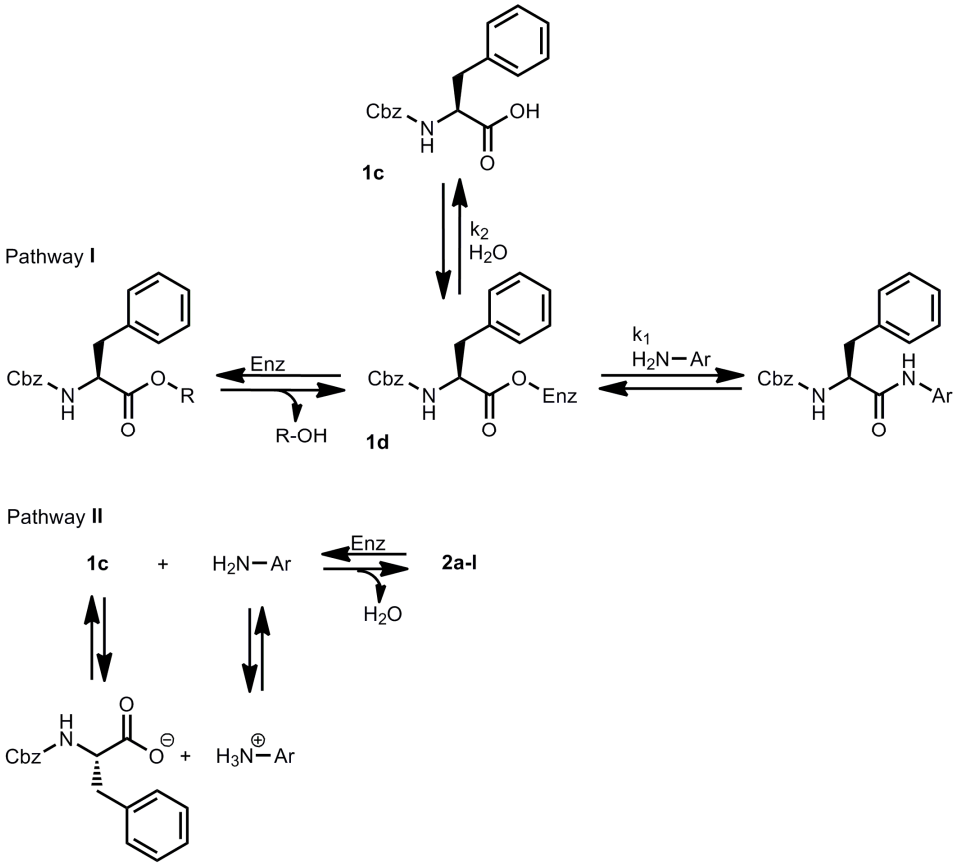
Arylamides, a class of carboxamides, are used in a wide variety of applications such as in polymers, peptidomimetics, dendrimers, scaffolds, inhibitors and as cell-signaling molecules.[1] A special class is formed by the *C*-terminal amino acid or peptide arylamides, which are often used as substrates in chromogenic, fluorogenic or amperogenic enzymatic assays.[2] For this application, the arylamides should be optically pure. *p*-Nitroaniline (*p*NA, absorbtion at 405 nm) is often used for chromogenic assays and 7-amino-4-methylcoumarin (AMC, emission at 518 nm) for fluorogenic assays. For instance, over 1000 *C*-terminal peptide *p*NA and AMC arylamide substrates have been used for the determination of the activity of enzymes involved in blood coagulation.[3] Chemical methods for their preparation have been investigated for decades and require highly reactive carboxylic acid derivatives for reaction with a free arylamine, due to its weak nucleophilicity. For example, the preparation of *N*-protected amino acid *p*NA derivatives required the use of $POCl_3$ in pyridine.[4] Often, most conventional coupling strategies, *e.g.* the DCC (*N*,*N*'-dicyclohexylcarbodiimide) method, do not result in satisfying yields.[5] Very commonly used in the past was the mixed anhydride method, which gave higher yields, but also a high degree of racemization of the *C*-terminal amino acid residue.[6] This racemization could only be partially suppressed by the addition of copper(II)chloride.[7] Other methods based on reactive condensing agents, *i.e.* reactive phosphorus or boron derivatives, also led to significant racemization.[8] It appeared especially difficult to couple electron deficient arylamines, such as those bearing a halogen, nitro or cyano substituent. Very recently, a three step protocol for the preparation of amino acid and peptide *C*-terminal electron deficient arylamides was described, based on the reaction of an azide with a selenocarboxylate.[9] Although high yields were obtained, some racemization proved to be inevitable. Evidently, each of the known chemical methods for the preparation of *C*-terminal arylamides of amino acid and peptides suffer from some drawbacks. Besides the low yield and the racemization issue, the condensing reagents used are often hazardous, expensive and consumed in stoichiometric amounts, leading to significant amounts of waste. Additionally, all reactive amino acid side chain functionalities have to be protected to prevent side reactions.

Compared to chemical approaches, chemo-enzymatic synthesis of arylamides may have several advantages such as the use of mild conditions, high selectivity and complete absence of racemization. Moreover the coupling reactions are catalyzed by (often recyclable) enzymes and no stoichiometric amounts of expensive coupling reagents are used.[10] Furthermore, no amino acid side chain protection is needed.

There are two approaches in chemo-enzymatic amide synthesis, *i.e.* the kinetically controlled pathway and the thermodynamically controlled pathway (pathway **I** and **II**, respectively, in Scheme 1).[11]

Pathway **I**, starting from an α-amino acid ester, is usually preferred in chemo-enzymatic amide synthesis, because the energy barrier for product formation is low. Reaction of the amine with the acyl-enzyme intermediate (**1d**) leads to the desired amide. However, after formation of **1d**, water can act as a nucleophile leading to undesired hydrolysis, so ideally $k_1 >> k_2$. Nevertheless, during the course of the amidation, the amide product is hydrolyzed to the free carboxylate compound. Hence, there is an optimum in the amount of product and

for a high product yield the reaction should be stopped after this optimum has been reached. In the thermodynamically controlled pathway **II**, the energy barrier is high and the equilibrium is usually on the side of the starting materials. Furthermore, reaction rates are generally low due to the equilibrium between the protonated and (unreactive) deprotonated carboxylic acid. Manipulation of the reaction conditions, *e.g.* by crystallization of the final product during the amidation, is essential to obtain an acceptable yield of the desired arylamide.



Scheme 1. Kinetically (pathway **I**) and thermodynamically (pathway **II**) controlled enzymatic peptide synthesis.

The thermodynamically controlled chemo-enzymatic synthesis of *C*-terminal α-amino arylamides has been published in the late thirties.[12] High yields were obtained using proteases in buffered aqueous systems due to rapid precipitation of the products. However, this method is limited to arylamines that are relatively strong nucleophiles such as aniline, and hydrophobic amino acids are required to obtain precipitation of the product. Furthermore, in the case that peptides are *C*-terminally arylamidated, simultaneous hydrolysis of the peptide bond occurs. Kato *et al.* describe[13] the kinetically controlled chemo-

enzymatic arylamidation with the less nucleophilic *p*NA, using Subtilisin A in organic solvents with a water content of 5%; however, their reported yields do not exceed 25%.
In this chapter a versatile, mild, high yielding, selective and racemization-free chemo-enzymatic strategy for the synthesis of amino acid and peptide *C*-terminal arylamides is described. This process is either kinetically or thermodynamically driven and uses the protease Subtilisin A, or the lipase *Candida antarctica* lipase-B (Cal B), both in organic solvents, or the protease Papain in a two phase system.

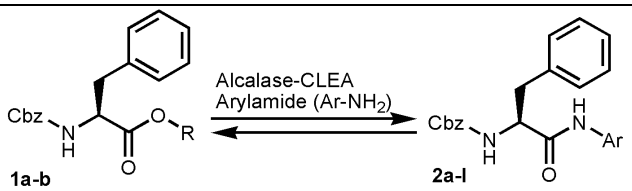
4

4.2 Results and Discussion

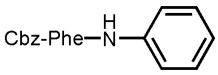
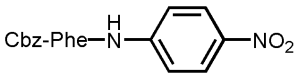
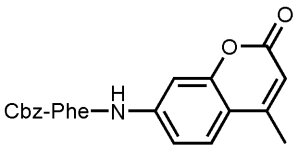
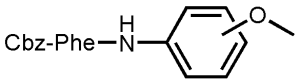
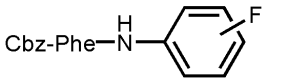
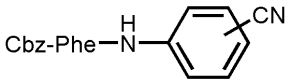
Subtilisin A (Alcalase),[14] a cheap industrial serine protease from *Bacillus licheniformis*, has been extensively used for various hydrolytic reactions and condensations. This biocatalyst displays a relatively broad substrate tolerance and has been used in kinetically controlled enzymatic peptide synthesis.[15] High yields are usually obtained due to the stability of the enzyme in nearly anhydrous organic solvents (water contents <1 wt%) thereby minimizing product and/or ester hydrolysis.[16] Therefore, Alcalase was selected in a first attempt to synthesize arylamides via the kinetically controlled pathway using Cbz-Phe-OMe (**1a**) as model substrate and aniline as arylamine.
When Alcalase was isolated and dried by precipitation with ^{t}BuOH as described by Chen *et al*.[17] followed by addition to Cbz-Phe-OMe (**1a**) in pure aniline, the yield of arylamide **2a** did not exceed 27% (based on HPLC analysis). An equilibrium mixture of arylamide **2a** and hydrolyzed starting material Cbz-Phe-OH (**1c**) was observed, and the water content proved to be critical for the yield of the arylamide. Surprisingly, the addition of 4 Å molecular sieves resulted in almost complete (>95%, HPLC analysis) conversion to arylamide **2a** with the enzyme still remaining active. The increased yield may be explained by the fact that not only water but also MeOH is trapped by 4 Å molecular sieves. After work-up, NMR analysis showed contaminations derived from the Alcalase solution. When Alcalase cross-linked enzyme aggregates (CLEA)[18] were used, equally high yields of **2a** could be obtained starting from **1a** or benzylester **1b** (Table 1, entries 1 and 2), without any contaminations in the final product. Sufficiently dry Alcalase-CLEA was obtained by washing once with ^{t}BuOH. After the reaction, Alcalase-CLEA was conveniently removed by simple filtration.
In subsequent amidations the amount of arylamine was decreased and the co-solvents toluene, tetrahydrofuran (THF) or methyl *tert*-butyl ether (MTBE) were used instead. Surprisingly, the thermodynamic approach (**II**) using the free carboxylic acid Cbz-Phe-OH (**1c**) and anhydrous aniline in the presence of 3 Å molecular sieves gave equally high yields of the arylamide **2a** (Table 1, entry 3). In addition, reaction times were comparable to those starting from an amino acid ester (entries 1 and 2). In order to broaden the scope of these amidation reactions, it was investigated whether the weakly nucleophilic *p*NA (pK_a = 1.0, vs. pK_a = 5.2 for aniline), and also the sterically demanding AMC derivative, as well as a number of methoxy-, fluoro- and cyano-substituted anilines could be used in the Alcalase-mediated arylamidations (see Table 1, entries 4-14). Satisfyingly, high yields of arylamides (**2b-l**) were obtained, although the efficiency of the coupling reaction in the presence of *p*NA and AMC was clearly lower than in the case of aniline. To verify the absence of racemization during the enzymatic arylamidation, e.e.'s was determined by chiral HPLC and compared to

chemically synthesized arylamides (see Table 1).[19] As expected, no detectable racemization was observed using the enzymatic reactions, in sharp contrast to the conventional chemical arylamidations using PCl_3 in pyridine, where significant racemization was observed (1-5%).

Table 1. Yields and enantiomeric excess (e.e.) of synthesized arylamides using Alcalase-CLEA as biocatalyst.



a: R = Me
b: R = Bn

Entry[a]	Precursor		Amide product		Yield (%)[b]	e.e. (%)[c], (%)[d]
1	Cbz-Phe-OMe	**1a**		**2a**	93	n.d.[e]
2	Cbz-Phe-OBn	**1b**		**2a**	94	n.d.
3	Cbz-Phe-OH	**1c**		**2a**	93	>99.5, 98.9
4[f]	Cbz-Phe-OH	**1c**	 (*p*NA)	**2b**	65	>99.5, 96.2
5	Cbz-Phe-OH	**1c**	 (AMC)	**2c**	51	>99.5, 95.7
6				**2d** (*o*)	87	n.d.
7	Cbz-Phe-OH	**1c**		**2e** (*m*)	86	n.d.
8				**2f** (*p*)	88	n.d.
9				**2g** (*o*)	85	n.d.
10	Cbz-Phe-OH	**1c**		**2h**(*m*)	79	n.d.
11				**2i** (*p*)	81	n.d.
12				**2j** (*o*)	70	>99.5, 97.6
13	Cbz-Phe-OH	**1c**		**2k**(*m*)	84	>99.5, 97.7
14				**2l** (*p*)	65	>99.5, 97.2

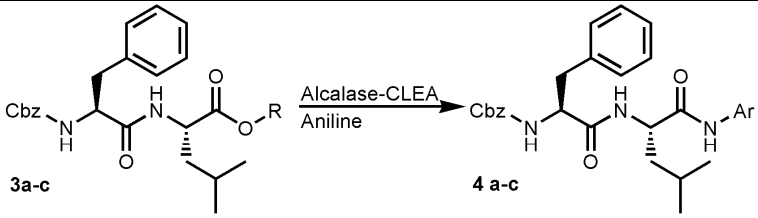
[a] Conditions: Alcalase-CLEA, MTBE, arylamide, 50°C, 16 h; [b] Isolated yields based on the acyl donor; [c] enzymatically synthesized arylamide; [d] chemically synthesized arylamide following the literature procedure;[19] [e] Not determined; [f] Reaction was performed in THF.

At this point it was important to address some industrially relevant aspects of these enzymatic conversions. Firstly, it was proved that, in order to obtain the required anhydrous conditions, the use of molecular sieves, which are unamenable for use at large scale, could be replaced by azeotropic distillation. For instance, the conversion of **1c** to **2a** using 1:1 (v/v) aniline/toluene proceeded to > 95% when toluene was continuously added and evaporated at reduced pressure. Secondly, it was demonstrated that Alcalase-CLEA, after converting **1c**

to **2a** could be easily separated from the reaction mixture by a rapid filtration and washing step.

Of even greater importance than amino acids, are *C*-terminal arylamides of peptides since they are pre-eminently used as substrates for enzymatic assays. Using both the thermodynamic (**II**) and the kinetic (**I**) approach, dipeptides **3a**-**c** were smoothly converted into the corresponding *C*-terminal anilides **4a-c** (Table 2, entries 1-5, 77-94% yield). No side products were detected by analytical HPLC, indicating that no peptidic bond hydrolysis nor transamidation had occurred in all cases. In addition, no detectable racemization of the Leu residue had taken place as was demonstrated by peptide hydrolysis followed by chiral HPLC of the resulting amino acids. This is in sharp contrast to the corresponding chemical conversions[19] (using PCl_3) were inevitable racemization occurs leading to e.e. values of 96% of each residue in the dipeptide.

Table 2. Yields of dipeptide arylamides and e.e.s of the amino acid residues.



3a-c

3a: R = Me
3b: R = Bn
3c: R = H

4 a-c

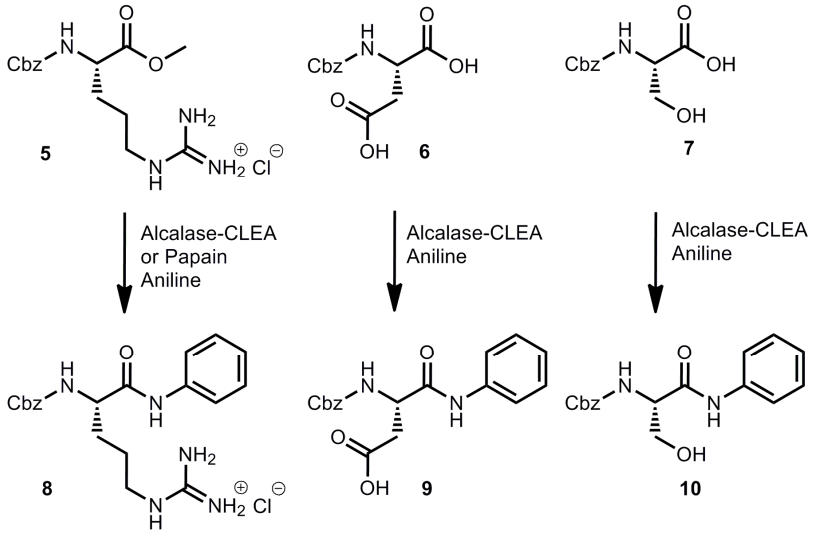
4a: Ar = anilide
4b: Ar = *p*NA
4c: Ar = AMC

Entry[a]	Precursor		Amide product		Yield (%)[b]	e.e. (%)[c], (%)[d]
1	Cbz-Phe-Leu-OMe	**3a**	Cbz-Phe-Leu-Anilide	**4a**	93	n.d.[e]
2	Cbz-Phe-Leu-OBn	**3b**	Cbz-Phe-Leu-Anilide	**4a**	94	n.d.
3	Cbz-Phe-Leu-OH	**3c**	Cbz-Phe-Leu-Anilide	**4a**	93	>99.5,[f] 95.7[g] >99.5,[f] 96.8[g]
4	Cbz-Phe-Leu-OH	**3c**	Cbz-Phe-Leu-*p*NA	**4b**	83	n.d.
5	Cbz-Phe-Leu-OH	**3c**	Cbz-Phe-Leu-AMC	**4c**	77	n.d.

[a] Conditions: Alcalase-CLEA, MTBE, aniline, 50°C, 16 h; [b] Isolated yields based on the acyl donor; [c] enzymatically synthesized arylamide; [d] chemically synthesized[19] arylamide; [e] Not determined; [f] Phe moiety; [g] Leu moiety.

Besides the absence of racemization, another advantage of the enzymatic arylamidation is that no protection of the amino acid side chains is required. This is again in contrast to chemical coupling methods where reactive side chain functionalities such as the β- or γ-carboxyl group of aspartic and glutamic acid respectively, the hydroxyl function of serine, threonine or tyrosine and the guanidino function of arginine needs protection. In chapter 2 and 3 it was shown that Alcalase-CLEA can be used for the regioselective esterification[20] and hydrolysis[21] of Asp and Glu derivatives. This chapter illustrates the smooth and regioselective conversion of Cbz-Arg-OMe **5**, Cbz-Asp-OH **6** and Cbz-Ser-OH **7** to the corresponding *C*-terminal anilides **8**, **9** and **10** (Table 3, entries 1-4).

Table 3. Yields of synthesized arylamides of Cbz-Arg-OMe, Cbz-Asp-OH and Cbz-Ser-OH.



Entry[a]	Precursor		Enzyme	Product	Yield (%)[b]
1	Cbz-Arg-OMe[c]	**5**	CLEA	**8**	54
2	Cbz-Arg-OMe	**5**	Papain	**8**	82
3	Cbz-Asp-OH	**6**	CLEA	**9**	91
4	Cbz-Ser-OH	**7**	CLEA	**10**	93

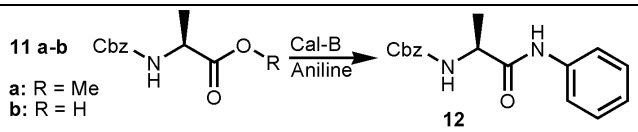
[a] Conditions: Alcalase-CLEA, THF (entry 1) or MTBE (entries 3 and 4), aniline, 50°C, 16 h; Papain (entry 2), phosphate buffer, aniline, 1,4-dithiothreitol, rt, 10 min; [b] Isolated yields are based on the acyl donor. [c] Cbz-Arg-OMe was used instead of Cbz-Arg-OH due to the low solubility of the latter in anhydrous organic solvents.

Very high yields of anilides **9** and **10** (Table 3, entries 3 and 4, 91-93%) were obtained using Alcalase-CLEA. For Cbz-Asp-OH (**6**) the α-*C*-terminal selectivity appeared to be 100% based on comparison (HPLC and NMR) with the chemically synthesized reference compound. In the arylamidation of Cbz-Ser-OH (**7**) only a small amount (2%) of the side chain ester was formed, as was demonstrated by LC-MS. However, Alcalase does not favor positively charged amino acids at the P_1 position[22] in the active site, resulting in only a moderate yield of **8** (Table 3, entry 1, 54%). To remedy this, the widely employed and commercially available cysteine protease Papain was used, which does favor positively charged amino acids at its P_1 position. The application of this enzyme in pure aniline did not result in any conversion of **5**, since Papain is not active in nearly anhydrous organic solvents. Therefore, a two-phase system consisting of aniline and phosphate buffer (pH = 7.5) was used and a high yield of anilide **8** (Table 3, 82%) was readily obtained after only 10 min reaction time.

In order to broaden the scope of this technology beyond proteases, also the use of lipases was explored. It was envisioned that Cal-B would be promising in this application since it has a broad substrate tolerance for aliphatic acids[23] and is known to be active in the transesterification of amino acids,[24] and it maintains a high activity in anhydrous organic solvents.[25] Hence, Cbz-Ala derivatives **11a** and **11b** were subjected to Cal-B and aniline

under anhydrous conditions. Indeed, the corresponding anilide **12** was obtained in high yield (Table 4, entries 1 and 2).

Table 4. Yields of synthesized Cbz-Ala arylamide using Cal-B.



Entry	Precursor		Product	Yield (%)[b]	e.e. (%)
1	Cbz-Ala-OMe	**11a**	**12**	92	>99.5
2	Cbz-Ala-OH	**11b**	**12**	77	>99.5

[a] Conditions: Cal-B, MTBE, aniline, 50°C, 16 h. [b] Isolated yields based on the acyl donor.

To conclude, in this chapter it was shown that *N*-terminal protected amino acids and peptides can be enzymatically converted in high yield into various *C*-arylamides under (nearly) anhydrous conditions, even if the arylamine has an extremely low nucleophilicity or is sterically very demanding. Although Alcalase readily accepted most *C*-terminal moieties in its active site, in some cases, for instance with *C*-terminal positively charged arginine residues, the use of Papain in a two-phase system gave more satisfactory results. Starting from either *C*-terminal free carboxylic acids or *C*-terminal alkyl esters, high yields were obtained and in case of dipeptides, no peptide bond hydrolysis or transamidation occurred. In contrast to the state-of-the-art chemical methods, no racemization occurred at the *C*-terminal position and no amino acid side chain protection was required. Thus, this chapter describes an enzymatic method which is a significant step forward toward the synthesis of this important class of compounds. Since the (nearly) anhydrous conditions can be obtained by (azeotropic) distillation this technology is also amenable to scale-up.

4.3 Experimental

General:

General experimental information is identical to chapter 2 and 3 with the following exceptions. Cal-B was purchased from Novozymes (immobilized Novozym® 435, LC 200204) and Papain from Merck (lyophilized powder from *Carica papaya*, Art 7144, 333 F677044). Melting points were determined on an automated cell coupled to a central processor. Samples were measured in triplo using a temperature gradient of 1°C/min from 150°C to 250°C. To determine the e.e. of the *N*-Cbz protected Phe and Ala residues in arylamide derivatives, the samples were suspended in excess 6 N HCl and kept at 80°C overnight. Chiral HPLC was performed on a crownether (+) column (150 × 4.0 mm, 5 µm particle size) at 25°C with 30 mM aqueous $HClO_4$ (pH = 2.0) as the eluent. UV detection was performed at 210 nm using a UV-VIS linear spectrometer. The flow was 1 mL/min. Injection volumes were 5 µL. R_t (D-Phe) = 6.90 min, R_t (L-Phe) = 8.82 min, R_t (D-Ala) = 2.22 min, R_t (L-Ala) = 2.77 min. To determine the e.e. of the Phe and Leu residues in samples of **4a-c**, the samples were suspended in 6 N HCl and refluxed overnight. Chiral HPLC was

performed on a crownether column (150 × 4.0 mm, 10 µm particle size) at 22°C with 50 mM $HClO_4$ (pH = 1.5) as the eluent. The flow was 0.8 mL/min. Post-column derivatization was carried out with 1,2-phthalic dicarboxyaldehyde and mercaptoethanol in borate buffer (pH = 9.5) at room temperature in a 1 mL reaction coil with spiralized PEEK, with a flow of 0.8 mL/min. Fluorescence detection was performed at 338 nm excitation wavelength and > 420 nm emission wavelength. Injection volumes were 5 µL. R_t (D-Leu) = 6.3 min, R_t (L-Leu) = 10.6 min, R_t (D-Phe) = 13.5 min, R_t (L-Phe) = 18.3 min. Optical rotations were measured at 20°C using a sodium ray, $[\alpha]^{20}_D$.

The analyses of Cbz-Phe-OMe (**1a**), Cbz-Phe-OBn (**1b**) Cbz-Phe-Leu-OMe (**3a**) and Cbz-Phe-Leu-OBn (**3b**) are described chapter 2.

Synthesis of arylamides 2a and 4a

Alcalase-CLEA (500 mg) was added to a mixture containing MTBE (4.5 mL), aniline (500 µL, 4.48 mmol), **1a-c** or **3a-c** (0.45 mmol) and 3 Å molecular sieves (200 mg). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. Then, the reaction mixture was filtered over a P4 glass filter and the solid enzyme particles were resuspended in EtOAc and removed by filtration; (50 mL, 3×). Distilled water (150 mL) was added to the combined filtrates and under vigorous stirring the pH was adjusted to 3.0 with 1 N HCl. After an additional 10 min of stirring, the two layers were separated. This procedure of washing the organic layer with an aqueous phase (pH 3.0) was repeated twice. Subsequently, the organic phase was washed with sat. aq. $NaHCO_3$ (100 mL, 3×), brine (100 mL), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was triturated with *n*-heptane (2 mL) and the resulting crystals were isolated by filtration and washed with cold *n*-heptane (5 mL, 2×).

Cbz-Phe-anilide (2a)[26a]

156 mg; 93%; R_t(HPLC) 19.65 min, Purity >99%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.55; Mp: 169°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.05-3.11 (m, 2H), 4.41-5.52 (m, 1H), 5.02 (s, 2H), 5.43 (d, *J* = 6.0 Hz, 1H), 7.00-7.27 (m, 15H), 7.59 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 38.6, 57.2, 67.3, 120.1, 124.6, 127.2, 128.0, 128.3, 128.6, 128.9, 129.3, 136.0, 136.3, 137.1, 169.1, 182.0; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{23}H_{23}N_2O_3$: 375.1703; found: 375.1692 IR(neat/cm^{-1}): 3285, 1686, 1654, 1600, 1531, 1494, 1445, 1284, 1260, 1243.

Cbz-Phe-Leu-anilide (4a)

205 mg; 93% yield; R_t(HPLC) 21.08 min, Purity 99%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.52; Mp: 225°C; $[\alpha]^{20}_D$ = +16.8 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.81 (dd, *J* = 6.0 Hz, 6H), 1.34-1.50 (m, 2H), 1.65-1.76 (m, 1H), 2.95-3.03 (m, 2H), 4.30-4.51 (m, 2H), 5.00 (s, 2H), 5.21 (d, *J* = 6.6 Hz, 1H), 6.25 (d, *J* = 7.2 Hz, 1H), 7.00-7.27 (m, 13H), 7.47 (d, *J* = 7.5 Hz, 2H), 8.25 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 21.7, 22.4, 24.5, 38.4, 41.0, 51.6, 51.9, 55.7, 66.6, 126.5, 127.6, 127.8, 128.2, 129.2, 136.1, 136.3, 155.9, 171.0, 172.7; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{29}H_{34}N_3O_4$: 488.2543; found: 488.2559; IR(neat/cm^{-1}): 3281, 1692, 1641, 1531, 1495, 1447, 1285, 1258, 1234.

Synthesis of arylamides 2b and 4b.
Alcalase-CLEA (500 mg) was added to a mixture containing THF (5 mL), *p*NA (500 mg, 3.6 mmol), **1c** or **3c** (0.33 mmol) and 3 Å molecular sieves (200 mg). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. The reaction mixture was filtered over a P4 glass filter and the solid enzyme particles were resuspended in EtOAc and removed by filtration (50 mL, 3×). The combined filtrate was concentrated *in vacuo* and purified by preparative HPLC.

Cbz-Phe-*p*NA (2b)[26b]
91 mg; 65% yield; R_t(HPLC) 20.29 min, Purity 99%; Rf(EtOAc/*n*-hexane, 1/1, v/v) 0.58; Mp: 159°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.98-3.07 (m, 2H), 4.43-4.51 (m, 1H), 4.98 (s, 2H), 5.37 (d, *J* = 7.8 Hz, 1H), 7.07 (dd, *J* = 2.1 and 5.4 Hz, 2H), 7.14-7.24 (m, 8H), 7.33 (d, *J* = 9.3 Hz, 2H), 7.96 (d, *J* = 9.0 Hz, 2H), 8.37 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 38.0, 57.3, 67.6, 119.2, 124.9, 127.4, 127.9, 128.5, 128.6, 129.0, 129.2, 135.6, 135.8, 143.0, 143.7, 156.6, 179.8; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]+ calcd for $C_{23}H_{21}N_3O_5Na$: 442.1373; found: 442.1364. IR(neat/cm^{-1}): 3305, 3270, 3033, 1696, 1681, 1533, 1509, 1495, 1347, 1249, 1214.

Cbz-Phe-Leu-*p*NA (4b)
147 mg; 83% yield; R_t(HPLC) 20.93 min, Purity 96%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.60; Mp: 196°C; $[\alpha]^{20}_D$ = +26.2 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 0.98 (dd, *J* = 6.3 and 10.2 Hz, 6H), 1.55-1.80 (m, 3H), 2.76--3.11 (m, 2H), 4.35-4.43 (m, 1H), 4.51-4.59 (m, 1H), 5.01 (s, 2H), 7.20-7.54 (m, 10H), 7.52 (d, *J* = 8.7 Hz, 1H), 7.93 (d, *J* = 9.0 Hz, 2H), 8.29 (d, *J* = 9.3 Hz, 1H), 8.36 (d, *J* = 7.5 Hz, 1H), 10.7 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 21.5, 22.8, 24.2, 37.2, 52.2, 55.7, 65.0, 118.9, 124.8, 126.1, 127.5, 127.8, 128.1, 129.1, 136.9, 137.8, 142.2, 144.9, 155.7, 171.6, 171.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{29}H_{33}N_4O_6$: 533.2395; found: 533.2411; IR(neat/cm^{-1}): 3277, 2958, 1691, 1647, 1532, 1515, 1341, 1300, 1254.

Synthesis of arylamides 2c-l and 4c.
Alcalase-CLEA (500 mg) was added to a mixture containing MTBE (4.5 mL), the corresponding arylamine (500 mg), **1c** or **3c** (100 mg, 0.33 mmol) and 3 Å molecular sieves (200 mg). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16h and subsequently filtered over a P4 glass filter. The solid enzyme particles were resuspended in EtOAc and removed by filtration (50 mL, 2×). The combined filtrate was concentrated *in vacuo* and the resulting residue was purified by preparative HPLC.

Cbz-Phe-AMC (2c)[26c]
77 mg; 51% yield; R_t(HPLC) 19.54 min, Purity >99%; R_f(MeOH/CH_2Cl_2, 1/20, v/v) 0.45; Mp: 218°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.43 (s, 3H), 2.87-3.12 (m, 2H), 4.41-4.52 (m, 1H), 5.01 (s, 2H), 6.30 (s, 1H), 7.19-7.40 (m, 10H), 7.50 (d, *J* = 8.4 Hz, 1H), 7.74-7.85 (m, 3H), 10.58 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 17.8, 37.2, 57.0, 65.3, 105.7, 112.3, 115.1, 115.2, 125.8, 126.3, 127.4, 127.6, 128.0, 128.1, 129.1, 136.8, 137.5, 142.0, 152.9, 153.5, 155.9, 160.0, 171.2; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{27}H_{25}N_2O_5$:

457.1757; found: 457.1773; IR(neat/cm^{-1}): 3310, 1698, 1618, 1583, 1521, 1497, 1390, 1255, 1216.

Cbz-Phe-2-methoxyanilide (2d)

116 mg; 87% yield; R_t(HPLC) 20.41 min, Purity 99%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.56; Mp: 160°C; $[\alpha]^{20}_D$ = +86.2 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.80--3.16 (m, 2H), 3.81 (s, 3H), 4.50-4.58 (m, 1H), 4.93-5.05 (m, 2H), 6.92-7.40 (m, 13H), 7.77 (d, *J* = 8.4 Hz, 1H), 8.01 (d, *J* = 8.4 Hz, 1H), 9.24 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 37.0, 55.7, 56.7, 65.2, 111.1, 120.2, 121.0, 124.3, 126.2, 126.9, 127.3, 127.6, 127.9, 128.2, 129.2, 136.8, 137.8, 149.1, 155.9, 170.2; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{24}H_{25}N_2O_4$: 405.1808; found: 405.1783; IR(neat/cm^{-1}): 3291, 1685, 1656, 1598, 1531, 1499, 1444, 1326, 1263, 1246, 1051.

Cbz-Phe-3-methoxyanilide (2e)

115 mg; 86% yield; R_t(HPLC) 19.72 min, Purity 98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.54; Mp: 156°C; $[\alpha]^{20}_D$ = +70.2 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.81-3.06 (m, 2H), 3.74 (s, 3H), 4.37-4.45 (m, 1H), 4.97 (s, 2H), 6.65 (d, *J* = 7.8 Hz, 1H), 7.12-7.35 (m, 13H), 7.69 (d, *J* = 8.4 Hz, 1H), 10.10 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 37.4, 54.9, 56.8, 65.2, 105.0, 108.8, 111.5, 126.2, 127.4, 127.6, 128.0, 128.2, 129.1, 129.4, 137.7, 139.9, 155.9, 159.4, 170.4; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{24}H_{25}N_2O_4$: 405.1808; found: 405.1829; IR(neat/cm^{-1}): 3313, 1687, 1662, 1611, 1532, 1454, 1256, 1222, 1038.

Cbz-Phe-4-methoxyanilide (2f)

117 mg; 88 % yield; R_t(HPLC) 19.06 min, Purity 98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.49; Mp: 167°C; $[\alpha]^{20}_D$ = +73.6 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.85-3.12 (m, 2H), 3.77 (s, 3H), 4.38-4.48 (m, 1H), 5.01 (s, 2H), 6.93 (d, *J* = 9.0 Hz, 2H), 7.22-7.41 (m, 10H), 7.54 (d, *J* = 8.7 Hz, 2H), 7.70 (d, *J* = 8.4 Hz, 1H), 10.00 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 37.5, 55.1, 56.7, 65.2, 113.7, 120.8, 126.2, 127.4, 127.6, 127.9, 128.2, 129.1, 131.8, 136.9, 137.8, 155.2, 155.8, 169.8; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{24}H_{25}N_2O_4$: 405.1808; found: 405.1793; IR(neat/cm^{-1}): 3286, 1690, 1658, 1526, 1512, 1282, 1239, 1028.

Cbz-Phe-2-fluoroanilide (2g)

110 mg; 85% yield; R_t(HPLC) 19.87 min, Purity 99%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.62; Mp: 161°C; $[\alpha]^{20}_D$ = +76.2 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.80-3.13 (m, 1H), 4.53-4.64 (m, 1H), 4.97 (s, 2H), 7.18-7.40 (m, 13H), 7.69 (d, *J* = 8.4 Hz, 1H), 7.84 (m, 1H), 9.92 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 37.3, 56.4, 65.2, 115.3, 115.5, 124.2, 125.3, 125.4, 125.6, 125.8, 126.2, 127.4, 127.6, 127.9, 128.2, 129.2, 136.8, 137.7, 152.0, 155.3, 155.9, 170.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{23}H_{22}N_2O_3F$: 393.1608; found: 393.1586; IR(neat/cm^{-1}): 3318, 1686, 1673, 1600, 1536, 1488, 1460, 1435, 1252, 1218, 1024.

Cbz-Phe-3-fluoroanilide (2h)
102 mg; 79% yield; R_t(HPLC) 20.27 min, Purity 99%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.59; Mp: 162°C; $[\alpha]^{20}_D$ = +12.4 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.80-3.05 (m, 2H), 4.35-4.43 (m, 1H), 4.96 (s, 2H), 6.85-6.93 (m, 1H), 7.15-7.38 (m, 12H), 7.58 (d, *J* = 11.7 Hz, 1H), 7.74 (d, *J* = 7.8 Hz, 1H), 10.33 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 37.3, 56.9, 65.3, 105.8, 106.2, 109.6, 109.9, 114.9, 115.0, 126.3, 127.4, 127.6, 128.0, 128.2, 129.1, 130.2, 130.3, 136.8, 137.6, 140.4, 140.5, 155.9, 160.4, 163.6; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{23}H_{22}N_2O_3F$: 393.1608; found: 393.1589; IR(neat/cm^{-1}): 3278, 1686, 1657, 1603, 1531, 1489, 1447, 1284, 1256, 1218.

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Cbz-Phe-4-fluoroanilide (2i)
104 mg; 81% yield; R_t(HPLC) 19.87 min, Purity 98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.57; Mp: 195°C; $[\alpha]^{20}_D$ = +77.8 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.80-3.06 (m, 2H), 4.35-4.43 (m, 1H), 4.96 (s, 2H), 7.10-7.35 (m, 12H), 7.57-7.62 (m, 2H), 7.70 (d, *J* = 8.4 Hz, 1H), 10.15 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 37.4, 56.8, 65.2, 115.0, 115.3, 121.0, 121.1, 126.3, 127.4, 127.6, 128.0, 128.2, 129.1, 135.1, 136.8, 137.7, 155.8, 156.4, 159.5, 170.3; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{23}H_{22}N_2O_3F$: 393.1608; found: 393.1592; IR(neat/cm^{-1}): 3301, 3260, 1690, 1656, 1534, 1509, 1284, 1260, 1217.

Cbz-Phe-2-cyanoanilide (2j)
92 mg; 70% yield; R_t(HPLC) 18.85 min, Purity 97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.56; Mp: 173°C; $[\alpha]^{20}_D$ = +64.6 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.85-3.18 (m, 2H), 4.48-4.56 (m, 1H), 4.97 (s, 2H), 7.18-7.43 (m, 11H), 7.56 (d, *J* = 8.1 Hz, 1H), 7.73 (q, *J* = 9.3 Hz, 2H), 7.83 (d, *J* = 7.5 Hz, 1H), 10.37 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 37.2, 56.4, 65.2, 107.2, 116.5, 125.4, 125.8, 126.3, 127.4, 127.6, 127.9, 128.0, 128.1 (2C), 128.2, 129.1, 133.1, 133.7, 136.8, 137.6, 139.7, 155.9, 171.0; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{24}H_{21}N_3O_3Na$: 422.1475; found: 422.1445; IR(neat/cm^{-1}): 3294, 3262, 2230, 1667, 1582, 1530, 1448, 1284, 1260, 1246, 1055, 1041.

Cbz-Phe-3-cyanoanilide (2k)
110 mg; 84% yield; R_t(HPLC) 19.33 min, Purity 98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.49; Mp: 169°C; $[\alpha]^{20}_D$ = +55.2 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.83-3.08 (m, 2H), 4.41-4.47 (m, 1H), 4.98 (s, 2H), 7.12-7.37 (m, 10H), 7.77-7.80 (m, 5H), 10.57 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 37.1, 57.0, 65.3, 105.1, 118.9, 119.2, 126.3, 127.4, 127.6, 128.0, 128.2, 129.1, 133.1, 136.8, 137.4, 142.9, 155.9, 171.3; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{24}H_{22}N_3O_3$: 400.1656; found: 400.1627; IR(neat/cm^{-1}): 3331, 3325, 2227, 1681, 1668, 1590, 1514, 1409, 1310, 1255, 1213, 1203, 1178, 1038.

Cbz-Phe-4-cyanoanilide (2l)[26d]
86 mg; 65% yield; R_t(HPLC) 19.31 min, Purity 97%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.48; Mp: 172°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.83-3.08 (m, 2H), 4.37-4.45 (m, 1H), 4.97 (s, 2H), 7.14-7.56 (m, 12H), 7.79 (d, *J* = 6.6 Hz, 2H), 8.09 (s, 1H), 10.46 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 37.2, 56.9, 65.3, 111.4, 118.5, 121.9, 123.8, 126.3, 126.9, 127.4, 127.6, 128.0, 128.2, 129.1, 130.1, 136.8, 137.5, 139.4, 155.9, 171.1; FIA-ESI(+)-TOF-MS: *m/z* [M +

H]$^+$ calcd for $C_{24}H_{22}N_2O_4$: 400.1656; found: 400.1659; IR(neat/cm^{-1}): 3293, 3279, 2231, 1696, 1674, 1586, 1531, 1428, 1266.

Cbz-Phe-Leu-AMC (4c)

146 mg; 77% yield; R_t(HPLC) 19.77 min, Purity 98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.33; Mp: 173°C; $[\alpha]^{20}_D$ = +26.8 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.82 (dd, *J* = 2.1 and 6.6 Hz, 6H), 1.38-1.77 (m, 4H), 2.32 (s, 3H), 3.02-3.06 (m, 2H), 4.37-4.54 (m, 2H), 5.02 (dd, *J* = 7.8 Hz, 2H), 5.26 (d, *J* = 6.0 Hz, 1H), 6.09 (d, *J* = 0.9 Hz, 1H), 6.39 (d, *J* = 7.8 Hz, 1H), 7.07-7.28 (m, 11H), 7.38-7.45 (m, 2H), 7.65 (s, 1H), 8.88 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 18.5, 21.6, 23.0, 24.7, 37.7, 39.9, 52.8, 56.6, 67.6, 107.4, 113.3, 115.8, 125.0, 127.4, 128.1, 128.4, 128.6, 129.0, 129.1, 135.5, 135.7, 141.4, 152.3, 154.0, 161.2, 170.1, 171.6; FIA-ESI(+)-TOF-MS: *m/z* [M + H]$^+$ calcd for $C_{33}H_{36}N_3O_6$: 570.2599; found: 570.2599; IR(neat/cm^{-1}): 3286, 2958, 1691, 1647, 1615, 1482, 1340, 1254.

Cbz-Arg-OMe·HCl (5)[26e]

Compound **5** was synthesized according to a procedure described in the literature.[26a] Thionylchloride (2 mL, 10.4 mmol) was added dropwise to cooled (-80°C) methanol (50 mL). Subsequently, Cbz-Arg-OH·HCl (2.5 g, 7.3 mmol) was added to this solution in small portions over 30 min with the temperature maintaining at -80°C. After the addition was complete the reaction mixture was allowed to warm at rt and was subsequently stirred for 16 h. Volatiles were removed by concentration *in vacuo* and the residue was co-evaporated with MeOH. 2.61 g; quantitative yield; R_t(HPLC) 7.69 and 8.11 min, Purity 99%; R_f(CH_2Cl_2/MeOH, 9/1, v/v) 0.24; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 1.42-1.72 (m, 4H), 3.02-3.11 (m, 2H), 3.61 (s, 3H), 3.97-4.06 (m, 1H), 5.01 (s, 2H), 7.00-7.40 (m, 9H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.85 (t, *J* = 5.4 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 25.1, 27.7, 51.8, 55.4, 65.4, 127..6, 127.7, 128.2, 136.8, 156.0, 157.0, 172.5; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{15}H_{23}N_4O_4$: 323.1714; found: 323.1736.

Cbz-Arg-anilide·HCl (8)

a) Alcalase-CLEA (500 mg) was added to a mixture containing THF (4.5 mL), aniline (500 µL, 4.5 mmol, 10 equiv), **5** (145 mg, 0.45 mmol, 1 equiv) and 3 Å molecular sieves (200 mg). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h and subsequently filtered over a P4 glass filter. The solid enzyme particles were resuspended in EtOAc and removed by filtration (50 mL, 3×). The combined filtrate was concentrated *in vacuo*. The residue was triturated with distilled water (20 mL) and the resulting crystals were isolated by filtration over a P4 glass filter, washed with water (50 mL, 2×), resuspended in toluene (50 mL) and this solution was concentrated *in vacuo*.

94 mg; 54% yield; R_t(HPLC) 9.46 min, Purity 98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.31; Mp: 56°C; $[\alpha]^{20}_D$ = +44.8 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 1.40-1.90 (m, 4H), 3.08-3.15 (m,2H), 4.15-4.23 (m, 1H), 5.04 (s, 2H), 7.00-7.48 (m, 12H), 7.64-7.86 (m, 3H), 7.93 (s, 1H), 10.28 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 25.1, 28.8, 54.8, 65.3, 119.1, 123.2, 127.6, 127.7, 128.2, 128.5, 136.9, 138.9, 155.9, 156.9, 170.7; FIA-ESI(+)-TOF-MS: *m/z* [M + H]$^+$ calcd for $C_{20}H_{26}N_5O_3$: 384.2030; found: 384.2041; IR(neat/cm^{-1}): 3292, 3260, 1748, 1597, 1553, 1498, 1488, 1434, 1368, 1321, 1262.

b) Papain (10 mg) was added to a mixture containing phosphate buffer (2.5 mL, 250 mM, pH 7.5), aniline (2.5 mL), 1,4-dithiothreitol (1 mg) and **5** (145 mg, 0.45 mmol). The mixture was shaken at ambient temperature for 10 min followed by the addition of EtOAc (100 mL) and distilled water (100 mL). Under vigorous stirring the pH was adjusted to 3.0 with 1 N HCl. After an additional 10 min of stirring the two layers were separated. This procedure of washing the organic layer with an aqueous phase (pH 3.0) was repeated twice. The organic phase was concentrated *in vacuo* and the residue was suspended in distilled water (20 mL). The resulting crystals were isolated by filtration over a P4 glass filter, washed with distilled water (50 mL, 2×), resuspended in toluene (100 mL) and the solution was concentrated *in vacuo*.

171 mg; 82% yield; R_t(HPLC) 9.44 min, Purity 96%; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 1.38-1.90 (m, 4H), 3.087-3.17 (m,2H), 4.14-4.25 (m, 1H), 5.03 (s, 2H), 7.02-7.50 (m, 12H), 7.60-7.83 (m, 3H), 7.90 (s, 1H), 10.30 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 25.2, 29.0, 54.9, 65.5, 119.3, 123.1, 127.8, 127.7, 128.2, 128.6, 137.1, 138.9, 156.0, 157.2, 170.9.

Synthesis of arylamides 9 and 10

Alcalase-CLEA (500 mg) was added to a mixture containing MTBE (4.5 mL), aniline (500 µL, 4.5 mmol, 10 equiv), **6** (120 mg, 0.45 mmol, 1 equiv) or **7** (108 mg, 0.45 mmol, 1 equiv) and 3 Å molecular sieves (200 mg). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. The reaction mixture was filtered over a P4 glass filter and the solid enzyme particles were resuspended in EtOAc and removed by filtration (50 mL, 3×). This enzyme washing procedure was repeated three times with 0.1 N HCl (50 mL). The two phases of the filtrate were separated and the organic layer was concentrated *in vacuo*. Compound **9** was purified by column chromatography (MeOH/CH_2Cl_2/AcOH, 15/84/1, v/v).

Cbz-Asp-anilide (9)[26f]

140 mg; 91% yield; R_t(HPLC) 14.37 min, Purity; 98%; R_f(MeOH/CH_2Cl_2/AcOH, 15/84/1, v/v) 0.16; Mp: 179°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.55-2.77 (m, 2H), 4.55-4.62 (m, 1H), 5.10 (s, 2H), 7.11 (t, *J* = 7.2 Hz, 1H), 7.28-7.44 (m, 7H), 7.66 (d, *J* = 7.8 Hz, 2H), 7.75 (d, *J* = 7.8 Hz, 1H), 10.11 (s, 1H), 12.41 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 36.2, 52.0, 65.4, 119.3, 123.2, 127.6, 127.7, 128.2, 128.5, 136.8, 138.8, 155.7, 169.5, 171.4; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{18}H_{19}N_2O_5$: 343.1288; found: 343.1274; IR(neat/cm^{-1}): 3292, 3064, 1693, 1678, 1531, 1444, 1276, 1243.

Cbz-Asp-anilide (9) (Chemical synthesis of reference compound)

Aniline (182 µL, 2 mmol, 2 equiv) was added in one portion to a mixture of pyridine (4 mL) and PCl_3 (86 µL, 1 mmol, 1 equiv) and stirred for 1 h at ambient temperature. Subsequently, a solution of Cbz-Asp(O^{t}Bu)-OH (323 mg, 1 mmol, 1 equiv) in pyridine (2.5 mL) was added in one portion and the mixture stirred at 40°C for 4 h. Subsequently, the mixture was concentrated *in vacuo* and the residue was resuspended in water (50 mL). The resulting precipitates were filtered off and washed with water (20 mL, 2×). The solids were resuspended in trifluoroacetic acid (TFA, 10 mL) in the presence of a drop of water as scavenger and this reaction mixture was stirred for 3 h at ambient temperature. Then, the reaction mixture was concentrated *in vacuo* and the residue was redissolved in CH_2Cl_2 (50

mL) and water (50 mL). The water layer was adjusted to pH = 8 with 1N NaOH and the mixture was stirred vigorously for 10 min followed by separation of the two layers. The pH of the water layer was adjusted to pH = 2 using 1 N HCl. The resulting crystals were removed by filtration and washed with water (20 mL, 2×), followed by co-evaporation with toluene (20 mL, 2×) and $CHCl_3$ (20 mL, 2×).
283 mg; 83% yield; R_f(HPLC) 14.40 min, Purity; 96%; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.53-2.77 (m, 2H), 4.52-4.64 (m, 1H), 5.11 (s, 2H), 7.11 (t, *J* = 7.2 Hz, 1H), 7.30-7.43 (m, 7H), 7.68 (d, *J* = 7.8 Hz, 2H), 7.75 (d, *J* = 7.8 Hz, 1H), 10.13 (s, 1H), 12.45 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 36.4, 52.0, 65.3, 119.3, 123.3, 127.7, 127.9, 128.0, 128.6, 137.0, 138.9, 155.5, 169.4, 171.5.

Cbz-Ser-anilide (10)[26g]
132 mg; 93% yield; R_f(HPLC) 20.36 min, Purity 98%; R_f(MeOH/CH_2Cl_2, 1/20, v/v) 0.19; Mp: 160°C; ^{1}H NMR (300 MHz, DMSO-d_6) 4.20-4.36 (m, 2H), 4.50-4.59 (m, 1H), 5.11 (s, 2H), 7.12 (t, *J* = 6.9 Hz, 1H), 7.28-7.44 (m, 7H), 7.64 (d, *J* = 7.5 Hz, 2H), 7.86 (d, *J* = 7.8 Hz, 1H), 10.24 (s, 1H); δ = ^{13}C NMR (75 MHz, DMSO-d_6) δ = 55.2, 55.3, 64.3, 65.7, 119.5, 123.6, 127.6, 127.7, 128.2, 128.6, 136.6, 138.4, 155.8, 167.1; IR(neat/cm^{-1}): 3282, 3061, 1694, 1677, 1668, 1601, 1537, 1498, 1445, 1298, 1250.

Cbz-Ala-OMe (11a)[26h]
Cbz-Ala-OH (**11b**, 223 mg, 1.0 mmol), 1-ethyl-3-(3-dimethylamino propyl)carbodiimide hydrochloride (EDC·HCl, 190 mg, 1 mmol, 1 equiv) and 1-hydroxy-7-azabenzotriazole (HOAt, 136 mg, 1 mmol, 1 equiv) were dissolved in a mixture of EtOAc (20 mL), MeOH (2 mL) and DIPEA (174 µL, 1 mmol, 1 equiv). The reaction mixture was stirred at ambient temperature for 16 h, washed with 0.1 N HCl (15 mL, 2×), brine (15 mL), sat. aq. Na_2CO_3 (15 mL, 2×), brine (15 mL), dried (Na_2SO_4), and concentrated *in vacuo*. R_f(HPLC) 13.89 min, 186 mg; 78% yield; Purity 99%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.39; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.33 (d, *J* = 7.2 Hz, 3H), 3.66 (s, 3H), 4.25-4.37 (m, 1H), 5.03 (s, 2H), 5.32 (d, *J* = 7.2 Hz, 1H), 7.10-7.35 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 18.6, 49.5, 52.3, 66.8, 128.0, 128.1, 128.4, 136.2, 155.5, 173.4; FIA-ESI(+)-TOF-MS: *m/z* [M + Na]$^+$ calcd for $C_{12}H_{15}NO_4Na$: 260.0893; found: 260.0949. Calcd for $C_{12}H_{16}NO_4$: 238.1073; found: 238.1073.

Cbz-Ala-anilide (12)[26i]
Cal-B (100 mg) was added to a mixture containing MTBE (4.5 mL), aniline (500 µL), **11a** or **11b** (100 mg) and 3 Å molecular sieves (200 mg). The obtained reation mixture was shaken at 50°C with 150 rpm for 16 h. The reaction mixture was filtered over a P4 glass filter and the solid enzyme particles were resuspended in EtOAc and removed by filtration (50 mL, 3×). Distilled water (150 mL) was added to the filtrate and under vigorous stirring, the pH was adjusted to 3.0 with 1 N HCl. After an additional 10 min of stirring, the two layers were separated. This procedure of washing the organic layer with an aqueous phase (pH 3.0) was repeated twice. Subsequently, the organic phase was washed with sat. aq. $NaHCO_3$ (100 mL, 2×), brine (100 mL), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was triturated with *n*-heptane (2 mL) and the resulting crystals were isolated by filtration and

washed with *n*-heptane (2 × 5 mL). 115 mg; 92% yield (from **11a**); 103 mg; 77% (from **11b**); R_t(HPLC) 15.43 min, Purity 98%; R_f(EtOAc/*n*-hexane, 1/1, v/v) 0.43; Mp: 152°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 1.29 (d, *J* = 7.2 Hz, 3H), 4.15-4.23 (m, 1H), 5.03 (s, 2H), 7.04 (t, *J* = 7.2 Hz, 1H), 7.08-7.40 (m, 7H), 7.59 (m, 3H), 9.96 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 17.9, 50.7, 65.3, 119.1, 123.1, 127.6, 127.7, 128.2, 128.5, 136.9, 138.9, 155.7, 171.4; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{17}H_{19}N_2O_3$: 299.1390; found: 299.1390; IR(neat/cm^{-1}): 3296, 1686, 1665, 1533, 1490, 1455, 1285, 1257, 1248, 1213, 1058.

4

4.4 References

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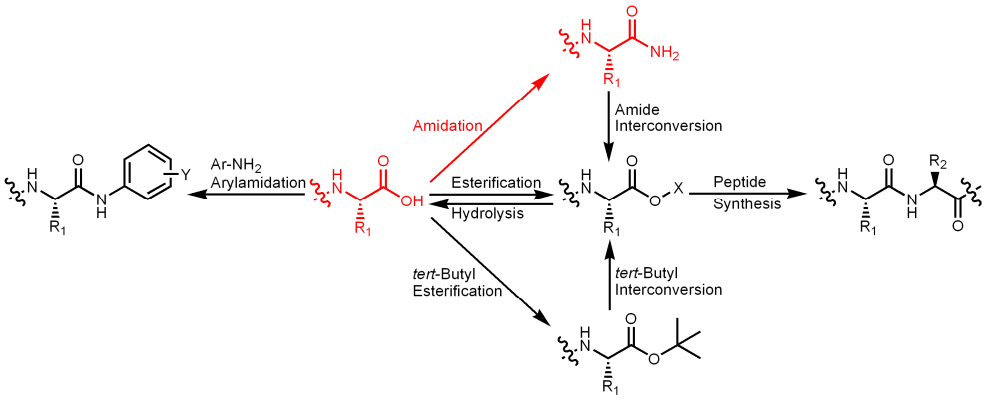
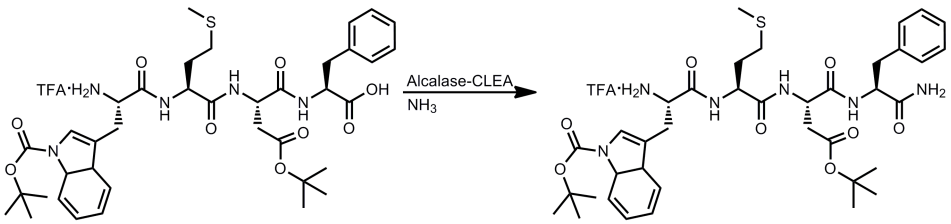
Chapter 5

Enzymatic synthesis of amino acid and peptide *C*-terminal α-carboxamides



In this chapter, two versatile and high yielding enzymatic approaches for the conversion of semi-protected amino acid and peptide *C*-terminal α-carboxylic acids into their corresponding carboxamides are described. The first approach uses the lipase *Candida antarctica* lipase-B, while the second approach uses the protease Subtilisin A. It was found that the application of the ammonium salt of the α-carboxylic acid, instead of a separate ammonia source, the enzymatic amidation reaction proceeded much faster without side reactions and gave near to quantitative yield.



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5.1 Introduction

Many peptide hormones and pharmaceutically relevant peptides contain a *C*-terminal primary amide functionality, which is not only important for their biological activity, but also increases their stability against exoproteases.[1] Furthermore, the primary amide functionality is often used as a (temporary) protecting group in chemical and enzymatic peptide synthesis; its cleavage can be effectuated by using peptide amidases such as the peptidase from the *flavedo* of oranges.[2] Solution phase synthesis of peptides bearing a *C*-terminal primary amide functionality usually starts from the *C*-terminal amino acid carboxamide and the desired peptide sequence is stepwise assembled in the *C* → *N* direction using *N*-carbamate protected amino acid building blocks to minimize racemization. However, the generally limited solubility of amino acid and peptide amides makes the synthesis of longer peptide amides rather laborious by solution phase methodology. Therefore, peptide amides are mostly synthesized by solid phase peptide synthesis (SPPS) using, among others, Rink Amide resin or 4-methylbenzhydrylamine (MBHA) resin in Fmoc- or Boc-SPPS, respectively.[3] However, these resins are expensive and not attractive for application on large scale. On the other hand the solid-phase synthesis of peptide acids is much more cost-efficient since cheap and industrially available resins can be used (such as the 2-chlorotritylchloride resin). Alternatively, several peptide acids are accessible via fermentative routes, whilst their *C*-terminal amides are the desired end product. Therefore, several chemical approaches have been developed to convert peptide acids into the corresponding amides. Unfortunately, these methods lack selectivity toward the *C*-terminus and usually cause racemization or prove incompatible with labile substrates or other protecting groups.[4]
In contrast to chemical approaches for modification of amino acids and peptides, enzymatic conversions proceed under mild conditions and are often chemo-, regio- and stereoselective. Several enzymes are interesting candidates to be used for the amidation of amino acids and peptide acids. For instance, few peptide amidases have been described in the literature, but they tend to be selective for a limited number of *C*-terminal amino acids and can not be produced on large scale.[2] Lipases, such as *Candida antarctica* lipase-B (Cal-B), have been described to perform esterification and subsequent amidation of aliphatic carboxylic acids, but amidation of peptides by Cal-B has so far not been reported.[5] The *C*-terminal amidation of amino acid *esters* and peptide *C*-terminal *esters* has been reported using the protease Subtilisin A.[6] Subtilisin A is a serine endoprotease produced from *Bacillus licheniformis* and commercially available as Alcalase in which it is the main constituent. Alcalase-mediated amidation reactions have recently been optimized using *C*-terminal peptide methyl esters and ammonium carbamate as amine source.[7] However, the chemical synthesis of the *C*-terminal peptide (methyl) esters that are required for the enzymatic reaction encounters the same restrictions as the direct chemical amidation, *i.e.* lack of *C*-terminal selectivity, racemization issues, and incompatibility with labile substrates or other protecting groups.
This chapter describes the direct and *C*-terminally selective enzymatic amidation of side chain protected as well as side chain unprotected amino acids and peptides. In the presence of either Cal-B or Subtilisin A, the desired amides were obtained in high yield and purity, starting from the corresponding carboxylic acids.

5.2 Results and Discussion

The direct amidation of *N*-protected amino acids and peptides is energetically unfavorable and should therefore be conducted in dry organic solvents with removal of the formed water to drive the equilibrium to the side of the amide. Additionally, the activation energy of the amidation reaction starting from the carboxylic acids is much higher than starting from the ester congeners. Therefore, longer reaction times are required and side reactions, such as hydrolysis or transamidation of the peptide backbone, can be expected. In fact, when the reported optimized amidation conditions with Alcalase[7] were applied on Cbz-Phe-Leu-Ala-OH as the substrate, 81% conversion of the starting material into the desired product was observed, but also the formation of a significant amount (15%) of side products caused by transamidation and hydrolysis could be identified, as shown in Figure 1.

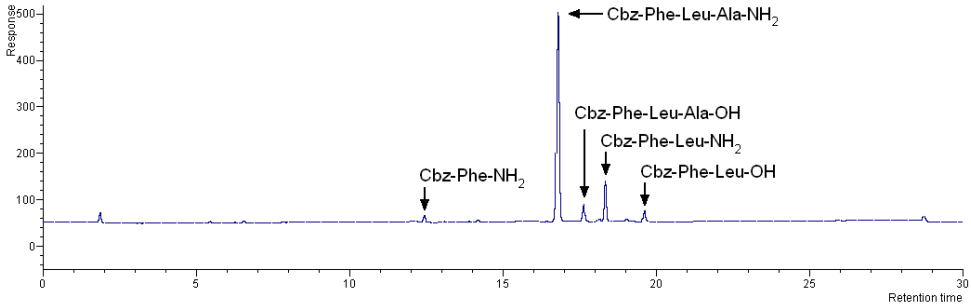
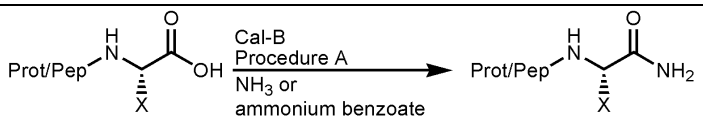


Figure 1. Amidation of Cbz-Phe-Leu-Ala-OH; LC-MS area distribution: 4% Cbz-Phe-Leu-Ala-OH (starting material), 81% Cbz-Phe-Leu-Ala-NH_2 (product), 9% Cbz-Phe-Leu-NH_2, 4% Cbz-Phe-Leu-OH and 2% Cbz-Phe-NH_2.

Based on these results, it was decided to test in first instance the versatility of lipases for the direct amidation, since these enzymes virtually lack the ability to hydrolyze or transamidate peptide bonds. Cal-B, a commercially available lipase which is active in organic solvents, proved to be the most promising candidate (see experimental section for lipase screening data). Gratifyingly, in case of Cal-B, no transamidation side products during the direct amidation of a number of amino acid and peptide acids were observed, as shown in Table 1. The Cal-B catalyzed amidations proceeded smoothly in toluene using ten equiv of ammonium benzoate. Up to quantitative HPLC yield was obtained in the presence of molecular sieves to absorb the liberated water during amide formation (Table 1, entries 1-5). After purification by column chromatography, Cbz-Ala-NH_2 (**1**, entry 2) could be obtained in a yield of 87%. Although no peptide-based side products were observed, the amidation of benzoic acid turned out to be a serious problem, especially when more challenging peptide substrates were used (Table 1, entries 6-9).

Table 1. *C*-terminal amidation of amino acids and peptides in the presence of Cal-B using ammonium benzoate or NH_3 gas in toluene.



X = Amino acid side chain
Pep = Peptide moiety
Prot = Protecting group

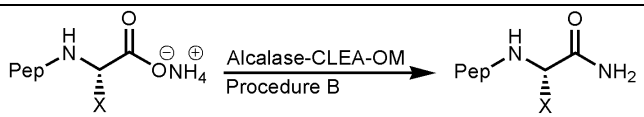
Entry[a]	Amide product[b]	Conversion[c] to amide (%) using ammonium benzoate	Conversion[c] to amide (%) using ammonia gas
1	Cbz-Pro-NH_2	99	-
2	Cbz-Ala-NH_2 (**1**)	95	-
3	Cbz-Gly-NH_2	80	-
4	Cbz-Met-NH_2	60	-
5	Cbz-Val-Pro-Pro-NH_2	90	-
6	Cbz-Gly-Leu-Ala-NH_2	65	-
7	Cbz-Gly-Phe-Ala-NH_2	60	82
8	Cbz-Gly-Ile-Ala-NH_2	52	85
9	Cbz-Val-Val-Pro-NH_2	12	56

[a] Conditions: Cal-B, ammonium benzoate or NH_3 in dioxane, 3 Å molecular sieves, toluene, 50°C, 16 h (Procedure A); [b] Product identity was confirmed using LC-MS analysis; [c] Conversions based on integration of the acyl donor starting material and the product, assuming that response factors are identical.

Therefore, the possibility of using a direct NH_3 source (*e.g.* NH_3 in dioxane) or the ammonium salt of the corresponding amino acid or peptide acid was investigated. Gratifyingly, the amidation reaction with Cal-B in the presence of only one equiv of NH_3 gas or NH_3/dioxane (via the amino acid or peptide ammonium salt) resulted in a much higher yield and proceeded much faster than in the presence of ammonium benzoate (Table 1, entries 7-9). However, Cal-B appeared rather restricted in accepting bulky side chains in its active site, so that phenylalanine, tyrosine, tryptophan or arginine and peptides containing these amino acids at the *C*-terminus, were not converted into the corresponding amides at all.

Hence, it was decided to investigate the use of Subtilisin A (Alcalase) in the direct amidation reaction of amino acid and peptide ammonium salts under anhydrous conditions. It is known[8] that this serine endoprotease has a very broad substrate tolerance and is also active in organic solvents. This biocatalyst was applied in the form of cross-linked enzyme aggregates (Alcalase-CLEA-OM, from CLEA Technologies designed for organic media)[9] to achieve maximal stability and activity in anhydrous organic solvents.

Table 2. *C*-terminal amidation of peptide ammonium salts using Alcalase-CLEA-OM.



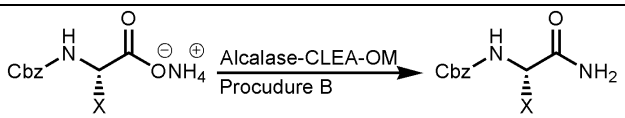
X = Amino acid side chain
Pep = Peptide moeity

Entry[a]	Amide product[b]	Conversion[c] (%)	Transamidation (%)
1	Cbz-Phe-Leu-Ala-NH_2	94	1
2	Cbz-Gly-Gly-Ala-NH_2	92	-
3	Cbz-Val-Ala-NH_2	91	-
4	Cbz-Ile-Met-NH_2	89	-
5	Cbz-Gly-Leu-Ala-NH_2	89	1
6	Cbz-Ala-Leu-NH_2	87	-
7	Cbz-Ala-Phe-NH_2	87	-
8	Cbz-Ala-Ala-NH_2	86	-
9	Cbz-Val-Met-NH_2	85	-
10	Cbz-Gly-Pro-Ala-NH_2	85	-
11	Cbz-Ala-Met-NH_2	81	-
12	Cbz-Tyr-Leu-NH_2	81	-
13	Cbz-Gly-Phe-NH_2	81	-
14	Cbz-Val-Phe-NH_2	80	-
15	Cbz-Leu-Phe-NH_2	79	-
16	Cbz-Gly-Ile-Ala-NH_2	78	-
17	Cbz-Phe-Leu-NH_2	77	-
18	Cbz-Ala-Pro-Leu-NH_2	68	-
19	Cbz-Pro-Leu-Gly-NH_2	53	4
20	Cbz-Gly-Tyr-NH_2	46	-
21	Cbz-Val-Gly-NH_2 (**2**)	30	-

[a] Conditions: Alcalase-CLEA-OM, NH_3 in dioxane, ^{t}BuOH/DMF, 50°C, 16 h (Procedure B); [b] Product identity was confirmed using LC-MS analysis; [c] Conversions based on integration of the acyl donor and the product.

As demonstrated in Table 2, a large number of peptide ammonium salts could be converted into their corresponding amides using Alcalase. Surprisingly, transamidation did not occur at all in most of the tested substrates (or to a very limited extent in a few). High conversions as identified by analytical HPLC were obtained for almost all substrates (products were verified by LC-MS analysis). By increasing the reaction time and/or the amount of enzyme, even the amidation of the more challenging substrates, such as Cbz-Val-Gly-NH_2 (**2**, entry 21), could be brought to full completion and obtained in an isolated yield of 89%. To avoid any hydrolysis, the amidation reactions were run in the presence of molecular sieves. To show the versatility of the amidation reaction, and to demonstrate the complete specificity toward α-carboxylic acids, Alcalase-CLEA-OM was also applied to amino acids containing reactive side chain functionalities, as shown in Table 3.

Table 3. α-Selective amidation of amino acid ammonium salts using Alcalase-CLEA-OM.



X = Amino acid sidechain

Entry[a]	Amide product[b]	Conversion (%)[c]
1	Cbz-Gln-NH_2	96
2	Cbz-Ser-NH_2	87
3	Cbz-Asn-NH_2	70
4	Cbz-Glu-NH_2	67
5	Cbz-Asp-NH_2	57

[a] Conditions: Alcalase-CLEA-OM, NH_3 in dioxane, ^{t}BuOH/DMF, 50°C, 16 h (Procedure B); [b] Product identity was confirmed using LC-MS analysis and commercially available reference materials; [c] Conversions based on integration of the acyl donor starting material and the product, assuming that response factors are identical.

5

The amidation of amino acids turned out to be slower than the amidation of peptide acids. Nevertheless, no side reactions occurred and full α-selectivity was observed when β- or γ-carboxylic acids were present (entry 4 and 5).

Finally, the direct amidation approach was applied to the tetrapeptide Trp-Met-Asp-Phe with several degrees of protection (Table 4). Both diagnostic peptides Sincalide and Pentagastrine contain the *C*-terminal sequence Trp-Met-Asp-Phe-NH_2[10] which is a general motif of gastrin-related peptides.

Table 4. *C*-terminal amidation of Trp-Met-Asp-Phe using Alcalase-CLEA-OM.

Entry[a]	Amide product		Yield (%)[b]
1	Ac-Trp(Boc)-Met-Asp(O^{t}Bu)-Phe-NH_2	**3**	77
2	TFA·H-Trp(Boc)-Met-Asp(O^{t}Bu)-Phe-NH_2	**4**	68
3	TFA·H-Trp(TFA)-Met-Asp-Phe-NH_2	**5**	63

[a] Conditions: Alcalase-CLEA-OM, NH_3 in dioxane, ^{t}BuOH/DMF, 50°C, 16 h (procedure C); [b] Isolated yield based on acyl donor starting material.

As a first approach the amidation of the fully protected peptide was examined (entry 1, Table 4). Its carboxylic acid congener is easily accessible by SPPS and is highly soluble in anhydrous organic solvents. Despite the presence of two bulky (side chain) protecting groups, the peptide acid was well recognized by Alcalase-CLEA-OM and smoothly converted into its corresponding amide. The same peptide sequence, with a free *N*-terminal amine (entry 2), was also amidated without side reactions. This result is important since side chain protected peptides with a *C*-terminal primary amide and an unprotected *N*-terminus are very useful building blocks for chemical peptide fragment condensation; for example in the synthesis of the pharmaceutical peptide products like Fuzeon and Exenatide.[11] Finally, the unprotected tetrapeptide (entry 3) was also amidated in a good yield, without amidation of the aspartic acid side chain (as proven by MS-MS analysis).

In conclusion, this chapter describes for the first time, that amino acids and peptide acids can be directly converted to their C-terminal amide congeners by using either Cal-B or Subtilisin A. The amidation is high yielding and competitive with other functional groups and is independent of the presence of any protecting group. This emphasizes the generality of the method since any peptide acid, either synthesized by classical solution methodology, SPPS, or fermentation may be used as starting material, while the obtained peptide amides are important starting compounds for the synthesis of therapeutic peptides.

5.3 Experimental

General:
For general experimental information and the synthesis of side chain protected peptide starting materials see chapter 2 and 3. Alcalase-CLEA type OM (organic media) was used (CLEA-Technologies, 580 U/g) and Cal-B was purchased from Novozymes (immobilized Novozym®-435, LC 200204). All amino acid and di- and tripeptide starting materials were obtained commercially.

General Procedure A: Amidation of amino acids using Cal-B
a) Using ammonium benzoate. Cal-B (100 mg) was added to a mixture of Cbz-Xxx-OH (50 mg), ammonium benzoate (10 equiv) and 3 Å molecular sieves (200 mg) in toluene (6 mL). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h.
b) Using a direct ammonium source. To a mixture of Cbz-Xxx-OH (50 mg) and 3 Å molecular sieves (200 mg) in toluene (6 mL) was added 2 N NH_3 in dioxane (1.1 equiv), or NH_3 gas was bubbled through the mixture for 30 s. Afterwards, Cal-B (100 mg) was added and the obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h.

Cbz-Ala-NH_2 (1)[12]
The reaction mixture of Cbz-Ala-OH was filtrated and the solid enzyme particles were resuspended in MeOH and removed by filtration (5 mL, 3×). The combined organic layers were concentrated *in vacuo*. The crude product was purified by column chromatography with MeOH/CH_2Cl_2 (7/93, v/v) as eluens. The pure fractions were combined, concentrated *in vacuo* and co-evaporated with toluene (20 mL, 2×) and $CHCl_3$ (10 mL, 2×). Cbz-Ala-NH_2 was obtained in a yield of 87% and its analytical data was compared to and in accordance with the commercially available reference compound. R_t(HPLC) 4.38 min, Purity 98%; R_f(MeOH/ CH_2Cl_2, 7/93, v/v) 0.24; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.40 (d, *J* = 7.2 Hz, 3H), 4.21-4.29 (m, 1H), 5.12 (s, 2H), 5.34 (d, *J* = 6.6 Hz, 1H), 5.52 (s, 1H), 6.05 (s, 1H), 7.26-7.35 (m, 5H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.2, 49.8, 65.2, 127.6, 127.7, 128.2, 137.0, 155.5, 174.4.

General Procedure B: Amidation of amino acids and peptides using Alcalase-CLEA-OM
Alcalase-CLEA-OM (50 mg) was added to a solution of the respective amino acid or peptide (0.1 mmol) in 2 N NH_3 in dioxane (1.1 equiv) with ^{t}BuOH/DMF (82.5/17.5 v/v) as the co-solvent. The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h.

Cbz-Val-Gly-NH_2 (2)

When twice the amount of enzyme was used and 72 h of reaction time for the amidation of Cbz-Val-Gly-OH (Procedure B, 100 mg Alcalase-CLEA-OM), the conversion was 98% as indicated by analytical HPLC. The reaction mixture was filtrated and the solid enzyme particles were resuspended in MeOH and removed by filtration (5 mL, 3×). This enzyme particle washing procedure was repeated with EtOAc (25 mL, 3×). The combined organic layers were washed with sat. aq. $NaHCO_3$ (50 mL), brine (50 mL), dried over Na_2SO_4, concentrated *in vacuo* and dried by co-evaporation with toluene (50 mL, 2×) and $CHCl_3$ (50 mL, 2×). Cbz-Val-Gly-NH_2 was obtained in a yield of 89%. R_t(HPLC) 17.98 min; Purity >97%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.85 (dd, *J* = 6.6 Hz, 6H), 1.93-2.01 (m, 1H), 3.60-3.66 (m, 2H), 3.82-3.87 (m, 1H), 5.03 (s, 2H), 7.04 (s, 1H), 7.22-7.40 (m, 7H), 8.16 (m, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.0, 19.1, 29.8, 41.7, 60.3, 65.4, 127.6, 127.7, 128.2, 136.9, 156.2, 170.6, 171.3; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{15}H_{22}N_3O_4$: 308.1605; found: 308.1622.

5

General Procedure C: Amidation of side chain protected peptides using Alcalase-CLEA-OM

Alcalase-CLEA-OM (500 mg) was added to a solution of the respective peptide (1 mmol) in 2 N NH_3 in dioxane (1.1 equiv and 2.2 equiv for compound **5**) with ^{t}BuOH/DMF (82.5/17.5 v/v) as the co-solvent. The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. The reaction mixture was filtrated and the solid enzyme particles were resuspended in DMF and removed by filtration (5 mL, 3×). This enzyme particle washing procedure was repeated with CH_2Cl_2 (25 mL, 3×). The combined organic layers were concentrated *in vacuo* and the resulting oil was purified by preparative HPLC.

General Procedure D: Solid phase synthesis of protected peptide starting materials

2-Chlorotritylchloride resin (1 g, loading = 1.2 mmol/g) was reacted with Fmoc-Phe-OH (2 mmol) and DIPEA (5 mmol) in CH_2Cl_2 (10 mL, 30 min) followed by washing with DMF (10 mL, 2×). Afterwards, the unreacted tritylchloride moieties were capped with MeOH/CH_2Cl_2/DIPEA (10 mL, 15/85/5, v/v/v) followed by washing the resin with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and NMP (10 mL, 2 min, 3×). The following Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[13] The desired peptide sequence was cleaved from the resin using 2.5 vol% TFA in CH_2Cl_2 (50 mL, 20 min). After filtration, the resin was washed with CH_2Cl_2 (50 mL) and the combined filtrates were concentrated *in vacuo* to half of the original volume. Subsequently, ^{i}PrOH/H_2O (50 mL, 1/3, v/v) was added followed by concentration of the volatiles *in vacuo* to one third of the original volume. The precipitated protected peptide was filtered off and subsequently washed with H_2O (20 mL, 2×) followed by lyophilization from CH_3CN/H_2O (3/1, v/v). The protected peptides were purified by preparative HPLC followed by lyophilization from CH_3CN/H_2O (3/1, v/v).

Screening of different lipases

A solution of Cbz-Ala-OH (0.05 mmol) in toluene (0.5 mL) containing 2 N NH_3 in dioxane (1.1 equiv) was added to lyophilized lipase (40 mg) in a 96 well format. The obtained reaction mixture was shaken at rt for 48 h followed by HPLC analysis. The following lipases proved less active than Cal-B; lipase lyophilizates from: *Rhizopus aarhizus, Aspergillus niger, Rhizopus delemar, Rhizopus niveus, Mucor javanicus*, porcine pancreas, *Candida rugosa, Rhizopus oryzae, Candida lipolytica, Mucor javanicus, Penicillium roqueforti, Humicola lanuginosa, Rhizopus delemar, Rhizopus niveus, Rhizopus japonicus, Burkholderia cepacia, Candida antarctica* (A), *Rhizomucor miehei, Candida cylindracea, Rhizopus oryzae, Aspergillus niger, Candida lipolytica, Rhizopus javanicus, Pseudomonas alcaligenes, Pseudomonas fluorescens, Candida lipolytica, Aspergillus asamii, Geotrichum candium* and *Pseudomonas fluorescens*.

Ac-Trp(Boc)-Met-Asp(O^{t}Bu)-Phe-NH_2 (3)

R_t(HPLC) 22.31 min, Purity 98%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.35 (s, 9H), 1.62 (s, 9H), 1.77-1.94 (m, 5H), 2.01 (s, 3H), 2.28-2.45 (m, 2H), 2.61-2.68 (m, 1H), 2.79-3.09 (m, 4H), 4.26-4.41 (m, 2H), 4.50-4.64 (m, 2H), 7.14-7.33 (m, 9H), 7.53 (s, 1H), 7.67 (d, *J* = 7.8 Hz, 1H), 7.84 (d, *J* = 8.1 Hz, 1H), 8.00 (d, *J* = 8.1 Hz, 1H), 8.17-8.24 (m, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 14.5, 27.6, 29.2, 32.5, 37.2, 49.4, 51.6, 51.8, 53.7, 80.2, 83.6, 113.3, 114.7, 119.4, 122.3, 124.4, 125.6, 126.2, 127.9, 129.0, 129.8, 134.8, 137.6, 148.9, 168.0, 169.2, 169.7, 170.1, 172.4; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{40}H_{54}N_6NaO_9S$: 817.3565; found: 817.3535.

TFA.H-Trp(Boc)-Met-Asp(O^{t}Bu)-Phe-NH_2 (4)

R_t(HPLC) 14.22 min, Purity 98%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.36 (s, 9H), 1.63 (s, 9H), 1.68-1.95 (m, 2H), 2.03 (s, 3H), 2.64-2.81 (m, 2H), 2.91-3.05 (m, 2H), 3.11-3.21 (m, 1H), 4.10-4.21 (m, 1H), 4.36-4.48 (m, 2H), 4.55-4.63 (m, 1H), 7.14-7.37 (m, 9H), 7.63 (s, 1H), 7.78 (d, *J* = 7.8 Hz, 1H), 7.93 (d, *J* = 8.1 Hz, 1H), 8.00-8.10 (m, 5H), 8.40 (d, *J* = 7.8 Hz, 1H), 8.90 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 14.5, 22.4, 26.9, 27.6, 29.3, 31.7, 37.3, 49.5, 52.0, 52.5, 53.8, 80.2, 83.4, 114.5, 116.6, 119.4, 122.3, 123.8, 124.2, 126.1, 128.0, 129.0, 130.2, 134.5, 137.7, 149.0, 169.3, 169.4, 169.7, 170.8, 171.5, 172.6; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{38}H_{53}N_6O_8S$: 753.3640; found: 753.3661.

TFA.H-Trp-Met-Asp-Phe-NH_2 (5)

R_t(HPLC) 5.41 min, Purity 98%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.68-1.90 (m, 2H), 1.96 (s, 3H), 2.80-2.92 (m, 2H), 3.01-3.13 (m, 2H), 3.14-3.20 (m, 1H), 3.94-4.05 (m, 1H), 4.28-4.40 (m, 2H), 4.46-4.53 (m, 1H), 6.95-7.32 (m, 11H), 7.62 (d, *J* = 7.8 Hz, 1H), 7.88-7.94 (m, 4H), 8.32 (d, *J* = 7.5 Hz, 1H), 8.75 (d, *J* = 8.1 Hz, 1H), 10.9 (s, 1H), 12.3 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 14.5, 16.8, 27.3, 29.3, 32.3, 35.9, 36.4, 37.3, 49.5, 51.8, 52.4, 53.8, 106.7, 111.4, 118.4, 121.0, 125.0, 126.1, 127.0, 128.0, 129.0, 136.2, 137.7, 168.4, 167.0, 170.3, 171.9, 172.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + Na]^+$ calcd for $C_{29}H_{37}N_6O_6S$: 597.2490; found: 597.2492. MS-MS: *m/z* calcd for $C_{29}H_{34}N_5O_6S^+$: 580; found 580; calcd for $C_{20}N_{25}N_4O_5S^+$: 433; found 433; calcd for $C_{16}H_{20}N_3O_2S^+$: 318; found 318; calcd for $C_{11}H_{11}N_2O^+$: 187; found 187.

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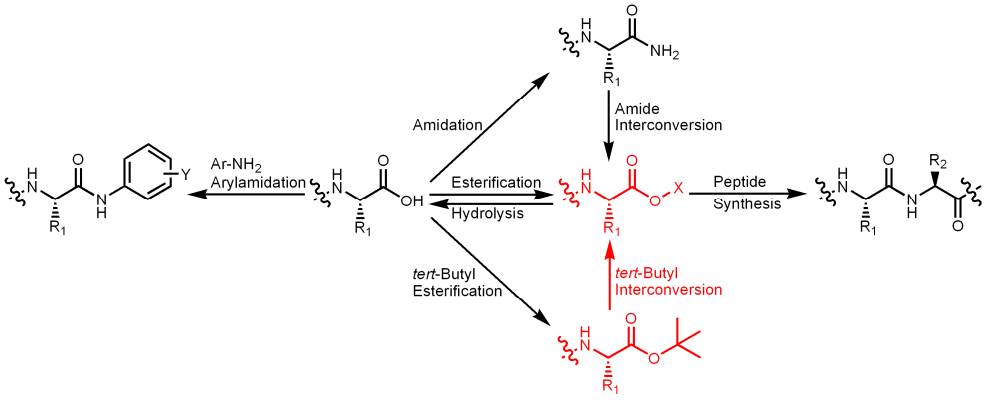
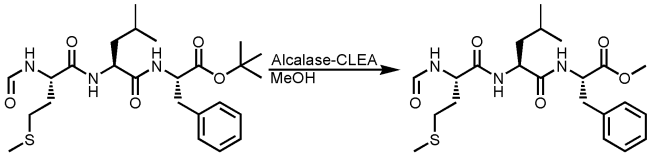
Chapter 6

Enzymatic peptide synthesis using *C*-terminal α-*tert*-butyl ester interconversion



In this chapter, a novel *C*-terminal ester interconversion catalyzed by the serine endopeptidase Subtilisin A is described. *C*-terminally protected peptide α-*tert*-butyl esters were enzymatically converted into primary alkyl esters in high yield and used directly in the next enzymatic peptide elongation step with another amino acid *tert*-butyl ester. This fully enzymatic *N* → *C* elongation strategy by *C*-terminal *tert*-butyl ester interconversion was applied toward the synthesis of three biologically active peptides up to the pentamer level.



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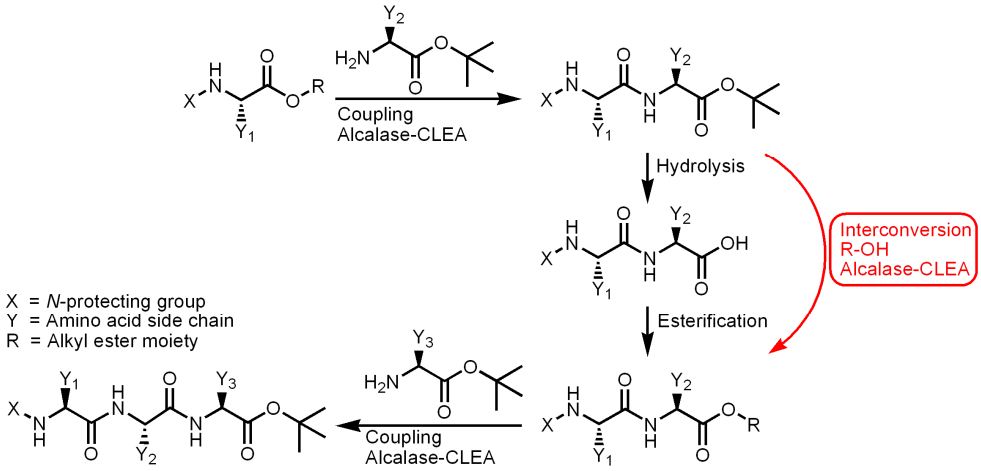
6.1 Introduction

Peptides, from simple dipeptides to complex oligopeptides, are increasingly abundant on the market as (pro)drugs or in clinical development.[1] Additionally, peptides have also gained importance as nutritional and cosmetic ingredients.[2] Therefore, during the past decades, an increased interest for peptide synthesis has arisen but this is still an expensive and time consuming procedure up to date.[3]

There are four main approaches available for peptide synthesis, *i.e.* fermentation, solid-phase or solution-phase chemical peptide synthesis, and chemo-enzymatic peptide synthesis.[4] Currently, fermentation is only well feasible for long peptides (>50 amino acids). Solid-phase and solution-phase chemical peptide synthesis are the most commonly used methods, but they require full protection of the side chain functionalities, except in the case of fragment ligation techniques where a *C*-terminal thioester is involved, which is difficult to introduce. Moreover, to suppress racemization, expensive and environmentally unfriendly coupling reagents are required in stoichiometric amounts. Chemo-enzymatic peptide synthesis, wherein peptides are stepwise elongated enzymatically, has been studied in academia during the past decades and proved to be suitable for certain short peptide sequences (up to 5 amino acids).[5] The amino acid side chains do not require protection and, most importantly, racemization is completely absent so that purification is potentially much easier. However, (de)protection of the *N*- and *C*-termini still require harsh reaction conditions.[6] When the peptide is elongated in the *C* → *N* direction, expensive *N*-protected and *C*-activated amino acid building blocks are employed and after each coupling step the *N*-protecting group should be removed. Elongation in the *N* → *C* direction (Scheme 1) would require the less expensive *C*-protected amino acid building blocks, such as carboxamides or *tert*-butyl (^{t}Bu) esters, but a selective *C*-deprotection should then be at hand.

Recently, it was discovered that the serine protease Subtilisin A (commercially available as Alcalase in which Subtilisin A is the main constituent)[7] can hydrolyze *C*-terminal peptide ^{t}Bu esters.[8] When combining the ^{t}Bu deprotection (hydrolysis) with activation (esterification, as described in chapter 2) and peptide coupling, a fully enzymatic peptide elongation strategy is obtained (Scheme 1).

However, upon ^{t}Bu ester deprotection, simultaneous hydrolysis of the peptide bonds is mostly inevitable. It was earlier reported by Liu *et al.*[9] that the amidolytic activity of Alcalase is significantly reduced in alcoholic solvents. It was envisioned that it should be possible to combine *C*-terminal deprotection and activation in one single step by interconverting ^{t}Bu esters into primary alkyl esters with Alcalase in organic solvents with an alcohol as the nucleophilic species (see Scheme 1), avoiding peptide bond hydrolysis since water is absent.

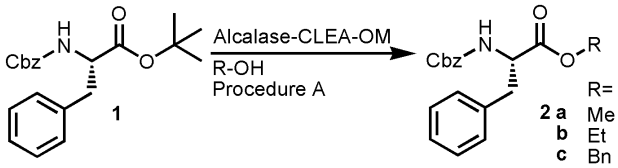


Scheme 1. Chemo-enzymatic peptide synthesis in the *N*→*C* direction using *C*-terminal ^{t}Bu ester interconversion

6.2 Results and Discussion

As a proof-of-principle for the interconversion reaction, Alcalase-CLEA-OM (cross-linked enzyme aggregates of Alcalase designed for organic media)[10] was used with Cbz-Phe-O^{t}Bu **1** as the model substrate, as shown in Table 1.

Table 1. Interconversion of Cbz-Phe-O^{t}Bu into primary alkyl esters.



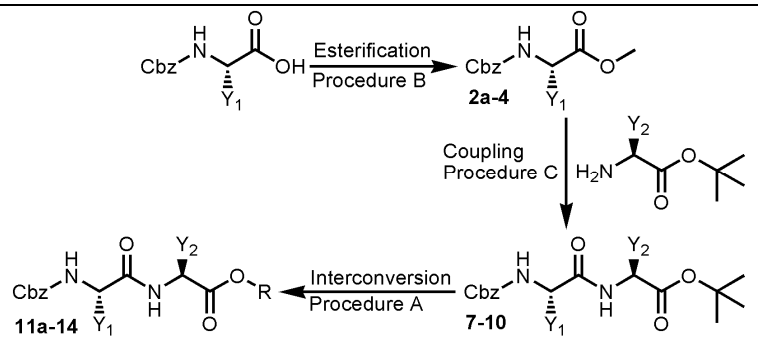
Entry[a]	Amide product		Yield (%)[b]
1	Cbz-Phe-OMe	**2a**	93
2	Cbz-Phe-OEt	**2b**	87
3	Cbz-Phe-OBn	**2c**	95

[a] Conditions: Alcalase-CLEA-OM, 3 Å molecular sieves, MTBE or THF or dioxane with the appropriate alcohol, 50°C, 48 h (Procedure A); [b] Yield based on acyl donor starting material.

Initial experiments showed that the interconversion of Cbz-Phe-O^{t}Bu (**1**, via procedure A) into primary alkyl esters (**2a-c**) indeed proceeded smoothly. When Alcalase-CLEA-OM was used in pure methanol (MeOH) or ethanol (EtOH), rapid deactivation of the enzyme occurred. However, when >90% of a cosolvent (e.g. tetrahydrofuran (THF) or methyl *tert*-butyl ether (MTBE)) was used along with the alcohol, quantitative conversion of the ^{t}Bu ester was observed on HPLC without detectable Alcalase-CLEA-OM deactivation. Some ^{t}Bu ester hydrolysis was observed, which could be prevented by adding 3 Å molecular sieves,

resulting in almost quantitative conversion of **1** into the primary alkyl esters **2a-c** (Table 1). Based on these promising results, this interconversion was applied on the dipeptide ^{t}Bu esters **7-10** as shown in Table 2, which were obtained in satisfactory yields by Alcalase-CLEA-OM mediated coupling of Cbz-protected methyl esters[11] **2a**, **3** and **4** in the presence of H-Phe-O^{t}Bu (**5**) or H-Leu-O^{t}Bu (**6**).

Table 2. Enzymatic dipeptide synthesis and *C*-terminal ^{t}Bu ester interconversion.



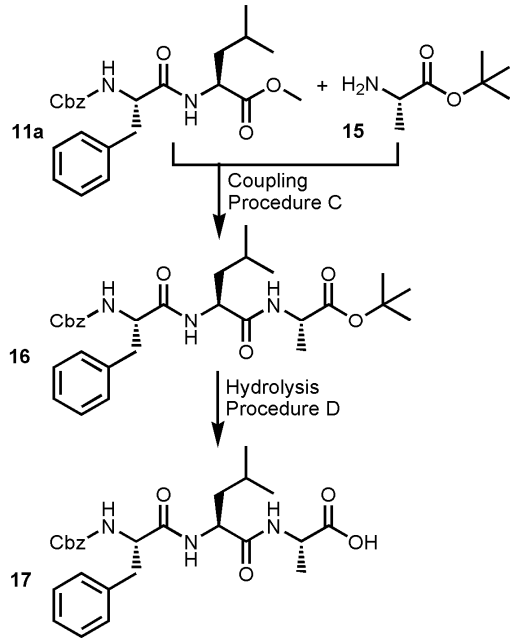
Entry[a]	Compound[b]		Enzyme	Yield (%)[c]	Procedure
1	Cbz-Phe-OMe	**2a**	Alcalase	93	B
2	Cbz-Phe-OEt	**2b**	Alcalase	87	B
3	Cbz-Phe-OBn	**2c**	Alcalase	95	B
4	Cbz-Ala-OMe	**3**	Alcalase	91	B
5	Cbz-Gly-OMe	**4**	Cal-B	93	B
6	Cbz-Phe-Leu-O^{t}Bu	**7**	Alcalase	77	C
7	Cbz-Ala-Leu-O^{t}Bu	**8**	Alcalase	85	C
8	Cbz-Ala-Phe-O^{t}Bu	**9**	Alcalase	86	C
9	Cbz-Gly-Phe-O^{t}Bu	**10**	Alcalase	81	C
10	Cbz-Phe-Leu-OMe	**11a**	Alcalase	93	A
11	Cbz-Phe-Leu-OEt	**11b**	Alcalase	94	A
12	Cbz-Phe-Leu-OBn	**11c**	Alcalase	89	A
13	Cbz-Ala-Leu-OMe	**12**	Alcalase	96	A
14	Cbz-Ala-Phe-OMe	**13**	Alcalase	91	A
15	Cbz-Gly-Phe-OMe	**14**	Alcalase	92	A

[a] Conditions: Alcalase-CLEA-OM, 3 Å molecular sieves, appropriate alcohol in MTBE or THF or dioxane (1/14, v/v), 50°C, 48 h (Procedure A); Alcalase-CLEA-OM or Cal-B, MeOH/MTBE (1/14, v/v), 3 Å molecular sieves, 50°C, 16h (Procedure B); Alcalase-CLEA-OM, 4 Å molecular sieves, amino acid nucleophile, MTBE or THF or DMF/THF (1/9, v/v), 50°C, 20 h (Procedure C); [b] Compounds **3-4**: methyl esterification, compounds **7-10**: coupling, compounds **11-14**: interconversion; [c] Isolated yield based on acyl donor starting material.

Since peptide bond formation and ^{t}Bu ester interconversion are catalyzed by the same enzyme, one would expect *tert*-butyl to methyl ester interconversion during peptide coupling where methanol (up to 1 equiv) is liberated leading to subsequent coupling of a second ^{t}Bu ester building block. Indeed this “double coupling” side reaction was observed in some cases, however, to little extent, since peptide bond formation is preferred over the much

slower interconversion. Furthermore, double coupling could always be minimized (<2%) when 4 Å molecular sieves were used to absorb the MeOH.
Gratifyingly, the dipeptide ^{t}Bu esters **7-10** were smoothly converted into the corresponding primary alkyl esters using Alcalase-CLEA-OM in high yields (Table 2, **11a-c**, **12-14**). Noteworthy, even the use of prolonged reaction times (48h) did not result in any detectable peptide bond cleavage according to HPLC analysis.
The applicability and versatility of this efficient and fully enzymatic peptide elongation strategy was further illustrated by the synthesis of three bioactive peptides, as shown in Tables 3 and 4, and Scheme 2.

Table 3. Fully enzymatic synthesis of the tripeptide substrate Cbz-Phe-Leu-Ala-OH which can be used in a thermolysine assay.[12]



Entry[a]	Compound		Yield (%)[b]	Procedure
1	Cbz-Phe-Leu-Ala-O^{t}Bu	**16**	82	C
2	Cbz-Phe-Leu-Ala-OH	**17**	79	D

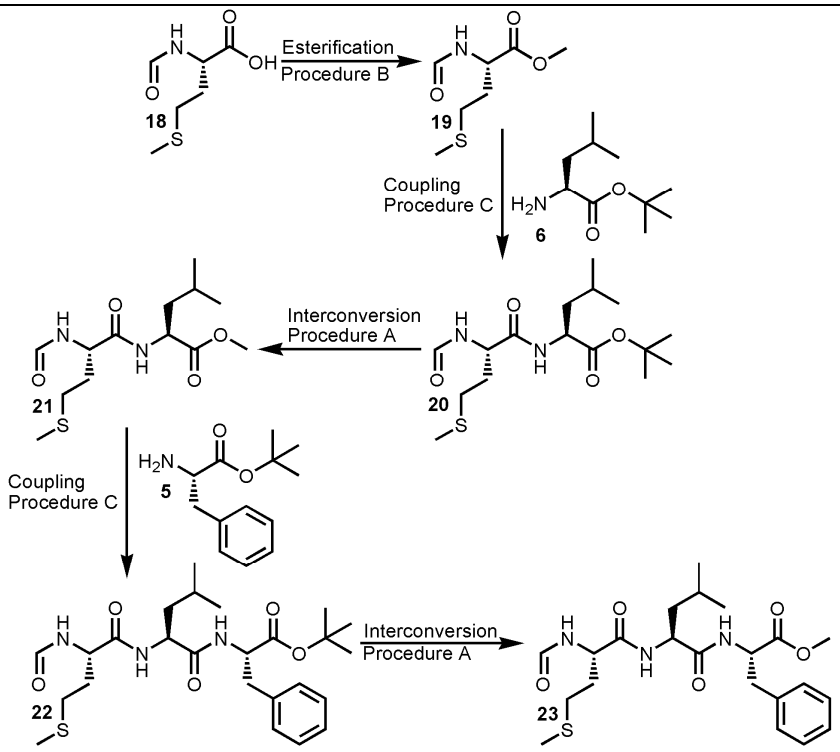
[a] Conditions: Alcalase-CLEA-OM, 4 Å molecular sieves, amino acid nucleophile, MTBE or THF or DMF/THF (1/9, v/v), 50°C, 20 h (Procedure C); Alcalase-CLEA-OM, dioxane/H_2O (8/2, v/v or DMF/H_2O, 7/3, v/v or THF/H_2O, 9/1, v/v), 37°C, 36 h (Procedure D); [b] Isolated yield based on acyl donor starting material.

The synthesis of the tripeptide substrate Cbz-Phe-Leu-Ala-OH[12] (**17**) (Table 3), which can be used in a thermolysine assay, was performed by condensing Cbz-Phe-Leu-OMe (**11a**) with H-Ala-O^{t}Bu (**15**) using Alcalase-CLEA-OM (82% yield), subsequently followed by hydrolysis of the ^{t}Bu ester of **16** giving the desired tripeptide **17** in 79% yield and 98% purity

(according to analytical HPLC). Along with the ^{t}Bu ester deprotection, 6% peptide bond hydrolysis was observed by HPLC.

The anti-inflammatory peptide For-Met-Leu-Phe-OMe[13] (**23**) (Table 4) was prepared analogously, by stepwise enzymatic elongation and interconversion using Alcalase-CLEA-OM for all individual steps. The synthesis[11] of methyl ester **19**, its coupling (to give **20** and **22**) and its interconversion (yielding product **21**) all proceeded smoothly and in high yield (85-92%). Finally, transesterification of tripeptide ^{t}Bu ester **22** into the corresponding methyl ester gave the anti-inflammatory tripeptide **23** in 83% yield and 97% purity (according to HPLC).

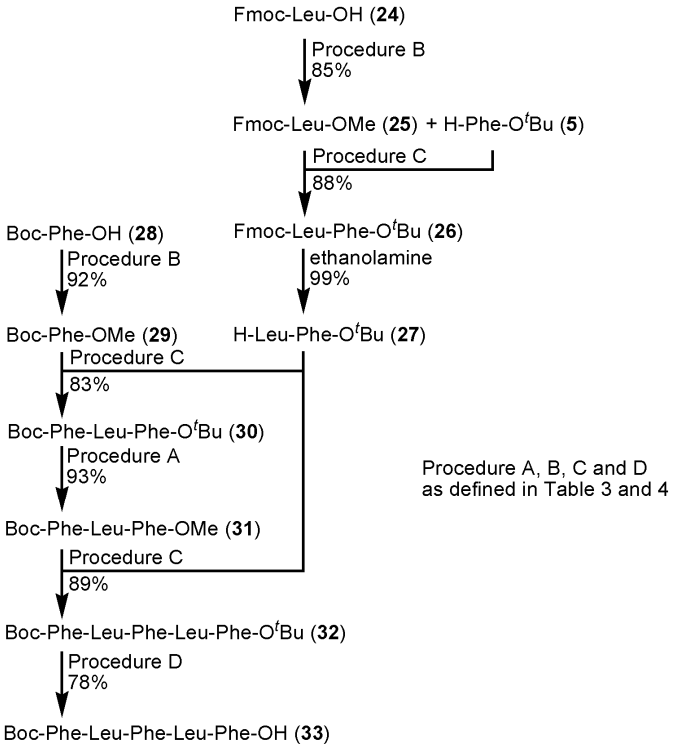
Table 4. Fully enzymatic synthesis of the anti-inflammatory tripeptide For-Met-Leu-Phe-OMe.[13]



Entry[a]	Compound		Yield (%)[b]	Procedure
1	For-Met-OMe	**19**	88	B
2	For-Met-Leu-O^{t}Bu	**20**	90	C
3	For-Met-Leu-OMe	**21**	92	A
4	For-Met-Leu-Phe-O^{t}Bu	**22**	85	C
5	For-Met-Leu-Phe-OMe	**23**	83	A

[a] Conditions: Alcalase-CLEA-OM, 3 Å molecular sieves, MeOH in MTBE or THF or dioxane (1/14, v/v), 50°C, 48 h (Procedure A); Alcalase-CLEA-OM or Cal-B, MeOH/MTBE (1/14, v/v), 3 Å molecular sieves, 50°C, 16h (Procedure B); Alcalase-CLEA-OM, 4 Å molecular sieves, amino acid nucleophile, MTBE or THF or DMF/THF (1/9, v/v), 50°C, 20 h (Procedure C); [b] Isolated yield based on acyl donor starting material.

Finally, the anti-inflammatory pentapeptide Boc-Phe-Leu-Phe-Leu-Phe-OH[13a-c] (**33**) (Scheme 2), a competitive antagonist of tripeptide **23**, was synthesized by means of Alcalase-CLEA-OM mediated interconversions and fragment condensations. The conversion of acids **24** and **28** into their corresponding methyl esters proceeded smoothly (85% and 92% yield, respectively) indicating that the nature of the *N*-protecting group seems to be irrelevant for substrate recognition. The fragment condensations (products **30** and **32**) proceeded even faster than the coupling of the single amino acids (giving **26**), which is in accordance with the fact that Alcalase is an endoprotease and thus prefers peptides over single amino acids. Transesterification as well as final hydrolysis of the ^{t}Bu ester functionality (in **30** and **32**, respectively), which is chemically usually not orthogonal to *N*-Boc deprotection, proved to be well feasible using Alcalase-CLEA-OM. The anti-inflammatory pentapeptide **33** was obtained 78% yield and 95% purity (according to analytical HPLC).



Scheme 2. Chemo-enzymatic synthesis of the anti-inflammatory pentapeptide Boc-Phe-Leu-Phe-Leu-Phe-OH.[13a-c]

In conclusion, this chapter shows for the first time the feasibility of fully enzymatic peptide synthesis in the *N* → *C*-terminal direction with simultaneous *C*-deprotection and activation of the growing oligopeptide. Cheap amino acid ^{t}Bu ester building blocks, as well as peptide fragments, could be used for the elongation. To synthesize the bioactive oligopeptides as shown in this chapter, the use of only one single enzyme, Alcalase-CLEA-OM, was sufficient.

6.3 Experimental

General

General experimental information is identical as described in chapter 2 and 3 with the following exceptions. Alcalase-CLEA type OM (organic media) was used (CLEA-Technologies, 580 U/g) and trifluoroacetic acid was used for the preparative HPLC buffers A and B. Cal-B was purchased from Novozymes (immobilized Novozym®-435, LC 200204).

General Procedure A: Transesterification of ^{t}Bu esters

Alcalase-CLEA-OM (1.0 g) and 3 Å molecular sieves (2 g) were added to a solution of *N*-protected amino acid or peptide ^{t}Bu ester (1.67 mmol) in MTBE ((or THF or dioxane) 28 mL) and MeOH (2 mL). The obtained reaction mixture was shaken at 50°C with 150 rpm for 48 h. After filtration, the solid enzyme particles were resuspended in MeOH and removed by filtration (50 mL, 3×). This enzyme particle washing procedure was repeated with EtOAc (50 mL, 2×) and CH_2Cl_2 (50 mL, 2×). The combined filtrates were concentrated *in vacuo* and the resulting residue was redissolved in EtOAc or CH_2Cl_2 (100 mL) and washed with sat. aq. $NaHCO_3$ (75 mL, 2×), 0.1 N HCl (75 mL, 2×), brine (75 mL) and dried (Na_2SO_4). The solution was concentrated *in vacuo* and the residue was lyophilized from CH_3CN/H_2O (3/1, v/v). Most methyl esters were obtained in a purity >95% as determined by analytical HPLC, if not, the resulting crude peptide was purified by preparative HPLC.

General Procedure B: Esterification of *N*-protected amino acids

a) Alcalase-CLEA-OM (300 mg) and 3 Å molecular sieves (200 mg) were added to a solution of *N*-protected amino acid (1.67 mmol) in a mixture of MTBE/MeOH (3 mL, 14/1, v/v). The obtained reaction mixture was shaken at 50^{o}C with 150 rpm for 16 h. After filtration, the solid enzyme particles were washed by resuspension in EtOAc and removed by filtration (20 mL, 3×). The combined organic filtrates were washed with sat. aq. $NaHCO_3$ (40 mL, 2×), 0.1 N HCl (40 mL, 2×), brine, (40 mL), dried (Na_2SO_4) and subsequently concentrated *in vacuo* and the residue was co-evaporated with toluene (20 mL, 2×) and $CHCl_3$ (20 mL, 2×). According to analytical HPLC analysis most compounds proved >95% pure.

b) Cal-B (100 mg) was added to a solution of Cbz-Gly-OH (1.67 mmol) in MTBE/MeOH (3 mL, 14/1, v/v) containing 3 Å molecular sieves (100 mg). The obtained reaction mixture was shaken at 50^{o}C with 150 rpm for 16 h. After filtration, the solid enzyme particles were resuspended in MeOH and removed by filtration (50 mL, 3×). This enzyme particle washing procedure was repeated with EtOAc (50 mL, 2×) and CH_2Cl_2 (50 mL, 2×). The combined filtrates were concentrated *in vacuo* and the resulting residue was redissolved in EtOAc (100 mL) and washed with sat. aq. $NaHCO_3$ (75 mL, 2×), 0.1 N HCl (75 mL, 2×), brine (75 mL) and dried (Na_2SO_4). The solution was concentrated *in vacuo* and the residue was lyophilized from CH_3CN/H_2O (3/1, v/v).

General Procedure C: Enzymatic peptide coupling reactions

Alcalase-CLEA-OM (0.5 g) and 4 Å molecular sieves (1.0 g) were added to a solution of *N*-protected amino acid or peptide methyl ester (1.3 mmol), or *N*-protected amino acid or

peptide ^{t}Bu ester derivatives (2.6 mmol, 2 equiv) in MTBE (30 mL, or THF or DMF/THF, 1/9, v/v). The obtained reaction mixture was shaken at 50°C with 150 rpm for 20 h. After filtration, the solid enzyme particles were resuspended in MeOH and removed by filtration (50 mL, 3×). This enzyme particle washing procedure was repeated with EtOAc (50 mL, 2×) and CH_2Cl_2 (50 mL, 2×). The combined filtrates were concentrated *in vacuo* and the resulting residue was redissolved in EtOAc or CH_2Cl_2 (100 mL) and washed with sat. aq. $NaHCO_3$ (75 mL, 2×), 0.1 N HCl (75 mL, 2×), brine (75 mL) and dried (Na_2SO_4). The solution was concentrated *in vacuo* and the residue was lyophilized. Most peptides were obtained in a purity >95% as determined by analytical HPLC, if not, the resulting crude peptide was purified by preparative HPLC.

General Procedure D: Hydrolysis of peptide ^{t}Bu esters

Alcalase-CLEA-OM (0.5 g) was added to a solution of peptide *C*-terminal ^{t}Bu ester (1 mmol) in dioxane/H_2O (20 mL, 8/2, v/v or DMF/H_2O, 7/3, v/v or THF/H_2O, 9/1, v/v). The obtained reaction mixture was shaken at 37°C with 150 rpm for 36 h. After filtration, the solid enzyme particles were resuspended in MeOH and removed by filtration (50 mL 3×). This enzyme particle washing procedure was repeated with EtOAc (50 mL, 2×) and CH_2Cl_2 (50 mL, 2×). The combined filtrates were concentrated *in vacuo* and the resulting crude acid was purified by preparative HPLC.

The analyses of Cbz-Phe-OMe (**2a**), Cbz-Phe-OEt (**2b**), Cbz-Phe-OBn (**2c**), Cbz-Phe-Leu-O^{t}Bu (**7**), Cbz-Phe-Leu-OMe (**11a**) and Cbz-Phe-Leu-OBn (**11c**) are described in chapter 2.

Cbz-Ala-OMe (3)[14a]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.32 (d, *J* = 7.2 Hz, 1H), 3.66 (s, 3H), 4.25-4.35 (m, 1H), 5.03 (s, 2H), 5.32 (d, *J* = 5.1 Hz, 1H), 7.20-7.28 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.6, 49.5, 52.3, 68.8, 128.0, 128.1, 128.4, 136.2, 155.5, 173.4.

Cbz-Gly-OMe (4)[14b]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 3.68 (s, 3H), 3.91 (d, *J* = 5.7 Hz, 3H), 5.06 (s, 2H), 5.12 (s, 1H), 7.19-7.28 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 42.6, 52.3, 67.1, 128.2, 128.5, 136.2, 156.2, 170.4.

Cbz-Ala-Leu-O^{t}Bu (8)[14c]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.84 (dd, *J* = 0.9 and 6.0 Hz, 6H), 1.32 (d, *J* = 7.2 Hz, 3H), 1.39 (s, 9H), 1.41-1.59 (m, 3H), 4.11-4.22 (m, 1H), 4.36-4.44 (m, 1H), 5.04 (s, 2H), 5.28 (d, *J* = 6.9 Hz, 1H), 6.23 (d, *J* = 7.2 Hz, 1H), 7.11-7.30 (m, 5H); 18.7, 22.1, 22.7, 24.9, 28.0, 41.8, 51.5, 67.0, 82.0, 125.3, 128.0, 128.2, 128.5, 136.2, 155.5, 171.7, 171.8.

Cbz-Ala-Phe-O^{t}Bu (9)[14d]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.27 (d, *J* = 7.2 Hz, 3H), 1.34 (s, 9H), 2.95-3.04 (m, 2H), 4.09-4.15 (m, 1H), 4.61-4.68 (m, 1H), 5.03 (s, 2H), 5.18 (d, *J* = 6.9 Hz, 1H), 6.32 (d, *J* = 6.9 Hz, 1H), 7.05-7.29 (m, 10 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.6, 27.9, 37.9, 50.5, 53.6, 67.0, 82.5, 127.0, 128.1, 128.2, 128.4, 128.5, 129.5, 136.0, 170.2, 171.5.

Cbz-Gly-Phe-O^{t}Bu (10)[14e]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.33 (s, 9H), 3.00 (d, *J* = 6.0 Hz, 2H), 3.73-3.84 (m, 2H), 4.65-4.41 (m, 1H), 5.05 (s, 2H), 5.32 (d, *J* = 6.6 Hz, 1H), 6.36 (d, *J* = 6.9 Hz, 1H), 7.04-7.29 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 27.9, 38.0, 44.5, 53.5, 67.2, 82.6, 127.0, 128.1, 128.2, 128.4, 128.5, 129.6, 135.9, 136.1, 156.4, 168.2, 170.3.

Cbz-Phe-Leu-OEt (11b)[14f]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.79 (dd, J = 5.1 and 9.0 Hz, 6H), 1.12-1.52 (m, 6H), 2.99 (d, *J* = 6.6 Hz, 2H), 4.00-4.08 (m, 2H), 4.35-4.52 (m, 2H), 4.99 (s, 2H), 5.35 (d, *J* = 8.1 Hz, 1H), 6.22 (d, *J* = 8.4 Hz, 1H), 7.05-7.26 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 14.0, 21.8, 22.6, 24.5, 38.7, 41.3, 50.7, 61.2, 66.9, 126.8, 127.9, 128.0, 128.4, 128.5, 128.6, 129.2, 136.4, 155.8, 170.6, 172.3.

Cbz-Ala-Leu-OMe (12)[14g]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.81 (dd, *J* = 5.7 Hz, 6H), 1.28 (d, *J* = 6.9 Hz, 3H), 1.40-1.58 (m, 3H), 3.61 (s, 3H), 4.22-4.32 (m, 1H), 4.47-4.54 (m, 1H), 5.01 (s, 2H), 5.72 (d, *J* = 7.2 Hz, 1H), 6.87 (d, *J* = 6.9 Hz, 1H), 7.22-7.26 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.5, 21.7, 22.6, 24.6, 41.1, 50.6, 52.0, 66.7, 127.8, 127.9, 128.3, 136.1, 155.8, 172.3, 173.1.

Cbz-Ala-Phe-OMe (13)[14d]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.25 (d, *J* = 6.9 Hz, 3H), 2.95-3.10 (m, 2H), 3.64 (s, 3H), 4.09-4.19 (m, 1H), 4.74-4.81 (m, 1H), 5.02 (s, 1H), 5.20 (d, *J* = 5.7 Hz, 1H), 6.41 (d, *J* = 5.7 Hz, 1H), 6.99-7.28 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.4, 37.8, 50.4, 52.3, 53.2, 67.0, 127.1, 128.0, 128.2, 128.5, 129.2, 135.6, 136.2, 155.8, 171.6, 171.7.

Cbz-Gly-Phe-OMe (14)[14h]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.99-3.07 (m, 2H), 3.64 (s, 3H), 3.70-3.83 (m, 2H), 5.05 (s, 2H), 5.29 (d, *J* = 6.3 Hz, 1H), 6.35 (d, *J* = 6.6 Hz, 1H), 6.99-7.29 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 37.8, 44.5, 52.4, 53.1, 67.2, 127.2, 128.0, 128.2, 128.5, 128.6, 129.2, 135.5, 136.1, 168.4, 171.6.

Cbz-Phe-Leu-Ala-O^{t}Bu (16)
R_t(HPLC) 22.95 min; Purity >97%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.79 (d, *J* = 5.7 Hz, 6H), 1.24 (d, *J* = 7.2 Hz, 3H), 1.55-1.33 (m, 12H), 3.02-2.90 (m, 2H), 4.42-4.29 (m, 3H), 5.00-4.90 (m, 2H), 5.64 (d, *J* = 8.4 Hz, 1H), 6.82 (d, *J* = 7.2 Hz, 1H), 6.89 (d, *J* = 6.9 Hz, 1H), 7.24-7.05 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.2, 22.1, 22.7, 24.5, 27.9, 38.4, 41.3, 48.6, 51.7, 56.0, 81.8, 126.9, 127.9, 128.0, 128.4, 128.5, 129.3, 136.3, 156.0, 171.0, 171.1, 171.7; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{30}H_{42}N_3O_6$: 540.3068; found: 540.3040.

Cbz-Phe-Leu-Ala-OH (17)[14i]
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.88 (dd, *J* = 6.6 and 11.4 Hz, 6H), 1.27 (d, *J* = 7.2 Hz, 3H), 1.45-1.69 (m, 3H), 2.68-3.03 (m, 2H), 4.11-4.41 (m, 3H), 4.94 (s, 2H), 7.08-7.47 (m, 11H), 8.02 (d, *J* = 8.1 Hz, 1H), 8.19 (d, *J* = 6.9 Hz, 1H), 12.49 (s, 1H); ^{13}C NMR (DMSO-d_6,

75 MHz): δ = 17.0, 21.6, 22.9, 23.9, 37.3, 41.0, 47.3, 50.6, 55.9, 65.1, 126.1, 127.3, 127.5, 127.9, 128.1, 129.1, 136.9, 138.0, 155.7, 171.1, 171.5, 173.8.

For-Met-OMe (19)[14j]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.88-2.20 (m, 5H), 2.47 (t, *J* = 7.5 Hz, 2H), 3.71 (s, 3H), 4.71-4.48 (m, 1H), 6.47 (d, *J* = 4.2 Hz, 1H), 8.17 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.4, 29.8, 31.6, 50.1, 52.6, 160.7, 163.9, 171.9.

For-Met-Leu-O^{t}Bu (20)

R_t(HPLC) 16.43 min; Purity >95%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.87 (dd, *J* = 3.9 and 6.0 Hz, 6H), 1.66-1.34 (m, 12H), 2.07-1.87 (m, 5H), 2.59-2.53 (m, 2H), 4.23-4.35 (m, 1H), 4.68 (q, *J* = 6.9 Hz, 1H), 6.51-6.46 (m, 2H), 8.13 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.0, 22.0, 22.7, 24.9, 28.0, 31.7, 41.5, 50.4, 51.7, 82.1, 160.6, 170.1, 171.4; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{16}H_{32}N_2O_4S$: 347.1999; found: 347.2003.

For-Met-Leu-OMe (21)

R_t(HPLC) 14.78 min; Purity >98%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.85 (dd, *J* = 3.3 and 5.7 Hz, 6H), 1.61-1.48 (m, 3H), 2.07-1.61 (m, 5H), 2.54 (dd, *J* = 7.2 Hz, 2H), 3.04 (s, 3H), 4.50-4.43 (m, 1H), 4.78 (q, *J* = 7.8 Hz, 1H), 7.24-7.18 (m, 2H), 8.10 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.0, 21.6, 22.6, 24.7, 29.6, 31.9, 40.7, 50.3, 50.9, 52.2, 161.0, 170.9, 172.8; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{13}H_{25}N_2O_4S$: 305.1530; found: 305.1539.

For-Met-Leu-Phe-O^{t}Bu (22)[14k]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.82 (dd, *J* = 6.3 and 8.7 Hz, 6H), 1.28 (s, 9H), 1.43-1.64 (m, 3H), 1.75-1.99 (m, 5H), 2.39 (t, *J* = 7.2 Hz, 2H), 2.95-3.00 (m, 2H), 4.64-4.71 (m, 1H), 4.75-4.83 (m, 1H), 4.92-4.99 (m, 1H), 7.08-7.21 (m, 5H), 7.81-7.87 (m, 3H), 8.05 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.2, 22.5, 22.6, 24.7, 27.8, 29.9, 33.1, 38.2, 42.0, 50.3, 51.6, 53.9, 82.0, 126.7, 128.2, 129.3, 136.2, 161.0, 170.5, 170.8, 171.7.

For-Met-Leu-Phe-OMe (23)[14l]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.83 (dd, *J* = 6.6 Hz, 6H), 1.42-1.65 (m, 3H), 1.80-1.99 (m, 5H), 2.37 (t, *J* = 7.5 Hz, 2H), 2.95-3.07 (m, 2H), 3.59 (s, 3H), 4.67-4.78 (m, 2H), 4.91-4.99 (m, 1H), 7.04-7.21 (m, 5H), 7.70 (d, *J* = 9.0 Hz, 1H), 6.78 (d, *J* = 7.2 Hz, 1H), 7.87 (d, *J* = 9.3 Hz, 1H), 8.04 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.2, 22.4, 22.6, 24.7, 29.8, 33.0, 37.9, 41.8, 50.3, 51.7, 52.1, 53.6, 126.9, 128.3, 129.0, 136.0, 161.9, 170.9, 171.8, 171.9.

Fmoc-Leu-OMe (25)

R_t(HPLC) 20.23 min; Purity >96%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.89-0.87 (dd, 6H), 1.63-1.47 (m, 3H), 3,67 (s, 2H), 4.16 (t, *J* = 6.9 Hz, 1H), 4.36-4.30 (m, 3H), 5.07 (d, *J* = 8.1 Hz, 1H), 7.35-7.18 (m, 5H), 7.54-7.51 (m, 2H), 7.69 (d, *J* = 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.8, 22.8, 24.7, 41.8, 47.2, 52.3, 67.0, 120.0, 125.1, 127.0, 127.7, 141.3, 143.8, 155.9, 173.9; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{22}H_{26}NO_4$: 368.1856; found: 368.1855.

Fmoc-Leu-Phe-O^{t}Bu (26)

R_t(HPLC) 23.62 min; Purity >97%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.84 (dd, *J* = 3.3 Hz, 6H), 1.50-1.25 (m, 12H), 2.99 (dd, *J* = 6.0 Hz, 2H), 4.14-4.10 (m, 2H), 4.38-4.22 (m, 2H), 4.64 (q, *J* = 7.5 Hz, 1H), 5.16 (d, *J* = 8.4 Hz, 1H), 6.38 (d, *J* = 7.2 Hz, 1H), 7.33-7.04 (m, 9H), 7.50 (d, *J* = 6.9 Hz, 2H), 7.67 (d, *J* = 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.9, 22.9, 24.6, 27.9, 38.0, 41.5, 47.1, 53.6, 67.0, 82.3, 119.9, 125.0, 125.1, 126.9, 127.0, 127.7, 128.3, 129.5, 136.0, 141.3, 143.8 (2C), 156.0, 170.2, 171.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{34}H_{41}N_2O_5$: 557.3010; found: 557.2982.

H-Leu-Phe-O^{t}Bu (27)

Fmoc-Leu-Phe-O^{t}Bu (1 g) was dissolved in CH_2Cl_2/ethanolamine (50 mL, 19/1, v/v) and the obtained reaction mixture was stirred at ambient temperature for 2 h. Then, the volatiles were removed *in vacuo* and the residue dissolved in DMF (10 mL). The obtained solution was added to cold MTBE/*n*-heptane (100 mL) followed by centrifugation (5000 rpm). The precipitates were washed with MTBE/*n*-heptane (100 mL) and lyophilized from CH_3CN/H_2O (3/1, v/v), giving H-Leu-Phe-O^{t}Bu in a yield of 99%. R_t(HPLC) 6.88 min; Purity >96%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.88 (dd, *J* = 6.9 Hz, 6H), 1.54-1.32 (m, 11H), 1.73-1.30 (m, 1H), 2.97 (dd, *J* = 2.4 and 7.8 Hz, 2H), 3.57-3.52 (m, 1H), 4.41 (q, *J* = 7.2 Hz, 1H), 7.24-7.20 (m, 5H), 8.65 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.7, 22.8, 23.5, 27.4, 36.7, 41.7, 51.3, 53.9, 80.7, 126.4, 128.1, 129.1, 136.8, 163.3, 170.0, 171.4; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{19}H_{31}N_2O_3$: 335.2329; found: 335.2351

Boc-Phe-OMe (29)[14m]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.25 (s, 9H), 2.86-2.99 (m, 2H), 3.54 (s, 3H), 4.35-4.44 (m, 1H), 4.79 (d, *J* = 6.6 Hz, 1H), 6.95-7.18 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 28.3, 38.4, 52.2, 54.4, 80.3, 127.0, 128.5, 129.3, 136.0, 172.3.

Boc-Phe-Leu-Phe-O^{t}Bu (30)

R_t(HPLC) 24.18 min; Purity >98%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.79 (dd, *J* = 6.0 Hz, 6H), 1.56-1.31 (m, 21H) 2.97 (dd, *J* = 6.3 Hz, 2H), 4.64-4.57 (m, 2H), 4.61 (q, *J* = 7.8 Hz, 1H), 4.98 (d, *J* = 7.8 Hz, 1H), 6.36 (d, *J* = 7.8 Hz, 1H), 6.49 (d, *J* = 7.8 Hz, 1H), 7.23-7.07 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 22.0, 22.8, 24.5, 27.9, 28.2, 38.1, 41.1, 51.7, 53.7, 55.6, 80.2, 82.2, 126.9, 128.3, 128.6, 129.3, 129.5, 136.1, 136.6, 155.4, 170.2, 170.9, 171.1; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{33}H_{48}N_3O_6$: 582.3538; found: 582.3572.

Boc-Phe-Leu-Phe-OMe (31)[14n]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.78 (dd, *J* = 5.7 Hz, 6H), 1.32-1.55 (m, 12H), 2.90-3.08 (m, 2H), 3.62 (s, 3H), 2.26-4.40 (m, 2H), 4.69-4.75 (m, 1H), 4.98 (d, *J* = 7.8 Hz, 1H), 6.40 (d, *J* = 7.8 Hz, 1H), 6.57 (d, *J* = 7.5 Hz, 1H), 7.02-7.23 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.9, 22.8, 24.5, 28.2, 37.8, 40.9, 51.7, 52.2, 53.3, 55.6, 80.3, 126.9, 127.1, 128.5, 128.6, 129.2, 129.3, 135.8, 136.5, 155.4, 171.2, 171.3, 171.6.

Boc-Phe-Leu-Phe-Leu-Phe-O^{t}Bu (32)

R_t(HPLC) 24.76 min; Purity >95%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.87-0.77 (m, 1H), 1.20-1.06 (m, 24H), 3.02-2.64 (m, 6H), 4.06-4.05 (m, 1H), 4.29-4.22 (m, 3H), 4.57-4.48 (m, 1H), 6.90 (d, *J* = 8.4 Hz, 1H), 7.29-7.11 (m, 15H), 7.99-7.86 (m, 3H), 8.22 (d, *J* = 7.2 Hz, 1H) ; ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.5, 22.9, 23.9, 27.4, 36.6, 37.0, 41.1, 41.2, 50.5, 50.8, 53.2, 54.0, 55.5, 77.9, 80.4, 125.9, 126.0, 126.3, 127.8, 128.0, 129.0 (2 C), 137.0, 137.5, 138.1, 155.0, 170.1, 170.2, 170.4, 171.2, 171.6; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{48}H_{68}N_5O_8$: 842.5062; found: 842.5035.

Boc-Phe-Leu-Phe-Leu-Phe-OH (33)[14o]

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.79-0.86 (m, 12H), 1.02-1.62 (m, 15H), 2.64-3.08 (m, 6H), 4.10-4.57 (m, 5H), 6.90 (d, *J* = 8.7 Hz, 1H), 7.18-7.25 (m, 15H), 7.87-8.11 (m, 4H), 12.85 (s, 1H) ; ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.5, 23.0, 23.8, 28.0, 36.5, 37.0, 41.1, 50.7, 50.8, 53.1, 55.5, 77.9, 126.0, 126.3, 127.8, 128.9, 129.0, 129.1, 137.3, 137.5, 138.1, 155.1, 170.3, 171.6, 172.5.

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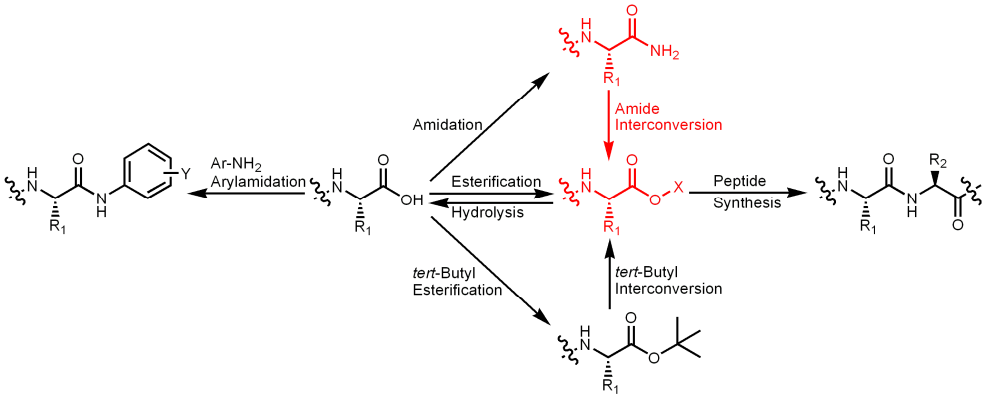
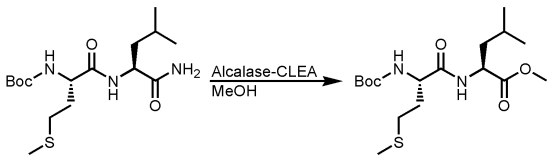
Chapter 7

Enzymatic peptide synthesis using *C*-terminal α-carboxamide to ester interconversion



In this chapter, a novel interconversion reaction of peptide *C*-terminal α-carboxamides into primary alkyl esters catalyzed by Subtilisin A is described as an approach to a fully enzymatic peptide synthesis strategy. For each peptide elongation step, a cost efficient amino acid α-carboxamide building block was used followed by its interconversion into the corresponding primary alkyl ester. These peptide esters are the starting materials for the next enzymatic peptide elongation step. This fully enzymatic *N* → *C* elongation strategy by *C*-terminal α-carboxamide to ester interconversion was applied toward the synthesis of two biologically active peptides.



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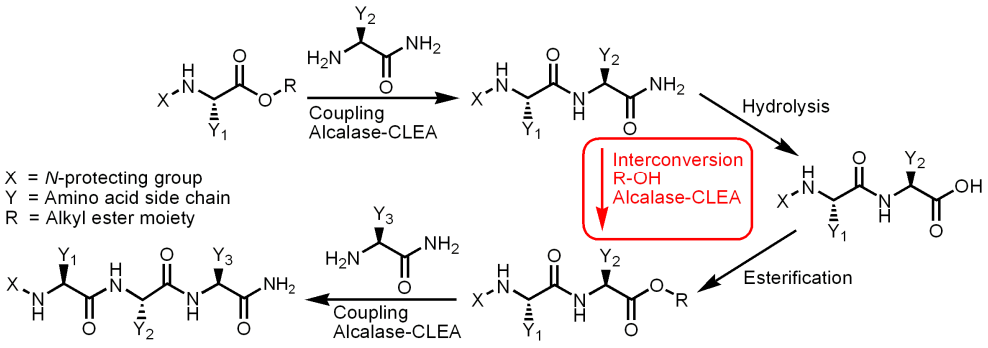
7.1 Introduction

Although an increasing amount of peptides is used as therapeutic, (pro)drug, nutritional or cosmetic ingredient,[1] their large-scale and cost-effective production remains a huge challenge.[2] Commonly used production methods are very time consuming and expensive.[3] There are four main approaches toward the synthesis of peptides, *i.e.* solid-phase or solution-phase chemical peptide synthesis, chemo-enzymatic peptide synthesis and fermentation. Mostly applied are solid-phase and solution-phase chemical peptide synthesis. However, both methods require full protection of the functionalized amino acid side chains. Furthermore, expensive coupling reagents have to be used in stoichiometric amounts and in case of fragment assembly uncontrolled racemization of the *C*-terminally activated amino acid residue occurs during the coupling reaction.

During the past two decades, chemo-enzymatic peptide synthesis proved to be useful for the cost-effective synthesis of certain peptide sequences (up to 5 amino acids) on a large scale.[2] Herein, the amino acids are coupled enzymatically in a stepwise manner wherein the functionalized amino acid side chains require no protection and, more importantly, racemization is completely absent during the fragment assembly. Nevertheless, the *N*- and *C*-termini still require protection and after each elongation step, a selective deprotection of one of the termini needs to be performed. When the peptide sequence is synthesized in the *C* → *N* terminal direction, expensive *N*-terminally protected and *C*-terminally activated amino acid building blocks are required and after each coupling step the *N*-terminal protecting group must be removed. Furthermore, with the growing peptide being the acyl acceptor which is generally applied in an excess over the acyl donor this strategy is highly cost-inefficient. Elongation of the peptide sequence in the *N* → *C*-terminal direction requires an excess of the much cheaper *C*-terminally protected amino acids as building blocks, such as α-carboxamides (Scheme 1) or tert-butyl (^{t}Bu) esters. After peptide elongation, the *C*-terminus must be selectively deprotected and subsequently activated for the next enzymatic coupling step. However, the acid-catalyzed hydrolysis of *C*-terminal ^{t}Bu esters or α-carboxamides requires rather harsh reaction conditions and is unselective.[4]



Scheme 1. Chemo-enzymatic peptide synthesis in the *N*→*C* terminal direction using *C*-terminal α-carboxamide to ester interconversion.

The enzymatic hydrolysis of ^{t}Bu esters under mild reaction conditions has been reported for a number of lipases and proteases.[5] Unfortunately, lipases most often do not recognize peptides as their substrates, while proteases are fraught with undesired peptide bond cleavage. In chapter 6, a fully enzymatic peptide synthesis strategy, in which *C*-terminal interconversion of peptide ^{t}Bu esters into primary alkyl esters by the industrial protease Subtilisin A as the key step, was described.[6] In any case, the preferred economical *C*-terminal protective group is the α-carboxamide, since such α-amide building blocks are easily accessible from amino acids *e.g.* in a one-pot process using an acid-catalyzed methyl ester formation followed by aminolysis (quenching by an excess of ammonia).

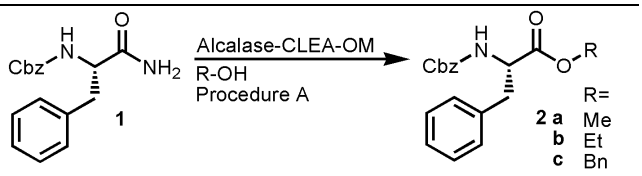
The enzymatic hydrolysis of peptide α-carboxamides is a known reaction. Selective peptide deamidases with a wide substrate tolerance have been identified, among others the peptide amidase from the *flavedo* of oranges (PAF).[7] In a single example, PAF has been used for the interconversion of peptide α-carboxamides into methyl esters,[8] albeit in a maximum yield of 40%. Moreover, PAF is not well characterized which makes fermentation by a micro-organism impossible, while extraction from orange peel requires 1000 kg to give 1 g of enzyme.

Recently, a versatile α-amidation of *C*-terminal peptide alkyl esters using ammonium salts as amine donor in the presence of the protease Subtilisin A was described.[9] It was envisioned that this reaction could be reversed yielding an interconversion reaction of *C*-terminal peptide α-carboxamides into alkyl esters. Subsequently, these esters can serve as an activated intermediate for further enzymatic peptide elongation, as shown in Scheme 1.

7.2 Results and Discussion

As a proof-of-principle for the interconversion reaction, Subtilisin A (commercially available as Alcalase in which Subtilisin A is the main constituent) was applied to Cbz-Phe-NH_2 (**1**) as model substrate in the presence of alcohols, as depicted in Table 1.

Table 1. Interconversion of Cbz-Phe-NH_2 into primary alkyl esters using Alcalase-CLEA-OM.



Entry[a]	Amide product		Yield (%)[b]
1	Cbz-Phe-OMe	**2a**	98
2	Cbz-Phe-OEt	**2b**	90
3	Cbz-Phe-OBn	**2c**	83

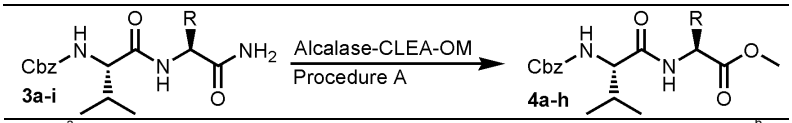
[a] Conditions: Alcalase-CLEA-OM, 3 Å and 5 Å molecular sieves MTBE (or THF or DMF/THF, 1/9, v/v), appropriate alcohol, 50°C, 48 h (Procedure A); [b] Isolated yield based on acyl donor starting material.

The interconversion of Cbz-Peh-NH_2 (**1**) as a model α-carboxamide into its corresponding primary alkyl esters (**2a-c**) proceeded smoothly and in high yield. Immobilized Alcalase

cross-linked enzyme aggregates (Alcalase-CLEA, type OM)[10] were used for optimal enzyme stability in anhydrous organic solvent but also for a convenient workup (filtration) and recycling. Performing this interconversion reaction in the presence of 3 Å molecular sieves nullified the hydrolysis of the α-carboxamide towards its carboxylic acid. The reverse reaction of the interconversion *i.e.* α-carboxamide synthesis from the alkyl ester, by the ammonia released during interconversion, could be minimized by using a large excess of the corresponding alcohol and 5 Å molecular sieves to absorb the ammonia. To avoid deactivation of the enzyme in anhydrous methanol (MeOH) or ethanol (EtOH), a co-solvent, *i.e.* tetrahydrofuran (THF) or methyl *tert*-butyl ether (MTBE), was used. When these optimal reaction conditions were applied, full conversion of the α-carboxamide into the corresponding esters was observed (HPLC analysis), and **2a-c** were isolated in good to excellent yield (83-98%).

To investigate the scope of this novel α-carboxamide interconversion reaction, several dipeptide *C*-terminal α-carboxamides with an unprotected side chain (**3a-i**) were converted into their corresponding methyl esters (**4a-h**), as is shown in Table 2.

Table 2. Interconversion of Cbz-Val-Xaa-NH_2 into their corresponding methyl esters using Alcalase-CLEA-OM.



Entry[a]	Starting material		Product		Yield (%)[b]
1	Cbz-Val-Ala-NH_2	**3a**	Cbz-Val-Ala-OMe	**4a**	97
2	Cbz-Val-Ser-NH_2	**3b**	Cbz-Val-Ser-OMe	**4b**	89
3	Cbz-Val-Leu-NH_2	**3c**	Cbz-Val-Leu-OMe	**4c**	88
4	Cbz-Val-Gln-NH_2	**3d**	Cbz-Val-Gln-OMe	**4d**	73
5	Cbz-Val-Phe-NH_2	**3e**	Cbz-Val-Phe-OMe	**4e**	71
6	Cbz-Val-Glu-NH_2	**3f**	Cbz-Val-Glu-OMe	**4f**	67
7	Cbz-Val-Asn-NH_2	**3g**	Cbz-Val-Asn-OMe	**4g**	36
8	Cbz-Val-Asp-NH_2	**3h**	Cbz-Val-Asp-OMe	**4h**	16
8	Cbz-Val-Gly-NH_2	**3i**	Cbz-Val-Gly-OMe	**4i**	Trace

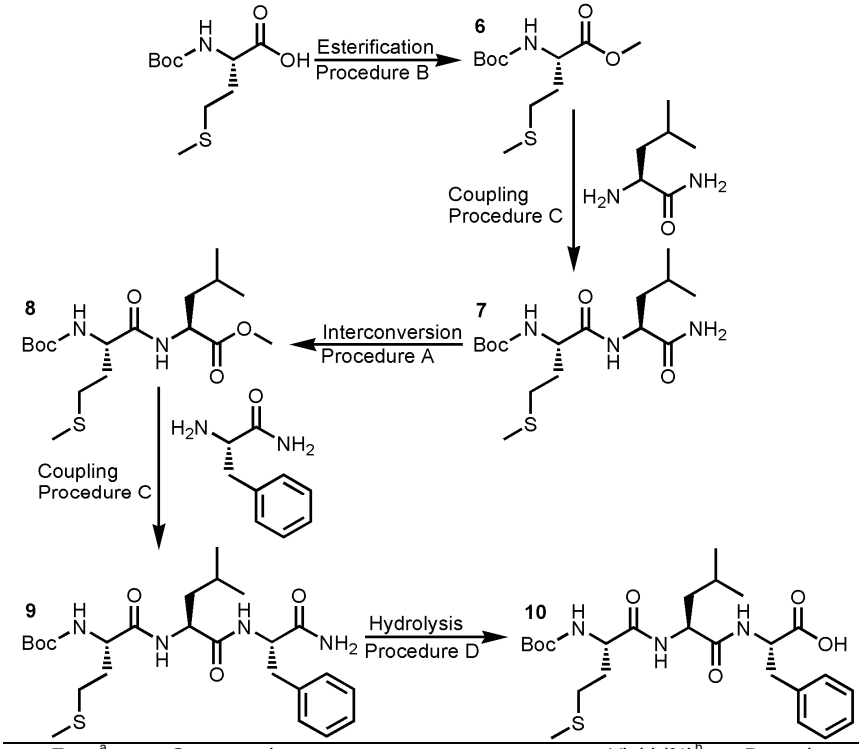
[a] Conditions: Alcalase-CLEA-OM, 3 Å and 5 Å molecular sieves MTBE (or THF or DMF/THF, 1/9, v/v), MeOH, 50°C, 48 h (Procedure A); [b] Isolated yield based on acyl donor starting material.

Gratifyingly, several dipeptide α-carboxamides were smoothly converted into their corresponding methyl esters in a good yield. It should be noted that also in case of moderate conversion after 72h, satisfactory yields could be obtained after prolonged reaction times. For instance, Cbz-Val-Asp-NH_2 (**3h**) was isolated in 78% yield after 15 days of stirring. Interestingly, the hydroxyl functionality of serine (**3b**), and even the β- and γ-carboxamides of asparagine and glutamine (**3d** and **3g**, respectively), were not affected during the α-carboxamide interconversion reaction. The dipeptide product Cbz-Val-Gln-OMe (**4d**) was compared with reference compounds by NMR and analytical HPLC, including Cbz-Val-Glu(OMe)-NH_2 (**5**), to verify that the amide interconversion was fully selective.[11]

The interconversion was also feasible with *Candida antarctica* lipase B (Cal-B) under identical reaction conditions with Cbz-Ala-NH_2 as substrate (93% yield). However, the interconversion of (di)peptide α-carboxamides catalyzed by Cal-B gave no or only low yields (data not shown).

To demonstrate the versatility of the fully enzymatic peptide elongation strategy by *C*-terminal α-carboxamide interconversion, two biologically interesting tripeptides were synthesized, as shown in Table 3 and 4.

Table 3. Fully enzymatic *N*→*C* directed synthesis of the chemotactic peptide antagonist Boc-Met-Leu-Phe-OH[12] (**10**).

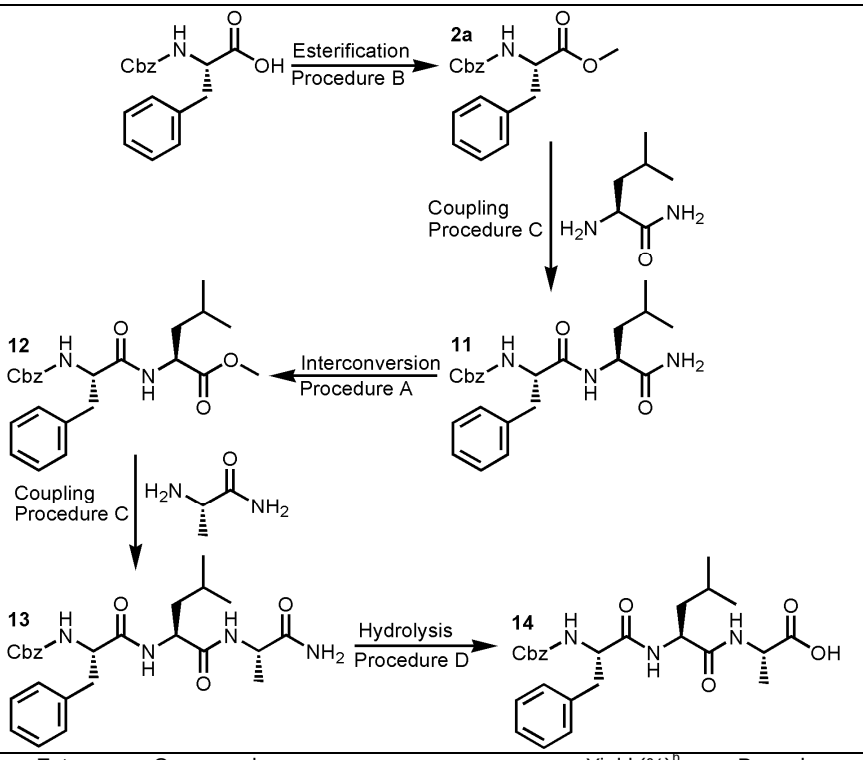


Entry[a]	Compound		Yield (%)[b]	Procedure
1	Boc-Met-OMe	**6**	91	B
2	Boc-Met-Leu-NH_2	**7**	78	C
3	Boc-Met-Leu-OMe	**8**	97	A
4	Boc-Met-Leu-Phe-NH_2	**9**	67	C
5	Boc-Met-Leu-Phe-OH	**10**	Quant.[c]	D

[a] Conditions: Alcalase-CLEA-OM, 3 Å and 5Å molecular sieves MTBE (or THF or DMF/THF, 1/9, v/v), MeOH, 50°C, 48 h (Procedure A); Alcalase-CLEA-OM, MeOH/MTBE (1/14, v/v), 3 Å molecular sieves, 50°C, 16h (Procedure B); Alcalase-CLEA-OM, 4 Å molecular sieves, amino acid nucleophile, MTBE or THF or DMF/THF (1/9, v/v), 50°C, 20 h (Procedure C); PAF, TRIS/HCl buffer (50 mM, pH 7.5) with DMF (1/1, v/v) (Procedure D); [b] Isolated yield based on acyl donor starting material; [c] According to LC-MS analysis.

As a first example, the synthesis of the chemotactic peptide antagonist[12] Boc-Met-Leu-Phe-OH (**10**) (Table 3) was performed using Alcalase-CLEA-OM for all individual reaction steps, except for the final hydrolysis of Boc-Met-Leu-Phe-NH_2 (**9**), which was performed with PAF (quantitative conversion according to HPLC analysis).[7] High yields were obtained for the esterification (**6**, 91%), peptide coupling (**7**, 78% and **9**, 67%) and the interconversion (**8**, 97%). The tripeptide Boc-Met-Leu-Phe-NH_2 (**9**) was isolated in a somewhat lower yield of 67%, since it appeared that the tetrapeptide Boc-Met-Leu-Phe-Phe-NH_2 (11%, verified by LC-MS analysis) had also been formed.

Table 4. Fully enzymatic *N*→*C* directed synthesis of the thermolysine assay substrate Cbz-Phe-Leu-Ala-OH[13] (**14**).



Entry	Compound		Yield (%)[b]	Procedure
1	Cbz-Phe-OMe	**2a**	94	B
2	Cbz-Phe-Leu-NH_2	**11**	83	C
3	Cbz-Phe-Leu-OMe	**12**	68	S
4	Cbz-Phe-Leu-Ala-NH_2	**13**	88	C
5	Cbz-Phe-Leu-Phe-OH	**14**	Quant.[c]	D

[a] Conditions: Alcalase-CLEA-OM, 3 Å and 5Å molecular sieves MTBE (or THF or DMF/THF, 1/9, v/v), MeOH, 50°C, 48 h (Procedure A); Alcalase-CLEA-OM, MeOH/MTBE (1/14, v/v), 3 Å molecular sieves, 50°C, 16h (Procedure B); Alcalase-CLEA-OM, 4 Å molecular sieves, amino acid nucleophile, MTBE or THF or DMF/THF (1/9, v/v), 50°C, 20 h (Procedure C); PAF, TRIS/HCl buffer (50 mM, pH 7.5) with DMF (1/1, v/v) (Procedure D); [b] Isolated yield based on acyl donor starting material; [c] According to LC-MS analysis.

In the second example, the thermolysine substrate[13] Cbz-Phe-Leu-Ala-OH (**14**) (Table 4) was synthesized analogously with high yields for all individual steps. The interconversion of Cbz-Phe-Leu-NH_2 (**11**) into Cbz-Phe-Leu-OMe (**12**) proved to be challenging due to alcoholysis of the dipeptide backbone into Cbz-Phe-OMe (**2a**, 10%, as shown by HPLC).
Since Alcalase has a very broad substrate tolerance,[14] the fully enzymatic peptide synthesis strategy via α-carboxamide to ester interconversion is expected to be applicable to a large variety of industrially interesting peptides. However, for a limited number of peptide sequences the application is hampered due to undesired alcoholysis of the backbone which may become more prominent for longer peptide sequences. Therefore it is thought that the stepwise *N* → *C*-directed peptide synthesis strategy will only be applicable on large scale for peptides up to five amino acids length.
In conclusion, this chapter describes a versatile and generally applicable fully enzymatic peptide synthesis strategy based on simultaneous *C*-terminal deprotection and activation of the growing oligopeptide, using the easily accessible amino acid α-carboxamides as building blocks, and Alcalase as the biocatalyst. It was also shown that in case of longer peptides side-chain groups were not affected.

7.3 Experimental

General

General experimental information is identical to chapter 2 and 3 with the following exceptions. Alcalase-CLEA type OM (organic media) was used from CLEA-Technologies (580 U/g), peptide amidase from the *flavedo* of oranges (PAF) was obtained from Codexis and trifluoroacetic acid was used for the preparative HPLC buffers A and B.

General Procedure A: Enzymatic α-carboxamide to ester interconversion

Alcalase-CLEA-OM (250 mg), 3 Å molecular sieves (400 mg) and 5 Å molecular sieves (200 mg) were added to a solution of *N*-protected amino acid or peptide α-carboxamide (0.84 mmol) in MTBE (14 mL, or THF or DMF/THF, 1/9, v/v) and the appropriate alcohol (1 mL). The obtained reaction mixture was shaken at 50°C with 150 rpm for 48 h. After filtration, the solid enzyme particles were resuspended in the appropriate alcohol and shaken at 50°C for 30 min and removed by filtration (50 mL, 3×). Subsequently, the enzyme particles were washed with EtOAc (50 mL, 2×) and CH_2Cl_2 (50 mL, 2×). The combined filtrates were concentrated *in vacuo* and the residue was lyophilized from CH_3CN/H_2O (3/1, v/v). Most methyl esters were obtained in a purity >95% as determined by HPLC, if not, the resulting crude peptide was purified by preparative HPLC.

General Procedure B: Enzymatic peptide coupling

Alcalase-CLEA-OM (0.5 g) and 4 Å molecular sieves (1.0 g) were added to a solution of either *N*-protected amino acid, peptide methyl ester (1.3 mmol) or amino acid or peptide ^{t}Bu ester (2.6 mmol, 2 equiv) in MTBE (30 mL, or THF or DMF/THF, 1/9, v/v). The obtained reaction mixture was shaken at 50°C with 150 rpm for 20 h. After filtration, the solid enzyme particles were resuspended in MeOH and removed by filtration (50 mL, 3×). This enzyme particle washing procedure was repeated with EtOAc (50 mL, 2×) and CH_2Cl_2 (50 mL, 2×).

The combined filtrates were concentrated *in vacuo* and the resulting residue was redissolved in EtOAc or CH_2Cl_2 (100 mL) and this solution washed with sat. aq. $NaHCO_3$ (75 mL, 2×), 0.1 N HCl (75 mL, 2×), brine (75 mL) and dried (Na_2SO_4). The organic layer was concentrated *in vacuo* and the residue was lyophilized. Most peptides were obtained in a purity >95% as determined by analytical HPLC, if not, the resulting crude peptide was purified by preparative HPLC.

General Procedure C: Esterification of *N*-protected amino acids

Alcalase-CLEA-OM (300 mg) and 3 Å molecular sieves (200 mg) were added to an *N*-protected amino acid (1.67 mmol) which was dissolved in a mixture of MTBE/MeOH (3 mL, 14/1, v/v). The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. After filtration, the solid enzyme particles were washed by resuspension in EtOAc and removed by filtration (20 mL, 3×). The combined filtrates were washed with sat. aq. $NaHCO_3$ (40 mL, 2×), 0.1 N HCl (40 mL, 2×), brine, (40 mL), dried (Na_2SO_4) and subsequently concentrated *in vacuo* and the residue was co-evaporated with toluene (20 mL, 2×) and $CHCl_3$ (20 mL, 2×). According to analytical HPLC analysis most compounds proved to be >95% pure.

7

General Procedure D: Hydrolysis of *C*-terminal α-carboxamides

To a solution of PAF (50 µg/mL) in TRIS HCl buffer/DMF (1 mL, 1/1, v/v, 50 mM, pH 7.5) tripeptide amide **9** or **14** (1 mg) was added. The obtained reaction mixture was shaken at 37°C with 150 rpm for 24 h. finally, the conversions were determined using analytical HPLC and product peaks were identified by LC-MS analysis.

Dipeptide α-carboxamide synthesis

Dipeptides **3a**-**3i** were synthesized on a Rink resin (1 g, 0.5 mmol/g) using standard Fmoc/^{t}Bu SPPS procedures.[15] Peptides were cleaved from the resin and deprotected using trifluoroacetic acid/H_2O (TFA/H_2O, 10 mL, 19/1, v/v, 60 min) followed by precipitation using cold MTBE/*n*-heptane (100 mL, 1/1, v/v) and finally lyophilized from CH_3CN/H_2O (3/1, v/v).

The analyses of Cbz-Phe-OMe (**2a**), Cbz-Phe-OEt (**2b**), Cbz-Phe-OBn (**2c**) and Cbz-Phe-Leu-OMe (**11**) are described in chapter 2.

Cbz-Val-Ala-NH_2 (3a)[16a]

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84 (dd, *J* = 6.9 and 11.4 Hz, 6H), 1.20 (d, *J* = 7.2 Hz, 3H), 1.93-2.05 (m, 1H), 3.83-3.89 (m, 1H), 4.17-4.28 (m, 1H), 5.03 (s, 2H), 6.98 (s, 1H), 7.31-7.38 (m, 7H), 7.90 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.8, 18.3, 19.1, 30.1, 47.7, 60.0, 65.3, 127.5, 127.6, 128.2, 136.9, 156.1, 170.5, 173.9.

Cbz-Val-Ser-NH_2 (3b)

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84 (dd, *J* = 6.9 and 11.4 Hz, 6H), 1.87-2.06 (m, 1H), 3.53-3.59 (m, 2H), 3.88-3.94 (m, 1H), 4.20-4.26 (m, 1H), 4.56 (s, 1H), 5.04 (s, 2H), 7.07 (s, 1H), 7.22 (s, 1H), 7.30-7.37 (m, 6H), 7.77 (d, *J* = 7.8 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 19.1, 30.1, 54.6, 60.2, 61.6, 65.3, 127.5, 127.6, 128.2, 136.9, 156.1, 170.8, 171.7.

Cbz-Val-Leu-NH_2 (3c)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.81-0.86 (m, 12H), 1.36-1.63 (m, 3H), 1.92-2.04 (m, 1H), 3.82-3.89 (m, 1H), 4.22-4.30 (m, 1H), 5.03 (s, 2H), 6.95 (s, 1H), 7.29-7.36 (m, 7H), 7.81 (d, *J* = 8.1 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.0, 19.1, 21.5, 22.9, 24.0, 30.1, 50.6, 60.3, 65.3, 127.5, 127.6, 128.2, 136.9, 156.0, 170.7, 173.8.

Cbz-Val-Gln-NH_2 (3d)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.85 (dd, *J* = 6.6 and 12.6 Hz, 6H), 1.67-2.14 (m, 5H), 3.84-3.90 (m, 1H), 4.15-4.22 (m, 1H), 5.03 (s, 2H), 6.74 (s, 1H), 7.03 (s, 1H), 7.24-7.36 (m, 8H), 7.88 (d, *J* = 7.8 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 19.1, 27.9, 30.1, 31.2, 51.9, 60.2, 65.3, 127.5, 127.6, 128.2, 136.9, 156.1, 170.8, 172.9, 173.6.

Cbz-Val-Phe-NH_2 (3e)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.74 (dd, *J* = 6.6 Hz, 6H), 1.82-1.93 (m, 1H), 2.72-3.01 (m, 2H), 3.77-3.82 (m, 1H), 4.44-4.52 (m, 1H), 5.03 (s, 2H), 7.05 (s, 1H), 7.10-7.36 (m, 12H), 7.90 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 19.0, 30.1, 53.4, 60.4, 65.3, 126.0, 127.5, 127.8, 128.2, 129.0, 136.9, 137.6, 156.0, 170.6, 172.6.

Cbz-Val-Glu-NH_2 (3f)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84 (dd, *J* = 6.9 and 8.7 Hz, 6H), 1.71-2.15 (m, 5H), 3.85-3.90 (m, 1H), 4.19-4.26 (m, 1H), 5.04 (s, 2H), 7.07 (s, 1H), 7.30-7.37 (m, 7H), 7.86 (d, *J* = 8.1 Hz, 1H), 12.03 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 19.1, 27.3, 29.9, 30.0, 51.4, 60.2, 65.3, 127.5, 127.6, 128.2, 136.9, 156.1, 170.9, 172.7, 173.8.

Cbz-Val-Asn-NH_2 (3g)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.85 (dd, *J* = 6.9 and 9.3 Hz, 6H), 1.91-2.03 (m, 1H), 3.81-3.86 (m, 1H), 4.42-4.50 (m, 1H), 5.03 (s, 2H), 6.87 (s, 1H), 7.04 (d, *J* = 5.1 Hz, 1H), 7.30-7.38 (m, 7H), 8.06 (d, *J* = 7.8 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 19.0, 30.0, 36.8, 49.4, 60.3, 65.4, 127.6, 128.2, 136.8, 156.2, 170.7, 171.4, 172.6.

Cbz-Val-Asp-NH_2 (3h)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84 (dd, *J* = 6.9 and 8.7 Hz, 6H), 1.90-2.03 (m, 1H), 2.50-2.73 (m, 2H), 3.81-3.86 (m, 1H), 4.44-4.51 (m, 1H), 5.02 (s, 2H), 7.11 (d, *J* = 7.8 Hz, 1H), 7.33-7.38 (m, 6H), 8.11 (d, *J* = 7.8 Hz, 1H), 12.02 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 19.0, 30.1, 35.9, 49.2, 60.3, 65.4, 127.6, 127.7, 128.2, 136.8, 156.3, 170.8, 171.7, 172.2.

Cbz-Val-Gly-NH_2 (3i)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.85 (dd, *J* = 6.6 Hz, 6H), 1.92-2.01 (m, 1H), 3.61-3.66 (m, 2H), 3.81-3.87 (m, 1H), 5.03 (s, 2H), 7.05 (s, 1H), 7.22-7.40 (m, 7H), 8.16 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.0, 19.1, 29.8, 41.7. 60.3. 65.4. 127.6. 127.7. 128.2. 136.9. 156.2. 170.6. 171.3.

Cbz-Val-Ala-OMe (4a)[16b]
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, J = 6.6 and 13.8 Hz, 6H), 1.26 (d, J = 7.5 Hz, 3H), 1.92-1.98 (m, 1H), 3.60 (s, 3H), 3.86-3.92 (m, 1H), 4.23-4.28 (m, 1H), 5.02 (s, 2H), 7.23-7.39 (m, 6H), 8.36 (d, J = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 16.7, 18.0, 18.9, 30.3, 47.4, 51.6, 59.6, 65.2, 127.5, 127.7, 128.2, 137.0, 156.0, 171.0, 172.8.

Cbz-Val-Ser-OMe (4b)[16c]
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, J = 6.6 and 13.5 Hz, 6H), 1.90-2.05 (m, 1H), 3.53-3.74 (m, 6H), 3.95-4.00 (m, 1H), 4.30-4.38 (m, 1H), 5.03 (s, 2H), 7.26-7.38 (m, 6H), 8.21 (d, J = 7.2 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 19.0, 30.4, 51.7, 54.6, 59.6, 61.1, 65.3, 127.5, 127.7, 128.2, 137.0, 156.0, 170.8, 171.3.

Cbz-Val-Leu-OMe (4c)[16d]
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.82-0.90 (m, 12H), 1.42-1.67 (m, 3H), 1.89-2.01 (m, 1H), 3.60 (s, 3H), 3.86-3.92 (m, 1H), 4.24-4.32 (m, 1H), 5.03 (s, 2H), 7.24-7.39 (m, 6H), 8.24 (d, J = 7.2 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.0, 19.0, 21.1, 22.6, 24.0, 30.2, 50.1, 51.6, 59.8, 65.2, 127.5, 127.6, 128.2, 137.0, 155.9, 171.3, 172.7.

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Cbz-Val-Gln-OMe (4d)
R_t(HPLC) 14.77 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, J = 6.6 and 12.3 Hz, 6H; CH3 2x), 1.73-2.16 (m, 5H; CH, CH2 2x), 3.60 (s, 3H; COOCH3), 3.88-3.93 (m, 1H; CH), 4.17-4.24 (m, 1H; CH), 5.02 (s, 2H; CH2), 6.78 (s, 1H; NH), 7.24-7.40 (m, 7H; Ph and NH 2x), 8.33 (d, J = 7.8 Hz, 1H; NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.0, 18.9, 26.4, 30.3, 30.9, 51.6, 59.6, 65.3, 127.5, 127.6, 128.2, 137.0, 156.0, 171.3, 172.1, 173.1; FIA-ESI(+)-TOF-MS: m/z [M + H]$^+$ calcd for $C_{19}H_{28}N_3O_6$: 394.1973; found: 394.1986.

Cbz-Val-Phe-OMe (4e)[16e]
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.80 (dd, J = 3.9 Hz, 6H), 1.80-1.95 (m, 1H), 2.88-3.05 (m, 2H), 3.55 (s, 3H), 3.85-3.91 (m, 1H), 4.44-4.51 (m, 1H), 5.02 (s, 2H), 7.18-7.40 (m, 11H), 8.35 (d, J = 7.2 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.0, 18.9, 30.3, 36.5, 51.6, 53.4, 59.8, 65.3, 126.4, 127.6, 127.7, 128.1, 128.2, 128.9, 137.0, 155.9, 171.2, 171.7.

Cbz-Val-Glu-OMe (4f)
R^t(HPLC) 15.69 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, J = 6.6 and 11.1 Hz, 6H), 1.74-2.02 (m, 3H), 2.28-2.33 (m, 2H), 3.60 (s, 3H), 3.86-3.91 (m, 1H), 4.25-4.28 (m, 1H), 5.03 (s, 2H), 7.27-7.39 (m, 6H), 8.30 (d, J = 7.2 Hz, 1H), 12.18 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.1, 19.0, 25.9, 29.7, 30.2, 51.1, 51.7, 59.8, 65.3, 127.6, 127.7, 128.2, 137.0, 156.0, 170.4, 172.0, 173.6; FIA-ESI(+)-TOF-MS: m/z [M + H]$^+$ calcd for $C_{19}H_{27}N_2O_7$: 395.1813; found: 395.1798.

Cbz-Val-Asn-OMe (4g)
R_t(HPLC) 14.24 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84 (dd, J = 6.6 and 11.1 Hz, 6H), 1.92-1.99 (m, 1H), 3.00 (s, 3H) 3.88-3.93 (m, 1H), 4.57-4.61 (m, 1H), 5.03 (s, 2H), 6.94 (s, 1H), 7.27-7.40 (m, 7H), 8.26 (d, J = 7.5 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.8, 18.9,

30.4, 36.4, 48.6, 51.7, 59.7, 65.3, 127.5, 127.7, 128.2, 137.0, 156.0, 170.7, 171.0, 171.7; FIA-ESI(+)-TOF-MS: m/z $[M + H]^+$ calcd for $C_{18}H_{26}N_3O_6$: 380.1816; found: 380.1813.

Cbz-Val-Asp-OMe (4h)

R_t(HPLC) 15.47 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, *J* = 6.6 and 11.1 Hz, 6H), 1.92-2.02 (m, 1H), 2.59-2.78 (m, 2H), 3.16 (s, 3H) 3.90-3.95 (m, 1H), 4.61-4.63 (m, 1H), 5.04 (s, 2H), 7.27-7.38 (m, 6H), 8.37 (d, *J* = 7.5 Hz, 1H), 12.66 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.8, 18.9, 30.4, 36.6, 48.4, 51.9, 59.7, 65.3, 127.5, 127.7, 128.2, 137.0, 156.0, 171.0, 171.2, 171.3; FIA-ESI(+)-TOF-MS: m/z $[M + H]^+$ calcd for $C_{18}H_{25}N_2O_7$: 381.1656; found: 381.1666.

Cbz-Val-Glu(OMe)-NH_2 (5)

R_t(HPLC) 14.52 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, *J* = 7.8 Hz, 6H), 1.76-2.33 (m, 5H), 3.57 (s, 3H) 3.84-3.89 (m, 1H), 4.19-4.27 (m, 1H), 5.03 (s, 2H), 7.08 (s, 1H), 7.30-7.49 (m, 7H), 8.01 (d, *J* = 7.8 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.0, 19.1, 27.2, 30.0, 51.2, 60.2, 65.3, 127.5, 127.6, 136.9, 156.1, 170.9, 172.7; FIA-ESI(+)-TOF-MS: m/z $[M + H]^+$ calcd for $C_{19}H_{28}N_3O_6$: 394.1973; found: 394.1998.

Boc-Met-OMe (6)[16f]

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.38 (s, 9H), 1.80-1.92 (m, 1H), 1.98-2.10 (m, 4H), 2.47 (t, *J* = 7.5 Hz, 2H), 3.69 (s, 3H), 4.34 (d, *J* = 4.5 Hz, 1H), 5.04 (d, *J* = 4.8 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.5, 28.3, 30.0, 32.2, 52.4, 52.8, 80.1, 156.3, 172.8.

Boc-Met-Leu-NH_2 (7)

R_t(HPLC) 16.07 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.86 (dd, *J* = 6.3 Hz, 6H), 1.38 (s, 9H), 1.38-1.47 (m, 3H), 1.73-1.83 (m, 2H), 1.96-2.04 (m, 3H), 2.51 (dd, *J* = 7.2 Hz, 2H), 4.11-4.17 (m, 1H), 4.38-4.45 (m, 1H), 5.23 (d, *J* = 3.9 Hz, 1H), 5.53 (s, 1H), 6.39 (s, 1H), 6.65 (d, *J* = 6.9 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.4, 21.7, 23.0, 24.8, 28.3, 30.3, 40.5, 51.3, 54.1, 80.7, 171.7, 174.2; FIA-ESI(+)-TOF-MS: m/z $[M + H]^+$ calcd for $C_{16}H_{32}N_3O_4S$: 362.2108; found: 362.2112.

Boc-Met-Leu-OMe (8)

R_t(HPLC) 18.63 min; 1H NMR ($CDCl_3$, 300 MHz): δ = 0.86 (d, *J* = 5.7 Hz, 6H), 1.38 (s, 9H), 1.40-1.64 (m, 3H), 1.81-2.06 (m, 5H), 2.54 (dd, *J* = 6.9 Hz, 2H), 3.66 (s, 3H), 4.15-4.30 (m, 1H), 4.50-4.57 (m, 1H), 5.08 (d, *J* = 7.5 Hz, 1H), 6.65 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.5, 22.2, 23.2, 25.1, 28.7, 30.5, 31.7, 41.8, 51.1, 52.7, 80.7, 171.6, 173.4; FIA-ESI(+)-TOF-MS: m/z $[M + H]^+$ calcd for $C_{17}H_{33}N_3O_5S$: 377.2105; found: 377.2099.

Boc-Met-Leu-Phe-NH_2 (9)

R_t(HPLC) 20.14 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.82 (dd, *J* = 5.4 and 14.1 Hz, 6H), 1.38-1.79 (m, 15H), 2.02 (s, 3H), 2.41-2.50 (m, 2H), 2.79-3.02 (m, 2H), 3.80-4.15 (m, 1H), 4.21-4.30 (m, 1H), 4.38-4.54 (m, 1H), 7.00-7.23 (m, 2H), 7.26-7.34 (m, 6H), 7.80-7.88 (m, 2H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 14.5, 21.5, 22.9, 23.8, 28.0, 29.6, 31.3, 51.0, 53.3,

53.4, 78.0, 126.1, 127.9, 129.0, 137.6, 155.2, 171.4 (2 C), 172.4; FIA-ESI(+)-TOF-MS: m/z $[M + H]^+$ calcd for $C_{25}H_{41}N_4O_5S$: 509.2792; found: 509.2820.

Boc-Met-Leu-Phe-OH (10)

R_t(HPLC) 19.52 min; LC-MS ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{25}H_{40}N_3O_6S$: 510; found: 510.

Cbz-Phe-Leu-NH_2 (11)[16g]

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.76 (dd, *J* = 6.3 and 16.5 Hz, 6H), 1.23-1.46 (m, 3H), 2.73-3.00 (m, 2H), 4.12-4.20 (m, 1H), 4.24-4.32 (m, 1H), 4.95 (s, 2H), 7.02 (s, 1H), 7.17-7.38 (m, 11H), 7.59 (d, *J* = 7.8 Hz, 1H), 8.23 (d, *J* = 8.7 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.3, 23.0, 23.9, 37.4, 50.8, 56.3, 65.2, 126.1, 127.4, 127.6, 127.9, 128.2, 129.1, 136.8, 137.6, 155.8, 171.1, 174.0.

Cbz-Phe-Leu-Ala-NH_2 (13)[16h]

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.87 (dd, *J* = 6.3 and 15.2 Hz, 6H), 1.20 (d, *J* = 6.9 Hz, 3H), 1.40-1.69 (m, 3H), 2.69-2.77 (m, 1H), 2.99-3.05 (m, 1H), 4.14-4.34 (m, 3H), 4.94 (s, 2H), 7.00 (s, 1H), 7.17-7.49 (m, 11H), 7.50 (d, *J* = 8.7 Hz, 1H), 7.86 (d, *J* = 7.5 Hz, 1H), 8.12 (d, *J* = 7.1 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.3, 21.5, 23.0, 24.0, 37.2, 47.7, 50.9, 56.1, 65.1, 126.1, 127.3, 127.6, 127.9, 128.2, 129.1, 136.9, 138.0, 155.8, 171.3, 171.4, 173.9.

Cbz-Phe-Leu-Ala-OH (14)

R_t(HPLC) 18.66 min; LC-MS ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{26}H_{34}N_3O_6$: 484; found : 484.

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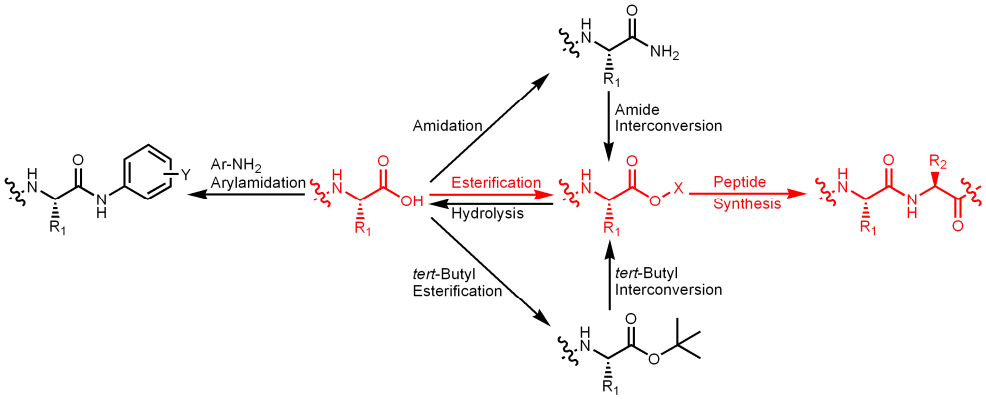
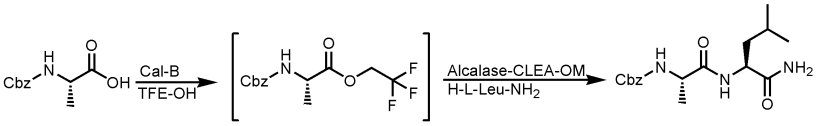
Chapter 8

Enzymatic synthesis of activated esters and their subsequent use in enzyme-based peptide synthesis



This chapter demonstrates that carboxamidomethyl and trifluoroethyl esters are very useful for Subtilisin A mediated peptide synthesis in case of sterically demanding and non-proteinogenic acyl donors, as well as poor nucleophiles, and combinations thereof. Furthermore, these esters can be efficiently synthesized by using either the lipase *Candida antartica* lipase B or the protease Subtilisin A. Finally, it is shown that ester synthesis by *Candida antartica* lipase B in combination with subsequent peptide synthesis by Subtilisin A can be performed simultaneously using a two-enzyme-one-pot approach with glycolamide or 2,2,2-trifluoroethanol as additive.



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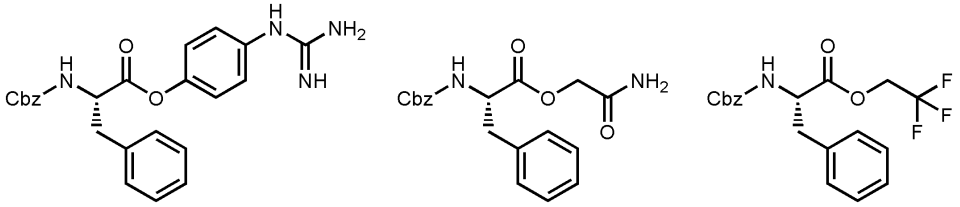
8.1 Introduction

In the past few decades, a large number of peptides has been introduced onto the market since they can be used either as a therapeutic or as prodrug,[1] and even an increasing number is in clinical trails. Additionally, peptides have found applications as nutritional additive or as a cosmetic ingredient.[2] Despite this demand for peptides, their production on large scale remains expensive and time consuming.[3] Common peptide synthesis approaches are fermentation, solid-phase or solution-phase chemical peptide synthesis, and chemo-enzymatic peptide synthesis.[4] Currently, the fermentative approach is well feasible for large peptides (>50 amino acid residues) and proteins containing natural fragments, however it still requires a large development effort for each individual peptide/protein. Solid phase peptide synthesis (SPPS) is the most commonly used method for medium-sized and long peptides (10-50 amino acid residues). However, SPPS requires besides full protection of the functionalized side chains, also expensive and environmentally unfriendly coupling reagents in at least stoichiometric amounts. Furthermore, the requirement of functionalized resins and the use of reagent excess makes SPPS an expensive method. Solution phase chemical peptide synthesis is most commonly used for the synthesis of small peptides containing two to ten amino acid residues. Also this approach requires expensive coupling reagents and of major concern is the uncontrolled *C*-terminal racemization during fragment assembly. Finally, chemo-enzymatic peptide synthesis, wherein peptide fragments are elongated enzymatically, has been studied in academia during the past decades and proved to be suitable for certain short peptide sequences up to five amino acid residues.[4] The application of enzymes as coupling reagent is a promising alternative since functionalized amino acid side chain do not require full protection and most importantly, *C*-terminal racemization is completely absent during fragment assembly, which is beneficial for the characterization and purification of the final peptide.

There are two approaches toward enzymatic peptide synthesis, either the thermodynamically, or the kinetically controlled approach.[5] In thermodynamically controlled peptide synthesis, an *N*-terminally protected acyl donor reacts with a *C*-terminally protected amino acid acceptor as the nucleophile, resulting in the formation of the peptide bond, and one water molecule is expelled. Thermodynamically controlled peptide synthesis is, however, rather slow and the thermodynamic equilibrium between product and starting materials needs to be shifted into the synthetic direction, for example by product precipitation, by water withdrawal, or by using organic solvents, to obtain a high product yield. This is in contrast to the kinetically controlled peptide synthesis approach, in which an *N*-terminally protected and *C*-terminally activated amino acid ester reacts preferentially with a *C*-terminally protected acyl acceptor (nucleophile), to give a high product yield in a generally shorter reaction time.

The high selectivity of enzymes restricts the number of amino acids that will be recognized. Therefore, coupling of sterically demanding amino acid as acyl donors (valine, isoleucine, threonine), notoriously weak nucleophiles (proline), or D- and other non-proteinogenic amino acid residues, remains rather challenging. To remedy the restricted access toward the primary specificity pocket, designed acyl donors with an ester moiety that is specifically recognized by the enzyme, among others, guanidinophenyl (Gp),[6] carboxamidomethyl

(Cam)[7] or trifluoroethyl (Tfe)[8] esters have been used (Figure 1). Thus, successful couplings of sterically demanding acyl donors decorated with these activated ester moieties have been reported. Also, the application of these activated esters, enzyme-mediated couplings of weak nucleophiles and non-proteinogenic amino acid residues, became feasible.[9] Although these active esters broaden the scope of enzymatic peptide synthesis, their chemical synthesis is however not straightforward[10] since highly reactive coupling reagents are required to couple the rather poor nucleophilic alcohol derivatives which increases the risk of racemization.[11]



Cbz-Phe-O(4-guanidinophenyl) Cbz-Phe-OCam Cbz-Phe-OTfe

Figure 1. Representative examples of acyl donors that are specifically recognized by certain proteases.

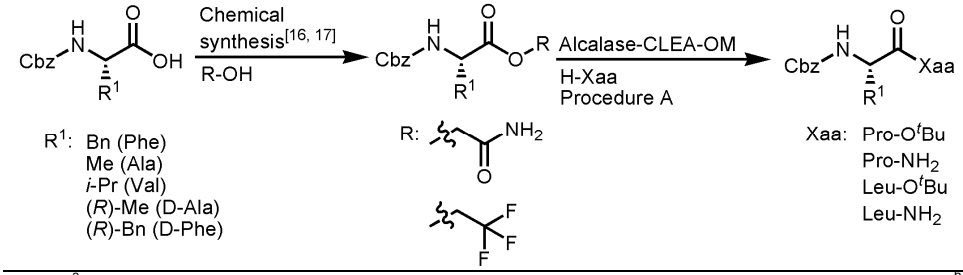
In this chapter, a highly promising enzymatic approach toward the synthesis of Cam and Tfe active esters by means of cross-linked enzyme aggregates of Subtilisin A (commercially available as Alcalase, Alcalase-CLEA-OM, specially designed for organic solvents)[12] and immobilized *Candida antartica* lipase B (Cal-B) is described. Additionally, application of these Cam and Tfe esters in an enzymatic peptide synthesis approach is demonstrated using a number of challenging acyl donors and nucleophiles to obtain dipeptides which are generally difficult to obtain. Furthermore, a 'two-enzyme-one-pot' approach was explored, in which the activated ester is synthesized by Cal-B and simultaneously used as a substrate by Alcalase-CLEA-OM to elongate the peptide sequence.

8.2 Results and Discussion

As described in chapter 6 and 7, Alcalase-CLEA-OM mediated peptide synthesis was highly efficient in anhydrous organic solvents for the coupling of *N*-terminally protected amino acid methyl esters with *C*-terminally protected amino acid nucleophiles.[13] Encouraged by these results, it was decided to explore the scope of coupling reactions in which the poor nucleophile proline was used. Although the Subtilisin-catalyzed coupling of proline was described in the literature,[14] others observed that proline as nucleophile gave no conversion at all.[15] In our hands, coupling of Cbz-L-Phe-OMe (**1**) with H-L-Pro-O^{t}Bu in the presence of Alcalase-CLEA-OM in anhydrous THF gave after 24 h an almost quantitative conversion (98%) to the dipeptide as judged by HPLC analysis, and dipeptide Cbz-L-Phe-L-Pro-O^{t}Bu was isolated in 92% yield. Noteworthy, only 50 mol% excess of H-L-Pro-O^{t}Bu was used, which is a rather small amount for enzymatic coupling reactions. Despite these promising data, in case of more challenging acyl donors like Cbz-D-Phe-OMe or Cbz-L-Val-OMe, the conversion toward dipeptides Cbz-D-Phe-Pro-O^{t}Bu and Cbz-L-Val-Pro-O^{t}Bu dropped significantly to 24% and 32%, respectively. In order to improve the coupling efficiency, the versatility of Cam and Tfe active esters in Alcalase-CLEA-OM mediated peptide synthesis in

the presence of a number of challenging acyl donors and nucleophiles was investigated, as shown in Table 1. Guanidinophenyl esters were not included in this study because they have poor solubility in anhydrous organic solvents and are recognized by arginine specific proteases, such as Trypsin and not by Alcalase.

Table 1. Alcalase-CLEA-OM mediated dipeptide synthesis using Cam and Tfe esters.



Entry[a]	Acyl donor		Nucleophile	Dipeptide		Yield (%)[b]
1	Cbz-L-Phe-OCam	**2**	H-L-Leu-O^{t}Bu	Cbz-L-Phe-L-Leu-O^{t}Bu	**10**	87
2	Cbz-L-Phe-OCam	**2**	H-L-Pro-O^{t}Bu	Cbz-L-Phe-L-Pro-O^{t}Bu	**11**	92
3	Cbz-L-Phe-OCam	**2**	H-L-Leu-NH_2	Cbz-L-Phe-L-Leu-NH_2	**12**	91
4	Cbz-L-Phe-OCam	**2**	H-L-Pro-NH_2	Cbz-L-Phe-L-Pro-NH_2	**13**	91
5	Cbz-L-Phe-OTfe	**3**	H-L-Pro-NH_2	Cbz-L-Phe-L-Pro-NH_2	**13**	90
6	Cbz-L-Phe-OTfe	**3**	H-L-Pro-NH_2	Cbz-L-Phe-L-Pro-NH_2	**13**	93
7	Cbz-L-Ala-OTfe	**4**	H-L-Leu-NH_2	Cbz-L-Ala-L-Leu-NH_2	**14**	94
8	Cbz-L-Ala-OTfe	**4**	H-L-Pro-NH_2	Cbz-L-Ala-L-Pro-NH_2	**15**	90
9	Cbz-D-Ala-OTfe	**5**	H-L-Leu-NH_2	Cbz-D-Ala-L-Leu-NH_2	**16**	45
10	Cbz-D-Ala-OCam	**6**	H-L-Leu-NH_2	Cbz-D-Ala-L-Leu-NH_2	**16**	92
11	Cbz-D-Ala-OCam	**6**	H-L-Pro-NH_2	Cbz-D-Ala-L-Pro-NH_2	**17**	69
12	Cbz-D-Phe-OTfe	**7**	H-L-Leu-NH_2	Cbz-D-Phe-L-Leu-NH_2	**17**	36
13	Cbz-D-Phe-OCam	**8**	H-L-Leu-NH_2	Cbz-D-Phe-L-Leu-NH_2	**18**	93
14	Cbz-D-Phe-OCam	**8**	H-L-Pro-NH_2	Cbz-D-Phe-L-Pro-NH_2	**19**	50
15	Cbz-L-Val-OCam	**9**	H-L-Leu-NH_2	Cbz-L-Val-L-Leu-NH_2	**20**	93
16	Cbz-L-Val-OCam	**9**	H-L-Pro-NH_2	Cbz-L-Val-L-Pro-NH_2	**21**	76

[a] Conditions: EDC·HCl, HOBt, CH_3CN, glycolamide;[16] DCC, DMAP, CH_2Cl_2, 2,2,2-trifluoroethanol;[17] Alcalase-CLEA-OM, MTBE or THF, 4 Å molecular sieves, amino acid nucleophile, 50°C, 16 h (Procedure A); [b] Isolated yield based on acyl donor starting material.

Evidently, Cam as well as Tfe active esters were found to be highly efficient acyl donors in Alcalase-CLEA-OM mediated dipeptide synthesis. Not only D-amino acids (entries 9-13) but also valine (entries 15 and 16) could be used in combination with the poor nucleophilic proline. There was almost no difference in efficiency in case of H-L-Leu-NH_2 or H-L-Pro-NH_2 as the nucleophilic species when acyl donors Cbz-L-Phe and Cbz-L-Ala were used irrespective of the active ester. However, in case of more challenging acyl donors such as Cbz-D-Phe, Cbz-D-Ala or Cbz-L-Val, the Cam esters turned out to be superior, while the coupling efficiency of proline was lower compared to leucine. Gratifyingly, the highly active Cam esters did not cause any racemization during the course of enzymatic peptide synthesis (Table 1, entry 1, Cbz-L-Phe-L-Leu-O^{t}Bu (**10**) e.e. L-Phe > 99.5 and e.e. L-Leu > 99.5)

Based on these results, the type of active ester was investigated in more detail in order to optimize such difficult coupling reactions. In order to optimize the efficiency of the enzyme-mediated dipeptide synthesis, the coupling of several amino acid methyl, Cam, and Tfe esters to H-L-Pro-NH_2 was investigated in the presence of Alcalase-CLEA-OM, as shown in Table 2.

Table 2. Alcalase-CLEA-OM mediated dipeptide synthesis with H-L-Pro-NH_2 as the nucleophile.

Entry[a]	Acyl donor		Initial rate[b]	Conversion[c]
1	Cbz-L-Ala-OMe	**22**	10.6	77[c]
2	Cbz-L-Ala-OTfe	**4**	11.9	90[c]
3	Cbz-L-Ala-OCam	**23**	12.8	95[c]
4	Cbz-L-Phe-OMe	**1**	11.0	81[c]
5	Cbz-L-Phe-OTfe	**3**	12.8	93[c]
6	Cbz-L-Phe-OCam	**2**	13.1	98[c]
7	Cbz-D-Ala-OMe	**24**	1.7	35[d]
8	Cbz-D-Ala-OTfe	**5**	2.8	52[d]
9	Cbz-D-Ala-OCam	**6**	5.1	97[d]
10	Cbz-D-Phe-OMe	**25**	0.8	12[d]
11	Cbz-D-Phe-OTfe	**7**	1.9	29[d]
12	Cbz-D-Phe-OCam	**8**	3.5	65[d]
13	Cbz-L-Val-OMe	**26**	0.9	11[d]
14	Cbz-L-Val-OTfe	**27**	1.4	21[d]
15	Cbz-L-Val-OCam	**9**	2.9	50[d]

[a] Conditions: Alcalase-CLEA-OM, MTBE or THF, 4Å molecular sieves, H-L-Pro-NH_2, 50°C, 16 h (Procedure A); [b] Initial rates were determined by HPLC and are expressed as %conversion per hour per 25 mg of Alcalase-CLEA-OM; [c] HPLC conversions to dipeptide product compared to acyl donor starting material after: [c]10h, [d]24h.

Gratifyingly, these results showed that the Cam esters, and to a lesser extent the Tfe esters, were in all cases more efficient acyl donors than the commonly used methyl ester.

As described in chapter 2, it was found that a large diversity of amino acid esters like methyl, ethyl, benzyl, as well as *tert*-butyl were accessible by Alcalase-CLEA-OM mediated ester synthesis. Therefore, it was tried to synthesize Cbz-L-Phe-OCam as well as Cbz-L-Phe-OTfe in the presence of the respective alcohols and Alcalase-CLEA-OM. Both esters could be obtained starting from Cbz L-Phe-OH, however, their formation was rather slow resulting in a relatively low yield (24% and 57% respectively, data not shown).

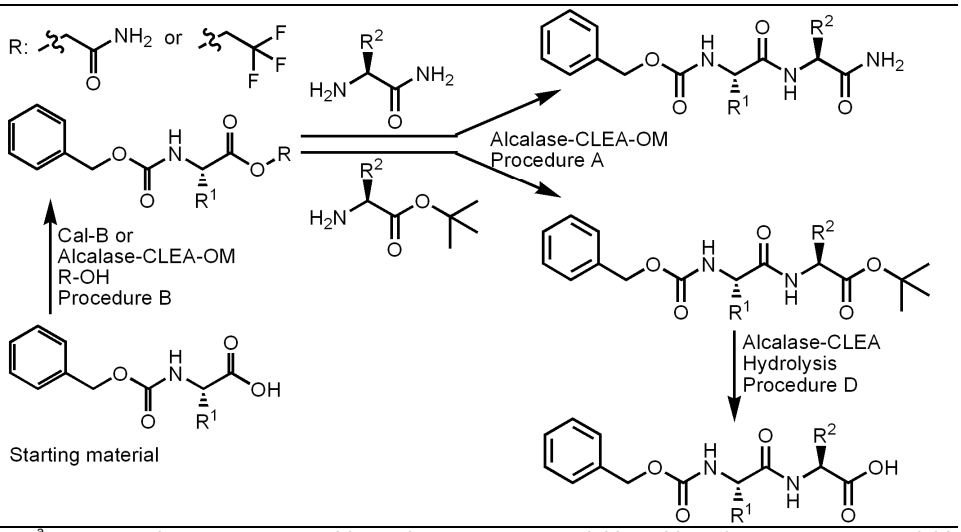
After an extensive screening for lipases (see experimental section for screening data) that were able to catalyzed ester synthesis in anhydrous organic solvents, Cal-B proved to be optimal for the desired Cam and Tfe active esters. It was found that Cal-B was highly efficient, and several amino acids as well as dipeptides were accepted as substrate, as shown in Table 3.

Table 3. Cal-B mediated synthesis of Tfe and Cam esters.

Entry[a]	Ester product		Yield (%)[b]
1	Cbz-Gly-OTfe	**28**	93
2	Cbz-L-Pro-OTfe	**29**	91
3	Cbz-L-Ala-OTfe	**4**	96
4	Cbz-L-Ala-OCam	**23**	80
5	Cbz-D-Ala-OTfe	**5**	93
6	Cbz-D-Ala-OCam	**6**	65
7	Cbz-L-Ala-L-Ala-OTfe	**30**	77

[a] Conditions: Cal-B, MTBE or CH_3CN or THF, 3 Å molecular sieves, glycolamide or 2,2,2-trifluoroethanol, 50°C, 16 h (Procedure B); [b] Isolated yield based on acyl donor starting material

Table 4. Fully enzymatic peptide synthesis using Cal-B and Alcalase-CLEA-OM.

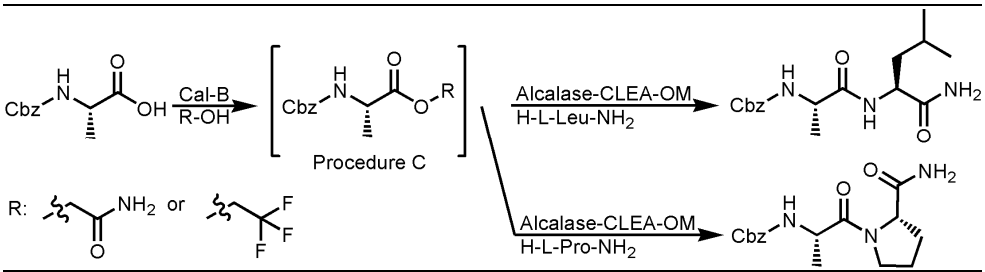


Entry[a]	Ester product		Peptide product		Yield (%)[a]	Acid product		Yield (%)[b]
1	Cbz-Gly-OTfe	**28**	Cbz-Gly-L-Phe-NH_2[18]	**31**	89			
2	Cbz-Gly-OTfe	**28**	Cbz-Gly-L-Leu-NH_2[19]	**32**	88			
3	Cbz-Gly-OTfe	**28**	Cbz-Gly-L-Phe-O^{t}Bu	**33**	90	Cbz-Gly-L-Phe-OH[20]	**38**	88
4	Cbz-L-Ala-OTfe	**4**	Cbz-L-Ala-L-Leu-O^{t}Bu	**34**	91	Cbz-L-Ala-L-Leu-OH[21]	**39**	86
5	Cbz-L-Ala-OTfe	**4**	Cbz-L-Ala-L-Phe-O^{t}Bu	**35**	92	Cbz-L-Ala-L-Phe-OH[22]	**40**	90
6	Cbz-L-Phe-OTfe	**3**	Cbz-L-Phe-L-Leu-O^{t}Bu	**10**	87	Cbz-L-Phe-L-Leu-OH[23]	**41**	84
7	Cbz-L-Ala-L-Ala-OTfe	**30**	Cbz-L-Ala-L-Ala-Gly-L-Phe-NH_2	**36**	85			
8	Cbz-D-Ala-OCam	**6**	Cbz-D-Ala-D-Ala-O^{t}Bu	**37**	75			

[a] Conditions: Alcalase-CLEA-OM, MTBE or THF, 4Å molecular sieves, amino acid nucleophile, 50°C, 16 h (Procedure A); Cal-B or Alcalase-CLEA-OM, MTBE or CH_3CN or THF, 3 Å molecular sieves, glycolamide or 2,2,2-trifluoroethanol, 50°C, 16 h (Procedure B); Alcalase-CLEA-OM, dioxane/H_2O (8/2, v/v or DMF/H_2O, 7/3, v/v or THF/H_2O, 9/1, v/v), 37°C, 36 h (Procedure D); [b] Isolated yield based on acyl donor starting material

Since the active esters were synthesized enzymatically, and on their turn can be used as versatile acyl donors in the next coupling step, the fully enzymatic synthesis of several biologically interesting peptides was explored,[18-23] as shown in Table 4.
Dipeptide amides **31**[18] and **32**[19] were easily accessible (entries 1-2), as well as dipeptide *tert*-butyl esters **10** and **33**-**35** (entries 3-6). With respect to the latter, enzymatic hydrolysis[24] by Alcalase-CLEA-OM gave the dipeptide acids **38**-**41**[20-23] that could be used as substrate for Cal-B to give the corresponding Tfe active esters as the dipeptide acyl donor for the next coupling step. Interestingly, also the dipeptide Tfe ester **30** was recognized by the enzyme and reacted smoothly with a dipeptide amide to give the tetrapeptide **36** in high yield (entry 7). Furthermore, also H-D-Ala-O^{t}Bu was recognized by the enzyme as nucleophile to give the all D-dipeptide, Cbz-D-Ala-D-Ala-O^{t}Bu (**37**, entry 8).
Finally, the possibility of performing the esterification and subsequent dipeptide formation in the presence of Cal-B and Alcalase-CLEA-OM in a one pot approach was investigated. Indeed, starting from an *N*-terminally protected amino acid as the acyl donor and an amino acid amide as the nucleophile, in the presence of both enzymes with either 2,2,2-trifluoroethanol or glycolamide as additive, the corresponding dipeptide was obtained, as shown in Table 5.

Table 5. Dipeptide synthesis using a Cal-B and Alcalase-CLEA-OM two-enzyme-one-pot approach with 2,2,2-trifluoroethanol or glycolamide as additive.



Entry[a]	Product	Enzyme(s)	Alcohol	Conversion to ester (%)[b]	Conversion to product (%)[b]
1	Cbz-L-Ala-L-Leu-NH_2 **14**	Alcalase-CLEA-OM	Tfe-OH	1	13
2	Cbz-L-Ala-L-Leu-NH_2 **14**	Cal-B	Tfe-OH	30	31
3	Cbz-L-Ala-L-Leu-NH_2 **14**	Cal-B and Alcalase-CLEA-OM	Tfe-OH	2	87
4	Cbz-L-Ala-L-Pro-NH_2 **15**	Alcalase-CLEA-OM	Tfe-OH	0	9
5	Cbz-L-Ala-L-Pro-NH_2 **15**	Cal-B	Tfe-OH	32	14
6	Cbz-L-Ala-L-Pro-NH_2 **15**	Cal-B and Alcalase-CLEA-OM	Tfe-OH	1	27
7	Cbz-L-Ala-L-Leu-NH_2 **14**	Alcalase-CLEA-OM	Cam-OH	0	15
8	Cbz-L-Ala-L-Leu-NH_2 **14**	Cal-B and Alcalase-CLEA-OM	Cam-OH	1	88
9	Cbz-L-Ala-L-Pro-NH_2 **15**	Alcalase-CLEA-OM	Cam-OH	0	11
10	Cbz-L-Ala-L-Pro-NH_2 **15**	Cal-B and Alcalase-CLEA-OM	Cam-OH	3	31

[a] Conditions: Alcalase-CLEA-OM, Cal-B, CH_3CN, 3 Å molecular sieves, glycolamide or 2,2,2-trifluoroethanol, amino acid nucleophile, 50°C, 16 h (Procedure C); [b] HPLC conversion based on carboxylic acid starting material

Although Alcalase-CLEA-OM and Cal-B were individually capable of catalyzing dipeptide formation, the obtained yield was significantly higher if both enzymes were combined, probably because the esterification equilibrium as mediated by Cal-B is shifted to the ester side since the ester is consumed in the Alcalase-CLEA-OM mediated coupling reaction. Without addition of an alcohol the yield of dipeptide formation remained low. No deactivation of the enzymes was observed, probably due to the fact both enzymes were immobilized and water was absent. For optimal results, only one equivalent of nucleophile was necessary. Thus, by applying this two-enzyme-one-pot approach, dipeptides were obtained in high yield starting from readily accessible amino acids with a free carboxylic acid functionality as acyl donor and an amino acid with a free N^{α}-amino moiety as nucleophile while its *C*-terminus is either an carboxamide or ^{t}Bu ester.

To conclude, this chapter demonstrates that Cam and Tfe active esters are very useful to achieve highly efficient Alcalase-CLEA-OM mediated peptide synthesis. These active esters allow the use of sterically demanding and non-proteinogenic acyl donors as well as poor nucleophiles, and combinations thereof. Furthermore, the Cam and Tfe active esters (based on amino acids) can be enzymatically synthesized by the lipase Cal-B. Finally, a fully enzymatic peptide synthesis approach was developed by combination of two enzymes in which the esterification is performed by Cal-B, while Alcalase-CLEA-OM is responsible for peptide synthesis, also in a two-enzyme-one-pot approach.

8.3 Experimental

8

General

General experimental information is identical to chapter 2, 3 and 4 with the following exceptions. Alcalase-CLEA type OM (organic media) was used (CLEA-Technologies, 580 U/g) and trifluoroacetic acid was used for the preparative HPLC buffers A and B. A stock solution of 2-hydroxyacetamide (Sigma-Aldrich) was prepared by dissolving 2-hydroxyacetamide (1.25 g) in HPLC grade CH_3CN (200 mL) and $MgHPO_4$ (5.0 g) was added. The obtained suspension was stirred for 30 min at room temperature followed by filtration and concentration of the filtrate *in vacuo*. Cal-B was purchased from Novozymes (immobilized Novozym®-435, LC 200204). Reference dipeptides[25] as well as *N*-Cbz-protected amino acid methyl,[26] Cam[27] and Tfe[28] esters were synthesized according to literature procedures.

General Procedure A: Enzymatic peptide synthesis

Alcalase-CLEA-OM (100 mg) was added to a mixture of MTBE or THF (3 mL) containing activated 4 Å molecular sieves (200 mg). To this suspension, the *N*-Cbz-protected amino acid or dipeptide ester (100 mg) followed by a *C*-terminally protected amino acid or dipeptide (1.5 equiv) were added. The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. After filtration, the remaining solid enzyme particles were washed with EtOAc (10 mL, 3×), CH_2Cl_2 (10 mL, 3×) and MeOH (10 mL, 3×). The combined filtrate was concentrated *in vacuo* and the residue was purified by one of the following three methods. In the first method, depending on the solubility of the product, the peptides were purified by column chromatography using EtOAc/n-heptane or CH_2Cl_2/MeOH as eluens. In the second method,

the residue was redissolved in EtOAc or CH_2Cl_2 (50 mL) and the solution was washed with sat. aq. $NaHCO_3$ (25 mL), 0.1 N HCl (25 mL), and brine (25 mL). The organic layer was dried (Na_2SO_4), concentrated *in vacuo*, and the volatiles were co-evaporated with toluene (20 mL, 2×) and $CHCl_3$ (20 mL, 2×). The third method consisted of a purification by preparative HPLC.

General Procedure B: Cam or Tfe ester synthesis

Alcalase-CLEA-OM or Cal-B (100 mg) was added to a mixture of MTBE or THF or CH_3CN (3 mL), 3 Å molecular sieves (200 mg), glycolamide or 2,2,2-trifluoroethanol (200 mg), and *N*-Cbz-protected amino acid (50 mg). The obtained reaction mixture was shaken at 50°C with 150 rpm for 72 h. Purification of the ester was performed as described in general procedure A.

General Procedure C: Dipeptide synthesis with simultaneous use of Alcalase-CLEA-OM and Cal-B

To a mixture of Alcalase-CLEA-OM (25 mg) and Cal-B (100 mg) in CH_3CN (3 mL), 3 Å molecular sieves (200 mg) and the appropriate alcohol (200 mg) were added. Then, the *N*-Cbz-protected amino acid (50 mg) followed by the amino acid amide (1.0 equiv) were added. The obtained reaction mixture was shaken at 50°C with 150 rpm for 16 h. Purification of the dipeptide was performed as described in general procedure A.

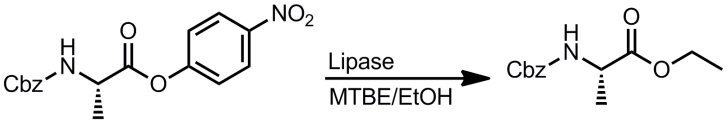
General procedure D: ^{t}Bu-ester hydrolysis using Alcalase-CLEA-OM

Alcalase-CLEA-OM (0.5 g) was added to a solution of peptide *C*-terminal ^{t}Bu ester (1 mmol) in dioxane/H_2O (20 mL, 8/2, v/v or DMF/H_2O, 7/3, v/v or THF/H_2O, 9/1, v/v). The obtained reaction mixture was shaken at 37°C with 150 rpm for 36 h. After filtration, the solid enzyme particles were resuspended in MeOH and removed by filtration (50 mL 3×). This enzyme particle washing procedure was repeated with EtOAc (50 mL, 2×) and CH_2Cl_2 (50 mL, 2×). The combined filtrates were concentrated *in vacuo* and the resulting crude acid was purified by preparative HPLC.

Screening of lipases

In first instance, a screening was performed to find lipases that were able to catalyze a transesterification reaction in anhydrous organic solvents. A solution of Cbz-Ala-*para*-nitrophenyl ester (0.05 mmol) in MTBE/EtOH (0.5 mL, 20/1, v/v) was added to 40 mg of lyophilized enzyme in a 96-well format. The mixtures were shaken at rt for 5 days and the conversion to Cbz-Ala-OEt was determined by analytical HPLC, as shown in Table 6. The lipases that showed activity in the first screening, were screened a second time using TfeOH instead of EtOH. After the second round, only Cal-B was found to be an efficient catalyst for Tfe ester synthesis.

Table 6. Screening of different lipases for catalyzing a transesterification reaction in anhydrous organic solvents.



Entry[a]	Lipase from	Conversion to Cbz-Ala-OEt (%)[b]
1	*Rhizopus aarhizus*	19
2	*Aspergillus niger*	0
3	*Rhizopus delemar*	67
4	*Rhizopus niveus*	0
5	*Mucor javanicus*	9
6	porcine pancreas	0
7	*Candida rugosa*	0
8	*Rhizopus oryzae*	0
9	*Candida lipolytica*	29
10	*Mucor javanicus*	0
11	*Penicillium roqueforti*	0
12	*Humicola lanuginosa*	0
13	*Rhizopus delemar*	0
14	*Rhizopus niveus*	0
15	*Rhizopus japonicus*	50
16	*Burkholderia cepacia*	67
17	*Candida antarctica* (A)	6
18	*Candida antarctica* (B)	92
19	*Rhizomucor miehei*	96
20	*Candida cylindracea*	0
21	*Rhizopus oryzae*	0
22	*Aspergillus niger*	0
23	*Candida lipolytica*	0
24	*Rhizopus javanicus*	0
25	*Pseudomonas alcaligenes*	0
26	*Alcaligenes sp.*	83
27	*Pseudomonas fluorescens*	0
28	*Pseudomonas* sp.	11
29	*Candida lipolytica*	0
30	*Aspergillus asamii*	0
31	*Geotrichum candium*	0
32	*Pseudomonas fluorescens*	40

[a] Conditions: Lipase, MTBE/EtOH (20/1, v/v), rt, 5 days; [b] HPLC conversion based on nitrophenyl ester starting material.

Analyses of Cbz-Phe-OMe (**1**) are described in chapter 2 and of Cbz-Phe-Leu-NH_2 (**12**) in chapter 7.

Cbz-L-Phe-OCam (2)[22a]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.98-3.09 (m, 2H), 4.42-4.56 (m, 3H), 5.00 (s, 2H), 5.28 (d, *J* = 6.9 Hz, 1H), 5.51 (s, 1H), 6.31 (s, 1H), 7.08-7.27 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 37.3, 55.3, 62.7, 127.3, 127.9, 128.2, 128.4, 128.8, 135.1, 135.6, 156.2, 169.0, 170.5.

Cbz-L-Phe-OTfe (3)[22a]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.99-3.10 (m, 2H), 4.36-4.46 (m, 2H), 4.65-4.72 (m, 1H) 5.02 (s, 2H), 5.08 (d, *J* = 8.1 Hz, 1H), 7.02-7.31 (m, 11H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 37.7, 54.5, 60.7, 67.0, 120.6, 124.3, 127.2, 127.9, 128.1, 128.3, 128.6, 128.9, 134.8, 135.8, 155.4, 170.0.

Cbz-L-Ala-OTfe (4)[22b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.39 (d, *J* = 7.2 Hz, 3H), 4.28-4.62 (m, 3H), 5.05 (s, 2H), 5.14 (d, *J* = 6.0 Hz, 1H), 7.19-7.28 (5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.2, 49.5, 60.6, 67.2, 120.8, 124.5, 128.1, 128.3, 128.6, 136.1, 155.6, 171.6.

Cbz-D-Ala-OTfe (5)[22b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.38 (d, *J* = 6.9 Hz, 3H), 4.32-4.60 (m, 3H), 5.05 (s, 2H), 5.18 (d, *J* = 5.7 Hz, 1H), 7.18-7.28 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.1, 49.4, 60.9, 67.0, 120.7, 124.4, 128.0, 128.1, 128.3, 128.4, 135.9, 155.4, 171.5.

Cbz-D-Ala-OCam (6)[22b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.40 (d, *J* = 7.2 Hz, 3H), 4.23-4.30 (m, 1H), 4.50-4.63 (m, 1H), 5.04 (s, 2H), 5.16 (d, *J* = 6.0 Hz, 1H), 5.40 (s, 1H), 6.64 (s, 1H), 7.19-7.30 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.2, 50.0, 62.9, 67.4, 128.1, 128.4, 128.6, 135.9, 171.8.

Cbz-D-Phe-OTfe (7)
This compound was synthesized starting from Cbz-D-Phe-OH according to a literature procedure.[28] R_t(HPLC) 22.37 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.99-3.10 (m, 2H), 4.37-4.46 (m, 2H), 4.65-4.71 (m, 1H), 5.02-5.09 (m, 3H), 7.02-7.30 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 37.8, 54.5, 60.6, 61.1, 67.1, 127.3, 128.0, 128.2, 128.4, 128.7, 129.0, 134.9, 170.1; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{19}H_{19}F_3NO_4$: 382.1261; found: 382.1249.

Cbz-D-Phe-OCam (8)[22a]
This compound was synthesized starting from Cbz-D-Phe-OH according to a literature procedure.[27] ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.97-3.08 (m, 2H), 4.45-4.52 (m, 2H), 5.01 (s, 2H), 5.21 (d, *J* = 6.3 Hz, 1H), 5.37 (s, 1H), 6.30 (s, 1H), 7.09-7.28 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 37.2, 55.1, 62.1, 67.1, 127.2, 127.8, 128.1, 128.6, 128.7, 135.0, 135.5, 156.1, 169.0, 170.5.

Cbz-L-Val-OCam (9)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.89 (dd, *J* = 6.9 Hz, 6H), 2.04-2.11 (m, 1H), 4.08-4.15 (m, 1H), 4.39-4.64 (m, 2H), 5.01 (s, 1H), 5.70 (d, *J* = 7.5 Hz, 1H), 6.26 (s, 1H), 6.71 (s, 1H),

7.19-7.25 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ =17.9, 18.9, 30.3, 59.8, 62.6, 67.2, 128.0, 128.2, 128.4, 135.9, 156.8, 169.9, 171.2.

Cbz-L-Phe-L-Leu-O^{t}Bu (10)

e.e. L-Phe > 99.5; e.e. L-Leu > 99.5; R_t(HPLC) 23.62 min; Purity >97%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.84 (dd, *J* = 3.3 Hz, 6H), 1.50-1.25 (m, 12H), 2.99 (dd, *J* = 6.0 Hz, 2H), 4.14-4.10 (m, 2H), 4.38-4.22 (m, 2H), 4.64 (q, *J* = 7.5 Hz, 1H), 5.16 (d, *J* = 8.4 Hz, 1H), 6.38 (d, *J* = 7.2 Hz, 1H), 7.33-7.04 (m, 9H), 7.50 (d, *J* = 6.9 Hz, 2H), 7.67 (d, *J* = 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.9, 22.9, 24.6, 27.9, 38.0, 41.5, 47.1, 53.6, 67.0, 82.3, 119.9, 125.0, 125.1, 126.9, 127.0, 127.7, 128.3, 129.5, 136.0, 141.3, 143.8 (2C), 156.0, 170.2, 171.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{34}H_{41}N_2O_5$: 557.3010; found: 557.2982.

Cbz-L-Phe-L-Pro-O^{t}Bu (11)[29c]

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.38 (s, 9H), 1.77-2.00 (m, 3H), 2.11-2.20 (m, 1H), 2.75-2.97 (m, 2H), 3.57-3.75 (m, 2H), 4.20-4.25 (m, 1H), 4.38-4.46 (m, 1H), 4.93 (s, 2H), 7.21-7.36 (m, 11H), 7.66 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 24.5, 27.5, 28.4, 36.1, 46.3, 54.2, 59.4, 65.1, 80.2, 126.2, 127.4, 127.6, 128.0, 128.1, 129.1, 136.9, 137.7, 155.8, 169.8, 170.8.

Cbz-L-Phe-L-Pro-NH$_2$ (13)

R_t(HPLC) 16.53 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.65-1.69 (m, 3H), 2.00-2.25 (m, 1H), 2.85-3.01 (m, 2H), 3.37-3.57 (m, 1H), 4.33-4.48 (m, 1H), 4.68 (q, *J* = 15.0 and 7.5 Hz, 1H), 5.00 (q, *J* = 18.9 and 11.4 Hz, 2H) 5.45 (s, 1H), 5.72 (d, *J* = 8.7 Hz, 1H), 6.32 (s, 1H), 7.15-7.26 (m, 11H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 24.4, 28.2, 38.4, 47.8, 54.6, 60.0, 67.1, 127.3, 127.9, 128.2, 128.5, 128.6, 129.3, 135.7, 136.1, 156.4, 171.4, 173.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{22}H_{26}N_3O_4$: 396.1918; found: 396.1925.

Cbz-L-Ala-L-Leu-NH$_2$ (14)[29b]

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.85 (dd, *J* = 6.3 and 14.4 Hz, 6H), 1.18 (d, *J* = 7.2 Hz, 3H), 1.42-1.62 (m, 3H), 4.00-4.09 (m, 1H), 4.15-4.24 (m, 1H), 5.01 (s, 2H), 7.01 (s, 1H), 7.23-7.36 (m, 6H), 7.44 (d, *J* = 7.2 Hz, 1H), 7.98 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 21.3, 22.9, 24.2, 50.1, 50.5, 65.3, 127.6, 127.7, 128.2, 136.8, 155.6, 172.2, 173.9.

Cbz-L-Ala-L-Pro-NH$_2$ (15)[29b]

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.19 (d, *J* = 6.9 Hz, 3H), 1.75-2.10 (m, 4H), 3.36-3.57 (m, 2H), 4.19-4.35 (m, 2H), 5.00 (s, 2H), 5.85 (s, 1H), 7.18-7.35 (m, 7H), 7.47 (d, *J* = 7.2 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 16.7, 24.3, 29.0, 46.4, 47.9, 59.4, 65.2, 127.6, 127.7, 128.2, 155.5, 170.7, 173.4.

Cbz-D-Ala-L-Leu-NH$_2$ (16)[29b]

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.83 (dd, *J* = 6.3 and 11.7 Hz, 6H), 1.19 (d, *J* = 7.2 Hz, 3H), 1.43-1.61 (m, 3H), 4.00-4.08 (m, 1H), 4.13-4.25 (m, 1H), 5.02 (s, 2H), 6.96 (s, 1H),

7.27-7.35 (m, 6H), 7.45 (d, *J* = 7.5 Hz, 1H), 7.75 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 21.5, 22.9, 24.1, 50.1, 50.6, 65.2, 127.5, 127.6, 128.2, 136.9, 172.0, 173.8.

Cbz-D-Ala-L-Pro-NH_2 (17)[29d]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.25 (d, *J* = 6.9 Hz, 3H), 1.80-2.33 (m, 4H), 3.33-3.42 (m, 1H), 3.72-3.82 (m, 1H), 4.33-4.47 (m, 2H), 4.99 (s, 2H), 5.61 (s, 1H), 5.91 (d, *J* = 6.6 Hz, 1H), 6.77 (s, 1H), 7.20-7.25 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.0, 24.4, 28.6, 47.0, 48.6, 60.2, 66.9, 127.9, 128.1, 128.5, 136.2, 156.3, 172.3, 173.8.

Cbz-D-Phe-L-Leu-NH_2 (18)
R_f(HPLC) 18.37 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.77 (dd, *J* = 6.3 and 10.2 Hz, 6H), 1.24-1.43 (m, 3H), 2.74-2.95 (m, 2H), 4.13-4.32 (m, 2H), 4.95 (s, 2H), 7.01 (s, 1H), 7.25-7.32 (m, 11H), 7.57 (d, *J* = 7.8 Hz, 1H), 8.14 (d, *J* = 8.1 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.2, 23.0, 23.9, 37.4, 50.6, 56.2, 65.2, 126.1, 127.4, 127.6, 127.9, 128.2, 129.1, 136.8, 137.5, 155.8, 171.0, 173.9 ; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{23}H_{30}N_3O_4$: 412.2231; found: 412.2215.

Cbz-D-Phe-L-Pro-NH_2 (19)
R_f(HPLC) 16.54 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.49-2.12 (m, 5H), 2.50 (q, *J* = 7.5 Hz, 1H), 2.93 (d, *J* = 6.6 Hz, 2H), 3.49-3.54 (m, 1H), 4.33-4.50 (m, 2H), 4.98 (d, *J* = 3.3 Hz, 2H), 5.41 (s, 1H), 5.85 (d, *J* = 6.6 Hz, 1H), 6.71 (s, 1H), 7.14-7.27 (m, 11H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 24.4, 28.2, 38.4, 47.8, 54.6, 60.0, 67.1, 127.3, 127.9, 128.2, 128.5, 128.6, 129.3, 135.7, 136.1, 156.4, 171.4, 173.5; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{22}H_{26}N_3O_4$: 396.1918; found: 396.1920.

Cbz-L-Val-L-Leu-NH_2 (20)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.86 (dd, *J* = 7.5 and 14.7 Hz, 6H), 1.40-1.65 (m, 3H), 1.95-2.03 (m, 1H), 3.83-3.89 (m, 1H), 4.24-4.31 (m, 1H), 5.05 (s, 2H), 6.97 (s, 1H), 7.29-7.38 (m, 7H), 7.90 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.0, 19.1, 21.5, 22.9, 24.0, 27.7, 30.1, 40.9, 50.7, 54.4, 60.3, 69.2, 127.4, 127.6, 128.2, 136.9, 155.3, 170.7, 173.8.

Cbz-L-Val-L-Pro-NH_2 (21)
R_f(HPLC) 14.52 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.90 (dd, *J* = 11.1 and 6.9 Hz, 6H), 1.87-2.31 (m, 6H), 3.50-3.58 (m, 1H), 3.64-3.70 (m, 1H), 4.24-4.29 (m, 1H), 4.50-4.54 (m, 1H), 5.02 (d, 2H), 5.45-5.51 (m, 2H), 6.74 (s, 1H), 7.19-7.29 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.3, 18.9, 24.4, 29.1, 29.7, 46.9, 57.8, 59.1, 65.3, 79.1, 127.5, 127.6, 128.2, 137.0, 156.1, 170.0, 173.3; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{18}H_{26}N_3O_4$: 348.1918; found: 348.1908.

Cbz-L-Ala-OMe (22)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.32 (d, *J* = 6.9 Hz, 3H), 3.66 (s, 3H), 4.25-4.37 (m, 1H), 5.02 (s, 2H), 5.23 (d, *J* = 7.5 Hz, 1H), 7.17-7.27 (5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.7, 49.6, 52.4, 66.9, 128.1, 128.3, 128.5, 136.3, 155.6, 173.4.

Cbz-L-Ala-OCam (23)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.39 (d, *J* = 7.2 Hz, 3H), 4.22-4.30 (m, 1H), 4.50-4.61 (m, 2H), 5.03 (s, 2H), 5.22 (d, *J* = 5.7 Hz, 1H), 5.53 (s, 1H), 6.66 (s, 1H), 7.19-7.29 (5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.2, 50.0, 62.9, 67.4, 128.1, 128.3, 128.4, 128.5, 128.6, 135.9, 156.5, 169.6, 171.8.

Cbz-D-Ala-OMe (24)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.33 (d, *J* = 7.2 Hz, 3H), 3.66 (s, 3H), 4.25-4.38 (m, 1H), 5.03 (s, 2H), 5.26 (d, *J* = 4.5 Hz, 1H), 7.19-7.28 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.5, 49.5, 49.6, 52.3, 66.8, 127.9, 128.0, 128.1, 128.2, 128.4, 136.2, 155.4, 173.3.

Cbz-D-Phe-OMe (25)[29e]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.95-3.10 (m, 2H), 3.64 (s, 3H), 4.56-4.62 (m, 1H), 5.01 (s, 2H), 5.13 (d, *J* = 7.8 Hz, 1H), 7.00-7.29 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 38.1, 52.1, 54.7, 66.8, 127.0, 128.0, 128.4, 129.1, 135.5, 136.1, 155.4, 155.5, 171.8.

Cbz-L-Val-OMe (26)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.86 (dd, *J* = 6.9 and 23.7 Hz, 6H), 2.11-2.18 (m, 1H), 3.31-3.48 (m, 2H), 4.54-4.61 (m, 1H), 5.05 (s, 2H), 5.13 (d, *J* = 5.4 Hz, 1H), 7.19-7.31 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.4, 18.8, 31.1, 59.0, 60.4, 60.9, 67.3, 128.1, 128.3, 128.6, 136.1, 156.2, 170.7.

Cbz-L-Val-OTfe (27)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.88 (dd, *J* = 6.9 and 36.3 Hz, 6H), 2.03-2.12 (m, 1H), 3.37 (s, 3H), 4.21-4.26 (m, 1H), 5.04 (s, 2H), 5.19 (d, *J* = 8.1 Hz, 1H), 7.19-7.30 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.4, 18.8, 31.1, 59.0, 60.4, 67.3, 128.1, 128.3, 128.6, 136.1, 156.2, 170.7.

Cbz-Gly-OTfe (28)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 4.02 (d, *J* = 5.7 Hz, 2H), 4.41-4.50 (m, 2H), 5.07 (s, 2H), 5.15 (d, *J* = 7.5 Hz, 1H), 7.19-7.28 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 42.4, 60.7, 61.1, 67.4, 128.1, 128.3, 128.6, 136.0, 156.2, 168.7.

Cbz-L-Pro-OTfe (29)[29b]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.83-1.99 (m, 3H), 2.15-2.28 (m, 1H), 3.36-3.62 (m, 2H), 4.20-4.28 (m, 1H), 4.32-4.59 (m, 2H), 4.97-5.13 (m, 2H), 7.18-7.28 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 23.3, 24.1, 29.7, 30.7, 46.2, 46.7, 58.4, 58.8, 67.0, 127.7, 127.8, 127.9, 128.3, 136.2, 136.4, 153.9, 154.7, 170.9, 171.1.

Cbz-L-Ala-L-Ala-OTfe (30)
R_f(HPLC) 18.06 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.30-1.36 (m, 6H), 4.21-4.59 (m, 4H), 5.03 (s, 2H), 5.37 (d, *J* = 6.6 Hz, 1H), 6.67 (s, 1H), 7.19-7.26 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.6, 18.4, 47.9, 50.3, 60.1, 60.6, 61.1, 61.6, 67.1, 67.2, 120.8, 124.5, 128.0,

128.2, 128.5, 136.1, 156.0, 171.2, 172.1; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{16}H_{20}F_3N_2O_5$: 377.1319; found: 377.1341.

Cbz-Gly-L-Phe-NH_2 (31)[29f]
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 2.75-3.02 (m, 2H), 3.48-3.69 (m, 2H), 4.41-4.48 (m, 1H), 5.02 (s, 2H), 7.10-7.42 (m, 13H), 7.96 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 37.5, 43.3, 53.5, 65.3, 126.1, 127.6, 127.7, 127.9, 128.2, 129.0, 136.9, 137.7, 156.3, 158.6, 172.7.

Cbz-Gly-L-Leu-NH_2 (32)[29g]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.98 (dd, *J* = 7.8 Hz, 6H), 1.50-1.70 (m, 3H), 3.80-3.95 (m, 2H), 4.43-4.51 (m, 1H), 5.11 (s, 2H), 5.69-5.82 (m, 2H), 6.54 (s, 1H), 6.82 (m, *J* = 6.9 Hz, 1H), 7.26-7.34 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.9, 22.9, 24.8, 40.7, 44.6, 51.4, 67.4, 128.1, 128.3, 128.6, 136.0, 156.8, 156.9, 169.5, 174.6.

Cbz-Gly-L-Phe-O^{t}Bu (33)[29h]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.33 (s, 9H), 2.99 (d, *J* = 6.0 Hz, 2H), 3.73-3.84 (m, 2H), 4.65-4.71 (m, 1H), 5.05 (s, 2H), 5.31 (d, *J* = 6.3 Hz, 1H), 6.36 (d, *J* = 6.0 Hz, 1H), 7.04-7.29 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 117.9, 38.0, 44.5, 67.2, 82.6, 127.0, 128.1, 128.2, 128.4, 128.5, 129.5, 135.9, 136.1, 156.4, 168.2, 170.3.

Cbz-L-Ala-L-Leu-O^{t}Bu (34)[29i]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.85 (dd, *J* = 6.0 Hz, 6H), 1.32 (d, *J* = 7.2 Hz, 3H), 1.39 (s, 9H), 1.44-1.59 (m, 3H), 4.14-4.22 (m, 1H), 4.36-4.44 (m, 1H), 5.04 (s, 2H), 5.24 (d, *J* = 7.5 Hz, 1H), 6.23 (d, *J* = 7.2 Hz, 1H), 7.11-7.33 (m, 7H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 22.1, 22.7, 24.9, 28.0, 41.8, 51.5, 67.0, 82.0, 125.3, 128.0, 128.2, 128.5, 136.2, 155.8, 171.7, 171.8.

Cbz-L-Ala-L-Phe-O^{t}Bu (35)[29j]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.29 (d, *J* = 7.2 Hz, 3H), 1.34 (s, 9H), 2.98-3.05 (m, 2H), 4.10-4.18 (m, 1H), 4.60-4.68 (m, 1H), 5.03 (d, 2H), 5.17 (d, *J* = 7.2 Hz, 1H), 6.33 (d, *J* = 6.9 Hz, 1H), 7.04-7.29 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.6, 27.9, 37.9, 53.6, 67.0, 82.5, 127.0, 128.1, 128.2, 128.4, 128.5, 129.5, 136.0, 170.2, 171.5.

Cbz-L-Ala-L-Ala-Gly-L-Phe-NH_2 (36)
R_f(HPLC) 14.28 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.16-1.29 (m, 6H), 2.74-3.06 (m, 2H), 3.56-3.77 (m, 2H), 4.05-4.12 (m, 1H), 4.22-4.46 (m, 1H), 5.02 (d, *J* = 6.9 Hz, 2H), 7.10-7.48 (m, 14H), 7.93-8.21 (m, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 48.2, 53.7, 65.3, 65.4, 126.1, 127.6, 127.9, 128.2, 129.0, 136.9, 137.9, 138.0, 155.5, 168.2, 168.3, 172.3, 172.6, 172.7; FIA-ESI(+)-TOF-MS: *m/z* $[M + H]^+$ calcd for $C_{25}H_{32}N_5O_6$: 498.2347; found: 498.2346.

Cbz-D-Ala-D-Ala-O^{t}Bu (37)[29k]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.26-1.31 (m, 6H), 1.44 (s, 9H), 4.10-4.18 (m, 2H), 5.06 (s, 2H), 7.35-7.45 (m, 6H), 8.19 (d, *J* = 6.9 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 16.8, 18.1, 27.5, 48.1, 49.5, 65.2, 79.0, 80.1, 127.5, 127.6, 128.2, 136.9, 155.5, 171.5, 172.2.

Cbz-Gly-L-Phe-OH (38)[29l]
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 2.85-3.07 (m, 2H), 3.57-3.63 (m, 2H), 4.41-4.46 (m, 1H), 5.02 (s, 2H), 7.19-7.39 (m, 11H), 8.10 (d, *J* = 8.1 Hz, 1H), 12.77 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 36.7, 43.1, 53.3, 65.3, 126.3, 127.5, 127.6, 128.0, 128.2, 129.0, 136.9, 137.3, 156.3, 168.8, 172.6.

Cbz-L-Ala-L-Leu-OH (39)[29m]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.90 (dd, *J* = 3.3 and 5.7 Hz, 6H), 1.35 (d, *J* = 7.2 Hz, 3H), 1.56-1.70 (m, 3H), 4.23-4.33 (m, 1H), 4.53-5.61 (m, 1H), 5.10 (s, 2H), 5.69 (d, *J* = 6.3 Hz, 1H), 6.78 (d, *J* = 6.6 Hz, 1H), 7.26-7.33 (m, 5H), 8.10 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.3, 21.8, 22.8, 24.8, 41.0, 50.9, 67.2, 128.0, 128.2, 128.5, 136.0, 156.2, 172.8, 175.9.

Cbz-L-Ala-L-Phe-OH (40)[29n]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.30 (d, *J* = 7.2 Hz, 3H), 3.00-3.29 (m, 2H), 4.19-4.30 (m, 1H), 4.80-5.88 (m, 1H), 5.06 (d, 2H), 5.43 (d, *J* = 5.6 Hz, 1H), 6.70 (d, *J* = 6.6 Hz, 1H), 7.14-7.35 (m, 10H), 7.92 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.1, 37.3, 50.4, 53.2, 67.3, 127.2, 128.1, 128.3, 128.6, 129.3, 135.6, 156.2, 172.4, 174.1.

Cbz-L-Phe-L-Leu-OH (41)[29o]
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.90 (dd, 6H, *J* = 6.0 Hz), 1.42-1.67 (m, 3H), 3.02-3.15 (m, 2H), 4.45-4.58 (m, 2H), 5.09 (s, 2H), 5.45 (d. *J* = 7.8 Hz, 1H), 6.29 (d. *J* = 7.8 Hz, 1H), 7.20-7.35 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.8, 22.7, 24.7, 38.2, 41.0, 50.9, 56.1, 67.3, 127.2, 128.0, 128.3, 128.6, 128.8, 129.3, 136.1, 156.1, 171.2, 175.4.

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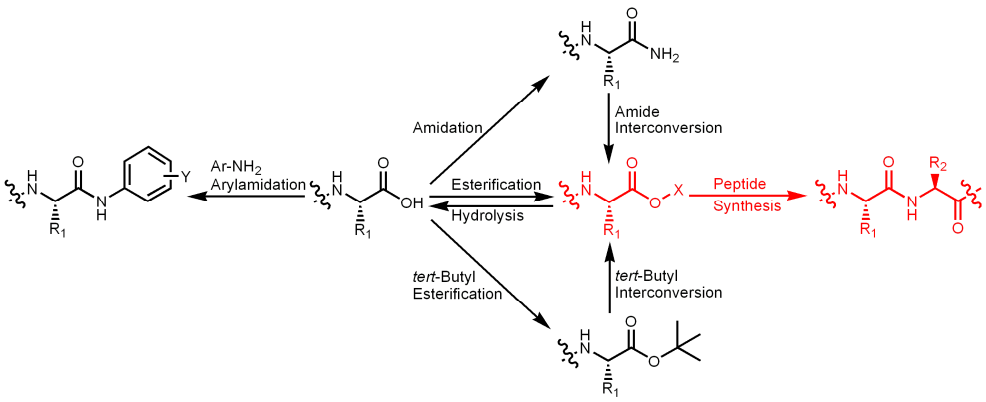
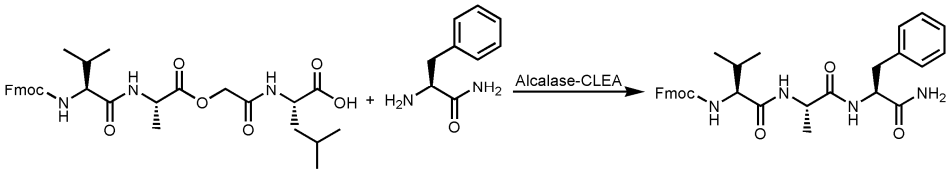
Chapter 9

Improving the carboxamidomethyl ester for Subtilisin A catalyzed peptide synthesis



In this chapter, a series of novel glycine esters was evaluated for their efficiency in Subtilisin A catalyzed peptide synthesis. The reactivity of the easily accessible carboxamidomethyl ester was further improved by elongating it with an amino acid residue thereby anticipating to increase the number of binding interactions for Subtilisin A.



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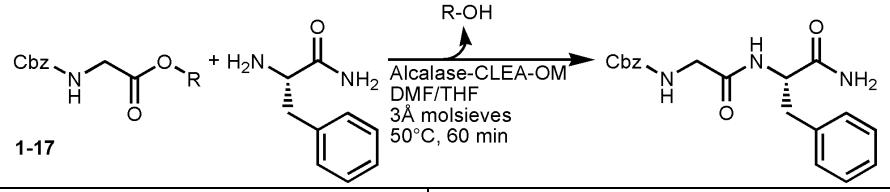
9.1 Introduction

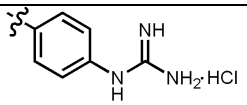
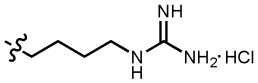
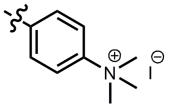
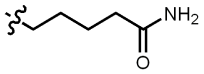
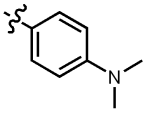
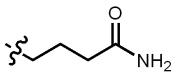
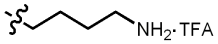
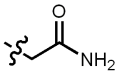
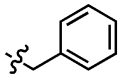
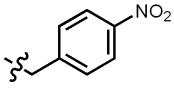
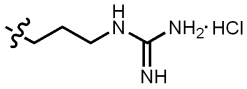
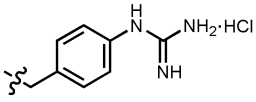
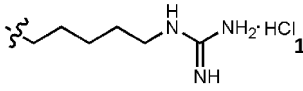
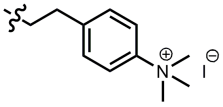
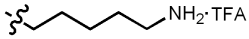
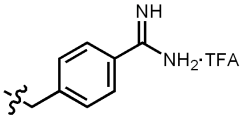
In chapter 8 the use of amino acid and peptide Cam-esters in Subtilisin A catalyzed peptide synthesis in anhydrous organic solvents was described.[1] Subtilisin A, which is a serine endoprotease, industrially available in the form of Alcalase, has a broad substrate tolerance and can therefore be applied for the synthesis of numerous peptides.[2] It was shown that the use of Cam esters broadened the scope of Alcalase catalyzed peptide synthesis even further and that the coupling of very challenging substrates became well feasible in high yields without any hydrolysis as side reaction. However, coupling reactions sometimes remained rather slow and a relatively large amount of enzyme is required, especially when longer peptide fragments are to be coupled. Clearly, there is room for improvement of the acyl donor ester for Alcalase catalyzed peptide synthesis.
In this chapter various new active esters for Alcalase catalyzed peptide synthesis in organic solvents are synthesized and compared, including Cam esters that have been elongated with any of the proteinogenic amino acid residues. These elongated Cam esters could be very conveniently synthesized in high yield and purity using specially designed solid-phase peptide synthesis techniques with readily available starting materials and resins.

9.2 Results and Discussion

To test the coupling efficiencies for the Alcalase catalyzed dipeptide synthesis, in which H-Phe-NH_2 functions as the nucleophile, a library of Cbz-Gly-OR esters was synthesized, in which R represents a moiety to increase binding interactions with the enzyme subsites. The results of this screening are given in Table 1. The library was synthesized using previously described chemical techniques and was originally designed to identify improved substrate mimetics for papain.[3] Alcalase cross-linked enzyme aggregates (CLEAs)[4] were used for convenient handling and workup. A solvent mixture of dimethylformamide/tetrahydrofuran (DMF/THF, 1/9, v/v) was used for good solubility of all starting materials. Molecular sieves were added to remove water from the reaction mixture, to prevent any hydrolytic side reactions.
Clearly, the substituted phenol esters are the most active species for the Alcalase-CLEA catalyzed peptide coupling followed by the Tfe and Cam ester derivatives (entries 1-5). This might be explained by their strong electron withdrawing properties, which make the carbonyl more susceptible to a nucleophilic attack by the active site serine of the protease while the corresponding alcohols being good leaving groups. Drawbacks of these esters are that, due to their high activation level, they are relatively difficult to synthesize, show spontaneous peptide coupling and other side reactions. The Cam-ester (entry 5), which is chemically[5] or enzymatically[1] much easier to synthesize and does not give any spontaneous peptide coupling nor racemization, also displays a very good activity. This activity cannot be only explained by its electron withdrawing properties.

Table 1. Relative activity of various Cbz-Gly-OR esters in Alcalase-CLEA catalyzed peptide coupling with H-Phe-NH_2.



Entry[a]	R =		Relative activity[b]	Entry[a]	R =		Relative activity[b]
1		**1**	100	10		**10**	23
2		**2**	95	11		**11**	11
3		**3**	70	12		**12**	9
4		**4**	68	13		**13**	8
5		**5**	60	14		**14**	8
6		**6**	34	15		**15**	7
7		**7**	31	16		**16**	4
8		**8**	30	17		**17**	4
9		**9**	28				

[a] Conditions: Alcalase-CLEA, H-Phe-NH_2, crushed 3 Å molecular sieves, THF/DMF (9/1, v/v), 50°C, 60 min; [b] The relative activity was calculated by dividing the amount of Cbz-Gly-Phe-NH_2 obtained by an enzymatic reaction with the amount of Cbz-Gly-Phe-NH_2 obtained by the enzymatic reaction using Cbz-Gly-OGp (**1**, entry 1) times 100%.

It is believed that the amide group of the Cam ester moiety binds to the enzyme via a hydrogen bond in the same fashion as an amide of a peptide backbone binds when it is recognized and cleaved by an endoprotease (Figure 1 A). Wells *et al.* already showed for

Subtiligase, a mutant of Subtilisin BPN wherein the active site serine is replaced by a cysteine, that the reactivity of the Cam ester (Figure 1 B) could be improved by elongating it with an amino acid amide and thereby creating additional binding interactions with the enzyme (Figure 1 C).[6]

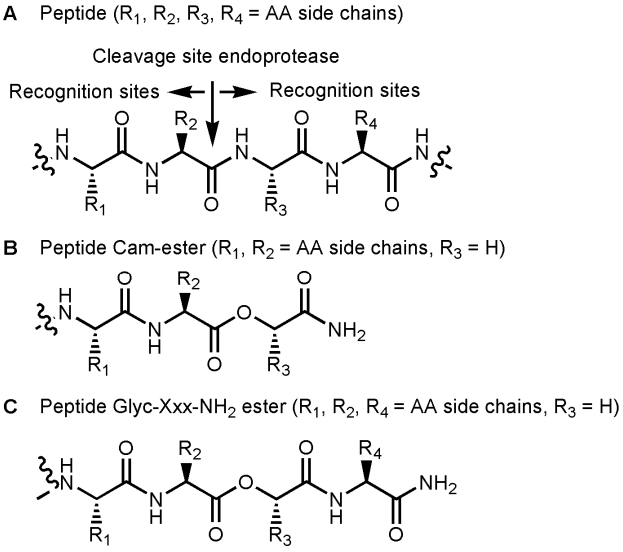
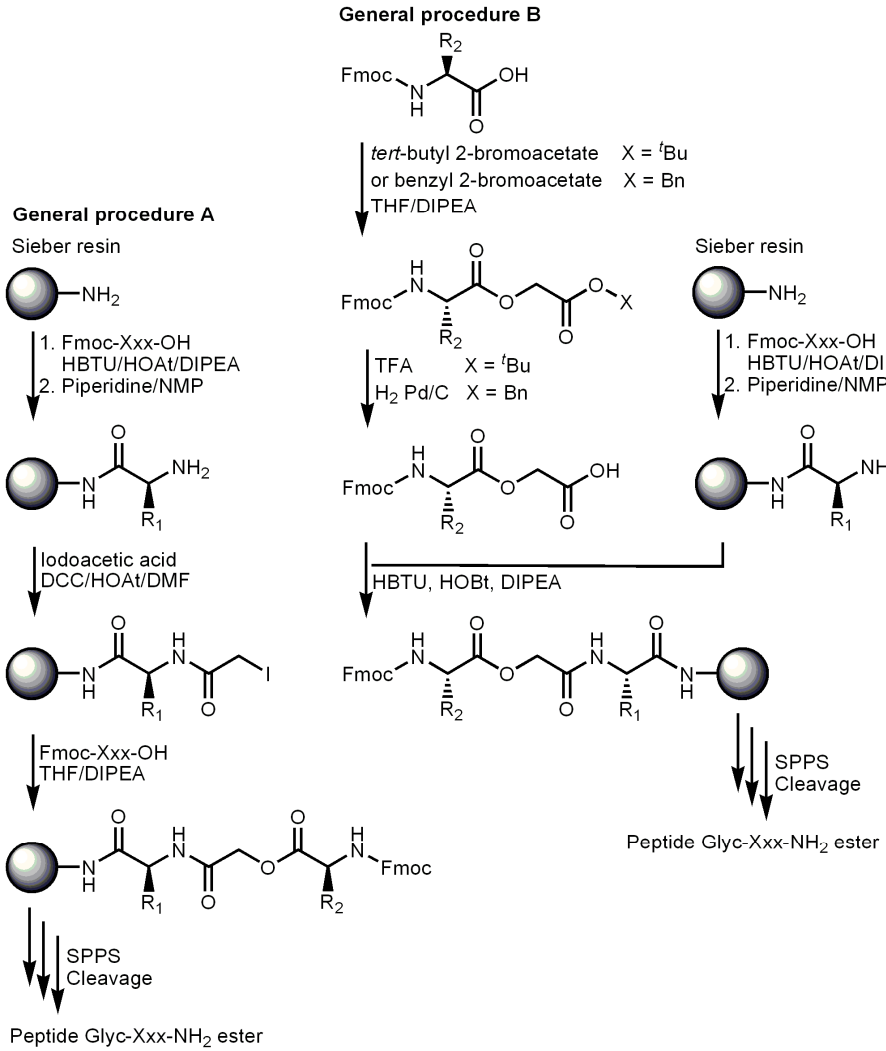


Figure 1. Similarity of the natural endopeptidase peptide cleavage site with the Cam and Glyc-Xxx-NH_2 ester.

9

To investigate if the Cam-ester could be further improved for Alcalase, a library of Fmoc-Val-Ala-Glyc-Xxx-NH_2 esters, wherein Xxx stands for all 20 proteinogenic amino acids, with an either protected or unprotected side chain functionality, was synthesized using specially designed SPPS techniques. Two methods were developed, one approach was based on the attachment of iodoacetic acid to a Sieber resin (Scheme 1 A) while the other method was based on the attachment of Fmoc-Xxx-OCH_2COOH building blocks to the same resin (Scheme 1 B).

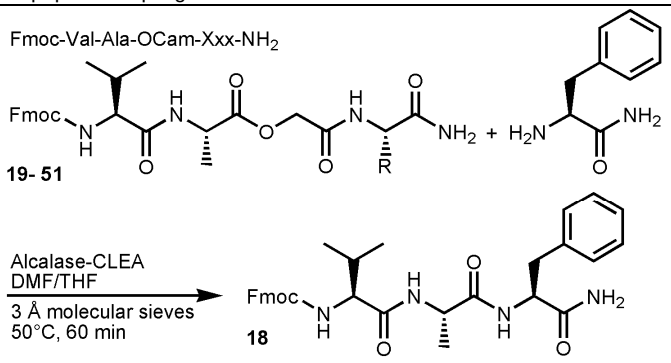
Both methods have their appealing characteristics. The advantage of method A is that no special amino acid building bocks have to be used, however, the esterification is performed at 50°C for 24 h, which is complicated for automated peptide synthesis. Although method B requires the use of special Fmoc amino acid building blocks, the advantage is that all reactions on the resin are performed using standard SPPS protocols at ambient temperature. Furthermore, the Fmoc-Xxx-OCH_2COOH building blocks are easily accessible. The dipeptide Glyc-Xxx-NH_2 esters were obtained in very high yield and purity and no side reactions were observed upon cleavage from the resin with trifluoroacetic acid.



Scheme 1. Two different solid phase methods developed for the synthesis of peptide Glyc-Xxx-NH_2 esters.

The relative activity of the Fmoc-Val-Ala-Glyc-Xxx-NH_2 esters was tested in an Alcalase-CLEA catalyzed peptide coupling using H-Phe-NH_2 as the nucleophile, as shown in Table 2. The product peak was analyzed using HPLC and the yields were calculated using a calibration curve of Fmoc-Val-Ala-Phe-NH_2 (**18**).There are large differences between the Glyc-Xxx-NH_2 esters and almost an equal part of them is better (entry 1-15) or worse (entry 17-33) than non-elongated Cam ester (entry 16). The best results were obtained using Phe and Tyr (**19** and **20**, entries 1 and 2), resulting in a two-fold enhancement.

Table 2. Relative activity of various Fmoc-Val-Ala-Glyc-Xxx-NH_2 esters in the Alcalase-CLEA catalyzed peptide coupling with H-Phe-NH_2.

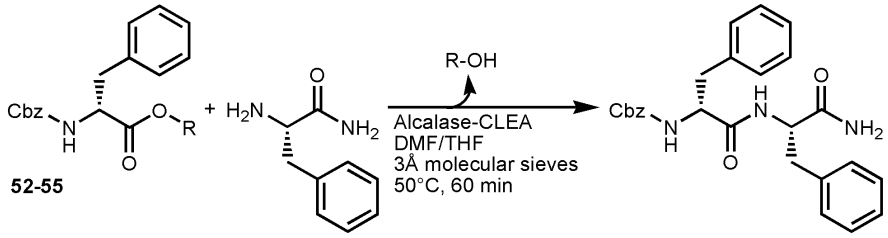


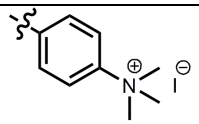
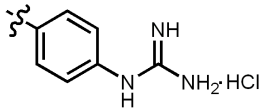
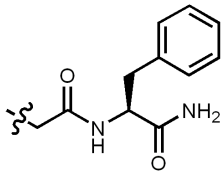
Entry[a]	Xxx =		Relative activity[b]	Entry	Xxx =		Relative activity[b]
1	Phe	**19**	204	18	Trp(Boc)	**36**	90
2	Tyr	**20**	198	19	Tyr(^{t}Bu)	**37**	88
3	Ser	**21**	169	20	Ile	**38**	88
4	Leu	**22**	165	21	Gln	**39**	84
5	Arg	**23**	163	22	Thr(^{t}Bu)	**40**	84
6	Asp(O^{t}Bu)	**24**	143	23	Lys	**41**	82
7	Ser(^{t}Bu)	**25**	143	24	Trp	**42**	63
8	Gln(Trt)	**26**	133	25	Met	**43**	53
9	Ala	**27**	131	26	Lys(Boc)	**44**	45
10	Gly	**28**	124	27	Asn(Trt)	**45**	41
11	Cys	**29**	114	28	Glu	**46**	37
12	Thr	**30**	108	29	Val	**47**	33
13	Glu(O^{t}Bu)	**31**	108	30	Asn	**48**	31
14	Arg(Pbf)	**32**	106	31	His	**49**	16
15	Pro	**33**	104	32	His(Trt)	**50**	16
16	--- (= Cam)	**34**	100	33	Asp	**51**	12
17	Cys(Trt)	**35**	92				

[a] Conditions: Alcalase-CLEA, H-Phe-NH_2, crushed 3 Å molecular sieves, THF/DMF (9/1, v/v), 50°C, 60 min; [b] The relative activity was calculated by dividing the amount of Fmoc-Val-Ala-Phe-NH_2 obtained by an enzymatic reaction with the amount of Fmoc-Val-Ala-Phe-NH_2 obtained by the enzymatic reaction using Fmoc-Val-Ala-OCam (**34**, entry 16) times 100%.

The optimal Glyc-Xxx-NH_2 ester (Table 2, entry 1, Glyc-Phe-NH_2, **19**) was compared to the most active substituted phenol esters from the first Cbz-Gly-OR screening (Table 1, entries 1, 2). To clearly discern the intrinsic reactivities, the challenging substrate Cbz-D-Phe was chosen as the acyl donor with H-Phe-NH_2 as the nucleophile (see Table 3). D-Amino acids are notoriously difficult substrates for Alcalase, in fact, Chen et. al. reported that no peptide product was obtained at all using Alcalase and Cbz-D-Phe as the acyl donor.[2]

Table 3. Relative activity of various Cbz-D-Phe-OR esters in Alcalase-CLEA catalyzed peptide coupling with H-Phe-NH_2.



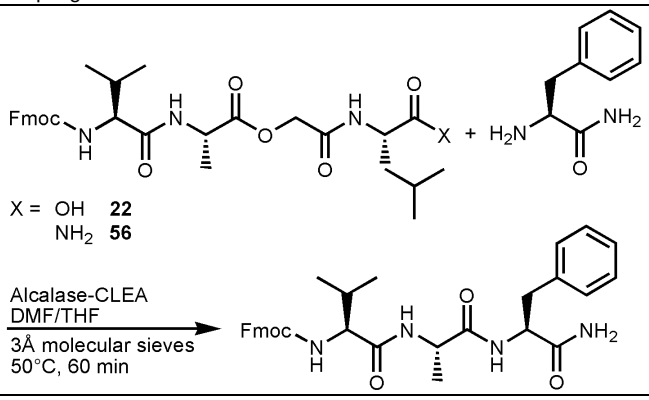
Entry[a]	R =		Conversion[b]	e.e. of Cbz-D-Phe
1		**52**	63	85.8
2		**53**	62	
3		**54**	57	>99.5
4		**55**	34	

[a] Conditions: Alcalase-CLEA, H-Phe-NH_2, crushed 3 Å molecular sieves, THF/DMF (9/1, v/v), 50°C, 60 min; [b] Conversions based on integration of the acyl donor starting material and the product, assuming that response factors are identical.

As is evident from Table 3, the elongated Cam ester (entry 3) shows a comparable reactivity as the substituted phenol esters (entry 1, 2). An equally active but more conveniently accessible ester was thus developed for Alcalase-CLEA catalyzed peptide synthesis. Another advantage is that no racemization occurred on the activated amino acid ester, *i.e.* D-Phe, using the Cbz-D-Phe-Glyc-Phe-NH_2 ester (**54**, entry 3, e.e. of D-Phe >99.5), this in contrast to the Cbz-D-Phe-OTMAP (**52**, entry 1, e.e. of D-Phe 85.8%) ester.

Although the Glyc-Xxx-NH_2 esters can be conveniently synthesized on the solid phase, the Sieber or Rink resins required to obtain the *C*-terminal amide functionality are relatively expensive. The resins which are commonly used to obtain a *C*-terminal carboxylic acid functionality (Wang or 2-chlorotritylchloride resin) are generally much cheaper and thus, better suited for large-scale applications. Therefore, the relative activity of a C-terminal carboxamide functionality (Fmoc-Val-Ala-Glyc-Leu-NH_2, **22**) was compared to a C-terminal carboxylic acid functionality (Fmoc-Val-Ala-Glyc-Leu-OH, **56**) in the Alcalase-CLEA catalyzed peptide coupling with H-Phe-NH_2 as the nucleophile. Gratifyingly, as demonstrated in Table 4, comparable reaction rates were obtained.

Table 4. Relative activity of Glyc-Leu-NH_2 and Glyc-Leu-OH esters in the Alcalase-CLEA catalyzed peptide coupling with H-Phe-NH_2.



Entry[a]	R =		Relative activity[b]
1	NH_2	**22**	91
2	OH	**56**	100

[a] Conditions: Alcalase-CLEA, H-Phe-NH_2, piperidine, crushed 3 Å molecular sieves, THF/DMF (9/1, v/v), 50°C, 60 min; [b] The relative activity was calculated by dividing the amount of Fmoc-Val-Ala-Phe-NH_2 obtained by an enzymatic reaction with the amount of Cbz-Val-Ala-Phe-NH_2 obtained by the enzymatic reaction using Fmoc-Val-Ala-Glyc-Leu-OH (**56**, entry 2) times 100%.

9

In conclusion, the performance of various novel and known *C*-terminal esters in Subtilisin A catalyzed peptide synthesis in an anhydrous organic solvent were investigated. It appeared that *C*-terminal phenolic esters were most active but they are difficult to synthesize in a chemically and stereochemically pure form. The slightly less reactive *C*-terminal carboxamidomethyl (Cam) esters, however, are easily accessible and are not prone to racemization. It was demonstrated that the activity of Cam esters can be increased to the level of the most active phenolic esters by elongating them with (apolar) amino acids and amino acid amides. Additionally, the convenient accessibility of these substituted Cam-esters via two different solid-phase strategies was demonstrated.

9.3 Experimental

General:
General experimental information is identical to chapter 2 with the following exceptions. For analytical HPLC chromatography a Phenomenex (C18, 5 μm particle size, 250 × 4.6 mm) column was used. The determination of the e.e. of Phe samples was identical to chapter 4.
For synthesis and analytical details of compounds **1**-**17** see literature reference.[3]
Synthesis and analytical data of Cbz-D-Phe-OCam (**55**) are described in chapter 8.

General procedure A: Synthesis of peptide Glyc-Xxx-NH_2 esters
The Fmoc protecting group was removed from a Sieber resin (1 g, loading = 0.5 mmol/g) using piperidine in *N*-methyl-2-pyrrolidone (NMP, 10 mL, 1/4, v/v) followed by washing steps with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and NMP (10 mL, 2 min, 3×). Fmoc-Xxx-OH was coupled to the amine using O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU, 2 mmol, 758 mg) 1-hydroxy-7-azabenzotriazole (HOAt, 2 mmol, 272 mg) and diisopropylethylamine (DIPEA, 4 mmol, 697 µL) in NMP (10 mL, 60 min). The resin was washed with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and NMP (10 mL, 2 min, 3×) followed by removal of the Fmoc protecting group using piperidine in NMP (10 mL, 1/4, v/v). After washing with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and *N*,*N*-dimethylformamide (DMF, 10 mL, 2 min, 3×) the free amine was reacted with iodoacetic acid (1 mmol, 186 mg), *N*,*N*-diisopropylcarbodiimide (DIC, 1 mmol, 155 µL) and HOAt (1 mmol, 136 mg) in DMF (10 mL, 30 min) followed by washing steps with DMF (10 mL, 2 min, 2×), CH_2Cl_2 (10 mL, 2 min, 2×), and DMF (10 mL, 2 min, 2×). Subsequently, Fmoc-Xxx-OH (2 mmol) and DIPEA (2.5 mmol, 436 µL) in DMF/tetrahydrofuran (DMF/THF, 1/1, v/v, 10 mL) were added and the mixture was shaken at 50°C with 200 rpm for 20 h. Afterwards, the resin was washed with 10 vol% H_2O in DMF (10 mL, 2 min, 2×) and DMF (10 mL, 2 min, 3×). Further Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[7] The peptide Cam ester was cleaved from the resin using 2.5 vol% trifluoroacetic acid (TFA) in CH_2Cl_2 (20 mL) for 30 min. After filtration, the filtrate was partly concentrated *in vacuo* to a volume of 10 mL. Subsequently, ^{i}PrOH in H_2O (1/3, v/v, 50 mL) was added followed by partial evaporation of the volatiles to a volume of 30 mL. The precipitates were removed by filtration and washed with H_2O (5 mL, 2×), followed by lyophilization from CH_3CN/H_2O (3/1, v/v).

General procedure B: Synthesis of peptide Glyc-Xxx-NH_2 esters
Coupling and Fmoc deprotection of the first amino acid was identical to general procedure A. Fmoc-Xxx-OCH_2COOH (1 mmol) (Fmoc-Val-Ala-OCH_2COOH for the library synthesis) was coupled to the free amine using HBTU (1 mmol, 379 mg), 1-Hydroxybenzotriazole (HOBt, 1 mmol, 136 mg) and DIPEA (2 mmol, 349 µL) in NMP (10 mL, 90 min). Further Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[7] After synthesis of the desired sequence, the peptide was cleaved from the resin and precipitated as described in general procedure A.

General procedure C: Side chain deprotection of Fmoc-Val-Ala-Glyc-Xxx-NH_2 peptides
Fmoc-Val-Ala-Glyc-Xxx-NH_2 (0.02 mmol) was dissolved in TFA/H_2O (1 mL, 95/5, v/v) and stirred for 1 h. Afterwards, the volatiles were concentrated by a nitrogen flow and the residue lyophilized from CH_3CN/H_2O (3/1, v/v). The lyophilized powders were dissolved in DMF (100 µL) and used as such for the relative activity determination assays described below. Additional piperidine (0.02 mmol, 2 µL) was added to the stock solutions of Fmoc-Val-Ala-Glyc-Asp-NH_2 and Fmoc-Val-Ala-Glyc-Glu-NH_2

General procedure D: Synthesis of peptide Glyc-Xxx-OH esters
2-Chlorotritylchloride resin (1 g, loading = 1.2 mmol/g) was reacted with Fmoc-Xxx-OH (2 mmol) and DIPEA (5 mmol) in CH_2Cl_2 (10 mL, 30 min) followed by washing with DMF (10 mL, 2×). Afterwards, the unreacted tritylchloride moieties were capped with

MeOH/CH_2Cl_2/DIPEA (10 mL, 15/85/5, v/v/v) followed by washing steps with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and NMP (10 mL, 2 min, 3×). Fmoc-Xxx-OCH_2COOH (1 mmol) was coupled to the free amine using HBTU (1 mmol, 379 mg), HOBt (1 mmol, 136 mg) and DIPEA (2 mmol, 349 µL) in NMP (10 mL, 90 min). Further Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[7] After synthesis of the desired sequence, the peptide was cleaved from the resin and precipitated as described in general procedure A.

General procedure E: Relative activity determination for Alcalase-CLEA catalyzed peptide coupling

To a suspension of Alcalase-CLEA (4.5 mg), H-Phe-NH_2 (0.54 mg) and crushed 3 Å molecular sieves (4.5 mL) in THF (900 µL), amino acid or peptide ester stock solution (20 mM) in DMF (100 µL) was added. The reaction mixture was shaken at 50°C with 200 rpm for 60 min. Afterwards, the reaction mixtures were filtrated and analyzed by analytical HPLC by integrating the peptide coupling product peak. Integration areas of different reactions were compared to each other to determine the relative activity (ester which gave the highest peptide product intergration area = 100%).

HCl.H-Ala-$OCH_2C(=O)O$-Bn

To a solution of Boc-Ala-OH (1 mmol, 189 mg) in THF (50 mL) was added DIPEA (2.5 mmol, 436µL) and benzyl iodoacetate (2.0 mmol, 552 mg). This mixture was shaken for 20 h at 50°C with 200 rpm and after this period of stirring, the reaction mixture was concentrated *in vacuo*. The residue was resuspended in EtOAc (100 mL) and this solution was washed with sat. aq. $NaHCO_3$ (100 mL, 2×), 0.1 N HCl (100 mL, 2×), brine (100 mL) and dried (Na_2SO_4). The solution was concentrated *in vacuo* and the residue was resuspended in 2 N HCl in dioxane (25 mL). After stirring for 1 h, cold diethylether (Et_2O, 100 mL) was added and the precipitate was filtered off and washed with cold Et_2O (20 mL, 2×). HCl.H-Ala-$OCH_2C(=O)O$-Bn was dried at 40°C *in vacuo* for 24 h and obtained in an overall yield of 87%. R_t(HPLC) 5.19 min; Purity = 98%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.62 (d, *J* = 6.9 Hz, 3H), 4.19-4.28 (m, 1H), 4.51-4.77 (m, 2H), 5.03 (s, 2H), 7.21 (s, 5H), 8.64 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 16.0, 49.2, 61.8, 67.4, 128.5, 128.6, 128.7, 134.8, 166.9, 169.7.

Fmoc-Val-Ala-OCH_2COOH

To a solution of Fmoc-Val-OH (1 mmol, 339 mg) in CH_2Cl_2 (50 mL) which was cooled on ice to 0°C, 1-ethyl-3(3-dimethylamino propyl)-carbodiimide hydrochloride (EDC·HCl, 1.1 mmol, 211 mg) and HOBt (1.1 mmol, 150 mg) were added. After stirring for 30 min, HCl.H-Ala-$OCH_2C(=O)O$-Bn (1.1 mmol, 300 mg) and DIPEA (1.1 mmol, 192 µL) were added. The obtained reaction mixture was stirred at ambient temperature for 6 h, followed by washing with sat. aq. $NaHCO_3$ (50 mL, 2×), 0.1 N HCl (50 mL, 2×), brine (50 mL). The CH_2Cl_2 solution was dried (Na_2SO_4) and subsequently concentrated *in vacuo* and the residue was resuspended in MeOH/toluene (1/1, v/v, 250 mL). Then, the mixture was hydrogenated using Pd/C (10 mol%) with 5 bar H_2 at 25°C for 24 h. Afterwards, the reaction mixture was filtrated over celite followed by concentration of the solvents *in vacuo* and purification of the residue by preparative HPLC. Fmoc-Val-Ala-OCH_2COOH was obtained in an overall yield of 73%. R_t(HPLC) 15.76 min; Purity = 99%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.88 (dd, *J* = 10.2 and 6.9 Hz, 6H), 1.34 (d, *J* = 7.2 Hz, 3H), 2.92-2.03 (m, 1H), 3.88-3.94 (m, 1H), 4.18-

4.39 (m, 4H), 4.51-4.65 (m, 2H), 7.29-7.43 (m, 5H), 7.75 (q, *J* = 3.6 and 3.3 Hz, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 8.42 (d, *J* = 6.6 Hz, 1H), 13.05 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 30.3, 46.6, 47.2, 59.6, 60.7, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 156.0, 168.7, 171.1, 172.0.

Fmoc-Val-Ala-Phe-NH_2 (18)

R_t(HPLC) 22.48 min; Purity = 99%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = = 0.82 (dd, *J* = 3.0 Hz, 6H), 1.15 (d, 3H, *J* = 7.2 Hz), 1.92-1.99 (m, 1H), 2.79-3.02 (m, 2H), 3.86 (q, 1H, *J* = 7.2 Hz and 1.2 Hz), 4.19-4.43 (m, 5H), 7.05 (s, 1H), 7.16-7.44 (m, 11H), 7.73 (t, 2H, *J* = 6.6 Hz), 7.84-8.00 (m, 4H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 19.0, 21.2, 22.7, 24.1, 30.3, 46.6, 47.5, 49.9, 59.6, 62.0, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.2, 171.3, 171.8, 173.5.

Fmoc-Val-Ala-Glyc-Phe-NH_2 (19)

R_t(HPLC) 16.85 min; Purity = 98%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.87 (dd, *J* = 7.5 Hz, 6H), 1.32 (d, *J* = 7.2 Hz, 3H), 1.87-2.15 (m, 1H), 2.77-3.05 (m, 2H), 3.86-3.92 (m, 1H), 4.20-4.46 (m, 7H), 7.11-7.46 (m, 11H), 7.72-7.76 (m, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 8.09 (d, *J* = 8.4 Hz, 1H), 8.44 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 30.3, 37.5, 46.6, 47.5, 53.5, 59.6, 62.1, 65.6, 120.0, 125.3, 126.1, 126.9, 127.5, 129.0, 137.7, 140.6, 143.7, 143.8, 166.0, 171.1, 171.7, 172.4

Fmoc-Val-Ala-Glyc-Leu-NH_2 (22)

R_t(HPLC) 16.60 min; Purity = 98%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.82-0.90 (m, 12H), 1.33-1.61 (m, 6H), 1.94-2.00 (m, 1H), 3.86-3.92 (m, 1H), 4.18-4.38 (m, 5H), 4.53 (m, 2H), 7.01 (s, 1H), 7.29-7.44 (m, 6H), 7.74 (q, *J* = 4.5 Hz and 2.4 Hz, 2H), 7.88-7.98 (m, 3H), 8.46 (d, *J* = 6.0 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 21.4, 22.9, 24.1, 30.3, 40.8, 46.6, 47.8, 50.5, 59.6, 62.2, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.0, 171.3, 171.9, 173.6.

Fmoc-Val-Ala-Glyc-Asp(O^{t}Bu)-NH_2 (24)

R_t(HPLC) 17.00 min; Purity = 98%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.87 (dd, *J* = 7.5 Hz, 6H), 1.32-1.37 (m, 12H), 1.93-2.00 (m, 1H), 2.41-2.70 (m, 2H), 3.87-3.93 (m, 1H), 4.17-4.58 (m, 7H), 7.15 (s, 1H), 7.31-7.44 (m, 6H), 7.73 (m, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 8.18 (d, *J* = 8.1 Hz, 1H), 8.45 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 27.5, 30.3, 31.2, 37.4, 46.6, 47.5, 49.0, 59.6, 62.2, 65.6, 80.0, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 156.0, 166.1, 169.3, 171.3, 171.8.

Fmoc-Val-Ala-Glyc-Ser(^{t}Bu)-NH_2 (25)

R_t(HPLC) 16.85 min; Purity = 97%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.87 (dd, *J* = 7.8 Hz, 6H), 1.10 (s, 9H), 1.35 (d, *J* = 7.2 Hz, 3H), 1.94-2.00 (m, 1H), 3.30-3.53 (m, 2H), 3.87-3.92 (m, 1H), 4.20-4.36 (m, 5H), 4.50-4.63 (m, 2H), 7.11 (s, 1H), 7.29-7.44 (m, 6H), 7.73-7.75 (m, 2H), 7.87-7.90 (m, 3H), 8.42 (d, *J* = 6.0 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 27.1, 30.3, 31.2, 46.6, 47.4, 52.9, 59.6, 61.7, 62.2, 65.6, 72.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 156.0, 166.1, 171.2, 171.2, 171.2.

Fmoc-Val-Ala-Glyc-Gln(Trt)-NH_2 (26)

R_t(HPLC) 19.63 min; Purity = 97%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.80 (dd, *J* = 6.6 Hz and 3.9 Hz, 6H), 1.26 (d, *J* = 7.2 Hz, 3H), 1.55-1.68 (m, 1H), 1.74-1.93 (m, 2H), 2.20-2.25 (m, 2H), 3.80-3.85 (m, 1H), 4.06-4.28 (m, 5H), 4.40-4.53 (m, 2H), 7.00-7.37 (m, 22H), 7.67 (q, *J* = 3.6 Hz, 2H), 7.82 (d, *J* = 7.5 Hz, 2H), 7.96 (d, *J* = 8.1 Hz, 1H), 8.37 (d, *J* = 6.6 Hz, 1H), 8.54 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.6, 18.1, 19.0, 27.2, 27.6, 30.3, 31.1, 46.6, 47.5, 51.3, 59.6, 62.2, 65.6, 79.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.2, 171.3, 171.5, 171.9, 172.6.

Fmoc-Val-Ala-Glyc-Ala-NH$_2$ (27)
R_t(HPLC) 14.69 min; Purity = 98%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.87 (dd, *J* = 7.2 Hz, 6H), 1.32 (d, *J* = 7.2 Hz, 3H), 1.87-2.15 (m, 4H), 2.77-2.85 (m, 1H), 2.99-3.05 (m, 1H), 3.86-3.92 (m, 1H), 4.20-4.61 (m, 6H), 7.12-7.46 (m, 11H), 7.72-7.76 (m, 2H), 7.90 (d, *J* = 7.5 Hz, 2H), 8.09 (d, *J* = 8.4 Hz, 1H), 8.44 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.6, 18.2, 19.0, 30.3, 46.6, 47.5, 47.7, 59.6, 62.3, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 165.8, 171.3, 171.9, 173.7.

Fmoc-Val-Ala-Glyc-Gly-NH$_2$ (28)
R_t(HPLC) 13.65 min; Purity = 92%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.88 (dd, *J* = 7.2 Hz, 6H), 1.34 (d, *J* = 6.9 Hz, 3H), 1.93-2.00 (m, 1H), 3.66 (d, *J* = 5.4 Hz, 2H), 3.87-3.93 (m, 1H), 4.22-4.59 (m, 6H), 7.08 (s, 1H), 7.30-7.44 (m, 6H), 7.74-7.90 (m, 4H), 8.12-8.16 (m, 1H), 8.47 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 30.3, 41.5, 46.6, 47.5, 59.6, 62.4, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 156.0, 166.6, 170.4, 171.3, 171.9.

Fmoc-Val-Ala-Glyc-Glu(O^{t}Bu)-NH$_2$ (31)
R_t(HPLC) 17.30 min; Purity = 98%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.88 (dd, *J* = 7.2 Hz, 6H), 1.33-1.39 (m, 12H), 1.66-1.79 (m, 1H), 1.87-2.02 (m, 2H), 2.17-2.27 (m, 2H), 3.87-3.93 (m, 1H), 4.17-4.37 (m, 5H), 4.48-4.60 (m, 2H), 7.11 (s, 1H), 7.29-7.44 (m, 6H), 7.74 (dd, *J* = 4.5 Hz and 2.7 Hz, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 8.00 (d, *J* = 8.1 Hz, 1H), 8.46 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 28.0, 30.3, 31.2, 32.5, 46.6, 47.5, 51.9, 59.6, 62.2, 65.6, 69.1, 120.0, 125.3, 126.2, 126.9, 127.3, 127.4, 127.5, 128.4, 140.6, 143.7, 143.8, 144.8, 166.1, 171.2, 171.3, 171.9, 172.0

Fmoc-Val-Ala-Glyc-Arg(Pbf)-NH$_2$ (32)
R_t(HPLC) 17.94 min; Purity = 94%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.86 (dd, *J* = 6.9 Hz, 6H), 1.10 (s, 3H), 1.32-1.40 (m, 9H), 1.96-2.02 (m, 4H), 2.94-3.03 (m, 3H), 3.80-3.92 (m, 1H), 4.17-4.35 (m, 5H), 4.53 (m, 2H), 7.07 (s, 1H), 7.28-7.43 (m, 5H), 7.75 (q, *J* = 4.8 Hz and 2.4 Hz, 2H), 7.88 (d, J = 7.5 Hz, 2H), 7.99 (d, *J* = 8.1 Hz, 1H), 8.45 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 12.1, 16.7, 17.5, 18.1, 18.8, 19.0, 28.2, 30.3, 31.2, 42.4, 46.6, 47.4, 51.6, 59.6, 62.2, 65.6, 86.2, 116.1, 120.0, 124.2, 125.3, 126.9, 127.5, 131.3, 137.2, 140.6, 143.7, 143.8, 156.0, 157.3, 166.1, 171.3, 171.9, 173.0.

Fmoc-Val-Ala-Glyc-Pro-NH$_2$ (33)
R_t(HPLC) 14.42 min; Purity = 97%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.87 (dd, *J* = 6.9 Hz, 6H), 1.36 (d, *J* = 7.2 Hz, 3H), 1.78-2.03 (m, 5H), 3.30-3.47 (m, 2H), 3.87-3.93 (m, 1H), 4.15-4.45 (m, 5H), 4.61-4.89 (m, 2H), 6.94 (s, 1H), 7.22-7.44 (m, 6H), 7.73-7.77 (m, 2H), 7.89 (d,

J = 7.5 Hz, 2H), 8.39 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.8, 18.2, 19.0, 24.0, 29.0, 30.3, 45.1, 46.6, 47.3, 59.6, 61.6, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 164.6, 171.1, 171.9, 173.3.

Fmoc-Val-Ala-OCam (34)
R_t(HPLC) 14.67 min; Purity = 98%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.88 (dd, *J* = 6.6 Hz, 6H), 1.33 (d, *J* = 7.2 Hz, 3H), 1.93-2.00 (m, 1H), 3.86-3.91 (m, 1H), 4.18-4.48 (m, 6H), 7.32-7.46 (m, 7H), 7.75 (q, *J* = 3.9 Hz and 2.7 Hz, 2H), 7.89 (d, *J* = 7.2 Hz, 2H), 8.49 (d, *J* = 6.6 Hz, 1H), ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.6, 18.2, 18.9, 30.3, 46.6, 47.6, 59.7, 62.2, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 156.0, 168.4, 171.4, 171.8.

Fmoc-Val-Ala-Glyc-Cys(Trt)-NH_2 (35)
R_t(HPLC) 20.91 min; Purity = 91%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.87 (dd, *J* = 6.9 Hz and 2.4 Hz, 6H), 1.34 (d, *J* = 7.2 Hz, 2H), 1.93-2.00 (m, 1H), 2.19-2.50 (m, 2H), 3.87-3.93 (m, 1H), 4.13-4.38 (m, 4H), 4.48-4.64 (m, 2H), 6.43-7.10 (m, 20H), 7.46-7.76 (m, 2H), 7.89 (d, *J* = 6.0 Hz, 2H), 8.20 (d, *J* = 8.4 Hz, 1H), 8.44 (d, *J* = 6.9 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 14.0, 14.3, 16.7, 18.1, 18.2, 19.0, 22.1, 30.3, 33.8, 46.6, 47.4, 55.7, 120.0, 125.3, 126.1, 126.6, 126.9, 127.4, 127.5, 127.7, 127.9, 128,2 128.9, 129.0, 140.6, 143.7, 143.8, 144.1, 166.0, 171.1, 171.8.

Fmoc-Val-Ala-Glyc-Trp(Boc)-NH_2 (36)
R_t(HPLC) 19.42 min; Purity = 92%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.84 (dd, *J* = 6.9 Hz, 6H), 1.31, (d, *J* = 7.2 Hz, 3H), 1.61 (s, 9H), 1.89-2.08 (m, 1H), 2.90-3.16 (m, 1H), 3.85-3.91 (m, 1H), 4.19-4.58 (m, 7H), 7.18-7.81 (m, 13H), 7.88 (d, *J* = 7.5 Hz, 2H), 8.02 (d, *J* = 8.1 Hz, 1H), 8.18 (d, *J* = 8.1 Hz, 1H), 8.44 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.6, 18.1, 18.9, 27.2, 27.6, 30.3, 46.6, 47.5, 52.0, 59.6, 62.1, 65.6, 83.3, 114.5, 116.4, 119.2, 120.0, 122.3, 123.7, 124.1, 125.6, 126.9, 127.5, 130.2, 134.5, 140.6, 143.7, 143.8, 148.9, 155.9, 166.1, 171.2, 171.7, 172.4.

Fmoc-Val-Ala-Glyc-Tyr(^{t}Bu)-NH_2 (37)
R_t(HPLC) 17.86 min; Purity = 94%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.87 (dd, *J* = 6.9 Hz, 6H), 1.25 (s, 9H), 1.32 (d, *J* = 7.5 Hz, 3H), 1.93-2.00 (m, 1H), 2.72-3.01 (m, 2H), 3.86-3.92 (m, 1H), 4.17-4.45 (m, 7H), 6.84 (d, *J* = 7.5 Hz, 2H), 6.98 (d, *J* = 7.5 Hz, 2H), 7.29-7.44 (m, 6H), 7.72-7.76 (m, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 8.05 (d, *J* = 8.4 Hz, 1H), 8.45 (d, *J* = 6.3, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.1, 17.6, 18.4, 27.9, 29.7, 46.0, 46.9, 52.9, 59.1, 61.5, 65.0, 76.9, 114.2, 119.3, 122.6, 124.7, 126.4, 127.0, 129.0, 129.4, 131.7, 140.0, 143.1, 143.2, 152.7, 155.4, 165.4, 170.7, 171.1, 171.9.

Fmoc-Val-Ala-Glyc-Ile-NH_2 (38)
R_t(HPLC) 16.46 min; Purity = 96%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.79-0.93 (m, 12H), 1.01-1.44 (m, 4H), 1.68-1.75 (m, 2H), 1.93-2.00 (m, 1H), 3.86-3.92 (m, 1H), 4.13-4.35 (m, 5H), 4.48-4.71 (m, 2H), 7.05 (s, 1H), 7.29-7.44 (m, 6H), 7.72-7.90 (m, 5H), 8.45 (d, *J* = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 11.0, 15.3, 16.7, 18.1, 190.0, 24.0, 30.3, 36.5, 46.6, 47.4, 56.3, 59.6, 62.2, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.0, 171.2, 171.9, 172.5.

Fmoc-Val-Ala-Glyc-Thr(^{t}Bu)-NH_2 (40)
R_t(HPLC) 17.32 min; Purity = 98%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.88 (dd, *J* = 6.9 Hz and 2.4 Hz, 6H), 1.00 (d, *J* = 6.3 Hz, 3H), 1.13 (s, 9H), 1.35 (d, *J* = 7.2 Hz, 3H), 1.94-2.00 (m, 1H), 3.84-3.98 (m, 2H), 4.13-4.36 (m, 5H), 4.52-4.69 (m, 2H), 7.16-7.44 (m, 7H), 7.62-7.76 (m, 3H), 7.89 (d, *J* = 7.5 Hz, 2H), 8.43 (d, *J* = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.8, 171.1, 19.0, 19.4, 27.9, 30.3, 46.6, 47.4, 57.3, 59.6, 62.3, 65.6, 66.7, 73.4, 120.0, 120.0, 121.3, 125.3, 126.9, 127.2, 127.5, 128.8, 140.6, 143.7, 143.8, 155.9, 166.2, 171.1, 171.2, 171.4, 171.7.

Fmoc-Val-Ala-Glyc-Met-NH_2 (43)
R_t(HPLC) 15.93 min; Purity = 91%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.87 (dd, *J* = 7.5 Hz, 6H), 1.33 (d, *J* = 6.9 Hz, 3H), 1.72-2.02 (m, 6H), 2.41-2.50 (m, 2H), 3.82-3.92 (m, 1H), 4.20-4.35 (m, 5H), 4.54 (m, 2H), 7.10 (s, 1H), 7.29-7.43 (m, 5H), 7.74 (dd, *J* = 3.6 Hz, 2H), 7.88 (d, *J* = 7.5 Hz, 2H), 8.04 (d, *J* = 8.1 Hz, 1H), 8.47 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 14.5, 16.6, 18.1, 19.0, 29.5, 30.3, 31.7, 46.5, 47.5, 51.3, 59.6, 62.3, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.3, 171.3, 172.0, 172.7.

Fmoc-Val-Ala-Glyc-Lys(Boc)-NH_2 (44)
R_t(HPLC) 17.06 min; Purity = 95%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.86 (dd, *J* = 7.5 Hz, 6H), 1.21-1.63 (m, 15H), 1.92-2.00 (m, 1H), 2.83-2.89 (m, 2H), 3.80-3.91 (m, 1H), 4.12-4.34 (m, 5H), 4.50-4.60 (m, 2H), 6.70-6.75 (m, 1H), 7.02 (s, 1H), 7.28-7.43 (m, 5H), 7.71-7.95 (m, 5H), 8.45 (d, *J* = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 12.1, 16.7, 17.5, 18.1, 18.8, 190.0, 22.5, 28.2, 29.1, 30.3, 31.1, 46.6, 47.5, 51.6, 59.6, 62.2, 65.6, 86.2, 120.0, 124.2, 125.3, 126.9, 127.5, 128.8, 131.3, 140.6, 143.7, 143.8, 156.0, 166.1, 171.3, 171.9, 173.0.

Fmoc-Val-Ala-Glyc-Asn(Trt)-NH_2 (45)
R_t(HPLC) 19.68 min; Purity = 96%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.88 (dd, *J* = 6.6 Hz and 5.4 Hz, 6H), 1.32 (d, *J* = 7.2 Hz, 2H), 1.97-2.02 (m, 1H), 2.63-2.65 (m, 2H), 3.79-3.94 (m, 1H), 4.17-4.33 (m, 4H), 4.44-4.61 (m, 3H), 7.11-7.43 (m, 21H), 7.74 (q, *J* = 4.5 Hz and 2.1 Hz, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 8.23 (d, *J* = 8.1 Hz, 1H), 8.46 (d, *J* = 6.3 Hz, 1H), 8.59 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 30.4, 46.6, 47.5 49.6, 62.1, 65.6, 69.3, 120.0, 125.3, 126.2, 126.9, 127.3, 127.5, 128.4, 140.6, 144.6, 166.0, 168.9, 171.3, 171.9, 172.0.

Fmoc-Val-Ala-Glyc-Val-NH_2 (47)
R_t(HPLC) 15.80 min; Purity = 98%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.81-0.90 (m, 12H), 1.34 (d, *J* = 7.2 Hz, 3H), 1.91-2.04 (m, 2H), 3.86-3.92 (m, 1H), 4.11-4.38 (m, 5H), 4.56 (m, 2H), 7.06 (s, 1H), 7.29-7.44 (m, 6H), 7.72-7.90 (m, 5H), 8.45 (d, *J* = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 17.7, 18.1, 19.0, 19.1, 30.3, 46.6, 47.4, 57.1, 59.6, 62.2, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.1, 171.2, 171.8, 172.5.

Fmoc-Val-Ala-Glyc-His(Trt)-NH_2 (50)
R_t(HPLC) 14.08 min; Purity = 90%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.85 (dd, *J* = 6.0 Hz, 6H), 1.30 (d, *J* = 7.2 Hz, 3H), 1.90-1.98 (m, 1H), 2.73-3.10 (m, 2H), 3.87-3.92 (m, 1H), 4.17-4.32 (m, 4H), 4.57-4.58 (m, 3H), 6.97 (s, 1H), 7.09-7.44 (m, 24H), 7.73 (dd, *J* = 4.5 and 2.4 Hz, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 8.14 (d, *J* = 8.1 Hz, 1H), 8.46 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR

(DMSO-d_6, 75 MHz) δ = 16.6, 18.1, 18.9, 30.3, 46.6, 47.5, 51.5, 55.7, 59.6, 62.1, 65.6, 120.0, 120.2, 125.3, 126.1, 126.5, 126.9, 127.4, 127.5, 127.6, 128.2, 128.4, 128.4, 128.9, 129.1, 136.7, 140.6, 140.8, 143.7, 143.8, 166.1, 171.3, 171.8, 171.8.

Cbz-D-Phe-Glyc-Phe-NH_2 (54)
e.e. D-Phe > 99.5; R_t(HPLC) 17.43 min; Purity = 97%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 2.79-2.89 (m, 2H), 3.01-3.16 (m, 2H), 4.37-4.57 (m, 5H), 4.98 (s, 2H), 7.14-7.49 (m, 17H), 7.86 (d, *J* = 8.1 Hz, 1H), 8.19 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 36.2, 37.3, 55.6, 55.2, 63.9, 65.3, 126.1, 126.4, 127.4, 127.6, 127.9, 128.1, 128.2, 129.0, 136.7, 137.4, 137.8, 156.0, 165.9, 171.1, 172.5.

Fmoc-Val-Ala-Glyc-Leu-OH (56)
R_t(HPLC) 21.45 min; Purity = 92%; ^{1}H NMR (DMSO-d_6, 300 mHz) δ = 0.85-0.87 (m, 12 H), 1.34 (d, *J* = 3.5 Hz, 3H), 1.54-1.60 (m, 3H), 1.98-2.02 (m, 1H), 3.87-3.92 (m, 1H), 4.22-4.36 (m, 5H), 4.46-4.60 (m, 2H), 7.32-7.44 (m, 5H), 7.74 (q, J = 4.5 and 2.7 Hz, 2H), 7.89 (d, *J* = 3.6 Hz, 2H), 8.15 (d, *J* = 3.6 Hz, 1H), 8.46 (d, *J* = 2.7 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 18.2, 19.0, 21.4, 22.9, 24.1, 30.3, 46.5, 50.5, 51.5, 59.7, 62.3, 65.6, 69.1, 120.0, 125.2, 126.2, 126.9, 127.3, 127.5, 128.2, 128.4, 140.6, 143.6, 143.8, 144.7, 156.0, 166.1, 170.9, 171.3, 171.6, 173.6.

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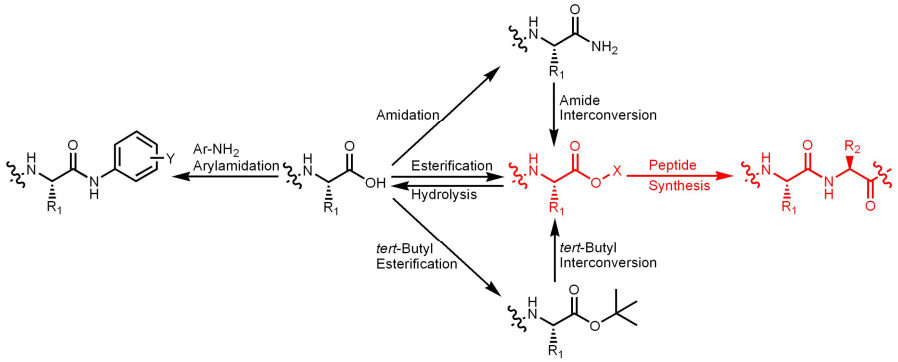
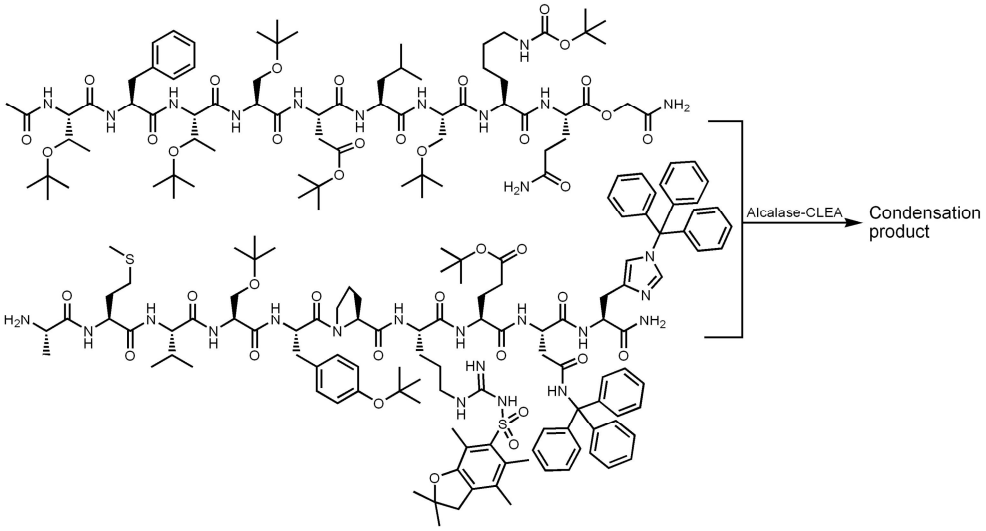
Chapter 10

Enzymatic fragment condensation of side chain protected peptides



In this chapter, the enzymatic condensation of side chain protected peptide fragments using Subtilisin A is described. A screening with dipeptide Cbz-Val-Xxx carboxamidomethyl esters with H-Xxx-Val-NH_2 nucleophiles was performed, wherein Xxx stands for every (side chain protected) amino acid residue, to investigate the scope and limitations of the enzymatic fragment condensation strategy. Finally, it was demonstrated that it is feasible to enzymatically condense larger peptide fragments (up to the 10-mer level) bearing multiple side chain protecting groups with very high conversion.



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10.1 Introduction

Although few pharmaceutically relevant peptides can be produced relatively cost-efficiently on large scale by fermentative approaches, most pharmaceutical peptides are synthesized by chemical means.[1] Solution phase chemical peptide synthesis is only feasible on large scale for short peptide sequences, whereas for medium-sized and long peptides (containing 10-50 amino acid residues), solid phase peptide synthesis (SPPS) is most commonly applied.[2] The main disadvantage of SPPS is that extremely high yields should be realized for each coupling and deprotection cycle.[3] For instance, when per step (coupling, Fmoc deprotection and final cleavage) a yield of 95% is realized in the synthesis of a decamer, an overall yield of only 34% of the crude peptide is obtained, which should be purified by a laborious and cost-inefficient HPLC method. Therefore, on industrial scale, peptides longer than 10-15 amino acids are not cost-efficiently synthesized on the solid phase. Furthermore, during synthesis, long side chain protected peptides tend to form tertiary structures (a process which is called "hydrophobic collapse")[4] making peptide elongation very troublesome so that a large excess of reagents and amino acid building blocks is needed. Additionally the purification of the final product is often cost-inefficient due to the presence of significant amounts of truncated peptides and peptides containing deletions.[5]

Due to these limitations, peptides longer than 10 amino acids are industrially usually produced using a hybrid approach, *i.e.* a combination of SPPS and solution phase peptide synthesis. Herein, side chain protected peptide fragments are synthesized on the solid phase and subsequently condensed in solution, *e.g.* by a 10 + 10 condensation, to make a peptide of 20 amino acids. The major drawback of chemical fragment condensation is that upon activation of the *C*-terminal amino acid residue racemization occurs, except when *C*-terminal Gly or Pro residues are used.[6] Therefore, the application of chemical fragment condensation is usually limited to *C*-terminally activated Gly and Pro residues, or one has to deal with a very difficult HPLC purification due to the formation of undesired diastereoisomers. Because Gly and Pro residues are often not equally distributed throughout the peptide sequence, or are not present at all, very asymmetric fragment condensation strategies (*e.g.* 3 + 17 or 5 + 15) have to be followed resulting in low overall yields and very high cost prices.

In contrast to chemical fragment condensation, the enzymatic coupling of peptide fragments is totally free of racemization.[7] Although many enzymes, *i.e.* proteases, have been applied to the synthesis of short peptide sequences in aqueous buffered solution, only very few examples of enzymatic peptide fragment condensations have been reported. The major drawback is that the presence water, which is essential for enzyme activity and stability, leads to undesired hydrolysis of the peptide backbone.[8] To ensure a fast coupling reaction and thereby reducing proteolysis, a peptide *C*-terminal ester has to be used as the acyl donor (following the kinetically controlled approach, see chapter 1). Special esters have been designed, so called substrate mimetics, which have such a high affinity for the enzyme so that side reactions could be virtually eliminated.[9] However, peptides containing such a *C*-terminal ester moiety are notoriously difficult to synthesize chemically in high stereoisomeric purity. In fact, the coupling reagents required to synthesize these substrate mimetics are often identical to those used for chemical peptide bond formation.[10] A few solid-phase methodologies have been reported for the synthesis of peptide substrate mimetics, but these strategies are complicated, low yielding and require the use of resins which are not suitable on industrial scale.[11] In chapter 9 the SPPS of peptide carboxamidomethyl (Cam)

esters, which are highly active in Subtilisin A catalyzed peptide synthesis, was described using industrially applicable resins. Besides these active Cam esters, anhydrous reaction conditions were required to eliminate any hydrolytic side reactions.
In this chapter, oligopeptide Cam esters under anhydrous reaction conditions are combined to condense peptide fragments with Subtilisin A. It is shown that fragment condensation is applicable to a large variety of Cam esters as the acyl donor and *N*-terminal peptide amines as nucleophiles. Surprisingly, also side chain protected peptides, which are highly soluble in organic solvents, are recognized by Subtilisin A and suitable as acyl donors and nucleophiles, giving high yield in fragment condensations. It appears advantageous to use acyl donors wherein the side chain functionality of the *C*-terminal amino acid residue is unprotected. Finally, it is demonstrated that this enzymatic fragment condensation of side chain protected peptides, up to the level of 10 + 9-mer couplings, is feasible with extremely high conversions.

10.2 Results and Discussion

Initially, it was investigated whether Cam esters could also be used to condense peptide fragments longer than dimers. As shown in Table 1, a number of peptide fragments, without any functionalized side chain could be successfully coupled by Subtilisin A (Alcalase) in anhydrous organic solvent (entries 1-5).

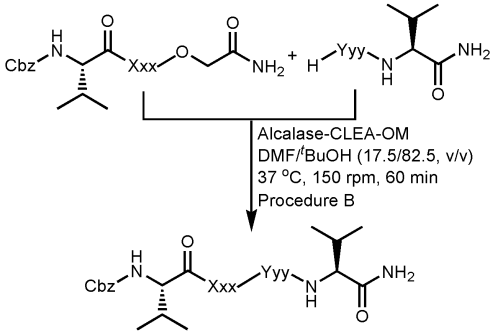
Table 1. Enzymatic fragment condensations.

Entry[a]	Acyl donor Cam ester		Nucleophile		Conversion to product (%)[b]
1	Cbz-Phe-Leu-Ala-OCam	**1**	H-Ala-Leu-Phe-NH_2	**6**	**12** (quant.)
2	Cbz-Ala-Leu-Phe-OCam	**2**	H-Leu-Phe-NH_2	**7**	**13** (95)
3	Cbz-Gly-Ile-Ala-OCam	**3**	H-Gly-Phe-NH_2	**8**	**14** (quant.)
4	Cbz-Ala-Pro-Leu-OCam	**4**	H-Gly-Phe-NH_2	**8**	**15** (87)
5	Cbz-Ala-Pro-Leu-OCam	**4**	H-Gly-Leu-Met-NH_2	**9**	**16** (quant.)
6	Cbz-Gly-Ile-Ala-OCam	**3**	H-Ser(^{t}Bu)-Leu-Leu-NH_2	**10**	**17** (95)
7	Cbz-Ala-Pro-Leu-OCam	**4**	H-Trp(Boc)-Met-Asp(O^{t}Bu)-Phe-NH_2	**11**	**18** (97)
8	Ac-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Trt)-Met-OCam	**5**	H-Leu-Phe-NH_2	**7**	**19** (96)

[a] Conditions: Alcalase-CLEA-OM, DMF/THF (1/9, v/v), 1.5 equiv nucleophile, 3 Å molecular sieves, 37°C, 16 h (Procedure A); [b] Conversions estimated using LC-MS by integration of the acyl donor starting material and the product peaks, assuming identical response factors.

Excellent conversions were observed by analytical HPLC with only 1.5 equiv of nucleophile. After work-up and purification, a yield of 73% could be obtained for Cbz-Phe-Leu-Ala-Ala-Leu-Phe-NH_2 (**12**). Unfortunately, peptide fragments containing amino acids with unprotected functional groups in the side chain, appeared to be very poorly soluble in organic solvents. Therefore, it was investigated whether peptides, containing amino acids with protected side chain functionalities, which are better soluble in organic solvents, were also accepted by Subtilisin A. Gratifyingly, side chain protected peptide fragments, up to the pentamer level, were well accepted by Subtilisin A and high conversions to the desired products were obtained, according to HPLC-MS analysis (Table 1, entry 6-8).

To explore the scope and limitations of this peptide fragment condensation strategy, two libraries of dipeptides were synthesized, *i.e.* a Cbz-Val-Xxx-OCam and an H-Yyy-Val-NH_2 library, in whitch Xxx and Yyy represent all proteinogenic amino acids with protected side chain functionalities. It was decided to take a Val residue at the penultimate positions because the steric hindrance minimizes side reactions such as transamidation of the peptide backbone. The efficiencies of all 400 possible coupling reactions were investigated as shown in Scheme 1 and Figure 1.



Xxx, Yyy =	#	Xxx, Yyy =	#	Xxx, Yyy =	#	Xxx, Yyy =	#
Ala	**20, 21**	Gln(Trt)	**30, 31**	Leu	**40, 41**	Ser(^{t}Bu)	**50, 51**
Arg(Pbf)	**22, 23**	Glu(O^{t}Bu)	**32, 33**	Lys(Boc)	**42, 43**	Thr(^{t}Bu)	**52, 53**
Asn(Trt)	**24, 25**	Gly	**34, 35**	Met	**44, 45**	Trp(Boc)	**54, 55**
Asp(O^{t}Bu)	**26, 27**	His(Trt)	**36, 37**	Phe	**46, 47**	Tyr(^{t}Bu)	**56, 57**
Cys(Trt)	**28, 29**	Ile	**38, 39**	Pro	**48, 49**	Val	**58, 59**

Scheme 1. Coupling efficiencies between Cbz-Val-Xxx-OCam and H-Yyy-Val-NH_2.

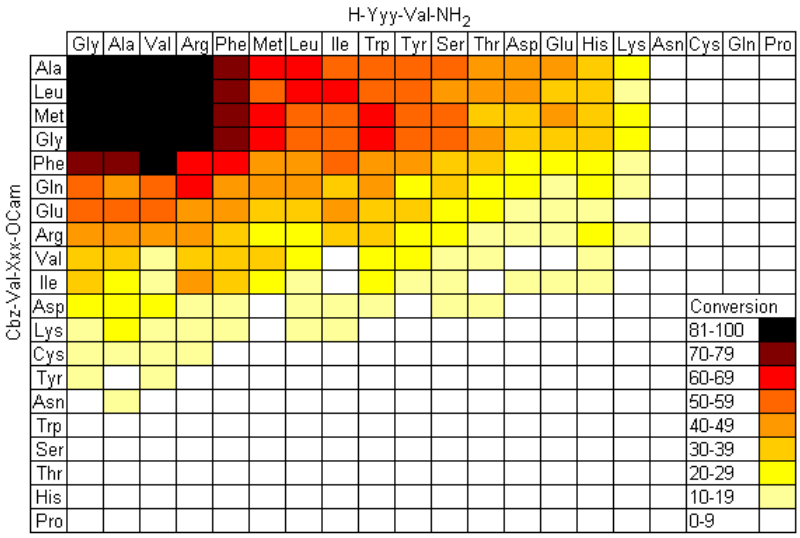


Figure 1. Comparison of coupling efficiencies (HPLC conversion) between Cbz-Val-Xxx-OCam and H-Yyy-Val-NH_2 dipeptides in which all functional side chains were protected (procedure B).

As is clear from Figure 1, the coupling reaction of a quite large number of dipeptide coupling combinations proceeds smoothly and high conversions were observed although Val

residues are located at the penultimate positions. With respect to the Cbz-Val-Xxx-OCam library, there is a clear preference for hydrophobic amino acids. However, also amino acids containing sterically demanding protecting groups, such as Gln(Trt) and Glu(O^{t}Bu), are very well accepted by the enzyme. It must be noted that, when reaction times were extended and/or more enzyme was applied, almost all reactions could be brought to complete conversion.

Table 2. Fragment condensation of some Cbz-Ala-Xxx-OCam with H-Xxx-Ala-NH_2 dipeptides with protected functional groups in the side chains of the amino acids.

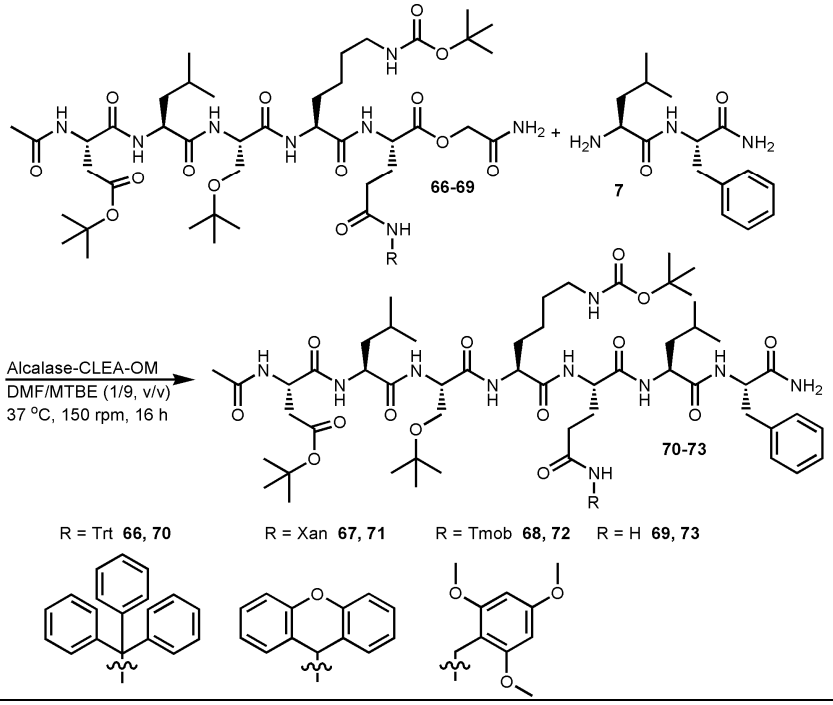
Entry[a]	Acyl donor Cam ester		Nucleophile		Product		Isolated yield (%)[d]
1[b]	Cbz-Ala-Gln(Trt)-OCam	**60**	H-Arg(Pbf)-Ala-NH_2	**62**	Cbz-Ala-Gln(Trt)-Arg(Pbf)-Ala-NH_2	**64**	69
2[c]	Cbz-Ala-Asn(Trt)-OCam	**61**	H-Lys(Boc)-Ala-NH_2	**63**	Cbz-Ala-Asn(Trt)-Lys(Boc)-Ala-NH_2	**65**	63

[a] Conditions: Alcalase-CLEA-OM, DMF/THF (1/9, v/v), 3 Å molecular sieves, 37°C; [b] 3 h; [c] 16 h; [d] Isolated yield based on acyl donor starting material.

As demonstrated in Table 2, when the sterically less demanding Ala is used in the penultimate positions instead of Val, also very difficult combinations, *i.e.* Cbz-Ala-Asn(Trt)-OCam with H-Lys(Boc)-Ala-NH_2, are very well feasible (entry 2). The coupling efficiency trends are still comparable: the coupling between Cbz-Ala-Gln(Trt)-OCam and H-Arg(Pbf)-Ala-NH_2 proceeded much faster using the same reaction conditions (entry 1). In other words, many more dipeptide coupling combinations than apparent from Figure 1 proceed rapidly and the reaction rate is also depending on the amino acid in the penultimate position.

Some acyl donors containing amino acids with sterically demanding protecting groups proved difficult to incorporate, such as Cbz-Val-Ser(^{t}Bu)-OCam and Cbz-Val-Thr(^{t}Bu)-OCam. Therefore, the influence of several protecting groups on the efficiency of the Subtilisin A catalyzed peptide fragment condensation was investigated. As a model, a pentapeptide with a *C*-terminal Gln residue containing several protecting groups was chosen, as shown in Table 3. Additionally, the side chain of the *C*-terminal Gln amino acid residue was left unprotected (entry 4).

Table 3. Fragment condensation with various side chain protected *C*-terminal glutamine Cam-esters.



Entry[a]	Pentamer Cam ester		Conversion to heptamer product (%)[b]
1	Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-**Gln(Trt)**-OCam	**66**	18 (**70**)
2	Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-**Gln(Xan)**-OCam	**67**	13 (**71**)
3	Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-**Gln(Tmob)**-OCam	**68**	37 (**72**)
4	Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-**Gln**-OCam	**69**	92 (**73**)

[a] Conditions: Alcalase-CLEA-OM, DMF/MTBE (1/9, v/v), 3 Å molecular sieves, 37°C, 16 h (Procedure A); [b] Conversions estimated using LC-MS by integration of the acyl donor starting material and the product peaks, assuming identical response factors.

Evidently, there were no large differences in coupling efficiency when different side chain protecting groups were used for the *C*-terminal Gln residue (entries 1-3). However, an unprotected Gln residue as *C*-terminal Cam-ester resulted in an excellent conversion of 92% to the desired heptamer. These semi-protected peptide starting materials can be relatively easily prepared via SPPS techniques because the γ-amide functionality is not very reactive as nucleophile.

Triggered by these observations, a second screening was performed wherein the Cbz-Val-Xxx-OCam library contained an unprotected amino acid residue at position Xxx, while the side chains of the H-Yyy-Val-NH_2 nucleophiles were still protected (see Figure 2).

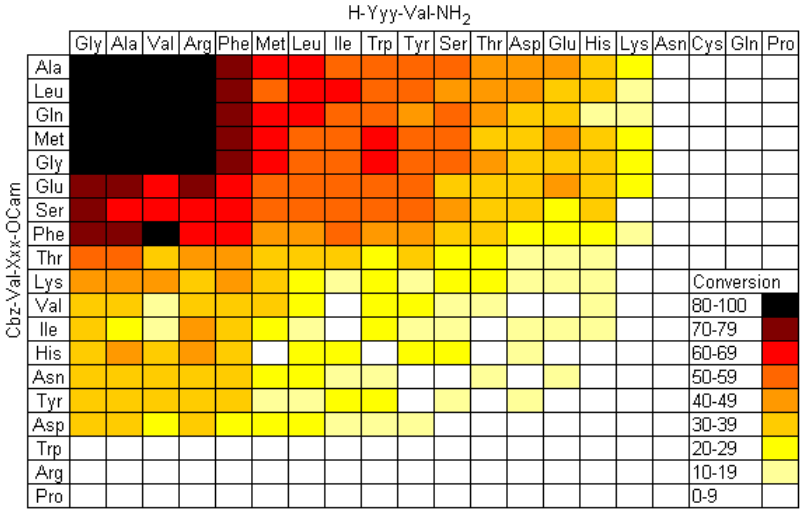


Figure 2. Comparison of coupling efficiencies of condensations between Cbz-Val-Xxx-OCam (unprotected side chains) and H-Yyy-Val-NH_2 (protected side chains) using procedure B.

The efficiency of several coupling reactions in case of acyl donors without protected side chain functionalities was clearly increased compared to the fully protected acyl donors. For instance, ^{t}Bu-protected Ser and Thr residues gave poor coupling efficiencies, while the unprotected analogues were readily accepted by Subtilisin A, thereby broadening the scope of the enzymatic peptide fragment condensation strategy. Another striking difference was observed for an unprotected Gln, which was found to be one of the most efficient acyl donors.

To show the versatility of this fragment condensation strategy, wherein only the *C*-terminal amino acid residue of the Cam ester was unprotected, two antimicrobial peptides containing ten amino acid residues were synthesized, as shown in Table 4.[12, 13] These peptides show activity against a number of gram-positive and gram-negative bacteria from the skin, the oral, the respiratory and the gastrointestinal tract.

Clearly, a good product yield was obtained for the peptide fragment condensation reactions. The semi-protected acyl donor with a *C*-terminal unprotected Ser residue (**75**) could be prepared by using a very acid labile protecting group, *i.e*. Trt, which was simultaneously deprotected during the acidic cleavage of the peptide from the solid support. During this mild acidic cleavage, the other side chain protecting groups were left in tact. After enzymatic fragment condensation, full deprotection of the decapeptides was performed chemically to obtain the natural antimicrobial peptides (**78**, **79**) in good yield (63, 55% respectively).

Table 4. Synthesis of two antimicrobial peptides using an acyl donor with an unprotected *C*-terminal residue.

Entry[a]	Acyl donor Cam ester	Nucleophile	Product after full deprotection[b]	Isolated yield (%)[c]
1	Boc-Leu-Asp(O^{t}Bu)-Gln(Trt)-Ser(^{t}Bu)-**Gln**-OCam (**74**)	H-Phe-Val-Gly-Ser(^{t}Bu)-Arg(Pbf)-NH_2 (**76**)	H-Leu-Asp-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2 (**78**)	63
2	Boc-His(Trt)-Lys(Boc)-Thr(^{t}Bu)-Asp(O^{t}Bu)-**Ser**-OCam (**75**)	H-Phe-Val-Gly-Leu-Met-NH_2 (**77**)	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 (**79**)	55

[a] Conditions: Alcalase-CLEA-OM, DMF/MTBE (1/9, v/v), 3 Å molecular sieves, 37°C, 16 h; [b] Conditions: TFA/TIS/H_2O (95/2.5/2.5, v/v/v); [c] Isolated yield based on acyl donor starting material.

After these successful 5 + 5 amino acid fragment condensations, it was investigated whether even longer peptide fragments could be condensed.

Table 5. Fragment condensation of side chain protected peptides of different lengths in DMF/MTBE (1/9, v/v) and CH_2Cl_2.

Entry[a]	Acyl donor Cam ester	Nucleophile	Conversion DMF/MTBE (%)[b]	Conversion CH_2Cl_2 (%)[b]
1	Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-**Gln**-OCam (5-mer, **69**)	H-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Ala-NH_2 (5-mer, **82**)	quant.	quant. (**84**)
2	Ac-Thr(^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-**Gln**-OCam (7-mer, **80**)	H-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Ala-NH_2 (5-mer, **82**)	62	98 (**85**)
3	Ac-Thr(^{t}Bu)-Phe-Thr(^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-**Gln**-OCam (9-mer, **81**)	H-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Ala-NH_2 (5-mer, **82**)	not soluble	78 (**86**)
4	Ac-Thr(^{t}Bu)-Phe-Thr(^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-**Gln**-OCam (9-mer, **81**)	H-Ala-Met-Val-Ser(^{t}Bu)-Tyr(^{t}Bu)-Pro-Arg(Pbf)-Glu(O^{t}Bu)-Asn(Trt)-His(Trt)-NH_2 (10-mer, **83**)	not soluble	95[c] (**87**)

[a] Conditions: Alcalase-CLEA-OM, DMF/MTBE (1/9, v/v) or CH_2Cl_2, 3 Å molecular sieves, 37°C, 16 h (Procedure A and C); [b] Conversions estimated using HPLC by integration of the acyl donor starting material and the product peaks, assuming identical response factors; [c] After 1 week

As demonstrated in Table 5, the solvent mixture suitable for the 5 + 5-mer (entry 1-2) fragment condensation, *i.e*. DMF/MTBE (1/9, v/v), was not applicable to longer fragment condensations (entry 3-4) due to the limited solubility of the peptides. However, CH_2Cl_2 was found to be a very good alternative, since protected peptides containing more than 5-7 amino acid residues tend to be highly soluble in this solvent. In fact, when CH_2Cl_2 was used as the solvent, nearly quantitative conversion was obtained for a number of different peptide

fragment condensations. When longer reaction times were applied, even an enzymatic 9 + 10-mer side chain protected peptide fragment condensation proved well feasible and an excellent conversion to the 19-mer product was observed (Table 5, entry 4). It was rather astonishing that such large peptides, bearing bulky side chain protecting groups, were still accepted by the enzyme and that the coupling proceeded smoothly, despite the anhydrous reaction conditions. This racemization free enzymatic side chain protected peptide fragment condensation strategy opens new doors for the (industrial) synthesis of long peptides.
In conclusion, this chapter describes a novel enzymatic fragment condensation strategy of side chain protected peptides up to the coupling of 10 + 9-mer fragments in neat organic solvent using Subtilisin A. It was shown that the strategy is broadly applicable, especially when the side chain functionality of the *C*-terminal amino acid cam ester as the acyl donor is unprotected.

10.3 Experimental

General:
General experimental methods were identical as described in chapter 2 with the following exceptions. For analytical HPLC chromatography a Phenomenex (C18, 5 μm particle size, 250 × 4.6 mm) column was used. Before use, 3 Å molecular sieves were crushed and passed through a 0.5 mm sieve.

General procedure A: Enzymatic peptide coupling reactions in DMF/THF or DMF/MTBE (1/9, v/v)
To a suspension of Alcalase-CLEA-OM (10 mg) and 3 Å molecular sieves (10 mg) in THF or MTBE (900 μL), a solution of the acyl donor Cam ester (30 μmol) and protected peptide nucleophile (45 μmol, 1.5 equiv) in DMF (100 μL) was added. The obtained reaction mixture was shaken at 37°C with 200 rpm for 16 h and subjected to LC-MS analysis.

General procedure B: Coupling efficiencies between Cbz-Val-Xxx-OCam and H-Yyy-Val-NH_2 with protected side chain functionalities
Fragment condensations were performed in a 96-wells format with glass vial inserts. To a glass insert was added a solution of Cbz-Val-Xxx-OCam (2.5 μmol) in DMF (45 μL) and a solution of H-Yyy-Val-NH_2 (5 μmol, 2 equiv) in DMF (45 μL), wherein Xxx and Yyy represent proteinogenic amino acids with a protected side chain functionality and proteinogenic amino acids without a side chain functionality. Subsequently, a suspension of crushed molecular sieves (5 mg) and Alcalase-CLEA-OM (4.5 mg) in ^{t}BuOH (410 μL) were added. The plate was covered with an alumina lid and shaken at 37°C with 150 rpm for 2 h. Afterwards, samples of the supernatant (300 μL) were taken and added to DMSO (700 μL) and analyzed by HPLC. The identity of the tetrapeptide products was confirmed by LC-MS analysis.

Synthesis of Cbz-Val-Xxx-OCam esters with unprotected side chain functionalities
The corresponding side chain protected Cbz-Val-Xxx-OCam peptide (5 μmol) was dissolved in TFA/H_2O (1 mL, 95/5, v/v) and stirred for 1 h at ambient temperature. Afterwards, the volatiles were removed by a nitrogen flow and the residue was lyophilized from CH_3CN/H_2O (3/1, v/v). The resulting lyophilized powders were dissolved in DMF (90 μL) containing

piperidine (10 µmol, 1 µL) and used as such for the coupling efficiency assays described in general procedure B.

Solid phase synthesis of side chain protected peptide Cam esters

The Fmoc protecting group was removed from a Sieber resin (1 g, loading = 0.5 mmol/g) using piperidine/NMP (10 mL, 1/4, v/v) followed by washing steps with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and DMF (10 mL, 2 min, 3×). The resin was reacted with a mixture of iodoacetic acid (1 mmol), dicyclohexylcarbodiimide (DCC, 1 mmol) and 1-hydroxy-7-azabenzotriazole (HOAt, 1 mmol) in DMF (10 mL, 30 min) followed by washing steps with DMF (10 mL, 2 min, 2×), CH_2Cl_2 (10 mL, 2 min, 2×), and DMF (10 mL, 2 min, 2×). Subsequently, a solution of Fmoc-Xxx-OH (2 mmol) and DIPEA (2.5 mmol) in DMF/THF (1/1, v/v, 10 mL) was added and the mixture was shaken for 20 h at 50°C with 200 rpm. Afterwards, the resin was washed with 10 vol% H_2O in DMF (10 mL, 2 min, 2×) and DMF (10 mL, 2 min, 3×). Consecutive Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[14] The peptide Cam ester was cleaved from the resin using 2.5 vol% trifluoroacetic acid (TFA) in CH_2Cl_2 (20 mL) for 30 min. After filtration the filtrate was concentrated *in vacuo* to a volume of 10 mL. Subsequently, $^{i}PrOH/H_2O$ (1/3, v/v, 50 mL) was added followed by partial evaporation of the volatiles to a volume of 30 mL. The precipitate was collected by filtration and washed with H_2O (5 mL, 2×), followed by lyophilization from CH_3CN/H_2O (3/1, v/v). The protected Cam ester was purified by preparative HPLC followed by lyophilization from CH_3CN/H_2O (3/1, v/v).

Solid phase synthesis of (side chain protected) peptide nucleophiles

Side chain protected peptide *C*-terminal carboxamides were synthesized on a Sieber resin (1 g, loading = 0.7 mmol/g) using standard SPPS protocols.[14] The desired peptide sequence was cleaved from the resin using 2.5 vol% TFA in CH_2Cl_2 (50 mL, 20 min). After filtration, the resin was washed with CH_2Cl_2 (50 mL) and the combined filtrates were washed with sat. aq. Na_2CO_3 (100 mL, 2×) and brine (100 mL). The organic layer was concentrated *in vacuo* to half of the original volume. Then, $^{i}PrOH/H_2O$ (50 mL, 1/3, v/v) was added and the suspension was concentrated *in vacuo* to one third of the original volume. The precipitated protected peptide was filtered off and subsequently washed with H_2O (20 mL, 2×) followed by lyophilization from CH_3CN/H_2O (3/1, v/v). If necessary, the protected peptide was purified by preparative HPLC followed by lyophilization from CH_3CN/H_2O (3/1, v/v). After lyophilisation the peptide was dissolved in CH_2Cl_2 (50 mL) and washed with sat. aq. Na_2CO_3 (100 mL, 2×) and brine (100 mL). The organic layer was concentrated *in vacuo* to half of the original volume. Then, $^{i}PrOH/H_2O$ (50 mL, 1/3, v/v) was added and the suspension was concentrated *in vacuo* to one third of the original volume. The precipitated protected peptide was filtered off and subsequently washed with H_2O (20 mL, 2×) followed by lyophilization from CH_3CN/H_2O (3/1, v/v).

General Procedure C: Enzymatic peptide coupling reactions in CH_2Cl_2

To a suspension of Alcalase-CLEA-OM (10 mg) and 3 Å molecular sieves (10 mg) in CH_2Cl_2 (500 µL), was added a solution of the peptide Cam ester (3.0 µmol) and the peptide nucleophile (4.5 µmol) in CH_2Cl_2 (500 µL). The obtained reaction mixture was shaken at

37°C with 200 rpm for 16 h, and the progress of the condensation reaction was monitored with LC-MS.

Cbz-Phe-Leu-Ala-OCam (1)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.88 (dd, *J* = 6.6 and 12.6 Hz, 6H), 1.33 (d, *J* = 7.2 Hz, 3H), 1.44-1.70 (m, 3H), 2.68-3.02 (m, 2H), 4.24-4.47 (m, 5H), 4.94 (s, 2H), 7.16-7.36 (m, 12H), 7.43 (d, *J* = 8.4 Hz, 1H), 8.04 (d, *J* = 8.1 Hz, 1H), 8.47 (d, *J* = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 22.3, 27.4, 28.6, 29.6, 43.0, 46.6, 53.2, 56.3, 61.6, 68.8, 70.8, 131.8, 133.0, 133.3, 133.6, 133.9, 134.8, 142.6, 143.7, 161.4, 174.1, 176.8, 177.5, 177.8.

Cbz-Ala-Leu-Phe-OCam (2)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.83 (dd, *J* = 6.3 and 13.8 Hz, 6H), 1.14 (d, *J* = 6.6 Hz, 3H), 1.36-1.59 (m, 3H), 2.93-3.18 (m, 2H), 3.99-4.02 (m, 1H), 4.09-4.43 (m, 3H), 4.48-4.62 (m, 1H), 5.00 (s, 2H), 7.17-7.19 (m, 13H), 7.83 (d, *J* = 7.2 Hz, 1H), 8.36 (d, *J* = 7.5 Hz, 1H) ; ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.5, 22.1, 23.3, 24.4, 36.6, 41.3, 50.3, 51.1, 53.6, 62.9, 65.7, 79.6, 126.6, 128.1, 128.1, 128.6, 128.7, 129.4, 137.4, 137.5, 156.0, 168.8, 171.0, 172.5, 172.7.

Cbz-Gly-Ile-Ala-OCam (3)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84 (dd, *J* = 8.4 Hz, 6H), 0.98-1.11 (m, 1H), 1.31-1.47 (m, 4H), 1.62-1.74 (m, 1H), 3.65 (d, *J* = 6.0 Hz, 2H), 4.21-4.42 (m, 4H), 5.03 (s, 2H), 7.30-7.45 (m, 8H), 7.80 (d, *J* = 9.0 Hz, 1H), 8.58 (d, *J* = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 11.3, 15.4, 17.02, 24.6, 37.4, 43.7, 56.6, 62.7, 65.8, 128.0, 128.1, 128.7, 137.5, 156.8, 168.9, 169.2, 171.6, 172.2.

Cbz-Ala-Pro-Leu-OCam (4)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, *J* = 6.3 and 16.8 Hz, 6H), 1.17 (d, *J* = 6.6 Hz, 3H), 1.56-2.07 (m, 7H), 3.35-3.64 (m, 2H), 4.23-4.41 (m, 5H), 5.00 (s, 2H), 7.27-7.50 (m, 8H), 8.24 (d, *J* = 7.2 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.2, 21.7, 23.4, 24.5, 24.8, 29.2, 47.0, 48.3, 50.8, 59.4, 62.7, 65.7, 128.1, 128.2, 128.7, 137.4, 156.0, 168.8, 171.2, 172.2, 172.3.

Ac-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Trt)-Met-OCam (5)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.85 (dd, *J* = 6.6 and 12.0 Hz, 6H), 1.11 (s, 9H), 1.17-2.03 (m, 29 H), 2.33 (t, *J* = 8.1 Hz, 2H), 2.87 (q, *J* = 6.6 Hz, 2H), 4.49 (d, *J* = 5.7 Hz, 2H), 4.16-4.50 (m, 7H), 6.74 (t, *J* = 5.1 Hz, 1H), 7.16-7.34 (m, 16H), 7.39 (s, 1H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.86-7.93 (m, 2H), 8.09 (d, *J* = 7.8 Hz, 1H), 8.27 (d, *J* = 7.5 Hz, 1H), 8.57 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 14.9, 22.0, 22.8, 23.4, 27.6, 28.6, 29.6, 51.4, 51.6, 52.8, 54.0, 61.7, 62.8, 69.6, 73.2, 126.7, 127.8, 128.9, 145.3, 168.7, 169.9, 171.4, 171.6, 171.7, 172.0, 172.8.

H-Ala-Leu-Phe-NH_2 (6)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, *J* = 6.3 and 9.9 Hz, 6H), 1.26 (d, *J* = 7.2 Hz, 3H), 1.35-1.63 (m, 3H), 2.78-3.03 (m, 2H), 3.77-3.84 (m, 1H), 4.27-4.35 (m, 1H), 4.41-4.48 (m, 1H), 7.07 (s, 1H), 7.14-7.27 (m, 5H), 7.40 (s, 1H), 7.93-7.96 (m, 3H), 8.41 (d, *J* = 8.1 Hz,

1H); ^{13}C NMR (DMSO-d_6, 75 MHz) : δ = 17.6, 22.0, 23.4, 24.4, 38.0, 41.3, 48.4, 51.7, 53.8, 126.6, 128.4, 129.5, 138.1, 169.7, 171.4, 172.9.

H-Leu-Phe-NH_2 (7)
CAS: 74214-38-3.

H-Gly-Phe-NH_2 (8)
CAS: 1510-04-7.

H-Gly-Leu-Met-NH_2 (9)
CAS: 40297-96-9.

H-Ser(^{t}Bu)-Leu-Leu-NH_2 (10)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84-0.92 (m, 12H), 1.14 (s, 9H), 1.35-1.66 (m, 6H), 3.49-3.65 (m, 2H), 3.90-3.93 (m, 1H), 4.21-4.29 (m, 1H), 4.40-4.48 (m, 1H), 6.97 (s, 1H), 7.32 (s, 1H), 8.02-8.09 (m, 4H), 8.52 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.5, 21.6, 22.9, 23.9, 24.1, 26.9, 50.6, 51.1, 52.6, 60.5, 73.3, 166.1, 170.9, 173.7.

H-Trp(Boc)-Met-Asp(O^{t}Bu)-Phe-NH_2 (11)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.36 (s, 9H), 1.69 (s, 9H), 1.70-3.03 (m, 5H), 2.64-3.22 (m, 5H), 4.08-4.15 (m, 1H), 4.36-4.47 (m, 2H), 4.55-4.63 (m, 1H), 7.14-7.36 (m, 9H), 7.64 (s, 1H), 7.78 (d, *J* = 7.5 Hz, 1H), 7.93 (d, *J* = 8.1 Hz, 1H), 8.04-8.11 (m, 5H), 8.40 (d, *J* = 7.8 Hz, 1H), 8.90 (d, *J* = 8.1 Hz, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 15.0, 22.9, 27.4, 28.0, 28.1, 29.8, 32.1, 37.5, 37.8, 50.0, 52.4, 52.9, 54.3, 80.7, 83.9, 115.0, 117.1, 119.8, 122.7, 124.3, 124.6, 126.6, 128.4, 129.4, 130.7, 135.0, 138.1, 149.4, 169.8, 169.9, 170.2, 171.2, 171.9, 172.8.

Cbz-Phe-Leu-Ala-Ala-Leu-Phe-NH_2 (12)
To a suspension of Alcalase-CLEA-OM (100 mg) and 3 Å molecular sieves (100 mg) in THF (9 mL) was added a solution of Cbz-Phe-Leu-Ala-OCam (**1**, 300 µmol, 162 mg) and H-Ala-Leu-Phe-NH_2 (**6**, 450 µmol, 157 mg, 1.5 equiv) in DMF (1 mL). The obtained reaction mixture was shaken at 37°C with 200 rpm for 16 h. Afterwards, the reaction mixture was filtrated and the solid enzyme particles washed with CH_2Cl_2 (10 mL, 2x) and DMF (5 mL, 2x). The combined organic phases were concentrated *in vacuo* till a volume of 10 mL and purified by preparative HPLC. The pure fractions were lyophilized yielding Cbz-Phe-Leu-Ala-Ala-Leu-Phe-NH_2 (**12**) as a white solid (176 mg, 73% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.77-0.95 (m, 12H), 1.20 (dd, *J* = 7.2 and 9.9 Hz, 6H), 1.30-1.67 (m, 6H), 2.68-2.85 (m, 2H), 2.98-3.04 (m, 2H), 4.13-4.44 (m, 6H), 4.94 (s, 2H), 7.09-7.32 (m, 17H), 7.48 (d, *J* = 8.4 Hz, 1H), 7.72 (d, *J* = 7.8 Hz, 1H), 7.83 (d, *J* = 7.8 Hz, 1H), 7.93 (d, *J* = 7.2 Hz, 1H), 8.05-8.09 (m, 2H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.7, 21.5, 22.8, 22.9, 23.9, 24.0, 37.4, 48.1, 51.0, 51.3, 53.3, 55.9, 65.1, 126.1, 127.3, 127.5, 127.9, 128.1, 129.0, 136.9, 137.6, 137.9, 155.7, 171.3, 171.4, 171.7, 171.9, 172.5; LC-MS: R_t = 22.07 min and *m/z* $[M + H]^+$ calcd for $C_{44}H_{60}N_7O_8^+$: 814; found: 814.

Cbz-Ala-Leu-Phe-Leu-Phe-NH_2 (13)
LC-MS: R_t = 22.05 min and *m/z* $[M + H]^+$ calcd for $C_{41}H_{55}N_6O_7^+$: 743; found: 743.

Cbz-Gly-Ile-Ala-Gly-Phe-NH_2 (14)
LC-MS: R_t = 21.36 min and *m/z* $[M + H]^+$ calcd for $C_{30}H_{41}N_6O_7^+$: 597; found: 597.

Cbz-Ala-Pro-Leu-Gly-Phe-NH_2 (15)
LC-MS: R_t = 20.98 min and *m/z* $[M + H]^+$ calcd for $C_{33}H45N_6O_7^+$: 637; found: 637.

Cbz-Ala-Pro-Leu-Gly-Leu-Met-NH_2 (16)
LC-MS: R_t = 23.02 min and *m/z* $[M + H]^+$ calcd for $C_{35}H_{56}N_7O_8S^+$: 734; found: 734.

Cbz-Gly-Ile-Ala-Ser(^{t}Bu)-Leu-Leu-NH_2 (17)
LC-MS: R_t = 22.54 min and *m/z* $[M + H]^+$ calcd for $C_{38}H_{64}N_7O_9^+$: 762; found: 763.

Cbz-Ala-Pro-Leu-Trp(Boc)-Met-Asp(O^{t}Bu)-Phe-NH_2 (18)
LC-MS: R_t = 23.68 min and *m/z* $[M + H]^+$ calcd for $C_{60}H_{83}N_9O_{13}S^+$: 1169; found: 1168.

Ac-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Trt)-Met-Leu-Phe-NH_2 (19)
LC-MS: R_t = 24.65 min and *m/z* $[M + H]^+$ calcd for $C_{70}H_{101}N_{10}O_{12}S^+$: 1306; found: 1306.

Cbz-Val-Ala-OCam (20)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.89 (dd, *J* = 4.8 and 6.6 Hz, 6H), 1.36 (dd, *J* = 3.9 Hz, 3H), 1.94-2.05 (m, 1H), 3.87-3.93 (m, 1H), 4.24-4.28 (m, 1H), 4.39-4.75 (m, 2H), 5.04 (s, 2H), 6.16 (d, *J* = 8.1 Hz, 1H), 6.39 (s, 1H), 6.85 (d, *J* = 3.3 Hz, 1H), 7.19-7.27 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.0, 18.5, 19.4, 31.1, 49.5, 60.9, 63.2, 67.5, 128.3, 128.6, 128.9, 136.6, 157.1, 170.3, 171.8, 173.4; LC-MS: R_t = 12.23 min and *m/z* $[M + H]^+$ calcd for $C_{18}H_{26}N_3O_6^+$: 380; found: 380.

H-Ala-Val-NH_2 (21)
LC-MS: R_t = 6.05 min and *m/z* $[M + H]^+$ calcd for $C_8H_{18}N_3O_2^+$: 188; found: 188.

Cbz-Val-Arg(Pbf)-OCam (22)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.87 (dd, *J* = 6.9 and 12.0 Hz, 6H), 1.12 (s, 6H), 1.41-2.01 (m, 13H), 2.44 (s, 3H), 2.97-3.06 (m, 4H), 3.88-3.93 (m, 1H), 4.19-4.48 (m, 4H), 5.05 (s, 2H), 6.17-6.95 (m, 3H), 7.33-7.37 (m, 8H), 8.41 (d, *J* = 6.9 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 12.6, 18.0, 18.6, 19.3, 19.4, 28.4, 30.7, 31.7, 42.8, 52.2, 60.3, 62.7, 65.8, 86.7, 116.6, 124.7, 128.0, 128.1, 128.7, 134.6, 137.6, 156.4, 157.8, 168.8, 171.6, 172.2; LC-MS: R_t = 22.35 min and *m/z* $[M + H]^+$ calcd for $C_{34}H_{49}N_6O_9S^+$: 717; found: 717.

H-Arg(Pbf)-Val-NH_2 (23)
LC-MS: R_t = 13.62 min and *m/z* $[M + H]^+$ calcd for $C_{24}H_{41}N_6O_5S^+$: 525; found: 525.

Cbz-Val-Asn(Trt)-OCam (24)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, J = 6.9 and 17.1 Hz, 6H), 1.98-2.05 (m, 1H), 2.86-3.10 (m, 2H), 4.01-4.05 (m, 1H), 4.27-4.54 (m, 2H), 4.70 (m, 2H), 4.97 (s, 2H), 4.53 (d, J = 8.4 Hz, 1H), 6.59 (s, 1H), 7.02-7.21 (m, 24H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.1, 19.6, 31.7, 38.9, 41.3, 49.5, 60.7, 63.6, 67.5, 71.5, 127.6, 128.5, 128.9, 129.0, 136.6, 144.3, 156.7, 169.9, 170.4, 170.5, 172.2; LC-MS: R_t = 21.10 min and m/z $[M + H]^+$ calcd for $C_{38}H_{41}N_4O_7^+$: 665; found: 665.

H-Asn(Trt)-Val-NH$_2$ (25)
LC-MS: R_t = 13.88 min and m/z $[M + H]^+$ calcd for $C_{28}H_{33}N_4O_3^+$: 473; found: 473.

Cbz-Val-Asp(O^{t}Bu)-OCam (26)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.89 (dd, J = 6.9 and 18.3 Hz, 6H), 1.37 (s, 9H), 2.05-2.16 (m, 1H), 2.69-2.98 (m, 2H), 3.95-4.00 (m, 1H), 4.57 (s, 2H), 4.70-4.78 (m, 1H), 5.04 (s, 2H), 5.60 (s, 1H), 6.91 (d, J = 7.2 Hz, 1H), 7.19-7.28 (m, 7H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.0, 19.5, 28.4, 31.3, 37.3, 49.5, 63.7, 67.7, 83.3, 128.4, 128.7, 129.0, 136.4, 169.9, 170.1, 171.4, 172.1; LC-MS: R_t = 14.77 min and m/z $[M + H]^+$ calcd for $C_{23}H_{34}N_3O_8^+$: 480; found: 480.

H-Asp(O^{t}Bu)-Val-NH$_2$ (27)
LC-MS: R_t = 8.90 min and m/z $[M + H]^+$ calcd for $C_{13}H_{26}N_3O_4^+$: 288; found: 288.

Cbz-Val-Cys(Trt)-OCam (28)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.89 (dd, J = 6.6 and 15.6 Hz, 6H), 1.89-2.01 (m, 1H), 2.59-2.76 (m, 2H), 3.06-3.09 (m, 1H), 3.77-3.97 (m, 2H), 4.39-4.55 (m, 2H), 5.01 (s, 2H), 5.48 (d, J = 8.4 Hz, 1H), 5.77 (s, 1H), 6.28 (d, J = 4.4 Hz, 1H), 6.79 (s, 1H), 7.16-7.35 (m, 20H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 16.02, 17.5, 29.4, 30.8, 41.6, 50.7, 58.4, 61.4, 62.2, 65.5, 66.0, 125.5, 126.3, 126.4, 126.6, 126.9, 127.4, 127.8, 138.5, 142.3, 154.8, 167.5, 167.9, 170.8; LC-MS: R_t = 24.97 min and m/z $[M + H]^+$ calcd for $C_{37}H_{40}N_3O_6S^+$: 654; found: 654.

H-Cys(Trt)-Val-NH$_2$ (29)
LC-MS: R_t = 18.21 min and m/z $[M + H]^+$ calcd for $C_{27}H_{32}N_3O_2S^+$: 462; found: 462.

Cbz-Val-Gln(Trt)-OCam (30)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.87 (dd, J = 6.9 and 9.0 Hz, 6H), 1.75-1.98 (m, 3H), 3.18-3.65 (m, 2H), 3.89- 3.94 (m, 1H), 4.27-4.43 (m, 3H), 4.94-5.05 (m, 2H), 7.17-7.34 (m, 23H), 8.43 (d, J = 6.6 Hz, 1H), 8.57 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.6, 19.5, 27.2, 30.7, 30.8, 32.6, 52.1, 60.3, 62.7, 65.7, 69.6, 126.7, 127.8, 128.0, 128.1, 128.7, 128.9, 137.4, 145.3, 156.5, 168.9, 171.5, 171.6, 172.2; LC-MS: R_t = 21.64 min and m/z $[M + H]^+$ calcd for $C_{39}H_{43}N_4O_7S^+$: 679; found: 679.

H-Gln(Trt)-Val-NH$_2$ (31)
LC-MS: R_t = 13.3 min and m/z $[M + H]^+$ calcd for $C_{29}H_{35}N_4O_3^+$: 487; found: 487.

Cbz-Val-Glu(O^{t}Bu)-OCam (32)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.96 (dd, *J* = 6.9 and 10.5 Hz, 6H), 1.46 (s, 9H), 2.05-2.19 (m, 3H), 2.34-2.53 (m, 2H), 4.01- 4.06 (m, 1H), 4.32-4.38 (m, 1H), 4.52-4.76 (m, 2H), 5.12 (s, 2H), 5.74 (d, *J* = 8.4 Hz, 1H), 5.98 (s, 1H), 7.12 (s, 1H), 7.27-7.37 (m, 6H), 7.64 (d, *J* = 4.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.5, 19.4, 25.6, 28.4, 31.4, 31.8, 53.7, 60.8, 63.2, 67.4, 82.1, 128.3, 128.5, 128.9, 136.7, 157.0, 170.5, 170.9, 173.4, 173.6; LC-MS: R_t = 14.87 min and *m/z* $[M + H]^+$ calcd for $C_{24}H_{36}N_3O_8^+$: 494; found: 494.

H-Glu(O^{t}Bu)-Val-NH$_2$ (33)
LC-MS: R_t = 8.93 min and *m/z* $[M + H]^+$ calcd for $C_{14}H_{28}N_3O_4^+$: 302; found: 302.

Cbz-Val-Gly-OCam (34)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.89 (dd, *J* = 8.7 Hz, 6H), 1.93-2.04 (m, 1H), 3.88-4.07 (m, 3H), 4.44 (s, 2H), 5.04 (s, 2H), 7.31-7.42 (m, 8H), 8.41 (m, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.5, 19.5, 30.6, 60.5, 62.6, 65.8, 128.0, 128.1, 128.7, 137.4, 156.5, 169.0, 169.6, 172.4; LC-MS: R_t = 9.56 min and *m/z* $[M + H]^+$ calcd for $C_{17}H_{24}N_3O_6^+$: 366; found: 366.

H-Gly-Val-NH$_2$ (35)
LC-MS: R_t = 5.25 min and *m/z* $[M + H]^+$ calcd for $C_7H_{16}N_3O_2^+$: 174; found: 174.

Cbz-Val-His(Trt)-OCam (36)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.83 (dd, *J* = 6.9 and 28.5 Hz, 6H), 1.92-2.11 (m, 1H), 2.90-3.16 (m, 2H), 3.93-3.97 (m, 1H), 4.30-4.35 (m, 1H), 4.64-4.72 (m, 2H), 4.89-5.01 (m, 2H), 5.29 (d, *J* = 8.4 Hz, 1H), 5.46 (s, 1H), 6.58 (s, 1H), 6.99-7.48 (m, 22H), 8.13 (s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.9, 19.4, 29.3, 31.5, 53.2, 60.5, 63.5, 67.4, 76.1, 120.7, 128.3, 128.6, 128.7, 128.9, 130.0, 135.5, 136.6, 139.0, 142.3, 156.6, 170.5, 170.6, 172.2; LC-MS: R_t = 14.24 min and *m/z* $[M + H]^+$ calcd for $C_{40}H_{42}N_5O_6^+$: 688; found: 288.

H-His(Trt)-Val-NH$_2$ (37)
LC-MS: R_t = 15.42 min and *m/z* $[M + H]^+$ calcd for $C_{30}H_{34}N_5O_2^+$: 496; found: 496.

Cbz-Val-Ile-OCam (38)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.83-0.90 (m, 12H), 1.09-1.23 (m, 1H), 1.36-1.51 (m, 1H), 1.63 (s, 1H), 1.72-1.88 (m, 1H), 1.96-2.07 (m, 1H), 3.89-3.94 (m, 1H), 4.16-4.21 (m, 1H), 4.34-4.79 (m, 2H), 5.04 (s, 2H), 5.84 (d, *J* = 8.1 Hz, 1H), 6.07 (s, 1H), 5.59 (d, *J* = 3.3 Hz, 1H), 6.94 (s, 1H), 6.99-7.48 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 11.6, 15.8, 18.5, 19.4, 25.9, 31.0, 36.7, 58.2, 60.9, 62.9, 67.5, 128.3, 128.6, 128.9, 136.6, 157.0, 170.3, 170.9, 173.5; LC-MS: R_t = 15.67 min and *m/z* $[M + H]^+$ calcd for $C_{21}H_{32}N_3O_6^+$: 422; found: 422.

H-Ile-Val-NH$_2$ (39)
LC-MS: R_t = 9.72 min and *m/z* $[M + H]^+$ calcd for $C_{11}H_{24}N_3O_2^+$: 230; found: 230.

Cbz-Val-Leu-OCam (40)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.88 (dd, *J* = 3.3 and 6.9 Hz, 6H), 1.50-1.71 (m, 2H), 1.93-2.05 (m, 1H), 3.89-3.93 (m, 1H), 4.24-4.36 (m, 2H), 4.76-4.82 (m, 1H), 5.04 (s, 2H), 6.13 (d, *J* = 7.8 Hz, 1H), 6.37 (s, 1H), 6.74 (d, *J* = 3.3 Hz, 1H), 6.99 (s, 1H), 7.19-7.28 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.5, 19.4, 22.3, 22.9, 25.3, 31.0, 40.2, 52.5, 63.0, 67.5, 128.3, 128.6, 128.9, 136.6, 157.0, 170.3, 170.9, 173.5; LC-MS: R_t = 15.53 min and *m/z* $[M + H]^+$ calcd for $C_{21}H_{32}N_3O_6^+$: 422; found: 422.

H-Leu-Val-NH_2 (41)
LC-MS: R_t = 9.78 min and *m/z* $[M + H]^+$ calcd for $C_{11}H_{24}N_3O_2^+$: 230; found: 230.

Cbz-Val-Lys(Boc)-OCam (42)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.88 (dd, *J* = 6.9 and 12.6 Hz, 6H), 1.30-1.41 (m, 13H), 1.70-1.82 (m, 2H), 2.09-2.20 (m, 1H), 2.95-3.07 (m, 2H), 3.94-4.07 (m, 1H), 4.08-4.17 (m, 1H), 4.30-4.71 (m, 2H), 5.03 (s, 2H), 5.75 (d, *J* = 7.5 Hz, 1H), 5.96 (s, 1H), 7.00 (m, 1H), 7.19-7.27 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.3, 19.5, 22.9, 28.8, 30.1, 31.5, 53.8, 63.1, 67.5, 79.9, 128.4, 128.6, 128.9, 136.6, 156.9, 170.2, 171.5, 173.4; LC-MS: R_t = 15.21 min and *m/z* $[M + H]^+$ calcd for $C_{26}H_{41}N_4O_8^+$: 537; found: 537.

H-Lys(Boc)-Val-NH_2 (43)
LC-MS: R_t = 9.90 min and *m/z* $[M + H]^+$ calcd for $C_{16}H_{33}N_4O_4^+$: 345; found: 345.

Cbz-Val-Met-OCam (44)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.87 (dd, *J* = 6.0 Hz, 6H), 1.93-2.15 (m, 6H), 2.02-2.57 (m, 2H), 3.91-3.97 (m, 1H), 4.40-4.79 (m, 3H), 5.03 (s, 2H), 5.89 (d, *J* = 4.5 Hz, 1H), 6.23 (s, 1H), 6.93 (s, 1H), 7.19-7.29 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.8, 18.5, 19.5, 27.4, 30.0, 31.0, 32.3, 63.3, 67.6, 128.3, 128.6, 129.0, 136.5, 157.1, 170.2, 173.0, 173.4; LC-MS: R_t = 10.87 min and *m/z* $[M + H]^+$ calcd for $C_{20}H_{30}N_3O_6S^+$: 440; found: 440.

H-Met-Val-NH_2 (45)
LC-MS: R_t = 6.34 min and *m/z* $[M + H]^+$ calcd for $C_{10}H_{22}N_3O_2S^+$: 248; found: 248.

Cbz-Val-Phe-OCam (46)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.81 (dd, *J* = 2.1 and 6.6 Hz, 6H), 1.85-2.00 (m, 1H), 2.92-3.19 (m, 2H), 3.85-3.91 (m, 1H), 4.34-4.49 (m, 2H), 4.57-4.65 (m, 1H), 5.04 (s, 2H), 7.17-7.37 (m, 13H), 8.43 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.5, 19.4, 30.7, 36.7, 53.7, 60.4, 62.8, 65.8, 126.9, 127.5, 128.0, 128.1, 128.6, 128.7, 129.5, 137.4, 156.4, 168.8, 171.2, 172.0; LC-MS: R_t = 15.59 min and *m/z* $[M + H]^+$ calcd for $C_{24}H_{30}N_3O_6^+$: 456; found: 456.

H-Phe-Val-NH_2 (47)
LC-MS: R_t = 9.68 min and *m/z* $[M + H]^+$ calcd for $C_{14}H_{22}N_3O_2^+$: 264; found: 264.

Cbz-Val-Pro-OCam (48)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.91 (dd, *J* = 6.9 Hz, 6H), 1.89-2.29 (m, 5H), 3.59-3.68 (m, 1H), 3.99-4.06 (m, 1H), 4.13-4.20 (m, 1H), 4.32-4.84 (m, 3H), 5.03 (s, 2H), 6.84-6.91 (m, 2H), 7.16-7.28 (m, 7H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.9, 19.3, 25.8, 29.6, 31.3, 48.3, 58.7, 60.0, 62.8, 67.1, 128.1, 128.3, 128.8, 137.0, 157.3, 170.9, 171.7, 173.4; LC-MS: R_t = 11.63 min and *m/z* $[M + H]^+$ calcd for $C_{20}H_{28}N_3O_6^+$: 406; found: 406.

H-Pro-Val-NH_2 (49)
LC-MS: R_t = 7.47 min and *m/z* $[M + H]^+$ calcd for $C_{10}H_{20}N_3O_2^+$: 214; found: 214.

Cbz-Val-Ser(^{t}Bu)-OCam (50)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.90 (dd, *J* = 6.9 and 12.9 Hz, 6H), 2.03-2.20 (m, 2H), 3.56-3.62 (m, 1H), 3.88-3.93 (m, 1H), 4.36-4.42 (m, 1H), 4.42-4.74 (m, 3H), 5.04 (s, 2H), 5.50 (s, 1H), 5.96 (s, 1H), 6.81-6.92 (m, 2H), 7.20-7.27 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.2, 19.5, 27.7, 31.4, 53.7, 60.7, 62.1, 63.3, 67.5, 74.9, 128.4, 128.6, 128.9, 136.6, 156.8, 169.6, 170.3, 172.4; LC-MS: R_t = 12.86 min and *m/z* $[M + H]^+$ calcd for $C_{22}H_{34}N_3O_7^+$: 452 found: 452.

H-Ser(^{t}Bu)-Val-NH_2 (51)
LC-MS: R_t = 10.02 min and *m/z* $[M + H]^+$ calcd for $C_{12}H_{26}N_3O_3^+$: 260; found: 260.

Cbz-Val-Thr(^{t}Bu)-OCam (52)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.90 (dd, *J* = 6.9 and 13.2 Hz, 6H), 0.94-1.20 (m, 11H), 2.04-2.15 (m, 1H), 3.99-4.15 (m, 2H), 4.31-4.87 (m, 3H), 5.05 (s, 2H), 5.34 (d, *J* = 8.1 Hz, 1H), 5.63 (s, 1H), 6.69 (d, *J* = 6.6 Hz, 1H), 7.19-7.28 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.1, 19.5, 20.1, 28.6, 31.3, 58.5, 60.8, 63.1, 67.3, 67.6, 75.7, 128.5, 128.7, 129.0, 136.5, 156.8, 169.9, 170.0, 172.5; LC-MS: R_t = 13.94 min and *m/z* $[M + H]^+$ calcd for $C_{23}H_{36}N_3O_7^+$: 466 found: 466.

H-Thr(^{t}Bu)-Val-NH_2 (53)
LC-MS: R_t = 8.44 min and *m/z* $[M + H]^+$ calcd for $C_{13}H_{28}N_3O_3^+$: 274; found: 274.

Cbz-Val-Trp(Boc)-OCam (54)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.79 (dd, *J* = 6.9 Hz, 6H), 1.60 (s, 9H), 1.81-1.97 (m, 1H), 3.04-3.37 (m, 2H), 3.85-3.90 (m, 1H), 4.36-4.48 (m, 2H), 4.67-4.87 (m, 1H), 4.94-5.06 (m, 2H), 7.19-7.62 (m, 12H), 8.05 (d, *J* = 8.1 Hz, 1H), 8.46 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.5, 19.4, 26.6, 28.1, 30.8, 52.1, 60.4, 62.9, 65.8, 83.9, 115.1, 116.2, 119.4, 122.9, 124.6, 124.8, 128.0, 128.7, 130.3, 135.1, 137.4, 149.4, 156.4, 168.8, 171.2, 172.0; LC-MS: R_t = 22.33 min and *m/z* $[M + H]^+$ calcd for $C_{31}H_{39}N_4O_8^+$: 595 found: 595.

H-Trp(Boc)-Val-NH_2 (55)
LC-MS: R_t = 13.22 min and *m/z* $[M + H]^+$ calcd for $C_{21}H_{31}N_4O_4^+$: 403; found: 403.

Cbz-Val-Tyr(^{t}Bu)-OCam (56)
^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.81 (dd, *J* = 6.9 and 21.0 Hz, 6H), 1.25 (s, 9H), 1.97-2.10 (m, 1H), 2.93-3.07 (m, 2H), 3.85-3.90 (m, 1H), 4.31-4.60 (m, 3H), 5.02 (s, 2H), 5.26 (d, *J* = 6.3 Hz, 1H), 5.50 (s, 1H), 6.39 (d, *J* = 5.1 Hz, 1H), 6.63 (s, 1H), 6.85-7.00 (m, 4H), 7.19-7.28 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.3, 19.4, 29.2, 31.0, 36.8, 54.9, 63.1, 67.6, 79.0, 124.9, 128.4, 128.7, 129.0, 129.8, 130.2, 136.5, 155.3, 156.9, 170.2, 170.9, 173.0; LC-MS: R_t = 20.49 min and *m/z* $[M + H]^+$ calcd for $C_{28}H_{38}N_3O_7^+$: 528 found: 528.

H-Tyr(^{t}Bu)-Val-NH$_2$ (57)
LC-MS: R_t = 12.96 min and *m/z* $[M + H]^+$ calcd for $C_{18}H_{30}N_3O_3^+$: 336; found: 336.

Cbz-Val-Val-OCam (58)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.85-0.95 (m, 12H), 1.90-2.20 (m, 2H), 3.95-4.00 (m, 1H), 4.27-4.50 (m, 3H), 5.04 (s, 2H), 7.28-7.43 (m, 8H), 8.19 (d, *J* = 4.8 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.4, 18.7, 19.2, 19.5, 30.1, 30.6, 57.7, 60.3, 62.7, 65.7, 128.0, 128.1, 128.7, 156.4, 168.8, 171.1, 172.3; LC-MS: R_t = 13.75 min and *m/z* $[M + H]^+$ calcd for $C_{20}H_{30}N_3O_6^+$: 408 found: 408.

H-Val-Val-NH$_2$ (59)
LC-MS: R_t = 8.78 min and *m/z* $[M + H]^+$ calcd for $C_{10}H_{22}N_3O_2^+$: 216; found: 216.

Cbz-Ala-Gln(Trt)-OCam (60)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.13 (d, *J* = 6.6 Hz, 3H), 1.71-1.89 (m, 2H), 2.66 (s, 1H), 2.82 (s, 1H), 3.85-4.03 (m, 1H), 4.12-4.36 (m, 3H), 4.84-4.96 (m, 2H), 7.11-7.31 (m, 25H), 8.28 (d, *J* = 6.6 Hz, 1H), 8.48 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.4, 27.2, 32.6, 50.2, 52.0, 62.7, 65.7, 69.6, 126.7, 127.8, 128.1, 128.7, 128.9, 137.4, 145.3, 156.0, 168.9, 171.5, 171.6, 173.5.

Cbz-Ala-Asn(Trt)-OCam (61)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.24 (d, *J* = 6.3 Hz, 3H), 2.76-2.92 (m, 2H), 4.12-4.17 (m, 1H), 4.3 (s, 2H), 4.60-4.68 (m, 1H), 5.03 (s, 2H), 7.17-7.47 (m, 23H), 8.35 (d, *J* = 7.2 Hz, 1H), 8.78 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 18.6, 38.0, 49.4, 50.3, 63.0, 65.8, 69.9, 126.7, 127.9, 128.1, 128.7, 128.9, 137.4, 145.0, 156.0, 168.9, 169.2, 171.1, 173.2.

H-Arg(Pbf)-Ala-NH$_2$ (62)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.25 (d, *J* = 6.6 Hz, 3H), 1.42-1.66 (m, 10H), 2.02 (s, 3H), 2.44 (s, 3H), 2.78-3.06 (m, 4H), 3.68-3.75 (m, 1H), 4.27-4.32 (m, 1H), 6.49-7.06 (m, 3H), 7.46 (s, 1H), 7.70 (s, 2H), 8.52 (d, *J* = 6.9 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) : δ = 12.6, 18.0, 18.8, 19.3, 24.9, 25.8, 28.7, 29.3, 42.9, 48.5, 52.5, 86.7, 116.7, 124.7, 131.8, 134.6, 137.7, 156.5, 157.8, 158.2, 158.7, 169.0, 174.0.

H-Lys(Boc)-Ala-NH$_2$ (63)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.25-1.39 (m, 15H), 1.40-1.71 (m, 2H), 1.89-2.22 (m, 1H), 2.85-2.97 (m, 2H), 3.63-3.78 (m, 1H), 4.26-4.31 (m, 1H), 6.76 (s, 1H), 7.04 (s, 1H),

7.44-7.60 (m, 3H), 8.49 (d, *J* = 7.2 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.6, 18.8, 22.0, 28.6, 29.4, 30.5, 31.7, 48.4, 48.9, 52.7, 77.8, 155.9, 169.2, 174.0.

Cbz-Ala-Gln(Trt)-Arg(Pbf)-Ala-NH$_2$ (64)

To a suspension of Alcalase-CLEA-OM (100 mg) and 3 Å molecular sieves (100 mg) in THF (9 mL), was added a solution of Cbz-Ala-Gln(Trt)-OCam (**60**, 300 µmol, 195 mg) and H-Arg(Pbf)-Ala-NH$_2$ (**62**, 450 µmol, 223 mg, 1.5 equiv) in DMF (1 mL). The obtained reaction mixture was shaken at 37°C with 200 rpm for 3 h. Afterwards, the reaction mixture was filtrated and the solid enzyme particles and molecular sieves washed with CH_2Cl_2 (10 mL, 2x) and DMF (5 mL, 2x). The combined organic phases were concentrated *in vacuo* till a volume of 10 mL followed by preparative HPLC purification. The pure fractions were pooled and lyophilized yielding Cbz-Ala-Gln(Trt)-Arg(Pbf)-Ala-NH$_2$ (**64**) as a white solid (221 mg, 69% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.23-1.25 (m, 6H), 1.46-2.06 (m, 16H), 3.01-3.07 (m, 4H), 4.10-4.31 (m, 4H), 4.97-5.09 (m, 2H), 7.00 (s, 1H), 7.21-7.54 (m, 24H), 7.87-8.12 (m, 3H), 8.58 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 12.1, 17.5, 18.8, 28.2, 47.9, 65.3, 69.1, 86.2, 124.2, 126.2, 127.3, 127.6, 128.2, 128.4, 131.3, 144.8, 155.9, 170.6, 171.3, 173.9.

Cbz-Ala-Asn(Trt)-Lys(Boc)-Ala-NH$_2$ (65)

To a suspension of Alcalase-CLEA-OM (100 mg) and 3 Å molecular sieves (100 mg) in THF (9 mL), was added a solution of Cbz-Ala-Asn(Trt)-OCam (**61**, 300 µmol, 191 mg) and H-Lys(Boc)-Ala-NH$_2$ (**63**, 450 µmol, 142 mg, 1.5 equiv) in DMF (1 mL). The obtained reaction mixture was shaken at 37°C with 200 rpm for 16 h. Afterwards, the reaction mixture was filtrated and the solid enzyme particles and molecular sieves washed with CH_2Cl_2 (10 mL, 2x) and DMF (5 mL, 2x). The combined organic phases were concentrated *in vacuo* till a volume of 10 mL followed by preparative HPLC purification. The pure fractions were pooled and lyophilized yielding Cbz-Ala-Asn(Trt)-Lys(Boc)-Ala-NH$_2$ (**65**) as a white solid (167 mg, 63% yield). ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.06 (d, *J* = 7.2 Hz, 3H), 1.21-1.69 (m, 18H), 2.69 (d, *J* = 6.6 Hz, 2H), 2.84 (q, *J* = 6.3 Hz, 2H), 4.02-4.15 (m, 3H), 4.50-4.58 (m, 1H), 5.01 (d, *J* = 7.8 Hz, 2H), 6.73 (t, *J* = 6.3 Hz, 1H), 6.90 (s, 1H), 7.16 (s, 1H), 7.19-7.45 (m, 22H), 7.76-7.84 (m, 2H), 8.23 (d, *J* = 7.5 Hz, 1H), 8.69 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.6, 28.1, 48.0, 50.0, 52.8, 65.3, 69.3, 77.2, 126.2, 127.3, 127.6, 128.2, 128.4, 136.8, 144.6, 169.0, 170.0, 171.0, 172.4, 173.5.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Trt)-OCam (66)

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84 (dd, *J* = 6.3 and 10.8 Hz, 6H), 1.09-2.04 (m, 43H), 2.41-2.79 (m, 4H), 2.91 (q, *J* = 6.6 Hz, 2H), 3.45 (d, *J* = 7.5 Hz, 2H), 4.28-4.54 (m, 6H), 4.61-4.69 (m, 1H), 6.77 (t, *J* = 7.8 Hz, 1H), 7.20-7-39 (m, 17H), 7.75 (d, *J* = 8.1 Hz, 2H), 7.88 (d, *J* = 7.5 Hz, 2H), 8.00 (d, *J* = 8.1 Hz, 1H), 8.20 (d, *J* = 8.1 Hz, 1H), 8.39 (d, *J* = 6.9 Hz, 1H), 8.62 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.5, 22.3, 22.9, 24.0, 27.1, 27.6, 28.2, 32.2, 37.2, 49.3, 51.1, 51.6, 52.0, 53.2, 61.3, 62.2, 69.1, 72.8, 77.2, 80.0, 126.2, 127.4, 128.4, 144.8, 155.4, 168,4, 169.0, 169.2, 170.5, 170.9, 171.0, 171.6, 171.7; LC-MS: R_t = 24.36 min and *m/z* $[M + H]^+$ calcd for $C_{60}H_{87}N_8O_{14}^+$: 1144; found: 1144.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Xan)-OCam (67)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.85 (dd, J = 6.6 and 12.6 Hz, 6H), 1.07-2.14 (m, 46H), 2.20-2.44 (m, 2H), 2.86 (q, J = 6.0 Hz, 2H), 3.46 (d, J = 5.4 Hz, 2H), 4.06 (s, 2H), 4.28-4.57 (m, 6H), 4.60-4.65 (m, 1H), 6.75-7.06 (m, 2H), 7.06-7.36 (m, 12H), 7.72 (d, J = 7.8 Hz, 1H), 7.85 (d, J = 6.6 Hz, 1H), 7.97 (d, J = 8.1 Hz, 1H), 8.18 (d, J = 8.1 Hz, 1H), 8.36 (d, J = 6.6 Hz, 2H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 22.0, 22.8, 23.4, 24.5, 27.3, 27.5, 28.0, 28.6, 31.5, 49.8, 52.1, 52.5, 61.8, 62.7, 73.3, 80.5, 116.4, 121.0, 123.6, 128.1, 129.5, 151.7, 155.9, 168.8, 169.7, 171.0, 171.4, 127.1, 172.3, 173.7; LC-MS: R_t = 22.96 min and m/z $[M + H]^+$ calcd for $C_{54}H_{81}N_8O_{15}^+$: 1082; found: 1081.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Tmob)-OCam (68)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.83 (dd, J = 6.3 and 11.4 Hz, 6H), 1.09-1.69 (m, 36H), 1.83-2.03 (m, 5H), 2.28 (t, J = 6.6 Hz, 2H), 2.35-2.66 (m, 2H), 2.86 (q, J = 6.6 Hz, 2H), 3.45 (d, J = 7.2 Hz, 2H), 3.74-3.76 (m, 9H), 4.13-4.46 (m, 8H), 4.53-4.57 (m, 1H), 6.61 (s, 2H), 6.71 (t, J = 4.5 Hz, 1H), 7.24-7.41 (m, 3H), 7.68 (d, J = 8.1 Hz, 1H), 7.83 (d, J = 7.5 Hz, 1H), 7.93 (d, J = 7.8 Hz, 1H), 8.15 (d, J = 8.1 Hz, 1H), 8.34 (d, J = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.5, 22.3, 22.9, 23.4, 27.0, 27.5, 28.1, 31.2, 36.5, 50.3, 55.1, 55.6, 72.7, 80.0, 90.6, 159.0, 160.5, 168.4, 169.1, 169.2, 170.4, 170.6, 171.0, 171.6; LC-MS: R_t = 21.98 min and m/z $[M + H]^+$ calcd for $C_{51}H_{85}N_8O_{17}^+$: 1082; found: 1082.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln-OCam (69)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.84 (dd, J = 6.3 and 11.4 Hz, 6H), 1.03-2.06 (m, 43H), 2.16 (t, J = 7.5 Hz, 2H), 2.85 (q, J = 6.6 Hz, 2H), 3.45 (d, J = 5.4 Hz, 2H), 4.22-4.48 (m, 6H), 4.55-4.63 (m, 1H), 6.70 (t, J = 5.1 Hz, 1H), 6.78 (s, 1H), 7.22 (s, 1H), 7.32 (d, J = 10.8 Hz, 2H), 7.69 (d, J = 8.1 Hz, 1H), 7.83 (d, J = 7.8 Hz, 1H), 7.95 (d, J = 8.1 Hz, 1H), 8.15 (d, J = 8.1 Hz, 1H), 8.34 (d, J = 7.2 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.5, 22.2, 22.3, 22.9, 26.5, 27.0, 27.6, 28.2, 29.1, 31.0, 37.2, 49.3, 51.2, 51.6, 52.0, 53.3, 61.3, 62.2, 72.8, 77.2, 80.0, 168.4, 169.1, 169.2, 170.5, 171.0, 171.6, 171.8, 176.2; LC-MS: R_t = 18.28 min and m/z $[M + H]^+$ calcd for $C_{41}H_{73}N_8O_{14}^+$: 902; found: 901.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Trt)-Leu-Phe-NH$_2$ (70)
LC-MS: R_t = 25.26 min and m/z $[M + H]^+$ calcd for $C_{73}H_{105}N_{10}O_{14}^+$: 1346; found: 1346.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Xan)-Leu-Phe-NH$_2$ (71)
LC-MS: R_t = 24.58 min and m/z $[M + H]^+$ calcd for $C_{67}H_{99}N_{10}O_{15}^+$: 1284; found: 1284.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln(Tmob)-Leu-Phe-NH$_2$ (72)
LC-MS: R_t = 23.74 min and m/z $[M + H]^+$ calcd for $C_{64}H_{103}N_{10}O_{17}^+$: 1284; found: 1284.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln-Leu-Phe-NH$_2$ (73)
LC-MS: R_t = 20.06 min and m/z $[M + H]^+$ calcd for $C_{54}H_{91}N_{10}O_{14}^+$: 1104; found: 1104.

Boc-Leu-Asp(O^{t}Bu)-Gln(Trt)-Ser(^{t}Bu)-Gln-OCam (74)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.86 (dd, J = 6.6 and 11.4 Hz, 6H), 1.25-2.21 (m, 18H), 2.52-3.11 (m, 5H), 4.15-5.65 (m, 6H), 6.76 (s, 1H), 7.11-7.36 (m, 16H), 7.73 (d, J = 7.8 Hz,

1H), 7.89-7.98 (m, 2H), 8.06 (d, *J* = 7.5 Hz, 1H), 8.33 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.9, 22.8, 23.3, 28.4, 31.7, 35.9, 51.5, 54.3, 126.6, 126.7, 128.4, 129.4, 129.5, 138.2, 170.0, 170.7, 171.5, 172.3, 173.0, 173.3, 174.2.

Boc-His(Trt)-Lys(Boc)-Thr(^{t}Bu)-Asp(O^{t}Bu)-Ser-OCam (75)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.02 (d, *J* = 6.3 Hz, 3H), 1.25-1.84 (m, 17H), 2.53-3.22 (m, 10H), 4.00-4.05 (m, 1H), 4.26-4.66 (m, 6H), 5.00-5.12 (m, 1H), 7.16-7.44 (m, 16H), 7.65-7.78 (m, 3H), 7.90-7.97 (m, 2H), 8.15-8.20 (m, 3H), 8.94 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 27.1, 28.0, 28.6, 32.7, 33.3, 33.8, 57.3, 58.1, 67.9, 74.8, 78.4, 85.9, 131.9, 132.0, 133.0, 134.0, 134.1, 134.6, 150.5, 174.0, 174.8, 175.5, 176.0, 176.5, 177.1, 178.1, 179.1.

H-Phe-Val-Gly-Ser(^{t}Bu)-Arg(Pbf)-NH$_2$ (76)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.87 (t, *J* = 6.6 Hz, 6H), 1.14 (s, 9H), 1.40-2.07 (m, 16H), 2.48-2.71 (m, 8H), 3.02-3.08 (m, 5H), 3.39-3.60 (m, 3H), 3.81 (d, *J* = 5.7 Hz, 2H), 4.14-4.25 (m, 2H), 4.35-4.44 (m, 1H), 6.44-6.75 (m, 2H), 7.08-7.36 (m, 8H), 7.97-8.09 (m, 3H), 8.38 (t, *J* = 8.7 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 12.6, 18.0, 18.3, 19.3, 19.5, 27.4, 28.7, 31.2, 42.8, 52.6, 53.7, 56.4, 57.5, 62.2, 73.5, 86.7, 116.6, 124.7, 126.5, 128.5, 129.7, 131.8, 137.6, 139.0, 156.4, 157.8, 169.3, 170.0, 171.7, 173.6, 174.5.

H-Phe-Val-Gly-Leu-Met-NH$_2$ (77)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.83-0.91 (m, 12H), 1.42-2.03 (m, 9H), 2.38-2.44 (m, 2H), 2.78-3.11 (m, 2H), 3.67-3.90 (m, 2H), 4.14-4.36 (m, 4H), 7.03 (s, 1H), 7.21-7.33 (m, 6H), 7.99-8.25 (m, 5H), 8.53 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 14.5, 18.1, 19.0, 21.5, 23.0, 24.0, 29.5, 30.7, 31.6, 36.9, 51.0, 51.6, 53.0, 57.7, 127.0, 128.4, 129.4, 134.6, 167.7, 168.3, 170.4, 171.8, 172.8.

H-Leu-Asp-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH$_2$ (78)
To a suspension of Alcalase-CLEA-OM (100 mg) and 3 Å molecular sieves (100 mg) MTBE (9 mL), was added a solution of Cam ester (**74**, 0.3 mmol) and nucleophile (**76**, 0.45 mmol, 1.5 equiv) in DMF (1 mL). The obtained reaction mixture was shaken at 37°C with 200 rpm for 16 h. Afterwards, the reaction mixture was filtrated and the solid enzyme particles and molecular sieves were washed with CH_2Cl_2 (100 mL, 5x) and DMF (5 mL, 2x). The combined organic phases were concentrated *in vacuo* and the residue dissolved in TFA/TIS/H_2O (10 mL, 95/2.5/2.5, v/v/v) and stirred at ambient temperature for 60 min. The reaction mixture was added to MTBE/*n*-heptane (100 mL, 1/1, v/v) and the precipitates were collected by centrifugation (4000 rpm, 10 min). The residue was purified by preparative HPLC, the pure fractions were pooled and lyophilized yielding H-Leu-Asp-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH$_2$ (**78**) as a white solid (214 mg, 63% yield). LC-MS: R_t = 13.67 min and *m/z* $[M + 2H]^{2+}$ calcd for $C_{48}H_{80}N_{16}O_{16}^{2+}$: 568; found: 567.

H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH$_2$ (79)
To a suspension of Alcalase-CLEA-OM (100 mg) and 3 Å molecular sieves (100 mg) in MTBE (9 mL), was added a solution of Cam ester (**75**, 0.3 mmol) and nucleophile (**77**, 0.45 mmol, 1.5 equiv) in DMF (1 mL). The obtained reaction mixture was shaken at 37°C with 200 rpm for 16 h. Afterwards, the reaction mixture was filtrated and the solid enzyme

particles and molecular sieves were washed with CH_2Cl_2 (100 mL, 5x) and DMF (5 mL, 2x). The combined organic phases were concentrated *in vacuo* and the residue dissolved in TFA/TIS/H_2O (10 mL, 95/2.5/2.5, v/v/v) and stirred at ambient temperature for 60 min. The reaction mixture was added to MTBE/*n*-heptane (100 mL, 1/1, v/v) and the precipitates were collected by centrifugation (4000 rpm, 10 min). The residue was purified by preparative HPLC, the pure fractions were pooled and lyophilized yielding H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 (**79**) as a white solid (187 mg, 55% yield). LC-MS: R_t = 10.23 min and *m/z* $[M + 3H]^{3+}$ calcd for $C_{50}H_{83}N_{14}O_{14}S^{3+}$: 379; found: 379.

Ac-Thr(^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln-OCam (80)
LC-MS: R_t = 23.09 min and *m/z* $[M + H]^+$ calcd for $C_{52}H_{93}N_{10}O_{18}^+$: 1146; found: 1146.

Ac-Thr(^{t}Bu)-Phe-Thr(^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln-OCam (81)
LC-MS: R_t = 25.01 min and *m/z* $[M + H]^+$ calcd for $C_{69}H_{117}N_{12}O_{21}^+$: 1450; found: 1450.

H-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Ala-NH_2 (82)
^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.13 (d, *J* = 6.9 Hz, 3H), 1.32 (s, 27H), 1.45-1.96 (m, 13H), 2.12-2.19 (m, 6H), 3.15-3.25 (m, 1H), 4.05-4.23 (m, 4H), 6.91 (s, 1H), 7.20 (s, 1H), 7.79 (d, *J* = 7.2 Hz, 1H), 7.91 (d, *J* = 7.5 Hz, 1H), 8.02 (d, *J* = 7.8 Hz, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 15.0, 18.6, 27.5, 28.0, 28.1, 30.2, 31.6, 35.0, 48.4, 51.8, 52.1, 54.2, 80.0, 170.7, 171.3, 171.5, 172.0, 172.1, 174.3, 175.2.

H-Ala-Met-Val-Ser(^{t}Bu)-Tyr(^{t}Bu)-Pro-Arg(Pbf)-Glu(O^{t}Bu)-Asn(Trt)-His(Trt)-NH_2 (83)
LC-MS: R_t = 17.15 min and *m/z* $[M + 2H]^{2+}$ calcd for $C_{114}H_{149}N_{17}O_{18}S_2^{2+}$: 1054; found: 1054.

Ac-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Ala-NH_2 (84)
LC-MS: R_t = 23.75 min and *m/z* $[M + H]^+$ calcd for $C_{74}H_{130}N_{13}O_{23}S^+$: 1601; found: 1600.

Ac-Thr(^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Ala-NH_2 (85)
LC-MS: R_t = 24.80 min and *m/z* $[M + H]^+$ calcd for $C_{89}H_{158}N_{15}O_{27}S^+$: 1901; found: 1900.

Ac-Thr(^{t}Bu)-Phe-Thr(^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Ala-NH_2 (86)
LC-MS: R_t = 24.96 min and *m/z* $[M + 2H]^{2+}$ calcd for $C_{106}H_{183}N_{17}O_{30}S^{2+}$: 1103; found: 1103.

Ac-Thr(^{t}Bu)-Phe-Thr(^{t}Bu)-Ser(^{t}Bu)-Asp(O^{t}Bu)-Leu-Ser(^{t}Bu)-Lys(Boc)-Gln-Ala-Met-Val-Ser(^{t}Bu)-Tyr(^{t}Bu)-Pro-Arg(Pbf)-Glu(O^{t}Bu)-Asn(Trt)-His(Trt)-NH_2 (87)
LC-MS: R_t = 25.08 min and *m/z* $[M + 3H]^{3+}$ calcd for $C_{185}H_{269}N_{28}O_{37}S_2^{3+}$: 1180; found: 1179.

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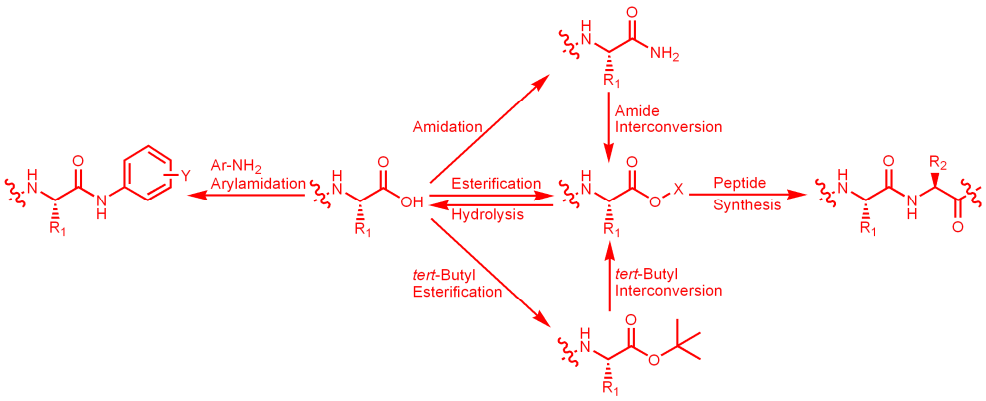
10

Chapter 11

Summary, discussion and perspectives



11.1 Summary

This thesis entails the development of generally applicable enzymatic approaches to functionalize the *C*-terminus of amino acids and peptides and to couple peptide fragments, particularly using Subtilisin A in (nearly) anhydrous organic solvents.

The current state-of-the-art of enzymatic peptide synthesis was described in **chapter 1**.

The start of this PhD project was triggered by the observation that a small percentage of side product was formed during the Subtilisin A catalyzed peptide coupling of Cbz-Phe-OMe with H-Leu-O^{t}Bu in *tert*-butanol. This side product proved to be the ^{t}Bu ester of the starting material, *i.e.* Cbz-Phe-O^{t}Bu and was a result of enzymatic transesterification. It was shown that this mild esterification could also be applied when starting from an *N*-terminally protected amino acid carboxylic acid, *e.g.* Cbz-Phe-OH, which could be brought to completion when the liberated water was removed by molecular sieves. In **chapter 2** it was shown that this enzymatic esterification reaction was independent on the *N*-terminal protecting group, and various esters such as methyl, ethyl, benzyl, allyl, trimethylsilylethyl or ^{t}Bu esters could be synthesized in high yield. Furthermore, it was demonstrated that Subtilisin A could esterify Asp and Glu derivatives, which also bear an additional β- and γ-carboxylic acid functionality, respectively, with complete α-regioselectivity. This is in sharp contrast to chemical approaches where discrimination between these two carboxyl functionalities is very difficult. Finally, the enzymatic esterification was successfully applied to peptides with various degrees of protection, which were surprisingly still accepted as a substrate by Subtilisin A.

Subtilisin A could also be applied for selective ester hydrolysis to obtain β-protected Asp and γ-protected Glu derivatives, as was described in **chapter 3**. These amino acid building blocks are difficult to obtain via chemical routes. Two strategies were developed: one comprises the chemical synthesis of Asp and Glu diesters followed by fully selective enzymatic hydrolysis of the α-ester, while the other method comprises a three step protocol using i) α-selective enzymatic methyl esterification, ii) chemical esterification of the side chain carboxylic acid, and finally iii) α-selective enzymatic methyl ester hydrolysis.

When other nucleophiles than alcohols were used in the synthesis direction (coupling reaction), various modifications of the peptide *C*-terminus could be realized using Subtilisin A. For instance, when using an arylamine (such as aniline), amino acid and peptide *C*-terminal arylamides could be obtained, as was demonstrated in **chapter 4**. Peptide *C*-terminal arylamides are useful substrates in chromogenic-, fluorogenic- or amperogenic-based enzymatic assays. Using Subtilisin A, the arylamides were accessible from the corresponding *C*-terminal methyl or benzyl esters or carboxylic acids, in high chemical and optical purity. Alternatively, the cysteine protease Papain and *Candida antarctica* lipase B (Cal-B) could also be applied to give the arylamides in high yield.

Furthermore, ammonia could be used as the nucleophile as well, to give access to peptide *C*-terminal amides, which constitute half of the currently known peptide pharmaceuticals. It was shown that the application of the amino acid or peptide α-carboxylic acid ammonium salt instead of separate ammonia sources like ammonium benzoate resulted in a much faster enzymatic amidation reaction without significant side reactions, as was described in **chapter 5**. Two approaches were developed, the first one using the lipase Cal-B, the second one using Subtilisin A.

Combining the newly developed methods as described above, a fully enzymatic *N* → *C* terminal peptide elongation strategy could be designed which was based on a Subtilisin A catalyzed methyl esterification followed by a peptide coupling and a protecting group (*i.e.* ^{t}Bu-ester) hydrolysis step. As drawback of this strategy it was found that in some peptide sequences the hydrolysis of the ^{t}Bu-esters was accompanied by hydrolysis of peptide bonds, especially when longer peptides were synthesized.
Since the transesterification of peptide *C*-terminal Me-esters into ^{t}Bu-esters in ^{t}BuOH had already been demonstrated, it was envisioned that the reverse reaction could be effectuated in the presence of methanol. This led to an improved strategy, wherein the peptide ^{t}Bu-ester was "reactivated" into the Me-ester in only one step and peptide backbone hydrolysis was avoided due to the absence of water. In **chapter 6** the applicability of this fully enzymatic peptide elongation strategy via ^{t}Bu-ester interconversion was demonstrated by the synthesis of a number of biologically active peptides up to the heptamer level.
During the course of this PhD project, the conversion of peptide *C*-terminal Me-esters to peptide *C*-terminal carboxamides by Subtilisin A was reported in the literature. Also in this case, it was envisioned that the reverse reaction could be effectuated to obtain a second fully enzymatic peptide elongation strategy, *i.e.* based on a carboxamide to Me-ester interconversion. Indeed, this interconversion of peptide carboxamides proved viable, and **chapter 7** described the application of this strategy to the synthesis of a number of (functionalized) biologically active peptides.
It was found that even highly activated esters, *e.g.* trifluoroethyl- (Tfe) and carbamoylmethyl- (Cam) esters, could be synthesized in high yield starting from either amino acids, peptide *C*-terminal carboxylic acids or their corresponding alkyl esters using Cal-B, as was described in **chapter 8**. These highly activated esters substantially broadened the scope of Subtilisin A catalyzed peptide synthesis and allowed the incorporation of non-proteinogenic, D-, and even sterically demanding amino acids, as well as weak nucleophiles (*e.g*. proline). Both enzymes could be combined in one pot, for the synthesis of the highly activated esters (by Cal-B) and subsequent peptide bond formation (by Subtilisin A) using an amine nucleophile.
Clearly, the type of activated ester was of significant influence on the efficiency of a peptide bond formation. In **chapter 9**, a series of novel esters was evaluated for their efficacy in Subtilisin A catalyzed peptide synthesis. The reactivity of the Cam ester was further enhanced by elongating it with an amino acid residue thereby creating more interaction sites for the enzyme.
Finally, side chain protected peptide *C*-terminal Cam-esters were used for Subtilisin A mediated peptide fragment condensation in anhydrous organic solvents, as was disclosed in **chapter 10**. The scope and limitations of this novel fragment condensation strategy was examined by coupling a series of 30 Cbz-Val-Xxx-OCam dipeptides with 20 H-Yyy-Val-NH_2 dipeptides, wherein Xxx stands for any proteinogenic amino acid with or without side chain protected functional groups and Yyy stands for any proteinogenic amino acid with side chain protected functional groups. It appeared advantageous to leave the side chain functionality of the *C*-terminal amino acid of the peptide Cam ester unprotected. Finally, it was demonstrated that it was feasible to enzymatically condense larger peptide fragments (up to the 10-mer level) bearing multiple side chain protection groups in very high yield catalyzed by Subtilisin A. This racemization free side chain protected peptide fragment condensation strategy opens new doors for the (industrial) synthesis of long peptides which are therapeutically relevant.

11.2 Discussion and Perspectives

A large number of papers was published in the last decades dealing with enzymatic formation of peptide bonds, enzymatic manipulation of protecting groups and enzymatic modification of peptides. However, at present more peptides are synthesized by chemical synthesis than by peptidase catalyzed processes. The use of peptide synthesizers, in addition to recent new developments in the field of chemical ligation procedures, still favor chemical methods over the enzymatic approach. However, there is no doubt that enzymatic methods have advantages, including the prevention of racemization, no need for time-consuming and expensive protection/deprotection procedures of side chain functional groups, the reduced use of (toxic) solvents and stoichiometrically required reagents, and possible reuse of the biocatalysts. Furthermore, much progress has been made in the cost efficient production of enzymes and immobilization has become routine, also on large scale. Furthermore, improving enzymes by (site-directed) mutagenesis is becoming more common practice.

There are three main reasons for enzymatic peptide synthesis and derivatization currently not (yet) being widely used. The first reason is that proteases are usually highly specific for the hydrolysis of certain sequences, which limits their application to bring about the condensation of any peptide fragment or the *C*-terminal derivatization of any peptide at will. The specificity can be significantly relaxed by the use of “substrate mimetics” such as guanidinophenyl esters combined with trypsin, but the preparation of such acyl donors is cost-inefficient and is usually accompanied by racemization. The second reason is that almost all reported enzymatic peptide coupling and derivatization reactions have been carried out in aqueous solution. This is the natural environment in which proteases carry out their normal function and in which they display high activities. Unfortunately this brings about that in the kinetically controlled reactions, hydrolysis of the acyl donor ester and within the peptide chains occurs predominantly, resulting in a difficult HPLC purification of a complex peptide mixture. In the thermodynamically controlled reactions, product yields are generally low because water drives the equilibrium towards the starting materials. The third reason is that, to perform enzymatic synthesis reactions, advanced knowledge of biocatalysis mandatory. Each individual enzymatic reaction needs optimization and quite some research and development is required when applying enzymes. This is in contrast to many organic synthetic reactions, which are often more generally applicable and easier to apply. Besides, the required enzymes used are not directly commercially available from the usual catalogue companies.

In this PhD project, the goal was to develop generally applicable enzymatic reactions with the final objective to apply these on industrial scale in the synthesis and derivatization of peptides. The approach was to use *anhydrous* organic solvents as the reaction medium in order to prevent hydrolysis of the acyl donor and of the peptide backbone or to drive thermodynamically controlled reactions towards product formation. This approach has been hitherto disregarded in academia and industry, firstly because the activity and stability of proteases in organic medium is generally very low, especially under the strictly anhydrous conditions that are required to prevent hydrolytic reactions, and secondly, it was not foreseen that peptide fragments or amino acids containing (multiple) side chain protecting groups - which are required to render them soluble in organic solvents – would be accepted

by proteases as the substrate. Subtilisin A is one of the few proteases (together with other enzymes from the class of Subtilisins) that still has significant activity in anhydrous organic solvents, in particular in apolar aprotic organic solvents. Therefore, and because of its rather broad substrate scope, which is crucial for the development of generally applicable methods, Subtilisin A was the enzyme of choice in the investigations described in this thesis. Besides, Subtilisin A is cheap and commercially available, even on industrial scale.

In this study, it was discovered that many amino acid and peptide *C*-terminal derivatization reactions, such as (trans)esterification, (aryl)amidation and carboxamide to ester interconversion can be run to full completion in a reasonable reaction time without significant side reactions using Subtilisin A, as long as the reaction conditions are strictly anhydrous. These dry conditions were for instance accomplished by using molecular sieves to capture the water which was released by the enzyme during the reaction. It was also found that the stability and activity of Subtilisin A was further preserved by immobilization. Compared to the enzymatic synthesis of primary esters of *N*-protected amino acids and peptides without functional groups in the side chain, chemical synthesis of these esters is usually easier, faster and more cost-efficient. However, when amino acids *with* a functional group in the side chain are used, such as Asp and Glu, the selectivity of enzymes has great benefits, as shown in chapter 2. The chemical synthesis of *N*-protected Asp and Glu α-carboxylic ester derivatives is most often performed using the internal anhydride method with a typical yield of around 50% requiring a (not easily scalable) silicagel column purification. The one step enzymatic synthesis, however, is much more straightforward and higher yielding. The *N*-protected Asp and Glu starting materials are readily available. Therefore, in academia, this α-selective esterification could be a great option for replacing the multi-step organic synthesis protocols and difficult purifications of α/β-ester mixtures. Besides the selective synthesis of primary α-esters, the enzymatic synthesis of ^{t}Bu-esters, as described in chapter 2, has great advantages over chemical synthesis, not only with respect to selectivity, but especially with respect to the extremely mild conditions used. Many substrates, including long peptides, containing acid labile (protecting) groups cannot be used in mineral acid catalysed ^{t}Bu-ester synthesis. The enzymatic reaction in ^{t}BuOH is safe and easy to perform, in contrast to the pressurized isobutene set-up used for chemical synthesis, and work-up is straightforward. Therefore, the methodology described in this thesis for the enzymatic synthesis of ^{t}Bu esters could be taken up by academia as the new standard. The enzymatic hydrolysis of *C*-terminal α-esters, as described in chapter 3, has the same advantages as the selective enzymatic α-esterification as described above: it is mild and selective. If enzymatic hydrolysis was more commonly applied, this would radically change the commonly used synthetic schemes and protecting group strategies. For instance, ^{t}Bu and Boc protection are fully orthogonal when using enzymes, while chemically, both groups are acid labile. Therefore, to simplify synthetic strategies, enzymes could be applied more often.

The *C*-terminal amidation of carboxylic acids and esters, as described in chapter 5, has no advantage on the amino acid level. Small peptides containing a *C*-terminal α-carboxamide functionality are easily synthesized via solution phase techniques in the *C*→*N* direction, using the carboxamide functionality as the *C*-terminal protecting group, although solubility is sometimes an issue. Longer peptide *C*-terminal carboxamides are easily synthesized on the solid phase containing *e.g.* a Rink, Sieber or Ramage linker. These linkers, however, are cost-inefficient on large scale, some are protected by patents and cleavage yields are often low. Hence, for longer peptide, the peptide *C*-terminal carboxamide functionality is most

often incorporated via the synthesis of a protected peptide on a 2-chlorotritylchloride linker resin, followed by a fragment condensation with an amino acid carboxamide, as shown in Figure 1 A, which is usually accompanied by significant epimerization of the penultimate residue. After the fragment condensation the peptide is deprotected and should be purified by a laborious preparative HPLC step to separate the diastereoisomers. Therefore, the one step enzymatic *C*-terminal amidation (Figure 1 B) of long peptides holds great promise for industry, especially when the peptide starting materials can be synthesized on an extremely cheap Wang linker resin.

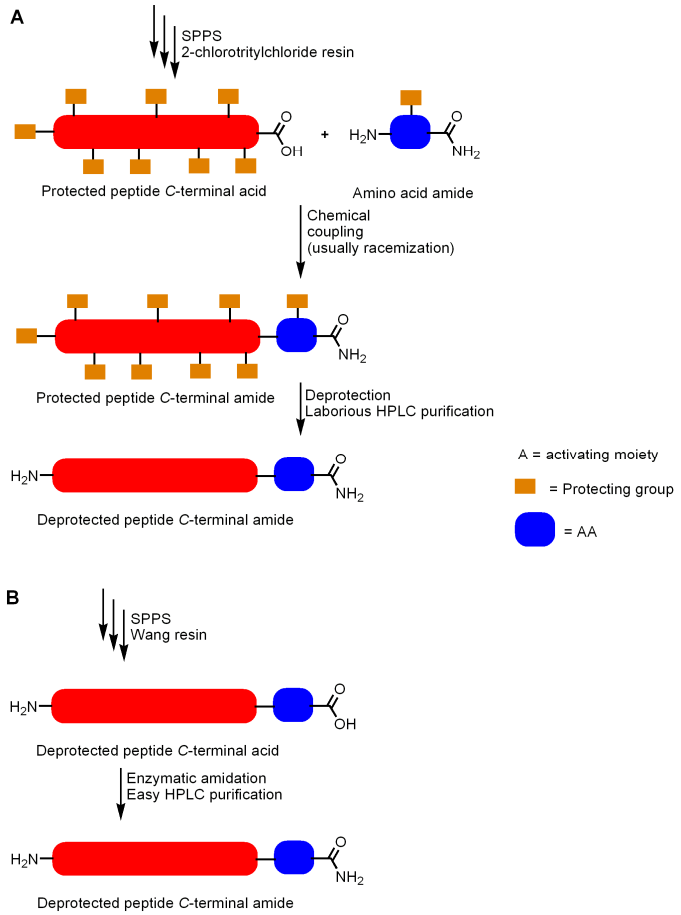


Figure 1. A) Industrial scale synthesis of a peptide *C*-terminal carboxamide; B) Proposed industrial scale enzymatic synthesis of a peptide *C*-terminal carboxamide.

The arylamidation technology, as developed in this thesis, can in principle be applied on industrial scale and would deliver, in line with the *C*-terminal amidation of peptides, a significant cost-price reduction since complex protection/deprotection procedures and racemization, inherent to chemical methods, are avoided. In academia, the chromogenic and fluorogenic *C*-terminal arylamide functionalities are very commonly applied. Their

enzymatic synthesis is less complicated than the chemical one, but is less generally applicable because not all peptide sequences are recognized by the enzyme. Moreover, most peptide *C*-terminal arylamide substrates used are short sequences (typically 3-4 amino acids), which can be chemically synthesized step-wise in the *C*→*N* terminal direction using the arylamide as a *C*-terminal protecting group. Therefore, in academia, there will be only a few cases where the enzymatic *C*-terminal arylamidation useful.

The (trans)esterification and carboxamide to ester interconversion reactions, as described in chapter 6 and 7, will enable a step-wise enzymatic peptide synthesis in the *N*→*C* terminal direction using cheap amino acid building blocks such as amino acid ^{t}Bu esters or amino acid carboxamides in excess over the growing peptide chain. This technology, when applied on industrial scale, would afford a significant cost-price advantage compared to solution-phase chemical stepwise peptide synthesis. The latter approach is limited to step-wise assembly in the *C*→*N* terminal direction and requires expensive *N*-protected building blocks and coupling reagents, because chemical elongation in the *N*→*C* terminal direction leads to racemization. An additional advantage of stepwise enzymatic peptide synthesis is that no side chain (de)protection reactions are required, as long as the side-chain unprotected peptide intermediates remain soluble in organic solvents. It should be noted that completely stepwise enzymatic peptide synthesis will only be industrially useful for relatively short peptide sequences (up to 4-5 amino acids) since purification of the intermediates is time consuming, especially when the peptides grow longer. Therefore, for peptides longer than 4-5 amino acids, convergent strategies are preferred over completely stepwise elongation, since the purification of the intermediates is most often much easier when fragments are condensed. For the chemical solution phase synthesis of peptides in the *C*→*N* terminal direction highly optimized and generally applicable protocols have been developed. In contrast, the stepwise enzymatic synthesis requires optimization for each individual peptide coupling and is therefore only advantageous in academia in very special cases.

In order to obtain a universal method to achieve peptide bond formation between amino acids and peptides in high yields, it appeared necessary to apply a more activated ester than a non-substituted alkyl ester as the acyl donor. Out of the examined activated esters, the carbamoylmethyl (Cam) ester appeared to be particularly useful, since it can be conveniently and efficiently prepared on the solid phase and can even be synthesized enzymatically as described in chapter 8. Cam esters elongated with an additional amino acid residue, can be synthesized on an industrially applicable 2-chlorotritylchloride resin. This is the first example of a solid phase synthesis of activated esters, which can directly be used for enzymatic coupling, using industrially applicable protocols and reagents. Using the (elongated or non-elongated) Cam-esters, it appeared feasible to couple amino acid sequences which are normally very difficult to incorporate in enzymatic peptide synthesis including non-proteinogenic, sterically demanding and side-chain protected amino acids or weak nucleophiles (*e.g.* proline) at the coupling positions, as long as strictly anhydrous conditions were retained, *e.g.*, by adding molecular sieves. Furthermore, the coupling reactions are faster then with less-activated esters and higher yields are obtained. Therefore, the enzymatic synthesis of small peptides using Cam esters, as described in this thesis, is a versatile method. However, the synthesis of small peptides on gram scale is chemically usually easier to develop. In contrast, the condensation of longer peptide fragments is chemically much more troublesome. Therefore, the Cam esters were investigated towards the enzymatic condensation of peptide fragments, as described in

chapter 10. It appeared feasible to couple almost any combination of amino acids on the 2 + 2 level using acyl donors with and without a protecting group on the side chain functionality. Using this technology, also longer peptide fragments (*e.g.*, up to the 9+10 level giving a 19-mer oligopeptide) could be condensed using Subtilisin A, even when (almost) all side-chain functionalities had been protected with sterically demanding protecting groups and even when only a slight excess of nucleophile was applied.

This enzymatic fragment condensation strategy holds great promise for the industrial production of long peptides (up to 50 amino acids length) if it is combined with SPPS of the fragments. Peptide fragments longer than 10-15 amino acids are difficult to synthesize on large scale via solid phase techniques because they tend to form tertiary structures (by so-called “hydrophobic collapse”) making peptide elongation very troublesome so that a large excess of reagents and amino acid building blocks is needed. Additionally, the purification of the final product is often cost-inefficient due to the presence of significant amounts of truncated peptides and peptides containing deletions. Therefore, peptides longer than 10 amino acids are often synthesized by the SPPS of side-chain protected oligopeptide fragments which are subsequently chemically condensed in solution, *e.g.* a 10+10 condensation to make a peptide of 20 amino acids. The major drawback of this chemical approach is that upon activation of the *C*-terminal amino acid residue significant racemization occurs, except when *C*-terminal Gly or Pro residues are used. Therefore, this chemical fragment condensation strategy is limited to using *C*-terminally activated Gly and Pro residues, or one has to deal with a very difficult purification due to the formation of (undesired) diastereoisomers. The limited choice of coupling positions in practice gives rise to very asymmetric fragments so that still long peptide fragments (> 15-20 amino acids) and undesirably short fragments (< 6 amino acid) should be synthesized by SPPS, as shown in Figure 2 A. This, in combination with the accidentally unfavorable solubility properties of the fragments, often results in a very high cost-price of the final peptide. In contrast, enzymatic fragment couplings are completely devoid of racemization allowing much more freedom to choose the coupling positions so that efficient convergent approaches become feasible, the fragment lengths can be limited to 10-15 amino acids and fragments with favorable solubility can be selected (Figure 2 B). Hence, it is expected that, using the enzymatic fragment condensation technology as described in this thesis, for various long pharmaceutical peptides, a significant cost-price reduction can be obtained on large scale, compared to the current state-of-the-art chemical fragment condensation.

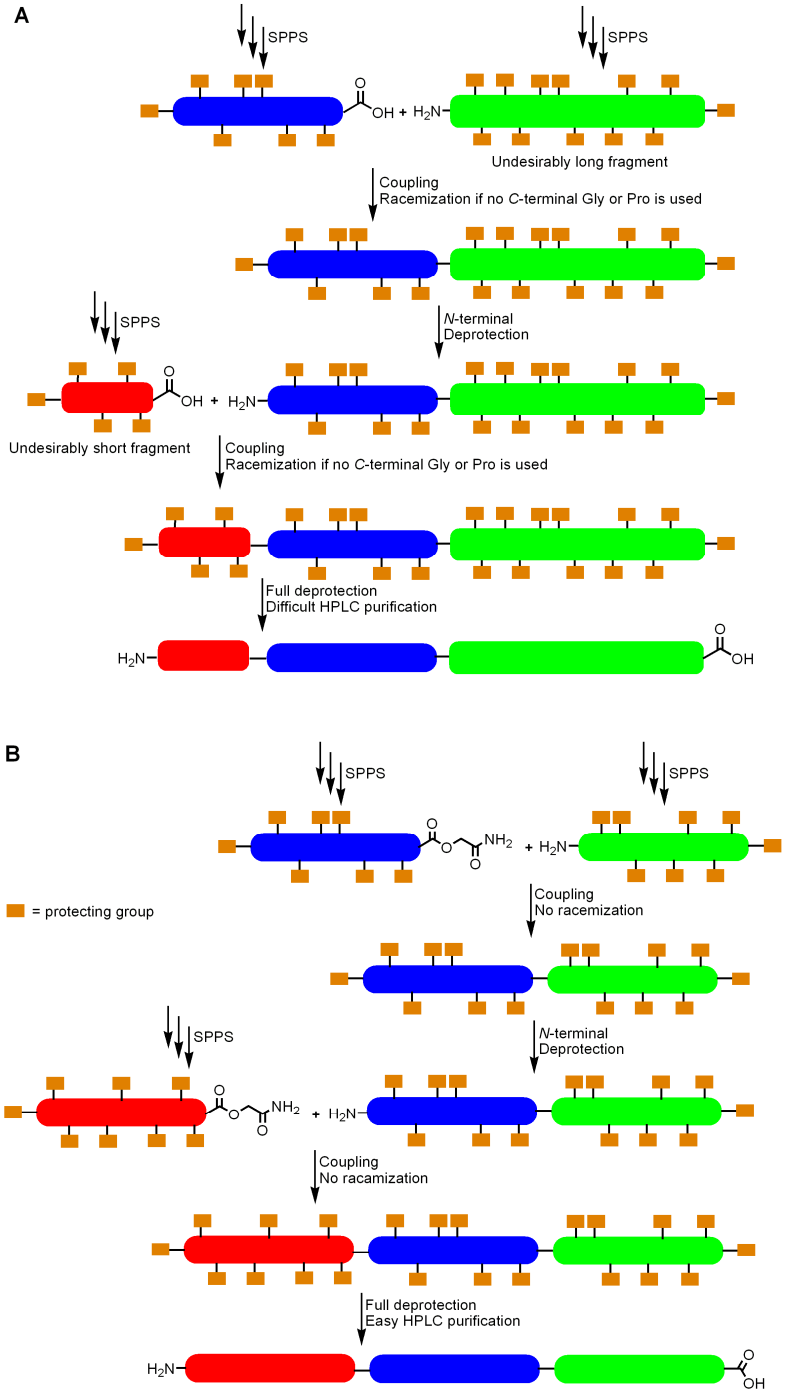


Figure 1. A) Industrial scale synthesis of a long peptide via the hybrid approach; B) Proposed industrial scale enzymatic synthesis of a long peptide via SPPS and enzymatic condensation.

In conclusion, many of the developed generally applicable technologies as described in this thesis, hold great promise for application on industrial scale and/or in academia, as summarized in Table 1.

Table 1. Potential areas of applicability of the various technologies both on industrial scale and on lab-scale (in academic research).

Ch.	Technology	Industrial applicability	Potential applicability in academia (lab-scale)
2	1^{o} ester synthesis	1) α-esters of Asp and Glu 2) α-esters of peptides containing Asp, Glu or acid-labile functionalities	1) α-esters of Asp and Glu 2) α-esters of peptides containing Asp, Glu or acid-labile functionalities
2	^{t}Bu ester synthesis	α-esterification of amino acids and peptides	α-esterification of amino acids and peptides
3	*C*-terminal α-ester hydrolysis	Asp and Glu β/γ-esters	1) Asp and Glu β/γ-esters 2) New orthogonality of protecting groups
4	*C*-terminal arylamide synthesis	Cost-efficient synthesis of diagnostic peptides	No advantage, only in the synthesis of long diagnostic peptides
5	*C*-terminal α-carboxamide synthesis	Cost-efficient synthesis of amides of peptides which have been prepared on the solid phase	No advantage over existing methods
6	*C*-terminal ^{t}Bu ester interconversion	Cost-efficient synthesis of small peptides	No advantage over existing methods
7	*C*-terminal α-carboxamide to ester interconversion	Cost-efficient synthesis of small peptides	No advantage over existing methods
8	Active ester and subsequent Peptide synthesis	Cost-efficient synthesis of small peptides	No advantage over existing methods
9	Elongated Cam ester	Active ester synthesis with industrially applicable resins, reagents and protocols	No advantage
10	Side-chain protected fragment condensation	Cost-efficient synthesis of medium-sized and long peptides (8-50 amino acids)	Sometimes easier and faster synthesis of certain difficult long peptide sequences

Appendices

Nederlandse samenvatting
List of abbreviations
Curriculum vitae
List of publications and awards
Dankwoord

Nederlandse samenvatting

Dit proefschrift omvat de ontwikkeling van algemeen toepasbare enzymatische methoden om de *C*-terminus van aminozuren en peptiden te functionaliseren en om peptide fragmenten te koppelen, in het bijzonder door gebruik te maken van Subtilisine A in droge organische oplosmiddelen.

De huidige status van enzymatische peptide synthese en potentiele toepassingen werden samengevat in **hoofdstuk één**.

De basis van dit promotie onderzoek is een gevolg van de toevallige ontdekking van een klein percentage bijproduct dat werd gevormd tijdens de koppeling van Cbz-Phe-OMe en H-Leu-O^{t}Bu in aanwezigheid van *tert*-butanol met het protease Subtilisine A. Dit bijproduct bleek de *tert*-butyl ester te zijn van het startmateriaal, namelijk Cbz-Phe-O^{t}Bu. Deze milde enzymatische verestering bleek ook mogelijk wanneer het aminozuur als carbonzuur met werd gebruikt, b.v. Cbz-Phe-OH, en de reactie verliep quantitatief wanneer het vrijgekomen water werd afgevangen met behulp van moleculaire zeven.

In **hoofdstuk twee** werd aangetoond dat deze enzymatische verestering kan worden toegepast op aminozuren met verschillende *N*-amino beschermgroepen en dat verscheidene esters onder meer methyl, ethyl, benzyl, allyl en trimethylsilylethyl esters in hoge opbrengst konden worden gesynthetiseerd. Daarnaast, werd aangetoond dat Subtilisine A de aminozuren Asp en Glu, die ook een carbonzuurgroep in de zijketen bevatten, selectief kan veresteren op de α-positie. Dit in tegenstelling tot chemische methoden waarbij onderscheid tussen deze twee carbonzuren erg moeilijk te maken is. Uiteindelijk werd de enzymatische verestering toegepast op peptiden met verschillende gradaties in bescherming die, verrassend genoeg, nog steeds als substraat geaccepteerd werden door Subtilisine A.

Naast voor de synthese, kon Subtilisine A ook gebruikt worden voor de hydrolyse van esters hetgeen werd toegepast voor het verkrijgen van β-beschermde Asp en γ-beschermde Glu aminozuur derivaten, zoals beschreven in **hoofdstuk drie**. Deze aminozuur bouwstenen zijn erg moeilijk te verkrijgen via chemische methoden. Twee strategieën werden ontwikkeld: de eerste was gebaseerd op de chemische synthese van Asp en Glu diesters gevolgd door de volledig selectieve enzymatische hydrolyse van de α-ester, de tweede strategie bestond uit drie stappen, t.w. i) een α-selective enzymatische methylester synthese, ii) een chemische β-verestering en iii) een α-selective enzymatische methylester hydrolyse.

Door het gebruik van andere nucleofielen dan alcoholen, was her mogelijk peptiden op verschillende manieren *C*-terminaal te modificeren. Zo werd er bijvoorbeeld een aniline derivaat gebruikt voor het verkrijgen van aminozuur en peptide arylamides, deze methodologie werd beschreven in **hoofdstuk vier**. *C*-terminale arylamiden van peptiden worden gebruikt als substraten voor enzymatische assays om bijvoorbeeld de activiteit van enzymen die verantwoordelijk voor bloed stolling zijn te bepalen. De arylamiden konden verkregen zowel uit *C*-terminale carbonzuren als uit methyl of benzyl esters en met een hoge chemische en optische zuiverheid. Naast Subtilisine A konden ook Papaïne en *Candida antarctica* lipase B (Cal-B) hiervoor gebruikt worden.

Bij gebruik van ammoniak als nucleofiel konden peptide *C*-terminale amides verkregen worden die veel voorkomen als pharmaceutische peptiden. Door het gebruik van het aminozuur of peptide ammonium zout in plaats van een separaat ammonium zout (zoals beschreven door anderen), verliep de amiderings reactie veel sneller zonder nevenreacties,

zoals werd beschreven in **hoofdstuk vijf**. Er werden twee methoden ontwikkeld; de ene maakt gebruik van Cal-B en de andere van Subtilisine A.
O.b.v. bovenstaande resultaten werd een volledig enzymatische *N* → *C* terminale elongatiestrategie voor peptiden ontworpen door gebruik te maken van Subtilisine A gekatalyseerde methylester synthese, gevolgd door een peptide koppeling en een beschermgroep (b.v. ^{t}Bu-ester) hydrolyse. Het nadeel van deze strategie is dat bij sommige (m.n. lange) peptidesequenties de hydrolyse van de ^{t}Bu-ester gepaard gaat met hydrolyse van peptide bindingen. O.b.v. de gevonden transverestering van *C*-terminale methylesters naar ^{t}Bu-esters in ^{t}BuOH, werd het mogelijk geacht om de tegenovergestelde reactie uit te voeren in de aanwezigheid van methanol. Zo zouden de ^{t}Bu-ester ontscherming en de daarop volgende activering naar de *C*-terminale methylester in één stap uitgevoerd worden en de hydrolyse van peptide bindingen worden vermeden omdat er geen water aanwezig is. In **hoofdstuk zes** werd deze volleding enzymatische stapsgewijze peptideverlenging door middel van ^{t}Bu-ester interconversie beschreven, en toegelicht aan de hand van de bereiding van verscheidene biologisch actieve peptiden.
Gedurende dit promotie onderzoek werd de synthese van *C*-terminal amides van peptiden vanuit de overeenkomstige methylesters beschreven door anderen. Opnieuw werd het mogelijk geacht om deze reactie in de tegenovergestelde richting uit te voeren om zo een tweede volledig enzymatische stapsgewijze synthesemethode te verkrijgen voor peptiden. Ditmaal gebaseerd op de interconversie van peptide amides naar methylesters. Dit bleek inderdaad mogelijk en in **hoofdstuk zeven** werd deze strategie toegepast op de synthese van enkele (gefunctionaliseerde) biologisch active peptiden.
Verder werd ontdekt dat zelfs sterk geactiveerde esters van peptiden, zoals trifluorethyl en carbamoylmethyl (Cam) esters, enzymatisch gemaakt konden worden vanuit de overeenkomstige *C*-terminale carbonzuren of alkyl esters, door gebruik te maken van Cal-B, zoals beschreven in **hoofdstuk acht**. Deze sterk geactiveerde esters verbreden de mogelijkheden van Subtilisine A gekatalyseerde peptide synthese. Zo konden de koppeling van onnatuurlijke, D- en sterisch gehinderde aminozuren en ook zwakke nucleofielen (b.v. proline) uitgevoerd worden. De twee enzymen konden gecombineerd worden in één pot zodat de synthese van de sterk geactiveerde esters (door Cal-B) en de peptidekoppeling (door Subtilisine A) met een amine nucleofiel tegelijk konden plaatsvinden.
Het werd duidelijk dat het type geactiveerde ester een grote invloed heeft op het verloop van een peptidekoppeling. Zoals getoond in **hoofdstuk negen** werden verschillende nieuwe esters getest op hun effectiviteit in Subtilisine A gekatalyseerde peptidesynthese. De activiteit van de gemakkelijk te verkrijgen Cam-ester werd nog verder verbeterd door hem uit te breiden met een aminozuur residu zodat er meer interacties werden gecreëerd voor herkenning door het enzym.
Tenslotte werden peptiden met beschermgroepen op de functionaliteiten in de zijketens van de aminozuren gebruikt voor enzymatische fragmentcondensatie in droge organische oplosmiddelen m.b.v. Subtilisine A, zoals beschreven in **hoofdstuk tien**. De mogelijkheden van deze nieuwe fragmentcondensatie strategie werden onderzocht door middel van een koppeling tussen een serie van 30 verschillende Cbz-Val-Xxx-OCam dipeptiden met 20 H-Yyy-Val-NH_2 dipeptiden, waarin Xxx voor alle natuurlijke aminozuren staat met en zonder beschermgroep op de functionele groepen in de zijketens en Yyy voor alle natuurlijke aminozuren staat met beschermgroep op de functionele groepen in de zijketens. Het bleek voordelig te zijn om het *C*-terminale aminozuur residu van de Cam-ester onbeschermd te

laten op de zijketen. Uiteindelijk werd aangetoond dat het mogelijk is om langere peptide fragmenten (tot en met tien aminozuren) aan elkaar te koppelen in hoge opbrengst terwijl ze meerdere beschermgroepen in de zijketens bevatten. Deze racemisatie vrije fragmentkoppelingsstrategie van beschermde peptiden biedt nieuwe mogelijkheden voor de (industriële) synthese van lange peptiden.

List of abbreviations

Å	Ångström
AA	amino acid
Ac	acetyl
AcOH	acetic acid
All	allyl
AllOH	allylalcohol
Ar	aryl
AMC	7-amino-4-methyl-coumarin
aq.	aqueous
Bn	benzyl
BnOH	benzylalcohol
Boc	*tert*-butyloxycarbonyl
Bz	benzoyl
Cal-B	*Candida antarctica* lipase-B
calcd	calculated
Cam	carbamoylmethyl
CamOH	glycolamide
Cbz	benzyloxycarbonyl
CLEA	cross-linked enzyme aggregate
CTC	2-chlorotritylchloride
d	doublet
DCC	*N,N'*-dicyclohexylcarbodiimide
dd	double doublet
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DOPA	3,4-dihydroxyphenylalanine
DTT	dithiothreitol
EDC	1-ethyl-3-(3-dimethylamino propyl)carbodiimide
equiv	equivalent
ESI	electrospray ionization
e.e.	enantiomeric excess
Et	ethyl
Et_2O	diethylether
EtOAc	ethylacetate
EtOH	ethanol
FIA	flow-injection analysis
Fmoc	9-fluorenylmethyl-oxycarbonyl
For	formyl
Glyc	glycolamide
Gp	guanidinophenyl
HBTU	*O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate
HOAt	1-hydroxy-7-azabenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
Hz	hertz
^{i}PrOH	*iso*-propylalcohol
IR	infra red
kV	kilovolt
LC-MS	liquid chromatography mass spectrometry
m	multiplet
m	meta
m/z	mass to charge ratio
MBHA	4-methylbenzhydrylamine
Me	methyl
MeOH	methanol
mp	melting point
MS	mass spectrometry
MSA	methanesulfonic acid
MTBE	methyl *tert*-butyl ether
N	normal
NMP	*N*-methyl-2-pyrrolidinone
NMR	nuclear magnetic resonance
o	ortho
OM	organic media
p	para
PAF	peptide amidase from the *flavedo* of oranges
Pbf	2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl
*p*NA	*p*-nitroaniline
Pr	Propyl
q	quartet
R_f	retention factor
rt	room temperature
R_t	retention time
SPPS	solid-phase peptide synthesis
t	triplet
TBAF	tetrabutylammonium fluoride
^{t}Bu	*tert*-butyl
^{t}BuOH	*tert*-butanol
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethyl
TFEOH	2,2,2-trifluoroethanol
THF	tetrahydrofuran
TIS	triisopropylsilane
TLC	thin layer chromatography
TMAP	trimethylaminophenol
TMS	tetramethylsilane
TMSE	trimethylsilylethyl
TMSEOH	trimethylsilylethylalcohol
TOF	time of flight
Trt	trityl
UV	ultra violet
v	volume
VIS	visible

Abbreviations of amino acids

Ala	A	L-alanine
Arg	R	L-arginine
Asp	D	L-aspartic acid
Asn	N	L-asparagine
Cys	C	L-cysteine
Gln	Q	L-glutamine
Glu	E	L-glutamic acid
Gly	G	glycine
His	H	L-histidine
Ile	I	L-isoleucine
Leu	L	L-leucine
Lys	K	L-lysine
Met	M	L-methionine
Phe	F	L-phenylalanine
Pro	P	L-proline
Ser	S	L-serine
Thr	T	L-threonine
Trp	W	L-tryptophan
Tyr	Y	L-tyrosine
Val	V	L-valine
Xxx and/or Yyy		arbitrary amino acid

Curriculum vitae

De auteur van dit proefschrift werd geboren op 28 november 1981 te Utrecht. Na het behalen van het VWO diploma in 2001 heeft hij anderhalf jaar gewerkt als kok in verscheidene restaurants. Vervolgens koos hij voor de studie scheikunde aan de Universiteit Utrecht waar het bachelor diploma werd behaald in 2006. De bachelor stage voerde hij uit bij de diciplinegroep Medicinal Chemistry & Chemical Biology onder begeleiding van dr. Alex J. Poot op het gebied van “selective enrichment of glycosylated peptides” hetgeen resulteerde in zijn eerste co-auteurschap. Vervolgens doorliep hij de master “drug innovation” en de major stage werd bij dezelfde groep uitgevoerd onder begeleiding van dr. Dirk-Jan van Zoelen. Het onderzoek richtte zich op “inhibition of Caspase-3 by TAC-based mimics of XIAP (X-linked inhibitor of apoptotic protein)”. De minor stage werd volbracht bij DSM in Geleen in de groep “Innovative Synthesis” onder begeleiding van dr. Claudia Cusan en dr. ir. Peter J.L.M. Quaedflieg. Hier kwam de auteur voor het eerst in aanraking met biokatalyse, te weten de enzymatische synthese en modificatie van peptiden. Het master diploma werd behaald in augustus 2007. In een nieuw samenwerkingsverband tussen Medicinal Chemistry & Chemical Biology en DSM Innovative Synthesis begon de auteur in oktober 2007 in Geleen als assistent in opleiding onder de begeleiding van dr. ir. Peter J.L.M. Quaedflieg, dr. ir. Dirk T.S. Rijkers, dr. ir. John A.W. Kruijtzer en prof. dr. Rob M.J. Liskamp. De behaalde onderzoeksresultaten staan in dit proefschrift beschreven en resulteerden onder meer in 9 wetenschappelijke artikelen en 6 patenten. Het onderzoek werd onder meer gepresenteerd op drie internationale wetenschappelijke congressen. Verder werd er nauw samengewerkt met prof. dr. Dick B. Janssen (Rijksuniversiteit Groningen), prof. dr. Floris P.J.T. Rutjes (Radboud Universiteit Nijmegen), prof. dr. ir. Hans Tramper (Wageningen Universiteit en Researchcentrum) en prof. dr. Rolf Breinbauer (Technische Universiteit Graz, Oostenrijk) hetgeen resulteerde in 5 co-auteurschappen, een patent en een hoofdstuk in het boek “Enzyme catalysis in organic synthesis”. Sinds oktober 2011 is de auteur werkzaam als scientist bij DSM Innovative Synthesis.

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Hopelijk kan ik ook in de toekomst zo nauw met je samenwerken en een keer de “geoptimaliseerde recepten” van je keuken labjournaal overnemen!!

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