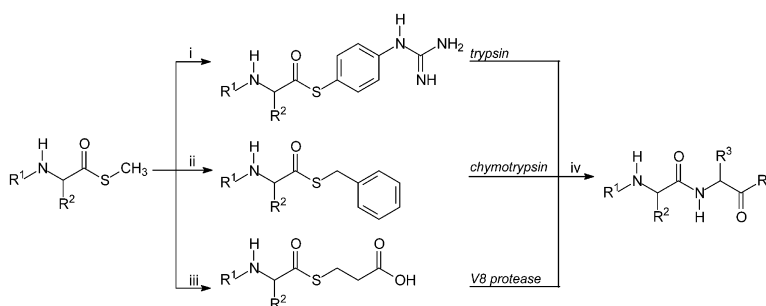Article

Reverse Proteolysis Promoted by in Situ Generated Peptide Ester Fragments

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Reverse Proteolysis Promoted by in Situ Generated Peptide Ester Fragments

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Abstract: In this contribution we describe a general synthesis concept for the in situ preparation of protease specific reactants using methyl thioesters as universal precursors. The precursor esters are readily available by standard synthesis procedures and can be used directly as reactants for protease-mediated peptide coupling reactions. Alternatively, they can serve as initial building blocks for the in situ preparation of various types of substrate mimetics. The synthesis of the latter is achieved by a one-pot spontaneous transthioesterification reaction of the parent thioester (Y-(Xaa)$_n$-SMe→Y-(Xaa)$_n$-SR; R: CH$_2$CH$_2$COOH, CH$_2$C$_6$H$_5$, C$_6$H$_4$NHC(:NH)NH$_2$), which proceeds efficiently in both a sequential manner and parallel to the subsequent enzymatic reaction. The resulting substrate mimetics act as efficient acyl donor components and show the typical behavior of substrate mimicry enabling irreversible reactions with originally nonspecific acyl moieties. Neither a workup of the substrate mimetic intermediate nor changes of the reaction conditions during the whole synthesis process are required. Model peptide syntheses using trypsin, α-chymotrypsin, and V8 protease as the biocatalysts proved the function of the approach and illustrated its synthetic value for protease-mediated reactions and the compatibility of the approach with state-of-the-art solid-phase peptide ester synthesis methods.

Introduction

Forced by successful enzyme, medium, and substrate engineering methods, proteases have gained in importance as regio- and stereospecific catalysts in organic synthesis.[2] Especially for applications that are based on their native hydrolysis activity, such as regiospecific ester hydrolysis or the kinetic resolution of racemates, proteases are now generally recognized as normal bench reagents. In principle, these engineering methods also allow for the reduction of competitive acyl donor hydrolysis, the alteration of the enzyme specificity and the minimization of undesired proteolytic cleavages, which are the main drawbacks when proteases are used as catalysts for reverse proteoly-

sis. The single or combined application of these approaches has made possible the use of proteases for the synthesis of short peptides,[3] the coupling of longer-chain peptide fragments,[4] and even the synthesis of fully active enzymes,[5] nonpeptidic carboxylic acid amides,[6] or peptidoglycans.[7] Despite these successful examples, it is still the enzyme's stringent specificity for both the acyl donor and acceptor component that hinders the universal synthetic use of the enzymatic approach. Substantial improvements to overcome the specificity problem for the acyl donor have been attained through the development of substrate mimetics.[8] Contrary to classical acyl donors, in substrate mimetics the site-specific amino acid moiety is transferred from the *C*-terminal of the peptide residue to the

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(1) Abbreviations: AM, amino methyl; Boc, *tert*-butoxycarbonyl; Bz, benzoyl; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; NEM, 4-ethylmorpholine; NMM, 4-methylmorpholine; OGp, 4-guanidinophenyl ester; PyBOP, (1*H*-benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; SBn, benzyl thioester; SCe, carboxyethyl thioester; SGp, 4-guanidinophenyl thioester; SMe, methyl thioester; TBTU, (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-benzotriazol-1-yl)uronium-tetrafluoroborate; THF, tetrahydrofuran; TIS, triisopropylsilane; TLCK, *N*^{α}-p-tosyl-L-lysine chloromethyl ketone; TPCK, *N*^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; V8 protease, Glu-specific endopeptidase from *Staphylococcus aureus* strain V8; Xaa, amino acid residue; Z, benzyloxycarbonyl.

(2) For recent review see: Bordusa, F. *Chem. Rev.* **2002**, *102*, 4817.

(3) For recent examples see: (a) Matsumoto, K.; Davis, B. G.; Jones, J. B. *Chem. Eur. J.* **2002**, *8*, 4129. (b) Sekizaki, H.; Itoh, K.; Toyota, E.; Tanizawa, K. *J. Peptide Sci.* **2002**, *8*, 521. (c) Günther, R.; Thust, S.; Hofmann, H.-J.; Bordusa, F. *Eur. J. Biochem.* **2000**, *267*, 3496. (d) Rival, S.; Aulnier, J.; Wallach, J. *Biocatal. Biotrans.* **2000**, *17*, 417.

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(8) (a) Schellenberger, V.; Jakubke, H.-D.; Zapevalova, N. P.; Mitin, Y. V. *Biotechnol. Bioeng.* **1991**, *38*, 104. (b) Sekizaki, H.; Itoh, K.; Toyota, E.; Tanizawa, K. *Chem. Pharm. Bull.* **1996**, *44*, 1585. (c) Bordusa, F.; Ullmann, D.; Elsner, C.; Jakubke, H.-D. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2473. (d) Thormann, M.; Thust, S.; Hofmann, H.-J.; Bordusa, F. *Biochemistry* **1999**, *38*, 6056.

Scheme 1. Basis Architecture of Common Acyl-Donor Components versus Substrate Mimetics
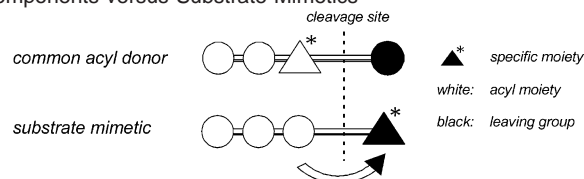
ester leaving group (Scheme 1). This replacement is accompanied by a shift in the enzyme activity, enabling proteases to couple nonspecific acyl moieties in an irreversible manner.[9] On the other hand, the approach requires the synthesis of specifically designed substrates bearing ester leaving groups that are adapted to the respective enzyme's S_1 subsite specificity. This requirement inevitably limits the choice of useable proteases to those which have similar S_1 subsite specificities. Thus, one must know in advance, which protease would be the most suitable catalyst for an intended synthesis, since the later replacement of an enzyme with another having a different S_1 specificity but better meets the demands of synthesis, requires a complete new chemical synthesis of the respective type of acyl donor. This general drawback complicates the practicability of the enzymatic approach and makes the screening for the most suitable biocatalyst inefficient and time-consuming. In addition, some of the substrate mimetic types developed are somewhat bulky in their fully protected forms, which can hamper their preparation especially on solid support.

This contribution reports on a novel general synthesis concept for both substrate mimetics and classical-type acyl donor esters that enhances the flexibility of the enzymatic approach as well as simplifies the chemical synthesis of the acyl donor and its use in enzymatic reactions. The concept is based on a single and uniform parent substrate thioester that itself can be used as a classical acyl donor component or, alternatively, can serve as a starting building block for the in situ preparation of different types of substrate mimetics. The synthesis of the latter is achieved by a one-pot spontaneous transthioesterification reaction of the parent thioester, which proceeds efficiently in both a sequential manner and parallel to the subsequent enzymatic reaction. Hence, neither a workup of the resulting substrate mimetics nor changes of the reaction conditions during the whole synthesis process are required. Model peptide couplings with three different enzymes, the parent substrate ester, and in situ generated substrate mimetics show no significant decrease in the reaction rate or the yield of synthesis due to the additional transthioesterification step. The universality of the approach was further demonstrated by model peptide fragment condensations, illustrating that the new synthesis concept is of high compatibility with state-of-the-art solid-phase peptide ester synthesis methods.

Results and Discussion

We initially selected a suitable parent thioester with respect to the following demands. To ensure efficient chemical synthesis, the thiol should be small, without additional functional

groups and should provide stable thioesters. Furthermore, it had to undergo an efficient thiol exchange when exposed to thiol-containing compounds and should be easily removable from the reaction mixture after transesterification. Conforming to these requirements, we chose methyl mercaptan as an interesting candidate whose respective methyl thioesters could be considered the smallest thioesters of all. Furthermore, methyl mercaptan should, due to its high vapor pressure, evaporate spontaneously from the reaction mixture after its liberation by transthioesterification. This should facilitate the purification of the synthesis products as well as mediate a rapid and complete thiol exchange. Suitable substrate mimetic leaving groups were selected from the scientific literature with respect to the three main important specificity classes of proteases, i.e., enzymes specific for Glu/Asp, Tyr/Phe, and Arg/Lys. For Glu/Asp-specific enzymes, a number of potential substrate mimetic thioesters have already been reported, from which we selected empirically the carboxyethyl thioester (SCe) type.[10] In the case of Arg/Lys-imitating substrate mimetics, the 4-guanidinophenyl ester (OGp) moiety is commonly used,[8,9] although it does not undergo efficient transesterification due to its nonthioester nature. Therefore, we used its structurally related 4-guanidino-mercaptophenol (SGp) analogue, for which we expected not only a similar kind of behavior as a substrate mimetic, but also a sufficient transesterification activity. Correspondingly, we have replaced the already established phenyl ester (OPh)-type substrate mimetics for Phe/Tyr-specific enzymes[4b,11] with the respective benzyl thioesters (SBn). The use of the structurally more related thiophenyl esters was avoided due to their high chemical reactivity that may cause undesired spontaneous acylations. Scheme 2 summarizes the ester structures selected and illustrates their formation from the parent methyl thioester by transthioesterification. The proof of concept was performed with five N^α-Z-protected amino acid methyl thioesters (Z-*Xaa*-SMe, *Xaa*: Ala, Phe, Pro, Lys, Glu) differing in the nature of their individual amino acid moiety. The synthesis of the esters can be easily achieved by well-known standard methods from readily available starting materials with excellent yields. To evaluate the transthioesterification behavior of the Z-Xaa-SMe esters, the influence of the nature and concentration of the added thiol on the rate and completeness of thiol exchange was investigated. Figure 1A shows the effect of the thiol concentration on the rate of spontaneous transthioesterification, using the example of Z-Ala-SMe with 3-mercaptopropionic acid and resulting in the formation of Z-Ala-SCe. Complete thiol exchange was achieved with a 10-fold excess of 3-mercapto-propionic acid over Z-Ala-SMe within 1 h, while lower concentrations of the added thiol led to a decrease in the rate of transthioesterification. Nevertheless, in all cases quantitative conversions could be reached as indicated by long-time experiments. By contrast, control reactions in which the evaporation of the released methyl mercaptan was restricted showed somewhat reduced reaction rates. Furthermore, no complete transesterifications could be observed in these cases which finally prove the importance of the evaporation of released methyl mercaptan for shifting the equilibrium toward the product ester. A shift to complete consumption of the parent thioester

(9) For recent examples see: (a) Sekizaki, H.; Itoh, K.; Toyota, E.; Tanizawa, K. *Chem. Pharm. Bull.* **1998**, *46*, 846. (b) Wehofsky, N.; Kirbach, S. W.; Haensler, M.; Jakubke, H.-D.; Wissmann, J.-D.; Bordusa, F. *Org. Lett.* **2000**, *2*, 2027. (c) Günther, R.; Stein, A.; Bordusa, F. *J. Org. Chem.* **2000**, *65*, 1672. (d) Sekizaki, H.; Itoh, K.; Toyota, E.; Tanizawa, K. *Amino Acids* **2001**, *21*, 175.

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Scheme 2. In Situ Generation of Different Types of Substrate Mimetics by Transthioesterification of Acyl Methyl Thioesters and Their Use for Enzymatic Peptide Synthesis[a]
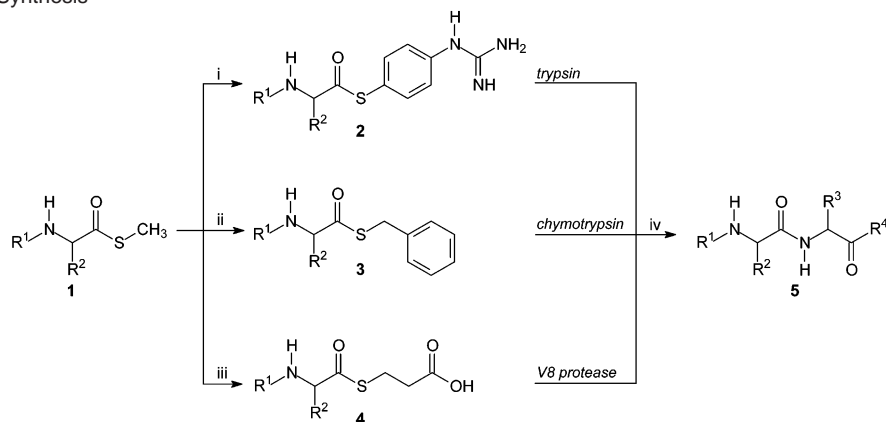
[a] **1**, acyl methyl thioester (SMe); **2**, acyl-4-guanidinophenyl thioester (SGp); **3**, acyl benzyl thioester (SBn); **4**, acyl carboxyethyl thioester (SCe); **5**, peptide product. Thiols for transthioesterification: i, 4-guanidinomercaptophenol; ii, benzyl mercaptan; iii, 3-mercaptopropionic acid. iv, *C*-terminal acceptor peptide.
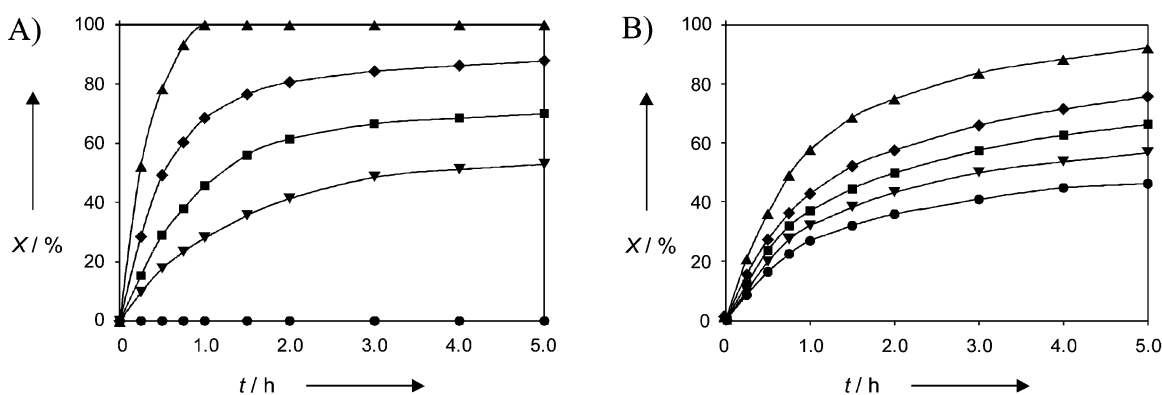

Figure 1. Effect of the concentration of 3-mercaptopropionic acid (A) and V8 protease (B) on the rate of spontaneous transthioesterification of Z-Ala-SMe to Z-Ala-SCe. Conditions: 0.2 M Hepes buffer, pH 8.0, 2.5% DMF, 37 °C, [Z-Ala-SMe]: 4 mM. (A) Concentration of 3-mercaptopropionic acid: (-●-), 0 mM; (-▼-), 4 mM; (-■-), 12 mM; (-◆-), 20 mM; (-▲-), 40 mM. (B) Concentration of V8 protease: (-●-), 0 μM; (-▼-), 1 μM; (-■-), 3 μM; (-◆-), 5 μM; (-▲-), 10 μM; concentration of 3-mercaptopropionic acid: 4 mM. X = concentration of Z-Ala-SCe.

can also be achieved by adding the enzyme to the transthioesterification mixture. As shown in Figure 1B for the Glu-specific V8 protease, the rate of thiol exchange increases with rising enzyme concentrations, enabling a nearly complete transthioesterification and subsequent hydrolysis under equimolar quantities of thiol and Z-Ala-SMe in about 5 h. By contrast, no hydrolysis of Z-Ala-SMe by V8 protease was found in control reactions in which 3-mercaptopropionic acid was lacking. This finding clearly proves that in the parallel approach a transthioesterification reaction also takes place and that the formation of Z-Ala-SCe is crucial for mediating the subsequent enzymatic reaction. Analysis of the kinetics of Z-Ala-SCe formation reveals however that only a negligible low concentration of the intermediate ester is virtually detectable during the course of reaction. This indicates an enzymatic hydrolysis of Z-Ala-SCe immediately after its formation, suggesting that the transthioesterification reaction is the slowest step. By contrast, the correlation between the rate of the whole reaction and the enzyme concentration indicates that the rate of Z-Ala-SCe formation is directly controlled by the rate of its own consumption by the enzyme that finally makes it likely that the enzymatic reaction is actually rate-limiting. In general, besides Z-Ala-SMe, similar results were found for 3-mercaptopropionic acid-mediated transthioesterifications of all other Z-Xaa-SMe esters. Despite the structural diversity, this also holds for reactions with benzyl mercaptan

and 4-guanidinomercaptophenol that used α-chymotrypsin and trypsin as the biocatalyst, respectively. Differences were limited solely to the rate of spontaneous (nonenzyme-coupled) transthioesterifications with 4-guanidinomercaptophenol, which proceeded about 5-fold slower than those with 3-mercaptopropionic acid and benzyl mercaptan. On one hand, these findings prove that beside the carboxyethyl thioesters the two aromatic ester types also behave as substrate mimetics; on the other hand, they suggest that the function of the transthioesterification approach is rather insensitive toward the acyl donor's sequence, the structure of the added thiol and the nature of the enzyme as well.

Next we have investigated the utility of the transthioesterification concept for enzymatic peptide synthesis. For this purpose, the general course of the sequential and parallel transthioesterification approach was first studied using the synthesis of Z-Ala-Leu-Gly-NH$_2$ from Z-Ala-SMe/Z-Ala-SCe and the dipeptide H-Leu-Gly-NH$_2$ catalyzed by V8 protease as an example reaction. The two syntheses were performed with identical reactant, thiol and enzyme concentrations and differed only in the time point of enzyme addition. Furthermore, two sets of control reactions, one without thiol and the other without enzyme were analyzed, which generally gave no products. The timelines of the enzyme- and thiol-containing reactions are illustrated in Figure 2, A and B, for the sequential and parallel
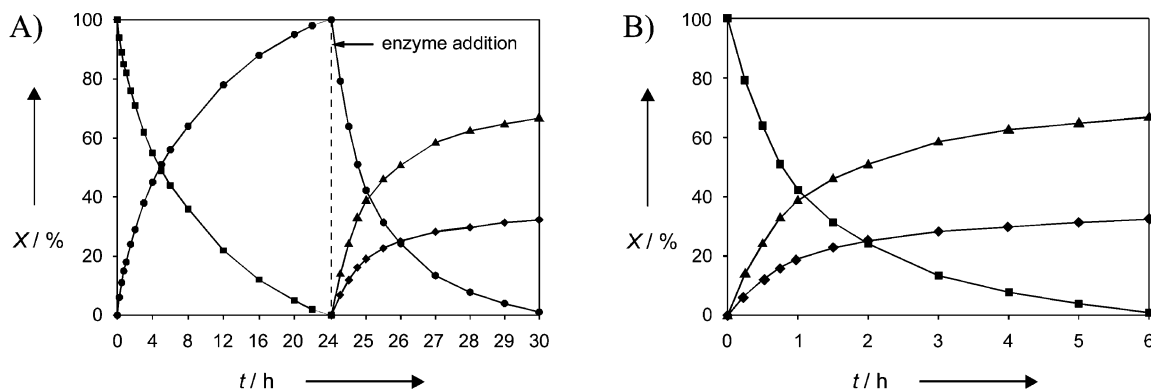
Figure 2. Course of the V8 protease-catalyzed coupling of Z-Ala-SMe/Z-Ala-SCe with H-Leu-Gly-NH$_2$ by using the sequential (A) and parallel (B) transthioesterification approach. Conditions: 0.2 M Hepes buffer, pH 8.0, 2.5% DMF, 37 °C, [Z-Ala-SMe]: 4 mM, [3-mercaptopropionic acid]: 4 mM, [H-Leu-Gly-NH$_2$]: 20 mM, [V8 protease]: 1 × 10^{-5} M. (-■-), Z-Ala-SMe; (-●-), Z-Ala-SCe; (-▲-), Z-Ala-Leu-Gly-NH$_2$; (-◆-), Z-Ala-OH. X = relative concentration.

approach, respectively. In the case of the sequential approach, the parent Z-Ala-SMe ester was preincubated with 3-mercaptopropionic acid, allowing spontaneous formation of Z-Ala-SCe. After about 24 h the thiol exchange and, hence, the formation of the substrate mimetic was found to be complete. The enzyme was then added, which resulted in the formation of the desired tripeptide product in about 70% yield after complete ester consumption in 6 h. Although this result proves the general function of the sequential approach to peptide synthesis, the reaction requires 30 h in total for completion, which is mainly the result of the rather slow spontaneous transthioesterification step. Acceleration of the latter can be achieved, however, by using an excess of the added thiol, as already shown above for the hydrolysis reactions (cf. Figure 1A), or simply by applying the parallel approach, as illustrated in Figure 2B. In fact, adding the enzyme parallel to the thiol at the beginning of the reaction results in a synthesis course that is virtually identical to that of the sequential approach but lacks the 24 h transthioesterification phase. Accordingly, the whole synthesis process is completed after 6 h and, hence, proceeds as fast as the reaction beginning with Z-Ala-SCe directly (cf. Figure 2A). In the same way, the product yields of the sequential and parallel approach are substantially identical. These findings indicate that for either approach, the additional transthioesterification step has no influence on the reaction yield. Moreover, in the case of the parallel approach, no effect of the preceding transthioesterification on the total rate of the synthesis reaction became evident, which in the end confirms that the thiol exchange is not rate-limiting. A further advantage of the parallel over the sequential synthesis is the ease of practicability since it requires only the control of the coupling reaction itself without the need for analyzing the progress of the preceding transthioesterification step.

To investigate the universality of the transthioesterification approach to peptide synthesis, we expanded our studies to the next Z-Xaa-SMe esters and the already specified enzyme/substrate mimetic systems. In addition, a respective specific amino acid-containing Z-Xaa-SMe ester was used for each enzyme to estimate whether the parent SMe esters could act directly as classical acyl donors without a preceding transthioesterification. As the acyl acceptors, several amino acid amides and peptides were exemplarily selected according to the already

known secondary specificities of the respective enzymes.[12] For reasons of comparison, analogous reactions were carried out using both the parallel and sequential approach. Although less efficient from a practical point of view, the latter allows for the separate quantification of the transthioesterification and the subsequent peptide coupling reaction. Hence, conclusions can be made on the influence of the transthioesterification step on the efficiency of the parallel approach to peptide synthesis simply by comparison of the two resulting data sets. To exclude potential interferences by spontaneous reactions, additional control experiments without thiol and enzyme were performed, which again gave no hints of any spontaneous product formation. The results obtained for the enzymatic reactions are summarized in Table 1. In general, the data document that with all enzyme/substrate mimetic systems and Z-Xaa-SMe esters, the expected peptide products could be synthesized and that the sequential and parallel approach gave identical yields. Peptide product formations were also found for reactions using the specific amino acid-containing esters, demonstrating the general capability of methyl thioesters to act as suitable conventional-type acyl donors in peptide synthesis. Differences in product yields of about 35%, caused by the individual amino acid moiety of the acyl donor, and of less than 10%, caused by the acyl acceptors used, were also found in previous studies for reactions with preformed substrate mimetics and, thus, are not typical for in situ generated substrate mimetics.[3c,8a,10a] On analysis of the effect of the additional transthioesterification step on the rate of reactions performed via the parallel approach, in neither case could a significant decrease compared to the corresponding sequential reactions be observed. In conclusion, these findings confirm the preliminary results already discussed above for the synthesis of Z-Ala-Leu-Gly-NH$_2$ and in the end indicate the general practicability of the transthioesterification concept for enzymatic peptide synthesis.

As a final step, we investigated the function of the approach to the ligation of elongated peptide fragments. The preceding chemical synthesis of the two starting fragments was achieved by using standard solid-phase Fmoc-peptide synthesis protocols. These utilized either standard Wang-resin[13] for the preparation

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(13) Wang, S. S. *J. Am. Chem. Soc.* **1973**, *95*, 1328.

Table 1. Peptide Coupling of in Situ Generated Substrate Mimetics and Specific Amino Acid-Containing Z-Xaa-SMe Esters Catalyzed by V8 Protease, Trypsin, and α-Chymotrypsin Using the Sequential and Parallel Transthioesterification Approach[a]

acyl donor	acyl acceptor	enzyme	product	yield [%][b]
Z-Glu-SMe[c]	Leu-Gly-NH2	V8[d]	Z-Glu-Leu-Gly-NH2	85[10a]
Z-Glu-SMe[c]	Leu-Ala-Ala-Ala-Gly	V8[d]	Z-Glu-Leu-Ala-Ala-Ala-Gly	92
Z-Pro-SMe/SCe	Leu-Gly-NH2	V8[d]	Z-Pro-Leu-Gly-NH2	59
Z-Pro-SMe/SCe	Leu-Ala-Ala-Ala-Gly	V8[d]	Z-Pro-Leu-Ala-Ala-Ala-Gly	56
Z-Phe-SMe/SCe	Leu-Gly-NH2	V8[d]	Z-Phe-Leu-Gly-NH2	89
Z-Phe-SMe/SCe	Leu-Ala-Ala-Ala-Gly	V8[d]	Z-Phe-Leu-Ala-Ala-Ala-Gly	84
Z-Lys-SMe/SCe	Leu-Gly-NH2	V8[d]	Z-Lys-Leu-Gly-NH2	56
Z-Lys-SMe/SCe	Leu-Ala-Ala-Ala-Gly	V8[d]	Z-Lys-Leu-Ala-Ala-Ala-Gly	55
Z-Ala-SMe/SCe	Leu-Gly-NH2	V8[d]	Z-Ala-Leu-Gly-NH2	67
Z-Ala-SMe/SCe	Leu-Ala-Ala-Ala-Gly	V8[d]	Z-Ala-Leu-Ala-Ala-Ala-Gly	67
Z-Lys-SMe[c]	Leu-NH2	tp[e]	Z-Lys-Leu-NH2	76
Z-Lys-SMe[c]	Met-Ala-Ala-Ala-Gly	tp[e]	Z-Lys-Met-Ala-Ala-Ala-Gly	83
Z-Pro-SMe/SGp	Leu-NH2	tp[e]	Z-Pro-Leu-NH2	82
Z-Pro-SMe/SGp	Met-Ala-Ala-Ala-Gly	tp[e]	Z-Pro-Met-Ala-Ala-Ala-Gly	91
Z-Phe-SMe/SGp	Leu-NH2	tp[e]	Z-Phe-Leu-NH2	79
Z-Phe-SMe/SGp	Met-Ala-Ala-Ala-Gly	tp[e]	Z-Phe-Met-Ala-Ala-Ala-Gly	86
Z-Glu-SMe/SGp	Leu-NH2	tp[e]	Z-Glu-Leu-NH2	75
Z-Glu-SMe/SGp	Met-Ala-Ala-Ala-Gly	tp[e]	Z-Glu-Met-Ala-Ala-Ala-Gly	82
Z-Ala-SMe/SGp	Leu-NH2	tp[e]	Z-Ala-Leu-NH2	68
Z-Ala-SMe/SGp	Met-Ala-Ala-Ala-Gly	tp[e]	Z-Ala-Met-Ala-Ala-Ala-Gly	71
Z-Phe-SMe[c]	Met-NH2	ct[f]	Z-Phe-Met-NH2	81
Z-Phe-SMe[c]	Arg-Ala-Ala-Ala-Gly	ct[f]	Z-Phe-Arg-Ala-Ala-Ala-Gly	92
Z-Pro-SMe/SBn	Met-NH2	ct[f]	Z-Pro-Met-NH2	91
Z-Pro-SMe/SBn	Arg-Ala-Ala-Ala-Gly	ct[f]	Z-Pro-Arg-Ala-Ala-Ala-Gly	94
Z-Lys-SMe/SBn	Met-NH2	ct[f]	Z-Lys-Met-NH2	87
Z-Lys-SMe/SBn	Arg-Ala-Ala-Ala-Gly	ct[f]	Z-Lys-Arg-Ala-Ala-Ala-Gly	92
Z-Glu-SMe/SBn	Met-NH2	ct[f]	Z-Glu-Met-NH2	89
Z-Glu-SMe/SBn	Arg-Ala-Ala-Ala-Gly	ct[f]	Z-Glu-Arg-Ala-Ala-Ala-Gly	93
Z-Ala-SMe/SBn	Met-NH2	ct[f]	Z-Ala-Met-NH2	81
Z-Ala-SMe/SBn	Arg-Ala-Ala-Ala-Gly	ct[f]	Z-Ala-Arg-Ala-Ala-Ala-Gly	87

[a] Reactions were performed in 0.2 M Hepes buffer, pH 8.0, 2.5% DMF at a concentration of 4 mM acyl donor, 20 mM acyl acceptor, 8 mM thiol (basic conditions). [b] Identical yields for the sequential and parallel approach were obtained in all cases. [c] Reactions were performed under kinetic control and were analyzed at the product optimum before the secondary hydrolysis of the products started. [d] V8, V8 protease; specific conditions: 37 °C, enzyme concentration between 3 and 10 x 10^{-6} M for substrate mimetics and 1×10^{-8} M for Z-Glu-SMe, thiol: 3-mercaptopropionic acid, reaction time of the parallel approach: 2−8 h, reaction time of transthioesterification: 16−24 h. [e] tp, trypsin; specific conditions: 0.1 M NaCl, 10 mM CaCl$_2$, 25 °C, concentrations of enzyme: 1.5×10^{-5} M for substrate mimetics and 1×10^{-8} M for Z-Lys-SMe, thiol: 4-guanidinomercaptophenol, reaction time of the parallel approach: 4−10 h, reaction time of transthioesterification: 48−120 h. [f] ct, α-chymotrypsin; specific conditions: 0.1 M NaCl, 10 mM CaCl$_2$, 25 °C, concentrations of enzyme: 2×10^{-5} M for substrate mimetics and 1×10^{-7} M for Z-Phe-SMe, thiol: benzyl mercaptan, reaction time of the parallel approach: 2−6 h, reaction time of transthioesterification: 16−24 h.

of the *C*-terminal peptide fragment or Kenner's 4-sulfamylbutyryl aminomethyl safety catch-resin[14] for synthesizing the *N*-terminal peptide methyl thioester. The latter allows for elegant esterification of the peptide's *C*-terminal carboxylate after safety catch-linker activation simultaneously to the release of the peptide from the solid support (Scheme 3). Because the esterification reaction is based on a nucleophilic attack on an electrophilic carbonyl-carbon atom, amines and thiols were found to give the highest product yields.[15] Our attempts to release the peptide with methyl mercaptan used in its solid sodium methanethiolate form, however, were hampered by the low solubility of the sodium salt in DMF, which only led to inefficient ester synthesis. By contrast, the respective amino acid methyl thioesters are completely soluble and gave quantitative yields of peptide methyl thioesters in preliminary model syntheses. Therefore, we preferred the use of a presynthesized amino acid methyl thioester as the nucleophile for peptide release in the synthesis of the target peptide ester. Scheme 3 specifies the peptide fragments selected and illustrates the general course of the reactions. To demonstrate the benefits of

the transthioesterification method, the same *N*- and *C*-terminal peptide fragment was used for all ligation reactions, differing only in the nature of the added thiol and enzyme, respectively. Such a constellation can be considered as typical within screening studies for investigating the most suitable biocatalyst for an intended synthesis. Solid-phase synthesis, peptide release, deprotection, and purification led to high yields of the two peptide fragments **6** and **10**, which were subsequently used for the enzymatic ligation reactions. The latter were performed as described above, using the parallel as well as the sequential transthioesterification approach. For synthesis-economy reasons, only the concentration of the acyl acceptor (**10**) was decreased from 20 to 10 mM, which corresponds to a ratio between **6** and **10** of 1 to 2.5. In the case of the parallel approach, after 2 h in the reaction with V8 protease, 3 h in that of trypsin and 1 h in the synthesis with α-chymotrypsin, complete consumption of **6** accompanied by the formation of the ligation product **11** was found. Figure 3 shows selected HPLC profiles that illustrate the well-defined course of catalysis, the example being the α-chymotrypsin-mediated synthesis. As for the efficiency of ligation reactions, with V8 protease 64%, with trypsin 68% and with α-chymotrypsin 75% of peptide **11** could be obtained, reflecting the distinct suitability of the individual enzymes for this ligation reaction. Nonquantitative coupling yields are based exclusively on competitive hydrolysis of **6**, while no further side products could be detected. Reactions using the sequential

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Scheme 3. Peptide Fragment Ligations via Combination of the Transthioesterification and Alkanesulfonamide Safety Catch Solid-Phase Peptide Synthesis Approach Using V8 Protease, α-Chymotrypsin and Trypsin as the Ligation Catalysts[a]
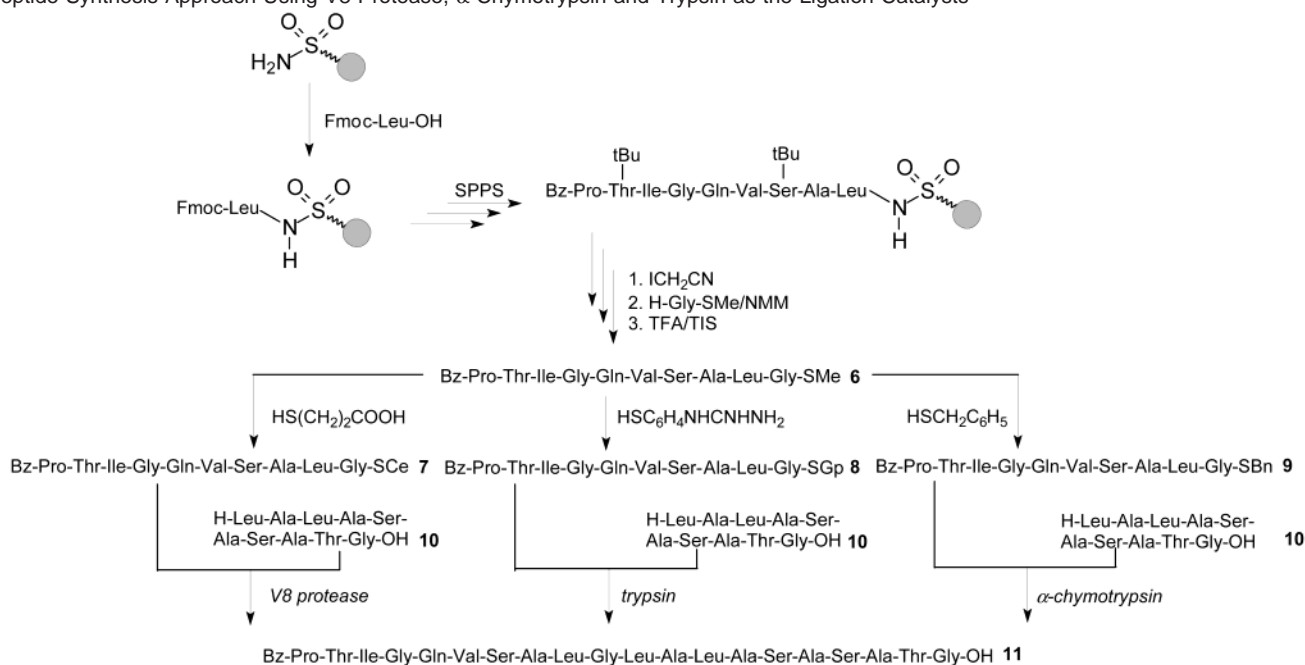
[a] Conditions of ligation reaction for V8 protease: 0.2 M Hepes buffer, pH 8.0, 2.5% DMF, 37 °C; for trypsin and α-chymotrypsin: 0.2 M Hepes buffer, pH 8.0, 0.1 M NaCl, 10 mM CaCl$_2$, 2.5% DMF, 25 °C. Concentration of reactants: [acyl donor]: 4 mM, [acyl acceptor]: 10 mM, [thiol]: 8 mM, [V8 protease]: 4×10^{-6} M, [α-chymotrypsin]: 2×10^{-5} M, [trypsin]: 1.5×10^{-5} M.
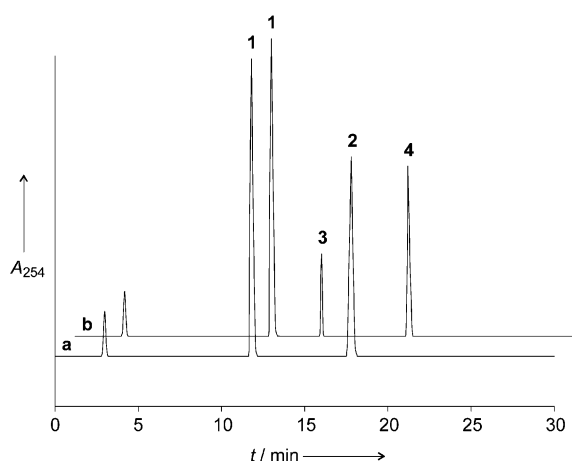
Figure 3. Analysis of the α-chymotrypsin-catalyzed coupling of Bz-Pro-Thr-Ile-Gly-Gln-Val-Ser-Ala-Leu-Gly-SMe/-SBn with H-Leu-Ala-Leu-Ala-Ser-Ala-Ser-Ala-Thr-Gly-OH by HPLC. (a) Before addition of enzyme; (b) after 1 h. (1) Benzyl mercaptan; (2) Bz-Pro-Thr-Ile-Gly-Gln-Val-Ser-Ala-Leu-Gly-SMe; (3) Bz-Pro-Thr-Ile-Gly-Gln-Val-Ser-Ala-Leu-Gly-OH; (4) Bz-Pro-Thr-Ile-Gly-Gln-Val-Ser-Ala-Leu-Gly-Leu-Ala-Leu-Ala-Ser-Ala-Ser-Ala-Thr-Gly-OH. A$_{254}$ = absorbance at 254 nm. Conditions: 0.2 M Hepes buffer, pH 8.0, 0.1 M NaCl, 10 mM CaCl$_2$, 2.5% DMF, 25 °C, [acyl donor]: 4 mM, [acyl acceptor]: 10 mM, [benzyl mercaptan]: 8 mM, [α-chymotrypsin]: 2×10^{-5} M.

transthioesterification approach led to identical product yields in all three cases, while the elongated reaction times due to the preceding spontaneous transthioesterification were again the only difference between the two approaches.

In summary, we have described an efficient synthesis concept for the in situ preparation of substrate mimetics using amino acid and peptide methyl thioesters as the universal precursors. The latter are readily available by standard chemical peptide synthesis procedures and can be used directly as classical acyl donor components for protease-catalyzed peptide synthesis.

Alternatively, they allow for efficient transthioesterification in sequential as well as parallel to the subsequent enzymatic coupling reaction. Independent of the transthioesterification approach used, the resulting spontaneously formed substrate mimetics act as efficient acyl donor components for all enzymes tested and show the typical behavior of substrate mimicry enabling the irreversible coupling of nonspecific native peptide bonds. In no case could an effect of the preceding transthioesterification reaction on the final product yield be observed. On the contrary, significant differences in the rate of reactions were found. While in sequential syntheses the thiol exchange is the rate-limiting step of reaction, the parallel approach enables reaction rates, which are virtually as fast as those starting from the respective preformed substrate mimetic directly. The consumption of the substrate mimetic by the enzyme after its in situ formation, which can be considered the driving force of the accelerated transthioesterification, simultaneously shortens the substrate mimetic's lifetime. This should allow for the use of stronger activated and, thus, higher reactive substrate mimetic types in this system, which would undergo a rapid hydrolysis under conventional conditions. From a synthetic point of view, the use of stronger activated esters should raise the synthesis rate and, furthermore, could allow for the efficient coupling of acyl donors containing originally slow-reacting acyl moieties. Despite the synthesis of higher activated esters, the need for synthesizing only one precursor ester generally reduces the synthetic efforts to a minimum and simplifies the screening for novel and more efficient acyl donor ester types significantly. Similarly, it allows for a fast screening of the most suitable biocatalyst for an intended synthesis as well, which is time-consuming and inefficient when conventional synthesis approaches are used. Besides the proteases selected, the spectrum of suitable enzymes can be easily enlarged, for example, by further wild-type enzymes such as subtilisin and thrombin,[8c]

by enzyme variants such as subtiligase, which is known for the acceptance of benzyl thioesters,[16] or by our recently optimized trypsin variants,[17] which react efficiently with SGp-esters. Furthermore, it can be expected that the approach is not only useful for enzymatic peptide synthesis but also for the kinetic resolution of racemic compounds. For the latter use, a significant influence of the nature of the ester leaving group on the enantioselectivity of proteases was found,[18] which could easily be specified with the approach presented. These characteristics qualify the method as a useful and broadly applicable synthesis concept in biocatalysis, addressing the main conflict of high specificity and limited universality of enzymatic reactions.

Experimental Section

Materials. TPCK-treated bovine trypsin (EC 3.4.21.4., product code (pc): T-1426), TLCK-treated bovine α-chymotrypsin (EC 3.4.21.1., pc: C-3142) and V8 protease (EC 3.4.21.19., pc: 45172) were obtained from Fluka or Sigma. Proteases were used without further purification. Amino acid derivatives, amides, 4-aminomercaptophenol, benzoic acid, benzyl mercaptan, coupling reagents, 3-mercaptopropionic acid, thiophenol, *N*,*N*′-bis-(*tert*-butoxycarbonyl)-*N*-(trifluoromethylsulfonyl)guanidine, sodium methanethiolate, and 4-sulfamylbutyryl AM resin were products of Bachem, Fluka, Merck, Aldrich or Novabiochem. If not otherwise stated, all reagents were of the highest available commercial purity. Solvents were purified and dried by usual methods.

Chemical Syntheses of Amino Acid Methyl Thioesters. N^α-Protected amino acid methyl thioesters were synthesized by coupling of the respective *N*-terminal and side-chain protected amino acid with sodium methanethiolate using the mixed anhydride method (isobutyl-chloroformiate/NEM).[19] Following removal of the side-chain protection, the products were precipitated and washed with dry diethyl ether. The yields of recovered esters ranged between 80 and 90%.

Z-Ala-SMe: ^{1}H NMR (300 MHz) δ 1.26 (d, 3H), 2.20 (s, 3H), 4.18 (m, 1H), 5.07 (s, 2H), 7.35 (m, 5H), 8.06 (d, 1H); elementary analysis calcd (%) for $C_{12}H_{15}NO_3S$ (253.3): C 56.90, H 5.97, N 5.53, found: C 57.10, H 5.77, N 5.52; MS, *m/z*: 252 $[M - H]^+$.

Z-Glu-SMe × 0.7 H₂O: ^{1}H NMR (300 MHz) δ 2.19 (s, 3H), 2.30 (m, 4H), 4.18 (m, 1H), 5.04 (s, 2H), 7.35 (m, 5H), 8.05 (d, 1H), 12.10 (s, 1H); elementary analysis calcd (%) for $C_{14}H_{18.4}NO_{5.7}S$ (324.0): C 51.90, H 5.72, N 4.32, found: C 52.09, H 5.83, N 4.11; MS, *m/z* calcd for $C_{14}H_{17}NO_5S$ (311.1): 312 $[M + H]^+$.

Z-Lys-SMe × 1 TFA × 1 H₂O: ^{1}H NMR (300 MHz) δ 1.53 (m, 6H), 2.20 (s, 3H), 2.74 (m, 2H), 4.08 (m, 1H), 5.08 (d, 2H), 7.34 (m, 5H), 7.68 (s, 2H), 8.04 (d, 1H); elementary analysis calcd (%) for $C_{17}H_{25}F_3N_2O_6S$ (442.5): C 46.15, H 5.70, N 6.33, found: C 45.50, H 5.57, N 6.59; MS, *m/z* calcd for $C_{15}H_{22}N_2O_3S$ (310.1): 309 $[M - H]^+$.

Z-Phe-SMe: ^{1}H NMR (300 MHz) δ 2.23 (s, 3H), 2.94 (m, 2H), 4.35 (m, 1H), 5.00 (s, 2H), 7.29 (m, 10H), 8.13 (d, 1H); elementary analysis calcd (%) for $C_{18}H_{19}NO_3S$ (329.4): C 65.63, H 5.81, N 4.25, found: C 65.70, H 5.62, N 4.12; MS, *m/z*: 328 $[M - H]^+$.

Z-Pro-SMe: ^{1}H NMR (300 MHz) δ 2.05 (m, 7H), 3.47 (m, 2H), 4.46 (m, 1H), 5.11 (s, 2H), 7.34 (m, 5H); elementary analysis calcd (%) for $C_{14}H_{17}NO_3S$ (279.4): C 60.19, H 6.13, N 5.01, found: C 59.50, H 6.01, N 5.04; MS, *m/z*: 278 $[M - H]^+$.

Boc-Gly-SMe: ^{1}H NMR (300 MHz) δ 1.34 (s, 9H), 2.19 (s, 3H), 3.90 (d, 2H), 7.99 (t, 1H); elementary analysis calcd (%) for C_8H_{15}-NO_3S (205.3): C 46.81, H 7.37, N 6.82, found: C 47.02, H 7.21, N 6.59; MS, *m/z*: 206 $[M + H]^+$.

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General Procedure for the Preparation of Peptides. The peptides MAAAG, RAAAG, LAAAG, and LALASASATG were synthesized with a semiautomatic batch synthesizer SP 650 (Labortech AG) using *p*-alkoxybenzyl alcohol resin, according to Wang[13] and standard Fmoc chemistry. Peptides were precipitated with dry diethyl ether. The identity and purity of all derivatives were verified by analytical HPLC (220 nm), NMR, thermospray mass spectroscopy, and elementary analysis. In all cases, satisfactory analytical data were found (±0.4% for C, H, N).

Protocol for the Synthesis of 4-Guanidinomercaptophenol. 4-Guanidinomercaptophenol was prepared from 4-[*N*′,*N*′′-bis(Boc)guanidino]-mercaptophenol by deprotection of the guanidino functionality with TFA and precipitation with dry diethyl ether. The latter was synthesized from *N*,*N*′-bis(Boc)-*N*-(trifluoromethylsulfonyl)guanidine (1 equiv) and 4-aminomercaptophenol (2 equiv) in absolute THF at room temperature. After complete consumption of the guanidine, the reaction mixture was evaporated, and the resulting crude product was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate (3/1) as the eluent.

4-[*N*′,*N*′′-Bis(Boc)guanidino]mercaptophenol: ^{1}H NMR (300 MHz) δ 1.43 (s, 9H), 1.53 (s, 9H), 5.46 (s, 1H), 7.30/7.44 (m/m, 4H), 9.96 (s, 1H), 11.43 (s, 1H); elementary analysis calcd (%) for $C_{17}H_{25}N_3O_4S$ (367.5): C 55.57, H 6.86, N 11.43, found: C 55.13, H 6.72, N 11.79; MS, *m/z*: 368 $[M + H]^+$.

4-Guanidinomercaptophenol × 1 TFA: ^{1}H NMR (300 MHz) δ 5.66 (s, 1H), 7.15/7.38 (m/m, 4H), 7.52 (s, 3H), 9.85 (s, 1H); elementary analysis calcd (%) for $C_9H_{10}F_3N_3O_2S$ (281.3): C 38.43, H 3.58, N 14.94, found: C 38.98, H 3.41, N 14.67; MS, *m/z* calcd for $C_7H_9N_3S$ (167.1): 168 $[M + H]^+$.

Preparation of Bz-Pro-Thr-Ile-Gly-Gln-Val-Ser-Ala-Leu-Gly-SMe. The peptide methyl thioester was synthesized using the alkane-sulfonamide safety catch-linker method.[14] The first amino acid, Fmoc-Leu-OH, was loaded to commercially available 4-sulfamylbutyryl aminomethyl resin by one standard PyBOP/DIEA coupling step resulting in a loading yield of 83%. All remaining amino acids were coupled by stepwise solid-phase method using PyBOP/NMM activation protocols. At the end of synthesis the N^α-amino group of the resin-bound peptide was deprotected with piperidine/DMF and subsequently benzoylated using benzoic acid. Alkylation of the linker's sulfonamide functionality was achieved using iodoacetonitrile according to the procedure of Backes et al.[15b,c] leading to the respective activated *N*,*N*-cyanomethylacylalkanesulfonamide ester. The peptide was liberated from the resin by adding a 5-fold excess of TFA·H-Gly-SMe, providing the fully protected peptide methyl thioester. The amino acid ester H-Gly-SMe was prepared from Boc-Gly-SMe by deprotection of the N^α-amino group with TFA and precipitation with dry diethyl ether. Neutralization of the amino acid trifluoroacetate was achieved by adding of NMM (1 equiv). Final deprotection of the peptide's side-chain functionalities by TFA/TIS/water treatment and purification of the crude product by preparative HPLC resulted in the N^α-Bz-protected decapeptide methyl thioester. Its identity and purity was checked by analytical HPLC (220 nm), elementary analysis and mass spectroscopy.

Bz-Pro-Thr-Ile-Gly-Gln-Val-Ser-Ala-Leu-Gly-SMe: elementary analysis calcd (%) for $C_{49}H_{77}N_{11}O_{14}S$ (1076.3): C 54.68, H 7.21, N 14.32, found: C 53.99, H 7.01, N 13.90; MS, *m/z*: 1076 $[M + H]^+$.

Transthioesterification Reactions. Spontaneous thiol exchange reactions were performed at 37 °C using an assay mixture containing 0.2 M Hepes buffer, pH 8.0 and 2.5% DMF which was added to mediate complete solubility of the methyl thioesters. The final concentration of esters was 4 mM and that of 3-mercaptopropionic acid varied between 4 and 40 mM. After thermal equilibration of the assay mixture, the reactions were started by addition of the thiol. Model V8 protease-catalyzed transthioesterification reactions were carried out in analogous reaction mixtures. After incubation of the assay mixtures for several minutes at 37 °C, the reactions were initiated by enzyme addition, resulting in V8 protease concentrations between 1 and 10 μM. The

rate of transthioesterification reactions was analyzed by RP-HPLC determining the disappearance of the methyl thioesters. For this purpose, at defined time intervals aliquots were withdrawn, diluted with a quenching solution containing 50% aqueous methanol and 5% trifluoroacetic acid, and immediately analyzed. The transthioesterification reactions with benzyl mercaptan and 4-guanidinomercaptophenol were performed analogously by using a slightly different buffer system adapted to the enzyme requirements (0.2 M Hepes buffer, pH 8.0, 0.1 M NaCl, 10 mM CaCl$_2$, 2.5% DMF) at 25 °C. Similar conditions were used for the respective enzymatic reactions in which trypsin and α-chymotrypsin were used in a concentration range between 0.2 and 20 μM.

Enzymatic Syntheses. Enzymatic peptide synthesis reactions were performed in 0.2 M Hepes buffer, pH 8.0 containing 0.1 M NaCl and 10 mM CaCl$_2$ at 25 °C (trypsin and α-chymotrypsin) and 0.2 M Hepes buffer, pH 8.0 at 37 °C (V8 protease). Stock solutions of acyl donor esters (16 mM) were prepared in distilled water containing 10% (v/v) DMF as cosolvent. Acyl acceptor components (stock solution 40 mM) and thiol additives (stock solution 100 mM) were dissolved in 0.4 M Hepes buffer, pH 8.0, 0.2 M NaCl, 20 mM CaCl$_2$ (trypsin and α-chymotrypsin), and 0.4 M Hepes buffer, pH 8.0 (V8 protease). To adjust a pH value of 8.0, appropriate equivalents of NaOH were added to the stock solutions of the acyl acceptors and thiols. If not otherwise stated, the final concentration of acyl donor, thiol component and acyl acceptor were 4, 8, and 20 mM, respectively. The latter was calculated as free, N^α-unprotected species according to the Henderson−Hasselbalch equation $[HN]_0 = [N]_0/(1 + 10^{pK-pH})$. The p$K$ values of the α-amino group of the acyl acceptor components were determined by inflection point titration on a Video titrator VIT 90 (Radiometer, Denmark). After thermal equilibration of assay mixtures, reactions using the parallel approach were initiated by addition of the respective enzyme at final concentrations between 3 and 10 μM for V8 protease, 15 μM for trypsin, and 20 μM in the case of α-chymotrypsin. For parallel syntheses, reaction times between 1 and 10 h led to complete ester consumption. In sequential syntheses, the methyl thioester was preincubated with the respective thiol. After complete transthioesterification, acyl acceptor and enzyme were added to the reaction mixture. Elongated reaction times between 17 h and up to 5 days led to complete ester consumption. Progress of all reactions was analyzed by RP-HPLC. For this purpose, at certain time intervals aliquots were withdrawn and diluted with a quenching solution containing 50% aqueous methanol and 5% trifluoroacetic acid. The control reactions lacking either the thiol or the enzyme were performed under identical conditions. The identities of the formed thioester intermediates and the resulting peptide products were established by thermospray mass spectroscopy. The data reported are the average of at least three independent reactions.

HPLC Analyses. Enzymatic reactions were analyzed by analytical reversed phase HPLC using RP C18 and C8 columns (Merck, 5 μm, 25 cm × 0.4 cm, Grom Capcell, 5 μm, 25 cm × 0.4 cm) and eluted with various mixtures of acetonitrile/water containing 0.1% trifluoroacetic acid under gradient conditions at flow rates of 1.0 mL min^{-1}. Detection was carried out at 254 nm. Reaction rates and product yields were calculated from peak areas of acyl donor esters, hydrolysis and aminolysis products, respectively. Peptide purification was performed on a HPLC system with 220 nm UV detection, using a preparative RP C8 column (Merck, 7 μm, 25 cm × 2.5 cm) and gradient elution at a flow rate of 15 mL min^{-1}.