Protease Catalysis Mediated by a Substrate Mimetic: A Novel Enzymatic Approach to the Synthesis of Carboxylic Acid Amides**

Robert Günther[b] and Frank Bordusa*[a, b]

Abstract: We present a protease-based method for the coupling of non-coded and non-amino-acid-derived amines with carboxy components. The key feature of this approach is the combination of the substrate-mimetic strategy with the ability of the cysteine protease clostripain to accept a wide spectrum of amines. Firstly, we tested the use of the 4-guanidinophenyl ester leaving group to mediate acceptance of non-coded and non-amino-acid-derived acyl residues. This employed β-amino acid and simple carboxylic acid moieties as acyl donors, and several amino acid and peptide units as acyl acceptors. The study was completed by the use of non-amino-acid-derived acyl acceptors comprising simple amines, amino alcohols, and diamines. The results indicate that the approach presented is a useful strategy for the synthesis of peptide isosteres, peptide analogues, and organic amides. These last open a new range of synthetic applications of proteases completely beyond peptide synthesis, achieving efficient and selective acylations of non-amino-acid-derived amines under extraordinarily mild reaction conditions.

Keywords: acylations · bioorganic chemistry · clostripain · enzyme catalysis · proteases

Introduction

Since proteases catalyze in vitro not only the hydrolysis of peptides but also the reverse process, these enzymes can be used as biocatalysts for peptide bond formation.[1–3] Owing to the high degree of stereo- and regiospecificity of such reactions, enzymatic peptide coupling offers advantages over chemical methods; for example, there is no need for side-chain protection, stereochemistry is controlled during synthesis, and therefore, no racemization occurs. However, despite these undisputed advantages, the high substrate specificity of proteases seriously limits the choice of amino acid residues between which a peptide bond can be synthesized. Most importantly, the high primary specificity towards the acyl donor usually prevents the coupling of non-coded or unnatural carboxy components.[4, 5] In contrast, the secondary specificity toward the acyl acceptor is frequently more relaxed, allowing the coupling of a wider spectrum of unnatural amino moieties.[6–9] Nevertheless, the product yields usually drop with increasing degree of modification of both the amino acid side chain and the backbone structure, particularly in the P_1' position (nomenclature according to ref. [10]). Therefore, only the coupling of acyl acceptors closely related to preferred amino acid residues is of practical relevance.[11–17] The enzymatic synthesis of peptide isosteres or analogues, however, requires strategies which allow the coupling of a broader variety of modified amino acid derivatives and also non-amino-acid-derived units. In this context, the use of esterases and particularly of lipases seems to be of higher synthetic utility.[18–21] Due to the broader substrate specificities of these enzymes, there is greater structural diversity among the amines and esters undergoing the esterase- and lipase-catalyzed aminolysis reaction. However, this synthetic activity needs the presence of anhydrous solvents, as reactivity in aqueous media is frequently lower. Furthermore, the organic solvent itself considerably affects the activity of these enzymes, as well as the yield and regio- and stereospecificity of such reactions.[22] Thus, a very careful and often time-consuming optimization of reaction conditions is required; this prevents the use of esterases and lipases from being easily and universally applicable.

[a] Dr. F. Bordusa
Max Planck Society
Research Unit "Enzymology of Protein Folding"
Weinbergweg 22, D-06120 Halle/Saale (Germany)
Fax: (+49) 341-973-6998
E-mail: bordusa@uni-leipzig.de

[b] Dipl.-Chem. R. Günther
Department of Biochemistry
Faculty of Biosciences, Pharmacy and Psychology
University of Leipzig, Talstrasse 33
D-04103 Leipzig (Germany)

[**] Abbreviations used in this paper: Bz: benzoyl; DCC: *N,N'*-dicyclohexylcarbodiimide; DMAP: 4-dimethylaminopyridine; DMF: *N,N*-dimethylformamide; DTT: DL-dithiothreitol; Fmoc: fluoren-9-ylmethoxycarbonyl; HEPES: *N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid]; OGp: 4-guanidinophenyl ester; Pbu: 4-phenylbutyric acid; Tos: *p*-toluenesulfonic acid; Z: benzyloxycarbonyl. Enzyme: clostripain (EC 3.4.22.8).

  0947-6539/00/0603-0463 $ 17.50+.50/0

In this paper an alternative protease-mediated approach to the coupling of non-coded and non-amino-acid-derived amines with esters is reported. The key feature of this method is the combination of the substrate-mimetic strategy[23–25] with the use of clostripain, which possesses a broad tolerance toward amines. The approach takes advantage of the characteristic of substrate mimetics to direct the enzyme to recognize an alternative site on the acyl donor, specifically, to the 4-guanidinophenyl ester leaving group, mediating the acceptance of originally poorly reactive acyl moieties (Figure 1). The synthetic utility of this approach was investigated by enzymatic coupling of esters derived from β-amino acids and simple carboxylic acids. As acyl acceptors a wide range of amines comprising amino acid amides, peptides, simple amines, diamines, and various amino alcohols were used.

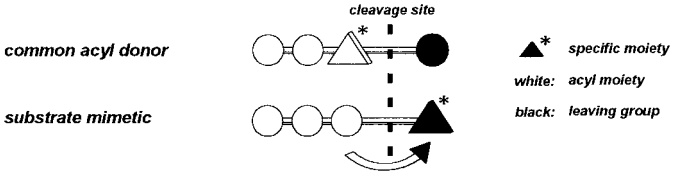
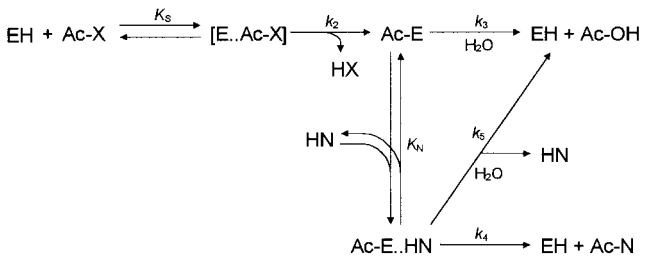


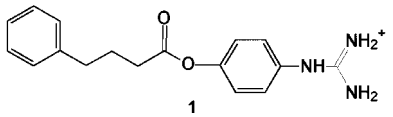
Figure 1. Schematic structure of common acyl-donor components versus reversed-type substrate mimetics.

Results and Discussion

Initially, the general ability of the 4-guanidinophenyl ester functionality to mediate the acceptance of non-coded and non-amino-acid-derived acyl components by clostripain was probed by model acyl-transfer experiments (Scheme 1). For this purpose reactions were performed using the acyl donors Pbu-OGp (**1**) and Bz-β-Ala-OGp (**2**), with several amino acid and peptide units as amino components. To control for spontaneous hydrolysis and aminolysis of the acyl-donor esters, parallel reactions without enzyme were analyzed. On the basis of these experiments nonenzymatic aminolysis could be ruled out and the extent of spontaneous hydrolysis was found to be less than 5%. The results observed for the enzyme-catalyzed reactions are summarized in Table 1, and Figure 2 illustrates typical time courses of these acyl transfer reactions. Generally the data show that productive binding and acylation of the enzyme, finally resulting in proteolytically stable amide products, occurs with the non-coded but naturally occurring β-Ala derivative as well as the non-amino-acid-derived 4-phenylbutyric acid ester. On analysis of the efficiency of catalysis, in all cases product yields within a range of 83 to 98% were reached, usually higher than 90%. Interestingly, comparison of the two acyl donors reveals differences in yields of only 7% at most. Thus, the efficiency of yield-determining deacylation of the acyl enzyme intermediate by the various amino components appears to be practically unaffected by the disparate nonspecific acyl residues. In the same way no differences were evident from data for analogous esters derived from coded amino acids,[26] indicating that clostripain generally does not discriminate between different acyl residues within the coupling reactions. This behavior also holds for coupling of proline amide (**4**, **11**) and proline-containing peptides (**5**, **6**, **12**, **13**) as amino



Scheme 1. Kinetic model for protease-catalyzed acyl transfer reaction. EH, free enzyme; Ac–X, acyl donor; HX, leaving group; Ac–E, acyl enzyme complex; Ac–OH, hydrolysis product; HN, acyl acceptor; Ac–N, aminolysis product.



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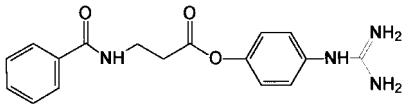

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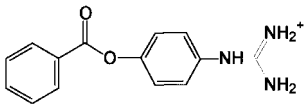

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Abstract in German: *Dieser Beitrag stellt ein Verfahren zur Protease-katalysierten Verknüpfung nichtcodierter und Nicht-aminosäure-artiger Amine als auch Carboxylkomponenten vor. Das wesentliche Merkmal dieses Ansatzes liegt in der Kombination der Substratmimetika-Strategie unter Verwendung der Cysteinprotease Clostripain, die ein breites Spektrum verschiedenster Amine akzeptiert. Zunächst wurde die Eignung der 4-Guanidinophenylester-Abgangsgruppe als „Vermittler“ für die Akzeptanz nichtnatürlicher Acylkomponenten durch die Protease untersucht. Hierzu wurden β-Aminosäure- und einfache Carbonsäure-Derivate als Carboxylkomponenten und ausgewählte Aminosäure- und Peptid-Derivate als Acylacceptoren eingesetzt. Komplettiert wurde die Studie durch Einsatz Nichtaminosäure-artiger Aminokomponenten wie einfache Amine, Aminoalkohole und Diamine. Die erhaltenen Ergebnisse belegen die Eignung des vorgestellten Ansatzes sowohl für die Synthese von Peptid-Isosteren und -Analoga als auch organischer Amide. Letzteres eröffnet ein völlig neues Anwendungsgebiet von Proteasen außerhalb der „klassischen“ Peptidsynthese zur effizienten und gleichzeitig selektiven Acylierung Nichtaminosäure-artiger Amine unter sehr milden Reaktionsbedingungen.*

0947-6539/00/0603-0464 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 3

Table 1. Clostripain-catalyzed coupling of Bz-β-Ala-OGp and Pbu-OGp with selected amino acid amids and peptides.[a]

Acyl donor	Acyl acceptor		Product		Yield [%]
Pbu-OGp	H-Leu-NH_2	(**1**)	Pbu-Leu-NH_2	(**15**)	98
Pbu-OGp	H-Lys-NH_2[b]	(**2**)	Pbu-Lys-NH_2	(**16**)	96
Pbu-OGp	H-Glu-NH_2[c]	(**3**)	Pbu-Glu-NH_2	(**17**)	87
Pbu-OGp	H-Pro-NH_2	(**4**)	Pbu-Pro-NH_2	(**18**)	83
Pbu-OGp	H-Ala-Pro-OH	(**5**)	Pbu-Ala-Pro-OH	(**19**)	93
Pbu-OGp	H-AAP-OH	(**6**)	Pbu-AAP-OH	(**20**)	86
Pbu-OGp	H-AFAAG-OH[d]	(**7**)	Pbu-AFAAG-OH	(**21**)	92
Bz-β-Ala-OGp	H-Leu-NH_2	(**8**)	Bz-β-Ala-Leu-NH_2	(**22**)	98
Bz-β-Ala-OGp	H-Lys-NH_2[b]	(**9**)	Bz-β-Ala-Lys-NH_2	(**23**)	93
Bz-β-Ala-OGp	H-Glu-NH_2[c]	(**10**)	Bz-β-Ala-Glu-NH_2	(**24**)	89
Bz-β-Ala-OGp	H-Pro-NH_2	(**11**)	Bz-β-Ala-Pro-NH_2	(**25**)	90
Bz-β-Ala-OGp	H-Ala-Pro-OH	(**12**)	Bz-β-Ala-Ala-Pro-OH	(**26**)	91
Bz-β-Ala-OGp	H-AAP-OH	(**13**)	Bz-β-Ala-AAP-OH	(**27**)	85
Bz-β-Ala-OGp	H-AFAAG-OH[d]	(**14**)	Bz-β-Ala-AFAAG-OH	(**28**)	93

[a] Conditions: 0.2 M HEPES buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 5% DMF, 25 °C, [acyl donor] = 2 mM, [acyl acceptor] = 25 mM. [b] [H-Lys-NH_2] = 15 mM. [c] [H-Glu-NH_2] = 50 mM. [d] [H-AFAAG-OH] = 4 mM.

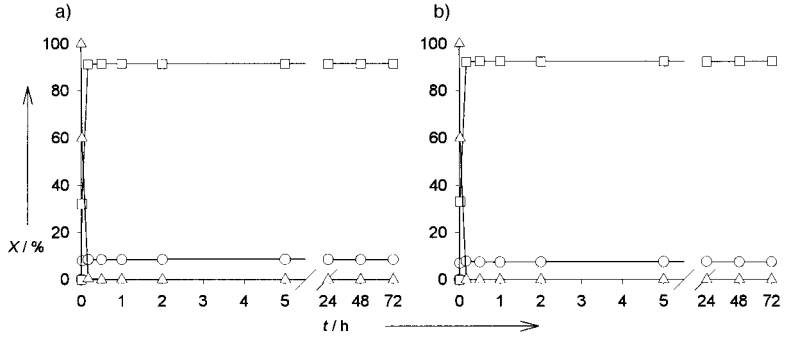

Figure 2. Course of the clostripain-catalyzed coupling of Bz-β-Ala-OGp and Pbu-OGp with H-Ala-Phe-Ala-Ala-Gly-OH. a) Bz-β-Ala-OGp; b) Pbu-OGp. △ Bz-β-Ala-OGp/Pbu-OGp; □ Bz-β-Ala-Ala-Phe-Ala-Ala-Gly-OH/Pbu-Ala-Phe-Ala-Ala-Gly-OH; ○ Bz-β-Ala-OH/Pbu-OH. Conditions: 0.2 M HEPES buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 5% DMF, 25 °C, [acyl donor] = 2 mM, [acyl acceptor] = 4 mM.

components. These are considerably more demanding than those of other amino acids because of the nonflexible and bulky nature of this secondary imino acid. Remarkably, a reduction in the excess of acyl acceptor, studied for reactions with the pentapeptide (**7**, **14**), also does not decrease the efficiency of coupling reactions with both esters. This atypical behavior indicates that achievement of satisfactory yields does not need a large excess of either reactant. This demonstrates the synthetic utility of clostripain for the coupling of more valuable carboxy and amino components too. Furthermore, in contrast to other biocatalytic and chemical methods, the synthetic utility was generally not constrained by the presence of additional unprotected functionalities, such as the ε-amino function of H-Lys-NH_2 or the γ-carboxylic acid group of H-Glu-NH_2. In both cases the expected amide products exclusively (**16**, **17**, and **23**, **24**) were formed, reflecting the high regiospecificity of the enzyme. In summary, the results of these model reactions show that non-coded and non-amino-acid-derived 4-guanidinophenyl esters are equally good substrates for clostripain-catalyzed acylations of amino acid and peptide derivatives.

Apart from the highly restricted primary specificity, clostripain possesses a broad tolerance toward the acyl acceptor. Besides the acceptance of practically all coded amino acids, this also includes the recognition of some D-configured counterparts.[27–30] Moreover, by the use of a wide range of non-coded acyl acceptors we found that, surprisingly, clostripain even tolerates amino components considerably different from coded amino acids, such as simple aliphatic, aromatic, or substituted amines including unnatural amino acids, amino alcohols, and diamines.[26] This unique specificity separates clostripain from most other known proteases, although only a little is known about the structural basis of this remarkable behavior. From the synthetic point of view, this broad specificity combined with the substrate-mimetic strategy might open an entirely new field of application of this protease, that of the synthesis of organic amides completely outside of peptide synthesis. To investigate whether this approach can be used in such a manner, enzymatic reactions were performed between 4-guanidinophenyl esters of benzoic acid (Bz-OGp, **3**) or 4-phenylbutyric acid (Pbu-OGp) and a number of non-amino-acid-derived amino components. The product yields of the appropriate reactions are listed in Table 2. Generally, the results convincingly show that clostripain is capable of catalyzing the coupling of both non-amino-acid-derived carboxy and amino components as indicated from the individual experiments. As found for reactions using coded amino-acid-derived acyl acceptors, the enzymatic acylation of unnatural amines also seems to be practically unaffected by the disparate acyl residues. As for the efficiency of catalysis, most enzymatic reactions lead to product yields higher than 70%. Accordingly, only small amounts of hydrolysis products (Bz-OH and Pbu-OH) were formed. These results are all the more impressive since only traces (0.22 to 3.7% depending on the respective p*K* value) of the amino components are unprotonated under the pH conditions used and, therefore, can serve as deacylating components. Unlike most chemical methods, in this case an increase of the pH value to deprotonate completely the amino group of the nucleophilic component is restricted by the limited intrinsic stability of enzyme and acyl donor ester. Figure 3 shows the dependence of the product yield on the concentration of pentylamine, which possesses the highest p*K* value (10.65) of all amines used. The plots for both acyl donor esters illustrate that even an initial nucleophile concentration of 4 mM, which corresponds to a concentration of unprotonated pentylamine of only 0.009 mM, already leads to product yields of approx-

Table 2. Clostripain-catalyzed coupling of non-amino-acid-derived carboxy and amino components.[a]

Acyl donor	Acyl acceptor		Product		Yield [%]
Pbu-OGp	H_2N	**(29)**	Pbu-NH	**(47)**	81
Pbu-OGp	H_2N	**(30)**	Pbu-NH	**(48)**	80
Pbu-OGp	H_2N	**(31)**	Pbu-NH	**(49)**	53
Pbu-OGp	H_2N, OH	**(32)**	Pbu-NH, OH	**(50)**	65
Pbu-OGp	H_2N, OH	**(33)**	Pbu-NH, OH	**(51)**	78
Pbu-OGp	H_2N, OH	**(34)**	Pbu-NH, OH	**(52)**	70
Pbu-OGp	H_2N, OH, OH	**(35)**	Pbu-NH, OH, OH	**(53)**	92
Pbu-OGp	H_2N, NH_2	**(36)**	Pbu-NH, NH_2	**(54)**	95
Pbu-OGp	HO	**(37)**	Pbu–O	**(55)**	n.s.
Bz-OGp	H_2N	**(38)**	Bz-NH	**(56)**	82
Bz-OGp	H_2N	**(39)**	Bz-NH	**(57)**	76
Bz-OGp	H_2N	**(40)**	Bz-NH	**(58)**	56
Bz-OGp	H_2N, OH	**(41)**	Bz-NH, OH	**(59)**	57
Bz-OGp	H_2N, OH	**(42)**	Bz-NH, OH	**(60)**	84
Bz-OGp	H_2N, OH	**(43)**	Bz-NH, OH	**(61)**	70
Bz-OGp	H_2N, OH, OH	**(44)**	Bz-NH, OH, OH	**(62)**	82
Bz-OGp	H_2N, NH_2	**(45)**	Bz-NH, NH_2	**(63)**	94
Bz-OGp	HO	**(46)**	Bz–O	**(64)**	n.s.

[a] Conditions: 0.2 M HEPES buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 5% DMF, 25 °C, [acyl donor] = 2 mM, [acyl acceptor] = 12 mM. n.s.: no synthesis.

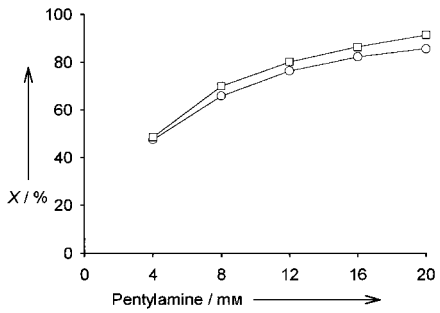

Figure 3. Influence of the pentylamine concentration on the product yield *x* of the clostripain-catalyzed coupling of Y-OGp with pentylamine. ○ Y = Bz; □ Y = Pbu. Conditions: 0.2 M HEPES buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 5% DMF, 25 °C, [acyl donor] = 2 mM; reaction time: about 15 min.

imately 50%. The data found for higher pentylamine concentrations reveal that an increase in the nucleophile excess results in nearly complete conversion of the acyl donor esters to the appropriate amides. Accordingly, from the synthetic point of view, the approach presented achieves highly efficient amide bond formation combined with extraordinarily mild reaction conditions. Moreover, neither additional hydroxy nor additional amine groups within the amino component seem to constrain this high synthetic utility. Only for the acylation of 2-aminoethanol (**32**, **41**) were slightly lower rates observed, whereas reactions with 3-amino-1,2-propanediol (**35**, **44**) as well as 1,3-diaminopentane (**36**, **45**) resulted in even higher product yields than found for the corresponding unsubstituted amines (**29**, **30** and **38**, **39**). Apart from this high efficiency, clostripain catalyzes acylations of substituted amines with a remarkably high degree of regiospecificity. As indicated by the lack of acceptance of 1-pentanol (**37**, **46**), for the amino alcohols no competitive formation of *O*-acylated products could be detected. In the same way, the acylation of ethanol, 1-propanol, and 1-octanol failed (data not shown), indicating that clostripain generally does not tolerate alcohols as deacylating components. Interestingly, the asymmetric 1,3-diaminopentane (**36**, **45**) was acylated by the enzyme exclusively on the amino group at position 1, with no acylation of the amino group at position 3. This finding indicates that clostripain accepts amines containing a neighboring branch less well than comparable nonbranched derivatives. Because of this distinct discrimination, clostripain should be a useful tool for selective acylations forming isomerically pure products, without great experimental efforts to protect additional functionalities.

In general, despite the serious limitations found for the classical approach of using proteases for selective C–N bond formation, the results demonstrate that the strategy of substrate mimetics enables clostripain to accept both non-coded and non-amino-acid-derived acyl components. On the basis of this strategy and the broad S′ subsite specificity of this enzyme, clostripain can be used as a suitable biocatalyst for the synthesis of peptide isosteres and analogues. Since organic amides can also be synthesized, the approach presented could even open up a new field of synthetic applications of proteases completely outside peptide synthesis. This offers efficient and selective organic amide bond formation under extraordinarily mild reaction conditions.

Experimental Section

Materials: Clostripain (EC 3.4.22.8) was a gift from Fluka Chemie (Switzerland) and had a specific activity of 100 U mg^{-1} (1 U = amount of enzyme that hydrolyzes 1 μmol min^{-1} N^{α}-Bz-L-arginine ethyl ester at pH 7.1 and 25 °C). Before use, the enzyme was activated for 2–3 h in the presence of 1.0 mM $CaCl_2$ containing 2.5 mM DTT. Amino acids, peptides, 4-aminophenol, DCC, DMAP, benzyl chloroformate (Z chloride), *S*-methylisothiourea, and Tos were purchased from commercial suppliers. All reagents were of the highest available commercial purity. Solvents were purified and dried by the usual methods. Mass spectra were recorded using thermospray ionization with a Hewlett Packard 5989 A instrument.

Chemical syntheses: 4-Guanidinophenyl esters were prepared by condensation of the appropriate carboxylic component and 4-[*N*′,*N*″-bis(Z)guanidino]phenol following the procedure described by Sekizaki et al.[31] Because of the more advantageous preparation and higher reactivity *N*,*N*′-bis(Z)-*S*-methylisothiourea was used for the amidination of 4-amino-

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phenol to synthesize 4-[*N*′,*N*″-bis(Z)guanidino]phenol[32] instead of 1-[*N*,*N*′-bis(Z)amidino]pyrazole. A final catalytic hydrogenation of the bis(Z)-protected esters results in the 4-*N*′,*N*″-deprotected 4-guanidinophenyl esters. The reaction yields were between 75 and 85%. Bz-*β*-Ala-OH was synthesized from the N^{α}-unprotected derivative by benzoylation using benzoyl chloride. The pentapeptide H-Ala-Phe-Ala-Ala-Gly-OH was prepared with a semiautomatic batch peptide synthesizer using *p*-alkoxybenzylalcohol resin, synthesized according to Wang[35] and standard Fmoc chemistry. The peptide was precipitated with dry diethyl ether. The identity and purity of all final products were checked by analytical HPLC at a wavelength of 220 nm, NMR, thermospray mass spectroscopy, and elemental analysis. In all cases, satisfactory elemental analysis data were found (±0.4% for C, H, N).

Enzymatic syntheses: Enzymatic reactions were performed in 0.2 M HEPES buffer, pH 8.0 containing 0.1 M NaCl, 0.01 M $CaCl_2$, and 5% DMF at 25 °C. Stock solutions of acyl donor esters (4 mM) were prepared in distilled water containing 10% DMF. Amino components were dissolved in 0.4 M HEPES buffer, pH 8.0, 0.2 M NaCl, and 0.01 M $CaCl_2$. To neutralize hydrochlorides or hydrobromides, appropriate equivalents of NaOH were added to the stock solutions of the amino components. If not otherwise stated, the final acyl donor concentration was 2 mM, and the final nucleophile concentration was 25 mM for amino acid and peptide derivatives and 12 mM in the case of the non-amino-acid-derived counterparts. The concentration of the amino-acid-derived acyl acceptors was calculated as free, $N\alpha$-unprotonated nucleophile concentration $[HN]_0$ according to the formalism of Henderson–Hasselbalch, $[HN]_0 = [N]_0/(1+10^{pK-pH})$.[34] The p*K* values of the non-amino-acid-derived acyl acceptors were not considered in this manner. After thermal equilibration of assay mixtures the reactions were started by addition of the enzyme to give an active enzyme concentration of 1.6×10^{-6} M. Reaction times of 5–20 min led to complete ester consumption. For the HPLC analysis aliquots were withdrawn and diluted with a quenching solution of 50% aqueous methanol containing 1% trifluoroacetic acid. For each acyl donor and acyl acceptor, an experiment without enzyme was carried out to determine the extent of spontaneous ester hydrolysis, which was always less than 5%. On the basis of the same control experiments, nonenzymatic aminolysis of the acyl donor esters was investigated and could be ruled out. The values reported are the average of at least three independent experiments. The identity of the formed peptide and amide products was established by thermospray and MALDI-ToF mass spectroscopy, respectively. NMR measurements were used for the examination of the enzymatically formed amide bonds.

HPLC analyses: Samples were analyzed by analytical reversed-phase HPLC with a SpectraSystem P2000 (ThermoSeparationProducts) on C18-polymer-coated columns [Vydac 218TP54, 5 μm, 300 Å, 25 × 0.4 cm, The Separations Group, and Grom Capcell, 5 μm, 300 Å, 25 × 0.4 cm, Shiseido] and a C4 reversed-phase column [Vydac 214TP, 10 μm, 300 Å, 25 × 0.4 cm, The Separations Group]. Columns were thermostated at 25 °C and eluted with various mixtures of water/acetonitrile containing 0.1% trifluoroacetic acid under gradient conditions. Detection was at 254 nm monitoring the aromatic chromophores within the acyl donors. Thus, the yields could be determined from the peak areas of the hydrolysis and aminolysis products, whereby Tos served as an internal standard. In the case of H-AFAAG-OH, which contains an additional chromophoric amino acid residue, the yields were checked from the lack of hydrolysis products by at least five independent experiments.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (JA 559/9-1 and INK 23) and Fonds der Chemischen Industrie (Liebig scholarship, F.B.). The authors would like to thank Prof. Dr. H.-D. Jakubke for inspiring discussions, Doris Haines and Regina Schaaf for skilful technical assistance, and Prof. P. Halling and Prof. D. Hilvert for reading and correcting the manuscript. The authors are grateful to ASTA Medica and Fluka for special chemicals.

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Received: June 30, 1999 [F1882]