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Comparative Study of Substrate- and Stereospecificity of Penicillin G Amidases from Different Sources and Hybrid Isoenzymes

Boris Galunsky, Karsten Lummer, and **Volker Kasche***

Arbeitsbereich Biotechnologie II, Technische Universität Hamburg-Harburg, D-21071 Hamburg, Germany

Summary. Four natural pencillin G amidase variants from different sources and two genetically constructed hybrid enzymes were produced and purified to homogeneity. The specificity constants of one enzyme (*E.* coli) were found to differ six orders of magnitude for hydrolytic transformations within a wide range of substrates. The substrate specificity of the homologous penicillin amidases was found to differ less than one order of magnitude for hydrolysis of the most specific and up to two orders of magnitude for the less specific substrates. The S_1'-substrate specificity in hydrolytic and transfer reactions (studied mainly with the *E. coli* enzyme) varied more than three orders of magnitude for the different substrates. The penicillin amidases were found to be *R*-specific in the S_1-binding site and *S*-specific in the S_1'-binding site. The S_1-stereoselectivity differs less than one order of magnitude for the different variants. The S_1'-stereoselectivity is more pronounced, increases with nucleophile specificity, and was found to differ up to three orders of magnitude in transfer reactions for the enzyme from *E. coli*. The observed variation of enatioselectivity for different penicillin amidases and one substrate can also be achieved by changes in temperature. Comparison of substrate- and stereospecificity of penicillin amidases from different sources and hybrid isoenzymes suggests that similar changes can be expected for enzyme variants derived by rational protein design or directed evolution.

Keywords. Homologous enzymes; Penicillin amidase (acylase); Stereospecificity; Substrate specificity.

Introduction

During the evolution, natural enzyme variants with a common function have been selected for favoured biological properties, e.g. high specificity (k_{cat}/K_m) and stereoselectivity for natural substrates at conditions in the main habitat of the microorganisms. Therefore, the enzyme properties differ for the homologous enzymes depending on their source. The evolution optimizes the biocatalysts with regard to their function *in vivo*, whereas their application as industrial biocatalysts requires tolerance to reaction conditions outside the physiological range and

* Corresponding author

transformation of substrates not encountered in nature [1]. During the last fifteen years much work has been done to optimize enzymes for industrial applications by rational protein design [2–5] or directed evolution [6–10]. Despite the progress in improving enzymes from a biotechnological point of view, fundamentally altered properties have hardly been achieved. The question on the relation between function and basic biocatalytic properties on one hand and structural parameters as the amino acid sequence on the other one still remains actual. Comparison of data on substrate- and stereoselectivity of homologous enzymes and mutation studies may provide insight into the role of selection in optimizing the kinetic behaviour of native enzymes and to what extent it could be modulated.

The family of pencillin G amidases (PA; EC 3.5.1.11) is suitable for such an analysis. These enzymes catalyze the hydrolysis of benzylpenicillin (**1**) to phenylacetic acid (**2**) and 6-aminopenicillanic acid (**3**) – one of the key intermediates for the production of semisynthetic β-lactam antibiotics [11]. PA can also catalyze the synthesis of these antibiotics [12, 13]. Due to the biotechnological interest, different natural and artificial variants of this enzyme have been produced [14–20]. The genes encoding penicillin G amidase in *E. coli*, *K. citrophila*, *P. rettgeri*, *A. faecalis*, and *A. viscosus* have been cloned and sequenced. The nucleotide sequences encode precursors that are processed to two nonidentical peptide chains (A and B) [11]. The homology in the primary structure of all compared enzymes decreases with increasing genetic distances between the different species which are supposed to be diverged from a common evolutionary ancestor [21]. Despite the large differences in the homology between the most distant variants (up to 70%), multiple sequence alignment data reveal in both A- and B-chains segments of strongly conserved amino acid sequences essential for the catalytic function of PA. The biological function of the penicillin amidase family is still unknown. The same applies for whether it is controlled by the same groups that control the biotechnical functions as a biocatalyst for benzylpenicillin hydrolysis and production of the penem nucleus (**3**) [22, 23]. The penicillin amidases have a wide range of substrate specificity and differ in properties important for existing and possible new industrial applications. The 3D-structure of the *E. coli* enzyme reveals a single amino acid (*Ser* B1) catalytic site [24], and supports its classification as an *Ntn*-hydrolase [25]. The 3D-structure confirms also the acyl-enzyme reaction mechanism [26, 27]. The same mechanism has been inferred for the other compared enzymes [11].

In this study we compare quantitative data on substrate- and stereoselectivity of different natural and genetically constructed hybrid enzyme variants. The results can yield information on how much these properties can be changed by directed or random mutagenesis.

Results and Discussion

The molar concentrations of the penicillin amidases were determined by active site titration [28]. Active site titration curves for all studied PA variants were almost identical which implies that only their N-terminal *Ser* B1 are accessible for acylation with phenylmethylsulfonyl fluoride (**4**).

Specificity constants in hydrolytic reactions

The S_1-subsite specificity[1] of penicilin G amidase is mainly restricted to phenylacetic acid and its derivatives [11], although other acyl moieties as acetyl, 2-benzoxazolon-3-yl-acetyl, or mandelyl are also accepted [30–33]. Most of the Pa-catalyzed transformations published so far have been done with the *E. coli* enzyme. For the substrates we studied with *E. coli* PA and presented in Fig. 1, the specificity constants for equilibrium controlled hydrolysis vary by 6 orders of magnitude. The observed differences between substrates varying in P_1 were predominantly due to larger differences in K_m. The S_1- and S'_1-subsite specificities of purified (homogeneous) natural and hybrid PA variants have been studied in detail for the hydrolysis of **1**, 6-nitro-3-phenylacetamido-benzoic acid (**11**) the less specific *R*-phenylglycine amide (**7**), *R*-phenylglycine methyl ester (**6**), *p*-hydroxy-*R*-phenylglycine amide (**9**), and 6-nitro-3-*R*-phenylglycylamidobenzoic acid (**10**). The results are shown in Fig. 2. The k_{cat}/K_m values for hydrolysis of **1** differ less than one order of magnitude for the different enzyme variants. The enzyme from *A. faecalis* has the highest specificity constant compared to the other natural enzymes. Similar data have been reported by other authors [34]. For the hydrolysis of **11**, **7**, and **6** the differences are larger – up to 2 orders of magnitude. Here differences in both the turnover number and the *Michaelis-Menten* constant account for the observed variation in the

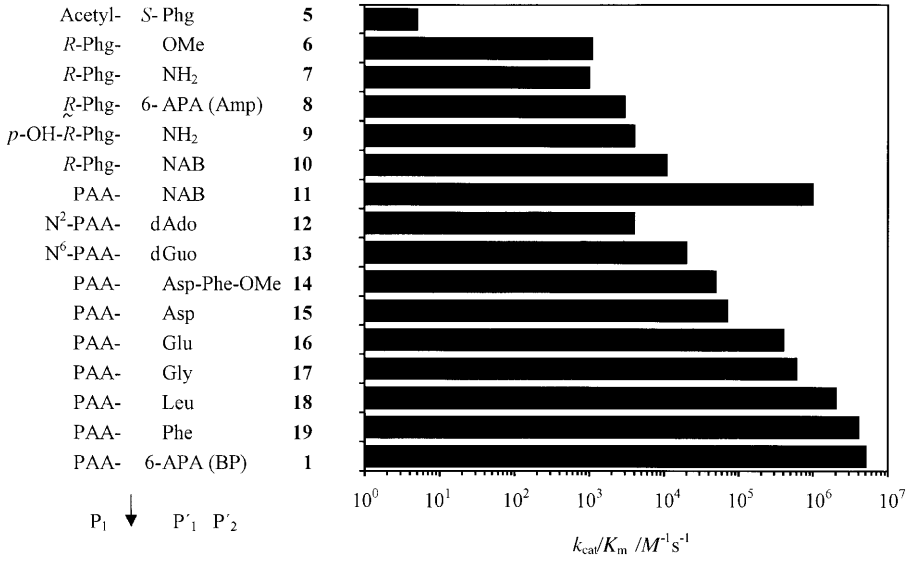

Fig. 1. Substrate specificity of affinity purified penicillin amidase from *E. coli* in equilibrium controlled hydrolytic reactions. Reaction conditions: phosphate buffer, $pH = 7.5$ ($I = 0.2$), 25°C. k_{cat} varies from 0.02 s^{-1} for **5** to 200 s^{-1} for **16**. K_m varies from 10 μ*M* for **1** to 30 m*M* for **6**. Abbreviations used: BP, benzylpenicillin; Amp, ampicillin; PAA, phenylacetic acid; Phg, phenylglycine; 6-APA, 6-aminopenicillanic acid; NAB, 6-nitro-3-aminobenzoic acid; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; three-letter symbols for the amino acids

[1] Using the convention for peptidases to designate the binding subsites [29]

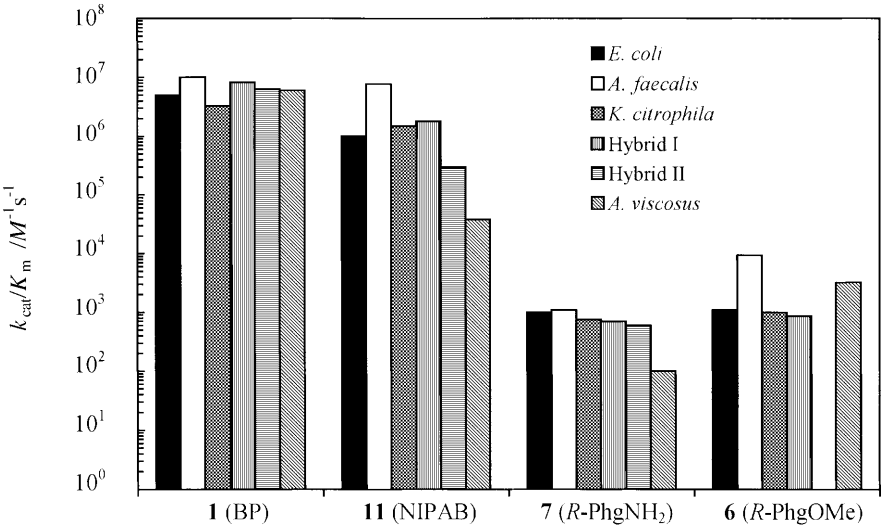

Fig. 2. Specificity constants of penicillin amidases from different sources and two genetically constructed hybrid enzymes (Hybrid I $A_{Kc}B_{Ec}$ and Hybrid II $A_{Ec}B_{Kc}$ for the hydrolysis of **1**, **11**, **7**, and **6**, at $pH = 7.5$ ($I = 0.2$) 25°C. All data were obtained with (affinity) purified enzymes. Both k_{cat} and K_m differ 3–4 fold for transformations of the same substrate catalyzed by different PA variants.
Abbreviations: for **1**, **6**, and **7**, cf. Fig. 1; NIPAB: 6-nitro-3-phenylacetamido-benzoic acid

specificity constants of the homologous enzymes. The specificity constants differ also for the different processed forms of one enzyme. For *E. coli* PA, the last processed form with $pI = 6.6$ ($PA_{6.7}$) and an A-chain which is by 8 amino acids shorter than $PA_{7.0}$[2], has a specificity constant for hydrolysis of **1** lower by 30% [35]. The last processed form of *A. faecalis* PA with $pI = 5.3$ ($PA_{5.3}$), with the A-chain shortened for 3 amino acids from the C-end and converted N-terminal *Gly* a 30% larger specificity constant for the same reaction as compared to $PA_{5.5}$[3] [36].

We found that genetically constructed hybrids are active and have specificity constants for the studied hydrolytic reactions similar to those of the wild type enzymes [18]. The similarity of the kinetic behaviour of the homologous PA is not surprising. Besides the identity of *Ser* B1, *Gln* B23, *Ala* B69, *Asn* B241, and *Arg* B263 forming the tetrahedral arrangements of ligands of O_γ and the α-amino group [24], the amino acid sequences in their vicinity are strongly conserved as well: of 32 residues within 10 Å of *Ser* B1 only 3 differ in the different variants [17]. Changes at larger distances from the active site (the C-terminus of the A-chain) can also result in small activity changes, as discussed for the different processed forms. The smaller differences between the specificity constants of the homologous enzymes for hydrolysis of **1** compared to **11**, **7**, and **6** suggest that the transformation of one of the most specific substrates is probably a reaction congruent to the biological function of PA, which has been optimized during the evolution. Thus, there are hardly to be expected large differences in the catalytic behaviour of mutant enzymes for the most specific substrates. For catalyzing reactions of less specific substrates (more distant

[2] The dominating processed form with C-terminal Ala_{209} of the A-chain and $pI = 7.0$
[3] The processed form with C-terminal Ala_{202} of the A-chain and $pI = 5.5$

from the biological function), mutant enzymes with improved properties are more probable. The few site directed or randomly created PA mutants have shown lower specificity constants for hydrolysis of **1** [39–40].

S'_1-site specificity

The S'_1-specificity reflects the ability of penicillin G amidase to catalyze acyl transfer reactions [12]. It is broader and varies from penem and cephem nuclei to α- and β-amino acids, their phosphonic and phosphonous analogs, peptides, aromatic and aliphatic amines and alcohols, and nucleosides. These have been used (also on an industrial scale) for the kinetically controlled synthesis of amoxicillin, ampicillin, cephadroxil, and cephalexin, for deprotection in peptide and nucleotide synthesis, and for pro-drug activation in cancer therapy [13, 40–46]. We studied the nucleophile specificity in both hydrolytic and synthetic directions mainly with the *E. coli* enzyme. In Fig. 1, the specificity constants for hydrolytic transformation of substrates with phenylacetic acid in P_1-position and different moieties in positions P'_1 and P'_2 are compared. The k_{cat}/K_m values vary by more than 3 orders of magnitude for the different leaving groups. The few quantitative data on S'_1-specificity published by other authors reveal specificity constants of the same order of magnitude [41].

Interactions in the S'_1-binding subsite depend on electrostatic interactions between the basic amino acid residue in S'_2-position (most probably *Arg* A145) and the unprotected carboxyl groups of amino acid and β-lactam structures [12, 41, 47]. This has been observed also with *A. faecalis* PA [33]. In kinetically controlled synthetic reactions where the enzyme catalyzes the transfer of an acyl moiety to a nucleophile, the measure for the S'_1-specificity is the transferase to hydrolase ratio k_T/k_H [12]. We determined its valuewith *E. coli* PA for transfer of phenylacetyl or phenylglycyl moieties from phenylacetyl-*Gly* **17**, **7**, and **6** to amino acids or peptides. For the different substrates this ratios differ up to 3 orders of magnitude (1×10^1–3×10^4). For one nonspecific acyl moiety, k_T/k_H was found to have a value typical for a transferase (2×10^5), [48]. The *E. coli* enzyme was shown to be better for the acyl transfer to cephem nuclei than the PA from *A. faecalis*. Here comparative studies with other natural and hybrid penicillin G amidases are necessary to analyze (together with 3D-structures of enzyme-substrate complexes) a possible improvement of the transferase properties of the enzymes. This can be relevant for the synthesis of β-lactam antibiotics and for new biotransformations where C–N and C–O bonds are synthesized or hydrolyzed.

Stereoselectivity

The enantioselectivity of an enzyme E [4] should be >100 or <0.01 to obtain pure and *R*-enantiomers, respectively, at the end-point in biotransformations [49, 50]. PA stereospecificity is also very important for its biotechnical application. *E. coli*

[4] The quantitative measure for enzyme stereospecificity is the enantioselectivity or enantiomeric ratio *E*. It is defined as the ratio of the specificity constants for hydrolysis of enantiomeric *S*- and *R*-substrates or as the ratio of the transferase to hydrolase ratios for acyl transfer to enantiomeric *S*- and *R*-nucleophiles.

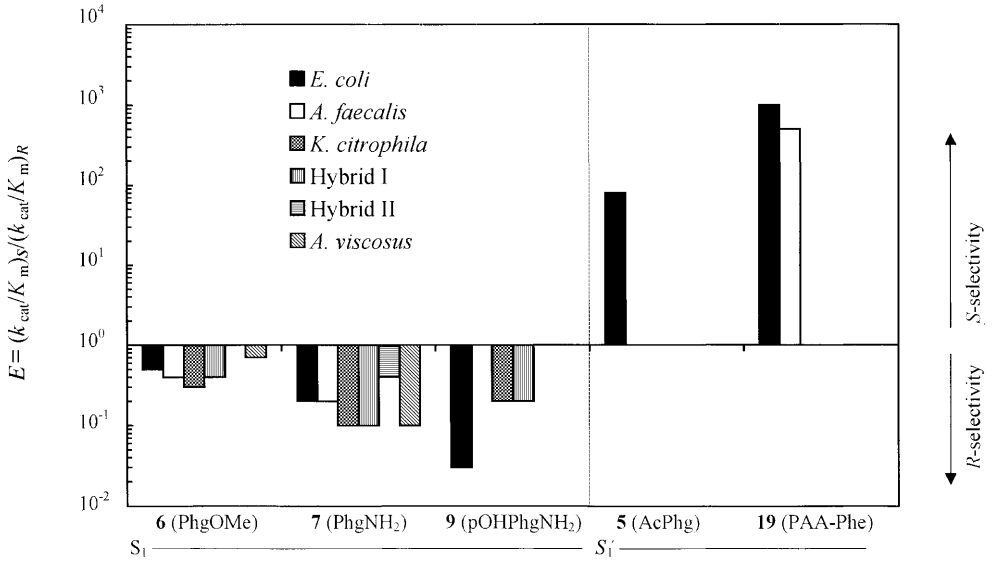

Fig. 3. S_1-subsite stereoselectivity of PA from different sources and two genetically constructed hybrid enzymes (Hybrid I $A_{Kc}B_{Ec}$ and Hybrid II $A_{Ec}B_{Kc}$), expressed as the enantiomeric ratio for various substrates hydrolysed at $pH = 7.5$ ($I = 0.2$) and 25°C. For comparison, the S'_1-subsite stereoselectivity is given for 2 substrates hydrolyzed at the same conditions by *E. coli* and *A. faecalis* enzymes. Abbreviations as in Fig. 1

PA has been used for the production of chiral synthons in peptide and nucleotide synthesis [41–44] and for kinetically controlled enantioselective acylation during loracarbef synthesis [51]. Considering the 3D-structure of *E. coli* PA and the sequence homology of the enzymes from different sources, very similar 3D-structures of all variants can be predicted [17]; thus, similar enantioselectivity could be expected.

S_1-enantioselectivity was studied with purified homologous penicillin G amidases only for a limited number of chiral substrates. The results are shown in Fig. 3. With the same enzyme, E was found to differ about one order of magnitude for the different substrates. A similar difference in enantioselectivity was observed for the transformation of one substrate by different PA variants. Differences mainly in k_{cat}, *i.e.* the activation energy, account for the observed changes.

S'_1-enantioselectivity was studied in both hydrolytic and transfer reactions, mainly with the *E. coli* enzyme. For comparison, in Fig. 3 the enantiomeric ratios for the hydrolytic transformations of two substrates catalyzed by two PA variants are given. S'_1-enantioselectivity is more pronounced and increases with nucleophile specificity. As for the S_1-stereoselectivity, differences mainly in k_{cat} account for differences in E. Literature data for the hydrolysis of phenylacetic acid derivatives of α-amino acids, peptides, β-amino acids, and alcohols reveal differences in E up to 3 orders of magnitude [41]. With the *E. coli* enzyme this was observed also in transfer reactions where $(k_T/k_H)_S/(k_T/k_H)_R$ differ up to 3 orders of magnitude (0.5–500) for the different substrates.

The penicillin G amidase was found to be *R*-specific in the S_1- and *S*- specific in the S'_1-binding subsites. This observation demonstrates that an enzyme consisting

of homochiral amino acids can simultaneously catalyze reactions of two opposite chiral centres. This applies for all studied PA variants. Since the most specific substrates of PA [41] have no chiral moieties in P_1-position, it may imply that S_1-subsite stereospecificity is not important for the biological function. S'_1-subsite stereospecificity is more pronounced, moreover many of the most specific substrates [41] possess chiral centres in the P'_1-position. For directed modulation of *E*, however, more information on the structural determinants of enantiomer discrimination for different enzyme variants is necessary. Directed evolution studies have shown only moderate improvement of the enantiomeric ratio [52], whereas temperature variation have resulted in larger (more than order of magnitude) influence [30, 53]. Thus, alternative modulation of *E* by the reaction conditions should be considered as well.

Conclusions

1. The specificity constants of a homogeneous PA from *E. coli* were found to vary by 6 orders of magnitude for different $P_1 - P'_1 - P'_2$-substrates. The differences between substrates varying in P_1 is predominantly due to larger differences in K_m. This indicates that the catalytic power of this enzyme is influenced both by substrate binding and lowering of the activation energy of the transition state.
2. The enantioselectivity of the S_1- (*R*-specific) and S'_1-(*S*-specific) binding sites of this enzyme varied by up to 3 orders of magnitude for the hydrolysis of $P_1 - P'_1$ substrates. The differences are larger in the S'_1-subsite and in both cases mainly due to changes in k_{cat}.
3. Four natural and two hybrid variants of PA were purified to homogeneity. They differ up to 70% in primary structure. There is, however, a large homology of amino acid residues that are located near the B-chain N-terminal *Ser* in the *E. coli* enzyme, acylated in the reactions catalyzed by this enzyme.
4. The specificity constants of the PA variants for hydrolysis of one $P_1 - P'_1 - P'_2$ substrate or kinetically controlled synthesis with one nucleophile were found to differ by maximal two orders of magnitude. The changes decrease with the specificity constants for the substrates and are due to changes both in k_{cat} and K_m.
5. The enantioselectivity of the S_1- and S'_1-binding subsites of the enzyme variants varied up to one order of magnitude. Such a change for one PA variant and one substrate can also be achieved by changes in temperature.
6. Based on this we conclude that changes in substrate- and stereospecificity of new active PA variants created by directed or random mutations will be of the order of magnitude observed for the homologous enzymes, *i.e.* less than two orders of magnitude for specificity constants (the changes decreasing with the specificity constants) and less than one order of magnitude for stereoselectivities.

Experimental

Substrates

R- and *S*-enantiomers of **5**, **6**, and **7** were kindly provided by Röhm (Dermstadt, Germany); *R*- and *S*-enantiomers of **9** were kindly provided by DSM Research (Geleen, The Netherlands); *S*-enantiomers of **15** and **16** were kindly provided by Dr. *I. Stoineva*, Institute of of Organic Chemistry, Bulgarian

Academy of Sciences, Sofia, Bulgaria); **17**, the *S*-enantiomer of **18**, and *S*-and *R*-enantiomers of **19** were synthesized as described in Ref. [53]; *S*,*S*-diastereomer of **14** was synthesized as described in Ref. [44]; **12** and **13** were synthesized as described in Ref. [43]; **11** was synthesized as described in Ref. [54]; **1**, **4**, and **8** were purchased from Sigma (Desenhofen, Germany); all other chemicals were of analytical grade.

Preparation of PA variants

Penicillin amidase from *E. coli* and Hybrid I (A-chain from *K. citrophila* and B-chain from *E. coli*) were produced in recombinant *E. coli* strains and isolated by affinity chromatography using a biospecific adsorbent with an immobilized monoclonal antibody directed against the B-chain of *E-coli* enzyme as described in Refs. [18, 56]. The enzymers from *K. citrophila* (the gene in the plasmid pYKH5 was kindly provided by Dr. *Barbero*, Antibioticos, Leon, Spain), *A. feacalis* (the gene was amplified by the PCR-technique from the total *DNA* prepared from strain NCTC 415 by DSMZ, Braunschweig, Germany), and Hybrid II (A-chain from *E. coli* and B-chain from *K. citrophila*) were produced in recombinant *E. coli* strains as described in Refs. [30, 36, 18]. The Enzyme from *A. viscosus* was prepared using *DNA* from the wild strain ATCC 15294. These PA variants were purified from a concentrated homogenate by ion exchange chromatography using the same procedure as described previously [35, 36]. The purity and homogeneity of the prepared enzymes from different sources was analyzed by activity and protein stains of isoelectric focusing gels [35]. *E. coli* and *A. faecalis* enzymes used here were the proteolytically processed forms with isoelectric points of 7.0 and 5.3.

Active site titration

The molar concentrations of the PA variants were determined by active site titration with **4** [28] using **11** as a substrate.

Determination of k_{cat} and K_m·

The substrates were dissolved and diluted to the desired concentration in phosphate buffer ($pH = 7.5$, $I = 0.2\ M$). The reaction was performed in this buffer at 25°C, initiated by addition of enzyme. The system was kept at constant temperature and *pH* value. The cleavage of **11** was followed spectrophotometrically at 380 nm by 6-nitro-3-aminobenzoic acid release [57]. With the other substrates, samples were withdrawn at different times and analysed by HPLC using a RP 8 column. The elution conditions and wavelengths used to quantitate the products are described in Refs. [58, 30, 44]. *Eadie-Hofstee* plots were used to determine k_{cat} and K_{m}.

Determination of k_T/k_H

The substrates (**6**, **7**, and **17**) and the nucleophiles (amino acids and peptides with free carboxyl function) were dissolved in carbonate buffer ($pH = 9$, $I = 0.2\ M$). The ratio was determined in this buffer at 25°C. Samples were withdrawn and analysed by HPLC using a RP 18 column. Gradient elution was used as described in Ref. [30]. The peak areas at 225 nm were used to quantitate the products. $k_{\mathrm{T}}/k_{\mathrm{H}}$ was calculated from the initial rates of condensation and hydrolysis product formation as described in Ref. [12].

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