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Versatile Peptide C-Terminal Functionalization via a Computationally Engineered Peptide Amidase

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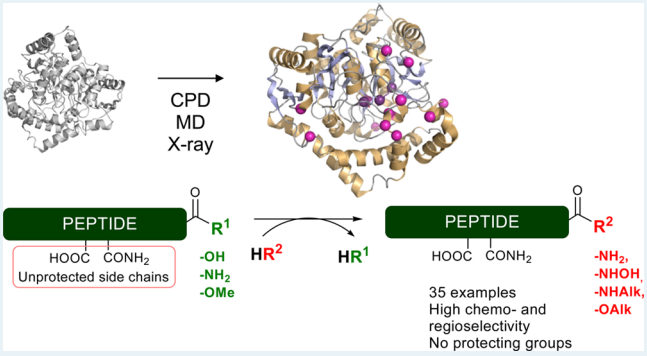
Supporting Information

ABSTRACT: The properties of synthetic peptides, including potency, stability, and bioavailability, are strongly influenced by modification of the peptide chain termini. Unfortunately, generally applicable methods for selective and mild C-terminal peptide functionalization are lacking. In this work, we explored the peptide amidase from *Stenotrophomonas maltophilia* as a versatile catalyst for diverse carboxy-terminal peptide modification reactions. Because the scope of application of the enzyme is hampered by its mediocre stability, we used computational protein engineering supported by energy calculations and molecular dynamics simulations to discover a number of stabilizing mutations. Twelve mutations were combined to yield a highly thermostable (ΔT_m = 23 °C) and solvent-compatible enzyme. Protein crystallography and molecular dynamics simulations revealed the biophysical effects of mutations contributing to the enhanced robustness. The resulting enzyme catalyzed the selective C-terminal modification of synthetic peptides with small nucleophiles such as ammonia, methylamine, and hydroxylamine in various organic (co)solvents. The use of a nonaqueous environment allowed modification of peptide free acids with >85% product yield under thermodynamic control. On the basis of the crystal structure, further mutagenesis gave a biocatalyst that favors introduction of larger functional groups. Thus, the use of computational and rational protein design provided a tool for diverse enzymatic peptide modification.



KEYWORDS: *computational protein engineering, peptide modification, enzymatic catalysis, protein stability, MD simulation*

INTRODUCTION

The discovery of novel bioactive peptides and the growing insight into their mode of action have strongly increased the level of interest in the development of peptide-based drugs in the past decade.[1] Currently, more than 60 approved peptide drugs are on the market, and intensive biomedical research of peptides will provide an effective pipeline for innovative therapeutic applications in the near future.[2] Other important applications of peptides are in medical diagnostics, nutritional supplements, and cosmetics.[3] The bioactivity and pharmacokinetics of peptides can be controlled by peptide engineering, e.g., by modification of the chain termini.[4] C-Terminal functionalization of peptides has significant effects on their biological properties.[5] The ability to selectively introduce functional groups, such as a fluorescent reporter or a synthetic polymer, onto a peptide under mild conditions would allow a broad array of peptide biophysical studies and applications.[6] Furthermore, efficient C-terminal protection, deprotection, and activation methods are crucial in chemoenzymatic peptide synthesis, particularly in cost-efficient N → C peptide elongation.[7] Therefore, methods for selective peptide carboxyl modification are of paramount importance.[8]

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As compared to chemical reactions, enzymatic methods for peptide modification may offer clear advantages, such as the use of mild conditions and high selectivity that obviates protection steps and prevents racemization.[9] Prominent enzymatic C-terminal functionalization methods involve catalysis by proteases. For instance, the industrial protease Alcalase has been used for C-terminal amide−ester interconversion[10] and arylamidation.[11] However, these proteases are not C-terminally selective, which often leads to undesired internal peptide bond cleavage. Thus, alternative enzymes that present a relaxed substrate specificity and a strict C-terminal regioselectivity are highly desirable.

In search of such a broadly applicable biocatalyst for C-terminal peptide modification, we examined peptide amidase (PAM) from *Stenotrophomonas maltophilia*. PAM is a serine hydrolase that cleaves the C-terminal amide bond of peptide amides.[12] The catalytic center of PAM is buried (Figure 1) and governs

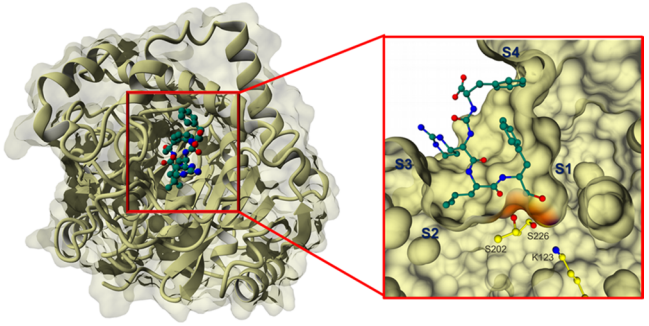

Figure 1. Peptide amidase structural features. Atom coordinates were taken from Protein Data Bank entry 1M21, in which the active site of PAM (brown) is bound with the inhibitor chymostatin (*N*-{[(*S*)-1-carboxy-2-phenylethyl]carbamoyl}-α-[2-iminohexahydro-4(*S*)-pyrimidyl]-L-leucyl-L-phenylalaninal, sea green). The side chains of catalytic residues (K123, S202, and S226) are colored yellow.

specificity by binding a peptide substrate in such way that only the C-terminal amide contacts the catalytic center, which is formed by a Ser-Ser-Lys triad.[13] Consequently, the enzyme exhibits an absolute C-terminal regioselectivity, discriminating among terminal, internal, and side chain amide bonds. The side chain amides of Asp and Gln are not processed by PAM.[14] The C-terminal residues of the substrate interact with the enzyme mainly through van der Waals interactions (Figure 1), making the substrate spectrum of PAM rather broad.[12] These structural features suggest that PAM could be an effective peptide modification biocatalyst. The catalytic mechanism involves formation of a covalent acyl (peptidyl)−enzyme intermediate,[13] and we anticipated that it would allow alternative reactions if water can be replaced by other nucleophiles in the hydrolytic half-reaction or if the reaction is reversed, which might be facilitated by performing conversions under anhydrous conditions. The use of PAM for one-step peptide C-terminal amide−ester interconversion has been reported.[15] However, the expansion of PAM-catalyzed reactions to broader peptide functionalization was restricted, because PAM has modest stability and low resistance to organic solvents while many modification reactions, e.g., functionalization of bioavailable peptide free acids, require a nonaqueous environment.

To overcome this severe limitation and to exploit the potential of PAM for alternative transformations, we embarked on the engineering of PAM into a robust biocatalyst for the selective modification of peptide C-termini with a wide substrate scope, with respect to both the peptide and the group to be attached

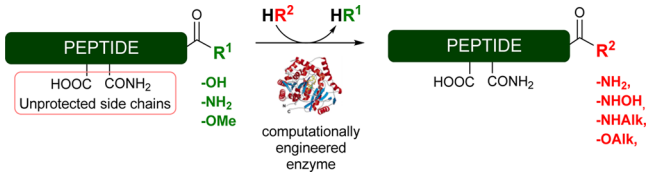

Figure 2. PAM-catalyzed regioselective peptide C-terminal functionalization. Computationally designed peptide amidase allows straightforward modifications of peptidic C-terminal acids, amides, and esters to a range of modified products.

(Figure 2). The recent development of powerful tools for computational protein design such as Rosetta[16] and FoldX[17] makes it possible to predict the effect of mutations on protein stability.[18,19] Such tools also allow computational library design to support the rapid engineering of mutant enzymes with thermostability enhanced by >15 °C[20−22] and with improved cosolvent compatibility.[21] Other types of mutations that might contribute to enhanced stability are substitutions of the consensus sequence, replacing at homologous positions rare amino acids with more common ones.[23] Here we report the use of computational library design to develop a PAM variant that is remarkably stable under nonaqueous conditions, rendering reactions that are not practical in the presence of water. This allows conversion of peptidic C-terminal acids, amides, and esters to a range of modified products, while avoiding hydrolysis of internal peptide bonds or side chain amide groups. By structure-guided mutagenesis, the substrate scope of PAM was expanded to accommodate more bulky functional groups. Together, the engineered variants may serve as a suitable platform for the development of versatile enzymatic peptide C-terminal modification.

RESULTS

Computational Engineering of a Robust PAM12A. To expand the applicability of PAM, we decided to engineer the enzyme into a more robust variant, anticipating that a stabilized PAM could tolerate harsh conditions, including the use of organic solvents, and allow peptide modification with various nucleophiles under kinetic and thermodynamic control. Engineering PAM robustness was approached through a computational workflow for enzyme stabilization called FRESCO.[20,21] It consists of four stages: (1) computational prediction of a large number of stabilizing mutations, (2) inspecting and ranking the predicted stabilizing mutations by molecular dynamics (MD) simulations, (3) validating predicted mutations experimentally, and (4) combining the best compatible stabilizing mutations. After all possible mutations along the whole protein sequence had been scanned, Rosetta ddg,[16] FoldX,[17] and consensus analysis[23] provided 458, 181, and 35 possibly beneficial mutations, respectively, with some overlap. In total, 616 unique mutations were screened by MD simulation and rationally guided selection to produce a library of 120 promising variants. Because correlations between thermostability and stability in organic solvents have been reported,[24,25] we used a rapid fluorescence-based thermal unfolding assay[26] to identify effective mutations, presuming that mutations that reduce the level of thermal unfolding will stabilize the enzyme in organic solvents, as well. Of the 120 designs that were tested experimentally, 12 well-expressed mutants (Table 1) displayed a higher stability (>1 °C increase in the apparent T_m). Rosetta ddg (64 designs), FoldX (44 designs), and consensus analysis (20 designs) provided 5, 6, and 2 beneficial mutations, respectively (D283P was proposed by both FoldX and consensus

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Table 1. Structural Effects of the Stabilizing Mutations

mutation	origin	mechanism of stabilization
R86H (T_m + 1.5 °C)	Rosetta ddg	Removes the positively charged side chain that is surrounded by three other arginines (R80 at 5.2 Å, R84 at 3.2 Å, and R190 at 5.2 Å). The stabilization reflects a decrease in the strength of repulsive electrostatic interactions in the folded state. The replacement of R86 with a histidine allows R80 to move toward H86 and to make a new salt bridge with D79 via its NH atoms and the *anti* orbitals of the carboxyl oxygen of D79. D80 now makes the new H-bond to the backbone carbonyl oxygen of V88. Both salt bridges and H-bonds can increase protein stability.[31−33] To allow these new interactions, the backbone of R80 shifted 1.5 Å toward H86, which in the wild-type structure would be highly unfavorable because of the repulsive positive charge of R86.
G142D (T_m + 1.5 °C)	Rosetta ddg	The replacement of glycines can stabilize proteins.[34,35] The introduced aspartate makes a new salt bridge via the *anti* orbital of a carboxylate oxygen to R144. R144 is shifted compared to the wild-type structure of PAM and makes a new salt bridge with E90. This elongates the existing salt bridge network of E90 with R185, which itself forms a salt bridge to D124, which itself forms a salt bridge to K164. Thus, a four-membered salt bridge network is converted into a six-membered salt bridge network. Salt bridge networks contribute to protein stability.
A171M (T_m + 6 °C)	FoldX	The introduced M171 fills a deeply buried cavity in the vicinity of the active site, which will make the folded state more stable and thus contribute to stability.[36] The Cε atom of M171 makes new hydrophobic interactions with the Cδ atom of I254, which rearranges to allow the interaction. The same Cε atom also makes hydrophobic contacts with the Cε3 atom of W170 and the C2 atom of Ile252. The Sδ atom of M171 makes hydrophobic interactions with the Cβ atom of Asn125, the Cδ1 atom of L167, and the Cβ atom of A135.
G175S (T_m + 1.5 °C)	consensus	This mutation leads to entropic stabilization, like G142D. The introduced serine makes a new H-bond to the backbone amide of N177 and to the *syn* orbital of a carboxylate oxygen of E406. There is also a backbone rearrangement of both residue 175 and its neighbor, N176, which is best explained by it allowing S175 to make the H-bond. Because the backbone dihedrals of residue 175 ($\phi = -145°$ and $\psi = 175°$ in the wild type; $\phi = -156°$ and $\psi = 145°$ in PAM12) were already in the allowed regions for any amino acid, it is unlikely that the rearrangement is due to the conformational preference of the original glycine.[37]
D177N (T_m + 3.5 °C)	Rosetta ddg	D177N appears to stabilize via local electrostatic interactions. In the immediate vicinity of D177, there are two glutamates, which causes repulsive electrostatic interactions (E406 at a distance of 5.9 Å, E464 at 4.5 Å). In the wild-type structure, there is a gap at residues 464−466, in which electron density was insufficient to model the protein. This gap is not present in the PAM12A structure, which suggests there is less disorder at this position in PAM12A.
I195P (T_m + 1 °C)	FoldX	Entropic stabilization due to a lower degree of freedom of a proline in the unfolded state. The original I195 was partially solvent exposed, and no hydrophobic contacts are lost because of the mutation. While prolines only fit well with a relatively restricted set of possible backbone conformations,[37] the backbone at position 195 did not change conformation as it already fell within the proper range for a proline ($\phi = -58°$ and $\psi = -11°$ in the wild type; $\phi = -62°$ and $\psi = -17°$ in PAM12A).
A261P (T_m + 1 °C)	FoldX	Mutation A261P causes entropic stabilization (see G142D and I195P) and also fills up a cavity (like A171M). The cavity in the wild-type structure contains a water molecule, which is no longer present in the mutant. This water release can also contribute to stabilization by entropy gain.[34]
D283P (T_m + 1.5 °C)	consensus/ FoldX	The mutation results in both entropic stabilization (see G142D and I195P) and more favorable electrostatic interactions. The mutation removes the repulsive interactions with its immediate neighbor in the sequence, which is D284. The mutations did not alter the backbone conformation.
T318V (T_m + 1.5 °C)	FoldX	The mutation improves hydrophobic interactions. The introduced Cγ atom of V318 makes new interactions with the Cβ atom of A452, and Cβ and both Cγ atoms of L316. As a result of these improved interactions, the surface-exposed backbone of residue 318 shifts 1.5 Å toward the protein.
Q352Y (T_m + 3.5 °C)	Rosetta ddg	The mutation results in improved hydrophobic interactions and new H-bonds. The original Q352 side chain lacked clear interactions. The new H-bonds in Q352Y are with the side chain *syn* orbital oxygens of E471 (2.7 Å oxygen−oxygen distance) and the amide nitrogen of A475 (N−O distance of 3.3 Å). New hydrophobic contacts include interactions of the Y352 aromatic carbon atoms with the Cγ atom of the also newly introduced V318, with one Cδ atom of L316, with both Cδ atoms of L349, with the Cβ atom of A475, and with the Cζ2 and Cη atoms of W355. The backbone Cα atom of Y352 has shifted 1.0 Å toward the center of the protein, which can be explained by these favorable new interactions.
G401A (T_m + 1.5 °C)	Rosetta ddg	The stabilizing effect is explained by entropic stabilization (see G175S and I195P). There is no significant effect on the conformation of the backbone, and the introduced alanine side chain makes no new interactions.
S463P (T_m + 2 °C)	FoldX	The mutation introduces a proline that causes entropic stabilization of the protein structure (see G175S and I195P). The newly introduced side chain makes no new interactions, and there is no effect on the backbone.

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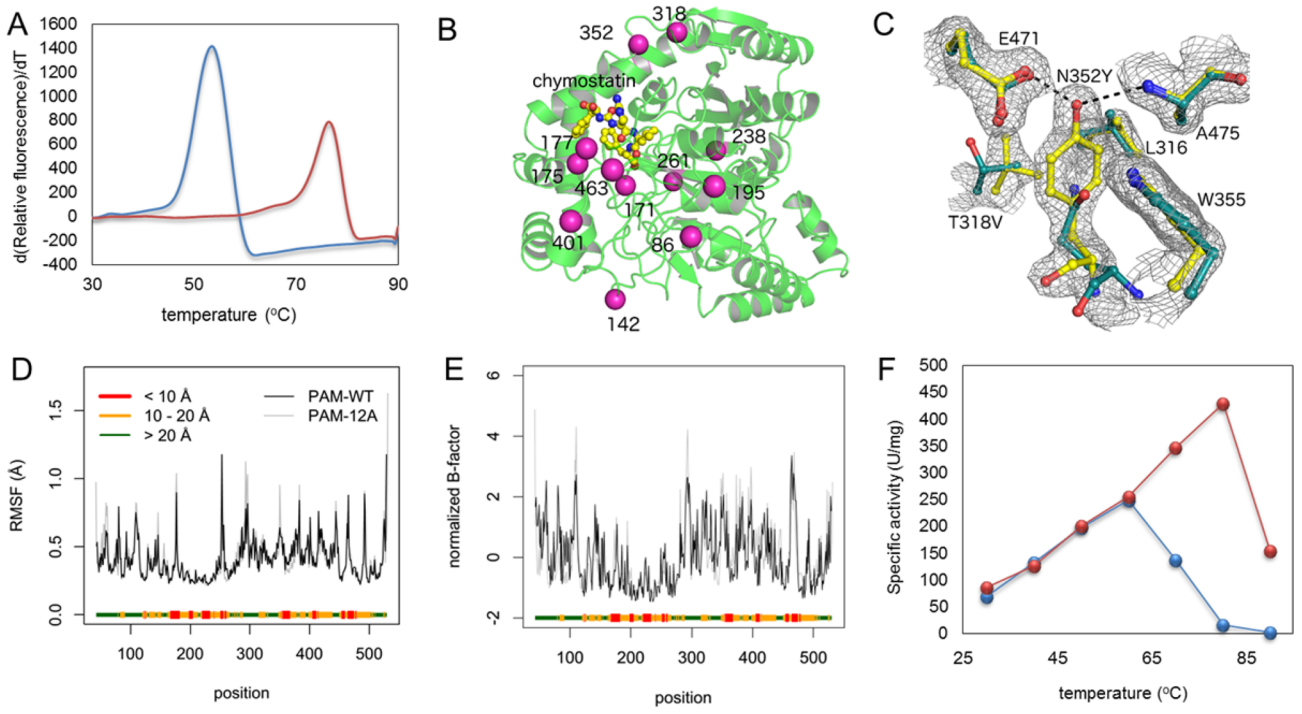

Figure 3. Computational engineering of PAM and investigating the source of its evolved robustness. (A) Apparent melting temperatures in phosphate buffer (20 mM, pH 7.5) measured by the thermofluor method: blue for wild type (WT) PAM and red for PAM12A. (B) Location of the stabilizing mutations in the crystal structure of PAM12A (PDB entry 5AC3). Atom coordinates of the inhibitor chymostatin were taken from WT-PAM (PDB entry 1M21). (C) Example of a stabilizing mutation. The PAM12A structure is shown with yellow carbon atoms, while the aligned WT structure has sea-green carbon atoms. The electron density is displayed at 1σ. The original Q352 side chain lacks clear interactions with its surroundings. The Q352Y mutation results in new H-bonds to the side chain of E471 and to the backbone amide of A475. (D) Atomic fluctuations obtained from MD simulations. Distances to the active site are indicated (see panel B). (E) Normalized *B* factors of the X-ray structures of WT PAM and PAM12A, indicating minor changes in protein flexibility after introduction of the 12 mutations. Normalized *B* factors are obtained from the equation $B_{norm} = (B_{orig} - B_{avg})/B_{SD}$, where B_{orig} is the original *B* factor, B_{avg} is the average *B* factor for all residues in the protein, and B_{SD} is the corresponding standard deviation. (F) Peptide amide hydrolysis activity of Z-Gly-Tyr-NH_2 in phosphate buffer (20 mM, pH 7.5) at different temperatures: blue for wild-type PAM and red for PAM12A.

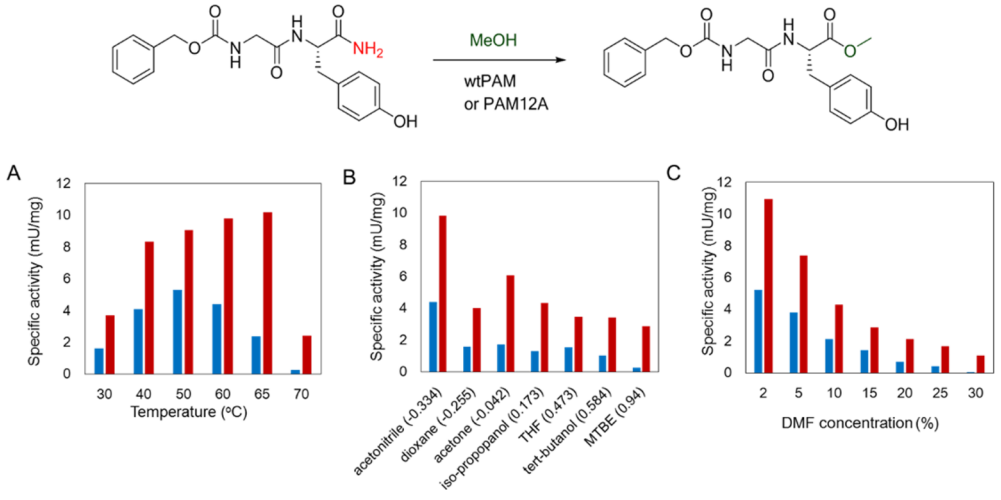

Figure 4. Comparison of activities of wild-type PAM (blue) and computationally engineered variant PAM12A (red) in nonaqueous solvents for one-step C-terminal deprotection and activation of peptides. (A) Specific activity in acetonitrile at different temperatures. (B) Specific activity in different organic solvents. The log *P* values of the solvents are given in parentheses. (C) Specific activity in an acetonitrile/DMF cosolvent system.

analysis). These results indicated that all three methods are useful computational tools for predicting stabilizing mutations with a reasonable success rate (~10%). The stabilizing mutations were stepwise combined into PAM (Table S1) to yield a highly stabilized variant (PAM12A) containing 12 mutations [R86H, G142D, A171M, G175S, D177N, I195P, A261P, D283P, T318V, Q352Y, G401A, and S463P (Figure S1)]. Compared to the wild-type enzyme, PAM12A exhibited a substantially higher apparent unfolding temperature [$\Delta T_{m,app}$ = 23 °C (Figure 3A)].

To gain insight into the structural basis of the enhanced robustness, we determined the crystal structure of PAM12A [1.8 Å resolution, Protein Data Bank (PDB) entry 5AC3 (Figure 3B)].

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The overall wild-type structure (PDB entry 1M21) was maintained in the mutant, with an average Cα root-mean-square deviation of 0.52 Å. An examination of the substitutions (Table 1) suggests that the robustness of the enzyme stems from improved electrostatic interactions (R86H, D177N, and D283P), enhanced hydrophobic interactions (T318V and Q352Y), introduction of new salt bridges (R86H and G142D) or new H-bonds [R86H, G175S, and Q352Y (Figure 3C)], elimination of internal cavities (A171M and A261P), release of bound water (A261P), and a decreased gain in unfolding entropy (G142D, G175S, I195P, A261P, D283P, G401A, and S463P).

Enzymes stabilized by protein engineering may exhibit loss of activity at lower temperatures, because of increased rigidity around the active site.[27,28] The influence of the mutations in PAM12A on flexibility was examined by MD simulations. To improve sampling of conformational space, we performed multiple independent MD simulations instead of a single long simulation.[29,30] The results indicated that the flexibility was maintained, which also emerged from an inspection of the *B* factors of the crystal structures (Figure 3E). The maintained flexibility of the robust PAM12A variant is in agreement with the preserved catalytic activity of the enzyme at lower temperatures. In aqueous buffer, PAM12A remained fully active in peptide amide hydrolysis at mild temperatures, whereas the engineered enzyme showed activity higher than that of the wild type at elevated temperatures (Figure 3F).

PAM12A-Catalyzed C-Terminal Deprotection and Activation of Peptides. To evaluate the applicability of PAM12A under non-natural conditions, we initially compared wild-type PAM and PAM12A for peptide C-terminal amide−ester interconversion, a useful conversion for cost-effective N → C chemoenzymatic peptide elongation. To prevent the hydrolytic side reaction, this conversion must be performed in nearly neat organic solvents. As desired, the higher thermal stability of PAM12A was paralleled by a remarkable increase in tolerance toward various organic solvents. In acetonitrile (<0.1% water content), PAM12A was several-fold more active than wild-type PAM at all temperatures tested (Figure 4A). In addition, PAM12A also showed superior robustness in other organic solvents [log *P* ranging from −0.334 to 0.94 (Figure 4B)].

We further studied whether PAM12A tolerates water-miscible cosolvents. The polar aprotic DMF is a very good cosolvent for dissolving peptides; however, it is also known to penetrate into enzyme active sites and induce detrimental secondary and tertiary structural changes.[38] Notably, PAM12A retained ∼15% activity in the presence of 30% DMF (Figure 4C), while the activity of wild-type PAM was almost abolished under this condition. The stabilized enzyme was judged to be sufficiently robust to explore peptide modification in various anhydrous solvents and water/cosolvent mixtures.

PAM12A Catalyzes C-Terminal Amidation of Peptide Acids. Food-derived peptides and protein hydrolysates have demonstrated diverse biological activities and serve as nutraceuticals and antimicrobial agents.[39,40] Most of these peptides have free carboxyl termini.[41] Modification of the C-terminus, e.g., by amidation, can increase peptide bioavailability and resistance to degradation by proteases.[5] Therefore, we explored the use of PAM12A for direct amidation of peptides with a free C-terminal carboxyl group. This reaction is challenging because formation of an acyl−enzyme complex from carboxylate groups is disfavored and conversion can be realized only under thermodynamic control. Because a high water activity will limit the accumulation of product, the robustness of the biocatalyst and its compatibility with nonaqueous solvents are of key importance for this process. A peptide amidase from orange flavedo could catalyze C-terminal peptide amidation,[42] but the enzyme is insufficiently stable in neat organic solvents to obtain a decent yield of peptide amide (>50%).

We tested several peptide free acids as the substrate for this reaction using PAM12A as a catalyst. The robustness of the enzyme made it possible to examine amide bond formation in nearly neat organic solvents, and conversions could proceed for several days until the reactions reached the thermodynamic equilibrium. As the water content was decreased to trace amounts (<0.1%), the PAM12A-catalyzed direct amidation of peptide free acids was brought almost to completion (Figure 5).

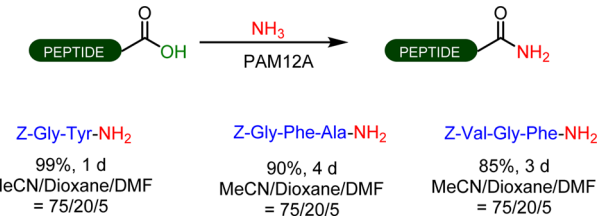

Figure 5. PAM12A-catalyzed C-terminal amidation of peptide acids. Detailed reaction conditions are described in the Supporting Information. Product yields were determined by high-performance liquid chromatography, and product identities were confirmed by mass spectrometry.

PAM12A-Catalyzed C-Terminal Amidation of Peptide Esters. For large-scale manufacturing of synthetic peptides, solution phase methods employing peptide esters are often preferred over solid phase synthesis because of the cost.[43] The better solubility of peptide esters in organic solvents as compared to that of amides or to that of C-terminally free peptides reduces the extent of product loss during extraction steps.[44] The subsequent transformation of peptide esters to amides can be catalyzed by subtilisin A, but yields are unsatisfactory because of substantial peptide ester hydrolysis.[45] To test the applicability of PAM12A for peptide ester−amide conversion, we examined reactions of several peptide esters with ammonia under nearly anhydrous conditions. As desired, several peptide esters were converted into the corresponding peptide amides smoothly in nearly neat organic solvents upon treatment with PAM12A and ammonia in almost quantitative high-performance liquid chromatography (HPLC) yield (Figure 6).

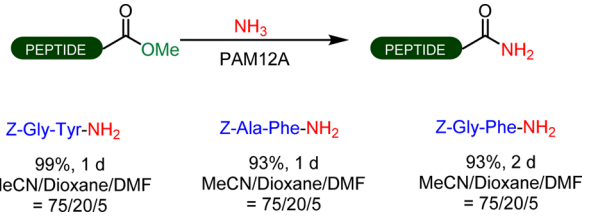

Figure 6. PAM12A-catalyzed C-terminal amidation of peptide esters. Detailed reaction conditions are described in the Supporting Information. Product yields were determined by HPLC, and product identities were confirmed by mass spectrometry.

PAM12A-Catalyzed Peptide C-Terminal Methylamidation and Hydroxylamidation. To evaluate the versatility of PAM12A-catalyzed peptide modification, the nucleophile scope of PAM12A was investigated. We first studied PAM12A-catalyzed methylamidation of different peptide substrates in organic solvents, either under thermodynamic control or under

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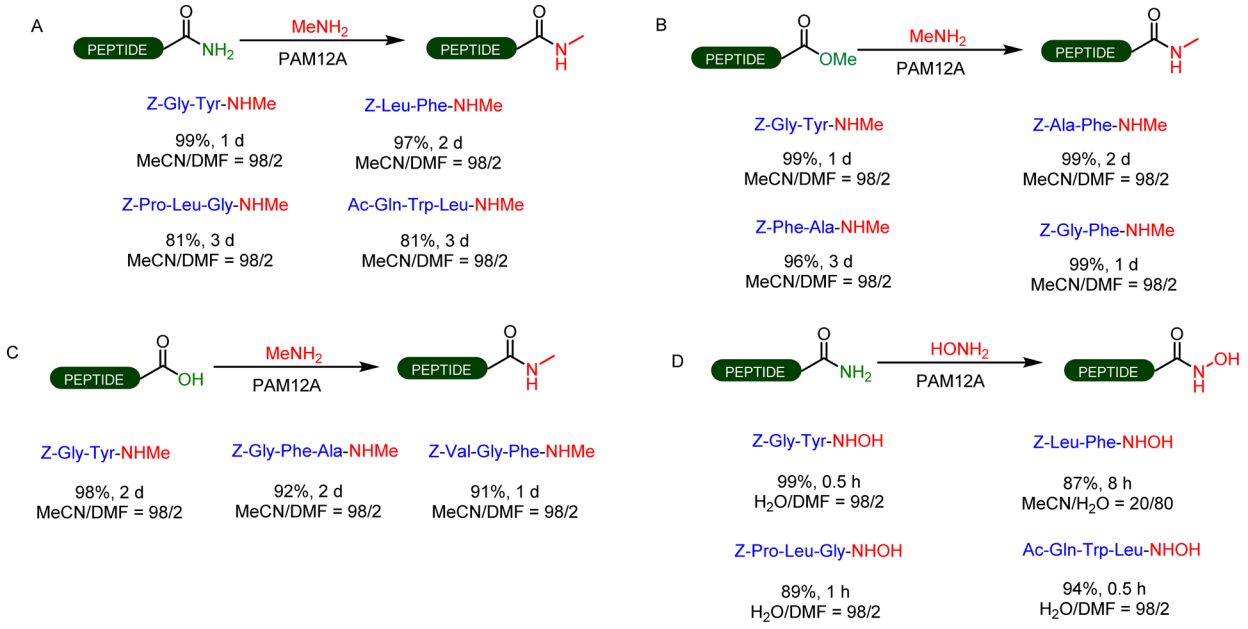

Figure 7. PAM12A-catalyzed C-terminal peptide modification with methylamine and hydroxylamine. Conversions include: methylamidation of (A) peptide amides, (B) peptide esters, and (C) peptide acids and (D) hydroxylamidation of peptide amides. Detailed reaction conditions are described in the Supporting Information. Product yields were determined by HPLC, and product identities were confirmed by mass spectrometry.

kinetic control. C-Terminal methylamidation can have a significant effect on the biological properties of a peptide, e.g., by increasing the lipophilicity or bioactivity.[46] Gratifyingly, all tested peptides were converted into the corresponding *N*-methylamide products in high HPLC yield (Figure 7A–C).

We next examined the use of PAM12A in the synthesis of peptide hydroxamic acids, an important class of compounds that has been widely explored as metal chelators and inhibitors of metalloenzymes.[47] PAM12A exhibited a substantial preference for hydroxylamine over water. When the peptide amides were treated with PAM12A in hydroxylamine·HCl buffer (pH 7.4), fast and almost complete conversions of the substrates to the corresponding peptide hydroxamic acids were observed (Figure 7D).

Modification of Pharmaceutical Peptides and a Long Peptide. After exploring the application scope with short peptides, we moved to assay the practical utility of PAM12A-mediated peptide modification with two pharmaceutical peptides: pentagastrin, a peptide amide, and leu-enkephalin, a peptide acid. Pentagastrin was efficiently converted to the corresponding *N*-methylamide and hydroxamic acid. The modifications of leu-enkephalin were performed via the thermodynamically controlled pathway, and this peptide was modified in good yield with ammonia, methylamine, and hydroxylamine (Figure 8).

Finally, we examined the applicability of PAM12A for modification of longer peptides. The 29-mer pharmaceutical peptide Sermorelin, which contains multiple unprotected reactive groups, such as Gln, Asp, Lys, etc., was selected as a model. The hydroxylaminolysis of Sermorelin was conducted in hydroxylamine·HCl buffer (5 wt %, pH 7.5) at room temperature for 15 min (Figure 9A). The HPLC data pointed to a single product with 99% yield. Electrospray ionization mass spectrometry (ESI-MS) analysis showed that this product was the monomodified Sermorelin on the basis of the 16 Da mass shift from unmodified substrate, and ESI-MS/MS analysis indicated that the modification was located at the C-terminal Arg residue (Figure 9B).

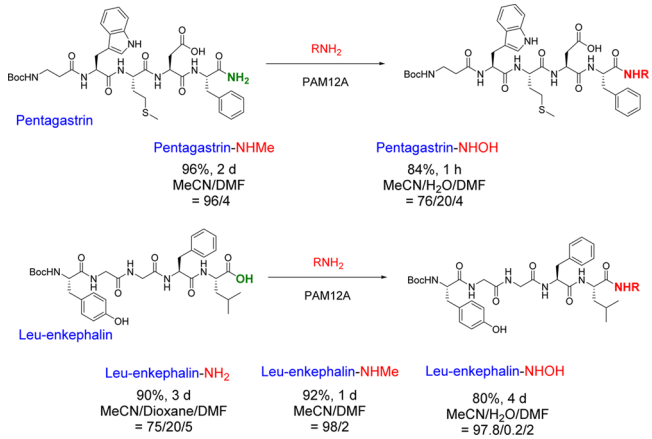

Figure 8. Examples of PAM12A-catalyzed pharmaceutical peptides modifications. Detailed reaction conditions are described in the Supporting Information. Product yields were determined by HPLC, and product identities were confirmed by mass spectrometry.

Expansion of Peptide Functionalization with PAM12B. The examples given above show that various peptide substrates are accessible to PAM12A-mediated reactions. Nevertheless, PAM12A showed a strong preference for small nucleophiles, and larger nucleophiles are either not processed or outcompeted by water, leading to a high degree of hydrolysis (Figure 10). To broaden its applicability, it would be attractive to expand the range of nucleophiles that are accepted. Because steric hindrance is a likely cause of the limited nucleophile spectrum, we reasoned that mutations creating more space in the active center may be beneficial for the reactions with larger nucleophiles. Because the side chain of position 171 lines the nucleophile binding pocket, we replaced Met171 with a glycine, resulting in mutant PAM12B (Figure 10A).

The catalytic performance of PAM12A and PAM12B in modifying a model peptide were compared for a variety of nucleophiles. While PAM12A-catalyzed peptide amidation was almost complete (>99%) in 1 day, the same reaction catalyzed

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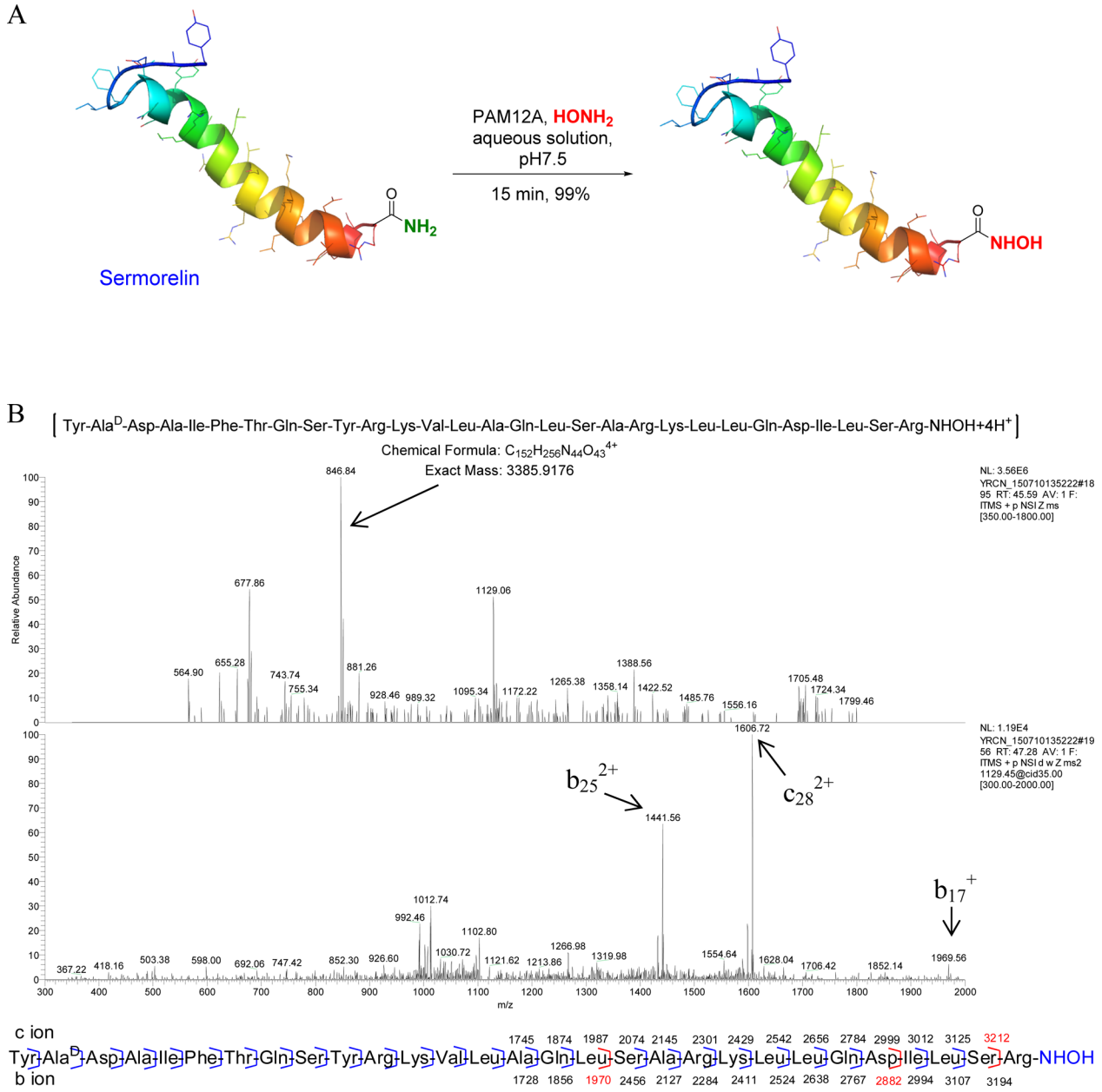

Figure 9. Example of PAM12A-catalyzed long peptide modification. (A) Sermorelin modification scheme. (B) ESI$^+$-MS and ESI$^+$-MS/MS of product Tyr-Ala^D-Asp-Ala-Ile-Phe-Thr-Gln-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-NHOH.

by PAM12B gave only 60% conversion, which suggests that PAM12B is suboptimal for modification with small nucleophiles (see the Supporting Information). On the other hand, PAM12B exhibited a preference for bulky nucleophiles such as benzyl amine (Figure 10B). Under the same reaction conditions, PAM12B is superior in terms of the synthesis/hydrolysis ratio (synthesis of desired product vs hydrolysis of amidated peptide substrate), and with all larger nucleophiles, the yields of the desired peptide derivatives were greater when using PAM12B (Figure 10B). These results demonstrate that the complementary nucleophile scope of PAM12A and PAM12B could provide broad substrate coverage for the regioselective preparation of diverse peptide derivatives.

DISCUSSION AND CONCLUSION

The effect of C-terminal modification on the functional properties of peptides has stimulated substantial efforts to develop suitable derivatization reactions. The selectivity of these methods usually relies on a pre-introduced C-terminal functional group, on a special recognition residue, or requires a specific amino acid sequence motif.[48−51] In contrast, the structural characteristics of peptide amidase, i.e., the nonspecific C-terminal binding tunnel and the buried active site, afford sequence-independent C-terminal peptide modification with absolute regioselectivity. Indeed, we observed that the amide or acid side chain (e.g., Ac-QWL-NH$_2$ or pentagastrin) was not processed by the enzyme. PAM12A exhibited a rather broad substrate spectrum. A range of unprotected peptides with an aliphatic (Ala or Phe), an aromatic (Phe, Tyr, or Trp), or a charged (Arg) residue at the C-terminus were selectively and efficiently modified. The crystal structure of PAM suggests that several C-terminal residues may make contact with the enzyme. Therefore, future mapping of the substrate profile of each binding subsite of PAM would reveal more details about the substrate−enzyme interactions. While the versatility of the synthetic applications was shown with short model peptides,

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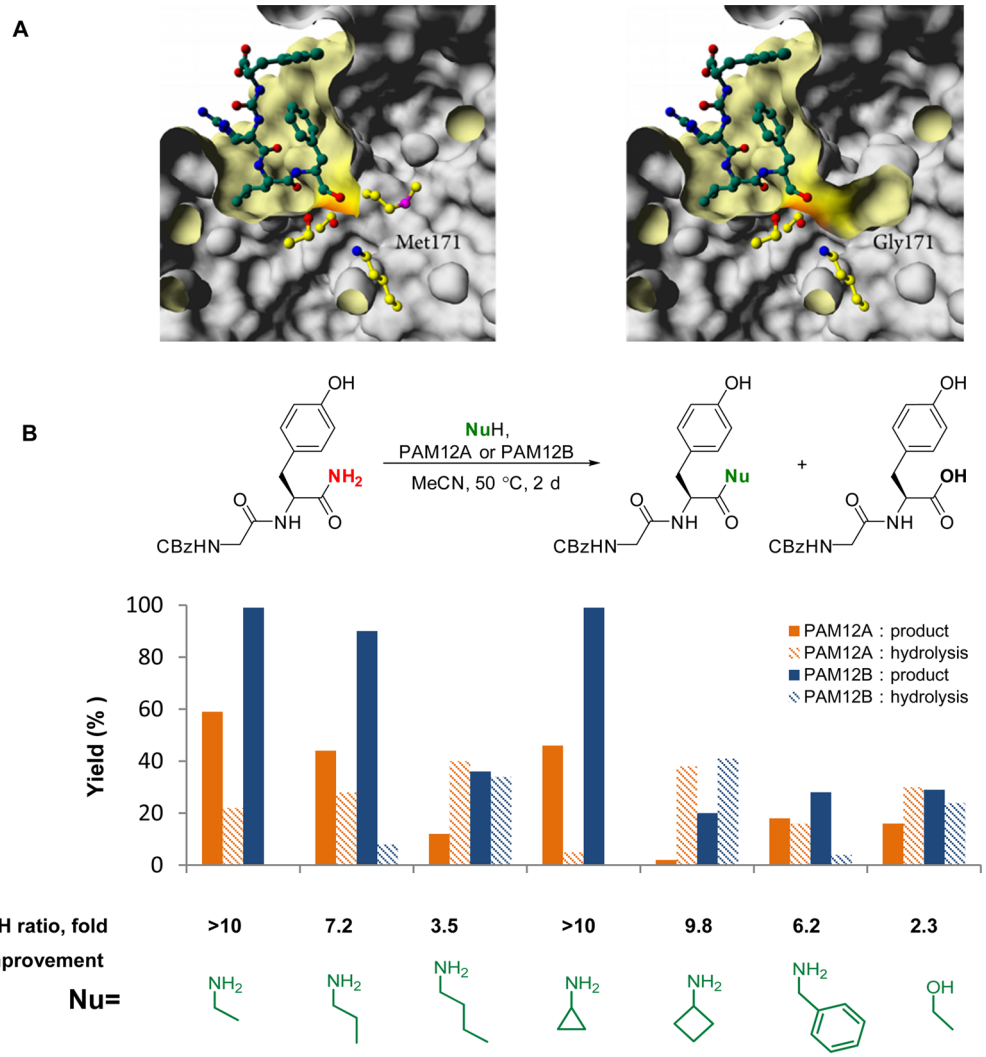

Figure 10. Expanded peptide functionalization using PAM12B. (A) Comparison of the active sites of PAM12A and PAM12B. The M171G mutation will enlarge the binding pocket for nucleophiles in PAM12B. The left panel shows the active site of PAM12A. Atom coordinates of the inhibitor chymostatin (sea green) were taken from PDB entry 1M21. The catalytic triad and Met171 are colored yellow. The right panel shows a model of the PAM12B active site, built with YASARA-Structure (www.yasara.org). (B) Comparison of PAM12A and PAM12B for modification of Z-Gly-Tyr-NH_2 with large nucleophiles. The S/H ratio is defined as the synthesis of desired product vs hydrolysis of the peptide substrate. Detailed reaction conditions are described in the Supporting Information. Product yields were determined by HPLC, and product identities were confirmed by MS.

we also demonstrated that PAM12A can be applied for bioactive peptides and long peptide modifications.

In addition to the enzyme's inherent virtues, the robustness of the redesigned PAM is a key feature allowing synthetic applications. The compatibility of PAM12A with diverse solvents, the tolerance to various nucleophiles without compromising selectivity, and the increased catalytic activity at elevated temperatures make it possible to use the enzyme under diverse conditions and allow a wide range of selective C-terminal functionalization. As with other enzymes used for peptide modification,[10,31] the catalytic activity displayed by PAM12A in organic solvents is much lower than in aqueous solution. Nevertheless, the relatively high enzyme loading is not a limiting factor for the process, because PAM12A can be produced at low cost by fermentation. The (co)solvent resistance is of special importance in view of the diversity of peptide properties and the necessity to use specific solvent mixtures for peptide solubilization. More importantly, the stability of PAM12A in nearly neat organic solvent allows high-yield conversions that do not proceed in water, particularly for the modification of peptide free acids. These conversions could be useful for various applications, such as functionalization of food-derived bioactive peptides and protein hydrolysates.

The stabilization of PAM was achieved by introducing a set of 12 mutations found by the FRESCO approach[20] for computational library design. Using conventional rational protein engineering or directed evolution, the swift discovery of such an intensively engineered variant would be troublesome. The crystal structures revealed that the introduced mutations stabilize the protein through diverse biophysical effects, which are also expected to prevent unfolding in the presence of organic solvents. It should be emphasized that the enhanced stability did not reduce activity, and there is no thermodynamic necessity for a protein to rigidify for its melting temperature to increase.[52] Furthermore, using the robust PAM12A as the template, a variant PAM12B that displays complementary substrate specificity was rationally designed.

In summary, we report the use of computational tools for developing a facile, regioselective, and broadly applicable method for C-terminal modification of peptides. The engineered enzymes

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catalyze a diversity of functionalization reactions of side chain unprotected peptides, with relaxed sequence and C-terminal functionality requirements. Depending on the desired reaction and substrate properties, they can be applied under aqueous conditions, in water/cosolvent mixtures, or in nearly anhydrous organic solvents. The robust peptide amidase variants may present a suitable platform for further engineering, enhancement of catalytic performance, and expansion of nucleophile specificity that would be particularly interesting.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.6b01062.

Experimental details and supplementary results, tables, and figures (PDF)

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Notes

The authors declare the following competing financial interest(s): T.N. and P.J.L.M.Q. are employees of Enzypep B.V. Enzypep B.V. develops chemoenzymatic processes for peptide synthesis.

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