

Catalysis of hydrazone and oxime peptide ligation by arginine

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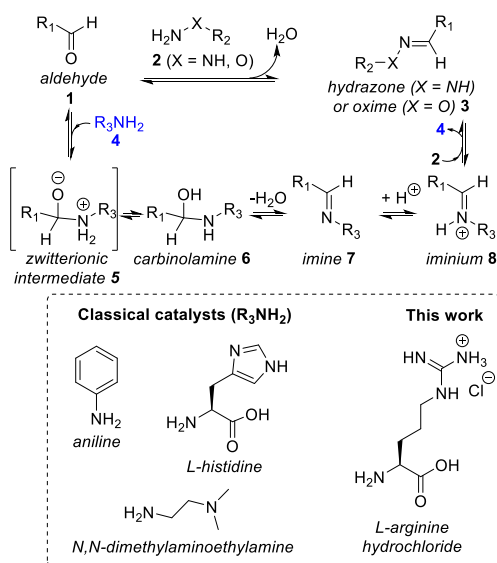
Supporting Information Placeholder



ABSTRACT: Hydrazone and oxime peptide ligations are catalyzed by arginine. The catalysis is assisted intramolecularly by the side-chain guanidinium group. Hydrazone ligation in the presence of arginine proceeds efficiently in phosphate buffer at neutral pH but is particularly powerful in bicarbonate/CO₂ buffer. In addition to acting as a catalyst, arginine prevents the aggregation of proteins during ligation. With its dual properties as nucleophilic catalyst and protein aggregation inhibitor, arginine hydrochloride is a useful addition to the hydrazone/oxime ligation toolbox.

Imine-based reactions leading to the formation of hydrazones or oximes (X = N or O respectively, Figure 1) are popular bioconjugation techniques recognized for their ease of

Figure 1. Principle of hydrazone or oxime ligations and of their nucleophilic catalysis by amine compounds. R₁, R₂ = Biomolecules, dyes, etc...



implementation, their high chemoselectivity in water and their compatibility with a wide range of substrates including polypeptides, nucleic acids and nanomaterials.^{1, 2} Hydrazone and oxime formation show useful reaction rates at mildly acidic pH (4-5) but are very slow processes in water at neutral pH, which is the preferred media for manipulating complex biomolecules such as proteins. As a consequence, many studies focused on accelerating these reactions by thermal activation,³ by freezing,⁴ by protein-protein complex formation,⁵ by addition of organic co-solvents³ or catalysts.^{1, 6-9} Nucleophilic catalysis by amine compounds such as aniline^{6, 8} (or derivatives thereof^{10, 11}) or *N,N*-dimethylaminoethylamine^{7, 12} has proven especially powerful in this regard (Figure 1).

In such a process, the amine catalyst **4** combines with the aldehyde **1** to produce imine **7**, which undergoes a transimination with hydrazine or hydroxylamine derivative **2** through iminium species **8**. The action of *N,N*-dimethylaminoethylamine involves an intramolecular acid-catalysis by the protonated *N,N*-dimethylamino group which considerably accelerates the formation of the imine product of type **7** by facilitating water elimination from carbinolamine **6** (Figure 1).⁷ In an analogous way, histidine has been shown to catalyze hydrazone or oxime bond formation, likely by favoring

the dehydration of carbinolamine **6** through intramolecular acid catalysis by the imidazolium side chain group.^{12, 13}

Despite a great interest with regard to reagent cost, operational advantages, low toxicity and adherence to sustainable chemistry principles,¹⁴ only a few works have examined the catalysis of hydrazone or oxime ligations by proteinogenic amino acids. Hereinafter, we report that the amino acid arginine catalyzes hydrazone or oxime bond formation in phosphate buffer at neutral pH. Detailed kinetic studies suggest that the side-chain guanidinium group plays a crucial role in the catalysis mechanism. We also show that the rate of arginine-catalyzed hydrazone ligation is dramatically enhanced in bicarbonate/CO₂ buffer, a classical buffer used for cell culture.

The model hydrazone ligations used for investigating the catalytic properties of arginine, added in the reactions as arginine hydrochloride, and other additives are shown in Figure 2. The hydrazide and α -oxo aldehyde functionalities were chosen for being popular and easily installed on peptides or proteins through synthetic or semi-synthetic methods.^{15, 16}

In a first series of experiments, hydrazone ligation was performed with model peptides **8a** and **9a** in pH 7.0 sodium phosphate buffer at 25 °C at 1 mM peptide concentration and 50 mM concentration for the additives. The progress of the reaction was monitored by measuring the absorbance of the hydrazone product **10a** at 285 nm. Kinetic data were fitted to extract the apparent second order rate constants. The reaction mixtures were also analyzed by LC-MS. Figure 3a shows that the catalytic potency of arginine hydrochloride is ~3 fold that of alanine. The relationship between arginine concentration and the rate of hydrazone formation is remarkably linear, with a break at ~400 mM (Figure 3b). At this catalyst concentration, hydrazone ligation is a fast process with a k_{app} of ~10 M⁻¹ min⁻¹. Arginine hydrochloride was found to catalyze ketoxime bond formation as well at pH 6.0 or 7.0 (see Supplementary Information).

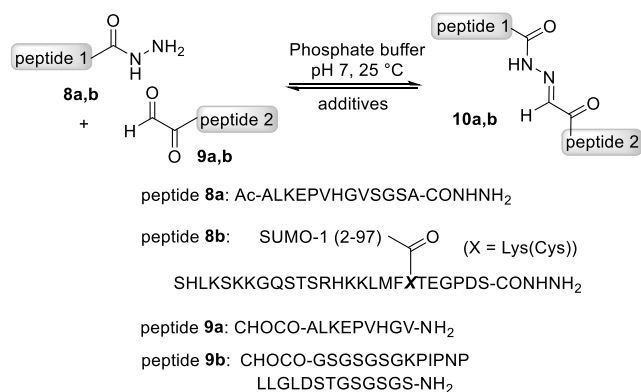


Figure 2. Model hydrazone ligations examined in this study.

The reaction between **8a** and **9a** was also catalyzed by histidine as reported for other types of aldehyde substrates.^{12, 13} In this case however, the reaction yielded a significant amount of side-product in addition to the expected hydrazone. This side-product was identified as the α -oxo aldehyde/histidine condensate, thereby discarding the use of histidine as a suitable

catalyst for hydrazone ligation with α -oxo aldehydes (see Supplementary Information). Note that the formation of cyclics adducts by reaction of histidine or histamine with aldehydes has been described by several groups.¹⁷⁻¹⁹ Therefore, arginine is the best proteinogenic amino acid described so far for catalyzing hydrazone formation with α -oxo aldehydes.

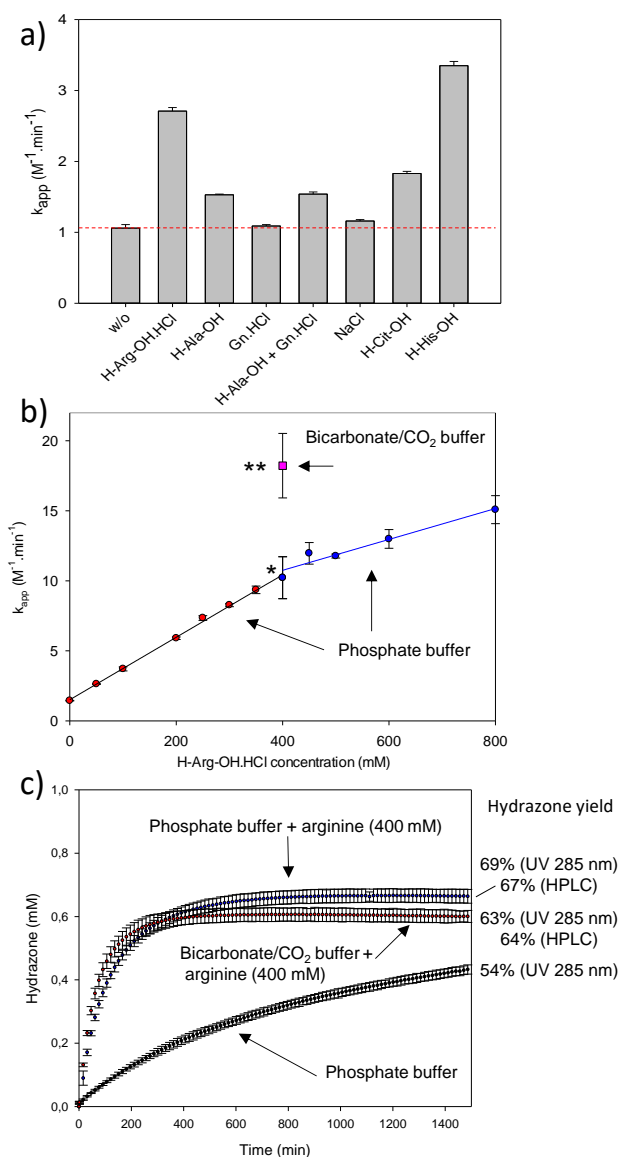


Figure 3. Apparent second order rate constant of hydrazone **10a** formation in the presence of amino acid and/or salt additives. Sodium phosphate buffer 0.1 M, ~1 mM final concentration for each peptide, pH ~7, 25 °C. The data correspond to the mean \pm standard error (95% confidence limit interval). a) The additive concentration was 50 mM. The reactions were performed in sextuplicate in one 96-well microtiter plate. The reaction with histidine is complicated by the formation of a covalent adduct between the CHOCO peptide and histidine catalyst in significant amounts (see Supplementary Information). b) Effect of H-Arg-OH.HCl concentration on the apparent second order rate constant of hydrazone formation in 0.1 M sodium phosphate (circles) or 40 mM bicarbonate/CO₂ buffer (square). * Mean of four independent experiments, each done in triplicate. ** Mean of five independent experiments, each done in triplicate. c) Time course of hydrazone

10a formation from **8a** (0.95 mM) and **9a** (0.99 mM) at 25 °C in phosphate buffer pH 7.04, phosphate buffer + arginine (400 mM) pH 7.01 or bicarbonate/CO₂ buffer + arginine (400 mM) pH 7.07.

At 50 mM catalyst concentration, we found that *N,N*-dimethylaminoethylamine ($6.46 \pm 0.05 \text{ M}^{-1}\cdot\text{min}^{-1}$) or aniline ($6.8 \pm 0.23 \text{ M}^{-1}\cdot\text{min}^{-1}$) perform better than arginine ($2.71 \pm 0.05 \text{ M}^{-1}\cdot\text{min}^{-1}$). However, raising arginine concentration to 200-250 mM enables to achieve a k_{app} of 6-7 $\text{M}^{-1}\cdot\text{min}^{-1}$ as well, with the advantage of using a catalyst of much lower toxicity. § Moreover, an interesting property of arginine hydrochloride is its well established capacity to inhibit protein aggregation.²⁰ It is a popular additive for protein refolding experiments and is frequently used at ~0.5 M concentration or higher for that purpose. Having a catalyst of hydrazone or oxime ligation that inhibits protein aggregation is a strong asset when it comes to work with complex protein targets (vide infra).

The data presented in Figure 3a show that sodium chloride, guanidine hydrochloride (Gn·HCl), or alanine plus Gn·HCl were all far below the catalytic level of arginine hydrochloride. The same was observed for citrulline which has a neutral urea group in its side-chain in lieu of a guanidinium group as in arginine. These series of experiments suggest that the catalysis of the hydrazone ligation by arginine is based on an intramolecular catalytic participation of the side-chain guanidinium group. Contrary to tertiary ammonium of imidazolium cations, guanidinium cations are very weak acids including those found in arginine (pK_{a} 13.8).²¹ Logically, evidence for a role of arginine residue as an acid catalyst in enzymes are scarce.^{22, 23} The occurrence of a general acid-catalysis of the carbinolamine dehydration step (**6** → **7** in Figure 1) by guanidinium cation, as observed for histidine or *N,N*-dimethylaminoethylamine, is therefore not obvious. Mechanisms other than general acid catalysis can impact imine formation. For example, some computational studies suggest that explicit water molecules can promote the dehydration of the carbinolamine **6** by facilitating an internal N-to-O proton shuffling mechanism.²⁴ Interestingly, arginine is strongly suspected to act as a relay for proton transfers occurring in bacteriorhodopsin,²⁵ pyridoxal 5'-phosphate synthase and other proteins.²⁶⁻²⁸ The question whether the side-chain guanidinium cation of arginine promotes hydrazone formation by facilitating proton transfers, by acting as a general acid catalyst or by another mean will require more investigations.

At high arginine hydrochloride concentrations (e. g. 400 mM), a few percent of a byproduct corresponding to the conversion of the α -oxo aldehyde moiety into a glycine residue was observed by LC-MS. We reasoned that the byproduct arose from a transamination of the α -oxo aldehyde peptide by the excess of added arginine, a reaction that is known to be promoted by trace metals. In accord with this hypothesis, byproduct formation could be suppressed by adding disodium ethylenediaminetetraacetate (EDTA, 1 mM) to the ligation mixture (see Supplementary Information).

After having scrutinized the arginine-catalyzed hydrazone ligation using model peptides **8a** and **9a**, we examined the reaction of protein hydrazide **8b** (0.5 mM) with α -oxo aldehyde peptide **9b** (2.5 mM) in the presence of arginine hydrochloride (400 mM) in phosphate buffer (pH 7.0). Protein **8b** is a fully

synthetic 14 kDa branched protein made of a SUMO-1 domain linked to a human p53 peptide. The sequence of peptide **9b** corresponds to V5 peptidic tag. The ligation was monitored by SDS-PAGE followed by Western-blot analysis (Figure 4). The anti-SUMO-1 antibody was used to visualize protein hydrazide **8b** and the hydrazone product **10b**, while anti-V5 antibody was used to reveal only the hydrazone product **10b**. These analyses show the successful formation of hydrazone product **10b** whose identity was confirmed by LC-MS and proteomic analysis (see Supplementary Information). The equilibrium was reached in about 300 min. The control experiment conducted in the absence of arginine catalyst failed to provide the expected protein hydrazone **10b** due to protein precipitation (see Supplementary Information). This example is a nice illustration of the dual properties of arginine which acts as a catalyst of hydrazone bond formation and as an inhibitor of protein aggregation.

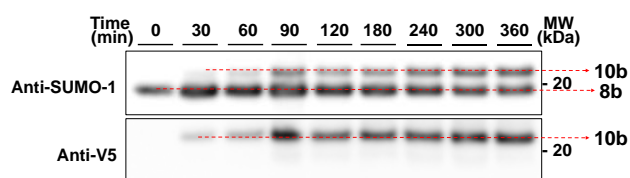


Figure 4. Western-blot analysis of hydrazone ligation between SUMO-1 p53 protein hydrazide **8b** and α -oxo aldehyde peptide **9b**. Synthesis of protein hydrazone **10b**. Conditions: **8b** 0.5 mM, **9b** 2.5 mM, arginine hydrochloride 400 mM, 0.1 M sodium phosphate buffer pH 7.0, 25 °C.

After having validated the interest of arginine catalysis in phosphate buffer at the protein level, we were interested to know if catalysis proceeds also in bicarbonate/CO₂ buffer (pH 7.2-7.4), which is a classical buffering system used for cell culture media. Toward this end, an aqueous solution of sodium bicarbonate (40 mM) was equilibrated for two days in a standard CO₂ incubator for cell culture (5% pCO₂) and then used to dissolve arginine hydrochloride at 400 mM. With a resulting pH of ~7.0, the solution was used directly for performing the hydrazone ligation of peptides **8a** and **9a**. We found that the rate constant for the arginine-catalyzed reaction in bicarbonate/CO₂ buffer ($18.2 \pm 2.3 \text{ M}^{-1}\cdot\text{min}^{-1}$) was almost twice that measured in phosphate buffer at the same pH (Figure 3b, pink square; Figure 3c). Note that we verified that phosphate buffer concentration (50-200 mM) had no effect on the rate of hydrazone formation whether arginine (400 mM) is present or not (see Figure S 6). Thus, contrary to phosphate buffer, bicarbonate/CO₂ buffer plays a major role in the reaction and its combination with arginine enables to achieve rates as fast as ~20 $\text{M}^{-1}\cdot\text{min}^{-1}$. It is tempting to suggest that bicarbonate ion might act through bifunctional acid-base catalysis and favor N to O proton transfers, as such mechanisms were observed for a few addition reactions occurring at carbonyls.²⁹⁻³¹ However, the precise understanding of arginine hydrochloride-bicarbonate/CO₂ system requires further investigations.

In conclusion, arginine hydrochloride catalyzes hydrazone and oxime bond formation in water at neutral pH. The reaction can be performed in phosphate buffer but proceeds at a much faster rate in bicarbonate/CO₂ buffer. Arginine hydrochloride

prevents the aggregation and precipitation of complex protein targets, is inexpensive, and shows low levels of toxicity. In that respect, arginine hydrochloride with its dual properties of nucleophilic catalyst and protein aggregation inhibitor is a useful addition to the hydrazone/oxime ligation toolbox.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, data fitting and characterization for all peptides (PDF file).

Data source file for kinetic studies (excel file)

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Author Contributions

NO, VA and OM wrote the manuscript. / NO, BS and RD performed the experiments. VA performed the data fitting. JV and OM supervised the work. OM conceived the study. / All authors have given approval to the final version of the manuscript.

Notes

§ *N,N*-dimethylaminoethylamine is classified as a corrosive and toxic substance with an LD50 in rat (1135 mg/kg) 10 fold lower than those of arginine hydrochloride (12000 mg/kg, Source United States Environment Protection Agency). Aniline (LD50 in rat 780 mg/kg, European Chemical Agency) is suspected to be carcinogenic and mutagenic and is classified as class 3 toxic substance.

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REFERENCES

1. Kölmel, D. K.; Kool, E. T., Oximes and Hydrazones in Bioconjugation: Mechanism and Catalysis. *Chem. Rev.* **2017**, *117*, 10358-10376.
2. Algar, W. R.; Prasuhn, D. E.; Stewart, M. H.; Jennings, T. L.; Blanco-Canosa, J. B.; Dawson, P. E.; Medintz, I. L., The Controlled Display of Biomolecules on Nanoparticles: A Challenge Suited to Bioorthogonal Chemistry. *Bioconjugate Chem.* **2011**, *22*, 825-858.
3. Shao, J.; Tam, J. P., Unprotected Peptides as Building Blocks for the Synthesis of Peptide Dendrimers with Oxime, Hydrazone, and Thiazolidine Linkages. *J. Am. Chem. Soc.* **1995**, *117*, 3893-3899.
4. Agten, S. M.; Suylen, D. P. L.; Hackeng, T. M., Oxime Catalysis by Freezing. *Bioconjugate Chem.* **2016**, *27*, 42-46.
5. Agten, S. M.; Koenen, R. R.; Ippel, H.; Eckardt, V.; von Hundelshausen, P.; Mayo, K. H.; Weber, C.; Hackeng, T. M., Probing Functional Heteromeric Chemokine Protein-Protein Interactions through Conformation-Assisted Oxime Ligation. *Angew. Chem. Int. Ed.* **2016**, *55*, 14963-14966.
6. Cordes, E. H.; Jencks, W. P., Nucleophilic Catalysis of Semicarbazone Formation by Anilines. *J. Am. Chem. Soc.* **1962**, *84*, 826-831.
7. Hine, J.; Cholod, M. S.; Chess, W. K., Kinetics of the Formation of Imines from Acetone and Primary Amines. Evidence for Internal Acid-Catalyzed Dehydration of Certain Intermediate Carbinolamines. *J. Am. Chem. Soc.* **1973**, *95*, 4270-4276.
8. Dirksen, A.; Hackeng, T. M.; Dawson, P. E., Nucleophilic Catalysis of Oxime Ligation. *Angew. Chem. Int. Ed.* **2006**, *45*, 7581-7584.
9. Dirksen, A.; Dirksen, S.; Hackeng, T. M.; Dawson, P. E., Nucleophilic Catalysis of Hydrazone Formation and Transimination: Implications for Dynamic Covalent Chemistry. *J. Am. Chem. Soc.* **2006**, *128*, 15602-15603.
10. Wendeler, M.; Grinberg, L.; Wang, X.; Dawson, P. E.; Baca, M., Enhanced Catalysis of Oxime-Based Bioconjugations by Substituted Anilines. *Bioconjugate Chem.* **2014**, *25*, 93-101.
11. Zhou, Y.; Piergentili, I.; Hong, J.; van der Helm, M. P.; Macchione, M.; Li, Y.; Eelkema, R.; Luo, S., Indoline Catalyzed Acylhydrazone/Oxime Condensation under Neutral Aqueous Conditions. *Org. Lett.* **2020**, *22*, 6035-6040.
12. Larsen, D.; Kietrys, A. M.; Clark, S. A.; Park, H. S.; Ekebergh, A.; Kool, E. T., Exceptionally Rapid Oxime and Hydrazone Formation Promoted by Catalytic Amine Buffers with Low Toxicity. *Chem. Sci.* **2018**, *9*, 5252-5259.
13. Larsen, D.; Pittelkow, M.; Karmakar, S.; Kool, E. T., New Organocatalyst Scaffolds with High Activity in Promoting Hydrazone and Oxime Formation at Neutral pH. *Org. Lett.* **2015**, *17*, 274-277.
14. Collins, T., Toward Sustainable Chemistry. *Science* **2001**, *291*, 48-49.
15. El-Mahdi, O.; Melnyk, O., Alpha-Oxo Aldehyde or Glyoxylal Group Chemistry in Peptide Bioconjugation. *Bioconjugate Chem.* **2013**, *24*, 735-765.
16. Zheng, J.-S.; Tang, S.; Qi, Y.-K.; Wang, Z.-P.; Liu, L., Chemical Synthesis of Proteins Using Peptide Hydrazides as Thioester Surrogates. *Nat. Protocols* **2013**, *8*, 2483-2495.
17. Rönnberg, A. L.; Hansson, C.; Drakenberg, T.; Håkanson, R., Reaction of Histamine with O-Phthalaldehyde: Isolation and Analysis of the Fluorophore. *Anal. Biochem.* **1984**, *139*, 329-337.
18. Kierska, D.; Sasiak, K.; Maśliński, C., Phosphopyridoxal Cyclic Compounds with Histamine and Histidine. 6: The Formation of Phosphopyridoxal Cyclic Compounds with Histamine and Histidine in the Presence of Biological Material. *Agents and Actions* **1978**, *8*, 470-473.
19. Robert, L.; Penaranda, F. S., Studies on Aldehyde-Protein Interactions. I. Reaction of Amino Acids with Lower Aldehydes. *J. Polym. Sci.* **1954**, *12*, 337-350.
20. Lange, C.; Rudolph, R., Suppression of Protein Aggregation by L-Arginine. *Curr. Pharm. Biotechnol.* **2009**, *10*, 408-414.
21. Fitch, C. A.; Platzer, G.; Okon, M.; Garcia-Moreno, B. E.; McIntosh, L. P., Arginine: Its pKa Value Revisited. *Protein Sci.* **2015**, *24*, 752-61.
22. Keenholz, R. A.; Mouw, K. W.; Boocock, M. R.; Li, N.-S.; Piccirilli, J. A.; Rice, P. A., Arginine as a General Acid Catalyst in Serine Recombinase-Mediated DNA Cleavage. *J. Biol. Chem.* **2013**, *288*, 29206-29214.
23. Silva, P. J.; Schulz, C.; Jahn, D.; Jahn, M.; Ramos, M. J., A Tale of Two Acids: When Arginine Is a More Appropriate Acid Than H₃O⁺. *J. Chem. Phys. B* **2010**, *114*, 8994-9001.
24. Hall, N. E.; Smith, B. J., High-Level ab Initio Molecular Orbital Calculations of Imine Formation. *J. Chem. Phys. A* **1998**, *102*, 4930-4938.
25. Ge, X.; Gunner, M. R., Unraveling the Mechanism of Proton Translocation in the Extracellular Half-Channel of Bacteriorhodopsin. *Proteins: Structure, Function, and Bioinformatics* **2016**, *84*, 639-654.
26. Moccand, C.; Kaufmann, M.; Fitzpatrick, T. B., It Takes Two to Tango: Defining an Essential Second Active Site in Pyridoxal 5'-Phosphate Synthase. *PLOS ONE* **2011**, *6*, e16042.
27. Chang, H.-C.; Kung, C. C. H.; Chang, T.-T.; Jao, S.-C.; Hsu, Y.-T.; Li, W.-S., Investigation of the Proton Relay System Operative in Human Cytosolic Aminopeptidase P. *PLOS ONE* **2018**, *13*, e0190816.
28. Jao, S.-C.; Huang, L.-F.; Hwang, S.-M.; Li, W.-S., Tyrosine 387 and Arginine 404 Are Critical in the Hydrolytic Mechanism of Escherichia coli Aminopeptidase P. *Biochemistry* **2006**, *45*, 1547-1553.

29. Barnett, R. E.; Jencks, W. P., Diffusion-Controlled Proton Transfer in Intramolecular Thiol Ester Aminolysis and Thiazoline Hydrolysis. *J. Am. Chem. Soc.* **1969**, *91*, 2358-2369.

30. Cunningham, B. A.; Schmir, G. L., Iminolactones. II. Catalytic Effects on the Nature of the Products of Hydrolysis. *J. Am. Chem. Soc.* **1966**, *88*, 551-558.

31. Eriksson, S. O., Hydrolysis of Anilides. IV. Hydroxylaminolysis, Hydrazinolysis, and General Acid-Catalysed Alkaline Hydrolysis of Trifluoroacetanilide. *Acta Chem. Scand.* **1968**, *22*, 892-906.