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Cleavable Amide Bond: Mechanistic Insight of Cleavable *4-Aminopyrazolyloxy Acetamide* at low pH

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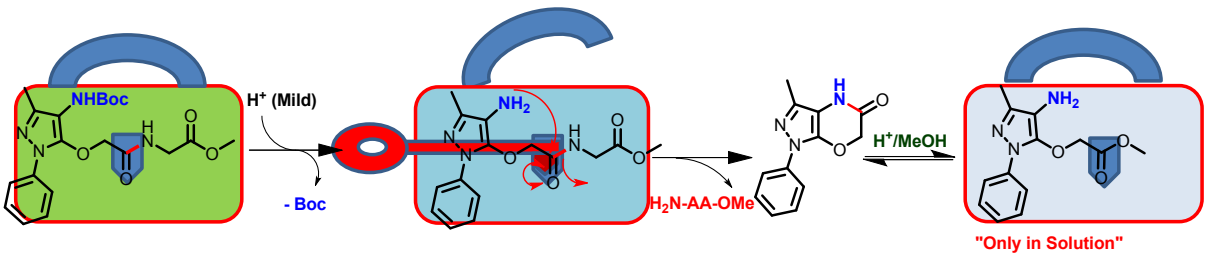
Abstract

The cleavage of amide bonds under mild acidic conditions are rare chemical event. *N*-acetamide bond of peptides is extremely stable even under the strongest organic acid TFMSA. This report mechanistically describes a new cleavable amide bond in 4-*aminopyrazolyloxy acetamide* peptide analogues under mild acidic conditions such as TFA (10-20%) or HCl (0.1 – 4.0 N) at room temperature, and the formation of unusual lactam from 4-*aminopyrazolyloxy acetic acid* after evaporation of solvent. This is a rare chemical event in peptide bond which could be explored as acid sensitive protecting group of free amines.



Introduction

Natural amides are extremely stable, and their half-life for spontaneous hydrolysis is estimated to be 350 to 600 years at neutral pH and room temperature.[1] The carbonyl group of amide are poor electrophiles because of the typical resonance stability. Though amides can readily be

cleaved/hydrolyzed by enzymes such as proteases. It is difficult to cleave the C-N bond of an amide selectively using synthetic chemistry. The cleavage/hydrolysis of amide bonds usually requires heating under strongly acidic or basic conditions. However, the cyclic amides, lactams, are easily cleavable as compare to the linear amides because of ring strained amides.[2] A large number of the highly strained lactams are synthesized and found as cleavable amide bond under mild conditions. Since the C=O bond of reactive lactams amide is arising to the strong electrophile as "ketonic" carbonyl because of the resonance decoupling through N-C=O torsion. Brown and co-workers have mechanistically confirmed that resonance decoupling induces the remarkable enhancement in hydrolysis rate by direct nucleophilic attack at the carbonyl of strained amide.[3] The most twisted amide as Trimethylazatricyclodecanone is highly strained lactam with large resonance decoupling through N-C bond, and found as readily hydrolysable. This twisted lactam also shows the dual reactivity of amide as nucleophilic character of amine and electrophilic nature of carbonyl. Booker-Milburn and co-workers have reported the solvolysis/substitution reaction of acyclic synthetic amides at room temperature and neutral conditions via the formation of ketene intermediates.[4] In literature, the cleavage of few natural amide bonds are precisely demonsterated through the formation of thiazolinone derivatives (by Edman degradation), oxazolinium intermediates and chemical modification of the amide backbone.[5] Most Importantly, Edman degradation method for cleavage of amide bond is foundation for protein sequencing technology.[5a] The presence of Zinc species in active sites of metalloproteases have inspired synthetic chemists to cleave amide bond using Zn^{2+}. Mashima and co-workers have reported the cleavage of amides bearing a β-hydroxyethyl group under mild conditions with Lewis acid $Zn(OTf)_2$.[6] Kostic and co-workers have prepared an artificial peptidase which cleavage of the sequence specific amide bonds as Pro-Met/Pro-His segments of peptide under mild conditions with Pd-catalyst.[7] In repertoire of the making reactive amide group, Hauk, Garg and co-workers have established the conversion of amide

functional group into ester group by cleaving C-N bond of amide using Ni-catalyst.[8] The conversion of an amide into another amide with different amine group, transamidation, is important chemical transformation reaction for ligation or removal of amino functionalized chemical moieties at carboxylate functionalized molecules.[9] Gellman, Stahl and co-workers have explored the amide groups for transamidation reaction, between secondary amines and tertiary amines, using Zr/Hf-catalyst.[9a] Other Metal catalyzed and metal free transamidation reactions are also reported.[9b, c, 10] Recently, we have also established the transamidation reaction in tropolonylalkylglycinate peptide derivatives with different amino acid/peptides.

We, recently, have found the instability of amide bonds containing *N*-Tropolonylaminoalkylglycinate (*Trag*) in short peptides under mild acidic conditions. The formation of stable *tropolonium cations* followed by the formation of ketene intermediate are main caused for the cleavage of *Trag*-amide bonds.[11] In repertoire of unnatural heterocyclic aromatic amino acids, we have explored the syntheses and supramolecular self-assembly studies of pyrazolone containing amino acid analogues. One of those analogue is 4-aminopyrazolonyl acetate has been conjugated with natural amino acid/short peptides for understanding the role of pyrazolonyl moiety in conformational changes of peptides.[12] However these conjugated peptides have shown an unusual reactivity under mild acidic conditions (Figure 1). To understand the role of 4-aminopyrazolonyl acetate in cleveage of amide bond, herein we report the syntheses and chemical instability of amide bond in simple non-functional amino acids and di-/tri-peptide containing aminopyrazolonyl acetyl residue under mild acidic conditions at room temperature by NMR and Mass analyses.

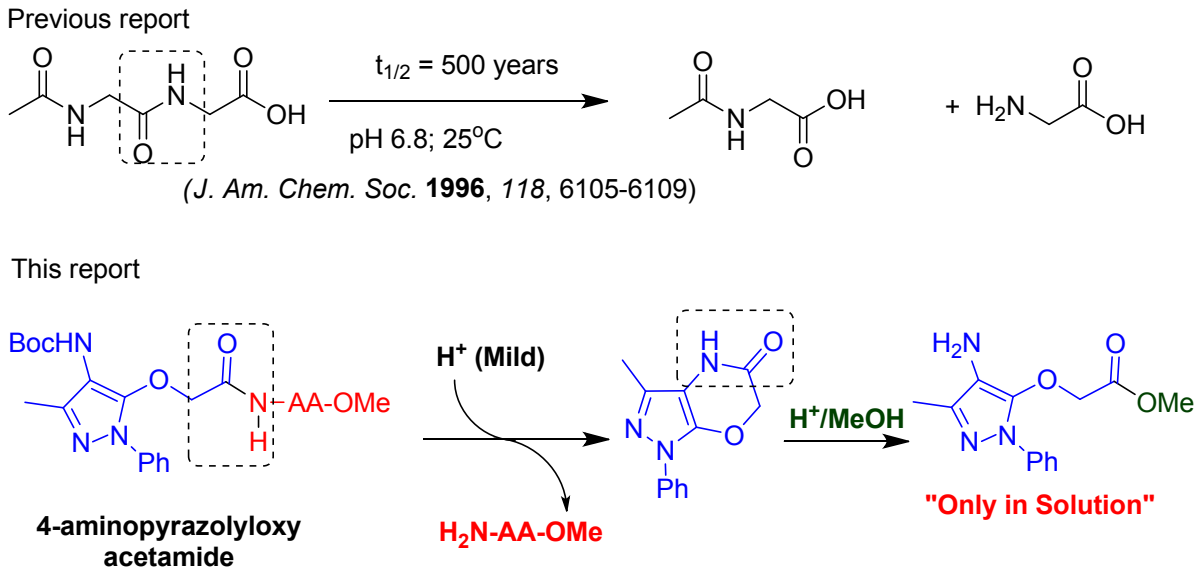
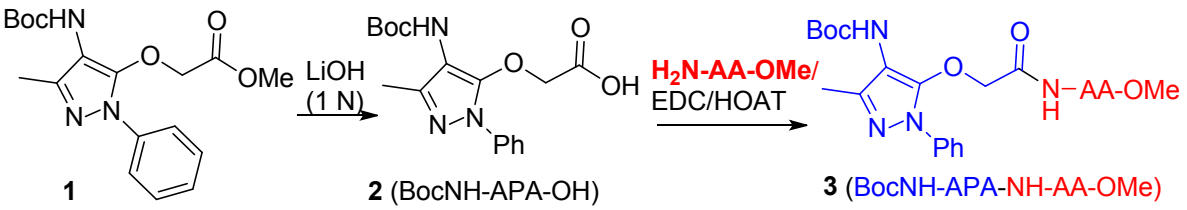


Figure 1. Hydrolyzable amide bonds: previous report (a); and this report (b).

Result and discussion

We used previously synthesized 4-aminopyrazolonyl amino acid (APA) ester derivative (**1**) for synthesis of conjugated peptides and their chemical behavior under mild acidic conditions. Ester (**1**) was hydrolyzed into 4-aminopyrazolonyl amino acid, APA acid (**2**) with LiOH (1.0N in THF), and then coupled with amino group of various natural amino acids/peptides (H_2N-AA-OMe) under peptide coupling reaction conditions which produced conjugated peptides **3** (APA-AA-OMe). Natural α-amino acid ester derivatives of Gly-OMe, Ala-OMe, Ile-OMe, and Phe-OMe produced unnatural respective conjugated dipeptides as BocNH-APA-Gly-OMe (**3a**), BocNH-APA-Ala-OMe (**3b**), BocNH-APA-Ile-OMe (**3c**), BocNH-APA-Phe-OMe. Further natural α-amino acid dipeptide esters, Gly-Ala-OMe, Ala-Gly-OMe, Gly-Ile-OMe and Ile-Gly-OMe gave respective unnatural conjugated tripeptides as BocNH-APA-Gly-Ala-OMe (**3e**), BocNH-APA-Ala-Gly-OMe (**3f**) BocNH-APA-Gly-Ile-OMe (**3g**), and BocNH-APA-Ile-Gly-OMe (**3h**) as shown in Scheme 1. The characterization data (^{1}H-/^{13}C{^{1}H}-NMR/Mass) of all synthesized peptides are provided in Supporting Information.

Scheme 1. Synthesis of *N*-Boc-aminopyrazolonyl amino acid and their peptides.



Peptides

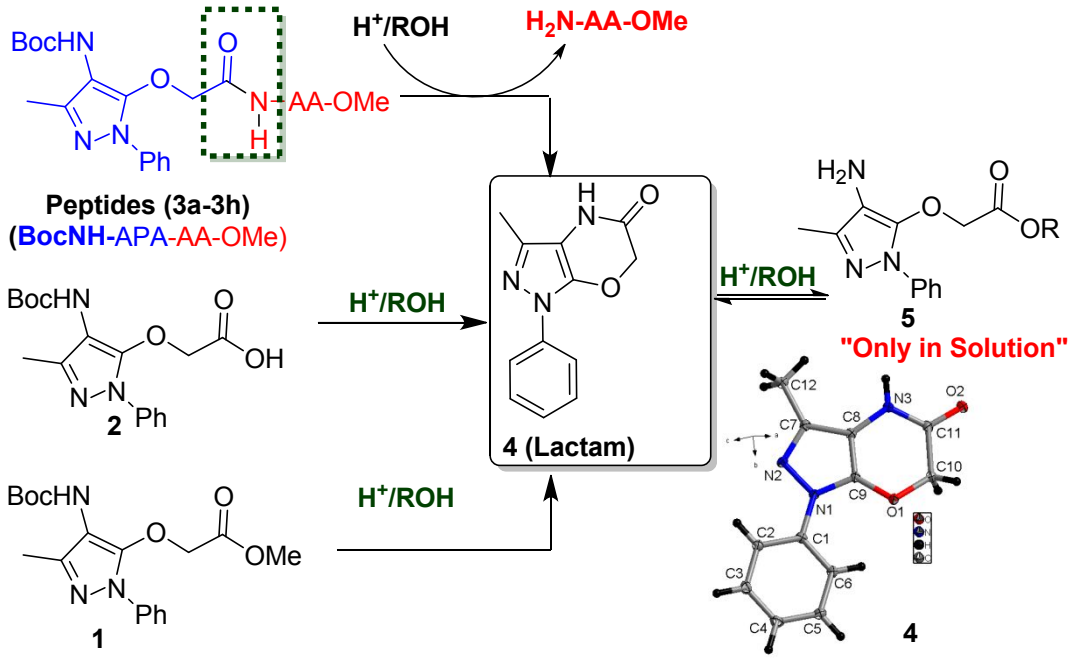
3a. BocNH-APA-Gly-OMe
3b. BocNH-APA-Ala-OMe
3c. BocNH-APA-Ile-OMe
3d. BocNH-APA-Phe-OMe
3e. BocNH-APA-Gly-Ala-OMe
3f. BocNH-APA-Ala-Gly-OMe
3g. BocNH-APA-Gly-Ile-OMe
3h. BocNH-APA-Ile-Gly-OMe

For further elongation of peptides at *N*-terminal of hybrid peptides **3a-3h**, the removal of *N*-Boc group was necessary. The removal of Boc group of *N*-Boc-aminopyrazolonyl amino acid containing peptides **3a-3h** was attempted with 20% TFA in DCM, unfortunately we could not achieve desired aminopyrazolonyl amino acid peptides (Scheme 2). Surprisingly, we noticed the cleavage of amide bond by ESI-Mass studies. Then we isolated a new crystalline product and characterized as aminopyrazolyl lactam **4** by NMR & HRMS analysis. This crystal was also studied by single crystal X-ray diffractometer. The X-ray studies confirmed the structure of lactam **4**, the ORTEP diagram is shown in Scheme 2. The cif file of crystal lactam **4** has deposited to CCDC with number CCDC 1890879. To ensure this cleavage of amide bond, we performed the similar acid treatment reaction with other hybrid di-/tri-peptides, **3b-3h** and then characterized their isolated products by NMR and Mass analyses (See Supporting Information). Irrespective of α-amino acid sequence, aminopyrazolyl amino acid containing amide bonds in peptides **3b-3h** were cleaved and produced the similar cyclized product as lactam **4**. In case of hybrid tripeptides **3e-3h**, other natural amide bonds, dipeptides (between two α-amino acids) were stable under that acidic conditions (20% TFA in DCM). To test the selective cleavage of 4-aminopyrazolyloxy acetamide bond in the presence of Boc group, we treated compound **3e** with 0.1 N HCl/1, 4-dioxane and studied with ESI-MS (Figure S20 in SI), Here the removal of Boc is very slow, under these conditions there are no significant representative peaks at m/z

348 (*N*-Boc acid) or 330 (*N*-Boc lactam) for selective cleavage products. We noticed the cleavage of 4-aminopyrazolyloxy acetamide bond is not cleavable until the Boc group is deprotected. Along with lactam **4**, we also observed *N*-trifluoroacylation with 20% TFA in ESI-MS, this side reaction was minimized/avoided by using 10% TFA or 4.0 N HCl in 1,4-dioxane (Supporting Information, Figure S13-S15). These results strongly suggest the cleavage of specific amide bond containing 4-aminopyrazolonyl amino acid residue at C-terminal of α-amino acid/peptides.

Further, we attempted to remove the Boc group of *N*-Boc-aminopyrazolonyl amino acid ester **1** and its carboxylic acid **2** under similar acidic conditions (10-20% TFA in $CDCl_3$) for conjugation of aminoprazolonyl moiety at C-terminal of above mentioned di-/tri-peptides. Surprisingly, we failed to generate amino functionalized amino ester (**1-NH$_2$**) and acid (**2-NH$_2$**). However, we isolated a new product and characterized by NMR and ESI-Mass analyses. Our NMR and Mass results supported that both ester **1** and its acid **2** gave the common cyclized product as lactam **4** under mild acidic conditions (Scheme 2). The synthesis of that lactam **4** is extremely unusual from ester **1**/acid **2**/amide **3** under acidic conditions at room temperature which are also unusual in peptide chemistry.

Scheme 2. Reaction of APA derivatives under mild acidic conditions.



The progress of amide cleavage reactions, under acidic conditions, are studied by ^{1}H-NMR technique. We recorded the time dependent ^{1}H-NMR spectra of peptide **3a** under 10% TFA in deuterated solvent $CDCl_3$. The array of the ^{1}H-NMR spectra with time is provided in Supporting Information, Figure S25. Most importantly, the gradual disappearance of NMR signal, singlet at δ 5.0 (ppm), is noticed in peptide **3a** with respect to time under that acidic conditions. A transient increase and decrease of signal at δ 4.5 (ppm) is also observed. Similar time dependent NMR experiments were performed under acidic conditions with another peptide **3e**. Their NMR data are provided in Supporting Information (Figure S27). After the NMR study the same samples were subjected to ESI-MS in solvent CH_3CN (neat). Their mass spectra exhibit the mass (m/z) peak at 230, in solvent CH_3CN, for both peptides **3a**/**3e**, which belongs to mass of lactam **4** as $[M+H]^+$ Supporting Information (Figure S26 & S28). These results (NMR and Mass) strongly support the involvement of α-CH_2 of aminopyrazolonyl residue in the cleavage of peptide **3a**/**3e** under acidic condition (10% TFA in $CDCl_3$) via the formation of stable lactam **4**. Further, we performed the similar NMR and Mass experiments

with *N*-Boc-aminopyrazolonyl amino acid ester **1** and its carboxylic acid **2**. Their NMR spectra, NMR profiles and mass spectra are provided in the Supporting Information. Our results suggest that the formation of lactam **4** is common for both aminopyrazolonyl ester **1** and its carboxylic acid **2** under mild acidic conditions (10% TFA in $CDCl_3$). We studied the ^{1}H-NMR kinetics of amide **3a**/acid **2**/ester **1** hydrolysis in $CDCl_3$. The relative concentrations were calculated in mole fractions (x) from relative integrations of –*O*-C**H**$_2$- peak of amide **3a**/acid **2**/ester **1** with gradually increasing lactam **4** with time. The plots of mole fraction (x) with time for APA amide **3a** and APA acid **2** is provided in Figure 2, and APA ester **1** is provided in supporting information (Figure S35A). We have also generated simulated kinetic plots from the sequential first-order reaction model with COPASI software program using experimentally obtained k_1 and k_2 values and then fitted with our experimental kinetic plots (Figure S41-S43).

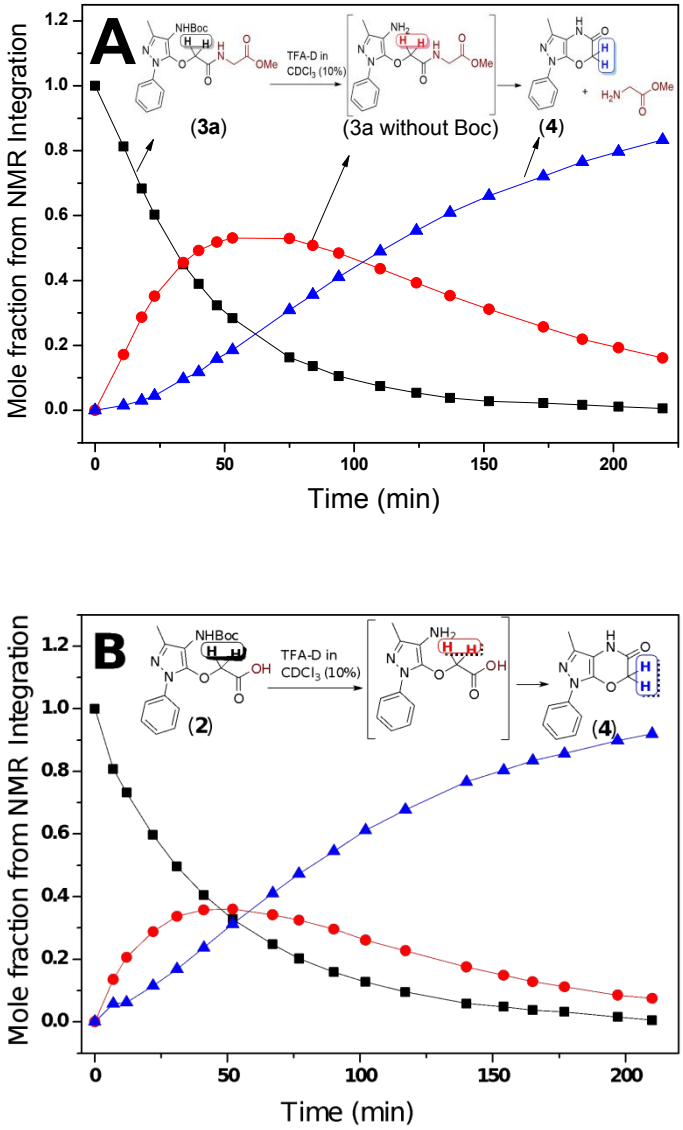


Figure 2. Time dependent ^{1}H-NMR studies of APA peptide **3a** (A) and APA acid **2** (B) in solvent $CDCl_3$ with 20 mM concentrations.

From the plots of concentration versus time, a transient increase and decrease of intermediate is also observed, this suggest that the cyclization of amide **3**/acid **2**/ester **1** is proceeding through an intermediate. After seeing these plots, we propose that compound **1**, **2**, & **3a** are following consecutive first order kinetic pathway and the rate laws are given by equations 1-3 in

supporting information. The plots of integral mole fraction versus time, (ln(x) vs time) are provided in Supporting Information (Figure S33-S35) and gave a straight line with negative slope which are consistent with literature reports for consecutive reaction pathway.[13] The rate constants k_1, k_2 and their respective half-lives ($t_{1/2}$) are calculated from these integral plots and provided in Table 1. The observed rate constants and half-lives from Table 1 suggest that the acid **2** is hydrolysed relatively faster than amide **3a** followed by ester **1**. This is possibly due to the intramolecular hydrogen bonding in APA peptide **3a** and acid **2,** between *H*-atom (-COOH-) or amide NH with *O*-atom (–***O***-*C*H_2-) of pyrazolonyl residue, which provide rigidity at carbonyl group and enhance the nucleophilic addition elimination reaction (Figure S44). Such hydrogen bonding is not possible with APA ester **1**.

Table 1. Kinetic data of 4-aminopyrazolyloxy derivatives from ^{1}H-NMR spectrum.

Compound	k_1/min^{-1}	k_2/min^{-1}	k_2/k_1	$t_{1/2}$ /min (for k_1)	$t_{1/2}$ /min (for k_2)
3a (Amide)	2.28 x 10^{-2}	0.83 x 10^{-2}	0.36	30.34	83.00
2 (Acid)	1.97 x 10^{-2}	1.09 x 10^{-2}	0.55	35.09	63.35
1 (Ester)	1.95 x 10^{-2}	0.78 x 10^{-2}	0.4	35.43	88.74

Interestingly, during the hydrolysis of amide bond in peptides **3a**-**3h**, we observed methanolysis of amide bond in lactam in mass spectrum and exist in methyl ester form in presence of MeOH (Supporting Information Figure S13-S18). To further understanding the reactivity of this amide bond, the lactam **4** is monitored in several aspects using mass spectroscopy (Supporting Information Figure S37), from these studies it is apparent that, acid and MeOH both are needed for the amide bond cleavage in lactam **4**. Moreover, there is no

complete disappearance of lactam in any of the cleavage conditions implemented during this studies. This cleavage is also monitored by time dependent ^{1}H-NMR in CD_3CN, CD_3OD and PTSA (~ 3.0 equivalents) at 293 K, from the array of spectra the reaction proceeds with the formation of hydrolysed methyl ester and remains constant after ~500 min without complete conversion of lactam **4**. The relative concentrations were calculated in mole fractions (x) from relative integrations of –*O*-C**H**$_2$- peak of lactam with gradually increasing methyl ester **4-OCD$_3$** with time, the resultant plots indicate the existence of equilibrium (Figure 3) and the rate law for this equilibrium is given by equation 4 in supporting information. The equilibrium constant (K) from the plot of mole ratio vs time has a value of 2.72 (Supporting Information Figure S36B). After NMR study, the same sample is subjected to ESI-MS, we observed the mass of both deuterated methyl ester (m/z 265) and lactam (Supporting Information Figure S37h). Importantly, we were not able to isolate this methyl ester. From these studies, we assume that the lactam **4** and its methyl ester **4-OCH$_3$** forms are in equilibrium in solution state in presence of acid and MeOH.

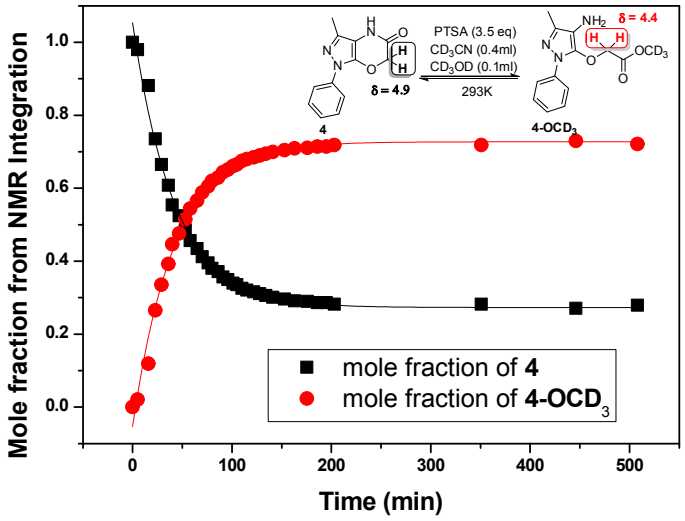


Figure 3. Time dependent ^{1}H-NMR studies of lactam **4** in CD_3CN, CD_3OD and PTSA.

Since aminopyrazolone is aromatic compound and UV active in nature. Thus we attempted to monitor the cleavage of lactam **4** into its amino ester derivative **4-OCH$_3$** under acidic condition by UV studies. We found that the UV spectrum of pure lactam **4** exhibited two characteristic peaks at wavelength (λ) 240 nm and 280 nm under neutral pH conditions (Supporting Information Figure S40). Then we recorded UV spectra of lactam **4** at different time intervals of times under acidic conditions (0.1 N HCl in MeOH) which exhibit the significant depletion of absorption intensity of lactam **4** at $\lambda_{240\ nm}$ and $\lambda_{280\ nm}$ (Figure 4). Such changes in UV spectra strongly support the formation of new species under that mild acidic conditions.

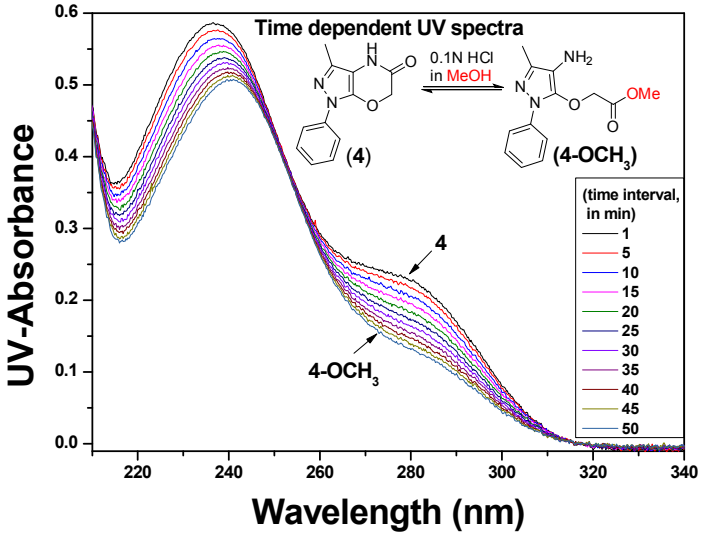


Figure 4. Time dependent UV-Spectra of lactam **4** under acidic conditions, time intervals in min. (right panel).

Recently, we have reported an unusual *syn* conformation between -***O***-***C***H$_2$–***C***O-***N***H- in crystal structures of peptides **3b**/**3h** and a weak intramolecular hydrogen bonding between *H*-atom of amide (-CONH-) and *O*-atom (–***O***-*C*H$_2$-).[12] Similar *syn* conformation is also noticed in crystal structure of APA ester **1** (Figure 5). Structural analyses of APA peptides **3b**/**3h** reveal that the

APA amide bond is twisted with tau (τ) ~ 5.5° (Supporting Information, Figure S39). We also noticed intramolecular hydrogen bonding between *H*-atom (-COOH-) and *O*-atom (–***O***-CH_2-) in APA acid **2** from NMR studies (Supporting Information, Figure S1-S4). The strong synergetic effects, such as electronegative –***O***-CH_2-, twisted amide bond (τ ~ 5.5°) and unfavourable *syn* conformation (ψ) weaken the stability of that amide bond. As a resultant, the favourable intramolecular attack of APA amine (6-exo-trig) led to the cleavage of that amide bond under mild acidic conditions.

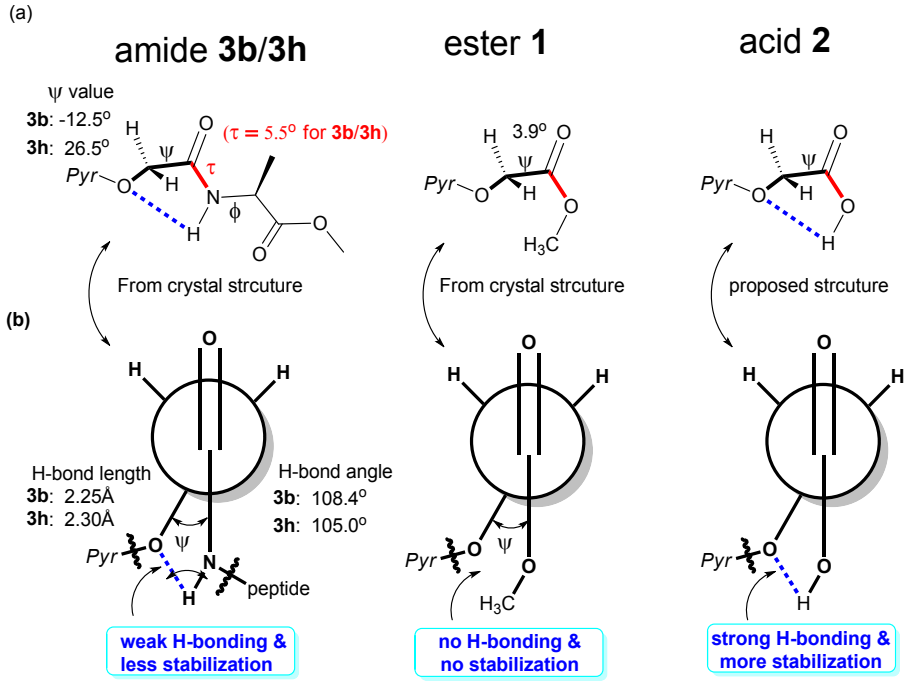


Figure 5. (a) Structures of amide **3b/3h**, ester **1**, acid **2**; (b) Their Newman Projection.

The removal of Boc group of aminopyrazolonyl derivatives **1/2/3** occurs under acidic conditions (10-20% TFA in DCM/CH_3CN) and generates the protonated amine intermediates (**a**) which may protonate the intramolecular carbonyl group of ester/acid/amide derivatives as intermediates (**b**). Subsequently the amine nucleophile possibly attacks at the protonated carbonyl electrophile as typical intramolecular nucleophilic addition-elimination reaction to

facilitate the formation of cyclic amide (lactam) derivative **4** (Figure 6). Most importantly, this lactam is stable in solvent free conditions and its structure is confirmed by single crystal X-ray analyses. The stability of this lactam **4** is further investigated under protic nucleophilic solvent (MeOH) under acidic conditions. Importantly, this lactam **4** is unstable in nucleophilic solvents (H_2O/MeOH) under acidic conditions and proceeds to amide hydrolysis possibly due to protonation of amide bond *N*-atom under acidic conditions. As resultant, lactam **4** hydrolyzes into acid/ester derivatives (**f**) with respective solvents H_2O/MeOH.

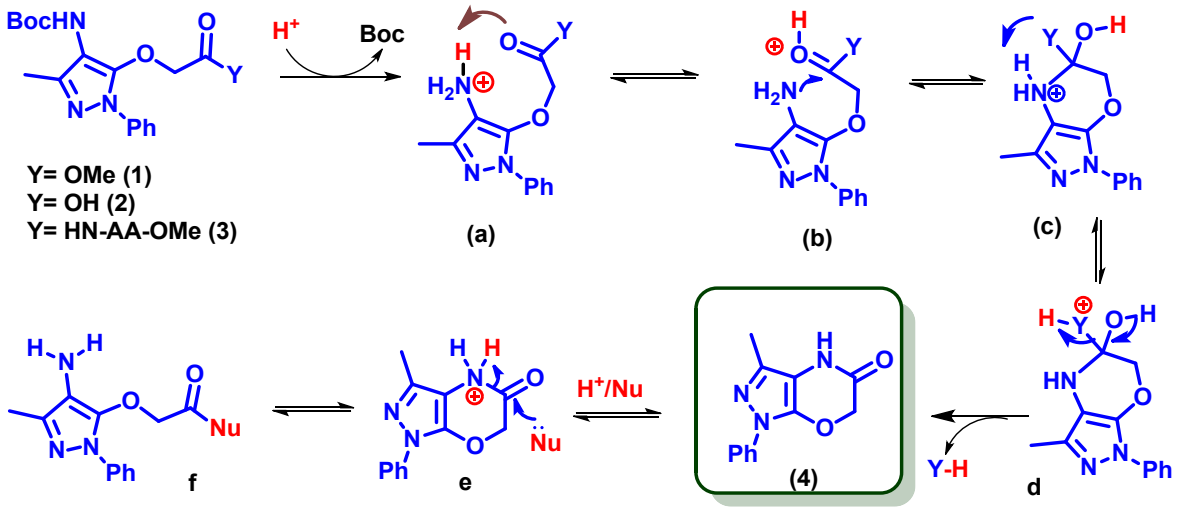


Figure 6. Plausible mechanism of amide hydrolysis.

Conclusion

In summary, 4-aminopyrazolyloxy acetate are successfully incorporated at *N*-terminal of natural di-/tri-peptides aminopyrazolyloxy acetamide derivatives which are hydrolysable under mild acidic conditions and such amide hydrolyses are rare. This unique character of amide bond is elaborated by NMR/UV/Mass/X-ray studies which helped to demonstrate the mechanism of reactions. Since aminopyrazoly rings and its derivatives are chromophores. Thus 4-aminopyrazolyloxy acetate could be employed in protection of free amine via acid sensitive

chromophoric amide bond unlike Boc group. Our future works are in progress to demonstrate the utility of this process, and have planned to study with other amino acids/longer peptides including functionalized amino acid/peptide derivatives.

EXPERIMENTAL SECTION

General methods**.** All required materials were obtained from commercial suppliers and used without any further purification. Dimethylformamide (DMF) was distilled over calcium hydride. Reactions were monitored by thin layer chromatography, visualized by UV and ninhydrin. Column chromatography was performed in 230-400 mesh silica. Mass spectra and HRMS were obtained from Bruker micrOTOF-Q II Spectrometer. ^{1}H NMR, ^{13}C{^{1}H} NMR, were recorded on Bruker AV-400 or 700 MHz at 298 K. ^{1}H and ^{13}C{^{1}H} NMR chemical shifts were recorded in ppm downfield from tetramethylsilane or residual solvent peak. Splitting patterns are abbreviated as: s, Singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; dq, doublet of quartet; m, multiplet.

Experimental procedure. Experimental procedure and their characterization data for compounds **1**, **3b-3d**, **3g-3h** are previously reported from our lab.[12]

N-phenyl, 4-aminopyrazolyloxy acetic acid, BocNH-APA-OH (**2**). Compound **1** (1.26 g, 3.52 mmol) is dissolved in THF (25 mL) and cooled to 0 ^{o}C, then added aqueous LiOH (1 M, 25mL) and at the same temperature stirred for 20 min. The solvents are evaporated under vacuum to half of its volume and adjusted the pH to 6-7 with 1 M HCl. The resulting aqueous layer extracted with EtOAc (3*30 mL) and dried over anhydrous Na_2SO_4 and concentrated under vacuum. The product is precipitated with chloroform and hexane, dried the solid to produce 1.0 g (90%) of title compound. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (OH, s, 1H), 7.63 (d, J = 7.8 Hz, 2H), 7.40 (t, J = 7.8 Hz, 2H), 7.27 (dd, J = 6.5, 4.7 Hz, 1H), 6.02 (NH, s, 1H), 4.67 (s,

2H), 2.15 (s, 3H), 1.49 (s, 9H). $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$) δ 172.6, 158.3, 147.4, 146.9, 138.2, 128.9, 126.7, 122.5, 101.2, 82.9, 67.8, 28.3. HRMS (ESI-TOF) m/z: $[M + H]^+$ Calcd for $C_{17}H_{22}N_3O_5$ 348.1554; Found 348.1567.

General experimental peptide coupling procedure for compounds, **3a-3h**:

Compound **2**, corresponding amine (TFA or HCl salt form) (1.5 equivalent), HOAT (1.5 equivalent) was dissolved in Dry DMF (1.5 M). After stirring for 10 min, N-methyl morpholine (3 equivalent) was added drop wise and cooled the temperature to 0 ^{o}C and added EDC.HCl (1.5 equivalent). After 20 min allowed to warm to rt followed by heating at 60 ^{o}C for 8 h. The crude reaction mixture was evaporated under reduced pressure. The resultant crude was purified by column chromatography with MeOH in CH_2Cl_2 (1-3%).

methyl 2-(2-((4-((tert-butoxycarbonyl)amino)-3-methyl-1-phenyl-1H-pyrazol-5-yl)oxy)acetamido)acetate, *BocNH-APA-Gly-OMe* (**3a**): R_f 0.25 (3% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, J = 7.8 Hz, 2H), 7.44 (t, J = 7.9 Hz, 2H), 7.30 (t, J = 7.4 Hz, 1H), 6.94 (t, 1H), 6.15 (s, 1H), 4.65 (s, 2H), 4.07 (d, J = 5.7 Hz, 2H), 3.77 (s, 3H), 2.19 (s, 3H), 1.48 (s, 9H). $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$) δ 170.0, 167.8, 155.1, 147.1 (two peaks merged), 138.1, 129.2, 126.9, 122.6, 102.6, 80.6, 70.7, 52.5, 40.5, 28.2, 11.8. HRMS (ESI-TOF) m/z: $[M + H]^+$ Calcd for $C_{20}H_{27}N_4O_6$ 419.1925; Found 419.1914.

(*S*)-methyl 2-(2-(2-((4-((tert-butoxycarbonyl)amino)-3-methyl-1-phenyl-1H-pyrazol-5-yl)oxy)acetamido)acetamido)propanoate *BocNH-APA-Gly-Ala-OMe* (**3e**): R_f 0.18 (3% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, J = 7.7 Hz, 2H), 7.42 (t, J = 7.6 Hz, 2H), 7.30 (d, J = 7.3 Hz, 1H), 7.16 (s, 1H), 6.69 (s, 1H), 6.48 (s, 1H), 4.63 (s, 2H), 4.62 – 4.53 (m, 1H), 3.98 (d, J = 5.3 Hz, 2H), 3.74 (s, 3H), 2.18 (s, 3H), 1.79 (d, J = 7.0 Hz, 2H), 1.46 (s, 9H), 1.41 (d, J = 7.1 Hz, 3H). $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$) δ 173.3, 168.3 (two peaks merged together), 155.3, 147.2 (two peaks merged together), 138.1, 129.1, 126.9, 122.5, 102.8, 80.4,

70.7, 52.5, 48.2, 42.0, 28.2, 17.9, 11.9. HRMS (ESI-TOF) m/z: $[M + H]^+$ Calcd for $C_{23}H_{30}N_5O_{7s}Na$ 512.2116; Found 512.2121.

(*S*)-methyl 2-(2-(2-((4-((tert-butoxycarbonyl)amino)-3-methyl-1-phenyl-1H-pyrazol-5-yl)oxy)acetamido)propanamido)acetate *BocNH-APA-Ala-Gly-OMe* (**3f**): R_f 0.32 (3% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, *J* = 7.8 Hz, 2H), 7.44 (t, *J* = 7.8 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 1H), 6.91 (d, *J* = 6.8 Hz, 1H), 6.78 (s, 1H), 6.32 (s, 1H), 4.63 (s, 2H), 4.59 – 4.49 (m, 1H), 4.09 – 3.92 (m, 2H), 3.74 (s, 3H), 2.18 (s, 3H), 1.47 (s, 9H), 1.34 (d, *J* = 7.0 Hz, 3H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 172.3, 170.3, 167.7, 155.3, 147.3, 147.2, 138.1, 129.3, 127.1, 122.7, 102.8, 80.6, 70.8, 52.4, 48.2, 41.2, 28.3, 17.9, 11.9. HRMS (ESI-TOF) m/z: $[M + H]^+$ Calcd for $C_{23}H_{31}N_5O_7$ 490.2296; Found 490.2295.

3-methyl-1-phenyl-4,6-dihydropyrazolo[3,4-b][1,4]oxazin-5(1H)-one, (**4**): 20% TFA in CH_2Cl_2 (5 mL) was added to **1** (0.50 g, 1.38 mmol) at room temperature and stirred for 1 h, then refluxed for an additional 1.5 h at 45 °C. The solvents were removed under vacuum and resulting pale orange residue was partitioned between EtOAc (25 mL) and saturated $NaHCO_3$ (10 mL). The aqueous layer was extracted with EtOAc (2 * 25 mL). The combined organic extracts were washed with saturated aqueous NaCl (10 mL), dried over anhydrous Na_2SO_4 and concentrated under vacuum. The residual viscous oil was purified via chromatography with 3% MeOH in CH_2Cl_2 to produce 0.293 g (92%) of **4** as a crystalline solid. *R_f 0.2* (0.3:9.7 MeOH/CH_2Cl_2); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.51 (s, 1H), 7.72 – 7.58 (m, 2H), 7.45 (dd, *J* = 10.8, 5.2 Hz, 2H), 7.25 (t, *J* = 7.4 Hz, 1H), 4.84 (s, 2H), 2.15 (s, 3H). ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 162.8, 140.2, 138.5, 136.0, 129.8, 126.3, 119.5, 106.5, 70.0, 12.2. HRMS (ESI-TOF) m/z: $[M + H]^+$ Calcd for $C_{12}H_{12}N_3O_2$ 230.0924; Found 230.0933. mp: 216-218 °C.

ASSOCIATED CONTENT

Supporting Information

NMR (^{1}H-/^{13}C{^{1}H})/HRMS/Time dependent NMR spectra of peptides; kinetic profiles of amide/acid/ester **3a**/**3h**/**1**/**2**; X-ray crystallography data, Crystallographic Information Framework (CIF) file and UV-spectra of lactam **4** are provided in the Supporting Information.

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