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Glutamic Acid Selective Chemical Cleavage of Peptide Bonds

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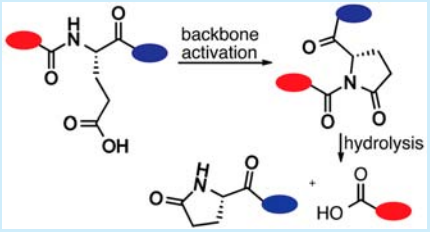
Supporting Information

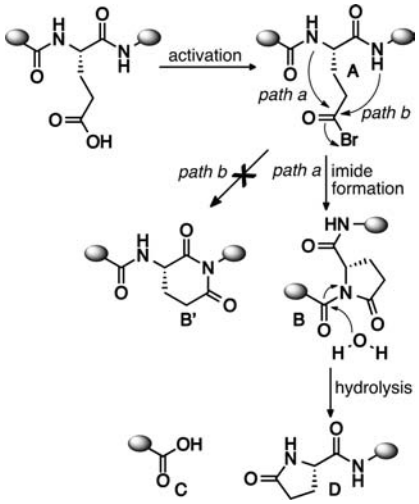
ABSTRACT: Site-specific hydrolysis of peptide bonds at glutamic acid under neutral aqueous conditions is reported. The method relies on the activation of the backbone amide chain at glutamic acid by the formation of a pyroglutamyl (pGlu) imide moiety. This activation increases the susceptibility of a peptide bond toward hydrolysis. The method is highly specific and demonstrates broad substrate scope including cleavage of various bioactive peptides with unnatural amino acid residues, which are unsuitable substrates for enzymatic hydrolysis.



Hydrolysis of peptide bonds at a specific residue is an important biochemical tool for correlating protein structure with activity,[1] designing new therapeutic agents,[2,3] converting proteins into fragments that are more amenable for sequencing, and various new bioanalytical and bioengineering applications.[4] These new methods require residue-specific cleavage of a peptide bond into large fragments. The peptide bond (i.e., the amide group), however, is highly stable toward hydrolysis, and the half-life for nonselective cleavage at room temperature and pH 4−8 is 500−1000 years.[5] Few enzymes and synthetic reagents are commonly used for selective hydrolysis of peptide bonds.[6] Most of these peptidases are residue-selective, such as trypsin, which is selective for cleavage at Arg and Lys; chymotrypsin cleaves at Phe, Trp, and Tyr; pepsin at Phe and Leu; endopeptidase Glu-C at Glu; and endopeptidase Lys-C at Lys. The selectivity of peptidases can be adjusted by varying the digestion time and the degree of prior unfolding. Nonetheless, peptidases are limited because they tend to produce short fragments ill-suited for bioanalytical applications, and they require tedious procedures of peptidase removal from the protein digest.[6] Moreover, these enzymes require narrow ranges of temperature and pH.

The existing chemical reagents, such as cyanogen bromide,[7] 2-nitro-5-thiocyanobenzoic acid,[8] and 2-iodosylbenzoic acid,[9] for cleaving peptide bonds often require harsh conditions. As a result, side reactions and the lack of specificity of amide-bond hydrolysis limits their scope in chemical biology and synthetic applications.[10] Recently, asparagine-selective cleavage of peptide bonds by using diacetoxyiodobenzene has been reported.[11] The use of metals for cleavage of peptide bonds has been intensively studied, but their practical use for protein analysis is still in its early stages.[12−18] New chemical reagents with high selectivity and improved efficiency are highly desirable for many emerging applications. For controlled and specific cleavage, a daunting task, a chemical reagent must selectively bind only to one particular amino acid in the peptide sequence and specifically cleave the peptide bond at the binding site. Based on this principle, we report a chemical methodology for site-selective cleavage of peptide bonds at glutamic acid with high efficiency under milder reaction conditions. Previous attempts utilized thionyl chloride for selective cleavage at Glu but were unsuccessful in achieving the desired goals.[19]

Scheme 1. Rationale for the Site-Specific Hydrolysis at Glutamic Acid through Backbone Amide Activation



The selective cleavage of a peptide bond at glutamic acid entails the strong activation of the side-chain carboxylate of Glu to generate intermediate (**A**) (Scheme 1). The nucleophilic attack of the amide nitrogen on the activated intermediate leads to the formation of the backbone pyroglutamyl (pGlu) imide moiety (**B**).[20] The formation of pGlu imide moiety (**B**) makes the C−N bond prone to hydrolysis and leads to the cleavage of the peptide chain into the N-terminal fragment (**C**) and pGlu imide-containing C-terminal fragment (**D**) (Scheme 1).

To implement this methodology of site-selective cleavage of peptides at glutamic acid, model hexapeptide Fmoc-Val-Ala-**Glu**-Arg-Phe-Ala-NH_2 (**1a**) was synthesized. For the formation of the

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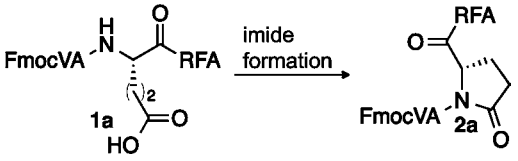
Table 1. Optimization of the Reaction Conditions on Peptide 1a for the Formation of pGlu Imide Moiety 2a[a]



entry	reagent (equiv)	base (equiv)	additive	time (h)	conv[b] (%)
1	DSC (40)	DIEA (40)		48	50
2	PyBrop (20)	DIEA (20)		24	20
3	PyBrop (20)	DIEA (20)		48	40
4	PyBrop (40)	DIEA (40)		48	60
5[c]	**PyBrop (20)**	**DIEA (20)**	**DMAP**	**24**	**99**

[a]Reaction conditions: peptide (1 equiv) was reacted with DSC/PyBrop (20−40 equiv) and DIEA (20−40 equiv) in DMF at room temperature. [b]Conversion to **2a** was calculated from the absorbance at 254 nm using HPLC. [c]A small crystal of DMAP was added to the reaction mixture. The entry in bold represents the optimized reaction conditions. DIEA = *N*,*N*-diisopropylethylamine, DMAP = 4-(*N*,*N*-dimethylamino)pyridine.

pGlu imide moiety, various acylation reagents were screened (Table 1). In principle, such activation of the Glu side-chain carboxylate could lead to not only the desired 5-membered ring (**B**) through path a but also a six-membered ring (**B′**) (path b, Scheme 1).[20] However, our studies suggest high specificity for the formation of the five-membered pGlu imide moiety (**B**), which is supported by NMR analysis (Supporting Information). Importantly, cleavage of peptides at glutamic acid (see below) provides the chemical proof for the formation of the five-membered ring (**B**, Scheme 1).

To optimize the cyclization at glutamic acid, various reaction conditions were explored on model hexapeptide **1a** (Table 1). In initial studies, *N*,*N′*-disuccinimidyl carbonate (DSC) was used for the formation of the pGlu imide moiety, but a large excess of reagents and longer reaction times were needed for the 50% conversion to the desired cyclized peptide **2a** (entry 1, Table 1). The yields for the conversion from **1a** to **2a** were calculated from HPLC data (Table 1). Next, bromotris(pyrrolidino)phosphonium hexafluorophosphate (PyBrOP) was used for the activation since it is one of the strongest coupling reagents and provides the corresponding acyl bromide. Initial reaction with uncrystallized PyBrop gave pyrrolide **2a′** as a side product with a mass 53 Da higher than the starting peptide **1a** (for details, see Figure S1, Supporting Information).[21−23] With recrystallized PyBrOP in hand, various other reaction conditions, such as base, additive, and time, were screened on a model hexapeptide, Fmoc-Val-Ala-Glu-Arg-Phe-Ala-NH_2 (**1a**) (entries 2−5, Table 1). The progress of the reaction was monitored by injecting the sample into an analytical HPLC after regular intervals of time (Figure 1). At time = 0 h, a sharp peak with retention time t_R = 13.4 min corresponding to the model hexapeptide **1a** was observed, as analyzed by mass spectrometer (MS) (Figure 1a). After 17 h, one sharp peak with a retention time t_R = 13.7 min was observed, indicating the formation of the pGlu imide moiety **2a**, as analyzed by MS (Figure 1b). After the modified peptide was incubated in phosphate buffer (pH 7.5) for 48 h, the sharp peak **2a** at 13.7 min disappeared and two new peaks appeared with retention times of 5.6 and 22.7 min (Figure 1c). MS analysis of these peaks corresponded to the cleavage products at the N-terminal side of glutamic acid, with N-terminal fragment **3a** = 22.7 min, and the

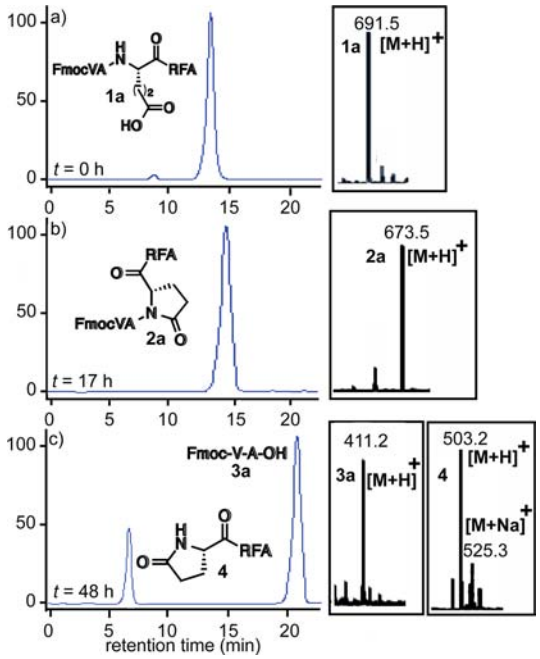
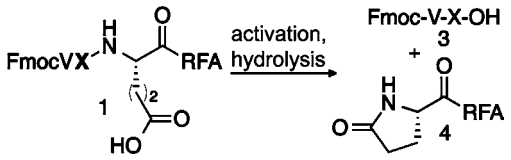


Figure 1. HPLC chromatogram of pGlu imide formation using optimized reaction conditions in Table 1, entry 5, at t = 0 h (top), at t = 17 h (middle), after hydrolysis under 0.1 M phosphate buffer (pH 7.5) at 25 °C, and at t = 48 h (bottom). Insets show the MS corresponding to retention times at 13.4 min (**1a**, top), 13.7 min (**2a**, middle), 22.7 min (**3a**, bottom), and 5.6 min (**4**, bottom).

pGlu imide moiety containing C-terminal fragment **4** = 5.6 min (Figure 1c) (Figure S2, Supporting Information).

To determine the effect of side-chain functionality of the amino acid adjacent to Glu in the activation of the backbone chain and hydrolysis, various peptides, Fmoc-Val-**X**-Glu-Arg-Phe-Ala-NH_2 (**1a−j**), with different amino acids at the **X** position were explored (Table 2). As a result, fragment **4** was obtained in

Table 2. Glu-Selective Amide Bond Cleavage of Fmoc-Val-X-Glu-Arg-Phe-Ala-NH_2 (1a−j)[a]



entry	substrate	X	conv[b] (%)
1	1a	Ala	99
2	1b	Gly	99
3	1c	Arg	99
4	1d	Met	80
5	1e	Asn	90
6	1f	His	90
7	1g	Phe	90
8	1h[c]	Tyr	90
9	1i[c]	Ile	65
10	1j	Asp	99

[a]Reaction conditions: peptide (**1a−j**, 1 equiv) was reacted with PyBrop, DIEA (20 equiv), and a crystal of DMAP in DMF at room temperature followed by hydrolysis with 0.1 M phosphate buffer (pH 7.5) at 25 °C for 48 h. [b]Conversion to N-terminal fragment, Fmoc-V-**X**-OH (**3a−j**), was calculated from the absorbance at 254 nm using HPLC. [c]Hydrolysis for 5 days.

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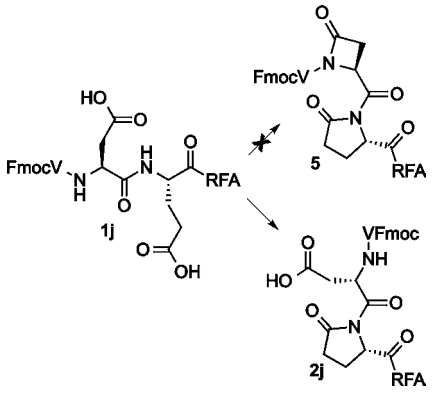
all cases after hydrolysis, with the HPLC conversion ranging from 65 to 99%. The results indicated that unprotected peptides with **X** = Gly (**1b**), Arg (**1c**), Met (**1d**), Asn (**1e**), His (**1f**), and Phe (**1g**) proceeded cleanly in a manner similar to that of **1a** (entries 2−7, Table 2). In contrast, substrates that contain X = Tyr (**1h**) and Ile (**1i**) with bulky side groups gave the cleaved products in good yields but required longer time (5 days) for cleavage (entries 8 and 9, Table 2).

In the case of peptide **1j**, containing Asp along with Glu, cleavage of the backbone peptide bond was observed only at the Glu residue (entry 10, Table 2). The contrasting reaction between Asp and Glu is due to the unlikely formation of the constrained 4-membered ring **5** at Asp, compared to the kinetically favorable 5-membered ring **2j** at Glu (Scheme 2 and Figure S3, Supporting Information). Therefore, this peptide bond cleavage method is highly Glu selective.

Scheme 2. Reactivity of Asp vs Glu toward the Backbone Activation for Peptide 1j



To determine the substrate scope of this method, it was further evaluated (Table 3). The reaction was applied for the hydrolysis

Table 3. Substrate Scope of Glu-Selective Amide Bond Hydrolysis[a]

entry	substrate	yield[b] (%)
1[c]	Fmoc-Ala-Val-Arg-**Glu**-Val-Ala-Phe-**Glu**-Arg-Phe-Gly-Phe-NH_2 (**7**)	80
2[c]	Fmoc-Arg-Ala-Gly-Ala-**Glu**-Val-Arg-Phe-Ala-**Glu**-Ala-Phe-Gly-NH_2 (**8**)	85
3	Fmoc-D-Val-D-Ala-D-**Glu**-D-Arg-D-Phe-D-Ala- NH_2 (**9**)	80
4	Fmoc-D-Val-Ala-**Glu**-D-Arg-D-Phe-Ala-NH_2 (**10**)	80
5	Fmoc-Cys-Gly-Arg-Arg-Ala-Cys-Gly-**Glu**-Phe-Ala-Gly-NH_2, disulfide bond (**11**)	75
6	Fmoc-Arg-Ala-**Glu**-Ala-Gly-**Ser**-Gly-Phe-NH_2 (**12**)	90

[a]Reaction conditions: peptide (1 equiv) was reacted with PyBrop (20 equiv), DIEA (20 equiv), and a crystal of DMAP in DMF followed by cleavage with 0.1 M phosphate buffer (pH 7.5) at 25 °C for 48 h unless otherwise noted. [b]Yield of N-terminal fragment with Fmoc group. [c]Three fragments were detected in the HPLC trace for cleavage at both Glu residues.

of longer 12-mer and 13-mer peptides (**7** and **8**) with two glutamic acid residues at internal positions. Peptides **7** and **8** underwent effortless cleavage at both glutamic acids and delivered three fragments in high yield (80−85%) (entries 1 and 2, Table 3, and Supporting Information). Next, we extended the current conditions to the scission of peptides with unnatural amino acid residues. Peptides **9** and **10**, which are made up of D-amino acids and a mixture of L- and D-amino acids, respectively, were cleaved successfully under the reaction conditions at Glu with ease and high yields (entries 3 and 4, Table 3). This chemical cleavage of unnatural D-amino acid residues containing peptides is a huge advantage over the conventional enzymatic method, where enzymes do not recognize and cleave these modified peptides. The conversion of natural L-amino acids to unnatural D-amino acids is a well-known mutation responsible for various age related disorders such as cataracts and Alzheimer's disease.[24] Thus, this method can be used as a diagnostic tool to determine different types of mutations in proteins and their role in the progression of diseases.

Peptide **11**, comprising an intramolecular disulfide bridge, afforded the cleavage product at glutamic acid with an intact disulfide bond (entry 5, Table 3). Thus, this methodology can be used to determine the position of disulfide pairing in a peptide chain, which is in contrast to other chemical reagents.[25,26]

Peptide **12**, containing a serine residue with a reactive hydroxymethyl group at the side chain, remained unreacted under the reaction conditions, and cleavage was observed only at Glu (entry 6, Table 3). This and Table 2 demonstrated the high specificity of this methodology toward glutamic acid. Next, this methodology was successfully applied for the scission of three bioactive peptides: **13**, a putative coproporphyrinogen III oxidase fragment, **16**, and Aβ (10−19)[11] **19**, a fragment of Alzheimer's disease associated amyloid-β peptide (entries 1−3, Table 4, and Figure S4).

Table 4. Glu-Selective Cleavage of Bioactive Peptides

entry	substrate	yield[a] (%)
1	Fmoc-Met-Gly-His-**Gln**-**Glu**-His-Leu-Pro-Tyr- NH_2 (**13**)	79
2	Fmoc-Leu-Pro-Arg-Leu-**Gln**-**Glu**-Ala-Trp-Gln- NH_2 (**16**)	75
3	Fmoc-**Tyr**-**Glu**-Val-His-His-Gln-Lys-Leu-Val-Phe-NH_2 (**19**)	80
4	Fmoc-Ala-Gly-Leu-**Pro**-**Glu**-Lys-Tyr-NH_2 (**22**)	82

[a]Yield of N-terminal fragment with Fmoc group.

Finally, this methodology was evaluated on a bioactive peptide, amyloid A protein fragment (*Homo sapiens*) **22**, with a proline residue next to Glu (entry 4, Table 4). Treatment of the peptide **22** with Pybrop for 17 h followed by hydrolysis under neutral aqueous buffer conditions cleaved the peptide at the **Pro-Glu** site (eq 4, Figure S4). This is in contrast to hydrolysis by proteases since the location of proline at a neighboring position nearly blocks the cleavage completely independent of the amino acid residue.[20,18] Thus, this method can potentially be used to selectively cleave a broad range of peptides/proteins at glutamic acid independent of the surrounding amino acid residues. Interestingly, peptides **19** and **22** with a free side-chain lysine only generated the kinetically favorable five-membered pGlu moiety (for details, see Figure S5, Supporting Information). High specificity, broad substrate scope, and easy purification demonstrate the widespread use of this methodology and its ability to determine the structure of unknown peptides/proteins.

Site-selective hydrolysis of unreactive peptide bonds under mild and metal-free reaction conditions has been developed. The methodology utilizes the activation of a backbone amide chain to cleave the peptide bond specifically at glutamic acid. The chemical reagents can be easily removed after the cleavage, unlike proteases. Disulfide bonds are stable toward the reaction

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conditions; thus, this methodology can be used to determine the position of disulfide pairing in peptides. This method exhibits broad substrate scope, including the cleavage of peptides at the Pro-Glu site, as such kind of site is resistant to enzymatic degradation. Since the hydrolysis of mutated peptides with unnatural amino acid residues such as D-amino acid, which are unsuitable substrates for enzymes, proceeded with ease under the reaction conditions, this methodology can be used to determine the mutations responsible for various diseases. This technology is highly specific for hydrolysis of peptides at one particular residue, which is one of the key requirements for semisynthesis and bioengineering of fusion proteins. These studies lay the groundwork for further studies aimed at developing artificial chemical proteases for the cleavage of target proteins responsible for various diseases and exploring this reaction for biotechnological applications. Work in this direction is currently underway in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00317.

Experimental procedures and full spectroscopic data for all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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