Selective copper-mediated cross-coupling of pyroglutamate post-translational modifications

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Supporting Information Placeholder

ABSTRACT: Pyroglutamate is a cyclic *N*-terminal posttranslational modification that occurs in both proteins and peptide hormones. The prevalence and biological roles of pyroglutamate are little understood, in part due to limited tools to identify, quantify, and manipulate its pyrrolidinone structure. Selective modification of pyroglutamate residues in complex polypeptides may provide unique tools to better understand its biological roles, and to allow late-stage diversification of biologically active pyroglutamate-containing sequences. This work describes a copper-catalyzed N–H cross-coupling of unprotected peptides that is selective for *N*-terminal pyroglutamate residues. The reaction is operationally simple under mild conditions, and tolerates almost all canonical residues. Mechanistic studies point to a key role for a multidentate copper-binding mode of the extended polypeptide structure in delivering the observed reactivity. The reaction allows direct labeling and identification of a pyroglutamate hormone present in porcine intestinal extracts.

Pyroglutamate is an important posttranslational modification (PTM) that formed by *N*-terminal cyclization of glutamine or glutamate residues to afford pyrrolidinone structures.[1–3] Pyroglutamate occurs both in proteins and in a variety of peptide hormones prevalent in the central nervous system and gastrointestinal tracts of mammals, including humans.[4–7] Both chemical and enzymatic pathways for pyroglutamate formation have been postulated, and there is evidence that pyroglutamate has diverse and extensive biological roles. Pyroglutamate is essential for the function of many neuropeptides, including the thyrotropin releasing hormone (TRH). Pyroglutamate formation accelerates aggregation of Aβ peptides and is observed at elevated levels in Alzheimer's disease.[8] Pyroglutamate formation protects sequences from degradation by aminopeptidases.[9] Altered regulation of the glutaminyl cyclase (QC) enzymes that control pyroglutamate levels are implicated in diverse human diseases, including Alzheimer's, Huntington's, periodontitis, and cancer.[10] Beyond its prevalence as a residue in polypeptides, the free pyroglutamate amino acid also has neurological effects,[11] and accumulates in the inherited metabolic diseases.[12]

Chemical methods to modify or profile post-translational modifications (PTMs) have been important additions to the arsenal of chemical biology in recent years.[13,14] In some cases, PTMs contain unique reactivity features. Phosphoserine reacts via a unique β-elimination/Michael addition sequence (Figure 1a),[15–17] and the secondary amine produced from lysine monomethylation reacts with diazonium reagents to produce a triazene. (Figure 1b).[18] Oxidation of cysteine also produces species with unique reactivity profiles.[13,14] Some other PTMs, including citrulline (Figure 1c), acetyllysine, and pyroglutamate are quite challenging targets for selective chemical manipulation because they have chemical reactivity that is extremely similar to the sea of biological amides. In the case of citrulline, condensation with phenylglyoxal under acidic conditions has been employed (Figure 1c).[19]

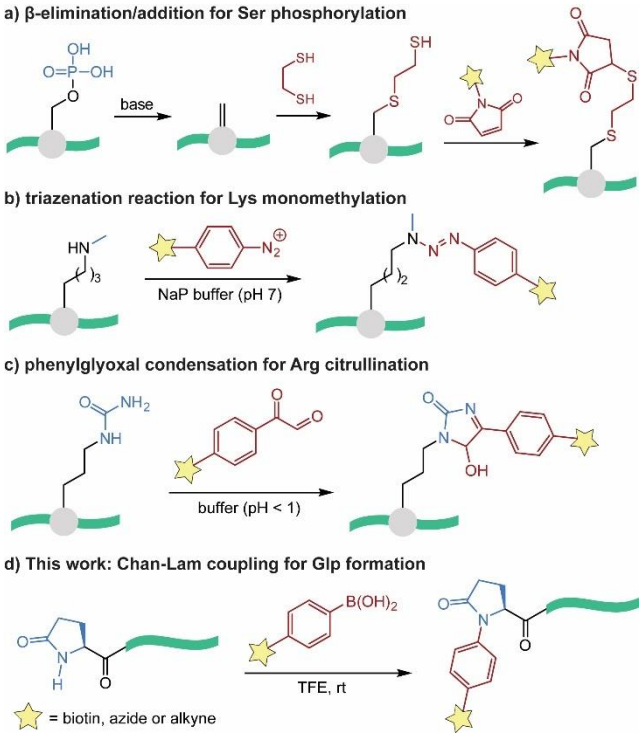

Figure 1. Representative chemical methods for profiling PTMs.

Methods to manipulate pyroglutamate residues are lacking, despite their frequent occurrence in natural polypeptides and proteins.[3,20] Indeed, the chemistry, prevalence, and biological function of pyroglutamate is poorly understood.[21] Regarding analysis, sensitive methods to identify or quantify pyroglutamate have been identified as a major factor limiting the understanding of pyroglutamate biology.[22] Traditional mass spec proteomics methods do not sample *N*-terminal pyroglutamate sequences effectively, and effective analysis may require special methods to enrich or select for *N*-terminal sequences.[23] The potential for chemical cyclization during analysis complicates these efforts in the case of pyroglutamate, and there is a lack of general antibody approaches to recognize pyroglutamate.

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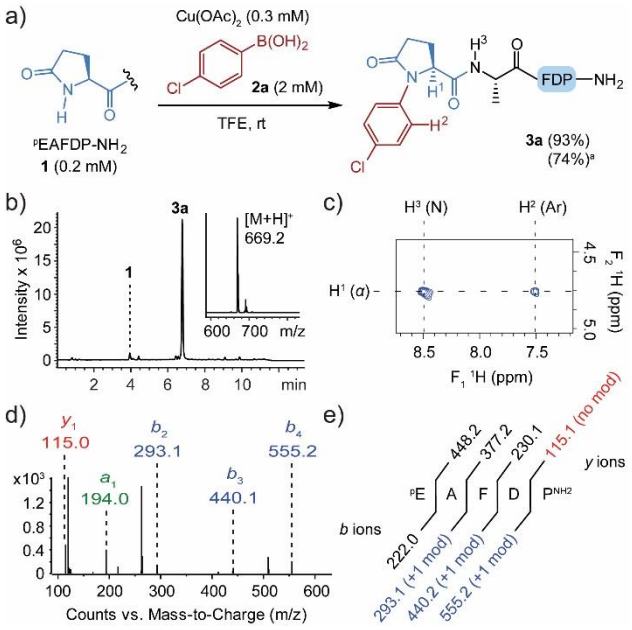

Figure 2. a) Modification of peptide **1** with boronic acid **2a**. Conditions: peptide **1** (0.2 mM), $Cu(OAc)_2$ (0.3 mM) and boronic acid **2a** (2 mM) in TFE at rt for 16 h. Conversion was determined by LC-MS. [a]Isolated yield. b) LC trace and MS spectrum (inset) of crude reaction. c) Partial ROESY spectrum of purified **3a**. d-e) MS/MS spectrum (d) and fragmentation ladder (e) of **3a**.

Given the prevalence of pyroglutamate in biologically active peptides and a limited understanding of the biological roles of pyroglutamate, chemical methods to modify pyroglutamate residues selectively could be broadly useful in late-stage diversification of peptides for medicinal chemistry applications and could provide useful chemical biology tools to identify, quantify, or characterize natural pyroglutamate-containing structures. In this paper, we report that pyroglutamate peptides are privileged structures for copper-catalyzed N–H cross-coupling, permitting efficient and predictable pyroglutamate arylation in complex polypeptides, in a process directed by multidentate backbone amide coordination of the peptide substrate (Figure 1d).

The first indications that pyroglutamate peptides are privileged structures for copper-catalyzed arylation came in our studies of histidine-directed N–H arylation in water, in which we observed that pyroglutamate-histidine (Glp-His) sequences reacted orders of magnitude faster than other Xaa-His sequences.[24,25] Given selectivity and reactivity challenges involved, we were surprised to find that, even without a neighboring histidine, pyroglutamate peptide **1** is reactive enough to allow N–H arylation with 4-chlorophenyl boronic acid **2a** in the presence of a $Cu(OAc)_2$ (Figure 2a).

Essential to this reactivity is the use of alcohol solvents; while histidine-directed reactivity proceeds efficiently in aqueous solution, no reaction with peptide **1** was observed in water. After some optimization (Table S2), the reaction with $Cu(OAc)_2$ in 2,2,2-trifluoroethanol (TFE) at room temperature provided a single arylation product **3a** with 93% conversion (Figure 2b). Isolation of **3a** by RP-HPLC allowed thorough NMR characterization (Figure S63-S65). A ROESY spectrum shows the expected cross peak ($H^1(\alpha)$ / $H^2(Ar)$) needed to establish the product structure (Figure 2c). MS/MS fragmentation also established pyroglutamate as the modification site for peptide **1** (Figure 2d, e) and for other substrates as well. In the course of our investigations, we have observed no evidence of by-products from arylation at any other sites.

With an initial concept in hand, we examined the scope of boronic acid reagents with peptide **1** (Figure 3). A variety of arylboronic acids with electronically diverse substituents (**2a**-**2u**) provided corresponding pyroglutamate N–H arylation products. Similar to histidine-directed N–H arylation reactions,[24,25] *ortho* substitution is not tolerated. The success of a broad range of arylboronic acids suggests electronic effects do not significantly affect the reaction efficiency (**2a**-**2u**). Reactions with alkenylboronic acids (**2v**-**2aa**) were also successful, although substituents on the alkene had a significant effect of reaction efficiency. Alkenylboronic acids bearing electron-donating groups (**2aa**) exhibit higher reactivities than those with electron-withdrawing substitutes (**2z**).

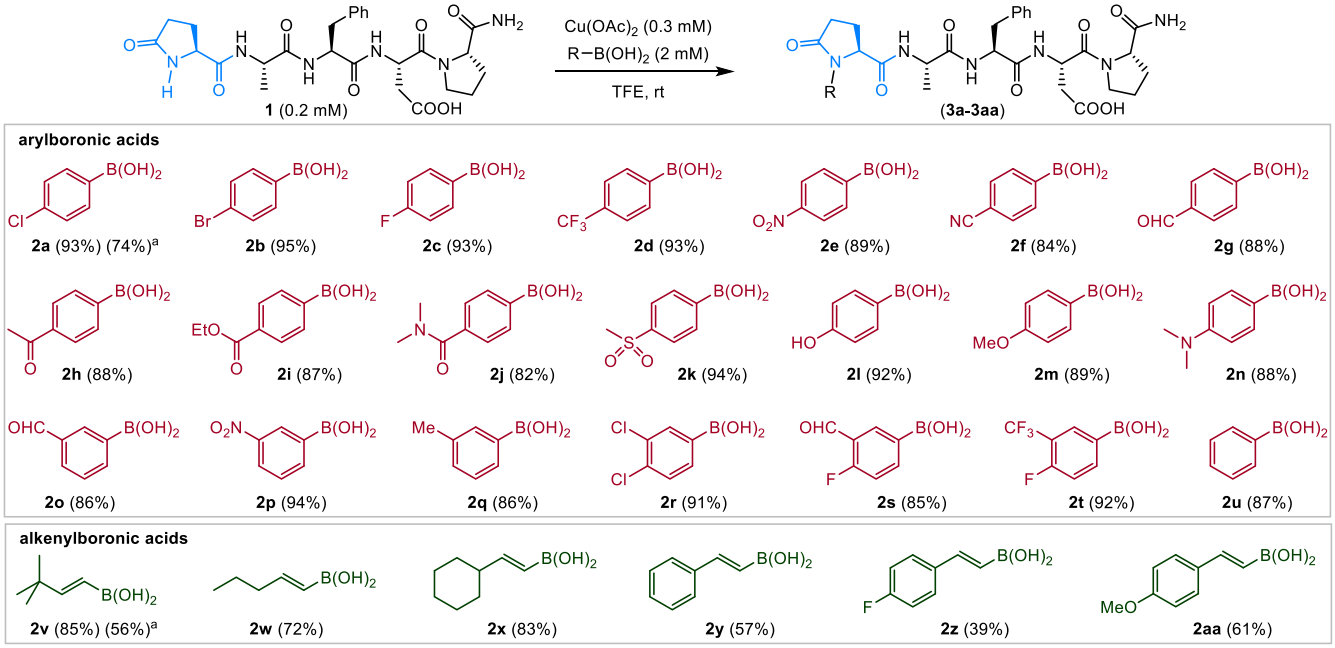

Figure 3. Boronic acid scope of pyroglutamate-selective peptide arylation and alkenylation. Conditions: peptide **1** (0.2 mM), $Cu(OAc)_2$ (0.3 mM) and boronic acids **2a**-**2aa** (2 mM) in TFE at rt for 16 h. Conversions were determined by LC-MS. [a]Isolated yields.

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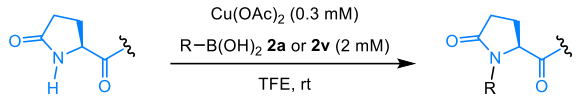
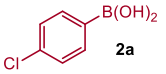
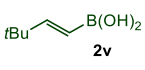
Table 1. N–H arylation/alkenylation of pyroglutamyl peptides



S4-S15 (0.2 mM) **4a-15a** or **4v-15v**

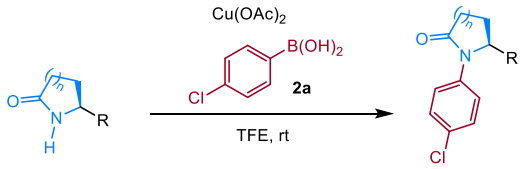
entry	peptide sequence	boronic acid:  **2a**	boronic acid:  **2v**
1	pEPARW^{NH2}	60%	19%
2	pEAPDPY^{NH2}	68%	72%
3	pEPPDPY^{NH2}	90%	85%
4	pEAFDPC^{NH2}	60%	61%
5	pEAYGNH^{NH2}	91%	88%
6	pEGVKTF^{NH2}	92%	67%
7	pEVRWSI^{NH2}	31%	46%
8	pEQELDFIG^{NH2}	37%	40%
9	pEGKRPWIL^{OH} (*xenopsin*)	76%	81%
10	pEWPRPQIPP^{OH} (*teprotide*)	53%	67%
11	pEGLPPRPKIPP^{OH} (*BPP-b*)	51%	85%
12[a]	pELSATGPOAIOGIO GIOGTOGPDGQOGTOG IKGEKGLOGL^{NH2}	33%	45%

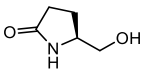
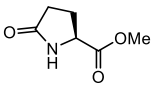
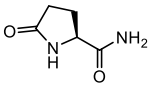
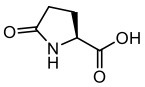
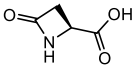
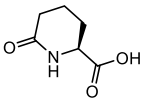
Conditions: peptide **S4**-**S15** (0.2 mM), $Cu(OAc)_2$ (0.3 mM) and boronic acids **2a** or **2v** (2 mM) in TFE at rt for 16 h. Conversions were determined by LC-MS. [a] Collagen-like domain of human C1qB (A4C). [26] O represents (2S,4R)-hydroxyproline.

The scope of pyroglutamyl peptides was then investigated. In general, N–H arylation and alkenylation was achieved with a range of unprotected polypeptides (Table 1). A structural basis for some variation in cross-coupling efficiency is difficult to ascertain, although steric bulk of a neighboring valine residue (entry 7) and poor solubility in TFE (entry 8) could be possible explanations for more moderate conversions in a few cases. The reaction tolerates a wide range of amino acids, including aromatic residues (tryptophan, tyrosine and histidine), charged resides (lysine, arginine, aspartate and glutamate) and other side chains potentially susceptible to cross-couplings (such as serine and threonine). To our surprise, cysteine is also compatible with the reaction conditions, although minor disulfide side products were observed (entry 4). The surprising reactivity of pyroglutamate in the presence of cysteine is consistent with our observations in histidine-directed catalysis,[25,27] and our reports that copper-catalyzed cysteine arylation requires boronic acids with electron-withdrawing substituents in the *ortho* position.[28,29] MS/MS analyses were used to verify pyroglutamate as the reactive site of several peptides (Figure S35, S38, S41 and S48).

No evidence of modification at other amide side chains (asparagine and glutamine), backbone or C-terminal amides were observed under the conditions. Several bioactive natural peptides were also found to be suitable substrates (entries 9–11), and even the 40-amino-acid collagen-like domain[26] of human C1qB served as a reasonable substrate for selective pyroglutamate modification (entry 12)).

Table 2. N–H arylation of pyroglutamate analogues



entry	substrate	condn A (yield)	condn B (yield)
1	pEAFDP^{NH2}	93%	–
2		0%	0%
3		0%	20%
4		0%	95%
5		2%	91%
6[a]		89%	>95%
7[a]		27%	>95%
8[a]		45%	67%

Conditions A: pyroglutamate analogues (0.2 mM), $Cu(OAc)_2$ (0.3 mM), and boronic acid **2a** (2 mM) in TFE at rt for 16 h. Yields were determined by RP-HPLC. Conditions B: pyroglutamate analogues (0.1 mmol), $Cu(OAc)_2{\cdot}H_2O$ (0.05 mmol), boronic acid **2a** (0.2 mmol), and 4 Å MS (100 mg) in TFE (1 mL) at rt for 16 h. [a]Reactions were performed with 1 equiv of Et_3N. Yields were determined by ^1H NMR.

To better understand the structural basis for pyroglutamate-specific arylation, we examined a variety of simple model pyrrolidinones. Most strikingly, simple pyrrolidinone and simple pyroglutamate analogues are dramatically less reactive than pyroglutaminyl under typical conditions for peptide modification (entries 1–5, "condn A"). The parent pyrrolidinone in particular (entry 2) is completely unreactive under all conditions examined. To explain the profound difference in reactivity, we hypothesized that the presence of a proximal carboxylic acid derivative in pyroglutamate could be essential, service as a Lewis base copper ligand. Indeed, uncharged pyroglutamate derivatives could undergo arylation to some extent under alternative reaction conditions far more concentrated than is typical for peptide reactions (entries 3–5, "condn B"). However, even the best model for a peptide backbone was only minimally reactive under our standard peptide conditions (entry 5, "condn A"). Interestingly, some arylation activity was

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recovered for carboxylic acid substrates by adding a base to the reaction, generating a carboxylate salt (entries 6–8). Although the 5-membered-ring structure of pyroglutamic acid was most efficient, analogous 4- and 6-membered-ring amino acids were also reactive and afforded N–H arylation products (entries 6–8).

The kinetics of copper-catalyzed C–O bond formation of boronic acids has been studied under similar conditions in alcohol solvents.[30,31] We examined the kinetics of pyroglutamate arylation and found that, in most respects, the kinetics mirror those of C–O bond formation (Figure 4): The reaction exhibits saturation kinetics for the boronic acid (Figure 4a), and Hammett analysis with different para-substituted boronic acids shows a negative slope as a function of σp (Figure 4b). In one important respect, the kinetics of the current system differ from the reported

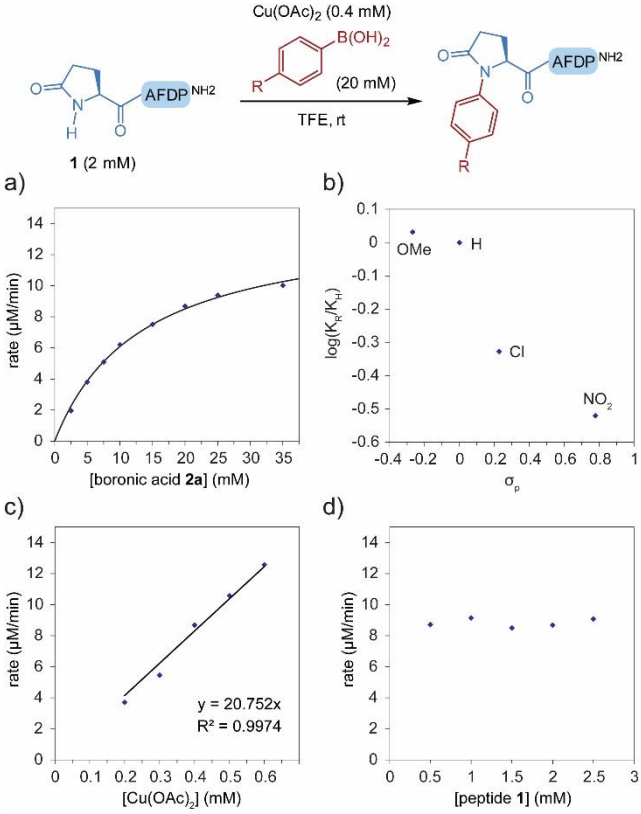

Figure 4. Kinetics analysis for reaction of peptide **1** with boronic acid **2a** measuring initial rates with varying concentrations of **2a** (a), Cu (c) and **1** (d). Conditions: **1** (0.5-2.5 mM), $Cu(OAc)_2$ (0.2-0.6 mM), and **2a** (2.5-35 mM) in TFE at rt. b) Hammett plot for pyroglutamate N–H arylation with electronically diverse boronic acids, based on initial reaction rates. Conditions: peptide **1** (2 mM), $Cu(OAc)_2$ (0.4 mM), and boronic acid **2a**, **2e**, **2m** or **2u** (20 mM) in TFE at rt.

methoxylation work, in which the reaction was found to be half-order with respect to copper catalyst. These kinetics were attributed to a dimeric copper resting state, which must dissociate prior to rate-limiting transmetallation. In contrast, we observed rates that are first-order in copper (Figure 4c) and also zero-order in peptide substrate (Figure 4d). These data are consistent with a monomeric copper resting state.

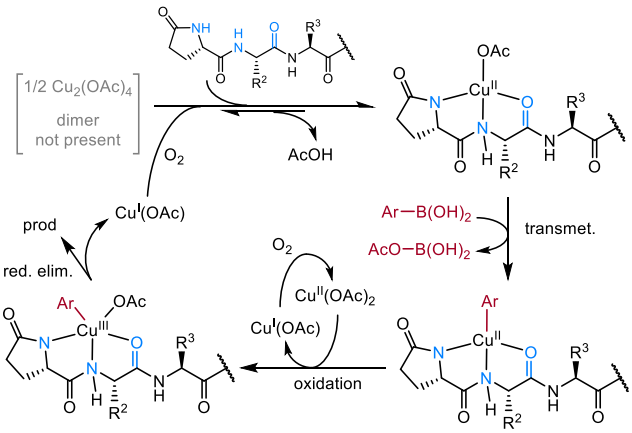

Figure 5. Proposed mechanism for selective pyroglutamate arylation.

We propose a mechanism (Figure 5) that proceeds via initial formation of a tridentate copper-peptide complex. A complete tridentate peptide ligand would more effectively maintain monomeric active copper, compared to simple pyrrolidone derivatives. The success of pyroglutamate-proline-proline sequences (e.g. Table 1, entry 3)—where no backbone N–H is present—imply N-coordination of a neutral amide at the middle binding site, and coordination of the third residue as a neutral ligand as well. Rate-limiting transmetallation with a boronic acid would then be followed by oxidation to Cu^{III} and reductive elimination to form the aryl amide product (Figure 5).

Chan-Lam coupling is formally oxidative, and for reactions under air, either O_2 or disproportionation of two Cu^{II} species is most typically assumed to be the oxidant on the catalytic cycle to form Cu^{III} before C–N bond-forming reductive elimination. The terminal oxidant for reactions run under air is typically assumed to be O_2. With catalytic amounts of copper, reactions under an inert atmosphere produced roughly a single catalyst turnover, consistent with O_2 as terminal oxidant. For reactions with stoichiometric quantities of copper, reactions under O_2 or inert atmosphere gave similar high conversions, consistent with Cu^{II} disproportionation as the oxidation pathway on the catalytic cycle (Figure S60).

We envisioned that it could be possible to use pyroglutamate-selective cross-coupling as a tool to profile pyroglutamyl peptides[5,6] in complex biological mixtures via a pull-down labeling approach. We targeted neurotensin, a hormone found in both brain and digestive tissue in mammals. Porcine intestine was homogenized and peptidic extracts were isolated according to established protocols.[32–34] Extracts were dissolved in TFE and treated with $Cu(OAc)_2$ and an arylboronic acid **16** containing a desthiobiotin handle for affinity purification. At the completion of the reaction, desthiobiotin-containing material was concentrated on and isolated from avidin beads (Figure 6a). Analysis of the pulled-down material indicated the presence of a single major peptide species, which could be identified as the arylated neurotensin[35] **17** (Figure 6c) on the basis of MS/MS (Figure 6 d,e) and validation with an authentic sample prepared from pure neurotensin. Comparison of LC traces of the initial extract and final pull-down material (Figure 6b, c) demonstrate the ability of our pyroglutamate profiling to identify minor components within a complex mixture.

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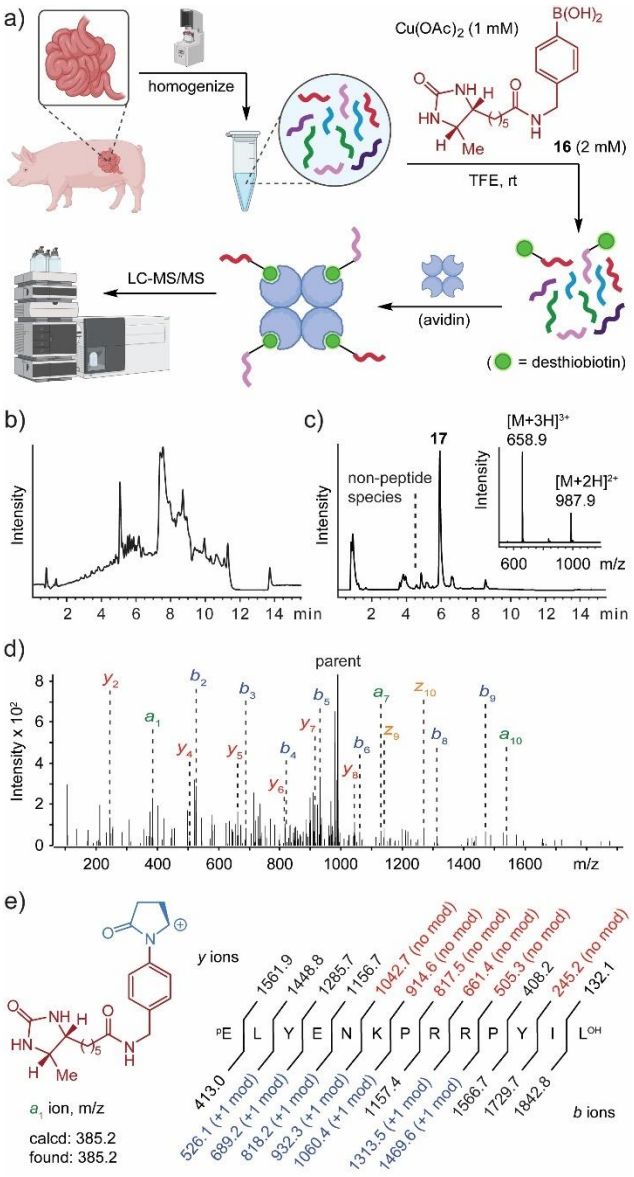

Figure 6. a) Schematic of modification, purification, and identification of pyroglutamate-containing peptides in pig intestine extracts. b) LC trace of pig intestine extracts. c) LC trace and MS spectrum (inset) of desthiobiotin-labeled peptide **17**. d-e) MS/MS spectrum (d), and fragmentation ladder (e) of peptide **17**.

CONCLUSION

We report a copper-mediated N–H arylation/alkenylation method. The method demonstrates that pyroglutamate can be considered a uniquely reactive sire for late-stage modification of complex polypeptides. Broad functional-group tolerance is observed, and simple, mild reaction conditions can be employed. Mechanistic/kinetic investigations point to tridentate binding of the Glp-containing polypeptide as a key to facilitating selective N–H arylation at a monomeric copper species. The remarkable selectivity observed points to the possibilities for multidentate binding within peptides to drive site-selective catalysis.

While pyroglutamate is a commonly recognized posttranslational modification, its prevalence and roles in biological systems is poorly understood, in part due to limited methods to identify, quantify, and or structurally manipulate the pyroglutamate modification. We demonstrate here that this method is suitable for profiling pyroglutaminyl peptides directly from extracts of living tissues. This result also demonstrates the use of this method in complex environments, and should prove useful as a tool to identify new pyroglutamyl peptides from living organisms. As pyroglutamate residues are present in many peptide hormones and neuropeptides, this reactivity may provide opportunities in the development of new drug candidates or therapeutic conjugates.

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