An electrochemical approach to designer peptide α-amides inspired by α-amidating monooxygenase enzymes

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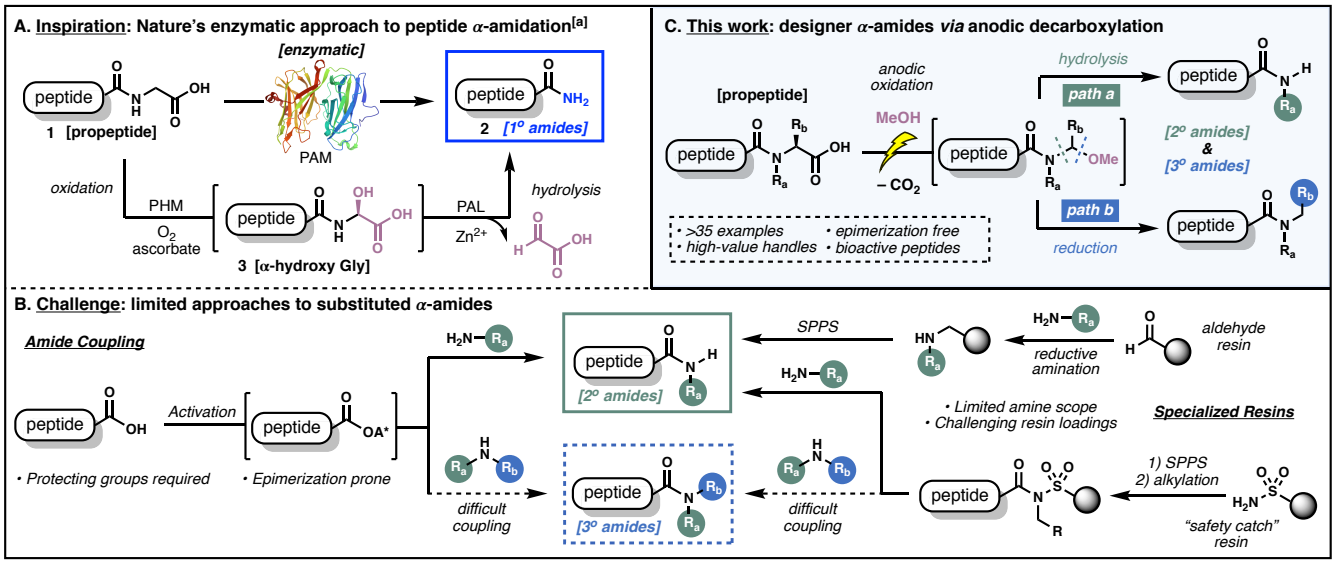
ABSTRACT: Designer C-terminal peptide amides are accessed in an efficient and epimerization-free approach by pairing an electrochemical oxidative decarboxylation with a tandem hydrolysis/reduction pathway. Resembling Nature's dual enzymatic approach to bioactive primary α-amides, this method delivers secondary and tertiary amides bearing high-value functional motifs, including isotope labels and handles for bioconjugation. The protocol leverages the inherent reactivity of C-terminal carboxylates, is compatible with the vast majority of proteinogenic functional groups, and proceeds in the absence of epimerization, thus addressing major limitations associated with conventional coupling-based approaches. The utility of the method is exemplified through the synthesis of natural product acidiphilamide A *via* a key diastereoselective reduction, as well as bioactive peptides and associated analogues, including an anti-HIV lead peptide and blockbuster cancer therapeutic leuprolide.

Introduction

Peptide α-amidation is a deceptively simple post-translational modification that characterizes ~50% of bioactive peptides and has profound impacts on function.[1] Relative to the corresponding C-terminal carboxylic acid, the α-amide functionality decreases peptide polarity and alters both isoelectric point and hydrogen bonding patterns, dramatically influencing binding and stability.[2] Nature's sophisticated α-amidating machinery leverages the dual catalytic ability of the enzyme peptidylglycine α-amidating monooxygenase (PAM) which converts a propeptide **1** bearing a C-terminal Gly extension into the corresponding truncated primary amide **2** *via* liberation of glyoxylic acid (Scheme 1A). Mechanistically, this oxidative cleavage involves two enzymatic processes, *oxidation* to the α-hydroxy Gly derivative **3** and subsequent *hydrolysis* to the target peptide α-amide.[3]

Scheme 1. (a) Biosynthetic approach to primary α-amides; (b) Contemporary strategies for C-terminal amidation; (c) Proposed electrochemical approach.



[a] PDB entry 5WJA was used to generate figure.

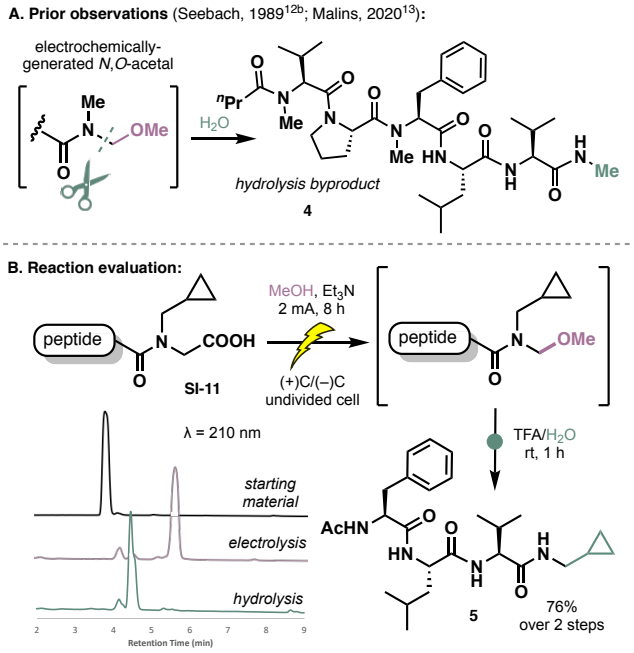
Although Nature's monooxygenase-mediated α-amidation strategy exclusively affords primary amides, there is considerable interest in the inherent tuneability of "designer" amides bearing more complex substitution patterns, including for applications in drug discovery.[2, 4] For example, a synthetic α-monomethyl amidated variant of human parathyroid hormone (hPTH) has shown superior binding capabilities relative to both the corresponding α-acid and primary α-amide.[5] Moreover, blockbuster cancer therapeutic leuprolide, a structural analogue of gonadotropin releasing hormone (GnRH), notably features a C-terminal *N*-ethylamide.[6]

Yet despite both the significance and simplicity of substituted amides, there are remarkably few chemical strategies enabling access to a broad diversity of α-amidated peptides,[7] particularly tertiary amides, which has limited critical evaluation of the structural and functional consequences of this modification. Direct late-stage amidation (Scheme 1B), through activation of a peptide C-terminal carboxylic acid followed by acyl substitution with an amine, requires orthogonal protecting group strategies and is notoriously plagued by epimerization at the C-terminal residue. Although considerable resources have been dedicated to the development and optimization of hundreds of new coupling reagents to address the latter challenge,[8] practitioners generally agree that there is no "one-size fits all" approach to overcoming epimerization in the activation process and to facilitating difficult couplings. Specialized resin linkers for the solid-phase peptide synthesis (SPPS) of C-terminally amidated peptides have also been explored.[2, 9] Aldehyde resins (Scheme 1B), for example, introduce the amide substituent at an early-stage *via* reductive amination.[4c, 10] Alternatively, latent resin linkages may be activated toward aminolysis to afford amide products (e.g., sulfonamide or "safety-catch" resins[4a, 11] can be alkylated with excess iodoacetonitrile or TMS-diazomethane). Though conceptually appealing, resin-based methods are often limited to non-bulky, nucleophilic amines, which considerably hampers access to tertiary amides. Furthermore, challenging acylation steps in the initial resin loading may contribute to lower yields. A robust approach to substituted α-amidated peptides would significantly accelerate the construction of C-terminally-modified peptides for myriad applications.

Building on the pioneering work of Seebach and coworkers,[12] we recently reported an arylation strategy which harnessed the reactivity of hydrophobic peptide *N,O*-acetals generated through direct electrolytic decarboxylation of C-terminal acids.[13] In the course of these studies, we identified hydrolysis of a sarcosine-derived *N,O*-acetal (Scheme 2A) as a prevalent side-pathway, which delivered a truncated *N*-methylamide variant of the natural product biseokeaniamide B **4** as a single diastereomer. This *oxidation-hydrolysis* pathway, previously noted by Seebach to afford simple primary amides,[12b] is reminiscent of the two step biosynthetic approach mediated by the enzyme PAM. In contrast to the primary amide products afforded by the enzymatic process, however, extending this chemistry to diverse *N*-alkylated variants presents an opportunity to access "designer" substituted amides in an epimerization free, bio-resemblant approach.

We therefore envisaged generalizing this simple decarboxylative strategy to designer C-terminal amides by pairing electrochemical *oxidation* with a tandem *hydrolysis* (path a), or alternatively, a facile *reduction* (path b) to deliver a suite of secondary and tertiary amides (Scheme 1C). This post-synthetic, divergent strategy would harness the inherent reactivity of unactivated C-terminal acids, in which "propeptide" substrates bearing C-terminal amino acid or *N*-alkyl amino acid extensions are converted to the corresponding truncated amides. The conceptual novelty lies in the ability to avoid entirely conventional activation chemistry (e.g., Scheme 1B), thus eliminating any risk of epimerization. At the outset of this endeavor, we were encouraged by recent work on decarboxylative peptide modifications,[14] including photocatalytic approaches to decarboxylative reduction[15] and peptide *N,O*-acetals.[16] Herein, we sought to exploit electrochemistry as a mild and tunable strategy[17] for site-specific oxidation. Indeed, a selection of recent methods has explored electrochemistry in the context of peptides,[18] although the majority of these studies rely on programmed oxidation of peptide electroauxiliaries or the reactivity of electrochemically-active small molecule reagents/catalysts. Moreover, our own studies[13] have focused exclusively on the electrochemical oxidation of peptides with hydrophobic side-chains. As such, the broad functional group compatibility of the proposed anodic decarboxylation remains to be explored. We therefore aimed to conveniently exploit the inherent reactivity of peptide C-terminal acids, including in the presence of diverse side-chain functionalities, to deliver high-value substituted amide products.

Scheme 2. (a) *N,O*-acetal hydrolysis; (b) Analysis of electrochemical oxidation-hydrolysis *via* UPLC-MS

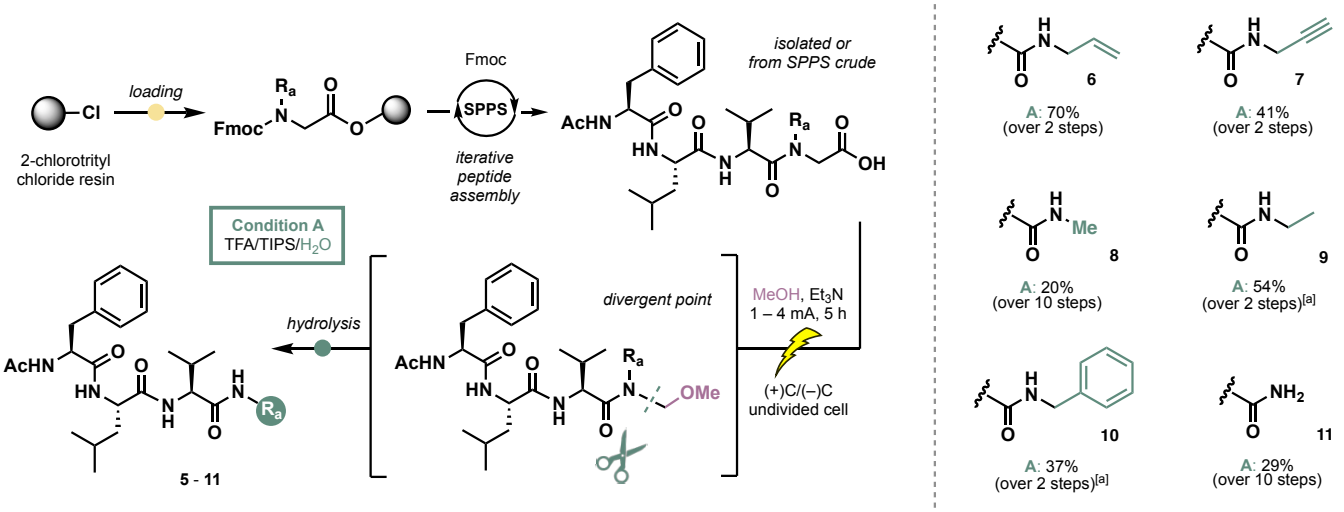


Results and discussion

Hydrolysis. To begin our investigations, model tetrapeptides bearing various *N*-alkylated glycine residues at the C-terminal position were designed as "propeptide" substrates to examine the generalization of the oxidation-hydrolysis pathway. Peptides were prepared using Fmoc-SPPS through initial loading

of Fmoc-*N*(R_a)-Gly-OH (R_a = hydrogen, methyl, ethyl, benzyl, propargyl, allyl, cyclopropylmethyl; R_b = H) and elongation using standard coupling protocols (see Scheme 3 and the supporting information for details). Following resin cleavage, peptides were dissolved in MeOH, treated with Et_3N, and oxidized at constant current in an undivided electrochemical cell equipped with carbon electrodes to deliver the intermediate *N*,*O*-acetals.[12b, 13] A rapid and facile TFA-promoted hydrolysis under standard peptide cleavage conditions (TFA/triisopropylsilane (TIPS)/H_2O, 90:5:5, v:v:v) then afforded the truncated *N*-alkylamides **5**-**11** (Scheme 3) in appreciable yields over 2 steps (from the starting peptide acid) or 10 steps (from the resin loading). UPLC-MS analysis of the oxidation-hydrolysis of *N*-cyclopropylmethyl glycine derivative **SI-11** (Scheme 2B), highlights the efficiency of the process, which afforded **5** in 76% yield over 2 steps. Peptides containing C-terminal alkenyl (**6**), alkynyl (**7**), *N*-methyl (**8**), *N*-ethyl (**9**), and *N*-benzyl (**10**) derivatives were readily accessible. Intriguingly, in the case of *N*-benzyl derivative **10**, standard conditions led to competitive reduction of the *N,O*-acetal affording the corresponding *N*-methyl-*N*-benzyl derivative; exclusion of TIPS afforded the desired *N*-benzyl product. Simple primary amide **11** was likewise accessible beginning with the quintessential PAM "propeptide" substrate (see Scheme 1A) bearing a single glycine extension at the C-terminus.

Scheme 3. Scope of electrochemical oxidation-hydrolysis



[a] Triisopropylsilane (TIPS) was excluded (see Supporting Information).

Although this bio-resemblant oxidation-hydrolysis strategy enables direct access to *N*-alkylamide variants, the method is not without limitations. Drawbacks include the need to prepare protected *N*-alkyl amino acids and difficulties encountered during acylation of the secondary amine of the *N*-alkyl glycine variants (c.f. the resin-based approaches outlined in Scheme 1B). In addition, SPPS yields of *N*-alkyl glycine-derived peptides were generally modest due to competitive diketopiperazine formation, which occurred upon elongation of the peptide.[19]

Reduction. Given the above considerations, we therefore aimed to exploit the availability, rich functional diversity, and facile couplings of amino acids bearing side-chain rather than *N*-alkyl substituents (e.g., R_a = H; R_b = alkyl, aryl, see Scheme 4). To this end, it was envisaged that electrochemical oxidation to deliver α-substituted *N*,*O*-acetals could be followed by a tandem reduction pathway,[20] to afford substituted amide products *via* retention of the side-chain functionality of the C-terminal amino acid (e.g., path b, Scheme 1C). Notably, the competitive, silane-mediated reduction observed in the preparation of *N*-benzyl peptide **10** (Scheme 3) lent credence to the viability of this pathway.

Accordingly, crude tetrapeptides with Gly, Ala and phenylglycine (Phg) residues at the C-terminus were directly subjected to an electrolysis/reduction sequence after cleavage from the resin. To our delight, amides **8** (*N*-Me), **9** (*N*-Et), and **10** (*N*-Bn) were smoothly obtained upon treatment of the crude *N*,*O*-acetal with BF_3•Et_2O/triethylsilane (TESH) or TFA/TESH—anhydrous reduction conditions which served to minimize hydrolysis (see SI, p. 44–45 for optimization tables). Importantly, unlike the *N*-alkylated peptide precursors required for the hydrolysis approach, the "propeptides" utilized in the oxidation-reduction were readily prepared by SPPS without complication (e.g., diketopiperazine formation) and able to be used without intermediary purification. Notably, amidated products were also obtained as single diastereomers. In contrast, attempts to synthesize *N*-Me amide **8** by simple coupling of the corresponding tripeptide C-terminal acid with *N*-methylamine using standard coupling reagents (EDC, PyBOP, DIC/Oxyma) led to considerable epimerization, and in some cases, complete loss of stereochemical integrity at the C-terminal position (see SI, p. 40).

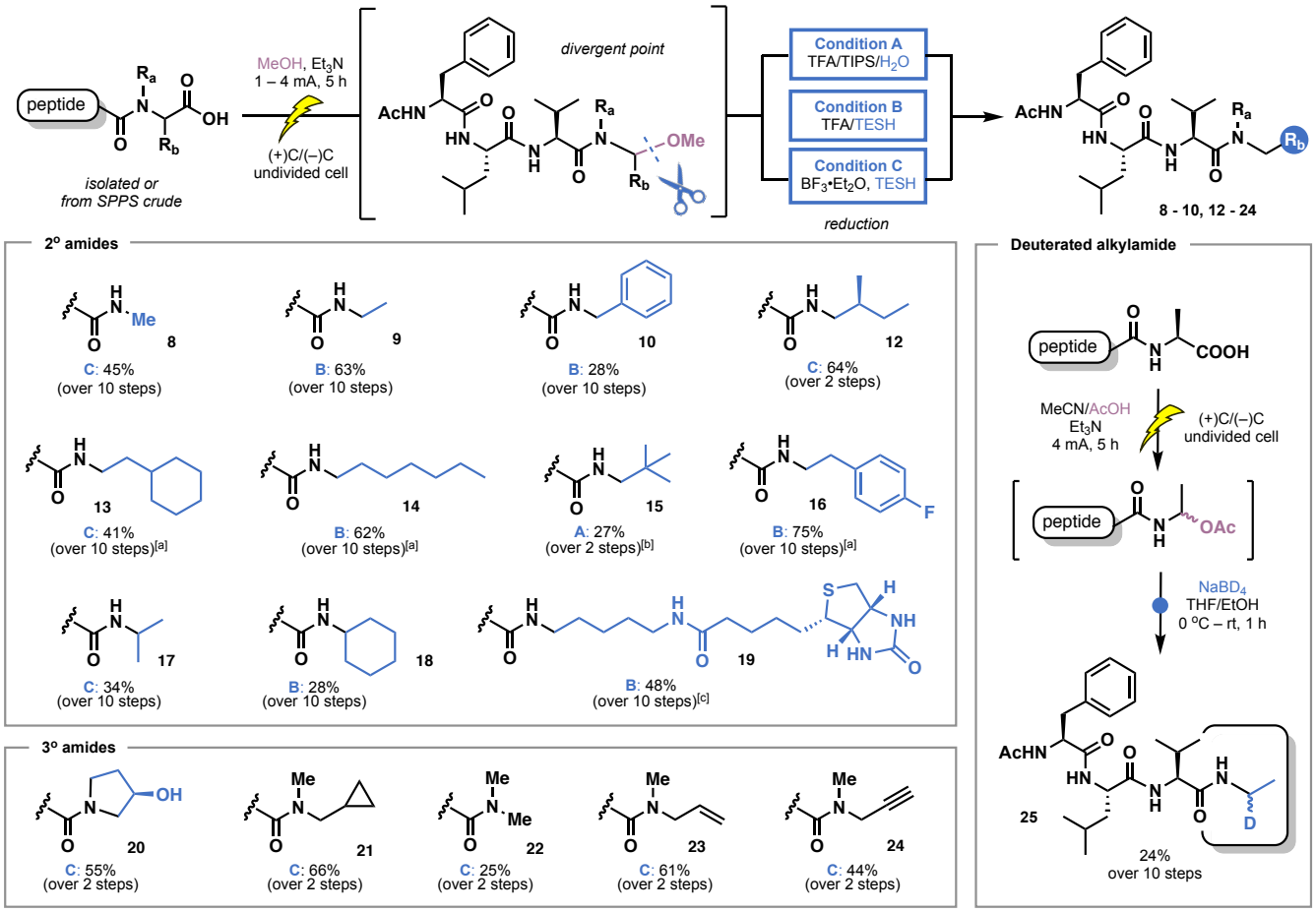
"Propeptides" containing a variety of other native or commercially available modified amino acids at their C-termini were also viable (**12**-**20**, Scheme 4). Highlights include **12** (derived from readily available Fmoc-Ile-OH), which would be difficult to synthesize *via* late-stage amidation given the cost of the requisite chiral amine (e.g. (*S*)-(–)-2-methylbutylamine = USD$156/g).[21] Notably, the α-stereochemistry of the C-terminal amino acid in the "propeptide" precursor is inconsequential, as reduction abolishes the stereocenter. Amino acids

with undefined α-chirality could therefore be readily incorporated (e.g., **13**, **14** and **16** were derived from C-terminal DL-amino acids). α,α-Disubstituted amino acids (e.g., **17** and **18**) were also successfully converted to secondary amides albeit in slightly lower yields, likely attributed to competitive hydrolysis of the corresponding substituted iminium intermediates which are more sterically encumbered and perhaps lead to slower rates of reduction. Surprisingly, hydrolysis was largely suppressed in the oxidation-reduction of a C-terminal *tert*-leucine peptide, which proceeded smoothly to afford **15**, even in the presence of water, with a standard peptide cleavage cocktail (TFA/TIPS/H_2O). Functional group tolerance is exemplified by the incorporation of a biotin handle from a commercially available biotin-lysine derivative. Initially, standard reduction with TFA/TESH afforded **19** in 13% yield over 10 steps based on the resin loading. We reasoned that thioether oxidation was a likely deleterious side-pathway in the electrochemical oxidation step. NH_4I was therefore added to the TFA/TESH reduction cocktail to effect concomitant sulfoxide reduction,[22] affording **19** in an optimized 48% yield.

Tertiary amides were also readily accessible (e.g., **20**, derived from Hyp).[23] Oxidation-reduction of the *N*-alkylglycine peptide precursors utilized in the hydrolysis approach (see Scheme 3) provided an additional path to valuable tertiary amides. For example, reduction of the *N*,*O*-acetal derived from *N*-cyclopropylmethyl glycine peptide **SI-11** (see Scheme 2), afforded the *N*-cyclopropylmethyl-*N*-methyl amide **21** in good yield (66% over 2 steps). *N*,*N*-dimethyl (**22**), *N*-allyl-*N*-methyl (**23**) and *N*-propargyl-*N*-methyl (**24**) peptides were also accessible; each of these substrates would be challenging, if not impossible, to prepare using standard solution- or solid-phase methods due to difficulties accessing the requisite amine precursor and challenges in forming the tertiary amide using conventional activation-based coupling approaches.

In another valuable extension of the method, site-selective deuteration was readily accomplished by adopting slightly modified oxidation-reduction conditions, allowing for the use of commercially available $NaBD_4$ as a reductant. This process provided **25** in good yield (24% over 10 steps) when the more reactive AcOH-derived *N*,*O*-acetal[13] was employed. The MeOH-derived *N*,*O*-acetal in this case afforded the desired product together with an inseparable byproduct. Importantly, the optimized protocol for late-stage isotope incorporation serves as a useful proof-of-principle study demonstrating facile access to labelled peptides, including for structural or biological assays.

Scheme 4. Scope of electrochemical oxidation-reduction



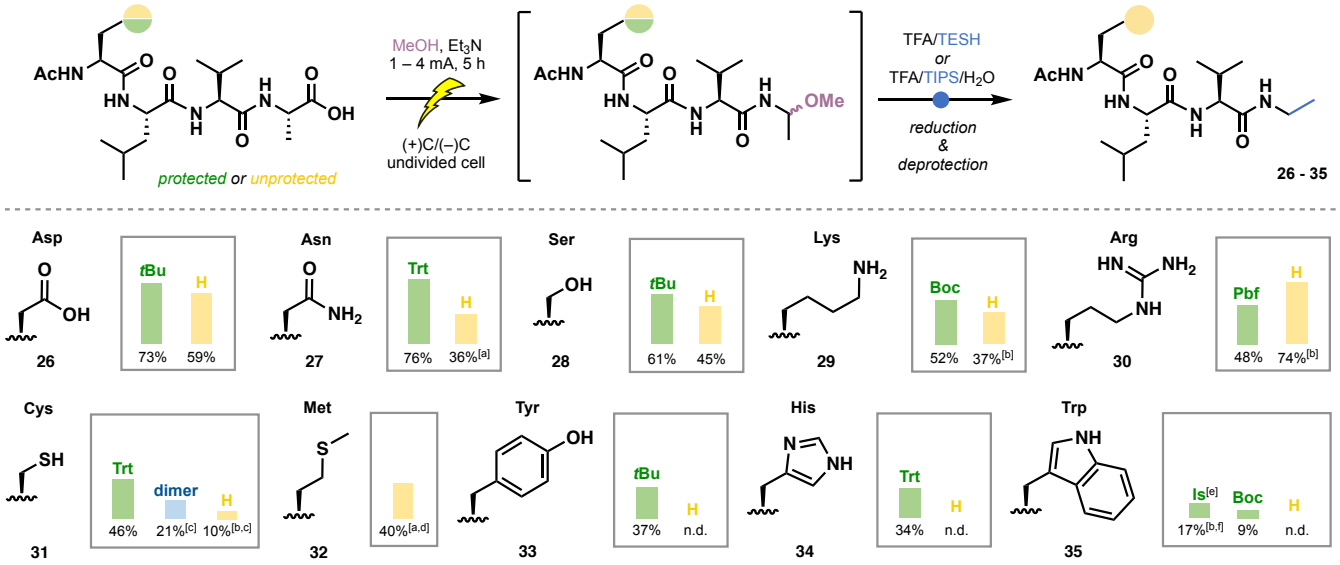
The following labels apply to the reaction scheme: [a] Peptide was prepared from a C-terminal DL-amino acid; [b] Hydrolysis conditions (TFA/TIPS/H_2O) led to the reduction product; [c] Additional NH_4I was added to the reduction (see Supporting Information).

Side-chain compatibility. Given that late-stage modification methods require broad compatibility with canonical amino acid side-chain functionalities,[24] a systematic evaluation of the side-chain tolerance of the oxidation-reduction pathway was next examined. To the best of our knowledge, an exhaustive evaluation of the side-chain tolerance of anodic oxidation of C-terminal peptide acids has never been undertaken.[25] Protected and unprotected model tetrapeptides bearing functionalized amino acids (e.g., Asp, Asn, Ser, Lys, Arg, Cys, Met, Tyr, His, Trp) at the *N*-terminal position were subjected to the standard oxidation-reduction conditions (Scheme 5, side-chain protected, green; side-chain unprotected, yellow). Notably, the acidic reduction conditions result in concomitant cleavage of side-chain protecting groups to deliver unprotected peptides, so both protected and unprotected peptides are treated equivalently and deliver identical peptide products. Pleasingly, the desired *N*-ethylamide product (e.g., **26**-**35**) was formed in all cases in the presence of protected residues. The majority of residues were well-tolerated (34-76%) while highly oxidizable aromatic side-chains, as expected, gave lower yields (e.g., Trp(Boc) **35**, 9%). The nature of the protecting groups played a critical role in modulating the oxidation potential of the side-chain functionalities. For example, oxidation-reduction of 2,4,6-triisopropylbenzenesulfonyl (Is)-protected Trp afforded the Trp-protected product in a remarkable 75% yield. We reasoned that the highly electron-withdrawing nature of the sulfonyl protecting group rendered the indole nucleus less prone to oxidation. Unfortunately, the use of sulfonyl-protected Trp is complicated by difficulties in removing the protecting group under standard acidolytic cleavage conditions. A suitable one-pot oxidation-reduction-deprotection protocol employing TMSBr[26] for the removal of the sulfone protecting group was devised but led to a significant reduction in yield (17% over 2 steps). In this case, there is a slight trade-off between the yield of product and the operational simplicity of the method.

In the presence of unprotected Asp, the protocol was selective for C-terminal decarboxylation, delivering **26** in 59% over 2 steps. Unprotected amides and alcohols (Asn **27**, Ser **28**) were well-tolerated. Basic residues (Lys **29**, Arg **30**) benefitted from the addition of acetic acid in the electrolysis step,[25a] with protonation reducing unwanted oxidation. Interestingly, unprotected Cys (e.g., **31**) did not preclude the desired transformation, likely owing to *in situ* oxidation to the disulfide during electrolysis (see SI). Markedly improved yield was obtained with the corresponding cystine substrate (**31**, from thiol: 10%; from disulfide: 21%). Oxidation-reduction of the Met thioether-containing peptide afforded **32** in 40% yield over 10 steps. As with the biotin substrate (**19**, see Scheme 4) the addition of NH_4I to the standard reduction conditions enabled concomitant reduction of side-chain sulfoxide formed in the electrochemical step.[22] Tyr, His, and Trp did not tolerate electrochemical oxidation in the absence of protecting groups.[27] Nevertheless, as protected peptides are easily accessible using common SPPS strategies and the acidic reduction conditions enable concurrent cleavage of side-chain blocking groups, we envisaged that the method would be readily extendable to more complex peptide systems with diverse side-chain functionalities.

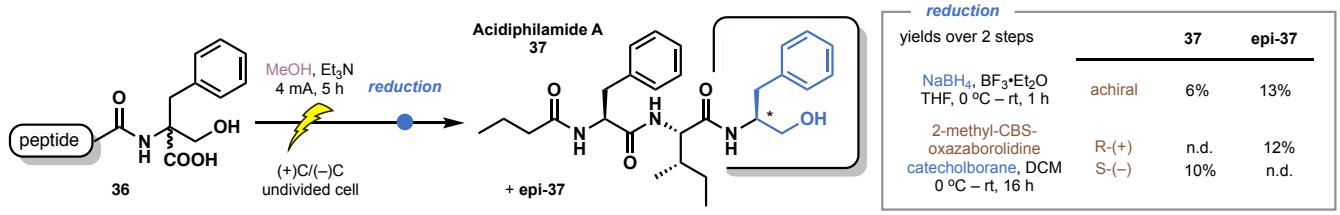
Applications. To further evaluate the method, the total synthesis of acidiphilamide A, a bioactive secondary metabolite from the rare actinobacterial genus *Streptacidiphilus*,[28] was pursued with an intent to construct the characteristic secondary amide using our electrochemical oxidation-reduction pathway. Though conceivably accessible *via* alternative approaches, we envisaged that acidiphilamide A would serve to

Scheme 5. Side-chain compatibility studies



The following labels apply to the reaction scheme: [a] Yield over 10 steps from resin loading; [b] MeOH/AcOH (10:1 v:v) was used for electrolysis; [c] Yield following an additional TCEP reduction step; [d] NH_4I was added to the reduction mixture (see SI); [e] Is = 2,4,6-triisopropylbenzenesulfonyl; [f] TMSBr/thioanisole/1,2-ethanedithiol cocktail was added for Is-deprotection.

Scheme 6. Synthesis of natural product acidiphilamide A

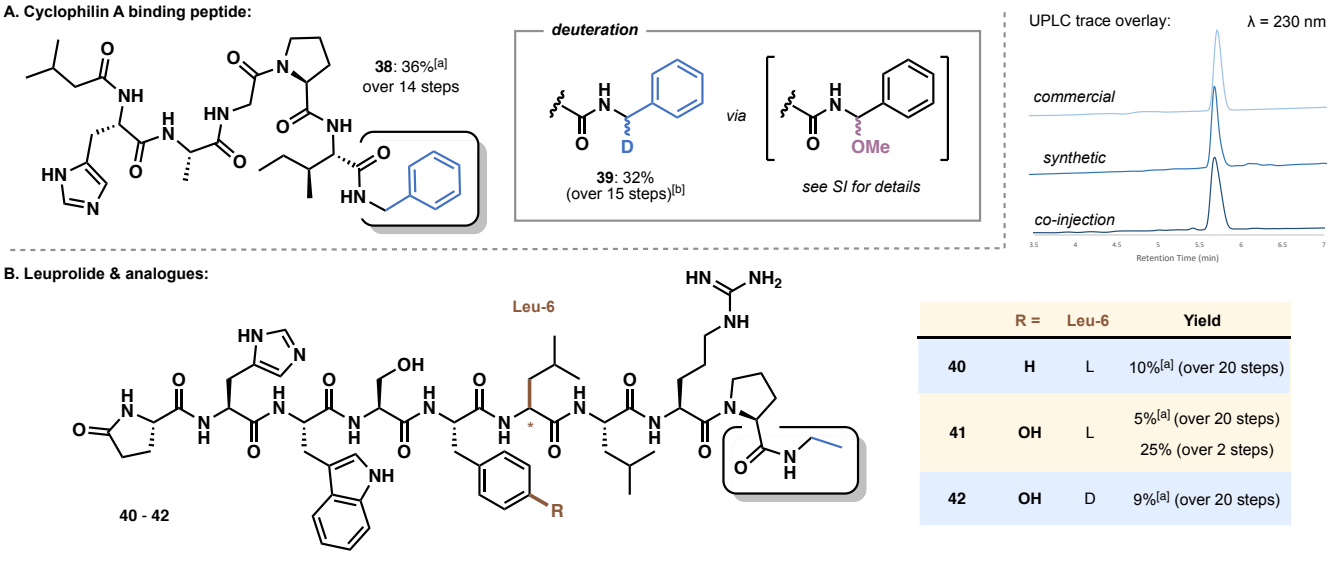


challenge our method due to the presence of an unprotected C-terminal alcohol and the obligatory intermediacy of a quaternary *N*,*O*-acetal. Pleasingly, following synthesis of the requisite "propeptide" incorporating commercially available DL-2-benzylserine at the C-terminus (**36**, see SI), standard electrolysis and subsequent reduction with the more reactive $NaBH_4$ afforded the natural product **37** and **epi-37** (*dr* 1:2.2) in synthetically useful yields (Scheme 6). In an effort to improve the diastereoselectivity of the reduction step, we next evaluated the use of a chiral reductant to direct the stereochemical outcome of the reduction. Accordingly, (*S*)- and (*R*)-CBS-oxazaborolidines were employed,[29] leading to selective formation of **37** and **epi-37**, respectively, and thus laying the framework for the future exploration of stereoselective reductions.

The method was finally applied to the synthesis of bioactive peptides. Pentapeptide **38** (Scheme 7A), bearing a C-terminal benzylamide, was reported to be a potent binder of cyclophilin A,[4a] which is implicated in HIV-1 replication. This therapeutic lead was readily accessible *via* oxidation-reduction of a phenylglycine (Phg) precursor in 36% yield over 14 steps (see SI). The deuterated variant **39** was likewise obtained in comparable yield (32% over 15 steps). In the context of drug discovery, facile isotope-labeling may serve as a valuable tool for understanding mechanisms of drug metabolism.[30]

The nonapeptide cancer therapeutic leuprolide and several structural variants (**40**-**42**) were also accessible. Leuprolide contains a C-terminal *N*-ethylamide and a number of challenging, oxidatively labile residues (His, Tyr, and Trp). Strikingly, the oxidation-reduction of the requisite protected "propeptides", each employing standard Boc/^{t}Bu-protecting group strategies (including the incorporation of Trp(Boc), see SI), successfully afforded the target *N*-ethylamide products over 20 steps, without the need for intermediary purification. Importantly, ^{1}H NMR analysis and UPLC-MS co-injection (Scheme 7B) demonstrated that synthetic leuprolide **42** was identical to a commercial sample of the drug. To further probe the efficiency of the two-step electrochemical oxidation-reduction, the purified [L-Leu^6] precursor peptide was subjected to the reaction conditions. This process delivered **41** in 25% isolated yield despite the presence of multiple oxidizable side-chains, notably outperforming our model tetrapeptide (e.g., the synthesis of **35** using Trp(Boc) protection, which proceeded in 9% yield). We postulate that the peptide microenvironment might influence side-chain oxidation potentials. In the case of leuprolide, the Trp residue is internally-located on the peptide chain, whereas model substrate **35** bears an N-terminal Trp. Future studies will aim to deconvolute the link between peptide sequence and propensity toward oxidation, and therefore explore opportunities for sequence-specific electrochemical modifications.

Scheme 7. Scope of bioactive peptides: (a) Potent pentapeptide binder of cyclophilin A; (b) Synthesis of leuprolide and structural analogues



[a] Yields based on resin loading. [b] An additional deprotection step was carried out following treatment with $NaBD_4$.

Conclusions

In summary, we disclose herein an electrochemically-enabled peptide C-terminal amidation strategy. Broadly inspired by the dual catalytic functionality of the α-amidating monooxygenase enzyme PAM, an electrochemical oxidative decarboxylation paired with a tandem hydrolysis or reduction facilitates post-synthetic conversion of C-terminally extended "propeptides" into *N*-alkylamides. A broad array of C-terminal alkylamides bearing valuable functional handles (e.g., alkene, alkyne, biotin, isotope labels) is readily accessible. The method is both highly functional group tolerant and by design, epimerization free, thus overcoming an unsolved challenge in the preparation of homogeneous, C-terminally-modified peptides. Several case studies have highlighted the preparation of bioactive peptides and associated analogues, and we have established proof-of-principle for the viability of a diastereoselective reduction protocol. We therefore envisage that this approach to designer C-terminal alkylamides will have broad application in the development and advancement of peptide-based therapies.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website. Experimental details and analytical data for all new compounds (PDF) are provided.

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ABBREVIATIONS

UPLC, ultra-performance liquid chromatography; TFA, trifluoroacetic acid; TESH, triethylsilane; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; DIC, *N*,*N*′-diisopropylcarbodiimide; Oxyma, Oxyma Pure®, ethyl (hydroxyimino)cyanoacetate; Hyp, *trans*-4-hydroxy-L-proline; TCEP, tris(2-carboxyethyl)phosphine hydrochloride.

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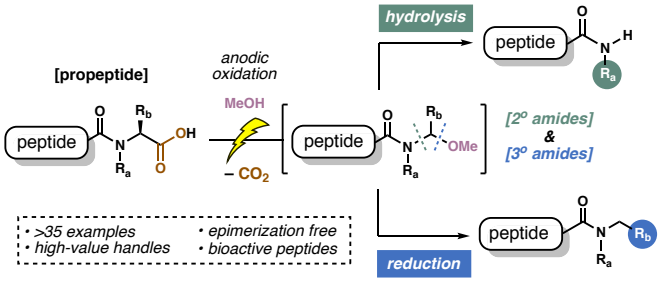
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