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Photochemically-enabled, post-translational production of C-terminal amides

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C-terminal α-amidated peptides are attractive therapeutic targets, but preparative methods to access amidated pharmaceuticals are limited both on lab and manufacturing-scale. Here we report a straightforward and scalable approach to the C-terminal α-amidation of peptides and proteins from cysteine-extended polypeptide precursors. This amidation protocol consists of three highly efficient steps: 1) selective cysteine thiol substitution with a photolabel, 2) photoinduced decarboxylative elimination and 3) enamide cleavage by simple acidolysis or inverse electron demand Diels-Alder reaction. We provide a blueprint for applying this protocol to the semi-recombinant production of therapeutically relevant targets where gram scale C-terminal α-amidation is achieved in a photoflow reactor on a recombinantly prepared peptide YY analogue and a GLP-1/amylin co-agonist precursor peptide. Robust performance of this reaction cascade in flow highlights the potential of this chemistry to enable amidated drug leads to enter development that would not be viable on commercial scale using existing technology.

Peptide therapeutics are currently enjoying a renaissance; with >50 drugs on the market and hundreds more in development, the pharmaceutical industry is capitalizing on a modality that offers high specificity and potency[1]. Over the past two decades, significant progress has been made in addressing limitations typically associated with therapeutic peptides, mainly rapid clearance and poor oral bioavailability. Advances in half-life protraction technologies and permeation enhancers have paved the way for the development of long-acting injectable and oral peptide drugs[2,3]. As interest and demand for peptide therapeutics continue to grow for serious chronic diseases with large patient populations, such as diabetes and obesity[4], there is an urgent need for scalable manufacturing processes to meet the high-volume production demands. At these scales, conventional solid-phase peptide synthesis (SPPS) has a significantly increased environmental burden relative to recombinant production and, depending on the length of the peptide, may not be feasible. Peptides containing a C-terminal α-amide are particularly challenging to prepare when SPPS is not suitable[5]. While C-terminal amidation is a common post-translational modification that occurs in half of all hormones and neuropeptides[6] and is critical for the full biological activity of peptides and of therapeutically relevant molecules that are derived from them[7,8], alternative technologies for making this peptide modification on scale remain inadequate despite its significance.

Modern semi-recombinant synthesis offers an attractive solution for the scalable preparation of peptide therapeutics. This approach combines the environmental benefits of microbial expression systems with a robust chemical or enzymatic modification that can install drug-like properties in a natural peptide[9]. Previously, semi-recombinant production of C-terminal α-amides has employed enzymatic methods where substrate specificity[10–12], limited scalability, and the requirement for enzyme expression in mammalian cultures add significant constraints to manufacturing processes[13]. In contrast, a selective chemical

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approach could provide unparalleled access to therapeutic C-terminal α-amides on scale. To date, however, no general process-friendly approach for the direct C-terminal amidation of a recombinantly expressed peptide or protein has been reported. Some of the most successful chemical amidations have relied on selective[14] formation of cyanocysteines[15–17] which often produce variable yields of amidated product mostly related to competitive formation of unwanted elimination and hydrolysis byproducts. The Davis group recently published an elegant and robust diboron-mediated C-terminal amidation protocol from dehydroalanine (DHA) intermediates[18], but the inefficient formation of DHA and the requirement for a large excess of electrophilic reagents present significant barriers to adapting the process to scale.

We postulated that a reliable and scalable amidation could be achieved through a biomimetic process targeting the formation of a carbinolamide, an intermediate that undergoes amidation in nature by peptidylglycine α-amidating monooxygenase (PAM)[19] enzyme (Fig. 1a). As an alternative to enzymatic cleavage, we recognized that mild, acid-promoted cleavage of the carbinolamide[20] would afford the desired C-terminal α-amide (Fig. 1b, Path A). To take advantage of the biosynthetic intermediate, we conceived of a C-terminal enamide as a masked C-terminal α-amide, whereby protonation of the nucleophilic olefin with an acid in aqueous solution would generate the key carbinolamide. Under the acidic conditions used to form the carbinolamide, concomitant cleavage to the C-terminal α-amide is expected. To further generalize the reaction, we envisioned leveraging the unique properties of the enamide to develop a complementary route to α-amides based on an inverse electron demand Diels-Alder (IEDDA)-mediated cleavage[21] (Fig. 1b, Path B). The IEDDA chemistry would enable the enamide cleavage to be performed in a neutral buffer for peptides and proteins sensitive to acid (e.g., glycopeptides). In addition, it provides an orthogonal route for the production of pharmaceutically relevant C-terminal amides that have a low pI, where crossing the pI is expected to result in solubility challenges on scale Cysteine was chosen as the enamide precursor based on its low natural abundance and decades of precedence for selective transformations in chemical biology[22]. We reasoned that recombinantly prepared C-terminal cysteine extended peptides could be converted to their corresponding C-terminal α-amides using this biomimetic approach, provided the enamide intermediate is readily accessible.

Recently, mild and efficient electrochemical[23] and photochemical[24,25] methods have been developed for the formation of alkylated C-terminal amides, including an intriguing C-terminal enamide disclosed by Baker[26]. Building on the mechanistic precedent proposed by the Baker group (Supplementary Fig. 52), we postulated that it would be feasible to selectively conjugate a C-terminal cysteine

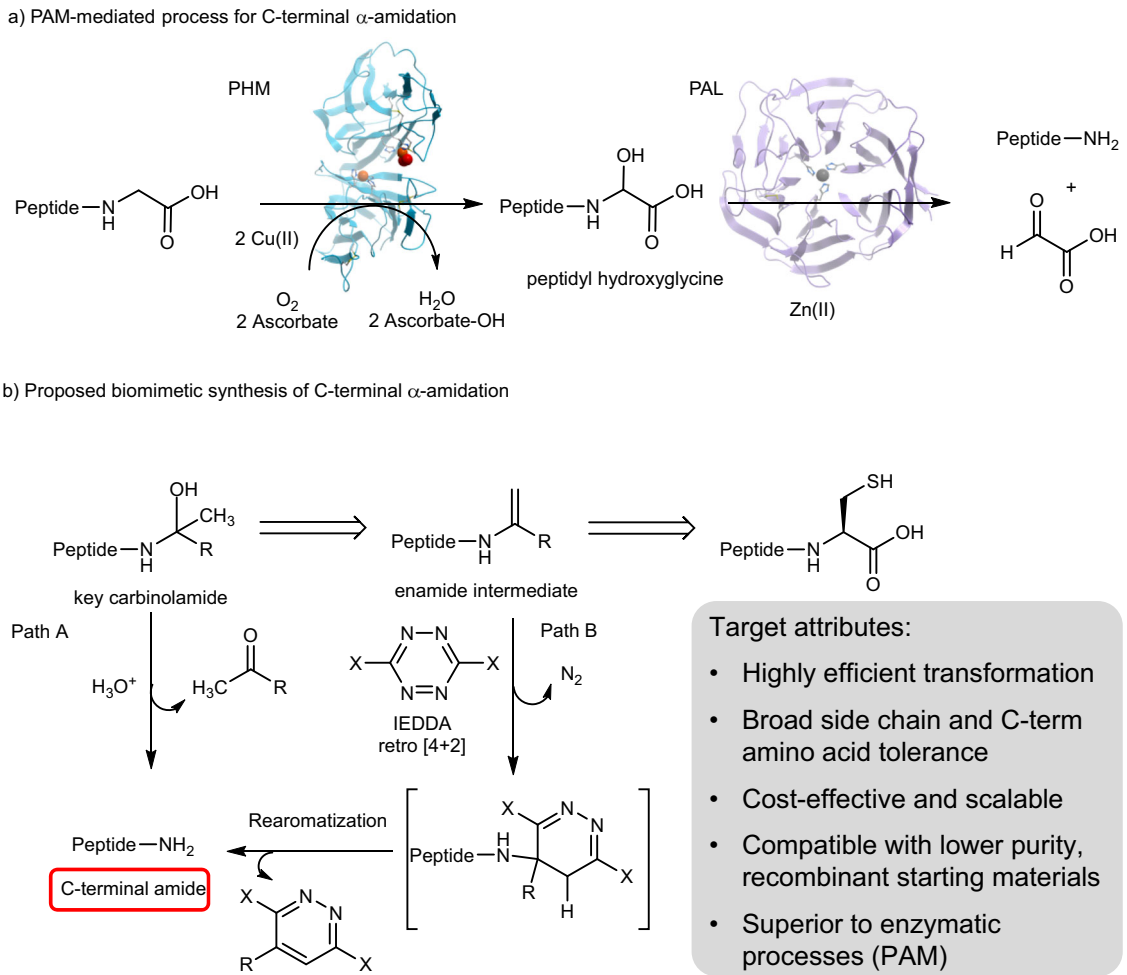

Fig. 1 | C-terminal α-amidation reactions. a Enzymatic (PAM) mediated α-amidation of peptides containing a C-terminal glycine residue. **b** Biomimetic C-terminal α-amidation of peptides containing a C-terminal cysteine residue (this work). The two reaction pathways provide complementary routes for accessing C-terminal α-amides that proceed through a common enamide intermediate, which can be converted to the amide under acidic conditions (Path A) via the biosynthetic carbinolamide intermediate, or under neutral conditions via an IEDDA reaction with a tetrazine (Path B).

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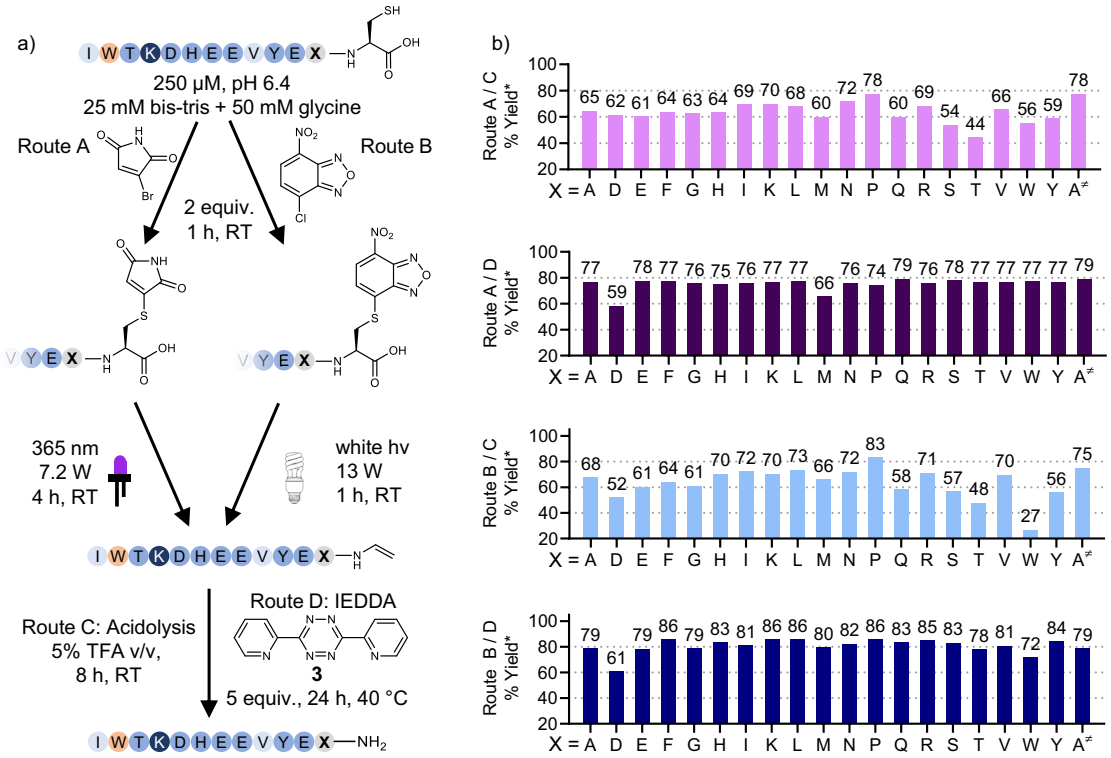

Fig. 2 | Evaluation of reaction conditions and scope of the α-amidation with respect to the penultimate position. a Generalized reaction scheme. **b** Yield of C-terminal α-amides obtained by substitution with bromomaleimide (Route A) or NBD-Cl (Route B), followed by enamide cleavage with acid (route C) or by IEDDA (Route D). *All yields were determined by extracted ion chromatography (XIC).≠ X = A, and the Trp residue at the 2-position is substituted to Phe (IFTKDHEEVYEA-NH_2).

thiol to a photolabile group that, upon irradiation, would undergo a decarboxylation/β-fragmentation[27] to afford a monosubstituted C-terminal enamide (Fig. 2). This intermediate was anticipated to have stereoelectronic properties more favorable to both acidolysis and IEDDA-mediated cleavage relative to a 1,1-disubstituted enamide, such as DHA[28].

In this work, we demonstrate the highly efficient photochemical conversion of C-terminal cysteine extended peptides to their corresponding C-terminal α-amides under mild reaction conditions. We show that the protocol developed herein can be readily applied to the synthesis of therapeutically relevant peptides. Scalability of the protocol is achieved with a photoflow process, which is utilized in the multi-gram semi-recombinant synthesis of a GLP-1/ amylin co-agonist precursor peptide.

Results

To test our hypothesis, we synthesized the C-terminal cysteine extended generic peptide H-IWTKDHEEVYEAC-OH **(1)**. This peptide was used to optimize reaction conditions for each step of the amidation process (Fig. 2a, X = A). Of the photolabile reagents evaluated (see Supplementary Table 1), bromomaleimide and NBD-Cl offered the best combination of cost, high conjugation yield, and facile conversion to C-terminal enamide. We found that the best yields for the photodecarboxylation step were obtained when temperature was controlled with a cooling fan, and when solvents were degassed with nitrogen prior to conjugation of the photolabile group. The efficiency of the acidolysis step was correlated to pKa, where weaker acids like acetic and oxalic acid were capable of converting enamide to the desired biosynthetic carbinolamide intermediate, but stronger acids were needed to promote efficient cleavage to the C-terminal α-amide. Complete conversion to the desired amide H-IWTKDHEEVYEA-NH_2 **(2)** was observed when trifluoracetic acid was employed, and no improvement in reaction yield was observed when stronger acids were used. In contrast, high yields of the IEDDA-mediated cleavage of the C-terminal enamide could only be achieved using 2-pyridyl substituted tetrazines, prompting us to choose commercially available **3** as our preferred reagent. For either enamide cleavage procedure, the addition of 1 mM methionine reduces oxidative byproducts and boosts the overall yields of the transformation. The yield of the acidolysis step can be further improved by adding 250 µM caffeic acid, which mitigates the formation of oxidation byproducts[29] (see Supplementary Table 4). Overall, the transformation is operationally simple and can be performed sequentially or in one pot using either visible light from a work light or a UV LED source and readily available chemical reagents found in most research labs.

With optimized reaction conditions in hand, we began a thorough evaluation of the scope of the amidation with respect to the penultimate amino acid (AA) position. In contrast to enzymatic amidation reactions where the identity of the penultimate AA can drastically affect reaction efficiency, our amidation protocol produced C-terminally α-amidated peptides in moderate to excellent yields for every AA at this position (Fig. 2b). Some variation in reaction performance was evident when comparing the use of bromomaleimide and NBD-Cl for generating the enamide intermediate (Fig. 2a, routes A and B), and between the two enamide cleavage protocols (routes C and D). Generally, our best yields were obtained when peptides were conjugated with NBD-Cl (route B) and when the enamide intermediate was cleaved by tetrazine-mediated IEDDA reaction (route D). The neutral conditions employed in the IEDDA chemistry reduce non-specific oxidation of the backbone as well as hydrolysis of the enamide intermediate to the C-terminal acid, a non-productive reaction that we found is promoted by the sidechains of penultimate serine, threonine, and aspartic acid residues (Supplementary Tables 5 and 7). For each set of conditions, peptides containing a penultimate tryptophan gave

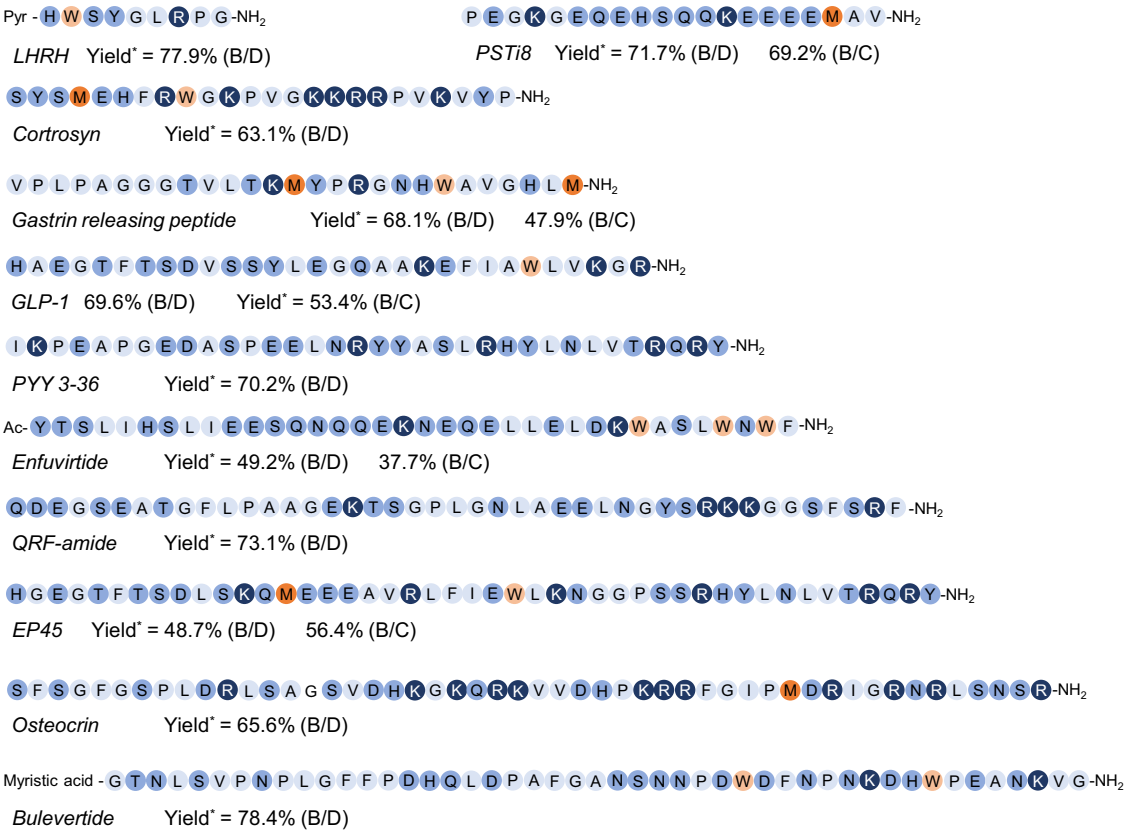

Fig. 3 | Demonstration of scope of the amidation on pharmaceutically relevant amidated peptides. All reactions were performed at a C-terminal cysteine-extended peptide concentration of 250 μM in 25 mM BisTris + 50 mM glycine buffer at pH 6.4. The choice of photolabel and enamide cleavage conditions are indicated by the letters following yields and correspond to the routes described in Fig. 2. *All yields were determined by extracted ion chromatography (XIC).

slower conversions and showed greater sensitivity to photon flux during the photocleavage step. Furthermore, the presence of tryptophan at any position led to an increased formation of M/z + 26 byproducts. However, when tryptophan was excluded from the peptide sequence, higher yields were achieved in the acidolysis step (Fig. 2, X = A ≠ , routes A/C and B/C).

Having developed a highly efficient and robust C-terminal amidation protocol, we synthesized a broad range of C-terminal cysteine extended peptides to demonstrate the application of our chemistry to the production of C-terminal amides of therapeutic relevance. The photochemical amidation protocol was successfully employed in the synthesis of a GLP-1R agonist,GLP-1(7-36)[30,31] which represents an important class of therapeutic peptides that include marketed drugs exenatide[32] and lixisenatide[33] (Fig. 3). Other obesity and diabetes targets, PSTi8[34], PYY[35], QRF-amide[36], and EP45[37], a GLP-1/NPY2R dual agonist were successfully prepared in good yields using our protocol. Osteocrin[38], a potential treatment for diabetic cardiomyopathy and cancer targeting peptide LHRH[39], is also formed in high yields, highlighting that the size of the target peptide therapeutic does not affect the reaction efficiency. Antiviral drugs Bulevertide[40] (Hepcludex) and Enfuvertide[5] (Fuzeon) can also be prepared in good yields by our photochemical amidation reaction, demonstrating the utility of this transformation as a platform technology for accessing biologically important peptides across therapeutic areas. A detailed analysis of the efficiency of the overall transformation for each biologically relevant peptide in Fig. 3 can be found in Supplementary Tables 9 and 10.

We next investigated the reactivity of peptides containing backbone cysteine residues with the aim of accessing the calcitonin family[41] of peptides and other[42] pharmaceutically relevant targets containing both a C-terminal α-amide and one or more internal disulfide bonds. A modified protocol was required for the successful amidation of this substrate class, as demonstrated for a model peptide **4** (IECTKSEGC EEVYEADHGEPC-OH (Fig. 4). This peptide was globally arylated with NBD-Cl and selective functionalization of the C-terminal cysteine residue was accomplished upon irradiation at 420 nm. Dearylation of the remaining NBD-conjugated cysteine residues to the free thiols and subsequent disulfide oxidation was achieved with the addition of a solution of cysteamine and cystamine. The enamide was then cleaved under standard acidic conditions to afford the desired product **5** (IECTKSEGCEEVYEADHGEP-NH_2) in 76% yield. We attempted to apply the modified protocol to a number of biologically relevant C-terminal amide peptides containing disulfides (e.g., pramlintide). However, we found that global Cys-arylation with NBD-Cl caused significant solubility issues that affected downstream chemistry for peptides within the calcitonin family. Nevertheless, our results provide proof of concept that a modified amidation protocol can, in principle, be utilized for the generation of disulfide-containing peptides, but we caution that reaction outcomes may be affected by substrate-specific solubility of poly-arylated intermediates.

With our C-terminal amidation methodology established on the laboratory scale, we turned our attention to scaling the reaction. As the high absorbance of our substrates would make a homogenous energy distribution challenging to attain with traditional batch setups, we moved our efforts to continuous-flow photochemistry systems. The application of flow microreactors for photochemical transformations allows for uniform irradiation of the entire reaction mixture[43,44]. We chose to pilot our photoflow process with an analog of PYY, an amidated gastrointestinal hormone that is involved in the regulation of appetite and satiety[35,45]. To be consistent with industrial manufacturing practices for semi-recombinant routes to therapeutic peptides, we developed an end-to-end process for the preparation of PYY analog **7**[43] based on the production of > 4 g of recombinant starting material **6**

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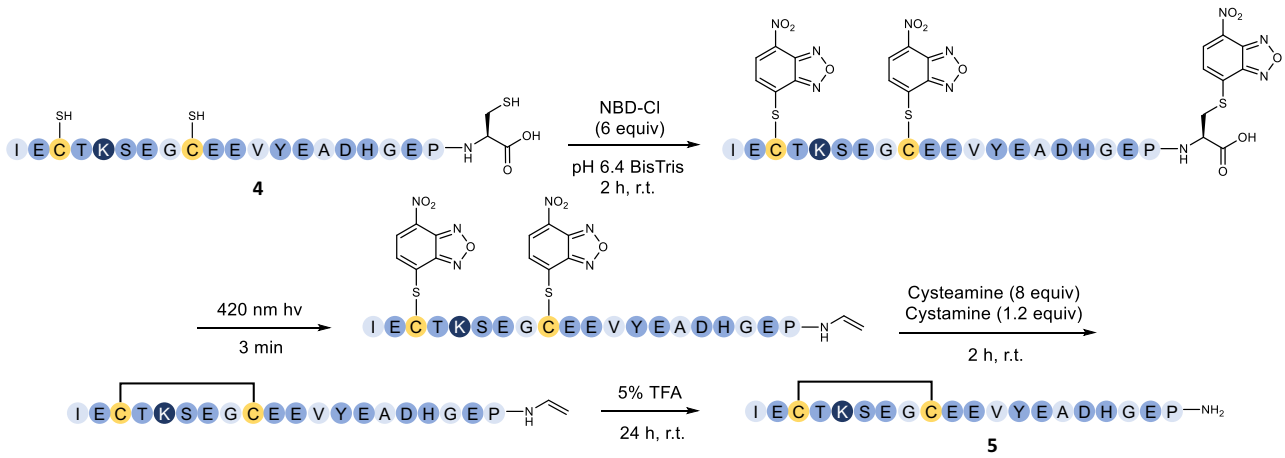

Fig. 4 | C-terminal α-amidation of a peptide containing a backbone disulfide bond. *Yield was determined by extracted ion chromatography (XIC).

(Fig. 5a). C-terminal cysteine extended **6** was reacted with NBD-Cl and subsequently subjected to photo-initiated cleavage reaction in a flow reactor. Full conversion to the enamide intermediate was achieved in a standard lab-scale photoflow system; the six-second residence time was encouraging for the prospect of large-scale production. Subsequent enzymatic digestion of the *N*-terminus and conversion of the enamide to the intended amidated product **7** was accomplished in 20% overall yield after a single final purification. Although IEDDA enamide removal had proven effective in earlier laboratory experiments, we transitioned to acidic hydrolysis for this substrate due to lower overall costs and ease of handling on scale. Phosphoric acid, in particular was identified as a suitable reagent in terms of both substrate solubility and scalable parameters such as equipment compatibility and handler safety. Finally, we measured the potency of **7** in a cell-based human Y2R assay and found that its activity was equal to that of PYY(3-36) prepared by SPPS (Supplementary Fig. 51).

As a final test of our methodology, we amidated a challenging target of therapeutic relevance that is difficult to prepare on scale using any other process. Using a setup mimicking large-scale API manufacturing, we converted 12 grams of a recombinant 81 amino acid GLP1R-amylinR co-agonist precursor peptide **8** (Fig. 5b) into the corresponding amide product (see supplementary information section VI). For this example, the previously described photo amidation protocol was carefully developed and optimized on parameters allowing for an efficient large-scale process such as volume yield (solubility/concentration), physical and chemical stability, and efficient reaction conversions (Supplementary Figs. 47–50). The recombinant starting material was made ready for processing using an optimized TCEP reduction diafiltration protocol. Subsequently, the cystenol was converted using an enhanced adaptation of the photochemical amidation protocol, at a concentration range of 7–16 mg/ml throughout the process, showcasing a high initial volume yield of the process. These concentrations, combined with sufficient stabilities and effective chemical conversions, suggest that this protocol can be adapted into a future process with an excellent space-time yield, paving the road for high-volume biopharmaceutical production. The individual chemical steps of our process (disulfide reduction, NBD arylation, in-flow photodecarboxylation, Phosphoric acid mediated enamide hydrolysis) proceeded in a rapid and clean manner, resulting in an overall yield of 78% of the target peptide **9**.

In summary, we have developed an end-to-end process for chemically converting peptides and proteins to their corresponding C-terminal α-amides. Our chemical conversion cascade is efficient, has a broad substrate scope, and can be used on synthetic or recombinantly prepared peptides. We have demonstrated that the transformation is robust on the benchtop and in a photoflow reactor, allowing for gram-scale preparation of C-terminal α-amides. The multigram production of a PYY analog and a GLP1R-amylinR co-agonist precursor peptide demonstrates the value of this technology for large-scale manufacturing of pharmaceutically relevant peptide amides, further enabling the development of this important compound class as current and future therapeutics.

Methods

Screening-scale reaction procedure for photochemical C-terminal amidation

C-terminal Cys conjugation to photo labile reagent. To minimize non-specific oxidation, an aqueous reaction buffer containing 25 mM bis-tris pH 6.4 with 50 mM glycine was degassed by bubbling nitrogen gas for 15 min. The C-terminal Cys-modified peptide is dissolved in the reaction buffer to generate a 1 mM stock solution. 25 µL of 1 mM stock is added to a well of a 96-well V-bottom assay plate, along with 65 µL of additional assay buffer. Either 2-bromomaleimide (3-Bromo-1H-pyrrole-2,5-dione, CAS: 98026-79-0) or NBD-chloride (4-chloro-7-nitrobenzofurazan, CAS: 10199-89-0) was dissolved in acetonitrile to produce a 5 mM stock solution. 10 µL of the 5 mM photo conjugate reagent is added to the well (500 µM final concentration, 2 equiv.), and allowed to incubate for 1 h at room temperature with shaking. If desired, conversion can be monitored by LC-MS.

Photochemical conversion to C-terminal N-vinyl amide. After 1 h, the plate is irradiated according to the photo aryl group utilized to generate the C-terminal N-vinyl amide. For 2-bromomaleimide, a 1.5-meter strip of 365 nm LEDs serves as the irradiation source, and the conversion proceeds for 4 h. A cooling fan is used to maintain the reaction mixture at room temperature. For NBD-chloride, irradiation can be conducted with a handheld white CFL lamp or a strip of 450 nm LEDs, and the conversion proceeds for 1 h at room temperature. If desired, conversion can be monitored by LC-MS.

Acidolysis of N-vinyl amide to C-terminal amide product. Acidolysis of the N-vinyl amide can proceed using a range of strong acids (see Supplementary Table 2). In general, the acid is added to the reaction mixture from a 10X aqueous stock solution. Trifluoroacetic acid appears to perform the fastest and was subsequently used for the majority of testing conditions. For TFA, 10 µL of a 50/50 solution of TFA/water is added to the reaction well (5% final TFA concentration), and the plate is shaken at room temperature for up to 24 h. When additives such as methionine, indole, or caffeic acid are utilized, the additive reagent is spiked from a 50X or 100X stock solution into the

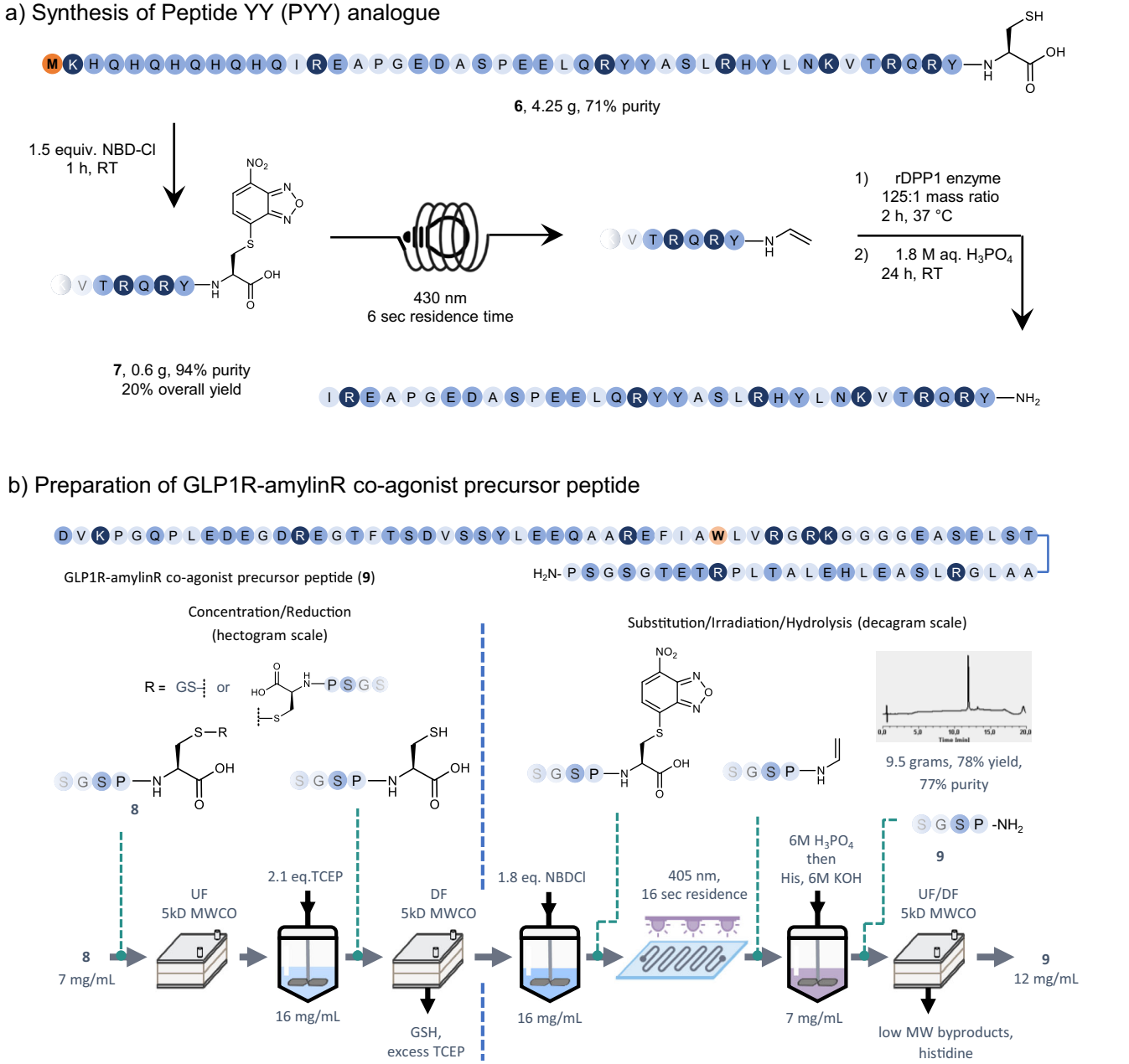

Fig. 5 | Demonstration of gram scale photo amidation using recombinant starting material. a Multi-step synthesis of peptide YY analog **7** utilizing a photoflow reactor. **b** Streamlined multigram photoamidation process of a GLP1R-amylinR co-agonist precursor mimicking biopharmaceutical manufacturing conditions. Isolated recombinant disulfide backbone **8** was converted to amide **9** using an optimized photoamidation process protocol with both high chemical yield and high-volume yield.

reaction prior to the addition of the acid. Conversion is monitored by LC-MS.

N-vinyl amide removal using inverse-electron demand Diels-Alder (IEDDA). The N-vinyl amide can also be removed using IEDDA chemistry with dipyridyl-tetrazine (3,6-Di-2-pyridyl-1,2,4,5-tetrazine, CAS: 1671-87-0). Dipyridyl-tetrazine is dissolved in a mixture of 80% acetonitrile and 20% 125 mM aq. HCl to produce a 12.5 mM stock solution (final concentration of HCl = 25 mM, 2 equiv. based on tetrazine conc.). Solubility of the dipyridyl-tetrazine is poor in acetonitrile alone and requires the addition of acid to protonate pyridyl groups and improve solubility. 10 µL of this stock solution is added to the reaction well (1.25 mM final tetrazine conc., 5 equiv. based on peptide conc.), and the reaction is incubated at 37 °C for 24 h. If desired, 1 mM methionine-HCl can be spiked into the reaction from a 100X aqueous stock solution to minimize non-specific peptide oxidation. Conversion is monitored by LC-MS.

Analysis of conversion by UPLC-MS. Reaction samples were directly analyzed by UPLC-MS analysis (Gradient: Hold at 95/5 A/B for 0.5 min, gradient from 95/5 A/B to 55/45 A/B over 7 min, followed by wash and re-equilibration). In several cases, it was not possible to achieve adequate chromatographic resolution to quantify conversion using integrated UV data at 214 nm. To quantify conversion, the calculated masses of all observed peptide starting materials, intermediates, side products, and desired products were input to generate an extracted ion chromatogram (XIC). The integrated peaks of these XICs were summed and normalized to 100%, and conversion to the desired

product was calculated based on the normalized integration. Calculated masses for each peptide species were calculated based on the most prevalent observed ion by LCMS and are provided in Supplementary Tables 11–13.

Data availability

The data generated in this study are provided in the Supplementary Information/ Source Data files. All data are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions

D.H., S.C.W., A.M., M.R.H., F.W., K.S.H, W.F.J.H., B.M.W., and A.R.M. performed and analyzed the experiments. M.R.H., D.H., K.S.H., F.W., N.C., and W.F.J.H. designed the experiments. M.R.H. conceived the project and drafted the manuscript with the help of D.H., F. W., K.S.H., W.F.J.H., and B.M.W. N.B.H. performed experiments supporting continuous-flow photochemistry. F.M. supported the design and analysis of the PYY purification. S.M. conducted experiments and analyzed data for the human PYYR2 cellular assay. All authors reviewed and edited the manuscript.

Competing interests

All authors except S. C. W. are employees and minor shareholders of Novo Nordisk A/S. D.H., F.W., W.F.J.H., S.C.W., B.M.W., N.C., A.R.M., M.R.H are co-inventors of a patent application (WO23105074A1) describing photochemically enabled amidations.

Additional information

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