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Novel electrochemically-mediated peptide dethiylation in processes relevant to native chemical ligation†

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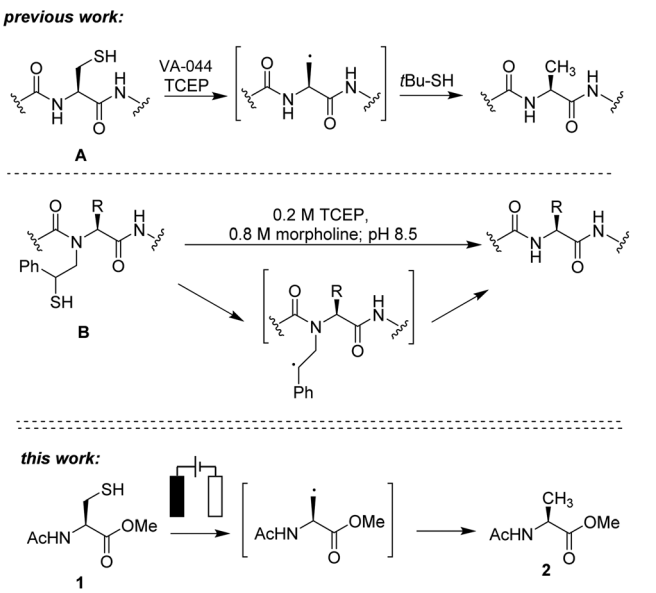
Here we explore electrochemical dethiylation in processes relevant to Native Chemical Ligation (NCL). NCL's reliance on the redox active amino acid cysteine and β-mercaptamine derivatives suggests a potential role for electrochemistry. We show that the application of a 1 V potential to platinum electrodes in 0.15 M TCEP solution is sufficient to convert Cys to Ala in cyclic peptides, and to cleave the 2-mercapto-2-phenethyl class of acyl transfer auxiliary.

Introduction

In recent years electrochemical processes have made a significant impact on organic synthesis, delivering novel, efficient, and environmentally benign reactions.[1] In the context of peptide chemistry, electrochemistry is less explored aside from well-known redox processes such as oxidation and reduction of disulfide bonds, reactions at aromatic sidechains and C-terminal decarboxylation/functionalization.[2] Owing to the heavy reliance on desulfurization chemistry in NCL-type processes, we became interested in exploring electrochemistry for this purpose.

Native chemical ligation is a powerful reaction for the synthesis of proteins,[3] and in its simplest guise unites one peptide component bearing a C-terminal thioester, with another containing an N-terminal cysteine. Occasionally a large target protein has cysteine residues suitably positioned along its backbone to allow ligation between synthetically accessible components of similar size. However, this situation is relatively rare. It can be overcome, in many cases, using synthetic β-mercapto analogues of naturally occurring amino acids,[4] and acyl transfer auxiliaries.[5] Whilst extremely powerful tools for protein total synthesis, disadvantages of these approaches include lengthy asymmetric syntheses of amino acid analogues, and acyl transfer auxiliaries often perform poorly at ligation junctions where neither amino acid (thioester, or auxiliary linked) are glycine.

A further popular way of solving this problem is to protect all naturally occurring cysteines in the protein throughout the synthesis and replace alanine, a far more frequently occurring amino acid and more likely to be suitably positioned in the sequence, with newly introduced cysteines. This allows ligations to take place under typical NCL conditions using a readily available naturally occurring amino acid. Once complete, all free cysteines are desulfurized to alanine and naturally occurring cysteine residues are then deprotected prior to protein folding. Ultimately desulfurization removes all traces of the ligation process (Scheme 1A).[6] This approach is equally suited for the



Scheme 1 Typical products of (A) NCL and (B) 2-mercapto-2-phenethyl acyl transfer auxiliary-mediated NCL, where initial desulfurization and radical formation reduces Cys to Ala and facilitates cleavage of the auxiliary. TCEP = tris-carboxyethyl phosphine. Here we explore the use of electricity for this purpose.

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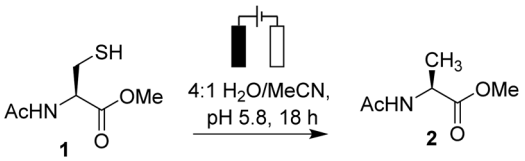
removal of the 2-mercapto-2-phenethyl class of acyl-transfer auxiliary (Scheme 1B) where desulfurization gives rise to a radical intermediate that initiates auxiliary cleavage.[6d,7]

Desulfurization often involves exposing peptides to highly flammable, noxious or malodorous chemicals and we were keen to investigate whether, using electrochemistry, we could discover a process that may remove or reduce the dependence on such reagents.

Table 1 Investigation of Ac-Cys-OMe reduction using different electrode materials[a]



Entry	Electrode material	Cell type	Potential/ V	[TCEP]/ M	Ratio **1** : **2**
1	Graphite	Divided	±1	0	1 : 0
2	Graphite	Undivided	1	0	1 : 0
3	Graphite	Undivided	1	0.2	1 : 0
4	Pt	Undivided	1	0	1 : 0
5	Pt	Divided	±1	0.05	1 : 0
6	Pt	Undivided	1	0.05	9 : 10
7	Pt	Undivided	1	0.1	6 : 13
8	**Pt**	**Undivided**	**1**	**0.15**	**0 : 1**
9	**Pt**	**Undivided**	**1**	**0.2**	**0 : 1**
10	Pt	Undivided	0	0.2	7 : 1
11	Stainless steel	Divided	+1	0	1 : 0
12	Stainless steel	Undivided	1	0	1 : 0
13	Stainless steel	Undivided	1	0.1	1 : 0
14	Cu	Divided	+1	0	1 : 0
15	Cu	Undivided	1	0	1 : 0
16	Cu	Undivided	1	0.1	1 : 0
17	Ti	Divided	+1	0	1 : 0
18	Ti	Undivided	1	0	1 : 0
19	Ti	Undivided	1	0.1	7 : 2
20	**Ti**	**Undivided**	**1**	**0.2**	**6 : 7**
21	Ti	Undivided	0	0.2	19 : 1

[a] All reactions employed a silver reference electrode and 10 mg **1** at a final concentration of 0.011 M in 4 : 1 H_2O/MeCN containing 0.1 M Na phosphate buffer; pH 5.8.

Results and discussion

We first examined reduction of available Ac-Cys-OMe (**1**) and a 1.0 V potential was employed as this extends beyond the redox potentials typically associated with oxidation and reduction of disulfide bonds. Initially, employing graphite electrodes in a divided electrochemical cell with NaCl as the electrolyte, various conditions were investigated. Notably, in an unbuffered reaction, the pH would drift over time to pH 14 leading to sample decomposition. Replacing water with 0.1 M Na phosphate buffer (pH 5.8) stabilized the reaction pH and replaced NaCl as the electrolyte but no conversion to Ac-Ala-OMe (**2**) was observed (Table 1, entries 1–3) after 18 h, prompting an investigation of alterative electrode materials (Table 1, entries 4–21). Successful desulfurization was clearly dependent on the application of the 1.0 V potential (Fig. 1).

Reaction progress was monitored by comparing the integration of CH_α protons in the crude reaction mixture (see ESI†). No reduction was observed when utilizing stainless steel or copper electrodes. Notably, in the case of platinum and titanium, reduction was taking place in the absence of the usual hydrogen donor (*t*-BuSH) and free radical initiator (VA-044). Overall, regardless of electrode material employed, or to which half-cell **1** was added to, no reduction was observed in a divided electrochemical cell. Comparing entries for platinum (8 and 10) and titanium (20 and 21), reduction was dependent on the application of a potential, with platinum proving to be the more effective material, showing complete reduction in 4 : 1 H_2O/MeCN in the presence of 0.15 M tris-carboxyethyl phosphine (TCEP). The progress of the reaction also appeared dependent on the presence of TCEP (entries 4 and 18),

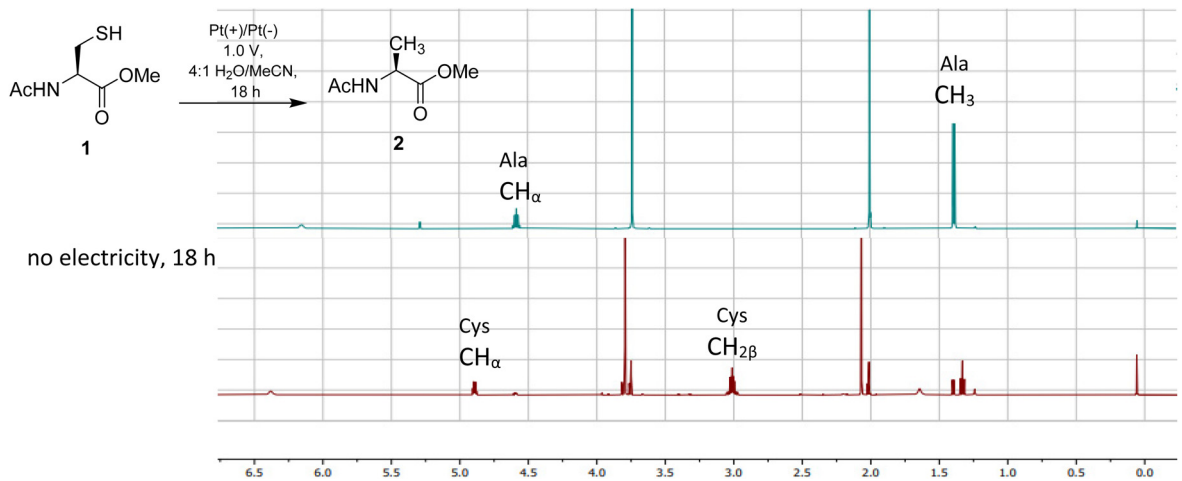


Fig. 1 ^{1}H NMR spectra of crude products obtained in the presence (upper trace) and absence (lower trace) of an applied potential. Only 5–10% conversion to the reduced product is observed in the control reactions where no electricity is applied.

although at significantly lower concentrations than those often reported in the literature (0.4–0.5 M). The reaction was performed on a larger scale such that Ac-Ala-OMe could be isolated (90% yield) and the optical rotation was determined to be −91.2, consistent with the literature value,[8] confirming that the stereochemical integrity of the starting material had been preserved. For subsequent reactions we employed platinum electrodes, a silver reference electrode, and conducted reactions in 4 : 1 H_2O/MeCN with 0.1 M and 0.15 M final concentrations of sodium phosphate buffer (pH 5.8) and TCEP respectively.

Due to an ongoing interest in peptide cyclisation *via* N → S acyl transfer we investigated desulfurization of short cyclic peptides including an Agardhipeptin A: cyclo(–His_1–Gly_2–Trp_3–Pro_4–Trp_5–Gly_6–Leu_7–)[9] analogue and crotogossamide: cyclo(–Gly_1–Ala_2–Ser_3–Gly_4–Leu_5–Asn_6–Gly_7–Ile_8–Phe_9–).[10] The parent peptide sequences were circularly permuted to afford linear precursors **3** and **4**, suitable for head-to-tail cyclisation *via* N → S acyl transfer (Fig. 2).[11] Cyclisation to afford **5** and **6** proceeded under typical reaction conditions. However **4** tended to precipitate upon heating to 60 °C, so the cyclisation was conducted in 3 M guanidine hydrochloride. Cyclic peptides **5** and **6** were isolated in yields of 55% and 57% respectively. In order to obtain a standard compound for comparison **5** was subjected to typical metal-free desulfurization reaction conditions utilizing VA-044, 0.2 M TCEP, and *t*BuSH at 37 °C. After 22 h the reduced peptide **7** was isolated in 54% yield. Next **5** and **6** were subjected to electrochemical reduction under identical conditions to reduction of **1** and the reduced peptides **7** and **8** accumulated over a period of 6 h at room temperature, and were isolated in yields of 33% and 27% respectively. Whilst yields were moderate for this class of molecule, it was encouraging that the reactions appeared to proceed smoothly, as determined by HPLC and LC-MS.

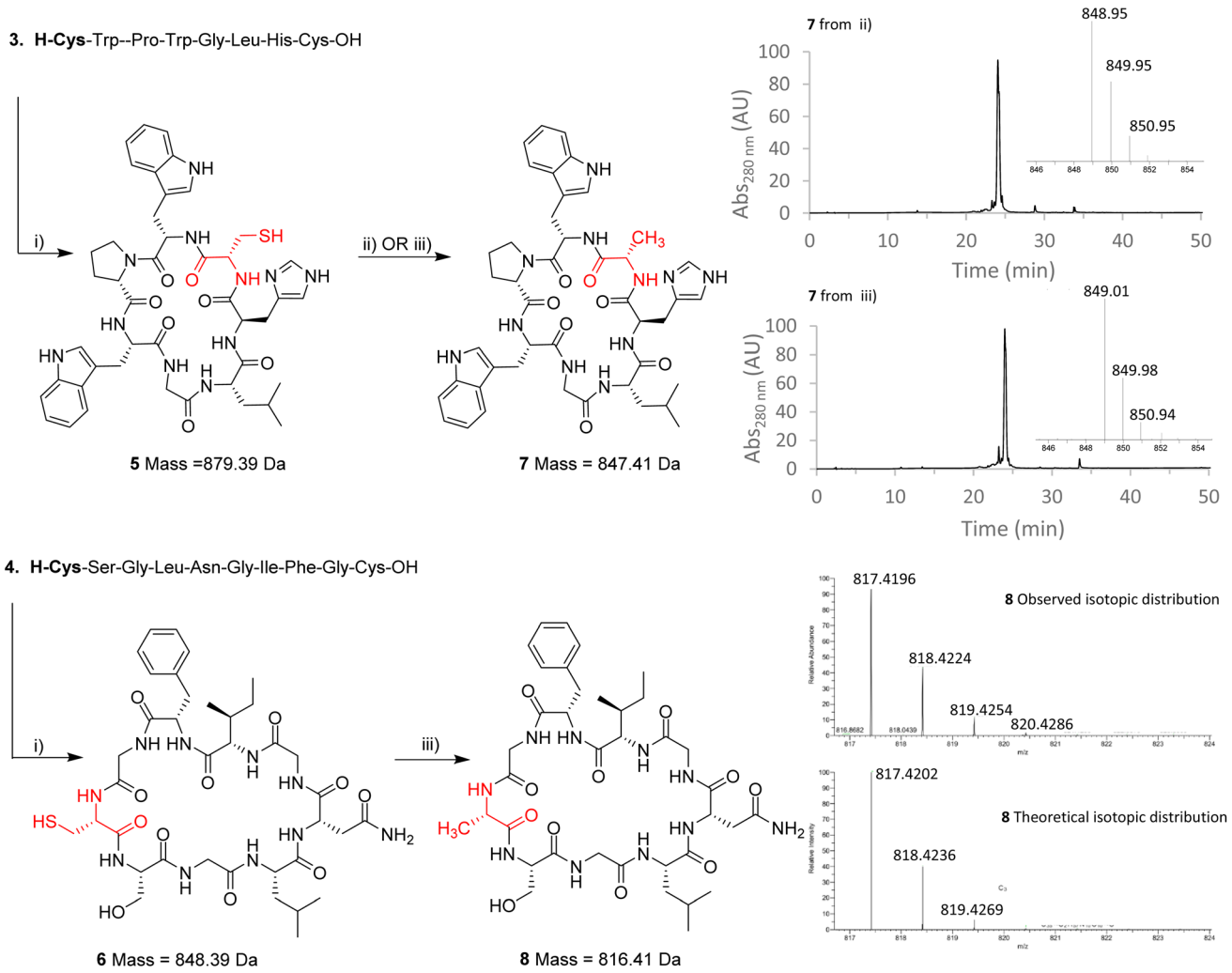
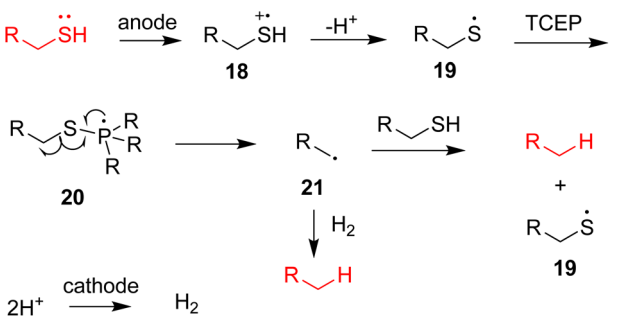


Fig. 2 Synthesis and desulfurization of cyclic peptides. *Reagents and conditions*: (i) 0.1 M Na phosphate buffer (pH 5.8), 10% w/v MESNa, 0.5% TCEP, 60 °C, 24 h. 55% (**5**), 57% (**6**) (ii) 0.2 M TCEP, *t*-BuSH, VA-044, pH 6, 37 °C, 22 h, 54%. (iii) Pt electrodes, undivided cell, 0.1 M sodium phosphate buffer; pH 5.8/MeCN (4 : 1), 0.15 M TCEP, 1.0 V, 6 h room temp. 33% (**7**), 27% (**8**). HPLC and LC-MS data confirm the identical nature of **7** produced by different methods and the identity of **8**.

However, there was some evidence of side-products accumulating over time during dethiylation of **5**. Based on significant literature precedent,[12] and the absence of this modification upon dethiylation of **6**, oxidation was presumed to have taken place on the indole ring of tryptophan. Mindful that peptide C_α-decarboxylation reactions are well known to electrochemistry and, to further explore the scope and limitations of this reaction we examined reduction of two further model peptides. The first was simply the linear precursor **3**, containing two cysteine residues. The fully-reduced peptide was isolated in 13% yield but Trp oxidation (presumed) and C_α-decarboxylation were also observed during the reaction by LC-MS. The second model peptide contained sulfur in the form of methionine in place of leucine in a further cyclic Agardhipeptin analogue. The reduced peptide was isolated in 25% yield, and once again possible oxidation of Trp was observed as the only significant byproduct (see ESI†).

Having demonstrated electrochemically induced dethiylation in peptides we were also keen to explore its application to cleavage of the 2-mercapto-2-phenethyl acyl transfer auxiliary.[8] We considered this reaction had potential to be more efficient than cysteine reduction as a consequence of the benzylic radical intermediate formed upon dethiylation. To prepare a model peptide for reaction first the auxiliary was prepared from phenyl acetic acid (**9**). In a procedure modified from the literature **9** was α-brominated using *N*-bromosuccinimide (NBS) and benzoyl peroxide to afford the α-bromo acid as the major product. The resulting mixture **9** and α-bromo acid was coupled to *N*,*O*-dimethylhydroxylamine hydrochloride to afford Weinreb amide **10**, which was isolated by column chromatography in 38% yield over two steps. The bromide was displaced by trityl thiol, and then the desired aldehyde **12** was obtained in 66% yield after reduction with $LiAlH_4$. A model peptide (sequence: GRAFS) was assembled on Rink amide resin using Fmoc-based solid phase peptide synthesis (SPPS) and the auxiliary was installed on the resin-bound peptide by reductive amination. The final auxiliary-linked peptide **13** was cleaved from the resin and obtained in 27% yield after HPLC purification. A thioester precursor peptide **14** was also readily assembled on solid support, isolated in 40% yield and converted to the 2-mercaptoethanesulfonic acid (MESNA) thioester **15** by N → S acyl transfer[11] in 22% yield (43% based on recovered starting material). **15** was ligated to **13** under typical NCL conditions and the product H–LYRAG(Aux)GRAFS–NH_2 **16** was



Scheme 2 Possible mechanism for electrochemical dethiylation.

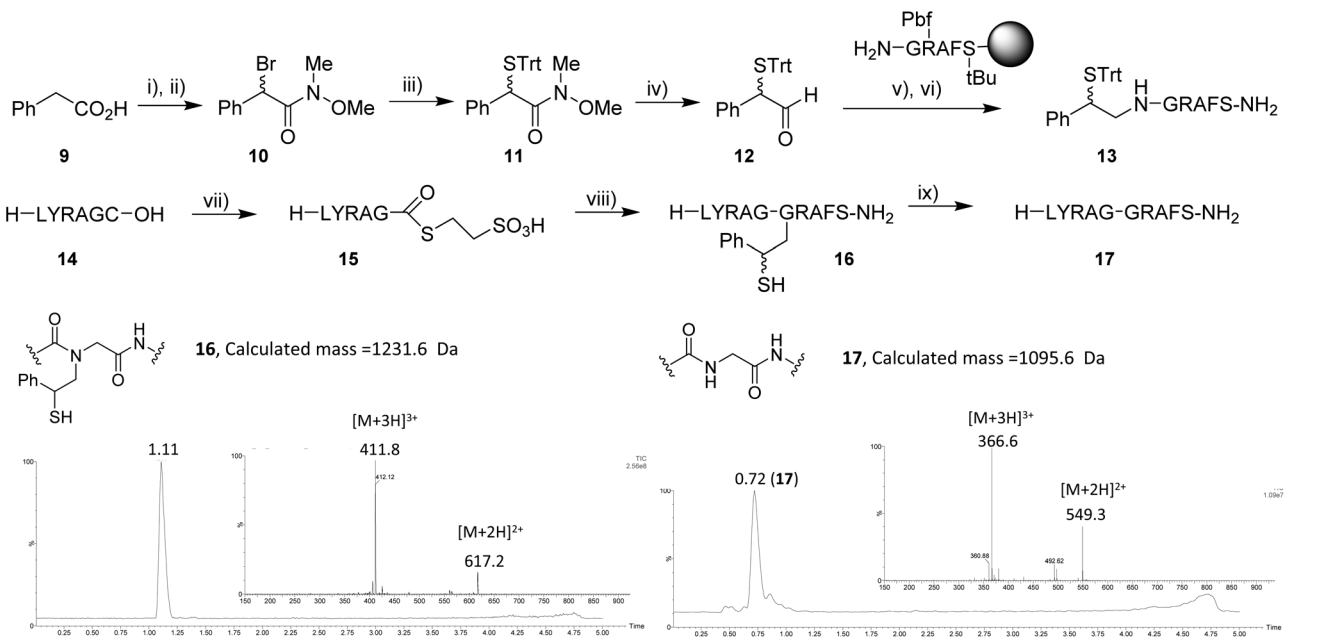


Fig. 3 Synthesis of model peptide and auxiliary cleavage investigation. *Reagents and conditions*: (i) NBS, $(PhCO_2)_2$, 1,2-DCE, reflux, 2 h. (ii) *N*,*O*-Dimethylhydroxylamine·HCl, EDCI, NEt_3, DCM, 0 °C–rt, 24 h (38% over 2 steps). (iii) TrtSH, K_2CO_3, DMF, 60 °C, 5 h, 21%. (iv) $LiAlH_4$, THF, −78 °C, 1 h, 66%. (v) Resin bound peptide, $NaCNBH_3$, 3 : 1 NMP/iPrOH, 5% AcOH, rt, 16 h. (vi) 95% TFA, 2.5% H_2O, 2.5% EDT, rt, 4 h, 27%. (vii) 0.1 M Na phosphate (pH 5.8), 10% w/v MESNA, 0.5% TCEP, 60 °C, 24 h, 22%. (viii) **13**, 50 mM sodium phosphate buffer (pH 8.0), 20 mM TCEP, 50 mM MPAA, rt, 2 h, 44%. (ix) Pt electrodes, undivided cell, 0.1 M sodium phosphate; pH 8.5/MeCN (4 : 1), 0.15 M TCEP, 1.0 V, 2 h rt, 60%.

isolated in 40% yield. Under our previously developed electrochemical conditions, we were then delighted to observe complete cleavage of the auxiliary within 2 h at room temperature to afford **17** in 60% yield (Fig. 3). The only minor byproduct observed in the reaction likely corresponded to the *N*-formylated peptide[7b] with an observed mass of 1124.5 Da (*M* +28 Da).

Conclusions

In summary, we have demonstrated a simple protocol for electrochemical dethiylation of peptides, and acyl transfer auxiliary cleavage. To the best of our knowledge this is the first time electrochemistry has been applied in this context. By reducing the number of required reagents, we have also raised the green credentials of the process. Initially we considered that application of a reducing potential may reduce the C–S bond directly. In fact, the reaction retains the need for TCEP and suggests initiation by anodic oxidation of the thiol to radical cation **18** (Scheme 2), which goes on to form sulfur centered radical **19** after deprotonation.[13] Platinum is known to demonstrate a greater tendency for one electron oxidation and radical desorption relative to other electrode materials *e.g.* carbon.[14] Interaction of the thiyl radical with TCEP to form intermediate **20**, followed by β-scission[6d] likely produces the desired carbon-centered radical which can abstract hydrogen from thiol starting material or from molecular hydrogen produced at the cathode.

Auxiliary cleavage is likely to follow a similar initial pathway to the benzylic radical. It is possible that β-scission of the benzylic radical to the amido radical precedes hydrogen abstraction. However, the likely presence of *N*-formylated material during the reaction, and in the absence of morpholine, agrees with recent mechanistic studies where the benzylic radical is first trapped by molecular oxygen prior to fragmentation.[7b]

Whilst we observe some of the known side-reactions that occur in densely functionalized peptides, further experiments employing lower potentials, rapid alternating polarity,[15] flow electrochemistry[16] and electrochemical mediators,[17] might all further increase chemoselectivity and product recovery. Interestingly, intercepting the carbon centred radical intermediates may also provide access to site specifically modified proteins. This was previously accomplished by addition of radicals to dehydroalanine (Dha)-containing peptides and proteins[18] yet this "reversed" protocol has the advantage that it can retain amino acid stereochemistry.

Experimental section

General experimental details

Chemicals were purchased from Sigma-Aldrich, Acros, Fischer Scientific and Nova Biochem, and were used without further purification. All nuclear magnetic resonance (NMR) experiments were recorded at room temperature on a Bruker AMX 600 MHz instrument. Preparative reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Dionex Ultimate 3000 system equipped with a Phenomenex Jupiter 10μ Proteo 90A, C_{12}, 250 × 21.2 mm column. A mobile phase of 0.1% TFA (v/v) in water (solvent A)/acetonitrile (solvent B) over a 5–60% acetonitrile gradient over 60 min, and were monitored at wavelengths 230 nm, 254 nm, and 280 nm. Analytical reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Dionex Ultimate 3000 equipped with a Phenomenex SphereClone 5μ ODS, C_{18} 250 × 4.6 mm column. Separations involved a mobile phase of 0.1% TFA (v/v) in water (solvent A)/acetonitrile (solvent B) over a 5–95% acetonitrile gradient, and were monitored at wavelengths 230 nm, 254 nm, and 280 nm. Analytical LC-MS was carried out on Waters uPLC/SQD-LC mass spectrometer instrument equipped with a C_{18}, 2.1 × 50 mm column. Separations were conducted with a linear gradient of 5–95% acetonitrile containing 0.1% formic acid over 10 min using a flow rate of 0.6 ml min^{-1}. High resolution mass spectrometry was obtained from a Q-Exactive Orbitrap mass spectrometer.

Electrochemical desulfurization of *N*-acetylcysteine methyl ester

N-Acetyl-L-cysteine methyl ester **1** (25 mg, 0.14 mmol) was dissolved in 4 mL of a solution comprised of 0.15 M TCEP (adjusted to pH 5.8 with 2 M NaOH) and 0.1 M pH 5.8 sodium phosphate buffer. Acetonitrile (1.0 mL) was added to ensure **1** was fully dissolved. The solution was transferred to a 25 mL three necked flask and platinum wire electrodes submerged 1 cm into the solution. A silver reference electrode was also inserted into the middle neck of the flask and all electrodes were connected to a Ivium Technologies Vertex model potentiostat operating in chronoamperometry mode. The solution was stirred vigorously at room temperature and a constant potential of 1.0 V was supplied. After 18 hours the electrical current was disconnected and the solution extracted with DCM (3× 5 mL). The organic fractions were combined, dried over $MgSO_4$, and concentrated under vacuum to produce a grey solid of *N*-acetyl-L-alanine methyl ester (18.5 mg, 0.13 mmol, 90%). **^{1}H NMR** (600 MHz, $CDCl_3$) δ_H/ppm 6.28 (1H, bs, NH), 4.56 (1H, quint, J = 6.8 Hz, MeC*H*), 3.72 (3H, s, OCH_3), 1.99 (3H, s, $NCOCH_3$), 1.37 (3H, d, J = 7.0 Hz, $CHCH_3$). **^{13}C NMR** (400 MHz, $CDCl_3$) δ_C/ppm 173.8, 169.7, 52.6, 48.1, 23.3, 18.6. $[\alpha]_D^{298K}$ = −91.2 deg cm^3 g^{-1} dm^{-1} (c = 1.0 g dcl^{-1} in H_2O).[9] **ESI-MS**: calculated m/z for $C_6H_{11}NO_3$ $[M]^+$: 145.07, observed $[M + Na]^+$ 168.20.

General peptide synthesis procedure

Model peptides were synthesised on a 0.05 mmol scale using either Novasyn TGT resin pre-loaded with Fmoc–Cys(Trt)–OH or Rink amide MBHA resin, and an ABI 433A automated peptide synthesiser following the Fastmoc protocol: 10 equivalents of Fmoc–Xaa–OH, HBTU/HOBt as the coupling reagents and DIPEA as base. After peptide synthesis the dry resin was suspended in a trifluoroacetic acid cleavage cocktail (5 mL, 95% TFA, 2.5% EDT, 2.5% water) then stirred for 4 h at room

temperature. The solution was then filtered to obtain a yellow solution to which diethyl ether (20 mL) was added to induce peptide precipitation. The sample was then centrifuged at 3500 rpm (4 °C) for 15 minutes and the supernatant discarded. The pellet was washed with diethyl ether (20 mL) then centrifuged again at 3500 rpm (4 °C). The supernatant was again discarded and the precipitated peptide dissolved in distilled water and acetonitrile (5 mL, 5–10% MeCN as required for solubility) then purified by preparative HPLC. The eluted fractions containing the peptide (determined by UV absorption and LC-MS) were combined and the acetonitrile removed *in vacuo*. Lyophilization produced a white fluffy solid.

Linear peptide 3. Linear peptide **3** (H–Cys–Trp–Pro–Trp–Gly–Leu–His–Cys–OH) was synthesised *via* our general solid phase peptide synthesis procedure, purified *via* preparative HPLC and lyophilized to produce a white fluffy solid (15 mg, 30%), t_R = 36.6 min. **ESI-MS**: calculated *m*/*z* for $C_{47}H_{60}N_{12}O_9S_2$ $[M]^+$: 1000.40, observed $[M + H]^+$ 1001.51.

Linear peptide 4. Linear peptide **4** (H–Cys–Ser–Gly–Leu–Asn–Gly–Ile–Phe–Gly–Cys–OH) was synthesised *via* our general solid phase peptide synthesis procedure, purified *via* preparative HPLC and lyophilized to produce a white fluffy solid **4**: (19.6 mg, 40%), t_R = 26.8 min. **ESI-MS**: calculated *m*/*z* for $C_{40}H_{63}N_{11}O_{13}S_2$ $[M]^+$ 969.40, observed $[M + H]^+$ 970.7.

Synthesis of cyclic peptide 5 *via* N → S acyl transfer. Peptide **3** (12.9 mg, 1.3 μmol) was dissolved to a concentration of 1 mg mL^{-1} in 0.1 M sodium phosphate buffer (pH 5.8) containing MESNa (10%, w/v) and TCEP (0.5%, w/v). The mixture was vortexed in a 15 mL Falcon tube and divided into Eppendorf tubes and heated in an Eppendorf thermomixer (750 rpm) for 24 h at 60 °C. After this time LC-MS confirmed the reaction was complete and the product was purified *via* semi-preparative HPLC. Lyophilization produced a fluffy white powder of peptide **5** (6.2 mg, 7.05 μmol, 55%), t_R = 39.8 min. **ESI-MS**: calculated *m*/*z* for $C_{44}H_{53}N_{11}O_7S$ $[M]^+$ 879.32, observed $[M + H]^+$ 880.93.

Chemical desulfurization of cyclic peptide 5. Peptide **5** (1 mg, 1.14 μmol) was dissolved in distilled water (200 μL) with 0.5 M TCEP·HCl solution (200 μL, neutralised to pH 6 with NEt_3), ^{t}BuSH (20 μL, 0.2 mmol) and 0.1 M VA-044 solution (10 μL) added. The mixture was vortexed then placed on a heated shaker at 37 °C. After 22 h, LC-MS showed no starting material remained and the mixture was centrifuged then purified *via* semi-preparative HPLC. Lyophilization resulted in a white powder of the desulfurized peptide **7** (0.5 mg, 54%). t_R = 38.8 min. **ESI-MS**: calculated *m*/*z* for $C_{44}H_{53}N_{11}O_7$ $[M]^+$ 847.41, observed $[M + H]^+$ 848.93.

Synthesis of cyclic peptide 6 *via* N → S acyl transfer. Synthesis of cyclic peptide **6** *via* N → S acyl transfer was conducted as above for cyclic peptide **5** except the reaction contained guanidinium hydrochloride at a final concentration of 3 M. The product, obtained from 9 mg (9.28 μmol) linear starting material, was purified *via* semi-preparative HPLC. Lyophilization produced a fluffy white powder of peptide **6** (4.5 mg, 57%), t_R = 35.3 min. **ESI-MS**: calculated *m*/*z* for $C_{37}H_{56}N_{10}O_{11}S$ $[M]^+$ 848.39, observed $[M + H]^+$ 849.80.

Electrochemical desulfurization of model peptide 5 was conducted as for Ac-Cys-OMe. 5 (5 mg, nmol) was dissolved to a concentration of 1 mg mL^{-1} in a buffered TCEP solution comprised of 0.15 M TCEP and 0.1 M pH 5.8 sodium phosphate buffer. To this a small volume of acetonitrile was added (1 mL) to ensure the peptide was fully dissolved. The solution was transferred to a 25 mL three necked flask and platinum wire electrodes submerged 1 cm into the solution. A silver reference electrode was also inserted into the middle neck of the flask and all electrodes were connected to an Ivium Technologies Vertex model potentiostat operating in chronoamperometry mode. The solution was stirred vigorously at room temperature and a constant potential of 1.0 V was supplied.

Desulfurization of **5** was complete after 6 h where LC-MS confirmed full consumption of the starting material and the electrical current was disconnected. The desulfurized peptide was purified directly from the solution *via* preparative HPLC and the eluted fractions freeze-dried to produce **7** (1.6 mg, 33%) as a fluffy white solid. **ESI-HRMS**: calculated *m*/*z* for $C_{44}H_{54}N_{11}O_7S$ $[M + H]^+$ 848.4208, observed $[M + H]^+$ 848.4200.

Electrochemical desulfurization of 6. 6 (4.2 mg, 4.9 μmol) was desulfurized as described above for **5** except, due to the poor water solubility of **6**, the reaction was conducted in 50% v/v MeCN. The product was purified directly from the solution *via* preparative HPLC and the eluted fractions freeze-dried to produce crotogossamide **8** (1.1 mg, 27%) as a fluffy white solid. **ESI-MS**: calculated *m*/*z* for $C_{37}H_{56}N_{10}O_{11}$ $[M]^+$ 816.41, observed $[M + H]^+$ 817.80.

Synthesis of the 2-mercapto-2-phenethyl auxiliary. The auxiliary was prepared in 4 steps using a route adapted significantly from ref. 7*a*. Benzoyl peroxide (0.45 g, 1.85 mmol) was added to a solution of phenyl acetic acid, **9** (5.0 g, 36.7 mmol) and NBS (7.26 g, 40.8 mmol) in 1,2-dichloroethane (60 ml). The mixture was heated under reflux for 2 h then allowed to cool to room temperature. The mixture was diluted with *n*-hexane (60 mL) and filtered through a Celite pad under vacuum. The filtrate was concentrated under vacuum and the resulting yellow oil dissolved in DCM (100 mL) the solution was cooled to 0 °C in an ice bath and *N*,*O*-dimethylhydroxylamine hydrochloride (2.07 g, 21.2 mmol), EDC (5.04 g, 26.3 mmol) and NEt_3 (1.82 mL, 25.3 mmol) were added. The solution was then warmed to room temperature and stirred for 24 h. After this time the mixture was washed with aqueous HCl (1 M, 3× 50 mL), saturated aqueous sodium bicarbonate (2× 50 mL) and saturated NaCl (1× 50 mL) then dried over $MgSO_4$. The crude product was concentrated under vacuum and purified *via* flash chromatography over silica with petroleum ether/ethyl acetate (4 : 1) as eluent to afford the Weinreb amide **10** as a pale-yellow oil (3.64 g, 14.1 mmol, 38% over 2 steps). **^{1}H NMR** (600 MHz, $CDCl_3$) δ_H/ppm 7.56 (1H, d, *J* = 8 Hz, ArH), 7.50 (1H, d, *J* = 8 Hz, ArH), 7.38–7.29 (3H, m, ArH), 5.99 (1H, s, BrCH), 3.57 (3H, s, $NOCH_3$), 3.21 (3H, s, NCH_3). **^{13}C NMR** (126 MHz, $CDCl_3$) δ_C/ppm 171.0, 136.7, 129.1, 129.0, 128.8, 128.4, 60.6, 35.3, 34.1. **ESI-MS**: calculated *m*/*z* for $C_{10}H_{12}BrNO_2$ $[M]^+$ 257.01, observed $[M + H]^+$ 258.39.

Bromide **10** (3.64 g, 14.1 mmol), trityl thiol (8.96 g, 32.4 mmol) and potassium carbonate (4.48 g, 32.4 mmol) were dissolved in dry DMF (30 mL) and stirred at 60 °C for 5 h. The reaction mixture was allowed to cool to room temperature and diluted with DCM (100 mL) and water (100 mL), then the organic phase separated. The water was extracted twice more with DCM (2× 50 mL) and the combined organic layers washed with water (4× 50 mL) and saturated NaCl (50 mL). The solution was then dried over $MgSO_4$, filtered, and concentration under vacuum gave the crude product as a yellow powder. This was purified *via* flash chromatography over silica with 4 : 1 pet. ether/EtOAc as the eluant to produce a yellow solid which was washed with diethyl ether and dried under vacuum, yielding **11** as a fluffy white powder (1.32 g, 2.91 mmol, 21%). **^{1}H NMR** (600 MHz, $CDCl_3$) δ_H/ppm 7.43–7.39 (m, 5H, ArH), 7.25–7.16 (m, 13H, ArH), 7.10–7.06 (m, 2H, ArH), 4.58 (bs, 1H, TrtSCH), 3.15 (bs, 3H, OCH_3), 2.97 (bs, 3H, NCH_3). **^{13}C NMR** (126 MHz, $CDCl_3$) δ_C/ppm 171.5, 144.5, 130.1, 129.4, 128.6, 128.5, 127.9, 127.5, 127.5, 126.8, 69.5, 61.2, 50.1, 32.5. **ESI-MS**: calculated *m*/*z* for $C_{29}H_{27}NO_2S$ $[M]^+$ 453.18, observed $[M + H]^+$ 454.31.

11 (1.32 g, 2.91 mmol) was dissolved in dry THF (50 mL) under nitrogen. The solution was then cooled to −78 °C in an acetone-dry ice bath. $LiAlH_4$ (1 M in THF, 3.5 mL, 3.5 mmol) was added dropwise over 5 min and the mixture stirred for 60 min. After this time $KHSO_4$ (4–5 drops, 5% w/v) was added dropwise and the mixture allowed to warm to room temperature. The mixture was diluted with DCM (100 mL) and washed with aqueous $KHSO_4$ (2× 100 mL, 5% w/v), dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified *via* flash chromatography with petroleum ether/ethyl acetate (1 : 2) as the eluant, to afford aldehyde **12** as a viscous yellow oil (756 mg, 1.92 mmol, 66%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ_H/ppm 9.01 (d, 1H, J = 3.4 Hz, CHO), 7.45–7.41 (m, 5H, ArH), 7.34–7.25 (m, 10H, ArH), 7.24–7.20 (m, 3H, ArH), 7.12–7.10 (m, 2H, ArH), 4.00 (d, 1H, J = 3.1 Hz, TrtSCH). **^{13}C NMR** (126 MHz, $CDCl_3$) 193.8, 147.0, 144.2, 134.8, 132.6, 130.2, 130.0, 129.9, 129.8, 129.6, 128.4, 128.3, 128.2, 128.1, 128.1, 127.9, 127.5, 127.4, 127.3, 127.2, 127.1, 69.5, 58.7. **ESI-MS**: calculated *m*/*z* for $C_{27}H_{22}OS$ $[M]^+$ 394.14, observed $[M + H]^+$ 395.15.

Linear peptide Aux–GRAFS–NH_2 (13). Linear peptide Aux–GRAFS–NH_2 (**13**) was synthesised on 0.05 mmol scale on Rink amide MBHA resin, initially *via* our general solid phase peptide synthesis procedure. Once complete to the terminal Gly the Fmoc was removed and aldehyde **12** (200 mg, 0.5 mmol) was dissolved in 2.0 mL 3 : 1 NMP/iPrOH containing 5% glacial AcOH. This solution was added to the resin followed by $NaCNBH_3$ (31.4 mg, 0.5 mmol) and the reaction was shaken at room temperature for 16 h. The reaction was drained, the resin was washed, and the peptide cleaved as described above and purified *via* preparative HPLC and lyophilized to produce a white fluffy solid (8.9 mg, 27%), t_R = 24.2 min. **ESI-MS**: calculated *m*/*z* for $C_{31}H_{45}N_9O_6S$ $[M]^+$: 671.3, observed $[M + H]^+$ 672.61.

Peptide H–LYRAGC–OH (14). Peptide H–LYRAGC–OH (**14**) was synthesised *via* our general solid phase peptide synthesis procedure, purified *via* preparative HPLC and lyophilized to produce a white fluffy solid (13.6 mg, 40%), t_R = 19.3 min. **ESI-MS**: calculated *m*/*z* for $C_{29}H_{47}N_9O_8S$ $[M]^+$: 681.33, observed $[M + H]^+$ 682.67.

Synthesis of thioester 15 *via* N → S acyl transfer. Synthesis of thioester **15** *via* N → S acyl transfer was conducted as above for cyclic peptide **5**. The product, obtained from 13 mg (0.0191 mmol) **15**, was purified *via* semi-preparative HPLC and lyophilization to produce a fluffy white powder (3.0 mg, 22%, 43% based on recovered starting material). t_R = 18.6 min. **ESI-MS**: calculated *m*/*z* for $C_{28}H_{46}N_8O_9S_2$ $[M]^+$ 702.28, observed $[M + H]^+$ 703.69.

Auxiliary mediated ligation. (3 mg, 4.47 μmol) Aux–GRAFS–NH (**13**) was dissolved in sodium phosphate buffer (50 mM, pH 8.0, 1 ml). This solution was used do dissolve lyophilised H–LYRAG–$SCH_2CH_2SO_3H$ (**15**, 2.6 mg, 3.7 μmol). TCEP (0.4 M) and MPAA (0.2 M) were added to final concentration of 20 mM and 50 mM respectively. The reaction was shaken at room temperature for 2 h, after which time LC-MS indicated that the reaction was complete. The product was isolated by semi-preparative HPLC and lyophilization to afford a white fluffy solid (2 mg, 44%), t_R = 25.9 min. **ESI-MS**: calculated *m*/*z* for $C_{57}H_{85}N_{17}O_{12}S$ $[M]^+$ 1231.6, observed $[M + 2H]^{2+}$ 617.2 and $[M + 3H]^{3+}$ 411.83.

Auxiliary cleavage. Auxiliary cleavage from **16** (2 mg, 1.6 μmol) to form **17** was conducted as for dethiylation of **5**, except at pH 8.5, and was complete after 2 h, where LC-MS confirmed full consumption of the starting material. The electrical current was disconnected and H–LYRAGGRAFS–NH_2 (**17**) was purified directly from the solution *via* preparative HPLC and the eluted fractions freeze-dried to produce **17** (1 mg, 60%) as a fluffy white solid. **ESI-HRMS**: calculated *m*/*z* for $C_{49}H_{78}N_{17}O_{12}$ $[M + H]^+$ 1096.6010, observed $[M + H]^+$ 1096.6019.

Conflicts of interest

There are no conflicts to declare.

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