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Depsipeptide substrates for sortase-mediated N-terminal protein ligation

Daniel J. Williamson, Michael E. Webb & W. Bruce Turnbull

School of Chemistry and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, UK. Correspondence should be addressed to M.E.W (m.e.webb@leeds.ac.uk) or W.B.T. (w.b.turnbull@leeds.ac.uk).

ABSTRACT

Technologies that allow the efficient chemical modification of proteins under mild conditions are widely sought after. Sortase-mediated peptide ligation provides a strategy for modifying the N- or C-termini of proteins. This protocol describes the use of depsipeptide substrates (containing an ester linkage) for sortase A (SrtA) to completely modify proteins carrying a single N-terminal glycine residue under mild conditions in 4-6 hours. The SrtA-mediated ligation reaction is reversible, so most labelling protocols using this enzyme require a large excess of both substrate and sortase to produce high yields of ligation product. In contrast, switching to depsipeptide substrates effectively renders the reaction irreversible allowing complete labelling of proteins with a small excess of substrate and catalytic quantities of sortase. Herein, we describe the synthesis of depsipeptide substrates that contain an ester linkage between a threonine and glycolic acid residue and an N-terminal FITC fluorophore appended via a thiourea linkage.

Ontology

Biological sciences / Biological techniques / Synthetic chemistry / Enzyme-catalysed reactions Biological sciences, Chemical biology, Chemical modification

Biological sciences / Systems biology / Protein engineering

Categories

Chemical modification

Solid phase peptide synthesis

Keywords

Sortase, transpeptidation-reaction, protein labeling, protein conjugation, N-terminal labeling, selective labeling, solid phase peptide synthesis, sortagging reaction, transacylation, peptide

synthesis, solid phase peptide synthesis, depsipeptide, Fmoc-threonine, alkylation, hydrogenolysis, FITC labelling, isothiocyanate labelling

INTRODUCTION

Chemical modification of proteins is widely used to optimise the properties of biopharmaceuticals and also provides chemical biologists with powerful strategies with which to study the molecular basis for life. It is essential that protein modification strategies are robust and efficient. Common strategies including: alkyne-azide cycloadditions[1,2], Staudinger ligation[3,4], maleimide conjugation etc., and all have associated challenges and limitations. For example, the necessity to incorporate non-natural amino acid residues into the protein to be labelled[5], the non-trivial synthesis of labelling reagents; risk of non-specific modification to the protein[6], and typically a requirement for a large excesses of reagents to ensure successful couplings. Herein, we report an optimised protocol that utilises Sortase and depsipeptide substrates to allow the efficient modification of proteins.

Sortases are a class of transpeptidase enzymes that are responsible for "sorting" and covalently anchoring virulence factors to the cell wall of gram-postive bacteria[7,8]. Sortase A (SrtA) is a type II membrane protein, native to Staphylococcus aureus, that ligates proteins carrying an LPXTG recognition motif to peptidoglycan substrates harbouring an N-terminal oligoglycine sequence[9]. The catalytic cysteine residue located in the active site of SrtA cleaves the amide bond between the threonine and glycine residues of the LPXTG substrate to form a thioacyl-enzyme intermediate[10]. The intermediate is then attacked by the oligoglycine substrate to form a new ligated product.

The advantage of using SrtA as a tool for protein modification became apparent once the catalytic core of the enzyme had been heterologously expressed nearly 15 years ago[7,9]. Since then the enzyme has been used extensively to modify proteins at both the N- and C-termini with a large variety of different labels[11,12]; including, biotin,[13] GPI mimics[14], fluorescent tags,[13] PEG chains[15] and other biologically important molecules [16,17]. The vast majority of examples of sortase-mediated ligation have used synthetic N-terminal oligoglycine peptides to label proteins harbouring a flexible C-terminal LPXTG motif. Alternatively, this approach can be reversed to allow the modification of proteins carrying an N-terminal oligoglycine sequence under the same mild conditions[18,19]. However, as the ligation reaction is reversible, a large excess of labelling reagent is normally required to achieve high levels of protein modification, which may not always be desirable or possible. The reaction can be rendered effectively irreversible if the scissile peptide bond (i.e. the amide bond between the threonine and glycine residues) is replaced by an ester linkage (Figure 1A).[20] In this regard, a methyl ester of the desired peptide can be used as a substrate for the N-terminal protein ligation; however, a high concentration of SrtA and a large excess of methyl ester labelling reagent are required to achieve a high ligation yield.

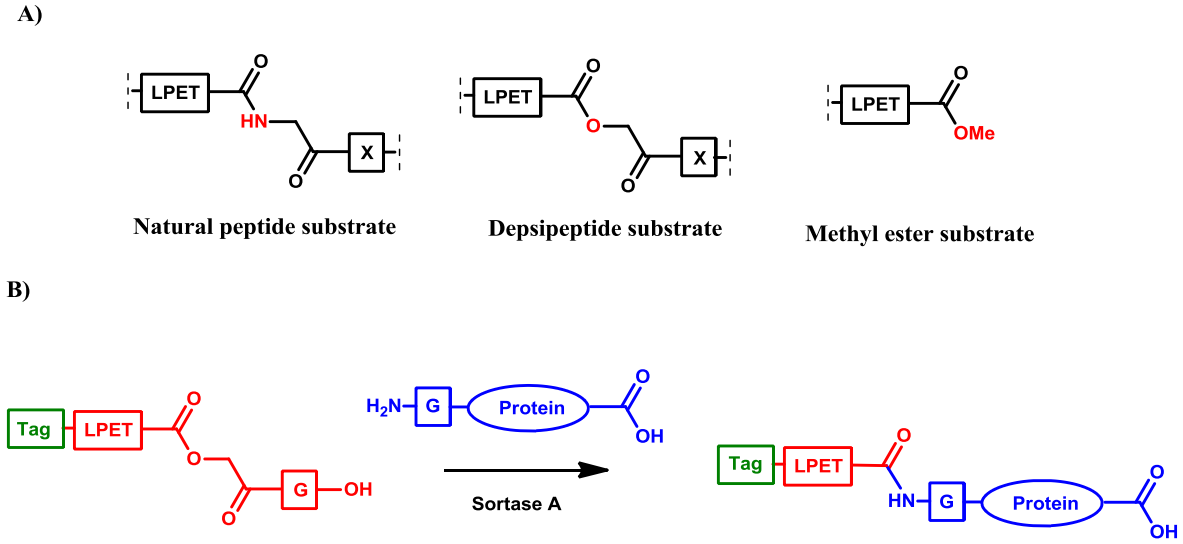

Figure 1: A) SrtA substrate variants. X = Any amino acid. B) SrtA-mediated modification of proteins using depsipeptide substrates.

In our laboratory, we have found that depsipeptide substrates can provide a more efficient approach to achieve quantitative labelling of proteins carrying a single N-terminal glycine residue (Figure 1B)[21]. The efficiency of the SrtA-mediated ligation reaction is significantly improved when using depsipeptide substrates such that typically only 1.5-3 molar equivalents of label and 0.1-0.2 molar equivalents of SrtA are required to completely modify proteins. Conversely, protocols for N-terminal labelling with unmodified peptides stipulate 20-50 equivalents of peptides and 2-3 equivalents of SrtA[22]. If the label is precious and/or difficult to acquire, this ability to maximise the yield of protein modification is highly desirable.

SrtA-mediated modification of proteins using:	Advantages	Limitations	References
Peptide substrates	Mild reaction conditions Site specific modification of N-terminal glycine residues Simple SPPS of labels	Large excess of label required for high level of protein modification High number of molar equivalents of SrtA required	22
Depsipeptide substrates	Mild reaction conditions Site specific modification of N-terminal glycine residues Small excess of label required for high level of protein modification Substoichiometric molar equivalents of SrtA required	Solution phase synthesis of depsipeptide precursors Depsipeptide susceptible to non-enzymatic hydrolysis	21

Synthesis of the depsipeptide amino acid

The protected depsipeptide amino acid is synthesised in two solution-phase steps in high yield (Figure 2): alkylation of a commercially available Fmoc-threonine derivative followed by hydrogenolysis of the C-terminal benzyl ester group. Although both synthetic steps are relatively simple, the hydrogenolysis of the benzyl protecting group requires careful monitoring. The resulting depsipeptide amino acid can be incorporated into a growing peptide chain using standard solid phase peptide synthesis (SPPS) methodologies to produce depsipeptide substrates for SrtA (Figure 2). Resins having 2-chlorotrityl or Rink amide linkers both provide high yields of depsipeptides[21].

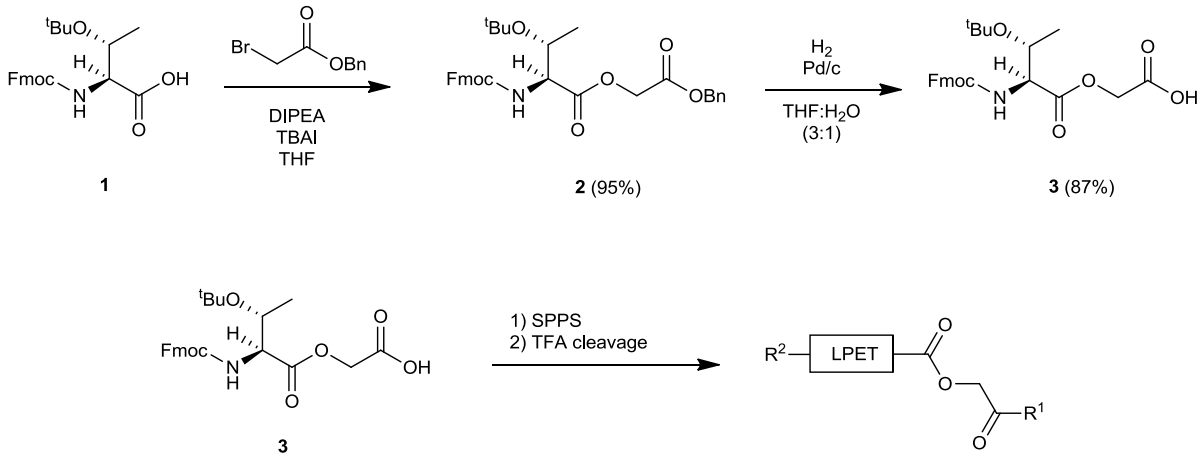

Figure 2: Synthetic route to depsipeptide substrates via Fmoc depsipeptide building block **3**. R^1, R^2 = any amino acid; DIPEA = diisopropylethylamine; TBAI = tetrabutylammonium iodide; THF = tetrahydrofuran; SPPS = solid phase peptide synthesis; TFA = trifluoroacetic acid.

We describe the synthesis on a 10 g scale, but there is no practical reason why this could not be scaled up or down by a factor of 2-5. The reaction thermodynamics have not however been thoroughly investigated so we would not recommend that larger scale reactions are attempted by non-specialists.

This synthetic strategy could feasibly be used to link any commercially available Fmoc-protected amino acid with glycolic acid to form a range of depsipeptide substrates. This was first demonstrated by Suich et al.[23] to make an Fmoc-Tyr(OtBu)-Gc-OH amino acid. Replacement of the glycine-mimicking analogue, glycolic acid is also feasible but would be dependent upon the availability of suitably-protected enantiomerically-enriched α-bromoalkanoic acids.

Depsipeptide incorporation into the peptide

The use of SPPS to prepare the depsipeptide substrate enables easy incorporation of a wide range of labels (e.g., fluorophores, affinity tags, bioorthogonal reactive groups), and, in general, the resulting structures are limited only by the availability of SPPS-compatible building blocks. The depsipeptide substrates can then be used directly to transform proteins carrying an N-terminal glycine residue under mild aqueous conditions within 4-6 hours.

Protein labeling

While only one glycine residue is necessary for ligation to occur successfully, it is essential that the glycine residue is sterically unhindered; typically a spacer of 3-4 amino acid residues from a globular domain is sufficient to allow ligation to occur. While a competing hydrolysis reaction may occur upon extended incubation with sortase, high ligation yields can usually be achieved with substrate protein concentrations >50 μM and 1.5-3 equivalents of depsipeptide label. Incorporation of an N-terminal glycine residue into a protein can be achieved in a number of different ways. For example, proteins are always expressed with either a methionine or an N-formylmethionine at their N-terminus, which is usually removed in vivo. In principle, a given protein's gene sequence could be modified to incorporate a glycine as the second N-terminal amino acid, which would be exposed after the methionine is lost. Alternatively, periplasmic targeting sequences can be designed to leave an N-terminal glycine residue once the sequence has been removed during co-translational protein export into the periplasm[24]. A third in vitro approach would be to engineer a suitable protease (e.g. TEV or Factor Xa) recognition sequence immediately before a glycine residue that would allow the release of a protein carrying an N-terminal glycine residue once the cleavage event has taken place.

In this protocol we describe detailed conditions for the synthesis of the required depsipeptide building block, the synthesis of an example labelling peptide using an N-terminal thiourea, and an example of the labelling of a galactose-binding variant of mannose-binding protein (gMBP). These protocols are freely adaptable for the preparation and labelling of any protein containing an N-terminal glycine and with essentially any material which can be chemically conjugated to the labelling peptide.

MATERIALS

CRITICAL All solvents used for reactions should be of the highest quality possible. Solvents for extractions and reaction workup should be at least reagent grade. All manipulations should be carried out in a fume hood. Solid and liquid waste generated must be disposed of in accordance with local environmental regulations.

REAGENTS:

- Fmoc-amino acids (Sigma Aldrich, VWR International or Cambridge Bioscience)
- Gly-2-Cl-trityl resin (Sigma, 92681-1G-F)
- O-(6-Chlorobenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HCTU) (Sigma Aldrich, 04936-25G-F)
- N,N-Diisopropylethylamine (DIPEA) (Sigma Aldrich, 550043-100ML)
- Fluorescein isothiocyanate isomer I (FITC) (Sigma Aldrich, F7250-1G)
- Trifluoroacetic acid (TFA) (Sigma Aldrich T6508-500ML)
- Triisopropylsilane (TIS) (Sigma Aldrich, 233781-10G)
- Benzyl 2-bromoacetate (Sigma Aldrich, 245631-50G)

- Tetrabutylammonium iodide (Sigma Aldrich, 86890-25G)
- Piperidine (Sigma Aldrich, 104094-2.5L)
- Triethylamine (Sigma Aldrich, 471283-100ML)
- Palladium on carbon (10 wt%) (Sigma Aldrich, 520888-10G)
- DMF (Sigma Aldrich,33120-2.5L)
- MeOH (Sigma Aldrich,33213-2.5L)
- THF(Sigma Aldrich, 34865-2.5L)
- EtOAc (VWR International, 23880.324)
- CH_2Cl_2 (VWR International, 23373.320)
- Acetonitrile (VWR International, 20060.320)
- Hexane (Sigma Aldrich, 34859-2.5L)
- Ether (VWR International, 83624.320)
- 1,4 dioxane (Sigma Aldrich, 360481-500ML)
- AcOH (Sigma Aldrich, 27221-2.5L)
- Geduran Si 60 for column chromatography (VWR International 1.11567.1000)
- Celite 577 fine (Sigma Aldrich, 22142-1KG)
- Sodium chloride (Sigma Aldrich, S5886-500G)
- Sodium thiosulfate (Sigma Aldrich, 217263-1KG)
- HEPES salts (Fisher Scientific Ltd, BPE310-1)
- $CaCl_2$ (Sigma Aldrich, C4901-1KG)
- Recombinant Sortase A from Staphylococcus aureus (We recommend a protocol similar to that described by Guimares et al.[25]except that we recommend that protein concentration is estimated using the UV absorption at 280 nm (theoretical extinction coefficient ε_{280} = 17420 cm^{-1} M^{-1})

REAGENT SETUP

Prepare a saturated aqueous solution of sodium chloride. Weigh approximately 400 g sodium chloride and add to a 1 L storage bottle. Fill the bottle to the 900 mL mark and shake to dissolve the sodium chloride. As the sodium chloride dissolves, top up the bottle to the 1 L mark. Approximately 10% of the sodium chloride will remain undissolved. Allow this to settle upon storage.

Prepare a 10% wt/vol solution of sodium thiosulfate. Weigh out 100g of sodium thiosulfate and add to a 1 L measuring beaker with a Teflon magnetic stirrer. Fill the measuring beaker to the 800 ml and stir the solution for 2-3 minutes until all the solid has dissolved. Fill the beaker to the 1 L mark with deionised water. Store the solution in a 1 L storage bottle.

Prepare ligation buffer. Weigh out HEPES (11.90 g), NaCl (8.70 g) and $CaCl_2$ (0.55 g) and add to a 1 L measuring beaker with a Teflon magnetic stirrer. Fill the measuring beaker up to the 800 ml mark with deionised water and stir the solution for 2-3 minutes until all the solid has dissolved. Adjust the pH of the solution to pH 7.50 by slowly adding NaOH solution (5 mM) with a pipette. Fill the beaker up to the 1 L mark with deionised water. The ligation buffer will consist of HEPES (50 mM), $CaCl_2$ (5 mM) and NaCl (150 mM) at pH 7.5. Store in a 1 L storage bottle for up to 3 weeks.

EQUIPMENT

- Fritted polypropylene tubes (10 ml) (Grace and Co)
- Stuart SB tube rotator
- Centrifuge
- Incubator
- NMR spectrometer
- Mass spec
- HPLC
- Rotary evaporator
- Single-neck round-bottom flasks (25–500 ml)
- 500 ml to 1 liter glass chromatography column (diameter >3 cm)
- Magnetic stir bars
- Hotplate/magnetic stirrer
- Separation funnels (500 ml to 1 liter)
- Storage bottles (1 liter)
- Freezer dryer
- High vacuum pump
- Water pump
- Vacuum manifold
- Thick walled balloon gas container connected to a two-way stopcock adapter with Luer fittings
- TLC plates
- 500 ml Buchner flask
- 14 ml neutral-glass sample tube
- Gilson p200 pipette
- 7 cm inner diameter fritted glass funnel

EQUIPMENT SETUP

HPLC Peptides are analysed using an Agilent 1290 affinity LC system equipped with an Ascentis Express 10 cm × 2.1 mm, 2.7 μm ES-C18 peptide column (0.5 ml min−1) and UV detection at 220 nm - 280 nm. Gradient from 0.1% TFA/5% MeCN (vol/vol) in H_2O to 0.1% TFA/95% MeCN in H_2O over 5 min 40 s.

HRMS Protein MS analysis is carried out using a Bruker HCT Ultra MS system equipped with an Agilent 1200 series autosampler. Protein is directly injected into the instrument after dilution in 0.1% TFA/50% MeCN in H_2O.

PROCEDURE

Stage 1: Synthesis of the precursor. Preparation of Fmoc-Thr(OtBu)-Gc-OBn ● TIMING: 19 h (overnight step)

1) Add 20 ml of THF to a 100 ml round bottom flask equipped with a magnetic stirrer bar. To this flask add sequentially 10 g (25 mmol) of Fmoc-Thr(tBu)-OH, 5.9 ml (38 mmol) of benzyl 2-bromoacetate and a catalytic amount of tetrabutylammonium iodide (3.7 g, 10 mmol) before adding 5.2 ml (30 mmol) of triethylamine. Stir the reaction mixture overnight at room temperature (18-25 °C). After a few minutes of stirring the solution will turn yellow and a solid should be seen to precipitate.

2) The following day, pour the reaction mixture into a 500 ml separating funnel and wash the flask out with 200 ml deionised H_2O and extract the crude product with 2 × 200 ml of ethyl acetate.

3) Combine the ethyl acetate extracts and wash in a 1 L separating funnel with 2 × 400 ml of 10% (wt/vol) sodium thiosulfate solution and 400 ml of saturated NaCl solution. Collect the ethyl acetate layer and add anhydrous sodium sulfate in approx. 3 g portions until the solid stops clumping together to ensure the solution is dry. Filter the mixture and reduce the filtrate to dryness by rotary evaporation at 40 °C (approx 4.0 Torr). The residue will be a yellow oil.

Purification of Fmoc-Thr(OtBu)-Gc-Obn by chromotgraphy ● TIMING: 2 h

4) Prepare a silica gel slurry by adding 300 ml of a 4:1 mixture of hexane/ethyl acetate to approx. 250 g silica gel 60A (40-60 µm particle size). The slurry should be added to a column with a 4.5-5 cm inner diameter and the column should be packed until the compressed silica layer reaches 30-35 cm in height.

5) Dissolve the crude product in a minimal volume of CH_2Cl_2. Apply to the column and elute with a 4:1 mixture of hexane/ethyl acetate. Collect fractions of 15-20 ml and analyse by thin-layer chromatography. The desired product can be visualised under a UV lamp and it should have an R_f of 0.53 in a mixture of 2:1 hexane/ethyl acetate. To speed up the elution of the product the solvent system may be ramped up to a 1:1 mixture of hexane/ethyl acetate after the product is initially detected.

7) Combine fractions containing the product and reduce these to dryness by rotary evaporation at 40 °C (approx 4.0 Torr) to leave a slightly coloured oil as the product. Dry the product to remove residual solvent using a high vacuum line (0.1 Torr) and store at -20 °C. The oil will form a glassy solid. Starting with 10 g of Fmoc-Thr(OtBu) will typically produce 13 g of product.

Preparation of Fmoc-Thr(OtBu)-Gc-OH ● Timing: 1-6 h

8) Add 12 ml of THF to a 100 ml round bottom flask equipped with a magnetic stirrer bar. To this flask add 1.0 g of Fmoc-Thr(OtBu)-Gc-OBn (1.80 mmol) and stir the solution until all the solid has dissolved before slowly adding 4 ml of H_2O. To the stirred solution add 100 mg of palladium on carbon (10 wt%) and seal the flask with an appropriate sized rubber Suba-seal.

9) Fit a two-way stopcock with Luer fittings to a thick-walled balloon and fill it with hydrogen. Attach a needle to the stopcock using the Luer fitting and insert the needle into the reaction vessel through the suba-seal.

! CAUTION: Hydrogen gas is extremely flammable and should be handled with extreme care in a ventilated fume hood fitted with blast shield. Inexperienced users must consult their safety advisor on the local rules for handling and dispensing hydrogen before attempting this part of the protocol.

Additional portions of the palladium on carbon catalyst must not be added to the system under hydrogen gas since it may spontaneously ignite..

10) Evacuate the air from the reaction vessel and purge three times with hydrogen. Stir the reaction mixture at room temperature under hydrogen for 1-6 h. Refill the balloon if necessary.

11) Remove 5-15 µL samples using a needle to allow the reaction to be monitored by TLC (4:1 hexane/ethyl acetate, 1% AcOH). The desired product should have an R_f of 0.23 in 23 in this solvent mixture.

Δ CRITICAL STEP: The hydrogenation step should be monitored carefully and if the reaction is very close or at completion it should be stopped immediately. Leaving the hydrogenation for prolonged periods will cause cleavage of the Fmoc protecting group making the subsequent purification step more difficult. The Fmoc cleavage byproduct will have an R_f of 0.78 in a mixture of 2:1 hexane/ethyl acetate

12) Once the reaction is complete the reaction vessel is purged with nitrogen and opened to the atmosphere.

13) Pack a pad of Celite (approx. 13 g) suspended in THF on a 7 cm inner diameter fritted glass funnel (approx. 3 cm high). Connect the funnel to a 500 ml Büchner flask attached to a water pump. Under reduced pressure wash the Celite pad with a mixture of 3:1 THF/H_2O until the filtration solution is clear and free from contaminants.

14) Filter the reaction mixture through the pad of Celite and wash twice with a 100 ml of 3:1 THF/H_2O solution.

15) Transfer the filtrate to a 500 ml round bottom flask and remove the solvent under reduced pressure using a rotary evaporator at 40 °C (approx 4.0 Torr). The crude product is obtained as an off-white foamy solid.

▪ **PAUSE POINT:** If no Fmoc-cleavage was detected during steps 10-11 then the crude product can be used without further purification. In this case, proceed to step 19. Trace amounts of Fmoc-Thr(OtBu)-Gc-OBn starting material does not appear to interfere with the solid phase peptide synthesis If Fmoc cleavage has occurred the crude product can be stored at least 3-4 days at - 20 °C before further purification is carried out.

Purification of Fmoc-Thr(OtBu)-Gc-OH TIMING: 2-3 h; plus, overnight freeze drying

16) Prepare a silica gel slurry by adding 100 ml of a 9:1 mixture of CH_2Cl_2/MeOH to appox. 40 g of silica gel 60A (40-60 µm particle size). The slurry should be added to a column with a 3.5-4.0 cm

inner diameter and the column should be packed until the compressed silica layer reaches 18-22 cm in height.

17) Dissolve the crude product in a minimal volume of CH_2Cl_2. Apply to the column and elute with 9:1 mixture of CH_2Cl_2/MeOH. Collect fractions of 5-10 ml and analyse by thin-layer chromatography. The desired product should have an R_f of 0.63 in a mixture of 9:1 CH_2Cl_2/MeOH and 1% AcOH.

18) Combine the elution fractions and concentrate to dryness using a rotary evaporator at 40 °C (approx 4.0 Torr) to leave a foamy white solid as the product.

19) The white solid is then dissolved in acetonitrile and freeze dried overnight to leave a white fluffy solid. Starting with 1.0 g of Fmoc-Thr(OtBu)-Gc-OBn it is possible to obtain ca. 700 mg of product.

▪ **PAUSE POINT:** The Fmoc-Thr(OtBu)-Gc-OH can be stored for at least a month at -20 °C

Part 2: Preparation of depsipeptides. Presynthesis preparation. TIMING 5 min

20) Prepare a 250 ml stock solution of 20% piperidine (v/v) in DMF for the removal of the Fmoc group and store in a screw capped glass bottle.

Resin preparation. TIMING: 30 min

21) Weigh 100 mg of Gly-2-Cl-trityl resin (0.11 mmol, loading capacity: 1.1 mmol g^{-1}) directly into a fritted 8 ml polypropylene reservoir fitted with a 2-way stopcock.

22) Add 6 ml of DMF to the resin, cap the reservoir and allow the resin to swell by rotation on a Stuart rotator tube spinner for 20 minutes.

LPETGcG depsipeptide synthesis. TIMING: 1 days

23) Weigh out 5 equivalents of Fmoc-Thr(OtBu)-Gc-OH (250 mg, 0.55 mmol) and 4.9 equivalents of HCTU (228 mg, 0.54 mmol) into a single 14 ml neutral-glass sample tube.

24) Dissolve the reagents in 4 ml of DMF and add 10 equivalents of DIPEA (203 µL, 1.10 mmol) to the solution. Mix the sample using a vortex mixer to ensure all the compounds are dissolved. After the addition of DIPEA the solution should turn a dark yellow/orange.

25) Connect the reservoir to a vacuum manifold and drain the DMF from the pre-swollen resin.

26) Add the amino acid solution to the pre-swollen resin and rotate for 1 h

27) Once an hour has elapsed, drain the amino acid solution from the resin using the vacuum manifold. Add 4 ml of DMF to the resin and agitate the resin by rotation for 2 minutes. Drain the DMF and repeat the procedure twice.

28) Remove the Fmoc group from the resin by rotating in 4 ml of 20% (v/v) piperidine/DMF solution for 2 minutes. Drain the cleavage mixture and repeat the procedure four more times.

29) Wash the resin thoroughly by rotating in 6 ml of DMF for 2 minutes. Drain the DMF and repeat the procedure four more times.

Δ CRITICAL STEP: It is imperative that all the residual piperidine is removed as it can interfere with the subsequent coupling step.

30) Repeat steps 23-29, substituting Fmoc-Thr(OtBu)-Gc-OH with Fmoc-Glu(OtBu)-OH.

31) Repeat steps 23-29, substituting Fmoc-Thr(OtBu)-Gc-OH with Fmoc-Pro-OH.

32) Repeat steps 23-27 using Fmoc-Leu-OH.

33) Repeat the coupling of Fmoc-Leu-OH by repeating steps 23-29to ensure maximum coupling efficiency to the slightly hindered proline residue.

34) Wash the resin by rotating in 4 ml of DMF for 2 minutes. Drain the DMF and repeat this wash step twice.

35) Wash the resin further by rotating in 4 ml of CH_2Cl_2 for 2 minutes. Drain the DCM and repeat this wash step twice.

36) Shrink the resin by rotating in 6 ml of MeOH for 2 minutes. Drain the MeOH and repeat this shrinking step twice. Leave the resin to partially dry for 15 minutes by exposing the reservoir to reduced pressure on the vacuum manifold.

37) Place the reservoir under high vacuum (0.1 Torr) for at least 3 hours to completely dry the resin.

▪ **PAUSE POINT:** The reservoir containing the dry depsipeptide-loaded resin can be sealed with parafilm and stored at – 20 °C for several months.

Peptide elongation. TIMING: Variable

38) Swell the depsipeptide-loaded resin by adding 6 ml of DMF and rotating for 20 minutes.

39) Repeat step 23-29with the desired Fmoc protected amino acid.

N-terminal fluorescent labelling using isothiocyanates TIMING: 20 h (overnight step)

40) Repeat step 23-29 using Fmoc-GABA-OH.

41) Weigh out 6 equivalents of FITC (219 mg, 0.66 mmol) into a 14 ml neutral-glass sample tube.

42) Dissolve the FITC in 900 µl of DMF and add 14 equivalents of DIPEA (319 µL, 1.54 mmol).

Δ CRITICAL STEP: It is important to dissolve the FITC in the minimal amount of solvent, as the coupling efficiency to the peptide is concentration dependent. FITC is more soluble once the DIPEA has been added, so ensure the solution is well mixed once all reagents have been added.

43) Add the FITC solution to the resin and rotate overnight at room temperature (18-25 °C)

Δ CRITICAL STEP: Completely cover the peptide reservoir in aluminium foil to protect the reaction from light.

▪ **PAUSE POINT:** The FITC-peptide conjugation is left overnight

44) The next day drain the FITC solution from the resin. Wash the resin thoroughly by rotating in 6 ml of DMF for 2 minutes. Drain the DMF and repeat the procedure four more times.

45) Repeat steps 15-18.

Depsipeptide cleavage TIMING: 20 h (overnight step)

46) Prepare a 3 ml cleavage-cocktail consisting of TFA:H_2O:TIS (95:2.5:2.5) in a 14 ml neutral-glass sample tube.

! CAUTION: TFA is a corrosive volatile solution and should only be handled in a fume hood with suitable ventilation.

47) Transfer 2 ml of the cleavage-cocktail to the dried resin and rotate for 2 hours to cleave the depsipeptide.

48) While the depsipeptide is being cleaved add 40 ml of ether to a falcon tube and cool it to -20 °C.

49) After 2 hours drain the cleavage cocktail into the pre-prepared cold ether to precipitate the depsipeptide. Store the ether suspension at -20 °C until step 51.

50) Transfer the remaining 1 ml of cleavage-cocktail to the resin and rotate for a further 5 minutes to ensure all the depsipeptide is removed the resin.

51) Drain the residual cleavage cocktail into the cold ether to precipitate any remaining depsipeptide.

52) Pellet the cleaved depsipeptide by centrifuging the ether layer for 10 minutes at 4000 × g.

53) Decant off the ether layer and resuspended the depsipeptide pellet in 40 ml of cold ether. Mix the sample using a vortex mixer to ensure complete resuspension and centrifuge for 10 minutes at 4000 × g. Repeat twice.

54) Decant off the ether layer and remove any residual ether with a glass pipette. Leave the depsipeptide pellet exposed to the atmosphere for one hour to ensure it is almost completely free from ether.

55) Dissolve the peptide pellet in a minimal amount of water (1-5 ml) and flash freeze in liquid nitrogen before freeze drying overnight to yield a fluffy solid.

- PAUSE POINT: Confirm the peptide identity by high resolution mass spectrometry (HRMS) and purity by HPLC

PROCEDURE Part 3: Typical protein labelling procedure. Preparation of ligation buffer solution: TIMING: 10 min

56)

▪ **PAUSE POINT:** The ligation buffer can be stored at room temperature for a few weeks before fresh buffer needs to be prepared.

Preparation of stock solution: TIMING: Variable

The following concentrations are for a typical protein labelling reaction in our lab, but it can be varied depending on the scale of the reaction.

56) Prepare a stock solution of 2 mM depsipeptide in ligation buffer and store in 1.5 ml eppendorf at -20 °C.

▪ **PAUSE POINT:** The depsipeptide stock solution should be stored at -20 °C, as the ester linkage is susceptible to hydrolysis.

57) Prepare a solution of 120 µM protein in ligation buffer and store in a 1.5 ml eppendorf at 4 °C.

58) Prepare a solution of 300 µM SrtA in ligation buffer and store in a 1.5 ml eppendorf at 4 °C.

▪ PAUSE POINT: SrtA can be stored for 2-3 weeks at 4 °C, but it can be flash frozen and stored at -80 °C for longer periods (6 months). The enzyme still functions after several cycles of flash freezing without addition of a cryoprotectant.

Typical protein labelling procedure: TIMING: 4-6 hours

59) In a clean 1.5 ml eppendorf add 50 µL of gMBP stock (60 µM), 3 µL of SrtA stock (6 µM, 0.1 mole equivalents, 6 µL of depsipeptide stock (120 µM, 2 equivalents) and 41 µL of ligation buffer.

60) Thoroughly mix the ligation solution with a Gilson p200 pipette and incubate at 37 °C.

PAUSE POINT: With larger volumes (approx. above 1 ml) it may be beneficial to agitate the ligation mixture to ensure complete mixing.

61) Monitor the progress of the ligation reaction using SDS-PAGE electrophoresis, or electrospray mass spectrometry (ESMS). (We routinely follow protein labelling reactions in real-time using a standard protein mass spectrometry methods as shown in figure 3).

PAUSE POINT: Protein modification is typically complete in 4-6 hours. However, if the ligation is not complete within 6 hours, add another 1.5-3 equivalents of depsipeptide substrate and increase the SrtA concentration to 0.2-0.3 mole equivalents. 62) Once the ligation is complete isolate the protein using a suitable affinity column. gMBP was isolated using a lactose-agarose affinity column.

Δ CRITICAL STEP: Leaving the ligation reaction for prolonged periods will cause SrtA to hydrolyse the label from the modified protein. The reaction mixture should either be isolated straight away or flash frozen to stop the reaction.

● TIMING

TIMING

Stage 1: Synthesis of the precursor. Preparation of Fmoc-Thr(OtBu)-Gc-OBn

Steps 1-3: 19 hours (Overnight step)

Purification of Fmoc-Thr(OtBu)-Gc-Obn by chromotgraphy

Steps 4-7: 2 hours

Preparation of Fmoc-Thr(OtBu)-Gc-OH

Steps 8-15: 1-6 hours

Purification of Fmoc-Thr(OtBu)-Gc-OH

Steps 16-19: 2-3 hours; plus, overnight freeze drying step

Part 2: Preparation of depsipeptides. Presynthesis preparation.

Steps 20-22: 35 minutes

LPETGcG depsipeptide synthesis.

Steps 23-37: 1 day

Peptide elongation.

Steps 38-39: variable

N-terminal fluorescent labelling using isothiocyanates.

Steps 40-45: 20 hours (overnight step)

Depsipeptide cleavage

Steps 46-55: 20 hours (overnight step)

Part 3:

Preparation of stock solution:

Steps 56-58-62: variable

Typical protein labelling procedure: TIMING: 4-6 hours

Steps 59-62: variable

ANTICIPATED RESULTS

Synthesis of Fmoc-Thr(OtBu)-Gc-OBn

As described, the synthesis will typically produce 95% of product.

Fmoc-Thr(OtBu)-Gc-OBn. Glassy Solid

$[\alpha]_D$ -0.4 (c 1, in CH_2Cl_2 at 22.7 °C)

TLC (Hexane-ethyl acetate 2:1 v/v) RF = 0.53

^{1}H NMR (500 MHz, CD_3OD): δ 1.15 (s, 9H), 1.26 (d, J = 6.2 Hz, 3H), 4.29 (m, 2H), 4.40 (m, 3H), 4.66 (d, J = 15.9 Hz, 1H), 4.76 (d, J =15.9 Hz, 1H,), 5.21 (s, 2H), 7.34 (m, 6H), 7.40 (m, 1H), 7.64 (m, 1H), 7.77 (d, J = 6.2 Hz, 1H).

^{13}C NMR (125 MHz, CD_3OD): δ 21.0 (CH_3), 28.5(CH_3), 47.2 (CH) 59.9 (CH), 61.4 (CH_2), 67.3 (CH), 67.4 (CH_2), 67.4 (CH_2), 79.4 (CH), 120.1 (CH), 125.2 (CH), 127.2 (CH), 127.8 (CH), 128.5(CH), 128.7(CH), 128.8 (CH), 141.4 (CH), 144.2 (CH), 156. 8 (C), 167.1(C), 170.6 (C).

IR (CH_2Cl_2, cm^{-1}) 3440, 2976, 1755, 1725, 1648.

HRMS: Found $[M+Na]^+$ 568.2321, (calculated, for $C_{32}H_{35}NO_7Na$ 568.2306)

Synthesis of Fmoc-Thr(OtBu)-Gc-OH

Fmoc-Thr(OtBu)-Gc-OH. White lyophilisate
$[\alpha]_D$ -0.5 (c 1 in CH_2Cl_2 at 22.5 °C)

TLC (CH_2Cl_2-methanol 9:1 and 1% acetic acid v/v) R_F = 0.63

^{1}H NMR (500 MHz,CD_3OD): δ 1.04 (s, 1H) 1.09 (d, J = 5.9 Hz, 1H), 4.16 (m, 2H), 4.26 (m, 1H), 4.36 (m, 3H), 4.55 (d, J = 15.9 Hz, 1H), 7.20 (m, 1H) 7.27 (m, 1H), 7.55 (m, 1H), 7.65 (d, J = 7.6 Hz, 1H),

^{13}C NMR (125 MHz, CD_3OD): δ 21.0 (CH_3), 28.5 (CH_3), 47.3 (CH), 60.3 (CH), 63.3 (CH_2), 67.0 (CH), 67.8 (CH_2), 74.2 (CH), 119.9 (CH), 125.3 (CH), 127.0 (CH), 127.6 (CH), 141.3 (CH), 143.9 (CH), 144.3 (CH), 157.6 (C), 170.5 (C), 174.1 (C).

IR (CH_2Cl_2, cm^{-1}) 3436, 3436, 2977, 1749, 1720.

HRMS: Found $[M+Na]^+$ 478.1837 (calculated, for $C_{25}H_{29}NO_7Na$ 478.1836)

The yield of this reaction can vary significantly depending on the rate of Fmoc-protecting group cleavage relative to hydrogenolysis of the benzyl group. Yields have ranged from 61 – 87%. This procedure can be scaled up.

Synthesis of depsipeptide substrates

The yields of the depsipeptide synthesis can vary depending on the sequence and length of the peptide. Yields have ranged from 52-89%. Expected retention time for described fluorescein-containing peptide in HPLC: 2.57 min. The N-terminal fluorescent labelling can be done with a range of fluorophores including rhodamine isothiocyanate and dansyl chloride. The depsipeptide should be able to be built on any base stable resin containing an N-terminal amine.

Sortase-mediated protein modification

The protein should be completely modified within 4-6 hours. If no protein ligation is detected then the protein's N-terminal glycine residues may be too sterically hindered for SrtA to access. If so, then an alternative N-terminally extended construct will be required.

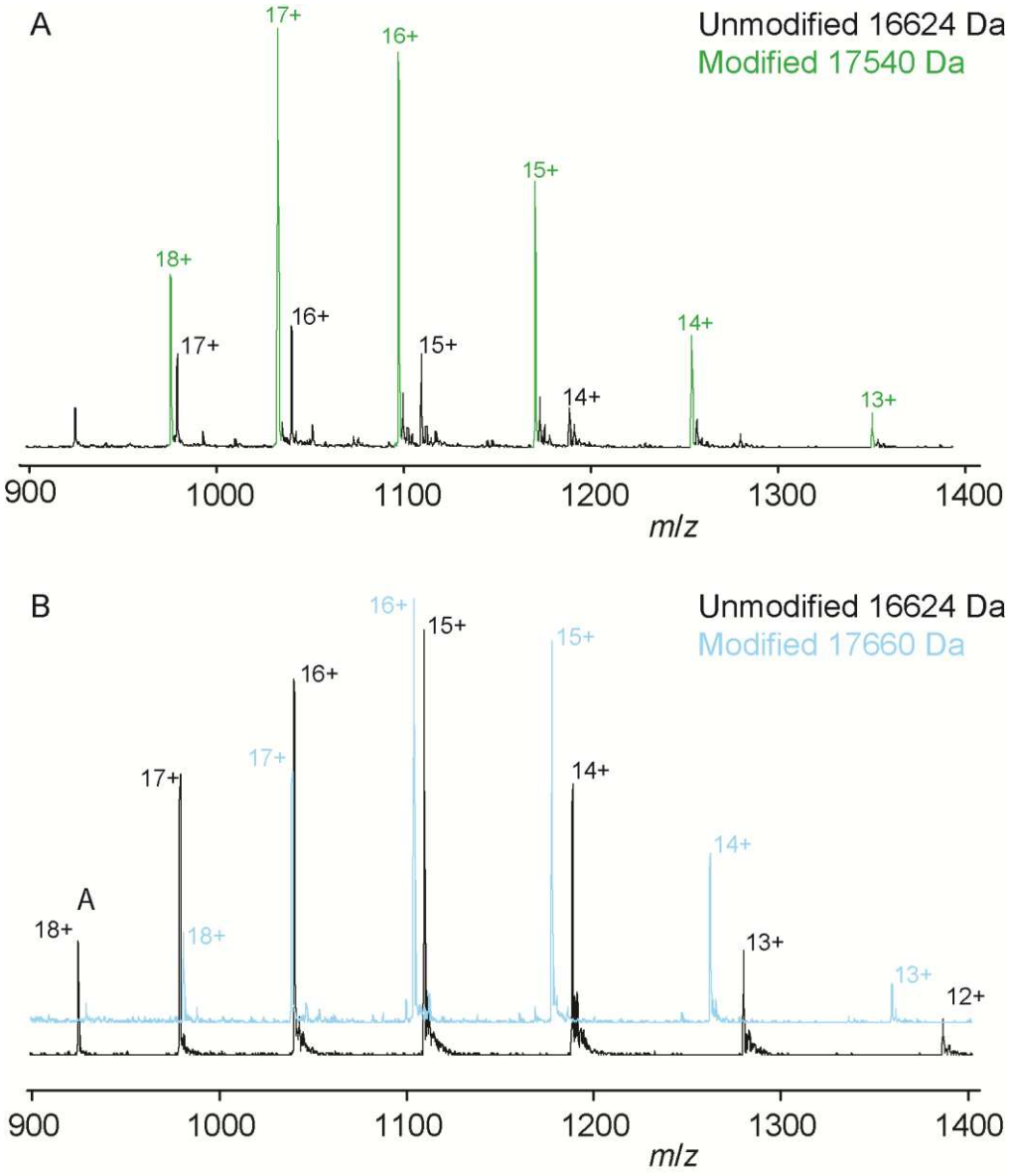

Figure 3: Examples of monitoring protein labelling reactions in real-time by electrospray mass spectrometry. **A** Analysis by mass spectrometry of the example labelling reaction of gMBP using the fluorescein-labelling reagent described in this manuscript – at this stage the reaction has not yet proceeded to completion. **B** Example of overlaid mass spectra for unlabelled gMBP (black) and gMBP 100% labelled with a dansyl-containing peptide (data reproduced from Williamson et al.[21])

?TROUBLESHOOTING

Table 1

Step	Problem	Possible reason	Solution
8			
	Precipitate formation after the addition of H_2O	Fmoc-Thr(OtBu)-Gc-OBn crashing out of soloution	Add more THF until the precipitate redissolves
11			
	Slow rate of hydrogenation	Inefficient and/or old Pd/C catalyst	The hydrogen balloon should be removed from the vessel and the reaction quenched by flushing with nitrogen gas. Once quenched a further 100-300 mg of palladium on carbon (10 wt%) should be added and the process (steps 9-11) repeated
15			
	Removal of solvent	Water removal can be slow using a standard rotary evaporator	The THF can be removed rapidly using the rotary evaporator at 40 °C, but removing the water can be problematic. To aid the removal of the water, add toluene to form an azeotropic solution that is removed more efficiently using the rotary evaporator at 50 °C
55			
	Depsipeptide solubility	Modified depsipeptides may be too hydrophobic to dissolve completely in water	The addition of acetonitrile or 1,4-dioxane can help dissolve peptides that are not completely soluble in water.
63			
	Hydrolysis of depsipeptide substrate	Under the conditions of the ligation reaction the depsipeptide substrate will slowly undergo non-enzymatic hydrolysis. The rate of hydrolysis can vary between different depsipeptide substrates	If hydrolysis is a problem then 3 equivalents of depsipeptide and 0.2 mole equivalents of SrtA can usually be used to achieve complete protein modification

Poor ligation efficiency	At very low protein concentrations (e.g. 10 μM) the efficiency of the ligation reaction can be greatly reduced	Using 3-5 equivalents of depsipeptide and 0.2-0.3 mole equivalents of SrtA will usually achieve complete protein modification

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AUTHOR CONTRIBUTIONS

All authors were involved in the design of the experiments; DJW conducted the experiments; all authors contributed to writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests.

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