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Diselenide–selenoester ligation for chemical protein synthesis

Sameer S. Kulkarni[1,3], Emma E. Watson[1,3], Bhavesh Premdjee[2], Kilian W. Conde-Frieboes[2] and Richard J. Payne[1*]

Chemoselective peptide ligation methods have provided synthetic access to numerous proteins, including those bearing native post-translational modifications and unnatural labels. This protocol outlines the chemical synthesis of proteins using a recently discovered reaction (diselenide–selenoester ligation (DSL)) in a rapid, additive-free manner. After ligation, the products can be chemoselectively deselenized to produce native peptide and protein products. We describe methods for the synthesis of suitably functionalized peptide diselenide and peptide selenoester fragments via Fmoc-solid-phase peptide synthesis (SPPS) protocols, fusion of these fragments by DSL, and the chemoselective deselenization of the ligation products to generate native synthetic proteins. We demonstrate the method's utility through the total chemical synthesis of the post-translationally modified collagenous domain of the hormone adiponectin via DSL–deselenization at selenocystine (the oxidized form of selenocysteine) and the rapid preparation of two tick-derived thrombin-inhibiting proteins by DSL–deselenization at β-selenoaspartate and γ-selenoglutamate. This method should find widespread use for the rapid synthesis of proteins, including cases in which other peptide ligation methods cannot be used (or cannot be used efficiently), e.g., at sterically hindered or deactivated acyl donors. The method's speed and efficiency may render it useful in the generation of synthetic protein libraries. Each protein discussed can be synthesized within 15 working days from resin loading and can be readily produced by practitioners with master's-level experience in organic chemistry. Each synthesis using these protocols was performed independently by two labs (one academic and one industrial), which attained comparable yields of the protein products.

Introduction

Chemical protein synthesis through the combination of SPPS and chemoselective peptide ligation methodologies has provided access to numerous challenging proteins, including those with site-selectively introduced post-translational modifications and/or other unnatural modifications, such as fluorescent labels and D-amino acids. Although native chemical ligation (NCL)[1] is currently the most widely used method for the chemical synthesis of proteins, some inherent limitations of this technique have led to the development of alternative chemoselective ligation techniques[2–6], most recently DSL. First reported in 2015 (ref. [7]), DSL offers a number of unique features that greatly expand the repertoire of ligation chemistry and, importantly, can be used in conjunction with NCL. DSL reactions are performed by simply mixing two suitably functionalized peptide fragments (a peptide bearing a C-terminal selenoester and another peptide dimer with an N-terminal diselenide moiety) in a denaturing buffer without the addition of any other reagents (which are required for other ligation techniques, Fig. 1). Owing to the enhanced reactivity of the reaction partners, ligations are typically complete within 1–10 min, including examples at sterically hindered junctions that would normally take 24–48 h (or longer) to reach completion by NCL[8]. In addition, owing to the lower pK_a value (logarithmic acid dissociation constant) of selenocysteine as compared with cysteine, these ligations can be performed over a broad pH range (pH 3–7) and, due to the absence of radical-quenching thiol additives (which are used for other ligation methods), allow one-pot deselenization post ligation. One additional benefit of the DSL chemistry is that deselenization can be performed chemoselectively under mild conditions in the presence of native unprotected cysteine residues to generate native amino acids. A review published in 2018 provides a comprehensive history of the DSL technology and its comparison to other ligation methods[9]. This protocol presents details of the synthesis of suitably functionalized peptide fragments (peptide selenoesters and peptide diselenide dimers) for use in DSL

[1]School of Chemistry, The University of Sydney, Sydney, New South Wales, Australia. [2]Department of Research Chemistry, Novo Nordisk A/S, Måløv, Denmark. [3]These authors contributed equally: Sameer S. Kulkarni, Emma E. Watson. *e-mail: richard.payne@sydney.edu.au

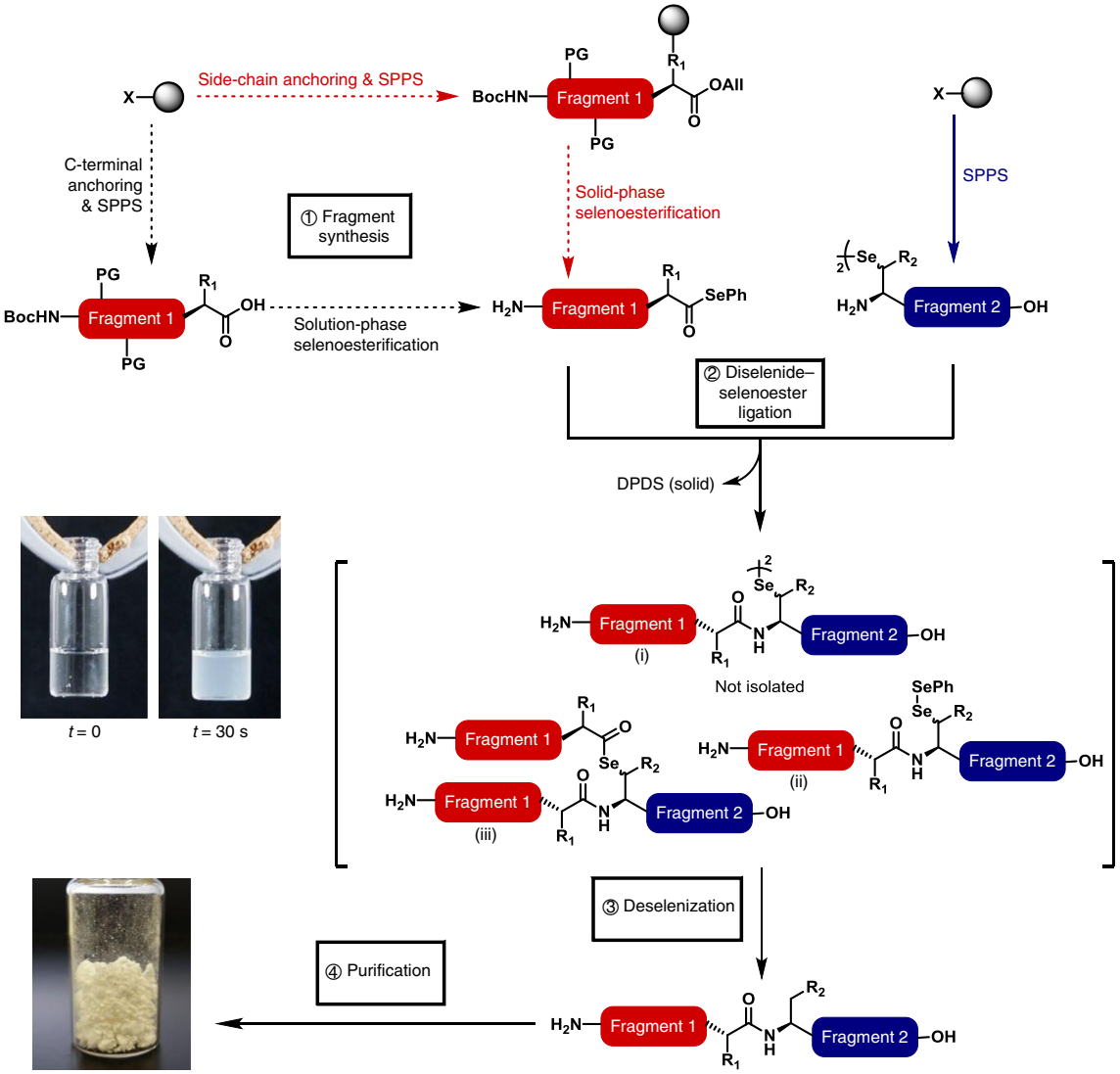

Fig. 1 | Overview of the DSL–deselenization protocol. The protocol begins with the synthesis of the component fragments via Fmoc-SPPS, followed by their functionalization as either selenoesters or diselenides to generate the two requisite fragments ready for ligation (1). The DSL reaction proceeds under neutral aqueous denaturing buffer conditions (2) without the need for additional reagents to generate a mixture of three ligation products: (i) a symmetric diselenide between ligation product molecules, (ii) an asymmetric diselenide between a product molecule and a molecule of phenylselenol liberated during the ligation and (iii) an unproductive ester that is generated by *trans*-selenoesterification of an additional N-terminal fragment by a product molecule. Insoluble DPDS is also generated during the reaction, resulting in the turbidity seen in the photograph taken at 30 s. Each of the three ligation products can subsequently undergo deselenization (3) in one pot to afford a given target protein in multi-milligram quantities following reverse-phase HPLC purification (4). X, the resin-bound functional group that reacts with the first amino acid in the loading step. BocHN, *tert*-butyloxycarbonyl protected amine; DPDS, diphenyldiselenide; PG, protecting group.

chemistry, as well as the procedures for one-pot DSL–deselenization chemistry for the synthesis of proteins at multi-milligram scales.

Experimental design

As with all ligation-based technologies, the first consideration in terms of synthetic access to a chosen target is the disconnection of the primary sequence at appropriate sites (called ligation junctions). In its initial form, the DSL–deselenization protocol required disconnection at Ala residues, which can be mutated to selenocysteine (Sec) for the purposes of the ligation step (and subsequently chemoselectively deselenized to regenerate the native Ala residue)[7]. In subsequent work, the method was extended to allow alternative ligation junctions at Ser (through an oxidative deselenization process from Sec)[10,11], as well as Asp[12], Glu[12], Leu[13], Phe[14] and Pro[15] (through ligation at

seleno-derived variants of the amino acids and deselenization to afford the native residues at the ligation junction). Attention should also be paid to the acyl donor residue of the ligation junction, with a select number of residues best avoided. These include Asp, Glu and Lys—because of their proclivity to cyclize via their side-chain functionalities and elimination of the C-terminal selenoester—as well as His, because of rapid C-terminal ester hydrolysis that can outcompete ligation. Unlike NCL and other ligation methodologies, DSL remains rapid at sterically hindered acyl donor residues, with the reactions still complete within 10 min for Val and Ile. Factors to be considered in choosing ligation junctions in NCL have recently been reviewed[16]. Many of these guidelines (with the notable exception of acyl donor sterics) are common to DSL.

With appropriate ligation junctions selected, attention can be turned to the generation of the requisite peptide fragments via Fmoc-SPPS and their subsequent functionalization. Generation of such fragments is possible through standard Fmoc-SPPS procedures, either via manual synthesis or through the use of automated peptide synthesizers. A detailed description of this process for the three case studies can be found within the 'Procedure' section of this protocol. After the generation of the required fragments, the N-terminal fragment can be functionalized at the C terminus as a selenoester either on resin or in solution, followed by global acidolytic deprotection and reverse-phase HPLC purification to produce the desired N-terminal fragments. Similarly, after assembly of the C-terminal fragment, a suitably protected seleno-derived amino acid residue can be coupled under standard conditions either as the free diselenide (in the case of Sec) or as a *p*-methoxybenzyl PMB-protected selenol moiety. Sec is typically coupled as the PMB-protected building block as this affords a monomeric peptide that can be easier to purify by HPLC. However, the unprotected diselenide (selenocystine) can be exploited for short fragments to help streamline the synthesis. Specifically, commercially available selenocystine [$(H_2N\text{-Sec-OH})_2$] can be incorporated into resin-bound peptides by SPPS following Boc-protection. Boc-Sec(PMB)-OH[11,17] and Fmoc-(β-SePMB)Asp-OH[12] have also recently become commercially available, whereas selenylated variants of Glu[12], Leu[13], Phe[14] and Pro[15] can be accessed via straightforward synthetic procedures previously described by ourselves and others. After global acidolytic deprotection and PMB deprotection (except for cases where unprotected Sec is used), the C-terminal fragments can be purified via reverse-phase HPLC to generate the desired fragments ready for fusion via DSL.

After synthesis of the requisite fragments, the practitioner can then consider the conditions required for the assembly of their peptide/protein target via DSL–deselenization. Standard conditions for the ligation reaction are the dissolution of the two requisite fragments to a concentration of 2.5 mM of the limiting peptide diselenide fragment in 6 M guanidine hydrochloride (Gdn.HCl), 0.1 M Na_2HPO_4, pH 7.0 buffer. Typically, the selenoester-functionalized fragment is used in slight excess (1.2–1.5 equiv.) to account for any ester hydrolysis during the ligation procedure, but up to 2.5 equiv. of the acyl donor has been used in cases where the fragments are highly sterically encumbered or hydrolysis prone[12]. In addition, for fragments which are poorly soluble or aggregation prone in aqueous buffer, up to 10 vol% of the buffer volume can be substituted with *N,N*-dimethylformamide (DMF) to help aid reaction progression. After completion of the DSL reaction (as judged by complete consumption of the limiting peptide diselenide fragment via ultra-performance liquid chromatography–mass spectrometry (UPLC–MS), the reaction mixture is then extracted with hexane (to remove the DPDS generated during the ligation), degassed and subjected to a deselenization cocktail containing 250 mM tris(2-carboxyethyl)phosphine (TCEP) as a phosphine reductant and 25 mM DTT (dithiothreitol) as an H-atom donor. For deselenization at β-selenoaspartate ((β-Se)-Asp) and γ-selenoglutamate ((γ-Se)-Glu) residues, the deselenization reaction is typically complete within 1–10 min, whereas for seleno-derived Leu, Phe and Pro, as well as Sec, this transformation typically proceeds over 6–8 h. Peptides and proteins remain stable under these mild conditions for at least 24 h, and it is common to allow the deselenization of these residues to proceed over 16 h if this is more convenient for the workflow.

The DSL–deselenization protocol has been shown to scale well, being conducted at up to 150-mg scale in an industrial setting. The typical academic scale for this reaction is between 1 and 10 mg of the limiting peptide diselenide fragment. We do not advise conducting ligations on scales smaller than this, as the small volume required for the reaction makes the process operationally difficult, as well as resulting in a lower yield. In the procedures we describe how to use DSL in the synthesis of three target proteins—adiponectin (19–107), haemathrin 1 and haemathrin 2—which enables us to demonstrate the utility of this technology at different ligation junctions.

Synthesis of the adiponectin collagenous domain (Procedure 1)

The collagenous domain of adiponectin comprises residues 19–107. This could be accessed via a single DSL reaction at a Sec junction, which could be converted in one pot to the native Ala residue at position 41 within the sequence following the ligation reaction. We chose adiponectin as a protein of interest because of its key role in diabetes and metabolic syndrome, and, in particular, its activity as an insulin sensitizer and anti-inflammatory agent, as well as the presence of extensive post-translational modifications of the protein, namely, proline hydroxylation, as well as hydroxylation and glycosylation of lysine[18]. To access the 89-residue collagenous domain of adiponectin, in this case bearing four hydroxyproline (Hyp) residues, the sequence was divided into two fragments, an N-terminal fragment comprising residues 19–40 of the adiponectin collagenous domain, and functionalized at the C terminus as a selenoester; and a C-terminal peptide dimer fragment comprising residues 41–107 and bearing a selenocystine residue at the N terminus and Hyp at positions 44, 47, 53 and 91. Each of these fragments could be readily accessed via automated Fmoc-SPPS, followed by C-terminal functionalization in the case of the N-terminal fragment (Supplementary Figs. 1 and 2). Cys36 was incorporated with an acetamidomethyl (Acm)-protected side chain to avoid unwanted thiolactonization of the selenoester being generated. The C-terminal fragment was synthesized by standard Fmoc-SPPS with the Hyp residues incorporated (residues 44, 47, 53 and 91) as commercially available Fmoc-Hyp(O*t*Bu)-OH and Boc-Sec(PMB)-OH as the N-terminal residue (Supplementary Fig. 3). With the required fragments in hand, ligation between the N- and C-terminal portions of the adiponectin collagenous domain proceeded smoothly under standard DSL conditions within 10 min. After hexane extraction and thorough degassing, the ligation product was submitted to standard deselenization conditions, which proceeded cleanly over 16 h at room temperature (~25 °C). At this point, the full-length collagenous domain was purified via reverse-phase HPLC to afford the Acm-protected material in good yield (2.5 mg, 47%; Supplementary Figs. 4 and 5). A final AgOAc-mediated Acm deprotection was then performed to afford the native adiponectin collagenous domain in good isolated yield after reverse-phase HPLC purification (0.7 mg, 74%) and high purity as assessed by analytical reverse-phase HPLC, ESI and MALDI-TOF mass spectrometry (Fig. 2 and Supplementary Figs. 6–8). Synthesis of adiponectin (19–107) was also performed in the Department of Research Chemistry at Novo Nordisk, with yields comparable to those achieved at the University of Sydney (DSL–deselenization: 1.1 mg, 33%; Acm deprotection: 0.6 mg, 55%; Supplementary Figs. 9–11).

Synthesis of haemathrin 1 and 2 (Procedures 2 and 3, respectively)

In addition to DSL reactions at Sec, DSL is now amenable to reactions at a range of other junctions through the use of synthetic seleno-derived amino acids that can be used as Sec surrogates. Of particular note are the (β-Se)-Asp and (γ-Se)-Glu residues, both of which have been shown to undergo deselenization at unprecedented rates, enabling rapid generation of native peptide sequences via DSL[12]. We chose to exploit this potential for rapid assembly of two salivary proteins from the hard tick species *Haemaphysalis bispinosa*, haemathrin 1 and haemathrin 2, which can be disconnected at glutamic and aspartic acid residues, respectively. As for adiponectin, each of these two proteins could be assembled through a single ligation from two suitably derivatized synthetic fragments. In the case of haemathrin 1, the requisite fragments included an N-terminal peptide comprising residues 1–23, functionalized as a C-terminal selenoester (Supplementary Figs. 12 and 13), and a C-terminal fragment comprising residues 24–59, bearing an N-terminal (γ-Se)-Glu residue (Supplementary Fig. 14). For haemathrin 2, the N-terminal fragment comprised residues 1–28, functionalized as a C-terminal selenoester (Supplementary Figs. 15 and 16), whereas the C-terminal fragment comprised residues 29–59, bearing an N-terminal (β-Se)-Asp (Supplementary Figs. 17 and 18). Although each of these fragments could be readily accessed through automated Fmoc-SPPS, the presence of a glutamine residue at the C terminus of each of the N-terminal fragments allowed the synthesis of the selenoesters to be performed using a side-chain anchoring strategy whereby Fmoc-Glu-OAll was loaded to Rink amide resin to generate the corresponding side-chain amide moiety upon cleavage. Using this strategy, we were able to access the selenoester-functionalized fragments by following an on-resin selenoesterification protocol[19], which afforded the desired fragments in excellent yields and in a faster and more operationally simple manner than traditional solution-phase selenoesterification protocols. With each of the required fragments in hand, attention could then be turned to their assembly via the DSL–deselenization manifold. For each of the haemathrins, the ligation reaction once again proceeded smoothly, cleanly producing the respective full-length constructs within 10 min under standard conditions. Although the same deselenization conditions were applied for each of the haemathrins as were used for adiponectin, deselenization of the seleno-derived aspartic and glutamic

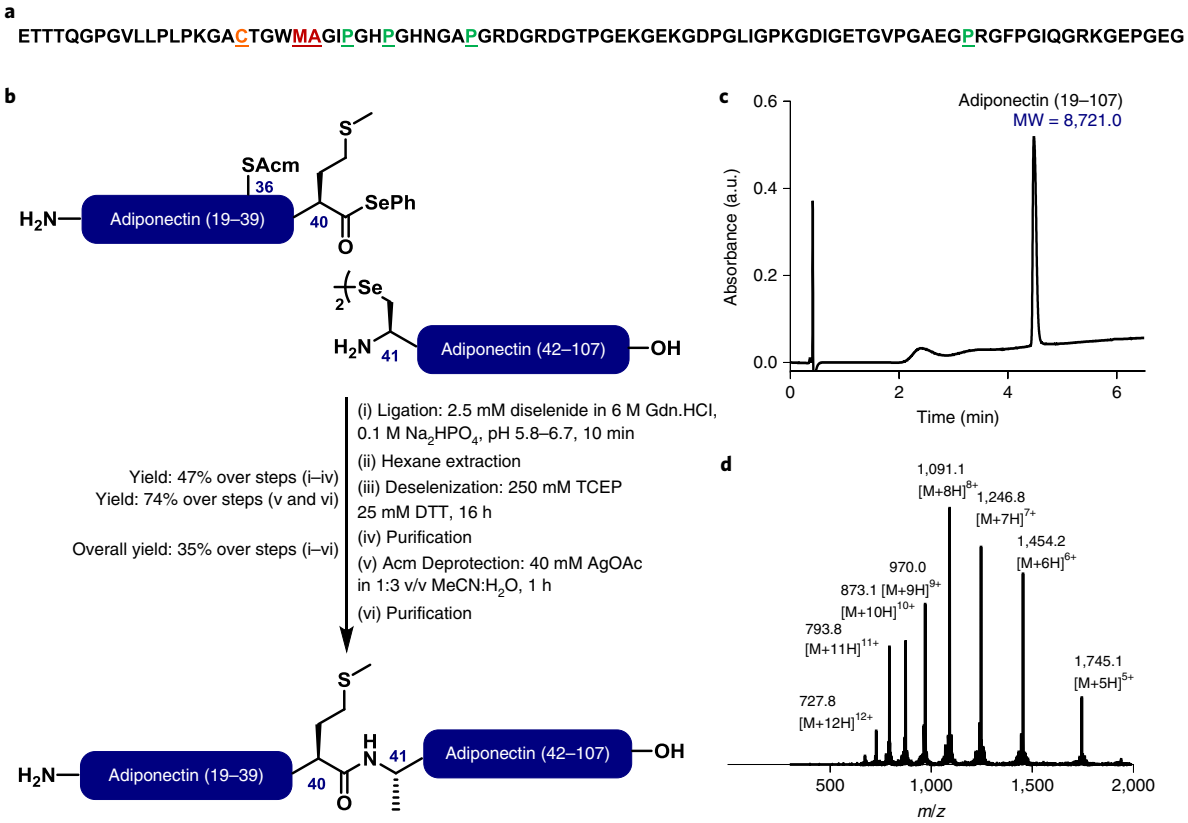

Fig. 2 | Synthesis of adiponectin (19–107) conducted in the Payne Laboratory, The University of Sydney. a, Sequence of the collagenous and variable domains of adiponectin (residues 19–107) showing the ligation junction in red and sites of proline hydroxylation in green. Cys36 (orange) is carried through the synthesis bearing an Acm protecting group. **b**, Reaction sequences for the assembly of adiponectin (19–107) from its component fragments. **c**,**d**, Analytical reverse-phase HPLC trace (**c**) and ESI(+) mass spectrum (**d**) of purified synthetic adiponectin (19–107). Mass found: 8,721.0 Da; mass calculated: 8,721.5 Da (see Supplementary Figs. 6–8 for additional data). This protocol was also performed in the Department of Research Chemistry, Novo Nordisk (see Supplementary Figs. 9–11 for data).

acid residues was complete within 10 min, meaning that each of these native full-length proteins was generated from their requisite fragments within 30 min. Each of the haemathrins was then purified via reverse-phase HPLC to afford the desired proteins in good yields over the two synthetic steps and high purity as assessed by analytical reverse-phase HPLC, ESI and MALDI-TOF mass spectrometry (Figs. 3 and 4). Synthesis of haemathrins 1 and 2 was also performed in the Department of Research Chemistry at Novo Nordisk, with yields comparable to those achieved at the University of Sydney (haemathrin 1—The University of Sydney: 5.0 mg, 61% (see Supplementary Figs. 19–21), Novo Nordisk: 8.2 mg, 52% (see Supplementary Figs. 22 and 23); haemathrin 2—The University of Sydney: 4.7 mg, 48% (see Supplementary Figs. 24–26), Novo Nordisk: 1.9 mg, 41% (see Supplementary Figs. 27 and 28)).

By using automated Fmoc-SPPS, each of the suitably functionalized peptide fragments described for use in DSL can be generated in between ~80 and 140 h (including synthesis, cleavage, functionalization, purification and lyophilization), constituting the major time investment of the protocol. This time is dependent on sequence length, difficulty (assessed on the basis of the number of double couplings and deprotections required) and the type of peptide synthesizer (or method of manual peptide synthesis) chosen. The DSL–deselenization and purification portions of the protocol take only 20 h (for the adiponectin (19–107) target where DSL is performed at Sec) or 2 h (for each of the haemathrins where DSL is performed at a (β-Se)-Asp or (γ-Se)-Glu residue). An additional 16 h of lyophilization is then required to generate the final haemathrins. It should be noted that, in the case of adiponectin, an additional 3 h is needed for Acm deprotection and purification, as well as a second 16-h lyophilization step if Cys protection is involved. We estimate that each of the target peptides can be generated within 15 d from resin loading, including the time required for lyophilization steps.

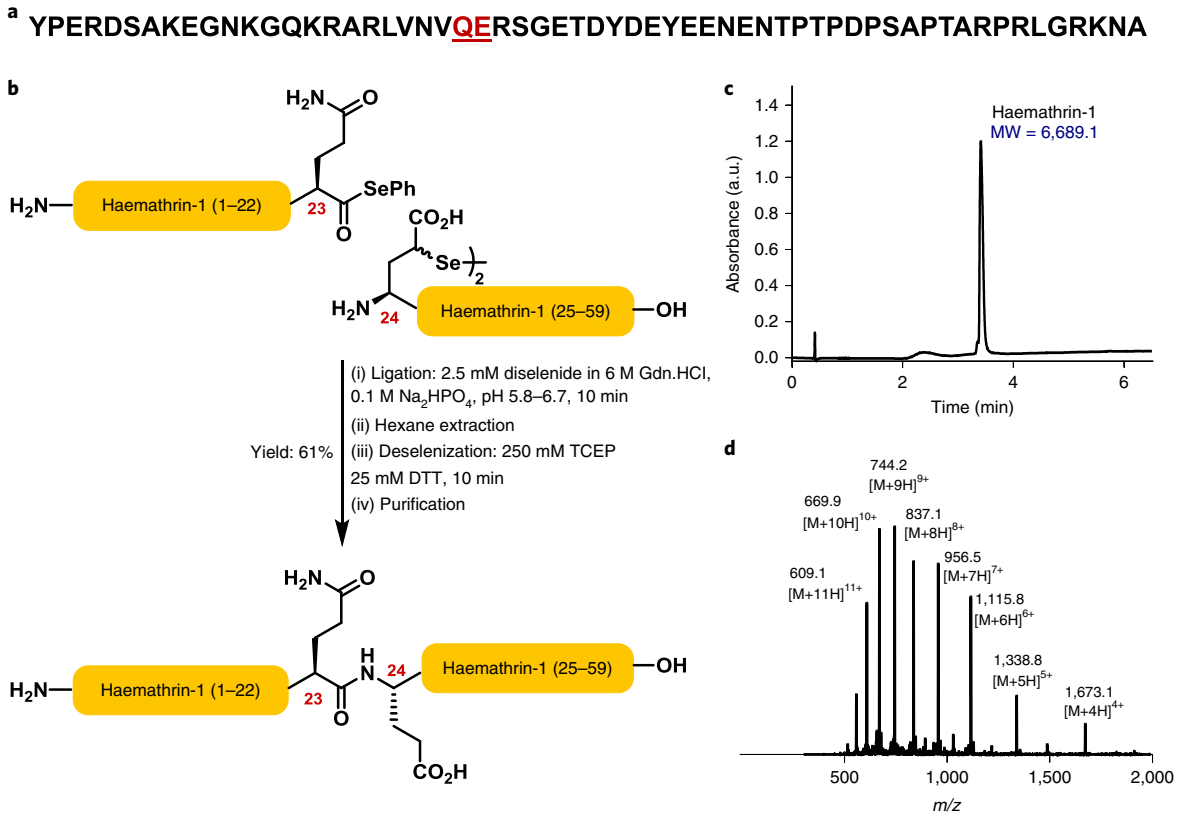

Fig. 3 | Synthesis of haemathrin 1 conducted in the Payne Laboratory, the University of Sydney. a, Sequence of haemathrin 1 showing the ligation junction in red. **b**, Reaction sequences for the assembly of haemathrin 1 from its component fragments. **c**,**d**, Analytical reverse-phase HPLC trace (**c**) and ESI(+) mass spectrum (**d**) of purified synthetic haemathrin 1. Mass found: 6,689.1 Da; mass calculated: 6,690.1 Da (see Supplementary Figs. 19–21 for additional data). This protocol was also performed in the Department of Research Chemistry at Novo Nordisk (see Supplementary Figs. 22 and 23 for data).

Materials

Reagents

! CAUTION Dichloromethane (DCM), DMF, *N*,*N*-diisopropylethylamine (DIPEA), piperidine, thioanisole, sinapinic acid and AgOAc are irritants and harmful upon ingestion, inhalation or skin contact. DPDS and *N*,*N*′-diisopropylcarbodiimide (DIC) are highly toxic. All the organic solvents are flammable, and diethyl ether, piperidine and Bu_3P are highly flammable. Bu_3P is also pyrophoric. Trifluoroacetic acid (TFA), acetic anhydride, hexafluoroisopropanol (HFIP), NaOH and HCl are corrosive. All such reagents should be used only in a fume cupboard and while wearing appropriate personal protective equipment (PPE), including safety glasses, lab coat and gloves.

- Dichloromethane (DCM; Merck, cat. no. 1060502500)
- *N*,*N*-dimethylformamide (DMF; Labscan, cat. no. AH1053-G4L (for preparation of solutions); AJAX, cat. no. 2217-2.5LGL (as wash solvent on peptide synthesizers))
- *N*,*N*-diisopropylethylamine (DIPEA; Merck, cat. no. 8008940250)
- *N*-acetylglycine (Bachem, cat. no. E.10550500)
- Acetic anhydride (AJAX, cat. no. 5-2.5LGL)
- Methanol (MeOH; Merck, cat. no. 1060092511)
- Piperidine (Merck, cat. no. 8222990500)
- Oxyma (Mimotopes, cat. no. 99969-0500)
- *N*,*N*’-diisopropylcarbodiimide (DIC; AK Scientific, cat. no. D053-500G)
- Trifluoroacetic acid (TFA; AJAX, cat. no. 2519-500mL)
- Triisopropylsilane (TIS; Sigma-Aldrich, cat. no. 233781-50G)
- Thioanisole (Fluka, cat. no. 88470-100ML)
- Hexafluoroisopropanol (HFIP, Procedure 1 only; Merck, cat. no. 8045150100)

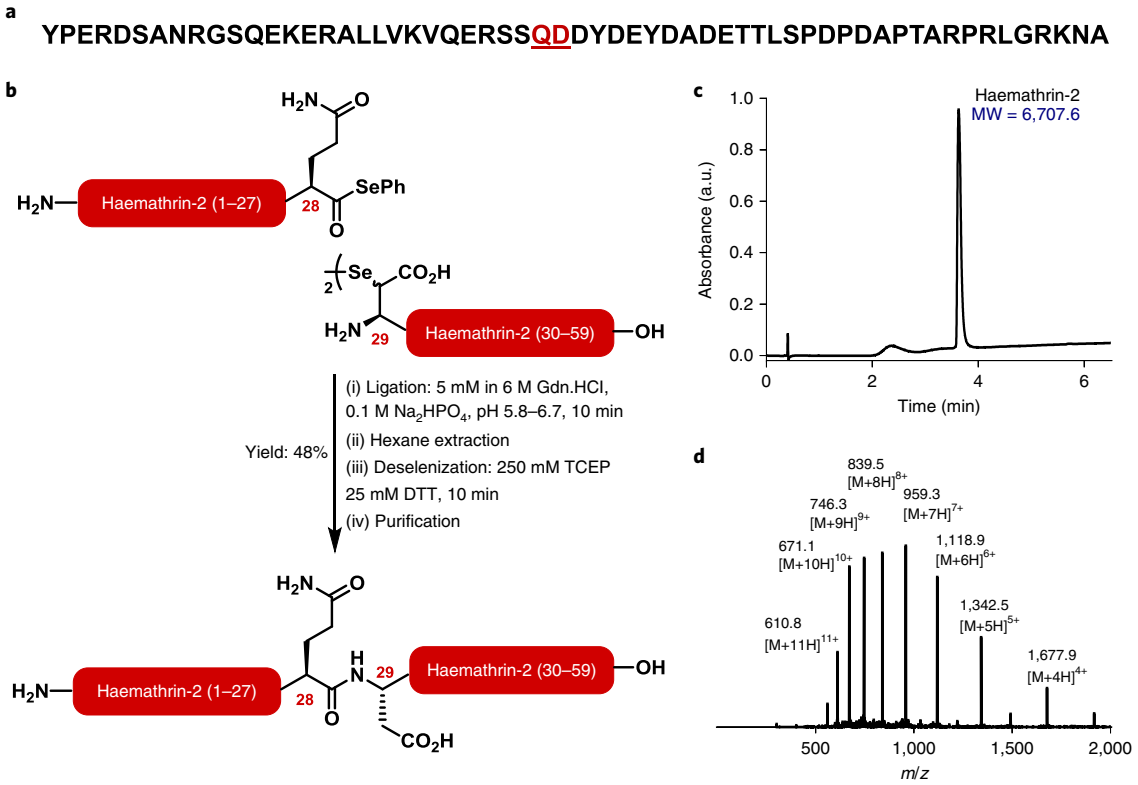

Fig. 4 | Synthesis of haemathrin 2 conducted in the Payne Laboratory, the University of Sydney. a, Sequence of haemathrin 2 showing the ligation junction in red. **b**, Reaction sequences for the assembly of haemathrin 2 from its component fragments. **c**,**d**, Analytical reverse-phase HPLC trace (**c**) and ESI(+) mass spectrum (**d**) of purified synthetic haemathrin 2. Mass found: 6,707.6 Da; mass calculated: 6,709.1 Da (see Supplementary Figs. 24–26 for additional data). This protocol was also performed in the Department of Research Chemistry at Novo Nordisk (see Supplementary Figs. 27 and 28 for data).

- Fmoc-Pro(O*t*Bu)-OH (Hyp) (Procedure 1 only; Mimotopes, cat. no. 21303-025)
- Guanidine hydrochloride (Gdn.HCl; Sigma-Aldrich, cat. no. G4505-1KG)
- Na_2HPO_4 (AJAX, cat. no. 621-500G)
- HEPES (Procedures 1 and 3 only; Sigma, cat. no. H4034-500G)
- TCEP (AK Scientific, cat. no. X4741-25G)
- DTT (AK Scientific, cat. no. X4647-25G)
- NaOH (Sigma-Aldrich, cat. no. 06306-2.5KG)
- HCl (AJAX, cat. no. 256-2.5LPL)
- Deionized water
- Acetonitrile (MeCN; Sigma-Aldrich, cat. no. 34851-4×4L)
- ProTide CTC resin (100–200 mesh, Procedure 1 only; John Morris, cat. no. 1249617-5G)
- Boc-Glu-(O*t*Bu)-OH (Procedure 1 only; Mimotopes, cat. no. 30605-025)
- Diphenyl diselenide (DPDS; Sigma-Aldrich, cat. no. 42944-10G)
- Bu_3P (ACROS, cat. no. 139345000)
- Diethyl ether (Sigma-Aldrich, cat. no. 346136-1L)
- HOAt (Mimotopes, cat. no. 00601-025)
- DMSO (Procedures 1 and 3 only; Sigma-Aldrich, cat. no. 276855-100mL)
- AgOAc (Procedure 1 only; ALFA, cat. no. 011660.14)
- Rink amide polystyrene resin (Procedure 2 only; Mimotopes, cat. no. 49001-25G)
- Fmoc-Glu-OAll (Procedures 2 and 3 only; Mimotopes, cat. no. 36613-025)
- Boc-Tyr(O*t*Bu)-OH (Procedures 2 and 3 only; Mimotopes, cat. no. 31809-025)
- $Pd(PPh_3)_4$ (Procedures 2 and 3 only; Sigma-Aldrich, cat. no. 216666-5G)
- Phenylsilane ($PhSiH_3$, Procedures 2 and 3 only; Sigma-Aldrich, cat. no. 335150-25G)
- 2,2′-Dithiobis(5-nitropyridine) (DTNP, Procedure 2 only; Sigma-Aldrich, cat. no. 158194-10G)
- ChemMatrix Rink amide resin (100–200 mesh, Procedure 3 only; Biotage GB, cat. no. 7-600-1310-25)

- 2-Chlorotrityl chloride polystyrene resin (100–200 mesh, 1 mol% divinylbenzene (DVB), Procedures 2 and 3 only; Novabiochem, cat. no. 8.55017.0025)
- Ethanol (Merck, cat. no. 1009832500)
- Sinapinic acid (Sigma-Aldrich, cat. no. D7927-5G)
- Hexane (Ajax, cat. no. 251-200L)
- Boc-L-Sec(Mob)-OH (Iris Biotech, cat. no. BAA3760)
- Boc-beta-(Mob-Se)-L-Asp(*t*Bu)-OH (Iris Biotech, cat. no. BAA3920)
- Thionyl chloride (Merck, cat. no. 8081540500)
- Hydrazine monohydrate (Merck, cat. no. 8046080250)

Standard Fmoc-protected amino acids (all residues below are L-amino acids)

- Fmoc-Ala-OH (Mimotopes, cat. no. 35001-500)
- Fmoc-Cys(Trt)-OH (Mimotopes, cat. no. 35202-100)
- Fmoc-Cys(Acm)-OH (Mimotopes, cat. no. 36302-100)
- Fmoc-Asp(O*t*Bu)-OH (Mimotopes, cat. no. 36501-100)
- Fmoc-Glu(O*t*Bu)-OH (Mimotopes, cat. no. 36607-100)
- Fmoc-Phe-OH (Mimotopes, cat. no. 35701-500)
- Fmoc-Gly-OH (Mimotopes, cat. no. 35301-500)
- Fmoc-His(Trt)-OH (Mimotopes, cat. no. 36701-100)
- Fmoc-Ile-OH (Mimotopes, cat. no. 35401-100)
- Fmoc-Lys(Boc)-OH (Mimotopes, cat. no. 36802-100)
- Fmoc-Leu-OH (Mimotopes, cat. no. 35501-100)
- Fmoc-Met-OH (Mimotopes, cat. no. 35601-500)
- Fmoc-Asn(Trt)-OH (Mimotopes, cat. no. 35102-100)
- Fmoc-Pro-OH (Mimotopes, cat. no. 35801-500)
- Fmoc-Gln(Trt)-OH (Mimotopes, cat. no. 36301-100)
- Fmoc-Arg(Pbf)-OH (Mimotopes, cat. no. 36401-100)
- Fmoc-Ser(O*t*Bu)-OH (Mimotopes, cat. no. 36102-100)
- Fmoc-Thr(O*t*Bu)-OH (Mimotopes, cat. no. 36202-100)
- Fmoc-Val-OH (Mimotopes, cat. no. 36001-100)
- Fmoc-Trp(Boc)-OH (Mimotopes, cat. no. 35903-100)
- Fmoc-Tyr(O*t*Bu)-OH (Mimotopes, cat. no. 36901-100)

Equipment

- Analytical balance (dual range; Mettler Toledo, model no. XS105)
- Magnetic stir bars (Rowe, cat. no. PM2358)
- Magnetic stirrer plate (Heidolph, model no. MR Hei-Tec)
- MALDI-TOF mass spectrometer (autoflex speed LRF MALDI-TOF mass spectrometer; Bruker, cat. no. 265320)
- Set of adjustable pipettes (2–20, 10–100, 20–200 and 200–1,000 µL; Gilson, cat. nos. F144056, F144057, F144058 and F144058)
- Pipette tips (2–20, 20–200 and 200–1,000 µL; Gilson, cat. nos. F161450, F161930 and F161670)
- Glass vials and caps (Lomb, cat. no. 33275751)
- Disposable polypropylene fritted reaction vessels (2.5, 5 and 10 mL; Torviq, cat. nos. SF-0250, SF-500 and SF-1000)
- Pressure caps for disposable reaction vessels (Torviq, cat. no. PC-SF)
- Needles (18 (blunt), 19 and 23 gauge; 32–38-mm; Livingstone, cat. nos. BD300204, DN19GX1.5LV and DN23GX1.25LV)
- Needles (21 gauge, 120 mm; Braun Sterican, cat. no. 4665643)
- Disposable polyethylene syringes (1, 3, 5, 10 and 20 mL; Livingstone, cat. nos. DSL001MLS, DSL003MLS, DSL005MLS and DSL010MLSC)
- Peptide synthesizers (Alstra Initiator+ peptide synthesizer, Biotage, model. no. 356018; Liberty Blue automated microwave peptide synthesizer (CEM, model no. 925610); or Symphony peptide synthesizer, Gyros Protein Technologies, model no. SMPS-230)
- Vessels for peptide synthesizers (CEM, part no. 167260; Biotage, cat. no. 356290; Gyros, cat. no. SMP-RV-500)

- Mechanical shaker (orbital shaker; Stuart, model no. SSL1)
- Centrifuges (Eppendorf, model no. 5430R with model no. F-35-6-30 rotor; and VWR, Ministar Silverline model)
- Preparative reverse-phase HPLC system (multisolvent delivery system, Waters, model no. 600E with a Rheodyne, model no. 7725i injection valve (4-mL loading loop) and Waters, model no. 500 pump with a Waters, model no. 490E programmable wavelength detector operating at 214, 230, 254 or 280 nm (Waters; model nos. 2535, 2489, 7725i))
- HPLC tubes (5 mL; Livingstone, cat. no. CT12X075B)
- Analytical UPLC system (UPLC system equipped with photodiode array (PDA) eλ detector (λ = 210–400 nm), Sample Manager FAN and Quaternary Solvent Manager (H-class) modules (Waters, Acquity H-Class plus model)
- UPLC vials (Waters, cat. no. 186002639)
- UPLC–MS system equipped with an LC-M20A pump, a Shimadzu 2020 ESI mass spectrometer operating in positive mode unless otherwise stated, and an SPD-M30A diode array detector (Shimadzu; model nos. LC-30AD, SPD-M30A, SIL-30AC, CTO-20A and CBM-20A)
- UPLC–MS vials (Finneran, cat. no. 30509P-1232)
- Lyophilizer (Labconco, FreeZone 4.5Plus model)
- Lyophilizer vessels (900, 300 and 150 mL; Labconco; cat. nos. 7540900, 7540600 and 7540400)
- UV spectrophotometer (UV–VIS spectrophotometer; Shimadzu, model no. UV-1280)
- Liquid nitrogen container (vacuum flask; Chemglass Life Sciences, model no. CG-1593-01)
- Sonicator (Elma, model no. Elmasonic S30)
- Rotary evaporator (Buchi, model no. Rotavapor R-210 with V-850 vacuum controller and V-700 vacuum pump)
- Vacuum pump (Edwards, model no. E2M1.5)
- pH meter (LAQUAtwin compact pH meter; Horiba Scientific, model no. pH-22)
- Eppendorf tubes (1.5, 2 mL; Eppendorf, cat. nos. 0030120086 and 0030120094)
- Rubber septa (Suba-Seal B10, B14, B19 and B24; Sigma-Aldrich, cat nos. Z124567, Z124591, Z124621, Z124656)
- Round-bottom flasks (10, 50, 100 mL; Rowe, cat. nos. GF0190, GF0032 and GQ3078)
- Volumetric flask (10 mL; Rowe, cat. no.GF9020)
- Micro-syringes (25, 100, 500 µL; Hamilton, cat. nos. 80200, 81000 and 81217)
- Paired quartz cuvettes (Starna., cat. no. 29-Q-10)
- Syringe filter (Luer lock PTFE, 25 mm × 0.22 µm; GS-Tek, part no. SP02555)
- Amber Schott bottles (Livingstone, cat. no. DB0250A)
- Parafilm (Livingstone, cat. no. PM996)
- Column (Sunfire; 19 × 150 mm, C18, 5 µm, 130 Å; Waters, cat. no. 186002568)
- Column (X-Bridge; 10 × 250 mm, C18, 5 µm, 300 Å; Waters, cat. no.186003946)
- Column (X-Bridge; 30 × 150 mm, C18, 5 µm, 130 Å; Waters, cat. no. 186002990)

Reagent setup

Fmoc deprotection solution: piperidine in DMF (20 vol%)

Mix 100 mL of piperidine with 400 mL of DMF. Once made, this solution can be stored for 1 month at room temperature if kept out of direct light. We find it best to store this solution in amber Schott bottles. **! CAUTION** Piperidine is flammable and toxic; DMF is flammable.

Capping solution for Cl-TCP(Cl) ProTide Resin and Novabiochem CTC resin

This solution is a 17:2:1 (vol/vol/vol) mixture of DCM, MeOH and DIPEA. Mix 170 mL of DCM with 20 mL of MeOH and 10 mL of DIPEA. The solution can be stored for 1 month at room temperature. **! CAUTION** MeOH and DIPEA are flammable.

Capping solution for manual synthesis and automated synthesis on Symphony and Biotage peptide synthesizers

This solution is 0.3 M acetic anhydride, 0.3 M DIPEA in DMF. Mix 5.65 mL of acetic anhydride and 10.45 mL of DIPEA with 184 mL of DMF. This solution can be stored for 1 week at room temperature. **! CAUTION** Acetic anhydride is corrosive; DIPEA and DMF are flammable.

Capping solution for automated peptide synthesis on a CEM peptide synthesizer
This solution is 0.2 M *N*-acetylglycine in DMF. Mix 4.7 g of *N*-acetylglycine with 200 mL of DMF. This solution is stable for 1 week at room temperature. **! CAUTION** DMF is flammable.

Oxyma in DMF (0.3 M)
Mix 10.6 g of Oxyma with 250 mL of DMF. This solution is stable for 1 month at room temperature. **! CAUTION** DMF is flammable.

Oxyma in DMF (0.5 M)
Mix 10.6 g of Oxyma with 150 mL of DMF. This solution is stable for 1 month at room temperature. **! CAUTION** DMF is flammable.

Oxyma in DMF (1 M)
Mix 10.6 g of Oxyma with 75 mL of DMF. This solution is stable for 1 month at room temperature. **! CAUTION** DMF is flammable.

DIC in DMF (0.3 M)
Mix 11.6 mL of DIC with 238 mL of DMF. This solution is stable for 1 month at room temperature. **! CAUTION** DIC is highly toxic and DMF is flammable.

DIC in DMF (0.5 M)
Mix 11.6 mL of DIC with 138 mL of DMF. This solution is stable for 1 month at room temperature. **! CAUTION** DIC is highly toxic and DMF is flammable.

TFA cleavage cocktail
This solution is 90:5:5 (vol/vol/vol) TFA/TIS/H_2O. Mix 4.5 mL of TFA with 0.25 mL of TIS and 0.25 mL of H_2O. **! CAUTION** TFA is corrosive. **▲ CRITICAL** Prepare fresh solution before use.

TFA cleavage cocktail containing thioanisole
This solution is 85:5:5:5 (vol/vol/vol/vol) TFA/TIS/H_2O/thioanisole. Mix 4.25 mL of TFA with 0.25 mL of TIS, 0.25 mL of thioanisole and 0.25 mL of H_2O. **! CAUTION** TFA is corrosive. **▲ CRITICAL** Prepare fresh solution before use.

HFIP cleavage cocktail
This solution is 30 vol% HFIP in DCM. Mix 1.5 mL of HFIP with 3.5 mL of DCM. **! CAUTION** HFIP is corrosive. **▲ CRITICAL** Prepare fresh solution before use.

Aqueous NaOH (6 M)
Mix 4.8 g of solid NaOH with 20 mL of H_2O. This solution is stable for 3 months at room temperature. **! CAUTION** Solid NaOH and solutions containing NaOH are corrosive. Dissolution of NaOH is exothermic; add small portions of the solid to water.

Aqueous NaOH (1 M)
Mix 40 g of solid NaOH with 1 L of H_2O. This solution is stable for 3 months at room temperature. **! CAUTION** Solid NaOH and solutions containing NaOH are corrosive. Dissolution of NaOH is exothermic, add small portions of the solid to water.

Aqueous HCl (1 M)
Mix 100 mL of concentrated HCl (32 vol%) with 900 mL of H_2O. This solution is stable for 3 months at room temperature. **! CAUTION** Concentrated HCl and solutions containing HCl are corrosive. Dissolution of HCl is exothermic; ensure that small volumes of the acid are added to the H_2O and not vice versa.

PMB deprotection buffer
This solution is 6 M Gdn.HCl, 0.2 M HEPES, pH 6.0. Mix 57.3 g of Gdn.HCl and 4.8 g of HEPES with 40 mL of deionized H_2O. Adjust the pH of the solution to pH 6.0 using 1 M NaOH and 1 M HCl. Bring the final volume of the solution up to 100 mL using deionized H_2O. Note that HEPES is used as

the buffer for PMB deprotection instead of phosphate because HEPES is more readily soluble in organic solvents. This solution is stable for 3 months at room temperature.

Ligation buffer
This solution is 6 M Gdn.HCl, 0.1 M Na_2HPO_4, pH 7.0. Mix 57.3 g of Gdn.HCl and 1.4 g of Na_2HPO_4 with 40 mL of deionized H_2O. Adjust the pH of the solution to pH 7.0 using 1 M NaOH and 1 M HCl. Bring the final volume of the solution up to 100 mL using deionized H_2O. This solution is stable for 3 months at room temperature.

Deselenization buffer
This solution is 250 mM TCEP, 25 mM DTT in ligation buffer. Mix 143 mg of TCEP and 7.7 mg of DTT in 1.5 mL of ligation buffer. Adjust the pH of the resulting solution to 4.5–5.5 using 6 M aqueous NaOH solution. Bring the final volume of the solution up to 2 mL using ligation buffer. ▲ **CRITICAL** Prepare fresh solution before use.

0.1 vol% TFA in H_2O
Mix 4 mL of TFA with 4 L of deionized water. This solution is stable for 1 month at room temperature. ! **CAUTION** TFA is corrosive.

0.1 vol% TFA in acetonitrile
Mix 4 mL of TFA with 4 L of HPLC-grade acetonitrile. This solution is stable for 1 month at room temperature. ! **CAUTION** TFA is corrosive and acetonitrile is flammable.

0.1 vol% TFA in 1:1 (vol/vol) H_2O/acetonitrile
Mix 100 mL of 0.1 vol% TFA in H_2O with 100 mL of 0.1 vol% TFA in acetonitrile. This solution is stable for 1 month at room temperature. ! **CAUTION** TFA is corrosive and acetonitrile is flammable.

Boc-Sec(PMB)-OH
Prepare this building block as reported in ref. [11]. This reagent is also commercially available as Boc-L-Sec(Mob)-OH from Iris Biotech (cat. no. BAA3760). This building block is stable for 6 months when stored at -20 °C.

Boc-(β-SePMB)-Asp(O*t*Bu)-OH
Prepare this building block as reported in ref. [12]. This reagent is also commercially available as Boc-beta-(Mob-Se)-L-Asp(*t*Bu)-OH from Iris Biotech (cat. no. BAA3920). This building block is stable for 6 months when stored at -20 °C.

Boc-(γ-SePMB)-Glu(O*t*Bu)-OH
Prepare this building block as reported in ref. [12]. ▲ **CRITICAL** Prepare this building block fresh from the allyl ester-protected precursor before use.

0.1 vol% Formic acid in H_2O
Mix 4 mL of formic acid (FA) with 4 L of deionized water. This solution is stable for 1 month at room temperature. ! **CAUTION** FA is corrosive.

0.1 vol% FA in acetonitrile
Mix 4 mL of FA with 4 L of HPLC-grade acetonitrile. This solution is stable for 1 month at room temperature. ! **CAUTION** FA is corrosive and acetonitrile is flammable.

25 vol% Acetonitrile in H_2O with 0.1 vol% TFA
Mix 2 mL of 0.1 vol% TFA in acetonitrile with 6 mL of 0.1 vol% TFA in deionized H_2O. The solution is stable for 1 month at room temperature. Degas the solution thoroughly before use. ! **CAUTION** Acetonitrile is flammable.

Matrix solution A
Prepare a saturated solution of sinapinic acid in 0.5 mL of ethanol. ▲ **CRITICAL** Prepare fresh solution before use. ! **CAUTION** Ethanol is flammable.

Matrix solution B

Prepare a saturated solution of sinapinic acid in 150 µL of acetonitrile and 350 µL of 0.1 vol% TFA in H_2O.
▲ **CRITICAL** Prepare fresh solution before use. ! **CAUTION** TFA is corrosive and acetonitrile is flammable.

Equipment setup

Biotage Alstra Initiator+ peptide synthesizer

Use an Initiator+ automated synthesizer running at a scale of 50–200 µmol, with volumes varying depending on scale. All steps are conducted at room temperature unless otherwise stated. Syntheses are conducted using standard Fmoc-SPPS protocols with the following reagents.

- *Coupling solutions*. Fmoc-protected amino acids (0.3 M in DMF), Oxyma (0.5 M in DMF) and DIC (0.5 M in DMF)
- *Capping solution*. DIPEA and acetic anhydride (each 0.3 M in DMF as a single solution)
- *Deprotection solution*. 20 vol% piperidine in DMF. A standard single-coupling synthesis program is as follows: 2 × 5 mL of 20 vol% piperidine in DMF, 5 min; 4 × 5 mL of DMF resin wash, 30 s; 1 × 2–6 mL of 1:1:1 molar ratio Oxyma/DIC/amino acid in 4 equiv. excess over the resin, 20 min at 50 °C; 4 × 5 mL of DMF resin wash, 30 s; 1 × 5 mL of 0.3 M acetic anhydride, 0.3 M DIPEA in DMF, 5 min; 4 × 5 mL of DMF resin wash, 30 s

CEM Liberty Blue automated microwave peptide synthesizer

Use a Liberty Blue automated synthesizer running at a scale of 50–200 µmol, with volumes varying depending on scale. All steps are conducted at room temperature unless otherwise stated. Syntheses are conducted using standard Fmoc-SPPS protocols with the following reagents:

- *Coupling solutions*. Fmoc-protected amino acids (0.2 M in DMF), Oxyma (1 M in DMF) and DIC (0.5 M in DMF)
- *Capping solution*. *N*-acetylglycine (0.2 M in DMF), Oxyma (1 M in DMF) and DIC (0.5 M in DMF)
- *Deprotection solution*. 20 vol% piperidine in DMF. A standard single-coupling synthesis program is as follows: 4–10 mL of 20 vol% piperidine in DMF, 1 × 1 min at 90 °C or 1 × 3 min at 75 °C or 2 × 3 min at 25 °C; 3 × 5–10 mL of DMF resin wash, 30 s; 1 × 4–8 mL of a 1:1:1 molar ratio Oxyma/DIC/amino acid in 5 equiv. excess over the resin, 20 min at 50 °C; 2 × 5 mL of DMF resin wash, 30 s; 1:1:1 (vol/vol/vol) molar ratio Oxyma/DIC/*N*-acetylglycine in 5 equiv. excess over the resin, 2 min at 50 °C; 3 × 5–10 mL DMF resin wash, 30 s

Gyros Protein Technologies Symphony peptide synthesizer

Use a Symphony automated synthesizer running at a scale of 200 µmol. All steps are conducted at room temperature.

- *Coupling solutions*. Fmoc-protected amino acids (0.3 M in DMF), Oxyma (0.3 M in DMF) and DIC (0.3 M in DMF)
- *Capping solution*. DIPEA and acetic anhydride (each 0.3 M in DMF as a single solution)
- *Deprotection solution*. 20 vol% piperidine in DMF. Single couplings are conducted for 1 × 45 min, whereas double couplings are conducted for 2 × 45 min. A standard single-coupling synthesis program is as follows: 2 × 5 mL of 20 vol% piperidine in DMF, 5 min; 4 × 5 mL of DMF resin wash, 30 s; 1 × 7.5 mL of a 1:1:1 molar ratio of Oxyma/DIC/amino acid in a 4 equiv. excess over the resin, 45 min; 4 × 5 mL of DMF resin wash, 30 s; 1 × 5 mL of 0.3 M acetic anhydride, 0.3 M DIPEA in DMF, 3 min; 4 × 5 mL of DMF resin wash, 3 min

Analytical UPLC–MS

Use a Shimadzu UPLC–MS instrument consisting of an LC-M20A pump and a SPD-M30A diode array detector coupled to a Shimadzu 2020 mass spectrometer with ESI operating in positive mode. Separations are performed using a Waters Acquity UPLC bridged ethylene hybrid (BEH) 1.7-µm, 300-Å, 2.1 × 50-mm column (C-18) at a flow rate of 0.6 mL/min. Separations are performed using a mobile phase of 0.1 vol% FA in H_2O (solvent A) and 0.1vol% FA in MeCN (solvent B) and a linear gradient of 0–50 vol% B over 8 min. All samples should be filtered with a 0.22-µm syringe filter before injection to prevent blockage of the system.

Analytical HPLC

Use a Waters Acquity UPLC system equipped with a PDA eλ detector (λ = 210–400 nm), Sample Manager FAN and Quaternary Solvent Manager (H-class) modules using an X-Bridge BEH 300-µm,

Table 1 | Conditions for the purification of target peptides

Fragment	Scale (flow rate)	Column	Gradient
Adipo. (19–40)	Prep (20 mL/min)	Sunfire 19 × 150 mm, C18, 5 μm, 130 Å	15–75 vol% B over 60 min
Adipo. (41–107)	Prep (7 mL/min)	Sunfire 19 × 150 mm, C18, 5 μm, 130 Å	0–50 vol% B over 70 min
Adipo. (19–107) C36Acm	Prep (7 mL/min)	Sunfire, 19 × 150 mm, C18, 5 μm, 130 Å	0–50 vol% B over 70 min
Adipo. (19–107)	Semi-prep (4 mL/min)	X-Bridge 10 × 250 mm, C18, 5 μm. 300 Å	0–50 vol% B over 50 min
Haem. 1 (1–23)	Super-prep (40 mL/min)	X-Bridge 30 × 150 mm, C18, 5 μm, 130 Å	0–40 vol% B over 40 min
Haem. 1 (24–59)	Prep (10 mL/min)	Sunfire 19 × 150 mm, C18, 5 μm, 130 Å	0–50 vol% B over 60 min
Haem. 1 (1–59)	Semi-prep (4 mL/min)	X-Bridge 10 × 250 mm, C18, 5 μm, 300 Å	0–40 vol% B over 40 min
Haem. 2 (1–28)	Super-prep (40 mL/min)	X-Bridge, 30 × 150 mm, C18, 5 μm, 130 Å	0–40 vol% B over 40 min
Haem. 2 (29–59)	Prep (10 mL/min)	Sunfire 19 × 150 mm, C18, 5 μm, 130 Å	0–50 vol% B over 50 min
Haem. 2 (1–59)	Semi-prep (4 mL/min)	X-Bridge 10 × 250 mm, C18, 5 μm, 300 Å	0–40 vol% B over 40 min

Adipo., adiponectin; Haem., haemathrin.

2.1 × 50-mm column (C-18). Samples are run at a flow rate of 0.6 mL/min using a mobile phase comprising 0.1 vol% TFA in H_2O (solvent A) and 0.1 vol% TFA in MeCN (solvent B) in a linear gradient of 0–50 vol% B over 5 min. Analysis of the chromatograms is conducted and retention time (Rt (min)) is recorded using Empower 3 Pro software at $\lambda = 214$ nm.

Semi-preparative HPLC

Use a Waters HPLC system with a 4-mL loading loop and Waters 500 pump with a Waters 490E programmable wavelength detector operating at 214, 230, 254 or 280 nm and a Waters X-Bridge-BEH300 wide-pore C18 column (300 Å, 5 μm, 10 × 250 mm) at a flow rate of 4 mL/min. For haemathrin final ligation products, use a mobile phase of 0.1 vol% FA in H_2O (solvent A) and 0.1 vol% FA in MeCN (solvent B) using the indicated linear gradient (Table 1). For all other semi-preparative HPLC procedures, use a mobile phase of 0.1 vol% TFA in H_2O (solvent A) and 0.1 vol% TFA in MeCN (solvent B) with the linear gradients specified (Table 1).

Preparative HPLC

Use a Waters HPLC system with a 4-mL loading loop and Waters 500 pump with a Waters 490E programmable wavelength detector operating at 214, 230, 254 or 280 nm and a Waters Sunfire C18 column (130 Å, 5 μm, 10 × 250 mm) at a flow rate of 7, 10 or 20 mL/min. Use a mobile phase of 0.1 vol% TFA in H_2O (solvent A) and 0.1 vol% TFA in MeCN (solvent B) with the linear gradients specified (Table 1).

Super-preparative HPLC

Use a Waters HPLC system with a 4-mL loading loop and Waters 500 pump with a Waters 490E programmable wavelength detector operating at 214, 230, 254 or 280 nm and a Waters X-Bridge C18 column (300 Å, 5 μm, 30 × 150 mm) at a flow rate of 40 mL/min. Use a mobile phase of 0.1vol% TFA in H_2O (solvent A) and 0.1 vol% TFA in MeCN (solvent B) with the linear gradients specified (Table 1).

Degassing apparatus for deselenization reaction mixture

Place a B10 Suba-Seal on top of the Eppendorf tube containing the deselenization reaction mixture, ensuring that the total liquid volume does not exceed half of the total capacity of the tube. Carefully pierce the seal with a 23-gauge × 32-mm needle and a 19-gauge × 120-mm needle. Position the needles so that the longer one is just submerged in the reaction mixture and the shorter one has only just penetrated the seal. Connect a balloon filled with $Ar_{(g)}$ to the inlet of the longer needle, using a 3-mL disposable syringe as an adaptor (see Fig. 5f for an image of this setup). Care should be taken so as not to expel the reaction mixture from the Eppendorf tube. The likelihood of this can be lowered by using only a single balloon (rather than multiple balloons, which are conventionally used in reaction setups) and only partially filling the balloon so as to reduce the pressure generated. The flow rate can also be controlled by pinching the neck of the balloon to achieve an ideal rate, at which a

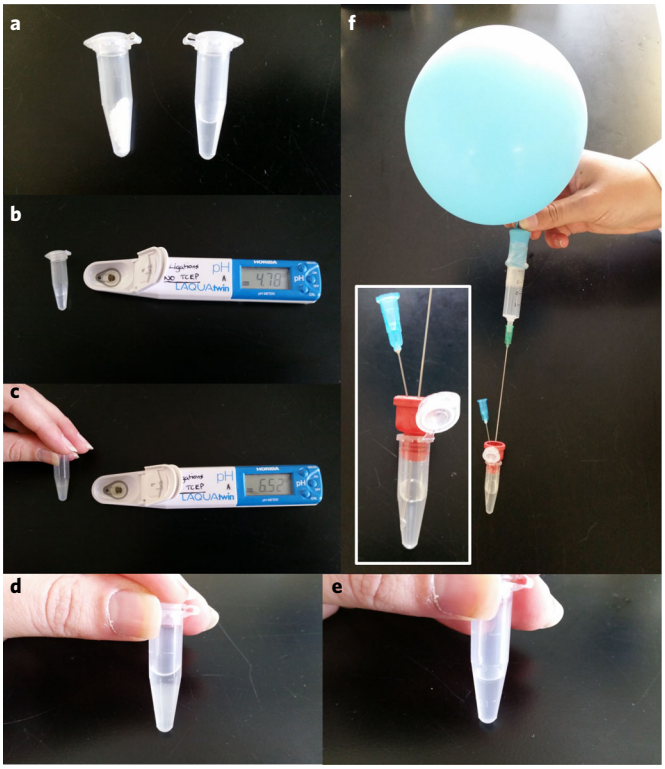

Fig. 5 | Photographic representation of the DSL–deselenization procedure. a, The ligation setup is conducted by first dissolving the diselenide fragment (right) in 6 M Gdn.HCl, 0.1 M Na_2HPO_4, pH 7.2 buffer to a concentration of 5 mM. This solution is then added to the selenoester-functionalized fragment (left). **b**, The initial pH of the ligation mixture is typically between pH 4.0 and 5.0 (depending on the peptide sequence), and the solution is clear. **c**, The pH of the ligation mixture is then adjusted to between pH 5.8 and 6.7, at which point the solution begins to go cloudy, indicating the formation of insoluble DPDS. **d**, After completion of the ligation, hexane is added, which forms an immiscible layer on top of the ligation mixture. The solution is mixed thoroughly, and any DPDS present is extracted in the organic layer, which is then removed. **e**, After the hexane extraction step, a clear, colorless solution is once again obtained. **f**, An equal volume of deselenization buffer (250 mM TCEP, 25 mM DTT in 6 M Gdn.HCl, 0.1 M Na_2HPO_4, pH 4.5–5.5) is then added and the solution and thoroughly degassed, using the apparatus described in the 'Equipment setup' section. Inset, close-up of the apparatus.

steady stream of bubbles can be seen in the reaction but not so strong that the reaction volume is being forced up the walls of the tube.

Procedure 1

Synthesis of adiponectin (19–40) selenoester, Part 1: loading to Cl-TCP(Cl) ProTide Resin

● Timing 18 h

1 Weigh 0.5 g (240 μmol) of Cl-TCP(Cl) ProTide Resin (0.48 mmol/g) into a 10-mL fritted disposable reaction vessel and allow to swell for 40 min in dry DCM (5 mL) while shaking at room temperature using a mechanical shaker.
2 Expel the liquid from the reaction vessel and wash the resin with 10 × 5 mL of DCM.
3 Dissolve 268 mg of Fmoc-Met-OH (0.72 mmol, 3 equiv.) in 3 mL of DCM in a 21-mL glass vial, using sonication to aid dissolution, if necessary.
4 Add 615 μL of DIPEA (3.6 mmol, 15 equiv.) using a micro-syringe, and agitate the vial for 30 s at room temperature.
5 Draw up the reaction solution into the fritted syringe bearing the resin and allow the syringe to shake on a mechanical shaker at room temperature for 16 h.
6 Expel the reaction solution from the syringe and wash the resin with 5 × 5 mL of DCM, 5 × 5 mL of DMF and 5 × 5 mL of DCM.
7 Add 5 mL of CTC resin capping solution (17:2:1 (vol/vol/vol) DCM/MeOH/DIPEA) to the resin and allow the syringe to shake at room temperature for 40 min.

8 Expel the solution from the syringe and wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF.

Synthesis of adiponectin (19–40) selenoester, Part 2: determination of resin-loading ● Timing 30 min

9 Treat the resin with 3 mL of Fmoc-deprotection solution (20 vol% piperidine in DMF) for 3 min. Expel the solution from the resin and retain for subsequent measurement. Repeat the treatment twice more, again retaining the deprotection solution. Wash the resin with a further 1 mL of deprotection solution and combine with the previously retained solutions.

10 Wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF and set the resin aside until the resin loading has been determined.

11 Dilute 100 µL of the retained Fmoc-deprotection solution in a further 10 mL of fresh Fmoc-deprotection solution, using a 10-mL volumetric flask.

12 Add 1 mL of fresh Fmoc-deprotection solution to a quartz cuvette and blank the spectrophotometer at $\lambda = 301$ nm.

13 Add 1 mL of diluted Fmoc-deprotection to the paired cuvette and measure the absorbance at $\lambda = 301$ nm.

14 Calculate the resin loading using the following formula:

Resin loading (µmol) = A_{301nm} × dilution volume (mL) × dilution factor × 1,000/7,800 M/cm

Which for the above volumes simplifies to the following:

Resin loading (µmol) = A_{301nm} × 10,000/78

For loading of 2-chlorotrityl chloride resins, we typically achieve loadings of ~70% of that stated by the manufacturer.

? TROUBLESHOOTING

Synthesis of adiponectin (19–40) selenoester, Part 3: fragment extension via automated SPPS ● Timing 20 h

▲ **CRITICAL** The adiponectin (19–40) fragment is synthesized on a CEM Liberty Blue peptide synthesizer at 50 °C, using microwave heating. Detailed synthesizer conditions can be found in the 'Equipment setup' section.

15 Place 100 µmol of the resin prepared in Step 10 into an appropriate synthesizer vessel.

16 Run the fragment sequence (TTTQGPGVLLPLPKGA**C**TGW) on an automated peptide synthesizer with heating, using appropriate coupling cycles. Note that the cysteine residue (Cys, C (boldface)) should be incorporated bearing side-chain Acm protection to prevent intramolecular transesterification of the selenoester by the thiol of the unprotected cysteine side chain, leading to the formation of the corresponding thiolactone. The thiolactone intermediate would be competent in a subsequent DSL reaction under additive conditions but at a substantially reduced rate.

▲ **CRITICAL STEP** Single couplings can be used for couplings that are not anticipated to be problematic, whereas double couplings should be used for couplings that are predicted to be difficult. Potentially difficult couplings are identified as those which involve coupling of or to (i) a β-branched amino acid (Fmoc-Val-OH, Fmoc-Thr(O*t*Bu)-OH, Fmoc-Ile-OH); (ii) a secondary amine (Fmoc-Pro-OH); or (iii) an amino acid bearing a bulky side-chain protecting group (Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH). During the course of all SPPSs, the efficiency of the synthesis should be monitored via analytical acidolytic cleavage (Box 1), specifically following non-standard steps such as coupling of specialty amino acids or other on-resin synthetic transformations.

17 Upon completion of the automated SPPS, transfer the resin to a new fritted reaction syringe and wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF.

18 Repeat Steps 9 and 10 to remove the N-terminal Fmoc-protecting group. It is not necessary to retain the deprotection solutions this time.

19 Dissolve 243 mg of Boc-Glu(O*t*Bu)-OH (800 µmol, 8 equiv.) and 114 mg of Oxyma (800 µmol, 8 equiv.) in 1 mL of DMF in a 21-mL glass vial, using sonication to aid the dissolution, if necessary.

20 Add 82 µL of DIC (800 µmol, 8 equiv.) to the vial using a micro-syringe and agitate for 30 s to ensure thorough mixing before adding the coupling solution to the resin from Step 18. Note that the N-terminal residue of any selenoester-functionalized fragment is coupled as a Boc-protected amino acid so that the N terminus can remain protected during the selenoesterification step and then be deprotected during the subsequent acidic global deprotection step.

Box 1 | Analytical acidolytic cleavage ● Timing 2 h

Procedure

1 Take a small amount of dry resin (~10 resin beads) and place it in a 3-mL fritted syringe. Note that this is best done on Fmoc-deprotected material, as the high UV absorbance of the Fmoc-protecting group can give an inaccurate representation of the peptide purity.
2 Draw up ~2 mL of TFA cleavage cocktail (90:5:5 (vol/vol/vol) TFA/TIS/H_2O) and seal the syringe with a pressure cap before placing it on a mechanical shaker for 1 h at room temperature.
! CAUTION TFA is corrosive. Note that for peptides containing oxidation-prone residues such as Met, a TFA cleavage cocktail containing thioanisole (85:5:5:5 (vol/vol/vol/vol) TFA/TIS/H_2O/thioanisole) should be used instead to prevent oxidation.
3 Expel the cleavage cocktail into a 21-mL glass vial and remove the solvent under vacuum, using a rotary evaporator.
4 Re-dissolve the crude residue in ~200 µL of 1:1 (vol/vol) MeCN/H_2O with 0.1 vol% TFA and analyze peptide purity via UPLC–MS analysis. See 'Equipment setup' section for specific details.

21 Allow the resin to shake at room temperature for 1 h, and then expel the coupling solution. Treat the resin with a fresh batch of coupling solution (prepared as described in Steps 19 and 20) for a further hour at room temperature with shaking.
22 Expel the solution from the syringe and wash the resin with 5 × 5 mL of DMF and 5 × 5 mL of DCM and dry the resin.
■ PAUSE POINT Dry resin can be stored at 4 °C under inert gas for several months. Any time a resin has been dried, it should be re-swelled in DCM for 5 min with shaking before recommencing synthesis. Resin can be dried and re-swelled as necessary.

Synthesis of adiponectin (19–40) selenoester, Part 4: solution-phase selenoesterification ● Timing 24 h

23 Split the resin to perform selenoesterification at a 50-µmol scale.
24 Wash the resin with 10 × 5 mL of DCM.
25 Treat the resin with 5 mL of HFIP cleavage cocktail for 40 min at room temperature with shaking.
26 Expel the cleavage solution from the resin into a 25-mL round-bottom flask and wash the resin with 3 × 3 mL of DCM, expelling the wash solutions into the same round-bottom flask.
27 Remove the solvent from the round-bottom flask, using a rotary evaporator until a viscous oil is obtained.
28 Dry the resulting oil under high vacuum for 1 h to ensure all HFIP has been removed.
29 Add 468 mg of DPDS (30 equiv.) to the round-bottom flask and dissolve the crude peptide residue and DPDS in 3 mL of dry DMF.
30 Exchange the headspace of the reaction vessel with argon gas and place the reaction flask in an ice bath and cool to 0 °C while stirring.
31 Add 375 µL of Bu_3P (30 equiv.) to the reaction vessel dropwise, using a micro-syringe, and allow the reaction to stir at 0 °C for 3 h under argon.
32 Remove DMF and Bu_3P under a nitrogen stream overnight.
33 Re-dissolve the crude residue in 6.5 mL of TFA cleavage cocktail containing thioanisole (85:5:5:5 (vol/vol/vol/vol) TFA/TIS/H_2O/thioanisole), keeping the reaction flask on ice during this process to prevent heating if any residual DMF is present.
34 Allow the reaction to stir at room temperature, using a magnetic stirrer and stirrer bar for 2 h.
35 After this time, transfer the solution to a 25-mL Falcon tube and concentrate under a nitrogen stream until the total volume is <2 mL. At this point, add ~40 mL of pre-chilled diethyl ether to precipitate the peptide fragment.
36 Isolate the peptide precipitate via centrifugation at ~4,000*g* at 4 °C for 10 min. Carefully decant the supernatant and dry the precipitate under a gentle nitrogen flow.

Synthesis of adiponectin (19–40) selenoester, Part 5: analysis and reverse-phase HPLC purification ● Timing 5 h + overnight lyophilization

37 Dissolve the peptide pellet in ~5 mL of 1:1 (vol/vol) MeCN/H_2O with 0.1 vol% TFA and dilute to a 10-mL total volume with H_2O (with 0.1 vol% TFA) to obtain a final solution that is ~25 vol% MeCN in H_2O. Filter the resulting solution using a 0.22-µm filter. Note that the concentration of

MeCN required for dissolution is dependent on the peptide length and the polarity of the amino acid sequence. The amount of MeCN can be adjusted as required on the basis of the peptide retention time from analytical UPLC–MS. If large volumes of MeCN are required for solubilization, the HPLC gradient should be left at 100 vol% H_2O (with 0.1 vol% TFA) for at least 5 min to allow the excess MeCN to elute before the gradient is started to prevent elution of the peptide in the solvent front.

38 Take a small aliquot (~40 μL) for analysis via UPLC–MS. See 'Equipment setup' section for details.

39 Purify the bulk of the solution 5 mL at a time, using reverse-phase HPLC with 0.1 vol% TFA each in buffer A (H_2O) and B (MeCN). See Table 1 for gradient and column details. Identify clean fractions via UPLC–MS (see 'Equipment setup' section for details), subject combined pure fractions to lyophilization to obtain pure adiponectin (19–40) selenoester fragment as a fluffy white solid. See Supplementary Fig. 1 for yield and characterization.

? TROUBLESHOOTING

■ PAUSE POINT Lyophilized peptide fragments can be stored at 4 °C under inert gas for several months.

Synthesis of adiponectin (41–107) diselenide, Part 1: peptide synthesis ● Timing 118.5 h

40 Load 285 mg of Fmoc-Gly-OH (960 μmol, 3 equiv.) to 0.67 g of Cl-TCP(Cl) ProTide Resin (320 μmol scale, loading 0.48 mmol/g), using 460 μL of DIPEA (4.8 mmol, 15 equiv.) as described in Steps 1–8.

41 Fmoc-deprotect and determine the resin loading as described in Steps 9–14.

▲ CRITICAL STEP The adiponectin (41–107) fragment is synthesized on a Biotage Alstra Initiator+ peptide synthesizer at 50 °C, using microwave heating. Detailed synthesizer conditions can be found in the 'Equipment setup' section.

42 Transfer the resin to an appropriate synthesizer vessel and run the fragment sequence (GI**P**G H**P**GHNGA**P**GRDGRDGTPGEKGEKGDPGLIGPKGDIGETGVPGAEG**P**RGFPGIQGRKGEPGE), using appropriate coupling cycles. Note that the boldface residues (P44, P47, P53 and P91) are incorporated as commercially available Fmoc-Hyp(O*t*Bu)-OH residues. It is worth mentioning that the coupling of any amino acid residue to Hyp is challenging and a double coupling with 10 equiv. of the next Fmoc-protected amino acid is required to avoid incomplete coupling, which results in formation of a truncated peptide, lowering the yield.

43 Repeat Steps 9 and 10 to remove the N-terminal Fmoc-protecting group. It is not necessary to retain the deprotection solutions this time.

44 Dry and split the resin, carrying forward 20 μmol for Boc-Sec(PMB)-OH coupling.

45 Dissolve 20 mg of Boc-Sec(PMB)-OH (50 μmol, 2.5 equiv.) and 6.8 mg of HOAt (50 μmol, 2.5 equiv.) in 1 mL of DMF in a 21-mL glass vial, using sonication to aid the dissolution, if necessary.

46 Add 8 μL of DIC (50 μmol, 2.5 equiv.) to the vial, using a micro-syringe, and agitate for 30 s to ensure thorough mixing before adding the coupling solution to the resin from Step 44. Allow the resin to shake at room temperature on a mechanical shaker for 16 h.

47 Expel the coupling solution and wash the resin with 5 × 5 mL of DMF and 5 × 5 mL of DCM, followed by drying of the resin.

■ PAUSE POINT The dry resin can be stored at 4 °C under inert gas for several months. Any time resin has been dried, it should be re-swelled in DCM for 5 min with shaking before recommencing synthesis. The resin can be dried and re-swelled as necessary.

Synthesis of adiponectin (41–107) diselenide, Part 2: acidolytic cleavage and purification ● Timing 2.5 h + overnight lyophilization

48 Wash the resin with 10 × 5 mL of DCM.

▲ CRITICAL STEP Care must be taken to wash the resin thoroughly with DCM before any acidic cleavage steps, as any remaining DMF will react with the acid exothermically, potentially causing hot acidic cocktail to be expelled from the syringe. The heat generated by this reaction also has the potential to cause the peptide to decompose, substantially reducing the synthetic yield.

49 Add 5 mL of TFA cleavage cocktail (90:5:5 (vol/vol/vol) TFA/TIS/H_2O) to the resin and allow the mixture to shake at room temperature for 2 h. After this time, expel the cleavage cocktail into a 25-mL Falcon tube and wash the resin with 3 × 3 mL of DCM, retaining the washings and combining them with the cleavage cocktail in the Falcon tube.

50 Concentrate the retained acidic cocktail to a total volume of <2 mL and isolate the crude peptide fragment via ether precipitation as described in Steps 35–36.

51 Re-dissolve the pellet in ~10 mL of H_2O with 0.1 vol% TFA, adding the minimum volume of MeCN to enable dissolution and then remove the solvent via lyophilization to afford the crude peptide as a fluffy white solid.

Synthesis of adiponectin (41–107) diselenide, Part 3: PMB deprotection and purification ● Timing 8 h + overnight lyophilization

52 Re-dissolve 10 mg of the crude peptide in 1.5 mL of freshly prepared PMB deprotection cocktail (3:1:1 (vol/vol/vol) TFA/DMSO/PMB deprotection buffer) and allow the mixture to agitate gently at room temperature for 30 min, monitoring completion of the reaction via UPLC–MS. See 'Equipment setup' section for details.

▲ **CRITICAL STEP** Note that the retention time of the PMB-protected material and the diselenide do not differ greatly, and thus the mass envelope must be examined in order to determine reaction completion. This step must be done carefully because any remaining PMB-protected peptide will be very difficult to separate from the diselenide via reverse-phase HPLC and thus it is essential that complete deprotection has occurred prior to purification.

? TROUBLESHOOTING

53 Dilute the deprotection cocktail with 3 mL of H_2O and purify immediately via reverse-phase HPLC

▲ **CRITICAL STEP** Side reactions, including peptide decomposition, can occur if peptide is left in this cocktail for extended periods. This reaction is also highly concentration dependent and must be carried out at a ~7 mg/mL concentration of peptide. As a result, this reaction should be carried out on several small batches rather than a single large reaction, as each 1.5 mL of deprotection solution must be diluted to ~5 mL prior to injection onto the HPLC system to prevent the Gdn.HCl present in the ligation buffer from causing the peptide to elute prematurely in the solvent front.

54 Identify pure fractions via UPLC–MS analysis (see 'Equipment setup' section for details) and subject combined pure fractions to lyophilization to obtain the adiponectin (41–107) diselenide fragment as a fluffy white solid. See Supplementary Fig. 3 for yield and characterization.

■ **PAUSE POINT** Lyophilized peptide fragments can be stored at 4 °C under inert gas for several months.

Synthesis of adiponectin (19–107) via DSL–deselenization, Part 1: synthesis of adiponectin (19–107) (C36Acm) via DSL ● Timing 20 min

55 Weigh out 4.0 mg of lyophilized adiponectin (41–107) diselenide fragment (0.61 μmol, 1.0 equiv.) into a 1.5-mL Eppendorf tube.

56 Weigh out 1.8 mg of lyophilized adiponectin (19-40) selenoester fragment (0.73 μmol, 1.2 equiv.) into another 1.5-mL Eppendorf tube.

57 Dissolve the diselenide fragment in ligation buffer (6 M Gdn.HCl, 0.1 M Na_2HPO_4, pH 7.2; 130 μL) to a concentration of 5 mM (with respect to the monomer, selenol), using sonication to aid the dissolution process, if necessary, to ensure a clear solution is formed. Spin down the Eppendorf tube (500*g*, room temperature, 15 s) to ensure all of the solution is at the bottom of the tube. DSL reactions can also be conducted in glass vials with no effect on the reaction; however, it is usually operationally easier to conduct the reactions in Eppendorf tubes so they can be centrifuged to remove solution adhered to the tube walls.

58 Add the diselenide solution to the pre-weighed selenoester fragment and mix well to make sure the selenoester dissolves completely, following with quick centrifugation (15 s, 500*g*, room temperature).

59 The pH of the ligation mixture is then measured and adjusted to 5.8–6.7. Note that DSL can be performed at a wider pH range (3–7) and the ligation is initiated as soon as both the fragments are in ligation buffer. However, based on our experience, the rate of ligation is optimal at around pH 6.0. Usually, aqueous NaOH (1 M or 0.1 M, depending on the system) is used to carefully adjust the pH of the ligation mixture to ~6.0.

▲ **CRITICAL STEP** Careful adjustment of pH is very important because selenoesters are highly prone to hydrolysis at pH 7 and above, which results in incomplete ligations and therefore lower yields. We typically conduct pH adjustments using a small-volume microchip-based pH probe such as that pictured in Fig. 5.

60 Incubate for 10 min at room temperature and then analyze the ligation by UPLC–MS. At this point, the ligation mixture often turns cloudy (this depends on the scale and it may not be

conspicuous on scales <1 mg), indicating formation of H_2O-insoluble DPDS, a by-product produced during ligation.

? TROUBLESHOOTING

61 For UPLC–MS analysis, take a 2-μL aliquot of the ligation mixture and dilute it to 50 μL with either H_2O (0.1 vol% TFA) or MeCN:H_2O (0.1 vol% TFA), depending on the solubility of the peptide or protein being analyzed.

62 Analyze a 10-μL injection of this solution by UPLC–MS, and evaluate the ligation carefully to ascertain reaction completion.

▲CRITICAL STEP Note that the ligation often results in a mixture of products (predominantly a symmetrical diselenide, an asymmetrical diselenide and an unproductive ester; see Fig. 1), which eventually merge to the desired final protein after the one-pot deselenization step. At this stage, complete conversion of the diselenide fragment (limiting substrate) implies that the ligation has reached completion.

Synthesis of adiponectin (19–107) via DSL-deselenization, Part 2: seselenization of adiponectin (19–107) (C36Acm) ● Timing 18 h + overnight lyophilization

63 Treat the ligation mixture with hexane (5 × 200 μL) to extract DPDS, which acts as a radical quencher, hampering the deselenization process. Upon each treatment, mix the resulting solution thoroughly and centrifuge (15 s, 500*g*, room temperature), pipetting off and discarding the resulting upper organic layer.

64 Degas the freshly prepared deselenization solution by sparging with argon for 10 min to completely remove dissolved oxygen.

▲CRITICAL STEP This is an important step; it ensures elimination of the undesired oxidative deselenization pathway.

65 Add an equal volume (130 μL) of the deselenization solution to the ligation mixture, resulting in a 2.5, 125 and 12.5 mM final concentration of the ligation product (as the monomeric selenol), TCEP and DTT, respectively.

66 Sparge the reaction mixture with argon for additional 5 min; then seal the Eppendorf tube with plastic film (e.g., Parafilm) and allow the deselenization to proceed for 16 h at room temperature.

67 For UPLC–MS analysis, take a 4-μL aliquot of the reaction mixture and dilute it to 50 μL with either H_2O (0.1 vol% TFA) or MeCN/H_2O (0.1 vol% TFA), depending on the solubility of the peptide or protein being analyzed.

68 Analyze a 10 μL injection of this solution via UPLC–MS to ascertain complete deselenization of the ligation products. Note that the ligation product unproductive ester (which is unreactive during deselenization) undergoes thiolysis with DTT to give the selenol, which then undergoes smooth deselenization.

? TROUBLESHOOTING

69 Dilute the reaction mixture with H_2O (or with ligation buffer, depending on the solubility of the peptide/protein being handled) to a final volume of ~ 4 mL and purify via reverse-phase HPLC (see Table 1 for conditions). Identify pure fractions via careful UPLC–MS analysis (see 'Equipment setup' section) and subject combined pure fractions to lyophilization to obtain adiponectin (19–107) bearing Cys36(Acm) as a fluffy white solid. See Supplementary Figs. 4 and 5 for yield and characterization.

Synthesis of adiponectin (19–107) via DSL-deselenization, Part 3: Acm deprotection of adiponectin (19–107) ● Timing 3 h + overnight lyophilization

70 Dissolve 1 mg of adiponectin (19–107) (C36Acm) in 1.5 mL of degassed 25 vol% acetonitrile in H_2O (0.1 vol% TFA).

71 Add 10 mg of AgOAc (40 mM), sonicating if necessary to aid in dissolution.

72 Incubate at room temperature for 1 h, monitoring the progress of the reaction via UPLC–MS. Before injection, UPLC–MS samples should be quenched with DTT (5 equiv. with respect to AgOAc), and the resulting precipitate should be removed via filtration.

73 Upon completion of the reaction, dilute to a final volume of 4 mL by adding additional deionized H_2O and purify via reverse-phase HPLC (see Table 1 for conditions). Identify pure fractions via UPLC–MS (see 'Equipment setup' section) and subject the combined pure fractions to

Box 2 | MALDI-TOF analysis of final proteins ● Timing 10 min

Procedure

▲ **CRITICAL** All final proteins should be analyzed via MALDI-TOF mass spectrometry to confirm identity and purity. Analysis is performed according to the Bruker Guide to MALDI[21]. A brief summary is provided below.

1 Deposit 0.5 µL of matrix solution A onto the MALDI target plate and allow it to dry.
2 Mix one part matrix solution B with one part analyte solution.
3 Deposit 0.5 µL of the matrix/analyte mixture onto the matrix spot and allow it to dry.
4 Perform analysis using linear, positive mode on a Bruker autoflex speed LRF MALDI-TOF mass spectrometer.

lyophilization to produce adiponectin (19–107) as a fluffy white solid. See Supplementary Figs. 6–8 for yield and characterization, including MALDI-TOF data for the final synthetic protein (see Box 2 for the MALDI-TOF analysis protocol).

Procedure 2

Synthesis of haemathrin 1 (1–23) selenoester, Part 1: loading to polystyrene Rink amide resin ● Timing 3.5 h

1 Weigh 0.7 g (400 µmol) of polystyrene Rink amide resin (0.57 mmol/g) into a 10-mL fritted disposable reaction vessel and swell for 40 min in dry DCM (5 mL) while shaking at room temperature using a mechanical shaker.
2 Expel the DCM and treat the resin with 5 mL of Fmoc-deprotection solution (20% (vol/vol) piperidine in DMF) for 3 min. Expel the solution from the resin and repeat the treatment once more. Wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF.
3 Dissolve 655 mg of Fmoc-Glu-OAll (1.6 mmol, 4 equiv.) and 227 mg of Oxyma (1.6 mmol, 4 equiv.) in 2 mL of DMF in a 21-mL glass vial, using sonication to aid the dissolution if necessary.
4 Add 164 µL of DIC (1.6 mmol, 4 equiv.) to the vial, using a micro-syringe, and agitate for 30 s to ensure thorough mixing before adding the coupling solution to the resin.
5 Allow the resin to shake at room temperature for 2 h and then expel the coupling solution.
6 Wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF and treat the resin with 4 mL of capping solution (0.3 M each of DIPEA and acetic anhydride in DMF) at room temperature for 5 min, with shaking.
7 Wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF and repeat the Fmoc-loading protocol described in Steps 9–14 of Procedure 1 above.

Synthesis of haemathrin 1 (1–23) selenoester, Part 2: peptide synthesis ● Timing 51 h

▲ **CRITICAL** The haemathrin 1 (1–23) fragment is synthesized on a Gyros Protein Technologies Symphony peptide synthesizer at room temperature. Detailed synthesizer conditions can be found in the 'Equipment setup' section.

8 Wash the resin with 5 × 5 mL of DMF, transfer the resin to an appropriate synthesizer vessel and run the fragment sequence (PERDSAKEGNKGQKRARLVNV) on an automated peptide synthesizer without heating, using appropriate coupling cycles.
9 Upon completion of the automated SPPS, transfer the resin to a new fritted reaction syringe and wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF.
10 Undertake Steps 9 and 10 of Procedure 1 to remove the N-terminal Fmoc-protecting group.
11 Dissolve 135 mg of Boc-Tyr(O*t*Bu)-OH (400 µmol, 8 equiv.) and 62.5 mg of Oxyma (400 µmol, 8 equiv.) in 1 mL of DMF in a 21-mL glass vial, using sonication to aid the dissolution, if necessary.
12 Add 70 µL of DIC (400 µmol, 8 equiv.) to the vial, using a micro-syringe, and agitate for 30 s to ensure thorough mixing before adding the coupling solution to the resin.
13 Allow the resin to shake at room temperature for 1 h and then expel the coupling solution. Treat the resin with a fresh batch of coupling solution (prepared as described in Steps 11 and 12) for a further hour at room temperature with shaking.
14 Expel the solution from the syringe and wash the resin with 5 × 5 mL of DMF and 5 × 5 mL of DCM and dry the resin.

■ **PAUSE POINT** Dry resin can be stored at 4 °C under inert gas for several months. Any time resin has been dried, it should be re-swelled in DCM for 5 min with shaking before recommencing synthesis. Resin can be dried and re-swelled as necessary.

Synthesis of haemathrin 1 (1–23) selenoester, Part 3: solid-phase selenoesterification

● Timing 5.5 h

15 Split the resin to perform selenoesterification at a 55-μmol scale.

16 Swell the resin in dry DCM for 30 min, followed by 5 × 5 mL washings with dry DCM.

17 Weigh out 58 mg of $Pd(PPh_3)_4$ (55 μmol, 1.0 equiv.) into a 21-mL vial and dissolve it in 1 mL of dry DCM.

18 Add $PhSiH_3$ (250 μL, 2 mmol, 40 equiv.) to this solution, using a micro-syringe, and mix it for 30 s.

19 Draw up the reaction solution into the fritted syringe containing the resin and allow the syringe to shake on a mechanical shaker at room temperature for 1 h.

▲CRITICAL STEP Note that the allyl deprotection reaction in this step generates $H_{2(g)}$ and therefore pressure may build up in the syringe during the course of the reaction. Care should be taken to seal the reaction vessel tightly with a pressure cap and, when opening the syringe at the completion of the reaction, the user is advised to wear a double layer of gloves and cover the cap of the syringe with a wad of paper towel while opening to prevent the solution from spraying.

20 Expel the solution, wash the resin with dry DCM (5 × 5 mL) and repeat Steps 17–19 once more.

21 Expel the solution and wash the resin thoroughly with DCM (10 × 5 mL).

22 Weigh out 468 mg of DPDS (1.5 mmol, 30 equiv.) into a 21-mL vial and dissolve it in 1 mL of dry DMF.

23 Add Bu_3P (375 μL, 1.5 mmol, 30 equiv.) to this mixture, cool it to 0 °C, draw up the resulting solution into the fritted syringe bearing the resin and allow the syringe to shake on a mechanical shaker at 0 °C for 3 h.

24 Expel the solution and wash the resin thoroughly with DMF (10 × 5 mL) and DCM (10 × 5 mL).

Synthesis of haemathrin 1 (1–23) selenoester, Part 4: acidolytic cleavage and purification

● Timing 4.5 h + overnight lyophilization

25 Add 7 mL of TFA cleavage cocktail (90:5:5 (vol/vol/vol) TFA/TIS/H_2O) to the resin and allow it to shake at room temperature for 2 h. After this time, expel the cleavage cocktail into a 25-mL Falcon tube and wash the resin with DCM (3 × 3 mL), retaining the washings and combining them with the cleavage cocktail in the Falcon tube.

26 Concentrate the retained acidic cocktail to a total volume of <3 mL and isolate the crude peptide fragment via ether precipitation as described earlier (Steps 35 and 36 of Procedure 1).

27 The purified haemathrin 1 (1–23) selenoester fragment is obtained after reverse-phase HPLC purification by following Steps 37–39 of Procedure 1. See Table 1 for gradients. See Supplementary Fig. 12 for yield and characterization

■PAUSE POINT Lyophilized peptide fragments can be stored at 4 °C under inert gas for several months.

Synthesis of haemathrin 1 (24–59) diselenide, Part 1: peptide synthesis ● Timing 115.5 h

28 Load 170 mg of Fmoc-Ala-OH (0.55 mmol, 0.5 equiv.) to 1 g of 2-chlorotrityl chloride resin (1.1 mmol/g) using 190 μL of DIPEA (1.1 mmol, 1 equiv.) as described in Steps 1–8 of Procedure 1.

29 Fmoc-deprotect and determine the resin loading as described in Steps 9–14 of Procedure 1.

▲CRITICAL STEP The haemathrin 1 (24–59) fragment is synthesized on a Gyros Protein Technologies Symphony peptide synthesizer at room temperature. Detailed synthesizer conditions can be found in the 'Equipment setup' section.

30 Split the resin, transfer 100 μmol to an appropriate synthesizer vessel and run the fragment sequence (RSGETDYDEYEENENTPTPDPSAPTARPRLGRKN), using appropriate coupling cycles.

31 Transfer the resin to a fritted syringe, wash with DCM (10 × 5 mL) and split the resin upon drying to perform the next coupling at a 27.5-μmol scale.

32 Repeat Steps 9 and 10 of Procedure 1 to remove the N-terminal Fmoc-protecting group, followed by DMF washes (5 × 5 mL).

33 Dissolve 28 mg of Boc-(γ-SePMB)-Glu(O*t*Bu)-OH (55 μmol, 2 equiv.) and 7.5 mg of HOAt (55 μmol, 2 equiv.) in 1 mL of DMF in a 21-mL glass vial, using sonication to aid the dissolution, if necessary.

34 Add 8 μL of DIC (55 μmol, 2 equiv.) to the vial, using a micro-syringe, and agitate for 30 s to ensure thorough mixing before adding the coupling solution to the resin from Step 32. Allow the resin to shake at room temperature on a mechanical shaker for 16 h.

▲ CRITICAL STEP The (γ-Se)-Glu building block has limited stability and should be used immediately after preparation from the allyl-protected variant, which can be stored at 4 °C for several months.

35 Expel the coupling solution and wash the resin with 5 × 5 mL of DMF and 5 × 5 mL of DCM, followed by drying the resin.

36 Subject a small amount of resin to acidolytic cleavage (see Box 1, 90:5:5 (vol/vol/vol) TFA/TIS/H_2O) to ensure complete coupling of Boc-(γ-SePMB)-Glu(O*t*Bu)-OH to the peptide.

■ PAUSE POINT The dry resin can be stored at 4 °C under inert gas for several months. Any time resin has been dried, it should be re-swelled in DCM for 5 min with shaking before recommencing synthesis. Resin can be dried and re-swelled as necessary.

Synthesis of haemathrin 1 (24–59) diselenide, Part 2: acidolytic cleavage and PMB deprotection ● Timing 8.5 h + overnight lyophilization

▲ CRITICAL As the conditions described for the PMB deprotection of the Sec(PMB) and β-Se(PMB)-Asp residues (TFA/DMSO/PMB deprotection buffer) result in the deselenization of γ-Se(PMB)-Glu residue, a different PMB deprotection approach (modified from Hondal et al.[20]) needs to be used.

37 Treat the resin from Step 35 (19-μmol scale) with 14 mL of TFA cleavage cocktail (92.5/5/2.5 (vol/vol/vol) TFA/TIS/H_2O) along with DTNP (10 equiv.) and Gdn.HCl (150 equiv.) for 2 h at room temperature, using a mechanical shaker.

38 Expel the cleavage cocktail into a 25-mL Falcon tube and wash the resin with 3 × 3 mL of DCM, retaining the washings and combining them with the cleavage cocktail in the Falcon tube.

39 Concentrate the retained acidic cocktail to a total volume of <2 mL and isolate the crude peptide fragment via ether precipitation as described in Steps 35 and 36 of Procedure 1.

40 Re-dissolve the pellet in 5 mL of 15 vol% MeCN in H_2O (with 0.1 vol% TFA) and subject this solution of crude peptide to reverse-phase HPLC purification (5 × 1 mL injections).

41 Identify pure fractions via careful UPLC–MS analysis and subject combined pure fractions to lyophilization to obtain the haemathrin 1 (24–59) diselenide fragment as a fluffy white solid. See Supplementary Fig. 14 for yield and characterization.

■ PAUSE POINT Lyophilized peptide fragments can be stored at 4 °C under inert gas for several months.

Synthesis of haemathrin 1 (1–59) via DSL–deselenization, Part 1: DSL of haemathrin 1 (1–59) ● Timing 20 min

42 Weigh out 5.1 mg of lyophilized haemathrin 1 (24–59) diselenide fragment (1.22 μmol, 1.0 equiv.) into a 1.5-mL Eppendorf tube.

43 Weigh out 4.1 mg of lyophilized haemathrin 1 (1–23) selenoester fragment (1.47 μmol, 1.2 equiv.) into another 1.5-mL Eppendorf tube.

44 Dissolve the diselenide fragment in ligation buffer (6 M Gdn.HCl, 0.1 M Na_2HPO_4, pH 7.2; 244 μL) to a concentration of 5 mM (with respect to the monomer, selenol). Add this solution to the Eppendorf tube containing the selenoester fragment.

45 Mix and adjust the pH of the ligation solution to pH 5.8–6.7 as described in Step 59 of Procedure 1 and monitor the reaction as described in Steps 60–62 of Procedure 1.

Synthesis of haemathrin 1 (1–59) via DSL–deselenization, Part 2: deselenization and purification of haemathrin 1 (1–59) ● Timing 1 h 40 min + overnight lyophilization

46 Repeat hexane extraction as described in Step 63 of Procedure 1.

47 Deselenize the ligation mixture as described in Steps 64–68 of Procedure 1, noting that the (γ-Se)-Glu residue used in haemathrin 1 deselenizes more rapidly than Sec and the reaction should be complete within 10 min.

▲ CRITICAL STEP Thorough degassing is not as important for deselenization at (γ-Se)-Glu or (β-Se)-Asp as compared to Sec, as these residues do not appear to be prone to oxidative deselenization. Although deselenization of both these residues has been shown to proceed in the absence of DTT[12] as an H-atom source, it is included to help speed up the rate of deselenization of the unproductive ester (via thiolysis to the selenol) as the unproductive ester itself cannot be directly deselenized. Similarly, hexane extraction of DPDS does not appear to be essential for deselenization at either of these residues.

48 Purify haemathrin 1 via reverse-phase HPLC (see Table 1 for details) as described in Step 69 of Procedure 1 to afford the desired full-length protein as a fluffy white solid upon lyophilization. See Supplementary Figs. 19–21 for yield and characterization.

Procedure 3

Synthesis of haemathrin 2 (1–28) selenoester, Part 1: loading to ChemMatrix Rink amide resin ● Timing 3.5 h

1 Weigh 0.33 g (170 μmol) of ChemMatrix Rink amide resin (0.5 mmol/g) into a 10-mL fritted disposable reaction vessel and swell for 40 min in dry DCM (5 mL) while shaking at room temperature using a mechanical shaker.
2 Expel the DCM and treat the resin with 5 mL of Fmoc-deprotection solution (20 vol% piperidine in DMF) for 3 min. Expel the solution from the resin and repeat the treatment once more.
3 Dissolve 270 mg of Fmoc-Glu-OAll (660 μmol, 4 equiv.) and 93.8 mg of Oxyma (660 μmol, 4 equiv.) in 2 mL of DMF in a 21-mL glass vial, using sonication to aid the dissolution, if necessary.
4 Add 68 μL of DIC (660 μmol, 4 equiv.) to the vial, using a micro-syringe, and agitate for 30 s to ensure thorough mixing before adding the coupling solution to the resin.
5 Allow the resin to shake at room temperature for 2 h and then expel the coupling solution.
6 Wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF, and treat the resin with 4 mL of capping solution (0.3 M each of DIPEA and acetic anhydride in DMF) at room temperature for 5 min, with shaking.
7 Wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF, and carry out the Fmoc-loading protocol described in Steps 9–14 of Procedure 1 above.

Synthesis of haemathrin 2 (1–28) selenoester, Part 2: peptide synthesis ● Timing 57 h

▲ **CRITICAL STEP** The haemathrin 2 (1–28) fragment is synthesized on a Gyros Protein Technologies Symphony peptide synthesizer at room temperature and a Biotage Alstra Initiator+ peptide synthesizer at 50 °C as described below. Detailed synthesizer conditions can be found in the 'Equipment setup' section.

8 Wash the resin with 5 × 5 mL DMF, transfer the resin to an appropriate synthesizer vessel and run the fragment sequence (QERSS) on an automated peptide synthesizer (87-μmol scale) without heating (Symphony), using appropriate coupling cycles.
9 Transfer the resin to an appropriate synthesizer vessel to run the difficult stretch (RALLVKV) of the peptide sequence on a Biotage Alstra peptide synthesizer with 50 °C double couplings and multiple Fmoc deprotection cycles (2–3 × 4 mL at room temperature; 1–2 × 4 mL at 50 °C).
10 Transfer the resin to an appropriate reaction vessel and complete the remaining sequence (PERDSANRGSQEKE) using appropriate room temperature coupling cycles on another automated peptide synthesizer (Symphony).
11 Upon completion of the automated SPPS, transfer the resin to a new fritted reaction syringe and wash the resin with 5 × 5 mL of DMF, 5 × 5 mL of DCM and 5 × 5 mL of DMF.
12 Carry out Steps 9 and 10 of Procedure 1 to remove the N-terminal Fmoc-protecting group.
13 Dissolve 108 mg of Boc-Tyr(O*t*Bu)-OH (320 μmol, 8 equiv.) and 50 mg of Oxyma (320 μmol, 8 equiv.) in 1 mL of DMF in a 21-mL glass vial, using sonication to aid the dissolution, if necessary.
14 Add 56 μL of DIC (320 μmol, 8 equiv.) to the vial, using a micro-syringe, and agitate for 30 s to ensure thorough mixing before adding the coupling solution to the resin.
15 Allow the resin to shake at room temperature for 1 h and then expel the coupling solution. Treat the resin with a fresh batch of coupling solution (prepared as described in Steps 12 and 13 of Procedure 1) for a further hour at room temperature with shaking.
16 Expel the solution from the syringe and wash the resin with 5 × 5 mL of DMF and 5 × 5 mL of DCM and dry the resin.

■ **PAUSE POINT** The dry resin can be stored at 4 °C under inert gas for several months. Any time resin has been dried, it should be swelled in DCM for 5 min of shaking before recommencing synthesis. The resin can be dried and re-swelled as necessary.

Synthesis of haemathrin 2 (1–28) selenoester, Part 3: solid-phase selenoesterification ● Timing 10 h + overnight lyophilization

17 Follow Steps 15–27 of Procedure 2 (43-μmol scale) for on-resin allyl deprotection, selenoesterification, acidolytic cleavage and reverse-phase HPLC purification (see Table 1 for details) to obtain the

pure haemathrin 2 (1–28) selenoester fragment upon lyophilization. See Supplementary Fig. 15 for yield and characterization.

■ **PAUSE POINT** Lyophilized peptide fragments can be stored at 4 °C under inert gas for several months.

Synthesis of haemathrin 2 (29–59) diselenide, Part 1: peptide synthesis ● Timing 107.5 h

▲ **CRITICAL** The haemathrin 2 (29–59) fragment is synthesized on a Gyros Protein Technologies Symphony peptide synthesizer at room temperature. Detailed synthesizer conditions can be found in the 'Equipment setup' section.

18 Transfer 110 μmol of resin from Step 29 of Procedure 2 to an appropriate synthesizer vessel and run the fragment sequence (DYDEYDADETTLSPDPDAPTARPRLGRKN), using appropriate coupling cycles.

19 Transfer the resin to a fritted syringe, wash with DCM (10 × 5 mL) and split the resin upon drying to perform the next coupling on 55-μmol scale.

20 Repeat Steps 9 and 10 of Procedure 1 to remove the N-terminal Fmoc-protecting group, followed by DMF washes (5 × 5 mL).

21 Dissolve Boc-(β-SePMB)-Asp(O*t*Bu)-OH (54 mg, 110 μmol, 2 equiv.) and HOAt (15 mg, 110 μmol, 2 equiv.) in 2 mL of DMF in a 21-mL glass vial, using sonication to aid the dissolution, if necessary.

22 Add DIC (17 μL, 110 μmol, 2 equiv.) to the vial, using a micro-syringe, and agitate for 30 s to ensure thorough mixing before adding the coupling solution to the resin from Step 20 of Procedure 3. Allow the resin to shake at room temperature on a mechanical shaker for 16 h.

23 Expel the coupling solution and wash the resin with 5 × 5 mL of DMF and 5 × 5 mL of DCM, followed by drying the resin.

24 Subject a small amount of resin to acidolytic cleavage (90:5:5 (vol/vol/vol) TFA/TIS/H_2O) to ensure complete coupling of Boc-(β-SePMB)-Asp(O*t*Bu)-OH to the peptide (Box 1).

■ **PAUSE POINT** The dry resin can be stored at 4 °C under inert gas for several months. Any time resin has been dried, it should be swelled in DCM for 5 min shaking before recommencing synthesis. The resin can be dried and re-swelled as necessary.

Synthesis of haemathrin 2 (29–59) diselenide, Part 2: acidolytic cleavage and PMB deprotection ● Timing 10.5 h + 2 × overnight lyophilizations

25 Follow Steps 48–54 of Procedure 1 to obtain pure haemathrin 2 (29–59) diselenide fragment as a fluffy white solid upon lyophilization. See Supplementary Fig. 17 for yield and characterization.

■ **PAUSE POINT** Lyophilized peptide fragments can be stored at 4 °C under inert gas for several months.

Synthesis of haemathrin 2 (1–59) via DSL–deselenization, Part 1: DSL of haemathrin 2 (1–59) ● Timing 20 min

26 Weigh out 5.2 mg of lyophilized haemathrin 2 (29–59) diselenide fragment (1.46 μmol, 1.0 equiv.) into a 1.5-mL Eppendorf tube.

27 Weigh out 5.9 mg of lyophilized haemathrin 2 (1–28) selenoester fragment (1.76 μmol, 1.2 equiv.) into another 1.5-mL Eppendorf tube.

28 Dissolve the diselenide fragment in ligation buffer (6 M Gdn.HCl, 0.1 M Na_2HPO_4, pH 7.2; 291 μL) to a concentration of 5 mM (with respect to the monomer, selenol). Add this solution to the Eppendorf tube containing the selenoester fragment.

29 Mix and adjust the pH of the ligation solution to pH 5.8–6.7 as described in Step 59 of Procedure 1 and monitor the reaction as described in Steps 60–62 of Procedure 1.

Synthesis of haemathrin 2 (1–59) via DSL–deselenization, Part 2: deselenization and purification of haemathrin 2 (1–59) ● Timing 1 h 40 min + overnight lyophilization

30 Carry out hexane extraction as described in Step 63 of Procedure 1.

31 Deselenize the ligation mixture as described in Steps 64–68 of Procedure 1, noting that the (β-Se)-Asp residue used in haemathrin 2 deselenizes more rapidly than Sec and should be complete within 10 min.

32 Purify haemathrin 2 via reverse-phase HPLC (Table 1) as described in Step 69 of Procedure 1 to afford the desired full-length protein as a fluffy white solid after lyophilization. See Supplementary Figs. 24–26 for yield and characterization.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step of Procedure 1	Problem	Possible reason	Solution
14	Poor loading to 2-chlorotrityl chloride resin	HCl produced from resin decomposition has neutralized the base in the loading step	Wash the resin more thoroughly with dry DCM (or 10 vol% DIPEA in DCM) before loading. Resin can also be regenerated by treating with 2 vol% thionyl chloride in DCM for 30 min and washing (5 × 5 mL) before loading.
39	Poor yield obtained for the synthesis of peptide fragments on 2-chlorotrityl chloride resin at elevated temperatures	Microwave heating has caused cleavage of the peptide from resin during synthesis	Add 0.1 M DIPEA to the Oxyma solution to help prevent cleavage
52	+16-Da mass observed after PMB deprotection	Oxidation of Met	Change to DTNP-based deprotection conditions described in Procedure 2, Steps 52–54 or those reported by Hondal et al.[20].
		Oxidation of Trp	Use conditions described in Procedure 1, Steps 52–54 without the addition of PMB-deprotection buffer.
60	Ligation proceeds slowly	pH of the ligation solution may be too low or fragments might not be sufficiently soluble	If pH is too low, use 1 M NaOH to adjust to between pH 6 and 6.5. If one of the fragments appears to be poorly soluble, a 10 vol% of the buffer volume can be replaced with DMF to aid in solubility.
	Diselenide fragment not fully consumed in ligation	Stoichiometry of the fragments may be incorrect if they are not completely dry or are hygroscopic. The pH of the ligation may be too high, causing selenoester hydrolysis. The unproductive ester may be the predominant product	Freeze-dry fragments again thoroughly before repeating the ligation. Repeat the ligation at a lower pH. Increase the number of equivalents of the selenoester fragment
	Direct aminolysis between selenoester fragments observed	Fragments may be aggregation prone, causing reaction due to proximity.	Replace 10 vol% of the buffer with DMF to aid in solubility
68	Incomplete deselenization	DPDS still present in the ligation mixture	Repeat the hexane extraction step and add a second portion of the same volume of deselenization buffer
	+16-Da mass observed after deselenization	Oxidative deselenization (Sec to Ser) has occurred	Make sure to sparge the reaction setup thoroughly with argon to remove any dissolved oxygen
	Unproductive ester product remains after other deselenization is complete	Unproductive ester is slow to thiolyze (more likely for hindered thioesters). Direct deselenization of the unproductive ester is not possible	Treat the ligation with 2 vol% hydrazine monohydrate (as an equal volume of a 4 vol% solution) to hydrazinolyze the unproductive ester to facilitate complete deselenization

Timing

Procedure 1

Steps 1–8, loading to Cl-TCP(Cl) ProTide Resin: 18 h
Steps 9–14, determination of resin loading: 30 min
Steps 15–22, synthesis of adiponectin (19–40) selenoester: 20 h
Steps 23–36, solution-phase selenoesterification of adiponectin (19-40): 24 h
Steps 37–39, analysis and purification of adiponectin (19–40) selenoester: 5 h + overnight lyophilization
Steps 40–47, synthesis of adiponectin (41–107) diselenide: 118.5 h
Steps 48–51, cleavage, deprotection and purification of PMB-protected adiponectin (41–107): 2.5 h + overnight lyophilization
Steps 52–54, PMB deprotection and purification of adiponectin (41–107): 8 h + overnight lyophilization
Steps 55–62, synthesis of adiponectin (19–107) (C36Acm) by DSL: 20 min

Steps 63–69, deselenization and purification of adiponectin (19–107) (C36Acm): 18.5 h + overnight lyophilization
Steps 70–73, Acm deprotection and purification of adiponectin (19–107): 3 h + overnight lyophilization

Procedure 2

Steps 1–7, loading to Rink amide resin: 3.5 h
Steps 8–14, synthesis of haemathrin 1 (1–23): 51 h
Steps 15–24, solid-phase selenoesterification of haemathrin 1 (1–23): 5.5 h
Steps 25–27, cleavage, purification and analysis of haemathrin 1 (1–23) selenoester: 4.5 h + overnight lyophilization
Steps 28–36, synthesis of haemathrin 1 (24–59): 115.5 h
Steps 37–41, cleavage, PMB deprotection, purification and analysis of haemathrin 1 (24–59): 8.5 h + overnight lyophilization
Steps 42–45, synthesis of haemathrin 1 (1–59) by DSL: 20 min
Steps 46–48, deselenization, purification and analysis of haemathrin 1 (1–59): 1 h 40 min + overnight lyophilization

Procedure 3

Steps 1–7, synthesis of haemathrin 2 (1–28), loading: 3.5 h
Steps 8–16, synthesis of haemathrin 2 (1–28): 57 h
Step 17, solid-phase selenoesterification, cleavage, purification and analysis of haemathrin 2 (1–28) selenoester: 10 h + overnight lyophilization
Steps 18–24, synthesis of haemathrin 2 (29–59): 107.5 h
Step 25, cleavage, PMB deprotection, purification and analysis of haemathrin 2 (29–59) diselenide: 10.5 h + 2× overnight lyophilizations
Steps 26–29, synthesis of haemathrin 2 (1–59) by DSL: 20 min
Steps 30–32, deselenization, purification and analysis of haemathrin 2 (1–59): 1 h 40 min + overnight lyophilization
Box 1, analytical acidolytic cleavage: 2 h
Box 2, MALDI-TOF analysis of final proteins: 10 min

Anticipated results

Information regarding the anticipated results for the synthesis of adiponectin, haemathrin 1 and haemathrin 2 can be found in Tables 3 and 4, 5 and 6, and 7 and 8, respectively. Examples of other proteins made via DSL–deselenization include:

- *Selenoprotein K*. Selenoprotein K is a 90-residue native selenoprotein involved in oxidative stress regulation that was synthesized from two segments via one ligation, followed by chemoselective deselenization of (β-Se)-Asp in the presence of the native Sec residue in a 40% overall yield[12].
- *Lumbricin-1*. The 62-residue anti-microbial protein lumbricin-1 was assembled in a 52% yield from two component fragments through a single, challenging Pro–Pro ligation junction[15].
- *UL22A*. Four differentially sulfated variants of the 83-residue chemokine binding protein UL22A were each synthesized from two component fragments in a 51–58% yield[13].
- *Chorismate mutase*. Chorismate mutase, an 83-residue enzyme from *Mycobacterium tuberculosis*, was synthesized from two fragments over a single ligation in a 57% overall yield, including folding[7].

Table 3 | Synthesis of adiponectin fragments

Product	Yield	Mass calculated	Mass found (ESI+)
Adiponectin (19–40) selenoester	25% (30%)	2,468.6	2,467.8
Adiponectin (41–107) diselenide	10%	13,121.8	13,120.2

First values listed represent yields and data from the University of Sydney, whereas the value in parentheses represents data from Novo Nordisk.

Table 4 | Synthesis of adiponectin (19–107) via DSL-deselenization

Product	Yield	Mass calculated	Mass found (ESI+)	Mass found (MALDI-TOF)
Adiponectin (19–107) C36Acm	47% (33%)	8,792.5	8,791.2 (8,791.5)	—
Adiponectin (19–107)	74% (55%)	8,721.5	8,721.0 (8,720.4)	8,722.0

First values listed represent yields and data from the University of Sydney, whereas values in parentheses represent yields and data from Novo Nordisk.

Table 5 | Synthesis of haemathrin 1 fragments

Product	Yield	Mass calculated	Mass found (ESI+)
Haemathrin 1 (1–23) selenoester	53% (45%)	2,783.9	2,782.8
Haemathrin 1 (24–59) diselenide	30%	8,286.4	8,283.0

First values listed represent yields and data from the University of Sydney, whereas the value in parentheses represents data from Novo Nordisk.

Table 6 | Synthesis of haemathrin 1 via DSL-deselenization

Product	Yield	Mass calculated	Mass found (ESI+)	Mass found (MALDI-TOF)
Haemathrin 1 (1–59)	61% (52%)	6,690.1	6,689.1 (6,688.5)	6,690.1

First values listed represent yields and data from the University of Sydney, whereas values in parentheses represent yields and data from Novo Nordisk.

Table 7 | Synthesis of haemathrin 2 fragments

Product	Yield	Mass calculated	Mass found (ESI+)
Haemathrin 2 (1–28) selenoester	27% (23%)	3,401.5	3,400.2
Haemathrin 2 (29–59) diselenide	8%	7,089.2	7,090.4

First values listed represent yields and data from the University of Sydney, whereas the value in parentheses represents data from Novo Nordisk.

Table 8 | Synthesis of haemathrin 2 via DSL-deselenization

Product	Yield	Mass calculated	Mass found (ESI+)	Mass found (MALDI-TOF)
Haemathrin 2 (1–59)	48% (41%)	6,709.1	6,707.6 (6,707.5)	6,709.6

First values listed represent yields and data from the University of Sydney, whereas values in parentheses represent yields and data from Novo Nordisk.
vol%

- *ESAT-6*. The 94-residue early secretory antigen ESAT-6 was synthesized in a 43% yield from three component fragments through a one-pot tandem DSL–deselenization–NCL–desulfurization protocol[7].

Data availability

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files

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Author contributions

S.S.K. and E.E.W. performed the experiments, compound characterization and data analysis at the University of Sydney, Sydney Australia. B.P. replicated the selenoesterification and ligation experiments, compound characterization and data analysis at Novo Nordisk A/S, Denmark. S.S.K., E.E.W., B.P., K.W.C.-F. and R.J.P. contributed to experimental design and writing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to R.J.P.

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