

Protocol for protein modification using oxalyl thioester-mediated chemoselective ligation

Francesco Terzani, Chen Wang, Simindokht Rostami, Rémi Desmet, Benoît Snella, Magalie Sénéchal, Birgit Wiltschi, Jérôme Vicogne, Oleg Melnyk, Vangelis Agouridas

▶ **To cite this version:**

Francesco Terzani, Chen Wang, Simindokht Rostami, Rémi Desmet, Benoît Snella, et al.. Protocol for protein modification using oxalyl thioester-mediated chemoselective ligation. STAR Protocols, 2024, 5 (4), pp.103390. 10.1016/j.xpro.2024.103390 . hal-04751542

HAL Id: hal-04751542
https://hal.science/hal-04751542v1

Submitted on 24 Oct 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



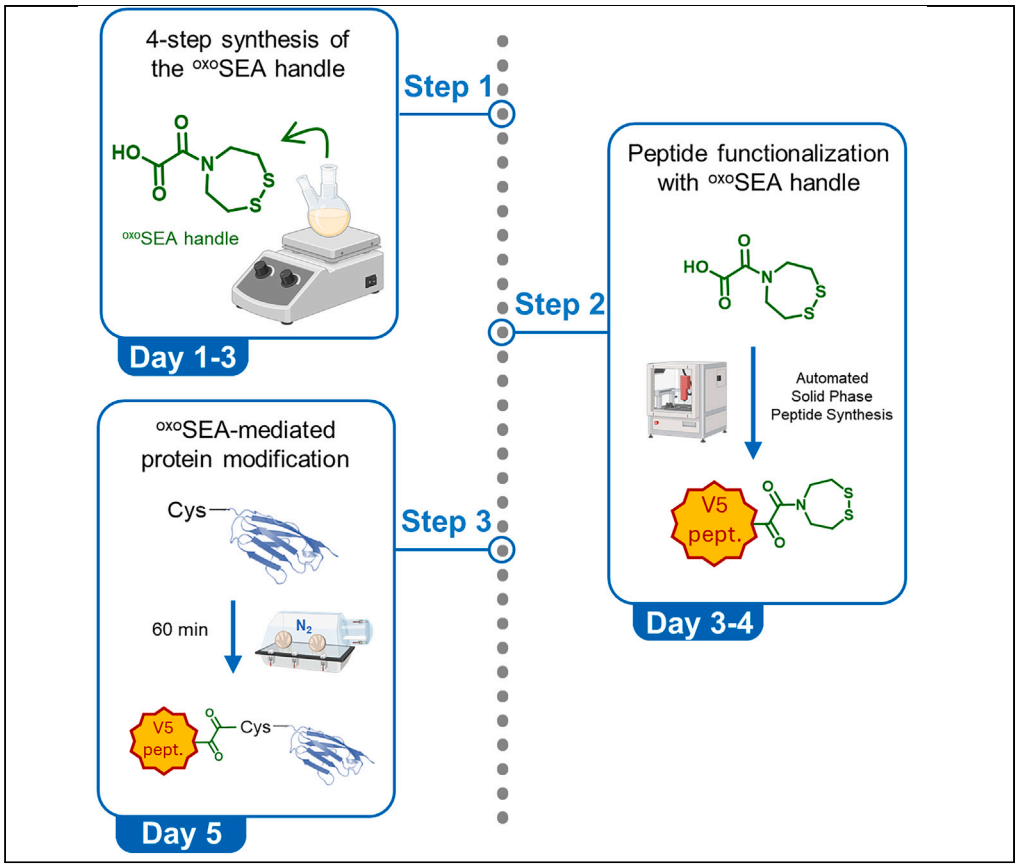
Distributed under a Creative Commons CC BY-NC-ND 4.0 - Attribution - Non-commercial use - No Derivative Works - International License

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for protein modification using oxalyl thioester-mediated chemoselective ligation



The development of fast ligation chemistries for the site-specific modification of proteins has become a major focus in chemical biology. We describe steps for preparing an oxalyl thioester precursor in the form of an *N*-oxalyl perhydro-1,2,5-dithiazepine handle, i.e., the ^{oxo}SEA group, and incorporating it into a peptide modifier using solid phase peptide synthesis. We then detail procedures for its application for the modification of an N-terminal Cys-containing B1 domain of the streptococcal G protein using the native chemical ligation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Francesco Terzani, Chen Wang, Simindokht Rostami, ..., Jérôme Vicogne, Oleg Melnyk, Vangelis Agouridas

vangelis.agouridas@ibl.cnrs.fr

Highlights

Four-step synthesis of the oxalyl thioester precursor handle (i.e., ^{oxo}SEA group)

Instructions for attaching the ^{oxo}SEA handle at the N terminus of a peptide

Steps for ligation of the ^{oxo}SEA peptide to the Cys-GB1 protein domain

Terzani et al., STAR Protocols 5, 103390
December 20, 2024 © 2024 The Author(s). Published by Elsevier Inc.
https://doi.org/10.1016/j.xpro.2024.103390

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for protein modification using oxalyl thioester-mediated chemoselective ligation

Francesco Terzani,[1] Chen Wang,[1] Simindokht Rostami,[2] Rémi Desmet,[1] Benoît Snella,[1,4] Magalie Sénéchal,[1] Birgit Wiltschi,[2] Jérôme Vicogne,[1] Oleg Melnyk,[1] and Vangelis Agouridas[1,3,5,*]

[1]Université de Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 9017, Center for Infection and Immunity of Lille, 59000 Lille, France

[2]University of Natural Resources and Life Sciences, Vienna, Department of Biotechnology, Institute of Bioprocess Science and Engineering, Muthgasse 18, 1190 Vienna, Austria

[3]Centrale Lille, 59000 Lille, France

[4]Technical contact

[5]Lead contact

*Correspondence: vangelis.agouridas@ibl.cnrs.fr
https://doi.org/10.1016/j.xpro.2024.103390

SUMMARY

The development of fast ligation chemistries for the site-specific modification of proteins has become a major focus in chemical biology. We describe steps for preparing an oxalyl thioester precursor in the form of an *N*-oxalyl perhydro-1,2,5-dithiazepine handle, i.e., the ^{oxo}SEA group, and incorporating it into a peptide modifier using solid phase peptide synthesis. We then detail procedures for its application for the modification of an N-terminal Cys-containing B1 domain of the streptococcal G protein using the native chemical ligation.

For complete details on the use and execution of this protocol, please refer to Snella et al.[1]

BEFORE YOU BEGIN

The modification of proteins through site-specific conjugation involves attaching custom-designed modifiers to precise locations on a protein of interest in order to modulate its biophysical properties, binding affinity or cellular functions.[2,3] The key to achieving this precise control lies in the development of fast and chemoselective ligation chemistries. The native chemical ligation (NCL[4,5]) reaction, a popular synthetic method for chemoselective peptide bond formation between a thioester and a cysteinyl (Cys) peptide, has a strong asset for this purpose, in that proteins featuring an N-terminal Cys residue are easily accessible through classical recombinant techniques. Unfortunately, this reaction suffers limitations when applied to the modification of proteins at low concentrations because of the modest reactivity of conventional thioesters that makes the chemistry poorly efficient in the low micromolar range. In search for a simple solution to this problem, we recently introduced an approach based on oxalyl thioester precursors relying on the *N*-oxalyl perhydro-1,2,5-dithiazepine scaffold[1,6] whose reactivity is reminiscent of the redox-controlled *bis*(2-sulfanylethyl)amide-based *N,S*-acyl transfer systems[7–9] (Figure 1). From a mechanistic perspective, a peptide equipped with a latent oxalyl thioester handle is activated upon exposure to a reductant. Reduction of the disulfide bond in the perhydro-1,2,5-dithiazepine cycle (Figure 1A in green) enables an intramolecular rearrangement to occur and results in the formation of a highly activated transient oxalyl thioester. The latter chemoselectively reacts with β-aminothiol motifs such as N-terminal cysteine residues present in target proteins. Owing to the outstanding reactivity of oxalyl thioesters, this approach allows protein conjugation to be performed at fast rates (~30 M^{-1} s^{-1}) and at reactant concentrations in the high nanomolar range. In addition, the reactivity of the ^{oxo}SEA group being redox-controlled, ligation and protein modification can be

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

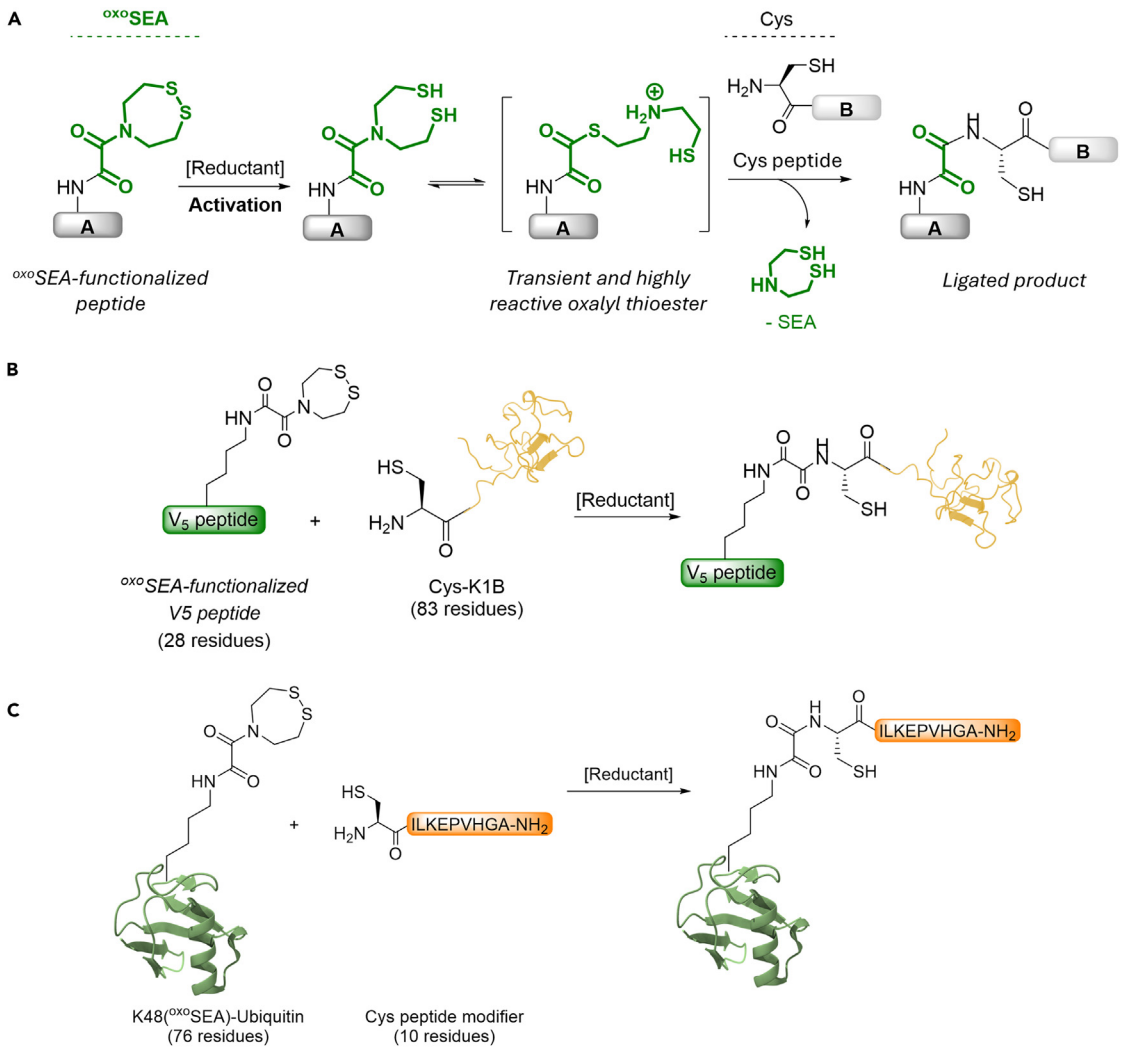


Figure 1. The chemistry of oxalyl thioester precursors

(A) General principle of the oxoSEA-mediated protein ligation.

(B) oxoSEA mediated modification of the K1 domain of HGF/SF equipped with an N-terminal cysteine residue.

(C) oxoSEA-mediated modification of Lys48 oxoSEA-functionalized ubiquitin.

triggered on demand upon exposure to thiol or phosphine reductants such as dithiothreitol (DTT) or *tris*(2-carboxyethyl)phosphine (TCEP).

In previous works, we showed that the oxoSEA group can be conveniently placed on the side chain of a lysine residue and incorporated within peptide modifiers accessed through conventional solid phase approaches (Figure 1B).[1] This strategy enabled the successful and highly efficient conjugation of the 10 kDa K1 domain of the hepatocyte growth factor (HGF-SF) with a V5 peptidic tag. Note that the two reactive functionalities are interchangeable, allowing for flexible design and broader applicability. For example, we also demonstrated that the oxoSEA group could be introduced within a protein partner by producing an oxoSEA-modified ubiquitin at Lys48 residue (Figure 1C).[10]

In the approach disclosed in this Protocol article, we describe a straightforward and high-yielding four step procedure for preparing a simplified oxalyl thioester precursor handle. This oxoSEA handle can be readily coupled at the N-terminus of peptide segments following peptide assembly and further serve as a latent thioester to modify N-terminal Cys-containing proteins. This last property

is specifically illustrated by the preparation of an ^{oxo}SEA-modified V5 peptide and its conjugation to the B1 domain of the streptococcal G protein capped by a Cys residue at its N-terminus.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides and recombinant proteins		
bis(2-chloroethyl)amine hydrochloride, 98%	Alfa Aesar	Cat#10224926
Triphenylmethanethiol, 97%	Acros Organics	Cat#A0429765
1,8-diazabicyclo[5.4.0]undec-7-ene, 98%	Alfa Aesar	Cat#10224458
Ethyl chlorooxoacetate, 98%	Thermo Scientific	Cat#A04439854
Iodine, 99.5+%	Thermo Scientific	Cat#A0459683
Sodium hydroxide (NaOH)	VWR Chemicals	Cat#28248.298
Phosphomolybdic acid hydrate	Fisher Scientific	Cat#11448670
Silica gel, ultrapure, 60–200 mm	Thermo Scientific	Cat#A0444730
Potassium dihydrogen phosphate (KH_2PO_4)	Acros Organics	Cat#A019523101
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma-Aldrich	Cat#S8875-500G
Sodium thiosulfate 98+% anhydrous ($Na_2S_2O_3$)	Thermo Scientific	Cat#10247149
Sodium chloride (NaCl)	VWR Chemicals	Cat#1027260015
Magnesium sulfate 97% anhydrous ($MgSO_4$)	Thermo Scientific	Cat#A0450590
N,*N*-Dimethylformamide	Carlo Erba	Cat# P0343521
Ethyl acetate	Fisher Chemical	Cat#2484809
Dichloromethane	Fisher Chemical	Cat#2376298
Tetrahydrofuran	Fisher Chemical	Cat#1549930
Cyclohexane	Carlo Erba	Cat#333751
Diethyl ether	Carlo Erba	Cat#447523
n-heptane	Carlo Erba	Cat#446785
1-methyl-2-pyrrolidinone (NMP)	Carlo Erba	Cat#M79603-1L-HON
Glacial acetic acid	Sigma-Aldrich	Cat#A6283-1L
Standard Fmoc protected L-amino acids: Fmoc-Gly-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH	Iris Biotech	(Respectively) Cat#FAA1050, Cat#FAA1090, Cat#FAA1125, Cat#FAA1185, Cat#FAA1110, Cat#FAA1015, Cat#FAA1120, Cat#FAA1020, Cat#FAA1210
Fmoc-Ala-Ser(ψ(Me, Me)Pro)-OH	Novabiochem	Cat#8521750001
1-[*Bis*(dimethylamino)methylene]-1*H*-1,2,3- triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide (HATU)	Novabiochem	Cat#8510130025
O-(1*H*-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU)	Novabiochem	Cat#8510120025
N,*N*-diisopropylethylamine (DIPEA)	Sigma-Aldrich	Cat#D125806-100ML
2,4,6-trinitrobenzene sulfonic acid (TNBS)	TCI Chemicals	Cat#P1447-10ML
Acetic anhydride > 99%	Sigma-Aldrich	Cat#320102-1L
Novasyn TGR resin (Rink Linker 0.25 mmol/g)	Novabiochem	Cat#855009
Trifluoroacetic acid	VWR	Cat#84578.290
Triisopropylsilane	Sigma-Aldrich	Cat#233781-50G
Cys-Gly-Ser-Gly-Ser-GB1	This study	N/A
^{oxo}SEA-V5 peptidic tag	This study	N/A
4-mercaptophenylacetic acid (MPAA)	Sigma-Aldrich	Cat#653152-5G
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl)	Sigma-Aldrich	Cat#C4706-2G
Other		
Thin-layer chromatography (TLC)	Machery-Nagel pre-coated TLC sheets ALUGRAM SIL G/UV_{254} 0.20 mm silica gel 60 with fluorescent indicator UV_{254} (Cat# 818133)	
Syringes	BD Discardit II (Cat#45887-QS2)	
Glassware	Schott Duran (borosilicate glass)	
Mechanical micropipettes 10, 20, 200, and 1,000 μL	Eppendorf Research plus	
Pipette tips for micropipettes (polypropylene)	Fisher	

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Protein low binding plastic tubes 1.5 mL	Eppendorf (Cat#0030108442)	
Protein low binding plastic tubes 5.0 mL	Eppendorf (Cat#0030108302)	
50 mL conical centrifuge tubes	VWR Cat#525-1098	
Electronic balance	Mettler Toledo XS105 dual range	
Automated high precision microbalance	Mettler Toledo XP6U	
Magnetic Stirrer	VELP Scientifica AREC.X heating magnetic stirrer equipped with VTF digital thermoregulator	
Constant temperature shaker	Eppendorf Thermomixer	
Peptide automated-synthesizer	Intavis Bioanalytical Instruments	
Shaker for manual peptide synthesis	Cat S50 (Cat#60285–0000)	
Centrifuge	Eppendorf centrifuge 5702 (Cat#5702000010)	
Vacuum pump	KNF Laboport N820.3 FT40.18	
Rotary evaporator	BUCHI rotavapor R-210	
Glove bag	Sigma-Aldrich Cat# Z530220-1EA	
IR spectrometer	Bruker FT-IR alpha	
NMR spectrometer	Bruker Advance-300 MHz	
LC-MS	Thermo Scientific HPLC UltiMate 3000 (UV d.a. rev.) coupled with MS detector LCQ fleet (ESI)	
Lyophilizer	Bioblock Scientific	
Melting point apparatus	Cole Parmer SMP10	

MATERIALS AND EQUIPMENT

- Analytical and semipreparative reverse phase-HPLC conditions used in this work.

	Analytical HPLC	Analytical LC-MS	Semipreparative HPLC
Columns	XBridge Peptide BEH C18 Column 300 Å, 3.5 µm 2.1 mm × 150 mm	ACQUITY UPLC Peptide BEH C18 Column 300 Å, 1.7 µm 2.1 mm × 150 mm	XBridge Peptide BEH C18 OBD Prep Column 300 Å, 5 µm 10 mm × 250 mm
Eluents	A: deionized H_2O containing 0.1% (v/v) TFA B: ACN containing 0.1% (v/v) TFA		
Flow rate	0.4 mL/min	0.4 mL/min	6 mL/min
Run time	30 min	20 min	variable
Injection volume	100 µL (maximum)	20 µL (maximum)	5 mL (maximum)
Detectors	UV detector (215 and 254 nm)	UV detector (215 and 254 nm) ESI-MS analyzer	UV-detector (215 and 254 nm)

STEP-BY-STEP METHOD DETAILS

Preparation of 2-(1,2,5-dithiazepan-5-yl)-2-oxoacetic acid or ^{oxo}SEA handle 1

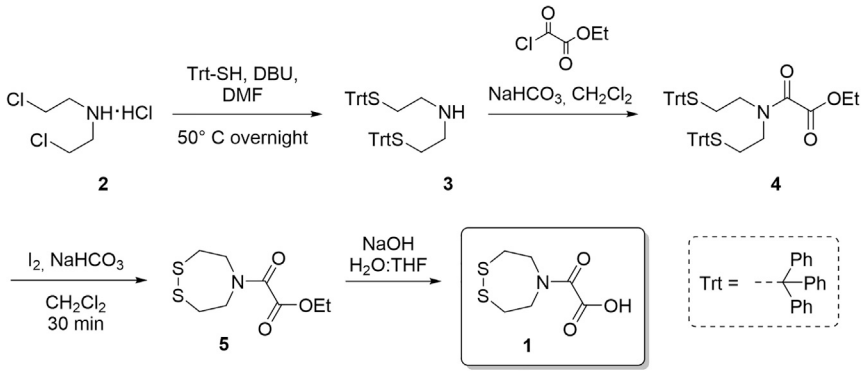
Timing: 27 h total expected time for the four-step preparation of 2-(1,2,5-dithiazepan-5-yl)-2-oxoacetic acid

Timing: 15 h (for step 1)

Timing: 4.5 h (for step 2)

Timing: 6 h (for step 3)

Timing: 3 h (for step 4)



Scheme 1. Synthetic approach towards ^{oxo}SEA handle 1

A streamlined method for preparing multigram quantities of 2-(1,2,5-dithiazepan-5-yl)-2-oxoacetic acid, i.e., ^{oxo}SEA handle **1**, from commercial reagents has been optimized (Scheme 1). This method minimizes the number of purification steps required to isolate the target product. The yield of each step is reported as an average value with standard error based on the yields obtained for 3 to 5 replicates (95% confidence limit). The amount of compound produced in one-single batch, typically 2.5 g, enables the production of ~40 different peptide modifiers, each on 50 μmol scale as done in this work.

1. Synthesize *bis*(2-(triphenylmethylthio)ethyl)amine **3**.
   a. Weigh 12.0 g (67.2 mmol) of *bis*(2-chloroethyl)amine hydrochloride **2** on a zeroed balance and add it in a 500 mL round bottom flask.

      Note: According to regulation (EC) No 1272/2008, *bis*(2-chloroethyl)amine hydrochloride is classified as being corrosive to metals (Category 1, H290), skin corrosive (sub-category 1B, H314), susceptible to have an acute toxicity if swallowed (Category 4, H302), and eye-damaging (Category 1, H318). Its adverse effects have however not to be confused with that of nitrogen mustards[11] or mustard gas[12] used in chemical warfare.

   b. Dissolve in 100 mL dimethylformamide (DMF).

      Note: Although the use of distilled DMF is not required for this reaction, the authors recommend the use of DMF from a freshly opened bottle. Old and degraded DMF batches can be contaminated by dimethylamine and result in the formation of byproducts. DMF is a flammable solvent (Category 3, H226), harmful if inhaled (Category 4, H332) or in contact with skin (Category 4, H312) and can cause serious eye irritation (Category 2, H319). It should be handled under an efficient fume hood and with appropriate personal protective equipment (lab coat, gloves and protective glasses).

   c. Weigh 37.2 g of triphenylmethanethiol (TrtSH, 135 mmol, 2.00 equiv) in a beaker on a zeroed balance and add it to the previous solution.
   d. Add an additional 150 mL of DMF.
   e. Cool the above DMF solution in an ice bath until the internal temperature reaches ~5°C. Check with a thermometer.
   f. Connect an addition funnel with pressure-equalization arm and fill it with 40.0 mL of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 258 mmol, 3.80 equiv).
   g. Add the DBU dropwise over 5–10 min. The reaction mixture turns from colorless to pale yellow.
   h. Remove the ice bath and the addition funnel. Connect an air reflux condenser on top of the flask.

i. Stir the reaction at 50°C overnight (approximately 12–14 h). The color of the reaction mixture gradually shifts to orange as the reaction proceeds.
j. Monitor the reaction.

Note: These steps describe how to monitor the reaction by thin-layer chromatography (TLC). While TLC can be performed at any time, early analyses may not provide much insight. We suggest checking the reaction's advancement after the initial 12–14 h stirring period at 50°C.

i. Microscale extraction – Use a 1 mL syringe to draw 0.25 mL of the crude reaction mixture and add the latter to a vial or test tube containing 1 mL of ethyl acetate (EtOAc) and 1 mL of a 5% w/v solution of KH_2PO_4 in deionized water.
ii. Shake vigorously, decant.
iii. Spot the upper organic layer on a TLC plate using a capillary. Also spot the triphenylmethanethiol and DBU reagents diluted in EtOAc. To avoid overstaining or undesirable migration patterns, especially of TrtSH, we suggest preparing diluted solutions where the analytes are present at concentrations similar to those found in the reaction mixture at the beginning.
iv. Place the plate in a TLC developing chamber using cyclohexane / EtOAc / TEA: 8 / 2 / 0.1 (v/v/v) as the mobile phase. Once the solvent front reaches ~6 cm, remove the plate from the chamber.
v. Check for reaction advancement at 254 nm and stain with an ethanolic solution of phosphomolybdic acid (prepared by dissolving 10 g of phosphomolybdic acid in 100 mL absolute ethanol). R_f = 0.43 (Figure 2).

k. Work-up the reaction.
i. Allow the crude mixture to cool to room temperature.

Note: The solution must be cooled to room temperature (~20°C) before continuing, as quenching the hot reaction mixture with water and ethyl acetate results in the formation of a heterogeneous solution by precipitation of material. The latter mixture becomes difficult to treat. This step typically takes 10–30 min, depending on the reaction scale (here ~20 min). The use of a bath to accelerate the cooling process is not recommended.

ii. Transfer the crude mixture into a 1 L separatory funnel.
iii. Add 400 mL of a 5% w/v aqueous solution of KH_2PO_4 and 200 mL of ethyl acetate.
iv. Close with a Teflon stopper and shake vigorously. Separate the layers after decantation.

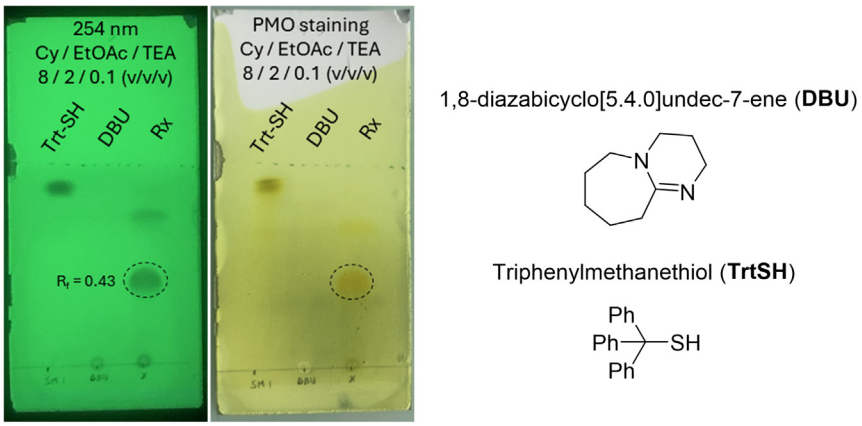


Figure 2. TLC-monitoring of the reaction
The expected product corresponds to the spot at R_f = 0.43.

v. Extract once the aqueous layer with 50 mL EtOAc and separate the layers. Combine organic layers.
vi. Wash the combined organic layers with 100 mL of a 5% w/v aqueous solution of KH_2PO_4.
vii. Separate the layers.
viii. Repeat steps vi. and vii. 6 times.
ix. Wash the organic layer with 50 mL brine and separate the layers.
x. Dry the organic layer over anhydrous magnesium sulfate.
xi. Magnesium sulfate is removed by suction filtration using a fritted glass funnel and is rinsed with 20 mL EtOAc.
xii. Concentrate the filtrate under reduced pressure (20 mbar, bath temperature 30°C) to obtain a yellowish powdery solid (Figure 3).
xiii. Based on five replicates performed from 12.0 to 15.0 g of *bis*(2-chloroethyl)amine hydrochloride **2**, the isolated yield of final product **3** was found to be 59 ± 12%.

l. Analyze the isolated product.

Note: Processed spectra are available in the supplementary material file (Figures S1–S4).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42–7.19 (m, 30H), 2.34–2.28 (m, 8H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 144.95, 129.72, 128.02, 126.78, 47.79, 32.06 ppm; IR (ATR) ν_{max}/cm^{-1} 3058, 3024, 2926, 2815, 1672, 1594, 1487, 1459, 1442, 753, 740, 695; MS (ESI, positive detection mode, direct infusion): $[M + H]^+$ m/z calcd. 622.90, found 622.33; m.p. (capillary method): 116°C–119°C; TLC: R_f = 0.43 in cyclohexane / EtOAc / TEA : 8 / 2.

2. Synthesize ethyl 2-(*bis*(2-(triphenylmethylthio)ethyl)amino)-2-oxoacetate **4**.
a. Weigh 12.4 g (19.9 mmol) of *bis*(2-(triphenylmethylthio)ethyl)amine **3** on a zeroed balance and introduce it in a 500 mL round bottom flask.
b. Dissolve in 200 mL dichloromethane (DCM).
c. Weigh 3.4 g (41 mmol, 2.1 equiv) of sodium bicarbonate on a zeroed balance and add it to the previously prepared solution.
d. Connect an addition funnel with pressure-equalization arm on top of the flask.
e. Add 4.2 mL of ethyl chlorooxoacetate (25 mmol, 1.3 equiv) within the funnel and close it with a rubber septum.

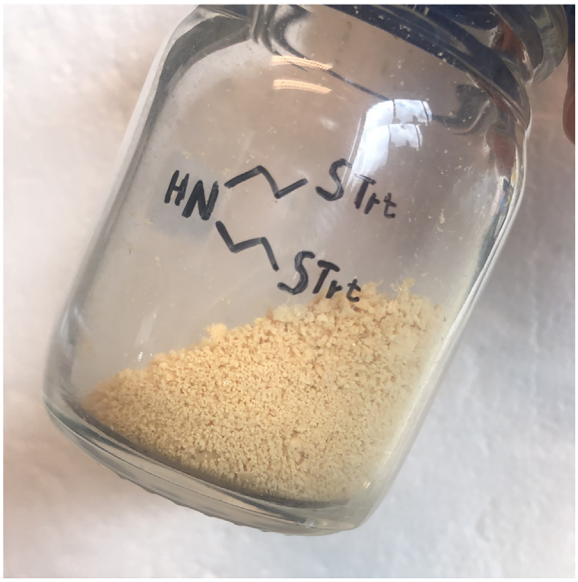


Figure 3. *Bis*(2-(triphenylmethylthio)ethyl)amine 3 is obtained as a yellowish powdery solid

f. Cool the suspension in an ice bath for 30 min.
g. Start the dropwise addition of the oxalyl chloride on the amine/$NaHCO_3$ suspension.

Note: In case of not properly cooled down solution or too fast oxalyl chloride addition, some fumes can be released in the flask.

h. Let the reaction proceed for 3 h at room temperature.
i. Monitor the reaction.

Note: Reaction monitoring can be performed at any time. We recommend a first analysis after 30–45 min and a second one after 3 h to appreciate the change in conversion.

   i. Spot the crude reaction mixture on a TLC plate, as well as the starting *bis*(2-(triphenylmethylthio)ethyl)amine **3** diluted in EtOAc.
   ii. Place the plate in a TLC developing chamber using cyclohexane / EtOAc: 8 / 2 (v/v) as the mobile phase. Once the solvent front reaches ~6 cm, remove the plate from the chamber.
   iii. Check for reaction advancement at 254 nm and stain with phosphomolybdic acid (R_f = 0.55, Figure 4).

j. Work-up the reaction.
   i. Quench the reaction with 50 mL of deionized water and add 100 mL of DCM.
   ii. Transfer the mixture in a 1 L separatory funnel.
   iii. Close with a Teflon stopper, shake vigorously. Separate the layers after decantation.
   iv. Extract once the aqueous layer with 50 mL DCM and separate the layers.
   v. Wash the combined organic layers with 50 mL of a saturated aqueous solution of $NaHCO_3$. Separate the layers.
   vi. Wash the organic layer with 50 mL brine and separate the layers.
   vii. Dry the organic layer over anhydrous magnesium sulfate.
   viii. Magnesium sulfate is removed by suction filtration using a fritted glass funnel and is rinsed with 15 mL DCM.
   ix. Concentrate the filtrate under reduced pressure (20 mbar, bath temperature 30°C).

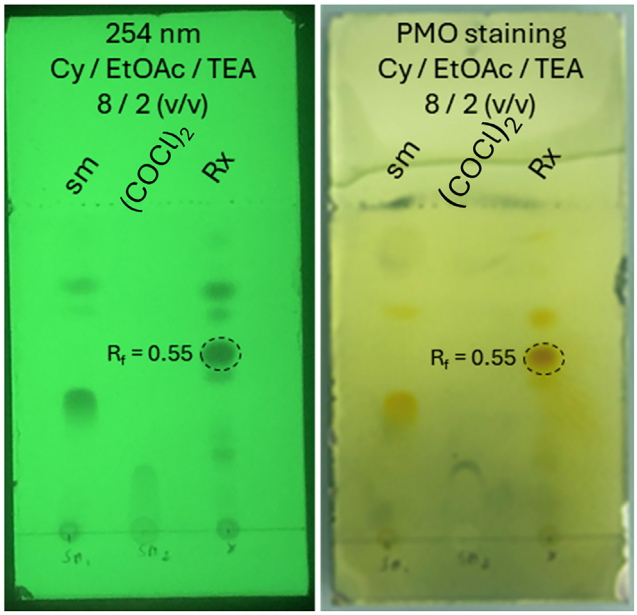


Figure 4. TLC-monitoring of the reaction (Sm = starting material; Rx = crude reaction mixture)
The expected product corresponds to the spot at R_f = 0.55.

x. The final product **4** is obtained as an orange glass. Based on four replicates performed from 10.0 to 20.0 g of *bis*(2-(triphenylmethylthio)ethyl)amine **3**), average isolated yield calculated for this synthetic step is 75 ± 8%.

Note: At this stage, the crude product can possibly be contaminated by triphenylmethyl-containing impurities. It can however be engaged as is in the next step which involves a silica gel column chromatography at the end.

k. Analyze the isolated product.

Note: Processed spectra are available in the supplementary material file (Figures S5–S7).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42–7.17 (m, 30H), 4.23 (q, 2H, *J* = 7.1), 2.89–2.84 (m, 2H), 2.78–2.73 (m, 2H), 2.32–2.22 (m, 4H), 1.28 (t, 3H, *J* = 7.1) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.43, 161.46, 144.70, 144.54, 129.72, 129.64, 128.16, 128.10, 128.07, 127.40, 127.04, 126.90, 67.20, 67.04, 62.24, 47.70, 44.79, 30.68, 29.14, 14.06 ppm; IR (ATR) ν_{max}/cm^{-1} 3056, 1735, 1658, 1488, 1444, 1265, 1153, 735, 698; TLC: R_f = 0.55 in cyclohexane / EtOAc: 8 / 2 (v/v); No mass analysis could be obtained for compound **4** (in-source decomposition).

3. Synthesize of ethyl 2-(1,2,5-dithiazepan-5-yl)-2-oxoacetate **5**.
   a. Weigh 14.4 g (19.9 mmol) of 2-(*bis*(2-(triphenylmethylthio)ethyl)amino)-2-oxoacetate **4** in a 1 L round bottom flask on a zeroed balance. Dissolve the colorless glass in 250 mL DCM.
   b. Weigh 5.0 g (60 mmol, 3.0 equiv) of sodium bicarbonate and add it to the previously prepared solution.
   c. In a separate round bottom flask, weigh 15.2 g (59.9 mmol, 3.01 equiv) of iodine on a zeroed balance and dissolve in 250 mL DCM.

   Note: Ensure that iodine is completely dissolved in DCM before pouring the I_2 solution on the oxalyl amide. This can be achieved by backlighting the bottom of the flask to notice the possible presence of undissolved solid. Introduction of less than 3 equivalents iodine can potentially result in longer reaction times and lower conversions.

   d. Pour the iodine solution on the 2-(*bis*(2-(triphenylmethylthio)ethyl)amino)-2-oxoacetate **4** / $NaHCO_3$ suspension using a glassmade liquid funnel.
   e. Stir the resulting purple mixture during 1 h at room temperature.
   f. Monitor the reaction.
      i. Microscale extraction – Use a 1 mL syringe to draw 0.25 mL of the crude reaction mixture and add the latter to a vial containing 1 mL of ethyl acetate (EtOAc) and 1 mL of a 1 M solution of sodium thiosulfate in deionized water.
      ii. Shake vigorously until complete fading of the purple color.
      iii. Using a capillary, spot the upper organic layer on a TLC plate, as well as the starting material diluted in EtOAc.
      iv. Place the plate in a TLC developing chamber using cyclohexane / EtOAc / DCM : 7.5 / 1.5 / 1 (v/v/v) as the mobile phase. Once the solvent front reaches ∼6 cm, remove the plate from the chamber.
      v. Check for reaction advancement at 254 nm and stain with phosphomolybdic acid (R_f = 0.32, Figure 5).
   g. Work-up the reaction.
      i. In an Erlenmeyer, prepare 400 mL of an aqueous solution of sodium thiosulfate 1 M.
      ii. Slowly pour the reaction mixture on the thiosulfate solution while stirring.
      iii. Transfer the colorless biphasic mixture into a 1 L separatory funnel and separate the layers.
      iv. Extract once the aqueous layer with 50 mL DCM and separate the layers.
      v. Wash the combined organic extracts with 50 mL brine. Separate the layers.

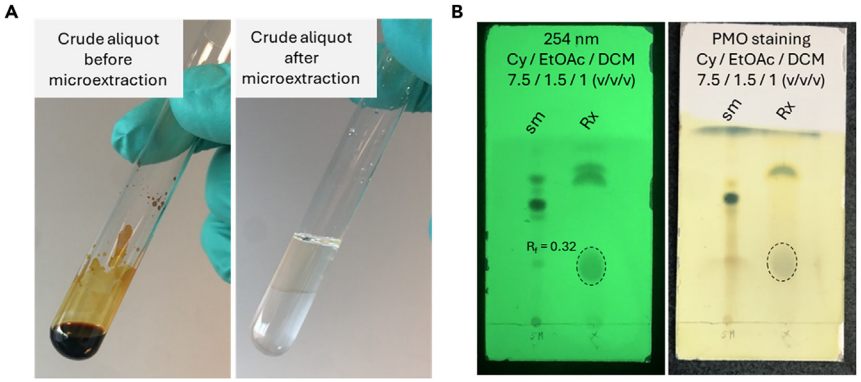


Figure 5. Monitoring of iodine-mediated trityl group cleavage

(A) Microextraction of the crude reaction. A colorless biphasic mixture is obtained after addition of ethyl acetate and a sodium thiosulfate aqueous solution.

(B) TLC-monitoring of the reaction (Sm = starting material ; Rx = crude reaction mixture). The expected product corresponds to the spot at R_f = 0.32.

vi. Dry the organic layer over anhydrous magnesium sulfate.
vii. Magnesium sulfate is removed by suction filtration using a fritted glass funnel and is rinsed with 25 mL DCM.
viii. Concentrate the filtrate under reduced pressure to a final residual volume of ~10–15 mL (20 mbar, bath temperature 30°C).

Note: Evaporating to dryness should be avoided as trityl byproducts formed during the reaction massively precipitate and severely complicate column loading.

h. Purify the crude mixture by silica gel column chromatography.
   i. Suspend 62 g of silica gel in 170 mL of eluent (cyclohexane / EtOAc / DCM : 7.5 / 1.5 / 1; v/v/v) in a 250 mL Erlenmeyer flask. Homogenize using a glass rod.
   ii. Using a plastic funnel, pour the slurry into a column having a fritted disk and backfill the extruded solvent into the column for 5 times under pressure until tight packing of the silica gel.
   iii. Load the solution containing the crude reaction mixture obtained at step 3.g.viii.
   iv. Add a layer of sand on the top (1–2 cm) and start eluting with cyclohexane / EtOAc / DCM : 7.5 / 1.5 / 1 (v/v/v) as eluent.
   v. Collect the fractions within test tubes (15–20 mL fraction size).
   vi. Check the content of the tubes by TLC using cyclohexane / EtOAc / DCM : 7.5 / 1.5 / 1 (v/v/v) as the mobile phase.
   vii. Pour the test tubes containing the desired product into round bottom flask and remove the solvent with a rotary evaporator.
   viii. Further remove the remaining solvent by using a high vacuum for 15 min.
   ix. The final product **5** is obtained as a yellowish oil (3.7 g, 80%). Based on three replicates performed from 14.5 g of 2-(*bis*(2-(triphenylmethylthio)ethyl)amino)-2-oxoacetate **4**, the average isolated yield obtained for this synthetic step was 81 $\pm$ 5%.

Note: Trityl byproducts might prove difficult to separate. If any remaining trityl derivatives are minimal (under 3% after purification as judged by NMR), the product can be engaged in the next step without further additional purification.

i. Analyze the isolated compound.

Note: Processed spectra are available in the supplementary material file (Figures S8–S11).

^{1}H NMR (300 MHz, $CDCl_3$) δ 4.34 (q, 2H, *J* = 7.1), 3.98 (t, 2H, J = 5.8), 3.79 (t, 2H, *J* = 5.8), 3.13–3.09 (m, 2H), 3.00–2.97 (m, 2H), 1.39 (t, 3H, J = 7.1) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.35, 161.91, 62.41, 53.18, 49.20, 40.23, 34.45, 14.13 ppm; IR (ATR) ν_{max}/cm^{-1} 2980, 2930, 1732, 1651, 1481, 1443, 1423, 1362, 1256, 1155, 1028, 887; MS (ESI, positive detection mode, direct infusion): $[M + H]^+$ m/z calcd. 236.32, found 236.08; TLC: R_f = 0.32 in cyclohexane / EtOAc / DCM : 7.5 / 1.5 / 1 (v/v/v).

4. Synthesize ethyl 2-(1,2,5-dithiazepan-5-yl)-2-oxoacetic acid **1**.
   a. Weigh 4.50 g (19.1 mmol) of ethyl 2-(1,2,5-dithiazepan-5-yl)-2-oxoacetate **5** in a 1 L round bottom flask on a zeroed balance. Dissolve the pale-yellow glass in 50 mL THF.
   b. In an Erlenmeyer, prepare 50 mL of an aqueous solution of sodium hydroxide 1 M.
   c. Pour the sodium hydroxide solution on the ethyl 2-(1,2,5-dithiazepan-5-yl)-2-oxoacetate **5** solution.
   d. Stir the resulting pale-yellow mixture during 3 h at room temperature.
   e. Monitor the reaction by TLC.

Note: Reaction monitoring can be performed at any time. We recommend a first analysis after 30 min and a second one after 3 h to better appreciate the change in intensity of the spot corresponding to the starting material.

   i. Microscale extraction – Use a 1 mL syringe to draw 0.25 mL of the crude reaction mixture and add the latter to a vial containing 1 mL of ethyl acetate (EtOAc) and 1 mL of a 1 M HCl solution.

⚠ CRITICAL: The pH of the aqueous layer must be strictly less than 2. If not, adjust by further adding 1 M HCl solution and control the pH using pH test paper. As predicted by MolGpka software,[13] ^{oxo}SEA's acid moiety pK_a is only 2.6.

   ii. Shake vigorously until formation of a biphasic colorless solution.
   iii. Using a capillary, spot the upper organic layer on a TLC plate, as well as the starting material diluted in EtOAc.
   iv. Place the plate in a TLC developing chamber using cyclohexane / EtOAc : 7 / 3 (v/v) as the mobile phase. Once the solvent front reaches ~6 cm, remove the plate from the chamber.
   v. Stain with an ethanolic solution of phosphomolybdic acid (Figure 6).

   f. Work-up the reaction.
      i. Slowly pour 200 mL of an aqueous 1 M solution of HCl on the reaction mixture while stirring.
      ii. Transfer the resulting colorless biphasic mixture into a 1 L separatory funnel and separate the layers.
      iii. Check that the pH of the aqueous layer is 2 or less. If not, adjust by adding more acid.
      iv. Extract twice the aqueous layer with 100 mL EtOAc and separate the layers.
      v. Combine the organic extracts and wash with 50 mL brine. Separate the layers.
      vi. Dry the organic layer over anhydrous $MgSO_4$.
      vii. $MgSO_4$ is removed by suction filtration using a fritted glass funnel and is rinsed with 25 mL EtOAc.
      viii. Concentrate the filtrate under reduced pressure (20 mbar, bath temperature 30°C).
      ix. Further remove the remaining solvent by using a high vacuum.
      x. The final product **1** is obtained as a white powder (2.51 g, 63%). Based on four replicates performed from 2.5 to 4.5 g of ethyl 2-(1,2,5-dithiazepan-5-yl)-2-oxoacetate **5**, the average isolated yield for this synthetic step is (60 ± 6%).

Note: Because the triphenylmethyl group is an intense UV chromophore, traces of residual trityl byproducts might be visible on control TLC plates (at R_f > 0.8) after final product isolation. However, as long as the level of this contamination is below 3%, as judged by the

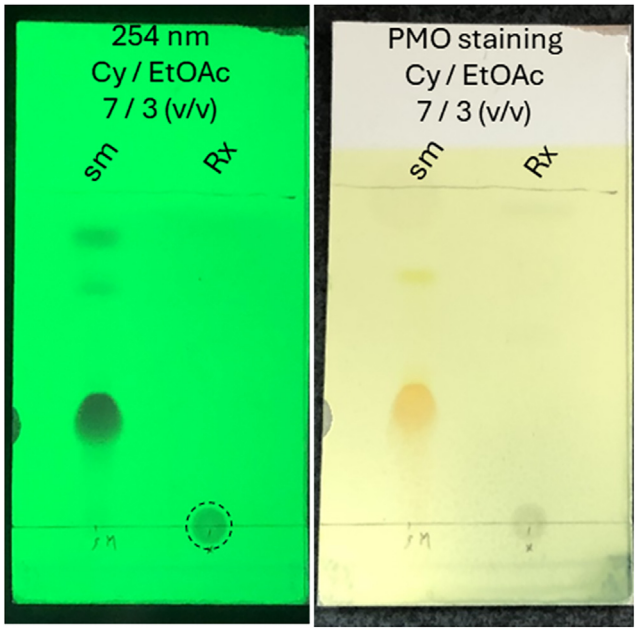


Figure 6. TLC-monitoring of the reaction (Sm = Starting material; Rx = crude reaction mixture)
Reaction completion is indicated by disappearance of the starting material spot (R_f = 0.32).

intensity of signals in the aromatic region of the ^{1}H NMR spectrum, the product can be used directly without further purification.

g. Analyze the isolated product.

Note: Processed spectra are available in the supplementary material file (Figures S12–S16).

^{1}H NMR (300 MHz, $CDCl_3$) δ 4.37 (s, 2H), 4.06 (dd, 2H, *J* = 6.18, 5.59), 3.11–3.06 (m, 4H) ppm. Note the presence of a broad multiplet at 4.37 ppm which corresponds to protons within the dithiepane cycle. This ^{1}H NMR pattern is likely due to the presence of conformers; ^{13}C NMR (75 MHz, $CDCl_3$) δ 54.91, 53.24, 38.93, 33.91 ppm. Despite varying relaxation time and number of scans in the ^{13}C-NMR experiment, signals attributable to carbonyl carbons in the ^{oxo}SEA handle **1** remain undetected; IR (ATR) ν_{max}/cm^{-1} 3389, 2925, 1924, 1673, 1625, 1446, 1425, 1366, 1353, 1322, 1290, 1215, 1195, 1157, 1031, 880, 699; MS (ESI, negative detection mode, direct infusion): $[M-H]^-$ m/z calcd. 206.26, found 206.17; m.p. (capillary method): 133°C–135°C.

Pause point: Ready to use ^{oxo}SEA handle **1** can be stored for several months at 4°C or below.

Preparation of ^{oxo}SEA-N-ter functionalized V5 peptide 6

Timing: 30 h total expected time for the peptide 6

Timing: 26 h (for step 5)

Timing: 1.5 h (for step 6)

Timing: 3 h (for step 7)

The ^{oxo}SEA handle **1** can be coupled at the N-terminus of any peptide during SPPS. The following section describes the preparation, the cleavage and the purification of a V5-derived peptide tag having the following sequence: ^{oxo}SEA-GSGSGSGKPIPNPLLGLDSTASGSK-NH_2 (Figure 7).

As fully automated assembly of this peptide sequence leads to the formation of complex crude mixtures containing capped peptide byproducts and results in low yield, the synthesis was optimized by manual incorporation of the AS diad as a pseudoproline dipeptide unit.[14] The procedure is described hereafter.

5. Prepare the GSGSGSGKPIPNPLLGLDSTASGSK peptide using Fmoc-SPPS (also refer Boll et al. (2015)[15]).
   a. Weigh 200 mg (0.050 mmol) of Novasyn-TGR resin (0.25 mmol/g) in the reactor.

   Note: The synthesis can be equally performed by hand using the above-described solutions and reaction times.

   b. Install the reactor in the automated batch peptide synthesizer.
   c. Program the sequence (GSK) to be assembled from the following commercial amino acid Fmoc-derivatives: Fmoc-Gly-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Lys(Boc)-OH.
   d. Use the method described in Table 1 to run the synthesizer.
   e. Transfer the resin into a manual peptide synthesis vessel and wash the latter with DMF. Drain the vessel.
   f. Weigh 87.7 mg of the pseudoproline dipeptide Fmoc-Ala-Ser(ψ(Me, Me)Pro)-OH (0.200 mmol, 4.0 equiv) in a low binding plastic tube and dissolve it in 1 mL DMF. Add 69.7 μL of DIPEA (0.401 mmol, 8.0 equiv) and 74.3 mg HATU (0.195 mmol, 3.9 equiv). Vortex for 45 s until the solution turns orange.

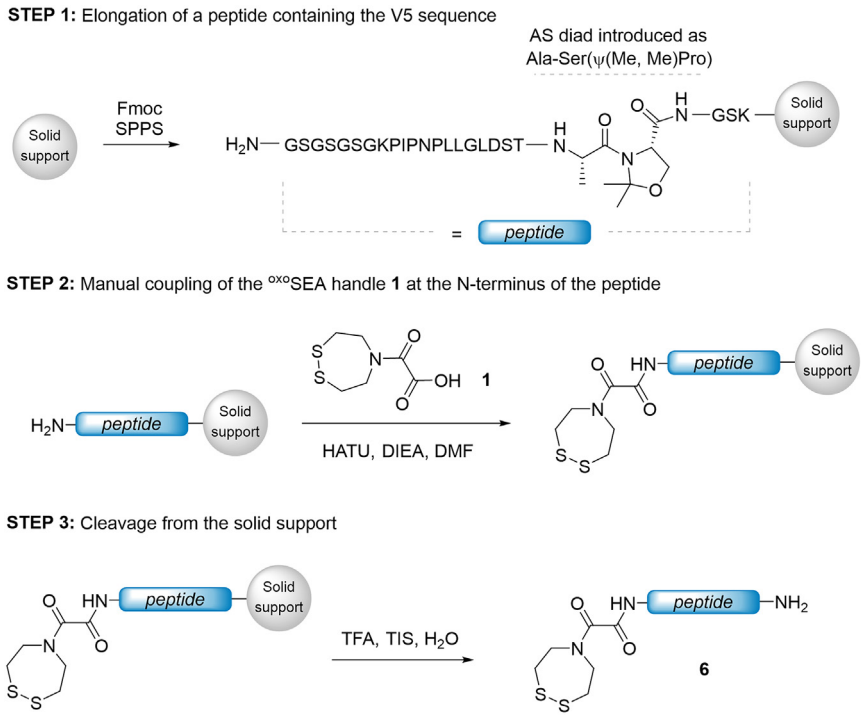


Figure 7. Synthetic approach for the preparation of an N-ter functionalized V5 tag-derived peptide with an ^{oxo}SEA handle

Table 1. Fmoc SPPS method used for assembling peptide 6

Preparation of the resin	
–	Wash reactor[a] with DMF (1.25 mL) Drain reactor
Cycle for n residues	
Fmoc deprotection *2 + 10 min per residue*	Set temperature to 25°C Add deprotection solution[b] (1.25 mL) and shake 2 min Drain the reactor Add deprotection solution (1.25 mL) and shake 10 min Drain the reactor
Column wash	Wash reactor with DMF (1.25 mL) Drain reactor Repeat last two steps 5 times
Amino acid coupling *2 × 30 min per residue*	Set temperature to 25°C Add 500 µL of activator solution[c], 125 µL of base solution[d] and 525 µL the amino acid derivative solution[e] Shake for 30 min Drain the reactor Proceed to a second coupling by repeating the last three steps once
Capping *2 min per residue*	Set temperature to 25°C Add capping solution[f] (1.25 mL) and shake 2 min
Column wash	Wash reactor with DMF (1.25 mL) Drain reactor Repeat last two steps 5 times Restart at step 2 for the following residue.
Final step	
Final Fmoc deprotection *2 + 8 + 8 min*	Add deprotection solution[b] (1.25 mL) and shake 2 min Drain the reactor Add deprotection solution[b] (1.25 mL) and shake 8 min Drain the reactor Repeat the above two steps once
Column wash	Wash reactor with DMF (1.25 mL) Drain reactor Repeat last two steps 5 times
Column wash	Wash reactor with DCM (1.25 mL) Drain reactor Repeat last two steps 3 times

[a]Reactor volume = 5 mL.
[b]The deprotection solution is a 20% (v/v) solution of piperidine in DMF.
[c]The activator solution is a 0.5 M solution of HCTU in DMF. Note 11.
[d]The base solution is a 37.5% (v/v) solution of DIPEA in 1-methyl-2-pyrrolidinone (NMP).
[e]Amino acid derivative solutions are 0.5 M solution of the appropriate Fmoc-protected amino acid in DMF.
[f]The capping solution is prepared as a mixture of DMF / acetic anhydride / DIPEA: 85:10:5 (v/v/v).

Note: HATU, as well as other uronium-based coupling agents used in SPPS (e.g. HBTU, TBTU, COMU…), are known for eliciting an allergic response upon skin contact.[16] They should be handled under an efficient fume hood and with appropriate personal protective equipment (lab coat, gloves and mask).

g. Add the previous solution in the peptide synthesis vessel containing the resin and shake for 4 h.
h. Drain the vessel and wash the resin with DMF. Repeat 5 times.
i. Perform a capping step by adding 1 mL of a DMF / acetic anhydride / DIPEA solution 85:10:5 (v/v/v). Shake for 15 min.
j. Drain the vessel and wash the resin with DMF. Repeat 5 times.
k. Transfer the resin into the reactor.
l. Program the rest of the sequence (GSGSGSGKPIPNPLLGLDST) to be assembled from the following commercial amino acid Fmoc-derivatives: Fmoc-Gly-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH

m. Use the method described in Table 1 to run the synthesizer.
n. Transfer the resin into a manual peptide synthesis vessel.
o. Drain the vessel and wash the resin with DMF. Repeat twice.

6. Couple the ^{oxo}SEA handle **1** on the supported peptide.
   a. Weigh 31 mg (0.15 mmol) of ^{oxo}SEA handle **1** in a 1.5 mL low binding plastic tube and dissolve in 0.5 mL DMF.
   b. Add 104 μL of DIPEA (0.598 mmol) in the above-prepared solution (solution 1).
   c. Weigh 55 mg of HATU (0.14 mmol) coupling agent in a separate 1.5 mL low binding plastic tube and dissolve in 0.5 mL DMF (solution 2).

⚠ CRITICAL: Pre-solubilization of the reagents in two separate solutions (solution 1 and 2) is crucial to the success of the coupling step. If solid HATU is added directly in the solution of the acid and DIPEA in DMF, the activation step will lead to the formation of byproducts and result in a low-yielding coupling to the peptide partner.

Note: Alternative coupling agents to HATU were investigated for the coupling of the ^{oxo}SEA handle **1** to lysine side chains or peptide N-terminus. Carbodiimide activating agents were entirely unsuccessful, while phosphonium salt-based reagents like PyBOP or PyBroP afforded some product, albeit in considerably lower yields compared to HATU.

   d. Drain the manual peptide synthesis vessel containing the elongated peptide supported on the resin beads and resuspend in 1 mL of DMF.
   e. Transfer solution 1 to the low binding plastic tube containing solution 2 and vortex for precisely 30 s. The resulting solution turns orange.

⚠ CRITICAL: For optimal results, activate oxalic acid derivative for no more than 30 s in order to minimize degradation of the activated intermediate.

   f. Transfer the above mixture into the peptide synthesis vessel containing the resin beads.
   g. Agitate with a laboratory flask shaker at room temperature for 30 min.
   h. Drain the vessel.
   i. Resuspend the resin in 5 mL DMF.
   j. Wash the resin by repeating steps h. and i. twice.
   k. Proceed to a second coupling by repeating all steps from a once again.

Note: At this step, a TNBS assay[17] can be performed to check for completeness of the ^{oxo}SEA handle coupling reaction to the supported peptide. The TNBS assay is a rapid and generalized test for the detection of free primary amines. The reaction of 2,4,6-trinitrobenzene sulfonic acid (TNBS) with free primary amines undergoes the formation of a highly chromogenic derivative. In the context of SPPS, a positive test result is indicated by a color change of the resin beads to orange-red, signifying the presence of unreacted primary amines (i.e. incomplete coupling). Conversely, the absence of a color change suggests successful coupling of the ^{oxo}SEA handle 1.

      i. Transfer a few resin beads to a small glass test tube and rinse with ethanol (by pipetting the supernatant and resuspending in ethanol). Finally resuspend in 0.5 mL of a 10% (v/v) DIPEA solution in DMF.
      ii. Add 2 drops of a 1% (w/v) solution of 2,4,6-trinitrobenzene sulfonic acid in DMF.
      iii. Gently agitate the tube and allow to stand for 3 min at room temperature.
      iv. Pipette the supernatant and resuspend in DMF. Observe the color of the beads.

7. Deprotect and cleave peptide **6** (also refer Boll et al. (2015)[15]).
   a. Drain the vessel.
   b. Resuspend the resin in 5 mL DCM.

c. Wash the resin by repeating steps a. and b. twice.
d. Drain the vessel.
e. Resuspend the resin in 5 mL Et_2O.
f. Wash the resin by repeating steps d. and e. twice.
g. Connect the vessel to a membrane pump for 30 min to dry the resin.
h. In a graduated cylinder, add 9.5 mL of TFA, 0.25 mL of deionized water and 0.25 mL of triisopropylsilane (TIS).

Note: TFA is strongly corrosive and should be handled inside a chemical fume hood while using appropriate personal protective equipment (lab coat, gloves and protective glasses).

i. Once the resin has been dried, add the TFA solution to the manual peptide synthesis vessel.
j. Agitate the resulting bead suspension with a laboratory flask shaker at room temperature for 2 h.
k. As the cleavage is being performed, prepare five 50-mL centrifuge tubes, each containing 40 mL of a 1:1 (v/v) mixture of Et_2O and *n*-heptane and cool in an ice-bath.
l. After 2 h, distribute the reactor's content equally into the 4 centrifuge tubes containing the ice-cold mixture of Et_2O and *n*-heptane (i.e., ~2.5 mL in each tube). The peptide precipitates immediately.

⚠ CRITICAL: To not impair the formation of the peptide precipitate, we recommend the volume of the Et_2O/*n*-heptane solution to be at least twenty times that of the TFA cleavage solution.

m. Add 1 mL of pure TFA in the manual peptide synthesis vessel and gently shake to rinse the beads.
n. Drain the vessel into the remaining 50-mL centrifuge tube.
o. Centrifuge the tubes at 4000 × *g* for 4 min at room temperature. Carefully discard the supernatant into an Erlenmeyer flask.
p. Add 25 mL of an ice-cold Et_2O/*n*-heptane solution (1:1 v/v) and resuspend the pellet by vortexing 30 s.
q. Centrifuge the tubes at 4000 × *g* for 4 min at room temperature and discard the supernatant.
r. Repeat steps p. and q. twice.
s. Evaporate the residual solvent under a slow stream of nitrogen gas.
t. Dissolve each crude peptide pellet in 5 mL deionized water, pool the peptide aqueous solutions and freeze in liquid nitrogen.
u. Place the tubes in a 600-mL lyophilizer flask and lyophilize for 24 h to obtain 29.2 mg of crude peptide **6** (22% yield).

Note: Despite achieving efficient ^{oxo}SEA handle **1** coupling as checked through microcleavage controls, the isolated yield for peptide **6** remains low (11%). This is likely attributable to its inherent hydrophobicity, which significantly complicates its recovery during precipitation and purification steps.

v. The crude peptide is dissolved in 4.5 mL of deionized water and purified on semipreparative HPLC according to the conditions described in the materials and equipment section with a 0%–50% eluent B linear gradient over 60 min.
w. Pool the tubes containing the peptide aqueous solutions and freeze in liquid nitrogen.
x. Freeze-dry during 24 h.
y. The final product **6** is obtained as a white fluffy solid (14.0 mg, 11% yield). Its identity is supported by UPLC-MC analysis (Figure 8).

Pause point: Peptide **6** can be stored for several months at −20°C.

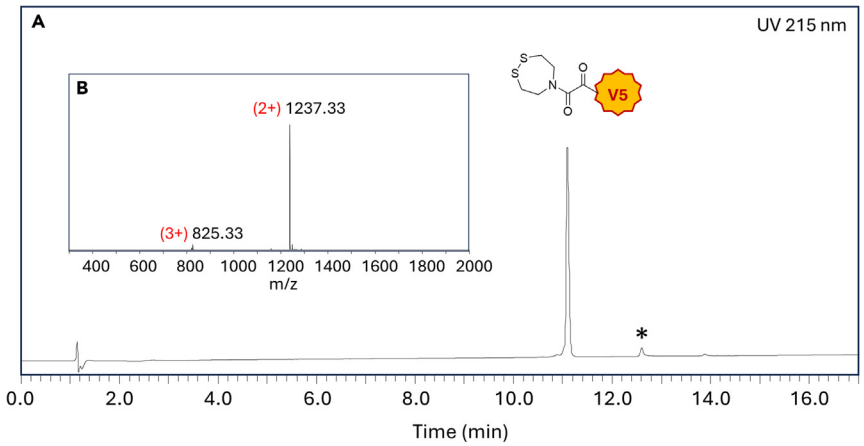


Figure 8. Analysis of purified V5-^{oxo}SEA peptide 6

(A) LC trace from UPLC-MS analysis: C18 BEH 300 Å, 1.7 µm, 2.1 × 100 mm column, eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH_3CN, gradient 0%–70% B in 20 min, 0.4 mL/min, T = 50°C, λ = 215 nm *: non-peptidic impurity at 12.8 min from the column.

(B) MS trace: $[M+2H]^{2+}$ m/z calcd. (av.) 1237.39, obs. 1237.33, $[M+3H]^{3+}$ m/z calcd. (av.) 825.26, obs. 825.33.

Conjugation of the Cys-B1 domain of the streptococcal G protein

Timing: 3 h

This section of the method details how ^{oxo}SEA ligation can be used to modify peptides and proteins. We describe herein a typical reaction between synthesized peptide **6** and an N-terminal cysteine-containing B1 domain of the streptococcal protein Cys-GSGS-GB1 (Figure 9). Here, the Cys-GSGS-GB1 protein was produced using recombinant expression in *E. coli* (full preparation details are available in the supplementary material file). More generally, the procedure can be adapted to any combination of reactants containing an ^{oxo}SEA group and a β-aminothiol motif, should they be peptidic or not. As a warning, note however that redox activation of the ^{oxo}SEA motif requires excess TCEP and that such conditions can lead to the unfolding of disulfide bond-stabilized proteins.

To showcase the power of the ^{oxo}SEA method, we chose reaction conditions with only 100 µM of the Cys-GSGS-GB1 substrate and 120 µM of the V5 modifier. Interestingly, ^{oxo}SEA-mediated ligation already achieves satisfying rates in the common millimolar concentration range. This reactivity positions oxalyl thioester precursors competitively with classical aryl thioesters for NCL reactions

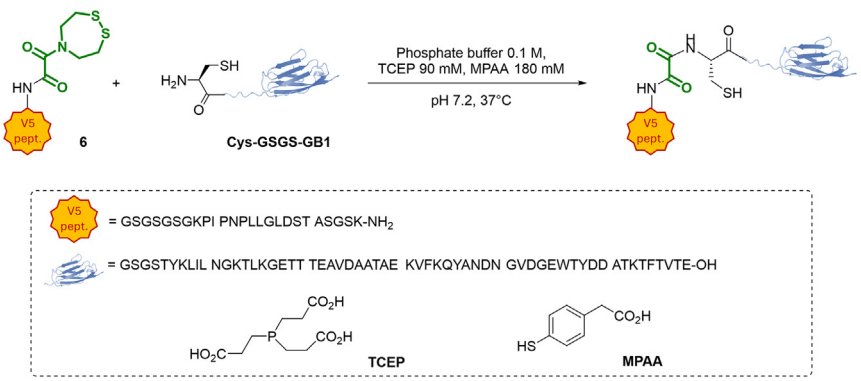


Figure 9. ^{oxo}SEA-mediated ligation of V5 peptide 6 with Cys-GSGS-GB1 protein

(~4 M^{-1} s^{-1}).[18,19] However, a surprising trend emerges when reactant concentrations, i.e., ^{oxo}SEA precursor and Cys peptide, are reduced to the micromolar range. The reaction rate can reach up to 30 M^{-1} s^{-1} from 100 μM reactant concentration and below. Therefore, such dilution conditions become particularly interesting, as they reveal a distinct advantage of our approach compared to classical NCL.

In addition, the original article by Snella et al. put forward the utilization of a nitrogen-regulated glovebox, which is not a common equipment in most chemistry and biology laboratories. Here, we present a more accessible alternative using a glovebag. This affordable option allows conducting sensitive reactions under an inert atmosphere.

8. Perform the ^{oxo}SEA-mediated ligation reaction.
   a. Prepare buffer solutions and weigh peptide/protein partners for the reaction.

   Note: The following steps (i-vii) are preparatory steps.

      i. Prepare 100 mL of a 0.1 M phosphate buffer pH 7.2 by weighing 0.396 g of monosodium phosphate monohydrate salt and 1.911 g of disodium hydrogen phosphate heptahydrate salt on a zeroed balance. Place the solids in a 100 mL-volumetric flask and add 50 mL deionized water. Once the solids dissolved, make up to the mark with deionized water. Check the pH with a pH-meter.
      ii. Weigh 114.8 mg of *tris*(2-carboxyethyl)phosphine (TCEP) in a 5-mL low binding plastic tube on a zeroed balance and dissolve in 4 mL of the phosphate buffer prepared in step 8.a.i. At this point, the concentration of TCEP is 100 mM.
      iii. Weigh 134.5 mg of 4-mercaptophenylacetic acid (MPAA) on weighing paper and add in the previous solution.
      iv. Measure the pH of the resulting suspension which should be close to a value of pH 3.
      v. Adjust the pH of the solution to 7.2 by adding 6 M sodium hydroxide. The solid will dissolve upon addition of NaOH to yield a homogeneous and colorless solution.

   Note: ~450 μL of 6 M NaOH are typically required to adjust the pH to 7.2. Following pH adjustment, TCEP and MPAA reach final concentrations of ~90 mM (from 100 mM) and ~180 mM, respectively.

      vi. Weigh 1087 μg of the Cys-GSGS-GB1·7 TFA protein (0.015 μmol) in a 1.5 mL conical low binding plastic tube on a high precision balance.
      vii. Weigh 486 μg of the ^{oxo}SEA-V5·2 TFA peptide **6** (0.018 μmol) in a 1.5 mL conical low binding plastic tube on a high precision balance.
      viii. Prepare 10 × 1 mL-low binding plastic tubes containing 90 μL of a 20% v/v acetic acid aqueous solution each.

   Note: This step is optional and required only if the kinetic monitoring of the reaction is planned.

   b. Prepare the glovebag.
      i. Place the glovebag flat on the workbench near a nitrogen inlet (Figure 10).
      ii. Introduce all the material required to perform the ligation reaction and the kinetic monitoring of the process (optional). The material is listed in Table 2.
      iii. Seal up any hole or opening using duct tape and zip the glovebag to close it.
      iv. Carefully vacuum the glovebag making sure that no sharp object is protruding.
      v. Inflate the chamber with nitrogen (> 99.98% pure N_2 from a 200-bar gas cylinder).
      vi. Open the low binding plastic tubes containing the reagents and solutions to allow them to equilibrate under the nitrogen atmosphere for 30 min.

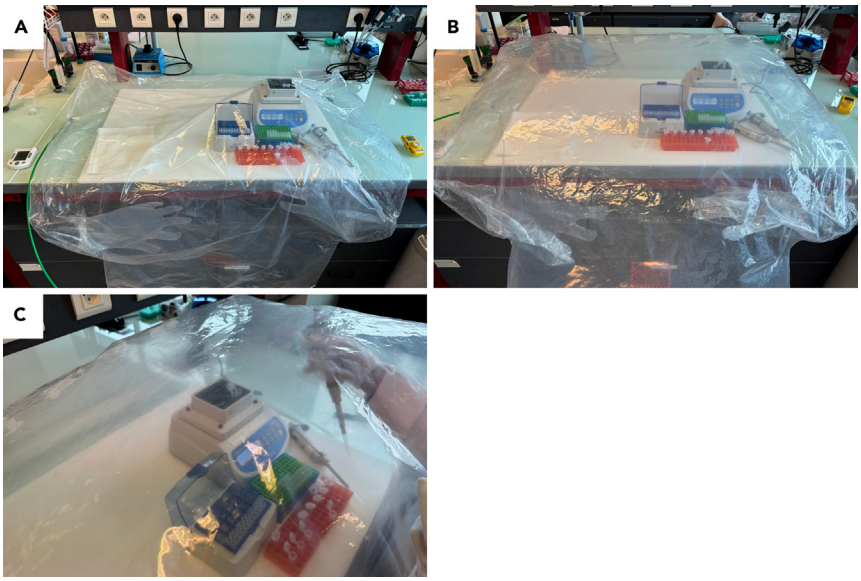


Figure 10. Glovebag installation and preparation

(A) All the required material is introduced within the glovebag (step 8.b.ii).

(B) Inflated glovebag (step 8.b.v).

(C) Manipulation inside the glovebag.

Note: The following steps (c-i) describe the ligation reaction procedure and the isolation of the conjugate.

c. Dissolve the Cys-GSGS-GB1 protein in 1.5 mL of phosphate buffer containing TCEP and MPAA (added as 2 × 750 µL using a 100–1000 µL automatic pipette).

⚠ CRITICAL: The Cys containing reaction partner, i.e. Cys-GSGS-GB1 in this case, should always be put first in contact with the reducing agents. Dissolving first the ^{oxo}SEA partner would result in premature activation of the handle and loss of material through hydrolysis.

d. Transfer this solution to the low binding plastic tube containing the ^{oxo}SEA-V5 peptide **6**.

e. Let the reaction proceed for at least 60 min at 37°C. At this point, a kinetic monitoring might be necessary to assess the progress of the reaction (Figure 11A). A detailed protocol is proposed below.

Table 2. Material to be introduced into the glovebag

REAGENT or RESOURCE
Reagents, Buffers
5 mL-low binding plastic tube containing 0.1 M phosphate buffer with 200 mM MPAA and 100 mM TCEP at pH 7.2
1.5 mL-low binding plastic tube containing the lyophilized Cys-GSGS-GB1 weighed powder
1.5 mL-low binding plastic tube containing the lyophilized ^{oxo}SEA-V5 **6** weighed powder
10 × 1.5 mL-low binding plastic tube containing 90 µL of a 20% v/v acetic acid aqueous solution each
Consumables
Pipette tips for automatic pipettes 20–200 µL and 100–1000 µL
Low binding plastic tube rack
Spatula
Small equipment
Mechanical micropipette 20–200 µL
Mechanical micropipette 100–1000 µL
Temperature control device (Eppendorf Thermomixer) or any heating bath at 37°C
100-mL beaker for used pipette tips

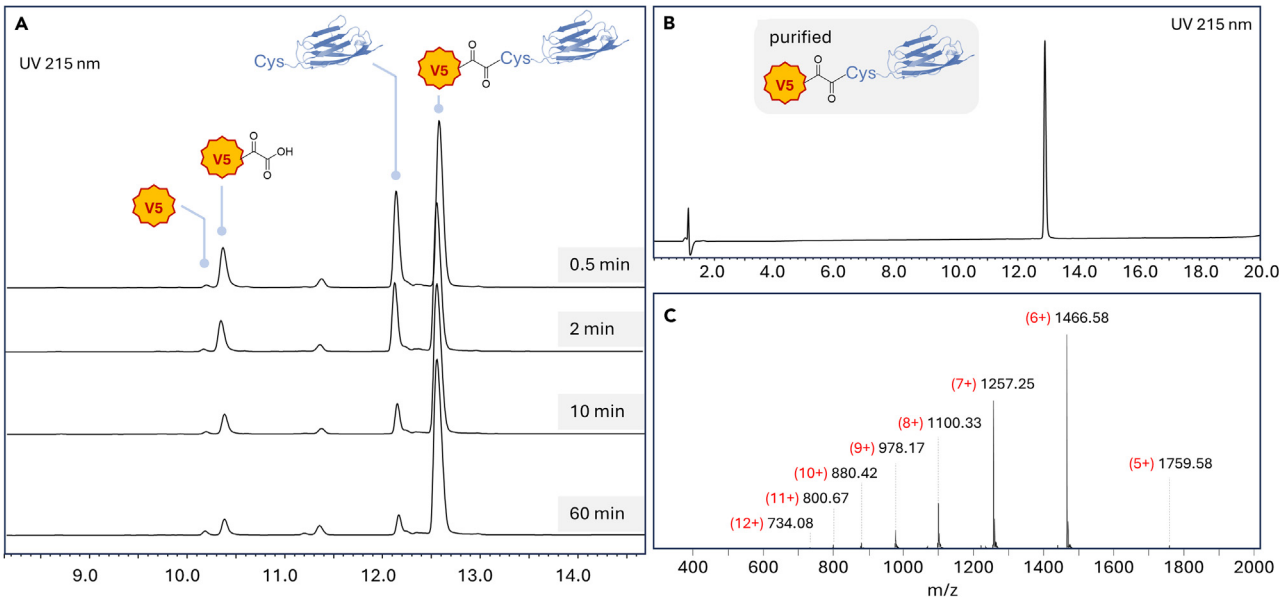


Figure 11. Synthesis of the V5-oxo-Cys-GSGS-GB1 conjugate

(A) Kinetic monitoring of the reaction by reverse phase HPLC at 215 nm

(B) Purified V5-oxo-Cys-GSGS-GB1 conjugate from UPLC-MS analysis. LC trace: C18 BEH 300 Å, 1.7 μm, 2.1 × 100 mm column, eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH_3CN, gradient 0%–70% B in 20 min, 0.4 mL/min, T = 50°C, λ = 215 nm

(C) MS trace: $[M+5H]^{5+}$ m/z calcd. (av.) 1759.71, obs. 1759.58, $[M+6H]^{6+}$ m/z calcd. (av.) 1466.59, obs. 1466.58, $[M+7H]^{7+}$ m/z calcd. (av.) 1257.22, obs. 1257.25, $[M+8H]^{8+}$ m/z calcd. (av.) 1100.20, obs. 1100.33, $[M+9H]^{9+}$ m/z calcd. (av.) 978.06, obs. 978.17, $[M+10H]^{10+}$ m/z calcd. (av.) 880.36, obs. 880.42, $[M+11H]^{11+}$ m/z calcd. (av.) 800.41, obs. 800.67, $[M+12H]^{12+}$ m/z calcd. (av.) 733.80, obs. 734.08.

f. Once the reaction finished, pour the reaction mixture in a 5 mL-low binding plastic tube containing 1 mL of a 20% v/v acetic acid aqueous solution.
g. Open the glovebag.
h. Purify by semipreparative HPLC (gradient 0%–50% eluent B in 60 min, 55°C column temperature).
i. Collect the fractions. Freeze in liquid nitrogen and lyophilize.
The V5-oxo-Cys-GSGS-GB1·8 TFA conjugate is obtained as a fluffy white solid (841 μg) with 57% isolated yield (Figures 11B and 11C).

Note: Figure 11A reveals the formation of a hydrolyzed V5 peptide peak at 10.4 min. Interestingly, the proportion of this byproduct appears to decrease throughout the chromatogram. This seemingly counterintuitive observation can be explained by the fact that this hydrolysis product primarily forms in the HPLC vial and not during the reaction itself. Additionally, the peak at 10.2 min corresponds to unmodified V5 peptide and stems from the hydrolysis product losing its N-terminal oxalic acid moiety.

Note: For comparison, the reaction was also performed under standard laboratory conditions (in air). Both reactions display remarkably similar profiles, reaching a plateau above 90% completion within 30 min (Figure 12). The apparent second order-rate constant determined by nonlinear regression is ~50 M^{-1} s^{-1}, a value which is consistent with former results obtained for an N-terminally positioned ^{oxo}SEA group.[1] High reductant concentrations (> 50 mM TCEP and 100 mM MPAA) mitigate the need for the application of rigorous anoxic conditions. However, for reactions requiring lower reductant excesses and concentrations, employing a glovebag is highly recommended to ensure consistent and reproducible outcomes.

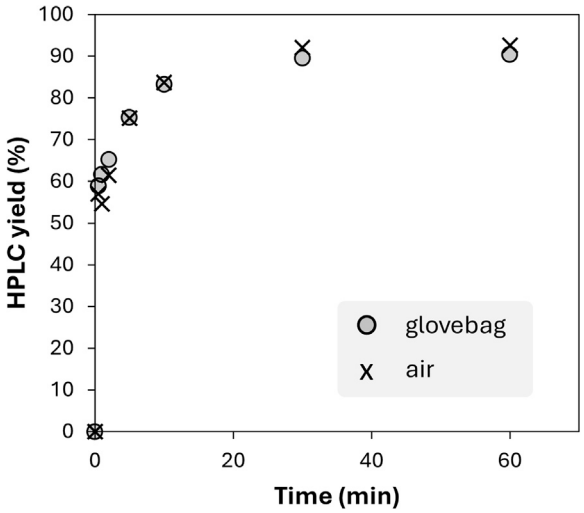


Figure 12. Kinetic monitoring of the ^{oxo}SEA-mediated ligation reactions between peptide 6 and Cys-GSGS-GB1 conducted in a glovebag (gray circles) or on the bench (black crosses)

HPLC yields were calculated from peak areas at 215 nm.

j. Monitor the ^{oxo}SEA-mediated ligation reaction overtime (**Optional**).
   i. At the desired time, sample 25 μL of the reaction mixture with an automatic pipette and add it to a low binding plastic tube containing 90 μL of a 20% v/v acetic acid aqueous solution (Figure 13A).
   ii. Gently agitate.
   iii. Repeat steps i. and ii. as many times as necessary.

⚠ CRITICAL: Crude diluted reaction samples should not be directly injected on analytical HPLC because of the presence of MPAA. The latter contaminates chromatograms and can potentially alter instrument performance upon precipitation under acidic conditions. An extraction step is recommended before injection.

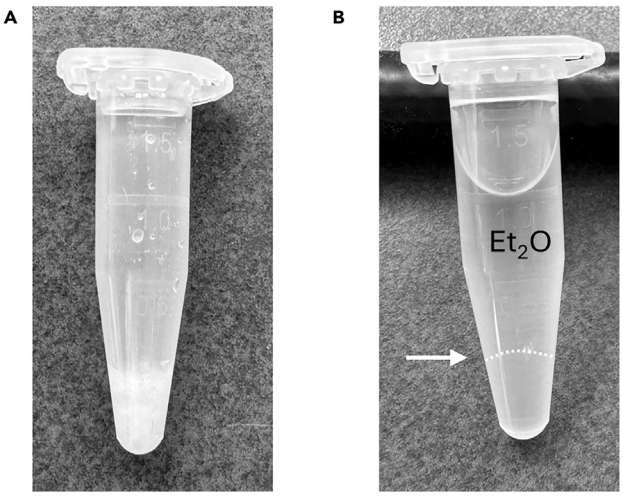


Figure 13. Extraction of MPAA before HPLC-analysis

(A) 25 μL of the reaction mixture are quenched in 90 μL of a 20% (v/v) aqueous acetic acid solution. This leads to the formation of a suspension due to MPAA precipitation.

(B) Addition of diethyl ether and vortexing results in the solubilization of MPAA in the upper ethereal phase which can be removed by pipetting.

iv. Once the reaction is complete, remove the samples from the glovebag.
v. In each low binding plastic tube, add ~1 mL of diethyl ether. Vortex, let decant (Figure 13B).
vi. Remove the upper ethereal layer using a Pasteur pipette.
vii. Repeat steps v. and vi. twice.
viii. Purge the plastic tube with nitrogen.
ix. Transfer into an HPLC-vial and analyze by HPLC (Figure 11A).

EXPECTED OUTCOMES

Application of this procedure is expected to yield ^{oxo}SEA handle **1**. Step-by-step analytical data are provided throughout the protocol. Additionally, processed NMR spectra are available in the supplementary material file for further comparison. The handle demonstrates versatility and can be attached not only at the N-terminus of a peptide segment as shown here but also to any aliphatic amine under similar reaction conditions.

The procedure provided herein also details how to perform an ^{oxo}SEA-mediated ligation reaction. Thanks to its chemoselectivity and efficiency, this process is suitable for preparative-scale modifications of N-terminal cysteine-containing proteins as well as *in situ* derivatization within complex mixtures as demonstrated by Snella et al.[1] It can be of course applied to any type of substrate harboring a β-aminothiol motif, with the constraint that redox activation of the ^{oxo}SEA motif requires excess TCEP and that such conditions can lead to the unfolding of disulfide bond-stabilized proteins.

LIMITATIONS

Decreasing the concentration of reducing agents to a few equivalents (low millimolar range) might be imposed by constraints such as sample sensitivity or analytical limitations. While the reaction is robust under excess reducing agents and can proceed smoothly even without special precautions (in air), its sensitivity to oxygen becomes apparent when reducing agent concentrations are lowered. In such a case, conversion rates can significantly decrease, and rigorous inert conditions are crucial to maintain reaction efficiency.

TROUBLESHOOTING

Problem 1

Triphenylmethyl derivatives are notoriously known to sequester solvent molecules. During the synthesis of intermediate **3**, ^{1}H NMR analysis may evidence product contamination by substantial amounts of solvents (DMF and EtOAc). This can impair crystallization after evaporation.

Potential solution

If ^{1}H NMR shows high contamination by DMF, dissolve the crude compound in EtOAc (~10 mL per gram of intermediate **3**). Wash the organic layer 6 to 8 times with 5% w/v aqueous solution of KH_2PO_4. Dry over $MgSO_4$, filter and concentrate under reduced pressure. If a solid is not obtained, redissolve the residue in dichloromethane and evaporate to dryness. Repeat the last step until a yellowish powder is isolated (3 times might be necessary).

Problem 2

During the synthesis of intermediate **5**, massive trityl byproducts precipitation can compromise efficient separation by silica gel column chromatography.

Potential solution

The crude mixture after extraction should be evaporated to a residual volume of ~10–15 mL. Evaporating the organic layer to dryness is not recommended as trityl byproducts formed during the reaction will massively precipitate and severely complicate column loading. Addition of 10% (v/v) of DCM in the eluent is also important to prevent precipitation of trityl derivatives during column

chromatography purification. For larger scale reactions (exceeding 30 mmol), consider increasing the DCM fraction in the eluent to 15% (v/v).

Problem 3

TLC analysis indicates complete conversion, yet the final product yield is low. This discrepancy suggests potential product loss during the final extraction of ^{oxo}SEA handle **1**.

Potential solution

^{oxo}SEA handle **1** can be discarded in the final aqueous layer after extraction. Ensure the pH of this layer is below 2. If necessary, adjust the pH with 4 M HCl instead of 1 M HCl for improved acidification and reduced aqueous layer volume.

Problem 4

The conversion of the ^{oxo}SEA-mediated ligation does not exceed 70%–80% at 100 μM and above reactant concentration.

Potential solution

The observed issue likely stems from a combination of factors. Here are two key areas to investigate.

- Stoichiometric imbalance: Insufficient starting quantities of a reagent can lead to its premature depletion, thus limiting conversion. Kinetic monitoring by HPLC, UV determination of peptide concentration[20] can reveal such depletion. In such a case, consider adjusting the stoichiometry.
- Inert atmosphere negligence: Failure to maintain an inert atmosphere during the reaction can lead to reactants oxidation. Notably, the activated acyclic ^{oxo}SEA moiety can revert back to its cyclic and inert form through the formation of an intramolecular disulfide bond (Figure 1). Also, the side chain thiol of Cys residues can be inactivated through the formation of disulfides with MPAA or other Cys residues. These side reactions effectively kill the process and form unwanted products/adducts detectable by HPLC analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Vangelis Agouridas (vangelis.agouridas@ibl.cnrs.fr).

Technical contact

Further information and requests regarding technical aspects of this work will be fulfilled by the technical contact, Benoît Snella (benoit.snella@ibl.cnrs.fr).

Materials availability

This study did not generate new unique reagents, all compounds have been described in the original article; see Snella et al.[1]

Data and code availability

The present article contains all datasets generated or analyzed during this study. Processed spectra and chromatograms can be found in the supplementary file.

ACKNOWLEDGMENTS

This research was supported by the Agence Nationale de la Recherche (ANR-21-CE44-0031) and the Austrian Science Fund (FWF I 5800-N).

AUTHOR CONTRIBUTIONS

V.A., J.V., and O.M. designed and wrote the protocol with inputs from all the authors. F.T., C.W., S.R., R.D., B.S., M.S., and B.W. performed the experimental data. V.A. and O.M. supervised the project. All authors approved the final version of the manuscript for submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103390.

REFERENCES

1. Snella, B., Grain, B., Vicogne, J., Capet, F., Wiltschi, B., Melnyk, O., and Agouridas, V. (2022). Fast Protein Modification in the Nanomolar Concentration Range Using an Oxalyl Amide as Latent Thioester. Angew. Chem. Int. Ed. *61*, e202204992. https://doi.org/10.1002/anie.202204992.

2. Boutureira, O., and Bernardes, G.J.L. (2015). Advances in Chemical Protein Modification. Chem. Rev. *115*, 2174–2195. https://doi.org/10.1021/cr500399p.

3. Hoyt, E.A., Cal, P.M.S.D., Oliveira, B.L., and Bernardes, G.J.L. (2019). Contemporary approaches to site-selective protein modification. Nat. Rev. Chem *3*, 147–171. https://doi.org/10.1038/s41570-019-0079-1.

4. Dawson, P.E., Muir, T.W., Clark-Lewis, I., and Kent, S.B. (1994). Synthesis of proteins by native chemical ligation. Science *266*, 776–779. https://doi.org/10.1126/science.7973629.

5. Agouridas, V., El Mahdi, O., Diemer, V., Cargoët, M., Monbaliu, J.C.M., and Melnyk, O. (2019). Native Chemical Ligation and Extended Methods: Mechanisms, Catalysis, Scope, and Limitations. Chem. Rev. *119*, 7328–7443. https://doi.org/10.1021/acs.chemrev.8b00712.

6. Melnyk, O., and Agouridas, V. (2014). Perhydro-1,2,5-dithiazepine. e-EROS, 1–3. https://doi.org/10.1002/047084289X.rn01723.

7. Ollivier, N., Dheur, J., Mhidia, R., Blanpain, A., and Melnyk, O. (2010). Bis(2-sulfanylethyl) amino native peptide ligation. Org. Lett. *12*, 5238–5241. https://doi.org/10.1021/ol102273u.

8. Agouridas, V., Ollivier, N., Vicogne, J., Diemer, V., and Melnyk, O. (2022). Redox-Controlled Chemical Protein Synthesis: Sundry Shades of Latency. Acc. Chem. Res. *55*, 2685–2697. https://doi.org/10.1021/acs.accounts.2c00436.

9. Diemer, V., Bouchenna, J., Kerdraon, F., Agouridas, V., and Melnyk, O. (2021). N,S- and N,Se-acyl transfer devices in protein synthesis. In Total Chemical Synthesis of Proteins, A. Brik, L. Liu, and P. Dawson, eds. (Wiley), pp. 59–85. https://doi.org/10.1002/9783527823567.ch3.

10. Grain, B., Desmet, R., Snella, B., Melnyk, O., and Agouridas, V. (2023). Incorporation of a Highly Reactive Oxalyl Thioester-Based Interacting Handle into Proteins. Org. Lett. *25*, 5117–5122. https://doi.org/10.1021/acs.orglett.3c01846.

11. Jabbour, R., Salem, H., and Sidell, F.R. (2014). Nitrogen Mustards. In Encyclopedia of Toxicology, Third Edition, P. Wexler, ed. (Academic Press), pp. 560–566. https://doi.org/10.1016/B978-0-12-386454-3.00636-9.

12. Reddy, G., and Lee, S.H. (2015). Chapter 33 - Wildlife Toxicity Assessment for Mustard Agents. In Wildlife Toxicity Assessments for Chemicals of Military Concern, M.A. Williams, G. Reddy, M.J. Quinn, and M.S. Johnson, eds. (Elsevier), pp. 617–636. https://doi.org/10.1016/B978-0-12-800020-5.00033-8.

13. Pan, X., Wang, H., Li, C., Zhang, J.Z.H., and Ji, C. (2021). MolGpka: A Web Server for Small Molecule pKa Prediction Using a Graph-Convolutional Neural Network. J. Chem. Inf. Model. *61*, 3159–3165. https://doi.org/10.1021/acs.jcim.1c00075.

14. Mutter, M., Nefzi, A., Sato, T., Sun, X., Wahl, F., and Wöhr, T. (1995). Pseudo-prolines (psi Pro) for accessing "inaccessible" peptides. Pept. Res. *8*, 145–153.

15. Boll, E., Drobecq, H., Ollivier, N., Blanpain, A., Raibaut, L., Desmet, R., Vicogne, J., and Melnyk, O. (2015). One-pot chemical synthesis of small ubiquitin-like modifier (SUMO) protein-peptide conjugates using bis(2-sulfanylethyl)amido peptide latent thioester surrogates. Nat. Protoc. *10*, 269–292. https://doi.org/10.1038/nprot.2015.013.

16. Graham, J.C., Trejo-Martin, A., Chilton, M.L., Kostal, J., Bercu, J., Beutner, G.L., Bruen, U.S., Dolan, D.G., Gomez, S., Hillegass, J., et al. (2022). An Evaluation of the Occupational Health Hazards of Peptide Couplers. Chem. Res. Toxicol. *35*, 1011–1022. https://doi.org/10.1021/acs.chemrestox.2c00031.

17. Hancock, W.S., and Battersby, J.E. (1976). A new micro-test for the detection of incomplete coupling reactions in solid-phase peptide synthesis using 2,4,6-trinitrobenzenesulphonic acid. Anal. Biochem. *71*, 260–264. https://doi.org/10.1016/0003-2697(76)90034-8.

18. Diemer, V., Ollivier, N., Leclercq, B., Drobecq, H., Vicogne, J., Agouridas, V., and Melnyk, O. (2020). A cysteine selenosulfide redox switch for protein chemical synthesis. Nat. Commun. *11*, 2558. https://doi.org/10.1038/s41467-020-16359-6.

19. Diemer, V., Firstova, O., Agouridas, V., and Melnyk, O. (2022). Pedal to the Metal: The Homogeneous Catalysis of the Native Chemical Ligation Reaction. Chem. Eur J. *28*, e202104229. https://doi.org/10.1002/chem.202104229.

20. Anthis, N.J., and Clore, G.M. (2013). Sequence-specific determination of protein and peptide concentrations by absorbance at 205 nm. Prot. Sci. *22*, 851–858. https://doi.org/10.1002/pro.2253.