Chapter 7

Improved Handling of Peptide Segments Using Side Chain-Based "Helping Hand" Solubilizing Tools

Michael T. Jacobsen, Paul Spaltenstein, Riley J. Giesler, Danny Hung-Chieh Chou, and Michael S. Kay

Abstract

Maintaining high, or even sufficient, solubility of every peptide segment in chemical protein synthesis (CPS) remains a critical challenge; insolubility of just a single peptide segment can thwart a total synthesis venture. Multiple approaches have been used to address this challenge, most commonly by employing a chemical tool to temporarily improve peptide solubility. In this chapter, we discuss chemical tools for introducing semipermanent solubilizing sequences (termed helping hands) at the side chains of Lys and Glu residues. We describe the synthesis, incorporation by Fmoc-SPPS, and cleavage conditions for utilizing these two tools. For Lys sites, we discuss the Fmoc-Ddap-OH dimedone-based linker, which is achiral, synthesized in one step, can be introduced directly at primary amines, and is removed using hydroxylamine (or hydrazine). For Glu sites, we detail the new Fmoc-SPPS building block, Fmoc-Glu(AlHx)-OH, which can be prepared in an efficient process over two purifications. Solubilizing sequences are introduced directly on-resin and later cleaved with palladium-catalyzed transfer under aqueous conditions to restore a native Glu side chain. These two chemical tools are straightforward to prepare and implement, and we anticipate continued usage in "difficult" peptide segments following the protocols described herein.

Key words Chemical protein synthesis, Native chemical ligation, Fmoc-SPPS, Helping Hand, Difficult peptides, Traceless linkers

1 Introduction

1.1 General Introduction to Chemical Protein Synthesis and Solubility Challenges

Chemoselective peptide ligation [1, 2] is a foundational technology toward building fully synthetic (and functional) proteins based on the connection of multiple peptide segments through Chemical Protein Synthesis (CPS). Typically, Boc- or Fmoc-SPPS is used to prepare peptides, which are then joined by Native Chemical Ligation (NCL) [3, 4]. Judicious planning of a total CPS project is critically important to success [5, 6]. There are multiple issues to consider, including but not limited to synthetic peptide quality [7, 8], peptide solubility [9], ligation kinetics [10, 11], orthogonal protecting groups [12, 13], and the method for generating the

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thioester [14–16]. Nevertheless, in our experience [6, 17–22] poor peptide solubility remains the primary bottleneck to routinely advancing large chemical protein synthesis projects.

We recently reviewed the topic of peptide solubility in the context of CPS in detail [23], highlighting solvent manipulation, isoacyl dipeptides, and semipermanent solubilizing tags as three prominent approaches. In this chapter, we focus on the third approach. Here we provide step-by-step procedures and notes to synthesize and implement two chemical tools for introducing semipermanent solubilizing sequences into challenging peptides. These tools can be used to improve the handling of poorly soluble and hard-to-purify peptides in CPS projects: Lys-based Fmoc-Ddap-OH and Glu-based Fmoc-Glu(AlHx)-OH (*see* **Note 1** for detailed names of specific abbreviations frequently used in this chapter).

1.2 Introduction to the Dde-Based Fmoc-Ddap-OH Solubilizing Tool

The protecting group Dde, and its more stable isovaleryl derivative ivDde, has served a valuable role in SPPS since its introduction in the 1990s [24–27]. Its compatibility with both Boc- and Fmoc-SPPS, as well as allylic ester (Allyl)/allyloxycarbonyl (Alloc) protecting groups [28–31], is key to its wide acceptance in the field. The operational ease of deprotection using dilute hydrazine or hydroxylamine solutions, in both organic and aqueous solvents, further cements its popularity. Many applications have been reported using Lys(Dde) building blocks for polyamine synthesis [32, 33], cyclic peptide synthesis [29, 34, 35], and even in the SPPS process itself [36, 37]. Additionally, biotin-Dde-based constructs [38] have been successfully employed as photoprobes for secretase activity [39], trifunctional reagents for protein enrichment [40], and in mapping of O-GlcNAc modifications [41].

In 2016 [19], in partnership with the V. Aucagne group, we applied the Dde protecting group concept (reversible reaction between an acyl dimedone and an amine) to install semipermanent solubilizing tags at Lys residues in difficult peptides. We introduced Fmoc-Ddae-OH as a tool to install solubilizing sequences (termed helping hands) at Lys positions in difficult peptides. This tool facilitated the purification of an intractable GroES-C peptide (Fig. 1a). GroES could then be assembled using a ligation-desulfurization approach, followed by hydrazine treatment to remove the solubilizing sequence. The refolded protein showed a native heptameric assembly and co-chaperone activity that matched a recombinant control.

In 2019 [21], we reported a second-generation linker, Fmoc-Ddap-OH, which possesses enhanced stability and can be directly formulated into a powder (rather than the viscous liquid of Fmoc-Ddae-OH). Application of a Ddap-linked solubilizing sequence was shown to simplify the production of a synthetically challenging and poorly soluble segment of the Shiga toxin B-subunit (StxB) protein (StxB-N, Fig. 1b). We also recently showed that both

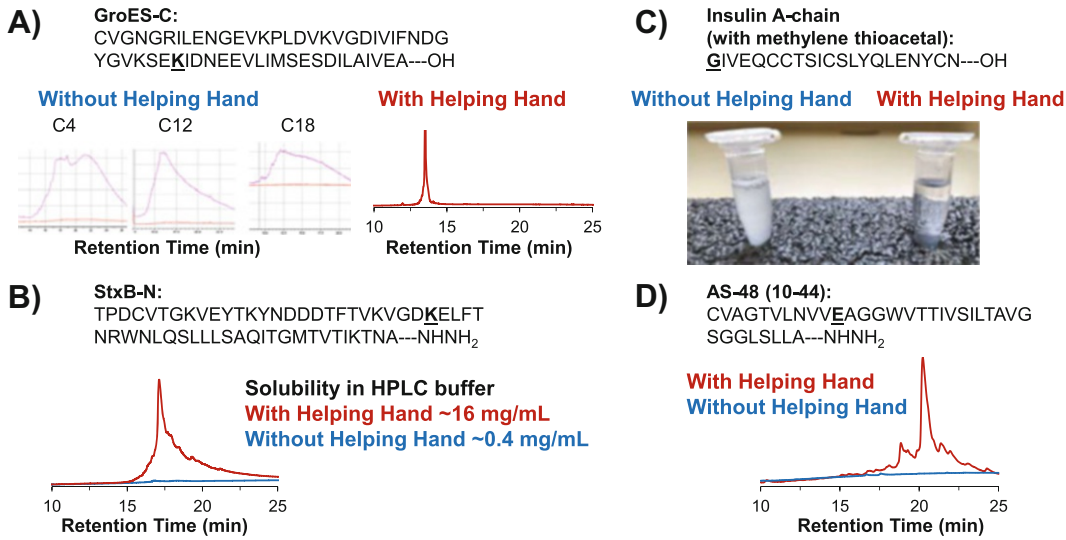

Fig. 1 Examples of Lys and Glu-based Helping Hands to solubilize difficult peptides. (**a**) Application of Fmoc-Ddae-OH linker in improving the HPLC resolution of GroES-C peptide; left panel, crude traces of unmodified GroES-C run on multiple stationary phases; right panel, purified GroES-C run on a C12 stationary phase. (**b**) Application of Fmoc-Ddap-OH linker in improving the resolution and solubility of StxB-N peptide. (**c**) Application of Fmoc-Ddap-OH in improving the solubility of human insulin A-chain with a methylene thioacetal linker between Cys6 and Cys11. (**d**) Utilization of Fmoc-Glu(AlHx)-OH to improve the solubility and HPLC resolution of AS-48 peptide segment. Underlined residues in the sequences indicate the attachment sites for the linkers. (**a** is adapted with permission from Jacobsen MT, Petersen ME, Ye X, Galibert M, Lorimer GH, Aucagne V, Kay MS *J Am Chem Soc* 2016, 138 (36): 11775–82, Copyright (2016) American Chemical Society. **b** is adapted with permission from Fulcher JM, Petersen ME, Giesler RJ, Cruz ZS, Eckert DM, Francis JN, Kawamoto EM, Jacobsen MT, Kay MS. *Org Biomol Chem* 2019, 17 (48): 10237–10244, Copyright (2019) Royal Society of Chemistry. **c** is adapted with permission from Zheng N, Karra P, VandenBerg MA, Kim JH, Webber MJ, Holland WL, Chou DHC *Journal of Medicinal Chemistry* 2019, 62 (24): 11437–11443, Copyright (2019) American Chemical Society. **d** is adapted with permission from Giesler RJ, Spaltenstein P, Jacobsen MT, Xu W, Maqueda M, Kay MS. *Org Biomol Chem* 2021, 19 (40): 8821–8829, Copyright (2021) Royal Society of Chemistry

Fmoc-Ddae-OH and Fmoc-Ddap-OH linkers could be used to simplify the synthesis of the widely recognized poorly soluble insulin A-chain [42]. A Lys_3 sequence was prepended to the N-terminus of chicken insulin using a Fmoc-Ddae-OH linker, resulting in improved handling and successful synthesis [43]. In this particular sequence, the established isoacyl dipeptide strategy [44] could not be used due to the absence of a Thr-Ser motif. Additionally, a Ddap-based Lys_6 sequence (installed at the N-terminus) was also used to prepare human A-chain with a stabilizing methylene thioacetal bond between the Cys6 and Cys11 positions [45] (Fig. 1c). Once again, the isoacyl dipeptide strategy failed due to peptide insolubility that arose during incorporation of the methylene thioacetal unit.

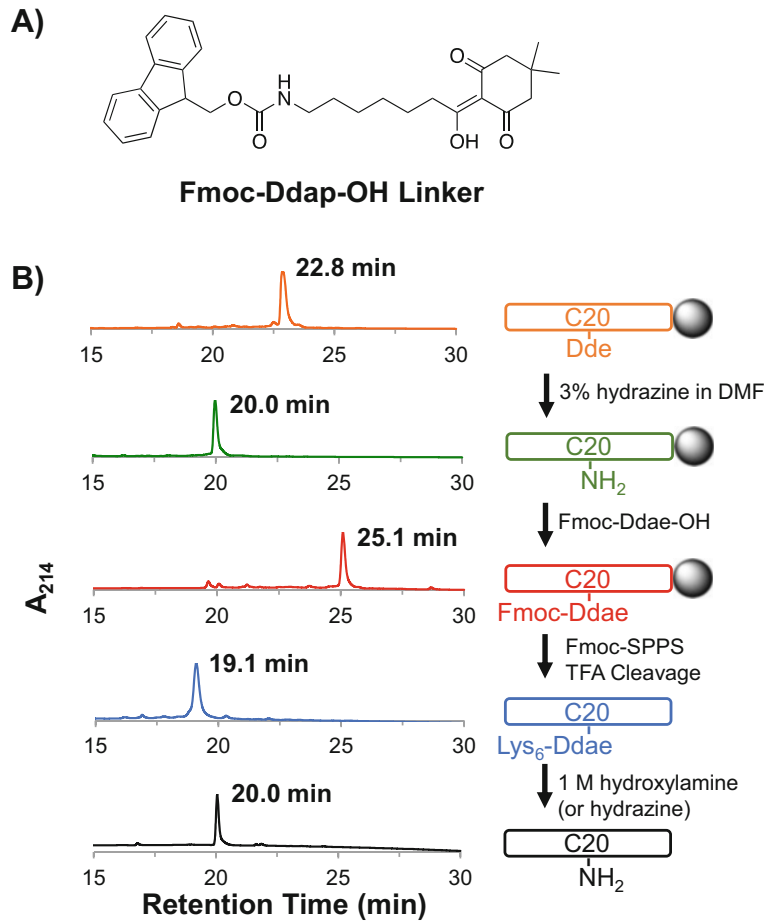

Fig. 2 Structure and incorporation of the Fmoc-Ddap-OH linker. (**a**) Fmoc-Ddap-OH. (**b**) Step-by-step process for installing and cleaving solubilizing sequences ("Helping Hands") in the C20 model peptide, using the analogous Fmoc-Ddae-OH linker. (**b** is adapted with permission from Jacobsen MT, Petersen ME, Ye X, Galibert M, Lorimer GH, Aucagne V, Kay MS *J Am Chem Soc* 2016, 138 (36): 11775–82, Copyright (2016) American Chemical Society)

The Fmoc-Ddap-OH (or Fmoc-Ddae-OH) linker (Fig. 2a) and a subsequent solubilizing sequence are installed in peptides on-resin (Fig. 2b), as demonstrated in the model peptide C20 (DWTKNITDKIDQIIHDFVDK, where K is the installation site). Briefly, the peptide of interest is first prepared by Fmoc-SPPS containing a single orthogonal protecting group. After completing synthesis of the linear sequence, this orthogonal protecting group is removed (Dde in this case), and the Fmoc-Ddap-OH linker is installed. Standard Fmoc-SPPS protocols can then be used to add the desired solubilizing sequence. Clean, traceless cleavage of the linker is achieved using bis-α nucleophiles.

1.3 Introduction to the Fmoc-Glu(AlHx)-OH Solubilizing Tool

In our efforts to deploy multiple chemical tools to facilitate CPS projects, we recognized that the original Lys-based tool, Fmoc-Ddap-OH, was insufficient despite its good abundance in proteins (5.6%) [46]. We encountered difficult sequences [22] that do not contain a Lys residue (or acceptable N-terminal residue, discussed below) for installing the original linker. Also, the original linkers possess two disadvantages when it comes to peptide solubility: (a) the native solubilizing Lys amine is temporarily masked on reaction with Fmoc-Ddap-OH and (b) the dimedone component of the linker is poorly water soluble. Multiple Lys residues must be incorporated to compensate for these solubility impairments.

We selected Glu as a prospective second site for introducing solubilizing sequences into difficult peptides for several reasons. First, the carboxylic acid side chain is easily derivatized for reversible modification, as shown by other CPS groups [47, 48]. Second, loss of a Glu charge (unlike a Lys charge) does not negatively affect solubility in acidic RP-HPLC conditions. Finally, Glu is a relatively common residue (6.6% in proteins) [46], which increases potential attachment sites across a wide variety of peptides.

In order to develop a Glu-based solubilizing tool, we first needed to find a suitable protecting group scaffold. We were inspired by the noteworthy achievements of H. Kunz [49, 50] and F. Albericio [51, 52] to apply the Allyl and Alloc protecting groups in peptide synthesis. Like the Dde protecting group, Allyl/Alloc are orthogonal with Fmoc removal and TFA cleavage conditions. Deprotection is accomplished through a palladium-catalyzed transfer to a nucleophilic scavenger [53]. Numerous examples of complex cyclic and glycopeptide syntheses have been reported using this orthogonal protecting group [54–56].

The new Glu tool, Fmoc-Glu(AlHx)-OH, was used to complete the synthesis of a noted [57, 58] challenging cyclic peptide AS-48 (Fig. 1d) [22]. Fmoc-Glu(AlHx)-OH (Fig. 3a) is inserted into peptides following standard Fmoc-SPPS protocols. As seen in the model peptide C20E (DWTKNITDEIDQIIHDFVDK, where E is the position of Glu(AlHx)) (Fig. 3b), upon completion of the linear sequence, the Dde group is removed, followed by Fmoc-SPPS installation of a solubilizing sequence. After assembly, the solubilizing sequence and AlHx linker are removed using aqueous Pd complexes [59–62], ultimately restoring the native Glu side chain.

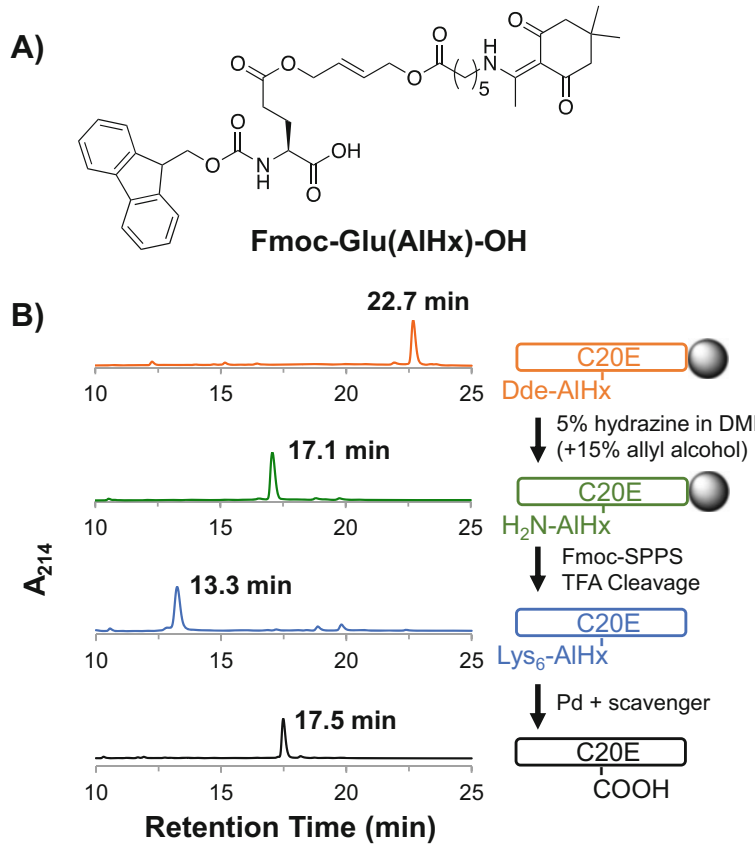

Fig. 3 Structure and incorporation of the Fmoc-Glu(AlHx)-OH building block. (**a**) Fmoc-Glu(AlHx)-OH. (**b**) Step-by-step process for installing and cleaving solubilizing sequences ("Helping Hands") in the C20E model peptide. (**b** is adapted with permission from Giesler RJ, Spaltenstein P, Jacobsen MT, Xu W, Maqueda M, Kay MS (2021) Submitted)

2 Materials

2.1 Buffers

1. Ligation buffer (100 mL): 6 M GuHCl, 200 mM phosphate, pH 7.

   To prepare 100 mL of ligation buffer, dissolve 57.25 g of guanidine hydrochloride (GuHCl, ≥99.5%) in a graduated beaker by adding 11.5 mL of 1 M Na_2HPO_4 and 8.5 mL of 1 M NaH_2PO_4. Stir the solution at room temperature until dissolved (use low heat if necessary, cover with aluminum foil). Add ddH_2O to a final volume of 100 mL while adjusting the pH to 7 and then pass through 0.45 μm filter. Ligation buffer is stable at room temperature; discard if any precipitates form in the buffer. For the AlHx removal reaction, degas the ligation buffer for 10 min prior to use.

2. Hydroxylamine cleavage buffer (10 mL): 2 M hydroxylamine in ligation buffer, pH 6.75.

   To prepare 10 mL of cleavage buffer, dissolve 1.39 g of hydroxylamine hydrochloride (ReagentPlus) in a 15 mL graduated Falcon tube and fill to 9 mL with ligation buffer. Vortex and sonicate the solution until the hydroxylamine is dissolved (*see* **Note 2** for dissolving hydroxylamine). Slowly adjust the pH of the solution to 6.75 and add ddH_2O to a final volume of 10 mL and then pass through 0.45 μm filter. Cleavage buffer should be freshly prepared (same day).

2.2 Synthesizing Fmoc-Ddap-OH

1. Fmoc-7-aminoheptanoic acid (≥98%).
2. Dimedone (99%).
3. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC-HCl, ≥98%).
4. 4-dimethylamino pyridine (DMAP, ≥99%).
5. Hydrochloric acid (HCl, ACS plus grade).
6. Sodium bicarbonate ($NaHCO_3$).
7. Magnesium sulfate ($MgSO_4$).

2.3 Synthesizing Fmoc-Glu(AlHx)-OH

1. 6-aminohexanoic acid (≥98%).
2. *N,N*-diisopropylethylamine (DIPEA, ReagentPlus grade).
3. 2-Acetyldimedone (Dde-OH, ≥98%).
4. Sodium sulfate (Na_2SO_4, ACS grade).
5. *trans*-2-Butene-1,4-diol (Toronto Research Chemicals).
6. Fmoc-(L)-Glu-OtBu (≥98%).
7. Trifluoroacetic acid (TFA, HPLC grade).

2.4 AlHx-Based Linker Removal

1. Allylpalladium(II) chloride dimer ($[Pd(allyl)Cl]_2$, 98%).
2. L-glutathione reduced (GSH, ≥98%).
3. Dithiothreitol (DTT, >99%).
4. Triphenylphosphine-3,3′,3″-trisulfonic acid trisodium salt (TPPTS, >95%).
5. Palladium(II) acetate ($Pd(OAc)_2$, 98%).
6. SiliaMetS Thiourea functionalized resin (1.3 mmol/g, Silicycle).

2.5 General Solvents

1. Methylene chloride (DCM, ACS grade).
2. Ethyl acetate (EtOAc, ACS grade).
3. Hexanes (ACS grade).
4. Methanol (MeOH, ACS grade).
5. Dimethylformamide (DMF, ACS grade).

6. N-methylpyrrolidone (NMP, ≥99.8%).
7. Acetonitrile (ACN, HPLC grade).
8. 0.1% formic acid in water or acetonitrile (Optima LC/MS grade).

3 Methods

3.1 Synthesis and Characterization of the Fmoc-Ddap-OH Linker

The Fmoc-Ddap-OH linker is preferably synthesized in a one-pot, two-step reaction involving an alkyl carboxylic acid, dimedone (or other appropriate cyclic 1,3-dione), coupling agent EDC-HCl, and DMAP to catalyze the O-C isomerization (Fries rearrangement) from the dimedone ester into the desired acyl dimedone [38, 63, 64]. This straightforward protocol has been used by our group successfully with multiple carboxylic acids [21]. The key procedural step is to distinguish the dimedone ester intermediate from the acyl dimedone product, as detailed below and shown in Fig. 4. The protocol described below is scaled for an input of 1 g of Fmoc-7-aminoheptanoic acid (2.7 mmol).

1. Place a 50 mL round bottom (rb) flask in ice-water bath.
2. To the rb flask, add 1.0 g (2.7 mmol) Fmoc-7-aminoheptanoic acid, 496 mg (1.3 eq) dimedone, and 548 mg EDC-HCl (1.05 eq). *See* **Note 3** on using different 1,3-diones, *see* **Note 4** on using different carboxylic acids.
3. Dissolve reagents in 10 mL DCM and cool for 10 min.
4. Add 432 mg DMAP (1.3 eq) and allow the solution to come to room temperature.
5. Typically, the reaction is complete in 5 h. Alternatively, it can be continued overnight, if required.
6. Monitor the reaction progress by HPLC and/or LCMS.
7. Important note on monitoring this reaction: It is critical to differentiate the dimedone (or enol) ester from the acyl dimedone product; these two species may be confused due to the same mass observed by ESI-MS. In the absence of DMAP, the acyl dimedone product is not obtained. Typically, the dimedone ester elutes slightly earlier, compared to the acyl dimedone, on a C18 RP-HPLC column. In case of uncertainty, multiple methods can be used to distinguish the two species:
   (a) NMR analysis (*see* **Note 5**).
   (b) Difference in 280 absorbance (*see* **Note 6** and Fig. 4b**)**.
   (c) Solution amine test (*see* **Note 7**).
   (d) On-resin amine test (*see* **Note 8**).

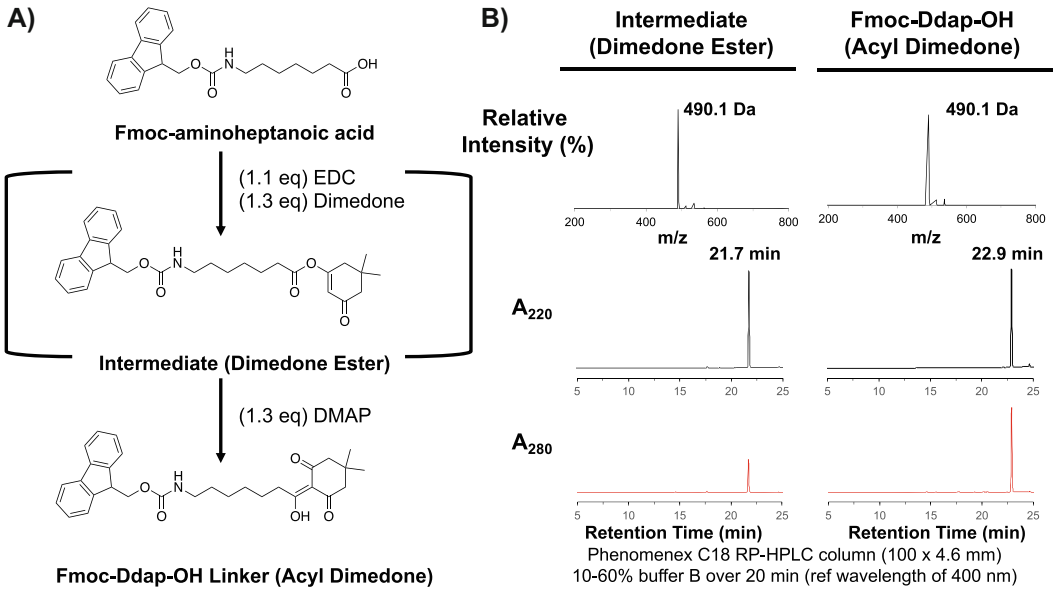

Fig. 4 Synthesis of Fmoc-Ddap-OH (**a**) Reaction scheme for synthesis of Fmoc-Ddap-OH, including the dimedone ester intermediate. (**b**) MS and HPLC analysis of the dimedone ester and acyl dimedone product (Fmoc-Ddap-OH)

8. The reaction is worked-up following dilution with ethyl acetate (60 mL) and washes with 1 M HCl (3 × 20 mL), 5% $NaHCO_3$ (3 × 20 mL), and saturated NaCl (3 × 20 mL).
9. Separate the organic layer from the aqueous layer, briefly dry over $MgSO_4$, filter out the $MgSO_4$, and concentrate the organic layer by rotavap.
10. Load the material onto a silica column and elute the product using a hexane/ethyl acetate gradient of 9:1 to 2:1 over multiple column volumes.
11. Pool fractions containing the acyl dimedone product and concentrate using rotavap, followed by vacuum drying.
12. For long-term storage, dissolve the dried sample in 90% acetonitrile in water, and then lyophilize, then store at 4 °C.
13. An expected yield of Fmoc-Ddap-OH (*N*-Fmoc-2-(7-amino-1-hydroxyheptylidene)-5,5-dimethylcyclohexane-1,3-dione) is ~50%.

3.2 Incorporation of Ddap-Based Helping Hands into Peptides on-Resin

The Fmoc-Ddap-OH linker can be incorporated into peptides on-resin after the Fmoc-SPPS assembly process. Here, the difficult peptide of interest is assembled on-resin using standard Fmoc-SPPS protocols. At the desired Lys position (or N-terminus, see discussion below), an orthogonal protecting group must be introduced, which will effectively be swapped with the Fmoc-Ddap-OH linker after completion of the linear peptide sequence. Subsequent incorporation of a solubilizing peptide sequence is then performed at the Fmoc-Ddap-OH site. The protocol detailed below is scaled for a 25 μmole peptide synthesis, typically at a resin loading density of 0.2–0.4 mmol/g.

1. Wash resin 3× with DCM, 3× with DMF, and swell in 5 mL DMF/DCM for >30 min (*see* **Note 9** on resins that have been used with Fmoc-Ddap-OH linkers, *see* **Note 10** on resin selection for performing quantitative evaluation of linker attachment).
2. Synthesize the peptide following standard Fmoc-SPPS protocols. The on-resin peptide should include a Lys with an orthogonal protecting group for subsequent Fmoc-Ddap-OH attachment. Preferably, Fmoc-Lys(Dde) is used in the growing chain due to the robustness and ease of Dde removal. Importantly, the N-terminus must be protected upon completion of the linear sequence (*see* **Note 11** on suggested approaches). Alternatively, the Fmoc-Ddap-OH linker can be incorporated at the N-terminus of the peptide chain; in this case, no other orthogonal protecting group is required.
3. After completing synthesis of the linear sequence (maintaining protection at the N-terminus as in **Note 11**), remove the orthogonal Dde protecting group with 3 × 10 min treatment with 3% hydrazine (*see* **Note 12** on removing Dde group on-resin; *see* **Note 13** on alternatives to Dde).
4. Wash resin thoroughly >5× with DMF and >5× with NMP to ensure that any residual hydrazine is removed.
5. Dissolve ~100 mg Fmoc-Ddap-OH in 1 mL NMP or DMF (~200 mM, 8 equivalents) and add directly to the peptide resin for 4 h at room temperature or 2 h at 37 °C (*see* **Note 14**).
6. If desired, any excess unreacted Fmoc-Ddap-OH linker can be collected through the filter and reused (*see* **Note 15** on reusing helping hand linkers).
7. Next, wash the resin 3× with DMF. Perform a Kaiser test (*see* **Note 16**) on the peptide resin to check the degree of amine modification or alternatively analyze a microcleavage sample to confirm linker attachment.

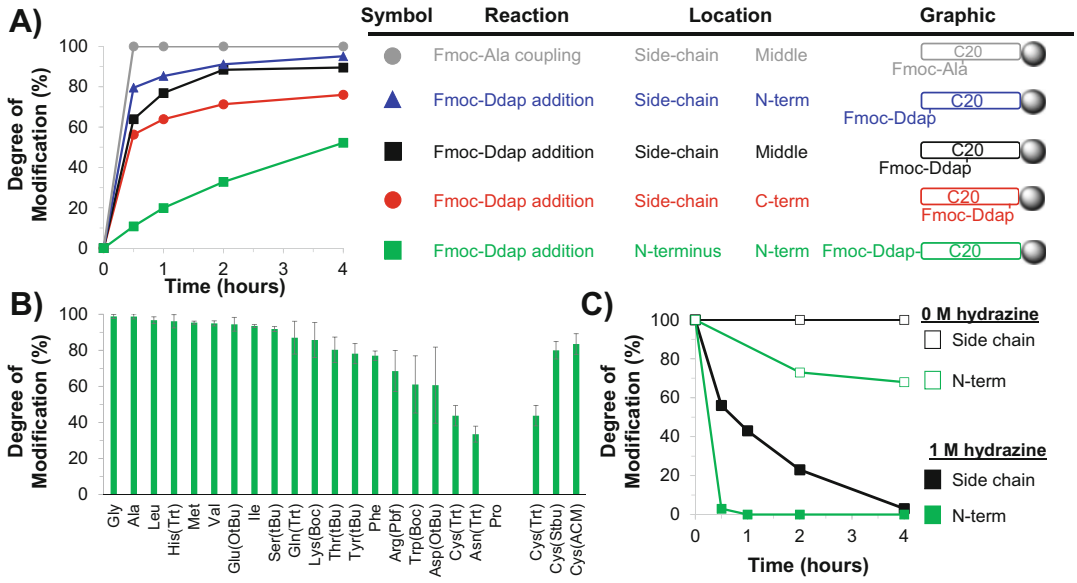

Fig. 5 Evaluation of Fmoc-Ddap incorporation kinetics. (**a**) Incorporation kinetics for Fmoc-Ddap-OH at different positions in model C20 peptide. (**b**) Degree of modification with Fmoc-Ddap-OH at the N-terminus after overnight treatment depending on the N-terminal residue. (**c**) Stability of Fmoc-Ddap at either side chain Lys or N-terminus (Asp residue), with and without 1 M hydrazine in denaturing ligation buffer

8. Important note on attachment (1): The kinetics and degree of linker attachment are highly dependent on the local environment around the amine (*see* **Note 17** and Fig. 5 for discussion and supporting data on this critical matter).
9. Important note on attachment (2): Although the original helping hand linker was intended to be used at Lys side chains, it can also be used at peptide N-termini. However, the degree of attachment and stability at the N-terminus is highly dependent on the residue at that position (*see* **Note 18** and Fig. 5 for discussion and supporting data). Generally, small and hydrophobic residues work well to tolerate the linker at the N-terminus; nevertheless, the stability at N-terminus is usually much lower than the Lys side chain (Fig. 5c).
10. If the degree of attachment is deemed unacceptable (based on a Kaiser test or microcleavage), then different strategies may be pursued to enhance the degree of modification, including:
    (a) Extend the reaction time, up to 16–18 h,
    (b) Increase the concentration and equivalents of Fmoc-Ddap-OH, up to 1 mL of 400 mM linker,
    (c) Change the solvent to affect resin swelling (linker can be incorporated in DMF/DCM solvent mixtures),
    (d) Add base to the reaction, such as 3–5 eq DIPEA,
    (e) Increase the reaction temperature (50 °C),

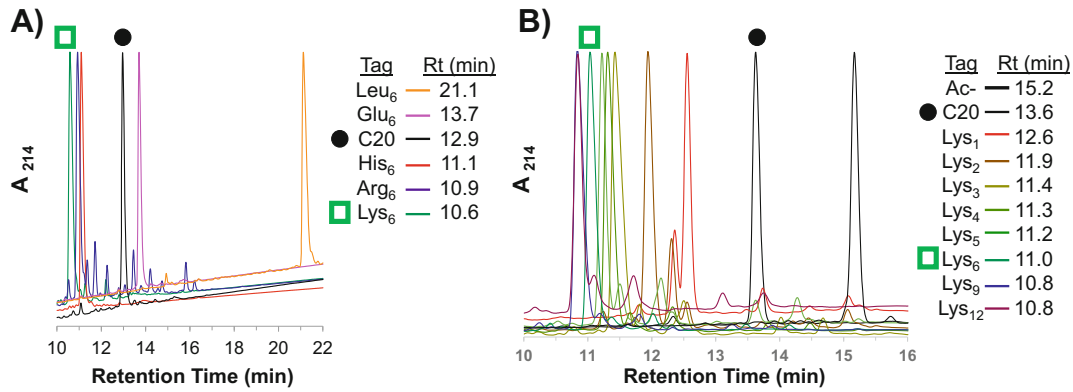

Fig. 6 Investigation into the most effective (polar) solubilizing sequences. Model C20 peptide was prepared by Fmoc-SPPS, and various sequences were attached to the N-terminus, followed by retention time evaluation by RP-HPLC on an analytical C18 column. (**a**) Evaluation of the impact of the composition of different repeating residue solubilizing sequences. (**b**) Evaluation of the number of Lys residues on the retention time

(f) If possible, resynthesize the peptide at lower density and include an aggregation-disrupting element, such as a pseudoproline dipeptide, or a backbone-substituted building block such as (Dmb)Gly, before (C-terminal to) the site of linker incorporation,

(g) If necessary, reposition the linker incorporation site to a more accessible position closer to the N-terminus (*see* **Note 19**).

11. After the linker has been incorporated and the resin washed with DMF, cap any unreacted sites with acetic anhydride (treat the resin for 10 min with 1 mL acetic anhydride and 1 mL 0.6 M NMM).
12. Wash resin >5× with DMF to ensure there is no residual acetic anhydride.
13. Apply standard Fmoc-SPPS conditions to introduce a solubilizing sequence, preferably a linear Lys_6 sequence, at the linker position (*see* **Note 20** on linear vs branched sequences, *see* **Note 21** and Fig. 6 for discussion and data on the composition of the solubilizing sequence).
14. The helping hand (or Lys_6-Ddap)-modified peptide resin can be cleaved from resin with standard TFA cleavage cocktails. Generally, 2 h of peptide cleavage followed by precipitation into ice-cold ether is adequate. *See* **Note 22** on tested cleavage cocktails.
15. Generally, no special handling steps are needed during the peptide dissolution and purification steps when working with helping hand (Lys_6-Ddap)-modified peptides, although it is recommended to freeze and lyophilize the purified peptides promptly to avoid any subtle leakage (or elimination of the linker) on storage. *See* refs. 19, 21 for data on linker stability in HPLC buffer.

3.3 Traceless Removal of Ddap-Based Helping Hands from Peptides in Solution

Cleavage, or removal, of the solubilizing sequence (Lys_6-Ddap) from the target peptide or protein is achieved by dilution in aqueous buffers containing hydrazine or hydroxylamine. The initial conditions used 1 M hydrazine or hydroxylamine aqueous (non-denaturing) buffers [65]. The protocol described below was optimized to rapidly cleave the linker and maximize peptide solubility in denaturing conditions prior to RP-HPLC purification or refolding.

1. Freshly prepare 2 M hydroxylamine in 6 M GuHCl, 100 mM sodium phosphate, pH 6.75 buffer (*see* Subheading 2 for buffer preparation, *see* **Note 23** on buffer dilution and **Note 24** on selection of hydroxylamine for cleavage).
2. When preparing this buffer, extensive vortexing and sonicating is required to dissolve the hydroxylamine (*see* **Note 2** for dissolving hydroxylamine).
3. Cleavage of the helping hand (Lys_6-Ddap)-modified peptide is usually performed after the complete linear target protein sequence has been assembled via NCL, and any other reactions (desulfurization, Acm-removal) have been completed. *See* **Notes 25–27** for compatibility of helping hands with processes involved in peptide and protein synthesis.
4. If the cleavage is to be performed directly on the lyophilized peptide, dissolve it in ligation buffer (*see* Subheading 2 for buffer preparation), aiming for a concentration ~2 mM depending on solubility (*see* **Note 28** on concentration determination of Ddap-containing peptides). Then, dilute the sample 1:1 with freshly prepared 2 M hydroxylamine buffer. *See* **Note 29** on adding reducing agents to the reaction.
5. Confirm the final pH is ~6.75 (subtle pH adjustments may be necessary) and rotate for 1–2 h at room temperature. *See* **Note 30** on potential hydroxylamine byproducts and **Note 31** on cleavage at N-terminal sites.
6. Take time points of the reaction for analysis by HPLC or LCMS. We generally dilute an aliquot 1:1 with 20% acetic acid in HPLC-grade water, vortex, and centrifuge at 18,000 × *g* for 10 min prior to injection. The cleaved sample almost always elutes later than the uncleaved sample on an RP-HPLC column.
7. After the reaction is complete, dilute 1:1 with 20% acetic acid in HPLC-grade water, vortex, filter (0.45 μm), and promptly purify by RP-HPLC.

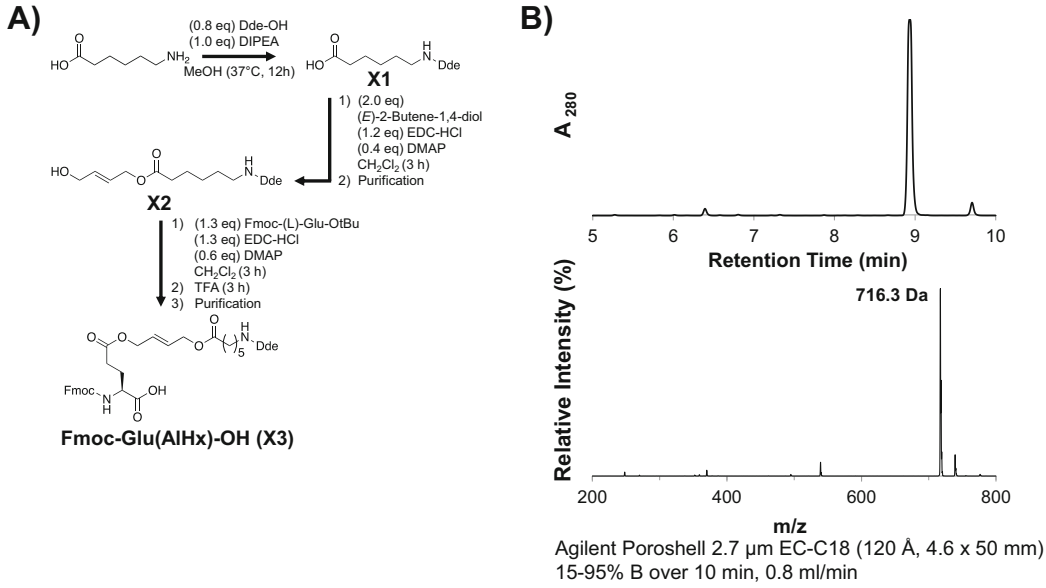

Fig. 7 Synthesis of Fmoc-Glu(AlHx)-OH (**a**) Reaction scheme for synthesis of Fmoc-Glu(AlHx)-OH. (**b**) HPLC and MS analysis of the final product. (**b** is adapted with permission from Giesler RJ, Spaltenstein P, Jacobsen MT, Xu W, Maqueda M, Kay MS (2021) Submitted)

3.4 Synthesis and Characterization of Fmoc-Glu(AlHx)-OH

To address challenging peptides without conveniently placed Lys residues, we recently introduced Fmoc-Glu(AlHx)-OH, a Glu-based traceless linker for the addition of a solubilizing tag. Just like the original Lys-based linker, it is compatible with standard Fmoc-SPPS and CPS conditions, enables chemoselective solubilizing cargo addition, and is easily removed in a traceless fashion. Moreover, we designed a straightforward and robust synthetic route from commercially available reagents. Fmoc-Glu(AlHx)-OH is synthesized in four steps and only requires two purifications (*see* Fig. 7). The amounts used below are for a multigram scale synthesis of Fmoc-Glu(AlHx)-OH.

The first reaction consists of protecting the amine of 6-aminohexanoic with Dde-OH:

1. In a round bottom flask (rb), suspend 6-aminohexanoic acid (18.7 g, 142.9 mmol) in 250 mL of methanol and DIPEA (18.4 g, 142.9 mmol). Stir the solution at room temperature for 5 min.
2. Add 2-Acetyldimedone (Dde-OH) (20 g, 109.9 mmol) to the rb flask and heat the reaction to 37 °C overnight.
3. Concentrate the solution by rotavap to remove the methanol. Resuspend the resulting mixture (oil) in water (white cloudy solution) and acidify with HCl (conc.) to pH 3–4.
4. Extract 6-(Dde-amino)hexanoic acid (**X1**) by adding methylene chloride (5 × 50 mL for a total of 250 mL) and separating the organic layer from the aqueous layer. Dry over Na_2SO_4,

filter out the Na_2SO_4, and concentrate by rotavap. The resulting white solid does not require additional purification and can be used directly in the next reaction.

The second reaction involves coupling **X1** with *trans*-2-butene-1,4-diol via a Steglich esterification [66]:

1. Dissolve **X1** (4.52 g, 15.34 mmol) in 25 mL of DCM in rb flask and stir the solution at room temperature.
2. Add EDC-HCl (3.5 g, 18.4 mmol) and DMAP (749 mg, 6.1 mmol) to the solution and stir for 5 min at room temperature.
3. To a separate flask with (*E*)-2-Butene-1,4-diol (2.7 g, 30.7 mmol) in 25 mL of DCM, slowly (dropwise) add the activated **X1** solution (*see* **Note 32** for preventing double esterification). Stir the combined mixture at room temperature for 3 h.
4. Concentrate the product by rotavap and purify by flash chromatography on silica (1–4% MeOH in DCM) to afford ((*E*)-4-hydroxybut-2-en-1-yl 6-(Dde)aminohexanoate) (**X2**, 3.9 g, 10.9 mmol, 55% yield).

The third reaction consists of coupling **X2** to the γ-carboxylate group of Fmoc-L-Glu-OtBu:

1. Dissolve Fmoc-L-Glu-OtBu (4.7 g, 10.9 mmol) in 35 mL of DCM in a rb flask and stir the solution at room temperature. *See* **Note 33** on chirality of the building block.
2. Add EDC-HCl (2.1 g, 10.9 mmol) and DMAP (789 mg, 6.54 mmol) to the flask and stir the mixture at room temperature for 5 min.
3. Add **X2** (3.1 g, 8.4 mmol) to the reaction and stir for 3 h at room temperature.
4. Concentrate the resulting Fmoc-Glu(AlHx)-OtBu (oil) by rotavap and use the crude material without purification.

In the fourth reaction the OtBu group is removed to yield Fmoc-Glu(AlHx)-OH:

1. Add 10 mL of TFA to the flask containing Fmoc-Glu(AlHx)-OtBu and stir for 3 h at room temperature to remove the OtBu protecting group.
2. Remove the TFA via evaporation by blowing air into the open reaction flask (a small amount of DCM (1–5 mL) can be added to the TFA to accelerate the evaporation) and then purify Fmoc-Glu(AlHx)-OH (**X3**) on silica (1% to 4% MeOH in DCM) Dissolve **X3** in acetonitrile and slowly dilute with 0.1% TFA in water to a final composition of 50% acetonitrile. Flash freeze the solution and lyophilize to afford a fine white powder (3.6 g, 5.0 mmol, 59%). Store at 4 °C.

3.5 Incorporation of Glu(AlHx) Helping Hand into Peptides During SPPS

Unlike the Lys Helping Hand, which is incorporated onto Lys side chains after completion of the linear chain and Dde removal, the Fmoc-Glu(AlHx)-OH was designed to be directly incorporated into the growing peptide chain during Fmoc-SPPS. Here, once the linear peptide is synthesized, the Dde on the AlHx linker is removed to enable site-specific incorporation of a solubilizing amino acid sequence, prior to cleavage of the peptide from the resin. The protocol below is scaled to a 25 μmole synthesis.

1. Wash resin 3× with DCM, 3× with DMF, and swell in 5 mL DMF/DCM for >30 min (*see* **Note 8** on resins that have been used with Fmoc-Glu(AlHx)-OH).
2. Synthesize the peptide following standard Fmoc-SPPS protocols. At the Glu site, substitute a Fmoc-Glu(OtBu)-OH typically used in traditional Fmoc-SPPS with a Fmoc-Glu(AlHx)-OH (*see* **Note 34** for improving coupling efficiency). Finally, note that the N-terminus must be protected upon completion of the linear sequence (*see* **Note 11** on suggested approaches).
3. After completing the synthesis of the linear sequence (maintaining protection at the N-terminus as in **Note 11**), remove the Dde protecting group on the AlHx linker with 3 × 5 min treatment with 1.7 mL of 5% hydrazine and 0.3 mL of allyl alcohol (*see* **Note 12** on removing Dde group on-resin and *see* **Note 35** on using allyl alcohol).
4. Wash resin thoroughly >3× with DMF to ensure that any residual hydrazine is removed.
5. Apply standard Fmoc-SPPS conditions to introduce a solubilizing sequence, preferably linear Lys_6, at the linker position (*see* **Note 20** on linear versus branched sequences and *see* **Note 21** and Fig. 6 for data and discussion on the composition of the solubilizing sequence).
6. Perform a Kaiser test (*see* **Note 16**) on the peptide resin to ensure complete incorporation of the solubilizing sequence or alternatively analyze a microcleavage sample.
7. The helping hand (Lys_6-AlHx)-modified peptide resin can be cleaved from resin with standard TFA cleavage cocktails. Generally, 2 h of peptide cleavage followed by precipitation into ice-cold ether is sufficient.
8. Generally, no special handling steps are needed during the dissolution and purification steps when working with helping hand (Lys_6-AlHx)-modified peptides, although it is recommended to freeze and lyophilize the purified peptides promptly to avoid any subtle leakage (or elimination of the linker) on storage. The rate of spontaneous AlHx linker cleavage has been carefully evaluated [22] and is practically insignificant.

3.6 Traceless Removal of AlHx-Based Helping Hand from Peptides in Solution

Once the solubility enhancement provided by the AlHx-based helping hand is no longer needed, the linker is cleaved to yield the native Glu residue. This reaction is based on the recent aqueous conditions from the A. Brik group to remove allyl protection at Glu and Asp residues [59]. The removal can be performed in one-pot directly following NCL or desulfurization. Alternatively, the AlHx group can be cleaved in a dedicated reaction after purification.

1. Dissolve the peptide (typically 1–2 mM) in sparged ligation buffer (*see* Subheading 2 for buffer preparation) at pH 7. Skip this step if the AlHx removal is performed after NCL or desulfurization.
2. Prepare the Pd and GSH solution by dissolving $[Pd(allyl)Cl]_2$ (25 eq) and GSH (25 eq, *see* **Note 36** on excess GSH) in sparged ligation buffer (25–100 μL).
3. Add the Pd/GSH solution to the peptide and adjust the pH to 8. Mix the reaction at 37 °C until complete.
4. Take time points of the reaction for analysis by HPLC or LCMS. First, add 60 eq of DTT (from a 500 mM stock solution in HPLC-grade water) to an aliquot of the reaction. This addition will chelate the metal and lead to Pd precipitation. Briefly mix for 10 min and centrifuge at 18,000 × *g* for 10 min. Remove the supernatant and dilute with HPLC buffer A or 5% acetic acid prior to injection. The cleaved sample almost always elutes later compared to the uncleaved sample on an RP-HPLC column.
5. To quench the completed reaction, add DTT (60 eq) to reduce and precipitate the Pd.
6. Centrifuge at 18,000 × *g* for 10 min to pellet the Pd, collect the supernatant, and purify it via RP-HPLC.

Alternatively, we have also demonstrated that the AlHx linker can be removed based on a method from the A. Okamoto lab [60], with slight variations.

1. Dissolve the peptide in sparged ligation buffer (*see* Subheading 2 for buffer preparation) at pH 7. Skip this step if the AlHx removal is performed in one-pot after NCL or desulfurization.
2. Prepare an 850 mM TPPTS solution in ddH_2O and a 188 mM $Pd(OAc)_2$ solution in degassed DMF.
3. Mix equal volumes of the TPPTS and $Pd(OAc)_2$ solutions to make a 100 mM $Pd(TPPTS)_4$ stock solution. Vortex briefly for 30 s.
4. Add the $Pd(TPPTS)_4$ to the peptide solution to attain 10 eq of Pd (with respect to concentration of AlHx). Stir the reaction at room temperature for 15–30 min.

5. Take time points of the reaction for analysis by HPLC or LCMS. First, add 15 equivalents of thiourea resin to an aliquot of the reaction. Briefly mix for 5 min and centrifuge at 18,000 $\times g$ for 10 min. Dilute the supernatant with HPLC buffer A or 5% acetic acid prior to injection. To capture the Pd and the cleaved AlHx linker, transfer the reaction to an Eppendorf tube containing 15 eq of thiourea resin and mix for 5 min (*see* **Note 37**).
6. Centrifuge at 18,000 $\times g$ for 10 min to pellet the resin, collect the supernatant, centrifuge the supernatant again to ensure removal of any resin particles, and purify via RP-HPLC.

4 Notes

1. The following protecting group and tool abbreviations are used multiple times throughout the chapter:

   Dde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-ethyl.

   ivDde: (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl).

   Fmoc-Ddae-OH: (*N*-Fmoc-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-[2-(2-aminoethoxy)ethoxy]-propan-1-ol).

   Fmoc-Ddap-OH: (*N*-Fmoc-2-(7-amino-1-hydroxyheptylidene)-5,5-dimethylcyclohexane-1,3-dione).

   Fmoc-Glu(AlHx)-OH: Fmoc-(L)-Glu((E)-4-hydroxybut-2-en-1-yl 6-((1-(Dde)amino)hexanoate))-OH.
2. To achieve 2 M hydroxylamine concentration, it is best to slowly add the hydroxylamine to the ligation buffer and vortex/sonicate repeatedly. If any precipitate forms through the process, pass the solution using a 0.45 μm filter.
3. Multiple 1,3-diones can be used to create acyl dimedones that are reactive to primary amines in peptides and can be removed by nitrogen nucleophiles. In our group, we have used both dimedone and 1,3-cyclohexanedione successfully; these two showing similar attachment and cleavage properties. Also, *see* refs. 64, 67, 68 for alternative 1,3-diones and an application in assembling long glycoproteins [65]. We additionally note the Nde (N-1-(4-nitro-l,3-dioxoindan-2-ylidene)ethyl) derivatives, which were reported to possess an additional advantage of visually evident deprotection [69]. Lastly, there is recent promising work on deploying (thio)barbiturate derivatives [70] as amine protecting groups, as well as aromatic derivatives Ddf and Ddp [71].
4. The reaction of dimedone with a variety of aliphatic and aromatic carboxylic acids has been comprehensively studied [64], with special attention on the relative ratios of o-acyl (ester) and c-acyl (acyl dimedone) species.

5. ^{1}H-NMR: The c-acyl product contains a diagnostic downfield peak (from the enol group) at ~18 ppm [19, 21].
6. With respect to the dimedone ester, the acyl dimedone product shows additional absorbance at 280 nm (*see* Fig. 4b). In cases of carboxylic acids with lower extinction coefficient at 280 nm, such as biotin, this difference can be used to clearly differentiate the product from the intermediate.
7. The two species (dimedone ester and acyl dimedone) can be dissolved in THF and treated with n-propylamine to distinguish the product from the intermediate. In a typical procedure, 0.05 mmole of either species is dissolved in 1 mL THF, and 0.15 mmole of n-propylamine is added. After 30 min, monitor the reaction by LCMS: the correct acyl dimedone product shows a +41 Da addition (observed mass of 531.2 Da), while the dimedone ester intermediate shows the amide product of 409.3 Da.
8. The two species can also be incorporated into peptides on-resin at primary amines following Subheading 3.2. The dimedone ester intermediate will form an amide bond with the primary amine on-resin, whereas the correct acyl dimedone product will form the expected (hydrazine-sensitive) Dde-bond.
9. Fmoc-Ddap-OH linkers and Fmoc-Glu(AlHx)-OH have been successfully incorporated into peptides on a variety of solid phase resins, including CTC (polystyrene), Tentagel, and Chemmatrix.
10. When carefully evaluating the kinetics of linker incorporation on-resin, it is best to use CTC-based polystyrene resins. We observed that the attachment kinetics can be affected by the age of the peptide PEG-resin (older peptide resins tend to show slower kinetics, despite extensive washing prior to linker incorporation). If PEG-based resins (e.g., Tentagel) are to be used, then it is advised to freshly prepare the linear peptide sequence and immediately, within one day, incorporate the Fmoc-Ddap-OH linker and solubilizing sequence.
11. The N-terminus of the peptide should be protected with Boc- or another protecting group that is insensitive to Fmoc deprotection conditions; alternatively, the N-terminus may be acetylated, depending on the functional relevance at that position. Preferably, a Boc-protected amino acid can be incorporated as the last (N-terminal) residue. Or, the N-terminus can be re-protected (after Fmoc deprotection) using Boc-anhydride and base (DIPEA or NMM). Importantly, Boc-anhydride should be properly stored at 4 °C and covered with inert gas in between use.
12. In model peptides, we have decreased the hydrazine exposure time to 2 min per cycle (3 × 2 min). However, in our experience, decreasing the hydrazine concentration to less than 1%

may be inadequate to quantitatively remove the Dde group on-resin. Others have noted that overly dilute hydrazine solutions can be inadequate at removing ivDde [30]. Alternatively, hydroxylamine-imidazole can be used to selectively remove Dde without removing Fmoc [72, 73]. In our hands, on-resin deprotection of Dde using n-propylamine [33, 74] is too sluggish to be practical.

13. Our preferred Lys derivative for selectively installing Fmoc-Ddap-OH linkers is Fmoc-Lys(Dde) due to its operationally simple and robust deprotection. However, if the target sequence also contains an isoacyl dipeptide, then we advise against using this derivative due to incompatibility between hydrazine and the isoacyl bond. A commercially available alternative, such as Fmoc-Lys(Mmt), Fmoc-Lys(Mtt), or Fmoc-Lys(Alloc) may be a suitable replacement depending on the context.
14. The standard incorporation protocol is 4 h at room temperature or 2 h at 37 °C. Additional optimization to either accelerate the kinetics or the degree of modification is elaborated elsewhere in the chapter.
15. The filtrate containing excess linker can be saved and recycled for subsequent incorporation into another peptide resin. The collected filtrate (linker dissolved in NMP) should be stored at −20 °C and reconcentrated following flash chromatography before reuse.
16. To monitor manual deprotections and couplings at primary amines, a Kaiser test [75] is an efficient method to detect free unreacted amines. To an Eppendorf tube containing 50 μL of Kaiser reagent (6% ninhydrin in ethanol), add a few beads of peptide resin and incubate at 90 °C for 5 min. If the beads remain yellow, it suggests that there are no primary amines (i.e., following successful Ddap coupling), while blue beads suggest the presence of unreacted primary amines (i.e., incomplete Ddap coupling or successful Dde removal).
17. The position and nature of the amine affects the rate of attachment and stability of the Fmoc-Ddap-OH linker. In Fig. 5a, different degrees and rates of attachment can be seen in model C20 peptides depending on the location of the amine. Less accessible positions closer to the resin tend to show slower attachment, whereas the N-terminal α-amine is significantly hindered compared to the ε-amine of Lys side chains.
18. The degree of attachment and stability of the Fmoc-Ddap-OH linker at the N-terminal α-amine is dependent on the residue at the N-terminus (Fig. 5b). Generally, small and hydrophobic residues can tolerate the linker, whereas residues with bulky functional groups at the β position are more difficult to modify. In these more difficult cases, substitution of the protecting

group with a less bulky variant can be productive, as the degree of attachment is higher in Cys(StBu) and Cys(Acm) compared to the bulkier Cys(Trt). Importantly, the solution stability of the linker when incorporated at the N-terminal α-amine of peptides is lower compared to the ε-amine of Lys side chain (Fig. 5c, [65]). We note that Iris Biotech (Marktredwitz, Germany) has addressed this incorporation problem by offering the preassembled Fmoc-Ddax-L-Ala-OH reagent, which is a building block containing Fmoc-Ddax (hexyl spacer) [21] already linked to an Ala residue. Multiple Helping Hand-based tools are available from Iris Biotech.

19. The relative position of the solubilizing sequence within the difficult peptide impacts its solubilizing effect, and this effect may need to be evaluated on a case-by-case basis. For example, during preliminary efforts to solubilize and purify the challenging GroES-C peptide [19], we observed that introduction of solubilizing sequences at the middle and C-terminal regions was more beneficial compared to the N-terminal region. More extensive discussion and experimental data on the complex nature of introducing beneficial solubilizing elements into difficult peptides was recently described by another group [76].
20. We prefer to install linear poly-Lys sequences over branched-Lys sequences to avoid the possibility of auto-elimination of the linker due to reactions at the Lys ε-amine. Generally, installing a linear Lys sequence, assembled with Fmoc-Lys (Boc), is a stable approach.
21. Multiple peptide sequences were tested to comprehensively decide on an optimal solubilizing sequence. Figure 6 shows data on HPLC retention times, using a C18 RP-HPLC column (a surrogate for the polarity and ultimate solubility), of various solubilizing sequences coupled to a model C20 (DWTKNITDKIDQIIHDFVDK) peptide. In Fig. 6a, Lys_6 shows the left-most peak compared to the other basic residues Arg_6 and His_6. Lys_6 is also preferred as we have occasionally experienced synthesis (incomplete coupling) and cleavage (incomplete Pbf deprotection) issues when trying to prepare poly-Arg sequences. The enhanced polarity of poly-Lys over poly-Arg has been reported elsewhere [77]. In Fig. 6b, the effect of changing the number of Lys residues was comprehensively evaluated. Generally, adding more Lys residues causes earlier retention times. However, this effect begins to level off at around Lys_6, which we identified as optimal.
22. We have used multiple cleavage cocktails, including those with thiols and ammonium iodide, without incident for peptides containing either the Lys(Ddap) or Glu(AlHx) linkers.

23. We recognized that equal volume dilution of the reaction with a pH-adjusted 2 M stock solution of either hydrazine or hydroxylamine was the most convenient method to cleave the Ddap linker. This approach minimizes the number of pH adjustments that may be required if directly adding hydrazine or hydroxylamine reagent to the reaction, which can be overly tedious when performing very small volume reactions ($\leq$1 mL).
24. Initially, we developed a cleavage protocol using 1 M hydrazine (stock of 2 M hydrazine) in denaturing buffer at pH 7.5. Although this works well to cleave the Fmoc-Ddae-OH linker to completion at around 4 h, we identified a faster protocol using 1 M hydroxylamine (stock of 2 M hydroxylamine) in denaturing buffer at pH 6.75. The latter shows ~19× faster cleavage kinetics [21].
25. During the synthesis of chicken insulin [43], the preformed intramolecular and intermolecular disulfides were retained during hydrazine cleavage of the Ddap linker. Hence, Dde-based helping hand linkers are suitable to solubilize difficult disulfide-containing synthetic intermediates, which is an important property in synthesizing these family members.
26. During the synthesis of chicken and human thioacetal insulin, the Dde linker was shown to be stable to 50% BME in DMF during on-resin StBu deprotection, as well as iodine-mediated Acm-deprotection and consequent disulfide formation.
27. Dde linkers are generally stable to all major processes of modern chemical protein synthesis [6, 21]: sodium nitrite-mediated hydrazide activation, thiol/thioester exchange, denaturing buffers at low and neutral pH, NCL, desulfurization, and acidic RP-HPLC conditions. However, as expected, the Dde linker is partially sensitive to Thz-ring opening conditions using methoxylamine hydrochloride [78], although this incompatibility could be addressed using alternative Pd-based methods [79].
28. The Ddap linker introduces significant absorbance at 280 nm in target peptides. We determined an extinction coefficient of ~14,600 M^{-1} cm^{-1} in ligation buffer [21]. This value is on-scale with the previously reported extinction coefficient of 15,020 M^{-1} cm^{-1} at 360 nm for the Dcm protecting group [25]. Importantly, this factor should be accounted for when measuring the concentrations of Ddap-modified peptides by UV absorbance. This property may be advantageous in accurately determining the concentrations of peptides that do not possess either Tyr or Trp residues.

29. Reducing agents (10–50 mM DTT or TCEP) may be needed to retain a singular, reduced synthetic product during the cleavage step. Be sure to readjust the final pH of the reaction after adding these reagents.
30. In our hands, we have not seen significant hydrazine or hydroxylamine-associated byproducts during the standard linker cleavage period, although hydrazide and hydroxamate modifications were seen after extended treatment (overnight) [21, 80].
31. As discussed above, the stability of Dde-based linkers at the N-terminus is lower compared to the Lys side chain. Indeed, during the synthesis of insulin peptides with N-terminal (at Gly) helping hands, only 30 min of 1 M hydrazine treatment was needed to cleave the linker [43, 45].
32. This reaction may lead to double esterification of the diol if not performed properly. It is important to slowly add **X1** to the diol and not add the diol to **X1**. This ensures that the diol remains in excess to minimize double esterification.
33. In the third reaction, Fmoc-L-Glu-OtBu can be substituted with Fmoc-D-Glu-OtBu for applications in solubilizing mirror-image D-peptides/proteins [81].
34. Although we have not encountered sequences that proved problematic for the incorporation of Fmoc-Glu(AlHx)-OH, standard methods can be used to improve coupling efficiency such as double couplings, increased concentrations, lower loading density, or minimizing on-resin aggregation by incorporating pseudoprolines dipeptides or backbone-substituted building block such as Fmoc-(Dmb)Gly before, or C-terminal, to the site of Glu(AlHx) incorporation.
35. We add allyl alcohol (15% v/v) to the hydrazine solution to preempt any reduction of the alkene, which has been advised by others when removing Dde groups in the presence of Allyl/Alloc-based protecting groups [28, 82].
36. Do not greatly exceed the equivalence of GSH during the AlHx removal reaction. We have noticed that excess GSH (40 eq) can stall the reaction (likely due to thiol poisoning of the catalyst).
37. In addition to thiourea resin, various thiols such as MPAA and GSH (reduced) can function as the Lys_6-AlHx scavenger. In this case, use the same amount (15 eq) of the thiol, however you must add 60 eq of DTT at the end of the reaction to quench the reaction. Unlike the first method however, this DTT-Pd complex is water soluble and will not precipitate. During HPLC purification, the DTT-Pd and TPPTS elute in the flow-through and should not interfere with peptide purification or analysis.

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