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Protocol

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On-resin assembly of cysteine-reactive linkers for controlled site-selective antibody bioconjugation

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Abstract

Antibody conjugates represent key advances in targeted biotherapeutics, combining the precision of antibodies with a range of functional payloads, including cytotoxic small molecules, enzymes and peptides, to achieve high selectivity, reduced off-target effects and an improved therapeutic window compared with conventional small-molecule drugs. Here we present a protocol for the solid-phase synthesis of tetra-divinylpyrimidine (tetraDVP) linkers, a modular and scalable strategy for generating cysteine-reactive linkers used in site-selective bioconjugation, enabling the rapid production of diverse antibody conjugates. This protocol describes a multistep synthetic workflow involving solution-phase intermediate synthesis, solid-phase assembly on resin, polyethylene glycol elongation, installation of divinylpyrimidine warheads, and mild cleavage conditions, enabling reproducible production of the final linker scaffold. TetraDVP linkers are designed to simultaneously rebridge all four interchain disulfide bonds of native IgG1 and IgG4 antibodies with a single molecule, enabling the controlled installation of functional payloads such as peptides, drugs or protein tags with controlled payload-to-antibody ratio. This approach provides the only reported strategy for conjugating a single payload to a native antibody without the need for chromatographic purification or genetic/glycan engineering. Compared with previous solution-phase routes, this solid-phase protocol improves yield, scalability and reproducibility while enabling rapid diversification of linker architecture. The complete procedure can be performed in ~2 weeks and provides a versatile platform for accessing tetraDVP linkers bearing a variety of functional handles for antibody conjugation.

Key points

- It is possible to introduce a single payload molecule into native IgG1 and IgG4 antibodies in a site-specific way without the need for genetic/glycan engineering, using a linker that bridges the conserved cysteine bonds.
- This protocol describes the solid-phase synthesis of tetra-divinylpyrimidine (tetraDVP) linker molecules and provides advice on how these can be adapted for the incorporation of different payloads, such as small-molecule cytotoxic drugs, fluorophores and peptides.

Key references

Krajcovicova, S. et al. *Chem. Sci.* **16**, 10602–10609 (2025): https://doi.org/10.1039/D5SC02286J

Wharton, T., Krajcovicova, S. & Spring, D. in *Molecular Biology: Bioconjugation Methods* (ed. Deiters, A.) (Springer, 2026)

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Introduction

Background

Antibody–drug conjugates (ADCs) are a rapidly growing class of biotherapeutics that harness the targeting specificity of monoclonal antibodies to deliver cytotoxic agents directly to diseased tissues. With multiple approved ADCs now in clinical use and many more under investigation, there is a growing emphasis on refining the underlying bioconjugation chemistry to optimize efficacy, safety and manufacturability. The properties of the linker and the conjugation strategy used are known to substantially impact ADC stability, pharmacokinetics and therapeutic index.

In recent years, a range of site-selective conjugation approaches has been developed to improve homogeneity and stability of ADCs. One promising class of strategies involves the rebridging of native interchain disulfide bonds, which allows the installation of functional payloads at defined sites while maintaining the antibody's native architecture. Various reagents, such as next-generation maleimides[1–3], pyridazinediones[4–8], pyrimidine nitriles[9], phosphonamidates[10–13] and divinylpyrimidines[14–18], have been successfully applied to site-selective modification[19].

Development and overview of the protocol

Traditional antibody conjugation approaches based on stochastic lysine modification often generate heterogeneous mixtures of products with variable drug-to-antibody ratios (DARs), which can negatively affect pharmacokinetics and therapeutic performance[20]. To address these limitations, our group previously developed divinylpyrimidine (DVP) reagents, a class of vinylheteroarene linkers[21,22] that selectively react with cysteine residues under mild aqueous conditions[17,18,20,23,24]. These reagents enable disulfide rebridging, whereby reduction of the interchain disulfides of an antibody exposes reactive cysteine residues that can be covalently reconnected by a bifunctional linker, restoring structural integrity while introducing a defined functional handle. This strategy enables site-selective modification of native antibodies without the need for genetic engineering or enzymatic manipulation[20]. Subsequent studies showed that DVP-based linkers can generate stable antibody conjugates and support installation of diverse payloads, including cytotoxic drugs and fluorescent probes, producing constructs with favorable stability and biological activity, including potent antitumor efficacy and tolerability in in vivo models[18]. In parallel, divinyltriazine reagents were developed as complementary cysteine-reactive linkers capable of efficient disulfide rebridging in antibodies and cysteine stapling in peptides[25,26]. The vinyl-heteroarene platform was further expanded through multifunctional DVP reagents enabling dual-functionalization of antibodies[17] and bispecific Fab constructs[24], and related strategies have also been applied to peptide stapling and macrocyclization[23,27,28], highlighting the versatility of this chemistry across protein and peptide bioconjugation applications.

Despite these advances, early DVP linker systems operate on individual antibody disulfide bonds independently, meaning that each of the four interchain disulfides of an IgG antibody is modified separately. This can result in incomplete or intrachain rebridging, producing 'half-antibody' species when cysteines within the same chain reconnect rather than forming the native interchain bridges[25]. In addition, multiple linker molecules are typically incorporated per antibody, producing conjugates with DAR values of ~4. While such payload loading can be advantageous for some ADCs, lower and precisely defined DAR values are desirable for highly potent cytotoxic payloads such as pyrrolobenzodiazepines, for which higher DAR values may increase systemic toxicity.

To address these limitations, tetra-divinylpyrimidine (tetraDVP) linkers were developed in our laboratory to enable simultaneous rebridging of all four interchain disulfide bonds of IgG1 and IgG4 antibodies in a single step[14–16]. This strategy introduces a single covalently integrated linker across the antibody scaffold, producing homogeneous, low-aggregation conjugates with preserved antibody function and a defined DAR of 1 (refs. 15,16). Owing to the symmetric architecture of IgG antibodies, conventional cysteine rebridging strategies typically

produce conjugates with even numbers of payloads (for example, a DAR of 2 or 4). As a result, single-payload conjugates (a DAR of 1) are difficult to access, although they are advantageous for highly potent cytotoxic payloads.

The original solution-phase synthesis of tetraDVP linkers[14,15] enabled proof-of-concept studies but suffered from various synthetic limitations, including low overall yields, multiple chromatographic purifications and limited flexibility for late-stage diversification. To address this, we developed a new solid-phase organic synthesis protocol that streamlines linker assembly while enhancing scalability, reproducibility and throughput[16]. Solid-phase synthesis offers distinct advantages in this context: it eliminates the need for intermediate purification, enables efficient removal of excess reagents by simple washing and supports iterative modification strategies that are otherwise laborious in solution. Using a commercially available H-Gly-OH preloaded 2-chlorotrityl chloride resin, we established a modular synthetic route involving orthogonal polyethylene glycol (PEG) extension and on-resin installation of the DVP warheads (Fig. 1a). This design is compatible with standard solid-phase synthesis platforms and enables gram-scale access to tetraDVP linkers, while terminal functionality (for example, azide or alkyne) can be introduced for late-stage conjugation via click chemistry. Overall, the protocol improves yield, reduces hands-on time and affords chemically uniform linkers suitable for diverse bioconjugation applications.

Applications of the method

The tetraDVP linker platform enables precise conjugation of various biomolecular payloads to IgG1 and IgG4 antibodies for applications (Fig. 1b) such as the following:

- Generation of antibody conjugates bearing SpyCatcher or peptide cargos, enabling orthogonal protein fusion[16]
- Preparation of single-payload ADCs[14,15]
- Imaging and analytical applications using fluorescent dyes[14]

As the DVP motif selectively reacts with reduced cysteines under mild aqueous conditions, this method is applicable to native IgG1 and IgG4 antibodies without the need for genetic/glycan engineering. Furthermore, by changing the PEG length or terminal handle, researchers can adapt this platform to a range of payload sizes and physicochemical properties. We anticipate its utility in the construction of degrader–antibody conjugates or bispecific antibodies.

Comparison to other methods

The majority of FDA-approved ADCs, such as brentuximab vedotin or trastuzumab deruxtecan, utilize maleimide conjugation chemistry[29,30], which, despite its widespread use, has a major limitation of retro-Michael addition, leading to payload deconjugation under physiological conditions[29–32]. Alternative cysteine-bridging approaches, such as pyridazinedione[4–8] and phosphonamidate[10–13] chemistries, have been developed to improve conjugate stability but typically generate multipayload constructs and therefore do not readily enable access to single-payload ADCs. Situations in which these chemistries remain advantageous are summarized in Table 1. TetraDVP enables irreversible cysteine rebridging with a four-arm configuration, ensuring complete rebridging of interchain disulfides, and the tetraDVP system represents the only method for accessing DAR 1 ADCs without the need for chromatographic purification or glycan/genetic engineering[33]. Moreover, the solid-phase synthesis presented here enables straightforward diversification of the linker scaffold, overcoming the batch-to-batch variability and low yield often associated with solution-phase routes (Fig. 2).

Experimental design

The procedure is separated into three sections:

1. Synthesis of building blocks
2. On-resin assembly of tetraDVP linkers
3. Bioconjugation of tetraDVP to antibodies

Conjugation with the antibody is described in more detail in a recent protocol[34].

This protocol also includes advice on how the resulting ADC is characterized using mass spectrometry (MS), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE),

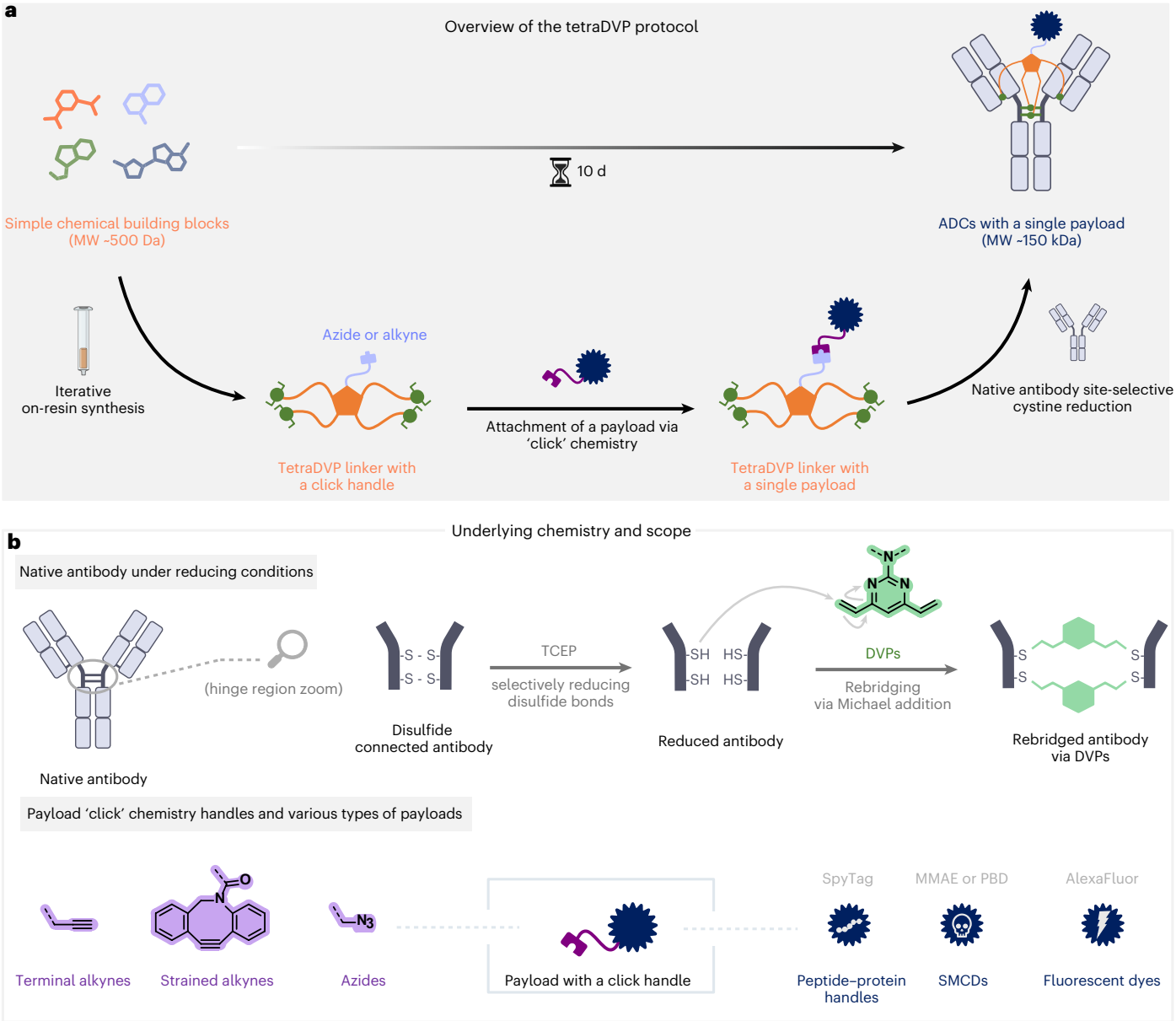


Fig. 1 | Overview and scope of the protocol. a, An overview of the present protocol. **b**, The underlying chemistry and scope of the protocol, illustrating the general reactivity of the DVP warhead and examples of compatible click handles and representative payloads that can be attached. MMAE, monomethyl auristatin E; PBD, pyrrolobenzodiazepine; SMCD, small-molecule cytotoxic drug; SpyTag, a short peptide derived from *S. pyogenes* that forms a stable isopeptide bond with its genetically encoded protein partner SpyCatcher.

hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC) techniques; and shows example data for a *Mycobacterium tuberculosis*-targeting Ab with 4× ValCit-linezolid after it has been clicked on.

The payload can be added to the tetraDVP construct either before[16] or after[14,15] bioconjugation to an antibody. Addition of the payload after bioconjugation allows facile diversification of the rebridged antibody, which allows different payloads to be added in single steps. Also, as the bioconjugation is done using a smaller known molecule the rebridging is more predictable, not being effected by the bulk or hydrophobicity of the payload. However, to ensure the click is successful a large excess of payload is required; if full click conversion

Table 1 | Overview of advantages and disadvantages of different bioconjugation methods

Method	Synthetic format	Typical accessible scale	Purification burden	Modularity and late-stage diversification	Ability to access single payload species	Typical use case
TetraDVP (solution-phase synthesis)	Solution	Gram scale (after route optimization)	High (multiple chromatographic purifications)	Limited (functional handles introduced early)	Yes (DAR 1)	Preparation of a single, optimized tetraDVP linker design
TetraDVP (solid-phase synthesis; this protocol)	Solid phase	100 mg–1 g resin scale (readily scalable with appropriate equipment)	Low (washing steps; final purification only)	High (variable PEG length, terminal handles, late-stage modification)	Yes (DAR 1)	Modular linker development, rapid analog generation, reproducible synthesis
Maleimide-based cysteine conjugation	Solution	Milligram to gram scale	High (instability)	High	No (DAR heterogeneity and instability common)	Routine ADC preparation where simplicity is prioritized
Pyridazinedione-based rebridging	Solution	Milligram scale	Moderate	High	No (typically DAR 2 or 4)	Stable cysteine rebridging
Phosphonamidate-based rebridging	Solution	Milligram scale	Moderate	High	No (typically DAR 4 or 8)	Site-selective cysteine modification for high DAR ADCs

is not achieved, this will lower the DAR and homogeneity. Therefore, the decision of when to add the payload is dependent on the number of variations desired, the physical properties of the payloads and the preciousness of the material. Successful addition of the payload can be determined by MS and liquid chromatography (LC) if done before bioconjugation, and MS and HIC if added after bioconjugation. To test that the antibody binding and payload efficacy are maintained after bioconjugation-relevant binding assays, payload release studies or affect on cells should be tested. As these studies will depend heavily on the type of antibody, the nature of the linker (cleavable or not) and the payload class, further details are not given here.

In this Protocol we describe the preparation of a linker that contains an azide group. This is suitable for the addition of a payload appended with an alkyne via strain-promoted (SPAAC) or copper-catalyzed azide–alkyne cycloaddition (CuAAC). We also describe the use of a double ended strained alkyne (dibenzocyclooctyne (DBCO)) to enable the joining of two azide containing molecules. If using an azide containing tetraDVP, bioconjugation should be caried out after the payload has been clicked on; this is to prevent tris(2-carboxyethyl)phosphine (TCEP) used in the bioconjugation from reducing the azide in a Staudinger reaction. While this reaction is very slow, it will hinder homogeneity[15]. Therefore, if click chemistry is desired after bioconjugation, an alkyne handle should be designed into the tetraDVP linker. While the use of other click systems, such as *trans*-cyclooctenes (TCOs) or bicyclononynes (BCNs), has not been explored on tetraDVP scaffolds, we believe they should be compatible. Payloads could also be attached via other chemistries, such as amide couplings; however, the order of synthesis may need to be adapted to avoid regioselectivity issues.

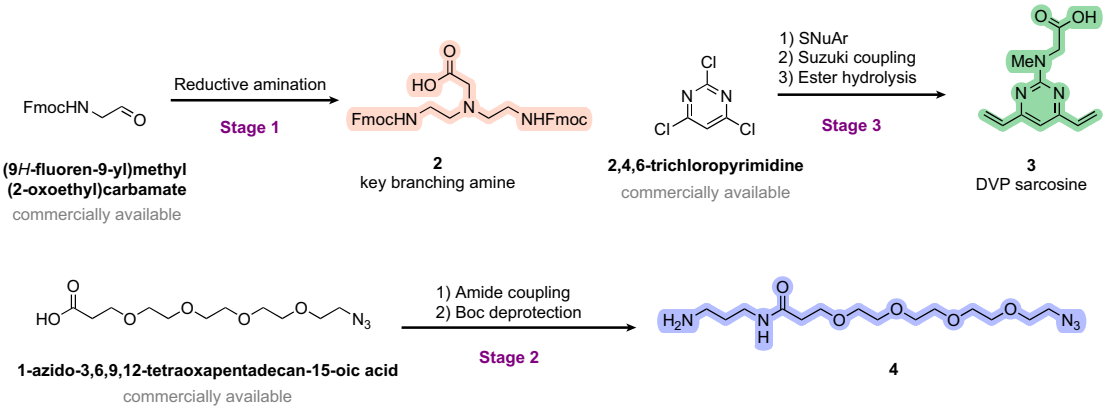
Choice of solvents

Dichloromethane was selected primarily for its excellent swelling properties with 2-chlorotrityl chloride resins and its compatibility with mild 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)-based cleavage conditions. For washing steps, an ethyl acetate/CH_2Cl_2 mixture can be used as a greener alternative, although reduced resin swelling may be observed. Other greener solvents commonly employed in solid-phase synthesis, such as 2-methyltetrahydrofuran; however, these were not evaluated. Users are encouraged to assess solvent substitutions on the basis of resin type and equipment availability.

Reactions in solution

Solution-phase reactions were carried out in round-bottom flasks sealed with rubber septa under a nitrogen atmosphere, in Ace pressure tubes (up to 10 mL), glass vials (up to 4 mL) or round bottomed flasks (up to 100 mL). Reaction progress was monitored by UV-LC–MS and/or thin-layer chromatography (TLC). Purification of products (**2, 3, 4, 9** and **10**) was achieved by

(Procedure 1) Synthesis of building blocks in solution (synthesized separately)



(Procedure 2) On-resin assembly of tetraDVP linkers

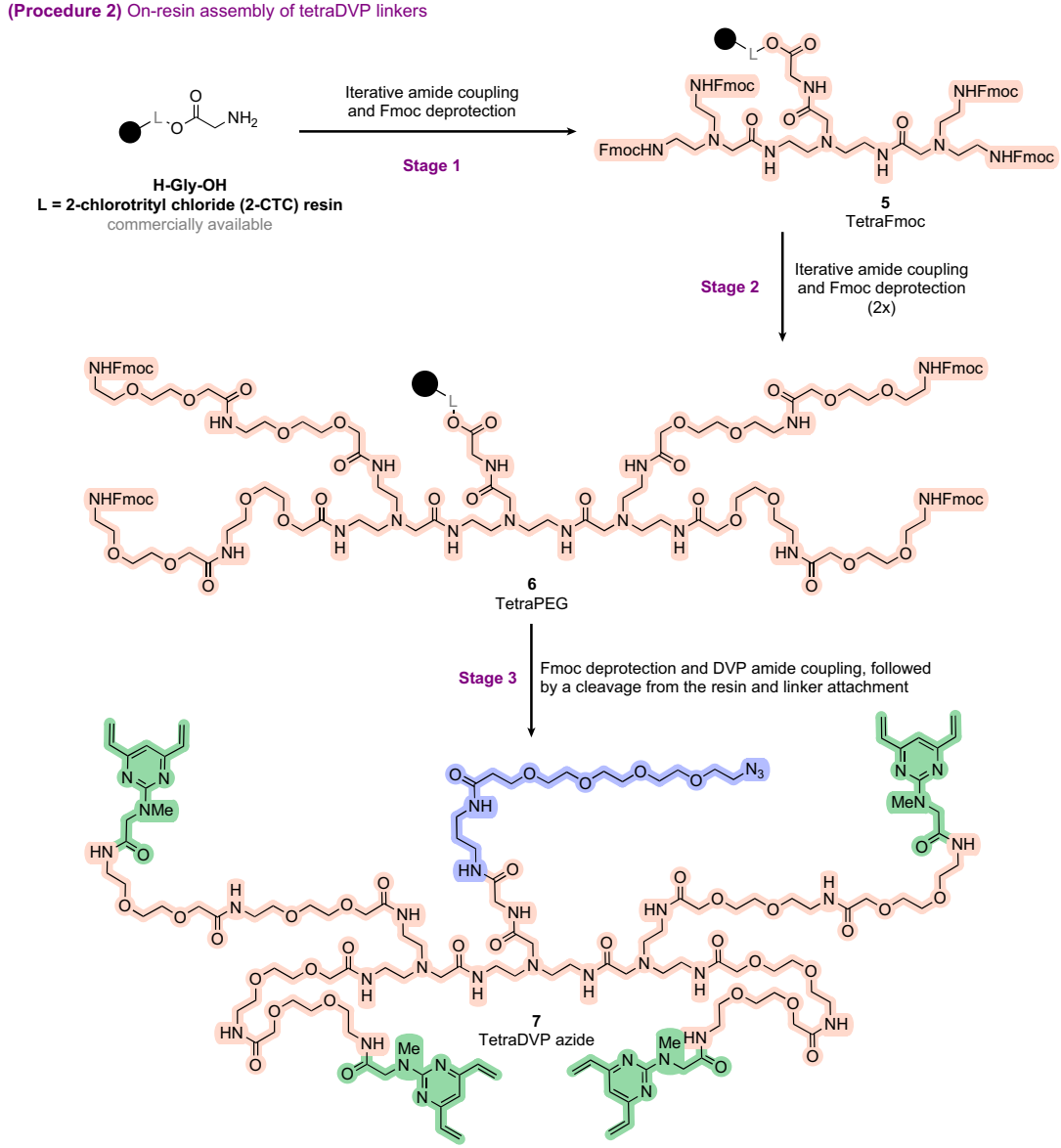


Fig. 2 | General synthetic scheme for the preparation of tetraDVP linkers. Procedure 1 (top) describes the synthesis of building blocks in solution. Procedure 2 (bottom) describes on-resin assembly of tetraDVP linkers. SNuAr, nucleophilic aromatic substitution.

flash column chromatography. As most compounds are oils and the reactions are carried out on relatively small scale, column chromatography is the advised purification method.

Reactions on resin

Manual solid-phase synthesis was performed using plastic syringes fitted with porous disks and loaded with H-Gly-OH premodified 2-chlorotrityl chloride resin (0.66 mmol/g). This acid-labile resin enables mild HFIP cleavage, preserving acid-sensitive DVP functionalities. Reagents were dissolved in glass beakers (25 mL for 2–10 mL volumes, 10 mL for ≤2 mL) and added to the resin in a single portion. Unless stated otherwise, 14 mL of solvent per 1 g of resin was used. Unless stated otherwise, for <100 mg of resin use a 3 mL syringe with porous frit, for 100–200 mg of resin use a 12 mL syringe with porous frit, for ≥ 250 mg use a 20 mL syringe with porous frit. All manual reactions were carried out at ambient temperature. For washing steps, the resin was agitated with fresh solvent for at least 10 s before solvent exchange. Reaction progress was monitored by UV-LC–MS after each step, except for deprotection of 9-fluorenylmethyloxycarbonyl (Fmoc).

Cleavage from the resin

For UV-LC–MS analysis during solid-phase synthesis, ~5 mg of resin was treated with CH_2Cl_2/HFIP (2:1, 1.5 mL) in an Eppendorf tube and shaken at room temperature (~20–25 °C), for 1 h. The solvent was evaporated under nitrogen and the cleaved material was dissolved in dimethyl sulfoxide (DMSO), filtered through ProFill HPLC syringe filters into an LC–MS vial and analyzed by UV-LC–MS to assess intermediate purity. For full cleavage of tetraDVP acids, CH_2Cl_2/HFIP (2:1, 20 mL) was used, typically in two 2 h cycles (4–5 h total). After evaporation under nitrogen, crude products were dissolved in DMSO and purified by preparative LC–MS/HPLC. Typical injections corresponded to the maximum amount of material that could be fully dissolved in the injection solvent. Unless otherwise stated, UV-LC–MS monitoring was employed by detection at 220–450 nm, with additional selective monitoring at 299 nm for Fmoc-containing species and 355 nm for DVP-containing species.

Scale and reproducibility

Synthesis was typically performed on a 250 mg resin scale (0.66 mmol/g loading; 0.165 mmol capacity), yielding 150–300 mg of crude linker depending on PEG length. The protocol is scalable to 1 g of resin without compromising yield or purity. For optimal reproducibility, monitor coupling efficiency after each amide coupling step by UV-LC–MS. Incomplete couplings can usually be resolved by increasing the acid equivalents. Ensure all required equipment and reagents are prepared before starting.

Design considerations and optimization

- PEG length: the number of PEG repeats can be adjusted to modulate solubility and the spatial separation between the antibody and the attached payload. Linkers with two {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (FAEEAA) units between the branching core and the DVP warhead were found optimal for most applications
- Terminal functionality: azido-PEG or alkyne groups can be introduced either on-resin or via postcleavage modification in solution, enabling orthogonal click conjugation to a wide range of cargo molecules. This Procedure describes the synthesis of an azide functionalized terminus that was added postcleavage in solution. The terminal functionality could be added on resin if an extra functional handle branch point (such as an orthogonally protected lysine) was incorporated into the linker that could be unmasked and the terminal functionality added before cleavage off the resin
- Payload compatibility: the platform supports conjugation to peptides (for example, SpyTag), fluorescent dyes and small molecules via SPAAC or CuAAC

- Quality indicators: successful synthesis is confirmed by recovery of a single major product, matching LC–MS mass, crude purity at least 80% and successful downstream derivatization (for example, SPAAC) as measured by LC–MS/HPLC of the subsequent step
- Process control: complete PEG elongation and crude purity above 90% should be ensured before DVP coupling, as truncation can result in linker heterogeneity and complications during HPLC purification
- Validation: we recommend using model antibodies such as trastuzumab to evaluate bioconjugation efficiency and confirm the integrity of the tetraDVP rebridged antibody product

Storage and stability

Cleaved tetraDVP acids and their functionalized analogs are stable for ≥9 months at −20 °C when stored under inert atmosphere as oils. Avoid repeated freeze–thaw–freeze cycles. If you cannot avoid them, we advise to distribute the compounds into multiple Eppendorf tubes and thaw them separately for each experiment. Intermediates containing DVP groups should be stored as solids where possible.

Expertise needed to implement the protocol

No further expertise beyond a graduate level of synthetic organic chemistry are required to successfully carry out this protocol.

Limitations

TetraDVP linkers are incompatible with strongly acidic environments (for example, TFA, HCl and stronger acids), which can degrade the DVP warhead and lead to polymerization.

The DVP motif is not generally stable under standard HATU (hexafluorophosphate azabenzotriazole tetramethyl uronium) and DIPEA (diisopropylethylamine) amide-coupling conditions.

The type of payload and linker should also be checked for suitability in the reaction conditions, such as stability to acid and DMSO. Payload and linker that are acid sensitive (for example, containing trityl group on imidazole, such as His(Trt)) or acid-cleavable self-immolative linkers such as hydrazones may be compromised during HFIP-mediated cleavage. DMSO-sensitive payloads may include highly electrophilic or redox-sensitive small molecules that undergo degradation or side reactions in polar aprotic solvents. If a desired payload is copper labile (for example, metalloproteins or copper-labile cofactors) or would react with azides or alkynes, then azide/alkyne cycloaddition should be avoided and other conjugation strategies (for example, amide coupling to the linker before bioconjugation or TCO/tetrazine) should be employed.

Resin-based synthesis is most effective for linker scales of 100–1,000 mg of resin per syringe; larger batches may require parallel synthesis. The purity of the final tetraDVP construct strongly influences bioconjugation efficiency and care should be taken to ensure high purity prior to antibody conjugation. Table 1 presents a comparison of different methods to guide individual needs.

Materials

Biological materials

- Trastuzumab (Roche, cat. no. 45-2317; RRID: AB_3669039; CAS 180288-69-1).

The results in this Protocol are based on trastuzumab. Other antibodies that have been successfully used include the commercially available IgG1 antibodies brentuximab (MedChemExpress; AB 3694976), cetuximab (MedChemExpress; AB 3694544) and durvalumab (MedChemExpress; AB 3695009) and the IgG4 antibody gemtuzumab (MedChemExpress; AB 3695574)[16]. This procedure has also been successfully caried out on novel antibodies expressed by biological research groups for various oncology and antimicrobial targets.

For in vivo applications the antibodies should be as pure as possible before bioconjugation; if there are contaminants, they will not be removed in the bioconjugation process and will make analysis of the product more difficult. Purity of starting antibody should be determined via HIC, SEC, SDS–PAGE and MS[34].

Reagents

▲ **CRITICAL** All reagents were of reagent grade and used without further purification. Solvents and chemicals were obtained from Merck, VWR, Thermo Fisher Scientific and Fluorochem. Anhydrous solvents were distilled over the chemicals specified in their respective entries listed under 'Solvents' (below).

▲ **CAUTION** Exercise care when handling organic materials. Always read safety information before handling the chemicals. A laboratory coat, gloves and safety goggles should be worn at all times. All synthetic operations should be performed in a chemical fume hood.

- 1,1′-Bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex ($Pd(dppf)Cl_2 \cdot CH_2Cl_2$) (Fluorochem, cat. no. F048593, CAS 95464-05-4)
- 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (Fluorochem, cat. no. F033415, CAS 6674-22-2)
- 1-Azido-3,6,9,12-tetraoxapentadecan-15-oic acid (Fluorochem, cat. no. F494260, CAS 1257063-35-6)
- 1-Hydroxybenzotriazole hydrate (HOBt) (Merck Sigma-Aldrich, cat. no. 54802, CAS 123333-53-9)
- 2,4,6-Trichloropyrimidine (Fluorochem, cat. no. F036437, CAS 3764-01-0)
- 2-Chlorotrityl chloride resin preloaded with H-Gly-OH (Iris Biotech, cat. no. RAA1040, particle size: 100–200 mesh)
- (9*H*-Fluoren-9-yl)methyl 2-oxoethylcarbamate (Fluorochem, cat. no. F235200, CAS 156939-62-7)
- Ammonium chloride, 98+% (NH_4Cl) (Thermo Fisher Scientific, cat. no. A15000.0I, CAS 12125-02-9)
- Brine (saturated aqueous NaCl; Fisher Scientific, cat. no. 10216410, CAS 7647-14-5)
- Dibutyltin dichloride (Bu_2SnCl_2) (Merck Sigma-Aldrich, cat. no. 205494, CAS 683-18-1)
- FAEEAA (Fluorochem, cat. no. F050302, CAS 166108-71-0)
- Glycine (Merck Sigma-Aldrich, cat. no. 410225, CAS 56-40-6)
- Lithium hydroxide monohydrate ($LiOH \cdot H_2O$) (Thermo Fisher Scientific, cat. no. 199540250, CAS 1310-66-3)
- Magnesium sulfate ($MgSO_4$) (Merck Sigma-Aldrich, cat. no. 63136, CAS 7487-88-9)
- *N*-Boc-1,3-propanediamine (Fluorochem, cat. no. F011409, CAS 75178-96-0)
- *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) (Merck Sigma-Aldrich, cat. no. E7750, CAS 25952-53-8)
- *N*-Hydroxysuccinimide (NHS) (Fluorochem, cat. no. F005022, CAS 6066-82-6)
- *N*,*N*′-Diisopropylcarbodiimide (DIC) (Fluorochem, cat. no. F132050, CAS 693-13-0)
- *N*,*N*-Diisopropylethylamine (DIPEA) (Merck Sigma-Aldrich, cat. no. 03439, CAS 7087-68-5)
- Phenylsilane ($PhSiH_3$) (Merck Sigma-Aldrich, cat. no. 335150, CAS 694-53-1)
- PNGase F (New England Biolabs, cat. no. P0704S)
- Potassium carbonate (K_2CO_3) (anhydrous; Fisher Scientific, cat. no. 11457967, CAS 584-08-7)
- Potassium vinyltrifluoroborate (Merck Sigma-Aldrich, cat. no. 655228, CAS 13682-77-4)
- Sarcosine ethyl ester hydrochloride (Fluorochem, cat. no. M06154, CAS 52605-49-9)
- Sodium hydrogen sulfate ($NaHSO_4$) (saturated aqueous; Thermo Fisher Scientific, cat. no. B25587.0B, CAS 7681-38-1)
- Triethylamine (Et_3N) (Fisher Scientific, cat. no. 10741301, CAS 121-44-8)
- Tris(2-carboxyethyl)phosphine (TCEP) (Fluorochem, cat. no. M02624, CAS 51805-45-9)

Solvents

- 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (Fluorochem, cat. no. F003409, CAS 920-66-1)
- 1,4-Dioxane (99.5%, extra dry over molecular sieve, stabilized, AcroSeal; Thermo Fisher Scientific, cat. no. 364341000, CAS 123-91-1)

- Acetone, laboratory reagent grade, for washing (Thermo Fisher Scientific, cat. no. 15681640, CAS 67-64-1)
- Acetone, for synthesis (99.8%, extra dry over molecular sieves, AcroSeal; Thermo Fisher Scientific, cat. no. 32680, CAS 67-64-1)
- Dichloromethane (CH_2Cl_2), laboratory reagent grade (distilled over CaH_2; Thermo Fisher Scientific, cat. no. 10616642, CAS 75-09-2)
- Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, cat. no. A13280.36, CAS 67-68-5)
- Ethyl acetate (EtOAc), laboratory reagent grade (distilled over CaH_2; Thermo Fisher Scientific, cat. no. 10332583, CAS 141-78-6)
- Methanol (MeOH), puriss. p.a. (distilled over CaH_2; Merck Sigma-Aldrich, cat. no. 32213-M, CAS 67-56-1)
- *N*,*N*-Dimethylformamide (DMF) for peptide synthesis (Merck-Supelco, cat. no. 1.00397, CAS 68-12-2)
- Petroleum ether, bp 40–60 °C, laboratory reagent grade (distilled over CaH_2; Fisher Scientific, cat. no. 16626153, CAS 64742-49-0)
- Phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4)
- Tetrahydrofuran (THF) (distilled over CaH_2 and $LiAlH_4$; Fisher Scientific, cat. no. 10578070, CAS 109-99-9)
- Trifluoroacetic acid (TFA), peptide grade (Fluorochem, cat. no. F001271, CAS 76-05-1)
- Tris-buffered saline (TBS) solution (25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM sodium chloride, 0.5 mM ethylenediaminetetraacetic acid (EDTA), adjusted to pH 8.0 with 1 M aq. NaOH)

HPLC and LC–MS solvents

- Acetonitrile (MeCN), LC–MS grade (Merck-Supelco, cat. no. 1.00029, CAS 75-05-8)
- Ammonium acetate (NH4OAc), LC–MS grade (Merck-Supelco, cat. no. 73594, CAS 631-61-8)
- Formic acid (FA), LC–MS grade (VWR, cat. no. 84865.260,CAS 64-18-6)
- Trifluoroacetic acid (TFA), peptide grade (Fluorochem, cat. no. F001271, CAS 76-05-1)
- Water (H_2O), LC–MS grade (Merck-Supelco, cat. no. 1.15333, CAS 7732-18-5)

Equipment

- Analytical HPLC: Agilent 1260 Infinity system with a reversed-phase Supelcosil ABZ+PLUS column (module model nos. G1322A, G1312B, G1329B, G1316A)
- Automated reverse-phase column chromatography: Teledyne ISCO Combiflash Rf 200; Redisep R_f Gold columns, 18-mL fraction tubes (model no. Rf 200)
- LC–MS UV detector (UV-LC–MS): Waters ACQUITY H-Class UPLC with an ESCi Multi-Mode Ionization Waters SQ Detector 2 (module models ACQUITY H-Class UPLC Column Manager, QSM, SQD2, PDA eλ, and FTN)
- LC–MS-HPLC: Waters AutoPurification mass-directed HPLC system with photodiode array detector, SQ Detector 2 and at-column dilution (module model nos. 3767, 2545, 515, 2998, SQD2)
- NMR: Bruker Avance III 500 MHz HD Smart Probe and Bruker TXO 700 MHz Cryo Probe spectrometers at magnetic field strengths of 11.75 T (500 MHz) and 16.44 T (700 MHz)
- Shaker: IKA VXR Basic Vibrax Shaker (2200 rpm; model Vibrax-VXR)
- Hot plate: IKA Plate (RCT digital) Magnetic Stirrer (1,500 rpm, 310 °C; model RCT digital)
- Freeze dryer (lyophilizer): Scanvac Coolsafe 100-9 Pro (cat. no 7001000617)
- Thermal shaker: Grant-bio PCMT Thermo-Shaker (model no. SI-0266)
- UV-Vis spectrometer: ThermoFisher NanoDrop One (model no. C)
- Centrifuge: VWR Micro Star 17 (model no. 17)

Basic laboratory equipment

- 10k Molecular weight cut-off (MWCO) Amicon Ultra Diafiltration columns (Merck, 0.5 mL, cat. no. UFC501024)
- 40k MWCO Zeba Spin Desalting columns (Thermo Fisher Scientific, 0.5 mL, cat. no. A57760)

- Analytical balances (Sartorius, model no. ME414S)
- Büchner funnel (120 mL, Merck Sigma-Aldrich, cat. no. Z247324; can also use sintered/Hirsch funnels)
- Celite S (Merck Sigma-Aldrich, cat. no. 06858)
- Centrifuge tubes (500 and 2,000 µL, cat. nos. 05-408-120 and 05-408-129, respectively)
- Clamps
- Column chromatography tubes (internal diameter 1.4 cm, 2.3 cm, SAMCO, cat. nos. TES1044 and TES1020, respectively)
- Column chromatography tubes rack (Merck Sigma-Aldrich, cat. no. Z693022)
- Cotton wool
- DrySyn heating blocks (Asynt, various sizes 10–100 mL, model no. MULTI-E, or analogous oil/sand baths)
- Eppendorf tube holder, plastic (Fisher Scientific, cat. no. 8860)
- Eppendorf tubes, microcentrifuge, non-sterile (Fisher Scientific, various sizes 1.5–2 mL, cat. no. 3434PK)
- Erlenmeyer flasks (Simax/SLS, various sizes, 250–2,000 mL, cat. no. FLA4070)
- Glass beakers (Simax/SLS, various sizes, 10–50 mL, cat. no. BEA100)
- Glass columns (Simax/SLS, internal diameter 3.0, 4.0 cm, cat. no. R1/40/400/2)
- Glass TLC chamber (Laboquip, 68 mm (L) × 68 mm (W) × 106 mm (H), cat. no. 5061017319412)
- Graduated cylinder (Simax, various sizes, 10–250 mL, such as cat. no. B8A73325)
- Graduated plastic Pasteur pipettes (DWK Life Sciences, 3 mL, cat. no. DWK711117F10S)
- Ice bath (a large bowl or Tupperware capable of containing a 50:50 ice/water mixture and wide enough to half submerge a 250-mL round-bottomed flask)
- LC–MS vials (Agilent, various sizes, 0.35–1.5 mL, such as cat. no. 1351010/01)
- Needles (Braun, Sterican G21 0.80 × 120 mm, cat. no. 4665643)
- Nitrogen source (dry)
- Pipette tips (Starlab, various sizes 20–1,000 µL, such as cat. no. S1111-0816)
- Pipettes, automatic (Gilson, various sizes 20–1,000 µL, such as cat. no. 12356132)
- Plastic syringes (Henke Sass Wolf, various sizes, 1–20 mL, such as cat. no. 17940251)
- Plastic syringes equipped with porous disks (Torviq, for 3–20 mL syringes, such as cat. no. SF-1000)
  ▲ **CRITICAL** Plastic syringes with porous disks were sourced from Torviq (http://www.torviq.com). Notably, we observed huge variability between suppliers and strongly recommend using Torviq's porous disks, as successful synthesis could not be consistently achieved with alternatives.
- Polyethylene plastic stoppers (Merck Sigma-Aldrich, various sizes, B14/23, B24/29, B29/32, such as cat. no. Z238015)
- Polypropylene powder funnels (Merck Sigma-Aldrich, cat. no. Z420042)
- ProFill HPLC syringe filters, PTFE membrane, diameter: 17 mm, pore size: 0.45 µm (Fisher Scientific, cat. no. 12636745)
- Reflux condenser
- Rotary evaporator (Büchi, Rotavapor R-210) with heating bath (B-491) (Merck Sigma-Aldrich, cat. no. Z565199)
- Round bottom flasks (Quickfit, Merck Sigma-Aldrich, various sizes, 5–1,000 mL, such as cat. no. Z302805)
- Rubber bands
- Rubber septa Suba-Seal (Merck Sigma-Aldrich, various sizes, B14/23, B24/29, B29/32, such as cat. no. Z124591)
- Separatory funnel with PTFE stopcock (Glassco, various sizes 100–1,000 mL, such as cat. no. 149.402.04)
- Silica gel (Merck-Supelco, cat. no. 60737, CAS 112926-00-8)
- Simax Screw Top Reagent Bottle with blue cap (Philip Harris/Simax, 1,000 mL, cat. no. B8A55955)
- Sonicator (ultrasonic bath, Fisher Scientific, cat. no. FB15055)

- Spatulas (micro and standard chattaway, Philip Harris, cat. no. PP00059927)
- Stirrer bars (magnetic, Merck Sigma-Aldrich, cat. no. Z744787)
- Syringe pressure caps (Merck Sigma-Aldrich, cat. no. Z120960)
- Timer
- TLC plates (Merck-Supelco, cat. no. 1.05715)
- Tweezers
- UV lamp (Spectroline, with short-wave ultraviolet 254 nm, long-wave ultraviolet 365 nm; Supelco Merck Sigma-Aldrich, cat. no. Z169641)

Equipment setup

UV-LC–MS analysis

In our laboratory UV-LC–MS analysis is carried out using a Waters ACQUITY H-Class UPLC with an ESCi Multi-Mode Ionization Waters SQ Detector 2 spectrometer using an ACQUITY UPLC CSH C18 (2.1 mm × 50 mm, 1.7 µM, 130 Å) column at 40 °C and flow rate 0.6 mL/min.

- Mobile phase is (A) 2 mM ammonium acetate in water/MeCN (95:5), (B) MeCN and (C) 2% aq. FA, linearly programmed from 5–95% solvent B over 1 or 3 min, then held at 95% solvent B for 1.5 min, with solvent C maintained at 5% throughout the run
- The electrospray ionization (ESI) source operates at a discharge current of 5 µA, vaporizer temperature of 350 °C and capillary temperature of 200 °C
- Chromatographs are monitored by UV absorbance using a photodiode array detector at a wavelength range of 210–800 nm, interval 1.2 nm

  For Fmoc-containing intermediates, chromatograms are monitored at 299 nm to selectively detect Fmoc-bearing species. For DVP-containing compounds, monitoring at 355 nm is used to selectively visualize DVP-containing products relative to other UV-active components.

Time (min)	Flow (mL/min)	%A	%B	%C
Analytical gradient method (fast)				
0.00	0.6	95.0	0.0	5.0
1.00	0.6	0.0	95.0	5.0
2.50	0.6	0.0	95.0	5.0
2.70	0.6	95.0	0.0	5.0
3.00	0.6	95.0	0.0	5.0
Analytical gradient method (slow)				
0.00	0.6	95.0	0.0	5.0
3.00	0.6	0.0	95.0	5.0
4.50	0.6	0.0	95.0	5.0
4.70	0.6	95.0	0.0	5.0
5.00	0.6	95.0	0.0	5.0

LC–MS-HPLC analysis

LC–MS-HPLC is carried out on a Waters AutoPurification mass-directed HPLC system with photodiode array detector, SQ Detector 2 and at-column dilution.

- The analytical runs use an X-Select CSH C18 column (4.6 × 150 mm, 5-µm particle size) and flow rate of 1.2 mL/min
- The preparative runs use an X-Select CSH Prep C18 OBD column (19 × 150 mm, 5-µm particle size) and flow rate of 20 mL/min

The machine uses a 1 min (analytical) or 1.4 min (preparative) isocratic hold before the gradient of solvent B in solvent A run over 15–20 min .

- For acidic runs, solvent A is water with 0.1% (vol/vol) FA and solvent B is MeCN with 0.1% (vol/vol) FA
- For neutral runs solvent A is water (no additives) and solvent B is MeCN (no additives)

For Fmoc-containing intermediates, chromatograms are monitored at 299 nm to selectively detect Fmoc-bearing species. For DVP-containing compounds, monitoring at 355 nm is used to selectively visualize DVP-containing products relative to other UV-active components.

Time (min)	Flow (mL/min)	%A	%B
Analytical gradient method			
0.00	1.20	95.0	5.0
1.00	1.20	95.0	5.0
15.00	1.20	5.0	95.0
18.00	1.20	0.0	100.0
25.00	0.00	0.0	100.0
Preparative gradient method			
0.00	20.0	95.0	5.0
1.40	20.0	95.0	5.0
16.00	20.0	5.0	95.0
19.00	20.0	0.0	100.0
25.00	0.0	0.0	100.0

Analytical HPLC

Analytical HPLC is carried out using an Agilent 1260 Infinity system with a reversed-phase Supelcosil ABZ+PLUS column (150 mm × 4.6 mm, 3 µm) eluting with a linear gradient system (5–95% B in A; solvent A: 0.05% (vol/vol) TFA in water; solvent B: 0.05% (vol/vol) TFA in MeCN) over 15 min, then an isocratic hold at 100% B for 4 min at a flow rate of 1 mL/min. Analytical HPLC is monitored by UV absorbance at 254 and 280 nm.

Time (min)	Flow (mL/min)	%A	%B
Analytical gradient method			
0.00	1.00	95.0	5.0
15.00	1.00	5.0	95.0
15.01	1.00	0.0	100.0
19.00	1.00	0.0	100.0
19.01	1.00	95.0	5.0

Automated reverse-phase flash column chromatography

Automated reverse-phase flash column chromatography is carried out on a Teledyne ISCO Combiflash Rf200 system with Redisep R_f Gold reverse-phase C18-silica flash columns (20–40 µm). All separations are carried out with mixtures of water and MeCN with the proportions of each changing overtime with a linear gradient. Some separations are carried out with acidic or basic additives.

NMR analysis

NMR spectra are recorded on Bruker Avance III 500 MHz HD Smart Probe and Bruker TXO 700 MHz Cryo Probe spectrometers at magnetic field strengths of 11.75 T (500 MHz) and 16.44 T (700 MHz) with operating frequencies 500.16 MHz and 700.13 MHz (for ^{1}H), 125.77 MHz and 176.04 MHz (for ^{13}C) at 23 °C, respectively.

- Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (*J*) are reported in Hertz (Hz)
- The ^{1}H and ^{13}C NMR chemical shifts (δ in ppm) are referenced to the residual signals of DMSO-*d*6 [2.50 (^{1}H) and 39.52 (^{13}C)], $CDCl_3$ [7.26 (^{1}H) and 77.16 (^{13}C)] or MeOD [3.31 (^{1}H) and 49.00 (^{13}C)]
- Structural assignment of resonances is performed with the help of 2D NMR gradient experiments (COSY, ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC, ^{1}H–^{15}N HSQC, ^{1}H–^{15}N HMBC, NOESY)

HRMS analysis

HRMS measurements were recorded on an Agilent 6230 LC/TOF HRMS system using ESI techniques. Mass values are reported within the 5 ppm error limit.

Antibody analytical LC–MS

Antibody conjugate analytical LC–MS is carried out on a Waters Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 µm, 2.1 × 50 mm).

- Solvent A (0.1% FA (aq.)) and solvent B (19:1 MeCN:0.1% FA (aq.)) are used as the mobile phase at a flow rate of 0.2 mL/min
- The gradient is programmed as follows: 95% A for 0.93 min, then a gradient to 100% B over 4.28 min, then 100% B for 1.04 min, then a gradient to 95% A over 1.04 min
- The electrospray source is operated with a capillary voltage of 2.0 kV and a cone voltage of 190 V
- Nitrogen is used as the desolvation gas at a total flow rate of 850 L/h
- Total mass spectra are reconstructed from the ion series using the MaxEnt 1 algorithm preinstalled on MassLynx 4.2 software according to the manufacturer's instructions
- Trastuzumab samples are deglycosylated with PNGase F (New England Biolabs) before LC–MS analysis. In our work, only the region of each total ion chromatogram containing protein signals was analyzed
- All calculated values for the masses of trastuzumab conjugates are based on the observed mass ion of native trastuzumab under the same preparation and ionization conditions (145,171 Da)

Antibody analytical SEC

Analytical SEC was carried out using an Agilent 1260 Infinity system with a Tosoh TSKgel G3000SWXL column (30 cm × 7.8 mm, 5 µm) eluting with sodium phosphate buffer (50 mM Na_3PO_4, 100 mM NaCl, 0.2% (wt/vol) sodium azide, pH 7) over 30 min at a flow rate of 0.5 mL/min. HPLC was monitored by UV absorbance at 280 nm, and extent of aggregation was determined on the basis of peak area.

Antibody analytical HIC

Analytical HIC was carried out using an Agilent 1260 Infinity system with a Tosoh TSKgel Butyl-NPR column (3.5 cm × 4.6 mm, 2.5 µm) eluting with a linear gradient of 100% solvent A for 3 min, then 0–100% solvent B in solvent A over 17 min (solvent A: 1.5 M ammonium sulphate, 25 mM Na_3PO_4, pH 7; solvent B: 25% (vol/vol) isopropyl alcohol in 25 mM Na_3PO_4, pH 7) at a flow rate of 0.6 mL/min. HPLC was monitored by UV absorbance at 280 nm, with conversion calculated by peak area.

Procedure

Section 1: synthesis of building blocks in solution

Stage 1: synthesis of key branching amine 2

● **TIMING 22 h (6 h hands-on time)**

Refer to Fig. 3.

1. Place a clean, dry 250-mL round-bottom flask (B24/32) on a hot plate. Insert a stirrer bar and fit the flask with a rubber septum. Set the stirring speed to 400 rpm.
2. Charge the flask with (9*H*-fluoren-9-yl)methyl(2-oxoethyl)carbamate (3 g, 10.65 mmol, 5 eq.) and add anhydrous dioxane (50 mL).
3. Use a sonicator to make the reaction mixture homogeneous.
4. Accurately weigh glycine (159 mg, 2.13 mmol, 1 eq.) and Bu_2SnCl_2 (645 mg, 2.13 mmol, 1 eq.) using an analytical balance. Use Eppendorf tubes for weighing each reagent separately.

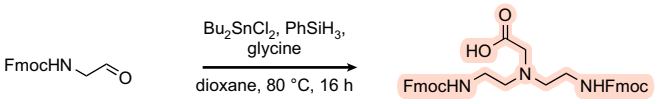


Fig. 3 | Synthesis of key branching amine 2.

▲ **CAUTION** Bu_2SnCl_2 is a skin sensitizer with a rather unpleasant odor. Always handle this reagent in a well-ventilated fume hood. Use gloves and other appropriate personal protective equipment at all times.

5. While stirring the solution at room temperature, add both reagents to the reaction mixture at once, as solids.
6. Add another 30 mL of anhydrous dioxane, remove the flask from the hot plate and use a sonicator to make the reaction mixture homogeneous.
7. Clamp the round-bottom flask back on the hot plate into a DrySyn heating block.
8. Under constant stirring at room temperature, add phenylsilane (1.31 mL, 10.65 mmol, 5 eq.) to the reaction mixture using a pipette or a syringe with needle.
9. Replace the rubber septum with a reflux condenser connected to a constant stream of cooling water and under a stream of nitrogen. Ensure the condenser is securely attached, the water flow is continuous and unobstructed and no leaks are present at the joints.
10. Set the hot plate temperature to 100 °C and the thermostat to 85 °C. Allow the reaction mixture to stir for at least 16 h, but no longer than 24 h. Ensure the setup remains sealed under nitrogen throughout the heating period.
11. After 16 h, allow the reaction flask to cool to room temperature. Using a pipette, transfer a 100-µL aliquot of the reaction mixture into an Eppendorf tube. Add 500 µL of ethyl acetate and 500 µL of saturated aqueous ammonium chloride solution. Vigorously shake the tube, then allow the phases to separate.
12. Using a plastic Pasteur pipette, carefully transfer the upper organic phase to a 5-mL round-bottom flask. Remove residual solvents using a rotary evaporator set to 100 mbar with a bath temperature of 45 °C.
13. Dissolve the residual oil in DMSO (1 mL) and analyze by UV-LC–MS to confirm full conversion to the desired product **2** (R_t = 2.45 min, when 5–95% solvent B over 3 min, followed by 1.5 min isocratic hold, with solvent C maintained at 5% throughout the run).
▲ **CRITICAL STEP** Ideally, the LC–MS chromatogram should show only three peaks: the solvent (DMSO; R_t = 0.52 min, when 5–95% solvent B over 3 min, followed by 1.5 min isocratic hold, with solvent C maintained at 5% throughout the run), the desired product (compound **2**), and any residual unreacted (9*H*-fluoren-9-yl)methyl(2-oxoethyl)carbamate (R_t = 2.24 min, when 5–95% solvent B over 3 min, followed by 1.5 min isocratic hold, with solvent C maintained at 5% throughout the run). Minor impurities below 10% are acceptable; however, it is essential to confirm the absence of partially reacted intermediates by LC–MS.
◆ **TROUBLESHOOTING**
14. Once the reaction is complete, transfer the mixture from a round-bottom flask into a 1,000-mL separatory funnel and add 400 mL of ethyl acetate. Wash the round-bottom flask with ~50 mL of ethyl acetate to transfer everything into the separatory funnel.
15. Add 400 mL of saturated aqueous ammonium chloride solution and shake the separatory funnel vigorously. Open the plastic stopcock and release the pressure.
16. Allow the mixture to settle and separate into two distinct layers. Carefully collect each phase into separate Erlenmeyer flasks (500 mL for the aqueous phase, 2,000 mL for the organic phase).
17. Return the aqueous layer to the separatory funnel, add a fresh 400 mL portion of ethyl acetate, and repeat the extraction three additional times. Combine all organic layers in a single Erlenmeyer flask.

18. Add ~1–2 g of anhydrous $MgSO_4$ per 50 mL of organic solvent to the Erlenmeyer flask containing the combined organic extracts and gently swirl or shake the flask by hand to dry the solution.
▲ **CRITICAL STEP** Upon swirling, the magnesium sulfate should move freely as a suspension in the Erlenmeyer flask, indicating that the solution is sufficiently dry. If it clumps or settles heavily, add additional spoons of $MgSO_4$ until free movement is observed.
19. Filter the solvent into a round-bottom flask (1,000 mL) using a polypropylene powder funnel stuffed with cotton wool.
20. Use a rotary evaporator to remove the solvent. Start at 700 mbar, bath temperature 45 °C and carefully reduce the pressure up to the point you see condensation on the rotary evaporator condenser (<200 mbar). Evaporate to dryness.
21. Meanwhile, prepare for column chromatography.
    - In a Simax screw-top reagent bottle (1,000 mL), mix 540 mL of CH_2Cl_2/MeOH in a 12.5:1 (vol/vol) ratio
    - In a separate glass beaker (500 mL), suspend silica gel in a portion of this solvent mixture, then carefully pour the slurry into a glass column
    - Allow the silica to settle then form a uniform packed bed using compressed air
22. The column details are as follows: silica (230–400 mesh), column length (total) 36 cm, column diameter 4.0 cm, silica height 18 cm, R_f = 0.18 (CH_2Cl_2/MeOH 10:1), fraction tube size 18 mL.
▲ **CAUTION** The particle size of silica gel can cause respiratory problems after prolonged exposure to, and inhalation of, the silica dust. Only handle silica gel in a ventilated fume hood.
23. To the round-bottom flask with crude product add ~200 mL of acetone and add ~30–50 g of silica gel.
24. Carefully evaporate to dryness. The crude product should be adsorbed on the silica. Drain the column until there is no solvent above the silica gel level.
25. Using a polypropylene powder funnel, transfer the adsorbed product on silica onto the column. Residual powder can be suspended in a small amount of chromatography solvent and added carefully on top of the silica bed.
26. Begin elution with CH_2Cl_2/MeOH (12.5:1, vol/vol), gradually increasing the polarity of the solvent system. Recommended gradient:
    - 540 mL of CH_2Cl_2/MeOH 12.5:1, followed by
    - 550 mL of CH_2Cl_2/MeOH 10:1, then
    - 570 mL of CH_2Cl_2/MeOH 7:1, then
    - 580 mL of CH_2Cl_2/MeOH 6:1, and then
    - 600 mL CH_2Cl_2/MeOH 5:1

    At this stage, product fractions (tube size 18 mL) should begin to elute around fraction 30. Monitor elution by TLC using UV light for detection.
27. Continue elution, gradually increasing polarity with each new 500 mL of solvents up to CH_2Cl_2/MeOH 1:2, until no further product is detected by TLC. Collect all fractions corresponding to the desired product.
▲ **CRITICAL STEP** Rapidly increasing the polarity of the eluent to speed up elution of compound **2** is strongly discouraged. The gradient described has been tested in over 10 independent runs and consistently provides high-purity material with excellent yield. Do not add acidic modifiers such as acetic acid to speed up the elution, as these have been found to interfere with downstream solid-phase synthesis. Patience during this step is critical for optimal results.
28. Collect all fractions containing product, transfer them to a round-bottom flask and evaporate to dryness. This affords the product **2** (1.27 g, 2.09 mmol, 98%) as a white amorphous solid after removal of the chromatography solvent.
29. Characterize the product via ^{1}H NMR, TLC, LC–MS and analytical HPLC.
TLC: R_f = 0.18, CH_2Cl_2/MeOH 10:1;
LC–MS: R_t = 2.24 min, when 5–95% solvent B over 3 min, followed by 1.5 min isocratic hold, with solvent C maintained at 5% throughout the run;
analytical HPLC: R_t = 11.81 min (5–95% B over 18 min);

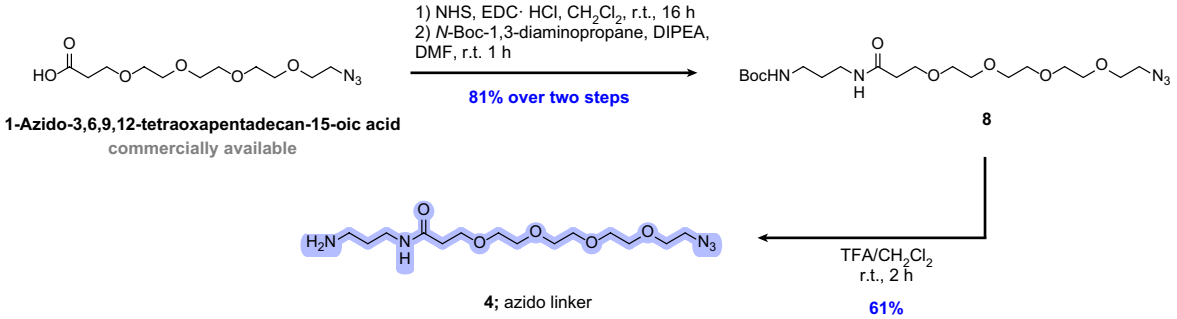


Fig. 4 | Synthesis of azido linker 4. Boc, tert-butyloxycarbonyl; r.t., room temperature.

^{1}H NMR (500 MHz, DMSO-d_6): δ 7.87 (d, J = 7.5 Hz, 4H), 7.66 (d, J = 7.5 Hz, 4H), 7.39 (t, J = 7.5 Hz, 4H), 7.30 (td, J = 7.5, 1.2 Hz, 4H), 7.22 (t, J = 5.7 Hz, 2H), 4.27 (d, J = 7.1 Hz, 4H), 4.19 (t, J = 7.0 Hz, 2H), 3.31 (s, 2H), 3.06 (q, J = 6.4 Hz, 4H), 2.67 (t, J = 6.7 Hz, 4H) ppm;
low-resolution mass spectrometry (LRMS) (ESI) m/z: 606.25 $[M + H]^+$.

■ **PAUSE POINT** Purified **2** can be stored at −20 °C for at least 12 months.

Stage 2: synthesis of azido linker 4 (via 8)

Refer to Fig. 4.

Stage 2a: synthesis of **8**

● **TIMING 24 h (3 h hands-on time) for 8**

30. Place a clean, dry round-bottom flask (100 mL, with Teflon-coated magnetic stirrer bar) on a hot plate. Fit the flask with a rubber septum and set the stirring speed to 400 rpm.
31. Charge the flask with CH_2Cl_2 (30 mL) followed by 15-azido-4,7,10,13-tetraoxapentadecanoic acid (1.50 g, 5.15 mmol, 1 eq.) and NHS (890 mg, 7.73 mmol, 1.5 eq.).
32. Add EDC·HCl (1.97 g, 10.3 mmol, 2 eq.) and stir the resulting solution for 16 h at ambient temperature.
33. Check that the reaction is complete by performing LC–MS: starting acid R_t = 1.13 min, activated ester = 1.22 min, no ionization for either species observed (5–95% solvent B over 1 min, followed by 1.5 min isocratic hold, with solvent C maintained at 5% throughout the run). Reaction mixture should be >90% activated ester.
34. Pour the reaction mixture into a separating funnel (100 mL) and rinse the reaction flask with CH_2Cl_2 (10 mL).
    - Wash the organic layer with saturated aqueous $NaHSO_4$ (30 mL), retaining the lower organic layer into a 250-mL conical flask
    - Discard the aqueous phase. Return the organic layer to the empty funnel and wash with brine (30 mL)
    - Collect the organic layer (lower)
35. Add ~1–2 g of $MgSO_4$ per 50 mL of organic solvent to the organic layer to dry it (Step 18).
    - Filter the solvent into a round-bottom flask (250 mL) using a polypropylene powder funnel stuffed with cotton wool
    - Rinse the $MgSO_4$ flask and wool with CH_2Cl_2 (20 mL)
36. Use a rotary evaporator to remove the solvent (40°C, ~400 mbar). When the solvent level drops below 20 mL, transfer to a clean, dry, round-bottomed flask (50 mL) and evaporate to dryness. Crude mass ~3 g (contains trapped solvent).
37. Dilute the resulting crude orange oil with DMF (10 mL) and add a Teflon-coated magnetic stirrer bar.
38. Add *N*-boc-1,3-diaminopropane (896 mg, 5.15 mmol, 1 eq.) and DIPEA (1.80 mL, 10.3 mmol, 2 eq.).
39. Stir for 1 h at ambient temperature.

40. Check that the reaction is complete by performing LC–MS: R_t = 1.27 min, (ESI) *m/z*: 492.4 $[M + HCOO]^-$ (5–95% solvent B over 1 min, followed by 1.5 min isocratic hold, with solvent C maintained at 5% throughout the run). Reaction mixture should be >90% product.
41. Dilute the reaction with EtOAc (50 mL) and extract with saturated aqueous NH_4Cl (30 mL) then 5% aqueous LiCl (4 × 30 mL) (keeping the organic layer (top) between washes).
42. Add ~1–2 g of $MgSO_4$ per 50 mL of organic solvent to the organic layer to dry it (Step 18).
43. Filter the solvent into a round-bottom flask (250 mL) using a polypropylene powder funnel stuffed with cotton wool. Rinse the $MgSO_4$ flask and wool with EtOAc (20 mL).
44. Use a rotary evaporator to remove the solvent (40°C, ~120 mbar). Evaporate to dryness to yield *tert*-butyl (1-azido-15-oxo-3,6,9,12-tetraoxa-16-azanonadecan-19-yl)carbamate **8** as a crude orange oil (1.86 g, 4.15 mmol, 81%).
45. Characterize the product **8** via NMR, TLC and LRMS.
    TLC: R_f = 0.92, CH_2Cl_2/MeOH 4:1;
    ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.77 (s, 1H), 5.15 (s, 1H), 3.71 (t, *J* = 5.7 Hz, 2H), 3.68 – 3.59 (m, 14H), 3.37 (t, *J* = 5.1 Hz, 2H), 3.28 (app. q, *J* = 6.3 Hz, 2H), 3.13 (app. q, *J* = 6.1 Hz, 2H), 2.46 (t, *J* = 5.8 Hz, 2H), 1.61 (app. qn, *J* = 6.3 Hz, 2H), 1.42 (s, 9H) ppm;
    ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.0, 156.4, 79.0, 70.71, 70.68, 70.6, 70.5, 70.4, 70.3, 70.0, 67.3, 50.7, 37.1, 37.0, 36.0, 30.1, 28.4 ppm;
    LRMS (ESI) *m/z*: 492.4 $[M + HCOO]^-$.
    PAUSE POINT Crude **8** can be stored at −20 °C for at least 12 months.

Stage 2b: synthesis of **4**

TIMING 4 h (2 h hands-on time) for 4

46. Dissolve **8** (300 mg, 0.67 mmol) in CH_2Cl_2 (5 mL) in a round-bottomed flask (25 mL, with Teflon-coated magnetic stirrer bar). Add trifluoracetic acid (1.25 mL) and stir for 2 h at room temperature. Completion of the Boc (tert-butyloxycarbonyl) deprotection can be monitored via TLC (R_f = 0.52, CH_2Cl_2/MeOH 4:1).
47. Remove the solvent under a stream of dry nitrogen gas. This can be done either by bubbling N_2 though the solution via a needle attached to a filled N_2 ballon or via a gas manifold/Schlenk line.
48. Purify the resulting oil via automated reverse phase flash column chromatography (50 g HP C18, 10–50% MeCN in aq. 0.1% NH_4OH over 20 min) and remove the solvent via lyophilization to yield **4** as a yellow oil (142 mg, 0.41 mmol, 61%).
49. Characterize and check the purity of the product via NMR and HRMS.
    ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.89 (app. s, 1H), 3.74 (t, *J* = 5.9 Hz, 2H), 3.71 – 3.61 (m, 14H), 3.40 (t, *J* = 5.0 Hz, 2H), 3.36 (app. q, *J* = 6.4 Hz, 2H), 2.78 (t, *J* = 6.5 Hz, 2H), 2.48 (t, *J* = 5.8 Hz, 2H), 1.65 (app. qn, *J* = 6.6 Hz, 4H) ppm;
    ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.6, 70.70, 70.68, 70.6, 70.5, 70.4, 70.3, 70.1, 67.4, 50.7, 39.8, 37.3, 37.1, 32.8 ppm;
    HRMS (ESI) *m/z*: found 348.2245 $[M + H]^+$, $C_{14}H_{30}N_5O_5^+$, calcd. 348.2241.

Stage 3: synthesis of DVP sarcosine 3 (via 9)

Refer to Fig. 5.

Stage 3a: synthesis of **9**

TIMING 5 h (4 h hands-on time) for 9

50. Add anhydrous acetone (100 mL) to a clean, dry round-bottomed flask (250 mL, fitted with a rubber septum and Teflon-coated magnetic stirrer bar).
51. Set up an ice bath on top of a stirrer hotplate. Half fill the bath with ice, and cover with water. Lower the reaction flask into the ice bath until it is half submerged. Ensure that the water does not overflow the bath and that the stirrer bar can rotate when the magnetic field is applied. Leave the flask to cool to 0 °C (~15 min).
52. Add 2,4,6-trichloropyrimidine (5.00 g, 27.5 mmol, 1 eq.), sarcosine ethyl ester hydrochloride (4.60 g, 30 mmol, 1.1 eq.), then Et_3N (9.50 mL, 68.7 mmol, 2.5 eq.) dropwise to maintain the reaction flask temperature. Stir for 1 h at 0 °C.

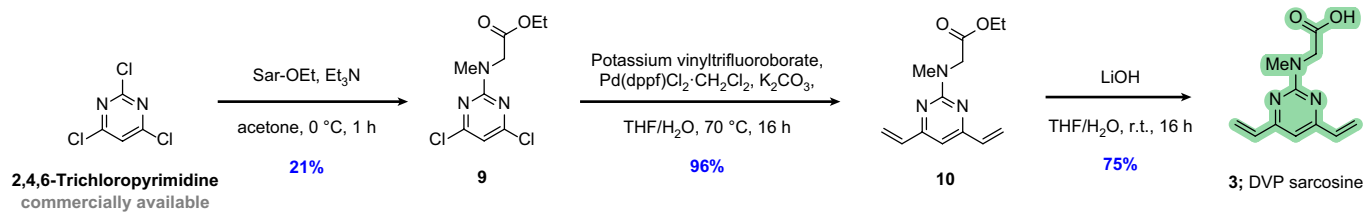


Fig. 5 | Synthesis of DVP sarcosine 3. dppf, 1,1′-bis(diphenylphosphino)ferrocene; Sar, sarcosine.

53. Monitor the reaction by TLC (product R_f = 0.46 EtOAc/PE 40–60 2:3) to ensure all sarcosine ethyl ester hydrochloride has been consumed (R_f = 0.50 EtOAc/PE 40–60 2:3). If not, continue the reaction, monitoring periodically.
▲ **CAUTION** Note that TLC analysis will show two product spots (desired R_f = 0.46 and by-product 0.13, EtOAc/PE 40–60 2:3). The S_NAr reaction yields a statistical mixture of 1- and 3- addition. As there are two chlorinated sites at the 3-positions, the desired 1-addition is the minor product. Although attempts to optimize this reaction have been unsuccessful, it remains robust and scalable, with successful execution on a 20-g scale.
54. Use a rotary evaporator to remove the solvent (40 °C, ~400 mbar). Evaporate to dryness.
55. Redissolve the crude in CH_2Cl_2 (100 mL) and transfer to a separating funnel (500 mL). Wash with water (100 mL) twice, collecting the lower organic layer in a conical flask (250 mL) each time and discard the aqueous layers.
56. Add $MgSO_4$ to the organic layer to dry it (Step 18). Filter the solvent into a round-bottom flask (250 mL) using a polypropylene powder funnel stuffed with cotton wool. Rinse the $MgSO_4$ flask and wool with CH_2Cl_2 (20 mL). Use a rotary evaporator to remove the solvent (40 °C, ~400 mbar). Evaporate to dryness.
57. Dissolve the crude oil in a minimum volume of CH_2Cl_2 (~10 mL), load onto a glass column and purify via flash column chromatography. Column details: silica (230–400 mesh), column length (total) 32 cm, column diameter 7.5 cm, silica height 18 cm, collecting 18 mL column fractions R_f = 0.46 EtOAc/PE 40–60 2:3).
▲ **CAUTION** The particle size of silica gel can cause respiratory problems after prolonged exposure to, and inhalation of, the silica dust. Only handle silica gel in a ventilated fume hood.
58. Run the column starting with one column volume of 100% petroleum ether 40–60, then further single column volumes of 2% EtOAc in petroleum ether 40–60 then 4%. Monitor by TLC until the starting material has eluted (R_f = 0.50 EtOAc/PE 40–60 2:3).
▲ **CRITICAL STEP** The starting material and product retention factors are very similar so care must be taken to only increase the polarity of the eluant slowly to ensure the product is successfully separated.
59. Increase the polarity of the eluant to 5% for 2–3 column volumes until the product has eluted (R_f = 0.46 EtOAc/PE 40–60 2:3).
60. Concentrate the product-containing fractions on a rotary evaporator at 40 °C to yield ethyl *N*-(4,6-dichloropyrimidin-2-yl)-*N*-methylglycinate **9** as a white solid (1.52 g, 5.8 mmol, 21%).
61. Characterize and check the purity of the product via NMR and LRMS.
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (s, 1H), 4.32 (s, 2H), 4.20 (q, J = 7.1 Hz, 2H), 3.22 (s, 3H), 1.27 (t, J = 7.1 Hz, 3H) ppm;
^{13}C NMR (101 MHz, $CDCl_3$) δ 169.3, 161.1, 161.3, 108.7, 61.3, 51.2, 36.6, 14.2 ppm;
LRMS (ESI) m/z: 264.0 $[M+H]^+$.
■ **PAUSE POINT** Purified **9** can be stored at −20 °C for at least 12 months.

Stage 3b: synthesis of **3**

● **TIMING 48 h (6 h hands-on time) for 3**

62. Set up a hotplate with a 100 mL DrySyn block or oil/sand bath.
63. Charge a clean, dry round-bottomed flask (100 mL, fitted with a large Teflon-coated magnetic stirrer bar) with intermediate **9** (1.4 g, 5.32 mmol, 1 eq.), $Pd(dppf)Cl_2·CH_2Cl_2$

(652 mg, 0.80 mmol, 0.15 eq.), potassium vinyltrifluoroborate (3.56 g, 26.6 mmol, 5 eq.), and K_2CO_3 (4.4 g, 31.9 mmol, 6 eq.).

▲ **CRITICAL STEP** This Suzuki reaction contains a large mass of solids and so requires effective stirring. Ensure that the largest reasonable stirrer bar is used.

▲ **CRITICAL STEP** The Suzuki must be oxygen free for a successful reaction so take care to thoroughly purge the system with N_2 before heating the reaction.

64. Attach a water cooled condenser (30 × 2.5 cm) fitted with a rubber septum or Schlenk line adaptor and purge the flask with anhydrous N_2. This can either be done by repeated (three times) vacuum evacuation/N_2 filling of the flask/condenser system on a Schlenk line, or by blowing N_2 over the solids using a balloon fitted with a needle for 20 min.
65. Purge a mixture of THF/H_2O (50 mL, 10:1) with N_2 before adding it the reaction flask via a syringe and needle. Evacuate/purge the system into N_2 three more times to ensure no air is present.
66. Heat the reaction at 70 °C for 16 h. On this scale the reaction mixture should retain an orange/red color throughout; smaller scales may go black over time. TLC should show the product with R_f = 0.65 EtOAc/PE 40–60 2:3 and some impurities with a yellow appearance on the plate with R_f = 0.4–0.5. This impurity should be negligible under UV, demonstrating the conversion of the starting dichloride.

◆ **TROUBLESHOOTING**

67. Load a 120-mL Büchner funnel with Celite and wash with CH_2Cl_2 (50 mL) to pack it; ensure the Celite does not dry out. Filter the reaction mixture through the Celite plug and wash the filter cake with MeOH (2 × 50 mL). Use a rotary evaporator to remove some solvent (room temperature, ~400 down to 50 mbar). Evaporate to ~30 mL, add 30 g silica and evaporate to dryness.

▲ **CRITICAL STEP** The vinyl groups in DVPs can polymerize if not handled correctly. Where possible, DVPs should be stored as solids to prevent polymerization. Molecule **10** is an oil and so should be concentrated without heating and characterized as soon as the solvent is removed. The oils should either be used in subsequent chemical steps or diluted before storage at −20 °C. Ideally, continue to the hydrolysis and leave it reacting overnight.

◆ **TROUBLESHOOTING**

68. Solid load the crude material onto a glass column as in Steps 24–25 and purify via flash column chromatography. Column details: silica (230–400 mesh), column length (total) 32 cm, column diameter 7.5 cm, silica height 18 cm, collecting 18 mL column fractions, R_f = 0.65 EtOAc/PE 40–60 2:3.

▲ **CAUTION** The particle size of silica gel can cause respiratory problems after prolonged exposure to, and inhalation of, the silica dust. Only handle silica gel in a ventilated fume hood.

69. Run the column starting with one column volume of 100% petroleum ether 40–60, then one column volume at 5% EtOAc. Increase the polarity of the eluant to 10% for 2–3 column volumes until the product has eluted (R_f = 0.65 EtOAc/PE 40–60 2:3).
70. Gently concentrate the product containing fractions on a rotary evaporator at room temperature to yield ethyl *N*-(4,6-divinylpyrimidin-2-yl)-*N*-methylglycinate **10** as a light-yellow oil (1.26 g, 5.10 mmol, 96%).
71. Characterize and check the purity of the product via NMR and LC–MS.
LC–MS: R_t = 2.06 min (5–95% solvent B over 3 min, with solvent C maintained at 5% throughout the run);
^{1}H NMR (500 MHz, $CDCl_3$) δ 6.58 (dd, *J* = 17.3, 10.5 Hz, 2H), 6.49 (s, 1H), 6.38 (d, *J* = 17.3 Hz, 2H), 5.53 (d, *J* = 10.5 Hz, 2H), 4.36 (s, 2H), 4.18 (q, *J* = 7.1 Hz, 2H), 3.32 (s, 3H), 1.23 (t, *J* = 7.1 Hz, 3H) ppm;
^{13}C NMR (126 MHz, $CDCl_3$) δ 170.9, 163.2, 162.0, 136.1, 121.1, 105.5, 60.7, 51.7, 36.4, 14.3 ppm;
LRMS (ESI) *m/z*: 248.3 $[M + H]^+$.
72. Charge a round-bottomed flask (25 mL, with Teflon-coated magnetic stirrer bar) with **10** (1.2 g, 4.86 mmol, 1 eq.) and dissolve in THF (10 mL). Add water (1 mL), LiOH·H_2O (408 mg, 9.72 mmol, 2 eq.) and stir for 16 h at ambient temperature.
73. Remove the THF on a rotary evaporator (40 °C, ~100 mbar) and then freeze and lyophilize off the water on a freeze drier. Suspend the remaining salts in DMF (5 mL) and sonicate

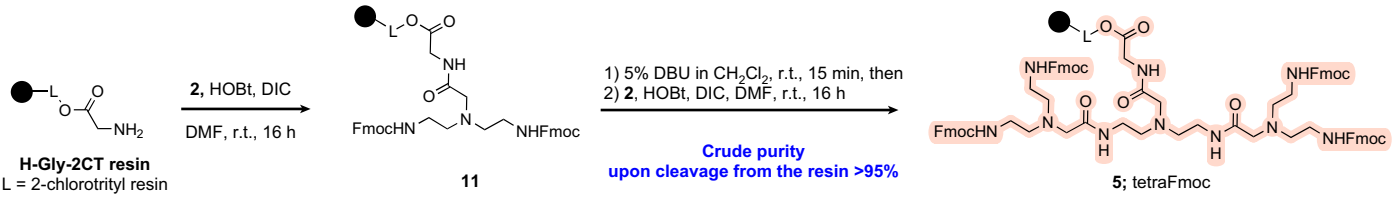


Fig. 6 | Synthesis of tetraFmoc intermediate 5.

to encourage dissolution for 10 min before filtering through a small Büchner funnel or syringe filter. Alternatively, the DVP can be extracted from the THF reaction mixture by diluting with water (20 mL) and washing with Et_2O (2 × 20 mL). The aqueous layer can then be acidified very carefully to pH 4 with dropwise addition of 1 M aq. HCl and extracted into CH_2Cl_2 (4 × 20 mL). The solvent can be removed by rotary evaporation at room temperature and the crude oil dissolved in DMF for purification.

74. Purify the filtrate via automated reverse phase column chromatography (80 g HP C18, 5–40% MeCN in H_2O over 10 min) and remove the solvent via lyophilization to afford an off-white solid (797 mg, 3.64 mmol, 75%).
▲ **CRITICAL STEP** As above, the DVP sarcosine **3** can polymerize if not handled carefully so avoid storing concentrated crude mixtures or decreasing the pH below pH 4.
75. Characterize and check the purity of the product via NMR and LRMS.
^{1}H NMR (500 MHz, MeOD) δ 6.67 – 6.59 (m, 3H), 6.40 (dd, J = 17.4, 1.8 Hz, 2H), 5.53 (dd, J = 10.6, 1.8 Hz, 2H), 4.27 (s, 2H), 3.27 (s, 3H) ppm;
^{13}C NMR (126 MHz, MeOD) δ 177.2, 163.3, 162.3, 136.2, 119.8, 103.5, 53.0, 35.1 ppm;
LRMS (ESI) m/z: 219.8 $[M + H]^+$.
■ **PAUSE POINT** Purified **3** can be stored at −20 °C for at least 12 months.

Section 2: on-resin assembly of tetraDVP linkers

Stage 1: synthesis of tetraFmoc intermediate 5

Refer to Fig. 6.

● **TIMING 36 h (4 h hands-on time) for 5**

76. Weigh 250 mg of H-Gly-OH preloaded 2-chlorotrityl chloride resin (loading 0.66 mmol/g, 100–200 mesh) into a plastic syringe (20 mL) fitted with a porous disk.
◆ **TROUBLESHOOTING**
77. Add 50 mL of CH_2Cl_2 to a 250-mL glass beaker and wash the resin by immersing the tip of the syringe in the solvent, drawing it up and fit with pressure cap.
    - Gently shake the syringe containing the resin slurry by hand for at least 10 s, then discard the solvent into appropriate waste
    - Repeat this washing step five times to ensure complete removal of the residual storage solvent (Extended Data Fig. 2d–f)

    ▲ **CRITICAL STEP** The resin should appear white or pale yellow in color and should noticeably swell in size compared to its dry form. Do not skip this step, as the dry form will not provide sufficient coupling efficiency.
78. In a 25-mL glass beaker, dissolve compound **2** (317 mg, 0.525 mmol, 0.15 M) in 3.5 mL of DMF. Add HOBt (80 mg, 0.525 mmol, 0.15 M) and use a sonicator to ensure complete dissolution. HOBt can be weighed into an Eppendorf tube, while compound **2** can be weighed directly into the beaker.
79. Use an automatic pipette to add DIC (82 µL, 0.525 mmol, 0.15 M) to the beaker, hand-stir briefly, then immediately draw up the solution to the syringe containing the resin.
▲ **CRITICAL STEP** In solid-phase synthesis, reagent concentration in the reaction mixture is more critical than the precise number of equivalents. Therefore, all reagent quantities should be calculated on the basis of the total volume of solvent used. While a typical guideline is to use 10 mL of solvent per gram of resin, we found that 14 mL per gram provides optimal results for tetraDVP linkers. For example, to achieve a 0.15 M concentration in 3.5 mL

of solvent (corresponding to 250 mg of resin), the required amount of reagent is calculated as: 3.5 × 0.15 = 0.525 mmol. This corresponds to ~3 equivalents relative to the resin loading. Always use a slight excess of reagents to drive the amide coupling to completion.

80. Expel air from the syringe and ensure the resin slurry is well mixed. Ideally one-quarter of the filled volume of the syringe should be air (Extended Data Fig. 2g). Seal the syringe with a cap and place it horizontally on a shaker and allow it to shake (600 rpm) for at least 12 h, but no longer than 24 h (Extended Data Figure 1a–c).
81. Expel the residual solvents from the syringe into waste. Wash the resin with CH_2Cl_2, DMF and CH_2Cl_2 again similarly as for Step 77. Wash at least five times with each solvent cycle. The final wash should always be CH_2Cl_2.
82. To analyze the completion of the reaction, carefully open the syringe and take ~5–10 mg of resin and transfer it to Eppendorf tube (Extended Data Fig. 2a–c).
83. Add cleavage cocktail CH_2Cl_2/HFIP (2:1, 1.5 mL) and put the Eppendorf on a shaker. Leave it for 1 h (600 rpm, room temperature). The beads should become red in color.
    ▲ **CAUTION** HFIP has a strong alcoholic odor. Use it in ventilated fume hood at all times.
    ◆ **TROUBLESHOOTING**
84. Remove the residual solvents under a gentle stream of nitrogen.
85. Suspend the resin in DMSO (1 mL) and use a plastic Pasteur pipette to transfer the slurry into a syringe (1 mL) equipped with a ProFill HPLC syringe filter (Extended Data Fig. 3a–d).
86. Filter it into an LC–MS vial. Measure UV-LC–MS to check purity of the intermediate.
    ▲ **CAUTION** Always make sure the solution is homogeneous before submitting to LC–MS measurement.
    ▲ **CRITICAL STEP** The UV chromatogram should show only the product **11** peak (R_t = 2.48 min, gradient 5–95% B over 3 min, followed by 1.5 min isocratic hold, solvent C maintained at 5% throughout the run) with a corresponding mass (calcd. for $C_{38}H_{39}N_4O_7$ *m/z* $[M + H]^+$ 663.28) and the DMSO solvent peak. Crude purity should exceed 95%.
    ◆ **TROUBLESHOOTING**
87. After confirming successful synthesis, wash the rest of the resin containing intermediate **11** with CH_2Cl_2 five times as in Step 77.
88. Prepare the Fmoc deprotection cocktail:
    - In a 25-mL beaker, add 3 mL CH_2Cl_2 using a graduated plastic pipette
    - Use an automatic pipette add 150-µL DBU
    - Rinse the pipette tip once or twice by drawing up and releasing a small volume of the solution to ensure complete transfer
89. Draw up the DBU solution into the syringe containing the resin-bound intermediate **11**.
90. Expel air from the syringe and ensure the resin slurry is well mixed. Ideally one-quarter of the filled volume of the syringe should be air (Extended Data Fig. 2g). Seal the syringe with a cap and place it horizontally on a shaker and allow it to shake (600 rpm) for 15 min at room temperature (Extended Data Figure 1a–c).
91. Meanwhile, prepare the next coupling solution of compound **2** and HOBt in DMF as described in Step 78.
92. After 15 min, discard the DBU solution. Wash the resin thoroughly with CH_2Cl_2 (5 × 10 mL), as in Step 77.
93. Add DIC as described in Step 79 to activate the coupling.
94. Expel air and place on a shaker as described in Step 90. Shake for at least 12 h, but no longer than 24 h.
95. Proceed with Steps 81–86 to cleave a sample and analyze purity.
    ▲ **CRITICAL STEP** The UV spectrum should contain only the peak of product **5** cleaved from the resin (LC–MS: R_t = 3.14 min in 5–95% solvent B over 3 min, followed by 1.5 min isocratic hold, with solvent C maintained at 5% throughout the run; analytical HPLC: R_t = 12.13 min (5–95% B over 18 min)) with a corresponding mass (calcd. for $C_{80}H_{85}N_{10}O_{13}$ *m/z* $[M + H]^+$ 1,393.63) and a peak of solvent (DMSO). Crude purity should exceed 95%. Ensure (by LC–MS) no partially reacted intermediates are present.
    ■ **PAUSE POINT** Resin-bound **5** can be stored at −20 °C for at least 12 months.

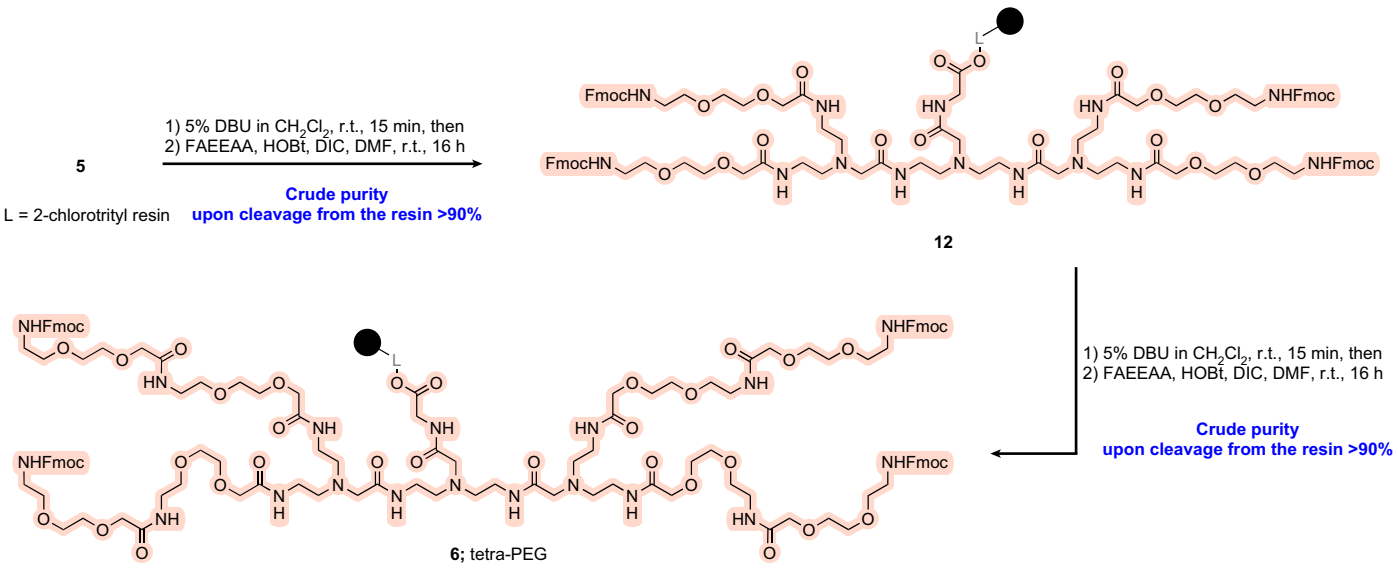


Fig. 7 | Synthesis of tetra-PEG intermediate 6.

Stage 2: synthesis of tetra-PEG intermediate 6

● **TIMING 36 h (4 h hands-on time) for 6**

Refer to Fig. 7.

96. Wash the resin as described in Step 77.
97. Prepare Fmoc deprotection cocktail as described in Step 88.
98. Draw up the DBU solution into the syringe containing the resin-bound intermediate **5**.
99. Expel air and place on a shaker as described in Step 90.
100. Meanwhile, in a 25-mL glass beaker, dissolve FAEEAA (202 mg, 0.525 mmol, 0.15 M) in 3.5 mL of DMF. Add HOBt (80 mg, 0.525 mmol, 0.15 M). Use a sonicator to ensure complete dissolution. HOBt can be weighed into an Eppendorf tube, while FAEEAA can be weighed directly into the beaker.
101. After 15 min, discard the DBU solution from the syringe to waste. Wash the resin thoroughly with CH_2Cl_2 five times as in Step 77.
102. Add DIC to the beaker as described in Step 79 to activate the coupling.
103. Expel air and place on a shaker as described in Step 90. Shake for at least 12 h, but no longer than 24 h (Extended Data Figure 1a–c).
104. Proceed with Steps 81–86 to cleave a sample and analyze purity.
▲ **CRITICAL STEP** From this step onward it is advised to use a LC–MS-HPLC machine to analyze analytical results. The UV spectrum should contain the peak of product **12** cleaved from the resin (LC–MS-HPLC: R_t = 11.50 min in 5–95% B over 15 min with 1 min isocratic hold before the gradient; analytical HPLC: R_t = 11.60 min (5–95% B over 18 min)) with a corresponding mass ($C_{104}H_{129}N_{14}O_{25}$ m/z $[M+H]^+$ 1,973.92, usually present as $[M+2H]^{2+}$ 988.22) and a peak of solvent (DMSO). Occasionally, residual HOBt might be present (R_t = 5.02). Crude purity should exceed 90%. Ensure no partially reacted intermediates are present.
105. After confirming successful synthesis, wash the resin containing intermediate **12** with CH_2Cl_2 five times as in Step 77.
106. Prepare Fmoc deprotection cocktail as described in Step 88.
107. Draw up the DBU solution into the syringe containing the resin-bound intermediate **12**.
108. Expel air and place on a shaker as described in Step 90.
109. Meanwhile, prepare the next coupling solution of FAEEAA and HOBt in DMF as described in Step 100.

110. After 15 min, discard the DBU solution from the syringe to the waste. Wash the resin thoroughly with CH_2Cl_2 five times as in Step 77.
111. Add DIC using an automatic pipette as described in Step 79 to activate the coupling.
112. Expel air and place on a shaker as described in Step 90. Shake for at least 12 h, but no longer than 24 h (Extended Data Figure 1a–c).
113. Proceed with Steps 81–86 to cleave a sample and analyze purity.
▲ **CRITICAL STEP** The UV spectrum should contain peak of product **6** cleaved from the resin (LC–MS-HPLC: R_t = 10.49 min in 5–95% B over 15 min with 1 min isocratic hold before the gradient; analytical HPLC: R_t = 11.15 min (5–95% B over 18 min)) with a corresponding mass ($C_{128}H_{173}N_{18}O_{37}$ *m/z* $[M + H]^+$ 2,554.22, usually present as $[M + 2H]^{2+}$ 1,278.33) and a peak of solvent (DMSO). Crude purity should exceed 90%. Ensure no partially reacted intermediates are present.
■ **PAUSE POINT** Resin-bound **6** can be stored at −20 °C for at least 12 months.

Stage 3: synthesis of tetraDVP azide 7

● **TIMING 2–3 d (8 h hands-on time) for 7**

Refer to Fig. 8.

114. Wash the resin as described in Step 77.
115. Prepare Fmoc deprotection cocktail as described in Step 88.
116. Draw up the DBU solution into the syringe containing the resin-bound intermediate **6**.
117. Expel air and place on a shaker as described in Step 90.
118. Meanwhile, prepare a solution of DVP sarcosine **3** (115 mg, 0.525 mmol, 0.15 M) in 3.5 mL of DMF. Add HOBt (80 mg, 0.525 mmol, 0.15 M). Use a sonicator to ensure complete dissolution. HOBt can be weighed into an Eppendorf tube, while compound **3** can be weighed directly into the beaker.
▲ **CRITICAL STEP** It is essential to use DVP sarcosine **3** of the highest purity, ideally prepared within the past 3 months. Prolonged storage may lead to polymerization and reduced solubility in organic solvents, which can negatively impact coupling efficiency.
119. After 15 min, discard the DBU solution from the syringe into the waste. Wash the resin thoroughly with CH_2Cl_2 five times as in Step 77.
120. Remove the syringe plunger and securely cap the bottom of the syringe.
121. Using a plastic Pasteur pipette, transfer the entire contents of the beaker into the syringe.
122. Using an automatic pipette, add DIC (82 µL, 0.525 mmol, 0.15 M) directly to the resin slurry. The solution should begin to turn dark brown after the addition of DIC.
123. Carefully attach the plunger to the syringe, gently release any built-up pressure by briefly removing the cap, and expel any residual air. Ideally one-quarter of the filled volume of the syringe should be air.
124. Re-cap the syringe and place it horizontally on a shaker. Shake for 16 h at room temperature (600 rpm). The reaction mixture should gradually darken to a deep brown color.
125. Expel the residual solvents from the syringe into waste. Wash the resin with DMSO, MeOH, water and CH_2Cl_2 similarly as for Step 77. Wash at least 7 times with each solvent cycle. The final wash should always be CH_2Cl_2.
◆ **TROUBLESHOOTING**
126. Proceed with Steps 81–86 to cleave a sample and analyze purity.
▲ **CRITICAL STEP** The UV spectrum should contain the peak of product **13** cleaved from the resin (LC–MS-HPLC: R_t = 8.20 min in 5–95% B over 15 min with 1 min isocratic hold before the gradient; analytical HPLC: Rt = 8.40 min (5–95% B over 18 min)) with a corresponding mass ($C_{112}H_{177}N_{30}O_{33}$ *m/z* 2,470.30 $[M + H]^+$, usually present as $[M + 2H]^{2+}$ 1,236.29) and a peak of solvent (DMSO). Crude purity should exceed 90%. Ensure (by LC–MS) no partially reacted intermediates are present.
■ **PAUSE POINT** TetraDVP acid **13** attached on resin can be stored at −20 °C for at least 12 months before final cleavage from the resin and purification.
127. After confirming the presence of tetraDVP acid **13**, wash the resin as described in Step 77.

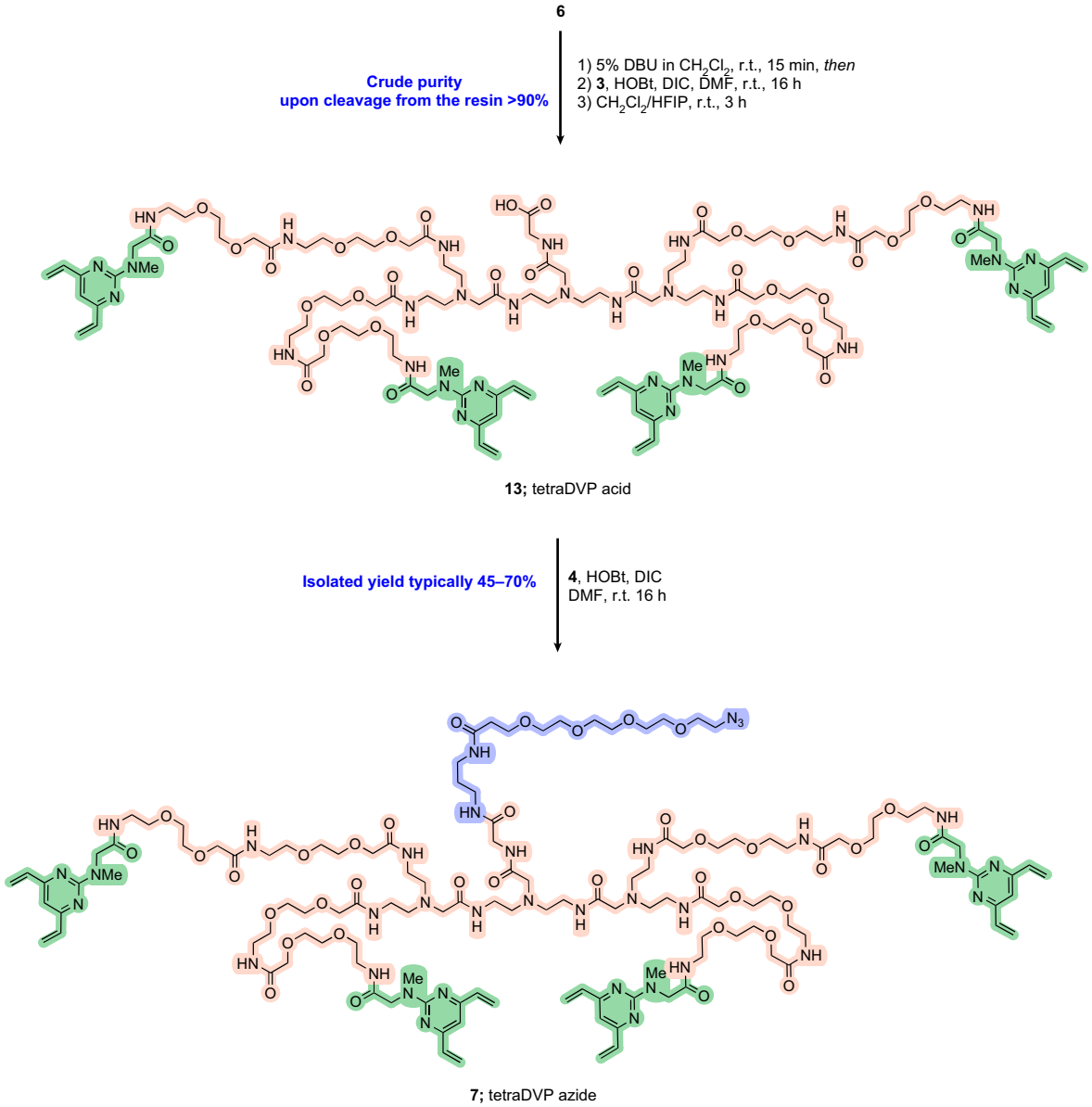


Fig. 8 | Synthesis of tetraDVP azide 7.

128. Prepare the cleavage solution for full resin cleavage: in a 100-mL glass beaker, mix 40 mL of CH_2Cl_2 with 20 mL of HFIP.
129. Draw up 15 mL of the cleavage solution to the syringe containing compound **13**. Expel residual air, cap the syringe, and place it horizontally on a shaker. Ideally one-quarter of the filled volume of the syringe should be air. Shake for 2 h at room temperature (600 rpm). The resin will turn deep red after addition of the cleavage cocktail.
130. After 2 h, empty the syringe contents into a 100-mL round-bottom flask. Wash the resin with CH_2Cl_2 (2 × 10 mL) as in Step 77 and combine the washes with the initial cleavage solution.
131. Repeat Steps 129–130.
132. Combine all cleavage solutions and evaporate the solvent under reduced pressure to yield crude product **13**.

133. Resuspend the resulting oil in a small volume of DMSO (no more than 5 mL).
134. Filter the suspension through a ProFill HPLC syringe filter into LC–MS vials and purify using an preparative LC–MS-HPLC using a neutral solvent system (R_t = 8.40 min when 5–95% B over 18 min, no solvent additives, collection of masses 618.72, 824.59 or 1,236.29).
▲ **CRITICAL STEP** Use a neutral solvent system during purification, without any acidic additives, as these may cause decomposition of tetraDVP acid **13** during lyophilization.
135. Combine the product containing fractions in a suitable preweighed container for lyophilization.
■ **PAUSE POINT** Dried tetraDVP acid **10** can be stored at −20 °C for at least 12 months.
136. Once dry, dissolve tetraDVP acid **13** (15 mg, 0.006 mmol, 1 eq.) in DMF (1 mL) and transfer to a round-bottom flask (5 mL) equipped with a magnetic stirrer bar on a hot plate (stirring at 200 rpm).
137. Weigh linker **4** (3 mg, 0.009 mmol, 1.5 eq.) and HOBt (16 mg, 0.102 mmol, 17 eq.) in separate Eppendorf tubes.
138. Dissolve each in 200 µL DMF.
139. Add both solutions to the flask containing compound **13**, followed by DIC (14 µL, 0.102 mmol, 17 eq.).
140. Stir the reaction mixture at room temperature for 16 h.
141. Take a 200-µL aliquot of the reaction mixture, dilute with 500 µL DMSO, filter through a ProFill HPLC syringe filter and analyze by UV-LC–MS to confirm formation of product **7**.
142. Purify the crude product **7** by preparative LC–MS-HPLC using a neutral solvent system (R_t = 8.79 min when 5–95% B over 15 min, no solvent additives, collection of mass 701.10).
143. Collect fractions and remove solvents by freeze drying.
144. TetraDVP azide **7** is typically obtained in 45–70% yield as a yellowish oil. Characterize and check the purity of the product via LC–MS.
LC–MS-HPLC R_t = 8.55 min (5–95% B over 15 min with 1 min isocratic hold before the gradient) LRMS m/z $[M + H]^{2+}$ 2,800.52, found $[M + 4H]^{2+}$ 701.10.

Section 3: bioconjugation to antibodies

● **TIMING 25 h (2 h hands-on time)**

145. Charge a 500-µL centrifuge tube with trastuzumab solution (TBS, 100 µL, ~10 µM). Record the UV-Vis absorbance at 280 nm and calculate the concentration using ε = 215,380.
146. Add 10 eq. of TCEP (5 mM in TBS) to the trastuzumab and incubate on a thermal shaker (1 h, 37 °C, 400 rpm).
147. Add 5 eq. of **7** (2.5 mM in DMSO) to the trastuzumab and incubate on a thermal shaker (23 h, 37 °C, 400 rpm).
148. Equilibrate a 0.5 mL 40k MWCO Zeba Spin desalting column into PBS according to the manufacturer's specification.
149. Pipette the antibody solution into the desalting column and centrifuge to the manufacturer's specification.
150. Transfer the filtrate into an 0.5-mL 10k Amicon diafiltration column and dilute with 300 µL PBS. Centrifuge the sample for 2 min at 14,000*g*. Add a further 300 µL PBS and spin-concentrate again. Repeat this process of dilution and concentration four times. Dilute a fifth time and centrifuge for 10 min to concentrate the sample to ~30 µL.
151. Recover the ADC by inverting the filter section of the Amicon into a clean tube and centrifuging for 2 min at 4,000*g*.
152. Record the final concentration via UV-Vis. Analyze by HIC, SEC, SDS–PAGE and MS.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting

Step	Problem	Possible reason	Possible solution
13 (Section 1, Stage 1)	Key branching amine is not the main product of the reaction	Less equivalents than suggested in the procedure have been used	It is possible to add more reagents to the existing reaction mixture; however, this does not substantially improve the conversion, in our experience. We therefore recommend setting up the reaction again using the suggested reagent amounts, which have been extensively optimized to ensure reliable results.
66 (Section 1, Stage 3)	Suzuki has not worked and reaction mixture has turned from orange to black	Palladium catalyst has been poisoned/hindered	Purify out starting dichloropyrimidine and try again by either (1) being more rigorous with N_2 purge. Oxygen has been present in the reaction or (2) acquiring a new stock of catalyst (store at −20 °C). Palladium catalyst is poisoned.
67 (Section 1, Stage 3)	DVP-containing oils or solids do not dissolve in any solvent	DVP polymerization. The DVP compound has been stored for too long as a concentrated oil or has been exposed to excessive heat or acidic environments	Once polymerized the DVP can not be regenerated. Add DMF to the DVP and sonicate for 10 min before filtering. The filtrate should contain any remaining nonpolymerized DVP.
76 (Section 2, Stage 1)	Commercial loading differs from 0.66 mmol/g	Different supplier or batch of the resin	Using a loading different from 0.66 mmol/g of H-Gly-OH 2-chlorotrityl resin does not cause any issues during the synthesis. For yield calculations, always refer to the specific loading information provided by the supplier for your batch.
83 (Section 2, Stage 1)	Resin did not turn red after the addition of CH_2Cl_2/HFIP cleavage cocktail on a small scale	Scavenging of the reactive resin intermediates	This does not cause any problems to the outcome of the mini cleavage.
86 (Section 2, Stage 1)	Crude purity of **11** is below 95%	Impure starting material **2**	Ensure that starting material **2** is of high purity, as confirmed by NMR, and free from any byproducts before subjected it to the resin.
125 (Section 2, Stage 3)	The syringe is stuck and impossible to wash with solvents	Polymerization by-products of DVP **3** clogged the resin	(1) Open the syringe containing the resin.
			(2) Add 10 mL of MeOH/water (1:1) to the resin.
			(3) Using a plastic Pasteur pipette, gently mix the slurry by drawing up and releasing the suspension four or five times.
			(4) Transfer the slurry into a clean syringe fitted with a porous disk.
			(5) After removing the solvent, proceed by adding a fresh solution of CH_2Cl_2/DMF (1:1) as described in Step 30.

Timing

Section 1: synthesis of building blocks in solution (Steps 1–75)

Stage 1 (Steps 1–29): 22 h (6 h hands-on time)
Stage 2a (Steps 30–45): 24 h (3 h hands-on time)
Stage 2b (Steps 46–49): 4 h (2 h hands-on time)
Stage 3a (Steps 50–61): 5 h (4 h hands-on time)
Stage 3b (Steps 62–75): 48 h (6 h hands-on time)

Section 2: on-resin assembly of tetraDVP linkers (Steps 76–144)

Stage 1 (Steps 76–95): 36 h (4 h hands-on time)
Stage 2 (Steps 96–113): 36 h (4 h hands-on time)
Stage 3 (Steps 114–144): 2–3 d (8 h hands-on time)

Section 3: bioconjugation to antibodies (Steps 145–152)

25 h (2 h hands-on time)

Anticipated results

All intermediates in the synthesis of the branching amine **2**, DVP sarcosine **3**, azido linker **4**, and solid-phase intermediates and products (for example, compounds **5**–**12**) described in this protocol have been previously isolated, purified and characterized[16]. The key branching intermediate **2** is obtained in excellent yield (95–98%) after column chromatography. The subsequent solid-phase steps proceed with crude purities typically exceeding 90%, and the final tetraDVP linkers are obtained in overall yields of 45–70% after preparative HPLC purification. Final linker compounds display the expected masses, and elution times on LC–MS are consistent across batches. No major side products or incomplete intermediates are usually observed when the protocol is followed as described.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data are publicly available as part of the supporting information of the primary research publication at https://pubs.rsc.org/en/content/articlelanding/2025/sc/d5sc02286j.

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Author contributions

S.K. and T.W. contributed intellectually and practically to the development of this protocol. All authors have been involved in writing and proof-reading of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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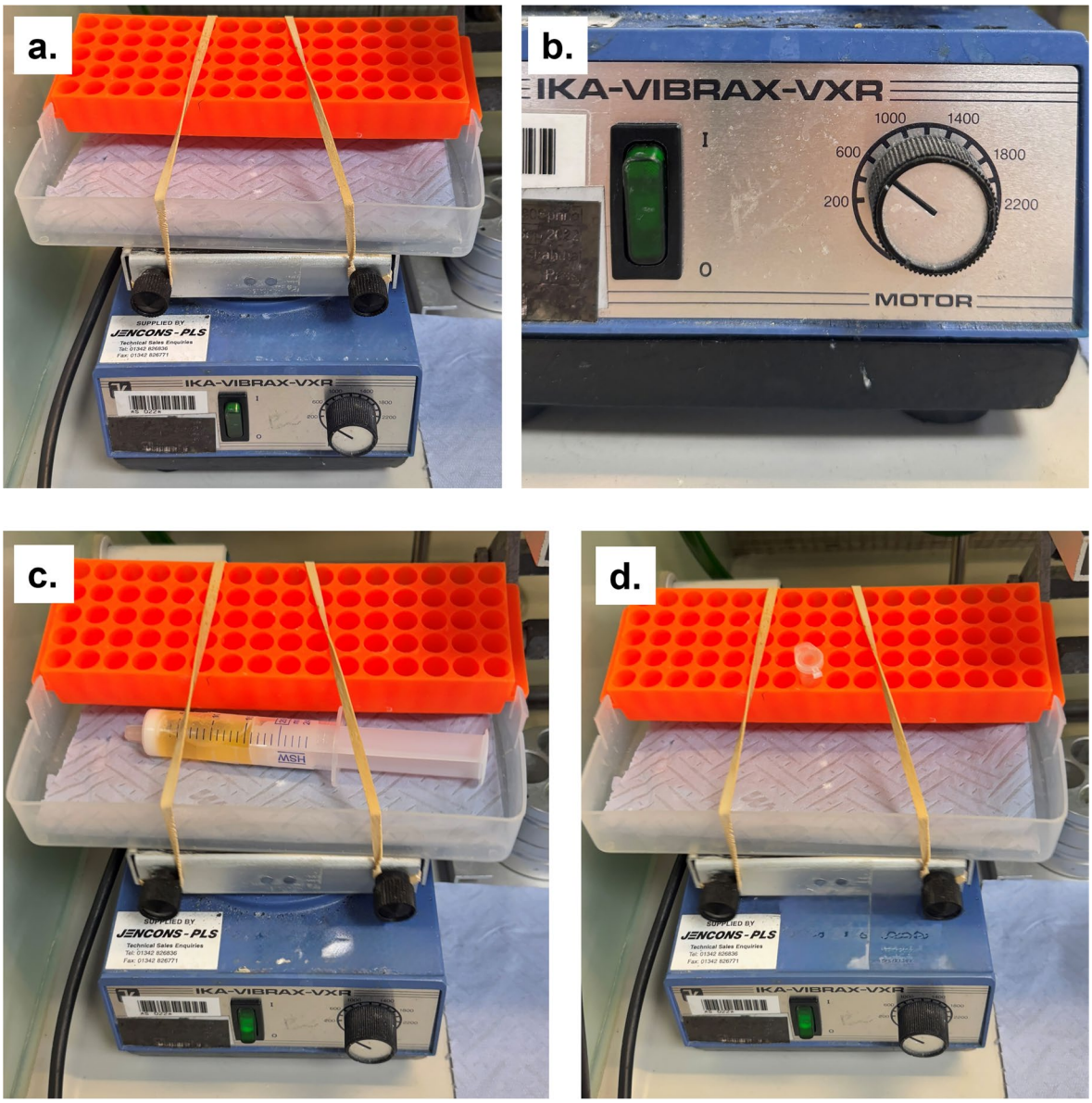
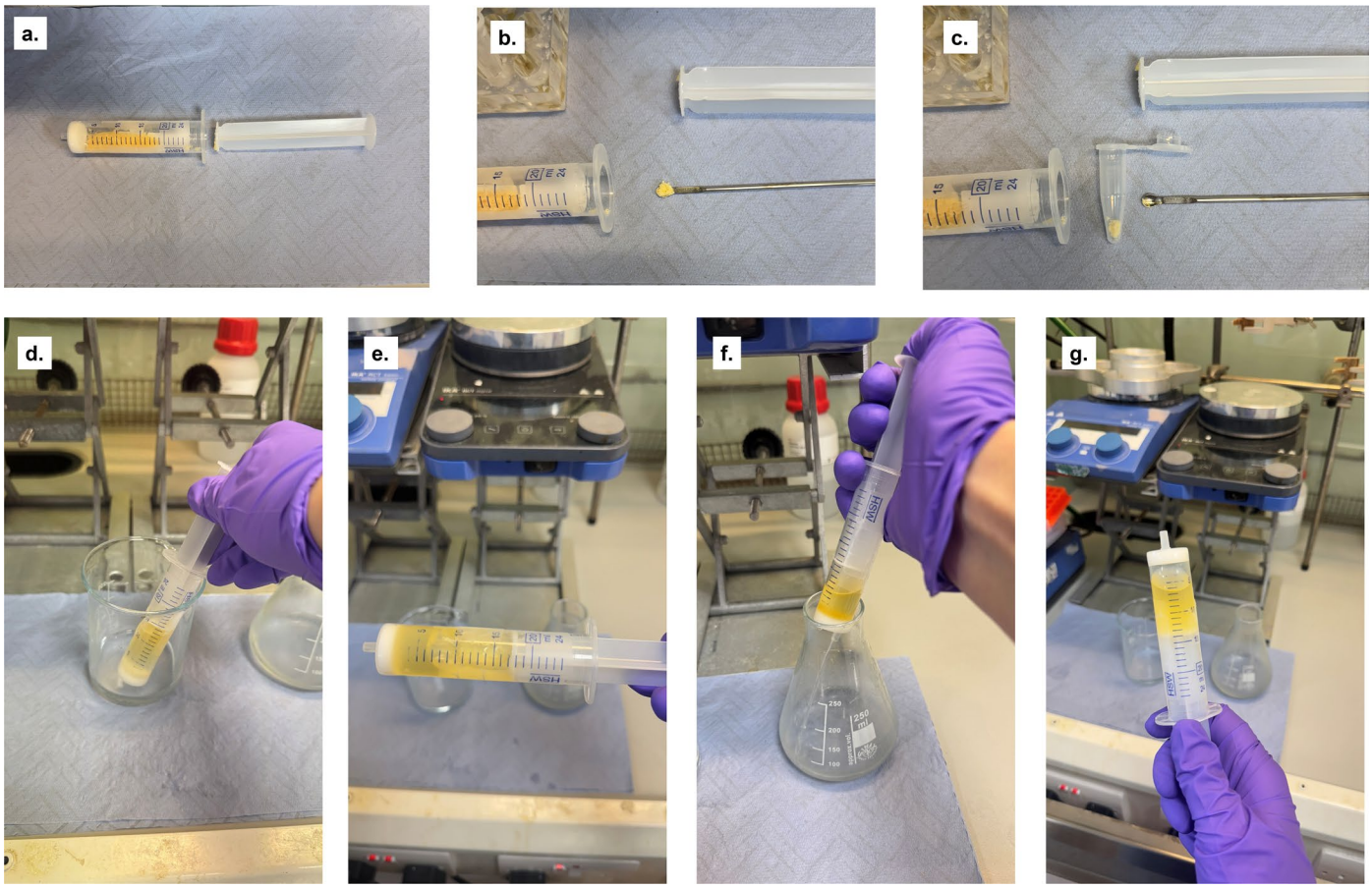
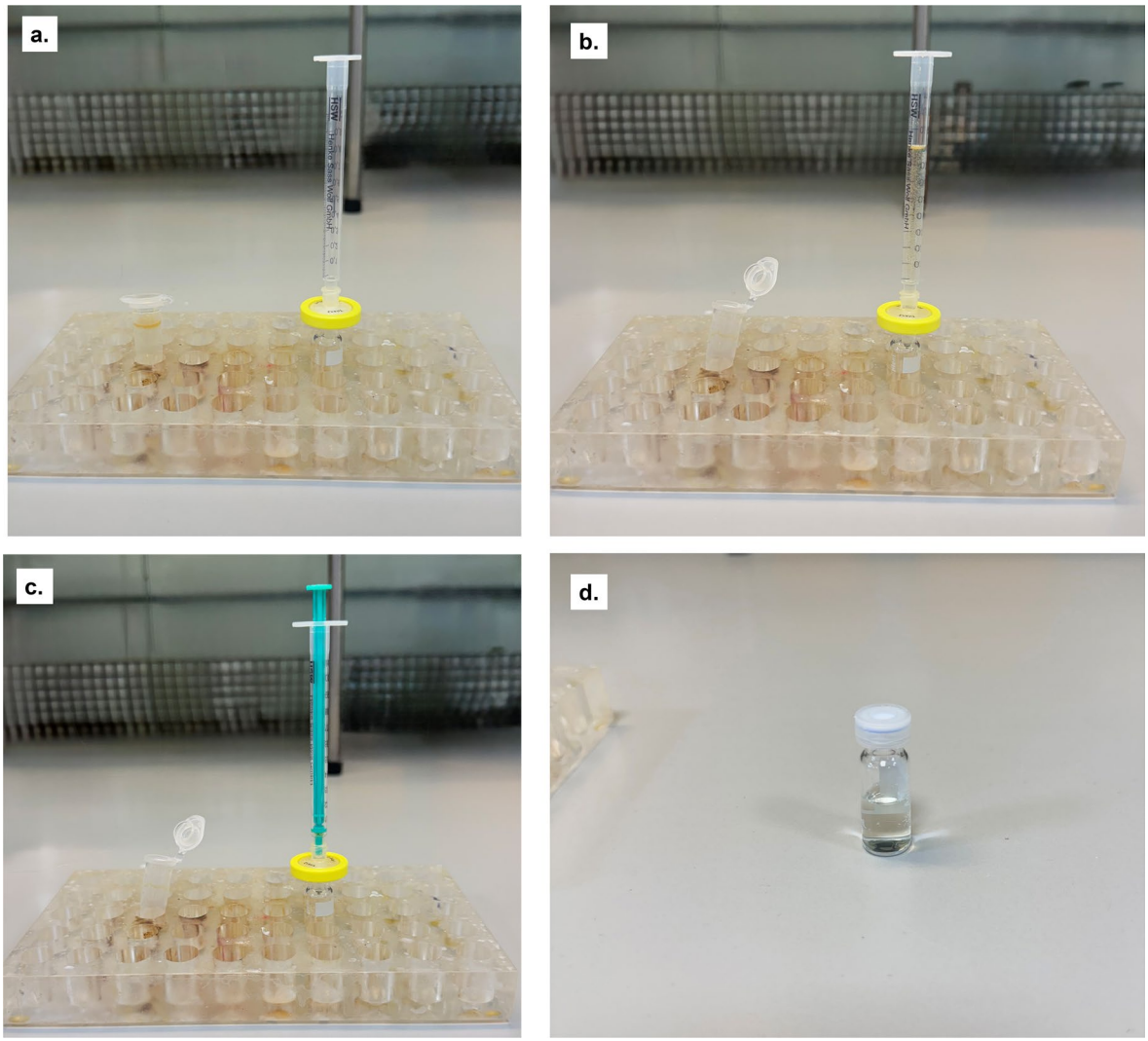


Extended Data Fig. 1 | Shaker set-up. (a) Shaker with extended Eppendorf rack; **(b)** Shaker set to 600 rpm; **(c)** Syringe placed horizontally and shaken at 600 rpm; **(d)** Eppendorf tube shaken at 600 rpm.



Extended Data Fig. 2 | Washing of resin and preparation for a small-scale analytical cleavage. (a) Open syringe containing resin; **(b)** Approximate amount of resin suitable for small cleavage taken from the syringe with a spatula; **(c)** Resin transferred to an Eppendorf tube; **(d)** Solvent drawn into the syringe; **(e)** Manual shaking of the resin in the syringe; **(f)** Residual solvent removed from the syringe into a waste container; **(g)** Air expelled from the syringe to approximately ¼ of the total volume.



Extended Data Fig. 3 | Preparation of an LC–MS sample. (a) HPLC filter attached to a 1 mL syringe; **(b)** Resin transferred from an Eppendorf tube into the syringe using a plastic pipette (not shown) and **(c)** passed through the HPLC filter; **(d)** Homogeneous LC–MS sample obtained.